Hepatoxicity Induced by Glyphosate-Based Herbicide Baron in Albino Rats

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Abstract: Baron is herbicide includes (48% glyphosate) widely used in Egypt. The present study assess cytotoxic and genotoxic effect of Baron on rats liver. Two groups of rats were treated orally with $1/10$ LD_{50} (275.49 mg kg^{-1}) and $1/40$ LD_{50} (68.86 mg kg^{-1}) glyphosate for 28 days compared with control group. Serum and liver tissues were taken at 14 and 28 days of treatment. An inhibition in Alanine aminotransferase (ALT) and Aspartate aminotransferase (AST) activites were recorded at both treatment periods and reduction in Total serum Protein (TP) and Albumin (ALB). However, non-significant changes in serum Acetylcholinesterase (AChE). Elevation in oxidative stress biomarker Malondyaldehyde (MDA) and decline in detoxification biomarkers total reduced Glutathione (GSH), Glutathione S-Transferase (GST) and Superoxide Dismutase (SOD) in liver tissue led to increase in percentage of DNA damage. Destruction in liver tissue architecture was observed. Although, Baron was classified in the safe category pesticides repeated exposure to small doses has great danger effect.

Key words: Glyphosate, liver toxicity, oxidative stress, DNA damage, commet assay

INTRODUCTION

Glyphosate [N-(phosphonomethyl) glycine] is a widely used non-selective post-emergence herbicide. It has been marketed since 1974 and its use is likely to increase further as it is one of the first herbicides against which crops have been genetically modified to increase their tolerance (Solomon et al., 2007). Glyphosate is an organophosphorated non-selective agrochemical widely used in many countries including Egypt. It is broad-leaf weeds, pastures and rice, corn and soy plantations (Smith and Oehme, 1992). Glyphosate inhibits plant growth through interference with the production of essential aromatic amino acids. Glyphosate is primarily a competitive inhibitor of the critical enzyme of the shikimate pathway, 5-enolpyruvylshikimate-3-phosphate synthase which is responsible for the synthesis of an intermediate in the biosynthesis of phenylalanine, tyrosine and tryptophan (Rubin et al., 1984; Malik et al., 1989). The resulting reduction in protein synthesis causes termination of growth and eventually cellular disruption and death. The effectiveness of glyphosate as a phytotoxin is due in part to its low molecular weight and high solubility in water which aid its rapid absorption and translocation by plant tissues. Its residues may thus enter the food chain and glyphosate and its metabolite such as Aminomethylphosphonic Acid (AMPA) and formaldehyde is found as a contaminant in environment such as soil and rivers (Temple and Smith, 1992; WHO, 2003). Animals injected with glyphosate showed depressed function of cytochrome P450 and two other enzymes which are vital to the body's processing of toxicants, the first involves cytochrome P450 enzymes and the second involves Glutathione S-Tranferases (GSTs) (Acquavella et al., 2004). Glyphosate is an Organophosphorus (OP) compounds are predicated irreversible inhibition of Acetylcholinesterase (AChE) and other serine hydrolases (Viragh et al., 1999). In vitro study for the determinations of the toxicity of glyphosate on the activities of serum Acetylcholinesterase (AChE), Lactate Dehydrogenase (LDH), Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), Alkaline Phosphatase (ALP) and Acid Phosphatase (ACP) are described as well as changes in electrophoretic patterns of serum proteins were also tested. Results revealed that glyphosate was effective on all enzymes, except ACP (El-Demerdash et al., 2001). On the the hand, (Benedetti et al., 2004) analyzed the hepatic effects of

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herbicide glyphosate-biocarb in Wistar rats they found that oral administration with 4.87, 48.7 or 487 mg kg^{-1} of glyphosate each 2 days during 75 days caused leakage of hepatic intracellular enzymes, Alanine aminotransferase (ALT) and Aspartate amiontransferase (AST). Excessive lipid peroxidation induced with glyphosate ingestion to pregnant rats leads to an overload of maternal and fetal antioxidant defense systems (Beuret *et al.*, 2005). *In vitro* studies with tissue cultures or aquatic organisms, several of the formulated products are more toxic than glyphosate AI (Giesy *et al.*, 2000; Williams *et al.*, 2000). Differences in the response of test organisms to the AI and the commercial formulation, e.g., round up are likely due to the toxicity of different formulates and surfactants contained in commercial products. There is a general agreement that adjuvants may be more toxic for animals than glyphosate itself (Giesy *et al.*, 2000; Williams *et al.*, 2000; Richard *et al.*, 2005). Many environmental pollutants may induce the formation of Reactive Oxygen Species (ROS) (Ahmad *et al.*, 2000; Sevgiler *et al.*, 2004) due to their high reactivity these species may cause damage to lipids, proteins, carbohydrates or nucleic acids (Fraga *et al.*, 1996; Parvez and Raisuddin, 2005). Pollutant-induced Lipid Peroxidation (LPO) as in the case of herbicides has been observed in several fish species (Sevgiler *et al.*, 2004). Variations in the activities of antioxidant enzymes have been proposed as indicators of pollutant mediated oxidative stress (Ahmad *et al.*, 2000; Li *et al.*, 2003).

