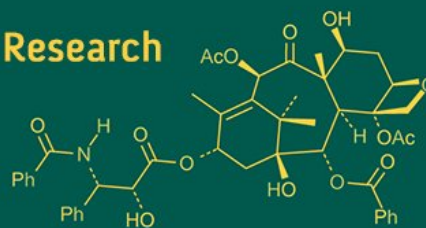


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Inclusion effect of onion (*Allium cepa*) and Neem (*Azadirachta indica*) powder in the diet of rohu (*Labeo rohita*): Growth, survival, immune response and disease resistance against *Aeromonas hydrophila*

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Abstract

The study was conducted to evaluate the effects of dietary supplementation with a mixture of onion (*Allium cepa*) and neem (*Azadirachta indica*) powders on the growth, survival, immunity, and disease resistance of *Labeo rohita* over 60 days. Diets were prepared with the mixture at 1%, 2%, 3%, and 4% inclusion levels (10, 20, 30, and 40g kg feed-1), along with a control diet. Growth performance indicators (mean weight, % weight gain, Specific growth rate, Feed conversion ratio), survival, hematological parameters (Total erythrocyte count, Total leucocyte count), respiratory burst activity, and resistance against *Aeromonas hydrophila* were assessed, while water quality remained within optimal ranges. Results showed that growth significantly improved at higher inclusion levels, with T₄ (4%) recording the highest mean weight, percentage weight gain, and Specific growth rate, along with the lowest Feed conversion ratio. Hematological analysis indicated enhanced immune responses in T₄, which showed the highest Total erythrocyte count, Total leucocyte count, and respiratory burst activity. Post-challenge tests revealed better survival in supplemented groups, with T₃ and T₄ showing the greatest resistance to *A. hydrophila*. Overall, inclusion of a 4% onion-neem powder mixture (20g kg-1) effectively enhanced growth, immune response, and disease resistance in *L. rohita*, suggesting its potential as a natural functional feed additive in aquaculture.

Keywords: *Labeo rohita*, onion and neem powder, immunostimulant, disease resistance, *Aeromonas hydrophila*

1. Introduction

Fisheries and aquaculture are vital for food security, nutrition and livelihoods in India, contributing nearly 8% to global fish production and ranking third after China (FAO, 2024; HoFS, 2022) [2]. The sector has shown rapid growth, recording a 9.03% increase from 2015-16 to 2020-21 (DOF, 2022) [2]. Globally, aquaculture has surpassed capture fisheries, producing 130.9 million tonnes in 2022, with inland aquaculture contributing more than 60% of farmed aquatic animal production (FAO, 2024) [4].

Rohu (*Labeo rohita*), one of the Indian major carps, is the most widely farmed freshwater species, contributing about 35% of total carp production in Asia (Khan *et al.*, 2004) [14]. Its strong growth potential, nutritional value and consumer preference make it a key species in carp polyculture systems of India and neighboring countries (Padmavathi & Veeraiah, 2009) [19]. However, disease outbreaks, particularly those caused by *Aeromonas hydrophila*, remain a serious problem, leading to heavy mortality and economic losses, with no effective vaccines currently available (Vivas *et al.*, 2004) [26].

Conventional approaches such as antibiotics and synthetic chemicals have been widely used for disease control, but their overuse raises concerns of antimicrobial resistance, environmental hazards and residue accumulation in fish. Vaccination can be prohibitively costly for fish farmers or may not be practical for all affected fish species (Raa, 1992; Sah *et al.*, 2018) [22, 20]. As a sustainable alternative, plant-based immunostimulants are increasingly being explored. These natural compounds, rich in bioactive substances such as phenolics and alkaloids, can improve growth, immunity, and disease resistance in fish while minimizing environmental risks (Rehman *et al.*, 2013; Cho *et al.*, 2012; Talpur *et al.*, 2013) [25, 8, 21].

