Effect Of Aqueous Methanol Leaf Extract Of Annona Muricata On SOD, CAT, And MDA Of Cadmium Induced Toxicant In Male Wistar Rat.

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CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

In the periodic table of elements, between zinc (Zn) and mercury (Hg), lies the naturally occurring metal cadium (Cd), which exhibits chemical properties akin to zinc (Zn). It is an element with supple, ductile, silvery white with bluish undertones, glossy, and electro positive characteristics. Cadmium has a melting point is 321 °C, boiling point is 765 °C, atomic number is 48, and atomic mass is 112. It has no taste or smell and is extremely toxic. Eight stable isotopes make up this metal: 106 Cd, 108 Cd, 110 Cd, 111 Cd, 112 Cd, 113 Cd, 114 Cd, and 116 Cd. 112 Cad and 114 Cad are the most prevalent isotopes (Adriano 2001). It typically appears as a divalent caption that has been complicated by additional elements (e.g., CdCl2). Since Cd is typically found as an impurity in deposits of zinc (Zn) or lead (Pb), it is primarily produced as a by-product of the smelting of these metals. Cd is present in the earth's crust at a rate of about 0.1 parts per million (Hans 1999).

Cadium is used in a variety of industrial applications, including television screens, lasers, batteries, paint pigments, alloys, coatings (electroplating), solar cells, plastic stabilizers, cosmetics, galvanizing steel, acting as a barrier in nuclear fission, and sealing lead water pipes before the 1960s with zinc. Each year, the United States produces roughly 600 metric tons, while about 150 metric tons are imported (Rolls 2012). Additionally, the amount of cadium varies widely based on dietary patterns and is found in the majority of meals.

Inhalation and ingestion are the main ways that humans are exposed to Cd. Depending on the particle size, ten to fifty percent of cadmium dust inhaled is absorbed. Skin contact has very little effect on absorption. Depending on the size of the particles, only 5–10% of the Cd that is consumed gets absorbed. When someone is iron, calcium, or zinc deficient, their intestinal absorption is higher (Nordberg et al 2007). Consuming contaminated food or water can expose one to cadmium and cause long-term health effects. Examples of contaminated food include crustaceans, organ meats, leafy vegetables, and rice from specific regions of Japan and China.

Contamination of drugs and dietary supplements may also be a source of contamination (Abernethy 2010). Smoking of cigarettes is considered to be the most significant source of human cadmium exposure (Friberg 1983). High levels of Cd in the blood and kidneys are continuously higher in smokers than in non-smokers. When working in environments that include welding or soldering, for example, inhalation from workplace exposure to chemicals can be significant and result in severe chemical pneumonitis (Nordberg et al 2007). The body's cardiovascular, renal, gastrointestinal, neurological, reproductive, and respiratory systems are all affected by cadmium exposure, which is also very toxic and has been linked to cancer.

Cell growth, differentiation, and death are all impacted by cadmium. These processes interact with the DNA repair pathway, produce reactive oxygen species (ROS), and cause apoptosis (Rani A et al, 2014). At low concentrations, cadmium binds to the mitochondria and can prevent both cellular respiration and oxidative phosphorylation (Patrick L. 2003). In cell lines, it causes DNA strand breakage, sister chromatid exchanges, chromosomal abnormalities, and

DNA-protein crosslinks. Potentially, cadmium causes chromosomal deletions and mutations (Joseph P. 2009). Its harmful effects include reduced glutathione (GSH) depletion, protein binding of sulfhydryl groups, and increased generation of reactive oxygen species (ROS), including superoxide ion, hydrogen peroxide, and hydroxyl radicals.

Cadmium also inhibits the activity of antioxidant enzymes, such as catalase, manganese-superoxide dismutase, and copper/zinc-dismutase (Filipic M. 2012).

In this study, we examine some of the advantages of complementary and alternative medicines (CAMs), especially annona muricata, as prospective agents for treating cadmium-induced toxicity. Graviola (*A. muricata*) is a part of the Annonaceae family of plants (Mishra *et al,* 2013) and can be found in many parts of the tropical and subtropical parts of the world, including some parts of the Americas, Asia and Afria (Adewole Caxton-Martins, 2006). The tropical plant is characterized as an evergreen and flowering tree that can stand up to 8 metres tall and that produces edible fruits (da Silvia, 2009). It is commonly known as 'Soursop' or 'Graviola'. Because of the 'custard-like texture' of its edible fruit, *Annona muricata* has been grouped with the 'Custard-Apple' plants of the Annonaceae family (Janick 2008 & Grin 2008). *Annona muricata (A. muricata)* is a medicinal plant with various types of phytochemicals and other natural products which have been widely employed traditionally in the treatment of many medical conditions like arthritic pain, dysentery, fever, malaria, neuralgia, arthritis, diarrhea, parasites, rheumatism, skin rushes and worms, and it is also eaten to elevate mother's milk after childbirth, while its leaves are employed to treat cystitis, diabetes, headaches and insomnia (Mishra *et al* 2013, Adewole *et al* 2006 & De souza *et al* 2006. According to popular belief, the crushed seeds offer

anti-helmintic properties that work against both external and internal worms and parasites. A. muricata leaves are used as an ethnomedicine to treat tumors and cancer in tropical Africa, including Nigeria (Adewole 2009). The leaves, barks, and roots of A. muricata have also been shown to have anti-inflammatory, hypoglycemic, sedative, smooth muscle relaxant, hypotensive, and antispasmodic properties (Abernethy 2010). They also act as antioxidant and antimutagenic agents. By modulating various mechanisms, such as cellular proliferation, differentiation, apoptosis, angiogenesis, and metastasis, the majority of anticancerous natural products (Annona muricata) prevent the onset, development, and progression of cancer [Gupta S. C. et al. 2010]. A muricata-derived compounds have been linked in studies to a number of anticancer effects, including cytotoxicity, the induction of apoptosis [Kuete V. et al 2016; Zorofchian M S. et al 2015], necrosis [Torres M. P. et al 2012], and the inhibition of proliferation [Sun S. et al 2016] on a variety of cancer cell lines. The primary bioactive substances that have been isolated from various A. muricata parts are referred to as annonaceous acetogenins (AGEs). These are long-chain (C32 or C34) fatty acid derivatives that are produced through the polyketide pathway [Liaw C. C., et al 2016].

According to research conducted by Moghadamtousi S. Z. and colleagues (2015), many of these derivatives are selectively toxic to cancer cells, including cancer cell lines that are resistant to multiple drugs. The oxidative phosphorylation and ATP synthesis processes that are carried out by mitochondrial complex I are inhibited by anonaceous acetogenins, at least in part, causing cytotoxicity [McLaughlin J. L. et al 2008]. Mitochondrial complex I inhibitors have potential as a cancer therapy because cancer cells require more ATP than

normal cells do [Deep G. et al 2016]. No extensive scientific research has been done to prove the plant's hypolipidemic and antioxidant benefits, despite claims that all of the plant's morphological parts have therapeutic use in conventional medicine.

Figure 1. *Annona muricata* L.; (A) the appearance of the whole plant (B) leaves; (C) flowers and (D) fruits (E). Source: (Wikipedia)

1.1.1. AIMS AND OBJECTIVES

This study's goal was to look at the kidney toxicity of cadmium in wistar rats. The goals of this examination were to:

To determine annona muricata's impact on superoxide dismutase alterations brought on by cadmium (SOD)

To determine annona muricata's impact on Catalase's cadmium-induced alterations (CAT)

to look into how annona muricata affected renal alterations in male wistar rats caused by cadmium-induced changes in malondialdelyde (MDA).

1.2. LITERATURE REVIEW

1.2.1. PHYSICAL AND CHEMICAL PROPERTIES OF CADMIUM

The periodic table's Group 12 (IIb, or zinc group) metals include cadmium (Cd), an element that belongs to this group.

Aspects of the Element

48th atomic position

112.414 for the atom

321 °C or 610 °F is the melting point.

765 °C (1,409 °F) is the boiling point.

