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Hepatic Antioxidant Status and Organ indices of *Oreochromis niloticus* Exposed to Sub-lethal Concentrations of *Anacardium occidentale* Linn; Aqueous Bark Extract

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Abstract

The hepatic antioxidant status and organ indices in Oreochromis niloticus exposed to sub-lethal concentrations of Anacardium occidentale aqueous bark extract (Piscicide) were assessed. Based on the 96h LC50 value of 0.775ml/L, three sub-lethal concentrations of 0.03875ml/L, 0.0775ml/L and 0.155ml/L corresponding to 1/20th, 1/10th and 1/5th respectively of the LC50 value, were selected for exposure to fish. Liver tissues of the fish were sampled on day 1, 7, and 14 for assessment of hepatic antioxidant responses and hepatosomatic indices. Results indicated that the effect of A. occidentale aqueous bark extract on the antioxidant enzymes examined were both dose and duration dependent. The levels of lipid peroxidation, superoxide dismutase, and catalase increased significantly ($p<0.05$) in a time and concentration dependent manner. Activities of glutathione peroxidase and reduced glutathione in the liver decreased with increase in concentration and time of exposure. The hepatosomatic index of the fish decreased significantly ($p<0.05$) with increased concentration and exposure duration of the piscicide. However, condition factor of the exposed fish were not affected. The results of this study show that the aqueous extract of A. occidentale bark extract enhances the production of reactive oxygen species and thus could lead to oxidative damage in exposed fish.

Keywords: Botanical piscicide, Condition factor, Hepatosomatic index, Lipid peroxidation

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Introduction

Recently, environmental contaminants have increased considerably due to industrial and agricultural activities (Gopi *et al.,* 2019). Rivers are highly prone to material loadings that can result in pollution. Biodiversity-rich freshwater ecosystems are currently declining faster than marine or land ecosystems, making them the world's most vulnerable habitats (WWFN, 2008). Their sustainability is being threatened by anthropogenic factors (WHO, 2003; UNFPA, 2003). Anthropogenic activities such as industrial, agricultural, domestic activities and urbanization processes give rise to pollutants, which are introduced into the surface waters through point and non-point sources mechanisms (UNFPA, 2003; Boyd, 2020). The addition of various kinds of pollutants and nutrients through agricultural runs-off into the water bodies brings about a series of changes in the physico-chemical characteristics of surface and ground water (Kumolu-Johnson et al., 2005; Boyd, 2020). This could be inimical to the aquatic lives there in, fish inclusive.

Fishermen in their quest to meet up with their needs have resort to the use of botanicals in hunting fish, and plants from different families have been applied for catching fish all over the world. As reported by Gabriel et al. (2009), most of these plants contain compounds of various classes that have insecticidal, piscicidal and molluscicidal properties. Phytochemical assays revealed that most of these plants contain flavonoids, saponins, tannins, glycosides and alkaloids (Obomanu *et al.,* 2005). The toxic parts of plants employed as fish poisons as reported by Fafioye (2005) include the roots, seeds, fruits, barks or leaves. Extracts from more than 60,000 species of plants are used for different purposes in the world (Audu, *et al.,* 2014) and approximately 1,190 pure chemical substances extracted from higher plants are used in medicine throughout the world (Fafioye, 2005). Ekpendu, *et al.* (2014) identified Anacardium occidentale as one of the piscicidal plants in Nigeria employed by fishermen for fish hunting. Although, most of these piscicidal plants are biodegradable, less severe than synthetic chemicals and are easily reversed in fish subjected to chronic concentration (Kela *et al.,* 1989), they are reported to modulate antioxidant defence systems and to cause oxidative stress through production of reactive oxygen species (ROS) (Dar et al., 2014; Nwani *et al.,* 2016). These free radicals at excess concentrations react with biological macromolecules to increase the level of lipid peroxidation, protein denaturation and alterations in the activities of antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD), Glutathione reductase (GR), Reduced Glutathione (GSH) and Glutathione Peroxidase (GPx). These

antioxidant enzymes are involved to counteract the toxicity of ROS (Orbea et al., 2002) thereby preventing the cells and tissues from oxidative damage. Therefore, the present study seeks to assess the effect of aqueous extract of A. occidentale bark on the responses of hepatic antioxidant enzymes in Oreochromis niloticus juveniles.