Among them, onion (*Allium cepa*) and neem (*Azadirachta indica*) are well-known for their health-promoting properties. Onion contains vitamins, minerals, and quercetin derivatives that enhance fish growth, appetite, and immunity (Harris *et al.*, 2001; Cho & Lee, 2012)^[8, 11], while neem has antimicrobial, antiviral and antioxidant effects, boosting nonspecific immune responses (Helmy, 2007; Talpur & Ikhwanuddin, 2013)^[25, 12]. In this context, the present study was designed with three objectives: to evaluate the effect of onion and neem powder mixture on the growth and survival of rohu, to assess its role in disease resistance against *A. hydrophila* and to examine its influence on the immune response of rohu (*L. rohita*)

2. Materials and Methods

2.1 Experimental Setup

The experiment was conducted for 60 days from 18th September to 16th November 2024 at the Hands-on Training Centre, Department of Aquaculture, College of Fisheries

Science, Kamdhenu University, Veraval. Healthy fry of *L. rohita*, measuring 3.00 ± 0.50 cm in length and weighing 0.53 ± 0.03 g, were procured from the Indian Major Carp hatchery and transported in oxygenated polyethylene bags to the experimental site. On arrival, the fish seeds were acclimatized in 500-L plastic tank with continuous aeration for 7 days and were provided with a basal diet during this period. All required feed ingredients were collected for diet formulation, and their proximate composition was analyzed following standard procedures (AOAC, 2019)^[6].

2.2 Preparation of Experimental Diets

Five treatment diets were prepared, including a control diet (T_0) without onion and neem powder mixture and four treatment diets (T_1 , T_2 , T_3 and T_4) supplemented with onion and neem powder mixture with 1% in T_1 , 2% in T_2 , 3% in T_3 , 4% in T_4 . All experimental diets were formulated to contain a 30% protein level, with ingredients measured precisely as outlined in Table 1.

Table 1: Formulated feed composition across treatments and their inclusion levels

Sr. No.	Ingredients	T ₁	T ₂	T ₃	T ₄
1	Ground nut oil cake (gm)	80.00	80.00	80.00	80.00
2	Wheat flour(gm)	10.00	10.00	10.00	10.00
3	Rice Bran(gm)	8.00	8.00	8.00	8.00
4	Vitamin & Mineral(gm)	2.00	2.00	2.00	2.00
5	Onion and Neem powder mixture (Supplement) in%	1.00	2.00	3.00	4.00

The ingredients were mixed in a plastic tray with the required amount of water to form a dough. The dough was thermally processed by steam cooking for 10-15 minutes at 121 °C and 15psi pressure. After cooking, the vitamin and mineral mixture was added and thoroughly incorporated into the feed. Onion and neem powder mixture were then added according to the treatment details and mixed evenly. The feed mixture was extruded into pellets using a mechanical pelletizer. The pellets were sun-dried until their moisture content was below 10%, then stored in properly labeled airtight plastic container

2.3 Experimental procedure

The experiment utilized 15 rectangular plastic tanks, each measuring 2×1×1 feet (55 L). Subsequently, all tanks were filled with filtered and disinfected freshwater and continuous aeration was maintained in each tank. The experiment followed a completely randomized design (CRD), with three replicates for each of the five treatments. Fish (*L. rohita*) were stocked in all 15 tanks with a stocking density of 15 nos. per tank. *L. rohita* were fed twice daily at a rate of their body weight throughout the experiment. Feeding occurred in the morning (9:00-9:30 a.m.) and evening (5:30-6:00 p.m.). All experimental diets contained 30% protein. The required feed amount was weighed before each feeding. Feed amounts were adjusted based on the growth of the fish, as determined by fortnightly sampling. Throughout the experiment, water quality parameters were regularly monitored every week. These included temperature, pH, dissolved oxygen (DO), total hardness, ammonia and total alkalinity. All analyses were performed according to the standard procedures outlined by APHA (2017). Growth parameters such as mean weight gain, percentage weight gain, specific growth rate (SGR), feed conversion ratio (FCR) and survival rate were measured at 15-days interval using standard procedures (Manivannan & Saravanan, 2012; El-Sayed, 1998)^[10, 17]. Immunological

parameters including total erythrocyte count (TEC) (Lal, 2010)^[16], total leukocyte count (TLC) (Shah & Altindag, 2004)^[24], and respiratory burst activity (Secombes & Fletcher, 1992)^[23] were also analyzed using standard methods. In addition, a challenge study against *A. hydrophila* was performed to evaluate disease resistance (Das *et al.*, 2015)^[9].

2.4 Statistical analysis

Completely Randomized Design (CRD) was employed, with five treatments replicated three times. Data were analyzed using one-way Analysis of Variance (ANOVA) to determine significant differences between treatments, followed by Duncan's Multiple Range Test (DMRT) for post-hoc comparisons. Statistical analyses were performed using IBM SPSS version 22, with a significance level of $p < 0.05$.

