

PARAMETERS OF GARLIC-VINE BASED TREATMENT IN AMAZON TAMBAQUIS GILL INFECTIONS

PARÂMETROS DE TRATAMENTO DE INFECÇÕES BRANQUIAIS EM TAMBAQUIS DA AMAZÔNIA USANDO CIPÓ-ALHO

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Recebido: (25/10/2022) / Publicado: (06/10/2025)

Resumo O objetivo deste estudo foi avaliar os efeitos das folhas de *A. alliaceum* no controle de monogêneas adicionado à dieta de juvenis de *C. macropomum* e avaliar os indicadores hematológicos. Foram testadas concentrações de 0, 30, 40 e 50 g de *A. alliaceum* kg⁻¹ na ração, durante 7, 15 e 30 dias e avaliados os parâmetros sanguíneos e parasitários. Houve diferença significativa no período 30 dias com o aumento do CHCM (Concentração de Hemoglobina Corpuscular Média) e diminuição do colesterol em relação aos outros períodos. No entanto, a glicose aumentou no período 15 dias e na comparação entre os tratamentos, os valores de CHCM, colesterol, proteína e glicose foram maiores com 40g de cipó alho kg⁻¹ em comparação ao controle. A prevalência dos monogêneas foi de 100% em todos os tratamentos e somente após 30 dias de alimentação as concentrações de 40 e 50 g de *A. alliaceum* kg⁻¹ apresentaram eficácia, com 70,59 e 62,21%, respectivamente. O cipó-alho mostrou-se promissor como tratamento fitoterápico contra monogêneas de tambaqui e este estudo mostra a necessidade de mais testes com esta planta.

Palavras-Chave: *Colossoma macropomum*; *Adenocalymma alliaceum*; Fitoterápico; Parasitas; Fisiologia.

Abstract This study evaluated *A. alliaceum* leaf powder effects controlling monogeneans added to the diet of juveniles of *C. macropomum* and assessed hematological indicators. Concentrations of 0, 30, 40 and 50 g of *A. alliaceum* kg⁻¹ were tested in the diet for 7, 15 and 30 days and blood and parasitic parameters were evaluated. There was a significant difference in the 30-day period with an increase in MCHC (Mean Corpuscular Hemoglobin Concentration) and a decrease in cholesterol in relation to other periods. However, glucose increased in a 15-days period and, in comparison between treatments, values of MCHC, cholesterol, protein and glucose were higher with 40g of garlic-vine kg⁻¹ compared to the control group. The prevalence of monogeneans was 100% in all treatments and, only after 30 days of feeding, concentrations of 40 and 50g of *A. alliaceum* kg⁻¹ showed efficacy, with 70.59 and 62.21%, respectively. Garlic-vine showed promise as an herbal treatment against tambaqui monogeneans and this study shows the need for further testing with this plant.

Keywords: *Colossoma macropomum*; *Adenocalymma alliaceum*; Herbal medicine; Parasites; Physiology

Introduction

Native *Serrasalminae Colossoma macropomum* is the most cultivated fish species in Brazil, due to huge advances in the fish productive chain in the last two decades (MPA, 2012). However, one of the obstacles for tambaqui production has been problems with parasitic diseases, including infections by monogeneans species (Andrade et al., 2016; Dias et al., 2015) which can compromise production and productivity (Lari et al., 2017).

Exposure to pathogens can cause physiological changes, which initiate reactions to stress. Hematological parameters are important tools to assist in the diagnosis of changes produced by stressor agents in fishes (Gonzales et al., 2020; Tavares-dias, 2015). Furthermore, indiscriminate use of products to control ectoparasitosis in cultivation systems can result in contamination of fishes and all organisms involved in the aquatic food chain, as well as environment pollution.

For the control of monogeneans, different chemical products (praziquantel, sodium chloride and others) have been used (Morales-Serna et al., 2018; Nogueira et al., 2019; Partridge et al., 2014), though these products, besides having limited efficacy, can bring toxicity to the fishes, contamination to the environment and even compromise human health.

These factors stimulated the search for new control strategies that could be used for monogeneans, due to phytotherapy being a great potential alternative for the control of these parasites (Ferreira et al., 2019). Many medicinal plants have been tested in the control of these parasites in fish, like *Bixa orellana* (Andrade et al., 2018, 2016), *Ocimum gratissimum* (Meneses et al., 2018), *Mentha piperita* (Anjos and Isaac, 2020), *Copaifera reticulata* (Malheiros et al., 2020) and *Allium sativum* (Militz et al., 2013; Fridman et al., 2014; Inoue et al., 2016).

