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Antioxidative status, immune response, and disease resistance of *Clarias gariepinus* fed on *Azolla pinnata* and *Moringa oleifera* supplemented diets

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ABSTRACT

The current study aims to supplement fish feed with two plants (*Azolla pinnata* and *Moringa oleifera*) in order to combat the disease caused by *Edwardsiella tarda* bacterium. Four groups of African catfish, *Clarias gariepinus*, were set up: fish fed on supplementary feed at 5% *Azolla pinnata* and 5% *Moringa oleifera*/kg diet for groups 1 and 2, the positive control, group 3, and negative control, group 4, received non-supplemented diets. After a two-week feeding period, all groups (except group 4) received an intraperitoneal injection containing a lethal dosage of *Edwardsiella tarda* isolated from a local outbreak with LD_{50} 3×10^4. Non-specific immune parameters and antioxidant indicators were estimated at the serum level of all experimental fish before and after the *Edwardsiella tarda* challenge. Furthermore, tissue expression levels of some immune and antioxidant-related genes were evaluated in the spleens of experimental fish before and after the *Edwardsiella tarda* challenge. Results recorded that supplemented feed groups showed relative percent survival of 75% and 66.67% for groups 1 and 2 against *Edwardsiella tarda* with normal serum levels of aspartate aminotransferase and alanine aminotransferase hepatic enzymes. The high survivability was accompanied by elevated serum levels of the measured non-specific immune parameters and antioxidant indicators, particularly after challenge. Also, transcription analyses showed upregulation of expression levels of GPX, SOD1, IL-1β, and MHC-IA genes in the spleens of experimental fish, indicating enhanced innate immune response of *Clarias gariepinus* fed on supplementary feed at 5 % *Azolla pinnata* and 5 % *Moringa oleifera* / kg diet for two weeks.

Introduction

One of the food production sectors with the greatest rate of growth in the world is aquaculture, and the goods it produces are a significant source of protein for human consumption (Ahmad *et al*., 2021). Aquaculture is rising rapidly in order to supply the increasing demand for fish products. However, because to their tendency to lower fish productivity, yield, and marketability, infectious illnesses pose a continuing threat to the sustainability of aquaculture (Lafferty *et al*., 2015; Irwin *et al*., 2024). Pathogens can travel great distances and spread quickly across populations in aquatic systems. Bidirectional transmission between cultivated and wild populations is also a possibility (Krkošek, 2017). Numerous bacterial pathogens, both Gram positive and Gram negative, are frequently found in aquaculture systems and pose serious risks (Sørum, 2006). *Edwardsiella tarda* (*E. tarda*) is one of these most common bacterial pathogens that affects African catfish (*Clarias gariepinus*) (*C. gariepinus*) in culture (Abdelazeem *et al*., 2022). Human and public health are at risk from consuming such infected cultured fish (Healey *et al*., 2021). Farmers in the aquaculture industry are compelled by this incident to use antibiotics often. Antimicrobial resistance (AMR) has emerged among the bacterial fish pathogens concurrently with the increase in antibiotic use in aquaculture as a part of therapy and prophylaxis (Preena *et al*., 2020). In order to restrict the formation and spread of antibiotic-resistant bacterial strains in aquaculture production systems, we must reduce the excessive use of antimicrobials and instead adopt other strategies. The creation of cost-effective vaccinations, the application of both specific and non-specific immune enhancers, and the use of probiotics and bioaugmentation to improve the quality of the aquatic environment are the main topics for more research and development addressing disease prevention in aquaculture. Another pertinent issue that needs to consider the proper provision of protein to improve fish health is the creation of fish meals and the use of nutritional supplements (Katheline *et al*., 2019). There are numerous studies that demonstrate the effectiveness of medicinal plants in enhancing fish immunity and shielding fish from the harmful effects of various contaminants on the aquatic environment (Verma *et al*., 2021; Reda *et al*., 2023). Recent research has concentrated on the use of medicinal plants in aqua-diets due to their low cost, high protein content, and local availability (Abdel-Latif *et al*., 2022; Brar *et al*., 2022). *Azolla pinnata* (*A. pinnata*) is a floating water fern that is found in many nations and is a member of the Azollaceae family. In stagnant wetlands, it is developing quickly, covering the water's surface and tripling its biomass in a matter of days (Korbekandi *et al*., 2014). While *Moringa oleifera* (*M. oleifera*) is found in Southwest Asia, Southwest Africa, Northeast Africa, and Madagascar, it is a member of the Moringaceae family of plants (Abd Rani *et al*., 2018). Flavonoids and phenolic compounds are among the many advantageous phytochemical active components found in *A. pinnata* and *M. oleifera* (Sankhalkar and Vernekar, 2016; Verma *et al*., 2021). Therefore, the purpose of this study was to examine the effects of *A. pinnata* and *M. oleifera* on immune and antioxidant functions as well as their potential to prevent *E. tarda* infection in the native African carnivore fish *C. gariepinus*, which has been introduced to many parts of the world.

Materials and methods

Plant collection

The *A. pinnata* plant was gathered in the Egyptian province of Beni Suef. Plants with healthy leaves were stripped off and given a distilled water wash. The leaves were then given time to dry before being ground into a fine powder using an automatic grinder (Krups). While *M. oleifera* leaves powder was commercially obtained (Imtenan, Egypt).

Ethical statement and fish rearing conditons

All animal tests were carried out at the Physiology Department, Faculty of Veterinary Medicine, Beni Suef University, Egypt, under ethical approval number 024-038 from the Institutional Animal Care and Use Committee (IACUC).

Fingerlings of *C. gariepinus* were obtained from a commercial fish farm in Beni Suef Province, Egypt, weighed 114.89±4.53 grams on average. Fish were housed in large plastic tanks with constant aeration and tap water free of chlorine. Before starting the trials, the fish had a two-week acclimation period during which their health was regularly checked. The fish were fed a simple meal at a rate of two percent of their body weight. Fish were re-admitted into smaller, 80-liter plastic aquariums after acclimatization in order to conduct the studies. The following water parameters were maintained throughout the entire experiment: water temperature of 30.0±1.0°C, pH of 7.5±0.5, dissolved oxygen content of 5.0±0.43 mg/L, NO_2 concentration of 0.018±0.006 mg/L, NH3 concentration of 0.06±0.004 mg/L, and a 12:12 dark to light schedule.

