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Research Article

Dietary *Tithonia diversifolia* improves hematology, antioxidant capacity, liver health, and disease resistance in hybrid of *Oreochromis mossambicus* × *Oreochromis niloticus*

Yuniel MÉNDEZ-MARTÍNEZ*, Pedro D. MENDOZA-CARRANZA, Juan P. ORDÓÑEZ-IGLESIA, Veronica del C. SEGOVIA-MONTESDEOCA, Ana N. MORENO-VERA, Melanie A. CASANOVA-ERAZO, Mishel E. MÉNDEZ-SEGOVIA

*Universidad Técnica Estatal de Quevedo (UTEQ), Facultad de Ciencias Pecuarias y Biológicas, Experimental Aquaculture Laboratory, Av. Quito Km. 1 1/2 vía a Santo Domingo de los Tsáchilas, Quevedo 120301, Los Ríos, Ecuador; ymendezm@uteq.edu.ec (*corresponding author); pedro.mendoza2014@uteq.edu.ec; jordonezi@uteq.edu.ec; vsegoviam@uteq.edu.ec; amoreno@uteq.edu.ec; melanie.casanova2014@uteq.edu.ec; mishel.mendez2014@uteq.edu.ec*

Abstract

Herbal medicines have been used for centuries for the treatment of various ailments and there has been a recent resurgence of interest in the use of natural products. This investigation was carried out to evaluate the role of *Tithonia diversifolia* (TD) on haematology, antioxidant and transaminases enzymes, liver histology and disease resistance in juvenile tilapia hybrids (*Oreochromis mossambicus* × *Oreochromis niloticus*). TD powder (0, 40, 80, 120 and 160 g kg^{-1} diet) was fed to cultured fish at a density of 15 fish/aquarium (n = 3 aquaria per treatment) for eight weeks (pre-challenge period). At the end of the feeding trial, 18 fish from each group were given intraperitoneal injections with a pathogenic strain of *Aeromonas hydrophila* to test the protective efficacy of TD against bacterial infection for seven days (post-challenge period). The results after eight weeks revealed significant differences ($P < 0.05$), with an increase in haematological variables and antioxidant activity, low levels of transaminases and an adequate hepatic histological response in the TD-fed fish. In the experimental challenge, the control group experienced higher mortality rates than the other groups after the IP injection with *A. hydrophila* at a dose of 0.25 ml for fish (1×10^6 CFU ml^{-1}) and the relative level of protection (RLP = 78.33%) was significantly higher ($P < 0.05$) with the treatment containing 160 $g.kg^{-1}$ of dietary TD. A correlation and principal component analysis (PCA) confirmed the results. In conclusion, these findings suggest that TD up to levels of 160 $g.kg^{-1}$, could serve as a natural phytobiotic to enhance tilapia health and disease resistance, potentially reducing reliance on synthetic products in aquaculture.

Keywords: bacterial infection; hepatoprotective effect; hybrid tilapia; phytobiotic; relative level of protection

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Introduction

Herbal medicinal stimulants have been used for centuries for the treatment of various ailments and diseases. But recently, interest is on the rise due to their combination of different phytochemicals that are synergistic in their action (Jiang *et al.*, 2020; Rajčević *et al.*, 2022) and reduced side effects compared to synthetic drugs (Pham *et al.*, 2020).

Polyphenolic compounds are abundant in plant-based functional foods, including fruits, vegetables, and legumes. Their health benefits have gained increasing attention (Rana *et al.*, 2022; Kamble *et al.*, 2024). These compounds contribute to antioxidant balance by enhancing enzymatic defenses (superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase) and neutralizing free radicals (Hu *et al.*, 2025; Rudrapal *et al.*, 2024). Additionally, they exert hepatoprotective effects by regulating metabolic enzymes, including transaminases (Jia *et al.*, 2019; Ahmadifar *et al.*, 2021). Despite their known benefits, the precise mechanisms by which herbal medicines modulate fish metabolism and immunity remain unclear (Ma *et al.*, 2024; Hu *et al.*, 2025).

One plant with biostimulatory properties is *Tithonia diversifolia* (TD), the Mexican sunflower, or golden button, is native to Mesoamerica, specifically to Mexico and Central America, which is a non-leguminous plant belonging to the family Asteraceae that is widely found in Asia, Africa and the Americas (Kriticos and Kriticos, 2021). It has characteristics that give it a high production potential, including tolerance to poor soils, resistance to water stress and cutting, with an approximate production of 55 t DM/ha/year (González-Castillo *et al.*, 2014). According to Vargas Velázquez *et al.* (2022) and Robinson *et al.* (2024), this plant contains more than 1,200 classes of secondary compounds. However, due to their high diversity, not all of them have been studied, thus few groups are known about, such as polyphenols, cyanogenic glycosides, saponins, steroids and phytohaemagglutinin (Gutiérrez *et al.*, 2015; Okuna, 2024). Its leaves and flowers are also good sources of high-quality protein and edible biomass (Buragohain and Rajkhowa, 2019).

Several studies have demonstrated that TD and its derivatives exhibit antioxidant, antibacterial, antiparasitic, anti-inflammatory, anti-obesity, anticancer, and neuroprotective activities (Mabou *et al.*, 2018; Chao *et al.*, 2022; Villarreal-Rivas *et al.*, 2022; Akeumbiwo *et al.*, 2023). Although research on TD in aquaculture remains limited, existing studies highlight notable benefits. For instance, Hahn-von-Hessberg *et al.* (2016) reported optimal digestibility of TD leaves in *Oreochromis niloticus*. Similarly, Nafiqoh *et al.* (2020) observed improvements in haematological parameters and innate immunity in *Clarias gariepinus*, while Méndez-Martínez *et al.* (2023a) documented enhanced growth and metabolic responses in red tilapia.

Currently, the aquaculture industry enjoys a favourable context for its growth. However, challenges persist that affect the efficiency of production systems, such as the limited availability of raw materials for feeds and the increase of pathogenic diseases caused by fungi, viruses and bacteria (Inslam *et al.*, 2024; Mkulo *et al.*, 2024). Aeromonas spp. are among the most common causative agents of bacterial infections in farmed fish (El Asely *et al.*, 2020; Moustafa *et al.*, 2020, Ofek *et al.*, 2023; Machuca *et al.*, 2024). Which have as their main virulence factor the ability to produce toxins and hydrolytic enzymes, which facilitate invasion and tissue damage in the host. (Abdella *et al.*, 2022; Andueza*et al.*, 2023; Ofek *et al.*, 2023), often resulting in high mortality rates, reduced growth performance, and increased production costs due to treatment and prevention measures (Nafiqoh *et al.*, 2020; Moustafa *et al.*, 2020). As a preventive measure, aquaculture has resorted to the use of prophylactic treatments and antibiotics to maximise the health and performance of species such as red tilapia (Moustafa *et al.*, 2020; Machuca *et al.*, 2024), which is farmed in intensive and semi-intensive systems and stands out for its biological and organoleptic characteristics (Méndez-Martínez *et al.*, 2021; 2023b; Karaket *et al.*, 2025).

