RESTORATIVE EFFECTS OF AZADIRACHTA INDICA ON CISPLATIN INDUCED NEPHROTOXICITY IN WISTER ALBINO RATS

BY

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DECLARATION

This research thesis is my original work and it has never been presented to any other institution. Where the work of other people has been included, acknowledgement of this has been made in a conference to the references noted.

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DEDICATION

I dedicate this thesis to my mother Mrs. Florence Khevali Mwangala, my sisters; Chilian Nerima, Ebby Ayuma, Mary Shisia, Edith Nakhone, Joyline Nafuna, Qureen Nafula.

ABSTRACT

Cisplatin is a cytotoxic drug commonly used worldwide in the treatment of cancer. Despite its effectiveness, studies have shown that it can cause nephrotoxicity in 20-30% of the population that use it, as a result, its administration is restricted. Few studies have been done to elucidate the restorative effects of Azadirachta indica in human patients experiencing nephrotoxicity secondary to cisplatin usage. In addition, there is paucity of data on the restorative effects of *azadirachta indica* on cisplatin induced nephrotoxicity. The broad objective of this study was to determine the restorative effects of azadirachta indica on cisplatin induced nephrotoxicity in Wister albino rats. The specific objectives were to evaluate the histo- architectural and histo stereological restorative effect of *azadirachta indica* on cisplatin induce nephrotoxicity at varied dosage, to determine the gross histo-morphological changes on cisplatin induced nephrotoxicity, to determine the restorative effect of *azadirachta indica* on cisplatin induced nephrotoxicity by assessing the renal biomarkers of acute kidney injury. The study used a posttest-only experimental design. Adult albino rats were bred under microbiologically controlled conditions for all the experiments and control groups. A modified human resource equation was used to determine the sample size, A total number of 25 adult Wister albino rats were selected randomly and used in the study. The 25 albino rats were randomly assigned into two main groups of 5 control and 20 experimental. The 20 experimental Wister albino rats were further assigned into four experimental subgroups of 5 each and received water, rats' pellets *ad libitum, Azadiracta indica* and cisplatin according to the experimental design below. The control group was not administered with any drug, received only water and rat pellets *ad libitum*. The group one from the experimental were only administered with cisplatin 0.28mg/kg at the beginning of the experiment. Group two, three and four were administered with cisplatin 0.28mg/kg at day one followed by low dose of 3.33mg/kg, medium dose 5.0mg/kg and high dose 6.67ml/kg of *Azadirachta indica* respectively at day five of the experiment, all animals were humanely sacrificed on day thirteen. The kidney tissues were prepared, stained using H & E and examined under 100X magnification using BP Olympus microscope. The photomicrographs were taken, uploaded and stored on a flash disk, Data were entered into the excel sheet and were analyzed using the SPPS version 27. There was a significance ($P< 0.0001$) increase in Urea & creatinine levels of the experimental GP1, 2 and 4 as compared to the control. There was significant ($P \leq 0.001$, 0.001 and 0.003 respectively) reduction in mean body weight of experimental GP1, 2, and 4. There was significant ($P < 0.0001$**) reduction in the mean length, width, volume, thickness and weight of the kidney for experimental group as correlated with the control group. The surface area of the glomerulus of experimental GP1, GP2 and GP4 had a significant ($P < 0.0001$**) reduction. The medium dose of *Azadiracta indica* of 5mg/kg was able to restore cisplatin-induced nephrotoxicity among the Wister albino rats. There was a significance increase in the surface area of the bowman's capsule, glomerulus space and reduction in glomerulus in cisplatin-induced nephrotoxicity among the Wister albino rats.

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LIST OF ABBREVIATIONS

AKI	Acute kidney injury
ANOVA	Analysis of variance
DF	Degree of freedom
KG	kilogram
LD_{50}	Lethal dosage
MG	Milligram
TNFR	Tumor necrosis receptors
TNFα	Tumor necrosis factor alpha
H & E	Haematoxylin and Eosin
SPSS	Statistical Package for Social Science
NGAL	Neutrophil gelatinase associated lipocalin
TIMP-1	Tissue inhibitor of metalloproteinase 1
GDF -15	Growth differentiation factor 15
KIM-1	Kidney injury molecule 1
DPX	Dibutylphthalate polysterene xylene

OPERATION DEFINATION OF TERMS

Nephrotoxicity : Is the fast act of the kidney to lose their function as a result of the toxic substance

Restorative : Bring back the original self after damage.

Azadirachta Indica : Local herb of indian origin that contain physiological properties that can attenuate or counteract Cisplatin induce nephrotoxicity

Cisplatin : Cytotoxic drug used in treatment of several tumors i.e neck cancers and it has been known to cause nephrotoxicity

Archimedes principle **:** The volume of water displaced is equal to the volume of the body that is completely submerged to it

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CHAPTER ONE

INTRODUCTION

1.1 Background Information

Restorative effects are regenerative process of a cell or tissue of an organ after it has been damaged by a toxic substance. So many factors have been associated with restoring kidney after it has been damaged such zinc and selenium following oxidative renal damage (Ping Xiao *et al.,* 2002), *Azadirachta Indica* has been associated with restoring the kidney following ibuprofen nephrotoxicity (Abireh *et al.,* 2020), physalis angulate has also been associated with restoring the damaged kidney caused by the allaxon (Adewoye *et al.,* 2016).

Neem tree (*Mwarubaini*) is a local herb traditionally used to treat asthma, constipation, coughing, diabetes, stomach ulcers, intestinal worms, skin diseases, indigestion and urinary tract infection. This herb is believed to be originating from India but is found in many parts of the world and in Kenya, it's found abundantly locally almost in all regions (Yao *et al.,* 2007). It has less side effects and should be used with precaution in pregnancy because it is associated with miscarriage. It also increases the sensitivity of the immune system therefore increasing the severity of the symptoms of the autoimmune diseases. Other side effects include drowsiness, seizures, loss of consciousness and coma (Wazarkar *et al.,* 2020).

The therapeutic effect of the *Azadirachta Indica* is dependent on its active components to treat specific ailments. The nimboline ascobate and the azadirachtin are responsible for the antidoxidant effect (Dubey & Kashyap, 2014).

Azadirachta Indica is significantly associated with the protection of the kidney from the cisplatin induced toxicity in the following way: it tends to reduce the urea, serum acid and creatinine levels in blood. It upholds the levels of the glutathione and activities of the glutathione s- transferase, superoxide dismutase, glutathione reductase, catalase and

glutathione peroxidase, it reduces the increase in nitric oxide and melondialdehyde then lastly reducing the expression of the nuclear factor kappa B hence reducing the apoptosis of in kidney (Ahmed *et al.,* 2014).

Cisplatin is commonly used in the Kenya and the worldwide. A cytotoxic drug is used alongside other drugs for chemotherapy purposes. Despites its clinical effectiveness, it is still restricted and is used in the treatment of ovarian cancer, breast cancer, esophageal cancer, Head and neck cancer, testicular cancer, cervical cancer, brain cancer, mesothelioma, lung tumors and neuroblastomas. It has side effects that include; kidney effects, hearing loss, change in taste, anemia, bleeding and the risk to acquire infections (Hanusova *et al.,* 2015).

Nephrotoxicity is the process of the kidney cells being damaged (Al-Naimi *et al.,* 2019). Renal damage is associated with many things such as food we take, medications that are metabolized there and other substances. Cisplatin causes nephrotoxicity by releasing free radical molecules that stimulate the distraction of the kidney cells (Miller *et al.,* 2010). This free radical molecule will cause the oxidative stress to the kidney hence achieving the nephrotoxicity (Gyurászová *et al.,* 2020).

1.2 Statement of the problem

Kidney disease is among the leading cause of death with a prevalence of 13% worldwide (Lv & Zhang, 2019) and over 4 million Kenyans are suffering from renal disease (Kabinga *et al.,* 2019) . Cisplatin is a cytotoxic drug used alongside other chemotherapy medications. Despite cisplatin being effective, 20-30% of patients using it develop kidney disease (Burns *et al*., 2021). So, cisplatin is used with precaution and sometimes restricted because of its known side effect of nephrotoxicity. Among the elderly, the incidence is high up to 65% of the patient’s taking cisplatin develop nephrotoxicity (Latcha *et al.,* 2016). Cisplatin produces free radical oxygen that damages the mitochondria at the tubules of the nephrons hence causing

nephrotoxicity (Cooper *et al.,* 2001). *Mwarubaini* destroys the free radical oxygen in the circulation thus helping to attenuate and counteract the effects of cisplatin. However, there is paucity of data showing the restorative histo-architectural and histo- morphological changes on damaged kidney mediated by *Mwarubaini* on cisplatin induced nephrotoxicity.

1.3 Justification

The physiological study of the azadiracta indica on the renoprotectivity activity following cisplatin induced nephrotoxicity has been done but there is inadequate information on restorative effects (Abdel Moneim *et al.,* 2014).

The restorative renal biochemical parameters are also missing on cisplatin induced nephrotoxicity mediated by azadiracta indica (*Mwarubaini)*

The Wister albino rats have a long-standing history of withholding medicinal scientific studies (Bailey *et al.,* 2014).

This study will help to show that *Azadirahcta indica* (*Mwarubaini*) can help to restore the kidney after it has been damaged by cisplatin. This will help to save the lives and complications of the people using the drug. This will increase the uptake of drugs within the population affected and also improve their quality of life.

1.4 Significance of the study.

The study confirmed the restorative effect of *Azadiracta Indica* on cisplatin induced nephrotoxicity, it is now of beneficial to the population using the cisplatin since neem tree is highly available locally.

1.5 Objectives

1.5.1 Broad objectives

To evaluate the restorative effects of *Azadiracta Indica* on cisplatin induced nephrotoxicity among Wister albino rats

1.5.2 Specific objectives

1. To determine the renal biochemical parameters following administration of cisplatin and *Azadiracta Indica* among the Wister albino rats.
2. To assess the gross, histo- morphological and histo-cyto-architectural injuries in cisplatin induced nephrotoxicity among the Wister albino rats.
3. To determine the gross and histomorphometry restorative of the kidney on administration of cisplatin followed by different doses of *Azadiracta Indica* among the Wister albino rats.
4. To determine the restorative histo- structural changes and stereological of the kidney on administration of cisplatin followed by different doses of *Azadiracta Indica* among the Wister albino rats.

1.6 Hypotheses

Ho_1: There is no significant change in renal biochemical parameters following administration of cisplatin and *Azadiracta Indica* among the Wister albino rats.

Ho_2: There is no change in the gross histo- morphological and histo-cyto-architectural injuries in cisplatin induced nephrotoxicity among the Wister albino rats.

Ho_3: There is no change in the gross, histomorphometries restorative of the kidney on administration of cisplatin followed by different doses of *Azadiracta Indica.*

Ho_4: There is no change in the restorative histo-stereological changes of the kidney on administration of cisplatin followed by different doses of *Azadiracta Indica.*

1.7 The study model Assumptions,

Adoption of the adult albino rats in the study would replicate the similar effect on human based on the documented close association in terms of biology, mechanism features and function.

1.7 Study limitation and de-limitation

Limitation of this study was that Wister albino rats was the only experimental animal used to evaluate the restorative effects of *Azadiracta Indica* on cisplatin induced nephrotoxicity. The delimitation is that even within the same species, similar disparities can be found among different sexes, breeds, age and weight ranges, and ethnic backgrounds.

CHAPTER TWO

LITERATURE REVIEW

2.1 Introduction

The cisplatin has been researched worldwide together with the azadiracta indica. The literature of the physiological properties of the azadiracta indica on how it can attenuate and nephroprotect the cisplatin induced nephrotoxicity is highly available

2.2 Cisplatin structure and mode of action in inducing nephrotoxicity

2.2.1 cisplatin structure

Cisplatin is also known as a cis-diamminedichloroplatinum (ii). Its molecular formula is $Cl_2H_6N_2Pt^{+2}$. Structurally is made up of two choro ligands that surrounds the central platinum ion, it also has two amine ligands that also surrounds the platinum ion.(Jeschonek *et al.,* 2012).

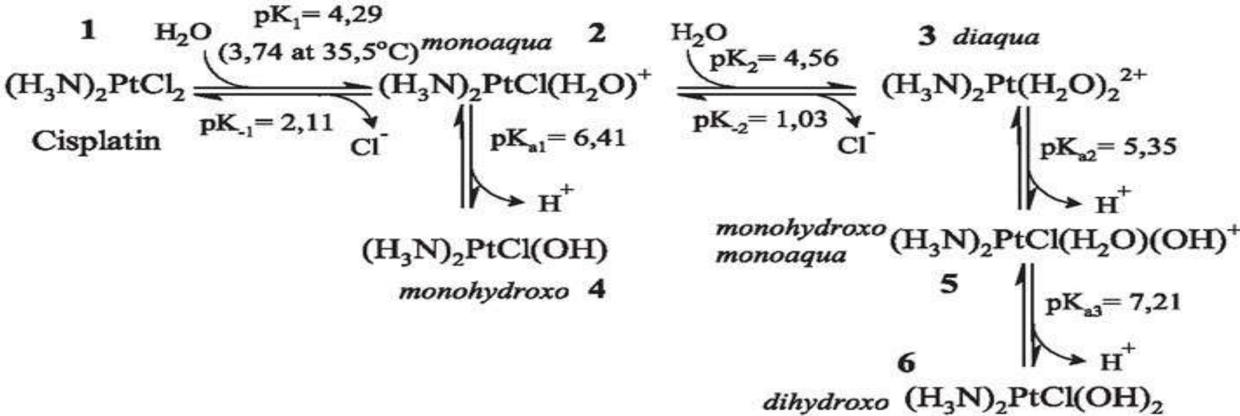

Figure 2.1: Showing the formation of cisplatin structure adopted from (Makovec, 2019).

