

IN VITRO ANTIBACTERIAL ACTIVITY AND IN VIVO EVALUATION OF TERMINALIA CHEBULA-SUPPLEMENTED FUNCTIONAL FEED AGAINST AEROMONAS HYDROPHILA IN CHANNA STRIATA

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ABSTRACT

Aquaculture intensification has increased interest in plant-derived feed additives as potential alternatives or complements to conventional approaches for managing bacterial diseases. This study evaluated a functional feed containing 2% methanolic Terminalia chebula fruit extract in juvenile Channa striata exposed to Aeromonas hydrophila. The formulated floating pellets had a mean diameter of 1.37 ± 0.21 mm, floating ability of 100%, water stability of $76.87 \pm 0.12\%$, pellet durability of $99.33 \pm 0.01\%$, and bulk density of 0.4393 ± 0.0003 g cm⁻³. Proximate analysis indicated 33.48% crude protein, 2.57% crude lipid, 6.37% crude fibre, 4.78% ash, and 10.64% moisture. Supporting in vitro assays demonstrated antibacterial activity against A. hydrophila following thermal exposure at 40, 60, and 80°C for 1 h and at 95°C for 1.5 h. Fish received the experimental diets for 20 days before bacterial challenge. The available growth summary descriptively favoured the methanolic-extract feed group; however, original fish-level and replicate-level growth records were unavailable for independent verification, so these indices require confirmation against primary records. Following bacterial challenge, mean mortality was 23.3% in the methanolic-extract feed group and 43.3% in the challenged positive control; mortality was also recorded in the unchallenged control. PCR detected the reported target in the bacterial isolate and yielded amplification bands in post-challenge tissue samples, although the target of the tissue assay requires confirmation from the original laboratory records. Overall, the findings characterise the physical and nutritional properties of the formulated feed and provide preliminary evidence of in vitro antibacterial activity and observed post-challenge responses. Further studies incorporating quantitative pathogen-load assessment and host-response analyses are warranted to establish the protective efficacy of T. chebula -supplemented functional feed.

KEYWORDS: Aeromonas hydrophila, aerA, Channa striata, Functional feed, Molecular confirmation, Terminalia chebula.

1. INTRODUCTION

Aquaculture production has increased rapidly, but illness remains a serious constraint, as bacterial outbreaks can cause mortality, growth restriction, and economic losses. Aeromonas hydrophila is a widespread freshwater bacterium that causes motile aeromonad septicemia and various fish illness syndromes. Its pathogenicity is multifaceted, involving aerolysins, hemolysins, proteases, adhesins, secretion systems, and other strain-specific virulence factors (Janda and Abbott, 2010; Rasmussen-Ivey et al., 2016). Antibiotic medication has historically been used to control bacterial infections, but the selection and spread of antibiotic resistance, as well as concerns about residues, have sparked an interest in preventive and complementary therapies. Current aquaculture strategies include better husbandry and biosecurity, immunization, probiotics, prebiotics, bacteriophages, and plant-based products (Bondad-Reantaso et al., 2023). Phytochemicals are particularly interesting because plant secondary metabolites can have antibacterial, antioxidant, and other biological properties, albeit responses are highly dependent on plant species, extract composition, dose, and delivery mode (Nek Rahimi et al., 2022; Wang et al., 2024).

Plant-derived products have received increasing attention as potential sources of bioactive compounds for aquaculture applications. Their biological activity may vary according to plant composition, extraction solvent, concentration and processing conditions. Terminalia chebula is a medicinal plant containing phenolic compounds, tannins and other phytochemicals of potential biological interest. Nevertheless, the identification of phytochemicals alone does not establish their contribution to antibacterial activity. Combining phytochemical characterisation with in vitro antibacterial testing provides an initial approach for assessing plant extracts against fish-associated bacterial pathogens. Agar diffusion assays are useful for comparing antimicrobial activity under defined laboratory conditions, although inhibition-zone measurements should not be interpreted as direct evidence of therapeutic efficacy in living fish (Balouiri et al., 2016).

The incorporation of plant extracts into functional feed offers a practical approach for delivering phytogetic materials through routine feeding. However, the suitability of such a feed depends on both the biological properties of the incorporated extract and the physical characteristics of the final pellets. Pellet size, buoyancy, durability and water stability influence feed handling and the retention of feed material in aquatic environments (Obaldo et al., 2002; Tacon, 1993). Processing conditions are also important because thermal exposure may alter plant-derived compounds and their biological activity. Therefore, evaluating antibacterial activity after heat treatment, alongside the physical and nutritional characteristics of the formulated feed, can provide useful information about its suitability for aquaculture applications.

Although in vitro antibacterial activity can support the selection of promising phytogetic materials, it cannot independently establish their effectiveness in fish. Experimental evaluation should also consider growth performance, clinical observations and mortality following pathogen exposure. Molecular detection of *A. hydrophila*, including PCR targeting the *aerA* gene associated with aerolysin, can provide additional evidence of bacterial presence. However, detection of this virulence-associated gene does not quantify bacterial load or independently establish the cause of mortality (Janda & Abbott, 2010; Rasmussen-Ivey et al., 2016). Integrating microbiological, feed-quality and fish-response assessments is therefore important when investigating the potential application of plant-derived functional feeds.

The present study evaluated a functional feed containing 2% methanolic *T. chebula* fruit extract in juvenile *Channa striata* experimentally exposed to *A. hydrophila*. The investigation included feed characterisation, evaluation of the extract's antibacterial activity following thermal exposure, monitoring of growth performance, recording of post-challenge clinical responses and mortality, and molecular detection of *A. hydrophila*. The study aimed to characterise the physical suitability of the prepared feed and document the growth and post-challenge responses associated with its use. By integrating these assessments, the study provides an experimental evaluation of *T. chebula* -supplemented functional feed while recognising that observed fish responses may also be influenced by environmental conditions and other factors unrelated to bacterial infection.

MATERIAL AND METHODS

Bacterial isolate and phenotypic identification

The collaborating microbiology facility at the Rajiv Gandhi Center for Aquaculture, Sirkali, Tamil Nadu, India, provided the *Aeromonas hydrophila* isolate used for the supporting in vitro assays. Form, color, margin, and elevation of the colonies were all noted. Hemolysis was seen on blood agar, and cellular Gram reaction and morphology were evaluated using Gram staining. Tests for catalase, oxidase, indole, methyl red, Voges-Proskauer, citrate utilization, urease, triple sugar iron (TSI), nitrate reduction, arginine dihydrolase, ornithine decarboxylase, and lysine decarboxylase were used to evaluate the biochemical profile. Standard bacteriological references were used to interpret the identification (Holt et al., 1994; Janda and Abbott, 2010).

Antibiotic susceptibility testing

A disc-diffusion method based on the standardized procedure outlined by Bauer et al. (1966) was used to evaluate antibiotic susceptibility. Ceftazidime, 21 mm; co-trimoxazole, 22 mm; enrofloxacin, 34 mm; doxycycline, 24 mm; ciprofloxacin, 34 mm; ampicillin, 0 mm; oxytetracycline, 24 mm; erythromycin, 15 mm; imipenem, 16 mm; meropenem, 28 mm; cefotaxime, 27 mm; sulphadiazine, 24 mm; azithromycin, 27 mm; and furazolidone, 24 mm. The measured diameters are reported directly because interpretive breakpoints depend on the organism, drug, and testing standard; categorical susceptibility labels should only be kept in cases where the original laboratory interpretation and relevant breakpoint standard are verified.

PCR confirmation of the bacterial isolate

The bacterial isolate was subjected to PCR analysis targeting the *aerA* gene, which encodes aerolysin, for molecular confirmation. The PCR result was assessed by agarose gel electrophoresis to determine the presence of the expected amplification product. The source manuscript reported an amplicon of approximately 309 bp for the isolate, with no amplification in the negative control. The original primer sequences, PCR reaction mixture, thermocycling conditions, and DNA polymerase details were not available in the source manuscript and have therefore not been specified.

Preparation of Terminalia chebula methanolic extract

Mature dried fruits of *Terminalia chebula* were cleaned and pulverized. The source laboratory record describes methanolic extraction at a plant-powder-to-solvent ratio of 1:10 (w/v), using 10 g powdered material with 100 mL methanol. After filtration, the filtrate was concentrated in a hot-air oven at 40°C for approximately three days, yielding about 3.4 mL of concentrated crude extract. The same extract was used for in vitro antibacterial screening and functional-feed preparation. Extraction methods and their effects on recovery can vary with solvent and processing conditions (Azwanida, 2015).

Qualitative phytochemical screening

Using standard color or precipitation reactions, the methanolic extract was qualitatively screened for alkaloids, terpenoids, sugars, proteins, phenols/tannins, steroids, flavonoids, and quinones. The initial laboratory record indicated that reactions were either absent (–), present (+), or strongly present (++). The screening process adhered to the general phytochemical methods outlined by Sofowora (2008) and Harborne (1998). These qualitative tests do not determine the identity or concentration of specific compounds; instead, they show general chemical classes.

