Evaluation of *Croton bonplandianum* Leaf Extract for the Control of Bacterial Pathogens and Growth Enhancement in *Labeo rohita*

RL Dhanya Mol and M Prabu

PG & Research Department of Zoology, Chikkaiah Government Arts and Science College (Affiliated to Bharathiyar University) Erode - 638 004, Tamil Nadu, India.

Abstract

The rapid expansion of aquaculture has been accompanied by an increased incidence of bacterial diseases, resulting in significant economic losses and reduced fish productivity. The present study investigated the antimicrobial potential of *Croton bonplandianum* leaf extracts against fish pathogenic bacteria and evaluated their effects on the growth performance of *Labeo rohita* (Rohu). Pathogenic bacteria were isolated from infected fish samples and identified using morphological and biochemical characterization. Leaves of *C. bonplandianum* were collected, processed, and extracted using ethanol, acetone, and petroleum ether solvents. Preliminary phytochemical screening revealed the presence of biologically active compounds including alkaloids, flavonoids, tannins, phenols, terpenoids, glycosides, and saponins. The bioactive constituents were further characterized using Thin Layer Chromatography (TLC), UV–Visible spectroscopy, and Fourier Transform Infrared (FTIR) spectroscopy. Antimicrobial activity of the crude extracts was assessed against selected fish pathogens using the agar well diffusion method. The extracts exhibited varying degrees of inhibitory activity against *Aeromonas hydrophila*, *Vibrio campbellii*, *Vibrio parahaemolyticus*, *Staphylococcus aureus*, *Escherichia coli*, and other bacterial isolates. A feeding trial was conducted using juvenile *Labeo rohita* supplemented with different concentrations of *C. bonplandianum* extract. Fish receiving the supplemented diets demonstrated improved growth performance, feed utilization efficiency, survival rate, and disease resistance compared with the control group. The findings suggest that *C. bonplandianum* possesses significant antimicrobial and growth-promoting properties and may serve as a sustainable phytotherapeutic alternative to conventional antibiotics in freshwater aquaculture. The study highlights the potential application of medicinal plant-based feed additives for enhancing fish health and promoting environmentally friendly aquaculture practices.

Keywords: *Croton bonplandianum*; *Labeo rohita*; Antimicrobial activity; Fish pathogenic bacteria; Growth performance

INTRODUCTION

Aquaculture is one of the fastest-growing food-producing sectors worldwide and plays a vital role in ensuring food security, nutritional sustainability, and economic development.

Freshwater aquaculture contributes significantly to global fish production, with Indian major carps accounting for a major share of inland fisheries production in India. Among these, *Labeo rohita* (Rohu) is one of the most commercially important freshwater fish species due to its rapid growth, consumer preference, high nutritional value, and adaptability to diverse culture systems. However, the intensification of aquaculture practices has increased the incidence of infectious diseases, particularly those caused by bacterial pathogens, resulting in substantial economic losses and reduced productivity (FAO, 2022; Pakhira et al., 2024). Recent studies have highlighted the emergence of virulent bacterial pathogens in cultured *L. rohita*, emphasizing the urgent need for effective disease management strategies in freshwater aquaculture.

Bacterial diseases caused by species of *Aeromonas*, *Vibrio*, *Staphylococcus*, *Streptococcus*, *Bacillus*, and *Salmonella* are among the most prevalent health problems affecting cultured fish. These pathogens can induce severe tissue damage, impaired growth, immune suppression, and high mortality rates. Traditionally, antibiotics have been widely used to control bacterial infections in aquaculture. However, the indiscriminate use of antibiotics has led to the emergence of antimicrobial resistance, environmental contamination, and the accumulation of drug residues in aquatic organisms. Consequently, the development of environmentally friendly and sustainable alternatives has become a major focus of aquaculture research. Recent investigations on bacterial infections in *L. rohita* have revealed increasing concerns regarding pathogen virulence and disease outbreaks, necessitating the exploration of natural antimicrobial agents for fish health management.

