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Nanoemulsions with oleoresin of *Copaifera reticulata* (Leguminosae) improve anthelmintic efficacy in the control of monogenean parasites when compared to oleoresin without nanoformulation

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Abstract

This study compared the *in vitro* anthelmintic activity of *Copaifera reticulata* oleoresin (200, 400, 600, 800 and 1,000 mg/L) and of nanoemulsions prepared with this oleoresin (50, 100, 150, 200 and 250 mg/L) against monogeneans on the gills of *Colossoma macropomum*. The major compounds present in the oleoresin of *C. reticulata* were γ-macrocarpene (14.2%), α-bergamotene (13.6%), β-selinene (13.4%) and β-caryophyllene (11.7%). All concentrations of the nanoemulsion and the oleoresin without nanoformulation showed anthelmintic efficacy against monogeneans, and higher concentrations led to more rapid parasite mortality. Structural damages to the tegument of the parasites exposed to *C. reticulata* oleoresin were observed with scanning electron microscopy. At two hours of exposure, fish showed 100% tolerance to all nanoemulsion concentrations used in the *in vitro* assays, whereas 100% mortality was shown in the fish exposed to the oleoresin without nanoformulation after one hour. The results of this study suggest that nanoemulsions with oleoresin of *C. reticulata* have advantages in the control and treatment of monogenean infections in *C. macropomum* when compared to the oleoresin without nanoformulation. In addition, since nanoemulsions with the *C. reticulata* oleoresin are safe to control monogeneans, the efficacy of these nanoformulations may be assayed in therapeutic baths to treat *C. macropomum* infected by monogeneans.

KEYWORDS
antiparasitic activity, aquaculture, nanotechnology, toxicity, treatment

1 | INTRODUCTION

Nanotechnology is an emerging technology with applications in industrial aquaculture, having the potential to enhance production and ultimately contribute to global food security (Luis, Campos, Oliveira, & Fraceto, 2019; Shah & Mraz, 2019; Valentim et al., 2018a, 2018b). Nanobiotechnology is among the recent advancements of nanotechnology and is used specifically in the treatment of diseases in aquaculture (Luis et al., 2019; Pimentel-Acosta et al., 2019; Shah & Mraz, 2019). Hence, nanotechnology can reduce economic losses caused by mass mortality in commercial aquaculture.

Chemical products are widely used to control diseases in aquaculture due to their efficacy, but can be toxic to other organisms and humans and can accumulate in the environment (Shah & Mraz, 2019; Valentim et al., 2018a, 2018b). As such, the management of parasites and diseases requires development of effective and eco-friendly alternatives. Phytotherapeutics have higher efficacy and lower toxicity when compared to chemotherapeutics and are considered as an alternative treatment in fish farming for the control of infections caused by monogeneans, which are the most prevalent parasites. Phytotherapeutics include oleoresins, essential oils and their metabolites with antiparasitic properties against monogeneans in fish (Costa et al., 2017; Tavares-Dias, 2018; Valentim et al., 2018b).

Monogenea is a diverse class of ectoparasitic worms with a direct life cycle, of which many species are highly pathogenic and resistant to treatment in fish farming (Morales-Serna et al., 2019; Pimentel-Acosta et al., 2019; Valentim et al., 2018a, 2018b). Monogeneans mainly affect the gills of fish and in high abundance cause epithelial and haematological alterations (Costa et al., 2017; Restiannasab, Hemmatzadeh, Khara, & Saljoghi, 2016). Outbreaks of monogeneans have led to mortalities of the entire fish stock and economic losses around the world (Costa et al., 2017; Morales-Serna et al., 2019; Valentim et al., 2018a, 2018b), which is a major limitation to sustainable fish farming. Therefore, global expansion of fish farming must include continuous monitoring of parasitic infections and development of treatments (Valentim et al., 2018a), specifically with the use of effective and functional phytotherapeutics to manage outbreaks of monogeneans. The use of phytotherapeutics based on nanotechnology has been suggested to facilitate the control of these ectoparasites in fish farming (Tavares-Dias, 2018; Valentim et al., 2018a, 2018b).

