Original Article

Braz. J. Vet. Parasitol., Jaboticabal, v. 24, n. 1, p. 66-71, jan.-mar. 2015
ISSN 0103-846X (Print) / ISSN 1984-2961 (Electronic)
Doi: http://dx.doi.org/10.1590/S1984-29612015004

Effect of dietary supplementation with propolis and *Aloe barbadensis* extracts on hematological parameters and parasitism in Nile tilapia

Efeito da suplementação dietária com extratos de própolis e *Aloe barbadensis* sobre parâmetros hematológicos e parasitismo em tilápia do Nilo

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Received December 5, 2014
Accepted December 8, 2014

Abstract

This study evaluated the influence of diet supplementation with propolis and *Aloe barbadensis* on hematological parameters and parasitism in tilapia. One hundred and eighty fish were distributed among 12 water tanks forming four treatments: fish supplemented with a 1:1 mixture of 0%, 0.5%, 1% and 2% propolis and aloe extracts. After the fish had been fed on the experimental diets for 15 and 21 days, blood samples were taken and parasites collected. The monogeneans *Cichlidogyrus sclerosus*, *C. halli*, *C. thurstonae* and *Scutogyrus longicornis* were identified in the gills. Between the sampling times, there were increases in the numbers of erythrocytes, leukocytes, thrombocytes and lymphocytes, as observed after 21 days, possibly due to the stress level over the course of the assay and/or accumulation of substances in the organism. Supplementation with the mixture of propolis and aloe for 15 days showed the highest efficacy against the parasites. This was possibly due to the association between the two compounds. The results demonstrated that supplementation with mixtures of extracts did not produce hematological alterations and also favored a significant reduction in the number of gill parasites. The best results were achieved after 15 days of feeding with a diet with 0.5% and 1% supplementation with the extract mixture, which increased efficiency by 83 and 85% respectively

Keywords: Hematology, parasitology, propolis, *Aloe barbadensis*.

Resumo

Este estudo avaliou a influência da alimentação de tilápias com dieta suplementada com extratos de própolis e *Aloe barbadensis* sobre os parâmetros hematológicos e parasitismo. Setenta e dois peixes foram distribuídos em 12 tanques, formando 4 tratamentos: peixes suplementados na dieta com extrato de própolis e aloe (0%, 0,5%, 1% e 2%). Após 15 e 21 dias de alimentação com dietas experimentais foram realizadas coletas de sangue e parasitos. Os monogeneas *Cichlidogyrus sclerosus*, *C. halli*, *C. thurstonae* e *Scutogyrus longicornis* foram identificados nas brânquias. Entre os períodos de coleta, observou-se aumento nos valores de eritrócitos, leucócitos, trombócitos e linfócitos, após 21 dias de alimentação, o que pode significar estresse ao longo do período experimental e/ou acúmulo dos produtos no organismo.A suplementação com a mistura de própolis e aloe, por 15 dias, apresentou melhor eficácia frente parasitos, possivelmente, devido à associação dos compostos. Os resultados demonstram que a suplementação com a mistura dos extratos não alterou os parâmetros hematológicos, além de favorecer redução significativa no número de parasitos branquiais. Os melhores resultados foram obtidos em 15 dias de alimentação com a dieta suplementada com 0,5% e 1% da mistura dos extratos, respectivamente por 83% e 85% de eficiência.

Palavras-chave: Hematologia, parasitologia, própolis, *Aloe barbadenensis*.

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Introduction

According to the Brazilian Health Surveillance Agency (ANVISA, 2003), phytotherapeutics are medicines derived from medicinal plants that comprise plant-derived drugs alone (in the form of extracts, tinctures, oils, waxes, exudates, juices and other presentations). Within aquaculture, these medicines have been gaining ground because they have several advantages in fish farms, such as lower environmental impact because they are biodegradable products, lower chemical waste in animals and potential for lower toxicity, because they are less concentrated. Moreover, they have different mechanisms of action, which means greater resistance and lower cultivation costs (COIMBRA et al., 2006).

