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Ameliorative Potentials of *Azadirachta indica* (Neem Leaf) on Effects of Diclofenac on Haematological Parameters and Growth Rate of *Heteroclarias*

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ABSTRACT: The uncontrolled release of diclofenac (DCF) effluents from the factories into the water bodies can cause physiological disorders in aquatic animals, especially fish. This study examined the haematological and growth responses of *Heteroclarias* exposed to six definitive concentrations (0.0. 4.0, 8.0, 12.0, 16.0 & 20.0 mg/l) of DCF for 96 h and evaluated the ameliorative potentials of *Azadirachta indica* (Neem leaf) through different percentage (0, 1, 2, 3, and 4%) inclusion in the fish diet for a period of 30 d. At the end of the exposure and ameliorative periods, the fish were sacrificed, and blood samples from both the control and DCF-exposed-groups were collected for haematological assay. The value of lethal concentration that caused 50% mortality during acute toxicity (96 h LC_{50}) was determined as 10.8 mg/L. In the DCF-exposed-fish, red blood cells (RBC), haemoglobin (HB), and packed cell volume (PCV) significantly ($P < 0.05$) decreased. In contrast, white blood cells (WBC), lymphocytes, and platelets showed significant ($P < 0.05$) increase as DCF concentration increased. During the chronic test, fish fed on a diet supplemented with 1% neem leaf revealed increased ($P < 0.05$) erythrocyte levels and reduced leukocyte levels with great improvement in the weight gain and the specific growth rates. These results indicate that DCF is toxic to *Heteroclarias*, but dietary supplementation with 1% neem leaf for 30 d can minimize the fish's physiological disorder by improving the erythrocyte levels and the specific growth rate.

Keywords: Diclofenac; Haematology; Growth; Neem Leaf; *Heteroclarias*

Introduction

Degradation of the aquatic environment caused a great decline in fish population as a result of chemical pollution. Among the chemical pollutants are the pharmaceutical effluents that are indiscriminately discharged from the pharmaceutical companies into the water bodies and uncontrolled dropping of unused drugs that eventually find their way into the water bodies through runoff. The occurrence of these pharmaceutical compounds in the water could lead to dissolved oxygen depletion and reduce the respiration rate in fish, thereby inducing oxidative stress. The pharmaceutical contaminants bio accumulates in the tissues of the fish including the blood stream, which could reduce the RBC that would lead to reduction of oxygen. This could alter the fish physiology and either increase or decrease the metabolic activities that may eventually lead to death, hence, reduction in the fish population and loss of biodiversity. To conserve the fish population, there may be the need for preventive measures that will help ameliorate the toxic effects of these pharmaceutical compounds and reduce stress in fish when in contact with chemical pollutants. Despite the benefits of drugs as naturally derived or synthetically created, for the prevention or treatment of health conditions in humans and animals, small concentrations, even in the microgram or nanogram per litre of water, can pose potential public health risks (Nava-Álvarez *et al*., 2014). Traces of active pharmaceutical ingredients (APIs) and their by-products have been found in treated and untreated sewage, groundwater, surface water, and drinking water, with concentrations ranging from nanograms to milligrams per litre (Gonzalez-Rey and Bebianno, 2014). Diclofenac (DCF), a widely used non-steroidal anti-inflammatory drug (NSAID) for treating pain and inflammation, has been shown

to be toxic to various aquatic organisms, including fish (Lee *et al*., 2011). Several studies have reported the toxicity levels of DCF to different fish species. Praskova *et al.* (2011) recorded the LC_{50} value of 167 mg/L in zebrafish, while Ajima *et al.* (2014) estimated the value of LC_{50} as 25.12 mg/L in *Clarias gariepinus*. Haematological parameters, such as red blood cells, white blood cells (WBC), haemoglobin levels, and packed cell volume, can be used to measure the response of fish to external stressors (Saravanan *et al*., 2012). Exposure of fish to DCF can significantly alter these haematological parameters in fish. Saravanan *et al.* (2011) recorded reduced RBC counts and increased WBC levels in common carp (*Cyprinus carpio*) exposed to DCF. Similarly, Ajima *et al.* (2014) reported decrease in haemoglobin, packed cell volume, and RBC counts, with an increase in the WBC counts in *Clarias gariepinus* exposed to DCF for 42 days. An insignificant change was recorded in haematological indices such as mean corpuscular volume (MCV) or mean corpuscular haemoglobin (MCH) decrease in the WBC counts in *Hoplias malabaricus* exposed to diclofenac (Ribas *et al.*, 2016). Exposure of fish to stress-induced contaminants could affect feeding behaviour, hence, poor assimilation and low feed conversion ratio that has effects on fish growth rates. Praskova *et al.* (2014) found that at concentrations of 15 mg/L and higher, DCF caused significant weight loss in zebrafish. Nassef *et al.* (2010) also reported that Japanese medaka showed reduced feeding behaviour when exposed to DCF at 1 mg/l, which could have far-reaching effects on growth, reproduction, and population success.

