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# ASSESSMENT OF THE ADVERSE EFFECTS OF THIOBENCARB AND DITHIOPYR HERBICIDES ON THE NILE TILAPIA, *OREOCHROMIS NILOTICUS*

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**Key words:** Nile tilapia, herbicides, Thiobencarb, Dithiopyr, tissues residue, biochemical parameters, histopathology, cytotoxicity.

## ABSTRACT

The herbicides Thiobencarb and Dithiopyr are largely used as weed killers, especially in the rice fields, where cultured tilapia can be exposed to the adverse effects of such herbicides. Leaching of the herbicides in the soil could also affect the wild fish in the natural habitats and so considered as chemical water pollutants. To assess the adverse effects of these herbicides on the Nile tilapia in this study; $L_{C50}$ was determined, and recorded as 720µg/l and 280µg/l for Thiobencarb and Dithiopyr respectively. Nile tilapia was exposed to acute and chronic exposures of Thiobencarb and Dithiopyr as single and mixed treatments. The residue of both herbicides in fish tissues was estimated, and revealed that the highest value was detected in the liver, while the lowest was in the brain.

Biochemical and genetic studies were applied to evaluate the toxicity and alterations due to Thiobencarb and Dithiopyr in Nile tilapia. Elevated values of serum and muscle total protein and alkaline phosphatase were observed in acute and chronic cases with a synergistic action in case of combination of the two herbicides. Concerning genetic studies, utilization of the apoptosis technique in the *in vitro* resulted apoptotic bands in the spleen, kidney and liver. This result was confirmed by the micronuclei findings, which indicated an alteration of the cell division in gills as a natural exposed organ to the polluted water in case of acute and chronic exposure. The histopathological studies revealed frequent degenerative changes in the parenchymetous organs, wide spread

necrosis and activation of the melanocytes and the severity of changes appeared in the long–term exposure to mixed Thiobencarb and Dithiopyr. However, this study confirmed the cytotoxicity of the two largely used weed controllers, Thiobencarb and Dithiopyr on the Nile tilapia.

## INTRODUCTION

Most environmental problems of concern today are attributed to the production and release of toxic chemicals capable of interacting with the environment and disrupting the ecosystem.

In recent years, large quantities of pesticides have been produced and discharged into the environment. Herbicides, a distinctive group of pesticides, are considered as selective chemical weed killer; hence they have been intensively used to destroy the unwanted plants, especially in agricultural settings.

The impact of chemical environmental contamination on fish health, consequently fish productivity is of economical relevance for fishes as well as aquaculture. Environmental pollutants have been reported to accumulate in fish and have threatened human health, either directly or indirectly through the food chain. Accumulations of toxic compounds which may be carcinogenic or mutagenic were manifested as hazards (Porte & Albaiges, 1994; Jacobs *et al.* 2002). However, the proper handling and use of herbicides in aquatic areas are especially critical, accidental spills or over dose can kill fish or cause other damage to its habitats that may lead to reduction in the fish population.

The biochemical processes represent the most sensitive and relatively early events of pollutant damage. Thus, it is important that pollutant effects be determined and interpreted in biochemical terms, to delineate mechanisms of pollutant action, and possibly ways to mitigate adverse effects (Begum, 2004). Knowledge of the sublethal effects of toxic compounds on biochemical, genetic and histopathological levels is very important for delineating fish health status and for understanding future ecological impact.

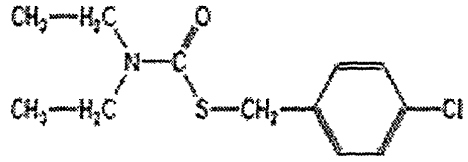
Thiobencarb and Dithiopyr are two herbicides used as weed killers which are heavily used in rice cultivation in Egypt, where tilapia fish is frequently cultured, although there are yet few reports on long-term effects of both herbicide.

In this respect, the present study aimed to investigate the biochemical, genetic and histopathological alterations in Nile tilapia in response to short (4 days) and prolonged (60 days) exposure to acute and

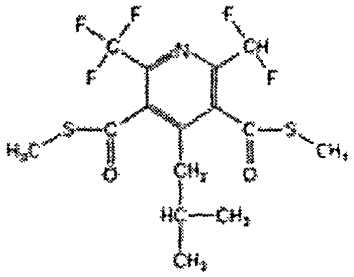
sublethal concentrations of two herbicides, Thiobencrb, Dithiopyr and their combination.

# MATERIALS AND METHODS

## 1-Chemicals:



Thiobencarb: is a thiocarbamate herbicide used in this study as commercial Preparation that contains 50% active ingredient.



Dithiopyr: is a pyridine herbicide used in this study as a commercial preparation that contains 25% active ingredient.

