STUDIES ON THE FFECT OF AQUEOUS EXTRACT OF *CYPERUS ESCULENTUS* ON THE LIVER OF CADMIUM EXPOSED MALE WISTAR RAT

BY

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DEPARTMENT OF BIOCHEMISTRY,

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CERTIFICATION

This is to certify that this project was carried out by **AKHABUE-OKAKA REBECCA** with the matriculation number **LSC1806248**, of the Department of Biochemistry, Faculty of Life Sciences, University of Benin, Benin-City in partial fulfilment for the award of Bachelor of Science (B.Sc.) Degree in Biochemistry

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Prof. (Mrs) M.A. Adaikpoh Date

(Project supervisor)

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Dr S.I Ojeaburu Date

(Project co-ordinator)

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Prof. E. C. Onyeneke Date

(Head of Department)

DEDICATION

To Almighty God; the giver of life, wisdom and knowledge. To my parent, Mrs. Okaka Beatrice, for their continuous financial and moral support which has kept me going. To my siblings for all the encouragement that is keeping me going. To my aunties and uncles for all the moral support.

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My gratitude goes to God Almighty for the gift of life, good health and strength to complete this report.

I greatly appreciate the efforts of Prof (Mrs) M.A. Adaikpoh, my project supervisor, thank you Ma for being a mother to me and imparting knowledge to me. I also appreciate Dr Ugbeni my Course adviser for your guidance, encouragement and patience. I also appreciate Dr S.I. Ojeaburu, my project coordinator, for his assistance.

I appreciate my mother, Mrs Beatrice Okaka, for all her prays, support and love that has no bounds. Thank you for always encouraging me in every height I ascertain. I will always make you proud. I love you mum. Thank you for being my stronghold, my motivator and above all my mother.

I want to appreciate my siblings, Mary, Pius, Cathrine, Timothy and Titus for being a source of motivation to me. A big thank you to my aunt Mrs Oamen Erebholo for being there for me through this journey. Thank you for making me see and realize how amazing biochemistry is. Thank you for the lengthy phone calls and being listening ears. To all my family members, thank you for your love, support and encouragement. I love you all.

This will not be complete without thanking my friends, Anwuli, Jeff and Best. Thank you for always being there for me through this process, thank you always answering my calls no matter how late it was. Thank you for being patient with me, I am highly grateful and indebted to you. I want to say a big thank you to all my friends and colleagues who had been of help in one way or the other, may God bless you all.

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ABSTRACT

Cadmium is a toxicant that is not just harmful to the gonads but also the liver. In this study, the ameliorating potentials of *Cyperus esculentus* on cadmium induced toxicity in Liver was evaluated. Thirty-five male albino wistar rats divided into five groups consisting of seven rats each were used for this experiment. All rats were treated orally via gavage for 28 days. The group 1 served as the control group was administered normal saline while group 2 was treated with Cadmium only (3 mg/kg body weight). Groups 3 and 4 were co-treated with cadmium and *Cyperus esculentus* extract at doses of 2 and 4 mL/kg body weight respectively. Following this, histopathology were analysed for all the rats. Biochemically, SOD, CAT and MDA activities were significantly altered ($p<0.05$) in cadmium treated group. Group 3 rats showed no significant alteration in SOD, CAT and MDA level. However, there was a significant increase in CAT activities in all rats. Histological section through the liver showed inflammation and necrosis in rats in group 2. However, the histology cross section in group 4 and 5 appeared to have recovered from the damages induced by cadmium. These results showed that *Cyperus esculentus* ameliorated the toxic effects induced by cadmium on the liver by restoring the morphology of the liver in a dose dependent manner.

CHAPTER ONE

INTRODUCTION

Cadmium is among the heavy metals which are environmental pollutants. It is mutagenic and genotoxic to human health and these has been a problem of increasing significance for ecological, evolutionary, nutritional and environmental issues. Cadmium is released into the environment by several human activities, such as mining, smelting, manufacturing batteries, pigments, and plastic stabilizers, and the use of phosphate fertilizers and compost. These activities release cadmium into the air, water and soil, where it can be taken up by plants and animals and enter the food chain (Nordberg *et al*., 2018). Cadmium toxicity has a few known mechanisms, including oxidative stress, mitochondrial dysfunction, and interference with calcium signaling. The primary mechanism of liver cellular damage provoked by cadmium include the induction of cysteine building proteins, reactive oxygen species (ROS) generation, steatosis leading to non-alcoholic steatohepatitis (NASH) and apoptosis, leading to liver dysfunction (Souza-Arroyo *et al*., 2022). Studies has shown that cadmium is responsible for a lot of human disorders and according to the International Agency for Research on Cancer (IARC), cadmium is considered one of the hazardous metals to human health. Some of the disorders include carcinogenesis, cardiovascular abnormality, osteotoxicity, renal disorder, hepatotoxicity, diabetes, hypertension etc (Sharma *et al*., 2015). Some methods currently being used to manage cadmium toxicity are soil remediation, waste water treatment and food processing (Haide *et al*., 2021). Also, using nanotechnology to detect and remove toxic metals, such as cadmium is another method currently being used to help to reduce the harmful effect of its contamination (Rahimzadeh *et al*., 2017). There have been a few challenges associated with these methods, firstly they can be expensive and time consuming. Secondly, these methods may not be effective at removing all the cadmium. Some research

has shown that nanoparticles affect tissues on long term exposure and it can be difficult to monitor since they are small and can easily spread in the environment.

Cyperus esculentus (Tiger nut) is a tuber belonging to the family, Cyperacae. They are cultivated for human and livestock consumption and feed respectively as their seed are edibles and has a slightly sweet nutty flavour. It is widely distributed in the tropical and subtropical regions and in Nigeria, is cultivated mainly in the Middle Belt and Northern regions (Barde *et al*., 2023). I chose *C. esculentus* as a potential ameliorating agent because it contains high levels of inulin which can help to offset the effects of cadmium toxicity on the gut. It has also been shown high levels of antioxidants, including polyphenols and flavonoids. These compounds can help reduce oxidative stress, inflammation, hepatotoxicity and protect the liver from damage. They can also increase levels of enzymes that protect the liver and decrease levels of enzymes that indicate liver damage. Several studies have also shown that most plants rich in antioxidants have the tendency to increase sperm count, motility and enhance oestrogen and testosterone production (Yakubu *et al*., 2011). Lastly, *C. esculentus* extract is relatively inexpensive and readily available, making it a practical and accessible treatment option. However, the research on the use of Tiger nut extract to manage cadmium toxicity is still in its early stages, hence more research is needed to fully understand its potential benefits and risks.

1.1 Aim and Objectives

Aim: The aim of this study is to investigate the effect of aqueous extract of *Cyperus esculentus* on the liver of cadmium exposed male wistar rat and provide answers to specific questions, such as:

1. To what extent can cadmium induce liver damage?
2. Can *Cyperus esculentus* attenuate liver injuries induced by cadmium and to what extent can it ameliorate these hepatic injuries?
3. Does *Cyperus esculentus* possess phytochemicals that can be used to treat cadmium toxicity?

Objective: The objective of this study is to assess the effects of Tiger nut extract on markers of oxidative stress and liver damage in rats exposed to cadmium. The study will measure levels of superoxide dismutase (SOD), catalase (CAT) and malondialdehyde (MDA) in liver before and after treatment with tiger nut extract. This study will also include histopathological assessment of the liver and study of the relative organ ratio and body weight of the rats.

1.2 Literature Review

1.2.1 Cadmium

Cadmium is a naturally occurring shiny silvery metal denoted by the chemical symbol Cd. It is melleable. It has an atomic number of 48 and it is in group 12, period 5 of the periodic table. It was discovered by German chemist Friedrick Strohmeyer in 1817 as a constituent of smithsonite ($ZnCO_3$) from zinc ore (Sharma *et al*., 2015).

Most of cadmium produced is obtained from zinc by products and recovered from spent nickel-cadmium batteries. It is an important metal in the production of nickel cadmium (Ni-Cd) rechargeable batteries as a corrosion-protection coating for iron and steel. It is also used industrially in batteries, alloys, coating (electroplating), solar cells, plastic stabilizers and pigments (WHO., 1992).

1.2.2 Properties of Cadmium

1.2.2.1 Physical Properties of Cadmium

Cadmium is an odorless, silvery-white melleable metal with a density of 8.7gcm^3, a boiling point of 766.8°C and a melting point of 321°C. It is inflammable and is a machinable heavy metal that cannot be found alone in nature (Hocaoglu-Ozyigit., 2020).

1.2.2.2 Chemical Properties of Cadmium

Cadmium has an atomic number of 48, atomic weight of 112.41gmol-1 and belong to Group 2b in the periodic table. It exists in two oxidative states +1 and +2 but almost all cadmium compounds have an oxidation state of +2. Cadmium is insoluble in water but soluble in dilute nitric acid, ammonium nitrate and hot sulfuric acid. It forms cadmium oxide fumes when heated and is slowly oxidized in moist air (IARC., 1993).

1.2.3 Production and Uses of Cadmium

1.2.3.1 Production of Cadmium

According to International Cadmium Association (ICdA), Cadmium minerals do not occur in concentrations and quantities that are sufficient enough for mining, they are by-product of zinc (ICdA., 2021). Cadmium is produced from the refining of zinc metal from sulfide ore concentrates. These zinc ores when crushed, ground and heated, are then treated by different flotation process to yield zinc concentrates which contain about 0.3% to 0.5% of cadmium (Llewellyn., 1989).

1.2.3.2 Uses of Cadmium

The earliest use if cadmium was primarily in sulphide form, in paint pigments and as dental almagams (IARC., 1993). Cadmium is now majoly used for the production of nickel-cadmium (Ni-Cd) batteries, it is also used as pigments, alloys, electroplating and coating, and as stabilizers for plastics. Cadmium can also form chloride, sulphide, sulphate, nitrate, oxides and hydroxides which have large variety of use industrially (IARC., 1993).

1.2.4 Sources of Exposure of Cadmium to Humans and The Environment

There are multiple sources of cadmium exposure in the general population. Food accounts for approximately 90% in the general, non-smoking population. The cadmium in crops is due to the uptake of cadmium from the soil. Less than 10% of the total cadmium exposures to the general population occurs due to inhalation of low levels of cadmium in ambient air and through drinking water (Nogawa *et al*., 2004). The amount of Cadmium added to the environment is directly proportional to the level of national industrialization.

Other sources of exposure include: industrial activities, smoking, mining activities etc. Cadmium exposure can be grouped into:

1.2.4.1 Exposure Through Food and Drinking Water

The cadmium contained in the soil and water can be taken up by certain crops and aquatic organisms and then accumulate in the food chain, therefore, food contains the main environment source of cadmium with the exception of smokers (WHO., 2019). The food categories that contribute to cadmium exposure in several countries are cereals or grains, meat, poultry organ meat, vegetables and some seafood that have been exposed to contamination from industries. When rice is grown on a cadmium polluted soil, it can accumulate high concentrations of cadmium (WHO., 2011).

On the other hand, contamination of drinking water may occur due to the presence of cadmium as an impurity in the zinc of galvanized pipes or cadmium containing soldersin fittings, water heaters, water coolers and taps. High levels of cadmium can also be found in areas supplied with soft water of low PH level and this could be more corrosive in plumbing systems containing cadmium (WHO., 2011).

1.2.5 Route Of Cadmium Exposure To The Body

The various was in which cadmium can be exposed to the human body include:

a. Inhalation route of exposure

b. Oral Route of exposure

c. Dermal Route of exposure

1.2.5.1 Inhalation Route of Exposure

One of the major inhalation route of cadmium exposure is occupational exposure because cadmium air levels are more greater in the workplace than the general environment (ATSDR., 2008). Inhalativly, resorbed cadmium reaches blood circulation usually in form of cadmium-cysteine complexes, hence workers exposed to cadmium-containing fumes have been reported to develop acute respiratory distress syndromes (ADS) (Godt *et al*., 2006). Studies has also shown that Cigarette smokers in the general population are exposed to cadmium through inhalation thus, Smokers typically have more cadmium blood and body burdens than nonsmokers (Godt *et al*., 2006).

1.2.5.2 Oral Route of Exposure

The major oral route of cadmium exposure is through food and water. Areas with high levels of cadmium in the soil are the most affected and crop uptake of cadmium in these areas can lead to significant dietary exposures to the people living nearby (Godt *et al*., 2006). For

example, Kobayashi *et al*., (2006) reported that in the Jinzu and Kakehashi river basins in Japan, there are areas with soil contaminated with cadmium. Rice absorbs the cadmium and a lifetime of eating this cadmium-contaminated rice can lead to a serious kidney and bone disorder called “Itai-Itai” disease, especially in women. Women have been shown to have higher blood levels of cadmium than men (ATSDR., 2008). 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 Also, cadmium in water is more easily absorbed than cadmium in food (ATSDR., 2008).