Materials and Methods

Procurement and management of the experimental fish

A total of 360 *O. niloticus* (Family: Cichlidae) of average ± SD weight 25.32±7.36g and length of 9.08±1.56cm were procured from Michael Okpara University of Agriculture Umudike (MOUAU) Fish Farm, Abia State, Nigeria. They were transported in a stress free manner to the Wet Lab, Department of Fisheries and Aquatic Resources Management, MOUAU where the research was carried out. The procured fish were treated with 0.05% KMnO4 as recommended by Nwani *et al.* (2016) for two minutes to avoid any dermal infections. The fish were acclimated for 2 weeks in fibre glass tank of 300 litres capacity before the commencement of the experiment. During the period of acclimation, the water in the tank was renewed after two days with well aerated borehole water. Commercial diets of 42% crude protein were fed to the fish twice per day at 1% body mass. Daily siphoning off of uneaten feed and fish metabolite was carried out to ensure hygienic condition of the acclimation media. Dead fish were also removed with plastic forceps to avoid possible deterioration of the water quality. Feeding was terminated 24hrs prior to range finding and acute toxicity tests to avoid interference of faeces (Ward and Parrish, 1982; Reish and Oshida, 1987).

Collection of plant material and preparation of aqueous stock solution

A. occidentale L. barks were collected in the morning hours between 6:00am and 10:00am before the commencement of photochemical reactions in the plant at noon (Ogunkule and Tonia, 2006). The sample was procured behind the senior staff club canteen MOUAU and was authenticated by a botanist in the Department of Forestry and Environmental Management, MOUAU.

The botanical was prepared according to the method of Ekpendu et al. (2016) by cutting the plant into smaller pieces and then oven drying at 75 0C to make it bristle before pounding into powder with clean mortar. The powder was sieved with a 40 micron sieve to obtain fine powder which was stored in an airtight container.

The stock solution of the botanical was prepared as described by Fafioye

(2005). Fifty (50) g of the botanical powder was dissolved in 1L of water in a transparent jerry-can of 5L volume and left to ferment for 3 days and finally filtered with a muslin cloth to obtain the aqueous extract.

Experimental procedure for sub-lethal exposure and tissue preparation

After acclimation and range finding, 12 plastic tubs (25l capacity each) were used to determine the LC50. Ten (10) litres of water was poured in each of the tubs. A total of 45 fish was randomly exposed to 3 different concentrations that were obtained after range finding test as suggested by Krishna and Hayashi (2000), without regard to sex. Another set of 15 fish was simultaneously maintained with equal amount of borehole water but without the test piscicide (placebo) and considered as the control. The experiment was performed in triplicate containing 5 fish in each replicate to determine the LC50 values of the test piscicide for the species at 24, 48, 72 and 96h using the probit method (Finney, 1971) which gave rise to 0.775ml/L. Based on this value, three different concentrations of A. occidentale L. aqueous extract; 0.03875ml/L, 0.0775ml/L and 0.155ml/L corresponding to 1/20th, 1/10th, and 1/5th of the 96h LC50 of the botanical respectively were used for the sub-chronic exposure. Each treatment was stocked with 30 fish in a 25l capacity transparent plastic tank and was set in triplicate. Another set of fish was also setup but without the test piscicide and considered as the control. The fish were exposed for 14 days and was sampled on day 1, 7 and 14. The physicochemical properties of the test media was analyzed on daily basis (APHA, 2005) and the means were found as follows: temperature 26.16 ± 1.10 0C, pH 7.21 ± 0.13, dissolved oxygen 6.94 ± 1.34 mg/L and electrical conductivity 271µS/cm. On each sampling day the fish sample to be sacrificed was carefully brought out of the test container with a hand net and anesthetized with tricaine methanesulfonate (MS 222) to minimize stress. It was weighed with a digital sensitive weighing balance (Mettler Toledo PL303) and the standard length (cm) was taken with a calibrated measuring board, the fish sample taken was dissected with a surgical blade and the liver carefully taken, weighed and stored in a sample bottle containing saline solution and taken to the laboratory for antioxidant assay.