However, chemical treatments have shown limitations, as they can accumulate in body tissues, affecting their quality and generating health problems (Elgendy *et al.*, 2024). In addition, antibiotics are critical both for their environmental impact and for the antimicrobial resistance they induce (Yang *et al.*, 2020). The ban on the use of antibiotics as growth promoters has prompted the search for alternatives that optimise cultivation

technologies, especially at early stages where diseases can be critical (Hemamalini *et al.*, 2022; Méndez-Martínez *et al.*, 2023b).

Based on the above, the use of medicinal plants like TD becomes relevant in terms of improving health, metabolisms and immune systems and preventing infection by pathogens as well as improving survival rates in tropical regions. This study focused on evaluating the effect of TD consumption on haematology antioxidant and transaminases enzymes, liver histology, and disease resistance in *Oreochromis mossambicus* × *Oreochromis niloticus*. The findings enhance our understanding of the potential application of TD in tilapia aquaculture.

Materials and Methods

Study site and experimental conditions

Fish with an average initial weight of 6.74 ± 0.50 g were purchased from a local fish farmer and transferred to the Aquaculture Laboratory at the Faculty of Livestock and Biological Sciences (01°03'18"S, 79°25′24"O) of Universidad Técnica Estatal de Quevedo (UTEQ). There, they underwent a seven-day acclimatisation period before the study began, which consisted of two experimental phases.

Trial 1 (pre-challenge): For this phase, fish were randomly distributed into 15 plastic aquaria (27.6 cm × 16.5 cm × 12.5 cm), with a density of 15 fish per aquarium and three aquaria assigned to each treatment. The aquaria were cleaned each morning before the fish were fed to remove faeces and food debris, and then the water was replaced. Fish were fed *ad libitum* twice a day (at 09:00 and 17:00 hours) for a period of eight weeks. Trial 2 (post-challenge): Of the fish that survived Trial 1, six individuals per aquarium (18 fish per treatment) were randomly selected for exposure to an experimental challenge with *A. hydrophila* for seven days. During the challenge trial, the same feeding regime was employed.

In both trial periods, the physicochemical parameters of the water were monitored to ensure that they were within the levels required for the species. Temperature values were measured with a mercury thermometer (0 to 50 °C) and ranged from 27.0 to -27.8 °C. Dissolved oxygen was measured using a digital oximeter (55-DO, YSI Incorporated, Yellow Springs, OH, USA) and ranged from 4.03 to 5.01 mg.L^{-1} and pH (7.26-7.34), NH4 (0.43-0.61 mg.L^{-1}), NO2 (0.29-1.13 mg.L^{-1}) and NO3 (0.47-0.89 mg.L^{-1}) measurements were taken with the aid of a colorimetric kit (Saltwater Master Test, OH, USA).

Formulation and bromatological analysis of diets

To produce TD powder, the plants were cultivated in Santo Domingo de los Tsachilas, Ecuador, at kilometre 12 on the road to El Carmen, Quevedo. The leaves were harvested at 70 days of growth, then dried at 80°C for 24 hours in an air-flow oven. Afterwards, they were ground using a hammer mill and sieved to 250 µm. The experimental diet formulations are presented in Table 1 and contain six different levels of TD leaf powder: 0, 40, 80, 120 and 160 g kg^{-1} of diet, for a total of five treatments, following the procedure used by Méndez-Martínez *et al.* (2023a).

All bromatological analyses were performed in triplicate. The bromatological composition of the experimental ingredients and diets (Table 1) was determined using the methods of the Association of Official Analytical Chemists (AOAC, 2005). Digestible energy (DE) was estimated theoretically from the conversion factors according to Ramanathan *et al.* (2015) and Botello *et al.* (2022).

Fish sampling

Trial 1 (pre-challenge): At the end of the experiment (eight weeks), the fish (final average live weight: 22.93 g) were fasted for 14 hours and then anaesthetised with 4-allyl-2-methoxyphenol (1: 10^4), to minimize pain in fish. Then, five experimental juvenile red tilapia were randomly selected from each tank (n = 15 per treatment), and blood samples were drawn from the fish by puncturing the caudal artery at the haemal arch

using disposable syringes (1 mL). Blood samples were stored at 4 °C until the analysis of antioxidant enzyme activity, transaminase and haematology. After blood collection, three juvenile red tilapia were randomly collected from each tank (n = 9 per treatment) for a liver examination and samples were immediately fixed by immersion in 10% neutral formalin for 24 hours for histological analysis. Test period 2 (post-challenge): Challenged fish were monitored daily for seven days for any signs of disease and mortality was also recorded.

Table 1. Formulation and chemical composition (g kg^{-1}, wet basis) of experimental diets with *Tithonia diversifolia* powder inclusion

Ingredient	*Tithonia diversifolia* flour levels (g kg^{-1})				
	0	**40**	**80**	**120**	**160**
Tithonia diversifolia powder [1]	0	40	80	120	160
Fish meal [2]	330	310	290	270	250
Soybean meal [3]	160	182	203	225	246
Wheat meal [4]	224	195	165	136	107
Corn meal [5]	210	197	186	173	161
Mineral premix [6*]	10	10	10	10	10
Vitamin premix [7**]	20	20	20	20	20
Grenetine [8]	20	20	20	20	20
Vitamin C [9]	1	1	1	1	1
Vegetable oil [10]	10	10	10	10	10
Fish oil [11]	15	15	15	15	15
Proximal composition					
Dry matter	925.4	925.4	925.3	925.2	925.2
Crude protein	336.4	336.0	335.2	334.8	334.1
Crude lipid	59.4	61.2	61.6	61.9	62.2
Ash	90.7	95.3	96.5	97.7	98.9
Nitrogen-free extract (NFE)	424.0	411.2	403.7	395.8	388.3
DE (MJ kg^{-1} of food) [12]	12.59	12.43	12.28	12.12	11.97
CP DE^{-1} (mg PC MJ^{-1}) [13]	26.72	27.04	27.31	27.62	27.90

Note: [1]Santo Domingo de los Tsáchilas, 12 km along to road the Carmen; [2] Commercial "El Gordillo" in Santo Domingo de los Tsáchilas, Ecuador; [3, 5, 8, 10,] Supermaxi, Quevedo, Ecuador; [4 6 9] Super Success, Quevedo, Ecuador; [7] Supplies AZ, La Paz, BCS, Mexico; [11] Fortidex S.A in Santa Elena; *mg kg^{-1}: Magnesium sulfate 5.1; Sodium chloride 2.4; Potassium chloride 2; Ferrous sulfate 1; Zinc sulfate 0.2; Cupric sulfate 0.0314; Manganous sulfate 0.1015; Cobalt sulfate 0.0191; Calcium iodate 0,0118; Chlorine chloride 0.051. **mg kg^{-1}: Thiamine 60; Riboflavin 25; Niacin 40; Vitamin B6 50; Pantothenic acid 75; Biotin 1; Folate 10; Vitamin B12 0.2; Hill 600; Myoinositol 400; Vitamin C 200; Vitamin A 5000 UI; Vitamin E 100; Vitamin D 0.1; Vitamin K 5. Calculated value: [12]MJ kg^{-1} of feed: digestible energy; [13]mg PC MJ^{-1}:protein ratio for each MJ of energy in the diet

