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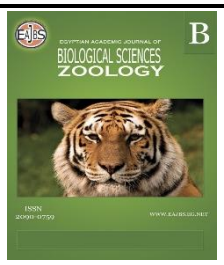

Protective Roles of Neem Leaves on Hormonal Levels in Heteroclarias Exposed to Diclofenac

Abdulkareem*, S.I. and Abdulkareem, A.O.
Department of Zoology, University of Ilorin, Ilorin, Nigeria.
***E-mail:** iyabodesaratu@yahoo.com /abdulkareem.si@unilorin.edu.ng

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ABSTRACT

Background: The release of the untreated effluents from the pharmaceutical factories has great potential adverse effects on the aquatic organisms like fish, which could induce stress and cause alterations in the hormonal levels of the fish. **Methods:** This study investigated the effects of lethal and sub-lethal concentrations of Diclofenac on hormonal levels of *Heteroclarias* for 96 h and 30 d. The Diclofenac-exposed-fish were fed on different percentages (0, 1, 2, 3, 4, 5 %) of Neem leaf to access the ameliorative potentials of the leaf against the toxicity of Diclofenac. After each experiment, blood was collected from both the control and the Diclofenac-exposed fish for hormonal assay. **Results:** The 96 h LC50 was estimated as 10.8 mg/l. The serum levels of hormones such as follicle-stimulating hormone (FSH); luteinizing hormone (LH); oestradiol; prolactin; testosterone; progesterone; triiodothyronine (T3); thyroxine (T4) and thyroid-stimulating hormone (TSH) were significantly ($P<0.05$) reduced in the Diclofenac-exposed-group compared to control and the reduction is concentration dependent. However, group C fed on 1 % Neem leaf significantly ($P<0.05$) increased compared to group B fed on 0 % Neem leaf. **Conclusion:** The study implies that 1 % Neem leaf inclusion in diet of *Heteroclarias* is capable of minimizing the toxic effect of Diclofenac and improve the hormonal levels of the fish.

INTRODUCTION

The increased pharmaceutical companies worldwide have great potential adverse effects on the aquatic organisms like fish (Chen *et al.*, 2013). The release of the untreated effluents from these factories and human through excretion into the aquatic environment induced stress that could lead to alterations in the hormonal levels of the fish thereby affecting the physiological activities such as reproduction and growth (Praskova *et al.*, 2014). The presence of these effluents in the water bodies bioaccumulated in the tissues of fish and could cause blockage in the gill filaments, which cause respiratory disorder and eventually lead to oxidative stress. The induced stress adversely affect the thyroid stimulating hormones and follicle stimulating hormones that are the major hormones that control the physiological and biochemical activities of the fish (Margiotta-Casaluci *et al.*, 2021 and Wang *et al.*, 2022). Pharmaceutical pollutions have become a matter of attention as being toxic in aquatic

environments, due to the fact that many of these compounds have been shown to reach considerably high concentrations within surface waters and sewage treatment plant (STP) effluents (Heberer, 2001). Most pharmaceuticals effluents are deposited in the aquatic environments from the factories without treatment and most often through human excretion. The effluents that are continuously discharged into water bodies lead to chronic exposure of aquatic organisms.

Diclofenac (DCF) is an important non-steroidal anti-inflammatory drug (NSAID) and used for the treatment of painful and inflammatory conditions (Barbosa *et al.*, 2016). Hormones are organic substances secreted by plants and animals that function in the regulation of physiological activities and in maintaining homeostasis (*McAninch and Bianco, 2014*). Types of fish hormones are the reproductive hormones, such as follicle-stimulating hormone (FSH), prolactin, leutinizing hormone, prosgesterone and testosterone. Follicle-stimulating hormone (FSH) is the most important and it is a type of gonadotropin; it is concerned with the regulation of the activity of the gonads, or sex organs, which are endocrine glands as well as the sources of eggs and sperm (Zhang *et al.*, 2014). The second types of fish hormones are the growth hormones, which are the thyroid-stimulating hormone (TSH), triiodothyronine (T3) and thyroxine (T4). Thyroid-stimulating hormone (TSH) is the most important growth hormone; it is a pituitary glycoprotein hormone, which stimulates the thyroid gland (Cheng *et al.*, 2017). The thyroid hormones (Triiodothyromine-T3 and Thyroxine-T_4) are known to regulate cell metabolism and growth. When fish are exposed to stressors, the level of the thyroid hormones was established to be decreased and chemical pollutants have been reported to be deleterious to the thyroid hormone status in a number of fish species (Xu *et al.*, 2002 and Brown *et al.*, 2004).