Concentration-dependent antibacterial assay

An agar-well diffusion method was employed in the previous in vitro antibacterial evaluation against *A. hydrophila*. After spreading the bacterial suspension on Mueller-Hinton agar, 50, 75, 100, and 125 mg/mL of methanolic *T. chebula* extract were added. The solvent control was methanol. Inhibition-zone diameters were measured in millimeters after incubation. The mean $\pm$ SD from three replicates was used to report the source results. Agar diffusion is not the same as determining the minimum inhibitory concentration; instead, it offers comparative inhibition-zone measurements (Balouiri et al., 2016).

Previously established GC–MS profile

The GC–MS data used in this work were derived from an earlier analysis of the same methanolic *T. chebula* extract. This was not a new analysis from the fish challenge experiment, but supporting chemical context. The previous study used mass-spectral library matching to describe 78 chromatographic components (Snehan et al., 2026). 1,2,3-benzenetriol, methylparaben, 5-hydroxymethylfurfural, 4-hydroxybenzoic acid, hentriacontane, methyl gallate, 3-hydroxybenzoic acid, and (24R)-stigmast-5-en-3 β -ol were among the representative reported matches. Library matches are preliminary identifications; they do not prove compound identity or establish which compound is responsible for antibacterial activity.

Experimental fish and acclimatization

On August 20, 2026, 130 young striped murels (*Channa striata*, Bloch, 1793) were purchased from Raghu Fish Farms in Walajapet, Tamil Nadu, India, and transferred alive to the Gill Research Institute at Guru Nanak College in Chennai. Fish were observed for swimming activity, eating habits, injuries, and mortality while they were acclimated to lab settings. Ten fish were lost during acclimation and were not included in estimations of treatment mortality that followed. Fishes were divided into four feeding groups with three experimental replicates according to their length and duration after acclimation. To enable individual monitoring and lessen hostile encounters, R3 fishes were kept in separate 2.5-L boxes with a 2 L working water volume, whereas R1 and R2 fishes were kept in 13-L rearing tanks with a 10 L working water volume.

Experimental design and feeding trial

After acclimatization, 120 fish were allocated to four groups with three replicates per group (10 fish per replicate): methanolic-extract feed (MEF), raw *T. chebula* plant-powder feed (RPF), challenged positive control receiving basal feed (PC), and unchallenged negative control receiving basal feed (NC). The experimental diets were administered for 20 days before bacterial challenge. R1 and R2 were held in 13-L tanks with a 10-L working volume; R3 fish were maintained in individual 2.5-L boxes containing 2 L water to permit closer observation. The recorded growth dataset contained treatment-level body weights at the beginning and end of a 15-day interval. Weight gain, relative weight gain, specific growth rate (SGR), and average daily gain (ADG) were calculated from these measurements. Statistical comparisons of growth parameters were not performed because replicate-level measurements were unavailable for statistical analysis.

Bacterial challenge and post-challenge observations

The PC, MEF, and RPF groups were exposed to *A. hydrophila* by immersion; NC fish were not challenged. The challenge record states that bacterial culture was added at 1 mL/L, with approximately 1 h of immersion exposure and aeration maintained during exposure. For the 10-L tank systems, 1 mL/L corresponds to 10 mL culture per tank; for 2-L boxes, the corresponding volume is 2 mL per box. After exposure, the water level was restored using filtered water. Clinical behaviour, visible injury, aggression and mortality were monitored. Because mortality also occurred in the unchallenged group and aggressive/cannibalistic interactions were observed, deaths were recorded as post-challenge mortality and were not automatically attributed solely to infection.

Water-quality monitoring

Water pH, temperature, total dissolved solids (TDS) and electrical conductivity (EC) were monitored during the pre-challenge and challenge periods. The available summary records pre-challenge pH of 6.43–7.13, temperature of 28.0–30.6°C and TDS of 915–1200 ppm; challenge-period records were pH 6.27–7.04, temperature 28.0–29.5°C and TDS 998–1208 ppm. A separately recorded reference-water observation was pH 5.91, 29.0°C, TDS 1174 ppm and EC 2354 μ S/cm. These are reported as observed ranges/reference measurements rather than as replicate means.

Growth indices and mortality calculations

Growth indices were defined as follows: weight gain = final body weight – initial body weight; relative weight gain (%) = [(final body weight – initial body weight)/initial body weight] $\times$ 100; specific growth rate (SGR, % day⁻¹) = [(ln final body weight – ln initial body weight)/duration in days] $\times$ 100; and average daily gain (ADG) = weight gain/duration in days (Crane et al., 2020). These formulas describe the calculations used for the reported summary values; however, the original individual and replicate-level growth records were unavailable for independent recalculation. Mortality (%) was calculated as (number of dead fish/number of fish at the beginning of the challenge) $\times$ 100 and summarized at replicate level where records were available. Mortality in the unchallenged group limits attribution of all deaths solely to bacterial infection.

Functional-feed formulation and preparation

A functional feed was developed with 2% (w/w) *T. chebula* methanolic extract. The formulation comprised fish meal 35%, soybean meal 26%, wheat flour 16%, maize gluten meal 10%, protein concentrate 5%, *T. chebula* methanolic extract 2%, corn starch 2%, fish oil 1%, sodium alginate 1%, vitamin-mineral premix 1%, and lecithin 1% (total 100%). The ingredients were weighed and carefully combined. The methanolic extract was included at

2% and applied to the prepared feed by spray coating using sodium alginate as the coating/binding component. The coated feed was dried at 40°C for 2 days and processed into floating pellets adequate for juvenile *C. striata*.

Feed physicochemical and proximate characterization

Pellet diameter, floating ability, water stability, pellet durability, and bulk density were all measured in triplicate using standard aquafeed physical-quality methods (Tacon, 1993; Obaldo et al., 2002; American Society of Agricultural Engineers, 2004). The Feed and Nutrition Laboratory, Directorate of Incubation and Vocational Training in Aquaculture, Tamil Nadu Dr. J. Jayalalithaa Fisheries University, Muttukadu, Chennai, estimated the approximate composition using standard AOAC protocols (AOAC International, 2023). Moisture, crude protein, crude fat, crude fiber, and ash were measured, and the nitrogen-free extract was derived using the difference.

Previous *in vitro* characterization of the same *T. chebula* methanolic extract was used as supporting evidence for the present feeding/challenge investigation. The earlier work included concentration-dependent agar-well diffusion against *A. hydrophila*, qualitative phytochemical screening, GC–MS library profiling, and antimicrobial susceptibility testing of the bacterial isolate. These supporting assays were conducted and reported separately from the fish feeding and challenge trial; their results are not treated as new measurements from the current *in vivo* experiment.

Thermal stability and antibacterial activity

Aliquots of the methanolic extract were subjected to temperatures of 40, 60, and 80°C for 1 hour, followed by 1.5 hours at 95°C. The treated extracts were cooled to room temperature and screened for *A. hydrophila* using agar-well diffusion. The crude extract was standardized at 135 mg/mL. The bacterial solution was inoculated into Mueller-Hinton agar, and extract quantities of 50, 75, 100, and 125 µL were examined. Plates were incubated at 37 ± 1°C for 24 hours, and inhibitory zone diameters were measured. The test was done in triplicate. Agar diffusion was employed as a comparative screening approach since it allows for direct determination of inhibition zone diameter while acknowledging that diffusion assays do not provide a quantifiable minimum inhibitory concentration (Balouiri et al., 2016).

Clinical observations and mortality

During the post-challenge period, fish were examined for changes in swimming, eating response, aggression, scratching, mucus secretion, skin discoloration, lesions, fin damage, aberrant posture, lethargy, and other externally evident indicators. Mortality was recorded individually, and dead fish were removed once documented. The 48-hour observation was excluded in cases where no experimental observation was undertaken.

Growth performance

Body weight and total length were recorded during the feeding trial. Weight gain, relative weight gain, ADG and SGR were defined using the formulas stated above. The available file did not contain verifiable original fish-level or replicate-level records, so the growth summary could not be independently recalculated. Accordingly, growth outcomes are treated as descriptive summaries only, and no inferential statistical comparisons are reported.

Post-challenge PCR

Following the 96-hour post-challenge observation period, selected fish from the positive-control group were analyzed for molecular detection of *A. hydrophila*. Intestinal and muscle tissues were collected aseptically and processed separately for genomic DNA extraction. PCR was performed using the tissue-assay primer set recorded by the laboratory, and amplified products were separated by agarose gel electrophoresis and visualized with a gel documentation system. The assay was treated as pathogen-target detection rather than a quantitative estimate of viable bacterial burden. The primer target and expected product size should be reported exactly as documented in the original laboratory record.

Statistical analysis

Where verified replicate-level data were available, results were summarized as mean ± standard deviation. Thermal-retention data were analysed using two-way ANOVA with temperature and extract volume as factors, as reported in the underlying analysis; these statistics should be retained only if the original assay dataset and model output are available for verification. Mortality was summarized by experimental replicate where records permitted. The original growth dataset was not available for independent verification; therefore, growth indices are presented descriptively, and no ANOVA or post-hoc comparisons are reported for growth performance. SGR was defined according to Crane et al. (2020). The author indicated that the original weighing records are unavailable. Accordingly, the reconstructed spreadsheet was not used to recalculate or validate growth statistics, and the existing growth estimates require verification against primary records before submission.

