Original article

In –vitro Cytotoxicity Bioassays of the Herbicide Glyphosate (Roundup) by Gills, and Liver Cell lines of the Nile tilapia (*Oreochromis nilotica*) V.S. *in- vivo* Bioassays

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Abstract

Four hundred of Nile tilapia (*O. niloticus*) juveniles with rang of body weight 2.6-12.6g and standard length of 5.0-11.0 cm were used for in- vivo bioassay to the herbicide Glyphosate and compared to in-vitro assay for gill and liver cell lines of the same *O. niloticus*. Doses tested to estimate the LC50 and LC90 were ranging between 3.6mg/l to 0.225 mg/l for both assessment .Probit analysis was adopted to estimate the lethal doses. Approved scientific methods were used first to establish primary cells and cell lines from samples taken from *O. niloticus* liver and gill. Eosin-Y uptake assay used to observe the dead cells and MTT method to evaluate the validity of the treated cells. Results recorded lethal concentrations to the toxicant less than what previous literature records. The LC50 value and LC90 of juveniles were found to be 2.277 mg/L and 3.405mg/L, respectively. LC50 value and LC90 for gill cells were 0.963mg/l and 3.53mg/l.and that of liver cells were 1.872mg/l and 4.129mg/l respectively. In-vitro test recorded damaged to liver and gill cell at concentrations far below what estimated by the *in-vivo* test using tilapia juveniles. These damages were breaking the cell membrane followed by damage to mitochondria and finally necrosis of the whole cell. The conclusion extracted from the experimental findings of the present study is that in spite of the benefits of Glyphosate as herbicides, its hazard is so great since it attacks the animal cells directly even by concentrations far below the lethal doses. Overall, the present findings highlighted the hazard of exposure to Glyphosate. Thus , the recommendation is to concentrate on adopting *in-vitro* bioassay with cells lines in managing herbicides and pesticides toxicity as they display damages cannot be recorded by the *in-vivo* bioassay

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1. Introduction

It is estimated that there are currently approximately 100,000 chemicals in commerce with 700 to 3000 new chemicals produced annually. Many of these chemicals eventually enter aquatic environments through rivers (Hongyan and Shicui , 2001) .Among those are pesticides and herpicides.

Some of these have drawbacks, such as potential toxicity to humans and other species. According to the Stockholm Convention on Persistent Organic Pollutants, 9 of the 12 most dangerous and persistent organic chemicals are organochlorine pesticides. Glyphosate among them and it is a broad-spectrum

systemic herbicide and crop disinfectant. The most common formulations is Glyphosate,(R). It contains polyoxyethylene amine as main surfactant.

Cell lines have been used in various aspect of scientific research. Many fish cell lines have been derived from detached adult and embryonic tissues. Primary cultures are usually short lived although require specific culture condition. Gills and liver cell lines have been used to understand mechanism of toxicity and evaluating the toxicity of environmental samples. *In vivo* bioassays are costly, time-consuming and requiring specially designed aquatic laboratory facilities .Thus *in vitro* bioassay trials are easier to achieve with cell line than whole organisms (Lee , et.al, 2009). .

1.1. Glyphosate

Glyphosate is an organophosphorus compound, specifically a phosphonate. It is the active component of many commonly used herbicides it can reach bodies of water through irrigated plantations. It is used to kill weeds, especially annual broad leaf weeds and grasses. Study, evaluated the fact of the cytoxcity of Glyphosate in established culture of the zebra fish hepatocyte cell line hF -observed after 24 and 48 h of exposure to concentrations of 50 and 3250 µg/L. a reduction in metabolic activity and lysosomal integrity, and an increase in cell number after 24 h of exposure at the highest concentration. Specific events in Glyphosate-induced cell death reported an increase in the number of early apoptotic cells at a low cytotoxicity level (15%), and a decrease, in favor of late apoptotic and necrotic cell rates for more severe cytotoxicity conditions. At the same time, results showed that the Glyphosate induced mitochondrial membrane potential disruption that could be a cause of apoptosis in keratinocytes cultures (Elie- Caille et.al.,2012) Eisenbauerr, (2012) reported that Glyphosate caused DNA and membrane damage and increase extracellular lactate dehydrogenase which indicate a membrane damage.

