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Antioxidants effects and resistance against pathogens of *Colossoma macropomum* (Serassalmidae) fed *Mentha piperita* essential oil

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ABSTRACT

Immunostimulants in farmed fish diets has been considered an effective approach to health management. This study aimed to assess antioxidant effects and resistance against pathogens of *Colossoma macropomum* (tambaqui) fed essential oil (EO) of *M. piperita* at 0%, 0.5%, 1.0% and 1.5% kg^{-1} commercial feed during 30 days. Tambaqui were challenged with *Aeromonas hydrophila* and did not present clinical signs of alterations. Higher hepatic catalase activity was observed in fish fed 1.0% of *M. piperita* EO, compared with 1.5%. Renal superoxide dismutase and glutathione peroxidase decreased in the fish fed *M. piperita* EO. Hepatic and renal lipid hydroperoxide increased among fish fed diets with 1.0 and 1.5% of EO. Tambaqui fed 1.0% of *M. piperita* EO showed respiratory activity increase, compared with 0.5%. Monocytes and PAS-GL were abundant in fish fed higher EO concentrations. Reduction in monogenoideans abundance was observed in fish fed 0.5 and 1.0% of EO. Although anti-helminth effects were observed, *M. piperita* EO in tambaqui diet did not promote leukocytes and lysozyme activity increases at the concentrations assessed. It can be suggested that other concentrations should be tested in future studies, as the combined use with other products, such as immunostimulants and probiotics.

1. Introduction

Diseases in particular have a considerable economic impact on aquaculture production. Globally, has been estimated economic losses of US$ 1.05 to US$ 9.58 billion/year due to mortality caused by diseases in aquaculture (Shinn et al., 2015). The agents that cause diseases among farmed fish may be bacteria, virus, parasites, poor nutrition and environmental stress (Talpur and Ikhwanuddin, 2012; Dias et al., 2016). In fish, several chemotherapeutic substances have been used to control and treat diseases. However, treatments against diseases are complex and usually inefficient (Malheiros et al., 2016). One alternative to use of chemotherapeutic products consists of using immunostimulants, probiotics and prebiotics in the diet of farmed fish (Nayak, 2010; Dotta et al., 2014; Van Hai, 2015; Feckaninová et al., 2017). The application of immunostimulants in fish diets has been considered a more effective approach to health management in aquaculture due to the enhancement of immune capability and diseases resistance (Sakai, 1999; Nya and Austin, 2009; Adel et al., 2015).

Peptides such as lysozymes, antibodies, complement factor and other lipid factors are the first line of defense for fish. These prevent adhesion and colonization by microorganisms, thus protecting against infectious diseases (Jha et al., 2007). High or increased phagocytic activity against various pathogens has been reported in fish after treatment with different types of medicinal plants, including *Mentha piperita* (peppermint), one of the herbs most widely used worldwide, with a long history of safe use in medicinal preparations (Harikrishnan et al., 2010a; Harikrishnan et al., 2010b). *Mentha piperita* is a promising plant that may offer low-cost alternative strategy for the use in medicine and in food industry (Adel et al., 2015), due to beneficial results for antioxidant properties. The use of *M. piperita* in diet of the fish *Rutilus frisii kutum* promoted growth performance and increased the main hematological and immune humoral parameters (Adel et al., 2015).

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Therefore, essential oil of *M. piperita* can be acceptance on the diet of fish, due to the action of its natural blend of bioactive components originate from plant metabolism (Bakkali et al., 2008; Ribeiro et al., 2016; Sutili et al., 2017). The present study aimed to assess the antioxidant effects and resistance against pathogens of *C. macropomum* fed with essential oil of *M. piperita*.