Several herbal medicines can be used for treating fish parasites. Among these, nin (CRUZ et al., 2004) and garlic (MARTINS et al., 2004) can be administered either as therapeutic baths or as dietary supplements. *Aloe barbadensis* Miller, which belongs to the Asphodelaceae family and is commonly known as the aloe plant, can also be used (WICHTL, 2004). The aloe plant is widely used in popular medicine due to its beneficial effects, such as in relation to treating burns, hepatitis and diabetes and controlling blood lipid levels (OKYAR et al., 2001), and as a healing agent (PAEZ et al., 2000), antiulcerative agent, antineoplastic agent (SAKAI, 1989; KOBAYASHI et al., 1993; MAEDA et al., 1998) and antiviral agent (KIM et al., 1999). Chemical compounds of parenchymal substances and aloe extract are responsible for their immunostimulant and healing properties (IMANISHI, 1993).

Another herbal product option is propolis, a substance of plant and animal origin derived from resinous gummy and balsamic substances that is collected by bees from flower buds and plant exudates and modified in the hive through addition of salivary secretions and wax (PINHEIRO-FILHO, 1998). Propolis is used by bees to protect the hive against attack by other insects and proliferation of microorganisms, including fungi and bacteria (GHISALBERTI, 1979; MARCUCCI, 1995). It has been demonstrated to be effective against Gram-positive bacteria and fungi, and can act as an immunostimulant (ORSI et al., 2005). The improvement in performance observed among animals treated with diets supplemented with propolis extract may be a consequence of the improvement in immune response, allied with the efficacy of its heterogeneous composition (MATSUNO, 1992). Great numbers of biological and chemical studies on propolis published during the last decade have demonstrated that flavonoids predominate in the composition of propolis. According to Marcucci (1995), more than 50 flavonoids have been discovered, including compounds belonging to the vegetable polyphenol, aromatic acid and oleic acid groups. In aquaculture, the action of these herbal medicines can be exploited mainly through stimulation of immune responses and treatment of parasitic infestation.

Parasitism occurs as a result of an interaction between host, parasite and environment (BUCHMANN & LINDENSTRØM, 2002). Some factors or substances are responsible for lowering the host immune response, thereby resulting in unbalanced host/parasite/environment interaction. Such factors include water temperature, stress level (XU et al., 2012), nutritional quality (CAVICHIOLO et al., 2002), age and natural immunity (BUCHMANN & LINDENSTRØM, 2002). In intensive farming systems, high fish density, low water flow and high organic matter concentration contribute towards the growth and reproduction of parasites (MORAES & MARTINS, 2004).

Monogenean ectoparasites of fish are characterized by the presence of sclerotized anchoring structures, and these parasites are preferentially located in the gills, nostrils, eyes and body surface. This feature enhances pathogenicity by causing tissue injury and behavior changes, with increased mucus production, skin hemorrhage, gill filament hyperplasia, anorexia and fish death (PAVANELLI et al., 2008). Moreover, in cases of less severe infection, the lesions produced serve as small doors that are open to secondary infections (MARTINS & ROMERO, 1996).

The present study was designed to test the efficacy of different concentrations of propolis and aloe extracts in supplements to the diet of tilapia, as immunomodulators of the primary immune response and thus as possible regulators of parasite infestation.

Material and Methods

Experimental conditions

A total of 180 juvenile Nile tilapias (mean weight: 18.13 ± 5.7 g; and mean total length: 9.94 ± 1.14 cm) from the same spawning at the Panamá fish farm, in Paulo Lopes, SC, Brazil, were acclimatized for seven days before assaying. After this period, the fish were distributed into 30 tanks of capacity 100 L of capacity with constant aeration, biological filtration and water quality maintained at pH 6.0 ± 1.5 (Alfakit, AT-350), temperature 24.0 ± 2.8 ºC, ammonia 0.08 ± 0.33 mg/L (Alfakit, colorimetric method) and dissolved oxygen 6.0 ± 0.0 mg/L (Hanna, HI 9146). The experiment comprised a completely randomized factorial design divided into 12 treatments with three replicates each (nine fish/treatment) and one control group, as follows: fish supplemented with propolis extract (0.5%, 1% and 2%); fish supplemented with aloe extract (0.5%, 1% and 2%); fish supplemented with 1:1 mixtures of propolis and aloe extracts (0.5%, 1% and 2%); and non-supplemented fish. After 15 and 20 days of feeding with experimental diets, blood samples were taken for parasite quantification.

