In Vitro Antibacterial Potential of Plant Extracts and in Vivo Ilex paraguariensis Effect on Oreochromis niloticus Physiology and Resistance to Aeromonas hydrophila

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Abstract: The aim of the study was to investigate the antibacterial potential of garlic (Allium sativum), pinus (Pinus elliottii), eucalyptus (Eucalyptus sp.), yerba mate (Ilex paraguariensis) and guava tree (Psidium guajava Linn.) in vitro and the effect of yerba mate supplementation on Oreochromis niloticus physiology and resistance to Aeromonas hydrophila. The in vitro antibacterial potential of the plants was verified by the minimum inhibitory concentration (MIC) method and by the antimicrobial sensitivity test (TSA). The in vivo assay was performed using diet supplementation with yerba mate and challenge with A. hydrophila. After the supplementation and challenge periods biochemical, immune and physiological parameters were evaluated. The in vitro assays showed that only yerba mate presented antibacterial potential against A. hydrophila and no one of the plant extracts were effective against Streptococcus agalactiae. The use of yerba mate in the diet did not influence the immune and metabolic status of O. niloticus. Also, the supplementation did not act as a growth promoter for the fishes. Considering the challenge of O. niloticus with A. hydrophila, the yerba mate did not improve the resistance of the fishes to the bacteria. The yerba mate extract presented potential to be used as an antibacterial agent for fish diseases. Also, more detailed studies are necessary to establish effective doses and times required for fish treatments.

Key words: Oreochromis niloticus, antibacterial potential, Ilex paraguariensis, fish metabolism, Aeromonas hydrophila.

1. Introduction

According to FAO (2016) [1], world fish farming in 2014 reached 49.8 million tons of fish and one of the most cultivated freshwater fish species in the world is tilapia (Oreochromis niloticus). According to FAO data, the production of this species of fish in 2011 was approximately 3,000,000 tons. Nile tilapia (O. niloticus) is a scaled fish belonging to the perciform order and to the Cichidae family. The tilapia has a high growth rate, great rusticity, easy handling, high carcass yield and good quality meat. Also, they are fish tolerant to the high stocking densities being cultivated in intensive regime [2].

The demand for animal protein, especially for fish, has been increasing significantly in recent decades worldwide. In order to meet this demand, there have been an intensification of fish farming and as a result, many fish diseases appeared and spread particularly among cultured fishes [3, 4]. When homeostasis is disturbed, fish become highly vulnerable to infections, which may be caused by opportunistic pathogens. The pathogens become virulent in special conditions, such as in cases of low immunity of fishes or they are stressed causing outbreaks in fish farms with high mortality rates and severe economic losses [3, 5].

Aeromonas hydrophila is widely distributed in aquaculture and is one of the most important microbial pathogens affecting cultured fishes...
worldwide. The bacterium causes haemorrhagic septicaemia or is associated with surface ulcerations on fish [6]. Besides *A. hydrophila*, other important pathogen that is responsible for mortalities of both cultured and wild fish species worldwide is *Streptococcus agalactiae* [7]. There are many reports of infections in marine and freshwater fish species, both in the wild and under cultivation [7, 8].

The most common strategy to fight aquaculture diseases is the use of antibiotics and disinfectants, however, such usage has been reported to have adverse effects. There is a growing concern about the use and abuse of the antibiotics in aquaculture, as they increase the selective pressure exerted on the microbes and encourage the emergence of resistant bacteria. Moreover, the use of antimicrobials tends to generate and accumulate drug residues in the treated fishes, besides having a negative impact on the environment [4].

In this view, the use of the so-called environment friendly approaches, natural products have been considered as an alternative to control bacterial infections in aquaculture. Some herbs are reported to have antimicrobial activity against several pathogenic bacteria and have been used as traditional medicines for the treatment of human diseases. Recently, attention has been focused on the effects of medicinal plants on animals, especially in fishes [9]. In this study, the objectives were to study the antibacterial potential of several herbs *in vitro* against *A. hydrophila* and *S. agalactiae* and *in vivo* in Nile tilapia.

