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Toxicity of diazepam on lipid peroxidation, biochemical and oxidative stress indicators on liver and gill tissues of African catfish *Clarias gariepinus* (Burchell, 1822)

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Abstract
The present study assessed the toxic effects of Diazepam (Valinex - 5) on liver and gill tissues of *Clarias gariepinus*. Fish were exposed to sublethal concentrations of 2.67, 5.34, 10.68, 21.36, and 42.72mg.L^{-1} for 28days. Liver and gill tissues were sampled on day 7, 14, 21 and 28. Results showed that lipid peroxidation (LPO), superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR) and glutathione peroxidase (GPx) in both tissues was significantly (p<0.05) elevated in the treated groups when compared to control. LPO, SOD, CAT and GPx showed tissue specific response to diazepam as their activities were more pronounced in the gill than the liver during the experimental run. Diazepam elicited in both tissues significantly (p<0.05) decreases in glucose and protein. Changes in the antioxidant enzyme activities indicated that Diazepam caused oxidative damage in the tissues. The formation of oxygen radicals might be a factor in the toxicity of Diazepam

Keywords: Diazepam; sub-lethal toxicity, oxidative-stress, biochemistry, *Clarias gariepinus*

1. Introduction
Diazepam, also known as Valium is a benzodiazepine, often prescribed by physicians in the management of anxiety, insomnia, seizures, alcohol withdrawal symptoms or muscle spasms. It is also used in conjunction with other medications to provide sedation before medical procedures. In animals, several authors recommended using diazepam in animal breeding, fish fry transport, and in veterinary practice [1, 2]. Psychiatric medicines and antidepressants are widely distributed around the world because of their frequent use in drug administration. Pharmaceuticals are gaining global attention as a class of environmental contaminants because of the massive use of these chemicals in human and animal care [3, 4, 5]. These types of pharmaceuticals are of great concern since their occurrence in the aquatic environment has been reported in various studies [20, 23]. They are continuously discharged into the aquatic environment and have been detected in rivers and streams [6]. Once discharged into the aquatic ecosystem, the molecules of these pollutants could adhere to materials in suspension, accumulate in the sediment or may be absorbed by aquatic flora and fauna. This could change their physiological responses including behaviour, morphological, enzymological, biochemical and antioxidant responses [7].
Aquatic ecosystems are probably the most affected by indiscriminate discharge of these drugs into the environment. Diazepam has been detected in concentrations up to 0.04ugL^{-1} in effluents from German treatment plants [57], while up to 2.13ngL^{-1} concentration has also be detected in the Po River in Italy [8]. In other parts of the world such as Slovenia, 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 [9]. Additional reasons for concern are associated with the detection of this drug in fish tissue [10]. 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} even when concentrations in sediments where the fish were collected was not detected [11]. This infers that diazepam can bioaccumulate, and chronic exposure could produce an effect on aquatic organisms even where concentrations in the environment are very low. 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. Under normal natural conditions in the ecosystem, animals maintain generation and neutralization of reactive oxygen species (ROS) [12], but when exposed to pollutants including diazepam, higher levels of free radicals such as superoxide (O2-), hydroxyl radicals (OH-) and hydrogen peroxide (H_2O_2) are generated [11]. Free radical generators and their biotransformation (xenobiotics) may lead to increased production of ROS which are highly toxic and may trigger oxidative and DNA damage in fish [13].

Reactive oxygen species at excess level was observed to react with biological macromolecules to increase the level of lipid peroxidation (thiobarbituric acid reactive substances, TBARS) [14], protein denaturation and changes in the activities of antioxidant enzymes including catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx) [15]. It may also affect the levels of other biochemical parameters such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), protein and glucose metabolism [14].

Studies have shown that diazepam can bind to GABA receptors also in fish brains and can affect behavioral responses [16, 17]. Anxiolytic effects of diazepam have been reported in various species of fish. For example in zebra fish, it was reported as reduced novel tank diving response [2], suppressed preference for the edge of experimental tank [20] and reduced typical dark avoidance behavior during light/dark preference test [21]. Reduction in schooling behavior has also been observed in medaka exposed to diazepam [17].

Literature on toxicological effects of diazepam on freshwater fishes is scanty. In Nigeria, no research to the best of our knowledge has been published on the effects of diazepam on freshwater fishes in the aquatic ecosystem. In this study, we set out to determine the toxicity of diazepam on lipid preoxidation, biochemical and oxidative stress indicators in liver and gill tissues of the African catfish *Clarias gariepinus*.

2. Materials and methods

2.1 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 (KMnO4) 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 devastate materials 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 and eminence. 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 run 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.

2.1.2 Experimental Design

Completely randomized design (CRD) was used for the experiment. One hundred and eighty fish were 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}, 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. One fish from each replicate (in both the treatment groups and control) was sacrificed after anesthetizing with tricaine methane sulfonate (MS-222) to minimize stress. This was carried out on days 7, 14, 21, and 28 during exposure period. Biochemical study was undertaken [23, 24] . The fish were dissected and liver and gill tissues were removed and washed in 0.9% sodium chloride (NaCl) solution, and blended in pre-chilled potassium phosphate buffer (1: 10 W/V,0.1 M, pH 7.0). One part of the homogenate was used for the estimation of thiobarbituric acid reactive substances (TBARS) while the other part was centrifuged for 20 minutes at 10,500 rpm under 4 ºC to obtain the supernatant which was stored at 4 ºC for enzyme assay. For each of the parameters, five determinations were made and the average recorded as mean±SE.