Adenocalymma alliaceum Miers (Bignoniaceae), a plant that occurs in Brazil, popularly known as garlic-vine, has similar properties to the *Allium sativum* (garlic), which has a broad spectrum of immunostimulants that can combat the parasites (Gabriel et al., 2019; Guo et al., 2012). Such anti-parasitic characteristics are attributed to the presence of sulfured composites like allyl trisulfide and diallyl disulfide, sulfur derivatives (Nya et al., 2010). Therefore, due to the necessity of product formulations based on medicinal plants as an alternative in the anti-parasitic control, the objective of this study was to evaluate the effectiveness of the garlic-vine added to the feed, in controlling tambaqui monogeneans parasite and evaluating blood indicators of fish health.

Material and methods

Fishes and acclimatization

Two hundred and sixteen juveniles of *Colossoma macropomum* (15.5 ± 0.7 cm and 124.0 ± 16.9 g) parasitized with monogeneans were obtained from commercial fish farming and acclimatized for 15 days in 250 L tanks. The fishes were fed, ad libitum, with commercial feed containing 36% of crude protein (CP), three times a day. Before the beginning of the experiments, branchial scraping was performed in some specimens to confirm the presence of monogenetics. Once this was done, the remaining fish were distributed in 36 tanks, corresponding to three replicates for each feed concentration (0, 30, 40 and 50 g kg⁻¹) and three experiment periods (7, 15 and 30 days) being deprived of feed for 24 h.

Water quality parameters

The physical and chemical variables of the water in the experimental tanks were determined daily: dissolved oxygen, temperature, conductivity and pH, determined with a multiparameter probe (YSI 550th, Tecnal, Ohio, USA). Water samples were collected for analysis of the total ammonia concentration (mg l⁻¹) (Verdouw et al., 1978) nitrite (mg l⁻¹), total alkalinity and total hardness (mg CaCO₃ l⁻¹) (Boyd and Tucker, 1992).

Adenocalymma alliaceum gathering, experimental diet elaboration and experimental design

A. alliaceum leaves were gathered at the Nilton Lins University garden (Manaus, AM, Brazil). They were manually removed and dried for seven days, then taken to an oven at 55 °C for two days to complete the dehydration, and then crushed until it became powder. A commercial feed containing 36% CP was utilized with the aim of adding *A. alliaceum* into the diet. This feed was crushed until turned into a powder, and after, manually mixed with the garlic-vine powder in the proportions of 0, 30, 40 and 50 g kg⁻¹, with three replicates, being three fishes for each replicate. The mix was moistened with water in the proportion of 500 ml kg⁻¹ of feed and the mass obtained was pelletized. The pellets were weighed according to the necessary quantity for each experiment day, considering the weight of the fishes. The same feed was offered for the control group, without the addition of the garlic-vine.

The medicated feed was provided to the fishes for 7, 15 and 30 days once a day by morning, and when not completely consumed, it was provided again by afternoon. Then, the fish received non-medicated feed until the apparent satiety.

Blood sampling and analysis

Samples of 1 ml of blood were collected by caudal vein puncture using 3 ml syringes, 27Gx½ needles, rinsed with EDTA anticoagulant (ethylenediaminetetraacetic acid) at 10% and stored under refrigeration at 8 °C. The fish were not anesthetized; however, the collection time was less than 1 minute. The total blood was destined to the hematocrit (Htc - %), determined by the microhematocrit method using a capillary tube and a microhematocrit centrifuge with rotation of 3000 RPM for 5 minutes; the erythrocyte count (RBC = number of erythrocytes µl⁻¹) was performed in a Neubauer® chamber diluted in Natt and Herrick solution (1952); the hemoglobin concentration (Hgb = g dl⁻¹) was determined by the cyanometahemoglobin colorimetric method in spectrophotometry (BIOPLUS2000) using a commercial kit (Doles®, Goiás, Brazil). The hematimetric indices, Mean Corpuscular Hemoglobin Concentration (MCHC = %), Mean Corpuscular Hemoglobin (MCH = pg) and Mean Corpuscular Volume (MCV = fl) were calculated from the values of Htc, RBC and Hgb, according to (Wintrobe, 1934). The blood plasma obtained by centrifugation was destined for the analysis of serum biochemistry. In this case, plasma glucose (mg dl⁻¹) was analyzed by the enzymatic-colorimetric glucose oxidase method (inVitro® kit, Goiás, Brazil), total plasma proteins (g dl⁻¹) by the biuret method (Human do Brazil® kit) and total cholesterol (mg dl⁻¹) by the enzymatic-trinder method (Labtest® kit). These analyses were performed in a UV/Visible spectrophotometer (BIOPLUS 2000).