Fish is an important tool in the assessment of toxic effect of contaminants in the aquatic environment (Stegeman *et al.*, 2000). *Heteroclarias spp* are very rugged and disease resistant, with highly efficient air-breathing organ, which allows them to survive in oxygen-depleted water, migrate for long distances as long as 1.2 km (Anyanwu, 2005). To conserve these characteristics in fish and other aquatic organisms, it is therefore necessary to establish protective means to minimize the toxic effects of this drug when accidentally released into the water bodies. Amongst the various medicinal plants, Neem leaf could have a great potential in ameliorating toxic effects of contaminants because of the presence of some phytochemical properties such as saponin, glycosides and flavonoids and amino acids (Tiwari *et al.*, 2011).

Several investigations have been carried out on the effects of various toxicants on different parameters in the fish. Amongst these are exposure of *Clarias gariepinus* to Dichlorvos ameliorated with Moringa leaf (Abdulkareem *et al.*, 2016); *Heteroclarias* to diesel oil (Owolabi and Abdulkareem, 2018); *Heteroclarias* to chlorpyrifos amelioated with vitamin E (Abdulkareem and Owolabi, 2021); *Clarias gariepinus* to *Carica papaya* ameliorated with *Mangifera indica* (Owolabi and Abdulkareem, 2021). While histopathological effects of Diclofenac residual was investigated in some fish species like in rainbow trout, Tench and Zebra fish (Praskova *et al*., 2014; Stancova *et al.*, 2014). However, these different research works only focused on the toxicological impacts of the contaminants but little or no results has been reported on the use of ameliorating agents as a protective measure for the fish against Diclofenac toxicity. This study therefore aimed at investigating the protective effect of Neem leaves on hormonal levels in African hybrid catfish (*Hetroclarias*) exposed to the Diclofenac.

MATERIALS AND METHODS

Experimental Setup:

Juveniles of hybrid catfish *Heteroclarias* with average weight 20.6±1.5 g and average length 10.13±0.6 cm were obtained from Raji Fish Farm in Ilorin, Kwara State, Nigeria. The fishes were transported in the early hours of the day in a well-aerated plastic aquarium to the laboratory in the Department of Zoology, Faculty of life Sciences, University of Ilorin, Nigeria. The fish were not fed until the following day, to avoid indigestion that could lead to death because of stress (Abdulkareem *et al.,* 2020). The fish were maintained in a large plastic aquarium with dechlorinated borehole water, under room temperature of 24 ^{o}C and fed commercial feeds (Durante 2 mm 35 % crude protein) twice daily at 3% of body weight (Abdulkareem *et al.,* 2020). They were then acclimated to the Laboratory condition for the period of 14 d (USEPA, 1996). Feeding was stopped 24 h before the commencement of the experiment (USEPA, 1996). The toxicant (Diclofenac) used was obtained from standard pharmaceutical centre in Ilorin, Kwara State, Nigeria.

Exposure Period:

Acute Toxicity Test:

At the end of acclimation period, the fish were subjected to range finding test. Based on the results, six different concentrations (0.0. 4.0, 8.0, 12.0, 16.0 and 20.0 mg/l) of Diclofenac were prepared through serial dilution (10-fold) in a static renewal bioassay for acute toxicity test (OECD, 1997) for the period of 96 h to determine the mortality rate. Sixty fish were randomly divided into six different groups of 10 fish each in an aquarium of 40-litre capacity containing 10 litres of chlorine-free bore-hole water. The experiment was set up in triplicate and fish fed 3 % body weight (Abdulkareem *et al.,* 2020). After the exposure period, the concentration that caused 50 % mortality was calculated using Arithmetic Karber method (Dede & Kaglo, 2001) and blood was collected from the caudal region of both the control and the experimental fish for hormonal assay.

Chronic Toxicity Test (Amelioration):

Following the results of the acute toxicity, fish was exposed to sub-lethal concentration Diclofenac (1/10 of the calculated LC_{50}) and fed diet supplemented with varying percentages of Neem leaf (1, 2, 3, 4, 5%) to evaluate the aameliorative potentials of Neem leaf on Diclofenac-exposed fish. The Neem leaves (ameliorative agent) were thoroughly washed, air-dried, ground and incorporated in to six experimental diets at the following inclusion levels; 0 % (control), 1%, 2%, 3%, 4% and 5% respectively.

