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Original Article

Immunomodulatory effects of indigenous herbal feed additives on innate immunity of common carp (*Cyprinus carpio*) challenged with *Aeromonas hydrophila*

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Abstract
The increasing incidence of bacterial diseases in aquaculture necessitates the development of sustainable immunostimulatory strategies as alternatives to antibiotics. The present study evaluated the immunomodulatory and growth-promoting effects of indigenous herbal feed additives on common carp (*Cyprinus carpio*) challenged with *Aeromonas hydrophila*. Fish were fed diets supplemented with neem (*Azadirachta indica*), tulsi (*Ocimum sanctum*), and their combination for 90 days under controlled conditions, followed by bacterial challenge. Hematological parameters, including hemoglobin (Hb), red blood cell (RBC) count, total leukocyte count (TLC), and packed cell volume (PCV), along with growth indices such as specific growth rate (SGR), weight gain, and feed conversion ratio (FCR), were evaluated. Results showed a significant improvement in hematological and immune parameters in all treated groups compared to control, with the combined supplementation (neem + tulsi) exhibiting the most pronounced effects. The highest Hb, RBC, TLC, and PCV values were observed in the combination group, indicating improved oxygen transport capacity and immune competence. Growth performance was also significantly improved, as indicated by higher SGR and

weight gain and lower FCR. Following bacterial challenge, treated groups exhibited increased resistance and survival, confirming the protective role of herbal supplementation. The findings demonstrate that dietary inclusion of indigenous herbal additives, particularly in combination, effectively enhances innate immunity, hematological health, and growth performance in common carp (*Cyprinus carpio*). This study highlights the potential of phytogenic feed additives as eco-friendly and sustainable alternatives for disease management in aquaculture systems.

Keywords: common carp; growth; haematology; immunomodulatory; neem; tulsi

1 | INTRODUCTION

Aquaculture plays a pivotal role in ensuring global food security, contributing significantly to protein supply and rural livelihoods (FAO 2020). However, the sustainability of aquaculture is often hindered by disease outbreaks, which can result in significant economic losses. Among freshwater species, common carp (*Cyprinus carpio*) is widely cultured due to its resilience and economic importance. Nevertheless, its susceptibility to bacterial infections remains a pressing concern. Among the bacterial pathogens, *Aeromonas hydrophila* is one of the most significant threats to aquaculture. The bacterium exhibits high virulence due to the production of several virulence factors, including hemolysins, proteases, lipases, and enterotoxins. These factors contribute to tissue damage, immune evasion, and systemic infections in fish. Infected fish often exhibit clinical signs such as reddening of the body, hemorrhagic lesions, exophthalmia, and abdominal distension, which can result in high mortality rates if not managed effectively (Baloch *et al*. 2021; Shrivastava *et al*. 2025). *Aeromonas hydrophila* is a gram-negative, facultative anaerobic bacterium widely distributed in aquatic environments, including freshwater and brackish water systems. It is an opportunistic pathogen that can infect a variety of fish species, causing diseases such as hemorrhagic septicemia, fin rot, tail rot, and ulcerative syndrome (Semwal *et al*. 2023).

This bacterium is responsible for conditions like bacterial enteritis and motile *A. septicemia*, resulting in substantial economic losses due to high mortality rates in fish populations (Mahmood *et al*. 2024). Aquaculture has expanded dramatically over the past thirty years to meet the rising nutritional demands of a growing human population. The shift toward intensive and semi-intensive farming systems, however, has heightened the incidence of disease outbreaks, often causing partial or total loss of fish yields (Pandey *et al*. 2025). For example, olive leaf extract enhanced digestive enzyme activity and growth-related gene expression in common carp (Zemheri-Navruz *et al*. 2020). Similarly, phytobiotic extracts of *Ginkgo biloba*, *Silybum marianum*, and *Myristica fragrans* promoted growth and immune responses in *Pangasianodon hypophthalmus* (Abd-Elaziz *et al*. 2023), while *Psidium guajava*, peppermint (*Mentha piperita*), neem (*Azadirachta indica*), and tulsi (*Ocimum sanctum*) enhanced immunity, antioxidant status, growth performance, and disease resistance in tilapia, common carp, and *Labeo rohita* (Ceballos-Francisco *et al*. 2020; Habib *et al*. 2025; Naz *et al*. 2025; Singh *et al*. 2026). In addition, diosin from *Dioscorea collettii* effectively controlled *Gyrodactylus kobayashii* infection in goldfish (*Carassius auratus*) (Zhou *et al*. 2021). Despite these advances, the widespread use of antibiotics and chemotherapeutic agents in aquaculture has accelerated the emergence of drug-resistant bacterial pathogens, reducing treatment efficacy and posing a major challenge to sustainable disease management.

Medicinal plants have been used for centuries as natural immunostimulants and possess diverse biological properties, including growth-promoting, antimicrobial, antifungal, antiviral, and immunomodulatory activities, making them cost-effective, environmentally friendly, and low-risk alternatives to antibiotics and chemotherapeutic agents for sustainable aquaculture (Zhu 2020). The excessive use of synthetic antibiotics and chemicals to manage bacterial infections has raised concerns regarding antimicrobial resistance, environmental pollution, and food safety (Okeke *et al*. 2022). Consequently, there is an increasing interest in exploring sustainable and eco-friendly alternatives to enhance fish immunity and health. Among these, the use of herbal immunostimulants has emerged as a promising approach in aquaculture. Herbal immunostimulants are natural products derived from plants, known for their bioactive compounds that can boost the immune system, promote growth, and mitigate infections (Dawood *et al*. 2021).

