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Research Paper

Growth, Haematology and Serum Biochemical Parameters of *Clarias gariepinus* Fed Diets of Neem (*Azadirachta indica*) Seed Meal and Challenged with *Aeromonas hydrophila*

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ABSTRACT: *The growth, haematology and serum biochemical properties of Clarias gariepinus fingerlings fed with soaked neem seed diets and challenged with Aeromonas hydrophilla was investigated. Five isonitrogenous diets (40% Crude protein) were formulated to contain 0%, 3%, 6%, 9% and 12% dietary levels of the soaked neem seed meal denoted by DT1, DT2, DT3, DT4 and DT5. Experimental fish were assigned randomly to the five diets at twenty-five (25) fish per treatment in a complete randomized design in triplicates. The fish were reared for 56 days and fed with the experimental diets at 5% body weight, twice daily and growth parameters and nutrient utilization indices were determined. At the end of the feeding trial ten fish per treatment were randomly challenged with A. hydrophila using intraperitoneal administration of 1ml of phosphate-buffered saline (PBS) containing 1 × 10^7 A. hydrophila and 1ml of phosphate-buffered saline only as negative control. The challenged fish were kept under observation for 7 days and fed their respective experimental diets. After 7 days of post treatment, the blood and serum were drawn from each of the treatment groups and haematological and serum biochemical parameters were evaluated. Initial growth trial showed that the mean final weight (MFW) and specific growth rate (SGR) did not differ significantly ($p>0.05$). The inclusion of soaked neem seed in the diets of C. gariepinus had no deleterious effect on growth. Post challenge haematology revealed significant differences ($P<0.05$) in blood parameters. Red blood cell (RBC) was lowest (0.95) in the positive control treatment (DT1) and highest (1.76) in the initial (negative control). Haemoglobin (Hb) was least in fish fed DT2 (4.83 g.dl^{-1}) and highest (5.52 g.dl^{-1}) in fish fed DT5. White blood cell ranged from 3750×10^0.l^{-1} (DT1) to 5100×10^0.l^{-1} (DT5). Higher values for haematological parameters recorded for the treatment groups over the positive control could be attributed to the immune response readiness induced by the neem seed to mitigate the infection of A. hydrophila.*

KEYWORDS: *neem, seed, haematology, biochemical, catfish*

I. INTRODUCTION

The continuous rise in the demand for fish has made aquaculture industry the fastest growing food producing sector in the world [1], hence, its continuous growth is largely dependent on the ability of fish feed industry to provide quality feed that are not just nutritionally balanced for best growth performance, but also promote the optimum health of the cultured fish.

The importance of fish feed cannot be over emphasized in the aquaculture industry, because aside from the fact that feed plays a major role in the viability and profitability of the industry [2], it also affects different physiological aspects of the cultured fish including growth, haematology, innate immunology and general health of the fish. In the light of this, researchers are searching into cost effective feedstuffs that are capable of ensuring profitability of the industry and ultimately promote optimum growth and health of the fish.

Neem (*Azardirachta indica*) seed meal is one of such feed ingredient with great potential in these facets, as it contains essential nutrients, immuno-stimulatory compounds and of low cost due to its local availability and no competitive demands from humans or the livestock industry [3]. Also, there are many reports about the antimicrobial, nematocidal, biopesticidal and immuno-modulatory activities of *A. indica* [4].

However, the major constraints to the use of neem seed meal is the antinutritional factors inherent in it. Although, neem extract is considered of low toxicity towards non-target aquatic life [5], however, there has been reported cases where water extracts of the bark of neem plant caused respiratory problems in *Tilapia zilli* [6], and long exposure to low concentrations of the crude extract of A. indica delayed the growth of cichlid fish [7].

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fortunately, these antinutritional factors can be drastically reduced or even eliminated through various processing techniques such as soaking, roasting, cooking, steaming, toasting, autoclaving. Processing methods are the effective way of achieving desirable changes, removal of undesirable components and effective utilization of the full potential of nonconventional feed stuff [8].

