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Assessment of *Melia Azedarach* Leaf Extract on Growth, Immune Responses, and Hematological Profile of *Cyprinus Carpio*

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Abstract
The objective of this study was to examine the effect of *Melia azedarach*, commonly known as chinaberry, on the growth, hematological characteristics, and serum immunological parameters of Common carp (*Cyprinus carpio*). This experimental study involved four groups: a control group fed with a basal diet having no *Melia azedarach* leaf extract addition, and three experimental groups, T1, T2, and T3, fed with 1%, 2% and 3% of *Melia azedarach* leaf extract, respectively. Each aquarium contained ten fish. The results obtained at the end of the feeding period indicated that the Common carp fed with diets comprising 1%, 2% and 3% *Melia azedarach* leaf extract had higher weight gain, SGR percentage, and feed conversion ratio compared to the control group. Hematological analyses revealed that Red blood cell (RBC) count (Control: 2.50×106/µL; T3: 4.15×106/µL) and hemoglobin (Hb) levels (Control: 6.80×µL; T3: 11.20µL) were high. Similarly, immunological parameter were also high in all treatment group i.e. Neutrophil (Control: 80%; T3: 85.5), Lymphocytes (Control: 6%; T2 and T3:8), Monocytes (Control: 3%; T2: 3.9) and Eosinophil (Control: 3%; T3: 2,4) were significantly higher in the treatments groups compare to the control group, suggesting a positive growth and health effect of the plant extract on grass carp. The study concludes that *Melia azedarach* leaf extract has a positive effect on the growth, hematology, and immunological parameters of common carp, therefore, safe in the current proportion as investigated in this experiment. Also, a cost-effective natural growth promoter and immunity booster being easily available in the local markets of Pakistan, *Melia azedarach* may as well be included in the diets of common carp in fish hatcheries across the country.

Keywords: Common Carp, *Melia azedarach*, Immunological parameters, Hematology, Immunity

Introduction
The common carp (*Cyprinus carpio*) belongs to the family Cyprinidae and the order Cypriniformes. This species typically avoids brackish water and predominantly resides in freshwater environments such as ponds, lakes, and rivers. It can often be found in streams, slow-moving rivers, and shallow wetlands (Yaqoob 2021). While *C. carpio* is originally native to Asia, it has been extensively introduced across Europe due to its popularity. Today, this species can be found on every continent except for Antarctica and northern (Parkos and Wahl 2014). Asia Fish farming serves as a crucial source of essential nutrients and sustainable protein