Feed Formulation:

The diet was formulated with the following ingredients: maize corn (30%), soya bean (20%), groundnut cake (18%), fish meal (30%), bone meal (0.4%), vitamin C (0.2%), methionine (0.4%), lysine (0.4%), salt (0.2%), premix (0.2%) which formed 100% balanced diet. The feedstuff were finely ground, mixed, pelleted through a mincer of 2 mm die, air-dried and packed in airtight polythene bags prior to use (Abdulkareem *et al.,* 2021). One hundred and eighty (180) fish were shared randomly into 18 aquaria in triplicates of six groups. The first group A is the control without toxicant and fed 0 % neem leaf and groups B, C, D, E & F were exposed to Diclofenac (1/10 of LC_{50}) and fed 1, 2, 3, 4 & 5 % Neem leaf respectively. Toxicant was renewed every 24 h to maintain toxicant concentration and good water quality, fish fed 3 % body weight and fed twice in a day. At the end of thirty-day of exposure to Diclofenac, the fish were sacrificed for hormonal assay. The hormonal levels in the serum were assessed on Triiodothyronine (T3), Thyroxine (T4), Thyroid-stimulating hormone (TSH), Follicle-stimulating hormone (FSH), luteinizing hormone (LH), estradiol, prolactin, testosterone, progesterone following the Environmental Impact Assessment (EIA) method of using omega diagnostic kit.

Water Quality Parameters:

Water quality parameters such as temperature, dissolved oxygen (DO), biochemical oxygen demand (BOD), conductivity, chemical oxygen demand (COD) pH. Total dissolved solid (TDS) and turbidity (TB) were continuously monitored following the procedure of (NESREA, 2011).

Statistical Analysis:

Analysis of variance (ANOVA) was carried out using a computerized IBM SPSS version 20 Programme and Duncan multiple range test were used to carry out the level of significance test at 95% ($P<0.05$) between the control and experimental means. All data were expressed as mean ± Stadard Error (SE).

RESULTS

Behavioural Responses:

The fish exposed to diclofenac showed immense behavioral and morphological changes such as erratic movement, respiratory distress, loss of balance, opercula movement, jumping, gasping for air, white colouration of fins and swollen of abdomen. The rate of mortality increased as the concentration of Diclofenac increased. Highest rate (100 %) of mortality occurred at 20.0 mg/l, while the lowest mortality rate (13 %) was recorded at 4.0 mg/l (Table 1). The lethal concentration Diclofenac that caused 50 % mortality in Diclofenac-exposed fish is 10.8 mg/l (Table 1).

Table 1: Estimation of LC_{50} value of *Heteroclarias* exposed to different concentration of Diclofenac for 96 h.

Concentration (mg/l)	Concentration Difference	∑Real & Replication Alive	∑Real & Replication Death	Mean Death	Mean Death x Concentration Difference
0	0	30	0	0	0
4	4	26	4	1	4
8	4	24	6	2	8
12	4	18	12	4	16
16	4	12	18	6	24
20	4	0	30	10	40
					92

$$LC_{50} = LC_{100} - \sum \frac{\text{Mean death x Conc. Difference}}{\text{No. of Organisms per group}}$$

$$= \left(20 - \frac{92}{10}\right) = 20 - 9.2 = 10.8\, mg/l$$ (Dede and Kaglo, 2001)

The serum levels of estradiol, progesterone and testosterone in *Heteroclarias* exposed to diclofenac for the period of 96 h significantly ($P < 0.05$) decreased as the concentration of diclofenac increased compared to that of control (Fig. 1). The fish exposed to the lowest concentration of diclofenac (4.00 mg/l) recorded the highest serum levels among the exposed groups while those exposed to the highest concentration (16.00 mg/l) recorded significant ($P < 0.05$) reduction in estradiol and progesterone except in the testosterone with an insignificant ($P > 0.05$) reduction (Figure 1). The hormonal levels of TSH, T3 and T4 also decreased significantly ($P < 0.05$) as the concentration of Diclofenac increased compared to control group. Among the Diclofenac-exposed-groups, highest level of hormones were recorded in the lowest (4.0 mg/l) concentration and highest hormonal levels in the highest concentration (Fig. 1).