Medicinal plants have long served as valuable sources of therapeutic compounds due to their rich diversity of secondary metabolites, including alkaloids, flavonoids, tannins, terpenoids, phenolics, and glycosides. Plant-derived bioactive compounds exhibit a broad spectrum of biological activities such as antimicrobial, antioxidant, anti-inflammatory, immunostimulatory, and growth-promoting effects. In recent years, phytotherapeutic approaches have gained increasing attention as sustainable alternatives to synthetic chemicals and antibiotics in aquaculture. Herbal feed additives and plant extracts have been reported to enhance disease resistance, improve immune responses, and promote growth performance in cultured fish species (Harikrishnan et al., 2011; Reverter et al., 2014).

Croton bonplandianum Baill., a member of the family Euphorbiaceae, is a medicinal herb widely distributed in tropical and subtropical regions. The plant has been traditionally used for the treatment of skin diseases, wounds, inflammation, liver disorders, bronchitis, and gastrointestinal ailments. Phytochemical investigations have demonstrated that *C. bonplandianum* contains numerous biologically active compounds, including flavonoids, phenols, terpenoids, steroids, tannins, and alkaloids, which are responsible for its pharmacological properties. Recent studies have confirmed its antimicrobial, antioxidant, antidiabetic, cytotoxic, and therapeutic potential, supporting its use as a promising natural source of bioactive compounds. Furthermore, contemporary reviews on the genus *Croton* have emphasized the remarkable diversity of phytochemicals and their potential applications in pharmaceutical and veterinary sciences.

Despite the growing evidence supporting the medicinal value of *Croton* species, information regarding the application of *C. bonplandianum* in aquaculture disease management remains limited. The utilization of plant-derived antimicrobial compounds as dietary supplements may provide an effective strategy to control bacterial infections while simultaneously enhancing fish growth and survival. In addition, phytochemical characterization using chromatographic and spectroscopic techniques can provide valuable insights into the bioactive constituents responsible for antimicrobial activity.

Therefore, the present study was undertaken to isolate and identify bacterial pathogens associated with cultured *Labeo rohita*, evaluate the phytochemical composition of *Croton bonplandianum* leaf extracts, determine their antimicrobial efficacy against selected fish pathogenic bacteria, and assess their effects on the growth performance and survival of *L. rohita*. The findings of this study may contribute to the development of environmentally sustainable phytotherapeutic approaches for disease prevention and health management in freshwater aquaculture.

MATERIALS AND METHODS

Collection of Fish Samples and Isolation of Bacterial Pathogens

Healthy and diseased specimens of *Labeo rohita* (Rohu) were collected from freshwater culture ponds located in Erode District, Tamil Nadu, India, during the study period. Water, sediment, gill, gut, and skin samples were collected aseptically at regular intervals throughout the culture period. Water samples were obtained in sterile screw-capped PVC bottles from just below the pond surface and transported immediately to the laboratory for microbiological analysis. Fish tissues including gill, gut, and skin were excised under sterile conditions and homogenized in physiological saline solution. The homogenates were serially diluted and inoculated onto nutrient agar plates using the spread plate technique. The plates were incubated at 30–37°C for 24 h, after which bacterial colonies were observed, counted, and purified for further analysis.

Pathogenic bacterial isolates were obtained from infected fish tissues following standard microbiological procedures. Tissue samples were enriched in sterile Alkaline Peptone Water and incubated for 24 h. The enriched samples were serially diluted and plated on nutrient agar medium. Distinct colonies were isolated, purified through repeated streaking, and maintained on nutrient agar slants. The purified isolates were subjected to morphological and biochemical characterization for identification up to species level using Bergey's Manual of Systematic Bacteriology.

Enumeration and Biochemical Characterization of Bacteria

The total heterotrophic bacterial (THB) count was determined using nutrient agar medium. Aliquots of serially diluted samples were spread evenly on sterile agar plates and incubated at 32°C for 24 h. Colony-forming units (CFU) were recorded and expressed as CFU mL^{-1} for water samples and CFU g^{-1} for tissue samples. Bacterial abundance and diversity were used as indicators of microbial prevalence in the culture environment.

The bacterial isolates were characterized using standard biochemical tests including Gram staining, motility, catalase, oxidase, indole production, methyl red, Voges–Proskauer, citrate utilization, triple sugar iron (TSI), urease activity, nitrate reduction, and carbohydrate fermentation tests using glucose, lactose, maltose, sucrose, and dextrose. The results obtained were compared with standard taxonomic descriptions for bacterial identification.