The physicochemical properties of the test water were monitored and analyzed daily [58], and the mean values were obtained as follows; temperature 27.02±0.07 ºC, dissolved oxygen 4.82± 0.03mgl^{-1}, pH 7.05±0.01, total alkalinity 32.75±0.77mgL^{-1}, salinity 0.31±0.25mgL^{-1}, conductivity 235mScm^{-1}, and total hardness 18.96±0.75 mgL^{-1}.

2.1.3 Estimation of lipid Peroxidation

Tissue lipid peroxidation (LPO) was determined by the estimation of thiobarbituric acid reactive substances (TBARS) [24]. The TBARS concentration was measured by the absorption at 535 nm at molar extinction coefficient of 156 mM/cm. Nanomoles was used as the unit of expression for specific activity of TBARS/mg protein.

2.1.4 Assessment of antioxidant enzymes and tissue biochemistry

Determination of glutathione peroxidase (GPx) activity was by using the rate of NADPH oxidation at 340 nm in coupled reaction with glutathione reductase. Extinction coefficient 6.22mMcm^{-1} was used to determine the specific activity [25]. The values obtained were expressed in unit/min/mg protein. Spectrophotometer was employed to determine tissue Catalase (CAT) activities by measuring the rate of H_2O_2 breakdown following a decrease in absorbance at 240 nm [26] and the unit of expression was in U/mg protein. Superoxide dismutase (SOD) activity was assayed at 420 nm [27]. The assay is derived from the oxidation of epinephrine-adrenochrome transition mediated by the enzyme and this is expressed in U/mg protein. Using bovine serum as a standard, spectrophotometer was used to estimate total protein in the tissues [59], while glucose levels were also estimated [28].

2.1.5 Data 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%.

3. Results

3.1 Effects of on Diazepam exposure lipid peroxidation and antioxidant enzyme activities

The effects of different chronic concentrations of Diazepam on lipid peroxidation in the form of TBARS formation and the activities of other antioxidant enzymes viz SOD, CAT, GR and GPx in liver and gill tissues of *C. gariepinus* are presented in Table 1 and 2. The LPO in both tissues was significantly (p<0.05) elevated in the treated group when compared to control. In the exposed fish, the elevation in LPO was more pronounced in the gill tissue than in the liver. The LPO inductions in the gill tissue were also duration dependent, but in the liver, it showed mixed trend except in 21.36mgL^{-1} concentration. In the highest concentration of 42.72mgL^{-1}Diazepam TBARS formation in the liver of treated fish, elevated by 4.71% and 46.74% in day 7 and 28 respectively, whereas in the gill, the percentage increases were 8.35% and 58.18% in similar time periods. The SOD in the gill and liver of treated *C. gariepinus* to Diazepam were significantly (p<0.05) increased when compared to control. The elevation was dose dependent in the gill tissue from day 14 – 28. As the experiment progressed over time, Diazepam elicited SOD increases with a mixed trend in both tissues. In the highest concentration of 42.72mgL^{-1} Diazepam induced in the liver, percentage SOD elevations of 5.01% and 28.38% in day 7 and 28 respectively, while in the gill, the percentage increases were 17.24% and 55.68% in similar durations. Equally the activity of SOD was more pronounced in gill than liver.

The CAT in the liver of treated *C. gariepinus* to Diazepam were significantly (p<0.05) increased in dose and duration dependent pattern in liver, but in gill tissue, CAT activity in the concentration of 2.67mgL^{-1}was comparable to control in all durations of exposure. In day 7 and 21, all treated group's elicited comparable CAT activity with control. However, in day14 and 28 there were significant (p<0.05) decreases in the groups treated to 5.34-42.72mgL^{-1}concentrations. In the gill tissue, the response was biphasic in two concentrations (5.34mgL^{-1}and 10.68mgL^{-1}). CAT activity in the fish exposed to 5.34mgL^{-1}concentration showed an increase of 4.88% in day 14 from day 7 (0.82 ± 0.04 - 0.86 ± 0.02), 5.81% reduction in day 21 from day 14 (0.86 ± 0.02 - 0.81 ± 0.04) and finally 9.88% increase in day 28 from day 21(0.81 ± 0.04 - 0.89 ± 0.00). The GR in both tissues was significantly (p<0.05) elevated in the treated group from day 14 – 28 in liver and day 7 – 28 in gill when compared to control. Looking at the highest concentration of 42.72mgL^{-1}, Diazepam induced in the liver, percentage GR elevations of 4.97% and 46.03% in day 7 and 28 respectively, while in the gill, the percentage increases were 10.09% and 40.02% in similar durations. In the liver of treated fish to Diazepam, GPx increased in day 7, but from day 14 – 28 it decreased when compared to control. However, in the gill tissue of exposed fish to the test drug, there were significant (p<0.05) induction of GPx activity

Table 1: Activity of lipid peroxidation (TBARS, nmol TBARS mg protein^{-1}), and superoxide dismutase (SOD, U mg protein^{-1}) in the liver and gill tissues of *C. gariepinus* exposed to sub-chronic concentrations (2.67, 5.34, 10.68, 21.36, 42.72mgL^{-1}) of Diazepam.