The use of medicinal plants to modulate the effects of environmental toxicants has gained interest in recent years. Neem (*Azadirachta indica*) leaves have medicinal properties and rich in flavonoids, glycosides and polyphenols, which are known for their antioxidant properties (Yadav *et al.*, 2016). The potential antioxidant properties of the medicinal plant could go a long way to protect the fish against the adverse effects of the toxicant. This could improve the stress resistance of the fish and counter the stress induced by the pollutant, thereby bringing the fish back to normalcy when in a polluted environment, hence, production of enabling environment for sustainability of the fish.

The potential ameliorative effects of medicinal plants prompted several studies on the effectiveness of medicinal plants in alleviating the negative impacts of environmental pollutants. Abdulkareem & Utuedor (2016) and Hamed & El-Sayed (2019) reported that *Moringa oleifera* leaf mitigated the adverse effects of dichlorvos and pendimethalin on *Clarias gariepinus* and *Oreochromis niloticus*, and improved their growth rates, respectively. Similarly, Owolabi and Abdulkareem (2021), reported increased erythrocytes and decreased leucocytes in atrazine intoxicated fish modulated with *Carica papaya* and *Mangifera indica*.

To solve the problem of adverse effects of pharmaceuticals on fish, there is therefore the need to investigate the toxicity of Diclofenac on *Heteroclarias* and treat the exposed fish with neem leaf through dietary supplementation.

Materials and methods

Experimental setup: *Heteroclarias* juveniles (average weight of 15 ± 0.5 g and average length of 10.25±1.5cm) were obtained from Raji Farm in Ilorin, Kwara State, Nigeria. The fish were transported during the early hours of the day to the Laboratory in the Department of Zoology, Faculty of Life Sciences, University of Ilorin in a well-aerated black tank containing water from the farm. The fish were not fed throughout the day to avoid indigestion that could cause mortality due to stress. The water was replaced with aerated de-chlorinated water, and the fish were allowed to acclimatize to the Laboratory conditions for 14 days (USEPA, 1996). The fish were fed twice daily with a standard commercial feed of 2 mm sized Durante commercial feed pellets at 3% body weight (Meyer *et al.* 1993). The water was renewed every 24 h to remove faecal materials and prevent contamination of the water and a 12 h light: 12 h dark photoperiod was maintained. The experimental fish were carefully handled in accordance with the regulations of the University of Ilorin Ethical Committee and based on the guidelines of the National Institute of Health on the care and use of laboratory animals. Feeding was stopped 24 h prior to the commencement of the experiment (USEPA, 1996).

Collection of Neem leaves: Fresh neem (*Azadirachta indica*) leaves (400 g) were collected from a farmland in the University of Ilorin main campus, identified and authenticated in the herbarium of the Department of Plant Biology, University of Ilorin and air-dried in the laboratory for seven (7) days. The dried leaves were grinded in an electric ground to make powder (300 g) and kept in a sealed polythene bag for later use in the formulation of fish feed.

Phytochemical analysis (*preparation of extract*): The neem powder (100 g) was soaked in 2000 ml of distilled water for 24 h and aqueous extract was filtered through muslin cloth and filter paper. The filtrate was then tested qualitatively with different reagents to determine the presence of saponin, tannin, phenolics, steroids, flavonoids, tripenoids, alkaloids, triterpenes, glycosides and coumarins following the standard methods of Trease and Evans (1989) and Harborne (1998).

Feed formulation: Formulated feeds with graded levels of neem (0, 1, 2, 3 and 4%) were prepared and fed to six different groups of fish for 30 days. The diet was formulated with various meal ingredients such as maize corn (30%), fish meal (30%), toasted soya bean (20%), groundnut cake (18%), vitamin C (0.2%), methionine (0.4%), lysine (0.4%), salt (0.2%), premix (0.2%), neem leaf (0%) that formed 100% complete diet for the first group, while the feedstuffs such as maize corn, fish meal, soya meal and groundnut cake were supplemented with 1, 2, 3 and 4% neem leaf each for the remaining four groups.. The ingredients were weighed, grinded, milled and pelleted with a mincer into 2mm sizes. The pelleted feeds were air-dried and kept in different airtight polythene bags for later use. The fish were divided into six (6) different groups and fed on different feeds that were supplemented with varying percentages of neem leaves to treat the diclofenac-exposed fish for 30 days. Group A was fed on normal diet (Basal) only without exposure to DCF; group B was exposed to DCF and fed on normal diet; group C was exposed to DCF and fed on diet supplemented with 1% neem leaf; group D was exposed to DCF and fed on diet supplemented with 2% neem leaf; while group E was exposed to DCF and fed on diet that was supplemented with 3% neem leaf and group F was exposed to DCF and fed on 4% neem leaf.

