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Research paper

Antiparasitic activity, histopathology and physiology of *Colossoma macropomum* (tambaqui) exposed to the essential oil of *Lippia sidoides* (Verbenaceae)

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ABSTRACT

In vivo and *in vitro* antiparasitic activity of the essential oil of *Lippia sidoides* and blood and histological alterations were assessed in *Colossoma macropomum* (tambaqui). Essential oil concentrations of 10, 20, 40, 80, 160 and 320 mg/L were assayed *in vitro* against monogenoideans *Anacanthorus spathulatus*, *Notozothecium janauachensis* and *Mymarothecium boegeri* from fish gills. *Lippia sidoides* essential oil concentrations of 320 and 160 mg/L were 100% effective against monogenoideans in 10 min and 1 h of exposure, respectively. However, the effectiveness of 100% concentrations of 80 mg/L and 40 mg/L occurred in 3 and 6 h, respectively. In the *in vivo* tests, juvenile fish were submitted to 60 min of baths with 10 mg/L and 15 min of baths with 20 mg/L of the essential oil of *L. sidoides*. These therapeutic baths were not efficient against *Ichthyophthirius multifiliis*, and monogenoideans present in the gills of *C. macropomum*. In addition, 10 and 20 mg/L of the essential oil of *L. sidoides* caused an anesthetic effect on the fish and did not influence total glucose and protein plasma levels; however, it decreased the number of total erythrocytes in fish exposed to the higher concentration of this essential oil. Severe alterations and irreversible damage were observed in the fish gills just after *L. sidoides* essential oil baths and after 24 h of recovery. The most recurrent lesions found were hyperplasia and fusion of the lamellar epithelium, vasodilation, detachment of the gill epithelium and lamellar aneurism, epithelial breakdown with hemorrhage, congestion, edema and necrosis, proliferation of the mucous cells and chloride cells and lamellar hypertrophy. Therefore, since the essential oil of *L. sidoides* has *in vitro* antiparasitic activity and low concentrations of it have shown toxic effects, the bioactive potential of its main chemical components should be investigated, as well as more efficient forms of its administration in therapeutic baths in order to eliminate fish parasites.

1. Introduction

Medicinal plants have become increasingly popular in aquaculture due to their diversity of chemical components that provide different actions, including therapeutic activity. In fish farming, the search for natural substances with antiparasitic action has gained importance due to their potential effects and because of the damage caused by chemotherapeutic agents, which can damage the environment, fish, and human health. Essential oils extracted from species of *Lippia* (Verbenaceae) have great bioactive potential, therefore, they could possibly be promising herbal medicines to use in fish farming. Species of *Lippia* have antimicrobial, antiparasitic, anesthetic, analgesic, anti-inflammatory and antitumor activities (Cunha et al., 2010; Becker et al., 2012; Soares and Tavares-Dias, 2013; Hashimoto et al., 2016; Soares et al., 2016).

Lippia sidoides Cham. 1832, known as alecrim-pimenta, alecrim-bravo, estrepa-cavalo and alecrim-grande, is popularly used to treat infections and other diseases, many of which had activities against

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infections proven scientifically (Silva et al., 2006; Lobo et al., 2014; Veras et al., 2014). It is an erect deciduous shrub, much branched and crumbly, typical of the semi-arid vegetation, growing in the Caatinga of the northeast region of Brazil. Leaves are aromatic and spicy, opposite, simple and petiolated. Its flowers are small and whitish, gathered in clusters of short axis in the leaf axils. Its fruits are tiny, producing very small seeds, which rarely germinate (Camurça-Vasconcelos et al., 2007; Fontenelle et al., 2007).

Studies have shown the essential oil of *L. sidoides* have antiparasitic action against ticks (Gomes et al., 2012, 2014), forms of *Leishmania* (Oliveira et al., 2009; Farias-Júnior et al., 2012), helminths from caprine and ovine animals (Camurça-Vasconcelos et al., 2007) and monogenoideans from *Oreochromis niloticus* (Hashimoto et al., 2016), and activity against fungi and bacteria (Fabri et al., 2011; Fernandes et al., 2012; Funari et al., 2012; Fontenelle et al., 2007). However, *L. sidoides* has not been used against monogenoideans from *Colossoma macropomum* Cuvier, 1818 (tambaqui), an important fish for the fish farming of the region of Amazon.

Colossoma macropomum is a species of Serassalmidae native to Amazon that, due to its rusticity and omnivorous feeding, has been widely cultured in different intensive systems, favoring the dissemination of parasitic diseases because of high densities used in these systems (Dias et al., 2015). As a result, it has often been parasitized by *Ichthyophthirius multifiliis* (Protozoa), *Anacanthorus spathulatus*, *Notozothecium janauachensis*, *Mymarothecium boegeri* and *Linguadactyloides brinkmanni* (Monogenoidea), which might cause economic loss (Boijink et al., 2015; Martins et al., 2002; Soares et al., 2016), not yet calculated. Due to the need to efficiently treat these parasites from *C. macropomum*, herbal therapy can be an alternative to chemotherapeutic products (Soares et al., 2016). Thus, because of the bioactive potential of the essential oil (EO) of *L. sidoides* against different pathogenic agents, it is important to study its action against *C. macropomum* ectoparasites. The objective of this study was to investigate the *in vivo* and *in vitro* antiparasitic activity of the EO of *L. sidoides*, and the possible blood and histopathological alterations in the gills of *C. macropomum*.

2. Materials and methods

2.1. Extraction and chemical compounds of the EO of L. sidoides

The cultivation of *L. sidoides* and the EO extraction were carried out at the Department of Medicinal Plants and Vegetables of Embrapa Western Amazon, in Manaus, State of Amazonas, Brazil. The essential oil was extracted from the leaves and inflorescences of *L. sidoides* by the hydrodistillation technique using a Clevenger apparatus. The chemical analysis of the EO was performed by gas chromatography connected to mass spectrometry. The chemical components of the EO of *L. sidoides* in this study are shown in Table 1.