Evaluation of haematological parameters

Haemoglobin was determined using Drabkin's reagent following cyanmethemoglobin methodology and a spectrophotometer reading at 546 nm. Total RBC (erythrocytes), count was calculated in a 0.0025 mm^2 Neubauer chamber (OPTIK-Labor, Germany) using acetic acid reagent and Hayem's solution as diluent in a ratio of 1:200 (Blaxhall and Daisley, 1973). The blood haematocrit value was measured using the standard microhematocrit method with the aid of a microcentrifuge. Erythrocyte indices of mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) were determined using the following equations (Blaxhall and Daisley, 1973):

$$MCV\ (fL) = 10 \times Hematocrit\ (\%) / Erythrocytes\ (10^{12}L^{-1})$$

$$MCH\ (pg) = 10 \times Hemoglobin\ (g.dL^{-1}) / Erythrocytes\ (10^{12}L^{-1})$$

$$MCHC\ (g.dL^{-1}) = 100 \times Hemoglobin\ (g.dL^{-1}) / Hematocrit\ (\%)$$

Transaminase and antioxidant enzyme analysis

Blood samples were centrifuged at 1,200 rpm for 10 min to separate plasma forms. Metabolic enzyme activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) were determined following the procedure of Bergmeyer *et al.* (1976) using a kit (Diagnostics Worldwide, Germany). Samples were incubated for 15 min at 37 °C for AST, 5 min at 37 °C for ALT and 6 min at 35 °C for ALP. Absorbance readings were performed with a spectrophotometer (SunostIk Plus, Kunshan Road, China) for three minutes at 340 nm ABS for AST and ALT, and two minutes at 405 nm ABS for ALP. The enzyme activities for these three enzymes were expressed as $U.L^{-1}$.

The antioxidant enzyme activities of superoxide dismutase (SOD), glutathione peroxidase (GPX) and catalase (CAT) were determined following the procedures of McCord and Fridovich (1969), Plagia and Valentine (1967) and Aebi (1984), respectively, using a kit (Ransel, Randox, Crumlin, Antrim, UK). Samples were incubated for 15 min at 37 °C for SOD and GPX, and at 25 °C for CAT. Absorbance readings were performed with a spectrophotometer for three minutes at 340 nm ABS for SOD and GPX, and one minute at 405 nm ABS for CAT. Enzyme activity for these three enzymes was expressed as $U\ ml^{-1}$. All assays were performed in triplicate to avoid errors as much as possible.

Liver morphohistology analysis

Livers were collected, fixed in 10% formalin, dehydrated in a series of ethanol solutions of graded concentrations (50-100%), rinsed with xylene and embedded in paraffin. From these samples, 4-5 µm thick sections were obtained and stained with haematoxylin and eosin (H&E). The images were captured at 100x magnification. The areas of hepatocytes, nuclei and cytoplasm in the liver tissues could be determined using the ImagenJ v1.54p software (NIH, Bethesda, MD, USA).

Bacterial preparation and challenge study

The *A. hydrophila* culture was grown in Trypticase-Soya Agar (TSA) nutrient broth for 24 h at 30 °C. The culture broth was centrifuged at 3000×g for 10 min to obtain a bacterial cell pellet which was resuspended in sterile phosphate-buffered saline (PBS, pH 7.4). The final bacterial concentration was adjusted to 1.0×10^6 CFU ml^{-1} using the serial dilution method.

Fish were infected intraperitoneally by injecting 0.25 ml/fish (1.0×10^6 CFU ml^{-1}) of previously prepared *A. hydrophila* bacterial suspension. The *A. hydrophila* were confirmed to be pathogenic for *O. mossambicus x O. niloticus* via the intraperitoneal (IP) route of injection, with LD_{50} values (lethal dose, the dose which kills 50% of the injected fish) estimated to be 1.0×10^6 CFU ml^{-1} for *A. hydrophila*. The challenged fish were monitored daily for seven days for any signs of disease. Dead fish were removed immediately. Mortality was also recorded to calculate the cumulative survival rate (SR) and the relative level of protection of challenged fish according to the equations (Ruangroupan *et al.* 1986):

SR (%) = 100 × (Number of fish still alive after challenge/Number of fish injected with pathogenic bacteria)

RLP (%) = 100 × (1 - (% mortality in TD-treated groups/% mortality of control group)

Statistical analysis

The statistical analysis of the results was presented as mean ± standard error of the group. Normality and homoscedasticity tests were applied to the collected data ($P < 0.05$). Subsequently, Duncan's comparison test with a significance level of 5% was used to identify significant differences between treatments using Minitab-v18 statistical software (Minitab LLC., Philadelphia, PA, USA). In addition, a Pearson´s correlation analysis, and Principal component analysis were performed using Python 3.10 software (Foundation, Wilmington, DE, USA).

Results

Haematology

Figure 1 shows the haematological results. Haemoglobin, erythrocyte count, and MCHC levels were significantly higher ($P < 0.05$) in fish fed TD-supplemented diets (T4 and T5) compared to the control group (T1). In contrast, MCH and MCV values were significantly lower in the T5 group, suggesting potential modifications in erythrocyte morphology and oxygen transport efficiency.

