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Effects of *Andrographis paniculata* Leaf Extract on Immune Response and Disease Resistance in Tilapia against *Aeromonas hydrophila* Infection

Faiz Al Faiz^a, and Rajendran Vijayakumar^a^aDepartment of Biology, College of Science in Zulfi, Majmaah University, Al Majmaah 11952, Saudi Arabia**Abstract**

The prolonged use of antibiotics in aquaculture has increase a chances of getting antimicrobial resistance and need necessitating effective alternatives. Medicinal plants, such as *Andrographis paniculata*, give a promising solution due to their antimicrobial and immunostimulatory properties. This study investigated the effects of dietary supplementation with *A. paniculata* leaf extract on the immune response and disease resistance of Mozambique tilapia, *Oreochromis mossambicus*. Fish were fed diets containing varying concentrations of aqueous and ethanolic leaf extracts for 30 days. A comprehensive analysis of non-specific immune parameters was subsequently conducted on serum samples. The extract demonstrated significant in vitro antibacterial and anti-biofilm activity against *A. hydrophila*. Dietary administration of *A. paniculata* significantly improved key cellular immune responses, which includes serum myeloperoxidase activity, respiratory burst activity, and nitric oxide production. Humoral immunity was also fortified, as evidenced by increased activities observed in serum anti-protease, natural haemolytic complement, lysozyme, and alkaline phosphatase. Furthermore, a marked increase in the antioxidant enzyme catalase and total serum protein was observed in treated groups. The immunostimulatory effects translated into superior disease resistance. Following a challenge with a lethal dose of *A. hydrophila*, fish fed the *A. paniculata*-supplemented diets demonstrated a significantly greater survival rate compared to the control group. The results conclusively demonstrate that *A. paniculata* leaf extract is a potent dietary immunostimulant that enhances both humoral and cellular immune mechanisms in *O. mossambicus*. Its application significantly improves survival against aeromoniasis, presenting a viable, natural strategy for sustainable health management in aquaculture.

Keywords: *Andrographis paniculata*; *Aeromonas hydrophila*; *Oreochromis mossambicus*; Tilapia fish

1. Introduction

Aquaculture is an important and rapidly growing sector for large-scale animal food production, supplying approximately 47% of the world's food fish (Ahmad et al., 2021). In Saudi Arabia, recognizing its importance for food security and proposed one of the important objective of Saudi Arabia's Vision 2030 and launched ambitious initiatives such as the 'Aquaculture Development Program'. In Saudi Arabia, total tilapia fish production reached approximately 45,200 tonnes in 2023, accounting for around 32% of national aquaculture output (Young et al., 2025). Tilapia production is essential to the national aquaculture sector for rural development and regional food security (Young et al., 2025). Currently, microbial infections in farm organisms pose a major problem in aquaculture. The spread of bacterial pathogens in aquatic environments is driven by various factors, including water quality, environmental conditions, stock density, and feed quality, all of which increase the susceptibility of cultured species to severe infections (Senthamarai et al., 2023). Bacterial diseases cause substantial economic losses and threaten the stability of production (Young et al., 2025). Among numerous fish pathogens causing infections in aquaculture, *Aeromonas hydrophila* is a major global concern. Antibiotics have been used extensively to control pathogens and improve growth performance; however, their prolonged usage has led to major antimicrobial resistance (Reverter et al., 2020). A concurrent challenge is the need to minimize the production cost, as aqua feed alone accounts for up to 70% of expenses in Saudi Arabia and worldwide (Young et al., 2025). Hence, to address these dual concerns of controlling bacterial infections and reducing the feed cost is very

important. In this situation, medicinal plants have gained considerable attention from researchers as sustainable alternatives. Recent reports are highly focused on utilizing plant extracts and their bioactive compounds in aquaculture to control bacterial infections and enhance immune responses of farm organisms (Dadras et al., 2023). They reported that the positive effects of single and combined phytochemicals observed on fish immunological parameters (Saleh et al., 2025; Liu et al., 2025).

In this context, the traditional herb *Andrographis paniculata* has been shown to improve growth and bolster immune capacity in cultured aquatic species (Palanikani et al., 2020). It is an annual herbaceous plant of the Acanthaceae family, often called creator green chiretta. It is indigenous to India and Sri Lanka and has been widely used as a medicinal plant in Asia, America and Africa for more than a century. It contains several photochemical components with distinct and remarkable biological characteristics (Vetvicka and Vannucci, 2021). It has been used to treat various diseases, including cancer, diabetes, bronchitis, skin diseases, colic, malaria and leprosy (Hossain et al., 2014). Consequently, recent research works demonstrated that *A. paniculata* extracts serve as an effective dietary supplement, enhancing disease resistance and promoting a healthier status in several fish species, including *Labeo rohita*, goldfish (*Carassius auratus*), and catfish (Palanikani et al., 2020; Thomas et al., 2023; Ear et al., 2024). However, no similar comprehensive studies in Saudi Arabia have investigated the use of *A. paniculata* as a dietary supplement for Mozambique Tilapia (*Oreochromis mossambicus*) or its effect on immunological response to enhance the cultivation of this economically valuable freshwater fish.

