BIOCHEMICAL AND HISTOPATHOLOGICAL STUDIES OF POSSIBLE CO-MORBIDITY OF CADMIUM, CHROMIUM AND LEAD IN ALBINO RATS

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CERTIFICATION

This is to certify that this work, "**Biochemical and Histopathological Studies of Possible Co-Morbidity of Cadmium, Chromium and Lead in Albino Rats,**" was carried out by Osoh, Kennedy (20104739428) in partial fulfillment for the award of the Degree of Doctor of Philosophy (Ph.D) in Environmental Biochemistry in the Department of Biochemistry, Federal University of Technology, Owerri.

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DEDICATION

I dedicate this work to the Most High God, the King of kings, the Lord of lords, the Creator of Heaven and Earth.

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ABSTRACT

Cadmium, chromium and lead are known environmental pollutants that induce toxicity in living organisms when exposed to them. The risk assessment of Cd, Cr and Pb is done individually while in reality living organisms are exposed to them jointly as they co-occur in the environment, food or water. This study was aimed at investigating their possible co-morbidity through biochemical and histopathological evaluations of albino rats exposed to them simultaneously. Seventy male albino rats were used in the study. Specified doses of 5, 10, and 20mg/kg body weight respectively, of these pollutants/metals were administered by gavage thrice weekly to 60 albino rats and 10 albino rats were used as control. There were four treatment groups Cd, Cr, Pb and Cd+Cr+Pb (i.e. Cd alone, Cr alone, Pb alone and Cd, Cr, Pb combined) per dose with five animals per treatment group. The treatments were for 90 days, and salt solutions of the metals (i.e. $CdSO_4$, $K_2Cr_2O_7$, and $Pb(NO_3)_2$) were used while the control received only distilled water. Body weights (BW) of the rats were measured and percentage BW gains of the rats were calculated. The animals were sacrificed after 90 days and blood samples were analyzed for alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), total bilirubin, urea, creatinine, potassium (K), chloride (Cl^-), inorganic phosphorus, malondialdehyde (MDA), reduced glutathione (GSH), ascorbic acid, superoxide dismutase (SOD), catalase (CAT), haemoglobin (Hb), packed cell volume (PCV), white blood cell (total) (WBC[T]), and red blood cell (RBC). Organ (liver, kidney, & heart) to BW ratio were measured. Histopathological evaluations of liver, kidney, and heart were done. Results of combined treatment showed hormetic response with regard to ALT, AST, total bilirubin, and urea as they were elevated in the low dose but decreased with high dose. This phenomenon was also observed in Cr individual treatment except in total bilirubin. The results showed that ALP, LDH, K, and inorganic phosphorus levels in the combined treatment group increased with increasing dose but was not significantly different ($p>0.05$) from that of the most hazardous metal in the individual treatments. Oxidative stress was observed in both the combined and individual treatments as MDA increased while GSH and ascorbic acid were reduced with increasing dose. SOD and CAT increased with increasing dose hence reducing the impact of the oxidative stress as observed. Less-than-additive interaction was observed in the 5mg/kg treatment group among the mixture components with regard to Hb and WBC. Combined treatment with the metals caused significant decrease in percentage BW gain but was not significantly different ($p>0.05$) from that of the most hazardous metal in the individual treatments. Histopathological evaluation showed tissue injury in liver and kidney in the 20mg/kg combined and individual treatment groups only probably due to high dose. Mortality was observed in the course of the study as two animals died due to the combined treatment (i.e. one at 10mg/kg group and one at 20mg/kg group) while three animals died due to the individual treatments (i.e. one at 10mg/kg Cd group, one at 20mg/kg Cr group and one at 20mg/kg Pb group). Conclusively, the results suggested that there was no significant health risk posed by simultaneous exposure to the metals beyond the risk already posed by the most hazardous individual metal for the endpoint of interest. Interactions where they occurred were predominantly less-than-additive. Hormesis should be considered in their risk assessment. The concept of effect addition (independent action) over-estimated the risk due to the combined treatments.

Keywords: Treatment, hormetic, combined, histopathological, hazardous, individual, interaction

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Humans are exposed to several environmental pollutants daily. Chemicals legislation in most parts of the world is based mainly on assessments carried out on individual substances but in reality humans and other living organisms (animals and plants) are exposed to a wide range of chemicals throughout their lives. While current assessment methods incorporate safety factors to take account of a range of uncertainties, the European Commissionand other developed countries are concerned with ensuring thattheirchemicals legislation takes proper account of the latest scientific information on mixture toxicity (European Union [EU], 2012; Hayes et al., 2019; Socianuet al., 2022). While all populations may be exposed to chemicals, workers tend to face exposure to higher doses and over longer time periods, increasing their risk of significant health effects (International Labour Organization [ILO], 2021).

Although some potential environmental hazards involve significant exposure to only a single compound, most instances of environmental pollution involve concurrent or sequential exposures to a mixture of compounds that may induce similar or dissimilar effects over exposure periods ranging from short-term to lifetime (Hernandez, Buha, Constantin& Tsatsakis, 2019). This may occur in the form of simultaneous exposure to mixtures of chemicals, where multiple chemicals occur in a given environmental medium. Exposures may also be cumulative, where multiple chemicals are encountered from multiple environmental media via multiple routes. These chemicals could be in air, food, water, soil, or consumer products. Exposures to mixtures of chemicals results from water, air and soil pollution from municipal incinerators, leakage from

hazardous waste facilities and uncontrolled waste sites, drinking water containing chemical substances formed during disinfection, and ground water around waste disposal sites. Exposure to residues of oxo-products of halogens, resulting from drinking water disinfection, could be carcinogenic. Recent national report on human exposure to environmental chemical, through the analyses of blood and urine samples, indicates that over 100 chemicals were found in the United States human population.

Two major sources of cadmium pollution are the production and consumption of cadmium and other non-ferrous materials, and the disposal of wastes containing cadmium. Increase in soil cadmium content leads to increase in plant uptake and consequently increase in cadmium concentration in foodstuffs. Edible free-living food organisms such as shellfish, crustaceans, and mushrooms are natural accumulators of cadmium. Cadmium present in some sea foods is as much as 10mg/kg wet weight and human consumption of these marine organisms has been linked to signs of kidney damage as food is the major route of exposure to cadmium for non-smoking general population (Genchi, Sinicropi, Lauria, Carocci & Catalano, 2020; Satarug, 2018). Long term exposure to cadmium causes renal tubular dysfunction with proteinuria, glycosuria, and aminoaciduria as well as histopathological changes in both experimental animals and humans (Genchiet al., 2020; Satarug, 2018). Oral exposure to cadmium can cause anaemia in humans and animals (ATSDR, 2012a; Fujiwara et al., 2020). Also, hepatic effects occur for higher oral doses of cadmium, usually for acute and intermediate duration.

Hexavalent chromium compounds such as potassium dichromate ($K_2Cr_2O_7$) are oxidizing agents capable of directly inducing tissue damage. They are found in the environment as a result of human activities resulting from industrial oxidation of mined chromium ores as well as manufacture and disposal of chromium containing materials. Examples of chromium containing

materials are chromium ore, baths, colours, lubricating oils, anti-corrosives (paints), wood preservation salts, cement, cleaning materials, textiles, and leather tanned with chromium. Most surface waters contain between 1 and 10µg of chromium per litre, and in general, the chromium content of surface waters reflects the extent of industrial activity (Tripathi & Chaurasia, 2020). The average concentration of chromium in rainwater is in the range 0.2-1g/litre. Hexavalent chromium is metabolized through reactions involving intracellular species such as ascorbate, and glutathione leading to generation of reactive oxygen species which may be responsible for deleterious effects of chromium on cells (Zhong, Zeng, Bian, Zhong & Xiao, 2017).

Lead containing paint is a primary source of lead exposure in children while the major route of exposure for the general population is from food and water as well as inhalation of lead oxide from exhaust fumes. Also, herbal medicines could be potential sources of lead exposure. Lead has been shown to affect virtually every organ and system in the body in both humans and animals. The effects of lead are similar across inhalation and oral routes of exposure and include neurological (particularly in children), hematological, cardiovascular and renal. Lead interferes with the synthesis of heme leading to reductions in blood hemoglobin and anaemia. Also, lead has been associated with chronic nephropathy in humans (Arkhipov, Garipova, Strizhacov, Bobkova & Tairova, 2022). Exposure to lead-contaminated soil and dust resulting from battery recycling and mining has caused mass lead poisoning and multiple deaths in young children in Nigeria, Senegal and other countries (World Health Organization [WHO], 2021). World Health Organization is currently developing guidelines on the prevention and management of lead poisoning, which will provide policy-makers, public health authorities and health professionals with evidence-based guidance on the measures that they can take to protect the health of children and adults from lead exposure (WHO, 2021).Cadmium, chromium, and lead mixture have been

chosen as the subject for this interaction study because it is a very frequently occurring ternary mixture at hazardous waste sites. This mixture was found in soil at 219 sites out of the 1,608 sites for which Agency for Toxic Substances and Disease Registry (ATSDR) has produced a Public Health Assessment (Agency for Toxic Substances and Disease Registry [ATSDR], 2004; Balali-Mood, Naseri, Tahergorabi, Khazdair & Sadeghi, 2021).

1.2 Problem Statement

An individual simultaneously exposed to cadmium, chromium, and lead through food, water or from occupational exposure is at risk of combined health effects of these chemicals. The presence of multiple chemicals within a biological system increases the potential for interactions that could enhance or diminish the toxicity of other chemical(s). These interactions could be additive, synergistic, or antagonistic. Although regulatory agencies and consensus standard setting bodies have recognized the existence of combined effects from mixed chemical exposures, in practice most exposures are regulated or controlled as if they occurred independent of any other substance exposures. Limitedinformation is available to government agencies responsible for setting standards on waste disposal and management on the effect of mixed chemical exposures. Thus, sometime in 2012, at Brussels Belgium, there was an international workshop tagged Metal Mixture Modelling Evaluation project,where scientists from Japan, USAand UK proposed various models for evaluation of metal mixtures toxicity in aquatic organisms(Farley et al., 2015).Less-than-additive toxicity was more difficult to describe with the available models because of limitations in the available datasets.Over the last several years, researchers have been developing promising new methods to solve this problem but there is still no clear consensus.As a result, conducting of targeted exposure studies to advance the understanding of metal mixture toxicity has been advocated by previous researchers(Farley &

Meyer, 2015).Research is, therefore, needed to provide sound scientific basis to describe interactions, and assist practitioners in applying appropriate algorithms for controlling exposures where antagonistic, additive, or synergistic effects may be predicted and expected.

1.3 Justification for the Study

The assessment of chemical mixtures is a complex topic for toxicologists, regulators, and the public, and the linkage between the science of toxicology and the needs of governmental regulatory agencies in the United States and other countries is continually explored. This is so because chemical mixtures have been, and continue to be, evaluated at hazardous waste sites around the world and for this reason the current U.S. Environmental Protection Agency (USEPA) guidelines for chemical mixtures assessment are also reviewed periodically (Monosson, 2005). In Nigeria, improper waste disposal and management is a common occurrence (Oluwafemi, Olukanni, & Justin, 2021), and regulatory framework for occupational exposure to hazardous substances is poor. It has been observed that farming of food crops and vegetables in contaminated environments is common in West Africa as small scale farmers do it to maximize yields due to the seemingly high organic contents of soils of waste dumpsites (Anyanwu, Ezejiofor, Igweze & Orisakwe, 2018).

Regulatory agency such as National Environmental Standards and Regulations Enforcement Agency (NESREA)have limited information on health risks associated with exposure to multiple chemicals whether simultaneously or consecutively to formulate adequate policies on environmental managementhence combined effect of Section 8(n)(p) of its Establishment Act 2007 (Laws of the Federation of Nigeria) empowers it to collect through publications and other appropriate means basic scientific data on chemical, physical and biological effects of various activities on the environment and other information pertaining to environmental standards.

This study is necessary as it shall provide information useful to interested relevant agencies (local or foreign)for formulation and management of policies for assessment of health risks, associated with exposure to chemical mixtures of hazardous substances, such as cadmium, chromium, and lead given that the ternary mixture of these metals was found in soil at 219 sites out of the 1,608 sites for which Agency for Toxic Substances and Disease Registry (ATSDR) has produced a Public Health Assessment (ATSDR, 2004). They were established in wastewater and soil samples obtained from some sites at Nairobi industrial area, Kenya (Kinuthia et al., 2020). In Kano, Nigeria, Abdullahi et al. (as cited in Anyanwuet al., 2018) reported 13.19mg/kg of Pb, 0.735mg/kg of Cd, and 12.89mg/kg of Cr in vegetables. Also, in Niger Delta, Nigeria, Ideriah et al. (as cited in Anyanwuet al., 2018) reported that concentrations of these heavy metals (Cd, Cr, Pb) in Abonnema shore line exceeded permissible limits set by the World Health Organization and hence posed a serious health concern.

Also, the current method of prediction of health risk from exposure to multiple chemicals based on mathematical models of dose-addition and response/effect-addition applies primarily to binary mixture. This study shall be used to test the suitability of these models for prediction of health risk of chemical mixtures of more than two components especially Cd, Cr and Pb. The study shall help to provide researchers data and fill the knowledge gap on whole mixture toxicity testing (EU, 2015) which is currently limited.

1.4.1 Aim of the Study

The aim of this study was to determine the possible co-morbidity of cadmium, chromium and lead in albino (Wistar) rats through biochemical and histopathological evaluations.

1.5 Objectives of the Study

The objectives of this study were to:

(i) Investigate if simultaneous exposure to cadmium, chromium, and lead produced a more adverse health effect on liver, kidney and blood than exposure to the individual metals.

(ii) Ascertain the nature of interaction (if any) among the mixture components (i.e. whether additive, less than additive/antagonistic, or greater than additive/synergistic interaction).

(iii) Compare the mode of action of the mixture to those of the individual metals.

(iv) Determine the organ(s) most affected (i.e. liver, kidney, or heart).

(v) Evaluate the dose-effect relationship of such interaction.

(vi) Enrich available library of metal mixture risk assessment beyond what is currently published by government and regulatory agencies.

1.6 Hypotheses

The first step in understanding the joint action of two or more chemicals (i.e., how they behave when they co-occur in an organism) is to formulate a testable null hypothesis based on what is known about the individual chemicals and to evaluate it empirically (Rider, Dinse, Umbach, Simmons & Hertzberg, 2018). The hypotheses for this study are:

Null hypothesis (H_o)

Exposure of albino rats to cadmium, chromium and lead simultaneously would produce same health/biologic effects as exposure of the rats to the metals singly.

Alternate hypothesis (H_A)

Exposure of albino rats to cadmium, chromium and lead simultaneously would not produce same health/biologic effects as exposure of the rats to the metals singly.

CHAPTER TWO

LITERATURE REVIEW

2.1 Toxicology of Chemical Mixture

2.1.1 Background

Long-term toxicity evaluations for chemicals used to set appropriate reference doses and presumed safe limits have been performed until now only for single chemicals (Docea, Calina & Tsatsakis, 2021). In this era of chemical advances, single chemical exposure is a myth. The entire living world is always being exposed to environmental chemical mixtures but the scarcity of toxicity data is a serious concern (Chatterjee & Roy, 2022). Thus, the assessment of toxicity of chemical mixture is one of the major challenges in the field of toxicology (Bart et al., 2022).Various regulatory authorities and the scientific world have come up with a handful of methodologies and guidelines for evaluating the harmful effects of the multi-component mixtures, though there is no such significant, standard and reliable approach for the toxicity evaluation of chemical mixtures and their management across diverse fields (Chatterjee & Roy, 2022).

2.1.2 Principles of toxicology of chemical mixture

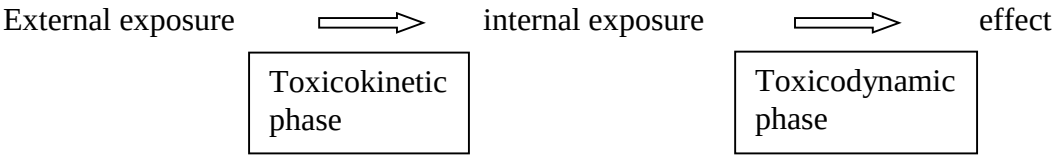
In practice, humans and animals are exposed to complex and variable combinations of chemical compounds. In a situation of multiple chemical exposures, the single chemicals may act independently as in a single exposure, or a number of the chemicals may interact to modulate the effects of the total multiple exposure. Significant questions exist when comparing single-to-mixture-chemical toxicity concerning additivity, synergism, potentiation, or antagonism (Hernandez et al., 2019).

In order to predict the toxicological properties of chemical mixtures, detailed information on the composition of the mixture, the mechanism of action and potency of each compound, as well as proper exposure data is required. Mostly, such detailed information is not available. *In vivo* data are often scarce since animal experiments are demanding. There is also a general policy to reduce the number of such studies due to animal welfare considerations (Kar & Leszczynski, 2019).

The main objective in the risk assessment of chemicals in mixtures is to establish or predict how the toxicological effects of the mixture might turn out, often in comparison with exposure to individual compounds. One of the main points to consider is whether chemicals in a mixture interact and produce an increased or decreased overall response compared to the expected sum of the effects if each chemical acts independently of each other (Vincenti & Filippini, 2021). Interactions may remain constant over the total dose-span, or there may be dose-dependent variations. Critical, limiting steps in toxicokinetic and/or toxicodynamic pathways may become saturated or overwhelmed, and responses may be altered in a non-linear manner with increasing dose. This may affect metabolic processes, endocrine regulation as well as cellular defense and repair mechanisms. An increase in the exposure dose may for example, shift additivity to synergism, toxic effects not seen without saturation of receptor or enzyme systems may appear or the metabolism of various chemical compounds may be modulated. For risk assessment of combined toxic effects of multiple chemical exposures, it is therefore of importance to know if dose-dependent variations in toxic effects occur, and if the variations take place at doses relevant to human exposure.

2.1.3 Mechanisms and cause of interaction

Interactions may take place in the chemical/chemical, toxicokinetic phase and/or in the toxicodynamic phase.



Alteration in the absorption, distribution, metabolism or excretion of a toxic compound related to exposure to another toxic compound is called toxicokinetic interaction. Interaction with absorption can occur when an active transport process is involved, such as absorption of iron and cadmium (ATSDR, 2012a; Upson et al., 2019). For instance, in iron-depleted subjects, an increased uptake of cadmium is seen because the expression of the transport protein is upregulated. However, most toxic compounds are absorbed via passive diffusion. After absorption, chemicals are distributed throughout the body via the blood circulation or the lymphatic system. For example, lipophilic compounds are protein-bound, and a more lipophilic compound can displace a less lipophilic one from the binding proteins in plasma.

The free concentration of the less lipophilic compound is then increased, and there is a possibility for a more severe toxic effect. This type of interaction is often seen with pharmaceuticals. For compounds, which are active as the parent compound, enzyme inhibition may reduce detoxication and thus enhance toxicity, whereas enzyme induction could enhance detoxication and thereby reduce toxicity. However, a majority of the chemicals which enter the body are metabolized (biotransformed). Metabolism can either increase or decrease the toxicity of a compound, and there are a number of possible interactions that can influence the outcomes.

Recalcitrant chemicals may compete for a given enzyme or co-factor and thus result in inhibition (Saito et al., 2022). Another scenario is interactions with the drug metabolizing enzymes, resulting in induction of the enzymes (increased amounts and activities). Inducers of the microsomal cytochrome P_{450} enzymes are well known to result in either increased production of active metabolites or reduced toxicity caused by increased detoxication, depending on which enzymes and pathways are affected and the biological activity of the parent compound and its metabolites. Interference (inhibition) with excretion of toxic compounds are mostly seen when active transport processes are involved and can enhance toxicity. Simultaneous exposure to a compound that either alters the pH of the urine, or act as an osmotic diuretic, can affect the excretion of chemicals and their metabolites.

Toxicodynamic interactions occur at cellular receptor/functional target level. Generally, the effect of combined action of two components at the same target is unlikely to result in synergism/potentiation. Competition for a receptor will usually result in addition of effects or antagonism (effect inhibition). An antagonist regulates negatively the activity of an agonist. Partial agonists, on the other hand, will act as agonist in the absence or at low concentrations of other ligands. Weak agonists may, however, function as antagonists by occupying the receptor preventing the binding of a more potent ligand (Richardson, 2020)**.** Dynamic interactions may also occur when two or more components act at different receptors/target sites or induce an increased or reduced antioxidant capacity (Liu, 2020).

2.1.4 Methods to assess combined actions and interactions of chemical mixtures

A number of different test strategies have been presented to obtain toxicological information on mixtures with a limited number of test groups (EU, 2015). They include:

(i) Testing of whole mixtures

Testing of the whole mixture as such may seem to be the proper way to evaluate the hazard of a mixture. A simple method of carrying out such a study is to evaluate the effects of mixture and of all individual constituents at one dose level. However, testing of whole mixtures will not provide data on combined actions and/or interactions between the individual components. This can only be achieved when information on dose-response for each single component is available. Therefore, this approach might be applied for assessing the combined toxicity of simple, defined chemical mixtures where the toxicological properties of each component are known or will be investigated. It may also be used for primary screening for potential adverse health effects (hazards) of mixtures that are not well characterized (Braeuninget al., 2022).

(ii) Independent action and dose addition (quantifiable responses)

More than 50 years ago, three basic types of action for combination of chemicals were defined (Rider*et* al., 2018) namely, similar action (dose or concentration addition), dissimilar action (independent action), and interactions. For mixtures of similarly acting chemicals the effects can be estimated directly from the sum of the doses or concentrations, scaled for relative toxicity (dose or concentration addition). For mixtures of independently acting chemicals the effects can be estimated directly from the probability of responses to the individual components (response addition) or the sum of biological responses (effect addition). Both concepts are based on the assumption that chemicals in a mixture do not influence each other's toxicity, i.e. they do not interact with each other at the biological target site. Such chemicals can either elicit similar responses by a common or similar mode of action, or they act independently and may have different endpoints and/or different target organs. Both concepts have been suggested as default approaches in regulatory risk assessment of chemical mixtures. In reality, however, chemical

mixtures are rarely composed of either only similarly or of only dissimilarly acting substances (Escher, Braun& Zafi, 2020; EU, 2012).

Independent action (response addition, effects addition) occurs if chemicals act independently from each other, usually through different modes of action that do not influence each other. This type of action is also referred to as simple dissimilar action. Response addition refers to the sum of probabilistic risks. Effects addition means the sum of biological responses. The toxicity of a mixture in terms of the probability of an individual being affected can be expressed as;

$$P_m = 1- (1-p_1) (1-p_2) (1-p_3)\dots (1-p_n) \quad (2.1)$$

With p_m being the response to the mixture and p_1, p_2, …, p_n being the responses due to exposure to the individual components C_1, C_2, …, C_n when present in a specified concentration.

This equation as stated by Backhaus et al. (2000) and Escheret al. (2020) can also be written as;

$$E(C_{mix}) = \prod_{i=1}^{n}(1-E(C_i)) \quad (2.2)$$

$E(C_i)$ is effect expressed as fraction = (effect of substance i at dose C/maximum possible effect).

$0\leq E(C_i) \geq 1$ or $0\%\leq E(C_i) \geq 100\%$

Where E=effect, C=dose, i=individual substance, mix=mixture of individual substances, n=number of substances in the mixture

The above equation means

$$E(C_{mix})=1- (1-E(C_{pb}))(1-E(C_{cd}))(1-E(C_{cr})) \quad (2.3)$$

$E(C_{mix})$ obtained by this calculation is termed $E(C_{mix})$predicted and is the expected effect of the mixture if no interaction (i.e. addition), while $E(C_{mix})$experimented is the effect of the mixture resulting from the experiment.

If $E(C_{mix})$experimented= $E(C_{mix})$predicted, additive effect

If $E(C_{mix})$experimented $\neq$ $E(C_{mix})$predicted, there is interaction

If $E(C_{mix})$experimented < $E(C_{mix})$predicted, the interaction is less than additive or antagonistic

If $E(C_{mix})$experimented > $E(C_{mix})$predicted, the interaction is more than additive or synergistic

According to the above equation, any substance for which $E(C_i)$ is equal to zero does not contribute to the joint effect of the mixture. Consequently, mixtures of independently acting chemicals pose no health concern, as long as the doses/concentrations of each individual component remain below their individual zero-effect levels (concentrations) (EU, 2012).

Dose/concentration addition (similar action, similar joint action) occurs if chemicals in a mixture act by the same mechanism/mode of action, and differ only in their potencies. Different methods exist for the dose/concentration approach, which mainly differ in the required knowledge about mode of actions and toxicological similarities of the mixture components. In principle, doses or concentrations of the single components are added after being multiplied by a scaling factor that accounts for differences in the potency of the individual substances. The mixture dose/concentration (D_{mix}) is the sum of the adjusted doses/concentrations (aD) of the individual components D_i;

$$D_{mix} = \sum_{i=1}^{n} aD_i \qquad (2.4)$$

The effect of a mixture of similarly acting compounds is equivalent to the effects of the sum of the potency-corrected (adjusted) doses/concentrations of each compound. Dose additivity is

assumed over the entire dose range, including doses/concentrations below the individual no observed adverse effect levels/concentrations (NOAEL/Cs) of the mixture components. It is noted that the dose-additivity approach relies on a current grouping of "similar" chemicals. Although guidance on grouping of chemicals has been issued, there is currently no general agreement on the scientifically best approach and grouping of chemicals is most often done by expert judgment on a case-by-case basis (EU, 2012).

Another concept/formula for dose addition is

$ICED = d_1 + (RPF_2 x d_2)$ (2.5)

Where d_1 = dose of chemical 1 present in a mixture, d_2= dose of chemical 2 present in a mixture (units must be consistent with dose d_1), ICED = index chemical equivalent dose based on relative potency estimates (units consistent with d_1 and d_2), RPF (relative potency factor) $=(ED10)_1/(ED10)_2$,

$(ED10)_1$ = dose of chemical 1 that results in a 10% response, either as a fraction of exposed test animals that respond, or as a fractional change in a measured physiological value. $(ED10)_2$= dose of chemical 2 that also results in the same 10% response

$f_1(ICED) = h(d_1,d_2)$ = mixture risk from chemicals 1 and 2 evaluated at the ICED of chemical 1

Where $f_1(ICED)$= dose-response function of the index chemical for the response(s) common to chemical 1 and chemical 2 (units consistent with d_1 and d_2), $h(d_1,d_2)$ = mixture risk from dose d_1 of chemical 1 and dose d_2 of chemical 2.

$ICED = d_1 + (RPF_2 x d_2) + \ldots + (RPF_n x d_n)$ (2.6)

where $RPF_n= ED_1/ED_n$, d_n = dose of chemical n, ED_n = dose of chemical n that also results in the same 10% response/effect as chemical1 (i.e. the index chemical).

It should be noted that any of the chemical component of the mixture could be used as the index chemical provided there is sufficient toxicologic information on the chemical (United States Environmental Protection Agency [USEPA], 2003).

2.1.5 Hormesis in mixture toxicology and risk assessment

Hormesis is a biphasic dose response relationship characterized by low-dose stimulation and high-dose inhibition (Calabrese et al., 2020). Biphasic hormetic dose responses classically start at a zero exposure level (i.e. control), with increasing exposure level and depending on the endpoint measured, the dose-response curve either switches from a decline in the endpoint to an increase (U- or J-shaped curve) or vice versa from stimulation at low doses to inhibition at high doses (inverted U-/J-shaped curve) (Belz & Duke, 2022). Hormesis refers to adaptive responses of biological systems to moderate environmental or self-imposed challenges through which the system improves its functionality and/or tolerance to more severe challenges (Calabrese & Mattson, 2017). Hormesis, therefore, is more than simply a dose-response relationship or a dose-time-response relationship but, rather, a quantitative manifestation of a reparative process that is adaptive in nature (Chattopadhyay, 2022). Hormesis challenges the way the toxicology community thinks about risk assessment. It indicates that the traditional NOAEL (no observed adverse effect level) is truly a misnomer and that biological activity occurs below this apparent threshold, hence various models are been proposed to quantitatively predict sub-NOAEL effects (Agathokleous, 2022; Belz & Duke, 2022). Scientists are challenged by the question of what will happen in the low concentration range when all or some of the chemicals in a mixture induce hormesis? Can the mixture effects still be predicted and can the size and concentration range of hormesis be predicted? (Belz, Cedergreen & Sorensen, 2008).

Hormesis is now generally accepted as a real and reproducible biological phenomenon, being highly generalized and independent of biological model, endpoint measured and chemical class/physical stressor. The hormetic dose-response model has been demonstrated to make far more accurate predictions of responses in low dose zones than either the threshold or linear at low dose models. It is expected that as low dose responses come to dominate toxicological research that risk assessment practices will incorporate hormetic concepts in the standard setting process (Calabrese, 2010). The current risk assessment approach for noncarcinogens is blind to the possibility that hormesis exists. Such a lack of consideration denies the risk assessor the opportunity to become informed on whether the hormetic response offers the affected population a response that enhances health, is harmful, or is uncertain. Such knowledge provides the risk assessor with a set of more flexible, data-based options that could be used to target uncertainty factor size (i.e. interindividual variation) for the optimal population response, an option that is currently ignored by regulatory agencies (Beck, Calabrese, Slayton & Ruddel, 2008). It is opined that hormesis should be considered when evaluating the effects and risks of some chemical mixtures (Agathokleus, Barcelo, Iavicoli, Tsatsakis & Calabrese, 2022).

Over the past several years, a general pattern has emerged suggesting a common mechanistic framework that may account for the generality of hormetic responses. It is opined that hormetic dose responses that are cytoprotective for chemicals and radiation are largely mediated following the redox activation of the transcription factor (TF) Nrf2. Nrf2 mediates a network of antioxidant defenses and initiates productive crosstalk with other TFs that not only act together with Nrf2 via dose-dependent hierarchal processes to enhance biological resilience but also conform to the quantitative features of the hormetic dose response. Stressors shift the redox homeostasis of cells toward the oxidative state and, in the process, activate Nrf2—a TF and redox sensor—to mediate

a host of integrated and cytoprotective responses, including antioxidation, detoxification, anti-inflammation, autophagy, and the facilitation of mitochondrial biogenesis. It is proposed that activation of Nrf2 is the general and dominate underlying mechanistic basis of hormetic dose responses. It accounts for the striking "generality" of hormetic dose responses, that is, they are independent of biological models, levels of biological organization (cell to whole organism), endpoints measured, and inducing agents as well as the occurrence of inter-individual variations in susceptibility to toxic substances, pharmaceuticals, and aging processes (Calabrese & Kozumbo, 2021).

An example of hormesis is that plant height, dry weight, leaf area, and chlorophyll content increased when honeysuckle was exposed to 10 mg kg−1 or 30 mg kg−1 Cd (low concentration), while in response to 150 mg kg−1 or 200 mg kg−1 Cd (high concentration) these growth parameters and chlorophyll content significantly decreased relative to untreated control plant groups (Li et al., 2022). Also, single and combined Cd and Pb induced hormesis in soil microbial populations. The mixture hormetic effects were related to the effect of single Cd or Pb. Thus, hormesis should be considered in Cd and Pb soil microbiota risk assessment (Fan et al., 2021). One of the benefits of hormesis is that administering a high toxic dose of a stressor to cells after (in preconditioning protocols) inducing hormesis with a low subtoxic dose of the stressor results in the protection of cells from damage induced by the high toxic dose (Calabrese & Kozumbo, 2021).

2.2 Background information on cadmium, lead, and chromium

2.2.1 Cadmium

Cadmium is a soft, silver-white transition metal often found in association with zinc ores and obtained primarily as a by-product of zinc preparation (Bernhoft, 2013**).** About 75% of cadmium produced is used in batteries, especially nickel-cadmium batteries. Because of its non-corrosive properties, cadmium has been used in electroplating or galvanizing alloys for corrosion resistance. It is also used as a color pigment for paints and plastics, in solders, as a barrier to control nuclear fission, as plastic stabilizer and in some special application alloys (Liu, Goyer & Waalkes, 2008). Cadmium is a top toxic substance.

(i) **Sources of cadmium exposure to the environment**.

The agricultural application of phosphate fertilizers represents a direct input of cadmium to the soil. The cadmium content of phosphate fertilizers varies widely and depend on the origin of the rock phosphate (Suciu, Vivo, Rizzati & Capri, 2022). It has been estimated that fertilizers of West African origin contain 160-255 g cadmium/tonne of phosphorus pentoxide, while those derived from the Southeastern USA contain only 35g cadmium/tonne (Roberts, 2014). The application of municipal sewage sludge to agricultural soil as a fertilizer can also be a significant source of cadmium (Jastrzebska, Saeid, Kostrzewska & Basladynska, 2018).

Municipal refuse is a waste related source, the cadmium being derived from discarded materials containing cadmium and solid wastes. Sources of cadmium release to the environment also include atmospheric emission and release of waste water from non-ferrous metal processing industries.

(ii) Route of exposure to cadmium

Food is the major source of cadmium for the general population. Many plants readily accumulate cadmium from soil. Shellfish accumulates relatively high levels of cadmium (1-20g/kg) and animal liver and kidney can have levels higher than 50µg Cd /kg. Cereal grains such as rice and wheat, and tobacco concentrate cadmium to levels of 10-150 µg/kg Cd/kg (Liu et al., 2008). Globally, cadmium in white rice varied from 4.9 to 3712 µg/kg (Shi, Carey, Andrew& Meharg, 2020). Occupations potentially at risk from cadmium exposure include those involved with refining zinc and lead ores, iron production, cement manufacture and fossil fuel combustion. Others include manufacture of paint pigments, cadmium-nickel batteries, and electroplating (ATSDR, 2012a; Zhang & Reynolds, 2019).

(iii) Toxicokinetics of cadmium

Gastrointestinal absorption of cadmium is limited to 5-10% of a given dose. Cadmium absorption can be increased by dietary deficiencies of calcium or iron and by diets low in protein (Upson et al., 2019). Once absorbed, cadmium is very poorly excreted and only about 0.001% of the body burden is excreted per day via urinary or faecal route (Liu et al., 2008).

Cadmium does not undergo metabolic conversion, but the cadmium ion can readily bind to anionic groups, especially sulfhydryl groups, in protein and other molecules (Meulenbelt, 2016). Cadmium is transported in blood by binding to albumin and other large molecular weight proteins. It is rapidly taken up by tissues and is primarily deposited in the liver and to a lesser extent in the kidney (Genchi, Sinicropi, Lauria, Carocci & Catalano, 2020). In the liver, kidney and other tissues, cadmium induces the synthesis of metallothionein (MT), a low molecular weight, high affinity metal-binding protein (Nordberg & Nordberg, 2022). Cadmium is stored primarily in the liver as cadmium-MT. Cadmium-MT may be released from the liver and

transported via blood to the kidney, where it is reabsorbed and degraded in the lysosomes of the renal tubules. This releases cadmium to induce more cadmium-MT complex or cause renal toxicity. Cadmium transport into cells is mediated through calcium channels (Yang & Shu, 2015) and through molecular mimicry (Thevenod &Wolff, 2016).

(iv) Toxicity of cadmium

The toxicity of cadmium has been widely investigated, and cadmium has been shown to nearly affect every organ system if the dose is high enough (Sassia, Amine, Nadia, Hadda & Smail, 2021). Cadmium is considered a cumulative toxicant (Tai et al., 2022). The human exposure scenarios of greatest concern are long-term oral exposures**.** Acute cadmium toxicity from ingestion of high concentration of cadmium in the form of heavily polluted beverages or food causes severe irritation to the gastrointestinal epithelium and symptoms include nausea, vomiting, and abdominal pain (Gillois, Leveque, Theodorou, Robert & Mercier-Bonin, 2018). Inhalation of cadmium fumes or other heated cadmium-containing materials may produce acute pneumonitis with pulmonary oedema (Rahimzadeh, Rahimzadeh, Kazemi & Moghadamnia, 2017**).** The major long term toxic effects of low-level cadmium exposure are renal injury, obstructive pulmonary disease, osteoporosis, and cardiovascular disease (Fatima, Raza, Hadi, Nigam& Mahdi, 2019**).** Cadmium is toxic to tubular cells and glomerulli, markedly impairing renal function. Pathologically, these lesions consist of initial tubular cell necrosis and degeneration, progressing to an interstitial inflammation and fibrosis. There appears to be a critical concentration of cadmium in the renal cortex that, once exceeded, is associated with tubular dysfunction (Prozialeck & Edwards, 2012**).** Renal effects have been seen in humans and animals by both inhalation and oral exposure, and are the most sensitive effects of chronic oral exposure, occurring at intakes as low as 0.0078mg/kg (ATSDR, 2004).

Some effects that are seen at moderately low levels of oral exposure are cardiovascular, haematological, neurological, and testicular effects. Oral exposure to cadmium can cause anaemia in humans and animals. Hepatic effects occur with higher oral doses of cadmium, usually for acute or intermediate durations (Genchi et al., 2020),

(v) Mechanism of action of cadmium

Cadmium accumulates in the kidney over the lifetime. Toxicity is thought to result when critical concentration of cadmium is reached in the kidney. Much of the cadmium in the kidney and in other tissues is bound to metallothionein, which is thought to sequester cadmium, preventing damage to cellular constituents, but which also retains cadmium in the cell. Metallothionein is thought to function in the storage of the essential metals zinc and copper, and to serve as an antioxidant. Details regarding the mechanism of cadmium renal toxicity are uncertain, renal damage is hypothesized to occur when an excessive concentration of free cadmium occurs intracellularly in the kidney, perhaps due to an insufficient rate of metallothionein synthesis to bind the intra renal cadmium. The free cadmium may bind to other intracellular ligands, including metalloenzymes, and may destabilize proximal tubule cell membranes (Zhang et al., 2021).

(vi) Treatment for cadmium poisoning

Chelation therapy for cadmium generally results in significant adverse effects such as depletion of essential metals (Gerhardsson, 2022). Cadmium intoxications need decontamination via gastrointestinal tract irrigation, supportive care, and chemical decontamination, the use of nanoparticles, traditional and new chelating agents and combination therapy (Rahimzadeh et al., 2017). Recently, palm oil has been shown to be useful in mitigating the effect of cadmium

poisoning in rats by reducing oxidative stress (Ichipi-Ifukor, Asagba, Nwose, Mordi & Oyem,. 2022)

2.2.2 Lead

Lead is a heavy, bluish-gray metal and, although it serves no biological purpose, is the most widely used non-ferrous metal (ATSDR, 2020). Lead and lead compounds have been used in many industrial applications, including batteries, ammunition, paints and varnishes, gasoline, pigments, radiation shields, medical equipment, solder, glass, and ceramic glazes **(**ATSDR, 2020). Lead in lead compounds primarily exists in the divalent form except organolead compounds which are dominated by the tetravalent form (Liu et al., 2008).

(i) Sources of lead to the environment

Lead can enter the environment through releases from mining lead and other metals, and from factories that make or use lead, lead alloys, or lead compounds. Lead is released into the air during burning coal, oil or waste (ATSDR, 2020; WHO, 2021). Landfills may contain waste from lead ore mining, ammunition manufacturing or battery production. Disposal of lead-containing products contribute to lead in municipal landfills (ATSDR, 2020; WHO, 2021). Most of the lead in inner city soils comes from old houses with paint containing lead and previous automotive exhaust emitted when gasoline contained lead (ATSDR, 2020; WHO, 2021).

The levels of lead may build up in plants and animals from areas where air, water, or soil are polluted with lead. It is important to note that lead released to air and water ultimately is deposited in soil or sediment. Although aquatic releases of lead from industrial facilities are expected to be small with respect to emissions to land and air, lead may be present in significant levels in drinking water (ATSDR, 2020). Lead has been identified in groundwater samples and in

surface water samples collected from several hazardous waste sites (Pazand, Khosravi, Ghaderi & Rezvanianzadeh, 2018).

(ii) Route of exposure to lead

A major route of exposure to lead for the general population is from food and water. Lead may be introduced into food through uptake from soil into plants or atmospheric deposition onto plant surfaces, during transport to market, processing, and kitchen preparation. Other factors such as absorption of lead from cooking water and cookware can influence the amount of lead in cooked vegetable (ATSDR, 2020). Consumption of canned foods is also source of lead exposure. Herbal medicines could be potential sources of lead exposure. Certain Ayurvedic herbal products were found to be polluted with lead ranging up to 37mg/g and over 55 cases of lead poisoning have been related to the ingestion of herbal medicines (Sakhartar, 2017).

(iii) Toxicokinetics of lead

Adults absorb 5-15% of ingested lead and usually retain less than 5% what is absorbed. Children absorb 42% of ingested lead with 32% retention (Liu et al., 2008). Lead absorption can be enhanced by low dietary iron and calcium, especially in children (Holstege, 2020; Stotaet al., 2021). Lead absorption by the lungs depends on the form, particle size, and concentration. Absorption of retained lead through alveoli is relatively efficient (Liu et al., 2008).

Lead in blood is primarily (99%) in erythrocytes bound to haemoglobin, only 1% of circulating lead in serum is available for tissue distribution (ATSDR, 2020). Lead is initially distributed to soft tissues such as kidney and liver, and then redistributed to skeleton and hair (Liu et al., 2008). Lead accumulates in bone. Inorganic lead is not known to be metabolized, but lead ions are complexed by macromolecules (ATSDR, 2004). Unabsorbed lead is excreted in the faeces, absorbed lead that is not retained is excreted through the urine and bile (ATSDR, 2020).

(iv) Toxicity or health effects of lead

The effects of lead are similar across inhalation and oral exposure. Lead has been shown to affect virtually every organ and system in both humans and animals. The most sensitive effects of lead appear to be neurological (particularly in children), hematological, renal and cardiovascular (Flora, Gupta& Tiwari, 2012). The toxic effects of lead range from inhibition of enzymes to the production of severe pathology or death (Charkiewicz & Backstrand, 2020).

Lead interferes with the synthesis of heme, resulting in accumulation of δ-aminolevulinic acid (ALA) in tissues and elevated excretion of ALA in urine, elevation of zinc-protoporphyrin in erythrocyte, reduction in blood hemoglobin, and in a hypochromic, normocytic anaemia at higher levels of exposure (Danziger, Dodge, Hu & Mukamal, 2022).

Acute lead nephrotoxicity consists of proximal tubular dysfunction and can be reversed by treatment with chelating agents. Chronic lead nephrotoxicity consists of interstitial fibrosis and progressive nephron loss, azotaemia and renal failure (ATSDR, 2020). A characteristic microscopic change is the presence of intranuclear inclusion bodies. By light microscopy the inclusions are dense, homogeneous, and are eosinophilic with hematoxylin and eosin staining (Liu et al., 2008). Lead colic is a major gastrointestinal symptom of severe lead poisoning, and is characterized by abdominal pain, nausea, vomiting, constipation, and cramps (ATSDR, 2020).

(v) Mechanism of action of lead

Lead can affect virtually every organ or system in the body through mechanisms that involve fundamental biochemical processes. These mechanisms include the ability of lead to inhibit or mimic the action of calcium and to interact with proteins (ATSDR, 2020). In the interaction with proteins, lead binds with virtually every available functional group, including sulfhydryl, amine,

phosphate, and carboxyl groups, with sulfhydryl having the highest affinity. In binding with sulfhydryl groups, lead may interfere with the activity of zinc metalloenzymes, as zinc binds to a sulfhydryl group at the active site (ATSDR, 2004). Lead also binds to metallothionein, a sulfhydryl-rich protein, but does not appear to displace cadmium or zinc. Metallothionein is induced by cadmium, zinc and arsenic, but apparently not by lead, although metallothionein sequesters lead in the cell. Another lead-binding protein is an acidic, carboxyl-rich protein found in the kidney and brain (ATSDR, 2020).

(vi) Treatment of lead poisoning

Lead poisoning could be treated by chelation therapy. The oral chelating agent dimercaptosuccinic acid (DMSA, also called Succimer) has advantages over EDTA in that it can be given orally and is effective in temporarily reducing blood lead level (George & Brady, 2022; Saed, Al-Kubaisi, Suleiman & Hassan, 2020).

2.2.3 Chromium

Chromium is a naturally-occurring element found in rocks, animals, plants, and soil, where it exists in combination with other elements to form various compounds (Sharma, Singh, Parakh & Tong, 2022). The name chromium is derived from the Greek word for “color” because most chromium compounds are brightly coloured (Gregersen, 2022). The three main forms of chromium are chromium (0), chromium (III), and chromium (VI). Small amounts of chromium (III) are needed for human health (ATSDR, 2012b). Hexavalent chromium is a by-product of various industrial processes and is a human carcinogen and produces a variety of toxic effects (Sharma et al., 2022). Chromium is widely used in manufacturing processes to make various metal alloys such as stainless steel (Korla & Mitra 2020). Chromium compounds are used for

plating, leather tanning, and the manufacture of dyes and pigments, cooking utensils and as wood preservatives.

(i) **Sources of chromium in the environment**.

Hexavalent chromium is derived from the industrial oxidation of mined chromium deposits and possibly from the combustion of fossil fuels, wood, paper, etc. Chromium fallouts from industrial activities are deposited in land and water, and eventually in sediments (Sharmaet al., 2022). The United States Environmental Protection Agency has found chromium in 1,127 out of 1699 hazardous waste sites evaluated. The hexavalent chromium compounds are also toxic to the ecosystem, and microbial and plant variants occur that adapt to high chromium levels in the eco-environment (Sharma et al, 2020).

(ii) Route of exposure to chromium

Exposure to hexavalent chromium could be by inhalation of polluted air from industries manufacturing or utilizing chromium compounds, or cigarette smoke. Exposure could also be by drinking water as chromium is occasionally detected in groundwater. The general population is most likely to be exposed to trace levels of chromium in the food that is eaten (Yaman, 2020).

(iii) Toxicokinetics of Chromium

The absorption of hexavalent chromium through the gastrointestinal tract after oral exposures of humans is about 2-10% for potassium chromate. The chromate anion can enter cells by facilitated diffusion through nonspecific anion channels, similar to phosphate and sulphate anions. Absorption efficiency appears to increase with increasing dose. Once in the blood, chromium is distributed to all organs of the body, preferential distribution to any particular organ does not appear to occur (Sun, Brocota & Costa, 2015). Hexavalent chromium does not appear to

accumulate in the body. It is unstable in body fluids and tissues, including the gastric juice, and is reduced to chromium (V), chromium (IV), and ultimately to chromium (III) by many substances, including ascorbate and glutathione (ATSDR, 2004). Absorbed chromium is excreted primarily in the urine. Minor pathways of excretion are through the hair and nails. Much of ingested hexavalent chromium passes through the body without being absorbed and is excreted in the faeces (ATSDR, 2012b).

(iv) Toxicity or health effects of chromium

Hexavalent chromium is acutely toxic, with most reports of human toxicity occurring as a result of accidental or intentional ingestion. The lethal oral dose of soluble chromates in humans is estimated to be in the range of 50 to 70 mg/kg. Symptoms of acute toxicity include vomiting and generalized gastrointestinal tract damage with gastrointestinal bleeding leading to cardiovascular shock (Merill, Morton, & Soileau, 2008). Hematological effects have been seen in rats and mice fed chromium (VI) in the diet for intermediate durations. Renal effects included accumulation of lipids and inhibition of membrane enzymes in rats given chromium (VI) at 13.5 mg/kg/day by gavage, and proteinuria in rats given chromium (VI) at 98 mg/kg/day from drinking water (Sharma et al., 2022).

(v) Mechanism of action of chromium

Chromium (VI) enters the cells through membrane channels that also admit sulphate and phosphate. Once in the cell, chromium (VI) is reduced to chromium (III) with chromium (V) and (IV) as intermediates. These intermediates have been shown to be involved in oxidative cycling, generating oxygen radical species. The formation of these radicals may be responsible for many of the deleterious effects of chromium on cells, which can be blocked by radical scavengers (Wu et al., 2020).

(vi) Treatment of chromium poisoning

Generally, treatments for reducing body burden of chromium are chelation therapies similar to those used to reduce body burdens of other metals, although the use of ascorbic acid is specific for chromium (Monga, Fulke & Dasgupta, 2022).

2.3 Clinical significance of biochemical parameters tested

Routinely performed clinical chemistry tests provide information concerning hepatocellular and biliary integrity and function, renal function, carbohydrate, lipid and protein metabolism, and mineral and electrolyte balance. Most of the common clinical chemistry assays developed for human testing is applicable, without modification, to animal clinical chemistry testing (Hall & Everds, 2008)

2.3.1 Alanine aminotransferase (ALT)

Serum activities of liver enzymes are used primarily to identify hepatocellular injury and cholestasis, with or without hepatobiliary injury. Serum activities of many enzymes normally present within hepatocytes are increased following hepatocellular injury (i.e. degeneration or necrosis). Serum ALT activity is the most frequently relied upon indicator of hepatoxic effects of substances, although it does not always correlate well with histopathology data (Aulbach & Amuzie, 2017). Histopathologic evidence of hepatocellular injury usually accompany very high test-article related increase in serum ALT activity while moderate increase in ALT activity may or may not have correlative findings (Hall & Everds, 2008). Elevation in ALT and AST in disproportion to elevations in alkaline phosphatase and bilirubin denotes a hepatocellular disease, whereas an, elevation in alkaline phosphatase and bilirubin in disproportion to ALT and AST would denote a cholestatic pattern (Lala, Zubair& Minter, 2022).

In general, ALT is the most useful enzyme for detection of hepatocellular injury in the majority of laboratory animal species. Although the enzyme is present in many tissues, its greatest concentration in most species is within hepatocytes, and, in general, significant elevation of serum ALT activity indicates release of ALT by hepatocytes. The magnitude of serum ALT activity elevation is proportional to the number of hepatocytes affected, and marked increases will reflect irreversible cell damage and necrosis, while mild increase may indicate mostly membrane blebbing and reversible cell damage. Biliary disease or toxicity and bile duct obstruction may cause increased serum ALT activity at least in part due to the effect of retained bile salts on the cell membranes of neighbouring hepatocytes (York, 2017). ALT increases in a dose-dependent manner with the body load of blood cadmium, lead and mercury within and above the normal range (Nowicki & Pizzorno, 2020).

2.3.2 Aspartate aminotransferase (AST) and Lactate dehydrogenase (LDH)

Serum AST activity tend to parallel serum ALT activity with respect to liver damage, but this enzyme is much less liver specific because of high concentrations in muscle and other tissues. Elevations in serum AST activity caused by hepatotoxicity are usually less pronounced than concurrent elevations in serum ALT activity. Because a portion of intracellular AST is located in mitochondria, a more severe injury may be necessary for the release of large quantities of AST.Marked increases in AST activity in the presence of minimal to mild change in ALT activity is indicative of increased release from nonhepatic sources such as skeletal muscle (York, 2017). Decreased serum activities of ALT and AST are occasionally observed in toxicology studies. Among the potential causes for these findings are decreased hepatocellular syntheses or release of the enzymes, inhibition or reduction of enzyme activity, and assay interference. Regardless of the mechanism involved, decreased serum activities of the aminotransferases are

generally not associated with toxicologically significant effects on the liver (Hall & Everds, 2008)

The enzyme lactate dehydrogenase (LDH) is distributed in tissues particularly heart, liver, muscle, and kidney. The enzyme found in circulation is a mixture of five isoenzymes based on their mobility. Elevated serum levels of LDH are found in serum in myocardial infarction, liver disease, renal disease, certain forms of anaemia, malignant diseases, and progressive muscle dystrophy (Klein, Nagy, Tothova & Chovanova, 2020). Elevated LDH is indicative of tissue damage but is not specific except an isoenzyme test is done (Farhana & Lappin, 2022) hence its result has to be correlated with other enzyme markers (e.g. ALT) to determine liver injury.

2.3.3 Alkaline phosphatase (ALP)

Checking ALP levels in the blood is a routine part of liver function and gallbladder tests. Abnormal levels of ALP in the blood most often indicate a health concern with the liver, gallbladder, or bones (Loewe, Sanvictores, Zubair, & John, 2022). Numerous things may cause increments of serum ALP activity but the most common being obstructive liver disease. Because of swelling and pressure obstruction of small bile ductules, primary hepatocellular toxicities often cause enough intrahepatic cholestasis to elevate serum ALP activity (Krupaa, Hariharan, Babu & Masthan, 2020).

2.3.4 Bilirubin

Bilirubin results from the breakdown of heme by cells of the mononuclear phagocyte system. Serum bilirubin concentration reflects the ability of hepatocytes to take up, conjugate, and secrete bilirubin, so it is functional marker rather than a marker of cellular integrity as reflected in serum liver enzymes. In the absence of biliary obstruction, total bilirubin is an insensitive

measure of liver function; due to the large reserve capacity of the liver for bilirubin processing, considerable hepatic injury must occur before alterations in total bilirubin are detectable. This means that by the time total bilirubin is elevated, substantial hepatic injury has occurred (Gwaltney-Brant, 2021). If normal levels of liver enzymes are observed alongside elevated bilirubin levels, it is called isolated hyperbilirubinemia **(**Lala et al., 2022).

2.3.5 Urea

Serum urea and creatinine concentration, are common tests used to evaluate renal function. Serum urea concentration is affected by rate of urea production, glomerular filtration rate, and flow rate of urine through the renal tubule. Mechanisms for increased serum urea are categorized as prerenal, renal or postrenal. Increased serum urea due to renal failure causes results from disease or toxicity of the renal parenchyma. Like the liver, kidneys have a large functional reserve capacity. In clinical practice, it is commonly said that serum urea concentration does not increase notably until approximately 75% of the kidneys' nephrons are nonfunctional. Increased urea concentration due to renal failure causes is generally accompanied by histopathologic evidence of renal damage, and the animals may exhibit signs of poor health such as inappetence, weight loss, or inactivity (Hall & Everds, 2008). Serum creatinine is a more accurate assessment of renal function than urea; however, urea is increased in renal disease (Gounden, Bhatt & Jialal, 2022).

2.3.6 Creatinine

Creatinine is a nonprotein nitrogenous waste product formed at a relatively constant rate by the nonenzymatic breakdown of creatine. Creatine is a breakdown product of phosphocreatine, a molecule that stores energy in muscle. Creatinine is freely filtered by the glomerulus, but unlike urea it is not reabsorbed by the tubules. Serum creatinine is usually a better reflection of

glomerular filtration than serum urea because it is influenced by fewer secondary factors (Hall & Everds, 2008). High serum creatinine levels may indicate kidney damage and poor kidney function (Goundenet al., 2022). If serum creatinine concentrations are increased in the absence of correlative effects on serum urea or renal histopathology, other analytical methods for creatinine (e.g. enzymatic) can be used to investigate the possibility of analytical interferences (Hall & Everds, 2008). About 50% of kidney function must be lost before a rise in serum creatinine can be detected. Thus, serum creatinine is a late marker of acute kidney injury (Goundenet al., 2022).

2.3.7 Inorganic phosphorus, chloride, and potassium

Other clinical chemistry findings sometimes observed when renal function is significantly impaired include increased serum inorganic phosphorus concentration and decreased serum sodium and chloride concentrations. Whereas increased inorganic phosphorus is primarily due to reduced filtration, decreased sodium and chloride result from loss of tubular function and normal reabsorption. Decreased serum inorganic phosphorus concentration observed in toxicology studies is most commonly associated with significantly reduced food consumption (Hall & Everds, 2008).

Chloride, the major extracellular anion in plasma, supports fluid homeostasis and balances cation secretion. Serum chloride concentrations are disproportionately affected in disorders affecting acid-base balance. Increases in serum chloride are occasionally observed secondarily to metabolic acidosis resulting from diarrhea (Hall & Everds, 2008).

Potassium is the major intracellular cation and has a critical role in neuromuscular and cardiac excitation. Increased serum potassium concentration may be observed with a variety of conditions causing acidosis because extracellular hydrogen ions are exchanged for intracellular

potassium ions. Severe tissue necrosis and anuric or oligouric renal failure are infrequent causes of increased serum potassium. Serum potassium is very sensitive to potassium intake, and decreased concentrations are often associated with anorexia (Hall & Everds, 2008). Elevated potassium level are often associated with renal failure, dehydration, shock or adrenal insufficiency while decreased potassium levels are associated with malnutrition, negative nitrogen balance, gastrointestinal fluid losses and hyperactivity of the renal cortex (Dhondup & Qian, 2017).

2.3.8 Haemoglobin (Hb), packed cell volume (PCV), red blood cell (RBC), and white blood cell (WBC)

Red cell mass is evaluated by RBC count, haemoglobin concentration, and hematocrit (PCV). These parameters may or may not change proportionally, depending on the cause of the decreased red cell mass and whether or not cell size and haemoglobin content are affected. Red cell mass may be increased or decreased as a result of experimental treatment. Decreases in red cell mass must be differentiated from anaemia. Anaemia is the condition of having a lower-than-normal number of red blood cells or quantity of hemoglobin. Anaemia diminishes the capacity of the blood to carry oxygen (Rhodes, Denault& Varacallo, 2022).

In many toxicology studies, animals in a treated group have lower RBC counts, Hb concentrations, or hematocrits (PCV) than those of the control animals, but the differences are less than those necessary to cause clinical signs or affect tissue oxygenation and are, therefore, not indicative of anaemia. For example, reductions from control values for these parameters up to approximately 10% are relatively mild and probably do not have an adverse effect on the health of the animal (Hall & Everds, 2008). Reductions from approximately 10-30% may be considered a moderate effect and may or may not be clinically adverse. Reductions of more than

30% are generally marked and clearly represent a clinically adverse anemic condition. It is important to understand that, although a 5% reduction in Hb concentration may be insufficient to adversely affect health, the cause of the reduction (e.g. liver toxicity, gastric ulceration, or immune-mediated red cell destruction) may be very adverse. Unless the differences for RBC count, Hb concentration, and hematocrit (PCV) are quite large, it is preferable to simply discuss the magnitude of the differences between the control and treated groups and avoid using the term anaemia (Hall & Everds, 2008).

White blood cells are useful in the defense of the body against invading harmful substances. There are five types of WBC namely neutrophils, lymphocytes, monocytes, eosinophils, and basophils. The responsibility of these WBC types varies in the body defense system. Total WBC count measures the total number of these cells without differentiating the respective cells. Increased total WBC count above normal upper limit is termed leukocytosis while decreased total WBC count below normal lower limit is termed leukopenia. The number of WBC in the blood is an indicator of disease as decreased WBC indicates the susceptibility of the animal to disease. Increase in WBC could be psychological hence caution in result interpretation (Hall & Everds, 2008).

2.3.9 Malondialdehyde (MDA), glutathione (GSH), and ascorbic acid

Normal cellular metabolism can result in production of reactive oxygen species (superoxide, hydrogen peroxide, singlet oxygen, and hydroxyl radical) and all cells contain defense systems to prevent or limit damage. Glutathione is a major component of this defense system, but ascorbic acid also plays important role (Boubakri, 2017). Substances responsible for forming reactive oxygen species are termed prooxidants while those responsible for mopping up reactive oxygen species are termed antioxidants. The imbalance between prooxidants and antioxidants is known

as oxidative stress (Ghazizadeh et al., 2020). Mitochondria are the main source of reactive oxygen species, which in turn, can lead to oxidative damage to mitochondrial DNA and cell death. Several studies about hepatic diseases indicate that overproduction of reactive oxygen species is common profile in these diseases (Andrade et al., 2015).

Reactive oxygen species degrade polyunsaturated lipids, forming malondialdehyde (Juan, Lastra, Plou& Perez-Labena, 2021). Malondialdehyde (MDA) is a stable end product of lipid peroxidation and therefore can be used as an indirect measure of the cumulative lipid peroxidation (Mao et al., 2019). Malondialdehyde is used as biomarker to measure the level of oxidative stress in an organism (Maurya et al., 2021). An increase in MDA is indicative of increase in lipid peroxidation and oxidative stress. MDA can be measured by thiobarbituric acid reactive substance (TBARS) method. Even though there remains a controversy cited in literature regarding the specificity of TBARS towards compounds other than MDA, it still remains the most widely employed assay used to determine lipid peroxidation (Senthilkumar, Amaresan & Sankaranarayanan, 2021).

Glutathione is an endogenous antioxidant. It is a tripeptide that is biosynthesized from the amino acids L-cysteine, L-glutamic acid, and glycine. The thiol group of cysteine in reduced glutathione is able to donate a reducing equivalent ($H+e^-$) to unstable molecules such as reactive oxygen species. In donating an electron, glutathione itself becomes reactive, and reacts with another reactive glutathione to form oxidized glutathione (GSSG). It participates directly in neutralization of free radicals and reactive oxygen species as well as maintaining ascorbic acid in its reduced form. A decrease in reduced glutathione is indicative of oxidative stress (Nuhu, Gordon, Sturmey, Seymour & Bhandari, 2020)

Ascorbic acid (vitamin C) is a six carbon lactone that is synthesized from glucose in the liver of most mammalian species but not by humans. Therefore, humans must obtain ascorbate in their diet in order to survive. Ascorbate ion is the predominant species at typical biological pH values. It is a mild reducing agent and an antioxidant. It is oxidized with loss of one electron to form a radical cation and then with loss of a second electron to form dehydroascorbic acid. It typically reacts with oxidants of the reactive oxygen species, such as the hydroxyl radical. Such radicals are damaging to animals and plants at the molecular level due to their possible interaction with nucleic acids, proteins, and lipids. Sometimes these radicals initiate chain reactions. Ascorbate can terminate these chain radical reactions by electron transfer. Its reducing power makes it a powerful antioxidant, rapidly scavenging many reactive oxygen species (Macan, Kraljevic & Raic-Malic, 2019). The oxidized forms of ascorbate are relatively unreactive and do not cause cellular damage. Ascorbic acid is critical for a variety of functions related to tissue growth and wound healing, neurotransmitter formation, blood cholesterol levels, as well as free radical neutralization. Decreased ascorbic acid level is indicative of oxidative stress and possible damage to cells. Dietary deficiency of ascorbic acid in humans leads to scurvy.

2.3.10 Catalase

Catalase is an ubiquitous antioxidant enzyme that is present in most aerobic cells. Catalase is involved in the detoxification of hydrogen peroxide, a reactive oxygen species, which is a toxic product of both normal aerobic metabolism and pathogenic reactive oxygen species production (Karakus, 2020).This enzyme catalyzes the conversion of two molecules of H_2O_2 to molecular oxygen and two molecules of water (catalytic activity). Catalase also demonstrates peroxidatic activity, in which low molecular weight alcohols can serve as electron donors. In humans, the highest levels of catalase are found in liver, kidney, and erythrocytes, where it is believed to

account for the majority of H_2O_2 decomposition **(**Boriskin, Deviatkin, Nikitin, Pavlova & Toporovskiy, 2019). Decreased serum level of catalase is indicative of oxidative stress and possible cells damage (Nandi, Yan, Jana & Das, 2019).

2.3.11 Superoxide dismutase (SOD)

Superoxide dismutase is a metalloenzyme that catalyzes the dismutation of the superoxide anion to molecular oxygen and hydrogen peroxide and thus form a crucial part of the cellular antioxidant defense mechanism (Sarka, Bhowmik, Sarkar, Sircar & Bhattacharya, 2022). SOD is widely distributed in both plants and animals. It occurs in high concentrations in brain, liver, heart, erythrocytes, and kidney. The amount of SOD present in cellular and extracellular environments is crucial for the prevention of diseases linked to oxidative stress. SOD also appears to be important in the prevention of other neurodegenerative disorders such as Alzheimer's, Parkinson's, and Huntington's diseases (Chang & Chen, 2020; Islam et al., 2022). The reaction catalyzed by SOD is extremely fast, having a turnover of $2x10^9$ $M^{-1}sec^{-1}$ and the presence of sufficient amounts of the enzyme in cells and tissues typically keeps the concentration of superoxide (O_2^-) very low (Marin-Garcia, 2014). Decreased SOD activity is associated with oxidative stress and could lead to diseases.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Materials

The materials used include cadmium sulphate (Riedel De Heaney), lead nitrate (M & B), potassium dichromate (Hopkin & William) and others listed in Appendix 1. The equipments used include Spectrophotometer (Spectrum lab. 7525 B. Bran-scientific), Microscope (Leica Dm750), Centrifuge 80 – 2 (made in UK) and others listed in Appendix 2.

3.2 Methods

3.2.1 Collection and preservation of animals

Seventy (70) male albino (Wistar) rats were used for the study. The animals were young males aged between 10 – 12 weeks. They were procured from the Veterinary Department, University of Nigeria, Nsukka (UNN). All the animals were kept in the animal house of the Biochemistry Department, Abia State University Uturu. They were subsequently allowed to acclimatize for fourteen (14) days. The animals were fed with animal feed (commercial rat chow) and had free access to water *ad libitum*.

Fourteen cages made from wood and wire mesh were used for the study. The animals were randomly selected into 14 groups and placed in the various cages. Each animal in each cage was weighed and the weight recorded. Each group/cage had 5 animals. Each animal in each cage was distinguished from the other using coloured permanent markers which were marked at their backs, tails or both. The cages were labelled 1 to 14. Animals in each cage received the same dose of the test substance except the control. The cages were cleaned daily.

3.2.2 Procedure for preparation of treatment solutions

Stock solution of 1% lead was prepared by weighing 1.60g $Pb(NO_3)_2$ using G & G electronic scale and dissolving the $Pb(NO_3)_2$ salt in 100ml distilled water. The salt was weighed using a beaker on the weighing balance. The beaker was removed from the balance and 20ml distilled water was added to the beaker and the solution was stirred until the salt was dissolved. The salt solution in the beaker was transferred into a 100ml measuring cylinder. The beaker was rinsed twice with distilled water and added to the measuring cylinder. The solution was made up to 100ml in the measuring cylinder with distilled water. Also, 3% lead stock solution was prepared by weighing 4.80g $Pb(NO_3)_2$ using the G & G electronic scale and dissolving the $Pb(NO_3)_2$ in 100ml distilled water as above. A solution of 0.5% lead was prepared by diluting the 1% lead stock solution with distilled water at a ratio of 1:1 while 0.25% lead solution was prepared by diluting the 1% stock solution with distilled water at a ratio of 1:3. Similarly,1.5% lead solution was prepared by diluting the 3% stock solution with distilled water at ratio of 1:1while 0.75% lead solution was prepared by diluting the 3% stock solution with distilled water at a ratio of 1:3.

Stock solution of 1% cadmium was prepared by weighing 1.85g $CdSO_4$ using G & G electronic scale and dissolving the $CdSO_4$ salt in 100ml distilled water. Also, stock solution of 3% cadmium was prepared by weighing 5.55g $CdSO_4$ salt using G & G electronic scale and dissolving the $CdSO_4$ in 100ml distilled water.A solution of 0.5% cadmium was prepared by diluting the 1% cadmium stock solution with distilled water at a ratio of 1:1 while 0.25% cadmium solution was prepared by diluting the 1% stock solution with distilled water at a ratio of 1:3. Similarly, 1.5% cadmium solution was prepared by diluting the 3% stock solution with

distilled water at ratio of 1:1 while 0.75% cadmium solution was prepared by diluting the 3% stock solution with distilled water at a ratio of 1:3.

Stock solution of 1% hexavalent chromium was prepared by weighing 2.83g $K_2Cr_2O_7$ using the G & G electronic scale and dissolving the $K_2Cr_2O_7$ in 100ml distilled water. Also, stock solution of 3% hexavalent chromium was prepared by weighing 8.49g $K_2Cr_2O_7$ salt using the G & G electronic scale and dissolving the $K_2Cr_2O_7$ in 100ml distilled water. A solution of 0.5% hexavalent chromium was prepared by diluting the 1% stock solution with distilled water at a ratio of 1:1 while 0.25% hexavalent chromium solution was prepared by diluting the 1% stock solution with distilled water at a ratio of 1:3. Similarly, 1.5% hexavalent chromium was prepared by diluting the 3% stock solution with distilled water at a ratio of 1:1 while 0.75% hexavalent chromium solution was prepared by diluting the 3% stock solution with distilled water at a ratio of 1:3.

All solutions prepared above were stored at room temperature for two weeks. Fresh solutions were prepared every two weeks.

3.2.3 Treatment

The animals were weighed weekly before commencement of treatment. The control group did not receive any test substance but received only distilled water**.** The animals were dosed by gavage using a combination of syringe and feeding tube (size 5). Treatment was done thrice weekly and for a period of 90 days.

The test substances for treatment were lead nitrate ($Pb(NO_3)_2$), for lead, cadmium sulphate ($CdSO_4$) for cadmium, and potassium dichromate ($K_2Cr_2O_7$) for hexavalent chromium. There were three treatment doses of each test substance as follows; (i) 5mg/kg body weight, (ii)

10mg/kg body weight, and (iii) 20mg/kg body weight. There were four treatment groups per dose level as follows; (i) lead singly, (ii) cadmium singly, (iii) chromium singly and (iv) lead – cadmium – chromium combined.

Various concentrations of test substances were used to ensure that animals of equivalent body weight received the same total volume of treatment solution irrespective of differences in treatment doses. A volume, 0.25% metal solution was used for dosing 5mg/kg single substance treatment group while 0.75% metal solution was used for dosing 5mg/kg combined substance treatment group. Also, 0.5% metal solution was used for dosing 10mg/kg single substance treatment group while 1.5% metal solution was used for dosing 10mg/kg combined substance treatment group. Similarly, 1% metal solution was used for dosing 20mg/kg single substance treatment group while 3% metal solution was used for dosing 20mg/kg combined substance treatment group.

3.2.4 Sample collection

In the 5mg/kg treatment group, no animal died before the end of the period of study. In the 10mg/kg treatment group, two animals died before the end of the study with one from the combined substances group (33 days from the commencement of treatment) and the other from the cadmium group (52 days from the commencement of treatment). In the 20mg/kg treatment group, three animals also died before the end of the study with one from the lead group (31 days from the commencement of treatment), the other from the chromium group (34 days from the commencement of treatment) and another from the combined substances group (42 days from the commencement of treatment).

The remaining 64 animals that survived till the end of the study were weighed using the Triple Beam balance and their final body weights recorded. Cotton wool was dipped in halothane and put in a dessicator and each animal was anaesthesized by placing it in the dessicator for a few seconds. Each animal was removed from the dessicator, sacrificed and blood samples were collected from the heart using syringe. One syringe was used for each animal.

One test tube was labelled for each animal indicating the cage and the code number of the animal. Two sample bottles were labelled as above for each animal. Part of the blood sample collected for each animal was collected in an EDTA bottle for hematological analysis while the other part was put in the designated test tube for centrifuging for obtaining of serum. The blood in the test tubes was allowed to stand for 30minutes and then centrifuged at 3000rpm for 10minutes. Serum was collected using a syringe and put in the second sample bottle. Also, liver, kidney and heart were excised from each sacrificed animal for histopathological evaluation. Each excised organ was weighed using the G and G electronic scale.

The three organs of each animal were put in a labelled sample bottle containing 10% formal saline. The organ samples were subsequently transferred to the Federal Medical Centre (FMC), Owerri, for histopatholigical evaluation after three days.

3.2.5 Biochemical Analysis

3.2.5.1 Assay of serum Alanine Aminotransferase (ALT) activity

This test was done using the method described by Reitman and Frankel (1957) as reported by Offor, Mbagwu and Orisakwe (2017).

Test Principle:

α - oxoglutarate + L – alanine $\xrightarrow{\text{GPT}}$ L – glutamate + pyruvate

Alanine aminotransferase is measured by monitoring the concentration of pyruvate hydrazone formed with 2, 4 – dinitrophemyl hydrazine.

Reagents:

- Reagent 1 (R1): ALT Buffer composed of phosphate buffer (100mmol/l), L–alanine (200mmol/l), and α - oxoglutarate (2.0mmol/l)
- Reagent 2 (R2): 2, 4 – dinitrophenylhydrazine (2.4mmol/l)
- Sodium hydroxide solution (0.4mol/l)
- Distilled water.

Test Sample:

Serum obtained from blood of albino rats was used in the study.

Test Procedure:

One test tube was labelled blank. Other test tubes were labelled according to the code number of the animal from which the serum to be analyzed was obtained. A volume,0.5ml of ALT Buffer was pipetted into each test tube. Another volume, 0.1ml of distilled water was pipetted into the test tube labelled blank while 0.1ml of the different serum samples was pipetted into the designated test tubes. The contents of each test tube were mixed and incubated in water bath at 37^{o}C for 30minutes. After that, the test tubes were brought out and 0.5ml of 2, 4 – dinitrophenylhydrazine was pipetted into each test tube. The contents of each test tube was mixed and allowed to stand for 20minutes at 25^{o}C.A volume**,** 5ml of sodium hydroxide solution was pipetted into each test tube. The test tubes were mixed thoroughly and absorbance read

against the reagent blank at 546nm wavelength after 5 minutes. The activity of ALT was obtained by calculating using the table and guidelines provided by the kit supplier.

3.2.5.2 Assay of serum Aspartate Aminotransferase (AST) activity

This test was done using the method described by Reitman and Frankel (1957) as reported by Offor, Mbagwu and Orisakwe (2017).

Test Principle:

α - oxoglutarate + L – aspartate $\xrightarrow{GOT}$ L–glutamate+ oxaloacetate

AST is measured by monitoring the concentration of oxloacetate hydrazone formed with 2, 4 – dinitrophenyl hydrazine.

Reagents:

- Reagent 1 (R1): AST Buffer composed of phosphate buffer (100mmol) L – aspartate (100mmol/l) and α - oxoglutarate (2mmol/l)
- Reagent 2 (R2): 2, 4 – dinitrophenylhydrazine (2mmol/l)
- Sodium hydroxide solution (0.4mol/l)
- Distilled water

Test Sample:

Serum obtained from blood of albino rats was used in the study.

Test Procedure:

One test tube was labelled blank. Other test tubes were labelled according to the code number of the animal from which the serum to be analyzed was obtained. A volume,0.5ml of AST Buffer was pipetted into each test tube. Another volume, 0.1ml of distilled water was pipetted into the test tube labelled blank while 0.1ml of the different serum samples was pipetted into the

designated test tubes. The contents of each test tube were mixed and incubated in water bath at 37^{o}C for 30minutes. After that, the test tubes were brought out and 0.5ml of 2, 4 – dinitrophenylhydrazine was pipetted into each test tube. The contents of each test tube was mixed and allowed to stand for 20minutes at 25^{o}C.A volume**,** 5ml of sodium hydroxide solution was pipetted into each test tube. The test tubes were mixed thoroughly and absorbance read against the reagent blank at 546nm wavelength after 5 minutes. The activity of AST was obtained by calculating using the table and guidelines provided by the kit supplier.

3.2.5.3 Assay of serum Alkaline Phosphatase (ALP) activity

This test was done using the method described by Roy (1970) as reported by Cury et al. (2005)

Test Principle:

Alkaline phosphatase acts upon AMP-buffered sodium thymolphthalein monophosphate. The addition of an alkaline reagent stops enzyme activity and simultaneously develops a blue chromogen, which is measured photometrically.

Reagents:

- Reagent 1 (R1): Alkaline phosphatase substrate composed of 3.6mM sodium Thymolphthalein in 0.2M 2 – Amino – 2 – methyl – 1 – propanol (AMP) buffer. $MgCl_2$ (1.0mM).
- Reagent 2 (R2): Alkaline phospatase color developer composed of 0.1M sodium hydroxide and 0.1M sodium carbonate.
- Standard: Alkaline phosphatase standard composed of thymolphthalein in n-propanol (0.5mM/l).
- Distilled water

Test Sample:

Serum obtained from blood of albino rats was used in the study.

Test Procedure:

One test tube was labelled blank and another labelled standard. Other test tubes were labelled according to the code number of the animal from which the serum to be analyzed was obtained. A volume, 0.5ml of Alkaline Phosphatase substrate was dispensed into each labelled test tube and was allowed to equilibrate to 37^{o}C for 3 minutes. Another volume,0.05ml (50ul) of ALP standard was added to the test tube labelled standard while 0.05ml (50ul) of distilled water was added to the test tube labelled blank. A volume, 0.05ml (50ul) of serum was added to the different designated test tubes. The content of each test tube was mixed gently and incubated for exactly 10minutes at 37^{o}C. Another volume, 2.5ml of Alkaline Phosphatate Color Developer was added to each test tube. The contents of each test tube were mixed well. The wavelength of the spectrophotometer was set at 590nm. The spectrophotometer was set to zero using the blank. The absorbance of the standard and samples were read and recorded. Alkaline phosphatase activity in the samples was calculated using the formula provided by the kit manufacturer.

$$\text{ALP} = \frac{\text{absorbance of sample}}{\text{absorbance of ALP standard}} \times \text{value of ALP standard (U/L)} \qquad (3.1)$$

3.2.5.4 Determination of serum Total Bilirubin concentration

This test was done using the colorimetric methoddescribed by Jendrassik and Grof (1938) as reported by Berrahal, Nehdi, Hajjaji, Gharbi and El-Fazaa (2007).

Test Principle:

Direct (conjugated) bilirubin reacts with diazotized sulphanilic acid in alkaline medium to form a blue coloured complex. Total Bilirubin is determined in the presence of caffeine, which releases albumin bound bilirubin, by the reaction with diazotized sulphanilic acid.

Reagents:

- R1 composed of sulphanilic acid (29 mmol/l) and hydrochloric acid (0.17N).
- R2 composed of sodium nitrite (38.5mmol/l)
- R3 composed of caffeine (0.2mol/l) and sodium benzoate (0.52mol/l).
- R4 composed of tartrate (0.92mol/l) and sodium hydroxide (1.9N).

Test Sample:

Serum obtained from blood of albino rats was used in the study.

Test Procedure

One test tube was labelled blank. Other test tubes were labelled according to the code number of the animal from which the serum to be analyzed was obtained. A volume, 200ul of R1 was pipetted into each test tube. Another volume, 50ul of R2 was pipetted into each test tube except the test tube labelled blank. A volume, 1000ul of R3 was pipetted into each test tube.Another volume, 200ul of the different serum samples were pipeted into the designated test tubes. A volume, 200ul of distilled water was pipetted into the test tube labelled blank. The contents of the different test tubes were mixed and allowed to stand for 10minutes at 25^{o}C.A volume, 1000ul of R4 was pipetted into each test tube. The contents of the test tubes were mixed and allowed to stand for 10minutes at 25^{o}C.The absorbance of the sample was read against the sample blank

(A_{TB}) at 578nm. Serum total bilirubin concentration was calculated using the formula provided by the kit manufacturer:

Total bilirubin (umol/l) = 185 x A_{TB} (578nm)

Total bilirubin (mg/dl) = 10.8 x A_{TB} (578nm)

3.2.5.5 Assay of serum Lactate Dehydrogenase (LDH) activity

This assay was done applying the method described by Henry, Chiamori, Golub and Berkman (1960) as reported by Agrawal, Gandhe, Gupta and Reddy (2016).

Test Principle:

Lactate dehydrogenase (LDH) catalyzes the reduction of pyruvate to lactate with simultaneous oxidation of reduced NADH to NAD^+. The rate of decrease in absorbance due to formation of NAD^+ is measured at 340nm and is proportional to the LDH activity in the sample.

$$\text{Pyruvate} + \text{NADH} + H^+ \xrightarrow{\text{LDH}} \text{lactate} + NAD^+$$

(NAD = nicotinamide adenine dinucleotide)

Reagents

- R1: buffer reagent composed of pyruvate (0.6mmol/l) and Tris buffer (50mmol/l).
- R2: Substrate/starter reagent (NADH 0.18mmol/l)

Preparation of working reagent

Four parts of R1 (buffer reagent) was added to one part of R2 (substrate) to constitute the working reagent.

Test Sample

Serum obtained from blood of albino rats was used in the study.

Test Procedure:

Test tubes were labelled according to the code number of the animal from which the serum to be analyzed was obtained. A volume,1000ul of the working reagent was pipetted into each test tube and incubated at 37ºC for 1 min. Another volume, 10ul of the different serum samples was pipetted to the designated test tubes.The contents of each test tube were mixed well.The spectrophotometer was set at 340nm and the reading zeroed with distilled water as blank.The absorbance of the samples was read after 60 seconds and repeated four times at 30 seconds interval at 340nm.

Mean change in absorbance per minute (ΔA/minute) was calculated. LDH activity was calculated as provided by kit manufacturer.

LDH activity (IU/L) = ΔA/minute x kinetic factor (3.2)

where kinetic factor (k) = 16030

3.2.5.6 Determination of serum Urea concentration

This test was done using the method described by Fawcett and Scott (1960) as reported by Offor, Mbagwu and Orisakwe (2017).

Test Principle:

Urea in serum is hydrolysed to ammonia in the presence of urease. The ammonia is then measured photometrically by Berthelot's reaction.

$$\text{Urea} + H_2O \xrightarrow{\text{urease}} 2NH_3 + CO_2$$

$$NH_3 + \text{Hypochlorite} + \text{phenol} \longrightarrow \text{indophenols (blue compound)}$$

Reagent:

- R1 composed of EDTA (116mmol/l), sodium nitroprusside (6mmol/l), and urease (1g/l)
- R2 composed of phenol (120mmol/l)
- R3 composed of sodium hypochlorite 27(mmol/l) and sodium hydroxide (0.14N)
- Urea standard (79.27mg/dl)

Preparation of reagents:

- R1 was supplied in a vial R1a and a bottle R1b. The content of the vial R1a was transferred into the bottle R1b and mixed gently.
- R2 was diluted with 660ml of distilled water.
- R3 was diluted with 750ml of distilled water.

Test Sample:

Serum obtained from blood of albino rats was used in the study.

Test Procedure:

One test tube was labelled blank and another labelled standard. Other test tubes were labelled according to the code number of the animal from which the serum to be analyzed was obtained. A volume, 10ul of distilled water was pipetted into the test tube labelled blank while 10ul of Urea standard was pipetted into the test tube labelled standard 10ul of the different scrum samples were pipetted into the designated test tubes. Another volume, 100ul of R1 was pipetted into each test tube. The contents of the eache test tube were mixed and incubated at 37^{o}C for 10minutes. A volume, 2.5ml of R2 was pipetted into each test tube. Another volume, 2.5ml of R3 was also pipetted into each test tube. The contents of each of the test tubes were mixed and incubated at 37^{o}C for 15minutes.The absorbance of the sample (A_{sample}) and standard ($A_{standard}$) was read against the blank at 546nm.

Serum urea concentration was obtained by calculation using the formula provided by the kit manufacturer.

$$\text{Urea concentration} = \frac{A_{sample}}{A_{standard}} \times \text{standard concentration (mg/dl)} \quad (3.3)$$

3.2.5.7 Determination of serum Creatinine concentration

This test was done applyingthe method described by Bartels, Bohmer and Heierli (1972) as reported by Osukoya, Oyinloye, Ajiboye, Olokode and Adeola (2021).

Test Principle:

Creatinine in alkaline solution reacts with picric acid to form a coloured complex. The amount of the complex formed is directly proportional to the creatinine concentration.

Reagent:

- R1 is composed of picric acid (35mmol/l)
- R2 is composed of sodium hydroxide (0.32mol/l)
- Creatinine standard (2.06mg/dl)

Preparation of working reagent

- 50ml of R1 was mixed with 50ml of R2 to constitute the working reagent.

Test Sample:

Serum obtained from blood of albino rats was used in the study.

Test Procedure:

One test tube was labelled standard while other test tubes were labelled according to the code number of the animal from which the serum to be analyzed was obtained. A volume, 1.0ml of working reagent was pipetted into each test tube. Another volume, 0.1ml of creatinine standard was pipetted into the test tube labelled standard. Similarly, 0.1ml of serum samples was pipetted

into the designated test tubes. The contents of each test tube was mixed and the absorbance A_1 of standard and samples was read at 492nm after 30seconds.

After 2 minutes, absorbance A_2 of standard and samples was read at 492nm.

$A_2 - A_1 = \Delta A_{sample}$ or $\Delta A_{standard}$.

Serum creatinine concentration was calculated using the formula provided by the kit manufacturer.

Creatinine concentration = $\frac{\Delta Asample}{\Delta Astandard} xstandardconcentration\left({}^{mg}/_{dl}\right)$ (3.4)

3.2.5.8 Determinaton of serum Potassium concentration

This test was done using the method described by Engelbrecht and McCoy (1956) as reported by Furukawa, Watanabe, Kimura and Kaneko (2012).

Test Principle:

The amount of potassium is determined by using sodium tetraphenylboron in a specifically prepared mixture to produce a colloidal suspension. The turbidity of which is proportional to potassium concentration in the range of 2 – 7mEq/l.

Reagents:

- Potassium reagent: sodium tetraphenylboron 2.1mM
- Potassium standard: equivalent to 4mEq/l.

Test Sample:

Serum obtained from blood of albino rats was used in the study.

Test Procedure:

One test tube was labelled blank and another labelled standard. Other test tubes were labelled according to the code number of the animal from which the serum to be analyzed was obtained. A volume, 1.0ml of potassium reagent was pipetted into each test tube. Another volume, 10ul of

distilled water was pipetted into the test tube labelled blank. Similarly, 10ul of potassium standard was pipetted into the test tube labelled standard. A volume, 10ul of the different serum samples was pipetted into the designated test tubes.The contents of each test tube were mixed and allowed to stand at room temperature for 3 minutes.

The sepectrophometer was set at 500nm and its reading zeroed with the blank.

The absorbance of the standard and sample was read at 500nm and recorded.

Potassium concentration in the samples was calculated using the formula provided by the kit manufacturer.

$$\frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times \text{concentration of standard} = \text{potassium concentration (mEq/l)} \qquad (3.5)$$

3.2.5.9 Determination of serum Chloride concentration

This test was done applying the method described by Iwasaki, Utsumi, and Ozawa (1952) as reported by Drienovska, Chovancova, Koudelakova, Damborsky and Chaloupkova (2012).

Test Principle:

Chloride ions form a soluble, non-ionized compound with mercuric ions and will displace thiocyanate ion from non-ionized mercuric thiocyanate. The released thiocyanate ions react with ferric ions to form a color complex that absorbs light at 480mm. The intensity of the colour produced is directly proportional to the chloride concentration.

$Hg(SCN)_2 + 2Cl^- \longrightarrow HgCl_2 + 2SCN^-$

$2SCN^- + Fe^{3+} \longrightarrow 4Fe(SCN)_3$ red complex

Reagents:

- Chloride reagent composed of mercuric nitrate (0.038mM), mercuric thiocyante (1.75mM), mercuric chloride (0.74mM) and ferric nitrate (22.3mM).

- Chloride calibrator: sodium chloride (100Eq/l).

Test Sample:

Serum obtained from blood of albino rats was used in the study.

Test Procedure

One test tube was labelled blank and another labelled calibrator. Other test tubes were labelled according to the code number of the animal from which the serum to be analyzed was obtained.

A volume, 1.5ml of chloride reagent was pipetted into each teat tube. Another volume, 10ul of distilled water was pipetted into the test tube labelled blank. Similarly, 10ul of chloride calibrator was pipetted into the test tube labelled calibrator and 10ul of the different serum samples was pipetted into the designated test tubes. The content of each test tube was mixed and incubated at room temperature for 5minutes.The spectrophotometer was set at 480nm and its reading zeroed with blank. The absorbance of the calibrator and samples was read at 480nm and recorded.

Chloride concentration in the samples was calculated using the formula provided by the kit manufacturer.

$$\frac{\text{Absorbance of sample}}{\text{Absorbance of calibrator}} \times \text{concentration of calibrator} = \text{chloride (mEq/l)} \qquad (3.6)$$

3.2.5.10 Determination of serum Phosphorus (Inorganic)concentration

This test was done applying the method described by Amador and Urban (1972) as reported by Pralle et al. (2022).

Test Principle

Inorganic phosphorus reacts with ammonium molybdate in the presence of sulphuric acid to form a phosphomolybdate complex which is measured at 340nm.

Reagents

- Blank reagent composed of sulphuric acid (0.36mmol/l), NaCl (0.154mol/l), and detergent (0.7%w/v).
- Molybdate composed of ammonium molybdate (3.9mmol/l), sulphuric acid (0.36mol/l) and NaCl (0.154mol/l).
- Phosphate standard (1.41mmol or 5mg/dl.

Preparation of working reagent

The content of 1 bottle of molybdate was added to 1 bottle of blank reagent and mixed to obtain a working reagent.

Test Sample

Serum obtained from blood of albino rats was used in the study.

Test Procedure:

One test of tube was labelled blank and another labelled standard. Other test tubes were labelled according to the code number of the animal from which the scrum to be analyzed was obtained.

