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Haemato-Immunological Modulation in *Pangasianodon hypophthalmus* (Sauvage, 1878) Fingerlings Fed *Syzygium cumini* Leaf Powder

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Abstract

This study evaluated the nutraceutical efficacy of dietary jamun leaf powder on haematological responses, biochemical activities, immune competence, and oxidative stress-related responses in *Pangasianodon hypophthalmus* fingerlings, followed by a challenge study with *Aeromonas hydrophila*. The experimental fish were allocated to five dietary treatments, each with three replicates, and fed diets containing 0, 0.5, 1.0, 1.5, and 2.0% jamun (*Syzygium cumini*) leaf powder for 60 days. After the 60-day feeding trial, haematological, biochemical, antioxidant, and immune parameters were assessed before and after the challenge test. In the pre-challenge condition, fish receiving 1% (T_2) exhibited significantly higher red blood cell (RBC) count, haemoglobin concentration, packed cell volume, serum protein levels, albumin, and globulin than the control ($p < 0.05$). Following *A. hydrophila* challenge, all parameters showed significant alterations compared with pre-challenge values ($p < 0.05$). Stress-related metabolites, including glucose, cholesterol, and triglyceride levels, were significantly higher in the control group after the challenge ($p < 0.05$), indicating stress. Antioxidant enzyme activities, including superoxide dismutase (SOD) and catalase (CAT), were significantly higher in the 1% (T_2) group during pre-challenge conditions. In contrast, hepatic enzyme activities were significantly elevated in the post-challenge control group ($p < 0.05$). Post-challenge immune parameters, including respiratory burst activity, lysozyme activity, and myeloperoxidase activity, were significantly higher in fish fed 1% leaf powder (T_2) ($p < 0.05$). Overall, the inclusion of 1% jamun leaf powder in the diet improved haematological and biochemical status, antioxidant capacity, and immune responses, indicating its potential as an effective phytogenic feed additive for sustainable aquaculture.

Keywords: *Aeromonas hydrophila*, haematology, Pangasius, phytobiotic, immunity

Introduction

Fisheries and aquaculture are integral to global food systems, contributing to food security, nutritional well-being, and the sustainable supply of aquatic animal protein. Production from these sectors reached a record total of 223.2 million tonnes (USD 472 billion) in 2022, with global aquaculture production at 130.9 million tonnes, valued at USD 312.8 billion (FAO, 2024). In India, freshwater aquaculture is predominantly driven by three Indian major carp species: catla (*Catla catla*), rohu (*Labeo rohita*), and mrigal (*Cirrhinus mrigala)*, which together account for about 80% of freshwater aquaculture production. Besides carps, pangasius (*Pangasianodon hypophthalmus*) and pacu (*Piaractus brachypomus*) have also been cultivated on a large scale in recent years (Jayasankar, Mohanta, & Ferosekhan, 2018). Catfishes are among the most

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promising and rapidly developing species in freshwater aquaculture (Segaran et al., 2023). Among catfish, *Pangasius* spp. are highly preferred candidate species for freshwater farming, with *P. hypophthalmus* emerging as one of the most widely cultivable species (Ferguson et al., 2001). It is now widely farmed in Andhra Pradesh, West Bengal, Kerala, and Odisha, positioning India as one of the leading producers of *P. hypophthalmus* (Singh & Lakra, 2012). Pangasius production of 124,716 metric tonnes was recorded in India from 11,489 hectares in 2022-23. Andhra Pradesh recorded the highest production of 99,322 metric tonnes of pangasius on 10,246 hectares (MPEDA, 2024).

Phytobiotics are a promising alternative for sustainable aquaculture as they are biodegradable, environmentally friendly, often readily available, and generally have minimal or no side effects on humans (Bhanja, Payr, & Mandal, 2023). Phytobiotics are natural, plant-derived compounds added to animal diets to boost productivity (Cristea et al., 2012). *S. cumini* (Java plum or jamun tree), a member of the Myrtaceae family, is one of the notable medicinal plants native to the Indian subcontinent, Southeast Asian countries, and eastern Africa (Srivastava & Chandra, 2013). Jamun leaves are particularly valuable due to their rich content of bioactive compounds such as alkaloids, anthocyanins, acylated flavanol glycosides, quercetin, myricetin, myricitrin, triterpenoids, tannins, galloyl carboxylase, ellagic acid, and kaempferol (Ayyanar & Subash-Babu, 2012). In shrimp, dietary jamun leaf powder strengthened non-specific defence mechanisms against bacterial diseases (Prabu et al., 2018).

Recent studies show that phytogenic feed additives and plant-derived bioactive compounds can enhance innate immunity, antioxidant defence mechanisms, and disease resistance in cultured fish species, thereby providing sustainable alternatives to conventional chemotherapeutics in aquaculture (Faheem et al., 2022; Rajan, Divya, & Mohan, 2023; Elgendy et al., 2024; Pudota et al., 2025). However, limited research has explored the potential

of Jamun as a phytobiotic to enhance growth, improve immunological responses, and increase resistance to pathogenic bacteria in fish. Prabu, Ebeneezar, Chandrasekar, Kavitha, and Vijayagopal (2021) found that feeding juvenile snubnose pompano (*Trachinotus blochii*) with a diet supplemented with 1% jamun leaf powder significantly reduced oxidative stress, improved immune response, and enhanced resistance to *Vibrio parahaemolyticus* infection. Kannan, Felix, Panigrahi, and Ahilan (2022) reported that *Oreochromis niloticus* (GIFT) fed diets supplemented with 10 g per kg jamun leaf extract resulted in significantly enhanced growth, feed utilisation, haemato-immunological responses, and reduced mortality against *A. hydrophila*. Giri et al. (2023) found that dietary jamun leaf extract at 10 g/kg significantly improved growth, immunity, and resistance to *A. hydrophila* in common carp (*C. carpio*). Collectively, these findings suggest that jamun leaves may serve as a potential phytobiotic to support fish health and strengthen disease resistance in aquaculture. Accordingly, this study investigated the effects of jamun leaf powder on haematological, biochemical, and immunological responses, and oxidative stress in *P. hypophthalmus* before and after challenge with *A. hydrophila*.

