HEMATOLOGICAL ALTERATIONS IN THE AFRICAN CATFISH (*Clarias gariepinus*) JUVENILES EXPOSED TO SUB-CHRONIC CONCENTRATIONS OF DIAZEPAM.

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Abstract
A 4-week toxicological study was conducted to ascertain the effect of diazepam drug on haematology of Clarias gariepinus Juvenile. A total of 180 (27.36±0.23cm and 197.39±2.34g) juveniles were exposed to diazepam drug at sub chronic concentrations; 2.67mg/l, 5.34mg/l, 10.68mg/l, 21.36mg/l, 42.72mg/l as treatments, and a control without diazepam substance. Blood samples were collected from each treatment on weekly basis for haematological examination. Significant decreases in red blood cells (RBC), haemologlobin count (Hb) and pack cell volume (PCV) were observed in diazepam treated groups at continued duration exposure when compared with the control. A reverse trend was observed for white blood cells. Significant increases ($p<0.05$) in red cell indices were observed among diazepam treated groups compared to the control at continued duration exposure. Result for leucocyte differentials revealed that there were no significant differences ($p>0.05$) among diapezam treated group compared to the control for monocytes, basophils and Eosinophils while significant differences ($p<0.05$) in neutrophil and lymphocyte values were observed among treatments compared to the control. Diazepam adversely altered blood parameters of the fish.
Key words: Diazepam, Sub-chronic exposure, Haematology, *Clarias gariepinus*

Introduction

Global attention has shifted to pharmaceuticals (drugs) as a class of ecological contaminants due to its frequent use in human and animal medicaments (Halling-sorensen *et al.*, 1998; Daughton and Ternes, 1999; Daughton and Ruhoy, 2009). Pharmaceuticals are largely used in hospitals and other medical outfits to prevent and treat all kinds of diseases (Daughton and Ternes, 1999; Kolpin *et al.*, 2002). Indiscriminate and unregulated use and discharge of these drugs into the aquatic environment has raised consciousness on their adverse impact on the environment (Steenbergen and Richardson 2011). Several studies have reported the occurrence of pharmaceuticals in the aquatic environment (Sharma and Krishna-murti, 1968; Steenbergn and Richardson, 2011). The presence of these chemical substances in aquatic ecosystem could impair growth, metabolic activity and reproduction in aquatic flora and fauna (Romero *et al.*, 2012).The chemical molecules of these contaminants could adhere to materials in suspension and absorbed by aquatic flora and fauna. When bio-accumulated in the systems of aquatic plants and animals, these chemical substances could change their physiological and biochemical responses (Romero *et al.*, 2012).

Diazepam (benzodiazepine), over the years has certified and used in the area of human and veterinary medicine to treat and manage ailments like insomnia, seizures, anxiety disorders and muscle spasms. Diazepam has been recommended by researchers in the area of animal breeding and fish fry transport and handling (Saravanan *et al.*, 2008; Bencan *et al.*, 2009). Presence of diazepam has been reported in liver samples of honey head turbot (*Pleuronichthys verticalis*) from Southern California coastal waters at concentrations of 23 and 110 ngL^{-1} without its detection in sediments where the fish were collected (Maruya *et al.*, 2012).

Diazepam was detected in rivers and wastewater treatment plant influent at about $20ngL^{-1}$ but the highest amounts ($111ngL^{-1}$) were found in hospital effluent in Slovenia (Kosjek *et al.*, 2012). Diazepam has also been detected in concentrations up to $0.04ugL^{-1}$ in effluents from German treatment plants (Lowry *et al.*, 1951), while up to $2.13ngL^{-1}$ concentration has also be detected in the Po River in Italy (Calemari *et al.*, 2013). Diazepam has been detected in fish tissue, implying that it can bioaccumulate in the system of fish and chronic exposure may possibly interfere with physiology and well being of aquatic organisms (Kwon *et al.*, 2008).