A volume, 10ul of distilled water was pipetted into the test tube labelled blank and 10ul of standard was also pipetted into the test tube labelled standard. Similarly, 10ul of the different serum samples was pipetted into the designated test tubes. A volume, 1000ul of the working reagent was pipetted into each test tube. The content of each test tube was mixed thoroughly and incubated for 10minutes at 25ºC. The absorbance of standard and samples was read against the blank at 340nm.

The concentration of Phosphorus was calculated using the formula provided by the kit manufacturer.

$$\text{Phosphorus concentration} = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times \text{concentration of standard} \quad (3.7)$$

3.2.5.11 Determination of serum Malondialdehyde (MDA) concentration

This test was done applying the method described by Wallin, Rosengren, Shetzer and Camejo (1993) as reported by Kumar, Chaitanya and Preedy (2017).

Test Principle:

Thiobarbituric acid (TBA) reacts with Malondialdehyde (MDA) to form a pink colour product (TBA – MDA adduct). The colour intensity at 532nm is directly proportional to the MDA concentration in the sample:

Reagents

- 17% Thiobarbituric acid (TBA)
- 25% Trichloroacetic (TCA)
- n – butanol

Test Sample:

Serum obtained from blood of albino rats was used in the study.

Test Procedure:

Test tubes were labelled according to the code of the animal from which the serum to be analyzed was obtained. A volume, 0.5ml of the different serum samples was pipetted into the designated test tubes. Another volume, 0.5ml of TCA was pipetted into each test tube. Similarly, 0.5ml of TBA was pipetted into each test tube. The test tubes were incubated in water bath at 100^{o}C for 15minutes. Thereafter, 2ml of n-butanol was pipetted into each test tube and mixed well. The tubes were centrifuged at 3000rpm for 10minutes.The supernatant butanol layer was collected and the absorbance of the samples read at 532nm.

The concentration of MDA was calculated using A = Ecl.

Where E = extinction coeffient = $1.5x10^5M^{-1}cm^{-1}$

1 = path length = 1cm, c = concentration of MDA in sample, A = absorbance of sample

3.2.5.12 Determination of serum reduced Glutathione (GSH) concentration

This test was done using method previously described by Ellman (1959) with slight modification as reported by Alisik, Neselioglu and Erel (2019).

Test Principle:

The general thiol reagent $5-5^{1}-$ dithiobis 2- Nitrobenzoic acid (DTNB, Ellman's reagent) reacts with glutathione (GSH) to form the chromophore, 5 – thionitrobenzoic acid (TNB) and GS – TNB. The absorbance at 420nm is directly proportional to the concentration of GSH.

Reagents

- Trichloroacetic acid, TCA, (25%)
- DTNB (10mM).
- Phosphate buffer (0.1M)

Test Sample:

Serum obtained from blood of albino rats was used in the study.

Test Procedure:

Test tubes were labelled according to the code of the animal from which the serum to be analyzed was obtained. A volume, 0.2ml of the different serum samples was pipetted into the designated test tubes. Another volume, 1.0ml TCA was pippetted into each test tube. Each test tube was centrifuged at 3000rpm for 10minutes. A volume, 0.2ml of the supernatant was reacted

with 1.0ml of DTNB in the presence of phosphate buffer. Absorbance of the samples was read at 420nm.

The concentration of reduced glutathione was calculated as follows:

Concentration (uM) of GSH = $\frac{A_{420}-b}{m}$

Where A_{420} = absorbance of samples at 420nm

b = 0.02058 m = 0.002045

b and m are intercept and slope obtained from a previous calibration graph by Boxy Tech – GSH 420.

3.2.5.13 Determination of serum Ascorbic Acid (Vitamin C) concentration

This test was done applying the method described byAssociation of Official Analytical Chemists(AOAC) (2005).

Test Principle:

The indicator, 2, 6 – dichloroindophenol (DCIP) is quite specific in its ability to oxidize only Vitamin C. Redox titration of Vitamin C with DCIP provides a quantitative measure of Vitamin C content in a sample. The solution stays colourless until all the ascorbic acid has been oxidized. After this point, further addition of DCIP will turn the solution pink. The amount of Vitamin C is found using its quantitative relationship to the standardized DCIP.

Reagents

- 2, 6 – dichloroindophenol (DCIP)
- Oxalic acid (0.5%)
- Ascorbic acid standard (5mg/100ml)

Test Sample:

Serum obtained from blood of albino rats was used in the study.

Test Procedure:

(1) A volume, 10ml of oxalic acid was pipetted into a 50ml conical flask labelled standard.

(2) Another volume, 0.1ml of ascorbic acid standard was pipetted into the conical flask.

(3) DCIP was added through a burette to the conical flask containing ascorbic acid and oxalic acid, drop wise and vortexed until the colour of the solution turned pink.

(4) The volume of DCIP used to titrate the ascorbic acid standard was recorded.

(5) A volume, 0.1ml of serum sample was pipetted into conical flask containing 10ml oxalic acid as above and titrated with DCIP and the titre recorded.

(6) This was repeated for each serum sample.

(7) The concentration of ascorbic acid in each sample was calculated as follows:

$$\text{Conc. of ascorbic acid in sample} = \frac{\text{volume of DCIP used to titrate sample}}{\text{volume of DCIP used to titrate standard}} \times \text{conc. of ascorbic acid standard used} \quad (3.8)$$

3.2.5.14 Assay of serum Superoxide Dismutase (SOD) activity

The method of Sun and Zigman (1978) was adopted with modifications as reported by Katerji, Filippova and Duerksen-Hughes (2019).

Test Principle:

Superoxide dismutase activity was assayed in terms of its ability to inhibit the radical mediated chain propagating autoxidation of epinephrine of pH 10.2. The enzyme assay is based on adenochrome absorption at 480nm.

Reagents

- Sodium carbonate buffer (0.05M, pH10.2)
- Epinephrine in 0.005N HCl.

Test sample:

Serum obtained from blood of albino rats was used in the study.

Test Procedure:

(1) A volume, 2.95ml of sodium carbonate buffer was pipetted in a cuvette.

(2) Another volume, 0.02ml of distilled water was pipetted into the curette.

(3) Similarly, 0.03ml of epinephrine in 0.005N HCl was pipetted into the cuvette.

(4) The absorbance of blank was read at 480nm and recorded.

(5) The cuvette was washed and cleaned.

(6) A volume, 2.95ml sodium carbonate buffer was pipetted into the cuvette.

(7) Another volume, 0.02ml of serum sample was pipetted into the cuvette.

(8) Similarly, 0.03ml of epinephrine in 0.005N HCl was pipetted into the cuvette.

(9) The absorbance of sample was read at 480nm and recorded.

(10) This was done for each serum sample.

(11) An extinction coefficient of $4020M^{-1}cm^{-1}$ for epinephrine at 480nm was used.

(12) One unit of SOD activity was defined as the amount ofprotein causing 50% inhibition of the autoxidation of epinephrine at 25°C.

(13) % inhibition = $\frac{(absorbanceofblank - absorbancesample)\ x\ 100\%}{absorbanceofblank}$ (3.9)

3.2.5.15 Assay of serum Catalase (CAT) activity

Serum catalase activity was determined according to the method of Beers and Sizer (1952) with modification as reported by Katerji, Filippova and Duerksen-Hughes (2019).

Test Principle:

Catalase catalyzes the decomposition of hydrogen peroxide (H_2O_2) into water and oxygen.

The rate of disintegration of hydrogen peroxide into water and oxygen is proportional to the concentration of catalase.

$2H_2O_2 \xrightarrow{\text{catalase}} 2H_2O + O_2$.

One unit of catalase will decompose 1.0micromole of hydrogen peroxide to oxygen and water per minute at pH 7.0 at 25°C at a substrate concentration of 50mM hydrogen peroxide. The absorbance of hydrogen peroxide is measured at 240nm.

Reagent

- Phosphate buffer (50mM, pH 7.0)
- Hydrogen peroxide in phosphate buffer (pH 7.0)

Test Sample:

Serum obtained from blood of albino rats was used in the study.

Test Procedure:

(1) A volume, 0.1ml of serum sample was pipetted into a cuvette.

(2) Another volume, 2.9ml of H_2O_2 in phosphate buffer was pipetted into the cuvette and mixed thoroughly.

(3) Initial absorbance of sample was read at 240nm and subsequently at 30 seconds interval for 120 seconds (2minutes).

(4) Each sample was run at a time.

(5) Catalase activity was calculated as follows:

Volume activity of catalase (U/ml) = $\frac{\Delta A samplex\ 3.0ml}{0.0436\ x\ 0.1ml}$

Where ΔAsample = change in absorbance per minute of sample.

0.0436 = extinction coefficient of H_2O_2 in $mM^{-1}cm^{-1}$.

3.0ml = volume of reaction mixture

0.1ml = volume of sample.

3.2.5.16 Determination of Haemoglobin (Hb) concentration

The cyanmethaemoglobin method was used as described by Drabkin and Austin (1935) with some modifications as reported by Karakochuk et al. (2019). This is a colorimetric method and is a more accurate method than other methods. A commercial cyanmethaemoglobin is used as standard.

Principle:

Haemoglobin is treated with a reagent containing potassium ferricyanide, potassium cyanide and potassium dihydrogen phosphate. The ferricyanide forms methaemgolobin which is converted to cyanmethaemeglobin by the cyanide. All forms of haemogblibin except sulfhaemoglobin are converted to cyanmethaemoglobin.

Reagents

- Drabkin's solution
- Cyanmethaemoglobin standard

Sample:

Whole blood obtained from albino rats was used in the study.

Procedure:

(1) One test tube was labelled blank and another labelled standard. Other test tubes were labelled according to the code number of the animal from which the blood to be analyzed was obtained.

(2) A volume, 5ml of Drabkin solution was pipetted into the test tube labelled blank. The blank was used to zero the spectrophotometer at 540nm.

(3) Another volume, 5ml of Drabkin's solution was pipetted into the test tube labelled standard and the respective blood samples.

(4) Then, a volume, 20ul of cyanmethaemmoglobin standard was pipetted into the test tube laballed standard. A fresh volume, 20ul of the respective blood samples was pipetted into the designated test tubes containing Drabkin's solution.

(5) The contents of each test tube were mixed thoroughly and allowed to stand for 10minutes.

(6) There absorbance was read at 540nm.

(7) Haemoglobin was calculated as follows:

$$\text{Hb (g/dl)} = \frac{\text{absorbance of sample}}{\text{absorbance of standard}} \times \frac{\text{concentration of standard}}{4} \quad (3.10)$$

3.2.5.17 Determination of Packed Cell Volume (PCV)

Packed cell volume (PCV) was determined using the method described by WHO (2000).

The PCV or haematocrit is a percentage of the total volume of whole blood occupied by packed red blood cells, when a known volume of whole blood is centrifuged at a constant speed for a constant period of time.

Procedure:

Special heparinized non-graduated glass capillary tubes of length 7cm and 1mm internal diameter were used.

(1) Each capillary tube was labelled according to the code number of the animal from which the blood to be analyzed was obtained.

(2) Each capillary tube was filled to two – third of its length with the specific thoroughly mixed blood samples.

(3) One end of each capillary tube was sealed with plasticine.

(4) The filled tubes were then placed in the microhaematocrit centrifuge and spun at 12000g for 5 minutes.

(5) The spun tubes were placed into a well designed scale (PCV reader) and the PCV was read as a percentage.

3.2.5.18 Determination of Red Blood Cells (RBC Manual)

Red blood cells count was determined manually using the method described by Kakel (2013).

Principle:

Whole blood is diluted appropriately using an isotonic diluent (Formol citrate) to avoid lyses of red cells. The number of red cells in a known volume and of known dilution is counted using a counting chamber.

Reagent:

- Formol citrate (diluent)

Sample:

Whole blood obtained from albino rats was used in the study.

Procedure:

(1) A volume, 0.02ml of EDTA anticoagulated blood was added to 3.98ml of the diluent.

(2) The Neubauer counting chamber was charged with the well mixed diluted blood.

(3) The cells were allowed to settle in a moist chamber for 3 to 5 minutes.

(4) The ruled area of the counting chamber under 10x objective of the microscope was located.

(5) The 16 square in the central ruled area of the chamber was counted.

(6) RBCs were obtained as follows:

Total number of cells counted x 10,000 = cells/mm^3

(7) Each blood sample was analyzed separately.

3.2.5.19 Determination of White Blood Cells (WBC Total)

Total white blood cells count was determined using the method described by Kakel (2013).

Principle:

Whole blood is diluted appropriately using a diluent (Turk's solution) which hemolyzes red cell, leaving all the nucleated cells intact. The number of white cells in a known volume and known dilution are counted using a counting chamber.

Reagent:

- Turk's solution (diluent)

Sample:

Whole blood obtained from albino rats was used in the study.

Procedure:

(1) A volume, 0.02ml of blood sample was added to 0.38ml of diluent.

(2) The Neubauer counting chamber was charged with the thoroughly mixed diluted blood.

(3) The cells were allowed to settle in a moist chamber for 3 to 5 minutes.

(4) The four large corner squares were located using 10x objective of the microscope.

(5) The total number of white cells in the four large corner squares was counted.

(6) WBC was obtained as follows:

Total number of cells counted x 50 = cells/mm^3.

(7) Each blood sample was analyzed separately.

3.2.6 Histopathological evaluation of heart, kidney and liver

Histological evaluation of liver, kidney and heart was done using the method described by Okoro (2002) with minor modifications.

(1) Organ collection and preservation:

The organs evaluated histologically were excised from the sacrificed animals used in the study. To preserve the tissues from degradation and maintain the structure of the cell and sub-cellular components, the organs were preserved in 10% buffered formalin in respective labelled sample bottles.

(2) Cut-up:

The organs were examined grossly and pieces of tissue cut out with surgical blade selected and placed in tissue cassette for histological processing.

(3) Dehydration:

The aim of this was to remove water from the tissues. Tissues were placed in four (4) increasing grades/concentrations of isopropyl alcohol (IPA) i.e. 70%, 80%, 90%, 100% for one (1) hour each.

(4) Clearing/Dealcoholization:

The tissues were placed in two (2) changes of xylene for one (1) hour each. This is to remove isppropyl alcohol.

(5) Impregnation/Infiltration:

The tissues were transferred into two (2) changes of wax bath containing molten paraffin wax, so that the xylene could diffuse out into the surrounding melted wax and the wax in turn replacing it.

(6) Embedding:

The tissues were embedded in molten paraffin wax using stainless embedding mould and then placed on ice block to solidify.

(7) Sectioning:

The tissue blocks were cut into thin sections of 5 micrometer using Leica RM 212 Rt Rotary microtome.

(8) Floatation:

Cut sections were floated on a thermostatically regulated water bath of 45°C to spread out and then picked with a clean grease free slide.

(9) Flattening:

The tissue sections on slides were placed on a thermostatically regulated hot plate of 65°C to allow the sections stick firmly on the slide

(10) Staining (HPE):

The tissue sections were stained using Haematoxylin (Harris alum Haematoxylin) and Eosin to demonstrate general tissue structure.

(11) Mounting:

Stained sections on slides were covered with cover slip (cover glass) using DPX mountant.

(12) Microscopy:

The sections were viewed and interpreted using Leica DM 750 Binocular microscope with photomicrographic facilities and then photomicrographed.

3.2.7. Statistical Analysis

Results of the study were presented as mean ± standard deviation and were analyzed using Stats Tester software and one way analysis of variance (ANOVA). Multiple t-test (with Bonferroni correction) was used to compare means at $p<0.05$.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1.1 Body weight gain and organs ratio

4.1.1.1 Percentage body weight gainof albino rats treated with cadmium (Cd), chromium (Cr) and lead (Pb) individually and as a mixture

Figures 4.1- 4.4 show the result of percentage body weight (BW) gain of albino rats treated with cadmium (Cd), chromium (Cr) and lead (Pb) individually and as a mixture.

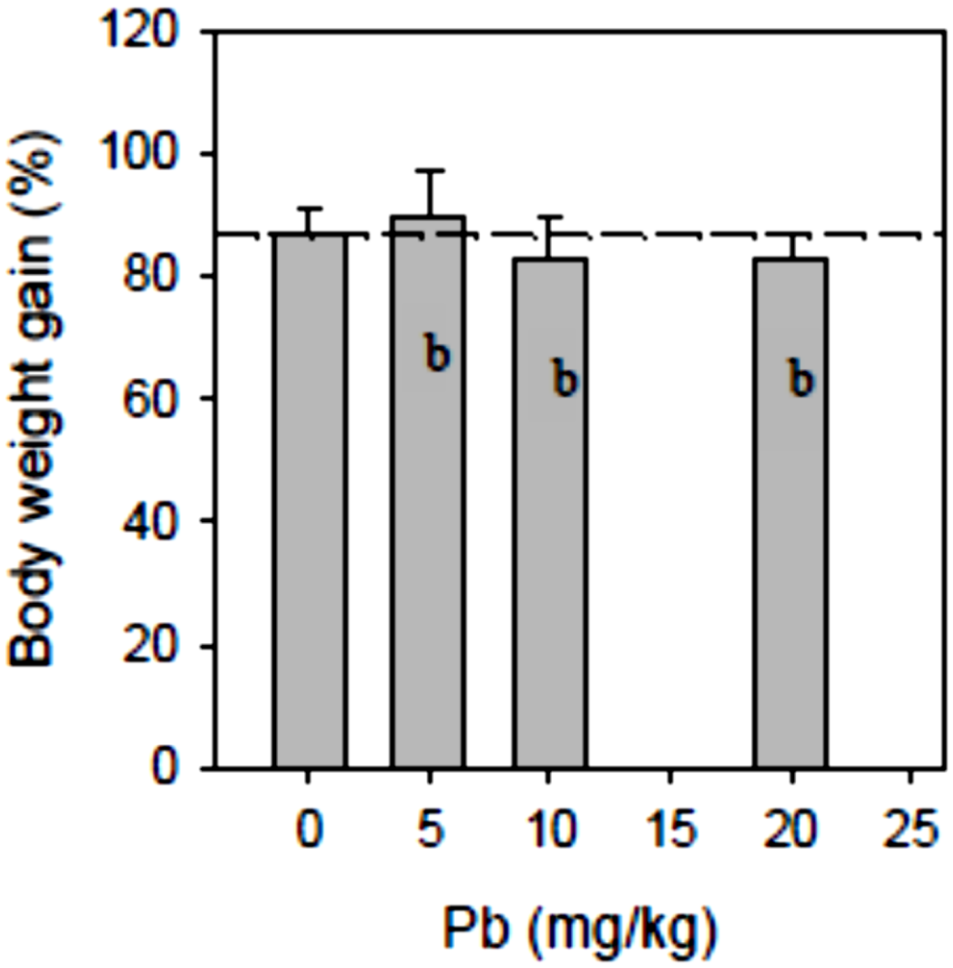


Figure 4.1: Dose-effect relationship for percentage body weight gain in albino rats treated with Pb. b = significantly different from mixture ($p<0.05$)

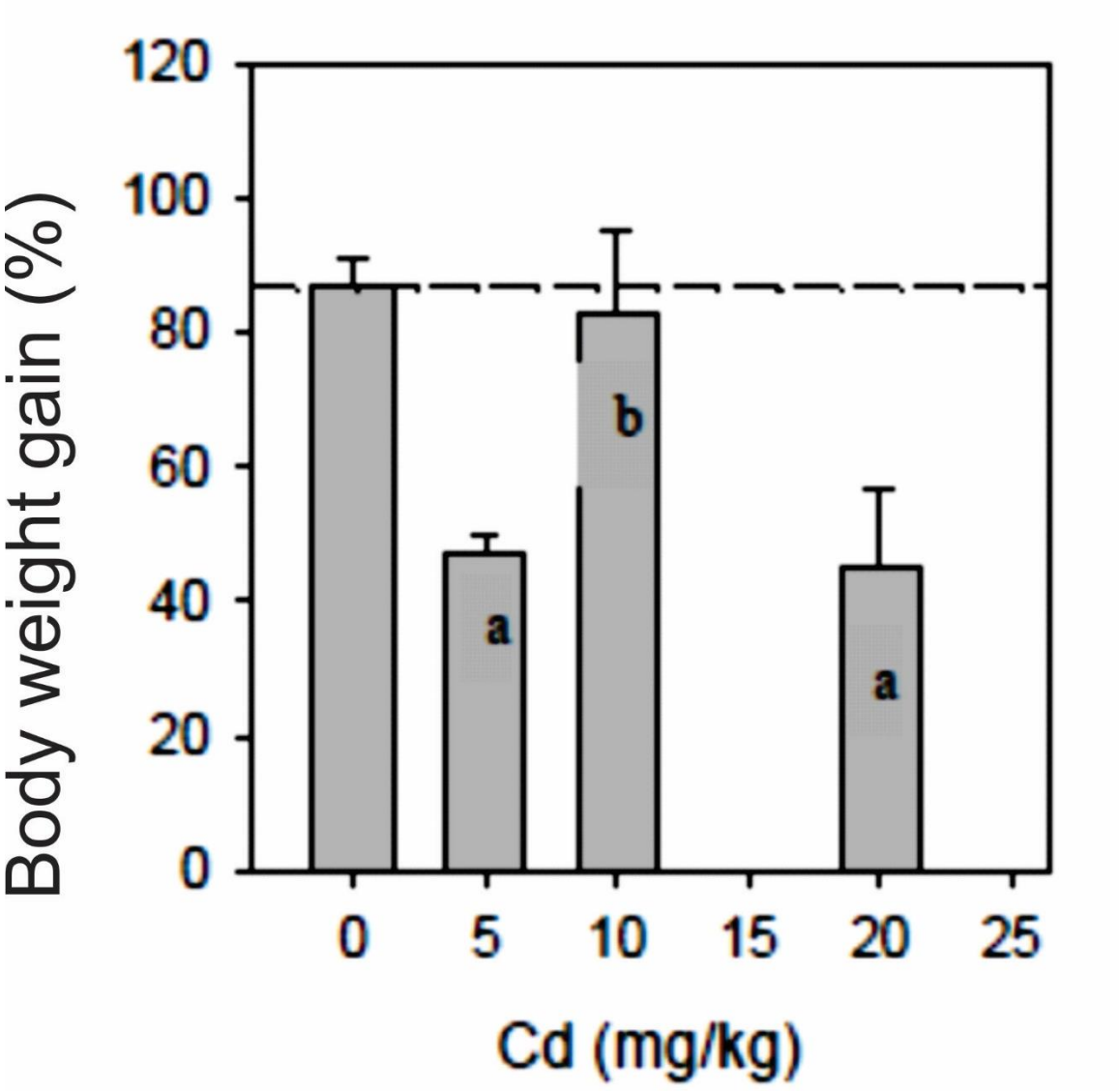


Figure 4.2: Dose-effect relationship for percentage body weight gain in albino rats treated with Cd. a = significantly different from control ($p<0.05$), b = significantly different from mixture ($p<0.05$)

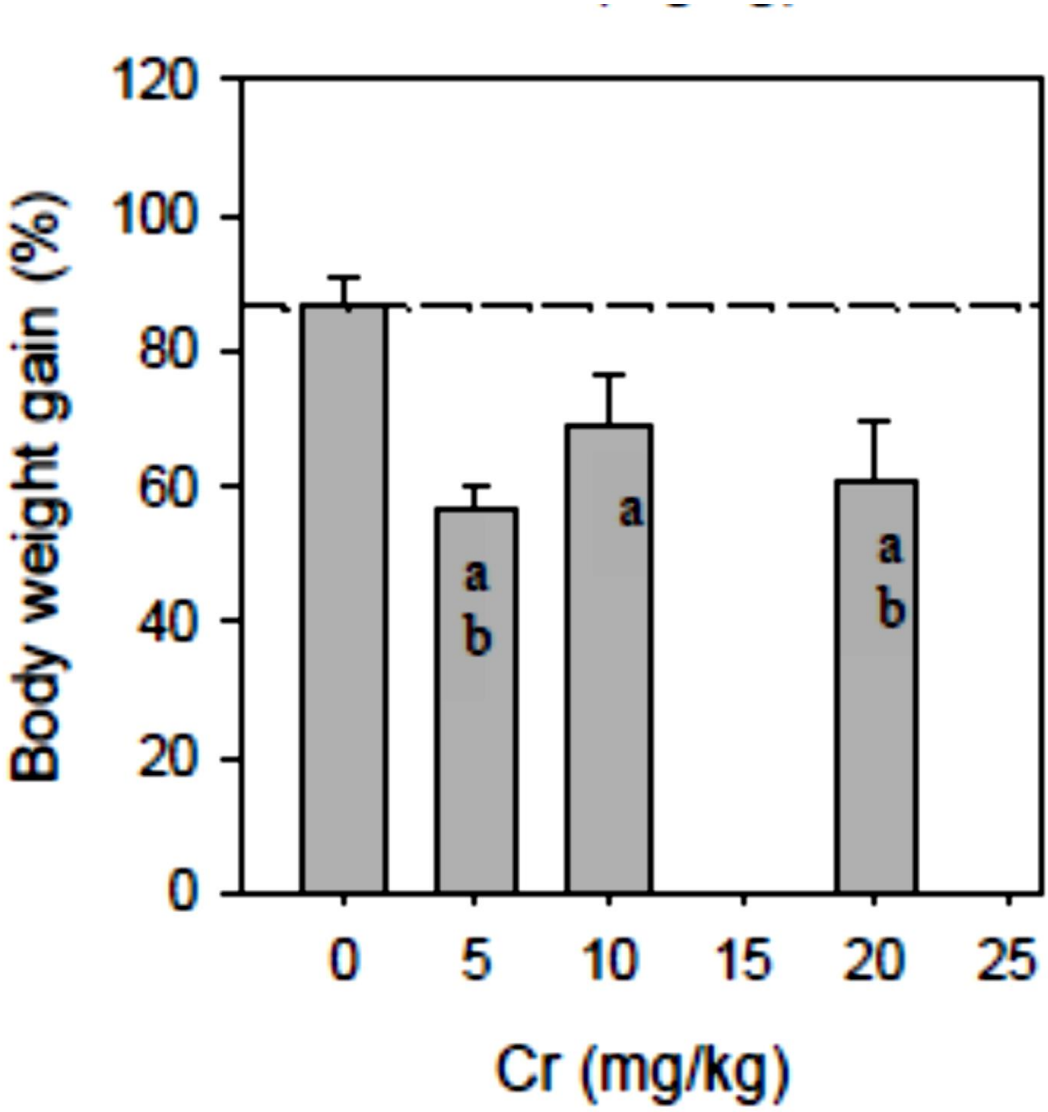


Figure 4.3: Dose-effect relationship for percentage body weight gain in albino rats treated with Cr. a = significantly different from control ($p<0.05$), b = significantly different from mixture ($p<0.05$)

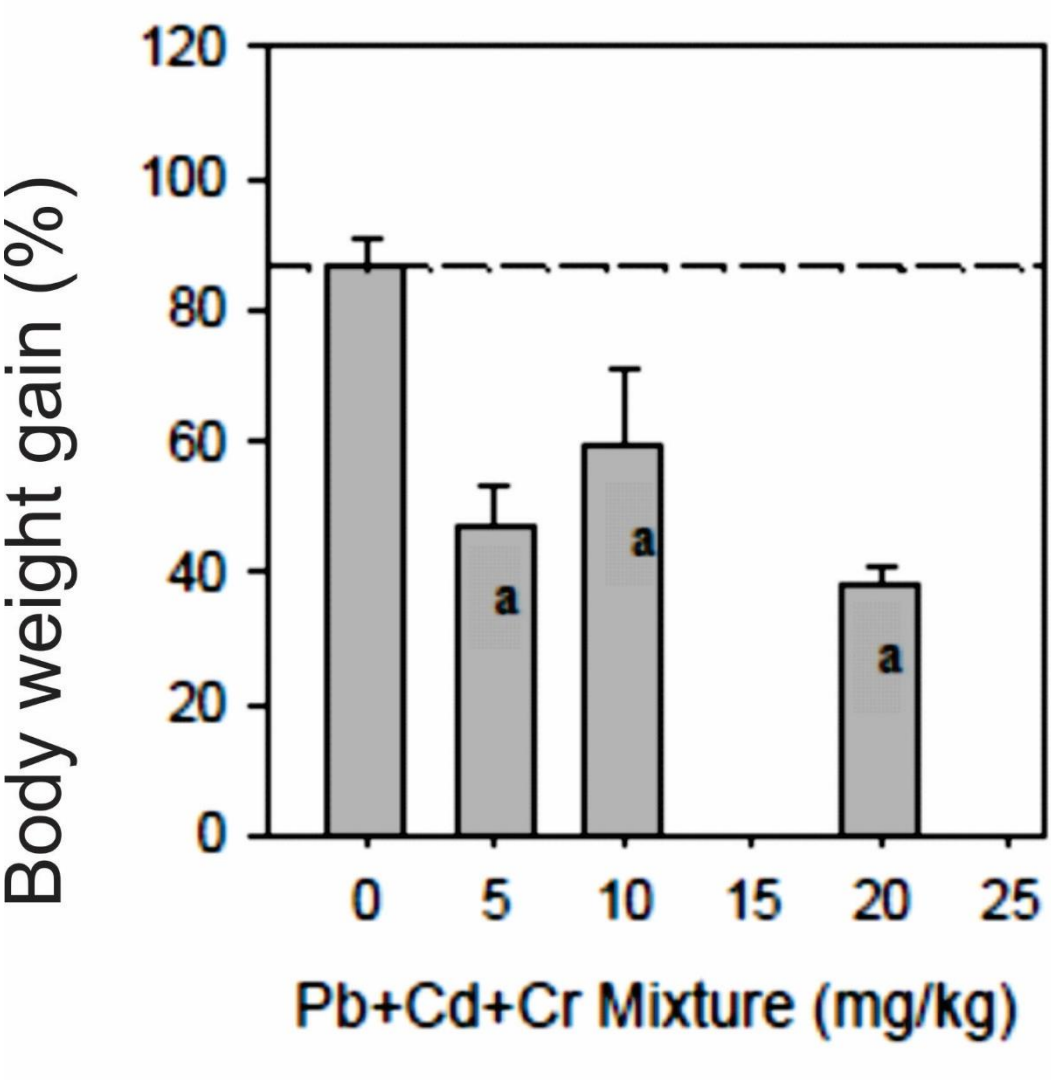


Figure 4.4: Dose-effect relationship for percentage body weight gain in albino rats treated with Pb, Cd and Cr as a mixture. a = significantly different from control ($p<0.05$).

Figures 4.1-4.4 showed that in the 5mg/kg treatment dose, there was significant decrease ($p<0.05$) in percentage body weight (BW) gain in Cd, Cr individual and combined treatment groups compared with control but there was no significant difference ($p>0.05$) in percentage body weight (BW) gain in Pb individual treatment group compared with control. Also, there was significant difference ($p<0.05$) in percentage body weight (BW) gain in Cr, Pb individual treatment groups compared with the combined treatment group but there was no significant difference ($p>0.05$) in percentage body weight (BW) gain in Cd individual treatment group compared with the combined treatment group. Percentage body weight (BW) gain in the combined treatment group was lower than that of Cr, Pb treatment group but was not significantly different ($p>0.05$) from that of Cd that produced the highest effect in the individual treatment group.

Figures 4.1-4.4 also showed that in the 10mg/kg treatment dose, there was significant decrease ($p<0.05$) in percentage body weight (BW) gain in Cr individual and combined treatment groups compared with control but there was no significant difference ($p>0.05$) in percentage body weight (BW) gain in Cd, Pb individual treatment groups compared with control. Also, there was no significant difference ($p>0.05$) in percentage body weight (BW) gain in Cr individual treatment group compared with the combined treatment group**.**

Figures 4.1-4.4 showed that in the 20mg/kg treatment dose, there was significant decrease ($p<0.05$) in percentage body weight (BW) gain in Cd, Cr individual and combined treatment groups compared with control but there was no significant difference ($p>0.05$) in percentage body weight (BW) gain in Pb individual treatment group compared with control. Also, there was significant difference ($p<0.05$) in percentage body weight (BW) gain in Cr, Pb individual treatment groups compared with the combined treatment group but there was no significant

difference ($p>0.05$) in percentage body weight (BW) gain in Cd individual treatment group compared with the combined treatment group. Percentage body weight (BW) gain in the combined treatment group was lower than that of Cr, Pb treatment group but was not significantly different ($p>0.05$) from that of Cd that produced the highest effect in the individual treatment group.

Treatment with Pb produced no significant difference ($p>0.05$) in percentage body weight gain compared with control in the three treatment doses (Figure 4.1).

4.1.1.2 Mean liver weight to body weight (BW) ratioof albino rats treated with cadmium (Cd), chromium (Cr) and lead (Pb) individually and as a mixture

Figures 4.5-4.8 show the result of liver to body weight (BW) ratio of albino rats treated with cadmium (Cd), chromium (Cr) and lead (Pb) individually and as a mixture.

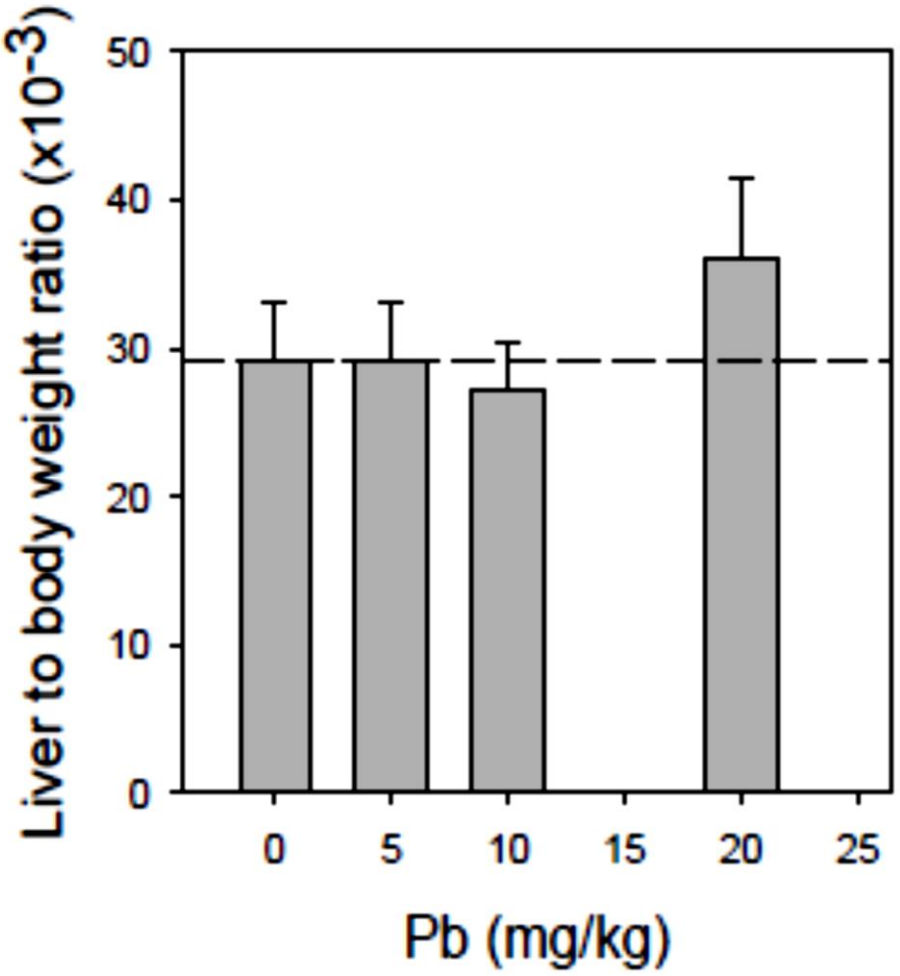


Figure 4.5: Dose-effect relationship for liver to body weight ratio in albino rats treated with Pb.

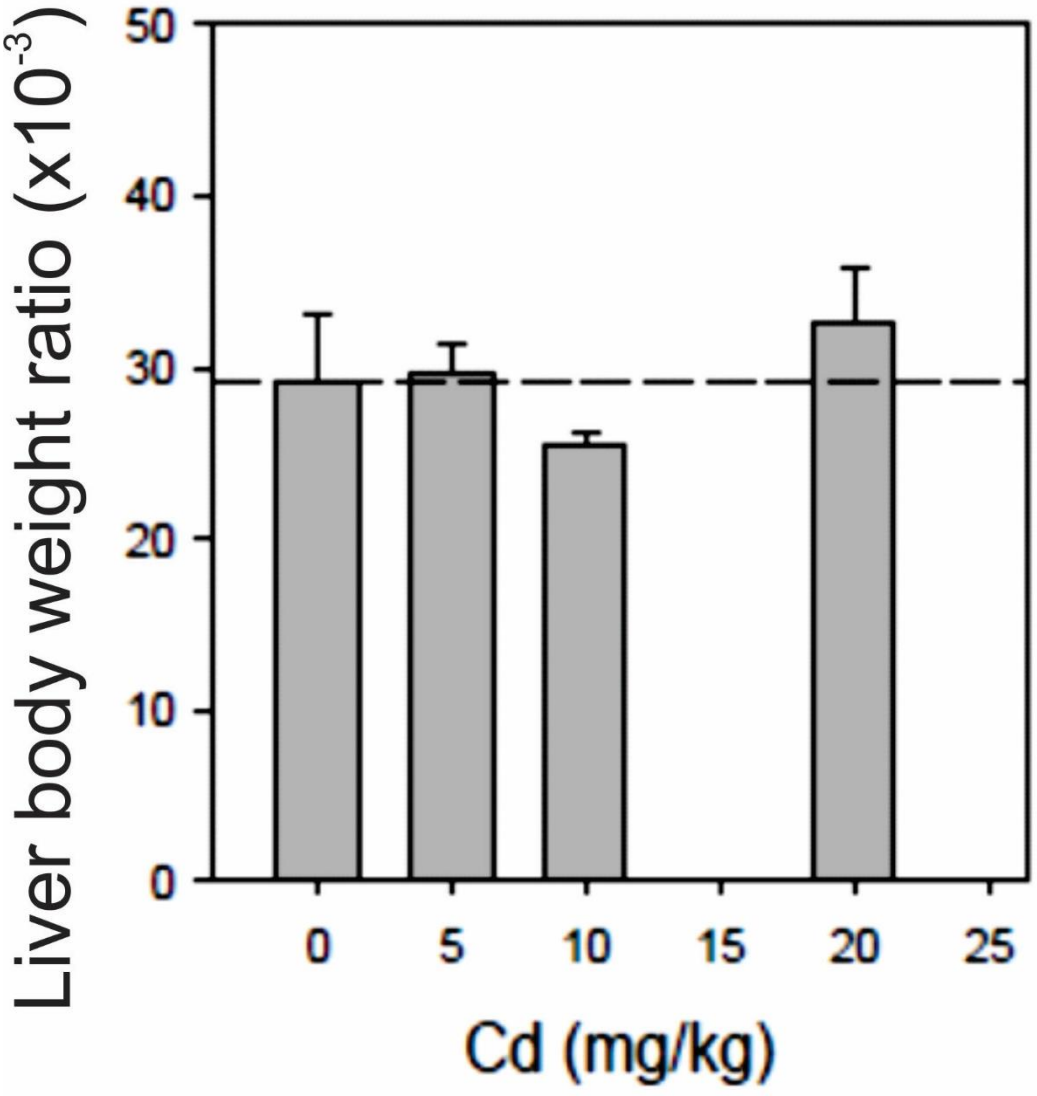


Figure 4.6: Dose-effect relationship for liver to body weight ratio in albino rats treated with Cd.

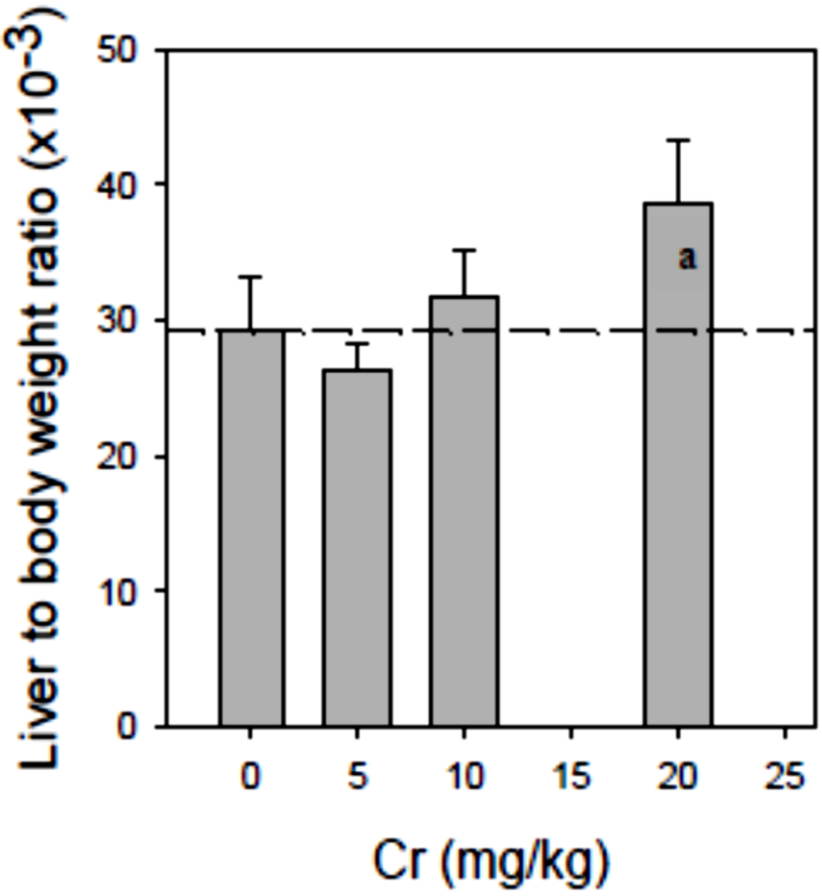


Figure 4.7: Dose-effect relationship for liver to body weight ratio in albino rats treated with Cr. a = significantly different from control ($p<0.05$).

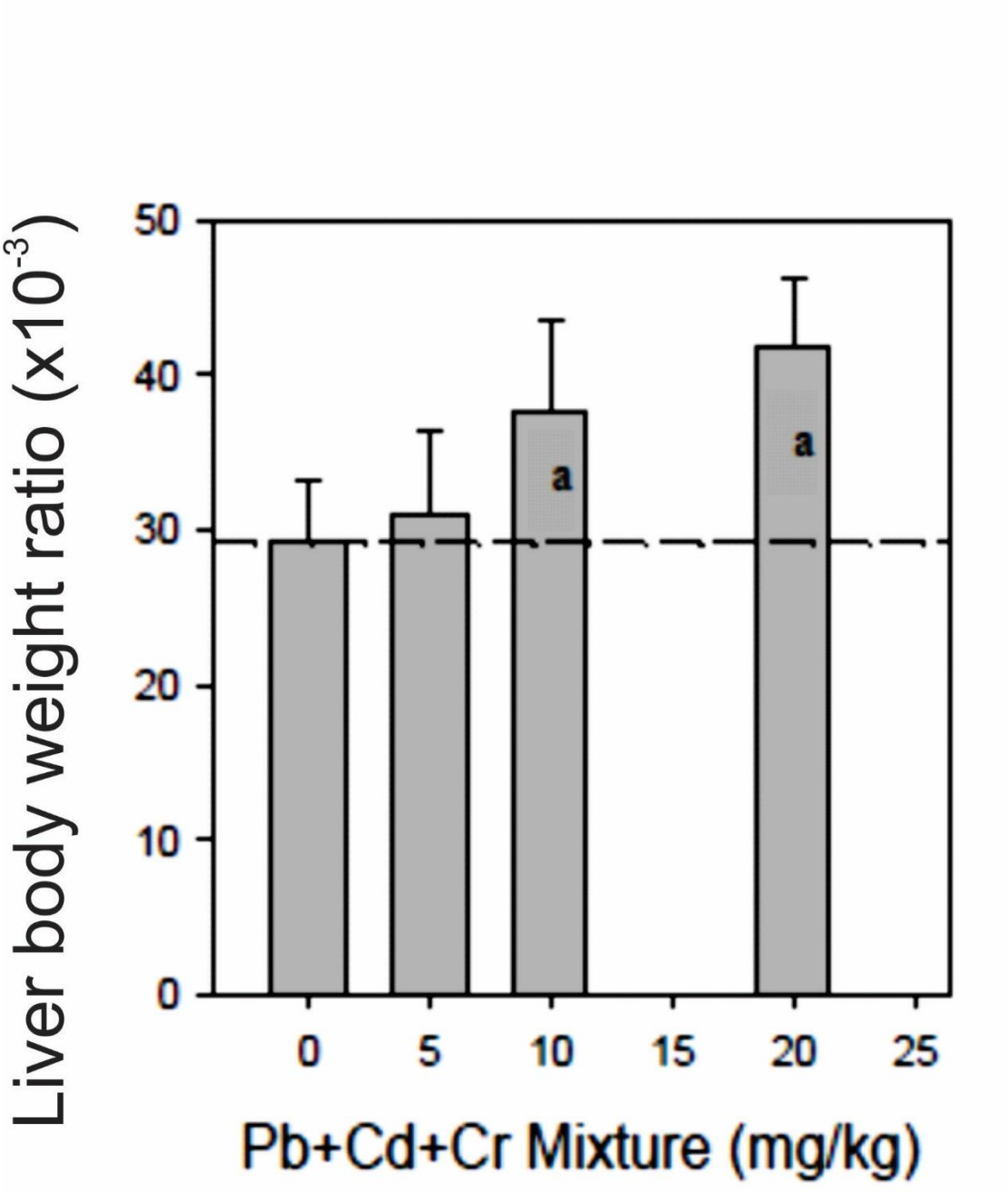


Figure 4.8: Dose-effect relationship for liver to body weight ratio in albino rats treated with Pb, Cd and Cr as a mixture. a = significantly different from control ($p<0.05$).

Figures 4.5-4.8 showed that in the 5mg/kg treatment dose, there was no significant difference ($p>0.05$) in liver to body weight (BW) ratio in Cd, Cr, Pb individual and combined treatment groups compared with control. Also, there was no significant difference ($p>0.05$) in liver to body weight (BW) ratio in Cd, Cr, Pb individual treatment groups compared with the combined treatment group.

Figures 4.5-4-8 also showed that in the 10mg/kg treatment dose, there was significant increase ($p<0.05$) in liver to body weight (BW) ratio in Cd, Cr, Pb combined treatment group compared with control but there was no significant difference ($p>0.05$) in liver to body weight (BW) ratio in Cd, Cr, Pb individual treatment groups compared with control.

Figures 4.5-4.8 showed that in the 20mg/kg treatment dose, there was significant increase ($p<0.05$) in liver to body weight (BW) ratio in Cr individual and combined treatment groups compared with control but there was no significant difference ($p>0.05$) in liver to body weight (BW) ratio in Cd, Pb individual treatment groups compared with control. Also, there was no significant difference ($p>0.05$) in liver to body weight (BW) ratio in Cr individual treatment group compared with the combined treatment group.

4.1.1.3 Mean kidney weight to body weight (BW) ratioof albino rats treated with cadmium (Cd), chromium (Cr) and lead (Pb) individually and as a mixture.

Figures 4.9-4.12 show the result of kidney to body weight (BW) ratio of albino rats treated with cadmium (Cd), chromium (Cr) and lead (Pb) individually and as a mixture.

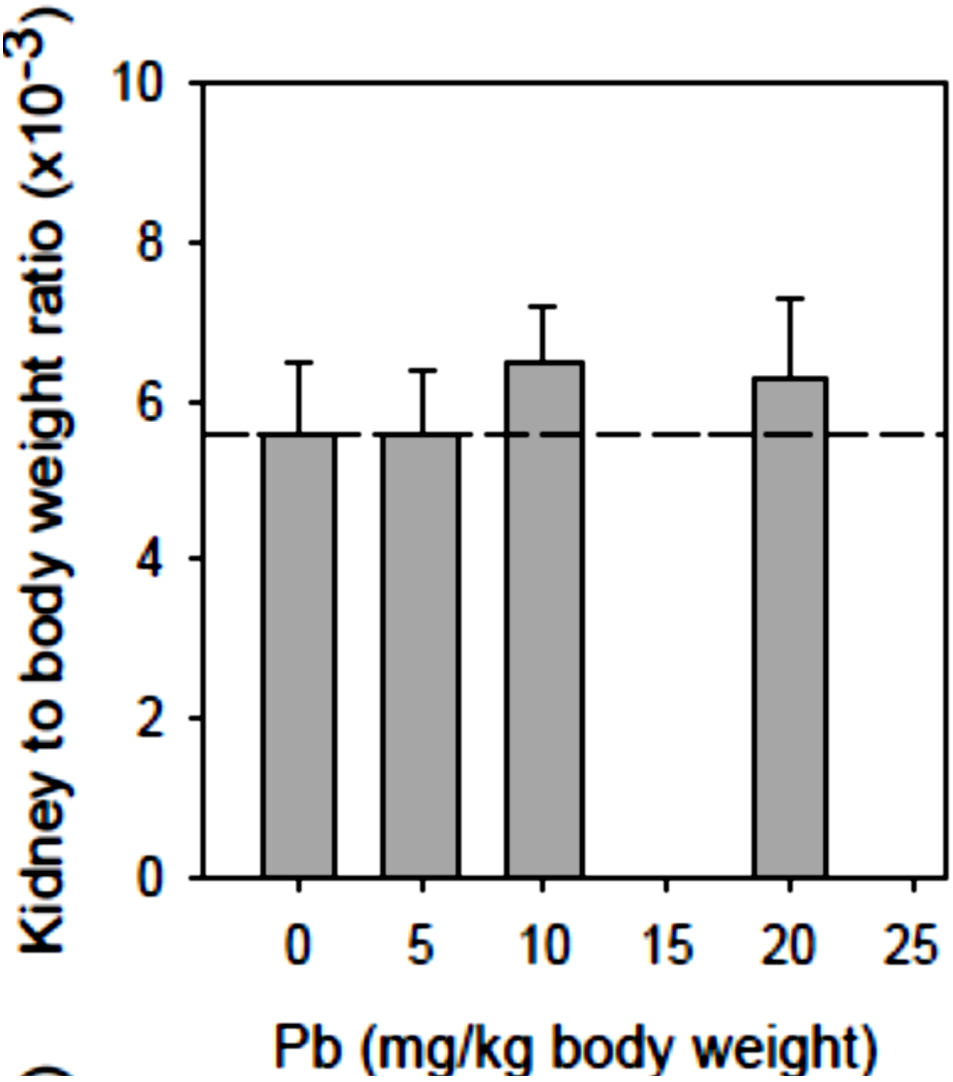


Figure 4.9: Dose-effect relationship for kidney to body weight ratio in albino rats treated with Pb.

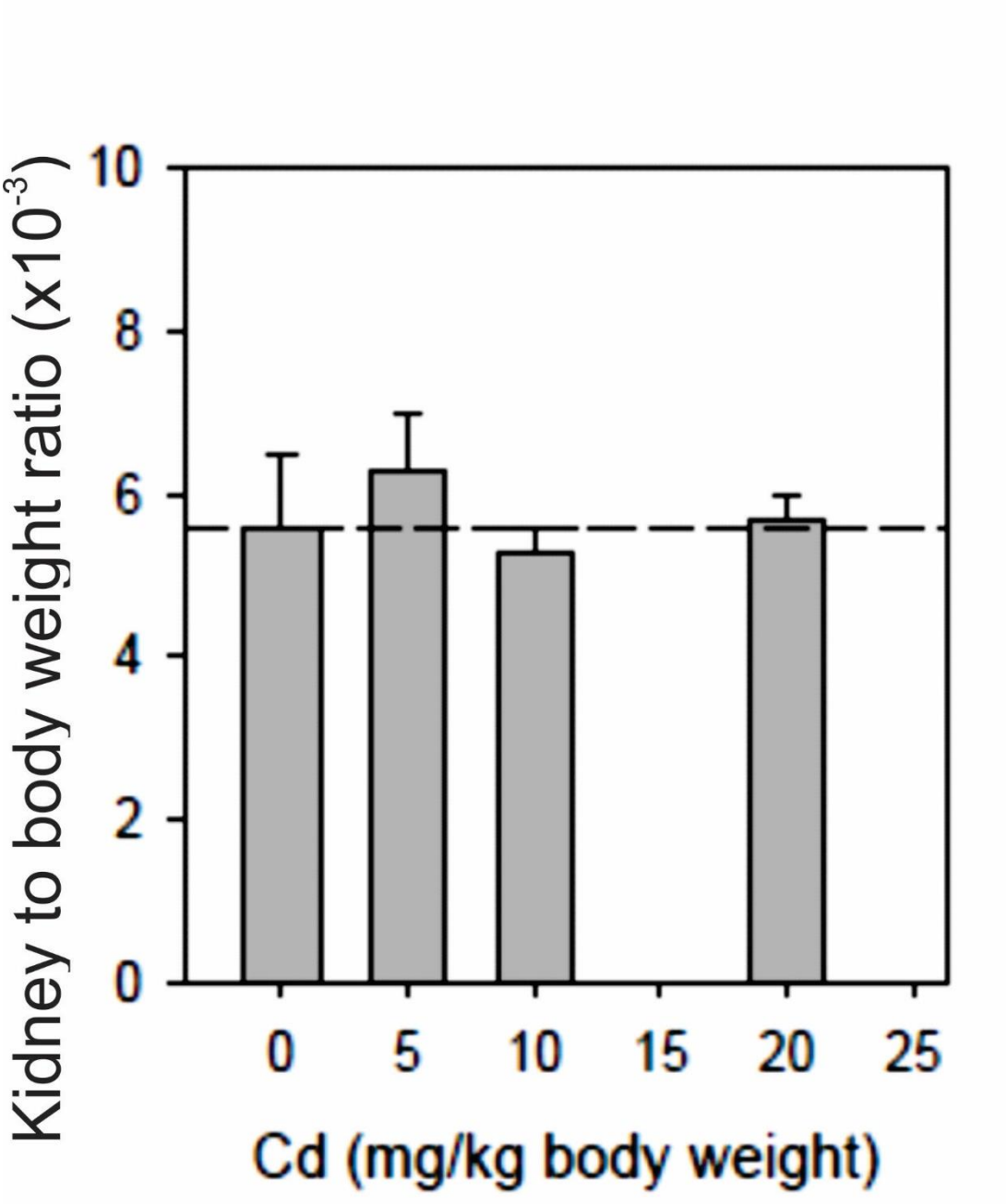


Figure 4.10: Dose-effect relationship for kidney to body weight ratio in albino rats treated with Cd.

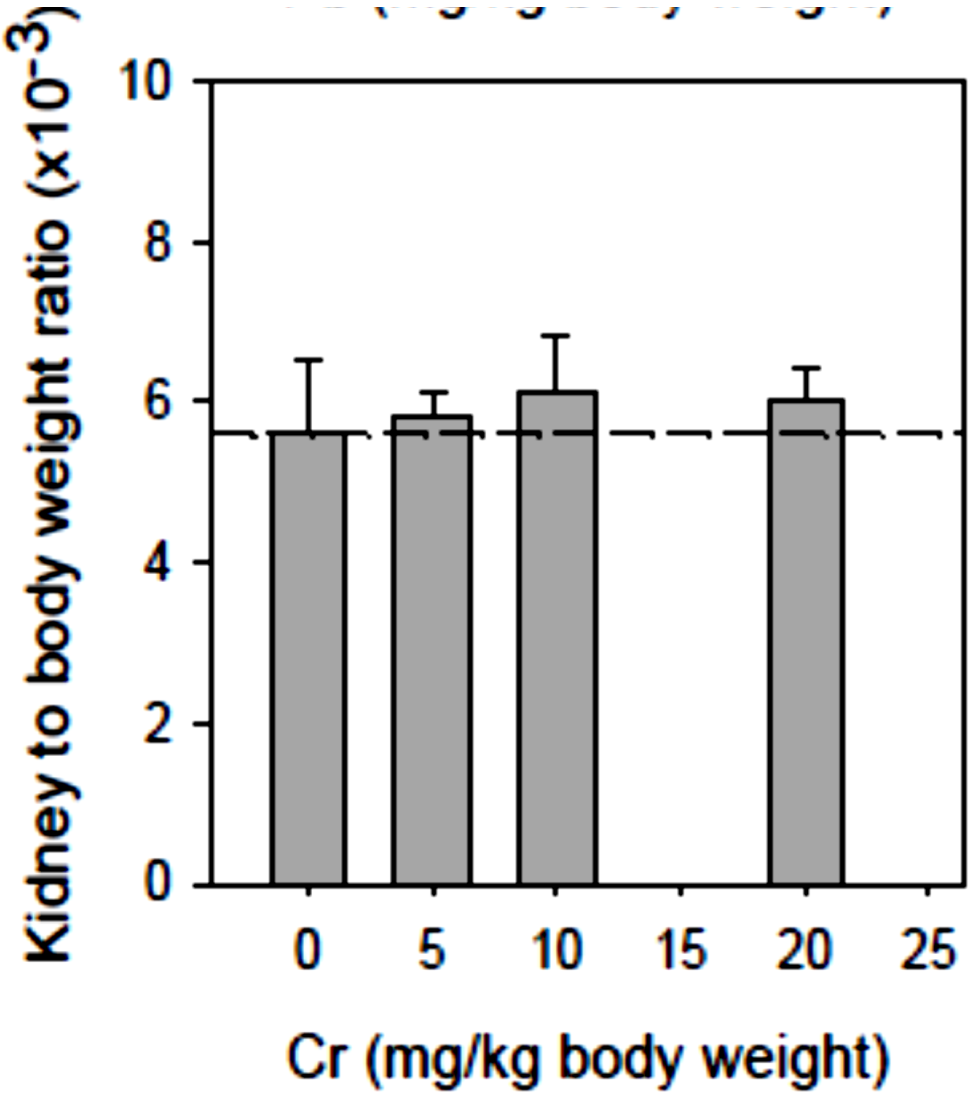


Figure 4.11: Dose-effect relationship for kidney to body weight ratio in albino rats treated with Cr.

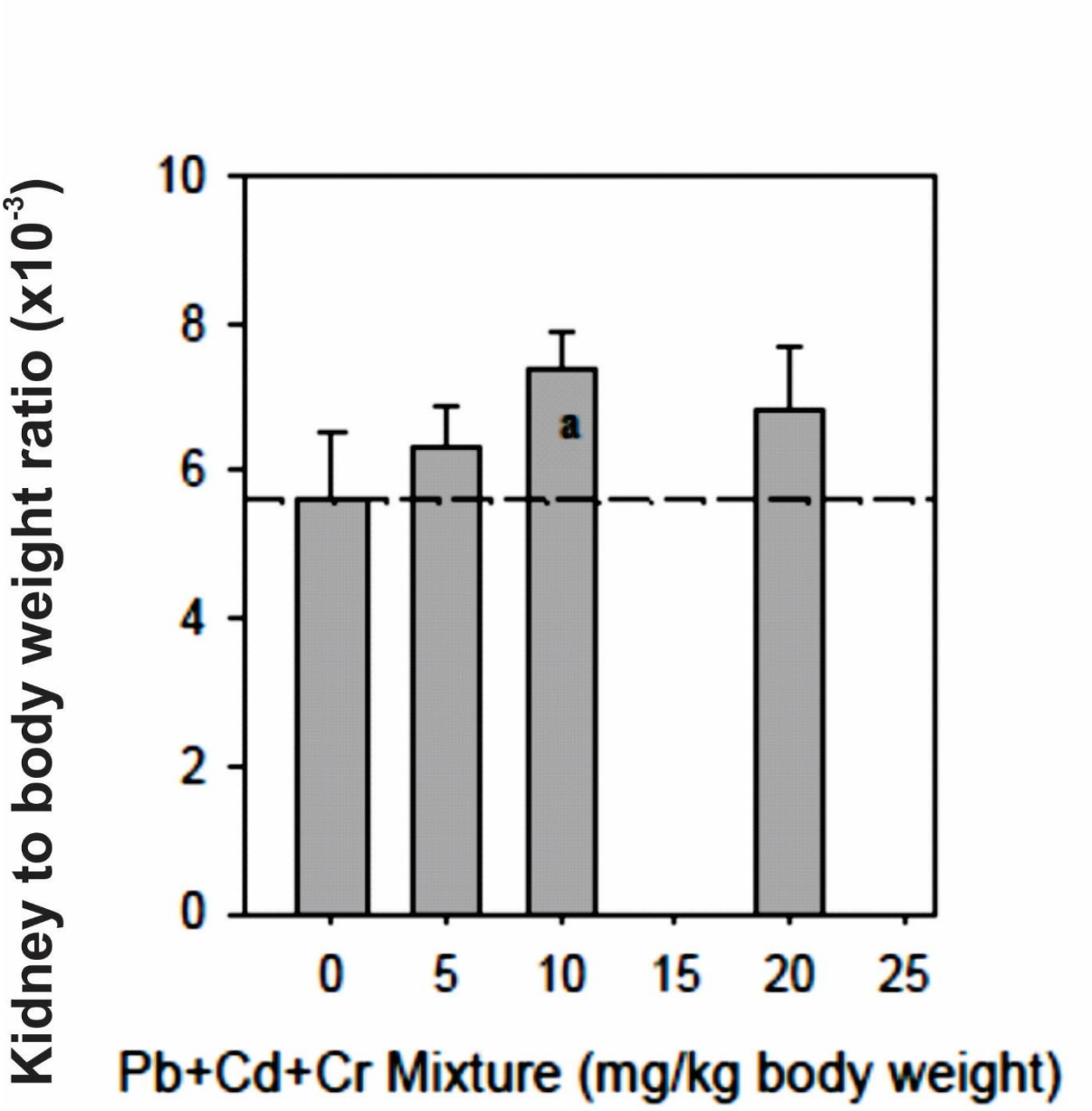


Figure 4.12: Dose-effect relationship for kidney to body weight ratio in albino rats treated with Pb, Cd and Cr and as a mixture. a = significantly different from control ($p<0.05$).

Figures 4.9-4.12 showed that in the 5mg/kg treatment dose, there was no significant difference ($p>0.05$) in kidney to body weight (BW) ratio in Cd, Cr, Pb individual and combined treatment groups compared with control. Also, there was no significant difference ($p>0.05$) in kidney to body weight (BW) ratio in Cd, Cr, Pb individual treatment groups compared with the combined treatment group.

Figures 4.9-4.12 also showed that in the 10mg/kg treatment dose, there was significant increase ($p<0.05$) in kidney to body weight (BW) ratio in Cd, Cr, Pb combined treatment group compared with control but there was no significant difference ($p>0.05$) in kidney to body weight (BW) ratio in Cd, Cr, Pb individual treatment groups compared with control.

Figure 4.9-4.12 showed that in the 20mg/kg treatment dose, there was no significant difference ($p>0.05$) in kidney to body weight (BW) ratio in Cd, Cr, Pb individual and combined treatment groups compared with control. Also, there was no significant difference ($p>0.05$) in kidney to body weight (BW) ratio in Cd, Cr, Pb individual treatment groups compared with the combined treatment group.

4.1.1.4 Mean heart to body weight (BW) ratioof albino rats treated with cadmium (Cd), chromium (Cr) and lead (Pb) individually and as a mixture

Figures 4.13-4.16 show the result of heart to body weight (BW) ratio of albino rats treated with cadmium (Cd), chromium (Cr) and lead (Pb)individually and as a mixture.

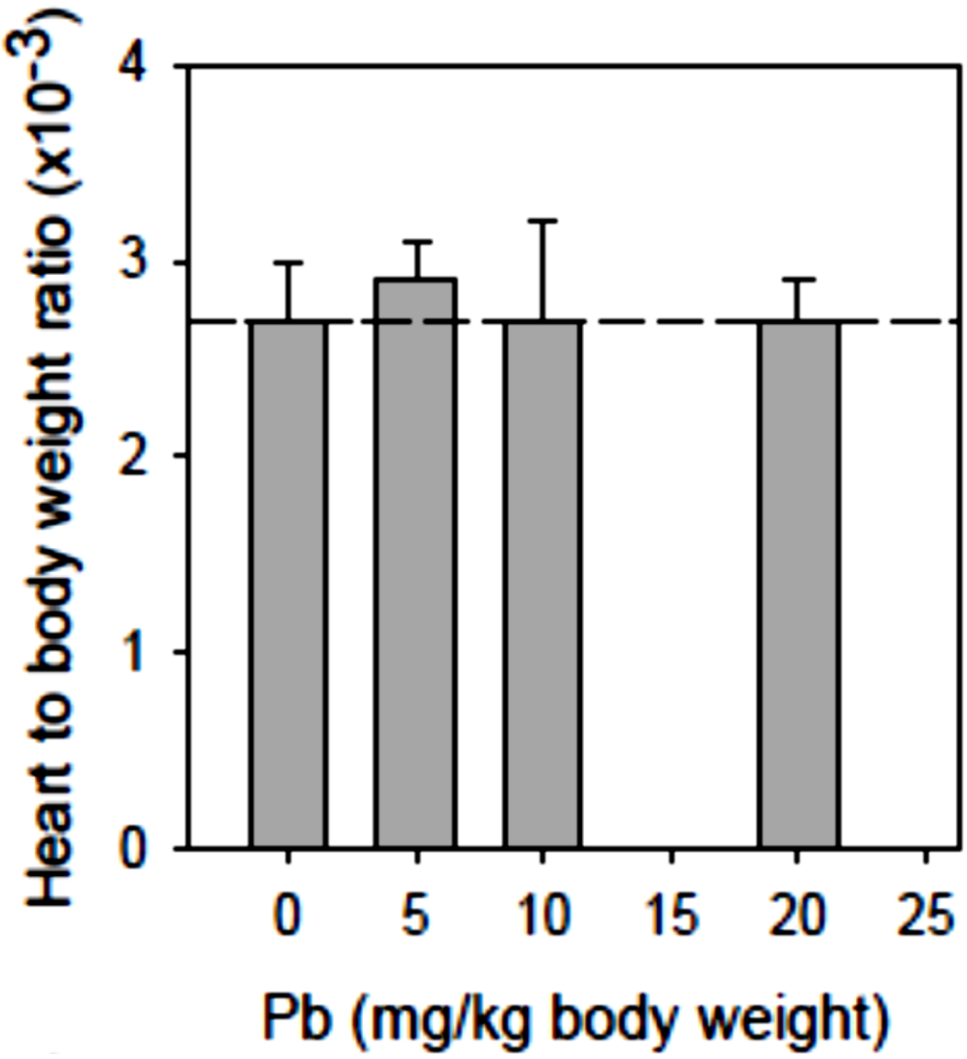


Figure 4.13: Dose-effect relationship for heart to body weight ratio in albino rats treated with Pb.

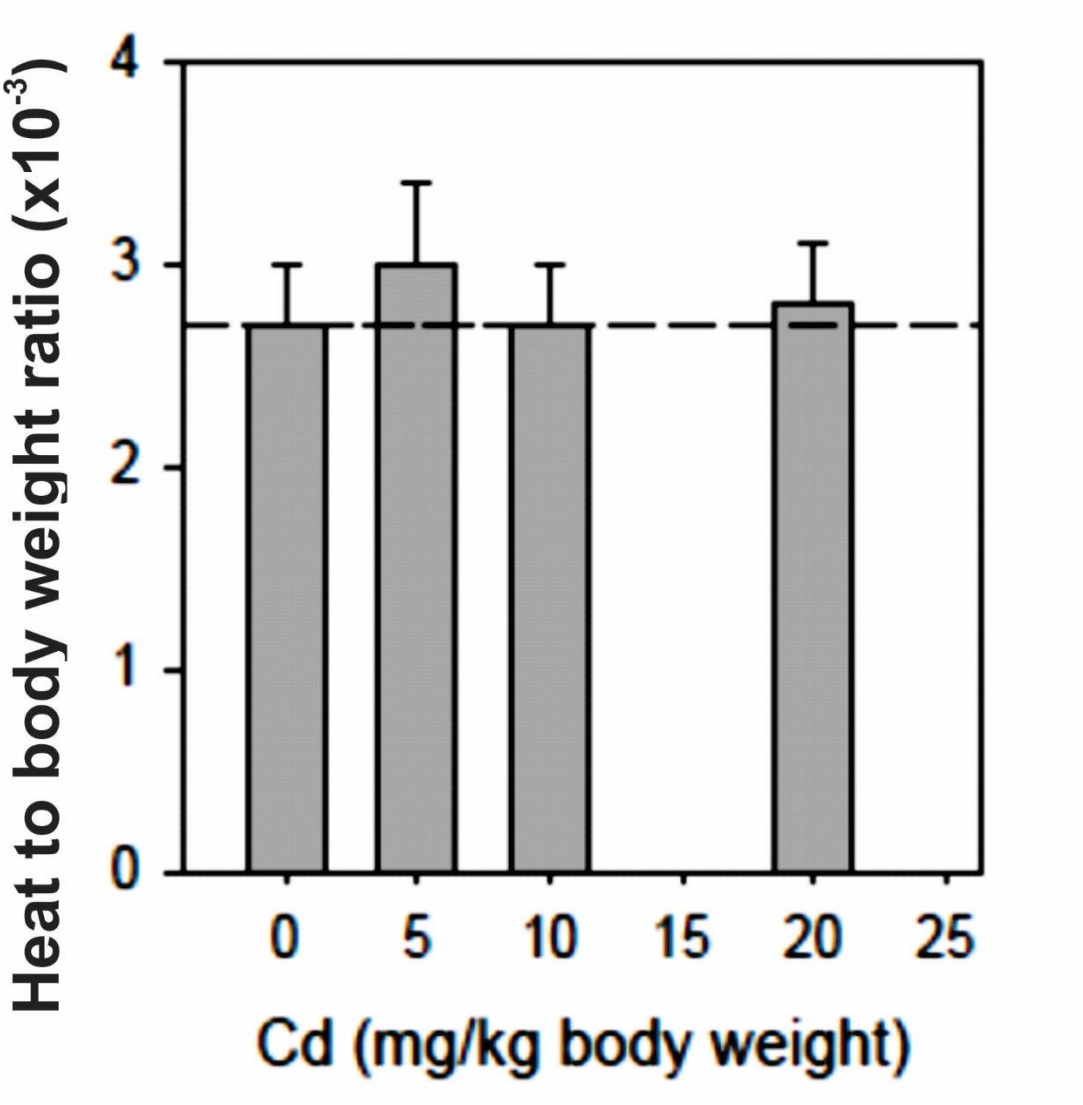


Figure 4.14: Dose-effect relationship for heart to body weight ratio in albino rats treated with Cd.

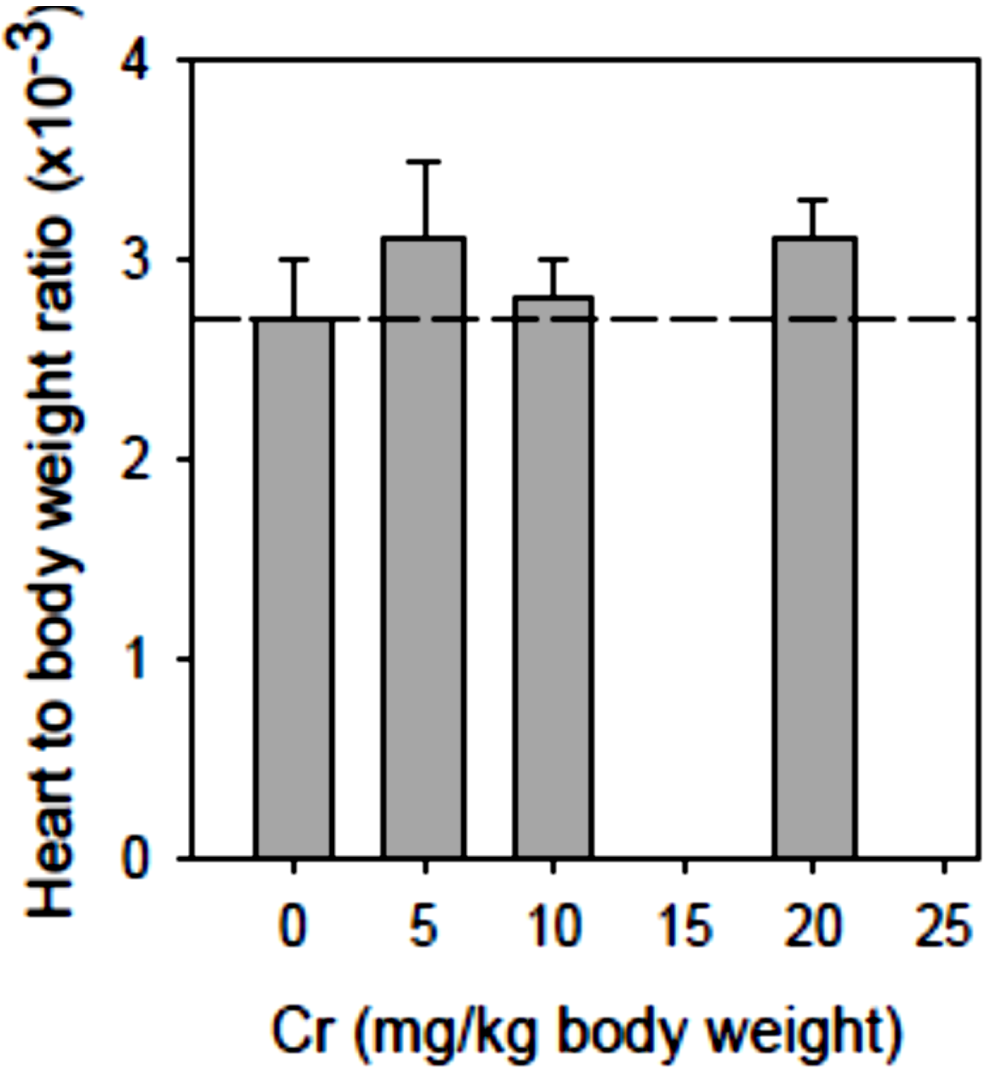


Figure 4.15: Dose-effect relationship for heart to body weight ratio in albino rats treated with Cr.

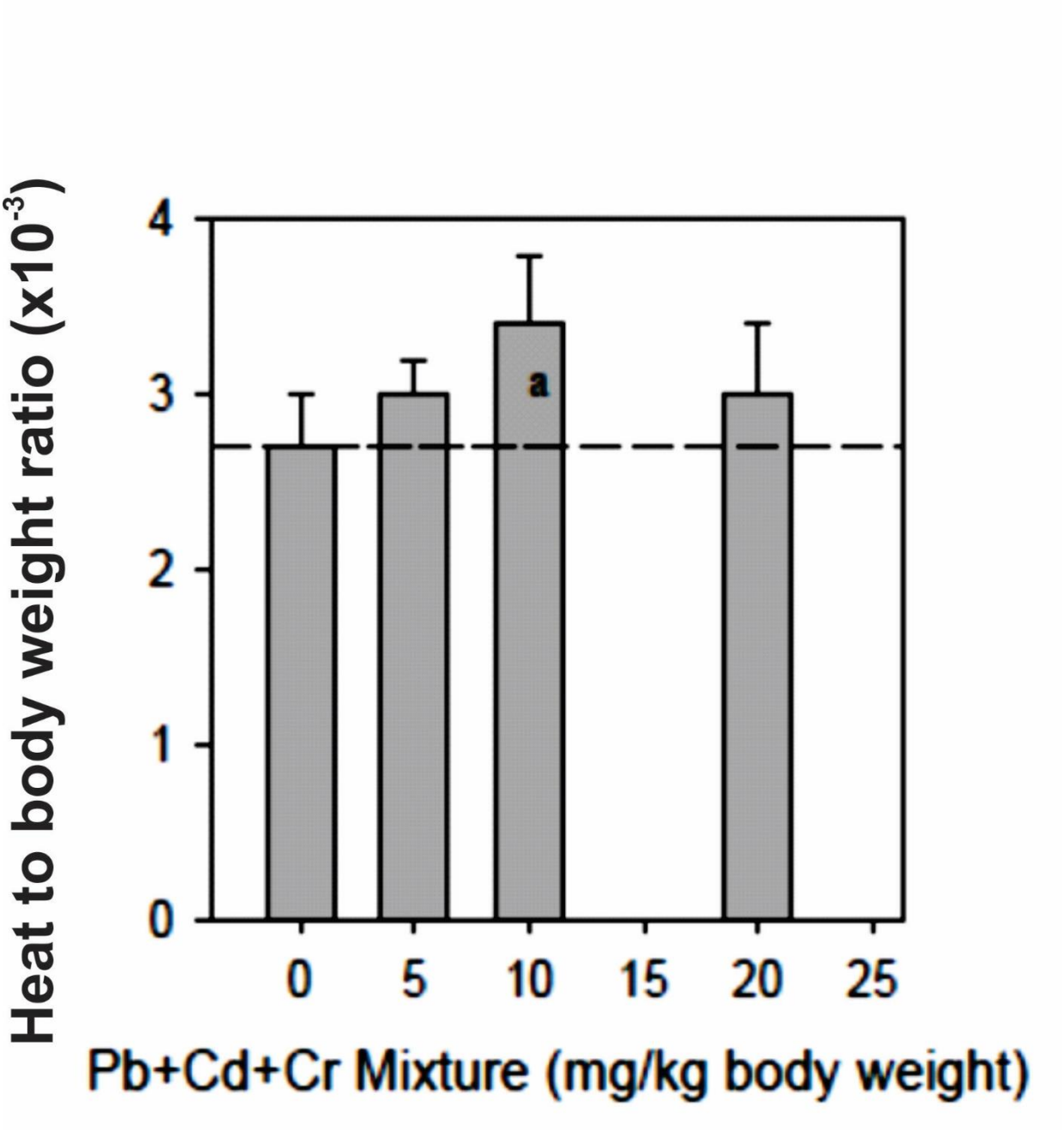


Figure 4.16: Dose-effect relationship for heart to body weight ratio in albino rats treated with Pb, Cd and Cr as a mixture. a = significantly different from control ($p<0.05$).

Figures 4.13-4.16 showed that in the 5mg/kg treatment dose, there was no significant difference ($p>0.05$) in heart to body weight (BW) ratio in Cd, Cr, Pb individual and combined treatment groups compared with control. Also, there was no significant difference ($p>0.05$) in heart to body weight (BW) ratio in Cd, Cr, Pb individual treatment groups compared with the combined treatment group.

Figures 4.13-4.16 also showed that in the 10mg/kg treatment dose, there was significant increase ($p<0.05$) in heart to body weight (BW) ratio in Cd, Cr, Pb combined treatment group compared with control but there was no significant difference ($p>0.05$) in heart to body weight (BW) ratio in Cd, Cr, Pb individual treatment groups compared with control.

Figures 4.13-4.16 showed that in the 20mg/kg treatment dose, there was no significant difference ($p>0.05$) in heart to body weight (BW) ratio in Cd, Cr, Pb individual and combined treatment groups compared with control. Also, there was no significant difference ($p>0.05$) in heart to body weight (BW) ratio in Cd, Cr, Pb individual treatment groups compared with the combined treatment group.

4.1.2 Liver function tests

4.1.2.1 Serum alanine aminotransferase (ALT) activities in albino rats treated with Pb, Cd and Cr individually and as a mixture

Figures 4.17-4.20 show dose-effect relationship for serum alanine aminotransferase (ALT) activity in albino rats treated with Pb, Cd and Cr individually and as a mixture.

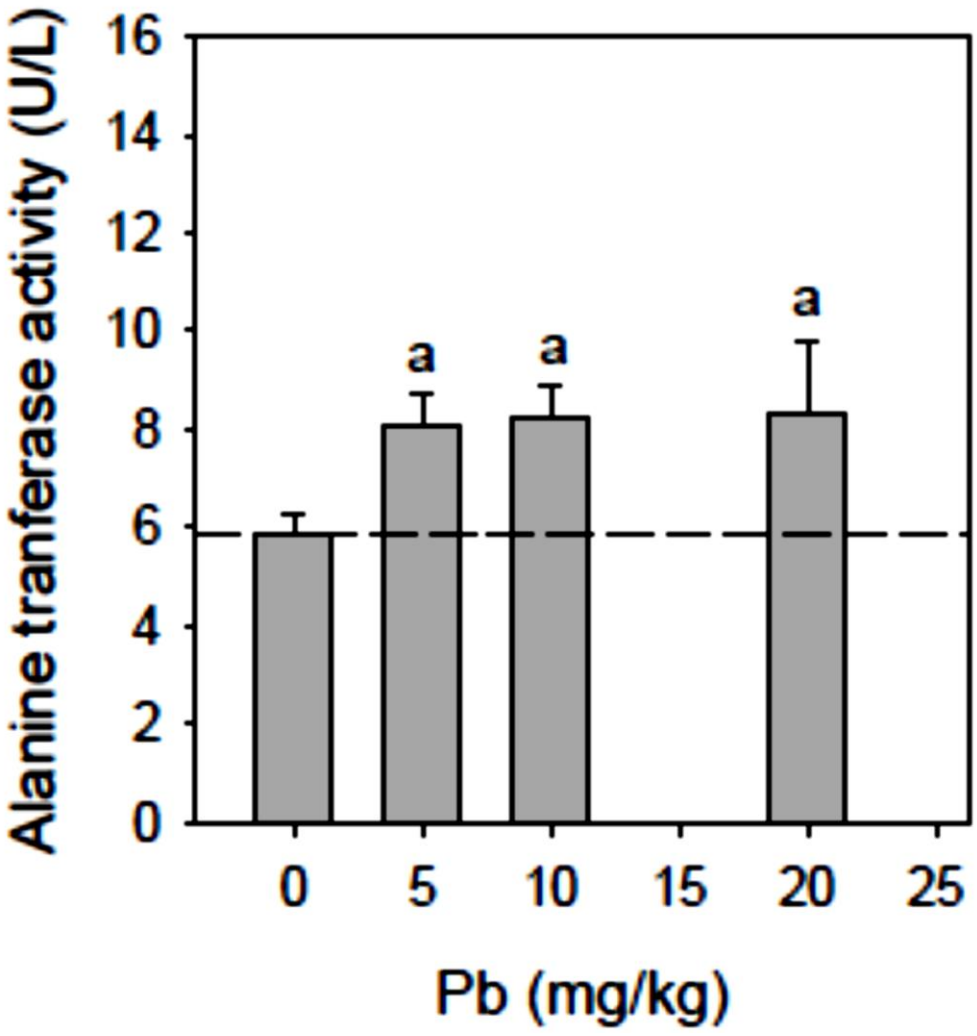


Figure 4.17: Dose-effect relationship for serum alanine aminotransferase (ALT) activity in albino rats treated with Pb. a = significantly different from control ($p<0.05$),

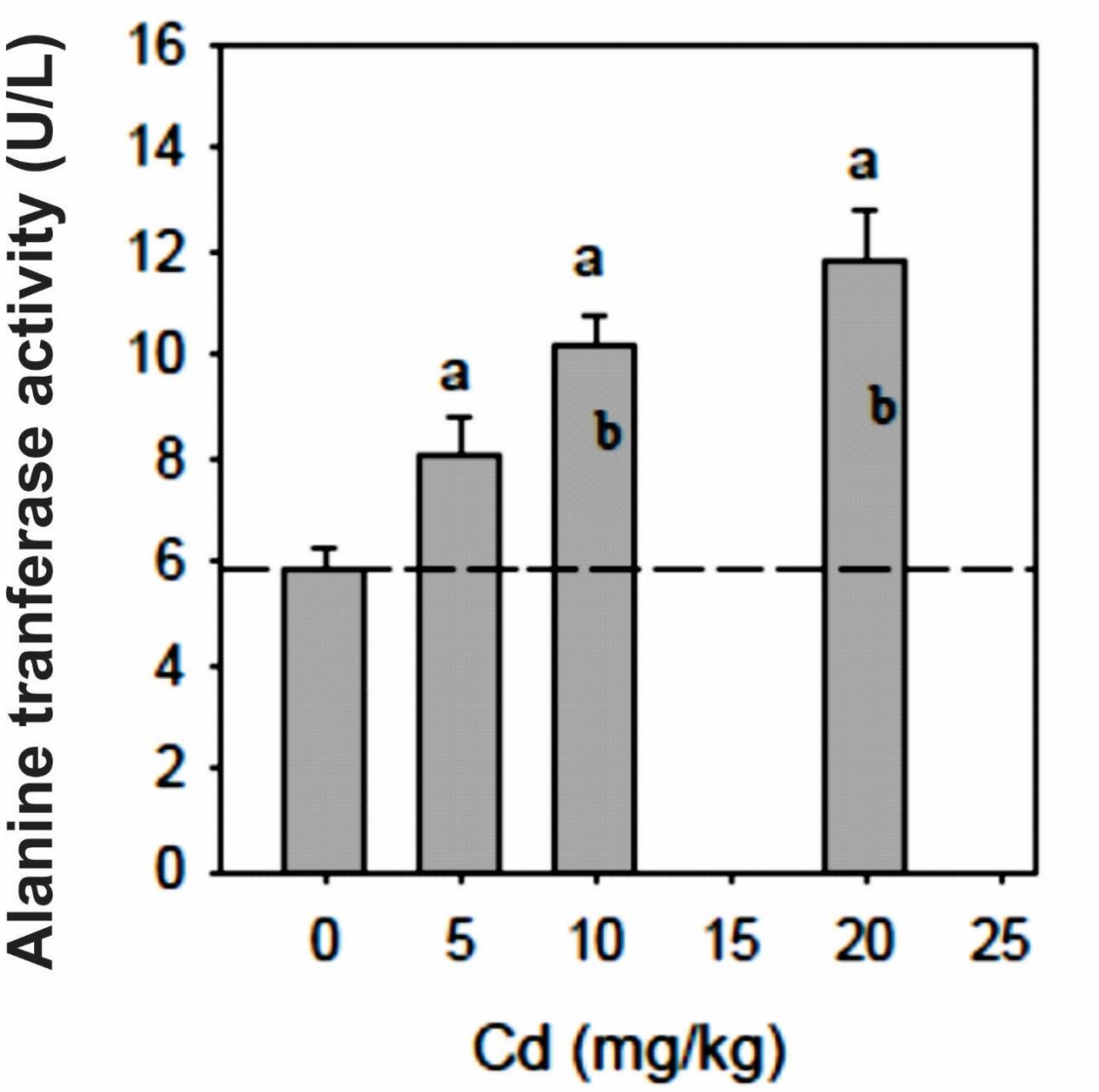


Figure 4.18: Dose-effect relationship for serum alanine aminotransferase (ALT) activity in albino rats treated with Cd. a = significantly different from control ($p<0.05$), b = significantly different from mixture ($p<0.05$).

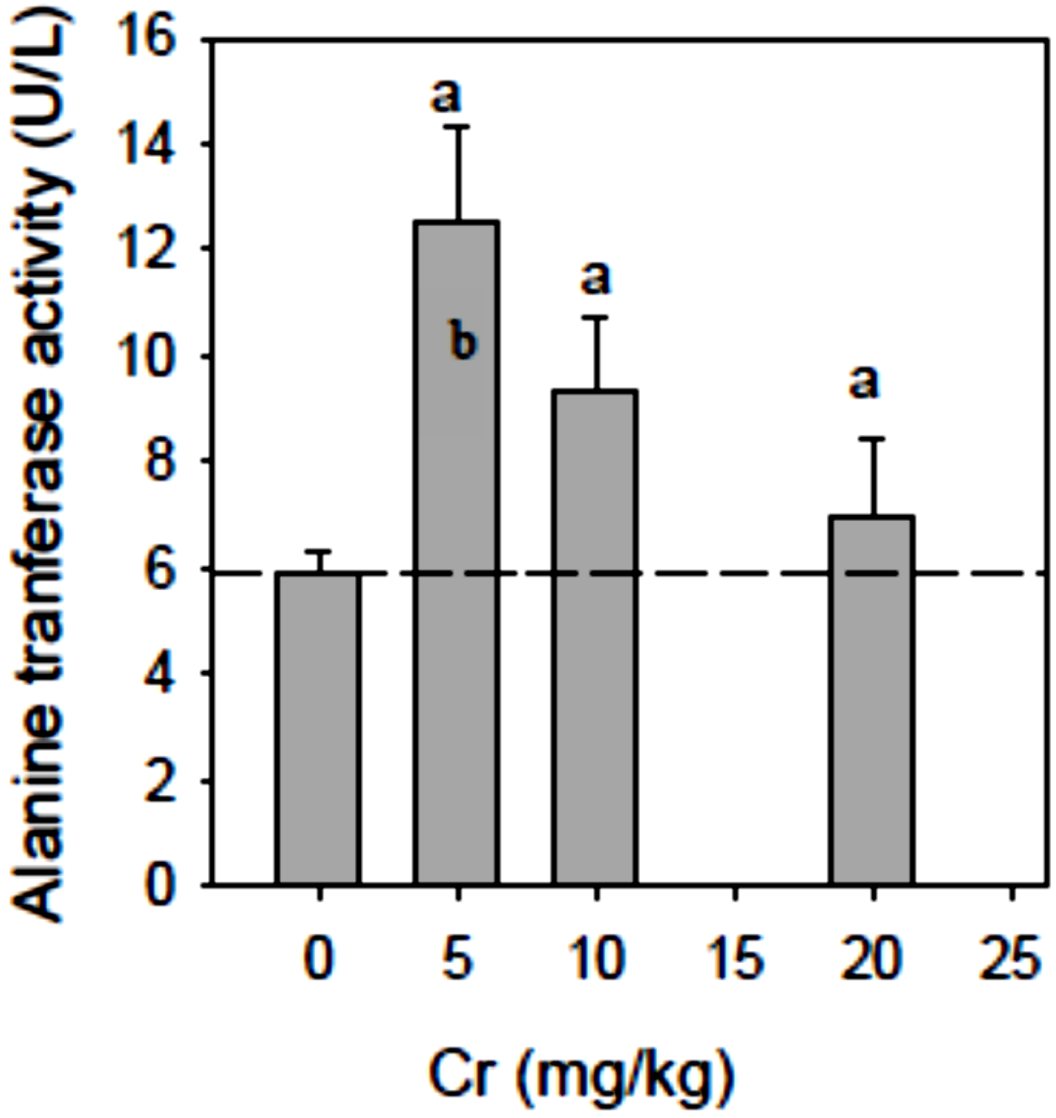


Figure 4.19: Dose-effect relationship for serum alanine aminotransferase (ALT) activity in albino rats treated with Cr. a = significantly different from control ($p<0.05$), b = significantly different from mixture ($p<0.05$).