Ethical considerations

There was no formal Institutional Animal Ethics Committee (IAEC) approval for the current investigation. Fish were kept and managed in a laboratory setting at the Gill Research Institute, Guru Nanak College, Chennai. Efforts were made to reduce needless handling, stress, and suffering, as well as to utilize the fewest animals possible for the experimental observations.

RESULTS

Morphological, biochemical and molecular characterization of the bacterial isolate

The isolate that produced smooth, round, creamy-white, convex colonies with a full margin was used in the supporting antibacterial tests. β-haemolysis was observed on blood agar, and the cells were Gram-negative rods. Catalase, oxidase, citrate utilization, urease, triple sugar iron (TSI), arginine dihydrolase, ornithine decarboxylase,

and lysine decarboxylase were all positive in the reported biochemical profile; indole, methyl red, Voges-Proskauer, and nitrate reduction were all negative (Table 1). The earlier isolate-characterization work is represented by these results.

Table 1. Morphological and biochemical characteristics reported for the *Aeromonas hydrophila* isolate.

Characteristic	Observation
Colony morphology	Smooth, circular, creamy-white
Colony margin	Entire
Colony elevation	Convex
Gram reaction	Gram-negative
Cell morphology	Rod-shaped
Haemolysis	β-haemolysis
Catalase	Positive
Oxidase	Positive
Citrate utilization	Positive
Urease	Positive
Triple sugar iron (TSI)	Positive
Arginine dihydrolase	Positive
Ornithine decarboxylase	Positive
Lysine decarboxylase	Positive
Indole production	Negative
Methyl red	Negative
Voges–Proskauer	Negative
Nitrate reduction	Negative

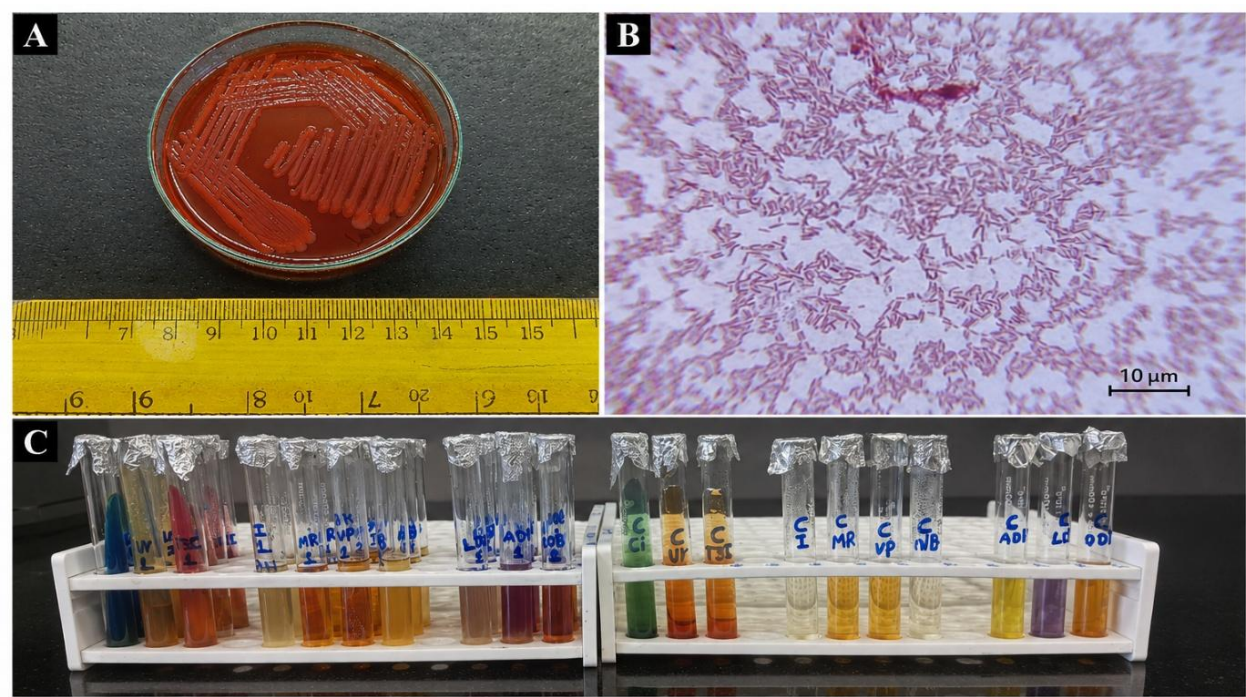


Figure 1. Characterization of *Aeromonas hydrophila* isolate. (A) β-hemolysis on blood agar; (B) Gram-stained smear showing Gram-negative, rod-shaped cells; (C) Biochemical test profile. Ci: Citrate, Ui: Urease, TSI: Triple Sugar Iron, MR: Methyl Red, VP: Voges–Proskauer, IND: Indole, ADH: Arginine dihydrolase, LDC: Lysine decarboxylase, OD: Ornithine decarboxylase.

Figure 1. Morphological and biochemical characterization of the *A. hydrophila* isolate, including colony appearance, Gram-stained cells, and biochemical test results.

The earlier isolate-confirmation assay targeting the *aerA* virulence-associated gene produced an approximately 309-bp amplicon, while no amplification was observed in the negative control. This result supports molecular confirmation of the isolate and is not a quantitative measure of bacterial abundance.

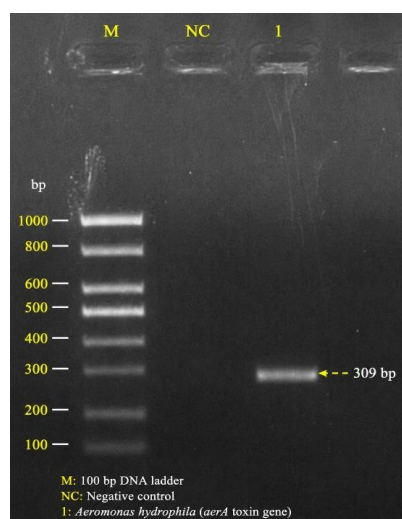


Figure 2. PCR amplification of the *aerA* gene in the bacterial isolate. Lane M: 100-bp DNA ladder; lane NC: negative control; lane 1: isolate with the reported approximately 309-bp product.

Antibiotic susceptibility profile

The recorded disc-diffusion results showed the largest inhibition zones for enrofloxacin and ciprofloxacin (34 mm each), followed by meropenem (28 mm), cefotaxime and azithromycin (27 mm each). No inhibition was recorded for ampicillin. Zone diameters are summarized in Table 2 and the plate images are shown in Figure 3. Susceptibility categories should be treated as those recorded in the source paper; independent interpretation requires verification of disc potency and applicable criteria.

Table 2. Previously recorded antibiotic susceptibility results for the *A. hydrophila* isolate.

Antibiotic	Disc potency (µg)	Zone (mm)	Recorded interpretation
Ceftazidime	30	21	Sensitive
Co-trimoxazole	25	22	Sensitive
Enrofloxacin	5	34	Sensitive
Doxycycline hydrochloride	30	24	Sensitive
Ciprofloxacin	5	34	Sensitive
Ampicillin	10	0	Resistant
Oxytetracycline	30	24	Sensitive
Erythromycin	15	15	Intermediate
Imipenem	10	16	Resistant
Meropenem	10	28	Sensitive
Cefotaxime	30	27	Sensitive
Sulphadiazine	300	24	Intermediate
Azithromycin	30	27	Sensitive
Furazolidone	100	24	Sensitive



Figure 3. Disc-diffusion antibiotic susceptibility plate images for the *A. hydrophila* isolate, reproduced from the earlier source manuscript.

Concentration-dependent antibacterial activity of *Terminalia chebula* extract

The earlier agar-well diffusion assay showed increasing inhibition-zone diameter with increasing concentration of methanolic *T. chebula* extract. Mean inhibition zones were 12.0 ± 0.5 , 14.0 ± 0.6 , 16.0 ± 0.4 and 18.0 ± 0.7 mm at 50, 75, 100 and 125 mg/mL, respectively (Figure 4). The methanol control produced no inhibition (Table 3). These results represent supporting in vitro findings from the earlier work, rather than outcomes of the fish challenge.

