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Evaluation of Cytochrome P_{450} and Glutathione S-Transferase as Biomarkers in Butachlor Exposed Rabbits

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ABSTRACT

The use of pesticides (herbicides) has increased worldwide over the years in other to secure food supply for the teaming global population.Butachlor(N-butoxymethyl)-2-chloro-2',6 diethylacetamide) is a selective systemic chloroacetamide herbicide used for the pre-emergent control of annual grasses and certain broad leaf weeds in rice, barley, wheat and some other leguminous crops. This study was carried out to evaluate the effect of an indirect subchronic exposure to butachlor using rabbit as animal model via the consumption of bean plant leaves. The plant leaves contained bioaccumulated butachlor of 0.10, 0.13 and 0.20ppm.The assessment of two biomarker enzymes; Cytochrome P_{450} and Glutathione-S-transferase was done with the post-mitochondria liver fractions. The result showed a significant increase ($p < 0.05$) in the liver Total Cytochrome P_{450} and Glutathione-S-transferase in the groups 3(0.13ppm) and 4(0.20ppm), when compared with the control; this was observed to be concentration and time-dependent. This study showed that the use of the butachlor herbicide concentration above the manufacturers recommended concentration of 2.6kg a.i/ha bio-accumulated in the plant leaves resulting to a significant effect on the two biomarker enzymes: Cytochrome P450 and Glutathione S-transferase, which are involved in the phase I and II xenobiotic detoxification, this indicative of induction and the certainty of animal exposure to xenobiotics ; these enzymes could however be overwhelmed when the concentration of herbicide is high and upon prolonged exposure, thus resulting to bioaccumulation and toxicity to animals.

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INTRODUCTION

Herbicides are frequently used in agriculture to enhance food production in both developed and developing countries with diverse impact. This is highly dependent on toxicity, time of spraying, persistence and the half-life of their products. Butachlor (N-butoxymethyl-2-chloro-2,6-diethylacetanilide) a chloroacetamide is a pre-emergence systemic herbicide commonly used in Asia and Africa to control a wide variety of grasses in rice, wheat and some leguminous crops. (Senseman , 2007). Butachlor is thought to inhibit the synthesis of long chain fatty acids, alcohols, isoprenoid and flavonoids in the target plants. (Ecobichon, 2001; Heydens *et al*., 2002). The increased application of the herbicide on weeds in rice, tea, wheat and other crops reportedly exerts detrimental effects on non-target organisms like earthworms (Muthukaruppan and Gunasekaran, 2010) and other organisms (Kumari *et al*., 2009). Ecotoxicological studies suggest that butachlor and their metabolites may be harmful to aquatic invertebrates. (Ateeq *et al*., 2005, 2006; Geng *et al*., 2005a, 2010; Mousa *et al*., 2007), microbial communities (Min *et al*., 2002) and possibly being carcinogenic in animals including humans (Panneerselvam *et al*.,1999; Geng *et al*., 2005a); Ou *et al*., 2000). Butachlor has been suggested to be mutagenic in primary rat tracheal epithelial and causes stomach tumors in rat cells. (Hill *et al*.,1997). Acute and Prolonged exposure to butachlor has been found to be toxic to freshwater fish (Tilapia Zillii and Channa punctata) and accumulate through the food chain. (Tilak *et al*.,2007; Nwani *et al*., 2013).

Pesticides (herbicides) are metabolized mainly in the liver and this may result in either increased or decreased toxicity of the chemical.

(Guengrich, 1990). The enzymic detoxification of xenobiotics in the liver has been classified into two distinct phases; Phase I and II, which all involve the conversion of a lipophilic non-polar xenobiotic

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into a more water-soluble and less toxic (in some cases more toxic) compounds which can be eliminated more easily from the cell. (Hogson and Levi, 1997; Sheehan *et al*.,2001). Phase I is catalyzed mainly by the Cytochrome P_{450} (CYP) system and is responsible for a range of reactions of which oxidation appears to be the most important (Guengerich, 1990). Glutathione S-transferase (GST) in the phase II, is a family of multi-functional proteins that catalyzes the formation of conjugates between reduced glutathione (GSH) and a wide variety of compounds including toxins, pesticides, and polyaromatic hydrocarbons.(Halliwell and Gutterdge, 2007; Salinas and Wong,1999). These reactions increase the water solubility of the substrate and facilitate its excretion.(Eaton and Bammler, 1999). Biomarkers are responses that reflect exposure to or effects of environmental pollutants in an organism (Peakall, 1994) and they are used as assessment tools to determine the degree of exposure to pollutants such as herbicides when they are directly or indirectly consumed by the organism. Cytochrome P_{450} and Glutathione S-transferase have been used as biomarkers of exposure to pollutants in aquatic system and other animals. (Bucheli and Font,1995;Van der Oost *et al*.,2003; Otitoju and Onwurah,2007).