Collection and Preparation of Plant Material

Fresh leaves of *Croton bonplandianum* were collected from different locations in Erode District, Tamil Nadu, India. The plant material was authenticated by qualified taxonomists, and a voucher specimen was deposited in the institutional herbarium for future reference. The collected leaves were thoroughly washed, shade-dried, and subsequently dried in a hot-air oven at 45°C. The dried material was ground into fine powder using a mechanical grinder and stored in airtight containers until extraction.

Solvent Extraction of Plant Material

The powdered leaf material was subjected to solvent extraction using ethanol, acetone, and petroleum ether. Approximately 25 g of leaf powder was mixed separately with 100 mL of each solvent and allowed to stand for five days with intermittent shaking. The extracts were filtered through Whatman No.1 filter paper, and the filtrates were concentrated by solvent evaporation. The resulting crude extracts were dissolved in dimethyl sulfoxide (DMSO) to prepare stock solutions for phytochemical and antimicrobial analyses.

Phytochemical Screening

Qualitative phytochemical analysis of the crude extracts was performed using standard protocols to detect the presence of bioactive compounds. Tests were conducted for tannins, saponins, steroids, terpenoids, flavonoids, alkaloids, phenols, glycosides, and anthraquinones. Characteristic colour changes, precipitate formation, or emulsion development were used as indicators for the presence of specific phytochemical constituents. The screening provided preliminary information regarding the medicinally important compounds present in *C. bonplandianum* leaves.

Thin Layer Chromatography (TLC)

The ethanol extract exhibiting significant biological activity was subjected to thin layer chromatography for compound separation. Silica gel-coated TLC plates were used as the stationary phase, while ethyl acetate:methanol:water solvent systems served as the mobile phase. The extract was applied as a thin streak along the baseline and developed in a saturated chromatographic chamber. Separated compounds were visualized under ultraviolet light at 254 and 365 nm. Distinct bands were scraped, eluted, and further purified for antimicrobial evaluation and characterization.

UV–Visible and FTIR Spectroscopic Analysis

The purified bioactive fractions were analyzed using UV–Visible spectroscopy to determine their absorption characteristics. Spectral measurements were recorded between 280 and 400 nm using quartz cuvettes with a path length of 1 cm. Functional groups present in the active

compounds were identified using Fourier Transform Infrared (FTIR) spectroscopy. Samples were analyzed over a wavelength range of 400–4000 cm^{-1}, and the resulting spectra were used to identify characteristic chemical bonds and functional groups associated with the bioactive constituents.

Antimicrobial Activity Assay

The antimicrobial activity of *C. bonplandianum* extracts was evaluated against fish pathogenic bacteria including *Staphylococcus aureus*, *Escherichia coli*, *Aeromonas hydrophila*, *Vibrio campbellii*, *Vibrio parahaemolyticus*, *Bacillus* spp., *Streptococcus* spp., and *Salmonella* spp. using the agar well diffusion method. Fresh bacterial cultures were grown in nutrient broth and spread uniformly on Mueller–Hinton agar plates. Wells were created in the agar and filled with predetermined concentrations of plant extracts. Standard antibiotic discs served as positive controls. Following incubation at 37°C for 24 h, antimicrobial activity was assessed by measuring the diameter of inhibition zones surrounding the wells.

Experimental Fish and Feeding Trial

Juvenile *Labeo rohita* were obtained from a local fish farm and acclimatized under laboratory conditions for five weeks prior to experimentation. Fish were maintained in aerated tanks under natural photoperiod conditions and fed a commercial diet containing 40% crude protein at 4% body weight per day. Water quality parameters were monitored regularly throughout the acclimatization and experimental periods.

The experimental diets were prepared by incorporating different concentrations of *C. bonplandianum* crude extract into commercial fish feed pellets. Four experimental groups were established: control diet, pathogen-challenged group, pathogen-challenged group supplemented with 0.25 mg extract, and pathogen-challenged group supplemented with 0.75 mg extract. The diets were prepared weekly and supplied twice daily during the experimental period.

Growth Performance Evaluation

Following acclimatization, fish with an average weight of approximately 4.5 g were randomly distributed into experimental net cages. Each treatment consisted of three replicates containing 25 fish per cage. The feeding trial was conducted for 150 days. Body weight and total length of fish were recorded at 15-day intervals. Growth performance parameters including weight gain (WG), length gain (LG), feed conversion ratio (FCR), and survival rate (SR) were calculated using standard formulae. These parameters were used to evaluate the effects of dietary supplementation with *C. bonplandianum* extract on fish growth, health, and survival under pathogen challenge conditions.