## 2-Determination of 96 hours half lethal concentration dose($L_{C50}$) for Thiobencarb and Dithiopyr

Preliminary screenings were carried out to estimate the concentration of the used herbicides which is most likely to cause 50% mortality ($L_{C50}$) for 96 hours exposure to determine the appropriate testing range of concentrations for each chemical. This task was done according to the procedure described by Behreus and Karbeur (1953). Sixty-six fish were distributed in eleven aquaria with 100 liter capacity (six fish for each aquarium). The aquaria were supplied with dechlorinated tap water and kept at constant aeration, temperature, pH and feeding. Ten concentrations of Thiobencarb and Dithiopyr were distributed in the aquaria (200, 300, 400, 500, 600, 700, 800, 900, 1000 and 1100) and (50, 100, 150, 200, 250, 300, 350, 400, 450 and 500) mg/l, respectively. The aquarium number 11 was kept as a control. All fish were observed for 96 hours to record the number of dead and active fish in each aquarium. The 96-hr $L_{C50}$ was calculated according to the following equation: $L_{C50}$ = highest dose - $\sum ab/n$ where,

a: is a constant factor between two successive concentrations.

b: is the mean of dead fish in two successive groups.

n: is the number of fish in each group (6 fish).

Toxicity tests showed that 96-hr $L_{C50}$ values of *Oreochromis niloticus* were 720 and 280 ppm for the herbicides Thiobencarb and Dithiopyr, respectively.

**3-Animals and experimental design:**

*Oreochromis niloticus* fish of 25.5±2.5 g body weight and 11.4±1.5 cm in total length were collected from a fish farm of the Central Laboratory for Aquaculture Research (CLAR). Abbasa, Sharkia Governorate, Egypt. A total of 300 tilapia fish sample were acclimated to laboratory conditions in aerated and dechlorinated tap water in 150 L aquaria for 1 week, at 26±1°C, under a natural photoperiod (light/dark hours = 12).

Fish were divided into three groups, the first is the control group (60 tilapia); second group (120 tilapia) was sub divided into 3 equal subgroups that were used for the short-term exposure group ($L_{C50}$ of Thiobencarb, Dithiopyr, and their combination for 4 days); and the third group (120 tilapia) was subdivided into 3 subgroups to be used for the long-term exposure group (1/10 $L_{C50}$ of Thiobencarb, Dithiopyr and their combination for 60 days). Fish samples were kept under observation along the period of experiment A sample of five fishes were taken daily in case of short-term exposure ($2^{nd}$ group) and in two weeks intervals in long-term exposure ($3^{rd}$ group).

**4-Biochemical and physiological studies:**

The serum alkaline phosphatase was determined according to the method described in Bergmeyer (1972). The serum total protein was determined according to the method of Doumas *et al.* (1971). The muscle total protein was determined using the semi-micro-kjeldahl method, as reported by Josyln (1950).

**5-Extraction and clean up of herbicides:**

The extraction procedure adopted by AOAC (1990) included both Thiobencarb and Dithiopyr, where their extracts were evaporated at 30°C to dryness. After clean up and dissolution in 1 ml methanol, then directed to HPLC analysis. These extracts were chromatographed with UV spectrophotometer detector and C18 stemless column 25 mm. The HPLC conditions for the two pesticides was recorded (Table 1).

**6-Genetic study:**

This study was conducted using two trends of investigation:

A- *In vivo* evaluation of DNA damage by micronucleus assay: in case of short and long term exposures:

At the end of the short exposure (4 days) and the long one (60 days), a drop of blood from the gills of tilapia fish exposed to the used herbicides was obtained. It was mixed with a drop of fetal calf serum on a clean dry slide and air-dried. The sample was fixed in methanol for 5 minutes. Slides were stained with 10% Giemsa stain for 10 minutes. One thousand erythrocytes were examined for each fish to determine the percentage of cells containing micronuclei (Al-Sabti & Metcalfe, 1995). Micronuclei (MN) were scored in 1000 binucleate cells per treatment (Fig.1).

B-*In vitro* evaluation of DNA damage by Apoptosis assay:

**Cell culture:**

Cells from the liver, spleen and kidneys were maintained in minimum essential medium supplemented with 5% fetal bovine serum. Cells were harvested using 2% trypsin and seeded onto 100 ml culture dishes. Cells were treated with 1/100 of the $L_{C50}$ for individual herbicides and mixture (1:1) of the two herbicides. The cells were then allowed to grow at 37 °C in 5% $CO_2$, 95% air humidified incubator for 72 hours.

**7-Genomic DNA isolation:**

DNA was extracted for apoptosis assay using salting out extraction method according to Aljanabi & Martinez (1997), 0.02 mg of the tissue was digested using 600 µl lysing buffer (50mM NaCl, 1mM $Na_2$ EDTA, 0.5% SDS, pH =8.3) overnight, centrifugation was carried out to precipitate the undigested materials, the supernatant was treated with saturated solution of NaCl (200 µl) to precipitate proteins. The supernatant was transmitted to a new eppendorf tube; DNA was extracted and precipitated using cold isopropanol, centrifuged for 10 min at 12.000 rpm. Pellets of the DNA was washed in 70% ethanol, dried and resuspended in 50µl TE buffer (10mMtris, 1mM EDTa, pH 8) for further use to detect the apoptotic bands.

**8-Apoptosis analysis:**

DNA was separated in 1.5% agarose gel, visualized by UV Trans illuminator after ethidium bromide staining, and photographed by a Polaroid camera.