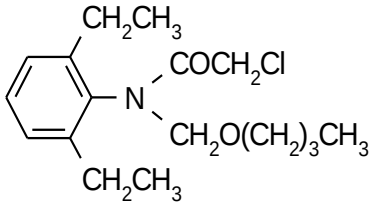

Fig. 1: Structure of Butachlor.

In developing countries, pesticides (herbicides) are routinely used in unsafe conditions; farmers or extension agents lack the technical skills for proper and effective use and also the resources to increase safety, thus resulting to many unfortunate consequences including human and livestock exposure (directly or indirectly) to pesticide poisoning, crop injuries and environmental pollution (Dugjie *et al*., 2008; Wang *et al*., 1996,Dearfield *et al*., 1999) .The herbicides could leach into groundwater, persist in the soil (Ayansina *et al*.,2003)or could be taken up by non-target crops, and may be metabolized to residues which may accumulate and ultimately consumed by animals or man via the food chain. In Nigeria, research work have centered mainly on crude oil and petroleum products toxicity on plants and aquatic organisms (Omoregie *et al*., 1990; Chindah *et al*., 2001) and also on pesticide toxicity on aquatic animals (Chindah *et al*.,2004). However studies on pesticide toxicity via the food chain are scanty; also information on butachlor is scanty. Thus, this research work was undertaken to assess the effect of butachlor upon subchronic exposure via the diet in rabbits by the assessment of two biomarker enzymes: Cytochrome P_{450} and Glutathione-S-transferase (GST) enzymes which are involved in detoxification.

MATERIALS AND METHODS

Chemicals:

Analytical grade butachlor standard (98%), Reduced Glutathione and 1 chloro-2, 4-dinitrobenzene solution (CDNB) were purchased from Zayo-Sigma Aldrich, USA. Total Cytochrome P_{450} Elisa kit (Antibodies Online Germany) and Butastar Herbicide (EC 50%butachlor as active ingredient) was obtained from an Agrochemical in Nsukka, Nigeria.

Plant material:

Beans (*phaseolus vulgaris*) seeds were purchased from the local market in Nsukka, Nigeria

Animals:

48Male rabbits with average weight of 1.2kg were obtained from the Department of Pharmacology, University of Nigeria, Nsukka. They were acclimatized for one week, under standard environmental conditions with approximately 12hours light/dark cycle maintained on regular feed (vital feed) and water *ad libitum*.

Field Study:

The Field experiment was conducted at the Research farm of the Department of Crop Science, University of Nigeria, Nsukka. Planting of the bean seeds was carried out in accordance with good agricultural practice (GAP) and the recommended application rate of butastar herbicide (EC 50% active ingredient-butachlor) for beans at pre-emergence was 2.6kg a.i/ha (Dugjie *et al*., 2008). Three (3) different application rates/concentrations were applied to the plots at pre-emergence; T_1- (2.6kg a.i/ha), T_{2-} (2.9kg a.i/ha), T_3- (3.2kg a.i/ha). At the beginning of the 6^{th} week, leave samples were collected in duplicates from the plots and analyzed for the presence of the butachlor herbicide using gas chromatography-mass spectrometry (GC-MS).

Experimental design:

Animal Protocol:

A total of forty eight (48) rabbits were divided into four (4) main groups with three (3) subgroups, each consisting of four (4) rabbits.

Group 1(Control): Animals were fed with the bean plant (leaves) cultivated without the application of butastar (butachlor) herbicide. 1,14 and 28days. While groups 2, 3 and 4 were fed with the bean plant (leaves) cultivated with butachlor at concentrations of 2.6, 2.9 and 3.2kg a.i/ha respectively for a period of 28days and water given to them ad libitum. They were fed with the diet of an average weight of 200g of the bean plant leaves/ rabbit daily. (Iyeghe-Erakpotobor and Muhammad, 2008) and water ad libitum.

