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Full Length Research paper

Toxicological evaluation of the effect of *Clarias gariepinus* (African catfish) cultivated in water contaminated with phthalate, benzene and cyclohexane on liver of albino rats

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Effect of consuming *Clarias gariepinus* cultivated in water contaminated with (10 µg/ml) respectively of phthalate, benzene and cyclohexane on the liver of rats was done. Serum concentrations of bilirubin, globulin and albumin were determined. Standard enzyme assays were conducted for selected liver enzymes followed by histological examination of liver section. Serum albumin and globulin concentrations were found to be significantly lower in rats fed with contaminated *C. gariepinus* than control ($p < 0.05$). Generally, activity of enzymes in the liver of experimental rats was found to be significantly lower than that of control ($p < 0.05$). Particularly, activities of alkaline phosphatase, acid phosphatase, alanine and aspartate transaminases in the liver of rats fed with *C. gariepinus* cultivated in phthalate contaminated water were found to be 9.1 ± 1.7, 113.8 ± 6, 13.4 ± 0.9 and 20.4 ± 0.9 nmol/min/mg protein respectively while those of control were 16.8 ± 2.2, 177.9 ± 5, 16.4 ± 1.3 and 23.7 ± 1.2 nmol/min/mg protein. Serum concentrations of direct and total bilirubin of rats fed with *C. gariepinus* cultivated in benzene contaminated water were found to be 3.52 ± 0.05 and 9.24 ± 0.50 mg/dl respectively while those of control were found to be 1.06 ± 0.02 and 4.93 ± 0.20 mg/dl respectively. Histological examination of section revealed distorted cellular arrangement in the liver section of rats fed with contaminated fish relative to control. Experimental evidences from this study suggest hepatotoxicity which may predispose to tissue failure. Increasing cases of liver problem in Nigeria may not be unconnected, *inta alia*, with consumption of fish from contaminated water.

Key words: Enzymes, bilirubin, phthalate, hepatotoxicity.

INTRODUCTION

Education and enlightenment programme on omega-3 fatty acids found in *Clarias gariepinus* (Penny et al., 2002) has prompted many farmers in Nigeria to cultivate the fish. As demand for *C. gariepinus* increased more people joined the league of farmers cultivating *C. gariepinus* and more ponds were constructed without consideration to water quality and intrusion by external water source (e.g. runoff and leachate from wastes) (Sunmonu and Oloyede, 2007).

There are several important common denominators to consider when building a pond, irrespective of whether the pond is built by hand for small scale aquaculture or by machines for large scale commercial farming. These are; type of wall, basic engineering principles, design and construction criteria for the water outlet and the drainage system and the exclusion of predators (Alegbeleye et al., 1991). The ponds found around do not conform to these guidelines; most ponds serve as recipient to runoff and leachate from wastes. Growing evidence revealed that animals placed on water contaminated with leachate from waste gain considerable body weight (Bakare et al., 1995; Oloyede et al., 2003a, 2003b; Oladiji et al., 2004; Adeyemi et al., 2007a). Mortality and weight are salient factors considered by most fish farmers in Nigeria. Once the fish do not die and are gaining weight, the farmers

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spend less money on feed and make more gain at the expense of public health.

Public health is endangered because components of waste leachate are hazardous and when allowed to contaminate water body the water become unfit for drinking (Adeyemi et al., 2007b). Components of waste leachate include heavy metals, organics such as phthalate, phenol, benzene, cyclohexane etc. (Brenaam, 2000; Johnson et al., 2000). Consumers need to be aware of both the benefits and risks of fish consumption for their particular stage of life. Children and pregnant and lactating women may be at increased risk for phthalate intoxication from fish consumption but also are at low risk for coronary heart diseases (CHD). Thus, avoidance of potentially contaminated fish is a higher priority for this group.