In view of the aforementioned, this work was aimed at investigating the effect of diets supplemented with processed neem seed meal on the growth, haematology and serum biochemical properties of *Clarias gariepinus* fingerlings challenged with *Aeromonas hydrophilla*.

II. MATERIALS AND METHODS

Feed Formulation and Production

The ripe neem seeds were collected, decorticated and soaked in water for three days to reduce the anti-nutritional factor in the seed before oven drying at 60^0C and grinding into meal.

Other feed ingredients were purchased and used in the formulation of five isonitrogenous diets at 40% crude protein containing 0%, 3%, 6%, 9% and 12% dietary levels of the soaked neem seed meal denoted by DT1, DT2, DT3, DT4 and DT5 respectively as shown in table 1. The formulated diets were produced into pellets, oven dried and stored (refrigerated) for use.

Experimental Set-up, Design and Management

Experimental fish were randomly assigned to five diets at twenty-five (25) fish per treatment in a complete randomized design in triplicates. Fish were reared in aquaria and fed with the experimental diets for eight (8) weeks at 5% body weight, twice daily. The fish were weighed weekly to determine weight gain and the quantity of feed was adjusted accordingly.

Measurement of growth and nutrient utilization parameters

Growth parameters measured included mean weight gain, percentage weight gain, specific growth rate, food conversion ratio, protein efficiency ratio, apparent net protein utilization and survival rate, and were calculated as follows:

Mean weight gained (MWG) = Mean Final Weight (MFW) - Mean Initial Weight (MIW)

Specific growth rate (SGR) = $\frac{LnW2-LnW1}{T} \times 100$

(Where: Ln = natural logarithm; W2 = final weight of fish; W1 = initial weight of fish and T = Period of time in days)

Protein efficiency ratio (PER) = $\frac{\text{mean weight gain of fish}}{weight\ of\ protein\ in\ feed}$

(Where protein fed = $\frac{percent\ protein\ in\ diet\ consumed\ X\ total\ feed\ comsumed}{100}$)

Apparent net protein utilization (ANPU) = $\frac{fish\ protein\ gained}{protein\ consumed} \times 100$

Feed conversion ratio (FCR) = $\frac{weight\ of\ feed}{weight\ gained\ by\ fish}$

Survival rate S (%) = $\frac{\text{number of fish stocked}}{number\ of\ fish\ harvested} \times 100$

Table 1: Gross Composition (g/kg) of neem seed meal supplemented diets

INGREDIENTS	DT1 (0%)	DT2 (3%)	DT3 (6%)	DT4 (9%)	DT5 (12%)
Neem seed meal	0	3	6	9	12
Fish meal	11.8	11.7	11.7	11.5	11.4
Soybean meal	35.7	35.2	34.9	34.5	34.3
Maize	20.2	18.1	15.6	13.5	11.0
Mineral premix	1	1	1	1	1
Vitamin premix	1	1	1	1	1
NaCl	0.5	0.5	0.5	0.5	0.5
CMC	2	2	2	2	2
Vegetable oil	3	3	3	3	3
L-Lysine	0.5	0.5	0.5	0.5	0.5
choline	0.5	0.5	0.5	0.5	0.5
Total	100	100	100	100	100

Analyses of proximate composition

The analyses of the proximate composition of the neem seed meal, experimental diets and samples of fish carcasses fed experimental diets at the start of the feeding trial and at the end of the experiment were carried out using the [9] standard method.

Aeromonas hydrophila **Challenge Test**.

Seven-day lethal dose 50 (LD_{50}) for *Aeromonas hydrophila* on the experimental fish was earlier determined to be 10^7 CFU/ml, and this strain was isolated from experimentally infected fish and identified using catalase, motility, indole, citrate utilisation and urease Tests [10].

At the termination of the feeding trial, 10 fish from each tank were randomly picked for the challenge test. The fish were injected intraperitoneally with 1ml of phosphate-buffered saline (PBS) containing 1×10^7 live *A. hydrophila* and 1ml of phosphate-buffered saline only as negative control. The experiment was in triplicates. The challenged fish were kept under observation for 7 days and fed their respective experimental diets. Mortality of the fish in each tank was observed. After 7 days of post treatment, the blood and serum were drawn from each of the treatment groups and haematological and serum biochemical parameters were evaluated.