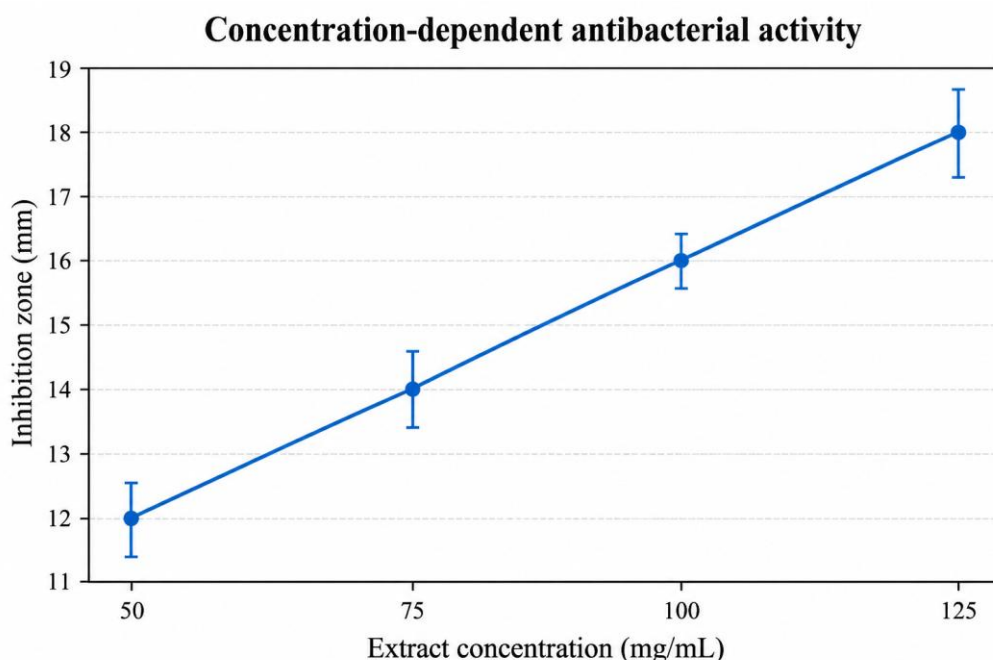


Figure 4. Concentration-dependent antibacterial activity of methanolic *T. chebula* extract against *A. hydrophila* (mean $\pm$ SD, $n = 3$).

Table 3. Previously recorded concentration-dependent antibacterial activity of methanolic *T. chebula* extract against *A. hydrophila*.

Extract concentration (mg/mL)	Inhibition zone (mm; mean $\pm$ SD)
50	12.0 ± 0.5
75	14.0 ± 0.6
100	16.0 ± 0.4
125	18.0 ± 0.7
Methanol control	No inhibition

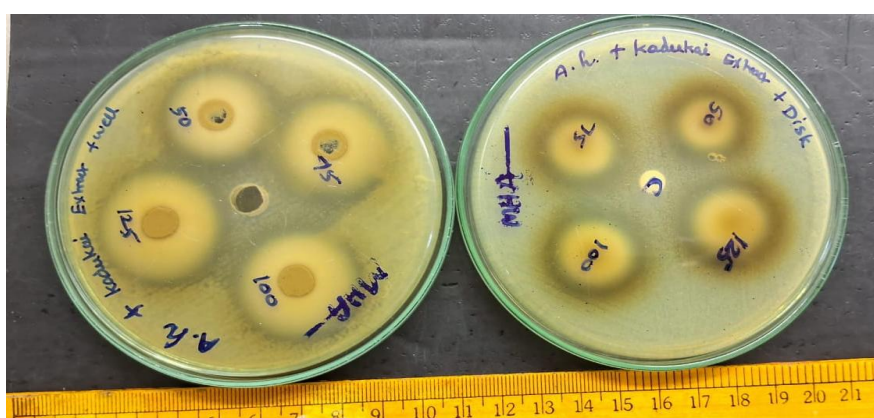


Figure 5. Agar-well diffusion image showing antibacterial activity of methanolic *T. chebula* extract against *A. hydrophila*.

Qualitative phytochemical screening

Qualitative screening indicated the presence of alkaloids, terpenoids, sugars, phenols/tannins, steroids, flavonoids, and quinones, while protein was not detected (Figure 6). These findings describe qualitative reactions and do not quantify individual phytochemical groups (Table 4).

Table 4. Qualitative phytochemical profile of methanolic *T. chebula* fruit extract.

Phytochemical group	Recorded result	Colour indication
Alkaloids	Present (++)	Reddish brown
Terpenoids	Present (+)	Red-brown
Sugars	Present (+)	Brown
Protein	Absent (–)	No significant colour change
Phenols and tannins	Present (++)	Deep blue
Steroids	Present (+)	Dark brown
Flavonoids	Present (+)	Dark pink
Quinones	Present (+)	Orange-brown



Figure 6. Qualitative phytochemical screening of the methanolic *T. chebula* fruit extract.

Previously established GC–MS profile

The previously completed GC–MS analysis of the same methanolic extract reported 78 chromatographic components with mass-spectral library matches (Figure 7). Representative annotations and their reported retention times, peak areas, and match factors are listed in Table 5. These are putative library-based annotations; they do not by themselves confirm compound identity or prove that any individual compound caused the antibacterial effect.

Table 5. Representative putative compound annotations from the previously established GC–MS profile of methanolic *T. chebula* extract.

Peak	Retention time	Peak area	Match (%)	Putative annotation
21	6.866	15,527,347	98.01	1,2,3-Benzenetriol
25	7.859	5,514,415	99.23	Methylparaben
9	4.964	1,170,342	97.69	5-Hydroxymethylfurfural
28	8.450	879,220.8	97.34	4-Hydroxybenzoic acid
71	28.847	439,374.4	89.46	Hentriacontane
52	12.764	360,031.6	92.82	Methyl gallate
26	8.113	390,356.7	95.06	3-Hydroxybenzoic acid
75	32.518	215,149.4	94.59	(24R)-Stigmast-5-en-3 β -ol

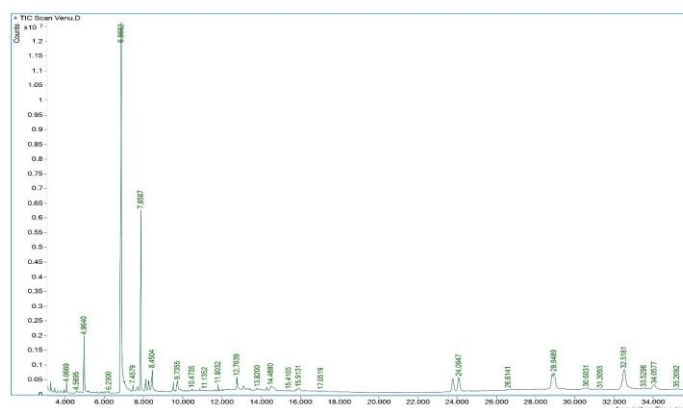


Figure 7. GC–MS chromatogram from the previously completed analysis of methanolic *T. chebula* extract; included as supporting evidence from the earlier study.

Thermal retention of antibacterial activity

After the extract was exposed to 40, 60, and 80°C for 1 hour and to 95°C for 1.5 hours, measurable inhibition of *A. hydrophila* was maintained. Inhibition-zone diameters generally increased with the applied extract volume. Following both 80°C and 95°C treatments, the largest reported mean zone was 25.67 ± 1.53 mm at 125 μ L, while the smallest was 16.67 ± 0.58 mm at 50 μ L following exposure to 40°C (Table 6). Two-way ANOVA indicated significant effects of temperature ($F_{3,32} = 20.85$, $P < 0.001$) and extract volume ($F_{3,32} = 52.03$, $P < 0.001$), whereas the temperature-by-volume interaction was not significant ($F_{9,32} = 0.365$, $P = 0.944$) (Figures 8 and 9).

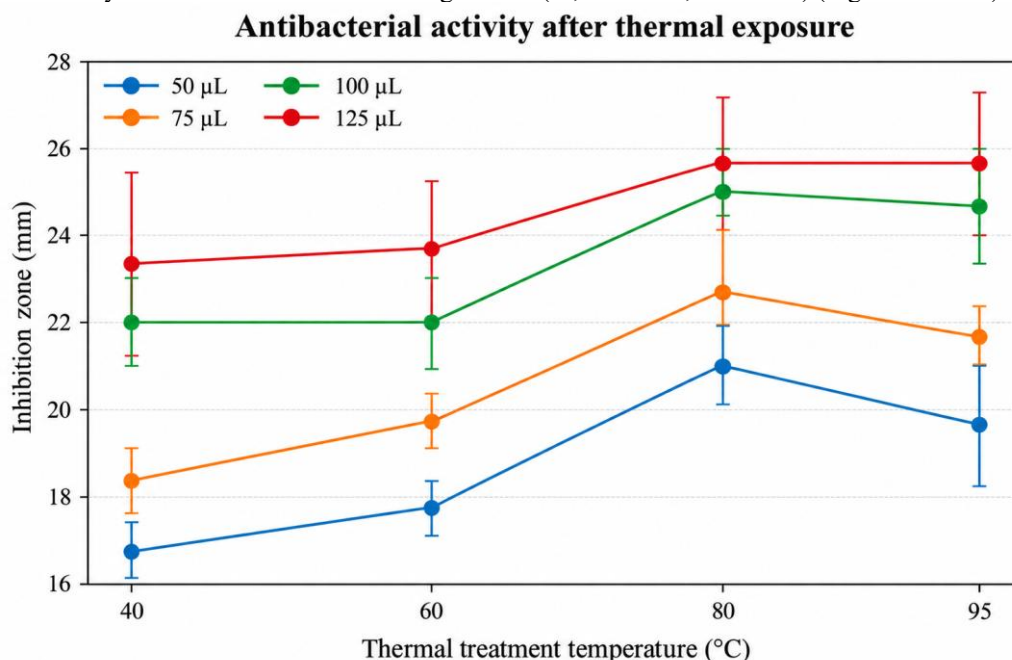


Figure 8. Antibacterial activity of methanolic *T. chebula* extract after thermal exposure at 40–95°C (previously reported data; mean $\pm$ SD).