Copaifera are large trees of the Leguminosae family found in the Amazon rainforest, and it has a resin with a non-volatile portion consisting mainly of acid diterpenes (e.g. kaurenoic, copalic and polyalthic acids) and sesquiterpenes, mainly caryophyllene, copaene and humulene. These compounds have shown biological activity in *Copaifera* spp. (Arruda et al., 2019; Trindade, Silva, & Setzer, 2018), including anthelmintic activity against monogeneans in fish (Costa et al., 2017; Valentim et al., 2018b). Hence, there exists a growing interest in the use of oleoresins and essential oils and their bioactive compounds for treating fish infected by monogeneans (Luis et al., 2019; Morales-Serna et al., 2019; Tavares-Dias, 2018). However, oleoresins and essential oils have low water solubility and low bioavailability, which are major disadvantages for use in fish farming (Costa et al., 2017; Tavares-Dias, 2018; Valentim et al., 2018a, 2018b). Thus, the administration of phytotherapeutics using a nanoemulsion-based delivery system may increase their effective use in the control of monogeneans in fish.

Nanoemulsions are generally oil-in-water-type substances, such as an oily (dispersed) phase and aqueous (dispersing) external phase, allowing lipophilic substances to be solubilized in water (Valentim et al., 2018a, 2018b). Such nanoformulations may improve water solubility of oleoresins and essential oils, thereby increasing therapeutic potency and reducing toxicity of phytotherapeutics when treating fish infected by monogeneans (Tavares-Dias, 2018; Valentim et al., 2018a, 2018b). In *Rhamdia quelen*, it has been demonstrated that the nanoencapsulation of *Melaleuca alternifolia* essential oil improved bactericidal activity when compared to the use of the oils without encapsulation (Souza, Baldissera, Santos, Raffin, & Baldisserotto, 2017). Nevertheless, research is scarce regarding nanoemulsions prepared with essential oils and oleoresins for the control and treatment of monogenean infections in fish (Valentim et al., 2018a, 2018b). Valentim et al. (2018a) investigated the in vitro antiparasitic activity of nanoemulsions prepared with *Copaifera officinalis* oleoresin and achieved 100% efficacy against monogeneans. Consequently, nanoemulsions have been suggested to facilitate the antiparasitic activity of oleoresins and essential oils against monogeneans of fish (Tavares-Dias, 2018; Valentim et al., 2018a, 2018b), since they are unaffected by hydrophobicity and other constraints characteristic of such compounds. Furthermore, no comparison has been performed between the use of nanoemulsions prepared with oleoresin and the use of oleoresin without nanoformulation. Thus, the present study compared the *in vitro* anthelmintic efficacy of *Copaifera reticulata* Ducke oleoresin and of nanoemulsions prepared with this oleoresin in the control of monogeneans on the gills of *Colossoma macropomum*, and also evaluated the tolerance of this fish species to both phytotherapeutics and verified whether the concentrations of nanoemulsions of oleoresin with in vitro efficacy against monogeneans are reduced when compared to the oleoresin without nanoformulation.

2 | MATERIALS AND METHODS

2.1 | Fish and acclimation

Colossoma macropomum fingerlings (*N* = 250) with an average weight of 30.0 ± 10.2 g were obtained from a commercial fish farm in Macapá (AP) and maintained in the Embrapa Amapá Aquaculture and Fishery Laboratory, Macapá, Amapá State, Brazil. The fish were kept in a 500-L tank with constant aeration and continuous water renewal (1.1 L/min) and were fed twice daily with a commercial feed containing 32% crude protein (Guabi®, Brazil). The present study used fish that were naturally infected by monogeneans for all assays. The following water parameters were monitored daily: mean temperature (28.8 ± 0.1°C), dissolved oxygen (5.5 ± 0.2 mg/L), pH (5.3 ± 0.2), ammonia (0.5 ± 0.2 mg/L), alkalinity (10.0 ± 0.001 mg/L) and hardness (10.0 ± 0 mg/L). The tank was siphoned weekly to remove accumulated organic matter, and the water was renewed.

2.2 | Obtainment of *Copaifera reticulata* oleoresin, and gas chromatography analysis

Oleoresin of *C. reticulata* was obtained from copaiba trees from the Jari Ecological Station (Esec Jari) in the municipality of Almeirim, Pará State, Brazil (0°27′24.18″S, 52°49′37.74″W), provided by the project Kamukaia III of Embrapa.

