INTERNATIONAL JOURNAL OF APPLIED BIOLOGY AND PHARMACEUTICAL TECHNOLOGY

www.ijabpt.com ISSN : 0976-4550

Volume-8, Issue-2, April-June-2017 Coden IJABFP-CAS-USA **Copyrights@2017**

Received: 24th Feb 2017 ***Revised: 27th Mar 2017*** ***Accepted: 28th Mar 2017***

DOI: 10.21276/Ijabpt, http://dx.doi.org/10.21276/ijabpt ***Research article***

A 90 DAY ORAL TOXICITY OF GLUFOSINATE AMMONIUM IN WISTAR RATS: BIOCHEMICAL AND HISTOPATHOLOGICAL INVESTIGATION OF LIVER

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ABSTRACT: Glufosinate Ammonium is a broad spectrum herbicide with systemic action for control of annual and perennial weeds and grasses. In the present study we investigated the potential repeated dose toxicity, providing useful information for assessing the toxicological relevance of herbicide in Wistar rats. Three groups of 20 rats each (10 male and 10 female) were administered with Glufosinate Ammonium orally at the dose level of 50 mg/kg b.wt (low dose), 250 mg/kg b.wt (intermediate dose) and 500 mg/kg b.wt (high dose) respectively for seven days a week for 90 days. General observation, mortality, feed intake, body weight were monitored throughout the experimental period. Biochemical parameters and histopathological changes were evaluated. The analysis showed increase in biochemical parameters i.e. ALT, AST, ALP and total bilirubin at terminal evaluation in high dose (500 mg/kg b.wt) and intermediate dose group(250 mg/kg b.wt) animals. Microscopical evaluation of all tissues of the treated and control group rats were carried out. However various microscopical changes were noticed in liver of intermediate dose (250 mg/kg b.wt) and high dose (500 mg/kg b.wt). It may be concluded that for 90 days repeated oral toxicity study, the "No observed Adverse Effect Level (NOAEL) for Glufosinate-ammonium came out as 50 mg/kg b.wt. It may therefore be inferred that day to day exposure of pesticides may exert hazardous effects on the liver.

Key words: Glufosinate-ammonium (GA), No observed Adverse Effect Level (NOAEL), Wistar rats, Liver toxicity

Abbreviations: Aspartate aminotransferase (AST), Alanine aminotransferase (ALT), Alkaline Phosphatase (ALP)

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INTRODUCTION

Fruits and vegetables are an essential part of a nutritious and healthy diet; however, the health benefits are compromised by consistent contamination with pesticide residues (Tahir et.al., 2009). With increasing need for food production from agriculture, pesticide use has been increasing. However, this has been occurring irregularly, without adequate control by government bodies (Koirala et.al., 2007).

One such organophosphate which has spurred interest is Glufosinate ammonium.Glufosinate is a broad-spectrum, contact herbicide (EPA, 2013), its short name for the ammonium salt, GA, which is derived from phosphinothricin, a natural microbial toxin isolated from two species of Streptomyces fungi.Glufosinate is a phosphorus-containing amino acid as it inhibits the activity of an enzyme, glutaminesynthetase, which is necessary for the production of the amino acid glutamine and for ammoniadetoxification (He Y et. al., 2007).

Glufosinate ammonium structurally resembles glutamate, a typical excitatory amino acid in thecentral nervous system. It is recognised that excess release of glutamate results in the death ofnerve cells in the brain (Jewell and Buffin, 2001). Ingestion of glufosinate affects the nervous system and evidence ofneurotoxicity has been found in most species of laboratory animals exposed to glufosinate (Peltzer PM et.al., 2013)

Glufosinate has been detected in soils, groundwater, surface water and sediments (Calas AG et. al., 2008).

Studies have reported that glufosinate is toxic to mouse embryos in vitro and causes growth retardation and neuroepithelial cell death. Paternal exposure to glufosinate in humans has been found to correlate with a possible risk in congenital malformations (Calas AG et. al., 2008).