		Exposure duration (Days)				
Parameter	**Tissues**	**Concentration (mg/L)**	**7**	**14**	**21**	**28**
LPO	Liver	Control	6.16 ± 0.01^{c1}	6.14 ± 0.33^{c1}	6.03 ± 0.01^{c1}	5.82 ± 0.32^{c1}
		2.67	6.32 ± 0.02^{bc2}	6.60 ± 0.01^{b12}	7.14±0.10^{a1}	6.16 ± 0.45^{c2}
		5.34	6.66 ± 0.16^{a12}	7.17 ± 0.14^{a1}	6.56± 0.22^{b2}	6.41 ± 0.16^{c2}
		10.68	6.37 ± 0.02^{bc2}	6.32 ± 0.09^{bc2}	7.19 ±0.13^{a1}	6.36 ± 0.01^{c2}
		21.36	6.26 ± 0.01^{bc2}	6.56 ± 0.21^{b2}	6.63 ± 0.13^{b2}	7.34 ± 0.03^{b1}
		42.72	6.45 ± 0.02^{b3}	6.39 ± 0.01^{bc3}	7.17 ± 0.01^{a2}	8.54 ± 0.09^{a1}
	Gill	Control	7.78 ±0.34^{b1}	7.78 ± 0.18^{c1}	7.57 ± 0.03^{d1}	7.48 ± 0.04^{d1}
		2.67	8.70 ±0.14^{a1}	8.73 ± 0.07^{b1}	8.79 ± 0.27^{c1}	8.03 ± 0.35^{c1}
		5.34	7.93 ± 0.42^{ab2}	9.08 ± 0.08^{a1}	9.25 ± 0.08^{b1}	9.48 ± 0.12^{b1}
		10.68	7.94 ± 0.15^{ab4}	8.49 ± 0.09^{b3}	9.40 ±0.01^{ab2}	9.89 ± 0.02^{ab1}
		21.36	8.66 ± 0.16^{a2}	9.06 ± 0.07^{a23}	9.27 ± 0.09^{ab2}	9.93 ± 0.28^{ab1}
		42.72	8.43 ± 0.38^{ab4}	9.29 ± 0.06^{a3}	9.65 ± 0.01^{a2}	10.25 ± 0.07^{a1}
SOD	Liver	Control	17.95 ± 0.25^{b1}	17.36 ± 0.01^{d1}	17.69 ± 0.15^{f1}	17.21 ± 0.45^{b1}
		2.67	18.65 ± 0.01^{ab3}	19.66 ± 0.12^{c2}	19.81 ± 0.08^{e2}	20.29 ± 0.72^{a1}
		5.34	18.17 ± 0.13^{ab3}	19.82 ± 0.08^{c2}	20.15 ± 0.02^{d12}	20.41 ± 0.15^{a1}
		10.68	18.84 ± 0.37^{a2}	20.59 ± 0.15^{b1}	20.80 ± 0.04^{c1}	20.79 ± 0.03^{a1}
		21.36	18.89 ± 0.29^{a3}	20.40 ± 0.01^{b2}	21.20 ± 0.12^{b1}	20.99 ± 0.18^{a12}
		42.72	18.85 ± 0.29^{a3}	21.31 ± 0.21^{a12}	21.84 ± 0.09^{a1}	20.81 ± 0.08^{a2}
	Gill	Control	10.50± 0.07^{c1}	10.19 ± 0.43^{d1}	10.40 ± 0.13^{d1}	10.16 ± 0.13^{e1}
		2.67	12.07 ± 0.46^{ab1}	11.30 ± 0.07^{cd12}	11.03 ± 0.31^{c2}	10.55 ± 0.08^{d2}
		5.34	12.43 ± 0.02^{a1}	12.21 ± 0.56^{bc1}	12.18 ± 0.11^{b1}	12.75 ± 0.21^{c1}
		10.68	12.28 ± 0.06^{a3}	12.89 ± 0.31^{ab2}	13.81 ± 0.08^{a1}	13.39 ± 0.12^{b12}
		21.36	11.51 ± 0.09^{b3}	13.06 ± 0.51^{ab2}	13.99 ± 0.09^{a1}	13.55 ± 0.08^{b12}
		42.72	12.31 ± 0.07^{a3}	13.54 ± 0.08^{a2}	13.93 ± 0.01^{a12}	14.26 ± 0.24^{a1}

Values with different alphabetic superscripts differ significantly (p<0.05) between concentrations within the same duration. Values with different numeric superscripts differ significantly (p<0.05) between durations within the same concentrations. Results are expressed as mean ± standard error of the mean.

Table 2: Activity of catalase (CAT, mmol min^{-1}mg $protein^{-1}$), glutathione reductase (GR, nmol min^{-1}mg $protein^{-1}$), glutathione peroxidase (GPx, nmol min^{-1}mg $protein^{-1}$), in the liver and gill tissues of *C. gariepinus* exposed to sub-chronic concentrations (2.67, 5.34, 10.68, 21.36, 42.72mgL^{-1}) of Diazepam.

