Received: 5 June 2017 | Revised: 4 September 2017 | Accepted: 6 September 2017
DOI: 10.1111/jfd.12739

ORIGINAL ARTICLE

WILEY Journal of Fish Diseases

Nanoemulsion from essential oil of *Pterodon emarginatus* (Fabaceae) shows in vitro efficacy against monogeneans of *Colossoma macropomum* (Pisces: Serrasalmidae)

D S S Valentim[1] | J L Duarte[2] | A E M F M Oliveira[2] | R A S Cruz[2] | J C T Carvalho[1,2] | E C Conceição[2] | C P Fernandes[1,2] | M Tavares-Dias[1,3]

[1]Postgraduate Program on Tropical Biodiversity (PPGBIO), Federal University of Amapá (UNIFAP), Macapá, AP, Brazil
[2]Phytopharmaceutical Nanobiotechnology Laboratory, Federal University of Amapá, Macapá, Brazil
[3]Embrapa Amapá, Macapá, AP, Brazil

Correspondence
M Tavares-Dias, Embrapa Amapá, Rodovia Juscelino Kubitschek, Macapá, AP, Brazil.
Email: marcos.tavares@embrapa.br

Funding information
Conselho Nacional de Desenvolvimento Científico e Tecnológico, Grant/Award Number: 303013/2015-0

Abstract

Outbreaks of diseases pose a major threat to sustainable aquaculture development worldwide. Application of herbal products to combat parasitic diseases provides an alternative approach for sustainable aquaculture. This study investigated the in vitro antiparasitic effects of an oil-in-water nanoemulsion prepared using the essential oil from *Pterodon emarginatus*, against monogeneans infesting *Colossoma macropomum*. Gill arches from *C. macropomum* (47.6 ± 14.5 g and 13.5 ± 1.4 cm) that were naturally parasitized by *Anacanthorus spathulatus*, *Notozothecium janauachensis* and *Mymarothecium boegeri* were immersed in different dispersions of the *P. emarginatus* nanoemulsions (0, 50, 100, 200, 400 and 600 mg/L). The major compounds presented in the essential oil of *P. emarginatus* were β-elemene, β-caryophyllene and α-humulene. Characterization of these nanoemulsions showed that they have a small mean droplet size and low polydispersity index, which is concordant with stable systems. In this in vitro trial, the *P. emarginatus* nanoemulsion concentrations of 100, 200, 400 and 600 mg/L presented 100% helminthic efficacy against monogeneans of the gills of *C. macropomum*. The highest two concentrations used (400 and 600 mg/L) were seen to immobilize the parasites after only 15 min. Therefore, it would be worthwhile testing these concentrations in therapeutic baths against monogeneans of *C. macropomum*.

KEYWORDS
fish, nanotechnology, parasites, *Pterodon emarginatus*

1 | INTRODUCTION

Phytotherapeutic products present bioactive properties that are obtained exclusively from medicinal plants. These products can be manufactured industrially or made up manually, both for human and for veterinary use. Essential oils are secondary metabolites produced by medicinal plants that can be extracted from a variety of plant sources. They are used for many purposes in the pharmaceutical and food industries (Anwer, Jamil, Ibnouf, & Shakeel, 2014), and for controlling and treating ectoparasites of fish (Zhang et al., 2014; Hashimoto et al., 2016; Soares et al., 2016; Valladão et al., 2016; Soares et al., 2017; Costa et al., 2017). Thus, use of medicinal plants has been increasing within aquaculture worldwide, given that these plants are easy to cultivate, have biodegradable action, do not give rise to accumulations in animal tissue and present low toxicity (Coimbra, Soares, Garrido, Sousa, & Ribeiro, 2006). However, it has been reported that the low dispersion of essential oils in water makes it difficult to use them in aquaculture (Hashimoto et al., 2016; Soares et al., 2016; Soares et al., 2017; Costa et al., 2017). Therefore, methods for facilitating their dispersion are required so that they can be used in therapeutic baths for fish. Furthermore, the loss of compounds caused by the volatility of these essential oils is a technological challenge for these products.