### 2. Materials and Methods

#### 2.1 Plant Material

Garlic (*Allium sativum*) extract (0.5% of allicin) was purchased from a commercial source (Active Pharmaceutics, São José, SC, Brazil). Pinus (*Pinus elliottii*), eucalyptus (*Eucalyptus* sp.), yerba mate (*Ilex paraguariensis*) and guava tree (*Psidium guajava* Linn.) leaves were collected at Federal University of Fronteira Sul, Campus Laranjeiras do Sul, PR, Brazil, during spring. The leaves were sanitized with distilled water. Native leaves collected were identified by Prof. Dr. Josimeire Aparecida Leandrin and archived as a voucher specimen in the Herbarium of the Federal University of Fronteira Sul, Campus Laranjeiras do Sul, PR.

#### 2.2 Preparation of the Plant Extracts

The pinus leaves extract was prepared according to Krishnaswamy and Orsat [10]. The eucalyptus extract was prepared using dried leaves according to the methodology of Ammer et al. [11]. For the guava tree extract, the fresh leaves were used and prepared according to Fernandes [12]. For the preparation of the yerba mate extract the methodology of Pereira et al. [13] was followed. All the powder extracts were dissolved (pinus and garlic in water and eucalyptus, yerba mate and guava tree in DMSO) at 80 mg/mL to be used in the *in vitro* and *in vivo* assays.

#### 2.3 Determination of the Minimum Inhibitory Concentration (MIC)

The antibacterial potential was verified by the MIC method. MIC was performed in 96-well microplates with Mueller Hinton broth, with serial dilutions initiating with 80 mg/mL of each plant extract. To each well in the microplate that contained plant extract was added 5 µL of the bacteria inoculum, *A. hydrophila* (CPQBA 228-08) or *S. agalactiae* (ATCC), in the concentration 5 × 10^7 CFU/mL. The crude extract of each plant and the solvent DMSO alone were used as controls. *A. hydrophila* and *S. agalactiae* inoculum alone, in the concentration 5 × 10^7 CFU/mL, were used as negative controls. The microplates were sealed with sealing film, incubated at 28 °C for 24 h. After this period, the reading was developed. The MIC is considered the concentration of the highest dilution microplate well in which there was no bacterial growth [14].

#### 2.4 Determination of the Antibacterial Potential of the Plant Extracts

The antimicrobial sensitivity test (TSA) was
performed by the well diffusion technique [15, 16] with modifications. It was considered the value of MIC using two concentrations above and two below it. Bacterial inoculums were prepared from fresh (18-24 h) cultures produced from a single colony grown on nutrient agar and adjusted to the 0.5 McFarland scale (1.5 $\times$ 10^8 CFU/mL). The inoculums of A. hydrophila were seeded on Mueller Hinton agar plates and S. agalactiae seeded on Mueller Hinton agar supplemented with 5% defibrinated sheep blood. Seven equidistant holes were filled with 40 $\mu$L chloramphenicol (positive control), DMSO (negative control) and the plant extracts at five different concentrations (5, 10, 20, 40, 80 mg/mL). The plates were incubated aerobically at 37 °C for 16-18 h. Inhibition halos (mm) were measured with a digital pachymeter considering the method proposed by Costa et al. [17] that considered halos above 10 mm as sensitive and under 10 mm as resistant.

2.5 Water Quality

Water pH, dissolved oxygen and salinity in the tanks were measured using a YSI oxygen meter (Model Y5512; YSI Inc., Yellow Springs, OH, USA). Ammonia levels were determined daily while nitrite levels were monitored each 15 d using commercial kits (Alfa kit).

2.6 Experimental Animals

Nile tilapia (O. niloticus) juveniles (8.5 ± 0.7 cm and 9.55 ± 2.1 g) were obtained from a commercial fish farmer (Akna Alevinos, Toledo, PR, Brazil). These juveniles did not present any apparent illness when they arrived at the Fish Pathology Laboratory, at Federal University of Fronteira Sul, Laranjeiras do Sul, PR, Brazil.

The fishes were randomly distributed in a continuously aerated 35 L tanks system, where they were maintained for a seven days’ acclimation period. Dead fish were removed daily. The feeding and swimming behavior of the juveniles were continuously observed throughout the acclimation period. The laboratory temperature was maintained by using an air conditioner (29 °C). All the animals were monitored carefully and maintained in accordance with the ethical recommendations of the Brazilian Veterinary Medicine Council and the Brazilian College of Animal Experimentation (Protocol CEUA/23205.004767/2016-82).

