

Efficacy of *Ocimum gratissimum* essential oil against the monogenean *Cichlidogyrus tilapiae* gill parasite of Nile tilapia

[Eficácia do óleo essencial de *Ocimum gratissimum* contra o monogenea *Cichlidogyrus tilapiae* parasita de brânquias de tilápia-do-nilo]

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ABSTRACT

The phytotherapy is an alternative to use of chemotherapeutical agents against monogenean infection. This study evaluated the anthelmintic activity of essential oil *Ocimum gratissimum* against monogenean *Cichlidogyrus tilapiae* as well as its acute toxicity in tilapia juveniles. The mean lethal concentration (LC_{50}) and different concentrations of the essential oil, both *in vitro* and *in vivo* assays (short and long-term baths) were assessed. The LC_{50} was 40.70mg.L^{-1} and in the *in vitro* assay this concentration showed 80% efficacy at the last two hours and in the *in vivo* assay 65.87% efficacy in long-term bath. However, it provoked morphological alterations on the gills such as hyperplasia and edema. The parasites exposure at the highest concentration (320mg.L^{-1}) showed 100% mortality after 2h exposure in the *in vitro* assay, whereas in the *in vivo* assay, short-term baths of 5min for 3 consecutive days showed an efficacy of 87.71% without gills damage. These results demonstrate the anthelmintic activity of essential oil *O. gratissimum* and the safety concentration to use in Nile tilapia.

Keywords: disease, freshwater fish, helminth, natural product, treatment

RESUMO

A fitoterapia é uma alternativa ao uso de agentes químicos contra infecções por parasitos monogenéticos. Este estudo avaliou a atividade anti-helmíntica do óleo essencial *Ocimum gratissimum* contra o monogenea *Cichlidogyrus tilapiae*, bem como sua toxicidade aguda e histopatologia em juvenis de tilápias. Foram avaliadas a concentração letal média (CL_{50}) e diferentes concentrações de óleo essencial, tanto *in vitro* como *in vivo* (banho de curta e longa duração). A CL_{50} foi de $40,70\text{mg.L}^{-1}$; no ensaio *in vitro*, essa concentração apresentou 80% de eficácia, e no ensaio *in vivo* 65,87% de eficácia em banho de exposição crônica. No entanto, provocou alterações morfológicas nas brânquias, como hiperplasia e edema. A exposição dos parasitas na concentração mais elevada (320mg.L^{-1}) mostrou 100% de mortalidade após duas horas de exposição no ensaio *in vitro*, enquanto no ensaio *in vivo*, em banho de curta duração de cinco minutos, durante três dias consecutivos, apresentou uma eficácia de 87,71%, sem danos às brânquias. Esses resultados demonstraram a atividade anti-helmíntica do óleo essencial de *O. gratissimum* e a concentração de segurança para uso na tilápia-do-nilo em banhos de curta duração.

Palavras-chave: doença, peixe de água doce, helmintos, produto natural, tratamento

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INTRODUCTION

Outbreak of diseases in production facilities can cause expressive financial losses to fish farmers (Faruk *et al.*, 2004), among these diseases, the monogenean helminths are the main cause of mortalities (Martins *et al.*, 2002; Pinto *et al.*, 2009) being considered an obstacle to productivity of fish farms in intensive systems.

Over the past years, the use of medicinal plants in aquaculture for diseases control has been prioritized because of its high therapeutic potential since observed to fishes and shrimps in several countries, such as Mexico (Ocampo and Jimenez, 1993), India (Chakraborty and Hancz, 2011), Thailand (Direkbusarakom *et al.*, 1996) and Brazil (Hashimoto *et al.*, 2016). Furthermore, the use of medicinal plants can avoid the use of chemotherapeutic agents which provoke resistant parasites and impacts on the environment (Doughari *et al.*, 2009).

The extracts and essential oil of plants have been used in controlling fish monogeneans, such as the *Artemisia annua* extract against the monogenean in *Heterobranchus longifilis* (Ekanem and Brisibe, 2010), tea tree essential oils against *Gyrodactylus* sp. in *Gasterosteus aculeatus* (Steverding *et al.*, 2005), cinnamon oil against monogenean in tambaqui *Colossoma macropomum* (Boijink *et al.*, 2015), *Mentha piperita* and *Lippia sidoides* essential oil against *Cichlidogyrus tilapia*, *C. thurstonae*, *C. halli*, and *Scutogyrus longicornis* gill parasites of Nile tilapia *Oreochromis niloticus* (Hashimoto *et al.*, 2016) and Cajeput (*Melaleuca cajuputi*) oil against *Gyrodactylus turnbulli* of guppies (*Poecilia reticulata*) (Schelkle *et al.*, 2015).