Preparing the supplemented diet

We used commercial feed (Nicoluzzi®) containing 28% crude protein, to which was added solutions of hydroalcoholic extract of propolis, aqueous extract of aloe or a mixture in the proportions of 1:1, to obtain final concentrations of 0.5; 1 and 2% of the total quantity of feed offered per day.

Hematological analysis

After the fish had been anesthetized in clove oil (75 $mg.L^{-1}$), blood samples were collected from the caudal vein using a syringe containing a drop of 10% EDTA solution (Ethics Committee no. 23080.009240/2011-93/CEUA/UFSC), in order to measure hematocrit (GOLDENFARB et al., 1971), red blood cell count in a Neubauer chamber, white blood cell count and thrombocyte

count (DOTTA et al., 2011). To obtain a differential leukocyte count, blood smears were stained with a combination of Giemsa/May-Grünwald (ROSENFELD, 1947), in which a hundred cells were counted to determine the cell percentage.

Parasitological analysis

After blood collection, the fish were sacrificed for parasitological examination and gross pathological examination in accordance with Ghiraldelli et al. (2006) to check the influence of treatments on the parasite fauna. Prevalence, mean intensity and abundance data were obtained in accordance with Bush et al. (1997). Using these data, the efficacy was determined using the formula: E = MNPCG- MNPTG x 100/ MNPCG (E: efficacy; MNPCG: mean number of parasites in control group; and MNPGT: mean number of parasites in treated group). The number of monogeneans in the gills was quantified as described by Ghiraldelli et al. (2006) and they were identified as described by Paperna & Thurston (1969), Ergens (1981), Douëllou (1993) and Pariselle & Euzet (1995).

Statistical analysis

The data were subjected to factorial analysis of variance (ANOVA) using Statsoft's STATISTICA 7.0. The Bartlett test was used to ascertain the homoscedasticity, while the Tukey test was used to compare the means. Data transformations were used according to pertinence.

Results

There were statistically significant increases in the hematocrit and in the red blood cell (RBC), white blood cell (WBC), thrombocyte and lymphocyte counts between the blood samples collected after 15 days and 21 days of feeding with the unsupplemented diet (Table 1). The experimental diet with 0.5% showed significant increase in all the hematological parameters studied after 21 days, except for red blood cells (RBC). On the other hand, there was no significant difference among the treatments over the same period. The experimental diet with 1% showed significant increase in white blood cells (WBC), lymphocytes and neutrophils after 21 days of feeding, while the highest monocyte counts among extract concentrations for this diet were shown after 15 days of feeding and remained at high values over the second data collection period. Fish supplemented with 2% did not show any significant difference between the feeding periods (Table 1).

The parasitological analysis revealed the presence of *Cichlidogyrus sclerosus*, *C. halli* Price and Kirk, 1967, *C. thurstonae* Ergens, 1981 and *Scutogyrus longicornis* Paperna and Thurston, 1969 (Monogenoidea: Dactylogyridae) on the gills of the tilapias examined. Table 2 shows that unsupplemented fish (0%) presented a significant increase in the mean intensity of parasites on the gills and that this difference continued until 21 days of feeding, with different concentrations of mixed extracts. One hundred percent prevalence was also found in un-supplemented fish (Table 2). The mean abundance of monogeneans was lower in the fish supplemented with the extracts, at both collection times (Figures 1, 2 and 3).

Discussion

This study demonstrates the need for and importance of knowledge regarding use of alternative products for maintenance of homeostasis and fish welfare, as well as activity against parasites shown by plant extracts. In this assay, the hematological variables did not show any variation between the different

Table 1. Hematological parameters of Nile tilapia fed supplemented diet with a mixture of propolis and *Aloe barbadensis* at concentrations of 0%, 0.5%, 1% and 2% for 15 and 21 days. Lowercase letters indicate significant differences among the concentrations of each extract and uppercase letters indicate significant differences between the different collection days for each concentration of the extract ($p < 0.05$) by Tukey test.