Neem, often referred to as the "village pharmacy," is a versatile tree native to the Indian subcontinent. It is known for its numerous medicinal properties, including antimicrobial, anti-inflammatory, and antioxidant effects. Research has shown that neem leaf extracts possess strong antibacterial and antifungal properties, which may help in managing bacterial infections in aquaculture (Islas *et al*. 2020). The bioactive compounds in neem, such as azadirachtin and nimbin, are believed to enhance fish immunity, promote growth, and reduce the incidence of diseases caused by pathogens like *A. hydrophila* (Islamy *et al*. 2024). Tulsi, also known as holy basil, is another plant with a long history of use in traditional medicine. It is recognized for its adaptogenic, antibacterial, antifungal, and antiviral properties (Kaur *et al*. 2020). In aquaculture, tulsi has been investigated for its ability to boost the immune response of fish, improve stress tolerance, and enhance

overall health. Tulsi extracts are rich in essential oils, phenolic compounds, and flavonoids that are thought to contribute to its therapeutic effects. Studies have demonstrated that tulsi supplementation in fish feed can help increase disease resistance, improve water quality, and reduce reliance on antibiotics in aquaculture settings (Semwal *et al*. 2023).

Both neem and tulsi have emerged as promising herbal supplements in aquaculture because of their eco-friendly nature and their ability to improve fish health. As the aquaculture sector faces increasing pressure to adopt sustainable practices, these medicinal plants offer viable alternatives to conventional therapeutics and support global efforts to reduce antibiotic use and minimize environmental impacts. Consequently, the use of plant-derived remedies can enhance fish health, stimulate immune responses, and contribute to more sustainable disease management in aquaculture (Stratev *et al*. 2018). In this study, the effects of aqueous leaf extracts of *A. indica* and *O. sanctum* administrated as a mixture with feed on the hematological profile with growth parameters and mortality rates in common carp against *A. hydrophila* were detected.

2 | METHODOLOGY

2.1 Experimental animals

The experimental fish were advanced common carp fingerlings with an average body weight of 9.0±1.0 g. The fish were procured from the local fish farm in Hisar, Haryana, India. The fish were transported in polythene packing with sufficient oxygen (50 fish per pack). On reaching the laboratory, they were carefully transferred to the plastic tubs (150 L) and were left undisturbed for the whole night. To ameliorate the handling stress, the fish were given a mild salt treatment followed by potassium permanganate at the rate of 2 ppm. The stock was acclimatized under aerated conditions for seven days and fed with commercial carp's diet.

2.2 Site of experiment

The experimental study was conducted in the wet laboratory of the Department of Aquatic Animal Health Management, College of Fisheries Science, CCS Haryana Agricultural University, Hisar, India (29°08′37.0″N 75°42′11.6″E). The feeding trial was carried out for 90 days, from May to July 2024.

2.3 Experimental design

Experimental fish were reared in 150 L rectangular plastic tubs containing 100 L of water and covered with green nets to prevent fish escape and external contamination. Continuous aeration was provided using air stones connected to an air pump to maintain adequate dissolved oxygen levels. Following a one-week acclimatization period, healthy, uniform-sized advanced common carp fingerlings were randomly stocked into 12 rearing tubs at a density of 10 fish per tub, with 30 fish allocated to each treatment (three replicates per treatment), after recording their initial average body weight and total length prior to the commencement of the feeding trial.

2.4 Preparation herbal extracts and fish diet

Fresh leaves of *A. indica* (Neem) and *O. sanctum* (Tulsi) were collected from neem trees and tulsi plants located in the Medicinal, Aromatic, and Potential Crops Section (GPB) at the CCSHAU campus in Hisar, India. The leaves were thoroughly washed with cold water followed by a 1 ppm potassium permanganate solution, then dried in a hot air oven at 60°C for 24 hours. Once dried, the leaves were ground into a fine powder using an electric mixer-grinder. For extract preparation, 5 grams of neem and tulsi leaf powder were separately mixed with 100 mL of distilled water and extracted using a Soxhlet apparatus at 60°C for 5–6 hours. The resulting extracts were filtered through sterile muslin cloth.

Four experimental diets were prepared: a control diet (T1) consisting of the basal diet (soybean meal, groundnut oil cake, rice bran, wheat flour, mineral mixture, and MOC), T2 (basal diet supplemented with 5% neem leaf extract), T3 (basal diet supplemented with 5% tulsi leaf extract), and T4 (basal diet supplemented with a 5% combination of neem and tulsi leaf extracts). All experimental diets were thoroughly mixed, pelleted, air-dried, and stored in airtight containers until use. The dietary inclusion level of 5% herbal extract was selected based on the findings of Abidin *et al*. (2022), who evaluated 5%, 7%, and 10% dietary neem (*A. indica*) leaf extract in rainbow trout (*Oncorhynchus mykiss*) and reported significant growth improvement at the 5% inclusion level, with an optimum predicted level of 7.5%. Therefore, a fixed inclusion level of 5% was adopted in the present study to evaluate the effects of neem, tulsi (*O. sanctum*), and their combination in common carp (*C. carpio*).

