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Optimizing the Effects of Dietary *Tridax Procumbens* leaf Extract on Growth Performance, Antioxidant Activity, Immune Response, and Disease Resistance Against *Aeromonas Hydrophila* in *Labeo rohita*

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Abstract

Asian ayurvedic herb *Tridax procumbens* (TP) has long been used for therapeutic purposes. However, the effects of TP on *Labeo rohita* remain ambiguous. Consequently, the present study investigated the impact of *T. procumbens* leaf extract (TPLE) on the growth performance, immune response, and disease resistance in the Indian major carp, *Labeo rohita,* across four treatment groups, followed by an anti-infection treatment against *Aeromonas hydrophila*. Fish were fed a basic diet fortified with 0%, 0.2%, 0.4%, and 0.8% of TPLE for 60 days. The results of this study demonstrate that the TLE0.4 and TLE0.8 diets considerably outperformed the control group in terms of growth performance, total protease, α-amylase activity, respiratory burst activity, lysozyme activity, myeloperoxidase activity, mucus, and serum bactericidal activity ($P<0.05$). Furthermore, the TLE0.4 and TLE0.8 extract-fed groups showed a significant decrease ($P<0.05$) in glucose content and a notable increase ($P<0.05$) in total protein, albumin, alkaline phosphatase, and superoxide dismutase compared to the control group. Moreover, the expression of pro-inflammatory cytokines (IL-1β, TNFα), antioxidant molecules (SOD, GPx, catalase), and pattern recognition molecules (TLR 22) was significantly upregulated by TLE0.8 supplemented diet. In contrast, the expression of cytokines such as IL-8, IL-10, and TGF-β was downregulated. The relative percentage of survival (RPS) analysis revealed that the TLE0.4 and TLE0.8 diet-fed groups had 83.30% and 77.73% of survival, respectively. Thus, our study demonstrated that incorporating TLE0.8 and TLE0.4 into the diets of *L. rohita* would enhance growth performance, antioxidant activity, immune response, and disease resistance against *A. hydrophila* infection.

Keywords: TPLE, *Labeo rohita*, growth performance, immune response, antioxidant activity, disease resistance.

Abbreviations: TPLE, *Tridax procumbens* leaf extract; TLE, *Tridax* leaf extract; RBA, respiratory burst activity; MPO, myeloperoxidase activity; ALP, alkaline phosphatase; SOD. superoxide dismutase; GPx, Glutathione peroxidase; CAT, Catalase; RPS, relative percentage of survival; FRP, fiberglass reinforced plastics; WGP, Weight gain percentage; FCR, Feed Conversion Ratio, FER, Feed Efficiency Rate; SGR, Specific Growth Rate; FW, final weight; IW, initial weight; DW, dry weight; WW, wet weight; ln, natural log; N, number of culture days; FBW, final body weight; WG, weight gain; PER, protein efficiency ratio; TP, total protein; Ig, globulin; TP, *T. procumbens*

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Introduction

Globally, aquaculture is acknowledged for its advantages in improving socioeconomic development, reducing poverty, and increasing food and nutritional security. In 2022, the combined output of fisheries and aquaculture hit a record-breaking 223.2 million tonnes, while the total amount produced worldwide hit a new high of 130.9 million tonnes, valued at USD 313 billion. The industry has been expanding at an average annual growth rate of 5.25% since 2000, ensuring food and livelihood security. Asia alone accounted for 91.4% of the world's aquaculture production (1,2). Indian major carp, *Labeo rohita,* is among the most coveted species for aquaculture and is one of the most extensively farmed fish in the Asia-Pacific region. In the modern era, increased aquaculture production from intensive farming has resulted in a highly stressed and crowded environment with degraded water quality, resulting in the frequent occurrence of diseases (3). Pathogens and the diseases they induce pose a significant challenge for the aquaculture industry to attain sustainability and reach its full potential (4). Opportunistic pathogens like *Aeromonas hydrophila* are major infectious disease-causing bacterial pathogens in *L. rohita* farming, incurring high incidences of mortalities and substantial financial loss, severely impeding aquaculture production (3–6). According to previous reports, *Aeromonas hydrophila* infections in *L. rohita* may result in various clinical manifestations like fin rot, tail rot, skin erosion, hemorrhages, abdominal swelling, and infection of internal organs such as the liver and spleen, as well as oxidative stress (5,7,8).

The prevention and treatment of diseases in aquafarming can be achieved through various means. These include immunostimulants, vaccinations, RNA and phage therapies, antimicrobial peptides (AMPs), phytotherapeutic substances, pre-, pro-, and postbiotics, chemicals, and recommended antibiotics (9). However, the inappropriate application of chemicals and antibiotics to treat bacterial infections in fish has led to serious issues like drug residues, contamination of the environment, and bacterial resistance (10). Further, the aquaculture supply chain may play a crucial role in the spread of bacteria resistant to antibiotics due to horizontal gene transfer from cultivated species to humans (11). To mitigate infectious diseases in fish, vaccines are employed; however, commercial vaccinations are expensive for fish producers. Therefore, it is necessary to adopt natural alternatives like phytochemical and phytobiotic medications in intensive and large-scale fish farming (12). As an alternative to traditional fish disease treatment, phytotherapy is natural, eco-friendly, biodegradable, and cost-effective against a wide range of bacterial diseases in aquaculture. Research has been centered on comprehending dietary supplements of phytotherapeutic substances that strengthen the aquatic species' innate immune defenses and elevate resistance to infections (13).

Over the past two decades, immunostimulatory and oxidative ingredients fortification or supplementation in the feed composition of aquatic animals has increased (14,15). This has sparked interest in biologists as this formulation has the potential to improve fish's immune systems. Phytochemicals containing active molecules such as flavonoids, steroids, alkaloids, saponins, terpenoids, tannins, glycosides, phenolic compounds, polysaccharides, pigments, organic acids, and essential volatile oils have been reported to exhibit growth-promoting, immunomodulatory activity, antibacterial, antiparasitic, antistress, appetite-stimulating in fish and shellfish that can enhance production performance and influence productivity in the aquaculture sector (13,14,16–19). Herbal or plant extracts mainly enhance the bactericidal effects, stimulate the phagocytic activity of cells, and support natural killer cell activity as well as improve the fish antibody responses (20). Several studies have monitored the immunological parameters after intraperitoneal injection or oral administration of plant extracts on distinct fish species and they found that treated fish showed increased haematological parameters, serum total protein (globulin and albumin), total immunoglobulin, myeloperoxidase activity, respiratory burst activity, bactericidal activity, complement activity, lysozyme activity, and phagocytic activity (21–24). Antioxidant and immune gene expression also get upregulated following herbal plant extract feeding in fish species, thereby protecting them from infectious pathogens (25–27). Enhancement of growth, antioxidative activity, haematological, nonspecific immune response, immune-related gene expression, disease resistance, and stress elimination has been reported in several fish species upon dietary supplementation of plant extract (25,28–30).

Tridax procumbens is a beneficial plant well-known for its bioactive components such as alkaloids, hydroxycinnamates, tannins, and phytosterols, which exhibit antioxidant, anti-inflammatory, anaesthetic, and anti-diabetic properties (31). A study revealed that *T. procumbens* incorporated diets enhanced serum protein profile, glucose levels, and bactericidal and lysozyme activity in *Nile tilapia* (32). The *T. procumbens* (63.3%) phytobiotics added to the *Nile tilapia* diet improved the feed efficiency, growth performance, immune response, and disease resistance against *A. hydrophila*. However, to the best of our knowledge, there is scant scientific evidence in favor of using *T. procumbens* in *L. rohita* as a dietary supplement. Therefore, our current investigation aimed to unravel the possible effects of regularly incorporating *T. procumbens* into *L. rohita* diets during their early life stages at doses T1: 2.0 g (0.20%); T2: 4 g (0.4%) and T3: 8 g (0.8%) extract kg $^{-1}$of basal feed, respectively, and its effect on growth, antioxidative activity, hematological, immune response, immune-related gene expression, and disease resistance.