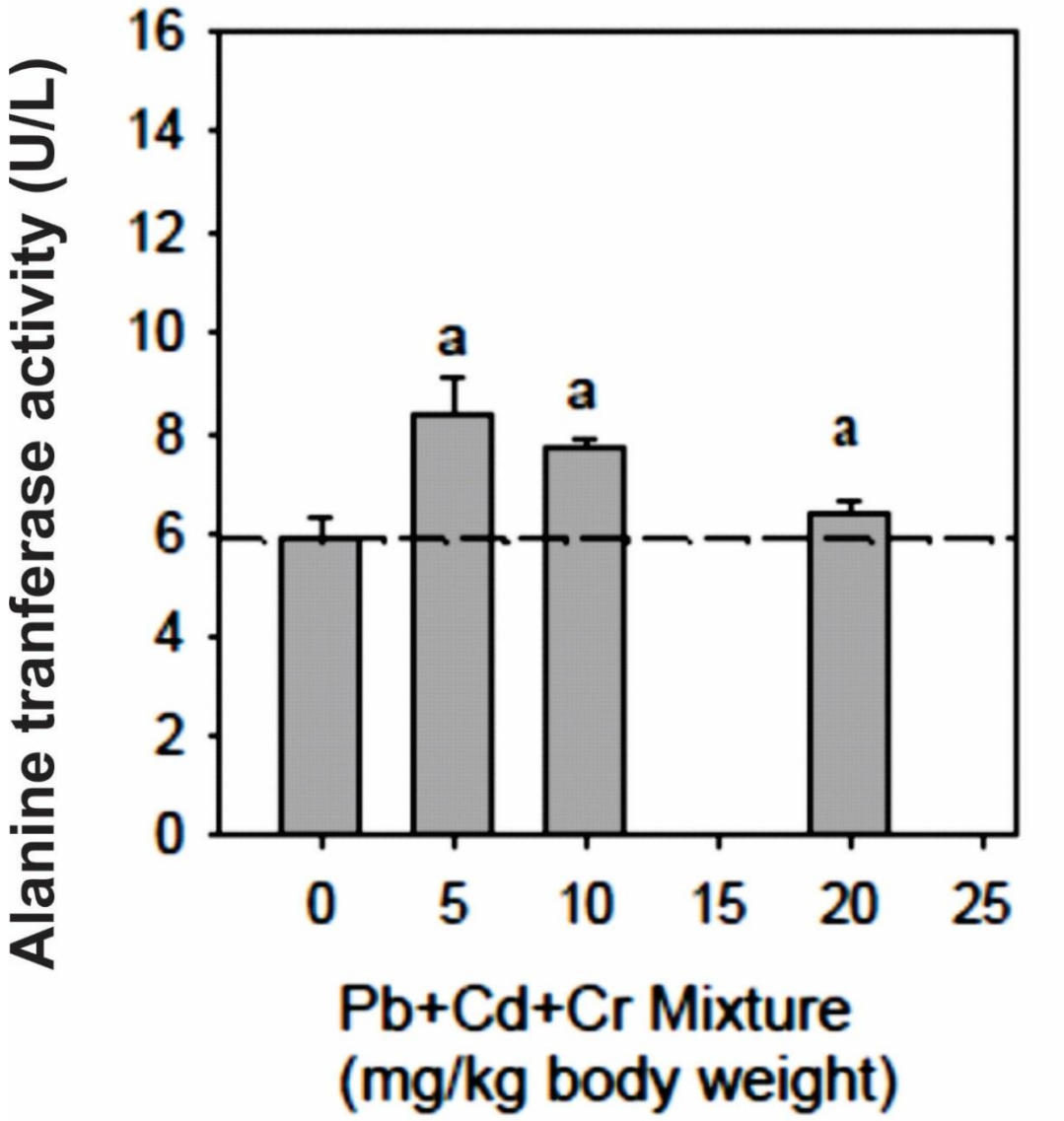


Figure 4.20: Dose-effect relationship for serum alanine aminotransferase (ALT) activity in albino rats treated with Pb, Cd and Cr as a mixture. a = significantly different from control ($p<0.05$).

Figuress 4.17-4.20 showed that in the 5mg/kg treatment dose, there was significant increase ($p<0.05$) in mean serum ALT activity in Cd, Cr, Pb individual and combined treatment groups compared with control. Also, there was significant difference ($p<0.05$) in mean serum ALT activity in Cr individual treatment group compared with the combined treatment group but there was no significant difference ($p>0.05$) in mean serum ALT activity in Cd, Pb individual treatment groups compared with the combined treatment group. Serum ALT activity in the combined treatment group was lower than that of Crtreatment group.

Figures 4.17-4.20 also showed that in the 10mg/kg treatment dose, there was significant increase ($p<0.05$) in mean serum ALT activity in Cd, Cr, Pb individual and combined treatment groups compared with control. Also, there was significant difference ($p<0.05$) in mean serum ALT activity in Cd individual treatment group compared with the combined treatment group but there was no significant difference ($p>0.05$) in mean serum ALT activity in Cr, Pb individual treatment groups compared with the combined treatment group. Serum ALT activity in the combined treatment group was lower than that of Cdtreatment group.

Figures 4.17-4.20 showed that in the 20mg/kg treatment dose, there was significant increase ($p<0.05$) in mean serum ALT activity in Cd, Cr, Pb individual and combined treatment groups compared with control. Also, there was significant difference ($p<0.05$) in mean serum ALT activity in Cd individual treatment group compared with the combined treatment group but there was no significant difference ($p>0.05$) in mean serum ALT activity in Cr, Pb individual treatment groups compared with the combined treatment group. Serum ALT activity in the combined treatment group was lower than that of Cdtreatment group.

In the Cr and combined treatment groups, an inverted U-shaped hormetic effect was observed with regard to serum ALT activity as treatment with the low dose (5mg/kg) produced a stimulating effect which was reduced at the high dose (20mg/kg). Treatment with Cd caused a dose dependent increase in mean serum ALT activity as the dose increased. Effect due Pb treatment was fairly constant as there was no significant difference ($p>0.05$) in mean serum ALT activity in the three treatment doses.

Table 4.1 Use of effect-addition in assessment/prediction of ALT activity in albino rats treated with Pb, Cd and Cr individually and as a mixture

Dose (C)	EC_{mix} (expt)	EC_{Pb}	EC_{Cd}	EC_{Cr}	EC_{mix}(pred) = [1- (1-EC_{pb}))(1-EC_{cd})(1-EC_{cr})]	Nature of interaction
5mg/kg	0.0894	0.0862	0.0862	0.1330	0.2767	Less than additive
10mg/kg	0.0819	0.0872	0.1085	0.0989	0.2667	Less than additive
20mg/kg	0.0681	0.0883	0.1255	0.0745	0.2621	Less than additive

$E(C_i)$ is effect expressed as fraction = (effect of substance i at dose C/maximum possible effect). $E(C_i)$ obtained with maximum possible ALT activity at 94U/L.

From Table 4.1, the use of effect-addition in assessment/prediction of ALT activity showed that the effect obtained from the experimented mixture was lower than the effect obtained by prediction using the addition of effect from the individual treatments, suggesting less than additive interaction of the mixture components in the three treatment doses with respect to ALT activity.

4.1.2.2 Serum aspartate aminotransferase (AST) activity

Figures 4.21-4.24 show dose-effect relationship for serumaspartate aminotransferase (AST) level in albino rats treated with Pb, Cd and Cr individually and as a mixture.

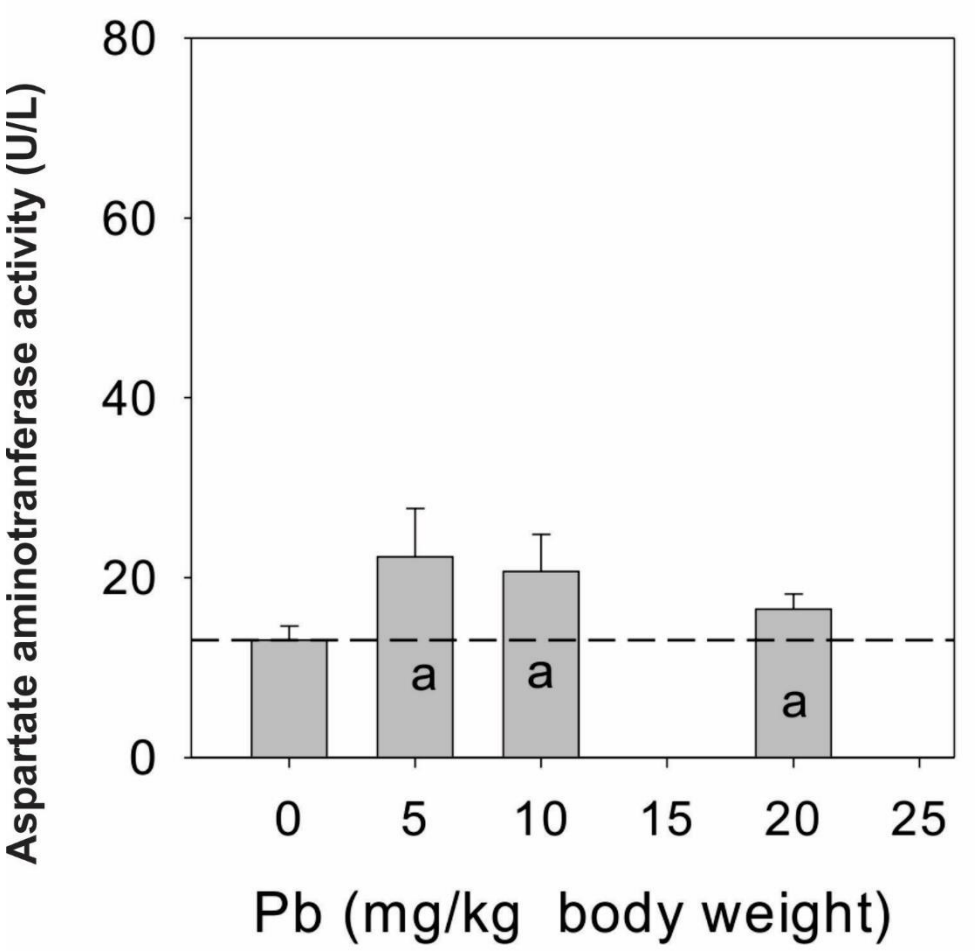


Figure 4.21: Dose-effect relationship for aspartate aminotransferase (AST) activity in albino rats treated with Pb. a = significantly different from control ($p<0.05$).

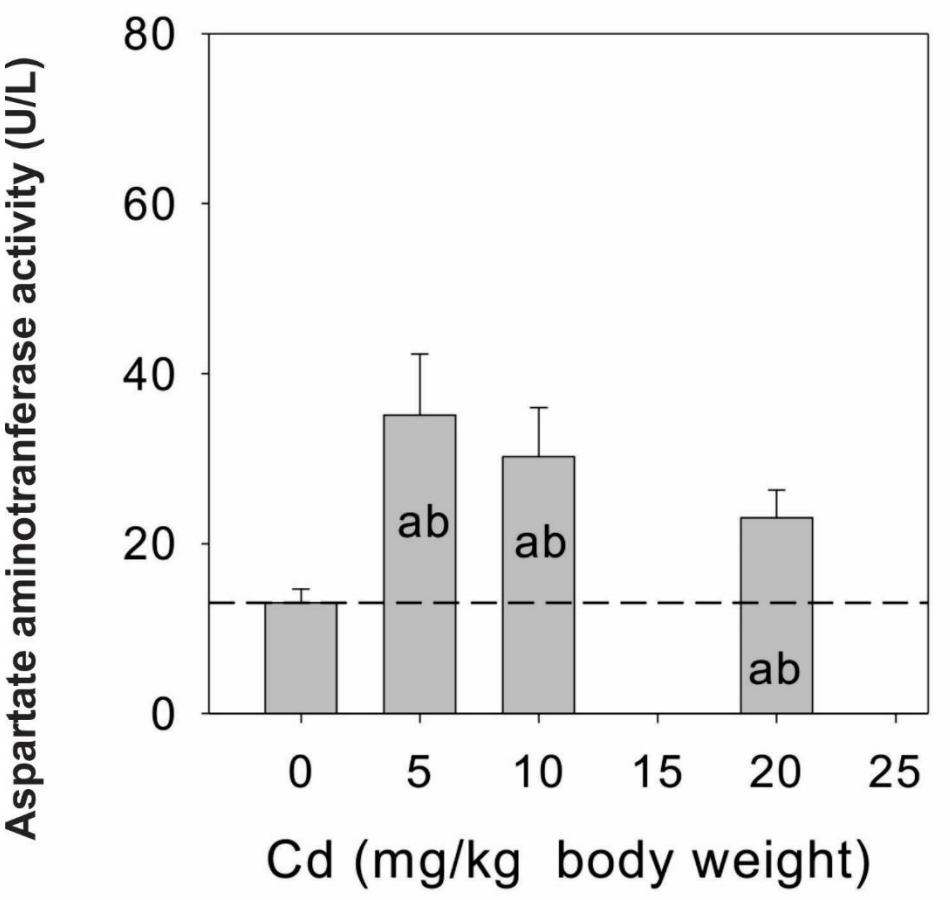


Figure 4.22: Dose-effect relationship for aspartate aminotransferase (AST) activity in albino rats treated with Cd. a = significantly different from control ($p<0.05$), b = significantly different from mixture ($p<0.05$).

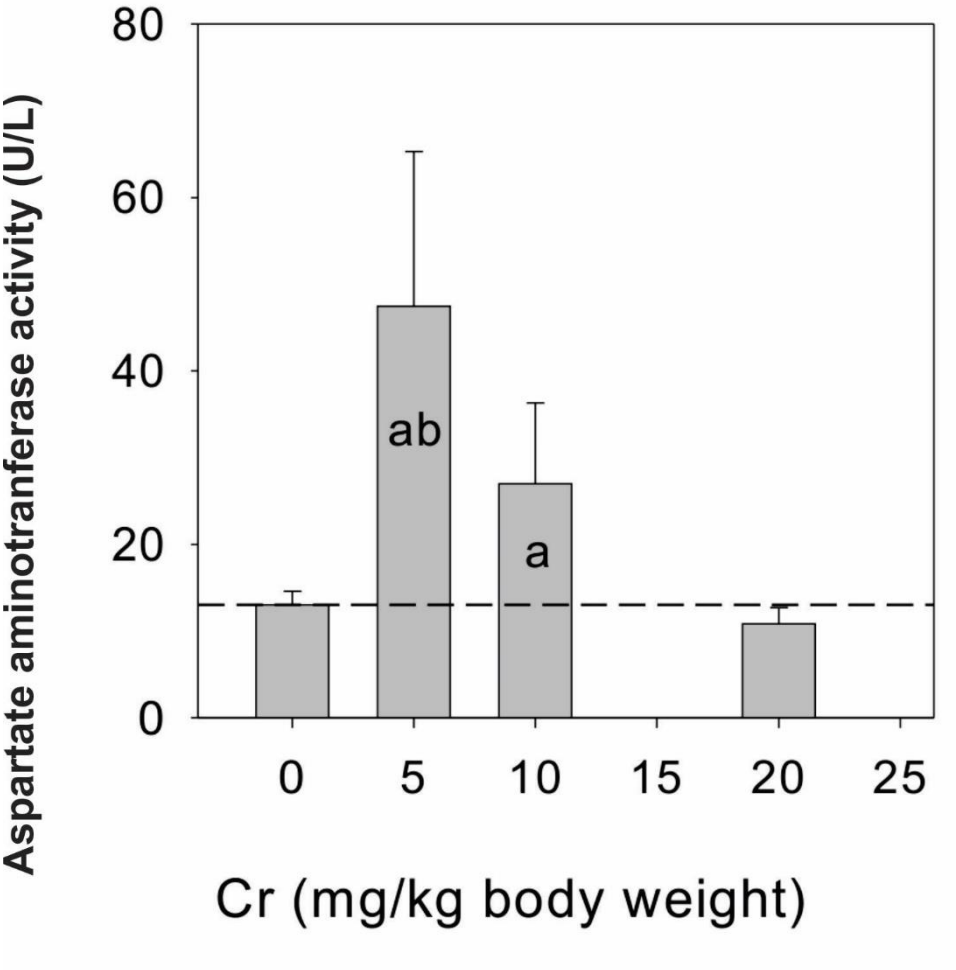


Figure 4.23: Dose-effect relationship for aspartate aminotransferase (AST) activity in albino rats treated with Cr. a = significantly different from control ($p<0.05$), b = significantly different from mixture ($p<0.05$).

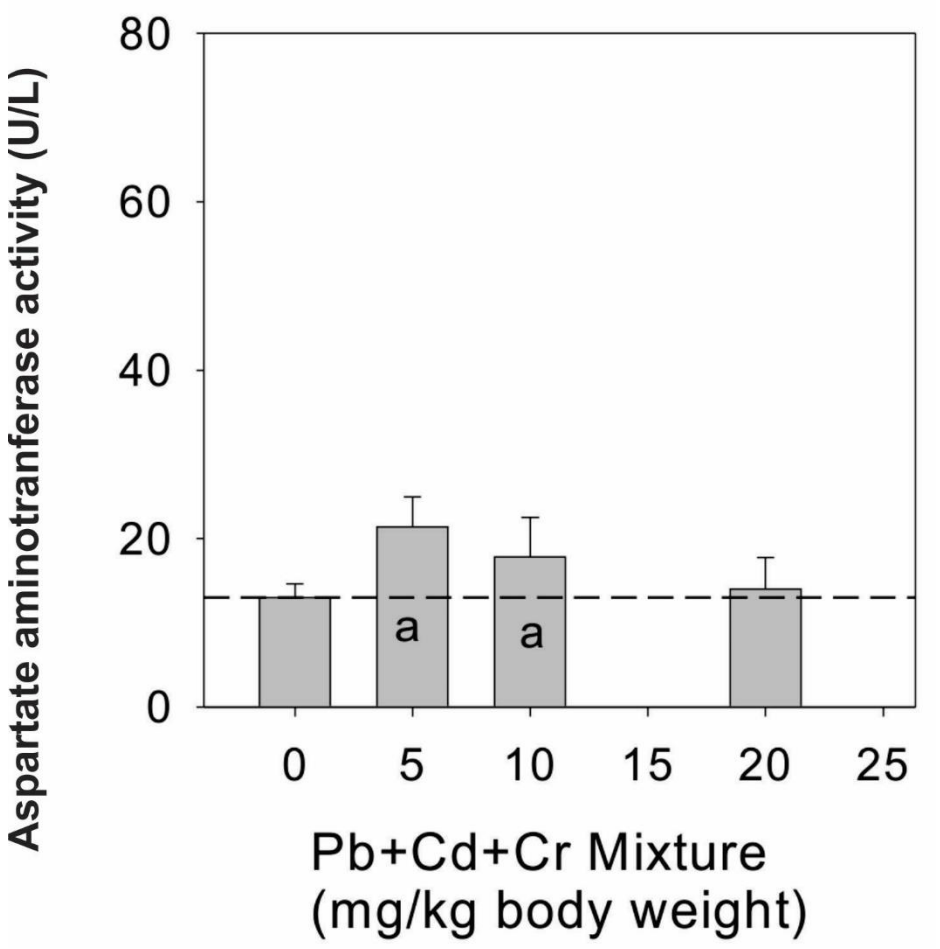


Figure 4.24: Dose-effect relationship for aspartate aminotransferase (AST) activity in albino rats treated with Pb, Cd and Cr as a mixture. a = significantly different from control ($p<0.05$).

Figures 4.21-4.24 showed that in the 5mg/kg treatment dose, there was significant increase ($p<0.05$) in mean serum AST activity in Cd, Cr, Pb individual and combined treatment groups compared with control.Also, there was significant difference ($p<0.05$) in mean serum AST activity in Cr, Cd individual treatment groups compared with the combined treatment group but there was no significant difference ($p>0.05$) in mean serum AST activity in Pb individual treatment group compared with the combined treatment group. Serum AST activity in the combined treatment group was lower than that of Cr, Cdtreatment groups.

Figures 4.21-4.24 also showed that in the 10mg/kg treatment dose,there was significant increase ($p<0.05$) in mean serum AST activity in Cd, Cr, Pb individual and combined treatment groups compared with control.Also, there was significant difference ($p<0.05$) in mean serum AST activity in Cd individual treatment group compared with the combined treatment group but there was no significant difference ($p>0.05$) in mean serum AST activity in Cr, Pb individual treatment groups compared with the combined treatment group. Serum AST activity in the combined treatment group was lower than that of Cdtreatment group.

Figures 4.21-4.24 showed that in the 20mg/kg treatment dose, there was significant increase ($p<0.05$) in mean serum AST activity in Cd, Pb individual treatment groups compared with control but there was no significant difference ($p>0.05$) in mean serum AST activity in Cr individual and combined treatment groups compared with control. Also, there was significant difference ($p<0.05$) in mean serum AST activity in Cd individual treatment group compared with the combined treatment group but there was no significant difference ($p>0.05$) in mean serum AST activity in Cr individual treatment group compared with the combined treatment group. Serum AST activity in the combined treatment group was lower than that of Cd treatment group.

In the Cd, Cr, Pb and combined treatment groups, an inverted U-shaped hormetic effect was observed with regard to serum AST activity as treatment with the low dose (5mg/kg) produced a stimulating effect which was reduced at the high dose (20mg/kg).

Table 4.2 Use of effect-addition in assessment/prediction of AST activityin albino rats treated with Pb, Cd and Cr individually and as a mixture

Dose (C)	EC_{mix} (expt)	EC_{Pb}	EC_{Cd}	EC_{Cr}	EC_{mix}(pred) = [1- (1-EC_{pb}))(1-EC_{cd})(1-EC_{cr})]	Nature of interaction
5mg/kg	0.2404	0.2506	0.3944	0.5326	0.7879	Less than additive
10mg/kg	0.2000	0.2326	0.3393	0.3034	0.6468	Less than additive
20mg/kg	0.1573	0.1854	0.2584	0.1213	0.4692	Less than additive

E(C_i) obtained with maximum possible AST activity at 89U/L.

From Table 4.2, the use of effect-addition in assessment/prediction of AST activity showed that the effect obtained from the experimented mixture was lower than the effect obtained by prediction using the addition of effect from the individual treatments, suggesting less than additive interaction of the mixture components in the three treatment doses with respect to AST activity.

4.1.2.3 Serum alkaline phosphatase (ALP) activity in albino rats treated with Pb, Cd and Cr individually and as a mixture

Figures 4.25-4.28 show dose-effect relationship forserum alkaline phosphatase (ALP) activity in albino rats treated with Pb, Cd and Cr individually and as a mixture.

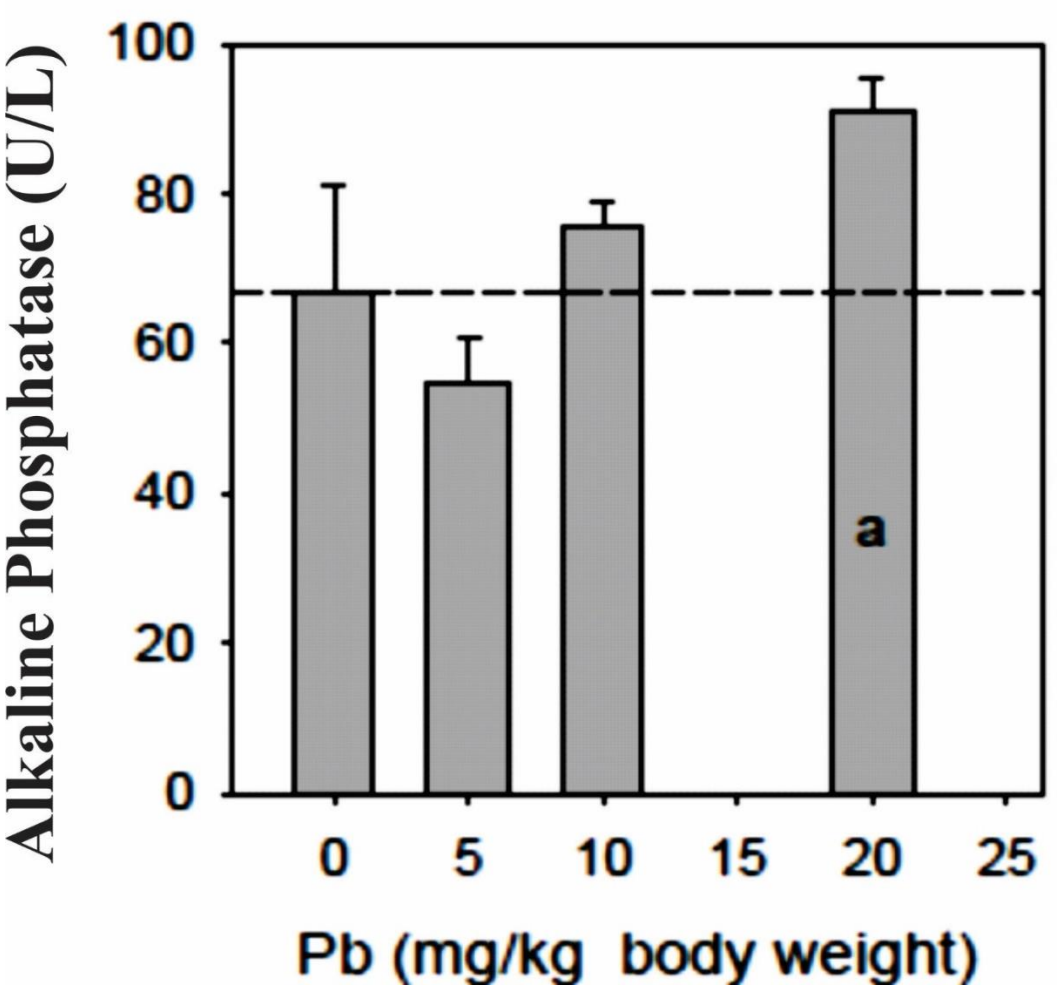


Figure 4.25: Dose-effect relationship for alkaline phosphatase (ALP) activity in albino rats treated with Pb. a = significantly different from control ($p<0.05$).

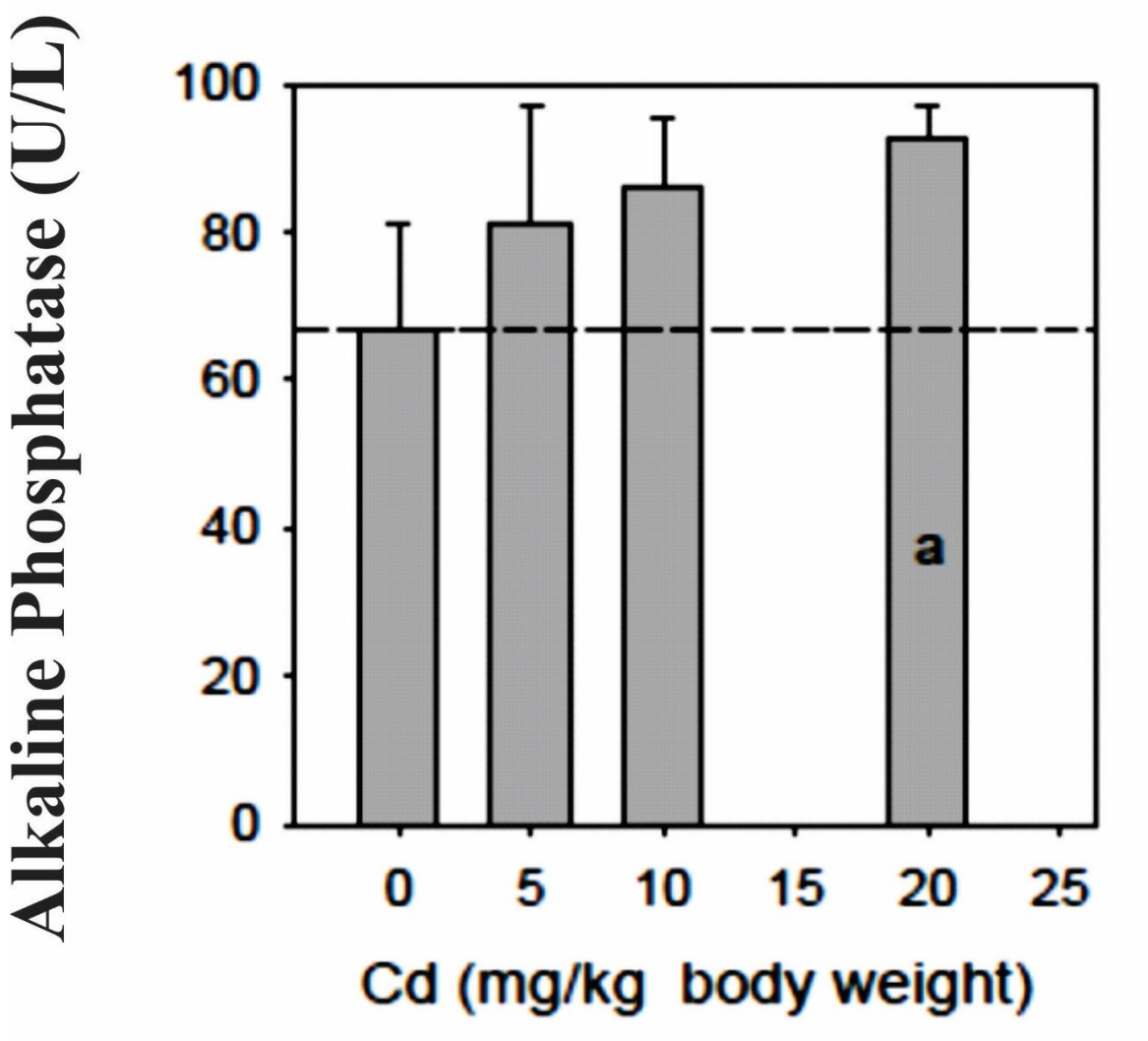


Figure 4.26: Dose-effect relationship for alkaline phosphatase (ALP) activity in albino rats treated with Cd. a = significantly different from control ($p<0.05$).

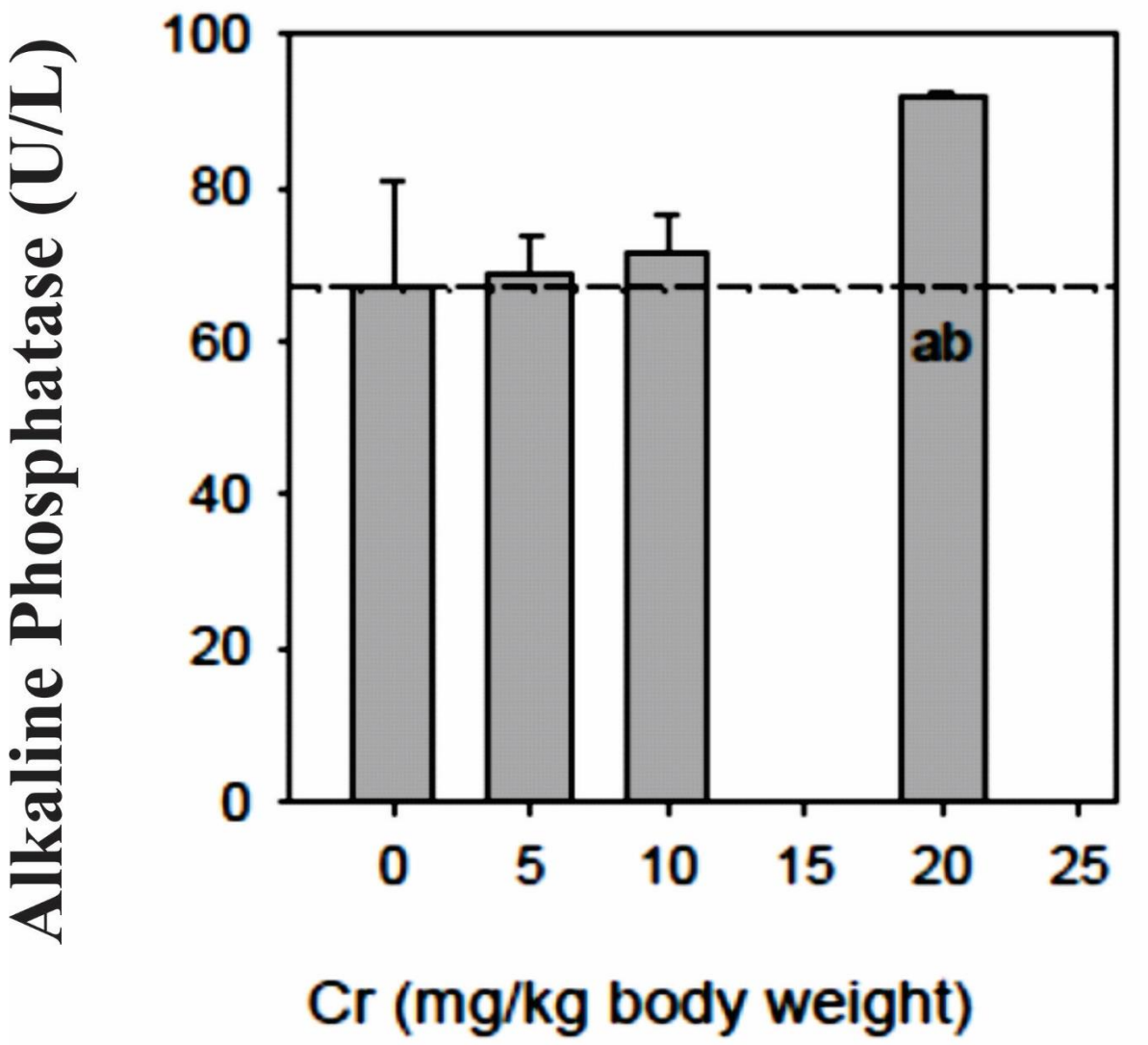


Figure 4.27: Dose-effect relationship for alkaline phosphatase (ALP) activity in albino rats treated with Cr. a = significantly different from control ($p<0.05$), b = significantly different from mixture ($p<0.05$).

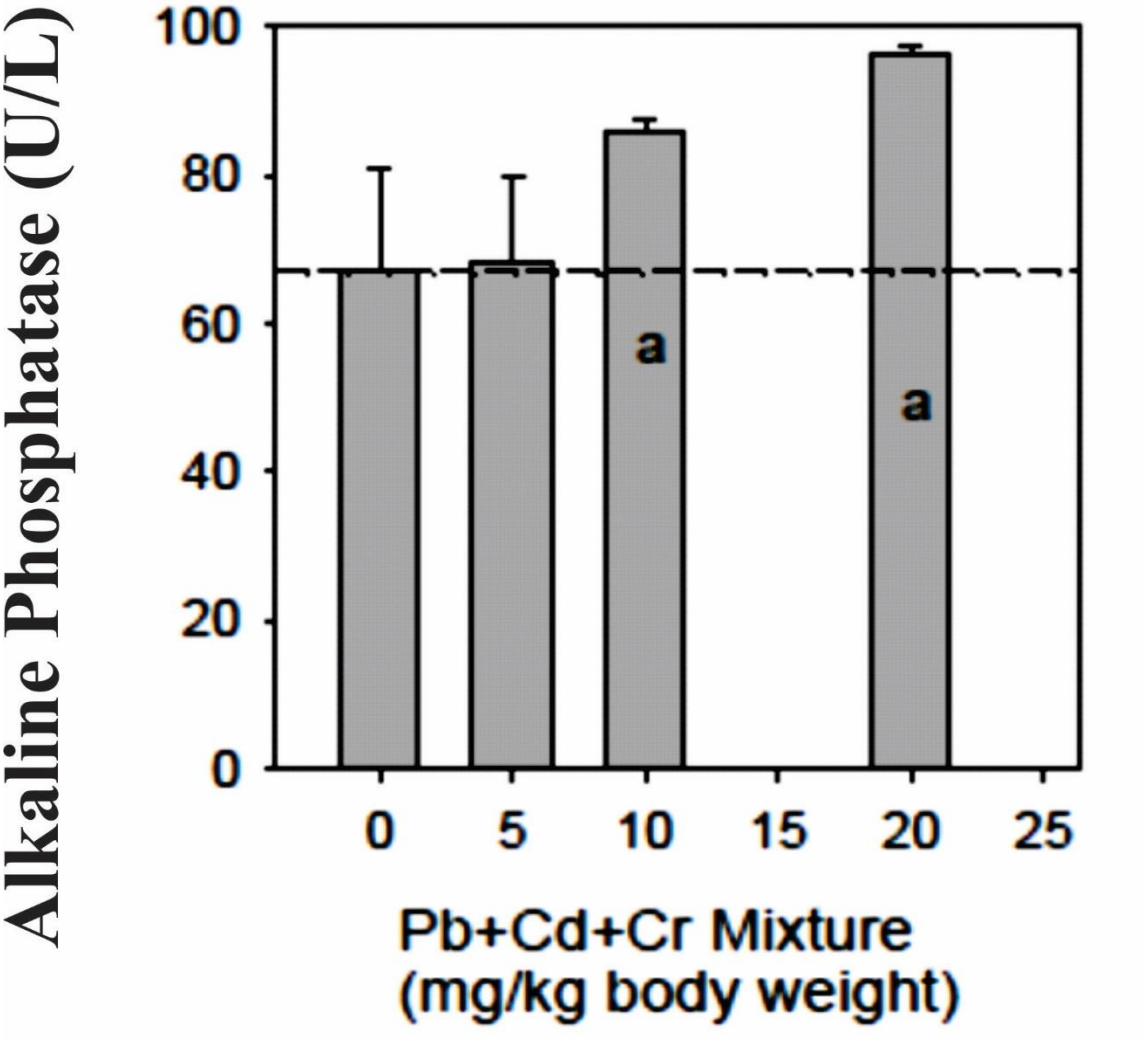


Figure 4.28: Dose-effect relationship for alkaline phosphatase (ALP) activity in albino rats treated with Pb, Cd and Cr as a mixture. a = significantly different from control ($p<0.05$).

Figures 4.25-4.28 showed that in the 5mg/kg treatment dose, there was no significant increase ($p>0.05$) in mean serum ALP activity inCd, Cr, Pb individual and combined treatment groups compared with control. There was no significant difference ($p>0.05$) in mean serum ALP activity in Cd, Cr, Pb individual treatment groups compared with the combined treatment group.

Figures 4.25-4.28 also showed that in the 10mg/kg treatment dose, there was significant difference ($p<0.05$) in mean serum ALP activity in Cd, Cr, Pb combined treatment group compared with control but there was no significant increase/difference ($p>0.05$) in mean serum ALP activity in Cd, Cr, Pb individual treatment groups compared with control.

Figures 4.25-4.28 showed that in the 20mg/kg treatment dose, there was significant increase ($p<0.05$) in mean serum ALP activity in Cd, Cr, Pb individual and combined treatment groups compared with control. Also, there was significant difference ($p<0.05$) in mean serum ALP activityin Cr individual treatment groups compared with the combined treatment group but there was no significant difference ($p>0.05$) in mean serum ALP activity in Cd, Pb individual treatment groups compared with the combined treatment group. Serum ALP activity in the combined treatment group was higher than that of Cr treatment group but was not significantly different ($p>0.05$) from that of Cd that produced the highest effect in the individual treatment group.

In Pb treatment group, a J-shaped hormetic effect was observed with regard to serum ALP activity as treatment with low dose (5mg/kg) produced a beneficial (not adverse) effect which was lower than control but changed to harmful (adverse) effect as the dose increased (20mg/kg). Treatment with Cd, Cr individually and the combined caused a dose dependent increase in mean serum ALP activity as the dose increased.

Table 4.3 Use of effect-addition in assessment of ALP activity in albino rats treated with Pb, Cd and Cr individually and as a mixture

Dose (C)	EC_{mix} (expt)	EC_{Pb}	EC_{Cd}	EC_{Cr}	EC_{mix}(pred) = [1- (1-EC_{pb}))(1-EC_{cd})(1-EC_{cr})]	Nature of interaction
5mg/kg	0.6840	0.5480	0.8090	0.6860	0.9729	Less than additive
10mg/kg	0.8560	0.7540	0.8630	0.7130	0.9903	Less than additive
20mg/kg	0.9610	0.9120	0.9270	0.9210	0.9995	Less than additive

$E(C_i)$ obtained with maximum possible ALP activity at 100 U/L.

From Table 4.3, the use of effect-addition in assessment of ALP activity showed that the effect obtained from the experimented mixture was lower than the effect obtained by prediction using the addition of effect from the individual treatments, suggesting less than additive interaction of the mixture components in the three treatment doses with respect to ALP activity.

4.1.2.4 Serum total bilirubin concentration

Figures 4.29-4.32 show dose-effect relationship forserum total bilirubin concentration in albino rats treated with Pb, Cd and Cr individually and as a mixture.

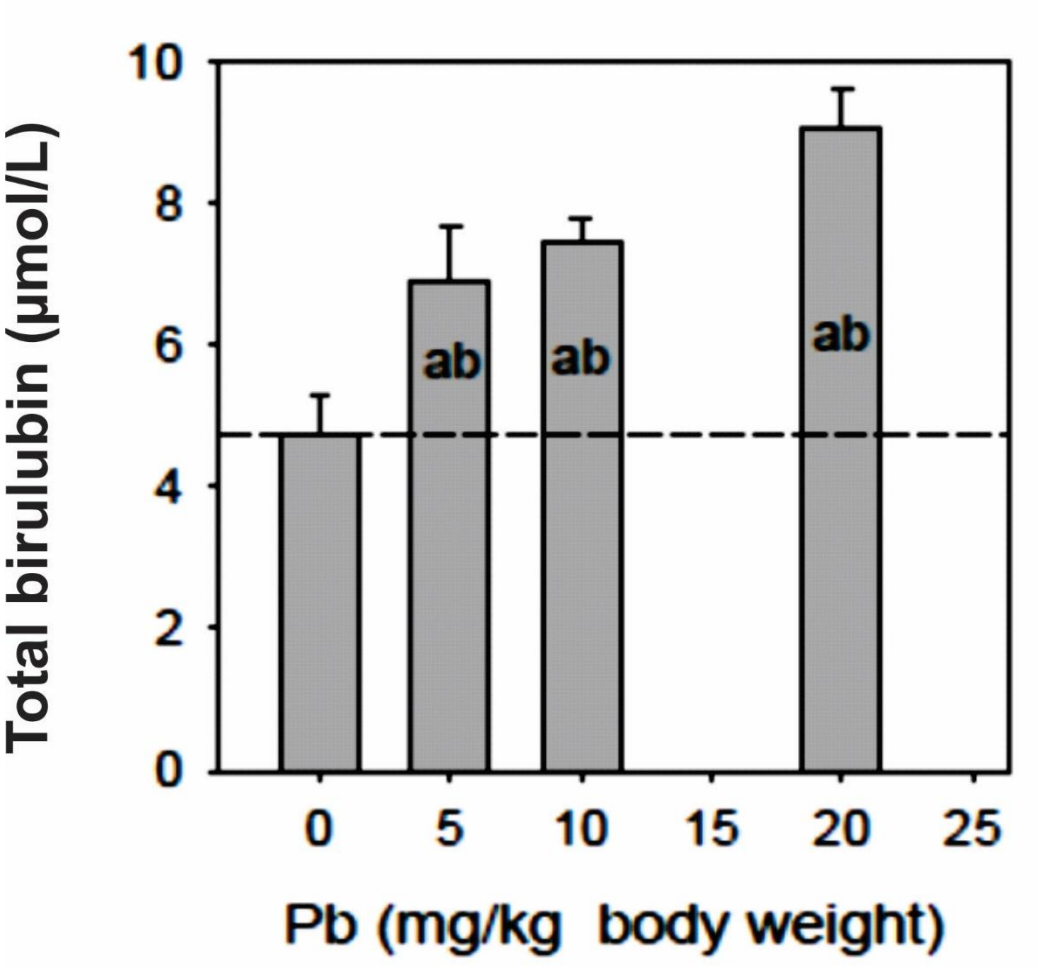


Figure 4.29: Dose-effect relationship for total bilirubin concentration in albino rats treated with Pb. a = significantly different from control ($p<0.05$), b = significantly different from mixture ($p<0.05$).

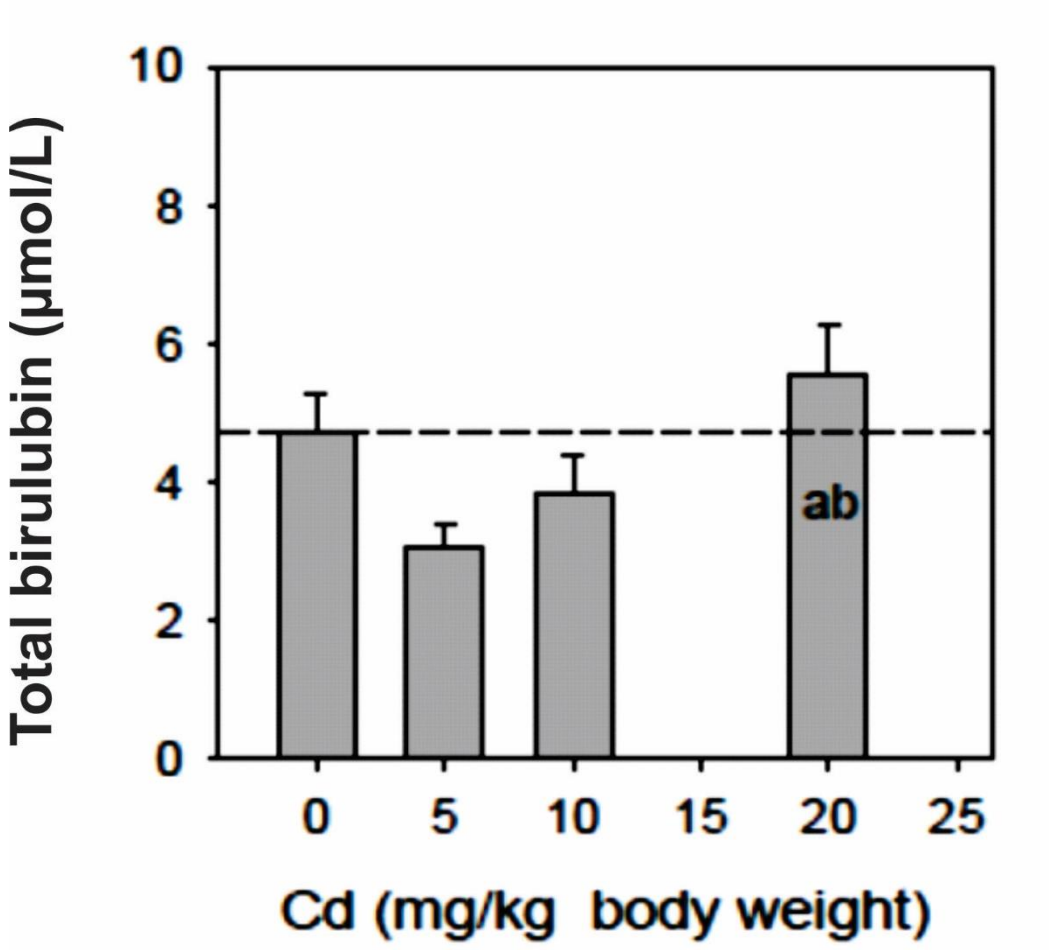


Figure 4.30: Dose-effect relationship for total bilirubin concentration in albino rats treated with Cd. a = significantly different from control ($p<0.05$), b = significantly different from mixture ($p<0.05$).

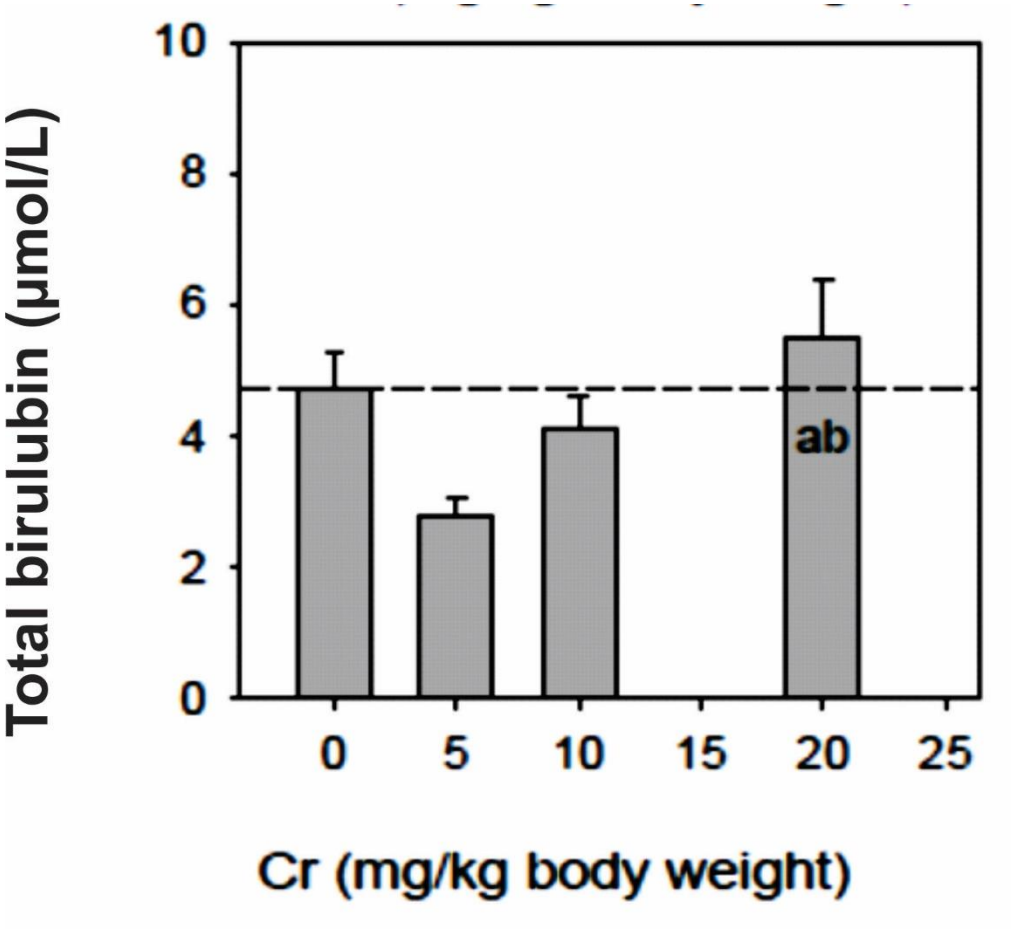


Figure 4.31: Dose-effect relationship for total bilirubin concentration in albino rats treated with Cr. a = significantly different from control ($p<0.05$), b = significantly different from mixture ($p<0.05$).

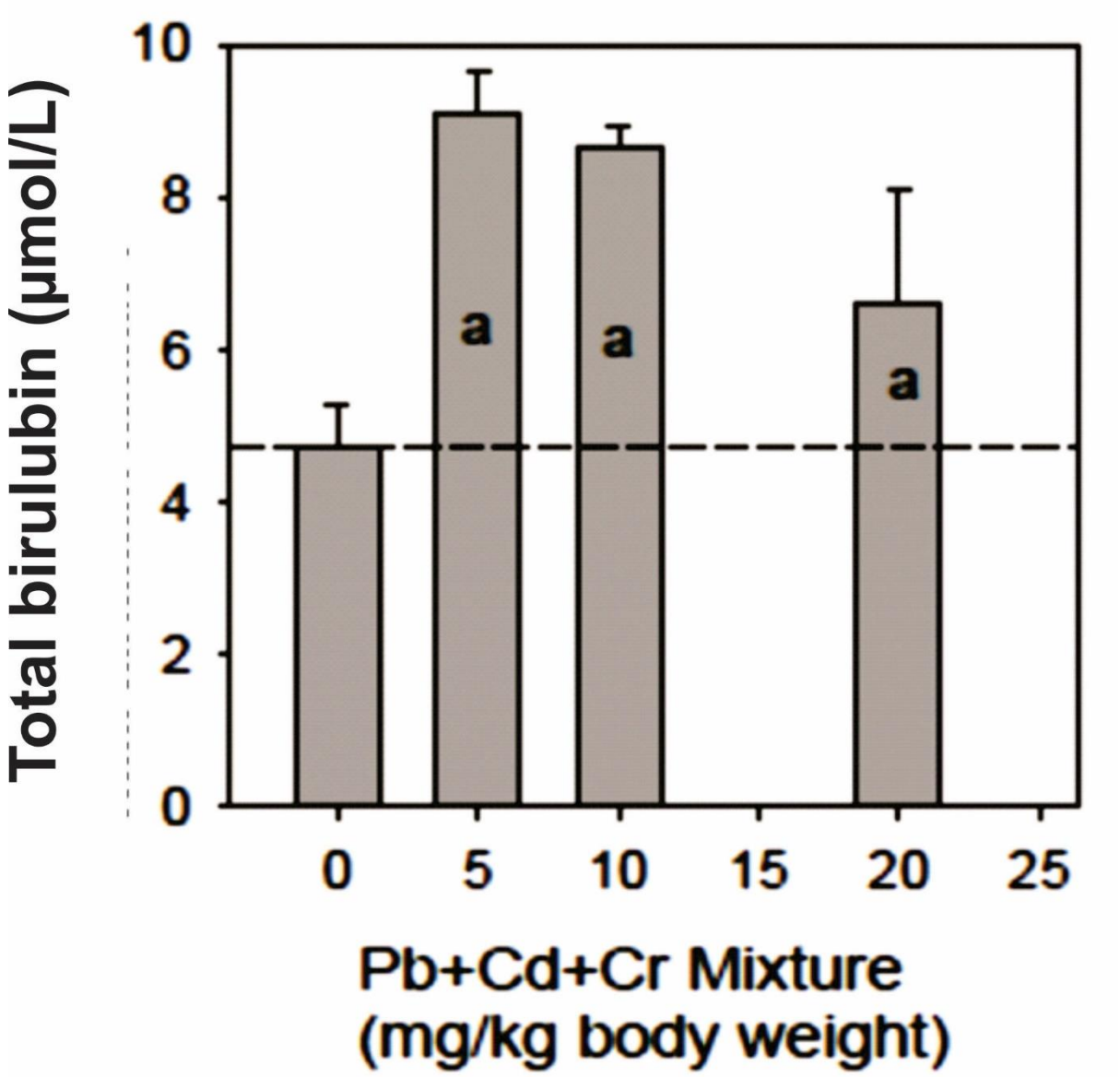


Figure 4.32: Dose-effect relationship for total bilirubin concentration in albino rats treated with Pb, Cd and Cr as a mixture. a = significantly different from control ($p<0.05$).

Figures 4.29-4.32 showed that in the 5mg/kg treatment dose, there was significant increase ($p<0.05$) in mean serum total bilirubin concentration in Pb individual and combined treatment groups compared with control but there was no significant difference ($p>0.05$) in mean serum total bilirubin concentration in Cd, Cr individual treatment group compared with control. Also, there was significant difference ($p<0.05$) in mean serum total bilirubin concentration in Pb individual treatment group compared with the combined treatment group. Serum total bilirubin concentration in the combined treatment group was higher than that of Pb treatment group.

Figures 4.29-4.32 also showed that in the 10mg/kg treatment dose, there was significant increase ($p<0.05$) in mean serum total bilirubin concentration in Pb individual and combined treatment groups compared with control but there was no significant difference ($p>0.05$) in mean serum total bilirubin concentration in Cd, Cr individual treatment group compared with control. Also, there was significant difference ($p<0.05$) in mean serum total bilirubin concentration in Pb individual treatment group compared with the combined treatment group. Serum total bilirubin concentration in the combined treatment group was higher than that of Pb treatment group.

Figures 4.29-4.32 showed that in the 20mg/kg treatment dose, there was significant increase ($p<0.05$) in mean serum total bilirubin concentration in Cd, Cr, Pb individual and combined treatment groups compared with control. Also, there was significant difference ($p<0.05$) in mean serum total bilirubin concentration in Cd, Cr, Pb individual treatment groups compared with the combined treatment group. Serum total bilirubin concentration in the combined treatment group was lower than that of Pb that produced the highest effect in the individual treatment group.

In Cd and Cr treatment groups, a J-shaped hormetic effect was observed with regard to serum total bilirubin concentration as treatment with low dose (5mg/kg) produced a beneficial (not

adverse) effect which was lower than control but changed to harmful (adverse) effect as the dose increased (20mg/kg). In the combined treatment group, an inverted U-shaped hormetic effect was observed with regard to serum total bilirubin concentration as treatment with the low dose (5mg/kg) produced a stimulating effect which was reduced at the high dose (20mg/kg). Treatment with Pb caused a dose dependent increase in mean serum total bilirubin concentration as the dose increased.

Table 4.4 Use of effect-addition in assessment of total bilirubin concentrationin albino rats treated with Pb, Cd and Cr individually and as a mixture

Dose (C)	EC_{mix} (expt)	EC_{Pb}	EC_{Cd}	EC_{Cr}	EC_{mix} (pred)	Nature of interaction
5mg/kg	0.0163	0.0124	0.0055	0.0050	0.0227	Less than additive
10mg/kg	0.0154	0.0133	0.0066	0.0073	0.0270	Less than additive
20mg/kg	0.0118	0.0162	0.0100	0.0098	0.0356	Less than additive

$E(C_i)$ obtained with maximum possible total bilirubin concentration at 560µmol/l.

From Table 4.4, the use of effect-addition in assessment of total bilirubin concentration showed that the effect obtained from the experimented mixture was lower than the effect obtained by prediction using the addition of effect from the individual treatments, suggesting less than

additive interaction of the mixture components in the three treatment doses with respect to total bilirubin concentration.

4.1.2.5 Serum lactate dehydrogenase (LDH) level in albino rats treated with Pb, Cd and Cr individually and as a mixture

Figures 4.33-4.36 show dose-effect relationship for lactate dehydrogenase (LDH) level in albino rats treated with Pb, Cd and Cr individually and as a mixture.

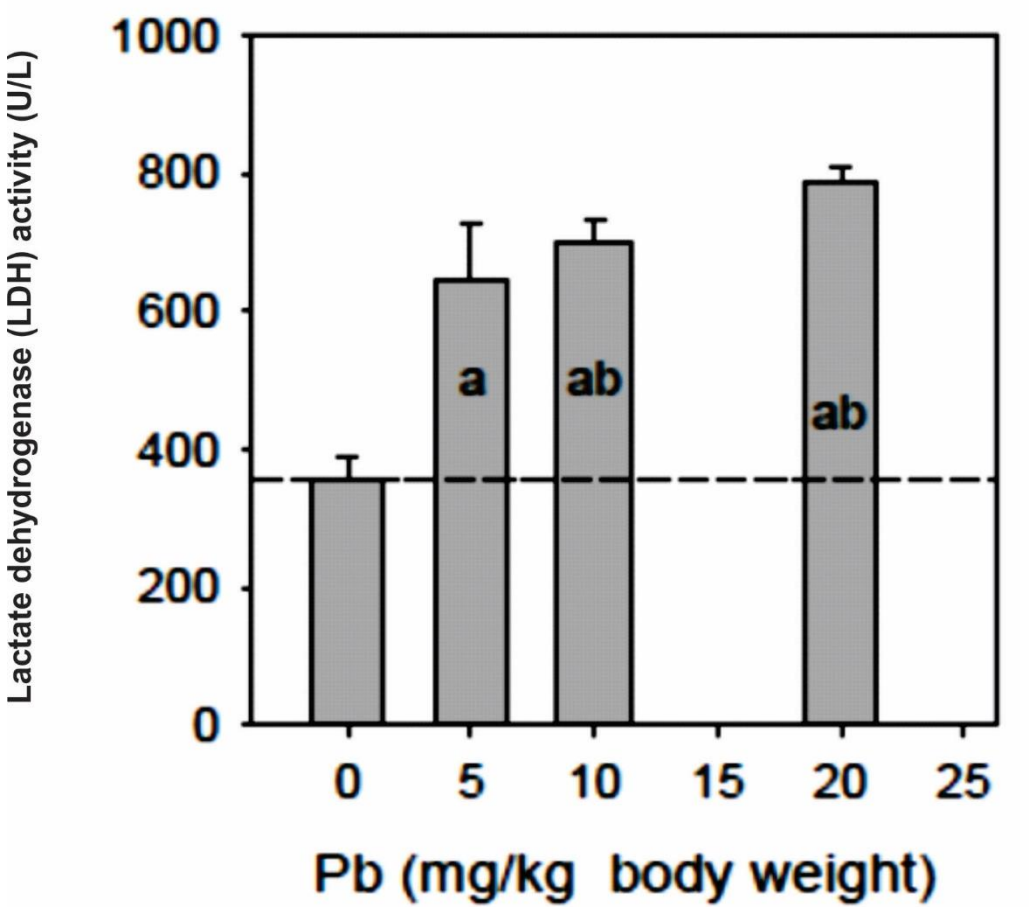


Figure 4.33: Dose-effect relationship for lactate dehydrogenase (LDH) activity in albino rats treated with Pb. a = significantly different from control ($p<0.05$), b = significantly different from mixture ($p<0.05$).

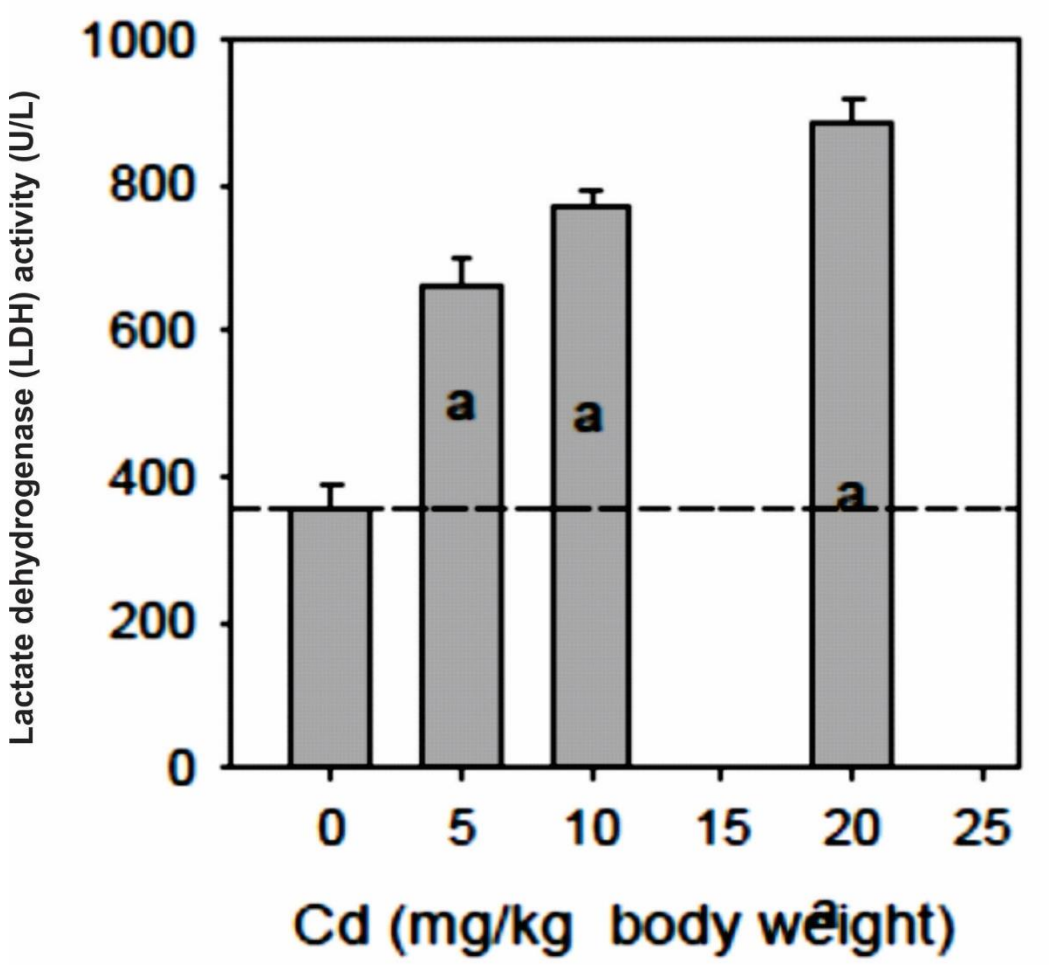


Figure 4.34: Dose-effect relationship for lactate dehydrogenase (LDH) activity in albino rats treated with Cd. a = significantly different from control ($p<0.05$).

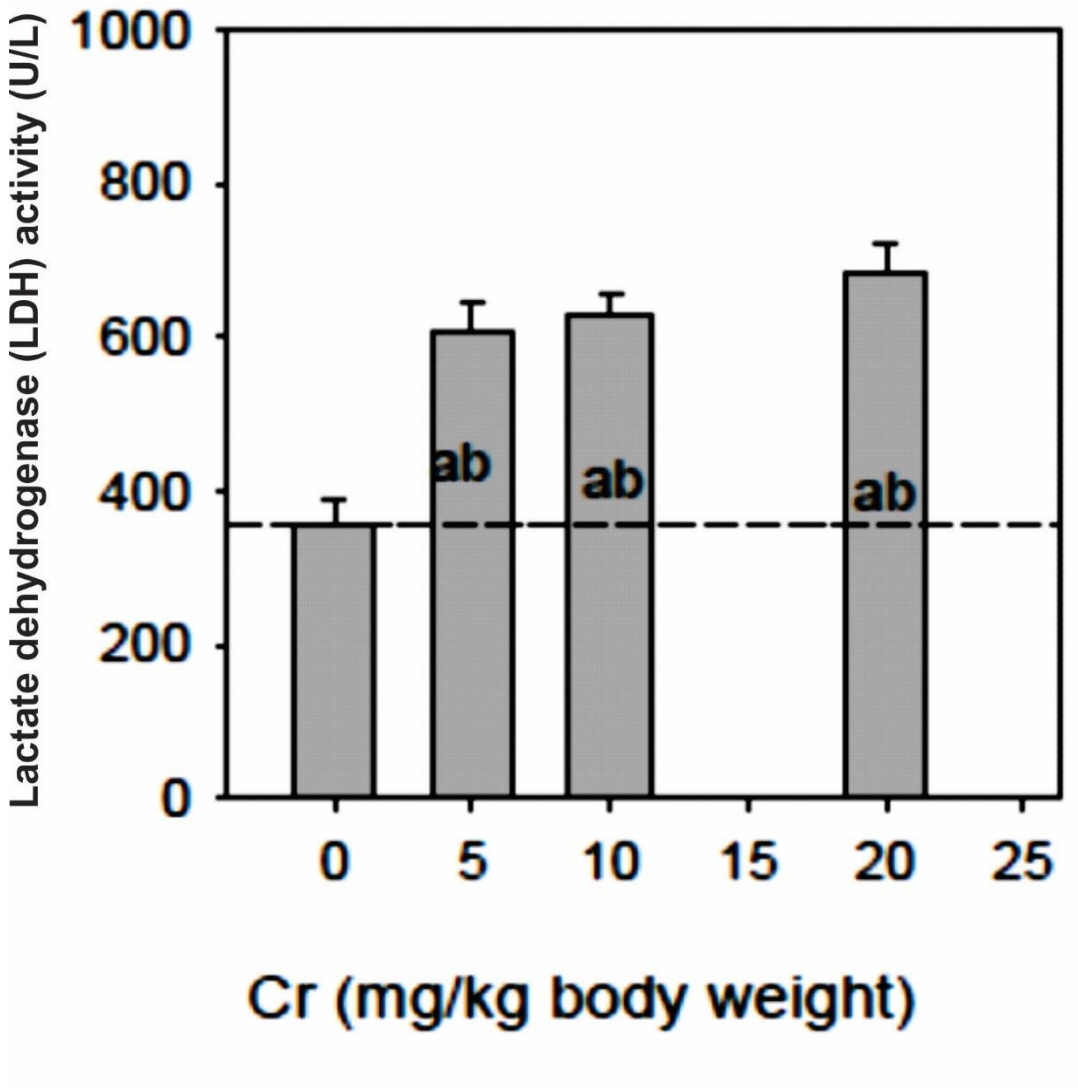


Figure 4.35: Dose-effect relationship for lactate dehydrogenase (LDH) activity in albino rats treated with Cr. a = significantly different from control ($p<0.05$), b = significantly different from mixture ($p<0.05$).

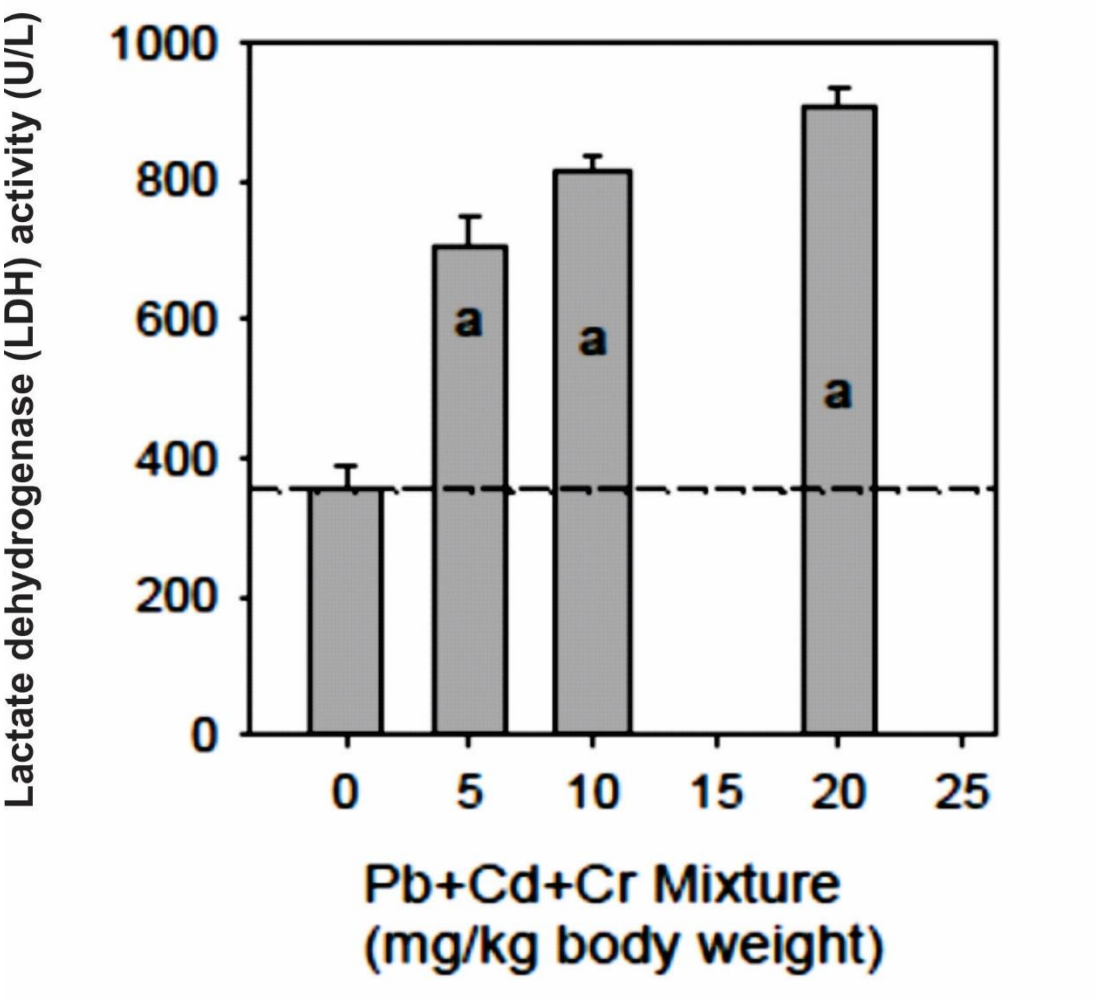


Figure 4.36: Dose-effect relationship for lactate dehydrogenase (LDH) activity in albino rats treated with Pb, Cd and Cr as a mixture. a = significantly different from control ($p<0.05$),

Figures 4.33-4.36 showed that in the 5mg/kg treatment dose, there was significant increase ($p<0.05$) in mean serum LDH activity in Cd, Cr, Pb individual and combined treatment groups compared with control. Also, there was significant difference ($p<0.05$) in mean serum LDH activity inCr individual treatment group compared with the combined treatment group but there was no significant difference ($p>0.05$) in mean serum LDH activity in Cd, Pb individual treatment groups compared with the combined treatment group. Serum LDH activity in the combined treatment group was higher than that of Cr treatment group but was not significantly different ($p>0.05$) from that of Cd that produced the highest effect in the individual treatment group.

Figures 4.33-4.36 also showed that in the 10mg/kg treatment dose, there was significant increase ($p<0.05$) in mean serum LDH activity in Cd, Cr, Pb individual and combined treatment groups compared with control. Also, there was significant difference ($p<0.05$) in mean serum LDH activity inCr, Pb individual treatment groups compared with the combined treatment group but there was no significant difference ($p>0.05$) in mean serum LDH activity in Cd individual treatment group compared with the combined treatment group. Serum LDH activity in the combined treatment group was higher than that of Cr, Pb treatment groups but was not significantly different ($p>0.05$) from that of Cd that produced the highest effect in the individual treatment group.

Figures 4.33-4.36 showed that in the 20mg/kg treatment dose, there was significant increase ($p<0.05$) in mean serum LDH activity in Cd, Cr, Pb individual and combined treatment groups compared with control. Also, there was significant difference ($p<0.05$) in mean serum LDH activity inCr, Pb individual treatment groups compared with the combined treatment group but there was no significant difference ($p>0.05$) in mean serum LDH activity in Cd individual

treatment group compared with the combined treatment group. Serum LDH activity in the combined treatment group was higher than that of Cr, Pb treatment groups but was not significantly different ($p>0.05$) from that of Cd that produced the highest effect in the individual treatment group.

Treatment with Cd, Cr, Pb individually and combined caused a dose dependent increase in mean serum LDH activity as the dose increased.

4.1.3 Renal function tests

4.1.3.1 Serum urea concentration

Figures 4.37-4.40 show dose-effect relationship for urea concentration in albino rats treated with Pb, Cd and Cr individually and as a mixture.

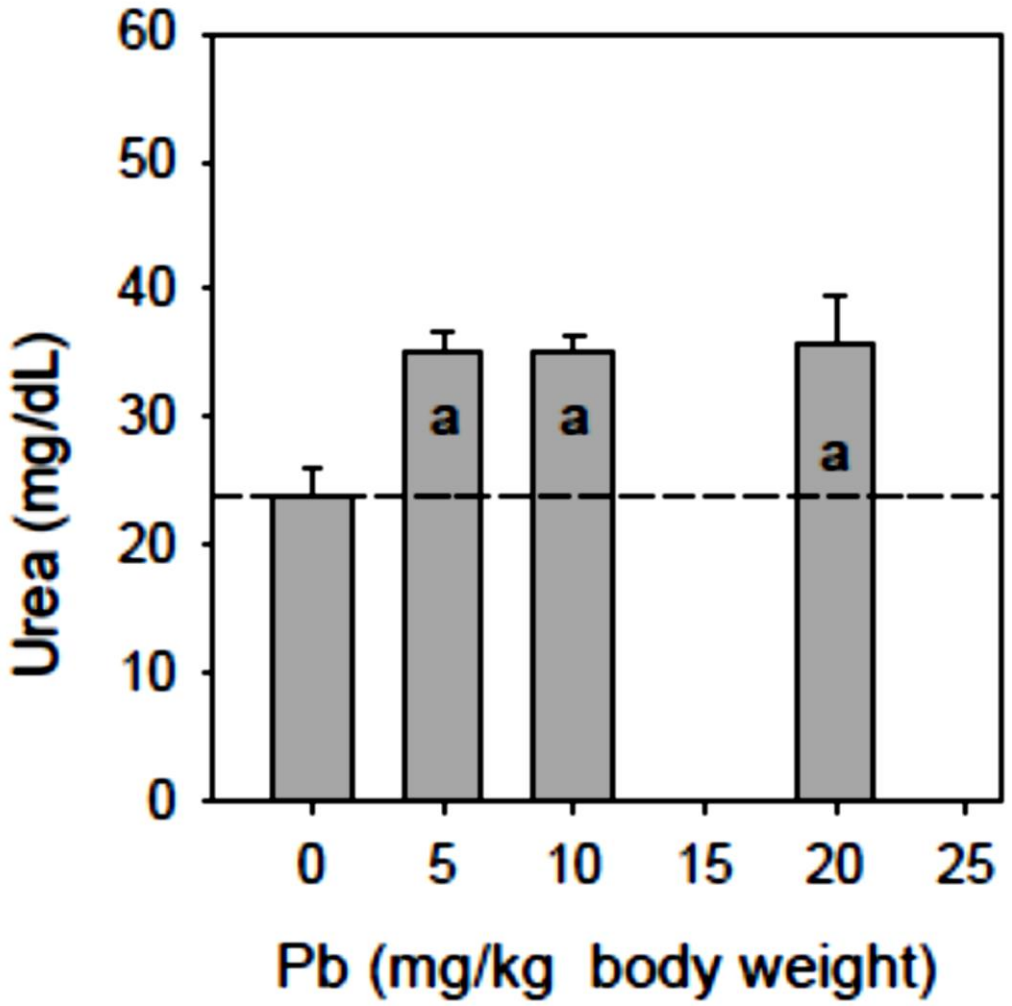


Figure 4.37: Dose-effect relationship for serum urea concentration in albino rats treated with Pb. a = significantly different from control ($p<0.05$).

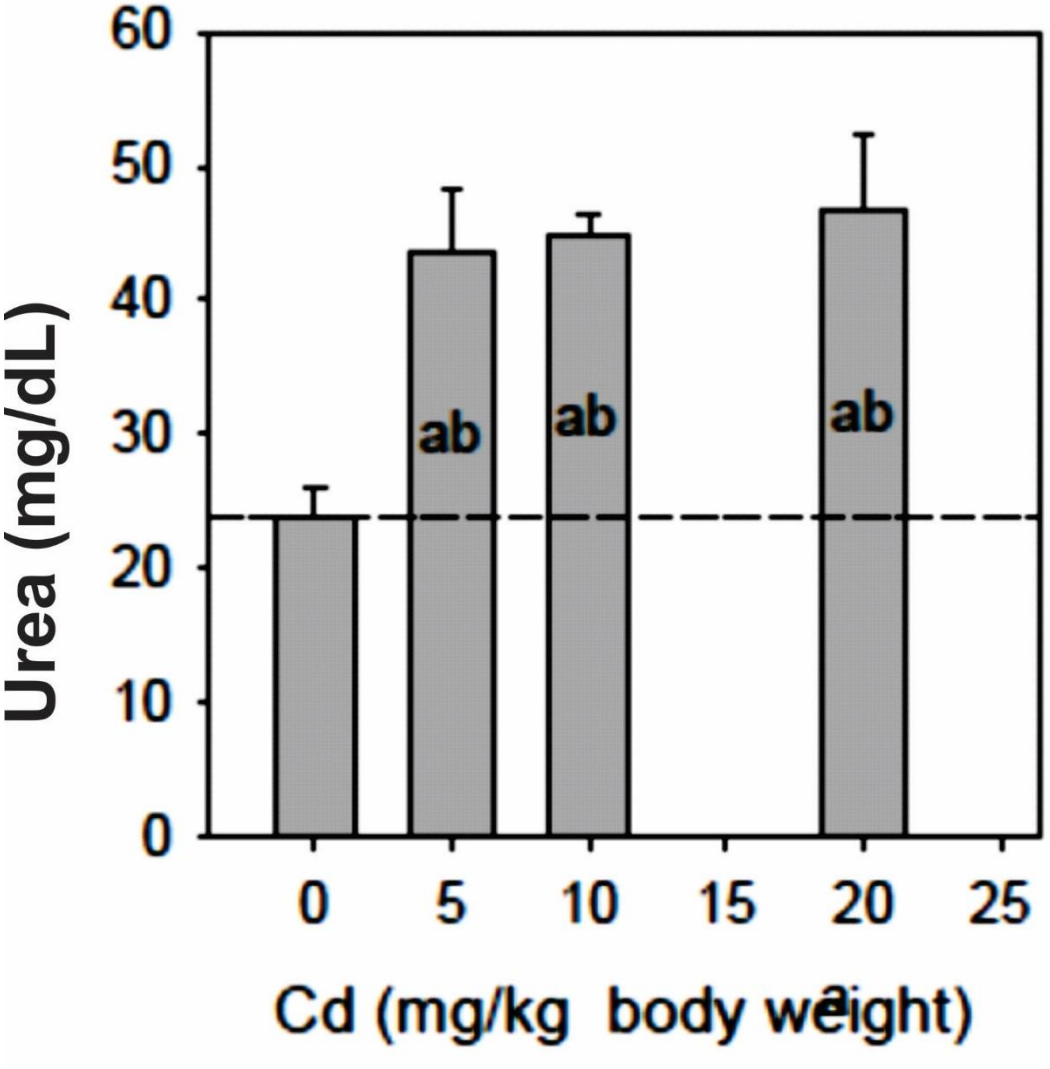


Figure 4.38: Dose-effect relationship for serum urea concentration in albino rats treated with Cd. a = significantly different from control ($p<0.05$), b = significantly different from mixture ($p<0.05$).

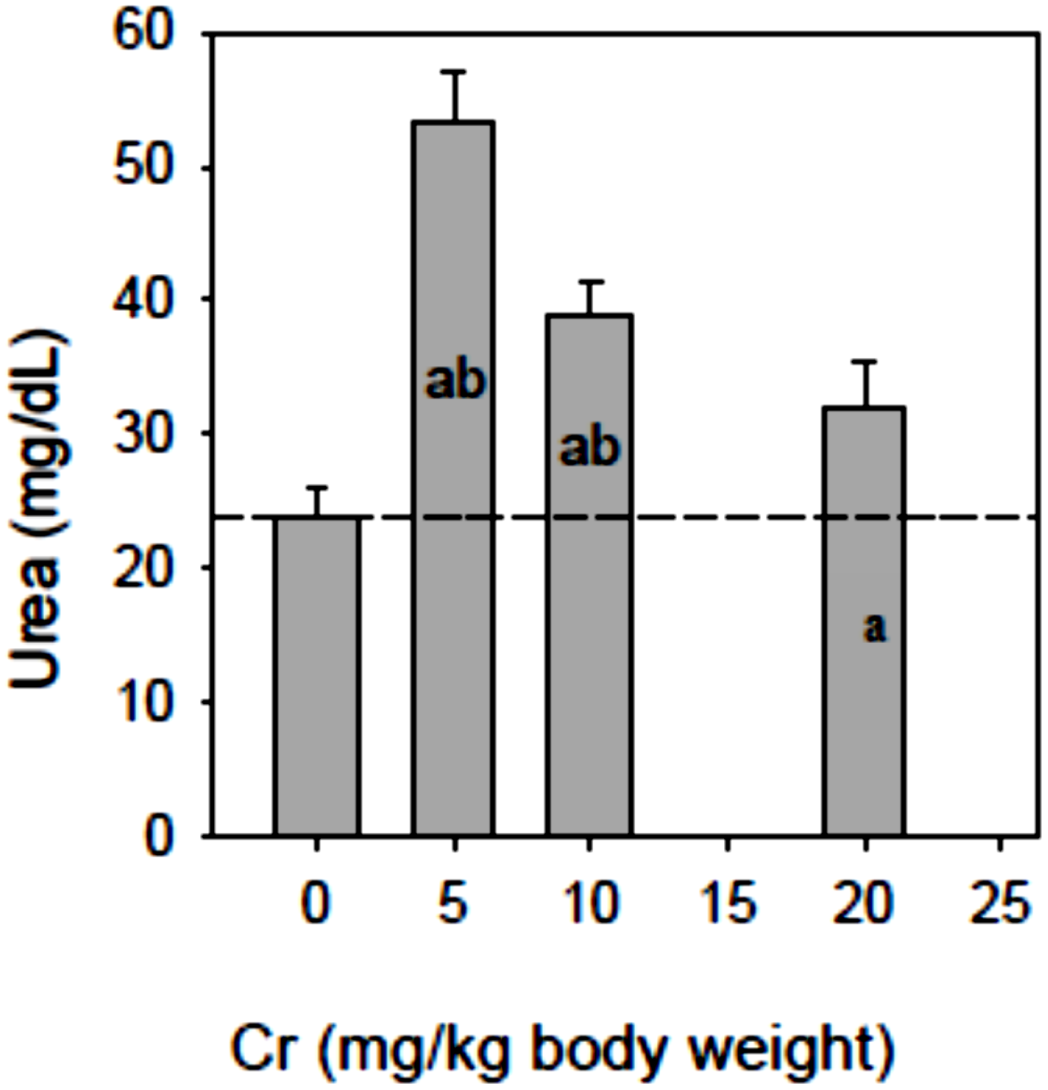


Figure 4.39: Dose-effect relationship for serum urea concentration in albino rats treated with Cr. a = significantly different from control ($p<0.05$), b = significantly different from mixture ($p<0.05$).