1.2.5.3 Dermal Route of Exposure

Absorption through the skin is not a significant route of cadmium entry; only about 0.5% of cadmium is absorbed by the skin therefore only little research has been done on the absorption of cadmium through dermal Route of exposure. Experiment on the resorption from cadmium-contaminated soil and water solutions by human cadaver skin in a diffusion cell model, performed by Wester *et al*., (1991) using shaved rats, showed hyperkeratosis and acanthosis with occasional ulcerative change, and an increase of the mitotic index of the skin cells. Also cadmium concentration in blood, liver and kidney increased, thus indicating percutaneous absorption (Wester *et al.*, 1991).

1.2.6. Mechanism of Cadmium Toxicity in the Body

Cadmium in the blood is transported through proteins, Albumin and Metallothionein (Godt *et al*., 2006). It reaches the liver first where it induces production of Metallothionein. After hepatocyte necrosis and apoptosis, Cd-Metallothionein complexes enter into the blood. From here, parts of the absorbed cadmium enter the entero-hepatical cycle via secretion into the biliary tract in form of Cadmium-Glutathione conjugates. It is then enzymatically degraded to cadmium-cysteine in the biliary tract and then re-enters the small intestine as shown in Figure

1.1 (Zalup., 2003). The blood concentration of cadmium serves as a reliable indicator for a recent exposition, while the urinary concentration reflects past exposure, body burden and renal accumulation. Excretion of Cadmium takes place via faeces and urine (Godt *et al*., 2006).

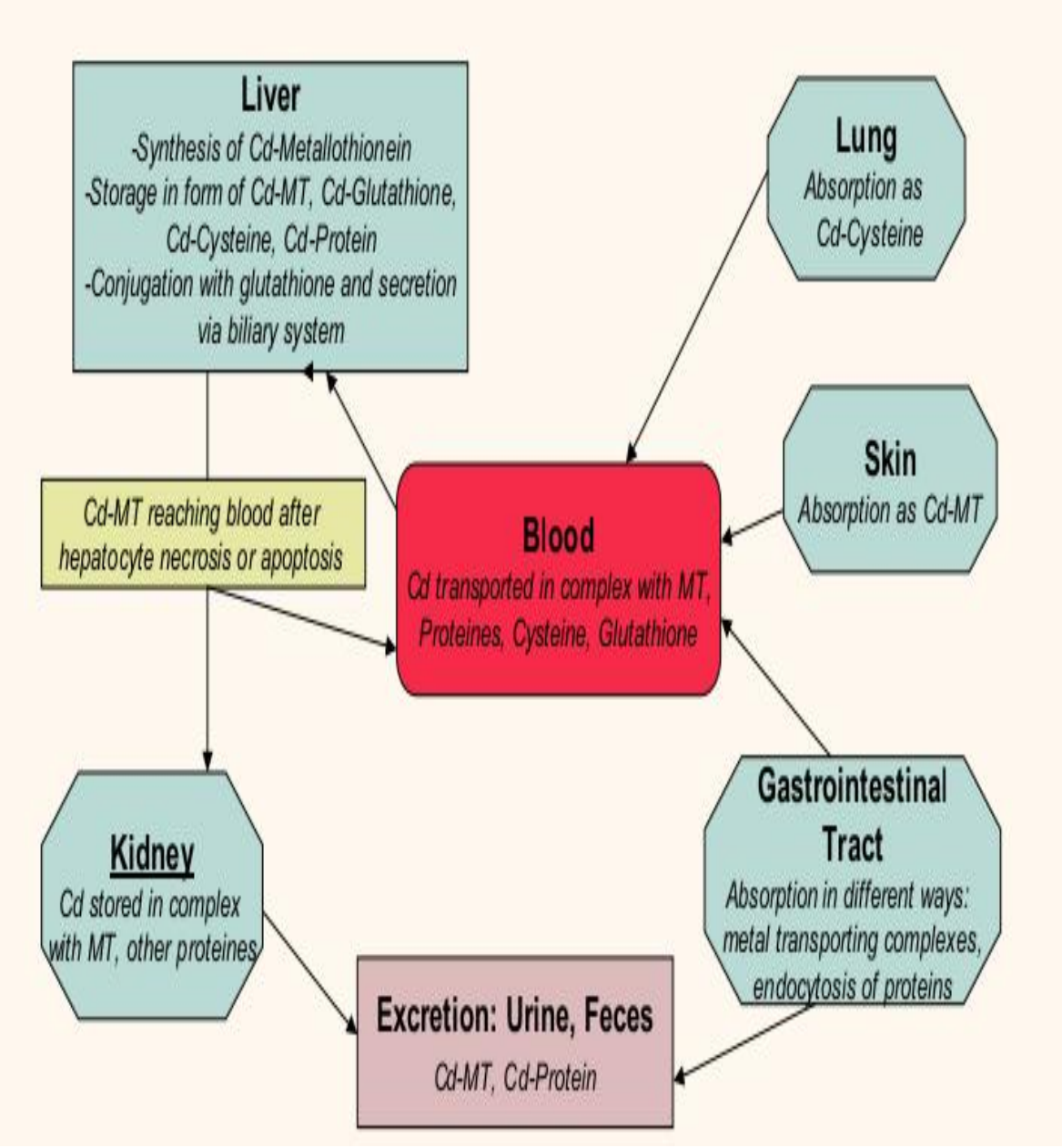


Figure 1.1. How cadmium is handled in the body: Metabolism, absorption and excretion of Cadmium.

Source: Zalup, 2003.

1.2.7 Cadmium Induced Toxicity

The toxicity of Cadmium in the human body has may be multifactorial. Cadmium exerts toxicity on the cells of various systems and tissues, such as the respiratory tract, the urinary, cardiovascular, gastrointestinal and nervous systems and the bones, by affecting their function either directly or indirectly. Some of the effects of Cadmium Toxicity include: Genotoxicity, osteotoxicity, nephrotoxicity, increase in oxidative stress, hepatotoxicity and reproductive toxicity.

1.2.7.1. Genotoxicity

Cadmium is known to affect the cell proliferation and differentiation, cell cycle progression, DNA synthesis, apoptosis and other cellular activities.

(a)Inhibition of DNA Repair

Cadmium can inhibit DNA Repair in several ways: base excision repair, nucleotide excision repair (NER), mismatch repair and the elimination of the premutagenic DNA precursor 8-oxodGTP. With regards to the base excision repair (BER), O'Connor *et al.*, (1993) reported that cadmium exposure inhibits and modifies some proteins of BER such as formamidopyrimidine glycosylase (Fpg): the substitution of a cysteine in the zinc finger localized in the C terminal of Fpg protein may inhibit the binding of the protein to DNA. Cadmium has also been shown to decrease hOGG1 activity, i.e. of the main DNA glycosylase activity responsible for the initiation of the base excision repair of 8-oxoguanine (an abundant

and mutagenic form of oxidized guanine). In nucleotide excision repair (NER), présence of Cadmium decreases the association and the dissociation of essential NER proteins and interferes with the removal of thymine dimers after UV irradiation by inhibiting the first step of this repair pathway. Cadmium also inhibits xeroderma pigmentosum (XPM), a protein. In respect to mix match repair (MMR), cadmium inhibits the adenosine triphosphate binding and hydrolysis of MSH2–MSH6 complex of MMR inhibition of MMR leads to the propagation of cellular errors, thus, the toxic effects of cadmium can be amplified in cells by creating mutations in genes that induce further faulty functions (Rani *et al.*, 2014).

(b) Cell Apoptosis

Apoptosis that is frequently observed in cadmium-exposed cells as a defence mechanism against the uncontrolled proliferation of mutated or transformed cells in the body. It has also been shown that cadmium transformed or adapted cells are characterized by increased resistance to apoptosis which may render them more prone to accumulation of mutations and neoplastic transformation. Cadmium enters into cells through the voltage-dependent Ca^{2+} channel and up-regulates IP3R1 (inositol 1,4,5-trisphosphate receptor) expression,and then Ca^{2+} release from endoplasmic reticulum (ER) is induced. Ca^{2+} activates calpain and induces DNA fragmentation and apoptosis. On the other hand, cadmium can possibly activate caspase-8 to induce apoptosis (Li *et al.*, 2000). Report has also shown that when treated with HL-60 cells, cadmium caused the appearance of cytochrome c (Cyt c) in the cytosol, a potent activator of caspase-9, which is an initiator caspase that is a potent activator of downstream effector caspases(e.g caspase-3). This resulted in cadmium-induced apoptosis by caspase-9(Rani *et al.*, 2014).

1.2.7.2 Osteotoxicity

Although cadmium accumulates in bone, the bone disease that results from excessive cadmium exposure is believed to be secondary to changes in calcium metabolism due to cadmiuminduced renal damage (ATSDR 1999). Clinically significant bone lesions usually occur late in severe chronic cadmium poisoning and include pseudofractures and other effects of osteomalacia and osteoporosis.

There are two proposed mechanisms by which cadmium is able to disrupt bone function. One mechanism is indirect, whereby cadmium damage to kidney or gastrointestinal organs produces a secondary effect on bone (Rani *et al*., 2014). Cadmium also causes the "Itai-itai" or ouch-ouch disease which was first described in postmenopausal Japanese women exposed to excessive levels of cadmium over their lifetimes. The women were exposed through their diet because the region of Japan in which they resided was contaminated with cadmium (Ikeda *et al.*,2000). It also causes severe osteoporosis and osteomalacia, severe renal distinction and anaemia (Nogawa *et al*., 2014).

1.2.7.3 Nephrotoxicity

The kidney is the principal organ targeted by chronic exposure to cadmium. Cadmium nephrotoxicity may follow chronic inhalation or ingestion. Data from human studies suggest a latency period of approximately 10 years before clinical onset of renal damage, depending on intensity of exposure. However, subtle alterations of renal function have been described after acute exposure in animals, and there are rare reports of renal cortical necrosis after acute high-dose exposure in humans. Several other studies have reported increased overall mortality in persons living in cadmium-polluted areas with elevated urinary cadmium levels and/or tubular proteinuria (Rani *et al*., 2014).

Prozialeck *et al*., (2012), reported that changes in proximal tubule cell–cell adhesion, cellular signalling cascades and autophagic responses that occur before the onset of necrosis or apoptosis of proximal tubule cells, occurs in the early stages of Cadmium nephrotoxicity. As soon as Cadmium is absorbed into the bloodstream, it is initially transported to the liver and taken up by hepatocytes and induces the synthesis of Metallothionein (MT), which binds to Cadmium and buffers its toxic effects in the cell. This causes the hepatocytes die off, either through normal turnover or as a result of Cd injury, the Cd–MT complex can be released into the blood stream. Cd–MT does not play a critical role in directly mediating the nephrotoxic effects of Cadmium, but it has the paradoxical effect of facilitating the delivery of Cadmium from the liver to the kidney (Rani et al., 2014). In addition, there is evidence for the uptake of lower molecular weight Cd-thiol conjugates (cysteine and GSH) by proximal tubule cells and Cadmium can enter renal tubular cells through a variety of channels and transporters for ions, such as Ca^{2+}, Fe^{2+} and Zn^{2+}(Bridges *et al.*, 2005). This shows that over time, Cadmium can accumulate in the epithelial cells of the proximal tubule. The traditional view has been that when the tissue levels of Cd exceed a critical concentration of about 150 μg/g tissue, intracellular defenses such as MT and GSH are overwhelmed and the cellsundergo injury and begin to die (Prozialeck *et al*., 2010).

1.2.7.4. Increase in Oxidative Stress

Transition metals are said to be catalysts in oxidative reactions of biological macromolecules. Either redox-active metals (like iron, copper and chromium) or redox-inactive metals (like lead, cadmium and mercury) may cause an increase in production of reactive oxygen species (ROS) such as hydroxyl radical (HO−), superoxide radical (O^{2-}) and hydrogen peroxide (H_2O_2). The two major enzymatic antioxidants are superoxide dismutase (SOD), which degrades O^{2-} and catalase, and the glutathione (GSH) redox system, which inactivates H_2O_2 and hydroperoxides. The hydrogen peroxide produced as a result of SOD

activity is detoxified by catalases and glutathione peroxidases (Das et al., 2019). The hydrogen peroxide is further broken down to water and molecular oxygen by either catalase (CAT) or glutathione peroxidase (GSH-Px). Glutathione reductase (GR) and GSH-Px work together to maintain a balance in glutathione (GSH) and its reduced product (GSSG). The presence of cadmium in cells interferes with these reactions by decreasing SOD, GSH and CAT thereby producing OH radicals by Haber-Weiss and Fenton reactions and accumulating free radicals. This is illustrated in Figure 1.2 (Das *et al.,* 2019).