20 °C (68 °F) specific gravity of 8.65

O2 + 2 electron configuration [Kr]

The chemical element calcium (Cd), which has the atomic numbers 48 and 112, as well as the melting and boiling points of 321 and 765 degrees Celsius respectively, is soft, ductile, silvery white with a bluish tint, glossy, and electropositive. It has no flavor or smell and is extremely toxic. There are eight stable isotopes of cadmium: 106, 108, 110, 111, 112, 113, 114, and 116. The most prevalent isotopes are 114 and 112 Cadmium (Adriano 2001). A wide range of complex organic amines, sulfur complexes, chloro complexes, and chelates are also formed by cadmium. Carbonates, arsenates, phosphates, and ferrocyanide compounds all react with cd ions to generate soluble salts. It can be generated alongside the manufacturing of zinc in a variety of commercial forms.

1.2.2. USES OF CADMIUM

Cadmium is used as alloys in electroplating (auto industries) and in production of pigments (cadmium sulfate, cadmium selenide), likewise as stabilizers for polyviyl plastic, and in batteries (rechargeable Ni-Cd batteries) (Adriano 2001 & Cobb 2008).Commercially, Cd is utilized in television screens, lasers, batteries, paint pigments, cosmetics, galvanizing steel, as a barrier in nuclear fission, and was previously used with zinc to weld seals in lead water pipes. Approximately 600 metric tons are produced annually in the United States, and about 150 metric tons are imported (Rolla 2012).

1.2.3. ROUTES OF EXPOSURE OF CADMIUM

Human normally absorb cadmium into the body either by ingestion or inhalation, dermal

exposure (uptake through the skin) is generally not regarded to be of significance (Lauwerys 1988).

INGESTION OF CADMIUM

Foods from the earth contain a significant amount of cadmium that is ingested and enters the body. This refers to meat from animals that have consumed plants cultivated in dirt or meat from plants grown in soil. The type of food crop planted, the agricultural methods used, and the atmospheric deposition of cadmium onto exposed plant parts all affect how much cadmium is present in terrestrial foods. This cadmium intake will inevitably be impacted by soil cadmium levels, which are mostly derived from natural sources, phosphate fertilizers, and sewage sludge

Ingesting polluted food or water, such as those found in specific regions of Japan and China's rice or in crustaceans, organ meats, green vegetables, or rice, can expose one to cadmium exposure, which can have long-term health repercussions

Contamination of drugs and dietary supplements may also be a source of contamination (Abernethy *et al* 2010)

INHALATION OF CADMIUM

Cadmium inhalation is a far smaller contributor to total cadmium body burden except in the cases of smokers or some highly exposed workers of the past. Smokers routinely have greater blood and kidney Cd levels than non-smokers. In work environments, inhalation brought on by industrial exposure can be considerable. For example, welding or soldering, and can produce severe chemical pneumonitis (Nordberg 2007)

Today, the inhalation route is well controlled in the occupational setting, and is

well-controlled from point sources such as those which directly pertain to the non-ferrous, cadmium or cadmium products industries. On the other hand, because of the considerable volumes of waste gases produced, atmospheric air emissions from fossil fuel power plants, the iron and steel sector, and other significant industries where cadmium may be present as a low concentration contaminant, may be significant.

Cadmium intake from Cigarette Smoking - Smokers take in levels of cadmium from cigarettes that are similiar to those from food, ranging from 1 to 3 micrograms each day. It has been reported that one cigarette contains about 1-2 microgram of cadmium and that about 10% of the cadmium content is inhaled when the cigarette is smoked (WHO 1992). However, in general, smoking cigarettes is a habit that can more than double the average person's daily cadmium intake. Cigarette construction, the use of filters, and variations in the cadmium content of tobaccos could decrease cadmium exposure by this route. Smokers who are additionally exposed to cadmium at work may consume considerably more cadmium overall.

Consumption of cadmium from occupational exposure In some workplaces, very high cadmium air exposure levels, sometimes as high as 1 mg/mg, were measured up to the 1960s. Since then, workplace exposures and limits have significantly dropped, and the majority of occupational exposure requirements are now in the 2 to 50 g/m3 range. The result has been that occupational exposures today are generally below 5 $\mu g/m^3$, and most cadmium workers are exposed at levels which are considered to be safe (ATSDR 1997).

Due to human activities like the burning of garbage and the usage of fossil fuels, cadmium is present in the environment in significant amounts. Sludge from sewage systems that has leaked into agricultural soil may contain cadmium compounds that have been absorbed by

plants and may be transferred to humans through various organ systems. Cigarette smoke is another another significant source of cadmium exposure.When cadmium was measured in smokers' blood samples, it showed that they had 4-5 times Cd levels in blood higher than the non-smokers (Liu *et al* 2009).

1.2.4. METABOLISM AND TOXICOKINETICS

1.2.4.1. ABSORPTION OF CADMIUM

The route of exposure seems to be the main determinant of how much cadmium is absorbed. After exposure, various variables, including age, gender, smoking, and nutritional status, determine how much cadmium is absorbed. Cadmium's biological load grows with age as a cumulative poison. Blood levels of cadmium have been found to be greater in women than in men. Typically women, with lower iron status, are believed to be at risk for greater absorption of cadmium after oral exposure (Olsson *et al* 2002).

INGESTION: Most orally ingested cadmium passes through the gastrointestinal tract unchanged as normal individuals absorb only about 6% of ingested cadmium, but up to 9% may be absorbed in those with iron deficiency (ATSDR 1999). Also, cadmium in water is more easily absorbed than cadmium in food (5% in water versus 2.5% in food) (IRIS 2006). Increased levels of zinc or chromium in the diet reduce cadmium uptake.

The type of cadmium compound, dose, frequency of administration, age or stage of development, pregnancy and breastfeeding, the presence or absence of medications, nutritional status, and interactions with different nutrients are some of the factors that determine how much cadmium is absorbed after consumption.

Studies in experimental animals have shown that 0.5-8% of cadmium nitrate or chloride is

absorbed after a single exposure (Friberg *et al.,* 1974). In humans given radioactive cadmium, the average amount absorbed is 5%(Flanagan *et al.*, 1978).

INHALATION: Once in the lungs, from 10% to 50% of an inhaled dose is absorbed, depending on particle size, solubility of the specific cadmium compound inhaled, and duration of exposure (Jarup 2002). Absorption is greatest for small (less than 0.1 m) and water-soluble particles and least for large (greater than 10 micrometers [m]) and water-insoluble particles. A high proportion of cadmium in cigarette smoke is absorbed because the cadmium particles found in that type of smoke are very small (ATSDR 1999).

DERMAL: Absorption through the skin is not a significant route of cadmium entry; only about 0.5% of cadmium is absorbed by the skin (ATSDR 1999).

1.2.4.2. DISTRIBUTION OF CADMIUM

Once absorbed, cadmium is transported in the blood, mainly in erythrocytes, and is bound intracellularly to protein fractions of low and high relative molecular mass (Nordberg, 1972). Like metallothionein, the fraction with a low relative molecular mass is present. Metallothioneins are low relative molecular mass metal-binding proteins with a high cysteine content that have a specific affinity for cadmium and can affect the toxicity of the metal. Cadmium is transported in large part by plasma metallothionein. According to Elinder and Nordberg (1985), it can have up to 11% of cadmium by weight bonded to sulfhydryl groups and is widely present in the liver, especially after cadmium exposure. Other tissues, such the kidneys, have metallothionein in various degrees, and its concentration is correlated with that of cadmium in these tissues. The low relative molecular mass of free metallothionein in plasma allows it to filter through the glomeruli and subsequently be reabsorbed in the

proximal tubules, which in turn results in selective accumulation of cadmium in the renal cortex (Nordberg, 1972). Transport of cadmium bound to metallothionein from blood to renal tubular cells is rapid and virtually complete, while free cadmium is not taken up by the kidneys to a similar extent (Johnson & Foulkes, 1980).