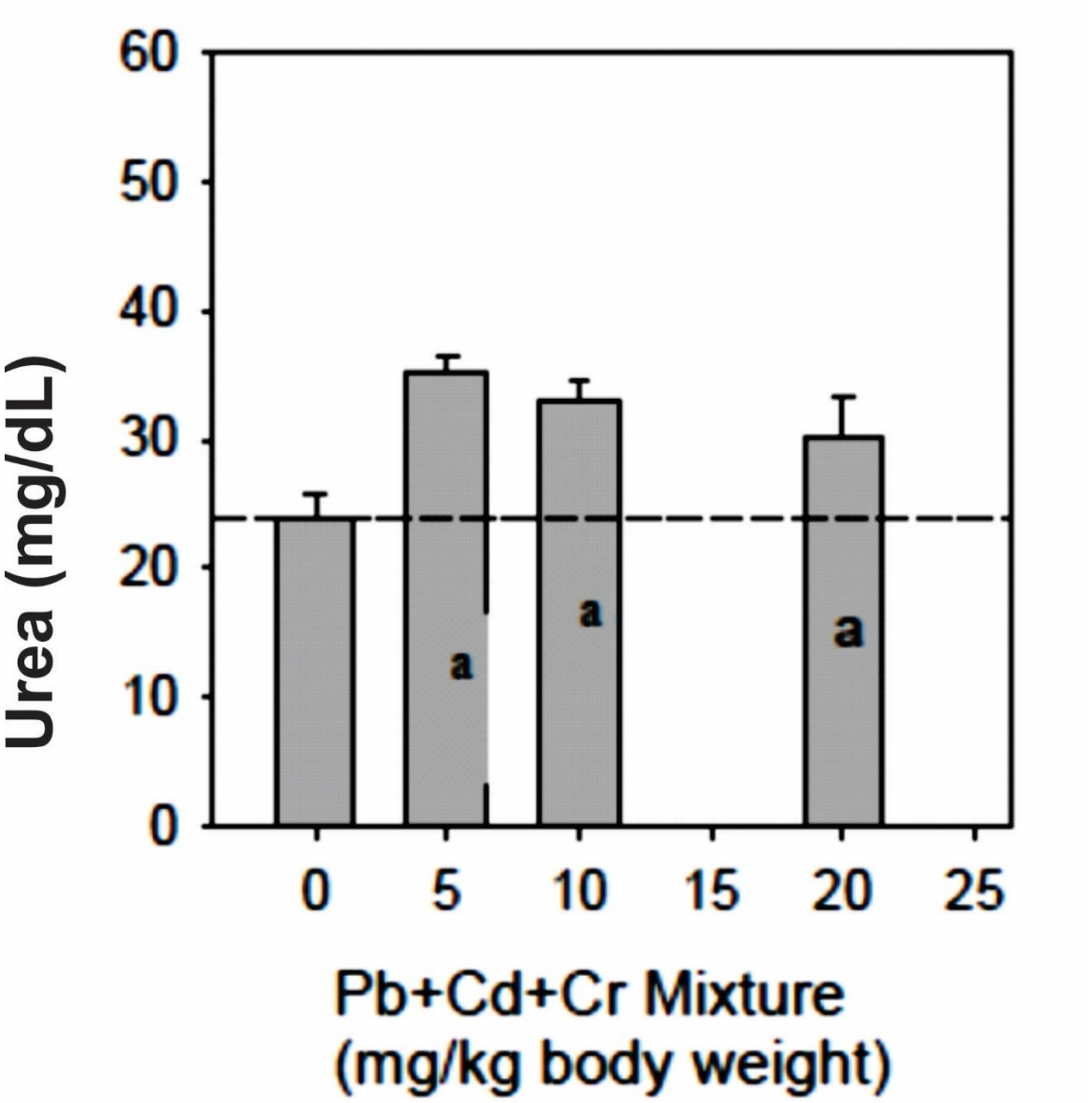


Figure 4.40: Dose-effect relationship for serum urea concentration in albino rats treated with Pb, Cd and Cr as a mixture. a = significantly different from control ($p<0.05$).

Figures 4.37-4.40 showed that in the 5mg/kg treatment dose, there was significant increase ($p<0.05$) in mean serum urea concentration in Cd, Cr, Pb individual and combined treatment groups compared with control. Also, there was significant difference ($p<0.05$) in mean serum urea concentration in Cd, Cr individual treatment groups compared with the combined treatment group but there was no significant difference ($p>0.05$) in mean serum urea concentration in Pb individual treatment group compared with the combined treatment group. Serum urea concentration in the combined treatment group was lower than that of Cd, Cr treatment groups.

Figures 4.37-4.40 also showed that in the 10mg/kg treatment dose, there was significant increase ($p<0.05$) in mean serum urea concentration in Cd, Cr, Pb individual and combined treatment groups compared with control. Also, there was significant difference ($p<0.05$) in mean serum urea concentration in Cd, Cr individual treatment groups compared with the combined treatment group but there was no significant difference ($p>0.05$) in mean serum urea concentration in Pb individual treatment group compared with the combined treatment group. Serum urea concentration in the combined treatment group was lower than that of Cd, Cr treatment groups.

Figures 4.37-4.40 showed that in the 20mg/kg treatment dose, there was significant increase ($p<0.05$) in mean serum urea concentration in Cd, Cr, Pb individual and combined treatment groups compared with control. Also, there was significant difference ($p<0.05$) in mean serum urea concentrationin Cd individual treatment group compared with the combined treatment group but there was no significant difference ($p>0.05$) in mean serum urea concentration in Cr, Pb individual treatment groups compared with the combined treatment group. Serum urea concentration in the combined treatment group was lower than that of Cdtreatment group.

In the Cr and combined treatment groups, an inverted U-shaped hormetic effect was observed with regard to serum urea concentration as treatment with the low dose (5mg/kg) produced a stimulating effect which was reduced at the high dose (20mg/kg). Treatment with Cd caused a dose dependent increase in mean serum urea concentration as the dose increased. Effect due Pb treatment was fairly constant as there was no significant difference ($p>0.05$) in mean serum urea concentration in the three treatment doses.

Table 4.5 Use of effect-addition in assessment/prediction of serum urea concentrationin albino rats treated with Pb, Cd and Cr individually and as a mixture

Dose (C)	EC_{mix} (expt)	EC_{Pb}	EC_{Cd}	EC_{Cr}	EC_{mix}(pred) = [1- (1-EC_{pb}))(1-EC_{cd})(1-EC_{cr})]	Nature of interaction
5mg/kg	0.1459	0.1463	0.1814	0.2211	0.4557	Less than additive
10mg/kg	0.1371	0.1458	0.1866	0.1615	0.4174	Less than additive
20mg/kg	0.1253	0.1480	0.1944	0.1324	0.4045	Less than additive

$E(C_i)$ obtained with maximum possible urea concentration at 241mg/dl.

From Table 4.5, the use of effect-addition in assessment of serum urea concentration showed that the effect obtained from the experimented mixture was lower than the effect obtained by prediction using the addition of effect from the individual treatments, suggesting less than additive interaction of the mixture components in the three treatment doses with respect to serum urea concentration.

4.1.3.2 Serum creatinine concentration in albino rats

Figures 4.41-4.44 show dose-effect relationship for serum creatinine concentration in albino rats treated with Pb, Cd and Cr individually and as a mixture.

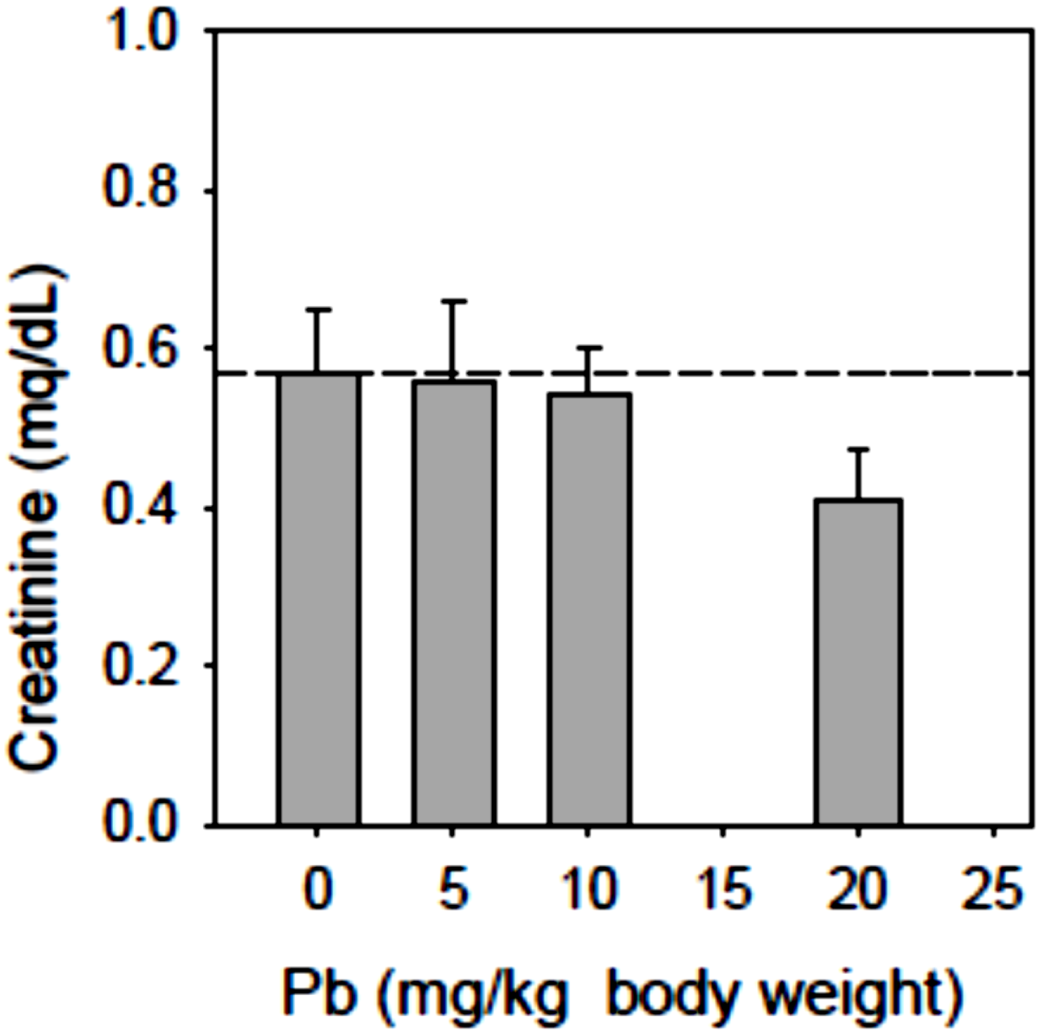


Figure 4.41: Dose-effect relationship for creatinine concentration in albino rats treated with Pb.

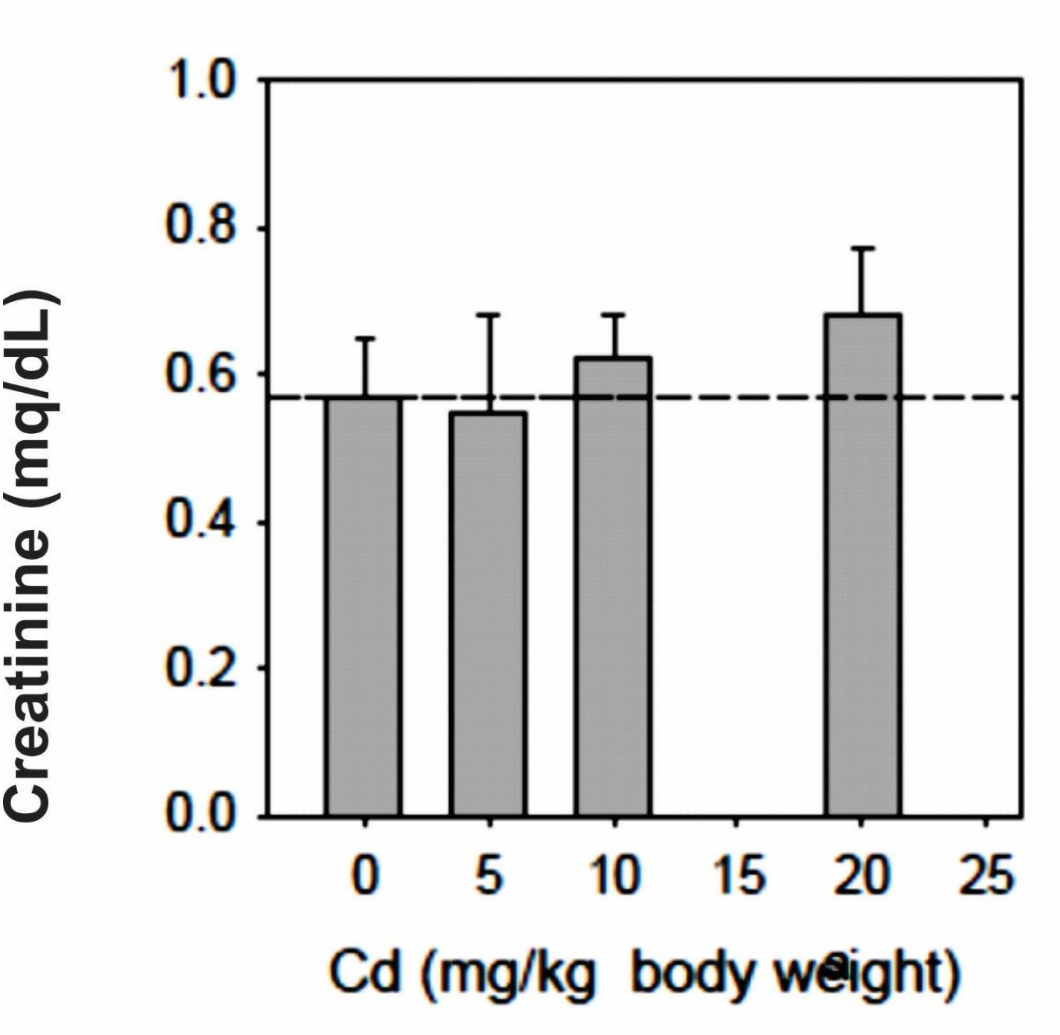


Figure 4.42: Dose-effect relationship for creatinine concentration in albino rats treated with Cd.

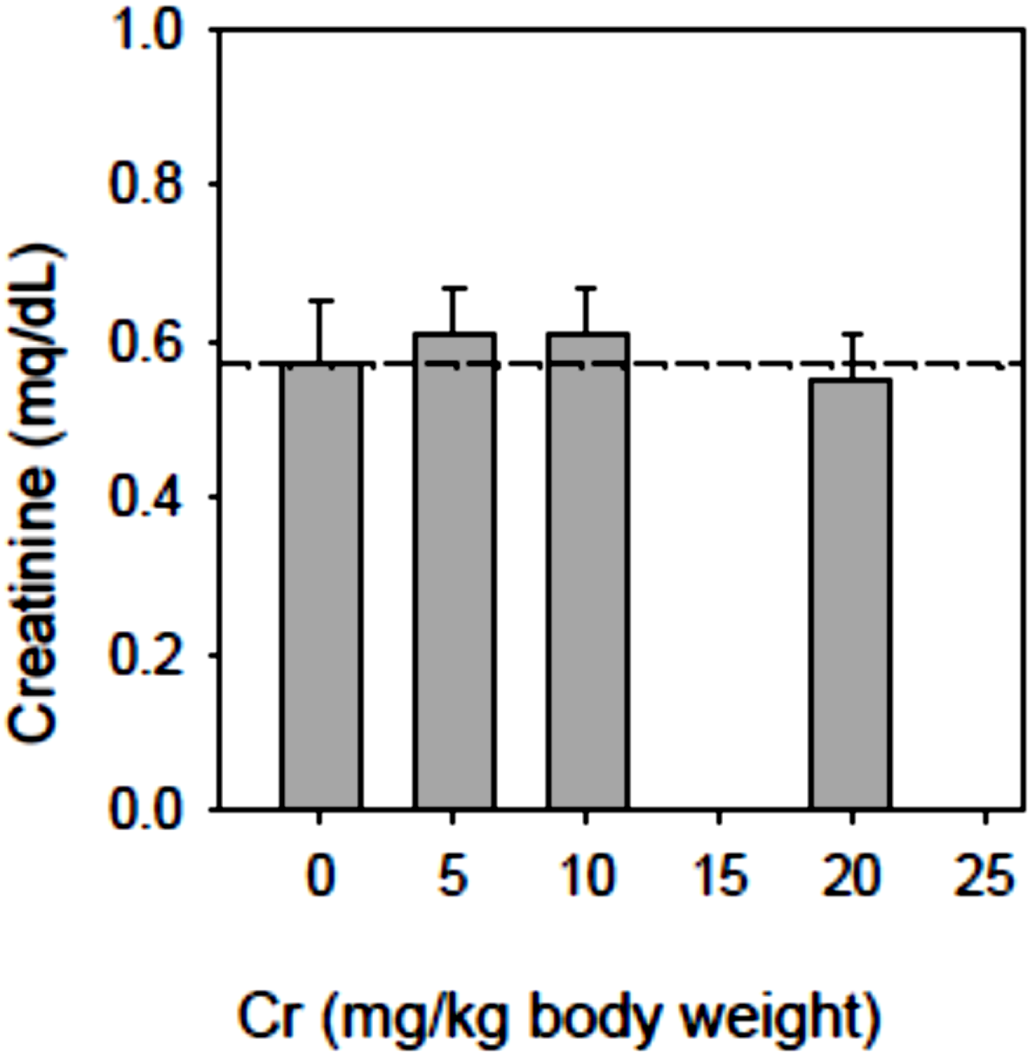


Figure 4.43: Dose-effect relationship for creatinine concentration in albino rats treated with Cr.

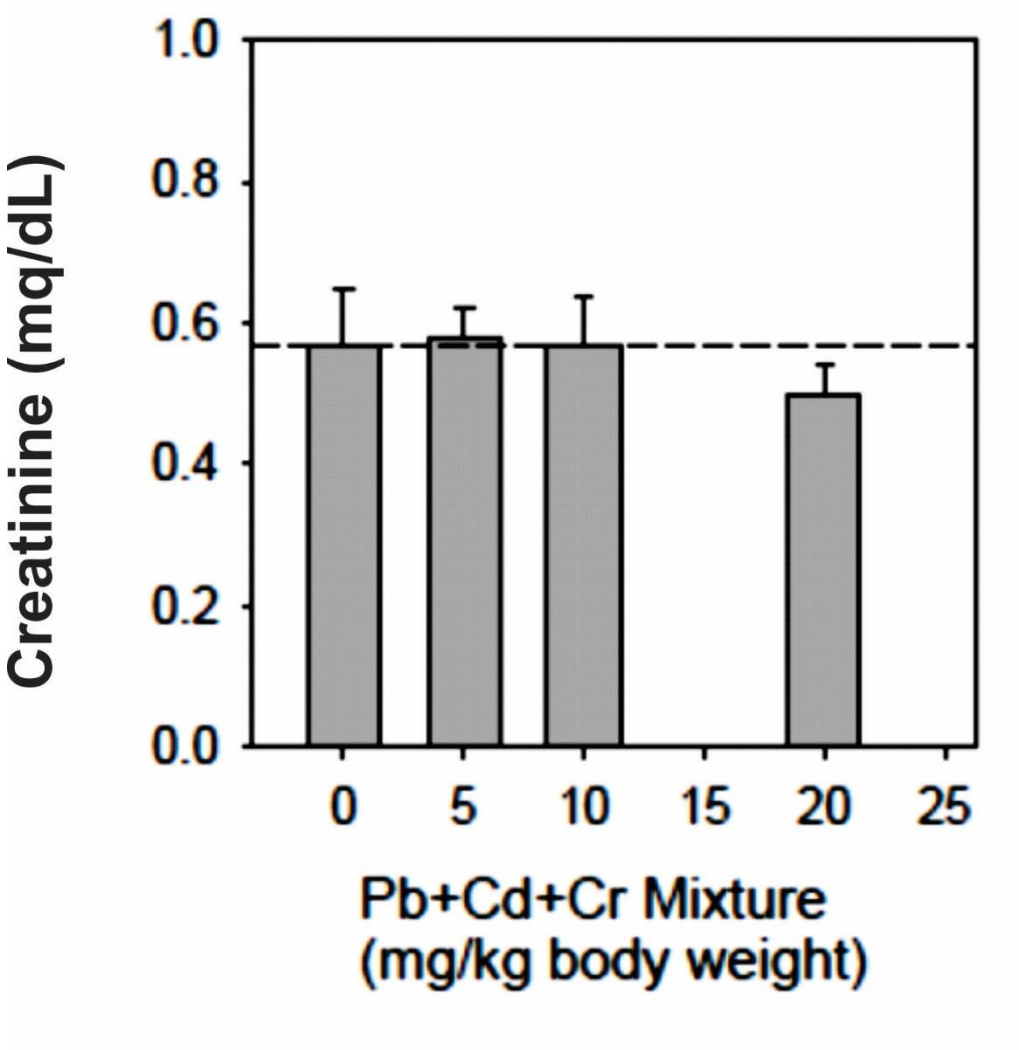


Figure 4.44: Dose-effect relationship for creatinine concentration in albino rats treated with Pb, Cd and Cr as a mixture

Figures 4.41-4.44 showed that in the 5mg/kg treatment dose, there was no significant difference ($p>0.05$) in mean serum creatinine concentrationin Cd, Cr, Pb individual and combined treatment groups compared with control. Also, there was no significant difference ($p>0.05$) in mean serum creatinine concentrationin Cd, Cr, Pb individual treatment groups compared with the combined treatment group.

Figures 4.41-4.44 also showed that in the 10mg/kg treatment dose, there was no significant difference ($p>0.05$) in mean serum creatinine concentration in Cd, Cr, Pb individual and combined treatment groups compared with control. Also, there was no significant difference ($p>0.05$) in mean serum creatinine concentration in Cd, Cr, Pb individual treatment groups compared with the combined treatment group.

Figures 4.41-4.44 showed that in the 20mg/kg treatment dose, there was no significant difference ($p>0.05$) in mean serum creatinine concentration in Cd, Cr, Pb individual and combined treatment groups compared with control. Also, there was no significant difference ($p>0.05$) in mean serum creatinine concentration in Cd, Cr, Pb individual treatment groups compared with the combined treatment group.

4.1.3.3 Serum potassium concentration in albino rats treated with Pb, Cd and Cr individually and as a mixture.

Figures 4.45-4.48 show dose-effect relationship for serum potassium concentration in albino rats treated with Pb, Cd and Cr individually and as a mixture.

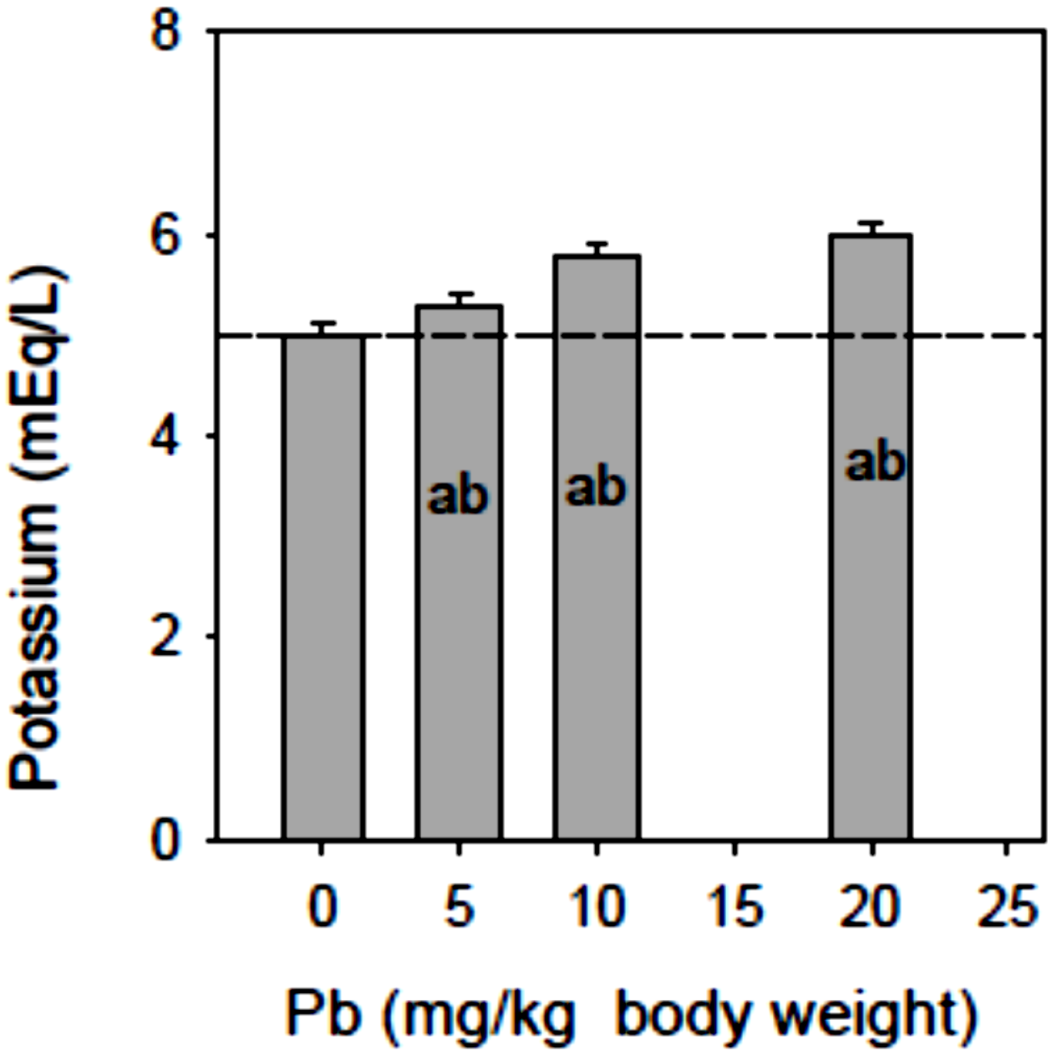


Figure 4.45: Dose-effect relationship for serum potassium concentration in albino rats treated with Pb. a = significantly different from control ($p<0.05$), b = significantly different from mixture ($p<0.05$).

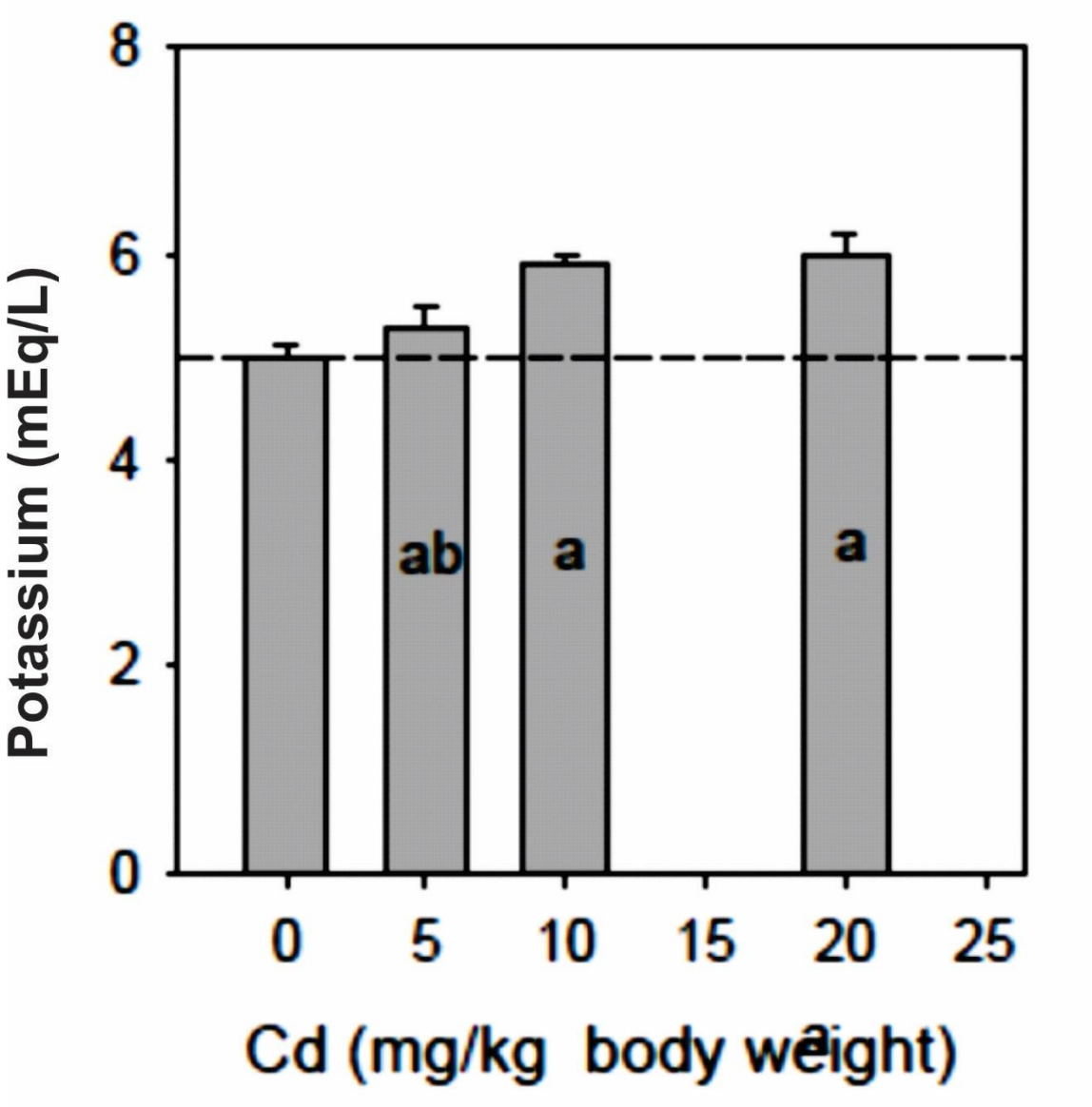


Figure 4.46: Dose-effect relationship for serum potassium concentration in albino rats treated with Cd. a = significantly different from control ($p<0.05$), b = significantly different from mixture ($p<0.05$).

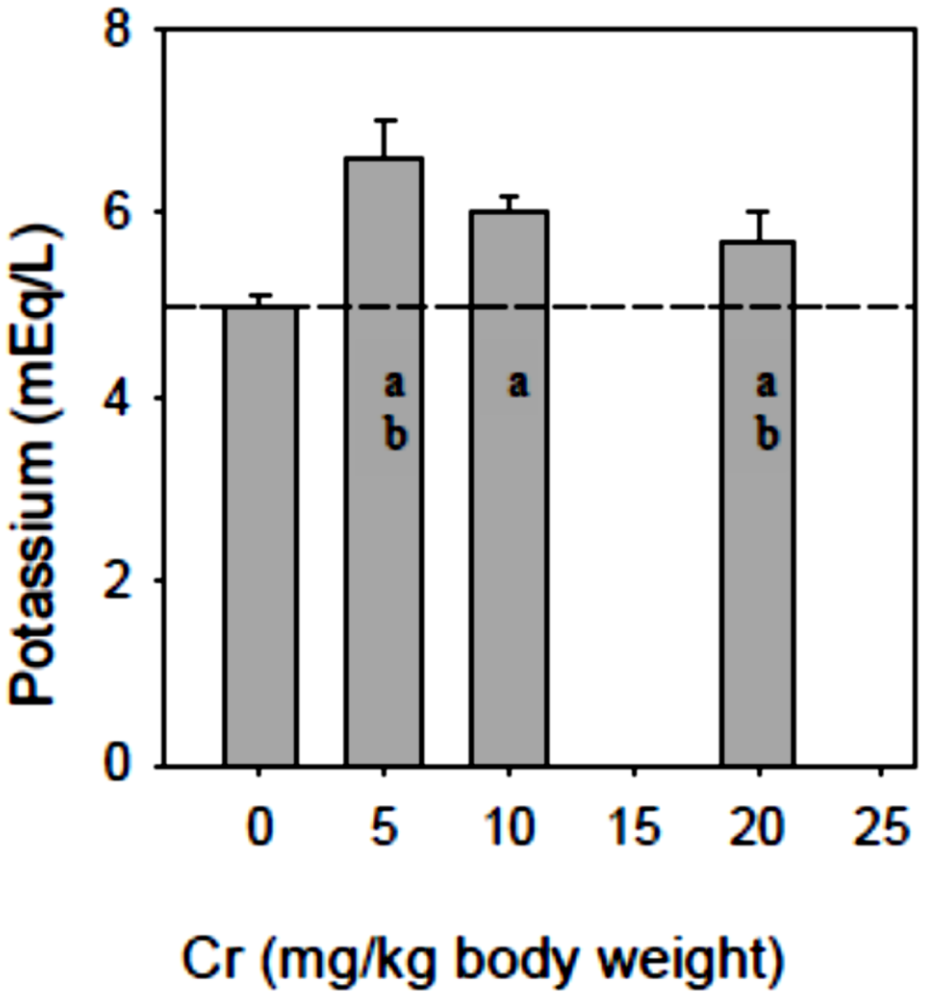


Figure 4.47: Dose-effect relationship for serum potassium concentration in albino rats treated with Cr. a = significantly different from control ($p<0.05$), b = significantly different from mixture ($p<0.05$).

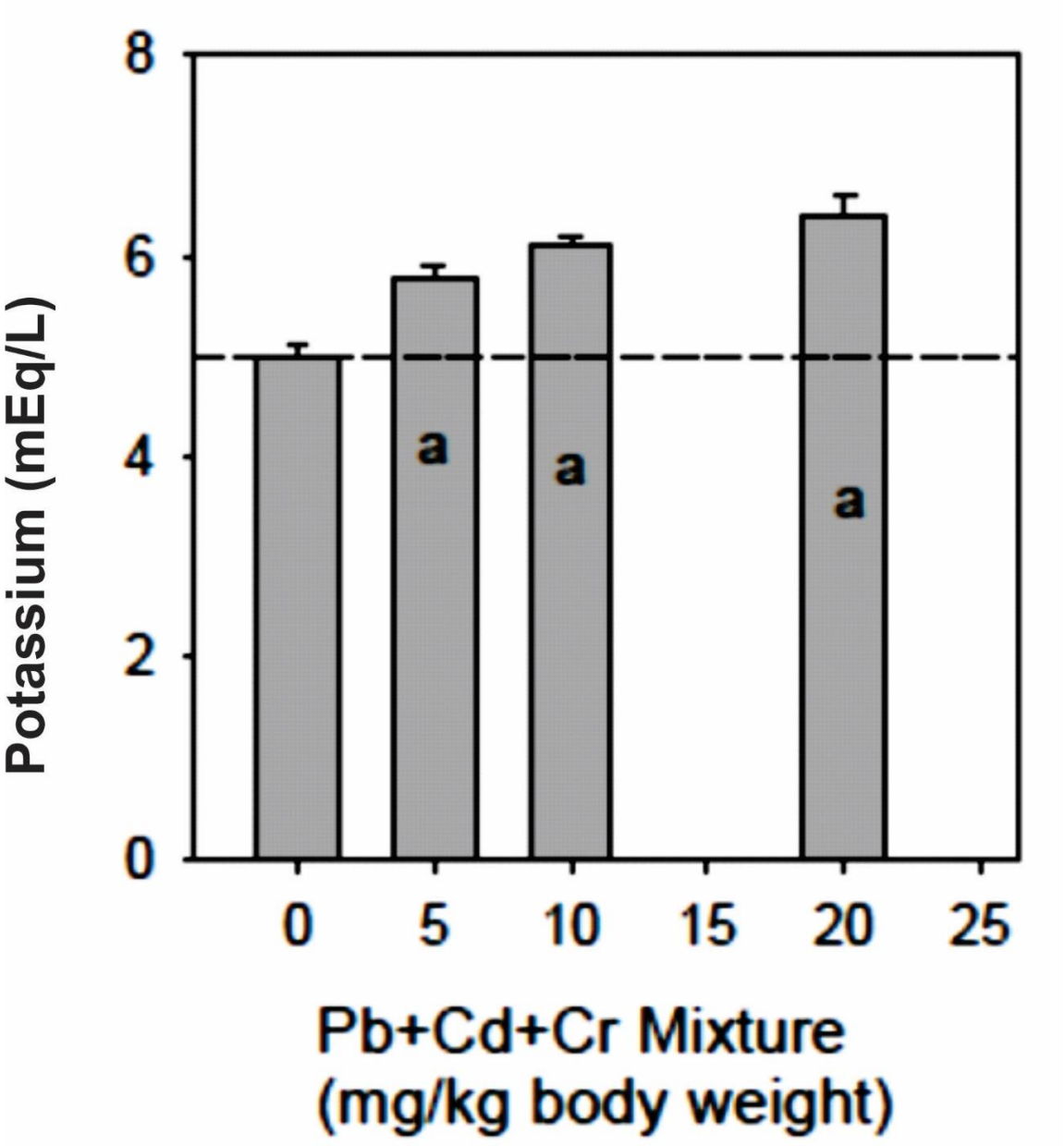


Figure 4.48: Dose-effect relationship for serum potassium concentration in albino rats treated with Pb, Cd and Cr as a mixture. a = significantly different from control ($p<0.05$).

Figures 4.45-4.48 showed that in the 5mg/kg treatment dose, there was significant increase ($p<0.05$) in mean serum potassium concentration in Cd, Cr, Pb individual and combined treatment groups compared with control. Also, there was significant difference ($p<0.05$) in mean serum potassium concentration in Cd, Cr, Pb individual treatment groups compared with the combined treatment group. Serum potassium concentration in the combined treatment group was lower than that of Cr treatment group that produced the highest effect in the individual treatments.

Figures 4.45-4.48 also showed that in the 10mg/kg treatment dose, there was significant increase ($p<0.05$) in mean serum potassium concentration in Cd, Cr, Pb individual and combined treatment groups compared with control. Also, there was significant difference ($p<0.05$) in mean serum potassium concentrationin Pb individual treatment group compared with the combined treatment group but there was no significant difference ($p>0.05$) in mean serum potassium concentrationin Cd, Cr individual treatment groups compared with the combined treatment group. Serum potassium concentration in the combined treatment group was higher than that of Pb treatment group but was not significantly different ($p>0.05$) from that of Cr that produced the highest effect in the individual treatment group.

Figure 4.45-4.48 also showed that in the 20mg/kg treatment dose, there was significant increase ($p<0.05$) in mean serum potassium concentration in Cd, Cr, Pb individual and combined treatment groups compared with control. Also, there was significant difference ($p<0.05$) in mean serum potassium concentration in Cr, Pb individual treatment groups compared with the combined treatment group but there was no significant difference ($p>0.05$) in mean serum potassium concentration in Cd individual treatment group compared with the combined treatment group. Serum potassium concentration in the combined treatment group was higher

than that of Pb, Cr treatment groups but was not significantly different ($p>0.05$) from that of Cd that produced the highest effect in the individual treatment group.

In the Cr treatment group, an inverted U-shaped hormetic effect was observed with regard to serum potassium concentration as treatment with the low dose (5mg/kg) produced a stimulating effect which was reduced at the high dose (20mg/kg). Treatment with Cd, Pb individually and the combined caused a dose dependent increase in mean serum potassium concentration as the dose increased.

Table 4.6 Use of effect-addition in assessment/prediction of serum potassium concentrationin albino rats treated with Pb, Cd and Cr individually and as a mixture

Dose(C)	EC_{mix}(expt)	EC_{Pb}	EC_{Cd}	EC_{Cr}	EC_{mix}(pred)	Nature of interaction
5mg/kg	0.8286	0.7571	0.7571	0.9429	0.9966	Less than additive
10mg/kg	0.8714	0.8286	0.8429	0.8571	0.9962	Less than additive
20mg/kg	0.9143	0.8571	0.9286	0.8142	0.9981	Less than additive

$E(C_i)$ obtained with maximum possible potassium concentration at 7 mEq/l.

From Table 4.6, the use of effect-addition in assessment of serum potassium concentration showed that the effect obtained from the experimented mixture was lower than the effect obtained by prediction using the addition of effect from the individual treatments, suggesting less

than additive interaction of the mixture components in the three treatment doses with respect to serum potassium concentration.

4.1.3.4 Serum chloride concentrationin albino rats treated with Pb, Cd and Cr individually and as a mixture

Figures 4.49-4.52 show dose-effect relationship for serum chloride concentration in albino rats treated with Pb, Cd and Cr individually and as a mixture.

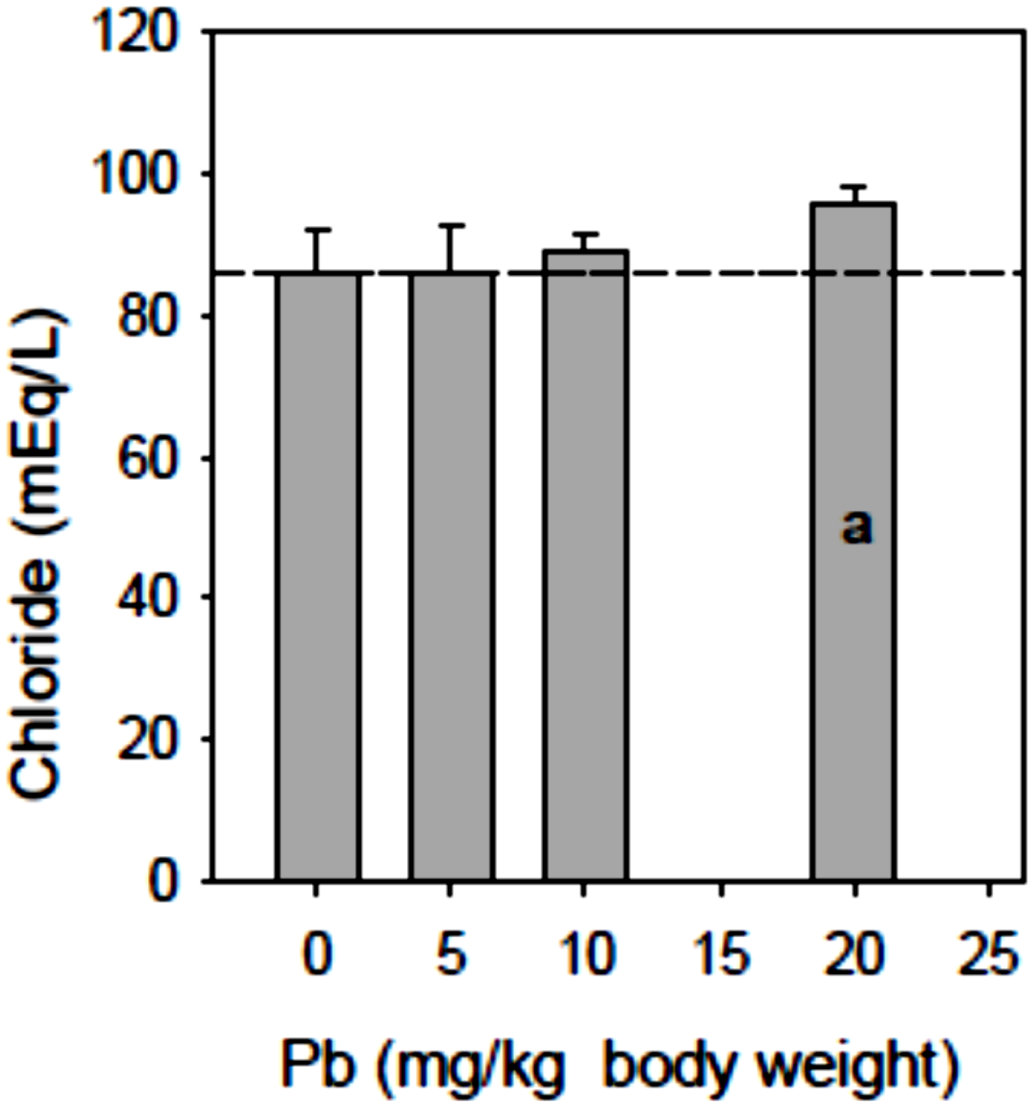


Figure 4.49: Dose-effect relationship for serum chloride concentration in albino rats treated with Pb. a = significantly different from control ($p<0.05$).

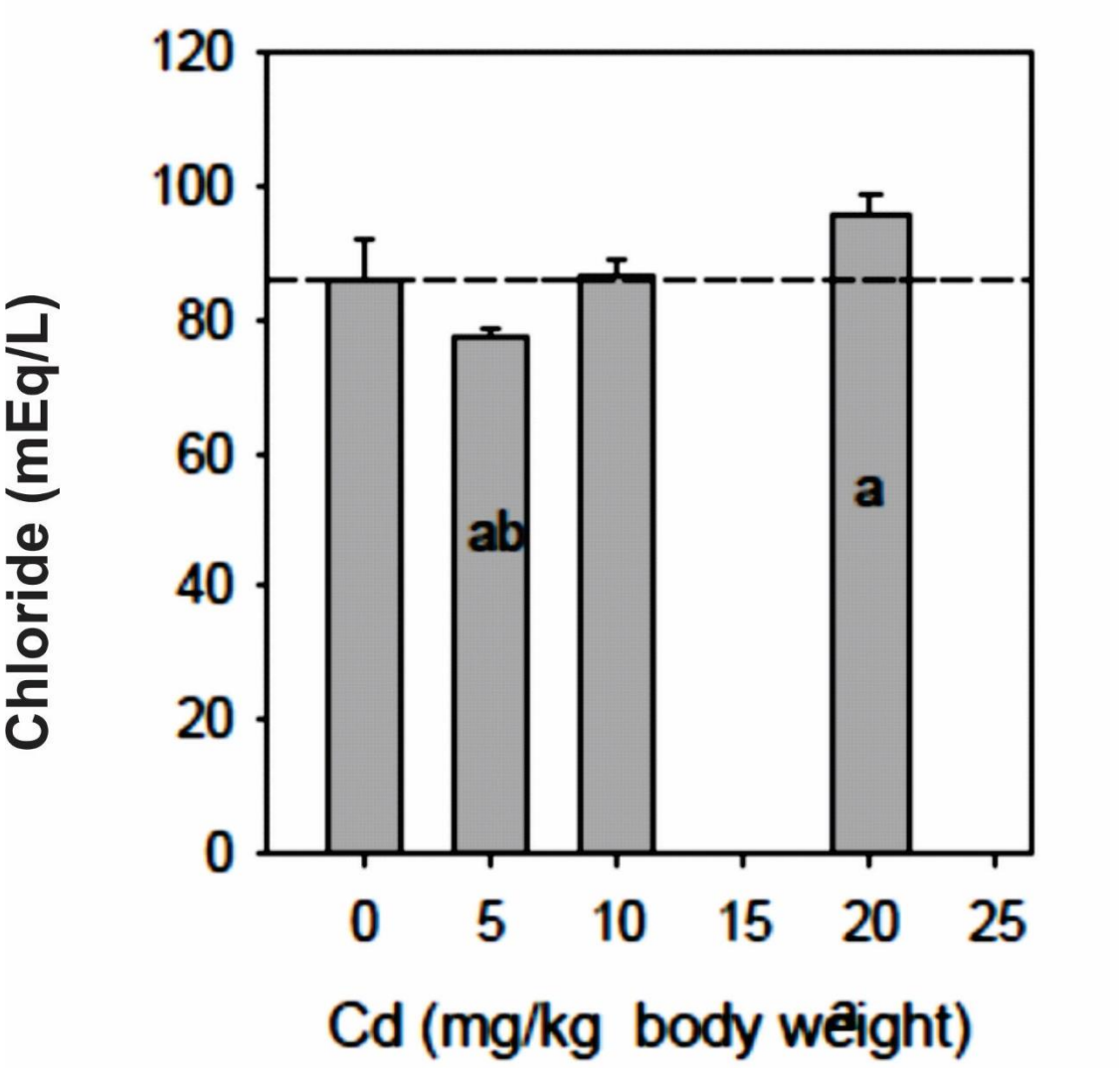


Figure 4.50: Dose-effect relationship for serum chloride concentration in albino rats treated with Cd. a = significantly different from control ($p<0.05$), b = significantly different from mixture ($p<0.05$).

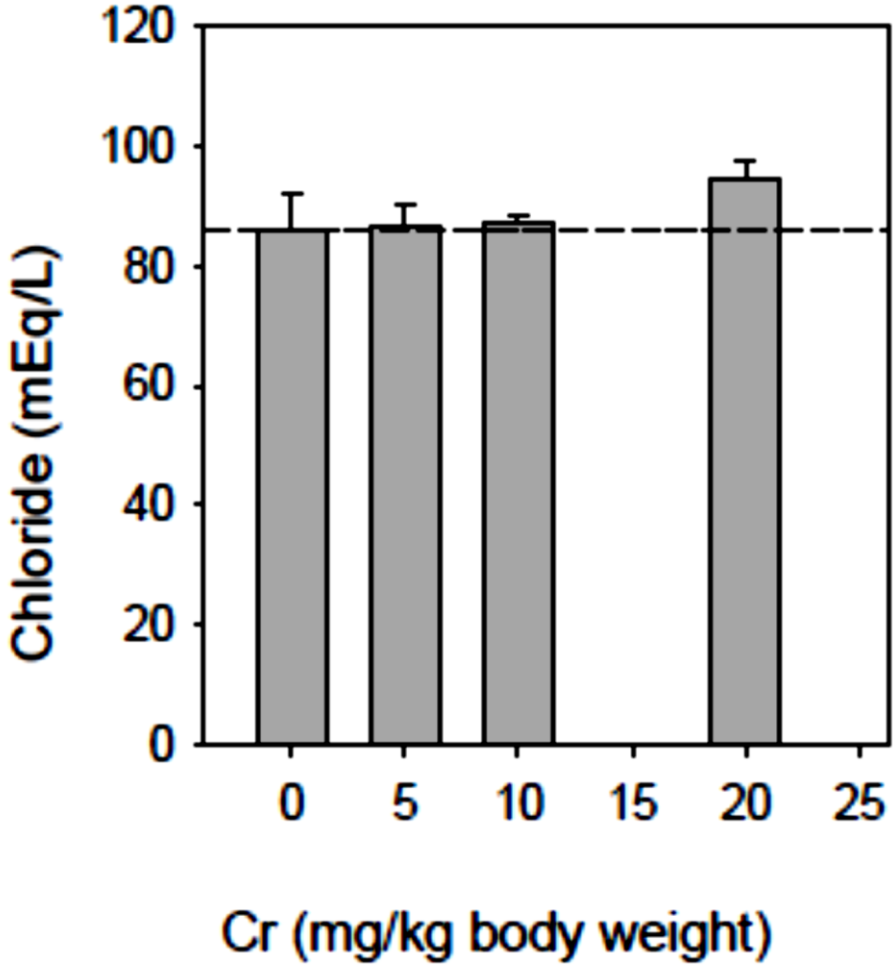


Figure 4.51: Dose-effect relationship for serum chloride concentration in albino rats treated with Cr individually and as a mixture

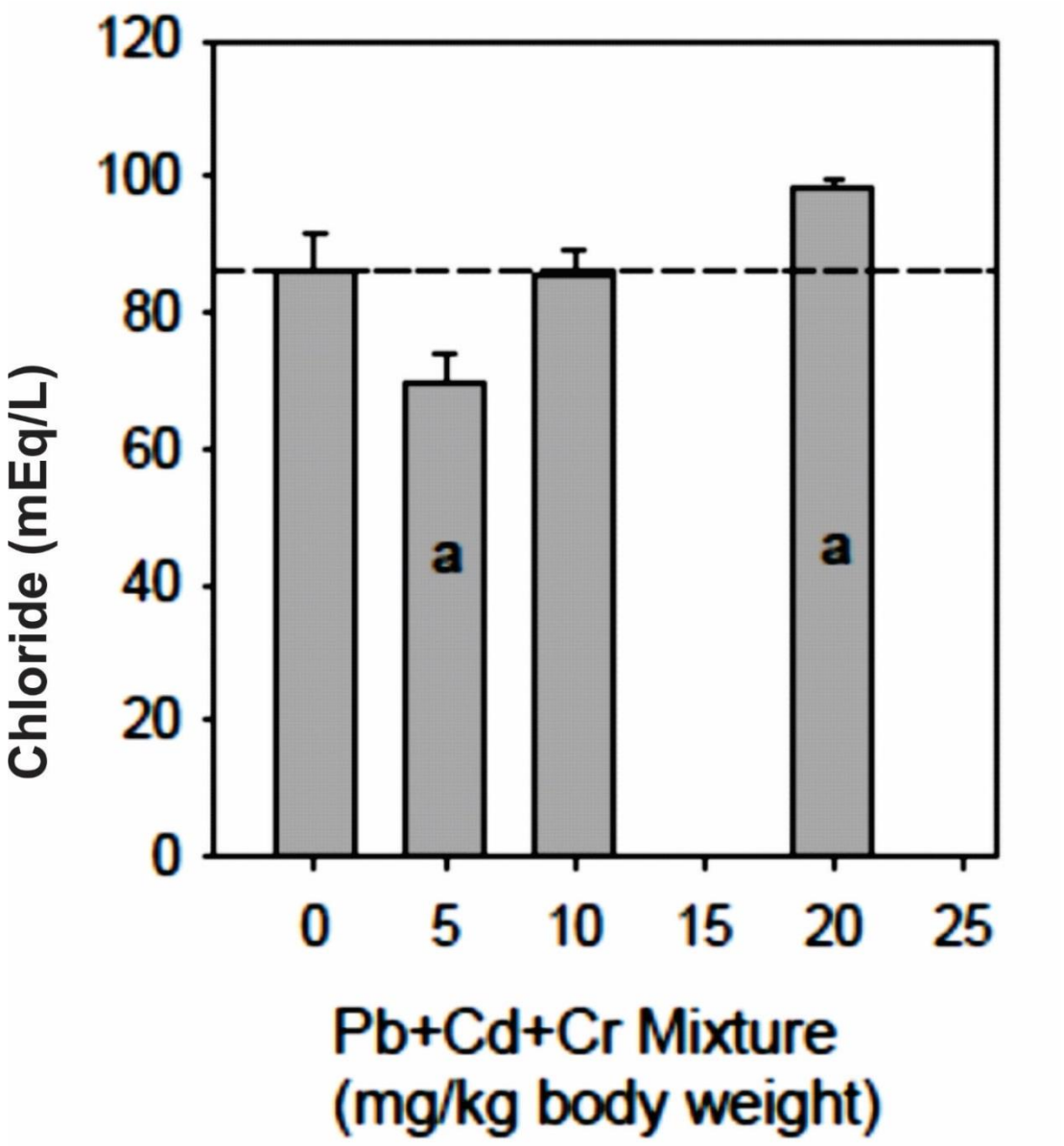


Figure 4.52: Dose-effect relationship for serum chloride concentration in albino rats treated with Pb, Cd and Cr as a mixture. a = significantly different from control ($p<0.05$).

Figures 4.49-4.52 showed that in the 5mg/kg treatment dose, there was significant difference ($p<0.05$) in mean serum chloride concentration in Cd individual and combined treatment groups compared with control but there was no significant difference ($p>0.05$) in mean serum chloride concentration in Cr, Pb individual treatment groups compared with control. Also, there was significant difference ($p<0.05$) in mean serum chloride concentration in Cd individual treatment group compared with the combined treatment group. Serum chloride concentration in the combined treatment group was significantly decreased ($p<0.05$) than that of Cd treatment group.

Figures 4.49-4.52 alsoshowed that in the 10mg/kg treatment dose, there was no significant difference ($p>0.05$) in mean serum chloride concentration in Cd, Cr, Pb individual and combined treatment groups compared with control. Also, there was no significant difference ($p>0.05$) in mean serum chloride concentration in Cd, Cr, Pb individual treatment groups compared with the combined treatment group.

Figures 4.49-4.52 showed that in the 20mg/kg treatment dose, there was significant increase ($p<0.05$) in mean serum chloride concentration in Cd, Pb individual and combined treatment groups compared with control but there was no significant difference ($p>0.05$) in mean serum chloride concentration in Cd, Pb individual treatment groups compared with the combined treatment group.

In the Cd and combined treatment groups, a hormetic effect was observed with regard to serum chloridr concentration as treatment with the low dose (5mg/kg) produced a stimulating effect which was reduced at the high dose (20mg/kg).

4.1.3.5 Serum inorganic phosphorus concentration in albino rats treated with Pb, Cd and Cr individually and as a mixture

Figures 4.53-4.56 show dose-effect relationship for serum inorganic phosphorus concentration in albino rats treated with Pb, Cd and Cr individually and as a mixture.

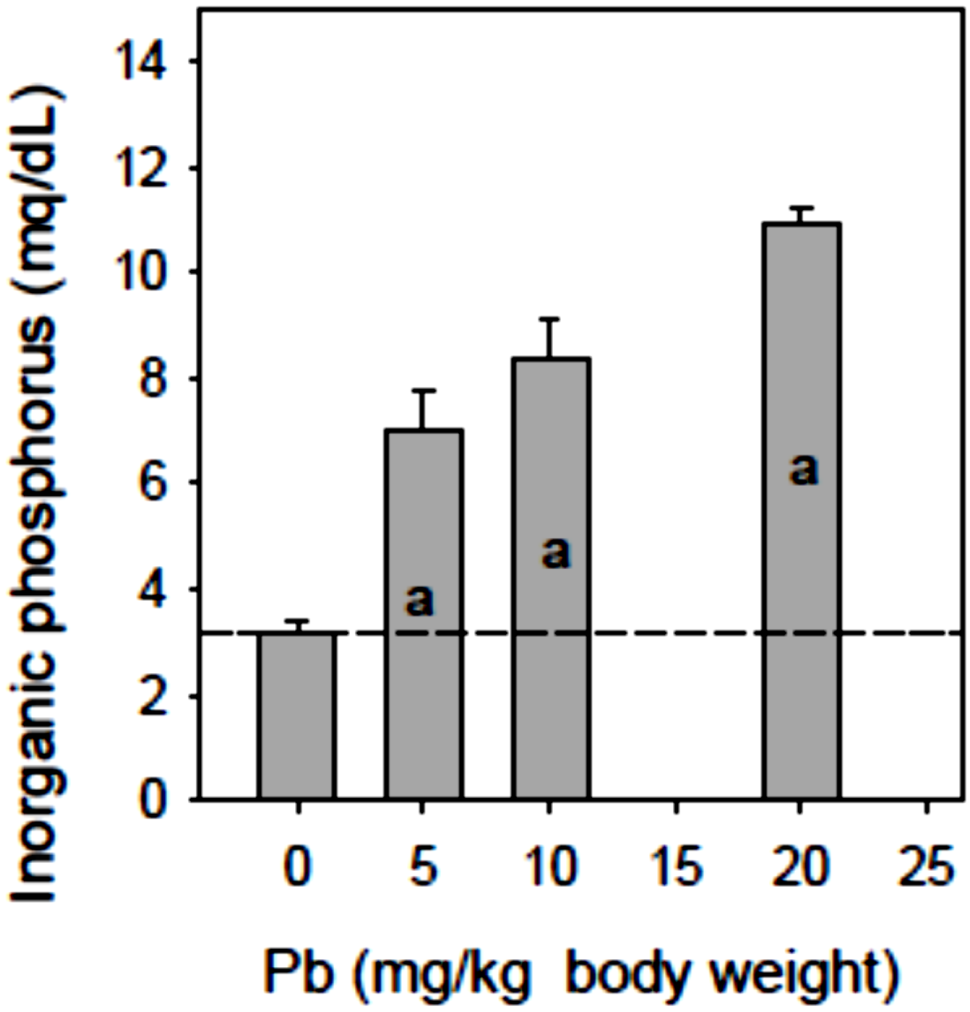


Figure 4.53: Dose-effect relationship for phosphorus concentration in albino rats treated with Pb. a = significantly different from control ($p<0.05$).

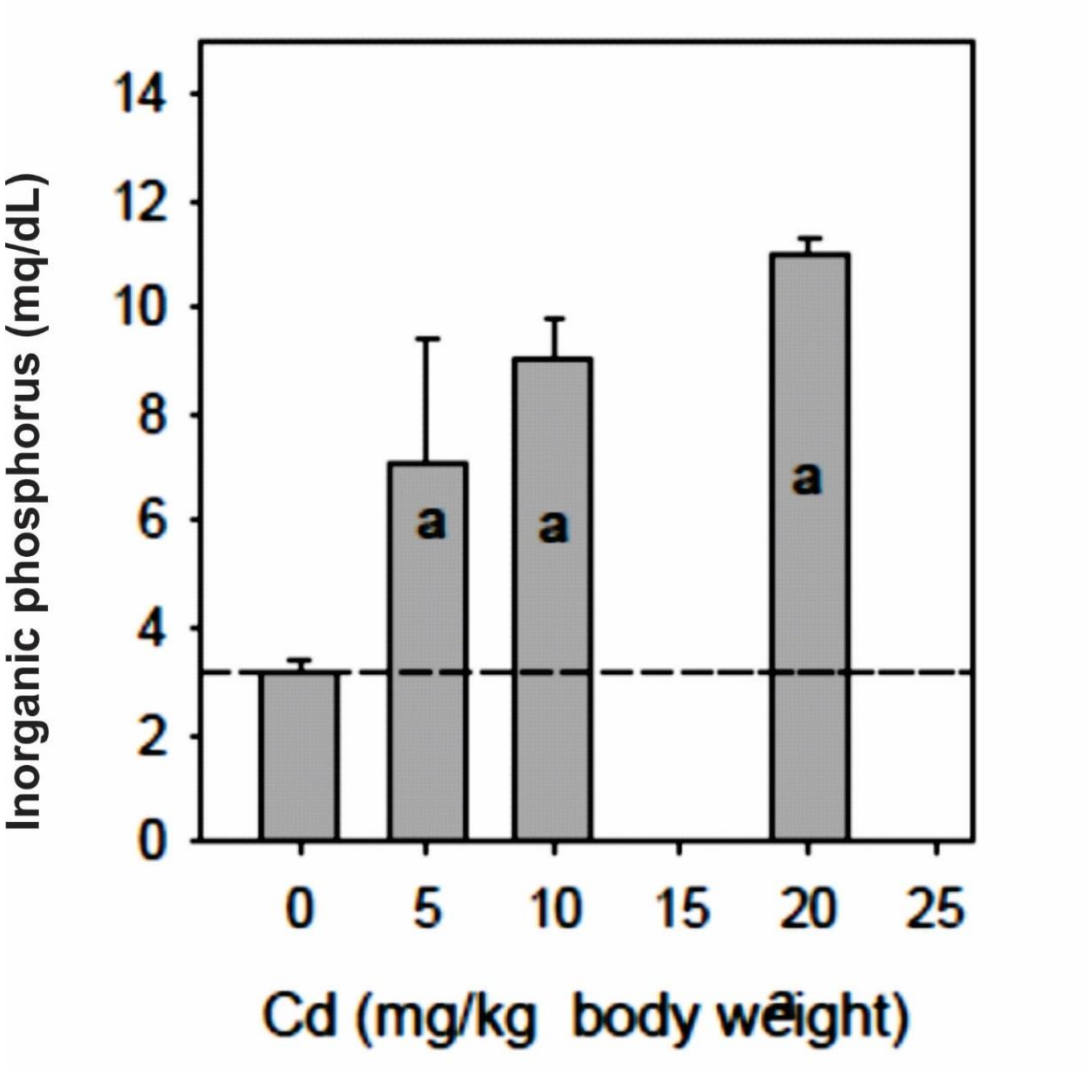


Figure 4.54: Dose-effect relationship for phosphorus concentration in albino rats treated with Pb, Cd and Cr individually and as a mixture. a = significantly different from control ($p<0.05$).

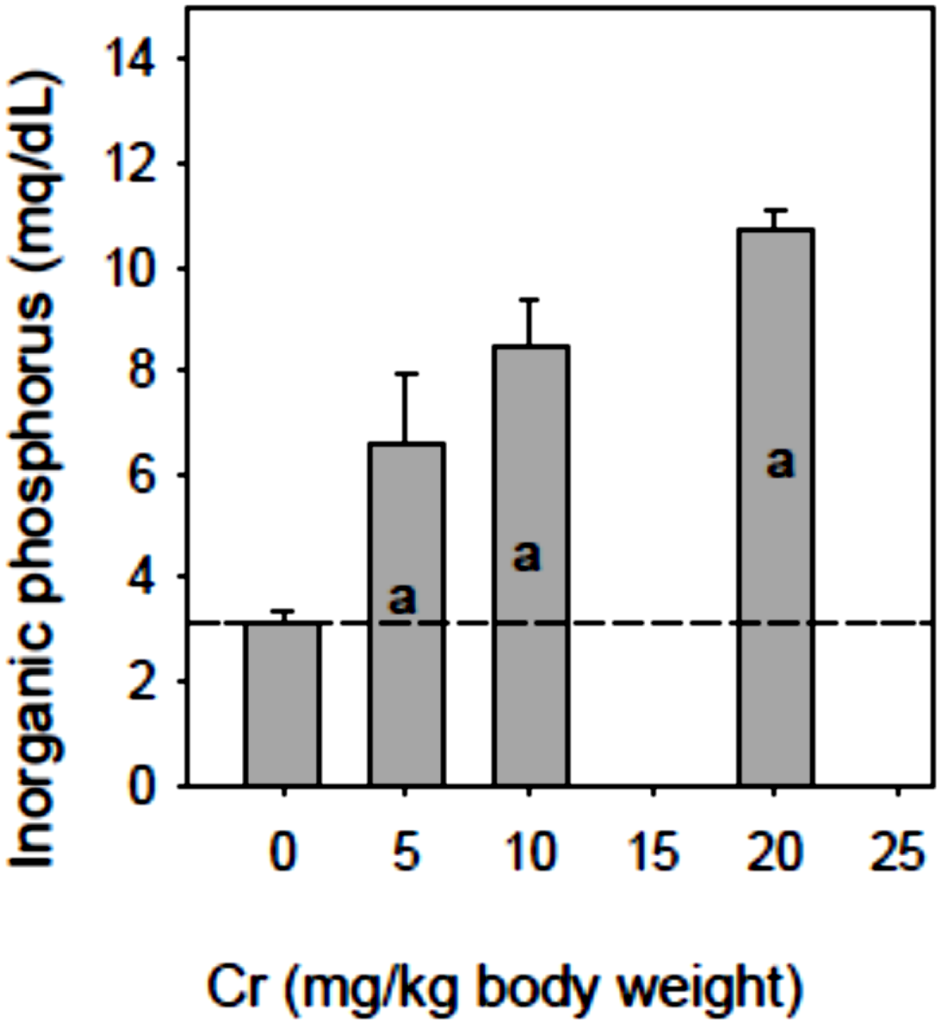


Figure 4.55: Dose-effect relationship for phosphorus concentration in albino rats treated with Cr. a = significantly different from control ($p<0.05$).

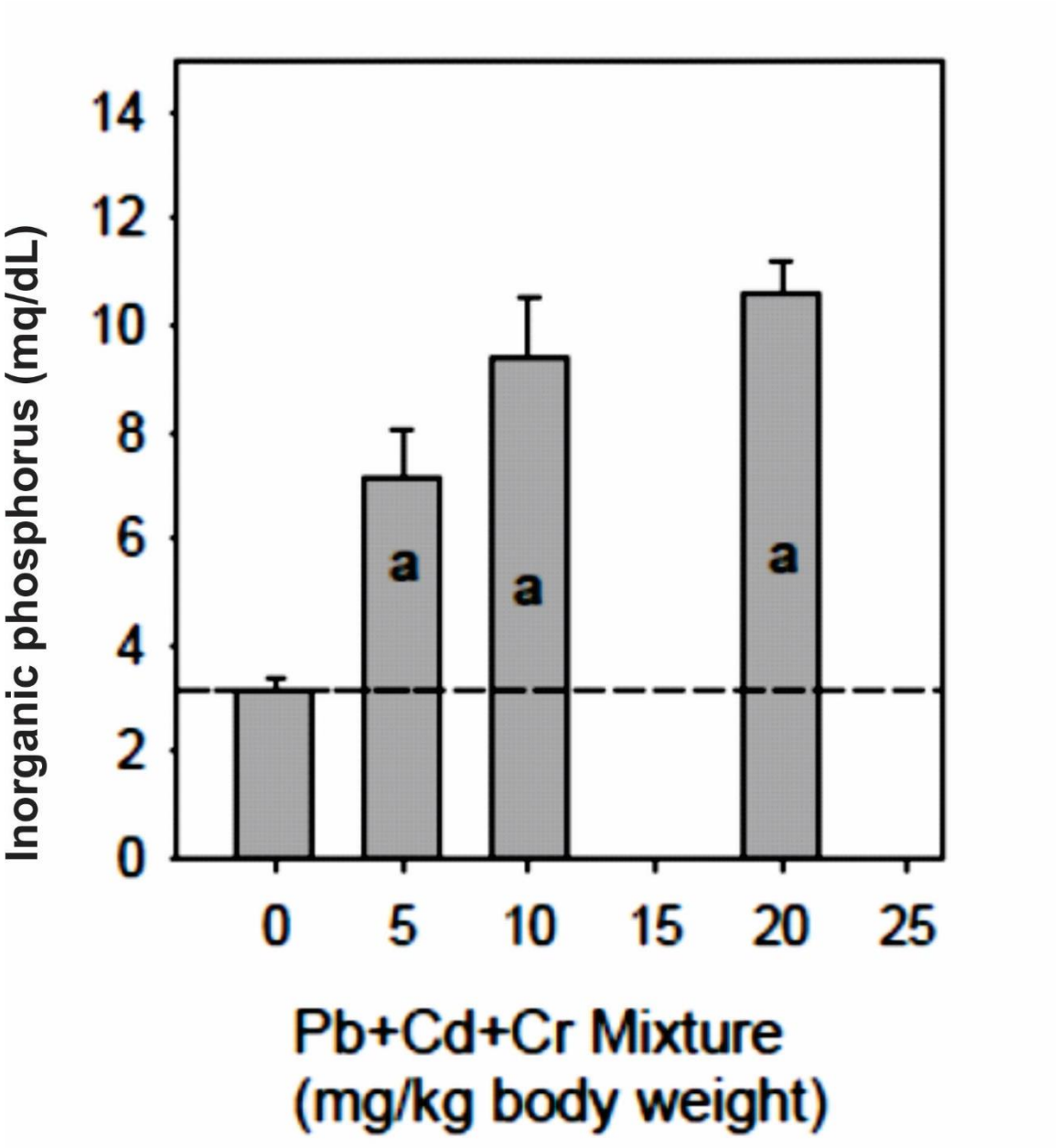


Figure 4.56: Dose-effect relationship for phosphorus concentration in albino rats treated with Pb, Cd and Cr as a mixture. a = significantly different from control ($p<0.05$).

Figures 4.53-4.56 showed that in the 5mg/kg treatment dose, there was significant increase ($p<0.05$) in mean serum inorganic phosphorus concentration in Cd, Cr, Pb individual and combined treatment groups compared with control but there was no significant difference ($p>0.05$) in mean serum inorganic phosphorus concentration in Cd, Cr, Pb individual treatment groups compared with the combined treatment group.

Figures 4.53-4.56 also showed that in the 10mg/kg treatment dose, there was significant increase ($p<0.05$) in mean serum inorganic phosphorus concentration in Cd, Cr, Pb individual and combined treatment groups compared with control but there was no significant difference ($p>0.05$) in mean serum inorganic phosphorus concentration in Cd, Cr, Pb individual treatment groups compared with the combined treatment group.

Figure 4.53-4.56 showed that in the 20mg/kg treatment dose, there was significant increase ($p<0.05$) in mean serum inorganic phosphorus concentration in Cd, Cr, Pb individual and combined treatment groups compared with control but there was no significant difference ($p>0.05$) in mean serum inorganic phosphorus concentration in Cd, Cr, Pb individual treatment groups compared with the combined treatment group.

Treatment with Cd, Cr, Pb individually and the combined caused a dose dependent increase in mean serum inorganic phosphorus concentration as the dose increased.

4.1.4 Oxidative stress markers

4.1.4.1 Serum malondialdehyde (MDA) concentration in albino rats treated with Pb, Cd and Cr individually and as a mixture

Figures 4.57-4.60 show dose-effect relationship for serum malondialdehyde (MDA) concentration in albino rats treated with Pb, Cd and Cr individually and as a mixture.

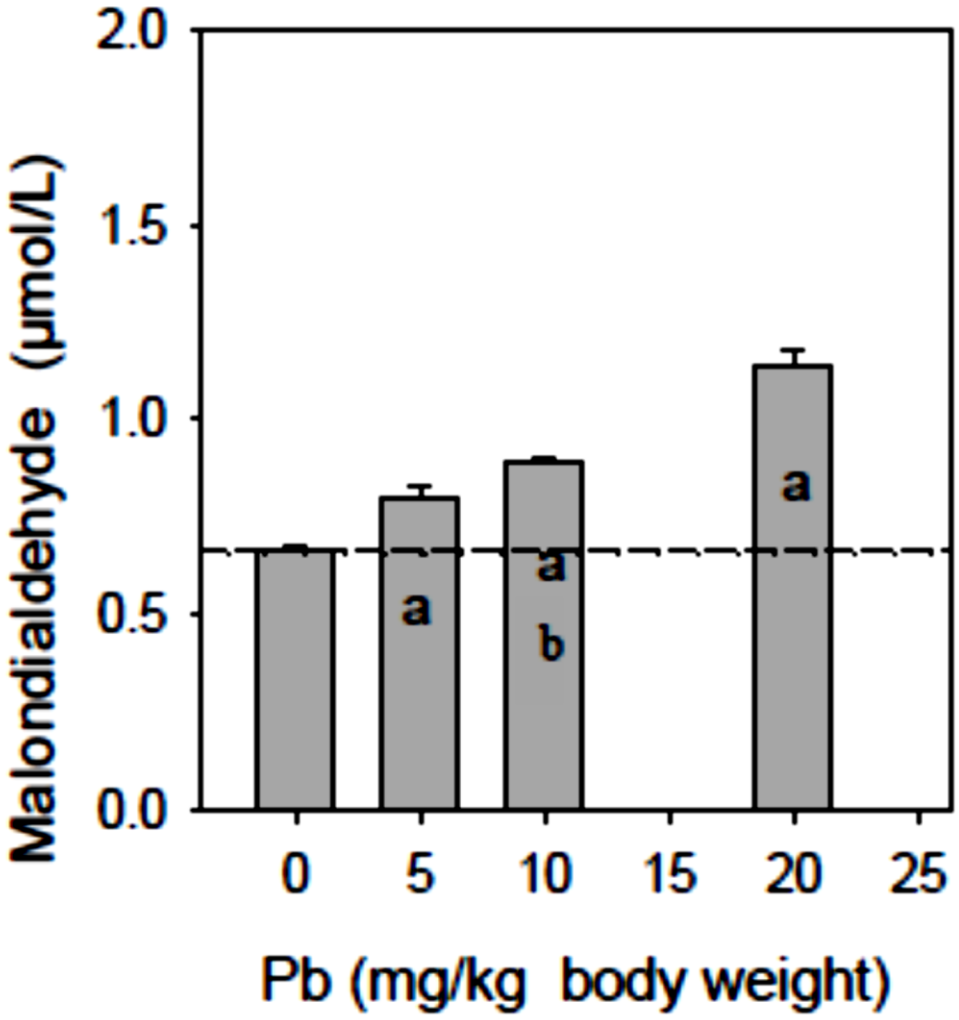


Figure 4.57: Dose-effect relationship for serum malondialdehyde (MDA) concentration in albino rats treated with Pb. a = significantly different from control ($p<0.05$), b = significantly different from mixture ($p<0.05$).

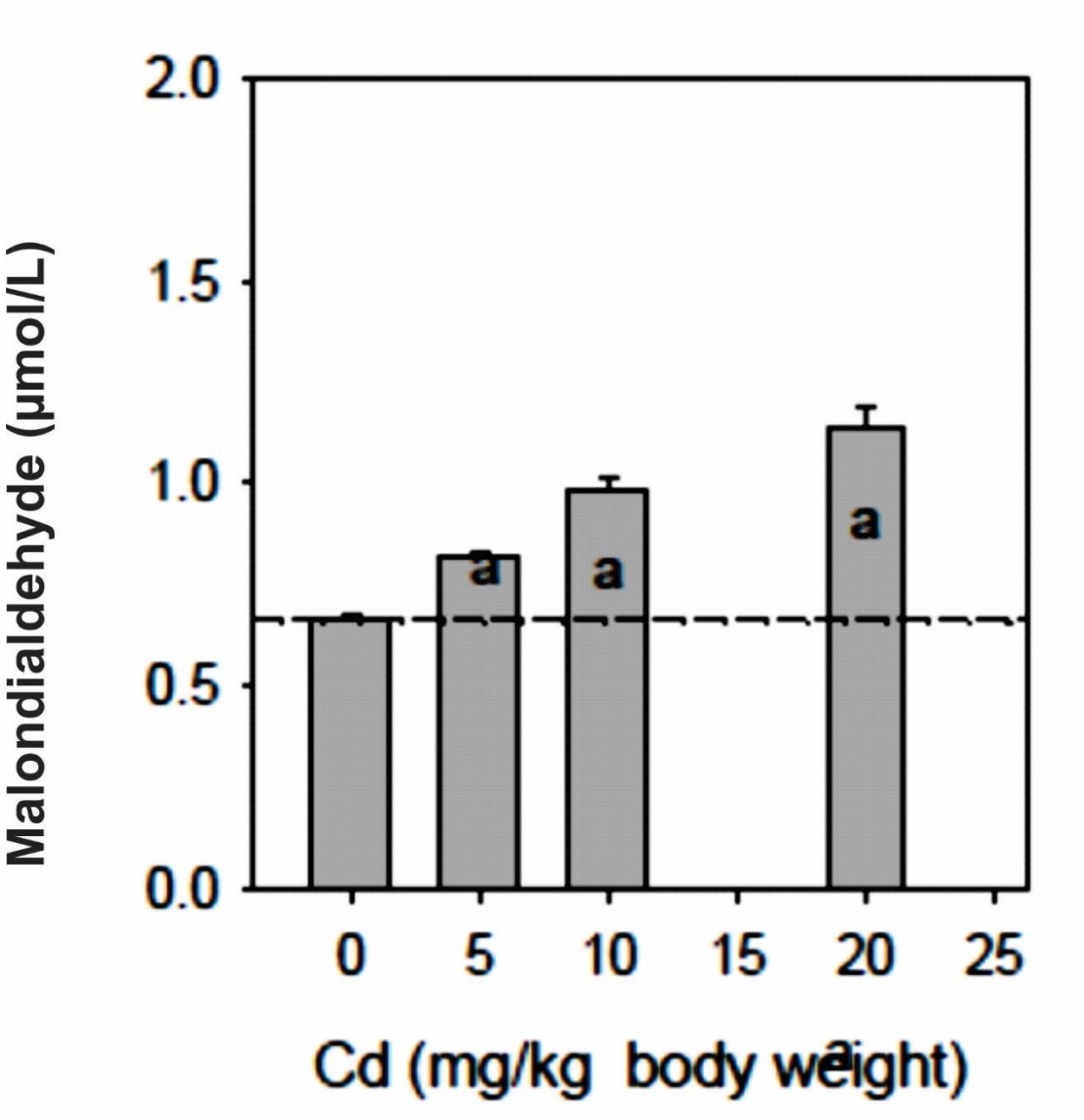


Figure 4.58: Dose-effect relationship for serum malondialdehyde (MDA) concentration in albino rats treated with Cd. a = significantly different from control ($p<0.05$).

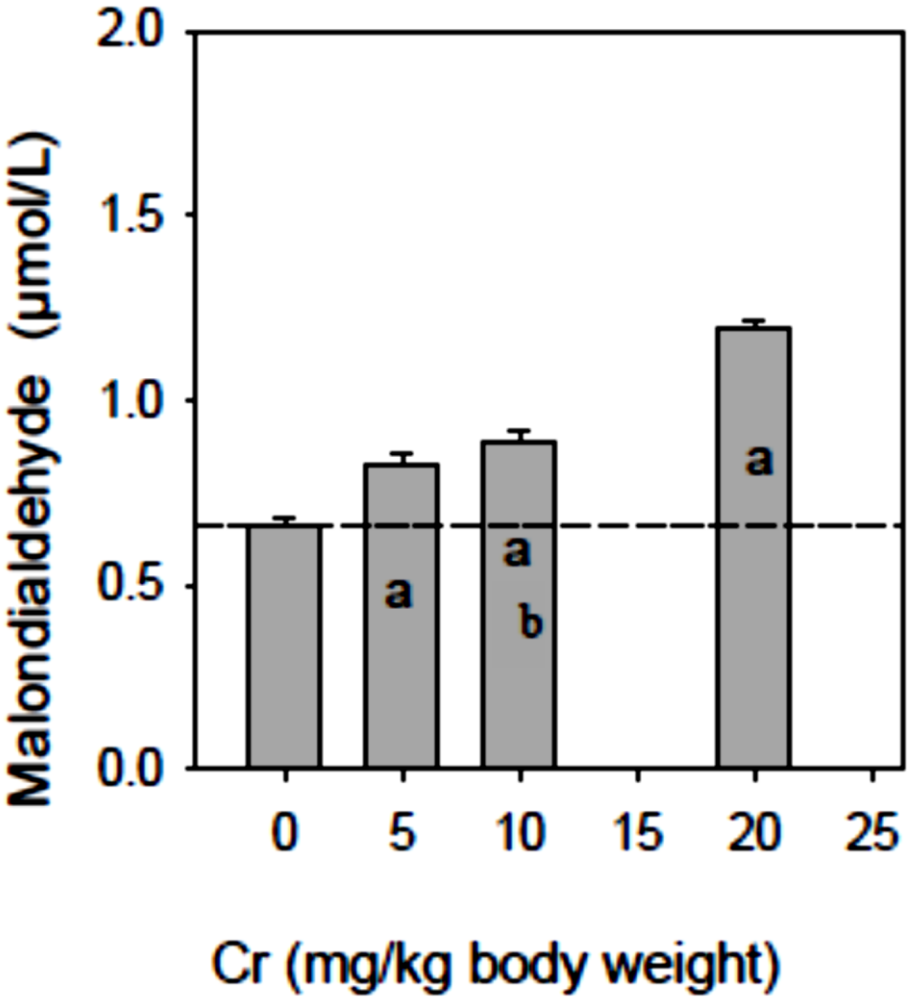


Figure 4.59: Dose-effect relationship for serum malondialdehyde (MDA) concentration in albino rats treated with Cr. a = significantly different from control ($p<0.05$), b = significantly different from mixture ($p<0.05$).

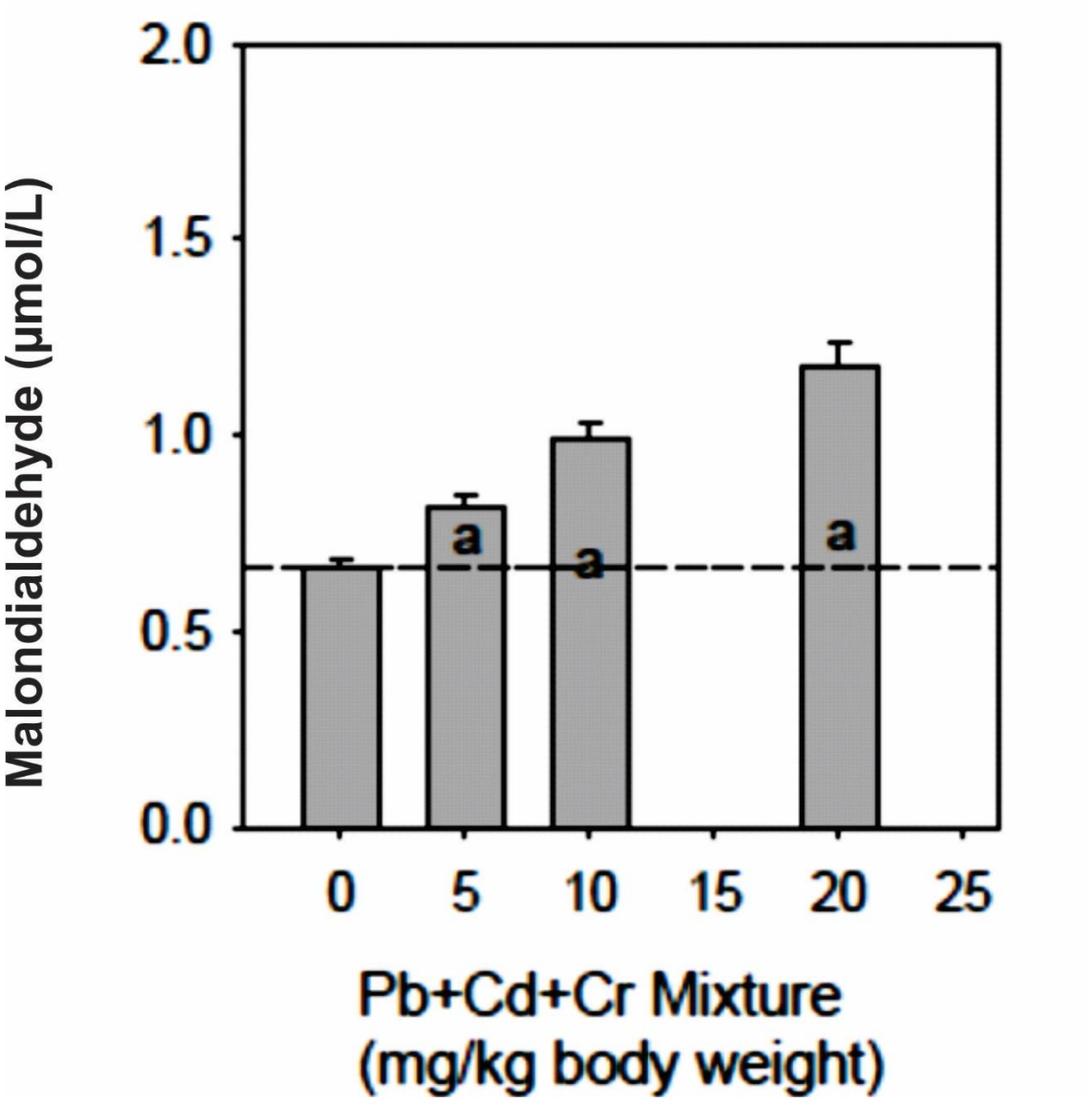


Figure 4.60: Dose-effect relationship for serum malondialdehyde (MDA) concentration in albino rats treated with Pb, Cd and Cr as a mixture. a = significantly different from control ($p<0.05$).

Figures 4.57-4.60 showed that in the 5mg/kg treatment dose, there was significant increase ($p<0.05$) in mean serum MDA concentration in Cd, Cr, Pb individual and combined treatment groups compared with control but there was no significant difference ($p>0.05$) in mean serum MDA concentration in Cd, Cr, Pb individual treatment groups compared with the combined treatment group.

Figures 4.57-4.60 also showed that in the 10mg/kg treatment dose, there was significant increase ($p<0.05$) in mean serum MDA concentration in Cd, Cr, Pb individual and combined treatment groups compared with control. Also, there was significant difference ($p<0.05$) in mean serum MDA concentration in Cr, Pb individual treatment groups compared with the combined treatment group but there was no significant difference ($p>0.05$) in mean serum MDA concentration in Cd individual treatment group compared with the combined treatment group. Serum MDA concentration in the combined treatment group was higher than that of Cr, Pb treatment groups but was not significantly different ($p>0.05$) from that of Cd that produced the highest effect in the individual treatment group.

Figures 4.57-4.60 showed that in the 20mg/kg treatment dose, there was significant increase ($p<0.05$) in mean serum MDA concentration in Cd, Cr, Pb individual and combined treatment groups compared with control but there was no significant difference ($p>0.05$) in mean serum MDA concentration in Cd, Cr, Pb individual treatment groups compared with the combined treatment group.

Treatment with Cd, Cr, Pb individually and the combined caused a dose dependent increase in mean serum MDA concentration as the dose increased.

4.1.4.2 Serum reduced glutathione (GSH) concentrationinalbino rats treated with Pb, Cd and Cr individually and as a mixture

Figures 4.61-4.64 show dose-effect relationship for reduced glutathione (GSH) concentration in albino rats treated with Pb, Cd and Cr individually and as a mixture.

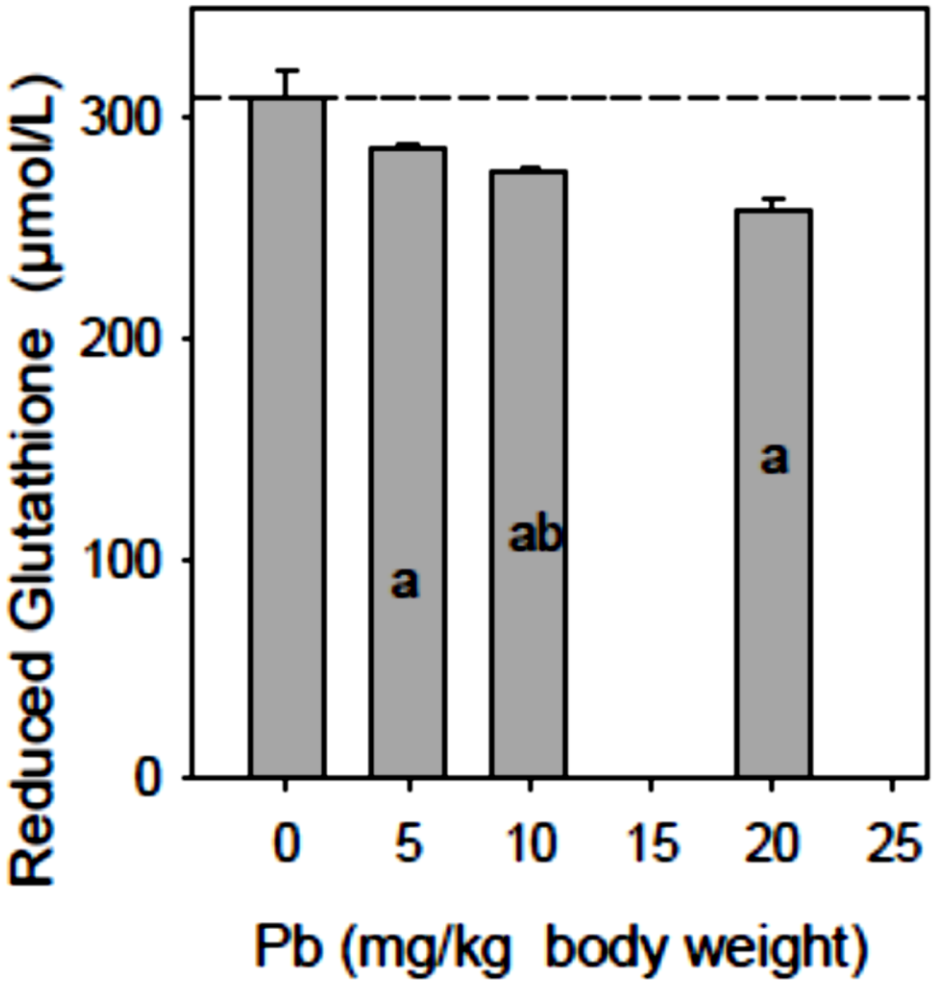


Figure 4.61: Dose-effect relationship for reduced glutathione (GSH) concentration in albino rats treated with Pb. a = significantly different from control ($p<0.05$), b = significantly different from mixture ($p<0.05$).

