CHRONIC TOXICOLOGICAL STUDIES ON THE INTERACTION OF FLUBENDIAMIDE AND LEAD FOLLOWING ORAL ADMINISTRATION IN BUFFALO CALVES

Dissertation

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(Minor Subject: Veterinary Biochemistry)

By

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CERTIFICATE I

This is to certify that the dissertation entitled, **"Chronic toxicological studies on the interaction of flubendiamide and lead following oral administration in buffalo calves"** submitted for the degree of **Ph.D.** in the subject of **Veterinary Pharmacology and Toxicology** (Minor Subject: Veterinary Biochemistry) of the Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, is a bonafide research work carried out by **Amita Ranjan (L-2009-V-6-D)** under my supervision and that no part of this dissertation has been submitted for any other degree.

The assistance and help received during the course of investigation have been fully acknowledged.

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CERTIFICATE II

This is to certify that the thesis entitled,**"Chronic toxicological studies on the interaction of flubendiamide and lead following oral administration in buffalo calves"** submitted by **Amita Ranjan (L-2009-V-6-D)** to the Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, in partial fulfillment of the requirements for the degree of **Ph.D.**, in the subject of **Veterinary Pharmacology and Toxicology** (Minor Subject: Veterinary Biochemistry) has been approved by the Student's Advisory Committee after an oral examination on the same, in collaboration with an external examiner.

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ABSTRACT

Toxic effects of flubendiamide, lead and their interaction were studied in buffalo calves. Flubendiamide administration (@ 0.024 mg/kg/day for 90 days) produced clinical signs including dullness, depression and poor weight gain. ALT, AST, BUN and AKLP increased significantly. Haematological parameters and blood mineral profile did not differ significantly. Increase in LPO, catalase and GST and decrease in SOD, catalase, GSH, GPx, GR, G6PD and TAA were observed, suggesting compromise in antioxidant defense. ALAD activity in blood declined marginally, but GFAP and aspartic acid (AA) in CSF increased. T_3 and T_4 declined, but TSH did not differ significantly. In lead acetate (9.2 mg/kg/day for 90 days) exposed calves ALT, AST, AKLP, GGTP, BUN and creatinine increased, but Hb and PCV decreased significantly. Significant increase in LPO and decline in antioxidant enzymes activities were noted. Significant decline in Ca, Fe, Zn and Cu and ALAD activity in blood were observed. In CSF, GFAP increased, while AA did not change significantly. T_3 and T_4 decreased, but TSH increased. Animals exposed to both flubendiamide and Pb revealed changes in biochemical profile similar to Pb exposed group. Haematological and oxidative stress parameters also revealed similar pattern of change. In CSF, GFAP were lower, while AA was higher. T_3 and T_4 were higher, but TSH was lower than Pb alone exposed animals. Present study revealed hepatotoxic, nephrotoxic and neurotoxic potential of Pb and flubendiamide, possibly associated with oxidative stress. Flubendiamide and Pb had additive effects on changes in biochemical and oxidative stress parameters.

Keywords: Buffalo- calves, Flubendiamide, Interaction, Lead, Toxicity

Signature of Major Advisor **Signature of the Student**

ABBREVIATIONS USED

AA	:	Aspartic acid
Ach	:	Acetylcholine
ACP	:	Acid phosphatase
AKLP	:	Alkaline phosphatase
ALAD	:	Delta- aminolevulinic acid dehydratase
ALT	:	Alanine aminotransferase
ANOVA	:	One way of analysis of variance
APVMA	:	Australian Pesticide and Veterinary Medicines Authority
AST	:	Aspartate aminotransferase
ATSDR	:	Agency for Toxic Substances and Disease Registry
b. wt.	:	Body weight
BUN	:	Blood urea nitrogen
Ca	:	Calcium
CAT	:	Catalase
Cd	:	Cadmium
CDNB	:	1-chloro-2,4 dinitrobenzene
CK	:	Creatine kinase
CNS	:	Central Nervous System
Conc.	:	Concentration
Cu	:	Copper
dl	:	Deciliter
DLC	:	Differential leukocyte count
DTNB	:	5-5’-dithiobis-(2-nitrobenzoic acid)
EAA	:	Excitatory Amino Acid
EDTA	:	Ethylenediamine tetra aceticacid
FAO	:	Food and Agricultural Organization
Fe	:	Iron
Fig.	:	Figure
fl	:	Femtolitre(s)
GADVASU	:	Guru Angad Dev Veterinary and Animal Sciences University
G6PD	:	Glucose 6 phosphate dehydrogenase
GFAP	:	Glial fibrillary acidic protein
GGT	:	Gamma-glutamyltranspeptidase

GPx	:	Glutathione peroxidase
GSSG	:	Oxidized glutathione
GSH	:	Glutathione
GST	:	Glutathione-s-transferase
H_2O_2	:	Hydrogen peroxide
Hb	:	Hemoglobin
HPLC	:	High performance liquid chromatography
hr	:	Hour
Ht	:	Hematocrit
INSA	:	Indian National Science Academy
IU	:	International units
LD_{50}	:	Lethal dose-50
LDH	:	Lactate dehydrogenase
LPO	:	Lipid peroxidase
MCH	:	Mean corpuscular haemoglobin
MCHC	:	Mean corpuscular haemoglobin concentration
MCV	:	Mean corpuscular volume
MDA	:	Malondialdehyde
mM	:	millimolar
Mn	:	Manganese
NADPH	:	Nicrotinamide adenine dinucleotide phosphate reduced form
ng	:	nanogram
nM	:	nanometer
NOEL	:	No observed effect level
NRC	:	National Research Council
O. D.	:	Optical density
Pb	:	Lead
P	:	Phosphorus
PCV	:	Packed cell volume
po	:	Per os
ppm	:	parts per million
RBC	:	Red blood cell
RyR	:	Ryanodine receptor
SE	:	Standard error

SGOT	:	Serum glutamate oxaloacetate transaminase
SGPT	:	Serum glutamate pyruvate transaminase
SPSS	:	Statistical Package for the social science
SOD	:	Superoxide dismutase
TAA	:	Total antioxidant activity
TBA	:	Thiobarbituric acid
TBARS	:	Thiobarbituric acid reactive substances
T_3	:	Triiodothyronine
T_4	:	Thyroxine
TEC	:	Total erythrocyte count
TLC	:	Total leucocyte count
TPP	:	Total plasma proteins
TSH	:	Thyroid Stimulating Hormone
USEPA	:	United States Environmental Protection Agency
WHO	:	World Health Organisation
Zn	:	Zinc
µg	:	Microgram
µl	:	Microliter
^{0}C	:	Degree Celsius
@	:	At the rate of
%	:	Per cent

LIST OF TABLES

LIST OF FIGURES

20. Comparative effect of chronic oral administration of flubendiamide, lead and their combination on monocyte percentage

21. Comparative effect of chronic oral administration of flubendiamide, lead and their combination on eosinophil percentage

22. Comparative effect of chronic oral administration of flubendiamide, lead and their combination basophil percentage

23. Comparative effect of chronic oral administration of flubendiamide, lead and their combination on lipid peroxides (LPO) level

24. Comparative effect of chronic oral administration of flubendiamide, lead and their combination on superoxide dismutase (SOD) activity

25 a. Blood zinc concentration and SOD activity in control animals

25 b. Blood zinc concentration and SOD activity in flubendiamide exposed animals

25 c. Blood zinc concentration and SOD activity in lead exposed animals

25 d. Blood zinc concentration and SOD activity in lead and flubendiamide exposed animals

26 a. Blood copper concentration and SOD activity in control animals

26 b. Blood copper concentration and SOD activity in flubendiamide exposed animals

26 c. Blood copper concentration and SOD activity in lead exposed animals

26 d. Blood copper concentration and SOD activity in lead and flubendiamide exposed animals

27 a. Blood manganese concentration and SOD activity in control animals

27 b. Blood manganese concentration and SOD activity in flubendiamide exposed animals

27 c. Blood manganese concentration and SOD activity in lead exposed animals

27 d. Blood manganese concentration and SOD activity in lead and flubendiamide exposed animals

28. Comparative effect of chronic oral administration of flubendiamide, lead and their combination on catalase activity

29. Comparative effect of chronic oral administration of flubendiamide, lead and their combination on glutathione (GSH) concentration

30. Comparative effect of chronic oral administration of flubendiamide, lead and their combination on glutathione peroxidase (GPx) activity

31. Comparative effect of chronic oral administration of flubendiamide, lead and their combination on glutathione reductase (GR) activity

32. Comparative effect of chronic oral administration of flubendiamide, lead and their combination on glutathione-s-transferase (GST) activity

33. Comparative effect of chronic oral administration of flubendiamide, lead and their combination on glucose-6-phosphate dehydrogenase (G6PD) activity

34. Comparative effect of chronic oral administration of flubendiamide, lead and their combination on total antioxidant activity (TAA)

35. Comparative effect of chronic oral administration of flubendiamide, lead and their combination on calcium concentration in blood

36. Comparative effect of chronic oral administration of flubendiamide, lead and their combination on phosphorus concentrations in blood

37. Comparative effect of chronic oral administration of flubendiamide, lead and their combination on iron concentrations in blood

38. Comparative effect of chronic oral administration of flubendiamide, lead and their combination on zinc concentrations in blood

39. Comparative effect of chronic oral administration of flubendiamide, lead and their combination copper concentrations in blood

40. Comparative effect of chronic oral administration of flubendiamide, lead and their combination on manganese concentration in blood

41. Comparative effect of chronic oral administration of flubendiamide, lead and their combination on lead concentrations in blood

42. Comparative effect of chronic oral administration of flubendiamide, lead and their combination on ALAD activity in blood

43 a. Blood lead concentration and ALAD activity in lead exposed calves

43 b. Blood lead concentration and ALAD activity in combined lead and flubendiamide exposed calves

44. Comparative effect of chronic oral administration of flubendiamide, lead and their combination on glial fibrillary acidic protein (GFAP) concentration in CSF

45 a. Lead and GFAP concentration in lead exposed calves

45 b. Lead and GFAP concentration in combined lead and flubendiamide exposed calves

46. Comparative effect of chronic oral administration of flubendiamide, lead and their combination on aspartic acid concentration in CSF

47 a. Lead and aspartic acid concentration in lead exposed calves

47 b. Lead and aspartic acid concentration in combined lead and flubendiamide exposed calves

48. Comparative effect of chronic oral administration of flubendiamide, lead and their combination on T_3 level in serum

49. Comparative effect of chronic oral administration of flubendiamide, lead and their combination on T_4 level in serum

50. Comparative effect of chronic oral administration of flubendiamide, lead and their combination on TSH level in serum

51 Cerebellum of lead exposed calf showing mild neuronal degeneration and purkinje cell degeneration in the molecular layer. H&E x 2000

52 Cerebrum of lead exposed calf showing marked vacuolar degeneration and neuronal degeneration (Inset: high power view). H&E x 2000

53 Liver of lead exposed calf showing swollen and degenerated hepatocytes with congested sinusoids. H&E x 2000

54 Lung of lead exposed calf showing haemorrhage and plenty of polymorphonuclear cells. H&E x 2000

55 Lung of lead exposed calf showing severe pneumonia. Note the presence of plenty of inflammatory exudates and polymorphonuclear cells. H&E x 2000

56 Spleen of lead exposed calf showing mild lymphoid depletion. H&E x 2000

57 Cerebellum of combined lead and flubendiamide exposed calf showing purkinje cell degeneration (Arrow). H&E x 2000

58 Cerebrum of combined lead and flubendiamide exposed calf showing mild neuronal degeneration and congested blood vessels. H&E x 2000

59 Cerebrum of flubendiamide exposed calf showing severe vacuolar degeneration, oedema and neuronal degeneration. H&E x 2000

60 Kidney of combined lead and flubendiamide exposed calf showing severe tubular degeneration, sloughing of epithelial cells of the proximal convoluted tubules and presence of proteinaceous casts in their lumen. H&E x 2000

61 Liver of combined lead and flubendiamide exposed calf showing severely swollen and degenerated hepatocytes and fatty infiltration. Note the absence of normal hepatocytic chord pattern. H&E x 2000

62 Heart of flubendiamide exposed calf showing myocardial degeneration. H&E x 2000

63 Kidney of flubendiamide exposed calf showing severe tubular degeneration. Note the occluded lumen of proximal convoluted tubules with proteinaceous casts and sloughed off cells H&E x 2000

64 Lung of flubendiamide exposed calf showing oedema and polymorphonuclear cells suggestive of pneumonia. H&E x 2000

CONTENTS

CHAPTER -I

INTRODUCTION

Use of pesticides and fertilizers in agriculture are essential for sustaining and increasing crop production to fulfill the demand of food by increasing human population. The consumption of pesticides in India has increased several folds in the last few decades. Punjab is among the top ranking states in terms of pesticide consumption. Nevertheless, an overall consumption of pesticides is expected to increase in near future (Abhilash and Singh 2009).

Though insecticides have been designed to selectively kill insect pests, they are not very selective in action. They are potentially hazardous to non-target species including man and animals and can induce acute or chronic toxicity. Domestic animals are often raised in the same premises where various crops are being cultivated and processed; hence they are highly vulnerable to pesticides exposure. Some pesticides have tendency to accumulate in the body and therefore can produce chronic as well as cumulative toxic effects. High levels of pesticide residues in blood, milk and other body fluids of human and animals have been reported from various states of India including Punjab (Kalra *et al* 1994; Mathur *et al* 2005). High pesticide burden in body is responsible for increase in incidence of nervous disorders, infertility, endocrine disruption and other health problems in man and animals (Flemmin *et al* 1994; Dich and Zahm 1997). These problems may aggravate in future with increase in pesticide consumption.

Development of resistance in pests against commonly used pesticides is another emerging problem of global significance. Many pesticides that were quite effective at the time of introduction have now become less effective or ineffective. To overcome this problem, scientists across the globe are trying to develop new pesticides with novel mode of action. Flubendiamide

belongs to a new class of insecticides phthalic acid diamides. It activates ryanodine receptors of insects leading to massive intracellular release of calcium ions. It has potent and broad spectrum activity against the insect order Lepidoptera and is expected to be a suitable agent for controlling these pests as a part of the insect resistance management (Tohnishi *et al* 2005; Lahm *et al* 2009). It does not activate mammalian ryanodine receptors and is thought very safe for non-target species. However, its mammalian toxicity has not been investigated much so far.

Increase in level of toxic/heavy metals in the environment is another health hazard prevalent in many countries. Various anthropogenic activities, such as burning of fossil fuel, mining and metallurgy, industries and transport sectors redistribute toxic heavy metals into the environment. They persist for a considerably long period and are translocated to different components of the environment including biotic segment. Heavy metal pollution and toxicity in animals has become more serious during the last two centuries due to rapid industrial expansion (I N S A 2011; Swarup and Dwivedi 2002). Lead is considered as one of the most hazardous and cumulative type of environmental pollutants that affect all biological systems through exposure via air, water and food sources (Patra and Swarup 2000). Lead poisoning is also known as plumbism or saturnism and its clinical symptoms that vary with dose and duration of exposure includes nervous signs, diarrhea, weight loss, anemia and death. Lead is accumulated in different body parts especially in bones, liver, kidney and brain. Lead toxicity is associated with renal damage, hypertension, male reproductive and neurological disorders, cognitive dysfunction and many blood biochemical and cerebrospinal fluid changes (Todd *et al* 1996). Reports of high lead concentration in feed, fodder, mineral-mixture and drinking water available for animals have raised the concern over the lead toxicity in animals (Bharathidhasani *et al* 2008; Dey *et al* 1996). High lead levels have been reported in blood and milk of animals reared in the vicinity of

industrial units (Swarup *et al* 2005). Increased body lead burden could be a contributing factor for poor health and production which is often reported in road- side pasture grazing cows (Dey *et al* 1999). Studies have revealed that in cattle and buffaloes, concentrations of lead and cadmium in maternal blood, umbilical cord blood, amniotic fluid and placenta were higher in cases of fetal death and abortion (Kaur 1989). It is believed that the toxic metals can damage the placenta and thus cause foetal death and abortion.

Mechanism of lead toxicity is not fully elucidated. Several mechanisms have been put forward which include ability of the Pb^{2+} to mimic and/ or inhibit the action of calcium (Ca^{2+}), activation of protein kinase, aberrant gene expression of glial fibrillary acidic protein (GFAP), reduced transthyretin production and increased synthesis of cytokines (TNF- α, IL- a, IL- 6) and gamma amino butyric acid (GABA) transaminase (Zheng *et al* 1996; Liu *et al* 2000 and Chen and Chen 2002). Among its many toxic effects, lead may adversely affect the pituitary-thyroid axis through an unknown mechanism. Perhaps, impaired uptake of iodine by the thyroid is an important mechanism of thyrotoxic effect of lead (Siegel *et al* 1989). However, perusal of available reports on occupational studies revealed conflicting results, suggesting varied response on thyroid function with dose and duration of lead exposure and age of the individual.

Oxidative stress supervenes whenever production of reactive oxygen species and free radicals occurs beyond the intrinsic capacity of cells to neutralize them (Santra *et al* 2000). Reactive oxygen species and free radicals in excess can damage cellular components like cell membrane, lipids, proteins and nucleic acids (Halliwell and Gutteridge 1999). Of late, oxidative stress has been implicated to play a role, at least in part, in pathogenesis of many heavy metal toxicities including lead, cadmium, mercury, arsenic and nickel (Valko *et al* 2005; Flora 2011). Beneficial effects of antioxidant supplementation in heavy metal toxicities further consolidate

this hypothesis (Vij *et al* 1998; Patra *et al* 2001; Kumar *et al* 2009). Moreover, developmental neurotoxicity of many pesticides and heavy metals have been found to be mediated by oxidative stress (Slotkin and Seidler 2009).

Aspartate is an excitatory neurotransmitter which is known to be toxic beyond physiological concentration (Chen *et al* 1991). Its level increases during inflammatory and degenerative conditions of brain (Stover *et al* 1997).

Glial fibrillary acidic proteins (GFAP) are synthesized in astroglial cells in all parts of the brain. In CSF, its level increases during structural changes in brain. Astrocytes are believed to serve as Pb^{+2} sink (Tiffany- Castiglioni and Qian 2001). In lead toxicity GFAP level increases in CSF, though the degree of change with dose of exposure and type of toxicity (Gong *et al* 1995).

Under natural conditions, man and animals are often exposed to more than one toxicant or pollutant. Toxic potential of one toxicant may increase due to simultaneous exposure of another toxicant. For example, exposure to a combination of chlorpyrifos (an organophosphorus pesticide) and lead is considered to be more dangerous than to an exposure of either alone (Krishna and Ramachandran 2009). Mixtures of heavy metals and pesticides present in the environment may elicit toxicity due to additive or synergistic effects among the constituents or the adverse outcome may be reduced by antagonistic interactions (Dondero *et al* 2011). Therefore, it is speculated that simultaneous exposure to flubendiamide and lead may alter the toxic effects of each other.

Buffalo is an important dairy animal with estimated population of 91 million in India. Asia accounts for one-third of the world's milk production, with India the largest regional producer at 103 million tons. Of this quantity, more than half (57 million tons) is from buffaloes. India accounts for two-thirds of the world’s entire production of buffalo milk (FAO 2009).

Keeping in view the above facts, the present study was designed in buffalo calves with the following objectives:

1. To study clinical, haematological, biochemical effects, plasma mineral profile and antioxidant status in chronic oral flubendiamide toxicity.
2. To study the chronic oral toxicity of lead and its effects on hematology and biochemical profile, plasma mineral and antioxidant status.
3. To evaluate the impact of flubendiamide-lead interaction on hematology and biochemical profile, plasma mineral and antioxidant status of buffalo calves.
4. To study the changes in the levels of aspartic acid (excitatory neurotransmitter) and glial fibrillary acidic protein level in cerebrospinal fluid during chronic oral lead, flubendiamide and their combined toxicity.
5. To study correlation between blood lead level and δ-amino-levulinic acid dehydratase activity.

CHAPTER -II

REVIEW OF LITERATURE

The use of insecticides in agriculture has grown tremendously in the last century. In India, pesticide consumption has increased by more than seventeen times since 1955, Punjab being one of the highest users. Though the state has only 1.5% landmass of the country, it accounts for about 17% of total pesticides consumed in India. The per hectare pesticide use is highest in Punjab (923g/ hectare) as compared to other agarian states like Haryana, Andhra Pradesh, Tamil Nadu, Karnataka and Gujarat (Tiwana *et al* 2007). The widespread and indiscriminate use of pesticides has resulted in high pesticide residues in human beings posing a serious health hazard across the state (Kalra *et al* 1994). Many pesticides are neurotoxicants and not very selective in their action, hence considered as a health hazard for non-target organisms. Their accidental exposure may result into acute poisoning and even low level exposure is responsible for many insidious diseases in man and animals. Moreover, the widespread use of these chemicals has triggered a rapid evolutionary response in many target species resulting into development of pesticide resistance. This has become a serious threat to agriculture productivity worldwide. Therefore, quest for new insecticides to replace old ineffective chemicals continues. Recently, a spate of new classes of insecticides have entered agricultural eve including neonicotinoids, fiprols, phenoxopyrazoles, spinosads etc. which have comparatively lesser mammalian toxicity.

Ancient Romans, Egyptians and Chinese used chemicals like sulphur, arsenic, mercury and lime in agricultural practices and animal husbandry to control invertebrates, vertebrates and microorganisms that continuously threatened the availability of food and fiber as well as posing a threat to human and animal health (Higley *et al* 1992). This was followed by use of botanical

products like Chrysanthemum and tobacco plants for control of undesirable insect-pests. Chlorinated hydrocarbons were the first major class of synthetic organic chemicals to become widely used as first generation insecticides. Majority of insecticides used till recently came from four classical groups of organochlorines, organophosphates, carbamates and pyrethyroids.

Flubendiamide

Phthalic acid diamides have emerged as one of the most promising new classes of insecticides due to their excellent insecticidal efficacy and high margins of mammalian safety. Chlorantraniliprole and flubendiamide are first two insecticides of this class. Flubendiamide is highly effective against a broad range of pests in the order Lepidoptera (Tohnishi *et al* 2005). Flubendiamide first acquired registration in year 2006 in Philippines. By 2008, it got registration in 10-15 countries including United States of America and Australia for use in fruit tree, grape, cotton, vegetables (Brassica, Cucurbits, fruiting and leafy), corn, tobacco and tree nuts (Lahm *et al* 2009). It is marketed with different trade names including Belt®, Fame™, Tenos™ and Synapse®. Flubendiamide shows a fast-acting efficacy and an excellent residual effect under greenhouse and field conditions. Its good rain and photo stability and the positive temperature coefficient suggest that it will be durable to the factors reducing the efficacy in the field (APVMA 2009). It does not exert toxic effects to beneficial arthropods at dose rates from 100 to 400 mg/ l suggesting its compatibility with Integrated Pest Management Programs (Ebbinghaus-Kintscher *et al* 2007).

Chemistry

Flubendiamide has a unique chemical structure (Fig. 1) that results from three parts with novel substituents; a heptafluoroisopropyl group in the anilide moiety, a sulfonylalkyl group in the aliphatic amide moiety, and an iodine atom at the 3-position of the phthalic acid moiety

(Tohnishi *et al* 2005). The lead compound of flubendiamide was discovered in a herbicide research program on pyrazinedicarboxamides. Although the insecticidal activity was low, the compound was attractive enough to continue the investigation because of its structural novelty and intriguing insecticidal symptoms. Introduction of unusual substituents, such as heptafluoroisopropyl and methylsulfonylalkyl groups into the aniline and alkylamine moieties respectively enhanced the insecticidal activity significantly and finally led to development of flubendiamide (Tsubata *et al* 2007). The identity, physical and chemical properties of flubendiamide is given in table 1.

Mechanism of action

Flubendiamide stabilizes insect ryanodine receptors (RyRs) to an open state, evoking massive calcium release from intracellular stores and hence continuous muscle contraction and death (Ebbinghaus- Kintscher *et al* 2006). This mode of action has been shown to be highly specific to insect RyRs and not to affect mammalian RyRs. Oral LD_{50} in rats is estimated to be more than 2000 mg/ kg body weight (APVMA 2009). Its no observed effect level (NOEL) in rats is 1 mg/ kg /day. Continuous intake of flubendiamide @ 0.01 mg/kg /day for one year is associated with hepatotoxicity and microcytic anaemia. However, manifestations of its chronic toxicity in other mammalian species are not known.

The ryanodine receptor (RyR) is composed of four identical subunits to form a non-voltage- gated calcium channel that regulates the release of calcium from intracellular stores. These channels coupled with various accessory proteins are localized in the sarcoplasmic reticulum of muscle and endoplasmic reticulum of non-muscle cells. Insects possess a single form of the RyR that are present on neurons, muscle and possibly other tissues. Therefore, the activity of flubendiamide on insect neuronal RyRs would be similar to that of insect muscle

receptors. Primary insecticidal action of flubendiamide is due to disruption of proper muscle function rather than effect on neuronal RyR (Ebbinghaus-Kintscher *et al* 2007). The application of flubendiamide results in gradual contraction, thickening and shortening of the insect body and rapid cessation of feeding without convulsions (Tohnishi *et al* 2005).

Unlike insects, mammals possess three isoforms of the RyR. RyR1 and RyR2 are predominately localized in skeletal and cardiac muscles respectively, whereas the more heterogeneously distributed RyR3 can be found in brain and smooth muscles. Flubendiamide is having almost no effect on all three different ryanodine receptor subtypes from vertebrates, which may be the possible reason behind the favourable mammalian profile of this compound (Ebbinghaus-Kintscher *et al* 2007).

Metabolism

Flubendiamide is moderately well absorbed by the oral route (greater than 20 per cent). Its dermal absorption is less than 2 per cent in monkeys. It is poorly metabolized and excreted predominately (about 93-99%) as unchanged parent compound through biliary excretion. It is lipophilic, hence accumulates in the fat. Highest tissue concentrations were found in the liver followed by intestines, kidneys and lungs.

Mammalian toxicity

There is paucity of data on mammalian toxicity of flubendiamide. The knowledge about its toxic effects in mammals is limited and based on some studies related to toxicokinetics and acute, short-term, sub-chronic and chronic studies in rats, dogs, fish and birds, (USEPA 2007; APVMA 2009). Flubendiamide is reported to have thyrotoxic effects in rats. Blood is also a target organ of toxicity, causing microcytic anemia in rats. Reproductive toxicity includes delay in perputial separation and enlarged eyeballs in offspring. However, in preliminary

investigations, it was found to have no genotoxic, carcinogenic and neurotoxic effects in mammals (APVMA 2009).

Flubendiamide and oxidative stress

Several studies have provided evidence that generation of excess free radicals and oxidative stress play an important role in pathogenesis of pesticide toxicity (Drechsel and Patel 2008, Symonds *et al* 2008). Oxidative stress and excitotoxicity are suspected to play important roles in neurotoxic effects of several organophosphate compounds (Slotkin and Seidler 2009). Cypermethrin, another widely used synthetic pyrethyroid insecticide is reported to cause neurotoxicity and oxidative stress (Shashikumar and Rajini 2010). However, there is no report available on the effect of flubendiamide on oxidative stress indices after its natural or experimental exposure in man and animals.

Lead

Lead is known to human kind for the last more than 7000 years. The atomic symbol for lead is Pb, which is derived from the Latin *plumbum*. It is a member of group IVB of the Periodic table of elements with atomic number 82, relative atomic mass 207.19 and specific gravity 11.34. It has four naturally occurring isotopes (208, 206, 207 and 204 in order of abundance). Lead occurs naturally in bedrock, soils, tills, sediments, surface waters, ground waters, and sea water (Reimann and de Caritat 1998).

Physical chemistry of lead

Lead is a bluish to silvery-grey metal that melts at 372^0 C and boils at 1744^0 C. It is soft, pliable and has no characteristic taste or smell; but pure metal is easily tarnished by an oxide film. Pb can exist in three forms: metallic lead, inorganic lead and lead compounds (or lead salts), and organic lead (containing carbon). Two oxidation states (Pb (II) and Pb (IV) are stable,

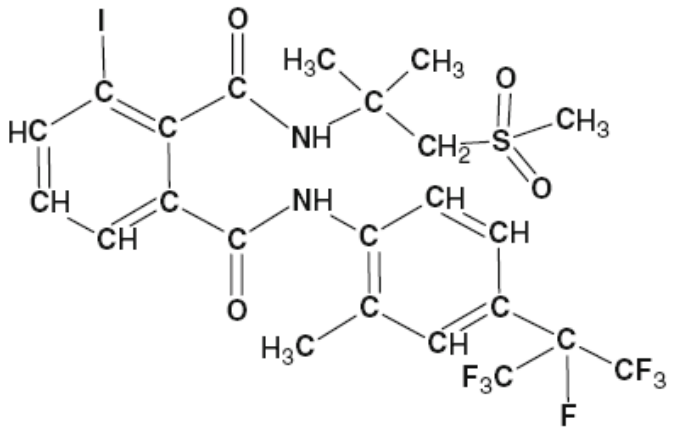

Fig 1. Chemical structure of flubendiamide (Gopal and Mishra 2008)

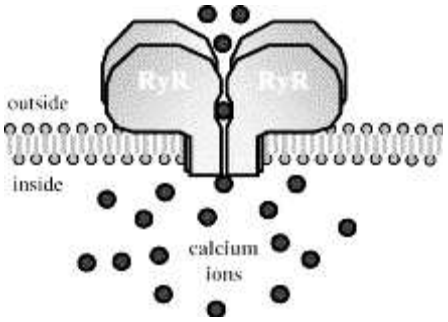

Fig 2. Structure of ryanodine receptor which is a tetramer consisting four identical subunits (Lahm *et al* 2009)

Table 1. Identity, physical and chemical properties of flubendiamide (APVMA 2009)

Common name	**Flubendiamide**
IUPAC Name	3-iodo-N'-(2-mesyl-1,1-dimethylethyl)-N-{4-[1,2,2,2-tetrafluoro-1-(trifluormethyl)ethyl- o-tolyl }]phthalamide
CAS Name	N^2-[1,1-dimethyl-2-(methylsulfony)ethyl]-3-iodo-N^1-[2-methyl-4-[1,2,2,2-tetrafluoro-1-(trifluoromethyl) ethyl]phenyl]-1-2-benzenedicarboxamide
Molecular formula	$C_{23}H_{22}F_7IN_2O_4S$
Molecular weight	682.4 g/mol
Colour	White crystalline powder (99.1%)
Physical state	Solid
Odour	No characteristic odour
Melting point	217.5- 220.70^{0}C (99.6% pure)
Boiling point	Not feasible due to thermal degradation
Relative Density @ 20^{0}C	1.659
Water Solubility (pH 4-10)	29.9±2.87 µg/L at pH 5.98 Solubility is not pH dependent in the range pH 4-10.
Solubility in Organic Solvents 20^{0}C	Methanol: 26.0 g/L Acetone: 102g/L n-heptane: 0.0008 g/L Ethyl acetate: 29.4 g/L 1,2-Dichloroethane: 8.12 g/L
Vapour Pressure @20^{0}C	<10 $^{-4}$ Pa (at 200^{0}C)
Partition Co-efficient octanol/water)@ 19^{0}C	log P_{ow} = 4.13±0.02 at pH 4 log P_{ow} = 4.2±0.02 at pH 6 log P_{ow} = 4.14±0.02 at pH 7 log P_{ow} = 4.11±0.04 at pH 9

but the plumbous ion (Pb^{+2}) is the dominant form of inorganic lead in the environment. Lead sulfide, lead oxide and most other inorganic salts of lead are poorly soluble in water with exception of nitrate, chlorate and chloride salts. Organic salts have variable solubility with lead oxalate being insoluble and acetate being highly soluble.

The most common ore is galena (PbS) which often contains appreciable concentrations of silver and zinc that adds to its economic value. Mixed lead and zinc ores account for about 70 per cent of total lead supplies from mining. Anglesite ($PbSO_4$), cerussite ($PbCO_3$) and lanarkite ($PbO.PbSO_4$) are some other ores of lead (Mahaffey *et al* 2000).

Lead usage and consumption

Lead, one of the seven metals known in ancient times, has been mined and used by man since at least the 4th millennium BC (Drasch 1982). Its use increased during Roman times to such an extent that it became a health hazard. Scientific evidences derived from carbon dating of certain samples recovered from Rajpura-Dariba mines indicated that Indians smelted lead and zinc nearly 2100-2500 years back. Widespread availability and many desirable properties like easy smelting, resistance to corrosion, alloy forming with other metals, high malleability and ductile nature were reasons for large-scale use for this metal for preparation of utensils, weapons, equipment etc. since early civilization. In addition, it was also used by ancient physicians including Charaka, Hippocrates, Galen and Dioscorides for treatment of various diseases. The Swiss physician and alchemist Paracelsus (1493-1541) discovered that Pb compounds can cure fistulas and cancer (Swarup and Dwivedi 2002).

Currently, production of lead-acid batteries is the dominant use of lead, accounting for about 83 per cent of reported lead consumption in year 2002 (Smith 2002). Other military and industrial use includes manufacturing of ammunition, pipes, cable covering, lead-sheet (used to

shield from radiation), solder and gasoline additives. Besides these various compounds, lead is also used for different purposes. For example, lead nitrate is used as a mordant in dyeing, textile printing, photographic sensitizer and manufacture of explosives and matches. Lead acetate is used in antifouling paints, hair dyes, dying industry, varnishes and manufacture of pesticides. Lead oxide is used in storage batteries, colouring rubber and manufacture of paints, inks, glass, ointments and plasters (Johnson 1998). Tetraethyl lead and tetramethyl lead (organolead compounds) are used as gasoline additives to increase the octane rating. However, they are not currently important industrial products and in India, use of leaded gasoline has been banned with effect from February 2000.

Australia, China, United States, Peru and Mexico are major lead producing countries. The estimated global use of refined lead in year 2010 was 9.11 million tons (Anonymous 2010). In terms of use, lead ranks fourth among the non-ferroly materials in India. Yet, the per capita intake of Pb in India (0.07 kg) is comparatively lower than many countries like USA (5.14 kg), West Germany (5.12 kg), China (0.219 kg) and Republic of Korea (1.08 kg). The lead consumption in India from different sources includes batteries (42%), Cables (22%), Paints (10%), Sheets/ Pipes (10%), Industrial alloys (8%), Miscellaneous (8%) (Kothari 1997).

Lead absorption and metabolism

Gastrointestinal absorption of lead occurs primarily in the duodenum. The efficiency of Pb absorption is influenced by a variety of factors including chemical form of lead, the level of other dietary constituents and the age and physiological state of the animal. Absorption increases with increase in solubility of lead compound, during pregnancy, lactation or deficiency of iron or calcium. Young animals absorb lead considerably more efficiently than older animals. Lead in

form of particulates can be absorbed by inhalation, but dermal absorption of inorganic lead is minimal.

Following absorption, lead enters the blood where more than 90 per cent is taken up by red blood cells (Cake *et al* 1996). Most of the lead in red blood cells is bound to lead-binding proteins, mainly ALAD and haemoglobin rather than erythrocyte membrane (Bergdahl *et al* 1996; Bergdahl *et al* 1997). Lead in plasma binds to albumin and γ-globulins and complexes with low molecular weight sulfhydryl compounds. Thereafter, lead enters into peripheral tissues where it binds predominantly to cytologic lead-binding proteins. Over time, lead redistributes from soft tissues to bones where it forms highly stable complexes with phosphate replacing calcium in hydroxyapatite. Transplacental transfer of lead to fetus also occurs if lead burden in mother is high (Goyer 1980).

Metabolism of inorganic lead consists primarily of reversible ligand reactions including the formation of complexes and thiols with free amino acids and proteins. Organolead compounds are actively metabolized in the liver by oxidative dealkylation catalyzed by cytochrome P-450. The half life for lead in blood and other soft tissues of adult human is about 1 month, but it is much longer for lead in bone (USEPA 2007).

Biological role of lead

Lead is not an essential nutrient for man and animals and does not participate in any known beneficial biochemical functions. However, few studies document beneficial effects of lead supplementation in rats and pigs in terms of improved growth rates and lipid metabolism (Reichlmayr-Lais and Kirchgessner 1981; Mazliah *et al* 1989; Kirchgessner *et al* 1991) and improved egg production in chicken (Mazliah *et al* 1989). The importance of lead in biology and medicine is mainly for its toxic action that occurs even at low doses of exposure.

Lead toxicity

Humankind have enjoyed usefulness as well as endured harmful and sometimes, devastating effects of lead since early civilization (Johnson 1998). The toxic effects of lead were recognized and recorded as early as 2000 BC. Hippocrates was probably the first to indicate lead as the cause of colic.

Lead toxicity has been reported from many countries as a cause of endemic chronic plumbism or saturnism in man (Bogden *et al* 1997) and animals (Swarup and Patra 2005). With industrial expansion in the last two centuries, the problem has become more serious as evident from the Antarctic and Arctic ice core data showing presence of lead in such far off places. Importance of Pb toxicity can be recognized by the fact that it remains the most widely investigated heavy metal toxicant. Reports suggest decline in Pb toxicity problem in developed countries due to implementation of strict environmental and occupational regulations (Howson *et al* 1996). On the contrary, it continues to pose serious health hazard in developing countries (Romieu *et al* 1997; Krishnaswamy and Kumar 1998).

Contamination of pasture from industrial emissions and other sources such as discarded batteries, empty paint tins and machinery grease are the major sources of lead exposure to domestic and wild mammals (Chowdhury and Naha 2002). Grazing animals suffer from plumbism by ingestion of contaminated herbage and soils. The toxicity is associated with high mortality in animals in polluted environment with no or few long term premonitory signs of depression, head pressing, violent movement, blindness and salivation (Dwivedi *et al* 2001). Lead toxicosis in buffaloes has been reported to occur naturally in cattle in Punjab reared around factories recycling lead from old batteries (Kwatra *et al* 1986; Sidhu *et al* 2006). Use of untreated sewage effluents for irrigation purposes is responsible for high concentration of heavy

metals including Pb, Cr, Cd and Ni in soil and vegetables grown in industrial areas of Ludhiana, Punjab (Brar *et al* 2000; Brar *et al* 2002). Kawatra and Bakhetia (2008) reported high Pb intake through food by women living in sewage irrigated areas around Ludhiana, Punjab.

Lead exposure could give rise to an array of toxic manifestations. The spectrum of toxic response ranges from acute or chronic toxicity to subclinical or subtle effects depending upon the dose, route and duration of exposure. Lead adversely affects the nervous, haematopoietic, gastrointestinal, immunological, cardiovascular, immune, renal, endocrine and reproductive systems in man and animals (Patrick 2006). It is estimated that health effects from environmental lead exposure account for 1 per cent of the global burden of disease, placing it in 16th position in terms of leading global health risk factors (Fewtrell *et al* 2004). Bone acts as a natural sink for long term sub-lethal lead exposure and account for about 95 per cent of all lead in the human body in adults and about 70 per cent in children (Todd *et al* 1996; Howard *et al* 1998). Proposed mechanisms for lead toxicity include its ability to interact with proteins and change their functions, inhibit or mimic the action of calcium, replace zinc as cofactor in enzymes and cause oxidative stress (NRC 2005).

Lead toxicity is associated with renal damage, hypertension, male reproductive and neurological disorders, cognitive dysfunction and alterations in many blood biochemical and cerebrospinal fluid changes (Todd *et al* 1996).

Plasma biochemistry

Administration of lead nitrate at the rate of 600 mg/ kg body weight orally once daily for a period of 90 days in rats has been reported to cause significant decrease in serum total protein, albumin and alkaline phosphatase, with significant increase in glucose, urea, creatinine, aspartate aminotransferase, alanine aminotransferase, bilirubin and cholesterol levels (Chandra *et al* 2010).

Brar et al (2000) observed significant increase in plasma levels of aspartate aminotransferase (70%), alanine aminotransferase (114.3%), gamma-glutamyltransferase (139.4%), lactate dehydrogenase (60.4%), alkaline phosphatase (71.9%), acid phosphatase (62.5%) and creatine kinase (226.7%) after single oral administration of lead acetate at the rate of 600 mg/ kg in buffalo calves. Rahman *et al* (2008) recorded decrease in total protein, albumin and globulin in broiler chicks given commercial diet along with 250 or 400 ppm lead acetate in drinking water.

Hamadouche-N *et al* (2009) found that exposure of lead acetate to rats resulted in a significant increase in blood lead concentration, ALP, cholesterol and a significant decrease in albumin concentration.

El-Hameed *et al* (2008) reported that after oral administration of lead acetate in pregnant Baladi goats, level of total protein, albumin and globulin decreased, while GGT, aminotransfereases activities and potassium, urea and creatinine concentrations in blood serum increased.

In rabbits, given lead acetate at the rate of 0.6 mg/ kg body weight in the drinking water for 3 weeks, significant increase in ALT, AST, urea and creatinine concentrations were recorded by Elayat and Bakheetf (2010) indicating hepatotoxic and nephrotoxic effects of lead.

Miranda *et al* (2006) did not find any significant change in serum markers of hepatic and renal functions in heifers that survived an accidental lead poisoning episode.

Haematology

Administration of lead nitrate at the rate of 600 mg/ kg body weight orally, once daily for a period of 90 days in rats has been reported to cause significant decrease in hemoglobin concentration (Hb), packed cell volume (PCV), total erythrocyte count (TEC), total leukocyte count (TLC), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean

corpuscular hemoglobin concentration (MCHC) (Chandra *et al* 2010). Almost similar changes in haematological picture were also observed by Sharma and Pandey (2010) in male albino mice following intraperitoneal administration of lead nitrate at the rate of 5 mg/ kg body weight once daily. In a study, Rahman *et al* (2008) observed decrease in Hb, PCV, TEC, MCV, MCH, MCHC, TLC, TLC and absolute lymphocyte count in broiler chicks given commercial diet with 250 or 400 ppm lead acetate in drinking water.

Longer *et al* (1984) reported decrease in PCV in calves after *ad libitum* feeding of a diet containing 500 and 1500 ppm lead as lead sulfate. However, he could not appreciate any change in Hb concentration in these animals.

Krishna and Ramchandaran (2009) observed decrease in RBC, Hb and PCV values in rats given lead acetate at the rate of 1000 mg Pb/ kg for 14 days.

In a study, Sujatha *et al* (2006) reported significant decrease in TEC, Hb and PCV levels in buffalo calves after administration of lead acetate for two months. They opined that it may be due to decreased life span of red blood corpuscles consequent to increase in osmotic fragility of erythrocytes. They further observed that TEC, Hb and PCV decrease with increase in blood Pb concentration.

Antioxidant status

There are growing evidences suggesting role of oxidative stress in pathogenesis of heavy metal toxicities particularly cadmium, mercury, arsenic, nickel and lead (Valko *et al* 2005; Flora 2011). Excess lead exposure produces diverse biochemical, physiological and behavioural alterations. Although several mechanism are involved in pathogenesis of lead toxicity, oxidative damage to lipids, nucleic acids and proteins also contribute, at least partly, to Pb-induced cellular and subcellular changes in various target organs (Hermes-Lima *et al* 1991; Patra *et al* 2011).

Lead exposure results into increase in lipid peroxidation and reduction in blood superoxide dismutase activity (Skoczynska and Smolik 1994; Shafiq 1984). Erythrocytes are highly susceptible to oxidative damage due to the high cell concentration of oxygen and hemoglobin, a powerful promoter of the oxidative process.

Exposure to lead through drinking water for a period of 10 days altered the levels of phospholipids and lipid peroxides in rats and the rate of lipid peroxidation increased in all brain regions in lead exposed rats (Shafiq 1984).

Ito *et al* (1985) found higher lipid peroxide (LPO) level in lead-exposed rats as well as in occupational workers exposed to lead.

Patra *et al* (2001) reported significantly higher lipid peroxides level in liver, kidney and brain in lead-exposed rats. The experimental studies on lead exposure in calves revealed an increase in lipid peroxides level and decrease in total, protein bound and non protein bound thiol content in erythrocytes (Patra and Swarup 2000).

Lead exposure in 14 days old chick embryos resulted in significant alteration in glutathione, glutathione peroxidase, catalase and superoxide dismutase activities in brain, liver and kidneys (Somashekaraiah *et al* 1992).

Sugawara *et al* (1991) reported a significant increase in lipid peroxidation (LPO) in erythrocyte and glutathione in lead-exposed workers.

Ercal *et al* (1997) recorded depletion of glutathione level, increase in oxidized glutathione (GSSG) and malonaldehyde (MDA) production in both liver and brain samples following five weeks of lead-exposure in mice. Lead-exposed goats had a significantly lower glutathion peroxidase (GPX), superoxide dismutase (SOD), catalase and total thiol content in erythrocytes than control animals (Moussa *et al* 2002).

Lead inhibits ALAD (delta-aminolevulinic acid dehydratase), leading to the accumulation of ALA (aminolevulinic acid), which is a potential endogenous source of free radicals (Bechara 1996). Aerobic oxidation of δ -aminolevulinic acid (δ -ALA) at physiological pH is responsible for production of reactive oxygen species (Costa *et al* 1997). Moreover, lead can also cause decrease in levels of free radical scavenging enzymes, such as glutathione reductase (GR), glutathione peroxidase (GPx), glutathione S-transferase (GST), superoxide dismutase (SOD) and catalase (CAT) (Chiba *et al* 1996; Hsu and Guo 2002; Olaleye *et al* 2007). The latter is mainly attributed to the high affinity of Pb for sulfhydryl groups or metal cofactors in these enzymes and molecules. Lead can also interfere with some essential metals needed for antioxidant enzymes activity (Flora *et al* 2003) and can interact directly with biological membranes causing oxidative damage and inducing lipid peroxidation (Villeda-Hernandez *et al* 2001). Various antioxidant enzymes including GPx, CAT and SOD require various essential trace elements for their synthesis and activity (Halliwell and Gutteridge 1999). Pb may inhibit their activity by acting as a competitive antagonist for these trace elements. Lead-associated reduction in selenium uptake may increase the susceptibility of cells to oxidative stress by affecting GPx activity (Schrauzer 1987). On the other hand, selenium supplementation before Pb exposure increased activity of SOD and glutathione reductase (GR) and glutathione (GSH) concentration in rats (Othman and Missiry 1998).

Several research reports documenting beneficial effects of antioxidant supplementation in terms of oxidative stress indices along with other parameters further substantiate the role of oxidative stress in pathogenesis of lead toxicity (Antonio-Gracia and Masso-Gonzalez 2008; Sharma *et al* 2010). Challa *et al* (2011) reported cytoprotective and antioxidant role of epigallocatechin 3-gallate (a tea polyphenol) against lead-induced toxicity in human

neuroblastoma cells. However, supplementation of antioxidants does not appear to have any significant effect on Pb accumulation in different tissues (Patra *et al* 2001).

Minerals in blood

Interrelationship among iron, zinc and lead and between lead and calcium are well established (Miller *et al* 1990). Lower levels of zinc and copper were found in rats that were fed added lead in their diet (Miller *et al* 1990; Bebe and Panemangalore 1996). In humans, the amount of zinc and iron chelated by dicalcium EDTA treatment is also influenced by the level of lead in the blood (Chisolm 1980). Interaction of zinc, lead and iron with identical proteins, possibly transport proteins that mediate metal uptake in intestine, seems the possible reason behind this interaction. Lead can competitively replace zinc in the catalytically active site of enzymes, such as ALAD and metallothionein (Simons, 1995; Warren *et al* 1998).

Calcium and phosphorus in the diet reduces lead absorption from the gastrointestinal tract (Fullmer 1991; Varnai *et al* 2001). Verster (2011) reported that oral co-administration of calcium carbonate at the rate of 50 mg/ kg and zinc sulfate at the rate of 0.2 mg/ kg body weight resulted in decrease in Pb absorption in cattle given lead acetate at the dose rate of 2 mg/ kg body weight.

Lead exerts it toxic effect by disruption of calcium-dependent mechanism (Pounds 1984). Diets deficient in calcium resulted in elevated levels of lead in children and experimental animals (Ziegler *et al* 1978; Cooper *et al* 1984). The possible reason behind this observation may be blockade of lead uptake through intestine by calcium ions (Miller *et al* 1990; Fullmer 1992), since lead is a potent blocker of calcium channels in excitable tissues (Bernal *et al* 1997). Lead and calcium compete for the same binding site on a group of ion binding proteins including calmodulin, S-100, calretinin, calbindin and parvalbumin (Pauls *et al* 1996). Pb forms an

extremely stable complex with potassium and is able to replace calcium in the primary crystalline matrix of bone, the calciumphosphate salt, hydroxyapatite (Swarup and Dwivedi 2002).

El-Hameed *et al* (2008) observed decrease in serum calcium and zinc concentration in pregnant Baladi goats following oral administration of lead acetate for a period of 14 weeks. Hamadouche *et al* (2009) found that exposure of lead acetate to albino rats results in a significant decrease in serum levels of calcium, magnesium and ascorbic acid.

Delta Aminolevulinic acid dehydratase (ALAD)

δ-aminolevulinic acid dehydratase (ALAD) is an essential enzyme in the biosynthetic pathway of heme and is required to maintain hemoglobin content in erythrocytes. Its level increases during increased erythropoiesis and iron deficiency and decreases in lead poisoning (Granick *et al* 1972). Inhibition of red blood cell ALAD has been accepted as a standard bioassay to detect acute and chronic lead exposure in humans (Hernberg *et al* 1970), other mammals (Mouw *et al* 1975) and birds (Ohi *et al* 1974; Dieter and Finley 1979).

When lead enters the blood stream, it is primarily taken up by the erythrocytes with usually less than one per cent of the lead remaining in the plasma. The accumulation of lead in erythrocytes has been ascribed to its affinity to lead-binding proteins (LBP) present inside erythrocytes. Previously, hemoglobin was considered as the major lead-binding protein, but later on ALAD was identified as the major LBP inside erythrocytes (Bergdahl *et al* 1996; Bergdahl *et al* 1997). Zinc acts as a competitive antagonist of lead for binding with ALAD (Border *et al* 1976).

Neurological effects of lead

Lead, a systemic toxicant affects virtually every organ system. However, brain in general and developing brain in particular is highly susceptible to its toxic effects. In USA, environmental lead exposures are thought to account for about 13 per cent of all cases of mild mental retardation (MMR) in children (Fewtrell *et al* 2004).

Lead crosses the blood-brain barrier rapidly and concentrates in the brain. The mechanism of neurotoxicity is complex and still not fully understood. Several hypotheses have been proposed to explain the mechanism of neurotoxic effects of lead compounds. The direct neurotoxic actions of lead include apoptosis (programmed cell death), excitotoxicity affecting neurotransmitter storage and release and altering neurotransmitter receptor, mitochondria, second messengers, cerebrovascular endothelial cells, and both astroglia and oligodendroglia (Garza *et al* 2006). Recent findings suggest that both Ca^{+2} dependent proteins and neurotransmitters receptors represent significant targets for Pb^{+2}. In particular, acute and chronic exposure to lead predominantly affects two specific protein complexes: protein kinase C and the N-methyl-D-aspartate subtype of glutamate receptor (Bressler *et al* 1999). These protein complexes are deeply involved in learning and cognitive functions are also thought to interact significantly with each other to mediate these functions (Marchetti 2003). Symptoms can appear immediately after exposure or may be delayed and include loss of memory, vision, cognitive and behavioral problems and brain damage/ mental retardation.

