IMMUNOSTIMULATORY EFFECT OF BETAL LEAF EXTRACT ON *Labeo catla* AND ITS RESISTANCE AGAINST *Aeromonas hydrophila*

ABSTRACT

Aquaculture plays a vital role in global fish production, but rising antibiotic resistance threatens the sustainability of this industry. This study explores the potential of betel leaf extract as a natural immunostimulant to improve fish health. The present experiment investigated the immunostimulatory effect of betel leaf extract on *Labeo catla* and its resistance against *Aeromonas hydrophila*. The experiment was conducted in 2024 at the Department of Aquaculture, College of Fisheries Science, Kamdhenu University, Veraval. It was conducted in rectangular plastic tanks using a Completely Randomized Design (CRD) with five treatments and four replications. T1, T2, T3, and T4 diets were prepared by incorporating betel leaf extract at 0.2%, 0.4%, 0.6%, and 0.8%, respectively, while T0 served as the control. The extract was directly supplemented into the feed and processed into feed to ensure uniform distribution. Fish were fed at 6% of their body weight twice daily for 45 days. Key parameters such as specific growth rate (SGR), feed conversion ratio (FCR), total erythrocyte count (TEC), total leucocyte count (TLC), respiratory burst activity, and disease resistance were assessed. The highest performance was observed in T4, which recorded an SGR of 1.49 ± 0.008, an FCR of 1.99 ± 0.02, and a survival rate of 93.33 ± 1.66% ($P < 0.05$). Immunological parameters were also significantly improved in T4, with TEC at 1.695 ± 0.006 cells/mm³ and TLC at 28.2 ± 0.06 cells/mm³ ($P < 0.05$). A challenge study against *A. hydrophila* confirmed that T4 exhibited the highest survival rate (93.33%). These findings suggest that incorporating betel leaf extract at 0.8% in fish feed enhances growth, immune response, and disease resistance, making it a viable natural alternative to antibiotics for sustainable aquaculture.

Keywords: Betel leaf extract, *Labeo catla*, *Aeromonas hydrophila*, Immunostimulant, Sustainable aquaculture.

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1. INTRODUCTION

Aquaculture is a critical sector that supplies over half of the global fish demand, contributing significantly to food security, economic growth, and employment (Action, 2020). With the increasing global population and the depletion of wild fish stocks, aquaculture provides a sustainable solution to meet the rising demand for fish protein. However, the rapid intensification of aquaculture practices has led to several challenges, including disease outbreaks, poor water quality, and the overuse of antibiotics (Dawood et al., 2020). Among these, bacterial infections, particularly those caused by *Aeromonas hydrophila*, severely threaten fish health and productivity, leading to substantial economic losses in aquaculture operations worldwide (Harikrishnan et al., 2011).

Aeromonas hydrophila is a gram-negative, opportunistic pathogen responsible for motile aeromonad septicemia (MAS) in various freshwater fish species. Infections caused by this pathogen can lead to high mortality rates, skin lesions, hemorrhages, and severe tissue damage, ultimately affecting fish growth and market value (Mariappan et al., 2023) Aquaculturists have traditionally relied on antibiotics and chemical treatments to combat such infections. However, the indiscriminate and excessive use of antibiotics has led to the emergence

of antimicrobial resistance (AMR), which threatens fish and human health through the food chain (Cabello et al., 2013). This has necessitated the urgent need for sustainable and eco-friendly alternatives to antibiotics in disease management.

One promising approach in aquaculture disease management is using natural plant-based immunostimulants. Medicinal plants have long been recognised for their bioactive compounds, which exhibit antimicrobial, anti-inflammatory, antioxidant, and immunostimulatory properties (Calow, 1985). Among these, betel leaf (*Piper betle*), a medicinal plant widely used in traditional medicine across Asia, has gained attention for its potential in aquaculture. Betel leaf contains alkaloids, flavonoids, tannins, phenols, and essential oils, which possess antibacterial, immunomodulatory, and growth-promoting effects (Tutu et al., 2022). These bioactive compounds can help enhance fish's immune system, improve resistance against bacterial infections, and support overall physiological health.