2.5 Bacterial strain and challenge study

A pure culture of *A. hydrophila* (MTCC No. 1739) was procured from the Microbial Type Culture Collection and Gene Bank (MTCC), CSIR–Institute of Microbial Technology, Chandigarh, India. The isolate was inoculated into nutrient broth (HiMedia, India) and incubated at 28±2°C for 24 h. Following incubation, the culture was centrifuged at 5000 rpm for 5 min, and the resulting pellet was washed three times with phosphate-buffered saline (PBS; pH 7.4) to remove residual media components. The bacterial concentration was adjusted spectrophotometrically to an optical density of 0.7 at 600 nm, corresponding to approximately 4.6×10^{8} cells mL^{-1}, as determined by serial dilution and viable plate count (Islas *et al.* 2020).

To establish the pathogenic dose of *A. hydrophila* for common carp, forty apparently healthy fish were random-

ly assigned to four aquaria (10 fish per group). Each group was intramuscularly injected with different concentrations of the bacterial suspension: 1×10^5, 1×10^6, 1×10^7, and 1×10^8 CFU mL^{-1}. Mortality was observed daily for 14 days post-injection. The highest mortality rate (70%) was recorded in the group injected with 1.0×10^8 CFU mL^{-1}, where 7 of 10 fish succumbed to infection. Following the feeding trial, conducted over 30 days with the experimental diets, fish were challenged intramuscularly with *A. hydrophila* at 1.0×10^8 CFU mL^{-1}. Haematological parameters were measured both before (day 30; pre-challenge) and after the bacterial challenge (post-challenge) to assess the influence of dietary treatments on immune responses.

2.6 Collection of blood sample

Fish were randomly selected from each experimental tub, including the control group, for blood sampling. Under anaesthesia induced with clove oil, blood was drawn from the caudal vein using a 1 mL sterile insulin syringe fitted with a superfine 31G short needle. The puncture was made along the lateral midline near the caudal or anal fin of *C. carpio* fingerlings. Collected blood was carefully transferred into sterilized Eppendorf tubes containing ethylene diamine tetra acetic acid (EDTA) as an anticoagulant and gently mixed to prevent clotting. The collected blood samples were subsequently used for haematological analyses following the standard procedures described in Dacie and Lewis Practical Haematology (Barbara *et al*. 2011).

2.7 Growth parameters

Several growth performance parameters were evaluated to assess the influence of different dietary treatments on fish growth. The initial and final body length and weight of the fish were measured to determine final mean body length and weight. Specific growth rate (SGR), percentage increase in length and weight, survival rate, and feed conversion ratio (FCR) were calculated using the following standard mathematical formulae:

Specific growth rate (SGR) = [(log final body weight – log initial body weight) / (Durations of days)] × 100

Percentage increment in length = [(Mean final length of fish – Mean initial length of fish) / (Mean initial length of fish)] × 100

Percentage increment in weight = [(Mean final weight of fish-mean initial weight of fish) / (Mean initial weight of fish)] × 100

Survivability = (Total number of fishes harvested – Total number of stocked) × 100

Feed Conversion Ratio (FCR) = (Feed given) / (Weight gain)

2.8 Haematological parameters

Red blood cell (RBC), white blood cell (WBC), and haemoglobin (Hb) concentrations were determined following the standard procedures described by Barbara *et al*. (2011). The packed cell volume (PCV) was measured using the micro haematocrit method with 25 mm heparinized capillary tubes. The mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), and mean corpuscular haemoglobin concentration (MCHC) were computed using the formulae outlined by Hu *et al*. (2005).

3 | RESULTS

3.1 Hemoglobin (Hb)

The hemoglobin concentration was significantly affected by treatment, sampling period, and their interaction ($p < 0.001$). All herb-supplemented groups exhibited higher hemoglobin values than the control throughout the experiment. The greatest concentrations were observed in the T4 group, reaching 8.30 g dL^{-1} at 30 days and 8.47 g dL^{-1} at 90 days. Conversely, the control group (T1) exhibited a pronounced decline in hemoglobin concentration at 60 days, recording a value of 4.97 g dL^{-1}, which suggests greater physiological stress under the non-supplemented condition (Figure 1). The progressive rise in Hb in T4 suggests enhanced erythropoietic stimulation over time. Overall, treated groups maintained higher hemoglobin levels than control throughout the experiment.

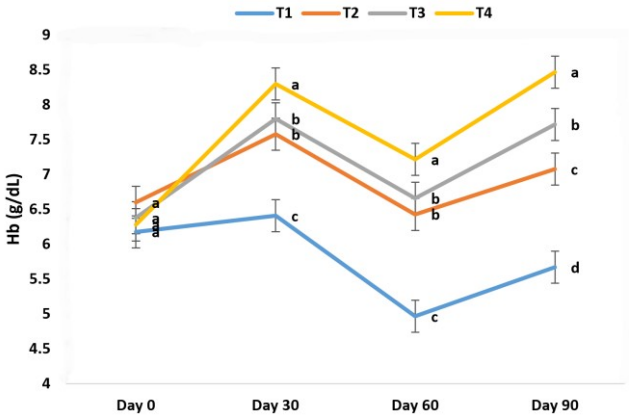

FIGURE 1 Effect of dietary treatments on hemoglobin (Hb) levels over the experimental period (0–90 days). Values are expressed as mean ± SE ($n = 3$). Different superscripts indicate significant differences ($p < 0.05$).