Glial fibrillay acidic proteins (GFAP)

GFAP are synthesized in astroglial cells in all parts of the central nervous system. Only low levels of GFAP are present in serum and hence structural damage to the brain causes a selective leakage of the GFAP into the cerebrospinal fluid irrespective of the blood-brain barrier dysfunction (Aurell *et al* 1991). High levels of the GFAP in the CSF has been observed in association with acute CNS injury (Aurell *et al* 1991), disintegration of astrogliosis (Rosengren

et al 1994) and CNS vasculitis (Nylen *et al* 2002). The GFAP is the structural subunit of the astroglial filaments, which are mainly found in the fibrillary astrocytes. Lead accumulates in brain tissues where its concentration does not fall rapidly even if the blood level of Pb^{2+} declines (Dyatlov *et al* 1998). In mature brain astroglial cells are believed to serve as Pb^{2+} sink (Tiffany-Castiglion and Qian 2001).

In a study, Gong et al (1995) observed that GFAP levels in brain show a biphasic response with decrease in level on day 7 and increase on day 14 following daily administration of 8 ppm trimethyl lead to rats in drinking water. They concluded that GFAP acts as sensitive indicator of role of astrocytes in lead-induced neurotoxicity. Struzynska *et al* (2007) observed that maximum increase in GFAP expression occurred in forebrain cortex, followed by hippocampus and cerebellum in rats after daily intraperitoneal administration of lead acetate @ 15 mg/kg body weight. They concluded that lead exposure in immature rat brain is associated with chronic glial activation with coexisting inflammatory and neurodegenerative features with variable extent in different parts of the brain.

Aspartic acid

Aspartate is an amino acid acting as a neurotransmitter. It is primarily localized to ventral spinal cord where it opens an ion-channel and is inactivated by reabsorption into the pre-synaptic membrane. Aspartate is an excitatory neurotransmitter which increases the likelihood of depolarization in the postsynaptic membrane. Aspartate and glycine form an excitatory/ inhibitory pair in the ventral spinal cord comparable to the excitatory/ inhibitory pair formed by glutamate and GABA in the brain. Both glutamate and aspartate are known to be neurotoxic at supraphysiologic concentration (Meldrum 1993; Chen *et al* 1991). Excessive increase in extracellualar glutamate and aspartate have been demonstrated after ischemia, hypoxia, head

injuries and prolonged seizures (Meldrum 1993; Hong *et al* 2001). Glutamate and aspartate levels are almost doubled in viral meningitis, acute multiple sclerosis and myelopathy (Stover *et al* 1997). They are believed to mediate excitotoxicity by acting as agonists at the NMDA receptors leading to cellular oedema and accumulation of intracellular Ca^{2+} and Na^{+} which leads to lethal and sublethal excitotoxic effects (Bullock and Fujisawa 1992). Aspartate level in CSF of patients with infantile spasms was found significantly higher, hence it was concluded that aspartate may have a role in triggering the spasms and development of neuronal dysfunctions in patients with infantile spasms (Ince *et al* 1997). However, a non-significant decrease in CSF aspartate levels was observed in adult epileptic patients and the level further declined following administration of valproic acid, an antiepileptic drug (Araki *et al* 1988).

Thyroid status

Among its many toxic effects, lead may adversely affect the pituitary-thyroid axis through an unknown mechanism. Perhaps, impaired uptake of iodine by the thyroid is an important mechanism of thyrotoxic effect of lead (Siegel *et al* 1989). However, perusal of available reports on occupational studies revealed conflicting results, suggesting response on thyroid function varies with dose and duration of lead exposure and age of the individual.

In a study, Robins *et al* (1983) observed low serum total T_4 and free T_4 levels with inappropriately normal serum TSH concentration in brass-foundry workers with high lead exposure.

Dundaro *et al* (2006) observed a negative correlation between blood lead levels and free T4 with no differences in serum TSH or T_3 in male adolescent automobile mechanics following long-term, low-level lead exposure. In another study, petrol pump workers and automobile mechanics with occupational exposures to high lead levels (mean blood lead level 52 mg/ dl),

had higher serum TSH than unexposed controls, but mean T_3 and T_4 levels did not differ (Singh *et al* 2000).

In a study, Wade *et al* (2002) observed a dose-related increase in thyrotoxic effects following exposure to a complex mixture of 16 organochlorines, lead and cadmium in rats. The study revealed alterations in thyroid gland morphology, serum hormone levels and hepatic thyroid hormone metabolism leading to significant alterations in thyroid homeostasis.

By contrast, a study on lead smelter workers did not demonstrate any thyroidal effects of moderate lead exposure for up to 10 years duration (Schumacher *et al* 1998).

El-Hameed *et al* (2008) observed that levels of T3 and T4 did not show any significant alterations in pregnant Baladi goats after oral administration of lead acetate at the rate of 4.5 and 6 mg/ kg body weight for 14 weeks.

Lopez *et al* (2000) reported positive correlation between TSH and blood lead levels at lower blood lead levels, while negative correlation with T_3 and T_4 at higher blood lead levels.

On the other hand, thyrotoxicosis may cause mobilization of accumulated bone lead stores resulting into clinically significant lead poisoning (Goldman *et al* 1994).

In a study in foundry workers, serum total thyroxine and free thyroxine were found to regress negatively with blood lead level (Robins *et al* 1983).

Katti and Sathyanesan (1987) reported impaired thyroid function in *Clarias batrachus* (catfish) after exposure of 5 ppm lead nitrate for a period of 150 days. They also observed histological changes in thyroid which included hypertrophy, increased cell height, vacuolation and reduction in colloid. They further reported decrease in I^{131} uptake by thyroid in lead-intoxicated fishes.

Lead pesticide interaction

Although scientists generally have a good understanding of the toxicity of individual chemical pollutants, knowledge about interactive effects of different pollutants is limited. There is a great need to bridge the gap between our understanding of the toxic effects of exposure to individual xenobiotics and those effects from exposure to mixtures of such chemicals including heavy metals and pesticides. The animal and human population in the industrialized world is ubiquitously exposed to complex mixtures of toxicants including heavy metals, pesticides and other natural or synthetic chemicals, rather than exposure to a single toxicant. The toxicity of a toxicant or chemical can be enhanced (positive interaction or synergism), reduced (negative interaction or antagonism) or remain unaffected (no interaction) by the presence of another toxicant (Cassee *et al* 1999). Lead has been reported to have synergistic interaction with mercury (Fernandez and Beiras 2001). Likewise, simultaneous exposure to a combination of lead and chlorpyrifos is considered more dangerous than exposure of either alone, due to long lasting inhibition of cholinesterase enzymes and impaired cognitive function of brain (Krishna and Ramachandran 2009). Formation of chelating complex between lead (metal) and chlorpyrifos (OP compound) and thereby bypassing or escaping of chlorpyrifos from detoxification mechanism of liver may be the possible reason behind their synergistic action (Tomlin 1997).

In a study in male rats, Wade et al (2002) concluded that exposure to a complex mixture of 16 organochlorines, lead and cadmium can alter hypothalamic-pituitary-thyroid axis, even at low doses of exposure.

Developmental neurotoxicological effects were more pronounced in rats treated with the combination of lead and dimethoate than those given lead or dimethoate alone (Nagymajtenyi *et al* 1998).

CHAPTER-III

MATERIALS AND METHODS

Experimental animals

The present study was carried out on sixteen healthy male buffalo calves of 8 to 12 months age and body weight in between 120-180 kg. They were procured from the University dairy farm or purchased from the local market, dewormed and acclimatized for two weeks in the animal shed of the department. The animals were maintained under identical managemental practices and provided green fodder, wheat straw and water *ad libitum*. The experimental protocol followed the ethical guidelines on the proper care and use of animals and was approved by the Institutional Animal Ethics Committee (IAEC) of GADVASU vide Memo no. VPT/2009/459, dated- 19.03.2009.

Insecticide and chemicals

Flubendiamide (Fame, Bayer Cropscience Limited, Sabarkanta, Gujarat) was purchased from Bayer Crop science Limited. Lead acetate was obtained from Merck India Ltd. 5-aminolevulinic acid hydrochloride, N-ethylmaleimide, and L-aspartic acid were procured from Sigma Chemicals Company, USA. Autopak kits for biochemical parameters were purchased from Bayer (Siemens Medical Solutions Diagnostics Ltd., Baroda). ELISA kit for Glial Fibrillary Acidic Protein estimation was obtained from Genxbio (Cusabio Biotech Co., Ltd., China). All other chemicals/ reagents used in this study were of analytical grade, obtained from reputed companies.

TECHNICAL PROGRAMME OF WORK

The experiments were performed under three different sections as follows:

I. Oral chronic toxicity studies of flubendiamide in buffalo calves

II. Oral chronic toxicity studies of lead in buffalo calves

III. Oral chronic toxicity of combined exposure of lead and flubendiamide in buffalo calves.

Following parameters were monitored in all the above experiments

a. Toxic symptoms

b. Oxidative stress parameters

c. Biochemical parameters and activity of delta-aminolevulinic acid dehydratase

d. Hematological parameters

e. Plasma calcium and inorganic phosphorous concentration

f. Concentration of lead, zinc, copper, manganese and iron in whole blood

g. Plasma T_3, T_4 and TSH concentration

h. Glial fibrillary acidic protein and aspartic acid concentration in CSF

Grouping of animals

The animals were divided into four groups of four animals each (Gr. I, II, III and IV).

Table 2: Experimental schedule for studies on toxic effects of lead, flubendiamide and their interaction in buffalo calves

Group	Drug	Dose	Route	Study conducted
I (n= 4)	Untreated control	-	-	Control
II (n= 4)	Flubendiamide	0.024 mg/kg/day	Oral	Chronic toxicity of flubendiamide
III (n= 4)	Lead acetate	9.2 mg/kg/day	Oral	Chronic toxicity of lead
IV (n= 4)	Lead acetate+ Flubendiamide	9.2 mg/kg/day + 0.024 mg/kg/day	Oral	Chronic toxicity of lead and flubendiamide combined exposure

Treatment

Group I animals did not receive any treatment and served as healthy control. Group II animals received sublethal dose of flubendiamide @ 0.024 mg/ kg/ day orally for a period of 90 days. Group III animals were given lead acetate @ 9.2 mg/ kg/ day orally, while group IV received both flubendiamide (@0.024 mg/ kg/ day) and lead acetate (@9.2 mg / kg/ day orally for 90 days. The requisite amount of flubendiamide and lead acetate were suspended in 50 ml of water and drenched to animals between 9.00 to 10.00 a.m. daily. All the animals were weighed weekly and doses of flubendiamide and lead acetate were adjusted according to changes in their body weight.

1. Clinical signs of toxicity

Buffalo calves administered with lead acetate, flubendiamide or lead acetate and flubendiamide combination were closely observed for appearance of clinical signs of toxicity, if any. The nature, degree and time of occurrence of various toxic symptoms were recorded during the experimental period.

Collection and processing of samples

Blood samples

Blood samples from experimental animals were collected by jugular venipuncture on 0, 30, 60, 90 days of treatment and day 30 post-treatment. Samples collected in heparinized vials were used for estimation of oxidative stress and biochemical parameters. For estimation of various hematological parameters blood samples were collected using disodium EDTA as anticoagulant. Plasma was separated from heparinized blood by centrifugation at 2300 g for 15 min and stored at -20^{0}C till analysis. The sediment (packed erythrocytes) left after separating plasma was washed thrice with normal saline solution. Thereafter, 10% hemolysate was prepared

by mixing 0.5 ml packed erythrocytes with 4.5 ml distilled water and used for estimation of antioxidant enzymes like superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), glutathione-*S*-transferase (GST) and Glucose-6-phosphate dehydrogenase (G6PD). Lipid peroxidase was estimated in 10% hemolysate prepared by mixing 0.5 ml packed erythrocytes with 4.5 ml lipid peroxide buffer. Glutathione was estimated in heparinized whole blood. Total antioxidant activity (AOA) was estimated in plasma.

Cerebrospinal Fluid (CSF)

Animal was restrained in lateral recumbancy and skin of the dorsal midline over the junction of the sixth lumber (L6) and first sacral (S1) vertebrae was surgically prepared. Sterile spinal needle (18 G, 4 inch long) was inserted between the dorsal spinous process of L6 cranially and S1 caudally and the two tuber sacrals laterally after anaesthetizing the site by administration of 2% lignocaine. The needle was inserted perpendicularly and advanced until the tip punctured the lumbosacral cistern and CSF starts coming. CSF obtained was collected in clean sterile 5 ml appendoff tubes. CSF was centrifuged at 2300 g for 10 min and the supernatant obtained was collected and stored at -80^{0}C till analysis.

2. Biochemical Parameters

(i) Aspartate aminotransferase (AST/ SGOT)

Aspartate aminotransferase (SGOT) level in blood was measured with Bayer Autopack kit on Photometer 5010 (Nicholas Piramal) by the method of International Federation of Clinical Chemistry and Laboratory Medicine (1976). AST catalyzes the transfer of amino group between L-aspartate and α-ketoglutarate to form oxaloacetate and glutamate. The oxaloacetate formed reacts with NADH in presence of malate dehydrogenase to form NAD. The rate of oxidation of NADH to NAD is measured as decrease in absorbance which is proportional to the AST activity in the sample. AST activity was expressed as U.L^{-1}.

$$\text{L-Aspartate} + \alpha\text{- Ketoglutarate} \xrightarrow{\text{AST}} \text{Oxaloacetate} + \text{L- Glutamate}$$

$$\text{Oxaloacetate} + \text{NADH} + H^{+} \xrightarrow{\text{MDH}} \text{L-Malate}$$

AST = Aspartate aminotransferase

MDH = Malate dehydrogenase

(ii) Alanine aminotransferase (ALT/ SGPT)

Alanine aminotransferase (SGPT) level in blood was measured with Bayer Autopack kit on Photometer 5010 (Nicholas Piramal) by the method of International Federation of Clinical Chemistry and Laboratory Medicine (1976). ALT catalyzes the transfer of amino group between L-alanine and α-ketoglutarate to form pyruvate and glutamate. The pyruvate formed reacts with NADH in presence of lactate dehydrogenase to form NAD. The rate of oxidation of NADH to NAD is measured as decrease in absorbance which is proportional to the ALT activity in sample. ALT activity was expressed as $U.L^{-1}$.

$$\text{L-Alanine} + \alpha\text{- ketoglutarate} \xrightleftharpoons{\text{ALT}} \text{Pyruvate} + \text{L- Glutamate}$$

$$\text{Pyruvate} + \text{NADH} + H^{+} \xrightleftharpoons{\text{LDH}} \text{L-Lactate} + NAD^{+}$$

ALT = Alanine aminotransferase

LDH = Lactate dehydrogenase

(iii) Alkaline phosphatase (ALKP)

Alkaline phosphatase activity was estimated by PNPP method using Bayer Autopack kits on Photometer 5010 (Nicholas Piramal) as described by Klin and Klin (1972). Alkaline phosphatase hydrolyses p-nitrophenyl phosphate (PNPP) into p-nitrophenol and phosphate. At the alkaline pH of the buffer medium, p-nitrophenol is yellow. The colour developed by

hydrolysis is measured at 405 nm and is proportional to the alkaline phosphatase activity. Alkaline phosphatase activity is expressed in U/ l.

$$\text{p-Nitrophenyl phosphate} + H_2O \xrightarrow{\text{ALKP}} \text{p-Nitrophenol} + \text{Phosphate}$$

(iv) Gamma-glutamyl transpeptidase (GGT)

Activity of gamma-glutamyl transpeptidase was estimated by kinetic method using Bayer Autopak kits on Photometer 5010 (Nicholas Piramal) as described by Szasz (1976). GGT catalyzes the transfer of the gamma-glutamyl group from the substrate gamma-glutamyl para-nitranilide to glycylglycine releasing free p-nitroaniline which absorbs light at 405 nm. Enzyme activity is proportional to the increase in absorbance at this wavelength.

$$\text{GPNA} + \text{Glycylglycine} \xrightarrow{\gamma\text{-GT}} \text{L-}\gamma\text{-Glutamyl- Glycylglycine} + \text{p-Nitroaniline}$$

GPNA = L-γ-Glutamyl-p-Nitroani

(v) Blood urea nitrogen (BUN)

Blood urea nitrogen was estimated by UV method using Bayer Autopack kits on Photometer 5010 (Nicholas Piramal) as per the method of Talke and Schubert (1965). Urea is hydrolyzed in the presence of water and urease to produce ammonia and carbon dioxide. The ammonia produced combines with α-ketoglutarate and NADH in the presence of glutamate dehydrogenase to yield glutamate and NAD. The amount of urea nitrogen was calculated by measuring the absorbance decrease per minute relative to urea nitrogen standard at 340 nm. Blood urea nitrogen concentration is expressed as mg/ dl.

(vi) Creatinine

Creatinine concentration was estimated by Picrate method using Bayer Autopack kits on Photometer 5010 (Nicholas Piramal) as described by Henry and Winkelman (1974). Creatinine

in alkaline solution reacts with picrate to form a red-orange compound. Under specific conditions of the assay, the rate of development of the colour is proportional to the concentration of creatinine in the sample when it is measured at 500 nm. Creatinine concentration is expressed as mg/ dl.

3. Haematological parameters

Parameters	**Units**
Hemoglobin concentration (Hb)	G %
Packed cell volume (PCV)	%
Total erythrocyte count (TEC)	X 10^6/ mm^3
Mean corpuscular volume (MCV)	fl
Mean corpuscular haemoglobin concentration (MCHC)	g/ dl
Mean corpuscular haemoglobin (MCH)	pg/ dl
Total leucocyte count (TLC)	X 10^3/ mm^3
Differential leucocyte count (DLC)	%

Following hematological parameters were analyzed by the method of Benjamin (1985)

The erythrocyte indices

(a) Mean Corpuscular Volume (MCV) expresses the average volume of individual erythrocyte and was calculated from the formula:

$$\text{MCV (fl)} = \frac{\text{Packed cell volume (\%)}}{\text{Total erythrocyte count (million/ µl)}} \text{ X } 10$$

(b) Mean Corpuscular Haemoglobin Concentration (MCHC) is the concentration of haemoglobin in the average erythrocyte or ratio of weight of haemoglobin to the volume in which it is contained and was calculated from the formula:

$$\text{MCHC (g/ dl)} = \frac{\text{Haemoglobin (g/ dl)}}{\text{Packed cell volume (\%)}} \times 100$$

(c) Mean Corpuscular Haemoglobin (MCH) is the amount of haemoglobin by the average erythrocyte and was calculated from the formula:

$$\text{MCH (pg/dl)} = \frac{\text{Haemoglobin (g/ 100 ml)}}{\text{Total erythrocyte count (million/ µl)}} \times 10$$

4. Antioxidant status

a. Lipid peroxides

Lipid peroxides in erythrocyte lysate was assayed by method of Stocks and Dormandy (1971). The method is based on the principle that the reaction of malondialdehyde (MDA), an end product of lipid peroxidation, with thiobarbituric acid (TBA) yielded a pink coloured trimethine complex exhibiting an absorption maximum at 532 nm.

Reagents

Phosphate buffered saline (0.1 M, pH 7.4)

40 mM sodium azide

28 per cent trichloroacetic acid (TCA)

1 per cent thiobarbituric acid (TBA)

40 mM hydrogen peroxide (H_2O_2)

Procedure

To 2 ml of 10 percent erythrocyte lysate, 1 ml of 40 mM H_2O_2 and 0.1 ml sodium azide were added in the test tube and incubated at 37^0 C for 1 h. After incubation the total volume was made to 4 ml with phosphate buffer saline in each tube and 2 ml of ice chilled TCA was added to stop the reaction. The tubes were centrifuged at 3000 g for 15 min. To 4 ml of supernatant, 1 ml of TBA was added and tubes were kept in boiling water bath for 15 min. Finally, the optical

density was measured at 532 nm against a blank (no H_2O_2 was added) after cooling the contents of tubes to room temperature. The values were expressed as nmol MDA produced/ g Hb/ h using a molar extinction coefficient of pure MDA as 1.56 X 10^5 (Esterbauer *et al* 1982).

b. Superoxide Dismutase

The activity of superoxide dismutase (SOD) in erythrocyte lysate was determined by the method of Marklund and Marklund (1974).

Reagents

Pyrogallol, 0.6 mM: 76 mg of pyrogallol in 100 ml of water, stored in brown bottle, solution was prepared fresh every hour.

EDTA, 6mM: 223 mg EDTA disodium salt in 100 ml distilled water.

Tris-HCl buffer, 100 mM: 1.21 g Tris in 80 ml of distilled water. pH adjusted to 8.2 with 10 mM HCl and volume made to 100 ml.

Procedure

In a cuvette, 1.5 ml of 100 mM Tris-HCl buffer, 0.5 ml of 6 mM EDTA and 1 ml of 0.6 mM pyrogallol solution were added. The rate of auto-oxidation of pyrogallol was taken from the increase in absorbance at 420 nm, every min after a lag of 30 sec up to 4 min. For the test, appropriate amount of enzyme was added to inhibit the auto-oxidation of pyrogallol to about 50 per cent. A unit of enzyme activity is defined as the amount of enzyme causing 50 per cent inhibition of the auto-oxidation of pyrogallol observed in blank.

c. Catalase

The activity of catalase in erythrocyte lysate was determined according to the method described by Aebi (1983).

Reagents

Phosphate buffer, 50 mM, pH 7.0

Hydrogen peroxide, 30 mM: 0.34 ml of 30% H_2O_2 was diluted with buffer. The optical density of diluted H_2O_2 at 240 nm should be around 1.5. Buffered H_2O_2 solution was prepared fresh.

Procedure

To 2 ml of phosphate buffer in quartz cuvette, added 20 µl of erythrocyte lysate and mixed well. The reaction was started by the addition of 1 ml of 30 mM H_2O_2 and the decrease in absorbance was recorded at every 10 sec interval for 1 min at 240 nm in a U.V. spectrophotometer. The results were expressed as µmol H_2O_2 decomposed per min per mg Hb using 36 as molar extinction coefficient of H_2O_2.

d. Glutathione

Glutathione (GSH) concentration in whole blood was estimated by the method of Beutler *et al* (1989).

Reagents

Precipitating solution: 1.67 g glacial metaphosphoric acid, 0.2 g disodium salt of EDTA and 30 g sodium chloride per 100 ml of distilled water. This solution is stable for approximately 3 weeks at 4^0C. A fine precipitate, which may form, probably consists of EDTA and does not interfere.

Phosphate solution (0.3M $Na_2HPO_4.2H_2O$): 13.35 g disodium hydrogen phosphate dihydrate in 250 ml of distilled water.

DTNB reagent: 40 mg of 5-5'-dithiobis-(2-nitrobenzoic acid) in 100 ml of 1per cent sodium citrate. The DTNB reagent is stable for at least 3 months at 4^0 C.

Standard GSH solution: 60 mg of reduced glutathione was dissolved in 100 ml distilled water. The solution is stable for 4 weeks at 4^0 C.

Procedure

To 0.2 ml of whole blood, 1.8 ml of distilled water was added to prepare hemolysate. 3 ml of the precipitating solution was added to the hemolysate. The mixture was allowed to stand for approximately 5 min and then centrifuged. To 2 ml of the supernatant, 8 ml of phosphate solution was added, followed by 1 ml of DTNB reagent and mixed well. A blank was prepared with 8 ml of phosphate solution, 2 ml of diluted precipitating solution (3:2 in distilled water) and 1 ml of DTNB reagent. The absorbance was recorded at 412 nm. A standard curve was prepared using different concentrations of glutathione in distilled water.

e. Glutathione Peroxidase

The activity of glutathione peroxidase in erythrocyte lysate was assayed by the method of Hafeman *et al* (1974).

Reagents

Glutathione, 20 mM: 9.2 mg GSH dissolved in 15 ml of distilled water.

Sodium phosphate buffer, 0.4 M, pH 7, containing 0.4 mM EDTA.

Sodium azide, 0.01 M: 65 mg sodium azide in 100 ml of distilled water.

Hydrogen peroxide, 1.2 mM: 0.012 ml 30% H_2O_2 in 100 ml of distilled water.

Disodium hydrogen phosphate (Na_2HPO_4), 0.4 M: 7.12 g Na_2HPO_4 in 100 ml distilled water.

M-phosphoric acid precipitation solution: 1.67 g m-phosphoric acid, 0.2 g EDTA and 30 g NaCl in 100 ml of distilled water.

DTNB reagent: 40 mg 5-5'-dithiobis (2-nitrobenzoic acid) in 100 ml of 1% trisodium citrate solution.

Procedure

To 0.1 ml erythrocyte lysate, 1 ml of glutathione 20 mM, 1 ml of phosphate buffer and 0.5 ml of sodium azide were added and volume was made to 4 ml with distilled water. After pre-incubation for five minutes, 1 ml of H_2O_2 (pre-warmed to 37^0C) was added. 1 ml aliquots of incubation mixture were removed after 1 min interval and added to 4 ml m-phosphoric acid precipitation solution. GSH in protein free filtrate was determined by mixing 2 ml of filtrate with 2 ml of Na_2HPO_4 and 1 ml of DTNB reagent and optical density was recorded at 412 nm within two minutes after mixing. The zero time GSH concentration was determined in the same manner using an aliquot from a sample treated similarly but containing water in place of H_2O_2.

Activity of GPx = 10 log Co/ C

Co = concentration of GSH at zero time.

C = concentration of GSH after one min incubation.

f. Glutathione reductase

The assay of glutathione reductase (GR) was performed according to the method described by Carlberg and Mannervik (1985).

Reagents

Potassium phosphate buffer, 0.2 M, pH 7.0, containing 2 mM EDTA.

NADPH, 2 mM in 10 mM Tris-HCl, pH 7.0

GSSG, 20 mM in water: 62 mg of oxidized glutathione in 5 ml of distilled water

Procedure

To a 3 ml cuvette, 2.6 ml phosphate buffer, 0.15 ml NADPH and 0.15 ml GSSG was added. The reaction was initiated by the addition of 0.1 ml of erythrocyte lysate to the cuvette

and decrease in absorbance at 340 nm was recorded at 30 second interval. A unit of glutathione reductase activity is defined as the amount of enzyme that catalyzes the oxidation of 1 µ mole of NADPH / min using 6.22 X 10^3 as molar extinction coefficient of NADPH.

g. Glutathione-S-transferase

The activity of glutathione-S-transferase (GST) in erythrocyte lysate was determined by the method of Habig *et al* (1974).

Reagents

Potassium phosphate buffer, 0.3 M, pH 6.5

GSH, 30 mM: 46 mg of reduced glutathione was dissolved in 5 ml of distilled water.

CDNB solution, 30 mM: 30 mg of 1-chloro-2, 4 dinitrobenzene in 5 ml of 95 per cent ethyl alcohol.

Procedure

To a 3 ml cuvette, 2.8 ml phosphate buffer and 0.1 ml GSH solution were added. To this mixture 10 µl of enzyme preparation was added and mixed. Then 0.1ml of CDNB solution was added to initiate the reaction. The increase in optical density at 340 nm was recorded every minute for 3 min, after a lag of 30 sec. (the increase in absorbance should be less than 0.05/min). The extinction coefficient of CDNB conjugate at 340 nm is 9.6/ mM/ Cm. The unit of enzyme activity is defined as the amount of enzyme that catalyzes the formation of 1 µmole of conjugate of GSH and CDNB per minute per mg of Hb.

h. Glucose-6-phosphate Dehydrogenase

Glucose-6-phosphate dehydrogenase (G6PD) activity was assayed by the method of Deutsch (1978). The assay is based upon the ability of this enzyme to catalyze the conversion of Glucose-6-phosphate and $NADP^+$ to 6-phosphogluconolactone and NADPH.

Reagents

3.8 mM NADP reagent

0.5M tris buffer (pH 7.5)

0.63M magnesium chloride

33 mM glucose-6-phosphate

Procedure

To a 3 ml cuvette, 1.7 ml distilled water, 0.3 ml NADP reagent, 0.3 ml Tris buffer, 0.3 ml magnesium chloride solution and 0.3 ml glucose 6 phosphate solution were added and mixed. To this mixture 0.1 ml of 10% erythrocyte lysate was added and optical density was estimated at 340 nm every 30 seconds for 4 minutes. The absorbance was set zero by using blank solution which contained all the reagents except 10 per cent erythrocyte lysate in equal volume as in test mixture. Increase in optical density was recorded. The activity of glucose-6-phosphate dehyrogenase was calculated according to the following equation.

$$\text{G6PD activity (U/ l)} = 8095 \times \frac{\text{Change in OD}}{\text{Unit Time}} \times \text{dilution factor}$$

g. Total Antioxidant Activity (TAA)

The antioxidant activity was determined by the method of Koracevic *et al* (2001) based on the principle that a standardized solution of Fe-EDTA complex reacts with hydrogen peroxide by a Fenton-type reaction, leading to the formation of hydroxyl radicals ($^{\cdot}OH$). These reactive oxygen species degrade benzoate, resulting in the release of thio-barbituric acid reactive substances (TBARS). Antioxidants from the added sample of calf plasma cause suppression of the production of TBARS. This reaction was measured spectrophotometrically and the inhibition of colour development is defined as the total antioxidant activity.

Reagents

0.1 M Sodium phosphate buffer (pH-7.4)

10 mM Sodium benzoate

50 mM NaOH

2mM EDTA

2 mM $Fe(NH_4)_2SO_4$

Fe-EDTA by mixing 2 mM EDTA and 2 mM $Fe(NH_4)_2SO_4$ in equal proportions

10 mM H_2O_2

20% acetic acid

0.8% TBA in 50mM NaOH

1mM Uric acid in 5mM NaOH

Last six solutions were prepared immediately before use. Phosphate buffer and sodium benzoate were stored in refrigerator (0-4 ^{0}C).

Procedure

Each sample (A_1) had its own control (A_0) in which the Fe-EDTA mixture and H_2O_2 was added after 20 per cent acetic acid. For each series of analysis, a negative control (K_1 and K_0) was prepared in duplicate, containing the same reagents as A_1 and A_0, except that plasma was replaced with phosphate buffer. Standards containing 1 mmol/ l uric acid (UA_1 and UA_0) were used for calibration**.**

Calculation: TAA (m mol/ l) = $C_{UA} \times \{K-A\}/ \{K-U_A\}$

Where: K= Absorbance of control (K_1-K_0)

C_{UA}= Concentration of uric acid (in m mol/ l)

A= Absorbance of sample (A_1-A_0)

UA= Absorbance of uric acid solution (UA_1-UA_0)

Protocol for measurement of total antioxidant activity

Reagent (µl)	**A1**	**A0**	**K1**	**K0**	**UA1**	**UA0**
Serum	10	10	-	-	-	-
Uric acid	-	-	-	-	10	10
TBARS buffer	490	490	500	500	490	490
Sodium benzoate	500	500	500	500	500	500
Acetic acid	-	1000	-	1000	-	1000
Iron EDTA	200	200	200	200	200	200
H_2O_2	200	200	200	200	200	200
Incubated at 37^0C for 60 minutes						
Acetic acid	1000	-	1000	-	1000	-
TBA	1000	1000	1000	1000	1000	-
Incubated for 10 minutes in boiling water bath. Cooled in an ice bath. Absorbance was measured at 532 nm against deionized water.						

5. Plasma calcium and inorganic phosphorous concentration

(a) Calcium

Calcium concentration was estimated by UV method by using Bayer Autopack kits on Photometer 5010 (Nicholas Piramal) as described by Baginski *et al* (1973). Calcium, in an alkaline medium, reacts with O-Cresolphthalein Complex-one to form an intense chromophore which absorbs light at 575 nm. Magnesium and iron are excluded from the reaction by

complexing with 8-Hydroxyquinolone. The original method of Baginski *et al* (1973) has been modified by surfactants and solvent system which minimize interference from lipemia. Calcium concentration is expressed as mg/ dl.

(b) Inorganic phosphorus

Inorganic phosphorus concentration was estimated as per the method described by Amador and Urban (1977) using Bayer Autopack inorganic phosphorus kit. The increase in formation of the unreduced phosphomolybdate complex, measured at 340 nm is directly proportional to the amount of inorganic phosphorus present. Inorganic phosphorus concentration is expressed as mg/ dl.

(c) Zinc, copper, manganese and iron concentration

The concentration of zinc, copper, manganese and iron in digested samples were measured by Atomic Absorption Spectrophotometer (Analyst 700, Perkin Elmer, USA) at suitable wavelength and lamp current using air-acetylene mixture as fuel-oxidant mixture. The concentrations of various minerals were calculated by multiplying with the corresponding dilution factor. The values were expressed in µg per ml (ppm) of blood.

(d) Lead concentration

Blood samples were wet digested as per the procedure described by Kolmer *et al* (1951). Briefly, 5 ml of blood was transferred to a 100 ml conical flask; 5 ml of concentrated nitric acid was added to it and kept overnight at room temperature. Next day, the mixture was heated below 80^0 C till the volume reduced to about 0.5 ml. Thereafter, 5 ml of double acid mixture consisting of 3 parts nitric acid and 1 part 70 per cent perchloric acid was added to each sample. The heating below 80^0 C was again continued till white fumes emanated and the volume reduced to 0.5 ml. The digested samples were cooled and diluted to 5 ml with triple glass distilled water.

Lead concentration in digested sample was estimated using Atomic Absorption Spectrophotometer (AAS, Analyst 700, Perkin Elmer, USA) equipped with a Deuterium background corrector and a graphite furnace. A wavelength of 283.3 nm and a spectral slit width of 0.7 nm were used. The values were expressed in ppb.

6. Delta-Aminolevulinic Acid Dehydratase (ALA-D)

Delta- Aminolevulinic acid dehydratase activity in erythrocytes was estimated by the method of Burch and Siegel (1971). The test is based on the principle that the enzyme catalyzes condensation of two moles of ALA to from one mole of Porphobilinogen (PBG) which forms a coloured complex with modified Ehrlich's reagent.

Reagents

Triton X-100

Buffered ALA substrate containing 0.01 mol/liter of ALA, pH 6.65

TCA Reagent containing 0.02 mol/ l of N-ethyl-maleimide

Modified Ehrlich's reagent

Procedure

In a test tube (test tube no. 1) 0.2 ml blood and 1.30 ml Titron X-100 reagent was taken. Thereafter, 1 ml of buffered ALA substrate was added and mixed properly. From the above mixture 1 ml was mixed with 1 ml of TCA reagent to serve as blank. In blank (test tube no. 2), the mixture was centrifuged at 3000 rpm for 10 minutes and 1 ml of clear supernatant was taken and used for colour reagent. The leftover content of test tube no. 1 was covered and incubated at 38^0 C for one hour after adding 1.5 ml of TCA reagent. Thereafter, the incubated mixture was centrifuged at 3000 rpm for 10 minutes and 1 ml of clear supernatant was taken in another test tube (test tube no. 3). In

test tube no. 2 and 3, 1 ml each of modified Ehrlich's reagent was added and kept at room temperature for 15 minutes for colour development. The absorbance of the colored mixture of test tube no. 3 was read within 10 minutes at 555 nm against blank, i.e. test tube no.2.

The activity of ALA-D was calculated using the formula as follows:

Units of ALAD activity = (Corrected absorbance X 100 X 12.5 X 10)/ PCV

Where 12.5 is the dilution factor of the blood.

Unit activity was defined as an increase in absorbance at 555 nm of 0.100 with a 1.0 cm light path/ ml erythrocytes/ hour at 38^0 C.

7. Glial fibrillary acidic protein (GFAP) in CSF

The quantitative determination of GFAP concentration in cerebrospinal fluid was done by microplate competitive enzyme immunoassay using standard kits of GenX-Bio (Cusabio Biotech Co., Ltd). The microtiter plates provided in this kit were pre-coated with an antibody specific to GFAP. Standards or samples were added to the appropriate microtiter plate wells with a biotin-conjugated antibody preparation specific for GFAP and Avidin conjugated to Horseradish Peroxidase (HRP) was added to each microplate well and incubated. Then a TMB (3, 3', 5, 5' tetramethyl-benzidine) substrate solution was added to each well. Only those wells that contained GFAP, biotin-conjugated antibody and enzyme-conjugated Avidin exhibited a change in color.

The enzyme-substrate reaction was terminated by the addition of sulphuric acid solution and the color change was measured spectrophotometrically at a wavelength of 450 nm ± 2 nm. The concentrations of GFAP in the samples were determined by comparing the OD of the samples with the standard curve (Fig 3).

8. Aspartic acid concentration in CSF

Aspartic acid concentration in cerebrospinal fluid (CSF) was determined using High Performance Liquid Chromatography (HPLC), Perkin Elmer (USA) as per the method of Zhang *et al* (2003) with slight modifications.

HPLC condition

HPLC system consisted of a single pump (Perkin Elmer, 200) and autosampler injector with 200 microlitre loop, fluorescence detector (Perkin Elmer, 200 series), and total chrom software® (version 6.1) for analysis. The excitation and emission wavelengths were 330 and 445 nm, respectively. Browniee Analytical Amino column (Perkin Elmer®, Particle size 5µ, and 4.6 X 150 mm; Catalog no-N9303504, USA) severed as a stationary phase. All HPLC solvents were filtered through 0.45 µm (pore size) filters and degassed. Mobile phase A was tetrahydrofuran-methanol-0.1 mol/ l sodium acetate (pH 7; 5:95:900 v/ v); mobile phase B was methanol. The gradient system was: 0 min, 0% mobile phase B, increased to 25% B at 5 min, to 35% B at 10 min, 55% B at 15 min and 95% B at 19 min, and held at 95% B until 22 min. The flow-rate was 1.0 ml/ min. The retention time of aspartic acid in CSF was 6.28 min.

Derivatization

CSF sample (stored at -80 ^{0}C, with de-proteinization using methanol immediately before assay, the methanol volume added to the CSF sample was two times that of the CSF) or calibration solution (50 µl) was taken into eppendof tube. 50 µl of derivatization reagent (OPA) and 450 µl borate buffer (0.4 mol/ l, pH 9.25) were added.

The mixture was shaken for 10 s and was centrifuged at 15,000 g for 1 min at room temperature; 20 µl of the reaction mixture were injected into the HPLC system after two minutes. The amino acid calibration solution was prepared by dissolving 2.46 µmol/ l aspartic acid in 0.5 mol/ l HCl. Derivatization reagent was prepared by dissolving 50 mg of OPA in 2.5

ml of methanol and adding 50 µl of mercaptoethanol, the mixture was kept at 4^0 C in the dark. The borate buffer contained the solution prepared by boric acid powder and distilled water, pH was then adjusted to 9.25 with NaOH. Fig. 4 shows chromatograms of aspartic acid in calibration solution (for standard curve preparation) and in CSF sample.

Preparation of standard curve:

The calibration curve for aspartic acid was constructed in the range of 0.625-2.50 µ mol/ l. The standard curve was plotted peak area against concentration of the aspartic acid. It was found linear with regression coefficient (R^2) 0.999. The limit of detection was 0.3125 µ mol/ l.

Quantification

The regression formula obtained from the calibration curve was used to quantify the concentration of aspartic acid in CSF. The limit of quantification was 0.625 µ mol/ l. The linear equation obtained was in the form of $Y=a + bX$

Where:

Y- Peak area

a- y intercept

b- Slope of the calibration curve

X- Concentration (µ mol/ l)

Precision of the method

The precision of the analytical method was determined by evaluating intra-day and inter-day variation. Intraday variation was determined by assaying three replicates of known concentration on three different occasions, at least 24 hours apart between each assay. Precision of the method was expressed as percent of co-efficient of variation (CV)/ RSD (Relative

Standard Deviation). The inter-day and intraday variations were found to be 8.2 % and 9.15 % respectively.

9. Thyroid hormones

(a) T_3 and T_4 levels

The quantitative determination of T_3 and T_4 concentration in plasma was done by microplate competitive enzyme immunoassay (Type 7) using ELISA kits of Monobind Inc., USA. The essential reagents required for an enzyme immunoassay included antibody, enzyme-antigen conjugate, native antigen and a substrate that produces colour. Upon mixing biotinylated antibody, enzyme-antigen conjugate and a serum containing the native antigen, a competition reaction resulted between the native antigen and the enzyme antigen conjugate for a limited number of antibody binding sites.

The interaction is illustrated by the following equation:

$$^{Enz}Ag + Ag + Ab_{Btn} \underset{K_{-a}}{\overset{K_a}{\leftrightarrow}} AgAb_{Btn} + {}^{Enz}AgAb_{Btn}$$

Ab_{CW} = Monospecific immobilized antibody (Constant quantity)

Ag = Native Antigen (Variable quantity)

^{Enz}Ag = Enzyme-antigen conjugate (Constant quantity)

$AgAb_{Btn}$ = Antigen-antibody complex

$^{Enz}Ag\ Ab_{Btn}$ = Enzyme-antigen conjugate -Antibody complex

K_a = Rate constant of association

K_{-a} = Rate constant of disassociation

$K = k_a / k_{-a}$ = Equilibrium constant

A simultaneous reaction between the biotin attached to the antibody and the streptavidin immobilized on the microwell occurred. This effected the separation of the antibody bound fraction after decantation or aspiration.

$AgAb_{Btn} + {}^{Enz}AgAb_{Btn} + Streptavidin_{CW} \Longrightarrow$ immobilized complex

$Streptavidin_{CW}$ = Streptavidin immobilized on well

Immobilized complex = Sandwich complex bound to the solid surface

The enzyme activity in the antibody bound fraction, measured by reaction with TMB (Tetramethyl benzidine), is inversely proportional to the native antigen concentration. By utilizing several different serum references of known antigen concentration, a dose response curve was generated from which the antigen concentration of an unknown was measured.

(b) TSH activity

Plasma TSH activity was measured by immunoenzymometric assay (TYPE 3) using ELISA kits of Monobind Inc., USA. The essential reagents required for an immunoenzymometric assay included high affinity and specificity antibodies (enzyme conjugated and immobilized) with different and distinct epitope recognition, in excess and native antigen. In this procedure, the immobilization takes place during the assay at the surface of a microplate well through the interaction of streptavidin coated in the well and exogenously added biotinylated monoclonal anti-TSH antibody. Upon mixing monoclonal biotinylated antibody, enzyme-labeled antibody and a serum containing the native antigen, a reaction resulted between the native antigen and the antibodies, without competition or steric hindrance, to form a soluble sandwich complex. The interaction is illustrated by the followed equation:

$$ {}^{Enz}Ab_{(p)} + Ag_{TSH} + {}^{Btn}Ab_{(m)} \underset{K_{-a}}{\overset{K_a}{\leftrightarrow}} {}^{Enz}Ab_{(p)} - Ag_{TSH} - {}^{Btn}Ab_{(m)} $$

$^{Btn}Ab_{(m)}$ = Biotinylated monoclonal antibody (Excess quantity)

Ag_{TSH} = Native antigen (Variable quantity)

$^{Enz}Ab_{(p)}$ = Enzyme-polyclonal antibody (Excess quantity)

K_a = Rate constant of association

K_{-a} = Rate constant of disassociation

$^{Enz}Ab_{(p)}$- Ag_{TSH}- $^{Btn}Ab_{(m)}$ = Antigen-antibody sandwich complex

Simultaneously, the complex was deposited to the well through the high affinity reaction of streptavidin and biotinylated antibody. This interaction is illustrated below:

$^{Enz}Ab_{(p)}$- Ag_{TSH}- $^{Btn}Ab_{(m)}$ + $Streptavidin_{cw}$ $\Longrightarrow$ immobilized complex

$Streptavidin_{CW}$ = Streptavidin immobilized on well

Immobilized complex = Sandwich complex bound to the solid surface

After equilibrium was attained, the antibody-bound fraction was separated from unbound antigen by decantation or aspiration. The enzyme activity in the antibody-bound fraction, measured by reaction with tetramethylbenzidine (TMB), was directly proportional to the native antigen concentration. By utilizing several different serum references of known antigen concentration, a dose response curve was generated, from which the antigen concentration in an unknown sample was estimated.

10. Histopathological examination

The tissue samples of brain, liver, kidney, lung and spleen were collected from the animals that died during the experiment. The formalin-fixed tissues were thoroughly washed in running tap water, dehydrated in ascending grades of alcohol and acetone, cleared in benzene and embedded in paraffin wax at 58^0 C. Sections of 5 micron thickness were cut from paraffin embedded tissues and stained with haematoxyline and eosin (H&E) stain (Lillie 1965). The

stained slides were examined using Research Photomicrographic Microscope system of Olympus Corporation, USA.

11. Statistical analysis

The data obtained were expressed as mean ± SE and analyzed using suitable tests (One way Analysis of Variance (ANOVA) or Student's t test) using SPSS® 16.0 software package. The significance was assessed at $P \leq 0.05$ (Singh *et al* 1991).

CHAPTER IV

RESULTS AND DISCUSSION

In the present investigation, chronic toxicity study of flubendiamide, lead and their interaction was undertaken in male buffalo calves. Effects of daily oral exposure of flubendiamide, lead and their combination for 90 consecutive days on various toxicological, haematological, biochemical and oxidative stress parameters, blood mineral profile, thyroid hormone level and aspartic acid and glial fibrillary acidic protein in cerebrospinal fluid were investigated.

I. Clinical Signs of Toxicity

A. Oral Chronic Flubendiamide Toxicity Study

The dose of flubendiamide for present study was selected on the basis of chronic reference dose as suggested by Lahm *et al* (2009) on the basis of liver toxicity across multiple species. Daily oral exposure of flubendiamide @ 0.024 mg/ kg/ day for 90 consecutive days produced mild toxicity in all four buffalo calves with signs of varying degree of dullness, depression, reduced feed consumption and lower body weight gain.

There is no published scientific report available on toxicity of flubendiamide in cattle or buffalo calves to substantiate the above findings. Available knowledge on clinical signs in flubendiamide toxicity in non-target organisms is mainly based upon unpublished reports of toxicity studies conducted on dogs wherein signs of reduced body weight gain and loose stool were observed (USEPA 2007).

B. Oral Chronic Lead Toxicity Study

Sub lethal dose of lead acetate was selected after perusal of the available literature (Patra and Swarup 2005). Daily oral exposure of lead acetate at the rate of 9.2 mg/ kg body weight for

90 consecutive days in four buffalo calves resulted into mild to moderate toxicity signs including dullness, depression, weakness and nervous signs like aimless wandering, partial vision loss, nystagmus and twitching of eyelid muscles. One calf died on day 28 after showing symptoms like muscle tremors of the face and rear legs, frothing at mouth, gnashing of teeth, bellowing, recumbency, convulsive seizures and death. Another calf died on day 92 after showing similar signs of toxicity which developed suddenly.

Similar to present findings, sudden death of some animals following clinical signs of muscle tremors, gnashing of teeth, bellowing and convulsions have been repeated in calves after lead exposure (Longer *et al* 1984; Sujatha *et al* 2006).

Acute lead poisoning is reported to be more common in young animals and is manifested predominantly as gastrointestinal and nervous signs including ataxia, blindness, salivation, spastic twitching of eyelids, jaw champing, bruxism, muscle tremors, and convulsions that develops within 24 to 48 hours after exposure (Siddiqui and Rajurkar 2008). Subacute lead poisoning, usually seen in sheep or older cattle, is characterized by anorexia, rumen stasis, colic, dullness, and transient constipation frequently followed by diarrhea, blindness, head pressing, bruxism, hyperesthesia, and incoordination (Kahn and Line 2005). Chronic lead poisoning, occasionally seen in cattle, is manifested as ill-thrift, emaciation, muscle wastage and developmental abnormalities in fetuses (Payne and Livesey 2010).

C. Oral Chronic Flubendiamide-Lead Interaction Study

Combined daily exposure of flubendiamide at the rate of 0.024 mg/ kg and lead acetate at the rate of 9.2 mg/ kg body weight once daily for 90 consecutive days resulted in mild to moderate toxicity signs and death of 2 out of 4 buffalo calves. Toxic signs were similar to those observed in lead alone treated group and included dullness, depression, weakness and nervous

signs like aimless wandering, partial vision loss, nystagmus and twitching of eyelid muscles. One calf died on day 55 after complete loss of appetite and thirst for few days. Another calf died on day 95 after rapid development of nervous sings like twitching of facial muscles, nystagmus, circling movement, recumbency, frothing at mouth and convulsive seizures. In general, intensity of toxic signs was more severe in animals receiving both flubendiamide and lead in comparison to animals receiving only lead acetate or flubendiamide. Appetite and feed consumption was also poor resulting into poor weight gain, diffused hair loss and muscle weakness in almost all animals of this group.

II. Biochemical Parameters

Plasma Aminotransferases

Changes in plasma alanine aminotransferase (ALT) and aspartate aminotransaminase (AST) in male buffalo calves receiving daily oral dose of flubendiamide, lead and their combination are presented in table 3 and figure 5 and 6.

A. Oral Chronic Flubendiamide Toxicity Study

Oral flubendiamide administration at the rate of 0.024 mg/ kg/day for 90 days resulted into significant increase (up to 59.77 per cent) in ALT activities on day 30 and 60. However, on day 90, slight decrease in ALT activity was observed. The ALT activity on day 30 post-treatment declined significantly than day 90 value. The ALT activities on different observation periods were significantly higher from corresponding values in control group, but it did not differ significantly from values observed in other treatment groups, except on day 90.

In comparison to day 0, plasma AST activity declined by 21.82 % on day 30, but increased significantly on day 60 and 90. On day 90, AST activity was 21.22 % higher than day 0 value. Slight non-significant decrease from day 90 AST activity was observed on day 30 post-

treatment. AST activities on different observation periods were significantly lower than corresponding values observed in group III and IV.

There is no published scientific report available to compare the results of the present study. However, the findings were in agreement with Kuwahara (2003) who observed perturbations in clinical chemistry and increased liver weight in male and female dogs after administration of flubendiamide @ 53-60 mg/ kg body weight/ day for 90 days. Likewise, in another study, he observed increased liver weights and plasma enzymes suggesting hepatotoxic effects of flubendiamide in dogs when given flubendiamide @ 35-38 mg/ kg/ day for a period up to one year (Kuwahara 2004).

Contrary to the present findings, significant decrease in ALT and AST activity was noted in rats after 2000 ppm flubendiamide administration in diet for four weeks (APVMA 2009).

B. Oral Chronic Lead Toxicity Study

Daily oral exposure of lead acetate @ 9.2 mg/ kg body weight in buffalo calves resulted into 34 % increase in ALT activity on day 30. The level further increased significantly on day 60 and 90. On day 90, the ALT activity was highest among corresponding values in all treatment groups. However, 30 days after last dosing of flubendiamide, the ALT activity declined by 27.28 % from day 90.

AST activity also increased, albeit non-significantly, on day 30. A steep significant rise in AST activity was noted thereafter, and the level increased from baseline value (day 0) by 56.95 % on day 60 and 128.72 % on day 90. However, on day 30 post treatment, the level significantly declined by 43.59 % from day 90 value. On different observation periods, the AST

activity were lower than corresponding values in group IV, but higher than corresponding values in group II.