LD_{50} determination of isolated E. tarda strain

In the summer of 2024, a strain of virulent *E. tarda* was isolated from an outbreak at a private catfish farm in the Beni Suef area of Egypt. Following biochemical identification, the isolated strain was placed in 50% glycerol solution and then exposed to molecular identification for the purpose of detecting the *gyr*B gene using specific primer sets, forward: GCATGGAGACCTTCAGCAAT and reverse: GCGGAGATTTTGCTCTTCTT as descriped by Wang *et al.* (2012). To create inoculums of approximately $3×10^8$ CFU/mL, a bacterial suspension in fish saline (0.65% NaCl) was adjusted to match McFarland tube 1 (bioM´erieux). The inoculums were then serially diluted to obtain dilutions ranging from $3x10^6$ to $3x10^2$ CFU/mL. The inoculums utilized rapidly for the pathogenicity and LD_{50} determination (Reed and Muench, 1938). One hundred and eight *C. gariepinus* were split into six groups, each with three replicates, and placed into eighteen plastic aquaria, each with a water capacity of 80 L. Six groups were assigned, five of which were labelled as experimental and the sixth as control. Eugenol (Sigma-Aldrich) mixed in dimethyl sulfoxide (DMSO, Sigma-Aldrich) at a concentration of 40 ppm was used to anesthetize fish (Roubach *et al*., 2005). Next, 150 µL of sequential bacterial suspensions in fish saline, ranging from $3x10^6$ to $3x10^2$ CFU/mL of *E. tarda*, were intraperitoneally (IP) injected into the fish. The sole thing given to the sixth subgroup (control) was 150 µL of fish saline. Fish of all groups observed for recovery and monitored until two weeks (experimental period) for clinical abnormalities and cumulative mortalities. Tissue samples from moribund fish, such as the liver and kidneys, were streaked over BHI and incubated at 28°C for 24 to 48 hours to re-isolate the injected pathogen. PCR assay used to concurrently identify re-isolated bacterial strains.

Diet preparation and feeding trial

Using a mortar, the commercially available fish diet pellets (containing 25% protein) (Table 1) were ground into a fine powder. To create two fish diets, the powders of *A. pinnata* and *M. oleifera* were combined directly with the fine powder that had been previously made. Diet 1 with 5% of *A. pinnata* / kg of feed and diet 2 with 5 % of *M. oleifera* / kg of feed. Groups 3 and 4 were designated as control positive and control negative groups, respectively. De-ionized water was added to the entire ingredients to create a homogeneous slurry. The mixture was run through a manually operated hand-minced machine (NAHA) to create extruded strings. These strings were then let to air dry for a day before being broken into 2-4 mm-long pellets and stored at 4°C for later use (Rattanachaikunsopon and Phumkhachorn, 2010). Seventy-two *C. gariepinus*, three replicates of six in each, were randomly assigned to each of the eighteen even groups. For 14 days, groups 1 and 2 received diet supplements consisting of diet 1 (*A. pinnata* 5%) and diet 2 (*M. oleifera* 5%) feed. Groups 3 and 4 received a plain food supplement for the same period. 2% of each experimental group's body weight was given to them at three regular intervals throughout the day.

Table 1. Composition of the commercial pelleted fish diet

Diet components (25% crude protein)	Percentage
Soya bean meal	34
Yellow corn	32
Fish meal	8
Rice polish	23
Common salt	0.5
Mono-calcium phosphate	1
Premix	1.5

Samples collection and challenge procedure

Blood samples were taken from the caudal vessels of the experimental fish (6 fish per group) after the feeding trial was completed. Anticoagulant was not used when collecting blood samples for serum separation (8000 g / 20 min at 4°C). Until analysis, the serum samples were stored at -20°C. After completing the blood sampling collection, the 6 fish / group were euthanized to obtain tissue samples. Spleens were incised, submerged in RNA later, and kept at -80°C directed to gene expression analyses. After that the remaining fish in groups 1, 2 and 3 were challenged by IP injection with lethal dose ($3×10^6$ CFU/mL) of *E. tarda* while group 4 was IP injected with PBS (Fig. 1). For a two-week observation period, clinical and post-mortem abnormalities, mortality rates, and relative percent survival (RPS) were calculated, as demonstrated by Ibrahim *et al.* (2022). Survival fish were sampled using non-anticoagulant blood in order to separate serum and harvest spleen tissues for expression analysis.

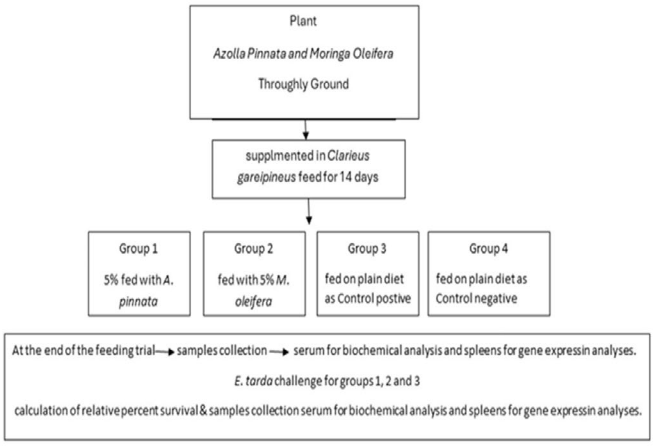

Fig.1. Experimental design.

Biochemical analyses

Liver function was evaluated through determination of the serum levels of aspartate aminotransferase (ALT) (U/L) and alanine aminotransferase (AST) (U/L) (Biotrend CliniSciences) following manufacturer's instructions. Immunological assays included estimation of the serum levels of nitric oxide (NO), lysozyme (LZM) and complement 5 (C-5). As mentioned by Attia *et al.* (2003), the serum nitric oxide level was determined using the Griess reagent. In a nutshell, each well of a flat bottom 96-well ELISA plate received 150 µl of serum sample and 150 µl of Griess reagent. For eight minutes, the mixture was incubated at 22°C. Using an ELISA reader, the prepared plate's absorbency was determined at 540 nm. By comparing the optical density values of the nitrite standard curve in a linear curve fit, the optical density of the tested samples was converted to micromolar (Mmol) of nitrite. Furthermore, lysozyme (LZM) activity (ng/L) and complement 5 (C-5) (nmol/L) (Cusabio Biotech) in addition to antioxidant indicators including superoxide dismutase (SOD) (U/mL) (Biotrend CliniSciences), catalase activity (CAT) (U/L) and reduced glutathione (GPx), (mmol/L) (Cusabio Biotech) were determined using assay kits according to manufacturer's instructions.