2.2.2 Mode of action of cisplatin in inducing nephrotoxicity

The molecular mechanism of the cisplatin inducing nephrotoxicity has been put in many ways. The high dose of cisplatin can cause necrosis of the tubular cells and the low concentration of the cisplatin causes apoptosis of the renal cells using cultured renal tubular cells (lieberthal *et al.,* 1996). In Vivo in animals, the relationship between apoptosis and necrosis in cisplatin induced tubular injury; necrosis is induced by direct toxic injury and it can be postmortem

result of apoptosis known as secondary apoptosis (Yao *et al.,* 2007)

Various pathways that trigger apoptosis of the tubular cells in cisplatin have been studied; the intrinsic and the extrinsic pathways. The extrinsic pathways is mediated by the death of the receptors due to binding of Ciplastin at the plasma membrane. This death of receptor leads to the activation of the caspases-8, that results in downward activation of the caspases to induce apoptosis. The receptor cells that die are tumor necrosis receptor 1 and two (TNFR), tumor necrosis factor α (TNFα) and Fas (Vickers *et al.,* 2004).

The intrinsic pathway also known as the mitochondrial pathway is mediated by the cellular stress resulting in the activation of the Bak and Bax proapoptotic Bcl-2 family protein. This protein family (Bak and Bax) makes the outer membrane of the mitochondria to be porous leading to the activation and release of the apoptogenic factors from the organelles. These apoptogenic factors; cytochrome c, AIF (apoptosis –inducing factor), Smac/DIABLO and endonuclease G binds to the adapter protein A paf- A and induces a conformational change. The conformational change leads to the activation of the caspases -9 which in turn leads to downward stream of caspase- dependent apoptosis. When Smac is released to the cytosol, it binds to the caspases inhibitor protein and IAPs antagonizes them (Townsend *et al.,* 2003)

Cell cycle regulation plays a major role in cisplatin nephrotoxicity. The cyclin –dependent kinase (cdk), its inhibitor and the P21 are key in cell cycle regulation. In cisplatin nephrotoxicity, there is inactive tubular cells cycle known as proliferating cell nuclear antigen staining and Brdu incorporation within the nucleus. The P12 is induced during the cisplatin nephropathy through a p53 – independent and dependent mechanism (Miller *et al.,* 2010).

2.3 Azadiracta indica components and postulated protective mechanism of the kidney cells

The azadiracta indica leave extract together with the flower, fruits and stem barks were evaluated and it found out that they contain high antioxidant activity (Smith *et al.,* 2005).

The azadiracta indica leave extract has many active components and that has specificity in terms of performing different function. The active compounds are; 6-desacetylnimbinene, nimbin, amino acid and n-hexacosanol, 17-hydroxyazadiradione, 7-desacetyl-7-benzoylazadirione, nimbanene, 7-desacetyl-7-benzoylgedunin, 17-hydroxyazadiradione, ascorbic acid, nimbandiol and nimbolide. This active component may work in combination or singularly to produce many health benefit (Biswas *et al.,* 2002).

Hossain et al 2013 discovered antioxidant activity of azadirachta indica that free radical molecules in the circulation and its repective phytochemical components. This free molecule in the circulation may be the genesis of various disease by attacking biological cells and at times causes organ damage. The active components in azadiracta indica responsible for the antioxidant activity are mainly nimboline, azadirachtin and ascorbate in the sequence of reductive potential.

Azadirachta indica plays a major role in regulation of apoptosis. The Bcl2, caspases -8 and the Bax protein play a role in inducing apoptosis in the tubular cell of the kidney (Othman *et al.,* 2011). The azadirahcta leaf extracts has been studied and has the potential to down regulate expression of Bcl-2 and it activates the caspases-8. This leads to reduction in apoptotic activity within the tubular cells (Vascularis, 2004).

There is paucity of data showing the restorative mechanism of azadirachta indica on restoring the kidney cells following cisplatin induced nephrotoxicity.

2.4 The patterns of nephrotoxicity metabolic pathway associated with cisplatin

The metabolism of the cisplatin takes place in the kidney and its dose administration is dependent on its nephrotoxicity. Different mechanisms and pathways have been investigated on how cisplatin causes nephrotoxity (Pinzani *et al.,* 1994).

High concentration of cisplatin induces necrosis of the inactive confluent monolayers of the tubular cells and the low dosage of the cisplatin induces cellular apoptosis via dependent pathway of caspase-9 (liberthal *et al.,* 1996). The inactive proximal tubule cells are highly affected as compared to the nonproliferating cells are less sensitive to DNA –toxic damaging agents (Fink *et al.,* 2000).

Rats have shown that the nephrotoxicity induced by the cisplatin can be inhibited by cysteine-s-congugate beta-lyase and gamma –glutamyl transpeptidase. These two enzymes activates the nephrotoxicity of a number of halogenated alkenes; tetrafluoroethylene, hexachloro-1, 3-butadiene and trichloroethylene (Cooper *et al.,* 2001). The metabolism of this halogenated alkenes compound is activated by the glutathione. The conjugate of glutathione cleaves to the cysteinylglycine-conjugates when passing on the surface of the tubular cells by gamma –glutamyl transpeptidase then bonds in the extracellular glutathione. Cysteinyl-glycine –conjugates are further broken down by the aminodipeptidase catalyzed reaction. The cysteine-conjugates are further taken to the proximal tubule cells to be metabolized into a highly reactive thiols by an enzyme called cysteine-s- conjugate beta-lyase. The high reactive thiol binds to the macromolecules in the proximal tubules cells causing an increase in free calcium in the cytosolic and this causes cell death (Chen *et al.,* 1992).

The nephrotoxicity that is associated with cisplatin, is involved with the alteration of the mitochondrial activity of the cortical and outer stripe of the medulla. The proximal tubules are

more affected than the distal tubules hence the maintaining the COX enzyme in human beings (Zsengellér *et al.,* 2012).

2.5. The comparative histo-morphological structure of the kidney of the rat and human

2.5.1 histo- morphological structure of the kidney of the rat

The histo – morphological structure of the kidney elucidated two parts, the inner medulla and the outer cortex. The renal pyramid is well arranged in between the cortex and medulla. Within distinct parts of the cortex and the medulla we got the nephrons. The first part of the nephron is found in the glomerulus, the second part is the Bowman's capsule, the third part is the proximal convoluted tubules and fourth part is the loop of Henle (Drees *et al.,* 2017).

The cortex consists of renal corpuscles which contain glomeruli, proximal convoluted tubules and distal convoluted tubules. The bowman capsule is composed of two layers, the outer inner visceral and inner parietal layer. The parietal layer is lined by the squamous epithelium that's continuous with the lumen of proximal convoluted tubule. The visceral layer of the cortex consists of the glomerular capillaries. (Vickers *et al.,* 2004)

2.5.2 Histo-morphological structure of the human kidney

The histological parenchymal cells of the kidneys are also made up of two parts; the medulla and the cortex. Almost the entire part of the parenchymal cells is made up of the urine collecting system called the nephrons. This nephron drains into the calyces. The cortex is dark in color and the medulla look striped. Within the medulla there is renal pyramids, when the renal pyramids are covered by cortical tissue they become renal lobe. Each renal lobe are divided into lobules that got a group of nephrons that empty specific places into the minor calyces. The minor calyces empty into the major calyces then into renal pelvis. The cortex has rich supply of blood and contains the renal capsule. The renal capsule contains the glomerular part of the nephron and the tubular system is located at the medulla (Rayner *et al.,* 2016).

The glomeruli have three key filtration apparatus; the visceral layer of the renal capsule, endothelium and the middle basement membrane. The basement membrane is complex and has three layers; lamina rara interna, lamina rara externa and a thick central lamina densa (Kang, 2013).

The tubular system is made up of three parts nephron loop, proximal and distal tubes. The proximal tube consist of the straight segments, its convoluted part is found within the renal cortex and all are covered by the simple cuboidal epithelium. The loop of henle is covered by the simple squamous epithelium with no microvilli (Ahmadmoradi *et al.,* 2012).

2.5.3 Histo- morphological comparative characteristics of the human kidney and rats when induced with cisplatin nephrotoxicity.

In a comparative study of the kidney of the rats and humans on cisplatin-induced nephrotoxicity, on rats it exhibited acute necrosis within the cortex and at the junction between the outer stripe of the medulla and the cortex. This necrosis was dependent on the vacuolation and swelling of epithelial lining and protrusion into the tubular lumina and tubular dilatation. The glomerulus were not affected but there was massive loss of the histoarchitectural and epithelial cells degeneration. In human the histological lesions were almost similar with the rats, however their alteration within the distal convoluted tubules in the cortex (Alisen *et al.,* 2004).

There is paucity of data suggesting the restorative histo-morphological and histo-stereological changes associated by cisplatin induced nephrotoxicity.

2.6. Restorative mechanism of Azadirachta indica

Low dose of azadirachta has been associated with reversal of the nephrotoxicity following abuse of non-steroidal anti-inflammatory drugs. Its resto-histological analysis showed normal renal capsule with no apparent histological changes alteration (Abireh *et al.,* 2020).

2.7 The biochemical indicators for nephrotoxicity induced my cisplatin

Several biomarkers have been studied and there changes have been associated as indicators for nephrotoxicity in either animal or human being, in vitro or vivo.

In vitro study; KM-1 TIMP-1 and calbindin protein level increase and NGAL, B_2-m, neutrophil gelatinase associated lipocalin, B_2 –microglobulin and cystatin were not affected in the condition media of HK-2 cells treated with cisplatin (Sohn *et al.,* 2013).

In vivo, leucine –rich- alpha-2- glycoprotein (LRG1), growth differentiation factor -15(GDF15) and secreted phosphoprotein 1 are potential biomarkers for acute kidney injury following cisplatin treatment. (Jiang *et al.,* 2020). KIM- 1 shows a positive association between the acute kidney injury and cisplatin treatment (Tanase *et al.,* 2019). Urinary KIM -1 is more sensitive to early development of acute kidney disease followed by the NGAL and cystastin, the serum creatinine is less good as compared those three detection of AKI following cisplatin treatment. (Abdelsalam *et al.,* 2018).

Presence of the cluterin and increased microalbumin in urine while reduction of albumin in serum. This implies injury to the proximal tubule as a result of the cisplatin induce nephrotoxicity.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study area

The study was done in Kenya at Maseno University located in Kisumu County situated at Maseno Kisumu – Busia Road. Breeding, weighing, handling and administration of azadiracta indica and cisplatin was done at the department of zoology in the school of biomedical sciences because of the well-established animal houses and expertise in handling the Wister (*Rattus norveginicus*) albino rats. The tissues were harvested and examined in the histology lab in the Department of human anatomy and blood sample examination was done in the Department of Clinical Studies, Faculty of Veterinary Medicine at University of Nairobi.

3.2 Study Design

An experimental study design where a post-test group of Wister albino rats (*Rattus norveginicus*) were used.

3.3 Study subject

The study subject was Wister albino rat's species of (*Rattus Norveginicus*) from a pure bread, the sample subjects were sourced from the department of zoology in Maseno University. The Wister albino rats (*Rattus norveginicus*) had long ears, short tail compared to their body size and their head was characterized to be wide as also suggested by (Pritchett & corning 2016).

The Wister albino rats (*Rattus norveginicus*) were preferred because of they; share 90% of the genome with human being, they have a relatively high survival rate, big body size as compared to mice but small and easy to take care of, they have a short gestational span (4 weeks) hence easier to find the study subjects, they are also resilient in withstanding most of the study medicine, male are always larger as compared to females approximately 450-650grams and 350-450 grams respectively (Bailey *et al.,* 2014).

3.4 Sample Size Determination

My sample size was calculated using the modified resource equation (Arifin & Zahiruddin, 2017)

$$DF = N - K = Kn - k = k\,(n-1)$$

by rearranging this formula n is given as: $n = DK/k + 1$

Where; N= total number of subjects, k = the total number of groups to be used in the study, n= is the total number of subjects to be used in a group and DF= is the acceptable range of degrees of freedom.

The study had five groups; my sample size was as follows:

I used the maximum DF of 20

Maximum $n = 20/5 + 1 = 5$, meaning that I had six Wister albino rats per group.

The total size of the sample population will 5 by 5 = 25.

3.5. Sampling methods

Simple random sampling method was used during sampling the population to select 25 Wister albino rats (*Rattus norveginicus*).

3.6 Selection criteria

3.6.1 Inclusion criteria

1. Healthy Wister albino rats (*Rattus norveginicus*).
2. All the animals that had attain the required weight for the study.

