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Activities of Acetylcholinesterase, Oxidative and Nitrosative Stress Markers in *Clarias gariepinus* Exposed to 'Uproot', a Glyphosate-Based Herbicide

Osioma E.^{1*} and Ejoh A.S.²

¹Department of Biochemistry, Faculty of Science, Federal University Otuoke, Bayelsa State, Nigeria

²Department of Biological Sciences, Covenant University Ota, Nigeria

*Corresponding Author

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Abstract: In this study, *Clarias gariepinus* were exposed to different concentrations (60, 80 and 100 mg/L) of *Uproot*, a glyphosate-based herbicide and the activities of acetylcholinesterase (AChE), hepatic enzyme markers, oxidative and nitrosative stress markers were determined in serum, gills, liver and brain by using standard assays. Results showed that AChE activity was not significantly inhibited. Activities of ALT, AST and ALP increased ($P < 0.05$) with increasing exposures. For SOD, elevated ($P < 0.05$) activities were observed in all tissues at 60 mg/L exposure, but decreased at 80 mg/L and 100 mg/L concentrations. The activity of CAT reduced significantly ($P < 0.05$) in brain at 100 mg/L exposure. Increased production of NO was observed in gill and brain whereas in serum it is increased. GPx was elevated in gills and reduced in brain and liver. Gills showed lower MDA concentrations. GST was elevated in liver and brain, while total protein reduced ($P < 0.05$) in serum, gill and liver with increasing concentrations of exposure. The impaired activities of antioxidant enzymes and induction of NO suggest a disruption of normal antioxidant response in *Clarias gariepinus* exposed to glyphosate, and these activities could be used as biomarkers in aquatic environmental contamination.

Keywords: *Uproot*, Glyphosate, *Clarias gariepinus*, Nitric oxide, Antioxidant enzymes

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Introduction

The use of agricultural herbicides in weed control in farms over the traditional weeding method cannot be over emphasized.

Herbicides applicability in crop fields to control weeds improves productivity of farm produce, leading to the cultivation of large

piece of land. However, such chemicals, when employed in farmland to contain weeds could also contaminate the environment; and for aquatic habitat, pollution by these herbicides is due to leaching and water run-off from treated areas which basically poses a great risk to non – target aquatic organism especially fishes of commercial importance (Oruc *et al.*, 2004; Dey *et al.*, 2016).

Exposure of aquatic lives to herbicides directly or indirectly, can induce an increase in the production of reactive oxygen species (ROS) and also modify the antioxidant defense. *Uproot*, is the conventional glyphosate-based herbicide, employed by farmers in Otuoke, Bayelsa State, Nigeria. Glyphosate is an isopropylamine salt of glycine, a non-selective herbicide which was introduced into the agricultural market in 1974 for the management of weeds in cultivars (Duke and Powles, 2008). As a result of the large-scale use of *Uproot* in our locality and the proximity of crop and fish culture areas, glyphosate which leached into aquatic environment could induce changes in several biochemical variables which could be employed to evaluate fish health. These modifications may be in the altered function of cells, tissues, as well as physiology and behavior of the organisms (Parvez and Raisuddin, 2005). Exposure to glyphosate can cause a greater amount of pro – oxidants than antioxidants, resulting in the intensity of ROS generation and/or altering antioxidant defense (Ahmad *et al.*, 2000; Monserrat *et al.*, 2007). To neutralize the effect of ROS, animals have an established antioxidant defense pathway of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GP_x), glutathione –S-transferase (GST) as well as non-enzymatic

antioxidants such as reduced glutathione (GSH).

Recently, nitric oxide has been reported as an antioxidant (Radi, 2018). It reacts with superoxide radical to form peroxynitrite (ONOO⁻) which is commonly associated with a pro-oxidant response and scavenge lipid peroxy radical (LOO^{*}) that is viewed as the antioxidant response (Goss *et al.*, 1997). Inhibition of acetylcholinesterase (AChE) activity by formulations containing glyphosate have been reported by several studies (Gluszczak *et al.*, 2006; Gluszczak *et al.*, 2007; Modesto and Martinez, 2010 a)

In the aquatic habitat, teleost, according to Sancho *et al.* (2000) has proven to be a good model to appraise the toxicity and consequences of contaminants on animals, since the biochemical responses are identical to those of mammals and other vertebrates. *Clarias gariepinus* found in rivers and swamp in the Niger Delta regions of Nigeria are of economic value, source of protein to many families and is also treated as a potential bio-indicator species (Osioma and Iniaghe, 2019). These biochemical markers established association of the sample with definite groups of chemical compounds and allows cause-effect relativity to be established at an initial stage of pollution (Osioma *et al.*, 2013). Thus, evaluation of changes in biochemical enzymes as biomarker in *Clarias gariepinus* exposed to different concentrations of glyphosate will support to provide a link between the effect of xenobiotic exposure and risk assessment process which could be utilized in documentary toxin interaction with biological system. Hence, in the present study, the activities of antioxidant enzymes (SOD, CAT, GP_x, GST), acetylcholinesterase (AChE), hepatic enzyme markers, alanine and

aspartate aminotransferase, and alkaline phosphatase (ALT, AST and ALP), concentrations of total protein (TP), nitric oxide (NO), reduced glutathione (GSH) and malondialdehyde (MDA) in serum and selected tissues (gills, liver and brain) of *Clarias gariepinus* exposed to different concentrations of glyphosate herbicide, were investigated.