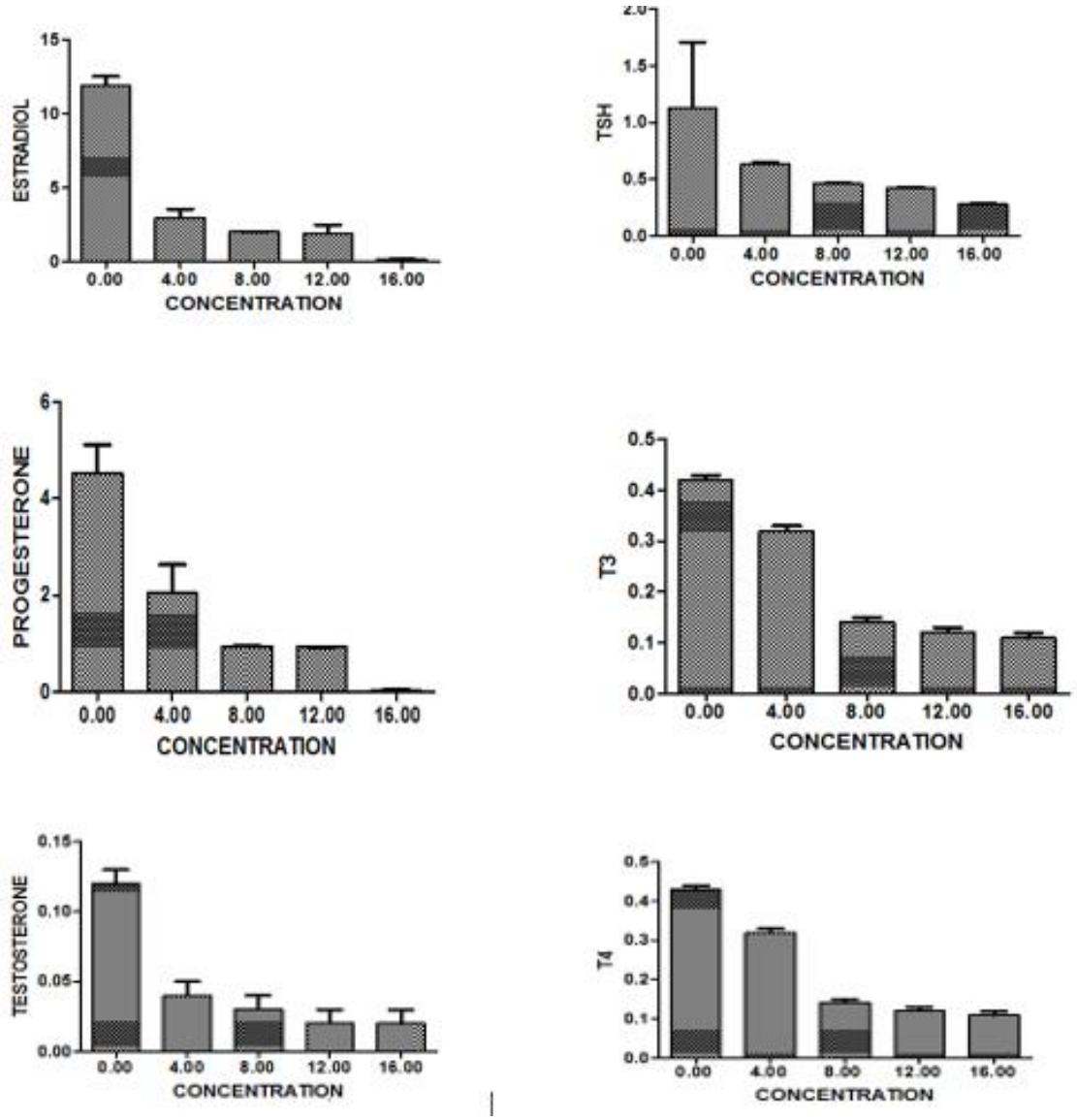

Fig. 1: Different hormonal levels in the serum of *Heteroclarias* exposed to varying concentrations of Diclofenac for 96.

KEY: Thyroid Stimulating Hormone = TSH; Follicle Stimulating Hormone = FSH; Triiodothyronine = T3; Thyroxine = T4

The serum levels of hormones FSH, LH, prolactin and estradiol in *Heteroclarias* juveniles exposed to sub-lethal concentration Diclofenac and fed on diet supplemented with different percentages of Neem leaf (Ameliorative agent) for the period of 30 d were illustrated in Figures 2, 3, 4 and 5. The levels of these hormones in group B exposed to Diclofenac and fed 0 % Neem leaf are significantly ($P < 0.05$) lower compared to those in group A (control) that were not exposed to Diclofenac and fed 0 % Neem leaf. While those in groups C, D, E, & F exposed to sub-lethal concentration of Diclofenac and fed different percentage inclusion (1, 2, 3 and 4 %)f Neem leaf were significantly ($P < 0.05$) higher respectively. However, the levels of the reproductive hormones decreased as the percentage inclusion of Neem leaf increased. The lowest level of hormones were recorded in-group F with the highest percentage of Neem leaf among the ameliorating groups (Figs. 2, 3, 4 and 5). The hormonal levels decreased as the exposure period increased from day 8 to day 30, the decrease was therefore concentration dependent.