Water quality parameters: During the test, the quality of the water was monitored for temperature, pH, conductivity, dissolved oxygen (DO), biological oxygen demand (BOD) and chemical oxygen demand (COD) following the Standard procedure in the methods described by the American Public Health Association (APHA, 2005).

Ethical approval: Ethical clearance on the use of laboratory animals was obtained from the University Ethical Review Committee (UERC) of University of Ilorin, Ilorin, Nigeria. The Experimental Animal approval number is UERC/ASN/2016/648.

Acute toxicity test: Following the results obtained from the presumptive test, the preliminary acute toxicity test was carried out according to the procedures of OECD (1992) to determine the 96 h LC_{50} value. Ten (10) of the acclimatized fish each were exposed to six (6) different definitive concentrations (0.0. 4.0, 8.0, 12.0, 16.0 and 20.0 mg/l) of DCF measured from the prepared stock solution. The experiment was set up in triplicate and in a constant renewal bioassay to maintain a consistent concentration of the toxicant for 96 h. The diclofenac-exposed fish were not fed 24 h before the experiment and during the experiment (USEPA, 1996). Fish behaviour and mortality with the physicochemical parameters were monitored according to the standard method of APHA, (2005). The lethal concentration that caused 50% mortality (LC_{50}) in diclofenac-exposed fish was calculated using the arithmetic method of Karber adopted by Dede and Igbigbi (1997).

$$LC_{50} = LC_{100} - \sum (Mean\ Death \times Conc.\ Diff) \div No\ of\ fish\ per\ group.$$

Ameliorative test: The ameliorative test was carried out by exposing ten (10) fish each to sub-lethal concentration, 1.08 mg/l DCF that is equivalent to 1/10 of 96 h LC_{50} value (Narra *et al*., 2011) in de-chlorinated borehole water media for 30 days in six (6) experimental groups: Group A, De-chlorinated borehole water and normal diet without neem leaf (positive Control group); Group B, DCF and normal diet with zero percent (0%) neem leaf (Negative Control group); Group C, DCF and diet with one percent (1%) neem leaf inclusion; Group D, DCF and diet with two percent (2%) neem leaf inclusion; Group E, DCF and diet with three percent (3%) neem leaf inclusion; Group F, DCF and diet with four percent (4%) neem leaf inclusion. Groups A and B serve as the control groups while groups C to F serve as the experimental groups. The experiment was performed in triplicate and the experimental water was renewed every 24 h. The fish were fed twice daily with Durante commercial feed pellets at 3% body weight of the fish. Feeding was suspended for 24 hours before the collection of blood for haematological assay to minimize gastrointestinal disturbance in the fish during the process of sacrifice (Garcia and Martinez, 2012). At the end of the experiment, the fish were sacrificed for haematological assay.

Haematological assay: Blood samples were collected from both the control and the exposed groups based on the procedure described by Owolabi and Abdulkareem, (2021). The right angle of the ventral part near the caudal artery was lacerated with a dissecting set to collect the blood. Two millilitre (2 ml) of blood was collected using a capillary tube and stored in a sample tube with ethylenediaminetetraacetic acid (EDTA) as an anticoagulant. The haematological parameters such as Red blood cells (RBCs) and white blood cells (WBCs) were estimated with the use of Neubauer haemocytometer as described by Dacie and Lewis (2001). Haemoglobin (HB) was evaluated by cyanmethemoglobin procedure (Blax hall and Daisley 1973), while packed cell volume (PCV) was determined using the microhaematocrit method (Jain 2000). Erythrocyte indices: mean cell volume (MCV), mean cell haemoglobin (MCH) and mean cell haemoglo bin concentration (MCHC) were evaluated through Dacie and Lewis's (2001) formulae.

Growth: The specific growth rate was estimated using the formula (Pack, 1991):

$$r^3 = \frac{logWt_2 - logWt_1}{t_2 - t_1} \times 100$$

Statistical analysis: The data was analysed with SPSS version 20, using analysis of variance (ANOVA) and Duncan's Multiple Range Test (DMRT) to test for the different treatment levels and to separate means (Ducan, 1995). Test of significance was at 95% ($p < 0.05$).