Acute, chronic and long term effects of chemical compounds on living systems could be studied by evaluating the biochemical and morphological changes in various organs especially the liver. The basic structural component of the liver is the liver cells or heaptocytes. The liver is the principal organ of metabolism and has a role to play in many body processes most especially detoxification of chemical compounds. Research studies have shown a variety of adverse effects on the hepatocytes of rats and catfish following exposure to environmentally toxic compounds (Oloyede et al., 2003b; Sunmonu and Oloyede, 2006). However, little or no attention has been given to effect of components of waste leachate on public health. In the present study, an attempt is made to assess the effect cat fish cultivated in water contaminated with some components of waste leachate on the hepatocytes and performance of rat.

MATERIALS AND METHODS

Reagents

Chemicals and solvents used are of analytical grade and most are products of Sigma-Aldrich Inc, St. Louis, U.S.A, while others are products of British Drug House, Poole, England.

Experimental water

The experimental water for the study was collected from the supply of Adekunle Ajasin University, Akungba-Akoko, Ondo State, Nigeria. The water was analysed using the method described by Apha (1992). The experimental water samples were contaminated with 10 µg/ml of phthalate (diethylphthalate) (CAS# 84-66-2), benzene (CAS# 71-43-2) and cyclohexane (CAS# 110-82-7) respectively and designated as follows;

A: water as collected from the University Supply (Control).
B: water contaminated with phthalate (10 µg/ml).
C: water contaminated with benzene (10 µg/ml).
D: water contaminated with cyclohexane (10 µg/ml).

The concentration of each pollutant is about ten times the recommended permissible limit (ACGIH, 1994) and a representation of the concentration found in a typical runoff from open dumps and leachate from landfills (Adeyemi et al., 2007b).

Table 1. Rat feed composition using *C. gariepinus* cultivated in water contaminated with phthalate, benzene and cyclohexane over a period of 56 days as protein source.

Nutrient	Weight (g)
Protein source (*C. gariepinus)*	200
Fat source (soy oil)	80
Sucrose	60
Cellulose	60
*Vitamin and mineral premix	50
Corn starch	550

*The vitamin/ mineral premix contains: Vit. A, 3,200,000.00 iu, Vit. D_5 600,000i u, Vit. E, 2,800 mg, Niacin 6,000 mg, Vit. B_1 800 mg, folic acid 70,000 mg, Cobalt 80 mg, iodine 499 mg, selenium 80 mg, Copper 1200 mg, Vit. B_{12} 4 mg, Vit. B_6 800 mg, Copper 1,200 mg, Folic acid 70,000 mg, Vit. K_3 600 mg, Panthothenic acid 2,200 mg, Vit. B_6 800 mg, Vit. B_2 1000mg, Iron 8,400 mg, Manganese 16,000 mg.

Experimental animals and management

Institutional and/ or national guides for the care and use of laboratory animals were followed.

Clarias gariepinus

Eighty (80) African catfish (*C. gariepinus*) of average weight 68.56 ± 6.92 g were obtained from the Department of Environmental Biology and Fishery, Adekunle Ajasin University, Akungba-Akoko, Ondo State, Nigeria. They were housed in transparent plastic containers and kept in a well ventilated laboratory. These fish were fed (3%w/w) with commercial feeds obtained from Livinco feeds, Jubilee road, Ikare Akoko, Ondo State, Nigeria. The experimental animals were kept inside a transparent plastic container assigned into eight (8) groups of ten (10) animals each. The first two groups of fish were cultivated in uncontaminated water from the University Supply and water contaminated with 10 µg/ml phthalate. The third and fourth groups were placed on water samples contaminated with 10 µg/ml benzene and 10 µg/ml cyclohexane respectively. A replica of the four groups was set-up as the last four groups. The feeding exercise lasted over a period of 56 days preceded by 14 days acclimatization period.

Two fish samples were taken from each group, dried in the oven at 40°C for 6 h daily over a period of 7 days after which they were used to compound feed for rat as shown in Table 1.

Rattus novergicus

Twenty albino rats (*Rattus novergicus*) of average weight 57.5 ± 3.25 g were used for the study. They were obtained from the Animal Holding of the Department of Biochemistry University of Ilorin, Ilorin, Nigeria. They were kept in wooden cages and fed, *ad libitum,* with the formulated diet over a period of 28 days. They were classified into four groups designated thus;

Group A - Rats fed with *C. gariepinus* propagated in water from University supply based diet (Control).
Group B - Rats fed with *C. gariepinus* propagated in phthalate

contaminated water based diet.
Group C - Rats fed with *C. gariepinus* propagated in benzene contaminated water based diet.
Group D - Rats fed with *C. gariepinus* propagated in cyclohexane contaminated water based diet.