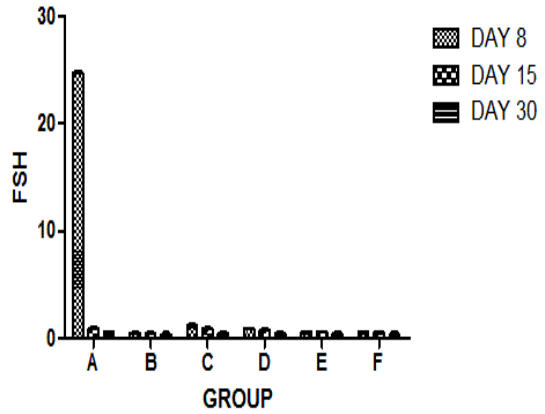

Fig. 2: Level of follicle stimulating hormones (FSH) in the serum of *Heteroclarias* exposed to sub-lethal concentration of Diclofenac and fed on neem leaves for 30 days.

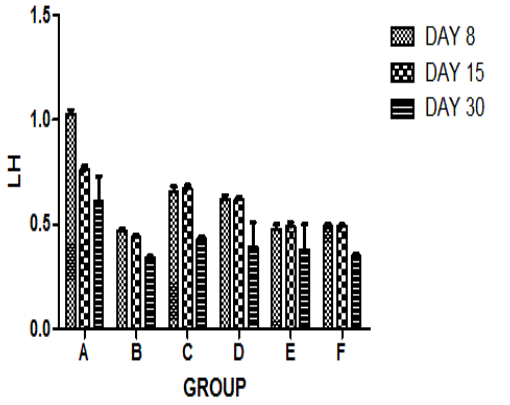

Fig. 3: Level of luteinizing hormones (LH) in the serum of *Heteroclarias* exposed to sub-lethal concentration of Diclofenac and fed on neem leaves for 30 days.

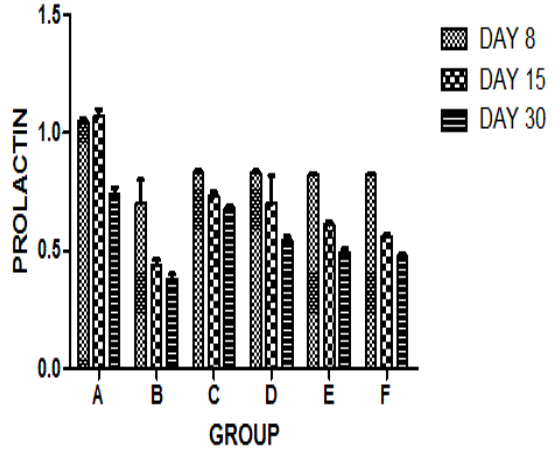

Fig. 4: Level of Prolactin in the serum of *Heteroclarias* exposed to sub-lethal concentration of Diclofenac and fed on neem leaves for 30days.

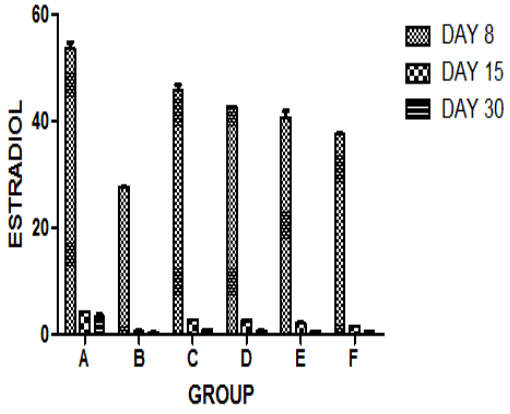

Fig. 5: Level of Estradiol in the serum of *Heteroclarias* exposed to sub-lethal concentration of Diclofenac and fed on neem leaves for 30 days.

Figures 6 and 7 revealed the serum levels of progesterone and testosterone in *Heteroclarias* exposed to sub-lethal concentration of Diclofenac and fed on diet incorporated with varying percentages of Neem leaf for 30 d. The levels of progesterone and testosterone in group B exposed to Diclofenac without Neem leaf recorded a significant ($P < 0.05$) reduction compared to that of group A (control group). However, progesterone showed no significant ($P > 0.05$) difference in groups C, D, E and F but a significant ($P < 0.05$) reduction was recorded in testosterone as the percentage inclusion of Neem leaf in the diet increased from groups C to F (Figs. 6 and 7).