Today, nanotechnology is helping to solve these problems while maintaining the therapeutic efficacy of these natural products, through use of nanoemulsions (Anwer et al., 2014; Bajerski et al., 2016). Oil-in-water nanoemulsions consist of fine dispersions of oil in water with a transparent or translucent appearance, bluish reflect and inner-phase droplets ranging in size from 50 to 200 nm. These systems are kinetically stable for long periods of time, without presenting any signs of coalescence or flocculation. Brownian motion alone is enough to overcome gravity and prevent instability. These systems have wide applications in the food, cosmetics and pharmaceutical industries due to their advantageous properties, such as enhancement of water solubility of compounds, potential improvement of bioavailability and others (Anwer et al., 2014; Bajerski et al., 2016; Oliveira, Carneiro, Albuquerque, & Marchetti, 2017). Previous studies demonstrated the versatility and advantages of nanoemulsions and their potential for delivering hydrophobic compounds (Oliveira et al., 2017), such as essential oils (Anwer et al., 2014). Recently, special attention has been given to essential oils that are used as the core in nanoemulsion preparation (Anwer et al., 2014; Bajerski et al., 2016). These systems are a promising potential means for delivering essential oils to combat infections, as was demonstrated by Anwer et al. (2014) with a nanoemulsion prepared from the essential oil of cloves, against bacteria.

Pterodon emarginatus Vogel 1837 is an arboreal species that grows to a height of up to 15 m in the Cerrado biome of Brazil. It is popularly known as the sucupira-branca or faveira. It has medicinal importance, as its seeds are used for treating rheumatism, inflammations and spinal problems. Its fruit is a major source of essential oils, which are commonly used to treat muscle pain, arthritis and arthrosis, and present anti-inflammatory and analgesic action. The seeds of *P. emarginatus* have also been reported to have antimicrobial, antiulcerogenic and leishmanicidal action (Dutra, Braga, Coimbra, Silva, & Barbosa, 2009). Tea made from the husk of the stalk is used to treat gynaecological infections (Alves et al., 2013; Bustamante et al., 2010; Leite de Almeida & Gottlieb, 1975; Lorenzi, 2002; Mors, Santos, Monteiro, & Gilbert, 1967; Santos et al., 2010). Oil from *P. emarginatus* has been used as an anti-inflammatory agent, especially in the form of a microemulsion (Pascoa, Diniz, Florentino, Costa, & Bara, 2015) and as a nanoemulsion for biological control over the larvae of *Aedes aegypti* (Oliveira et al., 2016). However, in relation to aquaculture, there are no studies on the use of nanoemulsions containing the essential oil of *P. emarginatus*.

Although *P. emarginatus* is considered to be an important source of phytotherapeutic material because of its phytochemical characteristics and bioactive properties (Machado et al., 2015), few studies have been conducted on the use of its essential oil (Alves et al., 2013; Dutra et al., 2009; Mors et al., 1967; Pascoa et al., 2015; Santos et al., 2010). Because of the characteristics and properties of *P. emarginatus*, this plant has been included in the list of species that are a conservation priority (Alves et al., 2013). Therefore, the essential oil of *P. emarginatus* should be tested against ectoparasites of *Colossoma macropomum* Cuvier 1816 (tambaqui), especially due to the fact that it can be extracted through a sustainable use of the biodiversity.

Colossoma macropomum is a neotropical member of Serrasalmidae that is native to the Amazon region. It is economically important within aquaculture in this region, where it is commonly consumed. Brazilian production of this species through aquaculture in 2014 was approximately 140,000 tons (IBGE, 2015; Valladão et al., 2016). However, one of the major problems in intensive farming of *C. macropomum* is infection caused by *Anacanthorus spathulatus*, *Notozothecium janauachensis* and *Mymarothecium boegeri* (Monogenea), which may compromise production (Soares et al., 2016; Cardoso et al., 2016; Soares et al., 2017).

Phytotherapy is one of the treatment alternatives, and this has shown promising antiparasitic effects, especially against monogeneans (Zhang et al., 2014; Hashimoto et al., 2016; Valladão et al., 2016; Costa et al., 2017; Soares et al., 2017). Few studies on the use of essential oils for controlling monogeneans that infest *C. macropomum* have been conducted to date (Soares et al., 2016; Soares et al., 2017), and nanoemulsions containing essential oils have not been tested on fish for this purpose. Thus, the objective of this study was to test the in vitro antiparasitic effects of a nanoemulsion from the essential oil of *P. emarginatus* against monogeneans infesting *C. macropomum*.