**9- Clinical investigation and post mortem examination:**

The exposed fish were kept under proper observation during the period of experiment for any external clinical signs, PM lesions or deaths according to Amlacher (1970).

**10-Histopathological study:**

Tissue specimens as gills, liver, kidney and spleen were taken from tilapia that were exposed to a combination of thiopencarb and Dithiopyr in water by the end of exposure. The specimens were fixed in 10% buffered formalin. They were processed by conventional method, sectioned at 4 um and stained with Haematoxylin and Eosin, (Bancroft *et al.*, 1996).

**11-Statistical analysis:**

The results were statistically analyzed using analysis of variance and Duncan's multiple comparison tests to evaluate the comparison between means at $P< 0.05$ (Duncan, 1955). Also percentage of change has been calculated compared to control fish to evaluate changes in parameters among the species due to effect of the exposure.

## RESULTS

**$LC_{50}$ and tissue residue in tilapia fish:**

The recorded $L_{C50}$ of Thiobencarb at 96hrs was 720 µg/ l water and 280 µg/ l water for Dithiopyr. Data in Table (2) indicated the presence of Thiobencarb and Dithiopyr residues in fish tissues after short-term and long-term exposures, with highest value in liver and lowest value recorded in the brain for all groups.

The recorded clinical signs in this study were more or less similar in both short-term and long-term herbicides exposed groups; severity was confirmed with the long term exposure in chronic case. Clinical signs were manifested in the form of nervous manifestations, abnormal swimming behavior in the form of erratic swimming, abnormal skin discoloration. The postmortem findings revealed congestion and haemorrhages in all internal organs in addition to pale anemic gills.

**Biochemical results:**

Changes in enzyme activity serum alkaline phosphatase, total protein and muscles total protein measurements (mean± *SD*) in Nile tilapia exposed to Thiobencarb and Dithiopyr, individually or/and mixed are presented in Tables (3 and 4).

The short-term exposure of tilapia to the herbicides revealed some significant increase in alkaline phosphatase at the 2$^{nd}$ day of exposure to Thiobencarb and Dithiopyr (T1 and T2), while the increase in mixture exposure(T3 ) was observed at 1$^{st}$ of exposure. The total protein in serum and muscles was increased at 1$^{st}$ day of exposure till the end of the experiment (Table 3).

The increase in the alkaline phosphatase in long-term exposure of tilapia to these herbicides was significant and apparent at the 4th week. The total protein was increased in blood at the 2nd week (T1 and T3) and 4th week for (T2), while that in muscles was increased at the 2nd week (T2 and T3) and at 6th week For (T1). The increase of these values was extended till the end of the experiment (Table 4).

**Genetic results:**

Slides of micronucleus analysis were examined at 1000x magnification using light microscope. Micronuclei (MN) were scored in 1000 binucleate cells per treatment. Fig.1 showed the MN main ±*SD* frequencies in individual and mixed exposures of Nile tilapia to the used herbicides; for short-term and long-term cases. Significant increase in the frequency of MN in all treated groups was compared to the control. The highest value of the MN frequency was detected with individual exposure to Thiobencarb compared to Dithiopyr in acute and chronic cases. An observed decrease in the MN frequencies in case of mixed exposure was demonstrated, an increased frequency of the MN was observed in the same type of exposure for the long- term compared to the short- term exposure. Figure 2 showed the induction of the MN by herbicides exposure.

To clarify the mode of cell death induced by the two used herbicides, DNA fragmentation analysis or the apoptosis assay was performed. Fig. 3 depicts the results of the agarose gel electrophoresis of cellular DNA showing that the in vitro exposure of tilapia tissue cells to the two used herbicides either individually or mixed, induced an oligosomal DNA ladder. Cultured and harvested spleen, kidney and liver cells were analyzed for the presence of apoptotic DNA and ladder formation. Apoptosis analysis showed an apparent DNA fragmentation in a ladder form by gel electrophoresis (Fig.3) for the treated spleen, kidney and liver cells, in case of individual and mixed treating at 200bp and its multiples.

**Histopathological results:**

In this study, gills were anemic and showed focal epithelial sloughing in the secondary lamellae, gills also exhibited telangiectasis of some secondary lamellae with degeneration of other lamellae (Plate 1, A).

The experimented fish of all treated groups, after the acute exposure, revealed degenerative changes in the parenchymatous organs (liver, kidney and spleen), mainly liver vacuolar degeneration (Plate 1,

D), and hepatic cells with piknotic nuclei (Plate 1, C). The spleen exhibited nuclear pyknosis of the lymphocytes (Plate 1, F).

The picture of the chronic exposure was more or less severe, where advanced degeneration and wide spread necrosis were evident in the parenchymatous organs together with either atrophy or activation of melanomacrophages. The spleen revealed also necrosis of lymphocytes and some melanomacrophages with marked lymphoid depletion (Plate1, E). Kidney showed defused tubular necrosis with activation of melanomacrophage centers (Plate 1, B).

No observed differences were found in case of combined exposure to Thiobencarb and Dithiopyr compared to the individual exposure.