The characterization of *C. reticulata* oleoresin was performed using gas chromatography coupled with mass spectrometry

(GC-MS-Shimadzu QP5050A). The separation was performed using a silica SPB-5 capillary column composed of 5% phenylmethylpolysiloxane (30 m length × 0.25 mm i.d. × 0.25 μm phase thickness). The sample was dissolved in dichloromethane and analysed according to the following experimental conditions: injection mode, split, 1:40; injector temperature, 250°C; carrier gas, helium; flow rate, 1.0 ml/min; and oven temperature, 100°C for 5 min and then increment to 260°C at a rate of 4°C/min, ending with an isothermal treatment of 20 min. Mass spectra were acquired in electron ionization mode at 70 eV with a scan range of 40–350 M/z and a sampling rate of 1.0 scans/s. The ion source temperature was 200°C, interface temperature was 250°C, and solvent cut time was 2.5 min (see Adams, 2007).

2.3 | Preparation of nanoemulsion with *Copaifera reticulata* oleoresin

The oil-in-water nanoemulsion with the *C. reticulata* oleoresin was prepared using a low-energy method. The nanoemulsion consisted of 5% *C. reticulata* oleoresin and 5% of the surfactant Tween-20 for the oily phase and 90% distilled water for the aqueous phase. Water was then added gradually with continuous homogenization using a vortex stirrer (Even, VX-38, Brazil) for approximately 5 min. The oil-to-surfactant ratio was 1:1, and the final mass of the nanoemulsion was 10 g. Dynamic light scattering (DLS) analysis and zeta potential measurements were performed on the particle size distribution using a Zetasizer Nano ZS (Malvern, UK) equipped with a 10-mW "red" laser (*k* = 632.8 nm), and samples were measured for size with a 90° scatter detection angle. The nanoemulsion was diluted in deionized water (1:10 g), and the analyses of the droplet size and polydispersity index were carried out in triplicate and expressed as mean ± standard deviation. Data were processed with Zetasizer 6.20® software (Malvern Instruments).

2.4 | *In vitro* assays with monogeneans of *Colossoma macropomum*

The gills of 19 *C. macropomum* fingerlings with an average weight of 44.8 ± 19.2 g and length of 15.3 ± 3.5 cm and naturally infected by monogeneans (*Anacanthorus spathulatus*, *Notozothecium janauachensis* and *Mymarothecium boegeri*) were removed and placed individually in Petri dishes. Each gill arch was immersed in different concentrations of oleoresin of *C. reticulata* (200, 400, 600, 800 and 1,000 mg/L) and of nanoemulsions prepared with this oleoresin (50, 100, 150, 200 and 250 mg/L), with three replicates for each treatment. The solvent dimethyl sulfoxide (DMSO) was used to dilute the oleoresin (1:10 g), and the surfactant Tween-20 was used in the nanoemulsions prepared with the *C. reticulata* oleoresin. The three control groups were as follows: cultivation tank water; cultivation tank water + DMSO (control for oleoresin); and cultivation tank water + Tween-20 (control for nanoemulsions).

In vitro assays were performed at ambient temperature (20°C). A stereomicroscope with cold-light illumination was used for analysis, and fields of view were selected that contained a minimum of 20 monogeneans for each replicate. After each gill arch was immersed in *C. reticulata* oleoresin and the nanoemulsions, the replicates were observed every 15 min to quantify the live and dead monogeneans. Parasites were considered dead when detached from the tissue or with no mobility while still attached (Soares et al., 2016). The efficacy of each treatment was calculated using the methods described in Zhang et al. (2014). The monogeneans were collected, fixed in formalin (5%) and prepared for identification as described in Eiras, Takemoto, and Pavanelli (2006).

2.5 | Tolerance of *Colossoma macropomum* to different concentrations of *Copaifera reticulata* oleoresin and nanoemulsions with this oleoresin

Fish tolerance was tested based on the *in vitro* results to determine the optimal concentration for therapeutic baths with oleoresin of *C. reticulata* and the nanoemulsions. The tolerance assays were carried out with 150 *C. macropomum* fingerlings (30.0 ± 6.6 g and 13.2 ± 1.0 cm). Each treatment consisted of 3 replicates with 5 fish per replicate (15 fish per treatment) using 100-L tanks. Treatments were carried out with different concentrations of the *C. reticulata* oleoresin (200, 400, 600, 800 and 1,000 mg/L) and of the nanoemulsions (50, 100, 150, 200 and 250 mg/L). Dimethyl sulfoxide (DMSO) was used to dilute the oleoresin (1:10 g), and Tween-20 was used in the nanoemulsions prepared with the *C. reticulata* oleoresin. The tanks used for the tolerance assays were maintained with no water renewal.

Changes in fish behaviour (i.e. opercular movement, caudal beat and response to mechanical stimuli) and mortality were observed for 2 hr of exposure to the *C. reticulata* oleoresin and nanoemulsions.