Results

Phytochemical properties of Neem: The results of the qualitative analysis of *Azadirachta india (*neem leaf*)* is shown in Table 1 revealed the presence of saponin, tannin, phenolics, steroids, flavonoids, tripenoids, alkaloids, triterpenes, glycosides and coumarins in aqueous extract of *A. indica*

Table 1: Phytochemical properties of Neem leaf (*Azadiracta indica*)

Components	Occurrence
Saponin	+
Tannin	+
Phenolics	+
Steroids	_
Flavonoids	+
Tripenoids	+
Alkaloids	+
Triterpenes	+
Terpenoids	+
Glycosides	+
Coumarins	_

Present (+); Absent (-)

Haematological parameters: Table 2 shows the variations of haematological parameters in *Heteroclarias* during exposure to lethal concentrations of DCF for 96 h. Compared to control, exposure of *Heteroclarias* to varying concentrations of DCF revealed significant ($p < 0.05$) reduction in the levels of RBC, HB, PCV, MCV, MCH and MCHC with an increase in the concentration of DCF (Table 2). The group of fish exposed to the lowest concentration of diclofenac showed the highest levels of erythrocytes among the exposed groups, while those groups exposed to the highest concentration recorded the lowest levels of erythrocytes. In contrast, the leucocytes- WBC, LYM and PLT showed a significant ($p < 0.05$) concentration-dependent increase in DCF-exposed fish compared to control group (Table 2). The highest ($p < 0.05$) level of leucocytes was recorded in the highest concentration (16.00 mg/l), while the lowest level of leucocytes was exhibited in the lowest concentration (4.00 mg/l).

Table 2: Haematological parameters of *Heteroclarias* exposed to Diclofenac for 96 h

Conc. (mg/l)	WBC (x 10^3/µl)	RBC (x10^6/µl)	HB (g/dl)	PCV (%)	MCV (fL)	MCH (pg)	MCHC (g/dl)	PLT (x10^3/µl)	LYM (%)
0.00	120.90 ± 57.74^{a}	2.25 ± 0.58^{c}	8.80 ± 0.58^{c}	22.70 ± 0.58^{d}	109.20 ± 0.12^{e}	42.70 ±0.58^{e}	39.43 ± 0.33^{c}	125.10 ± 57.74^{a}	96.30 ± 0.58^{a}
4.00	197.00 ± 0.58^{b}	2.06 ± 0.58^{c}	8.00 ± 0.58^{c}	22.50 ± 0.58^{d}	103.60 ± 0.17^{c}	38.20 ± 0.58^{bc}	38.00 ± 0.58^{b}	312.20 ± 57.74^{b}	96.40 ± 0.58^{a}
8.00	198.30 ± 0.65^{c}	1.96 ± 0.58^{b}	7.30 ± 0.58^{b}	20.30 ± 0.58^{c}	101.10 ± 0.58^{b}	37.40 ± 0.58^{cd}	36.00 ± 0.58^{c}	383.00 ± 57.74^{c}	97.50 ± 0.58^{ab}
12.00	207.40 ± 57.74^{d}	1.90 ± 0.58^{b}	7.30 ± 0.58^{b}	19.20 ± 0.58^{b}	100.60 ± 0.25^{b}	35.60 ± 0.58^{b}	35.13 ± 0.64^{b}	384.10 ± 57.74^{c}	97.50 ± 0.58^{ab}
16.00	215.00 ± 0.58^{e}	0.70 ± 0.01^{a}	0.32 ± 0.13^{a}	0.70 ± 0.06^{a}	97.00 ± 0.01^{a}	32.00 ± 0.58^{a}	29.80 ± 0.25^{a}	717.10 ± 1.15^{d}	98.10 ± 0.33^{b}

Means (mean ± SE, n=3) with the same superscript in the same column are not significantly ($P < 0.05$) different

NB: RBC = Red blood cells; HB = Haemoglobin Concentration; PCV = haematocrit; MCV = Mean corpuscular volume; MCH = Mean corpuscular haemoglobin; MCHC = Mean corpuscular haemoglobin concentration; WBC = White blood cells; PLT = Platelet; LYM = Lymphocytes