Thus, this study aims to evaluate the effects of dietary supplementation with *A. paniculata* leaf extract on the immune response and disease resistance of *O. mossambicus*. The expected outcome is to provide evidence supporting the use of plant compounds as dietary supplements to modulate immunological responses against fish pathogens. The specific objectives of the present work were to determine its impact on key serum parameters, including cellular immune markers (myeloperoxidase, respiratory burst, and nitric oxide synthase activities), humoral immune factors (anti-protease, natural haemolytic, lysozyme, and alkaline phosphatase activities), and antioxidant capacity (catalase activity, total protein). In addition, to assess the disease resistance by challenging fish with *Aeromonas hydrophila*.

2. Materials and Methods

2.1. Fish culture and experimental setup

Healthy tilapia fish (*Oreochromis mossambicus*) with an average weight of 18 to 22 gm and length of 8 to 12 cm were purchased from a local fish farm in the Al-Qassim region, Saudi Arabia. All the fish were carefully transported and cultured in a clean cement tank for 30 days at aquatic animal handling laboratory according to the standard protocol (Marathe et al., 2016). A complete panel of physicochemical parameters of the water such as temperature (maintained between 27 and 31°C), pH (ranges 5.4 to 8.5), total dissolved solids (0.2 -0.3 %), dissolved oxygen level (5.85 to 7.40 mg/L) in the culture tank was consistently maintained throughout the experimental period and the photoperiod (14:10 light: dark cycle) was maintained constantly. Fishes were fed with commercial food (Tairoun Feed Company, Taiwan) on daily basis and fecal matters and other wastes are often cleaned from the tank (Ma et al., 2020).

2.2. Preparation of *A. paniculata* extract

Fresh, undamaged leaves of *Andrographis paniculata* were collected, thoroughly rinsed with sterile distilled water, and shade-dried. The dried leaves were ground into a powder form using a mechanical grinder and stored in sterile containers at -20°C until further use (Jiang et al., 2023). Around 100 gm of leaf powder was immersed in 1000 ml of water and ethanol to make aqueous and ethanolic extract. The ingredients were well combined. Conical flasks were stored for one week at room temperature, wrapped firmly with aluminum foil, and stirred every day to guarantee thorough digestion (Naomi et al., 2022). The extracts were filtered through sterile muslin cloth, and the aqueous and ethanolic solvents were removed from the filtrate by concentration using a rotary evaporator. The resulting extract was dried to a powder and stored in a sterile, sealed glass container until further use (Bae et al., 2012).

2.3. *In vitro* antibacterial and anti-biofilm study

Antibacterial effect of plant extract was assessed against *A. hydrophila* by agar well-diffusion method with sterile Muller-Hinton agar. 10 µl of overnight grown culture was spread on the surface of agar plate and 5 mm size well was made using sterile cork-borer (Dedhia et al., 2018). Plant extracts at various concentrations (25 to 100 µg) were introduced into each well, and the plates were incubated at 37 °C for 24 hours. Following incubation, the formation of a clear zone of inhibition around any well was recorded.

The efficacy of *A. paniculata* leaf extract in controlling biofilm formation by the Gram-negative fish pathogen *A. hydrophila* was measured using a microtiter plate assay (Sternberg et al., 2014). Briefly, 500 µL of bacterial culture was added to 24-well plates containing sterile glass beads and 500 µL of nutrient broth, followed by incubation at 37 °C for 48 hours. Afterwards, 100 µL of *A. paniculata* extract at various concentrations (25–100 µg) was added to the test wells, while bovine serum albumin (BSA) was used in control wells. The plates were incubated at 37 °C for 24 hours on an orbital shaker at 100–150 rpm. After incubation, the broth was removed, and the biofilms were stained with 0.1% crystal violet for 30 minutes. The glass beads were then gently washed with sterile phosphate-buffered saline (PBS) and air-dried. Biofilm formation was quantified by measuring the intensity of the retained crystal violet stain under a microscope.

2.4. Study design

Initially fish were cultured in optimum and suitable environments, after the acclimatization period, fishes were grouped in a clean tank containing 80 L of water (tank capacity is 100L) and each group has 10 fish, and all the groups were cultured in same environment and maintain under same physiological parameters as that of acclimation time. This study comprises four experimental groups. During the experimental condition, all the groups were supplied with commercial fish feed in the frequency of once per day (De Bonville et al., 2024). The following experimental setup used for this study to incorporate *A. paniculata* extracts in per kg of commercial diet:

Group 1: 5 mg/g of *A. paniculata* leaf extract (water) with a commercial diet; Group 2: 10 mg/g of *A. paniculata* leaf extract (water) with a commercial diet. Group 3: 5 mg/g of *A. paniculata* in ethanol leaf extract with a commercial diet. Group 4: 10 mg/g of *A. paniculata* in ethanol leaf extract with a commercial diet.

The feeding trial was conducted in triplicate over 30 days. The fish were fed their respective experimental diets at a daily ration of 2% of their total body weight. (Jahja et al., 2023).

