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Copaifera duckei oleoresin as a novel alternative for treatment of monogenean infections in pacu *Piaractus mesopotamicus*

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ABSTRACT

Monogeneans are major parasites of fish and cause large economic losses in aquaculture. Treatment for this parasitic infection is done with products that are mostly toxic to fish and the environment. Essential oils (EOs) of *Melaleuca alternifolia* and *Mentha piperita* and the oleoresin (OR) of *Copaifera duckei* were tested for their *in vitro* anthelmintic activity against the monogenean parasites (*Anacanthorus penilabiatus* and *Mymarothecium viatorum*) of pacu *Piaractus mesopotamicus*. Naturally infected gills were bathed with the herbal solutions (100, 200, 400, 800, and 1600 mg/L) and monitored every 15 min for 4 h. Because of its greater efficacy *in vitro* ($p < 0.05$) compared to the other herbal medicines, *C. duckei* OR was selected for *in vivo* testing. The *in vivo* treatment consisted of 10 and 50 mg/L baths of *C. duckei* OR for 10 min. Parasitological, hematological, and histological analyses were conducted post-bath and seven days after treatment. Parasite loads decreased by approximately 45% in fish treated with 50 mg/L of *C. duckei* OR. No hematological changes caused by treatment with *C. duckei* OR at 10 and 50 mg/L were observed. Histology revealed branchial and hepatic alterations in fish from all groups, whereas spleen and kidney tissues were not affected. Histopathological alterations observed in all fish were due to parasitism or nutritional/farming conditions. Hematological and histological results showed that short baths were safe for fish. Based on the strong anthelmintic activity observed, *C. duckei* OR offers a promising alternative treatment against monogenean parasites.

1. Introduction

Monogenea are small parasitic flatworms (Platyhelminthes) of fish that in high numbers cause several epithelial and hematological alterations (Tavares-Dias et al., 2008; Araújo et al., 2009), predisposing the host to secondary infections and often leading to significant mortality (Huang et al., 2013; Ogawa, 2014). The use of therapeutic agents is necessary to minimize the economic losses caused by parasitic outbreaks in aquaculture (Valladão et al., 2015). The chemotherapeutic agents most used against monogeneans are hydrogen peroxide (Bowker et al., 2012; Benavides-González et al., 2015; Hirazawa et al., 2016), potassium permanganate (Umeda et al., 2006), formalin (Fajer-Ávila et al., 2003; Pahor-Filho et al., 2012), and praziquantel (Sharp et al., 2004; Sitjà-Bobadilla et al., 2006; Forwood et al., 2013). However, due to their negative effects on the environment (Rico and Van den Brink, 2014) and in animals and humans (Sapkota et al., 2008), the use of some of these molecules is discouraged. Phytotherapy is an alternative treatment that has shown promising antiparasitic effects, especially against monogeneans (Reverter et al., 2014; Valladão et al., 2015). Currently, there is no alternative treatments for the control of monogeneans infections in pacu *Piaractus mesopotamicus* (Holmberg, 1887) and *Copaifera duckei* OR had not yet been tested in fish for any given purpose.

Phytotherapeutic agents extracted from *Melaleuca alternifolia*, *Mentha piperita*, and *Copaifera* L. have been used for different therapeutic applications. *Melaleuca alternifolia* is an Australian native plant with broad antiseptic, antimicrobial, anti-inflammatory, and antiparasitic activity (Thomsen et al., 2011). Recent studies have shown that the

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essential oil (EO) of *M. alternifolia* has been successfully used against the platyhelminths *Gyrodactylus* spp. (Steverding et al., 2005) and the protozoan *Ichthyophthirius multifiliis* (Valladão et al., 2016a).

Mentha piperita has a broad spectrum antiseptic, antifungal, antispasmodic, and immunostimulant activity (McKay and Blumberg, 2006). Recently, the EO of this plant has shown promising potential against several platyhelminths such as *Dawestrema* spp. in *Arapaima gigas* (Schinz, 1822) (Malheiros et al., 2016), *Cichlidogyrus tilapiae*, *Cichlidogyrus thurstonae*, *Cichlidogyrus halli*, and *Scutogyrus longicornis* in *Oreochromis niloticus* (Linnaeus, 1758) (Hashimoto et al., 2016), and against the protozoan *I. multifiliis* in pacu *P. mesopotamicus* (Valladão et al., 2016a).

Trees in the genus *Copaifera* are Amazon plants known for their antibacterial (Bardají et al., 2016), anthelmintic (Gilbert et al., 1972), antiprotozoal (Santos et al., 2008; Dorneles et al., 2013; Izumi et al., 2013), and antioxidant (Paiva et al., 2004) properties. Plants in this genus are usually rich in oleoresin (OR), which is extracted directly from the tree trunk (Veiga and Pinto, 2002), but little is known about the use of OR in aquaculture. Some studies have described the effects of *Copaifera duckei* OR against major parasitic protozoans of mammals such as *Leishmania amazonensis* (Santos et al., 2008), *Trypanosoma cruzi* (Izumi et al., 2013), and *Trypanosoma evansi* (Dorneles et al., 2013).

