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Histopathological Alterations of *Heterobrancus bidorsalis* Exposed to Ricin Active Ingredient in *Ricinus communis* (Castor Oil Seed)

***Ajayi, C. T[1]., Jenyo-Oni, A[2]., Ajani, E. K.[2], and Oladosu-Ajayi, R. N.[1]**

[1]Department of Fisheries Technology, Federal college of Freshwater Fisheries Technology New Bussa, Niger State.

[2] Department of Aquaculture and Fisheries Management, University of Ibadan, Oyo State

Corresponding Author: acossyajayi@gmail.com, 08035311635

Abstract

Ricin an abundant protein component of *Ricinus communis*seed, a highly toxic plant, However dearth of information on the piscicidal potentials of its active ingredient Ricin. This study examines 96h LC_{50} toxicity, sub-lethal concentration and effect on histopathological parameters of exposed fish (*Heterobranchus bidorsalis)*. Ricin was extracted from seed of *Ricinus communis* using cold water maceration and *Heterobranchus bidorsalis* juveniles were subjected to a range finding test before 96-hours LC_{50} acute toxicity test was carried, four doses (100, 125, 160 and 200 mg/l) of ricin extract and the negative (0mg/l) control. *Heterobranchus bidorsalis* was afterwards exposed to sub-lethal concentrations corresponding to 10% (11.7mg/l), 20% (23.4mg/l), 30% (34.1mg/l) of the 96-h LC_{50} (117 mg/l), 0mg/l negative control and 4mg/l of Cyclophosphamide positive control for 21 days. Damages were observed in the different concentration samples collected against the control samples, this suggests ricin contained substances capable of inducing organs damage in fish.

Keywords:

Castor bean seed, Ricin, Piscicidal, and Histopathology

Introduction

Aquaculture is perceived as having the greatest potential to meeting the growing demand for aquatic food (FAO, 2018). According to Agbon, *et al.*(2010) fish is a heavily traded food commodity in the country and it's becoming a fastest growing farmed item in international markets. It is also cheap alternative source of protein in Nigeria (Omitoyin, 1995). Piscicidal plants have been widely used by fishermen in the rural communities all over the world as a means of catch enhancer. Some of these plants are non-selective in destruction, thereby interfering with ecological balance of the immediate environment. Piscicides are phytochemical substances that are poisonous to fish the plants contain different active ingredients such as Alkaloid, Micotine, Tannis, Saponins, Pipanne, Resin, Amide, Ricin, Glycoside, Carbazole, Curcin, Coumanin, Parkine, Resoranol and Cardol (Fafioye*et al*., 2009.) Useful effects of poisonous plants have also been reported In Nigeria, Cameron, and West Africa region, (Sogbesan 2015, Adediwura 2012, Agaie 2006 and Fafioye 2005).

Test like standardized acute and chronic toxicity tests lasting 96 hours, 21 days or more (Chronic test), is conducted to measure the effect of survival, effect on organs and physical look of the fish measured at each concentration in a gradient, along with a control test. (EPA 2001) (OECD, 1992).Several plant materials have shown to be toxic to aquatic organism, such phytospecies are *Ricinus communis*, *Thevetiamirifonia, Euphodiapaganorum, Balanitesaegyptiaca, Adeniumobesum*and*Azadirachtaindica* (Ayuba and Ofojekwu, 2002; Wade et al., 2002 and Fafioye, 2005 and Akinwande*et al.*, 2007).

Ricinus communis also known as castor oil plant is an important medicinal plant belonging to the family *Euphorbiaceae*. Its phytochemistry, biological and pharmacological activities, and ethnomedicinal uses have been reported. Adediwura and Ajibesin (2012), reported castor oil beans *Ricinus communis* as one of the toxic plants and plant part in a south western state in Nigeria, *R. communis* also showed free radical scavenging (helps protect cells from the damage caused by free radicals) and repellent properties.

M**aterials and Methods**

Study location and Experimental procedure

The experiment was carried out at lab.A in the Department of Aquaculture and Fisheries management in the faculty of Renewable Natural Resources, University of Ibadan.A completely randomized design was used in triplicate to test the toxicity level of ricin on the species. The 96h-LC50 value was determined for *H. bidorsalis,* toxicity data obtained from the study was estimated using probits.