2 | MATERIALS AND METHODS

2.1 | Extraction of *Pterodon emarginatus* essential oil

Pterodon emarginatus was identified by Dr. José Realino de Paula, and a voucher specimen was deposited at the Herbarium of the Federal University of Goiás (Brazil) under the register number 41714. *Pterodon emarginatus* fruits were crushed in distilled water and subjected to hydrodistillation using a Clevenger-type apparatus for 3 hr. After extraction, the essential oil was collected and stored at 4°C.

2.2 | Gas chromatography analysis

The chemical analysis on the oleoresin was performed using a gas chromatograph (GCMS-QP5000, Shimadzu) equipped with a mass spectrometer, using electron ionization, with the following experimental conditions: injector temperature, 200°C; detector temperature, 250°C; carrier gas, helium; flow rate, 1 ml/min; and split injection with split ratio 1:40. The oven temperature was programmed to start from 50°C (isothermal for 10 min), with an increase of 2°C/min to 200°C and then an increase of 10°C/min to 290°C (isothermal for 10 min). The RTx5-5MS column parameters were as follows: i.d. = 0.25 mm; length 30 m; and film thickness = 0.25 μm. The mass spectrometry conditions were as follows: ionization voltage, 70 eV; scan rate, 1 scan/s; and mass range, *m/z* 50-400.

2.3 | *Pterodon emarginatus* essential oil-based nanoemulsion

The oil-in-water nanoemulsion containing essential oil from *P. emarginatus* was prepared by means of a low-energy method (Oliveira et al., 2017), with some modifications. The oily phase, constituted by a non-ionic surfactant (polysorbate 80) and the essential oil, was vigorously mixed on a screw top glass vial using a vortex stirrer (Warmnest, Ionlab, Brazil) until a transparent homogeneous system was obtained. Deionized water was then added dropwise to this system under continuous stirring using a vortex stirrer (Warmnest, Ionlab, Brazil), being formed initially a high-viscous system, followed by an opaque white system, until the translucent nanoemulsion with the bluish reflect was formed. The final essential oil content was 5,000 mg/L, the surfactant-to-oil ratio was 1:1 and the nanoemulsion was prepared at a final mass of 10 g. Therefore, the nanoemulsion prepared with the essential oil from *P. emarginatus* contained 0.5% (w/w) of essential oil, 0.5% (w/w) of polysorbate 80 and 99% (w/w) of water. The characterization of the nanoemulsion, to verify the formation of nanodroplets, was carried out by dynamic light scattering (DLS) analysis using the Zetasizer Nano ZS (Malvern, United Kingdom) equipped with a 10 mW "red" laser (λ = 632.8 nm), and the sample size was measured at a 90° scatter detection angle. The nanoemulsion was diluted in deionized water (1:25), and the results of particle size distribution (droplet size and polydispersity index) were expressed as the mean ± standard deviation.

2.4 | Fish and acclimatization

Colossoma macropomum fingerlings were obtained from a commercial fish farm in Macapá, in the state of Amapá (Brazil), and were transported to the Aquatic Organism Health Laboratory of Embrapa Amapá, Macapá (Brazil). They were fed with fish food containing 32% crude protein, and the tanks were kept under constant water recirculation at a temperature of 30.2 ± 0.1°C, with dissolved oxygen 5.6 ± 0.2 mg/L, pH 5.4 ± 0.2, total ammonium 0.5 ± 0.2 mg/L, alkalinity 10.0 ± 0 mg/L and hardness 10.0 ± 0 mg/L. The organic matter that accumulated at the bottom of the tanks was removed once a day.