In Nigeria, there are no strong measures to monitor and regulate waste disposal into the aquatic environment from hospitals and pharmaceutical companies. Aquatic flora and fauna are highly susceptible to contamination of their environment with pharmaceuticals which enter streams and rivers as runoffs from farms, industries and hospitals. The effect of diazepam on blood

parameters of fish has not been studied. Therefore, this study seeks to provide information in this light.

Materials and Methods

Experimental fish specimen and drug

Juveniles of African catfish (*C. gariepinus*), (mean standard length of 27.36±0.23cm and mean weight of 197.39±2.34g) were procured from Regima Fish Farms Ltd, in Abakaliki urban and transported to Fisheries Wet Laboratory of the Department of Fisheries and Aquaculture, Federal University Ndufu Alike Ikwo, Ebonyi State Nigeria, Fish were subsequently subjected to a 2-min bath with 0.05% potassium permanganate ($KMnO_4$) to prevent skin infections.

The fish were acclimatized for two weeks in two plastic tanks of 500L capacity each and fed *ad libitum* on a daily basis with commercial feed (Coppens International Helmond Netherlands) containing 35% crude protein. In order to sustain a good hygienic condition and to avoid pollution, fecal waste and other pollutants were siphoned off every day. Deceased fishes were removed with the help of plastic forceps to prevent further or possible deterioration of the water quality. For the duration of acclimatization, water in the tanks was renewed every day with well aerated tap water. The feeding was discontinued 24 h before the experimental trial to prevent interference of feces. For the present study, commercial formulations of Diazepam with trade name ''Valinex - 5'' (NAFDAC REG No: 04-0063.Manufactured by VITABIOTICS (NIG) LTD, Lagos Nigeria, under licence from VITABIOTICS LTD London, England) containing Diazepam B.P. 5mg as the active ingredients were used as the stock solution.

Experimental Design

Completely randomized design (CRD) was used for the experiment. One hundred and eighty (180) fish were randomly distributed into eighteen glass aquaria tanks (60x30x30cm) and each treatment was triplicated with 10 fish per tank. Fish were exposed to different sublethal concentrations of diazepam as treatments. The different concentrations were 2.67, 5.34, 10.68, 21.36, and 42.72mg.L^{-1} (Ogueji *et al*., 2017), and a control with no toxicant. The different diazepam concentrations were measured and introduced into experimental aquaria tanks containing 40 litres of dechlorinated tap water. The mixture was allowed to stand for 30 minutes before introducing the fishes to be tested. The physicochemical properties of the test water were monitored and analyzed daily using water sample kit (Pro-Lab, Florida) and the mean values were obtained as follows; temperature 29.04±0.05^oC, dissolved oxygen 5.64± 0.06mgl^{-1}, pH 6.07±0.02, total alkalinity 35.65±0.77mgL^{-1}, salinity 0.45±0.25mgL^{-1}, conductivity 235$mScm^{-1}$, and total hardness 17.54±0.68 mgL^{-1}.

Blood collection and Hematological analysis

Blood was collected on day 1, 2, 3 and 4 of exposure to diazepam .On each sampling day, five fish from each replicate experiment were anesthetized with MS 222 to minimize stress. Blood was obtained by cardiac puncture using a hypodermic heparinized syringe. The collected blood sample was transferred into small vials, which were also previously rinsed with heparin.

Every sampled fish was removed from the experimental system to avoid getting blood a second time from the same fish.RBC counts were estimated using a Neubauer hemocytometer (Rusia and Sood, 1992). Briefly, 0.02mL of blood was pipetted from the blood sample and added to 4mL of the RBC diluting fluid (Toisson's solution) in a clean test tube to make a 1:200 dilution of the blood sample. The diluted blood sample was loaded onto a Neubauer counting chamber, and all RBCs in the five groups of 16 small squares in the central area of the Neubauer chamber was counted using a light microscope at 40x objective.