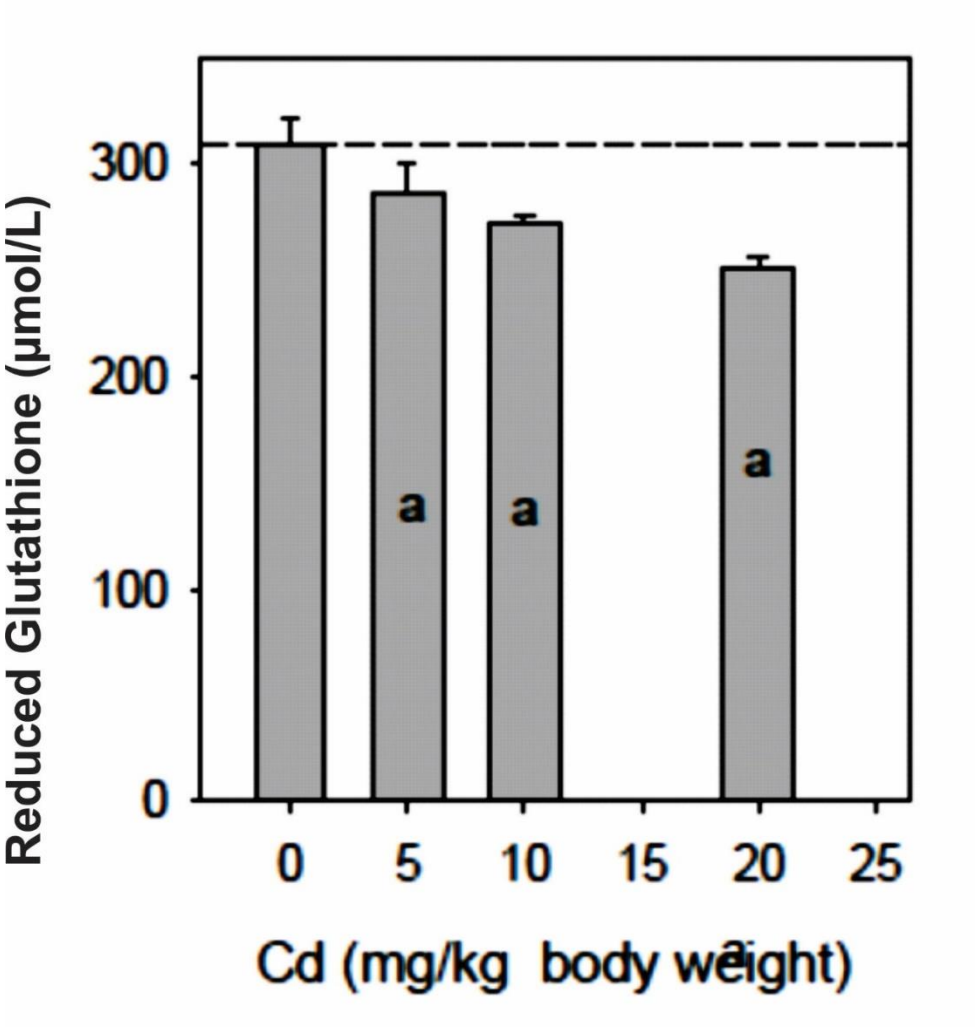


Figure 4.62: Dose-effect relationship for reduced glutathione (GSH) concentration in albino rats treated with Cd. a = significantly different from control ($p<0.05$).

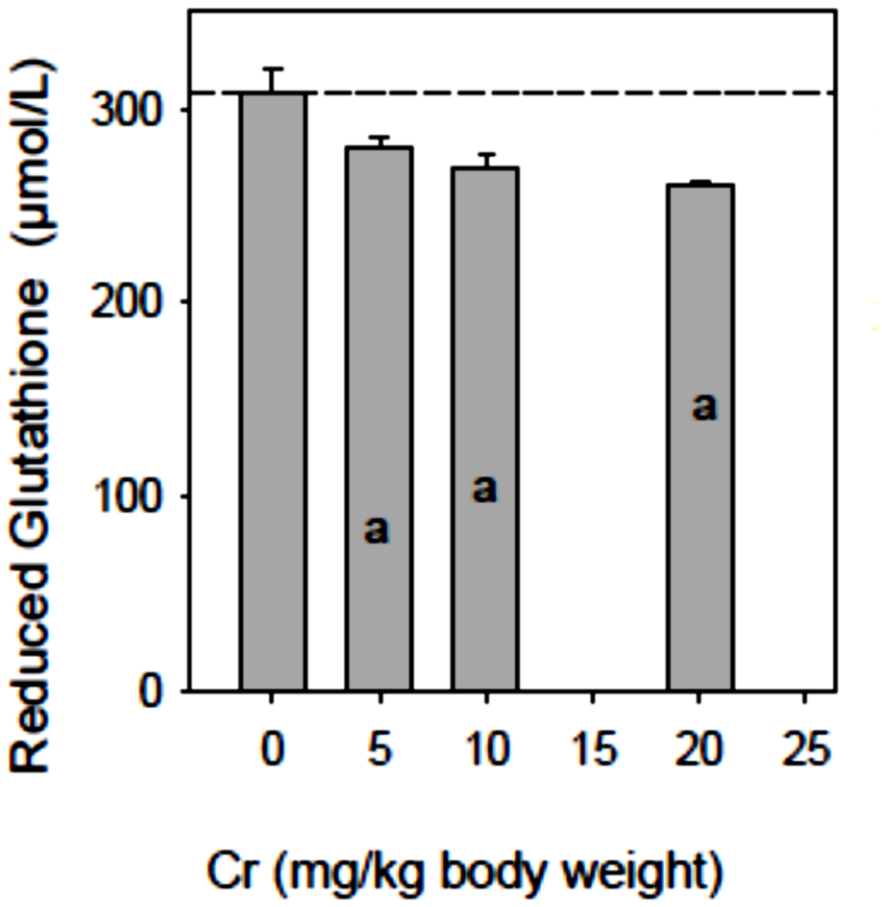


Figure 4.63: Dose-effect relationship for reduced glutathione (GSH) concentration in albino rats treated with Cr. a = significantly different from control ($p<0.05$).

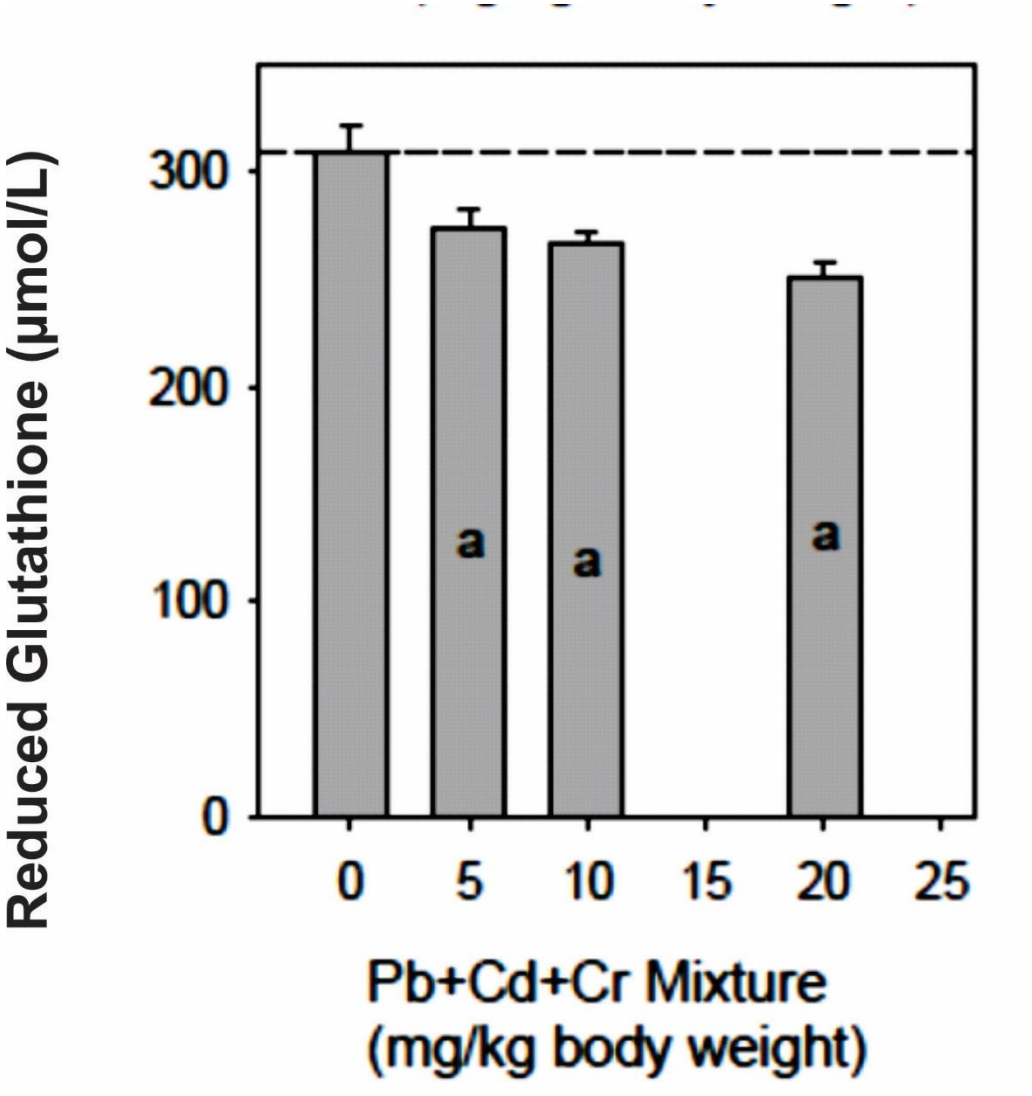


Figure 4.64: Dose-effect relationship for reduced glutathione (GSH) concentration in albino rats treated with Pb, Cd and Cr as a mixture. a = significantly different from control ($p<0.05$),

Figures 4.61-4.64 showed that in the 5mg/kg treatment dose, there was significant decrease ($p<0.05$) in mean serum GSH concentration in Cd, Cr, Pb individual and combined treatment groups compared with control but there was no significant difference ($p>0.05$) in mean serum GSH concentration in Cd, Cr, Pb individual treatment groups compared with the combined treatment group.

Figure 4.61-4.64 also showed that in the 10mg/kg treatment dose, there was significant decrease ($p<0.05$) in mean serum GSH concentration in Cd, Cr, Pb individual and combined treatment groups compared with control. Also, there was significant difference ($p<0.05$) in mean serum GSH concentration in Pb individual treatment group compared with the combined treatment group but there was no significant difference ($p>0.05$) in mean serum GSH concentration in Cd, Cr individual treatment groups compared with the combined treatment group. Serum GSH concentration in the combined treatment group was lower than that of Pb treatment group.

Figure 4.61-4.64 showed that in the 20mg/kg treatment dose, there was significant decrease ($p<0.05$) in mean serum GSH concentration in Cd, Cr, Pb individual and combined treatment groups compared with control but there was no significant difference ($p>0.05$) in mean serum GSH concentration in Cd, Cr, Pb individual treatment groups compared with the combined treatment group.

Treatment with Cd, Cr, Pb individually and the combined caused a dose dependent decrease in mean serum GSH concentration as the dose increased.

4.1.4.3 Serum ascorbic acid concentration in albino rats treated with Pb, Cd and Cr individually and as a mixture

Figures 4.65-4.68 show dose-effect relationship for serum ascorbic acid (AA) concentration in albino rats treated with Pb, Cd and Cr individually and as a mixture.

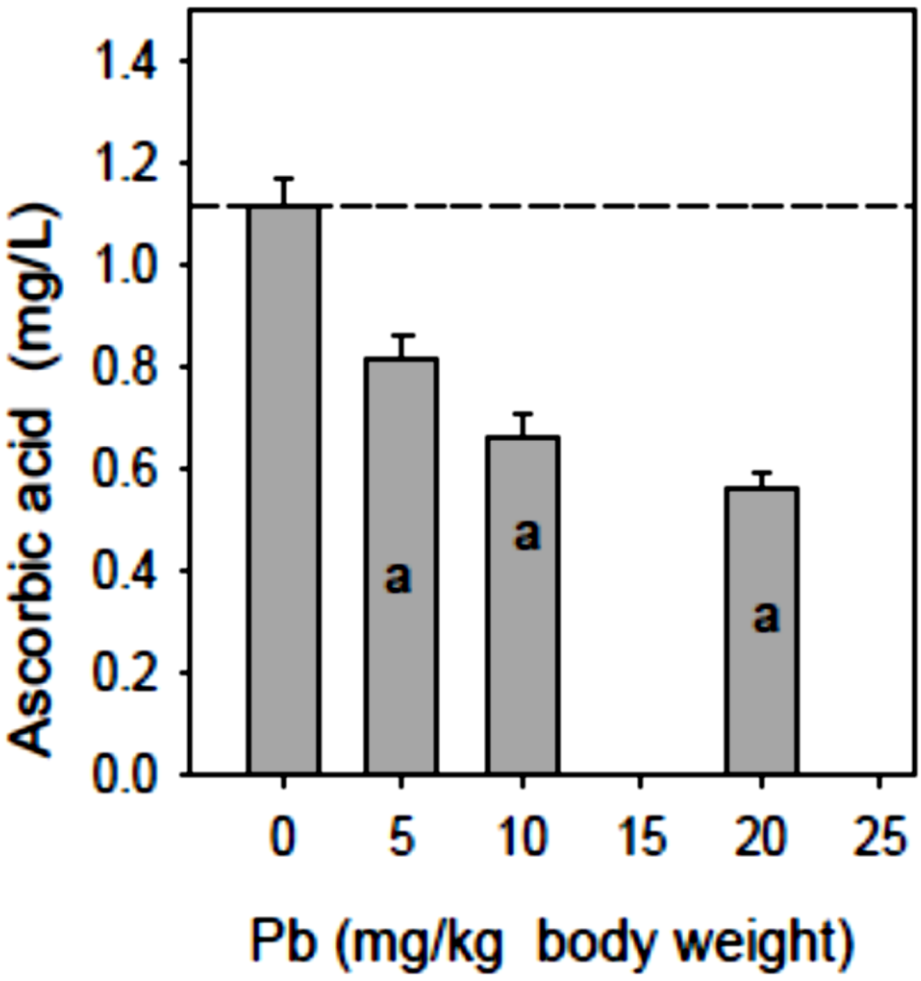


Figure 4.65: Dose-effect relationship for serum ascorbic acid (AA) concentration in albino rats treated with Pb. a = significantly different from control ($p<0.05$).

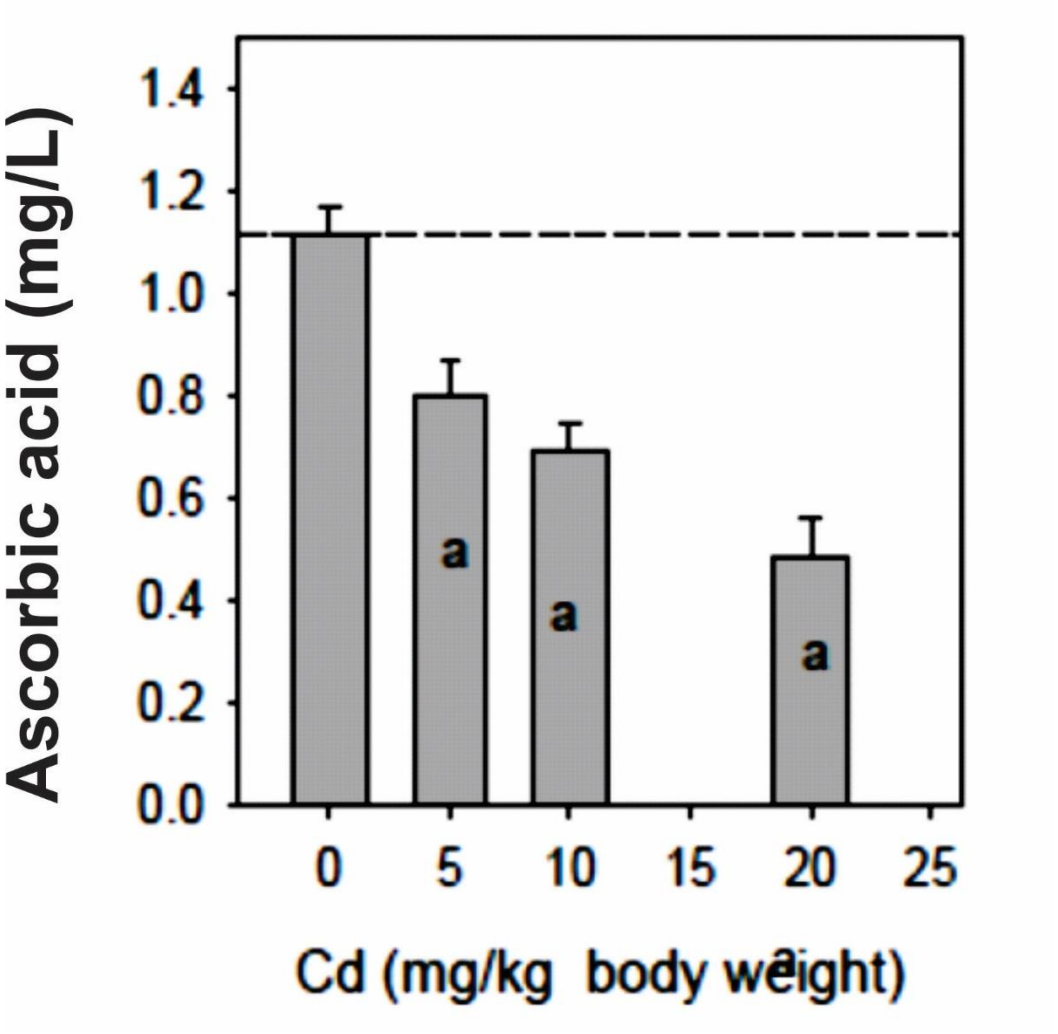


Figure 4.66: Dose-effect relationship for serum ascorbic acid (AA) concentration in albino rats treated with Cd. a = significantly different from control ($p<0.05$).

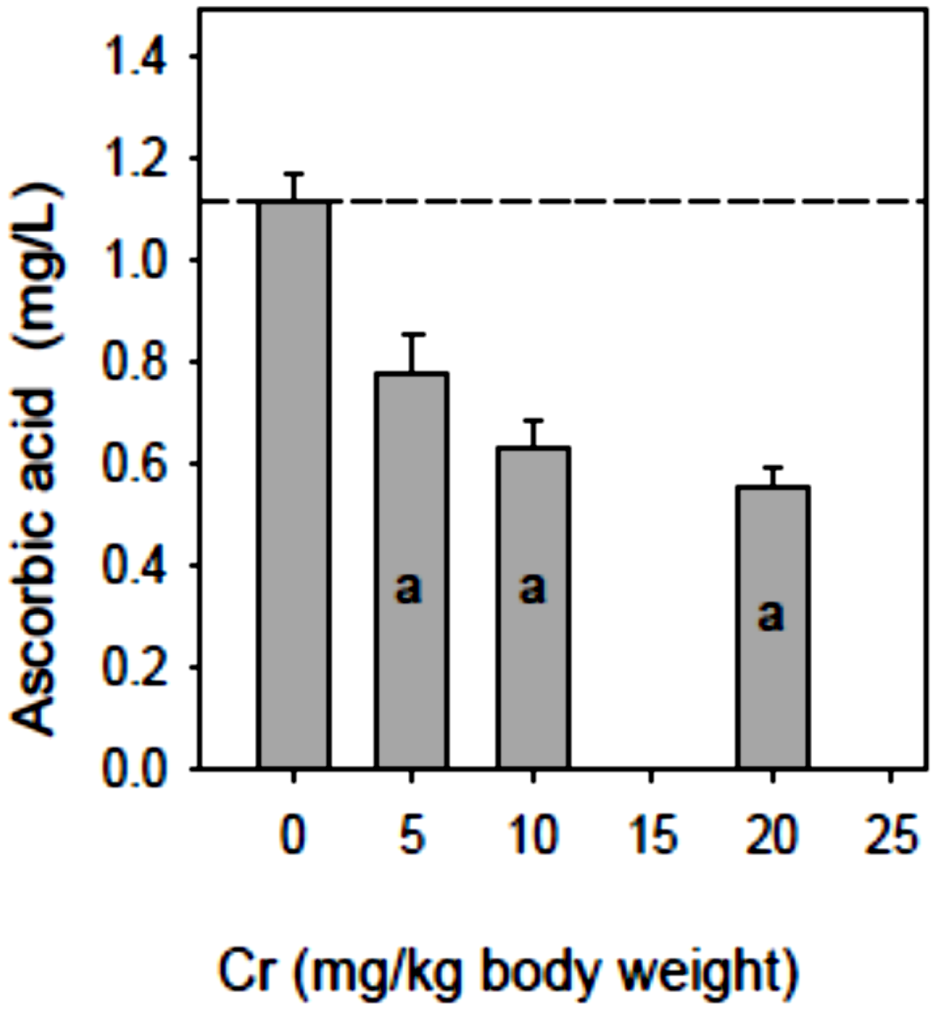


Figure 4.67: Dose-effect relationship for serum ascorbic acid (AA) concentration in albino rats treated with Cr. a = significantly different from control ($p<0.05$).

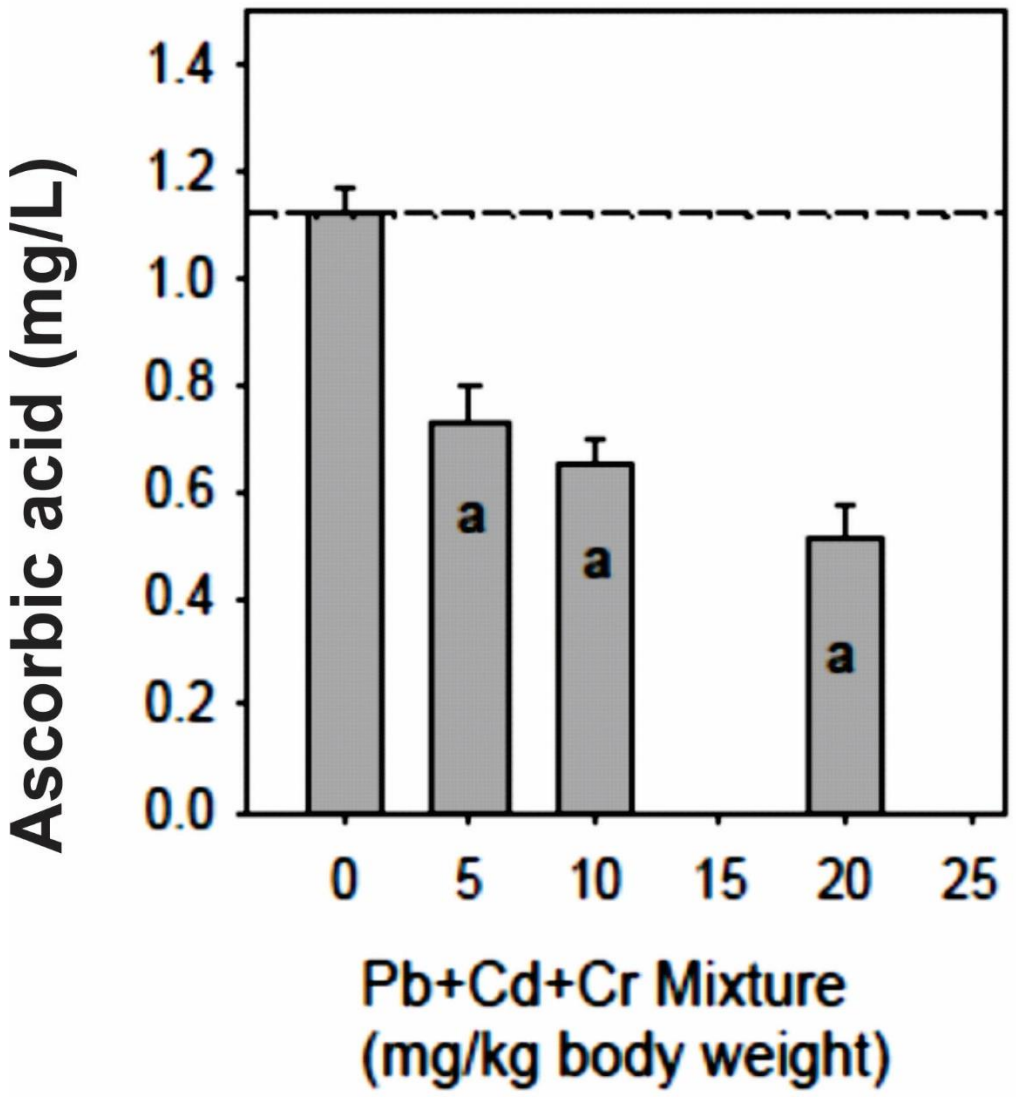


Figure 4.68: Dose-effect relationship for serum ascorbic acid (AA) concentration in albino rats treated with Pb, Cd and Cr as a mixture. a = significantly different from control ($p<0.05$).

Figures 4.65-4.68 showed that in the 5mg/kg treatment dose, there was significant decrease ($p<0.05$) in mean serum ascorbic acid concentration in Cd, Cr, Pb individual and combined treatment groups compared with control but there was no significant difference ($p>0.05$) in mean serum ascorbic acid concentration in Cd, Cr, Pb individual treatment groups compared with the combined treatment group.

Figures 4.65-4.68 also showed that in the 10mg/kg treatment dose, there was significant decrease ($p<0.05$) in mean serum ascorbic acid concentration in Cd, Cr, Pb individual and combined treatment groups compared with control but there was no significant difference ($p>0.05$) in mean serum ascorbic acid concentration in Cd, Cr, Pb individual treatment groups compared with the combined treatment group.

Figure 4.65-4.68 showed that in the 20mg/kg treatment dose, there was significant decrease ($p<0.05$) in mean serum ascorbic acid concentration in Cd, Cr, Pb individual and combined treatment groups compared with control but there was no significant difference ($p>0.05$) in mean serum ascorbic acid concentration in Cd, Cr, Pb individual treatment groups compared with the combined treatment group.

Treatment with Cd, Cr, Pb individually and the combined caused a dose dependent decrease in mean serum ascorbic acid concentration as the dose increased.

4.1.4.4 Serum superoxide dismutase (SOD) activity in albino rats treated with Pb, Cd and Cr individually and as a mixture

Figures 4.69-4.72 show dose-effect relationship for superoxide dismutase (SOD) activity in albino rats treated with Pb, Cd and Cr individually and as a mixture.

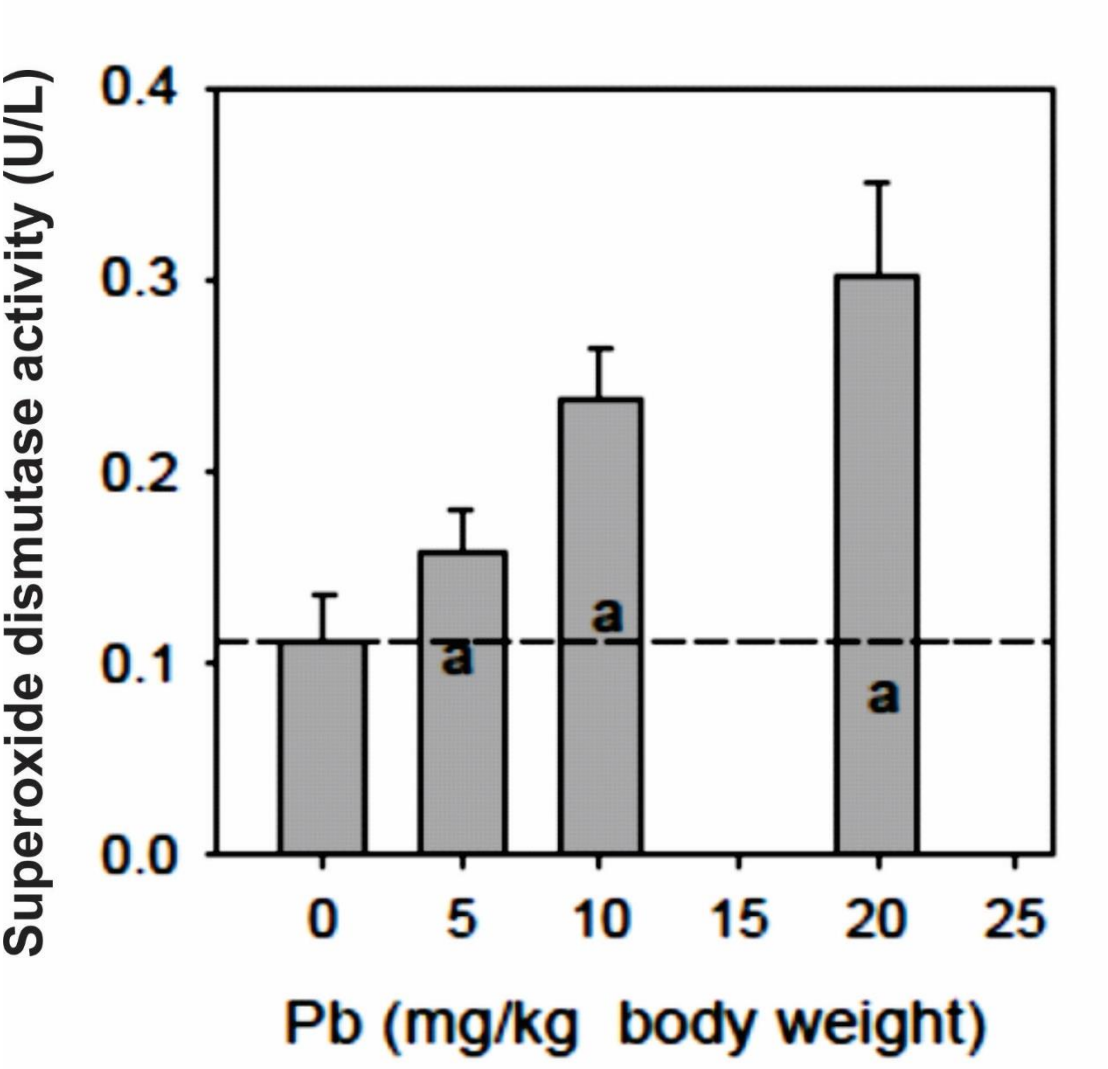


Figure 4.69: Dose-effect relationship for serum superoxide dismutase (SOD) activity in albino rats treated with Pb. a = significantly different from control ($p<0.05$).

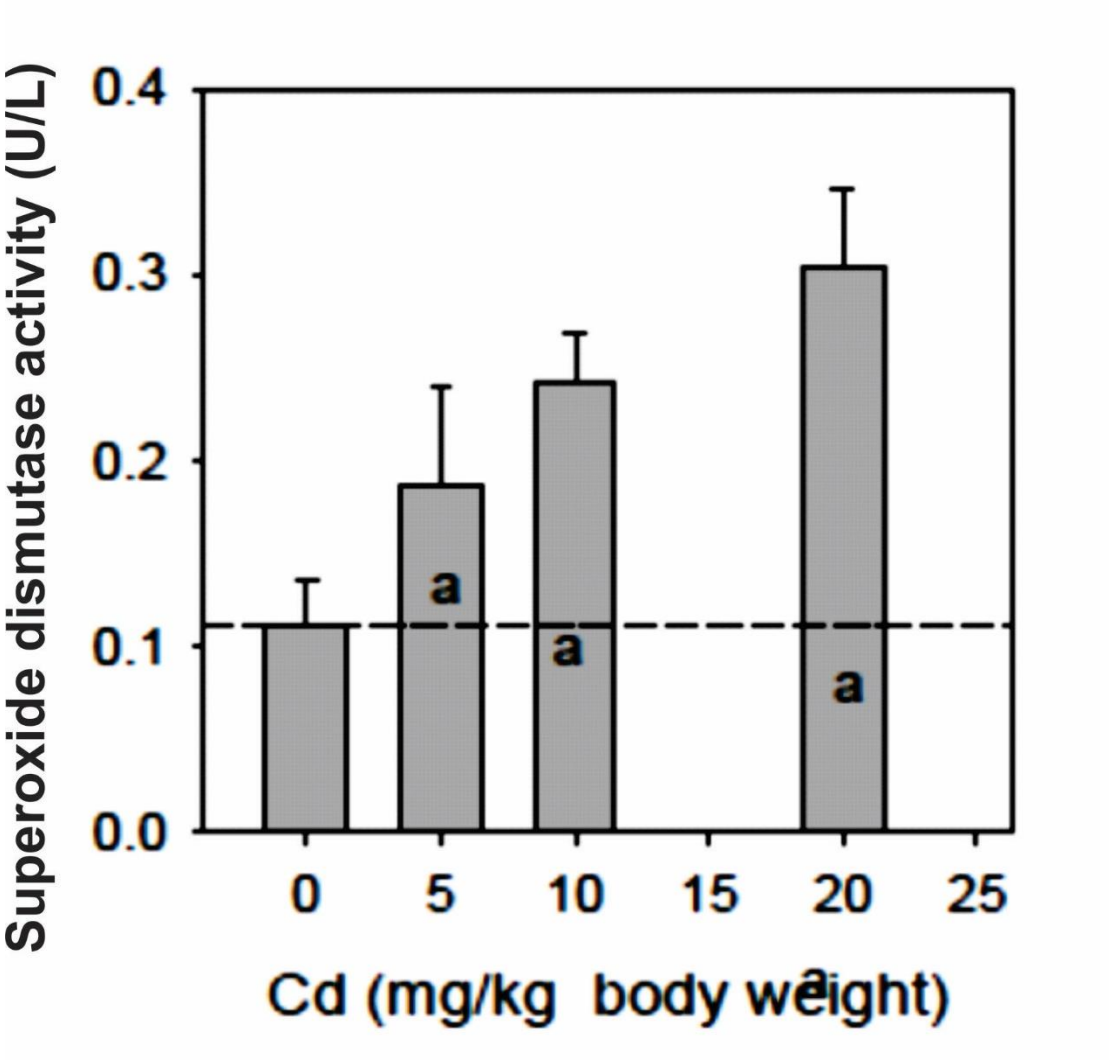


Figure 4.70: Dose-effect relationship for serum superoxide dismutase (SOD) activity in albino rats treated with Cd. a = significantly different from control ($p<0.05$).

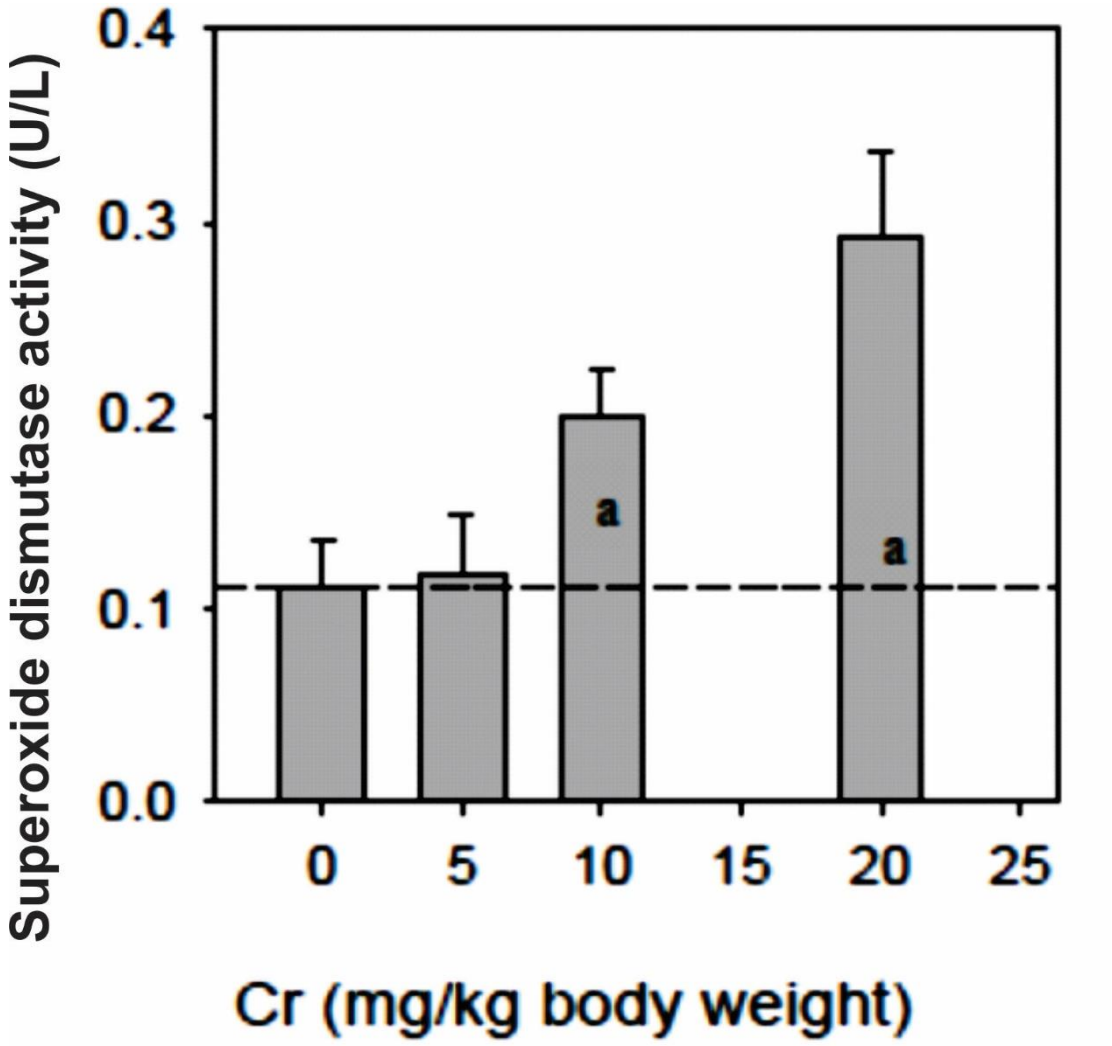


Figure 4.71: Dose-effect relationship for serum superoxide dismutase (SOD) activity in albino rats treated with Cr. a = significantly different from control ($p<0.05$).

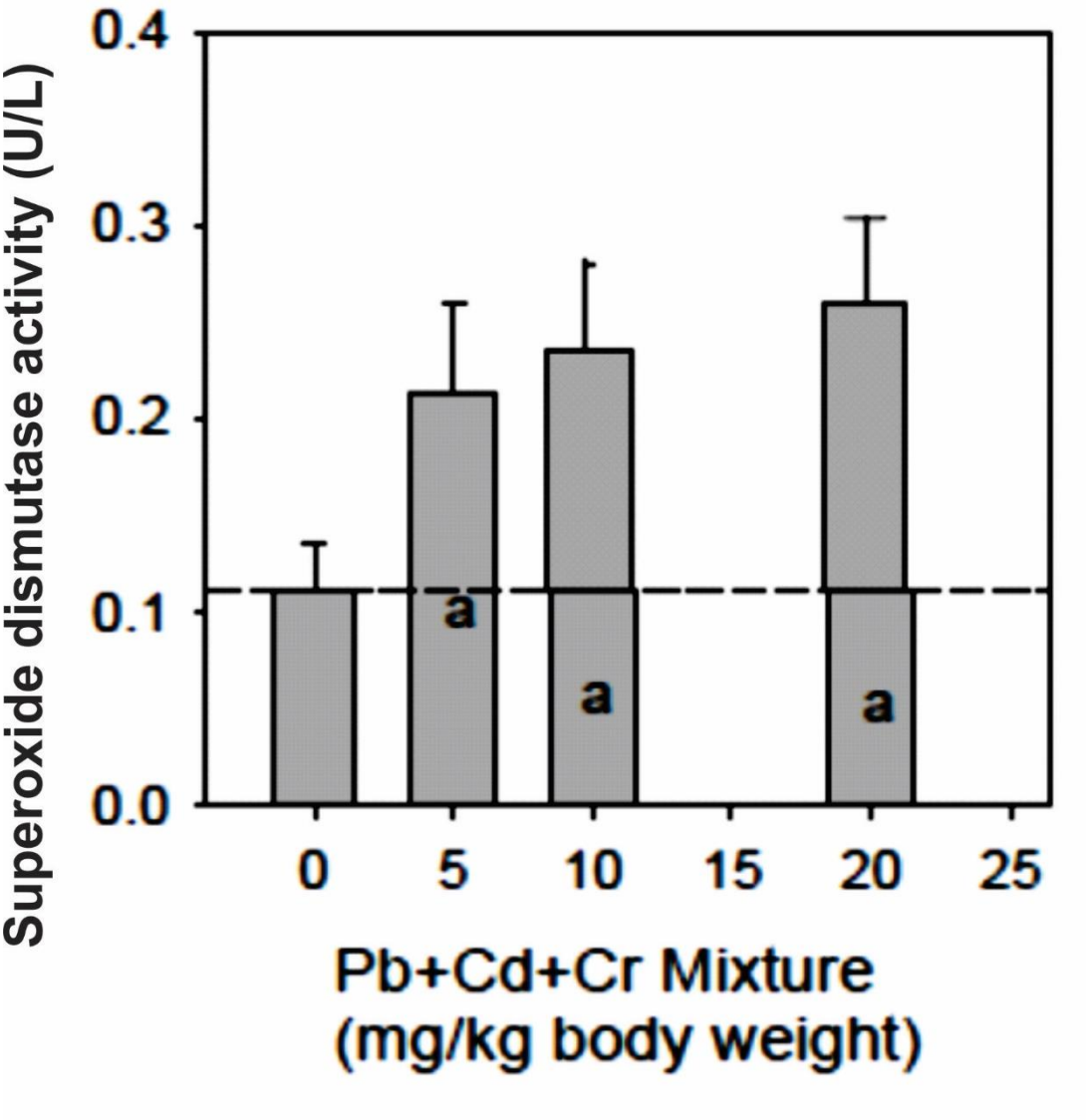


Figure 4.72: Dose-effect relationship for serum superoxide dismutase (SOD) activity in albino rats treated with Pb, Cd and Cr as a mixture. a = significantly different from control ($p<0.05$).

Figures 4.69-4.72 showed that in the 5mg/kg treatment dose, there was significant increase ($p<0.05$) in mean serum SOD activity in Cd, Pb individual and combined treatment groups compared with control but there was no significant difference ($p>0.05$) in mean serum SOD activity inCr individual treatment group compared with control. There was no significant difference ($p>0.05$) in mean serum SOD activity in Cd, Pb individual treatment groups compared with the combined treatment group.

Figures 4.69-4.72 also showed that in the 10mg/kg treatment dose, there was significant increase ($p<0.05$) in mean serum SOD activity in Cd, Cr, Pb individual and combined treatment groups compared with control but there was no significant difference ($p>0.05$) in mean serum SOD activity inCd, Cr, Pb individual treatment groups compared with the combined treatment group.

Figures 4.69-4.72 showed that in the 20mg/kg treatment dose, there was significant increase ($p<0.05$) in mean serum SOD activity in Cd, Cr, Pb individual and combined treatment groups compared with control but there was no significant difference ($p>0.05$) in mean serum SOD activity in Cd, Cr, Pb individual treatment groups compared with the combined treatment group.

Treatment with Cd, Cr, Pb individually and the combined caused a dose dependent increase in mean serum SOD activityas the dose increased.

4.1.4.5 Serum catalase (CAT) activity in albino rats treated with Pb, Cd and Cr individually and as a mixture

Figures 4.73-4.76 show dose-effect relationship for catalase (CAT) activity in albino rats treated with Pb, Cd and Cr individually and as a mixture.

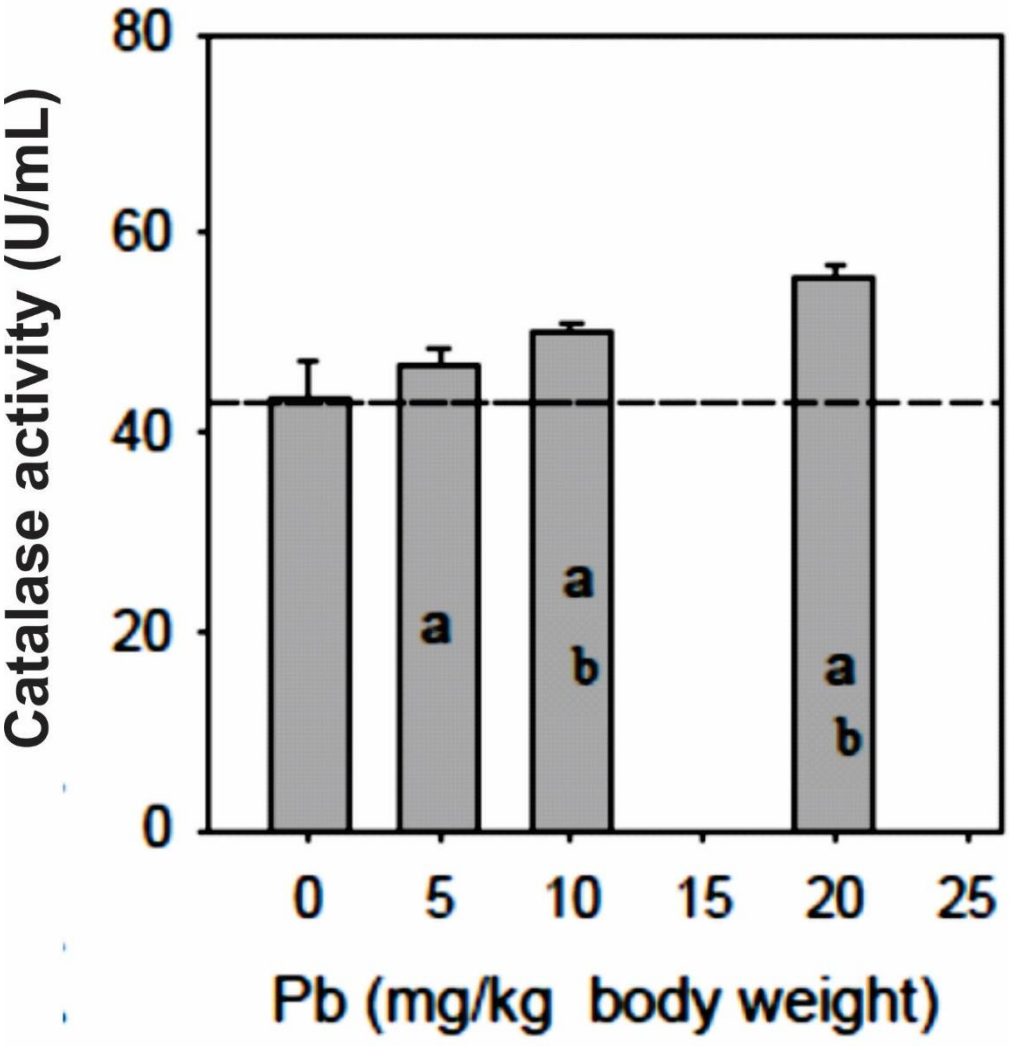


Figure 4.73: Dose-effect relationship for catalase (CAT) activity in albino rats treated with Pb. a = significantly different from control ($p<0.05$), b = significantly different from mixture ($p<0.05$).

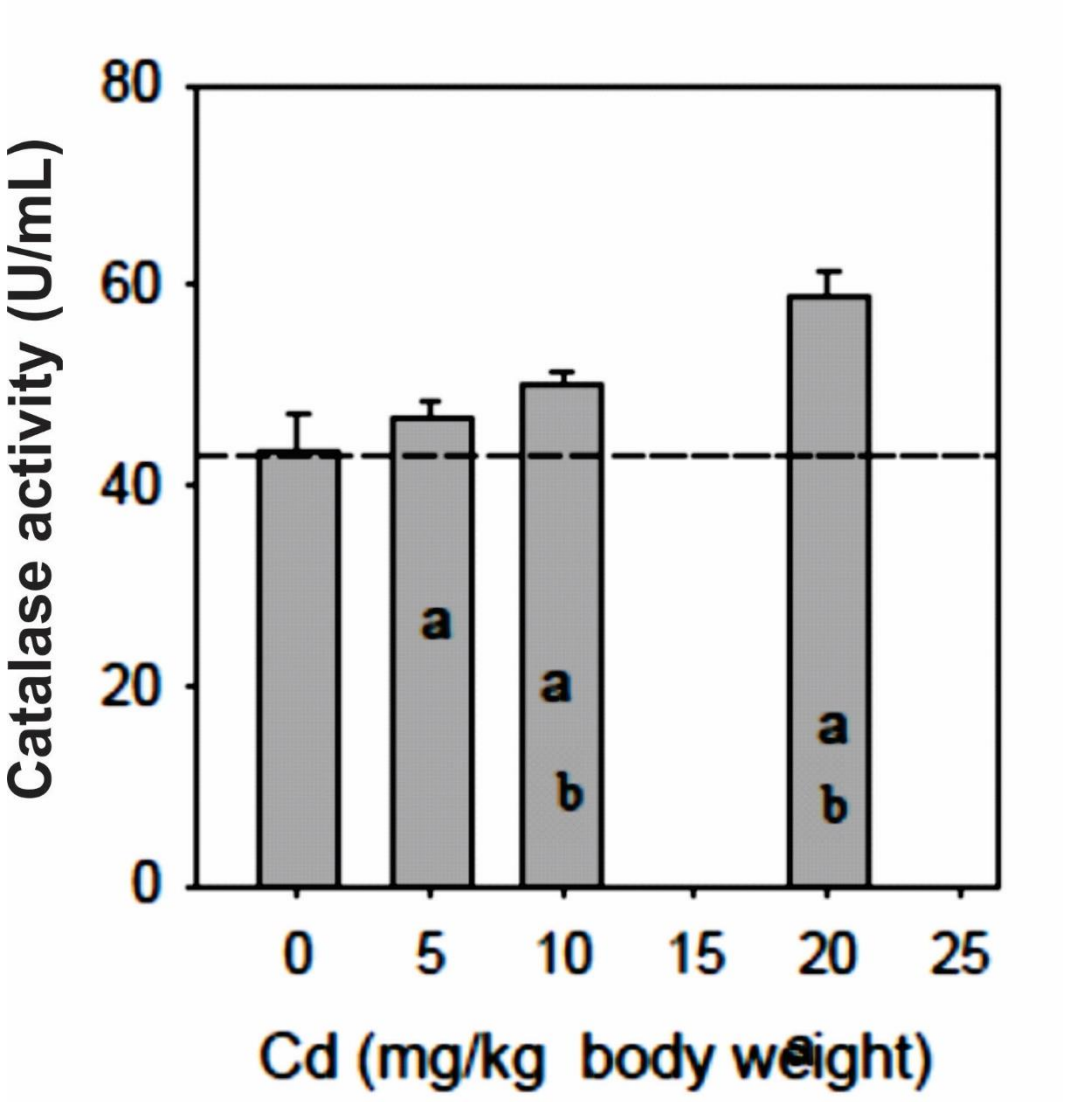


Figure 4.74: Dose-effect relationship for catalase (CAT) activity in albino rats treated with Cd. a = significantly different from control ($p<0.05$), b = significantly different from mixture ($p<0.05$).

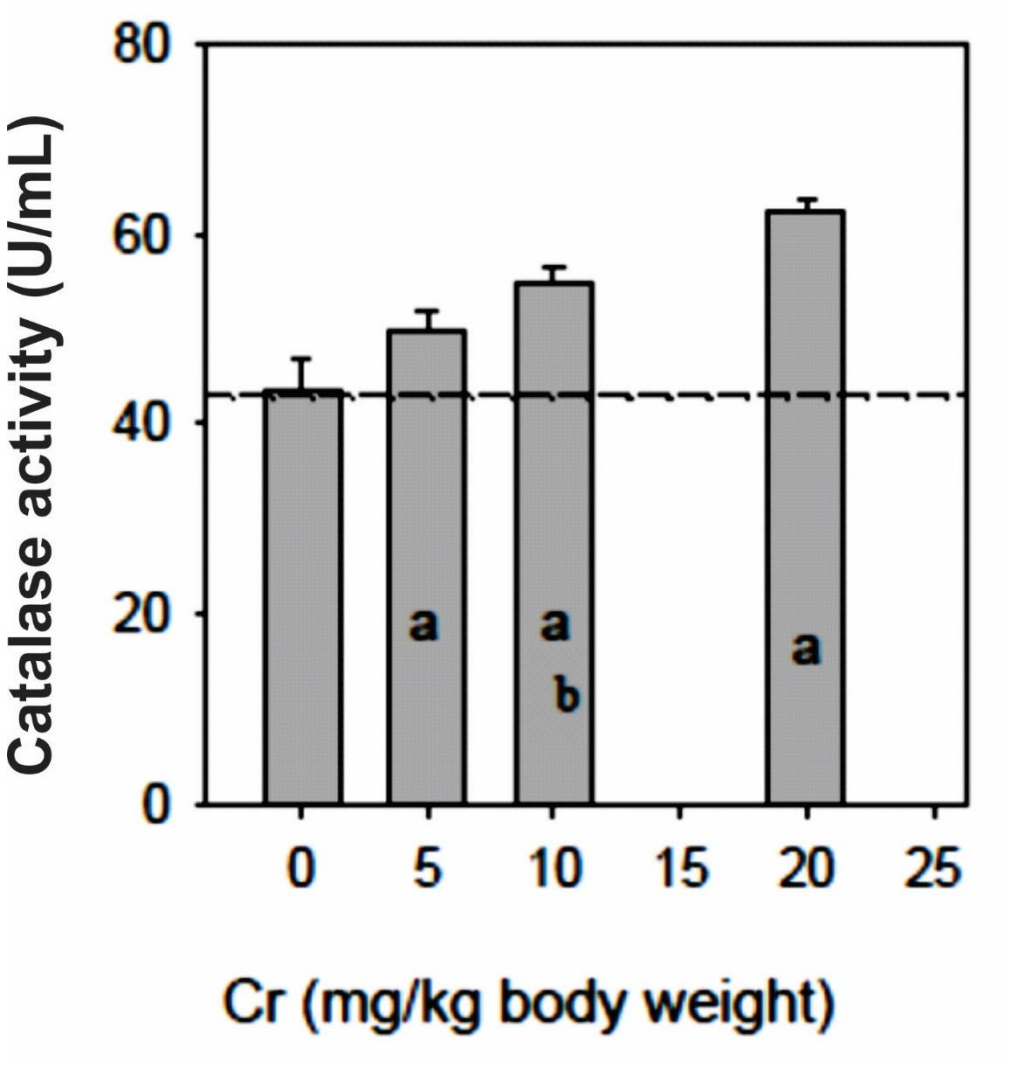


Figure 4.75: Dose-effect relationship for catalase (CAT) activity in albino rats treated with Cr. a = significantly different from control ($p<0.05$), b = significantly different from mixture ($p<0.05$).

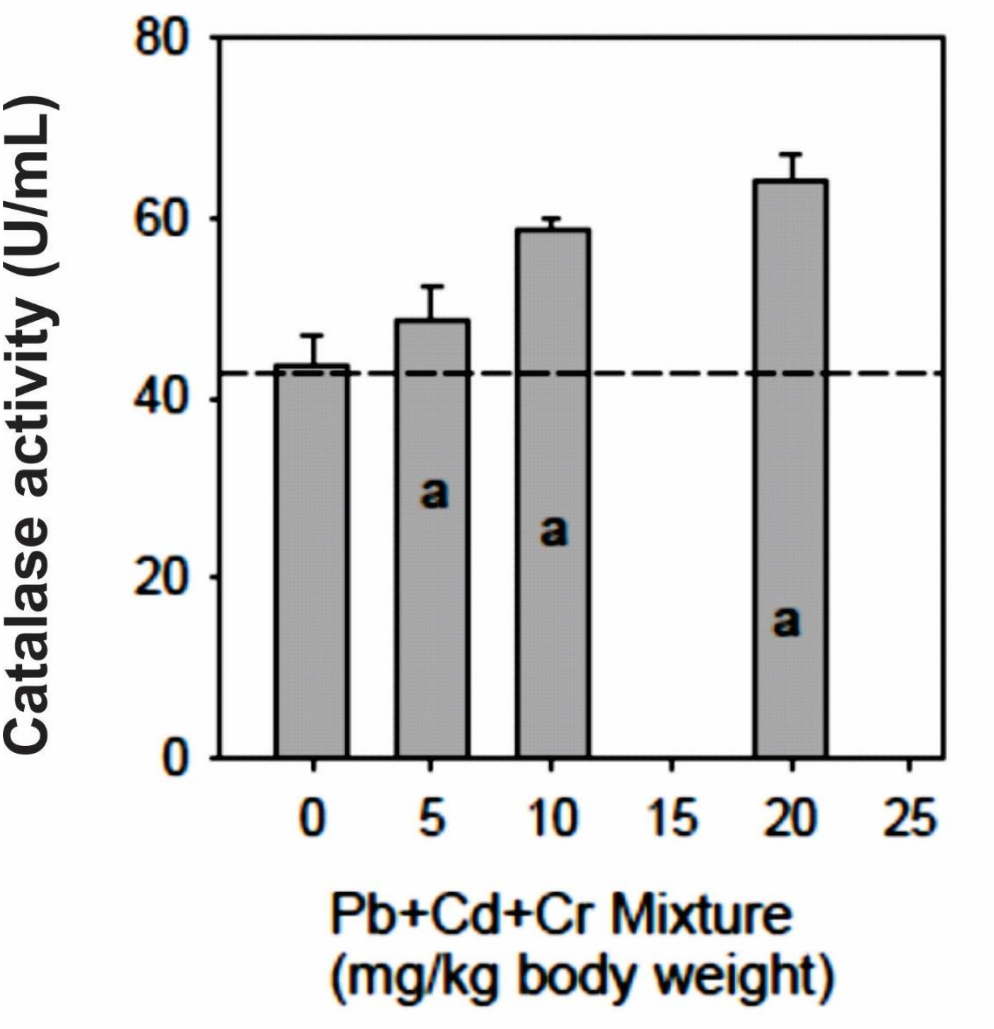


Figure 4.76: Dose-effect relationship for catalase (CAT) activity in albino rats treated with Pb, Cd and Cr as a mixture. a = significantly different from control ($p<0.05$).

Figures 4.73-4.76 showed that in the 5mg/kg treatment dose, there was significant increase ($p<0.05$) in mean serum CAT activity in Cd, Cr, Pb individual and combined treatment groups compared with control but there was no significant difference ($p>0.05$) in mean serum CAT activity in Cd, Cr, Pb individual treatment groups compared with the combined treatment group.

Figures 4.73-4.76 showed that in the 10mg/kg treatment dose, there was significant increase ($p<0.05$) in mean serum CAT activity in Cd, Cr, Pb individual and combined treatment groups compared with control. Also, there was significant difference ($p<0.05$) in mean serum CAT activity in Cd, Cr, Pb individual treatment groups compared with the combined treatment group. Serum CAT activity in the combined treatment group was higher than that of Cd, Cr, Pb individual treatment groups.

Figures 4.73-4.76 showed that in the 20mg/kg treatment dose, there was significant increase ($p<0.05$) in mean serum CAT activity in Cd, Cr, Pb individual and combined treatment groups compared with control. Also, there was significant difference ($p<0.05$) in mean serum CAT activityin Cd, Pb individual treatment groups compared with the combined treatment group but there was no significant difference ($p>0.05$) in mean serum CAT activityin Cr individual treatment group compared with the combined treatment group. Serum CAT activityin the combined treatment group was higher than that of Cd, Pb treatment group but was not significantly different ($p>0.05$) from that of Cr that produced the highest effect in the individual treatment group.

Treatment with Cd, Cr, Pb individually and the combined caused a dose dependent increase in mean serum CAT activityas the dose increased.

4.1.5 Hematological tests

4.1.5.1 Hemoglobin (Hb) concentration in albino rats treated with Pb, Cd and Cr individually and as a mixture

Figures 4.77-4.80 showed that there was no significant difference ($p>0.05$) in haemoglobin concentration between the treatment groups except in the 5mg/kg Cd and 5mg/kg Cr treatment groupscompared with the control**.**

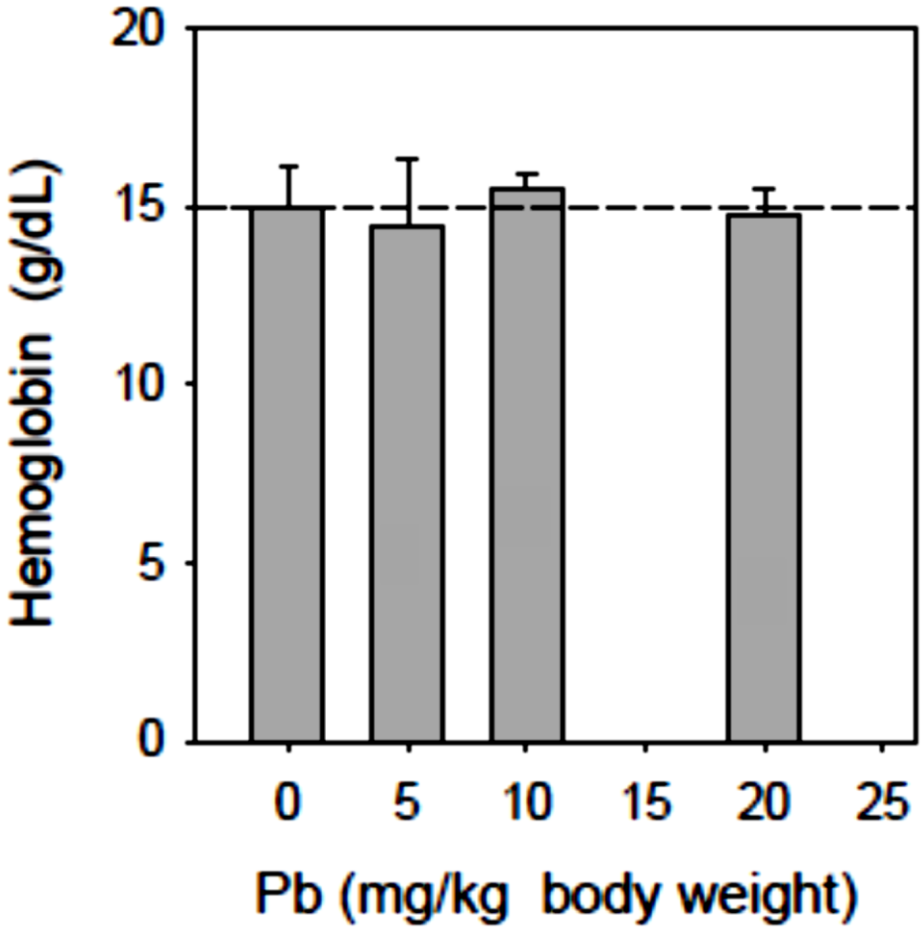


Figure 4.77: Dose-effect relationship for haemoglobin (Hb) concentration in albino rats treated with Pb.

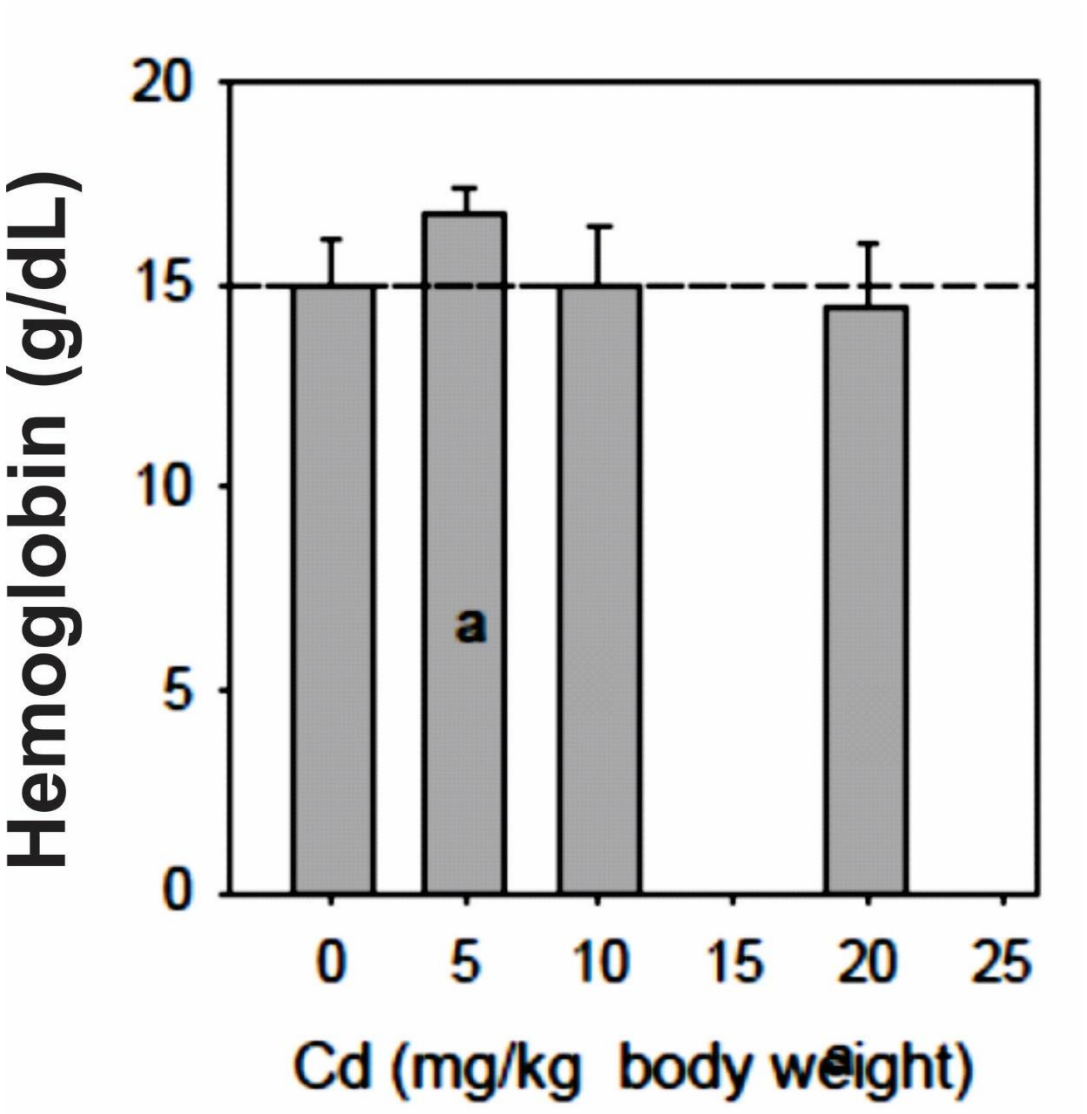


Figure 4.78: Dose-effect relationship for haemoglobin (Hb) concentration in albino rats treated with Cd. a = significantly different from control ($p<0.05$).

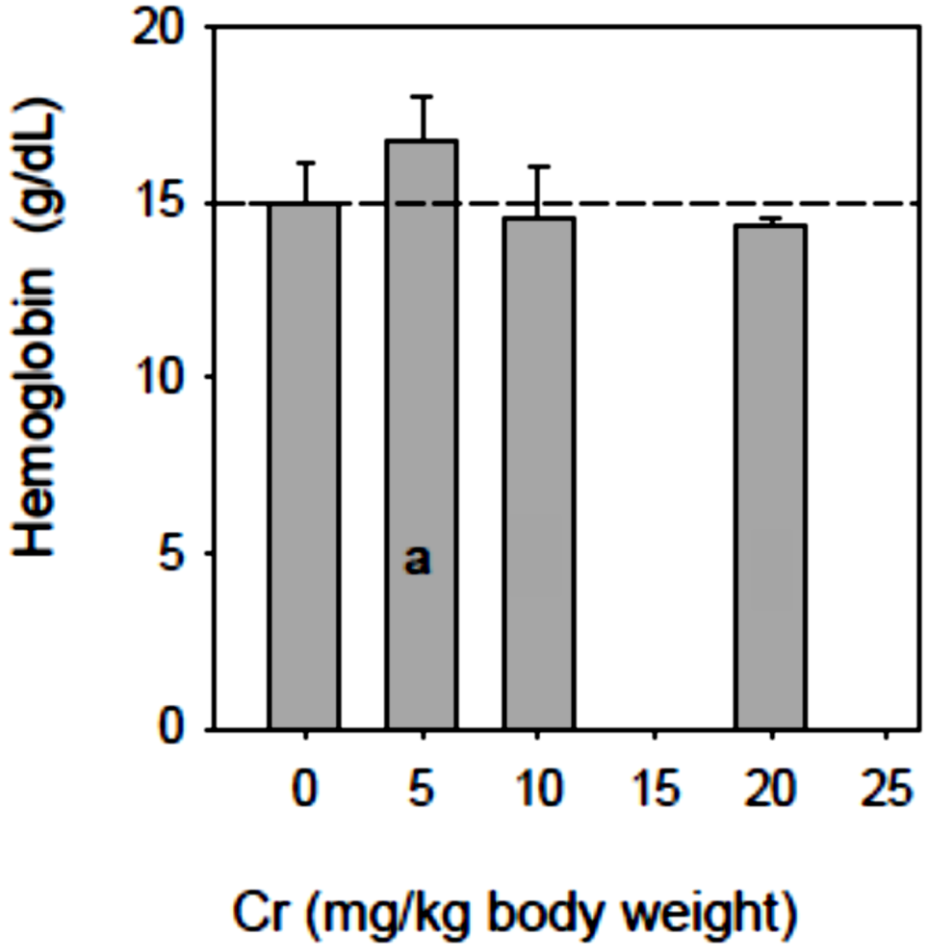


Figure 4.79: Dose-effect relationship for haemoglobin (Hb) concentration in albino rats treated with Cr. a = significantly different from control ($p<0.05$).

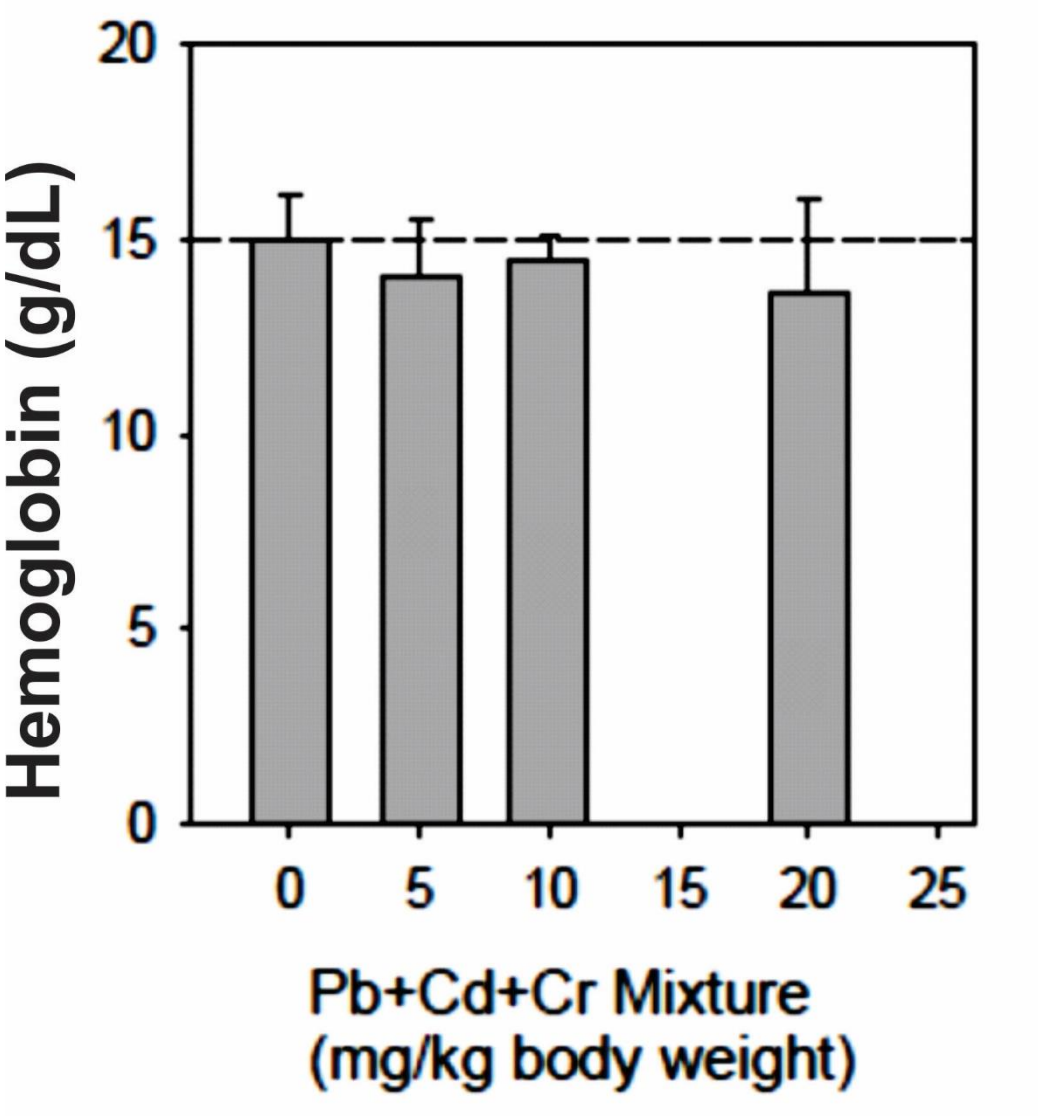


Figure 4.80: Dose-effect relationship for haemoglobin (Hb) concentration in albino rats treated with Pb, Cd and Cr as a mixture.

4.1.5.2 Levels of packed cell volume (PCV) in albino rats treated with Pb, Cd and Cr individually and as a mixture

Figures 4.81-4.84 showed that there was no significant difference ($p>0.05$) in PCV between the treatment groups compared with the control in the three treatment doses suggesting no adverse effect due to the metal treatments both singly and combined.

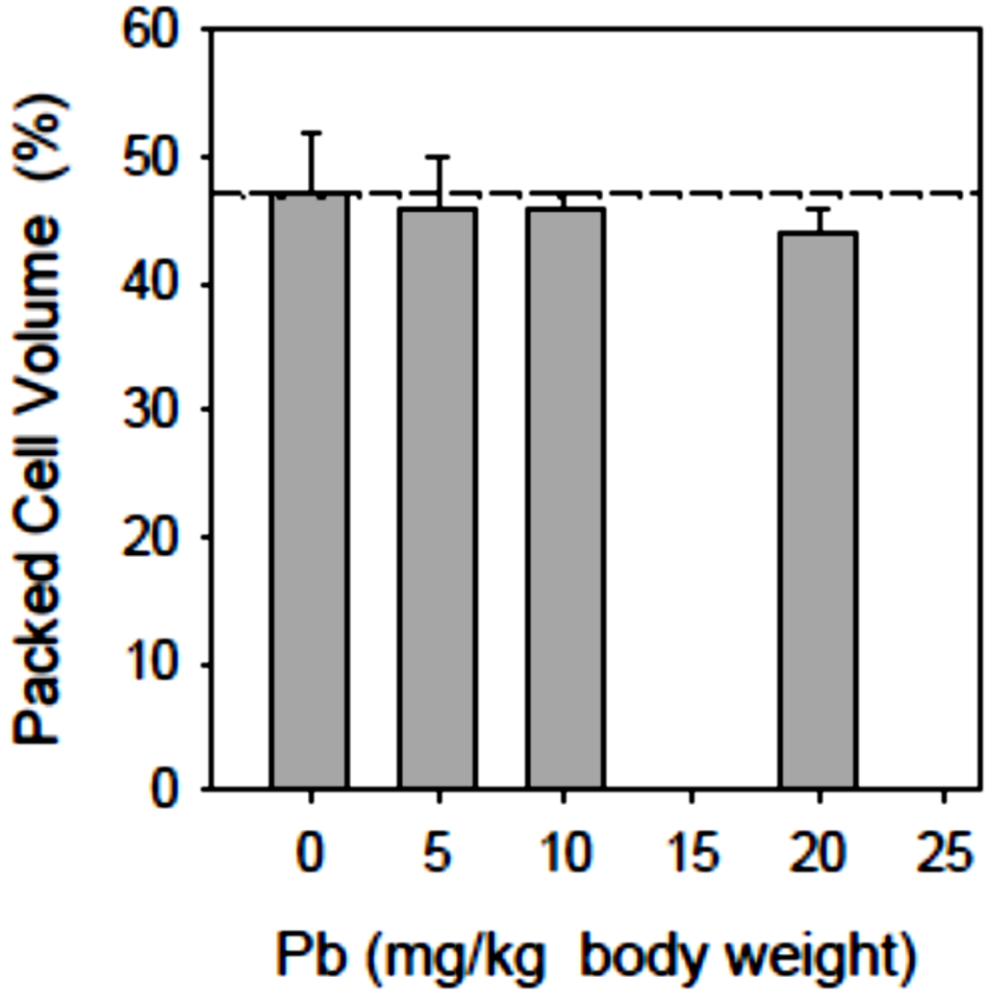


Figure 4.81: Dose-effect relationship for levels of packed cell volume (PCV) in albino rats treated with Pb.

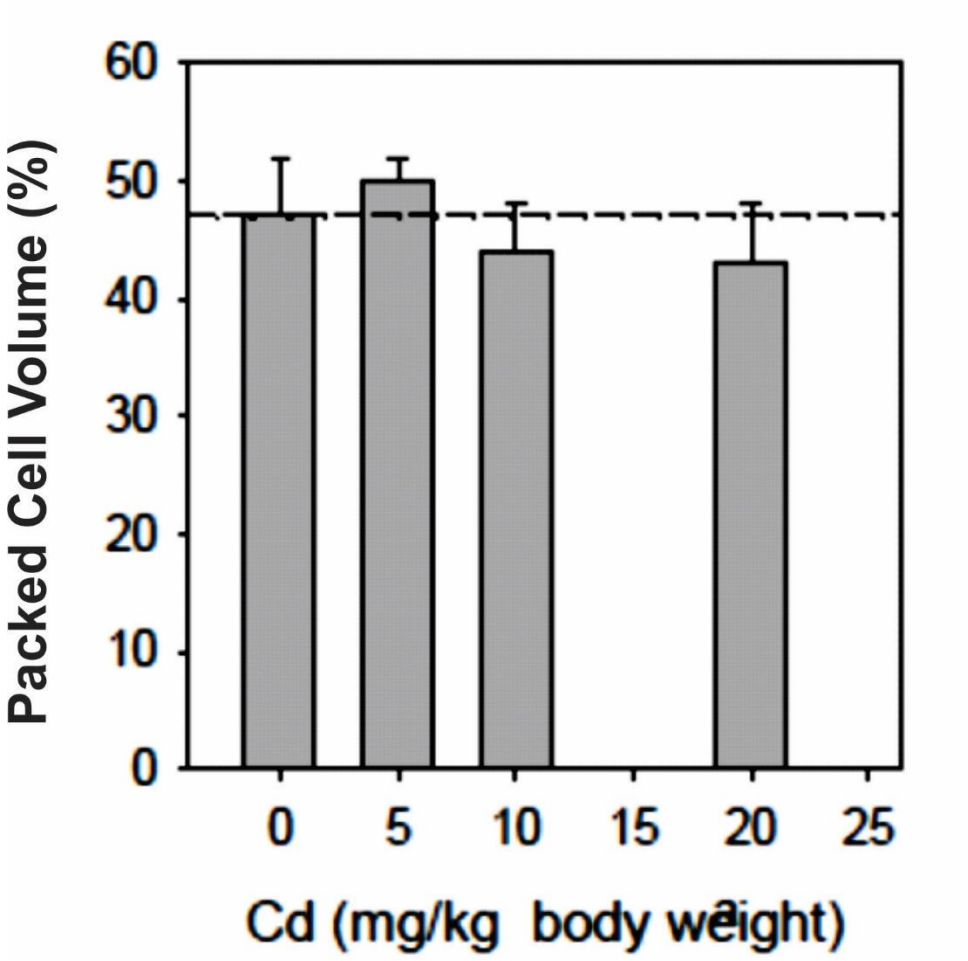


Figure 4.82: Dose-effect relationship for levels of packed cell volume (PCV) in albino rats treated with Cd.

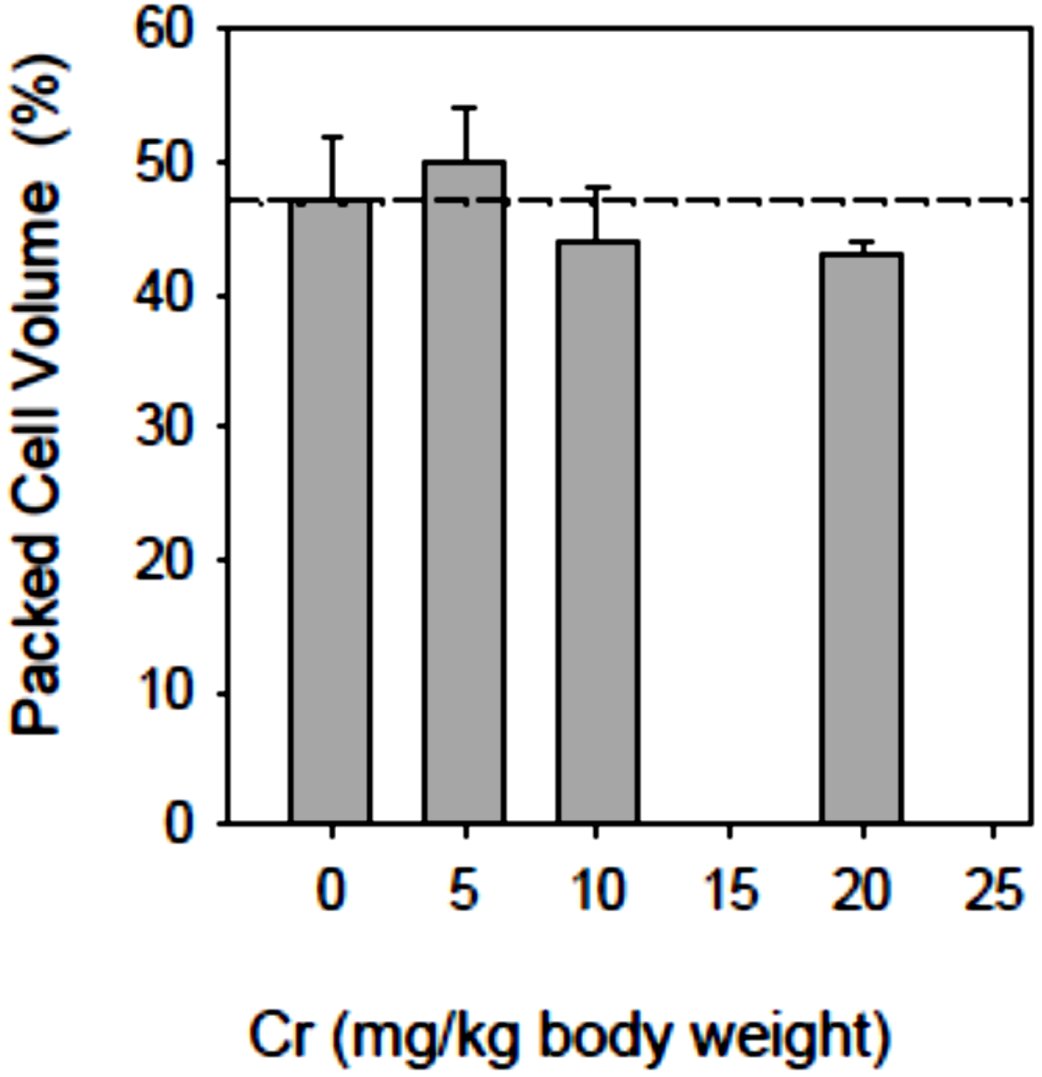


Figure 4.83: Dose-effect relationship for levels of packed cell volume (PCV) in albino rats treated with Cr.

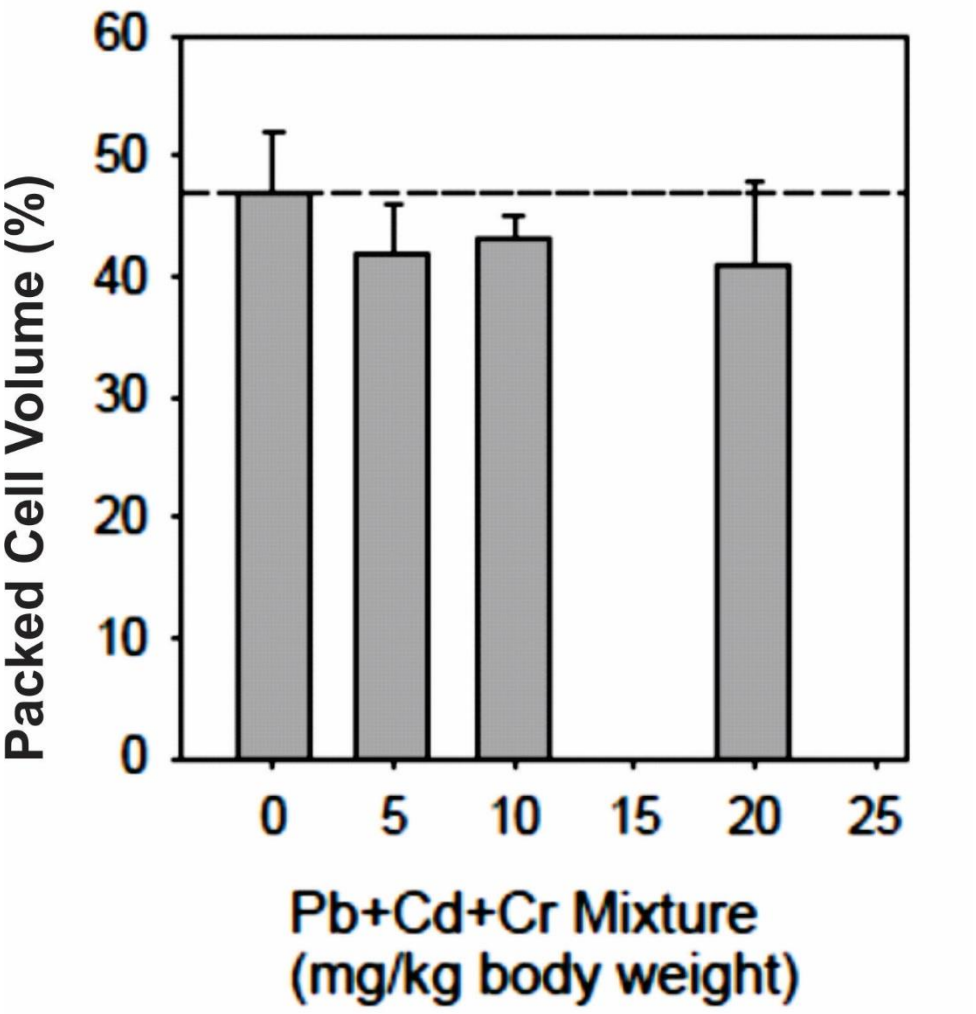


Figure 4.84: Dose-effect relationship for levels of packed cell volume (PCV) in albino rats treated with Pb, Cd and Cr as a mixture

4.1.5.3 Red blood cells (RBC)in albino rats treated with Pb, Cd and Cr individually and as a mixture

Figures 4.85-4.88 showed that there was significant difference ($p<0.05$) in RBC between the 5mg/kg (Cd and mixture) and 20mg/kg (Cr and mixture) treatment groups compared with control but there was no significant difference ($p>0.05$) between 5mg/kg Cd and 5mg/kg mixture as well as 20mg/kg Cr and 20mg/kg mixture treatment groups respectively.

