Assessment of Stomp® (Pendimethalin) toxicity on *Oreochromis niloticus*

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Abstract: In this study, the toxic effects of stomp® were evaluated by measuring growth performance, biochemichal parameters, histopathological findings and genotoxic effect in a commercially important fish species, *Oreochromis niloticus*. sixty *Oreochromis niloticus* with an average body weight (50.43 ± 0.20g.) were used and divided into 3 equal groups, the first group kept as control, while fish of the second and third groups were exposed to 10% and 5% (0.355 mg/l and 0.177 mg/l, respectively) of 96 hrs LC_{50} . The results revealed a significant decrease in body weight and weight gain while significant increase in serum glucose, aspartate amino transaferase (AST), alkaline phosphatase, total protein, cholestrol in fish exposed to 10% then 5% 96 hrs LC_{50} of stomp in a dose dependent manner compared to control. Stomp® produced genotoxic effects on the exposed fish.

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Keyword: Stomp®, Oreochromis niloticus, genotoxic effects, growth, biochemical parameters.

1. Introduction

Nile tilapia, *Oreochromis niloticus*, is one of the most common freshwater fishes used in toxicological studies *(Figueiredo-Fernandes et al., 2006a, b; Garcia-Santos et al., 2006*), because it presents a number of characteristics that may make it an appropriate model that can be used as indicator species in biomonitring programs *(Gadagbui et al., 1996).*

Pesticides, including herbicides, insecticides and fungicides, are used extensively to improve crop yields and as a result, they accumulate in the environment. More than 2.5 million tons of pesticides are applied every year to agricultural crops worldwide (*Van der Werf, 1996*). Pesticides tend to be very reactive compounds that can form covalent bonds with various nucleophilic centres of cellular biomolecules, including DNA (*Crosby, 1982*). The induction of DNA damage can potentially lead to adverse reproductive outcomes, induction of cancer and many other chronic diseases (*Ribas et al., 1996, Lander et al., 2000, Meinert et al., 2000 and Ji et al., 2001*).

Stomp® is liquid emulsive herbicide of the dinitroaniline type; its active ingredient is pendimethalin (*Fetvadjieva et al., 1994*). Pendimethalin is a widely-used herbicide for the control of annual grasses and certain broadleaf weeds in commercial crops. (*Engebretson et al., 2001).*

Direct overspray of a water body with a usual application rate of pendimethalin (2.4 kg/ha) can result in the concentrations severely toxic to algae, crustaceans, fish at a depth of 0.15 m (up to 1.6 mg/l) (*CICAD OAS, 2005*). Pendimethalin has been classified as persistent bioaccumulative toxic (PBT) and a group C carcinogen "possible human carcinogen" by the United States Environmental Protection Agency (*USEPA, 1997*). The herbicide Stomp® 33EC compound half-decay in the surface waters is 61days (*Doicheva etal., 2009*).

Dimitrov et al. (2006) showed an induction in chromosomal aberration and micronuclei in bone marrow cells of pendimethalin treated mice. However negative mutagenic effects were shown in mammalian and bacterial cells exposed to pendimethalin.

Data on stomp® (Pendimethalin) and its toxic effects on fish health, growth and genotoxic effect are scarce. Therefore, the aim of this study was to evaluate the hazardous effects of stomp® after long term toxicity on *Oreochromis niloticus.*

2. Material and Methods

1. Fish and experimental design

A total number of sixty *Oreochromis niloticus* with an average body weight (50.43 ± 0.20g.) were used. Fish were obtained from Abbassa Fish Hatchery, Sharkia Province. Fish were apparently healthy and free from any skin lesions and external parasites. They were maintained in glass aquaria (each, 80 x 40 x 30 cm capacity) with 60 liters of dechlorinated tap water. Each aquarium provided with aerator, thermostatically controlled heater and thermometer. Fish were acclimatized to water environment for two weeks before start of the experiment.