2.5 | In vitro trial using nanoemulsion from the essential oil of *Pterodon emarginatus*

Gill arches (47.6 ± 14.5 g and 13.5 ± 1.4 cm) of *C. macropomum* that were naturally parasitized by species of monogeneans were removed from specimens of *C. macropomum* and were placed individually in Petri dishes. For this trial, the gill arches were immersed in different nanoemulsion solutions of *P. emarginatus* oil (0, 50, 100, 200, 400 and 600 mg/L). There was a control group consisting only of use of water from the farming tank and five different concentrations of nanoemulsion from *P. emarginatus* oil, with three repetitions for each treatment. All the in vitro tests were performed at the ambient temperature of 23°C. Using cold-light stereomicroscopes, visual fields containing at least 15 monogeneans were selected for each repetition and, after immersion of the gill arches in the different concentrations of nanoemulsion from *P. emarginatus* oil or in tank water, the arches were then viewed every five min to quantify the numbers of monogeneans that were dead or alive on each Petri dish. The parasites were considered to be dead if they had detached from the gill tissue or if they had totally lost mobility while still attached (Hashimoto et al., 2016). After died, all monogeneans exposed to the nanoemulsion of *P. emarginatus* were fixed in 5% formalin and used for identification of the species. The efficacy of each treatment was then determined (Zhang et al., 2014).

3 | RESULTS

3.1 | Gas chromatography analysis

The chromatogram (Figure 1) obtained from qualitative gas chromatography analysis revealed the presence of some sesquiterpene chemical constituents of the essential oil of *P. emarginatus*, such as β-elemene (Rt = 43.180 min), β-caryophyllene (Rt = 44.820 min) and a-humulene (Rt = 46.870 min). The mass spectra of the sesquiterpenes identified are presented in Figure 2.

3.2 | Characterization of the nanoemulsion from *Pterodon emarginatus*

Figure 3 shows the particle size distribution profile of the nanoemulsion that was prepared from the essential oil of *P. emarginatus*. It was seen that the mean drop size and polydispersity index were

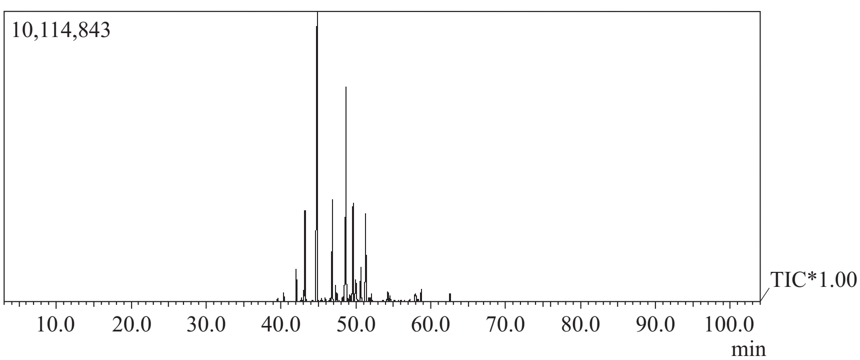

FIGURE 1 Total ion chromatogram of *Pterodon emarginatus* essential oil with a maximum peak relating to the sesquiterpene β-caryophyllene