Ocimum gratissimum commonly known as African basil, is widely distributed in tropical regions, and has the eugenol as the main compound presenting analgesic, antimicrobial, antifungal and immunostimulant activity (Silva *et al.*, 2012). The use of *O. gratissimum* leaf extracts or essential oil presented efficacy against *Haemonchus contortus*, helminth parasite from small ruminants (Pessoa *et al.*, 2002). However, its efficacy for monogenean parasites and toxicity for Nile tilapia has not been proven.

The current study evaluated the anthelmintic activity of *O. gratissimum* essential oil against

Cichlidogyrus tilapia as well as its acute toxicity and histopathological effect on Nile tilapia juveniles.

MATERIAL AND METHODS

This study was approved by the Embrapa Ethics Committee for Animal Experimentation (CEUA/02.12.01.020.00.06.006/2014).

Tilapia (*Oreochromis niloticus*) originated from a local fish farmer were transported to Embrapa Tabuleiros Costeiros Aquaculture Laboratory, acclimatized in polyethylene tanks (500L) and maintained for 10 days at $26.2\pm0.81^{\circ}\text{C}$ (YSI model 55-12FT), dissolved oxygen $6.45\pm0.58\text{mg.L}^{-1}$ (YSI model 55-12FT), pH 7.33 ± 0.7 (AKROM model KR20) and total ammonia $0.55\pm0.07\text{mg.L}^{-1}$ (HANNA model HI 93715) in recirculation system with artificial aeration. Extruded diet for omnivore fish (28% crude protein) was offered twice a day. Additionally three experimental assays were carried out: acute toxicity (mean lethal concentration, LC₅₀) to evaluate the toxic effects on tilapia juveniles, *in vitro* and *in vivo* assays to evaluate the efficacy of concentrations and strategies of application against monogenean.

The herbal *Ocimum gratissimum* was cultured in EMBRAPA Western Amazon, Manaus, AM, Brazil ($10^{\circ}56'45.7''$ S and $37^{\circ}02'58.5''$ W) and the shoots of one year old and reproductive stages were collected for extraction by hydro distillation using Clevenger-like equipment. The leaves (100g) were added to a flask (2L) with 1000mL distilled water. The essential oil samples were quantified and stored at a temperature of -20°C. After extraction, the essential oil showed 42.3% of eugenol, 20.4% of 1.8 cineole (eucalyptol) and 12.9% of β-selinene as major chemical components determined by gas chromatography according to Potzernheim *et al.* (2012) (Table 1). Prior to assays, the oil samples were diluted in 70% alcohol (1:10v:v) and maintained refrigerated at -18°C (Inoue *et al.*, 2005).

The methodology used to determine the mean lethal concentration was based on protocols defined by the US Environmental Protection Agency (EPA, 1996). The experiment used 72 tilapias (*O. niloticus*) (2.99g weight and 6.4cm total length) distributed into 18 recipients. The

recipients consisted of beakers containing 2L of water and fish biomass of 11.97 ± 1.75 g. The experiment was carried out in a static system, lasted 96h, evaluating six concentrations of the essential oil (0, 20, 40, 60, 80 and 100mg.L^{-1}) in triplicate. The mortality and behavior was evaluated every 6h with removal of the dead fish.

Table 1. Chemical compounds of essential oil of clove basil

RI	Identification	Proportion (%)
932	α -pinene	1.4
972	sabinene	0.8
976	β -pinene	3.8
1027	Limonene	0.7
1029	1,8-cineole	20.4
1100	Linalool	1.6
1165	δ -terpineol	0.6
1190	α -terpineol	1.6
1357	Eugenol	42.3
1418	(E)-caryophyllene	4.9
1450	α -humulene	0.8
1488	β -selinene	12.9
1494	α - selineno	3.2
1513	BHT	1.0
1582	Caryophyllene oxide	4.0

The monogeneans (*C. tilapia*) were collected from the gill arches from 10 fish (30-50g weight). The fish were euthanized by medullar section. An essential oil solution was prepared with $650\mu\text{l}$ of *O. gratissimum* oil and $350\mu\text{l}$ of 70% alcohol (Inoue *et al.*, 2005). The *in vitro* assay was carried out with 5 treatments in triplicate each as follows: control; control plus 70% alcohol and parasites exposed at concentrations of 40, 160 and 320mg.L^{-1} of essential oil. The monogeneans ($n=75$) were transferred to Petri dishes and mortalities were observed every hour for five hour aided by stereomicroscope (Hashimoto *et al.*, 2016). The monogenean were considered dead if they did not show any movement, even after tactile stimulation (Sutili *et al.*, 2014) and when it transferred to water without the oil.