The development of Glyphosate-resistant genetically modified organisms (GMO) has increased the use of herbicide Glyphosate by several magnitudes in recent years. It is now the most commonly used pesticide globally that affects aquatic habitats, especially fish.

Nile tilapia (*Oreochromis niloticus*)

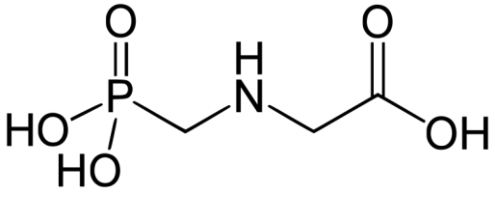
The Nile tilapia (*O. niloticus*) is a species of a cichlid fish native to Africa . It tolerates brackish water and survives temperatures between 8 and 42 °C (Froese and Pauly, 2015). Thus , *O. niloticus* is a traditionally and widely used species for fish farming in Africa and Asia.

1.2.Objective: to evaluate the adoption of *in -vitro* cell lines cytotoxcity bioassy versus the *in-vivo* bioassy of the herbicide Glyphosate on Nile tilapia *(Oreochromis niloticus*) juveniles and to check prospected hazards of Glyphosate at cocentrations below the lethal doses recorded by the *in- vivo* methode.

2. Material and Method:

2.1 Glyphosate:

The selected type is the most common formulations Glyphosate R (Roundup,).It contains polyoxyethylene amine as main surfactant.



Glyphosate chemical structure

2.2. Experimental animals and laboratory conditions:

Four hundred juvenile Nile tilapia (*O. niloticus*) with ranging of body weight 2.6-12.6g and standard length of 5.0-11.0 cm were brought from Fisheries Research Center / Ministery of Animal Resources and Fisheries. Collected fishes were transferred to fish laboratory in the National Center for Research where they kept in glass aquaria of dimensions 39.0 cm X 20.0 cm X 62.3cm. The fishes were acclimatized to the laboratory conditions for two weeks fed with commercial feed of the formula: Moisture 44.982%, ash 32.143%, proteins 19.250%, fat 4.816% and fibers 14.740% obtained from Fisheries Research Center. The feeding ration was at 5% of

their body weight The water temperature was at the range of 22-25^{0}C , PH was ranging between 6-7.4 and dissolved oxygen average concentration was 6.7± 3.7 ppm.

2.3 .Experimental Protocol:

2.3.1. Establishment of Gill and Liver Cell Primary Lines:

Cell culture media preparation :

RPMI-1640 medium prepared following Moore, and Woods, (1976). 16.0 g. Of powdered RPMI-1640 medium were dissolved in one litter Water at temperature 15-20 °C. and 2.0 g sodium bicarbonate was then added. The pH of the medium was adjusted to range of 1.0- 3.0. The medium sterilize immediately by filtration using a membrane with a porosity of 0.22 µm and kept in sterile container. RPMI medium kept at pH range of 6-7.

Culturing Gill and Liver Primary Cells

The cell culture carried out following a method modified by Gaurav, *et.al*.(2001). The tissue were cut with a sterile scissors and washed with chilled phosphate buffer solution (PBS) and transferred to a Petri dish containing 20% streptomycin and penicillin for 5min. Tissues were washed three time and cut to small fragments of 1.0 mm and washed twice with PBS .The pellet was suspended in 5.0ml PBS and trypsinized with 1.0 ml of 0.2 % chilled trypsin for 2 minutes using magnetic stirrer. 1.0 ml of fish serum was added to the cell suspension .Cell were harvested by filtering cell suspension through a sterile muslin cloth .The filtrate was centrifuged at 2000 rpm for 5 min and pellet was washed twice with culture medium. Finally the pellet was resuspended in culture medium and was seeded in 25cm3 tissue culture flask (Greiner) . The flasks were kept in plastic container where CO2 tension was given by burning out a candle .The container was kept in refrigerator at 28 ^{0} C. Tissue culture medium was changed after 15 days .

Establishment of cell line from primary cell was done after 120 days as sub cultures done in to Petri dishes contained RPMI, when the primary cell culture formed multiple layers reached up to 100% confluence.