1.2.4.3. EXCRETION OF CADMIUM

Most of the cadmium that has already been ingested is excreted from the body in urine. Because cadmium is still securely attached to metallothionein, which is almost entirely reabsorbed in the renal tubules, the rate of excretion is likely modest. Cadmium can accumulate significantly in the body since excretion is slow. Blood cadmium levels indicate recent exposure, whereas urine cadmium levels more accurately represent overall body load. The excretion rate increases significantly when renal impairment from cadmium exposure develops, and urine cadmium levels no longer represent body burden.

1.2.4.4. ACCUMULATION OF CADMIUM

The total cadmium body burden at birth is non-detectable (CDC 2005). It gradually increases with age to about 9.5 mg to 50 mg (ATSDR 1999). The kidneys and liver together contain about 50% of the body's accumulation of cadmium (HSDB 2006).

1.2.4.5. CADMIUM HALF-LIFE

The biologic half-life of cadmium in the kidney is estimated to be between 6 to 38 years; the half life of cadmium in the liver is between 4 and 19 years (ATSDR 1999). These lengthy half-lives are an indication that cadmium removal in humans is inefficient. In humans, cadmium has no known biological use. It appears that bioaccumulation is a side effect of

rising industrialization. Any significant buildup within the body needs to be considered poisonous.

1.2.4.6. MECHANISM OF TOXICITY:

Cell growth, differentiation, and death are all impacted by cadmium. These activities interact with DNA repair mechanism, the generation of reaction oxygen species (ROS) and the induction of apoptosis (Abernethy et al 2011). Cadmium binds to the mitochondria and can inhibit both cellular respiration and oxidative phosphorylation at low concentration (Munisamy 2013).

In cell lines, it causes DNA strand breaks, sister chromatid exchanges, chromosomal aberrations, and DNA-protein crosslinks. Potentially, cadmium causes chromosomal deletions and mutations (Joseph 2009). Its harmful effects include reduced glutathione (GSH) depletion, protein binding of sulfhydryl groups, and increased generation of reactive oxygen species (ROS) like superoxide ion, hydrogen peroxide, and hydroxyl radicals. The activity of antioxidant enzymes like catalase, manganese-superoxide dismutase, and copper/zinc-dismutase is also inhibited by cadmium (Filipic 2012). A protein that concentrates zinc and has 33% cysteine in it is called metallothionein. Additionally, metallicothionein has the ability to scavenge free radicals. It neutralizes superoxide and hydroxyl radicals (Liu et al 2009). Cells that have metallothioneins are typically resistant to the toxicity of cadmium. On the other hand, the cells that cannot synthesize metallothioneins are sensitive to cadmium intoxication (Han *et al* 2009). Cadmium can modulate the cellular level of Ca2+ and the activities of caspases and nitrogen - activated protein kinases (MRPKs) in the cells, in which these processes cause apoptosis indirectly (Brahma *et al* 2012).

Oxidative stress and ROS production can be induced by cadmium. The role of cadmium in organ toxicity, carcinogenicity, and apoptotic cell death may be expressed by this mechanism.

1.2.4.7. HEALTH MANIFESTATION OF CADMIUM

The information supplied below show how various cadmium chemical types have various clinical presentations and harmful effects.

CADMIUM TOXICITY RENEWAL DAMAGE: Although cadmium is primarily found in the kidney and liver, it can also be present in other tissues like bone and the placenta. Renal impairment has reportedly been linked to cadmium exposure in the workplace and environment (Jarup 2002). Early kidney injury, proteinuria, calcium loss, and tubular lesions can all be symptoms of cadmium exposure. Urine analysis may help to prove early signs of renal damage (Nordberg 2007). Generally, the glomerular filtration rate (GFR) and reserve filtration capacity will be diminished, and severe cadmium toxicity may induce nephrotoxicity with complications such as; glucosuria, aminoaciduria, hyperphosphaturia, hypercalciuria, polyuria and decreased buffering capacity (Gonick 2008). Calcium, amino acids, enzymes, and a rise in proteins were lost as a result of cellular damage and functional integrity in the proximal tubules. On the other hand, tubular proteinuria results from a reduced tubular reabsorption of a few low molecular weight proteins. The most common proteins in urine are beta 2- microglobulin, retinol-binding protein and alpha 1-microglobulin (Bernard 2004).

a. **Cadmium and reproductive system :** Several previous studies found that cadmium has the potential to affect reproduction and development in several mammalian species, and recent studies have also confirmed these findings (Thompson 2008). Compared to

animal studies, it is claimed that cadmium decreases density, volume and number of sperms, and increases immature sperm forms (Pizent 2021). A deficiency in spermatogenesis, sperm quality, and accessory gland secretory processes follow these issues. Besides, it deceases libido, fertility, and serum testosterone level (Saboblic 2006). In female reproductive system, the function of ovary and development of oocytes may be inhibited. Steroidogenesis is reduced under Cd toxicity and ovarian hemorrhage and necrosis can co-occur (Thompson 2008). It has been reported that the rate of spontaneous abortion and time of pregnancy are increased and rate of live births decreased (Pizent 2012).

1.2.5. EFFECT OF CADIUM ON KIDNEY

Due to its capacity to reabsorb and concentrate divalent ions and metals, the kidney is a target organ in the toxicology of heavy metals. The kind, dose, and length of exposure all affect how severely the kidneys are affected. In terms of the toxicity mechanism, acute kidney injury (AKI) caused by heavy metals differs from chronic kidney disease (CKD). For this reason, clinical features and a therapeutic approach are also peculiar (Rolla 2012).

Both protein-bound (non-diffusible) and complex/ionized (diffusible) forms of heavy metals can be found in plasma. Metals are rapidly removed from the circulation and deposited in a variety of tissues.

Both the bound form and the free form may be present in the luminal fluid in the early proximal tubule. The protein-bound, inactive form of chronic poisoning is conjugated with metallothionein and glutathione, which the liver and kidney then release into the blood. These compounds are subsequently reabsorbed through an endocytotic process in segment S1 of the

proximal tubule and can lead to chronic inflammation, fibrosis and renal failure (Sabobolic 2006 & Barbier et al 2005). The apical membrane of the first zone of the proximal tubule is the primary location of heavy metal reabsorption in acute poisoning, but the Henle loop and terminal segments can also take part. AKI is induced by the ionized form that produces direct cellular toxicity, cellular membrane disruption and uncoupling of the mitochondrial respiration pathway, with the release of numerous apoptosis signals such as cytokines and reactive oxygen species (Barbier *et al* 2005).

1.2.5.1. EXPOSURE AND TOXICITY SYMPTOMS

Toxic heavy metal exposure is typically divided into three categories: acute (1–14 days), intermediate (15–354 days), and chronic (>365 days). An abrupt or inadvertent exposure to or consumption of a high dosage of heavy metals results in acute poisoning. In contrast to chronic intoxication, which is typically uneventful, acute toxicity symptoms are severe, rapidly onset, and associated with exposure or ingestion. They include headache, vomiting, dyspnea, abdominal pain, and sweating. The medical past and particular testing can assist in identifying the specific agent.

1.2.5.2. EFFECT OF CADMIUM ON ANTIOXIDANT ENZYMES

Oxidative stress has been proposed as the most important mechanism underlying the toxic effects of Cd in many organs, including the brain (Figueiredo-pereira 1998 & Lopez *et al* 2005). A change in the equilibrium between oxidant and antioxidant agents in cells is how it is described. Several studies demonstrated that Cd itself is unable to generate free radicals directly, but it is responsible for indirect generation of reactive oxygen species (ROS), including superoxide radical and hydroxyl radical (Yang *et al* 2008*).* Antioxidant enzymes,

including superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidases (GPx) are involved in the defence against metal oxidative stress (Cuypers *et al* 2010). In the central nervous system, the levels of antioxidant enzymes were shown to be increased in astrocytes and cortical neurons treated with Cd (Yan et al 2012). Similarity, Cd administration to rats was demonstrated to lead to an increase in the activities of CAT, SOD, and GPx (Gupta *et al* 1993 & Antonio *et al* 2003). However, several other studies demonstrated that the activities of antioxidant enzymes decrease in rat brains treated with Cd (Shukla *et al* 1996,Nemmiches 2007 & Zhang *et al* 2009), indicating that the mechanisms underlying Cd-induced oxidative stress, cellular response, and the putative roles of antioxidant enzymes remain controversial (Cuypers *et al* 2010).