3. Result and Discussion

The growth parameters of rohu is presented in Table-2 At the end of the experiment, T_4 , T_3 and T_2 showed significantly greater body weights than the control group ($p < 0.05$). After 60 days of experiment, all treatments showed improvements over the control, with T_4 exhibited the highest weight gain, followed by treatment T_3 , T_2 , T_1 and T_0 . Among all the treatments, T_4 recorded the significantly highest percentage weight gain at 368.74 ± 7.02 , followed by T_3 , T_2 , T_1 and T_0 . Highest specific growth rate at 2.57 ± 0.025 recorded in T_4 , followed closely by T_3 at 2.49 ± 0.037 . In contrast, T_0 (2.27 ± 0.034) showed the lowest mean. The lowest (most efficient) FCR was observed in T_4 (2.17 ± 0.01), followed by T_3 (2.21 ± 0.02), T_2 (2.30 ± 0.03), T_1 (2.32 ± 0.01) and the highest FCR in the control group T_0 (2.43 ± 0.07). There was no significant differences in survival rate ($p > 0.05$) among the various treatment groups, including the one supplemented with a neem and onion powder mixture (equal ratio, 40g kg⁻¹).

Table 2: Growth parameters of rohu (*L. rohita*) in different inclusion level of onion and neem powder mixture in feed

Parameters	Treatments				
	T ₀	T ₁	T ₂	T ₃	T ₄
Initial body weight (BW _i) (gm)	0.52±0.017 ^a	0.54±0.011 ^a	0.52±0.006 ^a	0.53±0.017 ^a	0.54±0.015 ^a
Final body weight (BW _f) (gm)	2.03±0.043 ^d	2.14±0.065 ^{cd}	2.22±0.046 ^{bc}	2.36±0.040 ^b	2.53±0.058 ^a
Net weight gain (NWG) (gm)	1.51±0.026 ^d	1.60±0.053 ^{cd}	1.70±0.040 ^c	1.83±0.029 ^b	1.99±0.046 ^a
Percentage weight gain (PWG) (gm)	290.05±4.66 ^c	296.77±4.19 ^c	326.83±4.14 ^b	345.85±10.07 ^b	368.74±7.02 ^a
Specific growth rate (SGR) (%)	2.27±0.034 ^c	2.30±0.018 ^c	2.42±0.016 ^b	2.49±0.037 ^b	2.57±0.025 ^a
Feed conversion ratio (FCR)	2.43±0.07 ^a	2.32±0.01 ^{ab}	2.30±0.03 ^{abc}	2.21±0.02 ^{bc}	2.17±0.01 ^c
Survival	86.67±3.85 ^a	91.11±2.22 ^a	91.11±2.22 ^a	93.33±3.85 ^a	95.55±2.22 ^a

Note: Values (Mean±S.E.) with identical superscripts within a row are non-significance ($p \geq 0.05$)