Collection of blood and serum

Each fish was anaesthetized before blood was collected. Blood was drawn from the caudal peduncle vein as described by earlier reports [11] using a tuberculin syringe. Collected blood was immediately transferred to EDTA (ethylene diamine tetra acetic acid) test tubes and plain sample vials for haematological analysis and serum biochemical analysis respectively

Haematological Analysis

Haematological parameters such as white blood cells (WBC), red blood cells (RBC), haemoglobin (HB) and packed cell volume (PVC) were determined using Sysmex KX - 2IN automated haematology analyzer

Serum biochemical Parameters

Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were determined using diagnostic kit (Merck, Germany) based on Reitman and Frankel [12] method. Serum protein was estimated using the Merck, Germany test kit according to biuret method [13]. Albumin (ALB) was estimated by bromocresol green binding method [14]. Globulin content was obtained by calculating the difference between total serum protein and albumin values.

Data analysis

Data collected from the various experiments and analyses were analyzed using descriptive statistics and were presented as mean and standard error of mean. Data collected were also subjected to analysis of variance where the means were separated by least significant difference using Genstat package edition 12.

III. RESULTS

The proximate compositions of soaked neem seed meal and experimental diets are highlighted in table 2. The result shows that soaked neem seed meal has a crude protein content of 17.5% and ether extract of 63.73%. The table also shows that experimental diets were isonitrogenous with a crude protein content of approximately 40%.

The growth parameters of *Clarias gariepinus* fingerlings fed diets of soaked Neem seed meal is highlighted in Table 3. The table shows that mean initial weight (MIW) was statistically the same ($p>0.05$) across treatments. Mean Final Weight (MFW) did not differ significantly ($p>0.05$) with the range from 2.86 ± 0.17 (diet 2) to 3.42 ± 0.15 (diet 5). there was also no significant difference in specific growth rate (SGR) as values ranged between 1.61 ± 0.11 in diet 2 to 1.94 ± 0.08 in diet 5.

Table 2: Proximate Composition of soaked Neem Seed Meal and experimental diets

Parameters	Soaked neem seed meal	Experimental diets					P-Value
		Diet 1 (0:100)	Diet 2 (25:75)	Diet 3 (50:50)	Diet 4 (75:25)	Diet5 (100:0)	
Crude protein (%)	17.50±0.14	40.09±0.78^a	40.88±0.12^a	39.98±0.18^a	40.1±0.29^a	40.27±0.43^a	0.08
Ether extract (%)	63.73±0.27	8.83±0.23^a	8.45±028^a	8.54±0.51^a	8.77±0.24^a	8.19±0.13^a	1.07
Ash (%)	2.03±0.18	4.63±0.23^a	5.45±0.19^{bc}	5.24±0.19^b	5.96±0.05^c	4.74±0.15^a	0.03
Crude fibre (%)	4.48±0.11	5.92±0.09^b	4.44±0.11^a	4.95±0.29^a	6.04±0.18^c	6.06±0.18^c	0.01
Moisture (%)	5.85±0.07	4.93±0.09^b	4.23±0.10^a	4.88±0.1^b	3.91±010^a	4.22±0.09^a	0.04
NFE (%)	6.41±0.36	35.6±0.95^a	37.56±0.79^b	31.57±0.68^a	33.84±0.09^a	31.52±0.62^a	0.04

Means on the same row with different superscripts shows significant differences between experimental diets ($p<0.05$)

Table 3: Growth Parameters of *Clarias gariepinus* Fingerlings Fed Diets of Neem Seed Meal