Pacu *P. mesopotamicus* is a South American fish of great economic importance because it is one of the main fish species cultured in continental waters (Valladão et al., 2016b). Similar to several fish species cultivated worldwide, pacu is highly affected by monogenean infections and thus constitutes a good biological model for studying the *in vitro* and *in vivo* effect of herbal medicines on monogenean parasites.

This study evaluated the *in vitro* activity of *M. alternifolia*, *M. piperita*, and *C. duckei* against two monogenean species and the *in vivo* effect of *C. duckei* oleoresin on the parasites and on host health.

2. Materials and methods

2.1. Essential oils and chromatographic profile

The EOs of *M. alternifolia* and *M. piperita* were purchased from Phytoterapica® (São Paulo, SP, Brazil). The oleoresin of *C. duckei* was collected in a sustainable manner from a tree trunk in Pará, northern Brazil (1.9981° S, 54.9306° W). Extraction of plant material was authorized by the Chico Mendes Institute for Biodiversity Conservation (ICMBio) and the Brazilian Ministry of Environment (MMA), under number 35143–1. Two herbarium specimens were deposited in the ICMBio herbarium under code NID 03/2013.

The chemical characterization of the EOs was done using gas chromatography–mass spectrometry (GC–MS) as previously described in Baldin et al. (2013). The oleoresin was initially analyzed by high-performance liquid chromatography–mass spectrometry (HPLC-MS). Next, hydrodistillation was performed to obtain a volatile fraction using GC–MS as described by Santiago et al. (2015) and Bardají et al. (2016).

The stock solutions of each phytotherapeutic agent were prepared with 0.1 g of the plant extract solubilized in 2 mL of dimethyl sulfoxide (DMSO; Sigma-Aldrich®, St. Louis, MI, USA).

2.2. Host and parasites

Naturally infected pacu juveniles (weight: 75.25 ± 14.47 g and length: 16.29 ± 1.15 cm; mean ± SD) were obtained from a commercial fish farm. Fish were kept in 500-L tanks with constant aeration and continuous water flow. Water quality parameters were measured using an YSI Professional Plus® multiparameter probe (YSI Inc., Yellow Springs, OH, USA). Water quality parameters were not significantly different across treatments: water temperature (31.02 ± 0.15 °C), dissolved oxygen (6.26 ± 0.13 mg/L), pH (8.1 ± 0.05), and conductivity (157.32 ± 0.29 μS/cm).

Monogenean parasites were scrapped from the gills and counted under a stereomicroscope. The parasites were collected and preserved in 70% alcohol for identification using fish parasite identification keys (Thatcher, 2006). The monogenean species were identified as *Anacanthorus penilabiatus* (Boeger et al., 1995) and *Mymarothecium viatorum* (Boeger et al., 2002).

The experimental procedures were approved by the Ethics and Animal Welfare Committee (CEUA) of the School of Agricultural Sciences and Veterinary Medicine at São Paulo State University (UNESP), Jaboticabal, SP, Brazil, under protocol number 12291/15.

2.3. In vitro assay

The *in vitro* study was conducted to evaluate and compare the anthelmintic activity of the EOs of *M. alternifolia* and *M. piperita* and the OR of *C. duckei* on monogenean parasites.

Gill samples were placed in Petri dishes and filled with 20 mL of water from the fish tank. Five concentrations of each herbal stock solution (100, 200, 400, 800, and 1600 mg/L) were tested. Two control groups were tested: DMSO (solubilization solution) and water only. Parasites were observed every 15 min for 4 h. Mortality was recorded by verification of absence of movement when parasites were stimulated with a needle.

2.4. In vivo assay

Based on the results of the *in vitro* study, the OR of *C. duckei* was chosen for the *in vivo* test. Ninety-six naturally infected pacu juveniles were assigned to four treatments: fish not exposed to any substance (control), fish exposed to DMSO solution, and fish exposed to 10 and 50 mg/L of *C. duckei* OR. These concentrations were chosen from preliminary tests that showed no change in the fish behavior.

The experiment consisted of one short 10-min bath in plastic buckets containing 10 L of water. Fish were bathed two at a time and, at the end of each bath, one fish was collected for parasitological, histological, and hematological analyses, whereas the other fish returned to the tank (500 L) where it remained for seven days. Each treatment consisted of 12 replicates. Fish were anesthetized with benzocaine (0.1 g/L) and euthanized for tissue sampling.

2.4.1. Parasitological analysis

All branchial arches on one side of each fish were harvested and placed into Petri dishes for parasites counting under a stereomicroscope, whereas branchial arches on the other side were collected for histology. The result was doubled to estimate the total amount of parasite per fish. The prevalence and mean intensity of parasites was calculated according to Bush et al. (1997).