Transcription analysis of some antioxidant and immune genes

About 50 mg of spleen tissues from 2 fish per replicate (N = 6 per group) were used to separate total RNA using Trizol (1.5 mL) (Invitrogen). The attained amount of total RNA was assessed using a NanoDrop One UV-Vis Spectrophotometer (Thermo Scientific) to calculate RNA concentration. The cDNA was produced from the parted RNA managing Maxima First Strand cDNA Synthesis Kit (Thermo Scientific). Briefly, 10 µl RNA sample had 4 ng of total RNA, 5 µl of maximal enzyme mixture, 10 µl of 6X buffer reaction mix, and 25 µl of purified water were placed in 50-µl reaction tubes to generate the cDNA that kept at -80°C for furtherer use. Real-time quantitative PCR (RT-qPCR) were used for computing GPX, SOD1, IL-1β and MHC-IA genes in the spleens of experimental fish utilizing specific primers (ShineGene) for the above-mentioned genes (Table 3). Universal SYBR Green Master Kit (ROX) was utilized for real-time PCR procedures. 10 µl of Universal SYBR Green Master mix, 1 µl of forward and reverse specific primer sets, 2 µl of complement DNA (cDNA), and 11 µl of molecular purified water were used in each 25 µl qPCR tube. For the qPCR cycles, Real Time PCR Applied Biosystems was used. The thermal cycler was programmed to run 30 cycles for initial denaturation, denaturation, annealing, and extension, respectively, at 94°C for 10 min, 94°C for 30 s, 55°C for 45 s, and 74°C for 10 s. The transcripts of the genes that were evaluated were calculated as a relative fold change to the reference gene (β-actin) in accordance with Karsi *et al*. (2004).

Statistical analysis

GPX, SOD1, IL-1β, MHCIA, and other immune- and biochemical-associated gene data were examined using one-way analysis of variance (ANOVA) in SPSS 18 (SPSS, Chicago, Illinois, USA). A significance level of $P \leq 0.05$ was set for mean dissimilarities, which was the focus of Duncan's multiple range tests.

Results

Molecular recognition of isolated E. tarda strain

Virulence gene identification in *C. gariepinus* samples revealed that the isolated strain amplified at 415 bp for the *gyr*B gene (Fig. 2).

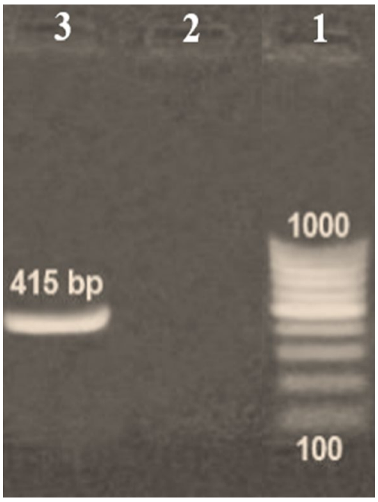

Fig. 2. PCR amplification product of *E. tarda gyr*B gene (415 bp). Lane (1): 100 bp DNA ladder, lanes (2) negative control and lane (3) isolated strain.

LD_{50} of the isolated E. tarda strain

The mortality rate of the *C. gariepinus* fingerlings that were experimentally infected was monitored for a period of two weeks following intraperitoneal injection of varying doses of virulent *E. tarda* isolate. The LD_{50} was 3×10^4 CFU/ml, and the fish died within the first week after injection (Fig. 3).

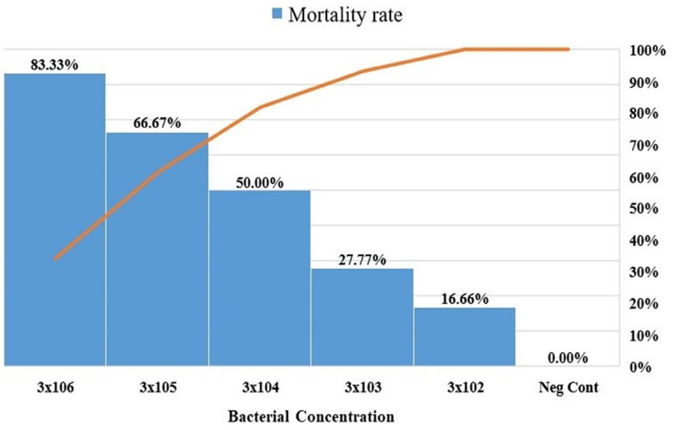

Fig. 3. Mortality percentage and LD_{50} of virulent *E. tarda* isolate from *C. gariepinus* outbreak.

Prevention of E. tarda infection in C. gariepinus fed on supplemented feed with A. pinnata and M. oleifera

Following intraperitoneal exposure to a virulent strain of *E. tarda*, group 1 supplemented with 5% *A. pinnata* per kg diet and had the highest relative percent of survival (75%). Group 2 supplemented with 5% *M. oleifera* per kg diet and had the second-highest relative percent of survival (66.67%). However, group 3 (control positive), which was given a plain diet before receiving an injection of the pathogenic *E. tarda* strain, had a 91.66% mortality rate. However, following an injection of physiological saline, the control negative group (group 4) fed a plain meal demonstrated 0% mortality (Fig. 4).