Materials and Methods

Collection of fish specimens

Thirty (30) specimens of African catfish (*Clarias gariepinus*) were procured from a commercial fish pond (Rubber Pond) along Azikoro Expressway, Yenagoa, Bayelsa State, Nigeria. They were transported on the same day to the laboratory in a well ventilated container with the pond water to avoid injury. Fish were left to acclimatize in the congenial laboratory condition for 14 days in aquaria of 200 L capacity. They were fed once a day with commercial fish pellets during both acclimation and exposure periods

Experimental design

Uproot, a herbicide containing isopropyl-ammonium salt of glyphosate as active ingredient (equivalent to 360 g/L) was purchased from a local trader at Otuoke, Bayelsa State, Nigeria. In preparing the required doses used for the experiment, 10 mL of the stock was taken and made up to 500 mL with distilled water. From this dilution, the required doses (60 mg/L, 80 mg/L and 100 mg/L) of glyphosate were calculated and multiplied by capacity of the aquaria, 20 L.

Treatment with herbicide commenced on the 3rd week. Fish were divided into four groups (A, B, C and D) comprising five fish per group (n = 5) and separated into 20 L capacity

aquaria. Group A served as control without glyphosate exposure. Groups B, C and D were exposed to 60 mg/L, 80 mg/L and 100 mg/L glyphosate, respectively. The water was changed every 24 h and exposure was repeated for 14 days. Experiments were conducted at natural photoperiod.

Preparation of serum and tissue supernatants

At the end of the experiment, fish were anaesthetized and blood collected by caudal arterial puncture from each fish with the help of a 5 mL syringe. A portion of the blood sample was put into EDTA container for the estimation of blood reduced glutathione, while the remaining blood sample was introduced into a 5 mL plain tube. The fish were euthanized by spinal section and decapitation, and then, dissected for tissue collection. Gills, liver and brain were washed in 0.75% saline solution, soaked with filter paper and stored at - 20 C for biochemical analysis.

The remaining portion of the collected fish blood was allowed to clot for about 20 min at room temperature (27 C). The clotted blood samples were dislodged and centrifuged at x5000 g for 15 min to obtain serum, which was stored frozen at - 20 C until analyzed.

0.5 g of each tissue (gills, liver and brain) was homogenized separately in 4.5 mL of ice cold 50 mM phosphate buffer (pH 7.2). The resulting homogenate was centrifuged at x4000 g for 10 min. The supernatant was decanted into a 5 mL sterilized plain tube. This was immediately stored in deep freezer at - 20 C for further biochemical analysis.

Biochemical analysis

Total protein determination

Total protein content in serum and tissue samples was determined spectrophoto-

metrically by the method of Doumas *et al.* (1981). The principle was based on the formation of purple complex when protein reacts with cupric ions in an alkaline solution. The intensity of the violet colour was proportional to the amount of protein present in the sample measured at 540 nm.

Superoxide dismutase assay

The superoxide dismutase activity was determined as described by Misra and Fridovich (1972). This method was based on inhibiting the radical superoxide reaction with epinephrine. The superoxide anion ($O_2^{\cdot-}$) substrate for SOD was generated in the oxidation of epinephrine at alkaline pH (pH 10.2) by the action of oxygen on epinephrine. Briefly, to 0.2 mL of homogenate, 2.5 mL of 0.05 M carbonate buffer (pH 10.2) was added. The reaction was initiated by adding 0.3 mL of freshly prepared 0.3 mM of epinephrine. This was mixed by inversion. The reference cuvette contained 2.5 mL of the buffer, 0.2 mL of distilled water and 0.3 mL of the substrate (epinephrine). The per cent inhibition (% I) is hyperbolic with respect to the SOD activity. Increase in absorbance was monitored at 480 nm at interval of 30 sec for 150 sec. The activity was expressed in unit/g tissue.

Catalase assay

The method of Kaplan and Groves (1972) was employed in the determination of catalase activity. This was carried out by monitoring the breakdown of hydrogen peroxide in the reaction mixture by changes in the absorbance at wavelength of 360 nm for 70 sec. To 2 mL of the prepared sample, 1 mL of H_2O_2 substrate was added to the reaction cuvette. The reference cuvette contained 1 mL of H_2O_2 and 2 mL of water. Catalase activity was expressed as unit/g tissue.