2. Materials and methods

This experiment was authorized by Embrapa (Protocol: 02001.002877/2014-64), in accordance with current Brazilian legislation and with the principles adopted by the Brazilian College of Animal Experimentation. Specimens of *C. macropomum* were acquired from a local fish farm, with a mean weight of 36.0 ± 7.7 g. These were transported to the farming shed of Embrapa Amapá (Macapá, AP) and were kept under laboratory conditions for 20 days. They were fed with commercial fish feed containing 32% crude protein (CP) (Guabi, Campinas, SP, Brazil) Fish were randomly distributed into 12 water tanks of capacity 500 L (n = 20 per tank), which were connected to a biological filter, with continuous water flow and constant aeration (closed system). Water physicochemical variables were monitored (Horiba Mod. U-5000, Kyoto, Japan) every day and kept constant: dissolved oxygen at 7.32 ± 0.58 mg L^{-1}, pH at 7.46 ± 0.46, temperature at 27.78 ± 0.52 °C, ammonia at 0.4 ± 0.1 mg L^{-1}, alkalinity at 26.43 ± 14.3 mg $CaCO_3$ L^{-1} and hardness at 16.67 ± 8.56 mg $CaCO_3$ L^{-1}. The water tanks were cleaned every three days to remove excess feed and feces.

3. *Mentha piperita* oil extraction

The leaves and inflorescences of *M. piperita* were collected from the Medical Plant and Vegetable Collection, and dried at room temperature, in an open shed, in the shade, and then stored for essential oil distillation, and processed at Laboratory of medicinal plants and phytochemical, both at Embrapa western Amazon in Manaus, Amazonas State, Brazil. Those plants were identified and deposited in the IAN Herbarium of the Botany Laboratory of Embrapa western Amazon, Belém, in Pará State (Brazil).

Essential oil from *M. piperita* were obtained by hydrodistillation in a Clevenger-type apparatus for 2 h. In each distillation 500 g of leaves and inflorescences or rhizomes were used. The essential oils collected were stored at 4 °C until analysis.

The analysis of the chemical components of the essential oil were determined by gas chromatography–mass spectrometry (GC–MS). The oils were analyzed in an Agilent (Palo Alto, USA) 6890 N gas chromatograph fitted with a 5% phenyl - 95% methylsilicone (HP5, 25 m 0.32 mm 0.25 mm) fused silica capillary column. The oven temperature was programmed from 60 °C to 240 °C at 3 °C min^{-1}, and hydrogen was used as carrier gas (1.4 mL min^{-1}). The oils were diluted to 1% in dichloromethane and 1.0 mL of this solution was injected in split mode (1:100). The injector was kept at 250 °C and the detector (FID) at 280 °C. Mass spectra were obtained in an Agilent 5973 N system operating in electronic ionization mode (EI) at 70 eV, with scan mass range of 40–500 *m/z*. The sampling rate was 3.15 scans s^{-1}. The ion source was kept at 230 °C, mass analyzer at 150 °C and transfer line at 260 °C. The mass detector was coupled to an Agilent 6890 gas chromatograph fitted with a low bleeding 5% phenyl 95% methylsilicone (HP-5MS, 30 m 0.25 mm 0.25 mm) fused silica capillary column. The injection procedure and oven temperature program were the same as above. Helium was the carrier gas, at 1.0 mL min^{-1}. Linear retention indices were measured by injecting a series of n-alkanes (C7–C26) in the same column and conditions indicated above for GC analyses. Identification of the oil components was based on a computer search using the Wiley 6th ed. library of mass spectral data and by comparison of their calculated linear retention indices with data from the literature (Adams, 2007).

4. Bioassay

Four diets were used: control (0%, no inclusion of oil); essential oil of *M. piperita* in the concentrations of 0.5%, 1.0% and 1.5% kg^{-1} of commercial feed. To incorporate the essential oil into the diet, solutions were prepared using grain alcohol (Dairiki et al., 2013). The essential oil of *M. piperita* was added as needed to the grain alcohol, and this mixture was sprayed over 1 kg of commercial fish feed (32% CP and grain size of 4 mm). Feed was kept at room temperature for 24 h to dry and was then refrigerated and stored. Treatments were performed in triplicate and the fish were fed until reaching apparent satiety twice a day (at 8 a.m. and 4 p.m.).

