IOSR Journal of Pharmacy and Biological Sciences (IOSR-JPBS)
e-ISSN:2278-3008, p-ISSN:2319-7676. Volume 13, Issue 3 Ver. V (May. – June. 2018), PP 12-19
www.iosrjournals.org

Hepatotoxicity in Rabbits resulting from sub-chronic Exposure to Bioaccumulated Herbicide, Butachlor® in Beans (*phaseolus vulgaris*) Leaf forages

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Abstract: *Bioaccumulation of herbicides is a potential hazard for man and animals. In a previous study conducted by our team of researchers, leaves from experimental beans that were treated with butachlor® at pre-emergent were found to have bioaccumulated residues of the herbicide at 6 weeks. The control beans without exposure to herbicide had no trace of butachlor® detected in the leaves but the test beans which were treated with 260mg/m^2 (recommended concentration) had 0.10 ppm while 290 and 320 mg/m^2 induced a correspondingly increased bioaccumulation value of 0.13 and 0.20ppm. Sub-chronic (28-day) exposure through feeding of experimental rabbits with bean forages containing the different concentrations of the bioaccumulatedbutachlor® was then conducted. Thereafter, assay of rabbit serum biomarkers of hepatotoxicity [alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP)] was carried out. Histopathological studies of liver samples from the separate test animal groups were also performed. There was a highly significant ($p<0.01$) concentration and time dependent increases in serum liver enzymes particularly AST and ALT of rabbits exposed to bioaccumulatedbutachlor® in bean leaf forages relative to the control. Evidence from histopathological studies on day 28 also corroborated with the results of the rabbit liver enzyme assays. Lesions of hepatocellular damage degenerated from cellular changes to necrosis of hepatocytes and initiation of fibrosis with consumption of 0.10, 0.13 and 0.20 ppm amounts of the herbicide residue in bean leaf forages respectively. Herbicide, butachlor® could bioaccumulate in beans leaves with a potential to cause hepatotoxicity in forage eating rabbits and probably other animals.*

Key words: *Herbicide, Bioaccumulation, Butachlor®, Hepatotoxicity, Lesions*

Date of Submission: 16-06-2018 Date of acceptance: 02-07-2018

I. Introduction

Globally, the wide use of herbicides to control weeds has supported large scale crop production and bumper harvests. In Nigeria however, the annual herbicide application rate is not closely monitored but there is significant increase within the last two decades in the use of herbicides even among rural communities.The ever-growing involvement of rural farmers with inadequate expertise encourages incessant and repeated application of herbicides thereby enhancing bioaccumulation.Herbicides do not only contaminate water, soil and air but also accumulate in crops to facilitate indirect human exposure through food chain [1].Bioaccumulation of herbicides and pesticides in our environment may presentdiverse health hazards to human and animal population.Over 350 different pesticides were detected in food and drinks within Europe in 2008 [2]. Active compounds from herbicides and pesticides may bioaccumulate in plants above the maximum residue limit and become toxic in food chains to organisms of higher trophic levels [1].

Butachlor® containing N-butoxymethyl-2-chloro-2,6-diethylacetamide was developed by the Monsanto Company, USA as a pre-emergent herbicide for the control of broadleaf weeds and annual grasses. It is in high demand in Asia, Africa, China and Japan. In Nigeria specifically, it is distributed in different domestic markets as anemulsifiable concentrate undervarious trade names (Machete®, USA; Butaforce/Butastar®, Nigeria). Butachlor is believed to inhibit the synthesis of lipids, alcohols, fatty acids, proteins, isoprenoidsand flavonoids in target plants[3, 4].

The present study evaluated potential hepatotoxicity in rabbits that consumed bean leaf forages containing different concentrations of bioaccumulated herbicide, butachlor® at sub-chronic level.

II. Materials and Methods

2.1. Reagents

Herbicide butachlor (98.4%purity) purchased from Monsanto, USA, serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) kits from QuimicaClinicaApplicada S.A. Spain, 10% formal saline, and alkaline phosphate (ALP) kit obtained from Randox Laboratory Ltd., UK were employed in the experiments.