Estimation of blood reduced glutathione

The reduced glutathione concentration of blood was estimated using the method of Beutler (1984). The sulfhydryl group of GSH reacted with DTNB (5,5-dithio-bis-2-nitrobenzoic acid) and produced a yellow coloured 5-thio-2-nitrobenzoic acid (TNB). The absorbance of TNB at 412 nm provided an estimation of GSH in the sample. Briefly, 1.8 mL of distilled water was added to 0.2 mL of blood sample and was followed by the addition of 3 mL of precipitating solution. The mixture was allowed to stand for 5 min and filtered. To 8 mL of phosphate solution was added 2 mL of the filtrate and 1 mL of DTNB solution. The blank was prepared with 8 mL of phosphate solution, 2 mL of dilute precipitating solution (3 parts to 2 parts of distilled water), and 1 mL of DTNB reagent. The absorbance was read at 412 nm against the reagent blank. The concentration of blood GSH was calculated as; Blood Reduced GSH (mg% in blood) = $310.4 \times \epsilon \times \text{Absorbance}$, Where, ϵ is the correction factor = 0.542.

Estimation of tissue reduced glutathione

The reduced glutathione levels in the supernatant of tissues were determined using the procedure of Ellman (1959). The principle is as outlined above for blood GSH. Tissue supernatant (0.5 mL) was added to 2 mL of 10% (w/v) trichloroacetic acid, mixed thoroughly and centrifuged at x5000 g. Then, 1 mL of supernatant was mixed with 0.5 mL Ellman's reagent and 3 mL of 0.2 M phosphate buffer (pH 8.0). The absorbance was read against the blank at 412 nm. A series of standards were prepared along with a blank containing 3.5 mL of buffer. The concentration of reduced GSH in 1 mole of GSH/g of wet

tissue was extrapolated from a standard calibration plot.

Glutathione peroxidase assay

The activity of GPx in tissue supernatant was evaluated spectrophotometrically as described by Moin (1986). The principle of the reaction was based on the production of oxidized glutathione (GSSG) upon reduction of organic peroxide by GPx, which was recycled to its reduced state by the enzyme glutathione reductase. The assay mixture contained 0.8 mL of 0.1 M Tris - HCl (pH 8.9) with 12 mM sodium azide and 6 mM EDTA, 0.2 mL of tissue supernatant, 0.1 mL of 0.01 M 5,5 - dithiobis -2- nitrobenzoic acid, 0.1 mL of 20 mM t - butylhydroperoxide, and 0.1 mL of 4.8 mM GSH. The decrease in absorbance at 412 nm was followed spectrophotometrically. GPx enzymatic activity was expressed as $\mu\text{mol}/\text{mg tissue}/\text{min}$.

Glutathione - S- transferase assay

The activities of glutathione S- transferase (GST) in the supernatant of gills, liver and brain tissues were assayed by the method of Habig *et al.* (1974). GST catalysed the conjugation of L-glutathione to 1-chloro-2,4-dinitrobenzene (CDNB) through the thiol group of the glutathione. The formation of the GS-DNB conjugate was proportional to the enzyme activity and can be used for photometric GST activity determination. The rate of increase in the absorption of GS-DNB conjugate at 340 nm was directly proportional to the GST activity in the sample. The reaction mixture was prepared by mixing 1.5 mL sodium phosphate buffer (0.1 M pH 6.5), 0.2 mL GSH (9.2 mM), 0.02 mL CDNB (0.1 M) and 0.1 mL of the sample (homogenate). The reaction solution without the supernatant was

used as the blank. GST activity was expressed as $\mu\text{mol}/\text{mg tissue}/\text{min}$.

Nitric oxide determination

Nitric oxide was determined in serum, gills, liver and brain tissues by the method of Green *et al.* (1982). The principle was based on the enzymatic conversion of nitrate to nitrite by nitrate reductase, which was followed by a 2-step diazotization reaction by Griess reaction. The formed nitrous acid diazotise sulphanilamide and the product was coupled with N- (1- naphthyl) ethylenediamine to form a bright reddish-purple colour which absorbed strongly at 540 nm. The assay mixture contains 0.5 mL of sample supernatant with equal volume of Griess reagent [(0.1 mL sulfanilic acid reagent (0.33% in 20% glacial acetic acid at room temperature for 5 min with 1 mL of naphthylethylenediamine dichloride, 0.1% w/v)]. The mixture was incubated at room temperature for 30 min and the absorbance of reddish-purple colour change was read at 540 nm.

Acetylcholinesterase assay

Acetylcholinesterase activity was performed according to the method of Ellman *et al.* (1961). The principle of this method was based on the measurement of the rate of producton of thiocholine as acetylthiocholine is hydrolyzed. This was accomplished by the continuous reaction of the thiol with 5', 5-dithiobis -2-nitrobenzoate ion to produce the yellow anion of 5- thio-2-nitro-benzoic acid (II). The rate of colour production read at 412 nm was directly proportional to acetylcholinesterase activity. Briefly, 25 μL of supernatant was added to a cuvette containing 2.925 μL of 0.1 M phosphate buffer (pH 8.0), 25 μL of 8 mM of DTNB and 25 μL of

45 mM acetylcholine iodide at room temperature (27 C). The contents in the cuvette were mixed, and the absorbance was read continuously at intervals of 30 sec for 2 min at 412 nm. Acetylcholinesterase activity was expressed as nmol/mg/min.