The aim of this study was to investigate cytotoxic and genotoxic effect of local commercial formulation Baron glyphosate based herbicide on the liver of albino rats treated orally with different doses for 28 days.

MATERIALS AND METHODS

Pesticide: Baron (glyphosate isopropyl ammonium 48% SL) was supplied by El Helb Company for pesticide industry, Egypt.

Acute oral medium Lethal Dose LD$_{50}$: The median Lethal Dose (LD$_{50}$) was determined according to the method of Weil (1952).

Experiments animals: About thirty male *Rattus noruogious* albino rats weighing between (200-250 g) were allotted for the present study. Animals were supplied by breeding unit of Egyptian organization for the Biology and Vaccine production A.R.E. These animals were clinically healthy and they were acclimatized to the experimental conditions for 2 weeks before start of the real experiment. Animals were fed on balanced ration and free access to tap water throughout the experimental period.

Experimental design: After 2 weeks of acclimatization period, animals were divided into 3 experimental groups as follows:

- The 1st group is control: rats were given distilled water (1 mL day^{-1}) by gastric intubation every other day for 4 weeks
- The 2nd group: rats were given 5 days/week an oral dose of Glyphosate equals 1/10 LD$_{50}$ (275.49 mg kg^{-1}) dissolved in distilled water for 4 weeks
- The 3rd group: rats were given 5 day/week an oral dose of glyphosate equals 1/40 (68.86 mg kg^{-1}) dissolved in distilled water for 4 weeks
- Five rats from each group were sacrificed after 14 and 28 days of experimental period after blood withdrawal

Blood samples: Blood has been collected from the retro-orbital plexus according to Schermer (1967) and immediately allowed to aliquots containing heparin. The blood test tubes were left 30 min then centrifuges at 3000 rpm for 15 min in refrigerated centrifuge to separate serum.

Tissue samples: Five rats from each group were sacrificed after 14 and 28 days of experimental period after blood withdrawal. The animals were dissected to obtain brain, liver, heart, lung, spleen, kidney and tests) were weighed. Liver tissue samples were taken for homogenized to determine MDA; GSH levels and GST; SOD activities. Other parts were kept in liquid nitrogen for studying DNA commet assay and other parts were kept in 10% buffered formalin for histopathological examination.

Biochemical analysis: Kinetic determination of Acetylcholinestrase (AChE) activity was done according to method by Ellman *et al.* (1961). Plasma glucose concentration was determined colorimetrically by the method adopted by Barham and Trinder (1972) using kits purchased from (Bio-Merieux, France). Colorimetric determination of AST and ALT activity was according to Reitman and Frankel (1957) using the kit of Stanbio (USA). Colorimetric determination of total protein in serum was according to Weichselbaum (1946) using the kit of Stanbio (USA). Colorimetric determination of total protein in serum was according to Dumas and Biggs (1971) using the kit of Stanbio (USA). Level of Malondialdehyde (MDA), marker of lipid peroxidation was estimated in liver tissues which was measured according to the method by Yoshioka *et al.* (1979). Determination of glutathione S-transferase activity was undertaken using the method by Habig *et al.* (1974). In this methods, the

activity was measured spectophotometrically by following the increase in the yellow color development as a result of conjugation of 1-Chloro 2, 4-Dinitrobenzene (CDNB) with GSH. Determination of reduced Glutathione (GSH) content was done using the method by Beutler *et al.* (1963). The spectrophotometric assay was based on the determination of the yellow color resulting from the reaction of 5, 5 Dithio-bis (2-nitrobenzoic acid) (DTNB) with the SH group of glutathione. Determination of Superoxide Dismutase (SOD) activity was carried out using the method by Masayasu and Hiroshi (1979). The method based on generation of superoxide anions by pyrogaloll auto-oxidation and detection of superoxide anions by Nitrobule Tetazolium (NBT) formazan color development.