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Materials and Methods

Methanolic extract of Tridax procumbens leaf extract

Extraction of TPLE was achieved following (33) with mere modifications. The plant taxonomy department at OUAT, Odisha, carefully identified fresh Tridax procumbens leaves collected at the ICAR-CIFA campus in Bhubaneswar, Odisha, ensuring the plant material's accuracy. The leaves were thoroughly washed with distilled water, followed by shade drying and occasional sun drying. After that, TPL samples were dried to constant weight at 70 °C. A mechanical grinder was used to grind the dried leaf materials into a fine powder, which was then kept for later usage at room temperature. After collecting the material, TPLE was crushed into a powder using an electric micro-pulverizer and then sieved through a 40-mesh sieve. After dissolving 50 g of dried sample in 500 mL of methanol, the samples were placed in a water bath at 37 °C for 48 hours. Following the completion of extraction in a rotary evaporator, the fine powder was labeled and kept for later use at 4 °C.

Fourier Transform Infrared (FTIR) characterization

The leaf extract of *Tridax procumbens* was characterized by FTIR (PerkinElmer, Spectrum II) using the central facility of OUAT, Bhubaneswar, India.

Gas chromatography-mass spectrometry (GC-MS) analysis

At the ICAR-National Rice Research Institute (ICAR-NRRI), Cuttack, India, a GC-MS apparatus (Shimadzu TQ8040, Shimadzu Corporation, Kyoto, Japan) with an Rxi-5-Sil MS (30 m × 0.25 mm, 0.25 µm) capillary column was used to evaluate the phytochemical composition of TP leaf extract. The TP leaf extract was diluted with methanol and then filtered by either direct injection into GC-MS or poly-tetrafluoroethylene. Utilizing helium as the carrier gas and keeping the GC injector temperature at 250ºC, the analysis was conducted in accordance with the phytochemical methodology (34). Each compound was identified by comparing the MS spectra from the reference library. Additionally, AMDIS software was employed for analyzing the data to determine the compounds, and KOV´ATS retention indices were computed to identify the phytochemicals based on peak area percentage and retention time, as represented in **Table 4**.

DPPH assay for in-vitro antioxidant activity

Using the method outlined by (35), the antioxidant capacity of tridax plant leaf extract was assessed by measuring the DPPH (1,1-diphenyl-2-picrylhydrazyl) free radical scavenging activity. In short, methanol was diluted with various amounts of Butyl Hydroxy Toluene (BHT) (10.0, 20.0, 30.0, 40.0, and 50.0 µg/ml). Further, 3.9 ml of a 0.2 mM DPPH solution and control were mixed with 0.1 ml of diluted leaf extract and BHT solutions.

Fish rearing and experimental conditions

The *L. rohita* fingerlings (n=180, average weight 12.24 g ± 0.5 g) were obtained from the carp culture unit, ICAR-CIFA, Bhubaneswar, India, without any prior history of infection or disease. With gentle aeration, the fish were acclimatized for three weeks in 500 L fiberglass reinforced plastics (FRP) tanks. Commercial feed was fed to the fish during this period, comprising 28 % protein at 2% body weight twice daily. After acclimatization, the healthy, energetic fish were randomly distributed into 12 independent FRP tanks with 400 L of fresh, aerated water that contained 15 fish per tank. Dietary interventions were created as follows: control (C): basic diet devoid of extract; T1: 2 g (0.20%) extract kg $^{-1}$ of basal feed; T2: 4 g (0.4%) extract kg $^{-1}$ of basal feed and T3: 8 g (0.8%) extract kg $^{-1}$of basal feed. The same amount of feed was added to each tank during the 60-day trial periods, and the ratio was divided into two equal daily portions (10:00 am and 4:00 pm). To maintain optimal water quality, the experiment was conducted in triplicate, with frequent exchanges of around thirty percent of the tank's water. Critical water quality indicators, including temperature, total alkalinity, dissolved oxygen, and total ammonia nitrogen, were measured and recorded at weekly intervals.

Herbal plant leaf collection and extract preparation

Fresh *Tridax procumbens* leaves were collected from ICAR-CIFA, Bhubaneswar campus, and meticulously identified by the plant taxonomy department of OUAT, Odisha, ensuring the accuracy of the plant material. To process the extraction, the leaves were carefully cleaned and dried in a 40 °C oven for 24 h. The leaves were mechanically grounded into tiny pieces and allowed to dry before being powdered. Subsequently, they were subjected to adding 50% methanol at a ratio of 1:10 (weight: volume). After 48 hours at room temperature and churning with a magnetic stirrer set at 120 rpm, the liquid was thoroughly mixed. Using an autoclaved funnel, the extract mixture was filtered using Whatman paper (No. 1, 42 µm pore size) to remove any minuscule particles. An oscillating evaporator with a 60 °C setting was used to extract the resulting alcohol from the filtered mixture (36). The powdered extract is stored at -4°C within an airtight glass container until further usage.

Preparation of experimental diet and feeding

Four experimental diets that were made with graded levels of leaf extract are described earlier. Inclusion levels were determined based on studies done on other fish (37). Feed ingredients that were readily available locally were used for the preparation of the basal feed **Table 1**. Ingredients

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were meticulously ground, weighed, mixed, and prepared as a dough, which was then used to prepare feed pellets. Before being fed to the fish, all manufactured pellets, including the various experimental and control feeds, were crushed to a 2-mm diameter, dried for the entire night, and then kept in storage at 4°C. The experimental and control groups were fed @ 3% of the fish's body weight for 60 days. Using the conventional protocol, the composition of the experimental diet was determined (38).

Table 1: The feed preparation (ingredients in g, used for 1 kg of feed) of the basal and experimental diet in rohu fingerlings a Composition of vitamin-mineral premix (PREEMIX PLUS) (quantity/2.5 kg).

Feed Ingredients (g)	Control (g kg^{-1} of feed)	0.2% TLE (g kg-1 of feed)	0.4% TLE (g kg-1 of feed)	0.8% TLE (g kg-1 of feed)
Fish meal	50	50	50	50
Soy meal	280	277.5	275	270
Ground nut oil cake (GNOC)	260	260	260	260
Rice bran	200	200	200	200
Corn flour	150	150	150	150
Oil mix (ml)	40	40	40	40
Vitamin and mineral mixture[a]	20	20	20	20
Extract (TPLE)	0	2	4	8

Vitamin A, 5500000 IU; Vitamin D3, 1100000 IU; Vitamin B2, 2000 mg; Vitamin E, 750 mg; Vitamin K, 1000 mg; Vitamin B6, 1000 mg; Vitamin B12, 6 mcg; Calcium Pantothenate, 2500 mg; Nicotinamide, 10 g; Choline Chloride, 150 g; Mn, 27,000 mg; I, 1000 mg; Fe, 7500 mg; Zn, 5000 mg; Cu, 2000 mg; Co, 450 mg; L- lysine, 10 g; DL- Methionine, 10 g; Selenium, 50 ppm; Satwari, 2500 mg.