The feeding exercise was over a period of 28 days preceded by 7 days acclimatization period. The rats were anaesthetized by placing them in a jar containing cotton wool soaked with chloroform before being sacrificed by jugular puncture. The blood was obtained through their jugular veins into non-heparinized bottles. The blood samples in non-heparinized bottles were spinned at 3500 b rpm for 10 mins and serum stored at - 8°C until required for use.

Preparation of homogenate

The isolated liver was weighed and a portion of it was cut out, chopped into very small pieces and then homogenized using pre-cooled pestle and mortar in a bowl of ice cubes. The liver homogenate was diluted using 0.25M sucrose solution to the tune of 1 in 30 dilutions. The diluted homogenate was stored at temperature of - 8°C until required for use. Another portion of the liver was cut out and fixed in 10% buffered neutral formaline (BNF) for 72 h at room temperature for histology study.
Protein Determination and Enzyme Assay

The method described by Tiez (1990) was adopted for albumin and bilirubin concentrations in the experimental rats. In this method, albumin reacts with the dye-Bromocresol green in an acidic medium to produce a greenish coloured complex the intensity of which is proportional to the amount of albumin present. The bilirubin in the serum is coupled with diazotized sulphanilic acid to form azobilirubin. The intensity of the purple colour that is formed is measured spectrophometrically at 520 nm. Globulin was calculated from the difference between total protein and albumin (Doumas, et al., 1971). Protein concentration was determined by the biuret reaction described by Gornall et al. (1949). In this method, copper ion (blue) is made to react with the peptide bond of protein to give a purple coloured complex, the intensity of which was measured spectrophotometrically at 540 nm.

Activities of aspartate transaminase (AST) and alanine transaminase (ALT) were determined using the method described by Reitman and Frankel (1957). The method measures spectrophotometrically the intensity of the red coloured hydrazone formed from the reaction of pyruvate with 2, 4 - dinitrophenylhydrazine at 546 nm. The method of Bessey et al. (1946) as modified by Wright et al. (1972) was employed in the determination of alkaline and acid phosphatases. In this method, the amount of phosphate ester that is split within a given period of time is taken as a measure of the phosphatase enzymes.

Histology

Histological study on tissues obtained from experimental rats was carried out following the method described by Drury and Wallington (1973).
Statistical analysis

Analysis of variance ANOVA; Duncan's multiple range test (DMRT) was the statistical analysis used (Duncan, 1955). $P < 0.05$ was regarded as significant.

RESULTS AND DISCUSSION

The present study is aimed at unveiling some of the health hazards associated with consumption of aquatic foods from contaminated aquatic habitats. The increasing liver disease in Nigeria is a cause for concern; this disease has its root partly from nutrition and partly from inheritance. The nutritional root is not unconnected with consumption of contaminated fresh water fish. Fresh water fish is on high demand because of its high omega-3 fatty acid content (Penny et al., 2002); however, proper care is not devoted to the quality of water where fresh water fish (e.g. *C. gariepinus*) is being propagated (Alegbeleye et al., 1991). This is, among other reasons, why investigation into effect of inclusion of *C. gariepinus* propagated in contaminated water in the diet demands a biochemical approach.

The liver-body weight ratio of experimental rats is presented in Figure 1. A significant decrease was observed in the liver-body weight ratio of experimental rats relative to the control ($p < 0.05$). Particularly, that of rats fed with *C. gariepinus* cultivated in water contaminated with phthalate was half that of control. The rats in the different groups ate their respective formulated feed freely. The reduced liver-body weight ratio of experimental rats observed in this study (Figure 1) is an indication of possible damage to the liver. This is in agreement with earlier report (Oloyede et al., 2003a; Adeyemi et al., 2008). However, further liver function tests will buttress this submission.