2.7 In Vivo Experiments

During the acclimation period, the juveniles were fed twice a day (08:00 a.m. and 05:00 p.m.) with a commercial diet (Supra tilapia, minimum guaranteed levels of crude protein 36%, Maringá-PR, Brazil) until apparent satiety. Uneaten food as well as other residues and feces were siphoned out 30 min after feeding. After acclimation, the animals were fed with commercial feed added with different concentrations of yerba mate extract and challenged with A. hydrophila. For the feed supplementation with yerba mate, the commercial feed was milled and the extract was added as powder and the feed was pelleted again and dried in the oven at 70 °C. The animals were divided into different groups based on the yerba mate in vitro results: control; T1 (0.005% of yerba mate), T2 (0.05% of yerba mate), T3 (0.5% of yerba mate) and T4 (5% of yerba mate). The animals received the feed supplemented with yerba mate for 25 d. At the end of this period, the animals were challenged with an intraperitoneal injection of 0.1 mL culture suspension of reference pathogenic A. hydrophila containing 1.5 $\times$ 10^7 CFU/mL. The non-challenged animals of all groups received 0.1 mL of saline solution. After the injection, the animals were followed for 5 d keeping the feed supplemented with plant extract [18].

2.8 Tissues and Blood Sampling

At the end of the experiment the fishes were anaesthetized by immersing the fish in water containing 50 mg/L of clove oil. Blood samples were
collected from the caudal vein of fish, by using needles previously rinsed in heparin for the hematological evaluation. For the plasma separation, the blood samples were centrifuged at 300× g for 10 min and the plasma was collected. The plasma was stored at -20 °C in microtubes until its use for biochemical and immune studies. After blood collection, the animals were killed by anesthetic deepening and tissue samples (liver and kidney) were removed to biochemical analysis.

2.9 Hematological Parameters

Blood collected with anticoagulant was used to measure the hematocrit percentage [19]. The hemoglobin (Hb) concentrations were estimated through a colorimetric kit (Bioclin, Belo Horizonte, Minas Gerais, Brazil) following the manufacturer’s instructions. Red blood cells (RBC) were counted in Neubauer chamber. Mean corpuscular volume (MCV), mean corpuscular Hb (MCH) and MCH concentration (MCHC) were calculated using standard formulas.

2.10 Lysozyme Levels

The serum and kidney lysozyme levels were measured spectrophotometrically according to the method of Ellis [20] with modifications [21] based in a calibration curve. The linear regression equation of the lysozyme calibration curve was used to determine the plasma and kidney lysozyme levels (µg/mL).

2.11 Quantification of Nitrite/Nitrate Levels

Quantification of the nitric oxide products in the kidney homogenates of the fishes was carried out according to the methodology described by Green et al. [22] and nitrite/nitrate concentrations expressed as µM were estimated by interpolation from a standard curve of sodium nitrite (0-150 µM) by colorimetric measurements at 540 nm in a microplate reader (Multiskan Go-Thermo Scientific, San Jose, CA, USA) [23].

2.12 Biochemical Parameters

For the biochemical parameters, plasma samples and tissues homogenates were used. The liver was homogenized in phosphate buffered saline (PBS), pH 7.2. The homogenate was centrifuged at 5,000× g for 10 min to eliminate nuclei and cell debris, and the supernatant fraction obtained was frozen at -70 °C for further measurements. The protein levels were estimated spectrophotometrically using the method described by Bradford [24] and bovine serum albumin was used as the standard. Plasma glucose levels were determined by the glucose oxidase method through commercial colorimetric kits (Gold Analisa®, Belo Horizonte, MG, Brazil) following the manufacturer’s instructions and expressed as mg/dL. The liver glycogen content was determined as described by Krisman [25] and expressed as mg glycogen/g tissue. The enzymatic activity of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) was quantified in aliquots of plasma and liver homogenate by using the colorimetric method and the results for tissues and plasma were expressed as U/L/mg protein or U/L, respectively.

2.13 Antioxidant Activity

The supernatant from the liver homogenate was used. Catalase (CAT) activity was determined by using the method described by Aebi [26], in which the disappearance of H₂O₂ is followed spectrophotometrically at 240 nm. The results were reported as mmol/mg protein/min. Glutathione S-transferase (GST) activity was measured using the methodology described by Habig et al. [27] and the enzyme activity was expressed as mM/min/mg prot. Reduced glutathione (GSH) levels were determined according to Beutler et al. [28] and the results were expressed in mM/mL. Lipid peroxidation was measured by thiobarbituric acid reactive substances (TBARS) using the method described by Federici et al.
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[29]. The results were reported as mmol TBARS/mg prot. The oxidative damage to proteins by carbonylation was determined by the method described by Levine et al. [30]. The carbonyl content was measured spectrophotometrically at 370 nm and the protein carbonyl concentration was expressed in μmol/mg protein.