for a large segment of the global population (Naylor, Goldburg et al. 2000). Small-scale aquaculture enterprises enhance the economic stability and food security of local populations in many developing nations (Béné, Barange et al. 2015). Intensive aquaculture systems designed for high-value fish species such as prawns and salmon have emerged as a key source of export revenue for various countries, aiding in their economic development and enhancing foreign exchange reserves (Anderson, Asche et al. 2017). The release of excess nutrients and organic matter from aquaculture facilities can cause eutrophication in nearby water bodies, negatively affecting water quality and aquatic life (Chopin, Buschmann et al. 2001). Integrated Multi-Trophic Aquaculture (IMTA) promotes the cultivation of multiple species in the same environment to maximize resource use and minimize waste (Reid, Gurney-Smith et al. 2019).
Melia azedarach, known as Persian lilac or chinaberry, is a deciduous tree belonging to the Meliaceae family and primarily found in South Asia (Iran, India, and South China). This species was introduced to the New World, where it has naturalized across tropical America, from Mexico to Argentina (Sharma and Paul 2013). The tree can reach heights of up to 45 meters and is valued for its shade and ornamental qualities. With its smooth greenish-brown bark that becomes grey and cracked with age, *M. azedarach* features alternate, bipinnate leaves and produces fragrant white to violet flowers. Its small, round golden drupes contain several black seeds surrounded by silky pulp (Al-Rubae 2009), (Asadujjaman, Saed et al. 2013). This plant has well-documented medicinal properties in Ayurvedic, Siddha, and Unani systems, classified as anthelmintic, astringent, and tonic, among others (Ahmad et al., 2004; Cropley & Hasegawa, 2007). Rich in terpenoids, flavonoids, and other compounds, *M. azedarach* extracts exhibit significant phytochemical diversity (Suresh, 2008; Sharma & Paul, 2013).
Medicinal plants have been utilized for thousands of years to treat various diseases, leading to increased scientific interest in safe phytomedicines and bioactive compounds derived from them (Sen and Batra 2012). *Melia azedarach*, known for its therapeutic properties, has effective ethanolic extracts that address fever, nausea, vomiting, thirst, and skin conditions, while also exhibiting antioxidant and analgesic effects. The fruits and leaves have shown antifeedant, cytotoxic, antifungal, antibacterial, and antimalarial properties (Asadujjaman et al., 2013). Additionally, fresh leaf extracts are applied externally for gingivitis and burns, and a mouthwash made from these extracts is used for oral hygiene. For the treatment of piles, a daily oral dose of 5 ml of leaf extract is recommended (Ramya, Jepachanderamohan et al. 2009).
Immune stimulants are substances that enhance resistance to pathogens by strengthening both specific and nonspecific defense mechanisms. Unlike vaccines, which target specific pathogens at the time of administration, herbal immune stimulants are rich in immune-boosting compounds that improve overall immune function (Galindo-Villegas and Hosokawa 2004). For instance, dietary supplementation of plant extracts in fish has been shown to significantly increase blood parameters indicating health, such as red blood cell counts and hemoglobin levels (Reverter, Bontemps et al. 2014). These extracts also enhance respiratory and phagocytic activities and overall plasma protein levels, contributing to fish immunity (Guo, Pu et al. 2021). The use of immune stimulants in aquaculture is expected to increase as feed additives, aiming to enhance fish health, reduce the reliance on antibiotics, and improve product safety (Midtlyng 1997). Moreover, historical usage of plant extracts in various ancient cultures highlights their recognized benefits, yet modern justification for their use in animal health requires empirical evidence of their positive effects (Greathead 2003). This review aims to explore the immune stimulant properties of *Melia azedarach* and its effects on common carp health.

Materials and Methods

The experiment was conducted at the Fisheries Research Farms within the Department of Fisheries and Aquaculture at the University of Veterinary and Animal Sciences in Lahore. Common carp (*Cyprinus carpio*) were obtained from the fish hatchery and placed in holding tanks with a continuous supply of aerated water. The fish were acclimated to their new environment for two weeks before the research trial began.

Acclimatization of Fish & Experimental Conditions

The experiment was carried out using 70-liter glass aquaria, each equipped with continuous aeration, and maintained under controlled laboratory conditions with consistent parameters. These conditions included a constant water temperature of 30±1.00°C, a pH level of 7.50±0.5, and a total hardness of 200±2.00 mg/L. To regulate the pH, NaOH was used to raise it when necessary, while HCl was employed to lower it. The total water hardness was managed by adding CaSO4 and MgSO4 salts to increase hardness and EDTA to reduce it. Throughout the entire experimental period, the fish experienced a 12-hour photoperiod. The physico-chemical characteristics of the test environment, including water temperature (in °C), pH, total hardness (in mgL-1), total ammonia (in mgL-1), dissolved oxygen (in mg/L), and carbon dioxide (in mgL-1) were measured at 12-hour intervals per the A.P.H.A. (1998) guidelines. Following acclimation, each glass aquarium with a 70-liter water capacity was randomly stocked with a group of 25 healthy fish of approximately the same size. There were four distinct treatments labeled as Control, Group 1, Group 2, and Group 3.