3.2 Red blood cell (RBC) count

Significant treatment, period and interaction effects were observed for RBC ($p < 0.01$). As shown in Figure 2, RBC values declined sharply in T1 at 60 days (1.10×10^6 cells mm^{-3}), whereas treated groups maintained relatively stable counts. T4 recorded the highest RBC values at 30 days

(1.80×10⁶ cells mm^{-3}) and 90 days (1.88×10⁶ cells mm^{-3}). The increasing trend in RBC in supplemented groups indicates improved erythropoiesis and hematological stability (Figure 2).

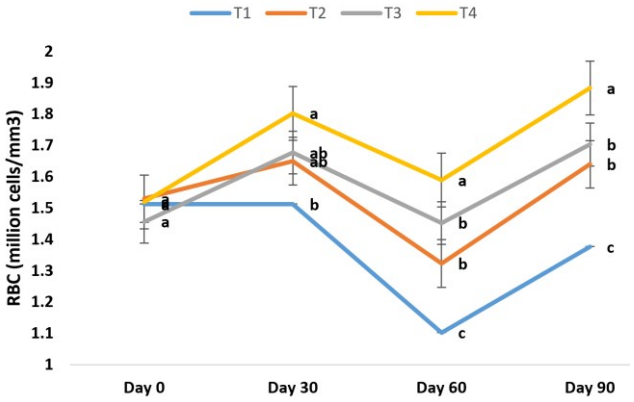

FIGURE 2 Effect of dietary treatments on red blood cell (RBC) levels over the experimental period (0–90 days). Values are expressed as mean ± SE (n = 3). Different superscripts indicate significant differences (p < 0.05).

3.3 Total leukocyte count (TLC)

TLC showed highly significant variation among treatments and periods (both p < 0.001). As depicted in Figure 3, leukocyte counts increased progressively in treated groups, particularly T4, which recorded the highest values at 60 days (84.67 ×10³ cells mm^{-3}) and 90 days (84.83 ×10³ cells mm^{-3}). The control group showed a decline at 90 days (55.17×10³ cells mm^{-3}), whereas supplemented groups maintained elevated immune cell counts, indicating enhanced immune response.

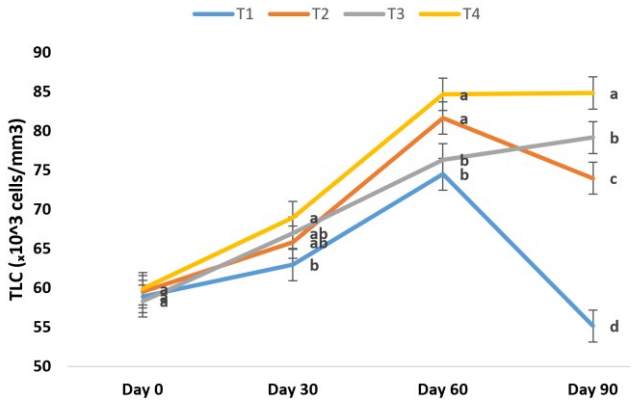

FIGURE 3 Effect of dietary treatments on total leukocyte count (TLC) levels over the experimental period (0–90 days). Values are expressed as mean ± SE (n = 3). Different superscripts indicate significant differences (p < 0.05).

3.4 Packed cell volume (PCV)

PCV values differed significantly across treatments and periods (p < 0.001). Figure 4 demonstrates that T4 consistently exhibited superior PCV values, reaching 25.10% at 90 days. The control group showed a marked decrease at 60 days (14.93%), suggesting reduced hematological stability. The increasing PCV trend in treated groups reflects improved oxygen transport capacity and circulatory efficiency.

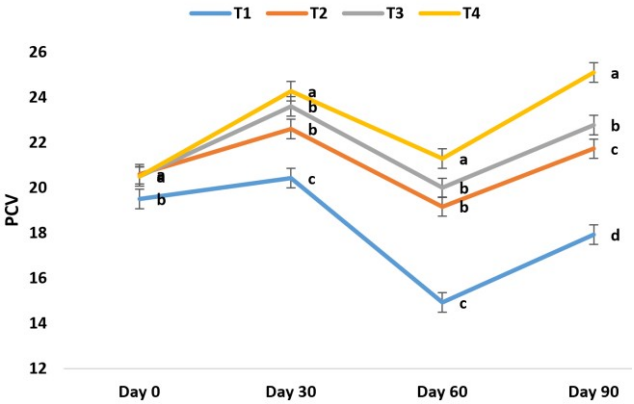

FIGURE 4 Effect of dietary treatments on packed cell volume (PCV) levels over the experimental period (0–90 days). Values are expressed as mean ± SE (n = 3). Different superscripts indicate significant differences (p < 0.05).

