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Effects Of Neem (*Azadirachta Indica* A. Juss) Seeds Oil On Reproductive Physiology Of *Oreochromis Niloticus* (Nile Tilapia)

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Abstract
The scarcity of cheap animal protein can be abated through tilapia aquaculture if uncontrolled reproduction and precocious maturity in tilapia can be combated. This research aims to explore natural reproductive inhibitory agents, specifically Neem seed oil, as a cost-effective method for managing tilapia populations. This study investigates the effects of varying dietary supplementation levels of Neem (Azadirachta indica) seed oil on the reproductive physiology of Nile tilapia (Oreochromis niloticus). The experiment was conducted for eight weeks using four treatment groups with Neem oil incorporated at 0% (T1), 1.25% (T2), 2.5% (T3), and 5% (T4) levels. Growth performance, feed intake, biochemical parameters, hematological profiles, histopathological effects, and hormonal assays were assessed to determine the impact of Neem oil on fish health and reproduction. Lower FCR values of 1.76 and 1.87 were recorded for T3 and T4 respectively with these two treatments having higher survival rate compared to other treatments. However, neem oil negatively impacted overall growth performance compared to the control group (T1). Biochemical analyses revealed enhanced protein and lipid profiles for T4 and T3 respectively. Hematological assessments also showed increased PCV and hemoglobin levels with T4 and T3 indicating improved oxygen-carrying capacity. Histopathological examinations revealed marked adverse effects on liver and kidney tissues of T1 and T4. In conclusion, this study suggests the inclusion rate of 2.5% (T3) of neem seed oil with potential as a natural reproductive inhibitor in Nile tilapia aquaculture without any adverse effect on the general health of the fish. Further research is recommended to optimize inclusion rates for maximizing growth while controlling reproduction effectively. This approach could contribute significantly to sustainable aquaculture practices in regions facing protein shortages.

Keywords: *neem oil, reproduction, Oreochromis niloticus, cheap protein*

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I. Introduction

One of the problems facing most developing tropical countries is the scarcity of cheap animal protein source for the teaming human population. The population of most countries continue to rise in synonyms with the inflation rate. These upsurge if not quickly combated, might expose some citizens, if not most, to protein deficiency. Thus, there is a need for the availability of cheap animal protein that is assessible and affordable for all and sundry.

Tilapia constitutes one of the most productive and internationally traded food fish in the world (Modadugu and Belen, 2004). Tilapia is a popular fish due to the quality of its protein and it's the second most farmed food fish after carps globally. They are omnivores and are easy to culture as they can feed on wide varieties of feeds like plants, planktons, detritus, etc (Lubna *et al.*, 2016). They feed low on the food chain resulting in low production cost compared to some carnivorous fishes. However, due to issues with premature maturity and uncontrolled reproduction, which frequently lead to an overabundance of immature, stunted fish in production ponds, tilapias have not yet reached their full aquaculture potential. There have been previous reviews on population control in farmed tilapias. The majority of these techniques are costly, call for specialized equipment, and need experienced labor (AftabUddin et al., 2017; Syed et al., 2021). In order to manage tilapia recruitment in ponds, it is necessary to investigate less costly, easily adoptable, and suitable technologies by utilizing natural reproductive inhibitory chemicals found in some plants.

It is well known that neem (*Azadirachta indica*) seed oil has antiovulatory and antifertility properties. Neem seed oil has also been shown to significantly alter the levels of related reproductive hormones and suppress folliculogenesis in albino rabbits (Oyedeji *et al.*, 2013). According to the phytochemical analysis, one of the plant's main flavonoids, quercetin, causes granulose cells to undergo apoptosis (Hadge *et al.*, 2022).This research work is therefore carried out to investigate the effects of varying dietary supplementation levels of Neem seed oil on reproductive physiology of tilapia fish.