Oreochromis niloticus were divided into 3 equal groups, each with two replicates (10 fish replicate $^{-1}$). The fish of the first group kept as control, while fish

of the second and third groups were exposed to 10% (0.355 mg/L) and 5% (0.177 mg/l) 96 hrs $LC_{50,}$ respectively according to *El-Nobi and Gihan (2010*) for 2 months. Fish were fed 3 times daily on a basal diet contained 35.4 % crude protein. The amount of food per day was 3 % of fish body weight.

2. Herbicide

Stomp® 50% EC

Stomp® 50% EC (BASF PLC) is an orange-yellow liquid emulsive herbicide of the dinitroaniline type, whose active ingredient is pendimethalin (*USEPA, 1997).* Stomp contains the inert components (50%); as petroleum solvents (naphthalene and ethylene dichloride).

Common Name: Pendimethalin

Chemical Name: n-(1-ethylpropyl)-3, 4-dimethyl-2, 6- dinitrobenzenamine)

Chemical Family: Dinitroaniline

Empirical Formula: $C_{13}H_{19}N_3O_4$

Trade and Other Names: Prowl, Squadron

Basic Manufacturer: American Cyanamid

Aqueous photolysis (half life): 60 days.

Mode of Action: A microtubule disruptor.

3. Sampling and analytical methods

3.1. Growth performance:

Fish of all replicate were counted and weighted individually after 2, 4, 6 and 8 weeks of the experiment and the growth parameter was calculated as follows:

Weight gain = $W_2 - W_1$ (*Jauncay and Ross, 1982*).

Specific growth rate (SGR) = 100 (In W_2 – In W_1) / T (*Castell and Tiews, 1980).*

where W1 and W_2 are the initial and final weight, respectively, and T is the number of days in the feeding period.

3.2. Survival rate:

Survivability was estimated during the experimental period by recording survivable in each aquarium.

3.3. Biochemical study:

Blood samples were collected from the caudal blood vessel (*Lucky, 1977*) using sterile syringes into Eppendorf tubes and centrifuged at 3000 r.p.m. for 15 minutes for serum separation which preserved at – 20°C in refrigerator till be analyze. Serum total protein (*Henry 1964*), glucose (*Tinder, 1969*), aspartate amino transaferase (AST) (*Reitmans and Frankel, 1957*), alkaline phosphatase (ALP) (*Kind and King, 1954*) and Cholesterol (*Watson, 1960*) were determined.

3.4. Genotoxic study:

3.4. A. Comet assay:

The DNA damaging potential single gel electrophoresis assessed using comet assay. Liver and gills tissues from stomp® exposed fish were analyzed by the comet assay according to the methods of *Singh et al. (1988)* with minor modifications. Briefly, liver and gills tissues were embedded between a layer of 1% normal melting point agarose and a layer of 0.5% low melting point agarose. After solidification, the slides were immersed in lysing solution (2.5 M Nacl, 200 mM Na_2EDTA, 10 mM Tris-Hcl, 10% DMSO and 1% Triton X-100) for 1 hour at 4°C to allow the DNA to unwind. The slides placed in alkaline buffer (0.3 M NaOH, 1mM Na_2EDTA, pH 12) for 30 minutes at 4°C and were electrophoresed (0.8 v/cm) for 30 minutes in freshly chilled alkaline buffer, then neutralized with Tris-Hcl buffer (400 mM, pH 7.4) and finally stained with a fluorophore (20 ug/ml propidium iodide). DNA damage was determined by measuring the tail length and tail moment of 50 cells/sample using a fluorescence microscope equipped with an automated digital imaging system running Comet Assay III ™ software (Perceptive Instruments, UK).

3.4. B. Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS- PAGE):

Protein fractionation was done using sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS – PAGE) *(Herzberg and Pasteur, 1975)*. The gels were stained with Comassie Brilliant Blue and the excess dye was washed using destaining solution (methanol and acetic acid). The gel bands were scanned using Hoefer 65 software (Hoefer Scientific Instruments, CA. USA).