Parameters	15 feeding days			
	0%	0.5%	1%	2%
Hematocrit (%)	31.2 ± 4.9^{aA}	28.2 ± 2.6^{aA}	29.7 ± 2.3^{aA}	27.3 ± 2.8^{aA}
RBC (x $10^6.\mu l^{-1}$)	1.58 ± 0.41^{aA}	1.43 ± 0.31^{aA}	1.65 ± 0.46^{aA}	1.71 ± 0.48^{aA}
WBC (x $10^3.\mu l^{-1}$)	54.4 ± 19.5^{aA}	35.8 ± 9.7^{aA}	45.3 ± 22.7^{aA}	52.8 ± 19.5^{aA}
Thrombocytes (x $10^3.\mu l^{-1}$)	22.9 ± 12^{aA}	24.8 ± 13.3^{aA}	19.1 ± 11.1^{aA}	25.5 ± 17.7^{aA}
Lymphocytes (x $10^3.\mu l^{-1}$)	44.1 ± 17.1^{aA}	30.5 ± 8.3^{aA}	36.4 ± 22.4^{aA}	45.1 ± 18.4^{aA}
Neutrophils (x $10^3.\mu l^{-1}$)	6.5 ± 7.3^{aB}	3.7 ± 2.8^{aA}	3.8 ± 2.6^{aA}	4.4 ± 3.7^{aA}
Monocytes (x $10^3.\mu l^{-1}$)	4.1 ± 4.8^{bA}	1.5 ± 0.5^{aA}	4.9 ± 5.1^{bA}	3.3 ± 1.5^{abA}
Parameters	**21 feeding days**			
	0%	**0.5%**	**1%**	**2%**
Hematocrit (%)	34.9 ± 7.2^{aB}	30.9 ± 8.18^{aB}	28.38 ± 4.83^{aA}	29.3 ± 6.5^{aA}
RBC (x $10^6.\mu l^{-1}$)	3.43 ± 0.86^{aB}	2.14 ± 1.13^{aA}	2.46 ± 1.27^{aA}	2.01 ± 0.84^{aA}
WBC (x $10^3.\mu l^{-1}$)	88.43 ± 22.42^{bB}	64.62 ± 40.81^{aB}	68.13 ± 41.57^{aB}	53.37 ± 32.30^{aA}
Thrombocytes (x $10^3.\mu l^{-1}$)	38.17 ± 9.62^{bB}	20.31 ± 26.80^{aA}	17.98 ± 11.05^{aA}	21.94 ± 13.50^{aA}
Lymphocytes (x $10^3.\mu l^{-1}$)	80.51 ± 38.62^{bB}	49.63 ± 36.70^{aB}	55.24 ± 34.45^{aB}	45.96 ± 29.80^{aA}
Neutrophils (x $10^3.\mu l^{-1}$)	4.0 ± 1.8^{aA}	10.86 ± 4.7^{bB}	7.8 ± 4.4^{abB}	4.2 ± 2.5^{aA}
Monocytes (x $10^3.\mu l^{-1}$)	3.8 ± 3.4^{aA}	4.1 ± 4.0^{aB}	4.7 ± 4.3^{aA}	3.1 ± 2.3^{aA}

Table 2. Mean values of prevalence and mean intensity of parasites in the gills of Nile tilapia fed supplemented diet with a mixture of propolis and *Aloe barbadenis* (P:A) at concentrations of 0%, 0.5%, 1% and 2% for 15 and 21 days. Lowercase letters indicate significant differences among the concentrations of extract and uppercase letters indicate significant differences between the different collection days for each concentration of the extract (p <0.05) by Tukey test.