II. Methodology

Experimental preparation

The experiment was conducted at the Agricultural Technology Research and Training Farm of The Federal Polytechnic, Ilaro, Ogun State. Ilaro is a popular town located in Yewa South Local Government Area, Ogun State, Southern Western Nigeria, Nigeria. It lies on latitude $6^{0}53^{1}11.5$"N and longitude $3^{0}1$'13.8"E. Commercially produced *Azadirachta indica* oil was purchased from a local market and was incorporated into the fish feeds, in partial replacement with groundnut oil, at 0%, 1.25%, 2.5% and 5% respectively for treatments 1,2 3 and 4. A total of 240 juveniles of Nile tilapia (*O. niloticus*) with average weight of 39.97±0.23g was utilized for the experiment. Four iso-nitrogenous and iso-lipidic diets of 30% crude protein and 5% oil, consisting of varying levels of Neem oil were produced and kept in zip lock bags until needed. Proximate analysis of experimental diets was determined prior to the experiment using the method of AOAC, 2000. The formulation and proximate composition of feed produced is displayed in Table 1 below

Table 1. Feed diet formulation and proximate composition of experimental feed

Ingredients	T1 control	T2	T3	T4
Fish meal	21.2	21.2	21.2	21.2
Wheat meal	25.3	25.3	25.3	25.3
Soya bean	21.2	21.2	21.2	21.2
Corn	25.3	25.3	25.3	25.3
Vitamin C	0.5	0.5	0.5	0.5
Bonemeal	1.0	1.0	1.0	1.0
Salt	0.5	0.5	0.5	0.5
Groundnut Oil	5.0	3.75	2.5	-
Neem oil	-	1.25	2.5	5
Total	100	100	100	100
CP	44.10	40.88	42.34	41.78
CF	13.54	11.84	12.16	11.99
MC	6.24	12.29	8.80	10.52
Crude fibre	4.80	3.98	4.23	4.41
Total ash	7.28	7.71	8.12	7.95
Carbohydrates	24.05	23.32	24.36	23.37

CP= Crude Protein, CF= Crude Fat, MC= Moisture Content

Experimental procedure

The experiment was carried out using twelve rectangular plastic tanks for a period of 8weeks. Each rectangular tank has a capacity of 30liters. The experimental design was completely randomized, consisting of 4 treatments replicated thrice at a stocking density of 20 fishes per tank. Fish were fed twice daily, early in the morning and in the evening. Mortalities are noted daily while growth development data are taken weekly. At the termination of the feeding trial, fish samples from each treatment are tested for biochemical analysis following the procedure of Lee *et al.*, (2020), hematological analysis using the method of Hammed *et al*., (2015), hormonal assay test following the procedure of Wartofsky &Handelsman, (2010) and histopathological test using the process of Slaoui & Fiette, 2011. Collected data were subjected to one way Analysis of Variance using SPSS for comparison of means. In cases of significant F-value, Tukey HSD was used to separate the means at $p<0.05$.

III. Results And Discussion

Growth

There is no significant difference in all parameters measured except for feed intake. Weight gain was greatest in the control group (10.01 g), while the group with 1.25% neem oil showed a marked reduction in weight gain (7.29 g). The groups with higher neem oil percentages (2.5% and 5%) performed better than the lowest concentration but still did not match the control. Fish with no neem oil consumed significantly more feed compared to those with added neem oil, particularly at the lowest concentration (1.25%). The FCR values suggest that as neem oil concentration increased, FCR improved. The best FCR and survival rate was observed in the group with a concentration of neem oil at 2.5% inclusion level.

Table 2: Growth parameters of *Oreochromis niloticus* fish fed varying levels of neem oil

Parameters	T1	T2	T3	T4
IBW (g)	46.56±0.49	37.90±4.15	35.53±4.24	41.65±1.76
FBW (g)	56.58±2.03	45.19±4.58	45.17±3.43	51.39±2.64
MWG (g)	10.01±1.65	7.29±2.73	9.64±0.91	9.74±0.89
FI (g)	21.71±1.47[a]	10.96±1.28[b]	16.73±0.27[a]	18.21±1.21[a]
FCR	2.24±0.24	1.86±0.48	1.76±0.15	1.87±0.05
SR (%)	86.67±3.33	83.33±3.33	93.33±3.33	90.00±10.00

IBW=Initial body weight, FBW= Final body weight, MWG= Mean weight gain, FI= Feed intake, FCR= Feed conversion ratio, SR= Survival rate. Means in each column with distinct superscripts indicate a significant difference ($p<0.05$)

Serum

The data provided includes various biochemical markers measured across the four treatments. T4 has the highest value for total protein, albumin, globulin (Glo), Blood Urea Nitrogen (BUN), creatinine, cholesterol, Total magnesium (Tmg), High density lipoprotein (HDL), Total bilirubin (TiBil), Glucose (GLU) and Lymphoid origin leukemic (LOL), followed by T3 in most cases while T2 has the least. For Aspartate Aminotransferase, Alanine Aminotransferase, Alkaline Phosphatase, T4 records the highest value, following the same trend as above, with T3 however, having the least in Aspartate Aminotransferase. This indicate that fish fed with highest level of neem oil perform best across all group for biochemical analysis.