Parasitological analysis and treatment efficacy

At the end of each experimental period, all fish were sacrificed and the gills were removed, with the approval of the Ethics Committee on Animal Experimentation (Comitê de Ética em Experimentação Animal - CEEA – Nilton Lins), according to the protocol 003/2012, for the counting of the monogeneans in the gills. The gathering, fixing and counting method of the parasites was that described by Eiras et al. (2006). Parasitary indices like: prevalence; intensity; average intensity and average abundance were calculated for the parasites quantitative analyses (Bush et al., 1997).

The equation to measure the efficacy of the feed with garlic-vine was calculated according to the mathematical expression: Efficacy = (mean of number of parasites of the control group - mean of number of parasites of the treated group) x 100 / mean of number of parasites of the control group (Onaka et al., 2003).

Statistical Analysis

The data on the number of parasites for each treatment were analyzed using a Kruskal-Wallis test for each period and as medians contrasted by the later Mann-Whitney test with

Bonferroni adjustment. The data on the blood parameters were analyzed by MANOVA with Hotelling T² later test, comparing periods and treatments. To eliminate the effect of multicollinearity of the blood parameters, the variance inflation factor (VIF) test was applied. The tests were performed in the R software, version 3.5.0 (R Core Team, 2020), using the packages ggplot2, multcomp, MASS, car and Hotelling.

Results

In the experimental tanks, the average water temperature ($29.21 \pm 0.22^{\circ}\text{C}$), dissolved oxygen levels ($6.08 \pm 0.55 \text{ mg l}^{-1}$), pH (5.32 ± 0.11), nitrite ($0.012 \pm 0.005 \text{ mg l}^{-1}$), alkalinity ($5.94 \pm 0.03 \text{ mgCaCO}_3 \text{ l}^{-1}$), hardness ($5.15 \pm 0.13 \text{ mgCaCO}_3 \text{ l}^{-1}$) and total ammonia ($0.163 \pm 0.01 \text{ mg l}^{-1}$) were similar ($p > 0.05$) and within tropical fish cultivation standards (table 1).

Table 1. Water Quality Parameters of each treatment in *Colossoma macropomum*.

	7 days				15 days				30 days			
	Garlic-vine (g kg ⁻¹)											
	0	30	40	50	0	30	40	50	0	30	40	50
DO	5.1	5.6	5.8	5.3	5.9	6.6	6.8	6.4	5.5	6.6	6.8	6.4
EC	28.80	28.30	27.80	28.43	25.67	25.33	24.67	26.00	28.17	25.33	24.67	26.00
T(°C)	29.1	28.8	29.1	28.9	29.4	29.3	29.5	29.2	28.8	29.3	29.5	29.2
pH	5.3	5.1	5.2	5.1	5.3	5.3	5.5	5.4	5.1	5.3	5.5	5.4
Hds	6.34	4.50	4.17	6.17	4.84	5.34	4.34	5.67	4.62	5.34	4.34	5.67
Alk	4.95	6.23	6.78	5.68	5.13	6.23	6.78	5.68	6.23	6.23	6.78	5.68
NH ₃	0.25	0.18	0.14	0.16	0.18	0.16	0.14	0.00	0.18	0.16	0.14	0.13
NO ₂	0.01	0.01	0.01	0.05	0.01	0.01	0.13	0.01	0.01	0.01	0.00	0.01

Period= days; DO = dissolved oxygen (mg l^{-1}); EC= Electrical Conductivity ($\mu\text{S cm}^{-1}$); T (°C) = temperature; Hds = Hardness ($\text{mg CaCO}_3 \text{ l}^{-1}$); Alk = Alkalinity ($\text{mg CaCO}_3 \text{ l}^{-1}$); NH₃ = Ammonia (mg l^{-1}); NO₂ = Nitrite (mg l^{-1}).

In the blood parameters of the present study, the treatment showed difference in the 30-day period, with increase of Hemoglobin and MCHC and decrease in Cholesterol when compared to the other periods, and increased Glucose in the 15-day period. Values of MCHC, Cholesterol, Protein and Glucose were higher in 40g garlic-vine kg^{-1} concentration comparing to the control treatment (table 2).