Table 6. Antibacterial activity after thermal exposure (mean inhibition-zone diameter $\pm$ SD, mm).

Temperature/duration	50 μ L	75 μ L	100 μ L	125 μ L
40°C / 1 h	16.67 $\pm$ 0.58	18.33 $\pm$ 0.58	22.00 $\pm$ 1.00	23.33 $\pm$ 2.08
60°C / 1 h	17.67 $\pm$ 0.58	19.67 $\pm$ 0.58	22.00 $\pm$ 1.00	23.67 $\pm$ 1.53
80°C / 1 h	21.00 $\pm$ 1.00	22.67 $\pm$ 2.08	25.00 $\pm$ 1.00	25.67 $\pm$ 1.53
95°C / 1.5 h	19.67 $\pm$ 1.53	21.67 $\pm$ 0.58	24.67 $\pm$ 1.53	25.67 $\pm$ 1.53

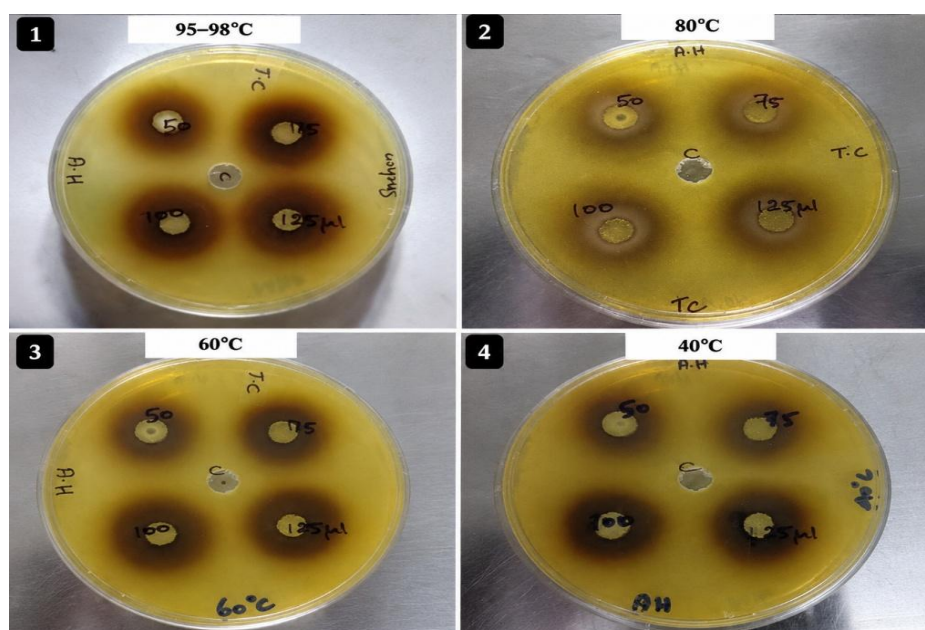


Figure 9. Representative agar-diffusion plates from the thermal-retention assay.

Functional-feed formulation and physicochemical characteristics

A 2% methanolic *T. chebula* extract was present in the functional feed (Figure 10). Tables 7 and 8 display the measured physical and proximate characteristics along with the ingredient formulation. Pellet durability was $99.33 \pm 0.01\%$, water stability was $76.87 \pm 0.12\%$, bulk density was $0.4393 \pm 0.0003 \text{ g cm}^{-3}$, and the mean diameter was $1.37 \pm 0.21 \text{ mm}$. 33.48% crude protein, 2.57% crude lipid, 6.37% crude fiber, 4.78% ash, 10.64% moisture, and 42.16% nitrogen-free extract were found in the proximate analysis.

Table 7. Ingredient composition of the *T. chebula* -supplemented functional feed.

Ingredient	Inclusion (%)
Fish meal	35.0
Soybean meal	26.0
Wheat flour	16.0
Maize gluten meal	10.0
Protein concentrate	5.0
Methanolic <i>T. chebula</i> extract	2.0
Corn starch	2.0
Fish oil	1.0
Sodium alginate	1.0
Vitamin–mineral premix	1.0
Lecithin	1.0
Total	100.0

Table 8. Physical and proximate characteristics of the formulated functional feed.

Parameter	Result
Pellet diameter (mm)	1.37 ± 0.21
Floating ability (%)	100.00 ± 0.00
Water stability (%)	76.87 ± 0.12
Pellet durability (%)	99.33 ± 0.01
Bulk density (g cm^{-3})	0.4393 ± 0.0003
Moisture (%)	10.64
Crude protein (%)	33.48
Crude lipid (%)	2.57
Crude fibre (%)	6.37
Ash (%)	4.78
Nitrogen-free extract (%)	42.16



Figure 10. Functional-feed pellets prepared with 2% methanolic *T. chebula* extract.

Water quality and growth performance

Water-quality records indicated a pre-challenge pH of 6.43–7.13, temperature of 28.0–30.6°C, and total dissolved solids (TDS) of 915–1200 ppm. On the challenge day, experimental tanks showed pH 6.27–7.04, temperature of 28.0–29.5°C, and TDS of 998–1208 ppm. The normal-water reference record was pH 5.91, temperature 29.0°C, TDS 1174 ppm, and electrical conductivity (EC) 2354 $\mu\text{S cm}^{-1}$ (Table 9). (Figure 11).

Recorded water-quality observations (ranges and reference value)

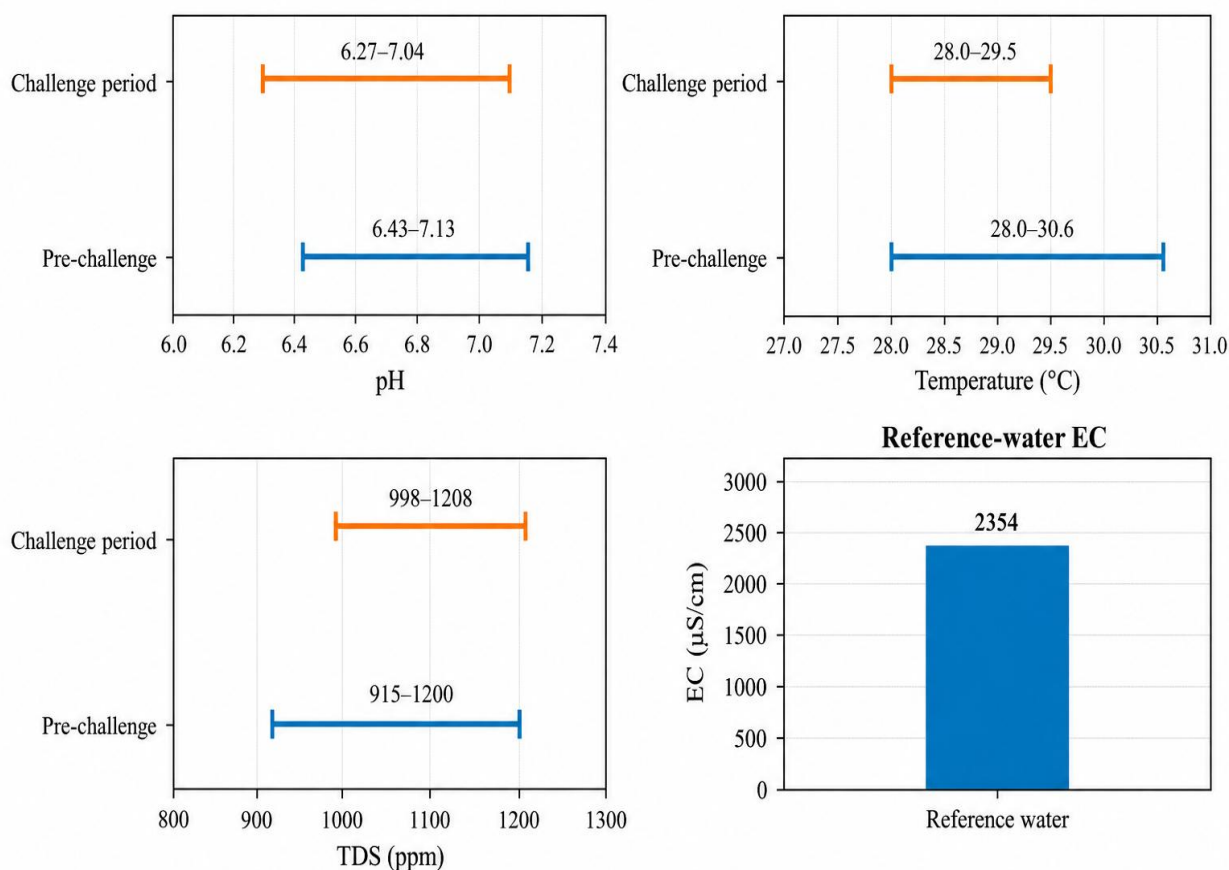


Figure 11. Recorded water-quality ranges during pre-challenge and challenge periods; electrical conductivity is shown only for the separately recorded reference-water observation. Values are ranges or a single reference measurement, not means.

Table 9. Summary of recorded water-quality observations during the feeding and challenge periods.