Three forms of SOD may be important: manganese SOD (which is located in mitochondria), Cu–Zn SOD (which resides in the cytoplasm) and extracellular SOD (which lines blood vessels). GSH is a cofactor for various enzymes that decrease oxidative stress and it has antioxidant capacity and forms intermolecular disulphide non-radical end-product-oxidized glutathione (GSSG). Since GSH is abundant in the liver, and is thought to be the first line of defence against Cd hepatotoxicity as Cd binds tightly to thiol groups, and depletion of hepatic GSH by diethyl maleate significantly enhances cadmium-induced hepatotoxicity. This enhances Cadmium in the bile suggesting that disruption of the cellular GSH system is a key element for cadmium-induced oxidative stress in the liver. Cadmium can also cause the depletion of selenium. Therefore, if there is less selenium to form GSH peroxidase, one of the body's main antioxidants, this will lead to the formation of greater levels of ROS and hydrogen peroxide (Liu *et al*., 2002).

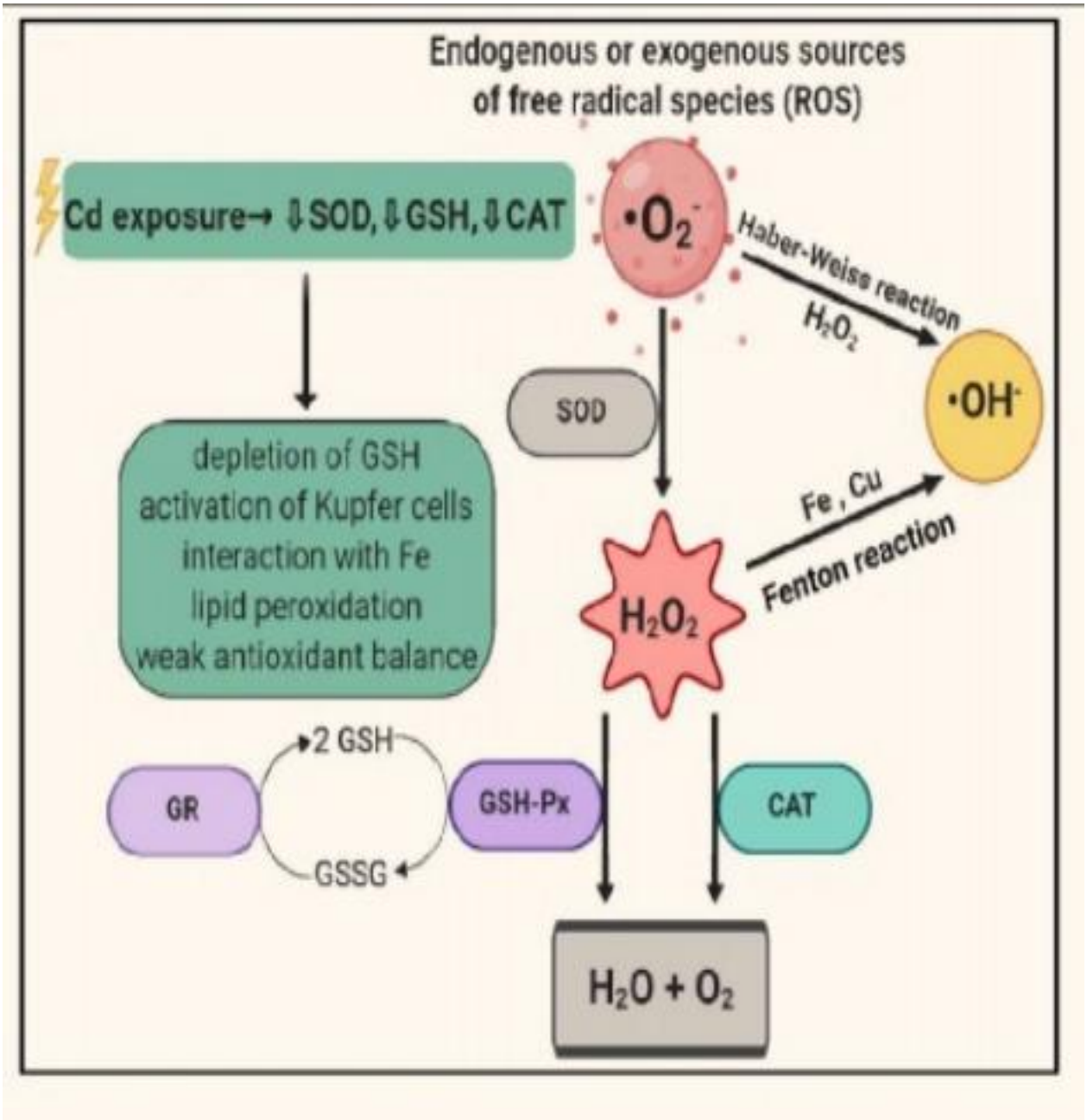


Figure 1.2. Illustration showing the how the presence of cadmium interferes with reactions to maintain redox balance in the cells.

Source: Das *et al*., 2019.

1.2.7.5 Reproductive Toxicity

Cadmium exposure can cause a lot of detrimental effects on mammalian reproduction. In female reproduction, studies have shown that Cadmium inhibits the biosynthesis of progesterone and affect the morphology of ovarian and the the reproductive tract. Cadmium has also shown decreased birth weights and premature birth in pregnant women (Miceli *et al*., 2005). Early pregnancy loss and implantation delay is caused by retardation of trophoblastic out growth and development, placental necrosis and suppression of steroid biosynthesis (Rani *et al*., 2014). In males, the mechanism of toxic effects of Cadmium in the testis including damage in the intracellular connections, vascular endothelium, Leydig and Sertoli cells, induction of oxidative stress, impaired antioxidant defence mechanism and high inflammatory response. This could result in functional and morphological changes like inhibitionof testosterone synthesis and spermatogenesis impairment (Taha *et al*., 2013). Studies has also shown that cadmium interferes with prostate function and cigarette smoking increases seminal plasma, decrease testis size, decrease sperm concentration and motility (Rani *et al*., 2014).

1.2.7.6 Effects of Cadmium Toxicity on Male Reproductive System

At least half of the cases of human male infertility are attributed to cadmium. Morphological changes Induced by cadmium include: Necrosis of the seminal tubules and intestinal edema, which leads to decreased testosterone synthesis and impaired spermatogenesis (Storlarczyk *et al*., 2017). Studies also showed that cadmium causes a decrease in testicle weight, in the amount of testosterone produced and in sperm count and activity. Ali *et al*., (2022) reported that the Sertoli cells (SCs), seminiferous tubules and Blood-Testis Barrier are severely structurally damaged by cadmium resulting in sperm loss. Cadmium also disturbs the

development and function of Leydig cells, the testis's vascular system, somatic cells and germ cells.

1.2.7.7 Hepatotoxicity

Majority of the cadmium absorbed are first delivered to the liver via portal circulation bound to albumin. There, it is taken up from the sinusoidal capillaries and then to the hepatocytes (Del Raso *et al*., 2003).

Acute hepatotoxicity caused by Cadmium exposure requires two pathways, the first one involves injury caused by direct effects of cadmium and the other involves a subsequent injury caused by inflammation. The initial injury are caused by the binding of Cd^{2+} to sulphydryl groups on critical molecules in mitochondria while the subsequent injury occurs from the activation of Kupffer cells and a cascade of events involving several types of liver cells and a large number of inflammatory and cytotoxic mediators (Rikans *et al*., 2000).

The major mechanisms found to be attributed to cadmium induced liver injury are: Production of inflammatory mediators and adhesion molecules, sulphydryl group inactivation, homeostasis of essential metals, oxidative stress and apoptosis (Rani *et al*., 2014).

Mechanism of Cadmium in the Liver

Cd binds with metallothionein (MT) proteins in the liver to form a Cd–MT complex, metallothionein proteins are involved in Cd and heavy metal trafficking, protecting against heavy metal toxicity, oxidative stress, and DNA damage. Earlier studies demonstrated that Cd induces DNA damage response by modulation of the ATR-CHK1-p53 pathway after DNA strand breaks. Cd exposure inhibits DNA repair, induces DNA methylation, interferes with gene expression, and also alters cell cycle regulation. Cd also induces oxidative stress in cells, and the rapid accumulation of Cd in the mitochondria upregulates the production of reactive oxygen species (ROS), inducing mitochondrial damage, inhibiting mitochondrial respiration,

and ultimately inducing apoptosis and necrosis. Cd-mediated induction of oxidative stress is one of the factors that activate antioxidant enzyme activities and alters redox status in cells (Niture *et al*., 2022). While the exact mechanism by which Kupffer cells contribute to the liver damage caused by cadmium is not fully understood, it is known that activated Kupffer cells release certain harmful substances that can directly damage liver cells. These substances include reactive oxygen species, nitric oxide, and cytokines. Researchers have focused on the role of tumor necrosis factor α (TNF-α) in the liver damage caused by cadmium (Arroyo et al., 2012). It has been shown that anti-TNF-α antibodies prevented Cd-induced secretion of acute phase proteins and gene expression of interleukin-1β (IL-1β), interleukin-6 (IL-6) and interleukin-8 (IL-8) in human hepatoma cell line HepG2 (Souza et al., 2004). ROS can also promote inflammation by activating transcription factors like nuclear factor-κB (NF-κB) and activator protein-1 (AP-1), which in turn trigger the expression of genes that promote inflammation and the attachment of cells to each other (figure 1.3). Since Cd poisoning severely affects most organs, including the liver, developing antidotes for Cd intoxication and limiting environmental exposure is critically important. Numerous studies suggest that chemo-preventive phytochemicals, chelating agents, 7 metals, natural drugs, and vitamins, can reverse Cd-induced hepatotoxicity as shown in Figure 1.3 (Arroyo *et al*., 2012).

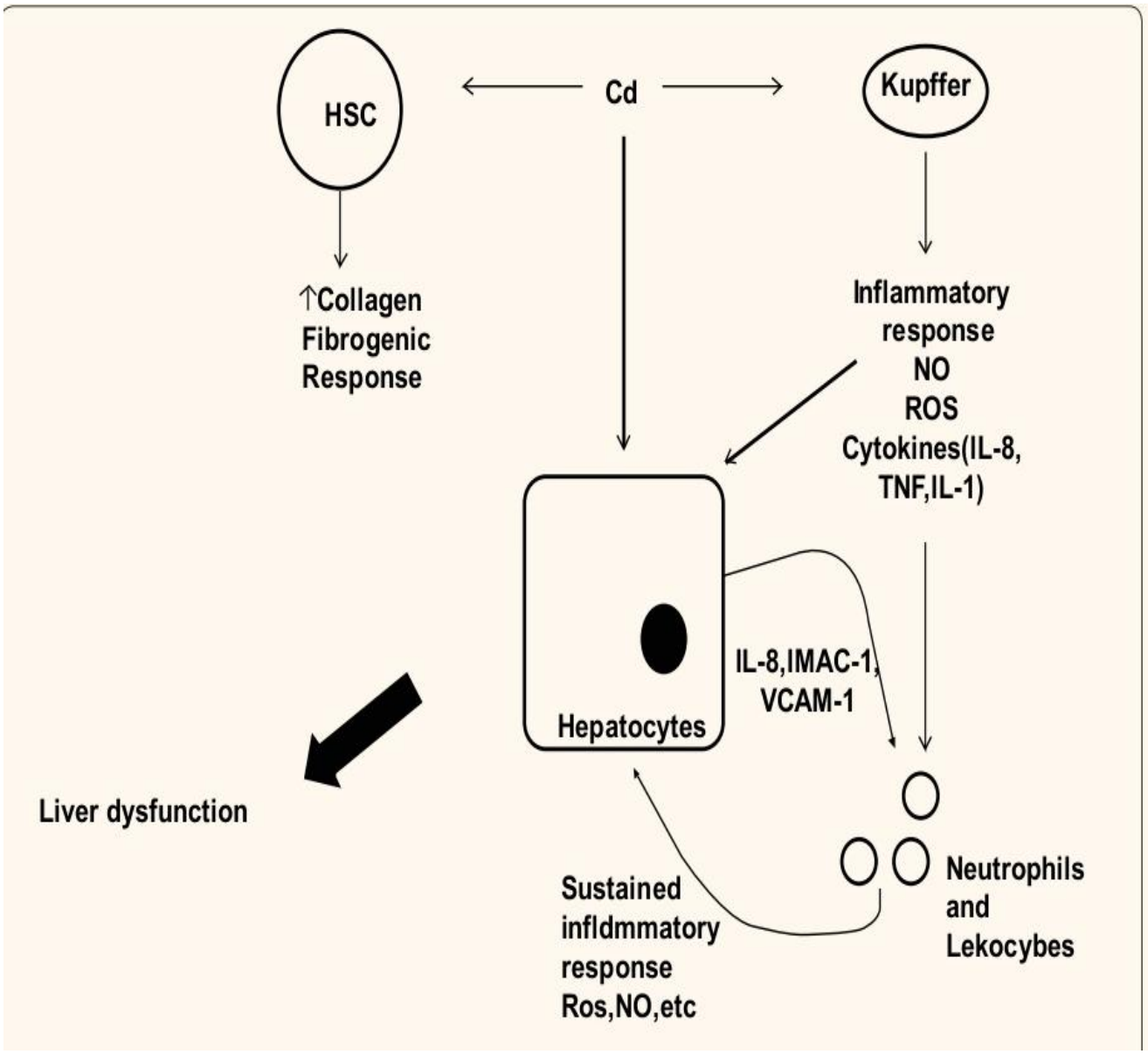


Figure 1.3: General mechanism of cadmium induced liver damage. Different hepatic cell types are involved in cadmium toxic response resulting in liver dysfunction.