Concentrations of some serum proteins of rats fed with diet formulated with *C. gariepinus* propagated in contaminated water are shown in Table 2. Generally, the concentrations of the serum albumin and globulin of rats fed with diet formulated with *C. gariepinus* propagated in contaminated water were found to be significantly lower than that of control ($p < 0.05$). Conversely, serum bilirubin concentration of rats fed with contaminated fish was found to be significantly higher than that of control ($p < 0.05$). Particularly, the concentrations of the serum albumin of rats fed with diet formulated with *C. gariepinus* propagated in water contaminated with phthalate and benzene were observed to be about ¼ that of control.

Serum bilirubin, albumin and globulin concentrations are some biochemical indices for monitoring liver function in the blood (Table 2). Abnormal levels of these proteins have been reported to be associated with haemolysis or increased breakdown of RBC and/ or liver damage (Islam et al., 2004). Bilirubin is a breakdown product of heme (a part of haemoglobin in red blood cells). The liver is responsible for clearing the blood of bilirubin. It does this by the following mechanism: bilirubin is taken up into hepatocytes, conjugated (modified to make it water-soluble) and secreted into the bile, which is excreted into the intestine. Increased total bilirubin causes jaundice and can signal a number of problems. Studies had shown that if direct (that is, conjugated) bilirubin is normal, then the problem is an excess of unconjugated bilirubin and the location of the problem is upstream of bilirubin excretion. Anemia, viral hepatitis, or cirrhosis can be suspected. If direct bilirubin is elevated, then the liver is

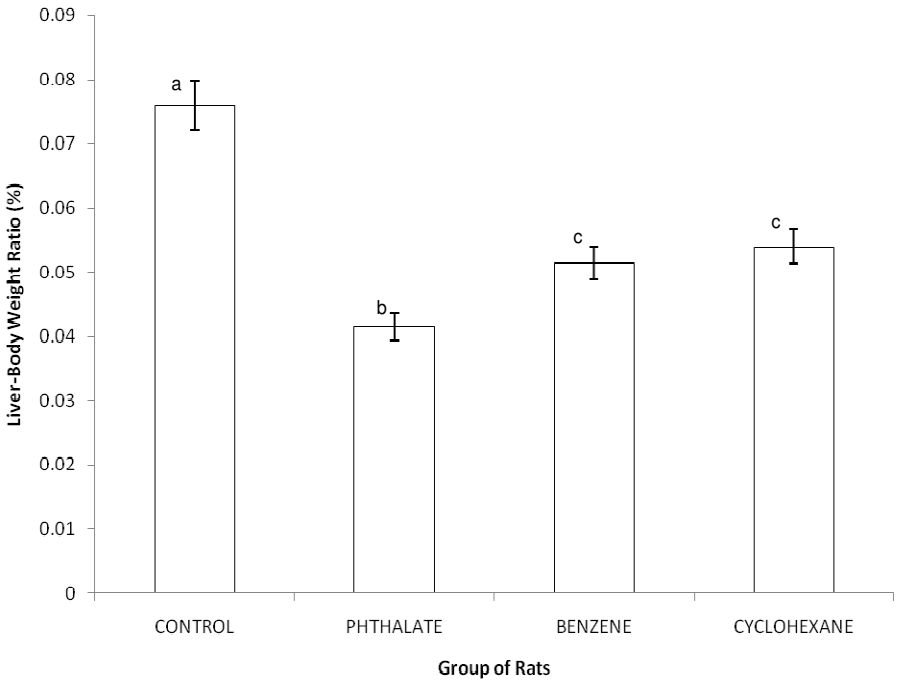

Figure 1. Liver-body weight ratio of rats fed with *C. gariepinus* cultivated in water contaminated with phthalate, benzene and cyclohexane. Results are means of five determinations ± SEM. Bars carrying different notations are significantly different ($p < 0.05$).

Table 2. Some serum indices of liver function of rats fed with *C. gariepinus* cultivated in water contaminated with phthalate, benzene and cyclohexane.