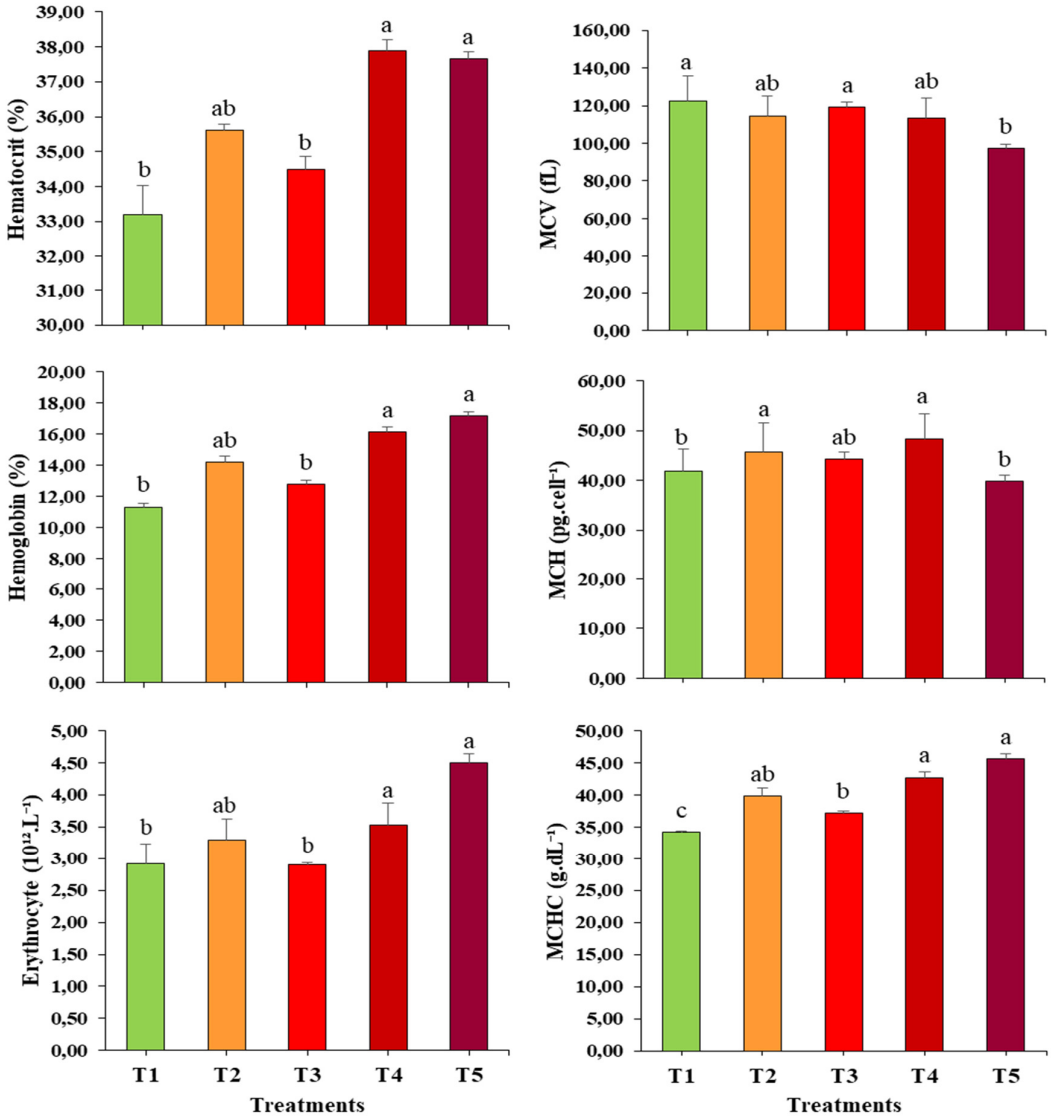

Figure 1. Effect of *Tithonia diversifolia* consumption on haematological parameters in *Oreochromis mossambicus* × *Oreochromis niloticus*

Note: Results are reported as mean ± standard error of three groups according to treatment (n = 3). [abc] Different letters in the denote significant differences ($P < 0.05$). MCV: Mean corpuscular volume, MCH: Mean corpuscular haemoglobin, MCHC: Mean corpuscular haemoglobin concentration

Antioxidant enzymes and transaminases

With respect to antioxidant enzymes and transaminases (Figure 2), significant differences ($P < 0.05$) were found between groups treated with TD powder and the control treatment. The groups of fish fed with TD (T4 and T5) in the diet showed significantly ($P < 0.05$) lower levels of SOD, CAT and GPX than the control treatment, indicating enhanced oxidative stress resistance, respectively. Additionally, ALT and AST levels were lower in TD treated groups, suggesting a hepatoprotective effect and reduced liver damage. While for ALP, a significantly ($P < 0.05$) higher level was found in T5 than in the rest of the treatments (Figure 2).

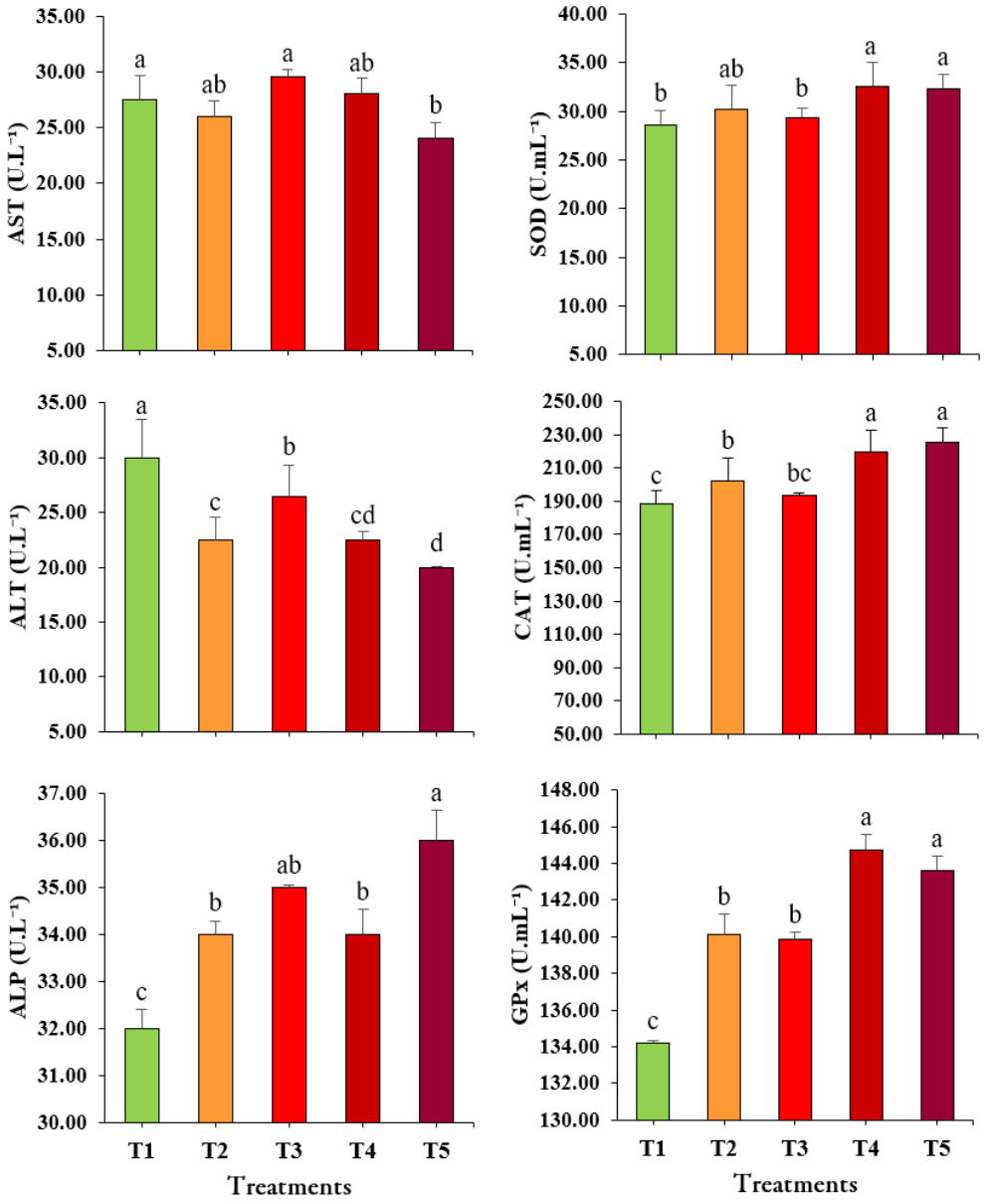

Figure 2. Effect of *Tithonia diversifolia* intake on transaminase enzymes and antioxidants in *Oreochromis mossambicus* × *Oreochromis niloticus*

Note: Results are reported as mean ± standard error of three groups according to treatment (n = 3). [abc] Different letters in the denote significant differences ($P < 0.05$). AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; SOD: Superoxide dismutase; CAT: Catalase, GPX: Glutathione peroxidase

Liver morphohistology

When examining the liver cross-section of red tilapia juveniles fed different levels of dietary TD (Figure 3), no pathological changes were observed in the fish livers. Hepatocytes were observed to form cords and plaques that establish continuous communication.