Malondialdehyde determination

The concentrations of malondialdehyde in serum and the supernatants of gill, liver and brain were evaluated using the method of Buege and Aust (1978). The thiobarbituric acid reactive substances (TBARS) assay measures lipid peroxides and aldehydes, such as malondialdehyde (MDA) in the cell, culture media and cell lysate. MDA combined with thiobarbituric acid (TBA) in a 1:2 ratio to form fluorescent adduct that is read at 530 nm. Briefly, to 1.0 mL of the sample (serum/supernatant) was added to 2.0 mL of TCA -TBA - HCl reagent [15 % (w/v), TCA, 0.375 % (w/v) TBA and 0.25 N HCl]. The contents were boiled for 15 min, cooled and centrifuged at 10,000 g for 10 min to remove the precipitate. The absorbance was read at 535 nm using the reagent blank. The concentration of MDA was expressed as unit/mg tissue

Hepatic enzyme markers and alkaline phosphatase assay

Alanine aminotransferase (ALT) and Aspartate aminotransferase (AST) activities were determined using the methods of Reitman and Frankel (1957). Aspartate aminotransferase catalyses the formation of oxaloacetate and glutamate from aspartate and α -oxoglutarate. The unstable oxaloacetate spontaneously decarboxylates to pyruvate. The pyruvate is reacted with 2, 4-dinitrophenylhydrazine. The absorbance of the resulting brown colour due to the

dinitrophenylhydrazone is read under alkaline condition at 546 nm.

ALT catalyzes L-alanine and α -ketoglutarate to form pyruvate and glutamate. The pyruvate is then reacted with 2, 4-dinitrophenyl-hydrazine to form 2,4-dinitrophenyl-hydrazone. The addition of sodium hydroxide dissolves this complex, and allows 2,4-dinitrophenyl-hydrazine to be read at 546 nm which is proportional to ALT activity.

Alkaline phosphatase was assayed with the method of Roy (1970). Alkaline phosphatase acts upon the 2-amino-2-methyl-1-propanol buffered sodium thymolphthalein monophosphate. The addition of an alkaline reagent stops enzyme activity and simultaneously develops a blue chromogen which is measured at 590 nm and is proportional to the enzyme activity. These assays (liver enzyme markers) were carried out using commercial kits (Randox Laboratories, Ardmore, UK).

Statistical analysis

The mean \pm SD of each group was calculated. Data were analyzed using One - way Analysis of Variance (ANOVA), followed by Duncan's Multiple Range Test (DMRT) to compare the treated groups with the control. Difference between means were considered significant when $p < 0.05$. Statistical analysis was performed using SPSS version 16 (SPSS, Inc - Chicago, Illinois, USA).

Results

The concentration of total protein in serum, gill and liver significantly reduced ($P < 0.05$) as glyphosate concentration increases as compared with the control. However, in the brain tissue, an increase in the total protein

concentration was observed. Although, this elevation in total protein concentration was only significant ($P < 0.05$) for fish exposed to 60 mg/L and 80 mg/L compared with the control (Table 1).

The activities of superoxide dismutase (SOD) were elevated ($P < 0.05$) in all tissues (gill, liver and brain) of *Clarias gariepinus* exposed to 60 mg/L, but decreased in fish exposed to 80 mg/L and 100 mg/L in these tissues (Table 2). Serum SOD activity significantly reduced ($P < 0.05$) with increased glyphosate concentration (Table 2).

Activities of catalase (CAT) in gills of experimental fish (groups B, C and D) were not altered significantly as compared to the control group. Liver CAT activity decreased ($P < 0.05$) in exposed fish as compared with the control. An initial insignificant increase in CAT activity of brain was observed in *Clarias gariepinus* exposed to 60 mg/L of glyphosate herbicide but significantly reduced ($P < 0.05$) as exposure to glyphosate increased to 80 mg/L and 100 mg/L (Table 2).

There was a significant reduction ($P < 0.05$) in the levels of reduced glutathione

Table 1: Concentration of total protein in serum, gills, liver and brain of *Clarias gariepinus* exposed to different concentrations of glyphosate herbicide

Groups	Serum (g/dl)	Gill (g/dl)	Liver (g/dl)	Brain (g/dl)
A	3.11±0.17 ^a	1.28±0.14 ^a	3.34±0.23 ^a	0.58±0.05 ^a
B	2.59±0.51 ^b	1.37±0.26 ^b	2.49±0.31 ^b	1.10±0.16 ^b
C	2.67±0.30 ^b	1.20±0.18 ^a	2.02±0.60 ^c	1.14±0.18 ^b
D	2.59±0.22 ^b	1.02±0.22 ^c	1.71±0.78 ^c	0.81±0.32 ^a

Values are expressed as mean ± standard deviation; n = 5. Means not showing the same superscript alphabet in a given column differ significantly at $P < 0.05$.

Group A = Control; Group B = *Clarias gariepinus* induced with 60 mg/L of glyphosate; Group C = *Clarias gariepinus* induced with 80 mg/L of glyphosate; Group D = *Clarias gariepinus* induced with 100 mg/L of glyphosate.