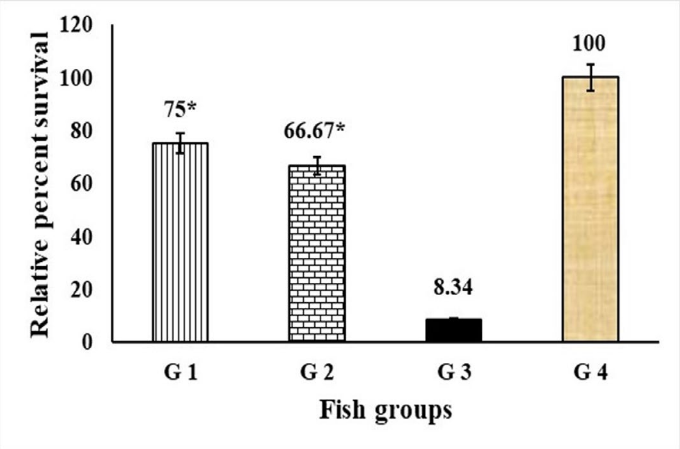

Fig. 4. Relative percent survival post feeding trial. G 1 and G 2 represent fish groups supplemented with 5 % *A. pinnata* and 5 % *M. oleifera*. G 3 and G 4 were supplemented with plain diet. All groups were injected with 3×10^6 CFU/mL of *E. tarda* except group 4 was injected with PBS. ⁎ Symbolizes to significant RPS $P \leq 0.05$.

Effect of supplemented feed with A. pinnata and M. oleifera on liver function, immune indicators and antioxidant biomarkers of C. gariepinus

Table 3 shows that prior to the *E. tarda* challenge, ALT and AST did not significantly change in any of the experimental groups. Groups 1 and 2 supplemented their feeds with 5% *A. pinnata* and *M. oleifera* / kg diet, exhibiting considerably greater levels of nitric oxide, lysozyme, complement, SOD, CAT, and GPx than the control groups (Fig. 5, Table 2). There were no appreciable differences between the groups that consumed diets supplemented with *A. pinnata* and *M. oleifera*. As seen in Table 2, group 3 had the highest levels of ALT and AST following the *E. tarda* challenge, while groups 1 and 2 maintained normal ranges. Group 3 had the greatest amounts of nitric oxide, lysozyme, complement, SOD, CAT, and GPx, followed by Groups 1 and 2 (Fig. 6, Table 2). It is clear to note that while both groups 1 and 2 had notable increases in the non-specific immunological measures and antioxidant indicators, there were no appreciable differences between them.

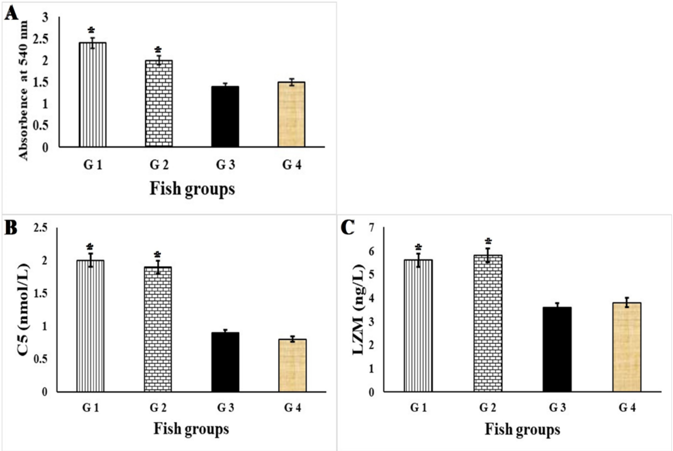

Fig. 5. (A) significant upsurge in NO production, (B) clear increases in serum C5 and (C), Significant elevation in LZM activity in fish G 1 and G 2 that supplemented with 5 % *A. pinnata* and *M. oleifera* in comparison to control groups G 3 and G 4 after the end of feeding trial and before *E. tarda* challenge. ∗ Symbolizes to significant elevation $P \leq 0.05$.

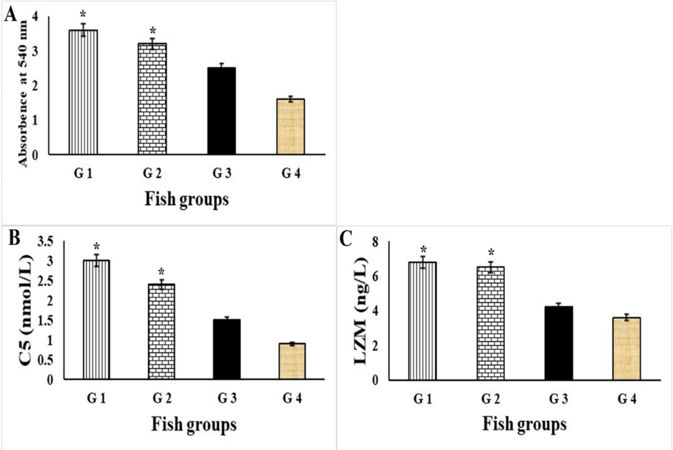

Fig. 6. (A) significant upsurge in NO production, (B) clear increases in serum C5 and (C), Significant elevation in LZM activity in fish G 1 and G 2 that supplemented with 5 % *A. pinnata* and *M. oleifera* in comparison to control groups G 3 and G 4 after the end of feeding trial and after *E. tarda* challenge. ∗ Symbolizes to significant elevation $P \leq 0.05$.

Effect of supplemented feed with A. pinnata and M. oleifera on transcription levels of some antioxidant and immune genes

At the level of antioxidant genes, GPX and SOD1, they showed upregulation expression levels both before and after challenge and the highest transcription level (Fold change 6.5) was observed in GPX gene in the group that fed on 5 % *A. pinnata* /kg feed after challenge with *E. tarda*. While the lowest upregulation expression level was detected for SOD1 (Fold change 1.5) in fish group which fed on 5 % *M. oleifera* / kg diet before challenge as cleared in (Fig. 7). At the level of immune related genes, IL-1β and MHC-IA, the same pattern also recorded with more marked upregulation transcription particularly for IL-1β with fold change 7 in the fish group that fed on 5 % *A. pinnata* /kg diet as shown in (Fig. 7).