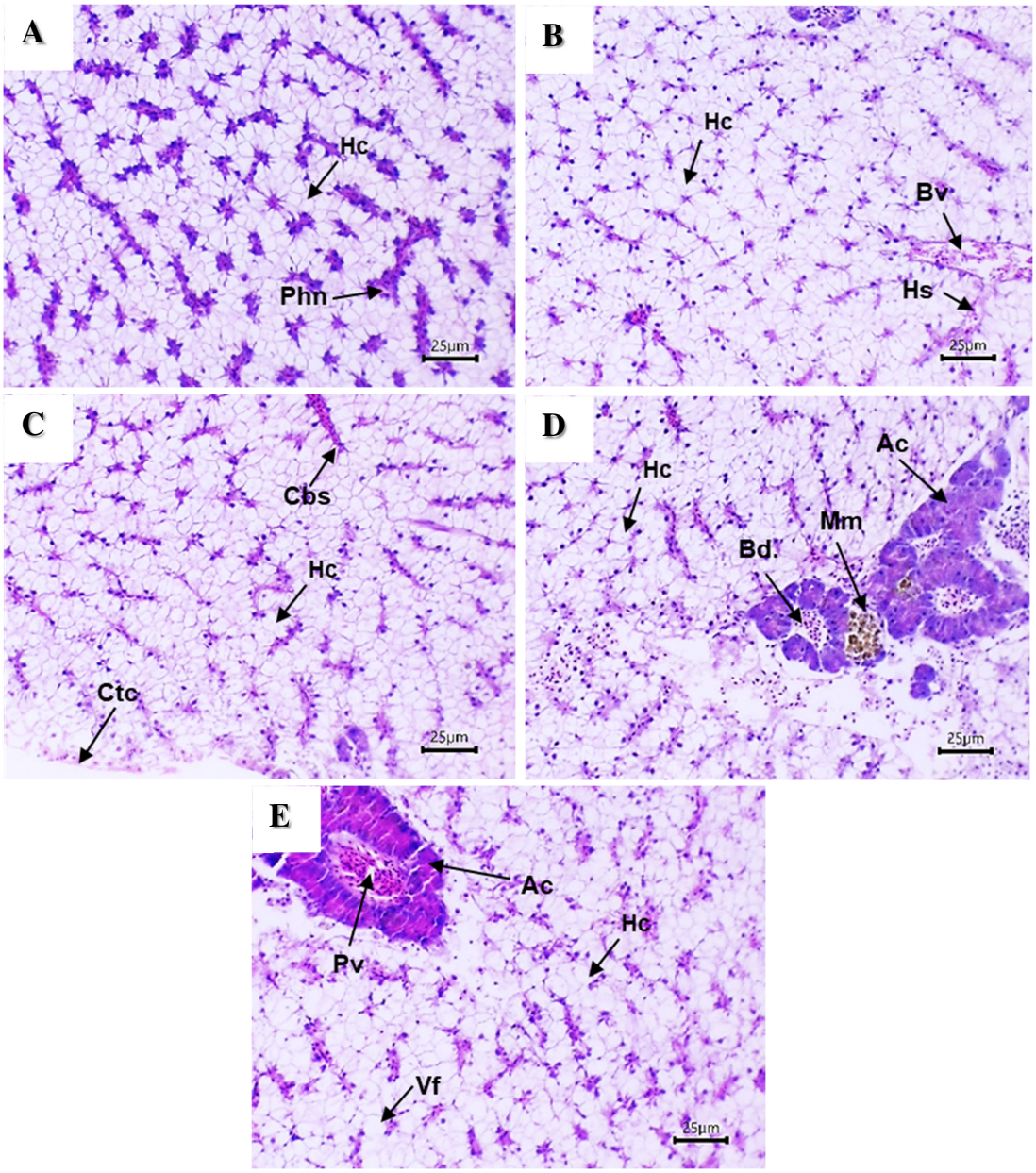

Figure 3. Photomicrograph of liver cross-sections of juvenile hybrid red tilapia *Oreochromis mossambicus* × *Oreochromis niloticus* fed with increasing amounts of *Tithonia diversifolia* in their diet

Note: Nph: number of pyknotic hepatic nuclei. Hc: Indicates hepatocyte cells, Bc: blood congestion, Ctc: connective tissue capsule composed of collagen and elastin fibers, Ac: Indicates pancreatic acinar cells that are organized around the portal vein, Pv: Portal Vein with presence of red blood cells, Bd: bile duct, Cbs: Congestion of blood in sinusoids, Vf: vacuole formation, Mm: melanomacrophages. H&E staining. 100 x magnification. Scale bar: 25 µm. T1 (A), T2 (B), T3 (C), T4 (D) and T5 (E)

Clearly defined, large, spherical nuclei containing nucleoli, and moderate eosinophilic cytoplasm were observed. The nucleoli were characteristically regular and abundant nucleoli were found to be located at the periphery of the hepatocyte cells. Kupffer cells, hepatic sinusoids and pancreatic acinar cell tissue in a normal state were also observed. However, in the hepatocytes of fish that received TD powder at T4 and T5, a cytoplasm with mild diffuse vacuolisation wasobserved.

Regarding histomorphometric parameters in hepatocytes (Figure 4), significant differences between treatments were recorded. The highest values of hepatocyte area, cytoplasm area and cytoplasm area/nuclear area ratio were found in TD treatments (T4 and T5), which differed significantly ($P < 0.05$) from the control treatment (T1). Meanwhile, the nucleus area reached its maximum with the T4 treatment, differing significantly ($P < 0.05$) from the remaining treatments.

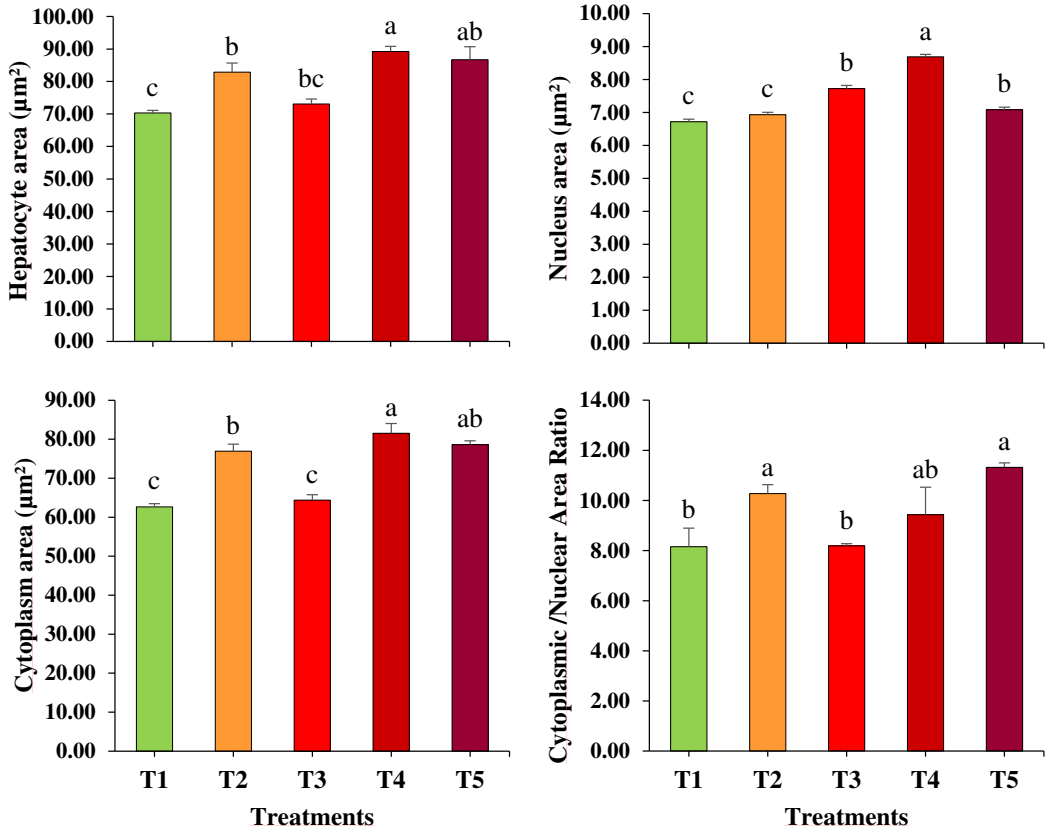

Figure 4. Effect of *Tithonia diversifolia* consumption on liver morphohistological parameters in *Oreochromis mossambicus* × *Oreochromis niloticus*