2.5. Sample preparation

On day 30, three fish were randomly tested from each tank. The fish were anaesthetized, and blood was collected by caudal venipuncture. The blood samples were immediately transferred to tubes containing an anticoagulant and centrifuged at 5000 rpm for 10 minutes to get plasma. Serum was obtained from the clotted blood and stored in sterile micro centrifuge tubes at -80 °C for subsequent analysis. All immunological parameters were assessed using this serum. Both humoral and non-specific cellular immune parameters were analyzed according to established protocols (Parasuraman et al., 2010; Garcia et al., 2013). The assessed parameters included nitric oxide production, serum anti-protease activity, myeloperoxidase activity, serum natural haemolytic complement activity, lysozyme activity, alkaline phosphatase activity, and the antioxidant enzyme catalase, in addition to total protein content.

2.6. Evaluation of immunological parameters in fish serum samples

2.6.1. Determination of myeloperoxidase (MPO) activity

MPO is the non-cellular immune parameters, the level of this enzyme in serum sample of *O. mossambicus* after treatment with *A. paniculata* leaf extract was measured. Briefly, 10µl of serum samples were collected and diluted 10 times with Hank balanced saline solution in 96 well plate, then 2mM solution of 3,3',5,5' – tetramethyl benzidine hydrochloride and 5mM solution hydrogen peroxide were added as substrate (Gururajan et al., 2009). Then, plate was incubated for 30 minutes

at 30°C, once the color has changed the reaction was interrupted by adding 4M H₂SO₄ and absorbance was read at 450 nm (Hoque et al., 2022).

2.6.2 Respiratory Burst (RBA) activity

RBA in serum samples was quantified using the nitroblue tetrazolium (NBT) reduction assay, adapted from the method described by Rakita et al. (1999). Briefly, 100 µL of serum from *O. mossambicus* was added to a 96-well plate and incubated at 30 °C for 1 hour to allow cell adhesion. After incubation, the supernatant was aspirated, and the adhered cells were washed gently with PBS (pH 7.4). Subsequently, NBT solution was added to the wells, and the plate was incubated for a further 60 minutes. The reaction was stopped by rinsing the wells with absolute and 30% methanol to fix the cells. After air-drying, 2N potassium hydroxide and dimethyl sulfoxide were added to dissolve the formazan crystals. The absorbance was finally measured at 540 nm.

2.6.3 Reactive nitrogen species production (NOSA)

Serum samples were combined with 10 mM sodium nitroprusside and incubated at 25°C for one hour. Following this incubation, Griess reagent was introduced to the mixture. After a subsequent 30-minute incubation period, the absorbance was read at 546 nm. This assay is called Griess reaction method, nitric oxide production was quantified by analyzing its stable metabolite called 'nitrite' (Bryan and Grisham, 2007).

2.7. Tests for Non-specific humoral immunological parameter

2.7.1. Serum anti-protease (SAP) activity

Sodium-benzoyl- DL-arginine-P-nitroanilide HCl (BAPNA) was exploited to measure the SAP in the serum sample by Bowden et al. (1997). Serum samples (10 µl) were added to the 0.1% of trypsin prepared in 0.1M tris HCl (pH 8.2) then 500 µl of substrate (BAPNA) were mixed to the serum sample and level up for 1ml by adding 0.1M tris HCl (pH 8.2). The mixture was incubated for 25 minutes at 22°C after incubation the effect of substrate activity was interrupted by adding 30 % of acetic acid solution and absorbance was measured at 415 nm.

2.7.2. Serum natural complements haemolytic (SNH) activity

A sheep red blood cell (RBC) assay was performed to analyze the haemolytic activity of fish serum. Firstly, sheep RBCs were washed with Hank's Balanced Salt Solution (HBSS) lacking phenol red. Serum samples were diluted in HBSS and mixed with the prepared RBC suspension. The reaction mixture was incubated at 22 °C for 90 minutes with intermittent gentle agitation applied at 15-minute intervals. The reaction was stopped by adding HBSS containing 10 mM EDTA. Following centrifugation to remove intact RBCs, the absorbance of the supernatant, representing haemoglobin release, was measured at 405 nm in a 96-well microplate reader (Chabannes et al., 2021).

2.7.3. Lysozyme (LYZ) activity

LYZ activity was determined using the turbidimetric assay described by Wang and Wang (2022). In this method, a suspension of *Micrococcus lysodeikticus* was prepared in 0.05 M PBS (pH 6.2). Serum samples were added to the bacterial suspension, and the reduction in turbidity was immediately monitored by checking the absorbance at 450 nm at timed intervals.

2.7.4. Alkaline phosphatase (AKP) activity

AKP activity in the serum sample was measured with p- nitro phenyl phosphate as substrate. Briefly, the serum sample was added to the ammonium bicarbonate solution and incubated for 15 minutes at 30 °C. Then to the mixture, p- nitro phenyl phosphate was added and optical density was checked at 405 nm (Kanta et al., 2021).

2.8. Antioxidant Activities

2.8.1. Catalase (CAT) activity

CAT activity was determined by measuring the breakdown of its substrate, hydrogen peroxide. The reaction rate was quantified by monitoring the decrease in absorbance of H₂O₂ at 240 nm using a microplate reader (Hadwan et al., 2024).

2.8.2. Total protein content

The total protein content was analyzed by dye-binding method described by Kielkopf et al., (2020). Blood serum was collected from the fish after herbal supplement of 30 days of incubation period. It was diluted with PBS and Bradford reagent further incubated for 2 minutes at room temperature. After that, absorbance was measured at 595 nm. BSA was used as standard solution and Bradford reagent was considered as blank.