		Exposure duration (Days)				
Parameter	**Tissues**	**Concentration (mg/L)**	**7**	**14**	**21**	**28**
CAT	Liver	Control	0.42 ± 0.00^{c12}	0.44 ± 0.03^{c1}	0.45 ± 0.02^{c1}	0.43 ± 0.03^{c1}
		2.67	0.52 ± 0.02^{b2}	0.60 ± 0.03^{ab12}	0.65 ± 0.01^{b1}	0.62 ± 0.03^{c1}
		5.34	0.57 ± 0.03^{b2}	0.59 ± 0.03^{ab2}	0.64 ± 0.00^{b12}	0.70 ± 0.02^{b1}
		10.68	0.52 ± 0.01^{b3}	0.61 ± 0.03^{ab2}	0.69 ± 0.02^{ab1}	0.72 ± 0.00^{ab1}
		21.36	0.55 ± 0.00^{b2}	0.57 ± 0.03^{b2}	0.68 ± 0.02^{ab1}	0.72 ± 0.01^{ab1}
		42.72	0.66 ± 0.01^{a3}	0.68 ± 0.00^{a23}	0.73 ± 0.00^{a2}	0.79 ± 0.03^{a1}
	Gill	Control	0.86 ± 0.02^{ab1}	0.95 ± 0.00^{a1}	0.81 ± 0.09^{a1}	0.93 ± 0.01^{a1}
		2.67	0.84 ± 0.00^{ab2}	0.90 ± 0.23^{ab1}	0.84 ± 0.00^{a2}	0.85 ± 0.01^{b2}
		5.34	0.82 ± 0.04^{ab1}	0.86 ± 0.02^{bc1}	0.81 ± 0.04^{a1}	0.89 ± 0.00^{ab1}
		10.68	0.79 ± 0.09^{b1}	0.84 ± 0.01^{cd1}	0.84 ± 0.01^{a1}	0.88 ± 0.01^{ab1}
		21.36	0.89 ± 0.05^{ab1}	0.85 ± 0.01^{bcd1}	0.86 ± 0.01^{a1}	0.86 ± 0.01^{b1}
		42.72	0.95 ± 0.02^{a1}	0.79 ± 0.03^{d2}	0.73 ± 0.00^{a2}	0.79 ± 0.03^{c2}
GR	Liver	Control	23.13 ± 0.01^{ab1}	23.06 ± 1.44^{d1}	21.39 ± 1.57^{c1}	20.88 ± 1.26^{d1}
		2.67	22.08 ± 0.82^{b3}	25.30 ± 022^{c2}	26.34 ± 0.02^{b12}	27.27 ± 1.59^{c1}
		5.34	23.89 ± 0.13^{a3}	26.40 ± 0.12^{bc2}	26.23 ± 1.05^{b2}	28.01 ± 1.35^{bc1}
		10.68	23.16 ± 1.45^{ab1}	27.19 ± 1.14^{ab12}	26.41 ± 1.02^{b2}	27.70 ± 1.28^{c1}
		21.36	24.64 ± 0.56^{a4}	27.44 ± 1.03^{ab2}	28.02 ± 0.35^{a12}	29.03 ± 1.22^{ab1}
		42.72	24.28 ± 0.19^{b3}	27.88 ± 0.04^{a2}	27.31 ± 0.06^{a3}	29.03 ± 0.02^{a1}
	Gill	Control	30.92 ± 0.42^{d1}	29.79 ± 0.49^{c1}	28.78 ± 0.79^{b1}	29.16 ± 0.29^{c1}
		2.67	33.09 ± 0.34^{b1}	33.91 ± 0.19^{b1}	33.91 ± 0.13^{a1}	34.34 ± 1.27^{b1}
		5.34	33.43 ± 0.02^{ab12}	32.95 ± 0.19^{b2}	33.42 ± 0.05^{a12}	34.43 ± 0.69^{b1}
		10.68	34.15 ± 0.27^{a2}	33.66 ± 0.59^{ab2}	33.89 ± 0.13^{a2}	36.46 ± 0.03^{a1}
		21.36	32.19 ± 0.12^{c3}	32.92 ± 0.29^{b3}	33.88 ± 0.15^{a2}	35.02 ± 0.35^{ab1}
		42.72	34.04 ± 0.23^{a2}	34.61 ± 0.13^{a2}	34.81 ± 0.50^{a2}	36.63 ± 0.12^{a1}
GPx	Liver	Control	14.30 ± 0.03^{b1}	14.33 ± 0.01^{a1}	14.13 ± 0.03^{a1}	14.23 ± 0.01^{a1}
		2.67	12.44 ± 1.02^{ab3}	13.68 ± 0.15^{a1}	13.86 ± 0.01^{a1}	13.03 ± 0.23^{bc2}
		5.34	12.51 ± 0.08^{a1}	12.04 ± 0.36^{b1}	12.25 ± 0.04^{b1}	12.66 ± 0.16^{cd1}
		10.68	12.43 ± 1.02^{ab1}	12.28 ± 0.63^{b1}	12.48 ± 0.09^{b1}	13.31 ± 0.21^{b1}
		21.36	12.54 ± 0.08^{a1}	11.76 ± 0.25^{b2}	11.75 ± 0.21^{e2}	12.39 ± 0.01^{d1}
		42.72	12.53 ± 0.06^{a12}	11.29 ± 0.06^{b3}	12.34 ± 0.01^{b2}	12.72 ± 0.06^{cd1}
	Gill	Control	16.66 ± 0.16^{b1}	15.87 ± 0.26^{d1}	16.45 ± 0.02^{d1}	16.56 ± 0.64^{d1}
		2.67	18.70 ± 0.16^{ab1}	18.87 ± 0.04^{c1}	16.88 ± 0.32^{d2}	18.44 ± 0.12^{c1}
		5.34	19.42 ± 0.02^{ab2}	19.79 ± 0.22^{bc1}	19.37 ± 0.01^{c2}	19.89 ± 0.02^{c1}
		10.68	17.84 ± 2.18^{ab2}	20.44 ± 0.14^{b12}	19.93 ± 0.15^{c12}	22.80 ± 0.82^{b1}
		21.36	19.89 ± 0.14^{a2}	20.80 ± 0.36^{b2}	20.69 ± 0.25^{b2}	22.89 ± 0.30^{b1}
		42.72	20.43 ± 0.14^{a3}	22.27 ± 0.66^{a2}	24.44 ± 0.14^{a1}	24.45 ± 0.46^{a1}

Values with different alphabetic superscripts differ significantly (p<0.05) between concentrations within the same duration. Values with different numeric superscripts differ significantly (p<0.05) between durations within the same concentrations. Results are expressed as mean ± standard error of the mean.