3.6.2 Exclusion criteria

i. The Wister albino rats (*Rattus norveginicus*) that showed signs and symptoms of not being health

ii. The Wister albino rats (*Rattus norveginicus*) that would develop hypersensitivity reaction after administering the cisplatin.

iii. Pregnant Wister albino rats (*Rattus norveginicus*).

3.7 Grouping of animals

The study used the cluster random sampling method to group the animals into five main groups. The first group was control group and the other four remaining groups were experimental groups

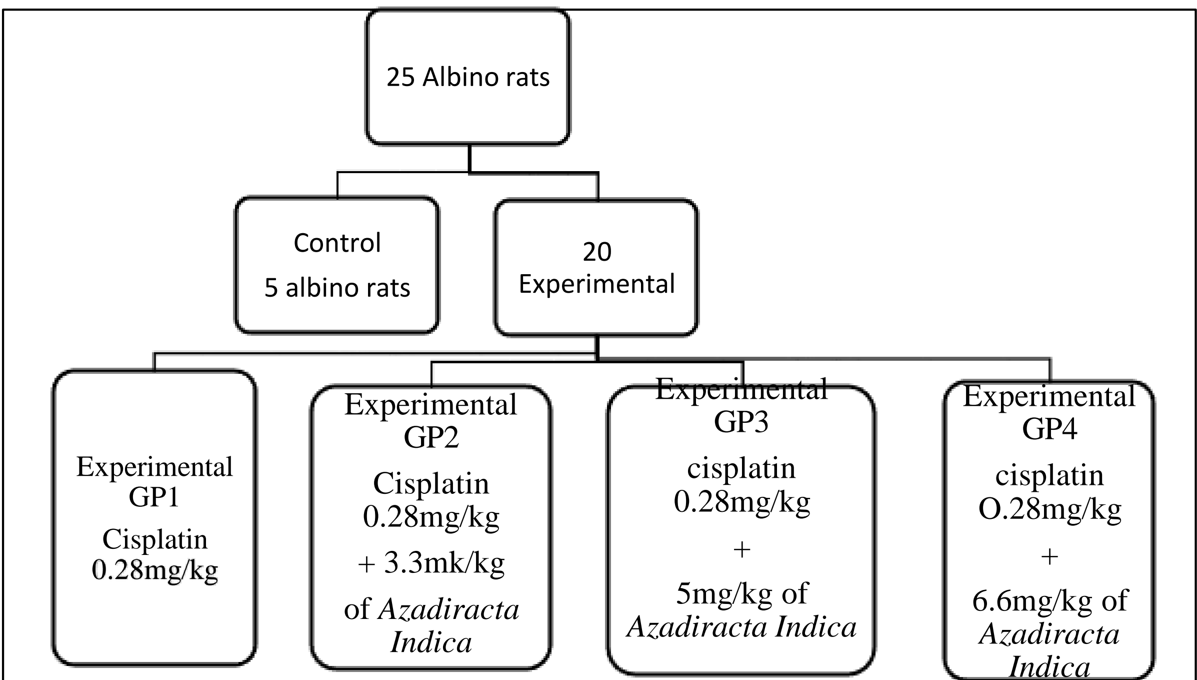

Figure 3.1: Grouping of animals (Source Author)

3.8 Handling of animals

Daily cleaning of the cages was done every morning, head count, examination taken and occupation safety measures were observed during the handling of the rats. Rats stayed in their cages for seven days to acclimatize before the beginning of the study. The feeding was also done every morning at around 0800hrs

3.8.1 Feeding of the rats

All the animals were fed on the standard rodent pellets, water and the *ad libitum.* The feeding was determined by the academy of nutrition and Dietetics that contain weight (g/100g); composing of 4% cellulose, 5% lipid (corn oil), 68% starch and the calories composed 72% carbohydrates, 20% proteins, 12% lipids and 54% zinc and water ad libitum

3.8.2 Drug acquisition

The experimental drugs used in this study were acquired from the Kakamega County Teaching and referral Hospital. The drugs contained the following batch numbers D 4565, ICSB20104E for both the *Azadirachta Indica* and Cisplatin respectively and were stored as instructed by the manufacturer. The drugs used had been verified by the Pharmacy and Poisons Board.

Figure 3.2: Mwarubaini (azadirachta indica) and cisplatin

3.8.3 Calculation of dosage

3.8.3.1 Cisplatin

The cisplatin is given intravenous once in every 3-to-4-week cycle. The nephrotocixity begins on the second week after administration of a single dose of 50-100mg/m^2 (FDA, 2009)

The minimum dose = $50mg/m^2$

Maximum dose = $100mg/m^2$

Average weight for a man is 60kgs

3.8.3.2 Maximum cisplatin dosage per kilogram

100mg/= 60kgs

? = 1 kg

The maximum HED will be 1.7mg/kg

Human equivalent dose (HED) (Mg/kg) = animal dose (mg/kg) multiplied by animal km

$1.7mg/m^2/kg$ =animal dose multiplied by 6

$1.7mg/m^2/kg=6x$

1.7/6=x

X=0.28mg/kg

Maximum animal dose will be $0.28mg/m^2/kg$.

3.8.3.3 Medium cisplatin dosage per kilogram

75mg=60kgs

? = 1kg

The medium dosage will be $1.3mg/m^2/kg$

1.3mg/kg =animal dose multiplied by 6

$$1.3mg/kg = 6x$$

$$1.3/6 = x$$

$$X = 0.22mg/kg$$

Median animal dose for cisplatin is $0.22mg/m^2/kg$

3.8.3.4 Low cisplatin dosage per kilogram

$$50mg = 60kgs$$

$$? \quad = 1kg$$

The low dosage will be 0.8mg/m^2/kg

0.8mg/m^2/kg =animal dose multiplied by 6

$$0.8mg/m2/kg = 6x$$

$$0.8/6 = x$$

$$X = 0.13mg/kg$$

Low animal dosage for cisplatin is 0.13mg/m^2/kg (Nair & Jacob, 2016).

Maximum dose of azadiracta indica will be used in the study: 0.28mg/m^2/kg

3.8.4.1 Mwarubaini (Azadiracta indica)

Human maximum dose= 40mg/kg

Medium dose =30mg/kg

Human low dose = 20mg/kg

Converting it back to Wister albino rat dosage will be Human equivalent dose (HED)

(Mg/kg) = animal dose (mg/kg) multiplied by animal km.

1kg= 40mg human dose.

40mg = 6x

40 ÷6 = 6.67mg/kg

3.8.4.2 Maximum animal dose

=6.67mg/kg.

30mg = 6 x

30 ÷6 = 5mg/kg

3.8.4.3 Medium animal dose

= 5mg/kg

20mg = 6 x

20 ÷6 = 3.33mg/kg

3.8.4.4 Low dose

= 3.33mg/kg

Adopted from the Neem foundation.

3.8.5 Administration of Cisplatin and Azadirachta

3.8.5.1 Material needed

1. Cisplatin
2. Azadiracta indica (*Mwarubaini*)
3. Needle gauge 23-25g
4. Table cloth
5. 5ml syringes
6. Gloves
7. Container
8. Absorbent pads
9. Disinfectant solution
10. Disposable gown and sleeves

3.8.5.2 The procedure of administering the cisplatin and Mwarubaini (azadirachta indica)

3.8.5.3 Procedure for cisplatin administration

Two-person technique was used.

i. The used 23-25 gauge of needle and draw volume less than 10ml/kg.
ii. 70% of alcohol and gauze was used for disinfection of multi-dose vial.
iii. Draw into the syringe the amount of solution that was required to be administered.
iv. Removed rats from the cages and restrained them firmly head – down.
v. Identified the anatomical landmarks on the abdomen.
vi. vi. Inserted the needle when the bevel facing up into the lower right quadrant of the abdomen towards the head at about 30-400 angle horizontally.
vii. Pulled back the plunger to confirm negative pressure before injecting, if there is negative pressure, then proceeded with the injection.
viii. Pulled out the needle straight and placed the syringe and the needle into the sharp container.
ix. Returned the animal back into the cages then observe for any arising complication.
x. The procedure adopted from (Andrews, 2014).

Figure 3. 3: Cisplatin and Mwarubaini administration.
Key: a- Cisplatin and b- Mwarubaini administration

3.8.5.4 Procedure for (Mwarubaini) azadirachta indica administration

Adopted from (Turner *et al.,* 2012)

i. The wister albino rats were held gently to immobilize the head and were put in a vertical position
ii. The gavage needle will be passed starting from the roof of the mouth, advanced into the esophagus then to the stomach.
iii. If there is any resistance during the procedure, can mean that there is an attempt of the needle to pass through the trachea.
iv. Observe for choking, a cough or struggling when the drug has been passed through the needle.

3.8.5.5 The sequence of drug administration

The drugs were administered in five groups, the control group was not administered with any drug. The Experimental GP1 were given cisplatin 0.28mg/kg only to induce the nephrotoxicity, Experimental GP2 were given a maximum dose 0.28mg/m^2/kg

intraperitoneally of cisplatin for induction of nephrotoxicity followed by maximum 6.6mg/kg of *Mwarubaini* (Azadirachta indica). Experimental GP3 were given 5mg/kg medium dose of *Mwarubaini* (azadirachta indica) and 0.28mg/kg of cisplatin then the Experimental GP4 were given 3.33mg/kg low dose of *Mwarubaini* (azadirachta indica) and 0.28mg/kg of cisplatin.

The *Mwarubaini* (azadirachta indica) for the experimental groups, were given on daily basis for one-week, five days past administration of cisplatin. cisplatin was administered once during the beginning of the study. In case of bites or exposure, immediate first aid and medical attention was taken. The animals were sacrificed on 13th day of the experiment (Pickett, 1987)

3.9 Weighing of rats

Before and after the beginning of the experiment, all the rats were weighed. Using (type of weighing scale, manufacturing company, % of error)

Figure 3. 4: Weighing of rats

3.10 The procedure for anaesthetizing

i. The cotton wool was soaked in chloroform or diethyl.

ii. The soaked cotton was introduced into the bell jar.

iii. The rats were put into a bell jar for 15-20 minutes for the rat to be anaesthetized.

iv. The rats were placed on the board, dorsal side on the board and mounted with mounting pins.

v. The rats were dissected using a pair of scissors and the forceps ventral media side, starting from the pubic symphysis until the sternal angle of the thoracic cage.

vi. A perfusion needles were inserted in the left ventricles of the heart

vii. The blood was cleared from the rats through the left ventricles using the normal saline of 0.85mol

viii. The firmness of the tail was checked, drip was removed together with the perfusion needle from the heart.

ix. The kidneys were excised and put in a fresh fixative.

The blood sample were tested for urea and creatinine

Figure 3. 5: anaesthetizing, dissecting and blood collection procedure.
***Key*: a- anaesthetizing, b – dissecting, c- blood sample collection, d- packaged blood**

3.11 Assessing the gross morphometric of the kidney

Immediately after harvesting the kidneys from the rats, they were washed with 5% normal saline. The lengths, thickness and widths were taken and determined using a caliper and a ruler.

Figure 3. 6: Measuring of gross morphometrics
Key: a- weighing of kidneys, b- measuring length of the kidney, c- measuring widths of the kidney, d- measuring volume.

3.12 Evaluation of the total kidney volume using Archimedes principle

The volume of the kidneys was estimated using the Archimedes principle (2018), in the way that the kidney was placed on a calibrated full of 5% normal saline. The saline that was displaced by the kidney was recoded as the kidney actual volume. The volume of the kidney was obtained and the standard deviation was attained among the group.

3.13. Histology for the Kidney

3.13.1 Material to be used for staining kidney sectioned for histology

The materials included; DPX mount, specimen bottles, Paraffin wax, slide holders, microtome, dropper, toluidine solution, formaldehyde 40% concentration, Rotary microtome, the specimen (the kidney), distilled water, glass staining square jars, zenkers solution (acetic acid 5mls and distilled water), xylene, wood blocs, isopropyl alcohol, beakers, egg albumin, dropper, cedar wood oil and heater and water bath container.

3.13.2 Procedure to be used for processing the kidney specimens for the light microscopy

i. The kidneys were fixed in formaldehyde solution for a period of 24 hours.

ii. The kidney tissues were fixed in a dehydrated ascending concentration of alcohol that is 50%>60%>70%>80%>90%>95>100% each for one hour.

iii. Was cleared with xylene.

iv. Was infiltrated with paraplast wax for about 12hours at 56%.

v. The infiltrated kidneys tissue was embedded on paraffin wax on a wood block.

vi. 5 micrometer thick sections were cut using leitz sledge rotary microtome.

vii. The cut section was floated on water at 37^0 to spread the tissue.

viii. The section was stacked onto glass slides firmly by micro- dropper.

ix. The slides were dried in an oven at about 37% for 24 hours.

x. The slides were stained with hemotoxylin and eosin (H&E).

3.13.3 Procedure to be followed in taking photomicrograph

i. The slides were mounted on the stage of the microscope.

ii. The focus was adjusted until the image to be photographed to be in focus.

iii. The field were magnified appropriately.

iv. The photographs of the regions were taken at best under the focus.

v. The photograph was transferred to the computer, stored and uploaded using adobe fireworks application.