Findings of the present study were in agreement with the observations of El-Hameed *et al* (2008) who reported significant increase in ALT and AST activities in pregnant Baladi goats, 12 weeks after daily oral administration of lead acetate @ 6 mg/ kg body weight. Gouda *et al* (1985) also reported almost similar observations in experimental lead toxicity in goats. Brar *et al* (2000) reported increase in plasma levels of AST (70%) and ALT (114.3%) after single oral dosing of lead acetate @ 600 mg/ kg body weight in buffalo calves. Hoffman *et al* (1981) reported significant increase in ALT and non-significant increase in AST in serum after lead shot ingestion by bald eagles (*Haliaeetus leucocephalus*). Moussa and Bashandy (2008) reported 64.43% increase in AST and 104.50 % increase in ALT activity from day 0 values after 3 months of lead acetate administration in rats. Elayat and Bakheetf (2010) reported about 50% increase in serum ALT and AST activities in rabbits receiving 0.6 and 0.9 mg/ kg body weight lead acetate in drinking water for 3 weeks. Swarup *et al* (2007) reported higher levels of ALT and AST in cows naturally exposed to lead and cadmium around different industrial areas.

C. Oral Chronic Flubendiamide-Lead Interaction Study

In buffalo calves given both lead and flubendiamide, ALT activities increased on day 30 by about 21%, on day 60 by 47.65% and on day 90 by 48.37% of the day 0 value. Much higher increase in AST activities from baseline values were recorded on different observation periods in this group (45.80, 118.26 and 182.18 % respectively on day 30, 60 and 90). ALT activity on day 90 was close to the corresponding value in group III and was significantly higher than control and flubendiamide-treated group. AST activity increased significantly up to 182.18 % from control on day 30, 60 and 90. On day 90, the AST activity was highest among corresponding

values in other treatment groups. However, on day 30 post-treatment, the AST activity declined significantly by 22.65 % from day 90 value.

Both ALT and AST are important enzymes involved in carbohydrate and amino acid metabolism. Aspartate aminotransferase (formerly known as serum glutamic-oxaloacetic transaminase, SGOT) is a cytoplasmic and mitochondrial enzyme that catalyses the transamination of L-aspartate and 2-oxoglutarate to oxaloacetate and glutamate. It is present in high concentration in liver and muscle tissues and is released into blood during degenerative changes in hepatocytes and myocytes (Evans and Health 1998). It is a non-specific but sensitive marker of liver damage in man and animals (Kramer and Hoffman 1997). In all domestic animals, the activity of AST is high in liver and the AST activity in blood increases during liver damage (Tennant 1997). Alanine aminotransferase (formerly known as serum glutamic-pyruvic transaminase, SGPT), is a cytoplasmic enzyme that is considered as liver specific in man, dogs and cats. However, in large domestic animal species including cattle and buffalo the activity of ALT in the liver tissue is low, hence its level does not increase much even during hepatic injury (Tennant 1997).

Increase in ALT and AST activities in present study may be due to increased cellular basal metabolic rate, irritability and the destructive changes in liver and skeletal muscle cells (El-Hameed *et al* 2008). The liver was found to be a significant target organ in repeat dose studies in all animal species (APVMA 2009). Flubendiamide induces cytochrome P 450 group of enzymes (Amanuma 2005). Likewise, lead is accumulated in liver and produces hepatotoxic effects (Swarup and Dwivedi 2002). However, hepatotoxic effects of lead and flubendiamide seem to be reversible up to some extent as evident by mild decrease in ALT and AST activities 30 days

post-treatment. Miranda *et al* (2006) also observed normal serum hepatic biomarkers after a long term follow up in heifers that survived an episode of acute lead poisoning.

The effect of lead on liver enzymes is attributable to binding of lead to sulfhydryl groups of enzymes containing cystein (Farag *et al* 2010).

Alkaline Phosphatase (AKLP)

Alteration in AKLP by chronic oral exposure of flubendiamide, lead and their combined exposure are presented in table 3 and fig. 7.

A. Oral Chronic Flubendiamide Toxicity Study

Slight decrease in plasma AKLP activity in buffalo calves given flubendiamide was recorded on day 30. However, in comparison to day 0, AKLP activity on day 60 and 90 increased by 12.61 % and 53.42 %, respectively. AKLP activities on day 90 in all three treatment groups were statistically comparable, but all were significantly higher than control. On day 30 post-treatment, the activity declined non-significantly from day 90 value.

In an unpublished research report, Kuwahara (2003) observed time-dependent increase in AKLP activity in dogs given ≥ 2000 ppm flubendiamide for 90 days. Increase in AKLP activity may be due to hepatotoxic effects of flubendiamide.

B. Oral Chronic Lead Toxicity Study

In lead treated group, AKLP activity increased on day 30, although non-significantly from day 0 value. However, on day 60 and 90 activities increased significantly by 46.08 and 51.81 % respectively from day 0 and the values were higher than corresponding values in two other treatment groups. AKLP activity decreased on day 30 post-treatment to reach a level statistically less than day 0.

Increase in AKLP activity in lead intoxicated rats was also observed by Nehru and Kaushal (1993) after a period of 60 to 90 days of lead administration. Moussa and Bashandy (2008) recorded significant increase (43.08 %) in plasma alkaline phosphatase activity in rats 3 months after exposure to 2 % lead acetate in drinking water. Likewise, Hamadouche *et al* (2009) reported significant increase in AKLP activity in rats exposed to lead by gavage @ 250 and 500 mg/l. Brar *et al* (2000) recorded 71.9 % increase in AKLP activity in buffalo calves given single oral dose of lead acetate @ 600 mg/ kg body weight.

On the contrary, Longer *et al* (1984) did not find any change in AKLP activity in calves given 500 and 1500 ppm lead in the diet for 7 weeks. Likewise, Chandra *et al* (2010) reported significant decrease in AKLP activity in Wistar rats given 100 and 900 mg/ kg body weight lead nitrate orally for 90 days.

C. Oral Chronic Flubendiamide-Lead Interaction Study

AKLP activity in lead and flubendiamide treated animals decreased by 18.46 % on day 30, but on day 60 and 90 activities increased to reach 30.60 and 42.90 % higher from the day 0 value. Day 90 value differed significantly from day 0, but no significant difference from day 0 value was evident on day 30 and 60. On day 30 post-treatment, activity decreased non-significantly from day 90 value.

Alkaline phosphatase is a zinc metallo-enzyme and its activity in blood is derived from a combination of isoenzymes produced in the liver, bone, kidney, platelets and intestine (Rosol and Capen 1997). Renal AKLP is generally not found in serum, whereas bone and hepatic enzymes have been identified in the serum of all animal species. Although, the actual physiological functions of AKLP are not fully understood, localization of the enzyme to cell surfaces suggest a role in the membrane transport like active secretion or absorption (Tennant 1997). In the present

study, lead intoxication resulted into significant increase in plasma AKLP activity. Flubendiamide intoxication was also associated with enhanced AKLP activity, though the degree of increase was less than those observed in group III (lead treated) and IV (lead plus flubendiamide treated) animals.

Gamma-Glutamyl Transpeptidase (GGT)

Effect of oral chronic exposure of flubendiamide, lead and their interaction on GGT levels are given in table 4 and figure 8.

A. Oral Chronic Flubendiamide Toxicity Study

In flubendiamide-intoxicated buffalo calves, progressive increase in GGT activity was observed. In comparison to day 0, activity on day 30, 60 and 90 increased by 7.27, 53.04 and 63.34 %, respectively. However, the values did not differ significantly from each other. On day 90, GGT activity was lowest in flubendiamide treated animals among all treatment groups. On day 30 post-treatment, the activity declined by 8.61 % of day 90 level.

Similar changes were observed in an unpublished study wherein administration of ≥ 200 ppm flubendiamide in rats resulted into significant increase in GGT activity (APVMA 2009). There seems to be no other published research report available to compare the findings of the present study.

B. Oral Chronic Lead Toxicity Study

In lead-intoxicated buffalo calves, plasma GGT activity increased non-significantly on day 30 and 60. However, on day 90 it significantly increased (by 150.78 %) from day 0 value. The activity on day 90 was numerically lower than that observed in group IV, but higher than group II. On day 30 post treatment, GGT activity declined by 29.71 % from day 90 value.

These findings were in corroboration with findings of Randhawa *et al* (1995) who reported significant increase in serum GGT activity during experimentally-induced lead toxicosis in crossbred calves. Similarly, El-Hameed *et al* (2008) also reported 80.39 % increase in GGT activity in pregnant Balady goats after oral administration of lead acetate @ 4.5 and 6.0 mg/ kg body weight for 20 weeks. Brar *et al* (2000) observed 139.4 % increase in GGT activity after single oral administration of lead acetate @ 600 mg/ kg body weight in buffalo calves.

C. Oral Chronic Flubendiamide-Lead Interaction Study

Buffalo calves intoxicated with both flubendiamide and lead revealed marginal decline in GGT activity on day 30. However, on day 60 and 90, activities were 63.45 and 150.16 % higher than day 0. On day 60 and 90, the GGT activity was higher than corresponding values in group II and III. The activity declined on day 30 post-treatment, but was still higher than day 0 level by 67.83 per cent.

Plasma or serum GGT activity is thought to be a sensitive indicator of liver insult (Pearson 1990). On the other hand, urinary GGT is considered as a good marker of renal damage (Braun *et al* 1983). GGT is a diameric carboxypeptidase that cleaves C-terminal glutamyl groups from synthetic substrates and transfers them to peptides and other suitable acceptors (Shaw 1983). In domestic animals, GGT is mainly located in the kidneys, pancreas and intestines. Its activity in liver is relatively high in cows, horses, sheep and goats and very low in dogs, cats and birds.

Increased GGT level in lead-treated animals may be due to hepatotoxic effects of lead. Flubendiamide is also hepatotoxic, but unlike lead, cirrhosis and increase in liver weight are major changes (Kuwahara 2003).

Blood Urea Nitrogen and Creatinine

Changes in blood urea nitrogen and creatinine in animals after exposure to flubendiamide, lead and their combination are given in table 4 and figure 9 and 10.

A. Oral Chronic Flubendiamide Toxicity Study

Repeated oral administration of flubendiamide at the dose rate of 0.024 mg/ kg body weight for 90 consecutive days resulted into significant increase in blood urea nitrogen (by 90.15 % from day 0 value) and non-significant increase in creatinine concentration (8.53%) on day 90. On day 30 post-treatment, non-significant decline from day 90 levels in blood urea nitrogen (16.90 %) and creatinine (4.28 %) concentrations were observed. On day 90, blood urea nitrogen concentration in flubendiamide-treated animals differed significantly from group IV animals, but no significant difference was observed with the corresponding value in group III animals. Creatinine levels on different observation days did not differ significantly from other treatment groups, except for day 90 value in group II.

Results of the present study indicated no significant effect of flubendiamide treatment on creatinine and blood urea nitrogen concentrations in buffalo calves. On the contrary, Enomoto (2004) observed nephrotoxicity in rats after exposure of 1000 ppm flubendiamide for two years with increase in kidney weights. However, no other report is available regarding effect on renal function during fubendiamide exposure in mammals.

B. Oral Chronic Lead Toxicity Study

Lead acetate treatment in buffalo calves resulted into significant increase in blood urea nitrogen levels on day 60 and 90 (51.31 and 125.02 % respectively) and creatinine level on day 90 (44.26 %) from day 0 values. BUN levels on different observation days did not differ significantly from corresponding values in other groups except for day 60; when it was

significantly lower than group IV. Likewise, creatinine levels differed significantly only on day 90 from group IV.

Findings of the present study were in agreement with the study of Elayat and Bakheetf (2010) and Swarup and Dwivedi (1992) who observed increase in BUN and creatinine concentration during experimental lead intoxication in rabbits and goats, respectively. Likewise, El-Hameed *et al* (2008) also observed significant increase in BUN and creatinine concentration in pregnant goats after 14 weeks of lead acetate administration.

Kidneys play an important role in eliminating lead from the body. Among different soft tissues, kidneys accumulate highest concentration of lead after its chronic exposure (Javed 2012). Lead is accumulated in the nuclei of proximal renal tubular epithelial cells as Pb-protein complex in the form of inclusions. Hence, prolonged exposure to Pb can cause chronic irreversible nephropathy, which is characterized by increase in urea nitrogen and creatinine (Swarup and Dwivedi 2002). However, Blood lead value of 60 µg/ dl is thought to be threshold for proximal tubular cell injury (Goyer *et al* 1987) and nephropathy may not be observed below this level. Lead nephropathy is associated with hyperplasia, cytomegaly, and dysplastic cellular changes in proximal tubular lining cells in man and experimental animals (Swarup and Dwivedi 2002).

C. Oral Chronic Flubendiamide-Lead Interaction Study

Combined exposure to flubendiamide and lead resulted into significant increase in BUN on day 30 (47.63 %), which further increased on day 60 (88.94 %) and 90 (138.54 %) from day 0 value. BUN level in this group was significantly higher than flubendiamide-treated group on day 90 and lead-treated group on day 60. Creatinine levels on different observation days were statistically comparable to each other, however the levels on day 60 (15.56 %) and 90 (25.19 %) were higher than day 0 level. Creatinine levels on different observation periods in this group did

not differ significantly from corresponding values in other groups. On day 30 post-treatment, BUN and creatinine levels declined by 12.34 and 21.30 % respectively, in comparison to day 90.

Animals exposed to both flubendiamide and lead suffered greater impairment in renal functions in comparison to animals exposed to lead or flubendiamide alone, as evident from higher increase in blood urea nitrogen and creatinine concentrations. Flubendiamide is very little excreted in urine (APVMA 2009), while most of the absorbed lead is eliminated through renal and biliary clearance (Swarup and Dwivedi 2002). The nephrotoxic effect of flubendiamide was reported by Enomoto (2004) in rats after exposure to 1000 ppm flubendiamide for two years. Nephrotoxicity in chronic lead poisoning is well established. Hence, it appears that flubendiamide and lead have some synergistic effect in terms of nephrotoxicity potential.

III. Haematology

Haemoglobin (Hb), Packed Cell Volume (PCV) and Total Erythrocyte Count (TEC):

Changes in Hb concentration, PCV and TEC in control and different treatment groups are given in table 5 and figure 11, 12 and 13.

A. Oral Chronic Flubendiamide Toxicity Study

In animals treated with flubendiamide, Hb and PCV on day 30 and 60 did not differ significantly; but on day 90, it was significantly lower (17.83 and 8.95 % respectively) than day 0 as well as the corresponding values in control. TEC, however, did not vary significantly on different observation days, though the values were numerically lower than corresponding values in control.

Results of the present study suggested moderate effect of flubendiamide administration on Hb, PCV, but not on TEC in buffalo calves.

B. Oral Chronic Lead Toxicity Study

Hb concentration, PCV and TEC on day 60 were lower than corresponding day 0 levels, though the difference was statistically significant for PCV. On day 90, all the three hematological indices were significantly lower than day 0 values within the group as well as corresponding values in control. A marginal increase in Hb, PCV and TEC level was observed on day 30 post treatment except for PCV, which increased significantly by 15.08 per cent from day 90.

Results of the present study was in agreement with the earlier report describing significant decrease in TEC, Hb and PCV levels in buffalo calves after administration of lead acetate for two months (Sujatha *et al* 2006). Longer *et al* (1984) also reported significant decline in Hb and PCV after 6 weeks of 1500 ppm lead administration as lead sulfate to Holstein calves. Alkahemal-Balawi *et al* (2011) reported decrease in Hb, PCV and TEC in *Clarias gariepinus* following exposure to 24.4 mg/ l lead acetate. In a survey in human infants and children, Hegazy *et al* (2010) observed that blood lead level ≥ 10 μg/ dl was significantly associated with anemia, decreased iron absorption and alterations in hematological parameters. High blood lead levels were associated with low serum iron concentration. In a study on Nile tilapia, significant decrease in Hb level along with non-significant decline in TEC and PCV were observed following exposure to 45 ppm lead nitrate for 28 days (Palipoch *et al* 2011). Krishna and Ramchandran (2009) observed no significant alterations in Hb, TEC and PCV in Wister rats after administration of lead acetate @ 100 mg/ kg for 15 days, though the values were numerically lower than that observed in control.

Anemia is an early manifestation of both acute and chronic lead toxicity. It may be the only clinical feature in chronic exposure to low levels of Pb (Goyer and Rhyne 1973). Anemia in Pb poisoning is supposed to be the end result of two basic defects, first is the shortened life span

of erythrocytes due to loss of membrane integrity (secondary to Na^+-K^+-ATPase inhibition) and increased osmotic fragility (Sujatha *et al* 2006). Second is the impaired heme synthesis owing to inhibitory effects of Pb on enzymes involved in the heme synthesis (George and Duncan 1981). Pb- induced anemia is generally normocytic-normochromic with a slight microcytic-hypochromic tendency and reticulocytosis (Jain 1986). Basophilic stippling of red blood cells is a common feature in peripheral blood film in case of chronic lead toxicity. It may be in the form of distinct granules (punctate) or web like (reticulate) materials (Jain 1986). The stippled cells are believed to be modified reticulocytes, an immature red cell that retains remains of cell organelles of ribosomal origin.

On the contrary, Kosai *et al* (2011) reported non-significant increase in haematocrit, Hb, TEC and TLC in Nile tilapia after exposure to sub-lethal dose of lead for 30 days. Likewise, Ergonul *et al* (2012) reported marked increase in RBC count and Hb levels without significant alteration in hematocrit in common carp (*Cyprinus carpio* L.). They concluded that it may be due to increase in newly formed immature RBC population and shortening of the life span of mature RBC following lead exposure or as a response to replace abnormal Hb which might have been oxidized or denatured by the metal that enters the RBC, which in turn stimulates erythropoietic tissues.

C. Oral Chronic Flubendiamide-Lead Interaction Study

On day 90, Hb, PCV and TEC were significantly lower than day 0 as well as day 90 value in control. However, the values were numerically higher than corresponding values in group III animals treated with lead alone. On day 30 post treatment, Hb and TEC increased non-significantly from day 90 values, but the PCV increased significantly by 16.55 per cent.

Results of the present study suggested no interactive effect of flubendiamide on lead induced haematological changes in buffalo calves.

Mean Corpuscular Volume (MCV), Mean corpuscular Haemoglobin Concentration (MCHC) and Mean Corpuscular Haemoglobin (MCH):

MCV, MCHC and MCH levels on different days of observation in treatment and control groups are given in table 6 and figures 14, 15 and 16.

A. Oral Chronic Flubendiamide Toxicity Study

MCV, MCHC and MCH values did not differ significantly from day 0 on different observation days, though values declined gradually. In comparison to day 90, MCV increased while MCHC and MCH decreased on day 30 post-treatment. On different observation days, MCV and MCH were numerically lower than corresponding values in control.

B. Oral Chronic Lead Toxicity Study

On day 30 MCV, MCHC and MCH values increased non-significantly from day 0. However, on subsequent observation days, values decreased, albeit non-significantly from day 0. On day 30-post treatment, all the three erythrocytic indices increased non-significantly from their day 90 values.

In agreement with present results, Palipoch *et al* (2011) observed significant decrease in MCH and MCHC, but non-significant increase in MCV in Nile tilapia after exposure to 45 ppm lead nitrate for 28 days. Rahman *et al* (2008) also reported decrease in MCV, MCH and MCHC in lead exposed broiler chicks given 250 or 400 ppm lead acetate in drinking water. Significant decrease in MCHC after lead exposure to swans and geese were observed by Katavolos *et al* (2007).

Contrary to the above, Ergonul et al. (2012) reported marked increase in RBC count and Hb levels without significant alteration in hematocrit in common carp (*Cyprinus carpio* L.). Significant increase in MCHC in fishes following exposure to sublethal dose of Pb was reported by Zaki *et al* (2008).

C. Oral Chronic Flubendiamide-Lead Interaction Study

Changes in MCV, MCHC and MCH in animals given both lead and flubendiamide did not exhibit any constant pattern as on day 30 and 60, some parameters increased while others decreased. However, on day 90, all the three parameters were lower than day 0 value within the group, though the difference did not reach statistical significance. On day 30 post-treatment, the values were higher than day 90 and statistically significant difference was observed only in MCV.

Total Leukocyte Count (TLC) and Differential Leukocyte Count (DLC)

Values of TLC and DLC in control and different treatment groups are given in tables 7 and 8 and figures 17, 18, 19, 20, 21 and 22.

A. Oral Chronic Flubendiamide Toxicity Study

TLC decreased on day 60 and 90 to reach a value 23.99 % lower than day 0. On day 90, TLC was significantly lower than corresponding values in control group as well as day 0 value within the group. Changes in neutrophil, lymphocyte, monocyte, eosinophil and basophil percentages did not show any constant pattern or significant difference within the group.

Flubendiamide exposure in buffalo calves did not induce any constant pattern of change in leukogram of buffalo calves suggesting paradoxical effects on haematological indices.

B. Oral Chronic Lead Toxicity Study

TLC decreased significantly on day 60 and 90 to reach a level 21.11 and 39.44 % lower than day 0. On day 30 post-treatment TLC increased but was still significantly lower than day 0 level. No significant change in neutrophil, eosinophil and basophil percentage were observed on different observation periods. The lymphocyte decreased, while monocyte increased significantly on day 90.

Contrary to the present findings, Palipoch *et al* (2011) observed non-significant increase in TLC and lymphocyte count, but decrease in neutrophil and monocyte percentage in *Nile tilapia* after exposure to lead nitrate for 28 days. Leukocytosis due to neutrophilia with a regenerative left shift possibly caused by increased bone marrow myeloid-erythroid ratio is reported to be a common finding in lead-induced haematological changes (Mitema *et al* 1980). Teijon *et al* (2000) reported decrease in total number of leukocytes following oral administration of lead but leukocytosis was reported after intra-peritoneal administration of lead indicating influence of the route of administration of lead on blood leukocyte counts. Epidemiological studies involving occupationally-exposed workers to toxic metals showed non-significant increase, in neutrophils (Pinkerton *et al* 1998). Another study revealed an increase in lymphocyte and decrease in neutrophil without any effect on the total of leukocyte count (Osfor *et al* 1998). These studies point to controversies in the peripheral blood leukocyte count in toxic metal-exposed subjects.

The alterations in neutrophil are attributed to multiple factors like inflammatory conditions metabolic disorders and physiological stress (Sacher and Mc Pherson 1992). Kuijpers *et al* (1999) suggested that lead might be acting like organisms that increase phagocytosis. Therefore, the leukocytic alteration in the present study might be due to the toxic effect of lead on spleen, thymus, bone marrow, lymph nodes and Payer's patches, involved in the regulation of

peripheral blood leukocytes. Teijon *et al* (2003) have indicated that lead administration through oral route caused histological modification including increase in number of lymphocytes as well as edema indicating spleenomegaly. Teijon *et al* (2000) indicated that spleen was clearly sensitive to lead, especially when it is administered intraperitonially. This was also associated with decrease in red blood cells and alterations in white blood cells.

C. Oral Chronic Flubendiamide-Lead Interaction Study

In animals treated with flubendiamide and lead, TLC decreased significantly on day 60 and 90 to become 21.92 and 49.80 % lower than day 0. On day 60 and 90, the count was lowest among corresponding values observed in other treatment groups and control. On day 30 post-treatment TLC increased to reach 48.15 % higher than day 90 value, but was still significantly lower than day 0. No significant change was observed in lymphocyte and basophil percentage on different observation days. However, monocyte and eosinophil showed an increasing trend while neutrophil showed a decreasing trend during flubendiamide and lead exposure. Flubendiamide and lead did not appear to have any interactive effects on leukogram in buffalo calves in the present study.

IV. Antioxidant status

Lipid peroxides

Changes in lipid peroxides in erythrocytes in calves following flubendiamide, lead and their combined exposure are depicted in figure 23 and table 9.

A. Oral Chronic Flubendiamide Toxicity Study

In comparison to day 0, LPO level increased significantly by 26.82 % on day 60 and by 45.30% on day 90 in animals treated with flubendiamide for 90 days. On day 30 post-treatment, the level decreased by 15.74% from day 90, but the value was still significantly higher than day

0. On day 60 and 90, the LPO level were significantly higher than corresponding values in control, but significantly lower than the values observed in group III and IV.

Significant increase in LPO levels suggested that flubendiamide administration induces oxidative stress and hence excessive peroxidation of membrane lipids in erythrocytes. There is no research report available on the effect of flubendiamide on lipid peroxides to compare the findings of the present study. However, some other pesticides like cypermethrin exposure have been reported to increase LPO level in Wistar rats (Raina *et al* 2009).

B. Oral Chronic Lead Toxicity Study

Treatment of lead acetate for 90 days resulted increase in LPO level on different observation days to the extent by 80.53 %. The degree of increase on different observation days was higher than the corresponding increase in group II animals. On day 30 post-treatment, the level declined significantly from day 90, but it was still higher than day 0 level.

Marked increase in LPO level in present study suggested increased oxidative damage of erythrocytic membrane lipids after lead exposure in calves. Significant correlation ($p = +0.713$) between blood lead level and LPO further substantiated this hypothesis. The propensity for lead to enhance lipid peroxidation has been demonstrated in multiple studies. Significant increase in LPO levels in erythrocytes was observed in human beings naturally exposed to lead (Sugawara *et al* 1991; Madhavi and Devi 2008). Patra and Swarup (2000) reported up to 20.69 % increase in erythrocytic LPO in calves given lead acetate @ 7.5 mg/ kg body weight for 28 days. Ahmed *et al* (2008) recorded 174.07 % higher erythrocytic LPO level in buffaloes reared near highways (with high blood lead level) in comparison to those reared in pollution free areas (with low blood lead level) in Egypt.

The erythrocyte membrane is rich in polyunsaturated fatty acid, hence prone to oxidative insult by pro-oxidants (Clemens and Waller 1987). Property of the lead to induce free radical reactions and subsequent increase in oxidative damage of erythrocytic membranes is thought to be a reason behind hemolysis observed in lead toxicity cases (Casado *et al* 2007).

C. Oral Chronic Flubendiamide-Lead Interaction Study

Combined exposure of flubendiamide and lead resulted into increase in LPO level to the extent of 93.42 % on day 90. The values on day 30, 60 and 90 were significantly higher than day 0. On day 90, the per cent increase in LPO level from day 0 was highest in this group. However, on day 30 post-treatment, the value declined, albeit non-significantly from day 90 (by 14.03 %), but was still 66.28 % higher than day 0.

Results of the present study indicated that combined exposure of flubendiamide and Pb resulted in higher increase in LPO level in comparison to lead- alone treated group. This was in agreement with the study of Ambali *et al* (2011) which reported significantly higher concentration of MDA in thyroid gland of Wistar rats exposed to both chlorpyriphos and lead in comparison to rats exposed to lead and chlorpyriphos alone. Co-exposure of organophosphorus pesticides has been reported to enhance lead toxicity in terms of changes in various biochemical parameters (Krishna and Ramchandran 2009). Farag *et al* (2010) reported higher increase in LPO in rats co-treated with lead and fenitrothion in comparison to those treated with either lead or fenitrothion suggesting additive effect of fenitrothion on lead-induced lipid peroxidation. Oxidative stress has been incriminated as a major mechanism behind toxic effects of many pesticides including chlorpyriphos (Oruc 2010) and cypermethrin (Shashikumar and Rajini 2010).

Lipid peroxidation is initiated when a hydrogen atom is abstracted from an unsaturated fatty acid by a free radical. This starts off a destructive chain reaction creating a heterogeneous group of compounds known as lipid peroxides (Benzie 1996) and results in the disruption of membranes and the production of reactive metabolites like malondialdehyde (MDA) and 4-hydroxy-2,3-transnonenal (4-HNE), and cellular dysfunction (Dey 2002). Excessive peroxidation of membrane lipids disrupts the bilayer arrangement, decreases membrane fluidity, increases membrane permeability and modifies membrane bound proteins (Halliwell and Gutteridge 1999).

Erythrocytes have a high affinity for lead binding 99 per cent of the lead in the blood-stream. Lead has a destabilizing effect on cellular membranes, inducing decrease in cell membrane fluidity and increase in the rate of erythrocyte lysis. Hemolysis appears to be the end result of ROS-generated lipid peroxidation in the RBC membrane (Lawton and Donaldson 1991). In an *in vitro* study Casado *et al* (2007) observed that lead-induced lipid peroxide formation is mediated by a metal-driven Fenton reaction, but without any direct involvement of hydroxyl radicals in this process. By contrast, addition of histidine, a singlet oxygen scavenger resulted in decrease in lead-induced hemolysis, suggesting that singlet oxygen plays an important role in lead-induced membrane damage and lysis of RBC.

Superoxide dismutase and Catalase

Table 9 and figures 24 and 28 show the influence of flubendiamide, lead and their interaction on erythrocytic superoxide dismutase and catalase activities. Figures 25 (a,b,c,d), 26 (a,b,c,d) and 27(a,b,c,d) shows the SOD activity and blood concentrations of zinc, copper and manganese respectively.

A. Oral Chronic Flubendiamide Toxicity Study

Flubendiamide treatment for 90 days resulted in non-significant decrease in SOD activity and non-significant increase in catalase activity on day 30 and 60. However, on day 90, SOD declined significantly by 18.19 %, while catalase increased significantly by 11.14 % from day 0. On day 30 post-treatment, the SOD level increased again by 15.51% and catalase decreased by 8.37 % from day 90 to become statistically comparable to day 0.

There is no report available to compare the results of the present study. Decrease in SOD and increase in catalase may be due to exhaustion or induction of antioxidant defense in response to flubendiamide-induced excess free radical production.

B. Oral Chronic Lead Toxicity Study

Administration of lead acetate in buffalo calves for 90 days resulted into rapid progressive decrease in erythrocytic SOD activity on day 30, 60 and 90 to the extent of 53.83 % from day 0 value. The SOD level on each observation period decreased significantly from its preceding value reaching to a minimum on day 90. The SOD activity on day 90 was lowest in comparison to values observed in other treatment groups. On day 30 post-treatment, the SOD activity increased significantly from day 90 by 40.25 %. The catalase activity, on the other hand increased on day 30 and 60 by 2.30 and 4.21 % respectively. On day 90, the value further increased to reach a level significantly different from day 0 (7.05 %). On day 30 post-treatment, the activity decreased by 2.27 % to become statistically comparable to day 90 as well as day 0. Significant decrease in SOD and CAT activities in blood and soft tissues (including brain, liver and kidney) after lead exposure has been reported in different animal species by several workers (Prasanthi *et al* 2010; Patra and Swarup 2000; Patra *et al* 2000). SOD which requires Cu^{2+} and Zn^{2+} for its activity was found lower in Pb administered buffalo calves. This could be due to the Pb-induced Cu and Zn deficiency as Pb competes and replaces Cu^{2+} and Zn^{2+} from their binding

sites (Mylorie *et al* 1984). The excessive accumulation of H_2O_2 might subsequently decrease the activity of SOD (Sivaprasad *et al* 2002). Decrease in blood level of Cu and Zn, as observed in the present study also supported this hypothesis. Mylroie *et al* (1984) reported that dietary Cu and Zn supplementation prevents Pb-induced decrease in SOD activity. Prasanthi *et al* (2010) reported that Zn supplementation had protective effect on Pb- induced oxidative stress in brain tissues of mice.

Catalase is responsible for breakdown of hydrogen peroxide, an important ROS, produced during metabolism. There are contradictory data available concerning the effects of lead on catalase activity. Some studies revealed elevated enzymatic activity which could play a significant role in protecting cells (Gurer *et al* 1998; Machartova *et al* 2000). However, few reports described decreased catalase activity that can be attributed to the reduced absorption of iron or the inhibition of heme biosynthesis (Mahaffey 1990; Chaurasia and Kaur 1997). In the present study, significant increase in catalase activity was observed in animals exposed to lead and/ or flubendiamide for 90 days. A possible explanation for this effect could be increase in the formation of erythrocytic lipid hydroperoxides in lead-intoxicated animals that acts as a signal to maintain higher levels of catalase to trigger the detoxification process for the metal. This hypothesis is supported by the study of Antonio-Gracia and Masso´-Gonzalez (2008) who observed increase in catalase activity in brain of lead-exposed rats, while catalase activity was comparable to control values in rats co-treated with antioxidants. Contrary to the present findings, Patra and Swarup (2000) reported decrease in erythrocytic catalase activity in calves exposed to lead acetate and concluded that it could be due to increased generation of hydrogen peroxide in lead-exposed calves due to accumulation of delta-ALA and because of the fact that lead has no direct effect on activities of catalase (Ariza *et al* 1998).

C. Oral Chronic Flubendiamide-Lead Interaction Study

Combined administration of flubendiamide and lead resulted into decrease in erythrocytic SOD activity on day 60 and 90 by 20.88 and 27.29 % respectively from day 0. However, the per cent decrease in SOD activity from preceding levels on different observation periods were lower than corresponding decrease in group III. On day 30 post-treatment, the activity increased to a level statistically comparable to day 0.

Concurrent administration of lead and flubendiamide for 90 days resulted into non-significant increase in catalase on day 30 and 60. However, on day 90 the activity increased by 11.02% to reach a value significantly higher from day 0 as well as day 30 and 60. Per cent increase in SOD activity on day 90 from day 0 value was slightly lower than group II, but higher than group III animals.

Results of the present study suggested that flubendiamide does not have any interactive effect on lead-induced changes in SOD and catalase activity. SOD exists as a copper/zinc containing SOD (Cu/ Zn-SOD; SOD1) and a manganese containing SOD (Mn-SOD/SOD2). The enzyme catalyzes the dismutation of superoxide to hydrogen peroxide and oxygen (Koek *et al* 2011). Hydrogen peroxide is further decomposed by catalase (Mates *et al* 1999) or reduced by GSH-dependent mechanism catalyzed by GPx. The importance of SOD as an antioxidant defense in erythrocyte is unclear. Under conditions where H_2O_2 catabolism is compromised, SOD can increase oxidant injury due to accelerated H_2O_2 generation (Scott *et al* 1989).

Catalase is responsible for breakdown of hydrogen peroxide, an important ROS produced during cellular metabolism. Except for dogs, all mammalian blood cells generally have high catalase activity (Suzuki *et al* 1984). Presence of catalase in erythrocytes also helps to

protect somatic cells exposed to high levels of H_2O_2 during active inflammation (Agar *et al* 1986).

Glutathione (GSH)

Effect of oral chronic exposure of flubendiamide, lead and their interaction on glutathione levels are given in table 10 and figure 29.

A. Oral Chronic Flubendiamide Toxicity Study

Flubendiamide administration in buffalo calves resulted into non-significant decrease in glutathione concentration on day 30 and 60. On day 90, the glutathione concentration further decreased to become significantly lower than day 0 (by 30.03 %) as well as corresponding value in control. However, on day 30 post-treatment, the level increased to become statistically comparable to day 0.

Results of the present study indicated decrease in blood glutathione concentration in chronic toxicity of flubendiamide. However, no report is available to substantiate the findings of the present study. Decrease in glutathione level may be due to its over-utilization to neutralize the free radicals generated in excess after flubendiamide administration.

B. Oral Chronic Lead Toxicity Study

Oral administration of lead acetate for 90 days in buffalo calves induced progressive decrease in blood glutathione concentration to the extent of 24.10 % on day 90. On day 30 post-treatment, the level increased by 16.37 % from day 90. The glutathione concentration was significantly lower on day 90 from day 0 as well as day 30 and 60, but levels on other days did not differ significantly from each other within the group.

Results of the present study are in agreement with the observations of Sharma *et al* (2010) who reported decrease in glutathione concentration in blood, kidney and brain tissues of

mice after chronic exposure to lead nitrate. Likewise, Moniem *et al* (2010) and Farag *et al* (2010) also reported decrease in glutathione concentration after lead-exposure in experimental animals. Kumar *et al* (2010) reported significant decrease in blood glutathione concentration in poultry following 4 weeks after lead exposure. Reduction in glutathione content in human neuroblastoma cells following lead-exposure was reported by Challa *et al* (2011).

Lead can exclusively bind to the -SH group, which decreases the GSH levels and can interfere with the antioxidant activity of GSH (Saxena *et al* 2005). The inhibition of ALAD by lead can be reversed by addition of zinc and reducing agents such as glutathione and dithiotheitol. Glutathione removes lead from sulfur groups on the enzymes and may reduce oxidized sulfur groups (Goering *et al* 1987).

C. Oral Chronic Flubendiamide-Lead Interaction Study

Combined exposure of flubendiamide and lead resulted into non significant decrease in blood glutathione concentration on day 30 and 60. But on day 90, the level decreased further to become significantly lower from day 0 (by 32.76 %). Levels on different observation periods did not differ significantly from corresponding values in control and other treatment groups, except on day 90 and day 30 post-treatment, when it was significantly lower than corresponding values in control.

The per cent decrease in glutathione concentration on day 90 was higher than the corresponding decrease in group II and III suggesting synergistic effect of lead and flubendiamide interaction on blood glutathione level.

Glutathione (GSH) is a cysteine-containing enzyme which is synthesized in all cells from its constituent amino acids (Meister and Alton 1988). GSH levels in cells reflect the dynamic equilibrium between its synthesis and utilization. The alteration in glutathione level is

considered as a sensitive indicator of oxidative stress (Gurbay and Hincal 2004). GSH provides first degree protection against oxidants in cells. The primary role of GSH in erythrocytes is to maintain hemoglobin in its native form in cells at higher concentrations and to bind with free hemin, thereby reducing its potential for membrane injury (Shviro and Shaklai 1987). It detoxifies reactive oxygen species produced in the mitochondrial electron transport chain (Okabe *et al* 1994). Antioxidant properties of GSH are linked to the thiol group in its cysteine moiety, which is a reducing agent and can be reversibly oxidized and reduced. Besides a direct role in protection against oxidative stress, GSH also functions as cofactor for a number of protective enzymes, such as glutathione peroxidase and glutathione-S-transferase. Under oxidative conditions, GSH is reversibly oxidized to glutathione disulfide (GSSG) that can pass through red cell membrane due to oxidative stress-induced membrane damage. This mechanism may be responsible for the decreased red cell GSH levels in oxidative stress condition (Dincer *et al* 2002). It is assumed that the capacity of GSH to neutralize oxidants is due to the nucleophilicity of the thiol group and its high reaction rate with oxidants (Manson 1979). It has also been observed that cells with low levels of GSH are more sensitive to the adverse effects of irradiation and stress than cells with normal levels of GSH (Pandey and Rizvi 2010).

The decline in the glutathione levels in the present study could be due to increased utilization of this intracellular antioxidant by GPx or GST. In addition, binding of lead to the -SH group and inhibiting the action of GSH or increased utilization of GSH for detoxification of lead or flubendiamide-induced free radicals may also be responsible for this observation.

Glutathione Peroxidase (GPx)

Effect of oral chronic exposure of flubendiamide, lead and their interaction on blood glutathione peroxidase activities are given in table 10 and figure 30.

A. Oral Chronic Flubendiamide Toxicity Study

Flubendiamide administration for 90 days resulted into decrease in GPx activity on day 30, 60 and 90 to the extent of 30.83 % from day 0 value. However, the difference was statistically significant only on day 90. The day 30 post-treatment activity was 7.81 % higher than day 90, but was significantly lower than day 0.

Results of the present study indicated that flubendiamide-exposure is associated with mild decline in GPx activity in buffalo calves. It may be due to its increased utilization to neutralize flubendiamide-induced excess free radical generation.

B. Oral Chronic Lead Toxicity Study

Administration of lead acetate for 90 days resulted into significant decrease in GPx activity by 13.87 % on day 60. The activity further decreased on day 90 to reach a level significantly lower than day 0 as well as day 60. On day 30 post-treatment, the activity increased albeit non-significantly from day 90 and it was still significantly lower than day 0, 30 and 60 values.

In agreement with the present findings Ashry *et al* (2010) also observed decrease in hepatic GPx level in rabbits following exposure to lead acetate. Besides, several other research reports including that of Berrahal *et al* (2007), Wang *et al* (2006) and Haleagrahara *et al* (2010) also showed decrease in GPx level following lead-exposure in experimental animals. Decrease in activity of GPx may be due to direct inhibitory action of Pb on this enzyme through binding with sulfhydryl groups of the enzyme.

C. Oral Chronic Flubendiamide-Lead Interaction Study

Combined exposure to flubendiamide and lead for 90 days caused non-significant decrease in GPx activities on day 30 (2.32 %) and 60 (8.20 %). On day 90, the activity decreased

by 35.74 % to become significantly lower than day 0, 30 and 60. On day 30 post treatment, the activity increased non-significantly from day 90 by 14.48 %. On different observation periods, GPX activities in different treatment groups did not differ significantly from each other. The activity on day 90 in this group was higher than group III, but lower than group II.

Results of the present study indicated that flubendiamide and lead did not have any significant interaction on GPx activity. Glutathione peroxidase system is important for the removal of hydrogen peroxide and comprises several enzymes including glutathione peroxidise, glutathione reductase along with NADPH as cofactor. GPx converts H_2O_2 to H_2O after dismutation of superoxides to H_2O_2 by SOD (Baskol *et al* 2007).

Glutathione Reductase (GR)

Table 10 and figure 31 depicts changes in blood glutathione reductase activities in calves after flubendiamide, lead and their combined exposure.

A. Oral Chronic Flubendiamide Toxicity Study

Flubendiamide exposure for 90 days resulted into mild increase in GR activity on day 30. However, on day 60 and 90 GR activities decreased to become 7.29 and 13.23 % lower than day 0. On day 30 post-treatment, the value again increased to become statistically comparable to day 0. On day 90, the activity was significantly higher than corresponding value in group III, but non-significantly higher than group IV.

The progressive decrease in GR activity may be due to over-utilization of this enzyme for reduction of glutathione disulfide (GSSG) back to the reduced glutathione (GSH) to protect the cells from oxidative damage. There is no related report available to compare results of the present study.

B. Oral Chronic Lead Toxicity Study

Lead exposure in buffalo calves resulted into non-significant increase in GR activity on day 30. But on subsequent observations, the activity declined significantly to become 13.64 and 27.28 % lower from day 0. On day 30 post-treatment, the activity again increased to become 16.41% higher than day 90. On day 90, the activity was lowest among different treatment groups. Also, the per cent decline from day 0 values within the group was maximum in animals treated with lead alone.

Decrease in GR activity after lead exposure, as observed in the present study, is in agreement with several previous studies (Ahmed *et al* 2008; Newairy and Abdou 2009; Moniem *et al* 2010). Lead can deactivate the enzyme GR (Lyn-Patrick 2006). Depressed levels of glutathione reductase GPx and GST were all found to correlate with depressed glutathione levels in occupationally lead-exposed workers (Hunaiti *et al* 1995).

C. Oral Chronic Flubendiamide-Lead Interaction Study

Combined exposure of flubendiamide and lead resulted into up to 20.71 % decline in GR activity on day 30, 60 and 90. The activity on day 60 was significantly lower than day 0, but it again increased on day 90 to become comparable to day 0 as well as day 60. On day 30 post-treatment, the activity increased by 6.87 % from day 90. On day 90 the level was lower than corresponding value in control but higher than group II and III.

Results of the present study suggested no significant interaction of flubendiamide and lead on activity of GR in buffalo calves.

Glutathione reductase, also known as GSR or GR reduces glutathione disulfide (GSSG) to the sulfhydryl from GSH which is an important cellular antioxidant (Meister and Alton 1988). The activity of GR is considered to be an important indicator of oxidative stress. GR activity has been reported to maintain high GSH/ GSSG ratio in normal red blood cells. In cells exposed to

high levels of oxidative stress, like red blood cells, up to 10 % of the glucose consumption may be directed to the pentose phosphate pathway (PPP) for production of the NADPH needed for this reaction. In erythrocytes, if the PPP is non-functional, the oxidative stress in the cell leads to cell lysis and anemia (Champe *et al* 2008).

Glutathione-S-Transferase

Table 11 figure 32 depicts changes in blood glutathione-s-transferase activities in calves after flubendiamide, lead and their combined exposure.

A. Oral Chronic Flubendiamide Toxicity Study

Oral administration of flubendiamide for 90 days caused non-significant increase in GST activity on day 30 and 60. However, on day 90 it increased significantly to reach a value 6.92 % higher than day 0. On day 60 and 90, GST activities were higher than corresponding level in control, but numerically lower than groups III and IV. GST activity declined on day 30 post-treatment to reach a level statistically comparable to day 0.

Increase in GST suggested up-regulation of antioxidant defense to counteract the oxidative stress associated with flubendiamide-exposure, as supported by changes in some other antioxidant parameters.

B. Oral Chronic Lead Toxicity Study

Administration of lead acetate in buffalo calves resulted into progressive increase in GST activity reaching its peak on day 90, when it was 14.40 % higher than day 0. On day 90, the GST activity was higher than corresponding values in control and group II, but lower than group IV. On day 30 post-treatment, the level declined non-significantly from day 90 by 5.73 per cent.

In agreement with present findings, Bokara *et al* (2009) after lead exposure in mice also observed increase in GST activity in brain tissues. Alghazal *et al* (2008) reported that in kidney

tissues, lower doses of Pb exposure resulted in decrease in GST activity, while higher dose evoked an increase in GST activity in mice.

C. Oral Chronic Flubendiamide-Lead Interaction Study

Concurrent exposure to flubendiamide and lead resulted into significant increase in GST activity on day 60 and 90 to become 9.76 % and 20.48 % higher than day 0 value. The activity on day 60 and 90 were higher than corresponding values in groups II and III, however the value differed significantly only on day 90. Maximum increase in GST activity was observed in this group suggesting a positive interaction between flubendiamide and lead on GST activity.

Glutathione-S- transferase is an enzyme present in both eukaryotes and prokaryotes and involved in a wide variety of biotransformations including xenobiotic detoxification, ligand binding, transport as well as synthesis and modification of prostaglandins, leukotriens and steroids (Mannervik 1987, Udomsinprasert *et al* 2005; Allocati *et al* 2009). It helps in detoxification process by facilitating the conjugation reactions between GSH and electorophilic centers of xenobiotics (Mulder *et al* 1990) and some endogenous compounds such as peroxidised lipids (Leaver and George 1998). This process helps in dissolution of compounds somewhat lipophilic in nature into the aqueous cellular and extracellular media and further out of the body.

Glucose-6-Phosphate Dehydrogenase (G6PD)

Table 11 and figure 33 depicts changes in blood G6PD activities in calves after flubendiamide, lead and their combined exposure.

A. Oral Chronic Flubendiamide Toxicity Study

Flubendiamide exposure for 90 days resulted into decrease in G6PD activity on day 60 and 90 by 5.96 and 17.72 % respectively from day 0 value. On day 30 post-treatment, the activity increased by 10.56 % from day 90. Values on different observation days did not differ

significantly from each other except for day 90, when it was significantly higher than day 0 and 30.

Results of the present study indicated decreasing trend in G6PD activity after flubendiamide-exposure that can be correlated with concurrent changes in other parameters suggesting existence of oxidative stress.

B. Oral Chronic Lead Toxicity Study

In animals exposed to lead acetate, increase in G6PD by 21.39 % was observed on day 30. But on day 60 and 90, values decreased significantly by 20.88 and 29.88 % respectively from day 0. The percent decrease in G6PD activity on day 90 was maximum among different treatment groups. On day 30 post-treatment, the activity increased by 30.51 % from day 90 to become statistically comparable to day 0.

Glucose 6-phosphate dehydrogenase (D-glucose 6-phosphate $NADP^+$ oxidoreductase) is the first enzyme in the pentose phosphate pathway (Beydemir *et al* 2003). G6PD contains many SH groups and supplies cells with most of the extra-mitochondrial NADPH through the oxidation of glucose-6-phosphate to 6-phosphogluconate. NADPH helps in cell membrane protection and cell detoxification from xenobiotics through the glutathione reductase-peroxidase system and the mixed-function oxidases (Barroso *et al* 1999 and Diez-Fernandez *et al* 1996). The major role of NADPH in erythrocytes is the regeneration of reduced glutathione which prevents haemoglobin denaturation, preserves the integrity of erythrocytic cell membrane sulfhydryl groups and detoxifies hydrogen peroxide and oxygen radicals in and on the red blood cells (Weksler *et al* 1990). G6PD activity has been shown to be inhibited by lead (Lachant *et al* 1984) which may be the reason behind decrease in G6PD activity in lead-exposed buffalo calves. On the contrary, some other studies documented increase in G6PD activity after lead-exposure

(Cocco *et al* 1995; Gurer *et al* 1998). The NADP-/NADPH ratio plays an important role in regulation of the pentose phosphate pathway and it changes in favour of the oxidized form under oxidative stress conditions. Therefore, lead-exposure results into an increase or decrease in G6PD activity depending on the concentration and duration of exposure, and magnitude of oxidative stress inside the cell (Gurer and Ercal 2000).

C. Oral Chronic Flubendiamide-Lead Interaction Study

Combined exposure of flubendiamide and lead resulted into non-significant increase in G6PD activity on day 30. However, on day 90 the activity decreased to become significantly lower than day 0 (by 24.04 %). On day 30 post-treatment the activity increased significantly from day 90 to reach a level statistically comparable to day 0.

Lower decrease in G6PD activity in group IV in comparison to groups II and III suggested no significant interactive effect of lead and flubendiamide on G6PD activity.

Total Antioxidant Activity (TAA)

Table 11 and figure 34 depicts changes in total antioxidant activity in blood of calves after flubendiamide, lead and their combined exposure.

A. Oral Chronic Flubendiamide Toxicity Study

Total antioxidant activities in flubendiamide- treated animals on day 30 and 60 were non-significantly lower than day 0. TAA further decreased on day 90 to become significantly lower from day 0 by 26 %. On day 30 post-treatment, the activity increased significantly from day 90 by 18.23 %. Values on day 0, 30 and 60 did not differ significantly on corresponding values in control and different treatment groups. However, on day 90, it was significantly lower than control, but higher than group IV.

Decrease in TAA as observed in the present study may be due to exhaustion of antioxidant defense due to flubendiamide-induced excess free radicals generation. However, there is no report available to compare the results.

B. Oral Chronic Lead Toxicity Study

Administration of lead acetate for 90 days resulted into progressive decrease in TAA reaching the lowest level on day 90, when it differed significantly from day 0 by 38.58 per cent. Level on day 90 was significantly lower than corresponding value in control, but it did not differ significantly from group II and group IV. TAA activity increased non-significantly by 25.62 % on day 30 post-treatment.

Results of the present study was in corroboration with the study of Wieloch *et al* (2012) who observed that total antioxidant activity was lower in volunteers from polluted area as compared with those from unpolluted area (0.731 Trolox-equivalents vs. 0.936 Trolox-equivalents, respectively). Likewise, Ahmed *et al* (2008) observed decrease in TAA in pregnant buffaloes reared besides highways, having elevated blood lead levels. Ghareeb *et al* (2010) recorded decrease in TAA in different parts of the rat brain after lead exposure. Decrease in TAA may be due to direct inhibitory action of lead on many antioxidant enzymes and/ or exhaustion of antioxidant defense in attempt to neutralize the effects of lead-induced oxidative stress.

C. Oral Chronic Flubendiamide-Lead Interaction Study

Combined exposure to flubendiamide and lead resulted into progressive non-significant decrease in TAA on day 30 and 60. On day 90, the TAA was lowest in comparison to corresponding values in groups II and III and it also differed significantly from control and group II animals. TAA increased significantly on day 30 post-treatment to reach a value comparable to day 0.