After the feeding period of 30 days, five fish from each experimental tank were sampled to collect blood through puncturing the caudal vein. The remaining specimens were challenged using intraperitoneal inoculation with *Aeromonas hydrophila* (ATCC 7966) at a concentration of 1.5×10^8 CFU mL^{-1} (Carraschi et al., 2012). After the infection with bacteria, fish were observed for 15 days, to identify mortality and any occurrence of clinical signs of aeromoniosis, such as petechial hemorrhage, distension of the abdominal cavity, ulcerative lesions on the surface of the body, hemorrhage on the fins, fish isolated from the school and darkened color, among others. Seven days after inoculation of the bacteria, a new blood sample was taken from nine fish per treatment, to determine the respiratory activity of the leukocytes and the lysozyme levels in the fish after challenge with *A. hydrophila*.

At the end of the feeding trial (30 days) and after seven days after inoculation, fish were deprived of food for 24 h before sampling. They were anesthetized with 0.1% benzocaine (Sigma-Aldrich, St. Louis, USA) and, blood samples were obtained from the caudal vein of the fish. Blood samples were immediately divided into two parts. One half was transferred to a tube containing anticoagulant (heparin) for studying the respiratory burst assay and make the hematological analysis. Blood smears were studied by light microscopy in order to make blood cell counts.

Other half of blood sample was transferred to non-heparinized tubes for biochemical and immunological studies. Serum samples were obtained by blood centrifugation (75 G, 10 min, Centrifuge 5424, Eppendorf, Hamburg, Germany) and stored at −80 °C until use.

5. Hematological and biochemical analysis

The serum obtained was used to determine lysozyme activity through a turbidimetric assay, following Ellis (1990, with adaptations). Absorbance readings were performed using a Biospectro (Mod. SP-220) spectrophotometer. Samples collected using heparin sodium anticoagulant (Hepamax-S, Blau Farmacêutica S.A.) were kept on ice to determine the respiratory activity of leukocytes, as described by Anderson and Siwicki (1995) and Biller-Takahashi et al. (2013). The respiratory activity of the leukocytes was based on determination of the reactive oxygen species (ROS) produced through the respiratory activity of macrophages, using a colorimetric assay consisting of reduction of the staining agent nitroblue tetrazolium (NBT, Sigma, St Louis, MO, USA), which forms insoluble precipitates of dark blue coloration inside phagocytes, called formazan granules. The optical density of the solution was determined in a Biospectro (SP-220) spectrophotometer at 540 nm.

To determine lysozyme activity, the serum samples were inactivated in a bain-marie at 56 °C for 30 min, with the intention of inactivating all proteins of the complement system, so that lysozyme action would be the only factor responsible for lysis of *Micrococcus lysodeikticus*. The preparations were made using inactivated serum with a sodium phosphate buffer (0.05 M; pH 6.2). This mixture was incubated at 26 °C in the spectrophotometer. A suspension of *M. lysodeikticus* was added to the mixture to observe reductions in optical density (ΔOD) at 450 nm and 26 °C. Lysozyme activity was expressed in U mL^{-1}, corresponding to the quantity of enzymes that produced an ΔOD of 0.001 min^{-1} at

450 nm (Won et al., 2004). Liver and kidney samples from the fish were taken and frozen to assess the state of oxidative stress. The immunostimulant and antioxidant potential of the essential oil in the diet was ascertained by analyzing the superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) enzyme activity and the lipid peroxidation level (LPO). Samples of hepatic and renal tissues were homogenized in 0.1 M phosphate buffer (pH 7.04; 10% w/v) in ice, and then centrifuged in a refrigerated centrifuge at 15,000 $\times g$ for 30 min at 4°C. The supernatant was separated and used in the analysis on the antioxidant enzymes activities and lipid peroxidation level. The superoxide dismutase enzyme activity was measured as described by Gao et al. (1998) and was expressed in units (U) of enzyme per mg of protein (U SOD mg of protein^{-1}). Catalase activity was determined in accordance with the technique described by Aebi (1984) and was expressed in μmol min^{-1} mg protein^{-1}. Glutathione peroxidase activity was determined based on the methodology proposed by Paglia and Valentine (1967). GPx activity was expressed in nmol min^{-1} mg protein^{-1}. The LPO level was determined in the homogenized tissues using the ferrous oxidation-xylenol assay (Jiang et al., 1992) and was expressed as nmol mg protein^{-1}. The protein concentration in the homogenized tissues was determined in accordance with the method of Bradford (1976), using bovine serum albumin (BSA) as the standard.