Long-term exposure to Cd increases lipid peroxidation and causes inhibition of SOD activity indicating oxidative damage in liver, kidney and testes (Patra *et al* 1999). Lipid peroxidation is a common and early sign of Cd exposure and has been linked to a variety of harmful effects Cd has on biological systems. The increase in lipid peroxidation caused by Cd toxicity has been linked to changes in the antioxidant defense system, which normally defends against free radical toxicity with enzymes like glutathione peroxidase (GPx), glutathione-S-transferase, superoxide dismutase (SOD), and catalase (CAT).

CHAPTER TWO

MATERIALS AND METHODOLOGY

2.1 MATERIALS

2.1.1. LIST OF EQUIPMENT/APPARATUS

The materials used in this study include; plastic cages with iron netting, saw dust (litter), animal feed (growers mash), laboratory coat and gloves, cotton wool, feeding trough, wire net cage, face masks, filter paper, test tubes, Centrifuge [Model 80-2 Harris England], Water bath [TT42D Multipurpose use, Techmel and Techmel, USA), UV/Visible spectrophotometer [Spectrum lab 22PC, England], Analytical weighing balance [Homgeek TL-Series, China], Hand held PH meter, slide cover, slide, Glass wares (pyrex, England), Dessicator(Pyrex, England), Needles and Syringes(First Care Plus Ventures, Nigeria), Plain bottles, Heparinized bottles, Wooden Cages, Glucometer,Micro Pipette, Refrigerator [Haier Thermocool HTF -66H], Dissecting set [Gold cross, England], chloroform chamber.

2.1.2 LIST OF CHEMICALS AND REAGENTS

Distilled water, Chloroform((Spectrum chemical Mfg.Corp,US), Diluted Sodium Hydroxide (NaOH), Phosphate buffer solution PH 7(0.05M), Methylated Spirit, 0.9% Normal saline, 1% Tween 80 (Haihang Industry Co. Ltd, China), formalin, Superoxide dismutase (SOD) chromogen solution, Superoxide dismutase (SOD) buffer, Hydrogen Peroxide (H_20_2), Potassium Permanganate ($KMnO_4$), sulfuric acid, Trichloroacetic acid (TCA), Hydrochloric acid, Thiobarbituric acid (TBA), SOD assay kit, (CAT) catalase kit.

2.1.3 CLASSIFICATION AND COLLECTION OF PLANTS

The fresh *Annona Muricata* leaves were gotten from within University of Benin and its environs here in Benin City, Edo State, Nigeria.The leaves were identified by Dr. A. Akinnibosun in the Department of Botany, Faculty of Life Sciences, University of Benin, Edo State, Nigeria. A voucher specimen was deposited at the Department (UBHa 0205).

2.2 METHODS

2.2.1. PREPARATION OF PLANT SAMPLE

After the collection of *Annona Muricata* leaves, they were sorted in order to separate the leaves from the stems, the leaves were rinsed with running water. They were then air dried at room temperature for 2 weeks. After drying, it was pulverized into fine powder with the use of a mechanical grinder in the Department of Pharmacognosy, Faculty of Pharmacy, University of Benin, Benin city, Edo state, Nigeria. Afterwards it was weighed and stored in an air tight container until used.

2.2.2. PREPARATION OF AQUEOUS-METHANOL EXTRACT OF ANONNA MURICATA LEAVES

The pulverized *Annona muricata* 1400 g was soaked in 14000 mL (14 L) of aqueous-methanol in the ratio (70:30%) in a glass jar, stirring periodically for 72 hours.The resulting mixture was then sieved with a cheese cloth, the filtrate was subjected to rotary evaporation and subsequently freeze dried using a freeze drier.

The first crude extract gotten was 300 g, and the second was 179 g. Hence, the extraction gave a total yield of 479 g of crude extract. The crude extract was stored at 4°c until use.

2.2.3. PREPARATION OF REAGENTS

2.2.3.1 PHOSPHATE BUFFER (0.05, PH 7.4)

Disodium hydrogen phosphate (4.36 g) and potassium dihydrogen phosphate (2.72 g) were dissolved in 500 mL of distilled water. The pH was adjusted to 7. 4 using 0.1M HCl. Then, the volume was made up to 1000 mL with distilled water.

2.2.3.2 1% TWEEN 80

Tween 80 (1mL) was pipetted into a volumetric flask and made up to 100 mL with diluted water.

2.2.3.3 NORMAL SALINE SOLUTION (0.9%)

Sodium chloride (0.9 g) was weighed into a beaker, dissolved in 200 mL of distilled water and made up to 1000 mL mark in a measuring cylinder with distilled water.

2.3 EXPERIMENTAL DESIGN/TREATMENT OF ANIMALS

Forty (40) male wistar albino rats weighing between 240 ± 50 g was used for this experiment and were obtained from the Department of Pharmacy, University of Benin, Edo state, Nigeria. The animals were acclimatized 7 days prior to the experimental start date. During acclimatization, the animals were housed in well ventilated cages (wooden sides,wire mesh bottom and wire mesh top) with free access to diet (standard rat chow) and water *ad libitum*. This was maintained in the animal care facility throughout the duration of the experiment. The animals were kept under controlled environment (at room temperature of about 20-25^{o}C and 12 hours light/dark cycle). This was maintained in the animal care facility throughout the duration of this experiment. The animals were kept under controlled environment (at room temperature of about 20-25^{o}C and 12 hours light/dark cycle).

After one (1) week of acclimatization, the animals were randomly divided into two five(5) groups of eight (8) rats each. Group 1 served as the control group while groups 2, 3 and 4, 5 served as the test groups.

Group 1 (control): The rats in this group were given extracts of 1% Tween 80 according to their body weights.

Group 2 (CD only): The rats in group 2 were given 3.5 mg/kg body weight of Cadmium only.

Group 3 (CDE_{100}): The rats in group 3 were given 3.5 mg/kg body weight of cadmium and 100 mg/kg body weight of aqueous-methanol extract of *A. muricata*.

Group 4 (CDE_{300}): The rats in group 4 were given 3.5 mg/kg body weight of cadmium and 300 mg/kg body weight of aqueous-methanol extract of *A. muricata*.

Group 5 (CDE_{500}): The rats in group 2 were given 3.5 mg/kg body weight of cadmium and 500 mg/kg body weight of aqueous-methanol extract of *A. muricata*.

The rats in group 1 (Control) were administered normal saline subcutaneously through the use of a syringe, while the rats in group 2,3,4,5 were administered their toxicant (Cadmium) subcutaneously through the use of a syringe. After a single administration of cadmium, rats in group 3, 4 & 5 (all given extract) were also administered their extract orally through the use of gavage, the administration was carried out by oral gavage. Cadmium was dissolved in normal saline while *A.muricata* extract was dissolved in 1% tween 80 (Sigma-Aldrich). The treatment duration was for 28 days (4 weeks) and physical observation was carried out on all animals twice everyday. All experimental animals were fed with standard rat chow and water *ad libitum*. Alterations in the skin and fur, mucous membranes, respiration, and animal

behaviour were monitored.

2.3.1. BODY WEIGHT MEASUREMENT

The body weight of experimental animals were taken at the beginning of the study. The recorded weight was used to calculate the amount of cadmium and *A.muricata* leave extract administered to the animals. Animals were weighed once every week and the amount of *A.muricata* leave extract, adjusted accordingly.

2.4. ANIMAL SACRIFICE/COLLECTION OF SAMPLE

On the 29th day of experiment, the final weights of the animals were measured, after which they were anaesthetized in chloroform saturated chamber, and then sacrificed via cervical dislocation. prior to the day of the sacrific, the rats were subjected to an overnight fast. After sacrificing, blood sample was taken from the inferior vena cava and were centrifuged to obtain plasma for laboratory analysis, and then the animals were rapidly dissected for further analysis. The vital organ, kidney were taken out and weighed so as to assess the effects of the toxicant.