Table 3. Serum Analysis of *Oreochromis niloticus* fish fed varying level of Neem Oil

PARAMETERS	T1	T2	T3	T4
Total Protein	3.40 ± 0.60^{ab}	2.65 ± 0.05^{b}	3.60 ± 0.20^{ab}	4.35 ± 0.15^{a}
Alb	0.65±0.15	0.50±0.00	0.70±0.00	0.75±0.15
Glo	2.75 ± 0.45^{ab}	2.15 ± 0.05^{b}	2.90 ± 0.20^{ab}	3.60 ± 0.00^{a}
AST	68.00 ± 29.00^{ab}	35.50±0.50	5.70±0.10	97.50±2.50
ALT	39.50 ± 7.50^{ab}	30.00 ± 0.00^{b}	49.00 ± 4.00^{ab}	56.50 ± 6.50^{a}
ALP	320.00 ± 69.00^{ab}	252.50 ± 7.50^{b}	369.00 ± 3.00^{ab}	418.50 ± 3.50^{a}
BUN	0.40±0.10	0.30±0.00	0.40±0.00	0.50±0.00
Creat	0.55±0.05	0.50±0.00	0.55±0.05	0.65±0.05
Chot	130.50±35.50	91.50±1.50	146.50±3.50	210.00±50.00
Tmg	69.00±17.00	52.00±5.00	69.00±6.00	89.50±9.50
HDL	76.50±16.50	56.50±6.50	84.50±2.50	111.50±26.50
TiBil	0.09±0.01	0.06±0.01	0.09±0.02	0.10±0.00
Glu	241.00±59.00	178.50±1.50	197.50±2.50	295.00±11.00
LOL	40.20 ± 15.60^{ab}	24.60 ± 6.00^{b}	48.20 ± 2.20^{ab}	80.60 ± 21.60^{a}

Alb= Albumin, Glo= Globulin, AST= Aspartate Aminotransferase, ALT= Alkaline Aminotransferase, ALP=Alkaline phosphatase, BUN= Blood Urea Nitrogen, Chot= Cholesterol, Tmg= Total magnisum, HDL= High Density lipoprotein, LOL= Lymphoid Origin Leukemic Means in each column with distinct superscripts indicate a significant difference ($p<0.05$)

Haematology analysis

There is a noticeable increase in PCV from T1 to T4, suggesting an improvement in red blood cell concentration (Table 4). Hemoglobin (Hb) levels also show an upward trend, indicating better oxygen carrying capacity of the blood in T4 compared to the other 3 treatments. There is variability in RBC counts, with T3 showing the highest count, which may suggest a response to treatment or a physiological change. WBC (White Blood Cells) counts are relatively stable, with T3 showing a peak. This could indicate an immune response. Lymphocytes varies from 55.5 to 63.5 across treatments. The percentage of lymphocytes is fairly consistent, suggesting stable immune function. Heterophils (Het) varies from 29.5 to 37.5. The percentage of heterophils shows some fluctuation, which may indicate changes in response to treatment or infection. Monocytes (Mon) shows a slight increase from 2.0 to 4.0. An increase in monocytes can indicate an ongoing immune response or recovery phase. Eosinophils range from 2.5 to 5.0, while basophils remain low, Eosinophil levels suggest a possible allergic response or parasitic infection while basophils are typically low in healthy individuals. MCV (Mean Corpuscular Volume) ranges from 131.7 to 148.1. Elevated MCV could indicate macrocytic anemia or other conditions affecting red blood cell size. MCHC (Mean Corpuscular Hemoglobin Concentration) parameters are stable across treatments.