Heteroclarias exposed to sub-lethal concentrations of DCF and fed on a diet with varying percentages inclusion of neem leaves for 15 days are shown in Table 3. There was a significant decrease ($p < 0.05$) in the levels of RBC, HB, PCV, MCV, MCH and MCHC in group B that was exposed to DCF and fed on diet with 0% neem leaf in comparison with the control group (Table 3). Treatment with diet supplemented with 1% neem leaf was able to significantly ($p < 0.05$) improve the erythrocytes levels in group C, but the DCF-induced reduction in the levels of erythrocytes in groups D, E & F were not significantly ($p < 0.05$) prevented from reduction (Table 3). The DCF-induced increase in the levels of the leucocytes WBC, LYM and PLT in group B was significantly ($p < 0.05$) reversed in group C that was fed on 1% neem leaf compared to normal control in group A. The rate of reverse in the levels of the leucocytes decreased as the percentage inclusion of the neem leaf in the diet increased from 2 to 4 % in groups D to F respectively (Table 3).

Table 3: Haematological parameters of *Heteroclarias* exposed to Diclofenac and ameliorated with neem leaves for 15 days

Group	WBC (x 10^3/µl)	RBC (x10^6/µl)	HB (g/dl)	PCV (%)	MCV (fL)	MCH (pg)	MCHC (g/dl)	PLT (x10^3/µl)	LYM (%)
A	166.60 ± 0.58^a	2.67 ± 0.58^b	9.30 ± 0.58^d	34.20 ± 0.58^c	102.60 ± 0.58^b	37.60 ± 0.58^c	36.80 ± 0.58^b	190.00 ± 0.58^a	3.40 ± 0.58^a
B	220.80 ± 0.58^f	1.96 ± 0.58^a	4.40 ± 0.58^a	17.80 ± 0.58^a	97.70 ± 0.58^a	29.90 ± 0.58^a	29.70 ± 0.58^a	851.00 ± 0.58^f	34.40 ± 0.58^e
C	199.27 ± 0.58^b	2.61 ± 0.58^b	9.10 ± 0.58^d	26.00 ± 0.58^b	103.40 ± 0.58^b	35.10 ± 0.58^b	35.00 ± 0.58^b	296.00 ± 0.58^b	6.40 ± 0.58^b
D	205.00 ± 0.58^c	2.33 ± 0.58^{ab}	8.00 ± 0.58^c	23.80 ± 0.58^{ab}	100.30 ± 0.58^b	34.60 ± 0.58^b	35.70 ± 0.58^b	425.00 ± 0.58^c	8.80 ± 0.58^c
E	208.60 ± 0.58^d	2.22 ± 0.58^{ab}	8.00 ± 0.58^c	21.40 ± 0.58^b	100.40 ± 0.58^b	34.50 ± 0.58^b	34.30 ± 0.58^{ab}	551.00 ± 0.58^d	9.60 ± 0.58^d
F	218.00 ± 0.58^e	2.10 ± 0.58^b	7.30 ± 0.58^b	21.10 ± 0.58^b	99.50 ± 0.58^a	34.80 ± 0.58^a	33.60 ± 0.58^b	726.00 ± 0.58^e	9.80 ± 0.58^d

Means (mean ± SE, n=3) with the same superscript in the same column are not significantly (P < 0.05) different
NB: RBC = Red blood cells; HB = Haemoglobin Concentration; PCV = haematocrit; MCV = Mean corpuscular volume; MCH = Mean corpuscular haemoglobin; MCHC = Mean corpuscular haemoglobin concentration; WBC = White blood cells; PLT = Platelet; LYM = Lymphocytes. N.B: A-Basal feed only; B- DCF without neem; C- DCF + 1% neem leaf; D- DCF + 2% neem leaf; E- DCF + 3% neem leaf and F- DCF + 4% neem leaf.

There was a progressive decrease and increase in the levels of erythrocytes (RBC, HB, PCV,) and leucocytes (WBC, LYM and PLT) in DCF-exposed *Heteroclarias* compared to control as the exposure period increased from 15 to 30 days (Table 4). The levels of erythrocytes in groups C, D, E, & F exposed to DCF and fed on diet supplemented with varying percentage inclusions (1%, 2%, 3% & 4%) of neem leaf respectively, are significantly (P < 0.05) lower than the control group A, but higher than those group B that was exposed to DCF only and fed on 0% neem leaf. While the leucocyte levels in group B were significantly (P < 0.05) higher than those in the control and ameliorative groups (Table 4). However, the levels of erythrocytes improved in groups D, E & F, with a significant (P < 0.05) improvement in group C that was fed on diet with 1% neem leaf inclusion. The higher values of leucocytes recorded in DCF-exposed fish were significantly reversed in group C that was fed on 1% neem leaf. Exposure to DCF only caused a significant (p < 0.05) reduction in the erythrocytes and increase in the leucocytes (Table 4). Treatment with diet supplemented with the lowest (1%) percentage of neem leaf was able to improve the reduced erythrocytes and reversed the increased leucocytes. While treatment with diets supplemented with the higher (2, 3 & 4%) percentages of neem leaf were not able to significantly (P < 0.05) reverse the DCF-induced alterations in the haematological parameters (Table 4).