Preparation of Feeding Diet

Germ-free leaves of the *Melia azedarach* plant were purchased and placed in a shady room for drying. Five to six days of shaded-dried leaves plant were then ground by grinder into fine powder form and then subjected to extraction using 80% methanol at room temperature, followed by filtration. The resulting filtrate was evaporated using a rotary evaporator, resulting in a dark-greenish residue referred to as the extract by following the method used by Azmat et al., 2021. This extract was then tested as a feed additive on experimental fish to assess its impact on *Cyprinus carpio* growth, hematology, and immunology. The experimental diets were formulated using locally available fish feed ingredients. The feed formulation is detailed as follows:

Table 1: Basal Diet Ingredients

Ingredients	Per 100g Mixture
Fish meal	35g
Corn	30g
Cottonseed meal	05g
Rice	10g
Wheat flour	10g
Sun flower oil	05g
Vitamin and Mineral Mixture	05g
Total	**100g**

Growth study

The control group received a basal diet containing 34% protein sourced from a local market. In contrast, treatments 1, 2, and 3 had 1%, 2%, and 3% *Melia azedarach* leaf extract added to their diets, respectively. Growth trials were conducted over 60 days, with observations made every two weeks on mean wet weight, fork length, overall length, feed consumption, feed conversion efficiency, and survival rate for each treatment group. At the end of each trial, five common carp samples were randomly selected from each treatment group to evaluate weight gain, feed conversion ratio, average daily weight gain, and survival rate, calculated using established formulas.

Weight gain (g/fish) = Final weight - Initial weight
Feed conversion ratio = Total feed / Total weight gain
Average daily gain (g/fish) = Final weight - Initial weight / Experimental days

Survival rate (%) = Number of fish at the end / Number of fish initially stocked x 100
Hematological tests were conducted to assess total leukocyte count (TLC), neutrophils, lymphocytes, monocytes, eosinophils, platelets, total red blood cell count, and hemoglobin levels after the feeding period. Each fish group underwent testing with three replicates, and average values were calculated. Blood samples were collected using a sterile plastic syringe from the caudal veins of randomly selected fish (Control, T1, T2, and T3) from each aquarium for hematological and immunological analysis. The samples were stored in anticoagulant (EDTA)-filled tubes for examination. Hematological parameters in fish can be influenced by environmental factors such as infections, diet, and water quality. Key red blood cell parameters include hemoglobin concentration (Hb) and erythrocyte count (RBC), while white blood cell parameters include TLC and differential leukocyte count (DLC), which measures different leukocyte types like neutrophils, lymphocytes, eosinophils, monocytes, and basophils. Analysis was performed using the Nihon Kohden Hematology Analyzer MEK-650, an automated device that provides results within 60 seconds.

Dropsy Disease Trails
Following the growth phase, each of the three aquariums and the control group was exposed to dropsy-diseased fish to assess their immune response. The evaluation of the fish's immune response, measured in terms of the percentage of diseased fish, was conducted using the methodology outlined by Rehman et al. in 2015.

Statistical Analysis
The impact of plant extract supplementation in the diet on growth, hematological factors and immunological parameters was assessed through one-way analysis of variance (ANOVA). Significance was evaluated at $p < 0.05$.

Results
Results of Growth Response
Growth parameters such as an increase in average weight gain, feed intake, and feed conversion ratio with different treatments, i.e., 1%, 2%, and 3% *Melia azedarach* leaf extract percentage, were recorded at the beginning and end of the experimental trial. The readings are stated in the table-

Table 2: Growth Response of Common carp fed with 1% plant extract (Treatment 1)

	Average Weight (g)	Increase in Average Weight (g)	Feed Intake (g)	Feed Conversion Ratio (%)
Start	21.16±1.16	-	-	-
End	52.98±1.09	7.46	23.20±0.19	3.11±0.09

Statistical significance level at $p \leq 0.05$

Table 2 indicates the average values for the growth parameters of Common carp, such as weight gain, feed intake, and feed conversion ratio, recorded at the beginning and at the end of the experimental trial for Treatment No. 1. Initial weight was recorded with average values of 21.16±1.16g. Final weight was recorded with average values of 52.98±1.09g. The average weight gain resulted in 7.46g. The feed conversion ratio, i.e., 3.11was recorded for treatment No.1.