## DISCUSSION

The indiscriminate use of pesticides in agriculture can cause environmental problems especially to the aquatic system by altering the quality of water and so affecting the physiology, biochemistry and cytology of non target organisms such as fish (Shakoori *et al.*, 1996).

Thiobencarb and Dithiopyr are two largely used weed killers herbicides in rice cultivation, while Nile tilapia is frequently cultured in the rice fields, hence, it is exposed to toxicity by the used chemicals. To assess the adverse effects of the two herbicides on tilapia fish experimentally, $L_{C50}$ of Thiobencarb was detected and recorded as 720 µg/ l, while it was 280 µg/l for Dithiopyr. This result is more or less similar to that of Tomlin (1997).

From results of toxins residues in fish muscles, it was clear that the highest bioaccumulation of the tested Thiobencarb and Dithiopyr exist in fish tissue following exposure. Mean residue levels of Thiobencarb, Dithiopyr as combination treatment are presented in Table (2). The high uptake and penetration within tissues of pesticides via integument of tilapia fish was observed by El-Shemy *et al.* (1991). The highest concentration of Thiobencarb and Dithiopyr as combination treatment was found in the liver, while the lowest was found in fish brain. Penetrability lead to higher residue levels of herbicide in fish treated with the combination of Thiobencarb and Dithiopyr formulation. Also, these results may be attributed to the chemical structure of the tested herbicides and this may be due to accumulation in the tissue because of their lipophilic nature (El-Sayed & Radwan, 2004).

Bioassay with herbicide mixture in short and long-term exposure resulted in more or less combined toxic effects that were slightly

synergistic. These results were not in agreement with findings from some studies, suggesting that the toxicity of many pesticide mixtures to fresh water organisms is rarely greater than additive (Marking, 1985).

Jyothi & Nrayan (1999) studied the effects of sub lethal concentrations of Carbyle and phorate in the serum of *Clarias batrachus.* They suggested that liver damages might have induced production of mitochondrial enzymes such as alkaline phosphatase were subsequently released into the blood. The toxic effects could be lipolytic in nature, as a result of which, the cell membrane, lysosomal membrane, and other organelles underwent dissolution, releasing the enzymes into the blood. Hence, the increased enzyme activities in the present investigation may be a result of cell necrosis in the liver which was confirmed by the pathological signs.

Concerning the genetic studies, two genotoxicity assays were applied on Nile tilapia exposed to two herbicides, Thiobencarb and Dithiopyr; the nuclear anomaly or micronucleus (MN) assay and the apoptosis assay which have been widely applied to the investigation of environmental genotoxic effects on fish. MNi are a whole or partial chromosomes which have been incorporated into the daughter nucleus during cell division and appears as small round dark stained structures, otherwise identical in appearance to the cell nucleus (Vincent *et al.*, 2000). MNi provide a convenient and reliable index of both chromosome breakage and loss, because MNi are expressed in cells that have completed nuclear division and are ideally scored in the binucleated stage of the cell cycle (Michael, 2000).

In this study, a picture of MNi frequency appeared in all treated groups, Thiobencarb could prove to be more genotoxic than Dithiopyr as revealed from the micronucleus assay. An exhibition of antagonistic action was demonstrated in case of combined exposure of the two used herbicides, that was indicated by the decreased frequency of the MNi in this case of exposure. The recorded increase in MNi frequencies in herbicide mixture long-term exposure group compared to short-term group could be explained by Teles (2003) who observed that the genotoxic response profile of *Anguilla anguilla* to naphthalene may reflect a considerable DNA repair capacity and or a metabolic adaptation providing an efficient naphthalene biotransformation and detoxification.

The nuclear morphology results are in agreement with many other studies which have found elevated erythrocyte MNi frequencies in fish

inhabiting contaminated sites and laboratory exposure studies (Al-Sabti & Metcalfe, 1995).

The observation that chromosome damage can be caused by exposure to carcinogenic chemicals was among the first reliable evidences that chemical agents can cause major alterations to the genetic material of eukaryotic cells, and chromosome abnormalities are a direct consequence and manifestation of damage at the DNA level (Michael, 2000).

The measurement of cytogenetic alterations *in vitro* is considered an initial step in the risk assessment procedures for genotoxic agents. The concern about genotoxic pollutants in natural fish population makes the use of fish-derived cells a useful tool for these purposes. Early study concerning prediction of carcinogenic/mutagenic potential revealed that the production of strand breaks is correlated with the carcinogenic and mutagenic properties of environmental contaminants with diverse structures (Sina *et al.*, 1983). It was also demonstrated that various isolated cells from aquatic species respond to a range of direct and indirect genotoxins by DNA Damage (Mitchelmore & Chipman, 1998).