6. Parasites analysis procedures in *C. macropomum*

After 30 days of feeding with 0, 0.5, 1.0 and 1.5% of *M. piperita* essential oil, five fish from each of the three repetitions of the different treatments were collected for quantification and identification of monogenoideans parasites on their gills. The gills were removed and fixed in 5% formalin. The parasites on the gills were prepared for identification following previous recommendations (Eiras et al., 2006). After quantification of the parasites, the prevalence and mean abundance of infection were calculated (Bush et al., 1997).

7. Statistical analysis

All data were initially evaluated regarding the assumptions of normality and homoscedasticity using the Shapiro-Wilk and Bartlett tests, respectively. Analysis of variance (one-way ANOVA) was applied to the data with normal distribution, followed by Tukey's test, to compare mean values. For data that did not follow this distribution pattern, the Kruskal-Wallis test was used, followed by Dunn's test, to compare medians. All tests were regarded as statistically significant when $p < 0.05$.

8. Results

The essential oil of *M. piperita* presented the following chemical components: alpha-pinene (1.1%), beta-pinene (1.9%), myrcene (1.1%), *p*-cymene (4.6%), limonene (3.1%), menthone (18.2%), isomenthone (13.4%), menthol (30.8%), 4-terpineol (1.5%), pulegone (13.7%) and menthyl acetate (9.7%).

Despite the various symptoms caused by the bacterium *Aeromonas* in fish due to this organism's virulence (Harikrishnan et al., 2009b), the tambaquis in the present study did not present signs of alterations caused by the experimental infection with *A. hydrophila*.

In relation to the antioxidant system, higher catalase activity was observed in the liver of the fish fed with the diet containing 1.0% of essential oil of *M. piperita*, compared with the level in the fish fed with a diet containing 1.5% of this essential oil. The concentration of lipid hydroperoxide increased among fish fed with diets containing 1.0 and 1.5% of *M. piperita* essential oil, compared with fish fed with a diet containing 0.5% of this essential oil. However, the concentration of superoxide dismutase did not present any significant alteration ($p > 0.05$) among the treatments.

The renal activity of superoxide dismutase decreased in the fish fed with diets containing 1.0 and 1.5% of *M. piperita* essential oil, compared with fish fed with a diet containing 0.5% of this essential oil. The activity of glutathione peroxidase decreased among fish that received diets with 0.5 and 1.0% of *M. piperita* essential oil, compared with control fish and fish that received a diet with 1.5% of this oil. The concentration of lipid hydroperoxide was higher among fish that received diets with higher levels of oil (1.0 and 1.5%). However, the catalase concentration did not show any significant alterations ($p > 0.05$) (Table 1).

Table 1
Concentrations of the antioxidant enzymes in the hepatic and renal tissues of *Colossoma macropomum* fed for 30 days on diets containing different concentrations of *Mentha piperita* essential oil.