Commet assay in liver tissues: Liver cell suspension was obtained by mincing briefly a part of the tissue with a pair of fine scissors in 1 mL of PBS and by pipetting up and down the finely minced tissue pieces. The conventional alkaline version of the comet assay was performed according to the method by Collins *et al.* (1993) with slight modifications. The slides were immersed in lysing buffer solution (2.5 M NaCl, 0.1 M Na2EDTA, 10 Mm Tris, freshly added 1% Triton X-100, PH 10 with NaOH) for 1 h at 40°C to remove proteins. The slides were then placed in a horizontal electrophoretic tank filled with freshly prepared cold alkaline buffer (40c.3 M NaOH, 1 mM Na2EDTA, pH 13.0) for 40 min to allow DNA unwinding and expression of alkali-labile sites. Electrophoresis was carried out in the former solution for 30 min (at 300 mA 0.65 v cm^{-1}) using a compact power supply. After electrophoresis, the slides were removed from the tank, immersed horizontally in neutralizing buffer (0.4 M Tris pH 7.5). After neutralization, slides can be stained with ethidium bromide (20 µg mL^{-1} distilled H$_2$O) for 1 h. After staining, the slides were rinsed with distilled water and kept in a closed box at 40°C. Stained slides were examined under a Carl zeiss fluorescence microscope at 40x magnification using filter 15 (Bp543/120 FT580, LP590). For each sample, 50 randomly selected cells, respectively were photographed using a 3-CCD camera and stored in a separate files. The acquired images were pre-processed to remove obtained artifacts. The cells with small heads and large fan-like tails were excluded under the principle that they represent apoptotic cells. The images were analyzed for measurement of DNA damage with scion image Software Version 3b (National Institute of Health, USA). For each cell, the length of DNA migration (comet tail length) was measured in micrometers from the center of nucleus to the end of the tail. The percentage of damaged DNA concentration in the comet tail was determined by measuring the total intensity of ethidium bromide fluorescence in the cells which was taken as 100% and determining what percentage of this total intensity measured only in the tail.

Histopathological studies: Samples were fixed in formaline 10%, tissues were trimmed in paraffin blocks and were cut at 4-6 µm thickness and the sections were stained with haematoxylin and eosin stain according to the technique used by Bancroft and Stevens (1977) and left for histopathological examination.

Statistical analysis: Data were statistically analyzed using Students t-test according to Snedecor and Cochran (1967). t-test was performed to evaluate the difference between mean values of the treated group and those of control group. Values are exposed as mean±SE.

RESULTS AND DISCUSSION

It has become common practice to use the medium Lethal Dose (LD$_{50}$) values to express and compare the toxicity of different pesticides. The calculated LD$_{50}$ of baron commercial formulation of glyphosate was 2636.33 mg kg^{-1} in male albino rats as demonstrated in Table 1. The depicted results in Table 2 revealed that intoxication with glyphosate (1/10 and 1/40 LD$_{50}$) for 28 days induced significant increase in liver, kidneys, lungs and testis weight at p<0.05 and p<0.01 pronounced at 28 days. Expressed data in Table 3 although, Baron (glyphosate-based) herbicide releated to the organophosphates, it had no significant effect on serum Acetyl cholinesterase (AChE) marker of Ops intoxication. However, a pronounced significant increase at p<0.01 in serum glucose level was recorded at the end of the experimental period. On the other hand, serum liver biomarkers Alanin aminotransferase (ALT), Aspartate aminotransferase (AST) recorded significant inhibition also, Total Protein (TP) and Albumin (ALB) recorded significant decrease in their levels at p<0.05 and p<0.01 in 1/40 LD$_{50}$ treated group at 28 days. Moreover, rats intoxicated with different dose levels of Baron (glyphosate-based) herbicide induced remarkable elevation in lipid peroxidation biomarker Malondyaldehyde (MDA) in liver tissues significant versus control at p<0.01 in both intoxicated groups throughout treatment periods. These findings concurrent with significant reduction in the defense store; total reduced Glutathione (GSH) in liver tissue. A Glutathione S-Transferase (GST) was recorded in all intoxicated groups significant versus control at p<0.05 and p<0.01.