In addition to its antimicrobial properties, betel leaf extract has been reported to improve fish feed utilization, growth performance, and stress tolerance (Zaeroni, Setyowati, & Azhar, 2022). The ability of betel leaf extract to enhance nonspecific immune responses, such as phagocytic activity and respiratory burst, further underscores its potential as an effective immunostimulant. Despite its known medicinal properties, the application of betel leaf extract in aquaculture remains relatively unexplored. Therefore, this study aims to evaluate the impact of betel leaf extract dietary supplementation on the growth, immune response, and disease resistance of *Labeo catla*, a commercially important freshwater fish species.

By incorporating betel leaf extract into fish diets, this study seeks to provide a sustainable alternative to antibiotics, addressing the concerns of AMR while improving fish health and productivity. The findings from this research could contribute significantly to the growing field of herbal immunostimulants in aquaculture, paving the way for developing novel, cost-effective, and eco-friendly strategies for fish disease management.

2. Materials and Methods

2.1 Experimental Setup: The experiment involved 20 tanks (2x1x1 feet, 50 L capacity), each assigned to one of five treatments (T0–T4) with four replicates. Fish were acclimatized for 10 days before stocking (15 fish per tank). Water quality parameters were maintained within optimal ranges, including temperature, dissolved oxygen, and pH.

2.2 Betel leaf extract

The fresh *Piper betle* leaves with bright green were bought from the local market. The leaves were washed in distilled water to remove all the contaminants. The betel leaf extract preparation involved washing the leaves, drying them, and grinding them into powdered form. It was then followed by maceration in 96% ethanol as a solvent. The mixture was then filtered thrice to obtain a concentrated extract. Finally, the extract was thickened and concentrated with a Rotary Vacuum Evaporator at 50°C (Rathee et al., 2006).

2.3 Feed formulation

All the experimental diets had a consistent protein level of 30%. Five experimental diets (T0 to T4) were formulated for the study. Among these, T1, T2, T3, and T4 were supplemented with betel leaf extract, while T0 acted as the control diet, which did not contain betel leaf extract. These ingredients were thoroughly mixed with water to make a dough in a plastic tray. The dough was then subjected to a thermal treatment, wherein it was steamed at

121°C under 15 lbs pressure for 15 minutes.

After that, vitamins and minerals were added to the mixture and blended evenly. Afterwards, the betel leaf extract was incorporated according to the treatment details and mixed thoroughly. The feed mixture was processed into pellets using a mechanical pelletizer, resulting in uniformly sized pellets. The pellets were spread out on steel trays and dried in a batch dryer at temperatures ranging from 50°C to 60°C until the moisture content dropped below 10%. Finally, the dried pellets were stored in airtight plastic containers and sealed to maintain freshness.

2.4 Growth Parameters

To evaluate the growth of *Labeo catla*, ten fish from each experimental tank were caught and weighed every 15 days. Before measurement, the fish were fasted overnight to obtain precise weight readings. Weights were measured with an electronic balance, and each fish was placed in a glass beaker filled with water to reduce stress during the weighing process. Throughout the treatment, care was taken to minimize the fish's stress.

2.5 Total erythrocyte count (TEC)

The total number of red blood cells was determined using the method outlined by Lal (2010). Blood samples were diluted with RBC diluting fluid (Hayem's fluid) at a 1:200 ratio. A fresh blood sample was drawn into an RBC pipette up to the 0.5 mark, after which Hayem's fluid was added to reach the 101 mark. The mixture was thoroughly agitated and placed on a hemocytometer for cell enumeration. Under a high-magnification microscope, the counting chamber was inspected, and red blood cells were counted in five specified areas: four small squares at the corners and one in the centre. The total TEC was calculated using the appropriate formula.

2.6 Total leucocyte count (TLC)

The method described by (Lugowska, Kondera, & Witeska, 2017) was followed for TLC. The blood samples were diluted 1:20 in Turk's solution. Blood was drawn to the 0.5 mark Turk's fluid up to also the 11 mark in another WBC pipette. The content was well mixed in the pipette bulb, and then the diluted blood was deposited on a hemocytometer for cell counting. The counting chamber was examined under a high-power microscope, and leucocytes were counted from four outer squares. Subsequently, the total number of TLC cells was calculated using the formula.

