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Effect of repeated oral administration of butachlor on haemato-biochemical parameters in wistar rats

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Abstract
Adult Wistar rats of either sex were purchased from the Indian Institute of Integrative Medicine, Jammu and maintained under standard experimental conditions with ad libitum feed and water. The animals were randomly divided into two groups with six rats in each group. The experiment was conducted for a period of 28 days and was given through oral gavage. Group I served as control and was provided with water only for drinking purposes. Group II animals were administered Butachlor (Mancheter EC) @ 262 mg / kg B.W/P.O. Administration of Butachlor @ 262 mg / kg alone produced haemato-biochemical changes. It produced significant ($p < 0.05$) decrease in levels of Hemoglobin, Packed cell volume (PCV), Total erythrocyte count (TEC), Lymphocytes, Monocytes, Basophils levels while significant ($p < 0.05$) increase was observed in Total leucocytic count (TLC), Erythrocyte sedimentation rate (ESR), Neutrophil, Eosinophils. Elevation in blood biochemical indices was observed like phosphatases (ACP & ALP), aminotransferases (AST & ALT), creatinine, BUN, Total cholesterol, Triglyceride, LDL but decreased the protein profile and HDL levels.

Keywords: Herbicides, butachlor, haematology, biochemical, wistar rat

Introduction
Agriculture scenario has changed drastically after first green revolution and since then, there has been steady growth in production of technical grade pesticides in India, from 5,000 metric tons in 1958 to 102,240 metric tons in 1998. In 1996-97 the demand for pesticides in term of value was estimated to be around Rs.22 billion (USD 0.5 billion), which is about 2 per cent of total world market. The pattern of pesticides usage in India differs from world in general. The demand for pesticides in India was 50583 tonnes in 2011-12 while in J&K the demand was 2468 MT during 2010 against 28 MT in 2005 (Economic survey., 2011) [7].
Butachlor (N-(butoxy-methyl)-2- chloro-2', 6'-diethyl acetanilide) is a widely recommended herbicide for use in rice cultivation. The mode of action of butachlor is by inhibiting the elongase that is responsible for elongation of very long chain fatty acids and geranyl pyrophosphate cyclization enzymes (Gotz and Boger, 2004) [11]. The consumption of butachlor is approximately 4.5×10^7 kg per years in Asia alone (Ateeq *et al*. 2002) [4]. In India, nearly 6750 metric tons of butachlor are sprayed annually, as it was the first rice herbicide to be introduced (Verma *et al*. 2014) [31].

Material and Methods
Adult Wistar rats of either sex were purchased from the Indian Institute of Integrative Medicine (Council of Scientific and Industrial Research Laboratory, Jammu) and maintained under standard experimental conditions with ad libitum feed and water. A daily cycle of 12 h of light and 12 h of darkness was provided to animals. Prior to the start of the experiment, the rats were acclimatized in the laboratory conditions for a period of 2-3 weeks. All the experimental-animals were kept under constant observation during entire period of study. The experimental protocols were duly approved by the institutional ethical committee and experimental design was approved by the University Animal Ethical Committee. The animals were randomly divided into two groups with six rats in each group. The experiment was conducted for a period of 28 days and administration of toxicant was undertaken in morning between 8:00-9:00 A.M. The toxicant to the animals was given through oral gavage. Group I served as control and was provided with water only for drinking purposes. Group II animals were administered Butachlor (Mancheter EC) @ 262 mg / kg B.W / P.O.

Blood samples of about 2ml were collected from retro-orbital sinus of rats on day 28th by using capillary-tubes in aliquots containing anticoagulant heparin strength @ 10 IU / ml of blood.

Haemoglobin: Drabkin solution (5ml) was mixed with whole blood (20 µl) and it was allowed to stand for 5 min. Absorbance was read at 540 nm.

Packed cell volume (PCV) / haematocrit value: Microhaematocrit method: A 7 cm long capillary tube with a bore of 1 mm was charged with blood up to ¾ of its length. Capillary tube was placed into slot of haematocrit centrifuge with the sealed end towards outside. Centrifuge was done for 8-10 minutes at 10,000 rpm. After centrifugation, the PCV was read using a PCV reader.