Greater decrease in TAA in group IV in comparison to groups II and III on different observation days suggested interactive effects of lead and flubendiamide on antioxidant defense in buffalo calves. Free radicals are highly reactive molecules that are produced during normal metabolism in the body or after exposure to toxicants and environmental pro-oxidants. Excess free radicals cause a dangerous chain reaction that can destroy nucleic acids, proteins, lipids and other cellular compounds (Halliwell and Gutteridge 1999). Body counteracts against ill effects of free radicals via antioxidant defense system that comprises antioxidant enzymes, other chemicals, vitamins and nutrients such as reduced glutathione, ubiquinone, vitamin C, vitamin E, zinc, folate and carotenoids. Oxidative stress supervenes when generated free radicals exceeds the capacity of antioxidant defense of the body (Santra *et al* 2000). The total antioxidant activity or total antioxidant capacity reflects combined capacity of different components of antioxidant defense of the body to counteract the effects of a pro-oxidant in natural or laboratory conditions. There are several methods for analyzing antioxidant activity/ capacity of biological fluids and tissues (Huang *et al* 2005). The method for estimation of total antioxidant activity used in the present study is based upon the measurement of the capacity of the biological fluids to inhibit the production of thiobarbituric acid reactive substances (TBARS) from sodium benzoate under influence of the free oxygen radicals derived from Fenton's reaction.

V. Minerals

Calcium

Table 12 and figure 35 show the influence of flubendiamide, lead and their interaction on blood calcium levels.

A. Oral Chronic Flubendiamide Toxicity Study

In buffalo calves given flubendiamide for 90 days, blood calcium level did not show any significant change on different observation days. Only a marginal decrease by 5.59 % from day 0 value was observed on day 90. The calcium concentration on day 30 post-treatment increased by 4.14 % from day 90 level.

There is no published research report available to compare findings of the present study. Flubendiamide stabilizes insect ryanodine receptors (RyRs) to an open state, evoking massive calcium release from intracellular stores and hence continuous muscle contraction and death (Ebbinghaus- Kintscher *et al* 2006). However, flubendiamide is having high affinity only for insect RyRs and do not affect mammalian RyRs. It seems that flubendiamide does not affect the calcium homeostasis in buffalo calves.

B. Oral Chronic Lead Toxicity Study

In lead intoxicated buffalo calves, calcium concentration declined non-significantly on day 30 and 60. However, on day 90 Ca concentration decreased by 15.08 % from day 0, to reach a level significantly lower than both day 0 and 30. The Ca concentration did not return to normal level even on day 30 post-treatment, when it was still 4.92 % lower than day 0 level.

Findings of the present study was in agreement with the observation of El-Hameed *et al* (2008) who reported decrease in serum calcium concentration in pregnant Baladi goats following oral administration of lead acetate for a period of 14 weeks. Likewise, Hamadouche *et al* (2009) also reported significant decrease in serum Ca in lead-intoxicated Albino rats.

Decrease in Ca level as observed in the present study may be a result of hypoproteinemia and/ or due to renal impairment and depressive effect of lead on parathyroid gland function (Ahmed and Shalaby 1991). In addition, hypocalcemia may also be a result of competitive absorption between lead and calcium at the level of intestinal epithelium (Bodgen *et al* 1995). In

a randomized placebo controlled trial, Ca supplementation was associated with modest reductions in blood lead when administered during pregnancy (Ettinger *et al* 2009).

Many toxic effects of lead are related to its ability to mimic or in some cases inhibit the action of calcium as a regulator of cell function (Bressler and Goldstein 1991). Lead and calcium compete for the same binding sites on a group of ion-binding proteins including calmodulin, S-100, calretinin, calbindin and parvalbumin (Pauls *et al* 1996). Calcium and phosphorus in diet reduce lead absorption from the gastrointestinal tract (Fullmer 1991; Varnai *et al* 2001). The possible reason behind this phenomenon may be blockade of lead uptake through the intestine by calcium ions (Miller *et al* 1990; Fullmer 1992). On the reverse, excessive lead in diet may also block calcium uptake by intestinal epithelium.

C. Oral Chronic Flubendiamide-Lead Interaction Study

In animals intoxicated with flubendiamide and lead, Ca concentration did not differ significantly from each other on different observation days. However, on day 90 calcium level was 11.33 % lower than day 0. On day 30 post-treatment, the level increased by 16.04 % from day 90, but was still lower than the baseline value.

It is well established that calcium homeostasis is disturbed in lead poisoning. Simultaneous exposure of flubendiamide and lead in present study did not appear to have any significant effect on blood Ca concentration as on different observation days values were statistically comparable to the corresponding level in animals treated with lead alone.

Phosphorus

Changes in blood phosphorus level in calves following flubendiamide, lead and their combined exposure are summarized in table 12 and figure 36.

A. Oral Chronic Flubendiamide Toxicity Study

Phosphorus concentration in flubendiamide-intoxicated animals did not show any significant change on different sampling days. In comparison to day 0, mild non-significant increase was observed on day 30 and 90, while on day 60, the level was non-significantly lower.

There is paucity of available literature to compare results of the present study. Fluctuations in phosphorus level may be secondary to toxic effects on liver and hence alterations in metabolism of different nutrients.

B. Oral Chronic Lead Toxicity Study

In animals given lead acetate for 90 days, the phosphorus concentration decreased by 7.09 % on day 60 and by 14.89 % on day 90. However, levels on both day 60 and 90 did not differ significantly from day 0. On day 30 post-treatment, the phosphorus concentration again increased to reach a level almost equal to day 0.

The observed changes in the present study are consistent with the findings of El-Hameed *et al* (2008). They reported no significant change in serum phosphorus level in pregnant Baladi goats following oral administration of lead acetate for a period of 14 weeks.

C. Oral Chronic Flubendiamide-Lead Interaction Study

In animals exposed to both flubendiamide and lead, phosphorus concentration increased non-significantly on day 30 by 2.73 %. However, in comparison to day 0, phosphorus concentration decreased, albeit non-significantly on day 60 as well as day 90. Again on day 30 post-treatment, the level increased by 30.03 % from day 90, but the values did not differ significantly both from day 0 as well as day 90.

In the present study, phosphorus level not altered significantly in flubendiamide-lead interaction group. Neither flubendiamide, nor the lead exposure had significant effect on phosphorus concentration in blood.

Iron

Table 12 and figure 37 show the influence of flubendiamide, lead and their interaction on blood iron levels.

A. Oral Chronic Flubendiamide Toxicity Study

Flubendiamide-exposure resulted into decrease in blood iron concentration non-significantly on day 30 (4.36 %) and 90 (7 %). However, on day 60, it was almost equal to the level observed on day 0. On day 30 post-treatment, the level of iron in blood increased by 6.55 % from day 90. The concentrations on different observation days within the group did not differ significantly from each other as well as from the corresponding values in other treatment groups. There is no report available on changes in iron concentration in flubendiamide toxicity to compare results of the present study. Flubendiamide treatment appears to have no significant effect on blood iron level.

B. Oral chronic Lead Toxicity Study

In animals exposed to lead alone, the iron concentration on day 30 and 60 decreased non-significantly by 8.79 and 13.87 % respectively from day 0 value. However, on day 90, it decreased by 30.71 % to reach a level significantly lower than day 0 as well as day 30. The day 90 value was significantly lower than control and group II, but did not differ significantly from corresponding value in group IV. On day 30 post-treatment, the level increased significantly from day 90 (36.43 %) to reach a level statistically comparable to day 0 and 30.

Hamadouche *et al* (2009) observed significant decline up to 78.48 % in plasma total iron concentration in Albinos rats following exposure to lead acetate for 90 days, which supported findings of the present study. In a survey in human infants and children, Hegazy *et al* (2010) observed that blood lead level $\geq$ 10 μg/ dl was significantly associated with anemia, decreased

iron absorption and alterations in hematological parameters. High blood lead levels were associated with low serum iron concentration. Swarup *et al* (2005) also observed that blood iron concentrations in animals reared around lead-zinc smelters were lower (8.21%) than control. However, the values did not differ significantly. El-Hameed *et al* (2008) did not find any significant change in plasma iron concentration in pregnant Baladi goats given lead acetate for 14 weeks.

Interaction between lead and iron has been established in several studies. Low dietary ingestion of calcium or iron predisposes animals to lead toxicity (Carpenter 1982; Hashmi *et al* 1989). Iron deficiency combined with lead exposure acts synergistically to impair heme synthesis and cell metabolism (Waxman and Rabinowitz 1966). In humans, the amount of zinc and iron chelated by dicalcium EDTA treatment is also influenced by the level of lead in blood (Chisolm 1980). Interaction of zinc, lead and iron with identical proteins, possibly transport proteins that mediate metal uptake in intestine, seems the possible reason behind this interaction.

C. Oral Chronic Flubendiamide-Lead Interaction Study

In animals given both lead and flubendiamide, the iron concentration on day 30 and 60 did not differ significantly from day 0, though the levels were numerically lower in comparison to day 0. On day 90, the iron concentration decreased significantly by 24.32 % from day 0 value. The concentration on day 30 post-treatment increased by 17.93 % from day 90 to reach a level statistically comparable to day 0. On different observation days, the concentration did not differ significantly from corresponding values in other treatment groups except on day 90, when it was significantly lower than control and flubendiamide-treated group.

The per cent decrease from preceding levels on different observation days were lower than group III, but higher than group II. Concurrent exposure to flubendiamide and lead seems to

have no interaction on blood iron level, as evident from the statistically comparable values observed on different observation periods in all treatment groups.

Zinc

Table 13 and figure 38 show the influence of flubendiamide, lead and their interaction on blood zinc levels.

A. Oral Chronic Flubendiamide Toxicity Study

In flubendiamide treated group, blood zinc concentration did not differ significantly from each other on different observation periods. On day 30 and 60, the levels differed marginally from day 0. On day 90, the concentration was 27.53 % lower than day 0, though the values did not differ significantly. On day 30 post-treatment, the level increased albeit non-significantly by 13.06 % from day 90. Zinc concentrations on different observation days did not differ significantly from corresponding values in all other groups.

Flubendiamide-exposure seems to have no significant effect on blood zinc concentration. However, no report is available to compare results of the present study.

B. Oral Chronic Lead Toxicity Study

In animals given lead acetate for 90 days, the blood zinc concentration declined significantly on day 90 by 36.36 % from day 0. However, on day 30 (12.94 %) and 60 (9.27 %) levels were non significantly lower than day 0. The level on day 30 post-treatment increased by 52.67 % from day 90 to become statistically comparable to values observed on day 0, 30 and 60.

Results of the present study were in corroboration with the findings of White *et al* (1985), who observed decrease in zinc levels in all tissues except tibia, muscle and brain in calves fed 1500 ppm lead. El-Hameed *et al* (2008) also observed significant decline in serum zinc concentration in pregnant Baladi goats after oral administration of lead acetate for 14 weeks.

On the contrary, Patra and Swarup (2005) did not find any significant change in blood zinc concentration in calves following administration of lead acetate at a daily dose of 7.5 mg/ kg body weight for 28 days.

The beneficial effect of high zinc level on lead toxicity has been observed in horses (Schmitt *et al* 1971, Willoughby *et al* 1972) and in rats (Cerklewski and Forbes 1976a), but in swine, high levels of zinc enhanced the toxicity of lead (Hsu *et al* 1975). Zinc is reported to inhibit lead absorption from gastrointestinal tract. Verster (2011) reported significant decrease in lead absorption following co-administration of zinc sulfate in cattle. About 80 % of the total blood zinc concentration is present inside erythrocytes. Zinc given in the diet with Pb can protect animals against toxic effects of Pb, possibly by displacing Pb from Pb-inhibited Zn-dependent enzyme such as aminolevulenic acid dehydratase (ALAD) inside erythrocytes (Swarup and Dwivedi 2002). ALAD is an allostering enzyme consisting of eight subunits each containing one zinc atom and eight sulfhydryl groups. Lead is believed to inactivate ALAD by replacing zinc in a stoichiometric manner.

C. Oral Chronic Flubendiamide-Lead Interaction Study

In animals co-treated with lead and flubendiamide, the zinc concentration increased marginally on both day 30 (7.80 %) and 60 (10.23 %) in comparison to day 0. However, on day 90 and day 30 post-treatment, levels were 8.59 and 16.35 % lower than day 0. On different observation days, values within a group or corresponding values in control and other treatment groups did not differ significantly from each other.

Results of the present study indicated that flubendiamide and lead do not have any interactive effect on blood zinc concentration, as Zn concentrations in different treatment groups did not differ significantly on different observation days.

Copper

Table 13 and figure 39 depicts changes in blood copper level in calves after flubendiamide, lead and their combined exposure.

A. Oral Chronic Flubendiamide Toxicity Study

In animals, given flubendiamide for 90 days, blood copper concentration decreased progressively to reach a value 2.54, 10.43 and 9.34 % lower than day 0 on day 30, 60 and 90 respectively. On day 30 post-treatment, the level increased by 7.56 % from day 90, but it was still 2.44 % lower than day 0. Within the group, Cu concentration did not differ significantly from each other on different observation periods. However, on day 90, the level was significantly higher from corresponding values in group III and IV, but statistically comparable to control group.

Results of the present study indicate that flubendiamide administration does not significantly alter the blood Cu concentrations. However, further study is required to validate this hypothesis, since no related research report is available so far.

B. Oral Chronic Lead Toxicity Study

Blood copper concentration in comparison to day 0 was 10.85 and 17.48 % lower on day 30 and 60 respectively, though all three values did not differ significantly from each other. A significant drop in Cu concentration was observed on day 90, when it was 38.04 % lower than day 0. The level on day 90 was significantly lower than corresponding levels in control and group II, but comparable to the value observed in group IV. On day 30 post-treatment, the concentration increased by 28.84 % from day 90 to reach a level statistically comparable to day 0, 30 and 60.

Results of the present study were in agreement with the findings of Doyle and Younger (1984) who observed decrease in copper levels in blood, liver and heart following lead ingestion by bovines. Teodorova *et al* (2003) reported the antagonistic interactions between copper and lead. Liver, brain and kidneys from hypocupremic lambs had low concentrations of copper but relatively high concentration of lead (Kaszubkiewicz *et al* 1984). Binot *et al* (1972) induced copper deficiency in grazing cattle by spraying lead nitrate along with iron and zinc solution on the pasture 15 times in a period of 5 months. Withdrawal of lead from the diet resulted in a significant increase in blood copper suggesting that lead inhibits copper absorption (Mehennaoui *et al* 1988). Patra and Swarup (2005) observed a non-significant decline in blood copper concentration in cattle calves given lead acetate @ 7.5 mg/ kg body weight once daily for 28 days. However, they observed increasing trend in blood copper concentration in these lead-exposed animals after treatment with calcium disodium EDTA.

C. Oral Chronic Flubendiamide-Lead Interaction Study

In animals receiving oral administration of both lead and flubendiamide, the copper concentration on day 30 and 60 was 13.76 and 12.47 % lower than day 0, but the values did not differ significantly from each other. On day 90, the Cu concentration was significantly lower (40.49 %) than day 0 as well as day 30 post-treatment. Moreover, the level on day 90 was significantly lower than corresponding levels in control and group II animals.

Results of the present study suggested no significant interaction between flubendiamide and lead on blood copper levels. However, large scale study is required to validate this hypothesis.

Manganese

Table 13 and figure 40 show the influence of flubendiamide, lead and their interaction on blood manganese levels.

A. Oral Chronic Flubendiamide Toxicity Study

Manganese concentration in flubendiamide-treated animals decreased non-significantly on day 30, 60 and 90 from day 0 value by 21.77, 13.60 and 22.45 %, respectively. However, the all four values did not differ significantly from each other. Day 90 level was significantly lower than corresponding value in control, but did not differ significantly from values observed in day 90 in other treatment groups. On day 30 post-treatment, the manganese concentration increased, albeit non-significantly by 23.68 % from day 90.

Flubendiamide administration had no significant effect on blood manganese levels on different observation days, as levels were statistically comparable to each other. Findings of the present study cannot be substantiated since no similar research report is available so far.

B. Oral Chronic Lead Toxicity Study

In animals treated with lead acetate for 90 days, the blood manganese concentration declined to reach a level up to 39.18 % lower than day 0. However, the values within a group on different observation days did not differ significantly from each other. On day 30 post-treatment, the concentration increased by 40.38 % from day 90, but again the difference was statistically non-significant.

Manganese in one of the least abundant trace elements in all livestock tissues (Underwood and Suttle 1999). Clays, silts, iron and manganese oxides, and soil organic matter can bind metals electrostatically (cation exchange) as well as chemically (specific adsorption) (Reed *et al* 1995). Therefore, marginal decrease in Mn concentration as observed in the present study may be due to binding of Pb with Mn in the diet reducing their bioavailability.

C. Oral Chronic Flubendiamide-Lead Interaction Study

Blood Mn concentrations in animals treated with flubendiamide and lead decreased non-significantly by 26.94 % on day 30. The level on day 60 and 90 decreased further to reach a level 43 and 52.33 % lower than day 0. Values observed on different observation period did not differ significantly from corresponding values in control and other treatment groups, except on day 90, when it was significantly lower than control. On day 30 post-treatment, the level increased by 43.47 % than day 90 to become statistically comparable to day 0.

Results of the present study indicated that flubendiamide and lead does not have any interactive effects on Mn level in blood.

Lead

Table 13 and figure 41 show the influence of flubendiamide, lead and their interaction on blood lead levels.

A. Oral Chronic Flubendiamide Toxicity Study

In animals given oral flubendiamide for 90 days, blood lead concentration did not differ significantly from day 0 on different observation days. The mean values varied between 0.070 ppm to 0.095 ppm which was close to the value observed in control animals. Results of the present study indicated that flubendiamide exposure does not have any effect on blood lead level.

B. Oral Chronic Lead Toxicity Study

Daily oral administration of lead acetate for 90 days resulted into progressive increase in blood lead concentration reaching peak on day 90, when it was 414.44 % higher than day 0. On day 30 post-treatment, the concentration decreased by 37.08 % from day 90 level. On day 60 and 90, levels were significantly higher than control and group II animals.

Results of the present study was in agreement with the findings of Patra and Swarup (2005) who observed mean blood Pb concentration of 0.93 µg/ ml following daily oral administration of lead acetate @ 7.5 mg/ kg body weight for 10 days. Likewise, Sahoo *et al* (2007) reported blood lead concentration to reach 1.05 ppm after administration of lead acetate @ 1 mg/ kg body weight once daily for 21 days in male cattle calves. The blood lead level of 0.35 µg/ ml is widely accepted as critical level of poisoning, but the death commence at 1.0 µg/ ml (Radostits *et al* 2000). However, the limit of blood lead concentration recommended by Agency for Toxic substance and Disease Registry is 60 µg/ dl in occupationally exposed human adults (ATSDR 2005). Sometimes, blood lead level above this critical level may not be manifested by characteristic clinical signs in animals (Koh and Babidge 1986), particularly in chronic Pb exposure. In the present investigation, most of the animals remained apparently normal up to one month of the Pb exposure and frank clinical signs of circling, blindness, head pressing, etc., suggestive of lead toxicity were not recorded. However, one calf died after one month of the experiment soon after showing nervous signs. On day 60, the mean value reached above the critical level and few animals started showing mild clinical signs of lead toxicity. The second calf died on day 93, i.e. 3 days after stopping lead acetate administration. Sujatha *et al* (2006) observed that when blood Pb level crosses 0.496 mg/ ml, early symptoms of lead poisoning, like mild to moderate anorexia starts and nervous signs and death occurs when the mean blood level reaches to 0.835 mg/ ml or more.

C. Oral Chronic Flubendiamide-Lead Interaction Study

Combined oral administration of flubendiamide and lead resulted into significant increase in blood lead concentration on day 60 and 90 by 604.28 and 975.71 % respectively from day 0. On day 60 and 90, per cent increase in blood lead concentration in comparison to day 0

were slightly higher than corresponding increase in group III, treated only with lead. On day 30 post-treatment, the concentration decreased by 34.26 % from day 90, but was still higher than the critical level as suggested by Radostits *et al* (2000).

Flubendiamide co-administration appeared not to have any effect on blood Pb concentration as increase in its level on different observation days were statistically comparable to corresponding values in animals receiving lead alone.

VI. Activity of Delta-Amino Levulinic Acid Dehydratase (ALAD)

Table 14 and figure 42 depicts changes in ALAD activity in blood of calves after flubendiamide, lead and their combined exposure. Figures 43 a and b show the blood lead concentration versus ALAD activity in lead and lead plus flubendiamide exposed animals.

A. Oral Chronic Flubendiamide Toxicity Study

Flubendiamide administration to buffalo calves resulted into non- significant increase in ALAD activity on day 30. However, on subsequent observation days the activity declined non-significantly to reach a level 9.23 and 11.33 % lower than day 0. Again a non-significant increase was noted on day 30 post-treatment.

Results of the present study suggested no significant effect of flubendiamide exposure in buffalo calves. There seems no research report available to compare the results of the present study.

B. Oral Chronic Lead Toxicity Study

Daily oral administration of lead acetate in buffalo calves resulted into non-significant decline (8.74 %) in ALAD activity on day 30. However, on day 60 and 90 the activities declined to become 34.57 and 56.11 % lower than day 0. The activity on day 60 and 90 were significantly

lower than corresponding activities in control and group II. On day 30 post-treatment, the activity increased to become statistically comparable to day 0.

In the present study, significant decline in ALAD activity was observed following oral exposure to lead in buffalo calves. This was in agreement with the study of Bratton *et al* (1986) who observed significant reduction in ALAD activity in cattle erythrocytes within 24 hours of lead administration. Likewise, Hoffman *et al* (1981) observed up to 80 % reduction in ALAD activity in bald eagles within 24 hours of lead shot ingestion. ALAD is an essential enzyme in the biosynthetic pathway of heme and is required to maintain hemoglobin content in erythrocytes. It is the second enzyme in the heme biosynthetic pathway, which is cytosolic and non-limiting in healthy cells. The enzyme catalyzes the condensation of two molecules of 5-aminolevulinic acid (ALA) to form one molecule of the monopyrrole porphobilinogen (PBG). Inhibition of red blood cell ALAD has become accepted as a standard bioassay to detect acute and chronic lead exposure in humans and other mammals (Hernberg *et al* 1970). Measurement of plasma ALAD activity has been found better indicator of Pb toxicity than ALAD activity in whole blood in cattle naturally exposed to Pb (Kang *et al* 2010). Gibson *et al* (1955) for the first time reported that activity of this enzyme is severely inhibited by lead. When lead enters the blood stream, over 99 per cent of the total quantity is taken up by the erythrocytes and less than one per cent remains in the plasma. Previously, the accumulation of lead in erythrocytes has been ascribed to its affinity for hemoglobin, but later on it was discovered that principal lead-binding protein in RBC is ALAD (Berghahl *et al* 1997). Inherited deficiency of ALAD associated with constant low activity of this enzyme and porphryia has also been reported in human beings (Gross *et al* 1998).

C. Oral Chronic Flubendiamide-Lead Interaction Study

Combined oral exposure to flubendiamide and lead caused non-significant alterations in ALAD activity on day 30. But on day 60 and 90, activities significantly decreased to become 28.90 and 42.65 % lower than day 0. On day 60 and 90, activities were significantly lower than corresponding values in control and group II, but comparable to group III values. On day 30 post-treatment, the activity increased by 38.79 % from day 90 to become statistically comparable to day 0.

Results of the present study suggested no significant interaction of flubendiamide with lead since ALAD activities in group III and IV on different observation days did not differ significantly from each other.

VII. Glial Fibrillary Acidic Protein (GFAP)

Influence of flubendiamide, lead and their interaction on concentration of GFAP in CSF is summarized in table 14 and figure 44. Figures 45 a and b depict lead concentration versus GFAP concentration in lead and lead plus flubendiamide exposed calves.

A. Oral Chronic Flubendiamide Toxicity Study

Daily oral flubendiamide administration for 90 days in buffalo calves did not induce any significant change in GFAP concentration on different observation days, though the values increased on day 30, 90 and post-treatment day 30. Moreover, values on different observation days did not differ significantly from corresponding values in control. But on day 60 and 90, values were significantly lower than the values observed in group III and IV.

There is no report available to compare results of the present study. Flubendiamide exposure did not appear to influence the GFAP level in CSF of buffalo calves. Glial fibrillary acidic protein (GFAP) is synthesized in astroglial cells in all parts of the central nervous system. GFAP is the structural subunit of the astroglial filaments which are mainly found in the fibrillary

astrocytes. Because only low levels of this protein is present in serum, structural damage to the brain causes a selective leakage of this protein from the brain tissue into the cerebrospinal fluid irrespective of the blood-brain barrier dysfunction. GFAP is not readily soluble but is highly susceptible to degradation to form water soluble products (De Armond *et al* 1983). Concentration of GFAP in CSF increases as a consequence of acute encephalomyelitis, encephalitis, meningitis, intracranial tumors, cerebrovascular damage and dementia (Lowenthal *et al* 1978; Aurell *et al* 1991). Besides acute brain disorders, level of GFAP also increases in chronic disorders with astrogliosis (Rosengren *et al* 1994).

B. Oral Chronic Lead Toxicity Study

Exposure to lead acetate in buffalo calves resulted into non-significant decrease in GFAP concentration on day 30. However, the level increased significantly from day 0 value on day 60 and 90 by 81.55 and 225 % respectively. On day 30 post-treatment, the level decreased significantly by 23.44 % from day 90 level, but it was still significantly higher than day 0 (by 148.81%).

Results of the present study was in agreement with the findings of Struzynska *et al* (2007) who observed increase in GFAP in immature rat brain following exposure to lead acetate. Likewise, Gong *et al* (1995) also observed increase in GFAP concentration in rats following exposure to trimethyl lead. Increase in GFAP in CSF in present study may be due to lead-induced activation of astroglial cells. Several studies have shown that astroglia can accumulate and store Pb and may function as a ‘‘Pb sink’’ (Holtzman *et al* 1984; Tiffany-Castiglioni *et al* 1986). Toxic Pb insult in the early period of embryonic development may impair both glial and neuronal function (Stoltenburg-Didinger *et al* 1996). The most important function of astroglial cells is maintenance of the homeostatic environment for proper functioning of neurons. Under

many pathological conditions including heavy metal toxicities, glial cells undergo rapid changes which have been described as reactive gliosis. One of the most important feature connected with this phenomenon is the increased expression of two glial markers, GFAP and S-100b protein. Over expression these two proteins occurs in response to neuronal damage (Griffin *et al* 1998).

C. Oral Chronic Flubendiamide-Lead Interaction Study

Combined exposure to flubendiamide and lead resulted into significant increase in GFAP concentration on day 60 and 90 by 53.57 and 173.98 % respectively from day 0. On day 30 post-treatment, the level declined non-significantly by 4.28 % from day 90, but it was 162.24 % higher than day 0. On day 60 and 90, per cent increase in GFAP concentration was lower than corresponding increase in group III, suggesting no significant effect of lead and flubendiamide co-administration on GFAP level in CSF.

VIII. Aspartic Acid (AA)

Effects of administration of flubendiamide, lead and their combination on concentration of aspartic acid in CSF is summarized in table 14 and figure 46. Figures 47 a and b depict lead concentration versus aspartic acid concentrations in lead and lead plus flubendiamide exposed calves.

A. Oral Chronic Flubendiamide Toxicity Study

Flubendiamide administration in buffalo calves resulted into progressive increase in AA concentration on day 30, 60 and 90 by 17.61, 55.11 and 73.29 % respectively. However, the values observed only on day 60 and 90 differed significantly from day 0. The level on day 30 post-treatment decreased non-significantly from day 90 level, but was still higher by 30.68% from day 0.

There seem no research report available documenting changes in AA level in CSF during flubendiamide exposure to compare results of the present study. Glutamate and aspartate are two excitatory amino acids (EAAs), necessary as neurotransmitters for normal function of

central nervous system. However, supraphysiological concentrations of glutamate and aspartate in the extracellular fluid are known to be neurotoxic (Meldrum 1993). Under physiologic conditions, large amounts of EAAs are stored in intracellular compartments and hence the EAA concentrations in the cytoplasm of brain cell are conspicuously higher than those in the extracellular space. But during traumatic brain injury, cerebral ischemia, anoxia of the brain and epilepsy concentrations of EAAs increases many fold (Albin and Greenamyre 1992; Baker *et al* 1993; Hong *et al* 2001). Higher concentrations of EAAs are related to excitotoxic brain damage and poor prognostic outcome in patients with brain injury (Hong *et al* 2001).

B. Oral Chronic Lead Toxicity Study

Daily oral administration of lead acetate resulted into increase in AA concentration on day 30, 60 and day 30 post-treatment by a level up to 28.95 per cent. However, the values within the group on different observation days did not differ significantly from each other. Moreover, the values on different observation days were also statistically comparable to corresponding values in control.

Findings of the present study suggest that Pb exposure did not have significant effect on aspartic acid concentration in CSF. Increase in aspartate concentration in CSF has been demonstrated in many neurodegenerative diseases. Stover *et al* (1997) demonstrated a 2 to 3 fold increase in levels of these two excitatory amino acids in multiple sclerosis patients during clinical relapse but not in patients with silent multiple sclerosis. The researchers observed similarly high glutamate and aspartate levels in the CSF samples from patients with viral

meningitis and myelopathy. Sarchielli *et al* (2003) reported increase in glutamate and aspartate levels in the CSF of patients with relapsing-remitting multiple sclerosis.

C. Oral Chronic Flubendiamide-Lead Interaction Study

Combined exposure of flubendiamide and lead induced progressive but non-significant increase in AA concentration on day 30, 60 and 90 by 44.58, 61.78 and 71.34 % respectively from day 0 level. The level on day 60 and 90 were significantly higher than corresponding values in control animals and group III, but did not differ significantly from group II.

Results of the present study suggest that combined exposure of flubendiamide and lead have no significant interactive effect on aspartic acid concentration in CSF.

IX. Thyroid Hormones

Table 15 and figures 48, 49 and 50 show the influence of flubendiamide, lead and their interaction on status of thyroid hormones.

A. Oral Chronic Flubendiamide Toxicity Study

Daily oral administration of flubendiamide @ 0.024 mg/ kg body weight for 90 days resulted into non-significant decrease in T_3 level on day 60 and 90. However, on day 30 post-treatment, T_3 level increased significantly from day 90 as well as day 0 values. On day 60, T_3 level was significantly lower than lead alone treated group. However, T_3 level on different observation days did not differ significantly from corresponding levels in other treatment groups as well as control. T_4 level declined significantly by 24.92 % on day 90 and again a marginal non-significant increase was observed on day 30 post-treatment. In comparison to control, the T_4 level was significantly lower on day 90 only. On other observation days, it did not differ significantly from corresponding values in control. TSH activity in flubendiamide treated animals did not differ significantly from each other on different observation periods, though the

value on day 90 was 6.5 % lower than day 0. Moreover, on day 90 the TSH activity was significantly lower than corresponding levels in group III and IV, but statistically comparable to the control value. The activity on day 30 post-treatment increased from day 90 by 15.62 %, but both did not differ significantly.

Results of the present study indicated thyrotoxic effects of flubendiamide administration in male buffalo calves. This was in agreement with findings of two unpublished studies on chronic flubendiamide toxicity. Exposure to 34 to 44 mg flubendiamide per kg body weight for two years induced thyrotoxic effects in rats (Enomoto 2004; Takeuchi 2004). Histopathological changes in flubendiamide-intoxicated rats included increased incidences of follicular cell hypertrophy with hydropic changes, increased large-size follicles and altered colloid. Identical changes in thyroid ultra-structure were noted in female rats after exposure to a dose 10 times higher than in male rats. Thus, males appear to be more susceptible for the thyrotoxic effects of flubendiamide (APVMA 2009).

B. Oral Chronic Lead Toxicity Study

T_3 level in animals given lead acetate increased marginally on day 30 and 60, but a non-significant decrease (by 16.27 %) from day 0 level was observed on day 90. On day 30 post-treatment, the level increased again but values on different observation periods did not differ significantly from each other. Moreover, T_3 levels were statistically comparable to those observed in other treatment groups and control on different observation periods except on day 60, when it was significantly higher than other groups. A decreasing trend in T_4 level with passage of time was observed, however only day 90 level was significantly lower (30 %) from the day 0 value. On day 60, T_4 level was significantly lower than all other groups and on day 90, it was significantly lower than control and group IV. TSH activity in lead-treated animals

revealed an increasing trend with passage of time. The activity on day 90 increased significantly to reach a value 31.95 % higher than day 0. On day 90, TSH activity was significantly higher than all other treatment groups and a marginal decrease was observed on day 30 post-treatment.

Results of the present study were in agreement with the report of Ibrahim *et al* (2011) who observed a non-significant decline in T_3 and T_4 levels in male Albino rats after lead-intoxication. Several studies have documented a pattern of low peripheral T_4 and serum TSH levels in moderate to high lead-exposed workers suggesting occurrence of secondary hypothyroidism. Low serum T_4 with almost normal TSH concentration was observed in a study on brass-foundry workers after high lead exposure (Robins *et al* 1983). In another study on long term low-level lead exposure in male automobile workers, a negative correlation between blood lead levels and free T_4 was shown but TSH and T_3 did not differ significantly (Dundaro *et al* 2006). Likewise, Singh *et al* (2000) observed significantly higher TSH in automobile workers with high blood lead levels (52 µg/ dl), but T_3 and T_4 levels did not differ significantly. On the other hand, Swarup *et al* (2007) reported significantly higher T_3 and T_4 levels in cows reared around a polluted industrial area that had mean blood lead level 0.51 µg/ ml. High degree of positive correlation was observed between blood lead level and T_3 and T_4 concentration in these cows.

Among its many toxic effects, lead may adversely affect the pituitary-thyroid axis through an unknown mechanism (Pearce and Braverman 2009). The response appears to vary with blood lead concentration, at lower blood lead concentrations thyroid markers may be elevated or show inconsistent pattern, but at higher blood lead concentration (> 50 to 98 µg/ dl), they show declining trend (USEPA 2007). The exact mechanism of thyrotoxic effects of lead remains unknown, though several hypotheses have been proposed. They include alteration in

thyroxin metabolism or binding to proteins (Robins *et al* 1983) and lowered iodine uptake by thyroid gland (Katti and Sathyanesan 1987).

C. Oral Chronic Flubendiamide-Lead Interaction Study

Changes in T_3 level were inconsistent and non-significant on different observation period in animals after concurrent exposure to flubendiamide and lead. On day 90, T_3 level was marginally lower (3.74 %), but it again increased on day 30 post-treatment to reach a level 32.71 % higher than day 0. In between different treatment groups, values did not differ significantly from each other on a given observation day. T_4 level also varied inconsistently and non-significantly on different observation days. Day 90, T_4 level was 4.16 % lower than day 0 value, but day 30 post-treatment level was 5.17 % higher than day 0. TSH level increased marginally on day 30 and 60 but differences were statistically non-significant. On day 90, TSH level increased significantly to reach a value 37.72 % higher than day 0. On day 30 post-treatment, TSH activity decreased to a level lower than day 0 value.

Results of the present study indicated that thyrotoxic effects of both lead and flubendiamide did not alter after their combined exposure in buffalo calves, indicating lack of interaction of these two toxicants on thyroid hormone status.

Histopathology

Organ samples including brain, liver, kidney, lung and spleen were collected from animals died (two from lead alone exposed group and two from combined flubendiamide and lead exposed group) during the experiment and processed for histopathological examination.

A. Oral Chronic Lead Toxicity Study

Figures 51-56 show alterations in organ tissues of animals following chronic oral lead exposure. Mild purkinje cell degeneration in the molecular layer were evident in cerebellum

(Fig. 51). Cerebrum also showed marked vacuolar degeneration of neurons (Fig. 52). Brain in general and developing brain in particular is highly susceptible to toxic effects of lead (Swarup and Dwivedi, 2002). Lead crosses the blood-brain barrier rapidly and concentrates in the brain. Lead accumulates in brain tissues where its concentration does not fall rapidly even if the blood level of Pb^{2+} declines (Dyatlov *et al* 1998). In mature brain astroglial cells are believed to serve as Pb^{2+} sink (Tiffany-Castiglion and Qian 2001). It is well known that functional and structural abnormalities of the brain accompany acute and chronic lead intoxication.

Photomicrograph of liver showed swollen and degenerated hepatocytes with congested sinusoids (Fig. 53). Lung tissues showed signs of severe pneumonia as evident from presence of inflammatory exudates, polymorphonuclear cells infiltration and haemorrhage (Fig. 54 and 55). Changes in blood biochemical profile as observed in the present study can be attributed to these parenchymal changes in different organs. Pneumonia may be secondary to infection set up in lung owing to lead exposure associated immunosupression. Lead toxicity is associated with renal damage, hypertension, male reproductive and neurological disorders (Todd *et al* 1996). In rabbits, given lead acetate significant increase in ALT, AST, urea and creatinine concentrations were recorded by Elayat and Bakheetf (2010) indicating hepatotoxic and nephrotoxic effects of lead. Lead is known to cause proximal renal tubular damage, characterized by generalized aminoaciduria, hypophosphatemia with relative hyperphosphaturia and glycosuria accompanied by nuclear inclusion bodies, mitochondrial changes and cytomegaly of the proximal tubular epithelial cells. Tubular effects are noted after relatively short term exposures and are generally reversible, whereas sclerotic changes and interstitial fibrosis, resulting in decreased kidney function and possible renal failure, require chronic exposure to high lead levels (Swarup and Dwivedi, 2002).

Contrary to the present findings Nehru and Kaushal (1993) observed no change in histoarchitecture of liver after lead exposure.

Spleen did not show marked effect and only mild lymphoid depletion was evident (Fig. 56). The effects of lead on the hemopoietic system result in decreased haemoglobin synthesis, and anemia has been observed in children at blood lead concentrations above 1.92 mmol/litre (40 mg/ dL). Hence lymphoid depletion in lead toxicity is expected.

B. Oral Chronic Flubendiamide-Lead Interaction Study

In present study, cerebellum of combined lead and flubendiamide exposed calves revealed purkinje cell degeneration (Fig. 57). Cerebrum also showed neuronal degeneration, oedema and congested blood vessels (Fig. 58 and 59). These changes suggested neurotoxic effects of flubendiamide and or lead. Since no tissue specimen from flubendiamide alone exposed animal was available, it is difficult to conclude whether these changes were due to lead toxicity or flubendiamide toxicity or a combination thereof. Changes in aspartic acid and glial fibrillary acid protein concentrations in CSF were observed in both lead alone and flubendiamide alone exposed animals, suggesting neurotoxic potential of both the toxicants. Hence it may be assumed that these changes may be result of the neurotoxic potential of both the toxicant. However, there is no report available on effect of flubendiamide on histomorphology of brain cells to compare results of the present study.

Kidney (Fig. 60) showed severe tubular degeneration, sloughing of epithelial cells of the proximal convoluted tubules and presence of proteinaceous casts in the lumen. Liver (Fig. 61) revealed severely swollen and degenerated hepatocytes and fatty infiltration. There were absence of normal hepatocytic chord pattern evident, suggesting heaptotoxicity might be a contributory factor to death. Histopathological changes in liver of rats suggesting hepatotoxic effects of

flubendiamide were also observed by Enomoto (2003) after its administration @ 200 ppm or 15 mg/ kg body weight/ day for a period of 28-90 days in rats. Hepatotoxicity in lead toxicity in well established. Hence, histopathological changes in liver as observed in present study can be attributed to combined effect of these two toxicants.

Heart tissues revealed mild myocardial degeneration (Fig. 62). Kidney showed severe tubular degeneration and occlusion of lumen of proximal convoluted tubules with proteinaceous casts and sloughed off cells (Fig 63). Lung of combined lead and flubendiamide exposed calf showed oedema and polymorphonuclear cells suggestive of pneumonia (Fig. 64). There are no research report available investigating effects of flubendiamide on changes in these soft tissues architecture. Therefore, it may be assumed that these changes may be either due to lead or a combined effect of lead and flubendiamide.

CHAPTER V

SUMMARY

Flubendiamide belongs to a new class of insecticides phthalic acid diamides, whose mammalian toxicity has not been widely investigated so far. In the research work, chronic oral toxicity study of flubendiamide, lead and their interaction was undertaken in buffalo calves.

Healthy 8 to 12 months old male buffalo calves (n = 16) with body weight in between 120-180 kg were divided into four equal groups. Group I animals received no treatment to serve as untreated control. Group II animals were drenched Flubendiamide (Fame, Bayer Cropscience Limited, Sabarkanta, Gujarat) at the dose rate of 0.024 mg/ kg body weight once daily. Group III animals received lead acetate (Merck,) at the dose rate of 9.2 mg/ kg body weight orally once daily. Group IV animals received single oral dosing of both flubendiamde (0.024 mg/ kg) and lead acetate (9.2 mg/ kg). The treatment was continued for 90 days. On day 0, 30, 60 and 90 day of treatment and one month post-treatment, blood samples were collected by jugular venipuncture and CSF samples were collected by lumbo-sacral spinal tapping.

Mild toxicity signs of varying degree of dullness, depression, reduced feed consumption and lower body weight gain were observed in group II animals. Group III animals given lead acetate, mild to moderate toxicity signs including dullness, depression, weakness and nervous signs like aimless wandering, partial vision loss, nystagmus and twitching of eyelid muscles were observed in all four animals. One calf died on day 28 after showing symptoms like muscle tremors of the face and rear legs, frothing at mouth, gnashing of teeth, bellowing, recumbency, convulsive seizures and death. Another calf died on day 92 after showing similar signs of toxicity which developed suddenly. Group IV animals showed mild to moderate toxicity signs and 2 calves died (one on day 55 and another on day 95) until completion of the experiment.

Toxic signs were similar to those observed in lead alone treated group and included dullness, depression, weakness and nervous signs like aimless wandering, partial vision loss, nystagmus and twitching of eyelid muscles. In general, intensity of toxic signs was more severe in animals receiving both flubendiamide and lead in comparison to animals receiving only lead acetate or flubendiamide.

Daily oral administration of flubendiamide resulted into significant increase in ALT (34.40 ± 1.39 to 53.13± 2.29 U/ L), AST (147.08±8.06 to 178.29±5.01U/L), AKLP (255.02 ±17.84 to 301.25±11.62 U/L) and BUN (15.34±1.28 to 29.17±1.37 mg/dl) levels while GGTP and creatinine levels did not alter significantly. Likewise, haemoglobin (Hb), total erythrocyte count (TEC), mean corpuscular volume (MCV), mean corpuscular haemoglobin concentration (MCHC) and mean corpuscular hemoglobin (MCH) did not differ significantly, but significant decrease in packed cell volume (PCV) was noted. Total leukocyte count (TLC) decreased (10.15±0.54 to 7.715±0.46 X 10^3/ µl) significantly, but differential leukocyte count did not show any significant alteration. Flubendiamide exposure resulted into significant increase in LPO (6.60±0.48 to 9.59±0.27 n mol MDA), catalase (3595.03±76.01 to 3995.55 ± 45.23 µmole H_2O_2) and GST (4595.03±76.01 to 4913.05±50.10) activity, but decrease in SOD (6.54±0.25 to 5.35±0.37), GPx(8.14±0.37 to 5.63±0.34 EU/ mg Hb), blood glutathione (265.55±4.75 to 185.79±7.23 µmole/ ml), TAA (2.00±0.08 to 1.48±0.06 µ mole/ l), glutathione reductase (8.09±0.29 to 7.02±0.20 µmole NADPH/ min/ g Hb) and G6PD (5.87±0.29 to 4.83±0.23 EU/ mg Hb) activities. Calcium, Phosphorus, iron, zinc, copper, lead and manganese levels in blood did not show any significant change on day 90. The activity of delta amino lavulinic acid and concentration of glial fibrillary acidic protein in CSF did not differ significantly from day 0 value. However, aspartic acid concentration in CSF increased (1.76±0.23 to 3.05±0.43)

significantly. Decrease in level of T_3 and T_4 suggested thyrotoxic effects of flubendiamide administration in buffalo calves.

Oral administration of lead for 90 days resulted into increase in ALT (33.52±0.86 to 62.82±1.67 U/L), AST (134.34±7.57 to 307.26±7.54 U/L), AKLP (261.57±19.90 to 397.08±8.10 U/L), GGTP (10.95±0.92 to 27.46±1.98 U/L), BUN (14.87±2.34 to 33.46±1.78 mg/dl) and creatinine (1.22±0.11 to 1.76±0.04 mg/dl) suggesting hepatotoxic and nephrotoxic effects of lead. Significant decrease in Hb (12.40±0.42 to 8.07±1.01 g/dl), PCV (36.49±0.73 to 25.33±0.50 %) and TEC (6.91±0.31 to 5.06±0.39 millions/ µl) was observed, which suggested suppression of hematopoietic system in lead exposed buffalo calves. Peripheral blood film of lead exposed buffalo calves also revealed anemic changes, unisocytosis, basophilic stipplings and presence of nucleated RBCs. Exposure to lead alone resulted into significant decline in TLC, though neutrophil, eosinophil and basophil percentage did not alter significantly. Lymphocyte percentage showed decreasing trend while monocyte percentage showed increasing trend. Significant increase in LPO (6.01±0.31 to 10.85±0.41), Catalase (3771.09±87.80 to 4037.14±28.24), but decline in SOD (6.78±0.18 to 3.13±0.18), Glutathione (262.87±12.94 to 232.18±11.81), glutathione peroxidase (8.22±0.26 to 4.49±0.31), glutathione reductase (8.21±0.33 to 5.97±0.11), G6PD (5.89±0.12 to 13±0.11) and TAA (1.97±0.05 to 1.21±.12) revealed that oxidative stress supervenes in Pb exposed animals. Pb administration resulted into decrease in Ca (9.15±0.23 to 7.77±0.22 mg/dl), P (4.23±0.22 to 3.60±0.40), Fe (122.497±9.975 to 84.880±3.884), Zn (2.372±0.242 to 1. 462±0.284), Cu (1.041±0.056 to 0.645±0.036) and Mn (0.171±0.020 to 0.104±0.006) levels in blood. ALAD activity also declined significantly (285.88±12.84 to 125.47±19.78) in Pb exposed animals. Level of GFAP increased (1.68±0.23 to

5.46±0.25 ηg / ml) while aspartic acid declined marginally in CSF. Decrease in T 3 and T4 and increase in TSH suggested thyrotoxic effects of Pb.

Combined oral administration of flubendiamide and lead resulted into increase in ALT (35.70±1.34 to 62.17±2.66 u/l), AST (135.23±2.68 to 381.59±10.65 u/l), AKLP (256.82±16.58 to 367.01±39.86 u/l), GGTP (12.56±1.69 to 31.42±2.27 u/ l), BUN (16.27±1.19 to 38.81±1.77 mg/dl) and creatinine (1.35±0.09 to 1.69±0.08 mg/dl) suggesting additive effects of the two toxicants in terms of their hepatootoxic and nephrotoxic potential. Significant decline in Hb (12.22±0.71 to 8.33±0.38 g/dl), PCV (35.37±0.74 to 27.07±0.38 %) and TEC (6.67±0.06 to .535±0.24 millions / µl) were recorded suggesting suppressive effects on hematopoietic system. Combined exposure of flubendiamide and Pb resulted into decrease in TLC and neutrophil percentage to a degree greater than those observed in Pb alone exposed group suggesting synergistic effects of the two toxicants. Levels of LPO (6.08±0.93 to 11.76±0.27) and catalase (3809.36±98.59 to 4229.10±81.08) increased, while SOD (6.56±0.23 to 4.77±0.25), glutathione (264.52±13.44 to 177.85±8.44), GPx (8.17±0.19 to 5.25±0.26), glutathione reductase (8.11±0.27 to 6.99±0.49) and G6PD (5.99±0.22 to 4.55±0.29) declined suggesting existence of oxidative stress in buffalo calves. Blood concentration of Ca, P, Fe, Zn, Cu and Mn declined significantly revealing negative balance of these minerals in lead and flubendiamide exposed animals. ALAD activity declined (271.09±15.18 to 155.46±26.37) in blood and levels of GFAP (1.96±0.08 to 5.37±0.28) and aspartic acid (1.57±0.26 to 2.69±0.16) in CSF increased suggesting neurotoxic effects of these two toxicants. Level of T_3 and T_4 did not differ significantly, but level of TSH increased from 4.56±0.23 to 6.28±0.38 µIU/ ml.

CONCLUSION

On the basis of results of the present study, it can be concluded that chronic flubendiamide and lead administration in buffalo calves causes increase in enzymes related to hepatic and renal functions. The toxicity is associated with neurotoxic effects, increase in oxidative stress and adverse effects on thyroid hormone function. Blood mineral profile is also altered during the toxicity. The two toxicants appear to have additive effects on various toxicological parameters recorded in the present study. However, further study is required to elucidate the mechanism and degree of interaction of the two toxicants.