2.2. Animals

Forty-eight male rabbits (1.0-1.4 kg) purchased from the Laboratory Animal Unit, Department of Biological Sciences, University of Nigeria, Nsukka (UNN) were used in the investigation. Test rabbits were housed in metabolic cages, maintained on growers mash diet and water *ad libitum*. Animals were allowed two weeks to acclimatize prior to commencement of experimentation. Ethical rules guiding the use of animals for experimentation were strictly adhered to; laboratory animals were used in accordance with laboratory practice regulation and the principle of humane care as documented by [5].

2.3. Plant Material

Bean seeds (*Phaseolus vulgaris)* were sourcedlocally within Nsukka municipality, Enugu State, Nigeria.

2.4. Hepatotoxicity study with sub-chronic (28-day) exposure of rabbits to bioaccumulated herbicide, butachlor in bean leaves

Sixteen (16) matured male rabbits 1200-1500 g), weighed individually and marked with 10% picric acid were randomly allocated to 4 groups (I-IV). Group I served as the control and was placed on a forage diet of bean leaves from Plot T_1 with zero (0) value of bioaccumulated herbicide. Group II rabbits fed on forage of bean leaves from Plot T_2 with 0.10 ppm herbicide; Group III was given forage bean leaves from Plot T_3 with 0.13 ppm herbicide while Group IV received bean leaves from Plot T_4 with 0.20 ppm herbicidein tissues. All animal groups were fed with equal quantities of forage at 200 g/kg body weight daily and according to the weekly changes in body weight while good drinking water provided *ad libitum* within the 28 days of the study.

2.4.1. Assay of serumbiomarkers of hepatotoxicity in rabbits following sub-chronic exposure to bioaccumulated herbicide,butachlor® in bean leaf forages

Blood samples were collected under light ether anaesthesia from the jugular vein of the rabbits on days 7, 14 and 28. The blood samples were allowed to coagulate and were centrifuged at 3000 rpm for 5 min. Serum samples were separated and stored at 4^oC in a refrigerator. Rabbit liver enzyme analyses were carried out for the measurement of serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) in each of the experimental animals. The mean change in the various rabbit serum liver enzyme for each group was determined relative to the control.

2.4.1. Histopathological studies of the rabbit liver following sub-chronic intoxication with bioaccumulated herbicide, butachlor®

Tissues samples from liver sections of rabbits in each group (I-IV) of the experiment were fixed in 10% formal saline for a minimum of 24 h and then dehydrated by washing in ascending grades of ethanol before clearing with xylene and embedding in paraffin wax. The samples were sectioned with a microtome, stained with haematoxyline and eosin (H and E) and mounted on Canada balsam. All sections were examined under light microscope (x10, x20 and x40) magnification. Photographs of the lesions would be taken with an Olympus photo microscope for observation and documentation of histopathologic lesions.

2.4. Statistical Analysis

Data obtained were subjected to One-way analysis of variance (ANOVA) andDuncan new multiple range post hoc test. Differences at $p < 0.05$ were considered significant; values were expressed as mean ± SE.