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

Peack	% content	Retention index	Identification
1	1.1	854	(*E*)-2-hexenal
2	0.9	928	Alpha-tujeno
3	2.0	989	Myrcene
4	1.1	1016	Alpha-terpinene
5	11.7	1024	p-cimeno
6	3.6	1059	Gamma-terpinene
7	1.4	1144	ipsdienol
8	1.2	1175	4-terpineol
9	1.1	1232	timil-methil-ether
10	4.6	1241	Carvone
11	64.5	1289	Thymol
12	4.9	1414	(E)-Beta-Caryophyllene
13	1.9	1576	Caryophyllene oxide
	Total identified (%): 100		

2.2. Fish and acclimatization

The experiments were carried out at the Laboratory of Aquaculture and Fishing of Embrapa Amapá, Macapá, State of Amapá, Brazil. Juveniles of *C. macropomum* (±30 g) were obtained from commercial fish farming. The fish were acclimated during seven days in 500 L water tanks and fed with fish feed containing 32% crude protein (CP). In the tanks, the constant system of water renewal was maintained, and the water parameters were monitored: average temperature of 30.7 ± 0.2 °C, dissolved oxygen of 5.6 ± 0.4 mg/L, pH of 5.3 ± 0.2, ammonium of 0.4 ± 0.2 mg/L, alkalinity of 10.0 ± 0 mg/L and hardness of 10.0 ± 0 mg/L. Removal of organic material accumulated at the bottom of the tanks was daily performed.

2.3. In vitro assay with the EO of L. sidoides and monogenoideans from C. macropomum

To assess the exposure time and concentrations of the EO of *L. sidoides* that cause mortality in species of monogenoideans from the gills of 24*C. macropomum* (15.7 ± 1.2 cm and 78.2 ± 10.7 g), *in vitro* assays were performed. In order to do that, two control groups were used, one with tank water and the other with tank water + absolute ethyl alcohol, and six different concentrations of the EO of *L. sidoides* (10, 20 40, 80, 160 and 320 mg/L), using three replicates for each treatment according to the methodology used by Soares et al. (2016), and in environment temperature of 17–18 °C. This solvent was used in the ratio 1:10.

From the *in vitro* results, the concentrations used in the therapeutic baths with the EO of *L. sidoides* were determined after a previous test of fish tolerance.

2.4. In vivo assay with C. macropomum

Juveniles (13.2 ± 1.1 cm and 42.4 ± 10.1 g), naturally parasitized, were randomly distributed in 12 tanks of 100 L in an open water system during 48 h. For this assay, four treatments and three replicates were used with 20 fish for each replicate, and the fish were maintained in a static water system (average temperature of 29.3 ± 0.1 °C, dissolved oxygen of 6.3 ± 0.06 mg/L, pH of 5.2 ± 0.09, ammonium of 0.3 ± 0.12 mg/L, alkalinity of 10.0 ± 0 mg/L and hardness of 10.0 ± 0 mg/L). The treatments were as follow: control groups with tank water or with water + absolute ethyl alcohol (1:10), the solvent used for diluting the EO, 10 and 20 mg/L of the EO of *L. sidoides*. The fish submitted to the concentration of 10 mg/L and 20 mg/L were exposed to the *L. sidoides* EO bath during 60 and 15 min, respectively, while the fish from the control treatments remained in the bath for 60 min. After the bath time, the tank water was maintained in continuous flow and 10 fish from each replicate, from the different treatments, were used for gill collection, fixed in 5% formalin, for parasite quantification and identification. The parasites were prepared for identification under the previous recommendations (Eiras et al., 2006). After parasite quantification, prevalence and mean abundance of infection were calculated (Bush et al., 1997). The efficacy of each treatment was calculated according to Zhang et al. (2014).

The other group of fish was used for blood and histopathological analyses.

In vitro concentrations, previously assayed, have shown low fish tolerance to the EO of *L. sidoides*. Thus, only concentrations of 10 and 20 mg/L could be used in the therapeutic baths for tambaqui.

2.5. Analysis procedures of the C. macropomum blood parameters after exposure to the EO of L. sidoides

After the therapeutic baths of 15 and 60 min with 20 and 10 mg/L of the EO of *L. sidoides*, respectively, and 60 min for the control group, five fish from each replicate (15 fish per treatment) were used for blood testing. From each fish, a blood sample was collected by caudal vein puncture, using syringes containing EDTA (10%), which were divided in two aliquots. An aliquot was used for counting total erythrocytes number in a hemocytometer, determining the hematocrit by using the microhematocrit method and concentration of hemoglobin by using the cyanmethemoglobin method. With the data, Wintrobe's indices were calculated: mean corpuscular volume (MCV) and mean corpuscular hemoglobin concentration (MCHC). Blood smears were prepared and panchromatically stained with a combination of May Grünwald-Giemsa-Wright (Ranzani-Paiva et al., 2013) stains for the leucocyte differential counting up to 200 cells in each blood smear. The identification and classification of the leucocyte populations were made according to the recommendations of 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).

The second blood aliquot was centrifuged at 75 G in order to obtain plasma and for the analysis of glucose levels and total plasma proteins. Glucose concentration was determined by the enzymatic colorimetric method of glucose oxidase by using a commercial kit (Biotécnica, MG, Brazil). The concentration of total plasma proteins was determined by the biuret method by using a commercial kit (Biotécnica, MG, Brazil). Both biochemical analyses were read in a spectrophotometer.

2.6. Procedures of histopathological analyses of the C. macropomum gills after exposure to the EO of L. sidoides

After the therapeutic baths of 15 and 60 min with 20 and 10 mg/L of the EO of *L. sidoides*, respectively, and 60 min for the control groups, 6 fish from each treatment (2 fish per each replicate) were used for the collection of gill arches for histopathological analyses. After 24 h of these therapeutic baths, other 6 fish per treatment (2 fish per each replicate) were used for the collection of gill arches for histopathological analyses (recovery). These fish used in the recovery were kept in continuous flow water tanks and were fed.

The first right gill arch of each fish was collected and fixed in buffered formalin (10%) for histopathological analyses. The gill arches were dehydrated by a gradual series of ethanol and xylol and, then, embedded in paraffin to obtain successive series of microtome sections. The histological sections were stained with hematoxylin and eosin (HE) and analyzed in a common light microscope (Soares et al., 2016).