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Table 3. Effect of chronic toxicity of flubendiamide, lead and their interaction on alanine aminotransferase, aspartate aminotransferase and alkaline phosphatase (Mean ± S. E.) activities in buffalo calves

Group	Day of observation				
	0	30	60	90	30 post-treatment
Alanine aminotransferase activity (U/ l)					
I	36.20±1.74^{aA}	34.33±0.83$^{a\ A}$	38.80±1.83^{aA}	34.97±2.78^{aA}	40.07±1.69^{aA}
II	34.40±1.39$^{\ aA}$	42.99±2.13^{bB}	54.96±1.62^{cB}	53.13±2.29^{cB}	49.16±2.45^{cB}
III	33.52±0.86$^{a\ A}$	44.98±2.09^{bB}	53.84±3.14^{cB}	62.82±1.67^{dC}	45.68±2.35^{bAB}
IV	35.70±1.34$^{a\ A}$	43.19±1.66^{bB}	52.71±2.73^{cB}	62.17±2.66^{dC}	52.97±1.84^{cB}
Aspartate aminotransferase activity (U/ l)					
I	130.30±2.92^{aA}	139.83±5.42^{aA}	127.55±9.33^{aA}	140.27±10.40^{aA}	133.33±8.54^{aA}
II	147.08±8.06$^{b\ A}$	114.99±6.29^{aA}	159.36±7.64^{bcA}	178.29±5.01^{cB}	170.08±5.61^{cB}
III	134.34±7.57$^{\ aA}$	165.71±12.62^{aB}	210.86±16.67^{bB}	307.26±7.54^{cC}	173.33±13.30^{aB}
IV	135.23±2.68^{aA}	197.16±8.36$^{b\ C}$	295.15±12.77^{cC}	381.59±10.65^{dD}	215.67±4.34^{bC}
Alkaline phosphatase (U/ l)					
I	251.07±17.20^{abA}	262.47±17.95^{abAB}	242.14±16.97^{aA}	298.04±5.58^{bA}	254.28±16.83^{abA}
II	255.02±17.84^{abA}	242.65±17.23^{aAB}	287.17±11.30^{abAB}	391.25±11.62^{cB}	289.50±12.24^{bA}
III	261.57±19.90^{aA}	272.53±14.88^{aB}	382.11±13.31^{bC}	397.08±8.10^{bB}	252.44±23.50^{bA}
IV	256.82±16.58^{abA}	209.41±17.61^{aA}	335.42±21.28^{bcBC}	367.01±39.86^{cB}	293.41±8.69^{cA}

Gr I: Control; Gr II: Flubendiamide; Gr III: Lead; Gr IV: Flubendiamide + Lead

Values bearing different superscript in small letters in a row and capital letters in a column differ significantly ($P<0.05$)

Table 4. Effect of chronic toxicity of flubendiamide, lead and their interaction on gamma glutamyl transpeptidase, blood urea nitrogen and creatinine (Mean ± S. E.) levels in buffalo calves

Gamma glutamyl transpeptidase (U/ l)					
Group	**Day of observation**				
	0	**30**	**60**	**90**	**30 post-treatment**
I	14.76±1.02^{aA}	15.89±2.28^{aAB}	15.23±1.94$^{a\ A}$	14.99±2.15^{aA}	14.25±1.80^{aA}
II	12.65±1.34$^{a\ A}$	13.57±1.92^{aAB}	19.36±1.09^{aA}	20.67±2.80^{aAB}	18.89±1.94$^{a\ A}$
III	10.95±0.92$^{a\ A}$	17.33±2.30^{abB}	19.15±1.17$^{ab\ A}$	27.46±1.98^{bBC}	19.30±0.47^{abA}
IV	12.56±1.69^{aA}	10.86±0.92$^{a\ A}$	20.53±3.03$^{ab\ A}$	31.42±2.27^{bC}	21.08±1.11^{abA}
Blood urea nitrogen (mg/ dl)					
I	15.64±2.35^{aA}	17.98±1.84^{aA}	15.54±1.78^{aA}	14.95±2.37^{aA}	14.38±1.76^{aA}
II	15.34±1.28^{aA}	21.23±1.69^{bAB}	24.19±1.98^{bcBC}	29.17±1.37^{cB}	24.24±2.13^{cB}
III	14.87±2.34^{aA}	19.72±0.88^{abAB}	22.50±2.91^{bcB}	33.46±1.78^{dBC}	27.75±2.23^{cdBC}
IV	16.27±1.19^{aA}	24.02±1.63^{bB}	30.74±1.66^{cC}	38.81±1.77^{dC}	34.02±2.87^{cdC}
Creatinine (mg/ dl)					
I	1.26±0.07^{aA}	1.25±0.11^{aA}	1.20±0.11^{aA}	1.26±0.11^{aA}	1.37±0.13^{aA}
II	1.29±0.13^{aA}	1.25±0.12^{aA}	1.29±0.11^{aAB}	1.40±0.09^{aAB}	1.34±0.1^{aA}
III	1.22±0.11^{aA}	1.21±0.10^{aA}	1.51±0.08^{abAB}	1.76±0.04^{bC}	1.46±0.00^{abA}
IV	1.35±0.09^{aA}	1.29±0.11^{aA}	1.56±0.06^{aB}	1.69±0.08^{aBC}	1.33±0.29^{aA}

Gr I: Control; Gr II: Flubendiamide; Gr III: Lead; Gr IV: Flubendiamide + Lead

Values bearing different superscript in small letters in a row and capital letters in a column differ significantly ($P<0.05$)

Table 5. Effect of chronic toxicity of flubendiamide, lead and their interaction on hemoglobin, packed cell volume and total erythrocyte count (Mean ± S. E.) in buffalo calves

Haemoglobin (g/ dl)					
Group	**Day of observation**				
	0	**30**	**60**	**90**	**30 post-treatment**
I	13.07 ± 0.82^{aA}	12.00 ± 0.85^{aA}	13.20 ± 0.63^{aB}	12.05 ± 1.08^{aB}	12.12 ± 0.13^{aB}
II	12.90 ± 0.40^{bA}	11.60 ± 0.92^{abA}	12.10 ± 0.59^{abAB}	10.60 ± 0.71^{aAB}	10.37 ± 0.37^{aA}
III	12.40 ± 0.42^{cA}	12.43 ± 0.34^{cA}	10.47 ± 0.49^{bcA}	8.07 ± 1.01^{aA}	9.50 ± 0.50^{abA}
IV	12.22 ± 0.71^{bA}	11.00 ± 0.57^{bA}	11.93 ± 0.74^{bAB}	8.33 ± 0.38^{aA}	10.40 ± 0.60^{abA}
Packed cell volume (per cent)					
I	37.17 ± 0.88^{abA}	36.35 ± 0.59^{abA}	38.85 ± 0.78^{bB}	35.92 ± 0.96^{aC}	38.92 ± 0.74^{bC}
II	35.88 ± 0.37^{bA}	34.30 ± 0.64^{abA}	34.14 ± 0.57^{abA}	32.67 ± 0.80^{aB}	34.45 ± 0.95^{abB}
III	36.49 ± 0.73^{dA}	34.71 ± 0.21^{cdA}	32.47 ± 1.23^{cA}	25.33 ± 0.50^{aA}	29.15 ± 0.35^{aB}
IV	35.37 ± 0.74^{cA}	36.58 ± 0.89^{cA}	33.87 ± 1.63^{bcA}	27.07 ± 0.38^{aA}	31.55 ± 0.95^{bAB}
Total erythrocyte count (millions/ µl)					
I	6.48 ± 0.22^{aA}	6.58 ± 0.49^{aA}	6.68 ± 0.21^{aB}	6.64 ± 0.24^{aB}	6.37 ± 0.21^{aC}
II	6.81 ± 0.43^{aA}	6.50 ± 0.32^{aA}	6.35 ± 0.25^{aB}	6.17 ± 0.12^{aB}	6.28 ± 0.22^{aBC}
III	6.91 ± 0.31^{bA}	5.59 ± 0.45^{aA}	5.22 ± 0.43^{aA}	5.06 ± 0.39^{aA}	5.46 ± 0.06^{aAB}
IV	6.67 ± 0.06^{cA}	6.23 ± 0.19^{bcA}	5.81 ± 0.06^{abAB}	5.35 ± 0.24^{aA}	5.36 ± 0.33^{aA}

Gr I: Control; Gr II: Flubendiamide; Gr III: Lead; Gr IV: Flubendiamide + Lead

Values bearing different superscript in small letters in a row and capital letters in a column differ significantly ($P<0.05$)

Table 6. Effect of chronic toxicity of flubendiamide, lead and their interaction on mean corpuscular volume, mean corpuscular hemoglobin concentration and mean corpuscular hemoglobin (Mean ± S. E.) in buffalo calves

Mean corpuscular volume (fl)					
Group	**Day of observation**				
	0	**30**	**60**	**90**	**30 post-treatment**
I	57.51±2.17^{aA}	55.89±3.09^{aA}	58.42±2.76^{aA}	54.37±2.64^{aA}	61.41±3.06^{aA}
II	53.25±2.86^{aA}	53.13±2.70^{aA}	54.02±2.25^{aA}	52.93±0.75^{aA}	55.13±2.89^{aA}
III	53.09±2.15^{aA}	63.06±5.81^{aA}	62.87±4.26^{aA}	50.53±2.82^{aA}	53.34±0.60^{aA}
IV	53.10±1.57^{abA}	58.81±1.93^{bA}	58.26±2.31^{abA}	50.74±1.68^{aA}	59.19±5.42^{bA}
Mean corpuscular haemoglobin concentration (MCHC) (g/dl)					
I	35.26±2.51^{aA}	33.01±2.30^{aA}	34.09±2.18^{aA}	33.76±3.75^{aA}	31.19±0.75^{aA}
II	35.95±1.06^{bA}	33.83±2.64^{abA}	35.40±1.26^{bA}	32.45±2.10^{abA}	28.81±2.09^{aA}
III	34.00±1.16^{aA}	35.82±0.90^{aA}	32.36±2.15^{aA}	31.83±3.98^{aA}	32.57±1.32^{aA}
IV	34.54±1.73^{aA}	30.08±1.41^{aA}	35.46±3.11^{aA}	30.81±1.59^{aA}	32.94±0.91^{aA}
Mean corpuscular haemoglobin (MCH) (pg)					
I	20.23±1.41^{aA}	18.54±2.02^{aA}	19.79±0.86^{aA}	18.18±1.55^{aA}	19.10±0.65^{aAB}
II	19.11±0.99^{aA}	18.18±2.44^{aA}	19.11±0.99^{aA}	17.15±0.99^{aA}	15.76±0.79^{aA}
III	18.06±1.02^{abA}	22.54±1.85^{bA}	20.23±1.24^{abA}	16.05±2.09^{aA}	17.37±0.71^{abAB}
IV	18.36±1.18^{abA}	17.63±0.62^{abA}	20.53±1.22^{bA}	15.63±0.89^{aA}	19.55±2.32^{abB}

Gr I: Control; Gr II: Flubendiamide; Gr III: Lead; Gr IV: Flubendiamide + Lead

Values bearing different superscript in small letters in a row and capital letters in a column differ significantly ($P<0.05$)

Table 7. Effect of chronic toxicity of flubendiamide, lead and their interaction on total leukocyte count, neutrophil percentage and lymphocyte percentage (Mean ± S. E.) in buffalo calves

Total leukocyte count (X 10^3/ µl)					
Group	**Day of observation**				
	0	**30**	**60**	**90**	**30 post-treatment**
I	10.57 ± 0.30^{aA}	11.19 ± 0.47^{aA}	10.39 ± 0.36^{aB}	9.80 ± 0.53^{aC}	10.69 ± 0.52^{aB}
II	10.15 ± 0.54^{bA}	10.505 ± 0.53^{bA}	9.38 ± 0.55^{bAB}	7.715 ± 0.46^{aB}	7.84 ± 0.44^{aA}
III	10.42 ± 0.55^{bA}	10.95 ± 0.54^{bA}	8.22 ± 0.47^{aA}	6.31 ± 0.64^{aAB}	8.30 ± 1.25^{aA}
IV	10.22 ± 0.42^{cA}	10.78 ± 0.41^{cA}	7.98 ± 0.35^{bA}	5.13 ± 0.20^{aA}	7.60 ± 0.85^{bA}
Neutrophil (percentage)					
I	27.25 ± 1.11^{aA}	28.75 ± 2.66^{aA}	26.50 ± 1.55^{aA}	27.00 ± 0.82^{aB}	28.50 ± 1.04^{aA}
II	26.50 ± 1.32^{aA}	28.50 ± 2.22^{aA}	26.50 ± 1.32^{aA}	25.50 ± 1.50^{aB}	28.25 ± 1.18^{aA}
III	28.25 ± 2.63^{aA}	26.67 ± 2.90^{aA}	23.67 ± 0.88^{aA}	24.33 ± 0.88^{aAB}	24.50 ± 0.50^{aA}
IV	29.00 ± 1.87^{bA}	27.50 ± 1.04^{bA}	24.67 ± 1.76^{abA}	21.33 ± 1.45^{aA}	25.00 ± 1.00^{abA}
Lymphocyte (percentage)					
I	67.50 ± 1.32^{aA}	66.25 ± 2.56^{aA}	68.00 ± 1.08^{aB}	67.25 ± 0.85^{aB}	65.75 ± 1.11^{aBC}
II	66.25 ± 1.38^{aA}	65.00 ± 1.78^{aA}	63.50 ± 1.66^{aAB}	68.25 ± 1.38^{aB}	66.75 ± 1.18^{aC}
III	66.75 ± 2.25^{bA}	65.00 ± 2.08^{abA}	62.00 ± 2.52^{abA}	57.67 ± 1.85^{aA}	59.50 ± 1.50^{abA}
IV	65.75 ± 1.75^{aA}	64.25 ± 1.89^{aA}	62.33 ± 1.76^{aAB}	63.33 ± 2.33^{aB}	61.50 ± 1.50^{aAB}

Gr I: Control; Gr II: Flubendiamide; Gr III: Lead; Gr IV: Flubendiamide + Lead

Values bearing different superscript in small letters in a row and capital letters in a column differ significantly ($P<0.05$)

Table 8. Effect of chronic toxicity of flubendiamide, lead and their interaction on monocyte, eosinophil and basophil percentage (Mean ± S. E.) in buffalo calves

Monocyte (percentage)					
Group	**Day of observation**				
	0	**30**	**60**	**90**	**30 post-treatment**
I	2.25 ± 0.25^{aA}	2.25 ± 0.48^{aA}	1.75 ± 0.48^{aA}	2.25 ± 0.63^{aA}	1.75 ± 0.48^{aA}
II	3.50 ± 0.87^{abA}	3.25 ± 0.75^{abA}	5.25 ± 1.25^{bAB}	3.25 ± 0.75^{abA}	2.25 ± 0.63^{aA}
III	2.50 ± 0.64^{aA}	4.33 ± 2.18^{abA}	7.00 ± 1.53^{abcB}	9.33 ± 1.45^{cB}	8.50 ± 0.50^{bcC}
IV	2.00 ± 0.58^{aA}	4.75 ± 0.75^{abA}	8.67 ± 2.85^{bB}	7.67 ± 0.88^{bB}	5.50 ± 1.50^{abB}
Eosinophil (percentage)					
I	2.00 ± 0.41^{aA}	1.75 ± 0.48^{aA}	2.00 ± 0.41^{aA}	2.00 ± 0.41^{aA}	2.25 ± 0.48^{aA}
II	2.75 ± 0.48^{aA}	1.50 ± 0.87^{aA}	2.75 ± 0.48^{aA}	1.00 ± 0.41^{aA}	1.50 ± 0.64^{aA}
III	1.50 ± 0.87^{aA}	2.67 ± 1.76^{aA}	4.67 ± 1.76^{aA}	5.00 ± 0.58^{aB}	3.50 ± 0.50^{aAB}
IV	2.00 ± 0.41^{aA}	1.75 ± 0.48^{aA}	3.00 ± 1.15^{abA}	6.33 ± 1.20^{cB}	5.00 ± 0.00^{bcB}
Basophil (percentage)					
I	1.00 ± 0.41^{aA}	1.00 ± 0.00^{aA}	1.75 ± 0.25^{aA}	1.50 ± 0.29^{aA}	1.75 ± 0.25^{aA}
II	1.00 ± 0.00^{aA}	1.75 ± 0.48^{aA}	2.00 ± 0.41^{aA}	2.00 ± 0.00^{aA}	1.25 ± 0.75^{aA}
III	1.00 ± 0.41^{aA}	1.67 ± 0.88^{abA}	2.67 ± 0.88^{abA}	3.67 ± 0.88^{abB}	4.00 ± 1.00^{bA}
IV	1.25 ± 0.25^{aA}	1.75 ± 0.25^{aA}	1.33 ± 0.88^{aA}	1.33 ± 0.67^{aA}	3.00 ± 2.00^{aA}

Gr I: Control; Gr II: Flubendiamide; Gr III: Lead; Gr IV: Flubendiamide + Lead

Values bearing different superscript in small letters in a row and capital letters in a column differ significantly ($P<0.05$)

Table 9. Effect of chronic toxicity of flubendiamide, lead and their interaction on lipid peroxides, superoxide dismutase and catalase (Mean ± S. E.) in erythrocytes in buffalo calves

Lipid peroxides (n moles MDA produced/ g Hb/ hr)					
Group	**Day of observation**				
	0	**30**	**60**	**90**	**30 post-treatment**
I	6.19±0.69^{aA}	6.67±0.33^{aA}	6.45±0.29^{aA}	6.42±0.30^{aA}	6.05±0.43^{aA}
II	6.60±0.48^{aA}	7.24±0.27^{abA}	8.37±0.34^{cB}	9.59±0.27^{dB}	8.08±0.21^{bcB}
III	6.01±0.31^{aA}	7.27±0.33^{aA}	9.50±0.40^{bcC}	10.85±0.41^{cC}	9.10±0.85^{bBC}
IV	6.08±0.93^{aA}	8.38±0.35^{bB}	9.98±0.37^{bcC}	11.76±0.27^{cC}	10.11±0.46^{bcC}
Superoxide dismutase (enzyme causing 50% inhibition of auto-oxidation of pyragallol)					
I	6.52±0.41^{aA}	6.34±0.16^{aA}	6.22±0.18^{aB}	6.52±0.39^{aC}	6.75±0.15^{aC}
II	6.54±0.25^{bA}	6.34±0.44^{ABa}	6.14±0.23^{abB}	5.35±0.37^{aB}	6.18±0.13^{abBC}
III	6.78±0.18^{dA}	5.81±0.24^{Ca}	4.38±0.35^{bA}	3.13±0.18^{aA}	4.39±0.47^{bA}
IV	6.56±0.23^{cA}	6.15±0.46^{bcA}	5.19±0.39^{abA}	4.77±0.25^{aB}	5.69±0.45^{abcB}
Catalase (µmole H_2O_2 decomposed /min/mg Hb)					
I	3667.04±54.13^{aA}	3688.90±71.06^{aA}	3795.02±70.26^{aAB}	3626.59±72.34^{aA}	3669.64±60.25^{aA}
II	3595.03±76.01^{aA}	3681.70±87.38^{aA}	3706.36±64.33^{aA}	3995.55±45.23^{bB}	3661.20±63.71^{aA}
III	3771.09±87.80^{aA}	3857.69±61.95^{abB}	3929.91±12.95^{abB}	4037.14±28.24^{bBC}	3945.47±53.81^{abB}
IV	3809.36±98.59^{aA}	3861.29±68.14^{aA}	3958.47±35.39^{aB}	4229.10±81.08^{bC}	3840.45±60.43^{aAB}

Gr I: Control; Gr II: Flubendiamide; Gr III: Lead; Gr IV: Flubendiamide + Lead

Values bearing different superscript in small letters in a row and capital letters in a column differ significantly ($P<0.05$)

Table 10. Effect of chronic toxicity of flubendiamide, lead and their interaction on glutathione, glutathion peroxidase and glutathione reductase (Mean ± S. E.) activities in erythrocytes in buffalo calves

Glutathione (µmole/ ml)					
Group	**Day of observation**				
	0	**30**	**60**	**90**	**30 post-treatment**
I	258.10±11.12^{aA}	254.53±19.22^{aA}	259.06±7.86^{aA}	262.87±6.14^{aB}	260.20±11.70^{aB}
II	265.55±4.75^{bA}	256.35±8.97^{bA}	237.64±17.73^{bA}	185.79±7.23^{aA}	247.79±5.42^{bB}
III	262.87±12.94^{bA}	253.58±3.91^{bA}	231.41±15.26^{abA}	199.52±4.92^{aA}	232.18±11.81^{abAB}
IV	264.52±13.44^{cA}	244.26±18.41^{bcA}	221.02±13.06^{abcA}	177.85±8.44^{aA}	208.59±6.22^{abA}
Glutathione peroxidase (EU/ mg Hb)					
I	8.04±0.17^{aA}	7.90±0.31 ^{aA}	8.05±0.29^{aA}	7.88±0.39^{aB}	7.77±0.24^{aC}
II	8.14±0.37^{bA}	7.49±0.32^{bA}	7.14±0.39^{bA}	5.63±0.34^{aA}	6.07±0.08^{aB}
III	8.22±0.26^{cA}	8.31±0.42^{cA}	7.08±0.35^{bA}	4.49±0.31^{aA}	5.20±0.30^{aA}
IV	8.17±0.19^{bA}	7.98±0.28^{bA}	7.50±0.22^{bA}	5.25±0.26^{aA}	6.01±0.12^{aB}
Glutathione reductase (Oxidation of µmol NADPH /min/ g Hb)					
I	8.20±0.37^{aA}	8.12±0.46^{aA}	8.30±0.39^{aB}	8.09±0.18^{aC}	8.05±0.27^{aAB}
II	8.09±0.29^{bcA}	8.11±0.32^{bcA}	7.50±0.44^{abAB}	7.02±0.20^{aB}	8.78±0.24^{CB}
III	8.21±0.33^{cA}	8.29±0.23^{cA}	7.09±0.13^{bAB}	5.97±0.11^{aA}	6.95±0.06^{bA}
IV	8.11±0.27^{bA}	7.93±0.25^{abA}	6.43±0.33^{aA}	6.99±0.49^{abB}	7.47±1.36^{abAB}

Gr I: Control; Gr II: Flubendiamide; Gr III: Lead; Gr IV: Flubendiamide + Lead

Values bearing different superscript in small letters in a row and capital letters in a column differ significantly ($P<0.05$)

Table 11. Effect of chronic toxicity of flubendiamide, lead and their interaction on glutathione, total antioxidant activity and glucose-6-phosphate dehydrogenase activity (Mean ± S. E.) in erythrocytes in buffalo calves

Glutathione-s-transferase (µmol of conjugate of GSH and CDNB formed/ min/ g Hb)					
Group	**Day of observation**				
	0	**30**	**60**	**90**	**30 post-treatment**
I	4491.54±44.79 ^{aA}	4489.90±95.07 ^{aA}	4435.02±78.90 ^{aA}	4393.09±70.08 ^{aA}	4444.64±32.67 ^{aA}
II	4595.03±76.01 ^{aA}	4637.45±29.96 ^{aA}	4746.36±45.23 ^{abB}	4913.05±50.10 ^{bB}	4636.45±69.82 ^{aA}
III	4548.59±126.87 ^{aA}	4656.36±39.88 ^{abA}	4896.57±57.47 ^{bcBC}	5203.81±113.86 ^{cC}	4905.47±13.81 ^{bcB}
IV	4517.61±84.32 ^{aA}	4667.29±55.62 ^{abA}	4958.47±35.39 ^{cC}	5459.10±60.57 ^{dD}	4870.45±109.56 ^{bcB}
Glucose-6-phosphate dehydrogenase (EU/ mg Hb)					
I	5.81±0.10^{aA}	5.84±0.14^{aA}	5.79±0.16^{aC}	5.83±0.26^{aB}	5.67±0.21^{aA}
II	5.87±0.29^{bA}	6.09±0.17^{bA}	5.52±0.26^{abBC}	4.83±0.23^{aA}	5.34±0.19^{abA}
III	5.89±0.12^{bA}	7.15±0.17^{cB}	4.66±0.11^{aA}	4.13±0.11^{aA}	5.39±0.46^{bA}
IV	5.99±0.22^{cdA}	6.73±0.14^{dB}	5.11±0.11^{abAB}	4.55±0.29^{aA}	5.39±0.53^{bcA}
Total antioxidant activity (micromole/ l)					
I	1.87±0.05^{aA}	1.91±0.12^{aA}	1.92±0.05^{aA}	1.97±0.14^{aC}	2.00±0.05^{aB}
II	2.00±0.08^{cA}	1.87±0.06^{bcA}	1.84±0.06^{bcA}	1.48±0.06^{aB}	1.75±0.02^{bAB}
III	1.97±0.05^{cA}	1.89±0.11^{bcA}	1.79±0.11^{bcA}	1.21±.12^{aAB}	1.52±0.28^{abA}
IV	1.99±0.05^{bA}	1.84±0.07^{bA}	1.73±0.10^{bA}	1.07±0.16^{aA}	1.72±0.17^{bAB}

Gr I: Control; Gr II: Flubendiamide; Gr III: Lead; Gr IV: Flubendiamide + Lead

Values bearing different superscript in small letters in a row and capital letters in a column differ significantly ($P<0.05$)

Table 12. Effect of chronic toxicity of flubendiamide, lead and their interaction on calcium, phosphorus and iron concentration (Mean ± S. E.) in plasma in buffalo calves

Calcium (mg/ dl)					
Group	**Day of observation**				
	0	**30**	**60**	**90**	**30 post-treatment**
I	8.87±0.25^{aA}	8.72±0.21^{aA}	8.75±0.30^{aA}	8.77±0.27^{aA}	8.87±0.22^{aA}
II	8.95±0.26^{aA}	9.05±0.17^{aA}	8.55±0.22^{aA}	8.45±0.37^{aA}	8.80±0.25^{aA}
III	9.15±0.23^{bA}	8.83±0.23^{bA}	8.53±0.35^{abA}	7.77±0.22^{aA}	8.70±0.50^{abA}
IV	8.65±0.32^{aA}	8.62±0.40^{aA}	8.37±0.26^{aA}	7.67±0.47^{aA}	8.90±0.30^{aA}
Phosphorus (mg/ dl)					
I	4.20±0.28^{aA}	4.35±0.38^{aA}	4.05±0.13^{aA}	4.42±0.23^{aA}	4.60±0.28^{aA}
II	4.30±0.44^{aA}	4.40±0.25^{aA}	4.22±0.36^{aA}	4.42±0.25^{aA}	4.35±0.17^{aA}
III	4.23±0.22^{aA}	4.33±0.18^{aA}	3.93±0.33^{aA}	3.60±0.40^{aA}	4.25±0.55^{aA}
IV	4.40±0.28^{aA}	4.52±0.20^{aA}	4.37±0.47^{aA}	3.73±0.26^{aA}	4.85±0.65^{aA}
Iron (mg/ l)					
I	124.487±8.511^{aA}	121.550±7.186^{aA}	127.280±5.537^{aB}	115.202±5.261^{aB}	121.207±4.838^{aA}
II	120.432±6.915^{aA}	115.180±6.760^{aA}	120.132±6.031^{aAB}	112.000±7.527^{aB}	119.340±4.319^{aA}
III	122.497±9.975^{bA}	111.727±8.542^{bA}	105.507±3.746^{abA}	84.880±3.884^{aA}	115.805±4.195^{bA}
IV	122.090±9.893^{bA}	121.502±3.738^{bA}	118.070±5.138^{bA}	92.400±2.512^{aA}	108.965±5.165^{abA}

Gr I: Control; Gr II: Flubendiamide; Gr III: Lead; Gr IV: Flubendiamide + Lead

Values bearing different superscript in small letters in a row and capital letters in a column differ significantly ($P<0.05$)

Table 13. Effect of chronic toxicity of flubendiamide, lead and their interaction on plasma zinc, copper, manganese and lead concentrations (Mean ± S. E.) in buffalo calves

Zinc (mg/ l)					
Group	**Day of observation**				
	0	**30**	**60**	**90**	**30 post-treatment**
I	2.101±0.367^{aA}	2.140±0.254^{aA}	2.116±0.10^{aA}	2.469±0.256^{aA}	2.174±0.355^{aA}
II	2.219±0.22^{aA}	2.324±0.154^{aA}	2.020±0.283^{aA}	1.608±0.392^{aA}	1.818±0.187^{aA}
III	2.372±0.242^{bA}	2.065±0.310^{abA}	2.152±0.161^{abA}	1.462±0.284^{aA}	2.232±0.131^{abA}
IV	2.141±0.112^{aA}	2.308±0.296^{aA}	2.360±0.198^{aA}	1.957±0.266^{aA}	1.791±0.544^{aA}
Copper (mg/ l)					
I	1.042±0.089^{aA}	1.062±0.059^{aA}	1.024±0.053^{aA}	1.051±0.086^{aB}	0.999±0.059^{aA}
II	1.064±0.09^{aA}	1.037±0.032^{aA}	0.953±0.053^{aA}	0.965±0.035^{aB}	1.038±0.035^{aA}
III	1.041±0.056^{bA}	0.928±0.049^{bA}	0.859±0.039^{abA}	0.645±0.036^{aA}	0.831±0.175^{abA}
IV	1.010±0.36^{bA}	0.871±0.108^{abA}	0.884±0.097^{abA}	0.601±0.117^{aA}	0.943±0.14^{bA}
Manganese (mg/ l)					
I	0.173±0.015^{aA}	0.132±0.026^{aA}	0.160±0.040^{aA}	0.182±0.027^{aB}	0.127±0.033^{aA}
II	0.147±0.021^{aA}	0.115±0.01^{aA}	0.127±0.017^{aA}	0.114±0.018^{aA}	0.141±0.023^{aA}
III	0.171±0.020^{aA}	0.170±0.027^{aA}	0.124±0.026^{aA}	0.104±0.006^{aA}	0.146±0.045^{aA}
IV	0.193±0.010^{bA}	0.141±0.036^{abA}	0.110±0.064^{aA}	0.092±0.014^{aA}	0.132±0.024^{abA}
Lead (µg / ml or ppm)					
I	0.075±0.014 ^{aA}	0.082±0.016^{aA}	0.092±0.023^{aA}	0.072±0.011$^{a\ A}$	0.070±0.011^{aA}
II	0.095±0.015^{aA}	0.075±0.006^{aA}	0.080±0.008^{aA}	0.070±0.010$^{a\ A}$	0.072±0.005^{aB}
III	0.090±0.019 ^{aA}	0.107±0.018$^{a\ A}$	0.463±0.081^{bB}	0.747±0.037$^{c\ B}$	0.470±0.090^{bB}
IV	0.070±0.020^{aA}	0.092±0.012 ^{aA}	0.493±0.175^{bB}	0.753±0.037$^{c\ B}$	0.495±0.015^{bB}

Gr I: Control; Gr II: Flubendiamide; Gr III: Lead; Gr IV: Flubendiamide + Lead

Values bearing different superscript in small letters in a row and capital letters in a column differ significantly ($P<0.05$)

Table 14. Effect of chronic toxicity of flubendiamide, lead and their interaction on ALAD activity in blood and glial fibrillary acidic protein and aspartic acid concentration in CSF (Mean ± S. E.) in blood in buffalo calves

ALAD (units/ ml erythrocytes/hour at 38^0C)					
Group	**Day of observation**				
	0	**30**	**60**	**90**	**30 post-treatment**
I	299.51 ± 7.15^{aA}	301.83 ± 5.73^{aA}	293.56 ± 12.97^{aB}	294.44 ± 10.80^{aB}	300.32 ± 4.78^{aB}
II	284.22 ± 13.46^{aA}	287.26 ± 15.10^{aA}	257.99 ± 24.47^{aB}	252.02 ± 19.14^{aB}	281.57 ± 13.23^{aB}
III	285.88 ± 12.84^{cA}	260.89 ± 26.33^{cA}	187.04 ± 9.04^{bA}	125.47 ± 19.78^{aA}	229.20 ± 20.18^{bcA}
IV	271.09 ± 15.18^{bA}	271.74 ± 22.11^{bA}	192.75 ± 5.94^{aA}	155.46 ± 26.37^{aA}	215.77 ± 15.32^{abA}
Glial fibrillary acidic protein (ηg / ml)					
I	1.82 ± 0.07^{aA}	1.78 ± 0.24^{aA}	1.86 ± 0.06^{aA}	1.75 ± 0.13^{aA}	1.68 ± 0.20^{aA}
II	1.72 ± 0.22^{aA}	1.74 ± 0.10^{aA}	1.64 ± 0.23^{aA}	1.84 ± 0.11^{aA}	1.99 ± 0.05^{aA}
III	1.68 ± 0.23^{aA}	1.57 ± 0.24^{aA}	3.05 ± 0.17^{bB}	5.46 ± 0.25^{dB}	4.18 ± 0.16^{cB}
IV	1.96 ± 0.08^{aA}	1.73 ± 0.21^{aA}	3.01 ± 0.08^{bB}	5.37 ± 0.28^{cB}	5.14 ± 0.31^{cC}
Aspartic acid (µmole/ l)					
I	1.90 ± 0.20^{aA}	1.75 ± 0.44^{aA}	1.38 ± 0.13^{aA}	1.53 ± 0.30^{aA}	1.83 ± 0.11^{aA}
II	1.76 ± 0.23^{aA}	2.07 ± 0.16^{abA}	2.73 ± 0.25^{bcB}	3.05 ± 0.43^{cB}	2.30 ± 0.13^{abcA}
III	1.52 ± 0.29^{aA}	1.96 ± 0.53^{aA}	1.71 ± 0.35^{aA}	1.16 ± 0.04^{aA}	1.76 ± 0.55^{aA}
IV	1.57 ± 0.26^{aA}	2.27 ± 0.59^{aA}	2.54 ± 0.26^{aB}	2.69 ± 0.16^{aB}	2.41 ± 0.40^{aA}

Gr I: Control; Gr II: Flubendiamide; Gr III: Lead; Gr IV: Flubendiamide + Lead

Values bearing different superscript in small letters in a row and capital letters in a column differ significantly ($P<0.05$)

Table 15. Effect of chronic toxicity of flubendiamide, lead and their interaction on thyroid hormones (Mean ± S. E.) in buffalo calves

T_3 (ng/ ml)					
Group	**Day of observation**				
	0	**30**	**60**	**90**	**30 post-treatment**
I	2.17±0.13^{aA}	2.19±0.24^{aA}	2.10±0.08^{aA}	2.20±0.05^{aA}	2.20±0.12^{aA}
II	2.18±0.18^{aA}	2.26±0.12^{abA}	2.05±0.09^{aA}	1.94±0.06^{aA}	2.67±0.20^{bA}
III	2.09±0.32$^{a\ A}$	2.23±0.23^{aA}	2.65±0.08$^{a\ B}$	1.75±0.36^{aA}	2.55±0.27^{aA}
IV	2.14±0.28$^{a\ A}$	2.10±0.19^{aA}	2.36±0.19^{aA}	2.06±0.38$^{a\ A}$	2.84±0.33$^{a\ A}$
T_4 (µg/ dl)					
I	5.27±0.22^{aA}	5.18±0.32$^{a\ A}$	5.50±0.19$^{a\ AB}$	5.27±0.16$^{a\ B}$	5.04±0.08^{aA}
II	5.25±0.24^{bA}	4.84±0.59^{abA}	5.63±0.22^{bB}	3.94±0.09$^{a\ A}$	4.67±0.21^{abA}
III	5.20±0.23^{bA}	5.13±0.28^{bA}	4.70±0.51^{abA}	3.64±0.27$^{a\ A}$	4.64±0.33^{abA}
IV	5.29±0.20^{aA}	5.24±0.31^{aA}	5.89±0.15^{aB}	5.07±0.39$^{a\ B}$	5.38±0.29^{aA}
TSH (µIU/ ml)					
I	4.69±0.23^{aA}	4.53±0.20^{aA}	4.88±0.12$^{a\ A}$	4.48±0.30$^{a\ A}$	5.02±0.41^{aA}
II	4.92±0.19^{aA}	4.59±0.28^{aA}	4.93±0.05^{aA}	4.60±0.21$^{a\ A}$	5.32±0.33^{aA}
III	4.65±0.22^{aA}	4.80±0.41$^{a\ A}$	5.66±0.27^{abB}	6.32±0.35^{bB}	5.42±0.40^{abA}
IV	4.56±0.23^{aA}	4.92±0.29$^{a\ A}$	5.46±0.29^{abAB}	6.28±0.38^{bB}	5.16±0.28^{aA}

Gr I: Control; Gr II: Flubendiamide; Gr III: Lead; Gr IV: Flubendiamide + Lead

Values bearing different superscript in small letters in a row and capital letters in a column differ significantly ($P<0.05$)

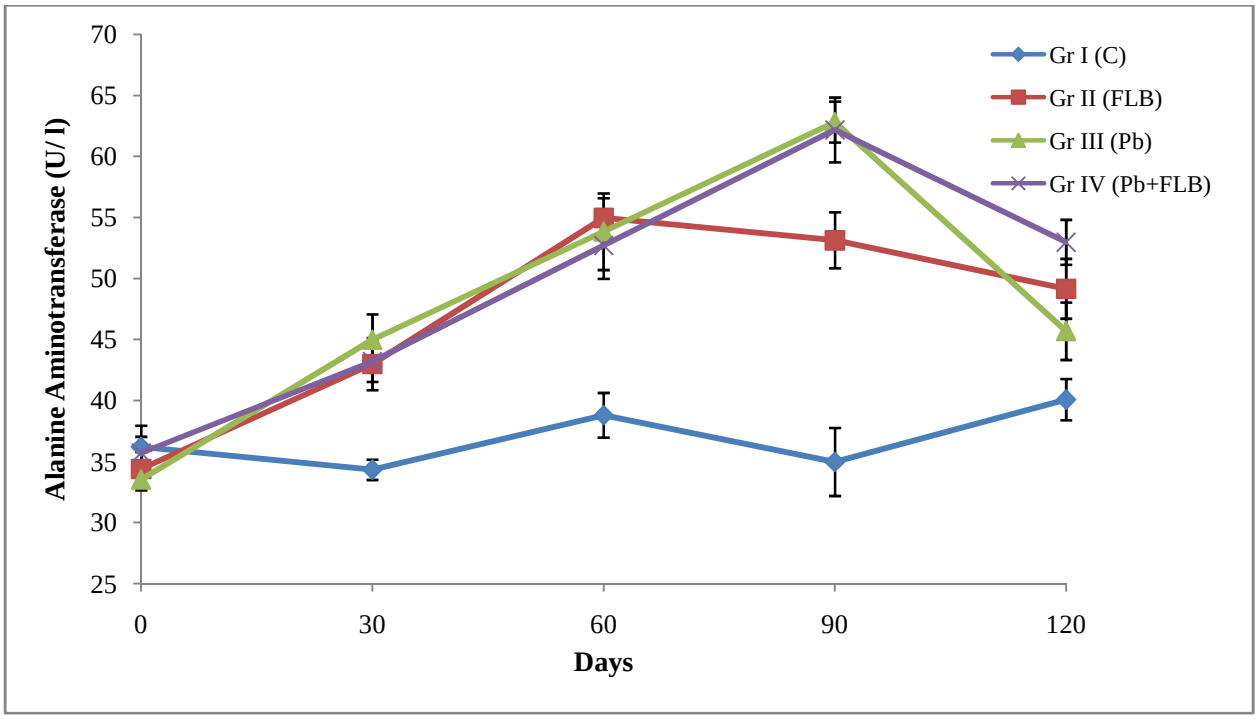

Fig. 5: Comparative effect of chronic oral administration of flubendiamide, lead and their combination on alanine aminotransferase (ALT) activity

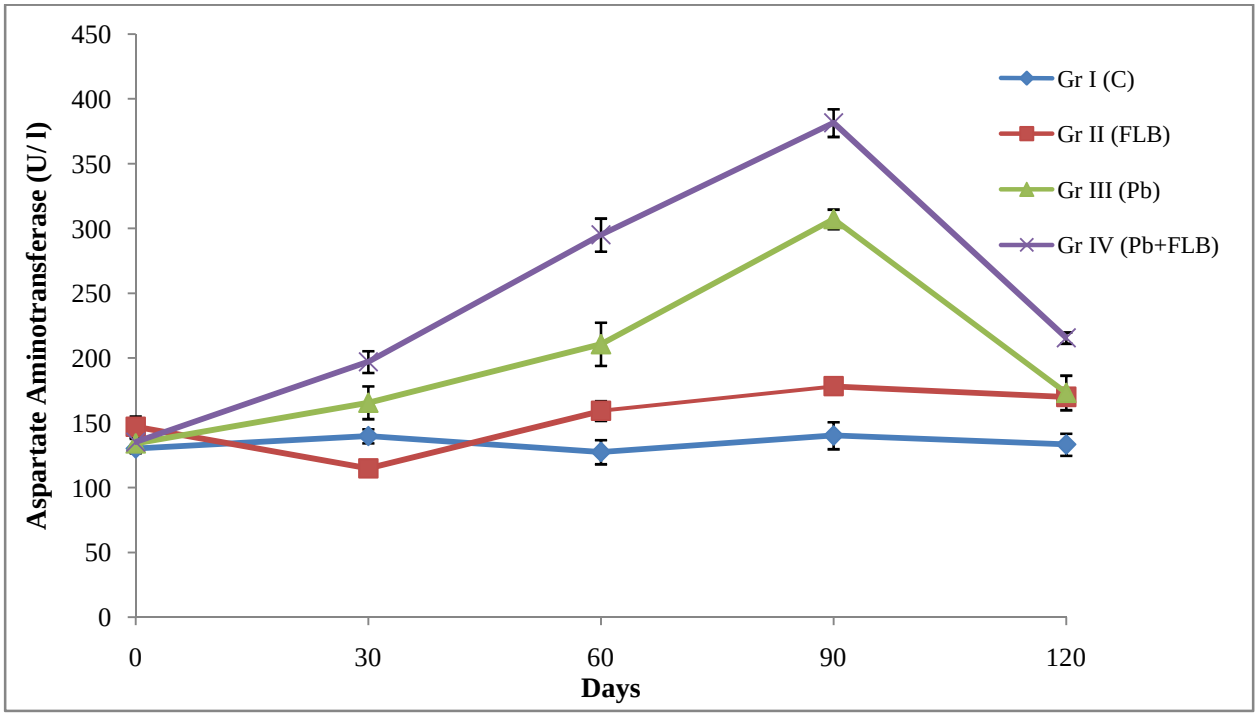

Fig. 6: Comparative effect of chronic oral administration of flubendiamide, lead and their combination on aspartate aminotransferase (AST) activity

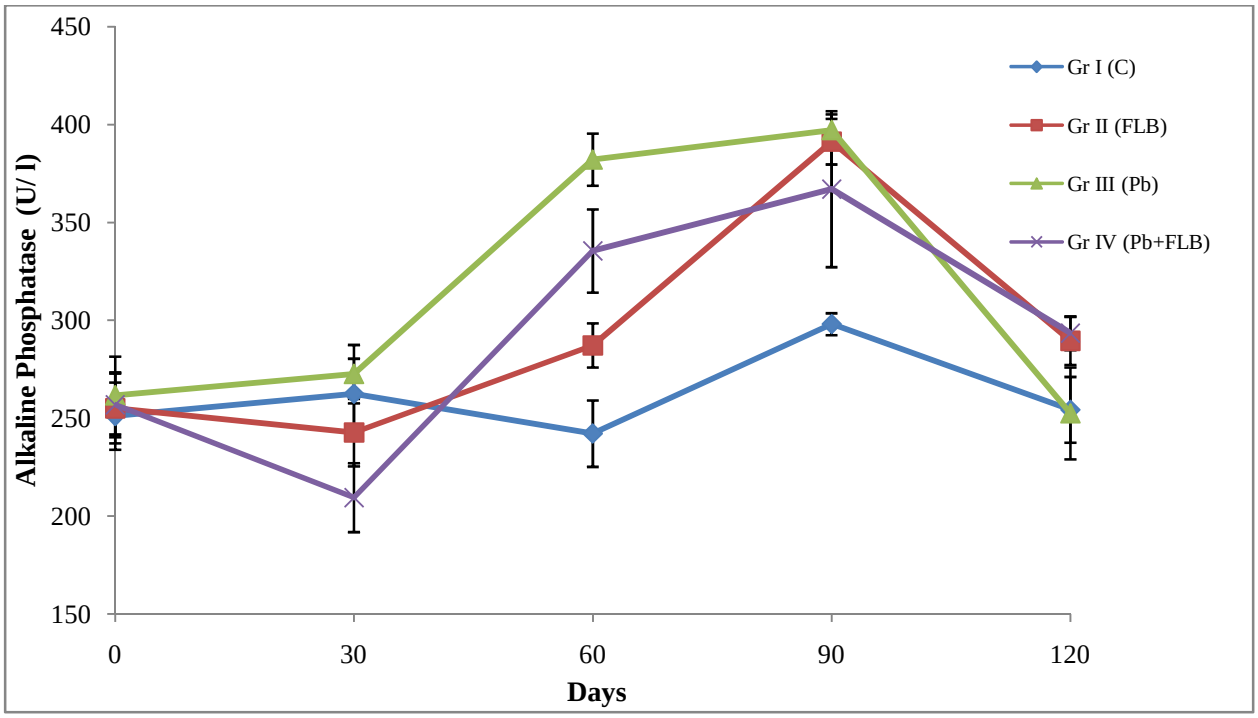

Fig. 7: Comparative effect of chronic oral administration of flubendiamide, lead and their combination on alkaline phosphatase (AKLP) activity

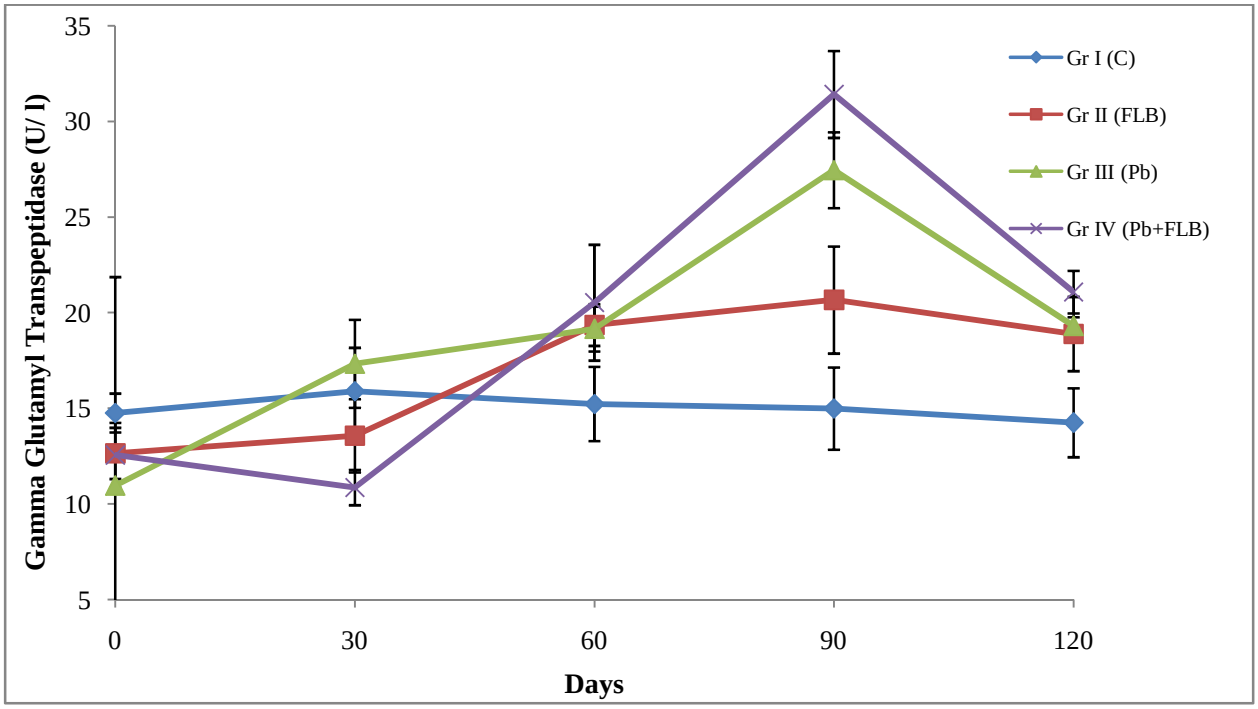

Fig. 8: Comparative effect of chronic oral administration of flubendiamide, lead and their combination on gamma glutamyl transpeptidase (GGT) activity

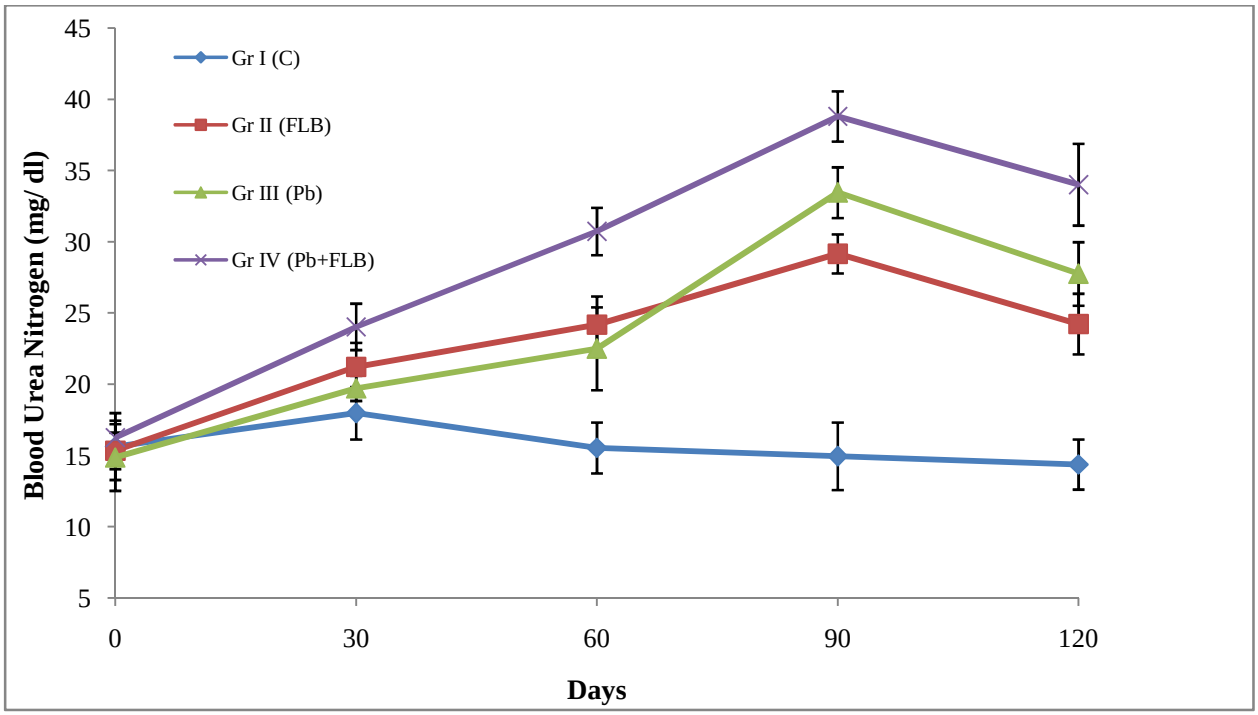

Fig. 9: Comparative effect of chronic oral administration of flubendiamide, lead and their combination on blood urea nitrogen (BUN) level

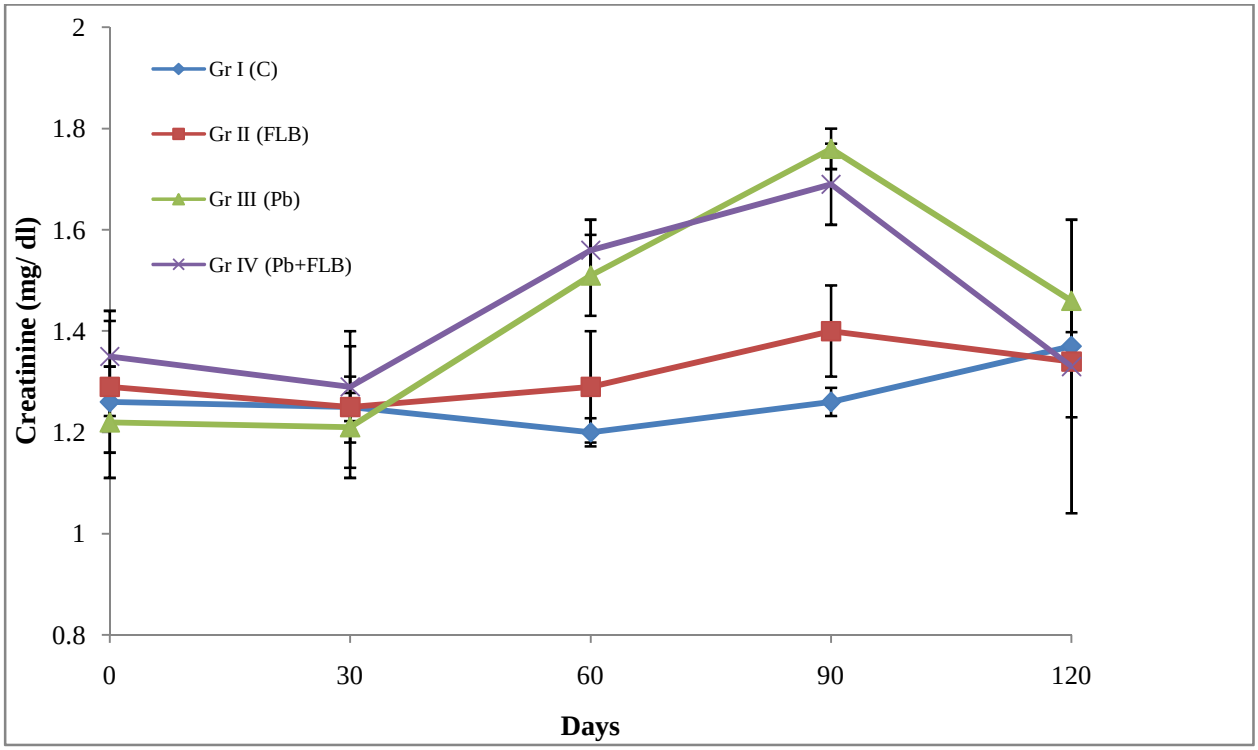

Fig. 10: Comparative effect of chronic oral administration of flubendiamide, lead and their combination on creatinine level