2.4.2. Effect of C. duckei OR on the host

After anesthesia, fish blood was taken at days 0 and 7 after treatment. Blood count (red blood cells (RBC), hematocrit, and hemoglobin) was performed using the entire blood sample diluted in 10 μL of heparin 5000 IU/mL. For RBC (%), 10 μL of blood was diluted in 2 mL of formalin-citrate solution for subsequent counting using a Neubauer chamber (Hesser, 1960). Hemoglobin (g dL^{-1}) was measured using a hemoglobin assay kit (Labtest® kit #43; Labtest, Lagoa Santa, MG, Brazil). Hematocrit percentage and RBC parameters mean corpuscular volume (MCV, in fL), mean corpuscular hemoglobin (MCH, pg), and mean corpuscular hemoglobin concentration (MCHC, %) were measured according to Ranzani-Paiva et al. (2013). Blood smears were stained with May-Grünwald Giemsa-Wright (MGGW) and used for total counts and differential leukocyte counts according to Ranzani-Paiva et al. (2013).

The serum was obtained by centrifugation (3000 rpm × 10 min at 4 °C) and stored (−20 °C) until use. Total protein and albumin concentrations (g dL^{-1}) were determined by spectrophotometry with 20 μL and 10 μL of serum using Labtest® kits #99 and #19, respectively.

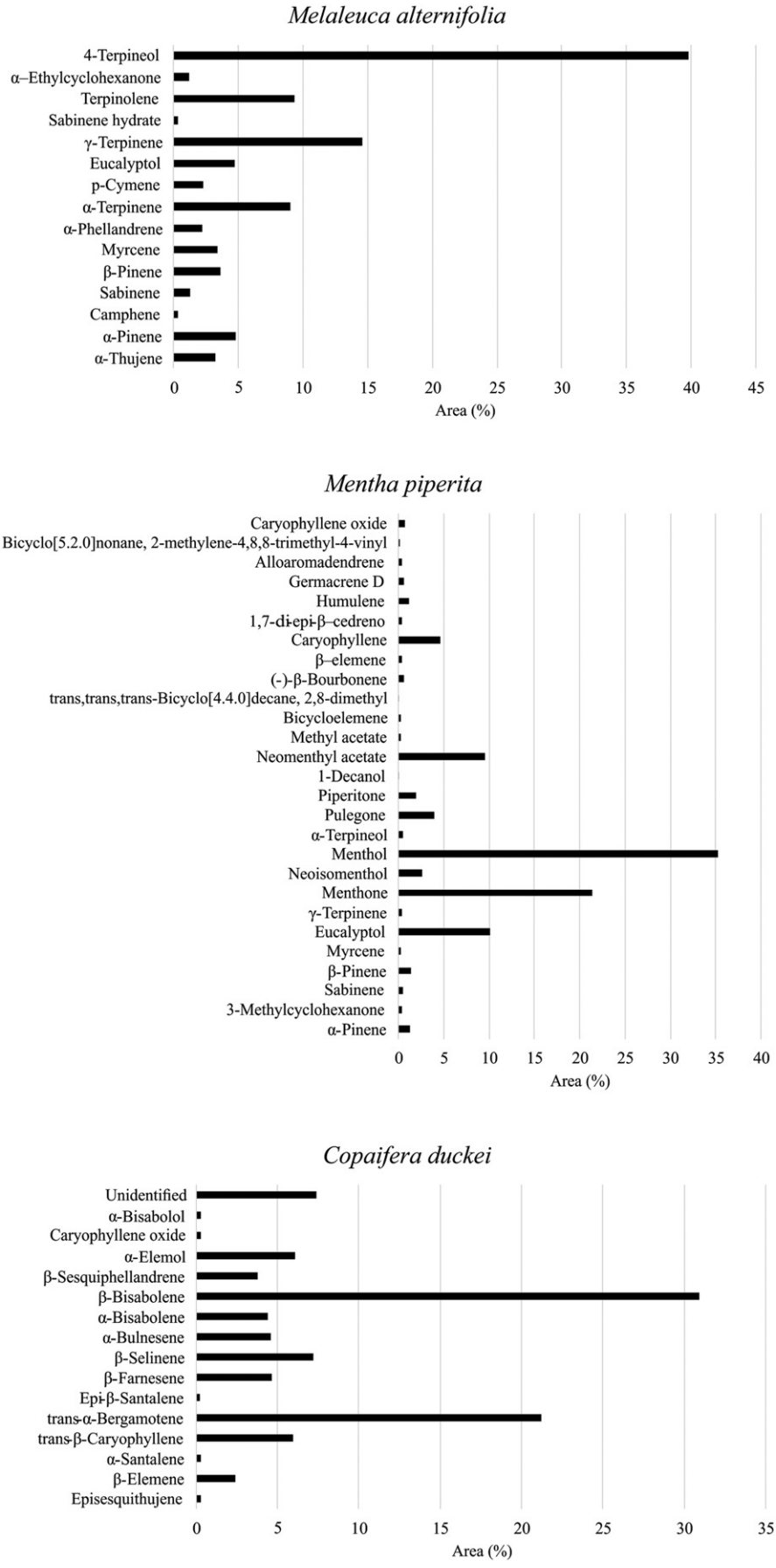

Fig. 1. Chemical composition of essential oils of *Melaleuca alternifolia* and *Mentha piperita* and *Copaifera duckei* oleoresin.