Experimental fish

Juveniles of *H. bidorsalis* (Geoffroy saint-Hilaire, 1809) was purchased from a reputable farm in Ibadan environs, they were weighed, with average weight of 21.12 ± 1.5g. They were left to acclimatize and fed with 2mm commercial feed under laboratory conditions in a static system for weeks before the commencement of the experiment. Temperature was measured by mercury-in-glass thermometer, pH insitu by meters, dissolved oxygen, and Nitrite and ammonia were determined for water on each sampling day by standard methods (APHA, (1985); WPCF, 2005).

Experimental plant (Collection of Plant samples)

R. communis seed(Family Euphorbiaceae) was collected from Ibadan metropolis and was taken to the Department of Botany, faculty of science, University of Ibadan, for proper identification and taxonomy.

Extraction procedures of pure Ricin (active ingredient) from castor beans

The ricin was extracted from the seed of *R. communis* by the method of Subramanian *et al*. (1971). Extraction of ricin was done, first harvesting the seed from the shrub and then air dried for those that are not totally dried. The seed was then separated from the shell and thoroughly washing and re-air dried. In preparing the protein material, the castor beans were first grounded into small particle size and were later soaked in n-hexane to remove the oil. After the oil has been removed, the pressed cake or pomace is extracted by agitating with water at a pH of 3.8+-0.1 at 25″ C as described by subramanan et al., 1971. The extraction was carried out in the pharmacological chemistry organic lab, of the University of Ibadan.

Test Procedure

A range finding test with space factor of 0.5 was carried out in order to determine the range of concentrations for the definitive test.

Definitive Test (Acute toxicity test)

Acute toxicity test was used to determine the short-term toxicity (4 days) of the active ingredient on the fish species, measuring lethality. It was conducted to determine the 24h-LC50 value of ricin with definitive test in a static system in a laboratory according to (standard methods of OECD guideline No 203 (OECD, 1992). Toxicity experiment was performed by the method of Singh and Agarwal, (1988). A set of 10 sample each were randomly exposed to each of the four concentrations of the active ingredient (Ricin) 100, 125, 160 and 200mg/l respectively , obtained after the range finding test and 0mg/l as control of the bioassay.

Determination of Sub-lethal Concentrations (In vivo Exposure Experiment)

20 juvenile each were distributed randomly among dose/exposure duration groups. Five 30 liters rectangle white plastic tanks were used for the exposure of various concentrations of the test Ricin; first plastic tank for negative control or control, second for solvent control (Cyclophosphamide 4mg/l), third, fourth and fifth Plastic tanks for 10%, 20% and 30% (11.7, 23.4, 35.1mg/l respectively) of Ricin extract. This study was conducted following the OECD guideline No 203 in the static renewal test conditions (OECD 1992). The exposure was continued up to 504hrs (21 days), The test fish were fed twice daily with 5% feed per body weight and the water for the treatment and control groups were replaced every 48 hrs to reduce volatilization of less stable substances in the ricin and maintain concentration of the ricin as well as reduce accumulation of metabolic wastes and remains of food particles. Blood sampling were done on the (21day) at the rate of five fishes (n=5) from each test concentrations. The specimens maintained in borehole water and those exposed to Cyclophosphamide 4mg/l were considered as the negative and solvent controls (positive), respectively. On each sampling day of (1, 7, 14 and 21 days) blood samples were collected and immediately processed for Micronucleus (MN). Blood sample collection was done by incising the lateral vein using 1ml heparinized syringe. (Tiano*et al*., 2000).

Histopathology sample collection

Histopathology first samples were taken on the 4^{th} day of the definitive test and on the 21^{st} day of the sub-lethal test the second samples were taken. One sample each from each of the treatments were taken, the gills, kidney, liver and brain were removed and kept in methanol in a test bottle and taken to the laboratory in the Department of Animal science of the University of Ibadan for analysis. All histological samples were prepared for light microscopy analysis (Gautier, 2011). The organs of sampled fish were fixed in 10% neutrally buffered formaldehyde for 24 h. The preserved samples were rinsed in distilled water, dehydrated in a series of solutions with increasing ethanol concentrations (70%, 90%, and 100%) for 2 h. Afterwards, they were cleared in xylene, infiltrated with liquid paraffin with a melting point of 54 – 56^0C and finally embedded in paraffin wax. The tested organs from each fish were sampled using a rotary microtome (Leica RM 2125 RTS, Leica Microsystems, Wetzlar, Germany); sections of 5 µm were stained with hematoxylin-eosin and viewed under a light microscope at x400 magnification.