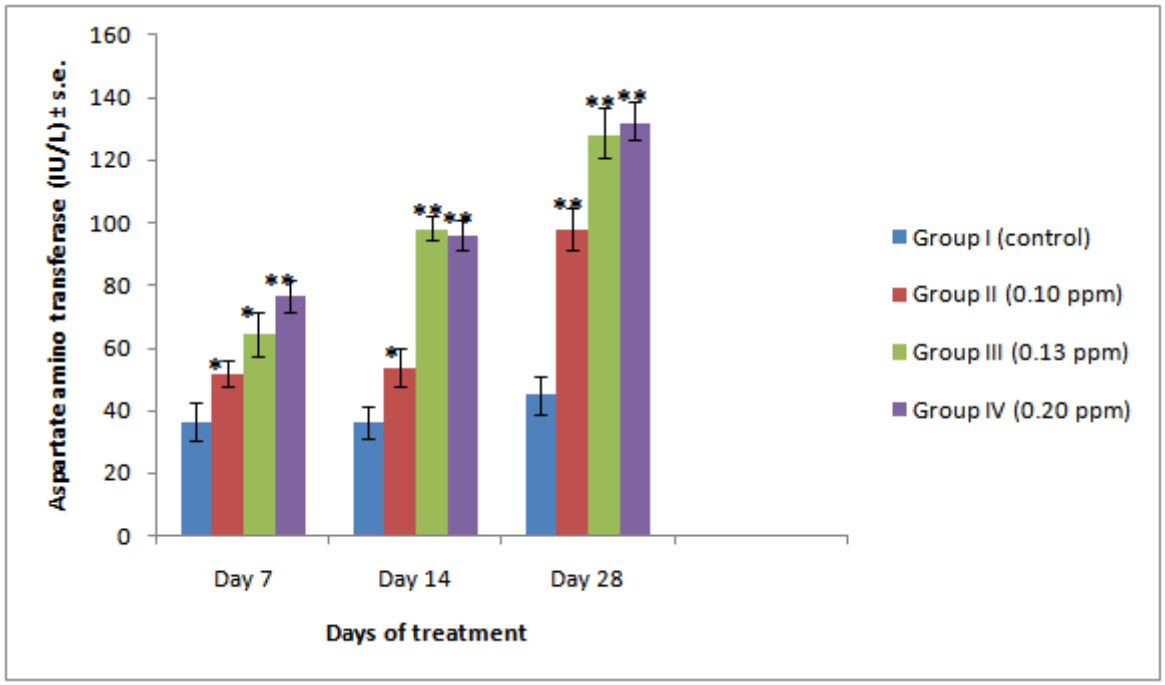
III. Results

3.1. A**ssay of rabbit serumbiomarkers of hepatotoxicity**

3.1.1.Aspartate aminotransferase (AST)

On day 7, Groups I and II rabbits which were fed bean forages with bioaccumulated herbicide butachlor values of 010 and 0.13 ppm had significant ($p<0.05$) increases in the serum concentration of 51.8 ± 4 and 64.3 ± 7 IU relative to control group with 36.3 ± 6 IU that received no exposure to the herbicide. In Group IV, forage with the highest bioaccumulated test value of 0.20 ppm induced a highly significant ($p<0.01$) comparative enzyme elevation of 76.5 ± 5.0 IU. Also on day 14 of the study, the rabbit AST level was

significantly ($p<0.05$) increased from 36 ± 5 to 53.8 ± 6 IU with intake of 0.10 ppm; the enzyme elevation became highly significant ($p<0.01$) with 0.13 and 0.20 ppm of the herbicide in the bean leaves (Groups III and IV) with values of 98.3 ± 7 and 96 ± 5 IU respectively. Similarly, on day 28 of the study, AST values of all the test rabbits (Groups II-IV) that fed on bean forages containing various concentrations (0.10, 0.13 and 0.20 ppm) of bioaccumulatedherbicide, butachlor® became highly increased significantly ($p<0.01$) with corresponding values of 98.0 ± 7., 128.5 ± 8 and 132.5 ± 6 IU compared to the control value of 45.0 ± 6 IU (Fig. 1).