Liver is a vital organ of metabolism and excretes virtually every drug and toxin introduced in the body. The liver being the primary site for biotransformation of foreign compounds is particularly vulnerable to chemical assaults (Nunez M et. al., 2006). Various pesticides cause pathological and biochemical alterations in the liver. Determination of the activity of the hepatic enzymes released into the blood by the damaged liver has become one of the most useful tools inthe study of hepatotoxicity (Papay JI et. al., 2009). Glufosinate can cause marked inhibition of the enzyme, glutamine synthetase in liver which interferes with ammonia detoxification (EFSA, 2005).

In the era of pesticides, Very little is known about the long-term effects of exposure to pesticides, and the toxicological classification basically reflects the acute toxicity and does not indicate the risks of diseases with prolonged evolution, such as neoplastic diseases and chronic hepatic diseases(Olson H et. al.,2000). The effects of chronic intoxication have not been adequately characterized, since they may only become apparent after many years of exposure (Dambach DM et. al., 2005).

Therefore the present study aimed to elucidate the chronic toxic effects of Glufosinate ammonium on liver, by evaluating biochemical and histopathological examinations in Wistar rats.

MATERIALS AND METHODS

Animals and Experimental design

Healthy Wistar rats used for the study were bred at the animal house facility of Shriram Institute for Industrial Research, Delhi. For the study, 5 to 8 weeks old Wistar rats weighing between 100 to 140 grams were used. Prior to starting the experiment, necessary approvals were taken from IAEC (Institutional animal ethics committee) for conducting the study (CPCSEA). The animals were housed (3 rats each cage) in an air conditioned room (12-15 air changes per hour) at the temperature 22 ± 3 ° C and 50-60 % relative humidity with a 12 hour light/ dark cycle. They were provided with standard laboratory animal diet (Amrut feed Ltd, Pune) and filtered water *ad-libitum*. The animals were acclimatized for five days prior to the initiation of experiment (OECD 408, 1998).

Grouping of Animals and Dose Selection

Prior to commencing the main study a dose range finding study was done to select the doses for the main study. Based on dose range findings and literature on Glufosinate, three dose levels of 50, 250 and 500 mg/kg b. wt. were selected. After 5 days of acclimatization, the animals were randomly assigned into six groups of ten animals each sex.

Group I: Control; administered only vehicle (distilled water)

Group II: Low dose; administered Glufosinate-ammonium orally, dissolved in water (w/v) (50 mg Glufosinate-ammonium /kg body weight/day)

Group III: Intermediate dose; administered Glufosinate-ammonium orally, dissolved in water (w/v) (250 mg Glufosinate-ammonium /kg body weight/day)

Group IV: High dose; administered Glufosinate-ammonium orally, dissolved in water (w/v) (500 mg Glufosinate-ammonium /kg body weight/day)

Group V: Satellite control; administered only vehicle (distilled water)

Group VI: Satellite High dose; administered, Glufosinate-ammonium orally, dissolved in water (w/v) (500 mg Glufosinate-ammonium /kg body weight/day)

Biochemical analysis

At the end of the treatment period, the rats were fasted before sacrifice; blood samples were drawn from orbital sinus by capillary tube under CO_2 anesthesia without addition of anticoagulant in clean dry test tubes and separated serum samples were kept in the vial under refrigeration at 20^oC till analysis for various biochemical parameters. Clinical chemistry parameters were determined by using Beckman Coulter haematology analyzer system.

ALT
Principle:

The principle employed was as described by (Bergmeyer et. al., 1986b).

L-alanine + α-ketoglutarate ⟶ Pyruvate + L-glutamate

Pyruvate + NADH + H^+ —LDH→ L- Lactate + NAD^+ + H2O

AST

Principle:
The principle employed was as described by (Bergmeyer et. al., 1986a).
AST
L-aspartate + α-ketoglutarate Oxaloacetate + L-glutamate
MDH
Oxaloacetate + NADH + H^+ L- malate + NAD^+ + H2O

Alkaline Phosphatase (ALP)
Principle:
The principle described by(Tietz et. al.,1983) as outlined below was followed:
ALP
P-nitrobenzol phosphate + H_2O ⟶ Phosphate + p-nitrophenol

Bilirubin ⟶
Principle:

The principle employed is as described by (Doumas et. al., 1973).

