Induced aerocystitis and hemato-immunological parameters in Nile tilapia fed supplemented diet with essential oil of *Lippia alba*

Aerocistite induzida e parâmetros hemato-imunológicos em tilápia do Nilo suplementada com óleo essencial de Lippia alba

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Abstract

This study evaluated the dietary supplementation with essential oil of *Lippia alba* on the hemato-immunological parameters of Nile tilapia (*Oreochromis niloticus*) submitted to acute inflammation induced by carrageenin injection in the swim bladder. For a period of 45 days, 96 fish were divided into four treatments in triplicate, as follows: (a) fish fed supplemented diet with essential oil of *L. alba* (4 mL kg^{-1} dry ration) injected with carrageenin; (b) fish fed supplemented diet with cereal alcohol injected with carrageenin; (c) fish fed unsupplemented diet with essential oil injected with carrageenin; (d) fish fed unsupplemented diet and noninjected. Cortisol levels, erythrogram, leukogram and the inflammatory infiltrate were analyzed 6 hours after an inflammatory stimulus. Carrageenin-injected fish showed an acute inflammatory reaction in the swim bladder characterized by higher infiltrate of neutrophils and monocytes. The circulating neutrophils number was significantly higher in fish fed *L. alba* when compared to other treatments. No difference in cortisol levels was found. For the dose, time and administration form tested, supplementation with essential oil of *L. alba* did not present anti-inflammatory activity. On the other hand, an influence of dietary supplementation was observed on the neutrophils number after induced aerocystitis highlighting its immunomodulatory characteristic.
Keywords: *Oreochromis niloticus*. Stress. Phytotherapics. Hematology. Acute inflammation.

Resumo

Este estudo avaliou a suplementação dietária com óleo essencial de *Lippia alba* sobre os parâmetros hematoimunológicos em tilápias do Nilo (*Oreochromis niloticus*) submetidas à inflamação aguda induzida por carragenina na bexiga natatória. Pelo período de 45 dias, 96 peixes foram divididos em quatro tratamentos em triplicata: (a) peixes suplementados com óleo essencial de *L. alba* (4 mL kg^{-1} de ração seca) injetados com carragenina; (b) peixes suplementados com álcool de cereais injetados com carragenina; (c) peixes não suplementados com óleo essencial injetados com carragenina; (d) peixes não suplementados e não injetados. Os níveis de cortisol, o eritrograma, o leucograma e o infiltrado inflamatório foram analisados seis horas após o estímulo inflamatório. Peixes injetados com carragenina apresentaram reação inflamatória aguda na bexiga natatória caracterizada pela maior concentração de infiltrado de neutrófilos e monócitos. O número de neutrófilos circulantes foi significativamente maior nos peixes suplementados com *L. alba*, numa comparação com os outros tratamentos. Não houve diferença nos níveis de cortisol. Para a dose, o tempo e a forma de administração testada, a suplementação com óleo essencial de *L. alba* não apresentou atividade anti-inflamatória. Por outro lado, foi constatada influência da suplementação dietária no número de neutrófilos após a aerocistite, enfatizando a sua característica imunomoduladora.
Palavras-chave: *Oreochromis niloticus*. Estresse. Fitoterápicos. Hematologia. Inflamação aguda.

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Introduction

Inflammation is characterized by a protection process of the organisms and involves the signaling and removal of bacteria, toxins or debris resulted from injuries, such as necrotic tissue. The process is complex and involves chemical and morphological alterations especially on the blood vessels and immunocompromised circulating cells (KUMAR et al., 2005). Studies on inflammatory responses and possible strategies for its control in aquaculture have gained attention since they give required scientific support to fish handling. Farmed fish are in constant contact with different agents capable of provoking inflammatory condition (MARTINS et al., 2001)

Inflammatory response has been evaluated under several factors such as fish fed supplemented diets containing vitamins (BELO et al., 2005; MARTINS et al., 2008a; BELO et al., 2012), probiotics (REQUE et al., 2010; DOTTA et al., 2011; CASTRO et al., 2014a), amino acids, minerals and polysaccharides (FALCO et al., 2012; CASTRO et al., 2014b; MACHADO et al., 2015), phytotherapics (DOTTA et al., 2015), stocking density (BELO et al., 2005) and the stressful effect on inflammatory response (MARTINS et al., 2004). Some phlogogen agents have been used to stimulate cell migration and the most used are carrageenin (MATUSHIMA; MARIANO, 1996; MARTINS et al., 2001; MARTINS et al., 2004; MARTINS et al., 2008a; MARTINS et al., 2009; DOTTA et al., 2011), thioglycolate (MARTINS et al., 2001; BOZZO et al., 2007; MARTINS et al., 2009; MORAES et al., 2012), lipopolysaccharides (LPS) (MARTINS et al., 2004; BOZZO et al., 2007; MARTINS et al., 2008a; MORAES et al., 2012), inactivated bacteria (BOZZO et al., 2007; REQUE et al., 2010; MORAES et al., 2012; CLAUDIANO et al., 2013; CASTRO et al., 2014b; MACHADO et al., 2015) and glass coverslip (BELO et al., 2005; BELO et al., 2012). In Brazil, the inflammatory process in fish was studied in pacu *Piaractus mesopotamicus* (BELO et al., 2005; BOZZO et al., 2007; GARCIA et al., 2007; GARCIA; MORAES, 2009; BELO et al., 2012; CLAUDIANO et al., 2013; MORAES et al., 2012; CASTRO et al., 2014b), Nile tilapia *Oreochromis niloticus* (MATUSHIMA; MARIANO, 1996; MARTINS et al., 2004; MARTINS et al., 2008a; REQUE et al., 2010; DOTTA et al., 2011) and in the hybrid tambacu (*P. mesopotamicus* male x *Colossoma macropomum* female) (MARTINS et al., 2001; MARTINS et al., 2009).