Table 2. Blood parameters of *Colossoma macropomum* fed with diets containing different amounts of *Adenocalymma alliaceum* (garlic-vine).

7 days	Garlic-vine (g kg ⁻¹)			
	0	30	40	50
Htc	25.1±6.2	22.3±2.6	25±2.2	20±3.6
RBC	1.9±0.4	1.7±0.3	1.5±0.4	1.5±0.3
Hgb	6.6±1.3	6.2±1.5	6.5±0.8	5.6±1.0
MCV	131.3±31.7	131.3±30.5	169.1±33.8	140.3±34.0
MCH	34.8±9.0	36.9±12.7	44.0±9.7	38.4±8.6
MCHC	26.7±4.3	27.8±6.2	26.1±2.7	27.6±2.3
Cholesterol	80.5±11.32	108.2±21.2	84.0±20.5	84.1±20.1
Protein	2.5±0.3	2.5±0.2	2.7±0.3	2.5±0.3
Glucose	45.7±19.8	41.9±9.2	51.2±19.2	43.2±13.7

15 days

Htc	23.2±3.4	23.7±1.9	23±4.6	21.6±2.6
RBC	1.8±0.4	1.6±0.3	1.8±0.3	1.6±0.3
Hgb	7.3±1.4	6.7±1.2	7.6±1.2	7.0±0.5
MCV	129.0±26.1	153.9±28.8	129.4±25.6	133.1±17.1
MCH	40.5±7.2	43.0±9.5	42.9±6.5	43.3±6.4
MCHC	31.9±5.2	28.2±4.7	33.7±5.2	32.6±3.1
Cholesterol	65.6±18.9	79.2±16.6	77.8±13.5	72.9±22.0
Protein	2.4±0.2	2.4±0.2	2.4±0.4	2.4±0.3
Glucose	52.0±6.0	58.9±9.8	56.7±9.2	57.9±14.3

30 days

Htc	24±3.3	22.6±3.3	24.8±3.7	24.6±2.8
RBC	1.7±0.1	1.5±0.2	1.7±0.1	1.7±0.2
Hgb	7.8±1.1	8.0±1.7	9.4±2.5	8.7±1.7
MCV	145.3±17.9	149.5±23.0	149.4±25.0	145.4±17.8
MCH	47.3±7.5	52.3±9.6	57.0±16.2	51.7±10.8
MCHC	32.6±3.7	35.2±5.5	37.9±7.2	35.3±4.0
Cholesterol	63.5±11.5	57.1±8.4	76.3±13.7	66.8±10.5
Protein	2.2±0.2	2.2±0.3	2.6±0.3	2.4±0.2
Glucose	46.7±7.6	43.3±14.3	54.4±19.2	39.6±8.4

Htc = Hematocrit (%); RBC = Red Blood Cells ($\times 10^6 \mu\text{l}^{-1}$); Hgb = Hemoglobin (g dl^{-1}); MCV = (fl); MCH = (pg); MCHC = Mean Corpuscular Hemoglobin Concentration (%); Cholesterol (mg dl^{-1}); Protein (g dl^{-1}); Glucose = (mg dl^{-1}).

Differences in blood parameters between the three time periods and the different concentrations of garlic-vine were noticed (Wilks test of MANOVA, $p < 0.0001$ periods and treatments $p < 0.01$) (table 3).

Table 3. Statistic values of the analysis of blood parameters multivariate variance between periods and treatments with T^2 Hotelling later test.

	Statistic Values				
	Wilks Lambda	Gl num.	Gl denum.	P val.	T^2 Hotelling
Period	0.41	12	194.00	< 0.0001	7days-15days* 7days-30days* 15days-30days*
Treatments	0.69	18	274.84	0.005	Control-40g/Kg*

* Significant for $p < 0.0125$ (Bonferroni adjustment). For MANOVA analysis, hemoglobin, MCV and MCH blood parameters were eliminated due to multicollinearity ($VIF > 5$).

In tambaqui gills, 100% infections by monogenean were found, with decrease observed in the mean intensity of these parasites in fish fed with 40 and 50g garlic-vine kg^{-1} diet. (table 4, figure 1).

Table 4. Monogenean parasitic population indices in tambaquis in each treatment period.