The histopathological analysis was performed semi qualitatively using the mean alteration value (MAV) (Schwaiger et al., 1997) and the histopathological alteration index (HAI) (Poleksic and Mitrovic-Tutundzic, 1994).

2.7. Statistical analyses

All data were previously assessed based on the assumptions of normality and homoscedasticity by using Shapiro-Wilk and Bartlett tests, respectively. For the data which did not follow any normal distribution pattern, Kruskal-Wallis analysis was used followed by the Tukey test ($p < 0.05$).

3. Results

3.1. In vitro antiparasitic action of the EO of L. sidoides

In the *in vitro* test, the EO of *L. sidoides* has shown 100% of anthelminthic activity against *A. spathulatus*, *N. janauachensis* and *M. boegeri* (Monogenoidea) from the gills of *C. macropomum* after 10 min of exposure in the concentration of 320 mg/L, and after 1 and 3 h of exposure in the concentrations of 160 and 80 mg/L, respectively, when total immobilization of parasites was observed. In the concentration of 40 mg/L, total immobilization of parasites occurred only in 6 h of *in vitro* exposure, while in lower concentrations, it occurred after 6 h of exposure. However, in the fish exposed only to water + alcohol (controls), the beginning of mortality of monogenoideans occurred in 3 h, while the fish exposed only to water from culture tanks (controls), it occurred in a 6 h of exposure. Total immobilization of parasites in both control groups occurred in more than 8 h of experiment (Fig. 1 and Table 2).

3.2. Antiparasitic action after C. macropomum exposure to the EO of L. sidoides

The fish gills exposed to the EO of *L. sidoides* were parasitized by *I. multifiliis*, *A. spalutathus*, *M. boegeri* and *N. janauachensis*, but there were no differences in terms of abundance and prevalence among the different treatments (Table 3).

In the therapeutic baths, the fish have shown the following behavior: normal behavior in the control with water, moderate excitement in the control with water + alcohol, lethargy in the concentration of 10 mg/L, and submersion in the bottom of the tanks in the concentration of 20 mg/L. When the continuous flow of water in the tanks was applied, for the elimination of the essential oil, the fish exposed to the EO of *L. sidoides* rapidly returned to normal swimming behavior, and there was no mortality during and after the experiment.

3.3. Effect of baths with EO of L. sidoides in the blood parameters

In *C. macropomum*, 60 min of baths with 10 mg/L and 15 min of baths with 20 mg/L of the EO of *L. sidoides* did not influence the glucose levels and total plasma proteins. The number of erythrocytes in the fish from the treatment with 20 mg/L was lower compared to the other treatments, but the hematocrit and hemoglobin of this treatment were similar to the treatments with water and 10 mg/L of the EO of *L. sidoides*. In relation to the MCV, the treatment with 20 mg/L of the EO of *L. sidoides* has shown higher values compared to the other treatments, but there was no difference between the values of MCHC (Table 4).

3.4. Histopathological effects in the C. macropomum gills exposed to the EO of L. sidoides

After the therapeutic baths, it was observed an increase in the MAV of the fish gills exposed to the EO of *L. sidoides* in both concentrations used, which were similar to the control with water + alcohol and different from the control with only water from the culture tanks. After 24 h of recovery, the gill MAV from the concentration of 20 mg/L was similar to the other treatments, but only the MAV from the concentration of 10 mg/L was different from the control group with water. A comparison between the two measurements of gill histopathological analyses showed a difference only between the MAV from the fish exposed to 20 mg/L and the control exposed only to water, after 24 h of recovery. But, there were similarities between the other treatments in the two periods analyzed (time zero and 24 h).

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

Time	Treatments	Mean of live parasites	Mortality (%)
	Water	25.3 ± 4.5	0
	Water + alcohol	22 ± 2.6	0
	10 mg/L	20 ± 0.0	0
	20 mg/L	20 ± 0.0	0
	40 mg/L	21.7 ± 2.9	0
	80 mg/L	21 ± 1.7	0
	160 mg/L	20.7 ± 12	0
0 h	320 mg/L	20.3 ± 06	0
	Water	25.3 ± 4.5	0
	Water + alcohol	22.0 ± 2.6	0
	10 mg/L	20.0 ± 0.0	0
	20 mg/L	20.0 ± 0.0	0
	40 mg/L	21.7 ± 2.9	0
	80 mg/L	19.3 ± 1.2	8.1
	160 mg/L	8.0 ± 7.5	61.3
10 min	320 mg/L	0.0 ± 0.0	100
	Water	25.3 ± 4.5	0
	Water + alcohol	22.0 ± 2.6	0
	10 mg/L	20.0 ± 0.0	0
	20 mg/L	19.7 ± 0.6	1.5
	40 mg/L	21.3 ± 3.2	1.8
	80 mg/L	19.3 ± 1.2	8.1
	160 mg/L	3.3 ± 3.5	84
20 min	320 mg/L	0.0 ± 0.0	100
	Water	25.3 ± 4.5	0
	Water + alcohol	22.0 ± 2.6	0
	10 mg/L	20.0 ± 0.0	0
	20 mg/L	19.7 ± 0.6	1.5
	40 mg/L	21.3 ± 3.2	1.8
	80 mg/L	19.0 ± 1.7	9.5
	160 mg/L	2.0 ± 3.5	90.3
30 min	320 mg/L	0.0 ± 0.0	100
	Water	25.3 ± 4.5	0
	Water + alcohol	22.0 ± 2.6	0
	10 mg/L	18.3 ± 1.5	8.5
	20 mg/L	19.7 ± 0.6	1.5
	40 mg/L	21.0 ± 2.6	3.2
	80 mg/L	16.0 ± 5.3	23.8
	160 mg/L	0.0 ± 0.0	100
1 h	320 mg/L	0.0 ± 0.0	100
	Water	25.0 ± 5.0	1.2
	Water + alcohol	20.0 ± 5.0	9.1
	10 mg/L	14.7 ± 6.8	26.5
	20 mg/L	17.3 ± 2.1	13.5
	40 mg/L	18.7 ± 3.1	13.8
	80 mg/L	0.0 ± 0.0	100
	160 mg/L	0.0 ± 0.0	100
3 h	320 mg/L	0.0 ± 0.0	100
	Water	4.7 ± 4.6	81.4
	Water + alcohol	12.0 ± 9.5	45.5
	10 mg/L	7.7 ± 4.9	61.5
	20 mg/L	3.0 ± 3.6	85
	40 mg/L	0.0 ± 0.0	100
	80 mg/L	0.0 ± 0.0	100
	160 mg/L	0.0 ± 0.0	100
6 h	320 mg/L	0.0 ± 0.0	100
	Water	1.3 ± 2.3	94.9
	Water + alcohol	1.3 ± 1.5	94.1
	10 mg/L	0.0 ± 0.0	100
	20 mg/L	0.0 ± 0.0	100
	40 mg/L	0.0 ± 0.0	100
	80 mg/L	0.0 ± 0.0	100
	160 mg/L	0.0 ± 0.0	100
8 h	320 mg/L	0.0 ± 0.0	100