Sampling of fish

Five fish were taken from each tank, randomly. After anesthetizing fish with 50 µl/L of clove oil (Himedia, India), samples of blood were extracted from the caudal vein by a 2 mL hypodermal sterile syringe. Collected blood was split into two microcentrifuge tubes, one of which had a thin film of the anticoagulant EDTA on it and the other without, so that the serum could be collected. Vials coated with EDTA were gently shaken to avoid hemolysis and blood coagulation. Serum was obtained and stored at a low temperature until required, following the procedure explained by (39). To get digestive tissues, a random sample of three fish was taken from each tank. The intestines of each fish were removed, and the adipose tissue was carefully cleaned. In addition, tissue samples were cleaned and homogenized using an electric blender at 8000 rpm for 30 s. Simultaneously, a cold solution of 50 mM tris-HCl with a pH of 8.1 was blended. To extract tissue fragments and lipids, the homogenate was centrifuged at 10,000 ×g (4 °C) for 20 min. The 1.5 mL Eppendorf vials containing the tissue extract supernatant were kept at −20 °C until the enzymatic analysis. In order to perform a whole-body proximate composition investigation **Table 2**, three extra fish were randomly picked from each tank, sliced, pooled, & frozen at -20 °C.

Growth indices

After the trial period of 60 days was over, four fish each from the three treatment groups and the control were taken out, and their growth indices were estimated as follows:

I. "Weight gain (%) (WGP) = (Final weight - Initial weight) X 100/ Initial weight,

II. Feed Conversion Ratio; FCR = Feed given (DW)/body weight gain (WW),

III. Feed Efficiency Rate; FER=1/FCR,

IV. Specific Growth Rate; SGR (%) = [ln (FW) − ln (IW)/N] × 100.

Where FW=final weight, IW=initial weight, DW=dry weight, WW=wet weight, ln=natural log, and N=number of culture days, respectively.

Estimation of non-specific immune parameters

The methods described by (40) were followed to estimate the serum lysozyme content. After an hour of blood collection, the respiratory burst activity (RBA) of the blood was determined in accordance with (41). The protocol described by (42) was followed to assess the serum myeloperoxidase (MPO) activity.

Estimation of biochemical parameters

Total serum albumin was assessed using the albumin kit (Merck, India) using the bromocresol green binding method as reported by (43)Total serum protein was estimated by following the manufacturer's instructions in the total protein kit (Merck, India). The serum albumin readings were subtracted from the total protein value to determine the serum globulin content. A commercial kit (Caymans Chemical, USA) was used to assess the antioxidant enzymes in the

Table 2. Proximate composition of diets. Data expressed as mean ± SE (n = 3)

Moisture (%)	Protein (%)	EE (%)	Ash (%)	Crude fibre (%)	NFE (%)	Energy (kcal/100 g)
8.42 ± 0.05	29.00 ± 0.03	7.89 ± .058	8.21 ± 1.13	4.98 ± 0.31	38.29 ± 1.00	421.72 ± 0.69

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serum, specifically the SOD and catalase activities following the manufacturer's instructions.

Blood and mucus sampling

The control and experimental animals were deprived for a full day on the 60th day of the experiment in order to collect samples of mucus and blood. 50 µg L^{-1} of clove oil was used to anesthetize five fish that were arbitrarily selected from each of the FRP tanks (5 fish from each of the replicas or 15 individuals from each treatment). Blood was drawn from every fish's caudal peduncle using a 2 mL disposable syringe. Blood was collected, and in order to get the serum, it was held for four hours at 4 °C for blood coagulation in a 1.5 mL microcentrifuge tube that had not been heparinized. Serum was extracted from the coagulated blood by centrifuging it at 2500 × g for 15 minutes at 4 °C. The serum samples from the treatment and control groups were stored at -80 °C prior to additional analysis (44). Furthermore, the technique described by (45) was followed to acquire skin mucus samples. Fish were placed in polyethylene bags with a 50 mM NaCl solution to collect mucus. Followed by gentle shaking of the fish inside the bags for a small duration to get mucus samples from the fish skin epidermal layer. The collected mucus samples were transferred to a sterilized 2 mL microcentrifuge tube, and centrifugation was done at 1300 rpm for 20 min in the refrigerated centrifuge, followed by the collection of supernatant and storage at – 80 °C in an ultra-freezer until further analysis (45).

Serum & mucus bactericidal activity test

A small modification to the (46) approach was followed to test the bactericidal activity of serum and mucus. After being incubated at 37 °C in a shaker incubator, the bacterial culture of *A. hydrophila* was obtained in a nutritional broth. The nutrient agar plate was then completely swabbed with 1.2 × 10^8 CFU mL^{-1} *A. hydrophila*. Following a 25-minute absorption period on 6 mm sterilized paper discs, 150 µl of serum and mucus samples were deposited on a solid agar plate (47). Plates were incubated at 37 °C for a single day. A digital caliper was used to assess the diameter of the growth inhibition zones after 24 h.

RNA extraction & cDNA synthesis

Following the manufacturer's directions, RNA was extracted from liver tissue samples from rohu using Thermo Fisher Scientific's TRIzol reagent. To get rid of any potential contamination from genomic DNA, isolated RNA was treated with DNase I (Takara, Japan). The quality and quantity of the extracted RNA were quantified using a NanoDrop ND1000 spectrophotometer (Thermo Scientific, USA). The oligo-dT primer was selected to synthesize the cDNA first-strand using the PrimeScript 1st strand cDNA Synthesis Kit (Takara, Japan).

Quantitative real-time PCR (RT-qPCR)

Using previously published primers, RT-qPCR was performed to examine the expression levels of genes related to pattern recognition molecule (TLR 22), pro-inflammatory cytokines (IL-1β, IL-8, TNF α), anti-inflammatory cytokines (IL-10), regulatory cytokines (TGF-β) and antioxidant molecules (SOD, GPx, catalase). Sigma-Aldrich provided the synthetic primers that were used in this process. **Table 3** is a list of the primers' specifications. The specificity of the primers was verified by semi-quantitative PCR. The qRT-PCR was performed using SYBR® Premix Ex TaqTM II (TliRNaseH Plus) (Takara Bio Inc., Japan) in the StepOnePlusTM Real-Time PCR system (Applied Biosystems, USA) in accordance with the manufacturer's instructions. In summary, a final 14 µl reaction mixture included 6.25 µl SYBR green master solution, 0.5 µl (10 µM) of each forward and reverse primer, 0.25 µL of ROX, and 2 µL of 100 ng cDNA. The qRT-PCR methodology included 40 cycles of amplification (denaturation for 10 s at 95 ◦C, annealing for 10 s at a predefined temperature, and extension for 10 s at 72 ◦C) after a preliminary denaturation at 95 ◦C for 10 min. Then, with cooling at 37 °C for 30 s, melt curve analysis was carried out at 95 °C for 15 s, 60 °C for 60 s, and 97 °C for 1 s. A triple control without a template was carried out for each gene. For each sample, the relative gene expression studies were performed in triplicate using β-actin as the reference gene. The $2^{-\Delta\Delta CT}$ approach was applied to ascertain the fold change in the experimental group (48).

Table 3: Information about primers used in the real-time gene expression analysis

Target Gene	Nucleotide base sequence (5′–3′)	Reference
β-actin	F- TTGGCAATGAGAGGTTCAGGT	-49
	R- TTGGCATACAGGTCCTTACGG	
TLR 22	F-TCACCCCATTTCGAGGCTAACAT	-50
	R-GAAGGCGTCGTACTGGAATGTC	
TNF α	F-CCA GGCTTTCACTTCAGG	-51
	R- GCCATAGGAATCGGAGTAG	
IL-1β	F- GTGACACTGACTGGAGGAA	-52
	R-AGTTTGGGCAAGGAAGA	
IL-8	F- AAAGGGTTCTTACTGG	-53
	R- TTTAGACATCTCGGACT	
IL-10	F- CTCATTTGTGGAGGGCTTTC	-54
	R- ATGCCAGATACTGCTCGATG	
TGF-β	F-ACGCTTTATTCCCAACCAAAC	-55
	R-GAAATCCTTGCTCTGCCTCA	
SOD	F-GTGGTTGTAATGTGTTCTGA	-56
	R- TCTGGAATGTTGTGAATTGG	
catalase	F-ACCTCTACAACGCCATCT	-56
	R- ATTCCACTTCCAGTTCTCAG	
GPx	F- TCAATGACATCAAGTGGAAC	
	R- ACAAGCTGACGGAAGTATT	-56