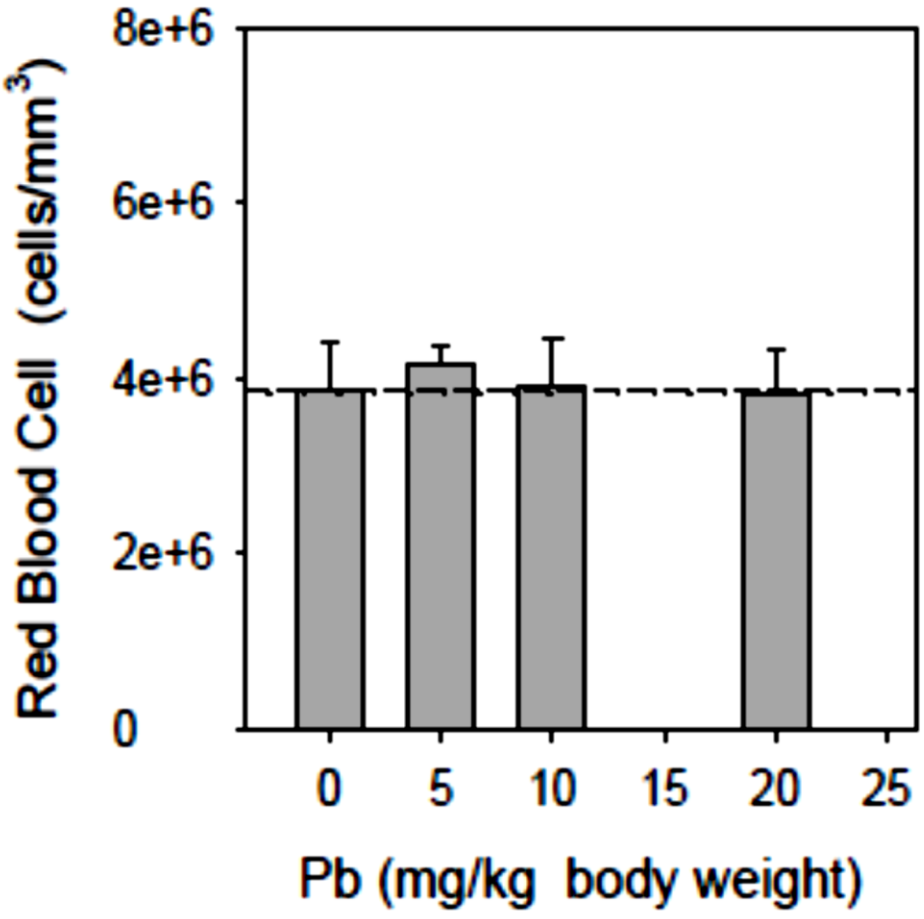


Figure 4.85: Dose-effect relationship for red blood cells (RBC)in albino rats treated with Pb.

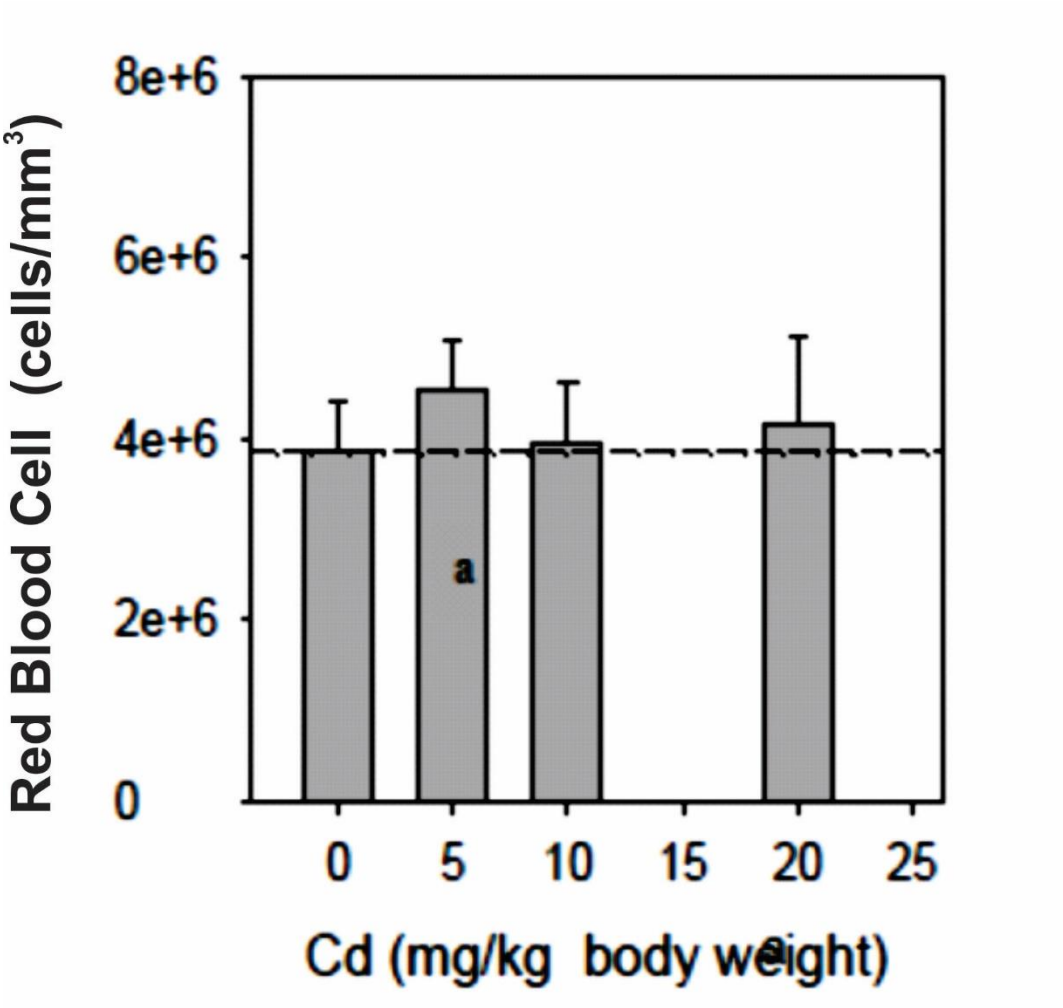


Figure 4.86: Dose-effect relationship for red blood cells (RBC)in albino rats treated with Cd. a = significantly different from control ($p<0.05$).

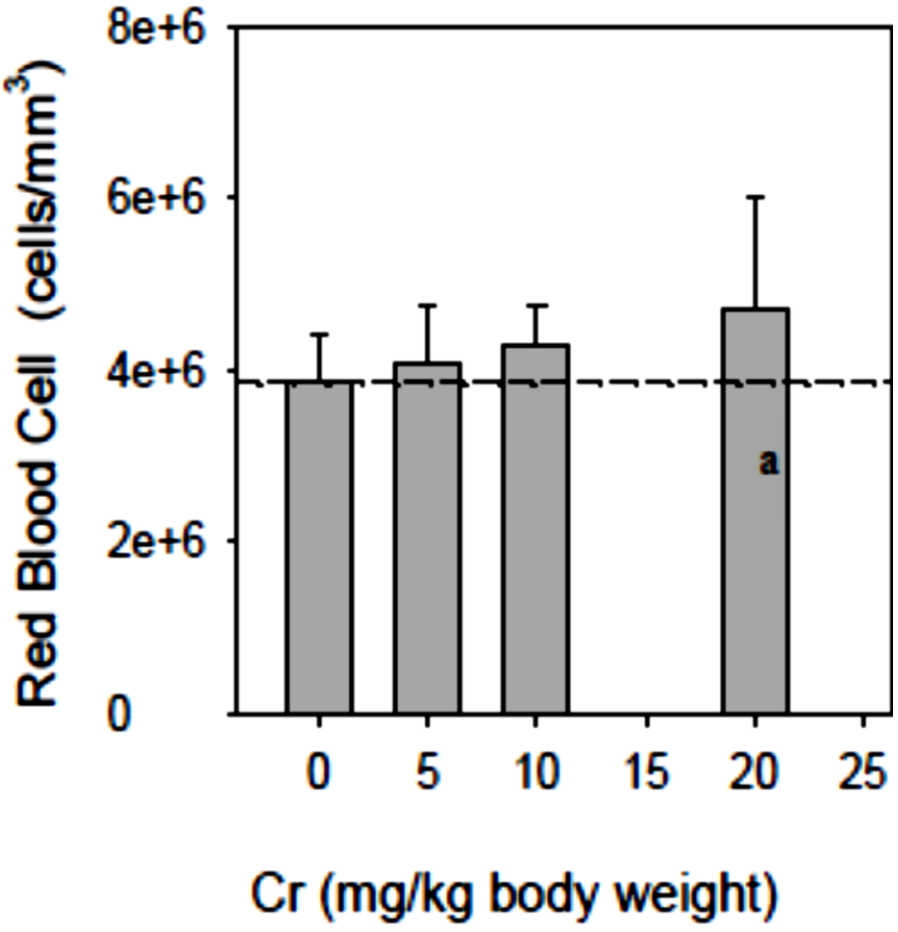


Figure 4.87: Dose-effect relationship for red blood cells (RBC)in albino rats treated with Cr. a = significantly different from control ($p<0.05$).

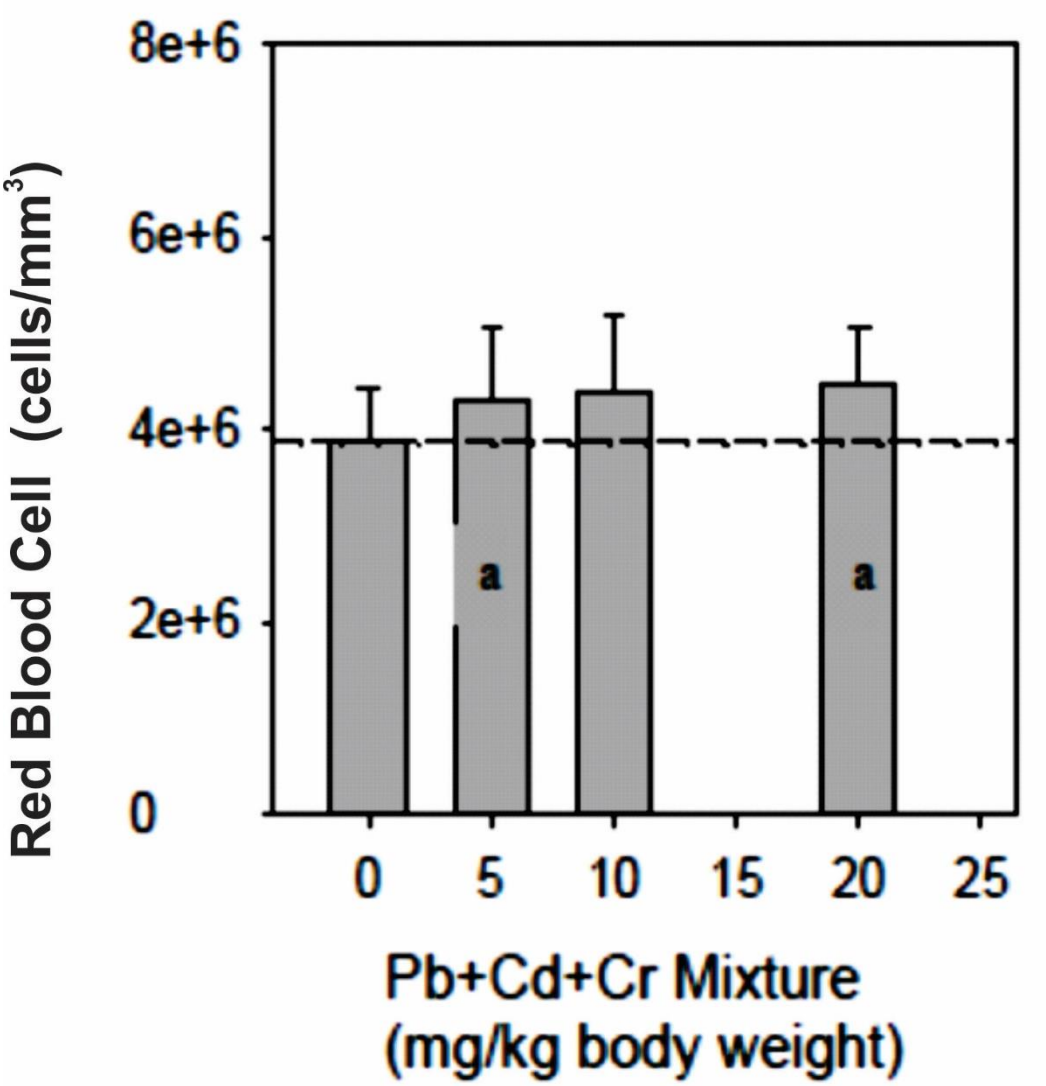


Figure 4.88: Dose-effect relationship for red blood cells (RBC)in albino rats treated with Pb, Cd and Cr as a mixture. a = significantly different from control ($p<0.05$).

4.1.5.4 White blood cells (total), WBC(T)in albino rats treated with Pb, Cd and Cr individually and as a mixture

Figures 4.89-4.92 showed that there was significant difference ($p<0.05$) in WBC between the 5mg/kg (Cd and Pb) and 10mg/kg mixture treatment groups compared with control. Also, there was significant difference ($p<0.05$) between 5mg/kg (Cd and Pb) treatment groupscompared with 5mg/kg mixture treatment group.

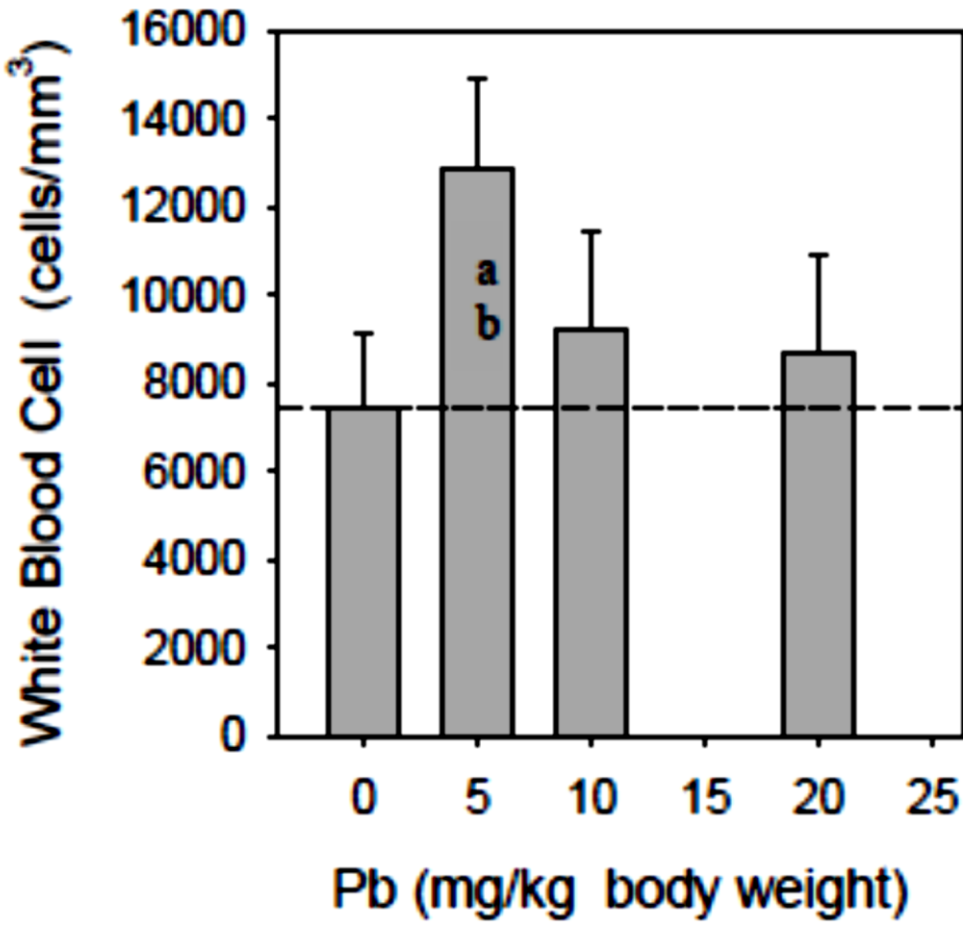


Figure 4.89: Dose-effect relationship for white blood cells (total) WBC(T) in albino rats treated with Pb. a = significantly different from control ($p<0.05$), b = significantly different from mixture ($p<0.05$).

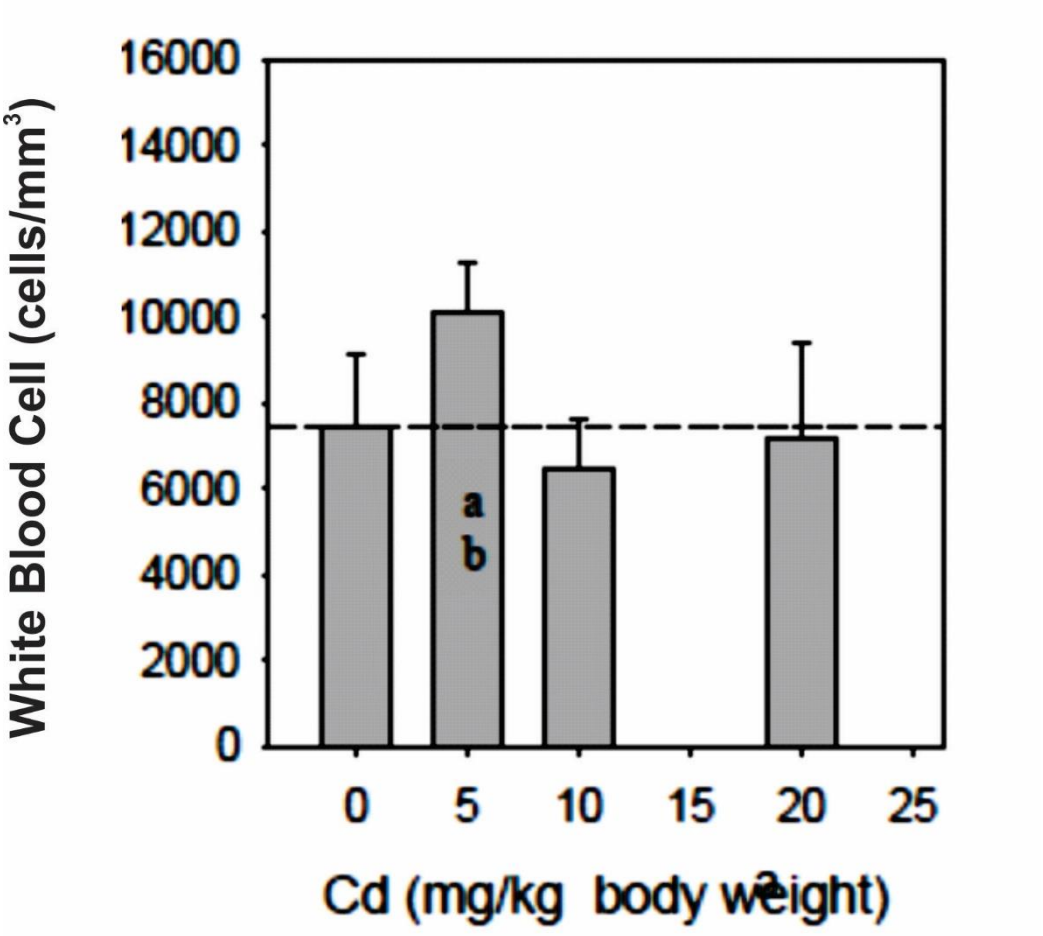


Figure 4.90: Dose-effect relationship for white blood cells (total) WBC(T) in albino rats treated with Cd. a = significantly different from control ($p<0.05$), b = significantly different from mixture ($p<0.05$).

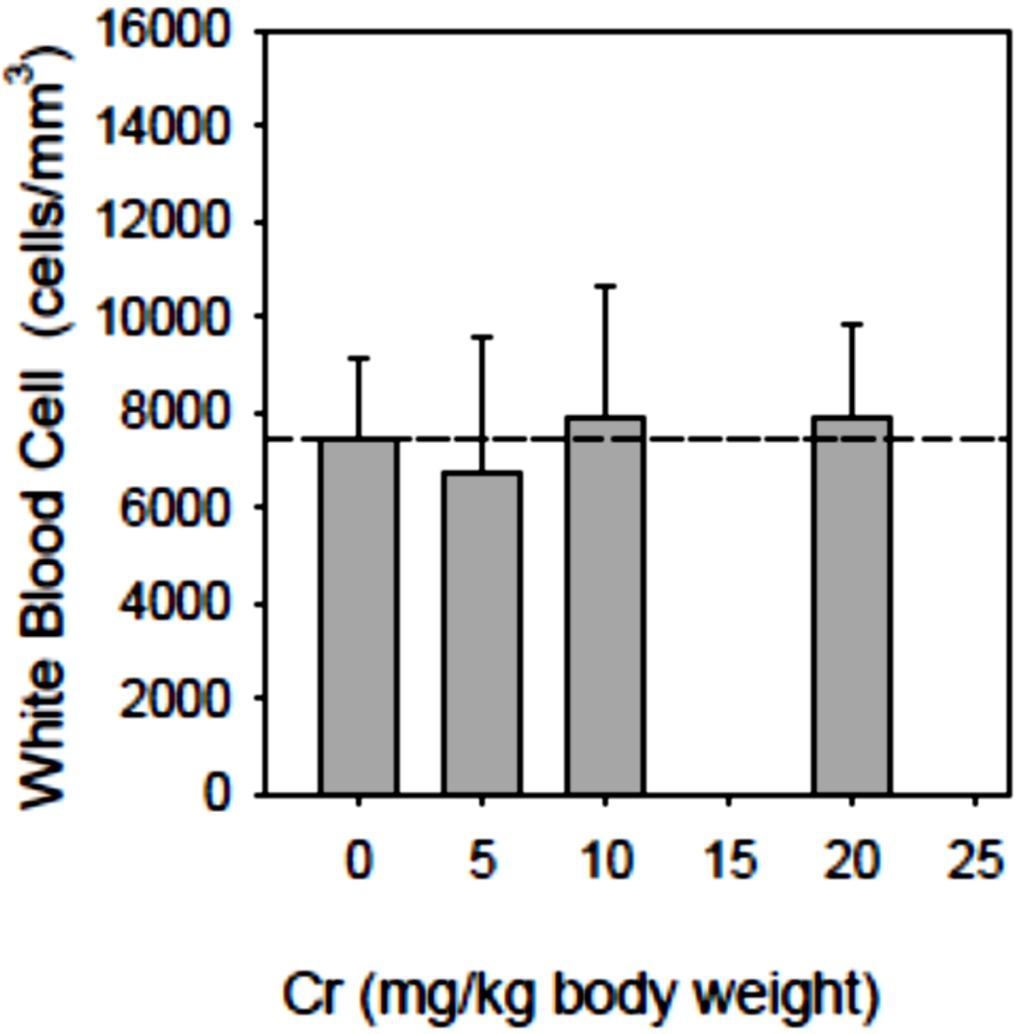


Figure 4.91: Dose-effect relationship for white blood cells (total) WBC(T) in albino rats treated with Cr.

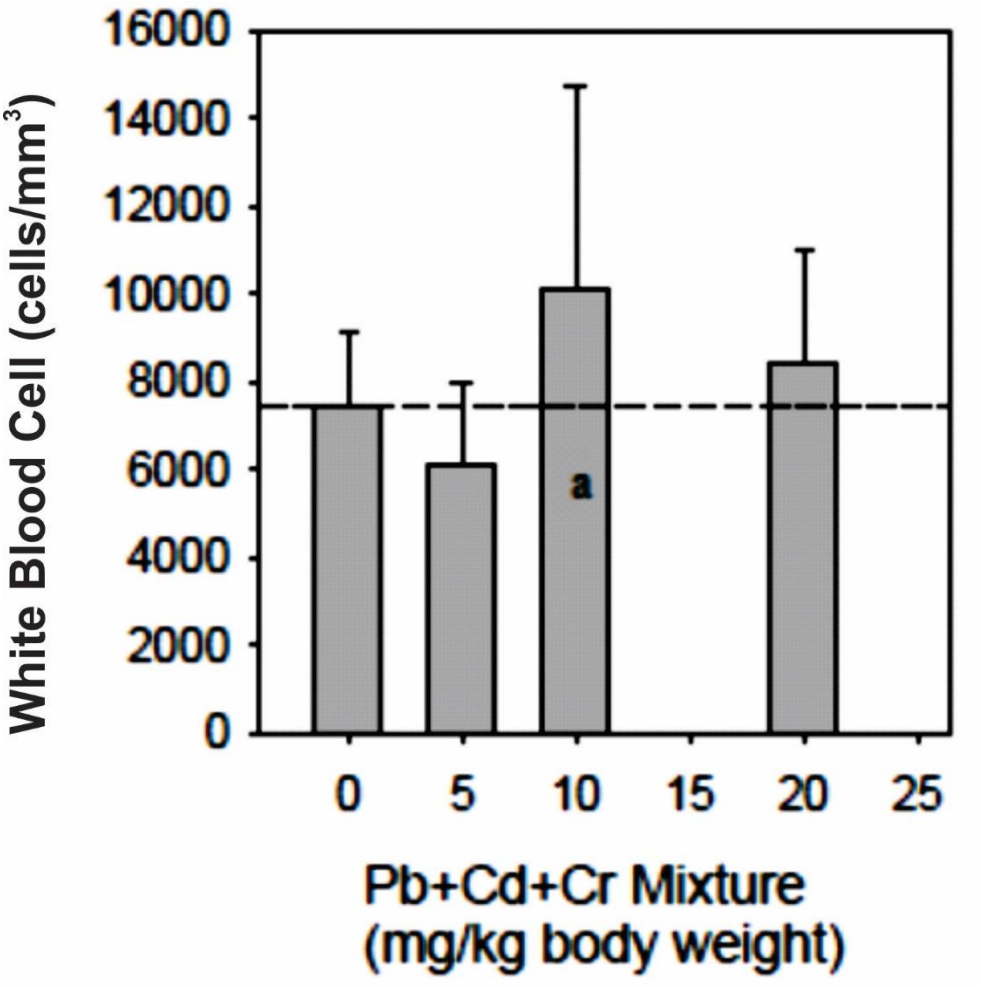


Figure 4.92: Dose-effect relationship for white blood cells (total) WBC(T) in albino rats treated with Pb, Cd and Cr as a mixture. a = significantly different from control ($p<0.05$).

4.1.6 Histopathological evaluations

4.1.6.1 Histopathological evaluation of heart of albino ratstreated with Pb, Cd and Cr individually and as a mixture

Histologic sections of hearts of albino rats (Plates 4.1- 4.13) in the three treatment dose groups and control showed normal myocardial tissue running in one direction with fibrocytes (stromal cells) exhibiting normal histology as summarized in Table 4.7.

Table 4.7 Summary of histopathological evaluation of heart of albino ratstreated with Pb, Cd and Cr individually and as a mixture

Dose	Slide reading				
	Control	Mixture (Pb, Cd, Cr)	Pb	Cd	Cr
5mg/kg	Normal	Normal	Normal	Normal	Normal
10mg/kg	Normal	Normal	Normal	Normal	Normal
20mg/kg	Normal	Normal	Normal	Normal	Normal

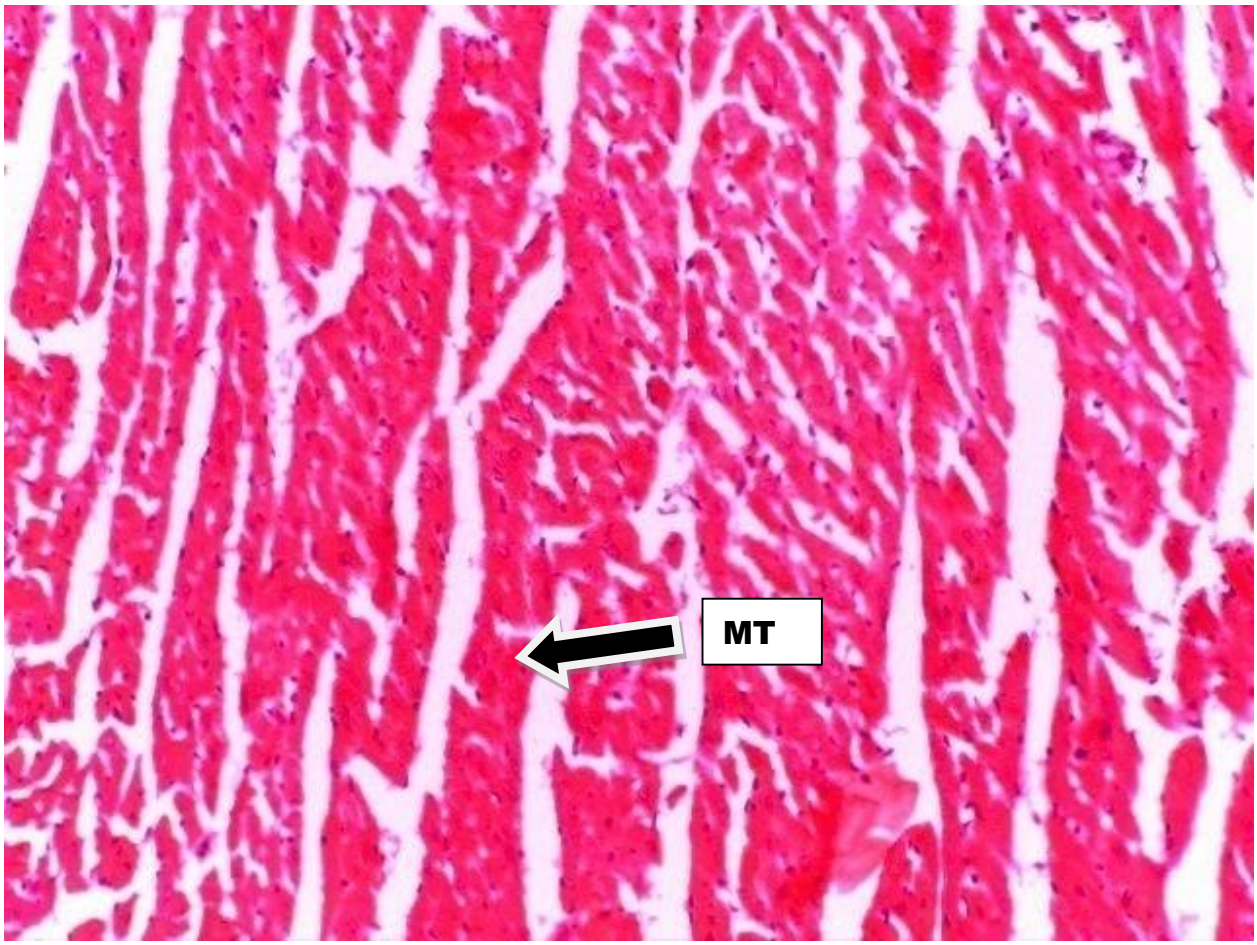


Plate 4.1: Photomicrograph of section of the heart of albino rat in the Control group for 90 days showing normal myocardial tissue (MT) running in one direction with fibrocytes (stromal cells) exhibiting normal histology (x400), Stain: H and E**.**

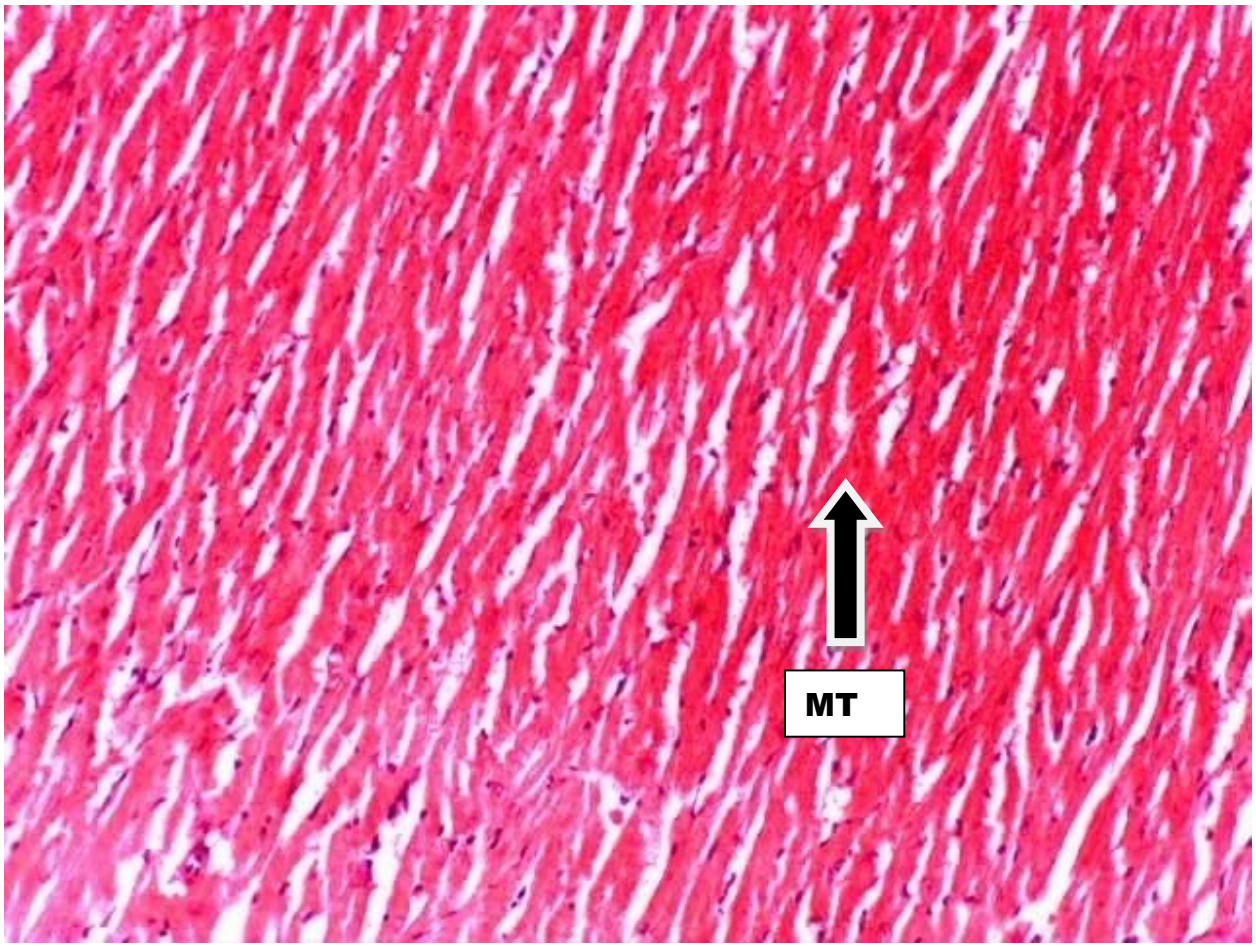


Plate 4.2: Photomicrograph of section of the heart of albino rat administered 5mg/kg body weight Cd for 90 days showing normal myocardial tissue (MT) running in one direction with fibrocytes (stromal cells) exhibiting normal histology (x400), Stain: H and E**.**

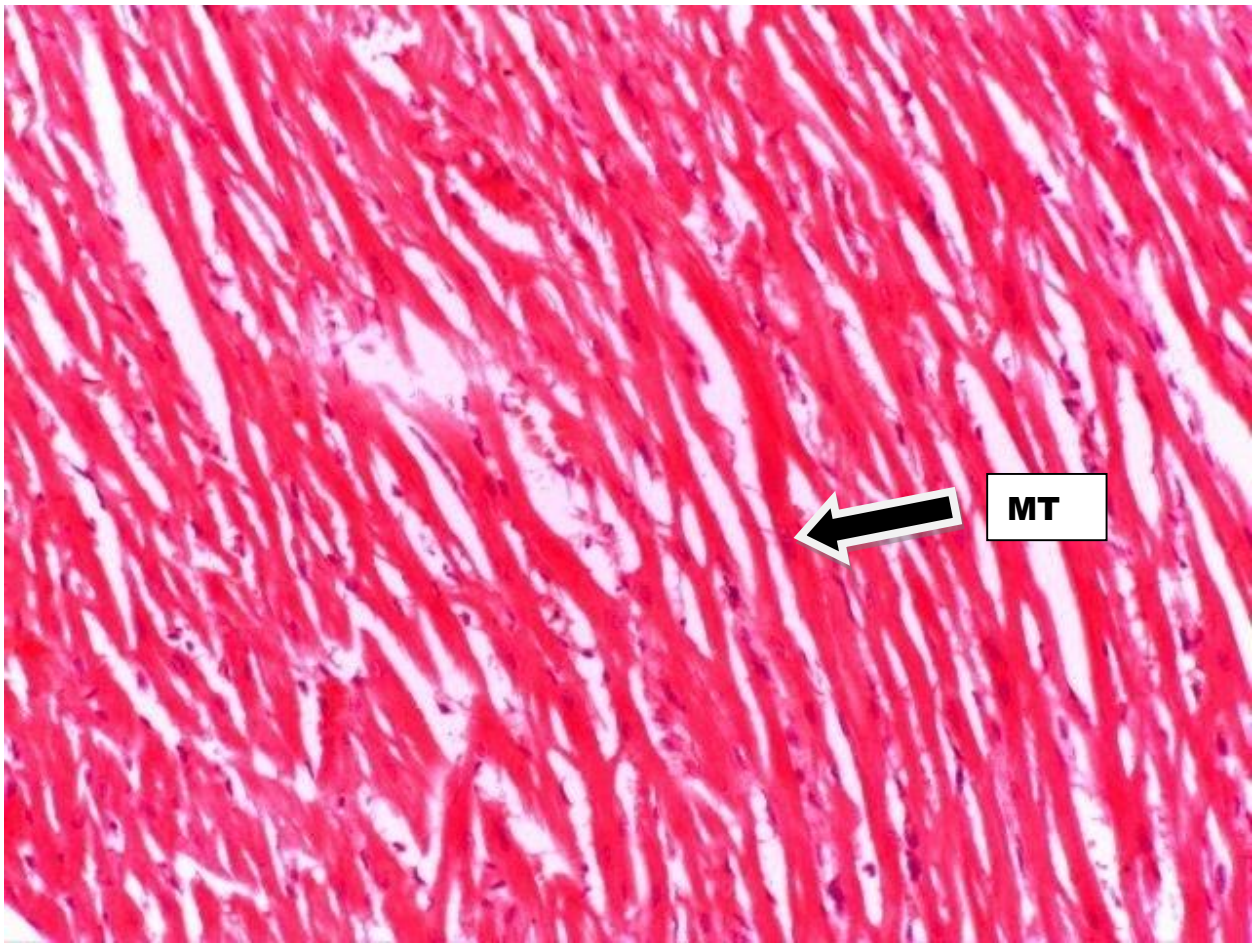


Plate 4.3: Photomicrograph of section of the heart of albino rat administered 5mg/kg body weight Cr for 90 days showing normal myocardial tissue (MT) running in one direction with fibrocytes (stromal cells) exhibiting normal histology (x400), Stain: H and E.

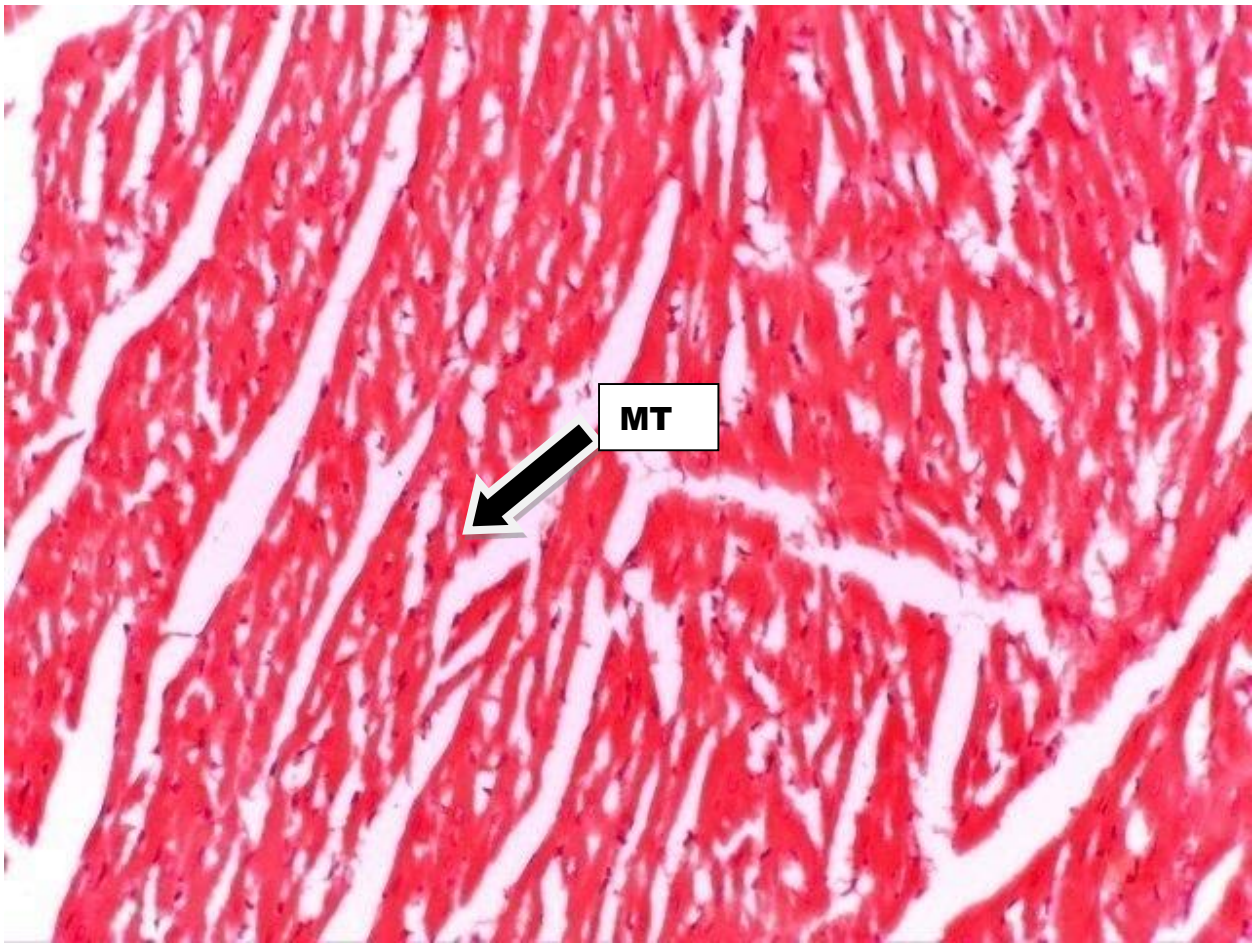


Plate 4.4: Photomicrograph of section of the heart of albino rat administered 5mg/kg body weight Pb for 90 days showing normal myocardial tissue (MT) running in one direction with fibrocytes (stromal cells) exhibiting normal histology (x400), Stain: H and E.

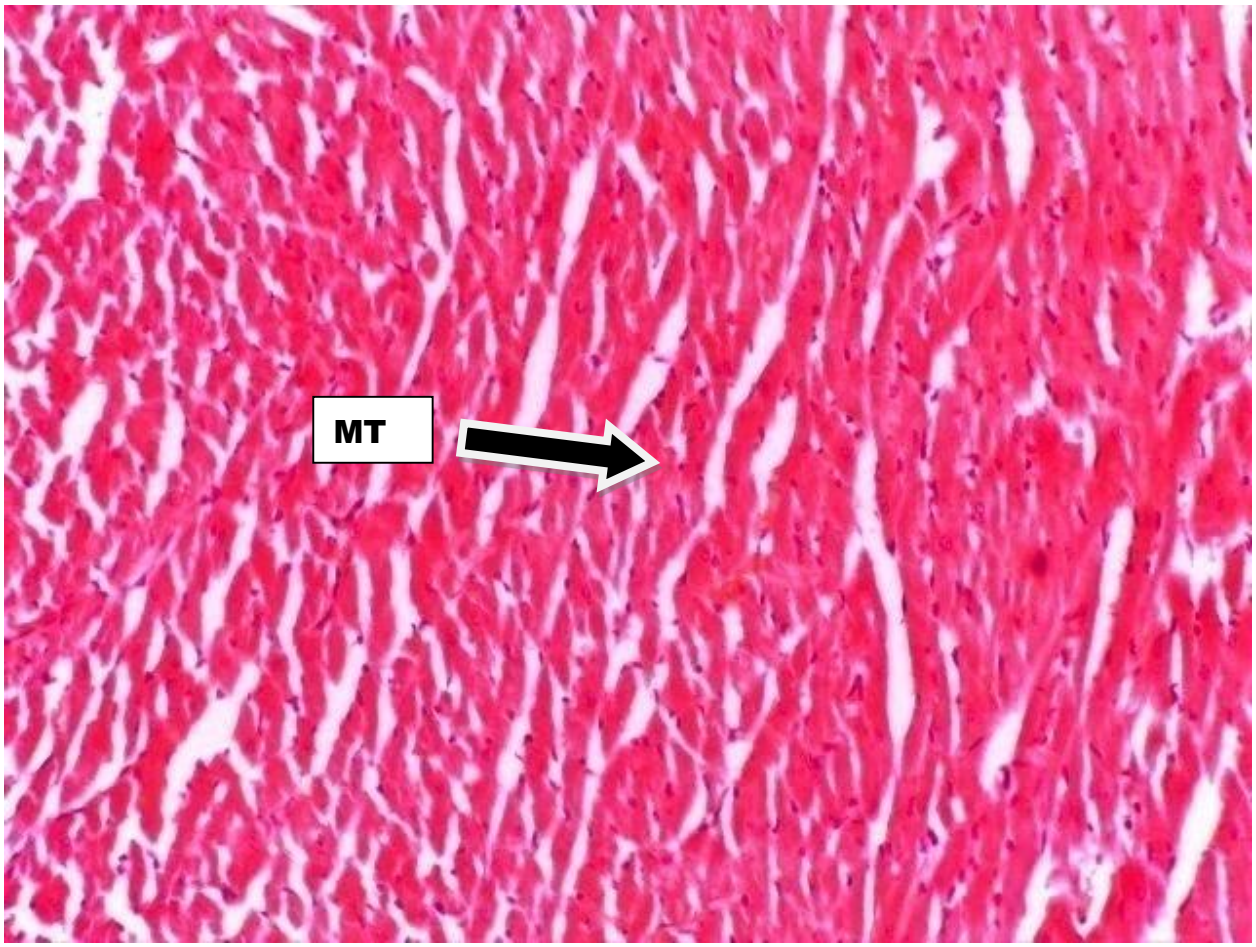


Plate 4.5: Photomicrograph of section of the heart of albino rat administered 5mg/kg body weight Cd, Cr, Pb mixture for 90 days showing normal myocardial tissue (MT) running in one direction with fibrocytes (stromal cells) exhibiting normal histology (x400), Stain: H and E.

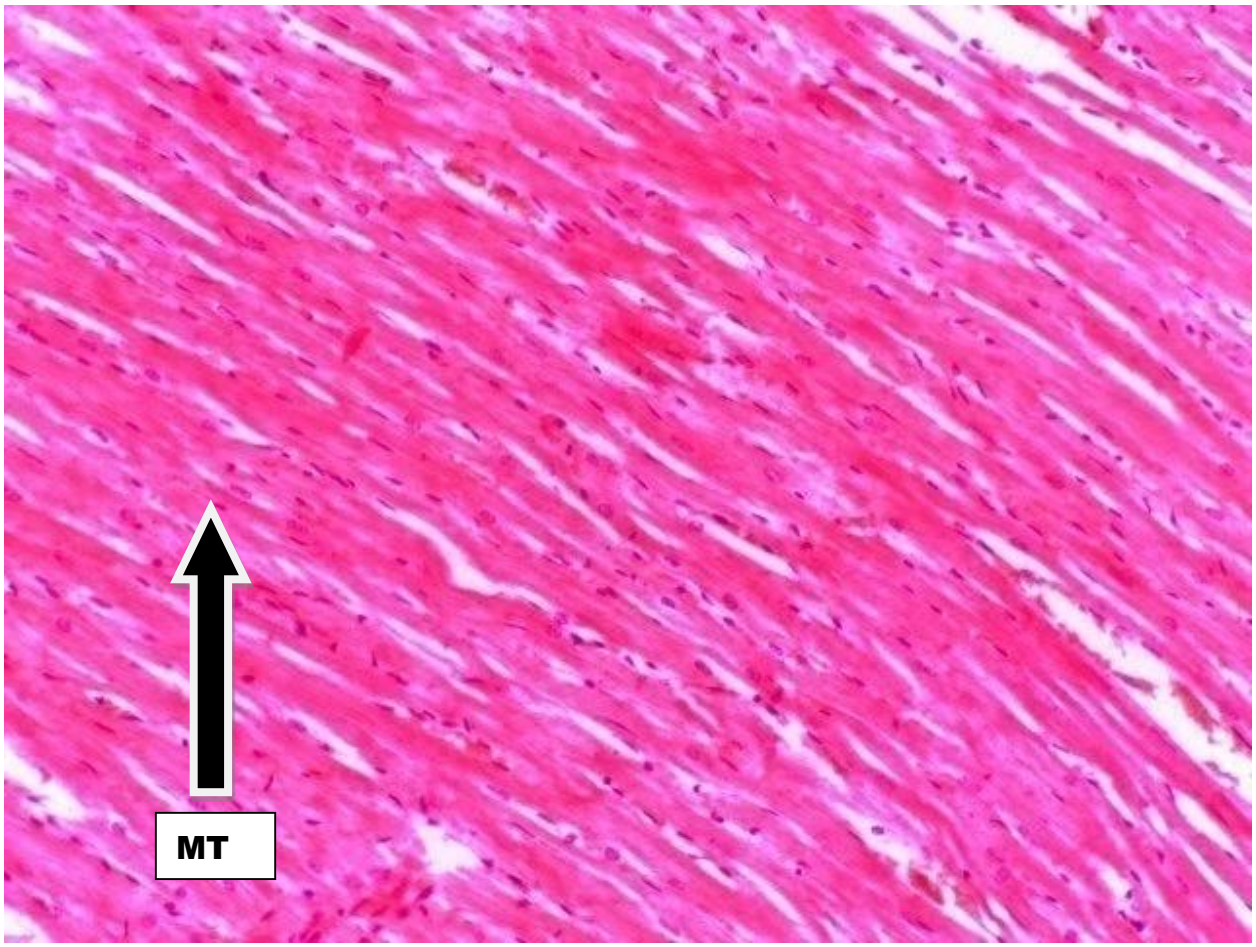


Plate 4.6: Photomicrograph of section of the heart of albino rat administered 10mg/kg body weight Cd for 90 days showing normal myocardial tissue (MT) running in one direction with fibrocytes (stromal cells) exhibiting normal histology (x400), Stain: H and E

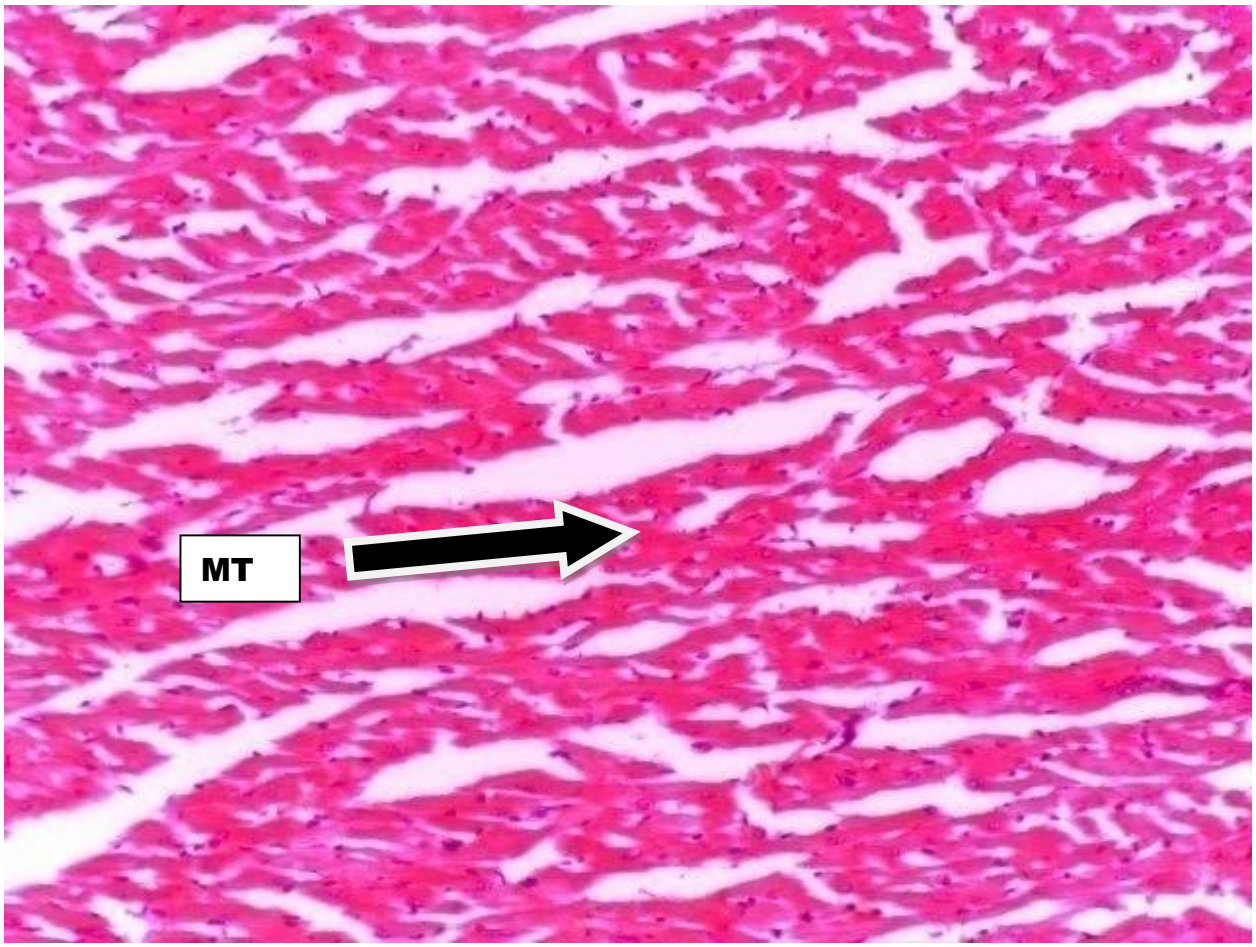


Plate 4.7: Photomicrograph of section of the heart of albino rat administered 10mg/kg body weight Cr for 90 days showing normal myocardial tissue (MT) running in one direction with fibrocytes (stromal cells) exhibiting normal histology (x400), Stain: H and E

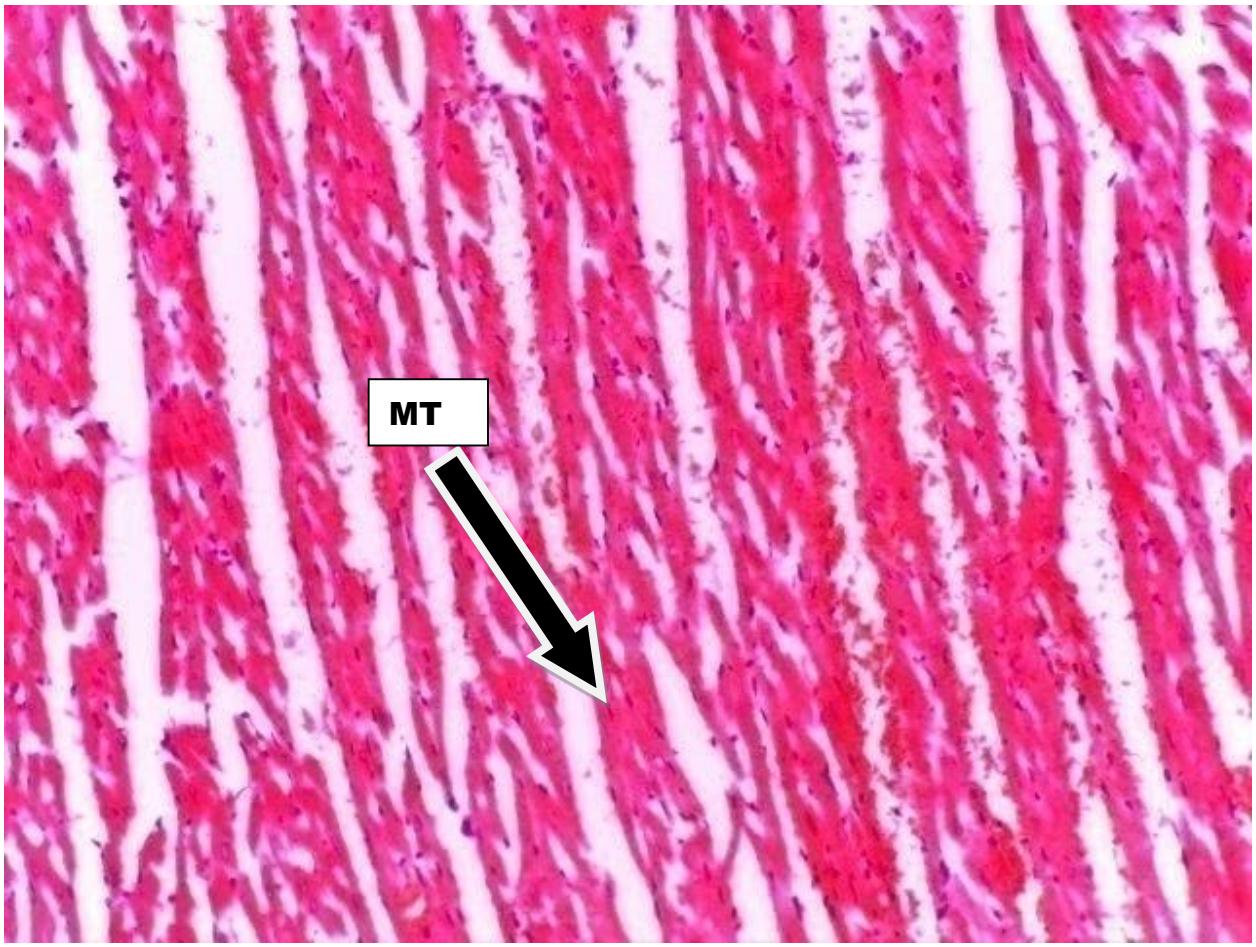


Plate 4.8: Photomicrograph of section of the heart of albino rat administered 10mg/kg body weight Pb for 90 days showing normal myocardial tissue (MT) running in one direction with fibrocytes (stromal cells) exhibiting normal histology (x400), Stain: H and E

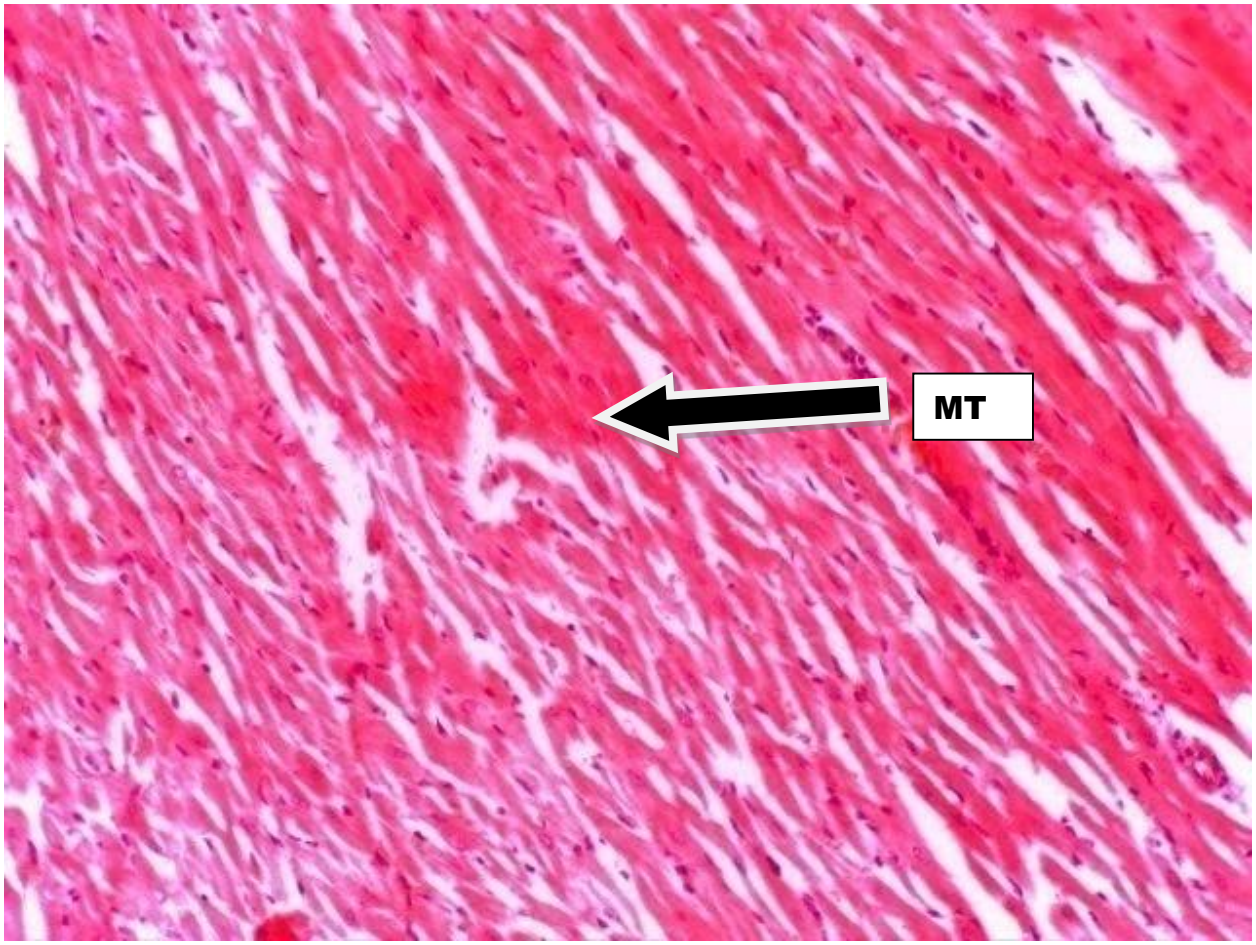


Plate 4.9: Photomicrograph of section of the heart of albino rat administered 10mg/kg body weight Cd, Cr, Pb mixture for 90 days showing normal myocardial tissue (MT) running in one direction with fibrocytes (stromal cells) exhibiting normal histology (x400), Stain: H and E

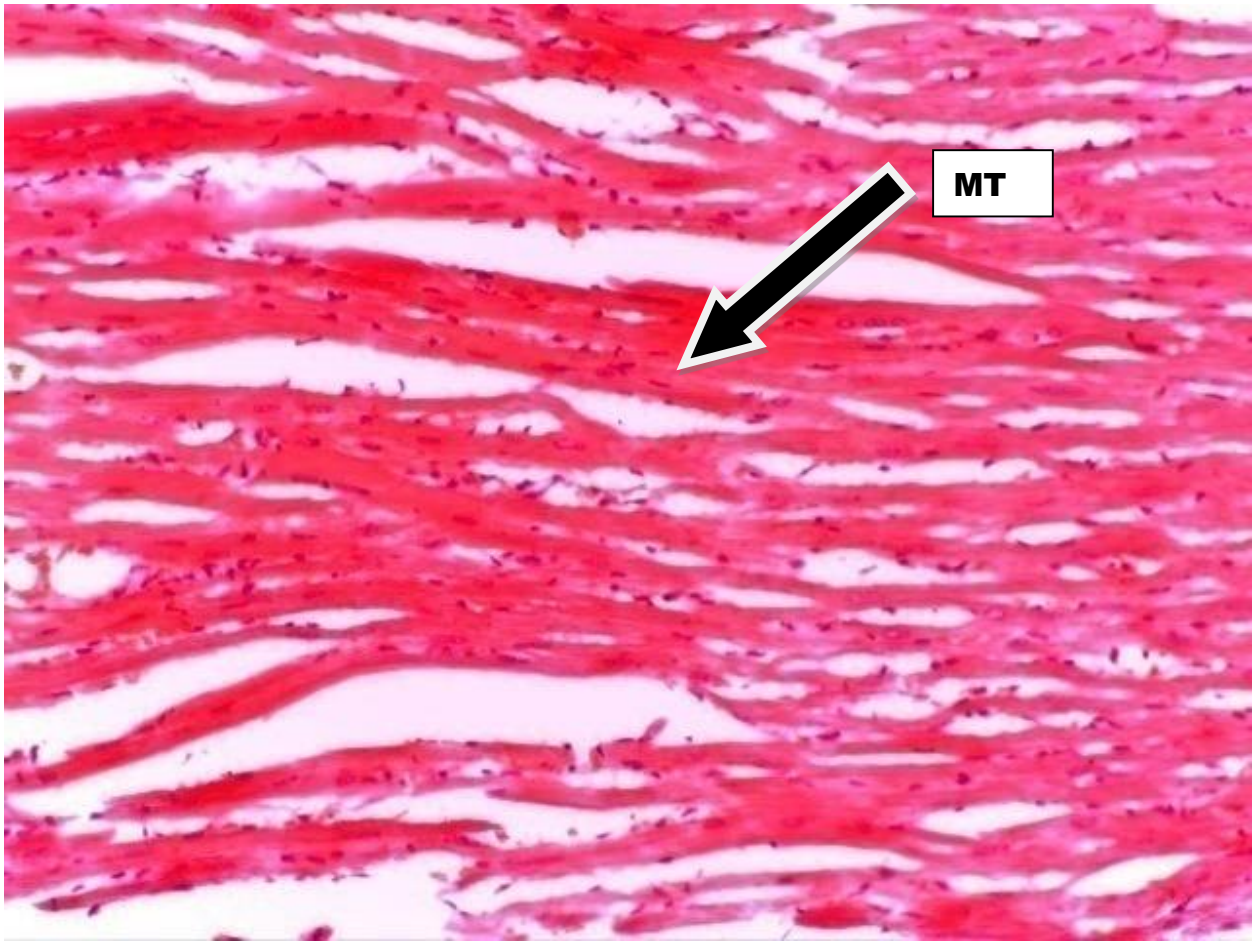


Plate 4.10: Photomicrograph of section of the heart of albino rat administered 20mg/kg body weight Cd for 90 days showing normal myocardial tissue (MT) running in one direction with fibrocytes (stromal cells) exhibiting normal histology (x400), Stain: H and E

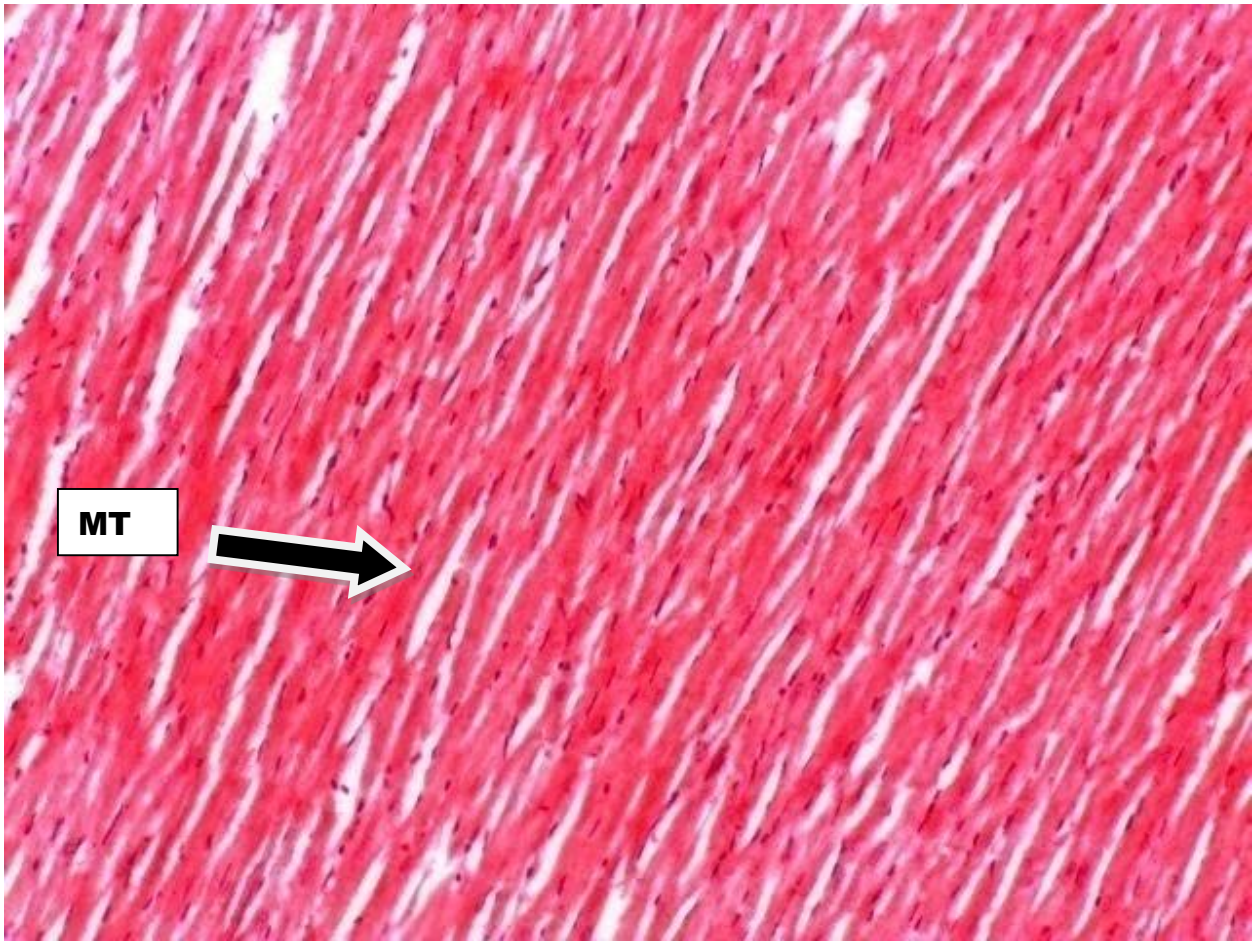


Plate 4.11: Photomicrograph of section of the heart of albino rat administered 20mg/kg body weight Cr for 90 days showing normal myocardial tissue (MT) running in one direction with fibrocytes (stromal cells) exhibiting normal histology (x400), Stain: H and E

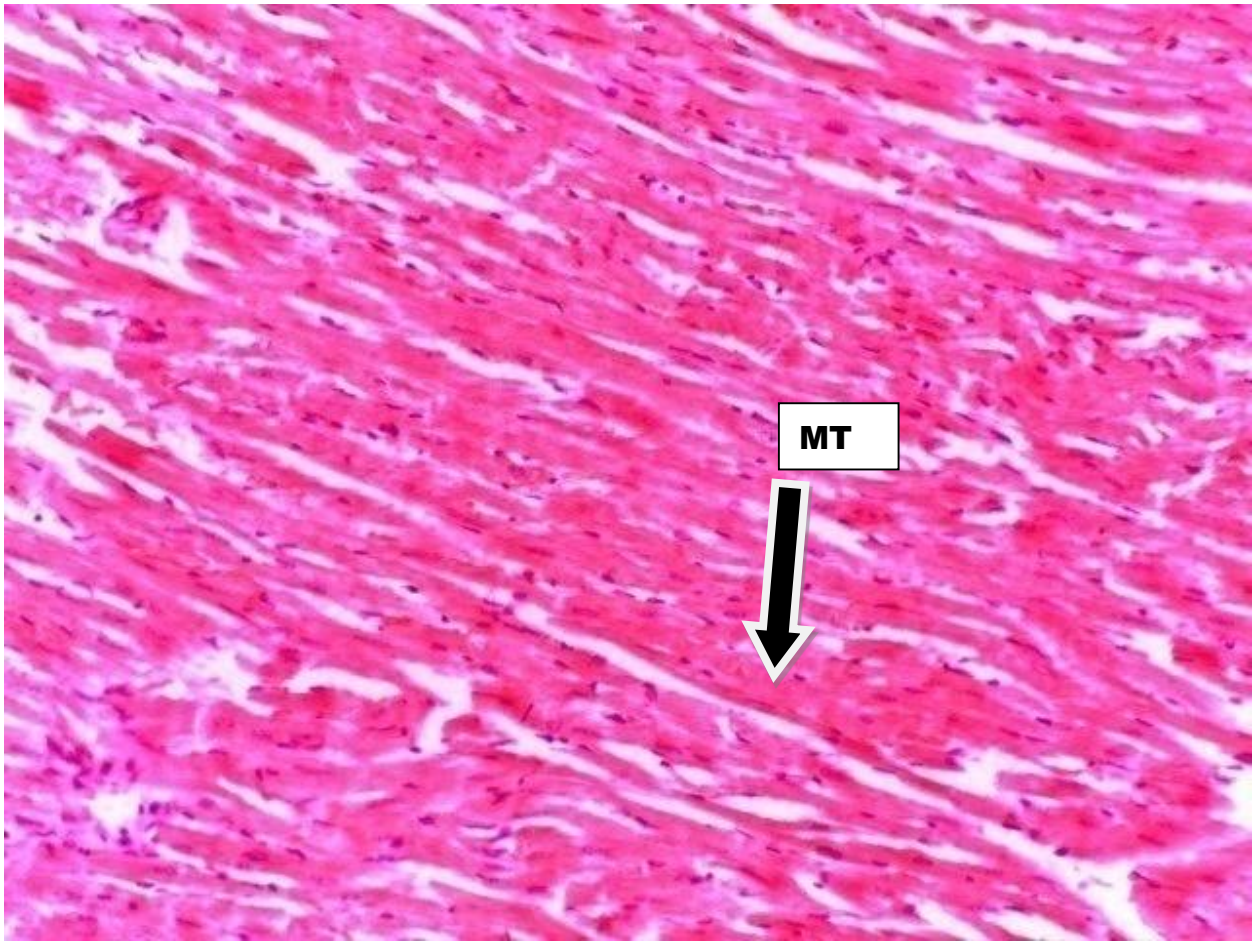


Plate 4.12: Photomicrograph of section of the heart of albino rat administered 20mg/kg body weight Pb for 90 days showing normal myocardial tissue (MT) running in one direction with fibrocytes (stromal cells) exhibiting normal histology (x400), Stain: H and E

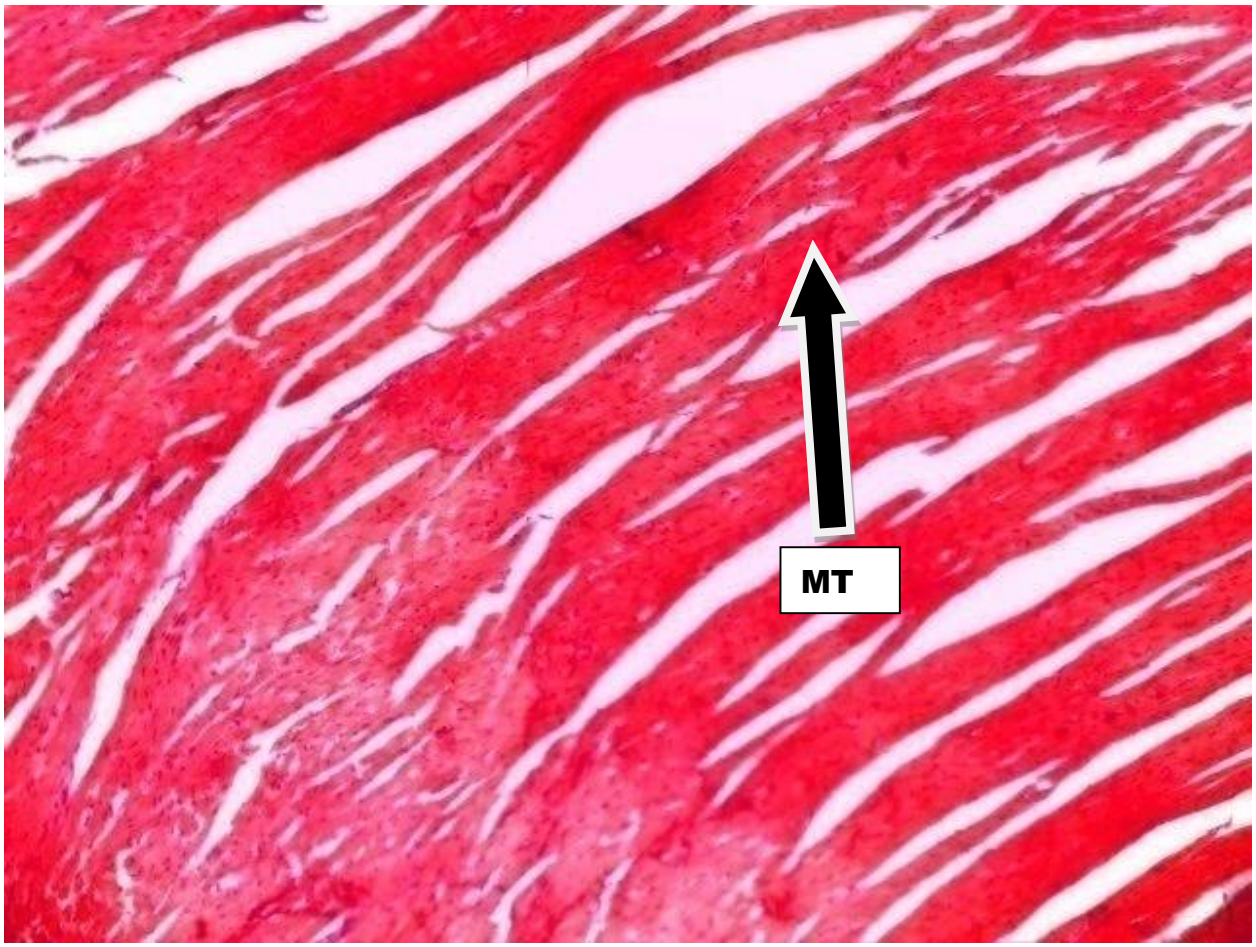


Plate 4.13: Photomicrograph of section of the heart of albino rat administered 20mg/kg body weight Cd, Cr, Pb mixture for 90 days showing normal myocardial tissue (MT) running in one direction with fibrocytes (stromal cells) exhibiting normal histology (x400), Stain: H and E

4.1.6.2 Histopathological evaluation of kidney of albino rats

Results of histological sections of kidneys of albino rats in the various treatment groups is summarized in Table 4.8. The 5mg/kg and 10mg/kg treatment dose groups compared with the control showed normal tissue architectural disposition (Plates 4.14 to 4.22). The glomeruli, Bowman's capsule, glomerular tuft, endothelial cells and tubes all appeared normal (Plates 4.14 to 4.22). The 20mg/kg treatment dose group was abnormal and showed some slightly and obviously shrunken glomeruli (atrophy) with increased Bowman's space (Plates 4.23 to 4.26). The interstitial tissue (stromal tissue) was fibrous with attempt at compressing some tubules and in some cases loss of identifiable tubules. The endothelial cells appeared normal. Also, there was cystically dilated space filled with eosinophilic materials.