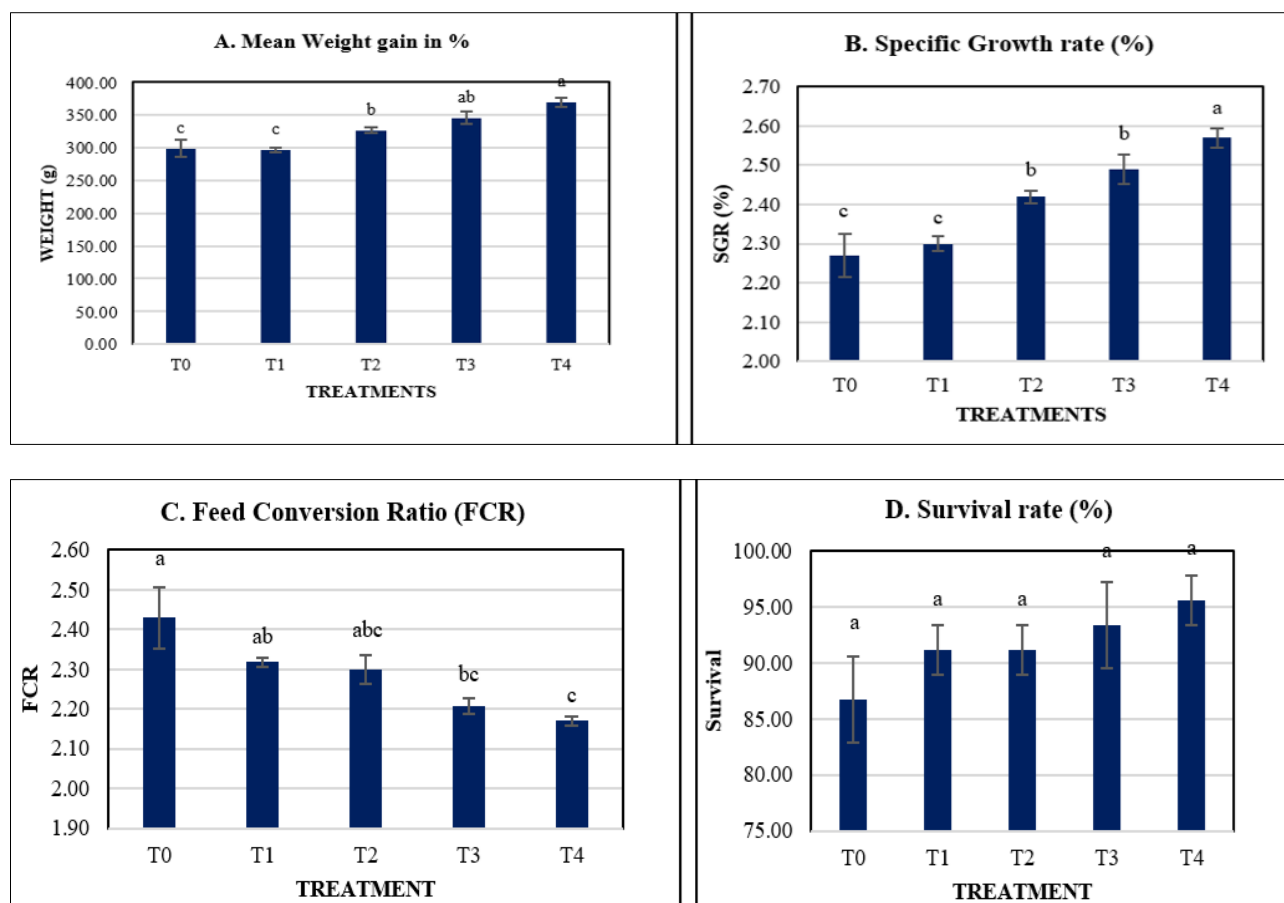


Fig 1: A-D. (A) Mean weight gain IN%, (B) Specific Growth rate (%), (C) Feed conversion ratio (FCR) and (D) Survival rate (%) of *Labeo rohita* recorded in different treatments at the end of 60 days.

The present study demonstrated that dietary supplementation of onion (*A. cepa*) and neem (*A. indica*) powders positively influenced the growth performance, survival, immune response, and disease resistance of *L. rohita*. A clear dose-dependent effect was observed, with higher inclusion levels showing more pronounced improvements in mean weight, mean weight gain, percentage weight gain, specific growth rate (SGR), feed conversion ratio (FCR), and survival rate. Among the treatments, T₄ (40 g kg⁻¹) exhibited the best performance, suggesting that combined supplementation of onion and neem powder at this concentration is particularly effective in enhancing growth and overall health status in *L. rohita*. The growth-promoting effects observed in the present experiment are consistent with earlier findings on the role of natural feed additives in aquaculture. Anwer *et al.* (2018) [5] reported significant improvements in growth and feed utilization parameters in grass carp (*Ctenopharyngodon idella*) fed onion powder at 1-2% levels. Similarly, Nazeer *et al.* (2020) [18] observed that neem leaf powder enhanced

body weight and SGR in *L. rohita*, attributing these results to its antimicrobial and immunomodulatory effects. The improved feed conversion ratio in the present study further indicates that both onion and neem supplementation enhance nutrient assimilation and metabolism, which may be attributed to their bioactive phytochemicals such as quercetin and azadirachtin.

Immune parameters of rohu (*L. rohita*) in different inclusion level of onion and neem powder mixture in feed is presented in Table 3. T₂ also showed improved TEC compared to T₀ and T₁, though to a lesser extent than T₃ and T₄. The highest TLC was observed in T₄, which was statistically different from T₀, T₁ and T₂ ($p < 0.05$). The highest TLC was observed in T₄, which was statistically different from T₀, T₁ and T₂ ($p < 0.05$). The group receiving T₄ (40g kg⁻¹ mixture of neem and onion powder in equal ratio) exhibited the highest survival rate at 80%, while the control group (T₀), which received a basal diet without supplementation, showed the lowest survival at 50%.