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Challenge Test

As previously described by (57), *A. hydrophila* obtained from the fish health and management division (FHMD), ICAR- CIFA, Bhubaneswar, India, was employed for experimental infection. Stock cultures were kept at −70°C in a Tryptic Soy Broth (TSB) solution with 15% glycerol. To prepare bacteria for the challenge test, *A. hydrophila* from stock was grown in TSB for 24 hours at 37 °C. The cells were centrifuged at 3,000 g for 15 minutes, and then they were cleaned three times using sterile phosphate-buffered saline (PBS, pH 7.2). The optical density of the bacterial suspension was determined to be around 10^8 CFU/mL at 540 nm. According to (58), six fish were randomly selected from each tank and eighteen from each treatment group, after 60 days of feeding with the experimental diets, and were intraperitoneally injected with *A. hydrophila* (10^8 CFU). The fish that had perished were taken out of the tanks after the challenge. The following formula (59) was used to calculate the relative percentage of survival (RPS) (%) = (no. of fish surviving after challenge/no. of fish injected with *A. hydrophila*) ×100.

Data analysis

The software SPSS ver. 14.0 (IBM, USA) was used to perform an ANOVA on the data in order to identify any significant differences between the means. Additionally, the data were examined for homogeneity of variance (Levene's test) and normality (Shapiro-Wilk test) (60). The average ± standard error (SE) is displayed for the data. $P < 0.05$ was found to be statistically significant.

Results

Infrared Characterization

The FTIR analysis exhibits absorbance peaks corresponding to various functional groups in the 4000 to 400 cm^{-1} range, as shown in **Fig 1**. The major bands in leaf extract were detected at 3293, 2931, 2206, 2171, 2103, 1995, 1683, 1628, 1607, 1515, 1445, 1366, 1270, 1183, 1165, 1102, 1071, 1042, 923, 898, 851, 813, 769, 504, 487, 452, 445, 426, 419 and 410 cm^{-1}. The strong and broad O-H stretch at 3293 cm^{-1} results from intramolecular hydrogen bonding in alcohols and phenols, while the band at 2931 cm^{-1} corresponds to C-H stretching vibrations. Additionally, alkynes (C≡C stretch) and nitriles (C≡N stretch) are characteristic of peaks at 2206 cm^{-1}, 2171 cm^{-1}, and 2103 cm^{-1}. Similarly, the peak at 1683 cm^{-1} reveals distinctive bands of carboxylic acids, whereas the peaks at 1628 cm^{-1},1607 cm^{-1}, and 1515 cm^{-1} represent amines observed in N-H bending. Furthermore, the C-H bending is represented by the peak in the fingerprint region at 1445 cm^{-1}, while C-O stretching and glycosidic linkage (C-O-C) are denoted by the peaks at 1183 cm-1, 1165 cm^{-1}, and 1102 cm^{-1}–1042 cm^{-1}, respectively. Multiple functional groups, commonly associated with methyl, phenol, carboxylic acid, alkynes, nitriles, polysaccharides, and amide groups, are detected in the leaf extract according to the FTIR data. These groups are components of different biomolecules. Thus, the *T. procumbens* leaf extract identified functional groups as characteristic of compounds like tannins, amides, flavonoids, amino acids, and polysaccharides.

GC-MS analysis of methanolic TP leaf extract

Table 4 lists the 30 main components that were identified by GC-MS profiling of methanolic TP leaf extract. **Table 4** lists the compound name, peak area, and peak area (%), retention time (min), and retention index (calculated and literature). The unique chromatogram of the leaf extract is displayed in **Fig. 2**. Methyl esters of hexadecanoic acid, 9-octadecenoic acid, γ-Sitosterol, Decane, and Phytol, among others, were significant constituents in the plant extract.

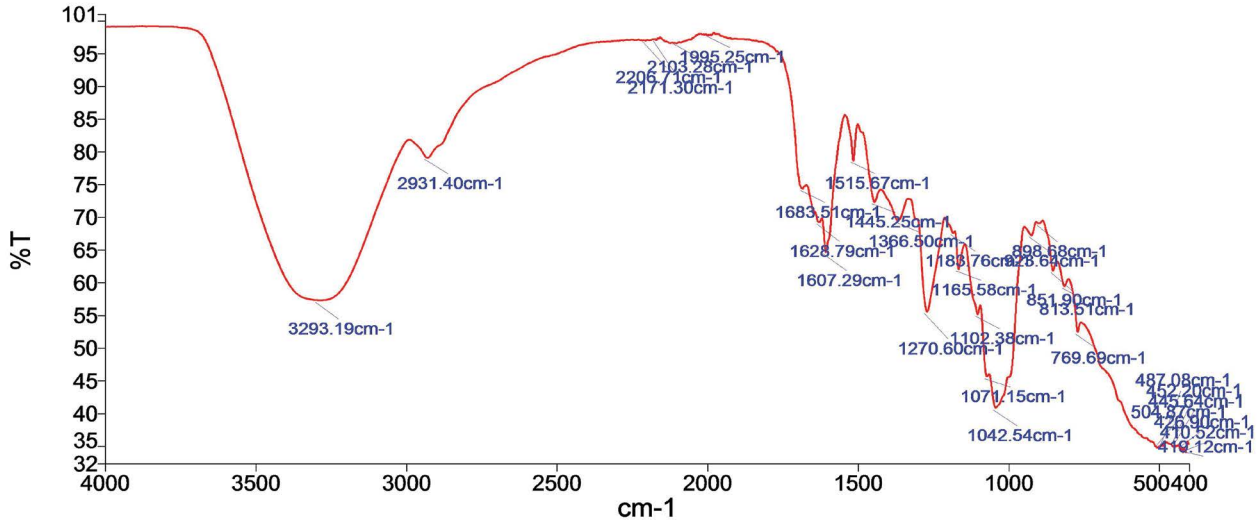

Figure 1: Fourier Transform Infrared (FTIR) spectrum of Tridax procumbens leaf extract

Citation: Shweta Priyadarshini Dash, Sasmita Mohanty, B Prince, Pushpa Choudhary, Chinmayee Muduli, Arabinda Das, Priyabrat Swain, Sudhansu Sekhar Mishra. Optimizing the Effects of Dietary *Tridax Procumbens* leaf Extract on Growth Performance, Antioxidant Activity, Immune Response, and Disease Resistance Against *Aeromonas Hydrophila* in *Labeo rohita*. Archives of Microbiology and Immunology. 9 (2025): 141-157.