2.6 | Scanning electron microscopy of monogeneans from *Colossoma macropomum* gills

At the end of the *in vitro* assays with tank water (control) and *C. reticulata* oleoresin (200, 400, 600, 800 and 1,000 mg/L), the gills with monogeneans were fixed in 2.5% glutaraldehyde and 0.1 M sodium cacodylate buffer (pH 7.2) and used for scanning electron microscopy analysis. The gill samples were then incubated with a 1% osmium tetroxide solution in 0.1 M sodium cacodylate buffer (pH 7.2) for 2 hr. The samples were subsequently incubated in 1% aqueous tannic acid solution for 45 min, washed twice with distilled water (20 min each), incubated again in 1% osmium tetroxide solution for 45 min and washed 3 times in distilled water (15 min each). The samples were then gradually dehydrated in 50%, 70% and 90% ethanol (two 30-min baths each) and 100% ethanol (three 30-min baths each). The samples were dried in a critical point chamber (BALZERS CPD 030, USA) using carbon dioxide, and were added to a stub using double-sided carbon tape and coated with a thin layer of platinum with a thickness of 20–30 nm ("SPUTTERING," ©LEICA EM SCD 500, Germany). The samples were analysed using a Quanta FEG 250 Field Emission Electron Microscope (FEI, Austria).

3 | RESULTS

3.1 | Compound composition of *Copaifera reticulata* oleoresin

The chemical compounds of the *C. reticulata* oleoresin are shown in Table 1. The major compounds present in the oleoresin were sesquiterpenes such as gamma-macrocarpene, alpha-trans-bergamotene, beta-selinene and beta-caryophyllene.

3.2 | In vitro efficacy of *Copaifera reticulata* oleoresin and nanoemulsions with this oleoresin against monogeneans of *Colossoma macropomum*

Monogeneans *A. spathulatus*, *N. janauachensis* and *M. boegeri* were alive or with slow movements after 1 hr and 45 min of exposure in the control groups with cultivation tank water and tank water + DMSO in the in vitro assays. However, 100% mortality occurred with the cultivation tank water at 8 hr of exposure and with tank water + DMSO at 6 hr of exposure. In the control group with tank water + Tween-20, 100% mortality occurred after 1 hr of exposure. Variation in the mortality time of monogeneans was found at the concentrations of 200, 400, 600, 800 and 1,000 mg/L of the *C. reticulata* oleoresin (Table 2 and Figure 1a). The highest concentration of oleoresin showed a high efficacy (97%) after 15 min of exposure, whereas the lowest concentration showed efficacy only after 1 hr. Similar to the oleoresin, the 250 mg/L nanoemulsion had a high efficacy (100%) after 15 min of exposure, while the lowest concentration (50 mg/L) showed efficacy after 1 hr and 45 min (Table 2 and Figure 1b).

When observing all concentrations of the *in vitro* assays, the bodies of the monogeneans contracted with the onset of exposure. The monogeneans then became paralysed, and over time, some parasites became swollen with a more globous body.

TABLE 1 Chemical composition of the *Copaifera reticulata* oleoresin

Peak	Retention index	% Content	Identification
1	1339	0.3	Delta-elemene
2	1367	1.56	Cyclosativene
3	1378	0.31	Alpha-copaene
4	1387	0.12	Beta-elemene
4	1395	7.49	Alpha-elemene
5	1402	0.39	Cyperene
6	1419	0.27	Alpha-cis-bergamotene
7	1423	11.72	Beta-caryophyllene
8	1442	13.64	Alpha-trans-bergamotene
9	1444	5.54	Aromadendrene
10	1448	1.71	6,9-Guaiadiene
11	1458	1.95	Alpha-humulene
12	1464	1.67	Sesquisabinene
13	1480	1.54	Gamma-gurjunene
14	1492	13.38	Beta-selinene
15	1502	9.07	Alpha-selinene
16	1514	3.33	Alpha-bulnesene
17	1521	14.23	Gamma-macrocarpene
18	1535	1.98	Gamma-cuprenene
19	1557	2.05	Germacrene B
	Total compounds identified (%)	92.2	–

3.3 | Morphological changes of monogeneans observed with scanning electron microscopy

Monogeneans exposed to tank water (controls) presented a defined body shape with shallow wrinkles on the surface (Figure 2a). In contrast, the parasites exposed to the *C. reticulata* oleoresin showed tegument with extensive damage due to perforation caused by the oleoresin (Figure 2b-d) and were covered with deep wrinkles (Figure 2c).

