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Serum Enzymes as a Result of *Azadirachta indica* Extract Injection to African Catfish *Clarias gariepinus* (Burchell, 1822)

Jessa E. Ochuko*, Kori-Siakpere Ovie and Ikhojie I. Faith

Department of Animal and Environmental Biology, Delta State University, P.M.B. 1, Abraka, Nigeria

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Abstract

The aim of this study is to evaluate the effect of the concentrations (0.0, 0.5, 1.0, 2.0 and 4.0g/L) of the leaves of *Azadirachta indica* for seven (7) days on the serum enzymes acid phosphatase (ACP), alkaline phosphate (ALP), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) of African catfish *Clarias gariepinus* which is widely cultured in Nigeria because of its remarkably fast growth rate and high market value. The level of serum ACP , ALP, ALT and AST revealed a significant decrease ($P<0.05$). In this study, the general inhibition of ALT, AST, ALP implies that there were hepatic disorders (liver disease) or renal injury, a disruption of the activity of the TCA cycle, respiratory process and glycolytic pathways. The present study, thus, tries to provide baseline information on the involvement of these biochemical parameters in toxicity assessment of aquatic system and also on the alteration of the enzymatic parameters maybe used in assessment of these plant extract.

Keywords: *Azadirachta indica*, African catfish, Serum, Enzymatic Parameters.

1. Introduction

The piscicidal plants are of a unique importance in the sense that their chemical compositions enhance their properties as medicinal plants, preservatives, insecticides, molluscidices, to mention a few, hence their usefulness to man and aquatic animals (Akinwade *et al*., 2007). Due to their narcotic, pesticidal and molluscidal properties, many fishermen and fish farmers indiscriminately use various parts of these plant extracts to weaken and kill the fishes for easy catch and clean up the aquatic systems of some pests. Invariably stronger concentrations than necessary are used and this could lead to a physiological disturbance of the aquatic organism and ultimately to a reduction in the aquatic productivity (Mondal *et al*., 2007). Some of the plants used are non-selective in their destruction, thereby interfering with the ecological balance of the immediate environment. The usefulness of these plants for piscicidal and medicinal purposes has been reported (Akobundu, 1987; Adewole *et al*., 2002).

The neem plant, *Azadirachta indica* (L) of the Family Meliaceae and native of eastern Asia is a known medicinal plant that contains margosine, eriterperenoid, azatin, rotinine and quinine among other active ingredients as reported by Ade-Serrano (1982) and Adewole *et al*. (2002). The leaves, barks, fruits and roots of the plant have been highly appraised for their medicinal purposes. As a natural insecticide, the plant contains tetranitroterpenoid compounds, known as meliatoxins that are highly toxic to insects and mammals (Ascher *et al*., 1993). Omoregie and Okpanachi (1992) and Oti (2003) reported on the sublethal and actute effects of water extract of the bark of *A. indica* on *Tilapia zillii*, mudcatfish hybrid and African pike. Neem is the vernacular name used in this part of the world; in Nigeria, it is 'dongoyaro' (Brahmachari, 2004). Martinez (2002) stated that aqueous extract of neem leaves and other neem-based products have been extensively used in fish-farms as an alternative for the control of fish parasites and fish fry predators like dragon-fly larvae.

The African Catfish *Clarias gariepinus* is the most suitable species for aquaculture in Africa. *C. gariepinus*, which is widely considered one of the most important tropical catfish species for aquaculture, has a Pan-African distribution, from Nile to West Africa and from Algeria to South Africa. The African catfish has a high growth rate; exposure of this catfish to these biocides may cause stress without necessarily leading to death. Stress response is characterized by biochemical and physiological changes which may be manifested in both acute and chronic toxicity tests (Singh and Singh, 2002; Tiwari and Singh, 2004). The disruption of the biochemical and physiological integrity is assessable by the changes in the enzyme activities in functional organs (de la Torre *et al*., 2000; Van Der Oost *et al*., 2003).

* Corresponding author. e-mail: ochukojessa@gmail.com.

