Journal of Environmental Biology
p-ISSN: 0254-8704 • e-ISSN: 2394-0379 • CODEN: JEBIDP

Original Research Paper
Volume 46 Issue 5 September 2025 pp. 659-668
DOI: http://doi.org/10.22438/jeb/46/5/MRN-5478

J. Environ. Biol., 2025
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Journal website : www.jeb.co.in ★ E-mail : editor@jeb.co.in

Synergistic effects of Neem and Moringa on fish (*Labeo rohita*) health: a challenge study with *Saprolegnia parasitica*

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Received: 29 July 2024 **Revised:** 30 October 2024 **Accepted:** 30 May 2025

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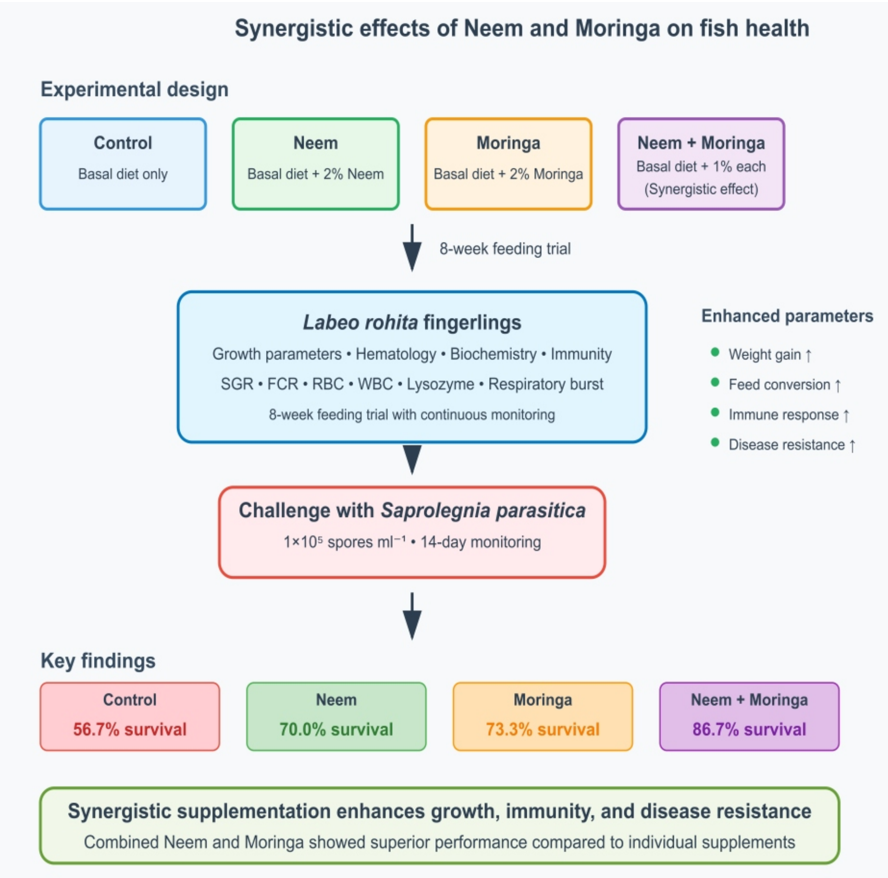
Abstract

Aim: To investigate the synergistic effects of Neem (*Azadirachta indica*) and Moringa (*Moringa oleifera*) leaves supplementation on the growth, health, immune response, and resistance to *Saprolegnia parasitica* infection in *Labeo rohita* under the agro-climatic conditions of Meghalaya, India.

Methodology: Four experimental diets (isonitrogenous and isocaloric) were tested over a 8-week feeding trial: Control (C), Neem-supplemented (N), *Moringa*-supplemented (M), and Neem+*Moringa*-supplemented (NM). Growth performance, feed utilization, hematological parameters and immune indices were measured. Fish were then challenged with *S. parasitica* (1×10^5 spores m l^{-1}) and monitored for 14 days.

Results: The NM group exhibited significantly improved growth performance, feed conversion ratio, hematological parameters, and immune indices compared to other groups. Following the challenge, the NM group showed the highest survival rate (86.7%) and lowest infection severity.

Interpretation: Combined supplementation of Neem and Moringa in *L. rohita* diets can enhance growth, immunity and disease resistance, offering a promising strategy for sustainable aquaculture in Meghalaya and potentially other regions with similar agro-climatic conditions.



Key words: Immunostimulation, *Labeo rohita*, *Moringa oleifera,* Neem, *Saprolegnia parasitica*, Synergistic effects

How to cite: Debnath, C.: Synergistic effects of Neem and Moringa on fish (*Labeo rohita*) health: a challenge study with *Saprolegnia parasitica*. *J. Environ. Biol.*, **46,** 659-668 (2025).