2.14 Data and Statistical Analysis

Data were expressed as mean ± SD. Bartlett and Shapiro-Wilk tests were used to determine the homogeneity of the variance and normality, respectively. One-way analysis of variance (ANOVA) and LSD test were used to verify significance of the mean differences among treatments. The minimum significance level was set at $p < 0.05$. All tests were performed with the Software R 3.4.3.

3. Results

3.1 In Vitro Antibacterial Activity of Plant Extracts

Extracts of five plants, garlic (A. sativum), pinus (P. elliottii), eucalyptus (Eucalyptus sp.), yerba mate (I. paraguariensis) and guava tree (P. guajava Linn.), were tested for antimicrobial activity against A. hydrophila and S. agalactiae. The results showed that the extract of yerba mate had the lowest MIC (20 mg/mL) while the extract of guava tree showed the highest value (40 mg/mL) considering the presence of A. hydrophila. The plant extracts did not present values of MIC for S. agalactiae in the concentrations studied. Based on that, it was performed the TSA for A. hydrophila (Table 1) and S. agalactiae. The five plants did not inhibit S. agalactiae growth in the concentrations tested (data not shown). As it can be seen, just yerba mate showed inhibitory activity against A. hydrophila at 80 mg/mL (inhibition zone diameter of 11.84 ± 1.5 mm). Since the ethanol extract of yerba mate presented the strongest inhibitory effect against A. hydrophila in both, the TSA and MIC determination, that extract was used in the in vivo experiment.

3.2 In Vivo Antibacterial Potential of Yerba Mate

3.2.1 Water Quality and Zootechnic Parameters

The water quality parameters remained constant and close to the reference levels for O. niloticus during the acclimation and the experimental periods (dissolved oxygen (4.8 ± 0.58 mg/L), temperature (27.22 ± 0.6 °C), pH (7.08 ± 0.19), salinity (2.02‰ ± 0.17‰), ammonia (0.37 ± 0.35 mg/L) and nitrite levels (0.108 ± 0.105 mg/L)). Also, there was no difference in terms of weight gain and growth parameters among the fish fed with yerba mate-supplemented diets, challenged fishes and control groups (data not shown). On the other hand, the non-challenged yerba mate-supplemented groups showed 90% (T1, T2 and T3) and 100% (T4) of survival while the challenged groups presented 100% (T1 and T4), 90% (T2) and 80% (T3) of survival. Considering the control groups (challenged or not) they presented 100% of survival at

| Table 1 | Antimicrobial activity of garlic (Allium sativum), pinus (Pinus elliottii), eucalyptus (Eucalyptus sp.), yerba mate (Ilex paraguariensis) and guava tree (Psidium guajava Linn.) against Aeromonas hydrophila (CPQBA 228-08) as determined by antimicrobial sensitivity test (TSA). |
|------------------------|---------------------------------------------------------------------------------------------------------------|------------------------|------------------------|------------------------|------------------------|------------------------|------------------------|
|                        | Control                                                                                                       | Extracts concentrations (mg/mL)                                                                 |
|                        | Chloramphenicol 3 mg/mL                                                                                       | 80                     | 40                     | 20                     | 10                     | 5                      |
| A. sativum             | 32.43 ± 1.6 AH                                                                                                 | AH                     | AH                     | AH                     | AH                     | AH                     |
| P. elliottii           | 31.80 ± 0.3 AH                                                                                                 | AH                     | AH                     | AH                     | AH                     | AH                     |
| Eucalyptus sp.         | 31.41 ± 0.3 AH                                                                                                 | AH                     | AH                     | AH                     | AH                     | AH                     |
| P. guajava             | 27.03 ± 0.4 AH                                                                                                 | AH                     | AH                     | AH                     | AH                     | AH                     |
| I. paraguariensis      | 33.84 ± 1.9 AH                                                                                                 | 11.84 ± 1.5 AH         | AH                     | AH                     | AH                     | AH                     |

AH: absent halo (below 10 mm); the data of chloramphenicol represents the value obtained from the plates of each extract as a positive control; inhibition halos measured in millimeters; data are presented as mean ± SD of three replicates.
the end of the period of study.

