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Protective and therapeutic effect of *Urtica dioica* Leaves against induce chronic renal failure and coincident anemia in male rats (*Rattus albicans*)

A Thesis

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in Partial Fulfillment of the Requirement for the Degree of Master of Science in Veterinary Medicine/Physiology

By

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بسم الله الرحمن الرحيم

﴿ وَقُلْ رَبِّ زِدْنِي عِلْماً ﴾

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Dedication

To the messenger of mercy, the Prophet Muhammad "Allah blessing and peace be upon him and his pure family" ...

To my homeland, Iraq, which is bleeding with martyrs…

To my loving father and mother, who were of help and support for me, and their blessed supplication had the greatest effect in facilitating the search ship to dock on this image…

To the most wonderful body of love in all its meanings to my husband for his great patience, which made my dream transformed into reality…

To those with whom I have shared all my life, and from whom I derive my pride and determination, my dear brothers and sister…

To my beloved son who was patient with me through hardship and fatigue… (AL fadl) ….

To family, friends and everyone who benefits from this work….

Fatima

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Fatima

Summary

This study is carried out to evaluate some coagulation factors, biochemical factors, histological changes of liver, kidney and bone marrow in adult male rats that caused chronic renal failure and the possible protective role of the treatment effect of *Urtica dioica* leaves on anemia induced by chronic renal failure.

Forty-five male rats were used in the study, divided randomly into five groups (9 mice per group) for 7 weeks, the first group (GI) control was with dimethyl sulfoxide (DMSO) by interaperitoneally for 4 weeks, the second group (GII) is the negative control was administrated *Urtica Dioica* leaves powder at given 4% mixed with diet. for 4 weeks, the third group (GIII) was administrated adenine dissolved by dimethyl sulfoxide (DMSO) interaperitoneally at dose 100 mg/kg.bw for 4 weeks for induction of renal failure, the fourth group (GIV) adenine dissolved by dimethyl sulfoxide (DMSO) was administrated interaperitoneally at dose 100mg/kg.bw for 4 weeks for induction of renal failure and then given 4% *Urtica dioica* leaves powder for 3 weeks mixed with diet, and the fifth group (GV) adenine dissolved by dimethyl sulfoxide (DMSO) was administrated interaperitoneally at dose 100mg/kg.bw for 4 weeks, and then given 4% *Urtica dioica* leaves powder mixed with diet at the same time. The results showed there were a significant elevation ($p \leq 0.01$) in the serum urea, serum creatinine, free erythrocyte protoprophyrine (FEP), white blood cells count (WBCs), serum ferritin and serum hepcidin in (GIII) adenine treated group and statistically significant decrease ($p \leq 0.01$) in red blood cells count, packed cell volume PCV, platelets, serum iron and hemoglobin (Hb) in adenine group (GIII) in comparison with the other groups, after *Urtica dioica* leaves administration we observe that there were a significant reduction in serum urea, creatinine, ferritin, FEP, hepcidin and WBCs in (GIV)

treated group and statistically significant ($p \leq 0.01$) increase in RBC count, PVC, PLT, Fe and Hb in forth group (GIV) in comparing to (GIII) group.

Histological changes in kidney, liver and bone marrow demonstrate that adenine treated group was damaged atrophied and degeneration especially the renal tubules that have the adenine crystalline precipitation in add to inflammatory infiltrate of inflammatory in comparing to control group however after *Urtica dioica* leaves administration the tissues are able minimize the inflammatory condition but not fully recovered. This study explains the induction of renal failure by adenine and how can chronic renal failure results in anemia also explains how can *Urtica dioica* leaves powder works on improvement of anemia.

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List of Abbreviations

Abbreviation	Meaning
UD	*Urtica dioica*
DMSO	Dimethyl sulfoxide
FEP	Free Erythrocyte Protoprophyrine
RBCs	Red blood corpusles
Hb	Hemoglobin
WBC	White blood cell
PCV	Packed cell volume
PLT	Platelets
µmol/L	Micromole per litter

ng/m	Nano gram per millimeter
H&E	Hematoxylin and Eosin stain
Fe	Iron
IDA	Iron deficiency anemia
EP	Erythrocyte protoporphyrin
CKD	Chronic kidney disease
EPO	Erythropioteine
GFR	Glomerular filtration rate
mL/min	Milliliter per minute
mg/dL	Milligrams per deciliter
g/dL	grams per deciliter
Fe/S	Iron-sulfur cluster
TNF	Tumor Necrosis Factor
ACD	Anemia of Chronic Disease
TF	Transferrin
FPN	Ferroportin
HJV	Hemojuvelin Coreceptor
ERFE	Erythroferrone
HIFs	Hypoxia-inducible factors
PPIX	Protoporphyrin IX
ALA	Aminolevulinic Acid
ESRD	End Stage Renal Disease
CsA	Cyclosporine
IMP	Inosine Monophosphate
ATP	Adenosine Triphosphate
BMP	Bone Morphogenet

DHA	Dihydroxyadenine
ROS	Reactive oxygen speIcies
mmol/L	Millimoles per litter
µl	Microliter
Ml	Milliter
Δ	Delta
(O.D)	Optical Density
IL-6	Interleukin-6
(STAT)3	Signal Transducer and Activvator Transcription 3
(TSAT)	Transferrin saturation
ESA	Erythropoiesis-Stimulating Agents
ID	Iron Deficiency
CRF	Chronic Rnal Failure
EDR	Endothelium-Dependent Relaxation
APRT	Adenine Phosphoribosyltransferase
(KDIGO)	Kidney Disease Improving Global Outcomes
GAE	Gaelic Acid Equivalent
(sTfR)	serum transferrin receptor
CRD	Chronic Rnal Disorder
TMPRSS6	Transmembrane serine protease
EDTA-tube	Ethylene Diamin Tetraacetic Acid
GLDH	Glutamate Dehydrogenase
NADH	Nicotinamide Adenine Dinuc
(α-KG)	*α*-ketoglutarate

Chapter one

Introduction

1. Introduction

Anemia is one of the most common medical conditions, and prevalent clinical characteristics of kidney disease cases and is associated with a longer period of active inflammation. Chronic kidney disease is a form of renal failure that develops under specific circumstances, characterized by a progressive loss of kidney function over time (Remuzzi *et al.,* 2002). The consequence of complicated metabolic processes involving the kidneys, bone marrow, and many molecules (hormones, growth factors, cytokines, vitamins, and so on) all work together to keep blood oxygen levels normal (Cernaro *et al.,* 2019). Anemia is a condition associated with erythropoietin insufficiency, decreased erythrocyte survival, uremic erythropoiesis inhibitors, and dysregulation of iron homeostasis. It is a disorder that manifests itself in a variety of ways a decrease in the number of erythrocytes or the amount of hemoglobin (Ganz and Nemeth, 2011).

Iron deficiency anemia (IDA) is a serious health condition that affects people all over the world (García-Cubillana et al., 1990; Şanlidağ *et al.,* 2016). The WHO, (2017), indicating that multiple nutrient deficiencies of both minerals and vitamins are causes of anemia, which occurs when a person's iron store is inadequate, is the most prevalent dietary in the world and the primary cause of anemia (Camaschella, 2017; Mbunga *et al.,* 2021). The researches Petry *et al.* (2016); DeLoughery, (2017) confirm that iron deficiency (ID) can be caused by a variety of causes.

Hepcidin is an iron metabolism inhibitor that increases with chronic inflammation (Ishikawa *et al.,* 2021). The endocrine regulating role of hepcidin in iron balance was addressed by Camaschella *et al.* (2020) in a recent review, during acute and chronic inflammation, proinflammatory cytokines like interleukin-6. The discovery of a liver-derived peptide hormone known as hepcidin began revolutionizing our understanding of anemia's relation to

a number of inflammatory diseases (Le and Richardson *et al.,* 2002; Ward and Kaplan, 2012). It is function in iron absorption proinflammatory cytokines, in particular, boost hepcidin output. Under the influence of erythrocyte generation, there is a reduction in the number of erythrocytes generated. It was discovered that a small amount of external or internal erythropoietin in rats, erythroferrone is unable to inhibit hepcidin (Kautz *et al.,* 2014). Anemia is increased by hepcidin-mediated iron restrictions, which reduces both intestinal iron absorption and the release of stored iron for erythropoiesis. (Atkinson and Warady, 2018). Anemia develops as the diseased kidney loses its ability to produce erythropoietin, which is required for the generation of hemoglobin (Robinson, 2006).

Urtica dioica (UD) leaves belongs to the family Urticaceae commonly known as 'stinging nettle'. It is an annual plant, the leaf of the UD has a long history as an herbal medicine and a nutrient-dense food (Joshi *et al.,* 2014; Augspole *et al.,* 2017). Herbal medicines have the potential to provide efficacious treatments for inflammatory disorders (Johnson *et al.,* 2013). It can be recommended as more suitable ingredients targeted for food enrichment owing to better retention of bio-active components (Đurović *et al.,* 2017; Nallan Chakravartula *et al.,* 2021). Nettles could be used in folk veterinary medicine (Benítez *et al.,* 2012; Disler *et al.,* 2014; De Vico *et al.,* 2018). Phytochemical researches showed the existence of several beneficial chemical compounds such as minerals [(especially iron), manganese, potassium, and calcium], and vitamins, including pro-vitamin A and vitamin C (Upton, 2013; Kregiel *et al.,* 2018; Shonte *et al.,* 2020), proteins, chlorophyll, polyphenols, and carotenoids which may have an anti-oxidant role (Bonetti *et al.,* 2016; Marchetti *et al.,* 2018; Maietti *et al.,* 2021; Paulauskienė *et al.,* 2021). *Urtica dioica* is widely utilized by the traditional medicinal practitioners for treating different pharmacological properties like blood purifier, diuretic, nasal and menstrual haemorrhage, rheumatism, eczema, anaemia, antibacterial, nephritis, antioxidant, analgesic,

anti‐ inflammatory, antiviral, immunomodulatory, hepato- protective, anticolitis and anticancer effects (De Vico *et al.*, 2018; Dhouibi *et al.*, 2020).

Aim of the Study

The present study was conducted to analyze protective and therapeutic effect of *Urtica dioica* leaves on anemia induced by chronic renal failure in male rats via performing the following objectives:

1- Evaluating the effect of the powder of *Urtica dioica* leaves as anti-anemia related with chronic renal failure in male rats.

2- to evaluate the Protective effect on hematology biochemical and histopathological changes of Urtica Dioica Leaves

3- Determining iron hemostasis test (Hepcidin, iron, ferritin, Free erythrocyte protoprophyrine).

Chapter Two

Literatures review

2. Literatures Review

2.1. Anemia

Anemia is a common feature of chronic kidney disease (CKD). It is a process related to erythropoietin deficiency, shortened erythrocyte survival, uremic erythropoiesis inhibitors, and disordered iron homeostasis. It caused by inflammation is a significant and common clinical problem (Nemeth and Ganz, 2014). Anemia is commonly observed in CKD patients (Estrela *et al.,* 2021). Chronic disease anemia is common in inflammatory states such as infections, inflammatory disorders, and some cancers (Goodnough, 2005; Keel and Abkowitz, 2009; Weiss and Hohaus *et al.,* 2010; Lee *et al.,* 2010). Anemia is prevalent in CKD patients and is linked to impaired renal and cardiovascular function (Santiago-Córdova *et al.,* 2013). Cardiovascular outcomes kidney damage reduces the production of erythropoietin (EPO), an anabolic hormone. erythropoiesis stimulating factor, which causes a decrease in the synthesis of red blood cells in the body bone marrow. As the EPO deficit is a determining cause of anemia in CKD, the kidney is the primary source of EPO (Babitt and Lin, 2012). Additionally, these patients frequently develop anemia as a result of decreased erythropoietin production, which is required to maintain iron homeostasis and regulate hemoglobin synthesis (Collister *et al.,* 2017; Gafter-Gvili *et al.,* 2019; Wong *et al.,* 2019). The absence of therapy for these diseases leads to a rise in oxidative stress, which aggravates the cardio-renal axis (Kopple *et al.,* 2001; Mcclellan *et al.,* 2004). Moreover, oxidative damage promotes eryptosis, which raises the chance of developing anemia (Bissinger *et al.,* 2019). When taken collectively, these factors may have a detrimental impact on microcirculation, resulting in undesired comorbidities such as cardiovascular disease.

From a functional standpoint, anemia is defined as an insufficient red blood cell mass to effectively transport oxygen to peripheral tissues (Greer *et al.*, 2009). It is diagnosed when the haemoglobin concentration in the blood falls below a range of age- and sex-specific cutoff levels (Pasricha *et al.*, 2018; Sachdev *et al.*, 2021). As a result, based on prior researchs establishing anemia as a disease that affects peak oxygen uptake in patients with CKD (Stray-Gundersen *et al.*, 2016). In addition, blood hemoglobin appears to decrease as CKD progresses (Clyne *et al.*, 1994).

2.2. Pathophysiology of Anemia of CRD

Anemia is associated with adverse outcomes in all disease states, including CKD (Borzych-Duzalka *et al.*, 2013; Hayashi *et al.*, 2019; van Swelm *et al.*, 2020). As kidney function decreases, anemia occurs gradually, with glomerular filtration rate (GFR) dropping to 70 mL/min in male patients and 50 mL/min in females (Hsu *et al.*, 2002). And recommend that the evaluation of anemia of CKD begin in patients with a serum creatinine ≥2mg/dL when the hemoglobin is <12 g/dL in adult males and postmenopausal females and <11 g/dL in premenopausal females (KDOQI, 2007).

Because failing kidneys generate less erythropoietin (EPO) than the body required for red blood cell formation, CKD causes anemia. EPO is an endogenous hormone generated by the renal cortex's peritubular fibroblasts (Donnelly, 2001). The kidney produces the majority of this hormone (90 percent), with hepatocytes producing the rest. Blood loss, lower oxygen tension, and increased oxygen affinity promote erythropoiesis, which leads to an increase in EPO synthesis via activation of the EPO gene. The detection of hypoxia by the kidney in healthy people can lead in a 1000-fold rise in EPO production (Ebert & Franklin, 1999).

In spite of anemia can have a variety of reasons, other options must be checked out before CKD anemia is diagnosed. A complete history and physical should be used to adapt testing to each unique circumstance.

Changes in iron homeostasis, the proliferation of erythroid progenitor cells, the generation of erythropoietin, and the life span of red cells are all induced by cytokines and cells of the reticuloendothelial system, all of which attribute to the pathophysiology of anemia. Anemia can be complicated by bleeding episodes, vitamin deficiencies, hypersplenism, autoimmune hemolysis, renal dysfunction, and radio-chemotherapeutic treatments (Groopman & Itri, 2000; Rodriguez *et al.*, 2001).

Although the reduction in erythropoietin production mediated by renal insufficiency and the antiproliferative effects of accumulated uremic toxins contribute significantly, anemia with chronic kidney illness holds some of the features of anemia with another chronic disease (Eschbach, 2002).

Anemia control should focus on treating inflammatory conditions and infectious diseases (Mbunga *et al.*, 2021).

2.3. Serum Iron

About 1-2 mg of iron are absorbed daily in the gut, compensating for an equal loss; the majority of iron (20-25 mg/day) is recycled by macrophages during erythrocyte phagocytosis (Camaschella *et al.*, 2020). The duodenum is the site of regulated non-heme iron uptake: heme iron absorbs more than non-heme iron, however the processes are unknown. Non-utilized iron in enterocytes is either retained in ferritin (and lost with mucosal shedding) or exported to plasma through basolateral membrane ferroportin (and lost with mucosal shedding). (Figure 2-1).