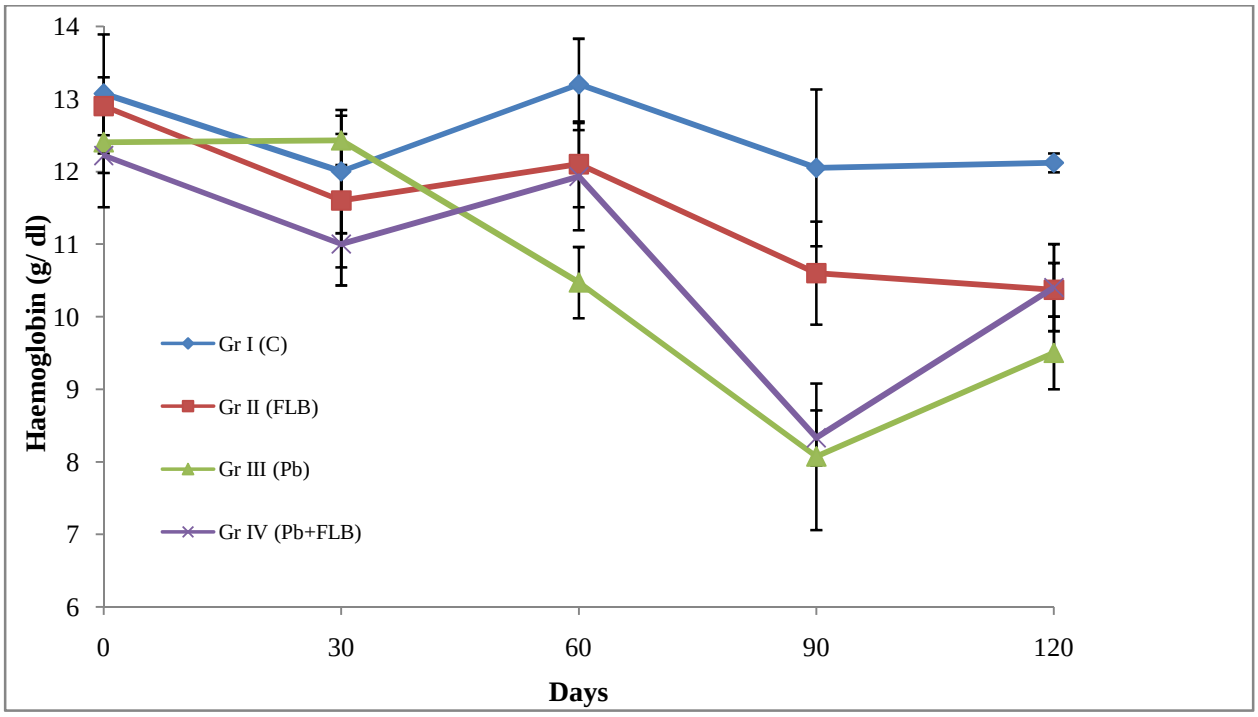

Fig. 11: Comparative effect of chronic oral administration of flubendiamide, lead and their combination on haemoglobin (Hb) concentration

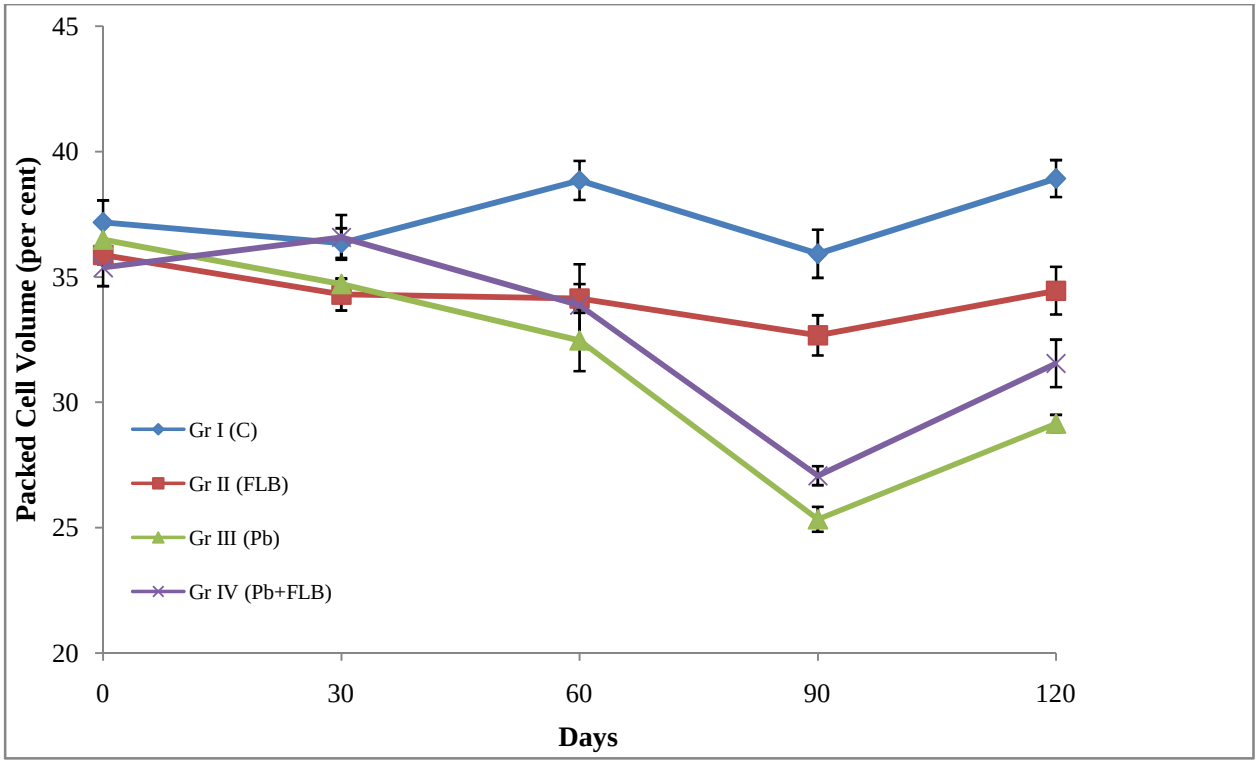

Fig. 12: Comparative effect of chronic oral administration of flubendiamide, lead and their combination on packed cell volume (PCV)

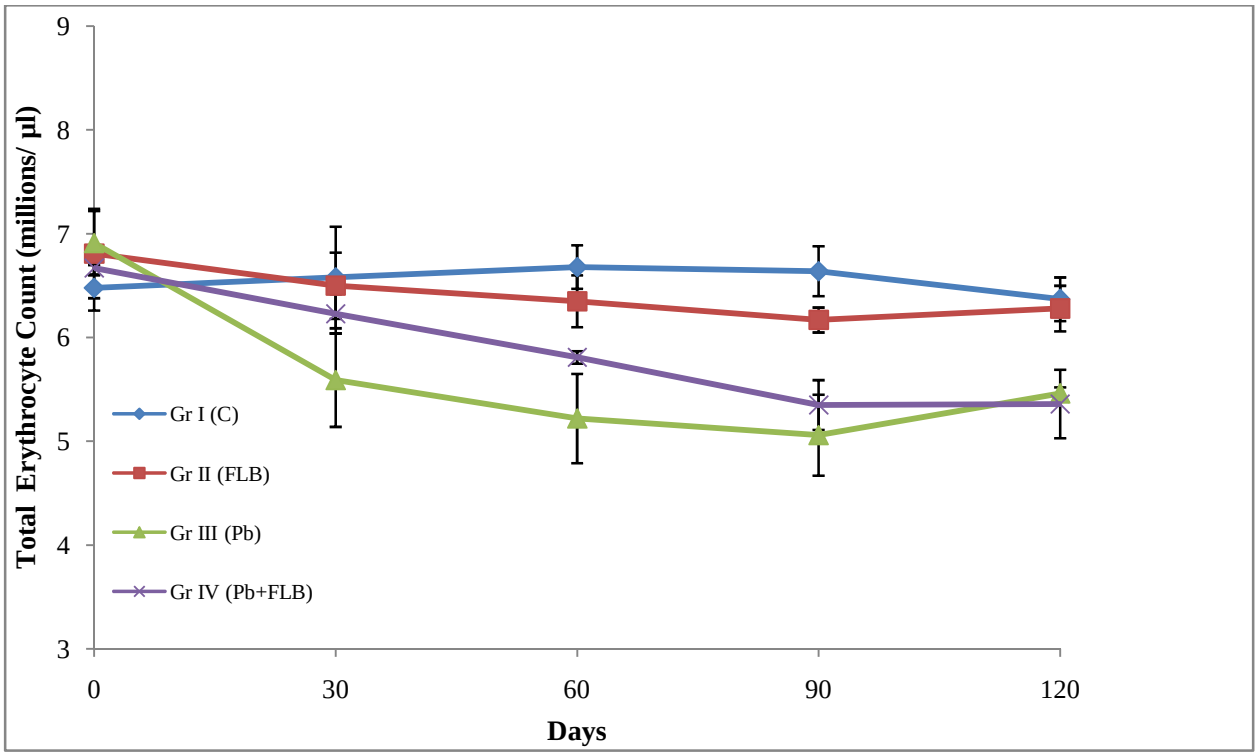

Fig. 13: Comparative effect of chronic oral administration of flubendiamide, lead and their combination on total erythrocyte count (TEC)

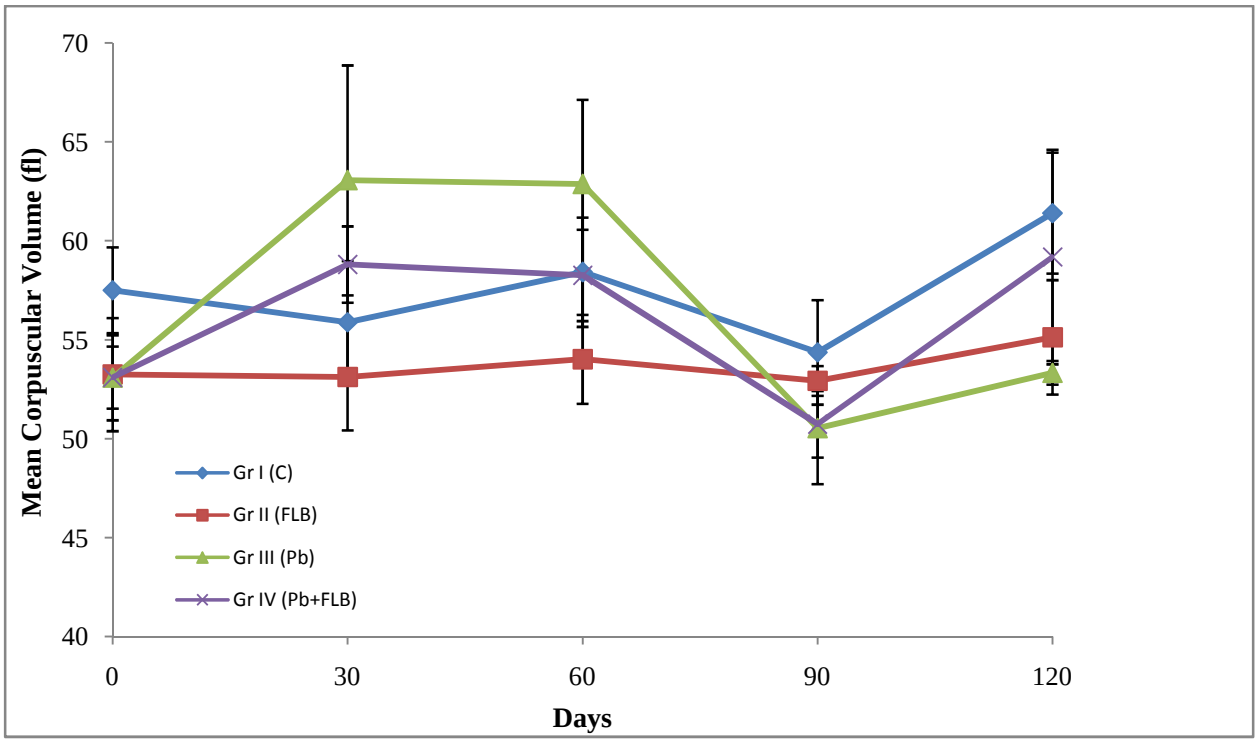

Fig. 14: Comparative effect of chronic oral administration of flubendiamide, lead and their combination on mean corpuscular volume (MCV)

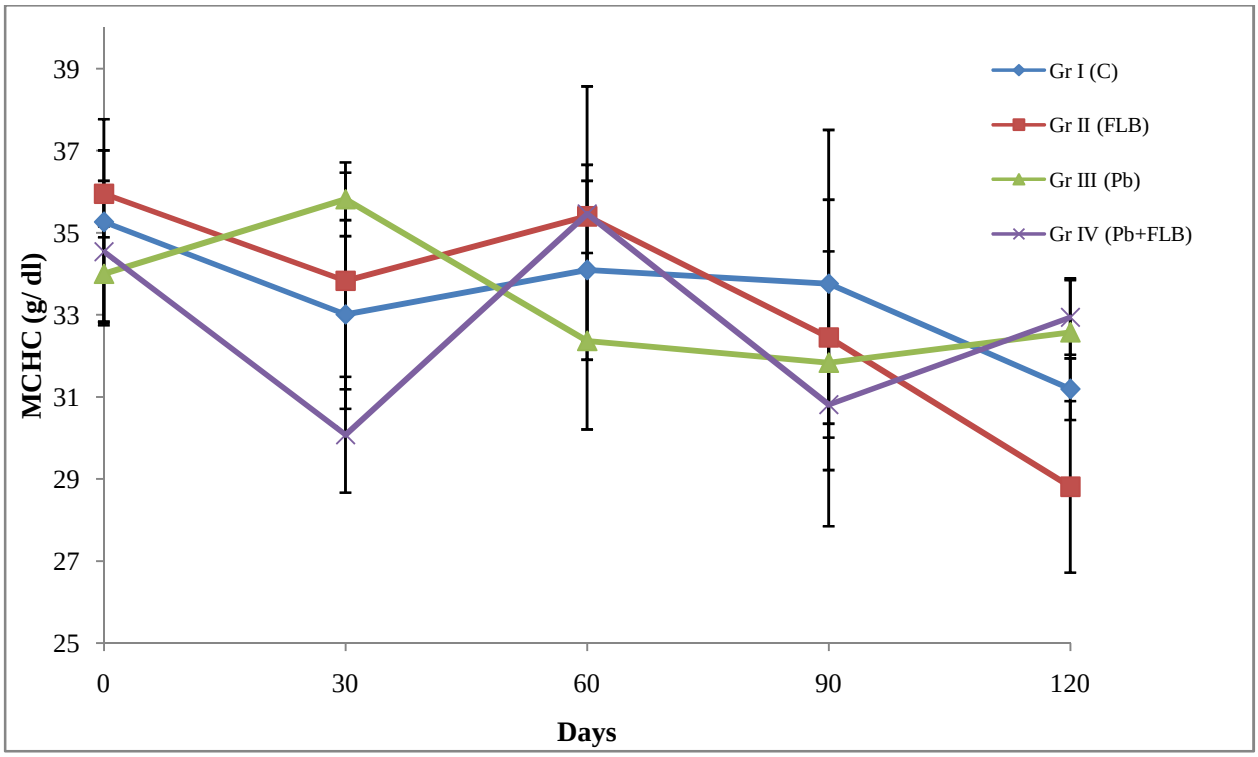

Fig. 15: Comparative effect of chronic oral administration of flubendiamide, lead and their combination on mean corpuscular hemoglobin concentration (MCHC)

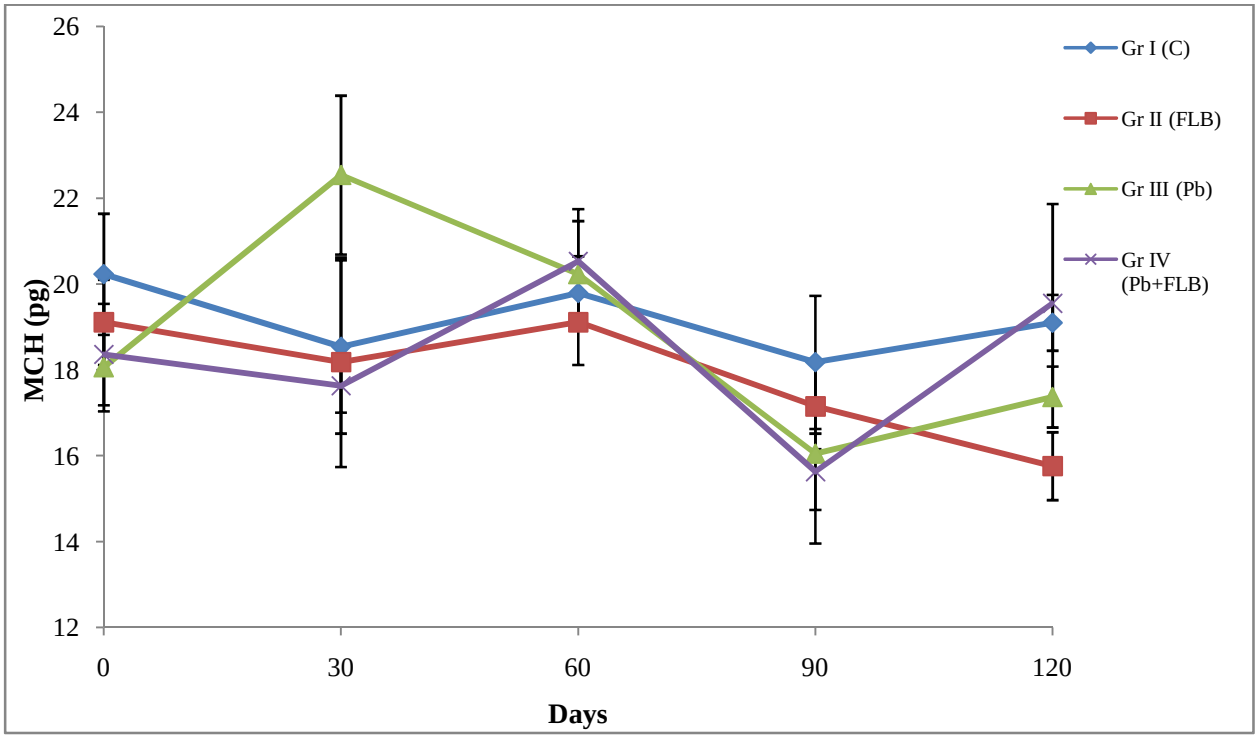

Fig. 16: Comparative effect of chronic oral administration of flubendiamide, lead and their combination on mean corpuscular hemoglobin (MCH)

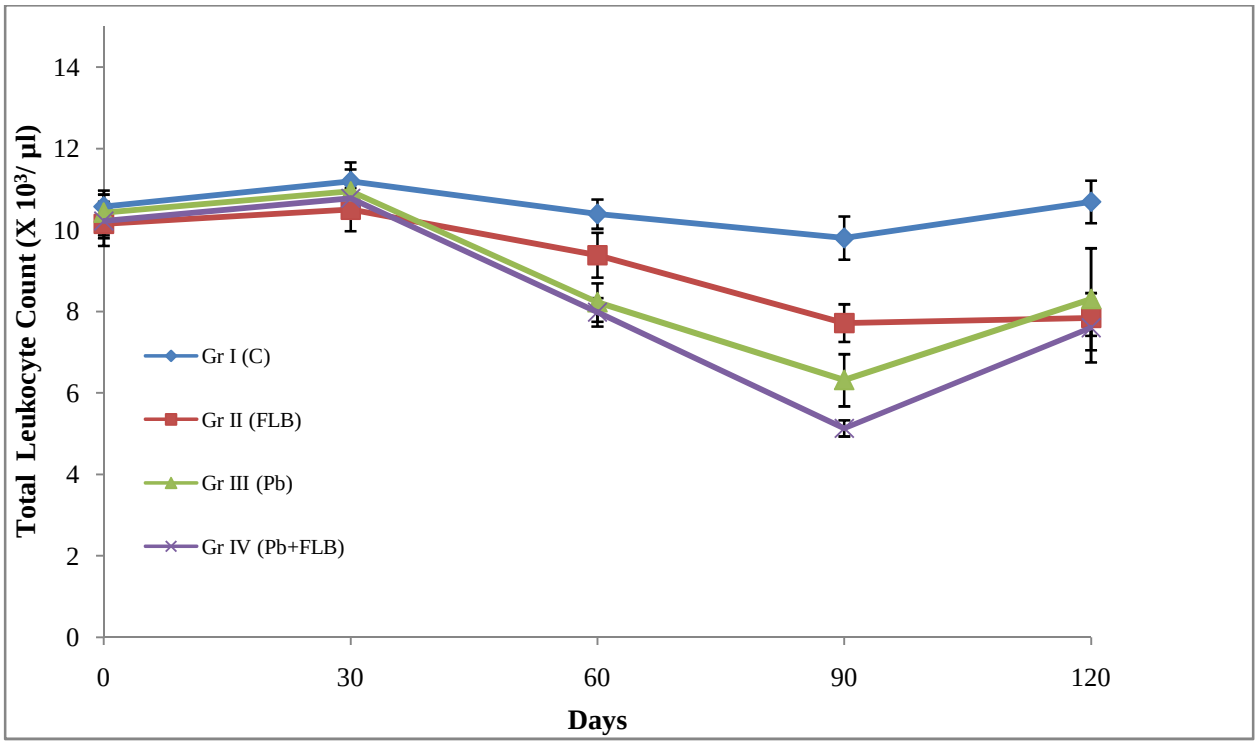

Fig. 17: Comparative effect of chronic oral administration of flubendiamide, lead and their combination on total leukocyte count (TLC)

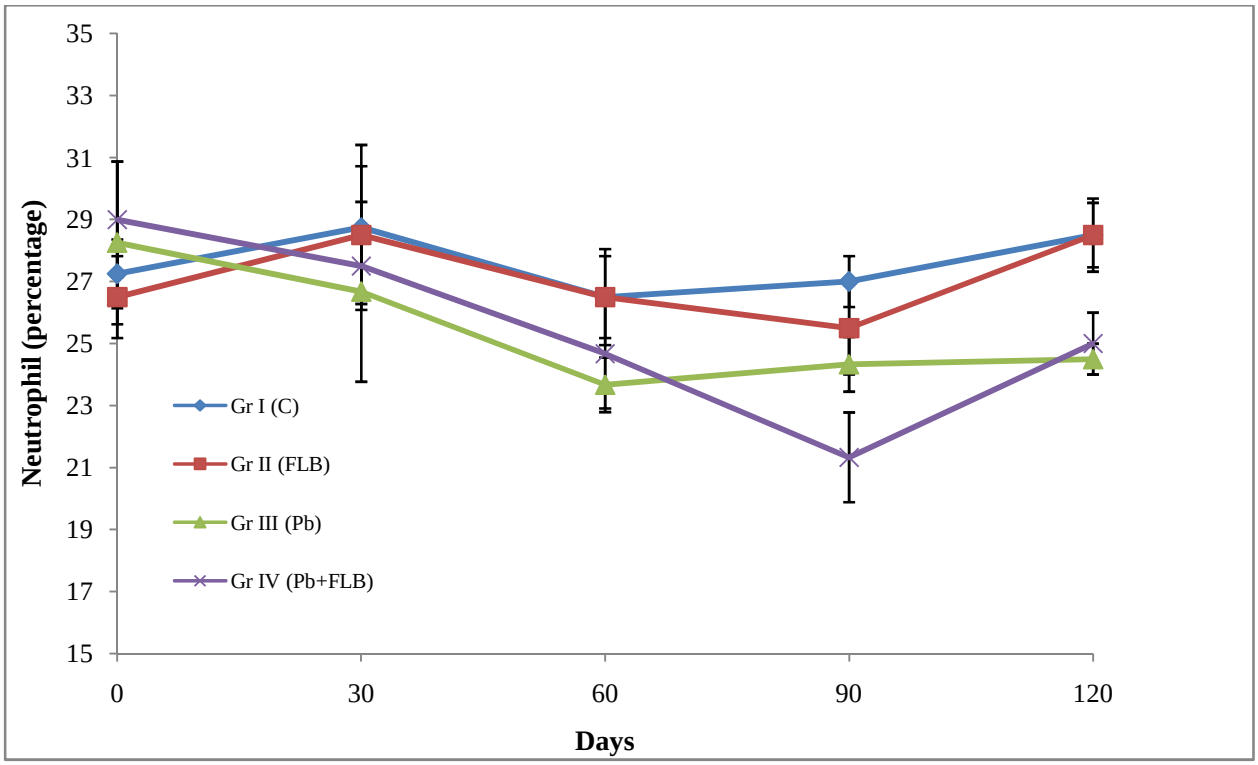

Fig. 18: Comparative effect of chronic oral administration of flubendiamide, lead and their combination on neutrophil percentage

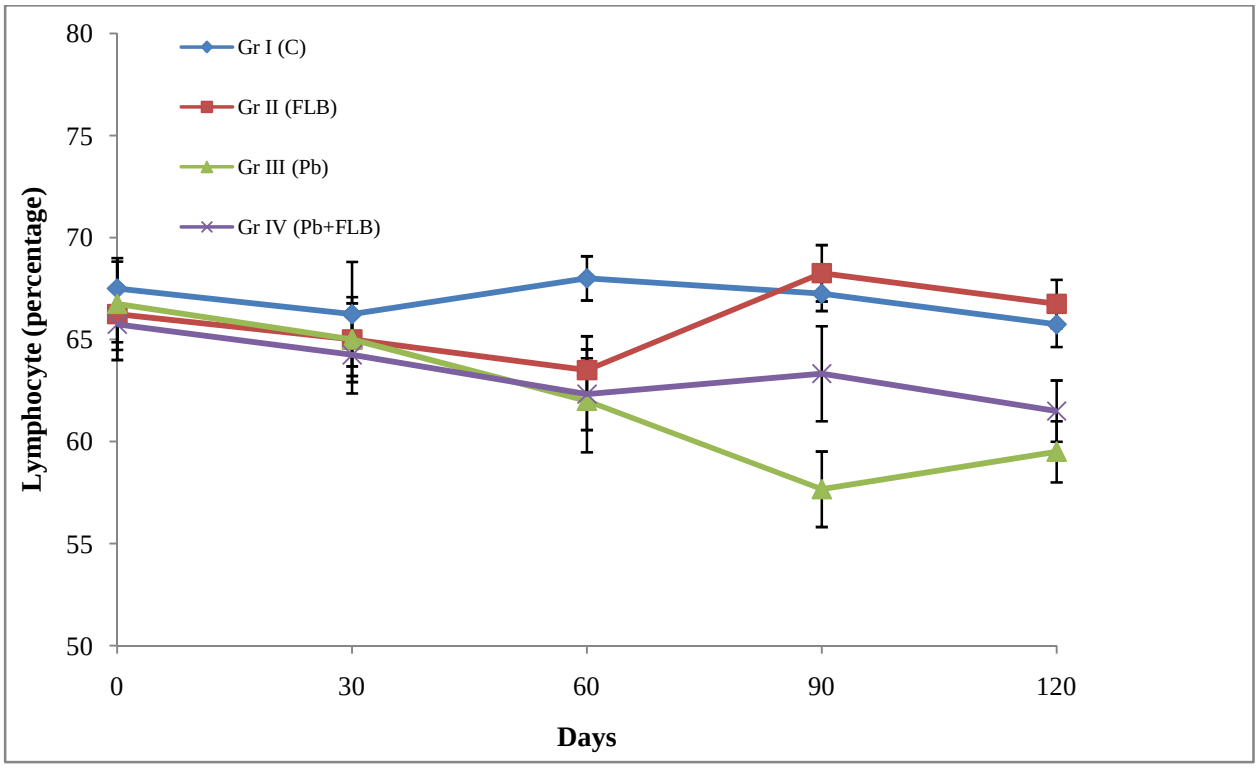

Fig. 19: Comparative effect of chronic oral administration of flubendiamide, lead and their combination on lymphocyte percentage

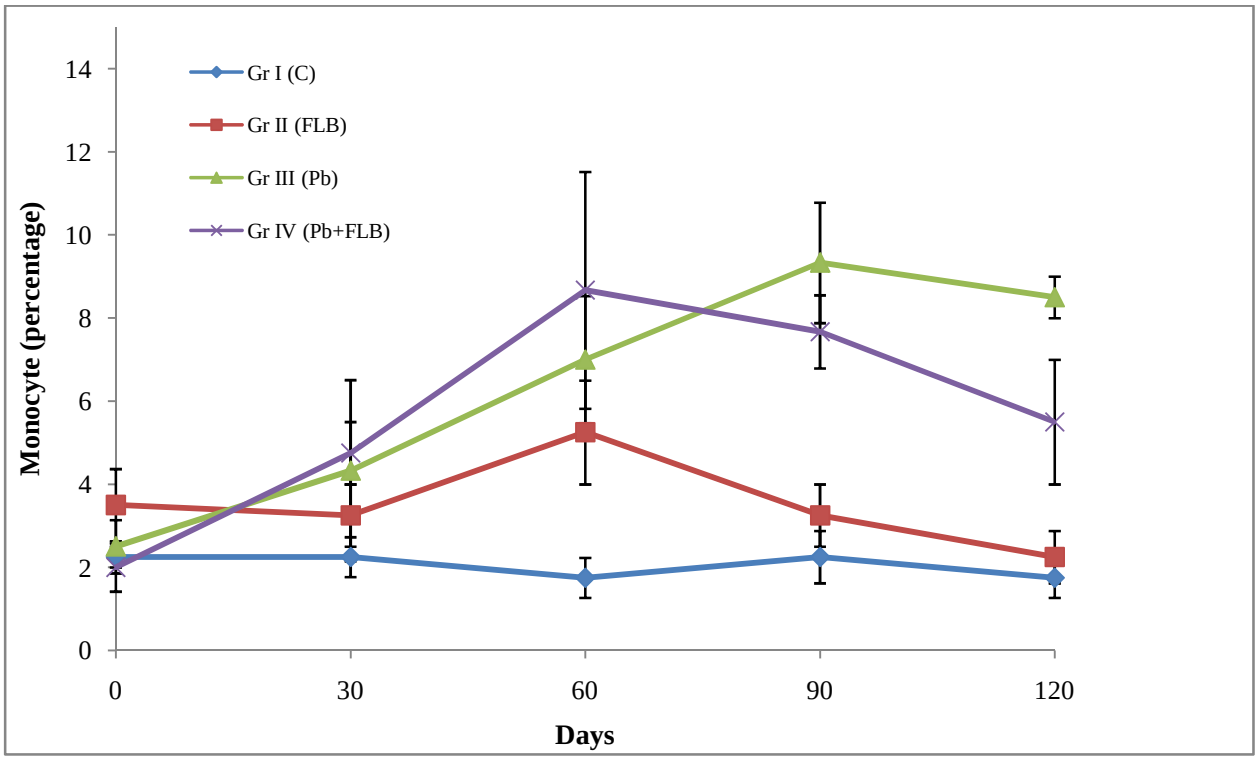

Fig. 20: Comparative effect of chronic oral administration of flubendiamide, lead and their combination on monocyte percentage

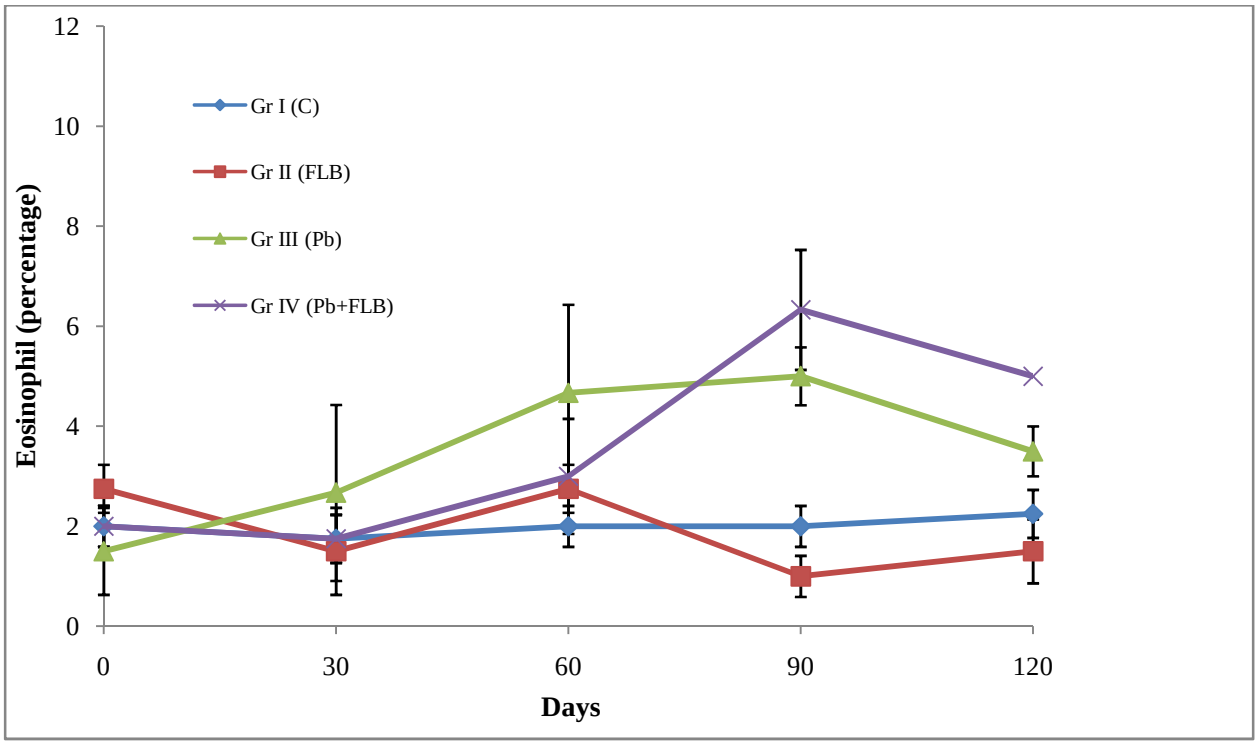

Fig. 21: Comparative effect of chronic oral administration of flubendiamide, lead and their combination on eosinophil percentage

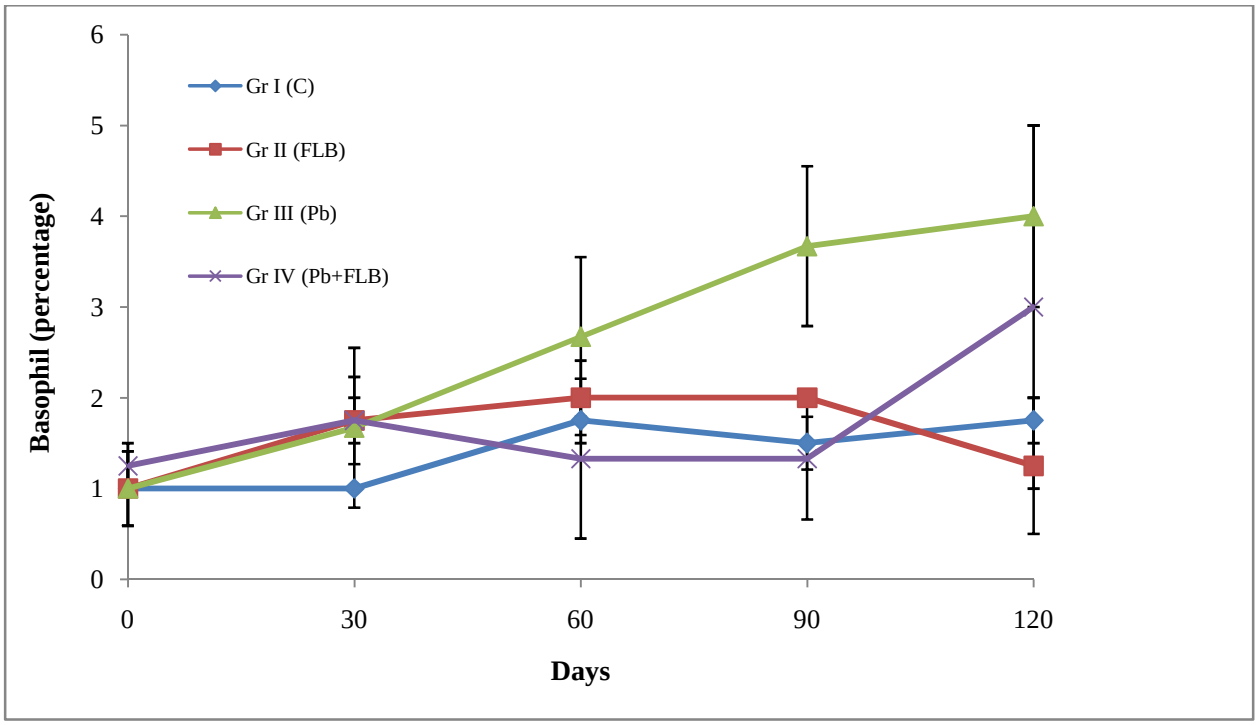

Fig. 22: Comparative effect of chronic oral administration of flubendiamide, lead and their combination on basophil percentage

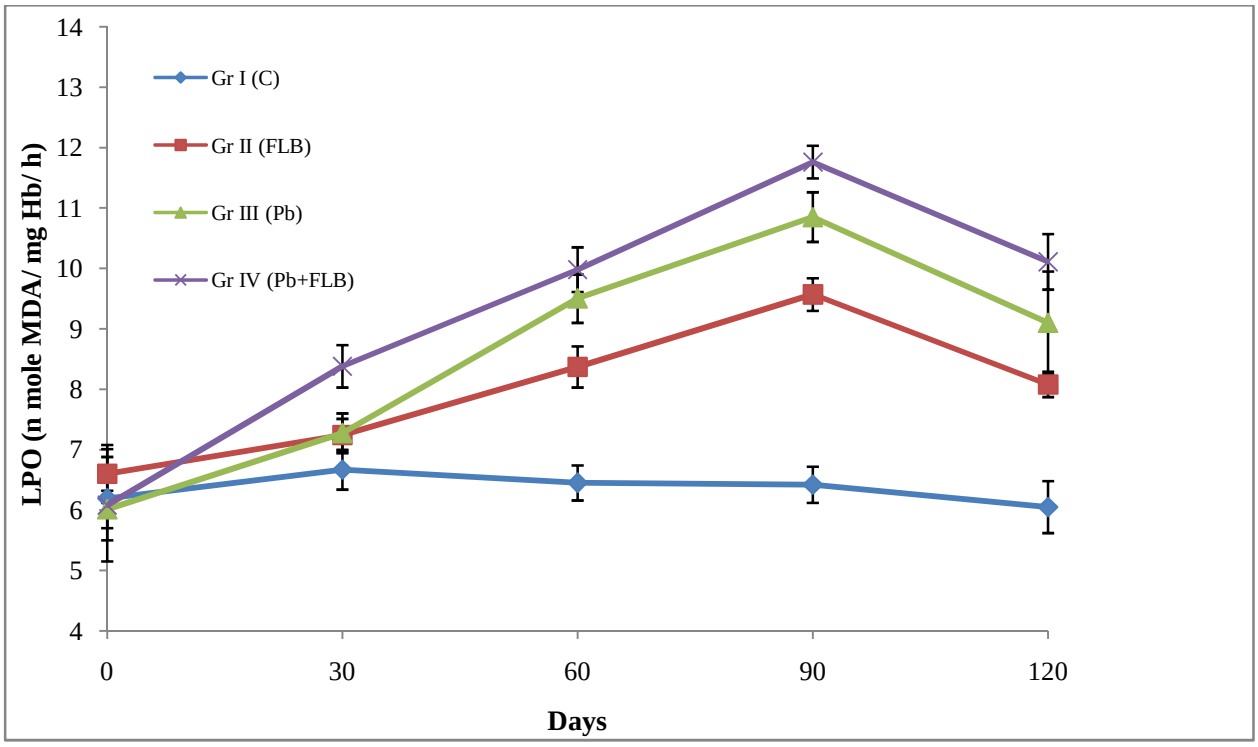

Fig. 23: Comparative effect of chronic oral administration of flubendiamide, lead and their combination on lipid peroxides (LPO) level

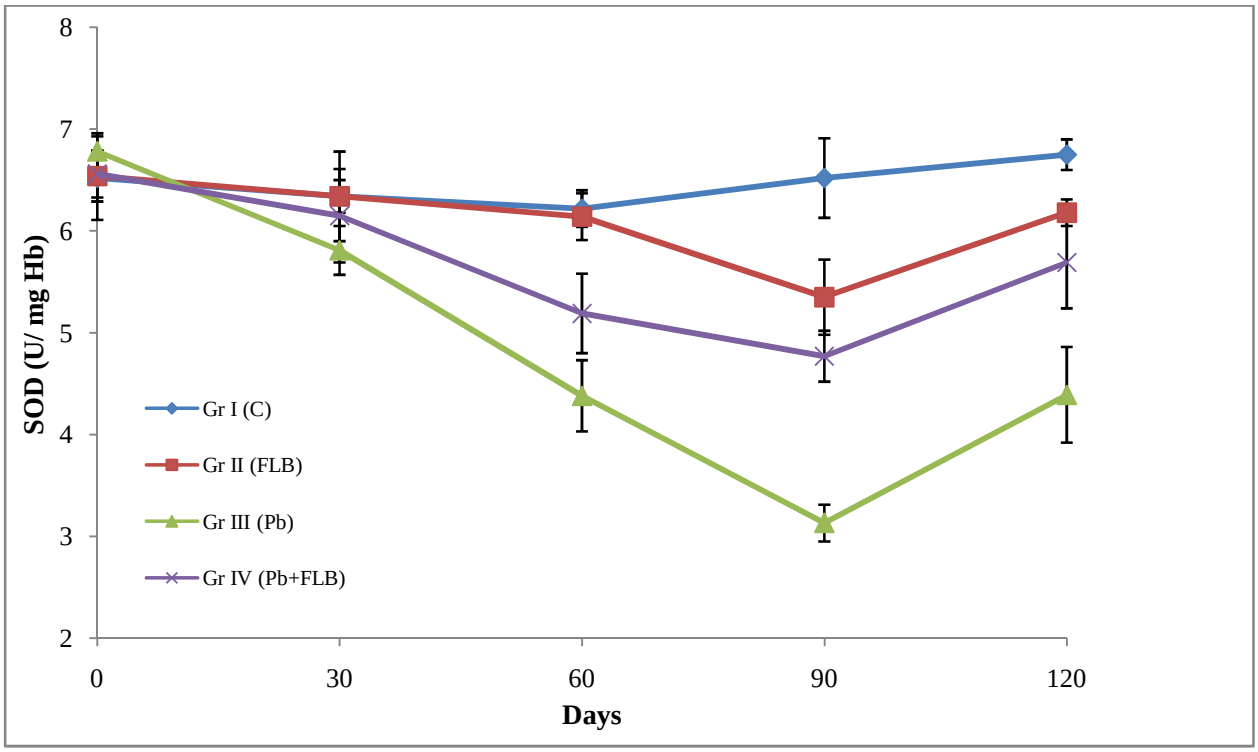

Fig. 24: Comparative effect of chronic oral administration of flubendiamide, lead and their combination on superoxide dismutase (SOD) activity

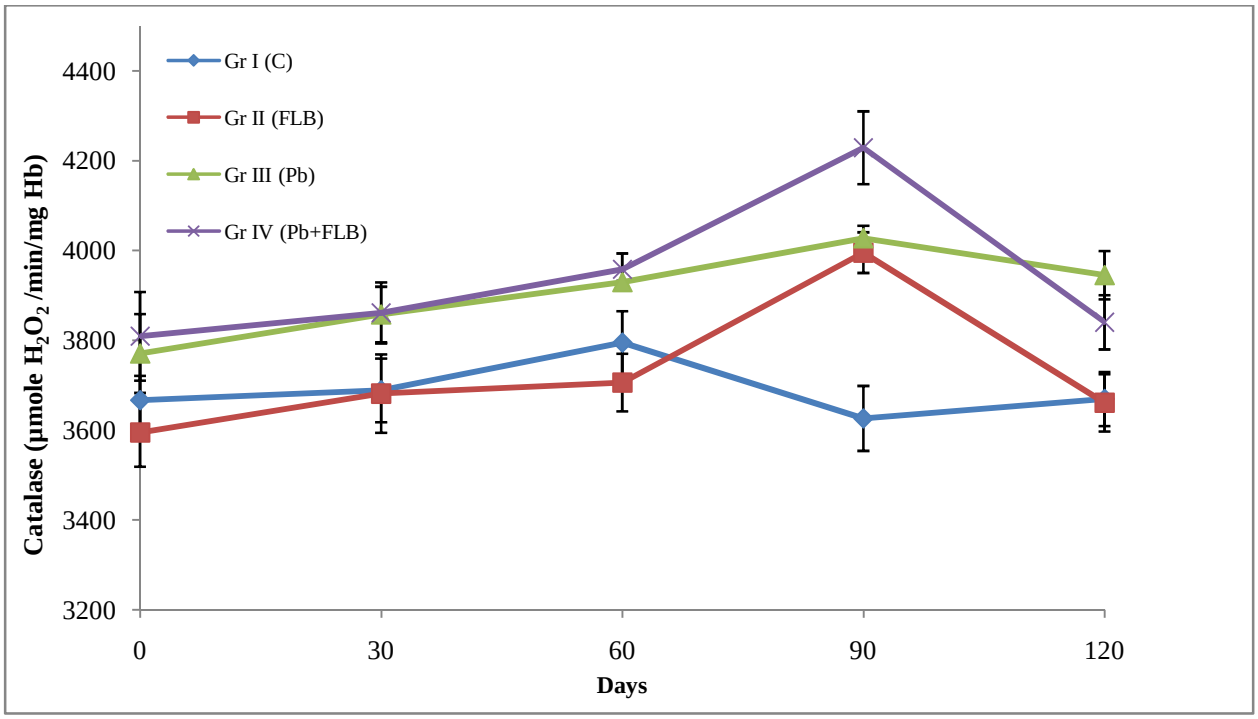

Fig. 28: Comparative effect of chronic oral administration of flubendiamide, lead and their combination on catalase activity

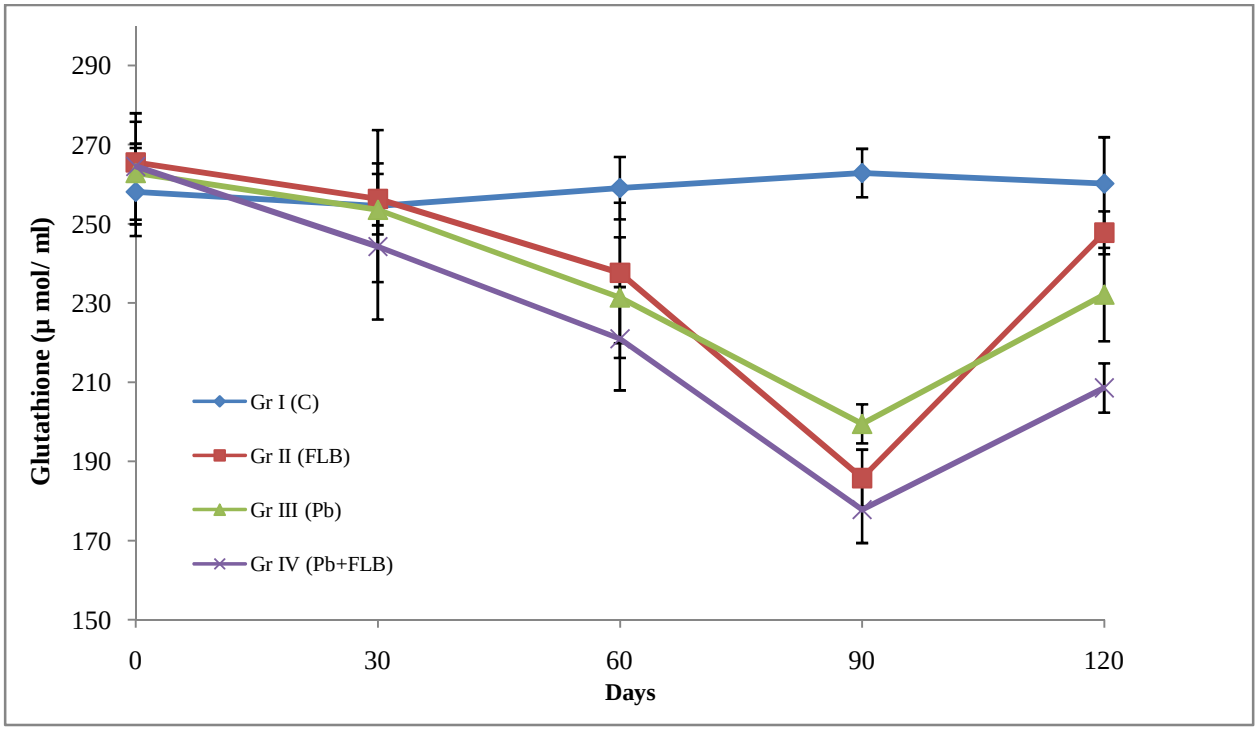

Fig. 29: Comparative effect of chronic oral administration of flubendiamide, lead and their combination on blood glutathione (GSH) concentration

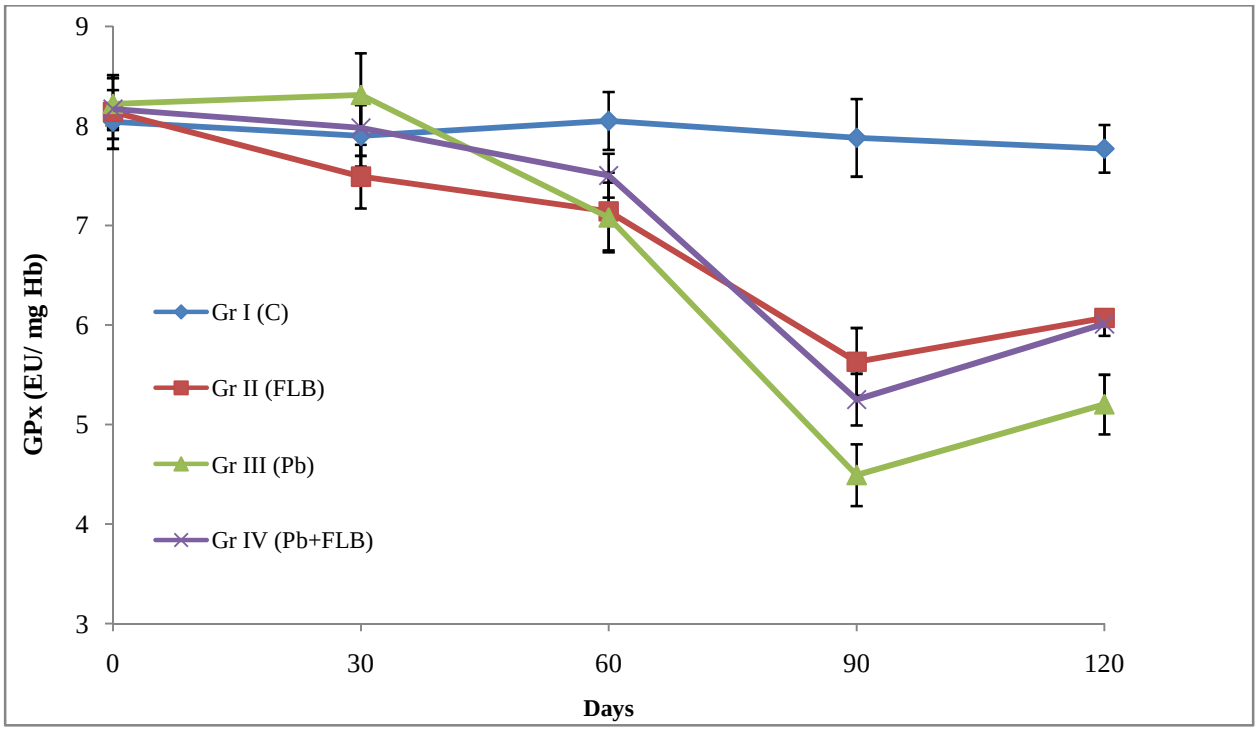

Fig. 30: Comparative effect of chronic oral administration of flubendiamide, lead and their combination on glutathione peroxidase (GPx) activity