3.1.2 Effect on biochemical parameters

Effects of Diazepam exposure on tissue glucose and protein levels in *C. gariepinus* are presented in Table 3. Diazepam elicited in the liver and gill tissues significantly (p<0.05) decreases in glucose and protein when compared to control. In both tissues, there was a duration dependent increase in protein with mixed trend. The changes in tissue ALP and ALT are presented in Figures 1 and 2. In day 14, 21 and 28 durations of exposure, liver and gill ALT activity significantly (p<0.05) elevated when compared to control. Day 7 exposure showed an increased activity of ALT that was not significant. ALP activity in day 7 in both tissues, were inhibited in fish treated to 2.67-21.36mgL^{-1}concentrations, but became comparable to control in the highest concentration of 42.72mgL^{-1}. In day 28, liver ALP activity elevated significantly (p<0.05) when compared to control. However, in the gill tissue, it showed mixed trend with significant (p<0.05) inhibitions in the lower sub-lethal concentrations of 2.67 and 5.34mg$L^{-1}$when compared to control. In day 14 and 21 durations of exposure, the activity of ALP showed mixed trend in both tissues when compared to control. Equally, significant (p<0.05) duration dependent increases were observed in ALT and ALP in the liver with 21.36mgL^{-1}concentration, while in other concentrations and in both tissues, elevations were biphasic with mixed trend.

Table 3: Mean values of glucose (mmol g tissue^{-1}) and protein (mg g tissue^{-1}) levels in the liver and gill tissues of *C. gariepinus* exposed to sub-chronic concentrations (2.67, 5.34, 10.68, 21.36, 42.72mgL^{-1}) of Diazepam.

		Exposure duration (Days)				
Parameter	**Tissues**	**Concentration (mg/L)**	**7**	**14**	**21**	**28**
Glucose	Liver	Control	92.00 ± 1.12^{a1}	89.00 ± 2.31^{a1}	88.33 ± 0.33^{a1}	91.67 ± 1.45^{a1}
		2.67	82.67 ± 0.88^{b1}	68.33 ± 0.33^{bc2}	63.00 ± 2.31^{ab3}	63.33 ± 1.45^{e3}
		5.34	76.67 ± 2.60^{c1}	66.00 ± 1.16^{c23}	63.00 ± 1.73^{ab3}	69.67 ± 0.88^{c2}
		10.68	71.67 ± 2.03^{d1}	68.33 ± 0.33^{bc12}	62.67 ± 0.88^{ab3}	64.67 ± 0.33^{de23}
		21.36	70.67 ± 0.88^{d2}	68.33 ± 1.45^{bc2}	67.00 ± 0.58^{b2}	75.00 ± 1.73^{b1}
		42.72	71.00 ± 0.58^{d1}	71.00 ± 1.73^{b1}	59.67 ± 0.88^{c2}	67.67 ± 0.33^{cd1}
	Gill	Control	92.00 ± 0.16^{a1}	89.00 ± 2.31^{a1}	88.33 ± 0.33^{a1}	91.67 ± 1.45^{a1}
		2.67	82.67 ± 0.88^{b1}	68.33 ± 0.33^{bc2}	63.00 ± 2.31^{bc3}	63.33 ± 1.45^{e3}
		5.34	76.67 ± 2.60^{c1}	62.00 ± 1.16^{c23}	63.00 ± 1.73^{bc3}	69.67 ± 0.08^{c2}
		10.68	71.67 ± 2.03^{d1}	68.33 ± 0.33^{bc12}	62.67 ± 0.88^{bc3}	64.67 ± 0.33^{de23}
		21.36	70.67 ± 0.88^{d2}	68.33 ± 1.45^{bc2}	67.00 ± 0.58^{b2}	75.00 ± 1.73^{b1}
		42.72	71.00 ± 0.58^{d1}	71.00 ± 1.73^{b1}	59.67 ± 0.88^{c2}	67.67 ± 0.33^{cd1}
Protein	Liver	Control	6.50 ± 0.17^{a1}	6.83 ± 0.03^{a1}	6.60 ± 0.12^{a1}	6.50 ± 0.06^{a1}
		2.67	4.40 ± 0.12^{a3}	5.50 ± 0.06^{b2}	6.70 ± 0.12^{b1}	6.70 ± 0.06^{b1}
		5.34	3.83 ± 0.03^{b3}	5.00 ± 0.52^{b2}	6.83 ± 0.32^{b1}	6.27 ± 0.09^{d1}
		10.68	3.70 ± 0.23^{b2}	3.20 ± 0.23^{cd2}	6.43 ± 0.20^{b1}	6.40 ± 0.58^{cd1}
		21.36	3.87 ± 0.03^{b2}	3.30 ± 0.12^{c2}	6.27 ± 0.45^{b1}	6.60 ± 0.12^{ab1}
		42.72	3.20 ± 0.15^{c3}	2.43 ± 0.02^{d4}	6.07 ± 0.03^{b1}	5.37 ± 0.33^{e1}
	Gill	Control	6.50 ± 0.17^{a1}	6.83 ± 0.03^{a1}	6.47 ± 0.03^{a1}	6.27 ± 0.09^{a1}
		2.67	4.40 ± 0.12^{a3}	5.50 ± 0.06^{b2}	6.10 ± 0.16^{b1}	5.37 ± 0.03^{b2}
		5.34	3.83 ± 0.33^{b2}	5.00 ± 0.52^{b1}	4.83 ± 0.03^{c12}	4.90 ± 0.35^{b1}
		10.68	3.70 ± 0.23^{b1}	3.20 ± 0.23^{cd1}	3.70 ± 0.06^{d1}	3.40 ± 0.12^{c1}
		21.36	3.87 ± 0.03^{b1}	3.30 ± 0.12^{c2}	2.67 ± 0.15^{e3}	3.17 ± 0.03^{e2}
		42.72	3.20 ± 0.15^{c1}	2.43 ± 0.20^{d2}	2.23 ± 0.09^{f2}	2.27 ± 0.17^{d2}

Values with different alphabetic superscripts differ significantly ($p<0.05$) between concentrations within the same duration. Values with different numeric superscripts differ significantly ($p<0.05$) between durations within the same concentrations. Results are expressed as mean ± standard error of the mean.