3.4 | Tolerance of *Colossoma macropomum* to different concentrations of *Copaifera reticulata* oleoresin and of nanoemulsions with this oleoresin

During the tolerance assays, the fish showed behavioural changes at all concentrations of the *C. reticulata* oleoresin (200, 400, 600, 800 and 1,000 mg/L), as shown in Table 3. All *C. reticulata* oleoresin concentrations presented fish mortalities after 1 hr of exposure and total mortality in less than 2 hr. However, fish exposed to 50, 100, 150, 200 and 250 mg/L of the nanoemulsions prepared with *C. reticulata* oleoresin only showed little behavioural changes, and no mortality was observed during 2 hr of exposure (Table 3).

4 | DISCUSSION

Oleoresins of *Copaifera* spp. generally have a complex chemical composition, and they vary between species regarding the presence and abundance of each chemical compound (Arruda et al., 2019; Veiga & Pinto, 2002). Nevertheless, these oleoresins are composed mostly of sesquiterpenes such as caryophyllene, copaene and humulene, and they interact with different target proteins in parasites (Arruda et al., 2019; Costa et al., 2017; Trindade et al., 2018; Veiga & Pinto,

TABLE 2 *In vitro* anthelmintic activity of different concentrations of the *Copaifera reticulata* oleoresin and the nanoemulsion with *Copaifera reticulata* oleoresin against monogeneans on the gills of *Colossoma macropomum*

Copaifera reticulata oleoresin				Nanoemulsion with oleoresin			
Time of exposure	Concentration mg/L	Live parasites	Dead parasites (%)	Time of exposure	Concentration mg/L	Live parasites	Dead parasites (%)
0	Tank water	34.0 ± 14.2	0	0	Tank water	34.0 ± 14.2	0
	DMSO	35.3 ± 17.0	0		Tween−20	22.7 ± 1.2	0
	200 mg/L	29.7 ± 4.7	0		50 mg/L	23.7 ± 3.3	0
	400 mg/L	26.3 ± 3.2	0		100 mg/L	21.3 ± 1.2	0
	600 mg/L	21.7 ± 2.9	0		150 mg/L	28.3 ± 11.9	0
	800 mg/L	26.0 ± 2.6	0		200 mg/L	24.7 ± 4.5	0
	1,000 mg/L	25.0 ± 5.0	0		250 mg/L	30.3 ± 2.5	0
15 min	Tank water	34.0 ± 14.2	0	15 min	Tank water	34.0 ± 14.2	0
	DMSO	35.3 ± 17.0	0		Tween−20	7.0 ± 3.0	69.2
	200 mg/L	28.0 ± 5.0	5.7		50 mg/L	23.3 ± 4.2	1.7
	400 mg/L	25.0 ± 4.4	4.9		100 mg/L	19.0 ± 3.0	10.8
	600 mg/L	14.7 ± 11.1	32.2		150 mg/L	3.3 ± 1.2	88.3
	800 mg/L	9.3 ± 11.0	60.0		200 mg/L	9.3 ± 9.5	62.3
	1,000 mg/L	0.7 ± 1.2	97.2		250 mg/L	0	100
30 min	Tank water	34.0 ± 14.2	0	30 min	Tank water	34.0 ± 14.2	0
	DMSO	35.3 ± 17.0	0		Tween−20	1.7 ± 0.6	92.5
	200 mg/L	24.3 ± 9.0	18.1		50 mg/L	22.3 ± 5.1	5.9
	400 mg/L	15.7 ± 13.2	40.3		100 mg/L	12.0 ± 1.0	43.7
	600 mg/L	0	100		150 mg/L	0.7 ± 0.58	97.5
	800 mg/L	0.3 ± 0.6	98.7		200 mg/L	0	100
	1,000 mg/L	0	100		250 mg/L	0	100
60 min	Tank water	33.7 ± 14.4	0.9	60 min	Tank water	33.7 ± 14.4	0.9
	DMSO	31.3 ± 14.4	11.3		Tween−20	0	100
	200 mg/L	0.3 ± 0.6	98.9		50 mg/L	7.3 ± 2.5	69.2
	400 mg/L	0.7 ± 1.2	97.3		100 mg/L	3.0 ± 1.7	85.9
	600 mg/L	0	100		150 mg/L	0	100
	800 mg/L	0	100		200 mg/L	0	100
	1,000 mg/L	0	100		250 mg/L	0	100
90 min	Tank water	32.3 ± 15.5	5.0	90 min	Tank water	32.3 ± 15.5	5.0
	DMSO	24.0 ± 9.8	32.0		Tween−20	0	100
	200 mg/L	0	100		50 mg/L	2.0 ± 2.6	91.6
	400 mg/L	0	100		100 mg/L	0	100
	600 mg/L	0	100		150 mg/L	0	100
	800 mg/L	0	100		200 mg/L	0	100
	1,000 mg/L	0	100		250 mg/L	0	100
105 min	Tank water	31.3 ± 16.1	8.0	105 min	Tank water	32.3 ± 15.5	5.0
	DMSO	24.0 ± 9.8	32.0		Tween−20	0	100
	200 mg/L	0	100		50 mg/L	0	100
	400 mg/L	0	100		100 mg/L	0	100
	600 mg/L	0	100		150 mg/L	0	100
	800 mg/L	0	100		200 mg/L	0	100
	1,000 mg/L	0	100		250 mg/L	0	100