Erythrocyte Sedimentation Rate (ESR): Win Trobe's method: The Win Trobe's or haematocrit tube was filled with blood up to '0' mark using a Win Trobe pipette. The tube was set in the Win Trobe stand in a vertical position at the constant temperature. The difference between final and initial readings was observed and results were estimated

Total Erythrocyte Count (TEC): Un-coagulated blood was taken up to 0.5 mark of R.B.C diluting pipette. Diluting fluid was sucked up to 101 mark, 3 or 4 drops of unmixed diluting fluid was discarded from the stem of pipette, and Cells were allowed to settle down for about 1-2 minutes. Chamber was focused under high power microscope. Let, N = the no. Of cells counted in 80 small squares. Then, N / 80 = the no. Of cells in 1 small squares. The area of 1 small square = 1 / 400 sq.mm and depth = 1 / 10 mm Volume of 1 small square = 1 / 400 × 1 / 10 cu mm. Then, result is given as the number of cells in 1 cubic mm. N / 80 multiplied by 4000. Besides, the blood was originally diluted 1 in 200. Therefore, No. of red cells / cu mm = N × 4000 × 200/80.

Total Leucocyte Count (TLC): A small, measured quantity of blood is diluted with suitable extender like diluting fluid, which will prevent hemolysis of erythrocytes but leave the leucocytes intact. Small drop of this mixture is placed on counting chamber of haemocytometer and number of leucocytes are counted in a known area under a high power microscope (100X) and then calculated in to number of leucocytes per mm^3 of blood, taking the dilution factor into consideration. The Un-coagulated blood is filling up to 0.5 mark of W.B.C diluting pipette. Diluting fluid was sucked up to 11 mark. Pipette was held at 45 ° angles to the surface of counting chamber and tip was placed close to the narrow slit between the counting chamber and the cover slip. Cells were allowed to settle down for about 1-2 minutes. Chamber was focused under high power and W.B.C number were counted in four large (1mm) corner squares of counting chamber under low power microscope with the condenser lowered. Light was adjusted so that leucocytes appear as round, slightly indented bodies with a definite outline. Leucocytes had definite cell outlines and defined nuclei. Platelets appeared as very small refractile bodies about 1/5th to 1/7th size of leucocytes without nuclei. Let the total number of W.B.C present in four squares of 1 sq. mm area of 1/10 mm depth i. e, 4 × 0.1 cu mm area = A1 + A2+ A3 +A4 = X. The no. of W.B.C present in 1 sq. mm area of 0.1 mm depth i.e., in 0.1 cu.mm. Volume = X/4.The no. of W.B.C present in 1 cu.mm volume = X / 4 × 10. The original blood sample is diluted 20 times that is 0.5 parts in 10 (11-1) parts. Therefore, 1 cu.mm of undiluted blood contains X / 4 ×10×20 i.e. X × 50.

Differential Count of Leucocytes (DLC): It may be defined as number of various types of leucocytes per hundred WBC counted.

Preparation of Blood Smear: A small drop of fresh or anticoagulant mixed blood was placed on clean, grease free, dry glass slide about 2cm away from one end. Another slide (spreader slide) was placed in front of the blood drop such that an angle of 30-45° was made between the two slides. With a single swift forward movement of spreader slide, the blood was spread along the surface of slide. Smear was dried immediately by waving the slide rapidly or exposing it to air. The rapid drying of smear prevents crenation of cells. The dried smears were stained on the staining rack with Leishman's stain or Wright stain**.** Stain was allowed to act for 1-2 minutes at room temp. Equal quantity of distill water or phosphate buffer was added and thoroughly mixed with the stain either by rocking movement or blowing air through a glass tube. Incubation was done for 5-10 minutes at room temperature. Stain solution was washed off gently with distilled water and then dried. Slide was focused under high power microscope to see the distribution of cells and nature of staining.