Results

4.1. Prevalence of Total Heterotrophic Bacteria (THB)

The prevalence of total heterotrophic bacteria (THB) in water samples collected from the culture ponds varied throughout the experimental period. The bacterial count ranged from 16

× 10^5 CFU mL^{-1} to 28 × 10^5 CFU mL^{-1}. The lowest count was recorded during Period II (61 days) with 16 × 10^5 CFU mL^{-1}, whereas the highest count was observed during Period V (151 days) with 28 × 10^5 CFU mL^{-1}. Similarly, the percentage occurrence of THB increased progressively from 15% in Period II to 25% in Period V, indicating a gradual increase in microbial abundance with the advancement of the culture period.

In sediment samples, THB counts exhibited considerable variation among the sampling periods. The bacterial population ranged between 17 × 10^5 and 27 × 10^5 CFU mL^{-1}. The highest bacterial load was recorded during Period III (91 days), reaching 27 × 10^5 CFU mL^{-1}, whereas the lowest count was observed during Period I (31 days) with 17 × 10^5 CFU mL^{-1}. The percentage occurrence followed a similar trend, increasing from 15% during the initial culture period to 25% during Period III, suggesting that sediment served as a major reservoir for bacterial proliferation.

The gill samples of *Labeo rohita* showed THB counts ranging from 12 × 10^5 to 27 × 10^5 CFU mL^{-1}. The maximum bacterial prevalence was recorded during Period V (151 days), with a count of 27 × 10^5 CFU mL^{-1} and a percentage occurrence of 30%. In contrast, the minimum count of 12 × 10^5 CFU mL^{-1} and 13% prevalence was observed during Period IV (121 days). These findings indicate that the gill tissue was highly susceptible to bacterial colonization, particularly during the later stages of culture.

In the gut samples, THB counts ranged from 12 × 10^5 to 22 × 10^5 CFU mL^{-1}. The highest bacterial load was observed during Period IV (121 days), reaching 22 × 10^5 CFU mL^{-1} with a prevalence of 26%, while the lowest count of 12 × 10^5 CFU mL^{-1} and 14% occurrence was recorded during Period II (61 days). The increased bacterial abundance in the gut during the later culture periods may be associated with changes in feeding activity and microbial colonization within the digestive tract.

The skin samples consistently exhibited higher bacterial populations than most other fish organs. THB counts ranged from 21 × 10^5 to 29 × 10^5 CFU mL^{-1} throughout the study period. The highest bacterial prevalence was observed during Period V (151 days), with a count of 29 × 10^5 CFU mL^{-1} and a percentage occurrence of 22%. The lowest count was recorded during Period II (61 days), with 21 × 10^5 CFU mL^{-1} and 16% prevalence. The results suggest that fish skin serves as a primary interface for microbial attachment and colonization in aquaculture environments.

Overall, comparison among different sample types revealed that the highest THB count (29 × 10^5 CFU mL^{-1}) was observed in fish skin during the final culture period, while the lowest count (12 × 10^5 CFU mL^{-1}) was recorded in both gill and gut samples during the early culture stages. Principal Component Analysis (PCA) further demonstrated substantial variation in THB distribution among sampling periods and sample types. The PCA results indicated that Axis I explained 56% of the total variance, followed by Axis II (24.7%), Axis III (16.4%), and Axis IV (3%). The clustering pattern suggested a strong association between increased bacterial prevalence and the later culture periods, particularly in sediment, gill, and skin samples (Table 1-5).