2.9. *Tilapia* challenge with *A. hydrophila*

On day 30 of the feeding trial, fish from both the control and *A. paniculata*-supplemented groups were challenged with *A. hydrophila*. Each fish received an intraperitoneal injection of 50 µL of an *A. hydrophila* suspension (approx. 10⁷ cells/mL). A negative control group was injected with the same volume of sterile PBS to account for mortality unrelated to the bacterial infection. Mortality was recorded daily for 14-day post-injection. To reaffirm *A. hydrophila* as the cause of mortality, bacterial strains were re-isolated from the kidneys of deceased fish using the pour plate technique on *Aeromonas* isolation medium, as per the protocol of Qosimah et al. (2023). A single colony from each sample was sub-cultured to obtain a pure isolate for confirmation.

At the end of 14 days observation period, the percentage survival rate of fishes was calculated for using formula is "Percentage of survival rate = [Number of surviving fish / Total number of challenged fish] × 100" (Jeyavani et al., 2025).

2.10. Data analysis and statistics

All biochemical assays were conducted in triplicate. Data were analyzed using Microsoft Excel and SPSS (Version 16). Values are expressed as mean ± standard deviation. Statistical significance among treatment groups was determined by one-way analysis of variance (ANOVA), with a p-value less than 0.05 considered statistically significant.

3. Results and Discussion

Dietary supplementation with *A. paniculata* phytocompounds serves as an effective immunostimulant, enhancing both humoral and cellular immune responses in cultured Mozambique tilapia fish. This variation of the immune system directly compares with improved disease resistance in aquatic farm organisms. The growing interest in phyto-genic feed additives stems from their multifaceted benefits, which support organismal health and contribute to sustainable production systems. In subsequent sections present detailed analyses of these immunological measurements and their relationship to disease outcomes.



Figure 1. Growth of *Aeromonas hydrophila* on Muller Hinton agar shows zone of inhibition of *A. paniculata* extract at various concentrations.

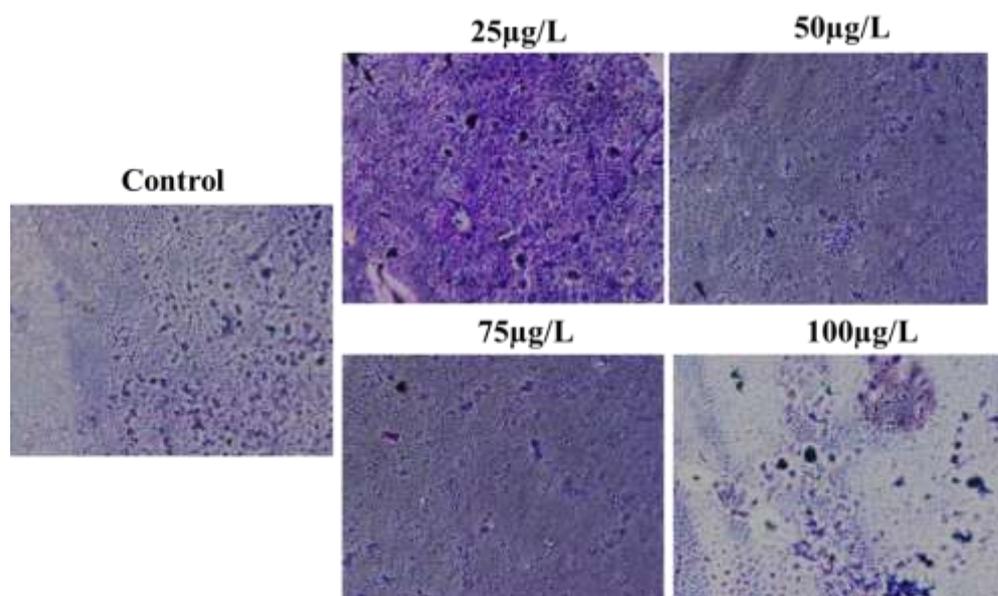


Figure 2. Inhibitory effect of *A. paniculata* extract on *Aeromonas hydrophila* biofilm biomass at various concentrations.

3.1 Antibacterial and anti-biofilm activities

The present study evaluated the effect of *A. paniculata* extract on Gram-negative *A. hydrophila* biofilms since biofilms are frequently found to support resistance to several antimicrobial drugs. *A. hydrophila* treated with *A. paniculata* extract exerts 80% of the toxicity rates in dark conditions and 95% below exposure to visible light during antibiofilm activity on bacterial colonies. The *A. paniculata* extract demonstrated significant, dose-dependent antibacterial activity against *A. hydrophila*. The zones of inhibition measured 14.5 ± 0.5 mm, 9 ± 0.5 mm, and 6 ± 0.5 mm at concentrations of 100 µg, 75 µg, and 50 µg, respectively (**Figure 1**).

Anti-biofilm activity of *A. paniculata* extract was shown in **Figure 2**. Compared to control group the *A. hydrophila* treated group shown the reduced biofilm formation where, the bacterial growth on the glass piece were detached at 100 µg/ml concentration. These findings support the minimum inhibitory concentrations of ethanol extract of *A. paniculata* determined to be 100 mg/ml which is comparable similar to the antibiotic ciprofloxacin (100 to 200 g/ml) as a reference. These results showed the *A. paniculata* extract has an excellent antimicrobial efficiency and may be used as an effective source to prevent the *A. hydrophila* infection.