Introduction

Aquaculture plays a crucial role in global food security and economic development, particularly in developing countries. In India, the freshwater fish *Labeo rohita,* commonly known as rohu, is one of the most important cultured species due to its fast growth rate and high market demand (FAO, 2020). However, intensification of aquaculture practices has led to increased disease outbreaks, causing significant economic losses and threatening the sustainability of the industry (Reverter *et al.,* 2020). In recent years, there has been a growing interest in using natural plant-based supplements to enhance fish health, growth, and disease resistance as an alternative to synthetic additives and antibiotics (Chakraborty and Hancz, 2011). Two plants that have shown promising results in various aquaculture species are Neem (*Azadirachta indica*) and Moringa (*Moringa oleifera*). Neem (*A. indica*), a medicinal tree indigenous to the Indian subcontinent, has been utilized for centuries in traditional medicine systems due to its diverse therapeutic properties (Ferreira *et al.,* 2024). In aquaculture applications, Neem extracts have garnered significant attention for their documented antimicrobial, antioxidant and immunostimulatory effects.

Research has consistently demonstrated the beneficial impacts of Neem supplementation across various fish species. Abidin *et al.* (2022) documented enhanced growth parameters and immune function in rainbow trout (*Oncorhynchus mykiss*) when feed was supplemented with 7.5% Neem leaf extract. Ubiogoro *et al.* (2019) reported similar growth improvements in catfish. Talpur and Ikhwanuddin (2013) observed increased immune response and survival rates in Asian seabass (*Lates calcarifer*) challenged with *Vibrio harveyi,* while Kumar *et al.* (2013) noted strengthened non-specific immunity and resistance against *Aeromonas hydrophila* in goldfish (*Carassius auratus*). The benefits of Neem-based supplementation extend beyond aquaculture to livestock production, particularly in poultry (Ampode *et al.,* 2021).

Moringa, often referred to as the "miracle tree," is rich in essential nutrients, vitamins, and minerals. It has shown potential in improving the growth performance and enhancing the immune system in various fish species (Adeshina *et al.,* 2018). Zhang *et al.* (2020) reported that partial replacement of fish meal with fermented *Moringa* leaves enhanced growth performance, antioxidant status and disease resistance against *A. hydrophila* in juvenile gibel carp (*C. auratus gibelio*). Additionally, Abd El-Gawad *et al.* (2020) found that dietary Moringa leaf powder supplementation improved immune responses and antioxidant status in Nile tilapia (*Oreochromis niloticus*), resulting in increased resistance to *A. hydrophila* infection. The agro-climatic conditions of Meghalaya, a state in the North-eastern India, present unique challenges for aquaculture. The region is characterized by high rainfall, subtropical climate and acidic soils, which can affect water quality and fish health (Department of Agriculture, Meghalaya, 2006). Therefore, developing locally adapted, sustainable aquaculture practices is crucial for the region's food security and economic development. *Saprolegnia parasitica* is a widespread water mold responsible for saprolegniasis (also called cotton wool disease), a disease that can cause significant fish mortality in freshwater environments, especially under stressful conditions (van West, 2006). Previous studies have shown that *S. parasitica* infection can significantly alter blood profiles of infected fish, leading to 45-50% mortality (Debnath *et al.,* 2017). This disease is particularly problematic in areas with unstable environmental conditions, like Meghalaya, where persistent cold weather and sudden fall in water temperature occurs. Infected fish typically succumb within 12-15 days of the infection's onset (Das *et al.,* 2012).

Recent studies have examined the potential of plant-based supplements for managing fungal infections in fish. Harikrishnan *et al.* (2009) found that a blend of herbal compounds, including azadirachtin, effectively inhibited the growth of *Aphanomyces invadans,* another pathogenic fungus, *in vitro* and boosted immune responses in *Cirrhina mrigala*. Later, Mehrabi *et al.* (2019) reported that 15 g kg^{-1} *Aloe vera* powder in rainbow trout diets enhanced the growth, immune responses and resistance to *S. parasitica.* Tandel *et al.* (2021) evaluated five natural plant extracts—Curcumin, Cinnamaldehyde, Eugenol, Stigmasterol and Morin—against two *Saprolegnia* species, finding that former two were particularly effective as phyto-additives. More recently, Elgendy *et al.* (2023) reported that 0.5% extract of *Allium cepa* improved Nile tilapia (*Oreochromis niloticus*) resistance to saprolegniasis.

While previous studies have examined the individual effects of Neem and *Moringa* on various fish species, there is limited research on their combined or synergistic effects, especially under specific agro-climatic conditions. Furthermore, the potential of these plant supplements in enhancing resistance to *S. parasitica* in *L. rohita* has not been thoroughly investigated. In view of the above, this study aimed to evaluate the synergistic effects of Neem and *Moringa* supplementation on the growth performance and health of *L. rohita* under the agro-climatic conditions of Meghalaya in order to assess the impact of these supplements on the immune response of *L. rohita,* and determine the efficiency of Neem and *Moringa* supplementation in enhancing resistance to *S. parasitica* infection.