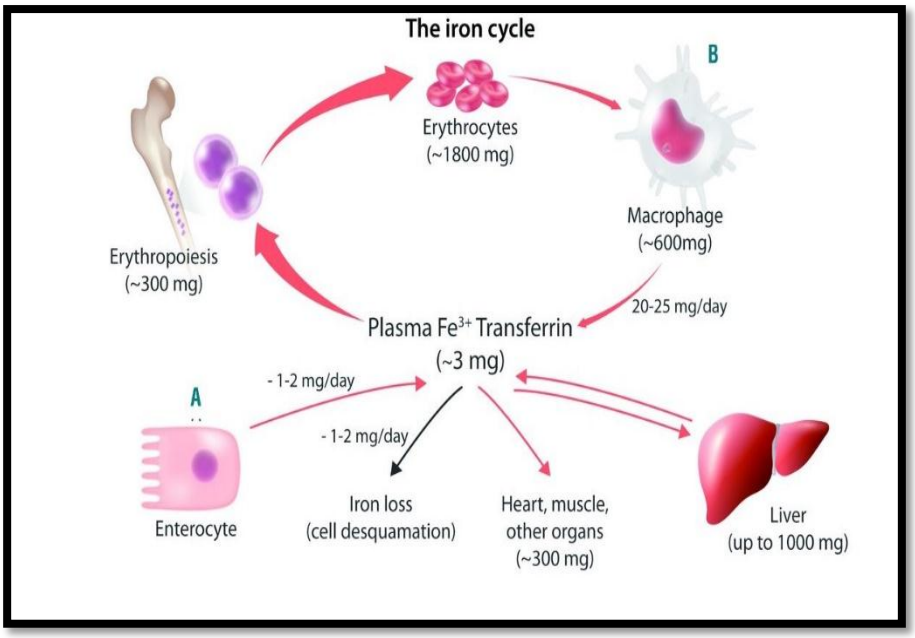


Figure (2-1): The iron cycle. (Camaschella *et al.*, 2020)

The percentage of total iron in the body that circulates bound largely to transferrin is known as the plasma or serum pool of iron. On transferrin, there are generally enough binding sites that saturation does not occur, and the typical saturation range is 35–45 percent of all binding sites. The iron in this pool is constantly changing and reflects iron in transit, such as from absorptive cells to erythrocytes forming in the bone marrow (Camaschella *et al.*, 2020). Upwards of 80% of the iron in plasma is consumed by erythroblasts in the bone marrow as they grow.

Transport of iron into cells: this implies that red cell development influences both the rate of plasma iron turnover and the iron content in plasma. This indicates that the concentration of iron in plasma, or serum, fluctuates rapidly due to the extremely dynamic movement of iron into the plasma pool from tissue (e.g. enterocytes, reticuloendothelial cells, hepatocytes, and others) and also the movement of iron out of the plasma pool into tissue (e.g. bone marrow, myocytes, blood brain barrier, etc.). (camaschella et al., 2020).

Intracellular iron is needed for a variety of activities; when not in use, it is stored in ferritin or exported via ferroportin to keep the labile iron pool within safe limits to avoid toxicity. Even though all cells may import, export, or store iron, some specialize in one or more of these functions: for example, erythroblasts specialize in iron absorption, macrophages and enterocytes specialize in iron export, and hepatocytes specialize in iron storage. The majority of iron in cells is transported to mitochondria for the synthesis of heme and Fe/S clusters. Heme is required for the production of hemoglobin, cytochromes, and enzyme activity. In erythroblasts, a "kiss and run" process between endosomes and mitochondria directs more than 80% of iron to mitochondria (Hamdi *et al.*, 2016).

The majority of the iron in the plasma pool comes from red blood cells that have been catabolized in the reticulo-endothelial system. Iron released by macrophages, and during cytokine responses in acute inflammation, causes significant variations in plasma iron content.

Infection and inflammation are two important biological processes that affect plasma iron content. During the inflammatory process, cytokines produced by immune cells have a strong influence on plasma iron content. Interleukin-6, interleukin-2, interleukin-10, and tumour necrosis factor (TNF) are all strong stimulators of iron transport from the plasma pool into macrophage storage sites (Lee *et al.*, 2005; Wrighting & Andrews, 2006).

The pace of “normalization” of this acute phase response differs between people, adding to the ambiguity surrounding the interpretation of plasma iron in inflammatory groups and individuals. For example, after an acute infection, plasma iron concentrations may recover to normal within 24–48 hours, but during chronic inflammatory conditions like arthritis, plasma iron concentrations may stay low for extended periods of time. As a result, the availability of iron to

cells is reduced, resulting in anemia of chronic disease (ACD), a disorder commonly observed in the elderly. Mohammed, et al, (2018).

2.3.1. Systemic Iron Homeostasis

Iron balance demands strict control at the cellular, systemic, and tissue levels. Reticuloendothelial macrophages are the major source of iron (Fe). that recycle iron from senescent red blood cells (RBCs), with dietary absorption and other body reserves contributing less (Figure 2-2). Iron circulates in the bloodstream mostly bound to transferrin (TF), and is stored in cells as ferritin. Hepcidin, a liver hormone, regulates systemic iron homeostasis by causing the iron exporter ferroportin (FPN) to degrade, limiting iron entry into plasma from dietary sources and body reserves. Hepcidin synthesis is suppressed by iron shortage and erythropoietic drive in order to supply enough iron for erythropoiesis and other important activities. Mohammed, et al, (2018).

Hepcidin is produced in response to iron overload and to reduce iron availability to pathogens. Iron stimulates liver endothelial cells to generate bone morphogenetic proteins (Wang & Babitt, 2019).

Iron shortage inhibits all of these processes, as well as increasing the activity of transmembrane serine protease 6 (TMPRSS6), which cleaves coreceptor hemojuvelin (HJV) and suppresses hepcidin further (Wang and Babitt, 2019). Erythropoietin (EPO) stimulates erythroid progenitor cells to generate erythroferrone (ERFE), which inhibits hepcidin by acting as a ligand trap to block the BMP signaling pathway under situations of increased erythropoietic activity (Arezes *et al.*, 2018). IL-6 and other inflammatory cytokines directly stimulate hepcidin transcription via a (STAT)-3 binding site in the hepcidin promoter during inflammation (Lee *et al.*, 2005; Wrighting and Andrews, 2006).

Hypoxia-inducible factors (HIFs), which are stabilized by low oxygen (O2) and low iron circumstances, regulate EPO synthesis in the kidney and contribute to iron homeostasis and erythropoiesis.

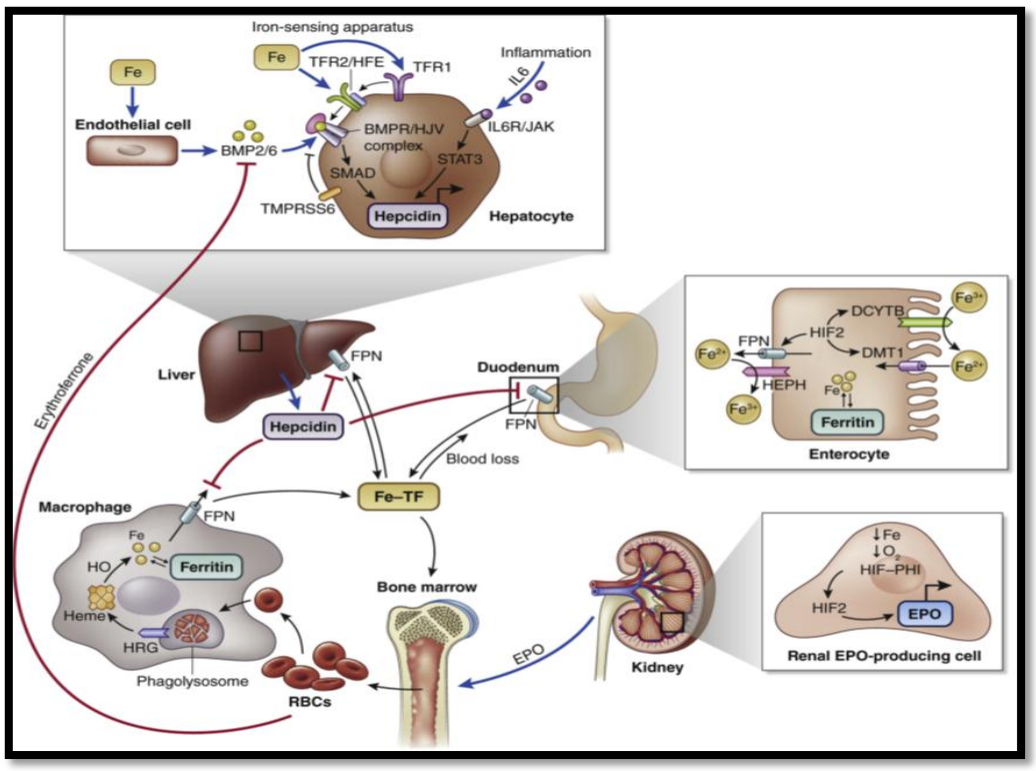


Figure (2-2): Direct and indirect regulation of systemic iron homeostasis. (Babitt *et al.*, 2021)

2.3.2. Iron Deficiency and Anemia in CKD

The definitions and diagnoses of iron deficiency and anemia in CKD are historically based on three parameters: hemoglobin (Hb); serum transferrin saturation (TSAT), an indicator of circulating iron; and serum ferritin, an indicator of stored iron. (Babitt et al., 2021)

Iron is a basic requirement of hemoglobin for erythropoiesis. CKD is related to a number of abnormalities in systemic iron homeostasis and this leading to an insufficient iron supply that may be divided into two types: absolute and functional iron insufficiency. Absolute iron deficiency is a total body iron deficiency characterized by low levels of both circulation and stored iron. While,

functional iron deficiency has been defined as a deficiency of circulating iron that limits erythropoiesis in spite of normal or elevated body iron stores. (Babitt et al., 2021)

The difference between absolute and functional iron deficiency is critical in establishing the cause of anemia and the best treatment strategy. The hepcidin, ferroportin axis, erythroferrone, and the involvement of hypoxia-inducible factor (HIFs) have all been discovered in the recent two decades, providing new perspectives into the control of systemic iron homeostasis and the pathophysiology of both absolute and functional iron deficiency in CKD (Lee *et al.*, 2005; Wang & Babitt, 2019). Due to decreased dietary intake, poor enteral absorption, and higher losses, advanced CKD is linked to a negative iron balance (Macdougall *et al.*, 2016).

Moreover, more accurately differentiating subgroups of "functional iron deficiency" resulting to inflammation/hepcidin-mediated iron sequestration vs kinetic iron shortfall from erythropoiesis-stimulating agents ESA-stimulated bursts of erythropoiesis may be clinically useful in guiding optimum therapy. Iron deficiency (ID), the leading cause of anemia and the most common nutritional deficiency globally (Mbunga *et al.*, 2021). Although anemia can be caused by a variety of causes, the research of (Petry *et al.*, 2016; DeLougher, 2017) indicates that iron insufficiency (ID), which occurs when a person's iron store is inadequate, is the most prevalent dietary cause (WHO, 2017; Camaschella, 2017). Nutritional treatments have been performed out in several poor nations for decades (WHO, 2017).

Iron is rapidly being considered as having an effect on host immunity through affecting immune cell proliferation and differentiation, as well as directly influencing cytokine production and antimicrobial immune effector pathways (Kortman *et al.*, 2017). Oral iron supplementation may also modify the gut microbiota, as well as, the gut and systemic metabolome, affecting intestinal

health, host immunity, and other systemic health outcomes (Kortman *et al.*, 2017).

2.4. Hepcidin

Is an iron regulating peptide hormone made in the liver. It controls the delivary of iron to blood plasma from intestinal cells iron, from erythrocyte-recycling macrophages, and from iron-storing hepatocytes (Nemeth and Ganz, 2014). the pathophysiology of the most prevalent iron diseases has been greatly simplified and explained since the discovery of hepcidin and its function in iron homeostasis (Ganz, 2011). Hepcidin is partially removed by glomerular filtration and degraded in the proximal tubules, leading blood hepcidin levels to rise as renal function declines (Zaritsky *et al.,* 2009). The process might have a role in the anemia and erythropoietin resistance seen in chronic renal disease (Ganz *et al.*, 2008; Ashby *et al.*, 2009; Bansal *et al.*, 2010).

Hepcidin is thought to play an important role in preventing the following iron fluxes into plasma: duodenal absorption, macrophage release (macrophages have an important function in iron homeostasis regulation), and mobilization of stored iron from hepatocytes, all of which are associated with inflammatory anemia (Nemeth and Ganz, 2014; Girelli *et al.*, 2016). moreover, high fat diet-induced hepcidin expression is linked to the development of steatosis and hepatocellular iron buildup (Meli *et al.*, 2013; Dongiovanni *et al.*, 2015).

Furthermore, the overproduction of hepcidin, a hormone generated by the liver and released into circulation that is important for maintaining systemic iron homeostasis, may explain the poor iron absorption found in many CKD patients (Babitt and Lin, 2010; Nakanishi *et al.*, 2019). Pro-inflammatory cytokines stimulate hepcidin as a defense mechanism against invading pathogens, resulting in iron sequestration and iron deficiency. Hepcidin-induced iron deficiency's relation to transient hyposideremia, anemia, and disease outcomes (Huang and Kuo, 2017).

2.5. Iron Deficiency Caused by Hepcidin is Associated with Anemia

Iron deficiency anemia, which is generally characterized by low serum ferritin levels, is frequently seen in CKD patients (Nissenson, 1997; Rocha *et al.*, 2009).

Hepcidin is essential for regulating both iron metabolism and the development of inflammatory anemia (Le and Richardson *et al.*, 2002; Ward and Kaplan, 2012). Ferroportin is internalized and destroyed after interacting with hepcidin, resulting in intracellular iron sequestration and reduced iron absorption (Nemeth, 2004). Ferroportin is currently the sole known mammalian iron exporter and is essential for iron transfer from one cell type to another (Nemeth, 2004). Hepcidin regulates not just iron absorption but also iron-restricted erythropoiesis.

In reality, in certain circumstances, because of the bias, the prevalence of ID based on ferritin may be underestimated ferritin concentrations approaching greater levels (Mbunga *et al.*, 2021).

2.6. Free Erythrocyte Protoporphyrin (FEP)

Free erythrocyte protoporphyrin (FEP), one of the outcomes of iron deficiency is an increase in erythrocyte protoporphyrin (EP) levels. Iron deficiency can raise FEP levels somewhat (Watson, 1950), but the greatest amounts are found in erythropoietic protoporphyria (Scholnick *et al.*, 1971), a rare hereditary disease, or in cases of severe lead poisoning (Watson *et al.*, 1958). The ideas basis for the measurement quantification measurement of protoporphyrin is a reduction of iron in the bone marrow for integration into newly synthesized globin and the protein porphyrin as the haemoglobin molecule is arriving its definitive steps in synthesis.

The pace at which EP levels in blood samples grow is related to the relative iron deficiency and the quantity of erythropoiesis that is taking place. Because the rate of erythropoiesis exceeds the availability of iron to the marrow, types of

haemolytic and aplastic anemia that lead to reticulocytosis are predicted to have a greater concentration of EP than normal.

The concentration of EP is reported to increase within 1–2 weeks of a shortage of iron in the bone marrow in simple iron insufficiency (Langer *et al.*, 1972; Labbe and Dewanji, 2004). After starting iron treatment, it takes more than a month to re-establish a normal EP concentration, and much longer to restore normal plasma iron kinetics.

During heme biosynthesis, protoporphyrin IX (PPIX) is produced from -aminolevulinic acid (ALA). It is colored because of its cyclic tetrapyrrole core structure, which absorbs in the visible part of the electromagnetic spectrum (Mochizuki *et al.*, 2010; Sachar *et al.*, 2016).

Porphyrins exist naturally as metal complexes, with the most well-known example being heme, the red pigment in blood cells. Hemes are iron-complexed cofactors of hemoproteins (e.g. hemoglobin, myoglobin) (Ordway and Garry, 2004; Immenschuh *et al.*, 2017). They are found all over the body. Because hemoproteins are involved in the movement of diatomic gases (respiration), chemical catalysis, and electron transfer, they are vital to life (Poulos, 2014).

Several intermediates are generated during heme production from glycine and succinyl-CoA, including -aminolevulinic acid (ALA), until protoporphyrin IX (PPIX) is converted to heme by insertion of a divalent iron [Fe (II), catalyzed by ferrochelatas] (Sachar *et al.*, 2016).

Protoporphyrin + Fe^{++} → ferrous protoporphyrin + globin → haemoglobin

2.7. Ferritin

Ferritin is a protein found in cells that stores iron for eventual use by the body. It may store up to 4,500 iron atoms in a shell-like structure made up of 24 chains, including both heavy (H) and light (L) chains with ferroxidase activity (Arosio *et al.*, 2015). Iron storage in ferritin protects against oxidative damage

while also preserving an important element for future needs. A ferritin test examines the amount of iron in the body in an indirect manner. The amount of ferritin in the body is proportional to the amount of iron it stores. (Haskins *et al.*, 1952).