3.13.3.1 Photography

3.13.3.1.1 Materials needed

i. Memory

ii. Histological glass slide

iii. Digital camera

3.13.3.1.2 Procedure that will be followed in taking photomicrographs

i. The prepared tissues were mounted on the stage of the microscope.

ii. Thereafter the images were focused and adjusted to be photographed.

iii. The images were viewed under the focus of the microscope and the photographs will be taken.

iv. The photographs were transferred into the memory cards.

v. The images were uploaded using the adobe application.

3.14 Histo- Stereological examination of the bowman's capsule, glomerulus space and glomerulus.

The images were uploaded on the stepanizer stereological tool, the spacing and section of the point prode was done and tossed randomly on to each section. The point that were hitting the intended regions were counted.

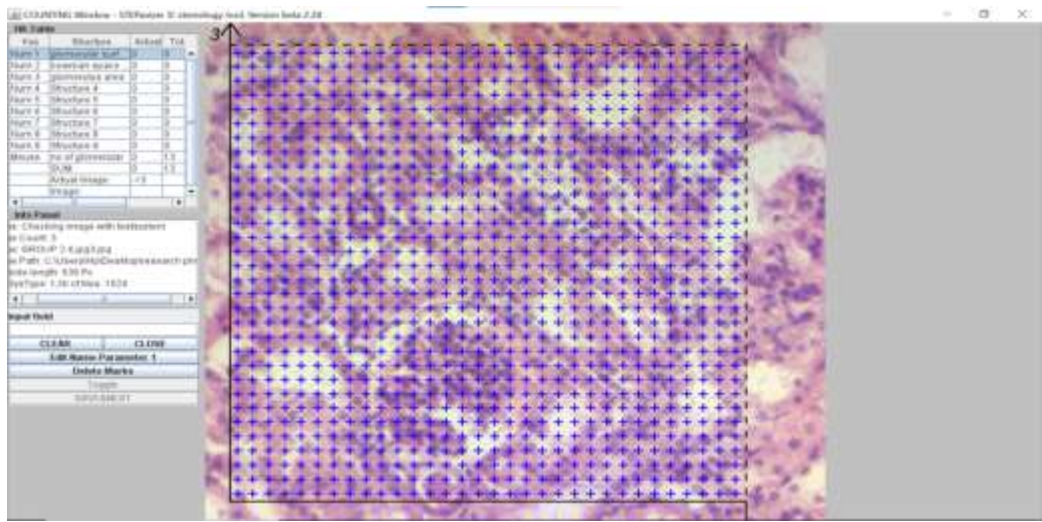

Figure 3.8: Showing a kidney tissue slide on a step analyzer ×100

The surface area was calculated as follows; the total number of point grid within the specified region × estimated surface area of the point grid

3.15 Data management and analysis

The data acquired through the gross, histological images and stereological were collected, uploaded using adobe fireworks and stored in flash disk for qualitative analysis. The descriptive gross and stereological data such as weight, height, width, thickness length was analyzed using the mean, standard error of mean and standard deviation. The uploaded histological images were put on adobe photo grid to calculate the his to-stereological for estimation of the surface area of normal and affected regions

The data of all the groups were analyzed using statistical package for social science (SPSS) version 27 for year 2021 Chicago Illinois. The data on renal bio-chemical parameters which will be used to assess acute kidney injury will be compared between the experimental and control groups. One-way analysis of variance (ANOVA) was used to compare the significance mean between control and experimental groups. The turkey post hoc t-test was used to assess for any significant difference between the groups and a P value of ≤ 0.05 will be considered significant at 95% confidence interval. The data was presented in tables, figures and images.

3.15 Dissemination

The disseminated study findings targeted the health policy makers, pharmaceutical companies, all health providers, my peers, scholars and funding agencies to support my finding. The study findings were disseminated through my final report, seminars, conferences, workshops and publication.

3.16 Ethical approval

The research topic and proposal were approved by the Department of Human Anatomy and the School of Medicine. The complete proposal was forwarded to the school of post graduate studies for approval and then to Maseno University Ethical committee approval. The proposal was also forwarded to Baraton University for Animal Ethical Committee for approval of using the Wister albino rats for collecting data. The proposal was further forwarded to National Commission for Science, Technology and Innovation for further approval and licencing. All the collected data were stored with confidentially and were used for the current research, the study followed the standards and procedures as per the guidelines and protocol for care and use of laboratory animals in biomedical research (guidelines & Kenya, 2016). The animals were sacrificed using humane end points during the study basing on the Leary, (2013) protocol.

CHAPTER FOUR

RESULTS

4.1: Introduction

This chapter will include detailed summary statistics of: the biochemical parameters which was based on urea and creatinine levels; the gross histomorphometry which was based on length, thickness, weight and width of the kidneys and total body weight; histo-stereological which was based on the surface area of glomerulus capillaries, glomerulus space, glomerulus capsule and then lastly the histo-architectural changes that was based on the parietal epithelium of the bowman's capsule , proximal and distal convoluted tubule, interstitial and vesicular part which were also examined histologically. One way ANOVA and post hoc test was used for comparison between the control and experimental groups in analysis.

4.2 Renal biochemical parameters following administration of Cisplatin and *Mwarubaini* among the Wister albino rats.

The renal biochemical parameters had the adopted reference ranges as follows, Urea 4.2 to 8.97mmol/ l and creatinine 0.2 to 0.8mg/dl.

4.2.1 Urea levels in control and the experimental groups

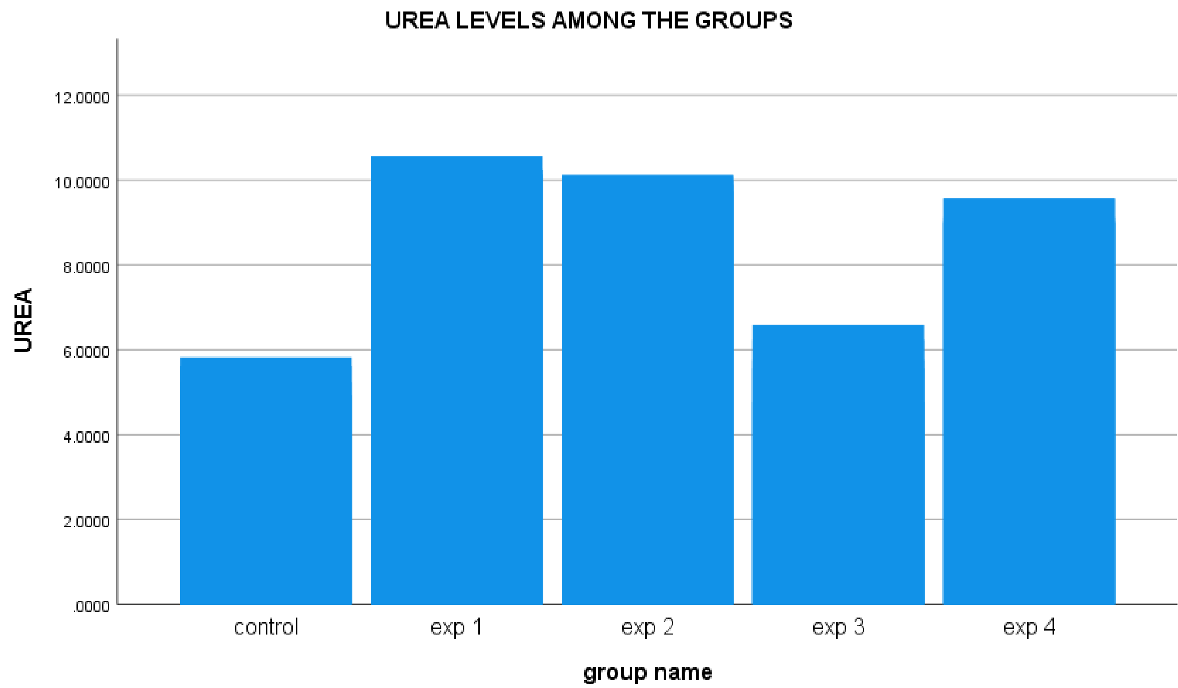

Figure 4. 1: Urea level of control and the experimental groups
Key: control (water+ feeds), exp 1- Experimental GP1 (0.28mg/kg Cisplain only), exp 2- Experimental GP2 0.28mg/kg Cisplatin + Mwarubaini 6.67mg/kg), exp 3- Experimental GP3 (0.28mg/kg Cisplatin + Mwarubaini 5mg/kg), exp 4- Experimental GP4 (0.28mg/kg Cisplatin + Mwarubaini 0.33mg/kg).

There was a significance ($P< 0.0001$) increase in Urea levels of the experimental GP1, 2 and 4 as compared to the control and their individual group means were above the normal reference range, with degree of freedom of 4 and f of 183.12 at 95% Confidence Interval. The mean for urea levels of control and experimental GP3 were within the normal reference range. (Figure 4.1).

4.2.2: Creatinine levels in control and the experimental group

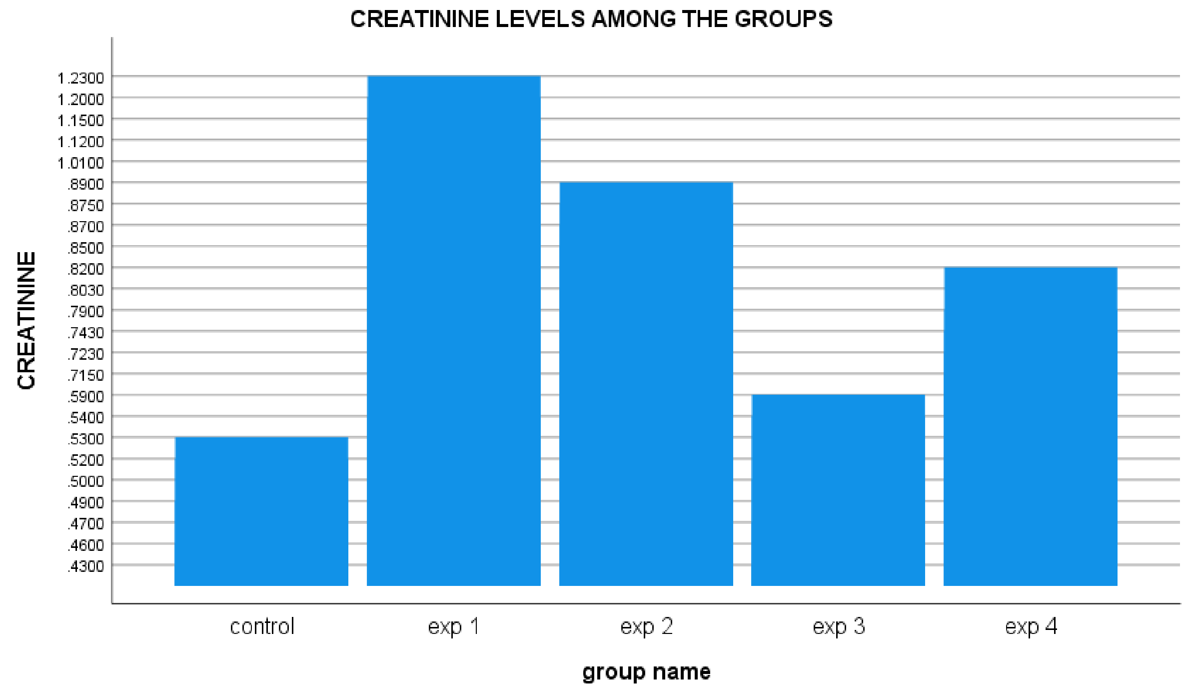

Figure 4.2: Creatinine level of control and the experimental groups.
key: control (water+ feeds), Experimental GP1 (0.28mg/kg Cisplain only), Experimental GP2 0.28mg/kg Cisplatin + Mwarubaini 6.67mg/kg), Experimental GP3 (0.28mg/kg Cisplatin + Mwarubaini 5mg/kg), Experimental GP4 (0.28mg/kg Cisplatin + Mwarubaini 0.33mg/kg).

The group mean for creatinine levels of control and experimental GP3 were within the normal reference range. There was a significance ($P< 0.0001$) increase in creatinine levels of the experimental GP1, 2 and 4 as compared to the control and their individual group means were above the normal reference range, with degree of freedom of 4 and ration of variance of 114.10 at 95% Confidence Interval. The group mean for creatinine levels of control and experimental GP3 were within the normal reference range (Figure4.2).

4.3: Gross histo-morphological and histo-cyto-architectural injuries in cisplatin induced nephrotoxicity among the Wister albino rats.

Immediately after humane sacrificial of Wister albino rats, the kidneys relations to other organs were observed and then kidney were extracted. The weight, width, length, thickness and volume were measured.

4.3.1: Observed gross anatomy of the kidney of Wister albino rat

On observation, the kidneys were bean shaped and reddish brownish in color located between the intestines anteriorly and the posterior aspect of the peritoneal cavity on sides of the vertebral column. The renal veins and the arteries were connected to the vena cava and the aorta respectively. The left kidneys were more caudal than the right ones and were covered by a connective tissue. The cortex , medulla and renal pelvis were normal in all the groups, this concured with the normal anatomy of winster rats. There were no abnormal variations observed in the kidney gross morphology (Figure 4.3).