3.5 Mean corpuscular hemoglobin (MCH)

MCH values were significantly affected by treatment and period (p < 0.05), with no significant interaction effect. As shown in Figure 5, the highest MCH values were observed in T3 at 30 and 60 days (46.58 and 45.89 pg, respectively). T2 showed a decline at 90 days (39.82 pg). Overall, erythrocyte hemoglobin content was moderately enhanced in T3 and T4.

3.6 Mean corpuscular hemoglobin concentration (MCHC)

MCHC was significantly influenced by sampling period and treatment × period interaction. Figure 6 shows peak values at 30 and 60 days across treatments. T4 exhibited relatively stable and higher MCHC values at later stages, suggesting maintenance of erythrocyte hemoglobin concentration.

3.7 Mean corpuscular volume (MCV)

MCV values were significantly affected by treatment and period (p < 0.05). As shown in Figure 7, T3 recorded the highest overall MCV values, particularly at 0 and 60 days (140.89 fL). A general decline was observed at 90 days across treatments, especially in T2. The relatively higher MCV in treated groups suggests improved erythrocyte development and maturation.

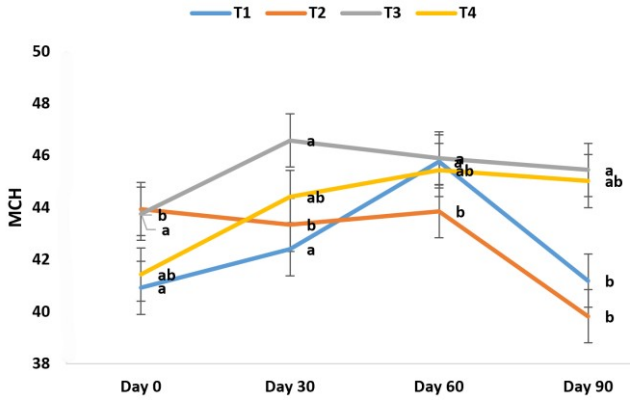

FIGURE 5 Effect of dietary treatments on Mean corpuscular hemoglobin (MCH) levels over the experimental period (0–90 days). Values are expressed as mean ± SE (n = 3). Different superscripts indicate significant differences (p < 0.05).

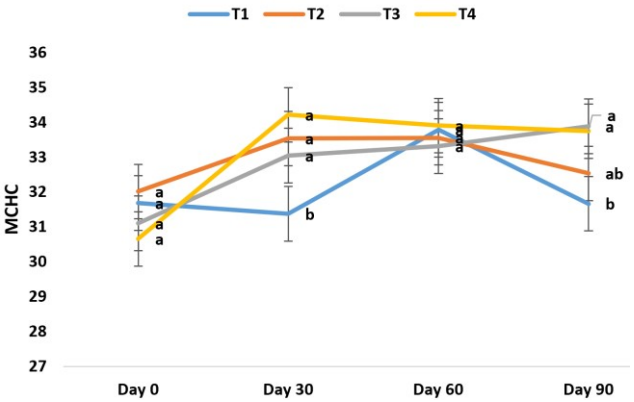

FIGURE 6 Effect of dietary treatments on mean corpuscular hemoglobin concentration (MCHC) levels over the experimental period (0–90 days). Values are expressed as mean ± SE (n = 3). Different superscripts indicate significant differences (p < 0.05).

3.8 Length gain

Length gain was significantly affected by the sampling period (p < 0.001), while neither treatment nor the treatment × period interaction showed a significant effect (p > 0.05). The highest mean length gain occurred during the initial phase (0–30 days), which was significantly greater than in the subsequent periods. No significant differences were observed among treatments at any sampling interval, indicating that all dietary treatments had comparable effects on length increment.

During the 0–30 day period, length gain did not differ significantly (p > 0.05) among treatments, reflecting uniform initial growth across experimental groups. However, numerically higher values were recorded in T2 (40.97±2.67 cm) and T3 (40.49±2.67 cm) compared to T1. In the later periods (30–60 and 60–90 days), length gain declined relative to the initial phase. Although differences among treatments remained statistically non-significant during these stages, T4 exhibited relatively higher length gain during 60–90 days (17.02±2.67 cm), suggesting better growth consistency under this treatment (Table 1).

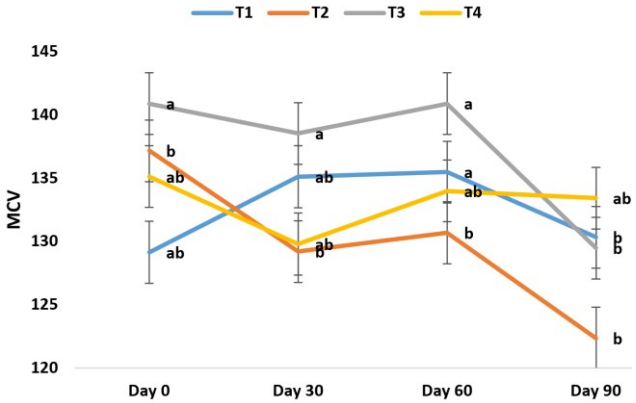

FIGURE 7 Effect of dietary treatments on mean corpuscular volume (MCV) levels over the experimental period (0–90 days). Values are expressed as mean ± SE (n = 3). Different superscripts indicate significant differences (p < 0.05).

TABLES 1 Effect of dietary treatments on length gain (cm) over the experimental period.