Absolute iron insufficiency is associated with serum ferritin less than 100 ng/mL, according to the kidney disease outcomes quality initiative National Kidney Foundation (K/DOQI, 2006). When serum ferritin levels are low, it is a sign of iron deficiency; when levels are high, it is a marker of iron overload/inflammation, indicating macrophage ferritin concentration. One theory is that cells may re-uptake released ferritin (Truman-Rosentsvit *et al.*, 2018), as an alternate method of iron recycling, such as when macrophage iron release is restricted in inflammation.

2.8. Renal Failure

Renal failure occurs when the kidneys suddenly become unable to filter waste products from the blood. End-products from the blood are removed, and the fluid, electrolyte, and pH levels are maintained. Extracellular fluids are in a state of equilibrium. It is possible that renal disease is the root of the problem. disease, systemic disease, or urologic problems that aren't caused by the kidneys (Mohmmed, 2018). The kidney plays a crucial function in the elimination of various substances and medications. As a result, renal failure may result in the storage of these chemicals, which can build up to lethal quantities over time (Hawkins, 2011). Uremia, high blood potassium, heart disease, high blood pressure, and uremia are some of the complications of kidney failure (Hovater *et al.*, 2008).

2.9. Chronic Renal Failure (CRF)

Chronic Renal Failure (CRF) is a clinical illness in which kidney function gradually deteriorates. CRF denotes permanent loss of renal function and can be

caused by either primary kidney disease or secondary kidney disease (Henry, 2003). Diabetes, hypertension, glomerulonephritis, and polycystic kidney disease are all diseases that cause permanent loss of nephrons, resulting in chronic renal failure. Renal failure signs and symptoms usually appear gradually and do not become apparent until the disease has progressed significantly. This is due to the kidneys' incredible compensating abilities. The remaining nephrons undergo structural and functional hypertrophy when kidney structures are damaged, each increasing its function to compensate for those that have been lost. Renal failure manifests itself only when the few surviving nephrons are eliminated.

Chronic renal failure causes the kidneys' glomerular filtration, tubular re-absorptive capacity, and endocrine functions to deteriorate over time, regardless of the reason. The glomerular filtration rate (GFR) is reduced in all forms of renal failure, indicating a drop in the number of functional nephrons. Chronic anemia is the most severe hematologic change that occurs as a result of renal failure. When the GFR falls below 40 mL/minute, anemia develops, which is common in people with end stage renal disease (ESRD). Anemia is caused by a number of causes in people with chronic renal failure, including a lack of erythropoietin, uremic toxins, and iron shortage (Mohmmed, 2018).

The hormone erythropoietin, which regulates red blood cell synthesis, is produced mostly in the kidneys. The buildup of uremic toxins in the bone marrow lowers red cell production even further, and the cells that do get formed have a shorter life span (Mohmmed, 2018).

2.10. Methods for Induction Renal Failure in Rats

Renal failure has been induced using a variety of methods. The majority of the researches used surgical procedures to impair renal function by lowering the parenchyma (Finkelstein and Hayslett, 1974), as a result of a reduction in blood flow (Kaye, 1974).

Schrooten *et al.* (1998) add SrCl2 to drinking water, strontium can be used to cause chronic renal failure.

Cyclosporine (CsA) effects vasopressor systems, including as catecholamine, the renin-angiotensin-aldosterone system, endothelin, and arachidonic acid metabolites. Endothelial damage and dysfunction has emerged as the fundamental cause of CsA-induced vascular, renal, and circulatory diseases, according to growing evidence, CsA appears to impede endothelium-dependent relaxation (EDR) (Vaziri *et al.*, 1998). In glycerol-treated rats, a well-established model for myoglobin uric acute renal failure, found a considerably greater suppression of renal catalase activity (Guidet *et al.*, 1989).

Administration adenine, which is converted into 2,8-dihydroxyadenine, which deposits in renal tubules and forms crystals, causing renal tissue injury (Diwan *et al.*, 2018).

2.11. Adenine

Adenine is made from the nucleotide inosine monophosphate (IMP), which is made from glycine, glutamine, and aspartic acid (amino acids) and fusion with the enzyme tetrahydrofolate on a pre-existing ribose phosphate via a specific pathway using substrates like glycine, glutamine, and aspartic acid (amino acids) (Wang *et al.*, 2002). It's an example of a nitrogen heterocycle (Figure 2-3). Excess adenine is converted to 2,8-dihydroxyadenine when oxidized by xanthine, and it appears at a very low level in the blood. In the kidney, there is a dehydrogenase enzyme. By connecting with ribose, adenine yields adenosine, a nucleoside, which is subsequently converted to adenosine triphosphate (ATP), a nucleotide, by adding three phosphate groups to adenosine. ATP is one of the most essential mechanisms of transmitting chemical energy across chemical reactions in cell metabolism, helping to maintain energy balance (Ereciñska and Wilson, 1982).

Furthermore, adenosine is a crucial signaling molecule that is produced in response to ischemia and hypoxic situations (Fredholm, 2007). As a result, increased adenosine in the kidney suggested renal lesions caused by ischemia or hypoxia, while decreasing adenosine in the kidney could indicate greater cell apoptosis, functional degradation, or ATP depletion. (Tang *et al.,* 2015).

For its low solubility, 2,8-dihydroxyadenine occurs in the kidney, especially in the nephron tubules (Yokozawa *et al.,* 1986). Compounds of waste the obstruction of renal tubule owing to 2,8-dihydroxyadenine prevents excretion from the kidney. As a result, the concentrations of creatinine and urea nitrogen in the blood will rise (Zhao *et al.,* 2013).

Adenine is created endogenously as a by-product of the polyamine pathway and is removed by the enzyme adenine phosphoribosyl transferase. When there is no functional APRT, adenine becomes a major substrate for xanthine dehydrogenase (XDH), which oxidizes adenine to 2,8-dihydroxyadenine (DHA) (Engle *et al.,* 1996; Stockelman *et al.*,1998).

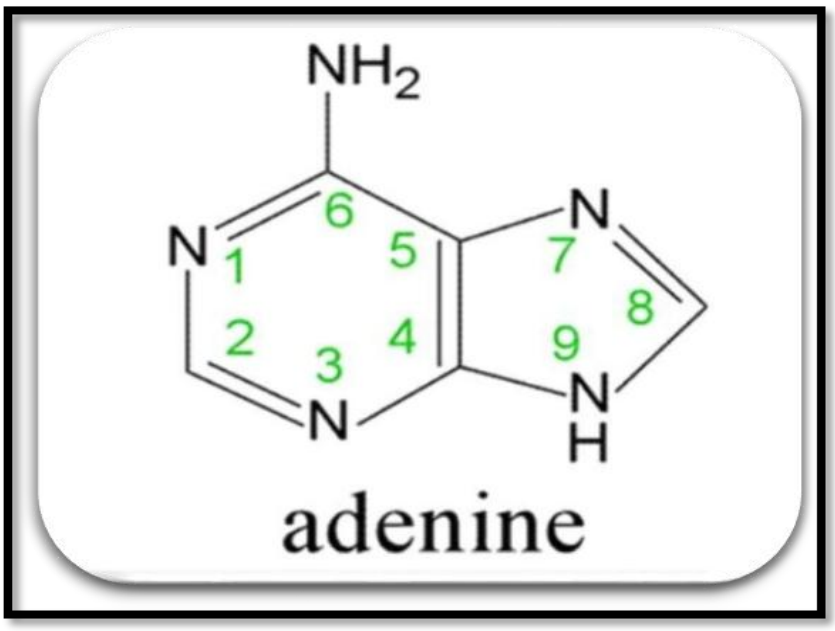


Figure (2-3): Chemical structure of adenine adapted by (Russo *et al.,* 1998)

2.12. Adenine Chronic Renal Failure Induction

Adenine induces chronic renal failure by converting it to 2,8-dihydoxyadenine, which deposits and solidifies in the proximal convoluted renal tubules. Kidney failure in rodents is characterized by blood in the urine, an increase in phosphate in the blood corresponding to hyperparathyroidism, and kidney anemia (Tamura *et al.*, 2009).

The models used to generate CKD in rats include mixing adenine with the meal or injecting it intraperitoneal at various doses for four weeks (Al Za'abi *et al.*, 2015). Intraperitoneal injection of adenine can thus be deemed a superior model than oral adenine for the induction of CKD. The advantages of this paradigm are that adenine enters the systemic circulation without having to go through any conceivable local (intestinal) direct physical interaction with any enteral ameliorating agent. It's also more practical, useful, and accurate (Ali *et al.*, 2014).

2.13. *Urtica dioica*

Nettles

Order: Rosales,

Family: Urticales - Urticaceae,

Sub-Family: Urticaceae,

Genus: *Urtica*.

Species: *U. dioica*

Species: The nettle is found in thirty different species across the world, with the exception of Madagascar and South Africa (Henning *et al.*, 2014).

Sructures of chemical constituents of *Urtica dioica* showed in (Figure 2-4).

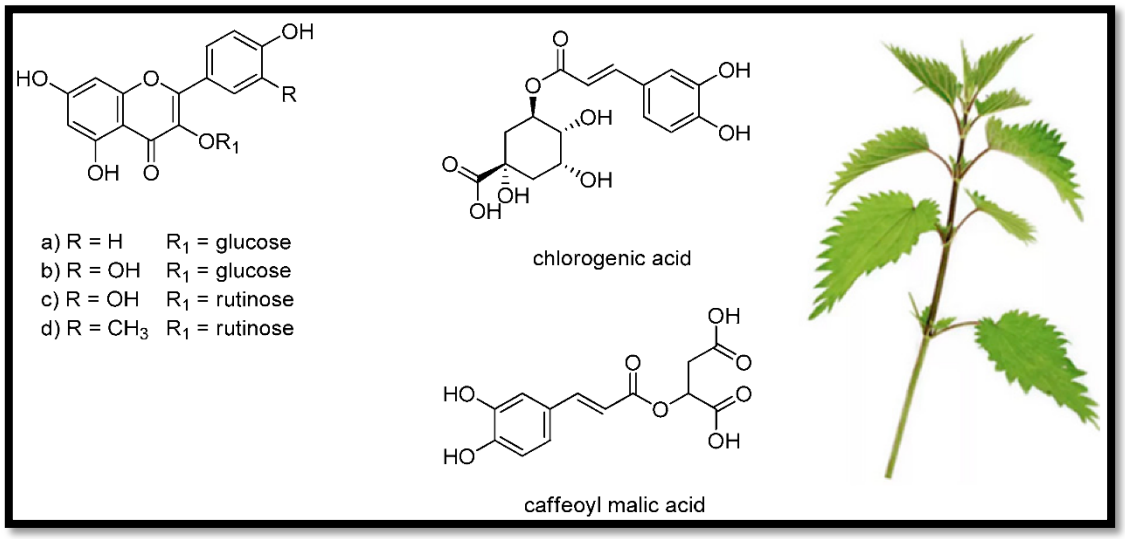


Figure (2-4): Structures of chemical constituents of *Urtica dioica* (Joshi *et al.*, 2014)

Nettle is a widely accessible plant that may be found in many parts of the world. It is a plant that can tolerate some shade. It has a dense covering of stinging hairs on its stems and leaves, which release potentially painful toxins (Taylor, 2009; Said *et al.*, 2015), as illustrated in (Figure 2-5)**.**

Urtica dioica is a herbaceous plant in the Urticaceae family that has been used to treat a range of diseases for ages. Nettle has a high nutritional value and a wide range of pharmacological effects, including anti-proliferative, anti-inflammatory, antioxidant, analgesic, immune-stimulatory, anti-infectious, hypotensive, antiulcer, and cardiovascular disease prevention due to its high content of nutrients and bioactive compounds like poly phenols, vitamins, and minerals. When eaten by mouth, up to 18 grams per day, stinging nettle is deemed harmless and has been proved to have no negative effects. The crude dried powder, dry extract, infusion (herbal tea), decoction, or fresh juice are the most frequent stinging nettle preparations. The root of the stinging nettle is used to treat mictional issues associated with benign prostatic hyperplasia, while the leaves are used to treat arthritis, rheumatism, and allergic rhinitis (Said *et al.*, 2015).

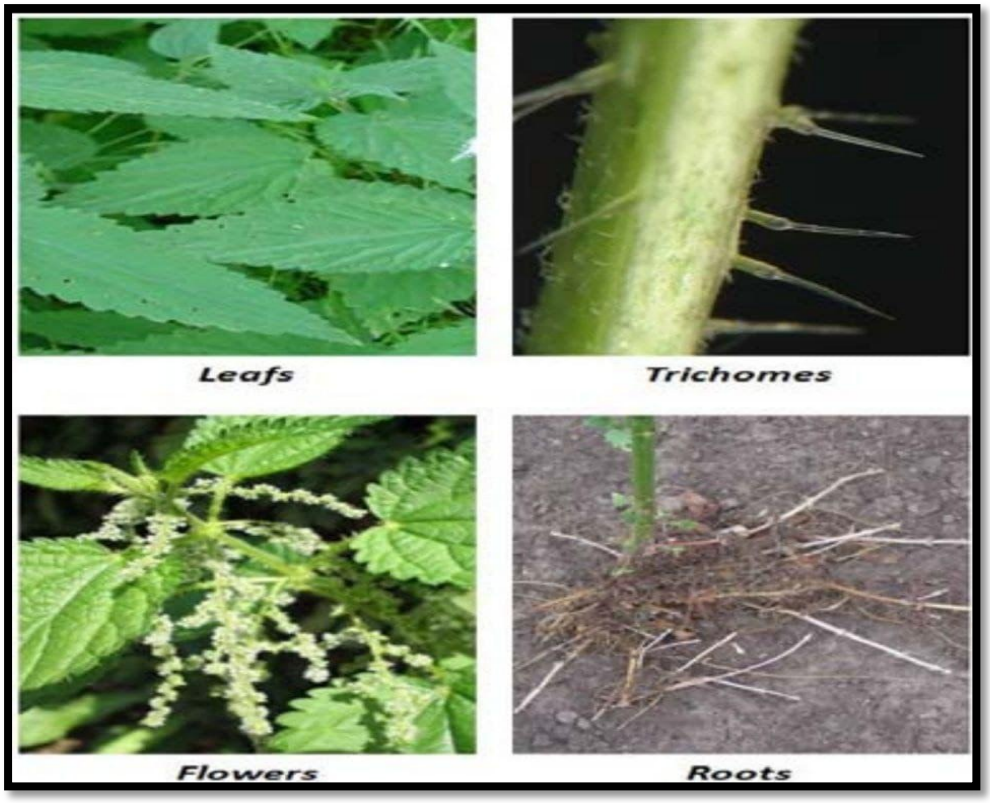


Figure (2-5): *Urtica dioica*, botanical aspects. (Said *et al.*, 2015)

2.13.1. Various Compounds and Uses of *Urtica dioica*

Natural products' medicinal potential is increasingly being sought to substitute manufactured medicines in today's society. *Urtica dioica* L. is a common green plant that may be found all over the world. It has been utilized in a variety of uses from ancient times to the current day, including alternative medicine, food, paint, fiber, manure, and cosmetics (Ak *et al.*, 2006). The key of healing has already been discovered in nature by traditional folk medicine. Nettles contain a significant number of biologically-active compounds: glycosides, tannins, phenolic substances, flavonoids, alkaloids, and proteins are all found in *U. dioica* extract (Safarinejad, 2006; Joshi *et al.*, 2014; Hajihashemi *et al.*, 2017). There are a high-nutrient, easily digestible food that is high in minerals (particularly iron) (Allardic, 1994). The fresh leaves contain high

concentrations of vitamins A, C, D, E, F, K and P, as well as, of vitamin B-complexes (Rutto *et al.*, 2013).

The elements selenium, zinc, iron, and magnesium are reported to be particularly abundant in the leaves. Rafajlovska *et al.* (2013) reported that the calcium content of stinging nettle leaves, stems, and roots was higher than the magnesium content, *Urtica* leaves in addition contain boron, sodium, iodine, chromium, copper and sulfur.