Enzymes are biochemical macromolecules that control the metabolic process of organisms, thus a slight variation in enzyme activities would affect the organism (Roy, 2002). They are indispensable for signal transduction and cell regulation, often via kinases and phosphatases. The activities of alkaline phosphatase, alanine aminotransferase, aspartate and aminotransferase, are useful marker enzymes of damage to the liver and kidney (Akanji *et. al*., 1993).

The complex of unspecified biochemical indicators of blood and organs reveals the general effect of pollutants and toxin on fish makes it possible to forecast the consequences of the long-term exposure to chemical pollutants (Adedeji *et al*., 2009). Moreover, evaluation of blood biochemistry was considered as a useful tool for the diagnosis of diseases and assessing the physiological status of fish (Stoskopf, 1993). Many studies have investigated changes in many physiological and biochemical, blood and organ indices induced by environmental conditions and the presence of contaminants (Kori-Siakpere *et al*., 2006; Maheswaran *et al*., 2008; Ololade and Oginni, 2010). The biochemical parameters in fish are valid for physio-pathological evaluation and sensitive for detecting potential adverse effects and relatively early events of pollutant damage (Almeida *et al*., 2002; Matos *et al*., 2007; Osman *et al*., 2010). Little attention has been given to the enzymatic effect of *A. indica* on the African catfish *C. gariepinus.*

Hence, the present study was conducted to evaluate the effect of the concentrations of *A. indica* on the serum enzymes of African catfish *C. gariepinus* which is widely cultured in Nigeria because of its remarkably fast growth rate and high market value.

2. Materials and Methods

2.1. Experimental Animals

Tank-raised *C. gariepinus* (mean total length 31.75 ± 0.47 cm; mean weight, 183.26 ± 13.85g) were obtained locally from a commercial fish farm. They were transferred to the Animal and Environmental Biology Research Laboratory, Delta State University, Abraka, Nigeria. The fishes were held in the laboratory in large plastic aquaria of 140L capacity with clean borehole water. They were then acclimatized for 14 days during which they were fed to satiation with commercial fish feed pellets (Coppens 4.0mm; 35% crude protein diet) twice on a daily basis. Uneaten food and faecal matters were removed daily during the acclimation and experimentation period. Dead fish were also promptly removed to avoid contamination. The percentage of death recorded during acclimatization was less than 2% as such the fishes were accepted as being adapted to the laboratory conditions.

2.2. Plant Material

Fresh leaves of *A. indica* were collected from within the campus of the Delta State University, Abraka and transported to the Department of Animal and Environmental Biology Laboratory. The plant was identified as *A. indica* by Dr (Mrs.) N. E. Edema of the Department of Botany, Delta State University, Abraka, Nigeria. They were air-dried for two weeks and later oven-dried for three hours at 60°C to a constant weight. The dried leaves were ground into powder with an electric blender (MX – 2071, Nakai Japan), sieved and the fine powder was stored in a dry airtight container. An aqueous extract was prepared by weighing out 200g of the milled powder leaves of *A. indica*, adding in 200ml of distilled water in a 500ml beaker and stirring vigorously with a glass rod. The combination was then allowed to settle for 3 hours using the infusion method. The extract was then filtered using Whatman No. 1filter paper. Soxhlet extraction was found to give a higher yield of the extract. The extract was then concentrated by evaporation to dryness using a rotary vacuum evaporator (RE52-2, Benjing China) at a temperature of 40^{o}C. A dark-grey colored mass was obtained and stored in airtight bottles at 4^{o}C in a refrigerator until ready for use.

2.3. Toxicant Preparation

The stored extract was reconstituted using distilled water to obtain extracts of stock solution of 10g/l of the aqueous solution of *A. indica.* From this stock, four test concentrations (0.5, 1.0, 2.0, and 4.0g/L) were prepared by serial dilution for injection of the fish.

2.4. Experimental Procedure

After acclimatization, the experimental fish were divided into six (6) groups (10 specimens per aquaria with replicates) to assess the sub-lethal effect of *A. indica* on serum enzymatic parameters. A dose of two ml of the extract for each concentration (0.0, 0.5, 1.0, 2.0 and 4.0g/L, respectively) was injected intramuscularly above the lateral line of the fish and then introduced into their respective aquaria. Fish in the control were injected with the same dose of distilled water. The aquaria used for the experiments were made of plastic having 140 L capacity. The injected fish and control fish were kept separately throughout the experimental period.