Period	pH	Temperature (°C)	TDS (ppm)	EC ($\mu\text{S cm}^{-1}$)
Pre-challenge observations	6.43–7.13	28.0–30.6	915–1200	Recorded concurrently
Pre-challenge, Day 19	6.53–7.04	28.0–30.6	915–1208	Recorded concurrently
Challenge day, experimental tanks	6.27–7.04	28.0–29.5	998–1208	Recorded concurrently
Challenge-day normal-water reference	5.91	29.0	1174	2354

The growth summary shows the methanolic-extract feed group had the highest reported growth indices, followed by the raw *T. chebula* feed and control groups. However, the primary weighing records were not available for independent verification, and the spreadsheet supplied was explicitly labelled as reconstructed. Consequently, the numerical growth indices and their associated variation cannot be treated as verified experimental estimates. No inferential comparison of growth among treatments is reported. The original measurements should be recovered and checked before these values are retained in a submission (Figure 12 and 13) (Table 10).

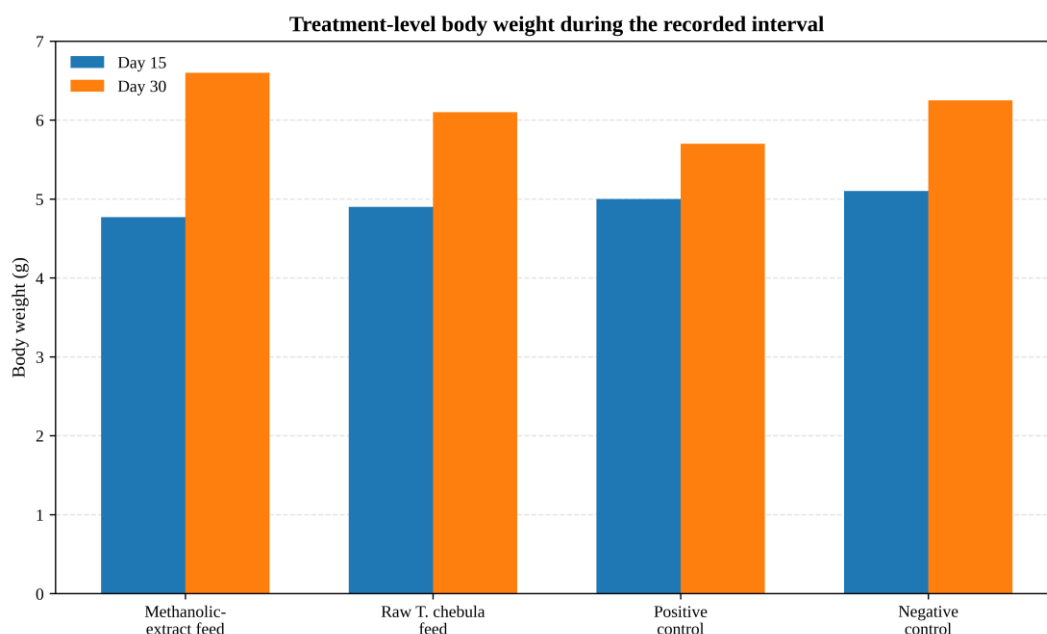


Figure 12. Treatment-level body weight of *Channa striata* on Days 15 and 30 across the four dietary treatments.

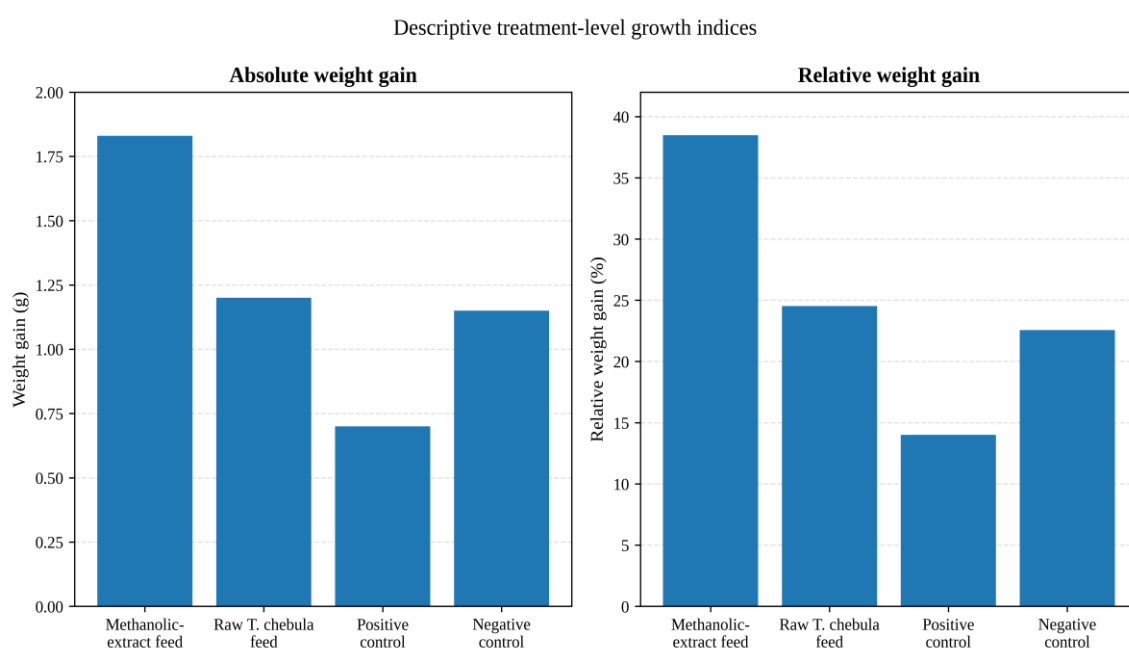


Figure 13. Absolute and relative weight gain of *Channa striata* across the four dietary treatments during the recorded interval.

Table 10. Treatment-level growth indices for *Channa striata* during the measured interval.

Treatment	Day 15 BW (g)	Day 30 BW (g)	Weight gain (g)	Weight gain (%)	SGR (% day ⁻¹)	ADG (g day ⁻¹)
Methanolic-extract feed	4.77 ± 0.10	6.60 ± 0.10	1.83 ± 0.03	38.48 ± 1.18	2.17 ± 0.06	0.122 ± 0.002
Raw T. chebula feed	4.90 ± 0.13	6.10 ± 0.10	1.20 ± 0.05	24.52 ± 1.61	1.46 ± 0.09	0.080 ± 0.003
Positive control	5.00 ± 0.10	5.70 ± 0.10	0.70 ± 0.00	14.00 ± 0.28	0.87 ± 0.02	0.047 ± 0.000
Negative control	5.10 ± 0.10	6.25 ± 0.05	1.15 ± 0.05	22.57 ± 1.42	1.36 ± 0.08	0.077 ± 0.003

Post-challenge clinical responses and mortality

Aggression, fin damage, skin disruption, mucus secretion, whitish appearance around the eyes or head, scratching, lesions, and decreased swimming activity were among the observations noted after immersion exposure. There were also reports of potentially cannibalistic behavior and aggressive interactions. Because deaths also happened in the unchallenged negative-control group, mortality is interpreted as a composite endpoint and cannot be solely attributed to bacterial infection. (Figure. 14).

Post-challenge mortality across replicates

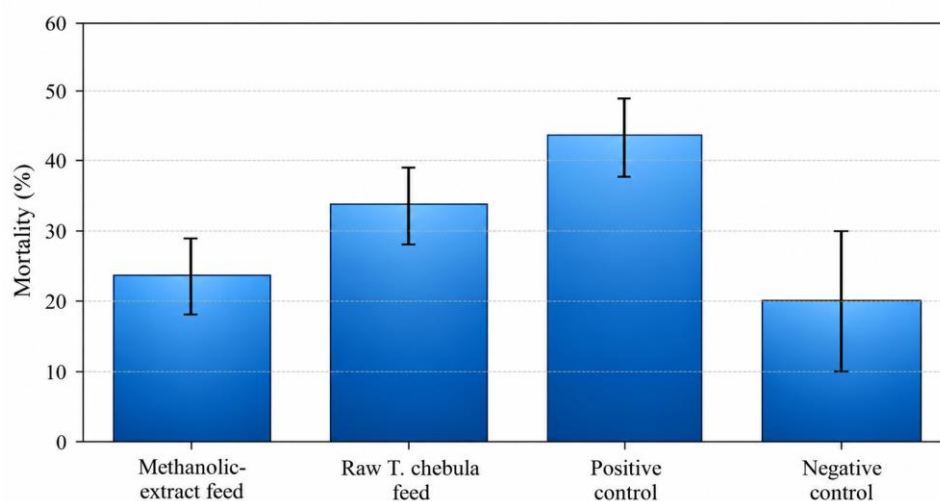


Figure 14. Mean post-challenge mortality (mean $\pm$ SD, three replicates per group). Mortality in the unchallenged control and observed aggressive interactions mean deaths cannot be attributed solely to *A. hydrophila*.

Table 11. Replicate-level mortality recorded after the challenge period.