The best results from *in vitro* assay (160 and 320mg.L^{-1}), the mean lethal concentration

($LC_{50}=40\text{mg.L}^{-1}$) and 9mg.L^{-1} of essential oil were used to evaluated the short and long-term therapeutic baths. Seventy five tilapia (mean weight 61.27 ± 25.81 g; mean total length 14.63 ± 2.06 cm) were distributed in 15 tanks (90L, static system and constant aeration). The experiment was carried out with 5 treatments (control; 9mg.L^{-1} ; 40mg.L^{-1} ; 160mg.L^{-1} and 320mg.L^{-1} essential oil) in triplicate.

The concentrations of 9mg.L^{-1} and 40mg.L^{-1} were applied as long-term baths lasted 48h by dilution of essential oil in the fish tanks. Further, the concentrations of 160mg.L^{-1} and 320mg.L^{-1} were applied as short-term baths of 10 and 5min, respectively, for three consecutive days. For the last two concentrations, the fish were removed from tanks and immersed on the concentrations of essential oil in recipients of 5L capacity. After the time exposure, the fish were reallocated in original tanks.

At the end of experimental period all fish were euthanized by medullar sectioning and their gills were collected and fixed in 10% neutral buffered formalin. The gill arches were submitted to histological analysis (Behmer *et al.*, 1976) and sections of $5\mu\text{m}$ were stained with hematoxylin-eosin (H&E). The lesions were evaluated semi-quantitatively by ranking tissue lesion according to the severity as suggested by Sirimongkolvorakul *et al.* (2012).

Other fragments were analyzed in Petri dishes for monogenean quantification using a stereomicroscope. Parasite indices of prevalence and mean intensity (Bush *et al.*, 1997), the total number of parasites, survival rates and the efficacy of the therapeutic baths (Martins *et al.*, 2001) were determined.

The normality and homogeneity of variances were analyzed by Shapiro Wilk and Bartlett tests, respectively. Data without normality distribution was transformed in arcsen (survival, total of parasites, prevalence, mean intensity, efficacy) and submitted to Analysis of Variance (ANOVA) and Tukey tests ($P<0.05$) (Zar, 1999). The lethal concentration (CL_{50} ; 96h) was determined by using the Trimmed Spearman Karber method (Hamilton *et al.*, 1977).

RESULTS

The lethal concentration (LC_{50} ; 96h) of *O. gratissimum* oil for Nile tilapia was 40.70mg.L⁻¹ with the highest and the lowest limits of 51.44mg.L⁻¹ and 32.20mg.L⁻¹, respectively. In the highest concentration, 83.3% mortality was found after 6h exposure. At the end of experimental period, the mortality rates were 33.33%, 50%, 75%, 83.33% and 100% at the concentrations of 20, 40, 60, 80 and 100mg.L⁻¹, respectively. Fish exposed to higher concentrations of *O. gratissimum* essential oil showed an equilibrium loss and reduced opercular movement.

During the first hour of exposure a monogenean mortality of 87% was observed in concentration of 320mg.L⁻¹ achieving a total mortality at the second hour. The concentration of 160mg.L⁻¹ provoked 33%, 87% and 100% mortality after the first, second and third hour, respectively. The concentration of 40mg.L⁻¹ showed 80% monogenean mortality during the last two hours. Nevertheless, no difference between controls and treatments was found after 2h ($P<0.05$) (Figure 1).