Table 1. Total heterotrophic bacteria in culture ponds in the water samples (in count 10^5 CFU/ml), percentage %

S. No	Bacterial Count	Water sample CFU/ml count X10^5 %				
		Period I	Period II	Period III	Period IV	Period V
1	THB CFU/ml	19	16	22	25	28
2	THB CFU/ml %	17	15	20	23	25

Table 2. Total heterotrophic bacteria in culture ponds in the sediment samples (in count 10^5 CFU/ml), percentage %

S. No	Bacterial Count	Sediment sample CFU/ml count X 10^5 %				
		Period I	Period II	Period III	Period IV	Period V
1	THB CFU/ml	17	24	27	19	23
2	THB CFU/ml %	15	22	25	17	21

Table 3. Total heterotrophic bacteria in culture ponds in the fish gill samples (in count 10^5 CFU/ml), percentage

S. No	Bacterial Count	Gill sample CFU/ml count X10^5 %				
		Period I	Period II	Period III	Period IV	Period V
1	THB CFU/ml	21	16	15	12	27
2	THB CFU/ml %	23	18	16	13	30

Table 4. Total heterotrophic bacteria in culture ponds in the fish gut samples (in count 10^5 CFU/ml), % percentage

S. No	Bacterial Count	Gut sample CFU/ml count X10^5 %				
		Period I	Period II	Period III	Period IV	Period V
1	THB CFU/ml	15	12	14	22	21
2	THB CFU/ml %	18	14	17	26	25

Table 5. Total heterotrophic bacteria in culture ponds in the fish skin samples (in count 10^5 CFU/ml)

S. No	Bacterial Count	Skin sample CFU/ml count X10^5 %

		Period I	Period II	Period III	Period IV	Period V
1	THB CFU/ml	26	21	27	28	29
2	THB CFU/ml %	20	16	21	21	22

4.2. Isolation and Identification of Pathogenic Bacteria

A total of eight bacterial strains were successfully isolated from infected *Labeo rohita* and identified based on their morphological and biochemical characteristics. The isolates were identified as *Staphylococcus aureus* (E1), *Escherichia coli* (E2), *Aeromonas hydrophila* (E3), *Vibrio campbellii* (E4), *Vibrio parahaemolyticus* (E5), *Bacillus* sp. (E6), *Streptococcus* sp. (E7), and *Salmonella* sp. (E8). The isolates exhibited distinct biochemical profiles with variations in catalase, oxidase, indole production, methyl red, Voges–Proskauer reaction, citrate utilization, urease activity, nitrate reduction, and carbohydrate fermentation patterns. Comparison of the observed characteristics with Bergey's Manual of Determinative Bacteriology confirmed the taxonomic identity of the bacterial isolates. These pathogenic bacteria were subsequently selected for antimicrobial screening studies(Table 6).

Table 6. Biochemical characterization of pathogenic bacteria from Rohu

	Biochemical Test	**E1**	**E2**	**E3**	**E4**	**E5**	**E6**	**E7**	**E8**
1	Morphology	**C**	**R**	R	R	R	R	**C**	**R**
2	Motility	+	-	+	-	+	+	+	-
3	Catalase	+	-	-	+	+	-	-	+
4	Oxidase	+	+	+	-	+	+	+	-
5	Indole	-	-	-	+	+	-	-	-
6	Methyl red	-	-	+	+	-	+	-	+
7	Vogesprouskaur	-	+	+	+	+	+	-	-
8	Citrate Utilization	+	+	+	+	+	+	-	-
9	TSI	+	+	-	-	-	-	+	-
10	Urease	+	-	-	+	+	-	-	-
11	Nitrate reduction	+	-	+	+	+	+	+	+
12	Dextrose	+	+	+	+	+	+	-	-
13	Glucose	+	-	-	+	+	-	-	-
14	Lactose	-	-	+	+	+	+	-	-
15	Maltose	+	-	-	+	+	-	-	-
16	Sucrose	-	-	-	+	+	-	-	+

4.3. Phytochemical Characterization of Croton bonplandianum Extracts

Qualitative phytochemical screening revealed the presence of several bioactive compounds in the leaf extracts of *Croton bonplandianum*. The ethanolic extract contained steroids, saponins, tannins, terpenoids, flavonoids, and phenolic compounds, indicating a rich phytochemical composition. The acetone extract showed the presence of saponins, tannins, flavonoids, and phenols, whereas the petroleum ether extract contained steroids, terpenoids, and tannins. Alkaloids, anthraquinones, and glycosides were absent in all three solvent extracts. The results suggest that ethanol was the most effective solvent for extracting biologically active phytochemicals from *C. bonplandianum* leaves.