Replicate	Treatment group	Initial fish	Deaths	Mortality (%)
R1	Methanolic-extract feed	10	2	20
R2	Methanolic-extract feed	10	2	20
R3	Methanolic-extract feed	10	3	30
R1	Raw T. chebula feed	10	3	30
R2	Raw T. chebula feed	10	4	40
R3	Raw T. chebula feed	10	3	30
R1	Positive control	10	4	40
R2	Positive control	10	5	50
R3	Positive control	10	4	40
R1	Negative control	10	2	20
R2	Negative control	10	3	30
R3	Negative control	10	1	10

Table 12. Summary of mortality across three experimental replicates.

Treatment group	Mean mortality (%)	SD (%)
Methanolic-extract feed	23.3	5.8
Raw T. chebula feed	33.3	5.8
Positive control	43.3	5.8
Negative control	20.0	10.0

Post-challenge molecular detection

Following the *Aeromonas hydrophila* challenge, affected *Channa striata* exhibited clinical lesions, and mortality was recorded during the observation period. Post-challenge PCR analysis of intestine and muscle tissue samples revealed visible amplification bands at approximately 226 bp (Figure 15), supporting detection of the assay target in both tissues. The identity and expected product size of the tissue-PCR primer set must be confirmed against the original laboratory record. These endpoint-PCR results do not quantify bacterial load or, by themselves, establish the cause of mortality.

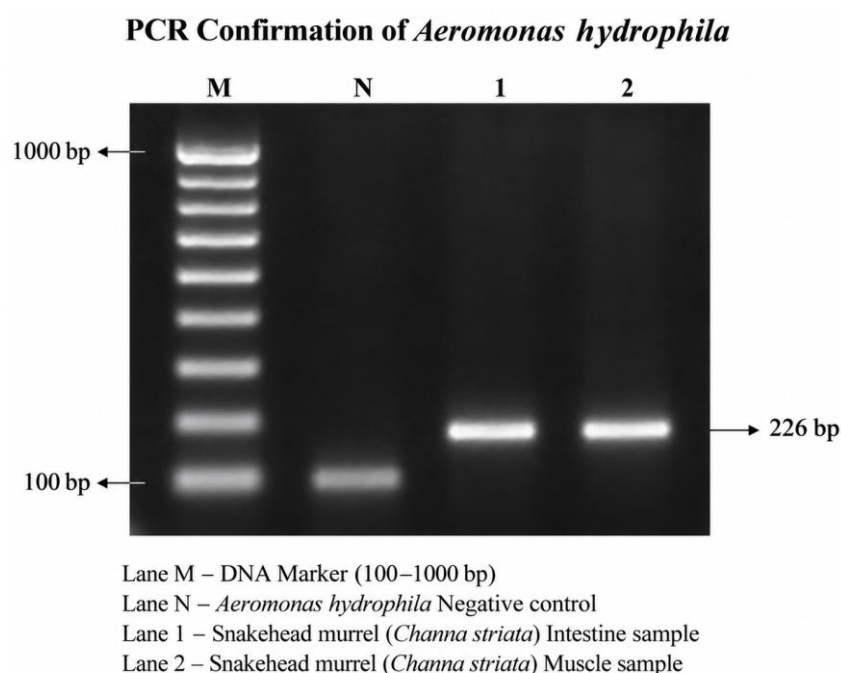


Figure 15. Post-challenge PCR analysis of *Channa striata* tissue samples. Lane M, DNA molecular-weight marker (100–1000 bp); lane N, negative control; lane 1, intestine sample; lane 2, muscle sample. Bands at approximately 226 bp are visible in the intestine and muscle samples.

DISCUSSION

The present study investigated the application of *Terminalia chebula* methanolic fruit extract as a functional-feed ingredient for juvenile *Channa striata* subjected to experimental challenge with *Aeromonas hydrophila*. The investigation combined antibacterial screening, thermal-retention assessment, functional-feed formulation, physical and proximate characterisation, growth monitoring, post-challenge clinical observations, mortality recording and molecular analysis. This integrated experimental approach provides information on the antibacterial properties of the extract, the characteristics of the formulated feed and the responses of fish following experimental bacterial exposure.

The antibacterial activity observed against *A. hydrophila* supports further investigation of *T. chebula* as a potential phytochemical ingredient for aquaculture applications. In the concentration-dependent agar-well diffusion assay, the mean inhibition zone increased from 12.0 ± 0.5 mm at 50 mg/mL to 18.0 ± 0.7 mm at 125 mg/mL. This pattern indicates greater inhibition under the higher-concentration test conditions. The antimicrobial potential of *T. chebula* has also been investigated in previous studies. Bag et al. (2013), for example, isolated and characterised a gallotannin from *T. chebula* and examined its activity against multidrug-resistant uropathogens. Although the bacterial targets differed from those examined in the present investigation, this work provides relevant context for exploring tannin-containing plant extracts as potential sources of antibacterial compounds. Nevertheless, the inhibition-zone measurements obtained here do not independently establish the contribution of individual phytochemicals or demonstrate therapeutic efficacy in living fish.

Thermal-retention testing demonstrated that antibacterial activity remained detectable after exposure of the extract to 40, 60 and 80°C for 1 h and 95°C for 1.5 h. At an extract volume of 125 µL, the largest reported inhibition zone was 25.67 ± 1.53 mm following both the 80°C and 95°C treatments. The reported two-way ANOVA indicated significant effects of temperature and extract volume, whereas their interaction was not significant. These findings demonstrate that detectable antibacterial activity was retained under the tested thermal conditions. This observation is relevant to the development of plant-based feed additives because processing and drying can expose feed ingredients to heat. However, retention of antibacterial activity does not necessarily indicate that all active constituents remained chemically unchanged. Further chemical characterisation before and after thermal exposure would help determine whether individual compounds were retained, degraded or transformed during treatment.

The functional feed containing 2% methanolic *T. chebula* extract exhibited measurable physical-quality characteristics. The mean pellet diameter was 1.37 ± 0.21 mm, floating ability was 100%, pellet durability was $99.33 \pm 0.01\%$, water stability was $76.87 \pm 0.12\%$, and bulk density was 0.4393 ± 0.0003 g cm⁻³. Proximate analysis revealed 33.48% crude protein, 2.57% crude lipid, 6.37% crude fibre, 4.78% ash and 10.64% moisture. These measurements characterise the physical properties and nutritional composition of the prepared feed. Obaldo et al. (2002) highlighted the importance of assessing feed stability in water when evaluating aquatic feeds. In the present study, the measured flotation, durability and water stability provide useful information about the prepared pellets and their physical integrity under the tested conditions. Nevertheless, these properties alone do not establish improved nutrient utilisation or nutritional superiority, which would require additional evidence from feed intake,

feed-conversion ratio and nutrient-utilisation measurements.

The available growth summary showed a numerical trend favouring the methanolic-extract feed group, followed by the raw *T. chebula* plant-powder feed and the control groups. This observation is relevant because the study examined two forms of plant-derived supplementation: methanolic extract and raw plant powder. The observed differences provide a basis for further investigation into whether the form of incorporation influences growth performance. However, the original fish-level and replicate-level weighing records were unavailable for independent verification. Therefore, the reported growth indices cannot be treated as independently validated estimates, and the numerical differences cannot establish a statistically significant treatment effect. The absence of ANOVA for these growth data does not invalidate the feeding experiment; rather, it limits the conclusions that can be drawn about differences among treatments. Future trials should retain complete individual and replicate-level measurements to enable reliable calculation of growth indices and appropriate statistical comparisons.

Post-challenge observations revealed clinical changes that included aggression, fin damage, skin disruption, mucus secretion, lesions, scratching, and reduced swimming activity. Mean recorded mortality was $23.3 \pm 5.8\%$ in the methanolic-extract feed group, $33.3 \pm 5.8\%$ in the raw-plant feed group, and $43.3 \pm 5.8\%$ in the challenged positive control. The unchallenged negative control recorded $20.0 \pm 10.0\%$ mortality. The lower observed mortality in the methanolic-extract feed group than in the challenged positive control is a noteworthy experimental observation. The numerical difference suggests a possible association between the extract-containing diet and the post-challenge response, although the present results do not establish that the difference was statistically significant or caused by the dietary treatment. Mortality in the unchallenged group, together with recorded aggression, injuries, and potentially cannibalistic interactions, also indicates that factors other than bacterial exposure may have contributed to some deaths. Accordingly, the mortality results should be interpreted in conjunction with the experimental challenge, clinical observations and molecular findings rather than as an isolated measure of protective efficacy.

Post-challenge PCR analysis performed with support from C. Abdul Hakeem College, Melvisharam, Tamil Nadu, revealed amplification bands in the examined intestine and muscle tissue samples at approximately 226 bp. The fish had been deliberately exposed to *A. hydrophila* by immersion as part of the experimental challenge. Consequently, the tissue-PCR findings are relevant to the assessment of the challenged fish, alongside their clinical responses and recorded mortality. However, the identity of the target amplified in the tissue samples and the expected product size must be confirmed against the collaborating laboratory's records before the bands can be definitively assigned to *A. hydrophila*. The earlier bacterial-isolate confirmation assay reportedly produced an approximately 309-bp product associated with the *aerA* target. The difference between the reported product sizes means that the tissue assay should not automatically be assumed to have used the same primer set. Once the tissue-PCR target is verified, the molecular findings can be interpreted more specifically in relation to the experimental challenge. Endpoint PCR can support detection of a validated target but does not quantify pathogen burden or independently establish the cause of every individual mortality event.