Table 4.8 Summary of histopathological evaluation of kidney of albino ratstreated with Pb, Cd and Cr individually and as a mixture

Dose	Slide reading				
	Control	Mixture (Pb, Cd, Cr)	Pb	Cd	Cr
5mg/kg	Normal	Normal	Normal	Normal	Normal
10mg/kg	Normal	Normal	Normal	Normal	Normal
20mg/kg	Normal	Abnormal	Abnormal	Abnormal	Abnormal

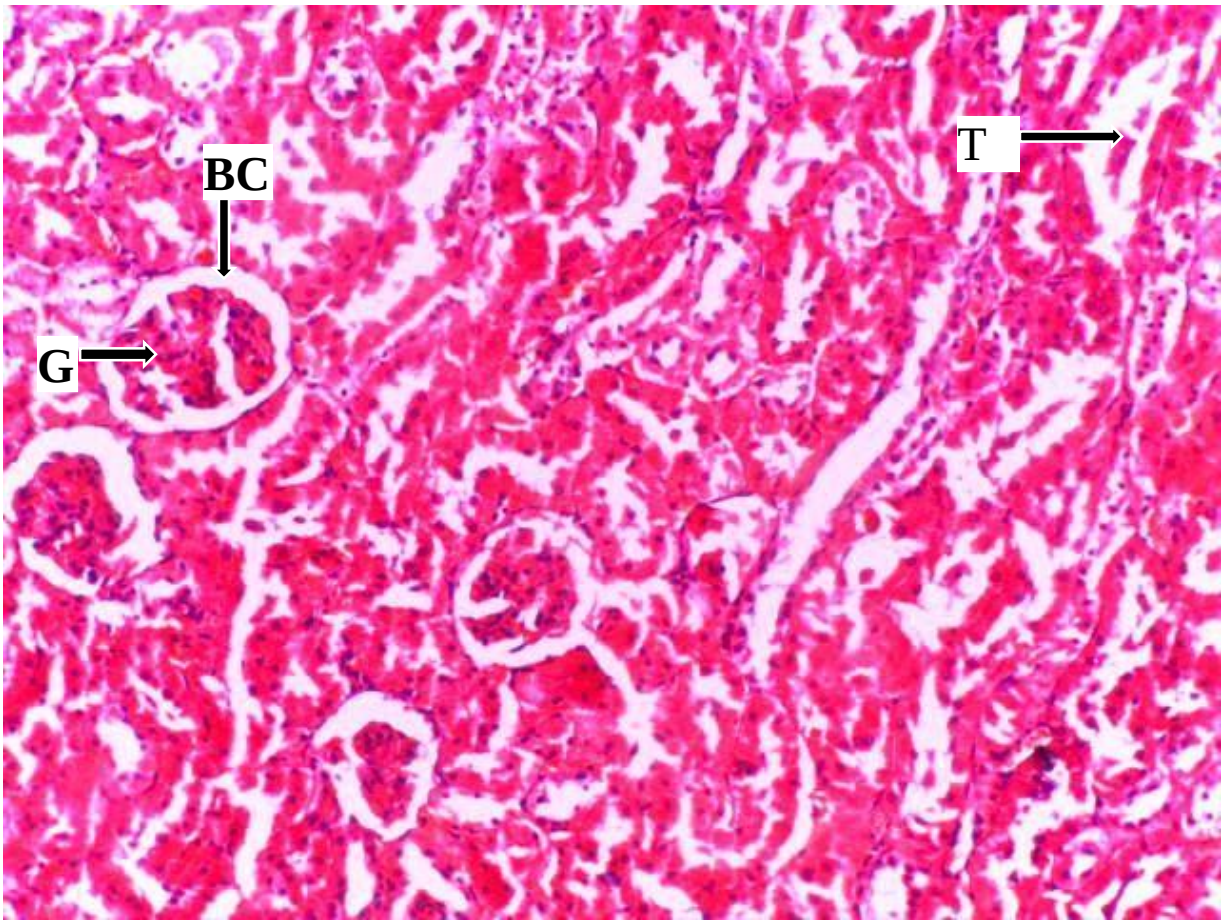


Plate 4.14: Photomicrograph of section of kidney of albino rat in the Control group for 90 days showing normal tissue architectural disposition. The glomeruli (G), Bowman's capsule (BC) (glomerular capsule), Glomerular tuft, Endothelial cells and Tubules (T) all appear normal (x400), Stain: H and E

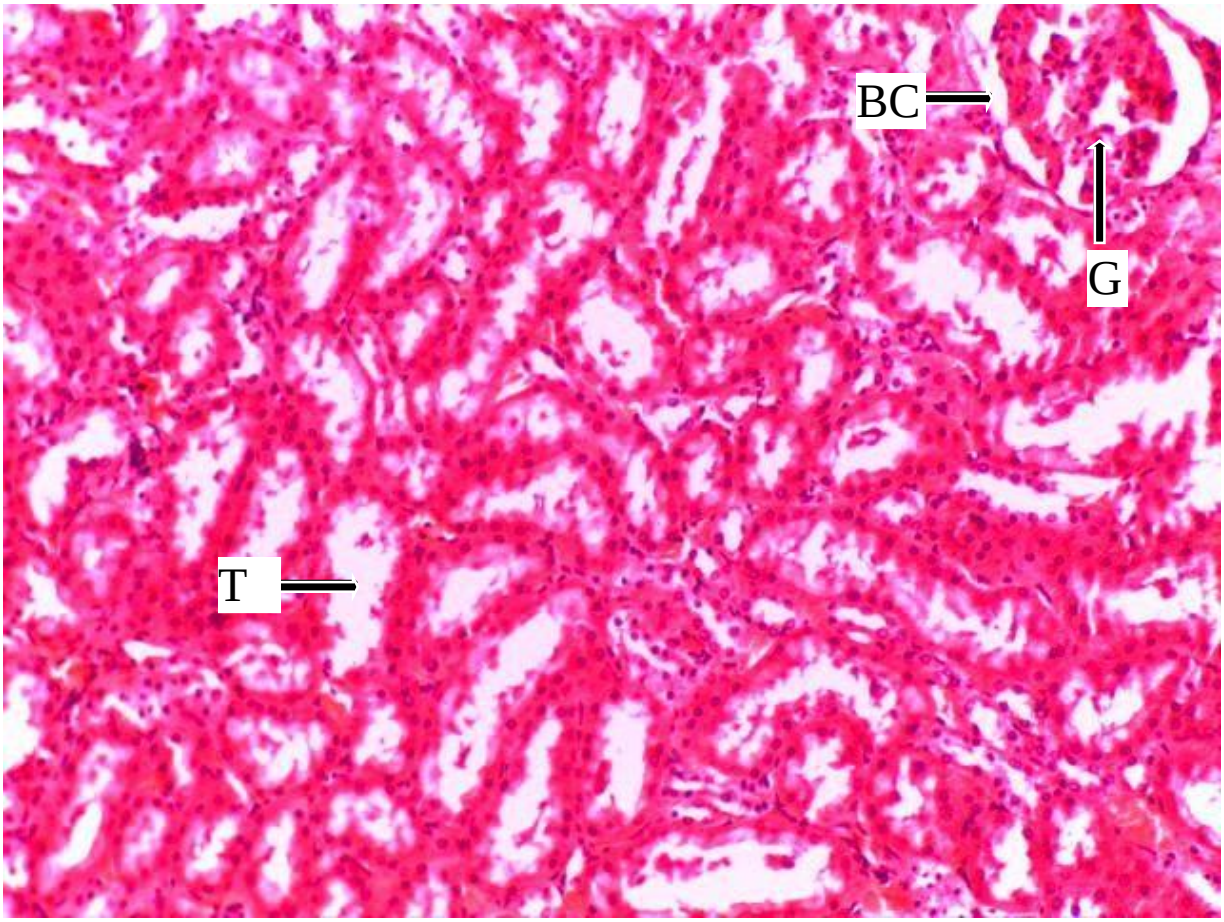


Plate 4.15: Photomicrograph of section of kidney of albino rat administered 5mg/kg body weight of Cd for 90 days showing normal tissue architectural disposition. The glomeruli (G), Bowman's capsule (BC) (glomerular capsule), Glomerular tuft, Endothelial cells and Tubules (T) all appear normal (x400), Stain: H and E

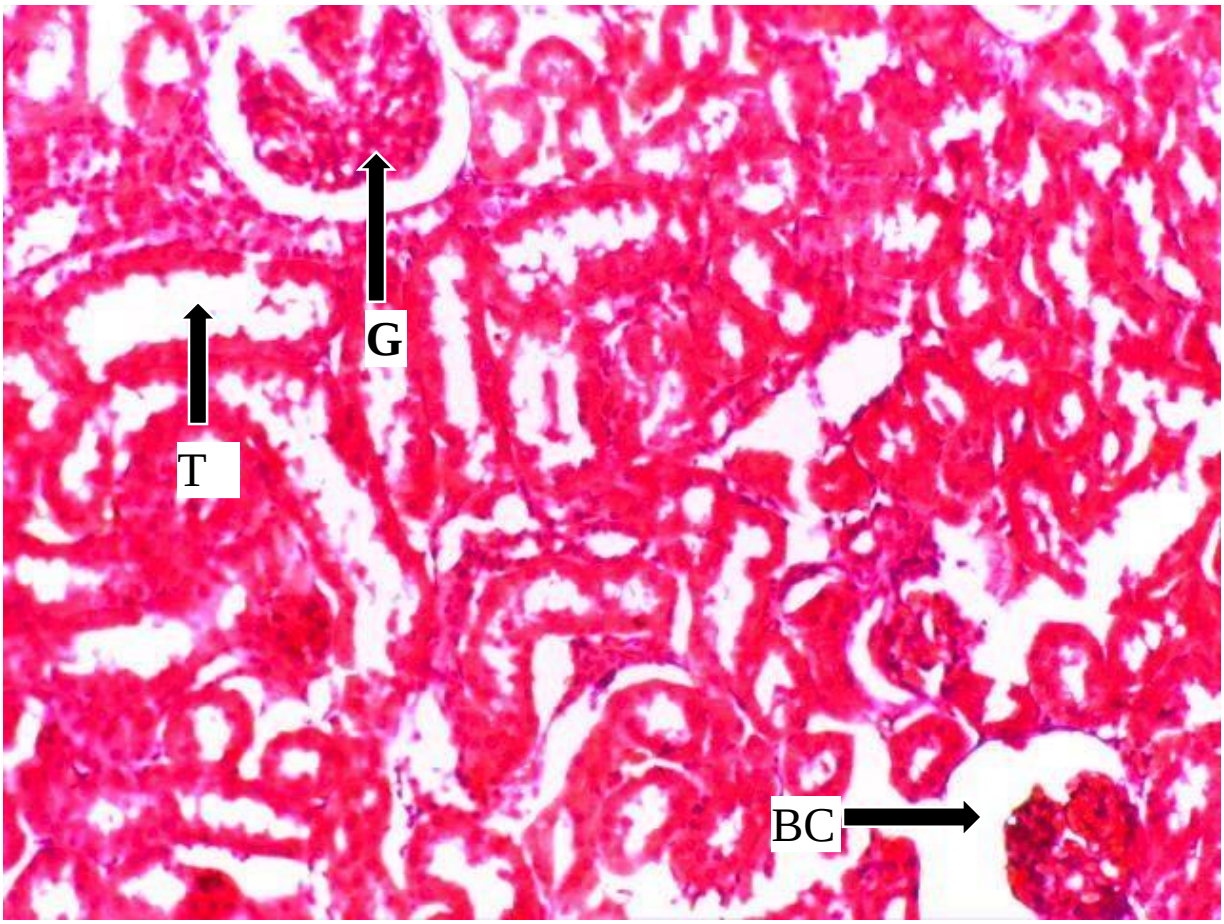


Plate 4.16: Photomicrograph of section of kidney of albino rat administered 5mg/kg body weight of Cr for 90 days showing normal tissue architectural disposition. The glomeruli (G), Bowman's capsule (BC) (glomerular capsule), Glomerular tuft, Endothelial cells and Tubules (T) all appear normal(x400), Stain: H and E

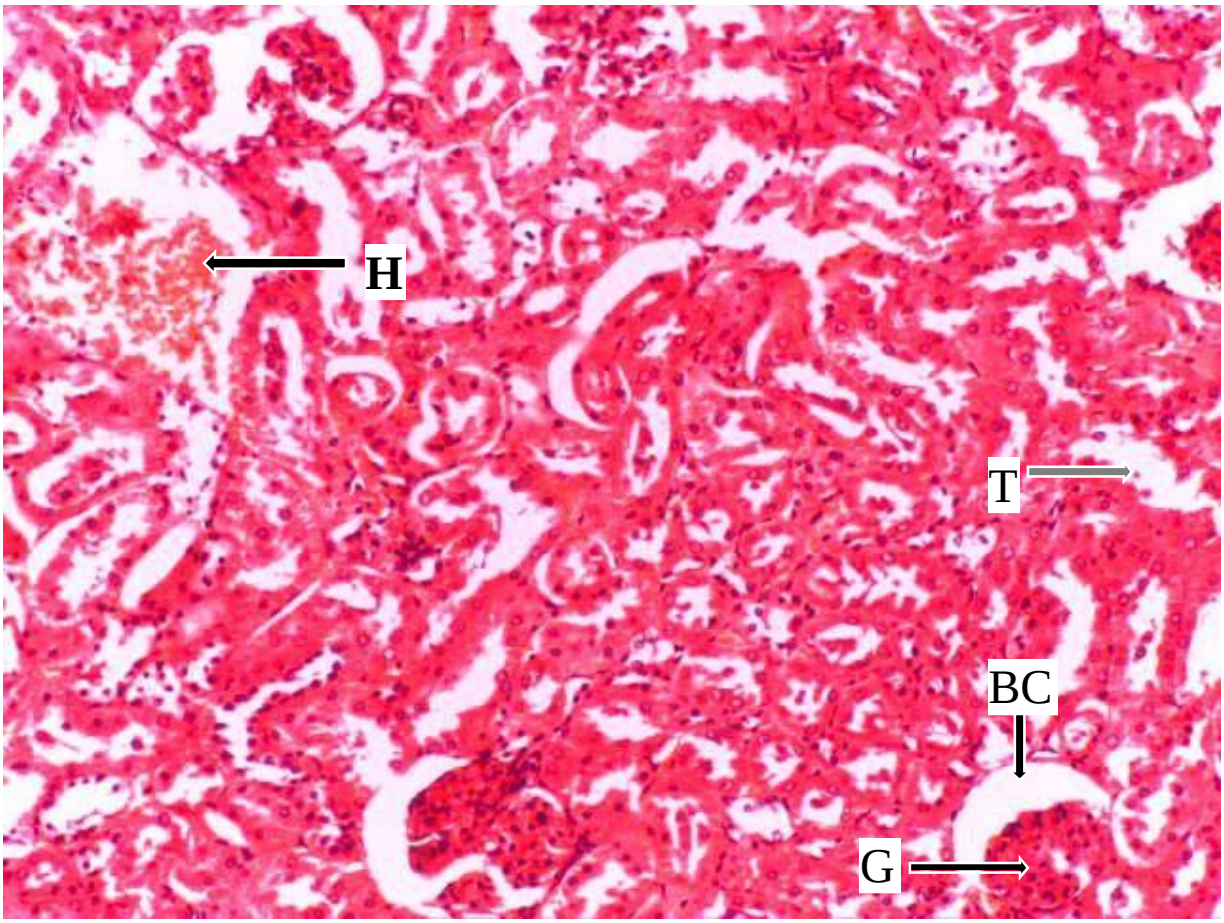


Plate 4.17: Photomicrograph of section of kidney of albino rat administered 5mg/kg body weight of Pb for 90 days showing normal tissue architectural disposition. The glomeruli (G), Bowman's capsule (BC) (glomerular capsule), Glomerular tuft, Endothelial cells and Tubules (T) all appear normal. H stands for hemorrhage(x400), Stain: H and E

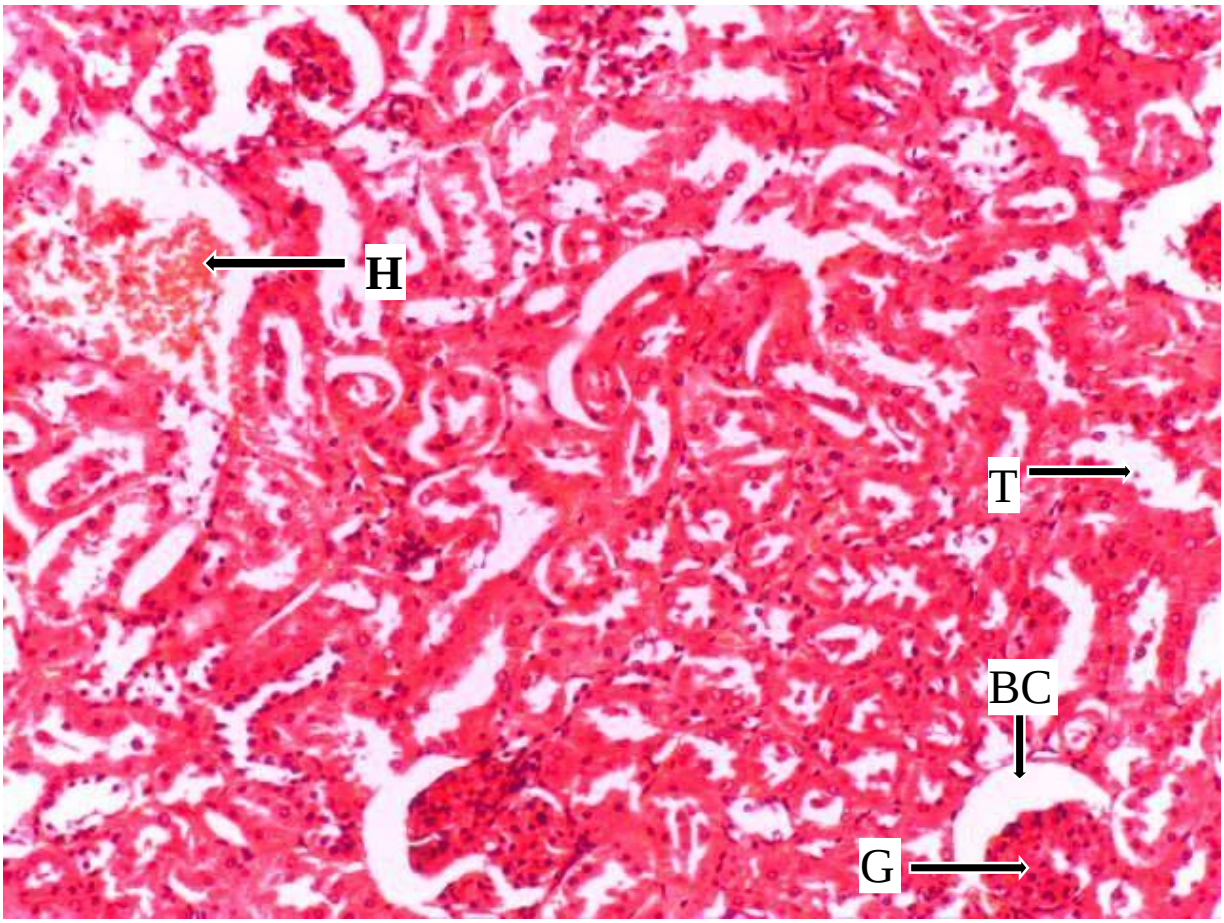


Plate 4.18: Photomicrograph of section of kidney of albino rat administered 5mg/kg body weight Cd, Cr, Pb mixture for 90 days showing normal tissue architectural disposition. The glomeruli (G), Bowman's capsule (BC) (glomerular capsule), Glomerular tuft, Endothelial cells and Tubules (T) all appear normal. H stands for hemorrhage (x400), Stain: H and E

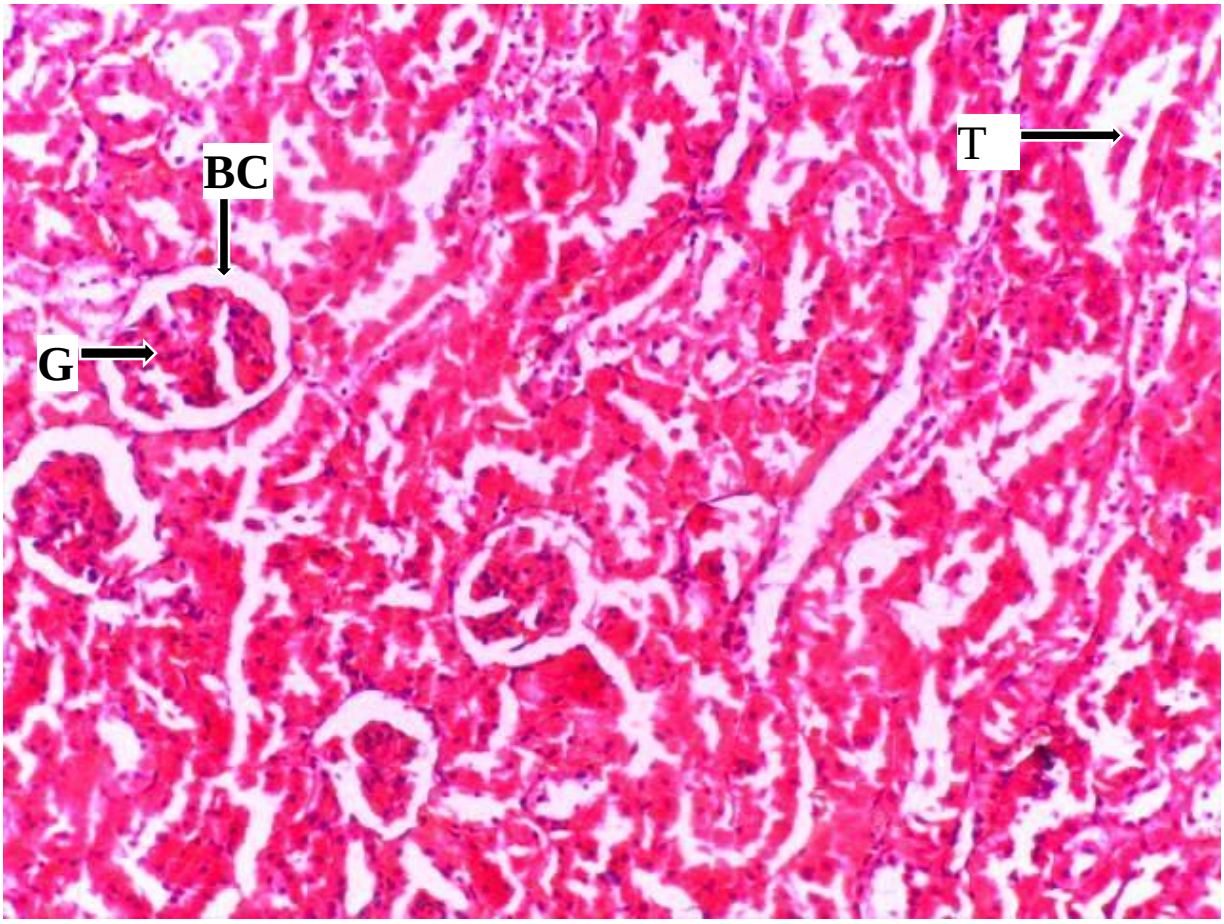


Plate 4.19: Photomicrograph of section of kidney of albino rat administered 10mg/kg body weight of Cd for 90 days showing normal tissue architectural disposition. The glomeruli (G), Bowman's capsule (BC) (glomerular capsule), Glomerular tuft, Endothelial cells and Tubules (T) all appear normal. (x400), Stain: H and E

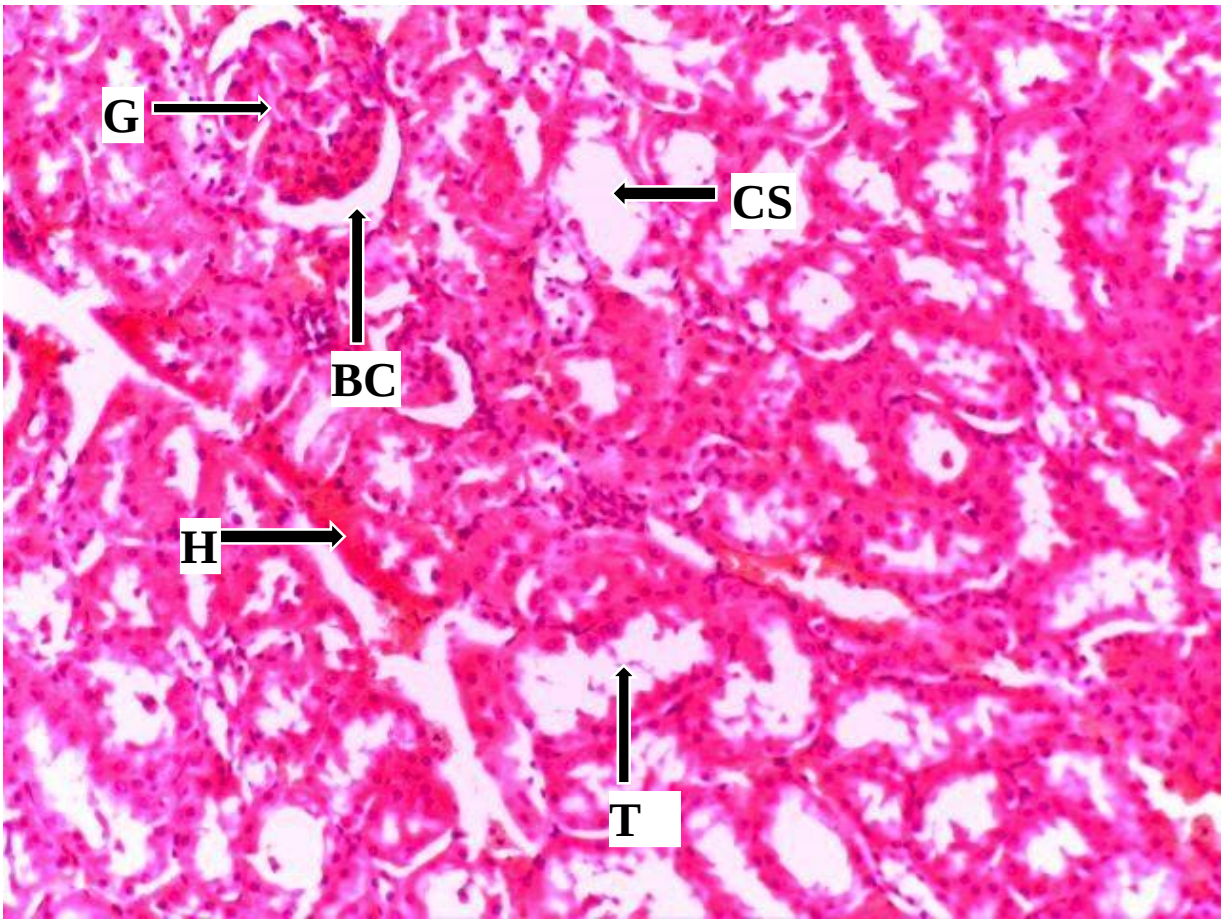


Plate 4.20: Photomicrograph of section of kidney of albino rat administered 10mg/kg body weight of Cr for 90 days showing normal tissue architectural disposition. The glomeruli (G), Bowman's capsule (BC) (glomerular capsule), Glomerular tuft, Endothelial cells and Tubules (T) all appear normal. H stands for hemorrhage. Also there is cystically dilated space (CS) filled with eosinophilic materials (x400), Stain: H and E

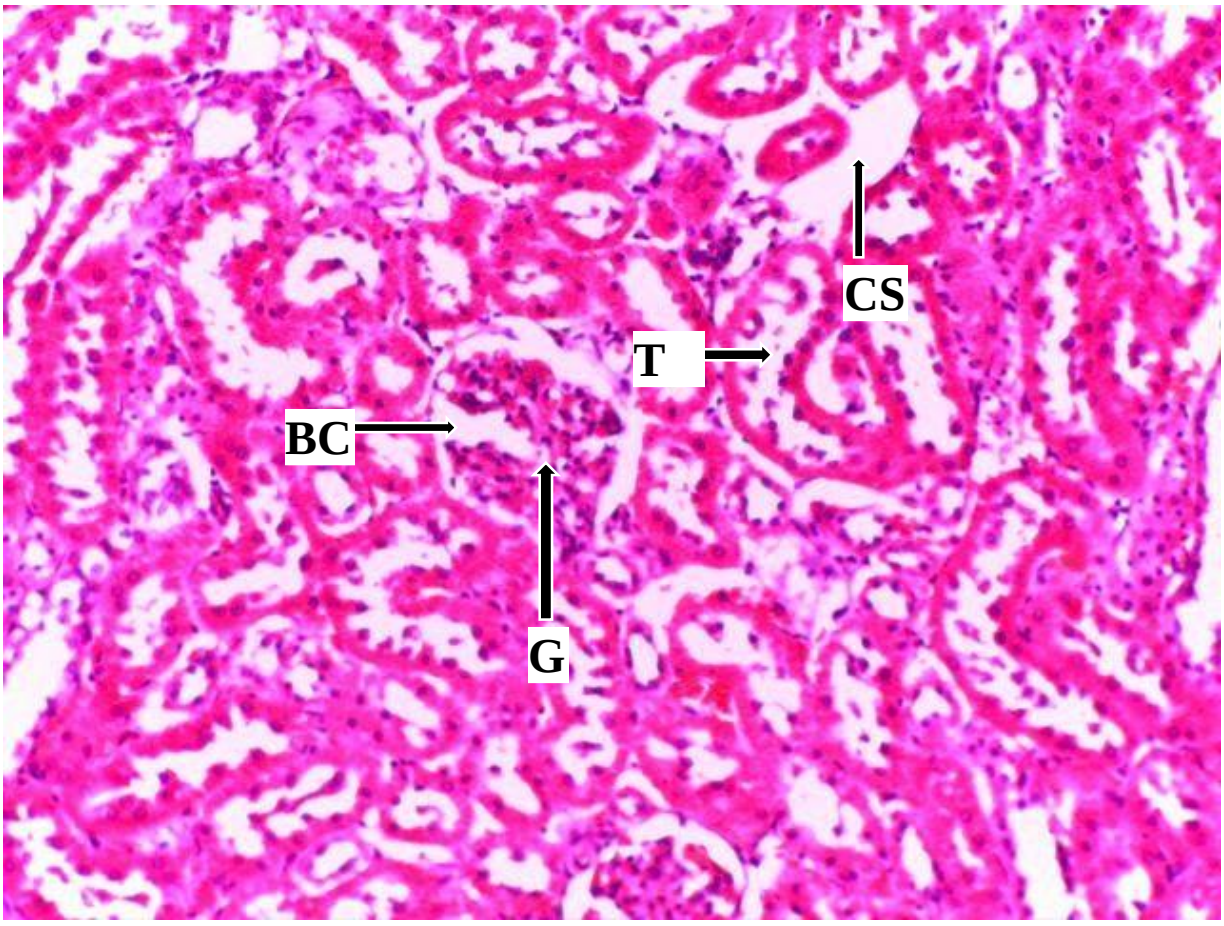


Plate 4.21: Photomicrograph of section of kidney of albino rat administered 10mg/kg body weight of Pb for 90 days showing normal tissue architectural disposition. The glomeruli (G), Bowman's capsule (BC) (glomerular capsule), Glomerular tuft, Endothelial cells and Tubules (T) all appear normal. Also there is cystically dilated space (CS) filled with eosinophilic materials (x400), Stain: H and E

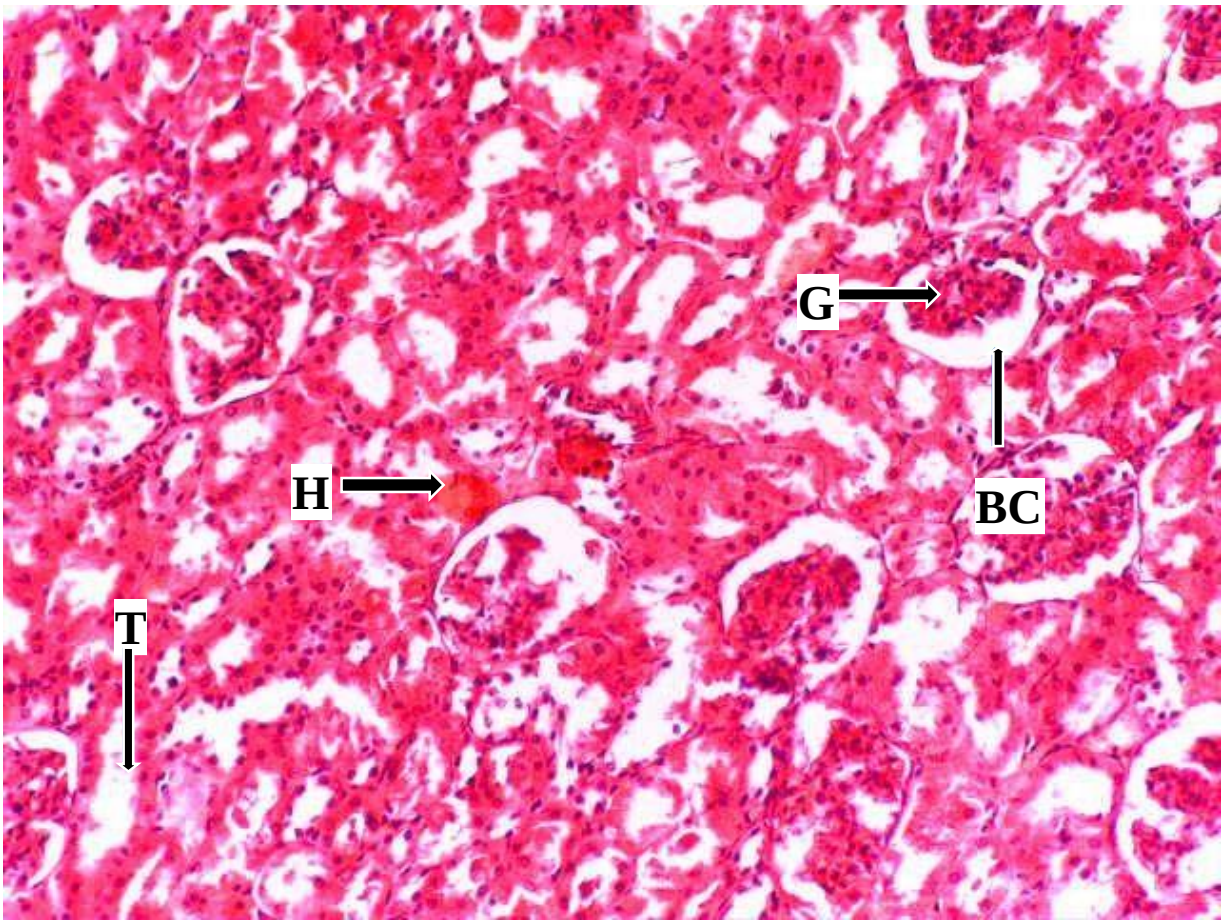


Plate 4.22: Photomicrograph of section of kidney of albino rat administered 10mg/kg body weight Cd, Cr, Pb mixture for 90 days showing normal tissue architectural disposition. The glomeruli (G), Bowman's capsule (BC) (glomerular capsule), Glomerular tuft, Endothelial cells and Tubules (T) all appear normal. H stands for hemorrhage (x400), Stain: H and E

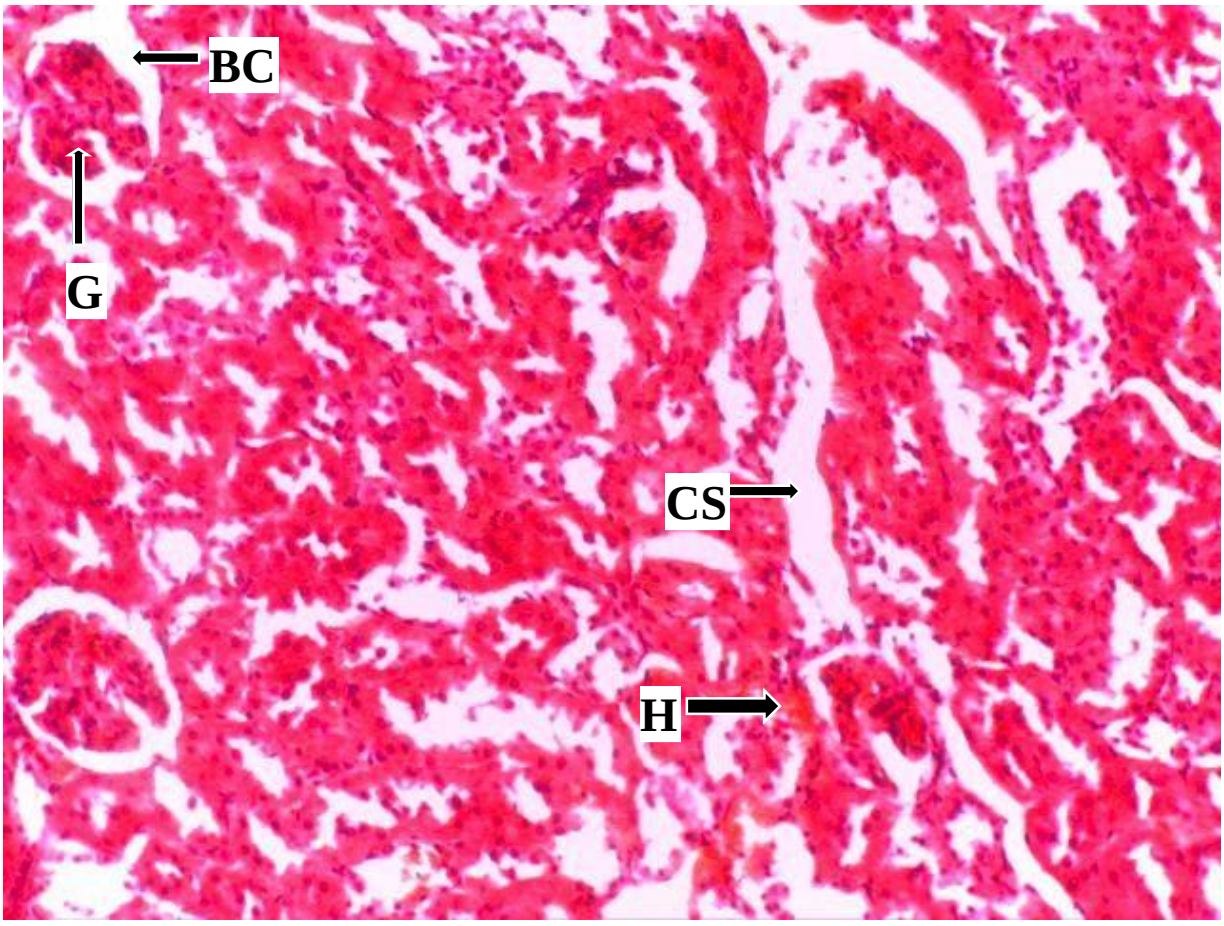


Plate 4.23: Photomicrograph of section of kidney of albino rat administered 20mg/kg body weight of Cd for 90 days showing some slightly and obviously shrunken glomeruli(atrophy) (G) with increased Bowman's space (BC). H stands for hemorrhage. The endothelial cells appear normal. Also there is cystically dilated space (CS) filled with eosinophilic materials (x400), Stain: H and E

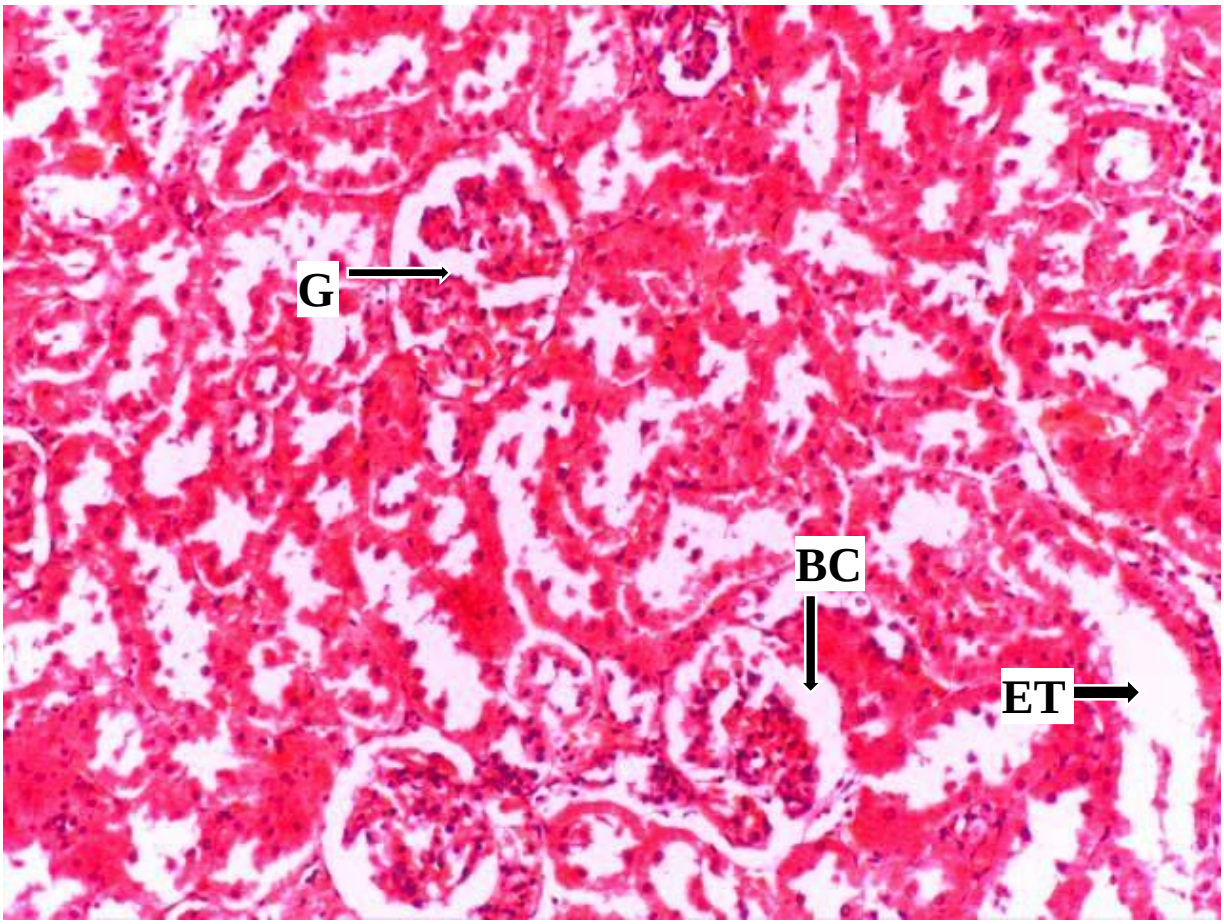


Plate 4.24: Photomicrograph of section of kidney of albino rat administered 20mg/kg body weight of Cr for 90 days showing some slightly and obviously shrunken glomeruli(atrophy) (G) with increased Bowman's space (BC). ET is enlarged tubule. The endothelial cells appear normal (x400), Stain: H and E

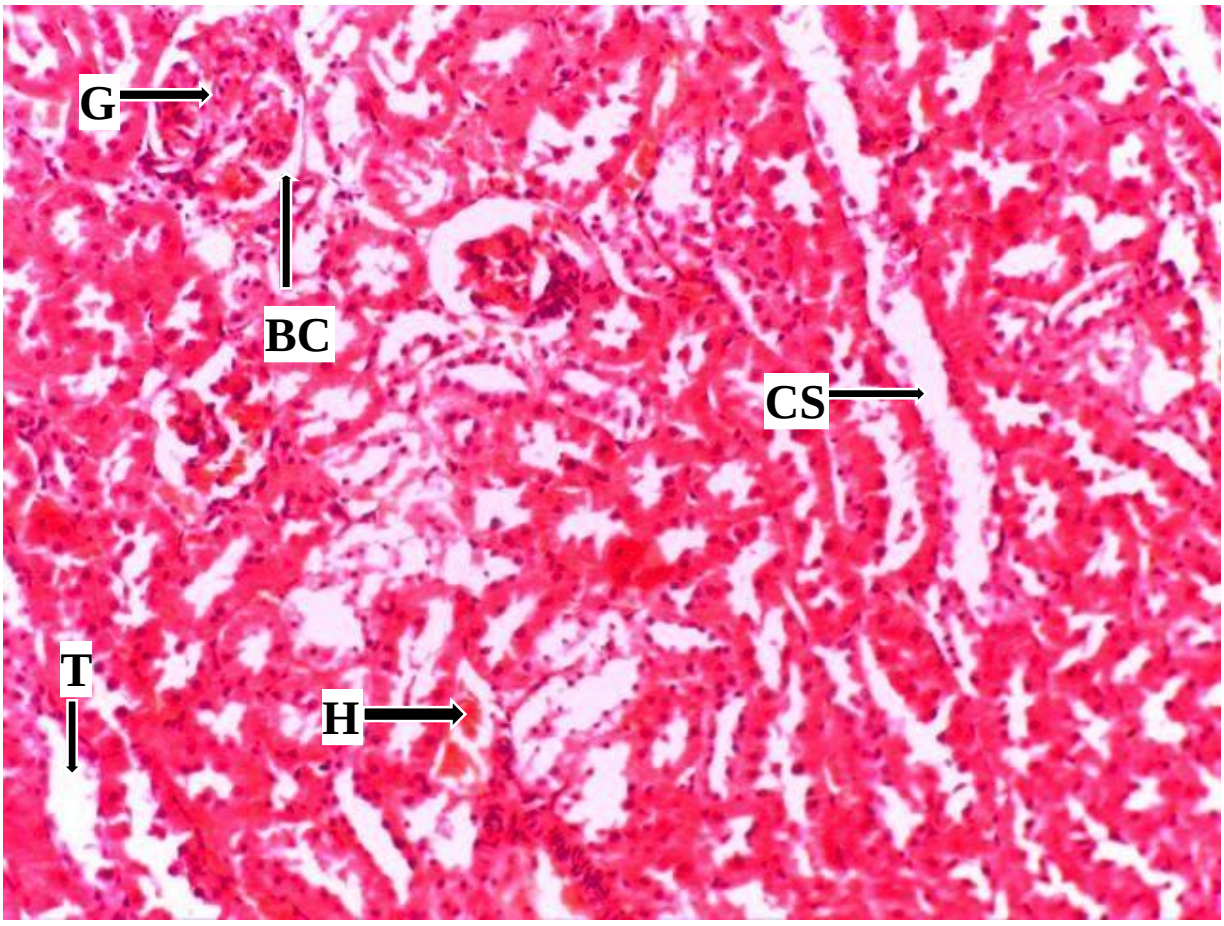


Plate 4.25: Photomicrograph of section of kidney of albino rat administered 20mg/kg body weight of Pb for 90 days showing some slightly and obviously shrunken glomeruli(atrophy) (G) with increased Bowman's space (BC). The interstitial tissue (stromal tissue) is fibrous with attempt at compressing some tubules (T) and in some cases loss of identifiable tubules. H stands for hemorrhage. The endothelial cells appear normal. Also there is cystically dilated space (CS) filled with eosinophilic materials (x400), Stain: H and E

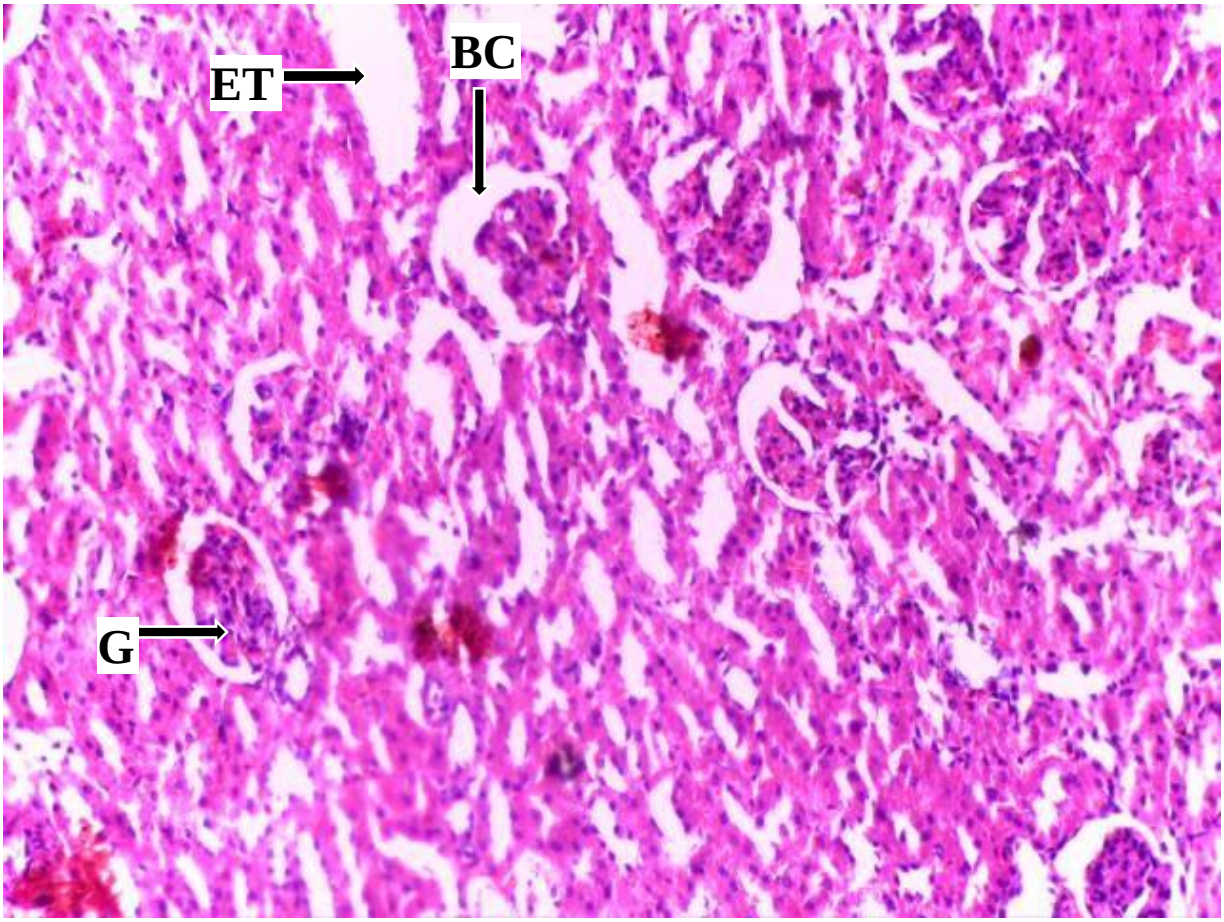


Plate 4.26: Photomicrograph of section of kidney of albino rat administered 20mg/kg body weight Cd, Cr, Pb mixture for 90 days showing some slightly and obviously shrunken glomeruli(atrophy) (G) with increased Bowman's space (BC). ET is enlarged tubule. The endothelial cells appear normal (x400), Stain: H and E

4.1.6.3 Histopathological evaluation of liver of albino rats treated with Pb, Cd and Cr individually and as a mixture

Result of histological sections of livers of albino ratstreated with Pb, Cd & Cr individually and as a mixture is summarized in Table 4.9. The 5mg/kg and 10mg/kg treatment dose groups compared with control showed normal tissue architecture. Within the interstitial tissue (stroma) were seen central vein, laminae (plate) of hepatocytes, sinusoids, hepatocytes and portal triad that appeared normal (Plates 4.27- 4.35). The 20mg/kg treatment group showed proliferation of stromal tissue (interstitial tissue) and hepatocytes with few hepatocytes that appeared swollen/enlarged (Plates 4.36- 4.39). The laminae (plate) of hepatocytes were not easily identifiable. The interstitial tissue was oedematous and the sinusoids were enlarged. In some areas there werefoci of necrosis (Plates 4.36- 4.39). This result suggests injury in the liver resulting from increased dose of the treatment metals in the combined and individual treatments.

Table 4.9 Summary of histopathological evaluation of liver of albino rats treated with Pb, Cd and Cr individually and as a mixture

Dose	Slide reading				
	Control	Mixture (Pb, Cd, Cr)	Pb	Cd	Cr
5mg/kg	Normal	Normal	Normal	Normal	Normal
10mg/kg	Normal	Normal	Normal	Normal	Normal
20mg/kg	Normal	Abnormal	Abnormal	Abnormal	Abnormal

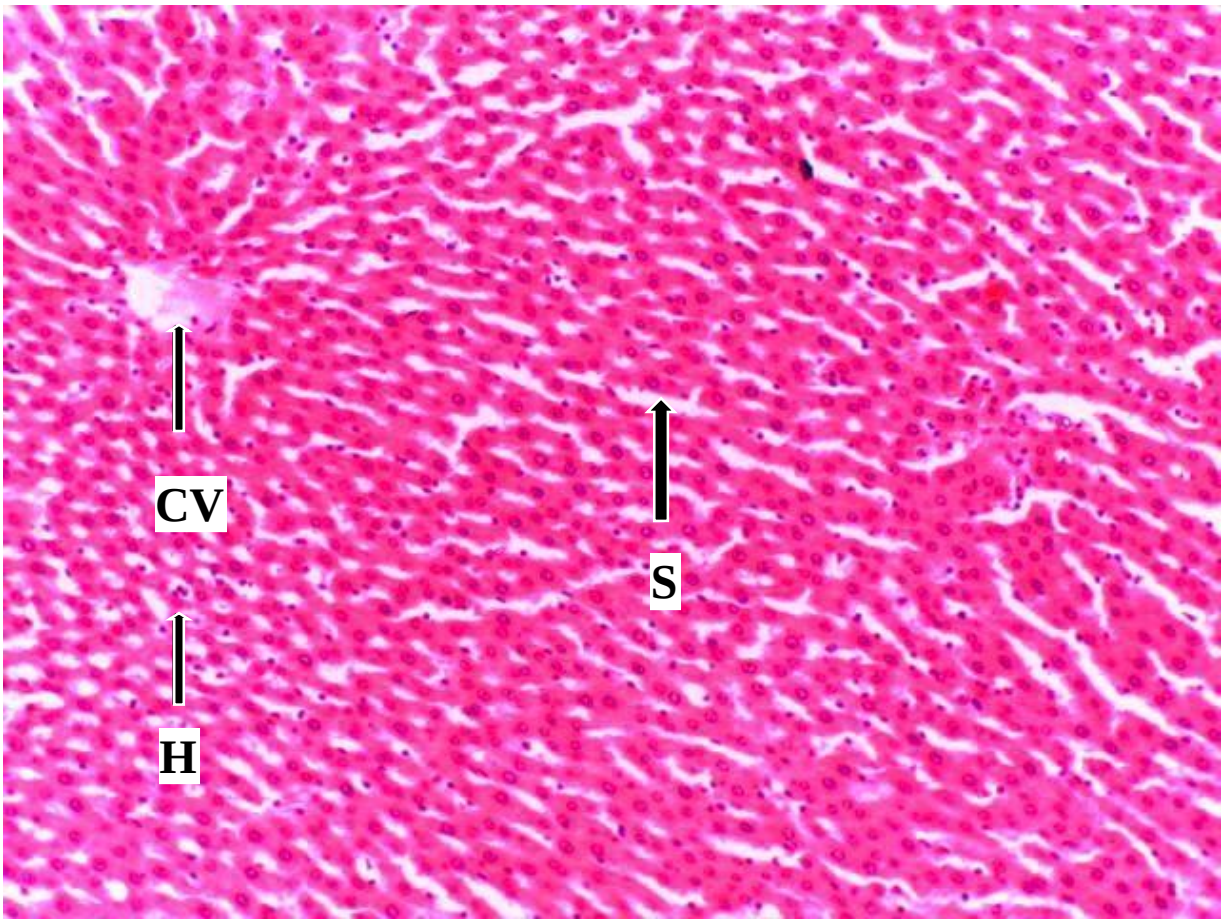


Plate 4.27: Photomicrograph of section of liver of albino rat in the Control group for 90 days showing normal tissue architecture. Within the interstitial tissue(stroma) are seen Central vein (CV), Laminae (plate) of hepatocytes (H), Sinusoids (S), capillary sinusoids (CS), Hepatocytes and Portal triad that appear normal (x400), Stain: H and E.

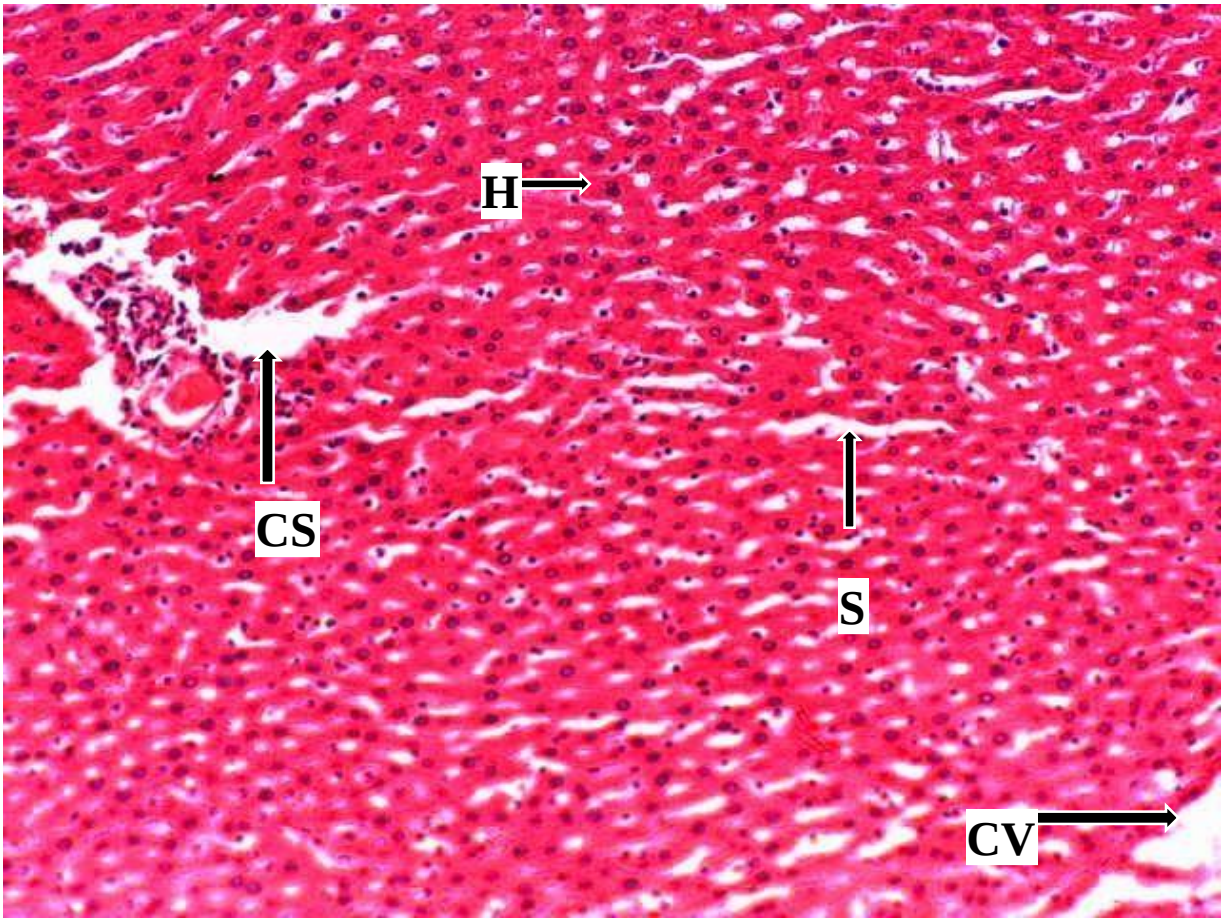


Plate 4.28: Photomicrograph of section of liver of albino rat administered 5mg/kg body weight of Cd for 90 days showing normal tissue architecture. Within the interstitial tissue(stroma) are seen Central vein (CV), Laminae (plate) of hepatocytes (H), Sinusoids (S), capillary sinusoids (CS), Hepatocytes and Portal triad that appear normal (x400), Stain: H and E.

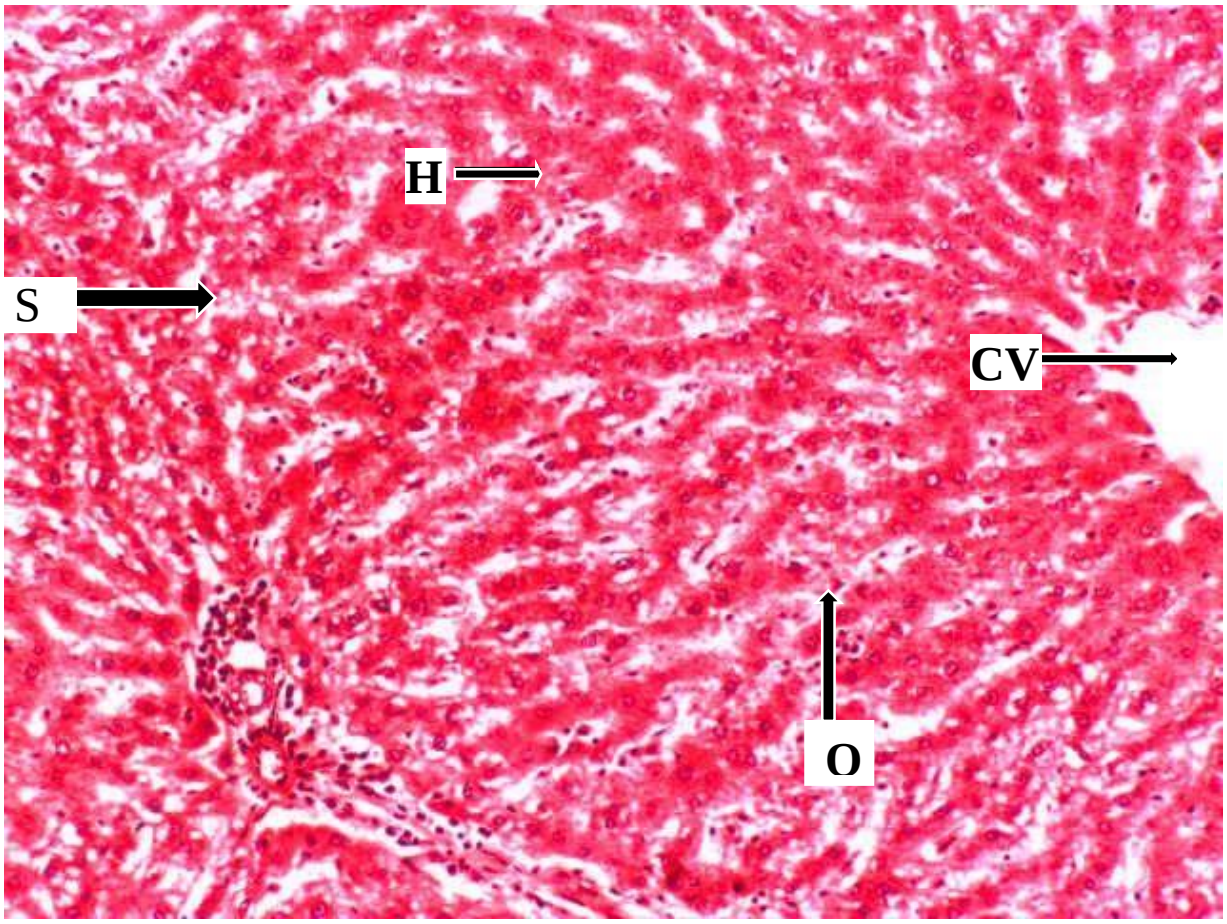


Plate 4.29: Photomicrograph of section of liver of albino rat administered 5mg/kg body weight of Cr for 90 days showing proliferation of stromal tissue (interstitial tissue) and hepatocytes (H) with few hepatocytes appearing swollen/enlarged. Within the interstitial tissue (stroma) are seen Central vein (CV). The laminae (plate) of hepatocytes are not easily identifiable. The interstitial tissue is oedematous (O) and the sinusoids (S) are enlarged. In some areas there are foci of necrosis (x400), Stain: H and E.

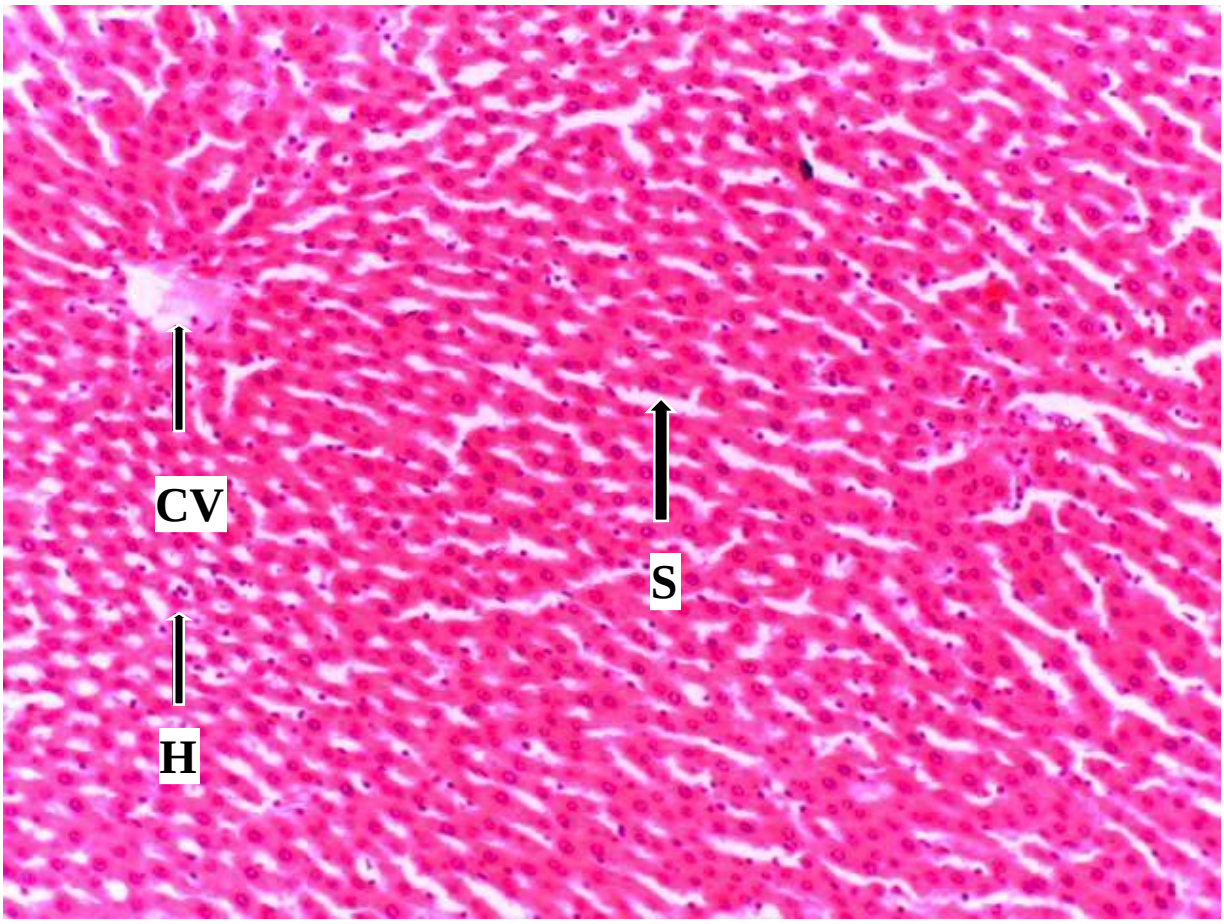


Plate 4.30: Photomicrograph of section of liver of albino rat administered 5mg/kg body weight of Pb for 90 days showing normal tissue architecture. Within the interstitial tissue(stroma) are seen Central vein (CV), Laminae (plate) of hepatocytes(H), Sinusoids(S), Hepatocytes and Portal triad that appear normal (x400), Stain: H and E.

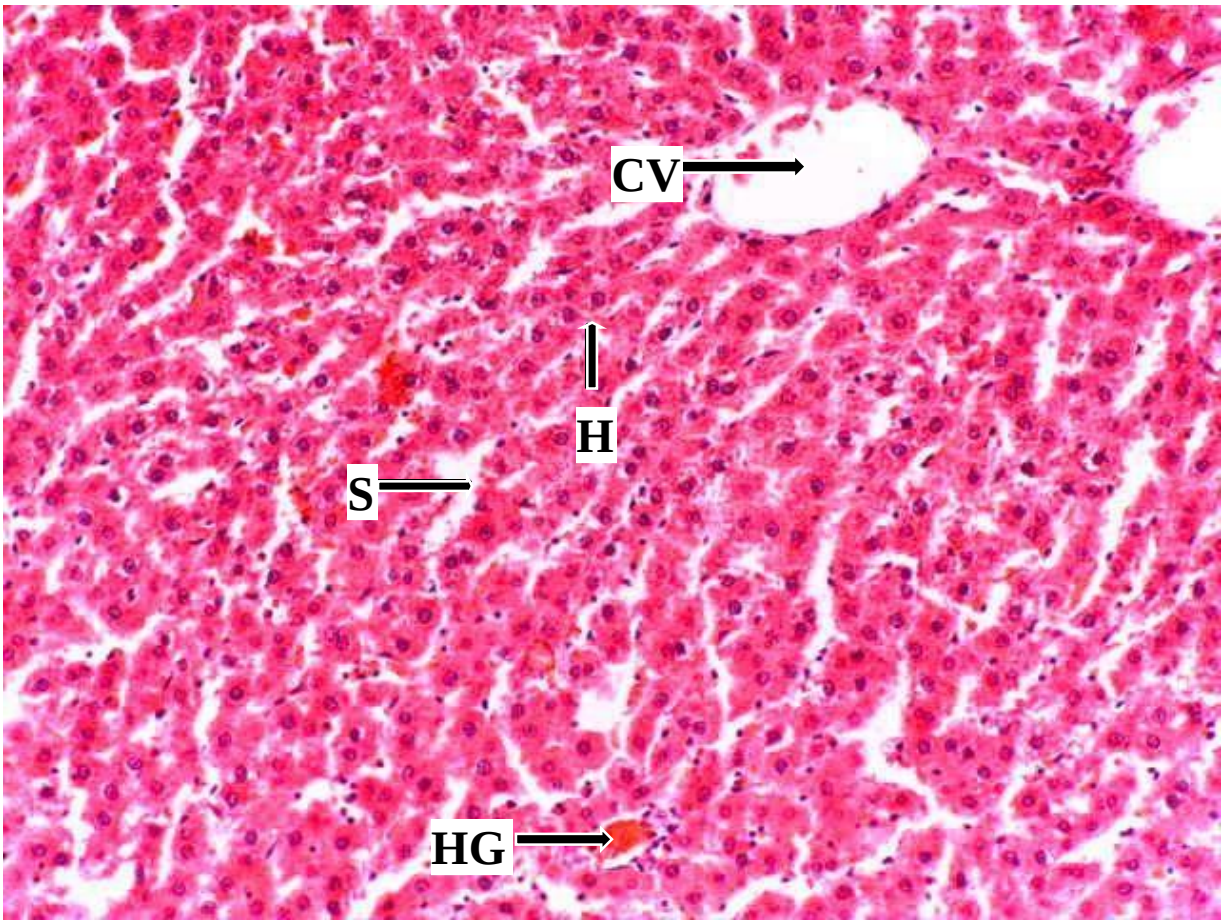


Plate 4.31: Photomicrograph of section of liver of albino rat administered 5mg/kg body weight Cd, Cr, Pb mixture for 90 days showing normal tissue architecture. Within the interstitial tissue (stroma) are seen Central vein (CV), Laminae (plate) of hepatocytes (H), Sinusoids (S), Hepatocytes and Portal triad that appear normal. HG stands for hemorrhage (x400), Stain: H and E.

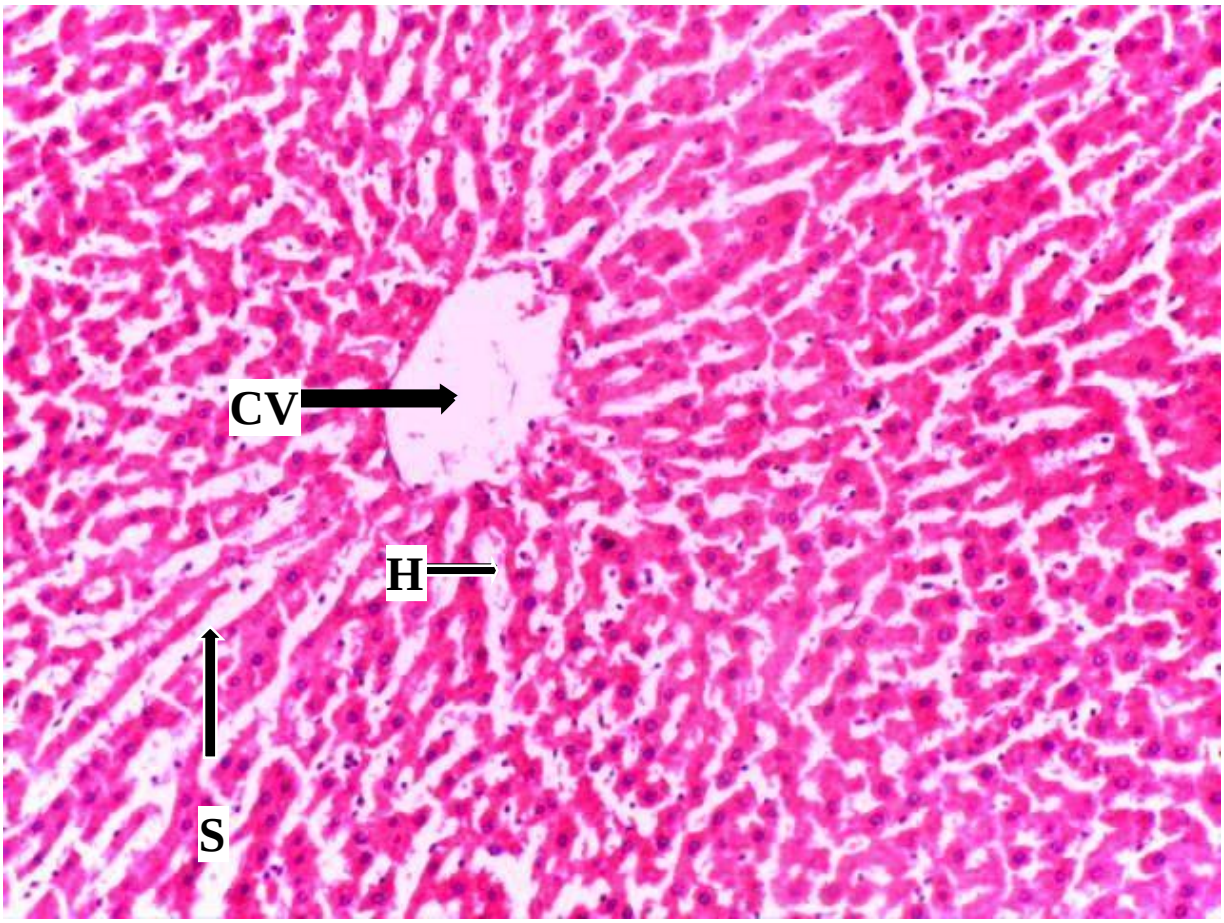


Plate 4.32: Photomicrograph of section of liver of albino rat administered 10mg/kg body weight of Cd for 90 days showing normal tissue architecture. Within the interstitial tissue(stroma) are seen Central vein (CV), Laminae (plate) of hepatocytes (H), Sinusoids(S), Hepatocytes and Portal triad that appear normal (x400), Stain: H and E.

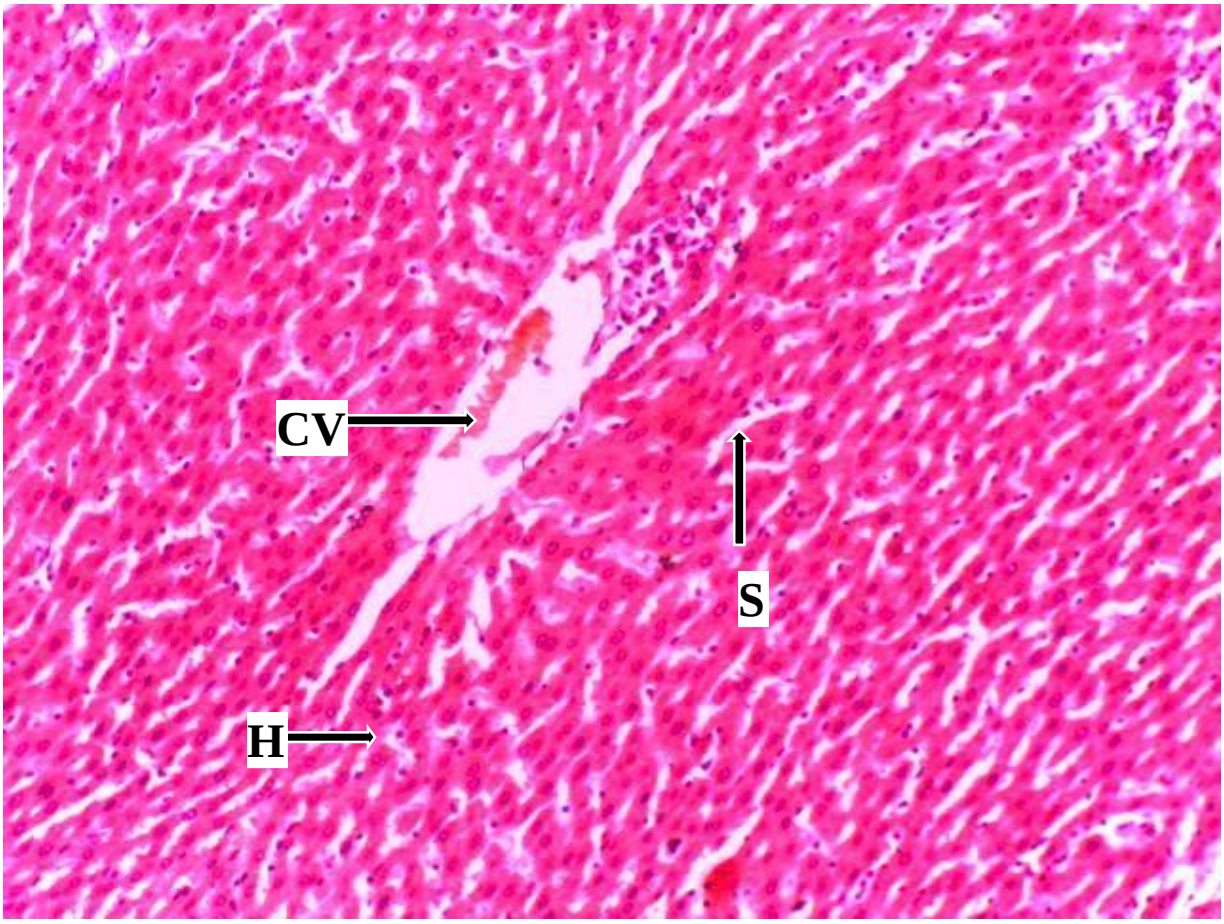


Plate 4.33: Photomicrograph of section of liver of albino rat administered 10mg/kg body weight of Cr for 90 days showing normal tissue architecture. Within the interstitial tissue (stroma) are seen Central vein (CV), Laminae (plate) of hepatocytes (H), Sinusoids (S), Hepatocytes and Portal triad that appear normal (x400), Stain: H and E.

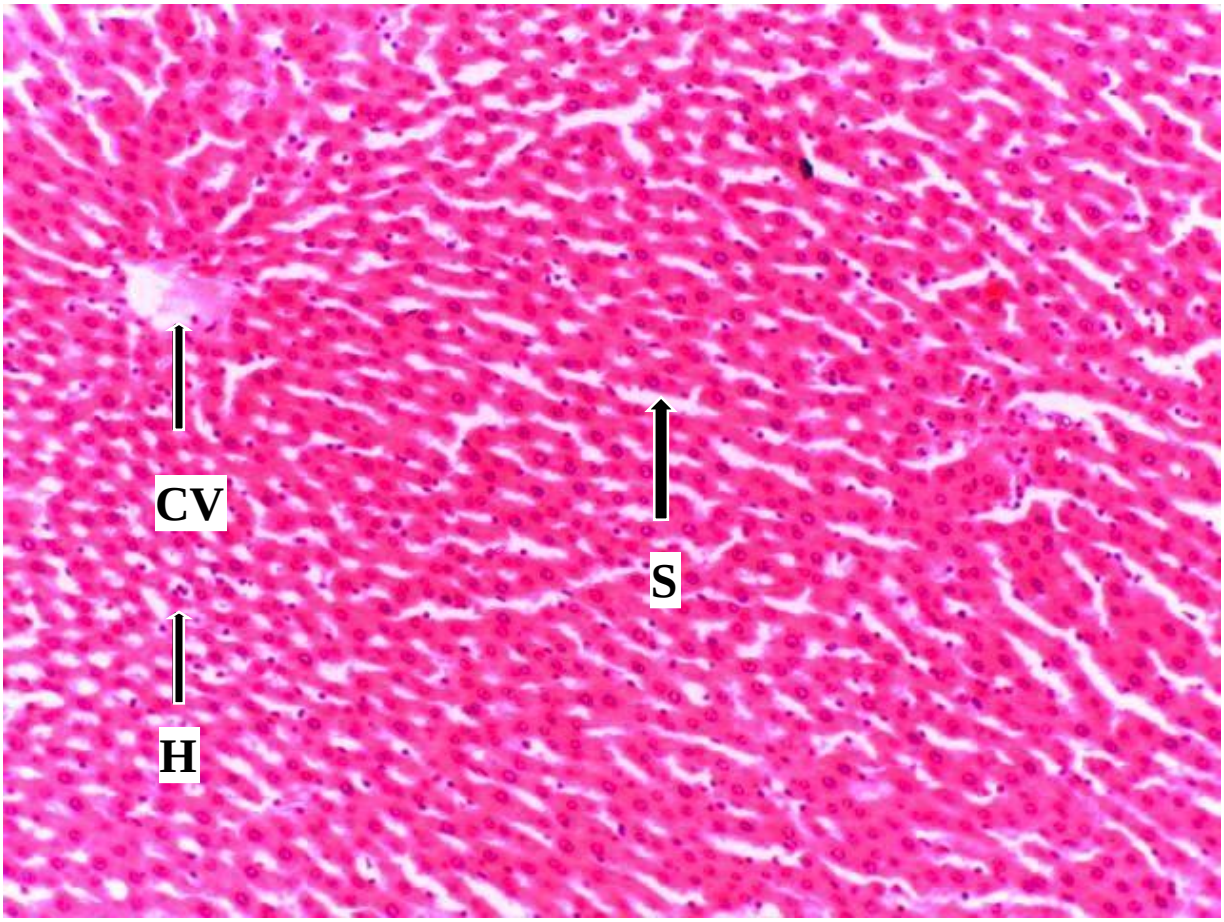


Plate 4.34: Photomicrograph of section of liver of albino rat administered 10mg/kg body weight of Pb for 90 days showing normal tissue architecture. Within the interstitial tissue(stroma) are seen Central vein (CV), Laminae (plate) of hepatocytes (H), Sinusoids (S), Hepatocytes and Portal triad that appear normal (x400), Stain: H and E

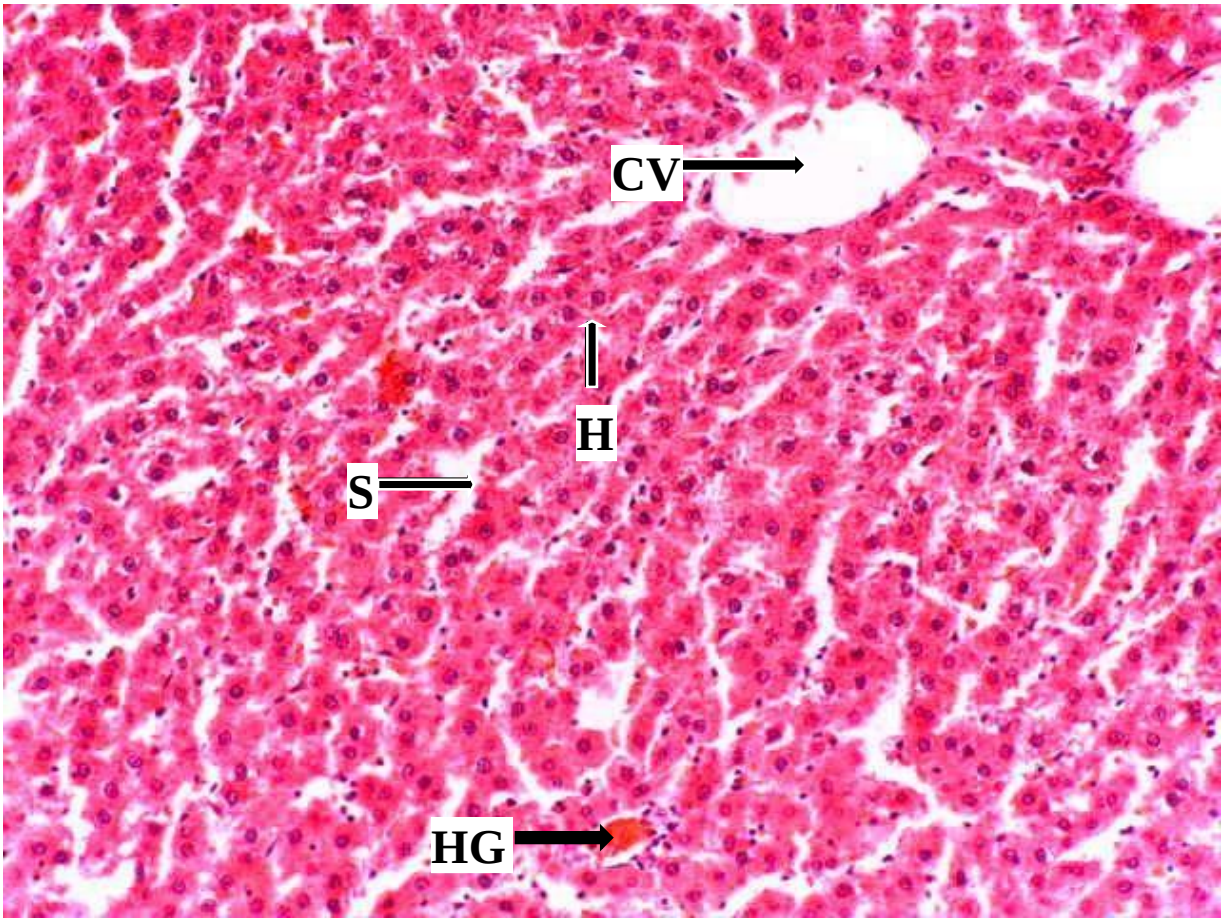


Plate 4.35: Photomicrograph of section of liver of albino rat administered 10mg/kg body weight Cd, Cr, Pb mixture for 90 days showing normal tissue architecture. Within the interstitial tissue (stroma) are seen Central vein (CV), Laminae (plate) of hepatocytes (H), Sinusoids (S), Hepatocytes and Portal triad that appear normal. HG stands for hemorrhage (x400), Stain: H and E.

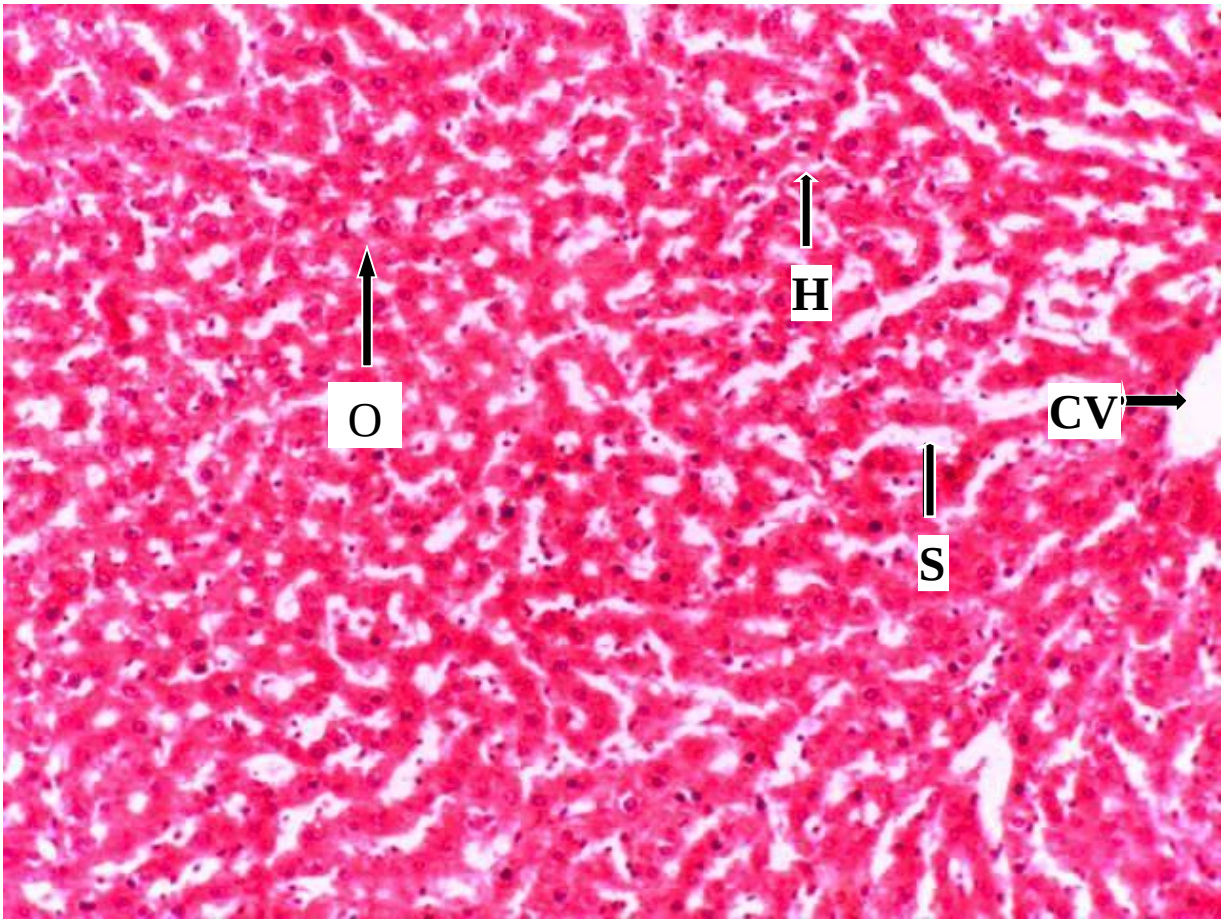


Plate 4.36: Photomicrograph of section of liver of albino rat administered 20mg/kg body weight of Cd for 90 days showing proliferation of stromal tissue (interstitial tissue) and hepatocytes (H) with few hepatocytes appearing swollen/enlarged. Within the interstitial tissue(stroma) are seen Central vein (CV). The laminae (plate) of hepatocytes are not easily identifiable. The interstitial tissue is oedematous (O) and the sinusoids (S) are enlarged. In some areas there are foci of necrosis (x400), Stain: H and E.

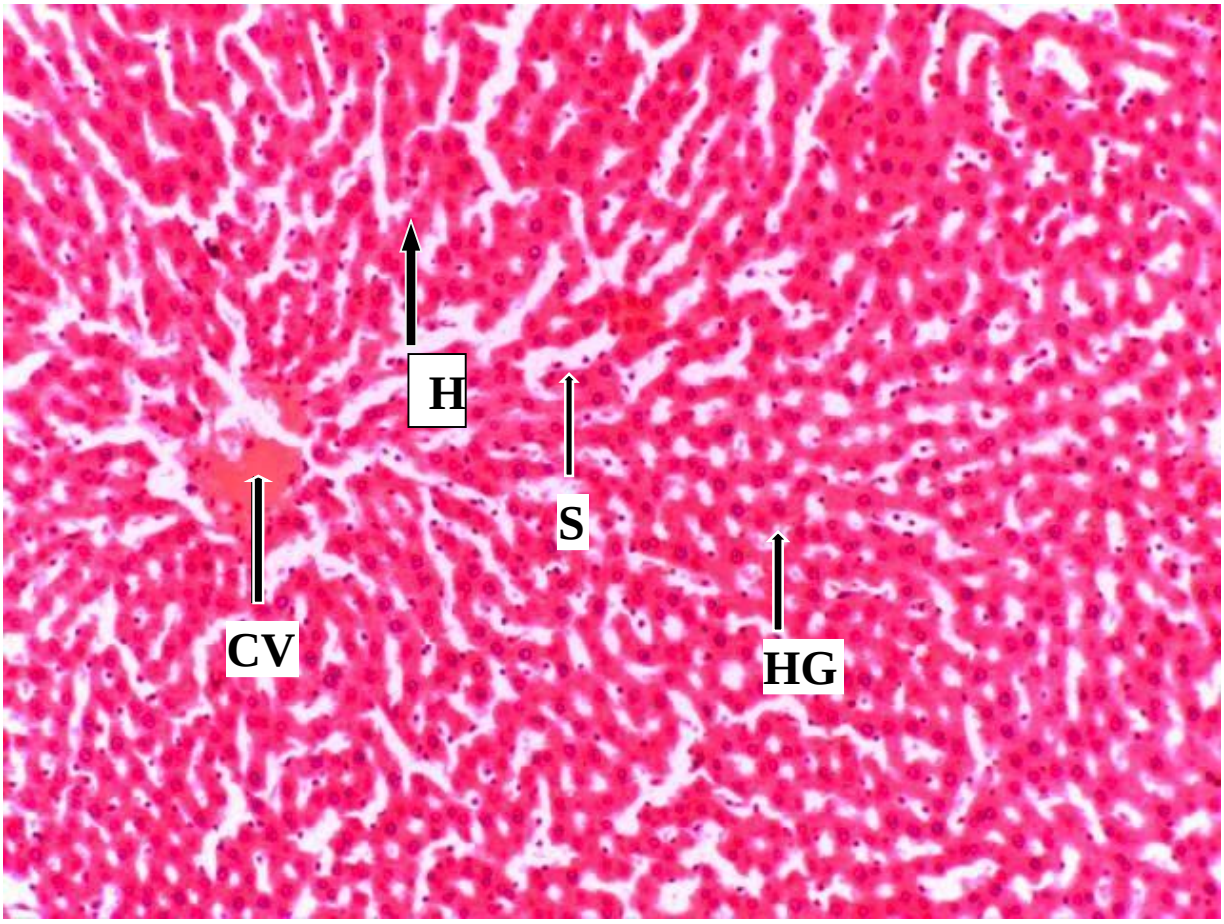


Plate 4.37: Photomicrograph of section of liver of albino rat administered 20mg/kg body weight of Cr for 90 days showing proliferation of stromal tissue (interstitial tissue) and hepatocytes (H) with few hepatocytes appearing swollen/enlarged. Within the interstitial tissue (stroma) are seen Central vein (CV). The laminae (plate) of hepatocytes are not easily identifiable. The sinusoids (S) are enlarged. In some areas there are foci of necrosis. HG stands for hemorrhage (x400), Stain: H and E.

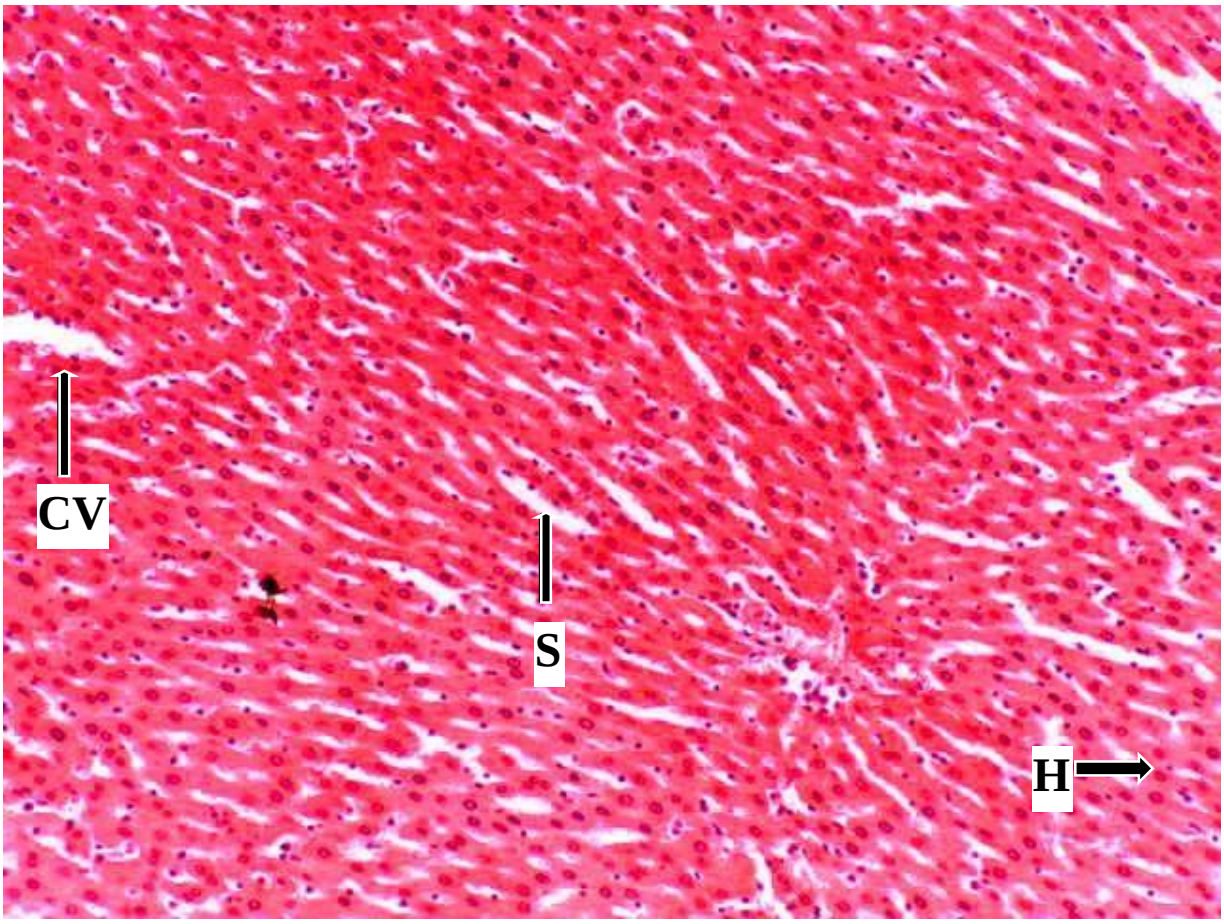


Plate 4.38: Photomicrograph of section of liver of albino rat administered 20mg/kg body weight of Pb for 90 days showing normal tissue architecture. Within the interstitial tissue (stroma) are seen Central vein (CV), Laminae (plate) of hepatocytes (H), Sinusoids (S), Hepatocytes and Portal triad that appear normal (x400), Stain: H and E.