Treatment	Days		
	0–30	30–60	60–90
T-1	38.58±2.67^{a}	13.75±2.67^{b}	15.80±2.67^{b}
T-2	40.97±2.67^{a}	13.58±2.67^{b}	16.78±2.67^{b}
T-3	40.49±2.67^{a}	14.67±2.67^{b}	15.18±2.67^{b}
T-4	40.38±2.67^{a}	14.77±2.67^{b}	17.02±2.67^{b}

Different superscripts letters along each column indicate significant differences (p < 0.05).

3.9 Daily weight gain (DWG)

Daily weight gain (DWG) was significantly influenced by treatment, sampling period, and their interaction (all p < 0.001). No significant differences among treatments were observed during the initial 0–30 day period. However, during 30–60 days, T4 recorded a significantly higher DWG compared to the other treatments. During the final phase of the experiment (60–90 days), fish fed the T3 and T2 diets maintained relatively higher daily weight gain (DWG) values than those in the control group (T1), whereas the T4 group exhibited a moderate decline, as presented in Table 2.

TABLE 2 Effect of dietary treatments on daily weight gain (g) over the experimental period.

Treatment	Days		
	0–30	30–60	60–90
T-1	0.17±0.02^{a}	0.08±0.02^{c}	0.09±0.02^{b}
T-2	0.16±0.02^{a}	0.15±0.02^{b}	0.14±0.02^{a}
T-3	0.16±0.02^{a}	0.14±0.02^{b}	0.15±0.02^{a}
T-4	0.19±0.02^{a}	0.20±0.02^{a}	0.10±0.02^{b}

Different superscripts letters along each column indicate significant differences (p < 0.05).

3.10 Weight gain

Weight gain was significantly influenced by treatment, sampling period, and their interaction (all $p < 0.01$). No significant differences among treatments were observed during the initial 0–30 day period. However, during 30–60 and 60–90 days, T4 exhibited significantly higher weight gain compared to the other treatments Consequently, the cumulative growth response under T4 was markedly superior at the end of the experimental period, as presented in Table 3.

TABLE 3 Effect of dietary treatments on weight gain (g) over the experimental period.

Treatment	Days		
	0–30	30–60	60–90
T-1	50.26±7.62^{a}	72.99±7.62^{c}	119.94±7.62^{b}
T-2	53.32±7.62^{a}	82.58±7.62^{bc}	132.45±7.62^{b}
T-3	47.66±7.62^{a}	89.21±7.62^{b}	132.76±7.62^{b}
T-4	54.44±7.62^{a}	112.11±7.62^{a}	177.03±7.62^{a}

Different superscripts letters along each column indicate significant differences ($p < 0.05$).

3.11 Specific growth rate (SGR)

The SGR was significantly influenced by treatment, sampling period, and their interaction (all $p < 0.01$). The highest SGR values were observed during the initial growth phase (0–30 days). During 30–60 days, T4 maintained a significantly higher SGR compared to the other treatments. Although SGR declined across all treatments during 60–90 days, T4 sustained relatively higher growth efficiency, as presented in Table 4.

TABLE 4 Effect of dietary treatments on specific Growth Rate (SGR) over the experimental period.

Treatment	Days		
	0–30	30–60	60–90
T-1	1.35±0.09^{ab}	0.47±0.09^{c}	0.80±0.09^{ab}
T-2	1.49±0.09^{a}	0.66±0.09^{bc}	0.72±0.09^{ab}
T-3	1.30±0.09^{b}	0.83±0.09^{b}	0.69±0.09^{b}
T-4	1.45±0.09^{ab}	1.06±0.09^{a}	0.89±0.09^{a}

Different superscripts letters along each column indicate significant differences ($p < 0.05$).

3.12 Feed conversion ratio (FCR)

Feed conversion ratio (FCR) showed a clear and significant improvement among treatments. T1 recorded the highest FCR (1.82±0.18), indicating poorer feed utilization efficiency, whereas T4 recorded the lowest FCR (1.28±0.03), followed by T3 (1.36±0.02) and T2 (1.48±0.02). The lower FCR observed in T4 indicates superior feed efficiency and a more effective conversion of feed into biomass, as presented in Table 5.

TABLE 5 Effect of dietary treatments on feed conversion ratio (FCR) over the experimental period.

Treatment	FCR (Mean ± SE)
T1	1.82±0.18^{a}
T2	1.48±0.02^{b}
T3	1.36±0.02^{b}
T4	1.28±0.03^{b}

Different superscripts letters along each column indicate significant differences ($p < 0.05$).

4 | DISCUSSION

4.1 Hemoglobin (Hb)

Hemoglobin concentration, a key indicator of oxygen-carrying capacity and physiological health in fish, was significantly affected by treatment, period, and their interaction ($p < 0.001$), revealing a time-dependent influence of dietary supplementation on erythropoietic activity. Treated groups, especially T–4, exhibited a progressive rise in hemoglobin levels, reflecting enhanced erythrocyte production and oxygen transport efficiency. In contrast, the control group showed comparatively lower values at 60 days, potentially signaling transient physiological stress or diminished hematopoietic drive. These results corroborate the findings of Habib *et al*. (2025), who reported significantly elevated hemoglobin levels in supplemented fish relative to controls. Elevated hemoglobin levels in supplemented groups may be attributed to bioactive compounds that stimulate erythropoiesis and enhance iron metabolism. Improved hemoglobin concentration reflects better metabolic activity and tissue oxygenation, which are essential for growth and stress resilience. The significant interaction between treatment and sampling period suggests that the hematological response intensified over time, indicating cumulative physiological benefits of dietary supplementation.