Note: Results are reported as mean ± standard error of three groups according to treatment (n = 3). [abc] Different letters denote significant differences ($P < 0.05$)

Challenge infection and relative level of protection

Mortalities were associated with characteristic signs of *A. hydrophila* infection, including external lesions (skin ulcers, reddening) and internal damage (hemorrhages, ascites), reinforcing the pathogenic impact of the bacterial challenge.

The survival rate of the challenged juvenile fish during a seven-day observation period showed significant differences ($P < 0.05$), specifically 20% for the control group, while the TD-treated groups had survival rates

of 53.33 to 80%, respectively, with the highest survival achieved at T5 (Figure 5). The RLP showed significant differences in the TD-treated groups, ranging from 39.44 to 78.33% as TD levels increased (Figure 5).

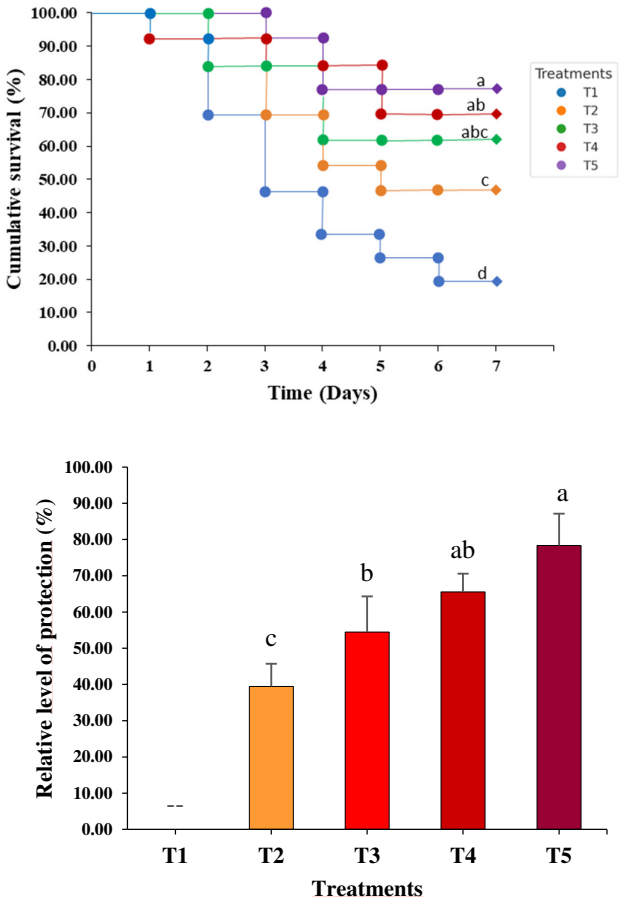

Figure 5. Effect of *Tithonia diversifolia* consumption on *Oreochromis mossambicus* × *Oreochromis niloticus* with *Aeromonas hydrophila*-induced infection.
Note: Results are reported as mean ± standard error of three groups according to treatment (n = 3). [abcd] Different letters denote significant differences (P < 0.05)

Pearson's correlation and Principal Component Analysis

The correlation analysis (Figure 6A) reveals significant interactions among various haematological, enzymatic, and morphological parameters. A strong positive correlation is observed between haemoglobin, haematocrit, and erythrocytes, suggesting a possible influence of *Tithonia diversifolia* on red blood cell production and oxygen transport capacity in fish. Additionally, antioxidant parameters such as SOD, CAT, and GPx exhibit variable correlations with hepatic enzymes (AST, ALT, and ALP), indicating a potential impact of *TD* on redox balance and liver function. Furthermore, cellular morphology, represented by hepatocyte and cytoplasmic area, is closely related to hepatic enzymes, which could imply structural changes in response to the experimental diet. On the other hand, the observed correlation between survival and various biomarkers suggests that the physiological state of the fish plays a crucial role in their adaptation to the diet containing *TD*. The relationship between hepatocyte ratio, nuclear area, and hepatic enzymes indicates that the diet may be modulating key cellular processes in fish metabolism.

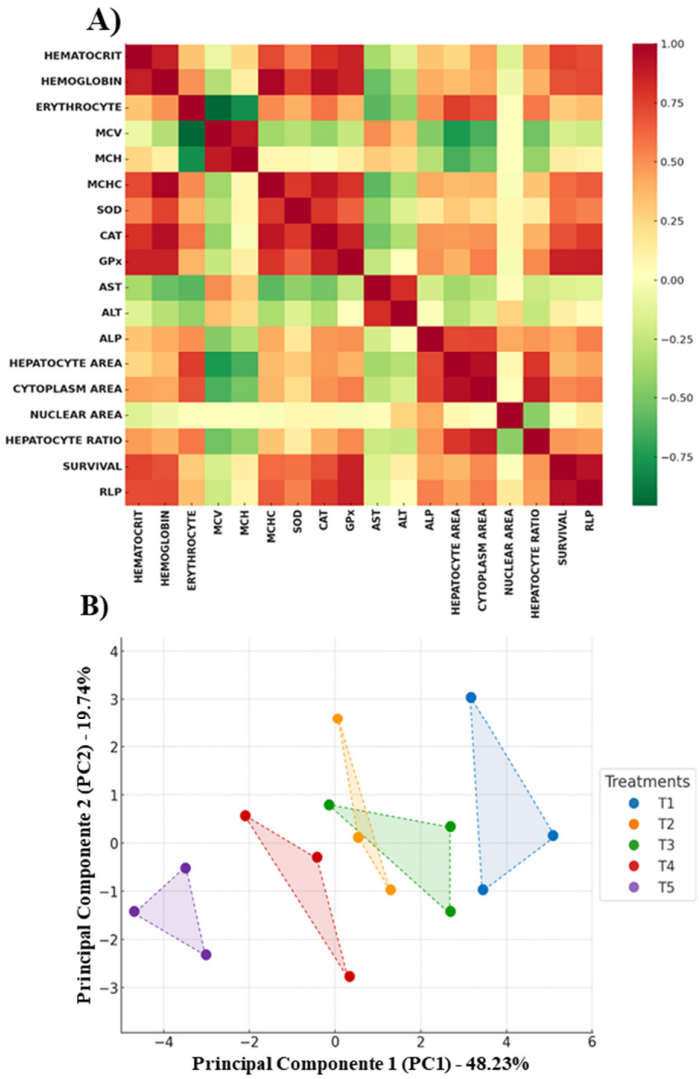

Figure 6. Pearson's correlation heatmap among the different study variables (A). Biplot of Principal Component Analysis (PCA), which allows for dimensionality reduction and pattern visualization in the treatments (B)