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Table 4: Components identified in GC-MS analysis of Tridax extract

Sl.No.	Retention time (Min)	Compound	Area	% Area	Calculated RI	Lit. RI
1	3.435	N-Methoxy-N-methylacetamide	537354	3.47	-	696
2	3.925	Propanoic acid, 2-oxo-, ethyl ester	161668	1.04	820	822
3	5.425	2-Furanmethanol	46372	0.3	880	885
4	8.805	Decane	244932	1.58	997	1000
5	12.86	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	242112	1.56	1136	1154
6	15.375	Benzofuran, 2,3-dihydro-	102564	0.66	1223	1223
7	19.325	n-Decanoic acid	20267	0.13	1368	1372
8	28.38	2-Propenoic acid, 3-(4-hydroxyphenyl)-, methyl ester	187979	1.21	1752	1698
9	28.55	Tetradecanoic acid	19933	0.13	1760	1769
10	29.29	2-Cyclohexen-1-one, 4-hydroxy-3,5,5-trimethyl-4-(3-oxo-1-butenyl)-	115514	0.75	1794	1751
11	31.9	Hexadecanoic acid, methyl ester	254233	1.64	1924	1912
12	32.61	n-Hexadecanoic acid	252130	1.63	1961	1968
13	34.835	9,12-Octadecadienoic acid (Z,Z)-, methyl ester	134718	0.87	2093	2093
14	34.935	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	360604	2.33	2099	2101
15	35.09	Phytol	770371	4.97	2111	2105
16	35.27	Methyl stearate	36824	0.24	2125	2113
17	35.375	9,12-Octadecadienoic acid (Z,Z)-	140739	0.91	2133	2134
18	35.475	9,12,15-Octadecatrienoic acid, (Z,Z,Z)-	511589	3.3	2141	2143
19	35.73	Octadecanoic acid	56265	0.36	2161	2161
20	36.71	Benzyl beta-d-glucoside	367895	2.37	2243	2461
21	39.32	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	470030	3.03	2509	2498
22	40.675	1-Hexacosanol	48693	0.31	2676	2848
23	41.005	Octadecanoic acid, 2,3-dihydroxypropyl ester	339338	2.19	2718	2681
24	41.22	γ-Sitosterol	1942555	12.54	2745	2731
25	41.38	Stigmastanol	74030	0.48	2765	2720
26	41.545	Stigmasta-5,24(28)-dien-3-ol, (3-beta,24Z)	229553	1.48	2786	2780
27	41.9	(9Z,12Z,15Z)-3,7-Dimethyloct-6-en-1-yl octadeca-9,12,15-trienoate	371684	2.4	2828	2896
28	42.91	9,19-Cyclolanost-24-en-3-ol, (3-beta)-	348782	2.25	2934	2816
29	44.27	(+)-γ-Tocopherol, O-methyl-	587946	3.8	3053	3004
30	44.575	γ-Sitostenone	2315852	14.95	3077	3483

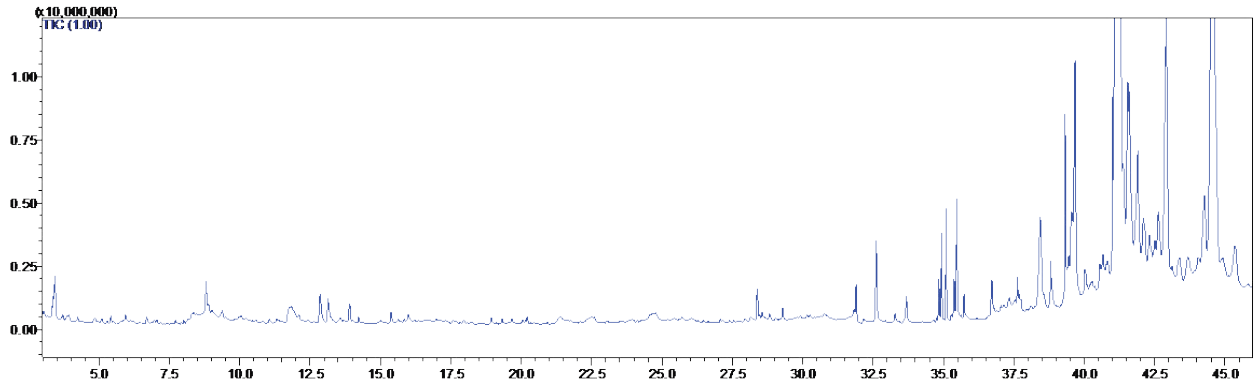

Figure 2: GC-MS Chromatogram of Tridax procumbens methanolic leaf extract

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DPPH free radical scavenging assay

Using butylated hydroxytoluene (BHT) as a reference, the *Tridax* leaf extract's in vitro antioxidant potential was assessed. Based on the calibration regression equation, it was determined that 100 µg/ml of leaf extract equates to 57.283 µg/ml of BHT activities as shown in **Fig 3**.

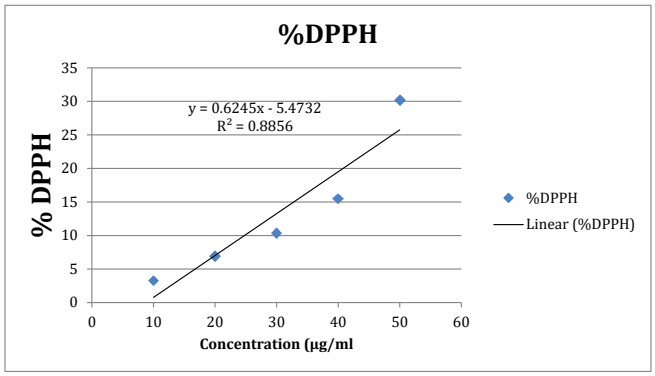

Figure 3: Calibration curve for determination of free radical scavenging ability of Tridax leaf extract by DPPH assay

Growth performance & feed utilization

The growth performance & feed utilization were analyzed upon feeding a selected concentration of *Tridax* leaf extract (TLE) to rohu. **Table 5** displays the rate of growth & feed consumption of *L. rohita* fingerlings. This study found a statistically significant difference in the FBW: final body weight, WG: weight gain, SGR: specific rate of growth, FCR: feed conversion ratio, & PER: protein efficiency ratio between the TLE0.2, TLE0.4, and TLE0.8 dietary & control groups ($P<0.05$). The ultimate body wt., WG, SGR, and PER of the fish-fed TLE0.8 and TLE0.4 groups were higher ($P<0.05$) than those of the control group. The group under control demonstrated reduced weight gain, SGR, and PER among the dietary categories. Fish fed TLE0.8 showed a reduced FCR, while fish in the control group showed a greater FCR.

Digestive enzyme

The changes in digestive enzyme levels were analysed upon feeding selected concentrations of different TLE to rohu. **Fig 4** displays the particular activities of α-amylase and total protease. Total protease & α-amylase activity in the gut of *L. rohita* fed with various TLE-supplemented meals showed a significant difference ($P<0.05$). Compared to the group under control, fish-fed with TLE showed increased activity of α-amylase & total protease, with fish-fed TLE 0.8 exhibiting the highest activity. Notably, the control group had significantly reduced levels of total protease and α-amylase activity.

Effects of T. procumbens leaf extract on non-specific immune parameters

The modulation of non-specific immune parameters in serum and mucus was analysed upon feeding selected different concentrations of TLE to rohu. **Table 6** shows the serum levels of MPO, lysozyme, & RBA in the treatment and control groups. The values of the fish-fed TLE0.4 and TLE0.8 diets were considerably higher ($P<0.05$) compared to those of the control. Mucus Lysozyme activity, & MPO at OD 450nm as represented in **Table 6.** It showed significantly higher differences in TLE0.8 and TLE0.4 diets when compared to the control group.