4.2 Red blood cell (RBC) count

The RBC count is a reliable proxy for erythropoietic activity and overall hematologic health. In the present study, treatment, sampling period, and their interaction each exerted a highly significant effect on RBC levels ($p < 0.01$), with the T4 diet consistently yielding the highest counts across the majority of sampling points. Conversely, the control group exhibited a pronounced decline in RBC numbers at the mid experimental interval, suggesting that the basal feed may have failed to mitigate environmental or metabolic stressors. The sustained elevation of RBCs in the T4 cohort indicates that the combined neem–tulsi supplementation enhances oxygen-carrying capacity and facilitates more efficient tissue respiration. The increase in erythrocyte production likely drives the observed gains in metabolic efficiency and stress tolerance. The significant treatment × time interaction further indicates that the dietary influence on erythropoiesis strengthens as the

trial progresses, becoming most apparent in the later sampling points. The present findings are consistent with previous reports demonstrating the hematological benefits of neem and tulsi in aquatic species. Similar improvements were also observed by Habib *et al*. (2025) in *C. carpio* fed tulsi-based diets. Collectively, these findings suggest that phytogenic additives can effectively modulate hematological parameters, thereby improving the physiological status of cultured carp. Overall, the results demonstrate that supplementation under T4 positively modulated erythropoietic function compared to other treatments and control.

4.3 Total leukocyte count (TLC)

Total leukocyte count is widely recognized as an important indicator of immune competence in fish. The present study demonstrated highly significant effects of treatment, period, and their interaction on TLC ($p < 0.001$). Treated groups, particularly T4, exhibited markedly elevated leukocyte levels at 60 and 90 days compared to control. The increase in total leukocyte count (TLC) observed in the phytochemical-supplemented groups indicates heightened immunostimulatory activity, reflecting a stronger innate immune response and greater disease-resistance potential. This pattern mirrors the leukocytic elevations reported by Misra *et al*. (2006) in *L. rohita* fed n−3 PUFA-enriched diets, suggesting that various bioactive compounds can activate similar immune pathways. Likewise, Pratheepa *et al*. (2010) found a significant rise in white-blood-cell counts in fish receiving Aegle marmelos leaf extract, with a pronounced post-infection surge comparable to the later-stage TLC increase seen in our trial. The decline in TLC for the control at the final sampling further underscores the immunocompromising effect of a diet lacking phytogenic additives. Together, these studies support the premise that phytogenic supplements modulate leukocyte dynamics, enhancing physiological adaptation and disease resistance in cultured carp. The significant treatment × period interaction indicates that immune enhancement was time-dependent and became more evident with prolonged supplementation. These findings support the immunomodulatory potential of the dietary intervention used in T4.

4.4 Packed cell volume (PCV)

Packed cell volume reflects the proportion of blood volume occupied by erythrocytes and is closely associated with oxygen transport efficiency. In this study, PCV was significantly influenced by treatment, period, and their interaction ($p < 0.001$). T4 consistently exhibited the highest PCV values across sampling intervals, whereas the control group showed a noticeable reduction at 60 days. Higher packed cell volume (PCV) in treated groups indicates enhanced erythrocyte production and hematological stability, while the decline in control fish suggests physiological stress or impaired hematopoietic efficiency. The progressive PCV increase under dietary supplementation reflects bolstered circulatory performance and metabolic vigor. These patterns align with Hammed *et al*. (2017), who observed reduced PCV in bacterial-infected fish relative to pre-infection baselines, with values recovering in groups fed herbal extracts—consistent with our evidence that supplementation mitigates infection-induced hematological disruptions. The significant interaction effect further highlights that the beneficial impact of supplementation on blood volume dynamics was time-dependent and more pronounced during later stages of the experiment.

4.5 Mean corpuscular hemoglobin (MCH)

Mean corpuscular hemoglobin represents the average hemoglobin content per erythrocyte and provides insight into red cell functionality. In the present study, MCH was significantly affected by treatment and period ($p < 0.05$), while their interaction was not significant. The relatively higher MCH values recorded in T3 and T4 suggest improved hemoglobin incorporation within erythrocytes. The peak values observed at 60 days across treatments indicate enhanced erythrocyte maturation during this phase. Since interaction effects were not significant, the influence of treatment on MCH appears consistent across sampling periods. These variations likely reflect differential effects of individual versus combined phytogenic ingredients on erythrocyte quality and are consistent with similar findings by Das *et al*. (2015). The results suggest that dietary supplementation moderately improved red blood cell functional quality, contributing to improved hematological efficiency.