Parameters	DT1	DT2	DT3	DT4	DT5	P-Value
MIW (g)	1.22±0.01[a]	1.2±0.02[a]	1.21±0.03[a]	1.21±0.04[a]	1.22±0.05[a]	0.67
MFW (g)	3.11±0.48[a]	2.86±0.17[a]	2.95±0.42[a]	3.11±0.62[a]	3.42±0.15[a]	0.64
MWG (g)	1.89±0.48[a]	1.65±0.17[a]	1.74±0.04[a]	1.89±0.62[a]	2.20±0.15[a]	0.65
%MWG	155.01±40.15[a]	135.17±13.72[a]	143.60±4.00[a]	155.87±51.72[a]	180.01±12.30[a]	0.67
SGR (%/day)	1.75±0.30[a]	1.61±0.11[a]	1.68±0.03[a]	1.75±0.38[a]	1.94±0.08[a]	0.68
FCR	2.58±0.82[a]	2.76±0.13[a]	2.48±0.05[a]	2.49±0.99[a]	1.90±0.14[a]	0.67
PER	0.94±0.22[a]	0.86±0.03[a]	0.82±0.01[a]	0.97±0.28[a]	0.98±0.05[a]	0.79
%Survival	95.00±4.20[a]	92.00±3.10[a]	91.00±4.00[a]	93.00±2.45[a]	91.00±3.31[a]	0.09

Means on the same row with same superscripts do not differ significantly (P>0.05)
Key: MIW = Mean Initial Weight; MFW = Mean Final Weight; MWG = Mean Weight Gain; %MWG = % Mean Weight Gain; SGR = Specific Growth Weight; FCR = Feed Conversion Ratio; PER = Protein Efficiency Rate; DT1 = control experiment with 0% neem seed meal; DT2 = 3% Neem Seed Meal; DT3 = 6% Neem Seed Meal; DT4 = 9% Neem Seed Meal; DT5 = 12% Neem Seed Meal

Weekly growth curve of *Clarias gariepinus* fingerlings fed Diets of neem seed meal revealed a progressive increment in mean weight over the weeks (Figure 1).