Table 3 Growth Response of Common carp fed with 2% plant (Treatment 2)

	Average Weight (g)	Increase in Average Weight (g)	Feed Intake (g)	Feed Conversion Ratio (%)

Start	22.14±2.13	-	-	-
End	59.24±3.44	10.09	15.67±0.24	1.65±0.06

Statistical significance level at $p \leq 0.05$

Table 3 represents the data recorded for Treatment No.2. The Initial weight of the Common carp in this treatment was recorded with average values of 22.14±2.13g. Final average weight was recorded as 59.24±3.44g. The average weight gain resulted in 10.09g. The feed conversion ratio, i.e., 1.65, was recorded for this treatment group.

Table 4 Growth Response of Common carp fed with 3% plant extract (Treatment 3)

	Average Weight (g)	Increase in Average Weight (g)	Feed Intake	Feed Conversion Ratio (%)
Start	22.39±3.10	-	-	-
End	49.40±3.17	9.92	21.87±0.03	2.29±0.05

Statistical significance level at $p \leq 0.05$

Table 4 represents the data recorded for growth response in treatment No.3 of Common carp fed with 3% plant extract. The initial weight of the Common carp in this treatment was recorded with average values of 22.39±3.10g. Final average weight was recorded as 49.40±3.17g. The average weight gain resulted in 9.92g. The feed conversion ratio, i.e., 2.29, was recorded for this treatment group.

Table 5: Growth Response of Common carp fed with Normal Basal Diet (Control group)

	Average Weight (g)	Increase in Average Weight (g)	Feed Intake (g)	Feed Conversion Ratio (%)
Start	21.30±4.23	-	-	-
End	51.44±4.32	5.94	10.10±0.10	1.70±0.04

Statistical significance level at $p \leq 0.05$

The fish in the control treatment were fed with a diet free from *Melia azedarach*. The growth parameters for the Common carp are recorded and presented in Table 4.4. Common carp with an average wet weight of 21.30±4.23g was stocked in an aquarium, and at the end of the experiment, the final average weight was recorded as 51.44±4.32g with the relevant feed intake and feed conversion ratio.

Table 6: Combined Representation of Growth Response of Common carp Fed with and without *Melia azedarach* Extract

	Average Increase in weight (g)	Average Feed intake (g)	Feed Conversion Ratio
Treatment 1	7.46±0.06	23.20±0.19	3.11±0.09
Treatment 2	10.09±0.51	15.67±0.24	1.65±0.06
Treatment 3	9.92±0.35	21.87±0.03	2.29±0.05
Control	5.94±0.33	10.50±0.10	1.70±0.04

The data regarding overall growth response in terms of average increase in fish weight, feed intake, and feed conversion ratio of both treated and control fish is presented in Table 4, which shows significant differences for the parameters among all treatments and the control group. The maximum weight gain among all the treatments was observed with a mean value of 10.09±0.51g in Treatment 2, while the minimum weight gain, 5.94±0.33g, was recorded in the control during the experimental period of 9 weeks. The maximum and minimum average feed intake was observed in Treatment 1 and control, with the average values 23.20±0.19g and 10.10±0.10g, respectively. Feed conversion ratio was recorded as poor in Treatment 2 i.e.1.65±0.06, while better for Treatment 1, i.e., 3.11±0.09.

Hematological & Immunological Parameters Results

Blood samples of fish collected from each treatment and control group with the help of syringes and put in vacutainers in which EDTA (anticoagulant) was already present. The following parameters were calculated for each treatment: TLC (total leukocyte count), Neutrophil, lymphocytes, monocytes, eosinophils, total RBC, and hemoglobin.