Factors like fish species, phlogogen agents, feeding and stressful conditions may influence cellular kinetics in acute inflammation (MARTINS et al., 2004; BOZZO et al., 2007). However, the use of phytotherapics as immune stimulant and against pathogens has been an environmental alternative (BULFON et al., 2015; HASHIMOTO et al., 2016) due to their rapid environmental degradation, low residue levels in the substrate and animals when applied correctly, low risks for human health, low cost and ease of use (SOARES; TAVARES-DIAS, 2013).

Beneficial effects of phytotherapics were compared in several studies especially antiparasitic, antimicrobial and immunomodulatory action (SOARES; TAVARES-DIAS, 2013; HASHIMOTO et al., 2016). For example, garlic *Alllium sativum* extract reduced 95% of the monogenean parasitism in *P. mesopotamicus* (MARTINS et al., 2002). In the gills of tambaqui *Colossoma macropomum* fed supplemented diet with essential oil of basil *Ocimum gratissimum*, a decrease in the number of monogeneans was related to an increase in the oil concentration in the diet (CHAGAS et al., 2012). Therapeutic baths with essential oil of peppermint *Mentha pipperita* at 40 mg/l^{-1} for 10 min reduced up to 41.63% the number of monogeneans in the gills of *O. niloticus* and no hematological alterations were found in treated fish (HASHIMOTO et al., 2016). To date, essential oil of *O. gratissimum* at 15 mg/l^{-1} for 15 min showed 100% efficacy against monogeneans in *C. macropomum* (BOIJINK et al., 2015). Regarding immunomodulatory activity, dietary supplementation with *A. sativum* has been shown to increase total number of circulating white blood cells (WBC) in Asian sea bass *Lates calcifer* (TALPUR; IKHWANUDDIN, 2012). Recently, an increase in circulating neutrophils and monocytes was related in *O. niloticus* fed supplemented diet containing 0.5% of propolis or *Aloe barbadensis* extracts for 15 days, as well as reduced mean abundance of monogenean parasites in the gills (DOTTA et al., 2015).

The uses of natural products have been highlighted in animal health due to bioactive substances that present

good protection against microorganisms and parasites, and their pharmacological residues do not present risk to both environment and human health. Even so, because of ease of production, plants of the genus *Lippia* have been explored in veterinary medicine and aquaculture (SOARES; TAVARES-DIAS, 2013). *Lippia alba,* commonly known as false melissa, water mint, lemon verbena, lemon balm (LORENZI; MATOS, 2002) has been used in popular medicine as tea, macerated, in compresses, baths or alcoholic extracts (JULIÃO et al., 2003). Their pharmacological properties include anti-inflammatory, antimicrobial, anesthetic, antipyretic, antiviral, cytostatic and anti-seizure activities (PASCUAL et al., 2001).

In aquaculture, essential oil of *L. alba* has been evaluated in silver catfish *Rhamdia quelen* fed 0; 0.25; 0.5; 1.0 or 2.0 mL essential oil/kg^{-1} dry ration for 60 days, recommended to diminish lipid peroxidation, increase glycogen stock and antioxidative tissue response (SACCOL et al., 2013). Anesthetic effect of essential oil of *L. alba* was found to be among 100 and 500 mg/l^{-1} in *R. quelen,* in addition to its ability to inhibit the increase of cortisol levels caused by fish management and with no noticeable consequences on sensorial analysis (odor and flavor) in fish fillet (CUNHA et al., 2010).

Few studies have evaluated the use of phytotherapy in fish and its relationship with both inflammatory and hemato-immunological parameters to suggest preventive strategies against diseases or to improve fish health status. This study tested three hypotheses: dietary supplementation with *L. alba* influences the acute inflammatory response and hemato-immunological parameters; dietary supplementation reduces cell migration to the inflammatory site in controlling the phlogogen stimulation and; fish fed supplemented diet show greater immunological capacity in combating induced acute inflammatory reaction. Juveniles of Nile tilapia were fed supplemented diet with essential oil of *L. alba* for 45 days to posterior induction of the acute inflammatory response by carrageenin injection evaluated by hematological parameters, cortisol levels and inflammatory infiltrate analysis.