Fresh nettle leaves have less sterols and more flavonol glycosides than dried nettle leaves. Carotenoids, namely -carotene, violaxanthin, xanthophylls, zeaxanthin, luteoxanthin, and lutein epoxide, are also found in the plant's leaves Upton, (2013). In addition, nettle leaves contain terpene diols, terpene diol glucosides, -tocopherol, and five monoterpenoid components (Kavalali, 2004).

Weglarz, (2000) and Roslon, (2001) investigated the polyphenolic acid content of leaves and rhizomes, they discovered that the male forms had larger levels of these chemicals, but that the chemical profiles of polyphenolic acids from female plants were significantly more varied. Furthermore, *U. dioica* is thought to be the sole plant with choline acetyl-transferase, an acetylcholine-synthesizing enzyme (Nasiri *et al.*, 2011).

Urtica plants' hairs contain an acrid fluid containing active ingredients such as acetylcholine, histamine, and formic acid, as well as, silica, serotonin, and 5-hydroxy tryptamine. Smooth muscle stimulants make up a large portion of these substances (Oliver *et al.*, 1991). *U. dioica* has a high amount of acetylcholine in its new hairs (Emmelin and Feldberg, 1948). Numerous studies have found that each nettle species, as well as each component of the plant (root, stem, or leaves), has a unique concentration and profile of bioactive chemicals. Different factors, such as variety, genotype, climate, soil, plant vegetative stage, harvest time, storage, processing, and treatment, all impact the phenolic content of plants (Angela and Meireles, 2009; Marrelli *et al.*, 2012).

2.13.2. Therapeutic Uses *Urtica dioica*

As a result, depending on their chemical properties, various species of nettle may have varied purposes (Otles and Yalcin, 2012). Many natural substances are now recognized to modulate physiological processes and biotransformation events involved in the detoxification process, providing protection from environmental toxicants' cytotoxic, genotoxic, and metabolic effects (Saha and Das, 2003). *Urticae* is described as a useful herb for various therapeutic applications by the World Health Organization (WHO) in its monographs on "selected medicinal plants" (WHO, 2004). Nettles, allowing them to be used in a wider range of food and pharmaceutical applications.

Furthermore, substances including nicotinamide, and synephrine have a role in anti-inflammatory properties. The vasorelaxation impact of nitric oxide and the inhibition of calcium channel activity are linked to the vasodilatory effects of UD (Upaganlawar *et al.*, 2006; Halder and Sharma, 2017). Orčić *et al.* (2014) measured several plant phenolics in methanol extracts of *U. dioica* flowers, roots, stems, and leaves collected from various places in Serbia.

Stinging nettle (*Urtica dioica* and *Urtica urens*) preparations have been used in nursing mothers orally as a postpartum as a "tonic" for treating anemia (Scott & Jacobson, 2005; Dennehy *et al.*, 2010).

Iron-promoted oxidation of phospholipids, linoleic acid, and deoxyribose is inhibited by aqueous infusions of *U. dioica* (Matsingou *et al.*, 2001).

Ratnam *et al.* (2006) reported an increase in urine production by 20% after 1g/kg oral dose in 10% decoction in rats. The diuretic effect of stinging nettle was approximately 25% of that achieved with hydrochlorothiazine (25 mg/kg) (Tita *et al.*, 1993).

Urtica spp. has no immediate or delayed adverse effect on human or animal health and has no negative impact on the environment, according to the study

(EC, 2017). Nettles are also employed in folk veterinary medicine due to their high amount of nutritional elements (Gülçin *et al.*, 2003; Viegi *et al.*, 2003; Safamehr *et al.*, 2012). *Urtica dioica* seed extract, according to Uyar *et al.* (2016), has a protective hepatorenal impact in broilers with aflatoxicosis, most likely through promoting antioxidant defense mechanisms. Many nutritional supplements based on *Urtica spp*. are currently available on the market. Their popularity can be attributed to their chemical composition, which is non-toxic, as well as their inexpensive cost and widespread availability.

2.13.3. Activity of Antioxidants of Nettles

Natural antioxidants have recently received a lot of attention for their potential to protect the human body against chronic disease caused by different ROS (Vives Corrons *et al.*, 1995; Diplock, 1991).

Antioxidants are emerging as prophylactic and therapeutic agents which scavenge free radicals or reactive oxygen species and prevent their damaging effect. Free radicals have been linked to the development of diseases such as cancer, diabetes, cardiovascular disease, autoimmune disease, and neurological disorders, as well as aging.

Many researches on plants and medicines show that they have anti-inflammatory and antioxidant properties that protect kidneys against renal-induced damage (Alan *et al.*, 2011; Dorai *et al.*, 2011; Roso *et al.*, 2012).

Ozen and Korkmaz, (2003) concluded that the use of *Urtica dioica* can have a significant effect on drug metabolism enzyme systems. Antioxidant enzyme levels may also be able to recover enough harmful free radicals generated during normal and pathological cellular metabolism (Marchetti *et al.*, 2018). The findings of Yener *et al.* (2009) study might have significant consequences for the chemopreventive and antioxidant profiles of this plant.

Nettles have been demonstrated to have strong efficacy in neutralizing generated reactive oxygen species (ROS) among these natural protectors. due to their efficacy in causing damage to biological tissues and contributing to disease phenotypes, ROS have been the subject of intensive research in recent years (Halliwell and Gutteridge, 1990 and 1992). The origin and mechanism (s) of their generation as well as reactivity been linked with biological macromolecules and inorganic/organic cofactors partaking of physiological metabolic functions in the majority of biota (Matsingou *et al.,* 2001).

Nettles are high in phenolic compounds, which are ascribed to hydrocinnamic acids, flavonoids, and tannins, and have a variety of biological activities (Adhikari *et al.,* 2016; Đurovi´c *et al.,* 2017; Movagharnejad *et al.,* 2019; Shonte *et al.,* 2020). However, Shonte *et al.* (2020) found greater phenol concentration in nettles oven-dried at 70 C.

Most of the antioxidant activity of plants is due to the presences of secondary metabolites like polyphenols and carotenoids. Nettle leaves (*Urtica dioica* variety) are high in phytoconstituents such as polyphenols, flavonoids (kaempferol, isorhamnetin, quercetin, isoquercitrin, and rutin), phenolic acids (caffeic acid and chlorogenic acid), and carotenoids (carotene, hydroxyl-carotene, luteoxanthin, lutein epoxide, and violaxanthin), but also essential oils and fatty acids (Joshi *et al.,* 2014). This went along with an increment in the concentration of lutein and -carotene, two important carotenoids involved in free-radical scavenging activity.

Nettle-enriched evidenced a total phenolic content and its exhibited antioxidant activity. The most interesting results were obtained for total phenols and antioxidant activity in UD. *Urtica dioica's* therapeutic benefits in the prevention of renal tubular damage and diseases have been identified, which is consistent with those data. Additionally, the enhanced antioxidant features might exert interesting nutritional benefits, such as protective effect. The findings of

the experiments proved that *Urtica dioica* has a nephroprotective effect in adenine-induced nephrotoxicity.

2.13.4. *Urtica dioica's* Effect on Anemia

Our findings confirm that the nettle leaves are a valuable ingredient for the development of enriched foods with improved nutritional and functional properties. This can be done on a large scale production.

Anemia due to iron deficiency is a severe health problem that affects individuals all over the world (Şanlidağ *et al.,* 2016). Multiple nutritional deficiencies of both minerals and vitamins are causes of anemia, according to the WHO, (2017) which is the most frequent dietary in the world and the major cause of anemia. Anemia arises when a person's iron reserve is inadequate (Camaschella, 2017; Mbunga *et al.,* 2021).

It is regarded that *Urtica dioica* leaves are a high-value resource, and numerous studies have shown that the vitamin and mineral content is exceptional, including iron, which is a crucial element for the development of anemia due to iron deficiency.

Urtica dioica manifest a high-nutrient, easily digestible food that is high in minerals (especially iron) (Allardic, 1994). It has been demonstrated that significantly increase of the level of fibers, calcium and copper, and also the iron content.

Selenium, zinc, iron, and magnesium are all shown to be abundant in the leaves. Vitamins A, C, D, E, F, K, and P, as well as vitamin B-complexes, are plentiful in the fresh leaves (Rutto *et al.,* 2013). Furthermore, aqueous nettle extracts showed a high in iron accumulation in the leaves (Peterson and Jensen, 1985; Mariˇci´c *et al.,* 2021).

Because of the improved preservation of bio-active components of nettle, it can be considered as a more appropriate ingredient for food enrichment (Urovi *et al.,* 2017; Nallan Chakravartula *et al.,* 2021).

In view of the fact that its health-promoting properties, nettles are one of the most widely used medicinal herbs in the world. Unlike pharmaceutical medicines, which have been created for a specific condition regardless of their side effects, the entire plant of *Urtica dioica* not only has no side effects, but also has several therapeutic qualities against a variety of diseases.

As a result, in spite of all its benefit, authors proposed that this plant be regarded an emerald in the medical kingdom, rather than just a simple weed (Fattahi *et al.,* 2016).

Chapter Three

Materials and Methods

3. Materials and Methods

3.1. Chemicals

Table (3-1) : Used chemicals according to the company and origin

No.	Chemical agents	Source
1	Adenine powder	Sigma Aldrich Company (USA)
2	Chloroform	Noorbrok, (England)
3	DMSO	LOBA, (Chi)
4	Formalin 10%	TEBIA Company, (USA)
5	Rat Ferritin (FE) ELISA Kit.	Biocellular Company, (China)
6	Rat Hepcidin (Hepcidin) ELISA Kit.	Biocellular Company, (China)
7	Eosin-hematoxylin stain	Merck, (Germany)
8	Rat FEP ELISA Kit.	Biocellular Company, (China)
9	Urea Kit	Biocellular Company, (Germany)
10	Creatinine Kit	Biocellular Company, (Germany)
11	Fe	Biocellular Company, (Germany)
12	*Urica dioica* Leaf powder	Bensina center

3.2. Devices and instruments

Table (3.2) the instruments and devices used in present study with manufacture company and Origin

No.	Tools type	Source
1	Automated hematology analyzer	China
2	Analytical sensitive balance	Sartorius /Germany
3	Digital camera (Canon)	Toup cam /China
4	EDTA tube	Jordan
5	Electric centrifuge (80-2)	China
6	Electric grinder	China
7	ELISA biotech	USA
8	Eppendorf tube	Biolabse /England
9	Freezer	Denka /China
10	Gel tube	Jordan
11	Glasses	China
12	Gloves	Malaysia
13	Incubator	Faithful /Malaysia
14	Insulin syringes	Italy
15	Rack for blood standing	China
16	Light microscope	Lieca /China

17	Masks	China
18	Micropipette	Biobase /China
19	Optical microscope	Italy
20	Semi-auto chemistry analyzer	GENEX XCHEM-S1/US
21	Sterile syringes (1, 3 and 5 ml)	China
22	Test tube	China
23	Feed press	China

3.3 Animals of the Study

the experiment was carried at the laboratory animal's facilities Faculty of veterinary medicine karbala University. forty _five Rattua albicans rats were useal in this study with an areange age range between 150-200g. These animals were kept in suitable environmental condition with a temperature of around 25-28 C°, relative lumirlity 40٪ - 60٪ conditions room with a 12:12 h/light light / dark cycle (Meyer et al., 1982). The animals were housed in aplastic cage with diameters of 50×35×15cm. The feed given were pellets. The animals were kept for at last 15 days for acclimatization before experiment began.

3.4. Preparation of Adenine

Adenine was obtained from Sigma Aldrich Company (USA).

3.5. Preparation of *Urtica dioica* Leaves Powder

The ready-made dried *Urtica dioica* leaves were obtained from Bensina center and then the samples were then grained by the electric grinder into powder 4% of *Urtica dioica* leave powder was mixed with the usual nutrition

ration of crushed feed diet mixed with tap water then made in shape of cookies then bake in the oven for 15 minutes in 180℃ (MAN *et al*,2019).

Figure (3-1): *Urtica dioica* leave powder

3.6. The Experimental Design

Forty-five male rats were divided randomly into five groups (9/group) as shown in Figure (3-2):

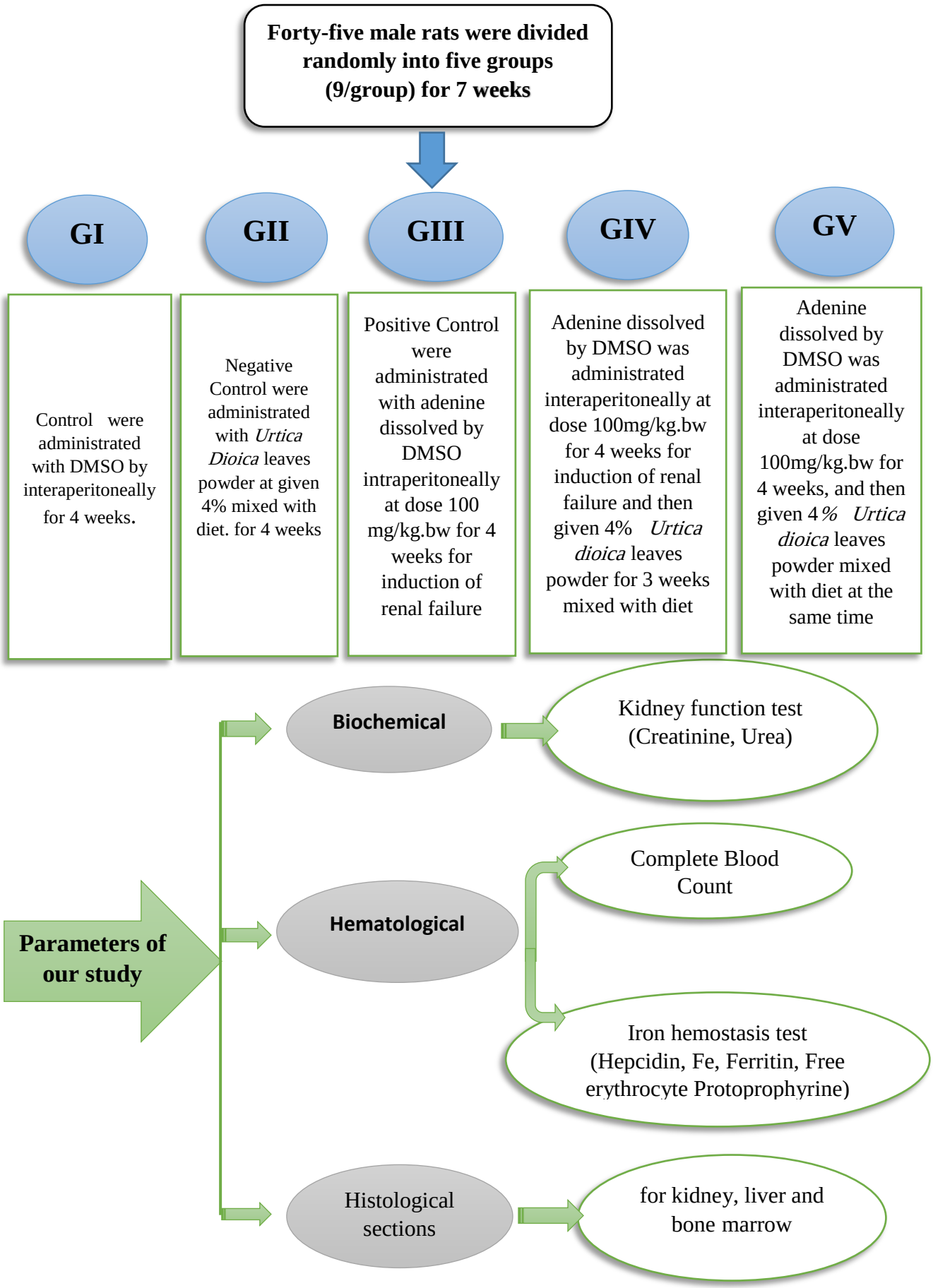
1- Group (G1) control was administrated with DMSO by interaperitoneally for 4 weeks.

2- Group (G2) negative control was administrated with *Urtica Dioica* leaves powder at given 4% mixed with diet. for 4 weeks (Turgeon, 2012).

3- Group (G3) positive control w administrated with adenine dissolved by DMSO intraperitoneally at dose 100 mg/kg.bw for 4 weeks for induction of renal failure (Rahman *et al.*, 2018).