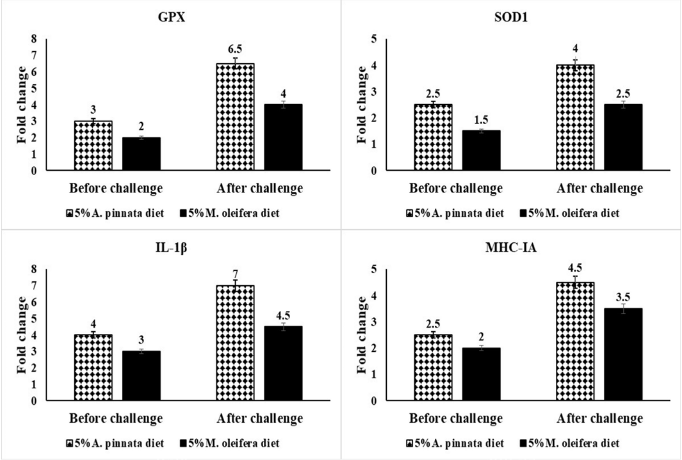

Fig. 7. Marked increases in upregulation transcription levels of GPX, SOD1, IL-1β and MHC-IA genes in fish groups that supplemented with 5 % *A. pinnata* and 5 % *M. oleifera* / kg diet for two weeks feeding period.

Discussion

Due to the medical value of whole plants as well as their distinct parts, diets of humans and animals have long included them. Additionally, the increased interest in the use of medicinal herbs to maintain animal and human health, as well as their source, affordability, lack of side effects, and lack of concern over the development of antibacterial resistance, are all contributing factors to the growing popularity of herbal medicines worldwide (Enerijiofi and Isola, 2019). Only a small portion of plant species have been thoroughly identified as having bioactive components up to this point. In addition, many new medications are developed by plant biological components or their active metabolites as economic causes (Esquer-Miranda *et al.*, 2016). According to earlier research, plants may have antibacterial properties against several Gram +ve and

Table 2. Impact of *A. pinnata* and *M. oleifera* on liver function and antioxidant activity indicators of *C. gariepinus* before and after *E. tarda* challenge.

Indicators		Fish groups			
		G 1	G 2	G 3	G 4
ALT (U/L)	Before	31.07±0.53^{a}	30.97±0.39^{a}	31.48±0.27^{a}	31.53±0.21^{a}
	After	32.03±0.56^{a}	31.47±0.45^{a}	43.87±0.32^{b}	30.78±0.19^{a}
AST (U/L)	Before	44.97±0.71^{a}	43.67±1.69^{a}	44.67±0.54^{a}	45.16±0.37^{a}
	After	48.16±0.51^{b}	49.11±1.78^{b}	59.34±0.28^{c}	47.36±0.17^{b}
SOD (U/mL)	Before	4.98±0.16^{b}	5.59±0.11^{b}	3.79±0.10^{a}	3.42±0.12^{a}
	After	8.16±1.06^{c}	7.91±0.71^{c}	12.65±1.18^{d}	4.22±0.22^{a}
CAT (U/L)	Before	187.00±1.00^{b}	189.00±3.00^{b}	169.00±2.00^{a}	170.00±2.00^{a}
	After	219±3.14^{c}	217.00±1.51^{c}	256.00±3.17^{d}	184.00±3.23^{b}
GPx (mmol/L)	Before	9.12±0.41^{b}	8.98±0.54^{b}	6.71±0.62^{a}	6.78±0.51^{a}
	After	16.33±2.73^{c}	14.23±1.14^{c}	21.71±2.62^{d}	8.52±1.50^{b}

Group 1 with 5% of *A. pinnata* / kg of feed and group 2 with 5 % of *M. oleifera* / kg of feed. While control groups (group 3 and group 4 represent control positive and control negative groups). The mean±standard error (±SE) is used to express values. Values in different superscript letters are substantially different at $P \leq 0.05$.

Table 3. Primers of some immune and antioxidant-related genes for the real-time quantitative PCR amplification.

Gene	Primer sequence	GenBank number	Reference
β-actin	F: ACCCCCGCCATGTACGTT R: CCGGAGTCCATGACGATACC	XR_002012167.1	Swaleh *et al*. (2020)
GPX	F: ACAACCAGGGACTACACTCAAGTG R: CACACCCAAAATAACGAGACCTT	GQ376155.1	Swaleh *et al*. (2020)
SOD1	F: TGCTCCCGTAGTGGTTAAAGGG R: TTCATCAAGTGGCCCACCATG	MK112879.1	Nasrullah *et al*. (2021)
IL-1β	F: TGCAGTGAATCCAAGAGCTACAGC R: CCACCTTTCAGAGTGAATGCCAGC	MH341527.1	Nasrullah *et al*. (2021)
MHC-IA	F: AACAAGTGGGATCCTGATAGTG R: AACAAGTGGGATCCTGATAGTG	MG545605.1	Nasrullah *et al*. (2021)

β-actin, beta actin; SOD, superoxide dismutase; CAT, catalase; 1β, interleukin and MHC- I, major histocompatibility complex.