The DNA lesion leads to incomplete transcription, cellular dysfunction, growth inhibition, aging, weakened immunity and diseases in the organism itself. Furthermore, it has created a severe problem within ecosystems and the food chains (Kurelec & Gupta, 1993). Apoptosis is a highly regulated process by which an organism eliminates unwanted cells without eliciting an inflammatory response. Apoptosis is triggered in response to various forms of stress and DNA damages (Smith & Fornace, 1996). Thiobencarb is a thiocarbamate herbicide; Dithiopyr is a pyridine carbamate herbicide; carbamate pesticides are readily converted into *N*-nitroso metabolites in the presence of nitrites or nitrogen oxides in the stomach under acidic conditions or in the colon by intestinal bacteria (Hugheset *et al.*, 2001). The nitroso derivatives of carbamate pesticides less the cholinesterase inhibiting properties of the parent compounds and is less toxic to mammals. However, they induce sister chromatid exchange, chromosomal aberrations, micronucleus formation and aneuploidy in vitro and in vivo (Chauhan *et al.*, 2000; Kurda *et al.*, 1992). In such study, the induction of mitotic toxicity and sister chromatid exchange were confirmed as a genotoxic impact of Thiobencarb herbicide on the Chinese hamster cell line. From our genetic study, the exposure of tilapia cells to Thiobencarb and Dithiopyr induced cell cycle arrest and apoptotic death of the cells. It is now recognized that total endogenous

nitrosation processes contribute significantly to a genotoxic burden for the fish population.

In the fish histopathological study, gills are the first target organ of several pollutants because of their very large interface area between external and internal fish environment, performing vital functions such as gas exchange and ion osmoregulation, the gills are particularly sensitive to adverse environmental conditions. In this study, gills were anemic and showed focal epithelial sloughing in the secondary lamellae; gills also exhibited telangiectasis of some secondary lamellae with degeneration of other lamellae.

The structural damage from the effects of pollutants in liver metabolism have been supported by the results of biochemical studies, which provide early warning indicators of toxicological responses. The fish kidney is composed of three distinct systems: endocrine, hemopeiotic, and excretory. Lesions that develop in the kidney may involve one or all of the three tissue systems, thus it is essential to study the changes that may occur in different cell types.

In the present study, the parenchymatous organs showed vacuolar degeneration, necrosis, and nuclear pyknosis of the spleen lymphocytes, in accordance with (Gingerich, 1982) who found that the most frequently encountered types of degenerative changes are those of hydropic degeneration, vaculization and focal necrosis in case of severe intoxication. The vaculization of hepatocytes might indicate an imbalance between the rate of synthesis of substances in the parenchymal cells and the rate of their release into the circulation system (Gingerich, 1982). Because the excretion of the divalent ions is a major function of the renal tubular epithelium, pollution with pesticides would be highly likely to affect these cells. The defused tubular necrosis appeared in the kidney exposed to the two used herbicides has been suggested to be an indicator of renal toxicity for a variety of chemicals, including pesticides and herbicides (Jiraungkoorskul *et al.*, 2003).

## CONCLUSION

This study has demonstrated that Thiobencarb and Dithiopyr have a drastic cytotoxic effects on Nile tilapia as manifested through the recorded histopathological and biochemical alterations and DNA damage. The current study of detection of DNA damage resulting from contaminant exposure may be a key tactic in assessing the general health of freshwater organisms, identifying the importance of genotoxicity as a

monitoring factor for predicting the pollution impact on fish populations in the aquatic ecosystem.

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Table (1): The HPLC standardization for the determination of Thiobencarb and Dithiopyr.

| Pesticides | Mobile phase | Flow rate | Retention time |
|---|---|---|---|
| **Thiobencarb** | Methanol 90/10AN | 1 ml/min | 3.106 |
| **Dithiopyr** | Methanol 70/30AN | 1 ml/min | 4.723 |

Table(2): Residues analysis of $L_{C50}$ and 1/10 $L_{C50}$ of combination between Thiobencarb(T1) and score(T2) in fish tissues after 1, 2, 3 and 4 days and after 15, 30, 45 and 60 days of exposures of exposures, respectively.

| Treatment | Acute ( µg/g ) | | | | | | | | Chronic ( µg/g ) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 day | | 2 day | | 3 day | | 4 day | | 15 day | | 30 day | | 45 day | | 60 day | |
| Pesticides | T1 | T2 | T1 | T2 | T1 | T2 | T1 | T2 | T1 | T2 | T1 | T2 | T1 | T2 | T1 | T2 |
| Brain | 11.37 | 0.37 | 15.05 | 0.41 | 18.23 | 0.80 | 20.87 | 0.88 | 16.71 | 1.13 | 18.53 | 2.20 | 20.19 | 3.94 | 27.21 | 4.14 |
| Kidney | 15.09 | 1.36 | 16.21 | 2.11 | 24.43 | 2.12 | 25.98 | 2.78 | 22.63 | 3.33 | 31.52 | 7.74 | 43.25 | 11.25 | 44.52 | 18.21 |
| Liver | 11.61 | 4.14 | 35.38 | 4.23 | 60.15 | 4.84 | 74.58 | 5.99 | 44.54 | 9.69 | 61.51 | 17.12 | 77.2 | 23.41 | 86.11 | 31.89 |
| Muscle | 16.33 | 0.37 | 11.67 | 5.58 | 20.27 | 5.97 | 24.02 | 7.42 | 29.77 | 11.26 | 36.87 | 24.18 | 51.75 | 28.52 | 69.15 | 30.89 |
| Gills | 23.08 | 2.12 | 24.15 | 2.16 | 26.26 | 2.47 | 26.30 | 3.40 | 31.13 | 7.81 | 43.30 | 15.36 | 54.82 | 21.24 | 73.14 | 23.82 |