4- Group (G4) adenine dissolved by DMSO was administrated interaperitoneally at dose 100mg/kg.bw for 4 weeks for induction of renal failure and then given 4% *Urtica dioica* leaves powder for 3 weeks mixed with diet (Gülçin *et al.*, 2004; Turgeon, 2012).

5- Group (G5) adenine dissolved by DMSO was administrated interaperitoneally at dose 100mg/kg.bw for 4 weeks, and then given 4% *Urtica dioica* leaves powder mixed with diet at the same time (Campbell *et al*., 2007).



(Figure 3-2): The Experimental design

3.7. Blood Collection and Tissue Preparation

Experimental animals (rats) get anaesthetized by putting them in covered jar include cotton rinsed with chloroform to be sedated for the next step which is blood via cardiac puncture in sterile syringes by needle prick in the heart draining 5ml of blood carefully;

Then separation of the blood collected into 1 ml drained in EDTA tube for the analysis of iron homeostasis tests quickly separate the blood in the centrifuge at 3500 rpm in 15 minutes and then set at Eppendorf tube;

While the rest of the blood drained into two separated parts; about 2 ml set in gel tube it is left about half hour at room temperature for properly agglutinated;

Then it would be separated at centrifuge at 3000 rpm for fifteen minutes to get the serum apart in Eppendorf tube;

Both of samples are hold in freezer at -20 ℃, while the remaining of the blood drained into EDTA-tube for hematological test liver, kidney was eradicated by abdominal surgical incision;

The femur was also removed by a surgical incision the vital organs which is kidney, liver and bone are transformed in to formalin (10%) to be ready for histological examination.

3.8. Biochemical Parameters

3.8.1. Kidney Function Test

3.8.1.1 Estimation of Serum Urea Concentration

Serum urea was measured by (Spectrum-urea kit, Egypt-IFUFCC40) by Semi-auto chemistry analyzer (Jing *et al.,* 2018), as shown appendix (I).

Principle

Colorimetric determination of urea activity is obtained according

to the following reactions:

1. Urea is hydrolyzed in the presence of water and urease to produce ammonia and carbon dioxide.

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

2. In the presence of glutamate dehydrogenase (GLDH) and reduce nicotinamide adenine dinucleotide (NADH), the ammonia combines with α-ketoglutarate (α-KG) to produce L- glutamate.

$$2NH_4 + 2\ \alpha\text{-KG} \xrightarrow{\text{GLDH}} 2\ \text{L-Glutamate}$$

$$+ \qquad\qquad +$$

$$2NAD + H_2O \qquad\qquad 2NADH$$

The rate decrease in the NADH concentration is directly proportional to the urea concentration in the specimen. It is determined by measuring the absorbance at 578 nm.

3.8.1.2. Estimation of Serum Creatinine Concentration

Creatinine concentration in serum was determined by using a special kit Spectrum–creatinine kit, Egypt-IFUFCC10, by using device spectrophotometer sesil, England measured by semi-auto chemistry.

analyzer (Dunn *et al.*, 2004). As shown in appendix (II).

Principle

Creatinine reacts with picric acid in alkaline solution to form a colored complex (Tietz, 1986).

$$\text{Creatinine} + \text{picrate} \xrightarrow{\text{NaOH}} \text{yellow red complex}$$

3.8.2. Hematological Parameters

3.8.2.1. Complete Blood Corpuscles

A complete blood count (CBC) is a blood test used to measure overall blood cell by automated hematology analyzer (George-Gay & Parker, 2003). As shown appendix (III).

3.8.3. Iron Homeostasis

3.8.3.1. Estimation of Serum Iron concentration

Iron concentration in serum was determined by using also, the mind ray device was used to determine the percentage of iron, and the instructions of the producing company were followed in the method of examination.

3.8.3.2 Estimation of Rat Serum Ferritin (FE) ELISA Kit.

This examination was done by preparing process from Biocellular Company (China) by using enzyme-linked immunosorbent assay method to determine the concentrations of FE in rat serum (Watanabe *et al.*, 2001) as shown appendix (IV)

Principle of the assay

The kit assay rat FE level in the sample, use purified rat FE antibody to coat microtiter plate wells, make solid-phase antibody, then add FE to the wells, combined antibody which with HRP labeled, become antibody-antigen-enzyme-antibody complex, after washing completely, add TMB substrate solution, TMB substrate becomes blue color at HRP enzyme-catalyzed, reaction is terminated by the addition of a sulphuric acid solution and the color change is measured spectrophotometrically at a wave length of 450 nm. The concentration of FE in the samples is then determined by comparing the O.D. of the samples to the standard curve.

3.8.3.3 Estimation of Rat Free Erythrocyte Protoporphyrin (FEP) ELISA Kit

This examination was done by preparing process from Biocellular company (China) by using a specific kit to measures rapidly and reliably free erythrocyte porphyrins in a blood sample as shown appendix (V).

Principle of Assay

This FEP ELISA kit is intended laboratory for research use only and is not for use in diagnostic or therapeutic procedures. The Stop Solution changes the color from blue to yellow and the intensity of the color is measured at 450 nm using a spectrophotometer. In order to measure the concentration of FEP in the sample, this FEP ELISA Kit includes a set of calibration standards. The calibration standards are assayed at the same time as the samples and allow the operator to produce a standard curve of optical density versus FEP concentration. The concentration of FEP in the samples is then determined by comparing the O.D. of the samples to the standard curve.

3.8.3.4 Estimation of Rat Serum Hepcidin (Hepcidin) ELISA Kit.

This examination was done by preparing process from Biocellular company (China) by using enzyme-linked immunosorbent assay method to determine the concentrations hepcidin in rat serum (Abbasi *et al.*, 2013) as shown in appendix (VIII).

Principle of the Assay

The kit assay rat hepcidin level in the sample purified rat hepcidin antibody to coat microtiter plate wells, make solid-phase antibody, then add hepcidin to the wells, combined antibody which with HRP labeled, become antibody-antigen-enzyme-antibody complex, after washing completely, add TMB substrate solution, TMB substrate becomes blue color at HRP enzyme-catalyzed, reaction is terminated by the addition of a sulphuric acid solution and

the color change is measured spectrophotometrically at a wave length of 450 nm. The concentration of hepcidin in the samples is then determined by comparing the O.D. of the samples to the standard curve.

3.9. Histological Study

The liver, kidney and bone of each animal were quickly removed and preserved in 10% formalin preparation of histological study according to (Mescher, 2010) as shown in appendix (IX).

3.10. Statistical Analysis

Statistical analysis data were analyzed using the software package SPSS version 24.00 where one way (ANOVA) was used to assess the significant changes between the groups' results. The data were expressed as mean ± standard error (SE). (Hau et al.,2002).

Chapter Four

Results

4. Results

4.1. Effect of Adenine and *Urtica dioica* Leaves on Some Blood Parameters of Male Rats

The reduction ($p \leq 0.01$) in RBCs count was recorded in adult male rats group three (3.145±0.501) was injected by adenine in comparison with other groups (Table 4-1). While, the RBCs count showed significant increased (6.312±0.822) in the value of adult male rats treated by *Urtica dioica* leaves in comparison with adenine group.

It was evident from table (4-1) that adenine group (8.35±1.48) had a lowest Hb concentration as compared with the control group. There were no significant differences observed in Hb values in group two, four and five respectively when compare with control group.

The PCV was showed account significant decrease ($p \leq 0.01$) in adenine group compared with the control group, but the treatment group showed significant increase in PCV account of group two treated by UD leaves approximately reach near the normal value of control group (Table 4-1). In addition, the PCV count showed significant increase in the value of adult male rats treated by *Urtica dioica* leaves in group four and five in comparison with adenine group.

A higher significant decrease ($p \leq 0.01$) in platelets count were noticed for adenine group (125.66±5.42) in comparison with other groups and showed significant increase in platelets count of group four that treated with adenine + *Urtica dioica* L. (Table 4-1).

The current results were showed a higher significant ($p \leq 0.05$) of the WBCs count in the group of adult male rats injected by adenine in comparison with control group. In group treated with *Urtica dioica* leaves observed

significant decrease in the value in comparison with control group (Table 4-1).

Table 4-1. Effect of Adenine Alone and in Combination with *Urtica dioica* Leaves on Some Blood Parameters in Male Rats (Mean±SE).

Groups / Parameters	GI Control	GII Negative Control + *Urtica dioica*	GIII positive Adenine	GIV Adenine + *Urtica dioica* Theraputic	GV Adenine + *Urtica dioica* Protective	Level of Significance
RBCs (cell*10^{12}/I)	7.598±0.34 A	6.312±0.822 A	3.145±0.501 C	5.385±0.602 B	4.97±0.411 B	0.01
Hb (mg/dl)	14.15±0.71 A	13.56±2.18 B	8.35±1.48 C	11.78±1.73 AB	10.88±0.59 AB	0.01
PCV %	50.33±0.83 A	48.13±2.34 B	21.27±1.80 C	40.33±0.714 B	35.418±3.77 B	0.01
PLT 10^9/I	504.5±86.97 A	496±43.799 B	125.66±5.42 C	462.5±70.34 A	384.7±47.408 B	0.01
WBCs 10^9/I	8.85±4.88 B	9.98±1.611 B	16.16±5.24 A	13.01±3.97 AB	11.02±0.817 B	0.05

-Values = Mean±SE

-Different letters represent significant (p≤0.05) differences between groups.

-Number of rats in each group = 9

4.2. Effect of Adenine and *Urtica dioica* Leaves on Some Serum Kidney Function Biomarker of Male Rats

4.2.1. Urea Concentration in Serum

The results in table (4-2) demonstrated highest ($p \leq 0.01$) urea concentration in serum were noticed for adenine and adenine treated group (96.4±1.69) in comparison with other groups. Furthermore, *Urtica dioica* L. group and combined adenine in group four and five at the same table showed a significant reduction comparing to adenine group but no significant as compared to control group (Table 4-2).

4.2.2. Creatinine Concentration in Serum

The serum creatinine in adenine treated group (2.28±0.034) recorded higher significant ($p \leq 0.05$) as compared to other groups (Table 4-2). Moreover, combined adenine plus *Urtica dioica* leaves (group four and five) showed significant decreased in the creatinine concentration in serum comparing to adenine group (Table 4-2).

Table 4-2. Effect of Adenine Alone and in Combination with *Urtica dioica* Leaves on Some Kidney Serum Function in Male Rats (Mean±SE).

Groups / parameters	GI Control	GII Negative Control + *Urtica dioica*	GIII positive Adenine	GIV Adenine + *Urtica dioica* Theraputic	GV Adenine + *Urtica dioica* Protective	Level of Significance
Urea mg/dl	36.6±7.99 B	38.25±4.57 B	96.4±1.69 A	69.8±2.69 AB	64.3±3.45 AB	0.01
Creatinine mg/dl	0.37±0.014 C	0.35±0.025 C	2.28±0.034 A	0.65±0.081 B	0.68±0.051 B	0.05

-Values = Mean±SE

-Different letters represent significant ($p \leq 0.05$) differences between groups.

-Number of rats in each group = 9

4.3. Effect of Adenine Alone and in Combination with *Urtica dioica* Leaves on Iron Homeostasis Parameters in Male Rats with Induced CRD

4.3.1. Free Erythrocyte Protoprophyrine (FEP)

The present study table (4-3) was exhibited a higher significant differences in free erythrocyte protoprophyrine in adenine treaded group (6.74±0.214) as compare with other groups. Moreover, the free erythrocyte protoprophyrine lacked to significance among two, four and five groups as compared to the control group (Table 4-3).

4.3.2. Serum Iron

A significant ($p \leq 0.02$) reduction in table (4-3) of serum iron was demonstrated in adenine treaded group (55.98±15.54) comparing to the other groups. Also the same table revealed a significant elevation in the groups treated adenine plus *Urtica dioica* leaves (group four and five) comparing to adenine group but not significant as compared to the control group.

4.3.3. Ferritin

The adenine treated group (8.306±1.803) recorded highest significant ($p \leq 0.035$) of serum ferritin, and the group five treated by adenine plus *Urtica dioic*a leaves (6.633±1.44) was the lowest (Table 4-3). On the other hand, there were no significant differences in group two (7.018±0.901) and four (7.761±1.22) in comparing to the control group (7.88±0.63).

4.3.4. Hepcidin

Higher significant differences in adenine treated group as compared with other groups (Table 4-3). Adenine + *Urtica dioica* leaves (group four) lead to the reduction of the value of hepcidin but that not significant differences was showed to value of control group.

Table 4-3. Effect of Adenine Alone and in Combination with *Urtica dioica* Leaves on Some Kidney Serum Function in Male Rats (Mean±SE).

Groups / Parameters	GI Control	GII Negative Control + *Urtica dioica*	GIII positive Adenine	GIV Adenine + *Urtica dioica* Theraputic	GV Adenine + *Urtica dioica* Protective	Level of Significance
FEP	**2.109±0.103 B**	**1.701±0.813 B**	**6.74±0.214 A**	**1.57±0.921 B**	**1.77±0.24 B**	**p≤0.86**
Fe mg/dI	**177.5±23.51 A**	**181.17±80.13 A**	**55.98±15.54 C**	**88.38±18.68 B**	**79.16±12.73 B**	**p≤0.02**
Ferritin	**7.88±0.63 B**	**7.018±0.901 B**	**8.306±1.803 A**	**7.761±1.22 B**	**6.633±1.44 B**	**p≤0.035**
Hepcidin	**19.37±3.39 B**	**20.66±1.93 B**	**25.03±5.11 A**	**19.35±5.31 B**	**20.66±2.31 B**	**p≤0.22**

-Values = Mean ± SE

-Different letters represent significant (p≤0.05) differences between groups.

-Number of rats in each group = 9

4.4. Histological Study:

4.4.1. Kidney

The histological section of the kidney is shown in figure (4-1) from the control group, which has a regular architectural appearance (renal tubules and glomeruli). The figure (4-2) group of rats treated with adenine stain by (H&E) showed sever necrosis and epithelial sloughing of the renal tubules with infiltration of inflammatory cells and exudate in glomeruli and significant dilation of renal tubules with epithelial necrosis. The histological changes in kidney of male rat in figure (4-3) treated with adenine and *Urtica dioica* leaves powder showed the glomerular, renal tubules and histological feature improve to normal (40X H and E).

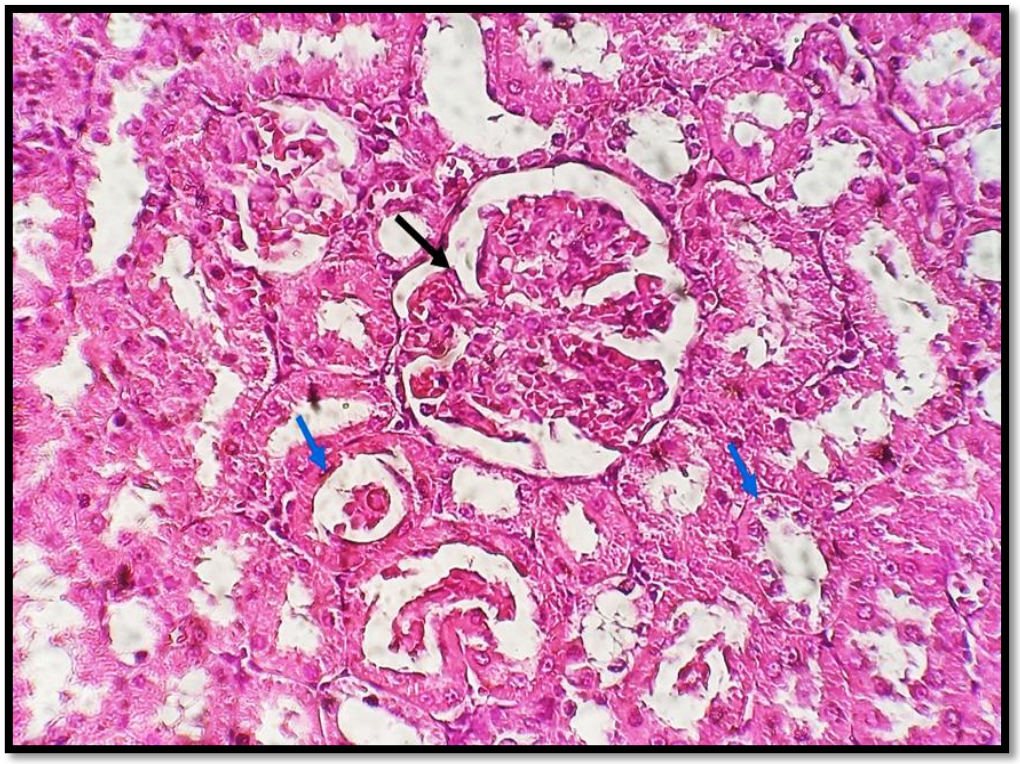


Figure (4-1): Light micrograph of histological section in kidney of male rat (control group) treated with DMSO only showed the normal renal glomerulus (black arrow) and showed the normal renal tubules (blue arrow). (Stain H&E). (40X).