Table 3: Immune parameters of rohu (*Labeo rohita*) under varying dietary inclusion levels of onion and neem powder mixture

Parameters	Treatments				
	T ₀	T ₁	T ₂	T ₃	T ₄
Total erythrocyte count (TEC)10 ³ cells/mm ³	1.08±0.035 ^c	1.19±0.037 ^c	1.34±0.044 ^b	1.52±0.050 ^a	1.63±0.047 ^a
Total leucocyte count (TLC)10 ³ cells/mm ³	40.64±0.981 ^d	42.42±0.658 ^{cd}	44.17±0.652 ^{bc}	46.29±0.336 ^{ab}	48.55±1.246 ^a
Respiratory burst activity	0.13±0.003 ^d	0.21±0.018 ^c	0.26±0.013 ^b	0.30±0.014 ^{ab}	0.33±0.014 ^a

Note: Mean values with different superscripts in the same row are significantly different ($p < 0.05$)

The immune-modulating potential of onion and neem supplementation was also evident in the present study. Total erythrocyte count (TEC) and total leucocyte count (TLC) were significantly elevated in treatment groups compared to the control (Table 3). These findings corroborate the observations of Akrami *et al.* (2015) [1], who demonstrated that onion powder improved haematological profiles in fish, likely due to the antioxidant and haematopoietic stimulating effects of quercetin. Likewise, Kumar *et al.* (2013) [15] reported that azadirachtin enhances erythropoiesis in goldfish, suggesting that neem supports red blood cell production and overall physiological resilience. In present study, the combination of onion and neem showed a synergistic effect on both TEC and TLC, indicating that their simultaneous inclusion may stimulate hematopoiesis

and immune defense more effectively than individual supplementation. Non-specific immune responses were also significantly improved, as evidenced by the elevated respiratory burst activity in onion and neem supplemented groups. The highest nitrobluetetrazolium (NBT) activity was recorded in T₄, which indicates enhanced phagocytic activation and oxidative defense mechanisms. This observation is in agreement with Akrami *et al.* (2015), who reported increased respiratory burst activity in fish fed onion powder, and Talpur and Ikhwanuddin (2013) [25], who found neem leaf extract to enhance superoxide anion production. The enhanced respiratory burst activity in the present study underscores the synergistic immunostimulatory potential of onion and neem in boosting innate immunity.