Group	D-BIL (mg/dl)	T-BIL (mg/dl)	albumin (g/dl)	globulin (g/dl)
Control	1.06 ± 0.02 [a]	4.93 ± 0.20 [a]	13.20 ± 2.50 [a]	8.80 ± 0.21 [a]
Phthalate	2.78 ± 0.01 [b]	7.31 ± 0.40 [b]	3.49 ± 0.70 [b]	2.14 ± 0.12 [b]
Benzene	3.52 ± 0.05 [c]	9.24 ± 0.50 [c]	4.75 ± 0.60 [c]	3.20 ± 0.34 [c]
Cyclohexane	1.05 ± 0.04 [a]	4.88 ± 0.20 [a]	8.68 ± 1.70 [d]	5.71 ± 0.32 [d]

Results are means of five determinations ± SEM. Values in the same column with different superscripts (a, b, c....) are significantly different ($p < 0.05$).

conjugating bilirubin normally, but is not able to excrete it. Bile duct obstruction by gallstones or cancer should be suspected (Sunmonu and Oloyede, 2007). In this study, rats fed with fish contaminated with phthalate and benzene presented elevated serum concentrations of both total and direct bilirubin suggesting that the liver is not able to excrete bilirubin which is an evidence of liver dysfunction.

Decreased serum concentrations of albumin and globulin as observed in this study lend credence to the submission that the liver function may be impaired. Both globulin and albumin are produced by the liver. If the liver is damaged, it can no longer produce these proteins. The results presented on serum proteins are consistent and all pointing to the fact that the liver may have been damaged by consumption of the contaminated fish.

Table 3 presents specific activity of selected liver enzymes of rats fed with contaminated fish. A general significant decrease of enzyme activity was observed in the liver of rats fed with contaminated fish relative to the control ($p < 0.05$). Additionally, enzyme activity was found to be least in the liver of rats fed with diet formulated with *C. gariepinus* cultivated in water contaminated with phthalate followed by rats fed with diet formulated with *C. gariepinus* cultivated in water contaminated with benzene.

Activity of enzymes assayed for in the liver of the experimental animals is consistent with the observation on serum protein concentrations. ALP catalyses the hydrolysis of organic phosphates at alkaline pH. ALP

Table 3. Specific activity (nmol/min/mg protein) of selected enzymes of the liver of rats fed with *C. gariepinus* cultivated in water contaminated with phthalate, benzene and cyclohexane.

Group	ALP	ACP	ALT	AST
Control	16.8 ± 2.2 [a]	177.9 ± 5 [a]	16.4 ± 1.3 [a]	23.7 ± 1.2 [a]
Phthalate	9.1 ± 1.7 [b]	113.8 ± 6 [b]	13.4 ± 0.9 [b]	20.4 ± 0.9 [b]
Benzene	12.4 ± 1.2 [c]	131.7 ± 4 [c]	12.4 ± 0.9 [b]	19.8 ± 1.0 [b]
Cyclohexane	12.6 ± 1.6 [c]	141.2 ± 4 [d]	14.6 ± 1.1 [ab]	22.6 ± 0.9 [c]

Results are means of five determinations ± SEM. Values in the same column with different superscripts (a,b,c...) are significantly different ($p < 0.05$).