Gram -ve bacterial species (Al-Nemari *et al*., 2020; Verma *et al*., 2021). Although synthetic antibiotics work faster and are more effective, frequent use of them can lead to bacterial resistance as well as a selective burden on the normal gut microbiota (Barbosa and Levy, 2000). For this reason, it is essential to use plant-based remedies to combat various bacterial illnesses in order to maintain environmental equilibrium. According to Zofia *et al*. (2020), active ingredients and other secondary metabolites found in plants play a crucial function in preventing bacterial infections. The phytochemical contents have been classified as alkaloids, phenols, quinones, saponins, xanthoproteins, tannins, proteins, carboxylic acids, carbohydrates, steroids, and coumarins in the light of the uses that *A. pinnata* and *M. oleifera* are capable (Oyama *et al*., 2019; Verma *et al*., 2021). Following the isolation of a virulent strain from a local epidemic, the present study assessed the potential of dietary integration of *A. pinnata* and *M. oleifera* to prevent *E. tarda* infection in *C. gariepinus*. The ability of *E. tarda* to infect epithelial cells, counterattack serum and phagocyte-mediated destruction, and produce toxins like dermatoxins, hemolysins, and cytotoxins to spread infection are the main components of its pathogenicity (Wang *et al*., 2012). In this research work, the molecular identity of the isolated *E. tarda* strain was validated by presence of *gyr*B, ATPase domain of DNA *gyr*ase, gene in conformity with the prevalence distribution patterns of the virulence-associated genes of *E. tarda* as reported by Wang *et al*. 2012. Most notably, the isolated strain's median lethal dose (LD_{50}) was 3×104. This finding supported the hypothesis that the pathogenicity of *E. tarda* is correlated with the presence of the virulent gene *gyr*B. According to earlier research, adding more plants to an animal's diet implied illness resistance and heightened immune responses (Galina *et al*., 2009). *A. pinnata* and *M. oleifera* powder, which had been fully dried and ground, were put to commercial fish feed at a concentration of 5 %. The control groups were started on the commercial diet without any supplements. As seen in Fig. 3, fish groups supplemented with 5 % *A. pinnata* and *M. oleifera* in the current study demonstrated 75.67% and 66.67% relative present survival (RPS) following challenge with a lethal dosage of *E. tarda*, respectively, in contrast to 8.34 % RPS in the control positive group. In a similar vein, Verma *et al*., 2021 found that 100 % RPS from an A. hydrophila induced infection could be obtained by supplementing the food of *C. gariepinus* with *A. pinnata* and ceratophyllum demersum at 5 % and 2.5 % for ten consecutive days. The *A. pinnata* and *M. oleifera* are weed plant and could have some detrimental impact on the fish when supplied into their diet. Therefore, the serum levels of AST and ALT were determined in order to assess this impact. Even after an *E. tarda* bacterial infection, no discernible changes were found in the experimental fish, G1 and G2, except for the control positive group, G3. This suggests that adding 5 % *A. pinnata* and *M. oleifera* to the plant's diet can lessen the negative effects of an *E. tarda* infection. Macrophages are essential to fish's innate immune response because they phagocytose bacteria and other foreign particles in line with the immune system's non-specific response. According to several research (Bricknell and Dalmo, 2005; Grayfer *et al*., 2018), macrophage activity can be used as an indication to evaluate the innate immune response in several fish species. Nitric oxide (NO), which is released by the anterior kidney cells when macrophages are involved, is an essential means of observing macrophage activity. Prior research shown that adding more plants to the diet increased their serum levels of nitric oxide, which suggested that this would boost their immune system and prevent disease (Kumar *et al*., 2019). Similarly, comparable to negative and positive control groups, fish groups fed diets supplemented with *A. pinnata* and *M. oleifera* also showed greater serum levels of NO in the current investigation, especially in the sera of fish who survived after challenge. In the current study, other non-specific immunological characteristics were also estimated. Fish serum lysozyme and complement activities have been shown to offer natural defence. Tables 3 and 4 show that groups fed on supplemented artificial feed with *A. pinnata* and *M. oleifera* at 5% concentration in their respective diets had increased levels of blood lysozyme and complement. This increase could be explained by neutrophils and macrophages becoming more activated following a bacterial assault (Saurabh and Sahoo, 2008; Carbone and Faggio, 2016). These results also coincide with the nitric oxide levels. Free oxygen radicals produced by microbial infections have the potential to damage essential macromolecules such as proteins, lipids, and DNA (Cadenas and Davies, 2000). Oxidative stress is caused by an excessive production of active free radicals within the host organism. The primary scavengers of these free radicals are the enzymes SOD, GPx, and CAT. Higher levels of SOD, GPx, and CAT were found in the groups treated with plant diet in the current investigation. This suggests that the fish had developed non-specific immune responses that were sensitized to deactivate reactive free radicals. Moreover, flavonoids, which are abundant in *A. pinnata* and *M. oleifera*, may be the cause of their antioxidant properties (Alhakmni *et al*., 2013). The current investigation examined the transcription of a few immunological and antioxidant genes in *C. gariepinus* and measured the expression of those genes in different tissues following a severe *E. tarda* challenge. According to Alejo and Tafalla, (2011), interleukins are the primary chemokines that regulate the immune system. They play a vital role in initiating inflammatory processes and supporting immunological responses. Additionally, MHC-IA is responsible for displaying antigens on the cell surface so that cytotoxic T lymphocytes can identify them (Fischer *et al*., 2005). In the current investigation, after the *E. tarda* challenge, both IL-1β and MHC-1A revealed higher elevated expression levels in the spleens of fish groups fed on 5% *A. pinnata* and *M. oleifera* / kg diet (Fig. 5). Following an *E. tarda* challenge, the mRNA transcription of SOD1 and GPX was likewise significantly up-regulated in a manner similar to those of IL-1β and MHC-1A. This increased expression may be due to the association with important roles of both antioxidant enzymes and cytokines in both innate and adaptive immune responses in the spleens and anterior kidneys following the microbial infection (Tort, 2011; Chen *et al*., 2014). These results are in line with those of Song *et al*. (2016) and Nasrullah *et al*. (2021), who found that after bacterial infection, the anterior spleens and anterior kidneys of both African catfish and channel catfish had increased transcription levels of IL-1β, MHC-1A, SOD1, and GPX genes.

Conclusion

To sum up, the overuse of antibiotics led to the emergence of numerous bacterial strains that are resistant to them. At a concentration of 5 %, the plants *A. pinnata* and *M. oleifera* might be added to artificial feed to improve the host's non-specific immune responses, which would strengthen the host's resistance to *E. tarda* infection. The field study and research on other fish species are recommended by the current study in order to make it economically viable and significantly boost aquaculture's bottom line.

Conflict of interest

The authors have no conflict of interest to declare.

References

Abd Rani, NZ., Husain, K., Kumolosasi, E., 2018. Moringa genus: a review of phytochemistry and pharmacology. Frontiers in Pharmacology 9. https://doi.org/10.3389/fphar.2018.00108.

Abdelazeem, M. Algammal., Mahmoud, Mabrok., Mahmoud, Ezzat., Khyreyah, J. Alfifi., Aboelkheir, M., Esawy, Nehal Elmasry., Reham, M. El-Tarabili., Prevalence., 2022. Antimicrobial resistance (AMR) pattern, virulence determinant and AMR genes of emerging multi-drug resistant *Edwardsiella tarda* in Nile tilapia and African catfish, Aquaculture 548, 737643. https://doi.org/10.1016/j.aquaculture.2021.737643.