The Principal Component Analysis (PCA) (Figure 6), with its first two principal components, explains a substantial proportion of the total variability (67%). PC1 (48.23%) accounts for nearly half of the total variance and is primarily influenced by haematological variables such as haematocrit, haemoglobin, and erythrocytes. Meanwhile, PC2 (19.73%) is mainly associated with hepatic and antioxidant factors, including GPx and ALT. In terms of clustering, T1 and T2 exhibit similarities, while T3 and T4 display greater dispersion, indicating a more variable response. In contrast, T5 is distinctly separated, suggesting a unique effect on the assessed parameters. The differentiation of treatments is driven by key variables: haematocrit, haemoglobin, and erythrocytes strongly influence PC1, whereas GPx, CAT, and SOD contribute notably to PC2. Additionally, important correlations are observed, such as a positive relationship between haematocrit and haemoglobin and an inverse correlation between MCV and erythrocytes. Overall, the PCA provides valuable insights into the differentiation of treatments, emphasising the impact of haematological, hepatic, and antioxidant variables on the observed variability.

Discussion

The TD supplementation significantly enhanced haematological parameters, including haematocrit, haemoglobin concentration, and erythrocyte count, suggesting improved oxygen transport efficiency. The increase in haematocrit levels is particularly relevant, as it enhances aerobic capacity and resilience to stress (Dagoudo *et al.*, 2021). Furthermore, elevated haemoglobin concentrations indicate improved protein synthesis, leading to better oxygenation at the tissue level and optimized metabolic performance. This may be attributed to effects generated by the bioactive content of TD, especially its phenolic compounds, flavonoids and terpenes (Maher *et al.*, 2021), which may stimulate erythropoiesis by enhancing bone marrow activity and mitigating oxidative stress (Nugroho *et al.*, 2016; Sogbesan and Ahmed, 2018).

The increase in total erythrocytes in TD-fed fish supports the hypothesis that plant bioactive compounds may be acting as modulators of gene expression or as precursors in the synthesis of cellular components, such as erythropoiesis, also providing protection of erythrocyte cell membranes from oxidative damage, thus improving their stability and lifespan. This is in line with previous research that reported similar haematological benefits of including antioxidant-rich plants in aquaculture diets (Sogbesan and Ahmed, 2018; Adeniyi *et al.*, 2024). Furthermore, the observed positive effects could be related to the high content of essential nutrients in TD, such as vitamins, minerals and high-quality proteins (Aye, 2016; Hahn-von-Hessberg *et al.*, 2016; Méndez-Martínez *et al.*, 2023a), which are essential for haemoglobin synthesis and erythrocyte formation (Pal *et al.*, 2018). The nutritional balance provided by this plant may optimise the physiological processes related to haematopoiesis, improving the overall health of the fish.

With respect to erythrocyte indices (MCV, MCH and MCHC), these were also significantly influenced by the TD powder, whereby our results were in agreement with those reported by Cardoso *et al.* (2020) and Kamble *et al.* (2024). The ability of TD to modulate the haematology of juvenile red tilapia fish may be linked to several biochemical-physiological mechanisms. For instance, flavonoids and phenolic compounds (Li *et al.*, 2016; Mansour *et al.*, 2022) possess antioxidant properties that reduce oxidative damage in cell membranes and erythrocyte organelles. Moreover, saponins (Tiwari *et al.*, 2018; Ahmadifar *et al.*, 2021, Kamble *et al.*, 2024) could act as modulators, promoting better immune response and environmental conditions. TD-phytobiotic seems to optimise the balance between cell volume and haemoglobin concentration, suggesting an improvement in the functional efficiency of cells.

Our study shows that the inclusion of TD in fish diets modulates the endogenous antioxidant system of the antioxidant enzymes SOD, CAT and GPX in blood. The increased activity of SOD, which is responsible for the dismutation of the superoxide radical to hydrogen peroxide and oxygen (Arockiaraj *et al.*, 2014), suggests that TD contributes to mitigate the accumulation of reactive oxygen species (ROS) generated by environmental or metabolic factors. This observation is consistent with previous research that associated the consumption of diets rich in phenolic and flavonoid compounds with increased SOD activity in fish and other organisms (Arockiaraj *et al.*, 2014; Jatayu *et al.*, 2018).

Likewise, the significant increase in the activity of CAT, a key enzyme for the breakdown of hydrogen peroxide into water and oxygen (Ortiz-Ordoñez *et al.*, 2011), supports the protective role of TD against oxidative damage. Studies in aquatic models have shown that supplementation with antioxidant-rich plants can stimulate the expression of genes related to CAT activity, promoting an effective reduction of oxidative stress (Rajabiesterabadi *et al.*, 2020). This mechanism could be associated with the high levels of bioactive compounds present in TD, such as sesquiterpene lactones and phenolic acids, which not only directly neutralise ROS but also act as modulators of the antioxidant system (Chagas-Paula *et al.*, 2011). GPX, moreover, showed a significant increase in fish on diets that included TD, indicating an increased ability to neutralise lipid peroxides and protect cell membranes from oxidative damage (Fadhlaoui and Couture, 2016). This result is consistent with previous studies that indicate that plant extracts with antioxidant properties enhance GPX activity by increasing the availability of reduced glutathione (GSH) in tissues (Cheng *et al.*, 2017; Rocha-Santos *et al.*, 2018).

It is worth noting that with the levels of TD inclusion used, no reduction in the activity of antioxidant enzymes was found. Therefore, the presence of secondary metabolites, such as alkaloids and tannins that may be present in TD (Olayinka *et al.*, 2015, Okuna 2024) did not generate prooxidant effects or interfere with antioxidant metabolic pathways. The inclusion of TD in the diet of juvenile tilapia was also found to be associated with a significant decrease in transaminase enzymes (ALT and AST) and an increase in alkaline phosphatase (ALP) levels in blood plasma. These findings highlight the hepatoprotective and metabolism-modulating potential of this plant on liver health in fish. The decrease in transaminases (ALT, AST) in TD-fed fish suggests hepatoprotective effects, potentially linked to antioxidant-mediated stabilization of hepatocyte membranes. Transaminases, particularly ALT and AST are important markers of liver damage in fish, as their levels tend to be elevated in the presence of oxidative stress, inflammation or necrosis of hepatocytes (Farag *et al.*, 2022; Méndez-Martínez *et al.*, 2023b).

Bioactive compounds present in TD, such as flavonoids, terpenes and phenolic compounds, have recognised antioxidant properties that probably help to protect cell membranes (Li *et al.*, 2016; Mansour *et al.*, 2022) and prevent lipid peroxidation in hepatocytes (Zarina and Tan, 2013). Meanwhile, the increase in ALP levels could reflect an improvement in liver and bone functions, as this enzyme plays a key role in detoxification, energy metabolism and nutrient transport (Jiang *et al.*, 2023). These results are agreement with previous studies that have reported a stimulatory effect of plants rich in antioxidant and hepatoprotective compounds on ALP activity in fish (Jiang *et al.*, 2023). Thus, TD powder may have enhanced hepatocyte regeneration and generated a more efficient metabolic environment in the liver. The combined effect of decreased transaminases and increased ALP may be attributed to the dual role of TD in reducing liver damage caused by factors such as oxidative stress and toxic compounds while optimising the metabolic functions of the liver. However, it is important to consider that excessive doses of TD could lead to adverse effects due to the accumulation of secondary metabolites.