Biochemical analysis: Immediately after collection of blood, samples were centrifuged at 3000rpm for 15 minutes to harvest the plasma and then kept in clean sterile glass test tubes and stored at -20 ºC for further biochemical analysis. Standard kits procured from Recon diagnostics Pvt. Ltd / Trans Asia Bio-Medicals were used for determination of all biochemical parameters in this study. Plasma aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities were determined by using standard kits from Trans Asia Bio-Medicals. The principle reaction of the calorimetric determination of AST and ALT activity is based on the reaction of aspartate or alanine with α-Ketoglutaric to form oxaloacetate or pyruvate, respectively. The oxaloacetate or pyruvate formed was measured by monitoring the concentration of oxaloacetate or pyruvate hydrazone formed with 2, 4-dinitrophenyl hydrazine. Plasma alkaline and acid phosphatase activity was measured at 405 nm by the formation of para-nitro phenol from para-nitro phenyl phosphate as a substrate. Urea in blood reacts with water in presence of urease and forms ammonia which again reacts with α-Ketoglutaric and NADH in presence of glutratedehydogenase to form glutamate and NAD which was measured spectrophotometric ally at 500-520nm. Creatinine reacts with alkaline picrate to produce an orange yellow colour, the absorbance is directly related to creatinine concentration and was measured spectrophotometric ally at 500-520nm.Total protein was estimated by Biuret method, in which plasma forms a blue coloured complex when treated with cupric ions in alkaline medium. The intensity of the blue colour is proportional to the protein concentration. Total cholesterol in plasma was estimated by CHOD-PAP method and High density lipoprotein cholesterol was estimated by phosphotungstic acid method. However, low-density-lipoproteins (LDL) in plasma were calculated as per the formula of LDL= TC-HDL-TG/5 by Friedewald *et al*., 1972 [8].

Results and Discussion

The administration of Butachlor at the rate of 262 mg / kg produced haemato-biochemical changes. It produced significant (p<0.05) decrease in levels of Hemoglobin by 13 per cent (12.69±0.80) as compared to the control group (14.70±0.96)., Packed cell volume (PCV) also showed non-significant decline by 5 per cent, Total erythrocyte count (TEC) reduced by 30 per cent (4.7±0.29) while the Lymphocytes, Monocytes and Basophils showed decreased levels by 14 per cent, 23 per cent, 22 per cent respectively. A significantly (p<0.05) increase was observed in Total leucocytic count (TLC) by 37 per cent in butachlor group, Erythrocyte sedimentation rate (ESR) increased by 44.73 per cent, Neutrophil (25 per cent) and Eosinophils showed a significant increase by 50 per cent when compared to control group depicted in table 01. Similar results were reported by Joshi *et al*. (2012) [13], following butachlor exposure in rats. Exposure of butachlor results in reduction of erythrocyte count which may be due to inhibition of production of red blood cells in bone marrow (Rezg, 2007) [21]. The decline in erythrocyte counts also may be due to the disruptive action of the insecticides on the erythropoietic tissues as a result of which viability of cells might be affected (Cakmak, 2003) [6]. Haematocrit percent (PCV) may be reduced due to reduction in the size of RBC (Rahman, 2006) [20].

Hussain, 2014 [12] showed a significant reduction in RBC counts, haemoglobin concentration and haematocrit percent in Japanese quail. The decline in RBC cell counts is attributed to the toxic effects of herbicide on circulating erythrocytes and the blood forming tissues. Haemoglobin concentration was also reduced which could be due to reduction in feed intake and deficiency of iron. The decrease in haemoglobin levels could be due to toxic effects of butachlor on maturating erythrocytes and inadequate iron supply (Hussian, 2014) [12] while decrease in haematocrit values is due to increase destruction or reduction in size of erythrocytes (Rahman, 2006) [20]. The decrease in haematological parameters may be due to reduced haematopoietin production, erythropoiesis and impaired proliferation of hematopoietic progenitor cells or direct RBC lysis (Salih, 2010) [25].

Another study of Ghaffar, (2015) [9] showed decrease in haemoglobin concentrations, packed cell volume, erythrocyte counts and lymphocytes significantly and increases in total leukocyte increased significantly in fish exposed to butachlor. The decrease values of haemoglobin, pack cell volume and erythrocyte was attributed to inability of fish to deliver sufficient oxygen to hematopoietic tissues suggesting poor physical activity (Nussey, 1995; Hussain *et al*. 2014) [18, 12].

ESR increased by 44 per cent and is negatively correlated with total erythrocyte count. A lower total erythrocyte count reciprocates with a higher ESR value (Agarwal and Chaurosia, 1989) [1]. An increase in ESR (mm / hr) may be due to an increase in the concentration of fibrinogen which results in fibrinogenemia as result of chlorpyrifos exposure (Singh and Bhatia, 1991) [2].

The term WBC pertains to all types of leukocytes, including granulocytes (neutrophils, eosinophil and basophils), lymphocytes and monocytes. The WBC count is usually reported in units of thousands per cubic millimeter. Activated neutrophils plays an essential role in free radical mediated injury by inducing extracellular release of super oxides and other free radicals (McCord *et al*. 1994) [32], which are toxic to host cells.