Borehole water was used throughout the acclimation and experimental periods. The water quality parameters of the exposure water used in the tests and control were determined by standard methods (APHA, 1998), as presented in Table 1.

Table 1. Water quality parameters

Parameter	Values
pH	7.58±0.32
Temperature (^{o}C)	28.30±1.3
Dissolved oxygen (mg L^{-}1)	8.32±1.04
Free carbon dioxide (mg L^{-1})	4.85±0.08
Alkalinity (mg L^{-1})	36.50±1.72
Hardness (mg L^{-1})	134.53±11.75

2.5. Sampling Procedure

At the end of the exposure period of seven (7) days, the fish were taken from the control and test tanks, sacrificed and subjected to the analysis.

Six fishes were caught individually in a small hand net from the containers. After the preliminary investigation of the length and weight, the fish were then placed belly upwards and blood samples obtained from the caudal

circulation with the aid of a heparinised 2cm^3 disposable plastic syringes and a 21 gauge disposable hypodermic needle. Serum was obtained from blood samples by centrifugation after coagulation and then drawn into a 1 cm^3 plastic syringe and transferred into a universal bottle, diluted 1:20 with deionised water. The diluted serum was then stored in a refrigerator and used later for analysis of serum enzymes: alkaline phosphatase, alanine aminotransferase, aspartate and aminotransferase. All determinations were carried out in duplicates for each sample.

2.6. Enzyme Analyses

The various serum enzymes: acid phosphatase, alkaline phosphate, alanine aminotransferase and aspartate aminotransferase were all determined spectrophotometrically, using Teco Diagnostic, Anahein, SA commercial kit, following the manufacturer's instruction with the aid of a spectrophotometer.

2.7. Data Analysis

The results obtained were subjected to analysis for mean and standard error. The mean values of the treatment were subjected to statistical analysis using one-way analysis of variance (ANOVA) to test the level of significance between the various concentrations of *A. indica.* Multiple comparisons of the means were analyzed with the Dunnet's Test. All statistical analysis was performed using the software programme (GraphPad Prism® Software version 5.0, San Diego, CA). Results were considered significant at the 95% confidence level $P < 0.05$.

3. Results

3.1. Acid Phosphatase

The level of serum acid phosphatase in *C. gariepinus* is presented in Figure 1. The level of acid phosphatase showed a significant decrease ($P<0.05$) in fish injected with 0.5g/L, 1.0g/L, 2.0g/L and 4g/L of the crude extract of the leaves when compared with the control using Dunnet's Multiple Comparism Test.

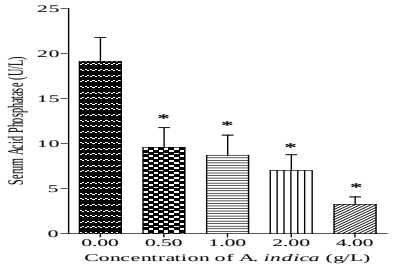

Figure 1. Mean values of acid phosphatase in the serum of *C.* Each column represents the mean value, and vertical bars indicate the standard error of the means. Asterisk represents the significant difference between the control and experimental groups at (*P<0.05*) level.

3.2. Alkaline Phosphatase

The level of serum alkaline phosphatase in *C. gariepinus* is presented in Fig. 2. The level of alkaline phosphatase showed an insignificant decrease (*P>0.05*) in fish injected with 0.5g/L, 1.0g/L, 2.0g/L and a significant decrease (*P<0.05*) in 4g/L of the crude extract of the leaves when compared with the control using Dunnet's Multiple Comparism Test.