Estimation of lipid peroxidation

Lipid peroxidation was determined by the estimation of thiobarbituric acid reactive substances (TBARS) as described by Sharma and Krishna-Murti (1968). The TBARS concentration was measured by the absorption at 535nm at molar extinction coefficient of 156nM/cm. The specific activity was expressed in nano moles of TBARS/mg protein.

Antioxidant enzymes assays

Preparation of post-mitochondrial supernatant (PMS): This was done according to Dabas *et al.,* (2012). Tissue samples of liver, kidney and gill were homogenized in chilled sodium phosphate buffer (0.1 M, pH 7.4) using Potter–Elvehjem homogenizer. The homogenate was centrifuged at 10,000g for 10 min at 4 0C using Sigma refrigerated centrifuge (model 3K30), and the resulting supernatant was centrifuged again at 12,500g for 10 min at 4 0C to prepare PMS, which was further used for various biochemical analyses. Biochemical measurements were taken using a dual-beam T70 UV/vis spectrophotometer (PG instrument Ltd.) at 25 ± 0.5 0C.

Reduced glutathione (GSH): (Brehe and Burch, 1976) Unit: µ mole/g

Procedure: 0.5ml of the supernatant was taken in a tube and similarly, blank and standards was prepared by taking 0.5ml of distilled water and 0.5ml of GSH standard respectively. To all the tubes, 4.5ml of tris buffer and 0.5 ml of DTNB solution was added. After 6 minutes OD was read.

$$\textbf{Calculation: } GSH = \frac{OD \text{ of unknown}}{OD \text{ of known}} \times 20$$

Superoxide dismutase (SOD): (Misra and Fridovich, 1972) Unit: U/mg protein

Procedure: Assay mixture containing 0.5ml bicarbonate buffer, 0.5ml EDTA, 50µl sample, and 1ml of distilled water was incubated for 5 minutes at room temperature. Reaction started by the addition of 0.3 ml epinephrine and absorbance was recorded at 480nm for 3 minutes. The activity expressed in U/mg protein.

$$\textbf{Calculation: } SOD = \frac{\Delta ODofControl - \Delta ODofTest}{\Delta ODofControl \text{ x } mgprotein} \times 100$$

Catalase (CAT): (Aebi, 1984) Unit: µ mole H202/min/mg protein.

Procedure: A typical reaction mixture containing 1ml 50nM potassium phosphate buffer (pH 7.4) was added to 25µ1 of the sample. Reaction was initiated by the addition of 1 ml H2O2. Amount of H2O2 consumed was determined by recording absorbance of solution at λ 240 nm and the activity was expressed as µ mole/min/mg protein. Catalase was calculated using the formula below:

$$Catalas\ e = \frac{\Delta \text{ OD of Test x total vloume x } 1000}{43.6 \text{ x } 0.05ml \text{ sample x mg protein}}$$

Organ indices

The condition factor (k) and hepatosomatic index (HSI) of the fish were calculated using the method described by White and Fletcher (1985) as follows:

K= Body weight (g)/Standard length (cm) 3 × 100

HSI = Liver weight (g)/Body weight (g) × 100

Statistical analysis

The data obtained were analyzed statistically using the statistical package SPSS computer program. The data were subjected to analysis of variance (ANOVA) to determine significant differences at ($p<0.05$). Means were separated using the Fisher's Least Significant Difference (LSD) at 5% level.

Results

Condition factor (K) and Hepatosomatic index (HSI) indices of O. niloticus exposed to sub-lethal concentrations of A. occidentale

The result of the effect of A. occidentale on the condition factor and HSI of O. niloticus is presented in table 1. The K-factor in A. occidentale exposed fish was not significantly different from the control ($p > 0.05$) throughout the duration of exposure (14 days). However, a significantly lower HSI ($p < 0.05$) was observed in the exposed fish in a dose and duration dependent manner.