Materials and Methods

Six hundred healthy fingerlings of pangasius (average length 7.5 ± 0.5 cm; weight 8.4 ± 0.45 g) were obtained for a preliminary study from a commercial nursery at Mollur (14.3024º N, 80.0842º E), Muthukur Mandal, SPSR Nellore District, Andhra Pradesh. The fish were moved to the wet laboratory at the College of Fishery Science, Muthukur, in an HDPE tank. The fish were subsequently placed in a circular flat-bottom polyvinyl chloride (PVC) reservoir tank with a volume of 5000 L and acclimatised for 30 days. Throughout the acclimatization period, the following water-quality parameters were maintained: temperature (25-28 °C), pH (7.0-7.2), dissolved oxygen (DO; 5.7-6.2 ppm), total alkalinity (112-118 ppm CaCO*f*), total hardness (173-182 ppm CaCO*f*), total ammonia nitrogen (TAN; 0.01-0.12 ppm), nitrite nitrogen (NO, -N; 0.00-0.024 ppm), and nitrate nitrogen (NO*f* -N; 0.01-0.26 ppm). During acclimatisation, fish were fed commercial pellet feed twice per day, at 08:00 and 17:00, until satiation. The leftover feed and faecal matter were removed by siphoning each day, followed by 5-10% water exchange.

Fresh, tender jamun leaves were obtained during the early summer season from a mature tree (15 years old) growing within the campus of College of Fishery Science, Muthukur, SPSR Nellore District, Andhra Pradesh, India (Latitude 14.299205° N, Longitude 80.088586° E). Approximately 2 kg of leaves (wet weight) were collected during the

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morning hours. The collected jamun leaves were rinsed thoroughly with fresh water to remove extraneous matter and shade-dried for several days. The dried leaves were pulverised and sieved to obtain a fine powder with a particle size of 100-150 ìm. The prepared leaf powder was stored in a clean, airtight container until its use in experimental diet preparation. The proximate composition of the jamun leaf powder was as follows: crude protein - 8%; moisture - 8%; crude fibre - 13%; crude fat - 1%; total Ash - 3%; and nitrogen-free extract - 65%.

Feed ingredients, namely fish meal, soya bean meal, groundnut oil cake (GNOC), de-oiled rice bran (DORB), wheat flour and fish oil were obtained from the local market. The vitamins-mineral mixture (Agrimin, India) was procured from a veterinary medical store. The feed ingredients were thoroughly dried and pulverised into a fine powder. Five experimental diets were formulated: a control diet (C) without jamun leaf powder and four supplemented diets (T_1, T_2, T_3, and T_4) containing 0.5, 1.0, 1.5, and 2.0% jamun leaf powder, respectively.

The trial-and-error method (Catacutan, 2002) was used for feed formulation. The crude protein content of all experimental diets was maintained at 30%. The pre-weighed ingredients were thoroughly mixed, and 300 mL of water was added per 1 kg of feed to form a dough. The dough was then cooked for half an hour in a pressure cooker. After cooking, it was allowed to cool to ambient temperature and then mixed with the vitamin-mineral mixture, fish oil, and jamun leaf powder. The dough was mixed properly and pressed through a pelletiser (La Monferrina model Dolly, Model no: 27/A - 14033) to get uniform-sized pellets. These moist pellets were dried in a hot-air oven overnight at 65 ! to reduce the moisture content below 10%. The dry pellets were stored in airtight containers and labelled according to their respective treatments. The composition of the control diet and experimental diets is given in Table 1.

The experiment was conducted in 15 collapsible tarpaulin tanks (R.S. Polymers, New Delhi) with dimensions of 0.8 m × 0.7 m (height × diameter), having a water-holding capacity of 270 L. Before starting the experiment, all the tanks were initially washed and filled with potassium permanganate solution (4 mg/L) and left overnight. The tanks were thoroughly cleaned, rinsed with fresh water, and sun-dried the following day. Each tank contained 200 L of water throughout the experimental period.

The 60-day feeding trial was conducted at the wet laboratory of the College of Fishery Science, Muthukur. Each of the five diets was tested in triplicate. Three hundred healthy and uniform-sized fingerlings of *P. hypophthalmus* (average length 10.65 ± 0.43 cm and average weight 12.56 ± 2 g) were distributed among 15 experimental tanks, with 20 fish in each tank. Approximately 10-15% of the water in each tank was replaced every other day. The fish in the experimental tanks were fed twice daily (8:00 and 17.00) at 5% body weight per day (Da, Lundh, Lindberg, & Berg, 2016) with the respective experimental diet. Fish readily consumed the feed, and no appreciable feed wastage was observed during the feeding period. During the 60-day feeding trial, the feeding rate was adjusted every two weeks based on changes in fish body weight. The haemato-immunological parameters of fish were analysed at the end of the feeding trial (pre-challenge) and at the end of the challenge study (post-challenge). All procedures followed the rules of the Committee for Control and Supervision of Experiments on Animals (CPCSEA), India, and the applicable animal welfare laws.

At the end of the feeding trial, six fish from each replicate tank were anaesthetised using MS-222 (Sigma-Aldrich Inc., St. Louis, MO, USA) at a concentration of 200 mg/L for the collection of both blood and serum to analyse various haematological and immunological parameters (Matthews & Varga, 2012; AVMA, 2020). For haematological analysis, blood samples were collected from the caudal vein of three fish per tank (n = 3) using a 2 mL syringe fitted with a 26-gauge needle. Before collection, the syringe was rinsed with 10% EDTA solution to prevent blood clotting. The collected blood was immediately transferred to EDTA-coated blood collection tubes. A 50 µL aliquot of the collected blood sample from each treatment and control was immediately analysed for red and white blood cell counts, neutrophil percentage, haemoglobin (Hb) concentration, haematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), and mean corpuscular haemoglobin concentration (MCHC). The haematological parameters were analysed using the haematology analyser (TRANSASIA H360, Automated 3 Part Differential Haematology Analyser, India), which has been calibrated and validated for use in fish blood analysis. The detailed workflow for the euthanisation

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of fish and the collection of blood and serum is provided in Fig. 1.

For immunology, blood was collected from the remaining three fish of each tank without using an anticoagulant and transferred to blood collection tubes. The samples were kept undisturbed at 4 °C for 4-5 h followed by centrifugation at 3000 rpm for 15 min (Biswas, Seoka, Takii, Maita, & Kumai, 2006). The supernatant serum was transferred to 1.5 mL Eppendorf tubes and stored at -20 °C until further use.