Material and methods

Fish and experimental design

A total of 96 healthy juveniles of Nile tilapia (27.05 ± 4.65 g mean initial weight and 10.94 ± 0.80 cm total length) from the same spawning harvested at "Empresa de Pesquisa Agropecuária e Extensão Rural de Santa Catarina", Camboriú were distributed in a completely randomized design in 12 tanks of 1,000-L capacity (useful volume 800 L) equipped with a recirculation system and provided with mechanical and biological filters. The fish were divided into four treatments with three replicates, composed of 24 fish in each: fish supplemented with essential oil of *L. alba* (4 ml/kg^{-1} ration) injected with carrageenin (*L. alba*/carrageenin); fish supplemented with cereal oil injected with carrageenin (alcohol/carrageenin); unsupplemented fish injected with carrageenin (ration/carrageenin); unsupplemented fish noninjected with carrageenin (ration).

During 10 days of acclimation period, all fish were fed commercial diet Guabi-Pirá® with 32% crude protein prior to experiment (BARCELLOS et al., 1997) and the water quality was maintained as follows: dissolved oxygen 5.67 ± 1.04 mg/l^{-1}; temperature 22.30 ± 1.97 °C and pH 8.06 ± 0.13 measured with a multiparameter Hanna® HI9829 (Hanna Instruments Brazil, SP). Fish management and sampling collections are according to the Ethic Committee on Animal Use (Ceua-UFSC PP00928).

Dietary supplementation

For *L. alba* supplemented fish, the essential oil was added to the diet weekly according to previous method (DAIRIKI et al., 2013). For each kilogram of Pirá® diet, 4 ml of essential oil was mixed with 100 ml of cereal alcohol and sprayed on the diet pellets. After that, the diet was air-dried for 24 h and maintained at –18 °C until use. For fish fed with the diluent, the pellets were sprayed only with cereal alcohol. Unsupplemented fish received only Pirá® diet. Fish were fed twice a day in a 3% of body weight for 45 days. Diet amount was adjusted weekly after biometry. At the end of the experiment, the fish showed 46.52 ± 9.36 g mean weight and 13.59 ± 0.88 cm total length.

Composition of the essential oil of Lippia alba

The plant was cultured in the Section of Medicinal Plants of Embrapa Western Amazon situated in Manaus, AM (3°6' 23.04" S and 60°1'35.14" W), mean altitude 50 m and mean air temperature 25.6 °C with annual rainfall of 2,200 mm. Leaves were collected in the morning for processing in the Medicinal Plants and Phytochemistry Laboratory of Embrapa Western Amazon, Manaus, Brazil. Oil extraction was made by hydro-distillation method using a Clevenger-like equipment and the oil was stocked in amber vials at

-18 °C. The chemical composition was analyzed using a gas chromatograph Agilent (Palo Alto, California, USA) 7890A equipped with capillary column HP-5 (5%-diphenyl-95%-dimethyl silicon 30 m × 0.32 mm × 0.25 µm) as previously detailed (HASHIMOTO et al., 016). Due to morphological and chemical variability, *L. alba* can be divided in chemotypes according to the predominant constituents present in the essential oil (PASCUAL et al., 2001). Essential oil used in this study showed carvone-limonene as the main constituent (Figure 1).