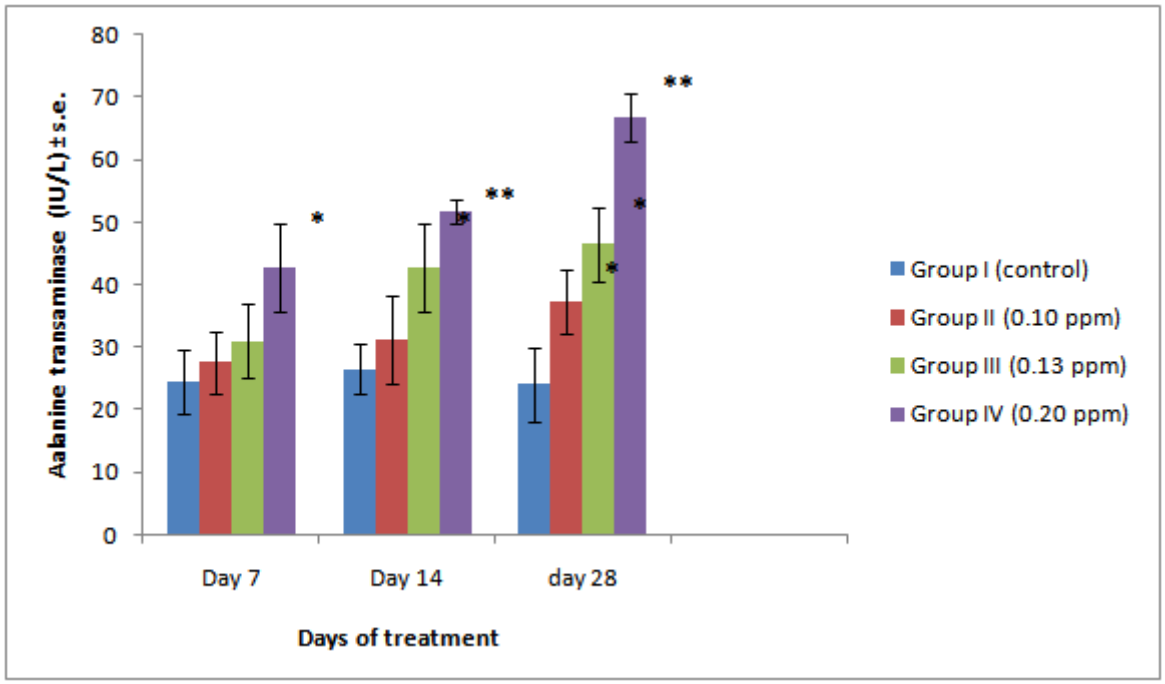


***, ****indicate significant differences at $p<0.05$ and $p<0.01$respectively compared to control.

Figure1: Serun aspartate amino transferaseofrabbits that fed on beans forages with varying concentrations of bioaccumulated herbicide,butachlor®.

3.1.2. Serum alanine transaminase (ALT)

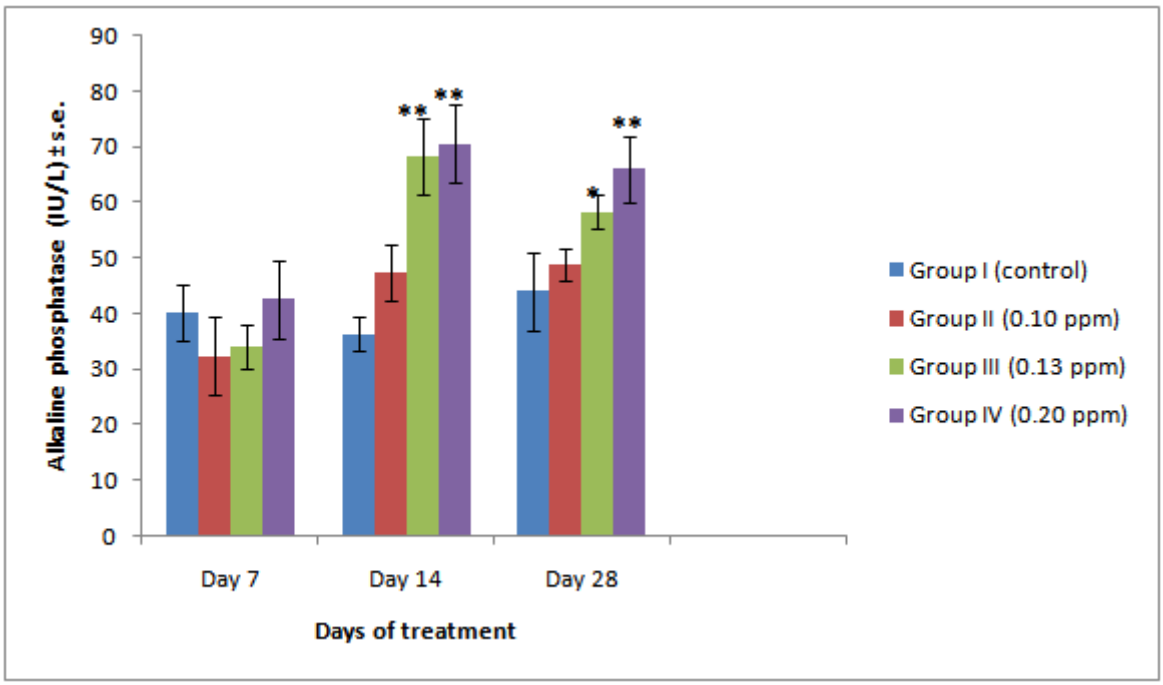
On day 7 of the investigation, intake of bioaccumulated herbicide butachlor at 0.10 and 0.13 ppm in bean forages (Groups II and III) did not cause significant ($p>0.05$) alteration in the rabbit ALT values different from the control; only 0.20 ppm induced a significant ($p<0.05$) increase in the enzyme level above that of the control.On day 14 however,two concentrations(0.13 and 0.20 ppm)of the herbicide residues in beans forage significantly ($p<0.05$) enhanced the rabbit enzyme production with 42.8 ± 7 and 51.8 ± 2 IU respectively above the control value (26.5 ± 4 IU).On day 28,all the three bioaccumulated herbicide concentrations (0.10, 0.13 and 0.20 ppm potentiated the rabbit enzyme production above the control but it was 0.20 ppm that exerted a highly significant ($p<0.01$)effect(Fig. 2).