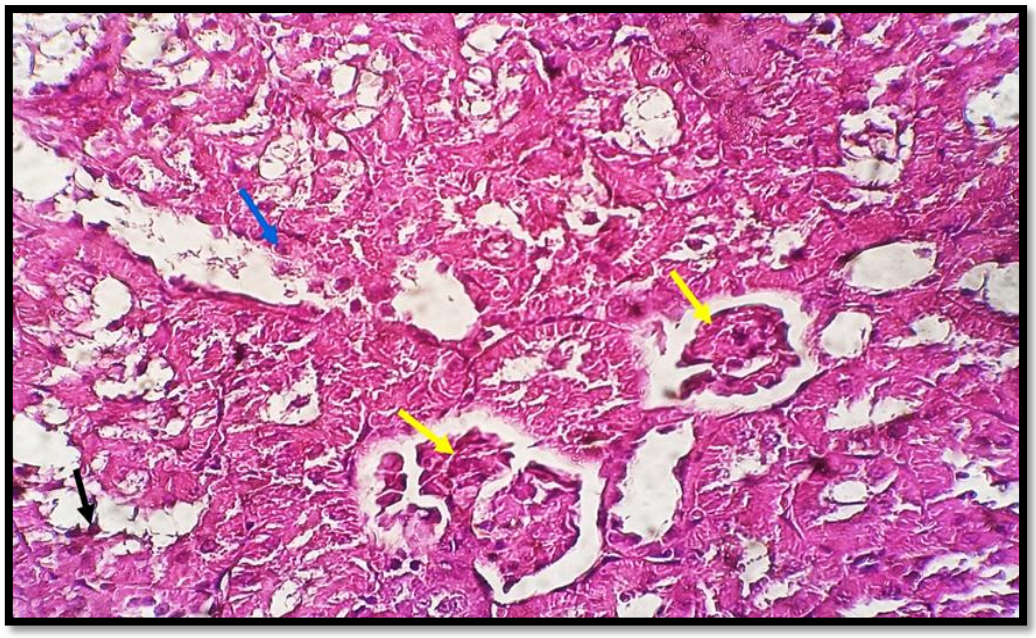


Figure (4-2): Light micrograph of histological changes in kidney of male rat, treated with adenine showed sever necrosis and epithelial sloughing of the renal tubules (white arrow) with infiltration of inflammatory cells and exudate in glomeruli (yellow arrow), significant dilation of renal tubules with epithelial necrosis (black arrow) (40X H and E).

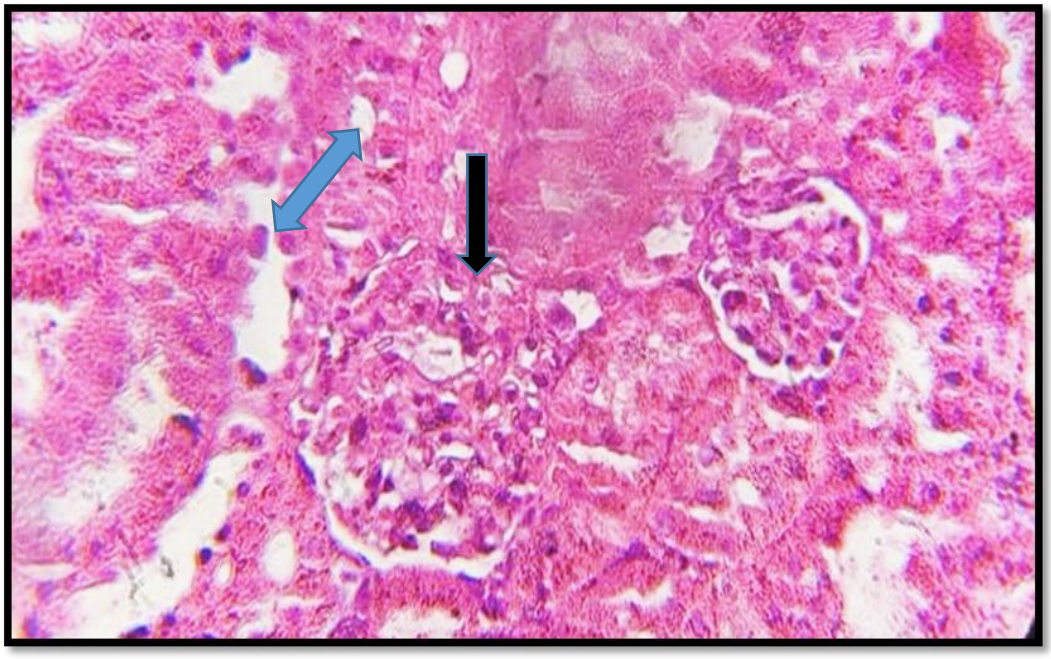


Figure (4-3): Light micrograph of histological changes in kidney of male rat, treated with adenine and *Urtica dioica* leaves powder showed the glomerular improve to normal (black arrow), normal renal tubules (blue arrow) and normal histological feature (40X H and E).

4.4.2. Liver

Liver tissue from the control groups (figure 4-4) stained with (H&E) showed normal hepatic architecture and normal hepatocytes cords with slight congestion of the hepatic central vein (40X H and E). The histological section in liver treated with adenine showed congestion of hepatic vein with infiltration of inflammatory cells, hyperplasia of the endothelial tissue of bile ductile and blood vessels, significant fibrosis with sever infiltration of fibroblasts and fibrous connective tissue and Kuepfer cells, hepatocytes necrosis (100X H and E) stain, figure (4-5). Histological section in liver treated with adenine + *Urtica dioica* leaves powder, liver showed reduced in inflammation hepatic architecture and reduced in inflammation hepatocytes cords with slight congestion of the hepatic central vein (40X H and E) stain, figure (4-6).

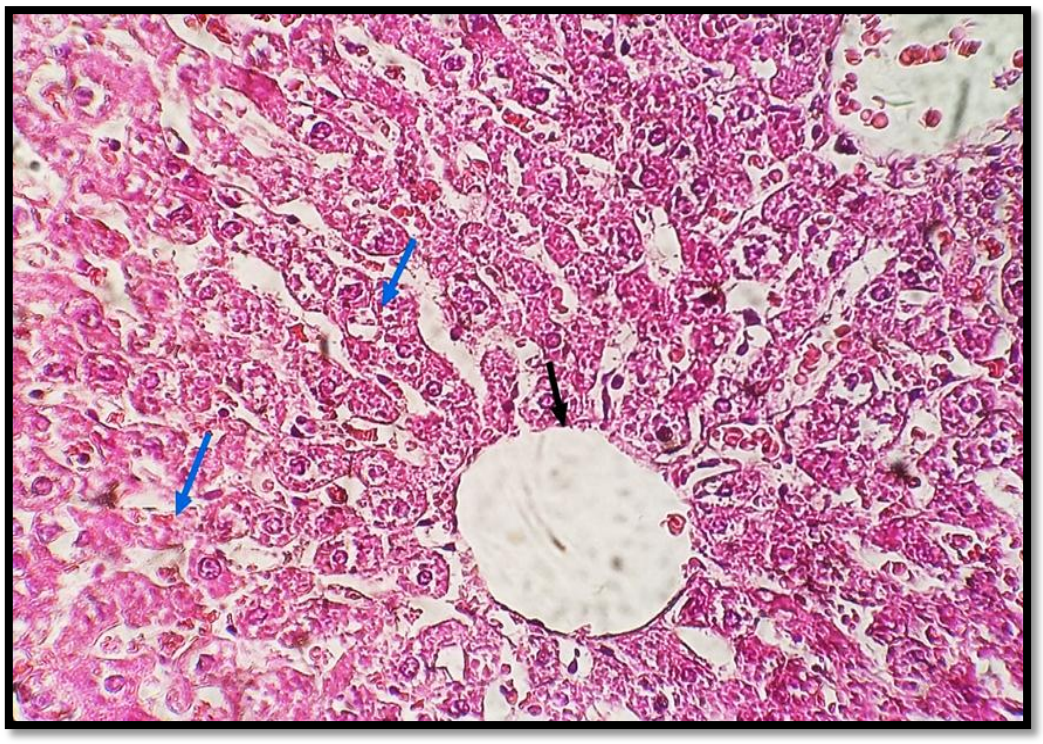


Figure (4-4): Photomicrograph of liver showed normal hepatic architecture and normal hepatocytes cords (white arrow) with slight congestion of the hepatic central vein (blue arrow). (40X H and E).

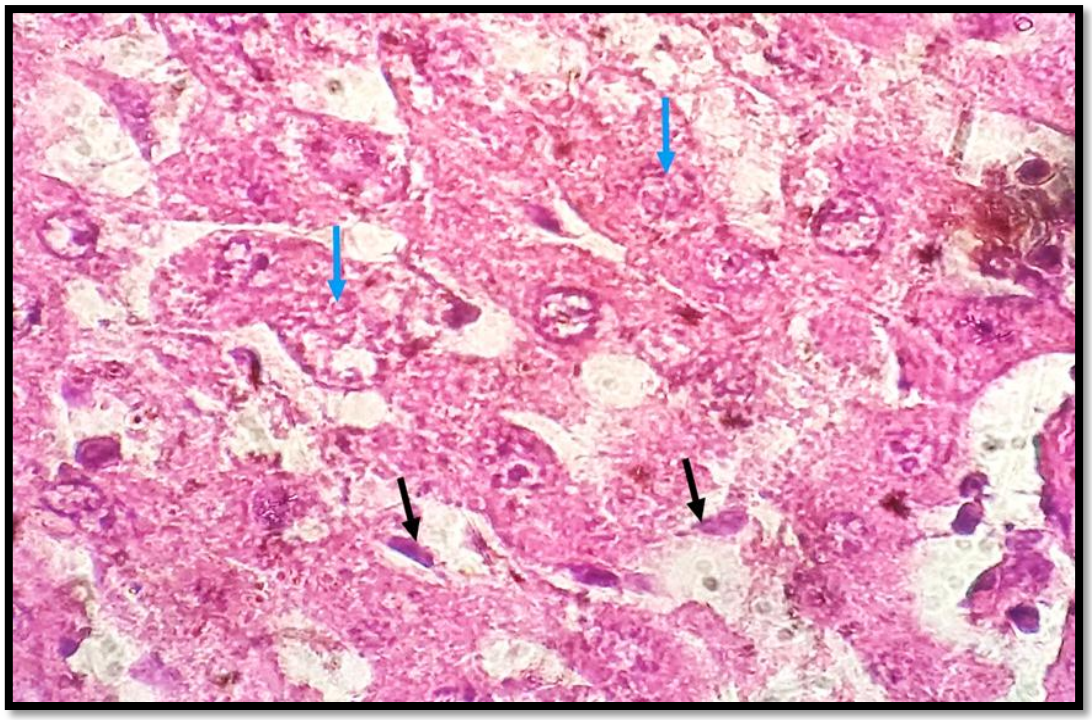


Figure (4-5): Microscopic section showed Kuepfer cells (black arrow) and hepatocytes necrosis (blue arrow) (100 X H and E).

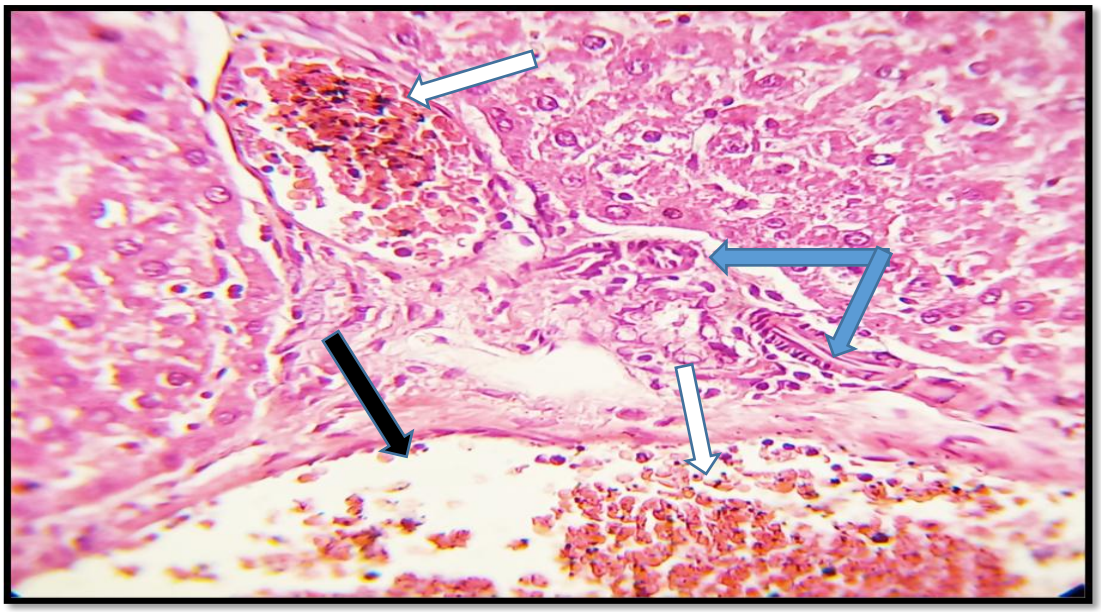


Figure (4-6): Microscopic section showed congestion of hepatic vein with infiltration of inflammatory cells (white arrow), hyperplasia of the endothelial tissue of bile ductile and blood vessels (blue arrow), significant fibrosis with sever infiltration of fibroblasts and fibrous connective tissue (black arrow) (40X H and E).

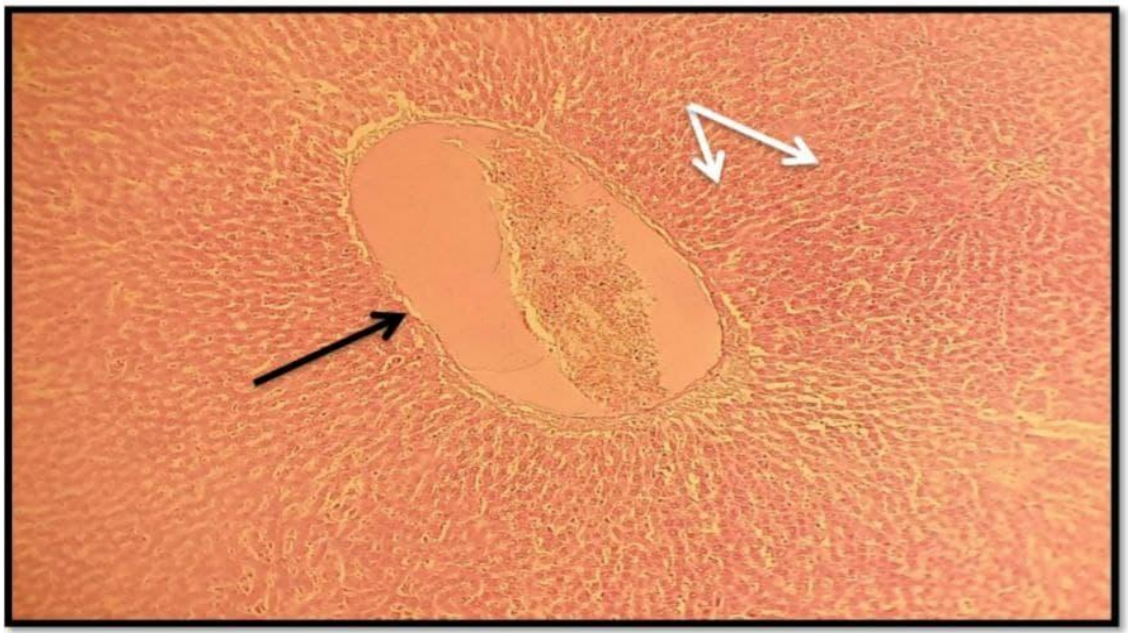


Figure (4-7): Microscopic section in liver treated with adenine + *Urtica dioica* leaves powder, liver showed reduced in inflammation hepatic architecture and reduced in inflammation hepatocytes cords (black arrow) with slight congestion of the hepatic central vein (White arrow) (10X H and E).