4.6 Mean corpuscular hemoglobin concentration (MCHC)

MCHC reflects hemoglobin concentration within erythrocytes and is indicative of cellular hemoglobin saturation. In this study, MCHC was significantly influenced by period and treatment × period interaction, whereas the main treatment effect was not significant. These findings imply that time exerted a greater influence on erythrocytic hemoglobin concentration than the dietary treatments themselves. Nonetheless, the significant treatment × time interaction reveals that particular supplements affected MCHC at distinct sampling points. The overall stability of MCHC across all groups suggests that erythrocyte structure and hemoglobin content remained intact, corroborating Kaur *et al*. (2020), who observed unchanged MCHC in neem-treated carp fry. These findings indicate that dietary supplementation maintained red cell hemoglobin concentration within physiological limits, without inducing abnormal erythrocyte morphology.

4.7 Mean corpuscular volume (MCV)

Mean corpuscular volume represents the average size of

erythrocytes and provides information on red cell morphology. In the present investigation, MCV was significantly affected by treatment and period ($p < 0.05$), while their interaction was not significant. Treatment T3 yielded the highest mean corpuscular volume (MCV) overall, indicative of superior erythrocyte development and maturation. Period-wise trends showed elevated MCV at initial and mid-sampling points, with slight stabilization by experiment's end. These observations align with Das *et al*. (2015), who reported comparable MCV elevations in supplemented fish, underscoring the role of dietary interventions in optimizing erythroid maturation. The absence of significant interaction suggests that treatment effects on erythrocyte size were consistent over time. Increased MCV in treated groups may reflect improved hematopoietic activity and efficient red cell production.

4.8 Growth

The present study demonstrated that T4 significantly enhanced growth performance parameters, particularly weight gain, daily weight gain, and specific growth rate during the mid and late experimental phases. The significant treatment × period interaction suggests that the growth-promoting effect of T4 was time-dependent and became more pronounced as the culture period progressed. The superior growth observed under the T4 regimen can be attributed to increased nutrient-assimilation efficiency, improved feed utilization, and activation of anabolic metabolic pathways. The markedly higher daily weight-gain (DWG) and specific growth-rate (SGR) in T4 fish indicate a more effective allocation of energy toward somatic tissue, reflecting robust anabolism. Throughout the 90-day trial, T4 consistently outperformed the control in total biomass gain, with the greatest increments occurring during the active growth phase (days 30–60). This performance is driven by enhanced feed conversion efficiency, allowing ingested nutrients to be converted into growth more readily. Moreover, the cumulative weight-gain curve under T4 showed a steady, progressive rise, suggesting sustained physiological adaptation and metabolic stability. These findings are in line with previous reports on herbal supplementation in carp. Habib *et al*. (2025) documented comparable weight-gain enhancements with Tulsi inclusion, while Ngugi *et al*. (2015) observed elevated SGR and survival in fish fed herb-based diets. The growth-promoting effects are plausibly linked to the diverse bioactive metabolites—tannins, alkaloids, terpenoids, saponins, phenolics, steroids, and flavonoids—present in the supplements, which are known to stimulate growth, immune function, and antibacterial and antistress responses in aquaculture species (Nazeemashahul *et al*. 2023). The absence of significant differences in length gain among treatments indicates that weight-related growth parameters were more responsive to dietary modulation than linear growth. This pattern is consistent with typical aquaculture growth dynamics, where weight gain is more sensitive to dietary interventions. Overall, T4 appears to promote superior growth performance by enhancing feed conversion efficiency and metabolic regulation, particularly during the rapid growth phase (30–60 days). The overall trend indicates that while linear growth was relatively similar among treatments, T4 maintained better length increment during the later growth phase, possibly due to improved nutrient assimilation or physiological efficiency. The superior performance of T4 suggests enhanced feed conversion, nutrient digestibility, and overall physiological growth response. Increased weight gain during later stages indicates sustained growth efficiency under T4 treatment conditions.

5 | CONCLUSIONS

The present findings demonstrate that dietary supplementation, particularly with the combination of 5% neem (*Azadirachta indica*) and tulsi (*Ocimum sanctum*) extracts, significantly enhanced the hematological and immunological status of the experimental fish. Improvements in HB, RBC, TLC, and PCV indicate stimulation of erythropoiesis and immune competence. Moderate enhancements in erythrocyte indices further support improved red cell functionality. The significant treatment × period interactions observed for major blood parameters confirm that the physiological benefits of supplementation were progressive and time-dependent. These results highlight the potential of the tested dietary intervention as a hematological and immunostimulatory enhancer in aquaculture species. The treatment enhanced growth rate, biomass accumulation, and feed conversion efficiency, particularly during the mid and late culture periods. The results indicate that combination of neem and tulsi provides a more favorable physiological and nutritional environment for optimal growth performance and immunity.

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ETHICAL APPROVAL

All applicable international, national and/or institutional guidelines for the care and use of animals were followed in this study.

CONFLICT OF INTEREST

The author declares no conflict of interest.

AUTHORS' CONTRIBUTION

Conceptualization: Gajender Singh; Methodology: R.K. Gupta; Investigation: Shivam Pandey & Asutosh Lowansi; Data Curation: Shivam Pandey & Domendra dhruve; Formal Analysis: Manoj Kumar; Writing – Original Draft: Shivam Pandey & Nitish Bansal; Writing – Review & Editing: Nitish Bansal. All authors have read and approved the final manuscript.

DATA AVAILABILITY STATEMENT

The author stated that the present work has not been previously published, nor is it before another journal for consideration.

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