Table 1: Median lethal doses in relation to the number of died animals in LD$_{50}$ calculation

Dose mg kg^{-1} body weight	No. of rat used	No. of dead rat	LD$_{50}$
4800	5	5	2636.33 mg kg^{-1}
3333.3	5	5	-
2777.7	5	3	-
2314.8	5	0	-

Table 2: Effect of repeated administration of glyphosate (1/10 LD$_{50}$ = 275.49 mg kg^{-1} and 1/40 LD$_{50}$ = 68.86 mg kg^{-1}) on organs weight ratio for 28 days (values are mean±SE)

	Periods					
	14 days			28 days		
Organs	Control	1/10 LD$_{50}$	1/40 LD$_{50}$	Control	1/10 LD$_{50}$	1/40 LD$_{50}$
Brain	5.358±0.080	4.700±0.063	5.400±0.148	5.07±0.097	4.990±0.15	5.144±0.211
Heart	3.426±0.067	3.406±0.060	3.640±0.080	3.34±0.054	3.360±0.07	3.490±0.051*
Liver	9.758±0.205	10.988±0.279*	10.240±0.164	9.80±0.130	10.928±0.50*	10.960±0.177**
Kidneys	4.900±0.130	5.010±0.030	4.692±0.128	4.80±0.050	4.930±0.09	5.730±0.161***
Lung	4.880±0.140	4.600±0.210	5.240±0.070*	4.59±0.040	4.550±0.13	4.760±0.070*
Spleen	3.240±0.120	3.820±0.550	3.590±0.237	3.71±0.160	2.900±0.10	3.235±0.082*
Tests	6.340±0.050	6.180±0.110	7.165±0.470	5.88±0.370	6.430±0.20	6.900±0.193*

Table 3: Effect of repeated administration of glyphosate (1/10 LD$_{50}$ = 275.49 mg kg^{-1} and 1/40 LD$_{50}$ = 68.86 mg kg^{-1}) on serum liver markers

Periods	Groups	AChE (U L^{-1})	Glucose (mg dL^{-1})	ALT (U L^{-1})	AST (U L^{-1})	TP (g dL^{-1})	ALB (g dL^{-1})
14 days	Control	347.50±24.01	156.630±11.58	36.00±1.90	25.00±1.53	10.70±0.32	7.700±0.68
	1/10LD$_{50}$	376.21±69.83	132.656±8.900	30.25±5.10	27.75±3.37	11.45±2.10	7.880±0.51
	1/40LD$_{50}$	415.88±23.49*	164.750±7.180	28.28±6.83	28.81±3.00	11.05±0.57	8.020±0.88
28 days	Control	382.35±31.40	135.030±6.310	35.55±1.91	30.10±1.90	10.67±.850	9.925±1.09
	1/10LD$_{50}$	398.08±39.62	141.470±11.13	27.60±4.10	30.16±2.10	10.44±0.89	8.900±1.15
	1/40LD$_{50}$	398.31±11.47	209.300±26.80**	25.13±0.45***	23.20±2.46*	9.00±0.34*	4.560±0.21**

Table 4: Effect of repeated administration of glyphosate (1/10 LD$_{50}$ = 275.49 mg kg^{-1} and 1/40 LD$_{50}$ = 68.86 mg kg^{-1}) on oxidative stress biomarkers and antioxidant enzymes in liver tissues

Periods	Groups	MDA (mg g^{-1} tissue)	GSH (mg g^{-1} tissue)	GST (µ mol/min/mg protein)	SOD (unit mg^{-1} protein)
14 days	Control	20.500±2.31	37.92±3.531	0.484±0.190	18.185±2.137
	1/10LD$_{50}$	51.152±1.28**	23.92±10.62**	0.137±0.029*	11.220±3.220**
	1/40LD$_{50}$	37.220±2.21**	29.77±6.960*	0.219±0.104	14.100±1.080*
28 days	Control	25.960±1.59	45.37±4.720	0.455±0.012	13.490±2.180
	1/10LD$_{50}$	61.300±2.43**	15.05±13.32**	0.103±0.013**	9.710±2.500**
	1/40LD$_{50}$	41.540±2.36**	23.71±6.600**	0.157±0.030**	10.250±4.510*

Data expressed as mean of 5 animals±SE; *Significance difference versus control at p<0.05; **Significance difference versus control at p<0.01

Meanwhile, activity of Superoxide Dismutase enzyme (SOD) recorded significant inhibition remarkable in all intoxicated group (Table 4).