Source: Arroyo *et al.,* 2012.

1.2.8 Cadmium Interaction with Vitamins and Enzymes

The reaction of Cadmium with protein causes oxidative stress, which is an imbalance of oxidants, enzymatic antioxidants and non-enzymatic antioxidants. The enzymatic antioxidants include Superoxide dismutase (SOD), Catalase (CAT), and Glutathione reductase (GSR), while the non- enzymatic antioxidants include such as vitamin E, vitamin C and Glutathione (GSH).

With regards to the enzymatic antioxidants, Superoxide dismutase works by catalyzing superoxide (·O2) to peroxide molecule, whereas CAT decomposes peroxide acid substrate into water and oxygen. Studies have shown that amino acid residues are involved in the mechanism of binding of cadmium with antioxidant enzymes. SOD binding sites with Cd were GLU-121 and SER-142; CAT binding sites were amino acid residues of HIS-230, ASP-228, HIS-181, HIS-109 and ASP-178; GSR binding sites were HIS-503 and TYR-502. Therefore, they can help increase oxidative stress caused by Cadmium (Komari *et al.*, 2020).

With the non-enzymatic antioxidants, Vitamin E (tocopherol) is the major lipid-soluble antioxidant, present in all cell membranes, which protects cells against lipid peroxidation, reaction with active radicals produces tocopheroxyl radicals, being further reduced to tocopherol via vitamin C or GSH, while Vitamin C (ascorbic acid) is a low-molecular antioxidant which protects cells against water-soluble oxygen and nitric radicals. Ascorbic acid can scavange radicals generated during lipid peroxidation or reduce tocopheroxyl radicals. Dehydroascorbic acid is produced in these reactions and can be reduced by GSH (Jurezuk *et al*., 2005). The concentrations of these vitamins can also be influenced by Ethanol (Leiber *et al*., 2000). Jurezuk *et al.*, (2005), was able to prove that Cadmium and Ethanol might influence the concentration of antioxidative vitamins in the organism via pathways not related with induction of oxidative stress, but they can reduce vitamin E through the induction of lipid peroxidation and decrease vitamin C through GSH deficiency.

1.2.9 Treatment of Cadmium Toxicity

Combining two or more chelating agents, dimercaptosuccinic acid (DMSA) and meso2,3-dimercaptosuccinic acid (MiADMSA), can be more effective at removing heavy metals from the body than using just one chelating agent. This combination therapy can reduce both the concentration of cadmium in the body and its toxic effects. DMSA and calcium trisodium diethylene triaminepentaacetate (CaDTPA), another chelating agent, have been shown to be effective when used together for treating acute oral cadmium toxicity (Rahimzadeh *et al.,* 2017). Studies have shown that antioxidants like vitamin C and vitamin E can protect against cadmium toxicity in different types of animals. When rats were given a combination of ascorbic acid, alpha-tocopherol, and selenium, it was found to be effective in reducing the effects of cadmium toxicity in the intestine. The combination of these antioxidants prevented an increase in lipid peroxidation and a decrease in glutathione levels in the intestine (Bolkent *et al.,* 2007). Also, zinc and magnesium are both elements with many clinical applications. Zinc is necessary for immune function and to prevent free radicals. Magnesium is a cofactor that is required for many enzyme systems. Zinc and magnesium may reverse the toxicity to the kidney caused by cadmium. Specifically, zinc and magnesium can help to counteract reactive oxygen species and lipid peroxidation, which are two harmful processes that cadmium toxicity can cause. The use of nanoparticles (AL2O3 and TiO3) and Plasma exchange-hemodialysis-plasmapheresis are also effective method for the treatment of cadmium poisoning (Rahimzadeh *et al.,* 2017).

1.2.10 Tiger Nut (*Cyperus Esculentus*)

The tiger nut, also known as the “underground walnut”, grows all over the world because of its high yield and broad prospects for comprehensive utilization. The tiger nut is the tiny tuber of *Cyperus esculentus* (Yu *et al.*, 2022). It is a rhizome spherical crop that can be eaten

raw, dry or processed. It is also called nutgrass, Chupa, nutsedge, earth almond, etc. It is native to most tropical and temperate regions of the world. It is majorly produced in Africa, Madagascar, Middle-East, Southern Europe and Indian subcontinent, the leading producing nations are Nigeria, Niger, Togo, Benin, United States, Iran, Iraq and Morocco. The plant can re-sprout severally, the leaves are very tough and fibrous, single plant can produce up to 2420 seeds, the tuber size ranges from 0.3 to 1.9 cm. It grows in large quantities in many West-African countries (Abdullahi *et al.*, 2022). It can be processed into wide varieties of foods are tiger nut milk, tiger nut oil and tiger nut flour. The Tiger nut milk also known as Kunun-aya, is a traditional non-alcoholic beverage commonly consumed in West-African countries, it is an aqueous extract of tiger nut, coconut, date and spices blend. Tiger nut milk is a good substitute for vegetarians and persons with lactose intolerance and it is known to contain low acid vegetable milk rich in protein and starch (Amponsah *et al.*, 2017). Récent studies has also shown that tiger nut could have detrimental effects when they are not properly stored and these effects could be due to microbial activities and Fermentation (Abdullahi *et al*., 2022).

Figure 1.4 Tiger nut

Sources Taha *et al.,* 2020

1.2.10.1 Chemical composition of tiger nut

Tiger nut has many nutrients that can be deeply explored, it contains 22.14–44.92% lipids, 3.28–8.45% proteins, 23.21–48.12% starch, 8.26–15.47% fibers and (45.73%) carbohydrate followed by oil content (30.01%). The protein, ash and crude fiber of tiger nut tubers are (5.08%), (2.23%) and (14.80%) respectively. The starch content of tiger nut tubers are (293.50 g/kg) followed by sucrose content (99.35g/kg) and reducing sugar (27.61g/kg) (Arafat, 2019). The high quantity of minerals in tiger nut tubers was potassium, phosphor, magnesium calcium, sodium which were 267.18 mg/100g, 158.86 mg/100g, 118.14 mg/100g, 43.36 mg/100g and 17.02 mg/100g respectively (Suleiman et al., 2018).

1.2.10.2 Health Benefits of Tiger Nut

Tiger nut contains bioactive substances such as organic acids, alkaloids and phenols. The tiger nut is a good source of edible oils that contain a lot of monounsaturated fatty acids. The nutritional value of tiger nut oil is similar to olive oil. It also contains a lot of starch—a renewable and low-cost food ingredient. The content of protein is relatively small, but it is found to be suitable for diabetic patients or those with digestive dysfunctions and may prevent heart disease after consumption. The dietary fiber in this tuber is effective in the prevention of colon cancer, obesity and gastrointestinal disorders. Due to the presence of flavonoids, the tiger nut has good antioxidant properties and can be used as a source of natural antioxidants (Yu *et al*., 2022).

Tiger nut has also been shown to be a good source of bioactive substances. It contains Polyphenols, flavones, minerals, essential fatty acids and vitamins C, D and E, etc. Therefore, it might possess medicinal properties. Assessment of the antioxidants of aqueous extracts Of the tiger nut showed 1,1-diphenyl-2 picrylhydrazyl (DPPH) and hydroxyl (OH) Radicals scavenging abilities, Fe2+-chelating ability, inhibition of Fe2+-induced MDA production And

inhibition of α-amylase and α-glucosidase activities by aqueous extracts of the tiger nut Are 9.63 ± 0.7 mg/mL, 3.01 ± 0.12 mg/mL, 0.72 ± 0.07 mg/mL, 2.09 ± 0.10 mg/mL and 0.76 ± 0.06 mg/mL, respectively. The findings support the hypothesis that tiger nut maybe beneficial in the management of type 2 diabetes. In other studies, the tiger nut powder was administered to the rats daily showed anti-inflammatory and anti-apoptotic effects to prevent testicular dysfunction, ameliorated male arousal, reduced diarrheal Symptoms in albino rats and reduced oxidative stress in liver and inflammatory with Atherosclerosis. The above results are most likely related to the presence of alkaloids, Quercetin, vitamins, steroids and zinc, etc., in tiger nuts. Therefore, in addition to be the Food and industrial materials, tiger nuts may also be developed into functional foods (Yu *et al.,* 2022).

CHAPTER TWO

MATERIALS AND METHODS

2.1. Materials

2.1.1 List of chemicals

Normal saline (Fidson Healthcare Plc, Lagos, Nigeria), EDTA (BHD Engl), Chloroform 200ml (Spectrum Chemical, USA), Epinephrine (JHD china).

2.1.2 List of equipment

Centrifuge (model 80 – 2, Harris, England), water bath (TT42D Multipurpose use, Techmel and Techmel, USA), spectrophotometer (Spectrum lab 22PC, England), refrigerator (Haier Thermocool, HTF-66H), dissecting set (Gold cross, England), analytical weighing balance (Homgeek TL-Series, China), histopathological slides (Pyrex Limited, USA), freeze-dryer, Pulverizing machine and glass wares (Pyrex Limited, USA).

2.1.3 Collection and Identification of Plant Material

Fresh *Cyperus esculentus* were bought from a uselu market in Benin City, Edo State. The Tiger nuts were identified and authenticated by Dr. H. A. Akinnibosun at the Department of Plant Biology and Biotechnology, Faculty of Life Sciences, University of Benin, Benin City, Edo State. A voucher specimen with the voucher number of UBH-C419 was deposited at the department.

2.2 Methods

2.2.1 Preparation of Plant Sample

The *Cyperus esculentus* were weighed and washed. The Tiger nuts (100 g) were blended with 100 mL of distilled water. The extract was then filtered by squeezing with a white cheese cloth and administered to the rats according to the calculated mL using a gavage.

2.2.2 Preparation of reagents

2.2.2.1 Phosphate Buffer (0.05M, pH 7.4)

Disodium hydrogen phosphate (4.36 g) and Potassium dihydrogen phosphate (2.72 g) were dissolved in 600 mL of distilled water. The pH was adjusted to 7.4 using 0.1M of HCL. The volume was then made up to 1000 mL using distilled water.

2.2.2.2 Carbonate Buffer (0.05M, pH 10.2)

Sodium Hydrogen Carbonate (2.604 g) was dissolved with 0.372 g of EDTA in 9000 mL of distilled water. After, the pH is adjusted to 10.2 using 0.1 M of HCL. Then the volume is made up to 100 mL with distilled water.

2.2.2.3 Normal Saline Solution (0.9%)

Sodium Chloride (9 g) was weighed into a beaker, dissolved in 200 mL of distilled water and made up to 1000 mL mark in the glass measuring cylinder with distilled water. For 500 mL, 4.5 g of NaCL are weighed and dissolved in 500 mL of distilled water.

2.2.2.4 Phosphate Buffer Hydrogen Peroxide (30mM)

Phosphate buffer solution (pH 7.4, 0.05 M) 250 mL was added in 500 mL volumetric flask. In addition, 30% Hydrogen Peroxide (1.53 mL) was added and made up to mark with the phosphate buffer solution.

2.2.2.5 Potassium permanganate KmnO4 (0.01 M) solution

Potassium permanganate (0.395 g) was weighed into a beaker, dissolved in 100 mL of distilled water and then made up to 250 mL mark with distilled water.

2.2.2.6 Hydrochloric acid solution HCL (0.05 M)

Concentrated HCL (100 mL) was added to 900 mL of distilled water and then made up to 1000 mL with distilled water.

2.2.2.7 Epinephrine (0.03 mM)

The epinephrine solution was prepared by dissolving 27.5 mg of 500 mL of 0.005 M of HCL.

2.2.2.8 Preparation of Reagent for Malondialdehyde (MDA) Assay

TBA 0.375%

A solution of Thiobarbituric acid (TBA) was prepared, by weighing 0.875 g of TBA, which was dissolved in 100 mL of distilled water.