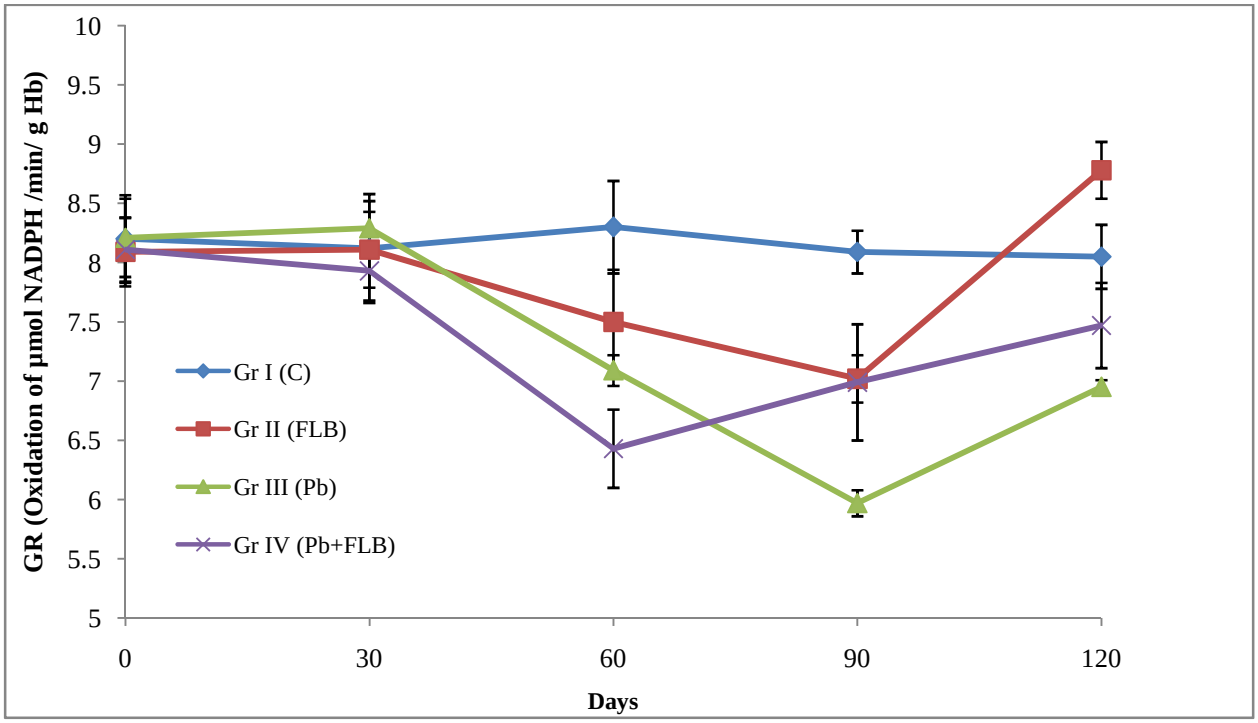

Fig. 31: Comparative effect of chronic oral administration of flubendiamide, lead and their combination on glutathione reductase (GR) activity

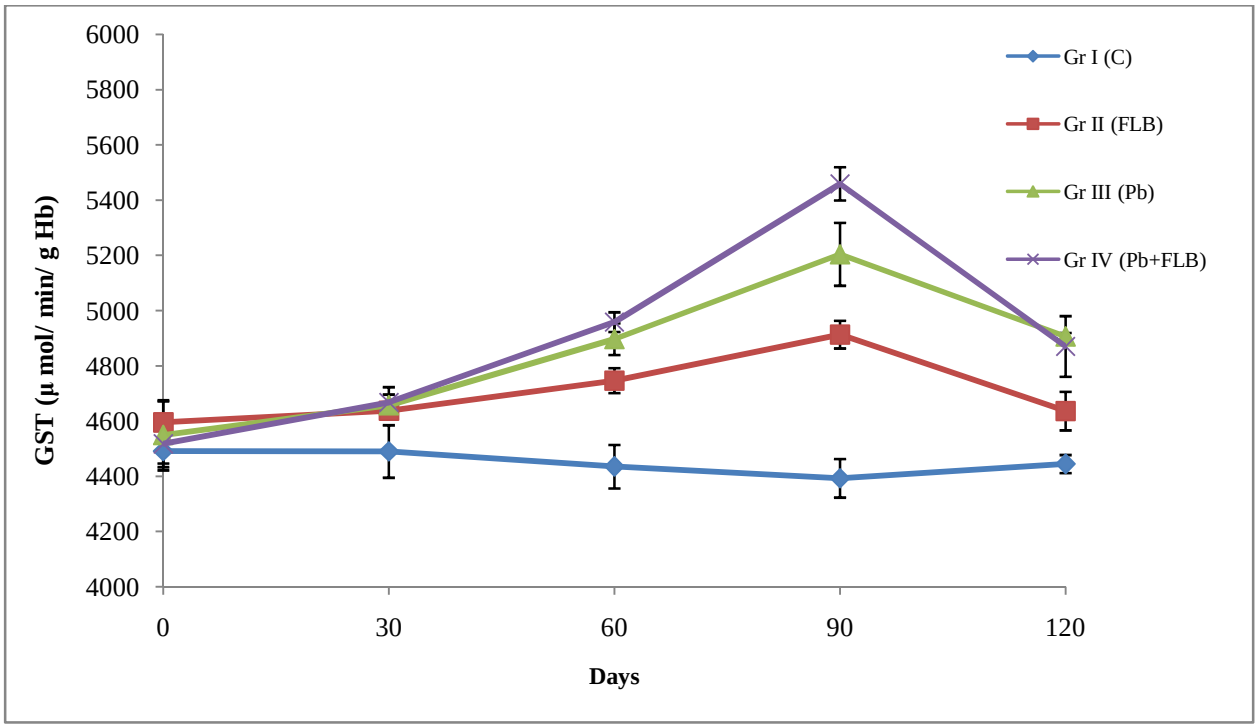

Fig. 32: Comparative effect of chronic oral administration of flubendiamide, lead and their combination on glutathione-s-transferase (GST) activity

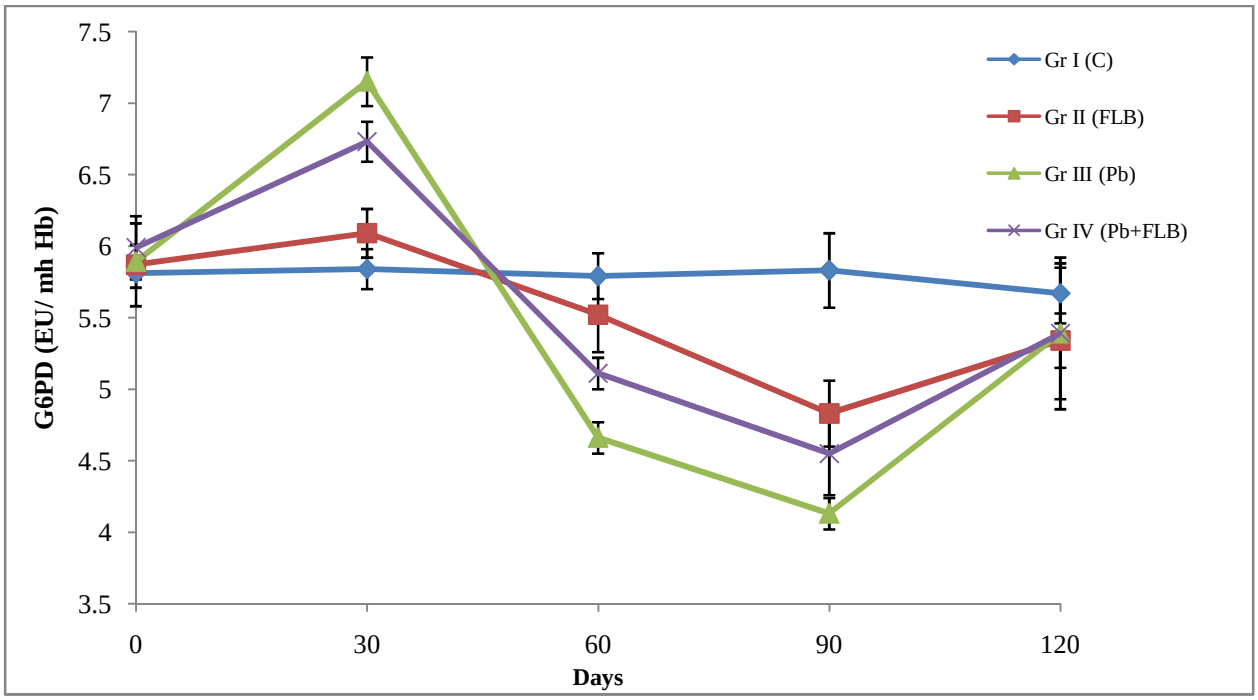

Fig. 33: Comparative effect of chronic oral administration of flubendiamide, lead and their combination on glucose-6-phosphate dehydrogenase (G6PD) activity

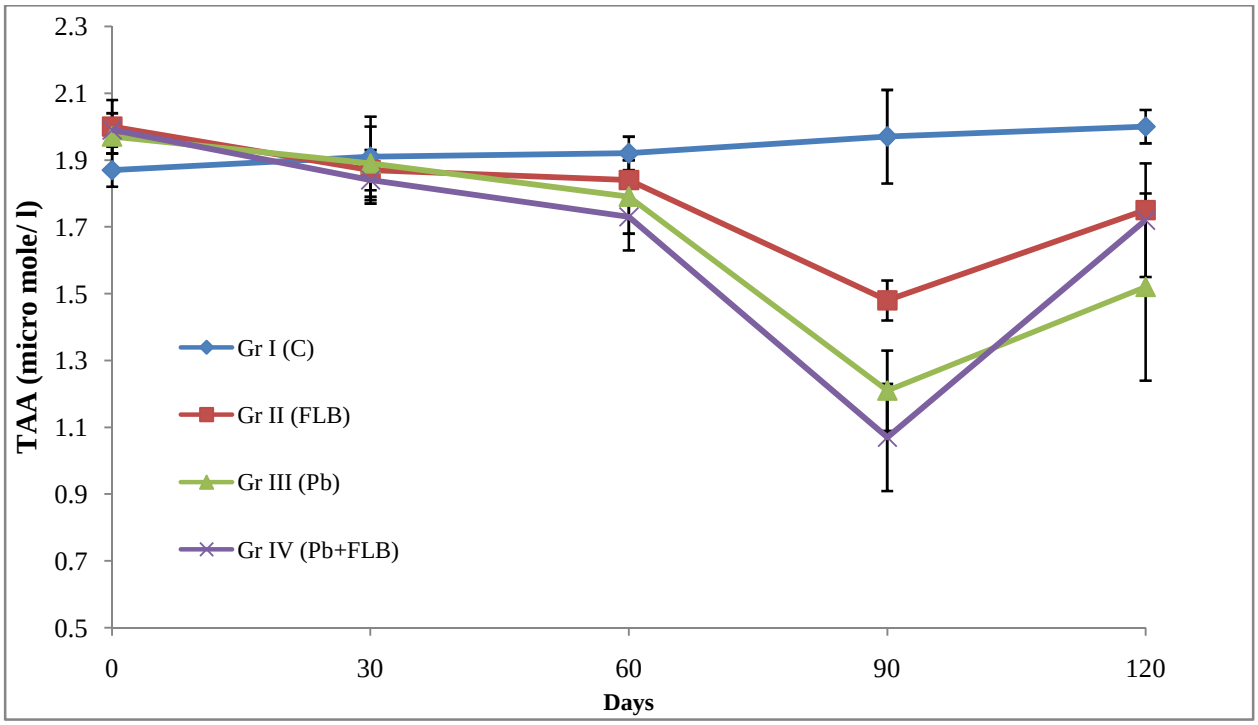

Fig. 34: Comparative effect of chronic oral administration of flubendiamide, lead and their combination on total antioxidant activity (TAA)

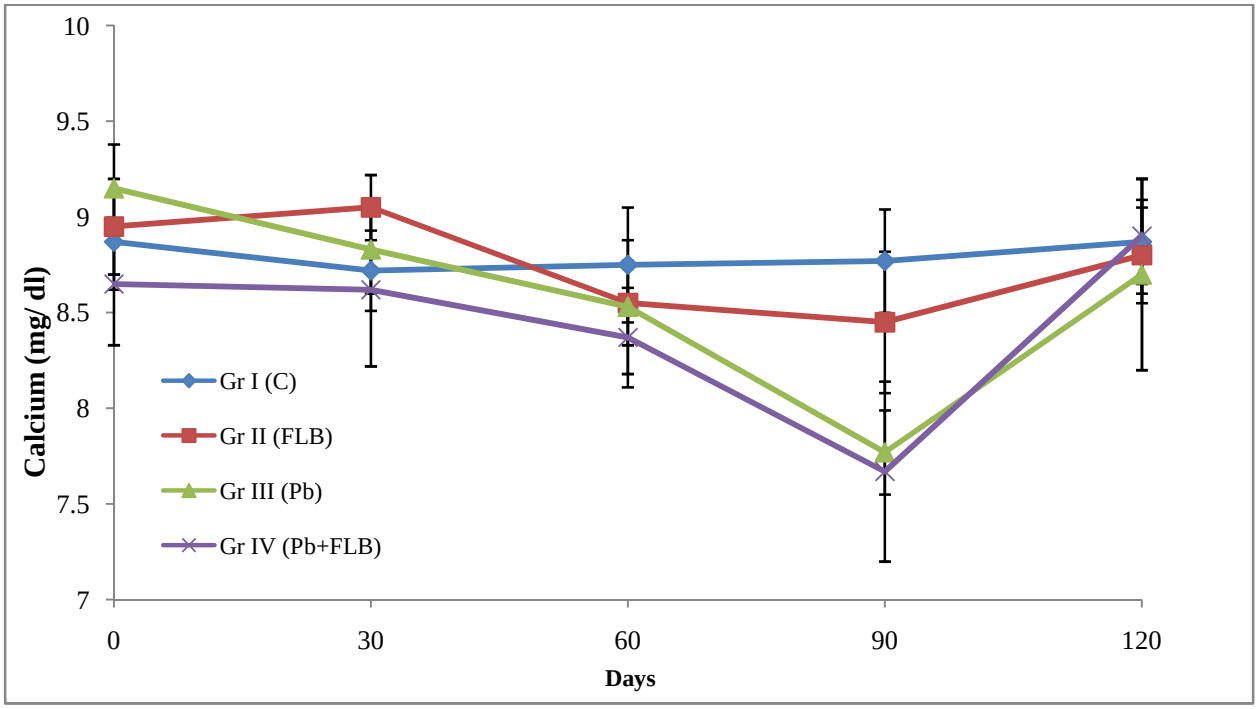

Fig. 35: Comparative effect of chronic oral administration of flubendiamide, lead and their combination on calcium concentration in blood

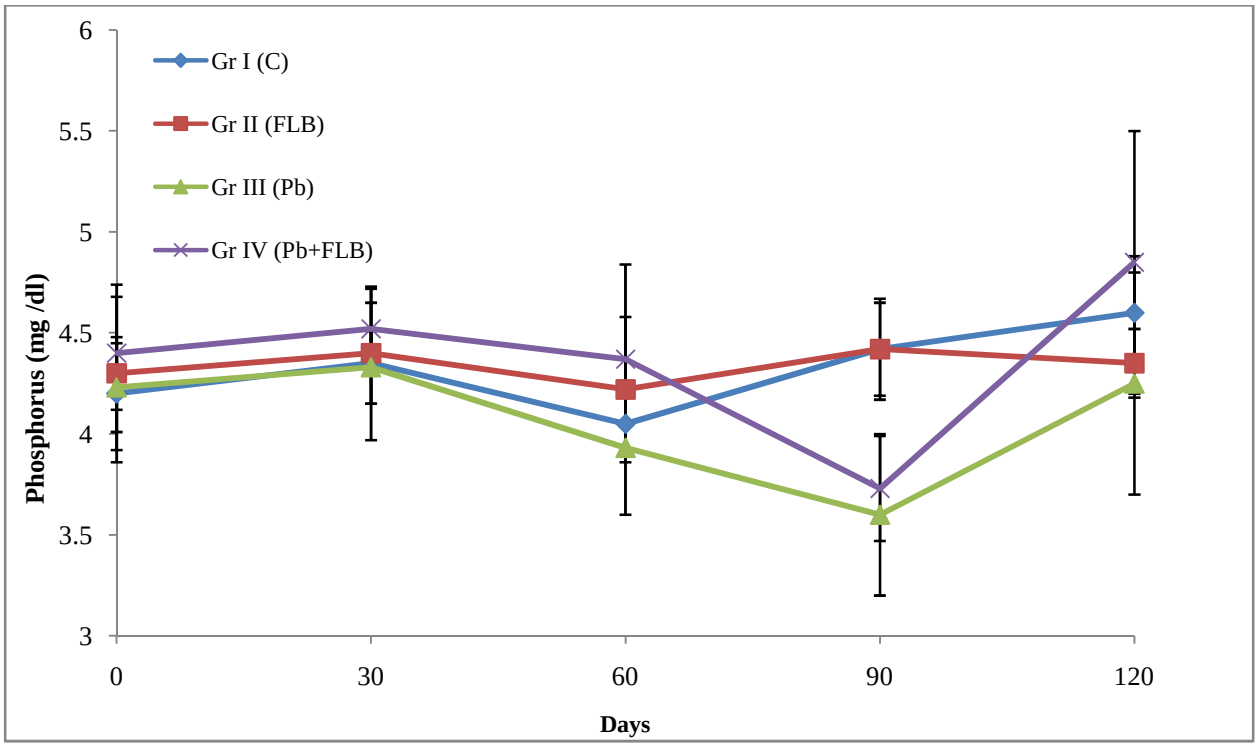

Fig. 36: Comparative effect of chronic oral administration of flubendiamide, lead and their combination on phosphorus concentration in blood

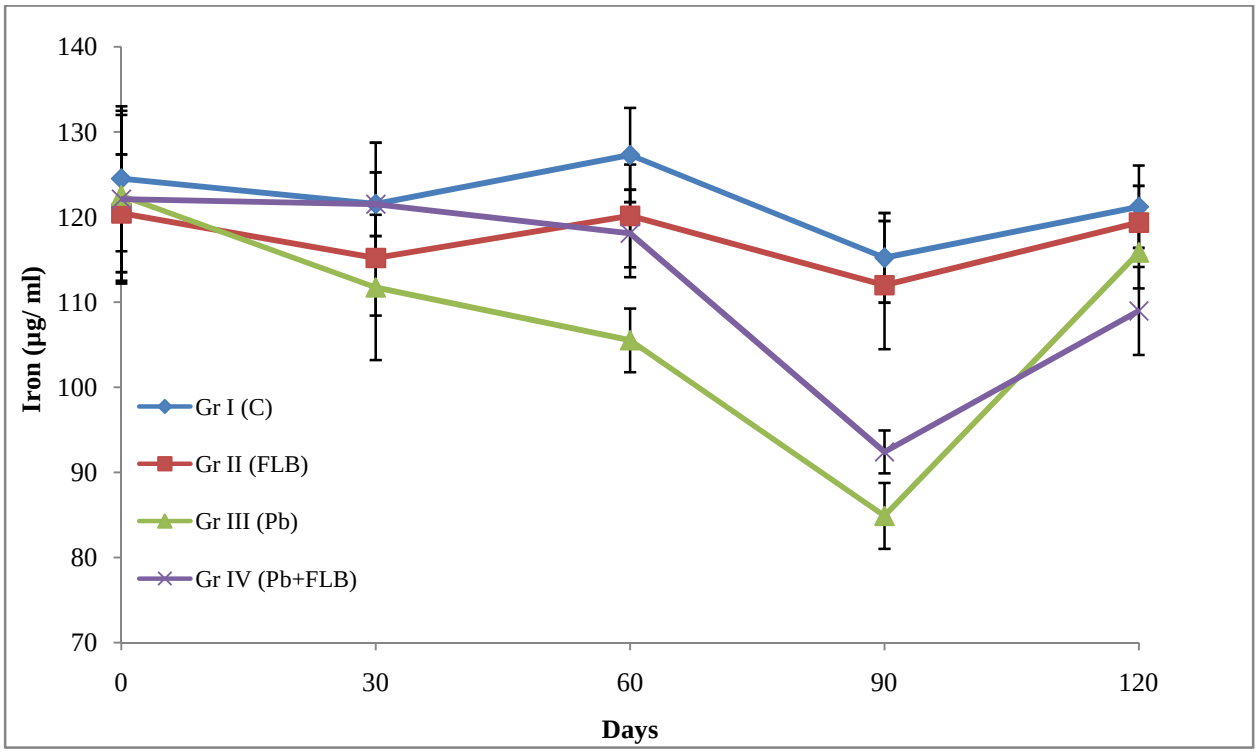

Fig. 37: Comparative effect of chronic oral administration of flubendiamide, lead and their combination on iron concentration in blood

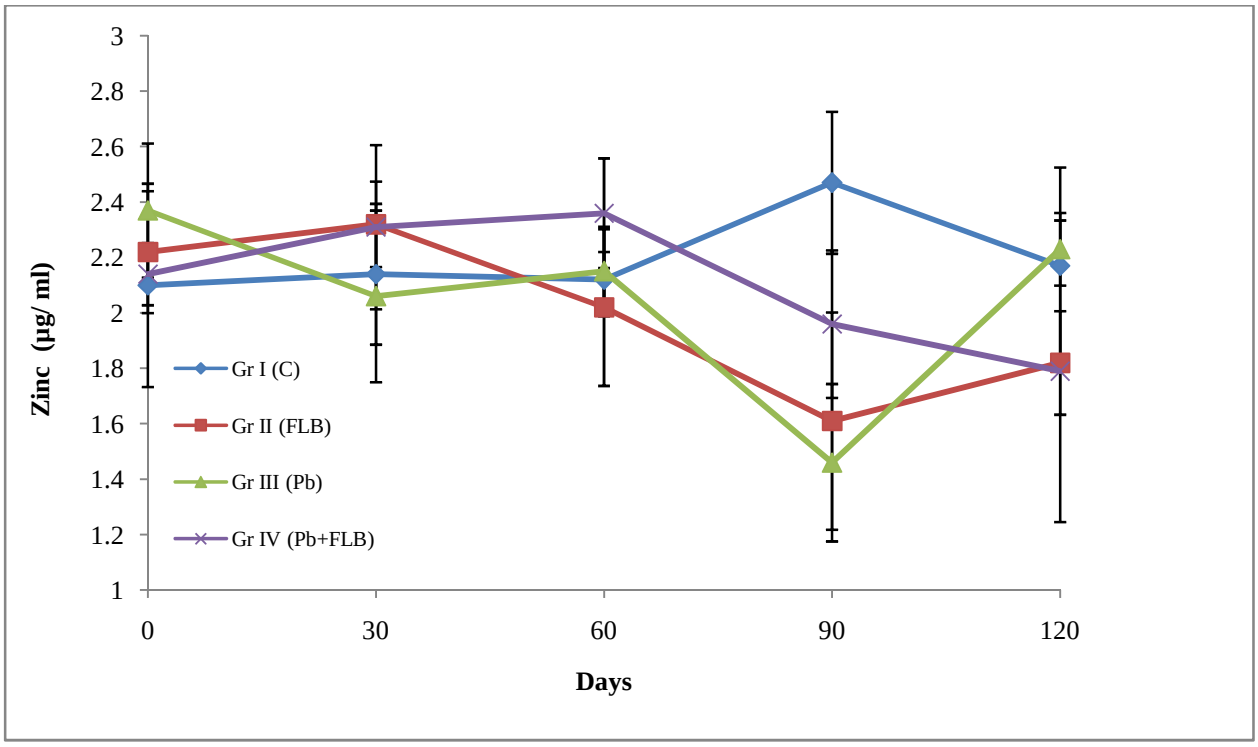

Fig. 38: Comparative effect of chronic oral administration of flubendiamide, lead and their combination on zinc concentration in blood

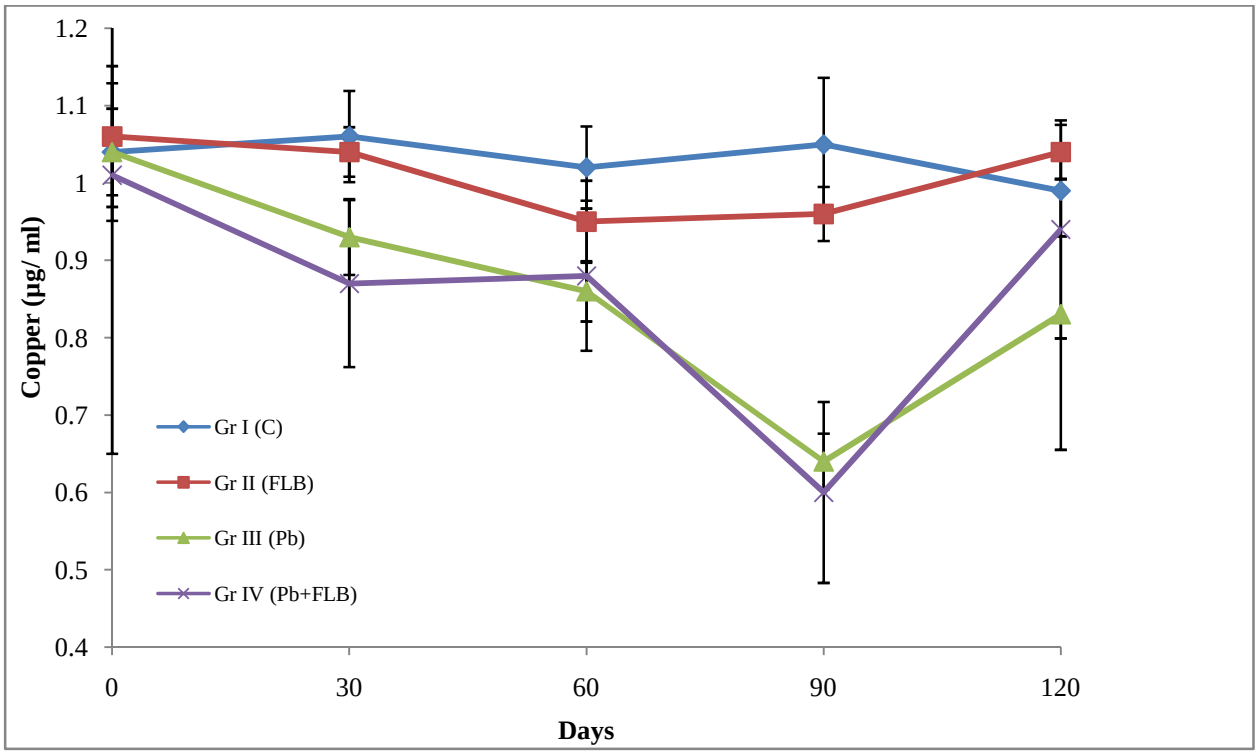

Fig. 39: Comparative effect of chronic oral administration of flubendiamide, lead and their combination on copper concentration in blood

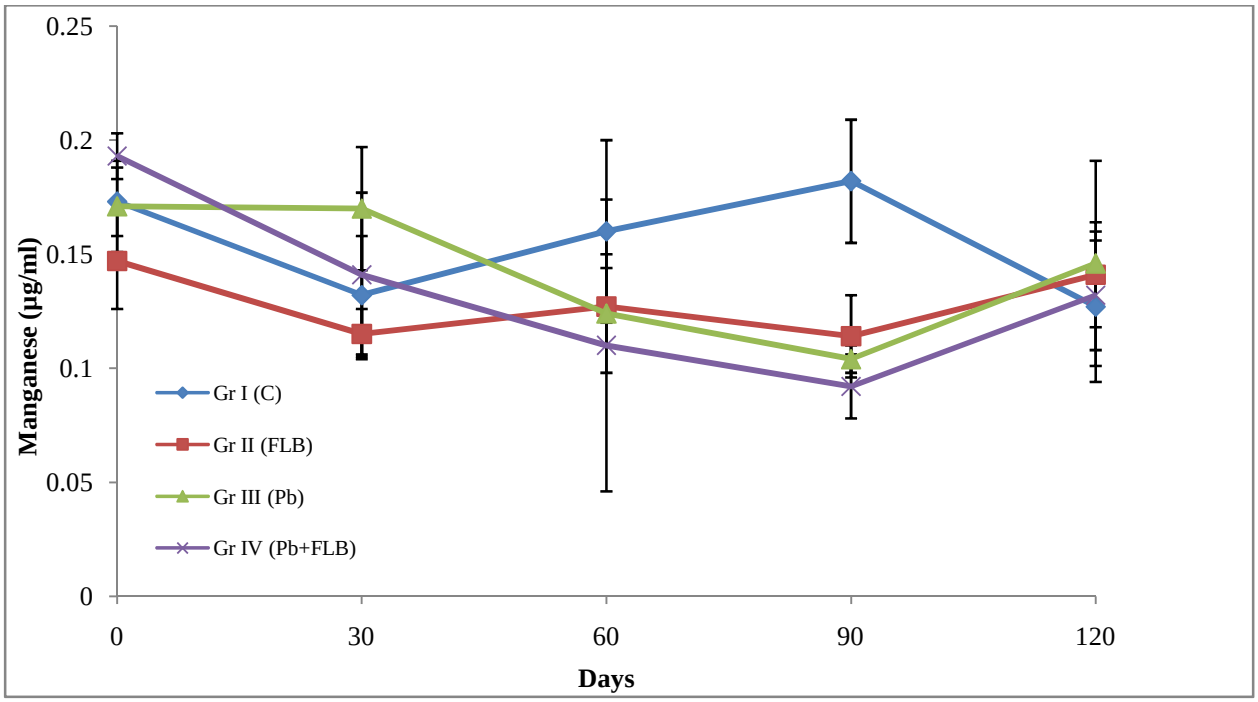

Fig. 40: Comparative effect of chronic oral administration of flubendiamide, lead and their combination on manganese concentration in blood

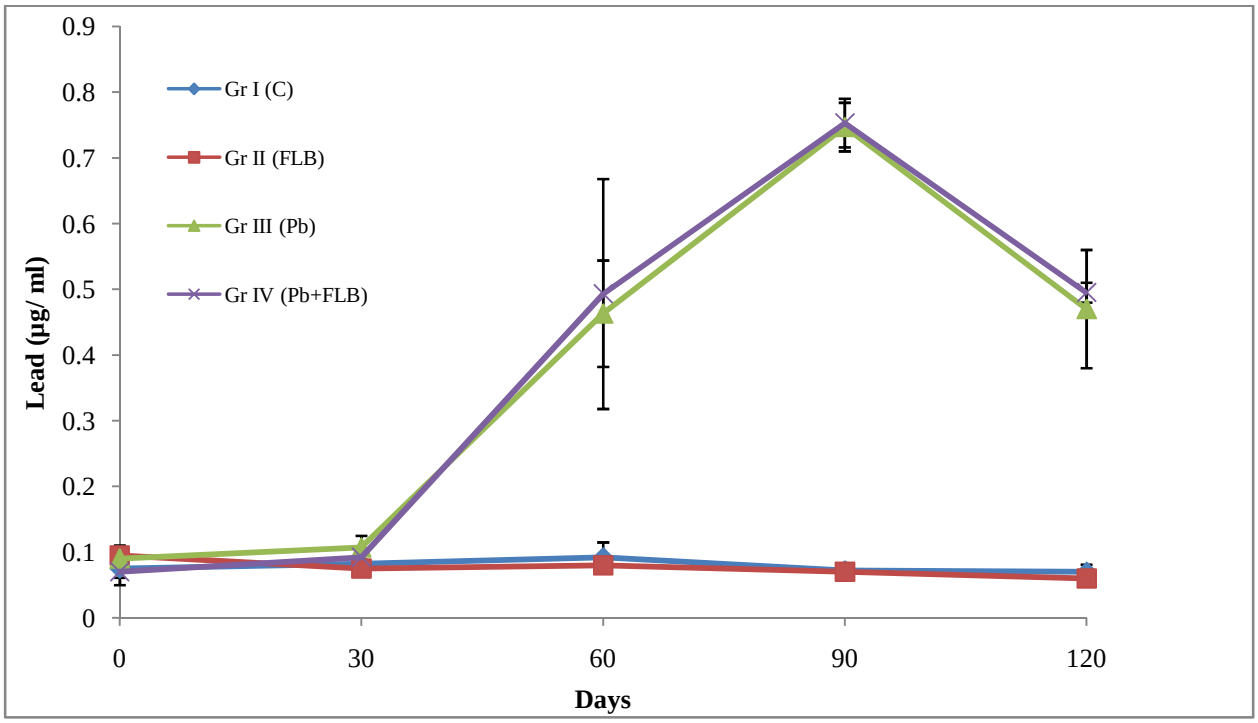

Fig. 41: Comparative effect of chronic oral administration of flubendiamide, lead and their combination on lead concentration in blood

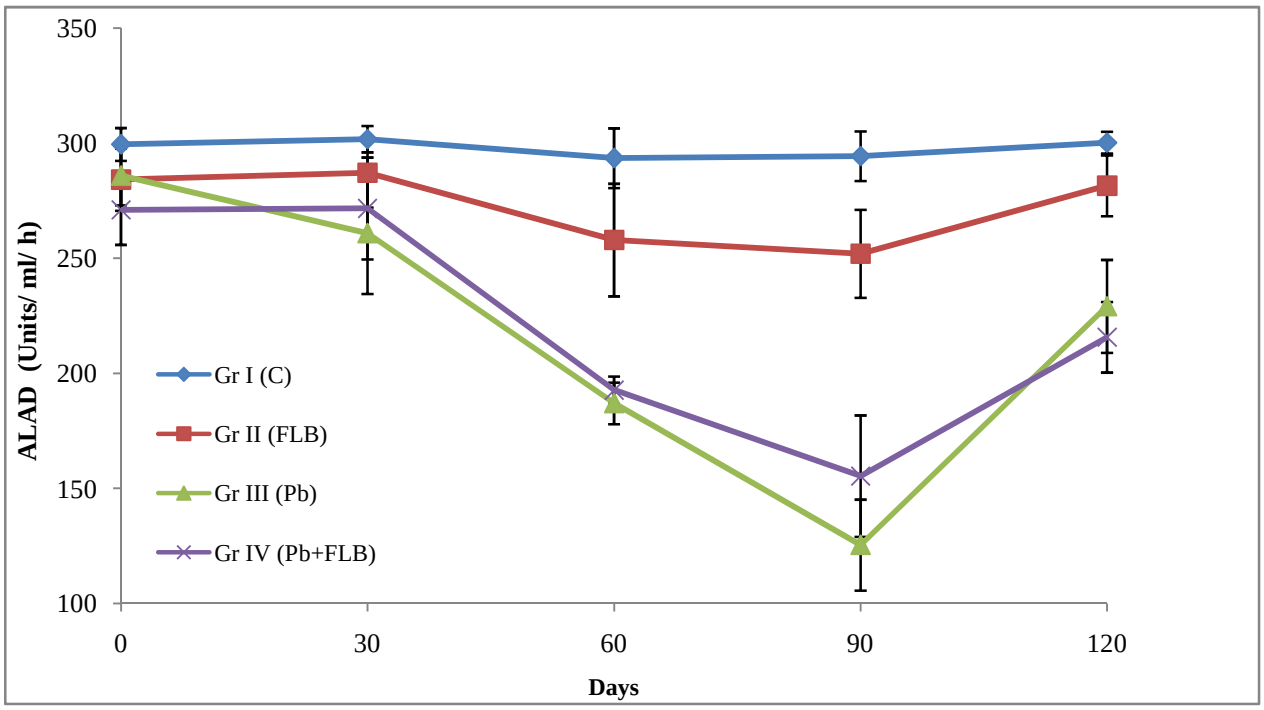

Fig. 42: Comparative effect of chronic oral administration of flubendiamide, lead and their combination on ALAD activity in blood

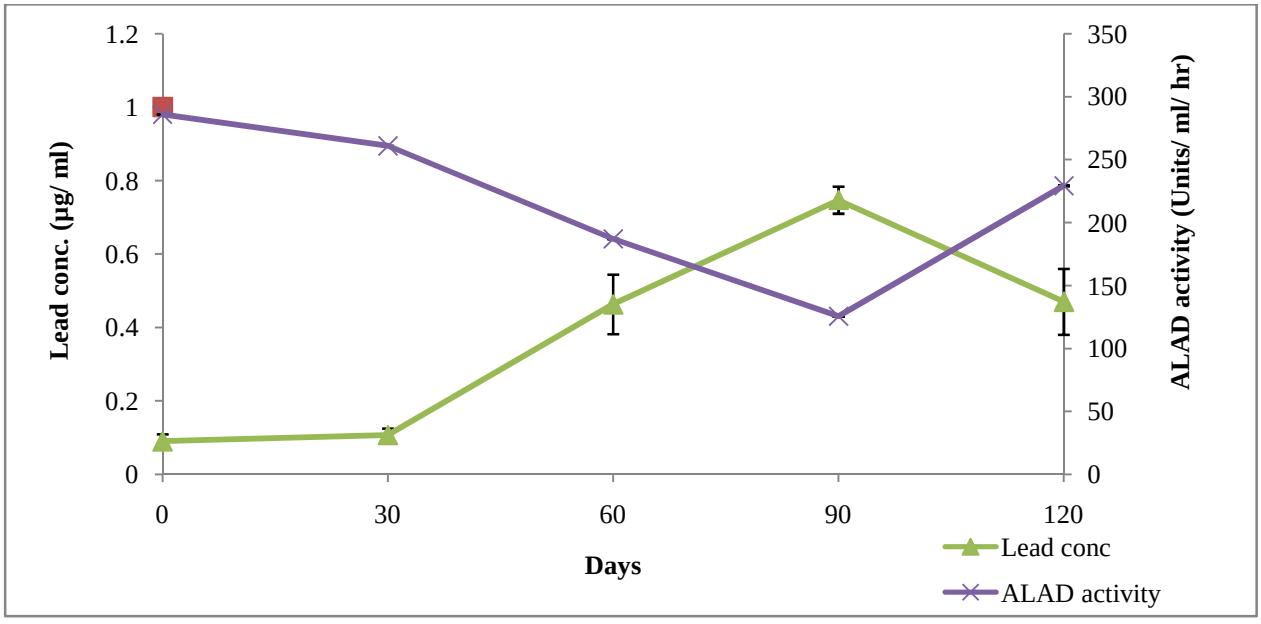

Fig. 43 a: Blood lead concentration and ALAD activity in lead exposed calves

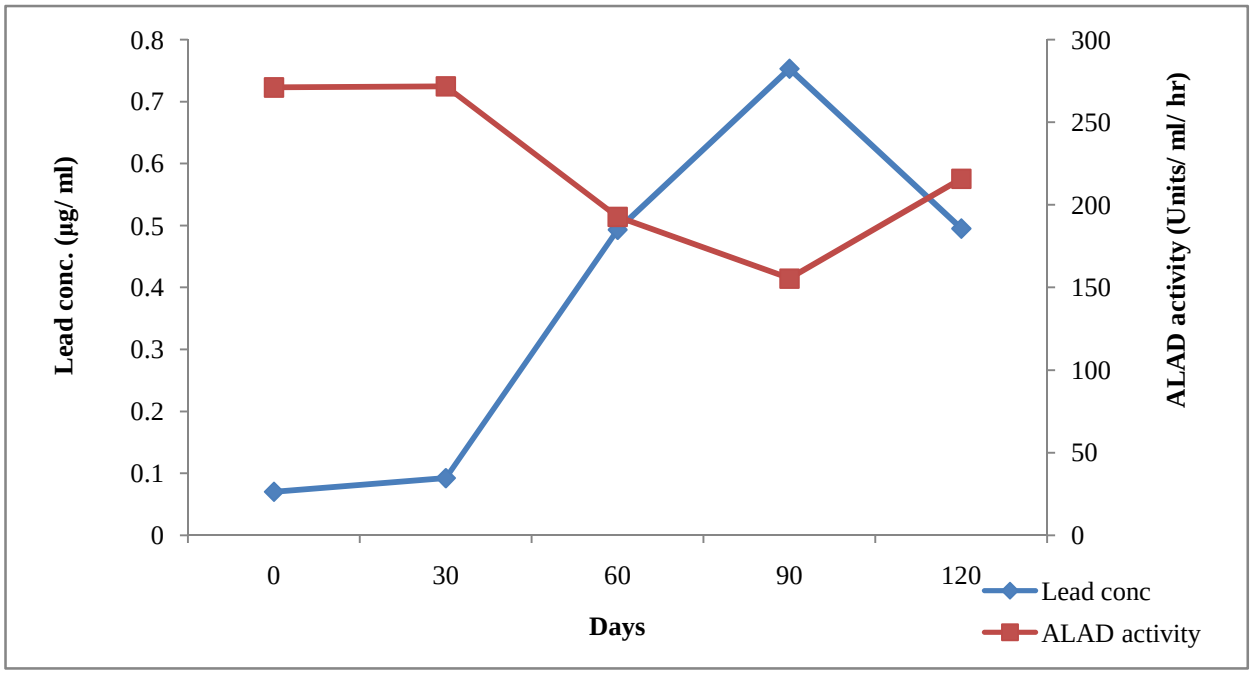

Fig. 43 b: Blood lead concentration and ALAD activity in combined lead and flubendiamide exposed calves

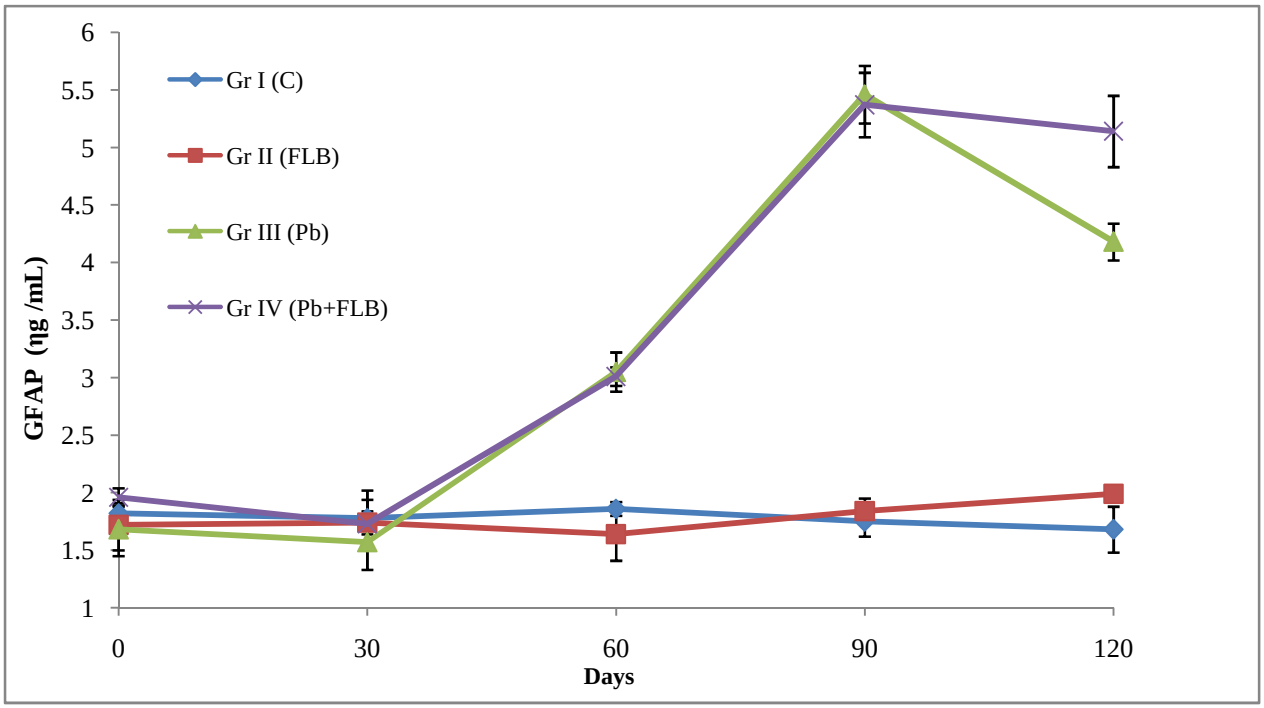

Fig. 44: Comparative effect of chronic oral administration of flubendiamide, lead and their combination on glial fibrillary acidic protein (GFAP) concentration in CSF

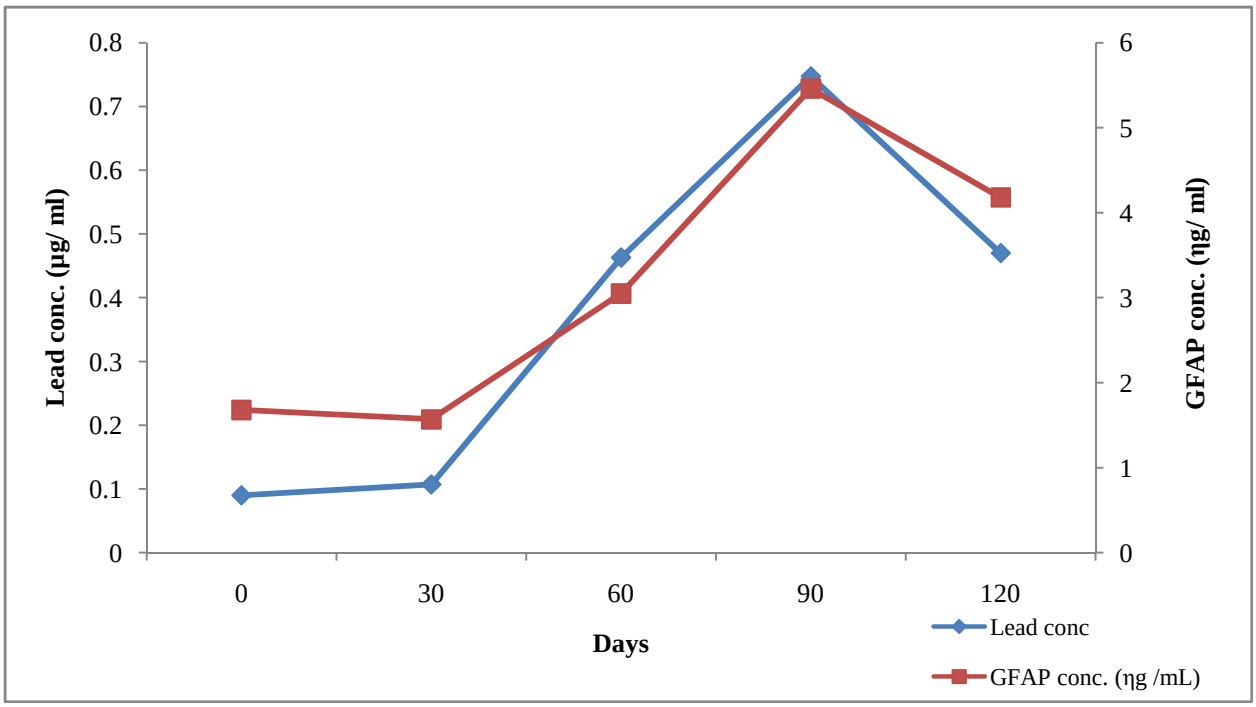

Fig. 45a: Lead and GFAP concentration in lead exposed calves

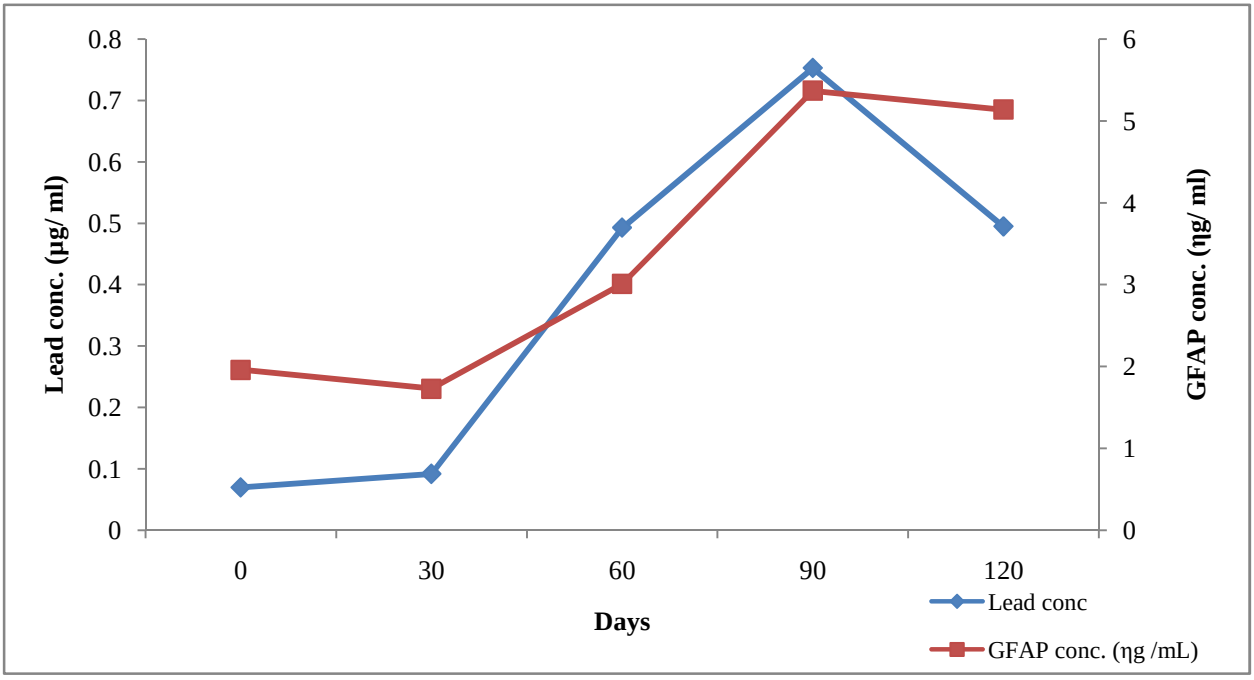

Fig. 45b: Lead and GFAP concentration in combined lead and flubendiamide exposed calves