Table 4: Haematological parameters of *Heteroclarias* exposed to Diclofenac and ameliorated with neem leaves for 30 days

Gro -up	WBC (x 10^3/µl)	RBC (x10^6/µl)	HB (g/dl)	PCV (%)	MCV (fL)	MCH (pg)``	MCHC (g/dl)	PLT (x10^3/µl)	LYM (%)
A	167.20 ± 0.58^a	2.61 ± 0.58^c	8.30 ± 0.58^d	27.40 ± 0.58^d	98.90 ± 0.58^d	34.00 ± 0.58^c	35.50 ± 0.58^d	81.00 ± 0.58^a	96.60 ± 0.58^a
B	236.30 ± 0.58^f	1.47 ± 0.58^a	3.20 ± 0.58^a	14.30 ± 0.58^a	60.30 ± 0.58^a	16.30 ± 0.58^a	18.50 ± 0.58^a	179.00 ± 0.58^e	97.90 ± 0.58^a
C	173.10 ± 0.58^b	2.44 ± 0.58^b	6.20 ± 0.58^c	25.70 ± 0.58^c	95.50 ± 0.58^c	27.90 ± 0.58^b	32.00 ± 0.58^c	83.00 ± 0.58^b	96.20 ± 0.58^a
D	216.90 ± 0.58^c	2.25 ± 0.58^b	5.80 ± 0.58^b	23.00 ± 0.58^b	93.30 ± 0.58^c	27.80 ± 0.58^b	27.60 ± 0.58^b	100.00 ± 0.58^c	97.30 ± 0.58^a
E	224.30 ± 0.58^d	2.13 ± 0.58^b	4.60 ± 0.58^{ab}	21.00 ± 0.58^b	87.40 ± 0.58^b	17.80 ± 0.58^a	19.00 ± 0.58^a	104.00 ± 0.58^c	97.30 ± 0.58^a
F	229.40 ± 0.58^e	2.09 ± 0.58^b	4.00 ± 0.58^{ab}	19.00 ± 0.58^b	88.50 ± 0.58^b	17.60 ± 0.58^a	17.90 ± 0.58^a	112.33 ± 0.58^d	97.70 ± 0.58^a

Means (mean ± SE, n=3) with the same superscript in the same column are not significantly (P < 0.05) different
NB: RBC = Red blood cells; HB = Haemoglobin Concentration; PCV = haematocrit; MCV = Mean corpuscular volume; MCH = Mean corpuscular haemoglobin; MCHC = Mean corpuscular haemoglobin concentration; WBC = White blood cells; PLT = Platelet; LYM = Lymphocytes
N.B: A-Basal feed only; B- DCF without neem; C- DCF + 1% neem leaf; D- DCF + 2% neem leaf; E- DCF + 3% neem leaf and F- DCF + 4% neem leaf.

Neem leaf diet ameliorates the growth reduction induced by DCF: Figure 1 revealed the percentage weight gain in *Heteroclarias* exposed to DCF and fed on varying neem leaves. The highest weight gain (14.05 g) was recorded in group A which was fed on normal diet (0% neem leaf) without toxicant, while the lowest weight gain (11.20 g) was recorded in group B exposed to DCF with 0% neem leaf. However, the growth rate decreased as the percentage inclusion of neem leaf in the fish diet increased (Figure 1). Among the DCF-exposed group, the highest percentage weight gain (93.86%) was recorded in group C that was exposed to DCF and fed on 1% neem leaf, while the lowest percentage weight gain was recorded in group B that was exposed to DCF and fed on 0% neem leaf. However, the percentage weight gain in groups D, E, & F were higher than that of group B, but not comparable with those in group C.
The specific growth rate of *Heteroclarias* exposed to DCF and ameliorated with neem leaf is shown in Figure 2. The highest specific growth rate (0.99) was recorded in group C, while the lowest specific growth rate (0.77) was revealed in groups B an F that were exposed to DCF and fed on 0% & 4% neem leaf respectively.