The levels of hormones T3, T4 & TSH in the fish exposed to sub-lethal concentration of Diclofenac for the period of 30 d were revealed in Figures 8, 9 and 10. The hormonal levels significantly ($P < 0.05$) increased in the Diclofenac-exposed group (B) compared to control (group A). While the hormonal levels in groups C, D, E and F fed on varying percentages of Neem leaf increased but reduced as the percentage inclusion of the

Neem leaf increased (Figs. 8, 9 and 10). There was also reduction in the levels of hormones as the period of exposure increased from day 8 to 30. The values of BOD, COD, CD, and TDS were significantly ($P < 0.05$) higher in group B that contained Diclofenac only without Neem leaf compared with the standard values. While the values of DO decreased significantly ($P < 0.05$), with insignificant ($P > 0.05$) differences in those of temperature and pHcompared to both national and international permisible levels (Table 2).

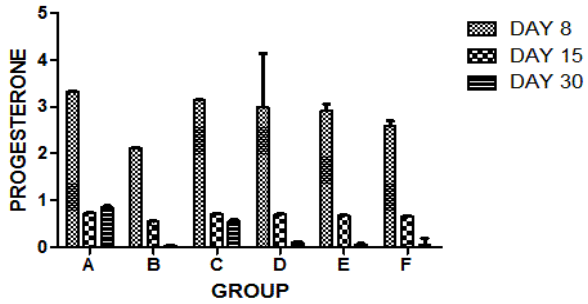

Fig. 6: Level of Progesterone in the serum of *Heteroclarias* exposed to sub-lethal concentration of Diclofenac and fed on neem leaves for 30 days.

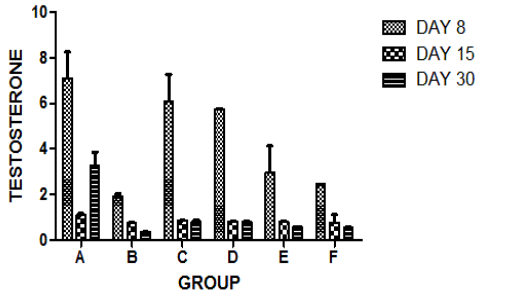

Fig. 7: Level of Testosterone in the serum of *Heteroclarias* exposed to sub-lethal concentration of Diclofenac and fed on neem leaves for 30 days.

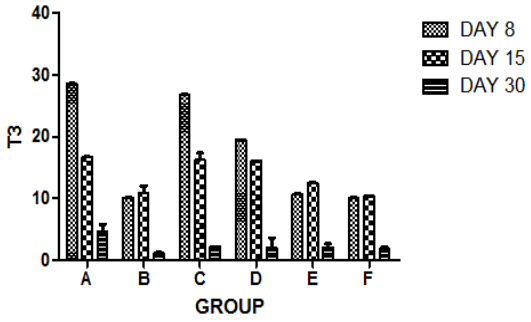

Fig. 8: Level of Triiodothyronine (T3) in the serum of *Heteroclarias* exposed to sub-lethal concentration of Diclofenac and fed on neem leaves for 30 days.

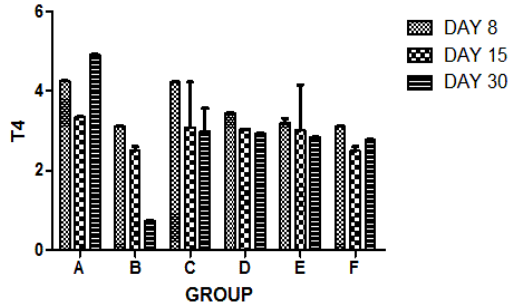

Fig. 9: Level of Thyroxine (T4) in the serum of *Heteroclarias* exposed to sub-lethal concentration of Diclofenac and fed on neem leaves for 30 days.