The experiment was carried out for twenty eight (28) days and day 1 was equivalent to 6weeks of the cultivation of the plant. At the end of the study, the rabbits were sacrificed by severing the jugular vein. Handling, management and use of animals for experimentation were in conformity with laboratory Animal Rights Regulation and the principle of laboratory animal care as documented by Zimmerman (1983).

Table 1: Butachlor Herbicide in plant leave extract n = 2,mean+ SD.

Concentrations of Butachlor Applied on soil (kg a.i/ha)	Concentration of Butachlor Herbicide bioaccumulated in Plant Leave (ppm)
Control	ND
T_1-2.6 (Standard Recommended conc.)	C_I 0.10±0.0002
T_2- 2.9	C_2 0.13±0.00014
T_3-3.2	C_3 0.20±0.007

$p < 0.05$

Herbicide/residue Identification and Quantification:

Extraction was done using acetone/n-hexane. 100mgL standard stock solution was prepared with n-hexane, and working standard solutions with concentration ranges of 0-2mg/l(ppm) were prepared by dissolving in appropriate volume of hexane. 1µl of each standard and samples were injected into the gas chromatography. The peak areas of the standards were plotted against the series of known concentrations and a standard curve and linear equation were obtained. The concentrations of the samples were then calculated. GC Conditions: Column Rt-5MS (30×0.25 mm i.d), Initial temp.:140^{o}C, initial time: 1min, Increasing temp.: 8^{o}C/min, Final temp,: 300^{o}C held for 5mins. Carrier gas: Helium, Injection Volume was 1ul (Chang *et al*., 2005).

Preparation of Post Mitochondrial Supernatant (PMS)/ S-9 Fraction:

Five gramm (5g) of liver tissue was homogenized with 20ml of cold 100mM sodium phosphate buffer (pH 7.4, 2mM EDTA) and the homogenate centrifuged for 20mins at 12,000g. The Supernatant, containing the microsomes and enzymes was carefully harvested and stored (-20^{o}C) in tubes for total protein and enzyme assays as described by (Nilsen *et al*., 1998).

Determination of Glutathione-S-transferase (GST):

The catalytic activity of liver GST was measured spectrophotometrically at 340nm by modified method of Habig *et al*., (1974) using 1 chloro2-4 dintrobenzene and GSH as substrates. 2.7ml of phosphate buffer (pH 7.0) and 0.1ml each of 3.0mM chlorodinitrobenzene (CDNB) and 0.1M reduced glutathione were pipetted into test-tubes, mixed and transferred into the cuvette of the spectrophotometer, then 0.1ml of the liver PMS was added, mixed and the change in absorbance was monitored every 60secs for 180secs. The Glutathione-S-transferase activity was expressed as U/ml enzyme.

Determination of Total Cytochrome P_{450} (CYP):

The liver enzyme Cytochrome P_{450} was assayed using a Total Cytochrome P_{450} ELISA kit as described by (Nilsen *et al*., 1998). The assay procedure was conducted as prescribed by the manufacturers guide**.** The Optical density was determined using a microplate reader at 450nm.The intensity of the color inversely proportional to the cytochrome P_{450} concentration in the standards and samples. A standard curve was obtained from a plot relating the Optical density (O.D) to the concentration of the standards and the sample concentrations were then calculated from the linear equation.

Statistical Analysis:

Values obtained were expressed as mean ± SD and subject to one-way and two-way analysis variance (ANOVA) and Duncan New multiple range. post hoc test. Values of $p< 0.05$ were regarded as statistically significantly.

Result:

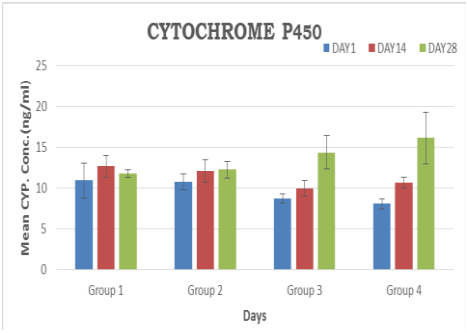

Fig. 2: Changes in Total Cytochrome P_{450} in liver samples of exposed rabbits via the diet.

n=4, Mean±SD. ($p < 0.05$)
Key:

Group1- Control: Animals fed with plants cultivated without the application of the Herbicide