2.7 Respiratory Burst Assay

Respiratory burst activity was determined using the NBT reduction assay of (Biller-Takahashi et al., 2013). using a plate reader to measure the amount of formazan produced to indicate superoxide anion formation. A 50-µl blood sample with anticoagulant was incubated at room temperature for 1 hour to allow cell attachment. The cells were washed with PBS three times to discard the supernatant after incubation. Then, an additional 50 µl of PBS containing 0.2% NBT solution was added for the next incubation period of 30 minutes. The NBT solution was removed, and methanol (100%) was added to fix the cells for 2- 3 minutes, washing with 30% methanol three times (Plate 5G). It was left to dry with air for a few minutes. The dried sample was treated with 60 µl of KOH and 70 µl of DMSO. A plate spectrophotometer measured the optical density (OD) of the 1:100 diluted sample at 630 nm.

2.8 Stock culture preparation of *Aeromonas hydrophila*

This stock culture of *Aeromonas hydrophila* was prepared by following the process described by (Maiti et al., 2023). In nutrient broth at 37°C, the bacteria grew for a full day. After incubation, the culture was centrifuged at 3000 rpm for ten minutes. The produced particle was washed with phosphate-buffered saline (PBS, pH 7.4), and the supernatant was discarded. The optical density was measured at 456nm using the spectrophotometer to standardize the bacterial cells and brought to 0.5,

equivalent to 1×10^6 cells/ml of bacterial cells.

2.8.1 Dose preparation: Serial dilutions were made with phosphate-buffered saline (PBS) to create doses of 1×10^6 cells/ml, 1×10^5 cells/ml, and 1×10^4 cells/ml. Fish were exposed to these doses to assess their susceptibility to infection over 24–48 hours. A 50% mortality rate was observed at the dose of 1×10^6 cells/ml. Consequently, this dose was selected for the challenge study in the experiment.

2.8.2 Challenge study: Thirty fish from each replicate and treatment group were randomly selected for the challenge study. Each fish was injected intramuscularly with a dose of 1×10^6 cells/ml of *Aeromonas hydrophila.* Over the following 10 days, the fish were monitored for signs of disease and mortality.

2.8 Statistical Analysis: The data were analyzed using one-way ANOVA followed by Tukey's post hoc test to determine significant differences between treatments ($P < 0.05$). All statistical analyses were performed using SPSS software.

3. RESULT AND DISCUSSION

3.1 Mean weight gain: In the 15 days, the experimental group T0 had the least weight gain compared to T1 (0.2% extract) and T2 (0.4% extract). The highest weight increase was observed in T3, which had a 0.6% extract, and T4, which had a 0.8% extract. In terms of weight gain at 30 days, T4 had the highest rate, followed by T3, and both these groups were significantly different from the lower concentration groups. At the end of the final week, the T4 gained more weight than the other treatments; the mean weight gain indicated a higher growth rate, thus superior to all other treatments.

3.2 Specific Growth Rate: After 45 days, treatment T4 with 0.8% betel leaf extract had the highest SGR of (1.490±0.008), which was significantly ($P < 0.05$) higher than all the other groups. T3 (1.382±0.006) and T2 (1.357±0.021) had moderate SGR, while T0 (1.230±0.047) and T1 (1.242±0.057) had the lowest SGR and were statistically similar.

3.3 Feed conversion ratio: The FCR value for T4 (0.8% betel leaf extract) was the lowest, which means it has the best feed efficiency among all the treatments. T3 and T2 had extremely close to slightly higher FCR values, but the two treatments did not differ significantly ($P > 0.05$) from T4. T1 and T0 presented the highest FCR values, statistically higher than T2, T3, and T4.

3.4 Survival Rate: The survival rate of *Labeo catla* was significantly influenced ($P <0.05$) by dietary supplementation with betel leaf extract. T4 (0.8% extract) recorded the highest survival rate, while T0 (control) had the lowest.