4.4.3. Bone marrow

Bone tissue from the control groups (figure 4-7) stained with (H&E), section in bone marrow showed bone with normal architecture and highly marrow cellularity, there was mild vascular congestion and highly erythroid cells maturation. Figure (4-8) group treated with adenine only showed trabecular exudation with inflammatory cell infiltration mainly mononuclear cells between bone marrow cells with bone marrow cells necrosis and congestion (40X H and E). Figure (4-9) histological section in bone marrow treated with adenine + *Urtica dioica* leaves powder, showed bone ameliorate architecture marrow cellularity, there is well distribution of megakaryocytes with well erythroid cells maturation (40X H and E).

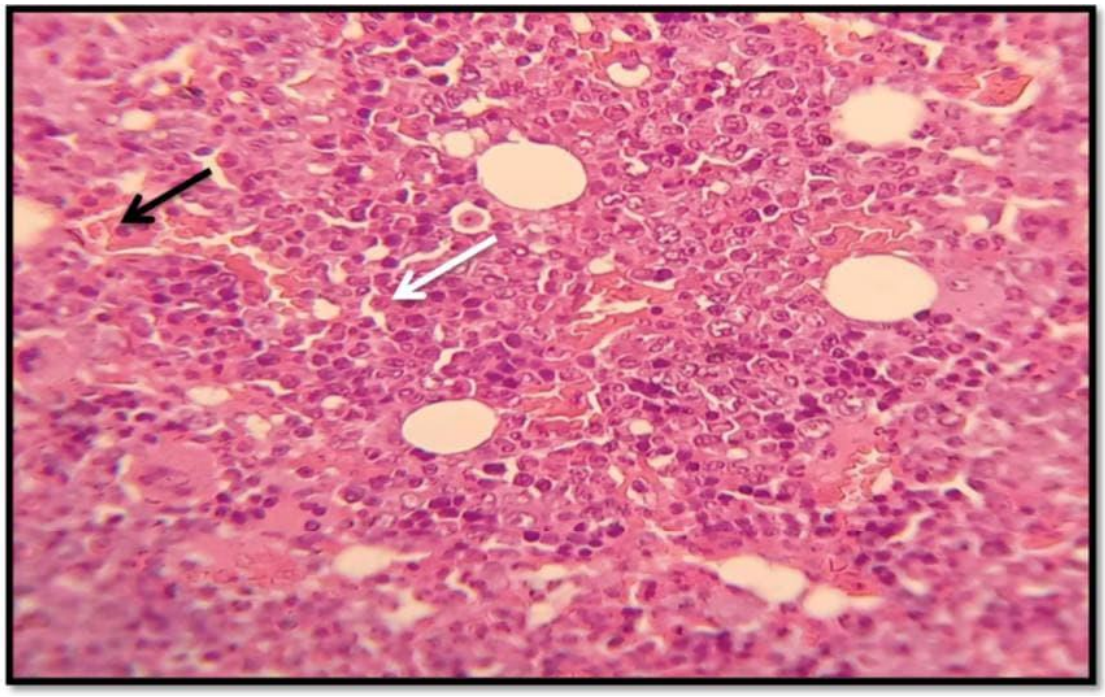


Figure (4-8): Light micrograph of histological section in bone marrow showed bone with normal architecture and highly marrow cellularity, there is mild vascular congestion (black arrow) and highly erythroid cells maturation (white arrow) (40X H and E).

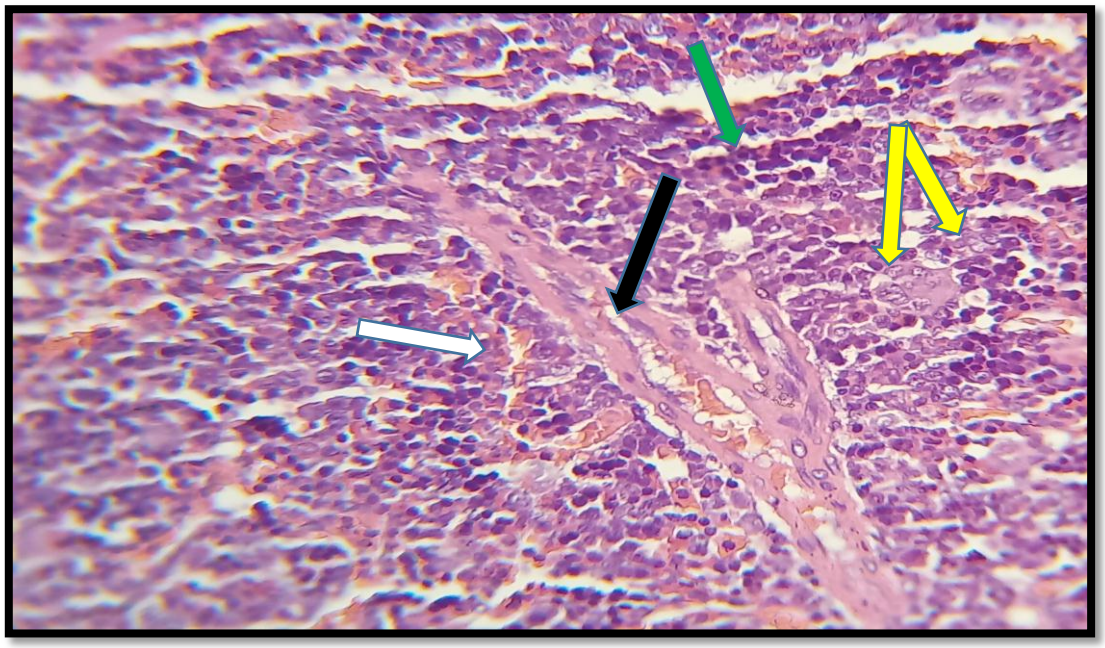


Figure (4-9): Photomicrograph of bone marrow treated with adenine only showed trabecular exudation (black arrow) with inflammatory cell infiltration mainly mononuclear cells between bone marrow cells (green arrow) with bone marrow cells necrosis (yellow arrow) and congestion (white arrow) (40X H and E).

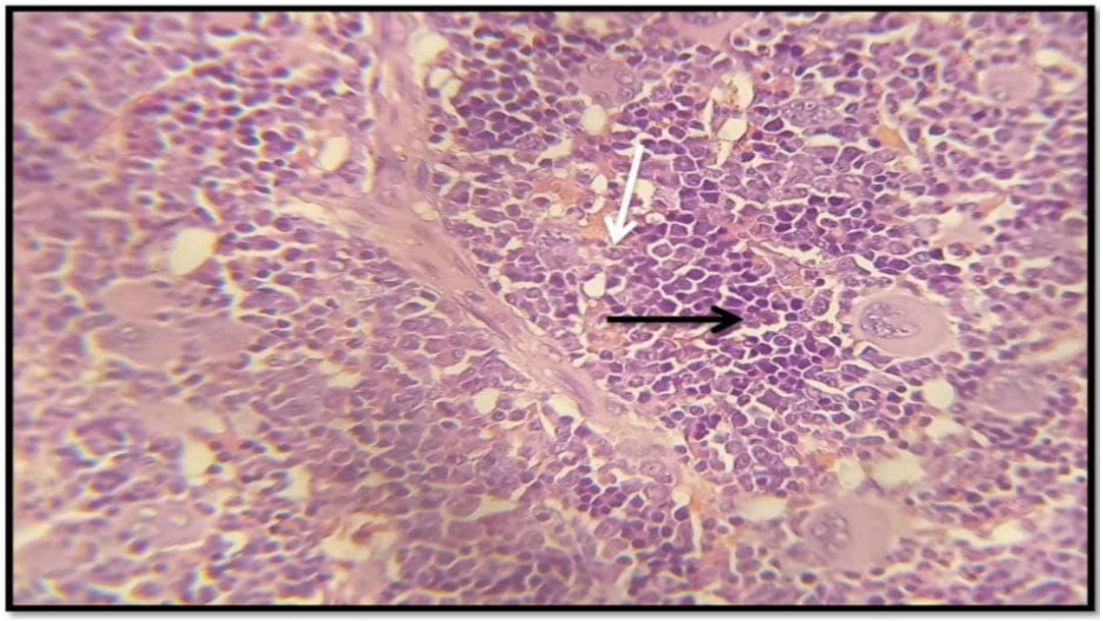


Figure (4-10): Light micrograph of histological section in bone marrow treated with adenine + *Urtica dioica* leaves powder, showed bone ameliorate architecture marrow cellularity, there is well distribution of megakaryocytes (black arrow) with well erythroid cells maturation (white arrow) (40X H and E).

Chapter Five

Discussion

5. Discussion

In the study Anemia worsens the quality of life in people with chronic kidney disease (CKD) and raises the risk of hospitalization and mortality (Babitt and Lin, 2012). The development of therapeutic tools requires a better understanding of the mechanism of anemia in CKD. Therefore, animal models are of considerable importance to investigate and find targets and new pharmacological approaches. As a result, the current study looked into the potential therapeutic benefits of *Urtica dioica* leaves on CKD damage in rats. Anemia control targets were determined using the Kidney Disease Improving Global Outcomes (KDIGO) recommendations (Ye *et al.,* 2018). Chronic kidney disease negatively affects the health status of the patient (Jhee *et al.,* 2020). According to the Global Burden of Disease Study, CKD was the 12th leading cause of mortality worldwide in 2015 (Fraser and Blakeman, 2016; Neuen *et al.,* 2017).

5.1. Effect of Adenine and *Urtica dioica* L. on Some Blood Parameters of Male Rats

The reduction ($p \leq 0.01$) on some blood parameters were recorded in adult male rats group three was injected by adenine in comparison with other groups (Table 4-1), in red blood cells count (RBCs), haemoglobin levels (Hb) and the percentage of packed cells volume (PCV).

The red blood cell indices, reticulocyte count (new red blood cell formation), and iron parameters are contributory to detect the cause of several anemias which are not due to EPO deficiency (Hillman and Finch, 1985). Anemia occurs in individuals with acute and chronic renal failure. It is traditionally defined as a drop in haemoglobin concentration in the blood this result agrees with (Daniel *et al.,* 2006; Greer *et al.,* 2009; Pasricha *et al.,* 2018). Several confirmations in patients with CKD, was noted with a low concentration of hemoglobin (Dorgalaleh *et al.,* 2013; da Silva *et al.,* 2021).

Adenine produces lipid peroxidation in experimental animals after administration. These reactive free radicals initiate cell damage through the mechanisms of covalent binding and lipid peroxidation (Fridovich, 1995). Increased ROS production occurs in inflammation, during radiation, or during metabolism of hormones, drugs, and environmental toxins (Vasilaki and McMillan, 2011). This might be one of the reasons for decreased RBC count, Hb and PCV level. Another reason might be the erythropoietin level, the formation of RBCs (erythropoiesis) is controlled by a circulating glycoprotein hormone called erythropoietin, which is secreted primarily by the kidney and liver (Silverberg *et al.*, 2001). It has been suggested that the ability to secrete erythropoietin in response to anemia is defective in many patients (Boshuizen *et al.*, 2018).

Decrease in platelet count agreement with study produced by Malyszko *et al.* (1996), decrease in granulocyte appropriate with (Fünfstück *et al.*, 2006), and decrease in lymphocyte agree with (Aguiar *et al.*, 2015; Habib *et al.*, 2017).

But elevated WBCs count agreement with study according to basic data by (Bash *et al.* 2009; Habib *et al.* 2017). All these result compared with control group. An abnormal white blood cell and/or platelet count might indicate a more serious problem with bone marrow function, such as malignancy or vasculitis. White blood cells provide the body with powerful defenses against tumors and viral, bacterial, and parasitic infections (Ganong, 1991). In this study, we found that repeated administration of adenine decreased the WBCs count in rats.

In this study, we demonstrated that UD treatments increased the lowered RBC, Hb value, and PCV. This result indicated that UD treatments might ameliorate the anemia and some mineral disturbances and increase the body's defense mechanism in adenine -treated rats.

Urtica dioica leaves include a significant number of biologically-active compounds. The leaves are rich sources of phenols, terpenoids, carotenoids and

fatty acids, in addition to various essential amino acids, chlorophyll, vitamins, tannins, carbohydrates, sterols, polysaccharides, isolectins and minerals (Tack and Verloo, 1996; Kara, 2009), the most important of which is iron. On the other hand, nettle leaf powders contain on average 30% proteins, 4% fats, 40% non-nitrogen compounds, 10% fiber and 15% ash (Kregiel *et al.*, 2018), The fresh leaves contain high concentrations of vitamins A, C, D, E, F, K and P, as well as of vitamin B-complexes (Rutto *et al.*, 2013), that may aid in the improvement of anemia in particular, as well as a general element that may be related to similar situations. In rats treated with CCl4, Meral and Kanter, (2003) found that *Urtica dioica* treatments might decrease the effects of Chemokine (C-C motif) ligand 4 (CCl4)-induced anemia, minerals, and the body's defensive mechanism. The results of (Ozkol *et al.* 2012) led us to conclude that nettle probably neutralizes Cisplatin induced damage hepatotoxicity, nephrotoxicity, and hepatic oxidative stress through its effects and antioxidant-free radical-defusing. These characteristics make nettles ideal for a variety of uses, including functional foods, dietary supplements, and pharmaceutical formulations. The liver has a central role in nutritional homeostasis and any liver disease leads to abnormalities in nutrient metabolism and subsequent malnutrition (Bavdekar *et al.*, 2002).

This traditional medicine works on endothelium, blood cells, angiogenesis, cellular proliferation, vascular dynamics and cell mediators to stop bleeding (Fisgin *et al.*, 2009).

5.2 Kidney Function Biomarkers

5.2.1. Urea

The concentration of urea in the adenine group is substantially higher than in the control group, as seen in the table (4-2). These findings agree with (Yonis and Hassan, 2020; Noushida *et al.*, 2020). The results by (Ormrod and Miller,

1980) who showed that uremia had a marked effect on erythroid formed elements.

The kidney normally excretes nitrogenous substances from the blood in the form of urine. Renal disease causes urea to accumulate in the bloodstream, which is observed in high amounts in chronic renal failure.

Adenine, on the other hand, generated a substantial increase in plasma urea and creatinine up to many days following injection, indicating reversible acute tubular necrosis. On day 2, the decreased bicarbonate level indicates a temporary acidosis due to compromised renal function, that uremia may result in immunosuppression. (Chalmers *et al.,* 1985).

Purine nucleotide phosphorylase converts adenosine into adenine, which is one of the products of nucleic acid breakdown through the uric acid pathway (Bender *et al.,* 2002). There is increased flux through this pathway, and an increase in concentration of these metabolites during tissue injury (Martinon, 2010). The association between tissue injury and increase in uric acid pathway metabolite concentrations makes them excellent candidates for co-coordinating adaptive responses to tissue injury.

On the basis of current data that *Urtica dioica* administration causes a considerable reduction in ($p \leq 0.05$) in concentration of urea in treatment group UD powder by diet when compared with adenine group as shown in table (4-2).

One gram of nettle powder has a total phenolic content of 129 mg GAE (Gaelic Acid Equivalent), which is two times greater than the phenolic content of 100 mL of cranberry juice (66.61 mg GAE) (Keskin-Šašić *et al.,* 2012).

Individual polyphenols in stinging nettles have been shown to be higher than in other wild plants (Augspole *et al.,* 2017). The presence of phenolic chemicals in this plant gives it antioxidant qualities.