Table (3): Serum biochemistry of experimented tilapia during the acute exposure to the $L_{C50}$ tested herbicides in comparison with the control group (Mean ±SD).

| Time/ exposure | Alk Phosphatase | | | T. protein (Blood) | | | T. protein (Muscles) | | |
|---|---|---|---|---|---|---|---|---|---|
| | T1 | T2 | T3 | T1 | T2 | T3 | T1 | T2 | T3 |
| Control | 11.80 ±0.92$^{a}$ | 11.17 ±0.81$^{a}$ | 12.79 ±1.12$^{a}$ | 8.60 ±0.28$^{a}$ | 8.33 ±0.45$^{a}$ | 8.13 ±0.38$^{a}$ | 16.75 ±0.22$^{a}$ | 16.7 ±0.16$^{a}$ | 16.76 ±0.18$^{a}$ |
| 1 day | 13.84 ±0.47$^{a}$ | 12.34 ±0.39$^{a}$ | 15.04 ±0.47$^{b}$ | 9.53 ±0.31$^{b}$ | 9.46 ±0.18$^{ab}$ | 9.97 ±0.34$^{b}$ | 16.10 ±0.13$^{ab}$ | 15.99 ±0.34$^{ab}$ | 16.10 ±0.32$^{b}$ |
| 2 days | 16.57 ±0.66$^{b}$ | 14.87 ±0.66$^{b}$ | 17.77 ±0.66$^{c}$ | 11.55 ±0.35$^{c}$ | 11.33 ±0.36$^{c}$ | 11.41 ±0.25$^{c}$ | 15.75 ±0.20$^{bc}$ | 15.45 ±0.55$^{bc}$ | 15.61 ±0.11$^{b}$ |
| 3 days | 19.36 ±1.03$^{c}$ | 17.66 ±1.12$^{c}$ | 20.56 ±2.05$^{d}$ | 12.29 ±0.30$^{c}$ | 10.77 ±0.61b$^{c}$ | 13.03 ±0.44$^{d}$ | 15.01 ±0.39$^{c}$ | 14.83 ±0.16$^{dc}$ | 14.78 ±0.19$^{c}$ |
| 4 days | 26.76 ±1.69$^{d}$ | 25.28 ±2.78$^{d}$ | 27.96 ±2.87$^{e}$ | 13.60 ±0.26$^{d}$ | 7.02 ±0.59$^{ad}$ | 14.12 ±0.38$^{e}$ | 13.65 ±0.30$^{d}$ | 14.27 ±0.07$^{d}$ | 13.12 ±0.16$^{d}$ |

T1= Thiobencarb; T2= Dithiopyr; T3= combined Thiobencarb and Dithiopyr

Mean with the same letters at the same column are not significantly different (p> 0.05)

Table (4): Serum biochemistry of experimented tilapia during the chronic exposure to the 1/10 $L_{C50}$ tested herbicides in comparison with the control group (Mean ± SE).

| Time/ Exposure | Alk Phosphatase | | | Total protein (Blood) | | | Total protein (Muscles) | | |
|---|---|---|---|---|---|---|---|---|---|
| | T1 | T2 | T3 | T1 | T2 | T3 | T1 | T2 | T3 |
| Control | 11.80 ±0.92$^{a}$ | 11.17 ±0.81$^{a}$ | 12.79 ±1.12$^{a}$ | 8.60 ±0.28$^{a}$ | 8.33 ±0.45$^{a}$ | 8.13 ±0.38$^{a}$ | 16.75 ±0.22$^{a}$ | 16.7 ±0.16$^{a}$ | 16.76 ±0.18$^{a}$ |
| 2 weeks | 11.74 ±0.39$^{a}$ | 11.24 ±0.43$^{a}$ | 13.44 ±0.76$^{a}$ | 9.01 ±0.26$^{ab}$ | 8.70 ±0.31$^{a}$ | 9.56 ±0.21$^{b}$ | 16.29 ±0.13$^{a}$ | 16.19 ±0.33$^{ab}$ | 16.33 ±0.28$^{ab}$ |
| 4 weeks | 14.27 ±0.66$^{b}$ | 13.77 ±0.71$^{b}$ | 15.80 ±0.86$^{b}$ | 9.63 ±0.17$^{b}$ | 9.64 ±0.36$^{ab}$ | 10.61 ±0.24$^{c}$ | 15.94 ±0.21$^{ab}$ | 15.65 ±0.56$^{bc}$ | 15.91 ±0.15$^{b}$ |
| 6 weeks | 17.06 ±1.03$^{c}$ | 16.56 ±1.09$^{c}$ | 18.75 ±2.03$^{c}$ | 10.51 ±0.33$^{c}$ | 10.34 ±0.24$^{b}$ | 11.83 ±0.30$^{d}$ | 15.35 ±0.38$^{b}$ | 15.13 ±0.17$^{cd}$ | 14.98 ±0.19$^{c}$ |
| 8 weeks | 24.68 ±0.79$^{d}$ | 24.18 ±0.93$^{d}$ | 26.37 ±1.37$^{d}$ | 12.04 ±0.39$^{d}$ | 10.86 ±0.11$^{bc}$ | 12.91 ±0.30$^{e}$ | 14.43 ±0.36$^{c}$ | 14.61 ±0.03$^{d}$ | 13.70 ±0.09$^{d}$ |