	Mentha piperita essential oil levels' diets			
	0%	0.5%	1.0%	1.5%
		Liver		
SOD	114.1 ± 24.9^{a}	121.9 ± 31.4^{a}	106.6 ± 14.3^{a}	97.2 ± 29.2^{a}
GPx	30.5 ± 2.3^{a}	32.0 ± 4.7^{a}	35.3 ± 2.7^{a}	32.6 ± 4.5^{a}
CAT	6.1 ± 1.3^{ab}	6.5 ± 1.3^{ab}	7.5 ± 2.3^{a}	5.0 ± 0.07^{b}
LPO	10.5 ± 3.0^{ab}	7.8 ± 1.5^{b}	13.0 ± 4.2^{a}	12.5 ± 3.2^{a}
		Kidney		
SOD	152.4 ± 31.1^{ab}	176.4 ± 50.0^{a}	117.9 ± 18.5^{b}	119.5 ± 16.9^{b}
GPx	139.6 ± 28.1^{c}	79.1 ± 12.6^{b}	77.0 ± 13.3^{b}	174.0 ± 25.2^{a}
CAT	30.1 ± 7.7^{a}	36.7 ± 9.4^{a}	33.4 ± 9.9^{a}	32.1 ± 9.5^{a}
LPO	10.8 ± 2.6^{bc}	8.1 ± 0.7^{b}	14.6 ± 2.8^{ac}	15.4 ± 3.0^{a}

Values expressed as mean ± standard deviation. Different lowercase letters on the same line indicate statistically significant differences ($p < 0.05$). ANOVA, Tukey *post hoc* test. n = 15. Superoxide dismutase, SOD (U mg of protein^{-1}), glutathione peroxidase, GPx (nmol min^{-1} mg of protein^{-1}), catalase, CAT (μmol min^{-1} mg of protein^{-1}), lipid peroxidation level, LPO (nmol mg of protein^{-1}).

The respiratory activity of leukocytes and lysozyme was determined among the tambaquis that received diets with different levels of inclusion of *M. piperita* essential oil, and which were challenged with *A. hydrophila*. The fish fed with diets containing 1.0% of the essential oil of *M. piperita* showed increased levels of respiratory activity, in comparison with those fed with 0.5% (Table 2). Inclusion of different concentrations of the essential oil of *M. piperita* in the diet of the tambaquis did not promote any alteration in the respiratory activity of leukocytes, either before or after challenge with *A. hydrophila*.

Among the fish fed on a diet containing 1% *M. piperita* essential oil, there was a reduction in the levels of lysozymes after challenge with *A. hydrophila*, compared with fish fed on diets containing 1.5% of this essential oil. However, there was an increase in the levels of lysozymes in all treatments after challenge with *A. hydrophila*, compared with the level prior to the challenge (Table 2).

The number of leukocytes, lymphocytes, neutrophils, eosinophils and thrombocytes did not present any alteration ($p > 0.05$) among fish fed on diets containing the essential oil of *M. piperita*. Monocytes were more abundant among fish fed on a diet containing 1.5% of the essential oil of *M. piperita* than among fish fed on a diet containing 0.5% of this substance. The Periodic Acid Schiff-positive granular leucocytes (PAS-GL) number was higher ($p < 0.05$) among fish fed on a diet containing 1.0 and 1.5% of *M. piperita* essential oil than among the control fish. The thrombocytes, monocytes, neutrophils and PAS-GL number did not alter among the fish in any treatment, but there was a reduction in the total leukocytes and lymphocytes number among the fish that received diets with 0%, 0.5% and 1.5% *M. piperita* essential oil (Table 3).

Anacanthorus spathulatus, *Notozothecium janauachensis* and *Mymarothecium boegeri* were found in the gills of tambaqui in all treatments. A reduction ($p < 0.05$) in the abundance of monogenoideans was observed among fish that received diets containing 0.5 and 1.0% of the essential oil of *M. piperita*, but this reduction was greater among fish fed with inclusion of 1.0% of this substance

Table 2
Respiratory burst and lysozyme activities among *Colossoma macropomum* fed for 30 days on diets containing different concentrations of *Mentha piperita* essential oil and after challenge through inoculation with *Aeromonas hydrophila*.