Abdel-Latif, HMR., Abdel-Daim, MM., Shukry, M., Nowosad, J., Kucharczyk, D., 2022. Benefits and applications of *Moringa oleifera* as a plant protein source in aquafeed: a review. Aquaculture 547,737369. https://doi.org/10.1016/j.aquaculture.2021.737369.

Ahmad, A., Sheikh Abdullah, S. R., Hasan, H. A., Othman, A. R. and Ismail, N., Izzati. 2021. Aquaculture industry: Supply and demand, best practices, effluent and its current issues and treatment technology. Journal of Environmental Management 287, e112271.

Alejo, A., Tafalla, C., 2011. Chemokines in teleost fish species. Developmental & Comparative Immunology 35,1215–1222. https://doi. org/10.1016/j.dci.2011.03.011.

Alhakmni, F., Kumar, S., Khan, SA., 2013. Estimation of total phenolic content in- vitro antioxidant and anti-inflammatory activity of flowers of *Moringa oleifera*. Asian Pacific Journal of Tropical Biomedicine 3, 623–627. https:// doi. org/ 10. 1016/ S2221- 1691(13) 60126-4.

Al-Nemari, A., Al-Senaidy, A., Semlali, M., Ismael, A.Y., Badjah-Hadj-Ahmed, A., Ben Bacha., 2020. GC-MS profiling and assessment of antioxidant, antibacterial, and anticancer properties of extracts of Annona squamosa L. leaves. Complementary Medicine and Therapies. 20, 296.

Attia, J., Hatala, R., Cook, D.J., Wong, J.G., 2003. The rational clinical examination: does this adult patient have acute meningitis?. JAMA psychiatry 282,175-181.

Barbosa, T.M., Levy, S.B., 2000. The impact of antibiotic use on resistance development and persistence, Drug Resistance Updates 3, 303–311.

Brar, S., Haugh, C., Robertson, N., Owuor, PM., Waterman, C., Fuchs, GJ III., Attia, SL., 2022. The impact of *Moringa oleifera* leaf supplementation on human and animal nutrition, growth, and milk production: a systematic review. Phytotherapy Research 36,1600–1615. https://doi.org/10.1002/ptr.7415.

Bricknell, I., Dalmo, R.A., 2005. The use of immunostimulants in fish larval aquaculture, Fish Shellfish Immunol. 19, 457–472.

Cadenas, E., Davies, K.J., 2000. Mitochondrial free radical generation, oxidative stress, and aging. Free Radical Biology and Medicine 29, 222–230.

Carbone, P.K., Faggio, C., 2016. Importance of prebiotics in aquaculture as immunostimulants. Effects on immune system of Sparus aurata and Dicentrarchus labrax, Fish Shellfish Immunol. 54, 172–178.

Chen, M., Wang, R., Li, L., Liang, W., Wang, Q., Huang, T., Li, C., Li, J., Gan, X., Lei, A., Huang, W., Luo, H., 2014. Immunological enhancement action of endotoxin-free tilapia heat shock protein 70 against Streptococcus iniae. Cellular Immunology 290,1–9. https://doi.org/10.1016/j.cellimm.2013.12.008.

Enerijiofi, KE., Isola, OB., 2019. Preliminary Phytochemical screening and in-vitro antibacterial activities of aqueous and ethanol extracts of Ageratum conyzoides L. Leaf, Stem, Flower and Root on some Bacterial isolates associated with Diarrhoea. Nigerian Journal of Pure and Applied Sciences 32, 3480–3489. Doi: https:// doi. org/ 10. 19240/ njpas. 2019.

Esquer-Miranda, M., Nieves-Soto, M.E., Rivas-Vega, A., Miranda-Baeza, P., Pina- Valdez., 2016. Effects of methanolic macroalgae extracts from Caulerpa sertularioides and Ulva lactuca on Litopenaeus vannamei survival in the presence of Vibrio bacteria. Fish Shellfish Immunol. 51, 346–350.

Fischer, U., Djikstra, JM., Kollner, B., Kiryu I, Koppang E, Hordvik I, Sawamoto Y, Ototake M., 2005. The ontogeny of MHC class I expression in rainbow trout (Oncorhynchus mykiss). Fish Shellfish Immunol. 18, 49–60. https://doi.org/10.1016/j.fsi.2004.05.006.

Galina, G., Yin, L., Ardo, Z., Jeney, L., 2009. The use of immune-stimulating herbs in fish. An Overview of Research, Fish Physiology and Biochemistry. 35, 669–676.

Grayfer, B., Kerimoglu, A., Yaparla, J.W., Hodgkinson, J., Xie, M., Belosevic, M., 2018. Mechanisms of fish macrophage antimicrobial immunity, Frontiers in Immunology 9, 1105.

Healey, K. D., Rifai, S. M., Rifai, A. O., Edmond, M., Baker, D. S., Rifai, K., 2021. *Edwardsiella tarda*: A Classic Presentation of a Rare Fatal Infection, with Possible New Background Risk Factors. The American Journal of Case Reports 22, e934347. https://doi.org/10.12659/AJCR.934347.

Ibrahim, Rowida., Elshopakey, Gehad., El-Rahman, Ghada., Ahmed, Amany., Altohamy, Dalia., Zaglool, Asmaa., Younis, Elsayed., Abdelwarith, Abdelwahab., Davies, Simon., Al-Harthi, Helal., Abdel Rahman, Afaf., 2022. Palliative role of colloidal silver nanoparticles synthetized by moringa against Saprolegnia spp. infection in Nile Tilapia: Biochemical, immuno-antioxidant response, gene expression, and histopathological investigation. Aquaculture Reports 26. 101318. 10.1016/j.aqrep.2022.101318.

Irwin, B.J., Tomamichel, M.M., Frischer, M.E., Hall, R.J., Davis, A.D., Bliss, T.H., Rohani, P. and Byers, J.E., 2024. Managing the threat of infectious disease in fisheries and aquaculture using structured decision making. Frontiers in Ecology and the Environment 22, e2695. https://doi.org/10.1002/fee.2695.

Karsi, A., Waldbieser, G.C., Small, B.C., Liu, Z., & Wolters, W.R. (2004). Molecular cloning of proopiomelanocortin cDNA and multi-tissue mRNA expression in channel catfish. General and Comparative Endocrinology 137, 312-21.