3.2.2 Metabolic Parameters from *O. niloticus* Supplemented with Yerba Mate and Challenged with *A. hydrophila*

In the present study the influence of the yerba mate supplementation and the challenge with *A. hydrophila* on fishes’ metabolism and homeostasis were analyzed. Fig. 1 shows that neither the yerba mate supplementation nor the bacteria challenge were able to influence significantly the activity of plasmatic AST and ALT of tilapias.

Also, the influence on glycemia and glycogen content were studied. As it can be seen at Fig. 2, there were not significant differences between the control groups, challenged or not. On the other hand, there was a significant reduction in glycemia in T1 non-challenged group compared with the control of the same group. There was, also, an increase in plasma glucose levels in T1 and T2 challenged groups compared with the respective non-challenged groups, however this variation seems to be related to the reduction of glycemia by the yerba mate supplementation in the non-challenged group. Although there were variations on blood glucose levels, the hepatic glycogen content was not significantly influenced neither by the supplementation nor by the bacteria.

In order to evaluate if the yerba mate supplementation and/or the challenge with *A. hydrophila* influence the tilapias’ antioxidant system the GST and CAT activities as well as the reduced GSH content, the lipid peroxidation and carbonyl protein levels were studied. As demonstrated at Table 2 there was an increase of GSH content in T2 group (non-challenged) when compared with the respective control group. Despite the increase in GSH content, there were no significant changes in the GST and CAT activities in all experimental groups (Table 2). Also, it was not verified any alteration in the hepatic lipid peroxidation as well as in the hepatic carbonyl protein levels after the supplementation period or the challenge with *A. hydrophila*.

3.2.3 Immune and Hematologic Parameters from *O. niloticus* Supplemented with Yerba Mate and Challenged with *A. hydrophila*

Fig. 3 shows the effect of the yerba mate supplementation and the *A. hydrophila* challenge on kidney nitrite/nitrate levels. As it can be noticed there was an increase in nitrite/nitrate levels in non-challenged T1 group compared with the non-challenged control group. Although the increase in nitrite/nitrate levels can indicate an improvement of the immune system, the renal and plasmatic lysozyme activity was not modified by the yerba mate supplementation and the *A. hydrophila* challenge (Fig. 4).

The hematological parameters of number of erythrocytes (RBC), Hb content, hematocrit (Hct) and the blood indices calculated (MCV, MCH and MCHC) were also studied (Table 3). The number of erythrocytes (RBC) from the groups that received yerba mate supplemented-diets and were challenged with *A. hydrophila* was significantly lower (*p* < 0.05) than the respective supplemented groups. The lowest number of erythrocytes (1.95 ± 0.44) was observed in the challenged group that received 0.005% yerba mate-added diet (T1). Also, it was observed an increase of the number of erythrocytes in T4 (5% yerba mate-added diet) challenged group when compared with the control challenged group. Additionally, there were statistical differences in the MHC values between non-challenged control and challenged control, non-challenged T2 and challenged T2 which showed increases in MHC. Also, there was significant reduction of MHC between challenged T4 and challenged control group. On the other hand, Hb content, Hct and the blood indices MCV and MCHC were not influenced neither by the yerba mate nor by the *A. hydrophila* challenge.
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Fig. 1 Effect of yerba mate supplemented diet and *Aeromonas hydrophila* challenge on plasma (a) alanine aminotransferase (ALT) and (b) aspartate aminotransferase (AST) activity from *Oreochromis niloticus* juveniles. Values are expressed as mean ± SD; n = 20.

Fig. 2 Effect of yerba mate supplemented diet and *A. hydrophila* challenge on (a) glycemia and (b) hepatic glycogen content from *O. niloticus* juveniles. Values are expressed as mean ± SD; n = 20.

Significant at *p* ≤ 0.05 in relation to non-challenged control group, †*p* ≤ 0.05 in relation to the respective non-challenged group.

Fig. 3 Effect of yerba mate supplemented diet and *A. hydrophila* challenge on plasma (a) and kidney (b) lysozyme levels from *O. niloticus* juveniles. Values are expressed as mean ± SD; n = 20.
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**Fig. 4** Effect of yerba mate supplemented diet and *A. hydrophila* challenge kidney nitrite/nitrate levels from *O. niloticus* juveniles.

Values are expressed as mean ± SD; n = 20.