Table 7: Hematological Parameters of the treated and Control groups

Parameters	Units	Treatment 1	Treatment 2	Treatment 3	Control
TLC	/µL	4.6×10^4	4.8×10^4	5.2×10^4	4.5×10^4
Total RBC	/µL	2.25×10^6	3.10×10^6	4.15×10^6	2.50×10^6
Hemoglobin	/µl	7.20	9.65	11.20	6.80
Neutrophil	%	83.9	84.5	85.8	80
Lymphocytes	%	5.9	8	8	6
Monocytes	%	2.8	3.9	3.6	3
Eosinophil	%	2.08	2.3	2.4	3

Concentration of TLC and RBC is reported higher in Treatment 3 among all treatments with values are 5.2×104/µL and 4.15×106/µL respectively. Concentration of the number of Neutrophil was also seen highest in Treatment 3with 85.8% while lowest in control group with 80%. Lymphocytes were recorded highest in Treatment 2 and 3 with equal value of 8% while the lowest values were recorded for Treatment 1, with 5.9%. Monocytes were calculated with the highest concentration of 3.90% in Treatment 2, while the lowest was in Treatment 1 with 2.8%. Eosinophils were recorded at 3% with the highest value in the control group, while the lowest was 2.08% in Treatment 1. The highest concentration of hemoglobin was observed in Treatment 3 and its value was 11.20/µl, whiles the lowest value, 6.80/µl, and was recorded in the Control group.

Results from the Challenge Trials with Dropsy Diseased

Fish After 9 weeks of the growth phase of experimental trial all, the three treatments and the control group was exposed to diseased fish to check immune response of fish. For this purpose dropsy was selected. Dropsy is a bacterial disease that causes abdominal swelling in fish with accumulation of fluid in body cavity. If one fish is suffered from dropsy all other fish living in the same environment are at the risk of same infection. Therefore, 12 diseased fish were obtained from a private fish farm and three diseased fish were placed in each aquarium, separately, containing healthy fish of three treatments and a control for a period of two weeks. The data computed during the challenge trials for diseased fish percentage is expressed in means ± SD and is presented in Table 4.18. Immunity of fish, which was fed with 1%, 2%, 3% *Melia azedarach* extract supplemented diet and control group without the extract was recorded in terms of the spreading of dropsy. During first two weeks no diseased fish was seen in the treatment groups. All treatment groups showed same patterns. The best results were obtained for Treatment 3, where the affected fish percentage was found to be 12% during second week of challenge trial. For Treatment groups 1 and 2, the diseased fish percentage recorded was 17 and 15% respectively. On the other hand Control groups showed diseased fish percentage from the start of the challenge trial. The values recorded were 20% during the first week and 70% in second week.

Table 8 Diseased Fish Percentage during Challenge Trials

	Diseased fish Percentage			
	Treatment 1	**Treatment 2**	**Treatment 3**	**Control**
Week 1	---	---	---	20%