Statistical analysis

One way analysis of variance (ANOVA) was used to analysis the results of Histopathology, for significant differences between the negative control and other treatments the result was further

subjected to Duncan Multiple Range Test (DMRT), to check the level of significant differences at $p<$ 0.05 using IBM SPSS statistics 20.

Results

Water quality parameters

The physical and chemical characteristics of the water samples during the acute and sub-lethal tests were taken and presented in Tables 1 and 2, the physical look of the water is a little cloudy few hour after application of chemical but no oil fume created on the surface of the water it was observed that ricin dissolves perfectly in the water. The dissolved oxygen was observed to be above the lethal concentration 4mg/l at all time during the experiment, temperature, pH, nitrate, nitrite and ammonia were all at favourable limits.

Determination of lethal concentration on Probit

The result of the acute test conducted was subjected to probit analysis to determine the lethal concentration of ricin that will kill at least 50% LC_{50} of the entire fish population in the bioassay the result of the LC_{50} of the experiment after probit analysiswas117.058mg/l

Table 1: Mean physico-chemical parameters of water during the 96 hours exposure of *H.bidorsalis* to varying concentrations of Ricin

Concentration (mg/l)	pH	Temperature (^{0}C)	Dissolved Oxygen (mg/l)	Nitrite (mg/l)	Ammonia (mg/l)
0.00 control	7.12 ± 0.02	25.20 ± 0.14	8.30 ± 0.04	0.14 ± 0.02	0.03 ± 0.09
100	6.80 ± 0.15	25.68 ± 0.12*	8.10± 0.04*	0.17 ± 0.04	0.54 ± 0.23*
125	6.60± 0.44*	26.10 ± 0.10*	7.52 ± 0.38*	0.37 ± 0.05*	0.65 ± 0.20*
160	6.30 ± 0.10*	26.50 ± 0.15*	7.33 ± 0.34*	0.52 ± 0.03*	0.69 ± 0.08*
200	6.10± 0.03*	26.70± 0.03*	6.80 ± 0.16*	0.59 ± 0.04*	0.79 ± 0.02*

* Values significantly different from control group (p<0.05)CYP = Cyclophosphamide

Table 2: Mean physico-chemical parameters of water during the 21 days exposure of *H. bidorsalis* to various concentrations of Ricin

Concentration (mg/l)	pH	Temperature (^{0}C)	Dissolved Oxygen (mg/l)	Nitrite (mg/l)	Ammonia (mg/l)
0.00	7.10 ± 0.00	27.99 ± 0.03	8.09 ± 0.03	0.14 ± 0.02	0.05 ± 0.00
11.7	7.00± 0.02	27.45 ± 0.01*	6.80 ± 0.01*	0.17 ± 0.01	0.47 ± 0.17*
23.4	6.71± 0.28*	26.30 ± 0.02*	6.34 ± 0.02*	0.30 ± 0.01*	0.51 ± 0.33*
35.1	6.69 ± 0.05*	26.22 ± 0.01*	5.86 ± 0.02*	0.33 ± 0.02*	0.68 ± 0.33*
Positive control (CYP- 4.00)	7.00 ± 0.01*	28.54 ± 0.01*	7.22 ± 0.13*	0.18 ± 0.01	0.06 ± 0.00

* Values significantly different from control group (p<0.05) CYP = Cyclophosphamide

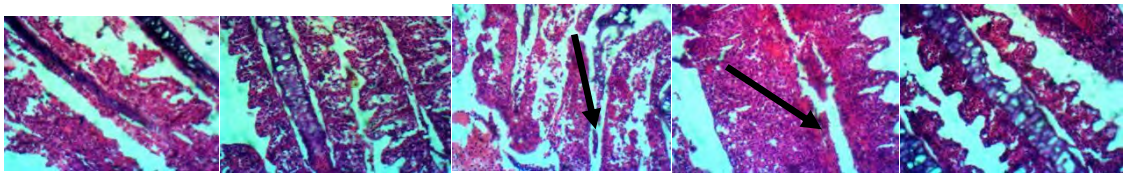
Table 3. Range finding assay of juvenile *H. bidorsalis* exposed to varying concentrations of ricin for 24 hrs.