Table 2: Activities of superoxide dismutase and catalase in serum, gills, liver and brain of *Clarias gariepinus* exposed to different concentration of glyphosate herbicide

Groups	Superoxide dismutase (Unit/g tissue)				Catalase (Unit/g tissue)		
	Serum	Gills	Liver	Brain	Gills	Liver	Brain
A	39.31±2.17 ^a	40.76±3.86 ^a	41.39±3.65 ^a	40.97±3.77 ^a	16.61±0.34 ^a	21.21±1.44 ^a	23.76±2.93 ^a
B	37.99±3.67 ^a	45.04±5.54 ^b	44.89±5.60 ^b	44.81±5.48 ^b	16.28±0.02 ^a	18.64±1.40 ^b	25.44±2.15 ^a
C	28.79±2.88 ^b	38.37±4.19 ^a	29.34±4.80 ^c	37.61±5.09 ^c	16.29±0.06 ^a	19.49±1.69 ^a	21.24±2.79 ^b
D	20.65±1.03 ^c	20.33±2.68 ^c	20.25±2.68 ^d	19.97±2.51 ^d	16.25±0.05 ^a	19.39±0.91 ^a	18.51±1.91 ^c

Values are expressed as mean ± standard deviation; n = 5. Means not showing the same superscript alphabet in a given column differ significantly at $P < 0.05$.

Group A = Control; Group B = *Clarias gariepinus* induced with 60 mg/L of glyphosate; Group C = *Clarias gariepinus* induced with 80 mg/L of glyphosate; Group D = *Clarias gariepinus* induced with 100 mg/L of glyphosate

Table 3: Levels of reduced glutathione in blood, gills, liver and brain of *Clarias gariepinus* exposed to glyphosate herbicide

	A	B	C	D
Blood (mg %)	100.34±2.20 ^a	73.01±1.78 ^b	74.31±3.37 ^b	72.17±4.96 ^b
Gills (µM)	8.63±0.47 ^a	5.95±0.93 ^b	7.61±0.54 ^c	5.48±0.37 ^b
Liver (µM)	4.74±0.33 ^a	5.60±1.00 ^b	5.89±0.89 ^c	4.44±0.90 ^a
Brain (µM)	9.56±0.78 ^a	5.62±1.30 ^b	6.21±1.26 ^b	5.48±0.38 ^b

All values are expressed as mean±SD of five replicates. Values followed by different alphabet superscript on the same row indicates that there is a significant difference at P < 0.05

Group A = Control; Group B = *Clarias gariepinus* induced with 60 mg/L of glyphosate; Group C = *Clarias gariepinus* induced with 80 mg/L of glyphosate; Group D = *Clarias gariepinus* induced with 100 mg/L of glyphosate

Table 4: Activities of glutathione peroxidase and glutathione-S-transferase in gills, liver and brain of *Clarias gariepinus* exposed to glyphosate herbicide

Tissues	Glutathione Peroxidase (µmol/mg tissue/min)			
	A	B	C	D
Gills	20.45±0.92 ^a	22.36±1.30 ^{ab}	25.63±2.78 ^{bc}	27.94±1.43 ^c
Liver	41.42±2.30 ^a	40.11±1.56 ^a	39.35±1.95 ^a	38.49±2.79 ^a
Brain	16.59±1.08 ^a	17.45±1.82 ^a	13.55±1.76 ^b	8.79±0.92 ^c

Tissues	Glutathione- S- transferase (µmol/mg tissue/minute)			
	A	B	C	D
Gills	39.26±0.99 ^a	38.13±0.82 ^a	35.12±0.77 ^b	36.39±0.44 ^b
Liver	33.52±1.75 ^a	35.79±2.22 ^{ab}	39.12±0.60 ^b	37.86±2.26 ^b
Brain	35.33±0.40 ^a	37.12±0.74 ^b	36.53±0.82 ^{ab}	37.48±0.96 ^b

All values are expressed as mean±SD of five replicates. Values followed by different alphabet superscript on the same row indicate that there is a significant difference at P < 0.05.

Group A = Control; Group B = *Clarias gariepinus* induced with 60 mg/L of glyphosate; Group C = *Clarias gariepinus* induced with 80 mg/L of glyphosate; Group D = *Clarias gariepinus* induced with 100 mg/L of glyphosate

(GSH) in the blood, gills, liver and brain of *Clarias gariepinus* exposed to varying concentrations of glyphosate herbicide as compared with the control fish (Table 3).

The activities of glutathione peroxidase (GP_x) in gill tissues increased (P<0.05) as compared with the control. However, a progressive reduction in the liver and brain GP_x activities has been recorded as compared with the control with respect to increased exposure to glyphosate concentration (Table 4).

Glutathione-S-transferase (GST) activity in gill decreased (P < 0.05) as compared with the control fish. Increase in the concentration of glyphosate from 60 mg/L to 100 mg/L led to a

significant elevation (P < 0.05) of liver and brain GST activities as compared with the control fish (Table 4).