2.4.1. PREPARATION OF PLASMA AND TISSUE SAMPLES

Blood samples were collected by cardiac puncture by means of a 5 mL hypodermic syringe and placed in heparinized bottles (to prevent coagulation). The blood was centrifuged at 3000 rpm for 15 min. The plasma sample were collected, which was placed in a plain bottle and then stored at temperature of -4°C after collection until required. The kidney was excised, weighed and placed in 10% formalin for histopathogical analysis.

2.4.2. PREPARATION OF KIDNEY HOMOGENATE

The kidney were weighed and homogenized in a pre-chilled mortar by grinding using pestle

and 5 mL of 0.9% normal saline. The homogenate was centrifuged at 3000rpm for 15 minutes and the supernatant was transferred into labeled containers and stored at -4°C until required for biochemical analysis.

2.5. BIOCHEMICAL ANALYSIS

Biochemical analysis was carried out to determine the activities of antioxidant enzymes such superoxide dismutase (SOD), Catalase (CAT), Malondialdelyde (MDA) was evaluated to determine the enzymatic activities of the kidney of the control and experimental groups. The activity of the enzymes were measured using commercially available kits according to the manufacturer's instruction.

2.5.1. SUPEROXIDE DISMUTASE (SOD) (E.C 1.15.1)

Superoxide is produced as a by product of oxygen metabolism and if not regulated causes many types of cell damage (hayyan *et al.* 2016). One of the most antioxidant enzymes is superoxide dismutase, which catalyzes the dismutation of the superoxide anion (O2) into hydrogen peroxide and molecular oxygen.

Principle

Pyrogallol's autoxidation can be stopped by the enzyme superoxide dismutase. In the presence of EDTA at pH 8.2, 50% of pyrogallol undergoes autoxidation. The pyrogallol autoxidation by O2 and the radical's dismutation by SOD compete for electrons in this method's basic working principle.

Calculation

Determine the change in absorbance of samples and blank using the following equation :

Change of absorbance of sample (Δs)= Absorbance of sample (1 min) - Absorbance of sample (initial)

Change in absorbance of blank (ΔC) = Absorbance of blank(1 min) - Absorbance (initial)

% Inhibition of pyrogallol autoxidation = × 100%

SOD activity in (U/mL) =

2.5.2. CATALASE (CAT) (E.C. 1.11.1.6)

The estimation of CAT activities in the kidney homogenate was done. A typical enzyme called catalase, which is present in practically all living things exposed to oxygen, catalyzes the breakdown of hydrogen peroxide into water and oxygen. Catalase protects the cells from oxidative damage by reactive oxygen specie.

Principle

When H_2O_2 is generated in large quantity, Catalase is used for its removal. Estimation of Catalase was based on the method of (Cohen *et al*. 1970).Catalase was determined by measuring the rate of decomposition or disappearance of hydrogen peroxide after the addition of the material containing the enzyme. It catalyzes the following reaction:

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

The amount of hydrogen peroxide that breaks down is directly correlated with the enzyme content in the sample. After reacting excess potassium tetraoxomanganate (VII) with hydrogen peroxide, the product is determined by spectrophotometrically analyzing the remaining KMnO4.

Procedure

Sample (0.5 mL) was added to ice cold test tube and 0.5 mL of distilled water was added into another ice cold tube as blank. The reaction was initiated by sequentially adding at fixed intervals 5 mL of cold 30 mM hydrogen peroxide H_2O_2. They were thoroughly mixed by inversion. After exactly 3 minutes, the reaction was stopped sequentially at the same fixed interval by rapidly adding 1 mL of 6 M H_2SO_4 and mixing quickly by inversion. The test and blank tubes were taken one at a time and 7 mL of 0.01M $KMnO_4$ was added and were mixed twice by inversion. The absorbance was read at 480 nm within 30 to 60 seconds of mixing. The photometric standard was prepared by adding 7 mL of 0.01M potassium permanganate $KMnO_4$ to a mixture of 5.5 mL phosphate buffer (pH 7.4) and 1 mL of 6M sulfuric acid H_2SO_4 solution. The spectrophotometer was zeroed with the distilled water.

Calculation:

$K = S_3 \times t$

Where; K = first order rate constant

S_0 = substrate concentration at zero times

S_3 = substrate concentration at 3 minutes

t = time interval(allowed) over which the reaction was measured.

To obtain S_0 (expressed in absorbance unit), subtract absorbance of the reaction system of the blank, S_0 from spectrophotometric standard S_t

Hence, $S_0 = S_t - S_3$

To obtain S_3, substrate absorbance of the reaction sample S_A from S_t

Hence, $S_3 = S_t - S_A$

2.5.3. MALONDIALDEHYDE (MDA)

The lipid peroxidation level of the kidney of cadmium-induced toxicity was assay for by the level of Malondialdehyde (MDA) concentration. Malondialdehyde (MDA) assay is used to estimate the level of lipid peroxidation in tissue, which is cause by the activities of toxic substances that effect membrane integrity of the organ.

Principle

Malondialdehyde formed from the degradation of polyunsaturated lipids by reactive oxygen species (pryor and Stanley, 1975), it is a reactive aldehyde which is one of the many reactive electrophile species that cause toxic stress in cells and form covalent protein addicts referred to as advanced lipoxidation end-products in analogy to advanced glycation end products (Farmer and Davoine, 2007). The production of this aldehyde is used as a biomarker to measure the level of oxidative stress in an organism (Moore and Roberts, 1998), Malondialdehyde is reactive and potentially mutagenic, (Hartman, 1983). When heated in acid conditions, it forms a pink coloured product which has a maximum absorbance at 535 nm.

Procedure

Malondialdehyde (MDA) level was estimated by the method of (Burege and Aust 1978).The aliquot of the kidney homogenate (1 ml) was add to 2 mL of (1:1:1 v/v/v) TCA (trichloroacetic acid), TBA (Thiobarbituric acid) and HCl reagent (hydrochloric acid) (Thiobarbituric acid 0.375% w/v, Trichloroacetic acid 15% w/v, and hydrochloric acid 0.25M) and mix thoroughly by swirling. After cooling down after being heated in a boiling water bath

for 15 minutes, the solution was centrifuged at 1000 g for 10 minutes to remove the flocculent precipitate. The absorbance of the clear supernatant was measured against a standard or reference blank at 535 nm.

The malondialdehyde (MDA) concentration was calculated by :

× concentration of standard(0.184mg/dl)

This calculation was now converted to mg/g tissue using the different individual weights of the tissues along side the volume of homogenate.

2.2.8 Analysis of data

The mean and standard deviation (Mean SD) were used to express the study's findings. A statistical analysis was carried out utilizing the Student's t-test to determine the significance of the differences between the control group and experimental group. data analysis

The mean and standard deviation were used to present the results of the investigation (Mean SD). It was evaluated whether the variations between the experimental group and control group were statistically significant using Student's t-test statistics. P 0.05 was used as the significant level.

CHAPTER THREE

RESULT

EFFECT OF *ANNONA MURICATA* EXTRACT ON CADMIUM (CD) INDUCED CHANGES IN GRAVIMETRIC PARAMETERS

The effect of Cadmium and *annona muricata* on change in weight and relative organ body weight are presented in (Table 3.1). The result showed weight loss for experimental group administered with *Annona muricata.* The change in weight of experimental groups were significantly ($p < 0.05$) lower than the change in body of control group. The weight of the kidney in experimental groups was significantly ($p < 0.05$) lower than the weight of the kidney in control group.

Table 3.1: Effect of Annona muricata extract on Cadmium Induced changes in gravimetric.