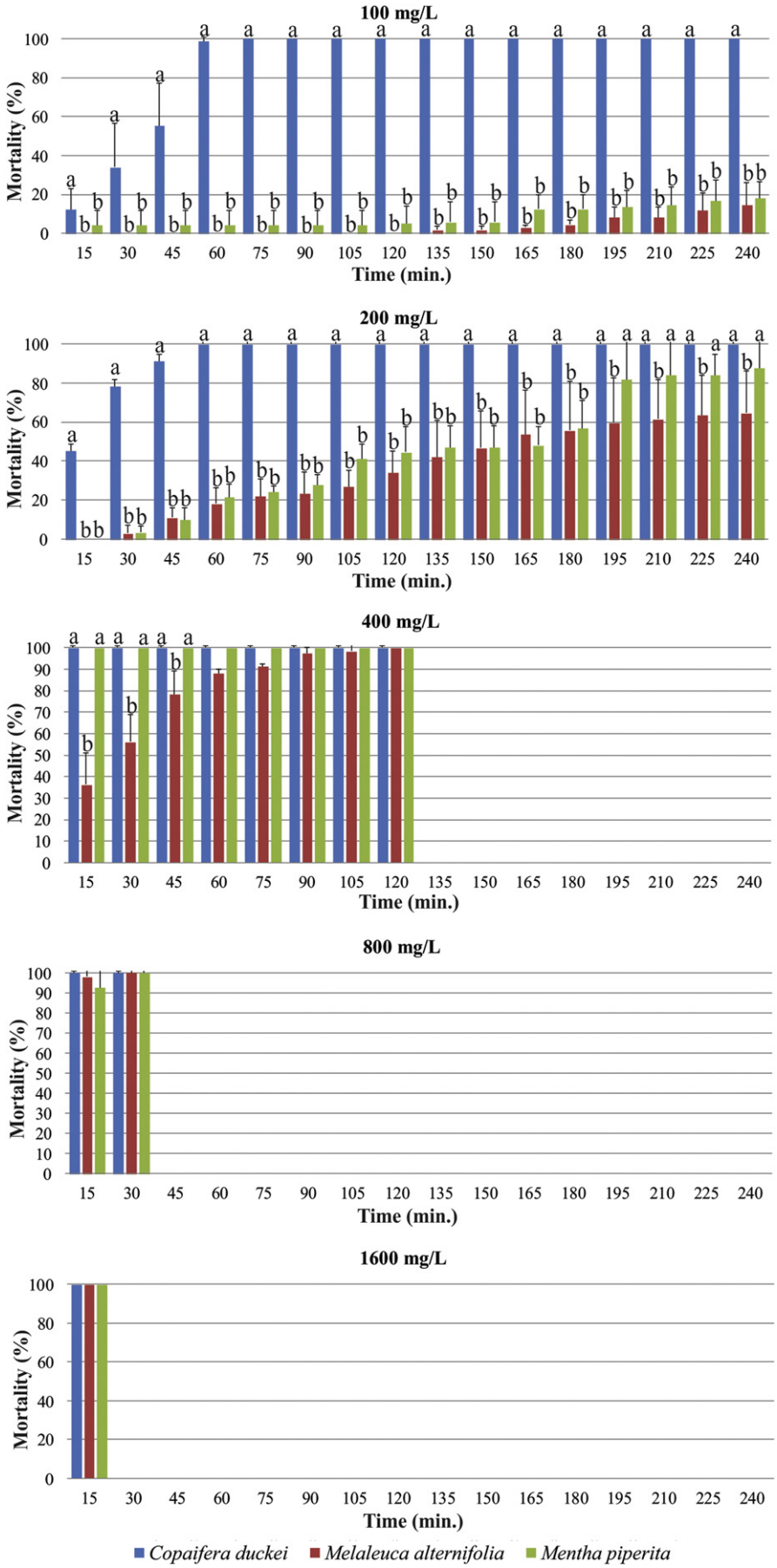

Fig. 2. *In vitro* mortality (%) of monogenean parasites exposed to essential oils of *Melaleuca alternifolia* and *Mentha piperita* and *Copaifera duckei* oleoresin at five concentrations for 240 min.

Table 1
Prevalence (%) and mean intensity (±standard deviation) of monogenean parasites in *Piaractus mesopotamicus* bathed with *Copaifera duckei oleoresin*.