Concentrations of nitric oxide increased significantly (P < 0.05). (NO) in gill (only at 100 mg/L) and brain tissues of fish exposed to glyphosate as compared to the control fish (Table 5). However, NO concentration was not significantly altered in the liver of *Clarias gariepinus*. In serum NO decreased significantly after 80 and 100 mg/L glyphosate (Table 5). Acetylcholinesterase (AChE) activities in the serum, gills and brain of *Clarias gariepinus* exposed to varying concentrations of glyphosate herbicide showed no significant

Table 5: Concentration of nitric oxide in serum, gills, liver and brain of *Clarias gariepinus* induced with glyphosate herbicide

	A	B	C	D
Serum (%)	25.57±4.26 ^a	24.70±3.61 ^a	20.47±1.42 ^b	15.91±3.75 ^b
Gill (%)	18.06±1.23 ^a	15.30±1.49 ^a	17.05±0.73 ^a	30.53±3.54 ^b
Liver (%)	28.88±2.05 ^a	28.67±1.63 ^a	29.26±1.87 ^a	31.36±2.22 ^a
Brain (%)	23.06±1.76 ^a	44.10±3.99 ^b	46.70±4.47 ^b	55.33±1.73 ^c

All values are expressed as mean±SD of five replicates. Values followed by different alphabet superscript on the same row indicates that there is a significant difference at $P < 0.05$.

Group A = Control; Group B = *Clarias gariepinus* induced with 60 mg/L of glyphosate; Group C = *Clarias gariepinus* induced with 80 mg/L of glyphosate; Group D = *Clarias gariepinus* induced with 100 mg/L of glyphosate

Table 6: Activities of acetylcholinesterase in serum, brain, liver and gill of *Clarias gariepinus* exposed to different concentrations of glyphosate herbicide

Groups	Serum (*)	Gill (*)	Liver (*)	Brain (*)
A	11.97±0.02 ^a	12.01±0.06 ^a	11.75±0.27 ^a	12.06±0.20 ^a
B	11.99±0.01 ^a	11.96±0.01 ^a	11.95±0.05 ^b	11.99±0.02 ^a
C	11.98±0.01 ^a	11.97±0.01 ^a	11.97±0.02 ^b	12.00±0.01 ^a
D	11.98±0.02 ^a	11.99±0.02 ^a	11.95±0.03 ^b	11.98±0.34 ^a

Values are expressed as mean ± standard deviation; n = 5. Means not showing the same superscript alphabet in a given column differ significantly at $P < 0.05$. (*) = nmol/mg/min

Group A = Control; Group B = *Clarias gariepinus* induced with 60 mg/L of glyphosate; Group C = *Clarias gariepinus* induced with 80 mg/L of glyphosate; Group D = *Clarias gariepinus* induced with 100 mg/L of glyphosate

change as compared with control fish. Significant ($P < 0.05$) inhibition of AChE was noticed in the liver of fish exposed to glyphosate as compared with the control (Table 6).

The concentrations of malondialdehyde (MDA) in the serum, liver and brain tissues of fish exposed to glyphosate were elevated ($P < 0.05$) as compared with the control fish (Table 7). MDA concentrations in the gills of fish exposed to 100 mg/L glyphosate herbicide were significantly lower ($P < 0.05$) as compared with the control fish (Table 7).

The activities of hepatic enzyme markers - alanine and aspartate aminotransferases (ALT and AST) and alkaline phosphatase (ALP)

were significantly ($P < 0.05$) elevated in the serum of *Clarias gariepinus* exposed to glyphosate herbicide as compared with the control fish.

Discussion

It is an agreed fact that agrochemicals contamination which includes a variety of herbicides is a crisis of worldwide attention, and aquatic habitats are continuously being exposed to herbicide pollutants especially glyphosate (Kelly *et al.*, 2018). This exposure is contemplated a low risk to aquatic ecosystems and may reach freshwater via spray drift, surface run-off and/or soil leachate (Siemering *et al.*, 2008; Contardo-Jara *et al.*, 2009). Fishes could perhaps, be exposed

Table 7: Concentration of malondialdehyde in serum, gills, liver and brain of *Clarias gariepinus* exposed to glyphosate herbicide

Groups	Concentration (Unit/mg tissue)			
	Gill	Liver	Brain	Serum
A	2.22±0.39 ^a	2.12±0.30 ^a	0.86±0.26 ^a	1.84±0.62 ^a
B	2.26±0.39 ^a	2.65±0.36 ^b	0.95±0.22 ^a	1.73±0.23 ^b
C	2.96±0.76 ^a	2.89±0.34 ^b	1.02±0.42 ^b	2.68±0.89 ^b
D	1.93±0.61 ^b	2.98±0.59 ^b	1.69±0.36 ^b	1.92±0.76 ^c

Values are expressed as mean ± standard deviation; n = 5. Means not showing the same superscript alphabet in a given column differ significantly at P < 0.05.