Group 2- (Animals fed with plants containing bioaccumulated butachlor concentration of 0.10ppm)

Group 3-(Animals fed with plants containing bioaccumulated butachlor concentration of 0.13ppm)

Group 4- (Animals fed with plants containing bioaccumulated butachlor concentration of 0.20ppm)

The mean ± SD of Cytochrome P_{450} levels for the groups 2 , 3, 4 and the control group 1 are presented in Figure 3. On day 1 of the experiment, there was no significant difference ($p>0.05$) in the CYP levels of group 2 when compared with the control group; however there was a reduction in CYP levels of group 3(not significant $p > 0.05$) and group4 (significant $p<0.05$) when compared with the control. There was no significant difference in the CYP level of group 2 on day 14, however there was a reduction in groups 3 which was significant ($p<0.05$) and group4 (not significant at $p>0.05$) when compared with the control. A significant increase ($p< 0.05$) was observed on day 28, after prolonged exposure in groups 3 and 4; when compared with the control group. The increase on day 28 was observed to be concentration dependent, implying that with the consumption of the higher concentrations of the bio accumulated butachlor, the level of CYP increased (Fig.3)**.** Within group2 there was an increase on day 14 and 28 though it was not significant but within groups 3 and 4 there was significant increase ($p<0.05$) on the 14th and 28th day and this was observed to be concentration and time-dependent; that is, as the days progressed, with increase in consumption of bio accumulated butachlor, the CYP increased.

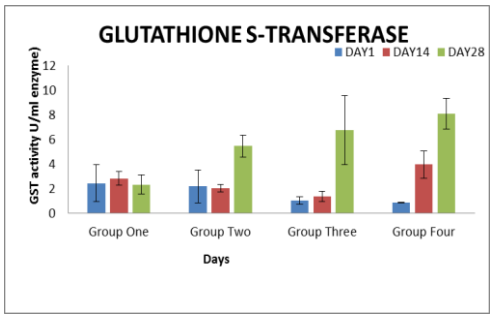

Fig. 3: Changes in Glutathione-s-transferase Activity in liver samples of exposed rabbits via the diet. n=4, Mean±SD. ($p < 0.05$).

The mean±SD GST activity for groups 2 , 3, 4 and the control group 1 are presented in Figure 4 .On day 1, there was a decrease observed in the GST activity in group 2 when compared with the control group, though not significant but there was significant decrease ($p<0.05$) in groups 3, 4 and the control group 1. On day 14, there was no significant difference between group2 and the control group ,but there was a significant decrease ($p<0.05$) in group 3 and a significant increase ($p<0.05$) in group 4 when compared with the control group 1. On the 28th Day, there was also a significant increase ($p<0.05$) in groups 2, 3 and 4 when compared with the control group1, with the highest in group 4-day 28. In groups 3 and 4 a similar trend could be observed, in that there was an initial decrease in GST on Day 1 of the experiment, and then on the 14th and 28th day there was a consistent increase ($p<0.05$) in the GST activity.

Discussion:

Studies have shown that the herbicide butachlor, persists in the aquatic system for a long period of time hence its toxicity to aquatics (Tilak *et al*., 2007). Persistence of butachlor have also been studied at two levels of application in different soils at three

different sites under three moisture regimes air-dry, field capacity and submergence(Prakash and Suseela, 2000). Animals that feed on plants may ingest one or more dietary pesticide/residue produced in or on the plant and this is subject to metabolism by the liver, the liver being the primary site of xenobiotic (pesticides) bio transformation for the purpose of facilitating clearance through excretion of water-soluble products via detoxification. However, the high level of oxidative metabolism in the liver makes this organ a possible target for more toxic metabolic products activation when detoxifying and protective mechanisms are overwhelmed.(Hogson and Levi,1997). The liver is also rich in xenobiotic metabolizing enzymes which include Cytochrome P_{450} and Glutathione S-transferase. Pesticides are known to be substrates for the enzymes as well as inducers or inhibitors. (Melancon, 1996).. From table 1, it could be observed that there was a 30% increase in the herbicide concentration of C_2 from C_1 (T_1 manufacturers recommended concentration) and also a 100% increase of C_3 from C_1. A statistical difference was observed between the C_1 and C_2 at 0.05 level of significance; between C_1 and C_3 and there was significant increase in C_2 and C_3 when compared with C_1 at 0.05 level of significance ;indicating that there was bioaccumulation and this was increasing with respect to the concentrations of herbicide applied.