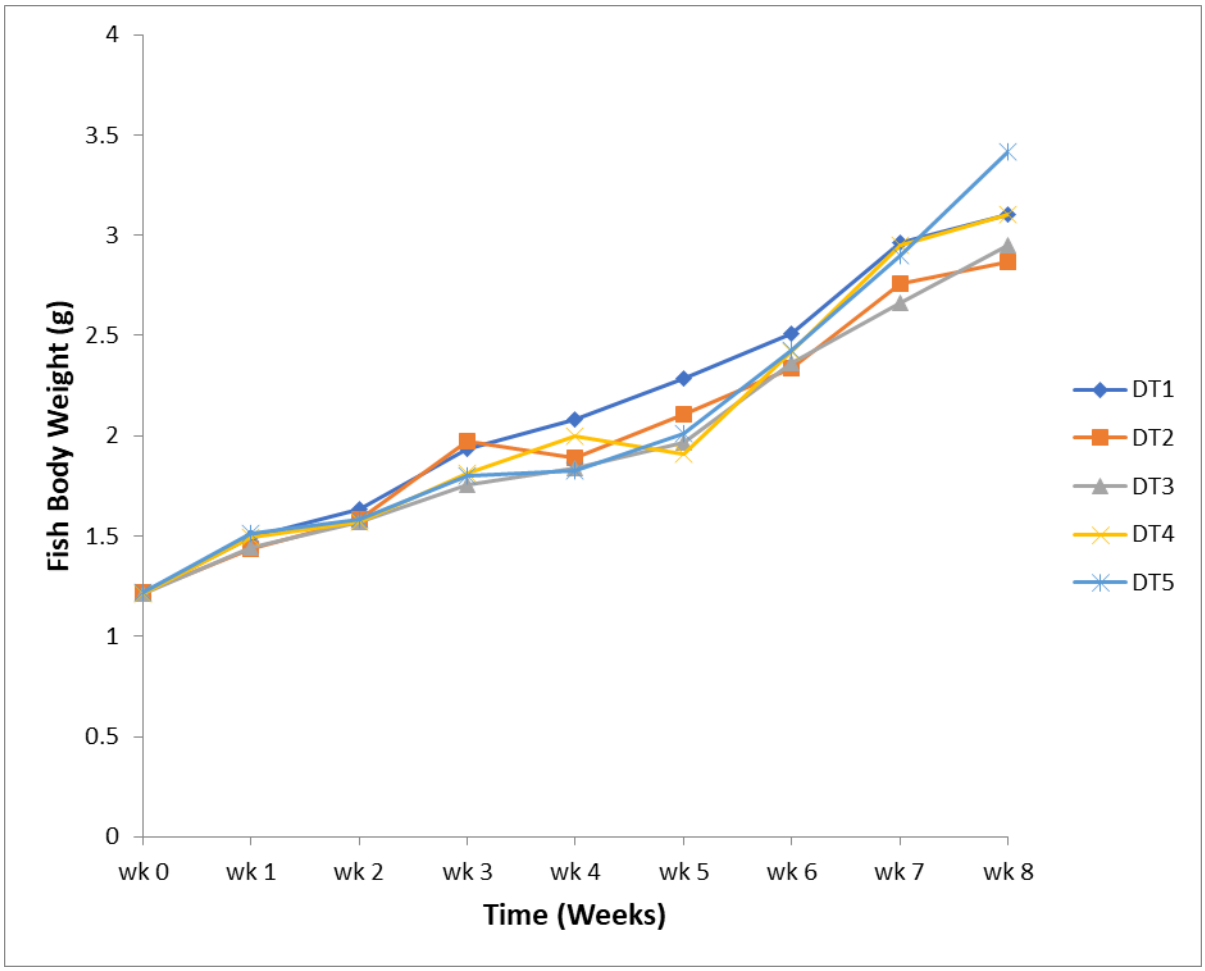

Figure 1: Weekly growth curve of *Clarias gariepinus* fingerlings fed Diets of soaked neem seed meal

Table 4 shows the haematological parameters of *Clarias gariepinus* before and after bacterial challenge test. The results reveal significant differences (P<0.05) in red blood cell values. Red blood cell was lowest (0.95) in the positive control treatment (DT1) and highest (1.76) in the initial (negative control). Haemoglobin (Hb) was also significantly different across treatments with DT2 recording the lowest value of 4.83 and DT5 recording the highest (5.52). white blood cell differed statistically ranging from 3750 (DT1) to 5100 (DT5). Survival rate was least in the positive control (72%) and maximum in the negative control (98%).

Table 4: Haematological parameters and survival rate of *Clarias gariepinus* before and after bacterial challenge

Parameter	Initial	Final Value					P-Value
		DT1	DT2	DT3	DT4	DT5	
PCV (%)	13.76±0.01^{a}	13.64±0.06^{a}	12.17±1.50^{a}	12.84±0.07^{a}	12.94±0.06^{a}	13.06±0.08^{a}	0.06
RBC ($\times10^{12}$.l^{-1})	1.76±0.01^{d}	0.95±0.06^{a}	1.64±0.07^{cd}	1.61±0.02^{c}	1.54±0.06^{b}	1.52±0.01^{b}	2.20×10^{-5}
Hb (g.dl^{-1})	4.98±0.04^{b}	4.84±0.06^{a}	4.83±0.07^{a}	5.06±0.08^{b}	5.43±0.04^{c}	5.52±0.70^{c}	0.01
WBC ($\times10^{0}$.l^{-1})	3800±70.71^{a}	3750±70.71^{a}	4200±141.4^{ab}	4500±141.4^{bc}	4800±141.4^{bc}	5100±141.4^{c}	2.10×10^{-4}
% Survival	98.00±2.00^{c}	72.00±3.00^{a}	87.00±4.00^{b}	90.00±2.00^{b}	90.00±2.50^{b}	92.00±1.00^{b}	0.03

Means on the same row with different superscripts differ significantly (P<0.05)

Key: PCV = Packed cell volumes; RBC =Red blood cell; Hb = Haemoglobin; WBC=White blood cell

DT1 = Positive control experiment with 0% neem seed meal; DT2 = 3% Neem Seed Meal; DT3 = 6% Neem Seed Meal; DT4 = 9% Neem Seed Meal; DT5 = 12% Neem Seed Meal; Initial =Negative control

Table 5 shows the biochemical parameters of fish fed diets of soaked neem seed meal. The result reveals significant differences (P<0.05) in Aspartate aminotransferase (AST) which was lowest in the initial/ negative control (0.26) and highest in DT5 (0.79). Alanine aminotransferase (ALT) showed significant differences (P<0.05) amongst treatments ranging from 0.09 in the initial (negative control) to 0.81 in DT5. Globulin (GLB) was also different significantly (P<0.05) with initial recording the least value (2.21) and DT5 recording the highest (2.54). Albumin (ALB) showed no difference statistically (P>0.05) between treatments.