Thin Layer Chromatography (TLC) analysis of the ethanolic extract revealed eight distinct compounds with Rf values ranging from 0.133 to 0.833. UV illumination and staining reactions indicated the presence of multiple bioactive fractions, including compounds associated with antimicrobial activity. UV–Visible spectroscopic analysis demonstrated characteristic absorption peaks at wavelengths between 345 and 787 nm, confirming the presence of diverse phytochemical constituents. FTIR analysis further revealed major absorption bands at 3399, 1618, 1510, 1395, 1245, 1075, 923, 773, and 620 cm^{-1}, corresponding to hydroxyl, carbonyl, aromatic, and other functional groups commonly associated with phenolic and flavonoid compounds (Table 7).

Table 7. Phytochemical analysis of extract of selected *C. bonplandianum* by standard protocols

Sl.No	Phytochemical constituents	Ethanol extract	Acetone extract	Petroleum ether extract
1	Alkaloid	-	-	-
2	Saponin	+	+	-
3	Steroids	+	-	+
4	Tannin	+	+	+
5	Terpenoids	+	-	+
6	Anthraquinones	-	-	-
7	Flavonoids	+	+	-
8	Glycosides	-	-	-
9	Phenols	+	+	-

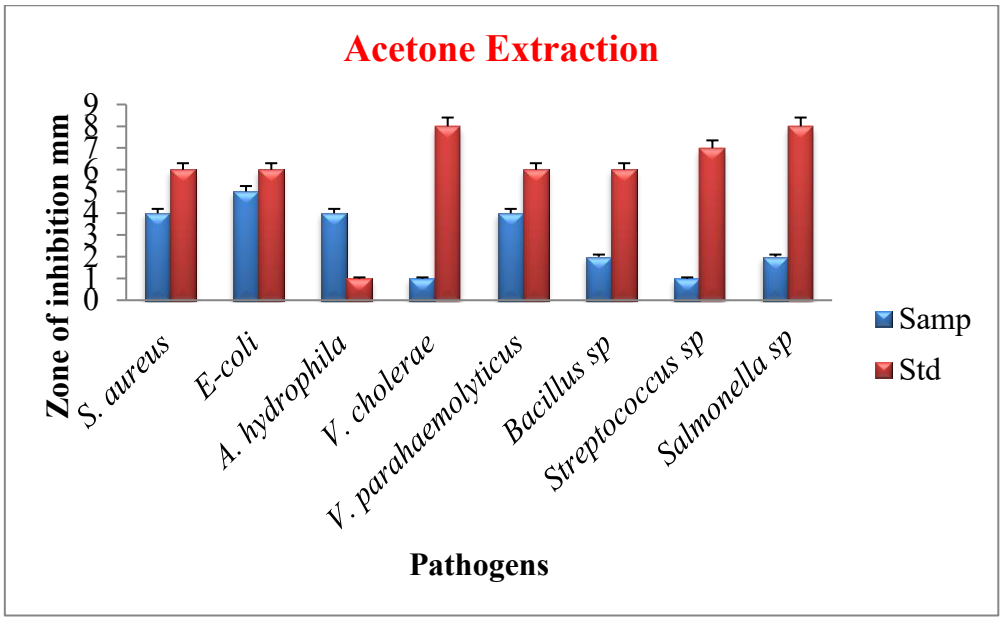
+ : Present; - : Absent

4.4. Antibacterial Activity of Croton bonplandianum Extracts

The antibacterial activity of *C. bonplandianum* extracts varied according to the extraction solvent and bacterial species tested. The ethanolic extract exhibited the strongest antibacterial activity, producing a maximum zone of inhibition of 5 mm against *Vibrio campbellii*, *Staphylococcus aureus*, and *Escherichia coli*. The lowest inhibition zone (1 mm) was observed against *Aeromonas hydrophila*. These findings indicate the superior efficacy of the ethanolic extract against fish pathogenic bacteria(Fig 1).

The acetone extract also demonstrated antibacterial activity, with the highest inhibition zone of 5 mm recorded against *Escherichia coli*, while *Streptococcus* sp. showed the least susceptibility with a zone of inhibition of only 1 mm. In contrast, the petroleum ether extract exhibited maximum antibacterial activity against *Salmonella* sp., producing a 5 mm inhibition zone, whereas *Aeromonas hydrophila* remained the least susceptible bacterial species. Overall, the results suggest that the antimicrobial potential of *C. bonplandianum* is strongly influenced by the extraction solvent, with ethanolic extracts displaying the broadest spectrum of antibacterial activity.