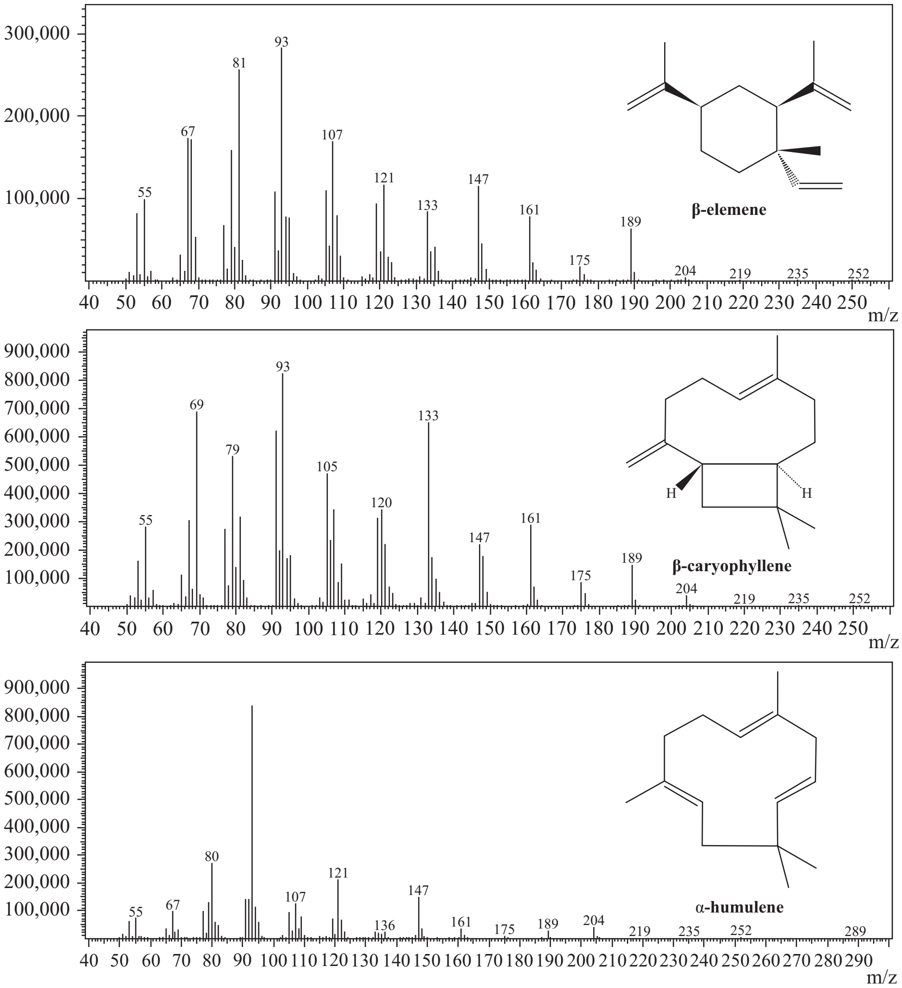

FIGURE 2 Mass spectra showing the fragmentation pattern of the sesquiterpenes β-elemene, β-caryophyllene and α-humulene in essential oils of *Pterodon emarginatus*

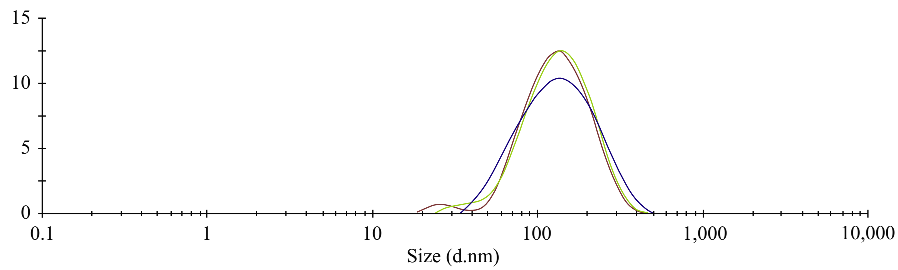

FIGURE 3 Particle size distribution of nanoemulsion prepared from *Pterodon emarginatus* [Colour figure can be viewed at wileyonlinelibrary.com]

small: 116.8 ± 0.3606 nm and 0.187 ± 0.008, respectively. The zeta potential value obtained was −19.9 ± 2.23 mV.

3.3 | In vitro antiparasitic efficacy of the nanoemulsion from *Pterodon emarginatus*

During the in vitro test, the concentrations of 100, 200, 400 and 600 mg/L of the nanoemulsion from *P. emarginatus* showed 100% antiparasitic activity against the monogeneans (*Anacanthorus spathulatus*, *Notozothecium janauachensis* and *Mymarothecium boegeri*) that were infesting the gills of *C. macropomum*. At the highest two concentrations, immobilization of the parasites was observed after only 5 min. On the other hand, at the concentration of 50 mg/L, immobilization of the parasites occurred after 5 h of exposure, which was similar to the controls (Table 1 and Figure 4).