Period	Garlic-Vine (g kg^{-1})	Efficacy (%)	Prevalence (%)	Mean Intesity	Mean Abundance
7	0		100	25.56	25.56

	30	-129.96	100	58.67	58.67
	40	-93.04	100	49.33	49.33
	50	-77.39	100	45.33	45.33
	0		100	28.89	28.89
15	30	-51.62	100	44	44
	40	-144.62	100	70.67	70.67
	50	-110.77	100	60.89	60.89
	0		100	136	136
30	30	32.79	100	92	92
	40	70.59	100	40	40
	50	62.21	100	51.56	51.56

Period = Treatment days

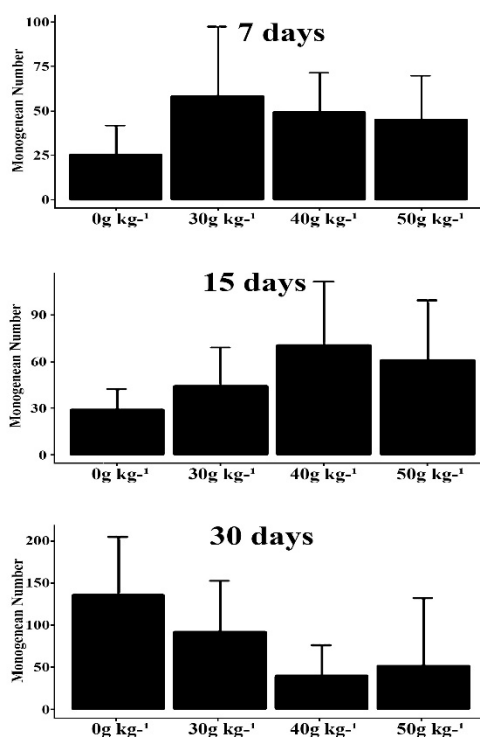


Figure 1. Number of monogeneans in tambaqui gills exposed to treatments for 7, 15 and 30 days. Each essay tested different concentrations of *A. alliaceum* per kilogram of feed: 0 g kg⁻¹, 30 g kg⁻¹, 40 g kg⁻¹ and 50 g kg⁻¹.

When comparing the treatments for each period, the means of monogenean were not statistically different between 7 and 15 days, however, for the 30-day period there was a significant difference (Chi2 = 14.4, $p < 0.05$), where the number of monogeneans was lower in the treatment with 40g garlic-vine kg⁻¹ (40±36.2) when compared to the control group (136±69.4) (Mann-Whitney test, $p < 0.0125$) (table 5).

Table 5. Average values of the number of parasites for each treatment with *Adenocalymma alliaceum* by period ± standard deviation and statistic values of Kruskal-Wallis proof and Mann-Whitney later test.

	Statistic Values
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Period	0 g kg ⁻¹	30 g kg ⁻¹	40 g kg ⁻¹	50 g kg ⁻¹	Chi ²	Gl.	P val.	Mann-Whitney
7 days	25.6±16.1	58.6±39	49.3±22.2	45.3±24.3	8.02	3	0.05	-
15 days	28.9±13.2	44±25.1	70.6±41.1	60.9±38.9	6.7	3	0.08	-
30 days	136±69.4	92±60.9	40±36.2	51.5±80.4	14.4	3	0.002*	0 - 40 g kg ⁻¹ **

* Significant for $p < 0.05$. ** Significant for $p < 0.0125$ (Bonferroni adjustment).

In the present study, efficacy was observed only after 30 days of fish feeding in the treatment of 40g garlic-vine kg⁻¹ (70.59%), and in the other experimental periods (7 and 15 days) due to parasitary indices of the treatment being higher than the control groups, the efficacy values were negative (table 4).

Discussion

Blood parameters have been an important tool to test the toxicity of a drug in fish disease treatments (Andrade et al., 2018; Chagas et al., 2016; Ferreira et al., 2019). In a study that investigated the performance, monogenean infestations and blood parameters of tambaqui maintained in net-cages and fed with diets containing garlic, Inoue et al. (2016) observed that there was no change in the plasma glucose levels, total plasma protein levels and red blood cells in tambaquis fed with 30g and 45g of garlic kg⁻¹ for 45 days.

Evaluating the impact of monogenean parasitism on the hematological parameters of fish in fish farming, Rocha et al. (2018) found 100% prevalence of parasites. This infection was able to debilitate the tambaquis causing hematological alterations, such as a correlation between abundance of monogeneans and glucose. Similar results were found by Gonzales et al. (2020) which detected increase in plasma glucose levels in tambaqui, however, this study obtained low efficacy against monogeneans using *Cymbopogon citratus* in baths. Nevertheless, according to the reference of blood parameters for cultivated fish Tavares-Dias (2015), mean values found in the present study are within the standards determined for tambaqui.