Regarding liver histology, the results show a preserved architecture with well-defined hepatocytes and an absence of significant alterations, such as necrosis or cellular infiltration, in treatments with optimal levels of TD inclusion. This suggests that the antioxidant compounds present in this plant may protect the liver against oxidative stress, which is a major cause of liver damage in intensively farmed fish (Tan *et al.*, 2018; Rajabiesterabadi *et al.*, 2020). However, in diets with higher inclusion levels, slight vacuolisation was observed in hepatocytes, which could indicate a metabolic stress effect associated with the consumption of secondary metabolites of TD, such as alkaloids or sesquiterpene lactones. This type of response has been reported in previous studies and is considered a reversible reaction that depends on the dose and the liver's ability to adapt (Ortiz-Ordoñez *et al.*, 2011; Amri *et al.*, 2022).

The increase in the size and number of hepatocytes observed in juvenile tilapia fed TD-enriched diets may be related to the hepatotrophic capacity of this plant. Previous studies have shown that their chemical composition, rich in flavonoids, tannins and phenolic compounds, can stimulate cell regeneration and improve liver metabolic activity (Tan *et al.*, 2018; Ortiz-Ordoñez *et al.*, 2011). What is more, these bioactive molecules could enhance protein synthesis and lipid metabolism, which are key processes in hepatocytes. This phenomenon is in line with studies that have shown an increase in hepatocyte mitotic activity when antioxidants or bioactive compounds are introduced in the diet (Sánchez-Nuño *et al.*, 2024; Zhang *et al.*, 2024). Furthermore, the lack of severe pathological changes indicates that the inclusion of TD at controlled levels does not compromise liver health but may promote a functional and metabolically efficient liver.

The results obtained in this study demonstrate that the inclusion of TD in the diet significantly increases the RLP and survival against *A. hydrophila* induced infections. These findings could be due to the presence of bioactive compounds present in TD, such as flavonoids, terpenoids and sesquiterpene lactones, which have been reported to have antimicrobial and immunomodulatory properties (Chagas-Paula *et al.*, 2011; Gutierrez *et al.*, 2015; Tagne *et al.*, 2018). These compounds probably strengthen the innate immune barriers of fish, such as the skin, mucosa and intestinal epithelium (Machuca *et al.*, 2024), limiting the ability of *A. hydrophila* to invade and colonise host tissues. On the other hand, the elevated RLP observed in TD treatments could be

attributed to the activation of specific immune mechanisms, including increased the phagocytic activity of macrophages and defence cells. Previous studies have shown that antioxidant-rich plants can enhance the production of proinflammatory cytokines and stimulate lymphocyte proliferation, which enhances the adaptive immune response against pathogens (Tripathi *et al.*, 2018; Maheshwari *et al.*, 2022).

Additionally, histopathological studies have shown that fish fed with plant-based diets rich in bioactive compounds exhibit reduced tissue damage from *A. hydrophila* infection (El Asely *et al.*, 2020; Moustafa *et al.*, 2020). This aligns with evidence supporting the ability of medicinal plants to modulate inflammation and accelerate tissue repair in aquatic organisms (Nafiqoh *et al.*, 2020). In this context, *TD* appears to not only enhance immune response but also provide protective effects against bacterial infections by mitigating oxidative stress and preserving cellular integrity. However, excessive inclusion levels could have adverse effects due to the accumulation of potentially toxic secondary metabolites (Chakraborty *et al.*, 2014), highlighting the importance of optimising dosage to maximise benefits while ensuring fish health.

The strong Pearson's correlation between haemoglobin, haematocrit, and erythrocytes suggests that TD may enhance erythropoiesis, potentially due to its bioactive compounds that promote red blood cell synthesis. This aligns with research indicating that plant-based diets improve haematological parameters by supplying essential micronutrients such as iron and folate (Adeniyi *et al.*, 2024; Kamble *et al.*, 2024). Furthermore, its antioxidant properties, reflected in the association between SOD, CAT, GPx, and hepatic enzymes, suggest a role in oxidative stress regulation, which is crucial for maintaining liver function under metabolic challenges (Jia *et al.*, 2019; Hu *et al.*, 2025). The link between survival rates and physiological biomarkers underscores the role of metabolic stability in dietary adaptation, reinforcing the hypothesis that plant-derived antioxidants contribute to stress resilience in aquaculture species (Chakraborty *et al.*, 2014; Ma *et al.*, 2024).

The PCA further substantiates these findings by demonstrating that haematological parameters predominantly contribute to PC1, reinforcing the role of TD in oxygen transport efficiency. Meanwhile, the distinct separation of T5 suggests a unique metabolic adaptation, likely driven by phytochemicals influencing liver metabolism and erythropoiesis pathways. Previous studies indicate that dietary polyphenols can modulate hepatic function and redox homeostasis, which may explain the clustering of antioxidant enzymes within PC2 (Cheng *et al.*, 2017; Jia *et al.*, 2019). While these results support the use of TD as a functional feed ingredient, further research is necessary to evaluate dose-dependent responses, long-term metabolic adaptations, and potential interactions with other dietary components (Chagas-Paula *et al.*, 2011). From a practical perspective, incorporating *TD* as an immunostimulant in aquaculture could reduce antibiotic dependency, thereby minimising the risk of antimicrobial resistance and the environmental impact associated with indiscriminate drug use.

Conclusions

The inclusion of TD in the diet of juvenile tilapia had a positive impact on various physiological and health parameters when up to 160 g kg^{-1} was included in the diet. An increase in haematological values was observed, indicating an improvement in the fish's oxygen-carrying capacity and general condition. In addition, an increase in antioxidant enzyme activity, together with a decrease in transaminases, showed protection against oxidative stress and the maintenance of liver function. Histological analyses confirmed a preserved liver structure, without significant damage, and an increased resistance against *A. hydrophila*-induced infections, highlighting the modulatory and protective potential of the phytochemicals present in TD. Our findings support the integration of TD as a natural phytobiotic in aquafeeds, potentially reducing reliance on antibiotics and enhancing sustainable aquaculture practices. Further research is needed to optimize dosage and evaluate long-term effects

Authors' Contributions

Conceptualization: Y.M-M.; Data curation: P.D.M-C., M.A.C-E., and M.E.M-S.; Formal analysis: Y.M-M.; Investigation: P.D.M-C., M.A.C-E., and M.E.M-S.; Methodology and Writing – original draft: Y.M-M.; Writing - review & editing: J.P.O-I., V.C.S-M., and A.N.M-V.; All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

This study was carried out without sacrificing organisms unnecessarily to reduce and perfect the use of animals used for scientific purposes. The proposed methods, use of animals and research practice (approval number: 091823) were examined and approved by the Scientific Ethics Committee at Universidad Técnica Estatal de Quevedo.

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Conflict of Interests

The authors declare they have no known competing financial interests or personal relationships could have appeared to influence the work reported in this paper.

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