2.4. Toxicity Bioassay

2.4.1. Tilapia Juveniles Toxicity Test

Experimental Design

Experiments were carried out in three replicates. The stocking rate was ten fishes per aquarium .Five doses was tested plus fishes kept as control .The different concentrations of Glyphosate R (Roundup) used were 3.6.0 mg/L, 1.80 mg/L,., 0.90 mg/L,., 0.45 mg/L, and 0.225 mg/L, for 48 hours and dead fishes was counted then LC50 and LC90 was computed. Data were subjected to Probit Analysis (Finney,1972) computed according to SPP program . The regression (Y = a + bx) as shown in figure (1) was the used method in this analyses. Lethal Concentrations that killed 50% and 90% of the population (LC50, LC90) were appointed graphically. Regression line together with of some associated terms were calculated.

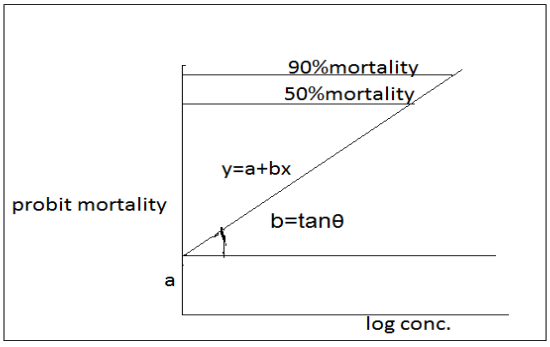


Figure (1) . Probit analysis graphical method.

2.4.2. Cytotoxicity of Glyphosate on Cell Lines:

Procedures followed were as noted below:

2.4.2.1. Eosin -Y uptake assay

When the cell line reached up to 80 -100 % confluencey, cells were detached by detachment solution (EDTA +PBS).Then the cell suspension was added to each of 18-well tissue culture plates each contained one of the five mentioned Glyphosate concentrations. a medium free from Glyphosate was used as control. The Plates were incubated at 20 ^{0} C for 48-h. These tests were replicated three times. Cultures were then tested for each concentration after 48 hour and the concentrations that

killed 50% and 90% of the population (LC50, LC 90) were determined by the uptake of Eosin -Y solution following Bjorndahl *et al.,* (2013).

2.4.2.2. Mitochondrial succinic dehydrogenase assay (MTT)

The method is due to the technique of Borenfreund *et al.,* (1988) that based on reduction of the soluble yellow tetrazolium salt (MTT) to a blue insoluble MTT formazan. The reduction done by the succinic dehydrogenase that produced from the mitochondria that had been injured by the toxicants i.e. Glyphosate.. After a 48-h exposure period, the tested medium was replaced by 20 ml of 5.0 mg/ml MTT in phosphate buffer solution (PBS). The solution was removed carefully after incubation for 4 h at 20^0 C and the cells were rapidly rinsed twice with PBS, and then 150 ml of dimethyl sulfoxide were added to each well to dissolve the purple formazan crystals that had produced. Absorbance of each well was measured at 490 nm and the concentrations of Glyphosate that killed 50% and 90% of the cells population (LC50, LC90) were determined.