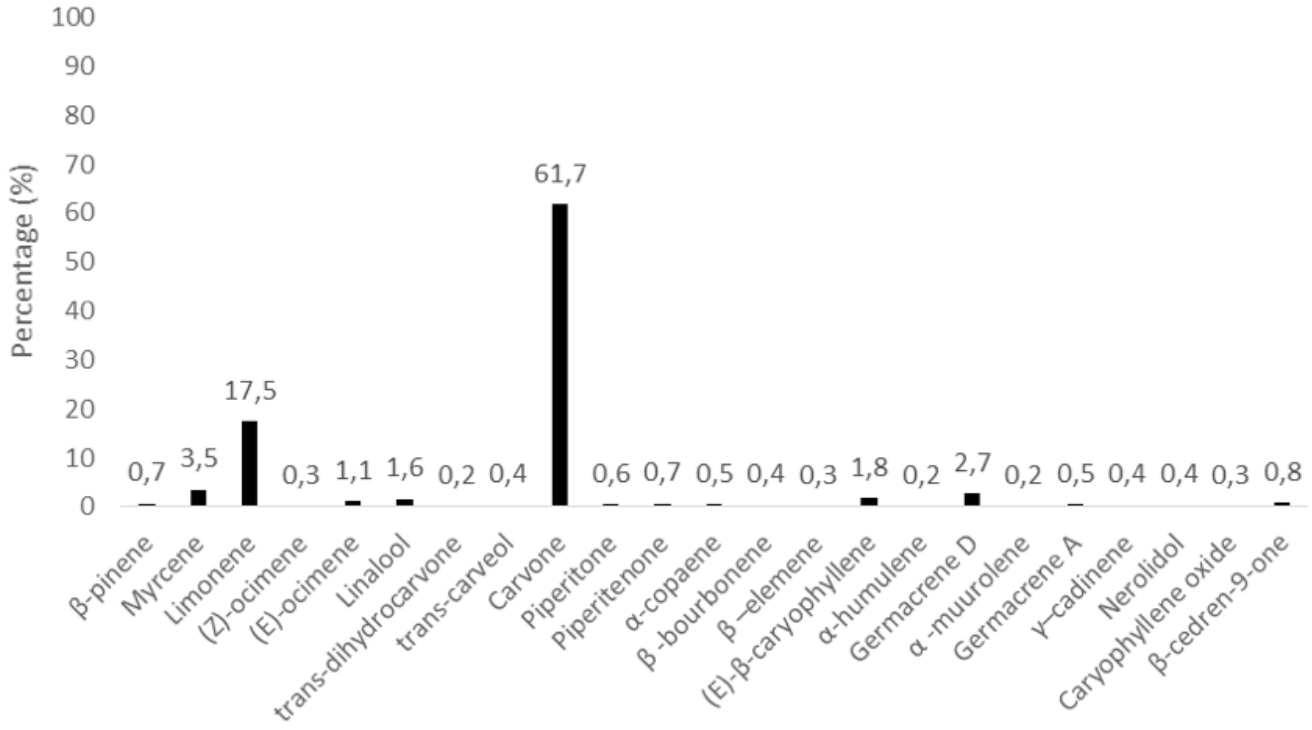

Figure 1 – Composition of essential oil of *Lippia alba* used in this study

Inflammatory induction

After feeding period, fish were anesthetized by immersion in eugenol (50 mg/l^{-1}) for induction of the inflammatory process in the swim bladder by injection of 500 µg carrageenin (Marine Colloids®) diluted in 0.5 ml sterile saline solution using 28-gauge insulin syringes. Six hours after injection, five fish from each replicate were anesthetized in eugenol for blood samples, euthanized, and the inflammatory exudate obtained by washing the swim bladder with buffered PBS solution added of 0.01 ml EDTA 10% and evaluated (MARTINS et al., 2001) slightly modified: dilution 1:5 in Dacie's fluid (BLAXHALL; DAISLEY, 1973).

Cortisol levels and hematological analysis

For immunological parameters and cortisol analysis, the blood from three fish per replicate was withdrawn from the caudal vein using 3 ml syringes without anticoagulant. After coagulation, the samples were centrifuged at 150 g for 10 min in a refrigerated centrifuge (4 °C) and the cortisol level measured with Cortisol ELISA kit (Enzo Life Sciences).

For hematological analysis, the blood was withdrawn using 3.0 ml syringes containing EDTA 10% to make blood smears that were stained with May-Grünwald/Giemsa/Wright for total count of leukocytes (WBC), total thrombocyte count and differential count of leukocytes (RANZANI-PAIVA et al., 2013). An aliquot was used to determine the hematocrit percentage, hemoglobin concentration and hematimetric parameters. Another aliquot was transferred to Eppendorf tubes and diluted 1:200 containing Dacie's fluid to determine the total number of erythrocytes (RBC) using a Neubauer chamber (RANZANI-PAIVA et al., 2013).

Immunological parameters

Serum total protein was measured with Total Protein kit (Lab Test®). The concentration of total immunoglobulin was measured (AMAR et al., 2000), where 50 µL of the serum

were mixed with 50 µL of a solution of 12% polyethylene glycol (Sigma-Aldrich) and the mixture was incubated at room temperature for 2 h to precipitate the immunoglobulin molecules. The immunoglobulin precipitate was removed by centrifugation (5.000 g at 4 °C for 10 min), the supernatant drawn, and the quantity of protein was also measured with the kit, using a bovine albumin to build the standard curve. The concentration of total immunoglobulin is expressed in mg/ml^{-1} and it is calculated by the formula:

Total Ig (mg/ml^{-1}) = total serum protein – protein treated with PEG

Serum agglutinating titer was made in U-bottom microplates in diluting 1:1 in PBS from the first to the 12th well. After that, 50 µL of the inactivated bacteria (*Streptococcus agalactiae* – GRS 2035) was added to all wells at an optical density of approximately 0.4 on the McFarland Scale, in 550 nm. The microplate was incubated at 25 °C for 18 h in a humidity chamber. The agglutination was confirmed visually by observing a button at the well's surface. The agglutination titer was considered reciprocal to the last well that presented agglutination (SILVA et al., 2009).