Fig 1. Antimicrobial activity of *C. bonplandianum* against fish pathogens



4.5. Growth Performance of Labeo rohita

Dietary supplementation with *C. bonplandianum* extract significantly enhanced the growth performance of *Labeo rohita*. At the end of the 150-day culture period, fish fed the diet containing 75 mg g^{-1} crude extract exhibited the highest weight gain (341 ± 5.3 mg), followed by fish receiving 25 mg g^{-1} extract (267 ± 5.2 mg). In comparison, the control group attained a final weight of 201 ± 2.7 mg, while pathogen-challenged fish without extract supplementation showed the lowest weight gain (89 ± 1.7 mg). These results clearly demonstrate the growth-promoting effect of *C. bonplandianum* supplementation.

Similarly, the highest length gain was recorded in fish fed the 75 mg g^{-1} extract diet, reaching 17.3 ± 5.3 cm at the end of the experiment. Fish receiving 25 mg g^{-1} extract attained a final length of 15.8 ± 3.4 cm, whereas control and pathogen-treated groups achieved only 10.3 ± 1.4 cm and 7.8 ± 0.7 cm, respectively. The enhanced growth performance observed in extract-supplemented fish may be attributed to improved nutrient utilization and overall health status.

Feed conversion ratio (FCR) was also positively influenced by dietary supplementation. The highest FCR value (2.8 ± 0.5) was observed in fish fed the 75 mg g^{-1} extract diet, followed by the 25 mg g^{-1} treatment (2.6 ± 0.3). Lower FCR values were recorded in the control (2.1 ± 0.1) and pathogen-challenged groups (1.3 ± 0.2). These findings indicate that *C. bonplandianum* supplementation enhanced feed utilization efficiency and promoted better growth performance in *L. rohita* under culture conditions (Table 8).

Table 8. Effect of feeding different doses of *C. bonplandianum* on FCR of Rohu

S. No	No. Days	Control	Treated II Pathogen	Treated III 25 Mg	Treated IV 75 Mg
1	1	1.2±0.2	1.3±0.1	1.5±0.4	1.6±0.4
2	15	1.3±0.2	1.1±0.1	1.4±0.4	1.5±0.5
3	30	1.6±0.1	1.2±0.1	1.5±0.3	1.8±0.5
4	45	1.7±0.2	1.2±0.1	1.6±0.3	1.7±0.5
5	60	1.6±0.3	1.3±0.2	1.8±0.4	2.1±0.4
6	75	1.8±0.1	1.2±0.2	2.1±0.4	2.2±0.5
7	90	1.9±0.3	1.3±0.1	2.3±0.3	2.4±0.4
8	105	2.1±0.2	1.2±0.1	2.2±0.4	2.6±0.5
9	120	2.2±0.1	1.4±0.2	2.4±0.4	2.5±0.4
10	135	2.4±0.2	1.5±0.1	2.5±0.4	2.7±0.5
11	150	2.1±0.1	1.3±0.2	2.6±0.3	2.8±0.5

5. Discussion

The present study demonstrated a significant prevalence of total heterotrophic bacteria (THB) in water, sediment, gill, gut, and skin samples collected from *Labeo rohita* culture ponds. Among the different sample types, fish skin and gill tissues exhibited higher bacterial loads during the later culture periods. This observation is consistent with previous reports indicating that fish skin and gills are continuously exposed to the surrounding aquatic environment and therefore serve as primary sites for microbial colonization (Austin & Austin, 2016). The gradual increase in THB counts observed during the culture period may

be attributed to increased organic matter accumulation, feed residues, fish excreta, and environmental fluctuations that favor bacterial growth. Similar findings have been reported by Das et al. (2022), who observed increased bacterial abundance in aquaculture ponds with prolonged culture duration and higher nutrient loading.

The sediment samples also showed considerable bacterial prevalence throughout the study. Sediments are recognized as reservoirs of diverse microbial communities because they accumulate organic debris and nutrients that support bacterial proliferation (Boyd & Tucker, 2012). The higher bacterial density observed in sediment compared to water during several sampling periods agrees with the findings of Banerjee et al. (2021), who reported that pond sediments harbor significantly greater bacterial populations than overlying water due to their rich organic content. The PCA analysis further confirmed the association between increasing bacterial abundance and culture duration, indicating that microbial dynamics are strongly influenced by environmental and management factors.