Materials and Methods

Experimental location and setup: The experiment was conducted at ICAR Research Complex for NEH Region, Umiam (Barapani), Meghalaya, India, located at 25.683964°N latitude, 91.913579°E longitude, 980 m above sea level. Twelve 1000 l cemented tanks were used, with four treatments replicated thrice. Each tank was stocked with 20 *L. rohita* fingerlings (initial average weight: 15 ± 0.5 g). Neem (*A. indica*) and Moringa (*M. oleifera*) leaves were harvested from the Institute's Horticulture Farm and authenticated by a Senior Scientist (Horticulture) at the institute. The leaves were thoroughly washed with clean water to remove debris, air-dried in shade for several days to preserve their nutritional content, and then grounded into a fine powder using a mechanical grinder.

Experimental diets and feeding: Four experimental diets were formulated (Table 1): Control (C): Basal diet without supplementation; Neem (N): Basal diet + 2% Neem leaf powder, *Moringa* (M): Basal diet + 2% *Moringa* leaf powder and Neem + Moringa (NM): Basal diet + 1% Neem leaf powder + 1% Moringa leaf powder. The basal diet was formulated to meet the nutritional requirements of *L. rohita.* Fish were fed twice daily at 3% of their body weight for 8 weeks.

The experimental feeds were prepared by mixing the rice bran, soybean meal, fishmeal and wheat flour to form a base. This mixture was then cooked to form a dough and allowed to cool. Subsequently, the leaf powders of 2% *Moringa* (M group), 2% Neem powder (N group), or 1% combination each (N+M group) was added to the respective treatment diets, along with a vitamin and mineral premix. The control diet was devoid of supplementation. The dough was then pelletized using a manual pellet machine with a 2 mm diameter die. The resulting pellets were dried at room temperature to reduce moisture content and stored in airtight plastic packets to maintain quality and nutritional integrity until feeding. The proximate composition of the feeds was analyzed prior to the commencement of feeding trials following the standard methods (AOAC, 1995). The crude protein content of the feeds ranged from 32.1-32.2%, and the lipid content ranged from 6-6.2%, indicating that the feeds were nearly isonitrogenous and isolipidic across all treatments (Table 1). The energy content of the diets ranged from 345-346 kcal 100 g^{-1}, ensuring that any difference in fish performance could be attributed to the herbal supplements rather than variations in macronutrient or energy content.

Water quality management: Water quality parameters (temperature, pH, dissolved oxygen, ammonia, and nitrite) of different treatment tanks were monitored daily following the standard methods of APHA (2023) and maintained within the optimal range for *L. rohita.* Partial water changes (30%) were performed weekly.

Growth performance and feed utilization: Fish were weighed biweekly to adjust feeding rates. At the end of 8-week feeding trial, the weight gain (WG), specific growth rate (SGR), feed conversion ratio (FCR) and condition factor (K) were calculated (Hopkins, 1992; Froese, 2006).

Blood sampling and analysis: At the end of 8-week feeding trial, blood samples were collected from six fish per tank (18 per treatment). Fish were anesthetized using MS-222 before blood collection from the caudal vein using heparinized and non-heparinized syringes. for analyzing following parameters.

Hematological parameters: Hematological analysis was performed following the standard methods of Blaxhall and Daisley (1973). RBC and WBC counts were determined using a

Table1: Composition of experimental feeds and proximate composition (% dry matter basis) of diets containing different levels of Neem (N), Moringa (M) and their combination (NM) for *Labeo rohita* fingerlings

Ingredient (g kg^{-1})	Control (C)	Neem (N)	*Moringa* (M)	Neem + *Moringa* (NM)
Fish meal	350	350	350	350
Soybean meal	280	280	280	280
Rice bran	200	180	180	180
Wheat flour	130	130	130	130
Fish oil	20	20	20	20
Vitamin-mineral premix*	20	20	20	20
Neem	-	20	-	10
Moringa	-	-	20	10
Total	1000	1000	1000	1000
Proximate composition (% dry matter basis)**				
Moisture (%)	8.8±0.3	8.7±0.5	8.7±0.3	8.7±0.4
Crude protein (%)	32.2±0.3	32.1±0.4	32.1±0.5	32.2±0.4
Crude lipid (%)	6.2±0.1	6.1±0.2	6.0±0.3	6.2±0.2
Ash (%)	8.5±0.2	8.4±0.3	8.5±0.2	8.3±0.5
Crude fibre (%)	4.8±0.4	4.7±0.5	4.6±0.3	4.7±0.6
Nitrogen free extract	39.5±0.4	40.0±0.4	40.1±0.4	39.9±0.4
Energy (kcal/100g)	344.6±5.0	345.3±5.0	344.5±5.0	346.2±5.0

*Composition of vitamin-mineral premix (quantity/2.5 kg): Vitamin A, 5500000 IU; Vitamin D3, 1100000 IU; Vitamin B2, 2000 mg; Vitamin E, 750 mg; Vitamin K, 1000 mg; Vitamin B6, 1000 mg; Vitamin B12, 6 mcg; Calcium pantothenate, 2500 mg; Nicotinamide, 10 g; Choline chloride, 150 g; Manganese, 27,000 mg; Iodine, 1000 mg; Iron, 7500 mg; Zinc, 5000 mg; Copper, 2000 mg; Cobalt, 450 mg; L- lysine, 10 g; DL- Methionine, 10 g; Selenium, 50 ppm; **mean of three replicates ± SD.