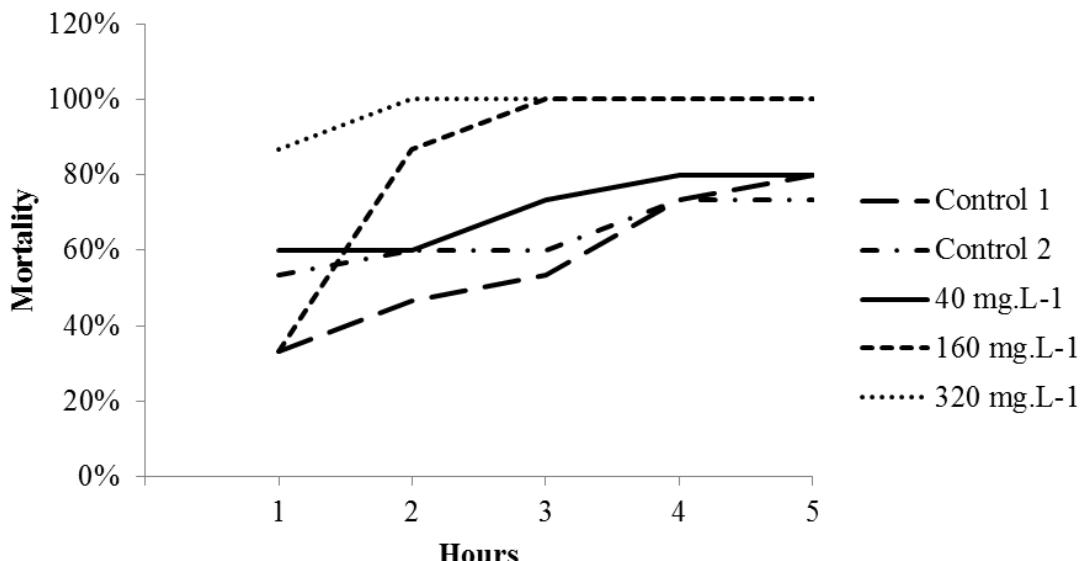


Figure 1. Monogenean mortality (%) after exposure at different concentrations of *Ocimum gratissimum* essential oil.*Difference by Tukey test ($P<0.05$).

Tilapia survival did not differ between the groups exposed to the essential oil and control (Figure 2A). However, anesthetic effects were observed in fish exposed to higher concentrations of *O. gratissimum* reflected by equilibrium loss and reduction of opercular movement.

Regarding parasite control, there was no significant difference ($P>0.05$) in prevalence among the treatments (Figure 2B). Nevertheless, significant difference ($P<0.05$) in the mean intensities was observed. The highest values for mean intensity were observed in control and the lowest value in the highest concentrations 160

and 320mg.L⁻¹ (Figure 2C). Fish exposure at 160mg.L⁻¹ and 320mg.L⁻¹ presented efficacy of 86.68% and 87.71% ($P<0.05$), respectively (Figure 2D). The long-term baths (9mg.L⁻¹ and 40mg.L⁻¹) presented a light reduction of parasitic infection with efficacy of 52.90% and 65.87%, respectively (Figure 2D), however, no difference was observed in the mean intensity.

Morphologically, the long-term baths (9 and 40mg.L⁻¹) caused hyperplasia on branchial epithelial cells of primary lamellae and epithelium lifting in the secondary lamellae with sub-epithelial edema (Figure 3, Table 2).