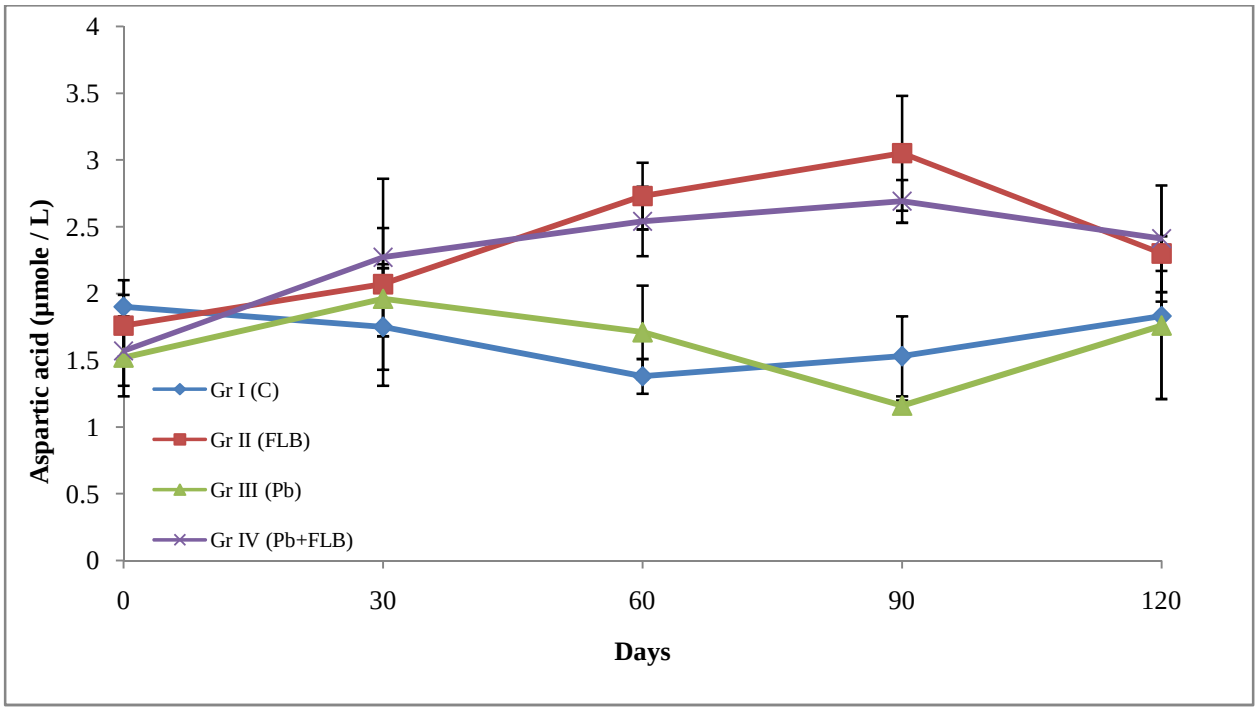

Fig. 46: Comparative effect of chronic oral administration of flubendiamide, lead and their combination on aspartic acid concentration in CSF

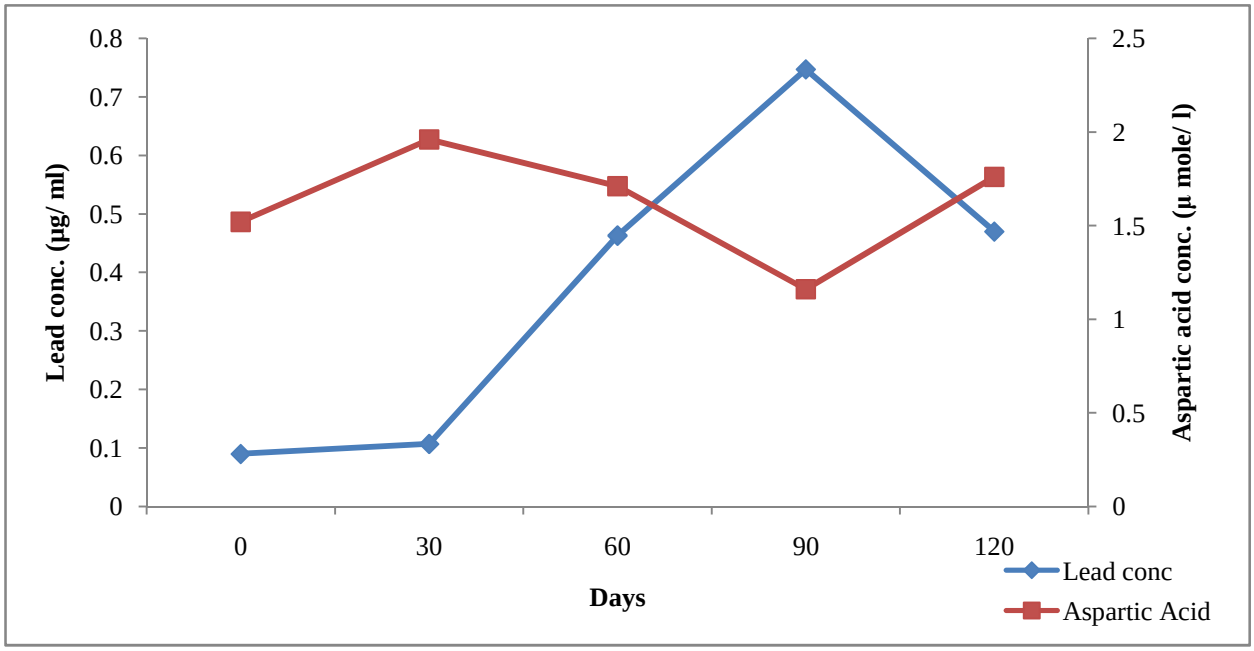

Fig. 47a: Lead and aspartic acid concentration in lead exposed calves

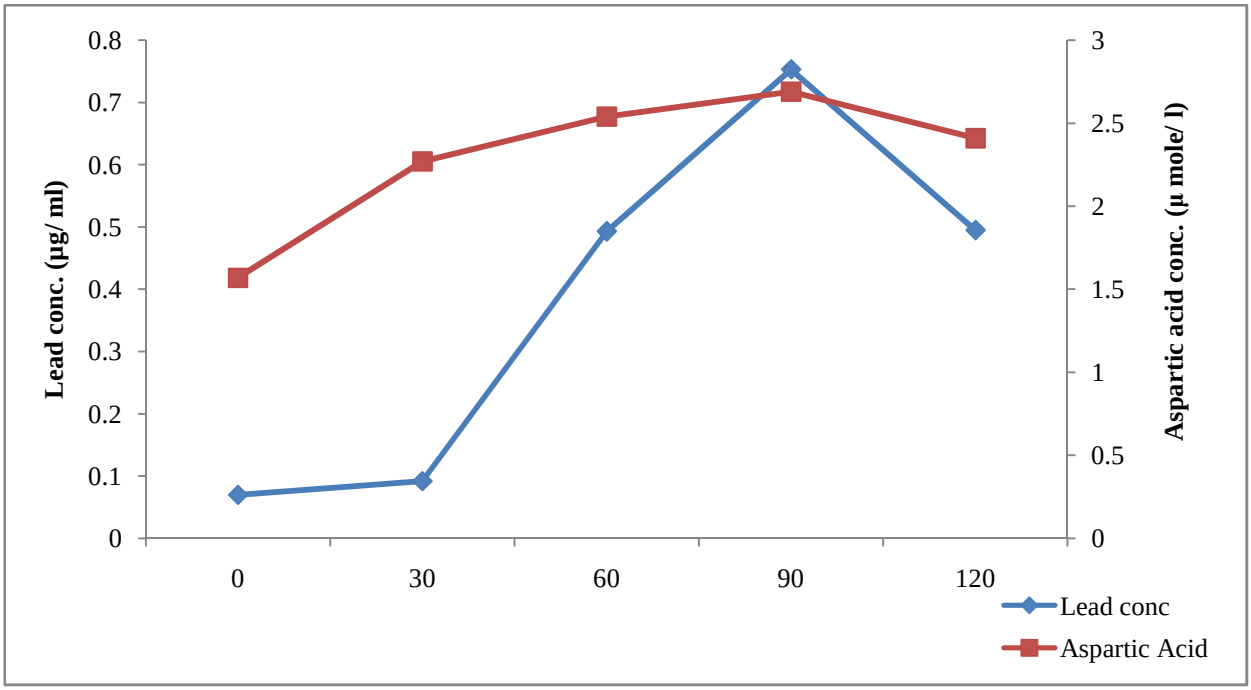

Fig. 47b: Lead and aspartic acid concentration in combined lead and flubendiamide exposed calves

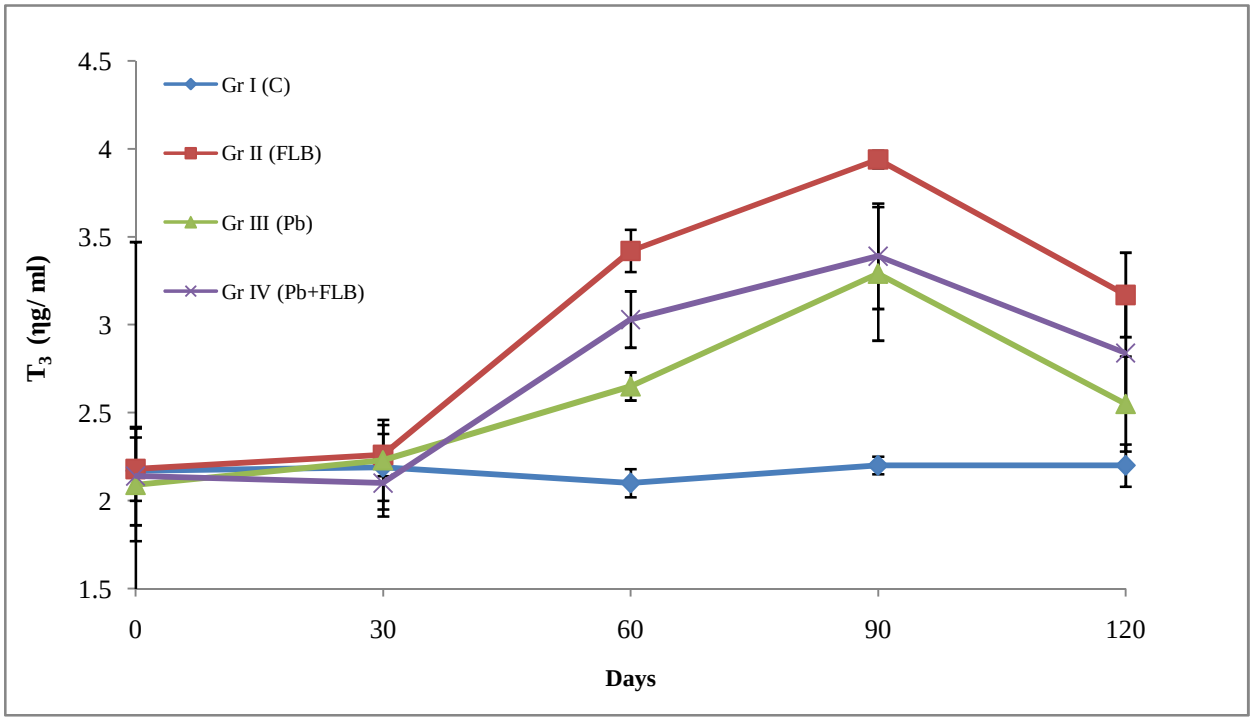

Fig. 48: Comparative effect of chronic oral administration of flubendiamide, lead and their combination on T_3 level in serum

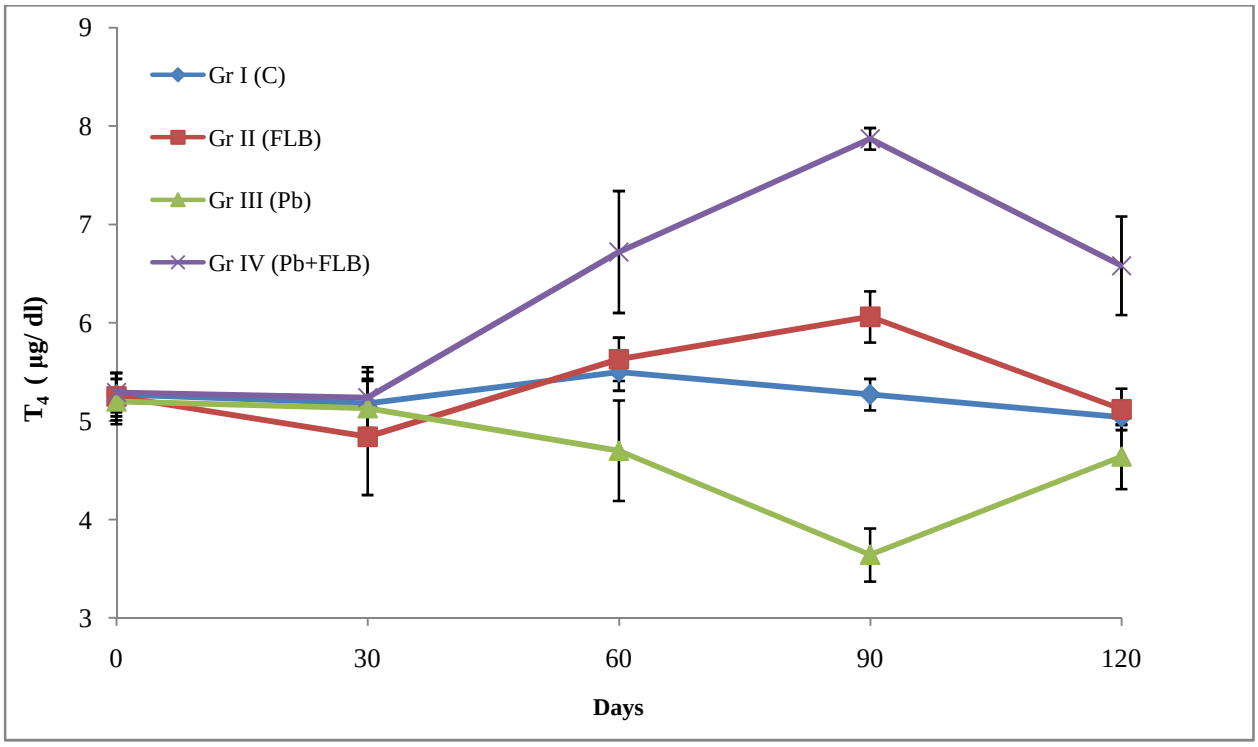

Fig. 49: Comparative effect of chronic oral administration of flubendiamide, lead and their combination on T_4 level in serum

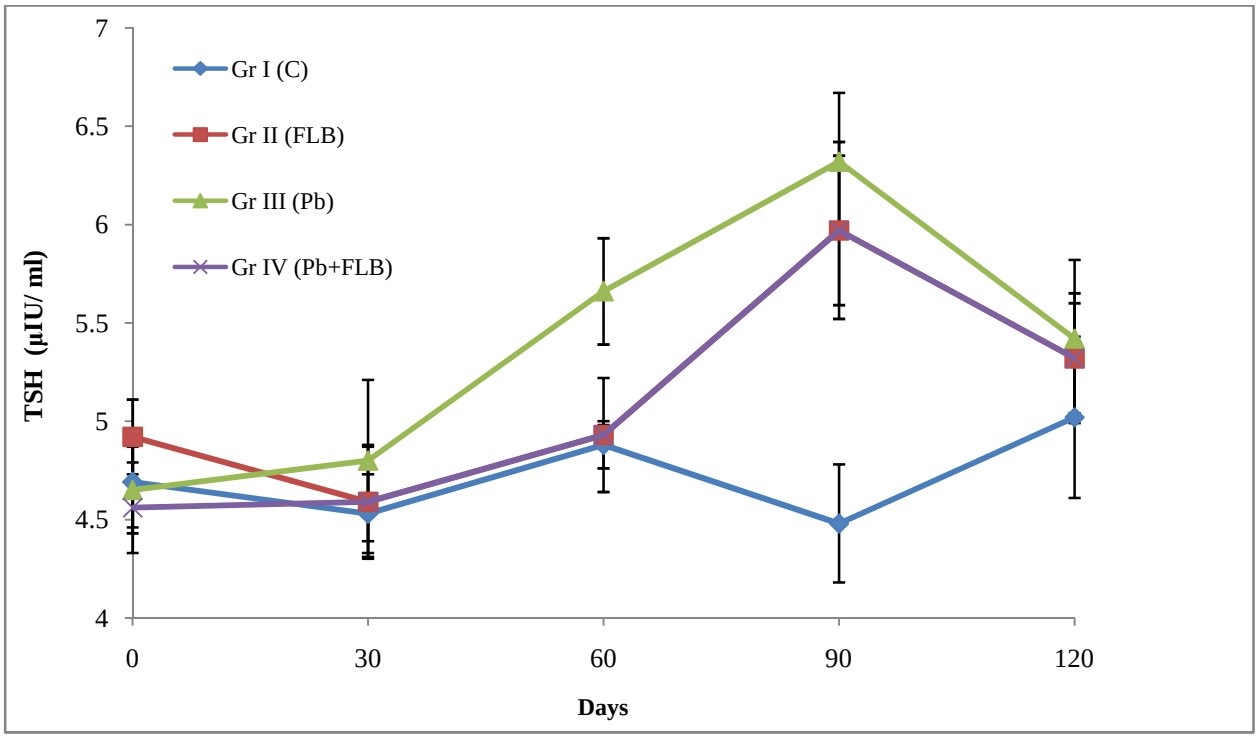

Fig. 50: Comparative effect of chronic oral administration of flubendiamide, lead and their combination on TSH level in serum

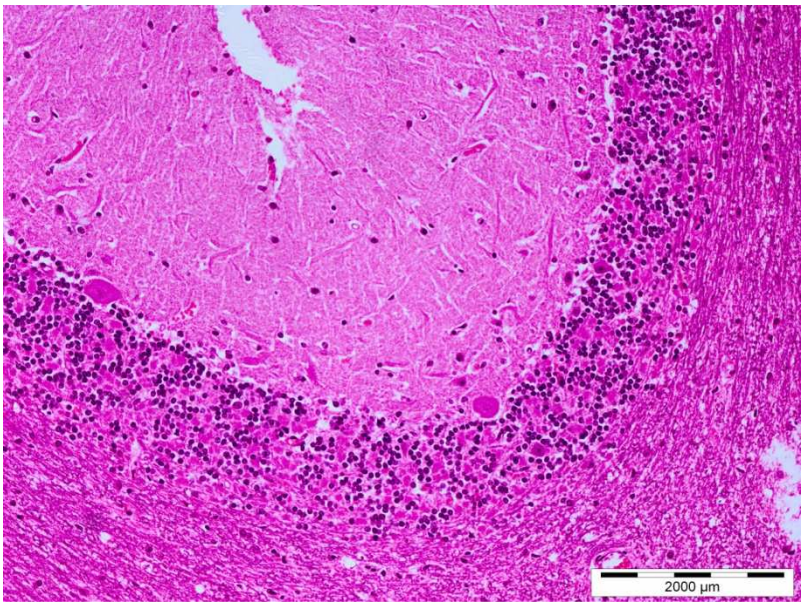

Fig 51. Cerebellum of lead exposed calf showing mild neuronal degeneration and purkinje cell degeneration in the molecular layer. H&E x 2000

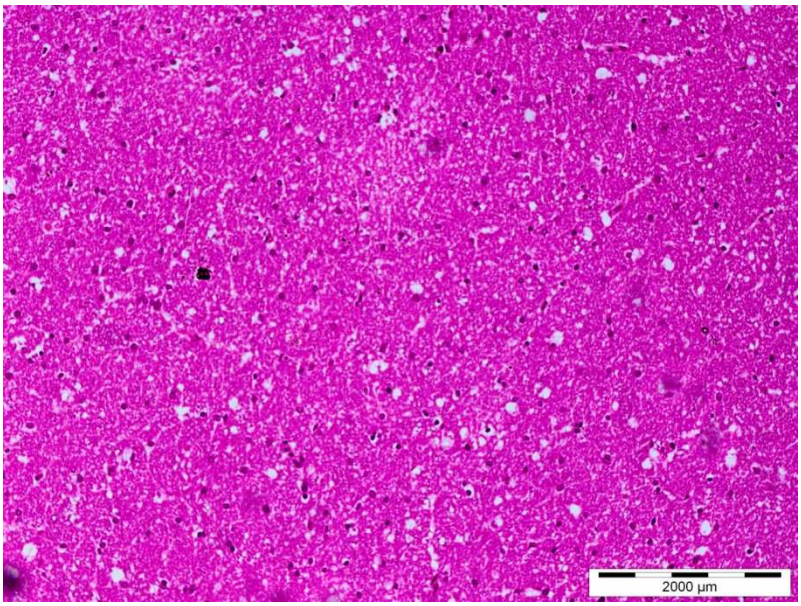

Fig 52. Cerebrum of lead exposed calf showing marked vacuolar degeneration and neuronal degeneration (Inset: high power view). H&E x 2000

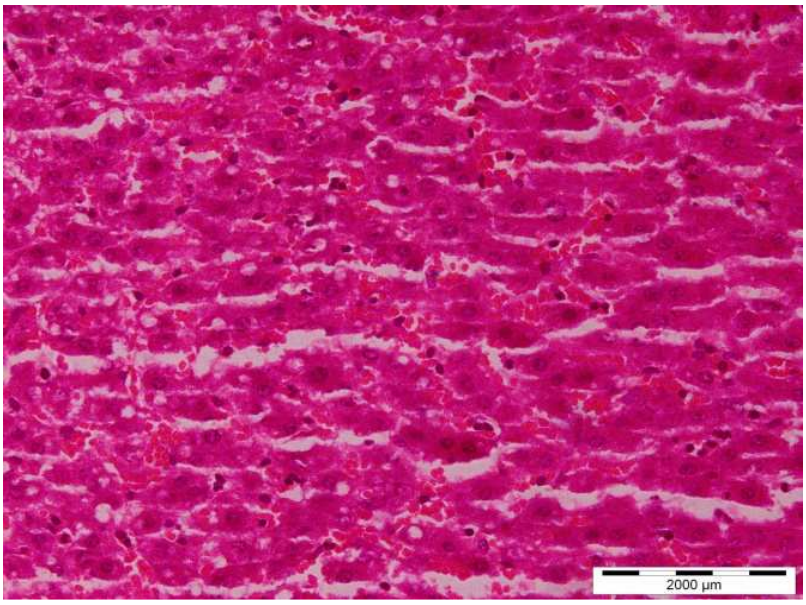

Fig 53. Liver of lead exposed calf showing swollen and degenerated hepatocytes with congested sinusoids. H&E x 2000

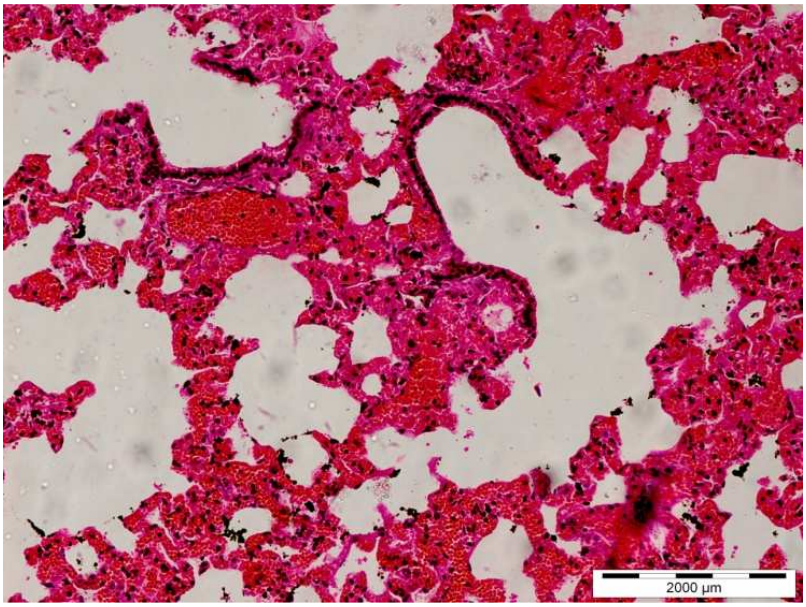

Fig 54. Lung of lead exposed calf showing haemorrhage and plenty of polymorphonuclear cells. H&E x 2000

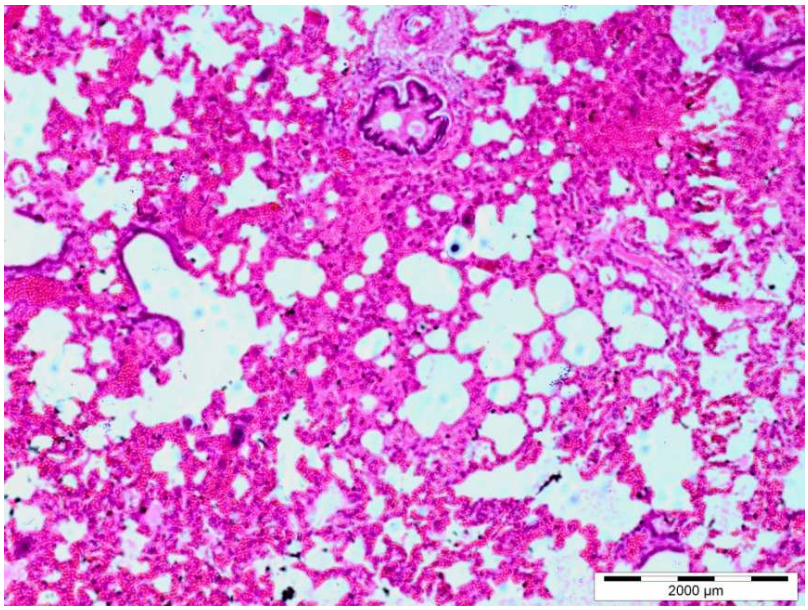

Fig 55. Lung of lead exposed calf showing severe pneumonia. Note the presence of plenty of inflammatory exudates and polymorphonuclear cells. H&E x 2000

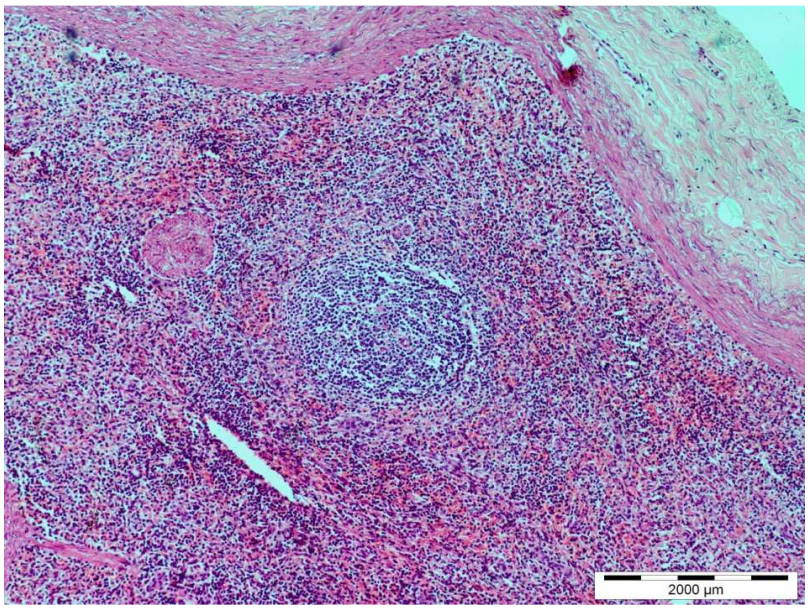

Fig 56. Spleen of lead exposed calf showing mild lymphoid depletion. H&E x 2000

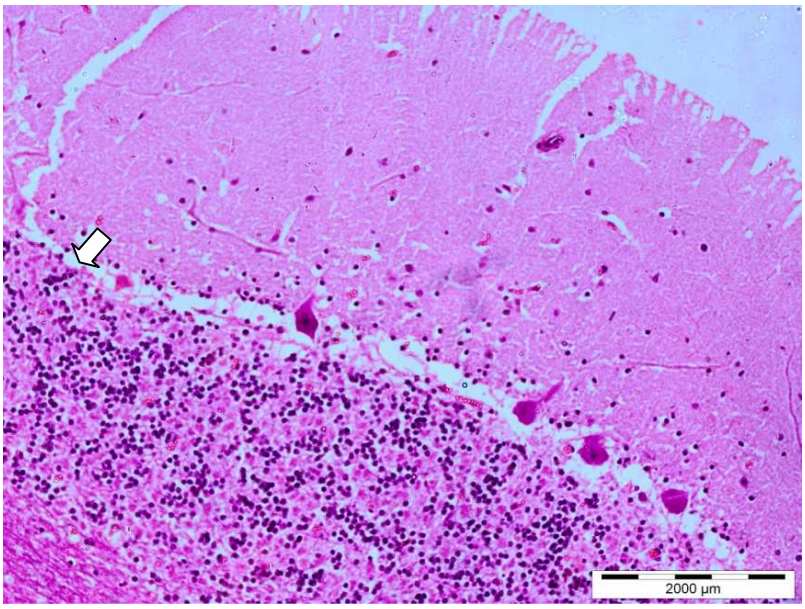

Fig 57. Cerebellum of combined lead and flubendiamide exposed calf showing purkinje cell degeneration (Arrow). H&E x 2000

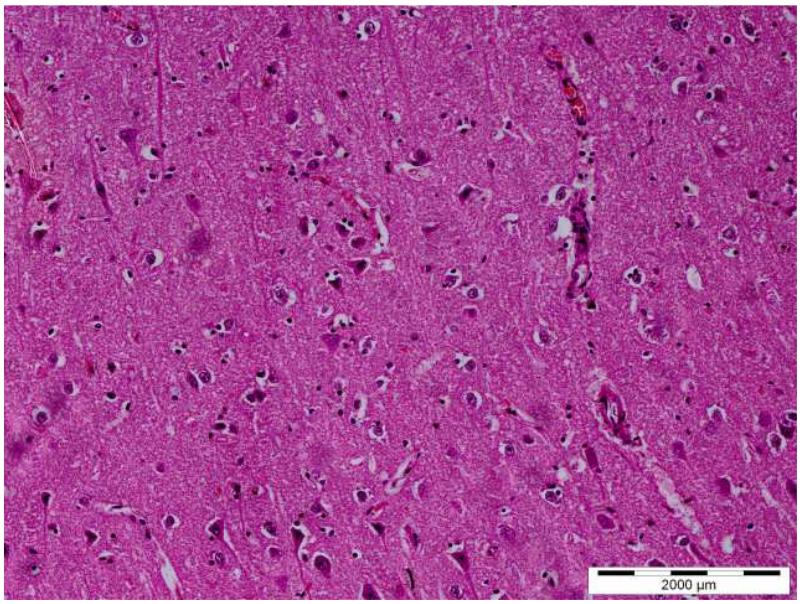

Fig 58. Cerebrum of combined lead and flubendiamide exposed calf showing mild neuronal degeneration and congested blood vessels. H&E x 2000

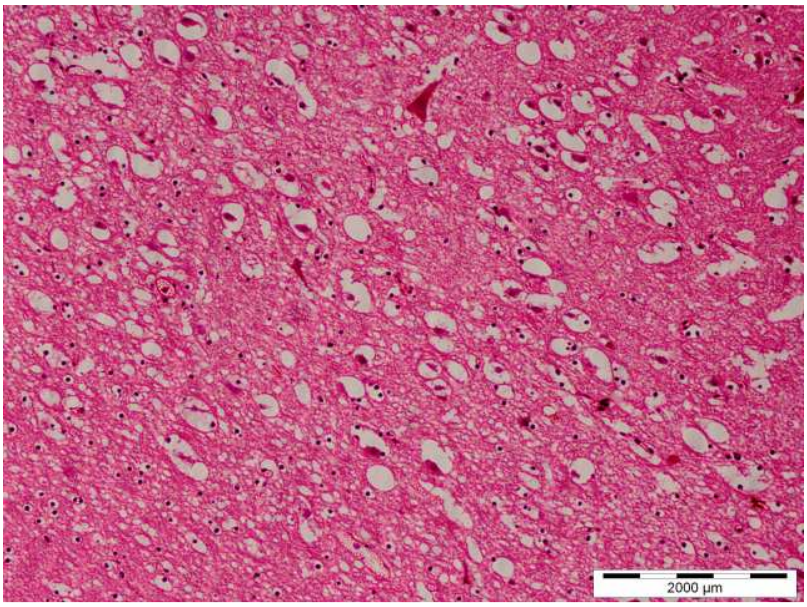

Fig 59. Cerebrum of flubendiamide and lead exposed calf showing severe vacuolar degeneration, oedema and neuronal degeneration. H&E x 2000

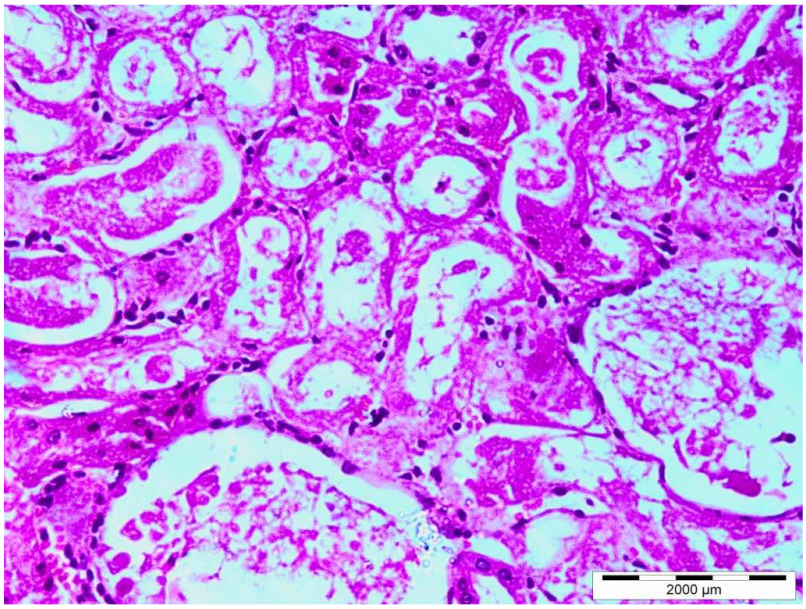

Fig 60. Kidney of combined lead and flubendiamide exposed calf showing severe tubular degeneration, sloughing of epithelial cells of the proximal convoluted tubules and presence of proteinaceous casts in their lumen. H&E x 2000

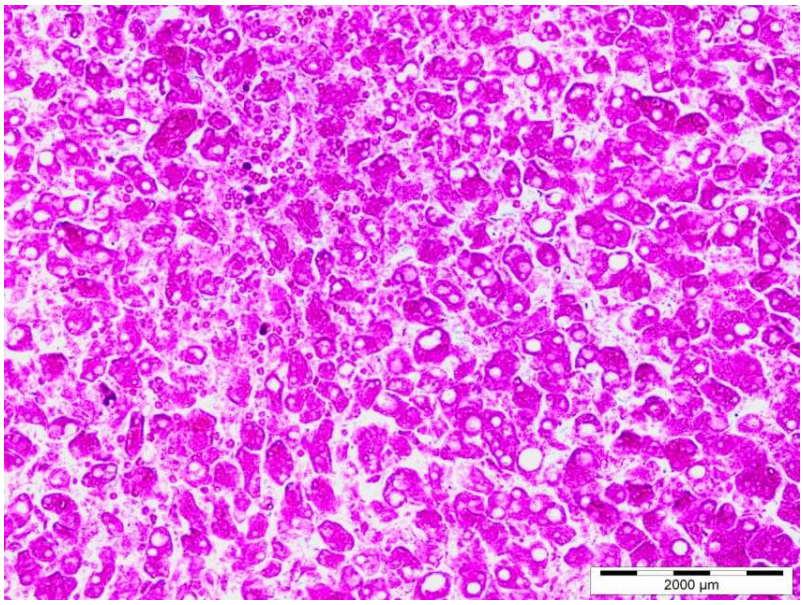

Fig 61. Liver of combined lead and flubendiamide exposed calf showing severely swollen and degenerated hepatocytes and fatty infiltration. Note the absence of normal hepatocytic chord pattern. H&E x 2000

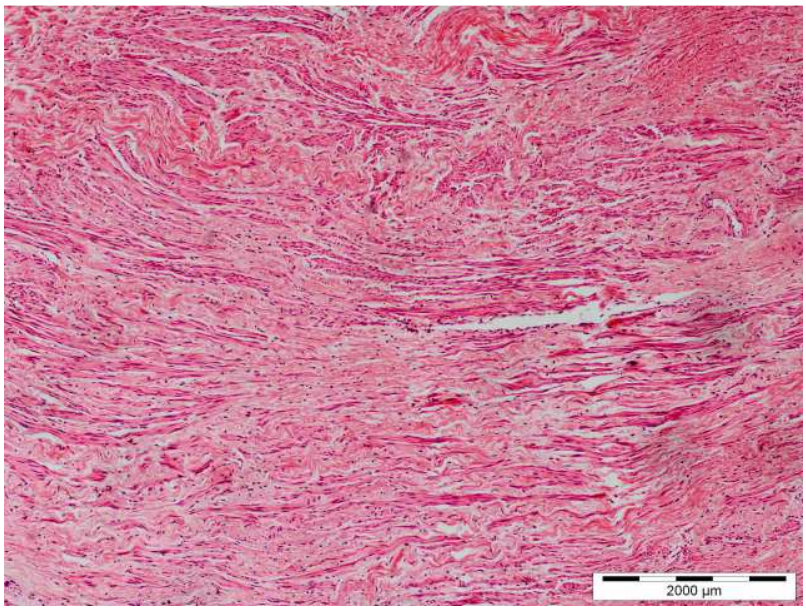

Fig 62. Heart of combined lead and flubendiamide exposed calf showing myocardial degeneration. H&E x 2000

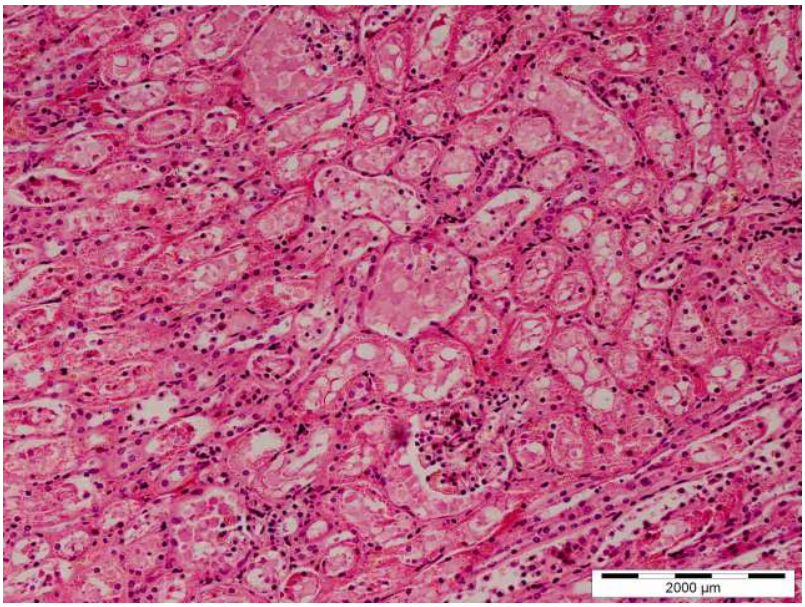

Fig 63. Kidney of combined lead and flubendiamide exposed calf showing severe tubular degeneration. Note the occluded lumen of proximal convoluted tubules with proteinaceous casts and sloughed off cells. H&E x 2000

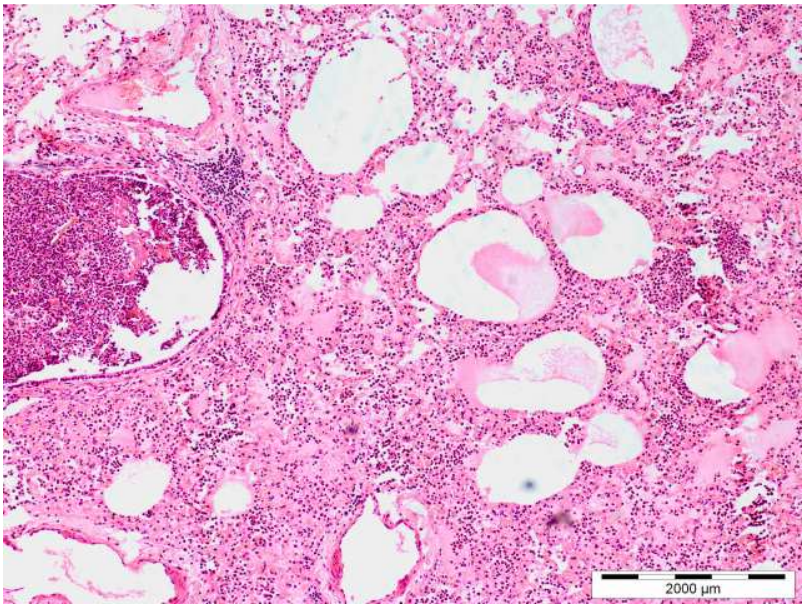

Fig 64. Lung of combined lead and flubendiamide exposed calf showing oedema and polymorphonuclear cells suggestive of pneumonia. H&E x 2000

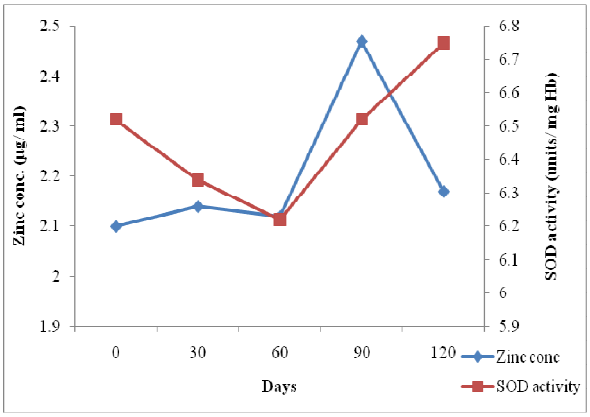

Fig. 25a. Blood zinc concentration and SOD activity in control animals

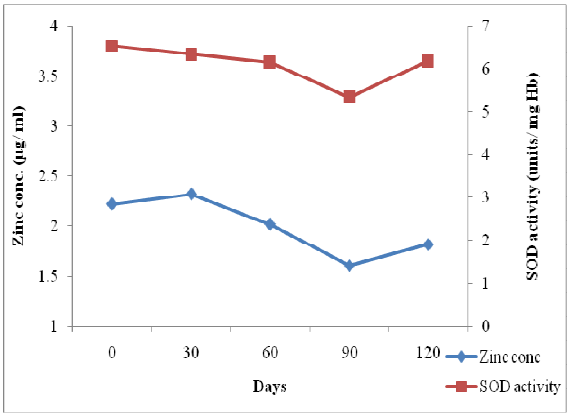

Fig. 25b. Blood zinc concentration and SOD activity in flubendiamide exposed animals

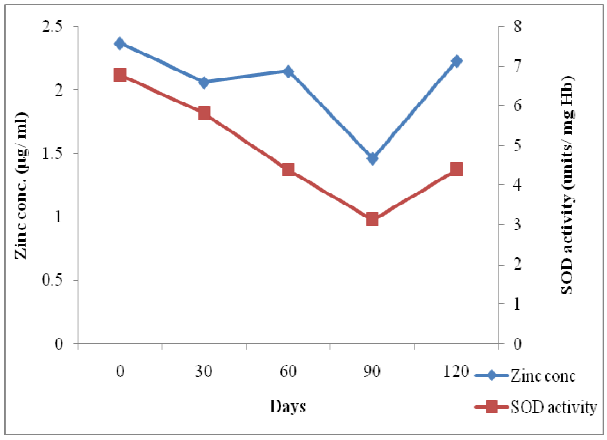

Fig. 25c. Blood zinc concentration and SOD activity in lead exposed animals

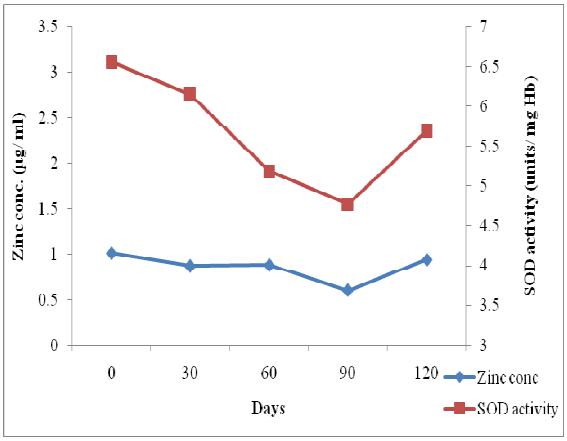

Fig. 25d. Blood zinc concentration and SOD activity in lead and flubendiamide exposed animals

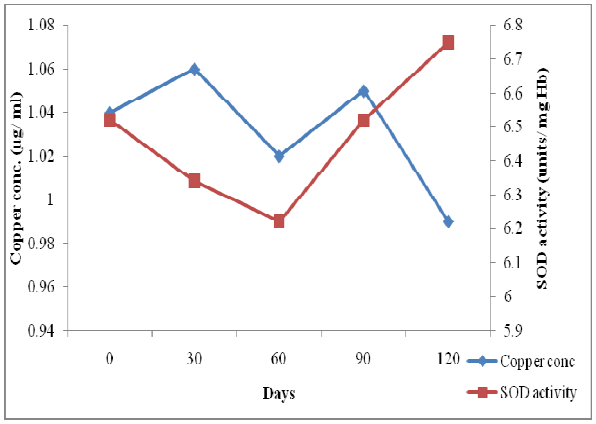

Fig. 26 a. Blood copper concentration and SOD activity in control animals

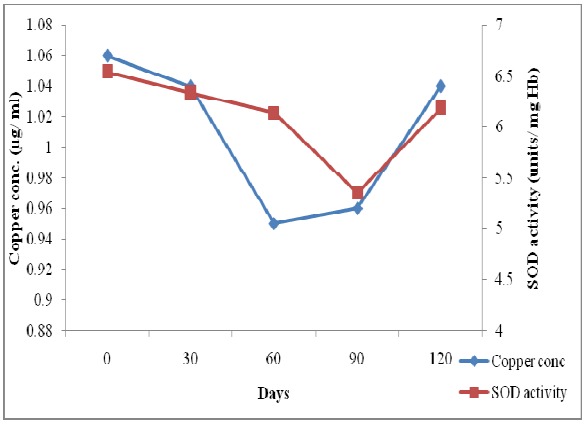

Fig. 26 b. Blood copper concentration and SOD activity in flubendiamide exposed animals

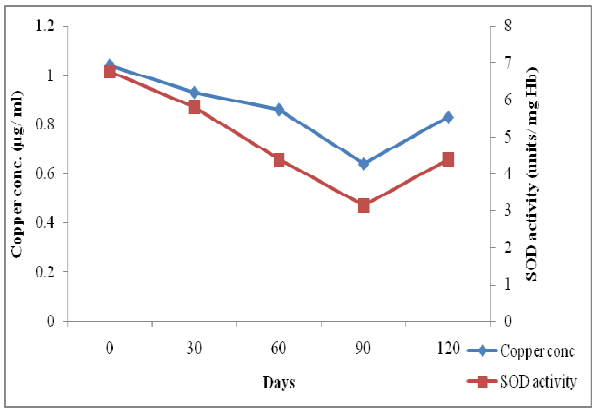

Fig. 26c. Blood copper concentration and SOD activity in lead exposed animals

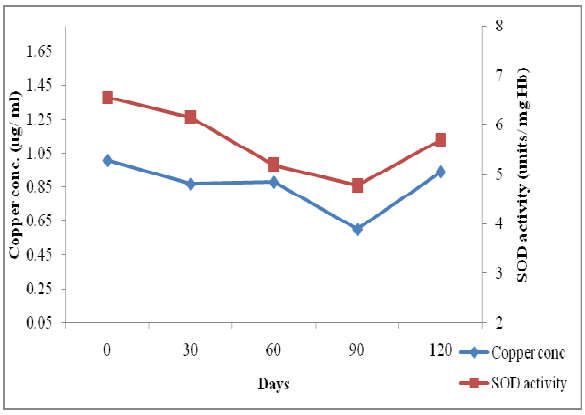

Fig. 26 d. Blood copper concentration and SOD activity in lead and flubendiamide exposed animals

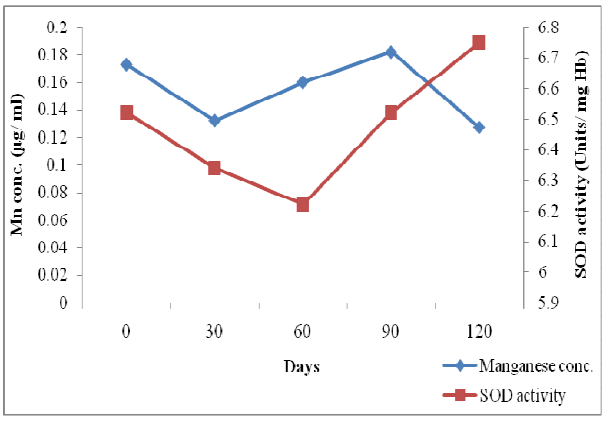

Fig. 27a. Blood manganese concentration and SOD activity in control animals

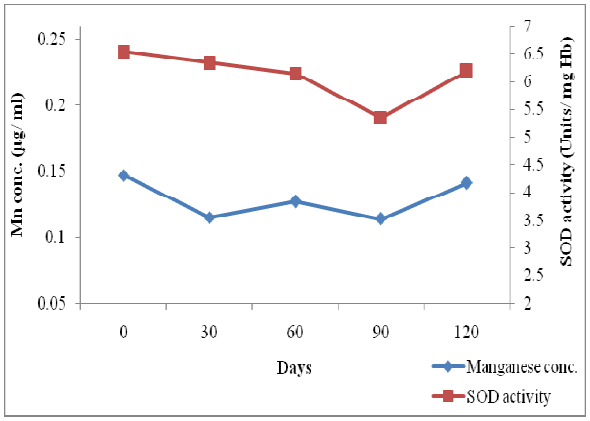

Fig. 27 b. Blood manganese concentration and SOD activity in flubendiamide exposed animals

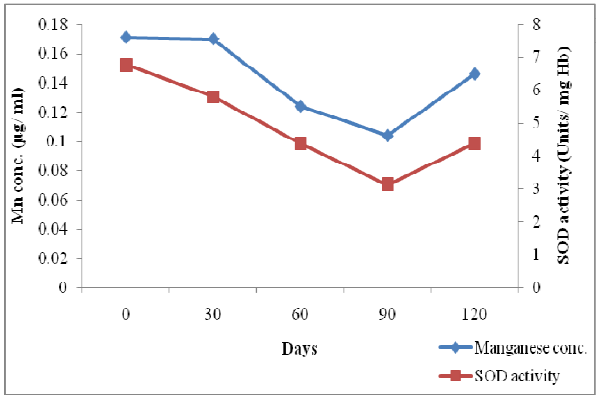

Fig. 27c. Blood manganese concentration and SOD activity in lead exposed animals

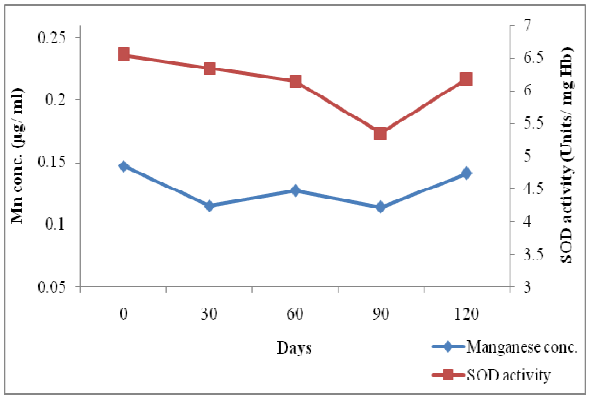

Fig. 27d. Blood manganese concentration and SOD activity in lead and flubendiamide exposed animals