Neubauer hemocytometer. Hemoglobin concentration was measured by the Cyanmethemoglobin method (Dacie and Lewis, 1984). Hematocrit was estimated with microhematocrit capillary tubes. MCV, MCH and MCHC were calculated using standard formulas (Dacie and Lewis, 1984).

Biochemical parameters: Serum was separated by centrifuging blood samples at 3000 rpm for 10 min. Total protein content was determined by the Biuret method (Gornall *et al.,* 1949). Albumin level was estimated by the Bromocresol green method (Doumas *et al.,* 1971). Globulin was calculated by subtracting the albumin content from the total protein content. Glucose was estimated by the Glucose oxidase method (Trinder, 1969). Cholesterol and triglycerides were determined by the enzymatic colorimetric methods (Allain *et al.,* 1974; Fossati and Prencipe, 1982).

Immune parameters: Lysozyme activity was estimated by turbidimetric assay as described by Ellis (1990), using *Micrococcus lysodeikticus* (ATCC) as a substrate. Respiratory burst activity was determined by the nitroblue tetrazolium (NBT) reduction assay following the method of Anderson and Siwicki (1995). Serum bactericidal activity was assessed following the method of Kajita *et al.* (1990), using *A. hydrophila* as a test organism. Myeloperoxidase activity was measured following the procedure outlined by Quade and Roth (1997). Total immunoglobulin levels were determined by the method described by Siwicki and Anderson (1993), which involved precipitation of immunoglobulins with polyethylene glycol.

Challenge study with *Saprolegnia parasitica*: The pathogenic water mold *S. parasitica* was isolated from the infected fish collected from local water bodies in Meghalaya. The isolate was cultured on glucose-yeast extract agar supplemented with penicillin and streptomycin to inhibit bacterial growth. Identification was based on the characteristic morphological features including cottony mycelial growth, aseptate hyphae, and the presence of sporangia containing motile zoospores (Willoughby, 1978). The species were confirmed through PCR amplification and sequencing of the internal transcribed spacer (ITS) region (Diéguez-Uribeondo *et al.,* 2007).

For the challenge study, zoospore production was induced by flooding 7-day-old cultures with sterile water and incubating at 10°C for 24 hrs. The zoospore suspension was adjusted to 1×10^5 spores m l^{-1} using a hemocytometer. After 8-week feeding trial, 15 fish from each tank were exposed to spore suspension for 30 min in separate tanks. A modified challenge protocol was followed where fish were subjected to controlled stress using the 'ami-momi' treatment method (Hoshiai, 1992; Hatai *et al.,* 1990), which involvedgently shaking the fish in a fan-shaped scoop net for 2 min. This challenge protocol was adapted from previous studies that have successfully demonstrated *S. parasitica* infection in Indian major carps (Debnath *et al.,* 2017; Das *et al.,* 2013). Following the stress treatment, fish were exposed to zoospore suspension (1×10^5 spores ml^{-1}) for 30 min before transferring back to pathogen-free tanks for 14-day observation period. Daily observations were made for clinical signs of infection, including cotton-like growths on the skin, fins and gills. Mortality was recorded daily, and dead fish were examined microscopically to confirm *S. parasitica* infection.

Statistical analysis: Data were analyzed by One-way ANOVA followed by Tukey's HSD test to determine significant differences among the treatments. All the statistical analyses were performed using SPSS software version 21.0, with significance set at $p<0.05$.

Results and Discussion

Water quality monitoring throughout the experimental period demonstrated that all measured parameters, including temperature, pH, dissolved oxygen, ammonia, and nitrite levels remained within the optimal range for *L. rohita* growth (Ayyappan *et al*., 2019), with no significant differences observed among treatments (Table 2). The maintenance of optimal water quality across all treatments indicated that the Neem and *Moringa* supplementation did not negatively impact the water quality, whichis crucial for sustainable aquaculture practices, especially in the regions of Meghalaya where water resources may be limited or sensitive to environmental changes. The findings of this study regarding water quality align with established research in the field. Specifically, Talpur and Ikhwanuddin's (2013) demonstrated that the neem leaf supplementation in the diet of Asian seabass maintained stable water quality parameters throughout the experimental period. This observation was further reinforced by Zhang *et al.* (2020), who documented similar results on incorporating fermented *Moringa* leaves into gibel carp diets, with no adverse effects on the water quality metrics.