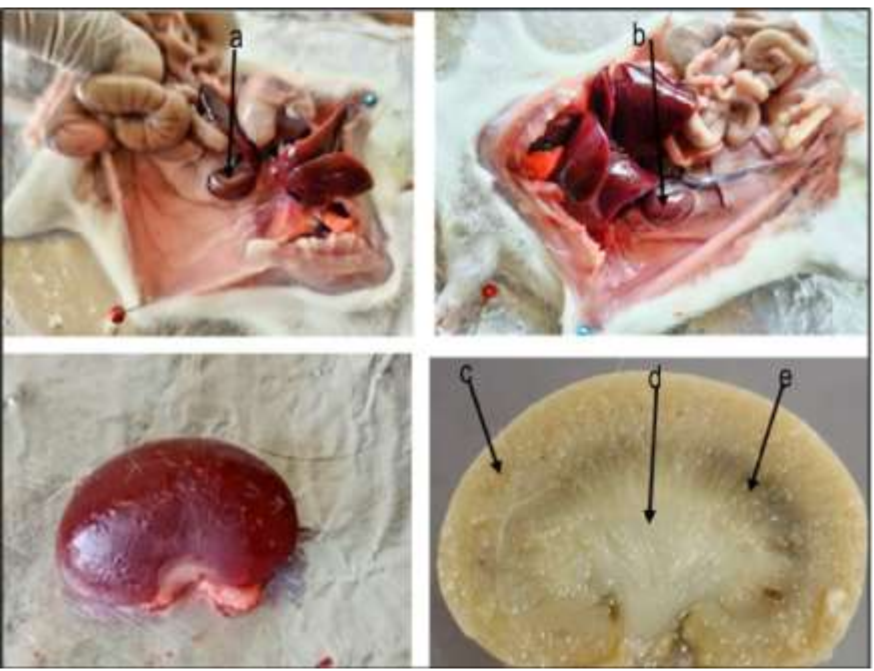

Figure 4.3: Observed gross anatomy of the Wister albino rats kidneys
Key: a- right kidney, b- left kidney, c- cortex, d- renal pelvis, e- medulla

4.3.2: Body weight of Wister albino rats.

The Wister albino rats were weighed before being sacrificed and their mean weight was used to plot a line graph.

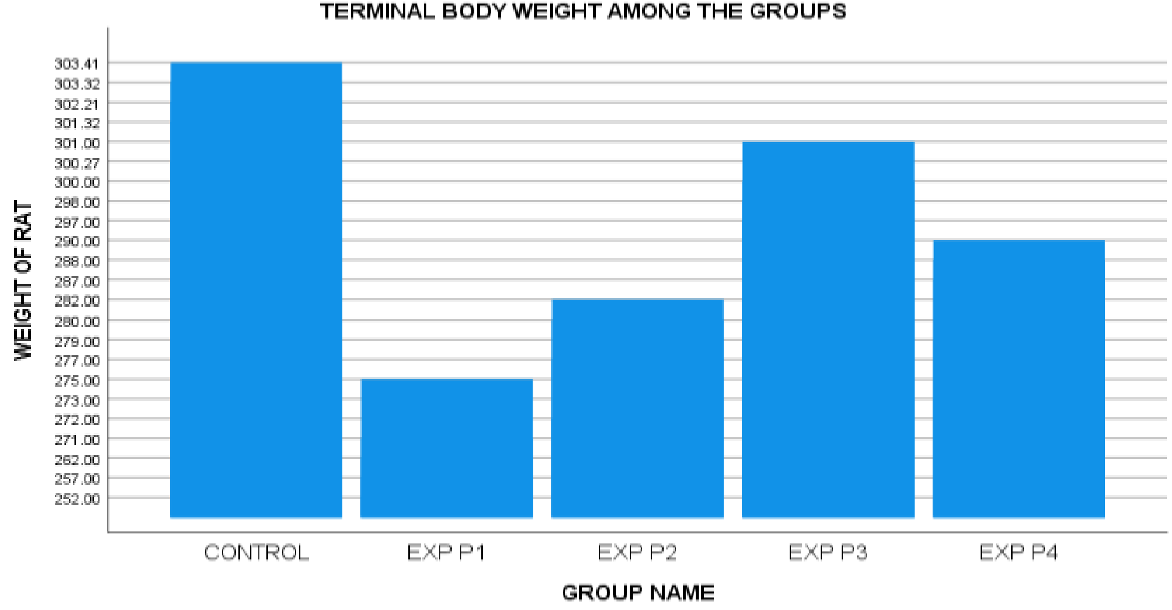

Figure 4.4: Summery statistics of body weight of Wister albino rats of the control vs experimental groups.
key: control (water+ feeds), exp 1- Experimental GP1 (0.28mg/kg Cisplain only), exp 2- Experimental GP2 0.28mg/kg Cisplatin + Mwarubaini 6.67mg/kg), exp 3- Experimental GP3 (0.28mg/kg Cisplatin + Mwarubaini 5mg/kg), exp 4- Experimental GP4 (0.28mg/kg Cisplatin + Mwarubaini 0.33mg/kg)

There was significant ($P \leq 0.001$, 0.001 and 0.003 respectively) reduction in mean body weight of experimental GP1, 2, and 4 body weight as shown I figure 4.4.

4.3.3 Gross morphometry of Wister albino rats

Table 4.1: The mean length, volume, weight, width and thickness between negative and positive controls of the right kidneys

Groups	mean weight right kidney Mean ± SEM	mean length right kidney Mean ± SEM	mean width right kidney Mean ± SEM	mean volume right kidney Mean ± SEM	mean thickness right kidney Mean ± SEM
Control (water+ feeds)	1.150 ± .02	18.25±.06	11.35±.10	2.30±.10	5.59±.03
Experimental GP1 (0.28mg/cispl ati)	0.894 ± .19	16.28±.65	9.53±.20	1.86±.45	3.39±.80
P Value	< 0.0001**	< 0.0001**	< 0.0001**	< 0.0001**	< 0.0001**

Key: 0.28mg/kg of cisplatin was given to Experimental GP1 to induce nephrotoxicity. SEM- Standard error of mean, P value less than 0.05 was considered statistically significant.

There was significant ($P < 0.0001$**) reduction in the mean length, width, volume, thickness and weight of the right kidney for experimental group as correlated with the control group. The P values were tested between the mean difference of the control (water + feeds) and Experimental GP1 (0.28mg/kg cisplatin) using one way ANOVA and post hoc test. (Table 4.1)

Table 4.2: The mean length, volume, weight, width and thickness between negative and positive controls of the left kidneys

Groups	mean weight left kidney Mean ± SEM	mean length left kidney Mean ± SEM	mean width left kidney Mean ± SEM	mean volume left kidney Mean ± SEM	mean thickness left kidney Mean ± SEM
Control (water+ feeds)	0.91±.01	14.82±.27	9.68±.12	1.61±0.38	4.29±.36
Experimental GP1 (0.28mg/kg Cisplatin)	0.61±.33	12.64±.16	7.68 ± .16	1.23±.31	3.42±.08
P Value	< 0.0001**	< 0.0001**	< 0.0001**	< 0.0001**	< **0.008**

Key: 0.28mg/kg of cisplatin was given to Experimental GP1 to induce nephrotoxicity, SEM- Standard error of mean, P value less than 0.05 was considered statistically significant.

There was statistical significance (*P* <0.0001, 0.0001, 0.0001, 0.0001 and 0.008 respectively) reduction in weight, length, width, volume and thickness of the left kidney for experimental group as correlated with the control group. The P value was tested between the mean difference of the control (water + feeds) and Experimental GP1 (0.28mg/kg cisplatin) using one way ANOVA and post hoc test (Table 4.2).

4.2.4: histo-architecture of the control group

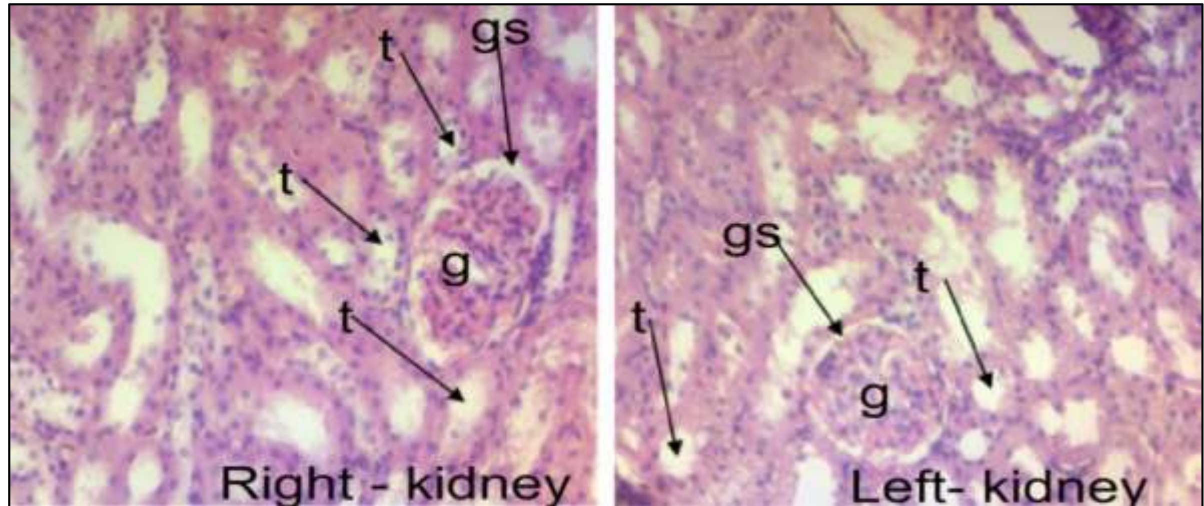

Figure 4.5: Photo-Micrograph of Control group of left and right kidney section H & E ×100 (only given water and feeds).

Key: g – glomerulus, gs- Glomerulus space, - Tubules

The renal capsule appeared normal with no necrotic or apoptotic signs in both the right and left kidney, the glomerulus was irregular in shape although with normal histoarchitecture. The tubules were normal with no dilatation of the epithelium in both the right and left kidney. The renal interstitial was normal with no vacuolation, necrotic, apoptotic cells and fibrosis in both the right and left kidney. The vesicular part is also normal in both the right and left kidney as shown in figure 4.5.

4.2.5: histo-architecture of experimental group 1

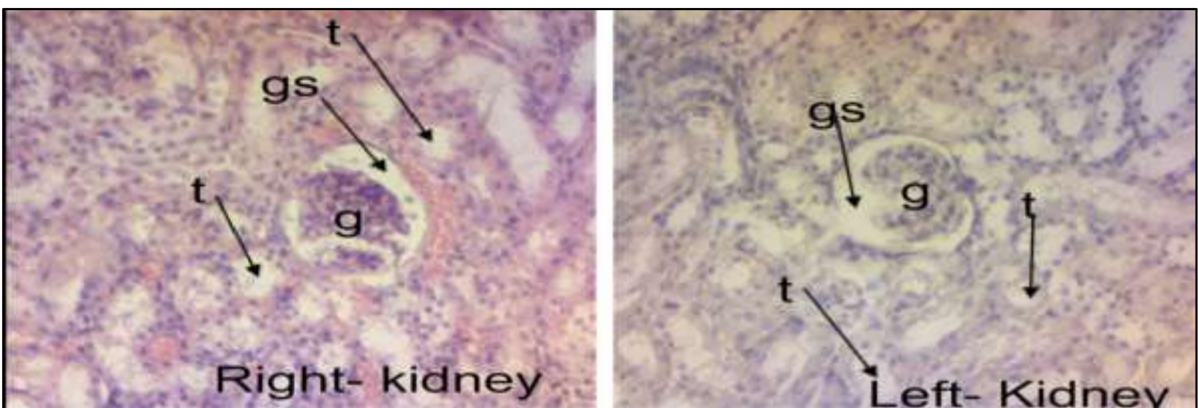

Figure 4.4: Photo-Micrograph of Experimental GP1 of left and right kidney section (given 0.28mg/kg of cisplatin to induce nephrotoxicity).
Key: g – glomerulus, gs- Glomerulus space, t- Tubules

There was tubular necrosis of the distal and proximal convoluted tubules, shown by shrinking of their epithelium and necrotic tissues both in the right and the left kidneys. There also was marked renal corpuscle involvement shown by necrosis of the parietal layer of the bowman's capsules and shrinking of the glomerulus capillaries mostly on the right kidney as compared to the left one. There was no involvement of the vesicular and the interstitial part of the kidney (Figure 4.6).

4.3: Gross restorative histomorphometries of the kidney on administration of cisplatin followed by different doses of Mwarubaini.

Table 4.3: Mean length, width, weight, volume and thickness experimental groups of the right kidneys.