Histopathological examination: The liver for control rats shows the normal histological structure of hepatic cords, hepatocyets, central veins and sinusoids (Table 1, Fig. 1a). Administration with 1/10 LD$_{50}$ of Baron (glyphosate-based) herbicide for 14 days showed congestion of blood vessels and hemorrhages as well as newly formed bile ductules and edema (Fig. 1b). The liver of rats treated with 1/10 LD$_{50}$ of Baron for 28 days showed edema as well as necrosis of hepatocytes (Fig. 1c). While the liver of rats administrated with 1/40 LD$_{50}$ of glyphosate for 14 days showed edema as well as necrosis of hepatocytes (Fig. 1d). Moreover, liver of rat treated with 1/40 LD$_{50}$ of glyphosate for 28 days showed thrombus formation of blood vessels and necrosis of some hepatocytes (Fig. 1e).

Genotoxicity studies: Depicted data in Table 5 and Fig. 2a-d revealed that intoxication with both doses of Baron (glyphosate-based) herbicide induced significant damage in DNA in liver cells. This expressed by increase in comet tail length and percentage of DNA damage studied by commet assay.

Glyphosate and its formulations have been extensively investigated for potential adverse effects in mammals (Williams *et al.*, 2000). The main objective of this study is to investigate cytotoxic and genotoxic effect of Baron commercial formulation of glyphosate herbicide. It has become common practice to use the medium Lethal Dose (LD$_{50}$) values to express and compare the toxicity of different pesticides. The calculated LD$_{50}$ of Baron commercial formulation of glyphosate was 2636.33 mg kg^{-1}. While LD$_{50}$ of glyphsate active ingredient was >5000 mg kg^{-1} (Bean, 2009). That is mean that baron commercial formulation is more toxic than glyphosate active ingredient these finding run with that previously recorded by Martinez *et al.* (2007) who reported that cytotoxicity of the commercial formulation round up to human peripheral mononuclear cells was 30 fold higher (LC$_{50}$ = 56 mg L^{-1}) than for the AI (LC$_{50}$ = 1640 mg L^{-1}). Remarkable increase in weight of liver, kidneys, lungs and testis recorded at the end of the experiment this finding was supported by Monsanto (1989) and Chan and Mahler (1992) who noticed a significant increase in liver, kidneys and testes weight after treatment with 20.000 ppm glyphosate for 24 months. Although, Baron glyphosate based herbicide is organophosphorous pesticides it has no effect on
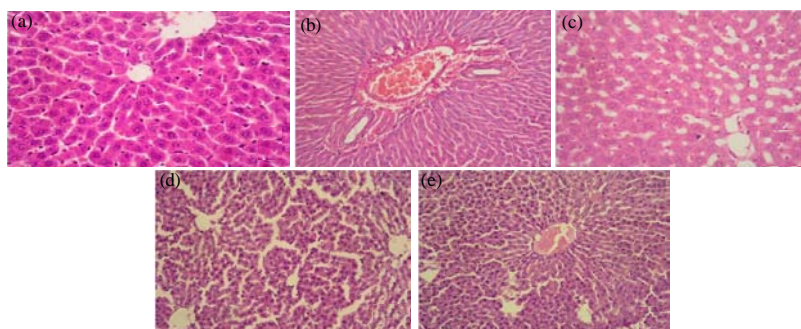
Fig. 1: a) Control rat showing normal structural of the liver; b) liver of glyphosate 1/10 LD_{50} at 14 days showing congestion of blood vessels as will as heamovages as well as neuly formed bile ductules and edema; c) liver of glyphosate 1/10 LD_{50} at 14 days showing congestion of blood vessels as will as heamovages as will as neuly formed bile ductules and edema; d) liver of glyphosate 1/40 LD_{50} at 14 days showing edema, necrosis of some hepatocytes; e) liver of glyphosate 1/40 LD_{50} at 28 days showing Thromlus of blood vessels formation, necrosis of some hepatocytes (H&E. X400)
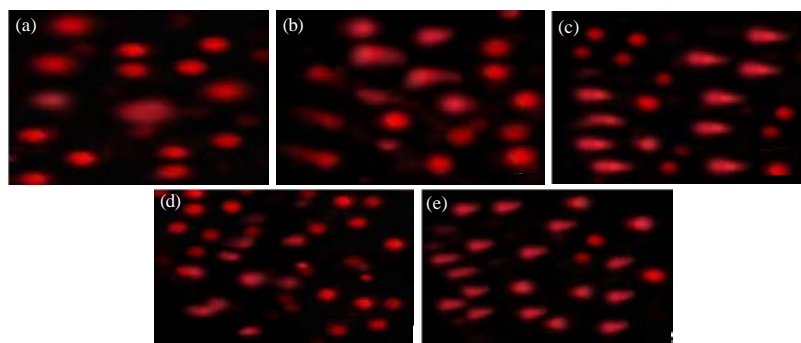