3.2. Non-specific cellular immunologic responses:

3.2.1. Myeloperoxidase (MPO) activity

Myeloperoxidase is highly present in white blood cells, specifically neutrophil as granules and plays key role in production of oxidative radicals to release hypochlorous acid which is important for destroying the pathogenic organism. In addition, myeloperoxidase enzyme is crucial to maintaining the redox homeostasis of immune cells via exclusion of hydrogen peroxide. The current investigation on MPO activity in *O. mossambicus* were done at the end of 28th day (**Figure 3a**), and the MPO level was increased in the *A. paniculata* extract group compared to the control group. The production of MPO regulates the halide synthesis pathway, as well as a variety of antimicrobial enzymes, all depend on the degranulation process. Similar to the obtained results, it is proved that, MPO activity of *A. hydrophila* challenged rainbow trout (*Oncorhynchus mykiss*) serum shows increased activity in fed meal containing *B. subtilis*, *P. hypophthalmus* supplied probiotic (*B. licheniformis*) Dahb1 at 10⁷ cfu/ml and catla fingerlings fed a supplemental diet containing a probiotic *B. amyloliquefaciens* at 10⁹ cfu/g all had higher serum levels (Das et al., 2013). Moreover, as per available literature, it is proved that after 80 days of feed, all treated groups those having the probiotics as supplements showed a substantial increase ($p < 0.05$) in the total myeloperoxidase content in common carp fry serum (Gupta et al., 2014).

3.2.2. Respiratory burst activity (RBA)

RBA is the crucial immune defense mechanism effectively involved in host-pathogen interactions via innate immune response, also create direct impact on host immune system from higher vertebrates to fish. In this study, the elevated level of RBA demonstrated that there is an activation of phagocytosis, a natural process to kill the pathogen. The activation of phagocytosis may be triggered by the production of oxidative metabolites including hydroxyl radical, superoxide ions and hydrogen peroxide. This observation is supported by previous research; for instance, dietary supplementation with andrographolide was shown to enhance non-specific immunological parameters and confer resistance against *A. hydrophila* in *Labeo rohita* (Ahamed Basha et al., 2013). Similarly, Jeyavani et al. (2025) reported comparable immunostimulatory effects in *O. mossambicus* using the probiotic *Bacillus licheniformis*. Similar findings were noticed in the current study with increased RBA in groups given *A. paniculata* extract. In this study, after treatment period, the RBA level was enhanced in *A. paniculata* extract treated *O. mossambicus* (**Figure 3b**). As per the available literatures, RBA represents a crucial oxygen-dependent defense mechanism in the phagocytic cells of vertebrates additionally an increased level of respiratory burst activity can be viewed as an increase in pathogen killing activities (Thornton Hampton et al., 2020). Based on the present findings, it is suggested that the increased level in NBT level effectively helps the fish by defending them against invasive pathogens.

3.2.3. Reactive nitrogen species production (RNSP)

Super-radical ions like nitric oxide are produced by macrophages which are highly toxic to pathogens and have effective anti-microbial activity. The increased level of nitric oxide in the host organism indicates the protection from pathogenic organisms. This nitric acid oxidation effectively alters the several signaling pathways in host and is implicated in granulocyte stimulation through innate immunological response. In this study, after treatment the nitric oxide production in *O. mossambicus* serum was found at the end of 28th day (**Figure 3c**).

The group which have the *A. paniculata* extract demonstrated increased resistance against *A. hydrophila* infection, as indicated by higher RNSP in these fishes, which may be related to the stimulation of a non-specific immunologic response. These findings align with previous research demonstrating the immunostimulatory effects of herbal supplements in aquatic species. Basha et al. (2013) reported enhanced immunity in *L. rohita* fed andrographolide, while Citarasu et al. (2003) documented similar benefits in *P. monodon* using a polyherbal formulation. Further supporting evidence comes from Thomas et al. (2023), who observed improved disease resistance in *Carassius auratus* supplemented with *A. paniculata*.