Total serum protein was measured using the Bradford method (Bradford, 1976). The globulin value was calculated by subtracting albumin from total serum protein. The albumin/globulin (A/G) ratio was calculated by dividing albumin by globulin. Stress-mitigating metabolic enzymes, such as glucose level, were assessed using a commercial kit (Sigma Diagnostics, Baroda, India) following the method of Trinder (1969). Serum cholesterol and triglyceride levels were estimated according to Parekh and Jung (1970) and Rice (1970), respectively.

Superoxide dismutase (SOD) activity and catalase (CAT) activity were measured according to the protocols described by Misra and Fridovich (1972) and Takahara et al. (1960). Alanine aminotransferase (ALT) (ERBA ALT KIT [Code Number - 120204]) and Aspartate aminotransferase (AST) (ERBA AST KIT [Code Number - 120207]) were determined using colourimetric assay kits from ERBA® Diagnostics (Mannheim, Germany), in accordance with the manufacturer's instructions (Patel, Verma, Krishnani, Sreedharan, & Chandrakant, 2022).

Respiratory burst activity (RBA) of phagocytes was evaluated through the Nitroblue tetrazolium (NBT) assay, following the procedure outlined by Stasiack and Bauman (1996). Lysozyme (LZY) activity was assessed using the turbidimetric method described by Ellis (2001). Myeloperoxidase (MPO) activity was measured based on the method described by Quade and Roth (1997).

A. hydrophila (ATCC 7966), obtained from Hi-Media Laboratories (Mumbai, India), was used for the challenge infection. The strain was subcultured in tryptone soya broth (TSB) with shaking at 37/ °C for 24 h. Bacterial growth was monitored by measuring optical density at 600/ nm (OD† • •) using a spectrophotometer (T60, LABINDA), followed by viable colony counting on tryptic soy agar (TSA). The bacterial suspension was centrifuged at 6,000 rpm for 10 min at 4 °C. The pellet was then washed twice using sterile 0.15 M phosphate-buffered saline (PBS). Subsequently, the bacterial cells were resuspended, dispensed into aliquots, and preserved at -70 °C in tryptic soy broth (TSB) containing 15% (v/v) glycerol until required. Before the challenge study, the lethal dose causing 50% (LD_{50}) mortality was estimated in pangasius fingerlings by injecting known concentrations of the bacterial pathogen

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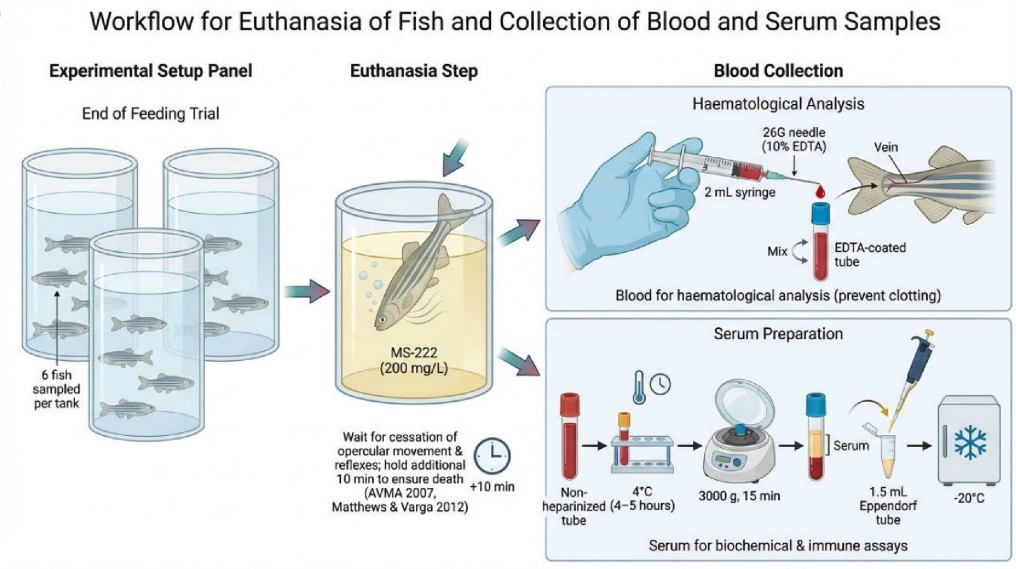


Fig. 1. Workflow for experimental set-up, sampling, euthanasia and blood collection from experimental fish

ranging from 2.3×10^3 to 2.3×10^9 CFU mL^{-1} through serial dilution. In the subsequent challenge trial, the fish were injected with the calculated LD_{50} concentration.

After 60 days of dietary administration of jamun leaf powder, ten fish per treatment were randomly selected and placed in separate tanks for the bacterial challenge. Each fish was administered an intraperitoneal injection of 0.1 mL of freshly prepared *A. hydrophila* suspension containing 2.3×10^7 CFU mL^{-1} (LD_{50}). Negative control fish received an intraperitoneal injection of 0.1 mL of sterile phosphate-buffered saline (PBS) following anaesthesia. Post-challenge, after 96 h, fish from each treatment were sampled for blood and serum for haematological, biochemical, and immunological studies as described above.

Statistical analysis of the experimental data was carried out using statistical software SPSS version 20 (IBM 2012). One-way analysis of variance (ANOVA) was used to test for significant differences. The statistical significance level was set at $p < 0.05$, and treatment means were compared using Duncan's multiple range test. Before performing ANOVA, the dataset was examined to verify normal distribution and homogeneity of variance using suitable statistical methods.

Results and Discussion

Haematological parameters of *P. hypophthalmus* fed diets supplemented with jamun (*S. cumini*) leaf powder were statistically analysed to determine significant differences between the dietary treatments and the control ($p < 0.05$) (Table 2). The effect of jamun leaf powder on the RBC of *P. hypophthalmus* fingerlings were evaluated at the end of the feeding trial and after the challenge study, with significant differences observed among the treatment groups ($p < 0.05$). After the feeding trial, RBC was significantly highest in the T_2 (3.8 ± 0.11**)** treatment, whereas the lowest value was observed in the control (1.4 ± 0.11). In the post-challenge study of intraperitoneal injection with *A. hydrophila*, the TEC was also significantly highest ($p < 0.05$) in the T_2 (2.87 ± 0.11) group, and lowest in the control group (0.6 ± 0.12).

After the feeding period, WBC counts were significantly elevated ($p < 0.05$) across all treatment groups relative to the control group. The highest WBC count was found in T_2 (17.3 ± 0.15), whereas the control group had its lowest value of 13.2 ± 0.06. After the challenge with *A. hydrophila*, WBC counts increased significantly ($p < 0.05$) in the treatment groups relative to the control group. T_2 group showed its highest WBC (37.4 ± 1.14), whereas the control group exhibited the lowest leukocyte count (17.7 ± 1.08) during the post-challenge study.