Concentrations (mg/l)	No of fish exposed	Mortality	% Mortality
0.0	5	0	0
1.00	5	2	40
10.00	5	5	100
100.00	5	5	100

Table 4. Acute toxicity assay of juvenile *H. bidorsalis* exposed to various concentrations of ricin for 96 h Lethal Median concentrations (LC_{50})

Treatments	1h	2h	6h	12h	24h	48h	72h	96h	%mortality	%survival	Total % mortality
T_1R_1	-	-	-	-	-	-	-	-	0	100	
T_1R_2	-	-	-	-	-	-	-	-	0	100	
T_1R_3	-	-	-	-	-	-	-	-	0	100	0
T_2R_1	-	-	-	-	-	-	-	-	0	100	
T_2R_2	-	-	-	-	-	1	-	-	10	90	
T_2R_3	-	-	-	-	-	-	-	-	0	100	3.33
T_3R_1	-	-	-	-	-	4	3	1	80	20	
T_3R_2	-	-	-	-	-	4	6		100	0	
T_3R_3	-	-	-	-	-	3	4		70	30	83.33
T_4R_1	-	-	-	-	2	7	-		90	10	
T_4R_2	-	-	-	-	1	4	5		100	0	
T_4R_3	-	-	-	-	3	7	-		100	0	96.67
T_5R_1	-	-	-	-	4	6	-		100	0	
T_5R_2	-	-	-	-	8	2	-		100	0	
T_5R_3	-	-	-	-	7	3	-		100	0	100

T_1= 0mg/l, T_2= 100mg/l, T_3 = 125mg/l, T_4= 160mg/l, T_5=200mg/l



A B C D E

Figure 1: Photomicrograph of gills of *H. bidorsalis* (HE x 400) after 96hrs (4days)acute exposure to ricin. A: (0mg/l)there is no observable lesion; B: (100mg/l)there is no observable lesion; C: (125mg/l) there is degeneration and necrosis of secondary lamellae; D: 160mg/l there is hyperplasia of secondary lamella; E: 200mg/l there is no observable lesion