Table2: Water quality parameters during the 8-week feeding trial of *Labeo rohita* fingerlings fed with different supplements

Parameters	Control	Neem	*Moringa*	Neem+*Moringa*
Temperature (°C)	26.5 ± 0.8^a	26.7 ± 0.7^a	26.4 ± 0.9^a	26.6 ± 0.8^a
pH	7.2 ± 0.2^a	7.3 ± 0.1^a	7.2 ± 0.2^a	7.3 ± 0.2^a
Dissolved oxygen (mg l^{-1})	6.8 ± 0.3^a	6.9 ± 0.2^a	6.7 ± 0.3^a	6.8 ± 0.2^a
Ammonia (mg l^{-1})	0.05 ± 0.02^a	0.04 ± 0.01^a	0.05 ± 0.02^a	0.04 ± 0.01^a
Nitrite (mg l^{-1})	0.02 ± 0.01^a	0.02 ± 0.01^a	0.02 ± 0.01^a	0.01 ± 0.01^a

Values are mean ± S.D. Different superscripts in the same row indicate significant differences ($p < 0.05$)

Table 3: Growth performance and feed utilization of *Labeo rohita* fingerlings fed with different supplements

Parameters	Control	Neem	*Moringa*	Neem+*Moringa*
Final weight (g)	45.2 ± 2.1^{c}	52.7 ± 1.8^{b}	54.3 ± 2.3^{b}	61.8 ± 2.5^{a}
Weight gain (g)	30.2 ± 1.9^{c}	37.7 ± 1.5^{b}	39.3 ± 2.1^{b}	46.8 ± 2.2^{a}
SGR (% day^{-1})	1.65 ± 0.08^{c}	1.92 ± 0.06^{b}	1.98 ± 0.09^{b}	2.24 ± 0.10^{a}
FCR	2.15 ± 0.12^{a}	1.87 ± 0.08^{b}	1.82 ± 0.10^{b}	1.61 ± 0.07^{c}
Condition Factor (K)	1.78 ± 0.05^{b}	1.85 ± 0.04^{ab}	1.87 ± 0.06^{ab}	1.93 ± 0.05^{a}

Values are mean ± S.D. Different superscripts in the same row indicate significant differences ($p < 0.05$)

Table 4: Hematological parameters of *Labeo rohita* fingerlings fed with different supplements

Parameters	Control	Neem	*Moringa*	Neem+*Moringa*
RBC ($\times 10^{6}$ µl^{-1})	1.85 ± 0.12^{c}	2.10 ± 0.15^{b}	2.15 ± 0.14^{b}	2.45 ± 0.18^{a}
WBC ($\times 10^{3}$ µl^{-1})	22.5 ± 1.8^{c}	26.7 ± 2.1^{b}	27.3 ± 2.3^{b}	31.8 ± 2.5^{a}
Hb (g dl^{-1})	7.2 ± 0.4^{c}	8.1 ± 0.5^{b}	8.3 ± 0.5^{b}	9.2 ± 0.6^{a}
Hct (%)	28.5 ± 1.5^{c}	32.7 ± 1.8^{b}	33.3 ± 2.0^{b}	36.8 ± 2.2^{a}
MCV (fl)	154 ± 5^{a}	156 ± 6^{a}	155 ± 5^{a}	150 ± 6^{a}
MCH (pg)	38.9 ± 1.2^{a}	38.6 ± 1.3	38.6 ± 1.1^{a}	37.6 ± 1.4^{a}
MCHC (g dl^{-1})	25.3 ± 0.8^{a}	24.8 ± 0.9^{a}	24.9 ± 0.8^{a}	25.0 ± 0.9

Values are mean ± SD. Different superscripts in the same row indicate significant differences ($p < 0.05$).

The consistency of water quality parameters observed in the present study, conducted under Meghalaya's distinct environmental conditions, provides compelling evidence that both neem and *Moringa* can be effectively integrated into aquaculture feed formulations without compromising water quality standards. This finding is particularly significant given the unique agro-climatic characteristics of the hill region, suggesting broader applicability of these plant-based supplements in diverse aquaculture settings. The neem-moringa fed group showed a significant increase ($p < 0.05$) in the final weight, weight gain, and SGR compared to all other groups (Table 3). Previous studies have shown that inclusion of 20–25% neem in feed does not affect the feed palatability or acceptance negatively (Mafouo Sonhafouo *et al.,* 2019). Namrah (2021) reported the optimal growth in *L. rohita* on including 15 g kg^{-1} neem in the feed. In this study, no reduction in feed intake was observed across any treatment group, including those containing neem powder. This was further evidenced by improved FCR values in neem-supplemented groups compared to the control, suggesting good feed acceptance and utilization.

The significantly lower FCR in the neem-moringa fed group (1.61 ± 0.07) as compared to the control group (2.15 ± 0.12) showed that the combination of neem and moringa not only maintained the feed palatability but also enhanced the feed utilization efficiency. The neem and moringa groups also showed significant improvements over the control group, with FCR values of 1.87 ± 0.08 and 1.82 ± 0.10 respectively, indicating better feed conversion compared to the control but not as efficient as the combined supplementation. This synergistic effect of neem and moringa on the growth performance is noteworthy and differs from previous studies that typically focused on these supplements such as neem and moringa individually (Ubiogoro *et al.*, 2019; Abd El-Gawad *et al.*, 2020; Zhang *et al.*, 2020; Abidin *et al.* 2022).