*, **indicate significant differences at p<0.05 and p<0.01respectively compared to control.
Figure2. Serumalanine transaminaseofrabbits that fed on beans leaf forage with increased concentrations of bioaccumulated herbicide,butachlor®.

3.1.3. Alkaline phosphatase (ALP)

The intake of 0.10 ppm bioaccumulated herbicide butachlor in beans forage diet did not cause (p>0.05) changes in the serum ALP of experimental rabbits relative to the control within the 28-day duration of the study. The other test concentrations (0.13 and 0.20 ppm) of the herbicide ingested in forage induced significant (p<0.05) increases above the control enzyme level. ALP values of 68.3 ± 7 and 58.3 ± 3 IU were elaborated with 0.13 ppm herbicide on days 14 and 28 while the enzyme concentrations of 70.5 ± 7 and 66 ± 6 IU became induced in rabbits with intake of 0.20 ppm bioaccumulated herbicide, respectively (Fig. 3).



*, **indicate significant differences at p<0.05 and p<0.01respectively compared to control.
Figure3. Serumalkaline phosphataseofrabbits that fed on beans forage with varying concentrations of bioaccumulated herbicide,butachlor®

3.1.4. Histopathological studies onliversof rabbits following sub-chronic (28 days) exposure to bioaccumulated herbicide,butachlor®in beans leaf forages

Group I (control): Hepatocytes were seen as numerous, large and polyhedral cells forming the parenchymal cells of the liver. Lobules consisted of hexagonal plates of hepatocytes stacked on each other. A lobule was roughly hexagonal in shape with portal triads at the vertices and a central vein in the middle. Hepatocytes contained between two to four nuclei and each nucleus had at least two nucleoli. Within each plate forming a lobule, hepatocytes were found to radiate outwards from a central vein. They became arranged into strips while extending towards the periphery (Plate 1).