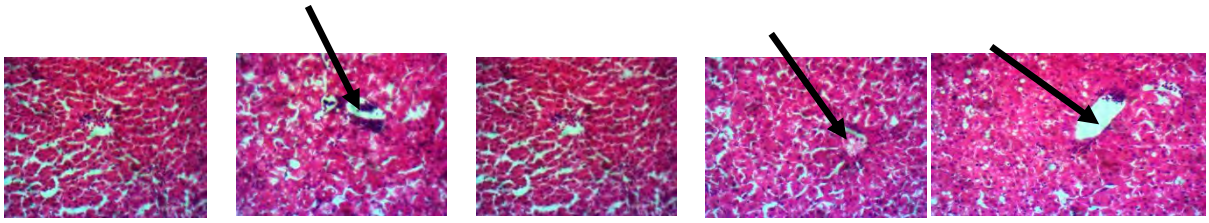


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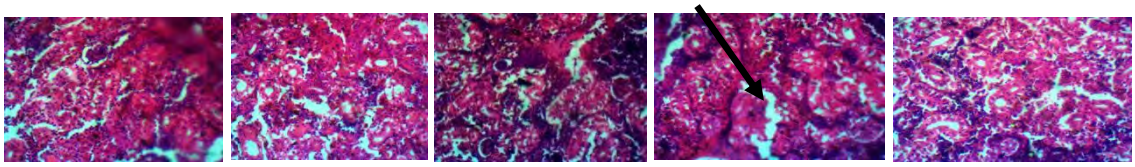
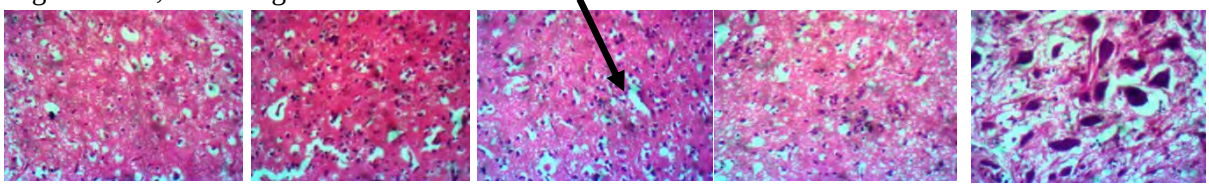
Figure 2: Photomicrograph of liver of *H. bidorsalis* (HE x 400) after 96hrs (4days)acute exposure to ricin. A: (0mg/l)there is no observable lesion; B: (100mg/l)there is centrilobular degeneration of hepatocytes; C: (125mg/l) there is no observable lesion; D: 160mg/l there is centrilobular hepatocellular coagulation necrosis; E: 200mg/l there is centrilobular hepatocellular vacuolar degeneration and coagulation necrosis



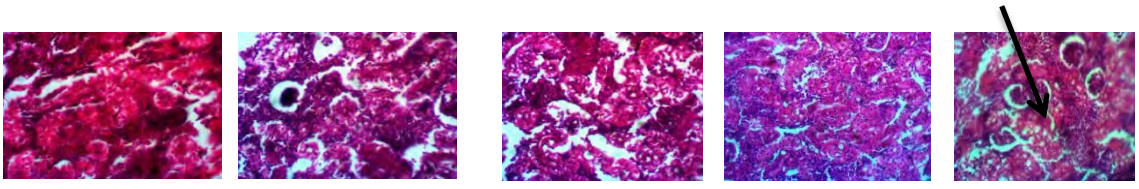
A B C D **E**

Figure 3: Photomicrograph of Kidney of *H. bidorsalis* (HE x 400) after 96hrs (4days)acute exposure to ricin; A: (0mg/l)there is no observable lesion; B: (100mg/l)there is no observable lesion; C: (125mg/l) there is no observable lesion; D: 160mg/l there is patchy tubular epithelial vacuolar degeneration; E: 200mg/l there is no observable lesion



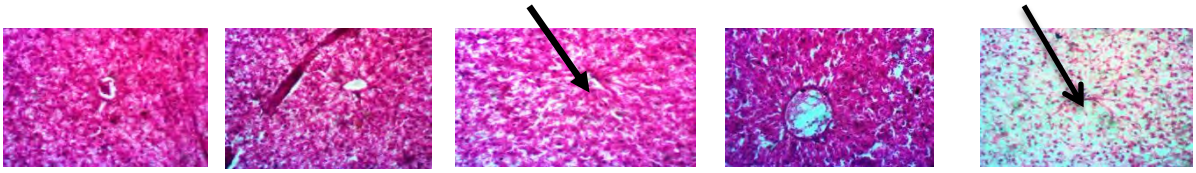
A **B** **C** **D** **E**

Figure 4: Photomicrograph of brain of *H. bidorsalis* (HE x 400) after 96hrs (4days) acute exposure to ricin; A: (0mg/l) there is no observable lesion; B: (100mg/l)there is no observable lesion; C: (125mg/l) there is no observable lesion; D: 160mg/l there is increased vacuolation of the neuropil and focal gliosis; E: 200mg/l there is no observable lesion



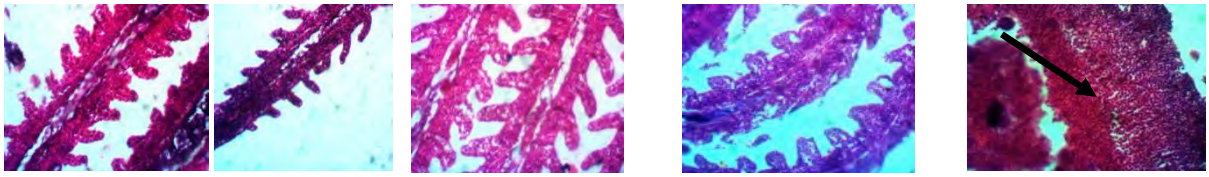
A **B** **C** **D** **E**

Figure 5: Photomicrograph of kidney of *H. bidorsalis* (HE x 400) after 504hrs (21days) sub-lethal exposure to ricin. A: (11.7mg/l) there is no observable lesion; B: (23.4mg/l) there is no observable lesion; C: (35.1mg/l)there is no observable lesion; D: There is no observable lesion; E:There is patchy tubular epithelial degeneration and necrosis.