Sulfanilic acid + HCL + $NaNO_2$ ⟶ Diazotized sulfanilic acid + NaCL

Total bilirubin + Diazotized Sulfanilic Acid ⟶ Azobilirubin (magenta)

Method: DPD (2, 5-dichlorophenyl diazoniumtetrafluoroborate)

Histopathology
At the end of the experiment (90 day), after collecting blood samplesfrom eyes, the animals were sacrificed by light CO_2 anesthesia and detailed postmortem examinations was carried out as per standard procedures. Livers were collected and fixed in 10% neutral buffered formalin and preserved for histopathological examination. Tissues collected for histopathology were processed and then embedded in paraffin wax and sectioned at 3-5 microns and stained with haematoxylin and eosin (Luna, 1968).

Statistical Analysis
Study parameters like Body weight, Feed consumption, and blood biochemistry data were evaluated using Statistical analysis. Study groups was analysed for level of significance with Parametric and non- parametric statistical method. All these Statistical analysis performed with statistical software IBM-SPSS 22.
The criteria for significance at 95% confidence are dependent on the p value.
If p value <0.05 = Significant
If p value >0.05 = Non significant

RESULTS

Clinical signs:

No mortality was observed in any of the dose groups. However the treatment related toxic signs i.e. Piloerection and soft faeces were noticed in group III from 67th day onwards till the end of dosing. Piloerection, aggressiveness, fighting, scratching, soft faeces, hyperasthesia were noticed from the 45thday ingroup IV and VI.

No toxic sign and symptoms were observed in the animals ofgroup II when compared with their control counterparts.

Feed Consumption

The feed consumed by the animals of low dose group (50 mg/kg B.wt.), intermediate dose group (250 mg/kg B.wt.) was similar to the control group of animals. A significant decline in the feed consumption from 9th week onwards was observed only in the high dose group animals (IV and VI) when compared to their control counterparts. (Table: 1)

Mean Body Weights

The mean and percentile body weights of the animals of II group and intermediate dose group were comparable to that of their control counterparts.

However, decreased body weights gain pattern were observed in the high dose (500 mg/kg b.wt.) groups, when compared to control group of animals, which was statistically significant from 9th week onwards in males and 7th week onwards in females.(Table:2).

Biochemical Evaluations

The activities of aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase and total bilirubin in serum samples were studied as biomarkers of liver function. In this study, the effects of Glufiosinate resulted in liver toxicity in rats as evidenced by a marked significant increase in serum AST, ALT, ALP and T.Bil, in group III, IV and VI when compared to control group. Changes in the serum liver function markers suggest increased incidence of lesions to the hepatocytes. (Table: 3)