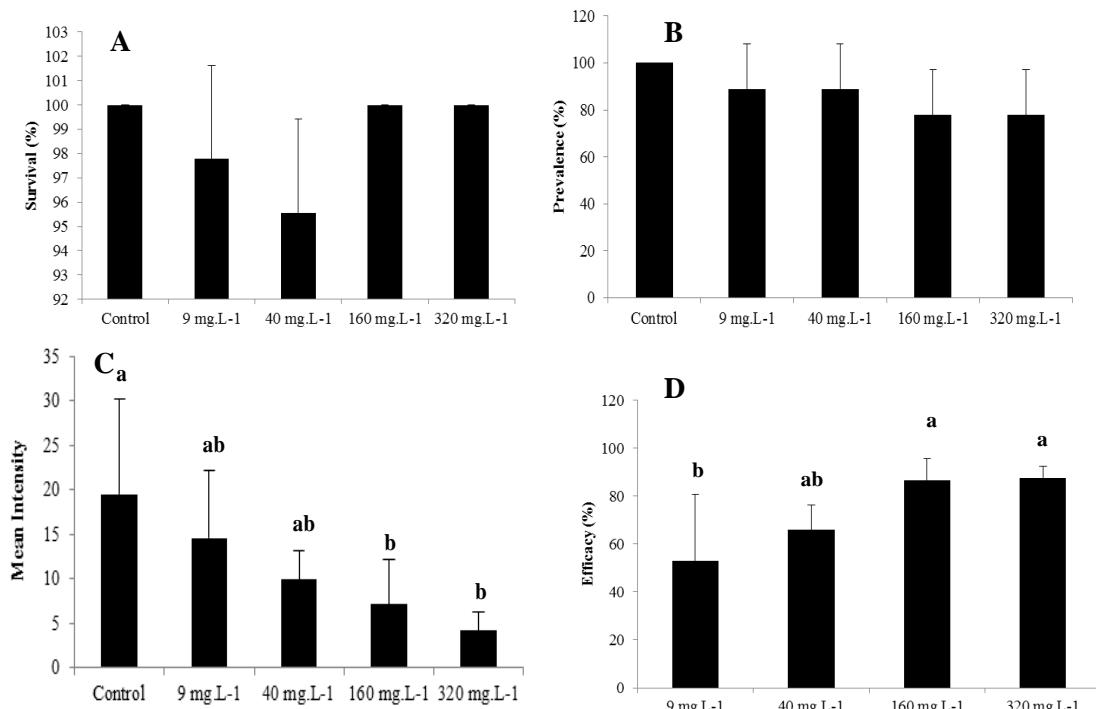


Figure 2. (A) Survival of fish after exposure at different concentrations of *O. gratissimum* essential oil. (B) Prevalence of monogenean. (C) Mean intensity of monogenean. (D) Efficacy against monogenean. Mean values (\pm SD) with the same letter did not differ statistically by Tukey test ($P>0.05$).

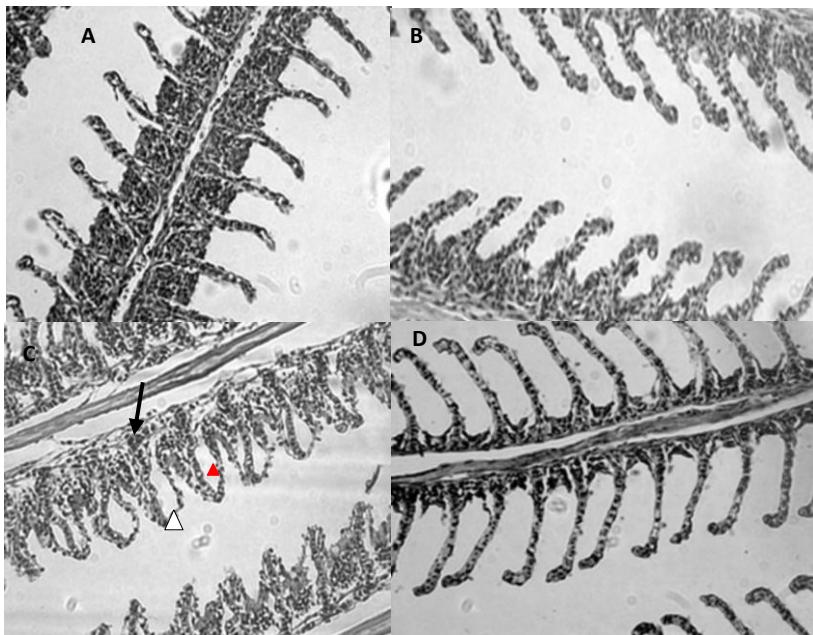


Figure 3. Histopathological gill analysis of *O. niloticus* after exposure using different concentration and application methods of *O. gratissimum* oil. (A) Control group: normal appearance of gills showing light hyperplasia. (B) Group exposure in long-term baths with 9mg.L^{-1} : gills displaying severe hyperplasia. (C) Group exposure in long-term baths with 40mg.L^{-1} : gills displaying severe hyperplasia (arrow) and epithelial lifting and subepithelial edema (arrow head). (D) Group exposures in short-term baths with 160 and 320mg.L^{-1} : gills showing normal appearance. Note: H & E stain and 40X.

Table 2. Histopathological gill analysis of *Oreochromis niloticus* after exposure using different concentrations and application methods of *Ocimum gratissimum* oil

Alterations	Control	9mg.L ⁻¹	40mg.L ⁻¹	160mg.L ⁻¹	320 mgL ⁻¹
Primary filament hyperplasia	+	++	+++	-	-
Epithelial lifting	-	-	++	-	-

(-) no histopathology; (+) histopathology in more than 20% of the fields; (++) histopathology in 20 to 60% of fields and (+++) histopathology in more than 60% of the fields.

