

# Evaluation of the antioxidant markers in clarias gariepinus heart exposed to mercury and ammonium chloride

## Abstract

ROS parameters and antioxidant enzyme activities in heart of *Clarias gariepinus* weighing ( $15 \pm 1.7$  g) in toxic exposure to mercury concentrations, 0.1 mg/ L group B, group C 10 mg/L ammonium chloride and group D 0.1 mg/L mercury and 10 mg/L ammonium chloride for 7 consecutive days at  $26 \pm 1$  °C temperature group A was exposed to water alone and serve as control. At the end of experimental period, Reduced glutathione (GSH), NO levels and the activities of Peroxidase, Superoxide dismutase (SOD) and ALT in heart were assayed. The result showed a significant change ( $P < 0.05$ ) in the activities of ALT, Peroxidase, and SOD. However a significant decrease in the levels of total protein content, GSH and NO were also observed. The results showed that the combined effect of mercury and ammonium had an adverse effect greater than that obtained individually.

## Introduction

Fish constitutes an important aspect of human food due to the high level of quality protein and essential amino acids for the proper growth and functioning of body muscles and tissues. Fish are commonly situated at the top of the food chain and therefore, they can accumulate large amount of toxicants (Alaa *et al.*, 2010). Fish are considered as one of the most susceptible aquatic organisms to toxic substances present in water (Enkeleda *et al.*, 2013). Fish are also usually considered as organism of choice for assessing the effects of environmental pollution on aquatic ecosystem. In nature, aquatic animals are constantly exposed to toxic substances (Ijeoma *et al.*, 2015). Rapid industrial development, as well as the use of metals in production processes has led to the increased discharges of toxic substances into the environment (Borham and Rahimeh, 2011).

Mercury occurs naturally as a mineral and is widely distributed throughout the environment as a result of natural and human activities. Inorganic mercury is the most common form of the metal released by industries in the environment. Mercury (Hg) is a famous contaminant to aquatic life (fish and marine organisms). Hg accumulates in many fish species and causes toxicity to various organs (Bano and Hasan 1990; Jalila *et al.*, 2012). On the other hand Ammonium Chloride ( $\text{NH}_4\text{Cl}$ ) is an inorganic, mildly acidic, white crystalline salt compound that is highly soluble in water. The toxicity of ammonia ( $\text{NH}_3$ ) and its compounds such as Ammonium chloride ( $\text{NH}_4\text{Cl}$ ) has been ascribed to the fact that it can readily diffuse through the gill membranes due to its high solubility (Hegazi and Hasanein 2010). Free radicals and ROS are produced by a variety of oxidative enzymes. Metabolic processes and metal catalyzed oxidation (Halliwell and Gutteridge, 1990). It is therefore of great significance to evaluate pollution effects on fish for both environmental protection and socio-economic reasons (Farombi *et al.*, 2007; Okomoda *et al.*, 2010). That is why this study investigated the toxicities of mercury and ammonium chloride on some antioxidant markers.

## 43 **Materials and methods**

### 44 **Chemicals**

45 Trishydroxymethylaminomethane Hydrochloric acid pyrogallol hydrogen peroxide sulphosalicylic acid  
46 DTNB Bovine Serum Albumin

### 47 **Fish husbandry**

48 *Clarias gariepinus* were obtained from the Niger Delta University fish farm. The fish were  
49 transported in plastic container. Fish of nearly equal size weighing ( $15 \pm 1.7$  g) (24) were  
50 distributed in 40 L plastic aquaria. Four fish were placed in every aquarium. The fish were  
51 acclimated to the laboratory conditions for one week. Group A (Control) Fish received palm  
52 fruits only as diet. Group B Fish received 10 mg/l of Ammonium chloride with palm fruits.  
53 Group C Fish received 0.1 mg/l of Mercury (II) nitrate with palm fruits. Group D fish received  
54 10 mg/l of Ammonium chloride and 0.1 mg/l Mercury II nitrate combined with palm fruits. The  
55 experiment lasted for 7 days. The fish were then sacrificed by a sharp blow on the head. The fish  
56 were immediately dissected and the heart was quickly removed and washed in ice-cold 1.15%,  
57 KCl solution blotted and weighed. They were then homogenized in 9 volumes of homogenizing  
58 buffer (50 mM Tris - HCl mixed with 1.15% KCl and pH adjusted to 7.4), using Teflon  
59 Homogenizer. The resulting homogenate was centrifuged at 10,000g for 20 min in a Beckman  
60 centrifuge at  $-40^{\circ}\text{C}$ . The supernatant was decanted and stored at  $-20^{\circ}\text{C}$  until biochemical  
61 analysis.