3.5. Histopathological examination:

Gills, liver and kidney specimens from both control and stomp® exposed fish were fixed in 10% buffered neutral formalin solution then processed for histopathological studies in ascending grades of ethyl alcohol, cleared in xylol, then embedded in paraffin wax. Paraffin sections of about 5-7 microns thickness were prepared and then stained with hematoxylin and eosin *(Robert, 1989).*

3.6. Statistical Analysis:

The data was analysed by One way analysis of variance (ANOVA) using SAS ANOVA procedure *(SAS, 1996).*

3. Results and Discussion

1. Clinical findings and mortality rate:

Oreochromis niloticus exposed to 10% and 5% 96 hrs LC_{50} of stomp® herbicide showed, abnormal

swimming movement, sluggish, did not respond well to escape reflex, dark coloration of the skin. Fish covered with thick layer of mucous which may be excreted under stress to increase not only the fish resistance, but they also help to survive unfavorable conditions as recorded in *Chlorella kessleri* which exposed to pendimethalin (*Doicheva et al., 2009*). The periphery of dorsal, caudal fins and belly colored in yellow from the herbicide. The respiratory rate was gradually slow and fish opened their mouth just prior to death which may be resulted from the inert components (50%); of the herbicide; as petroleum solvents (naphthalene and ethylene dichloride) (*Material safety data sheet, 2002*). Grossly, gills were congested with enlarged liver. This result agrees with *Garg et al. (1987)* who found liver damage, with increased its weight in dogs ingested 50 mg/kg pendimethalin or higher.

The mortality rate was 35% and 15% for fish exposed to 10% and 5% 96 hrs LC_{50} of Stomp®, respectively (Fig. 1). This result was nearly similar to that recorded by *El-Nobi and Gihan (2010*) who mentioned that the mortality rate of fish exposed to 10% and 5% 96 hrs LC_{50} of Stomp® for four weeks were 30% and 16.7%, respectively.

2. Growth performance:

Fig. 2, revealed that, there was a significant decrease in body weight, weight gain and specific growth rate in fish exposed to 10% followed by fish exposed to 5% 96 hrs LC50 of stomp®. These results agree with those previously recorded by *Bražėnaitė and Šakalienė (2006)* who found that 50% growth inhibition of aquatic microorganisms (*S. capricornutum*) green microalgae exposed to 52 µg/l pendimethalin. Similar findings were recorded in rats exposed to higher concentration of pendimethalin (*Material safety data sheet, 2002*). Decreased body weight may be attributed to critical effects of pendimethalin include short term toxicity for the liver and long term for thyroid of rats (*Zulalian, 1990; TOXNET, 2003 and European Commission, 2006*).

3. Biochemical study:

The results demonstrated in Fig. 3, revealed that a significant increase in serum glucose, AST, alkaline phosphatase and cholestrol in fish exposed to 10% then 5% 96 hrs LC_{50} of stomp while there was a significant decrease in total proteins compared to control group. The increase in blood glucose may be attributed to the increase in plasma catecholamines and corticosteroid hormones *(Pickering, 1981).* Moreover, the hyperglycemia induced by any toxicant might be explained by the inhibition of the neuroeffector sites in the adrenal medulla leading to hyper secreation of adrenaline, which stimulates the breakdown of glycogen to glucose *(Gupta, 1974).*

Serum aspartate aminotransferase (AST) and alkaline phosphatase, cholestrol belong to the plasma non-functional enzymes which are normally localized within the cells of liver, heart, gills, kidneys, muscle and other organs. Their presence in blood plasma may give information on tissue injury or organ dysfunction *(Wells et al., 1986).*

Serum protein significantly decreased which was dose dependent. This attributed to the inhibitory effect of dinitroanalin on the protein synthesis *(Ecobichon, 2001).*

4. Genotoxic study:

4. A. Comet assay:

The extent of DNA damage in liver and gills tissues varied among the three studied groups. Table, 1 and Figure, 4 showed that the mean tail DNA % was significantly different among the three groups of liver tissues, while in gills tissues there was differ significantly among the groups treated with 10% and 5% 96 hrs LC_{50} of Stomp® compared to control group and there was no significant difference between group treated with 10% and group treated with 5%. The gills tissues of group treated with 10% had the largest mean tail DNA % (5.21± 0.19), while the lowest mean tail DNA % (1.47± 0.02) was observed in control group of liver tissues.