Statistical analysis

To verify the normality and homoscedasticity, the data were submitted to Kolmogorov-Smirnov and Bartlett's tests and posteriorly submitted to unifactorial variance analysis (Anova). Average comparison was made by Tukey test using the software Statistica (Statsoft). Data transformations were used when necessary, discrete variables were transformed using square root and percentage variables were transformed using arcsine square root. The means were considered significantly different when $p < 0.05$.

Results

In this study, the fish showed elevated serum cortisol levels varying from 213.44 ± 45.28 ng ml^{-1} in alcohol/carrageenin fish to 287.72 ± 39.57 ng ml^{-1} in ration/carrageenin fish with no significant difference among the treatments (Figure 2). Hematological analysis did not show significant difference in the hemoglobin concentration, hematocrit percentage, RBC and thrombocyte counts, mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC) and mean corpuscular hemoglobin (MCH) values (Table 1). No difference among the treatments was found in WBC count, circulating lymphocytes and monocytes, but an increase in the circulating neutrophils was observed in fish fed *L. alba* essential oil injected with carrageenin (*L. alba*/carrageenin) when compared with other treatments (Table 2). No difference among the treatments was observed in the total protein, total immunoglobulin and agglutination titer (Table 3).

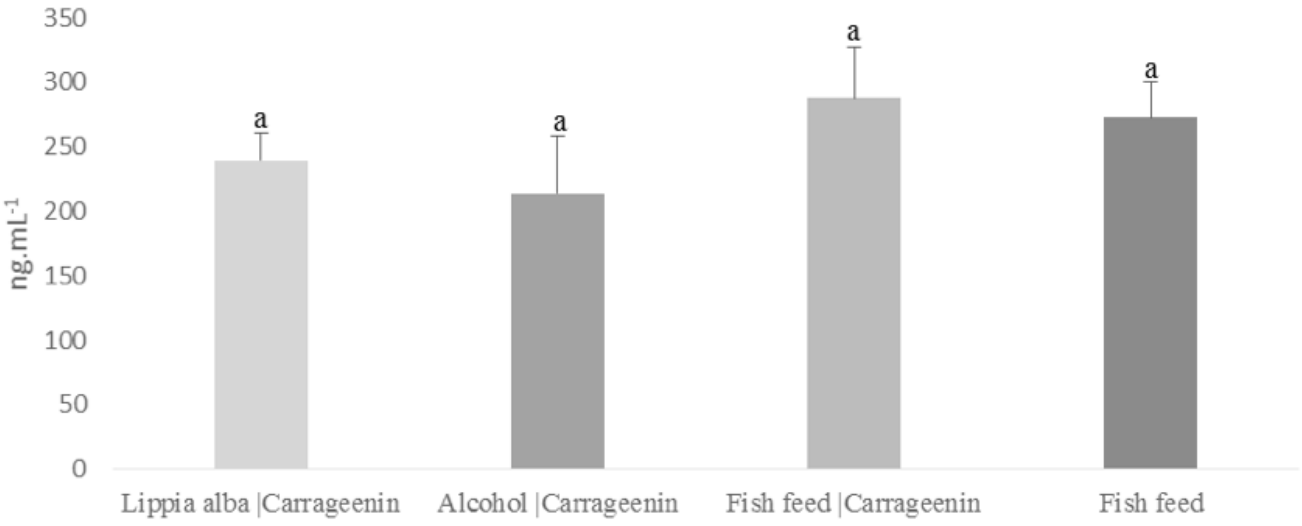

Figure 2 – Mean values ± standard deviation of cortisol in Nile tilapia fed supplemented diet with *Lippia alba* (*L. alba*-carrageenin), fed supplemented diet with cereal alcohol carrageenin-injected (alcohol-carrageenin), unsupplemented carrageenin-injected (fish feed-carrageenin) and unsupplemented noninjected fish (fish feed). Letters indicate no significant difference among treatments ($P > 0.05$)

Table 1 – Mean values ± standard deviation of the erythrogram in Nile tilapia non-supplemented and supplemented with *Lippia alba*, after induced aerocystitis. Supplemented with *L. alba* and carrageenin injected (*L. alba*-carrageenin), cereal alcohol and carrageenin-injected (alcohol-carrageenin), non-supplemented and carrageenin-injected (fish feed-carrageenin), non-supplemented and non-injected fish (fish feed)