### 62 **Protein content**

63 Protein precipitated from 0.1 ml of supernatant with an equal volume of 10% ice-cold trichloroacetic  
64 acid was solubilized in a known volume of 0.1 N NaOH solution. The quantity of protein in the sample  
65 was determined following the method of Lowry et al. (1951) using a standard curve prepared with bovine  
66 serum albumin.

### 67 **Peroxidase activity**

68 The peroxidase activity was measured following the method of Wadhwa et al. (1988). In the  
69 experimental set the reaction mixture included 2.0 ml of 0.1 M potassium phosphate buffer (pH  
70 7.0), 1.0 ml pyrogallol (0.005 M), 1.0 ml of a 0.05 M hydrogen peroxide " $\text{H}_2\text{O}_2$ " solution and 1.0  
71 ml heart supernatant (crude enzyme preparation) in a total volume of 5 ml. The reaction was  
72 started by adding 1 ml of supernatant to each of experimental. After 5 min at  $25^{\circ}\text{C}$ , the reaction  
73 was stopped by adding 0.5 ml  $\text{H}_2\text{SO}_4$  (5% v/v). The optical density was measured at 430 nm in a  
74 JASCO 7800 UV/visible spectrophotometer (Japan). After suitable corrections with the control,  
75 the enzyme activity was expressed as O.D. of purpurogallin formed/mg protein/5 min.

### 76 **Superoxide dismutase (SOD) activity**

77 The SOD activity was measured by the method of Marklund and Marklund (1974). The reaction  
78 mixture consisted of 2.875 ml Tris-HCl buffer (50 mM, pH 8.5), pyrogallol (24 mM in 10 mM  
79 HCl) and 100  $\mu\text{L}$  of heart homogenate in a total volume of 3 ml. The enzyme activity was  
80 measured at 420 nm and was expressed as units/mg protein. One unit of enzyme is defined as the  
81 enzyme activity that inhibits auto-oxidation of pyrogallol by 50%.

### 82 **AST Activity**

83 Briefly, 0.1ml of heart was mixed with phosphate buffer (100mmol/L, pH 7.4), L-aspartate  
84 (100mmol/L), and  $\alpha$ -oxoglutarate (2mmol/L) and the mixture incubated for exactly 30min at  
85  $37^{\circ}\text{C}$ . Then 0.5ml of 2, 4-dinitrophenylhydrazine (2mmol/L) was added to the reaction mixture  
86 and allowed to stand for exactly 20min at  $25^{\circ}\text{C}$ . Then 0.5 ml of NaOH (0.4mol/L) was added  
87 and the absorbance at 546nm read against the reagent blank (Reitman and Frankel 1957).

88

89

## 90 **Glutathione content**

91 The GSH content in liver homogenate was determined by the method of Jollow et al. (1974) in  
92 which 1.0 ml of PMS fraction (10%) was mixed with 1.0 ml of sulphosalicylic acid (4%). The  
93 samples were incubated at 4 °C for at least 1 h and then subjected to centrifugation at 1200g for  
94 15 min at 4 °C. The assay mixture contained 0.4 ml filtered aliquot, 2.2 ml phosphate buffer (0.1  
95 M, pH 7.4) and 0.4 ml DTNB (10 mM) in a total volume of 3.0 ml. The yellow color developed  
96 was read immediately at 412 nm on spectrophotometer. The GSH content was calculated as nmol  
97 of DTNB conjugate formed/g tissue using molar extinction coefficient of  $13.6 \times 10^3 \text{ M}^{-1} \text{ cm}$

## 98 **Nitrite estimation in plasma**

99 Nitrite assay was done using Griess reagent by the method of Green *et al.* 1982 with some  
100 modifications. In brief, 100 µL of Griess reagent (1:1 solution of 1% sulfanilamide in 5%  
101 phosphoric acid and 0.1% naphthylethylene diaminedihydrochloride in water) was added to 100  
102 µL of heart sample incubate for 10 min at room temperature protected from light.  
103 Purple/magenta color began to form immediately. Absorbance was measured at 546 nm, nitrite  
104 concentration was calculated using a standard curve for sodium nitrite, and nitrite levels were  
105 expressed as nmol/mg protein.