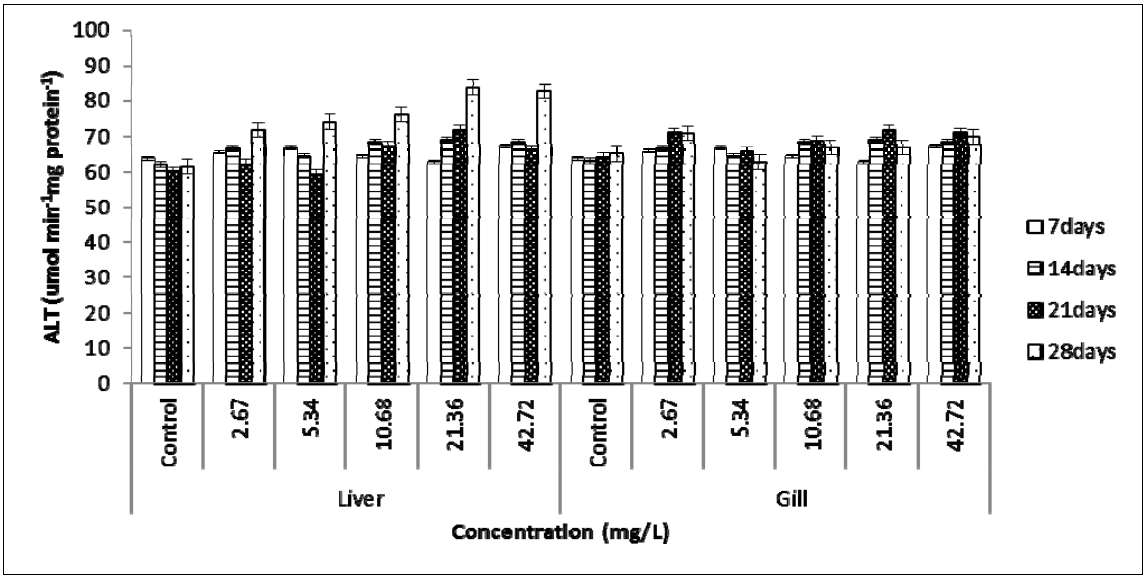

Fig 1: Changes in ALT (umol min^{-1}mg protein^{-1}) activities in liver and gill tissues of *C. gariepinus* exposed to sub-chronic concentrations (2.67, 5.34, 10.68, 21.36, 42.72mgL^{-1}) of Diazepam.

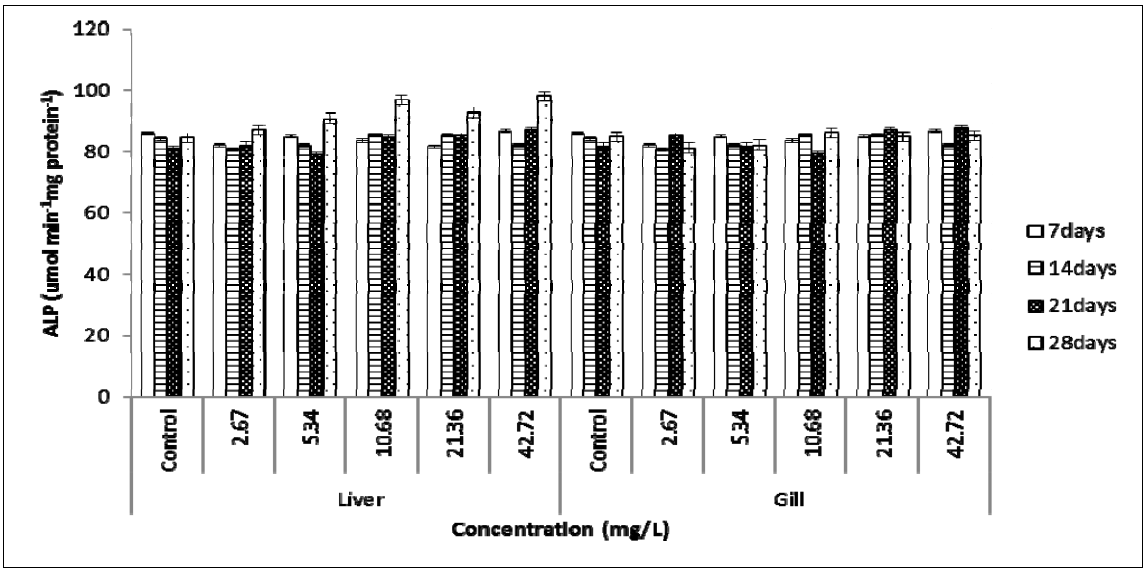

Fig 2: Changes in ALP (umol min^{-1}mg $protein^{-1}$) activities in liver and gill tissues of *C. gariepinus* exposed to sub-chronic concentrations (2.67, 5.34, 10.68, 21.36, 42.72mgL^{-1}) of Diazepam.

4. Discussion

4.1. Lipid peroxidation

The LPO in both tissues was significantly (p<0.05) elevated in the treated group when compared to control. However, the induction was more pronounced in the gill tissue than in the liver. In the highest concentration of 42.72mgL^{-1} Diazepam TBARS formation in the liver of treated fish, elevated by 4.71% and 46.74% in day 7 and 28 respectively, whereas in the gill, the percentage increases were 8.35% and 58.18% in similar time periods. The present data showed that reactive oxygen species (ROS) may be associated with the metabolism of diazepam leading to peroxidation of membrane lipids of the respective organs. This suggests that there is increased production of ROS in the liver and gill of *C. gariepinus* and the antioxidant defenses were not totally able to effectively scavenge them, thus leading to lipid peroxidation. [29] LPO activity is a popular biomarker for assessment of environmental pollution.[30, 42] Xenobiotics and their metabolites have been shown to induce oxidative stress, by producing free radicals that leads to damage to membrane lipids, DNA and proteins.