Treatment	Mean of initial weight (g)	Mean final weight (g)	Change in weight (g)	Organ body weight ratio
Control	256.49 ± 21.59	277.19 ± 21.25	20.70	$4.54 \times 10^{-3} \pm 3.36 \times 10^{-4}$
CD only	228.49 ± 18.88	208.89 ± 25.49[a]	-19.60[a]	$5.00 \times 10^{-3} \pm 6.67 \times 10^{-4}$ [a]
CDE_{100mg}	220.47 ± 17.09	199.60 ± 23.16[a]	-20.87[a]	$5.38 \times 10^{-3} \pm 8.95 \times 10^{-4}$ [a]
CDE_{300mg}	230.88 ± 20.14	226.39 ± 10.66[a]	-4.49[a]	$5.17 \times 10^{-3} \pm 6.50 \times 10^{-4}$ [a]
$CDE_{500mg.}$	215.35 ± 46.98	203.84 ± 29.75[a]	-11.51[a]	$5.03 \times 10^{-3} \pm 4.47 \times 10^{-4}$ [a]

Values are means ± SD

Values are statistically significant at p value ≤ 0.05

Values with 'a' superscripts on the same column differ significantly from control at ($p < 0.05$).

3.2 EFFECT OF CADMIUM AND *ANNONA MURICATA* ON SUPEROXIDE DISMUTASE, CATALASE AND MALONDIALDEHYDE

The effect of Cadmium and *annona muricata* on superoxide dismutase (SOD), Catalase (CAT) and Malondialdehyde (MDA) level is presented in Table 3.2. SOD, CAT, MDA levels significantly ($p < 0.05$) increased when compared to the control but not significantly ($p < 0.05$).

Table 3.2: Effect of Cadmium and *Annona muricata* on Superoxide dismutase, Catalase and Malondialdehyde.

TREATMENT	SOD	CAT	MDA
CONTROL	109.25 ± 9.41	7.33 ± 0.82.	$4.73 \times 10^{-3} \pm 2.66 \times 10^{-4}$
CD only	117.52 ± 2.27^{aa}	8.13 ± 1.42^{aa}	$5.94 \times 10^{-3} \pm 2.85 \times 10^{-4\ a}$
CDE_{100mg}	$125.09 \pm 5.82^{a.}$	8.65 ± 0.26	$7.89 \times 10^{-3} \pm 2.14 \times 10^{-4\ a}$
CDE_{300mg}	154.49 ± 27.59^{aa}	7.23 ± 0.38^{aa}	$7.29 \times 10^{-3} \pm 2.87 \times 10^{-4\ a}$
CDE_{500mg}	99.66 ± 22.77^{aa}	16.52 ± 0.14^{b}	$5.3 \times 10^{-3} \pm 1.18 \times 10^{-4}$

values are mean ± SD

Values with no superscript on the same column are statistically the same with control at ($p < 0.05$)

Values with 'a' superscript on the same column differ significantly from control at ($p < 0.05$)

Values with 'aa' superscript on the same column is not significantly different from control at ($p < 0.05$)

Values with 'b' superscript on the same column differ significantly ($p < 0.05$) from the DBP only

3.3 Histopathological Examination of Kidney Exposed to Cadmium

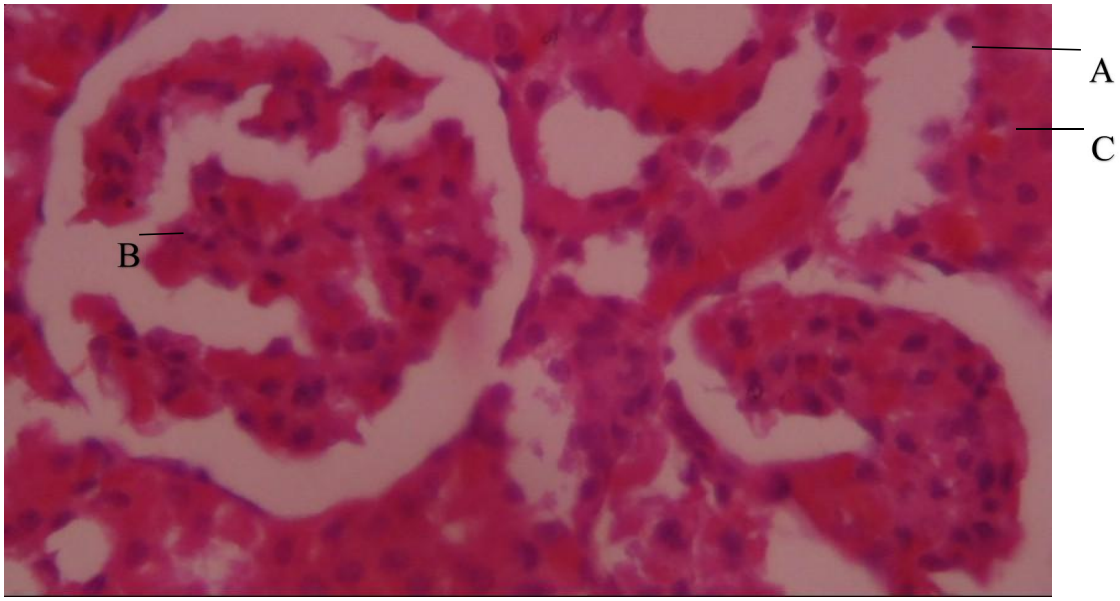

Plate 1: Rat kidney. Control. Composed of normal tissue architecture: A. tubules, B. glomeruli, C. interstitial space (H&E x 400)

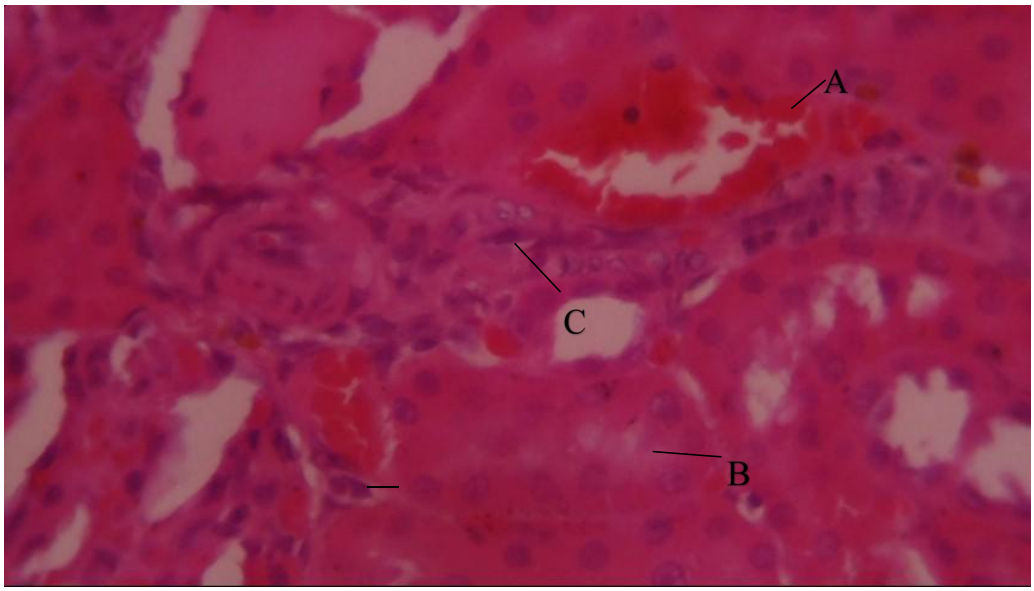

Plate 1a: Rat kidney given Cadmium showing: A. interstitial congestion, B. patchy tubular necrosis and C. vascular stenosis (H&E x 400)

In plate 1, the kidney cells showed normal tubules, glomeruli, interstitial space with no interstitial congestion, patchy tubular necrosis and vascular stenosis. In contrast, histopathological section showed in plate 1a shows interstitial congestion, patchy tubular necrosis and vascular stenosis indicating the effect of cadmium on the renal cells. (H&E x

400)