Parameters	Treatments			
	Lippia|carrageenin	Alcohol|carrageenin	Fish feed|carrageenin	Fish feed
Hemoglobin	7.57 ± 2.17	7.62 ± 2.43	9.76 ± 3.19	8.14 ± 3.26
Hematocrit (%)	30.00 ± 3.84	27.93 ± 6.17	32.30 ± 7.86	31.37 ± 4.55
RBC ($\times 10^6$. μL^{-1})	2.78 ± 0.90	2.16 ± 0.58	2.75 ± 0.75	3.00 ± 0.96
Thrombocytes ($\times 10^3.\mu L^{-1}$)	35.42 ± 13.96	75.27 ± 47.47	63.56 ± 38.34	30.09 ± 15.72
MCV (fL)	118.43 ± 38.56	134.43 ± 28.76	125.12 ± 44.72	115.93 ± 41.82
MCHC ($g.dL^{-1}$)	25.10 ± 6.07	27.59 ± 6.68	31.39 ± 11.41	26.07 ± 9.98
MCH (pg)	30.36 ± 14.74	36.96 ± 12.45	36.34 ± 9.29	29.31 ± 12.56

RBC: total red blood cells count; MCV: mean corpuscular volume; MCHC: mean corpuscular hemoglobin concentration; MCH: mean corpuscular hemoglobin. * Different letters indicate significant difference among treatments ($P < 0.05$)

Table 2 – Mean values ± standard deviation of the leukogram from the circulating blood of Nile tilapia non-supplemented and supplemented with *Lippia alba*, after induced aerocystitis. Supplemented with *L. alba* and carrageenin injected (*L. alba*-carrageenin), cereal alcohol and carrageenin-injected (alcohol-carrageenin), non-supplemented and carrageenin-injected (fish feed-carrageenin) and non-supplemented and non-injected fish (fish feed)

Parameters	Treatments			
	Lippia|carrageenin	Alcohol|carrageenin	Fish feed|carrageenin	Fish feed
WBC ($\times 10^3.\mu L^{-1}$)	105.46 ± 45.87[a]	88.21 ± 33.89[a]	94.52 ± 35.58[a]	100.78 ± 43.31[a]
Lymphocytes ($\times 10^3.\mu L^{-1}$)	71.00 ± 39.77[a]	68.96 ± 28.87[a]	71.83 ± 32.54[a]	89.95 ± 41.19[a]
Neutrophils ($\times 10^3.\mu L^{-1}$)	33.05 ± 14.72[a]	16.72 ± 7.70[b]	20.20 ± 12.52[b]	8.30 ± 3.03[b]
Monocytes ($\times 10^3.\mu L^{-1}$)	2.28 ± 1.22[a]	2.53 ± 1.39[a]	2.72 ± 1.27[a]	2.88 ± 1.99[a]

WBC: total leukocytes count. * Different letters indicate significant difference among treatments ($P < 0.05$)

Table 3 – Total protein (TP), total immunoglobulin (Ig) and agglutination titer (AT) of Nile tilapia non-supplemented and supplemented with *Lippia alba*, after induced aerocystitis. Supplemented with *L. alba* and carrageenin injected (*L. alba*-carrageenin), cereal alcohol and carrageenin-injected (alcohol-carrageenin), non-supplemented and carrageenin-injected (fish feed-carrageenin) and non-supplemented and non-injected fish (fish feed)

Parameters	Treatments				
	Lippia|carrageenin	Alcohol|carrageenin	Fish feed|carrageenin	Fish feed	P value
TP ($mg.mL^{-1}$)	33.95 ± 4.02	31.46 ± 2.61	33.92 ± 2.38	32.02 ± 2.55	0.1869
Ig ($mg.mL^{-1}$)	15.37 ± 4.07	16.73 ± 0.76	18.26 ± 2.37	14.87 ± 2.98	0.4954
AT	1.20 ± 0.25	1.10 ± 0.22	1.50 ± 0.30	1.30 ± 0.21	0.1503

Regarding inflammatory infiltrate analysis induced by carrageenin injection, the total leukocyte count was significantly higher in carrageenin-injected fish than unsupplemented and noninjected fish (Figure 3). Differential count of leukocytes showed no difference in the lymphocyte number but an increase in the neutrophil number was observed in fish fed *L. alba* supplemented diet. On the other hand, no difference was observed between unsupplemented carrageenin injected fish and cereal alcohol carrageenin injected fish.