Groups 1	mean weight Right kidney Mean ± SEM	mean length Right kidney Mean ± SEM	mean width Right kidney Mean ± SEM	mean volume Right kidney Mean ± SEM	mean thickness Right kidney Mean ± SEM
Experimental GP1(0.28mg/kg Cisplatin)	0.89±.02	16.28±.07	9.53 ± .20	1.85 ± .05	3.98±.08
Experimental GP2 0.28mg/kg Cisplatin + *Mwarubaini*6.67mg/kg	0.88±.01	16.12±.31	9.59 ± .18	1.86±.05	4.10±.45
Experimental GP3 0.28mg/kg Cisplatin + *Mwarubaini* 5mg/kg	1.15 ±.04	18.13 ± .06	11.26 ± .07	2.23±.02	5.54±0.52
Experimental GP 4 0.28mg/kg Cisplatin + *Mwarubaini*3.33mg/kg	0.93±02	17.14±.06	9.93±.25	1.84±.35	3.10 ± .55

Key: Experimental GP1 (cisplatin induced nephrotoxicity group) was being compared to restorative groups Experimental GP2,3 and 4). SEM- Standard error of mean

There was a statistically significant (*P* <0.0001**) reduction in experimental GP3 weight, width, thickness, length and volume as compared to right kidney of Experimental GP1. The P

value was tested between the mean difference of the Experimental GP1 and the Experimental G2, GP3 and GP 4 (water + feeds) (0.28mg/kg using one-way ANOVA and post hoc test.

(Table 4.3.)

Table 4.4: Mean length, width, weight, volume and thickness of experimental groups of the left kidneys.

Groups	mean weight left kidney Mean ± SEM	mean length left kidney Mean ± SEM	mean width left kidney Mean ± SEM	mean volume left kidney Mean ± SEM	mean thickness left kidney Mean ± SEM
Experimental GP1(0.28mg/kg Cisplatin)	0.61±.03	12.64±.16	7.68±.17	1.23±.03	3.42±.14
Experimental GP2 0.28mg/kg Cisplatin + *Mwarubaini* 6.67mg/kg	0.55±.04	13.26±.13	8.15±.89	1.25±.29	3.43±.08
Experimental GP3 0.28mg/kg Cisplatin + *Mwarubaini* 5mg/kg	0.90±.19	14.97±.24	9.41±.87	1.63±.39	4.10±.06
Experimental GP 4 0.28mg/kg Cisplatin + *Mwarubaini* 3.33mg/kg	0.65±.18	13.86±.31	8.20±.20	1.39±.54	3.41±.30

Key: Experimental GP1 (cisplatin induced nephrotoxicity group) was being compared to restorative groups Experimental GP2,3 and 4). SEM- Standard error of mean

There was a statistically significant (*P*< 0.0001**) reduction in experimental GP3 weight, width, thickness, length and volume as compared to left kidney of Experimental GP1. The P value was tested between the mean difference of the Experimental GP1 and the Experimental G2, GP3 and GP 4 (water + feeds) (0.28mg/kg using one way ANOVA and post hoc test (Table 4.4.)

4.4 Restorative histo- structural changes and histo- stereology of the kidney on administration of cisplatin followed by different doses of *Mwarubaini*.

The bowman's capsule, glomerulus and glomerulus space of the experimental GP1 were compared to experimental GP2, 3, and 4.

4.4.1: Histoarchitecture of experimental group 1

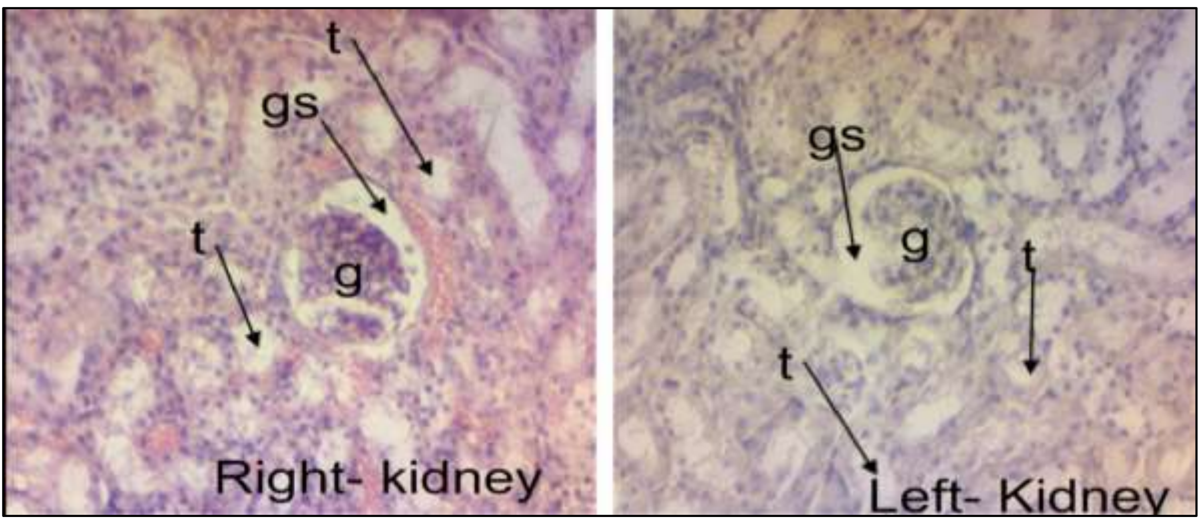

Figure 4.5: Photo-Micrograph of Experimental GP1 of left and right kidney section (given 0.28mg/kg of cisplatin to induce nephrotoxicity).

Key: g – glomerulus, gs- Glomerulus space, t- Tubules

There was tubular necrosis of the distal and proximal convoluted tubules, shown by shrinking of their epithelium and necrotic tissues both in the right and the left kidneys. There also was marked renal corpuscle involvement shown by necrosis of the parietal layer of the bowman's capsules and shrinking of the glomerulus capillaries mostly on the right kidney as compared to the left one. There was no involvement of the vesicular and the interstitial part of the kidney (Figure 4.7).

4.4.2: Histo-architecture of experimental group 2

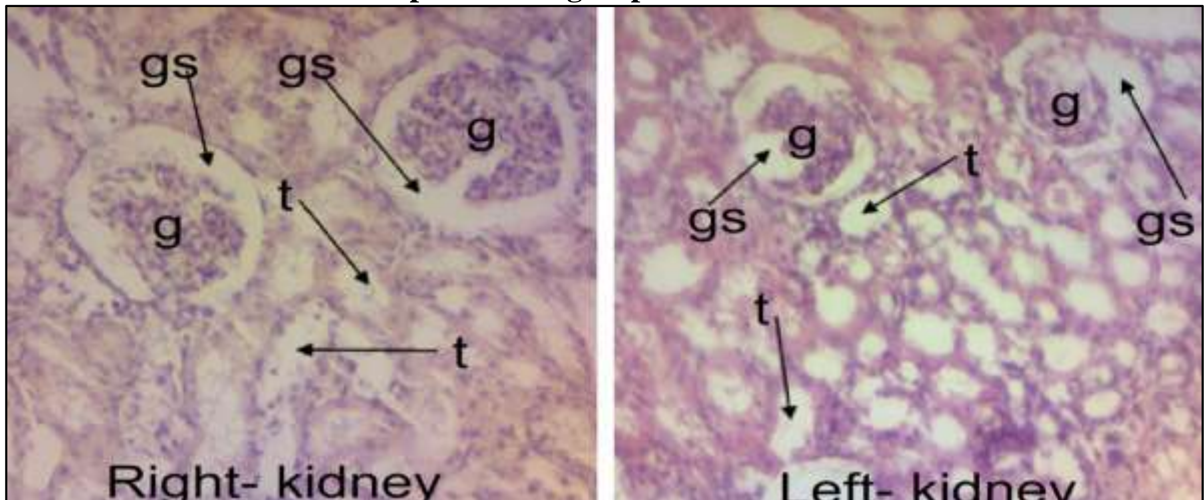

Figure 4.6: Photo-Micrograph of Experimental GP2 stained with H&E×100 of left and right kidney section (given 0.28mg/kg of cisplatin and 6.66mg/kg of Mwarubaini for restoration).
Key: g – glomerulus, gs- Glomerulus space, t- Tubules

There was also tubular necrosis of the distal and proximal convoluted tubules, shown by shrinking of their squamous epithelium and necrotic tissues more on the right as compared to the left kidneys. There was renal corpuscle involvement shown by necrosis of the parietal layer of the bowman's capsules and shrinking of the glomerulus capillaries more also on the right as compared to the left kidneys. There was no involvement of the interstitial and vesicular part of the kidney in both the right and left kidney (Figure 4.8).

4.4.3: Histo-architecture of experimental group 3

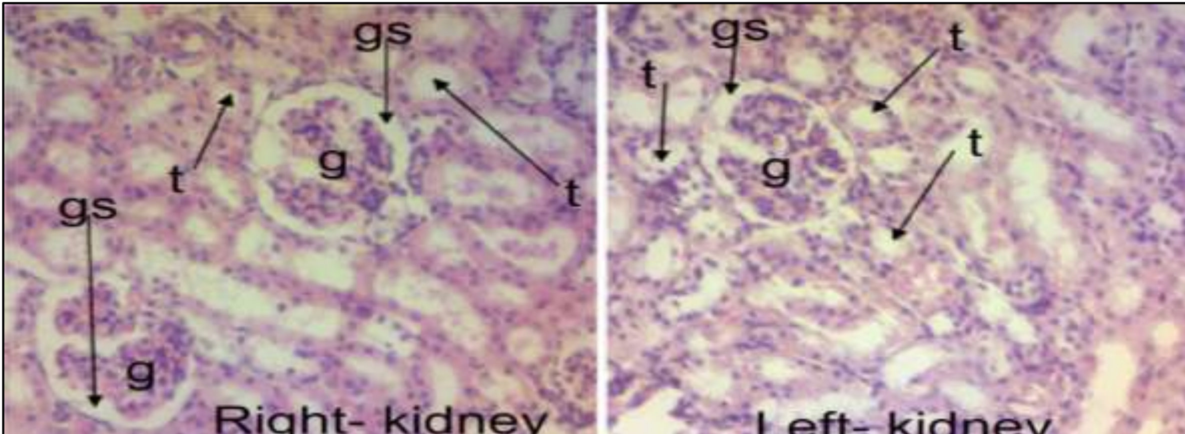

Figure 4.7: Photo-Macrograph of Experimental GP3 stained with H&E×100 of left and right kidney section (given 0.28mg/kg of cisplatin and 5mg/kg of Mwarubaini for restoration).
Key: g – glomerulus, gs- Glomerulus space, t- Tubules

There was minimal tubular necrosis of the distal and proximal convoluted tubules shown by less shrinking of their cuboidal epithelium both in the right and the left kidneys. There was also minimal renal corpuscle involvement shown by less necrosis of the parietal layer of the bowman's capsules and shrinking of the glomerulus capillaries and compared to the second and the third group. There was also no involvement of the interstitial and vesicular part of the kidney in the right and the left kidneys (Figure 4.9).

4.4.4: Histo-architecture of experimental group 4

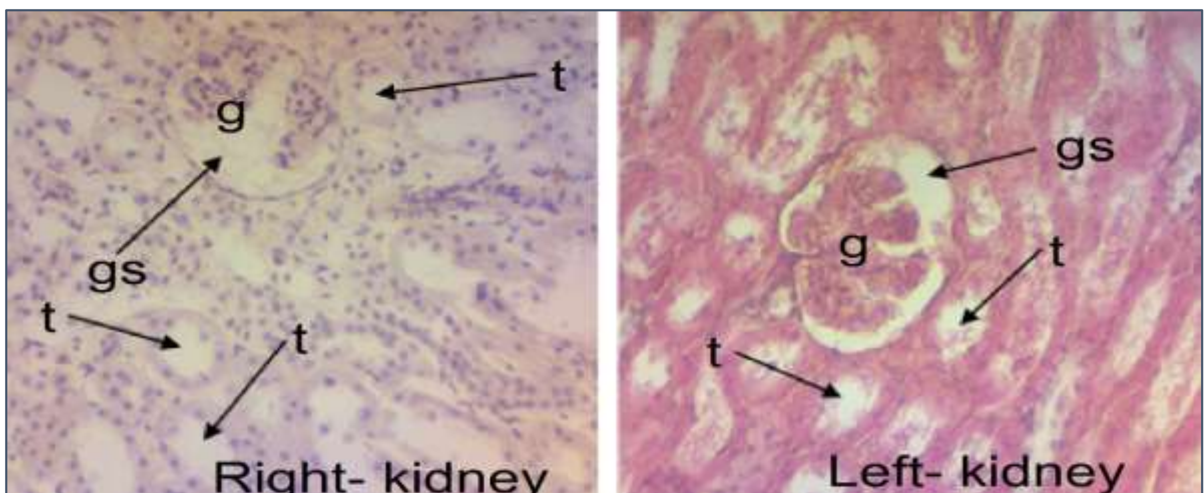

Figure 4. 8. Photo-Micrograph of experimental GP4 of left and right kidney section (given 0.28mg/kg of cisplatin and 3.33mg/kg of Mwarubaini for restoration).

Key: g – glomerulus, gs- Glomerulus space, t- Tubules

There was medium tubular necrosis of the distal and proximal convoluted tubules shown by medium shrinking of their squamous epithelium and necrotic tissues. There was renal corpuscle involvement shown by medium necrosis of the parietal layer of the bowman's capsules and shrinking of the glomerulus capillaries There was also no involvement of the interstitial and vesicular part of the kidney (Figure.4.10)

4.4.5: Histo -Stereological analysis

The surface area of the renal tubules of control and experimental group could not be measured as the epithelial cells of some of the tubules in study group were disintegrated and distorted.