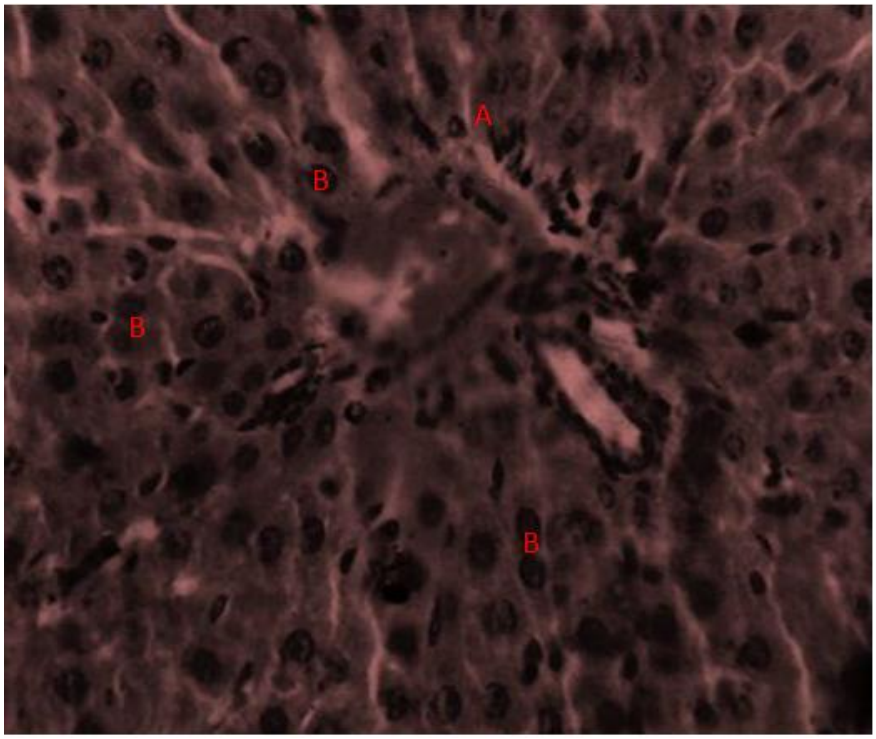


Plate1: Liver section of control rabbits that had no exposure to bean forages with bioaccumulated herbicide butachlor in on day 28 (H and E x400). **A**=Portal area; B = Plates of hepatocytes.

Group II (Intoxicated with 0.10 ppm bioaccumulated herbicide,butachlor®): On day 28, Degenerative changes resulting in necrosis and fewer numbers of hepatocytes compared to the control were seen in the liver of rabbits that fed on legume forage containing 0.10 ppm of the bioaccumulated herbicide (Plate 2).

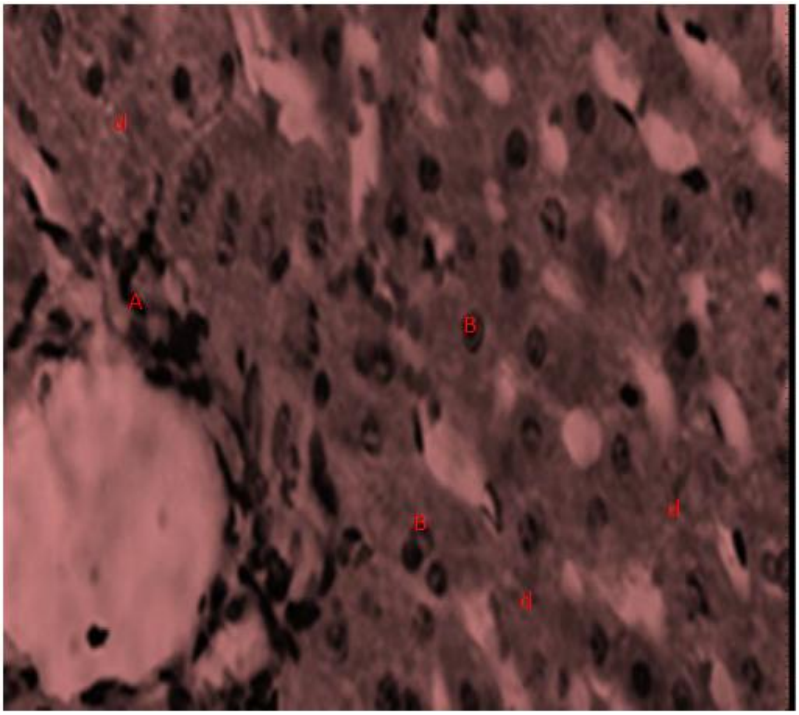


Plate 2: Liver section of rabbits exposed to 0.10 ppm bioaccumulated herbicide butachlor on day 28 showing degenerative changes in the liver parenchymal tissue resulting in fewer hepatocytes relative to the control (H and E x400). **A**=Portal area; B = Plates of hepatocytes; **d**= areas of degenerative changes without hepatocytes.

Group III (Intake of 0.13 ppm bioaccumulated herbicide,butachlor®): The hepatocellular architecture oftest rabbits placed on bean forages containing 0.13 ppm bioaccumulated herbicide butachlor on 28 day revealed a further sparse distribution of hepatocytes. The reduced number of hepatocytes may be due to the necrotic effect of the herbicide (Plate 3).