	Parasitism	Control	DMSO	*C. duckei* 10 mg/L	*C. duckei* 50 mg/L	*p*-Value
0 h post-bath	Prevalence (%)	100	100	100	100	–
	Mean intensity	121.17 ± 55.52a	94.33 ± 40.29ab	83.17 ± 35.38bc	54.33 ± 32.25c	<0.001
7 day post-bath	Prevalence (%)	100	100	100	100	–
	Mean intensity	21.64 ± 18.17*	31.0 ± 20.58*	32.67 ± 21.9*	30.5 ± 27.1*	0.347

Means followed by different letters in the same row indicate significant differences by the Tukey's test ($p < 0.05$).
* Indicates significant differences between times.

Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels (IU/L) were measured by ultraviolet-visible (UV–Vis) spectrophotometry with 50 μL of serum using Labtest® kits #108 and #109, respectively, according to the manufacturer's recommendations. Blood glucose (mg dL^{-1}) was measured using an Injex Sens II® glucose meter (Injex, Ourinhos, SP, Brazil).

2.5. Histological analysis

Immediately after the bath, the liver, spleen, kidney, and gills were collected and fixed in 10% buffered formalin. After 24 h, the tissues were transferred to 70% alcohol and processed for paraffin embedding. Histological sections (5–6 μm) were stained with hematoxylin-eosin (H&E) and gills were stained with periodic acid-Schiff. The alterations were analyzed with a Nikon Eclipse E200® optical microscope (Nikon, Tokyo, Japan) and images were captured with a Moticam 2300® camera (Motic, Hong Kong).

2.6. Statistical analysis

Data are expressed as mean ± standard deviation. The parasite mortality from each phytotherapeutic agent tested in the *in vitro* and *in vivo* studies was compared using one-way analysis of variance (ANOVA) and treatment means were compared using the Tukey's test (LSMEANS command). *p* values less than or equal to 0.05 were considered statistically significant. All analyses were performed using Statistical Analysis System (SAS Institute 2008).

3. Results

3.1. Essential oil and chromatographic profile

In total, 15, 27, and 15 volatile components were identified in *M. alternifolia*, *M. piperita*, and *C. duckei*, respectively (Fig. 1).

Terpinen-4-ol (39.8%) and γ-terpinene (14.6%) were the main constituents of *M. alternifolia* EO, whereas menthol (35.2%), menthone

Table 2
Hematological parameters (mean ± standard deviation) of *Piaractus mesopotamicus* bathed with *Copaifera duckei* oleoresin.