The number of cells counted for each sample was multiplied by 10, 000 to obtain the RBC count per micro liter of blood. Hematocrit (PCV) was determined using the micro-hematocrit method in which the capillary tubes were filled with blood and centrifuged for 5 minutes at 14, 000x g using a micro-hematocrit centrifuge (Hawkesley & Sons, Ltd, Lancing, UK) at room temperature (Nelson and Morris, 1989). Soon after centrifuging, the hematocrit was read using the micro-hematocrit reader. The result was expressed as the percentage of whole blood. Hb determination was done using the cyanmethemoglobin method (Blaxhall and Daisley, 1973). Briefly, 0.02mL of blood was mixed with4mL of Drabkin's solution. This was allowed to stand for10 minutes for full color development to occur. Absorbance was read at 540 nm with a Unican spectrophotometer against the blank. For determination of leucocytes, 0.02mL of blood was pipetted into a small test tube containing 0.38mL of WBC diluting fluid (Turk's solution) to make a 1:20 dilution of the blood sample. The diluted sample was loaded on to the Neubauer counting chamber, and all cells on the four corner squares were counted using a light microscope at 10x objective. The total number of WBCs was calculated inmm^3 x 10^4. While counting, the numbers of different types of leucocytes (neutrophils, monocytes, lymphocytes, eosinophilsand basophils) in the blood smears were identified (Hibiya, 1982; Chinabut *et al*., 1991). The number of each type of leukocytes was calculated as a percentage. Erythrocyte indices,

such as MCHC, MCH and MCV, were calculated from the results of RBC count, WBC count, Hb and PCV according to standard formulae (Dacie and Lewis, 1984):

$$MCV(fl) = \frac{\text{PCV}(\%) \times 10}{\text{RBC countin millions/mm}^3}$$

$$MCH(pg) = \frac{\text{Hb}(\frac{\text{g}}{\text{dl}}) \times 10}{\text{RBC countin millions/mm}^3}$$

$$MCHC(\frac{g}{dl}) = \frac{\text{Hb}\ (\frac{\text{g}}{\text{dl}}) \times 100}{\text{PCV}(\%)}$$

Statistical Analysis

The data obtained were analyzed using statistical package (IBM SPSS version 20). The data were subjected to one way ANOVA and means were separated by Duncan's multiple range tests. Significant difference was declared at 5%.

Results

Results on haematological responses of fish specimens exposed to sub-lethal concentrations of diazepam at week intervals (week 1 -4) are presented on Table 1. Significant difference ($p<0.05$) was observed in red blood cell (RBC) values among treatments compared to the control. Significant reductions in RBC values were noticed among treatments on continued duration exposure when compared to the control. Lowest RBC value was seen on the fourth week (Table 1). Haemoglobin (Hb) reduced significantly ($p<0.05$) in all treatment groups over the time (from week 1-4) when compared to the control. Lower Hb values were seen in 42.72mg/l treatment on the first, second and fourth week while on the third week, lowest Hb values was observed in 21.36mg/l. However, there was both a concentration and time dependent significant decrease ($p<0.05$) in pack cell volume (PCV) values in the treatment groups compared to the control for the period the experiment lasted. 42.72mg/l had the lowest PCV value during the whole experimental period. Exposure of fish specimens to sub-lethal concentrations of diazepam significantly increased ($p<0.05$) white blood cell (WBC) when compared to the control group all through the experimental period. Higher WBC value was noticed in 42.72mg/l treatment on first, second and third week while 5.34mg/l had the highest WBC value on the fourth week.