	Mentha piperita essential oil levels' diets			
	0%	0.5%	1.0%	1.5%
Respiratory burst activity	0.6 ± 0.1^{abA}	0.5 ± 0.1^{bA}	0.6 ± 0.1^{aA}	0.6 ± 0.1^{abA}
Lysozyme activity	63.3 ± 11.7^{aA}	67.6 ± 18.7^{aA}	69.8 ± 26.3^{aA}	119.2 ± 59.5^{aA}
	After *Aeromonas hydrophila* challenge			
Respiratory burst activity	0.6 ± 0.1^{aA}	0.6 ± 0.2^{aA}	0.5 ± 0.2^{aA}	0.6 ± 0.1^{aA}
Lysozyme activity	496.0 ± 238.7^{abB}	356.1 ± 136.1^{abB}	226.9 ± 101.6^{bB}	581.7 ± 200.0^{aB}

Values expressed as mean ± standard deviation. Different lowercase letters on the same line indicate statistically significant differences ($p < 0.05$). Different uppercase letters on the same column indicate differences from before to after the bacterial challenge. ANOVA, Tukey *post hoc* test. n = 15. Respiratory burst (optical density at 540 nm) and lysozyme (U mL^{-1}).

(Table 4).

9. Discussion

Increased catalase activity was observed in the liver of tambaquis fed on a diet containing 1.0% *M. piperita* essential oil, compared with fish fed on a diet containing 1.5% of this essential oil. However, both the liver and the kidneys of fish fed on a diet containing 1.0 and 1.5% *M. piperita* essential oil presented increases in lipid lipoperoxidation. Moreover, there was also decreases in the superoxide dismutase activity in renal tissues when increasing *M. piperita* oil in the tambaqui's diet. The decrease in this enzyme was concordant with the maintenance of leukocyte respiratory activity among the tambaquis in the present study. The leukocytes of the cephalic kidney of *Carassius auratus*, infected by *A. hydrophila*, presented increased production of superoxide anion after treatment with vaccines and diets enriched with immunostimulants (Harikrishnan et al., 2009a). Herbal *Hybanthus enneaspermus* aqueous extract supplemented in fish diet was reported to enhance superoxide dismutase enzyme activities in *Labeo rohita* (Giri et al., 2017).

Increased superoxide production by leukocytes could promote increased resistance (Karczewski et al., 1991), which determined the activation of leukocytes, an important part of any method for immunostimulation in treatments for fish (Ashida and Okimasu, 2005).

Table 4
Parasitological indices for Monogenoidea on the gills of *Colossoma macropomum* fed for 30 days on diets containing different concentrations of *Mentha piperita* essential oil.

Concentrations (%)	Prevalence (%)	Mean abundance
0	100	967.1 ± 361.6^{a}
0.5	100	379.6 ± 188.1^{b}
1.0	100	80.5 ± 38.7^{b}
1.5	100	936.1 ± 537.4^{a}

Different letters in the same column for abundance indicate differences through Dunn's test ($p < 0.05$).

The glutathione peroxidase activity was reduced in the kidneys of fish that received diets with 0.5 and 1.0% *M. piperita* essential oil, compared with the control fish and those that received a diet with 1.5% of the oil. Even with renal glutathione peroxidase increased in tambaqui fed with diet with 1.5% of *M. piperita* and, lipoperoxidation occurred, demonstrating that there was no reduction in stress response and oxidative damage.

Lysozyme and particularly the neutrophils, monocytes and macrophages found in various species of marine and freshwater fish are able to cause cell lysis in the peptidoglycan layer of the cell wall of bacteria in fish (Lie et al., 1989; Saurabh and Sahoo, 2008; Abreu et al., 2009). Lysozyme levels vary according to the type of tissue in the fish analyzed

Table 3
Leukocytes and thrombocytes number of *Colossoma macropomum* fed for 30 days on diets containing different concentrations of *Mentha piperita* essential oil and after challenge through inoculation with *Aeromonas hydrophila*.