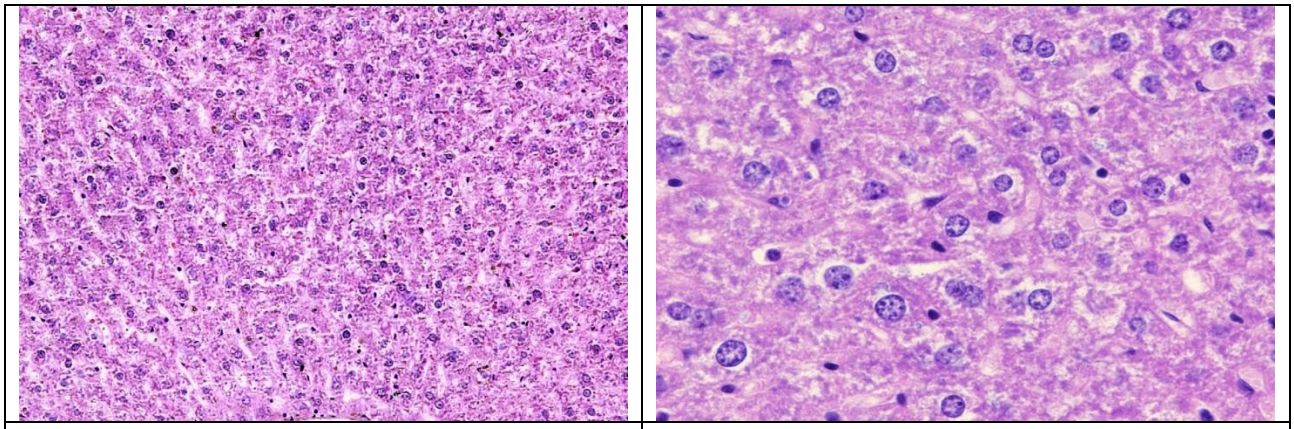
Histopathological Findings

Microscopical evaluation of tissues of all treated and control group rats were carried out. Various microscopical changes were noticed in liver of Intermediate and high dose group animals. Liver showed markedly enlarged sinusoidal space, mild fatty degeneration of hepatocytes, hemorrhage, and degeneration of hepatocytes.

All these microscopic findings were noticed in the intermediate and high dose group animals.

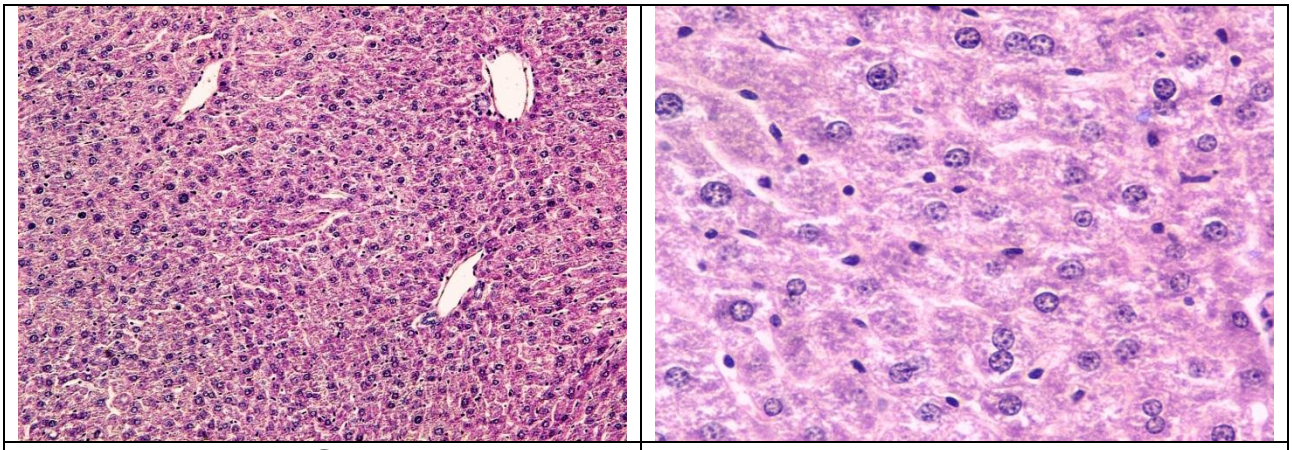
There were no histopathological changes in the animals of low dose group (50mg/kg B.wt.)

The histopathological alterations in the present study could be summarized as follows:



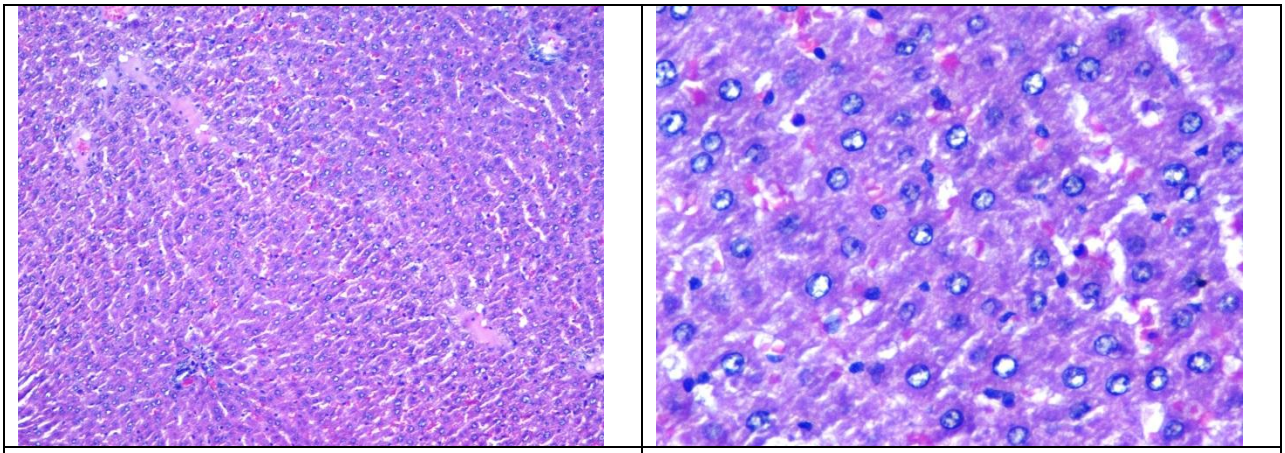
A B

Figure-1: Liver section of Group I rats showing normal histological appearance of the liver. (A). Low power photomicrograph of liver of control group showing normal parenchyma. (H & E, 100X). (B) High power photomicrograph of liver of control group showing normal hepatocytes. (H & E, 400X)



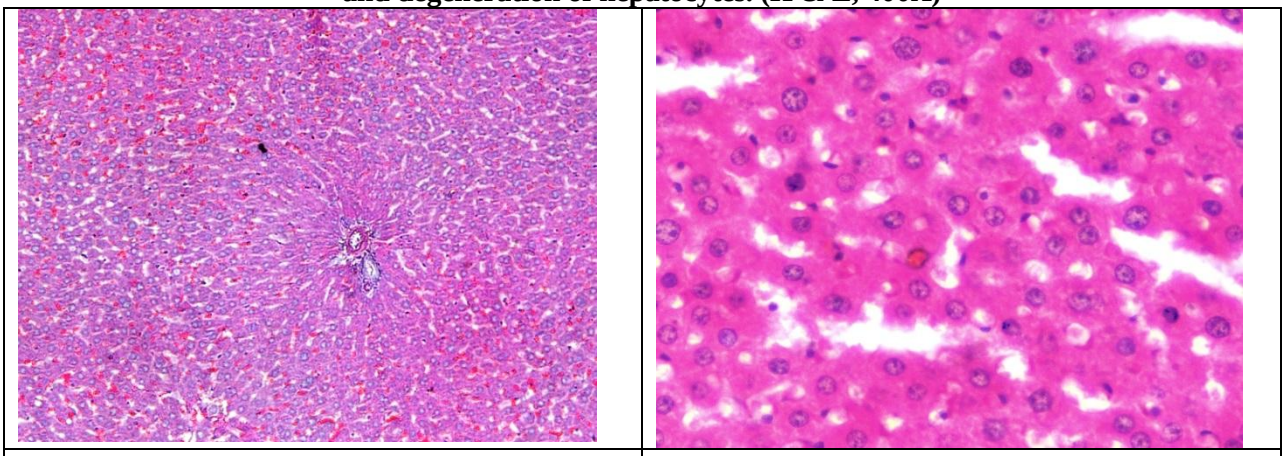
C D

Figure-2: Liver section of Group II rats showing normal histological appearance of the liver. (C). Low power photomicrograph of liver of Low Dose group showing normal parenchyma. (H& E, 100X) (D) High power photomicrograph of liver of Low Dose group showing normal hepatocytes. (H& E, 400X)