In the present study, the results of the comet assay indicated that gill cells were more sensitive than the liver cells to DNA damage caused by stomp®. A possible explanation for this difference between liver and gill cells would be that the repair system in gill cells are slower and consequently damaged cells could have remained longer in the gill tissue, resulting in an increased comet score than the liver cells *(Cavalcantea et al., 2008).* Genotoxic effects of stomp® in mice may be due to biosynthesis of genotoxic metabolites *(Dimitrov et al., 2006)*

4. B. Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS- PAGE):

In the present study, sublethal concentration of stomp® has showed variations on the electrophoretic patterns of proteins fractions in the serum protein of *O. niloticus.* In the serum protein of stomp exposed fish, three new protein fractions viz. 258.48 kD, 49.65 kD, 46.07 kD were observed in group exposed to 10% 96 hrs.LC50 and 175.63 kD, 11.84 kD, 10.42 kD were observed in group exposed to 5% 96 hrs.LC50 (Figure 5). This is confirmed by the decreased total proteins as shown in Fig. 3.

5. Histopathological findings

5.1. Gills

Fish exposed to 10% (0.355 mg/L) 96 hrs LC_{50} of Stomp® showed intense hyperplasia and hypertrophy of secondary lamellar epithelium forming sheets particularly on the tips of the primary filaments with congested bronchial blood vessels. The gill arch revealed edema, hemorrhages and round cell infiltration. The gill epithelium had changes to mucus secreting cells (Fig. 6- B). While in fish exposed to 5% (0.177 mg/l) 96 hrs LC_{50} showed hyperemic secondary lamellar capillaries with disassociated secondary lamellar epithelium and hemorrhages in primary lamellar filaments were common. A few eosinophil granular cells were seen scattered at the base of gill arches. Edema in gill rackers was exidents. Disorganization of lamellar cartilage of some gill filaments was seen (Fig. 6- C).

5.2. Liver

Liver in fish exposed to 10% (0.355 mg/L) 96 hrs LC_{50} of Stomp® showed intense degenerative or necrotic changes and inactivation of hepatopancreatic tissue with presence of numerous scattered melano macrophages. More melano macrophage centers could be seen scattered among degenerated hepatic parenchyma. Some portal areas had proliferative bile ductules and periductular fibrosis (Fig. 6-E). While in fish exposed to 5% (0.177 mg/l) 96 hrs LC_{50} of Stomp® showed swollen hepatic cell with granular or vacuolated cytoplasm with slightly activated hepatopancrease and congested blood vessels. Other hepatic cells revealed fatty damage and contained melanomacrophages .Telangectiasis of some hepatic blood vessels and dilated sinusoids could be countered. Numerous bile ductules or hyper plastic biliary epithelium were common accompanied by fibroblastic proliferations (Fig. 6-F). This incised with results recorde by *El-Nobi and Gihan (2010*). This may be attributing by the entrohepatic pathway of the stomp and confirmed by biochemical study as recorded an increase in serum AST, total protein. Moreover the technical grade pendimethalin is more toxic to oxidative phosphrylation of liver rat mitochondria than pure one (*Cetkauskait et al., 2006).*

5.3. Kidney

Fish exposed to 10% (0.355 mg/L) 96 hrs LC_{50} of Stomp showed focal destruction of renal parenchyma and replaced by melanomacrophage cells were common. The remaining renal tubules had necrotic or degenerative changes mainly hyaline droplets with depleted hemopoietic elements. Some glomeruli had contracted glomerular tufts and dilated Bowwman's spaces. Focal hemorrhages and hemisiderosis could be seen (Fig. 6-H). While in fish exposed to 5% (0.177 mg/l) 96 hrs LC_{50} of Stomp showed mild nephrotic changes varied from cloudy swelling to hydropic degeneration and melano macrophage centers were seen in the renal parenchyma. hemopoietic elements were depleted accompanied by mild edema or hemorrhage in renal tissue. Few eosinophil granular cells could be seen. Lobulated or destructed glomerular tufts were also noticed. Cellular or hyaline casts could be seen inside lamina of some renal tubules (Fig. 6- I).