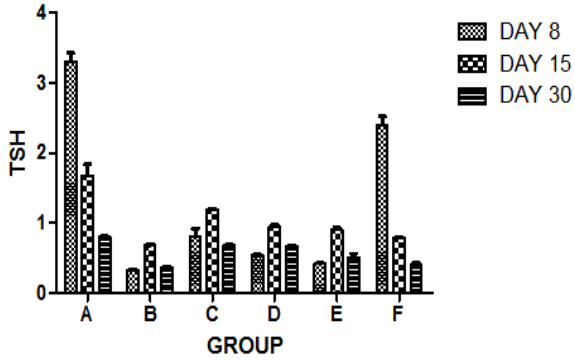

Fig. 10: Level of Thyroid Stimulating Hormone (TSH) in the serum of *Heteroclarias* exposed to sub-lethal concentration of Diclofenac and fed on neem leaves for 30days.

Values are means ±SE (n=3). *Significantly different from the control ($P < 0.05$).

NOTE: A: No toxicant + 0 % Neem leaf; B: Diclofenac (1/10 of LC_{50}) + 0 % Neem leaf C: Diclofenac (1/10 of LC_{50}) + 1% Neem Leaf ; D: Diclofenac (1/10 of LC_{50}) + 2% Neem Leaf ; E: Diclofenac (1/10 of LC_{50}) + 3% Neem Leaf; F: Diclofenac (1/10 of LC_{50}) + 4% Neem Leaf.

Table 2: Physico-chemical parameters of Diclofenac exposed to *Heteroclarias* and ameliorative effects of Neem leaf for 30 days.

Group	pH	TDS (mg/l)	COD (mg/l)	BOD (mg/l)	CD (ds/m)	TB (mg/l)	DO (mg/l)	T (°C)
A	6.10±0.46^{a}	127.00±5.24^{c}	3.10±0.26^{c}	20.20±0.06^{e}	85.00±5.77^{f}	0.40±0.02^{b}	9.40±5.71^{c}	28.50±5.17^{a}
B	5.30±0.07^{b}	199.00±5.47^{e}	6.40±0.08^{a}	22.42±0.16^{a}	99.00±5.17^{e}	0.50±0.47^{d}	1.20±5.22^{b}	32.80±5.23^{b}
C	6.60±0.15^{c}	107.00±5.72^{a}	2.80±0.25^{b}	19.08±0.05^{b}	79.00±5.23^{d}	0.39±0.58^{a}	9.90±5.18^{a}	28.50±5.55^{c}
D	6.80±0.28^{d}	129.00±5.20^{d}	3.40±0.08^{d}	20.65±0.01^{c}	90.00±5.04^{c}	0.41±0.20^{f}	9.00±5.11^{d}	28.47±5.72^{d}
E	6.10±0.15^{e}	137.00±5.12^{b}	3.80±0.11^{f}	21.10±0.034^{f}	93.00±5.58^{a}	0.47±0.14^{c}	1.60±5.54^{e}	28.57±5.77^{e}
F	5.40±0.09^{f}	139.00±5.56^{f}	4.40±0.21^{e}	21.40±0.15^{d}	94.00±5.72^{b}	0.60±0.26^{e}	1.21±5.22^{f}	28.30±5.16^{f}
FEPA	6.5 – 9.0	NA	30.0	4.00	NA	NA	5 – 6	20 – 33
WHO (2011)	6.5 – 8.5	500	NA	NA	1000	4.0	> 2.00	30 - 32
NESREA (2011)	6.0 – 9.0	5.00	90.0	50.00	NA	NA	4.00	NA
NSDWQ(2005)	6.5 – 8.5	500	NA	NA	NA	NA	7.5	30 – 32
USEPA (2011)	6,5 – 9.0	<1000	410	250	NA	NA	NA	NA

Means (± S.E, n=3) with the same superscript letters at the same column are not significantly different ($P > 0.05$)
NOTE: FEPA: Federal Environmental Protection Agency; NESREA: National Environmental Standard Regulation Enforcement Agency; NSDWQ: Nigerian Standard for Water Quality; WHO: World Health Organization; NA: Not Available. TDS = Total Dissolved Solid, COD = Chemical Oxygen Demand, BOD = Biochemical Oxygen Demand, T = Temperature, DO = dissolved Oxygen, CD= conductivity, TB= turbidity

DISCUSSION

Diclofenac triggered hormonal responses and endocrine responses in fish during the course of this research. Endocrine responses through integrative and early capacity may offer a potential indicator that may be useful in the detection and assessment of the sub-lethal toxic stress in fish exposed to Diclofenac (Brown *et al.,* 2004). Fish exposed to varying concentrations of Diclofenac exhibited some signs of stress, imbalance, erratic movement, over secretion of mucus, skin discoloration, gasping for air due to the neurotoxic effect of Diclofenac. The observed behavioral changes could signal respiratory impairment and this might be due to hyperactivity and oxidative stress because of the toxic effect of Diclofenac. The decrease in hormonal action might be due to the interference of the experimental toxicant with the synthesis, transport, metabolism and elimination of hormones thereby decreasing the concentration of naturally occurring hormones as reported by Gupta *et al*. (2006). Reduction in the levels of reproductive hormones (FSH, LH, prolactin, estradiol, progesterone and testosterone) could be due to the toxic effect of Diclofenac causing damage to the testes and ovary that lead to malfunction of the reproductive organs and hence reduction in the fish population. This is similar to the reports of Wang *et al*. (2011).