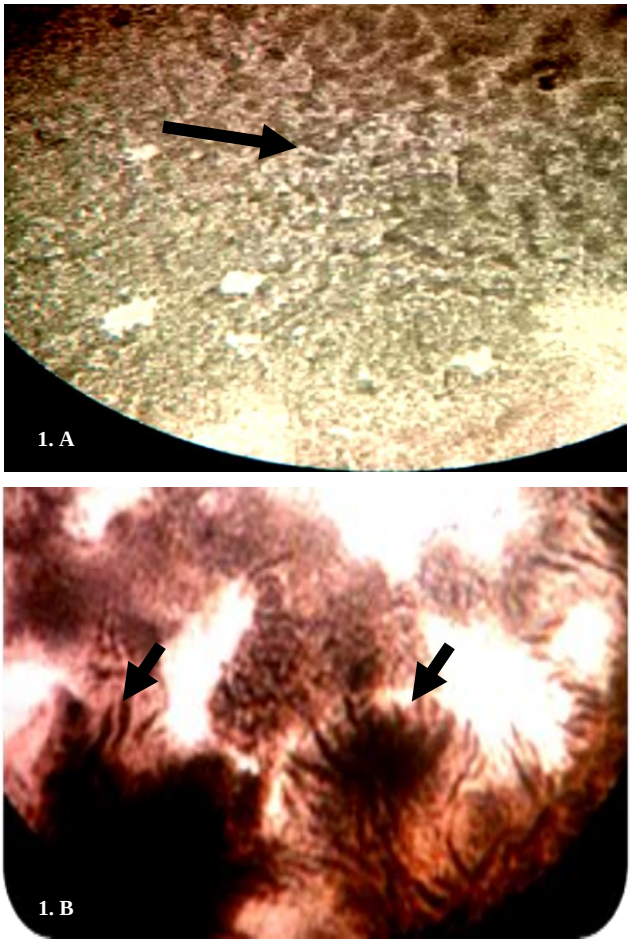
2.5. Statistical Analyses

Data were subjected to Probit Analysis. Lethal Concentrations that killed 50% and 90% of the some population (LC50, LC90) were appointed graphically. Regression line together with the mean of some associated terms i.e. the Coefficient of determination (r2), the Coefficient of correlation (r), Standard deviation, Standard Error, and 95% Confidence limits were calculated

3. Results

3.1. Primary Cell Culture and Cell Lines

Primary cells culture was been established from gill and liver in RPMI medium. Spindle formation in individual cells and clumps was seen on 5^{th}. day after which the cells were found to radiate from clumps and multiply. Individual cells showed elongation and multiplication to complete monolayer up to 100% influence on the 15^{th} .day for both liver and gill cells.. Sub cultures of primary cells were done in RPMI medium to established liver and gill cell lines which fully achieved after their growth for 15 days as shown in plates 1. (1.A &1.B).



Plates (1)1.A: Liver primary cell culture (10X40); 1. B. Gill primary cell culture (10X100) both grown in RPMI medium for 15 days .

3.2. Toxicity of Glyphosate on the Tilapia Juvenile: The toxicity of five different concentration of Glyphosate herbicide on tilapia juveniles showed an increase in mortality with rise of concentration of doses as shown in table (1).Results of Probit analysis that adopted to estimate the lethal concentrations for 50% and the 90% of treated fishes, i.e. LC50 and LC90, respectively were graphically plotted as shown on Fig.(2)

Table(1.) Toxicity Effect of Glyphosate Herbicide on tilapia Juveniles after 48 hours Treatmen

Conc. Glyphosate mg./L	Log 10 Conc.	Total No. ofish	No. of Dead fish	% of mortality	Corrected % mortality
3.60	0.556	30	30	100	100
1.8.0	0.255	30	5	16.7	13.8
0.90	-0.046	30	3	10.0	6.9
0.45	-0.347	30	3	10.0	6.9
0.225	-0.648	30	2	6.7	3.4

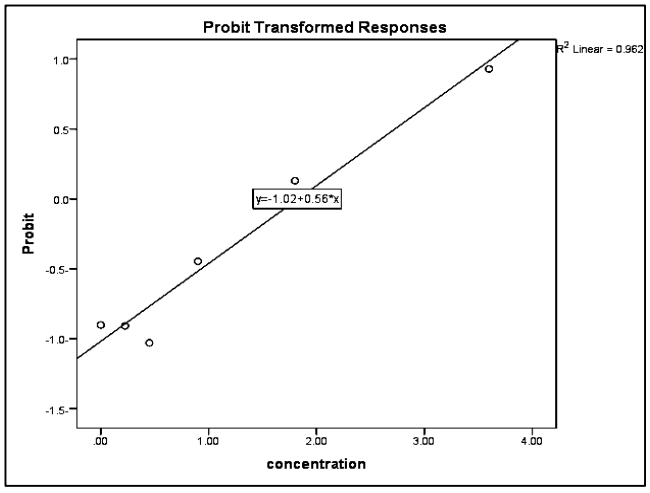


Fig.2.Probit plot for estimation to LC_{50} and LC_{90} doses of Glyphosate on tilapia fish juveniles

4.2. Cytotoxicity of Glyphosate Herbicide on Gill and Lliver Cells Lines of Nile tilapia :

The uptake of Eosin -Y solution methods were used to detected the cultured tilapia Gill and Liver Cells lines that died as result of Cytotoxicity of Glyphosate. The cells were treated by the descending concentrations of Glyphosate for 48 hour and the concentrations that killed 50% and 90% of the population (LC50, LC90) were determined by Probit analysis. Glyphosate induced cell membrane damage and accordingly, dead cells uptake Eosin -Y red color that diffused inside the cells through the damaged cell membranes. Plates 2 (2.A) and (2.C) demonstrated these effects in comparison to untreated gill cell (2.B.).