E F

Figure-3: Liver section of Group III rats showing histological appearance of the liver. (E).Low power photomicrograph of liver of Intermediate group showing mild haemorrhage and degeneration of hepatocytes. (H & E, 100X) (F) High power photomicrograph of liver of Intermediate group showing mild haemorrhage and degeneration of hepatocytes. (H & E, 400X)



G H

Figure-4: Liver section of Group IV rats showing histological appearance of the liver. (G). Low power photomicrograph of liver of High dose group showing mild enlargement of sinusoidal space and haemorrhage. (H & E, 100X) (H). High power photomicrograph of liver of High dose group showing slightly enlarged sinusoidal space and mild fatty degeneration of hepatocytes. (H & E, 400X)

Table-1: Effect of different dose levels of Glufosinate-ammonium on feed consumption in wistar rats

Groups	Male	Female
	Average Feed Consumption (g)	Average Feed Consumption (g)
Control (0 mg/kg b.wt.)	189.83 ±1.33	185.30±1.35
Low dose (50 mg/kg b.wt.)	189.67±0.82	187.23±3.36
Intermediate Dose (250 mg/kg b.wt.)	188.83±1.47	186.36±1.30
High dose (500 mg/kg b.wt.)	175.17*±2.56	173.26*±3.50
Satellite Control (0 mg/kg b.wt)	189.50±1.05	188.30±2.50
Satellite High dose (500 mg/kg b.wt)	174.23*±3.30	176.30*±2.60

Statistical analysis: T-test (p value > 0.05)

*: significant, If p value <0.05 = Significant, If p value >0.05 = Non significant

Table-2: Mean Weekly Body Weight Data of Male and Female wistar rats

Groups	Male		Female	
	Day 1	Terminal sacrifice	Day 1	Terminal sacrifice
Control (0 mg/kg b.wt.)	116.00 ± 1.76	278.50 ± 3.14	118.80 ± 0.79	260.70 ± 0.67
Low dose (50 mg/kg b.wt.)	114.20 ± 2.39	269.90 ± 0.88	113.40 ± 1.35	256.60 ± 0.97
Intermediate Dose (250 mg/kg b.wt.)	114.10 ± 0.88	269.70 ± 1.49	114.00 ± 0.82	257.60 ± 0.84
High dose (500 mg/kg b.wt.)	115.00 ± 3.20	243.00* ± 3.30	113.00 ± 1.83	240.10* ± 0.99
Satellite Control (0 mg/kg b.wt)	114.9 ± 1.73	271.1 ± 1.85	110.9 ± 2.18	259.1 ± 0.99
Satellite High dose (500 mg/kg b.wt)	115.10 ± 0.88	250.10* ± 0.88	114.90 ± 0.88	239.60* ± 0.97

Statistical analysis: T-test (p value > 0.05)

*: significant, If p value <0.05 = Significant, If p value >0.05 = Non significant

Table-3: Effect of Glufosinate ammonium on ALT,AST,ALP and Bilirubin

Groups	Dose level (mg/kg B.wt.per day)	SGOT (U/I) (Mean±S.D)	SGPT (U/I) (Mean±S.D)	ALP (U/I) (Mean±S.D)	T-BIL (mg/dl) (Mean±S.D)
I	0	93.24±1.90	57.56±3.21	86.20±1.10	0.27±0.04
II	50	96.10±2.25	50.10±2.23	85.30±2.21	0.30±0.03
III	250	200.25±3.56	93.20± 1.90	165.30±3.30	1.06±0.04
IV	500	225.20±4.25	115.3±3.36	195.20±2.20	1.50±0.09
V	0	95.53±2.25	55.60±2.31	90.20±3.31	0.25±0.10
VI	500	250.10± 3.31	125.30±3.30	190.50±3.30	1.45± 0.1

The criteria for significance at 95% confidence are dependent on the p value.