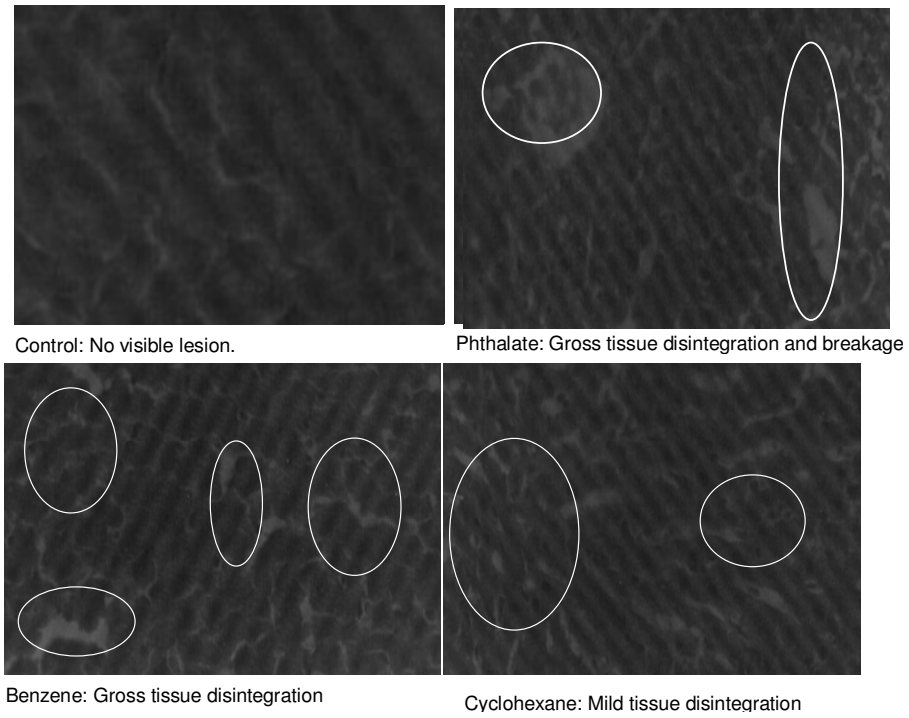

Figure 2. Light micrograph (×400) of liver of rats fed with *C. gariepinus* cultivated in water contaminated with phthalate, benzene and cyclohexane. Mild tissue disintegration to tissue breakage (see selected areas).

activity gives an indication of possibility of liver diseases (Nelson and Cox, 2000). Probable damage to the plasma membrane of the liver may be the reason for the reduced ALP activity in the liver of rats fed with *C. gariepinus* propagated in contaminated water (Table 3).

ACP catalyses the removal of phosphoryl group from a phosphate ester in an acidic medium. It is found throughout the body (Nelson and Cox, 2000). However, damage to tissues including liver, kidney, heart, red blood cells etc. causes a decrease in tissue level of ACP (Moul et al., 1998). The decrease in the ACP activity in the liver as observed in the present study also lend credence to liver dysfunction.

Increased ALT/AST ratio may be indicative of the extent of cellular damage (Adeyemi et al., 2008). Adeyemi et al. (2009) reported that loss of AST and ALT activity in tissues may be interpreted as a compromise of the tissues integrity. Although ALT and AST are “marker” enzymes for the liver, it is believed that any alteration at the subcellular level may affect the activity of these enzymes in other tissues (Adeyemi et al., 2008). The decrease in the ALT and AST activity in the liver as observed in this study (Table 3) suggests that consumption of *C. gariepinus* propagated in contaminated water possibly led to inhibition of ALT and AST activity. The experimental data revealed that inclusion of *C. gariepinus* propagated in contaminated water in diet may alter protein metabolism, amongst others, at the subcellular

level and this may predispose to impairment of the function of the liver.

Histology examination of liver of experimental rats is presented in Figure 2. No visible lesion was observed in the control rats, however, varying changes in cellular architecture was observed in the histology photograph of rats fed with contaminated fish. These changes range from mild tissue disintegration as observed in rats fed with cyclohexane contaminated fish to tissue breakage as observed for rats fed with phthalate contaminated fish.

Overall, all the data obtained as portrayed by changes in the biochemical indices for liver function and enzyme activities seem to be supported by the histological study (Figure 2) particularly for the rats fed with *C. gariepinus* cultivated in water contaminated with phthalate showed gross cellular disintegration and tissue breakage. The large open spaces in the histology photograph (marked by white circles) are indicative of tissue disintegration. These open spaces had been described as areas of tissue disintegration and breakage (Loekle et al., 1983; Sunmonu and Oloyede, 2007).

Conclusion

This study is significant as it has been able to show that consumption of *C. gariepinus* cultivated in water contaminated with component of waste leachate (such as phthalate, benzene and cyclohexane) is hazardous to health. Experimental evidence from this study reveals that consumption of such contaminated fish could damage the liver as evidenced by:

a. Reduced liver-body weight ratio.
b. Elevated serum bilirubin concentration.
c. Reduced serum albumin and globulin concentrations.
d. Decreased ALP, ACP, AST and ALT activity in the liver.
e. Gross tissue disintegration observed in the histology photograph.

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