A significant difference ($p<0.05$) in terms of mean cell volume (MCV) was observed among treated groups compared to the control. Significant increase in MCV values was noticed in 2.67mg/l treatment compared to other treatments and control throughout the experimental period. Mean cell haemoglobin (MCH) value increased significantly ($p<0.05$) in 2.67mg/l treatment on the first and second week while onward increment in MCH were seen in 5.34mg/l on the third week and 42.72mg/l on the fourth week. Result for Mean cell haemoglobin concentration (MCHC) revealed that there was significant difference ($p<0.05$) among treated groups with reference to duration exposure when compared to the control group. Highest MCHC value was seen in 2.67mg/l treatment on the first week. The control had the highest MCHC value on the second and third week while on the fourth week; MCHC value was found to be higher in 21.36mg/l and the control.

Effects of different sub-lethal concentrations of diazepam to WBC differentials on *Clarias gariepinus* are presented in Table 2. Significant increase in neutrophil value was observed in 21.36mg/l treatment on the first week. On the second and fourth week, neutrophil was found to higher in 42.72mg/l treatment while on the third week, the highest value was seen in 10.68mg/l treatment. Diazepam significantly increased lymphocyte value ($p<0.05$) in 2.67mg/l treatment on the first and third week while lymphocyte value was observed to be higher in 5.34mg/l treatment on the second week (Table 2).There was no significant difference ($p>0.05$) in lymphocyte when specimens were exposed to diazepam on the fourth week. Result for monocytes revealed that there was no significant difference ($p>0.05$) among treated groups compared to the control. This implies that diazepam applied at different sub-lethal concentration did not significantly increase or reduce monocytes throughout the experimental period. Significant decrease ($p<0.05$) in basophils was noticed in 21.36mg/l on the first week while onward exposure to diazepam showed no significant difference ($p>0.05$). Eosinophil was observed to be lower in 2.67mg/l and 21.36mg/l on the first week exposure to diazepam while further exposure (2^{nd} to 4^{th} week) showed no significant difference ($p>0.05$) in all the treated groups compared to the control.

Table 1: Sub-chronic effects of Diazepam on some hematological parameters of *C. gariepinus*