As to the HAI of the gills collected after the therapeutic baths with 10 mg/L of the EO of *L. sidoides*, it was similar to the other treatments of the same period, while the concentration of 20 mg/L was higher to both control treatments. After 24 h of recovery, the HAI from the treatments with EO was higher in the control group with water and similar to the control group with water + alcohol. A comparison between the two measurements of collection showed both treatments with oil after the bath were similar to both treatments with the EO of *L. sidoides* and control with water + alcohol, after 24 h of recovery (Table 5).

The main histological changes observed in the fish gills exposed to the EO of *L. sidoides* were: hyperplasia and fusion of the lamellar epithelium, vasodilation, detachment of the lamellar epithelium and lamellar aneurism, epithelial breakdown with hemorrhage, congestion, edema and necrosis (Fig. 2A–H), as well as proliferation of the mucous cells and chloride cells and lamellar hypertrophy, changes which occurred less frequently.

4. Discussion

The essential oil of *L. sidoides* used in this study presented thymol and *p*-cymene (76.2%) as major components, similar to what was described by Hashimoto et al. (2016), who found 83.0% of these two components. However, Veras et al. (2014) reported a higher concentration of thymol *p*-cymene (90.2%) in the EO of *L. sidoides* analyzed. During the therapeutic baths of *C. macropomum* with the EO of *L. sidoides*, an anesthetic effect was observed, similar to the effect reported on *O. niloticus* when exposed to this essential oil (Hashimoto et al., 2016). Thus, only two low concentrations of this EO could be used in the therapeutic baths for *C. macropomum*. Chemical analysis of natural products is essential, once extracts of the essential oil from the same species of plant can be different due to factors such as location, planting conditions, cut, seasonality, *etc*. (Soares and Tavares-Dias, 2013). However, since there were no studies about the antiparasitic activity of *L. sidoides* for *C. macropomum*, this has been the first report on it.

The *in vitro* antiparasitic test has shown the concentrations of 40, 80, 160 and 320 mg/L of the EO of *L. sidoides* had 100% efficacy against *A. spatulathus*, *M. boegeri* and *N. janauachensis* from *C. macropomum*, in different time, but lower concentrations compared to these ones had a low efficacy. These results corroborate the findings of Hashimoto et al. (2016), who found 100% effectiveness in *L. sidoides* against monogenoideans *Cichlidogyrus tilapiae*, *Cichlidogyrus thurstonae*, *Cichlidogyrus halli* and *Scutogyrus longicornis* from *O. niloticus* gills in the concentrations of 160 and 320 mg/L, whose time of parasite sensitivity depended on the concentration of the EO of *L. sidoides*. The essential oil of *L. sidoides* used in these two studies had a similar composition of thymol and *p*-cymene, substances responsible for the antiparasitic action (Oliveira et al., 2009). *In vitro* studies of the EO of *Lippia alba*, whose major components found were carvone and limonene, have shown 100% of activity against monogenoideans from *C. macropomum* after exposure in concentrations of 160, 320, 640, 1280 and 2560 mg/L (Soares et al., 2016).

After *in vitro* antiparasitic tests, *C. macropomum* were submitted to sensitivity tests with several concentrations of the EO of *L. sidoides,* to determine fish tolerance. The results have indicated a low tolerance of the EO of *L. sidoides*. Thus, only 10–20 mg/L of this oil could only be used for the *C. macropomum* exposure. As a consequence, there was no difference in the prevalence and abundance of *I. multifiliis*, *A. spatulathus*, *M. boegeri* and *N. janauachensis*. Hashimoto et al. (2016), after the sensitivity test of *O. niloticus*, the concentration of 20 mg/L of the EO of *L. sidoides* was adopted for the therapeutic baths and a 33.3% effectiveness was found against *C. tilapiae*, *C. thurstonae*, *C. halli* and *S. longicornis*. However, in *C.*

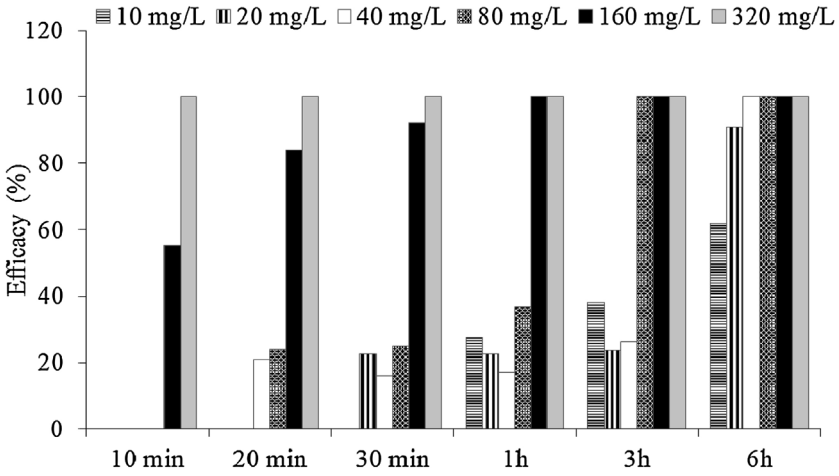

Fig. 1. *In vitro* efficacy of different concentrations of essential oil of *Lippia sidoides* against monogenoideans of *Colossoma macropomum*.