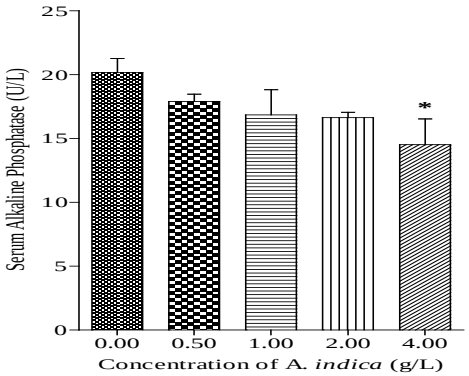

Figure 2. Mean values of alkaline phosphatase in the serum of *C. gariepinus*

3.3. Alanine Aminotransferase

The level of serum alanine aminotransferase in *C. gariepinus* is presented in Fig. 3. The level of alanine aminotransferase showed an insignificant decrease ($P>0.05$) in fish injected with 0.5g/L, 1.0g/L and a significant decrease ($P<0.05$) in 2.0g/L and 4.0g/L of the crude extract of the leaves when compared with the control using Dunnet's Multiple Comparism Test.

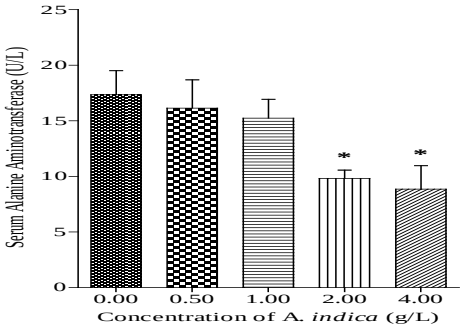

Figure 3. Mean values of alanine aminotransferase in the serum of *C. gariepinus*

3.4. Aspartate Aminotransferase

The level of serum aspartate aminotransferase in *C. gariepinus* is presented in Fig. 4. The level of aspartate aminotransferase showed a significant decrease ($P<0.05$) in fish injected with 0.5g/L, 1.0g/L, 2.0g/L and 4g/L of the crude extract of the leaves when compared with the control using Dunnet's Multiple Comparism Test.

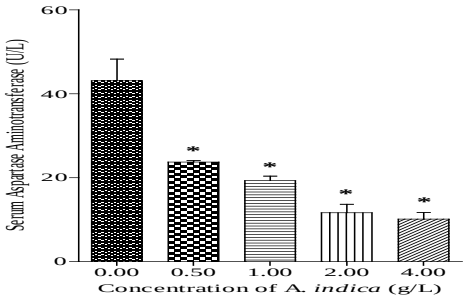

Figure 4: Mean values of aspartate aminotransferase in the serum of *C. gariepinus.*

4. Discussion

The enzymes considered in this study are useful marker enzymes that indicate the cellular damage long before revealing the structural damage by some other convectional techniques (Shahjahan *et al.*, 2004). The activities of the enzymes (alkaline phosphatase, alanine aminotransferase, aspartate and aminotransferase), considered in this study, are useful marker enzymes of damage in the liver and kidney (Akanji *et al.*, 1993).

Measurement of the enzymatic activities or marker enzymes in tissues plays a significant and well-known role in diagnostic, disease investigation and in the assessment of plant extract toxicant for safety toxicity risk. Enzymes serve a wide variety of functions inside living organisms. They are indispensable for signal transduction and cell regulation often via kinases and phosphatases (Hunter, 1995).

Enzyme (such as alkaline phosphate (ALP), aspartate transaminase (AST), alanine transaminase (ALT) and acid phosphatase (ACP)) assays are parts of standard laboratory test to detect abnormalities in animals (Ayalogu *et al*., 2001; Gabriel *et al*., 2010). Changes in these enzymes' activities, resulting from toxicant or contaminant effects in various organs of fish, have been reported (Mgbenka *et al*., 2005; Oliverira *et al*., 2006). Such alterations in fish are aimed at maintaining equilibrium in the presence of these toxicants which are known to disrupt physiological and biochemical processes (Winkler *et al.*, 2007).

Alkaline and acid phosphatase activities decreased as the concentrations of *A. indica.* Alkaline phosphate is a marker enzyme for the plasma membrane and endoplasmic reticulum (Wright, 1974). In this study, there was a significant decrease in the serum alkaline phosphatase and acid phosphatase activity of fish; this may be due to the inhibition of the enzyme by some components of the plant extracts (Akanji, 1993). This may result in a decrease in the phosphatase metabolism, an indication of the toxic effect of *A. indica*.