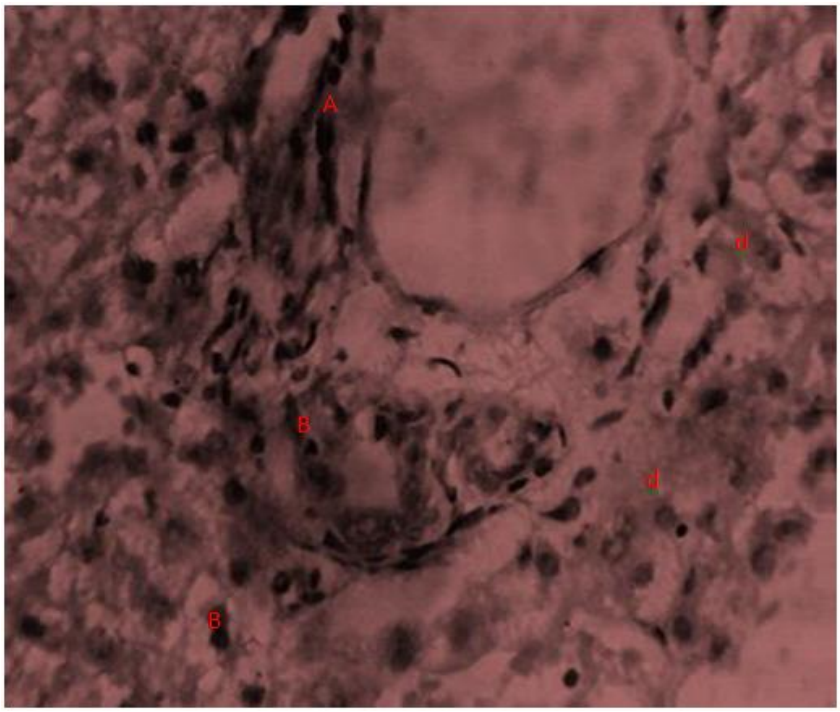


Plate 3: Liver section of rabbits that fed on bean forages containing 0.13 ppm bioaccumulated herbicide butachlor on day 28 showing further degenerative changes and necrosis of hepatocytes (H and E x400). **A**=Portal area; B = Plates of hepatocytes; d= Areas of degenerative and necrotic changes devoid of hepatocytes.

Group IV (Intoxicated with 0.20 ppm bioaccumulated herbicide,butachlor®): A late stage necrotic process and initiation of fibrosis prominence of stellate cells in the perisinusoidal spaces was observed (Plate 4). Hepatic stellate cells are responsible for fibrosis, since they secrete large amounts of collagen during liver injury.