Table 3
Prevalence (P) and mean abundance (MA) of parasites in *Colossoma macropomum* gills exposure to essential oil of *Lippia sidoides*.

	Water (n = 30) 60 min		Water + Alcohol (n = 30) 60 min		10 mg/L (n = 30) 60 min		20 mg/L (n = 30) 15 min	
Parasite species	P (%)	MA	P (%)	MA	P (%)	MA	P (%)	MA
Ichthyophthirius multifiliis	100	639.1 ± 561.3^{a}	100	451.3 ± 410.7^{a}	93.3	410.5 ± 329.3^{a}	96.7	469.4 ± 320.7^{a}
Anacanthorus spatulathus	100	112.0 ± 83.0^{a}	100	83.4 ± 68.8^{a}	100	131.5 ± 112.9^{a}	100	105.3 ± 78.2^{a}
Mymarothecium boegeri	83.3	16.5 ± 17.2^{a}	96.7	18.2 ± 21.8^{a}	86.7	6.6 ± 6.9^{a}	86.7	11.9 ± 13.0^{a}
Notozothecium janauachensis	96.7	118.2 ± 91.3^{a}	100	107.0 ± 73.8^{a}	100	151.5 ± 126.3^{a}	100	119.6 ± 68.0^{a}

Different letters on the same line indicate differences according to the test Tukey ($p < 0.05$).

Table 4
Blood parameters of *Colossoma macropomum* submitted to baths with essential oil of *Lippia sidoides*.

Parameters	Water	Water + Alcohol	10 mg/L	20 mg/L
Glucose (g/dL)	67.4 ± 12.1^{a}	80.2 ± 21.0^{a}	63.7 ± 15.8^{a}	64.6 ± 6.0^{a}
Total protein (mg/dL)	2.5 ± 0.4^{a}	2.4 ± 0.4^{a}	2.4 ± 0.2^{a}	2.3 ± 0.4^{a}
RBC ($x10^{6}/\mu L$)	1.3 ± 0.3^{a}	1.2 ± 0.3^{a}	1.0 ± 0.15^{a}	0.6 ± 0.3^{b}
Hemoglobin (g/dL)	6.9 ± 0.9^{ab}	7.0 ± 0.8^{a}	5.8 ± 0.6^{c}	6.2 ± 0.7^{bc}
Hematocrit (%)	30.1 ± 4.9^{ab}	30.2 ± 1.7^{a}	26.5 ± 2.0^{c}	26.9 ± 2.8^{bc}
MCV (fL)	278.6 ± 136.4^{a}	266.4 ± 56.3^{a}	267.0 ± 41.8^{a}	546.4 ± 355.8^{b}
MCHC (g/dL)	23.2 ± 3.5^{a}	23.3 ± 2.3^{a}	21.8 ± 2.2^{a}	23.2 ± 2.2^{a}

Data are expressed as mean ± standard deviation. Different letters in the same line indicate differences according to the Tukey test ($p < 0.05$). RBC: Red blood cells, MCV: Mean corpuscular volume, MCHC: Mean corpuscular hemoglobin concentration.

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

After 30 min therapeutic bath				
Treatments	N	MAV	HAI	Severity of the lesions according to the HAI
Water	6	1.0 ± 0.0^{aAB}	10.0 ± 6.3^{aB}	Normal functioning of the gills
Water + Alcohol	6	1.8 ± 0.4^{abAB}	86.0 ± 54.8^{cC}	Severe alterations to the gills
10 mg/L 60 min	6	2.0 ± 0.0^{bAB}	82.8 ± 53.1^{abcA}	Severe alterations to the gills
20 mg/L 15 min	6	2.3 ± 0.5^{bB}	119.5 ± 5.4^{bA}	Irreparable damage to the gills
After 24 h of recovery subsequent to therapeutic bath				
Water	6	1.0 ± 0.0^{bA}	8.5 ± 6.1^{aB}	Normal functioning of the gills
Water + Alcohol	6	1.7 ± 0.5^{abAB}	90.8 ± 54.2^{abA}	Severe alterations to the gills
10 mg/L 60 min	6	1.8 ± 0.4^{aAB}	94.0 ± 40.0^{bAC}	Severe alterations to the gills
20 mg/L 15 min	6	1.3 ± 0.5^{abAB}	96.8 ± 44.5^{bAC}	Severe 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 ($p < 0.05$).

macropomum, 100 and 150 mg/L of the EO of *L. alba*, during 30 min, showed a efficacy of 40.7% and 50.3%, respectively, in the treatment against *I. multifiliis* (Soares et al., 2016). Usually, an efficacy ≥50% in reduction of parasitic intensity is considered acceptable (Sommerville et al., 2016); however, we accept only an efficacy ≥70%.

Fish gills are organs responsible for the breathing, osmoregulation and excretion in fish (Fiuza et al., 2011; Kumar et al., 2010),