Table 4. Haematological profile of *Oreochromis niloticus* fish fed varying level of Neem Oil

PARAMETERS	T1	T2	T3	T4
PCV%	20.5 ± 0.5^{b}	21.5 ± 1.5^{ab}	21.0 ± 0.5^{ab}	25.0 ± 1.0^{a}
Hb gIU	6.7±0.4	7.0±0.4	6.9±0.4	8.0±0.5
RBC $x10^6$	1.4±0.0	1.5±0.1	1.4±0.1	1.9±0.3
WBC $x10^6$	15.45±1.9	14.88±1.7	16.38±0.07	15.2±30.9
Plat Ul	1.19±0.02	1.49±0.24	1.36±0.17	1.10±0.04
Lym %	59.5±0.5	57.5±6.5	55.5±0.5	63.5±1.5
Het %	33.5±0.5	35.0±7.0	37.5±2.5	29.5±1.5
Mono %	2.0 ± 0.0^{b}	2.5 ± 0.5^{b}	3.0 ± 0.0^{b}	4.0 ± 0.0^{a}
Eos %	5.0±1.0	5.0±0.0	4.0±2.0	2.5±0.5
Bas %	0.5±0.5	0.0±0.0	0.0±0.0	0.5±0.5
MCV fl	142.4±0.5	143.2±3.3	148.1±3.4	131.7±15.6
MCHC %	32.7±1.2	32.4±0.7	32.6±0.1	32.0±0.7

MCH pg	46.5±1.5	46.4±0.1	48.3±0.9	42.0±4.1

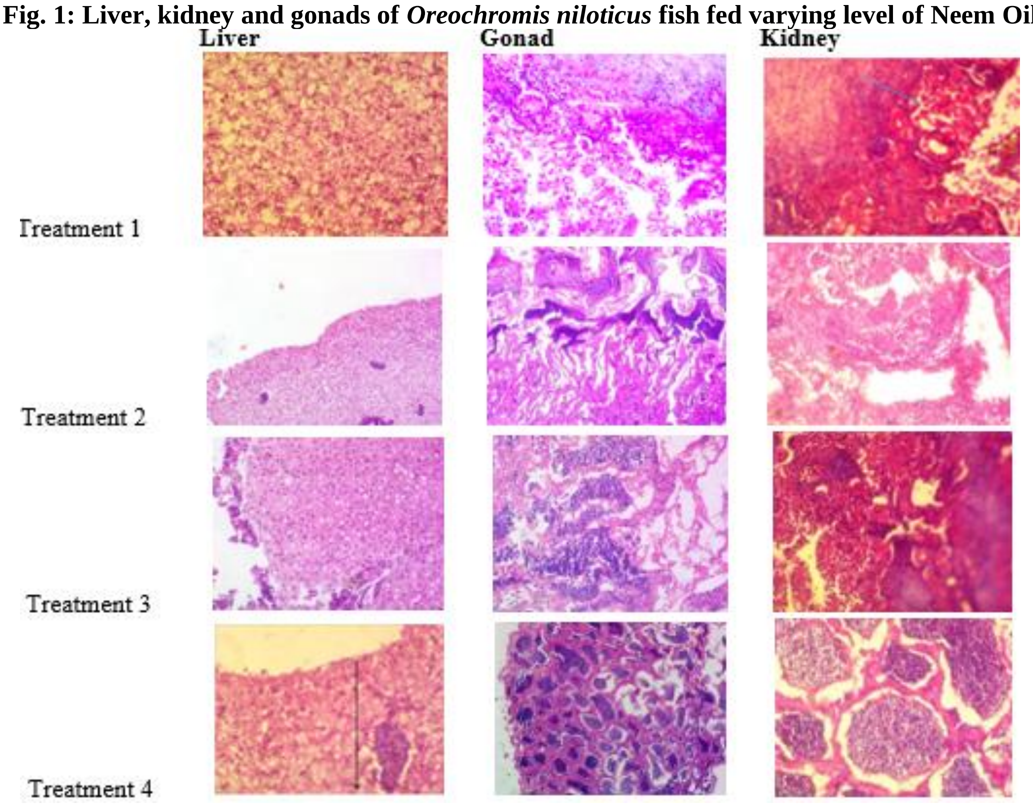
PCV= Packed Cell Volume, Hb= Hemoglobin, RBC= Red Blood Cell, WBC= White Blood Cell, Plat= Platelet, Lym= Lymphocyte, Het= Heterophil, Mono= Monocyte, Eos= Eosinophil, Bas= Basophil, MCV= Mean Corpuscular Volume, MCHC= , MCH= . Means in each column with distinct superscripts indicate a significant difference ($p<0.05$)

Histopathology analysis

There is significant hemorrhage within the cortex, accompanied by diffuse tubular epithelial necrosis in the kidney of T1. Central veins exhibit congestion in the liver, with marked hepatocellular evacuation. Prominent macrophagic centers are noted. Gonad is normal, with no significant abnormalities observed. There are no abnormalities noted in the hepatopancreas in T2 and the gonads appeared normal, with no disruption in spermatogenesis or spermatogonial formation. The architecture of the kidney also remain relatively preserved, with mild, diffuse tubular epithelial necrosis being observed.