Significant at *p ≤ 0.05 in relation to non-challenged control group.

**Table 2** Effect of yerba mate supplemented diet and *A. hydrophila* challenge on glutathione (GSH), protein carbonyl and thiobarbituric acid reactive substances (TBARS) levels and on glutathione S-transferase (GST) and Catalase (CAT) activity in liver from *Oreochromis niloticus* juveniles.

<table>
<thead>
<tr>
<th></th>
<th>GSH levels (mM/mL)</th>
<th>GST activity (mM/min/mg protein)</th>
<th>CAT activity (mM/min/mg protein)</th>
<th>Protein carbonyl levels (µM/mg protein)</th>
<th>TBARS levels (mM TBARS/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-challenged</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>9.75 ± 2.60</td>
<td>1.79 ± 1.05</td>
<td>0.09 ± 0.05</td>
<td>4.97 ± 1.44</td>
<td>2.37 ± 0.98</td>
</tr>
<tr>
<td>T1</td>
<td>10.55 ± 3.23</td>
<td>1.27 ± 0.49</td>
<td>0.07 ± 0.01</td>
<td>4.35 ± 2.94</td>
<td>2.83 ± 0.64</td>
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<tr>
<td>T2</td>
<td>11.75 ± 6.36</td>
<td>0.86 ± 0.42</td>
<td>0.09 ± 0.05</td>
<td>4.48 ± 1.55</td>
<td>2.97 ± 0.80</td>
</tr>
<tr>
<td>T3</td>
<td>9.44 ± 4.06</td>
<td>1.28 ± 1.06</td>
<td>0.09 ± 0.01</td>
<td>1.94 ± 1.19</td>
<td>2.94 ± 0.70</td>
</tr>
<tr>
<td>T4</td>
<td>8.44 ± 3.04</td>
<td>1.63 ± 1.58</td>
<td>0.09 ± 0.01</td>
<td>2.77 ± 1.46</td>
<td>2.90 ± 0.99</td>
</tr>
<tr>
<td>Challenged with <em>A. hydrophila</em></td>
<td></td>
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<tr>
<td>C BAC</td>
<td>6.29 ± 0.95</td>
<td>0.52 ± 0.12</td>
<td>0.11 ± 0.09</td>
<td>2.16 ± 1.56</td>
<td>2.44 ± 0.77</td>
</tr>
<tr>
<td>T1 BAC</td>
<td>8.92 ± 1.83</td>
<td>1.40 ± 1.28</td>
<td>0.15 ± 0.07</td>
<td>1.17 ± 0.72</td>
<td>2.74 ± 1.27</td>
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<tr>
<td>T2 BAC</td>
<td>10.90 ± 1.78</td>
<td>0.74 ± 0.36</td>
<td>0.09 ± 0.03</td>
<td>2.49 ± 1.50</td>
<td>2.79 ± 1.04</td>
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<tr>
<td>T3 BAC</td>
<td>10.77 ± 2.63</td>
<td>1.03 ± 0.28</td>
<td>0.06 ± 0.01</td>
<td>3.22 ± 1.10</td>
<td>2.84 ± 0.64</td>
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<td>T4 BAC</td>
<td>8.84 ± 2.34</td>
<td>1.27 ± 1.68</td>
<td>0.09 ± 0.01</td>
<td>1.73 ± 1.96</td>
<td>2.56 ± 0.89</td>
</tr>
</tbody>
</table>

BAC: experimental groups challenged with *A. hydrophila*; values are expressed as mean ± SD; n = 20.

Significant at *p ≤ 0.05 in relation to control group.
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Table 3  Effect of yerba mate supplemented diet and A. hydrophila challenge on hematological parameters of O. niloticus juveniles.