TCA 15%

Trichloroacetic acid solution was prepared by weighing 15 g of TCA which was dissolved in 10 mL of distilled water in volumetric flask. The solution was then made up to 100 mL

0.25M HCL

A solution of 0.25M HCL was prepared by measuring 2.15 mL of concentrated HCL, which was dissolved in 60 mL of distilled water and then made up to 100 mL of distilled water.

Stock TCA-TBA-HCL Reagent

Equal volume of Thiobarbituric acid 0.375% w/v, Trichloroacetic acid 15% w/v, and Hydrochloric acid 0.25M reagent were measured and mixed together to give the stock.

2.2.3 Preparation of the Cadmium Solution

Cadmium solution was prepared by dissolving 0.717g of $CdCl_2$ (Cadmium Chloride) in 100 mL of normal saline in order to give a stock concentration of 7.17 mg/mL. This was then

administered to the rats at a dose of 3 mg/kg body weight through subcutaneous route of administration.

2.2.4 Animal and Experimental Study

2.2.4.1 Experimental Design and Animal Treatment

All experiment were performed on 35 male wistar rats. Obtained from the Department of Pharmacy University of Benin. The animals were acclimatized for one week 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 (rat pellets) and water *ad libitum.* They were maintained in the animal care facility throughout the duration of the experiment. The animals were maintained at a temperature of 25°C ± 2°C and under a 12 hour light / 12 hour dark cycle.

GROUP 1 (CONTROL): Normal saline (vehicle).

GROUP 2 (Cd): Received Cadmium only.

GROUP 3 (CE4): Received 4 mL/kg body weight of *Cyperus esculentus* extract only.

GROUP 4 (Cd +CE2): Received 3 mg/kg body weight of Cadmium and 2 mL/kg body weight of *Cyperus esculentus* extract.

GROUP 5 (Cd + CE4): Received 3 mg/kg body weight of Cadmium and 4 mL/kg body weight of *Cyperus esculentus* extract.

Animals were treated with Cadmium (3 mg/kg) or its vehicle (normal saline) subcutaneously on day one of study. Administration was carried out by oral gavage. The treatment lasted for 28 days (4 weeks). All animals were weighed every week to determine the new weekly dosage per kilogram body weight.

2.2.5 Animal Sacrifice

The animals were sacrificed under Chloroform anesthesia twenty-four hours after the last treatment after fasting overnight. The thoracic and abdominal cavities were opened and the blood was collected. The Liver was excised and trimmed free from connective tissues and then weighed. The Liver of each group were collected in Bovine fluid and 10% formalin and then used for histopathological studies.

2.2.6 Preparation of Plasma and Tissue Homogenate

Blood samples were collected in plain bottles or containers and centrifuged at 3500 rpm for 15minutes. The supernatant plasma was separated from the red blood cells as soon as possible and stored at -4°C until it was used for the different assays (Cholesterol, bilirubin and total protein). A small portion of the liver was cut off and measured and then weighed to 1g and then homogenized in 5 mL normal saline solution. Thereafter, they were centrifuged at 3000 rpm and refrigerated at -4°C until it was used for the different assays (SOD, MDA and Catalase).

2.3 Biochemical Analysis

2.3.1 Superoxide Dismutase (SOD) (EC 1.15.1.1)

Principle

Superoxide dismutase was estimated according to the method of Misra and Fridovich (1972). Superoxide Dismutase (SODs) are metalloenzymes found in eukaryotes and some prokaryotes and are localized in the cytosol and mitochondria membrane, mitochondrial matrix, inner membrane and extracellular components. SOD catalyzes the conversion of superoxide anion free radical (O_2) to hydrogen peroxide (H_2O_2) and molecular oxygen (O_2). Hydrogen Peroxide can also generate another reactive oxygen species (ROS), the hydroxide

ion (OH) through the Fenton reaction in the presence of Fe^{2+}. Superoxide dismutase is the Antioxidant enzyme that responsible for the Dismutation of the highly reactive superoxide Anion to O_2 and to the less reactive species H_2O_2 (Arabaci *et al,* 2016).

Procedure

The Liver Homogenate (0.5 mL) was added to 2.5 mL of 0.05 M carbonate buffer (pH 10.2) and allowed to equilibrate. The reaction was initiated by the addition of 0.3mL of freshly prepared 0.03 mM epinephrine as substrate. The solution was mixed by inversion and the increase in absorbance was read at 420nm. The reference tube contained 2.7 mL of carbonate buffer and 0.3 mL of epinephrine, while the blank contained 2.5 mL of carbonate buffer, 0.5 mL of distilled water and 0.3 mL of 0.03mM epinephrine.

Calculations

The inhibited percent of standards were calculated using the formula below:

Change in absorbance of sample (ΔC) = Absorbance of sample (60sec) – Absorbance of sample (0sec)

$$\%\ \text{Inhibition of pyrogallol auto oxidation} = \frac{\Delta S}{\Delta C} \times \frac{100}{1}$$

$$\text{SOD Activity } (\mu/\text{mL}) \ = \frac{\%\ \text{inhibition of pyrogallol autooxidation}}{50}$$

$$\text{Where Y} = \frac{\text{vol of sample x wet weight of tissue}}{Total\ vol\ of\ sample}$$

$$\text{SOD Activity in } (\mu/mL) \text{ tissue weight} =$$

$$\frac{\text{SOD in} \frac{\mu}{\text{mL}}}{weight\ of\ coefficient}$$

2.3.2 Catalase (CAT) (EC1.11.1.6)

Principle

Estimation of catalase was based on the method of Cohen *et al* (1970). When Hydrogen Peroxide is produced in large amounts within a cell, catalase is used to break it down. Catalase is determined by the addition of the material containing the sample to a solution of H_2O. Then measure the rate of decomposition or how quickly the H_2O_2 is broken down into oxygen and water. The faster hydrogen peroxide is broken down, the more catalase is present in the sample. It catalyzes the following reactions:

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

The quantity of Hydrogen Peroxide decomposed is directly proportional to the concentration of the enzyme in the sample. The product of hydrogen peroxide is measured by reacting it with excess potassium tetraoxomanganate (VII) and then, measuring the residual $KMnO_4$ with a spectrophotometer.

Procedure

Reactions are carried out in an ice-water bath (0– 2°C) pH 7.0. The enzyme-catalyzed decomposition of H_2O_2 is measured. One person can handle up to 18 catalase sample using a micropipette for rapid delivery of the peroxide and acid l.

Duplicates of 0.5 mL aliquots of colder catalase samples were added to the test tubes and duplicates of blanks which consist of 0.5 mL distilled water were added to separate test tubes. The enzyme reactions was initiated sequentially at fixed intervals by adding 5 mL of cold 30mM H_2O_2 phosphate buffer and mixed thoroughly. After exactly 3minutes, the reactions was stopped sequentially at the same fixed intervals by rapidly adding 1M of 6M H_2SO_4 and mixing quickly by inversion.

The spectrophotometric standard is prepared by adding 7.0 mL of 0.01M $KMnO_4$ reagent and mixed thoroughly and quickly read at 480nm within 30-60seconds of mixing. On longer standing, turbidity develops and this could interfere with readings. To avoid precipitation of MnO_4 in the cell, flush with distilled water between samples.

The reference is prepared by adding 7 mL of 0.01M $KMnO_4$ to a mixture of 0.5 mL phosphate buffer (pH 7.4) and 1 mL of 6M H_2SO_4 solution. The spectrophotometer was zeroed with distilled water.

Calculations and Expression of Results

The decomposition of H_2O_2 by catalase follows first-order kinetics as given by the equation:

CAT (k) (units/mL) $k = \log(S_0/S_3) \times \frac{2.3}{t}$

$$CAT\ (k)(unit/g\ wet\ tissues) = \frac{CAT\ k(unit/mL)}{y\ (unit\ weight\ coefficient)}$$

$$y = \frac{volume\ of\ sample\ used \times weight\ of\ wet\ tissue}{total\ volume\ of\ sample\ aliquot}$$

2.3.3 Malondialdehyde (MDA)

Malondialdehyde was estimated by the method of Buege and Aust (1978).

This assay is used to estimate the level of lipid peroxidation in tissue, which is caused by the activities of toxic substances that effect membrane integrity of the organs

Principle

Malondialdehyde is formed when polyunsaturated fatty acids break down. It serves as a convenient index for determining the extent of the peroxidation reaction. Malondialdehyde

can react with thiobarbituric acid to form a pink -colored compound that absorbs light at 535nm. The amount of MDA produced was used as an index of lipid peroxidation.

Procedure

Reagents include: Stock TCA-TBA- HCL, 15%trichloroacetic acid, 0.375% thiobarbituric acid and 0.25 M Hydrochloric acid.

1 mL of the aliquots of the liver Homogenate was added to 2 mL of TCA -TBA-HCL reagent and mixed thoroughly. The solution is heated for 15 minutes in a boiling water bath. After cooling, the flocculant precipitate is removed by centrifugation at 1000 g for 10 minutes. The absorbance of the clear supernatant was measured against a standard or reference blank at 535nm.

Calculations

Absorbance of sample = Asample

Absorbance of standard = Astandard

MDA (mg/dl) = $\frac{Asample}{Astandard} \times 0.184\ (mg/dl)$

MDA (mg/g wet tissue) = $\frac{MDA\ (mg/dl)}{y\ (weight\ coefficient)}$

$$y = \frac{100ml \times weight\ of\ wet\ tissue}{total\ volume\ of\ sample\ (ml)}$$

2.4. Histopathological studies

The liver was exposed by mid-line incision then rapidly dissected from the surrounding structures and weighed. Liver specimens were obtained and fixed in buffered 10% formaldehyde solution for 24 hours and processed for paraffin sections of 5-micron thickness. The sections were stained with Hematoxylin and Eosin and was examined under light microscope (Ross *et al*, 1989).

2.5. Data Analysis

The data were analyzed by one-way analysis of variance (ANOVA) using IBM-SPSS ver.26. Duncan Post Hoc test was used to compare the difference between mean values of the groups. Values were considered significant at ($p<0.05$). The results are presented as mean ± standard deviation (SD).

CHAPTER THREE

RESULTS

3.1 Effect of Tiger nut *(Cyperus esculentus)* extract on gravimetric parameters in cadmium exposed rats.

Effect of *Cyperus esculentus* extract on weight change in cadmium exposed rats.

Effect of *C. esculentus* extract on the body weight in cadmium exposed rat is displayed in **Table 3.1**. Results statistically showed a significant decrease in weight change for groups treated with cadmium only relative to the control. Groups treated with *C. esculentus* extract only showed no significant decrease in weight with respect to control. However, there was a significant decrease in weight of rats co-treated with cadmium and 2 mL/kg *C. esculentus* extract and rats co-treated with cadmium and *C. esculentus* extract compared to the control. Rats co-treated with cadmium and 2 mL/kg *C. esculentus* extract and rats co-treated with cadmium and 4 mL/kg *C. esculentus* extract revealed significant weight reduction compared to cadmium only group.

Effect of *Cyperus esculentus* extract on the relative organ-body weight change in the Liver of cadmium exposed rats.

The effect of *C. esculentus* extract on the relative organ-body weight in cadmium exposed rats is dispayed in **Table 3.2.** The relative organ-body weight of rats liver treated with cadmium and *C.esculentus* extract was compared with control. Cadmium only administered rats showed an increase in their organ-body weight ratio when compared to that of control. Rats co-treated with

Table 3.1 Effect of *Cyperus esculentus* on body weight in cadmium exposed rats

Groups	**Change in Weight (g)**
Control (Normal Saline)	23.82 ± 1.63
Cd (3 mg/kg bwt)	-20.82 ± 3.72
C esculentus extract (4 mL/kg bwt)	18.51 ± 1.45[b]
Cd+ *C esculentus* extract (2 mL/kg bwt)	-7.78 ± 0.46[a]
Cd + *C esculentus* extract (4 mL/kg bwt)	-12.57 ± 2.13[a]

All values are expressed as mean ± standard deviation.

Values with different subscript along the same column are significantly different.

Values are statistically significant at P value ≤ 0.05

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

Values with 'b' superscript on the same column differ significantly from Cd-only treated group

Cd: Cadmium, bwt: body weight

cadmium and *C.esculentus* extract (2 ml and 4 mL/kg bwt) showed significant increase with respect to control and cadmium only groups. There was significant decrease in the groups treated with extract only compared to the control.

3.2 Results of Biochemical Analysis

The effects of *Cyperus esculentus* extract on markers of oxidative stress and liver damage in cadmium exposed rats are presented in **Table 3.3**.