Table 1: Hepatosomatic index and Condition factor of O. niloticus juveniles after exposure to sub-lethal concentrations of aqueous extract of A. occidentale bark for 14 days

		Duration (Days)		
Parameters	**Conc. (ml/L)**	**1**	**7**	**14**
HSI	Control (0.0)	1.91±0.16^{a1}	1.79±0.14^{a1}	1.90±0.18^{a1}
	0.03875	1.84±0.19^{a1}	1.38±0.29^{b2}	0.91±0.77^{b3}
	0.0775	1.92±0.14^{a1}	1.01±0.56^{b2}	0.61±0.46^{b3}
	0.155	1.94±0.18^{a1}	0.71±0.24^{c2}	0.45±0.77^{c3}
K	Control (0.0)	2.27±0.12^{a1}	2.13±0.11^{a1}	2.05±0.09^{a1}
	0.03875	2.28±0.22^{a1}	1.97±0.23^{a1}	2.14±0.78^{a1}
	0.0775	2.30±0.13^{a1}	2.12±0.17^{a1}	1.95±0.11^{a1}
	0.155	2.28±0.09^{a1}	2.02±0.27^{a1}	1.97±0.13^{a1}

Values with different alphabetic superscripts differ significantly ($p<0.05$) among concentrations within the same duration. Values with different numeric superscripts differ significantly ($p<0.05$) among duration within the same concentration.

Lipid peroxidation and antioxidant enzyme status of O. niloticus exposed to sub-lethal concentrations of A. occidentale

The effects of A. occidentale aqueous bark extract on the membrane LPO measured as the TBARS in the liver tissue of O. niloticus is presented in Table 2. The induction of LPO in the liver tissue of O. niloticus exposed to the piscicide was both duration and dose dependent with the lowest TBARS formation recorded on the first day (day 1) of the exposure. There was an increase of TBARS formation in the liver tissue of the fish exposed to 0.155 ml/L of A. occidentale aqueous bark extract from 7.28 nmol/protein on day 1 to 8.48 nmol/protein on day 14. The activities of CAT increased significantly ($p<0.05$) from 4.01 unit/mg protein on day 1 to 12.81 unit/mg protein on day 14 in the liver tissue of O. niloticus exposed to 0.155 ml/L concentration of A. occidentale aqueous bark extract. Although, the activities of SOD increased numerically with increase in concentration and duration of exposure, they were found not to be significantly ($p>0.05$) different when compared to the control. Compared to the control, GPx activities in the liver tissue of O. niloticus exposed to sub-lethal concentrations of A. occidentale decreased with increase in concentration and duration of exposure to the piscicide. The activities of the GSH in the tissue of fish exposed to the piscicide followed the same trend as that of GPx activity.

Table 2: Effects of *A. occidentale* aqueous bark extract on lipid peroxidation and antioxidant enzyme activities in the liver tissues of *O. niloticus*

		DURATION (DAYS)		
PARAMETER	**CONCENTRATION (ml/l)**	**1**	**7**	**14**
TBARS (nmol/protein)	Control (0.0)	3.76±2.65^{a1}	3.22±0.26^{a1}	3.49±0.12^{a1}
	0.3875	5.84±0.52^{b1}	6.11±0.31^{b2}	7.54±0.31^{b3}
	0.0775	7.09±0.98^{c1}	8.46±0.59^{c2}	8.66±0.44^{c2}
	0.155	7.28±1.26^{c1}	8.48±0.58^{c2}	8.48±0.68^{c2}
CAT (unit/mg protein)	Control (0.0)	3.40±0.10^{a1}	3.46±0.71^{a1}	4.11±0.53^{a1}
	0.03875	3.87±0.22^{b1}	3.98±0.96^{a1}	7.00±4.72^{b2}
	0.0775	3.94±0.12^{b1}	4.03±0.45^{a1}	8.95±4.36^{c2}
	0.155	4.01±0.26^{b1}	4.12±0.67^{a1}	12.81±0.51^{d2}
SOD(unit/mg protein)	Control (0.0)	10.71±0.06^{a1}	11.47±0.01^{a1}	11.48±0.08^{a1}
	0.03875	10.89±0.37^{a1}	11.48±0.01^{a1}	11.49±0.02^{a1}
	0.0775	10.92±0.13^{a1}	11.48±0.02^{a1}	11.53±0.08^{a1}
	0.155	11.11±0.03^{a1}	11.49±0.01^{a1}	11.59±0.06^{a1}
GPx(nmol/mg protein)	Control	147.32±28.64^{c1}	149.12±4.56^{c1}	146.32±32^{c1}
	0.03875	134.60±9.54^{b2}	127.46±12.80^{b1}	125.01±14.88^{b1}
	0.0775	132.20±9.48^{b3}	127.27±7.08^{b2}	122.40±4.08^{a1}
	0.155	129.70±5.07^{a2}	120.72±15.51^{a1}	120.17±6.20^{a1}
GSH (mg/protein)	Control (0.0)	2.71±0.46^{b1}	2.81±0.23^{b1}	2.60±0.26^{b1}
	0.03875	2.38±0.12^{a2}	2.00±0.50^{ab1}	1.96±0.16^{a1}
	0.0775	2.12±0.15^{a1}	9.99±0.40^{ab1}	1.77±0.41^{a1}
	0.155	1.93±0.06^{a2}	1.69±0.43^{a1}	1.56±0.47^{a1}