Effects of T. procumbens leaf extract on biochemical parameters

The changes in immune parameters, biochemical parameters, and antioxidant enzymes in serum and mucus were analysed upon feeding a selected concentration of TLE to rohu. Serum albumin, total protein (TP), and globulin (Ig) in the treatment and control groups are shown in **Table 7.** All three treatment groups, TLE0.2, TLE0.4, and TLE0.8 ($P<0.05$), showed significant differences from the control group. Serum antioxidant enzyme activity is shown in **Table 7** for catalase (CAT) and superoxide dismutase (SOD). The highest activities were observed in TLE0.4 &

Table 5: Effects of different doses of *Tridax procumbens* leaf extract on growth indices, feed utilization, and survival of rohu juveniles

Growth indices	Control	TLE0.2	TLE0.4	TLE0.8	P value
IBW (g fish^{-1})	18.17±0.60	18.03±1.03	17.83±1.18	19.13±0.31	0.719
FBW (g fish^{-1})	31.07±1.21^{a}	34.13±0.49^{b}	36.87±0.69^{b}	41.07±1.04^{c}	0
WG (g fish^{-1})	12.90±1.20^{a}	16.10±1.04^{ab}	19.03±0.82^{bc}	21.93±0.82^{c}	0.001
WG (%)	71.28±7.58^{a}	90.53±11.32^{ab}	108.21±11.39^{b}	114.60±3.46^{b}	0.037
SGR (%g day^{-1})	0.89±0.07^{a}	1.07±0.09^{ab}	1.22±0.09^{b}	1.27±0.03^{b}	0.033
FCR	2.59±0.20^{b}	2.05±0.15^{ab}	1.7±0.12^{a}	1.57±0.03^{a}	0.035
PER	1.45±0.09^{a}	1.8±0.23^{ab}	2.15±0.33^{b}	2.47±0.34^{b}	0.038
Survival (%)	100	100	100	100	-

Values presented as Mean ± SE (n=3); Mean values in the same row with different superscripts differ significantly ($P<0.05$). IBW: initial body weight, FBW: final body weight, WG: weight gain, SGR: specific rate of growth, FCR: feed conversion ratio, & PER: protein efficiency ratio

Citation: Shweta Priyadarshini Dash, Sasmita Mohanty, B Prince, Pushpa Choudhary, Chinmayee Muduli, Arabinda Das, Priyabrat Swain, Sudhansu Sekhar Mishra. Optimizing the Effects of Dietary *Tridax Procumbens* leaf Extract on Growth Performance, Antioxidant Activity, Immune Response, and Disease Resistance Against *Aeromonas Hydrophila* in *Labeo rohita*. Archives of Microbiology and Immunology. 9 (2025): 141-157.

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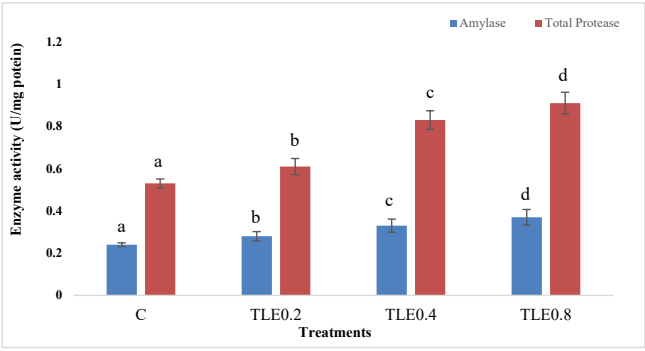

Figure 4: The specific activity of amylase and protease in rohu fed with a graded level of Tridax leaf extract (TLE). Bars presented as Mean ± SE. Different letters above the bar denote significant differences between dietary groups (P<0.05)

TLE0.8 (P<0.05) compared to the control group. TLE0.8 had significantly lower serum glucose activity (P<0.05) than the control group **(Table 7)**. Mucus TP showed a significant difference, as shown in **Table 7.** In all three treatment groups, TPL0.4, TLE0.8, and TLE0.2 (P<0.05) from the control group. Mucus Alkaline phosphatase (ALP) and protease activity showed higher values in fish fed TLE0.8, followed by TLE0.4 and TLE0.2, in comparison to the group under control, as represented in **Table 7.**

Mucus & Serum Bactericidal Activity

The bactericidal activity of serum and mucus was studied upon feeding selected different concentrations of TLE in rohu. It was observed that the fish fed with TLE0.2, TLE0.4, and TLE0.8 diets had the highest zone of inhibition in their serum and mucus samples compared to the group under control, with the control showing the lowest values (P<0.05). A larger zone of inhibition indicates that fish fed with TLE diets are more potent than the control fish group. TLE0.4, followed by TLE0.8 and 0.2, have higher mucus bactericidal activity than the control group (P<0.05) as presented in **Fig 5**. Additionally, Fish fed with TLE0.4, TLE0.8, and TLE0.2 diets at 1.65, 1.52, and 1.27, respectively, had the highest serum level of bactericidal activity. Similarly, the highest mucus bactericidal activity was seen in fish fed with TLE0.4, TLE0.8, and TLE0.2 diets with growth inhibition zone diameters of 2.52, 2.40, and 1.85nm, respectively.

Effects of T. procumbens leaf extract on expression profiles of innate immune and antioxidant genes

The expression of pattern recognition molecule (TLR 22), pro-inflammatory cytokines (IL-1β, IL-8, and TNF α), anti-inflammatory cytokines (IL-10), regulatory cytokines (TGF-β), and antioxidant molecules (SOD, GPx, and catalase) were studied upon feeding different concentration of TLE. The TLE0.8 significantly (P < 0.05) raised the expression of TLR 22, IL-1β, TNF α, SOD, GPx, and catalase as compared to the control. In contrast, gene expression of IL-8, IL-10, and TGF-β was down-regulated. As illustrated in **Fig 6A, B, and 7C**, the addition of TLE0.2, TLE0.4, and TLE0.8 significantly elevated the expression of proinflammatory cytokines and antioxidant gene expression in comparison to the control. However, the expression of some pro- (IL-8), anti- (IL-10), and regulatory (TGF-β) cytokines was downregulated.

Challenge Study

Post-challenge survival (%) of *L. rohita* challenged with *Aeromonas hydrophila* after feeding on TLE diets was observed for 60 days. The RPS rates of the treatment groups are shown in **Fig 7**. Different fish fed with TLE supplementation had a substantial impact on RPS rates. The fish-fed TLE0.8, TLE0.4, and TLE0.2 diet groups had considerably higher RPS rates (P<0.05) compared to the control group. The fish-fed TLE0.8 and TLE0.4 diet groups showed significantly greater RPS rates compared to the TLE0.2 group (P<0.05). The TLE0.8 diet group had the greatest RPS (83.30%), followed by the TLE0.4 (77.73%), TLE0.2 (61.06%), and the control group (27.73%) (P<0.05).

Table 6: Effects of different doses of *Tridax procumbens* leaf extract on serum immune parameters, antioxidants, and stress parameters of rohu juveniles. Data presented as Mean ±SE Different letters above the value denote significant differences between dietary groups (P<0.05).

Parameters	C	TLE 0.20	TLE 0.40	TLE 0.80	P value
RBA (OD 540)	0.26 ± 0.015^{a}	0.27± 0.005^{a}	0.31 ± 0.006^{b}	0.32 ± 0.011^{c}	0.004
MPO (OD 450)	0.24± 0.001^{a}	0.26± 0.007^{b}	0.30 ± 0.005^{c}	0.32 ±0.003^{cd}	0
Lysozyme	61.4 ± 3.2^{ab}	62 ± 0.88^{b}	65.3 ± 1.8^{b}	66.8 ± 1.3^{b}	0.033
Ig (mg/dl)	3.63 ± 0.07^{a}	3.65 ± 0.072^{a}	3.82 ± 0.21^{b}	3.7 ± 0.2^{a}	0.027
TP (mg/dl)	2.72 ± 0.02^{a}	2.75 ± 0.01^{ab}	2.8 ± 0.02^{bc}	2.85 ± 0.03^{c}	0.015
Albumin (mg/dl)	1.28 ± 0.02^{a}	1.36 ± 0.01^{ab}	1.45 ± 0.02^{b}	1.37 ± 0.03^{ab}	0.036
SOD (U/ml)	77.33 ± 1.2^{a}	81.33 ± 3.2^{ab}	85.66 ± 3.4^{ab}	88.9 ± 4.4^{b}	0.072
CAT (U/ml)	26.66 ± 3.2^{a}	31.33 ± 1.4^{ab}	38.33 ± 2.2^{b}	37.5 ± 2.2^{ab}	0.031
Glucose(mg/dl)	115.6 ± 2.9^{b}	104.0 ± 8.5^{b}	101.3 ± 3.2^{ab}	85.7 ± 4.7^{a}	0.025

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Table 7: Effects of different doses of *Tridax procumbens* leaf extract on mucus immune parameters of rohu juveniles. Data presented as Mean ±SE Different letters above the value denote significant differences between dietary groups (P<0.05).