Table 6 shows the Mean water quality parameters measured during the experimental feeding of *Clarias gariepinus* fed Diets of neem seed meal. The results reveal that there were no significant differences (P>0.05) in all water quality parameters across treatments.

Table 5: Serum biochemical parameters of fish fed diets of soaked neem seed meal

Parameters	Initial	Final values					P-value
		DT1	DT2	DT3	DT4	DT5	
AST	0.26±0.02^{a}	0.28±0.03^{a}	0.34±0.05^{ab}	0.44±0.05^{b}	0.61±0.01^{c}	0.79±0.07^{c}	1.2×10^{-4}
ALP	0.75±0.02^{ab}	0.83±0.04^{b}	0.63±0.04^{a}	0.63±0.04^{a}	0.72±0.02^{ab}	0.72±0.03^{ab}	0.01
ALT	0.09±0.31^{a}	0.11±0.01^{a}	0.51±0.56^{b}	0.22±0.21^{b}	0.42±0.2^{b}	0.81±0.01^{c}	0.04
ALB	1.01±0.06^{a}	1.13±0.04^{a}	1.12±0.01^{a}	1.12±0.01^{a}	1.18±0.01^{a}	1.19±0.01^{a}	0.06
GLB	2.21±0.02^{a}	2.35±0.07^{ab}	2.22±0.02^{a}	2.36±0.08^{ab}	2.48±0.07^{b}	2.54±0.06^{c}	0.01

Means on the same row with different superscripts differ significantly (P<0.05)

Key: AST = Aspartate aminotransferase; ALP =Alkaline phosphatase; ALT = Alanine aminotransferase; ALB = Albumin; GLB =Globulin

DT1 = Positive control experiment with 0% neem seed meal; DT2 = 3% Neem Seed Meal; DT3 = 6% Neem Seed Meal; DT4 = 9% Neem Seed Meal; DT5 = 12% Neem Seed Meal; Initial =Negative control

Table 6: Mean water quality parameters measured during the experimental feeding of *Clarias gariepinus* fed Diets of soaked neem seed meal

Parameters	DT1	DT2	DT3	DT4	DT5	P-Value
pH	7.70±1.22^{a}	7.64±1.17^{a}	7.55±1.10^{a}	7.72±1.31^{a}	7.68±1.24^{a}	0.99
DO	5.25±0.76^{a}	4.68±0.05^{a}	4.68±0.39^{a}	4.88±0.33^{a}	4.63±0.17^{a}	0.24
TDS	345.50±76.29^{a}	353.50±61.21^{a}	367.25±44.38^{a}	365.25±45.34^{a}	341.00±89.50^{a}	0.97
EC	654.50±121.09^{a}	665.25±73.67^{a}	688.50±35.35^{a}	689.00±41.64^{a}	592.75±161.53^{a}	0.64
Temp	26.43±0.90^{a}	26.40±1.04^{a}	26.33±0.84^{a}	26.35±0.87^{a}	26.40±0.87^{a}	0.99

Means on the same row with same superscripts do not differ significantly (P>0.05)

Key: EC = Electrical conductivity, TDS = Total Dissolved Solids, DO = Dissolved Oxygen

DT1 = control experiment with 0% neem seed meal; DT2 = 3% Neem Seed Meal; DT3 = 6% Neem Seed Meal; DT4 = 9% Neem Seed Meal; DT5 = 12% Neem Seed Meal

IV. DISCUSSION

The result of the effects of diets of soaked neem seed included at different levels on the growth of *C. gariepinus* showed that all treatments were statistically the same in all growth parameters with Diet 5 giving slightly higher values than the others. Maximum values of growth indices obtained in DT5 indicates that inclusion of soaked neem seed in the fish diet was beneficial to the fish. The continuous increase in weight exhibited by all treatment during the 8-week period as indicated by the growth curve shows that all the diets

supported fish growth. It also revealed that the inclusion of soaked neem seed in the diets of *C. gariepinus* had no deleterious effect on the growth of the cultured fish.