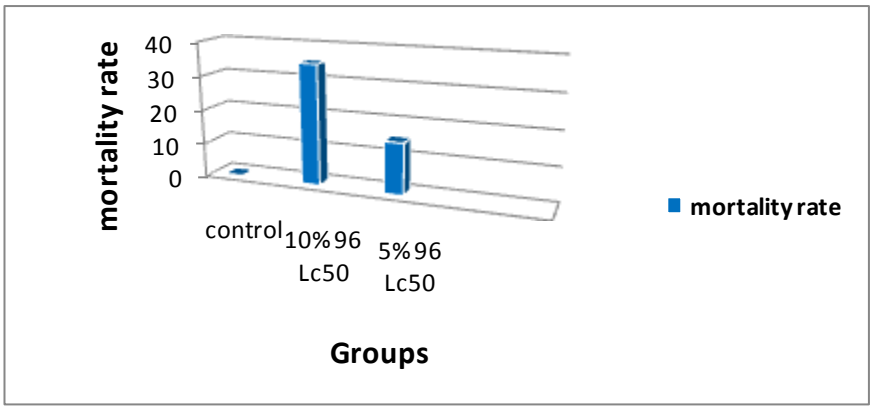

Fig. 1: Effect of 10% and 5%96 hrs. LC50 of stomp on mortality rate of *Oreochromis niloticus* after two months.

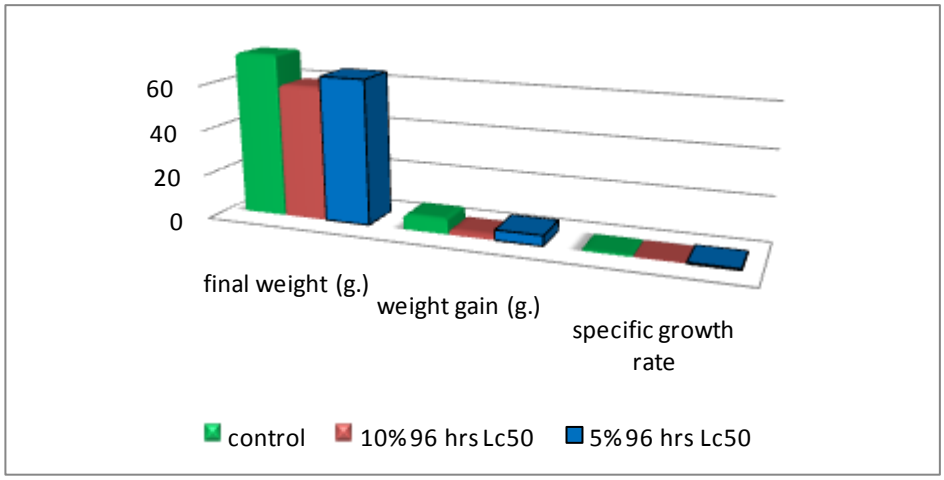

Fig. 2: Effect of 10% and 5%96 hrs. LC50 of stomp on growth performance of *Oreochromis niloticus* after two months (Mean ± S.E.).