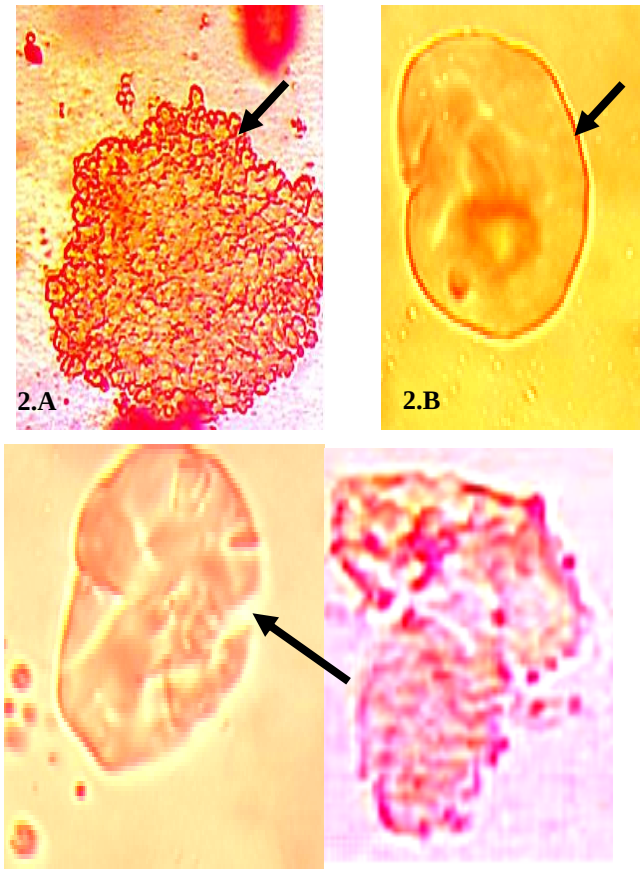


Plate 2: (2. A) The uptake of Eosin -Y solution by the dead liver cell when treated by the lethal concentrations of Glyphosate (3.6mg/L) for 48 hours. 2(B.) was the normal

liver cell, (2. C.) the live liver cell when treated with non-leathal dose 0.225mg/L of Glyphosate, the cell membrane was damaged and whole cell necrosed.

The toxicity of five different concentration of Glyphosate herbicide on tilapia liver and gill cell line were as showed in table (2). Probit analysis that adopted to estimate the lethal concentrations that kill the 50% and the 90% (LC50 and LC90 respectively) was as shown in Fig. (3) and (4).

The Probit analysis for the lethal L50 and L90 for the juvenile tilapia that as well as that liver and gill cells were shown on table (3).

Table(2.) Effect of Glyphosate herbicide on Nile tilapia gill and liver cell line after 48 hours Treatment.

Conc. Glyphosate. mg./L	% liver cell Mortality	Liver cell Corrected % mortality	% Gill cell mortality	Gill cell Corrected % mortality
3.60	82.40	78.40	89.60	87.00
1.8.0	55.20	45.10	72.40	65.50
0.90	32.80	17.60	48.30	35.40
0.45	15.20	3.70	44.00	30.00
0.225	18.20	0.25	40.90	25.00
0.00	18.40	0.00	20.00	0.00