The renal tubules for T3 appear normal, with the liver exhibiting normal architecture with minimal hepatocellular evacuation, representing an ideal healthy liver for this species. The gonads are relatively normal, with active spermatogenesis noted. Moderate to severe widespread hepatocellular vacuolation is evident in the liver of T4, though peripheral hepatocytes are less affected. The gonads appear normal, with no significant abnormalities observed. The kidney tissue is poorly processed with mild random tubular epithelial cell necrosis observed

Fig. 1: Liver, kidney and gonads of *Oreochromis niloticus* fish fed varying level of Neem Oil



Hormonal assay

Table 5 presents the hormonal assay results of *Oreochromis niloticus* fed with varying levels of neem oil (NO) in their diet. The hormones measured are testosterone, estrogen, and progesterone. Results from the experimental fish hormonal analysis revealed that values are not significantly different from each other. For testosterone, the values are relatively stable across all neem oil levels with T3 having the highest value. In contrast, estrogen levels show more pronounced changes with T3 having the least value with a relatively wide margin. Progesterone levels remain fairly consistent across all treatment with a slight increase above others noticed at T3.

Table 5: Hormonal assay of *Oreochromis niloticus* fed varying levels of neem oil (NO) in their diet

Hormone	T1	T2	T3	T4
Testosterone	30.36±1.09	29.86±0.89	31.87±0.13	29.99±0.89
Oestrogen	29.06±5.06	32.75±2.66	23.89±2.12	32.72±2.63
Progesterone	27.36±1.71	27.77±2.69	28.35±2.30	27.77±2.68

IV. Discussion

Crude protein is one of the most important supplements to consider in a potential feed because it is one of the most costly nutrients to provide and a lack of it can significantly affect fish growth performance (Mahaesar et al., 2010). The range of crude protein examined in this study is 40.88 to 44.10%. Table 1 displays the ash content of the fish diets, which ranges from 7.28 to 8.12%. This is consistent with the results of Ayuba and Iorkohol (2012), who found that the ash level of several fish feeds ranged from 5.33 to 9.45%. The amount of minerals determines the physico-chemical characteristics of feeds and inhibits the growth of microbes, which makes determining the ash content crucial. For ideal fish growth, a diet containing 5% lipid upward is advised. According to Hassan (2001), the main purpose of lipids in a diet is to optimize their ability to spare protein. Since it impacts the feeds' quality or value, it is crucial to keep an eye on the lipid content. Feeds contain carbohydrates, which provide heat and energy.

The three main factors used to evaluate fish production in an aquaculture business are growth, feeding rates, and FCR. Understanding how these factors relate to one another is essential for maximizing fish output (Mengistu et al., 2019). The result indicate that increasing levels of neem oil in feed negatively impacted growth performance in *Oreochromis niloticus*, particularly at lower concentrations (1.25%). The reduced final weight and weight gain in these groups suggest that high levels of neem oil may inhibit growth or affect palatability due to the bitter nature of the plant, leading to reduced feeding behavior. This resonates with the findings of Kumar et al., 2020 which indicates that high concentrations of neem oil can be toxic to fish due to its active compounds, leading to reduced feed intake and growth performance. The lower feed conversion recorded for the neem fed group support the research of Sahu et al., 2018 who suggest that low to moderate levels of neem oil has the ability to improve feed utilization.

Analyzing biochemical results across various treatments, it has been observed that higher levels of HDL cholesterol are associated with a decreased risk of cardiovascular disease, highlighting the potential for HDL as a therapeutic target (Barter *et al.*, 2007). Additionally, total bilirubin levels have been found to correlate with liver function, suggesting that monitoring TiBil can provide valuable insights into hepatic health (Lee *et al.*, 2012). These findings underscore the importance of these biomarkers in optimizing treatment strategies and improving patient outcomes.