Results in Superoxide dismutase (SOD) showed a significant increase in SOD activity in *C.esculentus* only treated rats compared with the control. However, there was a significant decrease of SOD activity in cadmium and *C. esculentus* (2 mL and 4 mL/kg bwt) extract co-treated rats compared to the control and cadmium only rats.

Results in Catalase (COD) showed no significant difference in CAT activity in the *C. esculentus* treated rats compared with the control. Meanwhile, there was significant elevation in catalase activity in cadmium and *C.esculentus* (2 mL and 4 ml/kg bwt) co-treated rats compared to the control and cadmium only treated rats.

Results in Malondialdehyde (MDA) showed no significant difference in MDA activity in *C. esculentus* treated rats compared to the control. However, there was a significant decrease in MDA activity in cadmium and *C. esculentus* 2 mL and 4 mL/kg bwt rats comparedto the control and cadmium.

Table 3.2 The effect of *Cyperus esculentus* extract on the relative organ body weight of Liver in cadmium exposed rat

Groups	**Relative Organ Weight (× 10^{-2})**
Control (Normal Saline)	1.62± 0.04
Cd (3 mg/kg bwt)	1.73 ± 0.07
C esculentus extract (4 mL/kg bwt)	1.35 ± 0.008[b]
Cd + *C esculentus* extract (2 mL/kg bwt)	2.49 ± 0.45[ab]
Cd + *C esculentus* extract (4 mL/kg bwt)	2.88 ± 0.07[ab]

All values are expressed as mean ± standard deviation.

Values are statistically significant at P value ≤ 0.05

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

Values with 'b' superscript on the same column differ significantly from Cd-only treated group

Cd: Cadmium, bwt: body weight

Table 3.3 The effect of *Cyperus esculentus* on markers of oxidative stress and liver damage in cadmium exposed rats.

Treatment	**SOD** **(unit/g tissue) $\times 10^{4}$**	**CAT** **(unit/g tissue) $\times 10^{-2}$**	**MDA** **(unit/g tissue) $\times 10^{-2}$**
CONTROL	5.52 ± 0.51	0.11 ± 0.008	0.70 ± 0.05
Cd-only	5.31 ± 0.27	0.07 ± 0.003	0.67 ± 0.02
CEE_4	6.17 ± 0.51^{b}	0.11 ± 0.008^{b}	0.8 ± 0.06^{ab}
$Cd + CEE_2$	3.49 ± 0.17^{ab}	0.17 ± 0.01^{ab}	0.55 ± 0.01^{ab}
$Cd + CEE_4$	1.62 ± 0.04^{ab}	0.13 ± 0.01^{ab}	0.55 ± 0.02^{ab}

All values are expressed as mean ± standard deviation.

Values are statistically significant at P value ≤ 0.05

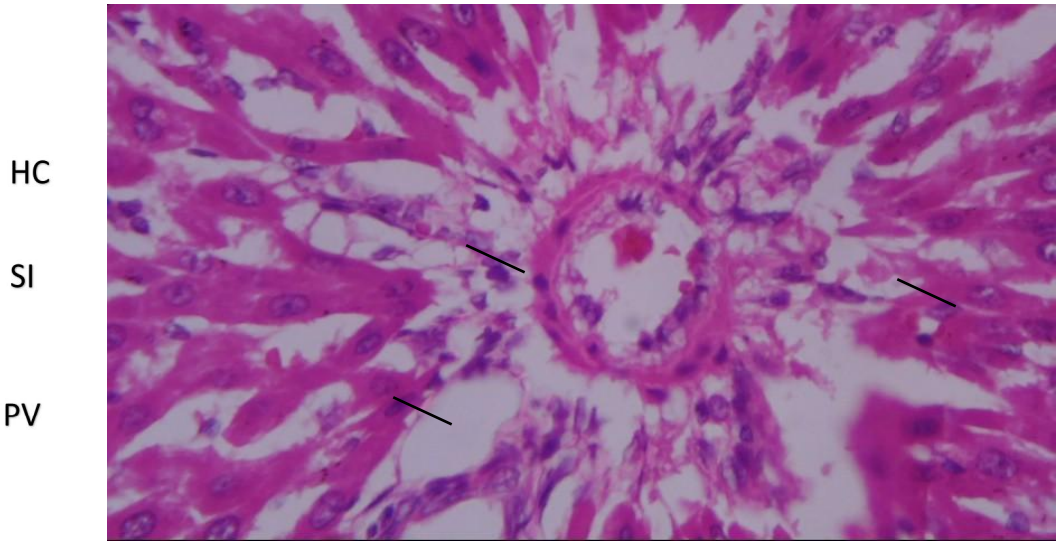
Values with 'a' superscript on the same column differ significantly from control at ($p \leq 0.05$)

Values with 'b' superscript on the same column differ significantly from Cd-only treated group

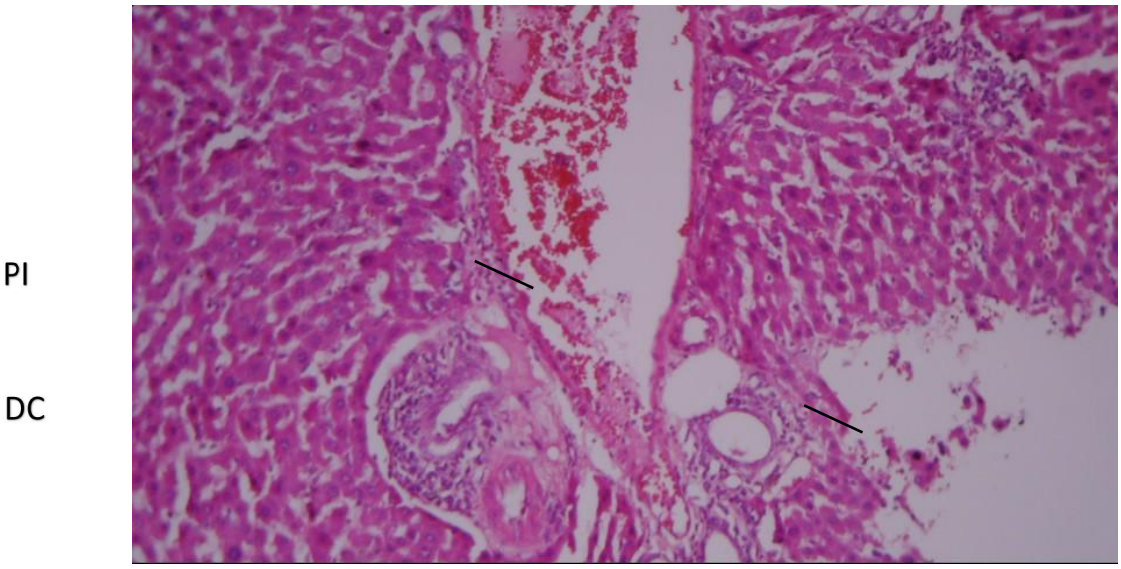
Cd: Cadmium, bwt: body weight, CEE: *C.esculentus* extract

3.3 Histopathological Evaluation of Rat Liver

Histopathological study showed that the treatment with cadmium caused severe liver damage including severe vascular ulceration, heavy periportal inflammatory infiltration and blood congestion when compared with control liver. The *cyperus esculentus* extract ameliorated hepatic damages caused by cadmium. However, 2ml/kg body-weight of the extract histopathological slide revealed mild periportal inflammation (hepatitis) and zonal necrosis relative to control. While, 4ml/kg body-weight completely ameliorated the hepatic damage induced by cadmium to its normal level relative to control. These histopathological alterations are indicated in plates 3.1 to 3.4.



(1)



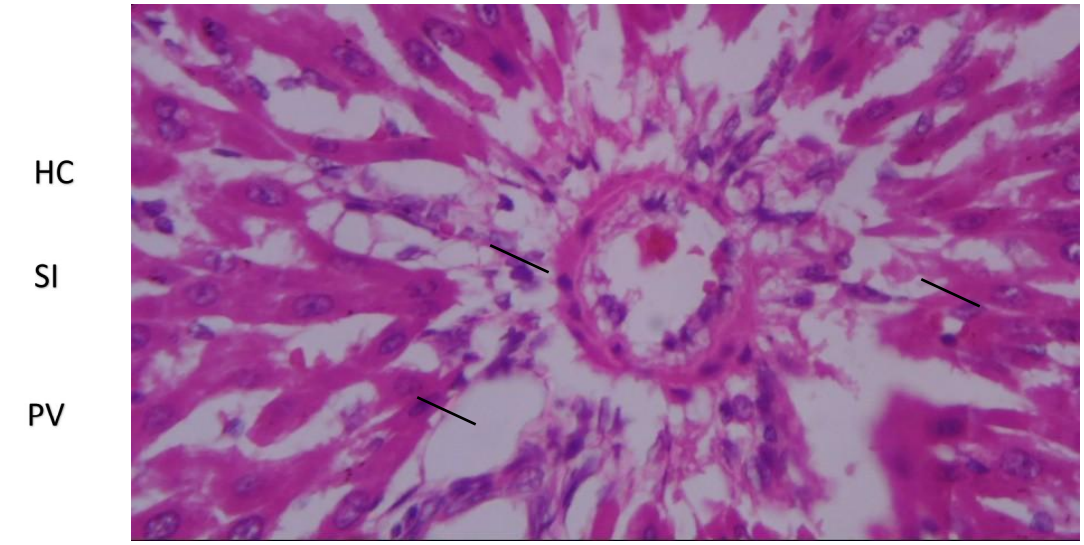
(2)

Plate 3.1 Histological section through the liver in normal rat (1) and in (2), rats orally treated with cadmium only (3mg/kg body weight).

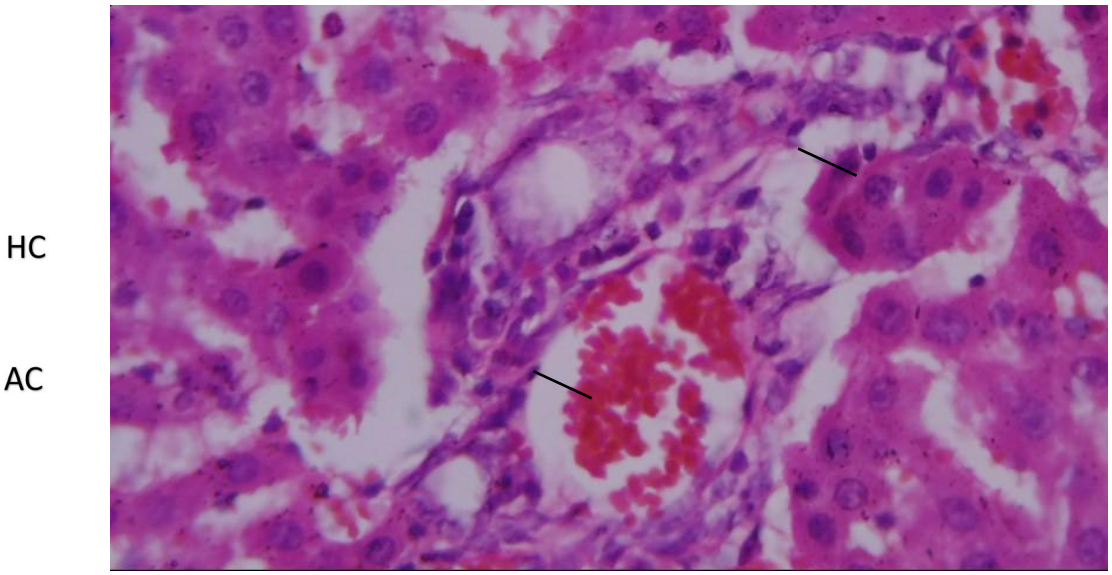
The hepatocytes, sinusoids and central vein were well preserved in control (1). In contrast histological section in (2), treated with cadmium only, show periportal infiltrates of inflammatory cells, zonal necrosis, vascular ulceration and blood congestion. (Hematoxylin & Eosin staining x 400)

Results showed in (1): HC: hepatocytes, SI: sinusoids and PV: portal vein. In contrast to (2):

PI: periportal infiltrates of inflammatory cells, DC: severe portal vascular dilatation and congestion.