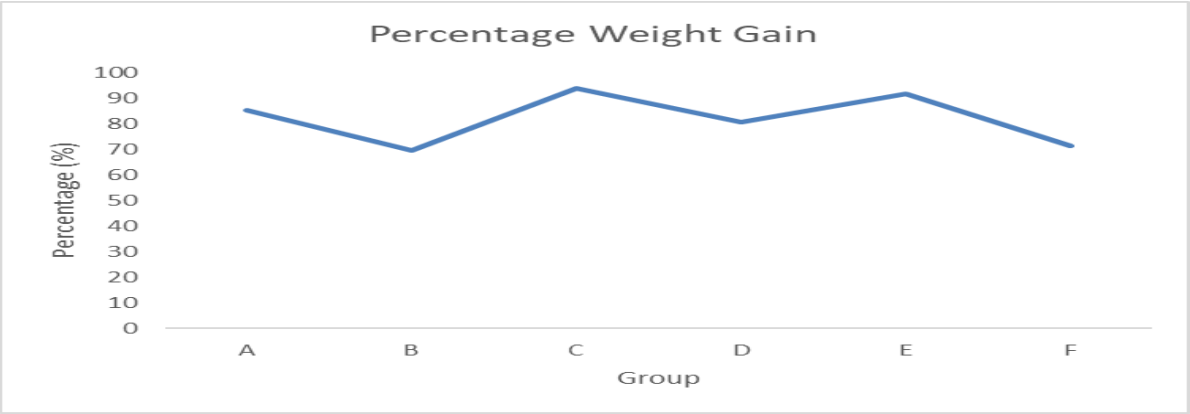

Figure 1: Percentage weight gain of *Heteroclarias* exposed to sub lethal concentration of DCF and ameliorative potentials of neem leaf for 30 days
N.B: A-Basal feed only; B- DCF without neem; C- DCF + 1% neem leaf; D- DCF + 2% neem leaf; E- DCF + 3% neem leaf and F- DCF + 4% neem leaf.

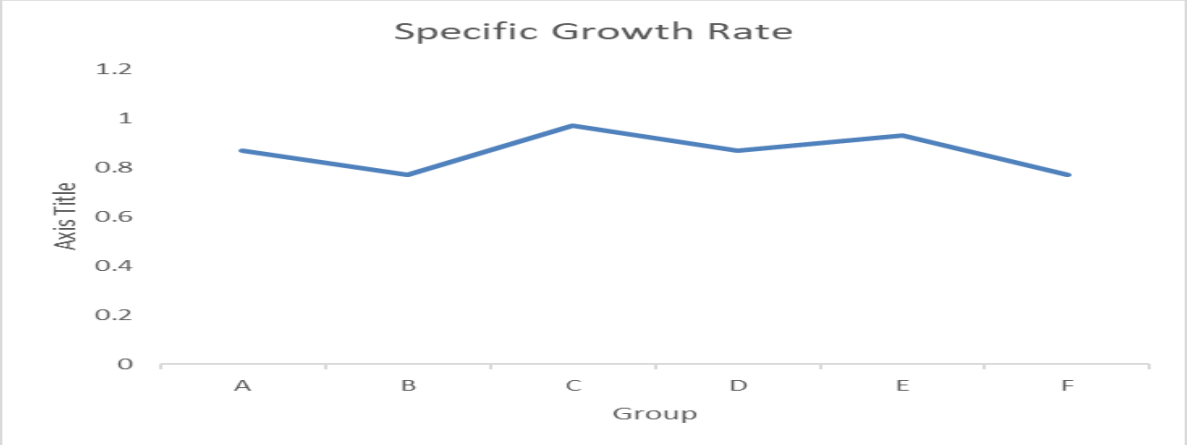

Figure 2: Specific growth rate of *Heteroclarias* exposed to sub lethal concentration of DCF and ameliorative potentials of neem leaf for 30 days
N.B: A-Basal feed only; B- DCF without neem; C- DCF + 1% neem leaf; D- DCF + 2% neem leaf; E- DCF + 3% neem leaf and F- DCF + 4% neem leaf.