Following the feeding period, Hb content showed a significant increase ($p < 0.05$) across all treatment groups relative to the control group. The maximum Hb level was found in the T_2 group (12.0 ± 0.14), while the control group had the lowest value of 8.2 ± 0.11. Haemoglobin levels dropped in all groups after the challenge trial but remained significantly higher ($p < 0.05$) in the jamun-fed groups.

PCV values significantly increased ($p < 0.05$) in the treatment groups relative to the control group, both at the end of the feeding trial and after the bacterial challenge. After the feeding trial, the highest PCV value of 38.1 ± 0.61 was observed in the T_2 group, whereas the control group had the lowest PCV of 27.4 ± 1.04. After the bacterial challenge, PCV levels decreased in all groups, with the control group experiencing the most significant ($p < 0.05$) reduction to 17.3 ± 0.83. In contrast, the T_2 group maintained the highest post-bacterial challenge PCV of 33.5 ± 0.79.

The MCV levels showed clear variations among the treatment groups and the control group. At the end of the feeding trial, the control group recorded the significantly highest ($p < 0.05$) MCV of 190.3 ± 7.02, and the T_2 group had the lowest MCV value (99.3 ± 1.73). After the bacterial challenge, MCV levels increased in all the experimental groups, with the highest ($p < 0.05$) post-bacterial challenge MCV (268.5 ± 37.17) observed in the control group and the lowest post-challenge MCV (116.7 ± 2.31) in T_2.

MCH values were significantly higher ($p < 0.05$) in the control group relative to the treatment groups, both at the end of the feeding trial and post-challenge. At the end of the feeding trial, the control group had the highest MCH (57.1 ± 3.55; $p < 0.05$), and T_2 had the lowest MCH (31.3 ± 0.76). After the bacterial challenge, MCH levels increased in all the experimental groups, with the highest ($p < 0.05$) post-challenge MCH (81.1 ± 12.93) observed in the control group, and the lowest post-bacterial challenge MCH (39.2 ± 1.15) in the T_2 group.

MCHC levels were not significantly different ($p > 0.05$) among all the experimental groups observed

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at the end of the feeding trial, with MCHC values ranging from 30.0 ± 0.70 to 31.6 ± 0.98. In the post-challenge, MCHC values increased in all experimental groups compared with their respective pre-challenge MCHC values. T_1 recorded the highest MCHC (34.3 ± 0.98), though not significantly different ($p > 0.05$) from the other jamun-supplemented groups, while the control group showed the lowest MCHC (30.10 ± 0.95).

Serum biochemical parameters, viz., total protein, albumin, and globulin content, along with the A/G ratio in *P. hypophthalmus* receiving jamun-supplemented diets were analysed both at the end of the feeding period and 96 h after challenge with *A. hydrophila*, and the results were statistically compared between the experimental groups for significant differences ($p < 0.05$) (Table 3). A general decline in serum protein, albumin, and globulin concentrations was observed after the bacterial challenge relative to pre-challenge levels. Significant differences ($p < 0.05$) were observed between pre- and post-challenge values.

The total serum protein levels showed a significant ($p < 0.05$) difference between treatment groups and control, both at the end of the feeding trial and post-challenge. During pre-challenge, T_2 exhibited significantly higher total serum protein levels of 5.5 ± 0.09. In the post-challenge, serum protein levels decreased in all treatments, showing significantly higher levels in the T_2 group (5.2 ± 0.09).

The albumin and globulin levels showed a significant ($p < 0.05$) difference between treatment groups and control, both at the end of the feeding trial and post-challenge. The highest albumin (2.2 ± 0.06 g dL^{-1}) was observed in the T_2 group, and the lowest (1.8 ± 0.06 g dL^{-1}) was observed in the control group under pre-challenge conditions. Similarly, the T_2 group exhibited the highest pre-challenge globulin content (3.2 ± 0.05 g dL{ 1). However, during post-challenge, albumin content decreased in all treatments, with significantly higher levels in the T_2 group (2.1 ± 0.06 g dL^{-1}). The highest post-challenge globulin was also recorded in the T_2 group (3.1 ± 0.04 g dL^{-1}). For the A/G ratio, the highest values were observed in the T_1 (2.3 ± 0.06) and control (0.8 ±/ 0.03) groups during pre-challenge and post-challenge with *A. hydrophila*.

Glucose levels varied among treatments under both pre- and post-challenge conditions. During pre-challenge, the highest glucose content was recorded in the control group (115.5 ± 2.41 mg dL^{-1}) compared with the jamun leaf-fed groups. In contrast, the post-challenge control group showed the highest glucose concentrations (124.3 ± 3.51 mg dL^{-1}), indicating stress due to bacterial infection. Although glucose levels increased in all groups after the challenge, the elevation was less pronounced in the jamun leaf-supplemented treatments than in the control group.

Cholesterol and triglyceride levels differed significantly ($p < 0.05$) between the treatment groups and

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Table 1. Composition of experimental diets

Ingredients	Quantity per kg diet				
	C	T_1	T_2	T_3	T_4
Fish meal (g)	100	100	100	100	100
Soya bean meal (g)	300	300	300	300	300
Groundnut oil cake (g)	150	150	150	150	150
De-oiled rice bran (g)	250	250	250	250	250
Wheat flour (g)	150	145	140	135	130
Vitamin-Mineral mix[1] (g)	20	20	20	20	20
Fish oil (ml)	30	30	30	30	30
S. cumini leaf powder (g)	0	5	10	15	20

[1]Composition of vitamin-mineral mix: Agrimin (quantity kg^{-1}): Vitamin A-625,000 IU; Vitamin D3- 62,500 IU; Vitamin E-250 mg; Nicotinamide-1g; Cu-312 mg; Co-45 mg; Mg-6 g; Fe-1.5 g; Zn-2.13 g; I-156 mg; Se-10 mg; Mn-1.2 g; Ca-247.34 g; P-114.68; S-12.2 g; Na-5.8mg; K-48.05 mg

the control, both before and after the challenge. Both were highest in the control group before (148.4 ± 4.21 mg dL{ 1 and 218.1 ± 3.11 mg dL{ 1, respectively) and after the challenge (177.1 ± 4.78 mg dL{ 1 and 254.3 ± 2.42 mg dL{ 1, respectively). Both parameters increased across all groups following the challenge, most markedly in the control group ($p < 0.05$).