	Parameter	Control	DMSO	*C. duckei* 10 mg/L	*C. duckei* 50 mg/L	*p*-Value
0 h post-bath	Hematocrit (%)	31.83 ± 3.81	32.66 ± 4.69	32.36 ± 3.55	31.91 ± 3.26	0.947
	Hemoglobin (g dL^{-1})	7.29 ± 0.72	7.57 ± 0.79	7.02 ± 0.83	7.36 ± 0.8	0.475
	Erythrocytes (×10^6 μL^{-1})	2.14 ± 0.46	2.28 ± 0.46	1.96 ± 0.34	2.01 ± 0.29	0.159
	MCV (fL)	155.94 ± 42.45	147.35 ± 33.95	171.90 ± 34.49	160.86 ± 21.41	0.382
	MCH (pg)	35.69 ± 8.31	34.19 ± 6.17	37.98 ± 8.08	38.67 ± 5.96	0.407
	MCHC (g dL^{-1})	23.38 ± 3.38	23.85 ± 4.33	22.36 ± 3.28	24.09 ± 2.51	0.646
	Glucose (mg dL^{-1})	182.41 ± 53.23	198.5 ± 51.37	193.33 ± 58.58	234.00 ± 53.35	0.123
	Leukocytes (×10^3 μL^{-1})	34.3 ± 19.1	32.67 ± 17.46	38.09 ± 2.71	30.27 ± 16.02	0.827
	Thrombocytes (×10^3 μL^{-1})	49.63 ± 35.55	45.43 ± 21.45	34.83 ± 17.79	33.61 ± 17.11	0.293
	Monocytes (×10^3 μL^{-1})	3.81 ± 3.48	4.11 ± 3.85	3.98 ± 4.42	3.44 ± 2.33	0.996
	Lymphocytes (×10^3 μL^{-1})	26.03 ± 17.34	30.55 ± 21.74	28.73 ± 25.43	24.84 ± 14.22	0.825
	Neutrophils (×10^3 μL^{-1})	2.09 ± 2.26	2.19 ± 3.13	1.99 ± 1.73	1.74 ± 1.34	0.882
	PAS-GL (×10^3 μL^{-1})	0.09 ± 0.17	0.12 ± 0.12	0.39 ± 0.67	0.26 ± 0.31	0.761
	Total protein (g dL^{-1})	2.79 ± 0.33	2.84 ± 0.39	2.84 ± 0.42	2.80 ± 0.42	0.987
	Albumin (g dL^{-1})	0.62 ± 0.18	0.64 ± 0.15	0.63 ± 0.13	0.66 ± 0.15	0.92
	ALT (U/L)	5.46 ± 1.81	6.15 ± 2.06	6.77 ± 1.89	6.57 ± 2.21	0.425
	AST (U/L)	41.05 ± 16.6	44.58 ± 16.71	44.57 ± 15.39	45.95 ± 12.6	0.894
7 day post-bath	Hematocrit (%)	31.58 ± 2.81	32.50 ± 2.06	32.16 ± 2.03	32.91 ± 2.93	0.612
	Hemoglobin (g dL^{-1})	7.7 ± 0.96	8.24 ± 0.74	7.97 ± 0.6	7.89 ± 0.61	0.375
	Erythrocytes (×10^6 μL^{-1})	1.35 ± 0.24*	1.45 ± 0.24*	1.44 ± 0.3*	1.41 ± 0.26*	0.758
	MCV (fL)	241.52 ± 49.21*	229.38 ± 40.14*	233.03 ± 58.5*	241.72 ± 53.28*	0.913
	MCH (pg)	58.73 ± 11.88*	58.03 ± 10.42*	57.18 ± 11.01*	57.34 ± 9.63*	0.984
	MCHC (g dL^{-1})	24.4 ± 2.3	25.47 ± 3.05	24.88 ± 2.49	24.16 ± 2.91	0.652
	Glucose (mg dL^{-1})	120.66 ± 38.07*	105.83 ± 18.9*	108.5 ± 23.33*	109.66 ± 25.48*	0.567
	Leukocytes (×10^3 μL^{-1})	18.65 ± 15.35	23.86 ± 7.88	16.84 ± 8.38	22.34 ± 11.96	0.409
	Thrombocytes (×10^3 μL^{-1})	22.09 ± 15.28	30.54 ± 13.16	27.02 ± 20.99	24.89 ± 11.60	0.606
	Monocytes (×10^3 μL^{-1})	0.48 ± 0.29*	1.06 ± 1.02*	0.81 ± 0.65*	0.74 ± 0.61	0.08
	Lymphocytes (×10^3 μL^{-1})	17.81 ± 15.49	22.23 ± 7.87	15.10 ± 8.53	20.58 ± 10.85	0.155
	Neutrophils (×10^3 μL^{-1})	0.29 ± 0.22*	0.43 ± 0.55	0.79 ± 1.26	0.34 ± 0.4	0.555
	PAS-GL (×10^3 μL^{-1})	0.08 ± 0.06	0.14 ± 0.21	0.15 ± 0.18	0.24 ± 0.32	0.135
	Total protein (g dL^{-1})	3.80 ± 0.31*	3.91 ± 0.42*	3.94 ± 0.48*	3.85 ± 0.43*	0.861
	Albumin (g dL^{-1})	0.84 ± 0.36	1.09 ± 0.34*	0.80 ± 0.32	1.13 ± 0.45*	0.084
	ALT (U/L)	3.63 ± 1.17*	3.56 ± 1.35*	3.60 ± 2.05*	3.31 ± 1.05*	0.949
	AST (U/L)	42.36 ± 16.33	48.19 ± 20.6	51.01 ± 41.39	50.59 ± 38.62	0.899

* Indicates significant differences between times.

(21.4%), and eucalyptol (10.1%) were the main components of *M. piperita* EO.

The volatile fraction of *C. duckei* OR was mainly composed of β-bisabolene (30.9%) and trans-α-bergamotene (21.9%). Analysis by HPLC-MS/MS of the OR also revealed the following metabolites: ent-agathic-15-methyl ester, ent-agathic acid, and ent-polyalthic acid.

3.2. In vitro assay

The EOs of *M. alternifolia* and *M. piperita* resulted in 100% parasite mortality at 400 mg/L, whereas *C. duckei* OR eliminated 100% of parasites even at the lowest concentration (100 mg/L). During this trial, we observed swelling and lysis of the parasites that were killed by the action of the phytotherapeutic agents.

No parasite mortality was observed in control groups (non-exposed fish and fish exposed to DMSO) during the 4 h of evaluation, indicating that parasites were not affected by baths with DMSO and water only.

At the lower concentration (100 mg/L) and in the first hour, *C. duckei* OR showed the highest efficacy ($p < 0.05$), with 98.7% of monogenean mortality *versus* 0 and 4.2% for *M. alternifolia* and *M. piperita*, respectively. At this concentration, after 4 h of evaluation, *C. duckei* OR continued to be more effective ($p < 0.05$) than the other essential oils (Fig. 2). At 200 mg/L, *C. duckei* OR showed greater efficacy ($p < 0.05$) than *M. alternifolia* and *M. piperita* EOs until 3 h of evaluation (Fig. 2). In addition, at 400 mg/L, *M. alternifolia* EO was less effective ($p < 0.05$) than *C. duckei* OR and *M. piperita* EO. Lastly, no significant differences in efficacy were observed over the trial period between the three phytotherapeutic agents at the highest concentrations (800 and 1600 mg/L) (Fig. 2).