Concentrations		15 days		21 days	
		Prevalence (%)	Mean intensity	Prevalence (%)	Mean intensity
P:A	**0%**	**100**	**10.56±11.39^{bA}**	**100**	**6.89±4.46^{bA}**
P:A	0.5%	89	2.25±2.00^{aA}	89	2.38±2.11^{aA}
P:A	1%	66	2.67±1.77^{aA}	89	3.88±3.44^{aA}
P:A	2%	78	3.14±2.44^{aA}	66	1.83±1.22^{aA}

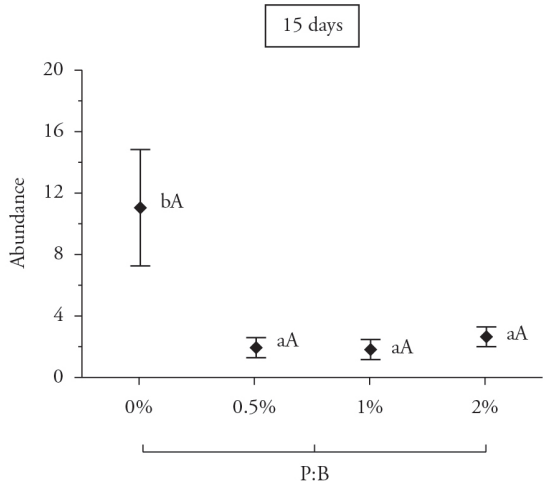

Figure 1. Abundance of parasites in the gills of Nile tilapia fed supplemented diet with mixture of propolis and *Aloe barbadensis* extracts (P:A) at concentrations of 0%, 0.5%, 1%, 2% for 15 days. Lowercase letters indicate significant differences among the concentrations of each extract and uppercase letters indicate significant differences between the different collection days for each concentration of the extract (p <0.05) by Tukey test.

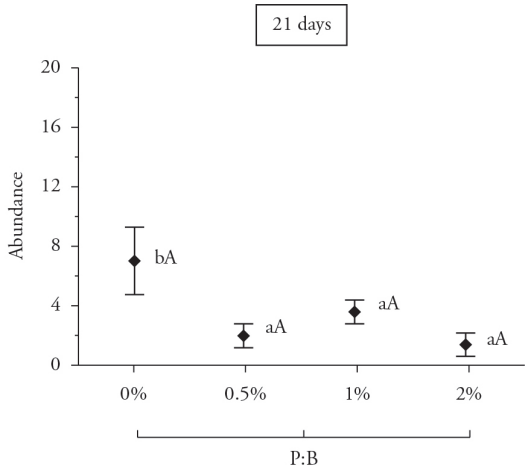

Figure 2. Abundance of parasites in the gills of Nile tilapia fed supplemented diet with mixture of propolis and *Aloe barbadensis* extracts (P:A) at concentrations of 0%, 0.5%, 1%, 2% for 21 days. Lowercase letters indicate significant differences among the concentrations of each extract and uppercase letters indicate significant differences between the different collection days for each concentration of the extract (p <0.05) by Tukey test.

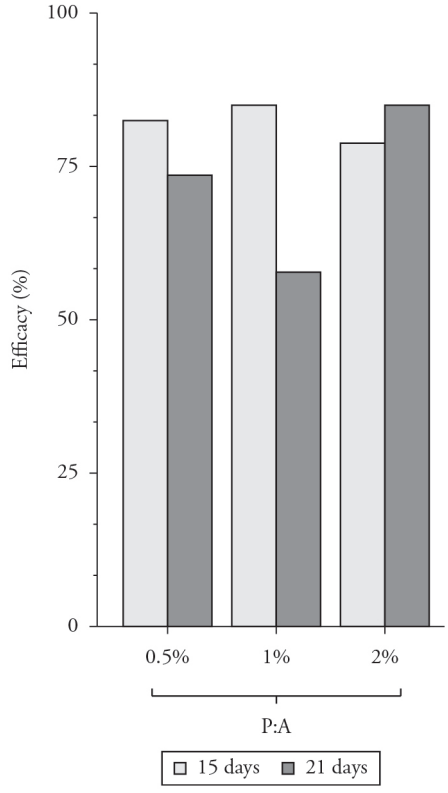

Figure 3. Efficacy of the mixture of propolis and *Aloe barbandensis* (P:A) at concentrations of 0%, 0.5%, 1%, 2% supplemented in the diet of Nile tilapia parasitized by *Cichlidogyrus sclerosus*, *Cichlidogyrus halli*, *Cichlidogyrus thurstonae* and *Scutogyrus longicornis* for 15 and 21 days.