The improved growth performance observed in the supplemented groups, particularly in the group fed with neem-moringa, can be attributed to the nutritional and bioactive compounds present in Neem and Moringa. Moringa is rich in essential amino acids, vitamins and minerals, which may have contributed to enhanced nutrient utilization and metabolism (Adeshina *et al.,* 2018). Neem contains various bioactive compounds, including azadirachtin, which have been found to improve the digestion and nutrient absorption in fish (Harikrishnan *et al.,* 2009). The findings of this study are in the line with Zhang *et al.* (2020), who observed improved growth performance and feed efficiency in gibel carp fed with fermented Moringa leaves. However, the present study demonstrates the synergistic effects of Neem and Moringa, which resulted in significant improvements in the growth parameters. The synergistic effect observed in the Neem-Moringa group suggests that the combination of these supplements may provide a more balanced and comprehensive nutritional profile, leading to optimal growth performance.

The improved FCR and condition factor (K) in supplemented groups, especially Neem-Moringa, indicates better

Table 5: Biochemical parameters of *Labeo rohita* fingerlings fed with different supplements

Parameters	**Control**	**Neem**	***Moringa***	**Neem+*Moringa***
Total Protein (g d l^{-1})	3.2 ± 0.2^{c}	3.7 ± 0.3^{b}	3.8 ± 0.3^{b}	4.3 ± 0.3^{a}
Albumin (g d l^{-1})	1.4 ± 0.1^{c}	1.6 ± 0.1^{b}	1.7 ± 0.1^{b}	1.9 ± 0.2^{a}
Globulin (g d l^{-1})	1.8 ± 0.2^{c}	2.1 ± 0.2^{b}	2.1 ± 0.2^{b}	2.4 ± 0.2^{a}
A/G Ratio	0.78 ± 0.05^{a}	0.76 ± 0.06^{a}	0.81 ± 0.07^{a}	0.79 ± 0.06^{a}
Glucose (mg d l^{-1})	75 ± 5^{a}	72 ± 6^{ab}	70 ± 5^{b}	68 ± 6^{b}
Cholesterol (mg d l^{-1})	165 ± 12^{a}	155 ± 10^{b}	152 ± 11^{b}	140 ± 9^{c}
Triglycerides (mg d l^{-1})	130 ± 10^{a}	120 ± 9^{b}	118 ± 8^{b}	105 ± 7^{c}

Values are mean ± S.D. Different superscripts in the same row indicate significant differences ($p < 0.05$).

Table 6: Immune parameters of *Labeo rohita* fingerlings fed with different supplements

Parameters	**Control**	**Neem**	***Moringa***	**Neem+*Moringa***
Lysozyme activity (µg m l^{-1})	18.5 ± 1.2^{c}	24.7 ± 1.5^{b}	25.3 ± 1.7^{b}	31.8 ± 2.1^{a}
Respiratory burst activity (O.D. 540 nm)	0.32 ± 0.03^{c}	0.45 ± 0.04^{b}	0.47 ± 0.05^{b}	0.58 ± 0.06^{a}
Serum bactericidal activity (%)	42.5 ± 3.1^{c}	53.7 ± 3.8^{b}	55.3 ± 4.2^{b}	64.8 ± 4.5^{a}
Myeloperoxidase activity (O.D. 450 nm)	1.25 ± 0.10^{c}	1.58 ± 0.12^{b}	1.62 ± 0.14^{b}	1.95 ± 0.16^{a}
Total Ig levels (mg m l^{-1})	12.5 ± 1.0^{c}	15.7 ± 1.2^{b}	16.3 ± 1.3^{b}	19.8 ± 1.5^{a}

Values are mean ± SD. Different superscripts in the same row indicate significant differences ($p < 0.05$)

feed utilization efficiency, which is crucial for sustainable and cost-effective aquaculture practices. This improvement could be due to enhanced activity of digestive enzymes and better nutrient absorption, as well as the potential prebiotic effects of *Moringa* enhancing the gut health and nutrient utilization. The group fed with Neem-Moringa feed showed significantly higher ($p < 0.05$) RBC, WBC, Hb and Hct values in comparison to other groups (Table 4).

The groups fed with neem and moringa showed similar trend, showing significant improvements over the control group. No significant differences were observed in the MCV, MCH and MCHC values among different treatment. The improved hematological parameters observed in the supplemented groups, specifically Neem-Moringa fed group showed the enhanced health status and physiological condition of the fish. The increased RBC count, hemoglobin concentration, and hematocrit in the Neem-Moringa group indicated improved oxygen-carrying capacity of the blood, which could contribute to better growth performance and overall health (Reverter *et al.,* 2014).

The findings of this study are in line with the reports of Mona *et al.* (2015), who reported improved hematological parameters in silver carp fed with neem leaf extract. However, the present study demonstrates that the combined Neem and Moringa feed provides even greater benefits than a single supplement alone. This synergistic effect on hematological parameters has not been previously reported in the context of *L. rohita* or in the unique environmental conditions of Meghalaya. The elevated WBC count in supplemented groups, especially Neem-Moringa fed group showed elevated immune system of the fish, corroborating the observed improvement in immune parameters (Tandel *et al.,* 2021). This increase in WBCs would provide better protection against pathogens like *S. parasitica* (Saha *et al.,* 2016). Fish fed with Neem-Moringa feed showed significant increase ($p < 0.05$) in total protein, albumin and globulin levels in comparison toother groups (Table 5). Glucose, cholesterol and triglyceride levels were significantly lower in fish fed with Neem-Moringa feed, indicating improved metabolic status.