3.3. In vivo assays

Before reporting on the parasitological, biochemical and histological analyses performed on the studied fish, it is important to note that neither treated fish nor non-treated fish showed any behavioral changes, anorexia, hypoxia, or any other changes as a result of treatments using *C. duckei* OR.

3.3.1. Parasitological analysis

Parasite numbers were significantly reduced ($p < 0.05$) after just one 10-min bath with *C. duckei* OR. Short baths with OR at 50 mg/L were more effective ($p < 0.05$) than the other groups, with approximately 45% reduction in the mean intensity of parasitism (Table 1). However, no significant difference in mean intensity of parasitism was observed across treatments seven days post-treatment (Table 1).

3.3.2. Effect of C. duckei OR on the host

Blood parameters of fish bathed with *C. duckei* OR at 10 and 50 mg/L and DMSO were not significantly altered compared to those of control fish ($p > 0.05$, Table 2). However, neutrophil counts were significantly reduced in the control group ($p < 0.05$) seven days post-treatment. MCV, MCH, total protein, and albumin levels were significantly higher ($p < 0.05$) seven days post-bath, whereas blood glucose and monocyte counts were significantly reduced ($p < 0.05$) in all groups (Table 2).

3.4. Histological analysis

Histological analysis revealed branchial and hepatic changes in fish from all groups, including the control group. Analysis of the gill tissue showed hyperplasia and hypertrophy, resulting in moderate fusion of secondary lamellae (Fig. 3). Hepatocytes showed moderate to extensive swelling and the loss of tissue organization in some areas is suggestive of focal necrosis (Fig. 3). No changes were observed in splenic and kidney tissues.

4. Discussion

Despite the histopathological changes observed in fish from all groups, none were caused by the therapeutic baths. The phytotherapeutic

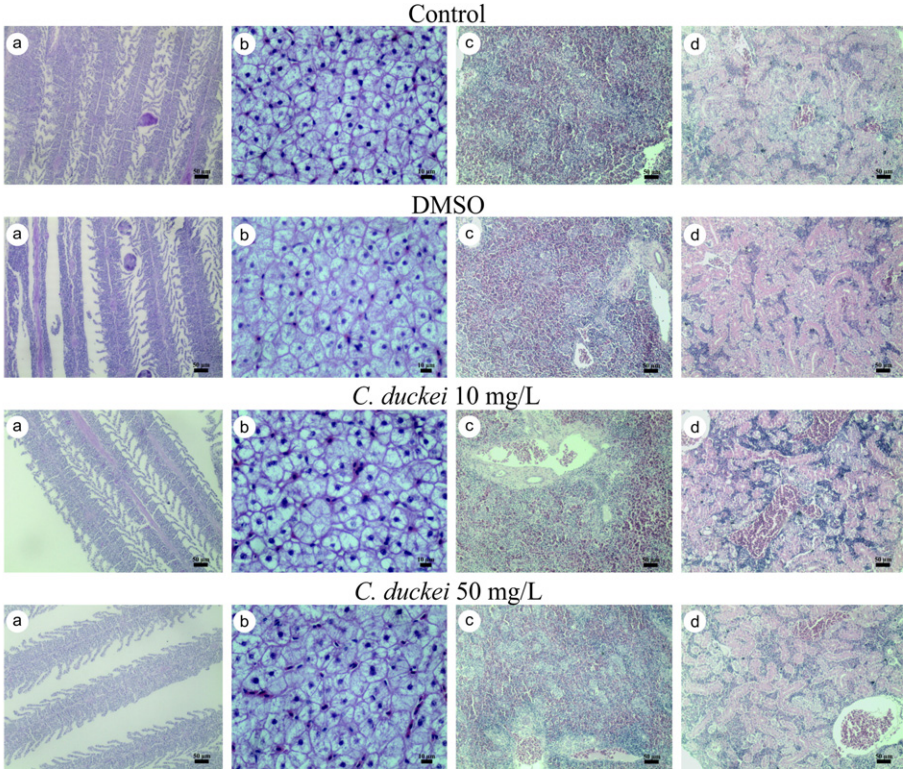

Fig. 3. Gill (a), liver (b), spleen (c), and kidney (d) of *Piaractus mesopotamicus* from the control, DMSO, and *Copaifera duckei* OR (10 mg/L and 50 mg/L) groups. Hyperplasia, hypertrophy, and fusion of secondary lamellae in gill sections from all groups (a). Moderate to extensive hydropic degeneration in liver sections from all groups (b). Healthy spleen and kidneys (c and d).

agents evaluated here, especially *C. duckei* OR, showed great potential for controlling monogenean parasites.