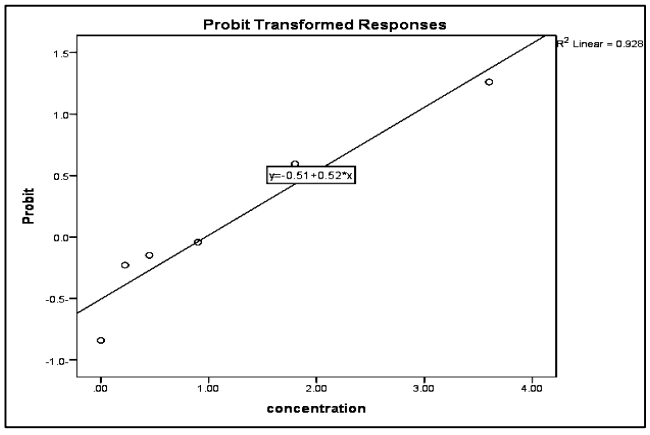


Fig.2. Probit plot for estimation to LC_{50} and LC_{90} doses of Glyphosate on tilapia fish gill cells

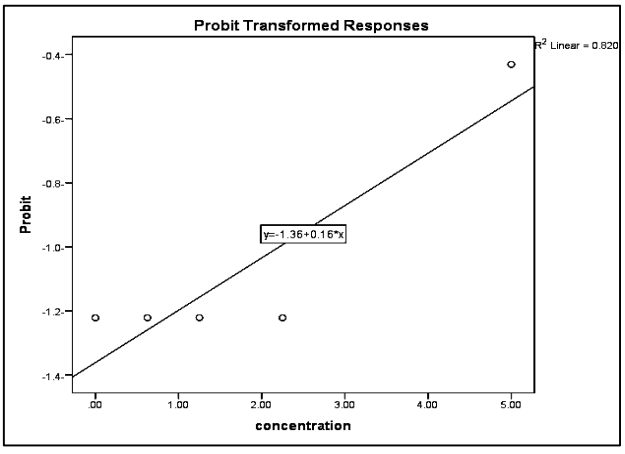


Fig.3. Probit plot for estimation to L_{50} and L_{90} doses of Glyphosate on tilapia fish liver cells.

Table (3.)The transformed Probit of the lethal effect of Glyphosate herbicide on tilapia juveniles , tilapia liver cells and tilapia gill cells after 48 hours Treatment

Conc. Glyphosate mg./L	Log $_{10}$Conc.	Probit of tilapia Juvenile	Probit of Liver cell	Probit of Gill cell
3.60	0.556	8.07	5.77	6.13
1.8.0	0.255	3.72	4.87	5.41
0.90	-0.046	3.52	4.08	4.61
0.45	-0.347	3.52	-	4.48
0.225	-0.648	3.12	-	4.33

Table (4). The estimated and the 95% Confidence Limits of lethal doses of Glyphosate toxicity on fingerlings, liver cell line and Gill cell lines of tilapia *O. niloticus*.

Subjects	Probability	Estimated toxcitant Conc.mg/L	95% lower limit Conc.mg/L	95% upper limit Conc.mg/L
Tilapia fish juveniles	500	2.227	1.557	3.980
	900	3.405	2.488	6.982
Liver cells	500	1.872	1.404	2.761
	900	4.129	3.110	6.468
Gill cells	500	0.963	0.437	1.401
	900	3.530	2.85	4.648

4.3. MTT Assay:

This colorimetric assay used reduction of tetrazolium salt as mean to measure cellular metabolic activity for cell viability. Viable cell contained NAD pH-dependent oxidoreductase enzyme which reduced the MTT reagent to formazan and the deep purple color was formed plate (3).

The of consequence of Glyphosate cytotoxcity on tilapia liver and gill line was slso expresed by degree cell viability was as shown in table (5)which show reduction of viable cells and thus reduction of formaza,

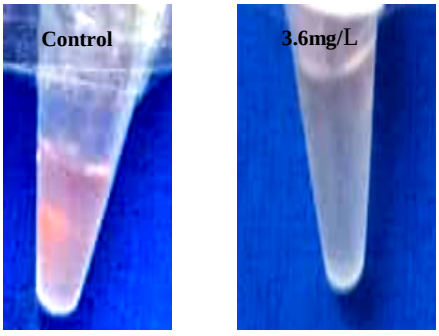


Plate (5) colorimetric assay show reduction of tetrazolium salt as measure for cellular metabolic activity of viable cells.