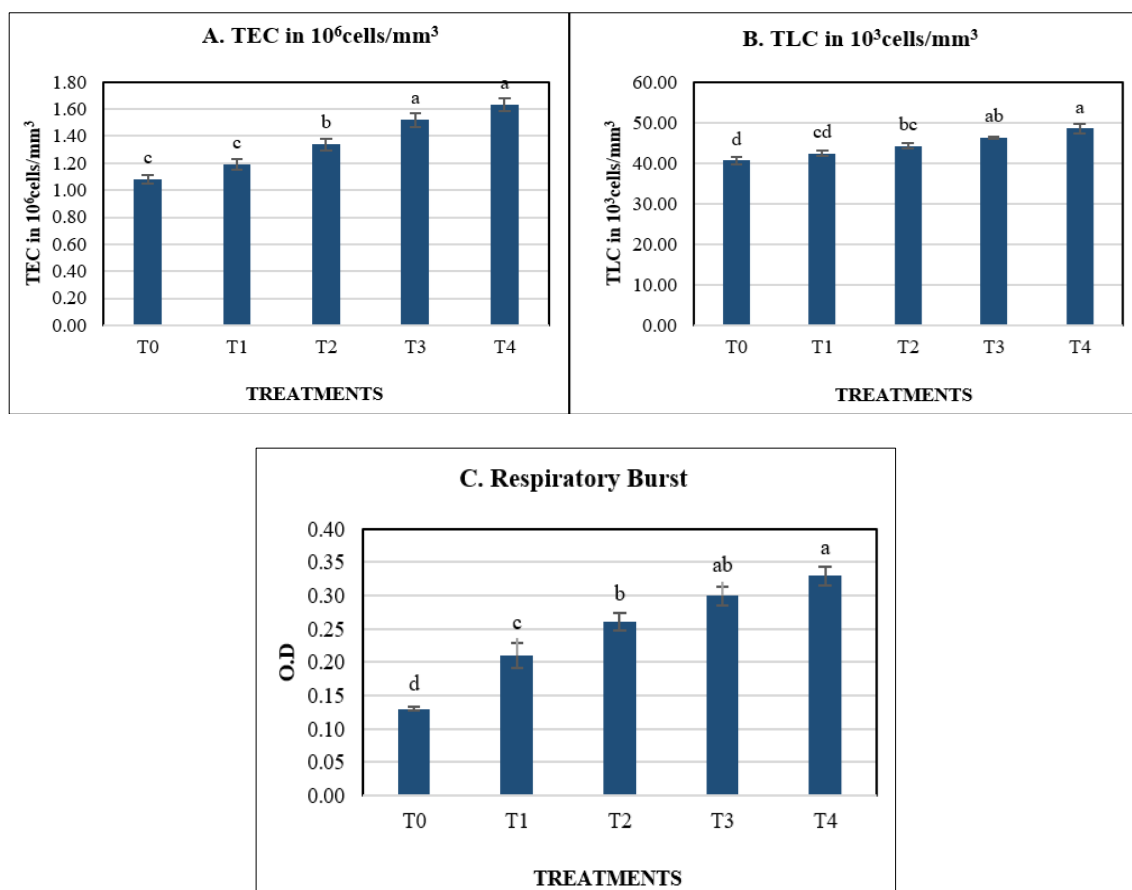


Fig 2: A-C. (A) TEC in 10⁶cells/mm³, (B) TLC in 10³cells/mm³, (C) Respiratory Burst of *Labeo rohita* recorded in different treatments at the end of 60 days.

The disease resistance trials further confirmed the protective effects of onion and neem supplementation. Fish fed higher inclusion levels of the mixture, particularly T₄, exhibited better survival following *A. hydrophila* challenge, although differences were not always statistically significant (Table 4). These results align with the findings of Younes *et al.* (2020) [27], who reported complete survival in fish fed

onion-supplemented diets after bacterial challenge, and Islamy *et al.* (2024) [13], who demonstrated neem extract's strong inhibitory activity against *A. hydrophila*. The enhanced survival may be attributed to the synergistic action of quercetin and azadirachtin, which collectively strengthen antioxidant defense, modulate immune responses, and inhibit pathogenic activity.

Table 4: Challenge study against *Aeromonas hydrophila*

Treatments	T ₀	T ₁	T ₂	T ₃	T ₄
Survival Rate (%) at 10 th day	50.00±0.00	60.00±0.00	70.00±0.00	70.00±0.00	80.00±0.00

Water quality parameters including temperature, pH, dissolved oxygen, total alkalinity, ammonia and total hardness were consistently monitored on a weekly basis throughout the experiment. The water temperature ranged from 25.2-30.1 °C, pH levels from 7.12-8.11, dissolved

oxygen between 5.1-6.8 ppm, total alkalinity 142.52-159.52 ppm, ammonia 162-280 ppm, and total hardness varied from 150-260 ppm (Table 5). All parameters remained within the optimal range for fish culture throughout the study.

Table 5: Physicochemical water quality parameters during the experimental period

Parameters	Treatments				
	T ₀	T ₁	T ₂	T ₃	T ₄
Temperature(°C)	25.2-28.4	25.3-29.5	26.4-29.9	25.5-28.0	26.8-30.1
pH	7.14-7.20	7.21-8.02	7.20-8.11	7.12-7.33	7.24-7.29
DO(ppm)	5.1-6.5	5.2-6.8	5.2-6.5	5.5-6.1	5.4-6.2
Total Alkalinity(ppm)	145.52-153.50	147.24-156.31	148.32-159.52	144.13-150.35	149.56-157.45
Ammonia(ppm)	0.06-0.15	0.05-0.09	0.05-0.11	0.08-0.14	0.07-0.09
Total Hardness(ppm)	165-210	177-240	182-235	175-260	184-280

Overall, the present study provides strong evidence that dietary inclusion of onion and neem mixture enhances growth, improves feed utilization, stimulates haematological and immune parameters, and strengthens disease resistance in *L. rohita*. These results highlight the potential of using natural plant-based feed additives as sustainable alternatives to synthetic growth promoters and antibiotics in aquaculture. The findings also confirm the consistency of the beneficial effects of onion and neem across multiple fish species, including *C. mrigala* and *C. idella*, indicating their broad applicability in aquaculture practices.

4. Conclusion

The present study showed that dietary inclusion of a 4% onion (*A. cepa*) and neem (*A. indica*) powder mixture (1:1 ratio) significantly improved growth performance, feed efficiency, and survival of *L. rohita*. Supplemented groups exhibited higher erythrocyte and leukocyte counts, enhanced respiratory burst activity, and greater resistance against *A. hydrophila*, indicating strong immunostimulatory and antioxidant effects. These findings highlight the potential of onion-neem powder mixture as a natural, plant-based alternative to synthetic additives for promoting growth, immunity, and disease resistance in aquaculture.

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