Parameters	C	TLE 0.20	TLE 0.40	TLE 0.80	P value
Lysozyme	44.70±1.46^{a}	48.63±2.0^{ab}	52.63±1.51^{bc}	54.78±1.32^{c}	0.009
MPO (OD 450)	0.27±0.008^{a}	0.34±0.012^{b}	0.38±0.003^{c}	0.40±0.008^{c}	0
TP (mg/dl)	2.38±0.035^{a}	2.55±0.024^{b}	2.71±0.04^{c}	2.82±0.018^{d}	0
ALP (IU/ml)	93.0±3.08^{a}	98.11±3.17^{ab}	102.10±2.42^{b}	103.47±3.52^{b}	0.013
Protease %	2.18±0.17^{a}	2.32±0.13^{a}	2.54±0.049^{ab}	2.79±0.079^{c}	0.024

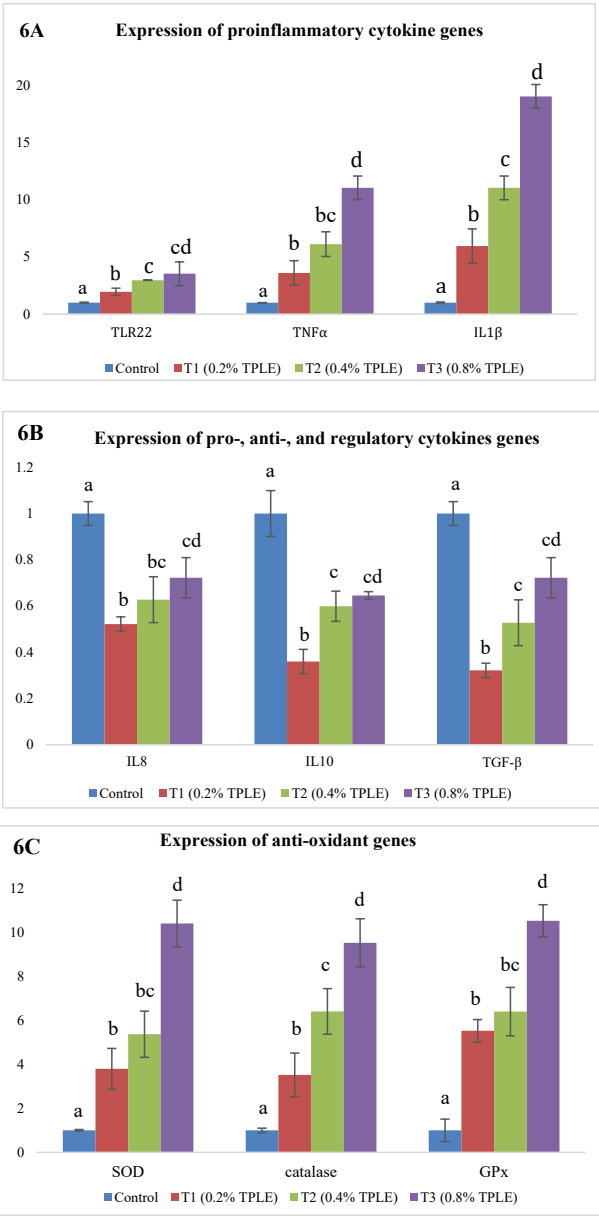

Figure 6 A, B, C: The expression modulation of pattern recognition molecule (TLR 22), pro-inflammatory (IL-1β, IL-8, TNFα), anti-inflammatory (IL-10), regulatory cytokine (TGF-β) and antioxidant molecules (SOD, GPx, catalase) in rohu fed with graded level of Tridax leaf extract (TLE). The bar is displayed as Mean ± SE for the three treatment and control groups.

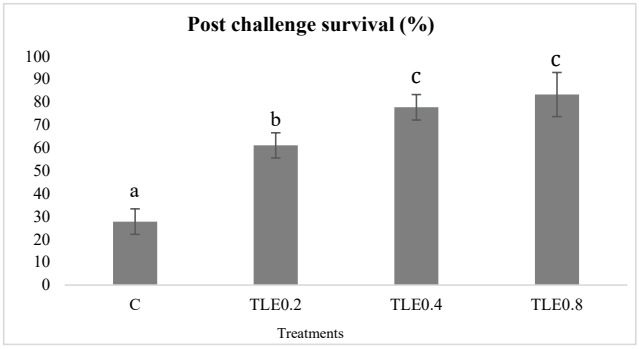

Figure 7: Post-challenge survival (%) of *L. rohita* challenged with *Aeromonas hydrophila* after feeding on Tridax leaf extract (TLE) diets. Significant differences between the dietary groups are denoted by different letters above the bar (P<0.05)

Discussion

Recent phytobiotic interventions in aquaculture have garnered interest as an environmentally friendly health management method and are being explored to promote fish growth, and immune responses, as well as to safeguard them against pathogenic ailments and stress (61–63). The present study describes the growth, immune, and antioxidant response-promoting effects of *T. procumbens* leaf extract (TLE) in *L. rohita,* revealing a more pronounced effect in the fish fed with TLE0.4 and TLE0.8 diet groups and significantly lower in the control group. It was observed that fish fed with 0.4% TPLE showed significantly lower body weight and Specific growth rate (SGR) % than the 0.8% TPLE group, but higher than the control group. This outcome could be a result of improved digestibility and feed intake, which would stimulate growth. Previous research has found that dietary supplementation with phytobiotics improves metabolism while also having antibacterial and anti-inflammatory properties (64). Our results are congruent with those of (65) African catfish were fed with varying levels of *T. procumbens* (TP) leaf meal (0.5, 1.0, 1.5, and 2.0 g/kg diet) for 56 days. They determined that performance deteriorated after reaching an elevated level at 1.0 g TP/kg diet. In a separate study (66) *Nile tilapia-fed* groups containing *T. procumbens* (TP) leaves at high doses (214.7, 322.1, 429.5, and 536.7 g/kg diet) for ten weeks revealed that the fish could sustain TP inclusion levels of up to 20% before slowing down their growth

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rate. The significant concentration of anti-growth and anti-nutritional components in the TP leaves diet may account for the reduced growth. However, the *T. procumbens* employed in our study was leaf extract rather than leaf powder, and its inclusion levels in meals were minimal, which has resulted in enhanced growth, demonstrating that low anti-growth and anti-nutritional ingredients.

A physicochemical analytical technique called Fourier transform infrared spectroscopy presents a precise representation of the metabolic makeup of leaves at a fixed period. It is considered one of the most effective methods for determining the functional groups that are present in compounds and for having a better understanding of their phytochemical properties (18,67). Thus, the purpose of the present investigation was to evaluate the effect on growth, immunity, and antioxidant activity when tridax extract is incorporated into feed. The extract's FTIR measurements revealed the presence of several functional groups, including methyl, phenol, carboxylic acid, alkynes, nitriles, polysaccharides, and amide groups. These compounds may be related to bioactive compounds like tannins and flavonoids, which play crucial roles in antioxidant activity (68,69), growth (70,71), and immunity (24,72). In our present investigation, the dietary inclusion of leaf extract may have contributed to the *Labeo rohita's* increased antioxidant activity and immunomodulation. According to the DPPH Assay, the leaf extract's antioxidant capacity was 57.283 µg/ml of equivalent BHT activities, which varies greatly among plants based on their source and growth conditions (73,74).