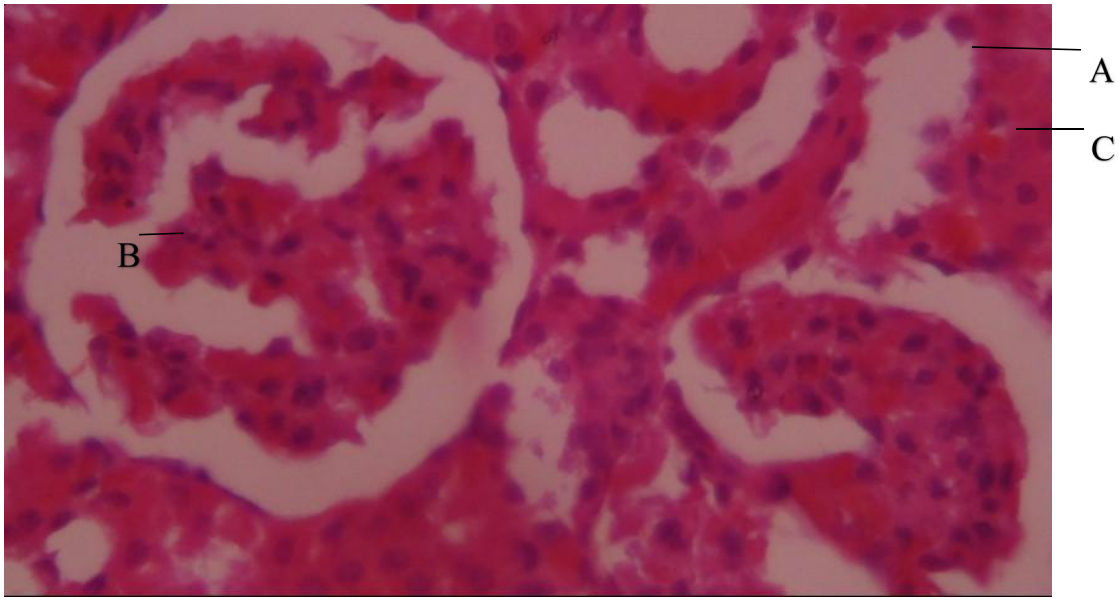

Plate 2: Rat kidney. Control. Composed of normal tissue architecture: A. tubules, B. glomeruli, C. interstitial space (H&E x 400)

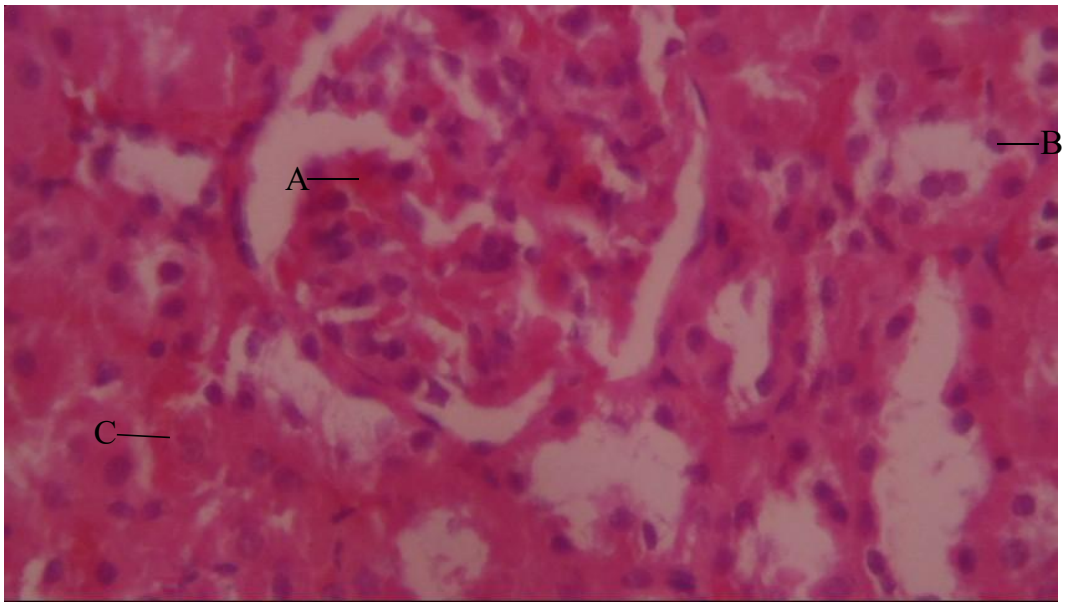

Plate 2a.: Rat kidney given cadmium + 100mg/kg extract showing normal architecture A. glomeruli, B. tubules and C. interstitial congestion (H&E x 400)

In plate 2, the kidney cells showed normal tubules, glomeruli, interstitial space with no interstitial congestion. In contrast, histopathological section shown in plate 2a shows glomeruli, tubules and interstitial congestion indicating the effect of cadmium on renal cells

treated with 100mg/kg extract. (H&E x 400)

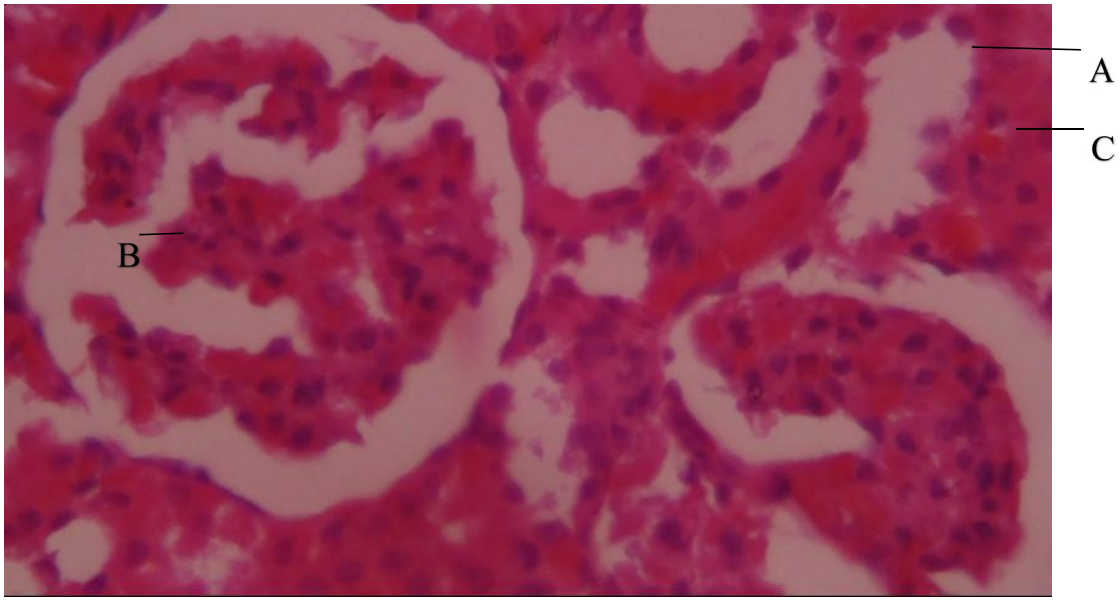

Plate 3: Rat kidney. Control. Composed of normal tissue architecture: A. tubules, B. glomeruli, C. interstitial space (H&E x 400)

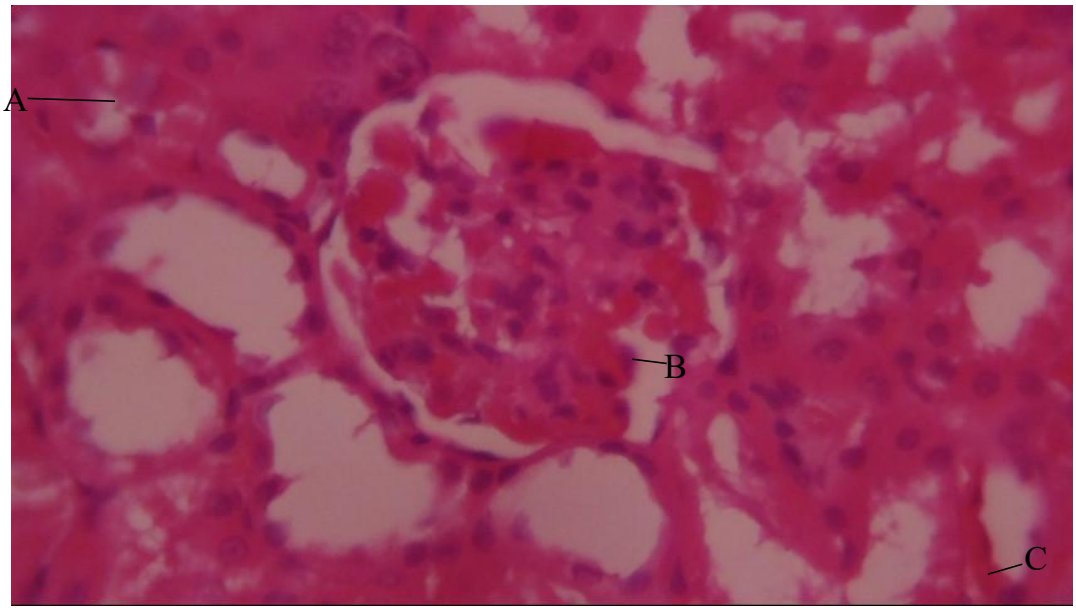

Plate 3a: Rat kidney given Cadmium + 300mg/kg extract showing normal architecture: A. tubules, B. glomeruli and C. interstitial space (H&E X 400)