Katheline, Hua., Jennifer, M. Cobcroft., Andrew, Cole., Kelly, Condon., Dean, R. Jerry., Arnold, Mangott., Christina, Praeger., Matthew, J. Vucko., Chaoshu, Zeng., Kyall, Zenger., Jan, M. Strugnell., 2019. The Future of Aquatic Protein: Implications for Protein Sources in Aquaculture Diets, One Earth 1, 316-329. https://doi.org/10.1016/j.oneear.2019.10.018.

Korbekandi, H., Chitsazi, M., Asghari, G., Najafi, R., Badii, A., Iravani, S., 2014. Green biosynthesis of silver nanoparticles using *Azolla pinnata* whole plant hydroalcoholic extract. Green Processing and Synthesis 3, 365-373. https://doi.org/10.1515/gps-2014-0042.

Krkošek, M., 2017. Population biology of infectious diseases shared by wild and farmed fish. Canadian Journal of Fisheries and Aquatic Sciences 74, 620–28.

Kumar, J., Sharma, S.P., Singh, A., Singh, V., Hari, Krishna, R., Chakrabarti, M., 2019. Validation of growth enhancing, immunostimulatory and disease resistance properties of Achyranthes aspera in Labeo rohita fry in pond conditions, Heliyon 5, 01246.

Lafferty K.D., Harvell C.D., Conrad J.M., Jon M. Conrad, Carolyn S. Friedman, Michael L. Kent, Armand M. Kuris, Eric N. Powell, Daniel Rondeau, Sonja M. Saksid, 2015. Infectious diseases affect marine fisheries and aquaculture economics. Annual Review of Marine Science 7, 471–96.

Nasrullah, H., Yanti, D.H., Faridah, N. Dian Hardiantho., Yanti I. Nababan., Sukenda S., Alimuddin A., 2021. Early immune gene development and expression in African catfish *Clarias gariepinus* after challenged with *Aeromonas hydrophila*. Aquaculture International 29, 595–607. https://doi.org/10.1007/s10499-021-00645-1.

Oyama, MO., Egbebi, AO., Akharaiyi, FC., 2019. Phytochemical analysis and antibacterial activities of some plant extracts on S*taphylococcus aureus* isolates from patients receiving hospital treatments in Ekiti State, Nigeria. Journal of Herbal Medicine and Pharmacology 8, 14–20.

Preena, P.G., Swaminathan, T.R., Kumar, V.J.R., Isaac S.B.S., 2020. Antimicrobial resistance in aquaculture: a crisis for concern. Biologia 75, 1497–1517. https://doi.org/10.2478/s11756-020-00456-4.

Rattanachaikunsopon, P., Phumkhachorn, P., 2010. Lactic acid bacteria: their antimicrobial compounds and their uses in food production. Annals of Biological Research 1, 218–228.

Reda, R.M., Helmy, R.M.A., Osman, A. Farag A Gh Ahmed., Gamila A. M. Kotb., Amir H. Abd El-Fattah., 2023. The potential effect of *Moringa oleifera* ethanolic leaf extract against oxidative stress, immune response disruption induced by abamectin exposure in Oreochromis niloticus. Environmental Science and Pollution Research 30, 58569–58587. https://doi.org/10.1007/s11356-023-26517-0.

Reed, L.J., Muench, H A.,1938. Simple method of estimating fifty percent endpoints. American Journal of Epidemiology 27, 493-497. https://doi.org/10.1093/oxfordjournals.aje.a118408.

Roubach, R., Gomes, L.C., Leao ˜ Fonseca, F.A., Val, A.L., 2005. Eugenol as an efficacious anaesthetic for tambaqui, *Colossoma macropomum* (Cuvier). Aquaculture Research 36, 1056–1061. https://doi:10.1111/j.1365-2109.2005.01319.x.

Sankhalkar, S., Vernekar, V., 2016. Quantitative and qualitative analysis of phenolic and flavonoid content in *Moringa oleifera* Lam and Ocimum tenuiforum L. Pharmacognosy Research 8,16–21. https://doi. org/10.4103/0974-8490.171095.

Saurabh, S., Sahoo, P.K., 2008. Lysozyme: an important defense molecule of fish innates immune system. Aquaculture Research 39, 223–239.

Song, L., Li, C., Xie, Y., Liu, S., Zhang, J., Yao, J., Jiang, C., Li, Y., Liu, Z., 2016. Genome-wide identification of Hsp70 genes in channel catfish and their regulated expression after bacterial infection. Fish Shellfish Immunol. 49, 154–162. https://doi.org/10.1016/j.fsi.2015.12.009.

Sørum, H., (2006) Antimicrobial drug resistance in fish pathogens. In: Aarestrup FM (ed) Antimicrobial resistance in bacteria of animal origin. ASM Press, Washington, DC, pp. 213–238.

Swaleh, S. B., Banday, U. Z., Asadi, M. A., Usmani, N., 2020. Biochemical profile and gene expression of *Clarias gariepinus* as a signature of heavy metal stress. Environmental pollution (Barking, Essex: 1987), 264, 114693. https://doi.org/10.1016/j.envpol.2020.114693.

Tort, L., 2011. Stress and immune modulation in fish. Developmental & Comparative Immunology 35, 1366–1375. https://doi.org/10. 1016/j.dci.2011.07.002.

Verma, V. K., Kumar, K. B., Sagar, K., Majumdar, S., Pal, S., Mehta, A., Vats, A., Rani, K. V., Sehgal, N., & Prakash, O., 2021. Amelioration of immune and digestive system through weed supplemented feed against Aeromonas hydrophila in *Clarias gariepinus*. Fish & Shellfish Immunology 115, 124–133. https://doi.org/10.1016/j.fsi.2021.05.016.

Wang, Xuepeng., Maocang, Yan., Qishuo, Wang., Lei, Ding., Fuchang, Li., 2012. Identification of *Edwardsiella tarda* isolated from duck and virulence genes detection. African Journal of Microbiology Research 6, 4970-4975.

Zofia, N.L., Martyna, Z.D., Aleksandra, Z., Tomasz, B., 2020. Comparison of the antiaging and protective properties of plants from the apiaceae family. Oxidative Medicine and Cellular Longevity 9, 5307614.