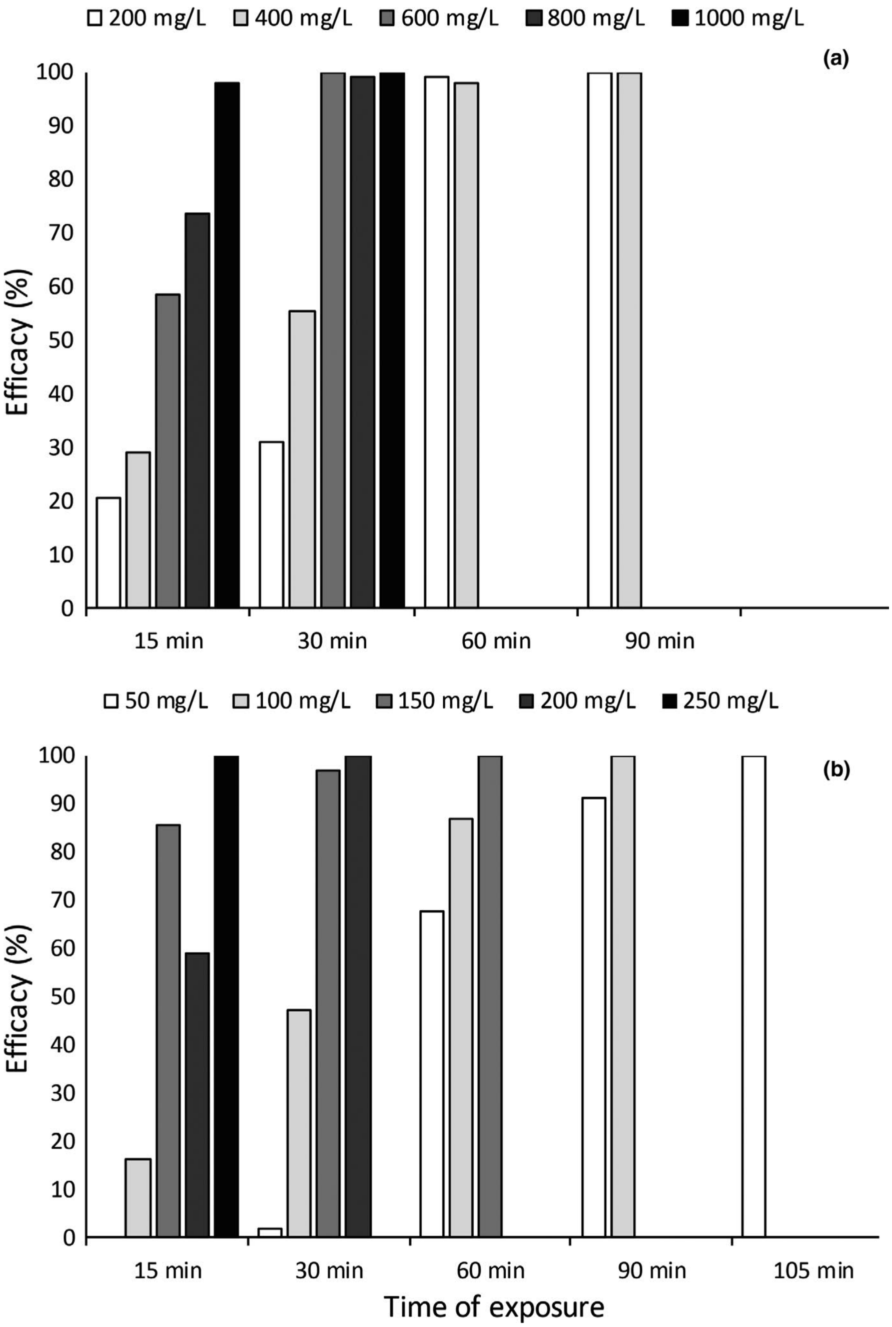

FIGURE 1 *In vitro* anthelmintic efficacy of different concentrations of *Copaifera reticulata* oleoresin (a) and of nanoemulsions prepared with this oleoresin (b) against monogeneans of *Colossoma macropomum*