Superoxide dismutase (SOD) and catalase (CAT) activities in *P. hypophthalmus* varied significantly ($p < 0.05$) among the dietary treatments (Table 4). Before the bacterial challenge, the T_2 group exhibited the highest SOD (17.7 ± 0.12 U mL{ 1) and CAT activities (8.7 ± 0.09 U mL{ 1), whereas the control group showed the lowest values (SOD: 6.8 ± 0.05; CAT: 2.8 ± 0.07). Following the challenge, both enzyme activities decreased significantly ($p < 0.05$) across all groups relative to their pre-challenge levels. Despite this reduction, the T_2 group retained significantly higher SOD (15.4 ± 0.15) and CAT (7.9 ± 0.11) activities, while the lowest activities were observed in the control group (4.1 ± 0.07 and 1.9 ± 0.04, respectively).

Alanine aminotransferase (ALT) (17.9 ± 0.22 U mL{ 1) and aspartate aminotransferase (AST) activities (38.7 ± 0.40 U mL{ 1) reached their highest levels in the pre-challenge control group, whereas the lowest activities were found in the T_2 group (13.5 ± 0.15 and 28.4 ± 0.29, respectively). Compared with pre-challenge enzyme activities, post-challenge enzyme activities increased significantly in all treatment groups, suggesting bacterial infection-induced stress. In the post-challenge phase, the control group exhibited the highest ALT (26.6 ± 0.30 U mL{ 1) and AST (47.5 ± 0.52 U mL{ 1) activities. Among the dietary treatment groups, fish receiving the T_2 diet showed the lowest enzyme activities (17.9 ± 0.21 and 34.6 ± 0.38, respectively) under post-challenge conditions.

The immune-related indices, including respiratory burst activity, myeloperoxidase activity, and serum lysozyme levels of *P. hypophthalmus* fed with Jamun leaf-supplemented diets, were analysed both following completion of the feeding trial and 96 h after challenge with *A. hydrophila,* and the results were statistically compared between the experimental groups for statistical significance ($p < 0.05$) (Table 5).

The RBA (OD† , •) assay results revealed a statistically significant variation ($p < 0.05$) among the dietary treatments and the control group. At the end of the feeding trial, T_2 recorded significantly higher RBA values (0.4 ± 0.01), whereas the control exhibited the lowest value (0.2 ± 0.01). In the post-bacterial challenge, RBA values showed a significant increase ($p < 0.05$) across the dietary treatments, with T_2 showing its highest RBA value (0.6 ± 0.01), whereas the lowest value was observed in the control (0.3 ± 0.01).

Serum lysozyme activity (g dL{ 1) differed significantly among the dietary treatments ($p < 0.05$) between the jamun leaf-powder-fed experimental groups and the control. After the feeding trial, T_2 showed the highest lysozyme activity (47.3 ± 0.90). Post-challenge, a marked rise in lysozyme activity was observed across the dietary treatments. T_2 recorded the significantly highest lysozyme activity (64.9 ± 1.75) ($p < 0.05$).

Myeloperoxidase activity (U mg{ 1) varied significantly among the experimental groups ($p < 0.05$).

Table 2. Haematological parameters of *P. hypophthalmus* fed diets containing graded levels of *S. cumini* leaf powder.

Parameters	C		T_1		T_2		T_3		T_4	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
RBC ($\times 10^6$ μL^{-1})	1.4^a±0.11	0.6^a±0.12	1.8^b±0.08	1.2^b±0.09	3.8^d±0.11	2.8^d±0.11	2.5^c±0.10	1.6^c±0.11	2.4^c±0.09	1.5^c±0.11
WBC ($\times 10^3$ μL^{-1})	15.2^a±0.06	17.7^a±0.11	15.2^a±0.06	26.5^b±0.12	15.3^a±0.15	37.4^e±0.11	15.3^a±0.11	32.2^d±0.12	15.2^a±0.10	31.9^c±0.09
Hb (g dL^{-1})	8.2^a±0.11	5.2^a±0.10	9.5^b±0.08	7.7^b±0.09	12.0^d±0.14	11.2^d±0.10	10.5^c±0.12	10.1^c±0.10	10.4^c±0.12	10.1^c±0.13
PCV (%)	27.4^a±1.04	17.3^a±0.83	30.5^b±0.67	22.5^b±0.89	38.1^d±0.61	33.5^d±0.79	34.1^c±0.92	30.5^c±0.91	33.5^c±0.92	29.5^c±1.14
MCV (fL)	190.3^d±7.02	268.5^c±7.17	162.7^c±3.79	178.6^b±5.51	99.3^a±1.73	116.7^a±2.31	135.0^b±1.73	183.4^b±7.37	136.6^b±1.53	191.3^b±5.86
MCH (pg)	57.1^d±3.55	81.1^c±2.93	50.8^c±1.78	61.4^b±3.64	31.3^a±0.76	39.2^a±1.15	41.7^b±1.11	61.0^b±3.50	42.4^b±1.95	65.6^b±3.72
MCHC (%)	30.0^a±0.70	30.1^a±0.95	31.2^a±0.42	34.3^b±0.98	31.6^a±0.98	33.5^b±0.59	30.9^a±0.50	33.2^b±0.72	31.1^a±1.18	34.1^b±0.91

RBC: red blood cells; WBC: white blood cells; Hb: haemoglobin; PCV: packed cell volume; MCHC: mean corpuscular haemoglobin concentration; MCH: mean corpuscular haemoglobin; MCV: mean corpuscular volume. Data are represented as mean ± SD (n=3). Means with different letters (a–e) in the same row indicate significant differences ($p < 0.05$)

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After the feeding trial, T_2 showed the highest myeloperoxidase activity (0.58 ± 0.01). Post-challenge, myeloperoxidase activity increased considerably across all dietary treatments, with the highest value ($p < 0.05$) observed in T_2 (0.74 ± 0.05).

Medicinal plants have emerged as a cost-effective and sustainable solution for disease management in aquaculture (Reverter, Tapissier-Bontemps, Sasal, & Saulnier, 2017). In fish and shrimp farming, various medicinal plants and their bioactive components have demonstrated beneficial effects, including growth promotion, antibacterial activity, immunostimulatory effects, antioxidant properties, and anti-stress effects (Naiel, Ismael, Negm, Ayyat, & Al-Sagheer, 2020). These attributes not only reduce the dependency on antibiotics but also enhance vaccine efficacy through their immunostimulatory effects, contributing to improved disease prevention and overall aquaculture sustainability (Raissy et al., 2018).