The dose-dependent inhibition of alkaline and acid phosphatase, observed in this investigation, is in agreement with the reports of many authors. Adamu (2009) reported a decreased value of plasma alkaline phosphatase in *Heteroclarias* (a Hybrid of *Heterobranchus Bidorsalis* and *Clarias gariepinus*) exposed to sublethal Effects of Tobacco (*Nicotiana Tobaccum*) Leaf Dust. Ogueji and Auta (2007) reported a reduced value of plasma alkaline phosphatase in African catfish *Clarias gariepinus* exposed to lambda-cyhalothrin. Sastry and Sharma (1980) reported alkaline phosphatase inhibition after 96 h of exposure to diazinon. Goel *et al*. (1982) reported plasma alkaline phosphatase inhibition by 15% in *Heteropneutes fossilis* resulting from the effect of malathion. Similarly, Das and Mukherjee (2003) reported a depletion of alkaline phosphatase due to sublethal exposure of *Labeo rohita* fingerlings to *cypermethrin.* Rashatwar and Hyas (1983) reported a significant decrease in alkaline phosphatase activity in freshwater fish *Nemachelius denisonii* (day) exposed to sublethal concentrations of Basalin.

The significant ($P<0.05$) decrease in the acid phosphatase (ACP) concentration with an increase in the concentration of the plant extract in this study is similar to that observed in *C. gariepinus* adults to acute effect of diazinon on blood plasma biochemistry (Adedeji, 2010) and this may support the assumption that the tissue of the experimental fish was markedly affected. Sastry and Sharma (1980) reported a decrease of activities in acid phosphatase in the brain of *Channa punctatus* following the effect of diazinon. Goel *et al.* (1982) reported that plasma acid phosphatase decreased by 15% in *Heteropneutes fossilis*, resulting from the effect of organophosphate malathion. The activities of acid phosphatase in blood plasma of *Cyprinus carpio* were almost identical in the control and test treatments following exposure to acute effect of diazinon (Luskova *et al.*, 2002).

Aminotransferases are gainfully used in the diagnosis of disease and tissue damage. They function as a link between carbohydrate and protein metabolism by catalyzing the inter conversion of strategic compounds, respectively (Martin *et al*., 1983). They are intracellular enzymes which exist only in a small amount of the plasma. Their presence in the plasma may give information on organ dysfunction (Wells *et al*., 1986; Gabriel and George, 2005). The aminotransferases occupy a central position in amino acid metabolism as they help in retaining amino group (to form a new amino acid) during the degradation of amino acid; they are also involved in the biochemical regulation of intracellular amino acid pool. They also help in providing necessary intermediates for gluconeogenesis. In this study, the activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) decreased significantly ($P<0.05$) in serum as the concentration of *A. Indica* increased; this indicates a stressed based tissue impairment (Svoboda, 2001). Under stress conditions, fish need more energy resulting in higher demand for carbohydrate and their precursors to keep the glycolytic pathway and TCA cycles at sustained levels (Tiwari and Singh, 2004). Similarly, in other studies (Ayalogu *et al*., 2001; Svoboda *et al.*, 2001; Tiwari and Singh, 2004), an alteration in the activities of ALT and AST was recorded indicating that there was an increased demand for energy due to tissue impairment. Studies carried out by Das *et al*. (2004) also showed that there was an alteration in the activity level of ALT and AST of Indian major carps exposed to nitritetoxicity, suggesting that the alteration of the aminotransferases is as a result of the diversion of the amino acids in the TCA cycle as keto acids to argument energy production. From the pattern of the results obtained in this serum aminotransferase, it is conceivable that the plant extract caused an increased energy demand by the exposed fish.

In summary, extracts of neem affected the liver function by decreasing the serum ACP, ALP and ALT, AST levels in African catfish and can be good indicators of deteriorating health in African catfish. Using some selected parameters, a presumptive prediction can be made on the health status and the possible problem (infection or toxicity). However, the parameter set may be, to some extent, case-dependent and requires information on the history of the fish.

Hence, this study was able to provide baseline information on the involvement of these biochemical parameters in toxicity assessment of aquatic system as well as in reporting organ dysfunction and the disease conditions of fish when exposed to *A. indica* extracts.

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