2002). In the present study, the major compounds present in the *C. reticulata* oleoresin were γ-macrocarpene (14.2%), α-bergamotene (13.6%), β-selinene (13.4%) and β-caryophyllene (11.7%), similar to the compositions reported in the literature (Arruda et al., 2019; Costa et al., 2017; Trindade et al., 2018). Despite the high presence of these compounds in the *C. reticulata* oleoresins, biological activities of the oleoresins may be synergistic or additive rather than being caused by individual compounds (Arruda et al., 2019).

There is a growing interest worldwide for new alternative practices to control parasites in fish farming. Many of the practices include the use of phytotherapeutics (Costa et al., 2017; Morales-Serna et al., 2019; Tavares-Dias, 2018), which have been applied in fish farming specifically to control and treat infections caused by monogeneans (Costa et al., 2017; Ling, Jiang, Liu, Li, & Wang, 2015; Tavares-Dias, 2018). In the present study, the in vitro assays with the *C. reticulata* oleoresin and assays with the nanoemulsion showed anthelmintic activity as indicated by the paralysis, swelling and death of monogeneans observed with all concentrations. The main compounds present in the *C. reticulata* oleoresin used in the present study were the sesquiterpenes γ-macrocarpene, α-bergamotene, β-selinene and β-caryophyllene, which present antiparasitic activities by damaging the tegument of the parasites (Arruda et al., 2019). Costa et al. (2017) observed that the use of *Copaifera duckei* oleoresin in *Piaractus mesopotamicus* culture had similar

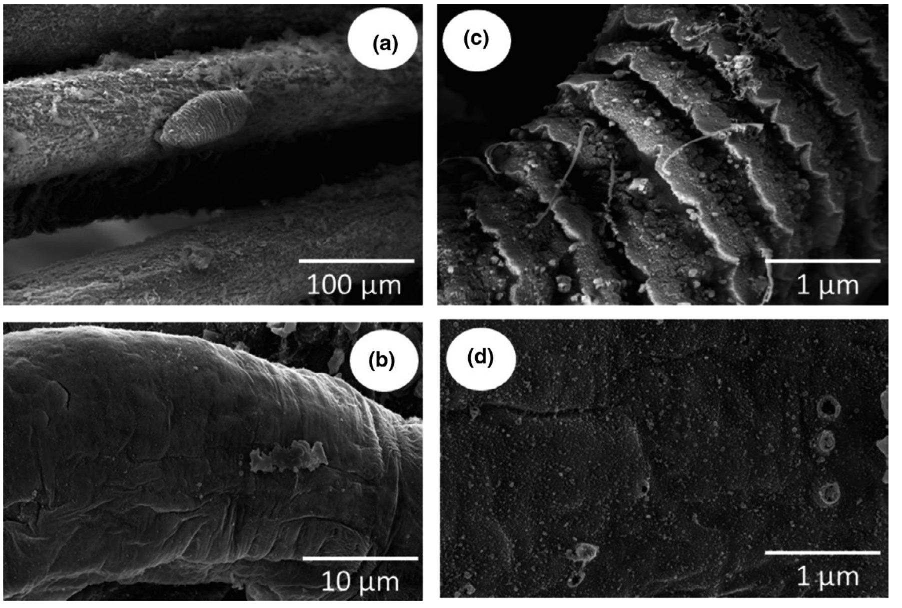

FIGURE 2 Scanning electron micrographs of monogeneans on *Colossoma macropomum* exposed to oleoresin of *Copaifera reticulata*. Parasites after 8 hr of exposure to tank water of cultivation (a). Parasites exposed to 400 mg/L *Copaifera reticulata* oleoresin (b-d) after 90 min and 800 mg/L *Copaifera reticulata* oleoresin after 60 min (c)

TABLE 3 Tolerance of *Colossoma macropomum* to different concentrations of *Copaifera reticulata* oleoresin and the nanoemulsion with this oleoresin, after one and two hours of exposure, respectively