The previously established GC–MS profile of the same methanolic extract provides additional chemical context for the investigation. The earlier analysis reported 78 chromatographic components with putative mass-spectral library annotations, including 1,2,3-benzenetriol and methyl gallate. These findings identify candidate compounds for further investigation but do not independently confirm their chemical identities or establish their individual contributions to antibacterial activity. In the present study, this earlier chemical characterisation was considered alongside antibacterial screening, thermal-retention testing, feed formulation and fish-response observations. The combined evidence connects the investigation of plant-derived antibacterial activity with the practical preparation of a functional feed for *C. striata*. Nevertheless, the individual contributions of the reported compounds to the observed fish responses remain to be established.

The principal contribution of the present investigation is the experimental evaluation of *T. chebula*-supplemented functional feed through the combined assessment of antibacterial activity, thermal retention, feed quality and post-challenge responses in juvenile *C. striata*. The study demonstrates that the extract-containing feed could be formulated and characterised and that antibacterial activity remained detectable under the tested thermal conditions. The observed mortality pattern and the available growth summary provide directions for further investigation, but they do not independently establish improved growth, immunomodulation, or definitive protection against *A. hydrophila*. Further research should incorporate verified replicate-level growth data, controlled challenge conditions, confirmation of the tissue-PCR target, quantitative pathogen-load assessment, histopathological examination, and host-response measurements. Such studies would help determine the reproducibility, biological significance, and potential practical application of *T. chebula* as a phytogetic ingredient in functional aquafeed.

CONCLUSION

The present study demonstrated the successful incorporation of 2% methanolic *Terminalia chebula* fruit extract into a functional feed for juvenile *Channa striata* and evaluated its antibacterial activity, feed quality, and associated fish responses following experimental challenge with *Aeromonas hydrophila*. The formulated feed exhibited favourable physical characteristics, including 100% floating ability, $99.33 \pm 0.01\%$ pellet durability and $76.87 \pm 0.12\%$ water stability, while the extract retained detectable antibacterial activity following the tested thermal treatments. The available growth results showed greater weight gain and higher growth indices in the

methanolic-extract feed group than in the raw *T. chebula* feed and control groups. Following bacterial challenge, the methanolic-extract feed group also recorded lower observed mortality ($23.3 \pm 5.8\%$) than the challenged positive control ($43.3 \pm 5.8\%$). These findings indicate the potential value of *T. chebula*-supplemented functional feed for improving growth performance and supporting fish health under the experimental conditions. Post-challenge PCR analysis further provided molecular findings for evaluating the challenged fish, although confirmation of the tissue-assay target is required for definitive interpretation. As the original growth records were unavailable for independent verification and statistical comparisons of growth were not performed, the reported growth differences should be interpreted as observed trends rather than statistically established treatment effects. Furthermore, mortality in the unchallenged control warrants caution when attributing all deaths exclusively to bacterial infection. Overall, the findings support further investigation of *T. chebula* methanolic extract as a promising phytogenic feed ingredient for *C. striata*, with additional controlled studies needed to confirm growth benefits and protective efficacy against *A. hydrophila*.

ACKNOWLEDGMENTS

The authors sincerely acknowledge the Microbiology Unit, Gill Research Institute, and the PG and Research Department of Zoology, Guru Nanak College, for laboratory and institutional support. The authors thank Raghu Fish Farms, Walajapet, Tamil Nadu, India, for providing experimental *Channa striata*; Dr. Abdul Majeed and Mr. Ajay Pathra, C. Abdul Hakeem College, Melvisharam, Tamil Nadu, India; Dr. Vasanth Albert R and Dr. Nithish, KCG College of Technology, Villupuram; Dr. G. Sathiyaraj, Rajiv Gandhi Centre for Aquaculture, Sirkali. Mr. N. Ranjith, Central Institute of Brackish Water Aquaculture, Santhome, Chennai, and Mr. Augustin, Research Scholar, Guru Nanak College, for technical assistance, guidance, and logistical support.

Funding

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

Conflicts of Interest

The authors declare no conflicts of interest related to this study.

Author Contributions

S. Snehan conducted the experiments, collected and analysed the data, and drafted the manuscript. S. Venu provided research supervision and contributed to the review and revision of the manuscript. Both authors reviewed and approved the final manuscript.

Data Availability

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

REFERENCES

1. American Society of Agricultural Engineers (2004). ASAE standard S269.4: Cubes, pellets, and crumbles—definitions and methods for determining density, durability, and moisture content. ASAE, St Joseph, MI, USA.
2. AOAC International (2023). Official methods of analysis of AOAC International, 22nd edn. AOAC International, Rockville, MD, USA.
3. Azwanida NN (2015). A review on the extraction methods use in medicinal plants, principle, strength and limitation. *Med. Aromat. Plants* 4:196. doi:10.4172/2167-0412.1000196.
4. Bag A, Bhattacharyya SK and Chattopadhyay RR (2013). Isolation and identification of a gallotannin 1,2,6-tri-O-galloyl- β -D-glucopyranose from hydroalcoholic extract of *Terminalia chebula* fruits effective against multidrug-resistant uropathogens. *J. Appl. Microbiol.* 115:390-397.
5. Balouiri M, Sadiki M and Ibnsouda SK (2016). Methods for in vitro evaluating antimicrobial activity: A review. *J. Pharm. Anal.* 6:71-79.
6. Bauer AW, Kirby WMM, Sherris JC and Turck M (1966). Antibiotic susceptibility testing by a standardized single disk method. *Am. J. Clin. Pathol.* 45:493-496. doi:10.1093/ajcp/45.4_ts.493.
7. Bondad-Reantaso MG, MacKinnon B, Karunasagar I, Fridman S, et al. (2023). Review of alternatives to antibiotic use in aquaculture. *Rev. Aquac.* 15:1421-1451.
8. Crane DP, Ogle DH and Shoup DE (2020). Use and misuse of a common growth metric: guidance for appropriately calculating and reporting specific growth rate. *Rev. Aquac.* 12:1542-1547.
9. Harborne JB (1998). *Phytochemical Methods: A Guide to Modern Techniques of Plant Analysis*, 3rd edn. Chapman and Hall, London, UK.
10. Holt JG, Krieg NR, Sneath PHA, Staley JT, et al. (1994). *Bergey's Manual of Determinative Bacteriology*, 9th edn. Williams & Wilkins, Baltimore, MD, USA.
11. Janda JM and Abbott SL (2010). The genus *Aeromonas*: taxonomy, pathogenicity, and infection. *Clin. Microbiol. Rev.* 23:35-73.
12. Kannan B, Felix N, Panigrahi A and Ahilan B (2022). Herbal extracts modulate growth, immune responses and resistance to *Aeromonas hydrophila* infection in GIFT tilapia (*Oreochromis niloticus*). *Aquac. Res.* 53:4627-4637.
13. Nek Rahimi NMM, Natrah I, Loh JY, Ranzil FKE, et al. (2022). Phytocompounds as an alternative antimicrobial approach in aquaculture. *Antibiotics* 11:469.

14. Obaldo LG, Divakaran S and Tacon AGJ (2002). Method for determining the physical stability of shrimp feeds in water. *Aquac. Res.* 33:369-377.
15. Rasmussen-Ivey CR, Figueras MJ, McGarey D and Liles MR (2016). Virulence factors of *Aeromonas hydrophila*: in the wake of reclassification. *Front. Microbiol.* 7:1337.
16. Sen K and Rodgers M (2004). Distribution of six virulence factors in *Aeromonas* species isolated from US drinking water supplies. *J. Appl. Microbiol.* 97:1077-1086.
17. Snehan S, Venu S, Sathiyaraj G and Mithunraj (2026). Extracting and exploring of bioactive compounds in *Terminalia chebula* through GC-MS profiling against fish pathogen *Aeromonas hydrophila*. *Int. J. Drug Deliv. Technol.* 16(58s). doi:10.25258/ijddt.16.58s.1.
18. Sofowora A (2008). *Medicinal Plants and Traditional Medicine in Africa*, 3rd edn. Spectrum Books Ltd., Ibadan, Nigeria.
19. Sultana MT, Anwar MJ, Imran M, Khalil I, et al. (2023). Phytochemical profile and pro-healthy properties of *Terminalia chebula*: a comprehensive review. *Int. J. Food Prop.* 26:526-551.
20. Tacon AGJ (1993). *Feed Ingredients for Warmwater Fish: Fish Meal and Other Processed Feedstuffs*. FAO, Rome, Italy.
21. Vijayaram S, Sun YZ, Zuorro A, Ghafarifarsani H, et al. (2022). Bioactive immunostimulants as health-promoting feed additives in aquaculture: a review. *Fish Shellfish Immunol.* 130:294-308.
22. Wang J, Deng L, Chen M, Che Y, et al. (2024). Phytogenic feed additives as natural antibiotic alternatives in animal health and production: a review of the literature of the last decade. *Anim. Nutr.* 17:244-264.