Haematological parameters are widely used to evaluate the health status, stress, and disease conditions of fish (Vazirzadeh et al., 2017) and to detect any abnormalities arising from the use of immunostimulants (Abdel-Tawwab, Adeshina, Jenyo-Oni, Ajani, & Emikpe, 2018). RBC, haemoglobin (Hb), and PCV levels were significantly elevated ($p < 0.05$) in fish receiving jamun leaf-incorporated diets relative to the control group, with the highest values observed in fish fed the 1% diet (T_2). This can be due to the positive effect of polyphenolic compounds present in the Jamun leaf powder at that dose, which contributed to the absorption of iron to a good extent (Layrisse et al., 2000). The lower values in T_3 and T_4 might be due to higher polyphenol concentrations at 1.5% and 2%, which can form complexes with iron and reduce its availability for absorption (Raes, Knockaert, Struijs, & Van Camp, 2014). Giri et al. (2024) investigated the effects of Jamun fruit extract supplementation at 0, 2, 3, 4, and 5 g/kg on the haematological parameters of common carp (*C. carpio*) juveniles. An increase in RBC levels in fish often indicates improved oxygen transport capacity in the bloodstream (Habib et al., 2022), suggesting enhanced oxygen delivery to tissues, which is vital for various physiological processes, including metabolism and overall health (Naz et al., 2024).

Following *A. hydrophila* challenge, RBC, Hb, and PCV levels declined in all groups, but a significant reduction ($p < 0.05$) was evident in the control group, and significantly higher values ($p < 0.05$) were recorded in T_2. Reduced RBC counts and Hb concentrations in infected fish indicate pathogen-induced stress (Pratheepa, Ramesh, & Sukumaran, 2010); the marked fall in the control group in this study therefore points to severe infection, stress, and anaemia. Previous studies on various plant leaves have also reported a significant reduction in RBC, Hb, and PCV during post-challenge in control groups. Chandravanshi et al. (2020) investigated the effects of dietary green tea (*Camellia sinensis*) leaf powder at concentrations of 0.5, 1, 1.5, and 2 g/kg on common carp (*C. carpio*) fingerlings, and after a post-challenge with *A. hydrophila*, RBC, Hb, and Hct

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Table 3. Biochemical parameters of *P. hypophthalmus* fed with graded levels of *S. cumini* leaf powder

Parameter	C		T_1		T_2		T_3		T_4	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
Total Protein (g dL^{-1})	4.2^{a}±0.11	3.1^{a}±0.10	4.7^{b}±0.14	4.2^{b}±0.09	5.5^{d}±0.12	5.2^{d}±0.09	5.2^{c}±0.10	4.9^{c}±0.08	5.2^{c}±0.07	4.8^{c}±0.12
Albumin (g dL^{-1})	1.8^{a}±0.06	1.4^{a}±0.07	1.9^{b}±0.06	1.9^{b}±0.08	2.2^{c}±0.14	2.1^{c}±0.06	2.1^{bc}±0.05	1.9^{b}±0.04	2.1^{bc}±0.05	1.9^{b}±0.04
Globulin (g dL^{-1})	2.3^{a}±0.07	1.6^{a}±0.08	2.7^{b}±0.05	2.3^{b}±0.06	3.2^{c}±0.05	3.1^{d}±0.04	3.1^{c}±0.09	2.9^{cd}±0.04	3.0^{c}±0.09	2.8^{c}±0.04
A/G ratio	0.7^{a}±0.02	0.8^{a}±0.03	0.7^{a}±0.03	0.8^{a}±0.04	0.6^{a}±0.03	0.6^{a}±0.03	0.6^{a}±0.05	0.6^{a}±0.04	0.6^{a}±0.03	0.6^{a}±0.02
GLU (mg dL^{-1})	115.5^{d}±2.41	124.3^{d}±3.51	104.9^{c}±2.73	107.2^{c}±3.59	83.6^{a}±2.07	87.8^{a}±1.87	86.7^{a}±1.96	90.3^{a}±2.36	93.4^{b}±3.14	98.6^{b}±2.23
CHO (mg dL^{-1})	148.4^{b}±4.21	177.1^{c}±4.78	110.0^{a}±2.41	134.3^{b}±3.62	106.2^{a}±3.47	124.5^{ba}±2.51	105.2^{a}±2.90	118.2^{a}±3.07	108.1^{a}±3.18	126.3^{ba}±3.21
TG (mg dL^{-1})	218.1^{b}±3.11	254.3^{c}±2.42	154.3^{a}±2.17	170.6^{b}±3.24	146.3^{a}±3.17	160.4^{a}±2.32	148.4^{a}±2.46	165.6^{ba}±3.02	152.7^{a}±2.33	166.1^{ba}±3.62

GLU: Glucose; CHO: Cholesterol; TG: Triglyceride. Data are represented as mean ± SD (n=3). Means with different letters (a–d) in the same row indicate significant differences ($p < 0.05$)

levels decreased in all treatments, with a significant decrease ($p < 0.05$) observed in the control group. Similarly, Neamat-Allah, Mahmoud, and Mahsoub (2021) found that during the feeding trial, white mulberry (*Morus alba*) leaf extract supplementation at 1 g/kg, 3 g/kg, and 5 g/kg decreased RBC, Hb, and Hct levels after the challenge with *A. hydrophila*.

After the feeding trial, the leukocyte count was significantly higher ($p < 0.05$) in jamun leaf-supplemented groups than in the control group, potentially reflecting the effects of bioactive constituents such as flavonoids and phenolic acids. According to Awaad, and Esteban (2015), quercetin, a flavonoid, can contribute to leukocyte stimulation and the activation of phagocytic functions. The highest WBC count in T_2, with lower counts in T_3 and T_4, suggests that moderate inclusion of Jamun leaf powder (1%) may enhance immune function more effectively than higher concentrations, possibly through optimal bioavailability of immunoactive compounds such as flavonoids and phenolic acids (Pudota et al., 2025). The lower WBC counts in T_3 and T_4 may reflect higher polyphenol concentrations, which could have high levels of anti-nutritional factors that interfere with the digestion and absorption of essential nutrients (Kannan et al., 2022), thereby hampering the immuno-stimulatory effects.