<table>
<thead>
<tr>
<th></th>
<th>RBC (×10⁶/mm³)</th>
<th>Hb (g/dL)</th>
<th>Hct (Hm%)</th>
<th>MCH (pg)</th>
<th>MCHC (g/dL)</th>
<th>MCV (fL)</th>
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<tr>
<td>C</td>
<td>3.27 ± 0.22</td>
<td>8.15 ± 0.71</td>
<td>35.88 ± 4.91</td>
<td>24.73 ± 2.81</td>
<td>22.95 ± 4.53</td>
<td>110.25 ± 14.26</td>
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<tr>
<td>T1</td>
<td>3.12 ± 0.27</td>
<td>8.49 ± 1.78</td>
<td>32.63 ± 6.84</td>
<td>29.05 ± 6.76</td>
<td>28.36 ± 5.24</td>
<td>99.79 ± 17.11</td>
</tr>
<tr>
<td>T2</td>
<td>3.03 ± 0.43</td>
<td>9.23 ± 1.60</td>
<td>32.22 ± 4.27</td>
<td>29.45 ± 5.35</td>
<td>27.09 ± 5.56</td>
<td>113.22 ± 9.26</td>
</tr>
<tr>
<td>T3</td>
<td>2.89 ± 0.40</td>
<td>9.14 ± 1.62</td>
<td>32.67 ± 7.28</td>
<td>32.67 ± 7.22</td>
<td>29.64 ± 5.35</td>
<td>107.87 ± 17.89</td>
</tr>
<tr>
<td>T4</td>
<td>2.95 ± 0.39</td>
<td>9.76 ± 2.18</td>
<td>32.33 ± 9.79</td>
<td>33.54 ± 7.18</td>
<td>31.31 ± 6.64</td>
<td>101.72 ± 24.98</td>
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<tr>
<td>Challenged with A. hydrophila</td>
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</tr>
<tr>
<td>C BAC</td>
<td>1.97 ± 0.28</td>
<td>7.08 ± 1.21</td>
<td>29.80 ± 5.12</td>
<td>37.52 ± 8.78</td>
<td>26.94 ± 4.34</td>
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<tr>
<td>T1 BAC</td>
<td>1.95 ± 0.44</td>
<td>8.13 ± 0.56</td>
<td>40.00 ± 2.31</td>
<td>36.87 ± 4.27</td>
<td>22.82 ± 4.40</td>
<td>177.72 ± 13.33</td>
</tr>
<tr>
<td>T2 BAC</td>
<td>2.34 ± 0.38</td>
<td>9.39 ± 1.84</td>
<td>34.80 ± 4.09</td>
<td>44.78 ± 9.37</td>
<td>32.99 ± 4.35</td>
<td>162.61 ± 25.75</td>
</tr>
<tr>
<td>T3 BAC</td>
<td>2.28 ± 0.48</td>
<td>7.63 ± 0.76</td>
<td>35.00 ± 8.37</td>
<td>34.49 ± 6.89</td>
<td>21.30 ± 5.28</td>
<td>125.69 ± 44.50</td>
</tr>
<tr>
<td>T4 BAC</td>
<td>2.87 ± 0.48</td>
<td>7.68 ± 1.08</td>
<td>30.13 ± 7.85</td>
<td>27.68 ± 7.37</td>
<td>27.13 ± 5.51</td>
<td>106.08 ± 9.17</td>
</tr>
</tbody>
</table>

Variables are red blood cell count (RBC), hemoglobin (Hb) concentration, hematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular Hb (MCH) and MCH concentration (MCHC); BAC: experimental groups challenged with A. hydrophila; values are expressed as mean ± SD; n = 20.

Significant at *p ≤ 0.05 in relation to control group; significant at #p ≤ 0.05 in relation to the respective non-challenged concentration; significant at @p ≤ 0.05 in relation to challenged control (C-BAC).

4. Discussion

Several plant-derived compounds have been used in the prevention and treatment of fish diseases since the plants present different biological properties and a great potential to be used as alternatives to the conventional treatments [9]. Although there is evidence of the benefits of using plant extracts in aquaculture, there are few studies on the effects of these extracts on Nile tilapia. This study showed the antibacterial potential of diverse plant extracts in vitro against S. agalactiae and A. hydrophila as well as the in vivo effect on Nile tilapia challenged with A. hydrophila.

The in vitro study demonstrated that among the tested extracts just yerba mate showed an antibacterial potential against A. hydrophila while none extract was effective against S. agalactiae. This result agrees with reports on the literature about the antibacterial effect of yerba mate on gram-negative bacteria [17, 31]. It is important to note that this is the first report of the effect of yerba mate on A. hydrophila, a gram-negative bacterium, widely spread in fish farms and a very common cause of fish diseases.

Considering the in vitro results obtained with yerba mate, this extract was chosen to study its antibacterial potential in vivo in Nile tilapia challenged with A. hydrophila. In the animals that received yerba mate supplemented-diet and challenged or not with the bacterium the extract has not acted as a growth promoter and did not influence the survival rates.