The surface area of the glomerulus, glomerulus space and the bowman capsule were determined on a standard grid using the (image J application).

Table 4.5: Surface area of the glomerulus, glomerulus space and the bowman capsule between control and experimental group.

Groups	Glomerulus surface area Mean ± SEM	Glomerulus space surface area Mean ± SEM	Bowman capsule surface area Mean ± SEM
Control **(water + feeds)**	0.017 ± 0.0004	0.008± 0.0010	0.024± 0.0006
Experimental GP1 (0.28mg/kg Cisplatin)	0.010± 0.0006	0.015± 0.0016	0.036± 0.0006
Experimental GP2 0.28mg/kg Cisplatin + *Mwarubaini* 6.67mg/kg)	0.013± 0.0005	0.012± 0.0016	0.032± 0.0066
Experimental GP3 0.28mg/kg Cisplatin + *Mwarubaini* 5mg/kg)	0.016± 0.0006	0.009± 0.0018	0.027± 0.0070
0.28mg/kg Cisplatin + ***Mwarubaini*** **3.33mg/kg)**	0.012± 0.0006	0.014± 0.0070	0.033± 0.0008

Key: Surface area was in µm^2, SEM- standard error of mean and mean (n = 5).

The surface area of the glomerulus of experimental GP1, GP2 and GP4 had a significant reduction (*P* <0.0001**). The surface area of the of the glomerulus space and bowman's capsule significantly (*P* <0.0001**) increase in Experimental GP1, GP2 and GP4, only glomerulus space of the Experimental GP2 had a significant increase (*P* 0.002). The P value was tested between the mean difference of surface area of the glomerulus, glomerulus space and the bowman's capsule for the control and Experimental GP1, GP2, GP3 and GP4 using one way ANOVA and post hoc test as shown in Table 4.5.

CHAPTER FIVE

DISCUSSION

5.1 Introduction

This chapter contains a detailed discussion of the summery findings and correlating them with the literature review. It starts by discussing the influence of *Mwarubaini* on cisplatin-induced nephrotoxicity on renal biochemical parameters such as Creatinine and Urea level, followed by gross histomorphometry such as the body weight of Wister albino rats, volume, thickness, width and length of the kidneys, then lastly microscopic and the stereological findings.

5.2 Influence of *Mwarubaini* on Cisplatin induced nephrotoxicity on Urea and Creatinine levels.

Urea and creatinine levels have been used as biomarkers in the early detection of nephrotoxicity in previous studies done in rats (Vlasakova *et al.,* 2014). In this current study, Cisplatin was able to induced nephrotoxicity marked by elevated urea and creatinine levels. Other studies that have also been able to achieve an induced nephrotoxicity have been reported by Canayakin *et al.,* (2016); Dedeke *et al.,* (2018); Kamal *et al.,* (2022) and they observed a significant increase in creatinine levels in paracetamol, carbon tetrachloride, and herbicide-induced nephrotoxicity. However, Hardick *et al.,* (2011) on the other hand, observed that carboplatin was not able to induce nephrotoxicity since the creatinine levels were within normal ranges. In this study, *Mwarubaini* had a restorative effect on cisplatin induced nephrotoxicity among Wister albino rats. This was evidenced by urea and creatinine levels in Experimental GP1, GP2, GP3, GP4 showing steady reversion to normal ranges. Other studies (Makokha *et al.,* 2023) that have also successfully restored an induced nephrotoxicity showed urea and creatinine level as reverting to near normal or normal. Besides urea and creatinine levels other studies have also used KIM-1 and NGAL, markers to confirm nephrotoxicity.

5.3 Influence of *Mwarubaini* on cisplatin induced nephrotoxicity on gross histomorphometry.

5.3.1 Influence on body and kidney weight outcome

There was a significant reduction of terminal body weight of experimental GP1, GP2, and GP4 compared to the control group, this may have been a result of secondary effects of renal toxicity including anemia and liver failure. Previous studies also reported a gradual reduction in weight in relation to higher doses of nephrotoxic agents (Chiang, 2014 and Harveen Kour *et al.,* 2023). The current findings demonstrated no significant change in daily body weight among the rats of the control group. The Experimental GP3 regained weight significantly (P=0.037) compared to experimental G1 after the introduction of the restorative agent (*Mwarubaini*), perhaps due to the physiological restoration of kidney functions.

5.2.2 Influence on volume, width, length and thickness of the kidneys

There was a significant reduction in the volume of the kidney for the experimental GP1, GP2, and GP4. This might be as a result of the distortion of the glomerulus and necrosis of the epithelium tissues of tubules. This study finding concurred with results of the Kunogi *et al.,* (2021). Who recorded a 25% reduction in volume following cisplatin-induced nephrotoxicity. There was no significant change in the volume of control and experimental GP3. There was a significant decrease in thickness, width, and length in the experimental GP1, GP2 and GP4 as compared to the control. There was no significance in the thickness, width, and length of the experimental GP3 as compared to the control.

5.3 Influence of *Mwarubaini* on cisplatin-induced nephrotoxicity on histo- architectural changes

Renal histo-architectural changes are associated with nephrotoxicity. In the current study, there was a significant shrinkage of the glomerulus, and dilatation of proximal and distal convoluted

tubules in the experimental GP1, GP2 and GP4 as compared to the control group. This might be due to the circulation of free oxygen radical, endoplasmic and intracellular stress, and mitochondrial damage induced by cisplatin toxicity that led to necrosis of the parietal layer of the bowman's capsules, destruction of the basement membrane of the glomerulus, and tubular necrosis. From the literature reviewed (Abdel *et al.,* 2014, Ukoha *et al.,* 2015, Fisch *et al.,* 2016; and Kumar Singh & Singh Karchuli., 2014), most authors recorded significant changes in the glomerulus, proximal, and distal convoluted tubules in cisplatin-induced nephrotoxicity, in line with the present findings. However, Albalawi *et al.,* (2023) reported contrary findings when they noted significant involvement of the interstitial part of the kidney as seen by the deposition of collagen fibrils following gentamycin-induced nephrotoxicity. This may be due to inflammation of the intestinal part as a result of gentamycin toxicity. There was no significant change in the histo-architecture of experimental GP3 as compared to the control group, this perhaps demonstrated restoration of the glomerulus, distal, and proximal convoluted tubules. This study's findings concurred with those of Abireh *et al.,* (2020) who recorded similar results after treatment of ibuprofen-induced nephrotoxicity with Mwarubaini (Plate 4.5)

5.4 Influence of Mwarubaini on cisplatin induced nephrotoxicity on histo-stereological outcomes.

The renal corpuscle, the surface area of the glomerulus, bowman's capsule and glomerulus space can be associated with the histo-stereological changes in assessing drug-induced nephrotoxicity. There was a significance increase in the surface area of the bowman's capsule in the experimental GP1, GP2 and GP4 as compared to the control group (Plate 4.3, 4.4 and 4.6). This may perhaps be due to the destruction of its parietal layer. The current study findings are in tandem with the results of

(Sobolev *et al.,* 2021 , Koca *et al.,* 2013 and Sasaki *et al.,* 2018) who recorded a significant increase in the surface area of bowman's capsule after introduction of -induction of a nephrotoxic agent. There was no significance increase in the surface area of the bowman's capsule of the experimental GP3 as compared to the control group and this may suggest restoration. There was a significance reduction in the surface area of the glomerulus in the experimental GP1, GP2, GP4 ((Plate 4.3, 4.4 and 4.6) as compared to the control. This may be due to the destruction of the basement membrane or loss of appetite that led to dehydration. Results from Dixit *et al.,* (2014) also recorded a significant reduction in the surface area of the glomerulus following monosodium glutamate-induced nephrotoxicity, which he suggested a reduction in glomeruli as a result of exudation of contents of capillaries, high cellular proliferation, and hyalinization.

There was a significance increase in the surface area of glomerulus space in the experimental GP1, GP2 and GP4 as compared to the control group. This could have been due to the shrinking of the glomerulus and dilatation of the bowman's capsule. Previous studies from the literature reviewed (Tobar *et al.,* 2013 and Owagboriaye *et al.,* 2022) also recorded a significance increase in the enlargement of glomerulus space as a result of hyperfiltration of the glomerulus space and degeneration of the glomerulus capillaries when exposed to nephrotoxic agents. There was no significance increase in the surface area of the glomerulus space of the experimental GP3 as compared to the control thus this may suggest reconstructive restoration (Table 4.6).

CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

1. Creatinine and urea levels were good markers for assessment of cisplatin induced nephrotoxicity and restoration effects of *Mwarubaini* among the Wister albino rats.
2. The gross morphometry parameters such as the length, width, thickness and volume of the kidney were able to demonstrate Cisplatin induced nephrotoxicity and restoration effects of *Mwarubaini* among the Wister albino rats.
3. Out of the three (Low, Medium & high) doses, the medium dose of *Mwarubaini* was able to restore cisplatin-induced nephrotoxicity among the Wister albino rats.
4. There was a significance increase in the surface area of the bowman's capsule, glomerulus space and reduction in glomerulus in cisplatin-induced nephrotoxicity among the Wister albino rats.

6.2 Recommendation

1. Creatinine and urea levels to be used in monitoring cisplatin induced nephrotoxicity and restoration effects of *Mwarubaini* among the Wister albino rats.
2. The gross morphometry parameters which are the length, width, thickness, and volume of the kidney to be used to demonstrate Cisplatin induced nephrotoxicity and restoration effects of *Mwarubaini* among the Wister albino rats.
3. The medium dose of *Mwarubaini* of 5mg/kg to be used to restore cisplatin-induced nephrotoxicity among the Wister albino rats.
4. The histo- structural changes and stereological of the kidney should be examined to demonstrate the restorative effect of *Mwarubaini* in cisplatin induced nephrotoxicity among the Wister albino rats.

6.3 Recommendation for future study

The study recommends the use of KIM-1 and NGAL markers to confirm nephrotoxicity because urea and creatinine levels are limited by delayed changes following kidney injury and have low sensitivity and specificity.

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APPENDICES

APPENDIX I: DATA ENTRY FORM

GROUP CODE:______________________________.

ID CODE:___________________________________.

WEIGHT

GROSS MORPHOMETRIES

Weight of left kidney	Volume of left kidney	Width of left kidney	Thickness of left kidney	Length of left kidney
Weight of right kidney	Volume of right kidney	Width of right kidney	Thickness of right kidney	Length of right kidney
HISTOSTEREOLOGICAL MEASUREMENTS.				
Surface area of the bowman's capsule	Surface area of the glomerulus	Surface area of the glomerulus space		

APPENDIX II: POST GRADUATE APPROVAL

MASENO UNIVERSITY
SCHOOL OF GRADUATE STUDIES
Office of the Dean

Our Ref: MSC/SM/00010/020

Private Bag, MASENO, KENYA
Tel:(057)351 22/351008/351011
FAX: 254-057-351153/351221
Email: sgs@maseno.ac.ke

Date: 25th August, 2022

TO WHOM IT MAY CONCERN

RE: PROPOSAL APPROVAL FOR WANJALA EDWIN— MSC/SM/00010/2020

The above named is registered in the programme of Master of Science in *Human* Anatomy in the School of Medicine Sciences, Maseno University. This is to confirm that his research proposal titled **"Evaluation of the Restorative Effects of Azadirachta Indica on Cisplastin Induced Nephrotoxicity in Wister Albino Rats"** has been approved for conduct of research subject to obtaining all other permissions/clearances that may be required beforehand.

for Prof. J.O. Agure
DEAN, SCHOOL OF GRADUATE STUDIES

Maseno University *ISO 9001:2008 Certified*

APPENDIX III: ANIMAL RESEARCH APPROVAL

OFFICE OF THE CHAIRPERSON
INSTITUTIONAL SCIENTIFIC ETHICS REVIEW COMMITTEE
UNIVERSITY OF EASTERN AFRICA, BARATON
P.O. BOX 2500-30100, Eldoret, Kenya, East Africa

B1019012023 January 19, 2023

TO: Wanjala U. Edwin
Department of Human Anatomy
Maseno University

Dear Edwin,

RE: Evaluation of the Restorative Effects of Azadirachta indica on Cisplastin Induced Nephrotxicity in Wister Albino Rats *(Rattus norvegicus)*

This is to inform you that the Institutional Scientific Ethics Review Committee (ISERC) of the University of Eastern Africa Baraton has reviewed and approved your above research proposal. Your application approval number is UEAB/ISERC/10/01/2023. The approval period is 19th January, 2023 – 19th January, 2024.

This approval is subject to compliance with the following requirements;

i. Only approved documents including (informed consents, study instruments, MTA) will be used.
ii. All changes including (amendments, deviations, and violations) are submitted for review and approval by the Institutional Scientific Ethics Review Committee (ISERC) of the University of Eastern Africa Baraton.
iii. Death and life threatening problems and serious adverse events or unexpected adverse events whether related or unrelated to the study must be reported to the Institutional Scientific Ethics Review Committee (ISERC) of the University of Eastern Africa Baraton within 72 hours of notification.
iv. Any changes, anticipated or otherwise that may increase the risks or affected safety or welfare of study participants and others or affect the integrity of the research must be reported to the Institutional Scientific Ethics Review Committee (ISERC) of the University of Eastern Africa Baraton within 72 hours.
v. Clearance for export of biological specimens must be obtained from relevant institutions.
vi. Submission of a request for renewal of approval at least 60 days prior to expiry of the approval period. Attach a comprehensive progress report to support the renewal.
vii. Submission of an executive summary report within 90 days upon completion of the study to the Institutional Scientific Ethics Review Committee (ISERC) of the University of Eastern Africa Baraton.