Mentha piperita essential oil levels' diets				
	0%	0.5%	1.0%	1.5%
Thrombocytes ($\times 10^3$ μL^{-1})	25.1 ± 12.4^{aA}	24.4 ± 11.9^{aA}	25.4 ± 18.1^{aA}	35.2 ± 9.4^{aA}
Leukocytes ($\times 10^3$ μL^{-1})	71.9 ± 34.5^{aA}	54.5 ± 14.6^{aA}	80.7 ± 51.7^{aA}	113.2 ± 58.7^{aA}
Lymphocytes ($\times 10^3$ μL^{-1})	52.3 ± 26.0^{aA}	43.5 ± 12.0^{aA}	45.8 ± 23.8^{aA}	77.9 ± 34.1^{aA}
Monocytes ($\times 10^3$ μL^{-1})	18.7 ± 11.3^{abA}	9.8 ± 3.4^{bA}	26.8 ± 21.5^{abA}	29.2 ± 22.4^{aA}
Neutrophils ($\times 10^3$ μL^{-1})	0.4 ± 0.5^{aA}	0.6 ± 1.1^{aA}	3.8 ± 3.9^{aA}	2.5 ± 2.2^{aA}
PAS-GL ($\times 10^3$ μL^{-1})	0.07 ± 0.2^{bA}	0.3 ± 0.4^{abA}	1.9 ± 0.6^{bA}	1.4 ± 1.7^{aA}
Eosinophils ($\times 10^3$ μL^{-1})	0.4 ± 0.6^{aA}	0.3 ± 0.6^{aA}	2.5 ± 2.4^{aA}	2.1 ± 2.1^{aA}
After *Aeromonas hydrophila* challenge				
	0%	0.5%	1.0%	1.5%
Thrombocytes ($\times 10^3$ μL^{-1})	30.9 ± 9.0^{aA}	31.0 ± 12.4^{aA}	37.3 ± 14.2^{aA}	33.3 ± 11.4^{aA}
Leukocytes ($\times 10^3$ μL^{-1})	30.5 ± 9.1^{bB}	34.2 ± 8.2^{bB}	56.2 ± 7.2^{aA}	47.4 ± 13.2^{abB}
Lymphocytes ($\times 10^3$ μL^{-1})	24.7 ± 7.7^{bB}	25.9 ± 5.8^{bB}	43.0 ± 5.4^{aA}	34.8 ± 11.1^{abB}
Monocytes ($\times 10^3$ μL^{-1})	5.0 ± 2.0^{bA}	7.8 ± 2.1^{abA}	10.8 ± 1.6^{aA}	10.1 ± 4.7^{abA}
Neutrophils ($\times 10^3$ μL^{-1})	0.5 ± 0.5^{aA}	0.4 ± 0.8^{aA}	1.8 ± 1.2^{aA}	2.4 ± 2.1^{aA}
PAS-GL ($\times 10^3$ μL^{-1})	0.1 ± 0.2^{aA}	0 ± 0^{aA}	0.5 ± 0.7^{aA}	0.1 ± 0.2^{aA}
Eosinophils ($\times 10^3$ μL^{-1})	0.3 ± 0.4^{aA}	0 ± 0^{aA}	0 ± 0^{aA}	0 ± 0^{aA}

Values expressed as mean ± standard deviation. Different lowercase letters on the same line indicate statistically significant differences ($p < 0.05$). Different uppercase letters on the same column indicate differences from before to after the bacterial challenge.

(Tort et al., 2003). Renal tissue seems to present higher concentrations of this enzyme, due to higher concentrations of leukocytes (Lie et al., 1989; Balfry and Iwama, 2004).