This is in line with the reports of Abidin, et al. [15] who reported that inclusion of extract of neem leaf was beneficial to the growth of rainbow trout and gave higher values of growth indices as compared to the control. A similar report was made by Ubiogoro, et al. [16] and Kaur, et al. [3] who also ascertained that supplementation of fish feed with neem leaf extract gave better growth parameters than the control. However, this is contrary to the observation made by Omoregie and Okpanachi [6], who observed that a low quantity of *A. indica* extracts delayed the growth of cichlid fish.

The haematological assessment before and after the *A. hydrophila* test showed higher haematological values (Red blood cell, Haemoglobin and white blood cell) of fish fed the neem seed supplemented diets over the positive control. This could be attributed to the immune response readiness induced by the neem seed to mitigate the infestation stress of *A. hydrophila*. A similar observation was made by Kaur, et al. [11] when neem leaf extract was administered to common carp.

The negative control had higher haematological values (Red blood cell, Haemoglobin and white blood cells) than the positive control indicating the negative effects of *A. hydrophila* infestation on the haematology of the fish. This is in accordance with the reported work of Kumar, et al. [4] who concluded that Argulus infestation altered marked haematological and serum biochemical parameters of goldfish. The PCV did not differ significantly in treatment groups, concurring the earlier work of Kumar, et al. [17] who also reported no differences in PCV in the treatment groups compared to control groups when azadirachtin was orally administered to goldfish (*Carassius auratus*).

Observations from the biochemical parameters in the present work reveals that ALT and AST were significantly higher in the treatment groups than the control groups. The result shows an increasing trend in these biochemical parameters with DT5 recording the highest value.

Aspartate aminotransferase (AST) also known as serum glutamate oxaloacetate transaminase (SGOT) and alanine amino transferase (ALT) also called serum glutamate pyruvate transaminase (SGPT) are both enzymes present in the cytosol of the hepatocytes. Increased activity of serum levels of AST and ALT in the absence of acute necrosis or ischaemia of other organs such as myocardium, suggests liver cell damage and leaching of these enzymes into blood [4]. Thus, the higher levels of AST and ALT in treatment groups when compared to the control groups may signify hepatocellular stress of fish induced by the neem seed diet. In line with the result of the present work Kumar, et al. [4] reported similar trend of higher AST and ALT values in the treatment groups when compared to the control groups during the experimental oral administration of azadirachtin (a compound in neem) to goldfish. On the contrary, Ubiogoro, et al. [16] recorded a progressive decrease in AST and ALT with increase in levels of neem leaf extract which suggested hepato-protective activity of Neem leaves meal

Globulin was significantly higher in the treatment groups than the control group while Albumin values were slightly higher in treatment groups than the control group, although this difference was insignificant. This is suggestive of the immune stimulatory effects of neem seed.

Albumin and globulins are the two groups of serum proteins. Globulins are essential for maintaining a healthy immune system. The albumin is an easily available protein reserve and a protein transporter [18]. Increase in albumin and globulin levels are considered a strong innate response in fishes [19]. increased globulin level in treatment groups may enhance the immune response and preparatory stage for stress mitigation of the fish [4].

V. CONCLUSION

The inclusion of soaked neem seed in the diet of *C. gariepinus* promoted growth and nutrient utilisation of the fish comparatively as the control. It also positively affected the haematological and some serum biochemical indices of the fish better than the control, suggesting immune-stimulatory effects of the soaked neem seed. Infestation by *A. hydrophila* had a negative effect on the haematology and serum biochemistry of the fish. Despite its numerous benefits, neem seed should be used only when necessary and with caution in fish culture due to its tendency to increase hepatocelluular activity.

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