If p value <0.05 = Significant

If p value >0.05 = Non significant

DISCUSSION

Pesticides have been one of the most effective weapons discovered by man to protect agricultural products from the attack of Pests (Guengerich et.al., 2008). Among these glufosinate is an extensively used organophosphate pesticide. Due to its wide-spread use it poses potential harm to nontarget organisms including humans (Hoerlein Get.al., 1994).

In this study, we investigated the toxicological effects of the agrochemical i.e. Glufosinate ammonium using biochemical evaluation histopathological changes in the liver of rats. In toxicological studies, body weight, feed intake, clinical sign are important criteria for evaluation of pesticide toxicity. In the current study, we observed a reduction of body weight in treated rats and is possibly attributed to lower feed intake from toxic effects of Glufosinate ammonium.

Among the most sensitive and widely used liver enzymes are the aminotransferases. They include aspartate aminotransferase (AST) and alanine aminotransferase (ALT).The aminotransferases catalyze chemical reactions in which an amino group is transferred from a donor molecule to a recipient molecule. AST (SGOT) is normally found in many tissues including liver, heart, muscle, kidney, and brain. It is released into serum when any one of these tissues is damaged. ALT (SGPT) is, by contrast, normally found largely in the liver. This is not to say that it is exclusively located in liver, but that is where it is most concentrated. It is released into the blood stream as a result of liver injury. It therefore serves as a fairly specific indicator of liver status. Increased activity of ALT and AST, were recorded. The enzyme (GOT) and (GPT) gain entrance into the serum and consequently their concentration rises markedly in hepatocellular damage (Momoh and Damazio, 2014).The result from this study showed significant increase ($P<0.05$) in the serum levels of AST and ALT (Table: 2) in intermediate and high dose group animals.

Alkaline Phosphatase (ALP) is an important enzyme which helps in body metabolismin various ways. Though it is present in all tissues, maximum amount of alkaline phosphatase is found in liver and in bones.

The activities of ALT, AST and ALP enzyme are the most sensitive biomarkers directly implicated in the extent of hepatic damage and toxicity (Patrick-Iwuanyanwu.et.al., 2007). In our findings we examined that Glufosinate administered to rats observed a marked elevation in serum AST, ALT and ALP parameters which indicate hepatocellular damage .This elevation could potentially be attributed to the release of these enzymes from the cytoplasm into blood circulation, indicating necrosis and inflammatory reactions.

Bilirubin is the major breakdown product that results from the destruction of old red blood cells. It is removed from the blood by the liver; hence it is a good indicator of the function of the liver (Sunmonuand Oloyede, 2007). The total bilirubin levels increased significantly ($P<0.05$) inintermediate and high dose group animals.Increased bilirubin level in the Glufosinate treated Wistar rats indicates hepatic injury.

The histopathological findings, evident in the photomicrographs, elucidated the deleterious effect of Glufosinate ammonium on the liver of animals in group III and group IV (Figure: 3 &4). In the control group animals liver sections showed normal hepatic cells with well preserved cytoplasm, prominentnucleus, nucleolus and central vein (Figure :1).The administration of Glufosinate ammonium at the dose level of 250 and 500 mg/kg b.wt induces severe histopathological damages in the liver with significant degeneration of cells, fatty changes in rat hepatocytes signifying the existence of hepatosteatosis,enlarged sinusoidal space and liver lesions were observed in intermediate and high dose group animals.

The biochemical alterations accompanied by histopathological changes resulted after Glufosinate administration attenuates hepatotoxicity.

CONCLUSION

Since all biochemical examinations and histopathological evaluations play an important physiological role by coordinating with oneanother. In the present work all the values in high dose group significantly increased as compared to control group animalswhich can cause adverse effects on hepatic system. Hence it is concluded from the present findings thatuse of Glufosinate ammonium as an herbicide is hazardous to non-target species as well. Further research is required to examine the treatment or reversal of hepatotoxicity with naturally occurring herbal products.

ACKNOWLEDGMENTS

The authors are thankful to the Shriram Institute for Industrial Research, Delhi for providing the facilities for this research work.

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ISSN : 0976-4550

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