4 | DISCUSSION

The chemical characterization showed that the *P. emarginatus* oil consists of sesquiterpenes, of which β-elemene, β-caryophyllene and α-humulene were the representative. Similarly, the presence of these compounds was reported in other studies using the essential oil of *P. emarginatus* (Alves et al., 2013; Dutra et al., 2009). However, high

TABLE 1 In vitro antiparasitic activity of nanoemulsion from the essential oil of *Pterodon emarginatus* at different concentrations, against parasites on the gills of *Colossoma macropomum*, as a function of duration of exposure

Time of exposure	Concentration (mg/L)	Live parasites	Dead parasites (%)
0 h	0	21.7 ± 5.8	0
	50	18.3 ± 2.9	0
	100	15.7 ± 0.6	0
	200	18.0 ± 4.4	0
	400	19.0 ± 6.9	0
	600	17.0 ± 6.1	0
15 min	0	21.7 ± 5.8	0
	50	18.3 ± 2.9	0
	100	14.3 ± 0.6	8.5
	200	1.3 ± 1.2	92.6
	400	0 ± 0	100
	600	0 ± 0	100
40 min	0	21.7 ± 5.8	0
	50	18.3 ± 2.9	0
	100	13.7 ± 0.6	12.8
	200	0 ± 0	100
	400	0 ± 0	100
	600	0 ± 0	100
70 min	0	21.7 ± 5.8	0
	50	18.3 ± 2.9	0
	100	8.7 ± 1.5	44.7
	200	0 ± 0	100
	400	0 ± 0	100
	600	0 ± 0	100
90 min	0	21.7 ± 5.8	0
	50	18.0 ± 2.6	1.8
	100	0 ± 0	100
	200	0 ± 0	100
	400	0 ± 0	100
	600	0 ± 0	100
2 h	0	14.0 ± 1.7	58.5
	50	0 ± 0	100
	100	0 ± 0	100
	200	0 ± 0	100
	400	0 ± 0	100
	600	0 ± 0	100
5 h	0	0 ± 0	100
	50	0 ± 0	100
	100	0 ± 0	100
	150	0 ± 0	100
	200	0 ± 0	100
	300	0 ± 0	100

variability of the chemical composition of the essential oil of *P. emarginatus* may occur, depending on the plant collection locality and other factors (Alves et al., 2013).

Nanoemulsions are kinetically stable due to their steric balance, especially when formulated with non-ionic surfactants. Due to their small-size droplets, they are resistant to being physically affected by creaming and instability of sedimentation (Bajerski et al., 2016; Oliveira et al., 2017). Our nanoemulsion from *P. emarginatus* presented particles of mean size below 200 nm and narrow size distribution. The zeta potential enables prediction of the particle stability, given that repulsion forces between the drops predominate (Heurtault, Saulnier, Pech, Proust, & Benoit, 2003). Therefore, we can conclude that our satisfactory results suggest that this nanoemulsion has potential to achieve appropriate stability. The small size of the nanoemulsion drops, which can be achieved even with low concentrations of surfactant, is directly associated with several advantages of this colloidal system, such as kinetic stability and fine appearance (Bajerski et al., 2016; Solè, Solans, Maestro, González, & Gutiérrez, 2012). The viability of the low-energy input method for preparing nanoemulsions from essential oils has also been reported from using clove oil (Anwer et al., 2014). The absence of any heating stage can be considered an advantage, given that this may diminish the loss of compounds, in the light of the volatile nature of the constituents of essential oils. As the nanoemulsion from *P. emarginatus* essential oil that was formulated here had parameters in suspension in accordance with expected physical stable nanoemulsions (Solans, Izquierdo, Nolla, & Azemar, 2005; Solè et al., 2012), it was therefore used in the in vitro antiparasitic treatment against monogeneans infesting *C. macropomum*.

Outbreaks of diseases pose a major threat for sustainable aquaculture development in Brazil. Application of herbal products in nanoemulsions to combat parasitic diseases provides an alternative approach for sustainable aquaculture. Thus, over the last few years, these systems have been extensively investigated as new means for improving the dissolvability, efficacy and biological capability of therapeutic substances (Anwer et al., 2014; Bajerski et al., 2016; Oliveira et al., 2017). In the present study, 400 and 600 mg/L of a nanoemulsion from the essential oil of *P. emarginatus* showed 100% efficacy against monogeneans (*A. spathulatus*, *N. janauachensis* and *M. boegeri*) infesting *C. macropomum*, after 15 min of exposure. Essential oil obtained from the fruit of *Pterodon pubescens* also showed therapeutic action in combating cercariae of *Schistosoma mansoni* (Mahajan & Monteiro, 1973; Mors et al., 1967). Similar studies have also reported in vitro anthelminthic activity presented by the essential oil of *Lippia alba* (Soares et al., 2016) and *Lippia origanoides* (Soares et al., 2017) against monogeneans infesting *C. macropomum*, and by the oleoresin of *Copaifera duckei* against monogeneans of *Piaractus mesopotamicus* (Costa et al., 2017). Hashimoto et al. (2016) reported that the essential oils of *Mentha piperita* and *Lippia sidoides* had 100% efficacy in vitro against monogeneans infesting *Oreochromis niloticus*. Although these different essential oils provided promising results in vitro against