Decrease in the levels of the growth hormones (TSH, T3 & T4) could probably be due to hyperactivity and stress which led to reduction in the feeding rate induced by the toxic effect of Diclofenac. Reduction in the thyroid hormones or absence of production of thyroid hormones are known to reduce the basal metabolic rate (BMR) and cause the muscles to become extremely sluggish in animals (Mullur *et al.,* 2014). Great reductions in T4 and TSH hormone levels indicating reduced thyroid function and reduced metabolic activity (Freeman and Sangalong, 2000). The recorded reduction in thyroid hormone levels was similar to the reports of Jenkins *et al*. (2003) who exposed *Cyprinus carpio* to sub-lethal endosulfan, and Thangavel *et al*. (2005) who exposed *Sarotherodon mossambicus* to dimecron.

The increase in hormonal levels of *Heteroclarias* at the early period (Day 8) of exposure to diclofenac and the subsequent decrease in their levels on day 15 and 30 could probably suggest the onset of an increased metabolic activity triggered by Diclofenac during the initial period followed by an adaptive lowering of the metabolic rate upon prolonged exposure. The reduction in thyroxine level could be due to the competitive binding of pesticide with the thyroxine (thereby making it inactive) or transthyretin (a plasma T4 binding protein), thereby reducing the hormone activity. The reduction in TSH could be due to the synthesis and secretion of TSH hormone by the anterior pituitary in response to a negative feedback mechanism involving concentrations of free plasma T3 level (Saravanan *et al.,*

2007).The increased levels of the hormones recorded in the groups of *Heteroclarias* exposed to Diclofenac and fed Neem leaf could be because of the presence of saponin, glycosides, and flavonoids with ameliorating properties, which signifies the protective effect of Neem leaf on the hormonal level of *Heteroclarias*. Great increase recorded in group C fed 1 % Neem leaf was an indication that 1 % inclusion in the diet of *Heteroclarias* exposed to Diclofenac was capable of neutralizing the toxic effects of Diclofenac and normalizing the activity of the endocrine gland for hormones production. While the drastic reduction in the hormonal levels in the group fed 4 %, Neem leaf produced adverse effect. The diclofenac-exposed fish were able to withstand the fluctuated water quality parameters since they were within the tolerable range as suggested by WHO (2011). The recorded BOD and COD in the diclofenac-exposed groups were above FEPA (1991) and WHO (2011) limits, which probably lead to the low rate of growth in the fish exposed to diclofenac. The rate of TDS, turbidity level and electric conductivity were highly above the recommended limits for fish habitat. This could prevent the fish from extracting the dissolved oxygen from the environment. The highest DO record in group C fed 1 % Neem leaf indicates that 1% neem leaf supplementation in the fish diet is capable of minimizing stress in fish, hence reduction in oxygen consumption by the fish. This result corroborated the report of Chapman (1991) that DO below 2 mg/l may lead to fish death.

CONCLUSION

Lethal and sub-lethal concentrations of Diclofenac altered the hormonal levels in *Heteroclarias* but supplementation of diet with 1 % Neem leaf was able to ameliorate the hormonal alteration. This implies that Neem leaf used as ameliorative agent showed protective capability on the hormonal levels of *Heteroclarias* exposed to Diclofenac. However, 1% of Neem leaf is capable of reducing the toxic effect of Diclofenac on growth and reproductive hormone in *Heteroclarias.* High levels of BOD and COD with decrease in the level of OD in the diclofenac-exposed group induced stress which led to alteration in the hormonal levels that caused reduction in the growth rate of the fish, but supplementation of the fish diet was able to improve the dissolved oxygen and reduced stress.

Declarations:

Ethical Approval: This study has been granted by Faculty of Life Sciences Ethical Research Committee of University of Ilorin, Ilorin, Nigeria in accordance with university guidelines for the care of animals with approval number UERC/ASN/2016/648.

Competing interests: The authors declare there are no conflicts of interest regarding the publication of this article.

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