Repeated exposure of butachlor to rat resulted in significant increase in leucocyte count and these observations are in agreement to the study of butachlor by Joshi *et al*., 2012 [13]. Reduction of WBC may be a consequence of androgens, as butachlor appears to target leukocytes (Milla *et al*. 2001) [17]. Justified by Ahmadivand, 2014 [2] who studied reduction in lymphocyte values and explained it as an anti-androgenic endocrine disruptor, because androgen plays an important role in haematological homeostasis by mediating lymphocyte proliferation (Milla *et al*. 2001) [17]. Conversely butachlor exposure resulted in an enhancing effect on neutrophil values in fish. This may be due to ability of butachlor to induce an immune response. It has been shown that neutrophil and macrophages activator gene IL-1b is significantly induced by butachlor in embryonic zebra fish (Tu *et al*, 2013) [28]. Ghaffar, 2015 [9] observed increased values of total leukocyte counts, neutrophils in fish by butachlor could be due to the inflammatory reactions after injurious stimuli. Increased monocyte values and decrease in lymphocyte count in present study could be due to sensitivity of the immune system against the stress condition due to butachlor herbicide.

Table 1: Effect of oral administration of Butachlor on blood profile in Wistar rats (n=6)

GRP	Treatment	HB	PCV	TLC	TEC	ESR
I	Control	14.70^{c}±0.96	44.13^{d}±4.04	9.33^{a}±0.66	6.8^{e}±0.30	2.3^{a}±0.21
II	Butachlor @ 262 mg / kg	12.69^{bc}±0.80	38.07^{bcd} ± 2.42	15.0^{cd}±0.73	4.7^{c}±0.29	3.8^{b}±0.40

Gr.	Treatment	Lymphocytes	Neutrophils	Monocytes	Eosinophils	Basophils
I	Control	68.3^{d} ±4.07	24.66^{a}±4.2	2.8^{d}±0.16	3.0^{a}±0.25	1.16^{a}±0.16
II	Butachlor @ 262 mg / kg	59.6^{abcd} ±3.09	32.16^{abcd}±3.1	2.1^{bc}±0.10	4.5^{bc}± 0.22	1.5^{ab}±0.22

Elevation in blood biochemical indices was observed like phosphatases (ACP and ALP), aminotransferases (AST and ALT), creatinine, BUN, Total cholesterol, Triglyceride, LDL but decreased the protein profile and HDL levels.

Plasma Phosphatases

Acid phosphatase enzyme (ACP) plays an important role in the process of cell metabolism, autolysis, differentiation and many related processes (Sugar, 1997) [26]. In this study, 33.36 per cent of increase in acid phosphatase enzyme was observed. The increase in acid phosphatase enzyme activity could be explained on the basis of enhancement of cell membrane permeability with disturbance in the Trans phosphorylation process as a result of cellular degeneration (Verma *et al*. 1984) [29].

Alkaline phosphatase (AKP) is a membrane bound enzyme at bile pole of hepatocytes and also found in Pinocytosis vesicle and Golgi complex. A significant decrease by 28.22 per cent was observed as shown in table 02 and decrease in AKP activity may be taken as an index of hepatic parenchymal damage and hepatocyte necrosis (Onikienko, 1963) [19]. Inhibition of AKP reflects alterations in protein synthesis and uncoupling of oxidative phosphorylation (Verma *et al*. 1984) [29]. This observation is in agreement to earlier reports of

Shalan *et al*, 2005 [24]. Similar results were recorded in previous studies following repeated Chlorpyrifos exposure (Ambali *et al*. 2007) [3].

Plasma aminotransferases

When the liver is damaged, Alanine transaminase (ALT) get elevated and released in the bloodstream. It is more sensitive marker to hepatocellular damage than AST (Lin *et al*. 1997) [15]. A drastic increase in AST levels by 30.83 per cent was observed as shown in table 02 and it may indicate liver damage or disease. The increase in the activity of plasma ALT is mainly due to leakage of this enzyme from liver cytosol into the blood stream, which reflects liver damage and disruption of normal liver function (Shakoori *et al*. 1994) [23]. Aspartate transaminase is the mitochondrial enzyme, predominantly found in the liver, skeletal muscles and kidneys. A significant increase by 22.25 per cent indicates deleterious effect of butachlor on liver as alteration of these enzyme drastically shows toxic responses occur more in liver as compared to other organs (Dar, 2009) [6]. Similar results were found by Hussain, 2014 [12], with Butachlor in Japanese quail, the enzyme AST and ALT.