In plate 3, the kidney cells showed normal tubules, glomeruli, interstitial space. Plate 3a; given Cadmium + 300mg/kg shows tubules, glomeruli and interstitial space indicating treated effect of the extract on the renal cell. (H&E X 400)

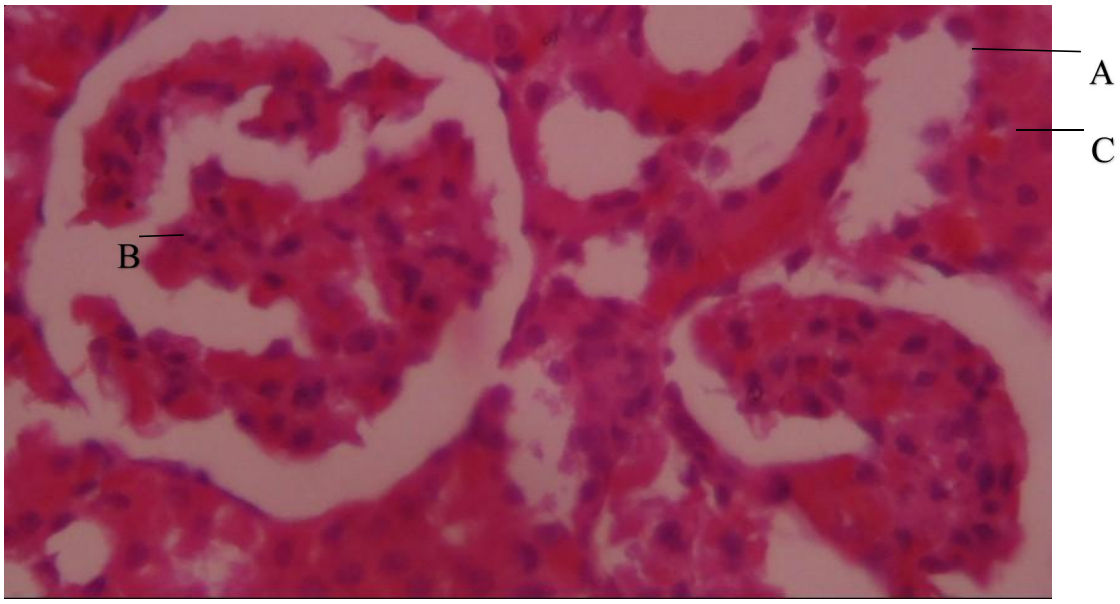

Plate 4: Rat kidney. Control. Composed of normal tissue architecture: A. tubules, B. glomeruli, C. interstitial space (H&E x 400)

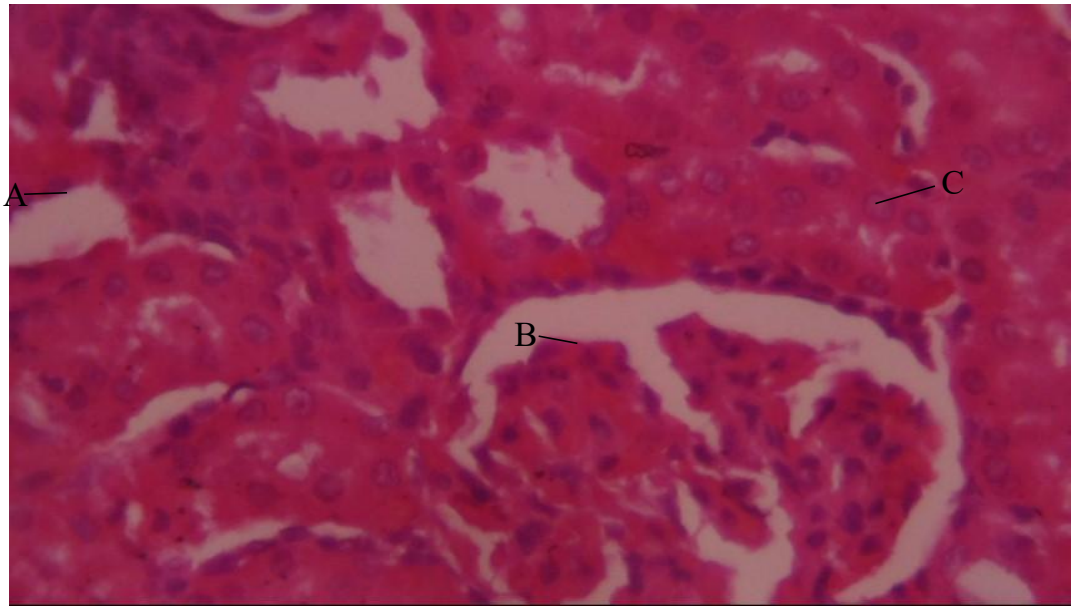

Plate 4a: Rat kidney given Cadmium + 500mg/kg extract showing: A. normal tubules, B. glomeruli, C. tubular necrosis (H&E x 100)

In plate 4, the kidney cells showed normal: tubules, glomeruli, interstitial space with no tubular necrosis. In contrast, histological sections shown in plate 1a shows: normal tubules, glomeruli, tubular necrosis indicating the effect cadmium had on renal cells treated with 500mg/kg extract. (H&E x 100)

CHAPTER 4

DISCUSSION

DISCUSSION

This present study indicates that effects in cadmium had on renal cells of rats exposed to the toxicant after 21 days and 28 days , cadmium cause acute toxicity in several specie including humans, it primarily affects tissues.

The change in body weight is used as a marker to check the animal health status,a reduction in body weight is an indication of worsening of experimental rats during the experimental period.

At the end of the 21 days experiment, results showed that there was a decrease in change in body weight as well as organ body weight of the group treated with cadmium. However, the difference in their decrease was significant.

Cadmium exposure on kidney cells is well established on the elevated antioxidant levels and selected plasma metabolites indicating the cellular leakage and loss of functional renal membrane architecture .

In this study, antioxidant enzyme activity of Superoxide dismutase (SOD), Catalase (CAT), Malonialdehyde (MDA) were evaluated to determine the effects cadmium had on rat kidney after 28 days of exposure to the toxicant both SOD and CAT have been thought to be the biological marker of antioxidant stress, the antioxidant enzymes are the first line of cellular defense against oxidative injury. While SOD and CAT mutually function in Reactive oxygen specie (ROS) and Reactive nitrogen species (RNS) elimination.

Malondialdehyde (MDA) is an indicator of lipid peroxidation and is used as a marker od

oxidative stress – induced kidney impairment in cadmium exposed rats ,it is a stable end product of lipid peroxidation arising from oxidative stress and from the results it was observed that after 28 days of exposure there was elevated SOD and CAT dat has difference that was not significant and decreased MDA levels with difference that was not significant as well when compared with control , the elevated SOD and CAT levels showed elevated oxidative stress. Therefore this further confirmed in the histopathological examination which showed histological alteration in kidney section of the rat indicating interstitial congestion, patchy tubular necrosis and vascular stenosis.

CONCLUSION

The administration of ameliorating at 100mg/kg extract showed a normal architecture and interstitial congestion of the kidney. The administration of ameliorating at 300mg/kg extract showed a normal architecture of the kidney, more effect. The administration of ameliorating at 300mg/kg extract showed a normal architecture and tubular necrosis of the kidney.