In fish, elevated levels of total protein, albumin, and globulins are widely recognised as indicators of a robust innate immune system and are essential for maintaining overall immune function (Barui et al., 2024). In this study, the T_2 treatment exhibited significantly elevated total protein, albumin, and globulin concentrations. Similarly, dietary supplementation with 1% jamun leaf powder increased total protein levels in common carp (Giri et al., 2023) and snubnose pompano (Prabu et al., 2021). Maiti et al. (2023) reported that dietary inclusion of *A. paniculata* led to an increase in total protein, albumin, and globulin levels compared to the control in *P. hypophthalmus*. Total serum protein is also a valuable biomarker for assessing fish health, as it reflects not only immune competence but also nutritional status and physiological stress levels (Hoseini & Tarkhani, 2013). The decrease in serum total protein following experimental infection suggests leakage of proteins through the vascular walls due to increased permeability, along with impaired synthesis and non-specific proteolysis (Misra et al., 2006).

Glucose plays a vital role in cellular respiration, acting as a primary energy source that supports ATP production for bodily tissues and is commonly recognised as a sensitive physiological indicator of stress in aquatic organisms. In this study, the control fish displayed significantly higher blood glucose concentrations than fish receiving jamun leaf powder-supplemented diets, indicating a possible stress-reducing effect of dietary supplementation. After challenge with *A. hydrophila*, glucose levels rose in all experimental groups; however, the increase was less pronounced in the jamun leaf-fed treatments, indicating its efficacy in reducing pathogen-induced oxidative stress. Similar findings were reported in *P. hypophthalmus* fed on a fucoidan-supplemented diet, which exhibited lower blood glucose levels compared to the control group and the Jamun leaf powder-supplemented diet in *T. blochii* (Prabu, Sahu, Pal, Dasgupta, & Narendra, 2016; Prabu et al., 2021). Elevated levels of glucose in the bloodstream typically reflect a stress response, during which stored glycogen in the liver and muscles is rapidly mobilised (Vosylienë & Kazlauskienë, 1999). Jamun leaves are known to possess hypoglycaemic properties attributed to their rich bioactive compound content, which has been shown to reduce blood

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Table 4. Serum enzyme activities of *P. hypophthalmus* fed with graded levels of *S. cumini* leaf powder

Parameters	C		T_1		T_2		T_3		T_4	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
SOD(U mL^{-1})	$6.8^{a}\pm0.05$	$4.1^{a}\pm0.07$	$9.4^{b}\pm0.08$	$7.6^{b}\pm0.09$	$17.7^{d}\pm0.12$	$15.4^{d}\pm0.15$	$15.5^{c}\pm0.09$	$13.1^{c}\pm0.10$	$13.1^{c}\pm0.09$	$10.3^{c}\pm0.11$
CAT(U mL^{-1})	$2.8^{a}\pm0.07$	$1.9^{a}\pm0.04$	$4.3^{b}\pm0.05$	$3.1^{b}\pm0.06$	$8.7^{d}\pm0.09$	$7.9^{d}\pm0.11$	$8.2^{c}\pm0.09$	$7.2^{c}\pm0.07$	$6.4^{c}\pm0.06$	$5.8^{c}\pm0.08$
ALT (U mL^{-1})	$17.9^{d}\pm0.22$	$24.6^{d}\pm0.30$	$16.3^{c}\pm0.18$	$21.7^{c}\pm0.26$	$13.5^{a}\pm0.15$	$17.9^{a}\pm0.21$	$14.4^{b}\pm0.16$	$19.2^{b}\pm0.20$	$14.9^{b}\pm0.17$	$19.8^{b}\pm0.24$
AST(U mL^{-1})	$38.7^{d}\pm0.40$	$47.5^{d}\pm0.52$	$35.2^{c}\pm0.36$	$43.1^{c}\pm0.47$	$28.4^{a}\pm0.29$	$34.6^{a}\pm0.38$	$30.5^{b}\pm0.31$	$36.8^{b}\pm0.40$	$31.1^{b}\pm0.33$	$37.2^{b}\pm0.41$

SOD: superoxide dismutase; CAT: catalase; ALT: alanine aminotransferase; AST: aspartate aminotransferase. Data are represented as mean ± SD (n=3). Means with different letters (a-d) in the same row indicate significant differences ($p < 0.05$)

glucose concentrations and oxidative damage while also improving lipid metabolism (Prabu et al., 2018). Increased cholesterol and triglyceride concentrations may be associated with impaired hepatic and renal function, potentially affecting lipid metabolism and circulating lipid levels. Since the liver plays a central role in lipid metabolism, such increases are often considered indicative of hepatic impairment (Öner, Atli, & Canli, 2008).

Oxidative stress refers to a state in which the production of free radicals and reactive metabolites exceeds the capacity of the body's antioxidant defence systems to neutralise and eliminate them (Yonar, Sakin, Yonar, Ispir, & Kirici, 2011). Antioxidant enzymes such as catalase and superoxide dismutase constitute the primary line of defence against oxidative stress by eliminating reactive oxygen species (ROS) (Ighodaro & Akinloye, 2018). The results of this study indicated that *P. hypophthalmus* receiving jamun leaf powder-supplemented diets showed significantly greater antioxidant capacity than the control group. These results are consistent with previous studies, which also demonstrated enhanced antioxidant responses following dietary inclusion of Jamun (Kannan et al., 2022; Giri et al., 2023). This improvement in antioxidant activity could be associated with phenolic and flavonoid constituents present in the plant-based supplements. These phytochemicals can contribute to antioxidant activity through their reducing capacity, which is related to the hydroxyl groups and unsaturated bonds in their molecular structures. Among them, phenolic acids, particularly gallic acid, are regarded as potent antioxidants capable of significantly improving the oxidative status of aquatic organisms (Rufchaei, Mirvaghefi, Hoseinifar, Valipour, & Nedaei, 2020).

The enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are widely recognised as key indicators of liver function and stress in fish (Khan et al., 2018). In the present study, fish receiving diets supplemented with jamun leaf powder exhibited lower ALT and AST activities than the control group under both pre- and post-challenge conditions. The T_2 treatment showed a significant reduction in these enzyme activities, indicating improved hepatic function and a possible decrease in cellular damage. These findings align with an earlier study, which also reported lowered AST and ALT levels in *Penaeus vannamei* fed diets enriched with jamun leaf powder (Prabu et al., 2018). A decline in these hepatic enzymes was also observed in *T. blochii* fed 1% jamun leaf powder, both before and after bacterial challenge (Prabu et al., 2021). Under normal conditions, ALT is mainly found around hepatic cells and bile ducts, while AST is located within hepatocyte mitochondria (Hassaan et al., 2019). However, liver damage can lead to their leakage into the bloodstream due to membrane disruption or increased enzyme synthesis (Yang & Chen, 2003). Elevated levels of AST and ALT enzymes have been associated with oxidative stress and lipid accumulation in the liver, making them valuable biomarkers for assessing hepatic health and physiological stress in fish (Satheeshkumar, Ananthan, Senthilkumar, Khan, & Jeevanantham, 2012). This hepatoprotective effect was attributed to the presence of flavonoids, which act as potent natural antioxidants, enhancing the liver's defence against oxidative stress and cellular damage (Li, Zhu, Tian, Liu, & Wang, 2019). Supporting the present findings, dietary supplementation with a seaweed extract mixture (*Ulva lactuca*, *Jania rubens*, and *Pterocladia capillacea*) significantly elevated antioxidant enzyme activities (SOD

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Table 5. Immunological parameters of *P. hypophthalmus* fed graded levels of *S. cumini* leaf powder

Parameters	C		T_1		T_2		T_3		T_4	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
RBA(OD_{620})	0.2^{a}±0.01	0.3^{a}±0.01	0.3^{b}±0.01	0.4^{b}±0.01	0.4^{e}±0.01	0.6^{e}±0.01	0.3^{d}±0.01	0.5^{d}±0.01	0.3^{c}±0.01	0.5^{c}±0.01
[1]LZY activity (U L^{-1})	32.4^{a}±1.00	37.6^{a}±0.82	38.2^{b}±0.93	45.6^{b}±1.03	47.3^{e}±0.90	64.9^{e}±1.75	43.4^{d}±0.76	56.6^{d}±0.99	41.3^{c}±0.69	52.3^{c}±0.86
[2]MPO(U L^{-1})	0.34^{a}±0.01	0.49^{a}±0.07	0.43^{b}±0.01	0.57^{b}±0.06	0.58^{d}±0.06	0.74^{d}±0.05	0.52^{c}±0.15	0.68^{c}±0.09	0.48^{c}±0.04	0.62^{c}±0.03

RBA: Respiratory Burst Activity; LZY: Lysozyme; MPO: Myeloperoxidase activity. Data are represented as mean ± SD (n = 3). Means with different letters (a–e) in the same row indicate significant differences ($p < 0.05$). [1]One unit of lysozyme will produce a decrease in absorbance (Delta A_450) of 0.001 per minute at pH 6.24 at 25 °C, using a suspension of Micrococcus lysodeikticus as the substrate. [2]Amount of protein that decomposes 1 micromole (μmol) of hydrogen peroxide per minute

and CAT) and lowered serum ALT and AST levels in *P. hypophthalmus* (Abdelhamid, Ayoub, El-Gawad, Abdelghany, & Abdel-Tawwab, 2021).

NBT activity reflects the oxygen-dependent bactericidal capacity of immune cells. The higher RBA in the treatment groups, particularly T_2, is consistent with earlier reports of enhanced RBA levels in *T. blochii* and *C. carpio* following dietary supplementation with jamun leaf (Kannan et al., 2022; Giri et al., 2023). The observed increase in NBT levels appeared beneficial in enhancing the fish's defence against invading pathogens (Solem, Jørgensen, & Robertsen, 1995). Lysozyme is an essential component of non-specific humoral immunity, acting before the activation of cellular defence mechanisms. In this study, dietary supplementation with jamun leaf powder resulted in a significant increase in lysozyme activity across the treatment groups, with the T_2 group exhibiting the highest activity before and after the challenge. Similarly, significantly higher lysozyme activity was reported in a 1% jamun leaf extract-supplemented diet in *C. carpio* (Giri et al., 2023). The higher MPO activity is in line with previous studies that reported similar increases in *O. niloticus* and *C. carpio* fed jamun leaf extract (Kannan et al., 2022; Giri et al., 2023). *P. hypophthalmus* fed with *A. paniculata* leaf extract exhibited significantly enhanced pre- and post-challenge lysozyme and NBT activity (Maiti et al., 2023). Similarly, plant extract-based diets significantly increased myeloperoxidase and lysozyme activity compared with the control group in *P. hypophthalmus* (Abd-Elaziz, Shukry, Abdel-Latif, & Saleh, 2023). The observed increase in RBA, lysozyme activity, and MPO levels suggests a strengthened innate immune response, which may confer enhanced protection against infectious diseases (Srivastava & Pandey, 2015).

The beneficial effects of jamun leaf powder observed in the present study may be associated with its diverse array of bioactive phytochemicals, such as flavonoids and phenolic compounds, tannins, anthocyanins, along with other antioxidant compounds. Phenolic compounds and flavonoids can scavenge reactive oxygen species and enhance endogenous antioxidant defence systems, thereby reducing oxidative stress and protecting immune cells from damage (Pudota et al., 2025). Tannins and other polyphenols have also been reported to modulate innate immune responses by stimulating phagocytic activity, lysozyme production, and leukocyte function. The combined action of these constituents, which are known to stimulate immune responses and support overall immune health in fish (Ahmadifar et al., 2021), may explain the improved antioxidant status, enhanced immune competence, and increased resistance against *A. hydrophila* infection observed in fish fed jamun-supplemented diets. The phytochemical composition of jamun may vary seasonally due to differences in environmental conditions, geographical location, plant maturity, and harvesting period. Therefore, further investigations should assess the influence of jamun leaf powder supplementation on growth performance, feed efficiency, economic viability to further establish its potential application in commercial aquaculture systems. In addition, studies are also required to evaluate seasonal effects on the phytochemical profile and efficacy of jamun leaf powder as a feed additive.

The findings of this investigation indicate that supplementation with 1% jamun leaf powder led to significant improvements in haemato-biochemical indices, enhanced immunological responses, strengthened the antioxidant defence system, and mitigated oxidative damage, thereby supporting innate immune responses to *A. hydrophila* infection. Such beneficial effects may be associated with the combined effects of bioactive constituents, including flavonoids, terpenoids, and phenolic acids, which are known for their immunostimulatory and antimicrobial properties. While the present study demonstrates the in vivo efficacy of jamun leaf powder against *A. hydrophila* in a controlled experimental setting, additional in vitro investigations and field-based trials are needed to confirm its antibacterial activity and determine its practical effectiveness and suitability under commercial farming conditions. These findings suggest that jamun leaf powder could be orally administered as an effective phytobiotic in pangasius culture.

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