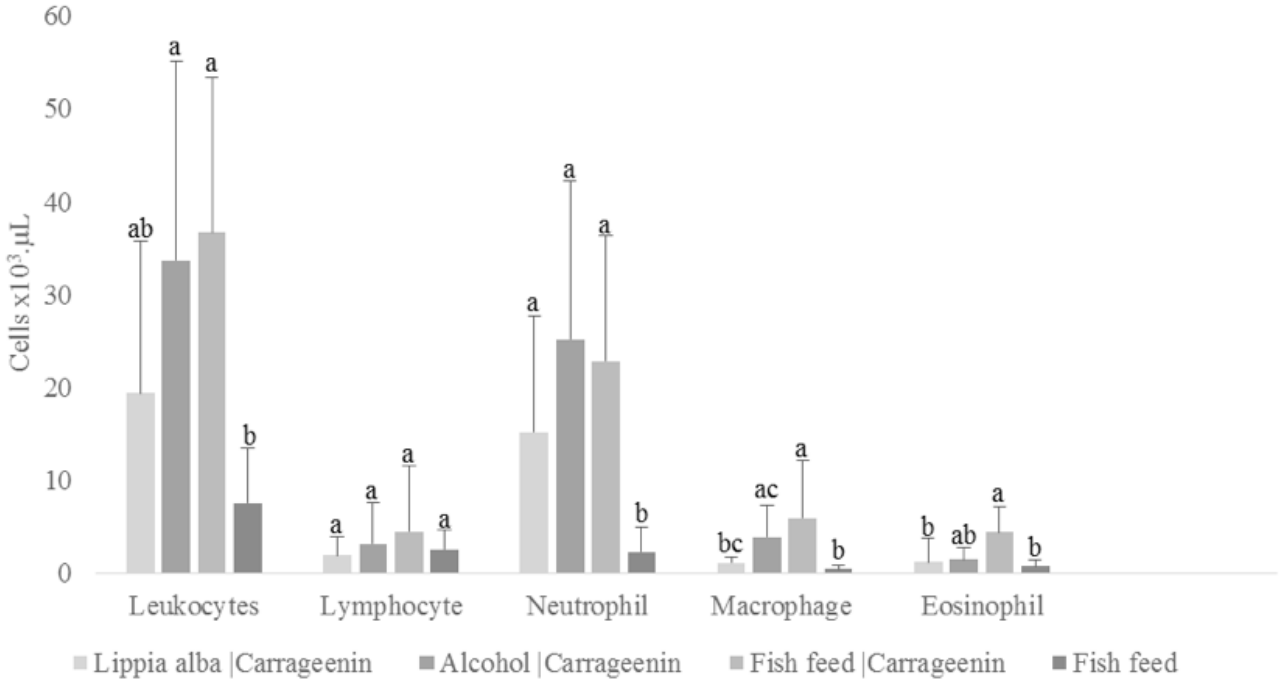

Figure 3 – Mean values + standard deviation of total and differential leukocytes counts from the inflammatory infiltrate of Nile tilapia fed supplemented diet with *Lippia alba* (*L. alba*-carrageenin), fed supplemented diet with cereal alcohol carrageenin-injected (alcohol-carrageenin), unsupplemented carrageenin-injected (fish feed-carrageenin) and unsupplemented noninjected fish (fish feed). Different letters indicate significant difference among treatments ($P < 0.05$)

Macrophage number in the exudate was higher in unsupplemented carrageenin-injected fish than that found in unsupplemented noninjected fish. Fish fed supplemented diet with the diluent cereal alcohol carrageenin injected did not show significant difference when compared with fish fed *L. alba* supplemented diet carrageenin injected. The number of eosinophils was increased in unsupplemented carrageenin-injected fish compared to that observed in unsupplemented noninjected and *L. alba* supplemented carrageenin-injected fish (Figure 3).

Discussion

Cortisol is one of the hormones produced in stressful conditions (BALM et al., 1989; WENDELAAR, 1997) and is used as a bioindicator to evaluate physiological responses. Cortisol levels found in this study were greater than that previously related for Nile tilapia exposed to a single and consecutive stress injected with different phlogogens, including carrageenin (MARTINS et al., 2004) and vitamin C and E supplemented tilapia after induced aerocystitis with carrageenin and lipopolysaccharide (LPS) (MARTINS et al., 2008a). The present results suggest no influence of the carrageenin injection on cortisol levels since no difference was found when compared to noninjected fish. Increased cortisol levels could be associated with two factors: fish handling prior to blood samples and air exposition (BARCELLOS et al., 1999; SILVA et al., 2012), and due to high-responsive individuals in the same fish population subjected to confinement stress as previously related (FEVOLDEN; RØED, 1993). It must be emphasized that the fish management for blood samples followed the same methods used previously (MORAES et al., 2012) and the increased cortisol levels did not influence the inflammatory response.

The erythrocytic and thrombocytic parameters were not affected by supplementation with essential oil. Comparable results were observed in Nile tilapia fed for 15 days with probiotic *Lactobacillus plantarum* in the diet and induced to an aerocystitis by the carrageenin injection (DOTTA et al., 2011) showing no damage to the hematopoiesis. In contrast to that observed in this study, aerocystitis induced by *Enterococcus* sp. (1×10^3 and 1×10^6 UFC mL^{-1}) injection caused an increase in the hematocrit percentage and thrombocytes number in the circulating blood (MARTINS et al., 2008b).