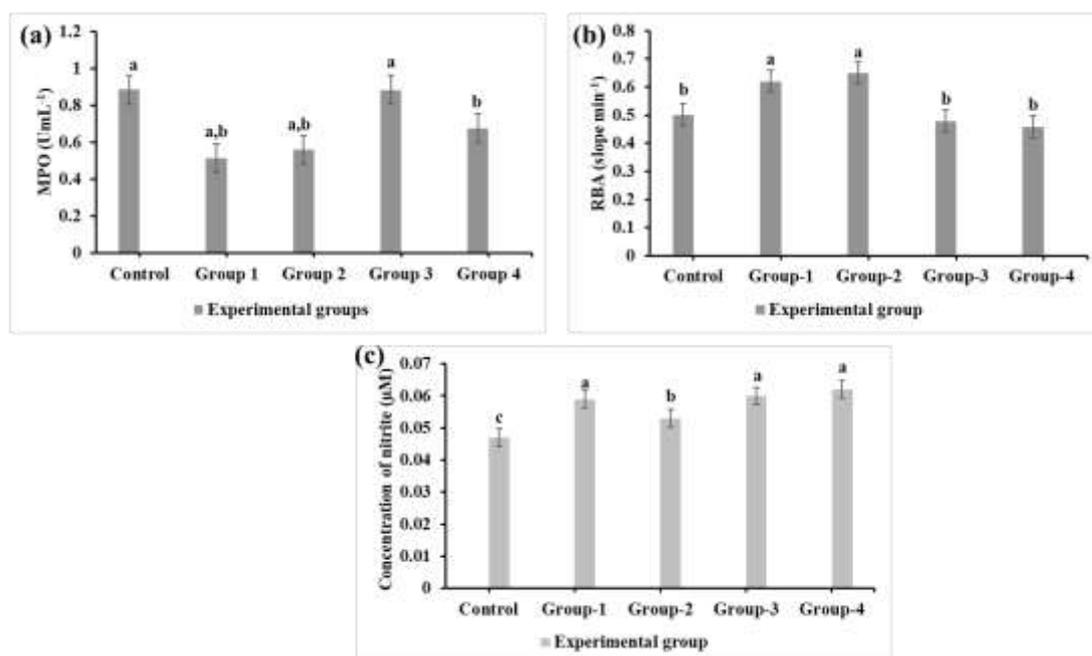


Figure 3. Myeloperoxidase activity (a), Respiratory burst activity (b), Nitric oxide scavenging activity (c) after treatment *A. paniculata* leaf extract in *O. mossambicus*. Data are presented as the mean \pm SD and results were analyzed with one-way ANOVA.

3.3 Non-specific humoral immune parameter

3.3.1 Serum natural complements haemolytic (SNCH) activity

SNCH is the unique and prominent component of immune system and plays a critical role in the humoral immunological response. The soluble plasma proteins present in this system are crucial for both innate and adaptive immune response. It acts as primary defense mechanism and is effectively involved in the protective function against several types of pathogenic microorganism via membrane attack complex (MAC) or phagocytosis. Hence, the level of immunological parameters and the activation of complement system of in the treated groups after the desired incubation period were analyzed, and noticed that increased complement of SNCH in *A. paniculata* extract treated serum of *O. mossambicus* (Figure. 4b). Our results are consistent with recent findings by Aguiar et al. (2023), who observed elevated level of SNCH activity in Nile tilapia fed diets containing 250 mg/kg peppermint and tea tree extracts. This correlation confirms that activating the complement system through phyto-genic supplementation represents a promising strategy for developing functional aqua feeds. The complement cascade serves as a vital bridge between innate and adaptive immunity, making its enhancement particularly valuable for comprehensive disease resistance in cultured fish.

3.3.2 Serum anti -protease (SAP) activity

From the analysis of SAP activity, fish serum comprises alpha 1, 2 anti-protease and alpha macroglobulin, both are crucial to defeating the protease activity of pathogens to limit the capacity of bacteria to penetrate and proliferate in fish. The present study revealed that, dietary supplement of *A. paniculata* extract in *O. mossambicus* significantly improved the SAP activity in the treated groups, whereas in the control groups no significant changes were observed (Figure 4a and b). Consistent with our observations, dietary supplementation with *Eclipta alba* aqueous extract was shown to enhance serum anti-protease activity in *O. mossambicus* within two to three weeks (Christyapita et al., 2007).

3.3.3 Lysozyme (LYZ) activity

Lysozyme is a crucial antimicrobial enzyme in body fluids that hydrolyzes bacterial cell walls, leading to pathogen lysis. It also functions as an opsonin, enhancing phagocytosis to facilitate pathogen clearance by host immune cells. In this study, there is enhanced level of lysozyme activity in *A. paniculata* extract treated groups in *O. mossambicus* compared to control groups. Lysozyme activity increased in a dose-dependent manner, with higher extract concentrations yielding greater

enzymatic activity (Figure 4c). Our results are comparable with similar studies done by various authors (Gupta et al., 2014; Thomas et al., 2023; Jeyavani et al., 2025).