		Exposure duration (Weeks)			
Parameter	**Concentration (mg/L)**	**1**	**2**	**3**	**4**
RBC (*10^{12})	Control	10.05 ± 0.21^{a3}	11.33 ± 0.20^{a1}	10.54 ± 0.08^{a23}	10.80 ± 0.69^{a2}
	2.67	7.37 ± 0.06^{b1}	5.89 ± 0.28^{c2}	6.87 ± 0.25^{b1}	5.76 ± 0.36^{b2}
	5.34	7.74 ± 0.32^{b1}	6.52 ± 0.08^{b2}	6.28 ± 0.06^{c2}	5.18 ± 0.00^{c3}
	10.68	7.93 ± 0.43^{b1}	6.41 ± 0.58^{bc2}	6.39 ± 0.01^{c2}	5.39 ± 0.01^{bc3}
	21.36	7.34 ± 0.04^{b1}	6.04 ± 0.25^{bc23}	6.24 ± 0.38^{c2}	5.64 ± 0.12^{bc3}
	42.72	7.15 ± 0.31^{b1}	6.54 ± 0.04^{b2}	5.99 ± 0.81^{c2}	4.54 ± 0.08^{d3}
Hb (g/dL)	Control	11.30 ± 0.06^{a3}	11.80 ± 0.12^{a2}	12.27 ± 0.15^{a1}	11.73 ± 0.18^{a2}
	2.67	10.50 ± 0.12^{b1}	7.20 ± 0.06^{c3}	7.80 ± 0.06^{b2}	6.60 ± 0.12^{bc4}
	5.34	9.30 ± 0.06^{c1}	7.50 ± 0.12^{b3}	7.87 ± 0.03^{b2}	6.07 ± 0.03^{cd4}
	10.68	8.10 ± 0.06^{d1}	6.93 ± 0.09^{c2}	6.77 ± 0.03^{c23}	6.10 ± 0.46^{cd3}
	21.36	7.87 ± 0.32^{d1}	6.60 ± 0.12^{d2}	6.03 ± 0.03^{d3}	6.80 ± 0.00^{b2}
	42.72	7.57 ± 0.26^{d1}	6.03 ± 0.03^{e2}	6.27 ± 0.27^{d2}	5.73 ± 0.03^{d2}
PCV (%)	Control	32.00 ± 1.16^{a1}	33.33 ± 0.33^{a1}	31.67 ± 0.33^{a1}	31.67 ± 0.88^{a1}
	2.67	26.33 ± 0.88^{b1}	25.67 ± 0.33^{b1}	25.33 ± 0.33^{b1}	25.00 ± 0.58^{b1}
	5.34	25.00 ± 0.58^{bc1}	22.67 ± 0.33^{c2}	21.33 ± 0.33^{c3}	20.00 ± 0.00^{c4}
	10.68	24.00 ± 0.58^{c1}	19.67 ± 0.33^{d2}	20.67 ± 0.33^{c2}	20.00 ± 0.00^{c2}
	21.36	23.67 ± 0.33^{c1}	20.67 ± 0.33^{d2}	19.33 ± 0.33^{d3}	18.00 ± 0.00^{d4}
	42.72	22.67 ± 0.33^{c1}	18.33 ± 0.33^{c2}	17.67 ± 0.33^{e2}	17.00 ± 0.00^{d2}
WBC(*10^{9})	Control	9.45 ± 0.03^{c2}	9.15 ± 0.38^{d2}	9.85 ± 0.03^{c12}	10.55 ± 0.09^{c1}
	2.67	11.35 ± 0.26^{a1}	12.10 ± 0.17^{ab1}	12.03 ± 0.32^{a1}	11.85 ± 0.03^{ab1}
	5.34	11.30 ± 0.06^{a2}	11.55 ± 0.03^{bc2}	12.15 ± 0.38^{a1}	12.20 ± 0.17^{a1}