Fig. 2: a) Showing comets (from liver cells) of control rats have non-distructive DNA; band c) showing DNA damage in single cells (Comet assay) of liver of rats intoxicated with 1/10 LD_{50} (275.49 mg kg^{-1}) glyphosate at 14 and 28 days, stained with ethidium bromide; d and e) showing DNA damage in single cells (Comet assay) of liver of rats intoxicated with 1/40 LD_{50} (68.86 mg kg^{-1}) glyphosate at 14 and 28 days, stained with ethidium bromide and 2200x (40x obj)

serum Acetylcholinesterase (AChE) as shown in the present study. Meanwhile that recorded by Acquavella *et al.* (2004) glyphosate is assumed by regulators to have no neurological effects so that the US EPA did not require neurotoxicity studies to be carried out for the registration of round up. Otherwise, several studies have also shown that formulations containing glyphosate can inhibit AChE activity in fishes (Glusczak *et al.*, 2006, 2007; Modesto and Martinez, 2010). Hepatotoxicity was monitored by quantitative analysis of the serum ALT, AST activities as well as total protein and albumin levels which were used as the biochemical markers of liver damages. Significant reduction in the mentioned parameters was recorded in the present study. The results of the present study were similar to Benedetti *et al.* (2004) and Caglar and Kolankaya

(2008) who studied the hepatotoxic effects of the glyphosate-biocarb or round up other commercial forms of glyphosate in Wistar rats. In their studies they found that starting from the lowest dose of glyphosate induced the hepatic intracellular enzymes, ALT and AST suggesting irreversible damage in hepatocytes. Histopathological studies in liver tissues in the present study confirm the previous findings where tissue damage was recorded. There is severe injury in liver tissue. This may impair its regular function due to xenobiotic modification in detoxification processes and its formulation products may act synergically on the liver metabolism and/or injury and the commercial formulation of glyphosate possess potential risknfor human health (Williams *et al.*, 2000; Caglar and Kolankaya, 2008). Many xenobiotics such as pesticides can produce Reactive

Oxygen Species (ROS) via several mechanisms such as interference in electron transport in the mitochondrial membrane and subsequent accumulation of reactive intermediates, inactivation of antioxidant enzymes, depletion of non-enzymatic antioxidants and membrane lipid peroxidation (Winston and Di Giulio, 1991). ROS production associated with the presence of pollutants and the establishment of oxidative stress has been imputed as a possible mechanism of toxicity in aquatic organisms exposed to pesticides (Oropesa *et al.*, 2008). The equilibrium between antioxidant defenses and the generation of oxygen Reactive Species (ROS) is fundamental for the animal's homeostasis. When there is an imbalance between prooxidants and antioxidant defenses, the situation known as oxidative stress can be established in which the excessive generation of ROS may lead to irreversible impairment of DNA and other macromolecules and even to death (Ahmad *et al.*, 2000). Damage to among the non-enzymatic antioxidants is the tripeptide glutathione in its reduced form (GSH) which acts as the main antioxidant in the cell and is a cofactor for the action of GST and Gpx (Maran *et al.*, 2009). Glutathione S-Transferase (GST) is an enzyme that acts in the process of biotransformation, catalyzing the conjugation of a variety of metabolites including xenobiotic metabolites and lipoperoxidation products with GSH, transforming the toxic compound into more easily excretable one GST contribute to the detoxification of oxidative stress products (Maran *et al.*, 2009). Results run parallel with these demonstrated facts where intoxication with both doses of Baron elicit the induction of oxidative stress represented by elevation of Malondyalehyde (MDA), reduction of GSH level and inhibition in the activity antioxidant enzymes GST and SOD in liver tissues. These findings explain DNA damage investigated by increasing commet assay parameters, measure of commet tail and DNA%. Cavalcante *et al.* (2008) reported the increased DNA impairment of erythrocytes of *P. lineatus* exposed to 10 mg L^{-1} of RD for 96 h studied by comet assay.

CONCLUSION

Baron is one of the commercial formulations of glyphosate herbicide used in Egypt. It was classified according to the median Lethal Dose (LD50) as low toxic herbicide but repeated exposure of experimental animals to it inhibited serum liver biomarkers ALT, AST, TP and ALB. Also, it induced damage in liver cells, induction of oxidative stress and inhibition in detoxifying system that leads to DNA damage.

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