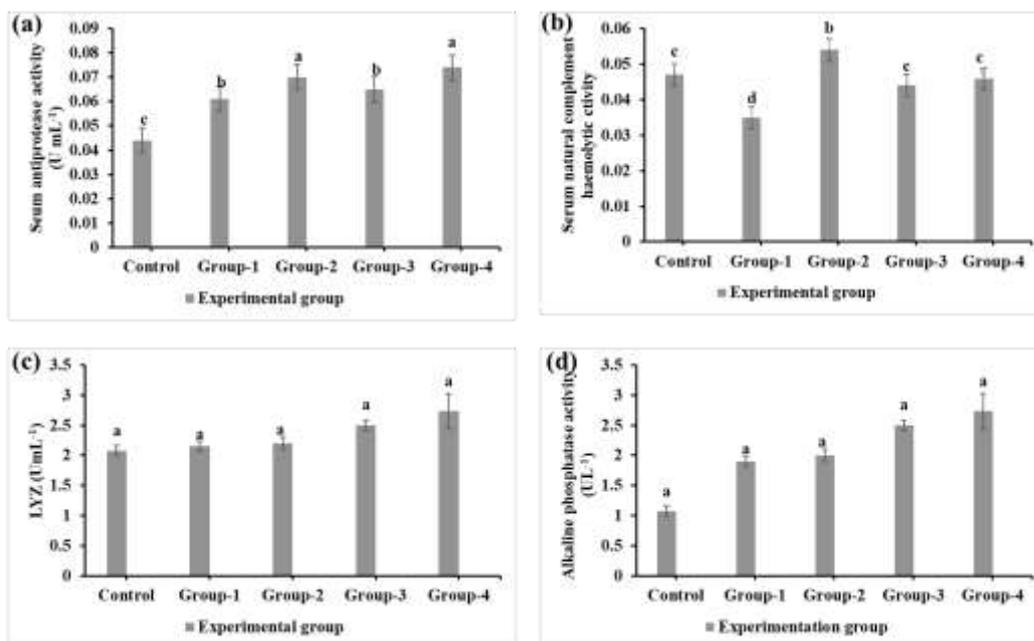


Figure 4. Estimation of anti-oxidant enzyme activity after treatment specially at 28th day after dietary supplemented with *A. paniculata* extract in *O. mossambicus*.

3.3.4 Alkaline phosphatase (AKP) activity

AKP is a member of under-lysosome enzymes which play an important role in the activation of macrophages by which it protects the fish from stress, parasite infection, and wound healing by acting as an antibacterial agent and protects the host from number of pathogenic organisms. In this study, the extract of *O. mossambicus* in dietary supplements effectively increase the levels of alkaline phosphatase and its activity at 28th days of treatment. Compared to control groups the treated groups show significantly increased activity (Figure 4d). This was evidenced by similar kind of increased level of AKP results were observed from the studies with *Ocimum gratissimum* supplementation to African catfish (Abdel-Tawwab et al., 2018) and Caspian white fish with peppermint (Adel et al., 2015). It notably elevated both total protein level and alkaline phosphatase activity via non-specific immune responses. In addition, it significantly activates the macrophages which are important for host protection.

3.3.5 Catalase (CAT) activity

Catalase is one of the key enzymes that play a crucial role in antioxidant defense mechanisms and protect the cells from oxidative stress conditions, including phagocytosis and highly increase the scavenging effect by enhancing the superoxide ion level, hydrogen peroxide and hydroxyl ions. Hence, we investigated the supplement of *A. paniculata* extract in diet of *O. mossambicus*, and we noticed an increased CAT activity (Figure 5). This enhanced activity might have the impact of the increased level of hydrogen peroxide, which is potentially involved in defense mechanism especially protect from oxidative stress and leads phagocytosis process. Similar findings were reported in African catfish by Abdel-Tawwab and his team member (Abdel-Tawwab et al, 2018) with clove basil.

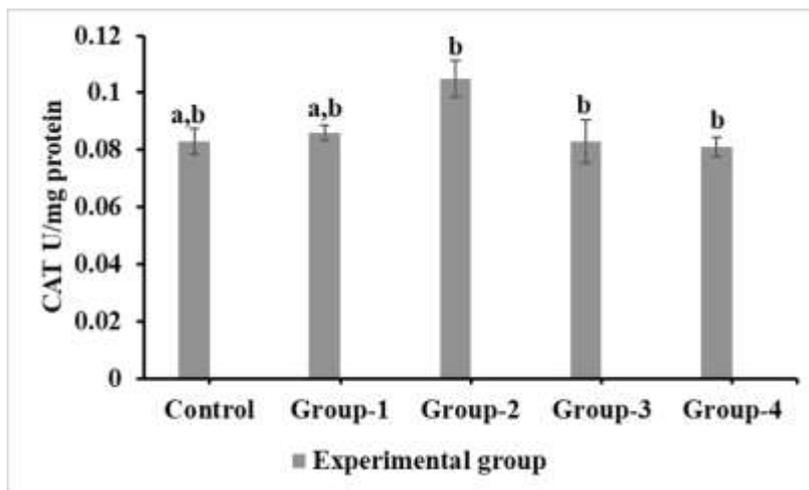


Figure 5. Serum catalase activity in *O. mossambicus* after 28 days of dietary supplementation with *A. paniculata* extract.

3.3.6 Total serum protein content analysis

After 28 days of the treatment, we have evaluated the total serum protein content of both control and treated groups, there was considerable increase in total protein in treated groups (**Figure 6**), which is supported by previous reports, the application of Clove basil, *Ocimum gratissimum* supplements also increased the serum and mucus protein content (Abdel-Tawwab et al., 2018). This study reported that the *A. paniculata* extract can be used as a potent dietary supplement for *O. mossambicus* which significantly increases the cellular immune response at end of 28th day via enhancing the by the production of radical ions including superoxide ions, H₂O₂, hydroxyl ions and nitric oxide and effectively scavenging the reactive oxygen radicals in *O. mossambicus*.