Discussion

The observed decrease in red blood cell counts in the erythrocytes of diclofenac-exposed fish could be because of accumulation of the pharmaceuticals in the blood stream which cause damage to the blood cells and reductions in RBC could limit the oxygen supply to tissues. This is in conformity with the findings of Saravanan *et al.* (2011) and Ajima *et al.* (2014), who reported reduction in the RBC counts in *Cyprinus carpio* and *Clarias gariepinus,* exposed to diclofenac.
The reduction in haemoglobin observed in the erythrocytes of DCF-exposed fish could be a sign of anaemia due to the toxic effect of DCF, similar to what Saravanan *et al*. (2011) found in *Cyprinus carpio* exposed to 1 µg/L, 10 µg/L, and 100 µg/L of DCF over 96 h. This effect has also been documented with other toxic substances, like heavy metals (Lavanya *et al*., 2011). Alternatively, the decrease in haemoglobin levels may result from haemodilution, a process in which the organism dilutes its blood to lower the concentration of circulating toxins (Takasusuki *et al*., 2004). Haemodilution can impair oxygen transport to tissues, potentially causing temporary anaemia and reducing physical activity (Nussey *et al*., 1995). It may also be due to the destruction of red blood cells or a reduction in their production, inhibiting their capacity to transport oxygen, as observed by Dethloff *et al*. (2001). Similar haemoglobin reductions were reported in *Clarias gariepinus* and *Cyprinus carpio* exposed to diclofenac in studies by Saravanan *et al*. (2011) and Ajima *et al*. (2014) respectively.

The decrease in the RBC, HB and PCV may be attributed to decrease in the life span of RBC and suppression of the bone marrow stem cell activity. These findings concur with the previous observations of Abdulkareem & Owolabi, (2014) and Owolabi & Abdulkareem, (2021) who reported a significant increase in RBC, HB and PCV in *Heteroclarias* and *Clarias gariepinus* exposed to monocrotophos and atrazine respectively.

The low levels of mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) observed in the DCF-exposed fish could be an anaemic response that could be attributed to destruction or the inhibition of erythrocyte production, haemodilution and destruction of intestinal cells induced by the DCF (Aster, 2004). The excessive destruction of RBC and decrease in the level of HB which caused marked reduction in the packed cell volume (PCV), thereby inhibiting the PCV in the transport of oxygen and nutrients round the body, which eventually induced stress in the fish (Saravanan *et al*., 2011 and Isaac *et al*., 2013).

The increase in the WBC could be due to production of reactive oxygen species (ROS) to help in the recruitment of WBC as a response to attack the contaminant (DCF) in order to give more strength to the affected cells. The increased WBC may signal the fish's ability to combat damages and other immune challenges (Soetan *et al*., 2013). Increased platelets could also be due to the production of ROS that induced the production of platelets to the site of injury for cell repair. The increase in WBC count observed during the acute and chronic phases in this study is similar to the reports of Saravanan *et al*. (2011) and Ajima *et al*. (2014), where *Cyprinus carpio* and *Clarias gariepinus* showed significant increases in white blood cells after DCF exposure.

Increase in the level of platelets and lymphocytes indicated adaptive responses to repair injured gills and liver cells and a coping strategy to enhance the fish immune system in response to free radicals induced-stress by DCF, thus stimulating the immune defence system. This is in contrast with the reports of Akinrotimi *et al*. (2013) who reported decline in the level of lymphocytes of *Tilapia guineensis* exposed to industrial effluents.

However, the simultaneous treatment of DCF-exposed *Heteroclarias* with varying percentages of neem leaf inclusion in the fish diet was able to prevent the reduction in the levels of erythrocytes and increase in the leucocytes near to their respective levels. The lowest percentage (1%) inclusion in the diet of the DCF-exposed fish reversed the toxic effects of DCF and improved the haematological parameters of DCF-exposed *Heteroclarias.*

Reduction in the percentage growth rate and the specific growth rate in the DCF-exposed fish could be due to excessive use of glucose and protein needed for the development of new cells as energy during hyperactivity exhibited by the fish in an attempt to jump out of the DCF-contaminated water. This result is in accordance with the results of Nassef *et al.* (2010) and Praskova *et al.* (2014), who exposed zebrafish and Japanese medaka to DCF respectively. The highest percentage weight gain and specific growth rate recorded in the group fed on 1% neem leaf imply that 1% neem leaf incorporation in the fish diet is capable of neutralizing the toxic effect of DCF in *Heteroclarias.* The marked reduction in group F that was exposed to DCF and fed on the highest (4%) percentage neem leaf could be attributed to excessive ameliorative agents contained in the neem leaf, which could in turn destroy cellular haemoglobin and reduce oxygen carrying capacity that could results in stress and insufficient utilization of assimilated food.

Conclusion

The results from this study showed that DCF induced alterations in haematological parameters and inhibited growth in *Heteroclarias*, but 1% neem leaf inclusion in the diet was able to neutralize the toxic effects by improving the erythrocyte levels and reducing the levels of WBC. The neem leaf supplemented in the diet was able to restore the physiological disorder and elevate the growth rate of the DCF-exposed-fish. The use of neem leaf in the modulation of induced-stress in DCF-exposed fish could be deployed under the fish management strategy for sustainable development.

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