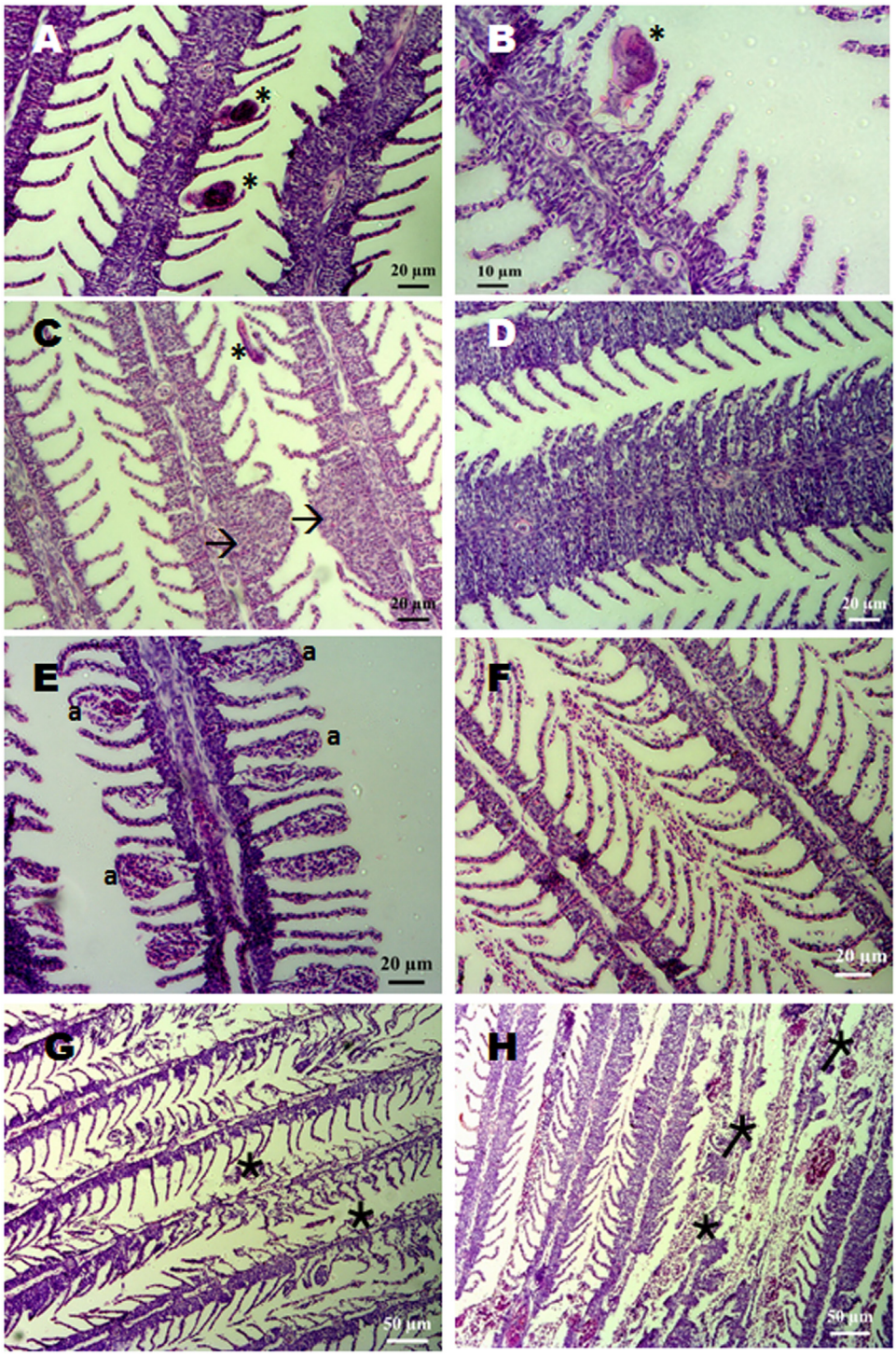

Fig. 2. A–H A Histological alterations in the gills of *Colossoma macropomum* exposed to two concentrations of the essential oil of *Lippia sidoides*, 10 and 20 mg/L, during 60 and 15 min, respectively. (A) Monogenoidean (*) in the fish gill from the control treatment with water. (B) Monogenoidean (*), in the gill from the control treatment with water. (C) Monogenoidean (*) and regions with epithelium hyperplasia with lamellar fusion (arrows), in the gill from the control treatment with water. (D) Epithelium hyperplasia generalized with regions of lamellar fusion and detachment of the lamellar epithelium in the gills of fish exposed to 20 mg/L. (E) Aneurisms (a) in the gills of control fish exposed to water + alcohol. (F) Epithelial breakdown with lamellar hemorrhage in the gills of fish exposed to 10 mg/L. (G) Lamellar necrosis (stars) with epithelial breakdown and hemorrhage in the gills of fish exposed to 20 mg/L. (H) Lamellar necrosis (stars), with regions of lamellar hyperplasia and epithelial breakdown with hemorrhage in the gills of fish exposed to 20 mg/L.

thus, they respond to exposure of different natural compounds. In *C. macropomum*, the results of the histological analysis have shown the therapeutic baths from the treatments with water + alcohol, 10 and concentration of 20 mg/L of the EO of *L. sidoides* caused severe alterations in the gills, such as hyperplasia and fusion of the lamellar gill epithelium, vasodilation, detachment of the lamellar epithelium and lamellar aneurism, epithelial breakdown with hemorrhage, congestion, edema and necrosis. Similar studies with therapeutic baths with concentrations of 100 and 150 mg/L of *L. alba* have also observed severe alterations in the fish gills, caused

by the alcohol of the EO (Soares et al., 2016). Veras et al. (2014) reported thymol and *p*-cymene to possess topic anti-inflammatory activity, but prolonged use of this EO causes inflammatory effect. Oliveira et al. (2014), evaluating the cutaneous inflammatory action of the EO of *L. sidoides* in mice, observed this oil caused an increase in skin thickness, edema and cutaneous erythema in various levels in mice, and its effect being dose-dependent, but there was no delay in wound healing. In addition, there was a cytotoxic effect on monocytic cells exposed to 100 μg/mL of the EO of *L. sidoides* and only 57.8% of the cells were viable after exposure (Rondon et al., 2012).

The therapeutic baths with concentrations of 10 and 20 mg/L of the EO of *L. sidoides* did not influence the glucose levels and total plasma proteins, but the MCV increased because of a decrease in the number of erythrocytes, hematocrit and hemoglobin in the fish exposed to 20 mg/L. Such decrease in the number of erythrocytes, hemoglobin and hematocrit can be related to hemorrhagic gill lesions, once in the treatments with the EO of *L. sidoides* the HAI varied, with severe injury in the gills to and irreparable injury. Similar results in blood parameters and gill injuries also occurred in *C. macropomum* exposed to concentrations of 100 and 150 mg/L of the EO of *L. alba*, during therapeutic baths of 30 min (Soares et al., 2016). Therefore, as the EOs of *Lippia* spp. can cause gill lesions and alterations in blood parameters in fish, they should not be used sparingly in therapeutic baths.