Table 1: Mean weight gain (g) of *Labeo catla* recorded in all treatments after 45 days

Replication	R1	R2	R3	R4	Mean±SE
T0	1.82	1.92	1.90	1.85	1.8725 ± 0.045^{d}
T1	1.73	2.04	1.97	1.92	1.9150 ± 0.025^{d}
T2	2.11	2.12	2.17	2.19	2.1475 ± 0.038^{c}
T3	2.17	2.23	2.23	2.25	2.2200 ± 0.034^{b}
T4	2.46	2.45	2.47	2.46	2.4600 ± 0.008^{a}

Table 2: Specific growth rate (SGR) of *Labeo catla* recorded in all after 45 days

Replication	R1	R2	R3	R4	MEAN±SE
T0	1.23	1.34	1.24	1.11	1.227 ± 0.017^{c}
T1	1.08	1.35	1.29	1.25	1.242 ± 0.031^{c}
T2	1.31	1.34	1.37	1.41	1.357 ± 0.021^{b}
T3	1.38	1.38	1.37	1.40	1.382 ± 0.006^{b}
T4	1.49	1.47	1.51	1.49	1.490 ± 0.008^{a}

Table 3: Feed conversion ratio of *Labeo catla* recorded in all treatments after 45 days

Replication	R1	R2	R3	R4	MEAN±SE
T0	2.86	2.48	2.59	2.44	2.59 ± 0.09^{c}
T1	2.62	2.48	2.48	2.62	2.55 ± 0.04^{c}
T2	2.37	2.50	2.29	2.13	2.32 ± 0.07^{b}
T3	2.31	2.07	2.24	2.21	2.20 ± 0.05^{b}
T4	2.07	1.97	1.98	1.97	1.99 ± 0.02^{a}

Table 4: Survival (%) of *Labeo catla* recorded in all treatment during after 45 days

Replication	R1	R2	R3	R4	MEAN±SE
T0	11	13	14	10	80 ± 0.24^{b}
T1	13	14	12	13	86.66 ± 0.20^{ab}
T2	14	13	13	12	86.66 ± 0.23^{ab}
T3	12	14	13	13	86.66 ± 0.25^{ab}
T4	15	13	13	14	93.33 ± 0.12^{a}

3.5 Respiratory Burst Assay: The higher superoxide anion production was recorded in treatment T4 (0.55+0.004) followed by T3 (0.52±0.009). There was a significant difference of ($P < 0.05$) in treatment T4 compared to the other table. Meanwhile, lower values were recorded in T0 (0.34+ 0.004), TI (0.36+0.006), and T2 (0.44+0.004). This enhanced RBA at progressively higher concentrations of betel leaf extract supports the immunomodulator function of betel leaves. The innate immune response mechanism called respiratory burst involves the fast generation of ROS needed to destroy pathogens, such as macrophages and neutrophils, in the body. In *Labeo catla*, RBA is an efficient biomarker for judging herbal immunomodulator's immune competence and effectiveness (Biller-Takahashi et al., 2013). The complex bioactive, including eugenol, chavicol, and flavonoids found in betel leaf, result in reinforced phagocytic activity and ROS production concurrent with a heightened immune response (Datta et al., 2011). These compounds collectively enhance the overall degradation of pathogens and raise

the organism's ability to combat infections (Fawole et al., 2024).

3.6 Total erythrocyte count (TEC): The TEC was also significantly raised in T4 to (1.695±0.006 × 10^6cells/mm^3) than the other groups ($P < 0.05$). Betel leaf extract enhances the formation of red blood cells since hematopoiesis is promoted by utilizing an antioxidant that minimizes damage to red blood cell formation. It also improves the bioavailability and utilization of important micro elements, such as iron, copper, and folic acid, which are important for haemoglobin formation and erythropoiesis (Saputra et al., 2023). Also, it has an antimicrobial effect that helps in maintaining gut health, and there is minimal nutrient loss through infections; its anti-inflammatory and immunostimulant properties decrease stress and, with it, inflammation, enhancing the production of red blood cells (Singh & Patel, 2018; Gupta et al., 2020).

3.7 Total leucocyte count (TLC): The counts were determined per cubic meter. Treatment T4 exhibited the highest total leucocyte count (28.2 ± 0.06). According to (Nafiqoh et al., 2020). betel leaf extract contains compounds that function as immunostimulants, enhancing the non-specific immune defenses in fish. The primary active components of betel leaf include essential oils, vitamins, organic acids, amino acids, sugars, tannins, fats, starches, and carbohydrates, which possess antibacterial, antiseptic, and immune-boosting properties. These compounds help strengthen the fish's immune system, increasing leucocyte counts, as white blood cells are crucial for the body's defense mechanisms.