T1= Thiobencarb; T2= Dithiopyr; T3= combined Thiobencarb and Dithiopyr

Mean with the same letters at the same column are not significantly different (p> 0.05)

# LEGEND AND FIGURES

**Figure (1)**: The MN frequency in the acute, chronic; individual and mixture exposure of tilapia to Thiobencarb and Dithiopyr herbicides.

A = Control group.

B= MN frequency in case of acute exposure to Thiobencarb herbicide.

C= MN frequency in case of chronic exposure to Thiobencarb herbicide.

D= MN frequency in case of acute exposure to Dithiopyr herbicide.

E= MN frequency in case of chronic exposure to Dithiopyr herbicide.

F= MN frequency in case of acute exposure to mixed herbicides.

G= MN frequency in case of chronic exposure to mixed herbicides.

**Figure (2) :** Micronuclei: Arrow shows micronucleus(MN).

**Figure (3) :** Apoptotic bands revealed from in vitro exposure of tilapia cells to individually and mixed used herbicides.

**S1,S2,S3=** Spleen cells exposed to Thiobencarb, Dithiopyr and the mixture of the two herbicides respectively.

**K1,K2,K3=** Kidney cells exposed to Thiobencarb, Dithiopyr and the mixture of the two herbicides respectively.

**L1,L2,L3 =** Liver cells exposed to Thiobencarb, Dithiopyr and the mixture of the two herbicides respectively.

**Figure (4) :**Light micrographs of a transverse section of Nile tilapia exposed to mixture of Thiobencarb and Dithiopyr herbicides ( H & E stain, X 400) showing:

A: Anemic gills with secondary gill lamellae showed focal sloughing and vacuolation in their epithelial lining.

B: Kidney showed defused tubular necrosis with activation of melanomacrophage centers.

C: Liver showing Spread necrosis with numerous activated melanomacrophage cells, other hepatic cells exhibited pycnotic nuclei.

D: Liver showed advanced vacuolar degeneration of hepatocytes with nuclear pycnosis, focal aggregation of melanomacrophageswas evident.

E: Spleen showing marked degeneration and necrosis of melanomacrophage centers with focal lymphoid depletion.

F: Spleen revealed nuclear pycnosis of lymphocytes with parenchymal dema , atrophy and necrosis of melanomacrophges.

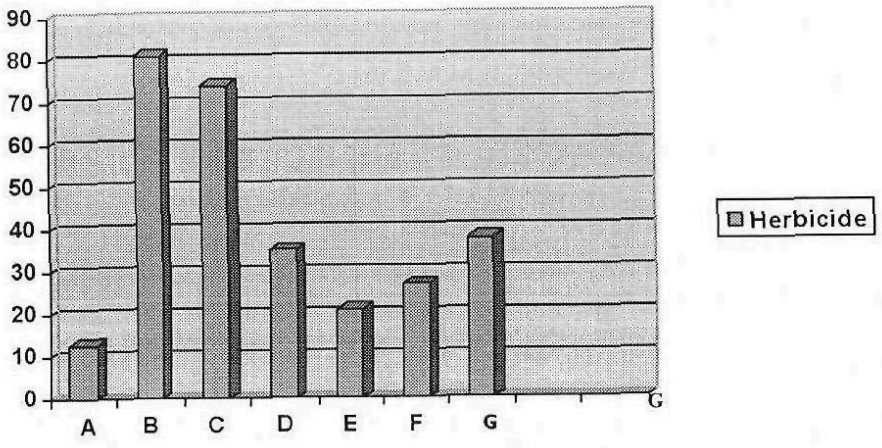


Fig. (1)

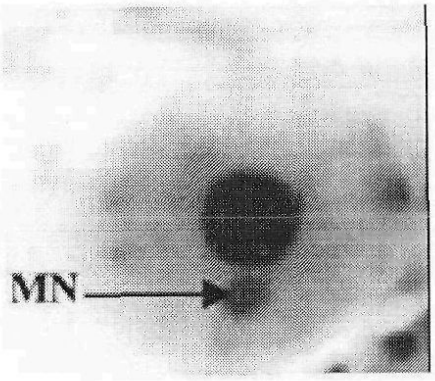


Fig. (2)