Group A = Control; Group B = *Clarias gariepinus* induced with 60 mg/L of glyphosate; Group C = *Clarias gariepinus* induced with 80 mg/L of glyphosate; Group D = *Clarias gariepinus* induced with 100 mg/L of glyphosate

Table 8: Activities of alanine aminotransferase, aspartate aminotransferase and alkaline phosphatase in serum of *Clarias gariepinus* exposed to glyphosate herbicide

Groups	Concentration (U/L)		
	Alanine aminotransferase	Aspartate aminotransferase	Alkaline phosphatase
A	7.79±1.19 ^a	8.36±1.09 ^a	846.42±13.66 ^a
B	23.28±2.89 ^b	9.28±1.29 ^b	1122.67±12.12 ^b
C	23.22±4.69 ^b	8.90±1.38 ^a	959.92±20.83 ^c
D	21.16±3.66 ^c	11.00±1.59 ^c	1210.90±23.13 ^d

Values are expressed as mean ± standard deviation; n = 5. Means not showing the same superscript alphabet in a given column differ significantly at P < 0.05.

Group A = Control; Group B = *Clarias gariepinus* induced with 60 mg/L of glyphosate; Group C = *Clarias gariepinus* induced with 80 mg/L of glyphosate; Group D = *Clarias gariepinus* induced with 100 mg/L of glyphosate

to contamination doses of glyphosate which is used in different times on different crop patterns in the same regions and therefore, rivers/streams/ponds/swamps receive this contaminant continuously. In the aquatic environment, glyphosate may induce disturbances at cellular and biochemical levels in fish, and assessment of such changes in biochemical enzymes activities as biomarkers could serve as an association between the effects of glyphosate exposure and risk assessment (Dey *et al.*, 2016). Toxicity of glyphosate-based herbicides in fish including metabolic, oxidative, neurotoxic, genotoxic and haematological parameters has been

reported (Modesto and Martinez, 2010 a; Guilherme *et al.*, 2014; Samanta *et al.*, 2014; Sinhoin *et al.*, 2014; Braz -Mota, *et al.*, 2015; Dey *et al.*, 2016; Sobjak *et al.*, 2017; Kurhaluk, 2019). However, the dose-dependant effect of *Uproot* in fish have not been reported.

In this present study, *Clarias gariepinus* was exposed to 60 mg/L, 80 mg/L and 100 mg/L of glyphosate herbicide for 14 days in ambient environment our laboratory and some biochemical parameters including the activities of acetylcholinesterase (AChE), antioxidant defense enzymes, nitric oxide (NO), hepatic enzyme markers (alanine

aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP)) as well as protein content was evaluated in serum, gills, liver and brain tissues of fish. Total protein concentration in serum, gills and liver of exposed fish were significantly reduced in fish exposed to different concentrations of glyphosate as compared to the control fish. However, the protein content of the brain tissues were elevated in exposed fish. The fundamental role of protein in the architecture and physiology of living organism has been reported (Adams *et al.*, 1990). Protein gives information on the regular energy mobilization of an animal and show relationship with the effect of contamination in these organisms and its catabolism is activated in energy production in fish (David *et al.*, 2004), Reduction in protein level in the serum, gills and liver tissues of *Clarias gariepinus* after glyphosate exposure could indicate protein catabolism for meeting the high energy demand for augmentation of defense mechanism of fish against herbicidal stress as a compensatory response. Again, since carbohydrate reserve is decreased during stress condition to accommodate the high energy demand (Sudhanshu and Ajay, 2009) and fish have very limited amount of carbohydrate, the next alternative source of energy is protein to meet the increased energy requirement occasioned by a pollutant. Exposure to glyphosate may have initiated metabolic stress in fish and consequently higher energy demands for metabolic purposes. Impaired food intake, increased energy cost of homeostasis, tissue repair and detoxification mechanism during stress could also cause reduction in total protein content of an organism (Neff, 1995).

Alkaline phosphatase (ALP) together with alanine aminotransferase (ALT) and aspartate aminotransferase (AST) provide an indicator of the degree of inflammation, possible causes of hepatocellular damage, and distortion of the plasma membrane and endoplasmic reticulum (Whitehead *et al.*, 1999; Cappo *et al.*, 2002). The present study indicated that exposure of *Clarias gariepinus* to *Uproot* significantly increased the activities of these enzymes (ALP, ALT and AST) in serum of the fish which may be partly due to hepatic damage resulting from glyphosate induced-oxidative insults in the hepatocytes.