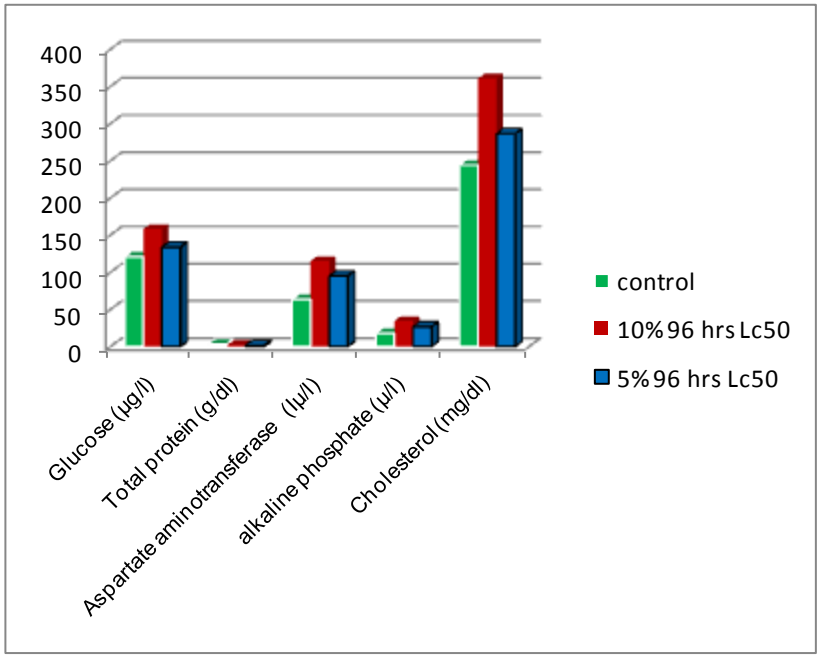

Fig. 3: Effect 10% and 5% 96 hrs. LC50 of stomp on some biochemical parameters in serum of *Oreochromis niloticus* after two months (Mean ± S.E.)

Table 1. Effect 10% and 5% 96 hrs. LC_{50} of stomp on DNA damage of *oreochromis niloticus* after two months by comet assay (Mean ± S.E.)

Group (n=20) ***Tail DNA %***	**Group (1)** **Control**	**Group (2)** **$^1/_{10}$ LC_{50}** **(**0.355 mg/L**)**	**Group (3)** **$^1/_{20}$ LC_{50}** **(**0.177 mg/l**)**
Liver	1.47 ± 0.02^{c}	3.85 ± 0.04^{b}	4.65 ± 0.02^{a}
Gills	2.56 ± 0.01^{b}	5.21 ± 0.19^{a}	5.16 ± 0.01^{a}

Means within the same row bearing different superscripts are significant by different at $p \leq 0.05$.

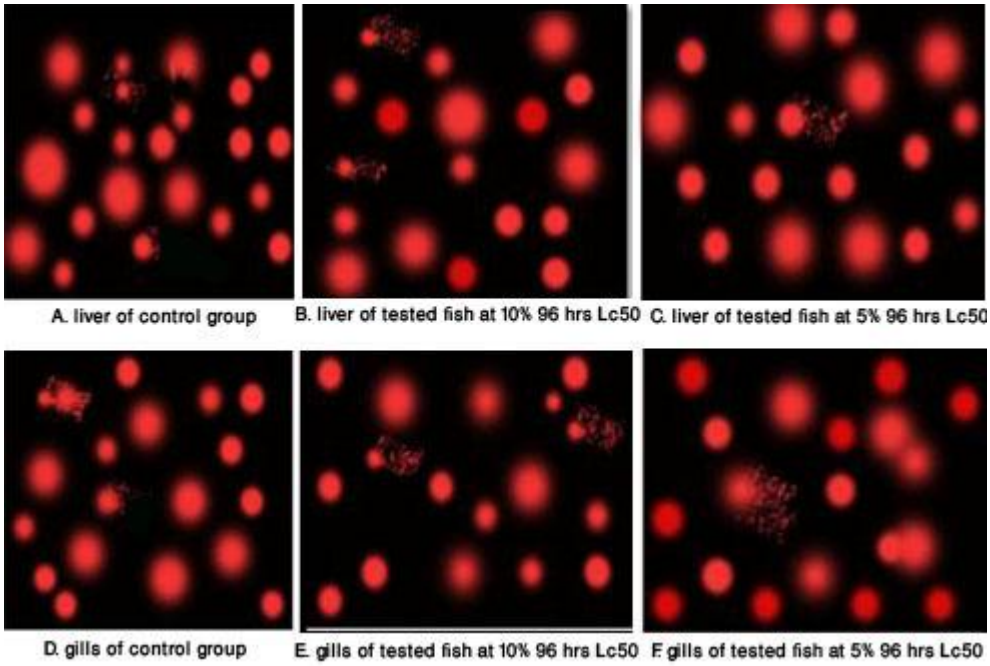

Fig. 4: Photomicrographs representative DNA damage (Comet assay) in liver and gills of *Oreochromis niloticus* exposed to 10% and 5% 96 hrs. LC50 of stomp®.