Table (5.) Effect of five different concentrations of Glyphosate herbicide on Viability of cells of tilapia liver and gill lines

Glyphosate Conc. (mg./L)	Absorption of liver cell	%Viable cells of liver cell	Absorption of gill cell	%Viable cells of Gill cell
3.60	**0.295**	**49.9**	**0.175**	**40.7**
1.8.0	**0.300**	**50.8**	**0.389**	**90.5**
0.90	**0.365**	**61.8**	**0.2**	**46.5**
0.45	**0.369**	**62.4**	**0.40**	**93.0**
0.225	**0.430**	**72.8**	**0.40**	**93.0**
0.00	**0.591**	**100.0**	**0.430**	**100.0**

4. Discussion:

The present study noted that the treatment with descending concentrations ranging between 3.8mg/L to 2.225 mg/L herbicide Glyphosate to juvenile *O. niloticus* for 48 hours exhibited respiratory disturbance, erratic swimming, loss of equilibrium, and sudden fish death varied greatly with differences in concentration of the toxicant and that mortality increases with an increase in concentration. The acute toxicity effects of Glyphosate on *O. niloticus* agreed with Muhammad, *et.al.*(2021); Selvarani *et al.* (2019), and Micah *et al.* (2017). The present study reported the LC50 value and LC90 of juveniles were found to be 2.277 mg/L and 3.405mg/L, respectively. These concentrations were far below for what recorded for the bioassay experiments performed in a static renewal regime with *O niloticus* exposing to varying acute toxicity concentrations of Glyphosate viz., 15.33, 30.67, 61.34, 122.68 and 245.36 mg/l for 96 hrs. In that study (Selvarani *et. al.* 2019), 100 % mortality was observed in concentrations of 122.68 and 245.36 mg/l of Glyphosate. The LC50 was determined to be 49.22 mg/l after 96 hrs of exposure. However, the present study results were in agreement with the study of toxicity effect of glyphosate on fingerlings of *Heteroclarias* (Micah et.al.(2017) when fishes were exposed to series of lethal concentration of 0.00 mg/L, 5.40 mg/L, 7.20 mg/L, 9.00 mg/L, 10.80 mg/L and 12.60 mg/L for 96 hours in a renewal bioassay. This procedure showed that the 96 hours LC50 was 6.838mg/L.

Furthermore, the present results showed that *in vitro* bioassess of Glyphosate cytotoxicity on liver and gills cell line demonstrated hazard at concentration of Glyphosateat very low comparing to *in vivo* bioassy. These concentration caused mortality and damage to treated liver cell and gill cells were varying between 0.963-1.872mg/L for LC50 and 3.530-4.129 mg/L for LC90.

These concentrations induced cell membrane damaged and decrease cell viability. Those results are agreeing with and Lopes and Souza (2018) who reported Glyphosate increase inactive mitochondria and apoptotic cells, necrotic cells and damage in cell line .Similarly, Eli-Caille *et al*.(2012) reported that the Glyphosate cytotoxic effects on cultured human keratinocytes were their morphological and functional cell characteristics. It got effects on cellular end points. This agree with Chaufan, *et.al.*(2014) who reported Glyphosate effects on oxidative balance and cellular end points.

Eisenbauerr, (2012) reported that Glyphosate caused DNA and membrane damage and increase extracellular lactate dehydrogenase which indicate a membrane damage. Glyphosate in present study have effect on cell viability at

concentration of glyphosate of 0.45mg/L for gill cells and 0.225mg/L for liver cells. That agree with Lopes and Souza (2018) on viability of human dermal fibroblasts and toxicity on human dermal fibroblasts and also agree with Hongfei , *et. al.* (2019) who reported that glyphosate caused cytotoxicity and decrease cell viability.

Pure glyphosate and Roundup showed similar non-monotonic toxicological profiles at low dose exposure from 10 µg/ml(10 mg/l), whereas Wipe out demonstrated a monotonic reduction in cell viability from a threshold concentration of 50 µg/ml (50 mg/l), when tested in whole blood.

Conclusion and recommendations

The conclusion of the present study is that in spite of the benefits of Glyphosate as herbicides, its hazard is great since it attacks the animal cells directly and in low concentration far below the lethal doses recorded by *in-vivo* assayes. Thus, the recommendation is to concentrate on adopting *in-vitro* bioassay in managing the uses these types of herbicides and pesticides.

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