Increase in LPO has been reported to be due to ibuprofen induced oxidative stress in liver and gill of *C. carpio* [31]. Increases in LPO leading to oxidative stress have also been reported in *C. gariepinus* by other xenobiotics like fenthion [32]. The elevated LPO resulting from ROS generated by the diazepam may lead to cell apoptosis [33]. ROS and oxidative stress have been demonstrated to be triggers of apoptosis. Each secondary gill lamella of *C. gariepinus* contains a thin-walled gill sinusoid that allows for the continuous exchange of respiratory gases such as oxygen and soluble metabolic wastes such as carbon dioxide and ammonia. In addition the respiratory epithelium of the secondary lamellae contains specialized chloride cells that assist with osmoregulation by excreting chloride, potassium and sodium ions. Increased LPO may alter gill architecture and lead to impairment in its osmoregulatory and ionoregulatory functions. Dose and duration dependent cyto-architectural degenerations of the liver hepatocytes exposed to sublethal concentrations of Chlorpyrifos-ethyl and Lambda-cyhalothrin have been reported to be characterized by: infiltration of inflammatory cells, aggregated macrophages with haemosiderosis activity, dialated sinusoidal spaces, pyknotic nuclei, yellow-brown cytoplasmic pigment (Bile) and coagulation necrosis of the hepatocytes [34].

SOD is the first defensive free radical scavenger; diazepam triggered an induction response in SOD activity in both organs of treated *C. gariepinus* in a dose and duration dependent manner.

In the highest concentration of 42.72mgL^{-1} Diazepam induced in the liver, percentage SOD elevations of 5.01% and 28.38% in day 7 and 28 respectively, while in the gill, the percentage increases were 17.24% and 55.68% in similar durations, indicating higher activity of SOD in gill than liver. Increased SOD activity in liver and kidney of common carp (*Cyprinus capio* L) after exposure to sub-lethal concentrations of heavy metals has been reported [35, 36]. Neuroactive pharmaceutical drugs (diazepam, carbamazepine and phenytonin) induced oxidative stress in some organs viz; gills, liver and digestive tract of *Lepomis gibbosus* (pumpkinseed sunfish) [31]. Highest increase in SOD activity in liver was observed after 12 h of exposure of *C. carpio* to ibuprofen. The induction of SOD activity in the organs indicates that more protein is required to protect cells against superoxide radicals. The accumulation and binding of diazepam in cell membranes, cytoplasm, and mitochondria may cause degeneration and disintegration of cells, leading to the release of SOD enzyme into the blood circulation [37, 38]. The SOD-CAT system provides the first defense against oxygen toxicity. SOD catalyzes the dismutation of the superoxide anion radical into water and hydrogen peroxide, which is detoxified by CAT activity. The target function of catalase is to protect the cells from the accumulation of H_2O_2 by dismuting it to form H_2O and O_2 or by using it as an oxidant where it works as a peroxidase [35].

The CAT in the liver of treated *C. gariepinus* to diazepam were significantly (p<0.05) increased in dose and duration dependent pattern in liver, but the response of gill tissue was different from that of liver, the response of gill was biphasic in that in day 7 and 21, all treated groups elicited comparable CAT activity with control, with a non significant increase in 42.72mgL^{-1} and 21.36 mgL^{-1} exposure concentrations in days 7 and 21 respectively. However, in day14 and 28 there were significant (p<0.05) decreases in the groups treated to 5.34-42.72mgL^{-1} concentrations.

The high levels of CAT in the liver tissue could be attributed to high production of peroxide radicals. The increase may be an adaptive response to protect fish from diazepam induced free radical toxicity. Previous study [53] revealed biphasic responses with an increase in CAT activity from day 7 to 14 of *Clarias gariepinu*s treated to sublethal concentration of fenthion at 8.0mg/L in gill, and a decline of its activity in day 21. The decrease in CAT activity could be due to the flux of superoxide radicals, or activated metabolites generated by diazepam on the cell membrane of treated fish, which have been reported to inhibit CAT activity [39]. The same decreased activity of CAT was observed in tissues of *Channa punctatus* Bloch exposure to Deltamethrin [40, 41] Reduced CAT activity was seen in freshwater fish *Labeo rohita* exposed to Endosulfan and Fenvalerate. Glutathione reductase (GR) catalyzes the reduction of glutathione disulfide (GSSG) to the sulfhydryl form glutathione (GSH), which is a critical molecule in resisting oxidative stress and maintaining the reducing environment of the cell. The enzyme glutathione peroxidase utilizes reduced glutathione to eliminate hydrogen peroxide: $2\ GSH + H_2O_2 \longrightarrow GSSG + 2\ H_2O$