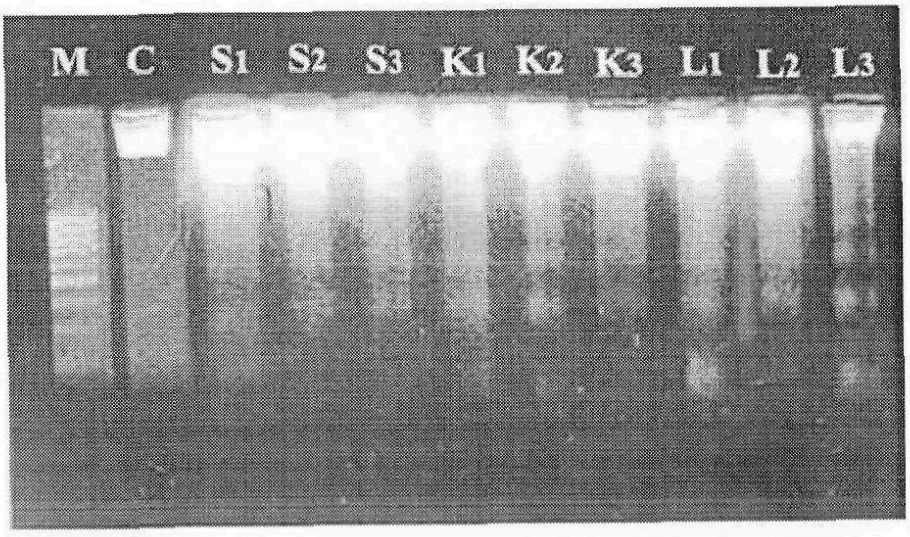


Fig. (3)

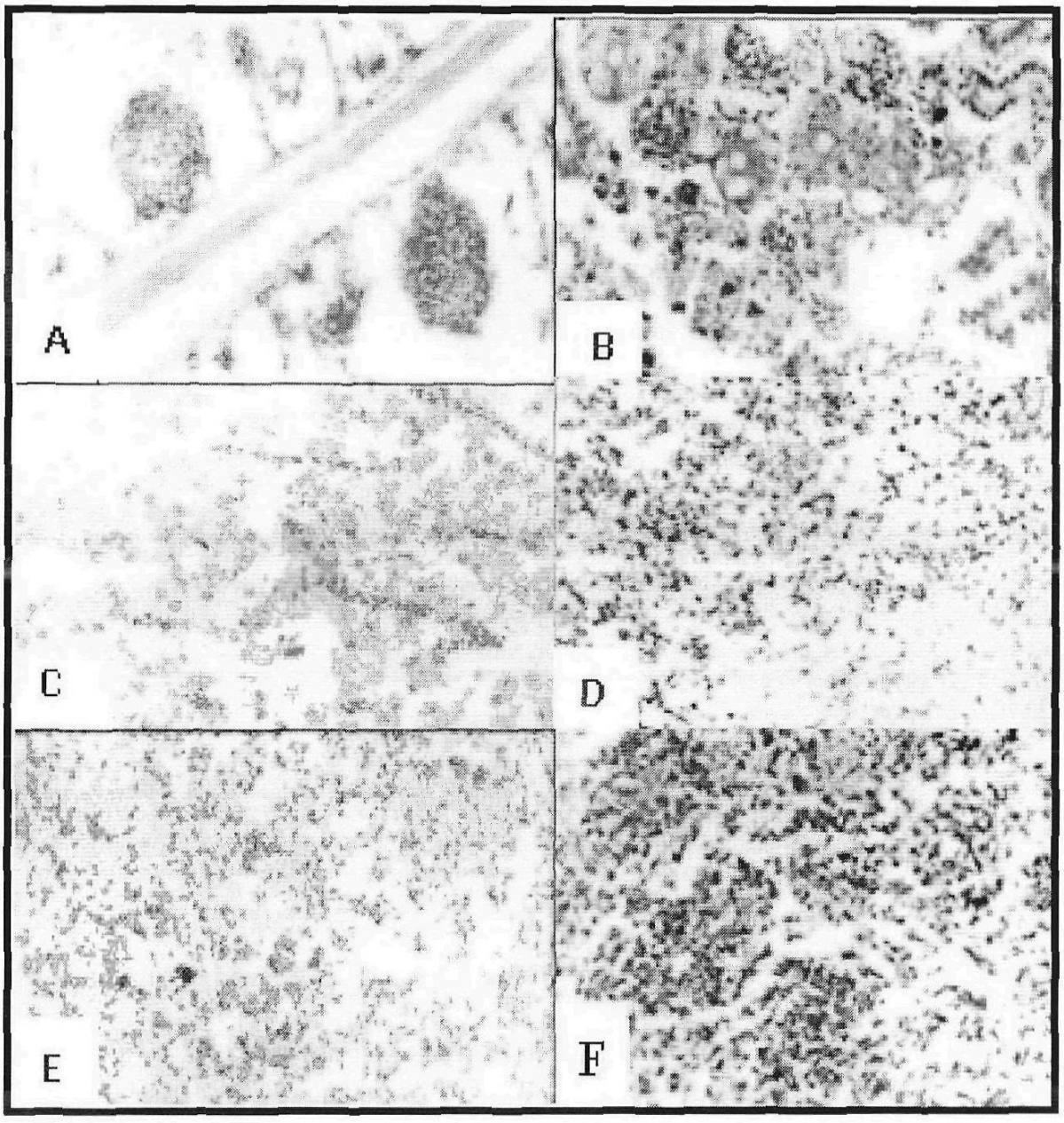


Fig. (4)