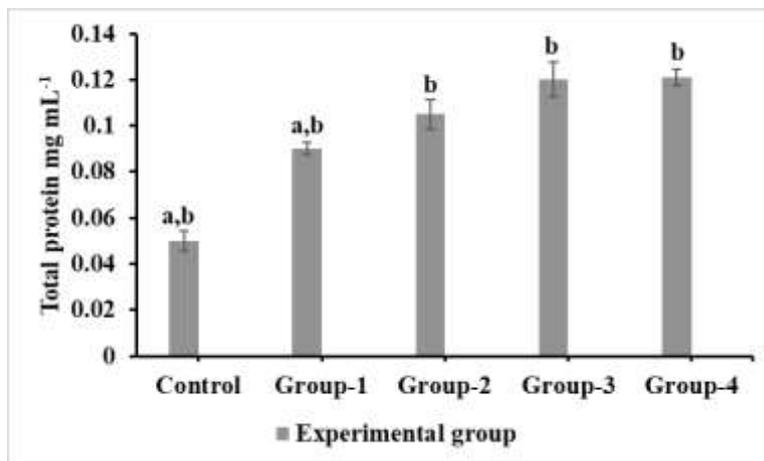


Figure 6. Total serum protein content in *O. mossambicus* after 28 days of dietary supplementation with *A. paniculata* extract compared to control group, all the treated groups show increased total protein content.

3.4 Aeromonas hydrophila challenge study

To evaluate the pathogenicity challenge against aquatic bacterial infection specifically against *A. hydrophila*, after *A. paniculata* supplement, 28th day treatment, both the treated and control groups were injected with *A. hydrophila* and their survival rates were assessed up to 14 days. From **Figure 7**, it was noticed that, compared to control groups the treated groups show a significant increase in survival group 2 (85.19%), group 3 (77.78%) and group 3 (51.85%), whereas in control groups shows 22.22%. in negative control no death was monitored. In addition, it should be noticed that hemorrhagic lesions on skin of dead fishes in the control groups. The same findings were reported in Nile tilapia, which has improved resistance against *Streptococcus agalactiae* when treated with clove basil and ginger fed as dietary supplements (Brum et al., 2017).

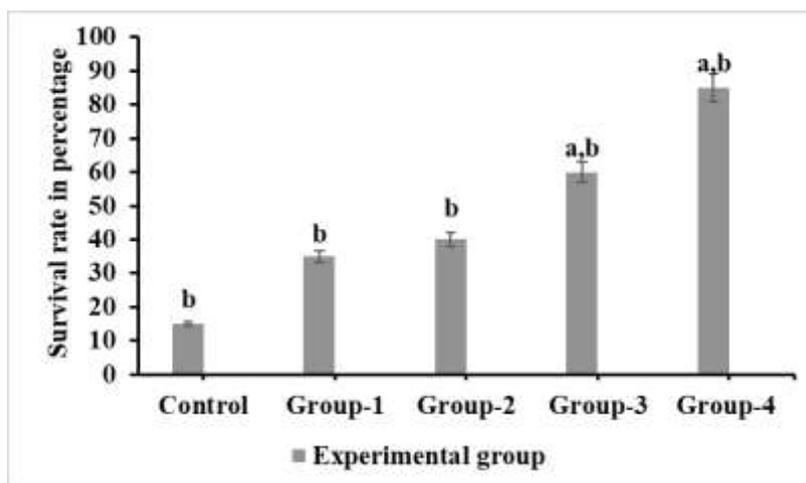


Figure 7. Survival rate of *O. mossambicus* following *A. hydrophila* challenge after dietary supplementation with *A. paniculata* extract. Compared to control group 4 shows increased survival rate.

4. Conclusions

This present investigation specifically examines the immunomodulatory potential of *A. paniculata* leaf extracts in *Oreochromis mossambicus* through comprehensive serum analysis. The study quantified important immunological parameters including cellular immune markers (myeloperoxidase activity, respiratory burst activity, nitric oxide production), humoral immune factors (serum anti-protease activity, natural haemolytic activity, lysozyme activity, alkaline phosphatase activity), and antioxidant capacity (catalase activity), along with total serum protein content. These serum enzymes including lysozyme, myeloperoxidase, and anti-proteases play crucial roles in piscine defense mechanisms against bacterial pathogens. The evaluation of these immune parameters provides critical insight into the host's immunological status and its capacity to respond to pathogenic threats. This study also pointed out the purposeful importance of these immunological measurements was further measured through a disease challenge model using the major aquatic pathogen '*Aeromonas hydrophila*'.

This study strongly demonstrates that dietary supplementation with *Andrographis paniculata* leaf extracts significantly enhances the health and disease resistance of *Oreochromis mossambicus*. Over a 28-day period, treated fish exhibited strengthened immune function, marked by significant improvements in both key humoral and cellular parameters. This immunostimulatory effect translated into practical benefits, as evidenced by a substantially higher survival rate following a lethal challenge with *A. hydrophila* compared to the control group. Complementary in vitro assays confirmed the extract's direct antimicrobial and anti-biofilm efficacy against the pathogen.

In conclusion, *A. paniculata* leaf extracts considered as a potent, natural immunostimulant could be used for sustainable aquaculture. Its combination into fish feed signifies a viable strategy for improving innate immunity and managing *Aeromonas* infections without relying on conventional antibiotics. The research data could help address the problem of controlling the antimicrobial resistance and reduce the feed costs in aquaculture. However, future research should focus on isolating the active compounds responsible for these effects and developing standardized, commercially viable feed formulations to optimize fish health and production.

Conflicts of Interest

There are no conflicts to declare

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