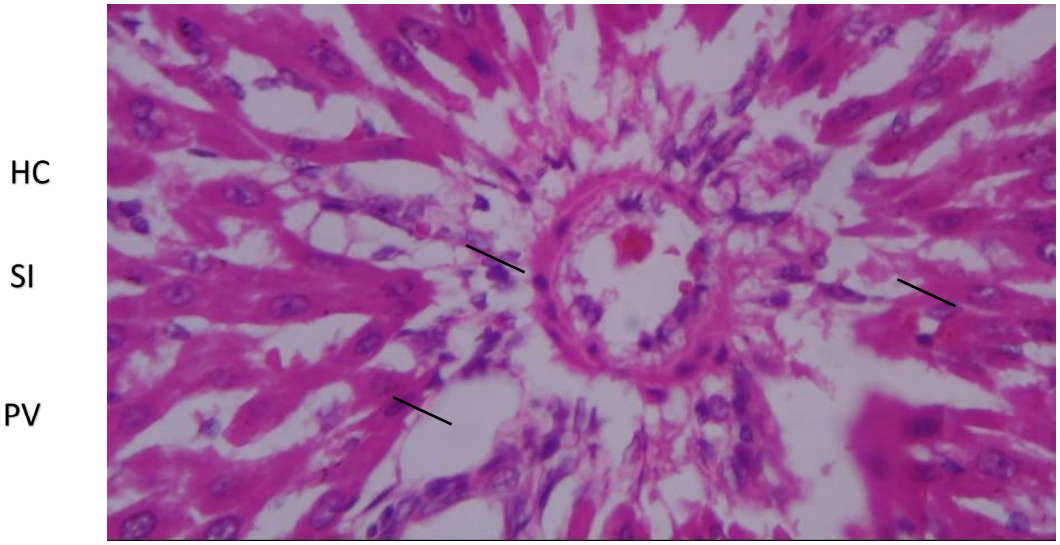


(1)

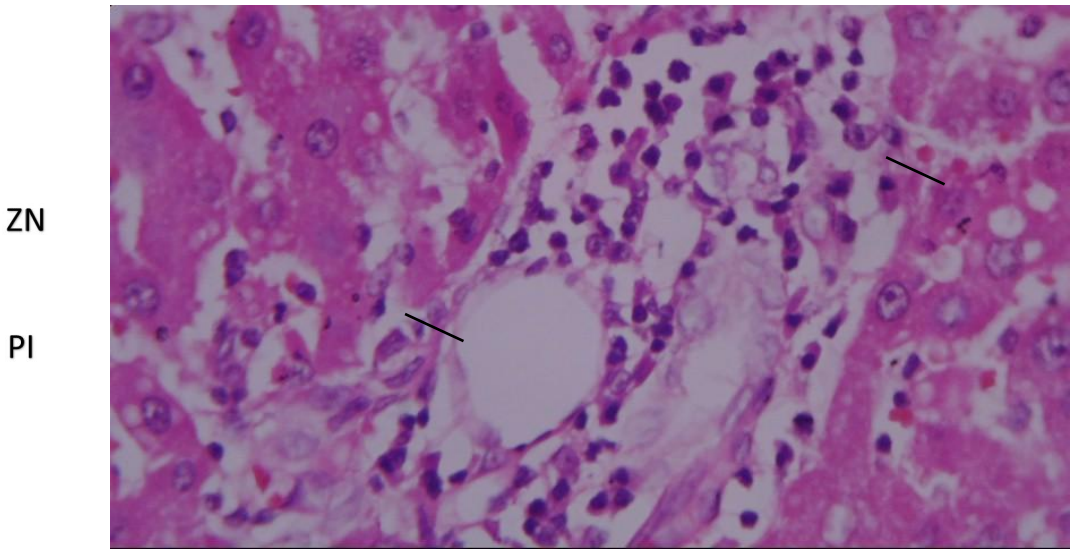


(3)

Plate 3.2 Histological section through the liver in normal rat (1) and in (3), rats orally treated with *Cyperus esculentus* only (4mL/kg body weight)



(1)

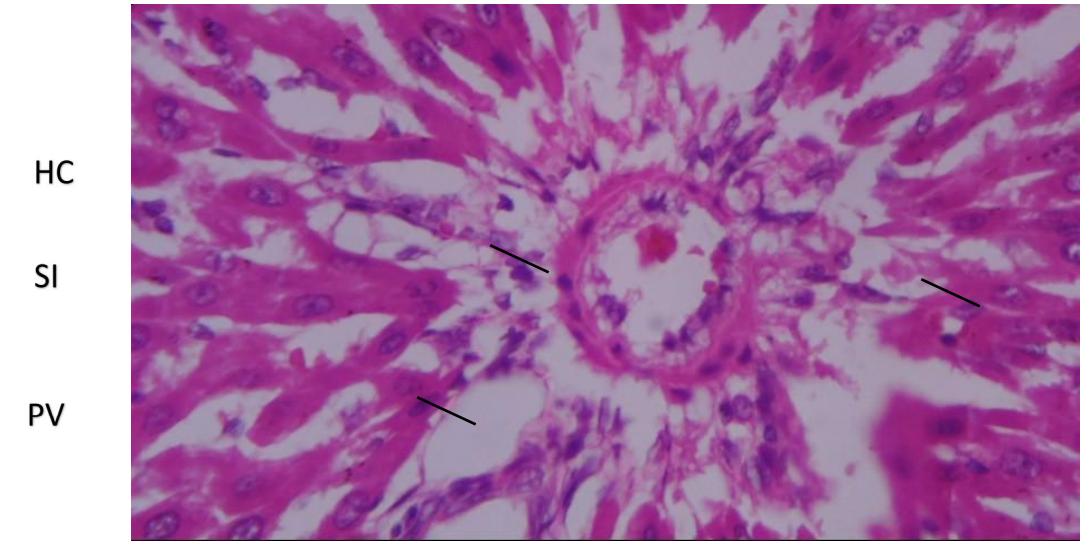


(4)

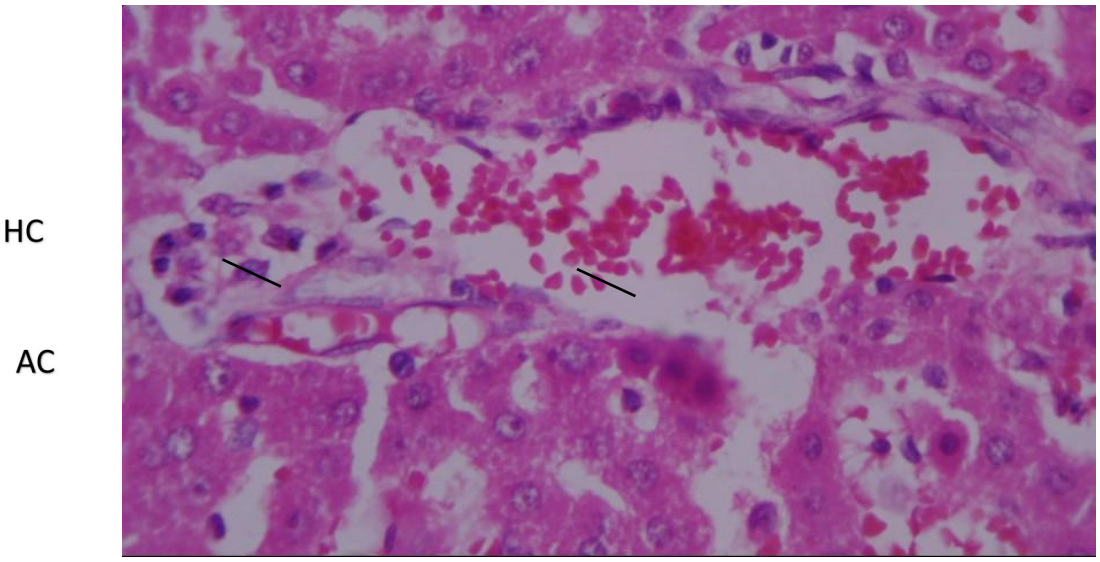
Plate 3.3: Histology of the liver in normal rat (1); in rats orally cotreated with cadmium and 2ml/kg body weight extract (4) (Hematoxylin and Eosin x 400)

The hepatocytes, sinusoids and central vein were well preserved in control (1). In contrast histological section in (4), rat cotreated with cadmium and 2mL extract, showed a mild reduction in inflammation, kupffer cell activation and zonal necrosis.

Results showed in (1): A: hepatocytes, SI: sinusoids and PV: portal vein. In contrast to (4): PI: mild periportal infiltrates: ZN: zonal necrosis.



(1)



(5)

Plate 3.4: Histology of the liver in normal rat (1); in rats orally cotreated with cadmium and 4mL/kg body weight extract (5) (Hematoxylin and Eosin x 400)

The hepatocytes, sinusoids and central vein were well preserved in control (1). In contrast histological section in (5), rat cotreated with cadmium and 4mL extract, showed normal hepatocyte, no hepatic inflammation and normal vascular architecture.

Results showed in (1): HC: hepatocytes, SI: sinusoids and PV: portal vein. In contrast to (5): HC: normal hepatocytes and AC: vascular architecture.

CHAPTER FOUR

DISCUSSION

Cadmium is a toxicant that has been shown to be toxic to the liver. Cadmium induces hepatotoxicity through injury in the liver cells and causes apoptosis and oxidative stress (Rani *et al.* 2014).

It has been suggested that measuring the weights of organs and comparing them to the weight of the entire body as a way to better understand how various experimental conditions affect the health of the test animal (Adeyemi *et al.,* 2015). In the study, there was a significant decrease in the liver weight at the end of the experiment in rats treated with cadmium. This could be caused by oxidative stress in the liver, which can lead to a decrease in the activity of enzymes involved in energy production and metabolism. This can cause a reduction in the production of ATP (adenosine triphosphate), which is the main source of energy for cells. A decrease in ATP production can lead to a decrease in cell viability and, ultimately, a reduction in liver weight. Another reason could also be caused by the induction of cell apoptosis (cell death) and autophagy (a process that breaks down and recycles cellular components) in the liver due to cadmium exposure. Additionally, cadmium exposure can cause changes in the expression of genes involved in lipid metabolism, which could lead to a decrease in liver weight. Finally, cadmium has been shown to inhibit protein synthesis in the liver, which could also contribute to a reduction in liver weight (Andjelkovic *et al.,* 2019). The rats in the groups co-treated with cadmium and *Cyperus esculentus* extract (2 mL and 4 mL/kg body weight) showed a significant Increase in their relative liver weight relative to cadmium treated. This could be because cadmium causes an increase in hepatocyte (liver cell) hyperplasia, which is an increase in the number of cells in the liver. Another possibility is that cadmium causes an increase in hepatocyte hypertrophy, which is an increase in the size of the liver cells. It's also possible that cadmium causes an increase in the amount of collagen

in the liver, which can cause an increase in relative liver weight (Samir *et al.,* 2013). Cellular enzymatic antioxidants such as SOD and CAT are the first line of defense suppressing the formation of free radicals inside the cells during Cadmium induced toxicity. Cadmium contributes to the adverse effects of organisms probably because of its ability to induce oxidative stress through alterations in the activities of antioxidant enzymes Catalase and Superoxide dismutase. The reduced level of SOD, observed in the Cadmium only treated group may be due to the fact that SOD being metalloenzyme, its Zn2+ ions has been replaced by Cadmium. The significant reduction in the levels of SOD and CAT in the Cd administered group may be attributed to a devastating oxidative alteration of enzymatic proteins and bio-membrane lipids by reactive oxygen species (ROS). Treatment with aqueous extract of *C. esculentus* resulted in a significant elevation in the activities of these antioxidant enzymes. Tiger nuts contains high amount of Vitamin E and C which are important antioxidants hence may have been responsible for the observed antioxidant effects recorded in this study. A significant increase in Catalase activity in *C esculentus* treated groups might because the extract is having a protective effect on the liver. This increase could be due to the antioxidant properties of tiger nut extract, which help to reduce oxidative stress and prevent damage to the liver. It's also possible that tiger nut extract could increase the production of catalase, leading to an increase in the enzyme's activity. This result is in contrast to the findings of Innih *et al.,* (2021) who reported a decrease in Catalase activity probably due to the interaction of a nitrogen atom of the imidazole ring with Cd^{2+}ion. However, more research is needed to confirm these possible mechanisms.

Specifically, the increased levels of MDA, which is a marker of lipid peroxidation observed in this study match the findings of other studies that have measured MDA levels in rats exposed to cadmium (Huang *et al.,* 2019). An increase in free oxygen radicals can cause excessive lipid peroxidation, causing oxygen damage in the tissues (Hursitoglu *et al.,* 2021),

this may be the cause of the significant increase in MDA activities of the cadmium only treated group. The groups treated with *C esculentus* extract showed a significant decrease in MDA activity, a decrease in MDA activity suggests that the liver is not being damaged by oxidative stress, which is likely due to the antioxidant properties of the tiger nut extract. In other words, the tiger nut extract may be helping to protect the liver from the damaging effects of cadmium. The liver histological section of animals co-treated with cadmium and 4mL/kg body weight extract revealed normal hepatocytes, vascular architecture and no hepatic inflammation. *Cyperus esculentus* was shown to attenuate the hepatotoxicity of cadmium in a dose dependent fashion. The mechanism by which this effect was exerted is unknown. However, it can be attributed to the presence of certain phytochemicals which have hepatoprotective properties. These properties may be attributed to the presence of flavonoids and polyphenols in the plants (Johnson *et al,* 2003).