	10.68	11.55 ± 0.29^{a1}	11.50 ± 0.23^{bc1}	11.80 ± 0.26^{ab1}	11.80 ± 0.23^{ab1}
	21.36	10.75 ± 0.20^{b1}	10.85 ± 0.43^{c1}	11.20 ± 0.28^{b1}	11.55 ± 0.20^{b1}
	42.72	11.40 ± 0.06^{a3}	12.50 ± 0.5^{a1}	11.30 ± 0.57^{a1}	11.70 ± 0.58^{b2}
MCV(fL/cell)	Control	31.82 ± 0.47^{b1}	29.45 ± 0.79^{c2}	30.03 ± 0.17^{c12}	29.33 ± 1.00^{c2}
	2.67	35.72 ± 0.89^{a2}	43.75 ± 2.21^{a1}	36.99 ± 1.81^{a2}	43.62 ± 1.72^{a1}
	5.34	32.36 ± 0.59^{b3}	34.76 ± 0.26^{b2}	32.32 ± 0.82^{b23}	38.59 ± 0.02^{b1}
	10.68	30.67 ± 0.30^{b2}	30.68 ± 0.59^{bc2}	30.97 ± 0.49^{bc2}	37.08 ± 0.06^{b1}
	21.36	32.26 ± 0.98^{b1}	34.37 ± 1.91^{b1}	29.50 ± 0.70^{c1}	31.92 ± 0.07^{c1}
	42.72	31.79 ± 0.81^{b2}	28.02 ± 0.37^{c3}	40.44 ± 0.49^{c3}	37.39 ± 0.63^{b1}
MCH(pg/cell)	Control	11.25 ± 0.18^{bc12}	10.42 ± 0.08^{c3}	11.63 ± 0.05^{b1}	10.87 ± 0.23^{b23}
	2.67	14.25 ± 0.03^{a1}	12.26 ± 0.49^{b2}	11.38 ± 0.50^{b2}	11.52 ± 0.52^{ab2}
	5.34	12.06 ± 0.43^{b12}	11.50 ± 0.03^{a1}	12.53 ± 0.13^{a1}	11.70 ± 0.07^{ab2}
	10.68	10.28 ± 0.64^{c1}	10.82 ± 0.05^{c1}	10.58 ± 0.05^{c1}	11.31 ± 0.84^{ab1}
	21.36	10.73 ± 0.49^{c2}	10.95 ± 0.12^{bc2}	9.66 ± 0.12^{d3}	12.05 ± 0.02^{ab1}
	42.72	10.59 ± 0.09^{c2}	9.22 ± 0.23^{d3}	10.45 ± 0.23^{c2}	17.63 ± 0.23^{a1}
MCHC(g/dL)	Control	35.39 ± 1.09^{b2}	35.41 ± 0.67^{a2}	38.74 ± 0.18^{a1}	37.08 ± 0.48^{b12}
	2.67	39.93 ± 0.89^{a1}	28.06 ± 0.43^{c3}	30.79 ± 0.24^{c2}	26.41 ± 0.15^{d3}
	5.34	37.23 ± 0.63^{ab1}	33.091 ± 0.26^{b2}	36.89 ± 0.51^{ab1}	30.33 ± 0.17^{c3}
	10.68	33.80 ± 1.05^{bc12}	35.27 ± 0.68^{a1}	32.76 ± 0.63^{c12}	30.50 ± 2.31^{c2}
	21.36	33.29 ± 1.77^{c2}	31.97 ± 1.05^{a1}	31.22 ± 0.36^{c2}	37.78 ± 0.00^{a1}
	42.72	33.67 ± 0.74^{c1}	32.93 ± 0.69^{b2}	35.47 ± 1.29^{b1}	33.80 ± 1.15^{bc1}