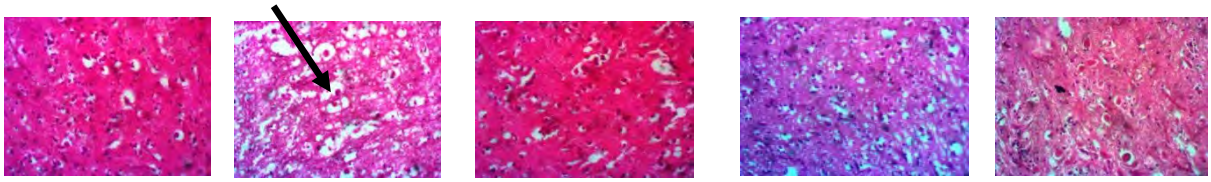
A B C D E

Figure 6: Photomicrograph of liver of *H. bidorsalis* (HE x 400) after 504hrs (21days) sub-lethal exposure to ricin; A: (11.7mg/l) there is no observable lesion. B: (23.4mg/l) there is no observable lesion; C (35.1mg/l) there is centrilobular hepatocellular degeneration; D:There is no observable lesion; E:There is moderate diffuse atrophy and coagulation of hepatocytes



A B C D E

Figure 7: Photomicrograph of gill of *H. bidorsalis* (HE x 400) after 504hrs (21days) sub-lethal exposure to ricin. A: (11.7mg/l) there is no observable lesion; B: (23.4mg/l) here is no observable lesion; C: (35.1mg/l)there is no observable lesion; D: (0.0mg/l) there is no observable lesion; E: (+ve control)There is moderate secondary lamellae hyperplasia



A B C D E

Figure 8: Photomicrograph of brain of *H. bidorsalis* (HE x 400) after 504hrs (21days) sub-lethal exposure to ricin. A: (11.7mg/l) there is no observable lesion; B: (23.4mg/l) there is vacuolar degeneration of the neuropil; C: (35.1mg/l) there is no observable lesion; D:(0.0mg/l) there is no observable lesion; E: (+ve control) there is no observable lesion

Histopathological alterations

The conducted histopathological study of the gill, liver, kidney and brain of *H. bidorsalis* exposed to varying concentrations of ricin. in 21days sub-lethal tests are presented in Figures 1-8. The 21 days sub-lethal test the negative control (0.0mg/l) showed no significance changes in the organs observed but there were significant changes in the kidney, liver and gill in the positive control (Cyclophosphamide 4.00mg/l) that showed a patchy tubular epithelial degeneration and necrosis, a moderate diffuse atrophy and coagulation of hepatocytes and a moderate secondary lamellae hyperplasia respectively (Figures 1-8) and no histopatological changes observed in the brain of the positive control test. There were no major changes in the ricin test concentration, but a patchy tubular epithelial degeneration and necrosis in the brain of 23.4mg/l concentration of the ricin.

Discussion (Water quality parameters)

Xenobiotics are usually known to contaminate water bodies and several studies have highlighted their persistence and effects on aquatic systems (Richardson, 1988). Moreover, studies have shown that

environmental factors such as temperature, pH, alkalinity, conductivity, dissolved oxygen, turbidity and salinity of a water body can significantly influence the quantity, quality and rate at which these pollutants enter the system (Olufayo, 2009). Water quality variables were within acceptable ranges (APHA, 1985) (FAO, 1999; Bhatnagar and Devi, 2013), it is assumed that they may not have acted synergistically with the toxicant to affect the behaviour as well as mortalities recorded in the fish species.