Concentration (mg/L)		Mortality (%)	Behavioural changes of fish
Oleoresin of *Copaifera reticulata*	200	100	Agitation, jumps, convulsions, accelerated opercular beat, irregular swimming, tipping over, lethargy and death
	400	100	
	600	100	
	800	100	
	1,000	100	
Nanoemulsion with *Copaifera reticulata* oleoresin	50	0	Accelerated opercular beat, shallow breathing, lethargy and tipping
	100	0	
	150	0	Agitation and jumping
	200	0	
	250	0	

anthelmintic effects on the monogeneans *Anacanthorus penilabiatus* and *Mymarothecium viatorum*. In addition, morphological analyses with scanning electron microscopy (SEM) showed that the *C. reticulata* oleoresin caused significant damages to the tegument of monogeneans such as disruption, wrinkling and holes when compared to untreated monogeneans. Similar observations were reported for the monogenean *Dactylogyrus intermedius* in goldfish *Carassius auratus* exposed to cinnamaldehyde (Ling et al., 2015).

Nanotechnology has been the focus of aquaculture research due to the need of effective delivery systems for certain phytotherapeutic agents (Luis et al., 2019; Shah & Mraz, 2019). The most recent approach to the control and treatment of monogenean infections relies on plant-derived phytotherapeutics to prepare nanoemulsions. The nanoscale optimizes the biological activities and the chemical and physical stability of the phytotherapeutics. Nanoformulations based on phytotherapeutic agents have shown 100% efficacy in the control of parasites in fish farming (Valentim et al., 2018a, 2018b). In the present study, both non-nanostructured oleoresin and nanoemulsions of *C. reticulata* had a similar efficacy against monogeneans of *C. macropomum*. However, all concentrations of the *C. reticulata* oleoresin without nanoformulation caused 100% mortality of the fish within two hours of exposure, whereas no mortality was observed for fish exposed to the different concentrations of nanoemulsions with oleoresin. Therefore, C. *macropomum* showed tolerance to all nanoemulsion concentrations, which were low when compared to the concentrations of the *C. reticulata* oleoresin without nanoformulation. Nanoemulsions are advantageous because they improve bioavailability, solubility and biological accessibility of phytotherapeutics (Luis et al., 2019; Shah & Mraz, 2019), while having low toxicity to the fish. The anthelmintic efficacy of the *C. reticulata* oleoresin was notably inhibited by poor water solubility, which reduces bioavailability. The increased stability and bioavailability of the *C. reticulata* oleoresin may permit a decrease in the concentration of the phytotherapeutic for controlling and treating monogeneans, benefitting the fish in terms of possible adverse effects and mortality. Thus, the present study indicates the use of nanoemulsions with *C. reticulata* oleoresin rather than the *C. reticulata* oleoresin without nanoformulation for therapeutic baths to control monogeneans in

C. macropomum. Further studies should be carried out focusing on better antiparasitic efficacy.

In conclusion, 100% efficacy was shown *in vitro* with oleoresin of *C. reticulata* and the nanoemulsions with this oleoresin, and the nanoemulsions showed promising results for application in aquaculture since they decrease the concentrations of oleoresin used for controlling monogeneans. The anthelmintic activity observed with the *C. reticulata* oleoresin and the nanoemulsion may be attributed to the terpenes macrocarpene, α-bergamotene, β-selinene and β-caryophyllene. Nanoemulsions containing phytotherapeutic agents, such as oleoresins and essential oils, are an innovative strategy of nanobiotechnology to manage parasitic diseases in fish farming. Antiparasitic nanoemulsions are novel water-in-oil formulations that are stabilized by the addition of small amounts of surfactants, and therefore have high efficacy due to the increased solubility and bioavailability of herbal oils, making them ideal vehicles for the treatment of monogenean infections in aquaculture. Nanoemulsions containing oleoresin of *C. reticulata* offer advantages when compared to the use of oleoresin without nanoformulation, and their application is currently the preferred management strategy to control and treat infections caused by monogeneans in fish. Therefore, the nanoemulsions should be considered for therapeutic baths to control such parasites in *C. macropomum*.

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CONFLICT OF INTEREST

The authors declare they have no conflict of interest.

ETHICAL DISCLOSURES

This study was developed in accordance with the principles adopted by the Brazilian College of Animal Experimentation (COBEA) and with authorization from the Ethics Committee in the Use of Animals of Embrapa Amapá (Protocol No. 013-CEUA/CPAFAP).

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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