Values with different alphabetic superscripts (**a,b,c…**) differ significantly ($p < 0.05$) between concentrations within the same duration. Values with different numeric (**1, 2, 3 …**) superscripts differ significantly ($p < 0.05$) between durations within the same concentrations. Results are expressed as mean ± SE of the mean (N=30).

Table 2: Percentage change (mean ± SE) in the leucocyte differentials in juvenile *Clarias gariepinus* after exposure to sub-chronic concentrations of Diazepam

		Exposure duration (Weeks)			
Parameter	**Conc. (mg/L)**	**1**	**2**	**3**	**4**
Neutrophils	Control	18.00± 1.16^{b2}	19.00±0.58^{bc2}	19.00±1.73^{bc2}	25.67± 2.60^{a1}
	2.67	15.00± 2.89^{b2}	22.67 ± 1.45^{b1}	14.00 ± 2.31^{d2}	22.00± 1.16^{a1}
	5.34	16.00± 1.16^{b2}	17.00± 0.58^{c12}	22.00 ± 1.73^{b1}	20.00±2.31^{a12}
	10.68	17.00± 0.58^{b3}	18.33 ± 0.88^{c3}	29.00 ± 0.58^{a1}	21.00± 0.58^{a2}
	21.36	32.00± 0.16^{a1}	20.00±2.31^{bc23}	18.00 ± 1.16^{c3}	25.00± 2.89^{a2}
	42.72	17.00± 1.16^{b2}	29.00 ± 0.58^{a1}	25.00± 2.89^{ab1}	26.67± 2.03^{a1}
Lymphocytes	Control	78.00±1.16^{b12}	78.33±1.45^{ab1}	79.67±1.45^{ab1}	72.00± 2.89^{a2}
	2.67	84.00± 2.31^{a1}	76.00 ± 0.16^{b2}	83.00 ±1.73^{a1}	77.33 ±1.45^{a2}
	5.34	79.00±0.58^{b12}	81.67 ±0.33^{a1}	77.00 ±1.73^{b2}	78.00±1.16^{a12}
	10.68	80.67±0.33^{ab1}	79.33±0.58^{ab1}	69.00 ± 0.58^{c3}	76.00± 0.58^{a2}
	21.36	67.67 ±1.45^{c2}	77.00±2.89^{ab1}	76.33 ±0.88^{b1}	74.00±2.31^{a12}
	42.72	79.33± 0.33^{b1}	70.33±0.33^{c2}	74.33±3.18^{bc12}	11.67±2.03^{a12}
Monocytes	Control	1.67 ± 0.33^{a1}	1.33 ± 0.33^{a1}	0.33 ± 0.33^{a2}	1.33 ± 0.33^{a1}
	2.67	0.33 ± 0.33^{a1}	1.33 ±0.33^{a1}	1.00 ± 0.58^{a1}	0.33 ± 0.33^{a1}
	5.34	2.00 ± 0.58^{a1}	0.33 ±0.33^{ab1}	0.67 ± 0.33^{a1}	0.67 ± 0.67^{a1}
	10.68	1.33 ± 0.33^{a1}	1.33 ±0.33^{a1}	0.33 ± 0.33^{a1}	1.67 ± 0.67^{a1}
	21.36	0.67± 0.33^{a1}	1.33 ± 0.33^{a1}	1.33 ± 0.88^{a1}	0.33 ± 0.33^{a1}
	42.72	1.33 ± 0.88^{a1}	1.00 ±0.00^{ab1}	0.33 ± 0.33^{a1}	1.00 ± 0.58^{a1}
Basophils	Control	0.67±0.33^{ab1}	0.33 ± 0.33^{a1}	0.33 ±0.33^{a1}	0.67 ± 0.33^{a1}
	2.67	0.33 ±0.33^{ab1}	0.33 ±0.00^{a1}	0.67 ±0.33^{a1}	0.33 ±0.33^{a1}
	5.34	1.00 ±0.00^{a1}	0.33 ±0.33^{a1}	0.61 ±0.00^{a1}	0.63 ±0.00^{a1}
	10.68	0.67 ±0.33^{ab1}	0.33 ±0.33^{a1}	0.67 ±0.00^{a1}	0.59±0.00^{a1}
	21.36	1.00 ±0.00^{a1}	0.33 ±0.33^{a1}	1.33±0.88^{a1}	0.65±0.00^{a1}
	42.72	0.33 ± 0.33^{ab1}	0.00 ±0.00^{a2}	0.33 ±0.33^{a1}	0.33 ±0.33^{a1}
Eosinophils	Control	1.67 ±0.33^{a1}	0.33 ±0.33^{a2}	0.33 ±0.33^{ab2}	0.33 ±0.33^{a2}
	2.67	1.00 ±0.00^{ab1}	1.00 ±0.00^{a2}	0.67 ±0.33^{ab1}	0.00 ±0.00^{a2}
	5.34	1.33 ±0.33^{ab1}	0.33 ±0.33^{a2}	1.00 ±0.00^{ab2}	0.33 ±0.33^{a2}
	10.68	1.17 ±0.33^{ab1}	0.33 ±0.33^{a1}	1.33 ±0.33^{a1}	0.67 ±0.67^{a1}
	21.36	1.00 ±0.00^{ab1}	0.67 ±0.33^{a12}	1.33 ±0.33^{a1}	0.33 ±0.33^{a2}
	42.72	1.33 ±0.33^{ab1}	0.33 ±0.33^{a2}	0.33 ±0.33^{ab2}	0.00 ±0.00^{a2}

Values with different alphabetic superscripts (**a,b,c…**) differ significantly ($p < 0.05$) between concentrations within the same duration. Values with different numeric (**1, 2, 3 …)** superscripts differ significantly ($p < 0.05$) between durations within the same concentrations. Results are expressed as mean ± SE of the mean (N=30).