106

## 107 **Histopathology**

108 The heart tissues were excised, flushed with saline, cut open longitudinally along the main axis,  
109 and then again washed with saline. These heart sections were fixed in 10% buffered formalin for  
110 at least 24 h and after fixation, the specimens were dehydrated in ascending grades of ethanol,  
111 cleared in benzene, and embedded in paraffin wax. Blocks were made and 5µm thick sections  
112 were cut from the heart. The paraffin embedded liver tissue sections were deparaffinized using  
113 xylene and ethanol. The slides were washed with phosphate buffered saline (PBS) and  
114 permeabilized with permeabilization solution (0.1M citrate, 0.1% TritonX-100). These sections  
115 stained with haematoxylin and eosin and were observed under light microscope at 40x  
116 magnifications to investigate the histoarchitecture of heart.

## 117 **Statistical analyses**

118 Results are presented as means±standard deviation. The statistical evaluation of all data was  
119 done using one-way analysis of variance (ANOVA) followed by Dunnett's test. P values < 0.05  
120 were regarded as statistically significant.

121

## 122 **Results**

### 123 **Table 1**

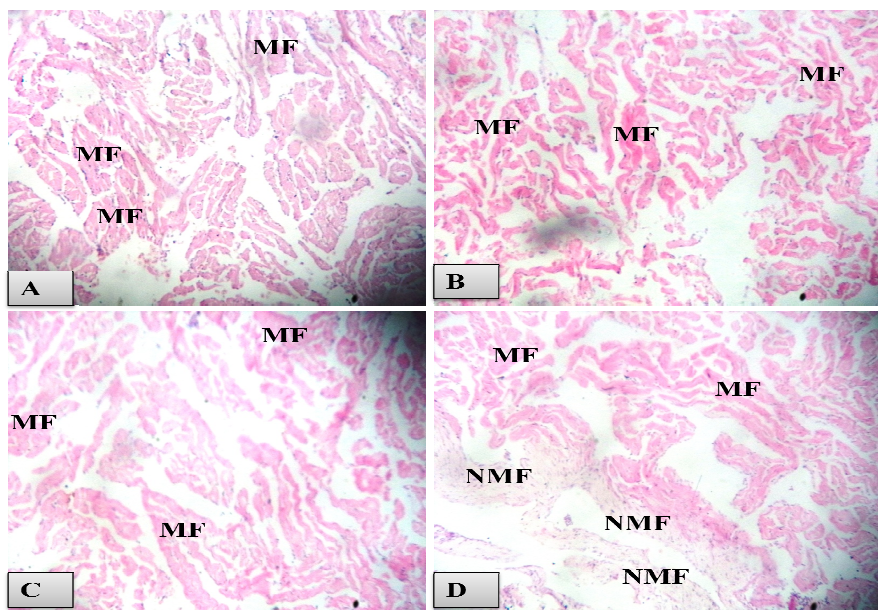
124 Glutathione and nitric oxide levels, Peroxidase, superoxide dismutase and ALT activities in heart  
125 of clarias gariepinus exposed to ammonium chloride and mercury nitrate.

Groups	Protein (mg/ml)	ALT (U/L)	GSH (nmol DTNB/mg protein)	Peroxidase O.D purpurogallin/mg protein	NO (nmol/mg protein)	SOD (U/mg protein)
A	1.03±0.08 <sup>a</sup>	66.92±7.84 <sup>a</sup>	4.08±0.107 <sup>a</sup>	6.13±0.16 <sup>a</sup>	22.19±3.19 <sup>a</sup>	13.45±0.10 <sup>a</sup>
B	0.76±0.16 <sup>b</sup>	89.46±8.61 <sup>b</sup>	1.18±0.28 <sup>b</sup>	6.41±0.70 <sup>a</sup>	44.48±14.71 <sup>b</sup>	7.88±0.19 <sup>b</sup>
C	0.66±0.00 <sup>b</sup>	92.2±5.83 <sup>b</sup>	1.17±0.03 <sup>b</sup>	6.57±0.096 <sup>a</sup>	44.98±0.62 <sup>b</sup>	9.56±0.16 <sup>b</sup>
D	0.49±0.01 <sup>b</sup>	81.20±5.33 <sup>b</sup>	0.63±0.06 <sup>b</sup>	8.52±0.08 <sup>b</sup>	58.16±1.78 <sup>a</sup>	5.65±0.03 <sup>b</sup>