Histopathological response of fish

The study showed severe histopathological alterations in the gills of the test fish in progressively higher concentrations of the test chemical. There were varying levels of cellular and tissue modifications like enlargement of the filament core and globular protrusions of the lamellae tips due to blood congestion, epithelial hyperplasia, hypertrophy, degeneration of filaments and lamellae, atrophy, severe lesion, tissue pigmentation, malignancy, inclusion bodies, telangiectasis, necrosis as well as other discernible cellular irregularities.

Gills are vital organs which serve various functions such as respiration, ammonia excretion, transfer and exchange of ions and water (osmoregulation). Gills are in close contact with the external aquatic environment and are therefore prime targets of contaminants and changes in water quality. It is quite apparent that the damage caused to the gills results in impairments in gaseous exchange capacity of the gills, which ultimately lead to respiratory distress. Similar view was presented by Rahman*et al*., (2002); Omitoyin*et al.* (2006) and Ezemonye and Ogbomida (2010) for the damage of gills in the fish treated with toxicants and support to present findings. Studies have shown that the exposure of fish to contaminants will result in a number of behavioural and histopathological alterations in different tissues (gills, liver, kidneys and brain) of fish (Nero, *et al*., 2006; Camargo, *et al.*, 2007; Peyghan, *et al.*, 2007 and Chezhian, *et al.*, 2010) this behaviour and histopathological changes were experienced in the study which further confirm the Piscicidal propensity of ricin. hypoxia or anoxia, which is as a result of oxygen intake reduction is a result of cellular alterations creating increase in the distance between the blood in the gills and the water thereby prejudicing the oxygen uptake capabilities of the gill (Camargo, *et al.*, 2007). Similar alterations in the architecture of the gills have also been reported by Nero *et al.*, (2006).

Liver is an important organ for assessing the pollutant's effects as the chemicals are stored in it. Liver is also considered as major site for the biotransformation of chemicals, thus reduce their toxicity and make the excretion easy. Necrosis observed especially in the fourth treatment (23.4mg/l) in the liver of exposed fish can be attributed to the excessive burden of work for the detoxification of extract from the body. Similar thought has been postulated for the liver necrosis by Rahman*et al.*, (2002) in fish treated with toxicant. Biagianti-Risbourg and Bastide (1995); Velmurugan*et al*., (2007) and Ikele*et al.*, (2011) have reported necrosis of tubular epithelium and pyknotic nuclei in the liver of fish exposed to chemicals and this further confirm our investigation. The pathological alterations observed were also supported by the work of Ayoola and Ajani, (2008) that reported that exposure of cypermethrin causes pathological effects on gills, liver and kidneys of catfish, this they said caused fusion of tubules and condensation of glomeruli content

In this study patchy tubular epithelial degeneration and necrosis were observed in the kidney of the test fish (*Heterobranchus bidorsalis)*, this confirms the work conducted by Ikele*et al*., (2011) and Ortiz *et al.*, (2003) that observed a Pyknotic nucleus, destruction of fusion of tubules, condensation of nuclei and sloughing of the epithelium and necrosis in the walls of tubules due to the toxic effects of lindane

respectively. The brain of the fish which is the driving house of the entire body system was also examined for possible damage, after exposure of *H. bidorsalis* juveniles to *Ricinus communis* active ingredient (ricin), the brain showed an increased vacuolation of the neuropil and focal gliosis and vacuolar degeneration of the neuropil. This is believed to have damaging effect on the activities of the fish; these changes observed in the fish shows that the fish is highly sensitive to the toxin introduced to the water as supported by the study of Al-Sawafi*et al.,* (2017) which found histological changes in the brain and muscles of zebra fish exposed to cadmium.

Conclusion

Conclusively the project work is an eye opener to the histopathological effect of ricin and a need to tackle it, otherwise it residual effect could be a course of worry in the near future, also there is a need for more study directed towards the seed utilization in feed formulation as a replacement for some ingredients in fish feed production .The need for proper screening of the process of it utilization in feed production should also be effectively monitored and ensure that the poison is full extracted from the seed before it is used for feed production. The study further brought to the fore the effect on the organs of the tested fish, and further confirm is classification as a biological weapon.

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