Conclusion

From this study, it can be concluded that cadmium is highly toxic and can cause injury to the liver. The hepatotoxicity of cadmium is linked to increased oxidative stress, cell apoptosis and zonal necrosis with a little to no alteration in plasma liver enzymes. The Tiger Nut extract helped in ameliorating hepatotoxicity induced by cadmium by restoring the morphology of the liver in a dose dependent manner. This study found that treatment with medium and high doses of tiger nut extract can help to prevent oxidative stress and damage to the liver caused by cadmium toxicity. This suggests that tiger nut extract has the potential to protect against the negative health effects of cadmium exposure.

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APPENDICES

Appendix A

Stock preparation of Cadmium (Cd)

The salt used is Cadmium Chloride Monohydride $CdCl_2.H_2O$

Cadmium = 112.4 g

Chloride = 71 g

H_2O = 18 g

Total = 201.4 g

112.40 = 201.4~~g of $CdCl_2$~~.H_2O

3 mg Cd = x

$x = \frac{3mg \times 201.4g}{112.40g}$

x =5.38 mg of $CdCl_2H_2O$

Stock Solution:

1 mL = 5.38mg (Cd) in normal saline

10 mL = x

x = 10 × 5.38

= 53.8 mg

= 0.054 (Cd) in 10 mL normal saline

Conc = 3mg /kg bwt.

A rat of 1000 g = 3 mg of Cd

200 g = x = 200× 3/1000 =0.6 mg

1mL = 5.38 mg Cd

Z = 0.6 mg

z =1mL ×0.6 mg /5.38 mg

z = 0.111 mL

1 mL = 100 units

0.111m = a

a =11.1 units

Cadmium Dosage

Weight (g) = x

Amount of Cd (mg) = x = 0.003x

Amount of cd in mL Y = 0.003x/5.38

Amount in unit = 0.003x/5.38×100

Extract Dosage

Weight (y)

Administration dose (mL) = 4×y/1000

Dose in units = 4×y/1000×100

Appendix B

Statictical Weight Of Rat

T	LABEL	INITIAL	WEEK 2	WEEK 3	WEEK 4	FINAL
GROUP 1 CONTROL	H	194.11	201.44	205.12	192.22	192.77
	RH/LH	126.97	148.14	157.75	151.69	150.18
	H/LH	181.38	186.63	197.30	185.15	182.58
	ABD	192.46	221.75	241.46	239.37	230.38
	RH	157.30	170.46	181.76	185.03	183.34
	RL	184.46	199.58	216.09	200.74	206.66
	ULB	168.37	186.98	200.41	184.42	187.34
GROUP 2 Cd only	H/B	182.42	197.88	206.81	192.22	188.23
	H/T	145.80	176.50	176.40	181.33	170.11
	H/ABD	195.87	176.07	185.16	185.97	179.97
	H/RH	150.22	102.78	110.83	119.44	125.21
	H/LL	190.56	183.53	186.45	184.12	189.01
	H/RL	182/86	180.17	181.66	175.27	181.23
	B	142.89	162.73	177.27	181.50	165.37
GROUP 3 CE_4	RH/RL	172.14	202.44	211.28	231.46	238.97
	RH/B	125.46	132.78	137.83	150.07	161.54
	RH/T	168.69	171.94	173.94	187.33	185.62
	RH/LL	162.55	168.85	167.42	174.48	180.71
	ULB	190.74	193.76	194.17	208.76	211.18
	RH/ABD	168.83	181.46	185.12	186.82	202.13
	T	204.30	204.79	213.42	220.58	231.42

GROUP 4 Cd + CE_2	H	164.24	158.39	165.46	161.40	157.03
	T	193.99	178.08	177.73	183.38	185.65
	B	167.81	151.84	144.79	154.71	164.37
	H/T	203.97	195.33	217.89	237.89	242.03
	B/ABD	159.98	154.85	157.79	156.57	162.49
	RH/LL	116.01	118.37	120.69	119.44	112.60
	RH/RL	184.03	189.57	190.45	190.89	190.36
GROUP 5 Cd + CE_4	RL/H	153.48	142.03	152.65	156.59	162.36
	RL/B	194.64	181.49	178.48	167.79	130.14
	RL/T	149.90	158.55	156.54	146.75	145.59
	H/ABD	208.30	190.36	197.73	199.58	197.43
	B/RH	181.35	157.74	162.08	159.25	165.77
	ULB	152.80	137.03	157.05	133.65	141.54
	RH/LL	169.79	173.82	169.74	156.02	161.97

Appendix C

Statistical Analysis

SOD

SOD	**Control**	**Cd**	**CEE**	**CEE_2**	**CEE_4**
	5.37	5.21	5.45	3.73	1.57
	6.21	5.69	6.58	3.34	1.67
	4.99	5.04	6.48	3.41	1.63
(X)	5.52	5.31	6.17	3.49	1.62
S.D	0.51	0.27	0.51	0.17	0.04
ΣX	16.57	15.94	18.51	10.48	4.87
ΣX^2	92.3	84.9	114.9	36.7	7.91

$CT = \frac{(\Sigma\Sigma X)2}{n} = 293.6$

$TSS = \Sigma X^2 - CT = 43.11$

$TrSS = \frac{\Sigma\Sigma X^2}{n} - CT = 41.3$

ESS = TSS – TrSS = 1.81

Degree Of Freedom df

Total df = N - 1

Tdf = 15 – 1 = 14

Trdf = r -1

= 5 – 1 = 4

Edf = Tdf – Trdf = 14 - 4 = 10

Mean Square MS

TrMS = $\frac{TrSS}{Trdf} = \frac{41.3}{4} = 10.3$

EMS = $\frac{ESS}{Edf} = \frac{1.81}{10} = 0.181$

F.value = $\frac{TrMS}{EMS} = \frac{10.3}{0.181} = 56.90$

ANOVA TABLE

Sources of Variation	SS	**Df**	**MS**	**F**	**F.crit**
Between	4.13	4	10.3	56.90	3.48
Within	1.81	10	0.181		
Total	5.94	14	10.481		

F critical > F value

There is significant difference and the null hypothesis is rejected

LSD

LSD = $\sqrt{\frac{2(0.181)^2}{3}} \times 2.228 = 0.327$

From t table,

$t_{(p=0.05)(Edf)} = 2.228$

For Comparison,

G1 and G2, G1 and G3, G1 and G4, and G1 and G5 are being compared

G1 and G2,

5.52 – 5.31 = 0.21 (No significant difference)

G1 and G3,

6.17 – 5.52 = 0.65 significant difference

G1 and G4,

5.52 – 3.49 = 2.03 Significant difference

G1 and G5,

5.52 – 1.63 = 3.89 Significant difference

Comparing with group 2,

G2 and G3,

6.17 – 5.31 = 0.86

G2 and G4,

5.31 – 3.49 = 1.82 Significant difference

G2 and G5,

5.31 – 1.62 = 3.69 Significant difference

CAT

CAT	**Control**	**Cd**	**CEE**	**CEE_2**	**CEE_4**
	0.12	0.076	0.12	0.18	0.14
	0.11	0.068	0.12	0.16	0.13
	0.12	0.069	0.11	0.18	0.14
(X)	0.11	0.07	0.11	0.17	013
S.D	0.008	0.003	0.008	0.01	0.01
ΣX	0.35	0.21	0.35	0.52	0.41
ΣX^2	0.04	0.01	0.04	0.09	0.05

CT = $\frac{(\Sigma\Sigma X)^2}{n}$ = 0.22

TSS = ΣX^2 – CT = 0.02

TrSS = $\frac{\Sigma\Sigma X^2}{n}$ – CT = 0.016

ESS = TSS – TrSS = 0.0005

Degree Of Freedom df

Total df = N - 1

Tdf = 15 – 1 = 14

Trdf = r -1

= 5 – 1 = 4

Edf = Tdf – Trdf = 14 - 4 = 10

Mean Square MS

TrMS = $\frac{TrSS}{Trdf} = 0.004$

EMS = $\frac{ESS}{Edf} = 5.05$

F.value = $\frac{TrMS}{EMS} = \frac{0.004}{5.05} = 81.80$

ANOVA TABLE

Sources of Variation	SS	**Df**	**MS**	**F**	**F.crit**
Between	0.016	4	0.004	81.80	3.48
Within	0.0005	10	5.05		
Total	0.021	14	5.054		

F critical > F value

There is significant difference and the null hypothesis is rejected

LSD

LSD = $\sqrt{\frac{2(5.05)^2}{3}} \times 2.228 = 9.19$

From t table,

$t_{(p=0.05)(Edf)} = 2.228$

For Comparison,

G1 and G2, G1 and G3, G1 and G4, and G1 and G5 are being compared

G1 and G2

0.11- 0.07 = 0.04 No significant difference

G1 and G3,

0.11 – 0.11 = 0 No significant difference

G1 and G4,

0.11 – 0.17 = -0.06

G1 and G5,

0.11 – 0.13 = -0.02

Comparing with G2,

G2 and G3,

0.07 – 0.11 = -0.04

G2 and G4

0.07 – 0.17 = -0.1

G2 and G4,

0.07 and 0.13 = -0.06.

MDA

MDA	**Control**	**Cd**	**CEE**	**CEE_2**	**CEE_4**
	0.63	0.67	0.76	0.54	0.55
	0.77	0.71	0.89	0.56	0.55
	0.72	0.65	0.76	0.55	0.52
(X)	0.70	0.67	0.8	0.55	0.55
S.D	0.05	0.02	0.06	0.01	0.02
ΣX	2.12	2.03	2.4	1.65	1.65
ΣX^2	1.51	1.37	1.93	0.90	1.65

CT = $\frac{(\Sigma\Sigma X)^2}{n}$ = 6.46

TSS = ΣX^2 – CT = 0.9

TrSS = $\frac{\Sigma\Sigma X^2}{n}$ – CT = 0.1386

ESS = TSS – TrSS = 0.026

Degree Of Freedom df

Total df = N - 1

Tdf = 15 – 1 = 14

Trdf = r -1

= 5 – 1 = 4

Edf = Tdf – Trdf = 14 - 4 = 10

Mean Square MS

TrMS = $\frac{TrSS}{Trdf} = 0.03465$

EMS = $\frac{ESS}{Edf} = 0.0026$

F.value = $\frac{TrMS}{EMS} = 13.3$

ANOVA TABLE

Sources of Variation	SS	**Df**	**MS**	**F**	**F.crit**
Between	0.1386	4	0.034	13.3	3.48
Within	0.026	10	0.0026		
Total	0.1646	14	0.0366		

F critical > F value

There is significant difference and the null hypothesis is rejected

LSD

LSD = $\sqrt{\frac{2(0.026)^2}{3}} \times 2.228 = 0.047$

From t table,

$t_{(p=0.05)(Edf)} = 2.228$

For Comparison,

G1 and G2, G1 and G3, G1 and G4, and G1 and G5 are being compared.

G1 and G2,

0.70 – 0.67 = 0.03 No significant difference

G1 and G3,

0.70 – 0.8 = - 0.1 Significant difference

G1 and G4,

0.70 – 0.55 = 0.15 Significant difference

G1 and G5,

0.70 – 0.55 = 0.15 Significant difference

Statistical Analysis of Relative Liver Body Ratio

R.W	**Control**	**Cd**	**CEE**	**CEE_2**	**CEE_4**
	1.57	1.63	1.34	2.81	2.96
	1.61	1.79	1.35	2.81	2.91
	1.68	1.79	1.36	1.85	2.79
(X)	1.62	1.73	1.35	2.49	2.88
S.D	0.04	0.07	0.008	0.45	0.07
ΣX	4.86	5.21	4.05	7.47	8.66
ΣX^2	7.86	9.06	5.46	19.2	25.0

$CT = \frac{(\Sigma\Sigma X)^2}{n} = 4.44$

$TSS = \Sigma X^2 - CT = 62.16$

$TrSS = \frac{\Sigma\Sigma X^2}{n} - CT = 61.46$

$ESS = TSS - TrSS = 0.7$

Degree Of Freedom df

Total df = N - 1

Tdf = 15 – 1 = 14

Trdf = r -1

= 5 – 1 = 4

Edf = Tdf – Trdf = 14 - 4 = 10

Mean Square MS

TrMS = $\frac{TrSS}{Trdf} = 15.365$

EMS = $\frac{ESS}{Edf} = \frac{1.81}{10} = 0.07$

F.value = $\frac{TrMS}{EMS} = 219.5$

ANOVA TABLE

Sources of Variation	SS	**Df**	**MS**	**F**	**F.crit**
Between	61.46	4	15.365	219.5	3.48
Within	0.7	10	0.07		
Total	62.16	14	13.435		