3.8 Disease resistance: The T4 treatment group exhibited the highest survival rate (94±0.00%), $P < 0.05$), attributed to betel leaf extract's immunostimulatory, antioxidant, antimicrobial, and anti-inflammatory properties. Bioactive compounds like flavonoids and phenolics enhanced innate and adaptive immune responses, strengthening resistance to *Aeromonas hydrophila* infections (Biswas et al., 2022).

Table 5: Respiratory burst activity of *Labeo catla* recorded in all treatments

Respiratory Burst Activity					
Treatment	**T0**	**T1**	**T2**	**T3**	**T4**
Mean±SE	$0.34+0.004^{d}$	$0.36+0.006^{c}$	$0.46+0.004^{b}$	$0.47±0.009^{b}$	$0.55+0.00^{a}$

Total Erythrocytes count 10^6cells mm3					
TREATMENT	**T0**	**T1**	**T2**	**T3**	**T4**
MEAN±SE	$1.405±0.006^{d}$	$1.465±0.006^{c}$	$1.615±0.006^{b}$	$1.625±0.006^{b}$	$1.695±0.006^{a}$

Table 6: Impact of betel leaf extract on total erythrocyte count (TEC) of *Labeo catla*

Table 7: Impact of betel leaf extract on total leucocytes count (TLC) of *Labeo catla*

Total Leucocytes count 10^3 cells mm3					
TREATMENT	**T0**	**T1**	**T2**	**T3**	**T4**
Mean±SE	$21.8±0.06^{d}$	$23.2±0.09^{c}$	$25.8±0.02^{b}$	$26.2±0.04^{b}$	$28.2±0.06^{a}$

Table 8: Impact of betel leaf extract in on survival of *Labeo catla* after the challenge test

Treatment	T0	T1	T2	T3	T4
Survival Rate (%) at 10th day	66.66±0.75[d]	73.33±0.26[c]	80±0.14[b]	86.66±0.52[b]	93.33±0.77[a]

4. DISCUSSION

Comparison with Other Herbal Immunostimulants

The immunostimulatory effects of betel leaf extract observed in this study align with findings on other plant-derived immunostimulants such as garlic (*Allium sativum*), turmeric (*Curcuma longa*), and neem (*Azadirachta indica*). Studies have shown that these herbs enhance fish immune responses by increasing white blood cell count, respiratory burst activity, and antioxidant enzyme activity. Compared to these, betel leaf extract demonstrated superior enhancement in TEC, TLC, and disease resistance, making it a promising alternative for large-scale fish farming.

Growth and Survival

T4 (0.8% betel leaf extract) significantly improved growth parameters, with the highest weight gain (2.46 g) and SGR (1.49 ± 0.008, $P < 0.05$). The improved FCR (1.99 ± 0.02, $P < 0.05$) suggests better feed utilization efficiency. These results highlight the potential of betel leaf extract to promote growth while reducing feed costs.

Immunological Enhancement

Respiratory burst activity, an essential marker of innate immunity, was highest in T4 (0.55 ± 0.00, $P < 0.05$), confirming the immunostimulatory effects of betel leaf extract. TEC and TLC were also significantly elevated in T4 ($P < 0.05$), supporting enhanced immune function and disease resistance.

Disease Resistance

The challenge study against *A. hydrophila* showed that T4 had the highest survival rate (93.33%, $P < 0.05$), significantly higher than T0 (66.66%). This improvement is attributed to betel leaf compounds' antimicrobial and immunomodulatory properties such as eugenol and hydroxychavicol.

5. CONCLUSION

The research potential established that betel leaf has immunostimulatory properties and can increase disease resistance besides stimulating immunity *in Labeo catla*. Betel leaf extract contains compounds that enhance growth and stimulate appetite, possess wide spectrum antimicrobial properties, and may benefit carp culture in several ways. Among the treatments, T4 containing 0.8% betel leaf extract was found to be most effective in immunostimulant ability and enhancing the general resistance against *Aeromonas hydrophila*. Betel leaf extract at 0.8% significantly enhances growth performance, immune response, and disease resistance in *Labeo catla*. Its bioactive compounds provide a natural, eco-friendly alternative to antibiotics in aquaculture. Further studies are recommended to explore the long-term effects and optimal dosage levels for large-scale applications.

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