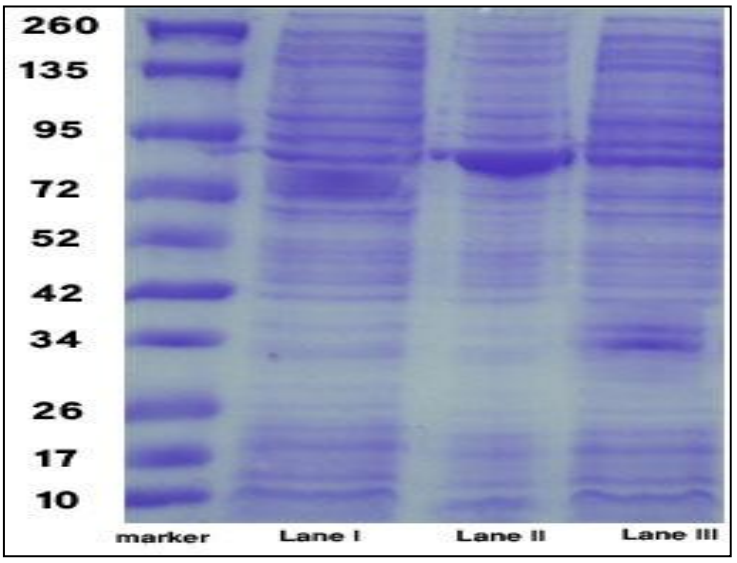

Fig. 5: SDS-PAGE of serum protein fractionation of *O. niloticus* exposed to10% and 5% 96 hrs LC50 of stomp. Lane I: protein fractionation of control group. Lane II: protein fractionation of *O. niloticus* exposed to10% 96 hrs LC50 of stomp®. Lane III: protein fractionation of *O. niloticus* exposed to 5% 96 hrs LC50 of stomp.