Monogeneans are highly pathogenic and they have very efficient dispersal means, because their transmission occurs by contact between hosts, being transported passively by the water stream (Eiras et al., 2010; Thatcher, 2006).

In places that have high fish density, changes in water quality and poor nutritional quality, these parasites can cause intense infections with high mortality rate and despite the study environment being controlled, it was still possible to observe increase in average intensity of monogeneans of control groups over 7 (25.56), 15 (28.89) and 30 days (136) (fig. 1).

The major compounds isolated from *Adenocalymma alliaceum* (Syn. *Mansoa alliacea*) were: tannin, flavonoids, terpenes, alkaloid, cumarin, saponin, p-coumaric acid, ferulic acid, resveratrol, and sulfur compounds (disulfide, trisulfide and diallyl tetrasulfide) (Fanela et al., 2016; Pires et al., 2016; Venkataraman and Srilakshmi, 2018).

With this, their biological capacities are antifungal (Aswini et al., 2010; Sudirga et al., 2019), insecticidal (Fanela et al., 2016), antibacterial (Frank et al., 2014; Rachel et al., 2012), inhibitor of antiallergic histamine release, antiviral, anti-inflammatory, antioxidant, suppressor of tumor growth (Pires et al., 2016) and activity in an inflammatory pain similar to those arthritis-induced (Hamann et al., 2019). However, the studies proved the anthelmintic properties of garlic-vine are rare, and it is necessary to compare the present study with *Allium sativum* results.

Boijink et al. (2011), evaluating the anthelmintic activity of *Adenocalymma alliaceum* added to feed over 45 days, reached 52% efficacy with 30g of garlic-vine kg⁻¹ and 63% adding 45g kg⁻¹ in feed. Examining the effects of dietary supplementation with allicin-containing garlic extract as preventive against monogenean infection, on farmed barramundi, *Lates calcarifer*, Militz et al. (2013) obtained similar results to the present study, decreasing 70% of parasite infection with 50 ml

kg⁻¹ of garlic-vine in a 30-day diet. The allicin present in *Adenocalymma alliaceum* and *Allium sativum* is a lipid-soluble sulfur compound which, according to Schelkle et al. (2013) and Batiha et al. (2020), might not be very efficacious because it can be easily degraded by cooking, and oven drying is needed to prepare the feed. These previous studies and our data suggest that, by extending the in-feed treatment period (30 to 45 days), the removal of monogeneans infestation will increase.

In a study comparing the efficacy of baths using garlic products and components as alternative treatments against *Gyrodactylus* infections on guppies, *Poecilia reticulata*, Schelkle et al. (2013) observed efficacy increased for all three treatments, with the mean of 96% to Chinese freeze dried garlic flakes (0.33 mg ml⁻¹), 95% to freeze dried garlic flakes (1 mg ml⁻¹), and 95% to allyl disulphide (0.5 mg ml⁻¹). However, at higher doses, garlic induced slight damage the fin edges. In another work with guppies, Fridman et al. (2014) observed that bath exposure to the lower concentration of 1500 mg fresh garlic l⁻¹ for 1 hour, reduced the infection prevalence to 12.3% from the original 80%.

Conclusion

Under the conditions in this study, garlic-vine can be evaluated as promising due to its capacity to reduce the amount of fish parasites, revealing itself as an efficacious handling strategy controlling monogeneans without compromising blood parameters. There is scarce information about the use of garlic-vine in fish-farming diseases treatment and future studies evaluating its toxicity are needed.

Acknowledgements

To the National Council for Research and Technological Development (Conselho Nacional de Pesquisa e Desenvolvimento Tecnológico - CNPq) and to the Financier of Studies and Projects (Financiadora de Estudos e Projetos - FINEP) for the financial support, and to the Coordination for the Improvement of Higher Education Personnel (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Capes) for the granting of the Master's scholarship.

Funding: This work was supported by the National Council for Research and Technological Development (Conselho Nacional de Pesquisa e Desenvolvimento Tecnológico) CNPq [grant numbers MCT/CNPq/CT-Hidro/CT-Aqua N° 19°2009 Legal Amazon / Notice n° 19/2009 – Axis 2, effective from 2009 to 2012] and the Financier of Studies and Projects (Financiadora de Estudos e Projetos - FINEP).

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