concentrations of the extracts in the diet. Hematological analysis on fish is a tool of fundamental importance for biological and biochemical knowledge of normal and pathological conditions (FUJIMOTO et al., 2012). Hematological changes in the levels of erythrocytes, leukocytes, thrombocytes and lymphocytes were observed after 21 days of treatment in the groups supplemented with 0.5% and 1%, possibly caused by the stress of confinement under the experimental conditions. This was also observed among the unsupplemented fish. According to Ranzani-Paiva & Silva-Souza (2004), changes in the hematological parameters of fish can be observed when they are infected or subjected to stress, or when their food is changed.

Supplementation with the mixture of propolis and *Aloe vera* extracts showed excellent efficacy outcomes in this study. Studies on mammals have demonstrated that propolis presents effective action against *Trypanosoma*, a pathogenic microorganism that causes human and animal diseases (SALOMÃO et al. 2004). Ayres et al. (2007) reported that, *in vitro*, there was a decrease in *Leishmania amazonensis* infection after propolis administration. Oda et al. (2011) explained that the mechanisms through which propolis extracts exert their leishmanicidal effect *in vivo* are unclear, but activation of macrophages has been suggested as a mechanism. Macrophages are involved in functions such as phagocytosis, enzyme release, generation of free radicals and mediation of inflammatory processes. It has been suggested that propolis may act towards production of microbicidal substances by macrophages (ORSI et al., 2000), through stimulating the response or decreasing the diameter of the lesions caused by the presence of parasites in the tissues of the host organism (ODA et al., 2011).

The good results achieved with regard to combating monogeneans in this study can be attributed to the combination of aloe and propolis. Stevens (1999) stressed that the resin located in the channels below the epidermis of the leaves of *A. barbadensis* presents anthraquinones (aloin and emodin), which are substances with cathartic and laxative activity in animals and humans (STEVENS, 1999). These compounds may cause damage to the intestinal epithelium if used as supplements for long periods or at high concentrations. Nevertheless, the antifungal, anti-inflammatory, hypoglycemic, hemagglutination and mitogenic activity towards lymphocytes remains the main feature of interest in studying this product (CHOI & CHUNG, 2003; TAI-NIN CHOW et al., 2005; MURAKAMI et al., 2009). Several polysaccharides have been detected and isolated from the pulp of aloe, including mannose, galactose, arabinose, glucuronic acid and pectic acid (NI et al., 2004). Other polysaccharides present in the gel of *A. barbadensis* include glucomannan and acemannan. According to Vega et al. (2005), acemannan is a substance that acts by increasing the strength of the immune system of the organism against parasites, bacteria and viruses.

Supplementation with the mixture of propolis and *A. barbadensis* for 15 days showed the best efficacy against parasites. The combination of these two compounds provided in the diet for 21 days, negatively influenced the results from the present study, possibly due to stress containment and/or accumulation of the products. However, it is known that one of the main factors that influence fish health is dietary balance, such that a combination of compounds is needed in order to meet the appropriate nutritional requirements. Dietary supplements can also stimulate the action of nonspecific defense mechanisms and immune responses in fish.

The results demonstrated that supplementation with mixtures of the extracts did not show any alterations in the hematological parameters and, moreover, this favored significant reduction in the number of gill parasites. The best results were achieved after 15 days of feeding with diet supplemented with 0.5% and 1% of the mixture of extracts, which respectively presented 83 and 85% efficacy.

Acknowledgements

The authors thank CNPq (National Council for Scientific and Technological Development, Brazil) for financial support and Grant to M.L. Martins (302493/2010-7); to REUNI program (Restructuring and Expansion of Federal Universities) for Doctoral scholarship to G. Dotta; to Nicoluzzi Rations Ltda, for ration during the experiment.

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