Tambaqui fed on diets containing the essential oil of *M. piperita* demonstrated increased lysozyme activity after being challenged with *A. hydrophila*, because the concentrations of these enzymes increased rapidly in the blood of the fish after infection (Harikrishnan et al., 2009a). In *Oreochromis niloticus*, lysozyme activity increased after they were fed on a diet containing 1% of aqueous extract of *Cratoxylum formosum* and after they were infected with *Streptococcus agalactiae* (Rattanachaikunsopon and Phumkhachorn, 2010). *Rutilus frisii kutum* fed on a diet containing 2 and 3% dried aerial parts of *M. piperita* demonstrated increased lysozyme activity (Adel et al., 2015). Therefore, the concentration of lysozymes could be useful as an indicator for the innate immune response of fish (Tort et al., 2003; Abreu et al., 2009). The innate or nonspecific immune system includes various components of an organism (antimicrobial enzymes, interleukins, interferon, and defense cells such as granulocytes, monocytes and macrophages) that act as the first line of defense. This system acts faster than the specific immune system (Magnadóttir, 2006; Saurabh and Sahoo, 2008). Thus, granulocytes are the first defense cells to arrive at an inflammation site, since they are responsible for destruction of pathogens through phagocytosis (Wang et al., 2016).

The respiratory burst and number of leukocytes, lymphocytes, neutrophils, eosinophils and thrombocytes in tambaquis were not influenced either by the different levels of *M. piperita* essential oil in the fish diet or after infection by *A. hydrophila*. However, after challenge with *A. hydrophila*, the total leukocytes and lymphocytes number decreased among the fish fed on diets containing the essential oil of *M. piperita*, probably due to migration of these cells to the tissues. The increased leukocytes and erythrocytes number may be attributed to the effective antioxidant role of 1.0% of the essential oil from *M. piperita* (Ribeiro et al., 2016), that was related by Giri et al. (2017) when applied *H. enneraspermus* in L. *rohita*.

Conversely, *R. frisii kutum* fed with a diet containing 1, 2 and 3% *M. piperita* presented an increase in respiratory burst (Adel et al., 2015). Likewise, *Piaractus mesopotamicus* infected with *A. hydrophila* also presented an increase in respiratory burst (Garcia et al., 2007; Biller-Takahashi et al., 2013). The respiratory burst of leukocytes is related to cytokine release and the inflammatory response in fish (Neumann et al., 2001; Rieger and Barreda, 2011). In *O. niloticus*, the respiratory burst and phagocytic activity increased after being fed on a diet containing 1% *C. formosum* and being infected by *S. agalactiae*, demonstrating that this immunostimulation effect can be used to prevent such infections in this species of fish (Rattanachaikunsopon and Phumkhachorn, 2010).

In fish farming, several chemotherapeutic substances (*e.g.* sodium chloride, praziquantel, levamisole, formalin, mebendazole, copper sulfate, and others) have been used on fish to control and treat monogenoidean infections. In addition, the most of these chemical products are toxic and compromise the gills, integument and liver, and also are potential pollutants of the environment and may cause risks to human health (Malheiros et al., 2016). However, herbal substances have several advantages, such as reduced environmental impact, biodegradability, lower residue levels in fish and low toxicity, resulting in low likelihood of causing resistance and low cost for farmers (Ribeiro et al., 2016; Hashimoto et al., 2016).

Abundance of the monogenoideans *A. spathulatus*, *N. janauachensis* and *M. boegeri* on the gills of *C. macropomum* decreased when fed 0.5 and 1.0% *M. piperita* essential oil, but these anti-helminth effects were twelve times higher in fish that received a diet containing 1.0% of this essential oil. Similarly, *in vitro* studies using 160 and 320 mg L^{-1} of *M. piperita* essential oil also demonstrated anti-helminth action against the monogenoideans *Cichlidogyrus tilapiae*, *Cichlidogyrus thurstonae*, *Cichlidogyrus halli* and *Scutogyrus longicornis* in *Oreochromis niloticus* (Hashimoto et al., 2016), and against *Dawestrema cycloancistrium* and *Dawestrema cycloancistrioides* in *Arapaima gigas* (Malheiros et al., 2016).

Although anti-helminth effects were observed, use of the essential oil of *M. piperita* in the diet of tambaqui did not promote increases in leukocyte count and lysozyme activity, at the concentrations assessed. It can be suggested that other concentrations should be tested in future studies, as the use of this substance combined with other products, such as immunostimulants and probiotics.

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