The present study evaluated the non-specific immunological parameters of *L. rohita*. The RBA, MPO, and lysozyme are crucial innate immunological metrics used to evaluate the innate immunity of fish (75,76). According to several studies, fish with diets including plant extracts or powder had higher lysozyme and RBA indices than control (21–25,27–30,70,77) and all the variables have aided in defending against the invasion of pathogens. Similarly, our findings observed a significantly higher RBA, MPO, and lysozyme activity in the fish fed with the TLE0.4 and TLE0.8 diet when compared with the control. The activation of RBA and MPO results in the synthesis of oxidants, which lead to the oxidation of biomolecules, subsequently promoting inflammation and oxidative stress. Serum lysozyme is an essential antibacterial effector molecule that stimulates phagocytes and the complement system. Elevation of lysozyme level signifies the existence of many humoral components that can protect the host against the introduction of pathogens (22). According to GC-MS profiling, the presence of methyl esters of hexadecanoic acid and 9-octadecenoic acid appears to be linked to the enhanced lysozyme activity in our investigation. This suggests that the TLE0.4 and TLE0.8 diets have the capacity to boost the immune response and provide protection from several diseases in rohu. Furthermore, dietary supplementation with TPLE has a significant impact on the biochemical properties. The present study evaluated biochemical indices involving total protein with other critical indices such as AST, ALP, ALT, SOD, CAT, and glucose. According to (78), blood glucose levels are regarded as indicators of stress reactions since they are produced as secondary responses during stress to comply with enhanced energy requirements. Based on the current investigation, the fish fed with phytobiotics had lower glucose levels than the control group. This suggested that adding TPLE to *L. rohita*'s diet provided additional stress reduction benefits. The TLE0.4 or 0.8-fed group showed significantly higher levels ($P > 0.05$) of total protein, albumin, ALP, and SOD compared to the control group. However, previous data (79,80) reported that feeding yellow perch and *Nile tilapia* 10 g/kg licorice showed reduced AST, ALT, and ALP activity when compared to the control group. The effects may be attributed to several TPLE bioactive components and secondary metabolites possessing antioxidant characteristics (81).

TPLE supplementation also had a significant impact on skin mucus immunological parameters in addition to serum immune parameters. Mucus on fish skin serves as the initial line of defense against pathogen invasion and is protected from infections by a thin physical, chemical, and biological barrier called the skin mucus layer, which also contains immune system components such as lectins, complement, immunoglobulins, and lysozyme (82,83) whereas Ig functions as an immune effector molecule in fish blood and fish skin, lysozyme is critical for mediating protection against microbial invasion (84) The most widely distributed immunoglobulin, IgM is necessary for systemic immune responses (85). In this study, the TLE0.8 diet-fed group had significantly higher serum and mucosal IgM and lysozyme activity. Similar to our findings, in zebrafish (86) and *C. carpio* (87,88) upon dietary supplementation of 0.8% ginger, 20g kg−1 of myrtle (Myrtus communis), and 200 mL/kg of DPFE stimulated the mucosal immune response. Seabream's skin mucosal IgM levels were raised by dietary fenugreek seeds (89). However, fenugreek seeds did not significantly affect the total Ig level in common carp skin mucus (90). Moreover, fish health can be precisely and noninvasively monitored by measuring changes in mucus protein levels. In the present study, mucosal lysozyme activity was considerably higher in the TLE0.4 and TLE0.8 fed groups than in the controls, with TLE0.8 being the most active. Furthermore, mucosal protein levels were higher in TLE0.4 and TLE0.8 than in the control ($P < 0.05$), with TLE0.8 having the highest level. Although the difference was only significant in TLE0.8, there was a higher level of protease activity in the TLE0.4-fed groups compared to the control group.

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In order to reveal the potential mechanism of immune stimulation following TPLE feeding in rohu, the expression of toll-like receptors, pro-, anti-, and regulatory cytokines, and anti-oxidant molecules was evaluated. In this study, the expression of pro-inflammatory cytokines (IL-1β, TNFα), antioxidant molecules (SOD, GPx, catalase), and pattern recognition molecules (TLR 22) was all significantly upregulated by the TLE 0.2, 0.4, and 0.8 supplemented diet with TLE0.8 having the highest level. Contradictorily, the expression of some pro- (IL-8), anti- (IL-10), and regulatory- (TGF-β) cytokines was downregulated compared to the control group, with TLE0.2 having the lowest level. In order to reduce oxidative stress in fish, GPx, SOD, and catalase enzymes are essential. These enzymes contribute to the enhancement of reactive oxygen species imbalance and the preservation of normal redox equilibrium in biological systems (81). Previous studies have demonstrated the modulation of immune and antioxidant genes upon supplementation of herbal ingredients in fish diets (12,25,28–30,77). In agreement with the previous studies, our results demonstrated that fish fed with a TLE0.8 diet increased the expression of antioxidant molecules (SOD, GPx, and catalase) which suggests that incorporating the TLE0.8 in to fish diet can lessen oxidative damage. The presence of phenolic substances such as tannins, phenol-carboxylic acids, and flavonoids may be linked to the improved antioxidant status in the rohu, as evidenced by the study's enhanced SOD activity in TLE0.8 ($P < 0.05$) (33,91) and can also be related to the FTIR results of our research. It has been reported that TPLE is rich in flavonoids, sterols, polysaccharides, phenolic content, and tannins (92). Therefore, based on in-vitro DPPH activity, tridax methanolic leaf extract can be regarded as a good antioxidant that has enhanced the SOD antioxidant enzyme status in rohu through diet.

Fish-specific TLR22 plays a crucial role in mucosal and systemic defense against bacterial infections (93,94). Elevation of TLR22 in rohu upon TPLE feeding indicates the immunostimulatory activity of TPLE. Earlier studies reported that *Astragalus* treatment elevated the production of pro-inflammatory cytokines TNF-α and IL-1β in common carp and yellow catfish (28). Similar to our study, feeding guava leaves downregulated the expression of IL-10 and TGF-β (93). Feeding of olive leaf (*Olea europea* L.) extract downregulated the IL-8 expression (95). Further, another study reported the immunomodulatory characteristics of the ethanol-insoluble fraction of *Tridax procumbens,* which also revealed that its incorporation can improve growth, enhance immunity, antioxidant status, and resistance in the *Nile tilapia*, *Oreochromis niloticus* (96). These findings suggest that TPLE could be used to boost immunity and promote disease resistance in *Labeo rohita*. Mortality due to bacterial infection is an integral barrier to aquaculture, resulting in larger economic losses. The post-challenge survival rate may determine host health and evaluate the potency of immunostimulants like phytobiotics (58). The present study corroborates the research outcomes of *Nile tilapia,* indicating that the addition of *T. procumbens* enhances the antioxidant, immunity, & resistance (65). Further, tilapia fed with the TPLE diet exhibited a 10% reduction in fish mortality when compared to the control group (65). In our current investigation, RPS rates were significantly greater in the TLE diet-fed groups in the order of TLE0.8, followed by TLE0.4, and TLE0.2. Thus, TPLE administration may have improved immunological responses in challenged fish, leading to higher survival rates.

Conclusions

The present study reinforces the prospect for the functional feed additive application of *T. procumbens* leaves extract (TPLE) that can help strengthen the immune system and effectively enhance *L. rohita's* capacity for growth, immunological responses, & disease resistance, with the RPS rate being highest in the TLE0.4- 0.8 diet group when challenged with *Aeromonas hydrophila,* indicating no detrimental effects on fish welfare.

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CRediT authorship contribution statement

Shweta Priyadarshini Dash: writing of the original draft manuscript and investigation.

Pushpa Choudhary: feed preparation and feed data analysis, investigation, formal analysis, and supervision.

Sasmita Mohanty: investigation.

B. Prince: Writing- review & editing

Chinmayee Muduli: formal analysis and final manuscript draft preparation.

Arabinda Das: formal analysis.

Priyabrat Swain and **Sudhansu Sekhar Mishra:** visualization, resource acquisition, and supervision. All the authors have given consent for the final submission to the journal.

Data availability

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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