In terms of metabolism, the supplementation and the challenge were not able to change the AST/ALT activities indicating that the extract did not cause acute toxicity. Also, the hepatic glycogen content was not modified by yerba mate. It is well known that in stressing conditions there is an increase of cortisol in the blood stream and the hormone acts especially at the liver stimulating glucose release to the blood increasing its levels [32]. It is interesting to note that it was observed an increase in glycemia in the T1 and T2 challenged groups, although there were not alterations in liver glycogen levels indicating that probably the increase in glycemia is maintained by the activation of hepatic gluconeogenesis [32, 33]. This type of response, increase in cortisol and glycemia, was already described for tilapia challenged with S. agalactiae [34]. Considering that, this physiological
condition denotes that the presence of *A. hydrophila* induced a stress condition with metabolic adaptations and this was not prevented by yerba mate.

It is well known that the body homeostasis is crucial to the fishes’ development during the cultivation and any type of stressing condition can influence the homeostasis. These conditions can induce the production of reactive species, especially those derived from oxygen (EROs), leading to an increase in oxidative stress and damaging lipids, proteins and DNA [35]. The challenge with *A. hydrophila* represents a source of stress for fishes and the use of yerba mate could improve the antioxidant system or even avoid EROs cellular damage. Although the yerba mate is known as a natural antioxidant [36] and *A. hydrophila* challenge works as a source of stress for fishes [37] it was not observed neither increases in EROs cellular damage nor improvements in the antioxidant system, although there was a punctual increase in GSH. It seems that the period of time used for the supplementation and for challenge was not enough to destabilize the antioxidant status and induce defense responses [18].

Nonspecific immunity is a fundamental defense mechanism in fish and has a key role in the immune responses and homeostasis. Several endogenous and exogenous factors can influence innate immune response such as temperature changes, stress management and density that may have suppressive effects on this type of response, while several food additives and immunostimulants can enhance their efficiency [18, 38]. Considering that, it was evaluated if yerba mate was able to improve tilapia’s immune response. The supplementation with yerba mate stimulated an increase in kidney nitrite/nitrate levels in T1 non-challenged group, which could indicate a stimulus of the NO production and an improvement of the immune capacity although this increase was not observed in the challenged groups. On the other hand, there were no changes in plasma and renal lysozyme activity. The kidney is considered the largest site of hematopoiesis in fishes producing RBCs and granulocytes such as macrophages and neutrophils. These cells remove bacteria mainly by the production of reactive oxygen species during a respiratory burst. In addition, they have lysozyme and other hydrolytic enzymes in their lysosomes that altogether contribute to kill bacteria [38]. Considering that is probable that there was an increase in the number of granulocytes as well as in their activity in kidney stimulated by yerba mate and in the presence of *A. hydrophila* these cells were sent to the blood stream to face the infection. Also, the absence of activity of lysozyme could be related with the activation of other mechanisms of defense, such as the NO production and free radicals, growth inhibitors, complement pathways antibodies, cytokines, chemokines and antibacterial peptides [38].

The hematological parameters are an important tool of diagnosis that reveals the state of health of fish and may vary according to stressor stimulus, treatment, parasitic or infectious diseases. Since the circulating system of a fish is in close association with the external environment and with every tissue, it is sensitive to foreign stimuli and reflects the homeostasis of the animal. Changes in the blood physiology can thus be used as criteria of the systemic response to such stimuli [39].

The results showed a significant reduction in erythrocytes in the groups challenged with *A. hydrophila*. Decreased erythrocyte count indicates that probably RBCs were being destroyed by the leucocytic activity resulting in an erythrocytic anemia caused by the bacteria. Also, the increase in MCH values shows that there is an adaptative response to the infection, involving the migration of immature cells such as erythroblasts into the blood stream [40, 41].

5. Conclusions

The yerba mate extract presented potential to be used as an antibacterial agent for fish diseases. It is important to note that this is the first report on the
effect of *I. paraguariensis* as an antibacterial agent for fish diseases. Considering that the use of medicinal plants or natural compounds in aquaculture represents a cost-effective alternative to treat fish diseases, this is a very prominent research field. Furthermore, more detailed studies are necessary to establish effective doses and times required for fish treatments, especially considering *I. paraguariensis* applications.

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**References**


In Vitro Antibacterial Potential of Plant Extracts and in Vivo Ilex paraguariensis Effect on Oreochromis niloticus Physiology and Resistance to Aeromonas hydrophila