Apart from the present results, an increase in the hematocrit percentage, hemoglobin and RBC number was reported in carp *Cyprinus carpio* fed 0.5 and 1% crude

extract of *Aloe vera* for 60 days (ALISHAHI; ABDY, 2013). Additionally, increased RBC number and hemoglobin concentration was also found in *Rutilus frisii kutum* fed 2 and 3% with extracts of *Mentha piperita* for 8 weeks (ADEL et al., 2015). No alterations found in this assay agree with other studies using vitamin C (500 mg kg^{-1}) and inulin (5 g kg^{-1}) in Nile tilapia for up to 60 days of feeding (IBRAHEM et al., 2010). Different concentrations of *L. alba* (0, 0.25, 0.5, 1.0, 2.0 ml/kg^{-1} ration) used in the diet for juveniles of *R. quelen* for 60 days did not influence hematological parameters (SACCOL et al., 2013). When analyzing these previous studies and the present results, *L. alba* supplementation to the diet of Nile tilapia kept at this water temperature showed no deleterious effect. No difference in the hematological parameters observed in this study might be strongly associated with the water temperature of 22.3 °C in this study and 21.5 °C (SACCOL et al., 2013) when compared to previous studies that kept fish at 28.4 °C (CASTRO et al., 2014a) and 28.9 °C (MARTINS et al., 2009).

Contrary to results found in this study, inflammatory response in Nile tilapia fed vitamin C and E for 30 days was characterized by an increase and RBC number and hematocrit and a decrease in the circulating thrombocytes (MARTINS et al., 2008a). Apart from the migration of thrombocytes to the inflammatory site observed in this assay, the values did not differ between injected and noninjected fish. Migration of these cells to the inflammatory site occurs under influence of chemotactic molecules by the process named diapedesis (TIZARD, 2002). The classic inflammatory induction used in this study possibly was not enough to provoke alterations in the WBC, thrombocytes and lymphocytes. Two factors could be associated with the lack of responsiveness: the time that the inflammatory response was evaluated (6 h) and, mainly, water temperature.

Similar to that reported in this assay, an increase in the circulating neutrophils number was anteriorly observed in Nile tilapia fed supplemented diet containing the probiotic *L. plantarum* and induced to aerocystitis by the carrageenin injection (DOTTA et al., 2011). Nonspecific immunological response has been led by the phagocytosis process (RANZANI-PAIVA; SILVA-SOUZA, 2004) and the neutrophils participation activates the innate defense system against pathogens (RANZANI-PAIVA et al., 2013). Despite no influence in WBC number, a beneficial effect of the *L. alba* supplementation could be suggested when analyzing the neutrophilia after inflammatory response demonstrating improved capacity under phlogogen stimulus. Several natural products may activate the specific and nonspecific defense system in teleost fishes (SAKAI, 1999).

The inflammatory reaction provoked by the carrageenin injection in the swim bladder of Nile tilapia used in this study was successful when observing the efficiency in provoking cell migration to the inflammatory site, corroborating previous reports (MATUSHIMA; MARIANO, 1996; MARTINS et al., 2001; MARTINS et al., 2009; DOTTA et al., 2011).

Independent of diet supplementation, 6h after injection the increased number of neutrophils found in the inflammatory infiltrate corroborate the acute process (RANZANI-PAIVA; SILVA-SOUZA, 2004), which was characterized by neutrophils migration followed by macrophages. The inflammatory infiltrate found in this study differed from other findings that reported macrophages and granulocytes as the main cells involved in the inflammatory process (DOTTA et al., 2011). Different from that observed in this assay, *P. mesopotamicus* fed supplemented diet with chrome (18 and 36 mg/kg^{-1} rations) for 90 days showed an increase in the number of lymphocytes and thrombocytes in the swim bladder exudate (BOZZO et al., 2007), as well as an increased number of phagocytic cells in the inflammatory exudate of tilapia fed soy oil, flaxseed oil or LPS of *Saccharomyces cerevisiae* (SAKABE et al., 2013).

Lippia alba did not show anti-inflammatory action as also reported previously (CLAUDIANO et al., 2013). These results may suggest the use of different time, water temperature and associated anti-inflammatory drugs. Anti-inflammatory drugs (dexamethasone, indomethacin and meloxicam) was administrated in *P. mesopotamicus* 30 min before the inflammatory induction with inactivated *A. hydrophila* in the swim bladder, and a decrease in the inflammatory infiltrate was observed 24 h after injection (CLAUDIANO et al., 2013).

Anti-inflammatory action of drugs is related to several factors like posology, site of action and locale of inflammatory investigation, and also other non-drug variables to be tested. Moreover, the elimination of agents or products from the tissue lesion is essential for successful inflammatory process[1] justifying the use of substances to improve cell responsiveness.

Conclusions

Supplementation with essential oil of *L. alba* did not show anti-inflammatory activity in Nile tilapia. On the other hand, its supplementation had immunomodulatory action when observing increased number of neutrophils in the swim bladder exudate after induced aerocystitis. At the dose tested, *L. alba* could be used with no damage to fish physiology. Also, in inflammatory assays we suggest an increase in fish maintenance to magnify the inflammatory response.

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