The improved biochemical parameters in supplemented groups, particularly Neem-Moringa fed fish showed enhanced nutritional status and metabolic efficiency. Higher total protein, albumin, and globulin levels suggest improved protein synthesis and immune function, as these proteins play crucial roles in maintaining the osmotic balance and immune responses (Kumar *et al.,* 2013). The findings of this study are consistent with those of Elgendy *et al.* (2021), who observed improved biochemical parameters in Nile tilapia fed with Moringa oleifera-supplemented diets. However, in the present study combined supplement of Neem and Moringa showed a significant improvement in the biochemical parameters, particularly in the context of *L. rohita* and the environmental conditions of Meghalaya.

The lower glucose, cholesterol and triglyceride levels in the fish fed with neem-moringa supplement showed improved energy utilization and lipid metabolism, which contributed significantly improved the growth performance and feed utilization efficiency due to improved insulin sensitivity, lipid-lowering effects, and enhanced metabolic efficiency (Xiao *et al.,* 2017). All the groups fed with supplements showed significantly

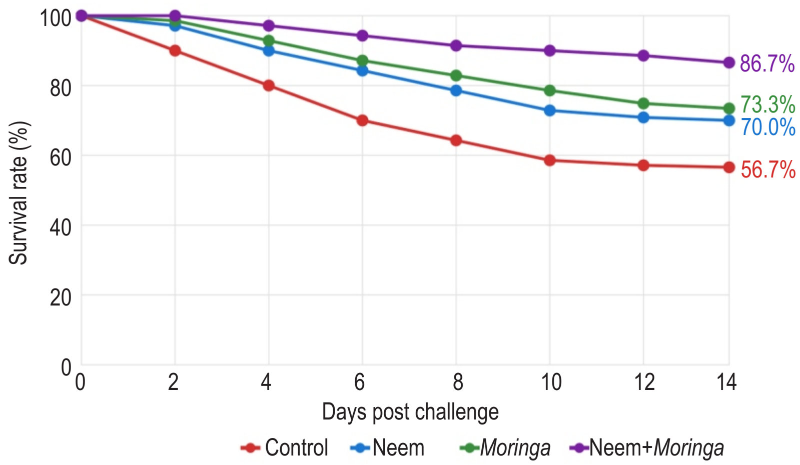


Fig. 1: Kaplan-Meier survival curves of *Labeo rohita* fingerlings challenged with Saprolegniaparasitica (1×10^5 spores ml^{-1}) after 8-week feeding with different dietary supplements. Control (red), Neem (blue), *Moringa* (green), and Neem+*Moringa* (purple) groups showed final survival rates of 56.7%, 70.0%, 73.3%, and 86.7%, respectively."

enhanced immune parameters as compared to the control (Table 6). The group fed with neem-moringademonstrated the highest values for all measured immune parameters, indicating a synergistic effect of Neem and Moringa supplementation.

The enhanced immune parameters observed in the supplemented groups, specifically Neem-Moringa, highlight the immunostimulatory properties of Neem and *Moringa*. The synergistic effect observed in the Neem-Moringa group suggests that the combination of these plant supplements may activate multiple immune pathways, resulting in a more robust immune response. These results corroborate with the previous study of Sahoo *et al.* (2017), who demonstrated that dietary immunostimulants can significantly enhance both hematological and innate immune parameters in Indian major carps, including improvement in the lysozyme activity, respiratory burst and serum bactericidal activity. The enhanced innate immune responses observed in this study are particularly significant in the context of fungal disease management. Earlier, Yadav *et al.* (2014) demonstrated that developing specific antibody responses against fungal pathogens through vaccination presents significant challenges, improving innate immunity through immunomodulation may be a more practical approach for protecting fish against fungal infections. Our findings are consistent with the previous studies that have demonstrated the immunostimulatory effects of Neem and Moringa individually. For instance, Kumar *et al.* (2013) reported enhanced immune responses in goldfish fed with azadirachtin-supplemented diets, while Abd El-Gawad *et al.* (2020) observed improved immune parameters in Nile tilapia fed with *M. oleifera* leaf powder.