Week 2	17%	15%	13%	70%

Discussion

In recent years, there has been a significant focus on the utilization of plant extracts as immune stimulants and promoters of growth in aquaculture. Research indicates that these extracts can boost immune responses, thereby reducing mortality caused by various pathogenic agents. Additionally, they have demonstrated the capacity to enhance the growth responses of fish (Citarasu 2010, Matiullah, Bhatti et al. 2016) conducted a study investigating the impact of black pepper seeds (BPS) on the growth performance of Labeorohita. They utilized varying levels of BPS (0, 0.5, 1, and 2%) and found that the group fed with 0.5% BPS exhibited a notably higher percentage weight gain, a finding significant at the in the control group. These findings align closely with the results obtained in the present study. The hematology of fish was found to be enhanced by the supplementation of *Melia azedarach* leaf extract in the feed, a result akin to the study conducted by (Javed et al. 2012) Javed and colleagues explored the impact of various schedules of administration of water-based infusions, including Aloe vera gel, Allium sativum, Berberis lycium, Silybum marianum, Caram copticum, and Trigonella foenumgreacum, on the hematology of broiler chicks. Their findings suggested that a mixed infusion of these plants at a rate of 20ml/L of drinking water on a daily basis effectively improved hemoglobin and packed cell volume in broiler chicks. This outcome bear's resemblance to the hematological results observed in common carp in our study. In particular, total leukocytes (TLC), total RBCs, and lymphocytes were significantly higher in Treatment 3 (3% extract) and Treatment 2 (2% extract), while Treatment 1 (1% extract) demonstrated better results than the control group. This aligns with the research of (Pratheepa and Sukumaran 2014) who reported a positive response to the immunostimulant effect of Euphorbia hirta plant extract. Pratheepa also concluded that plant extracts aid in eliminating certain pathogenic microbes from the kidney and enhance blood function (Awad and Awaad 2017) describes wide range of medicinal plants such as herbs, seeds, and spices with different forms such as crude, extracts, mixed and active compounds, used as immunostimulants and resulting in a marked enhancement in the immune system of fish to prevent and control microbial diseases (Awad & Awaad, 2017). The given study is similar to the present study in which *Melia azedarach* leaf extract were used as source of food and examine the growth hematology and immunity of common corp. The result of both studies is almost similar and supports one another. Another study was done in which the effects of supplementing diets with acetone extract (1% w/w) from four medicinal plants Bermuda grass *Cynodondactylon*, beal Aegle marmelos, winter cherry Withaniasomnifera and ginger Zingiber officinale on growth, the non-specific immune response and ability to resist pathogen infection in tilapia *Oreochromis mossambicus* were assessed. The result shows that when Oreochromis mossambicus were fed 5% of their body mass per day for 45 days, and those fed the experimental diets showed a greater increase in mass over the 45 days compared to those that received the control diet. Furthermore, Leucocrit value, phagocytic index and lysozyme activity were enhanced in fish fed the plant extract supplemented diets. A challenge test with V. vulnificus showed 100% mortality in O. mossambicus fed the control diet by day 15, whereas the fish fed the experimental diets registered only 63–80% mortality at the end of challenge experiment (30 days) (Immanuel, Uma et al. 2009). Similar result was obtained from the present study in which all the three-treatment group fed with *Melia azedarach* leaf extract show increase in weight gain compared to the control group. In present study immunological and hematological parameter were also found increased in all treatment groups that is T1, T2, and T3 compared to control group which is fed without *Melia azedarach* leaf extract. In present study challenge trial were done with Dropsy disease fish, in which T3 shows best result with 12% which is similar result obtain from previous study done by Immanuel et al., (2009). The present study was carried out by (Rajeshwari, Rajan, Pavaraj, & Sevarkodiyone, 2016) to check the effect of leaf extract of *Melia azedarach* at different concentration, such as 1.0g, 1.5g and 2.0g formulated diet against

0.1 ml of CFU/ ml 105 cells Aeromonas hydrophila on catlacatla. The physiological parameters, such as Survival and Mortality, Antibody response, Phagocytic activity, Oxygen consumption, opercular movement, and Growth rate were studied. Result revealed that control fish showed 60% mortality and 20%, mortality at 1.0g concentration of plant extract, 10% mortality at 1.5g concentration of plant extract 30% mortality at 2.0g concentration of plant extract was observed. In the above study akin to the present study in which experimental group exhibit high number of white blood cells count and red blood cells which is corresponded to the above study. The introduction of diseased fish into the aquarium presented a challenge for the common carp in combating dropsy disease. Results revealed a lower percentage of dropsy spread in fish that were fed with *Melia azedarach* leaf extract, suggesting an enhancement of the immune system in common carp. This finding aligns with Das et al., 2015 report, which similarly highlighted the improved immune response in fingerlings of labeorohita when supplemented with the extract of Ocimum sanctum leaf against Aeromonas hydrophila infection. The study involved varying concentrations of the plant extract in the feed, and subsequent challenges against A. hydrophila showed increased contents in treatment groups compared to the control group. Notably, at a 2% concentration, a higher relative percentage of survival against A. hydrophila infection was observed, indicating that the leaf extract boosted immunity and conferred increased resistance to the infectious bacteria in labeorohita.

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