Eight pathogenic bacterial species, namely *Staphylococcus aureus*, *Escherichia coli*, *Aeromonas hydrophila*, *Vibrio campbellii*, *Vibrio parahaemolyticus*, *Bacillus* sp., *Streptococcus* sp., and *Salmonella* sp., were isolated and identified from infected *L. rohita*. Among these, *Aeromonas* and *Vibrio* species are recognized as important opportunistic pathogens responsible for severe disease outbreaks in freshwater and brackish water aquaculture systems (Harikrishnan et al., 2023). The occurrence of these bacterial pathogens confirms their widespread distribution in culture environments and their potential role in reducing fish health and productivity. Similar bacterial communities have been reported from diseased carp species in Indian aquaculture systems (Pakhira et al., 2024).

Phytochemical screening revealed that the ethanolic extract of *Croton bonplandianum* contained several bioactive compounds, including flavonoids, tannins, saponins, terpenoids, steroids, and phenolic compounds. These phytochemicals are known to possess antimicrobial, antioxidant, anti-inflammatory, and immunostimulatory properties (Saxena et al., 2013). The higher number of phytoconstituents detected in the ethanolic extract compared to acetone and petroleum ether extracts suggests that ethanol is a more efficient solvent for extracting polar bioactive compounds. Similar observations have been reported by Kumar et al. (2024), who demonstrated that ethanolic extracts of *Croton* species contain higher concentrations of phenolic and flavonoid compounds than non-polar solvent extracts.

TLC, UV–Visible spectroscopy, and FTIR analyses confirmed the presence of multiple bioactive constituents in the crude extract. The FTIR absorption bands corresponding to hydroxyl, carbonyl, and aromatic functional groups indicate the presence of phenolic and flavonoid derivatives, which are frequently associated with antimicrobial activity (Silverstein et al., 2014). The occurrence of these compounds supports the traditional medicinal use of *C. bonplandianum* and provides evidence for its potential application as a natural therapeutic agent in aquaculture.

The antibacterial assays revealed that the ethanolic extract exhibited greater inhibitory activity against fish pathogenic bacteria than acetone and petroleum ether extracts. The strongest antibacterial effect was observed against *Vibrio campbellii*, *Staphylococcus aureus*,

and *Escherichia coli*. These findings suggest that the active phytochemicals present in the ethanolic extract effectively disrupt bacterial growth and metabolism. Previous studies have shown that flavonoids and phenolic compounds can alter bacterial cell membrane permeability, inhibit enzyme activity, and interfere with nucleic acid synthesis, leading to bacterial growth suppression (Cushnie & Lamb, 2011). Similar antimicrobial effects of *Croton* species against Gram-positive and Gram-negative bacteria have been reported by Rios and Recio (2005) and Kumar et al. (2024).

One of the most significant findings of the present investigation was the enhanced growth performance of *L. rohita* following dietary supplementation with *C. bonplandianum* extract. Fish receiving the 75 mg extract diet exhibited the highest weight gain, length gain, and feed conversion ratio compared to the control and pathogen-challenged groups. The improved growth may be attributed to the presence of bioactive phytochemicals that enhance digestion, nutrient absorption, immune function, and metabolic efficiency. Herbal feed additives have been widely reported to stimulate digestive enzyme activity and improve feed utilization in cultured fish species (Reverter et al., 2014).

The poor growth performance observed in pathogen-challenged fish confirms the detrimental effects of bacterial infections on fish metabolism and nutrient utilization. In contrast, extract-supplemented fish showed improved resistance and better growth performance, suggesting that *C. bonplandianum* may possess immunoprotective properties. Similar improvements in growth and disease resistance have been reported in carp and tilapia fed diets supplemented with medicinal plant extracts rich in flavonoids and phenolic compounds (Awad & Awaad, 2017). Overall, the present study demonstrates that *Croton bonplandianum* possesses significant antimicrobial and growth-promoting properties. The presence of diverse phytochemicals, coupled with strong antibacterial activity and enhanced growth performance, highlights its potential as a sustainable phytotherapeutic alternative to synthetic antibiotics in aquaculture. The use of plant-based feed additives may contribute to improved fish health, reduced disease incidence, and environmentally friendly aquaculture practices.

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