Based on the haematological analysis results from the four treatments (T1 to T4), several conclusions can be drawn regarding the effects of the treatments on the experimental fish. The increase in Packed Cell Volume (PCV) from T1 (20.5±0.5) to T4 (25.0±1.0) indicates an enhancement in the concentration of red blood cells in circulation. Increased PCV is generally associated with improved oxygen-carrying capacity and overall vitality, as seen in other studies on hematological improvements from various treatments (Thrall *et al.*, 2012). This is further supported by the rise in Hemoglobin levels from 6.7±0.4 in T1 to 8.0±0.5 in T4, suggesting improved oxygen carrying capacity, which is essential for cellular and systemic vitality (Weiss & Wardrop, 2010). There is a significant difference in the Red Blood Cell (RBC) across the treatments, which implies that the observed increases in PCV and hemoglobin are not due to an increase in the number of red blood cells but rather an improvement in the quality or functionality of the existing red blood cells (Smith *et al.*, 2000). Studies have shown that an increase in hemoglobin concentration without a rise in RBC count could indicate better erythrocyte function rather than quantity, potentially benefiting overall metabolic performance (Campbell, 2015).The fluctuations in White Blood Cell (WBC) counts, particularly the decrease from 15450.0±1900.0 in T1 to 15225.0±975.0 in T4, may indicate a shift in the immune response. This could suggest a normalization of immune function as the treatments progressed, potentially reducing inflammation or stress on the immune system (Tizard, 2004). In terms of the differential leukocyte count, the decrease in lymphocytes from T1 to T4, coupled with a reduction in heterophils, indicates a possible modulation of the immune response, aligning with findings that certain treatments can reduce leukocyte counts as a response to lower inflammatory demand (Thrall *et al.*, 2012). The increase in monocytes suggests an active immune recovery process, possibly linked to tissue repair and phagocytic activity, a pattern also seen in other studies of immune modulation (Abbas *et al.*, 2014). Changes in eosinophils and basophils point towards a reduction in allergic or parasitic activity, which could indicate an overall stabilization in immune response.

In laboratory trials, histopathological analyses can be employed as sensitive biomonitoring methods for health in toxicity studies to identify direct impacts of chemical substances within fish target organs, especially for chronic and sublethal effects (Authman *et al.*, 2012, 2013). According to Van der Oost *et al.* (2003), the liver is the main organ involved in metabolism, xenobiotic detoxification, and the elimination of toxic chemicals. Additionally, it is among the organs most impacted by waterborne pollutants (Camargo and Martinez, 2007). Significant hepatocytic vacoulation was seen in T1 and T4 in the current investigation. Significant kidney degenerative changes are also evident in all treatments, however they are more noticeable in T1 and T4. Since T3 had a perfectly healthy kidney and liver, it was possible to conclude that adding neem oil at lower quantities had a therapeutic effect on the fish's kidney and liver. However, at a high concentration of 5% inclusion, neem oil become poisonous, causing significant harm to the kidney and liver. The current findings are consistent with those of *Prochilodus lineatus* treated to neem leaf extract (Winkaler *et al.,* 2007), which showed glomerular enlargement leading to granular degeneration, cytoplasmic vacuolation, Bowman's space reduction, and tubular lumen constriction.

The male testes and the female ovaries are the gonads, the main reproductive organs. These organs are in charge of creating the ova and sperm, also secrete hormones and are considered to be endocrine glands. In the present study, the gonads appear to be normal across all treatment including the control group, showing neem oil does not induces any change in gonadal development and spermatogenesis. Estrogen and progesterone are steroid hormones that regulate female reproduction. The estrogen and progesterone levels are least in T3 with more marked reduction shown for estrogen compared to other treatment groups. Testosterone values are relatively stable across all treatments with T3 having the highest value. The increase in testosterone level coupled with a decrease in both estrogen and progesterone for same treatment suggests the efficacy of neem oil in disrupting or inhibiting folliculogenesis in *Orechromis niloticus*.

V. Conclusion

The replicate responses to the treatment indicate varying levels of effectiveness among the different therapies. T4 consistently yielded the best response rates across the measured parameters for haematology and serum biochemistry of *Orechromis niloticus,* and even for growth after the control. T3 showed more promising results having the best FCR and survival rate. From these results, this study suggests neem oil as a dietary supplement for enhanced fish health and growth. However, caution needs to be taken in dosage application, as inclusion at 5% rate became lethal to fish organs, negatively impacting the kidney and liver.

Inclusion of neem oil at moderate percentages (2.5%) recorded for T3 also has the potential to disrupt or reduce the fecundity of female fish, without impacting its health negatively. Overall, this research suggests the moderate usage of neem oil (2.5 - <5%) to inhibit or reduce reproduction in *Oreochromis niloticus*, without compromising its overall health conditions.

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