Cytochrome P_{450} are enzymes involved in phase 1 metabolism of xenobiotics. There was no significant difference ($p>0.05$) in the total cytochrome P_{450} levels when group 2 was compared with the control group, on the first day of the experiment, although there was a reduction in the levels of CYP. Similarly there was a non-significant($p>0.05$); reduction in CYP of group 3 but decreased significantly ($p<0.05$) in group 4 when compared with that of the control group, on day 1.This may be attributed to the presence of small quantities of the butachlor(higher concentration) which have been consumed from the plant on day 1 and 14, hence there was an immediate utilization of the detoxifying enzyme cytochrome P_{450} in the body system of the rabbits causing a reduction, however, as the days went by, with more quantities of the plant with higher butachlor concentration being consumed, there was an induction of synthesis/production of the detoxifying enzyme, as could be observed by the increase (significant at $p< 0.05$) on the 28^{th} day after prolonged exposure for groups 3 and 4, when compared with the control group on day 28 as well as within the group. The increase could be observed to be time-dependent. The result obtained showed that a higher concentration of butachlor that bioaccumulated in plants consumed in group4 (0.20ppm) resulted to an increase in the levels of liver CYP by 37% and also in group 3 by 22%when compared to the control group (1); while in group 1 which contained no herbicide, there was no induction and in group 2 there was little or no induction of CYP. Such was also observed in an acute study by (Pogrmic-Majkic *et al*., 2012), where there was a similar effect of atrazine herbicide on the liver CYP however in this case, a higher concentration of atrazine increased the levels of CYP by 56% when compared with the control. This result is also in agreement with some other acute and chronic studies of direct exposure to butachlor and atrazine. (Islam *et al*., 2002; Farombi *et al*.,2008)

From Fig4 .It was observed in group 2 on the Day 1 that there was little or no reduction in the GST activity when compared with the control group(1); likewise on the 14^{th} Day, probably due to the fact that herbicide present for detoxification was in small quantities (0.10ppm). However there was an increase on the 28^{th} day but not as much as in group 3 and 4 which had higher concentration of herbicide. In groups 3 and 4 a similar trend could be observed in that there was an initial decrease in GST activity, which was significant when compared with the control on the day 1 of the experiment, probably indicating a rapid usage of the GST in detoxification of the herbicide; and then on the 14 and 28 day there was a consistent increase (significant $p<0.05$) in the GST activity. This may be due to the prolonged exposure to the xenobiotic, such that the enzyme was induced in other to cope with the detoxification of the herbicide in the liver. The concentration and time dependent increase in GST as observed in the present study is in agreement with some other research findings involving a direct exposure in several fish species (Shalaby *et al*., 2007; Otto and Moon ,1996). Also an exposure to atrazine herbicide has been shown to cause the induction of GST in hepatic microsomes and cytosol of Fisher rats (Islam *et al*., 2002). In vitro incubation of liver and kidney fractions with butachlor showed that butachlor was first biotransformed by conjugation with GSH by the enzyme GST to form butachlor glutathione conjugate which was further transported to the kidneys to form mercapturic acid by N- acetylation. However it was also reported that in the absence of acetyl CoA, the GSH conjugate was metabolized to butachlor cysteine conjugate (Qu and Lin 1992). It is possible that an increase in the activity of GST contributes to the the elimination of reactive oxygen species, induced by the herbicide from the cell. (Jin *et al*., 2010).The increased GST activity could also suggest that the enzyme may be responsible for the conversion of butachlor to more hydrophilic metabolites which could increase the elimination from the organism.

Conclusion:

The result from the present study revealed that a subchronic exposure to butachlor at the two different concentrations of 0.13 and 0.20ppm in the diet led to an increase in the two pesticide biomarker enzymes,

in a concentration and time-dependent pattern. This, indicative of their induction and the certainty of animal exposure to xenobiotics. This study showed that there is the possibility of a more toxic response upon chronic exposure to butachlor when the herbicide is improperly applied to soil resulting to increased bioaccumulation and consumption.

Recommendation:

Hence it is recommended that farmers or agriculturalist adhere to the standard concentration of this herbicide usage as stated by the manufacturers, so as to prevent increased herbicide/residue accumulation in plant parts and subsequent consumption by animals.

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