Discussion

Haematological characteristics are essential tools that are used as indicators for monitoring physiological status and changes in fish (Erhunmunse and Ainerua, 2013). Haematological studies have provided reliable information on health status, metabolic disorders and chronic stress status before and after clinic examination of specimens (Bahmani *et al*., 2001). The findings of the current study revealed that the exposure of *Clarias gariepinus* to diazepam can evoke toxicological repercussion such as disruption of normal physiological function of the fish. The

continued decrease in RBC values in *C. gariepinus* treated with diazepam indicates an impairment of erythropoietic process (Table 1). Velisek *et al.*, (2011) reported a significant reduction in RBCs when rainbow trout (*Oncorhynchus mykiss*) were exposed to verapamil. Significant reductions in PCV and Hb values were also observed across diazepam treated fish specimens when compared to the control. The reduction in PCV values after duration exposure to diazepam might be as a result of significant decrease in hematopoietic activity. Thus, the decrease in Hb values could be as a result of the adverse effect on Hb biosynthesis. Nwani *et al.,* (2013) opined that Hb biosynthesis when adversely altered, could limit the oxygen-carrying capacity of the fish blood. The respective significant reductions in blood parameters indicate immune suppression induced by diazepam. This is evident following the alterations in the values of the examined blood parameters of the treated fish groups when compared to the control (Table 1). Influx of pharmaceutical drugs and their metabolites into the aquatic environment can have negative impact on aquatic organisms (Ghelfi *et al*., 2015). Ajima *et al.,* (2016) stated that *O. niloticus* juvenile exposed to varying concentrations of verapamil significantly reduced RBC, Hb and PCV values.

WBCs have been reported to be involved in immune function regulation in many organisms (Nwani *et al.,* 2013). A significant increase in WBC values is as a result of the corresponding increase in duration exposure to diazepam was seen among treated fish specimens compared to the control. The increase of WBC values in the blood of the diazepam-treated fish infers an immune system response to the toxic effect of diazepam. Significant increase in WBCs was observed in *Cirrhinus mrigala* after exposure to various concentrations of ibuprofen drug (Saravanan *et al.*, 2012). Similar result has been reported (Ajima *et al.,* 2016) when *O. niloticus* were exposed to verapamil. Red cell indices (MCV, MCH and MCHC) are important for the diagnosis of anemia in most animals including fish (Cole, 1986). Significant increase or decrease in red cell indices may indicate macrocytic or microcytic anemia (Dacie and Lewis, 2011; Iheanacho *et al.,* 2017). Continued exposure of fish specimens to diazepam significantly increased ($p<0.05$) MCV, MCH and MCHC although in mixed trend (Table 1). Increase in red cell indices (MCV, MCH and MCHC) as observed in the current study implies possible macrocytic anemia. An increase in MCV, MCH, HcT and Hb values when *Oncorhynchus mykiss* was exposed to pharmaceutical drug verapamil (Cole, 1986). Significant increase was observed in MCV and MCH values when Indian major carp (*Cirrhinus mrigala*) were exposed to Ibuprofen drug (Saravanan *et al*., 2012).

Alteration in WBC differential count is an insightful indicator of environmental stress (Cole *et al*., 2001). Significant increases ($p<0.05$) in neutrophil and lymphocytes contents were seen in diazepam treated fish above the control expect in the fourth week (Table 2). Similar finding was observed when *C. gariepinus* were exposed to chlorinfenicol drug (Nwani *et al.,* 2013). Significant increase in neutrophil values was observed when Zebra fish (*Danio* rerio) larva were exposed to oxytetracycline (Barros-Beeker *et al.,* 2012). Diazepam did not significantly ($p>0.05$) affect the monocytes, eosinophils and basophils in the study. Previous studies have reported no significant differences in these WBC differentials when different fish species were exposed to various concentrations of pharmaceutical drugs (Nelson and Morris, 1989; Mahammad *et al.,* 2012; Roy and Nath, 2012).

Conclusion

Pharmaceutical drugs pose serious danger to aquatic biota and habitats as revealed in this study. The findings of this study revealed that diazepam drug administered at various sub-chronic concentrations altered hematological indices in *Clarias gariepinus* juvenile.

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