The Kaplan-Meier survival analysis of post-challenge data revealed significant differences between the treatment groups (log-rank test, $p < 0.05$), with the group fed with Neem-Moringa showing the highest survival rate (86.7%), followed by fish fed with Moringa (73.3%), Neem (70.0%) and control (56.7%) group (Fig. 1). This is a crucial finding of this study, as it demonstrates the practical implications of Neem and Moringa supplementation in enhancing disease resistance against *S. parasitica.* The significant separation of survival curves confirms that the protective effect of the combined Neem-Moringa supplement was superior to either supplement alone. It's important to note that this is the first study to examine the combined effect of Neem and Moringa on *Saprolegnia* resistance, specifically in the unique hill climate of Meghalaya. This geographical context is significant, as the environmental conditions in Meghalaya may influence both fish physiology and pathogen virulence. The findings of this study suggest that this combination of supplements could be particularly beneficial for aquaculture in similar hill regions. The immuno-stimulatory effects observed in this study align with the previous research of Das *et al.* (2009), who demonstrated that appropriate immunostimulants can enhance key immune parameters including lysozyme, bactericidal and NBT activities in fish. Their findings showed that such enhancement of immune parameters directly correlate with improved disease resistance, supporting our observations regarding the protective effects of Neem and *Moringa* supplementation.

The improved survival rates in this study are more pronounced than those reported in previous research on individual supplements. Harikrishnan *et al.* (2009) found that a tri-herbal compound containing azadirachtin (from neem) provided protection against *A. invadans* in *C. mrigala,* but with lower survival rates than observed with the Neem-Moringa combination in this study. The results of this study also differ from those of Ibrahim *et al.* (2022), who used *Moringa*-synthesized silver nanoparticles to control *Saprolegnia* spp. infection in Nile tilapia.

While they observed improved survival and immune parameters, the present study demonstrated that a more natural, potentially more sustainable approach using dietary supplementation can achieve similar or better results. The reduced severity of clinical signs in the Neem-Moringa group is also noteworthy, which suggests that the combination of Neem and Moringa not only enhances survival but also mitigates the pathological effects of *S. parasitica* infection. The consistently superior performance of the Neem-Moringa group across all measured parameters demonstrates the powerful synergistic effects of combining Neem and *Moringa* supplementation, particularly under Meghalaya's distinct agro-climatic conditions. This synergy operates through multiple complementary mechanisms: at digestive level, Neem's enhancement of digestive enzyme activity (Alzohairy, 2016) works in concert with *Moringa*'s proteolytic enzymes (Abdull Razis *et al.,* 2014) to optimize nutrient utilization, while their effects on gut health are complementary–Neem's antimicrobial properties control pathogenic bacteria (Biswas *et al.,* 2002) as *Moringa*'s prebiotic effects promote beneficial gut microbiota (Sangkitikomol *et al.,* 2014). The combined antioxidant properties of both plants (Nahak and Sahu, 2010; Gopalakrishnan *et al.,* 2016) create a robust defense against oxidative stress, enhanced by their shared anti-inflammatory properties (Subapriya and Nagini, 2005; Mbikay, 2012), allowing more metabolic energy to be directed towards growth and immunity.

This metabolic optimization is further supported by their complementary immunological effects, where the bioactive compounds of neem (including azadirachtin, nimbidin, and quercetin) provide immunostimulant and antimicrobial properties (Harikrishnan *et al.,* 2009), while *Moringa*'s comprehensive nutrient profile of vitamins, minerals and bioactive compounds like flavonoids and phenolics (Gopalakrishnan *et al.,* 2016) support both innate and adaptive immune responses. Additionally, the hepatoprotective effects of both plants (Subapriya and Nagini, 2005; Fakurazi *et al.,* 2008) work synergistically to enhance metabolic efficiency and detoxification processes, collectively resulting in improved growth performance, enhanced immune function, and increased disease resistance, particularly valuable for aquaculture operations in regions with challenging environmental conditions.

This study provides a strong evidence for the synergistic effects of Neem and *Moringa* supplementation on the growth, health, and disease resistance of *L. rohita* under the agro-climatic conditions of Meghalaya. The findings of this study have several important implications for sustainable aquaculture practices in Meghalaya and potentially other regions with similar agro-climatic conditions. These findings will contribute to the development of sustainable, eco-friendly aquaculture practices tailored to the specific needs of the Meghalaya region and potentially other areas with similar agro-climatic conditions. Future research should focus on optimizing the dosage and ratio of Neem and Moringa supplementation, investigating the long-term effects of these supplements on fish health and environmental impact, and exploring their efficacy against other common pathogens in the region. Additionally, field trials in various aquaculture systems across Meghalaya would be valuable to validate the results of this controlled study under diverse real-world conditions.

Acknowledgments

The author expresses sincere gratitude to the Director of ICAR Research Complex for NEH Region, Umiam, Meghalaya, and Dr. S.K. Das, Principal Scientist (Fisheries Science) for their support and encouragement. Additionally, the author appreciate the efforts of all field staff of Fisheries Research Farm, for their comprehensive assistance in the experimental setup, fish procurement, acclimatization and stocking, collection of plant materials, processing and feed preparation, fish feeding, and sampling for the analysis of various growth and blood parameters.

Authors' contribution: C. Debnath: Conceptualization, methodology, writing - original draft, supervision.

Funding: This research was supported by the Institute's contingency fund.

Research content: It is original and not published elsewhere.

Ethical approval: The study was conducted following the standard methods and protocols.

Conflict of interest: The authors declare no conflict of interest.

Data availability: The data that support the findings of this study are available from the Corresponding author upon reasonable request.

Consent to publish: All authors have consented to the publication of this research.

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