The control group using water + alcohol differed from the control group using tank water only as to gill lesions, because the alcohol used as a solvent of the essential oil can influence in the results. *Colossoma macropomum* exposed to water + alcohol has shown severe alterations in the gills, while the fish exposed to water only did not present functional alterations in the gills. Other studies have also shown the influence of different solvents, such as alcohol (Soares et al., 2016), DMSO (Hashimoto et al., 2016) and Tween-80 (Steverding et al., 2005) in experimental studies about antiparasitic activity using plant extracts. Therefore, such solvents can potentialize the action of the essential oils used in therapeutic baths.

5. Conclusions

In vitro efficacy of the essential oil of *L. sidoides* was dose-dependent and even the low concentrations used in the therapeutic baths showed toxicity, causing histopathological alterations. In addition to that, the solvent used in the essential oil causes damage to the fish gills, resulting in blood and histological alterations, without satisfactory tissue recovery in 24 h. Therefore, the concentrations of the essential oil of *L. sidoides* and the exposure time used in this study cannot be indicated in the antiparasitic treatment against *I. multifiliis* and monogenoideans from *C. macropomum* yet. However, herbal therapy is an alternative therapeutic resource in fish farming, but studies to evaluate the bioactivity of the major compounds of the essential oil of *L. sidoides* should be conducted, besides testing more efficient forms of administration in fish.

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References

Becker, A.G., Parodi, T.V., Heldwein, C.G., Zeppenfeld, C.C., Heinzmann, B.M., Baldisserotto, B., 2012. Transportation of silver catfish, *Rhamdia quelen*, in water with eugenol and the essential oil of *Lippia alba*. Fish Physiol. Biochem. 38, 789–796.

Boijink, C.L., Miranda, W.S.C., Chagas, E.C., Dairiki, J.K., Inoue, L.A.K.A., 2015. Anthelmintic activity of eugenol in tambaquis with monogenean gill infection. Aquaculture 438, 138–140.

Bush, A.O., Lafferty, K.D., Lotz, J.M., Shostack, A.W., 1997. Parasitology meets ecology on its own terms: Margolis et al. revisited. J. Parasitol. 83 (4), 575–583.

Camurça-Vasconcelos, A.L.F., Bevilaqua, C.M.L., Morais, S.M., Maciel, M.V., Costa, C.T.C., Macedo, I.T.F., Oliveira, L.M.B., 2007. Anthelmintic activity of *Croton zehntneri* and *Lippia sidoides essential* oils. Vet. Parasitol. 148, 288–294.

Cunha, M.A., Barros, F.M.C., Garcia, L.O., Veeck, A.P.L., Heinzmann, B.M., Loro, V.L., Emanuelli, T., Baldisserotto, B., 2010. Essential oil of *Lippia alba*: a new anesthetic for silver catfish, *Rhamdia quelen*. Aquaculture 306, 403–406.

Dias, M.K.R., Neves, L.R., Marinho, R.G.B., Tavares-Dias, M., 2015. Parasitic infections in tambaqui from eight fish farms in Northern Brazil. Arq. Brasil. Med. Vet. Zoot. 67 (4), 1070–1076.

Eiras, J.C., Takemoto, R.M., Pavanelli, G.C., 2006. Métodos de estudos e técnicas laboratoriais em parasitologia de peixes. Editora UEM, Maringá, 173 pp.

Fabri, R.L., Nogueira, M.S., Moreira, J.R., Bouzada, M.L.M., Scio, E., 2011. Identification of antioxidant and antimicrobial compounds of *Lippia* species by bioautography. J. Med. Food 14 (7/8), 840–846.

Farias-Júnior, P.A., Rios, M.C., Moura, T.A., Almeida, R.P., Alves, P.B., Blank, A.F., Fernandes, R.P.M., Scher, R., 2012. Leishmanicidal activity of carvacrol-rich essential oil from *Lippia sidoides* Cham. Biol. Res. 45, 399–402.

Fernandes, L.P., Candido, R.C., Oliveira, W., 2012. Spray drying microencapsulation of *Lippia sidoides* extracts in carbohydrate blends. Food Bioprod. Proc. 90, 425–432.

Fiuza, T.S., Silva, P.C., Paula, J.R., Tresvenzol, L.M.F., Souto, M.E.D., Sabóia-Morais, S.M.T., 2011. Análise tecidual e celular das brânquias de *Oreochromis niloticus* L. tratadas com extrato etanólico bruto e frações das folhas da pitanga (*Eugenia uniflora* L.) -Myrtaceae. Rev. Brasil Plantas Med. 13 (4), 389–395.

Fontenelle, R.O.S., Morais, S.M., Brito, E.H.S., Kerntopf, M.R., Brilhante, R.S.N., Cordeiro, R.A., Tomé, A.R., Queiroz, M.G.R., Nascimento, N.R.F., Sidrim, J.J.C., Rocha, M.F.G., 2007. Chemical composition, toxicological aspects and antifungal activity of essential oil from *Lippia sidoides* Cham. J. Antimicrob. Chemother. 59, 934–940.

Funari, C.S.F., Gullo, P., Napolitano, A., Carneiro, R.L., Mendes-Giannini, M.J.S., Fusco-Almeida, A.M., Piacente, S., Pizza, C., Silva, D.H.S., 2012. Chemical and antifungal investigations of six *Lippia* species (Verbenaceae) from Brazil. Food Chem. 135, 2086–2094.

Gomes, G.A., Monteiro, C.M.O., Senra, T.O.S., Zeringota, V., Calmon, F., Matos, R.S., Daemon, E., Gois, R.W.S., Santiago, G.M.P., Carvalho, M.G., 2012. Chemical composition and acaricidal activity of essential oil from *Lippia sidoides* on larvae of *Dermacentor nitens* (Acari: Ixodidae) and larvae and engorged females of *Rhipicephalus microplus* (Acari: Ixodidae). Parasitol. Res. 111, 2423–2430.

Gomes, G.A., Monteiro, C.M.O., Julião, L.S., Maturano, R., Senra, T.O.S., Zeringóta, V., Calmon, F., Matos, R.S., Daemon, E., Carvalho, M.G., 2014. Acaricidal activity of essential oil from *Lippia sidoides* on unengorged larvae and nymphs of *Rhipicephalus sanguineus* (Acari: Ixodidae) and *Amblyomma cajennense* (Acari: Ixodidae). Exp. Parasitol. 137, 41–45.