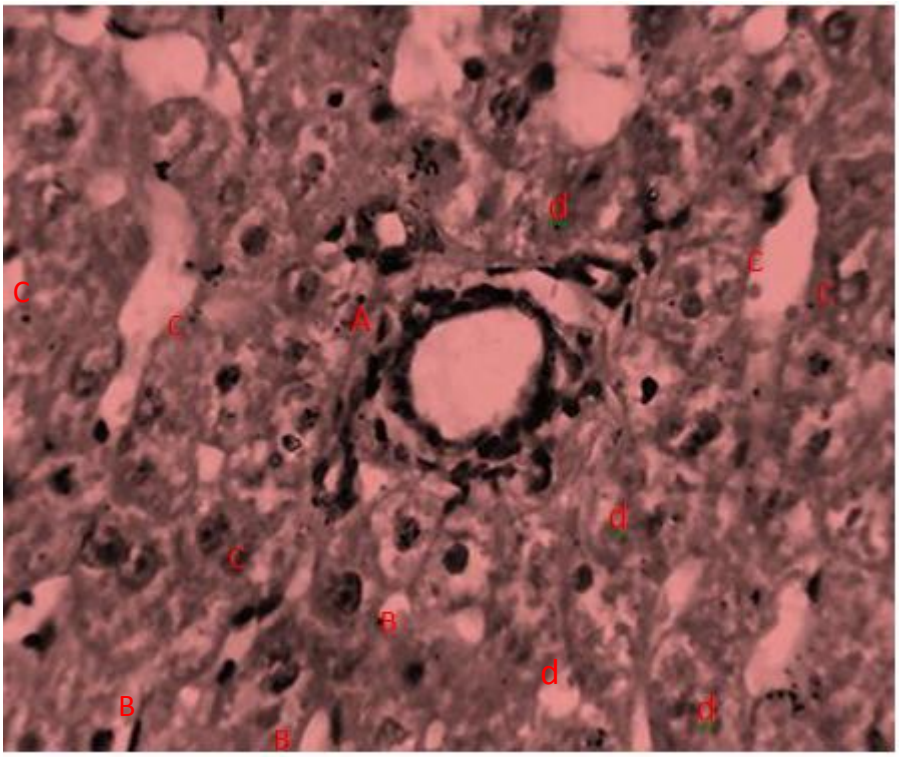


Plate 4: Liver section of rabbits that fed on bean forages containing 0.20 ppm bioaccumulated herbicide butachlor on day 28 showing evidence of cellular degenerative changes, reduction in the number of hepatocytes, and initiation of fibrotic processes with elaboration of stellate cells. **A**=Portal area; B = Plates of hepatocytes; C= stellate cells; d=fibrotic areas with complete replacement of hepatocytes.

IV. Discussion

The results of the study revealed that sub-chronic exposure of rabbits to the least test concentration of 0.10 ppm bioaccumulated herbicide in the legume forage was deleterious liver parenchymal cells. A deteriorating hepatocellular injury of the rabbit liver was observed withintake of increased concentration of the herbicide residue. On day 7 of the study, control ALT of 24.5 ± 5 IUL^{-1}became elevated to 42.8 ± 7.0 IUL^{-1} with 0.20 ppm butachlor® content in theforage while the control enzyme level of 24.0 ± 6 IUL^{-1}on day 28 got boosted significantly ($p<0.05$) to 66.8 ± 4 IUL^{-1}. The elevation in serum ALT and AST were indications of hepatocellular damage. Injury to hepatic cells induces leakage of liver enzymes into the blood, where they can be measured as indicators of cell damage [6]. Although serum levels of ALT, AST and ALP become elevated whenever disease processes affect liver cells, ALT is the more liver-specific enzyme [6].

The histopathological findings (Plates 1-4) corroborated with elevations in the rabbit serum biomarkers of hepatotoxicity.. Rabbits that consumed forages with 0.10 ppm of the herbicide had lesions suggestive of degenerative changes in the liver parenchyma; 0.13 ppm of the herbicide depleted the rodent hepatocytes while exposure to 0.20 ppm of the bioaccumulated herbicide initiated fibrotic processes as evidenced in elaborated stellate cells. Hepatic fibrosis is a scarring process that progresses to cirrhosis in the absence of an intervention [7].

Bioaccumulation of toxicants in edible crops could be hazardous to human and animal life. Incessant and indiscriminate application of the herbicide coupled with accidental spillage could cause ecotoxicity and harmwild life and aquatic animals[8,9].The toxic effects of exposure to bioaccumulated chemical agents within food chain and water reserves may contribute to increased cases of cancer, infertility, hypertension, renal failure and neuro-degenerative diseases. A review of about 200 epidemiological studies reported a consistent association between low consumption of fruits and vegetables and cancer incidence at many target sites [10]. Again, US Environmental Protection Agency [11] reported that tetrachlorodibenzo-p-dioxin (TCDD), the most potent rodent carcinogen bioaccumulates through the food chain because of its lipophilicity, and more than 90% of human intake is from animal fats in the diet. The analysis of pesticides and their residues had in the past aided

objective re-evaluation and reassessment of these substances on a benefit-risk analysis basis and their subsequent withdrawal from use when found to be hazardous to human health and the environment [12].There is therefore need for restriction of herbicide applicationto crops and the practicebe enforced to be performed only by professionals to reduce problems of bioaccumulation in crops andavailability of pesticides residues in food chains.

V. Conclusion

Sub-chronic exposure of experimental rabbits to bioaccumulated herbicide, butachlor® induced a corresponding severity in hepatocellular lesion to the concentration of the herbicide in the forage. The findings from the study provided evidence that bioaccumulated herbicide in legume forage could be hepatotoxic to rabbits.

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Enefe, Ndidi G., "Hepatotoxicity in Rabbits resulting from sub-chronic Exposure to Bioaccumulated Herbicide, Butachlor® in Beans (phaseolus vulgaris) Leaf forages''. IOSR Journal of Pharmacy and Biological Sciences (IOSR-JPBS) 13.3 (2018): 12-19.