126

127 Values are expressed as mean ±standard deviation of triplet determination. Mean with same  
128 superscript letters on a column are not significantly different (P <0.05). The result showed a

129 significant change ( $P < 0.05$ ) in the activities of ALT, Peroxidase, and SOD. However a  
130 significant decrease in the levels of total protein content, GSH and NO were also observed  
131



132  
133  
134 **Figure 1:** Photomicrograph of Heart: Group A (Control) shows normal cardiac muscle fibres  
135 (MF). Similarly, Groups B and C also displayed normal cardiac tissue architecture. On the  
136 contrary group D show focal area of necrosis (NMF). H&E x100

137  
138 **Discussion**

139 Due to the industrial development, many dangerous chemicals have been released directly or  
140 indirectly which resulted in the polluted water bodies. The African cat fish (*Clarias gariepinus*)  
141 shows high sensitivity towards dissolving toxicants, hence this fish has been utilized as a  
142 biomarker to indicate the existence of toxicant exposure. The present study revealed a significant  
143 variation in the activities of Alanine amino transferase (ALT), reduced glutathione (GSH), Nitric  
144 oxide (NO), peroxidase and Superoxide dismutase (SOD). The changes in these biochemical  
145 parameters indicate that they can be used as indicators of related stress in fish on exposure to  
146 toxic chemicals such as ammonium chloride and mercury (II) nitrate.

147  
148 Peroxidases are haem-containing enzymes that use hydrogen peroxide as the electron acceptor to  
149 catalyse a number of oxidative reactions in this experiment the activity of peroxidase decreases  
150 although catalase is another enzyme that can carry out the same function the results are in line  
151 with the work reported by Wadhwa et al., 1988

152  
153 GSH is a tripeptide, a cellular antioxidant. It protects the heart cells from oxidative stress and  
154 plays an important role in the scavenging reactions by acting both as a nucleophilic of various  
155 undesired compounds and their toxic metabolites and as a specific substrate for the enzymes GPx

156 and GST. The present study reveals that there was a significantly different ( $P < 0.05$ ) from group  
157 A the levels of GSH were high as compared to the other groups but the decrease in the levels of  
158 GSH is more pronounced in group D may be the combined effects of mercury and ammonium.  
159 These results are in line with the research results of Hegazi et al., 2010

160  
161 Elevated activities of heart ALT are a common sign of heart disease. The levels of heart enzymes  
162 are used as diagnostic indicators of heart injury. ALT is one of the most sensitive tests employed  
163 in the diagnosis of heart diseases. The activity of ALT increased significantly from group A –D  
164 this shows that there is an injury and since the activity of ALT is more in group D the injury is  
165 much in D.

166  
167 Superoxide anion formed from aerobic respiration is scavenged by Super Oxide Dismutase  
168 (SOD) leading to the formation of hydrogen peroxide ( $H_2O_2$ ) and in the presence of reduced  
169 transition metals (such as iron) reactive hydroxyl radical ( $OH\bullet$ ) is produce through the Fenton  
170 reaction. The decreased activity of SOD in groups B-D indicates that there are more ROS in the  
171 form of super oxide that has no been scavenged by this enzyme due to the toxicity of mercury or  
172 ammonium or both. These findings are also in tune with the recent works of Hegazi et al., 2010

173  
174 NO (nitric oxide) is another important mediator in the pathogenesis of inflammatory diseases.  
175 The levels of this molecule are higher in groups B-D these results shows that many inflammatory  
176 enzymes are produced but that less of these molecules are produced in group A. this shows that  
177 the toxicity of mercury, ammonium or the combination of both is lined with inflammation. These  
178 are in consonant with the work reported by Eboh et al 2016 even though nitric oxide was assayed  
179 on the skin of rats.

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