Values with different alphabetic superscript (lower case) differ significantly ($p < 0.05$) among different concentrations. Values with different numeric superscripts differ significantly ($p < 0.05$) among exposure durations.

Discussion

Several physiological and biochemical alterations have been reported in fish exposed to differentbotanical extracts (Devados *et al*., 2013, Audu *et al.,* 2015). Results of the present study indicated that exposure to sub-lethal concentrations of *O. niloticus* aqueous bark extract significantly increased levels of LPO in the liver of *A. occidentale,* thus reflecting oxidative damage in the liver tissue of the exposed fish. The formation of TBARS is as a result of concentration increase and the LPO lowest TBARS formation was observed on the first day of exposure which is comparable to the control. This finding is in agreement with the report of Nwani *et al.* (2016) when they exposed *C. gariepinus* to *Psychotoria macrophylla.* Piner and Uner (2013) had similar report. The elevations in the activities of CAT observed in this study could be an adaptive mechanism against stress and thus suggests the ability of *A. occidentale* aqueous bark extract to induce stress in *O. niloticus* (Gate *et al.,* 1999; Nwani *et al.,* 2015). Gate *et al.* (1999) reported that the increase in CAT activity in exposed animals to toxicant is a response to the damaging effect of H_2O_2 which results from degradation of anion superoxide by SOD. This fact may also be a plausible explanation to the increased activity of CAT in the exposed *O. niloticus*. Though SOD increased continuously throughout the experiment, however, it was unable to eliminate ROS and thus, suggest that SOD may have been suppressed in the liver and also as a result of reduction in GPx (Nwani *et al.,* 2015). The reduction in GPx in this study may possibly be due to its increased utilization in combating excessive oxidative stress. Dabas *et al.* (2012) reported that GPx decreases as CAT activities increased and the reduction in the GSH and induction of

LPO as observed in the present study is an indication of oxidative damage resulting from cell damages. Similar results have been documented by many authors (Stephensen *et al.,* 2002; Dabas *et al.,* 2012; Srikanth *et al.,* 2013; Dar *et al.,* 2014). This therefore, suggest that *A. occidentale* can increase oxidative stress and may react with bio-molecules to continually produce ROS and nitrogen, thus, reducing antioxidant status which was supposed to scavenge these free ROS that are continually produced in metabolic cells such as the liver in response to stress (Nwani *et al.,* 2015). In this study, condition factor (K) of the exposed fish decreased which shows that *A. occidentale* has a negative effect on its well-being. The HSI and VSI increased as experiment progresses which may indicate that the overall condition of the fish may be affected by *A. occidentale*.

In conclusion, even though *A. occidentale* has the potential to stupefy fish for greater catch, the results of the present investigation indicate that *A. occidentale* is toxic to fish and has the potential to impair the physiological activities of *O. niloticus.* Hence, suggesting that presence of *A. occidentale* in the aquatic ecosystem may be potentially harmful to the fish population and other aquatic biota. Therefore the indiscriminate use of *A. occidentale* and other botanicals should be strongly controlled and carefully monitored to avoid the possible damage it would cause the aquatic environment. Further studies on the constant effect of *A. occidentale* should be carried out.

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