An excellent measure of the condition of fish is the assessment of the oxidative stress level (Sinha *et al.*, 2014) which is understood as a disturbance in the pro- and antioxidant balance (Yadav *et al.*, 2015) and could lead to unfavourable changes such as increases in the level of lipid peroxidation processes as well as oxidative damage to protein and DNA (Kolakowska and Bartosz, 2010). During oxidative stress, ROS such as superoxide radical and hydroxyl radical are produced. The first line of defense antioxidants to curb the menace of ROS is superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) (Ighodaro and Akinloye, 2018). SOD catalytically scavenges superoxide anion and catalase, hydrogen peroxide. In this study, a decrease in the activities of SOD was observed in all tissues with increase in the rate/concentration of exposure to the glyphosate herbicide, while catalase was relatively stable except in the brain tissue where its activity was significantly reduced in fish exposed to 100mg/L glyphosate compared with the control animal. Reduction in SOD activity with respect to exposure to higher glyphosate

concentrations may be related to over utilization of SOD due to the high production of superoxide anions. Increased production of nitric oxide (NO) in fish tissues exposed to glyphosate has been noticed in this study which could also lead to a possible decline in the activities of SOD, since NO can react with superoxide radical to form peroxynitrite anion (Sawyer and Valentine, 1981), which could also lead to the nitration of SOD (Ischinopoulos *et al.*, 1992). The rivalry of SOD and NO for superoxide radical exists, and of critical concern is the rate constant for the reaction of superoxide radical and NO to produce peroxynitrite which is one order of magnitude surpassing that of SOD – catalysed superoxide radical dismutation (Radi, 2018). Therefore, it could be argued that at higher concentration of *Uproot* exposure, the NO produced reacted more with superoxide anion, thus reducing the induction of SOD activity. Peroxynitrite is also viewed as a strong oxidant (Beckman, 1990; Wang and Zweier, 1996) which encourages both nitration and hydroxylation of different bio-organic molecules (Beckman and Koppenol, 1996).

The dismutation product, hydrogen peroxide from the reaction of SOD, is reduced to water and oxygen by CAT (McCord and Fridovich, 1969). CAT activities in the gills and liver of exposed fish were comparable with the control fish. This could be as a result of the inhibition of CAT by high production of superoxide anion (Kono and Fridovich, 1982; Bagnyukova *et al.*, 2006). Superoxide anion is small enough to gain entry to the hemes of catalase and would convert the resting enzyme to the ferro-oxy state (compound III) which is known to be inactive (Chance, 1949).

The high activities of glutathione peroxidase (GPx) in these tissues (gills and brain) could also explain the reduction of CAT, since GPx has been reported to catalysed the reduction of lipid peroxide to corresponding alcohols and free hydrogen peroxide to water by using reduced glutathione (GSH) (Sedaghatfard *et al.*, 2016). From this study it is evident that reduced glutathione was being utilized in all tissues investigated. GPx contains a molecule of selenocysteine in its active site, which is considered to participate directly in electron donation to the peroxide substrate and become oxidized in the process. GSH is used to reproduce the reduced form of the selenocystein by GPx (Ursini *et al.*, 1995).

The potency of the scavenging abilities of the antioxidant enzymes are more pronounced in gills of exposed fish which showed lower concentration of malondialdehyde as compared with the control. MDA has been reported as a marker/measure of free radical damage to lipid, oxidative stress with cytotoxic, genotoxic, mutagenic and carcinogenic properties (Leliuna, 2010; Pocwierz – Kotus *et al.*, 2013).

Glutathione S-transferase (GST) activity plays a vital role in the detoxification of oxidative stress products as well as in the conjugation of glutathione to xenobiotic metabolite to aid their excretion (Schlenk *et al.*, 2008). An elevated activity of GST was observed in liver and brain tissues of exposed fish. This could indicate a defense response to chemical or oxidative stress in cells (Van der Oost *et al.*, 2003). Reduced GST activity in the gills of fish exposed to higher concentrations of the glyphosate herbicide – *Uproot*, may be connected with over utilization of GSH in that tissue; since the gill remains the first contact

point with the external environment and the greatest surface area of the fish in contact with the external environment.

Acetylcholinesterase (AChE) activity is a well confirmed specific biomarker of exposure of fish to organophosphate and carbamate insecticides (Monserrat *et al*, 2002; Pfeifera *et al.*, 2005). Several studies also have shown that formulation containing glyphosate can also inhibit AChE activity in fish (Gluszczak *et al.*, 2006; 2007; Modesto and Martinez, 2010 b). Results of this study indicate that AChE activity was only inhibited in hepatic tissues, but were comparable in the serum, gill and brain tissues, although, with lower values. The length of time of exposure to the herbicide may be of importance when considering the inhibitory effect of glyphosate on fish.

Conclusion

In this study, it was established that the various concentrations (60, 80, and 100 mg/L) of *Uproot* used in the experiment led to significant reduction in total protein concentration. Increased production of NO in tissues of exposed fish may have led to the decrease SOD activity, since NO reacts with superoxide radical anion at a faster rate than SOD. GPx enzyme showed more effectiveness in scavenging hydrogen peroxide than catalase whose activities seemed to have been inhibited by the superoxide anion. GST tended to detoxify glyphosate in gills as evident in its utilization of reduced GSH, while exposure of fish to glyphosate did not appreciably inhibit acetylcholinesterase activity. This may be due to the duration of exposure. In all, the induction of NO and impaired activities of the antioxidant enzymes suggested a disruption of normal antioxidant response in *Clarias gariepinus* exposed to glyphosate, and these

biomarkers could be used as indicators of aquatic environmental contamination.

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