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

ABSTRACT

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 in the Department of Aquaculture at the College of Fisheries Science, Kamdhenu University Veraval. This experiment was done in rectangular plastic tanks with five treatments and put in a completely randomized CRD design. T1, T2, T3, and T4 diets were prepared by incorporating betel leaf extract 0.2%, 0.4%, 0.6%, and 0.8%, respectively, and T0 was the control. The experiment had four replications for each treatment. The fish were fed at 10% of their body weight twice daily for 45 days. Important parameters such as specific growth rate (SGR), feed conversion ratio (FCR), total erythrocyte count (TEC), total leukocyte count (TLC), respiratory burst activity, and disease resistance were assessed. The T4 treatment with 0.8% fish showed the highest SGR of 1.49 ± 0.008, the lowest FCR of 1.99 ± 0.02, and the highest survival rate of 93.33± 1.66%. Immunological parameters were also significantly improved in T4, where TEC was 1.695 ± 0.006 cells/mm³ and TLC was 28.2 ± 0.06 cells/mm³. In the challenge study against *A. hydrophila*, the highest survival rate was in T4 at 93.33%. It is suggested that the betel leaf extract should be included at a level of 0.8% for sustainable aquaculture.

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

1. INTRODUCTION

Today, aquaculture supplies more than half of the global fish demand. The world's rapidly growing food production sub-sector will continue providing solutions to food, income, and genuine livelihood crises by 2024 (FAO). Food fish production from aquaculture stood at 76,934 thousand tons in 2022, thus making the segment the only practical solution for meeting future demand while being less harmful to wild fish stocks. The other major carp in India is *Labeo catla* (Catla) since they mature fast, tolerate different cultural practices, and are most preferred in the market. This species is grown mainly in South Asia and is a significant component of world aquaculture. However, the aquaculture industry is threatened by serious economic threats resulting from *Aeromonas hydrophila* infectious diseases. This Gram-negative, opportunistic pathogen and the disease result in high mortality and loss in carp farming, causing MAS. Increased use of antibiotics to treat such diseases has led to AMR and, therefore, constitutes one of the biggest threats to humans, animals, and the environment. To overcome these challenges, there is growing attention to finding natural plant-based products that can replace antibiotics. Medicinal plants also have immunological, antibacterial, and growth-promoting activities, which have been widely acclaimed. Of these, the betel leaf (*Piper betle*), which is used in Southeast Asian herbal medicine, has come out to be highly promising. Being rich with alkaloids, flavonoids, and essential oils, the extract derived from betel leaf has been found to possess antibacterial and immunomodulatory properties that could effectively improve fish health and stress tolerance. This research aims to analyze the impact of betel leaf extract as a feed supplement for *Labeo Catla* in terms of growth

performance, survival rate, immune response, and disease tolerance against *A. hydrophila,* and an eco-friendly solution is proposed for aquaculture.

2. Materials and Methods

2.1 Experimental tanks: The research was conducted using 20 tanks, each with dimensions of 2x1x1 feet and a volume of 50 Liters. Before use, the tanks were disinfected with a potassium permanganate (KMnO4) solution at a concentration of 4 ppm, followed by sun-drying. The tanks were allocated to five treatments, with four replicates per treatment. They were subsequently filled with filtered and disinfected freshwater and provided with continuous aeration. *Labeo catla* was brought from a commercial hatchery. They were acclimatized in a 500-liter FRP tank for 5 days with constant aeration to ensure adaptability to experimental conditions. After acclimatization, fish were stocked in experimental tanks using a completely randomized (CRD) experiment. There were 300 fish divided as follows for treatment tanks: 15 fish were stocked in each experimental tank.

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 a temperature of 50°C (Setiawan et al., 2016).

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. Afterward, 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 Shah & Altindag (2004) 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 Secombes and Fletcher (1992) as modified by Stasiack and Baumann (1996), 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 Das et al. (2015). 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.

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	91	92	92	93	80 ± 0.24^{b}
T1	93	94	93	92	86.66 ± 0.20^{ab}
T2	95	96	96	96	86.66 ± 0.23^{ab}
T3	97	96	97	98	86.66 ± 0.25^{ab}
T4	99	98	98	98	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 (Bongaerts & Severijnen, 1994). 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 (Maheswari, 2008). These compounds collectively enhance the overall degradation of pathogens and raise the organism's ability to combat infections (Harikrishnan et al., 2011).

3.6 Total erythrocyte count (TEC): The TEC was also significantly raised in T4 to ($1.695\pm0.006 \times 10^{6}$cells/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 microelements, such as iron, copper, and folic acid, which are important for hemoglobin formation and erythropoiesis (Sarma et al., 2016). 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 Zainuddin et al. (2018), 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 (Dey, 2015).

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 10^{th} 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

The Specific Growth Rate (SGR) demonstrated a notable upward trend in growth performance with increasing concentrations of betel leaf extract, as observed in treatments T2, T3, and T4. Among these, T4 exhibited the highest mean SGR (1.490±0.0081), followed by T3 (1.382±0.0062), T2 (1.357±0.0213), T1 (1.242±0.0318), and the control group T0 (1.227±0.0170). This improvement in SGR with the addition of betel leaf extract to the diet can be attributed to the bioactive components such as hydroxychavicol and eugenol, which are known for their beneficial effects on fish health and growth (Shah et al., 2016). Fish treated with T4 (0.8% BLE) displayed significantly enhanced immune responses, as indicated by higher total erythrocyte count (TEC), total leucocyte count (TLC), and respiratory burst activity. These improvements are linked to the antibacterial and immunomodulatory actions of eugenol and chavicol, key constituents of betel leaf extract, which enhance resistance to infections and reduce stress, lowering disease risk (Davis & Gaylord, 2011).

T4 also observed improved growth because energy was allocated better than in T3. This greater immune response in the group mitigated the energy cost of fighting infections and left more nutrients and energy for growth and maintenance (Zhou et al., 2018). Because diseases act less frequently in the T4 group, the fish focused on body growth rather than having a time-consuming recovery process. This

led to enhanced specific incremental rates and increased general physiological upkeep. Furthermore, the anti-stress property of eugenol in stabilizing physiological functions improved metabolic features and prepared the fish for handling environmental adversities (Wendelaar Bonga, 1997).

The T4 group had the lowest feed conversion ratio, which shows efficient feed conversion into body tissues. Eugenol, an anti-stress agent obtained from betel leaf extract, is expected to enhance metabolic mobilization, better digestive efficiency, and better feed intake (Wendelaar Bonga, 1997). This combination of enhanced immunity and efficient metabolism enabled the fish in T4 to convert feed into body mass more effectively. The beneficial effects of betel leaf extract (*Piper betle*) are mainly attributed to its abundant bioactive compounds, including hydroxychavicol, eugenol, phenolics, and antioxidants like vitamin A. They exhibit high activity against bacteria and microbial activity, and they are important for enhanced growth, survival rate, immunological function, and disease tolerance in *Labeo catla* (Shah et al., 2016). Among the compounds, hydroxychavicol has evidenced strong antibacterial effects and might explain the increase in the survival rate after infection in T4 fish.

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*. This study's higher concentration of extract containing active antibacterial compounds resulted in improved durability, increased weight gain, low FCR, and high SGR. Having determined these, it is suggested that the dietary inclusion level of 8 g/kg betel leaf extract be used as the optimal and economical inclusion rate for enhancing growth, immune, and disease resistance of *Labeo catla.*

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