Table 2: Effect of oral administration of Butachlor on hepatic biomarkers in Wistar rats (n = 6)

Gr.	Treatment	AST	ALT	ACP	ALP
I	Control	114.77^{a}±6.76	52.32^{a}±2.8	41.05^{a}±3.9	270.60^{a}±25.35
II	Butachlor @ 262 mg / kg	147.63^{cd}±7.40	75.65^{bc}±4.7	61.60^{cd}±3.4	377.03^{cd}±25.12

Blood urea nitrogen and Creatinine

Creatinine is produced by degradation of creatinine and creatinine-phosphate, energy storing molecule mainly present in skeletal muscle, and its catabolism to creatinine occurs at a steady state. It mainly circulates in a free form in the plasma and is distributed into whole body water compartment (Watson *et al*. 2002) [30]. There was a significant increase in the levels by 40 per cent. Similar results were indicated by Dong, 2015 who studied toxic effects of butachlor exposure on goldfish (*Carassius auratus*) and found drastic elevation in tissue and serum creatinine along with moderate degeneration of renal tubules which indicated renal function compromise. A significant drastic increase in the levels of BUN by 58.80 per cent was observed as shown in table 03 and similar changes were reported by Joshi *et al*., 2012 [13]. Increase in the BUN and creatinine levels suggest impairment in glomerular function and reduced ability of kidney to eliminate the toxic metabolic substances (George *et al*. 2014) [10].

Table 3: Effect of oral administration of Butachlor on renal biomarkers in Wistar rats (n = 6)

Groups	Treatment	BUN (mg / dl)	Creatinine (mg / dl)
I	Control	23.08^{a}±2.17	0.593^{a}±0.036
II	Butachlor @ 262 mg / kg	56.02^{bc}±3.42	0.992^{b}±0.041

Plasma Proteins

Plasma proteins are significant indicator of health status, metabolic and production features of the organism because of numerous roles in the physiology (Kaneko *et al*. 1997) [14]. Hypoproteinemia was recorded in present study with the exposure of Butachlor by 19 per cent as shown in table 04. Similar findings have been observed by workers earlier following low dose of Chlorpyrifos exposure i.e.10.6 mg kg (Ambali *et al*. 2007) [3]. It may be due to increased catabolism of proteins and their decreased synthesis.

Table 4: Effect of oral administration of Butachlor on Protein profile in Wistar rats (n = 6)

Gr.	Treatment	Total protein	Albumin	Globulin	A/G ratio
I	Control	6.672^{b}±0.334	4.11^{c}±0.25	2.260±0.163	1.872^{b}±0.180
II	Butachlor @ 262 mg / kg	5.115^{a}±0.208	3.28^{b}±0.15	2.072±0.110	1.610^{ab}±0.118

Plasma Lipids

Detoxification is accomplished generally by hydroxylation's catalyzed by mixed function oxidases of endoplasmic reticulum membrane. Thus, disorders of fat metabolism are primary biochemical alterations of insecticides use resulting in an increase in cholesterol level and reduction in serum triglyceride concentration.

In present study a significant increase in plasma cholesterol ($p < 0.05$) by 20.25 per cent and decreased HDL by 30.20 per cent was observed in the rats exposed to butachlor as shown in table 05. Earlier workers reported mobilization and lipolysis of fats from adipose tissue on oral administration of pendimethalin in rats leading to increase in plasma cholesterol concentration (Shah *et al*. 1997) [22].

Table 5: Effect of oral administration of Butachlor on Lipid Profile in Wistar rats (n = 6)

Treatment	Total cholesterol	HDL	Triglyceride	LDL
Control	63.33^{a}±4.00	32.98^{e}±2.8	40.99^{a}±2.6	22.14^{a}±3.69
Butachlor @ 262 mg / kg	79.81^{b}±3.7	23.02^{cd}±1.6	49.17^{b}±2.4	46.95^{c}±3.97

Conclusions

The extensive use of pesticides leads to exposure risk of general population and several studies have shown the presence of pesticides and their residues in the environment, in food and in human tissues. The present study was undertaken due to extensive and un-prescribed use of butachlor in Jammu and Kashmir State, with aim to determine its toxicological effects on the hematopoietic system and marked biochemical changes.

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