Previous studies have shown the efficacy of *M. alternifolia* (Steverding et al., 2005) and *M. piperita* (Hashimoto et al., 2016; Malheiros et al., 2016) against monogenean parasites, but this is the first time that the effect of *C. duckei* OR has been evaluated against these fish pathogens. Results of the *in vitro* study revealed that *C. duckei* was more effective than *M. alternifolia* and *M. piperita*. Specifically, *C. duckei* OR caused swelling and lysis of monogenean parasites, suggesting that its mode of action is similar to that of other plant extracts that have been tested against protozoan parasites (Zhang et al., 2013; Valladão et al., 2016a), in which the permeabilization of the membrane lead to swelling and lysis of the parasite. The main constituents of *C. duckei* OR identified in the current study were β-bisabolene and trans-α-bergamotene.

In the *in vivo* study, baths with *C. duckei* OR at 50 mg/L for 10 min were effective in killing monogenean parasites, reducing by 45% the intensity of parasitism. Seven days after the baths, fish from all groups had lower parasite infestation rates, which was probably due to the handling of fish (moving fish from tanks to buckets for treatment) and reduction of stocking density. The *in vivo* treatment strategies used in previous studies included short or long as well as single or repeated baths, making comparisons across studies difficult (Valladão et al., 2015). Indeed, different types of herbal medicines, EOs, extracts, and isolated compounds have been tested before against fish monogeneans. Levy et al. (2015) showed in *Poecilia reticulata* (Peters, 1859) that short 30-min baths with ethanol extract of *Zingiber officinale* significantly reduced parasitism by *Gyrodactylus turnbulli*. Boijink et al. (2016) showed in *Colossoma macropomum* (Cuvier, 1818) that short 15-min baths with *Ocimum gratissimum* EO were effective in killing 100% of monogenean parasites. In long bath (2 h or 12 h) using *Bixa orellana* seed extract at 125 and 250 mg/L, Andrade et al. (2016) obtained 100% of efficacy against monogenean of *C. macropomum*. However, nothing is known about the use of OR in aquaculture. The results of this study will enable the development of novel treatment strategies using *C. duckei* OR against other major fish pathogens and in other hosts. In addition, innovations in the solubilization of OR in water can further enhance the action of this herbal medicine. For instance, Rodrigues et al. (2014) reported that *C. duckei* OR showed the best solubilization using nanoemulsion, which is essential for the stability of its constituents.

Therapeutic baths with *C. duckei* OR did not alter the hematological parameters of fish either immediately after treatment or seven days post-treatment, indicating that treatment with this OR is safe for pacu. The unaltered MCV, MCH, total protein, and albumin levels, the increased glucose values, and lower monocyte counts in all groups at day 7 probably occurred because of the handling of fish and not due to treatments. Other studies have described hematological changes in fish treated with different herbal medicines. For instance, Hashimoto et al. (2016) reported a reduction in RBC and thrombocyte counts and an increase in hematocrit, neutrophil, and glucose levels in Nile tilapia *Oreochromis niloticus* (Linnaeus, 1758) given three baths with 40 mg/L of *Lippia sidoides* EO for 10 min with a 24-h interval between baths. Further, *C. macropomum* bathed for 30 min with 150 mg/L of *Lippia alba* EO showed reduced RBC and hematocrit values and increased glucose, MCHC, and thrombocyte levels due to stress (Soares et al., 2016).

Histopathology analysis revealed that fish from all groups had gill alterations, which were related to the intensity of parasitism. In fact, baths with 50 mg/L of *C. duckei* OR reduced parasite loads by approximately 45% without causing extra damage to the gill tissue. This is particularly interesting given the lack of studies phytotherapeutic potential harmful effects on host health and their association with dose and exposure time, as highlighted by (Valladão et al., 2015). Recently, Malheiros et al. (2016) showed that baths with 160 mg/L of *M. piperita* EO for 4 h caused epithelial displacement, fusion of secondary lamellae, hypertrophy, aneurysm, and necrosis in the gills of *Arapaima gigas*. Soares et al. (2016) reported that 30-min baths with 100 and 150 mg/L of L. *alba* EO caused hyperplasia, fusion of the lamellar epithelium, capillary dilatation, displacement of the lamellar epithelium, aneurysm, epithelial disruption with hemorrhage, congestion, and necrosis in *C. macropomum* gills. The liver alterations were observed in both untreated and treated fish, it may be related to fish cultivation or diet conditions, but were not worsened by treatment with *C. duckei* OR. Indeed, this phytotherapeutic agent did not cause any detectable changes to spleen and kidney tissues, suggesting that its use is safe under the tested conditions.

Copaifera duckei OR showed strong anthelmintic activity in *in vitro* and *in vivo* tests against monogenean parasites of pacu, which are among the major parasitic platyhelminths in aquaculture worldwide. Under the conditions tested, this herbal medicine was safe for use in pacu due to its rapid action even in low concentrations, which reduces costs and facilitates the implementation of the treatment protocol. Nevertheless, further testing with different doses and exposure times is warranted to improve the treatment including testing other doses and times that could be even more effective. The use of *C. duckei* OR offers an environmentally friendly alternative for treating parasitic infections in aquaculture by directly and/or indirectly reducing the use of chemotherapy and antibiotics.

Conflict of interest

The authors declare no conflicts of interest.

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