DISCUSSION

Studies on *O. gratissimum* toxicity in fish are scarce. Crude leaves extract from this plant present LC₅₀ of 1.271,22mg.L⁻¹ for fish (Kone *et al.*, 2013) higher value than that found in present study. In fact, lower concentrations of essential oil provoked similar effect, which demonstrates the higher toxicity of the essential oil compared to extract.

Eugenol, the major compound of the *O. gratissimum* essential oil (Silva *et al.*, 2012), was found to be LC₅₀ of 71.68mg.L⁻¹ (Pratte-Santos and Carrielo, 2010), 184.26mg.L⁻¹ (Vidal *et al.*, 2008) for tilapia larvae (0.44g) and fingerlings (5.34g), respectively. The *O. gratissimum* essential oil was more toxic for tilapia than eugenol, demonstrating that other compounds of essential oil can affect the toxicity for fish. However, for the other two major components, 1.8 cineole and β-selinene, no information on the toxicity for fish is available.

The anthelmintic activity of essential oil of *O. gratissimum* was also observed in monogenean of tambaqui (*Colossoma macropomum*) at 10 and 15mg.L⁻¹ after one week of exposure (Boijink *et al.*, 2016). The authors assigned the anthelmintic effect to the eugenol, the main compound of *O. gratissimum* essential oil. An anthelmintic activity has also been reported for 1.8 cineole (Oliveira *et al.*, 2014) the second major compound of *O. gratissimum* essential oil. Furthermore, the β-selinene is present in the essential oil of many plants and it was the third major compound of *O. gratissimum* oil, but no anthelmintic activity has been reported.

The eugenol presented efficacies of 80 and 90% against monogenean *Gyrodactylus* sp., parasite of jundiá (*Rhamdia quelen*), when exposed to 5

and 10mg.L⁻¹ for 1h baths (Sutili *et al.*, 2014). Compared to *O. gratissimum* oil, similar efficacies were observed in therapeutic short-term baths at 160 and 320mg L⁻¹ for 10 and 5 minutes, respectively. These concentrations presented higher values of eugenol equivalent concentrations of 67.2mg.L⁻¹ and 134.4mg.L⁻¹, respectively.

In long-term baths (48h), lower efficacies of 52.90% and 65.87% were observed at concentrations of 9mg.L⁻¹ (3.6mg.L⁻¹ eugenol equivalent) and 40mg.L⁻¹ of essential oil (16.8mg.L⁻¹ eugenol equivalent), respectively. The lower efficacy values in long-term baths is probably due to a toxicological antagonism effect among eugenol and 1.8 cineole. However, this hypothesis remains to be proven. Moreover, the sensitivity of monogenean is species-specific and concentration and exposure time affect the efficacy of the treatments (Boijink *et al.*, 2016) and the fish survival.

Higher concentrations as 300mg.L⁻¹ could be utilized in short-term baths (4min) to anesthetize without physiological damage or fish mortalities (Silva *et al.*, 2012). The short-term therapeutic baths (5 and 10min) with 160 and 320mg.L⁻¹ of *O. gratissimum* oil presented the best results of efficacy without morphological gill damage.

The hyperplasia and edema found in long-term baths are common responses to pollutant and metal contaminations representing an adaptive response to maintain physiological functions of fish (Sirimongkolvorakul *et al.*, 2012). Concentrations above 30mg.L⁻¹ of essential oil of *O. gratissimum* for long period cause fish mortalities (Silva *et al.*, 2012) as observed in treatment with 9 and 40mg.L⁻¹. These long-term baths with essential oil aggravated the

morphological damage and probably fish more sensitive died due to those alterations.

An anesthetic effect of essential oil of *O. gratissimum* were reported to tambaqui (*C. macropomum*) and matrinxã (*Brycon amazonicus*) at concentrations of 50-100mg.L⁻¹ for 10min (Boijink *et al.*, 2016). No stress was reported in tilapia exposed to eugenol until 80mg.L⁻¹ (Deriggi *et al.*, 2006). Therefore, a strategic measure of the essential oil of *O. gratissimum* combining the anesthetic and parasite control effects must be considered as a prophylactic method and would be an interesting measure benefiting the animal welfare.

CONCLUSION

The essential oil of *O. gratissimum* can be safely used as anthelmintic against gill monogenean *Cichlidogyrus tilapiae* when applied 160mg.L⁻¹ in short term baths (10min).

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