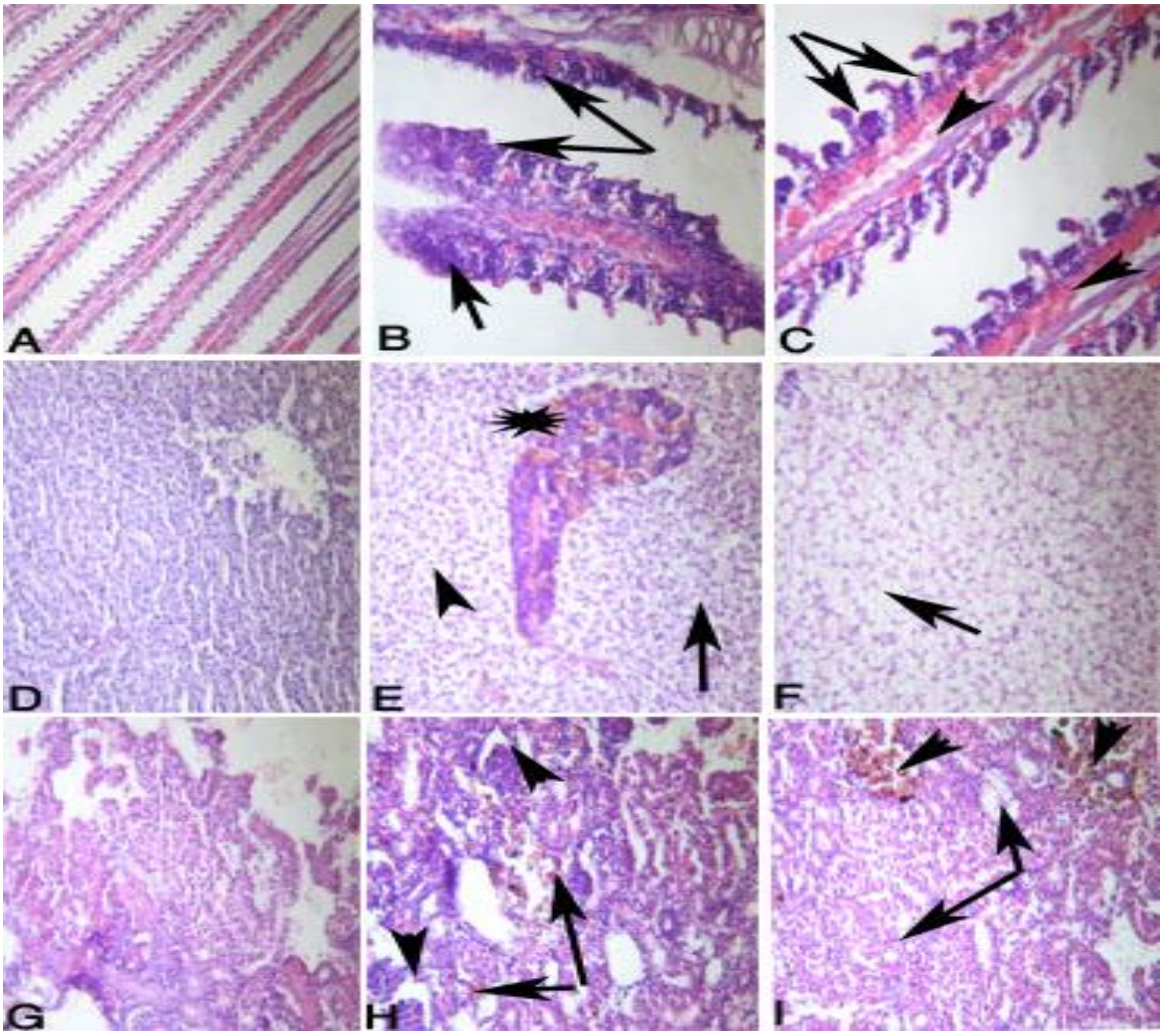

Fig.6: A) Gills of Tilapia nilotica of control group (H &E X120). B) Gills of Tilapia nilotica exposed to 10% 96 hrs. LC50 for two months showing hyperplasia and hypertrophy of secondary lamellar epithelium forming sheets "arrow" (H &E X300). C) Gills of Tilapia nilotica exposed to 5% 96 hrs. LC50 for two months showing desquamation of secondary lamellar epithelium" arrow" and hemorrhage "head of arrow" with gill filaments .(H &E X300). D) Liver of Tilapia nilotica of control group (H &E X300). E) Liver of Tilapia nilotica exposed to 10% 96 hrs. LC50 for two months showing degenerated "arrow" or necrotic "head of arrow" hepatic cells with numerous melanomacrophages "starched star" in hepatopancrease. (H &E X300). F) Hepatic cells revealed fatty changes and contained melanomacrophages G) kidney of Tilapia nilotica of control group (H &E X300) "arrow". H) Posterior kidney of Tilapia nilotica exposed to 10% 96 hrs. LC50 for two months showing focal replacement of renal parenchyma with numerous melanomacrophages "arrow" and contracted glomerular tufts "head of arrow" (H &E X300). F) Posterior kidney of Tilapia nilotica exposed to 5% 96 hrs. LC50 for two months showing reversible nephritic changes "arrow" and melanomacrophages "head of arrow" in the renal parenchyma. (H &E X300)

Conclusion:

The results of this work showed a significant decrease in body weight and weight gain and significant increase in serum glucose, AST, alkaline phosphatase, cholestrol in fish exposed to 10% then 5% 96 hrs LC_{50} of stomp compared to control group. Stomp produced genotoxic effects on the fish species Tilapia nilotica.

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