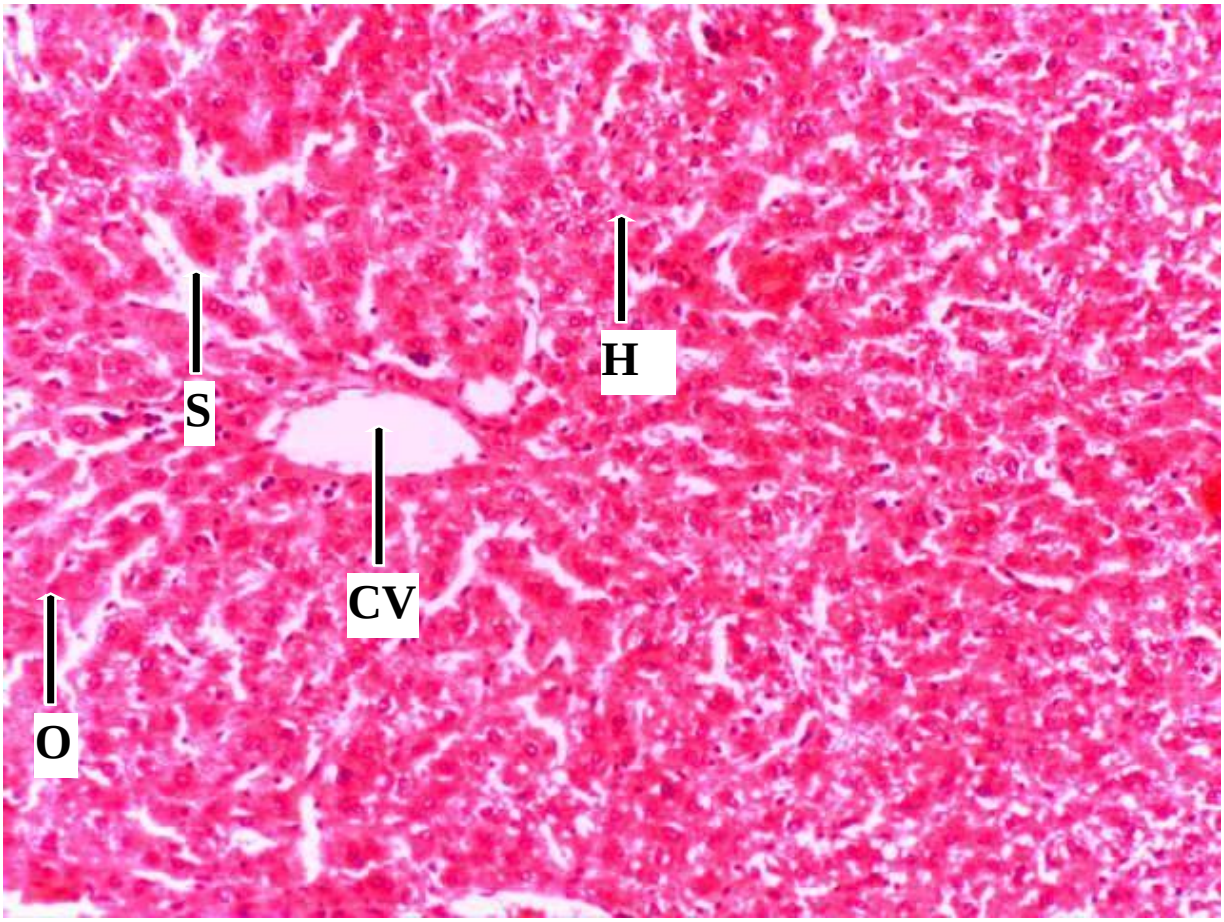


Plate 4.39: Photomicrograph of section of liver of albino rat administered 20mg/kg body weight Cd, Cr, Pb mixture for 90 days showing proliferation of stromal tissue (interstitial tissue) and hepatocytes (H) with few hepatocytes appearing swollen/enlarged. Within the interstitial tissue (stroma) are seen Central vein (CV). The laminae (plate) of hepatocytes are not easily identifiable. The interstitial tissue is oedematous (O) and the sinusoids (S) are enlarged. In some areas there arefoci of necrosis (x400), Stain: H and E.

4.2 Discussion

Decrease in percentage body weight gain of the albino rats compared to control suggests a significant weight loss due to treatments with the metals. This weight loss could be due to loss of appetite. From this study, treatments with 5mg/kg and 20mg/kg of the metals individually and combined caused significant decrease in percentage body weight gain relative to control except treatment with Pb. But the effect due to the combined treatment was not significantly different from that caused by cadmium treatment. This is suggestive of no interaction and that the effect from the combined treatment could be as a result of cadmium in the mixture. Recent study has shown that cadmium caused significant reduction in body weights in animals (Poli et al., 2022). Also, in the 10mg/kg dose, Cr and combined treatments caused significant decrease in percentage body weight gain but there was no significant difference in percentage body weight gain between combined treatment and Cr treatment. This is suggestive of no interaction as the effect observed in the mixture is due to chromium being the only metal that produced significant decrease in percentage body weight gain in 10mg/kg treatment dose. This result agrees with an earlier conclusion by Saha, Choudhuri, and Choudhuri (2017), which reported that exposure of male albino rats to low dose of Cr (VI) caused decrease in body weight gain. The observation that treatment with Pb caused no significant difference ($p>0.05$) in percentage body weight gain compared to control in the three treatment doses agrees with a recent study by Okon et al. (2022).

Also, from the results of this study, there were no adverse effects on relative kidney and heart weights (i.e. organ to body weight ratio) for individual and combined treatments with the metals. For the liver, in the 10mg/kg dose, only combined treatment caused significant increase in liver to body weight ratio but there was no significant difference in liver to body weight ratio between

combined treatment and Cr treatment. In the 20mg/kg dose, Cr and combined treatment caused significant increase in liver to body weight ratio but there was no significant difference in liver to body weight ratio between combined treatment and Cr treatment. Thus, it could be said that the effect observed in the mixture is that due to the Cr component of the mixture. This suggests no interaction amongst the mixture components in the 10mg/kg and 20mg/kg treatment doses with regard to liver to body weight ratio. With the foregoing, we accept the hypothesis that treatment with Cd, Cr, Pb mixture would produce the same effect as treatment with the individual metals with regard to percentage body weight gain and organ (liver, kidney, heart) to body weight ratio.

The results obtained from this study suggest hepatotoxicity by the metals (Cd, Cr, Pb) both singly and in combination (as a mixture). This was manifested by the effects on the levels of biomarkers of hepatotoxicity such as serum ALT, AST, ALP, LDH activities and serum total bilirubin concentration. Simultaneous exposure to these three toxicants (Cd, Cr, Pb) was expected to produce increased adverse hepatic effect but this was not the observation. Instead, the observed hepatotoxic effect of the mixture was either same as one of the individual metals or less than that of the individual metals. This suggests an interaction (less than additive) in which the mode of action of one metal is affected by the other metal(s). The decrease in adverse effect observed in the metal mixture (combined) treatment with increasing dose with respect to serum ALT, AST, total bilirubin in the three treatment doses is hormetic.It is proposed that the mixture at low dose caused a stimulating effect which may have shifted the redox status of the liver cell and activated the cell defense and repair mechanism through the Nrf2 transcription factor as stated below (renal effects) leading to decrease in adverse effect observed at high dose of the metal mixture (combined) treatment (Calabrese & Kozumbo, 2021). This was also observed for Cr individual treatment group with respect to ALT and AST suggesting that the Cr component of

the mixture may be responsible for the shift in the redox status of the cell as Cr mechanism for toxicity had been said to include production of reactive oxygen species following its reduction from hexavalent Cr to trivalent Cr (Singh et al., 2022). This is similar to the observation of Fan et al. (2021) which reported that single and combined Cd and Pb treatment induced hormesis in soil microbial populations and that the mixture hormetic effects were related to the effect of single Cd or Pb.

Earlier reports have suggested renal toxicity due to exposure to Cd, Pb, or Cr (Balali-Moodet al., 2021; ATSDR, 2020; Ceuvas-Magana et al., 2022). The results obtained from this studyagree with these findings as renal toxicity was observed in treatments with the metals both singly and in combination (as a mixture). This was manifested by the effects on the levels of biomarkers of renal toxicity such as serum urea concentration, serum inorganic phosphorus concentration, serum potassium concentration, and serum chloride concentration (5mg/kg dose). Simultaneous exposure to these three renal toxicants was expected to produce increased adverse renal effect but this was not the observation. Instead, the observed renal effect of the mixture was either same as one of the individual metals or less than that of the individual metals. This suggests an interaction in which the mode of action of one metal is affected by the other metal(s). The point of interaction could be at the toxicokinetic or toxicodynamic phase of the metals' metabolism.

The sequestration of lead in intranuclear inclusion bodies in the kidney may limit or prevent toxic interactions with other molecular targets of lead. These bodies have a profound effect on the intracellular disposition of lead in the kidney (ATSDR, 2020). Lead exposure appears to produce oxidative stressas a mechanism of its renal toxicity (Abdelrazek et al., 2022)**.** From the foregoing, it could be adduced that Pb renal toxicity occurs when a critical concentration is reached for which the sequestration in the intranuclear inclusion bodies is no longer possible thus

leading to renal cellular degeneration resulting from lead-induced oxidative stress. The renal effects observed in Pb alone treatment suggests that this critical concentration had been exceeded in the three treatment doses with regard to serum urea, potassium, and inorganic phosphorus concentrations leading to the observed increases.

Cadmium complexed with metallothionein from the liver can redistribute to the kidney. In the kidneys, exogenous metallothionein is degraded in lysosomes and released cadmium is sequestered by endogenous metallothionein as well as other proteins. Early work indicated that metallothionein binding decreased toxicity of cadmium. Renal damage is believed to occur when the localization of cadmium, or an excessive concentration of cadmium, is unbound to metallothionein (Nordberg & Nordberg, 2022). From the foregoing, the renal effects observed in Cd alone treatment suggests that this critical concentration had been exceeded in the three treatment doses with regard to serum urea, potassium, inorganic phosphorus and chloride (5mg/kg only) concentrations leading to the observed increases.

Peak plasma concentrations of chromium occur within 2 hours following an oral dose of soluble chromium (e.g. $K_2Cr_2O_7$), suggesting that absorption occurred. Absorbed chromium distributes to nearly all tissues with the highest concentrations found in kidney and liver (ATSDR, 2012b). Chromium (VI) is unstable in the body and is reduced to chromium (III). Reduction of chromium (VI) to chromium (III) can give rise to reactive intermediates, chromium adducts with proteins and DNA, and secondary free radicals (Singh et al., 2022). From the result of this study, treatment with chromium (VI) produced remarkable renal effect. This suggests that the absorbed and distributed chromium is chromium (VI) and its reduction in the kidney tissue could be responsible for the renal effect observed in Cr alone treatment with regard to serum urea, potassium, and inorganic phosphorus concentrations leading to the observed increases. The

observed adverse renal effect decreased with increasing dose of Cr for urea and potassium. The less-than-additive interaction observed in the mixture treatment with regard to serum urea for the three treatment doses may be due to the Cr component of the mixture as the effect due to the mixture also decreased with increasing mixture dose just as observed in Cr. This dose response phenomenon is known as hormesis.

Hormesis refers to adaptive responses of biological systems to moderate environmental or self-imposed challenges through which the system improves its functionality and/or tolerance to more severe challenges (Calabrese & Mattson, 2017). It is opined that hormetic dose responses that are cytoprotective for chemicals and radiation are largely mediated following the redox activation of the transcription factor (TF) Nrf2. Nrf2 mediates a network of antioxidant defenses and initiates productive crosstalk with other TFs that not only act together with Nrf2 via dose-dependent hierarchal processes to enhance biological resilience but also conform to the quantitative features of the hormetic dose response. Stressors shift the redox homeostasis of cells toward the oxidative state and, in the process, activate Nrf2—a TF and redox sensor—to mediate a host of integrated and cytoprotective responses, including antioxidation, detoxification, anti-inflammation, autophagy, and the facilitation of mitochondrial biogenesis. It is proposed that activation of Nrf2 is the general and dominate underlying mechanistic basis of hormetic dose responses (Calabrese & Kozumbo, 2021). From this study, the decrease in adverse effect observed in the metal mixture (combined) treatment with increasing dose with respect to serum urea concentration (a biomarker of kidney injury) is hormetic.It is proposed that the Cr component of the mixture at low dose may have caused a stimulating effect by shifting the redox status of the cell(Chakraborty et al., 2022) which may have activated the cell defense and repair mechanism through the Nrf2 transcription factor as postulated above leading to decrease in adverse effect

observed at high dose of the metal mixture (combined) treatment.This is responsible for the decreased adverse effect observed as the mixture dose increased contrary to the increased adverse effect observed in the individual treatments(Cd and Pb)**.**

Cadmium exposure affects different organs and tissues and its deleterious effects can be linked to indirect reactive oxygen species (ROS) generation (Branca et al., 2020). The mechanism of lead-induced oxidative stress involves the effects of Pb on the membranes, DNA, and antioxidant defense systems of cells (Arslanet al., 2022). The reduction of chromium (VI) to chromium (III) is responsible for the generation of reactive oxygen species and chromium intermediate species, such as Cr (V) and Cr (IV) (Singh et al., 2022). From the result of this study, there was concurrence with the results of earlier studies as above that the mechanism of toxic action of these metals individually and in combination includes induction of oxidative stress. This was manifest by the elevation of serum MDA concentration (a biomarker of lipid peroxidation) by both individual and combined treatments of the metals as well as the depletion of serum GSH and serum ascorbic acid concentrations (both cellular antioxidants) as observed.It was alsoobserved from this study that there was the induction, instead of decreased activity, of antioxidant enzymes like SOD and CAT, which was manifest in their elevation in serum in both individual and combined treatments of the metals. This could be responsible for the decrease in the severity of the adverse effects caused by these metal treatments. This may have been enabled by the activation of Nrf2 transcription factor which mediates a network of antioxidant defenses and initiates productive crosstalk with other transcription factors for a compensatory and reparative response by the cells to the metal stressors as earlier discussed (Calabrese & Kozumbo, 2021; Peluso, 2022).

From the results of this study, the increase observed in hemoglobin concentration (5mg/kg Cd, Cr individual treatments), red blood cell count (5mg/kg Cd individual and combined treatment groups; 20mg/kg Cr individual and combined treatment groups) and white blood cell count (5mg/kg Cd, Pb individual treatments and 10mg/kg combined treatment group) may be due to increased syntheses resulting from enzyme inductions caused by activation of cellular defense system by Nrf2 transcription factor as a result of increased production of reactive oxygen species by the metals (Calabrese & Kozumbo, 2021). This agrees with Saha et al. (2017) whoreported significant increase in RBC and WBC in rats treated with 0.5 and 1mg/kg Cr for 90 days compared with control. However, this is contrary to the report of a study by Andjelkovic et al. (2019) in which they reported a significant decrease in all these aforementioned parameters for male Wistar rats treated with a single dose of Cd and Pb individually and combined.

Results of histopathological evaluation indicated no tissue injury in the heart in both combined and individual treatments of the metals for the three treatment doses. Also, results of histopathological evaluation indicated no tissue injury in the kidney in both combined and individual treatments of the metals (Cd, Cr, Pb) for 5mg/kg and 10mg/kg treatment doses as tissue architecture appeared normal while tissue injury was observed in the kidney in both combined and individual treatments of the metals for 20mg/kg treatment dose.This is contrary to the observation of Yasmin and Hussain (2021) who treated albino rats with 10mg/kg Cr (VI) orally daily for 42 days. They observed obliteration of the Bowman's space due to mesangial proliferation in glomeruli. The difference may be because we treated the rats thrice weekly as against their daily dosing and as such the bioavailability of Cr in the kidney may have been altered by earlier clearance during the intervals of treatment thereby altering its adverse effect on the kidney.Also, results of histopathological evaluation indicated no tissue injury in the liver in

both combined and individual treatments of the metals for 5 & 10mg/kg treatment dose but tissue injury was observed in the liver in both combined and individual treatments of the metals for 20mg/kg treatment dose. This suggests that there is no significant difference between tissue injury due to combined and individual treatments of the metals and hence our null hypothesis is accepted. It also suggests dose-dependence of injury.

There was inconsistency in direction of effect as some effects increased with increasing dose (e.g. oxidative stress) while some effects decreased with increasing dose (e.g. hepatocellular). The decrease in effect observed with increasing dose was due tohormesis as earlier explained. Also, inconsistencies were observed in the use of dose-effect relationship to determine the mode of action of the mixture as it varied between the 3 metals across endpoints. This calls for further study.

Researchers in numerous fields (e.g. pharmacology, entomology, toxicology, and epidemiology) have attempted to model the joint action of chemicals using simple formula based only on knowledge of individual chemical toxicity or pharmacologic effect. In toxicology, additivity-based predictions are often compared to observed mixture data to assess the presence and magnitude of interactions (greater-than-additive or less-than-additive) among chemicals (Rider etal., 2018). One of these methods is effect-addition (independent action)used by international organizations (e.g. USEPA, ATDSR, EU etc.) for assessing risk due to exposure to chemical mixtures for which whole mixture toxicity data is limited. The effects of the mixture in this study were predicted using the concept of independent action using/from/following its basic equation as stated by (Backhaus et al., 2000; Escher et al., 2020). This is from addition of individual effects of the mixture components with regard to the endpoint/parameter of concern. From this study, for the various parameters measured and for the three dose levels, it could be seen using

effect-addition (independent action) that the predicted effects were higher than those obtained from the whole mixture in the experiment thus a deviation from additivity thereby suggesting a less-than-additive interaction amongst the mixture components. Given that the observed effect of the mixture was less than the predicted effect, this method could be applied in risk assessment of this mixture at other doses or of similar mixtures as it over stated the risk rather than under estimate it, and in risk assessment, it is safer to over-estimate than to under estimate risk.

In the 5mg/kg treatment group, no animal died before the end of the period of study. In the 10mg/kg treatment group, two animals died before the end of the study with one from the cadmium group and the other from the combined substances group. In the 20mg/kg treatment group, three animals also died before the end of the study with one from the lead group, the other from the chromium group and another from the combined substances group.

ATSDR (2020), had reported that one study had shown a significant association between blood lead (PbB) and mortality due to disease of blood and blood-forming organs while several studies had shown increased risk of death from chronic or unspecified nephritis or non-malignant kidney disease associated with PbB>10ug/dl. From the result of this study with regard to hematological parameters, it could be assumed that the cause of death in the 20mg/kg Pb group could not have been from disease of blood but more likely from unspecified nephritis or some other cause.

ATSDR (2012b), reported LD_{50} values for chromium(V1) compounds (sodium chromate, sodium dichromate, potassium dichromate, and ammonium dichromate) range from 13 to 19 mg chromium(VI)/kg in female rats and from 21 to 28 mg chromium(VI)/kg in male rats which correlates with the death observed in the 20mg/kg Cr group. Given that only one animal died in the 20mg/kg combined treatment group, it could be assumed that either the Pb or Cr component

of the mixture caused the death and no interaction or less than additive interaction hence only one death. This view is supported with the observation in the 10mg/kg treatment group, in which one death was recorded in the Cd treatment group and one death in the combined metal treatment group suggesting that the cause of death in the mixture group is probably the Cd component of the mixture. Correlating this observation with histopathological evaluations, it could be assumed that increased dose of the treatment metals both individual and combined caused the death except in the Cd treatment group.

With the foregoing, it could be assumed that increased dose of the treatment caused mortality both in the individual and combined treatments suggesting that the cause of death in the combined treatment group was from the metal that caused death in the individual treatment (i.e. Cd for 10mg/kg treatment group, and Pb or Cr for the 20mg/kg treatment group).

There were 23 biochemical parameters measured in this study in the 3 treatment doses (5, 10, and 20mg/kg). This translated to 69 measurements. The results obtained could be classified into three categories namely; (i) no interaction (ii) less than additive interaction, and (iii) additive interaction. Out of the 69 measurements, 47 measurements indicated no interaction, 12 measurements indicated less than additive interaction, while 10 measurements indicated additive interaction. From Appendix 26 and by use of weight of evidence (Organization for Economic Co-operation and Development [OECD], 2019), it could be suggested that the nature of interaction of the metals is principally less-than-additive for hepatic, renal effects, and no interaction for hematological, oxidative stress and body/organ weight effects. On dose-dependence of the interactions, it could also be suggested using the application of weight of evidence that the nature of interaction was not dose-dependent.

CHAPTER FIVE

CONCLUSION AND RECOMMENDATION

5.1 Conclusion

This study has shown that exposure of albino rats to cadmium, chromium and lead simultaneously has adverse health effect on some organs of the animals. Biochemical analysis of the liver and kidney showed adverse effect at low dose that was ameliorated at high dose based on the phenomenon called hormesis. Oxidative stress was observed as one of the mechanism of toxic action of the metals (individually and combined).

Simultaneous exposure to the metals caused body weight loss and increased liver to body weight ratio in the animals, and the effect was same as observed in individual treatment. Increase in hematological parameters (RBC and WBC) was observed in combined treatment and same was also observed in individual treatment.

The use of effect addition (independent action) to predict the toxicity of the mixture overestimated the risk as the result of the whole mixture tested was less than the predicted effect from the addition of the effect of the individual metals. Predominantly, less than additive interaction effects amongst the mixture components were observed in most of the endpoints measured.

Conclusively, this study has shown that simultaneous exposure to these metals (Cd. Cr and Pb) posed no greater health risk than that posed by the exposure to the individual metals as the

Observed adverse effects in the mixture were similar to those of individual metals. Thus, the null hypothesis was not rejected.

5.2 Recommendation

Further to this study, the following are recommended:

1. Due to the high cost of the study and its relevance to public health, relevant government agencies in Nigeria should sponsor more research on the toxicity of chemical mixtures in line with the new trend across developed countries.
2. The study should be repeated for a longer duration (i.e. 12 months or more) across more dose range and with more animals because the clinical chemistry parameters did not show adverse effects clearly in increasing or decreasing dose direction but the histopathological evaluation suggested tissue injury in increasing dose direction. This calls for further studies across more dose range to determine the true direction of dose-effect relationship of the mixture for the various endpoints assayed.
3. More studies should be carried out on the mode of action of the mixture because the mode of action of the mixture varied across various end points and thus requires further study.
4. The assessment of risk due to this mixture and similar mixtures could be done using toxicological information (where available) on the most hazardous mixture component for the endpoint of concern.
5. Hormetic concept (non threshold approach) is recommended to be included in the risk assessment of simultaneous exposure to these metals.

5.3 Contribution to knowledge

- The observations from this study will help to fill the knowledge gap on toxicity of this ternary mixture.
- And it willassist in any future risk assessment of similar mixture by relevant government and regulatory agencies.
- It will also provide needed data for *in silico* modeling of toxicity of heavy metal mixtures in the environment.

REFERENCES

Abdelrazek, F., Salama, D.A., Alharthi, A., Asiri, S.A., Khodeer, D.M., Qarmush, M.M., … Ibrahim, M. (2022). Glycine betaine relieves lead-induced hepatic and renal toxicity in albino rats. *Toxics,10*(5), 271. https://doi.org/10.3390/toxics10050271

Agathokleous, E. (2022). On the meta-analysis of hormetic effects. *Science of the total environment, 15*, 852. doi:10.1016/j.scitotenv.2022.158273.

Agathokleus, E., Barcelo, D., Iavicoli, I., Tsatsakis, A., & Calabrese, E. J. (2022). Disinfectant-induced hormesis: An unknown environmental threat of the application of disinfectants to prevent SARS-CoV-2 infection during the COVID-19 pandemic? *Environmental Pollution, 282*, 118429. Doi: 10.1016/j.envpol.2021.118429.

Agency for Toxic Substances and Disease Registry (ATSDR). (2004). *Interaction Profile for Arsenic, Cadmium, Chromium, and Lead.*Retrieved from https://www.atsdr.cdc.gov/interactionprofiles/ip-metals1/ip04-c5.pdf Last accessed 7/72022

Agency for Toxic Substances and Disease Registry (ATSDR). (2012a). *Toxicological Profile for Cadmium*.Retrieved from https://www.atsdr.cdc.gov/toxprofiles/tp5.pdf. Last accessed7/7/2022

Agency for Toxic Substances and Disease Registry (ATSDR). (2012b). *Toxicological Profile for Chromium*.Retrieved from https://www.atsdr.cdc.gov/toxprofiles/tp7.pdf. Last accessed7/7/2022

Agency for Toxic Substances and Disease Registry (ATSDR). (2020). *Toxicological Profile for Lead*. Retrieved from https://www.atsdr.cdc.gov/ToxProfiles/tp13.pdf. Last accessed15/4/2021

Agrawal, A., Gandhe, M.B., Gupta, D., & Reddy, M.V.R. (2016). Preliminary study on serum lactate dehydrogenase (LDH)-prognostic biomarker in carcinoma breast. *Journal of Clinical and Diagnostic Research 10*(3), 6-8. DOI :10.7860/JCDR/2016/17111.7364

Alisik, M., Neselioglu, S., & Erel, O. (2019). A colorimetric method to measure oxidized, reduced and total glutathione levels in erythrocytes. *Journal of Laboratory Medicine43*(5), 269-277. https://doi.org/10.1515/labmed-2019-0098

Amador, E., & Urban, J. (1972). Simplified serum phosphorus analyses by continuous-flow ultraviolet spectrophotometry. *Clinincal Chemistry, 18*(7), 601-604.

Andjelkovic, M., Djordjevic, A.B., Antonijevic, E., Antonijevic, B., Stanic, M., Kotur-Stevuljevic, J., ... Bulat, Z. (2019). Toxic effect of acute cadmium and lead exposure in rat blood, liver, and kidney. *International Journal of Environmental Research and Public Health , 16*(2), 274. https://doi.org/10.3390/ijerph16020274

Andrade, K.Q.D., Moura, F.A., Santos, J.M.D., Araujo, O.R.P.D., Santos, J.C.D.F., & Goulart, M.O.F. (2015). Oxidative stress and inflammation in hepatic diseases: Therapeutic possibilities of N-acetylcysteine. *Journal of Molecular Science,16*(12),30269-30308.

Anyanwu, B.O., Ezejiofor, A.N., Igweze, Z.N., & Orisakwe, O.E. (2018). Heavy metal mixture exposure and effects in developing nations; An update. *Toxics, 6,* 65.doi:10.3390/toxics6040065

Arkhipov, E.V., Garipova, R.V., Strizhacov, L.A., Bobkova, I.N., & Tairova, N. (2022). [Kidney damage caused by lead exposure: Historical aspects]. *Ter Arkh, 94*(6), 777-780. Published in English and Russian languages

Arslan, A.S,, Seven, I., Mutlu, S.I., Arkali, G., Birben, N., & Seven, P.T. (2022). Potential ameliorative effect of dietary quercetin against lead-induced oxidative stress, biochemical changes, and apoptosis in laying Japanese quails. *Ecotology and Environmental Safety, 231*, 113200

Association of Official Analytical Chemists(AOAC). (2005). Vitamin C in juices and vitamin preparations: Official Method 967.21. In W. Horwitz (Ed.), *Official Methods of Analysis of AOAC International* (18th ed., p.45). Gaithersburg, MD: AOAC International.

Aulbach, A.D., & Amuzie, C.J. (2017). Biomarkers in nonclinical drug development. In A.S. Faqi (Ed.), *A Comprehensive guide to toxicology in nonclinical drug development* (2nd ed., pp. 447-471). Cambridge, MA: Academic Press.https://doi.org/10.1016/B978-0-12-803620-4.00017-7

Backhaus, T., Altenburger, R., Boedeker, W., Faust, M., Scholze, M., & Grimme, L.H. (2000). Predictability of the toxicity of multiple mixtures of dissimilarly acting chemicals to *VibrioFischeri.Environmental Toxicology and Chemistry, 19*, 2348-2356.

Balali-Mood, M., Naseri, K., Tahergorabi, Z., Khazdair, M.R., & Sadeghi, M. (2021). Toxic mechanism of five heavy metals: Mercury, lead, chromium, cadmium and arsenic. *Frontiers in Pharmacology*. https://doi.org/10.3389/fphar2021.643972

Bart, S., Short, S., Jager, T., Eagles, E.J., Robinson, A., Badder, C., … Ashauer, R. (2022). How to analyse and account for interactions in mixture toxicity with toxicokinetic-toxicodynamic modes. *Science of the total environment, 843*,157048. https://doi.org/10.1016/j.scitotenv.2022.157048

Bartels, H., Bohmer, M., & Heierli, C. (1972). Serum creatinine determination without protein precipitation. *Clinica Chimica Acta,37*, 193-197.

Beck, B.D., Calabrese, E.J., Slayton, T.M., & Ruddel, R. (2008). The use of toxicology in the regulatory process. In A.W. Hayes (Ed.), *Principles and Methods of Toxicology* (5th ed., pp. 88-89). New York, NY: Informa Healthcare.

Beers, R. F. Jr., & Sizer, I. W. (1952). A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. *Journal of Biological Chemistry, 195*(1), 133-140.

Belz, R.G., Cedergreen, N., & Sorensen, H. (2008). Hormesis in mixtures -- can it be predicted? *Science of Total Environment, 404*(1), 77-87.

Belz, R.G., & Duke, S.O. (2022). Modelling biphasic hormetic dose responses to predict sub-NOAEL effects using plant biology as an example. *Current Opinion in Toxicology, 29,* 36-42. https://doi.org/10.1016/j.cotox.2022.01.003

Bernhoft, R.A. (2013). Cadmium toxicity and treatment. *The Scientific World Journal,2013,*7.

Berrahal, A.A., Nehdi, A., Hajjaji, N., Gharbi, N., & El-Fazaa, S. (2007). Antioxidant enzymes activities and bilirubin level in adult rat treated with lead. *Comptes Rendus Biologies, 330*(8), 581-588. https://doi.org/10.1016/j.crvi.2007.05.007. Published in English and French.

Boriskin, P., Deviatkin, A., Nikitin, A., Pavlova, O., & Toporovskiy, A. (2019). Relationship of catalase activity distribution in serum and tissues of small experimental animals. *IOP Conference Series Earth and Environmental Sciences, 403*(1), 012113.

Boubakri, H. (2017). The role of ascorbic acid in plant-pathogen interactions. In M.A. Hossain, S. Munne-Bosch, D.J. Burritt, P.D. Vivancos, M. Fujita & A. Lorence (Eds.), *Ascorbic acid in plant growth, development and stress tolerance* (pp. 255-271). Cham, Denmark: Springer.

Braeuning, A., Bloch, D., Karaca, M., Kneuer, C., Rotter, S., Tralau, T., & Marx-Stoelting, P. (2022). An approach for mixture testing and prioritization based on common kinetic groups. *Archives of Toxicology, 96,* 1661-1671.

Branca, J.J.V., Florillo, C., Carrion, D., Paternostro, F., Taddei, N., Gulisano, M., … Becatti, M. (2020). Cadmium-induced oxidative stress: Focus on the central nervous system. *Antioxidants, 9*(6). 462. https://doi.org/10.3390/antiox9060492

Calabrese, E.J. (2010). Hormesis is central to toxicology, pharmacology and risk assessment. *Human & Experimental Toxicology, 29(*4), 249-261. doi: 10.1177/0960327109363973.

Calabrese, E.J., & Kozumbo, W.J. (2021). The hormetic dose-response mechanism: Nrf2 activation. *Pharmacological Research,* 167, 105526. https://doi.org/10.1016/j.phrs.2021.105526.

Calabrese, E.J., & Mattson, M.P. (2017). How does hormesis impact biology, toxicology, and medicine? *npj Aging and Mechanisms of Disease, 3,* 13. https://doi.org/10.1038/s41514-017-0013-z

Calabrese, E.J., Mattson, M.P., Dhawan, G., Kapoor, R., Calabrese, V., & Giordano, J. (2020). Chapter Ten – Hormesis: A potential strategic approach to the treatment of neurodegenerative disease. In G. Soderbom, R. Esterline, J. Oscarsson, & M.P. Mattson (Eds.), *International Review of Neurobiology* (volume 155, pp. 271-301). Cambridge, MA: Academic Press.

Ceuvas-Magana, M.Y., Vega-Garcia, C.C., Leon-Contreras, J.C., Hernandez-Pando, R., Zazueta, C., & Garcia-Nino, W.R. (2022). Ellagic acid ameliorates hexavalent chromium-induced renal toxicity by attenuating oxidative stress, suppressing TNF-α and protecting mitochondria. *Toxicology and Applied Pharmacology, 454,* 116242.

Chakraborty, R., Renu, K., Eladl, M.A., El-Sherbiny, M., Elsherbini, D.M.A. Mirza, A.K., … Gopalakrishnan, A.V. (2022). Mechanism of chromium-induced toxicity in lungs, liver, and kidney and their ameliorative agents. *Biomedicine & Pharmacotherapy, 151,* 113119.

Chang, K., & Chen, C. (2020). The role of oxidative stress in Parkinson's disease. *Antioxidants, 9*(7), 597.

Charkiewicz, A. E., & Backstrand, J.R. (2020). Lead toxicity and pollution in Poland. *International Journal of Environmental Research and Public Health,17,* 1-14.

Chatterjee, M., & Roy, K. (2022). Computational modeling of mixture toxicity. *Methods in Molecular Biology, 2425,* 561-585. doi:10.1007/978-1-0716-1960-5_22. PMID:35188647

Chattopadhyay, M.K. (2022). Hormesis: A fundamental concept in biology. *Resonance, 27,*1903-1912. https://doi.org/10.1007/s12045-0221488-x

Cury, M.C., Guimaraes, M.P., Lima, W.S., Caldeira, M.C.M., Couto, T.R., Murta, K., … Baptista, J.M.B. (2005). Biochemical serum profiles in dogs experimentally infected with *Angiostrongylus vasorum* (Baillet, 1866). *Veterinary Parasitology, 128*(2), 121-127. https://doi.org/10.1016/j.vetpar.2004.11.009

Danziger, J., Dodge, L.E. Hu, H., & Mukamal, K.J. (2022). Susceptibility to environmental heavy metal toxicity among Americans with kidney disease. *Kidney360, 3*(7), 1191-1196.

Dhondup, T., & Qian, Q. (2017). Electrolyte and acid-base disorders in chronic kidney disease and end stage kidney failure. *Blood Purification,43,* 179-188.

Docea, A.O., Calina, D., & Tsatsakis, A.M. (2021). Chapter 1- Mixture toxicity evaluation in modern toxicology. In A.M. Tsatsakis (Ed.), *Toxicological Risk Assessment and Multi-System Health Impacts from Exposure* (pp. 3-12). London, United Kingdom: Academic Press.

Drabkin, D.L., & Austin, J.H. (1935). Spectrophotometric studies. II. Preparations from washed blood cells; nitric oxide hemoglobin and sulfhemoglobin. *Journal of Biological Chemistry, 112*,51.

Drienovska, I., Chovancova, E., Koudelakova, T., Damborsky, J., & Chaloupkova, R. (2012). Biochemical characterization of a novel haloalkane dehalogenase from a cold-adapted bacterium. *Applied and environmental microbiology*, *78*(14), 4995-4998

Ellman, G. L. (1959). Tissue sulfhydryl groups. *Archives of Biochemistry and Biophysics*, *82*, 70-77.

Engelbrecht, R.M., & McCoy, F.A. (1956). Determination of potassium by tetraphenyborate method. *Analytical Chemistry*, *28*, 1772-1773.

Escher, B., Braun, G., & Zafi, C. (2020). Exploring the concepts of concentration addition and independent action using a linear low-effect mixture model. *Environmental Toxicology and Chemistry, 39*(12), 2552-2559.

European Union(EU). (2012). *Toxicity and Assessment of Chemical Mixtures.*Retrieved from https://ec.europa.eu/health/scientific_committees/environmental_risks/docs/scher_o_155.pdf. Last accessed 15/7/2022

European Union (EU). (2015). *Scientific methodologies for the assessment of combined effects of chemicals – a survey and literature review*. Retrieved from https://publications.jrc.ec.europa.eu/repository/bitstream/JRC97522/jrc_tech_rep_sci%20meth%20for%20mix_final.pdf. Last accessed 15/7/2022

Fan, D., Sun, J., Liu, C., Wang, S., Han, J., Agathokleous, E., & Zhu, Y. (2021). Measurement and modeling of hormesis in soil bacteria and fungi under single and combined treatments of Cd and Pb. *Science of the Total Environment, 783*, 147494

Farhana, A., & Lappin, S.L. (2022). Biochemistry, lactate dehydrogenase. In *StatPearls [Internet]*. Treasure Island, FL: StatPearls. Retrieved from https://www.ncbi.nlm.nih.gov/books/NBK557536/#_NBK557536_pubdet_

Farley, K.J., & Meyer, J.S., (2015). Metal mixture modeling evaluation project: 3. Lessons learned and steps forward. *Environmental Toxicology and Chemistry, 34*(4), 821-832. https://doi.org/10.1002/etc.2837

Farley, K.J., Meyer, J.S., Balistrieri, L.S., De Schamphelaere, K.A.C., Iwasaki, Y., Janssen, C.R., … Tipping, E. (2015). Metal mixture modeling evaluation project: 2. Comparison of four modeling approaches. *Environmental Toxicology and Chemistry, 34*(4), 741-753. https://doi.org/10.1002/etc.2820

Fatima, G., Raza, A.M., Hadi, N., Nigam, N., & Mahdi, A.A. (2019). Cadmium in human diseases: It's more than just a mere metal. *Indian Journal of Clinical Biochemistry, 34* (4), 371-378.

Fawcett, J.K., & Scott, J.E. (1960). A rapid and precise method for the determination of urea. *Journal of Clinical Pathology, 13,* 156.

Flora, G., Gupta, D., & Tiwari, A. (2012). Toxicity of lead: A review with recent updates. *Interdisciplinary Toxicology, 5*(2), 47-58.

Fujiwara, Y., Lee, J., Banno, H., Imai, S., Tokumoto, M., Hasegawa, T., …Satoh, M. (2020). Cadmium induces iron deficiency anemia through the suppression of iron transport in the duodenum. *Toxicology Letters,332,* 130-139.

Furukawa, F., Watanabe, S., Kimura, S., & Kaneko, T. (2012). Potassium excretion through ROMK potassium channel expressed in gill mitochondrion-rich cells of Mozambique tilapia. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, 302*(5), R568-R576

Genchi, G., Sinicropi, M.S., Lauria, G., Carocci, A., & Catalano, A. (2020). The effects of cadmium toxicity. *International Journal of Environmental Research and Public Health,17,* 11.

George, T., & Brady, M.F. (2022). Ethylenediaminetetraacetic acid (EDTA). In *StatPearls [Internet]*. Treasure Island, FL: StatPearls

Gerhardsson, L. (2022). Chapter 28 – Diagnosis and treatment of metal poisoning general aspects. In G.F. Nordberg & M. Costa (Eds.), *Handbook on the Toxicology of Metals* (5thed., pp. 663-684). London, United Kingdom: Academic press.

Ghazizadeh, H., Saberi-Karimian, M., Aghasizadeh, M., Sahebi, R., Ghazavi, H., Khedmatgozar, H., … Ghayour-Mobarhan, M. (2020), Pro-oxidant—antioxidant balance (PAB) as a prognostic index in assessing the cardiovascular risk factors: A narrative review. *Obesity medicine, 19,* 100272.

Gillois, K., Leveque, M., Theodorou, V., Robert, H., & Mercier-Bonin, M. (2018). Mucus: An underestimated gut target for environmental pollutants and food additives. *Microorganisms, 6*(53), 4-5.

Gounden, V., Bhatt, H., & Jialal, I. (2022). Renal function tests. In *StatPearls [Internet]*. Treasure Island, FL: StatPearls. Retrieved from https:/www.ncbi.nlm.nih.gov/books/NBK507821/#_NBK507821_pubdet_ Last accessed 20/7/2023

Gregersen, E. (2022). *Chromium chemical element*. Retrieved from https://www.britannica.com/science/chromium. Last accessed 29/7/2022

Gwaltney-Brant, S.M. (2021). Nutraceuticals in hepatic diseases. In R.C. Gupta, J. Lall, & A. Srivastava (Eds.), *Nutraceuticals* (2nd ed., pp. 117-129). Cambridge, MA: Academic Press. https://doi.org/10.1016/B978-0-12-821038-3.00008-2.

Hall**,** R.L. & Everds, N.E. (2008). Principles of clinical pathology for toxicology studies. In A.W. Ha**y**es (Ed.), *Principles and methods of toxicology* (5th ed., pp. 1318-1350). New York, NY: Informa Healthcare.

Hayes**,** A.W., Li, R., Hoeng, J., Iskandar, A., Peistch, M.C., & Dourson, M.L. (2019). New approaches to risk assessment of chemical mixtures. *Toxicology Research and Application, 3,* 1-10.

Henry, R. J., Chiamori, N., Golub, O. J., & Berkman, S. (1960). Revised spectrophotometric methods for the determination of glutamic-oxaloacetic transaminase, glutamic-pyruvic transaminase and lactic acid dehydrogenase. *American Journal of Clinical Pathology*, *34*, 381-396.

Hernandez, A.F., Buha, A., Constantin, C., Wallace, D.R., Sarigiannis, D., Neagu, M., … Tsatsakis, A. (2019). Critical assessment and integration of separate lines of evidence for risk assessment of chemical mixtures. *Archives of Toxicology, 93,* 2741-2757.

Holstege, C.P. (2020). *Pathophysiology and etiology of lead toxicity*. Retrieved from https://emedicine.medscape.com/article/2060369-overview#a1 Last accessed 18/7/2023

Ichipi-Ifukor, P.C., Asagba, S.O., Nwose, C., Mordi, J.C., & Oyem, J.C. (2022). Palm oil extracts protected against cadmium chloride poisoning via inhibition of oxidative stress in rats. *Bulletin of the National Research Centre, 46*(5). https://doi.org/10.1186/s42269-021-00688-7

International Labour Organization (ILO). (2021). *Exposure to hazardous chemicals at work and resulting health impacts: A global review.* Retrieved from https://ilo.org/wcmsp5/groups/public-ed_dialogue/-lab_admin/documents/publication/wcms_795460.pdf Last accessed 18/7/2023

Islam, M.N., Rauf, A., Fahad, F.I., Bin-Emran, T., Olatunde, A., Shariati, M.A., … Mubarak, M.S. (2022). Superoxide dismutase: An updated review on its health benefits and industrial applications. *Critical Reviews on Food Science & Nutrition, 62*(26), 7282-7300.

Iwasaki, I., Utsumi, S., & Ozawa, T. (1952). New colorimetric determination of chloride using mercuric thiocyanate and ferric ion. *Bulletin of the Chemical Society of Japan, 25,*226.

Jastrzebska, M., Saeid, A., Kostrzewska, M.K., & Basladynska, S. (2018). New phosphorus biofertilizers from renewable raw materials in the aspect of cadmium and lead contents in soil and plants P-biofertilizers from waste and Cd and Pb in soil and plant. *Open Chemistry, 16,* 1.

Jendrassik**,** L., & Grof, P. (1938). Simplified photometric methods for the determination of bilirubin. *Biochemical Journal, 297,* 81-89.

Juan, C.A., Lastra, J.M.P., Plou, F.J., & Perez-Labena, E. (2021). The chemistry of reactive oxygen species (ROS) revisited: Outlining their role in biological macromolecules (DNA, lipids and protein) and induced pathologies. *Journal of Molecular Sciences, 22*(9), 4642.

Kakel, S. J. (2013). The evaluation of traditional and automatic Coulter method in estimation of haematological parameters in adult rats. *Beni-Suef University Journal of Basic and Applied Sciences,2*(1), 31-35.

Kar, S., & Leszczynski, J. (2019). Exploration of computational approaches to predict the toxicity of chemical mixtures. *Toxics, 7(*1), 15.

Karakochuk, C.D., Hess, S.Y., Moorthy, D., Namaste, S., Parker, M.E., Rappaport, A.I., … Dary, O. (2019). Measurement and interpretation of hemoglobin concentration in clinical and field settings: A narrative review. *Annals of the New York Academy of Sciences,1450*(1): 126-146. https://doi.org/10.1111/nyas.14003

Karakus, Y.Y. (2020). *Typical catalases; Structure and function*. Retrieved from https://www.intechopen.com/chapters/70428 Last accessed 18/7/2023

Katerji, M., Filippova, M., & Duerksen-Hughes, P. (2019). Approaches and methods to measure oxidative stress in clinical samples: Research applications in the cancer field. *Oxidative Medicine and Cellular Longevity, 2019,* 1-29. https://doi.org/10.1155/2019/1279250

Kinuthia, G.K, Ngure, V., Beti, D., Lugalia, R., Wangila, A., & Kamau, L. (2020). Levels of heavy metals in waste water and soil samples from open drainage channels in Nairobi, Kenya: community health implication. *Scientific Report,* 10, 8434. https://doi.org/10.1038/s41598-020-65359-5

Klein, R., Nagy, O., Tothova, C., & Chovanova, F. (2020). Clinical and diagnostic significance of lactate dehydrogenase and its isoenzymes in animals. *Veterinary Medicine International*. Doi: 10.1155/2020/5346483

Korla, K., & Mitra, C.K. (2020). Biochemical hazards associated with unsafe disposal of electrical and electronic items. In M.N.V. Prada, M. Vithanage, & A. Borthakur (Eds.), *Handbook of Electronic waste management* (pp. 55-80). Oxford, England: Butterworth Heineman.

Krupaa, R.J., Hariharan, R., Babu, N.A., & Masthan, K.M.K. (2020). Alkaline phosphatase and its clinical importance-A review. *European Journal of Molecular & Clinical Medicine, 7*(5), 1409-1413.

Kumar, S., Chaitanya, R.K., & Preedy, V.R. (2017). Chapter 20 - Assessment of antioxidant potential of dietary components. In V. R. Preedy and R.R. Watson (Eds.), *HIV/AIDS: Oxidative Stress and Dietary Antioxidants* (1st ed., pp 239-253). Cambridge, MA: Academic Press

Lala, V., Zubair, M., & Minter, D.A. (2022). Liver function tests. In *SteatPearls[Internet]*. Treasure Island, FL: SteatPearls. Retrieved from https://www.ncbi.nlm.nih.gov/books/NBK482489/#_NBK482489_pubdet_ Last accessed 18/7/2023

Li, C., Tang, Y., Gu, F., Wang, X., Yang, W., Han, Y., & Ruan, Y. (2022). Phytochemical analysis reveals an antioxidant defense response in Lonicera japonica to cadmium-induced oxidative stress. *Scientific Reports, 12*, 6840.

Liu, L. (2020). Cellular responses to toxicants. In C.N. Pope & J. Liu (Eds.), *An Introduction to Interdisciplinary Toxicology* (pp. 59-68). New York, NY: Elsevier.

Liu, J., Goyer, R.A., & Waalkes, M.P. (2008). Toxic effects of metals. In C.D. Klaassen (Ed.), *Cassaret & Doull's toxicology: The basic science of poison* (7thed., pp. 931-971). New York, NY: The McGraw Hill Companies.

Loewe, D., Sanvictores, T., Zubair, M., & John, S. (2022). Alkaline phosphatase. In *StatPearls [Internet]*. Treasure Island, FL: StatPearls. Retrieved from https://www.ncbi.nlm.nih.gov/books/NBK459201/ Last accessed 17/7/2023

Macan, A.M., Kraljevic, T.G., & Raic-Malic, S. (2019). Therapeutic perspective of vitamin C and its derivatives. *Antioxidants, 8*(8), 247.

Mao, C., Yuan, J., Lv, Y., Gao, X., Yin, Z., Kraus, V.B., … Shi, X. (2019). Associations between superoxide dismutase, malondialdehyde and all-cause mortality in older adults: a community based cohort study. *BMC Geriatrics, 19,* 104.https://doi.org/10.1186/s12877-019-1109-z

Marin-Garcia, J. (2014). Oxidative stress and cell death in cardiovascular disease. In *Pos-Genomic Cardiology* (2nd ed., pp. 471-498). New York, NY: Elsevier

Maurya, R.P., Prajapat, M.K., Singh, V.P., Roy, M., Todi, R., Bosak, S., … Morekar, S.R. (2021). Serum malondialdehyde as a biomarker of oxidative stress in patients with primary ocular carcinoma: Impact on response to chemotherapy. *Clinical Opthalmology, 15*, 871-879.

Merill, J.C., Morton, J.P.J., & Soileau, S.D. (2008). Metals. In A.W. Hayes (Ed.), *Principles and methods of toxicology* (5th ed., pp. 847-875). New York, NY: Informa Healthcare.

Monga, A., Fulke, A.B., & Dasgupta, D. (2022). Recent developments in essentiality of trivalent chromium and toxicity of hexavalent chromium: Implications on human health and remediation strategies. *Journal of Hazardous Materials Advances, 2*, 100113.

Monosson, E. (2005). Chemical mixtures: Considering the evolution of toxicology and chemical assessment. *Environmental Health Perspectives,113*(4), 383-390.

Meulenbelt, J. (2016). Cadmium. In: J. Brent, K. Burkhart, P. Dargan, B. Hatten, B. Megarbane & R. Palmer (Eds.), *Critical Care Toxicology* (pp. 1-7). Cham, Denmark: Springer. https://doi.org/10.1007/978-3-319-20790-2_45-1

National Environmental Standards and Regulations Enforcement Agency (Establishment) Act (2007). Section 8(n)(p*).* Laws of the Federation of Nigeria No. 25of 2007.Retrieved from https://www.placng.org. Last accessed 18/7/2023

Nandi, A., Yan, L., Jana, C.K., & Das, N. (2019). *Role of catalase in oxidative stress- and age-associated degenerative disease.* Retrieved from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6885225/ Last accessed 17/7/2023

Nordberg, M., & Nordberg, G.F. (2022). Metallothionein and cadmium toxicology-historical review and commentary. *Biomolecules, 12*(3), 360. https://doi.org/10.3390/biom12030360

Nowicki, J., & Pizzorno, J.E. (2020). 200- Nonalcoholic fatty liver disease and nonalcoholic steatohepatitis. In J.E. Pizzorno & M.T. Murray (Eds.), *Textbook of Natural Medicine* (5th ed., pp. 1600-1604.e3). London, England: Churchill Livingstone.

Nuhu, F., Gordon, A., Sturmey, R., Seymour, A., & Bhandari, S. (2020). *Measurement of glutathione as a tool for oxidative stress studies by high performance liquid chromatography.*Retrieved from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7571047/#_ffn_sectitle. Last accessed 21/7/2022

Offor, S.J., Mbagwu, H.O.C., & Orisakwe, O.E. (2017). Lead induced hepato-renal damage in male albino rats and effects of activated charcoal. *Frontiers in Pharmacology, 8,* 107.https://doi.org/10.3389/fphar.2017.00107

Oluwafemi, J., Olukanni, D., & Justin, D.L. (2021). Improper waste disposal in Ota, Ogun State-a proposed waste segregation approach. *Journal of Physics: Conference Series, 1734.*

Okon, A.J., Olanrewaju, O.E., Okon, I.J., Inah, S.A., Aluko, O.O., & Dania, A.V. (2022). Protective effect of Carica papaya extracts againstlead bioaccumulation in albino rats. *Research Square*. https://doi.org/10.21203/rs.3.rs-2050296/v1

Okoro, I. (2002). *Manual of practical of histology* (2nd ed., pp 4-9). Owerri, Nigeria: Peace.

Organization for Economic Co-operation and Development (OECD). (2019). *Guiding principles and key elements for establishing a weight of evidence for chemical assessment*. Series on testing and assessment No. 311, Environment, Health and Safety Division, Environmental Directorate. Retrieved from www.oecd.org/chemicals safety/ Last accessed 17/7/2023

Osukoya, O.A., Oyinloye, B.E., Ajiboye, B.O., Olokode, K.A., & Adeola, H.A. (2021). Nephroprotective and anti-inflammatory potential of aqueous extract from *Persea americana* seeds against cadmium-induced nephrotoxocity in Wistar rats. *Biometals, 34,* 1141-1153

Pazand, K., Khosravi, D., Ghaderi, M.R., & Rezvanianzadeh, M.R. (2018). Hydrogeochemistry and lead contamination of groundwater in the north part of Esfahan province, Iran. *Journal of Water Health, 16*(4), 622-634.

Peluso, I. (2022). Diet and exercise in lifestyle medicine: The hormetic effects of bioactive compounds on human health. *Current Opinion in Toxicology, 30,*10032. https://doi.org/10.1016/j.cotox.2022.03.003

Poli, V., Madduru, R., Aparna, Y., Kandukuri, V., & Motireddy, S.R. (2022). Amelioration of Cadmium-Induced Oxidative Damage in Wistar Rats by Vitamin C, Zinc and N-Acetylcysteine.*Medical Sciences (Basel)10*(1), 7.

Pralle, R.S., Holdorf, H.T., Oliveira, R.C., Seely, C.R., Kendall, S.J., & White, H.M. (2022). Prediction of liver triglyceride content in early lactation multiparous Holstein cows using blood metabolite, mineral, and protein biomarker concentrations. *Animals (Basel), 12*(19), 2556

Prozialeck, W.C., & Edwards, J.R. (2012). Mechanisms of cadmium-induced proximal tubule injury: New insights with implications for biomonitoring and therapeutic interventions. *The Journal of Pharmacology and Experimental Therapeutics, 343*(1), 2-12.

Rahimzadeh, M.R., Rahimzadeh, M.R., Kazemi, S., & Moghadamnia, A. (2017). Cadmium toxicity and treatment: An update. *Caspian Journal of Internal Medicine, 8*(3), 135-145.

Reitman, S., & Frankel, S. (1957**)**. A colorimetric method of determination of serum glutamic oxaloacetic and glutamic pyruvic transaminase. *American Journal of Clinical Pathology, 28*(1), 56-63.

Rhodes, C.E., Denault, D., & Varacallo, M. (2022). Physiology, oxygen transport. In *StatPearls [Internet]*. Treasure Island, FL: StatPearls. Retrieved from https://www.ncbi.nlm.nih.gov/books/NBK538336/ Last accessed 17/7/2023

Richardson, R.J. (2020). Toxicant interactions with macromolecular targets. In C.N. Pope & J. Liu (Eds.), *An Introduction to Interdisciplinary Toxicology* (pp. 45-57). New York, NY: Elsevier.

Rider, C.V., Dinse, G.E., Umbach, D.M., Simmons, J.E., & Hertzberg, R.C. (2018). Predicting mixture toxicity with models of addivity. In C.V. Rider, G.E., Dinse, D.M., Umbach, and J.E. Simmons (Eds.), *Chemical mixtures and combined chemical and nonchemical stressors*(pp.235-270). New York, NY: Springer International Publishing,

Roberts, T.L. (2014). Cadmium and phosphorus fertilizers: the issues and the science. *Procedia Engineering*, *83*, 52-59.

Roy, A.V. (1970). Rapid method for determining alkaline phosphatase activity in serum with thymolphthalein monophosphate. *Clinical Chemistry, 16*, 431-436.

Saed, O.S., Al-Kubaisi, S.M.A., Suleiman, J., & Hassan, M.S. (2020). Treatment of induced lead toxicity by using DMSA in puppies. *Indian Journal of Forensic Medicine and Toxicology*, *14*(1), 479-482.

Saha, J., Choudhuri, S., & Choudhuri, D. (2017). Effect of subchronic exposure to chromium on hematological and biochemical parameters of male albino rat. *Asia Journal of Pharmaceutical and Clinical Research,10*(5), 345.

Saito, T., Nishida, Y., Tabata, M., Isobayashi, A., Tomizawa, H., Miyahara.,& Sugizaki, Y. (2022). Molecular interactions between an enzyme and its inhibitor for selective detection of limonene. *Analytical Chemistry, 94*(21), 7692-7702.

Sakhartar, P. (2017). Herbal medications. *Indian Journal of Clinical Biochemistry*, 32(4), 500-501.

Sarka, R.K., Bhowmik, M., Sarkar, M.B., Sircar, G., & Bhattacharya, K. (2022). Comprehensive characterization and molecular insights into the salt tolerance of a Cu, Zn-superoxide dismutase from an Indian Mangrove, *Avicennia marina. Scientific Reports, 12*, 1745

Sassia, S., Amine, B., Nadia, B., Hadda, A., & Smail, M. (2021). Investigation of single and combined effects of repeated oral cadmium and lead administration in ewes. *Scientific African, 13*.

Satarug**,** S. (2018). Dietary cadmium intake and its effects on kidney. *Toxics*,*6*, 15.

Senthilkumar, M., Amaresan, N., & Sankaranarayanan, A. (2021). Estimation of malondialdehyde (MDA) by thiobarbituric acid (TBA) assay. In *Plant-Microbe Interactions* (pp. 100-105). New York, NY: Springer Protocols Handbooks. https://doi.org/10.1007/978-1-0716-1080-0_25

Sharma, P., Singh, S.P., Parakh, S.K., & Tong, Y.W. (2022). Health hazards of hexavalent chromium (Cr (VI)) and its microbial reduction. *Bioengineered,13*(3), 4923-4938.

Sharma, A., Kapoor, D., Wang, J., Shahzad, B., Kumar, V., Shahzad, B., … Yan, D. (2020). Chromium bioaccumulation and its impacts on plants: An overview. *Plants, 9*(1), 100.

Shi, Z., Carey, M., Andrew, A., & Meharg, C. (2020). Rice grain cadmium concentrations in the global supply-chain. *Exposure Health, 12*, 869-876.

Singh, V., Singh, N., Verma, M., Kamal, R., Tiwari, R., Chivate, M.S., … Mishra, V. (2022). Hexavalent-chromium-induced oxidative stress and the protective role of antioxidants against cellular toxicity. *Antioxidants, 11*(12), 2375.https://doi.org/10.3390/antiox11122375

Socianu, S., Bopp, S.K., Govarts, E., Gilles, L., Buekers, J., Kolossa-Gehring. M., …, Franco, A. (2022). Chemical mixtures in the EU population: Composition and potential risks. *International Journal of Environmental Research and Public Health, 19,* 6121. https://doi.org/10.3390/ijerph19106121

Stota, M., Wasik, M., Stoltny, T., Machon-Grecka, A., Kasperczyk, A., Bellanti, F., … Kasperczyk, S. (2021). Relationship between lead absorption and iron status and its association with oxidative stress markers in lead-exposed workers.*Journal of Trace Elements in Medicine and Biology, 68,* 126841.

Suciu, N.A., Vivo, R.D., Rizzati, N., & Capri, E. (2022). Cd content in phosphate fertilizer: Which potential risk for the environment and human health? *Current Opinion in Environmental Science and Health, 30,* 100392. https://doi.org/10.1016/j.coesh.2022.100392

Sun, H., Brocota, J., & Costa, M. (2015). Oral chromium exposure and toxicity. *Current Environmental Health Reports, 2*, 295-303.

Sun, M., & Zigman, S. (1978). An improved spectrophotometric assay for superoxide dismutase based on epinephrine autoxidation. *Analytical Biochemistry, 90*(1), 81-89.

Tai, Y., Chou, S., Cheng, C., Ho, C., Lin, H., Jung, S., … Ko, F. (2022). The preferential accumulation of cadmium ions among various tissues in mice. *Toxicology Reports, 9,* 111-119.

Thevenod, F., & Wolff, N.A. (2016). Iron transport in the kidney: Implications for physiology and cadmium nephrotoxicity. *Metallomics, 8,* 17-42.

Tripathi, S.M., & Chaurasia, S. (2020). Detection of chromium in surface and groundwater and its bio-absorption using bio-wastes and vermiculite. *Engineering Science and Technology, an International Journal, 23,* 115-1161.

United States Environmental Protection Agency (USEPA). (2003). *Developing Relative Potency Factor for Pesticide Mixtures: Biostatistical Analysis of Joint Dose Response*. Retrieved from https://cfpub.epa.gov/ncea/risk/recordisplay.cfm?deid=66273 Last accessed 17/7/2023

Upson, K., Filippini, T., Vincenti, M., Wegienka, G., Wise, L., Tokar, E., & Donna, B. (2019). Deficient calcium, zinc, and iron intake on absorption of cadmium from diet. *Environmental Epidemiology, 3,* 402-403.

Vincenti, S.R. & Filippini, T. (2021). Public health and public law issues for the toxicological risk assessment of chemical mixtures. *Public Health Toxicology, 1*(2), 6.

Wallin, B., Rosengren, B., Shetzer, H.G., & Camejo, G. (1993). Lipoprotein oxidation and measurement of thiobarbituric acid reacting substances (TBARS) formation in a single microlitre plate: its use for evaluation of antioxidants. *Annual Journal of Biochemistry,208,* 10-15.

WorldHealth Organization (WHO). (2000). *Recommended method for the determination of packed cell volume by centrifugation*.Retrieved from https://apps.who.int/iris/handle/10665/6620 Last accessed 17/7/2023

WorldHealth Organization (WHO).(2021). *Lead poisoning*. Retrieved from https://who.int/news-room/fact-sheets/detail/lead-poisoning-and-health Last accessed 17/7/2023

Wu, Y., Ling, J., Wang, T., Lin, T., Yen, M., Liu, Y., … Yeh, I. (2020). Hexavalent chromium intoxication induces intrinsic and extrinsic apoptosis in human renal cells. *Molecular Medicine Reports, 21*(2), 851-857.

Yaman, B. (2020). Health effects of chromium and its concentrations in cereal foods together with sulfur. *Exposure and Health, 12,* 153-161.

Yang, H., & Shu, Y. (2015). Cadmium transporters in the kidney and cadmium-induced nephrotoxicity. *International Journal of Molecular Science, 16*(5), 1484-1494

Yasmin, N., & Hussain, M.S. (2021). Histopathological changes in the kidney of albino rat due to chromium and the ameliorative role of α-tocopherol. *Journal of Clinical and Diagnostic Research, 15*(1), AC05-AC08.

York, M.J. (2017). Cinical pathology. In A.S. Faqi (Ed.), *A Comprehensive guide to toxicology in nonclinical drug development* (2nd ed., pp. 325-374). Cambridge, MA: Academic Press.https://doi.org/10.1016/B978-0-12-803620-4.00014-1

Zhang, H., & Reynolds, M. (2019). Cadmium exposure in living organisms: A short review. *Science of the Total Environment, 678,* 761-767.

Zhang, Y., Liu, Z., He, Q., Wu, F., Xiao, Y., Chen, W., … Wang, Q. (2021). *Construction of mode of action for cadmium-induced renal tubular dysfunction based on a toxicity pathway-oriented approach*. Retrieved from https://www.frontiersin.org/articles/10.3389/fgene.2021.696892/full Last accessed 17/7/2023

Zhong, X., Zeng, M., Bian, H., Zhong, C., & Xiao, F. (2017). An evaluation of protective role of vitamin C in reactive oxygen species-induced hepatoxicity due to hexavalent chromium *in vitro* and *in vivo*. *Journal of Occupational Medicine and Toxicology*, 12, 15.https://doi.org/10.1186/s12995-017-0161-x

APPENDICES

Appendix 1: CHEMICALS/MATERIALS	SOURCES
Alanine aminotransferase (ALT) buffer	Randox
Alkaline phosphatase color developer	Teco
Alkaline phosphatase standard	Teco
Alkaline phosphatase substrate	Teco
Ascorbic acid	
Aspartate aminotransferase (AST) buffer	Randox
Cadmium sulphate	Riedel De Heaney
Caffeine	Randox
Chloride calibrator	Teco
Chloride reagent	Teco
Creatinine standard	Randox
Cyanmethaemoglobin standard	
2, 6 – dichloroindophenol	
2, 4 – dinitrophenyl hydrazine	Randox
Drabkin's reagent	
Ellman's reagent (DTNB)	
Eosin	
Formol citrate	
Growers mash	Topfeed
Haematoxylin	
Halothane B.P.	Raman & Weil

Hydrogen peroxide	
Isopropyl alcohol	
Lactate dehydrogenase buffer	Liquid gold
Lactate dehydrogenase substrate	Liquid gold
Lead nitrate	M & B
Molybdate	Fortress
n-butanol	
Oxalic acid	
Phenol concentrate	Randox
Phosphate buffer	
Phosphate standard	Fortress
Phosphorus blank reagent	Fortress
Picric acid	Randox
Potassium dichromate	Hopkin & William
Potassium standard	Teco
Sodium chloride solution	
Sodium hydroxide	Randox
Sodium hydroxide solution	
Sodium hypochlorite	Randox
Sodium nitrite	Randox
Sodium nitroprusside	Randox
Sodium tetraphenylboron	Teco
Sulphanilic acid	Randox

Tartrate	Randox
Thiobarbituric acid	
Trichloroacetic acid	
Turk's solution	
Urease	Randox
Urea standard	Randox
Xylene	

Appendix 2: APPARATUS

Beakers

Conical flasks

Cover glass (slips)

Dessicator

Feeding tube (size 5)

Filter papers

Forceps

Heparinized glass capillary tubes

Magnetic stirrer

Measuring cylinders

Permanent marker pens

Pipettes

Reagent bottles

Sample bottles

Scale i.e. PCV reader

Scissor

Slides

Stainless steel bowls

Stirring rod

Syringes (2ml, 5ml)

Test tube racks

Test tubes

Tissue cassette

Titration set (burette, stand, clamp, funnel)

Water bath

Wooden cage with wire mesh

Centrifuge 80 – 2 (made in UK)

Digital photocolorimeter (E.I)

G & G electronic scale

Hot plate

Microhaematocrit centrifuge (Newlife med. Inst. England)

Microscope (Leica Dm750)

Microtome (Leica Rm 212 Rt. Rotary microtome)

Olympus microscope

Refrigerator (LG)

Spectrophotometer (Spectrum lab. 7525 B. Bran-scientific)

Spectrophotometer (Spectrum lab. 7555 Search tech)

Timer (Samsung GT – S7562)

Triple beam balance (Techmel USA)

Appendix 3: Summary of percentage body weight (BW) gain of albino rats treated with Cd, Cr and Pb individually and as a mixture

Treatment	Initial BW (g)	Final BW (g)	BW gain (g) (Final BW-initial BW)	% BW gain (BW gain/initial BW)x100
Control	162.2±6.4	302.5±28.8	140.3±29.5	86.5±4.6
5mg/kg Pb	161.3±5.6	306.2±39.2	144.9±39.6	89.8±7.1[b]
5mg/kg Cd	161.6±7.0	238.3±16.4	76.7±17.8	47.5±2.5[a]
5mg/kg Cr	160.8±4.7	251.3±17.2	90.5±17.8	56.3±3.8[ab]
5mg/kg Mixture	162.5±3.2	238.8±20.6	76.3±20.8	47.0±6.5[a]
10mg/kg Pb	164.0±3.7	299.6±26.0	135.6±26.3	82.7±7.1[b]
10mg/kg Cd	160.6±4.9	293.4±60.4	132.8±60.6	82.7±12.4[b]
10mg/kg Cr	161.9±4.6	263.8±34.3	101.9±34.6	62.9±7.5[a]
10mg/kg Mixture	162.5±3.7	258.6±44.4	96.1±44.6	59.1±12.1[a]
20mg/kg Pb	162.7±6.1	297.4±21.8	134.7±22.6	82.8±3.7[b]
20m/kg gCd	159.5±2.8	231.6±31.9	72.1±32.0	45.2±11.4[a]
20m/kg gCr	161.0±5.9	258.4±55.0	97.4±55.3	60.5±9.4[ab]
20mg/kg Mixture	160.8±4.7	222.2±13.0	61.4±13.8	38.2±2.9[a]

a = significantly different from control ($p<0.05$)

b = significantly different from mixture ($p<0.05$)

Appendix 4: Summary of mean liver to body weight (BW) ratio of albino rats treated with Cd, Cr and Pb individually and as a mixture

Treatment	Liver weight (g)	Final BW (g)	Liver to body weight ratio ($x10^{-3}$) (Liver wt./final BW)
Control	8.82±1.15	302.5±28.8	29.2±4.0
5mg/kg Pb	8.93±1.58	306.2±39.2	29.2±4.0
5mg/kg Cd	7.07±0.29	238.3±16.4	29.7±1.8
5mg/kg Cr	6.64±0.34	251.3±17.2	26.4±2.0
5mg/kg Mixture	7.38±1.15	238.8±20.6	30.9±5.6
10mg/kg Pb	8.15±0.86	299.6±26.0	27.2±3.3
10mg/kg Cd	7.45±0.46	293.4±60.4	25.4±0.8
10mg/kg Cr	8.36±1.15	263.8±34.3	31.7±3.4
10mg/kg Mixture	9.73±2.61	258.6±44.4	37.6±5.9[a]
20mg/kg Pb	10.73±1.18	297.4±21.8	36.0±5.4
20mg/kg Cd	7.58±1.03	231.6±31.9	32.7±3.2
20mg/kg Cr	9.94±2.66	258.4±55.0	38.5±4.8[a]
20mg/kg Mixture	9.28±0.58	222.2±13.0	41.8±4.5[a]

a = significantly different from control ($p<0.05$)

Appendix 5: Summary of mean kidney to body weight (BW) ratio of albino rats treated with Cd, Cr and Pb individually and as a mixture

Treatment	Kidney weight (g)	Final BW (g)	Kidney to body wt. ratio ($x10^{-3}$) (Kidney wt./final BW)
Control	1.70±0.27	302.5±28.8	5.6±0.9
5mg/kg Pb	1.70±0.33	306.2±39.2	5.6±0.8
5mg/kg Cd	1.51±0.11	238.3±16.4	6.3±0.7
5mg/kg Cr	1.46±0.06	251.3±17.2	5.8±0.3
5mg/kg Mixture	1.51±0.13	238.8±20.6	6.3±0.6
10mg/kg Pb	1.96±0.18	299.6±26.0	6.5±0.7
10mg/kg Cd	1.55±0.19	293.4±60.4	5.3±0.3
10mg/kg Cr	1.61±0.25	263.8±34.3	6.1±0.7
10mg/kg Mixture	1.92±0.20	258.6±44.4	7.4±0.5[a]
20mg/kg Pb	1.88±0.21	297.4±21.8	6.3±1.0
20mg/kg Cd	1.33±0.10	231.6±31.9	5.7±0.3
20mg/kg Cr	1.54±0.21	258.4±55.0	6.0±0.4
20mg/kg Mixture	1.50±0.12	222.2±13.0	6.8±0.9

a = significantly different from control ($p<0.05$)

Appendix 6: Summary of mean heart to body weight (BW) ratio of albino rats treated with Cd, Cr and Pb individually and as a mixture

Treatment	Heart weight (g)	Final BW (g)	Heart to body weight ratio ($x10^{-3}$) (Heart wt/final BW)
Control	0.83±0.10	302.5±28.8	2.7±0.3
5mg/kg Pb	0.89±0.06	306.2±39.2	2.9±0.2
5mg/kg Cd	0.72±0.06	238.3±16.4	3.0±0.4
5mg/kg Cr	0.78±0.07	251.3±17.2	3.1±0.4
5mg/kg Mixture	0.72±0.04	238.8±20.6	3.0±0.2
10mg/kg Pb	0.80±0.12	299.6±26.0	2.7±0.5
10mg/kg Cd	0.80±0.20	293.4±60.4	2.7±0.3
10mg/kg Cr	0.73±0.06	263.8±34.3	2.8±0.2
10mg/kg Mixture	0.88±0.18	258.6±44.4	3.4±0.4[a]
20mg/kg Pb	0.80±0.04	297.4±21.8	2.7±0.2
20mg/kg Cd	0.65±0.09	231.6±31.9	2.8±0.3
20mg/kg Cr	0.79±0.12	258.4±55.0	3.1±0.2
20mg/kg Mixture	0.66±0.05	222.2±13.0	3.0±0.4

a = significantly different from control ($p<0.05$)

Appendix 7: Serum alanine aminotransferase (ALT) levels in albino rats treated with Cd, Cr and Pb individually and as a mixture

Dose	ALT (U/L)				
	Control	Mixture (Pb, Cd, Cr)	Pb	Cd	Cr
5mg/kg	5.9±0.4	8.4±0.7[a]	8.1±0.6[a]	8.1±0.7[a]	12.5±1.8[ab]
10mg/kg	5.9±0.4	7.7±0.2[a]	8.2±0.7[a]	10.2±0.6[ab]	9.3±1.4[a]
20mg/kg	5.9±0.4	6.4±0.3[a]	8.3±1.5[a]	11.8±1.0[ab]	7.0±1.4[a]

a = significantly different from control ($p<0.05$)

b = significantly different from mixture ($p<0.05$)

Appendix 8: Serum aspartate aminotransferase (AST) levels in albino rats treated with Cd, Cr and Pb individually and as a mixture

Dose	AST (U/L)				
	Control	Mixture (Pb, Cd, Cr)	Pb	Cd	Cr
5mg/kg	13.0±1.6	21.4±3.5[a]	22.3±5.4[a]	35.1±7.2[ab]	47.4±17.9[ab]
10mg/kg	13.0±1.6	17.8±4.7[a]	20.7±4.1[a]	30.2±5.8[ab]	27.0±9.3[a]
20mg/kg	13.0±1.6	14.0±3.7	16.5±1.7[a]	23.0±3.3[ab]	10.8±1.9

a = significantly different from control ($p<0.05$)

b = significantly different from mixture ($p<0.05$)

Appendix 9: Serum alkaline phosphatase (ALP) level in albino rats treated with Cd, Cr and Pb individually and as a mixture

Dose	ALP (U/L)				
	Control	Mixture (Pb, Cd, Cr)	Pb	Cd	Cr
5mg/kg	67.0±13.9	68.4±11.4	54.8±6.2	80.9±15.9	68.6±4.9
10mg/kg	67.0±13.9	85.6±2.1[a]	75.4±3.7	86.3±8.9	71.3±5.1
20mg/kg	67.0±13.9	96.1±1.5[a]	91.2±4.1[a]	92.7±4.1[a]	92.1±0.3[ab]

a = significantly different from control ($p<0.05$)

b = significantly different from mixture ($p<0.05$)

Appendix 10: Serum total bilirubin concentration in albino rats treated with Cd, Cr and Pb individually and as a mixture

Dose	Total Bilirubin (umol/l)				
	Control	Mixture (Pb, Cd, Cr)	Pb	Cd	Cr
5mg/kg	4.73±0.55	9.10±0.58[a]	6.92±0.76[ab]	3.07±0.34	2.78±0.29
10mg/kg	4.73±0.55	8.65±0.28[a]	7.44±0.33[ab]	3.84±0.55	4.11±0.51
20mg/kg	4.73±0.55	6.62±1.49[a]	9.07±0.52[ab]	5.59±0.72[ab]	5.51±0.85[ab]

a = significantly different from control ($p<0.05$)

b = significantly different from mixture ($p<0.05$)

Appendix 11: Serum lactate dehydrogenase (LDH) levels in albino rats treated with Cd, Cr and Pb individually and as a mixture

Dose	LDH (U/l)				
	Control	Mixture (Pb, Cd, Cr)	Pb	Cd	Cr
5mg	355±32	705±47[a]	647±80[a]	663±39[a]	609±34[ab]
10mg	355±32	813±24[a]	702±29[ab]	769±26[a]	631±24[ab]
20mg	355±32	906±28[a]	785±26[ab]	884±34[a]	681±40[ab]

a = significantly different from control ($p<0.05$)

b = significantly different from mixture ($p<0.05$)

Appendix 12: Serum urea concentration in albino rats treated with Cd, Cr and Pb individually and as a mixture

Dose	Urea (mg/dl)				
	Control	Mixture (Pb, Cd, Cr)	Pb	Cd	Cr
5mg	23.95 ±1.96	35.16 ±1.37[a]	35.27 ±1.51[a]	43.72 ±4.62[ab]	53.29 ±3.89[ab]
10mg	23.95 ±1.96	33.05 ±1.37[a]	35.14 ±1.40[a]	44.97 ±1.51[ab]	38.93 ±2.52[ab]
20mg	23.95 ±1.96	30.19 ±3.25[a]	35.66 ±3.91[a]	46.86 ±5.39[ab]	31.90 ±3.64[a]

a = significantly different from control ($p<0.05$)

b = significantly different from mixture ($p<0.05$)

Appendix 13: Serum creatinine concentration in albino rats treated with Cd, Cr and Pb individually and as a mixture

Dose	Creatinine (mg/dl)				
	Control	Mixture (Pb, Cd, Cr)	Pb	Cd	Cr
5mg/kg	0.57±0.08	0.58±0.04	0.56±0.10	0.55±0.13	0.61±0.06
10mg/kg	0.57±0.08	0.57±0.07	0.54±0.06	0.62±0.06	0.61±0.06
20mg/kg	0.57±0.08	0.50±0.04	0.41±0.06	0.68±0.09	0.55±0.06

Appendix 14: Serum potassium concentration in albino rats treated with Cd, Cr and Pb individually and as a mixture

Dose	Potassium (mEq/l)				
	Control	Mixture (Pb, Cd, Cr)	Pb	Cd	Cr
5mg	5.0±0.1	5.8±0.1[a]	5.3±0.1[ab]	5.3±0.2[ab]	6.6±0.4[ab]
10mg	5.0±0.1	6.1±0.1[a]	5.8±0.1[ab]	5.9±0.1[a]	6.0±0.2[a]
20mg	5.0±0.1	6.4±0.2[a]	6.0±0.1[ab]	6.5±0.2[a]	5.7±0.3[ab]

a = significantly different from control ($p<0.05$)

b = significantly different from mixture ($p<0.05$)

Appendix 15: Serum chloride concentration in albino rats treated with Cd, Cr and Pb individually and as a mixture

Dose	Chloride (mEq/l)				
	Control	Mixture (Pb, Cd, Cr)	Pb	Cd	Cr
5mg	86.2±5.7	69.7±4.4[a]	86.0±6.8	77.2±1.2[ab]	86.7±3.7
10mg	86.2±5.7	85.8±3.1	89.3±2.3	86.4±2.7	87±1.3
20mg	86.2±5.7	98.2±1.5[a]	95.9±2.2[a]	95.6±3.3[a]	94.4±3.1

a = significantly different from control ($p<0.05$)

b = significantly different from mixture ($p<0.05$)

Appendix 16: Serum inorganic phosphorus concentration in albino rats treated with Cd, Cr and Pb individually and as a mixture

Dose	Phosphorus (mg/dl)				
	Control	Mixture (Pb, Cd, Cr)	Pb	Cd	Cr
5mg	3.16±0.23	7.14±0.93[a]	6.99±0.81[a]	7.06±2.33[a]	6.61±1.34[a]
10mg	3.16±0.23	9.39±1.16[a]	8.34±0.76[a]	9.07±0.74[a]	8.46±0.89[a]
20mg	3.16±0.23	10.57±.65[a]	10.95±0.27a	11.03±0.29[a]	10.71±0.39[a]

a = significantly different from control ($p<0.05$)

Appendix 17: Serum malondialdehyde (MDA) concentration in albino rats treated with Cd, Cr and Pb individually and as a mixture

Dose	MDA (umol/l)				
	Control	Mixture (Pb, Cd, Cr)	Pb	Cd	Cr
5mg	0.661 ±0.020	0.815 ±0.032[a]	0.804 ±0.028[a]	0.816 ±0.017[a]	0.829 ±0.029[a]
10mg	0.661 ±0.020	0.988 ±0.040[a]	0.887 ±0.020[ab]	0.984 ±0.028[a]	0.884 ±0.030[ab]
20mg	0.661 ±0.020	1.180 ±0.057[a]	1.140 ±0.035[a]	1.137 ±0.057[a]	1.194 ±0.022[a]

a = significantly different from control ($p<0.05$)

b = significantly different from mixture ($p<0.05$)

Appendix 18: Serum glutathione (GSH) concentration in albino rats treated with Cd, Cr and Pb individually and as a mixture

Dose	GSH (µM)				
	Control	Mixture (Pb, Cd, Cr)	Pb	Cd	Cr
5mg	308.9±11.9	273.3±9.7[a]	285.3±1.7[a]	285.9±13.3[a]	279.1±6.8[a]
10mg	308.9±11.9	266.2±6.0[a]	275.5±2.1[ab]	272.4±3.3[a]	269.6±7.1[a]
20mg	308.9±11.9	250.0±8.5[a]	257.7±4.8[a]	251.2±5.7[a]	261.0±2.1[a]

a = significantly different from control ($p<0.05$)

b = significantly different from mixture ($p<0.05$)

Appendix 19: Serum ascorbic acid concentration in albino rats treated with Cd, Cr and Pb individually and as a mixture

Dose	Ascorbic acid (mg/dl)				
	Control	Mixture (Pb, Cd, Cr)	Pb	Cd	Cr
5mg	1.118 ±0.051	0.730 ±0.067[a]	0.812 ±0.049[a]	0.800 ±0.067[a]	0.777 ±0.077[a]
10mg	1.118 ±0.051	0.647 ±0.048[a]	0.659 ±0.049[a]	0.691 ±0.056[a]	0.635 ±0.049[a]
20mg	1.118 ±0.051	0.515 ±0.056[a]	0.559 ±0.034[a]	0.482 ±0.076[a]	0.559 ±0.034[a]

a = significantly different from control ($p<0.05$)

Appendix 20: Serum superoxide dismutase (SOD) levels in albino rats treated with Cd, Cr and Pb individually and as a mixture

Dose	SOD (U/L)				
	Control	Mixture (Pb, Cd, Cr)	Pb	Cd	Cr
5mg	0.112 ±0.023	0.212 ±0.048[a]	0.159 ±0.022[a]	0.186 ±0.054[a]	0.117 ±0.031
10mg	0.112 ±0.023	0.240 ±0.015[a]	0.238 ±0.026[a]	0.241 ±0.027[a]	0.199 ±0.025[a]
20mg	0.112 ±0.023	0.268 ±0.009[a]	0.301 ±0.049[a]	0.305 ±0.041[a]	0.293 ±0.044[a]

a = significantly different from control ($p<0.05$)

Appendix 21: Serum catalase (CAT) levels in albino rats treated with Cd, Cr and Pb individually and as a mixture

Dose	CAT (U/ml)				
	Control	Mixture (Pb, Cd, Cr)	Pb	Cd	Cr
5mg	43.0±3.5	48.7±3.9[a]	46.9±1.4[a]	46.9±1.7[a]	49.8±2.0[a]
10mg	43.0±3.5	58.7±1.4[a]	50.0±1.1[ab]	50.1±1.2[ab]	54.9±1.5[ab]
20mg	43.0±3.5	64.3±2.8[a]	55.7±1.2[ab]	59.0±2.4[ab]	62.3±1.4[a]

a = significantly different from control ($p<0.05$)

b = significantly different from mixture ($p<0.05$)

Appendix 22: Haemoglobin (Hb) concentration ofalbino rats treated with Cd, Cr and Pb individually and as a mixture

Dose	Haemoglobin (g/dl)				
	Control	Mixture (Pb, Cd, Cr)	Pb	Cd	Cr
5mg/kg	15.0±1.1	14.0±1.5	14.4±1.9	16.7±0.7[a]	16,7±1.3[a]
10mg/kg	15.0±1.1	14.5±0.6	15.5±0.4	15.0±1.4	14.5±1.5
20mg/kg	15.0±1.1	13.6±2.4	14.7±0.8	14.4±1.6	14.3±0.2

a = significantly different from control ($p<0.05$)

Appendix 23: Levels of packed cell volume (PCV) ofalbino rats treated with Cd, Cr and Pb individually and as a mixture

Dose	PCV (%)				
	Control	Mixture (Pb, Cd, Cr)	Pb	Cd	Cr
5mg/kg	47±5	42±4	46±4	50±2	50±4
10mg/kg	47±5	43±2	46±1	44±4	43±4
20mg/kg	47±5	41±7	44±2	43±5	43±1

Appendix 24: Red blood cells (RBC) of albino rats treated with Cd, Cr and Pb individually and as a mixture

Dose	RBC (cells/mm^3)				
	Control	Mixture (Pb, Cd, Cr)	Pb	Cd	Cr
5mg/kg	3,855,556 ±575,394	4,310,000 ±756,307[a]	4,152,000 ±240,740	4,556,000 ±550,209[a]	4,072,000 ±689,906
10mg/kg	3,855,556 ±575,394	4,400,000 ±787,062	3,920,000 ±534,556	3,940,000 ±702,756	4,266,000 ±479,354
20mg/kg	3,855,556 ±575,394	4,482,500 ±571,569[a]	3,842,500 ±476,961	4,160,000 ±993,680	4,715,000 ±1,299,756[a]

a = significantly different from control ($p<0.05$)

Appendix 25: White blood cells (total) WBC(T) of albino rats treated with Cd, Cr and Pb individually and as a mixture

Dose	WBC(T) (cells/mm^3)				
	Control	Mixture (Pb, Cd, Cr)	Pb	Cd	Cr
5mg/kg	7,467 ±1,616	6,140 ±1,795	12,880 ±2,008[ab]	10,060 ±1,159[ab]	6,740 ±2,814
10mg/kg	7,467 ±1,616	10,075 ±4,647[a]	9,200 ±2,206	8,450 ±1,170	7,860 ±2,792
20mg/kg	7,467 ±1,616	8,375 ±2,642	8,700 ±2,232	7,200 ±2,162	7,850 ±1,960

a = significantly different from control ($p<0.05$)

b = significantly different from mixture ($p<0.05$)

Appendix 26: Summary of nature of interaction of the mixture components in albino rats treated with Cd, Cr and Pb individually and as a mixture

		Nature of interaction per dose group		
Endpoint	Parameter	5mg/kg	10mg/kg	20mg/kg
Hepatic	ALT (U/L)	Less than additive	Less than additive	Less than additive
	AST	Less than additive	Less than additive	Less than additive
	ALP	No interaction	Additive	No interaction
	Tbil	Additive	Additive	Less than additive
	LDH	No interaction	No interaction	No interaction
Renal	Urea	Less than additive	Less than additive	Less than additive
	Creatinine	No interaction	No interaction	No interaction
	Potassium	Less than additive	No interaction	No interaction
	Chloride	Additive	No interaction	No interaction
	Inorganic phosphorus	No interaction	No interaction	No interaction
Oxidative stress	MDA	No interaction	No interaction	No interaction
	GSH	No interaction	Less than additive	No interaction
	Ascorbic acid	No interaction	No interaction	No interaction
	SOD	No interaction	No interaction	No interaction
	CAT	No interaction	Additive	No interaction
Body/organ weight	Body weight gain	No interaction	No interaction	No interaction
	Relative Organ weight (liver)	No interaction	Additive	No interaction
	Relative Organ weight (kidney)	No interaction	Additive	No interaction
	Relative Organ weight (heart)	No interaction	Additive	No interaction
hematological	Hb	No interaction	No interaction	No interaction
	PCV	No interaction	No interaction	No interaction
	WBC	No interaction	No interaction	No interaction
	RBC	No interaction	No interaction	No interaction

(i) No interaction means that the effect observed in the combined treatment is not significantly different ($p>0.05$) from that observed in the individual treatment and is due to one component of the ternary mixture. (ii) Less-than-additive interaction means that the effect due to treatment with the mixture (combined treatment) is significantly ($p<0.05$) less than that due to individual

treatmentwith highest effect. (iii) Additive interaction is when the effect due to treatment with the mixture (combined treatment) is significantly ($p<0.05$) more than that due to the individual treatment with highest effect.