Prior to commencing your study, you will be expected to obtain a research license from National Commission for Science, Technology and Innovation (NACOSTI) https://oris.nacosti.go.ke and also obtain other clearances needed.

Sincerely yours,

Prof. Jackie K. Obey, PhD
Chairperson, Institutional Scientific Ethics Review Committee

University of Eastern Africa, Baraton
19 JAN 2023
P. O. Box 2500-30100, ELDORET
Research Ethics Committee

A SEVENTH-DAY ADVENTIST INSTITUTION OF HIGHER LEARNING
CHARTERED 1991

APPENDIX IV: NACOSTI LICENCE

REPUBLIC OF KENYA

NATIONAL COMMISSION FOR SCIENCE,TECHNOLOGY & INNOVATION

Ref No: **614664**

Date of Issue: **28/February/2023**

RESEARCH LICENSE

This is to Certify that Mr.. edwin uluma wanjala of Maseno University, has been licensed to conduct research as per the provision of the Science, Technology and Innovation Act, 2013 (Rev.2014) in Kisumu on the topic: EVALUATION OF THE RESTORATIVE EFFECTS OF AZADIRACHTA INDICA ON CISPLASTIN INDUCED NEPHROTOXICITY IN WISTER ALBINO RATS (RATTUS NORVEGICUS) for the period ending : 28/February/2024.

License No: **NACOSTI/P/23/23376**

614664

Applicant Identification Number

Director General
NATIONAL COMMISSION FOR SCIENCE,TECHNOLOGY & INNOVATION

Verification QR Code

NOTE: This is a computer generated License. To verify the authenticity of this document, Scan the QR Code using QR scanner application.

See overleaf for conditions

National Commision for Science, Technology and Innovation -

THE SCIENCE, TECHNOLOGY AND INNOVATION ACT, 2013 (Rev. 2014)
Legal Notice No. 108: The Science, Technology and Innovation (Research Licensing) Regulations, 2014

The National Commission for Science, Technology and Innovation, hereafter referred to as the Commission, was the established under the Science, Technology and Innovation Act 2013 (Revised 2014) herein after referred to as the Act. The objective of the Commission shall be to regulate and assure quality in the science, technology and innovation sector and advise the Government in matters related thereto.

CONDITIONS OF THE RESEARCH LICENSE

1. The License is granted subject to provisions of the Constitution of Kenya, the Science, Technology and Innovation Act, and other relevant laws, policies and regulations. Accordingly, the licensee shall adhere to such procedures, standards, code of ethics and guidelines as may be prescribed by regulations made under the Act, or prescribed by provisions of International treaties of which Kenya is a signatory to
2. The research and its related activities as well as outcomes shall be beneficial to the country and shall not in any way;
   i. Endanger national security
   ii. Adversely affect the lives of Kenyans
   iii. Be in contravention of Kenya's international obligations including Biological Weapons Convention (BWC), Comprehensive Nuclear-Test-Ban Treaty Organization (CTBTO), Chemical, Biological, Radiological and Nuclear (CBRN).
   iv. Result in exploitation of intellectual property rights of communities in Kenya
   v. Adversely affect the environment
   vi. Adversely affect the rights of communities
   vii. Endanger public safety and national cohesion
   viii. Plagiarize someone else's work
3. The License is valid for the proposed research, location and specified period.
4. The license any rights thereunder are non-transferable
5. The Commission reserves the right to cancel the research at any time during the research period if in the opinion of the Commission the research is not implemented in conformity with the provisions of the Act or any other written law.
6. The Licensee shall inform the relevant County Director of Education, County Commissioner and County Governor before commencement of the research.
7. Excavation, filming, movement, and collection of specimens are subject to further necessary clearance from relevant Government Agencies.
8. The License does not give authority to transfer research materials.
9. The Commission may monitor and evaluate the licensed research project for the purpose of assessing and evaluating compliance with the conditions of the License.
10. The Licensee shall submit one hard copy, and upload a soft copy of their final report (thesis) onto a platform designated by the Commission within one year of completion of the research.
11. The Commission reserves the right to modify the conditions of the License including cancellation without prior notice.
12. Research, findings and information regarding research systems shall be stored or disseminated, utilized or applied in such a manner as may be prescribed by the Commission from time to time.
13. The Licensee shall disclose to the Commission, the relevant Institutional Scientific and Ethical Review Committee, and the relevant national agencies any inventions and discoveries that are of National strategic importance.
14. The Commission shall have powers to acquire from any person the right in, or to, any scientific innovation, invention or patent of strategic importance to the country.
15. Relevant Institutional Scientific and Ethical Review Committee shall monitor and evaluate the research periodically, and make a report of its findings to the Commission for necessary action.

National Commission for Science, Technology and
Innovation(NACOSTI),
Off Waiyaki Way, Upper Kabete,
P. O. Box 30623 - 00100 Nairobi, KENYA
Telephone: 020 4007000, 0713788787, 0735404245
E-mail: dg@nacosti.go.ke
Website: www.nacosti.go.ke

APPENDIX V: OUT-PUT OF SPSS DATA ANLYSIS

```
ONEWAY UREA CREATININE BY groupname
  /POLYNOMIAL=1
  /STATISTICS DESCRIPTIVES HOMOGENEITY BROWNFORSYTHE WELCH
  /PLOT MEANS
  /MISSING ANALYSIS
  /CRITERIA=CILEVEL(0.05)
  /POSTHOC=BONFERRONI ALPHA(0.95).
```

Oneway

Notes

Output Created		19-APR-2023 18:19:54
Comments		
Input	Data	C:\Users\BOSIRE\OneDrive\Documents\My Data Sources\EDU 4.sav
	Active Dataset	DataSet2
	Filter	<none>
	Weight	<none>
	Split File	<none
	N of Rows in Working Data File	25
Missing Value Handling	Definition of Missing	User-defined missing values are treated as missing.
	Cases Used	Statistics for each analysis are based on cases with no missing data for any variable in the analysis.
Syntax		ONEWAY UREA CREATININE BY groupname /POLYNOMIAL=1 /STATISTICS DESCRIPTIVES HOMOGENEITY BROWNFORSYTHE WELCH /PLOT MEANS /MISSING ANALYSIS /CRITERIA=CILEVEL(0.05) /POSTHOC=BONFERRONI ALPHA(0.95).
Resources	Processor Time	00:00:01.52
	Elapsed Time	00:00:02.00

Descriptives

		N	Mean	Std. Deviation	Std. Error	5% Confidence Interval for Mean Lower Bound
UREA	control	5	5.30800	.398836	.178365	5.29610
	exp 1	5	10.26200	.182401	.081572	10.25656
	exp 2	5	9.96600	.161028	.072014	9.96119
	exp 3	5	5.78400	.686826	.307158	5.76350
	exp 4	5	9.25000	.287402	.128530	9.24142
	Total	25	8.11400	2.201085	.440217	8.08611
CREATININE	control	5	.48600	.041593	.018601	.48476
	exp 1	5	1.14200	.085264	.038131	1.13946
	exp 2	5	.85760	.033709	.015075	.85659
	exp 3	5	.50600	.061887	.027677	.50415
	exp 4	5	.75820	.045185	.020207	.75685
	Total	25	.74996	.253043	.050609	.74675

Descriptives

		5% Confidence Interval for Mean Upper Bound	Minimum	Maximum
UREA	control	5.31990	4.930	5.820
	exp 1	10.26744	10.120	10.570
	exp 2	9.97081	9.730	10.120
	exp 3	5.80450	4.780	6.580
	exp 4	9.25858	8.980	9.560
	Total	8.14189	4.780	10.570
CREATININE	control	.48724	.430	.530
	exp 1	1.14454	1.010	1.230
	exp 2	.85861	.803	.890
	exp 3	.50785	.430	.590
	exp 4	.75955	.715	.820
	Total	.75317	.430	1.230

Tests of Homogeneity of Variances

		Levene Statistic	df1	df2	Sig.
UREA	Based on Mean	2.816	4	20	.053
	Based on Median	2.189	4	20	.107
	Based on Median and with adjusted df	2.189	4	9.636	.146
	Based on trimmed mean	2.883	4	20	.049
CREATININE	Based on Mean	1.088	4	20	.389
	Based on Median	.856	4	20	.507
	Based on Median and with adjusted df	.856	4	13.453	.515
	Based on trimmed mean	1.042	4	20	.410

ANOVA

				Sum of Squares	df
UREA	Between Groups	(Combined)		113.184	4
		Linear Term	Contrast	5.800	1
			Deviation	107.384	3
	Within Groups			3.090	20
	Total			116.275	24
CREATININE	Between Groups	(Combined)		1.473	4
		Linear Term	Contrast	.004	1
			Deviation	1.469	3
	Within Groups			.064	20
	Total			1.537	24

ANOVA

				Mean Square	F	Sig.
UREA	Between Groups	(Combined)		28.296	183.122	.000
		Linear Term	Contrast	5.800	37.538	.000
			Deviation	35.795	231.650	.000
	Within Groups			.155		
	Total					
CREATININE	Between Groups	(Combined)		.368	114.998	.000
		Linear Term	Contrast	.004	1.310	.266
			Deviation	.490	152.893	.000
	Within Groups			.003		
	Total					

Robust Tests of Equality of Means

		Statistic[a]	df1	df2	Sig.
UREA	Welch	171.987	4	9.624	.000
	Brown-Forsythe	183.122	4	9.311	.000
CREATININE	Welch	88.742	4	9.814	.000
	Brown-Forsythe	114.998	4	13.492	.000

a. Asymptotically F distributed.

Post Hoc Tests

Multiple Comparisons

Bonferroni

Dependent Variable	(I) group name	(J) group name	Mean Difference (I-J)	Std. Error	Sig.	5% Confidence Interval	
						Lower Bound	Upper Bound
UREA	control	exp 1	-4.954000*	.248612	.000	-5.38970	-4.51830
		exp 2	-4.658000*	.248612	.000	-5.09370	-4.22230
		exp 3	-.476000*	.248612	.700	-.91170	-.04030
		exp 4	-3.942000*	.248612	.000	-4.37770	-3.50630
	exp 1	control	4.954000*	.248612	.000	4.51830	5.38970
		exp 2	.296000	.248612	1.000	-.13970	.73170
		exp 3	4.478000*	.248612	.000	4.04230	4.91370
		exp 4	1.012000*	.248612	.006	.57630	1.44770
	exp 2	control	4.658000*	.248612	.000	4.22230	5.09370
		exp 1	-.296000	.248612	1.000	-.73170	.13970
		exp 3	4.182000*	.248612	.000	3.74630	4.61770
		exp 4	.716000*	.248612	.093	.28030	1.15170
	exp 3	control	.476000*	.248612	.700	.04030	.91170
		exp 1	-4.478000*	.248612	.000	-4.91370	-4.04230
		exp 2	-4.182000*	.248612	.000	-4.61770	-3.74630
		exp 4	-3.466000*	.248612	.000	-3.90170	-3.03030
	exp 4	control	3.942000*	.248612	.000	3.50630	4.37770
		exp 1	-1.012000*	.248612	.006	-1.44770	-.57630
		exp 2	-.716000*	.248612	.093	-1.15170	-.28030
		exp 3	3.466000*	.248612	.000	3.03030	3.90170
CREATININE	control	exp 1	-.656000*	.035786	.000	-.71872	-.59328

	exp 2	-.371600*	.035786	.000	-.43432	-.30888
	exp 3	-.020000	.035786	1.000	-.08272	.04272
	exp 4	-.272200*	.035786	.000	-.33492	-.20948
exp 1	control	.656000*	.035786	.000	.59328	.71872
	exp 2	.284400*	.035786	.000	.22168	.34712
	exp 3	.636000*	.035786	.000	.57328	.69872
	exp 4	.383800*	.035786	.000	.32108	.44652
exp 2	control	.371600*	.035786	.000	.30888	.43432
	exp 1	-.284400*	.035786	.000	-.34712	-.22168
	exp 3	.351600*	.035786	.000	.28888	.41432
	exp 4	.099400*	.035786	.116	.03668	.16212
exp 3	control	.020000	.035786	1.000	-.04272	.08272
	exp 1	-.636000*	.035786	.000	-.69872	-.57328
	exp 2	-.351600*	.035786	.000	-.41432	-.28888
	exp 4	-.252200*	.035786	.000	-.31492	-.18948
exp 4	control	.272200*	.035786	.000	.20948	.33492
	exp 1	-.383800*	.035786	.000	-.44652	-.32108
	exp 2	-.099400*	.035786	.116	-.16212	-.03668
	exp 3	.252200*	.035786	.000	.18948	.31492

*. The mean difference is significant at the 0.95 level.