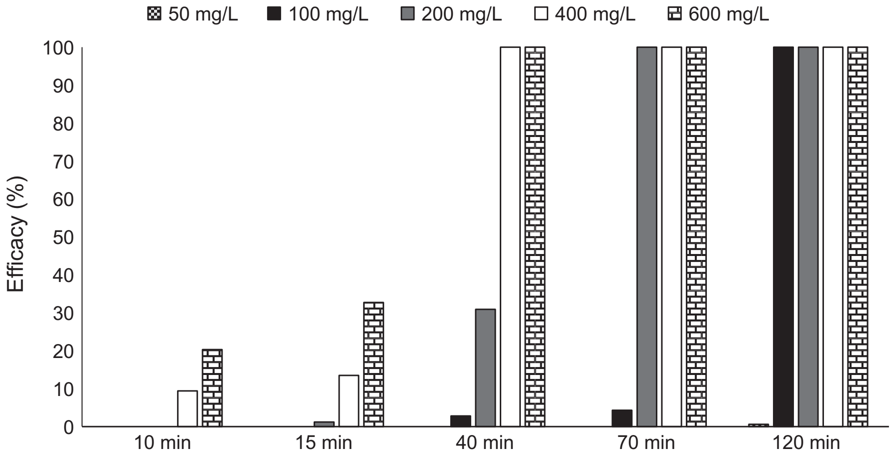

FIGURE 4 In vitro efficacy of different concentrations of nanoemulsion prepared from *Pterodon emarginatus* essential oils, against monogeneans on the gills of *Colossoma macropomum*

monogeneans infections in fish, their toxicity limited their use for prevention and treatment in therapeutic baths (Costa et al., 2017; Hashimoto et al., 2016; Soares et al., 2016). However, Anwer et al. (2014) showed that a nanoemulsion from the essential oil of cloves presented enhanced antibacterial effects in comparison with pure essential oil. Therefore, it might be possible to use nanoemulsions containing the abovementioned essential oils, at low therapeutic concentrations, for controlling monogeneans in these fish and for treating these fish, but further investigation would be needed.

In summary, nanoemulsions containing 400 and 600 mg/L of essential oil of *P. emarginatus* showed antiparasitic efficacy against monogeneans infesting *C. macropomum*, because of the biological potential and stability of the nanoformulations. These nanoemulsions from *P. emarginatus* demonstrated anthelmintic efficacy against monogeneans infesting *C. macropomum*, and this therefore opens up research on applications for controlling and preventing these parasites. Thus, these nanoformulations containing *P. emarginatus* may constitute a promising alternative treatment against monogeneans infesting *C. macropomum*. However, they need to be tested in therapeutic baths in order to determine the best concentration and means of application. Lastly, these results contribute towards reinforcing the role of nanotechnology as a significant means for revolutionizing the use of promising drugs such as essential oils.

ACKNOWLEDGEMENTS

The authors thank the Coordination Office for Improvement of Higher Education Personnel (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, CAPES) for the Master`s bursary granted to the first author, and the National Council for Scientific and Technological Development (Conselho Nacional de Desenvolvimento Científico e Tecnológico, CNPq) for the research productivity bursary granted to Marcos Tavares-Dias (#303013/2015-0).

ORCID

M Tavares-Dias http://orcid.org/0000-0002-8376-1846

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How to cite this article: Valentim DSS, Duarte JL, Oliveira AEMFM, et al. Nanoemulsion from essential oil of *Pterodon emarginatus* (Fabaceae) shows in vitro efficacy against monogeneans of *Colossoma macropomum* (Pisces: Serrasalmidae). *J Fish Dis*. 2018;41:443–449. https://doi.org/10.1111/jfd.12739