Hashimoto, G.S.O., Neto, F.M., Ruiz, M.L., Achille, M., Chagas, E.C., Chaves, F.C.M., Martins, M.L., 2016. Essential oils of *Lippia sidoides* and *Mentha piperita* against monogenean parasites and their influence on the hematology of Nile tilapia. Aquaculture 450, 182–186.

Kumar, A., Prasad, M.R., Srivastava, K., Tripathi, S., Srivastav, A.K., 2010. Branchial histopathological study of catfish *Heteropneustes fossilis* following exposure to purified neem extract, azadirachtin. World J. Zool. 5 (4), 239–243.

Lobo, P.L.D., Fonteles, C.S.R., Marques, L.A.R.V., Jamacaru, F.V.F., Fonseca, S.G.C., Carvalho, C.B.M., Moraes, M.E.A., 2014. The efficacy of three formulations of *Lippia sidoides* Cham. essential oil in the reduction of salivary *Streptococcus mutans* in children withcaries: a randomized double-blind, controlled study. Phytomedicine 21, 1043–1047.

Martins, M.L., Moraes, F.R., Fujimoto, R.Y., Nomura, D.T., Fenerick Jr., J., 2002. Respostas do híbrido tambacu (*Piaractus mesopotamicus* Holmberg, 1887 macho X *Colossoma macropomum* Cuvier, 1818 fêmea) a estímulos simples ou consecutivos de captura. Bol. Inst. Pesca 28 (2), 195–204.

Oliveira, V.C.S., Moura, D.M.S., Lopes, J.A.D., Andrade, P.P., Silva, N.H., Figueiredo, C.B.Q., 2009. Effects of essential oils from *Cymbopogon citratus* (DC) Stapf., *Lippia sidoides* Cham., and *Ocimum gratissimum* L. on growth and ultrastructure of *Leishmania chagasi* promastigotes. Parasitol. Res. 104, 1053–1059.

Oliveira, M.L.M., Bezerra, B.M.O., Leite, L.O., Girão, V.C.C., Nunes-Pinheiro, D.C.S., 2014. Topical continuous use of *Lippia sidoides* Cham. essential oil induces cutaneous inflammatory response, but does not delay wound healing process. J. Ethnopharmacol. 153, 283–289.

Poleksic, V., Mitrovic-Tutundzic, V., 1994. Fish gills as a monitor of sublethal and chronic effects of pollution. In: Muller, R., Lloyd, R. (Eds.), Sublethal and Chronic Effects of Pollutants on Freshwater Fish. Fishing News Books, Oxford, pp. 339–352.

Ranzani-Paiva, M.J.T., Padua, S.B., Tavares-Dias, M., Egami, M.I., 2013. Métodos para análises hematológicas em peixes. EDUEM, Maringá, 135 pp.

Rondon, F.C.M., Bevilaqua, C.M.L., Accioly, M.P., Morais, S.M., Andrade-Júnior, H.F., Carvalho, C.A., Lima, J.C., Magalhães, H.C.R., 2012. *In vitro* efficacy of *Coriandrum sativum*, *Lippia sidoides* and *Copaifera reticulata* against *Leishmania chagasi*. Rev. Brasil. Parasitol. Vet. 21 (3), 185–191.

Schwaiger, J., Wanke, R., Adam, S., Pawert, M., Honnen, W., Triebskorn, R., 1997. The use of histopathological indicators to evaluate contaminant-related stress in fish. J. Aquat. Ecosyst. Stress Recov. 6, 75–86.

Silva, M.I., Gondim, A.P.S., Nunes, I.F.S., Sousa, F.C.F., 2006. Utilização de fitoterápicos nas unidades básicas de atenção à saúde da família no município de Maracanaú (CE). Rev. Bras. Farmacogn. 16 (4), 455–462.

Soares, B.V., Tavares-Dias, M., 2013. Espécies de *Lippia* (Verbenaceae), seu potencial bioativo e importância na medicina veterinária e aquicultura. Biota Amaz. 3 (1), 109–123.

Soares, B.V., Neves, L.R., Oliveira, M.S.B., Chaves, F.C.M., Dias, M.K.R., Chagas, E.C., Tavares-Dias, M., 2016. Antiparasitic activity of the essential oil of *Lippia alba* on ectoparasites of *Colossoma macropomum* (tambaqui) and its physiological and histopathological effects. Aquaculture 452, 107–114.

Sommerville, C., Endris, R., Bell, T.A., Ogawa, K., Buchmann, K., Sweeney, D., 2016. World association for the advancement of veterinary parasitology (WAAVP) guideline for testing the efficacy of ectoparasiticides for fish. Vet. Parasitol. 219, 84–99.

Steverding, D., Morgan, E., Tkacynski Walder, F., Tinsley, R., 2005. Effect of Australian tea tree oil on *Gyrodactylus* spp.: infection of the three-spined stickleback *Gasterosteus aculeatus*. Dis. Aquat. Org. 66, 29–32.

Tavares-Dias, M., Sandrin, E.F.S., Campos Filho, E., 1999. Características hematológicas do tambaqui *Colossoma macropomum* Cuvier (Osteichthyes: Characidae) em sistema de monocultivo intensivo. II. Leucócitos. Rev. Brasil. Zool. 16, 175–184.

Veras, H.N.H., Rodrigues, F.F.G., Botelho, M.A., Menezes, I.R.A., Coutinho, H.D.M., Costa da, L.G.M., 2014. Antimicrobial Effect of Lippia sidoides and thymol on *Enterococcus faecalis* biofilm of the bacterium isolated from Root Canals. Sci. World J., http://dx.doi.org/10.1155/2014/471580.

Zhang, X.P., Li, W.X., Ai, T.S., Zou, H., Wu, S.G., Wang, G.T., 2014. The efficacy of four common anthelmintic drugs and traditional Chinese medicinal plant extracts to control *Dactylogyrus vastator* (Monogenea). Aquaculture 420–421, 302–307.