Glutathione reductase then adds hydrogens to the oxidized glutathione (GSSG) to regenerate reduced glutathione (GSH). The ratio of GSSH/GSH present in the cell is a key factor in properly maintaining the oxidative balance of the cell, that is, it is critical that the cell maintains high levels of the reduced glutathione and a low level of the oxidized Glutathione disulfide. This narrow balance is maintained by glutathione reductase, which catalyzes the reduction of GSSG to GSH. In this study, there were significant (p<0.05) elevation in GR activity in the gill throughout the duration of the experiment when compared to control. The activity of GR in the liver was similar to the gill, from day 14-28 exposure period. In day 7, the activity of GR in the liver was comparable to control [42]. Significant increase in GR activity was observed in embryo-larval stages of common carp (*Cyprinus carpio*) exposed for 30 days to 0.52 $mg.L^{-1}$ of terbuthylazine and 0.9, 4, and 14 $mg.L^{-1}$ of metribuzin compared to control. The higher GR values obtained could be a demonstration that the ability of the enzyme to sustain the recycling of GSSG to GSH was not compromised [35]. Glutathione peroxidase (GPx) is the most important peroxidase that has been postulated to protect the erythrocytes from damage by H_2O_2. It is strictly linked with the concentration of GSH because it catalyses the reaction between glutathione and hydrogen peroxide, resulting in the formation of glutathione disulphide (GSSG). It is hypothesized that this enzyme may protect tissues against oxidative damage due to lipid peroxidation.

In this study, diazepam caused a significant (p<0.05) decrease and significant (p<0.05) elevation in the GPx activity in the liver and gill tissues respectively throughout the period of exposure.

GPx showed a tissue-specific response to diazepam exposure. Differences in antioxidant responses between the gill and liver may be related to different physiological functions of the tissues [43]. Heavy metal Cadmium (Cd) showed a tissue specific response in the tissues of Nile Tilapia *Oreochromis niloticus*. GPx activity was stimulated in the gill of fish exposed to 0.016, 0.08 and 0.4 mg/L of Cd but not in fish exposed to 2 mg/L Cd [43, 43] On the other hand, Cd significantly inhibited GPx activities in the fish livers of all the Cd treatment groups. The decreased activity of GPx in the liver indicated its reduced capacity to scavenge H_2O_2 and lipid hydroperoxides, as it reduced the GSH conversion to GSSG. The decreased activity of GPx may be the result of $^{\bullet}O^{-}_2$ production [44]. Significant increase in GPx activity as observed predominantly in the gill indicates the protective role of the enzyme against lipid peroxidation, as it sustained the conversion GSSG to GSH. In humans, sex-specific differences in pharmacokinetics have been identified for many drugs, including benzodiazepines [45, 46]. It has been reported that trichlorfon and methidathion caused a decrease in the GPx activity.

In general environmental contaminants in aquatic media induce significant changes in biochemical parameters of aquatic organisms. In the current study, Diazepam elicited in the liver and gill tissues significant (p<0.05) decreases in glucose and protein in all durations of exposure when compared to control. The reported concentration dependent hypoglycemia may be due to Kidney failure, where damaged kidneys releases glucose into the urine or impairments in Glycogenolysis and or gluconeogenesis as a result of liver dysfunction. In both tissues, there was a duration dependent increase in protein with mixed trend [47]. Hypoglycemia have been reported to be as a result of exposure to meclofenamic acid (MFA) while the inhibition of glucose suggests that more energy was needed to achieve the positive survival value.

Significant inhibition of protein in all durations of exposure may be due to metabolic utilization of the ketoacids to glucogenesis pathway for synthesis of glucose to compensate for the high energy requirement or high rate of protein degradation or impaired incorporation of amino acids into polypeptide chains [48]. Decreased protein may also be attributed to necrosis of cells and consequent impairment in protein synthesis machinery. Liver disorder due to toxicant stress may also lead to decrease in protein levels [49]. High serum protein levels have been reported to be indicative of osmoregulatory dysfunction, haemodilution, or tissue damage surrounding blood vessels [50]. This recent finding is consistent with the report of some previous authors [51, 52].

Determinations of ALT and ALP enzymes in blood plasma are a sensitive indicator of cellular damage, organ malfunction and water pollution [53]. In this study, plasma ALT and ALP activities were elevated in liver and gill tissues indicating the disorder in Kreb's cycle caused by the drug diazepam. Damage in hepatic cells may have been responsible for the significant increases in the enzymes. The elevations of ALT and ALP activity indicated that the fish tried to mitigate the drug induced stress. The elevations were due to damage in hepatic cells (necrosis, apoptosis or both) [54]. Increase in serum ALP activity suggest leakage of the enzyme into the blood as a result of tissue necrosis. High serum ALP as in the present study was also reported in the teleost *Clarias batrachus* treated with endosulphan and kelthane, suggesting cell necrosis and an increase in lysosomal mobilization [55]. Increased GOT and GPT activity in *O. mykiss* exposed to verapamil indicates amplified transamination processes [56]. However, ALP activity in day 7 in both tissues, were inhibited in fish treated to 2.67-21.36mgL^{-1} concentrations, but became comparable to control in the highest concentration of 42.72mgL^{-1}. The decrease indicates that detoxification mechanism may not be sufficiently effective to prevent the action of diazepam on the system in day 7 duration of exposure. The accumulation and binding of the drug in the tissues of the exposed fish could induce stressful conditions and subsequently results in the elevation of the transamination pathway to counter the resultant energy crisis.

5. Conclusion

The activities of antioxidant enzymes, such as SOD, CAT, GR, and GPx changed in gill and liver tissues of *C. gariepinus* under diazepam stress as indicated by increase in LPO. This suggested that reactive oxygen species may be involved in the toxic effects of diazepam. The increase in the activities of biochemical enzymes is evident that diazepam induced damage on liver and gill tissues. The integrated use of biochemical and oxidative stress biomarkers using fish model may be useful to the regulatory agencies in risk assessment of pharmaceuticals in aquatic ecosystem. Further studies on the toxicokinetics and dynamics of diazepam are necessary for a greater insight on the mechanisms of action that results in induction of oxidative stress.

6. Conflict of interest statement

None Declared.

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