5.2.2. Creatinine

As seen in the table (4-2), the creatinine concentration in the adenine group is significantly greater than in the control group. In clinical practice, creatinine is widely applied as an indication of kidney dysfunction (Bohra *et al.*, 2013). Creatinine is a waste product excreted mostly by glomerular filtration through the kidney. When the value of this product rises, it means that excretion has reduced or that renal function has degraded (Feehally *et al.*, 2007). The glomerular filtration rate may be estimated using creatinine clearance. These findings corroborate previous research (Martínez-Salgado *et al.*, 2007; Abd-Elhamid *et al.*, 2018; Al_Shammari, 2019; Babaeenezhad *et al.*, 2021). Adenine is well known to promote ROS overproduction, which has a main role in the pathogenesis of adenine-induced CRD. The imbalance between the formation of ROS and antioxidant protection causes renal cellular injury (Quiros *et al.*, 2010). Consistent with different studies (Ali *et al.*, 2013; La *et al.*, 2018) we observed that adenine caused a significant elevation in creatinine levels in the serum and kidney. Bendich *et al.* (1950) confirmed the suggestion that 2,8- dioxyadenine (DHA) crystals were deposited in the kidneys following adenine administration; this focused subsequent interest of toxicologists on the kidney and its function. The oxidation of adenine in animal tissues, which was previously considered to be caused by the activity of a mammalian adenase, has been demonstrated to be caused by the action of xanthine oxidase and the product to be percent dihydroxyadenine (Wyngaardenl and John, 1957). Philips *et al.*, (1952) observed stippling, pitting, and softening of the kidney cortex in rats after very high adenine dosage. Stevens *et al.*, (1979) found that these patients had reduced renal function due to 2,8 dihydroxyadenine renal calculi.

Adenine and DHA precipitate in renal tubules as they have low solubility (Yokozawa *et al.*, 1986 and Shuvy *et al.*, 2011), lead to disturbance in filtration and finally lead to increase serum creatinine concentration. If the levels of urea

and creatinine rise, it might suggest a disruption in epithelial cell transportation in collecting tubules, as well as a widespread impairment in the function of proximal convoluted tubules (Gowda and Ledoux, 2008).

Increased ROS generation has been correlated to cellular damage and necrosis in proximal tubule cells. As a result, free radicals impact glomerular mesangial cells, causing them to contract, depressing the filtration coefficient and, as a result, decreasing the glomerular filtration rate (Polat *et al.*, 2006).

There is significant decrease ($p \leq 0.05$) in serum creatinine concentration in group treated with *Urtica dioica* powder compare with adenine as shown in table (4-2), these effects of phenols are related with the maintenance of the oxidative stress, which raised the antioxidant capacity of the body and conservation of the permeability of the cell membrane (Yilmaz *et al.*,2018). From the results obtained, UD treatment showed an ameliorative impact on adenine nephrotoxicity in rats.

In a previous study, it was shown that the antioxidant compounds such as resveratrol could increase GFR by inhibiting mesangial cells' contraction. The removal of free radicals improved creatinine clearance. (Morales *et al.,* 2006). *Urtica dioica* powder contains antioxidant and free radical scavenging characteristics due to the presence of phenolic components such as caffeic and malic acid (Halder and Sharma, 2017). In this study, administration of UD leaves inhibited oxidative stress induced by adenine as shown by a decline in plasma creatinine concentration in treated rats.

Nettles are one of the most commonly-used medicinal plants in the world, due to their health-enhancing qualities. Because of their high content of nutritive substances (Joshi *et al.,* 2014). The presence of phenolic chemicals in this plant gives it antioxidant capabilities. The vasorelaxation impact of nitric oxide and the inhibition of calcium channel activity are linked to the vasodilatory characteristics of UD (Upaganlawar *et al.*, 2006; Halder and Sharma, 2017).

The production of reactive oxygen species contributes to cellular damage (Atanassova *et al.*, 2011), and data suggests that oxidative stress is a more severe factor in the pathophysiology and toxic consequences of antioxidant medications. Many complications of renal disorders are mediated by oxidative stress, oxidative stress-associated mediators, and inflammation, and oxidative stress play a key role in their pathogenesis (Chade *et al.*, 2002). The kidney is a rather susceptible organ to ROS-induced degeneration, possibly due to the presence of long-chain polyunsaturated fatty acids in renal lipids (Ozbek, 2012).

5.3. Effect of Adenine Alone and in Combination with *Urtica dioica* Leaves on Iron Homeostasis Parameters in Male Rats with Induced CRF

5.3.1. Free Erythrocyte Protoprophyrine (FEP)

Free erythrocyte protoporphyrin (FEP), one of the outcomes of iron deficiency is an increase in erythrocyte protoporphyrin (EP) levels. The ideas basis for the measurement quantification measurement of protoporphyrin is a reduction of iron in the bone marrow for integration into newly synthesized globin and the protein porphyrin as the haemoglobin molecule is arriving its definitive steps in synthesis. (Hastka *et al.* 1993) determined that EP can be used beneficially to assess intensity of the degree of reduced iron transport to a marrow in a limited sample of individuals with chronic inflammatory disorders. Increased EP concentrations, as well as the ratio of EP to haemoglobin (Hb), are good indicators of a lack of iron to meet the normal demands of the bone marrow (Labbe *et al.*, 1999). The prevalence of iron deficiency was much greater when the erythrocyte protoprophyrine (EP) concentration was used to assess the prevalence than when the ferritin concentration was utilized as the only indication of iron deficiency, according to the research. Reticulocyte production in response to iron treatment was associated to iron stores in the bone marrow, although ferritin concentration, EP and the serum transferrin receptor (sTfR) to ferritin ratio all showed a substantial predictive value in distinguishing

iron deficiency anemia (IDA) from non-deficiency anemia (non-IDA). Hastka *et al.* (1993) found that EP can be used to assess the degree of reduced iron transport to the marrow in a limited sample of patients with chronic inflammatory diseases. The bone marrow response is blocked or diminished by inflammatory or renal disease (Braun, 1999). The nature, intensity, and duration of this inflammatory response appear to be contributing factors in the lack of consensus on the use of detecting EP concentration in clinically complicated settings. Because it cannot discriminate between the many causes of a shortage of iron in the bone marrow, the EP concentration does not offer a precise indication of iron insufficiency. In situations when iron shortage occurs along with inflammatory diseases and infection, a field experiment was recently undertaken to see if EP, sTfR, or ferritin concentrations were better predictors of iron status Asobayire *et al.*, (2001).

Since EP is not sensitive to acute inflammation and it is not time consuming or expensive to measure the concentration, there is some strong appeal for its use in screening patients.

Mei *et al.* (2003) recently conducted a study that looked at the sensitivity and specificity of Hb and EP concentrations in children and adult women for diagnosing iron deficiency.

Meanwhile, in the *Urtica dioica*-treated adenine-induced CRF group four, FEP levels are higher see in table (4-2). Numerous studies have proved *Urtica dioica's* beneficial properties over the world. Understanding the mechanisms that underpin positive effects can lead to a new therapeutic approaches.

(Serdar *et al.* 2000) revealed the reverse connection between Hb levels and free erythrocyte protoprophyrine concentrations detected in the research population. This relationship is theoretically expected, because an increase in unconjugated protoporphyrin in erythrocytes would be the result of an

insufficient supply of iron at the cellular level, therefore, be insufficient for the formation of Hb.

Sergio *et al.* (1973) showed the clinical significance of the positive free erythrocyte protoprophyrine (FEP) tests in patients, it appears probable that these might be due to iron deficiency anemia.

5.3.2. Serum Iron

A significant ($p \leq 0.02$) reduction in table (4-3) of serum iron was demonstrated in adenine treaded group comparing to the other groups. Iron is critical for Hb synthesis. Consequently, patients should be carefully evaluated for the availability of iron, by measuring the serum iron. The serum iron reflects the amount of iron immediately available for hemoglobin synthesis.

Iron stores in the body range from 800 to 1,200 mg Council on Food and Nutrition, (1968). If the original Hct is reduced by 25% and the target Hct is raised to 35%, the amount of additional iron necessary is required. Both iron and erythropoietin are required for effective erythropoiesis. Anemia occurs when CKD patients do not have enough of one or both of these substances.

So several CKD patients have functional iron deficiency, which is defined as impaired iron release from body stores that is insufficient to meet the demand for erythropoiesis (also called reticuloendothelial cell iron blockade). These patients have low serum transferrin saturation (a measure of circulating iron) and normal or high serum ferritin (a marker of body iron stores).

Iron treatment beneficial may be lower disease activity in end-stage renal disease by decreasing the production of TNF- *a*. (Kaltwasser *et al.*, 2001; Weiss *et al.*, 2003).

Based on existing evidence, supplementary iron treatment should be given to patient with chronic disease anemia and absolute iron deficiency (Rizzo *et al.*, 2002). Phytochemical researches showed the existence of several beneficial

chemical compounds in the *Urtica dioica* such as minerals [(especially iron), manganese, potassium, and calcium], and vitamins, including pro-vitamin A and vitamin C (Kregiel *et al.,* 2018; Shonte *et al.,* 2020).

5.3.3. Ferritin

The adenine treated group recorded highest significant ($p \leq 0.035$) of serum ferritin. The serum ferritin reflects total body iron stores in liver, spleen, and bone marrow reticuloendothelial cells, in addition to reflecting body iron stores, is also an acute phase reactant. As a result, it can increase in the presence of acute or chronic inflammation. It considers iron that is readily available for erythropoiesis.

Ferritin levels are elevated (Table 4-3), indicating enhanced iron storage and retention in the reticuloendothelial system, as well as elevated ferritin levels associated to immunological activity.

The high fluctuation of SF and Hb concentrations that arise from pathological changes, such as infectious processes, has been documented in reports (Cook, 1999). Anemia would not appear until the body's iron reserves were depleted to dangerously low levels.

5.3.4. Hepcidin

Higher significant differences in adenine treated group as compared with other groups (Table 4-3). Anemia has been seen in patients with chronic infectious, inflammatory, and neoplastic diseases (van den Berge *et al.,* 2008). Low hemoglobin levels are a good indication of anemia; in all of our CKD models, we look for low hemoglobin levels decreased hemoglobin levels were discovered, along with low serum ferritin levels, which may suggest a deficiency of iron Estrela *et al.,* (2021). Indeed, hepcidin, a hormone generated in the liver that is crucial for regulating iron homeostasis (Nakanishi *et al.,* 2019; Babitt and Lin, 2010), has been linked to iron deficiency. Excess hepcidin has

been shown to impede dietary iron absorption in many CKD patients, implying that anemia induction in our CKD models might be due to this mechanism. The relative lack of erythropoietin (EPO) production is the cause of anemia, erythroid cell proliferation and differentiation are impaired by the blunted EPO effect and by iron limitation via hepcidin and cytokines (Weiss *et al.*, 2019).

Anemia of chronic disease (ACD) is one of the most prevalent medical disorders. Increased production of cytokines that mediate the immunological or inflammatory response, like tumor necrosis factor, interleukin 1 and interferons, is a characteristic of ACD diseases. These cytokines are responsible for all of the processes associated with the development of ACD, which include reduced red cell survival, a blunted erythropoietin response to anemia, impaired erythroid colony formation in response to erythropoietin, and abnormal mobilization of reticulo- endothelial iron stores (Means, 1995).

Increased hepcidin levels are linked to low ferroportin expression on duodenal enterocytes and macrophages, as well as impaired dietary iron absorption and iron retention in macrophages (Theurl *et al.*, 2006, 2009) resulting in decreased iron delivery for erythropoiesis in animal models of inflammatory anemia (AI) and patients with inflammatory diseases (Weiss *et al.*, 2019).

The endocrine regulating role of hepcidin in iron balance was addressed by Camaschella *et al.* (2020) in a recent review, during acute and chronic inflammation, proinflammatory cytokines like interleukin-6 raise hepcidin levels, according to their study. This results in iron-restricted erythropoiesis caused by inflammatory anemia. In addition to anemia, particularly in people with iron-loading anemias or chronic anemia: hepcidin levels are low, the inhibition of, or control is lost.

5.4. Histological changes

5.4.1. Kidney

In the current study, histological examination revealed that adenine induced epithelial sloughing of the renal tubules, with infiltration of inflammatory cells and exudate in glomeruli, significant dilation of renal tubules with epithelial necrosis showed in figure (4-2).

Adenine also increased creatinine, urea, numerous inflammatory cytokines in plasma, and oxidative stress indicators, as well as causing histological damage in the kidneys.

In this investigation, we discovered that the kidney of the adenine + UD group rats (figure 4-3) the kidneys showed the glomerular improve to normal renal tubules and normal histological feature.

In line with earlier plant research (Kahraman *et al.,* 2003; Singh *et al.,* 2004; Singh and Chopra, 2004), this finding demonstrate that UD treatment avoided damage and revealed normal glomeruli as well as little mononuclear cell infiltration. In the renal cortical tissues, there was also renal corpuscle atrophy, glomerular shrinkage, significantly localized mononuclear cell infiltrations, and interstitium widening.

Several tissue repair processes are capable of restoring function after an acute injury kidney function. This wound healing process consists of consecutive events, such as acute inflammation and resolution, extracellular matrix synthesis (fibrinogenesis), epithelial cell de-differentiation, proliferation, and reform of the tubular structure (Hewitson *et al.,* 2017; Sato *et al.,* 2020). If the healing is effective, acute kidney damage can occur when processes are interrupted or incorrect, or when the injury-causing shock progresses to a chronic disease characterized by persistent organ remodeling, which leads to fibrosis and

dysfunction of the organ (Hewitson,2009; Venkatachalam *et al.*, 2015; He *et al.*, 2017).

According to histopathological results in renal tissue figure (4-5 and 6), concurrent therapy with UD leaves in their diet and adenine might be regarded a possible protective strategy to avoid tubular, vascular, and glomerular damage. The reactivity of proliferating cells in the renal cortical tissues was significantly improved after treatment with UD.

Sayhan *et al.* (2012) demonstrated that UD inhibits apoptotic cell death in proximal and distal tubular cells and provided strong evidence that *Urtica dioica* has a protective effect against proximal tubule damage after ischemia/reperfusion injury in the rat kidney.

A previous histological research from the kidney section revealed that nettle has a preventive effect in the prevention of renal pathological alterations (Moghaddam *et al.*, 2010; Manikanadan *et al.*, 2011). *Urtica dioica's* defensive properties may be attributed to phenols. Due to their capacity to scavenge free radicals and active oxygen species such as single oxygen, free radicals, and hydroxyl radicals, phenolic compounds have antioxidant characteristics (Lakshmi *et al.*, 2009; Nale *et al.*, 2012).

5.4.2. Liver

Hepatic architecture and hepatocyte cords are normal in the control groups' liver tissue (figure 4-4) stained with (H&E), with minor congestion of the hepatic central vein.

However, there were pathological changes in liver rats that treated with adenine in figure (4-5). The congestion of the hepatic vein with infiltration of inflammatory cells, hyperplasia of the endothelial tissue of bile ductile and blood vessels, significant fibrosis with severe infiltration of fibroblasts and fibrous connective tissue and Kuepfer cells, and hepatocytes necrosis. This

most likely implies some liver tissue damage in adenine-treated rats, which is inconsistent with the severity of the results obtained by (Feere *et al.* 2015). This alteration in the liver might be caused by prolonged renal failure or the toxic impact of adenine on hepatic cells directly. Adenine, via the adenine receptor, induces morphological change in liver (Watanabe *et al.*, 2012).

In a histological section of the liver treated with adenine + *Urtica dioica* leaves powder, the liver revealed decreased inflammatory hepatic architecture and hepatocyte cords, as well as mild congestion of the hepatic central vein, figure (4-6).

Treatment of *Urtica dioica* markedly reduced vacuolization and sinusoidal congestion in the liver. These results show the beneficial effects of UD on hepatic regeneration at the cellular level.

The *Urtica dioica* has been proven to have potent antioxidative and cytoprotective properties. It has several pharmacological properties such as anti-inflammatory, antimicrobial, antioxidative activities and hepatoprotective effects (Gulcin *et al.*, 2004; Kanter *et al.*, 2005; El Haouari *et al.*, 2006). Hepatoprotection of leaves extract of UD is the ability to prevent damage to the liver, prevent the liver affections prophylactically and maintains balance in liver enzymes. The *Urtica dioica* extract has shown significant hepatoprotective effect in isolated rat hepatocytes (in-vitro) and same in rabbits (in-vivo) with protective effect against hepatocellular degeneration and necrotic changes. (Daba and Abdel Rahman, 1998; Turkdogan *et al.*, 2003; Kanter *et al.*, 2005).

Studies have suggested that the hepatoprotective effect of *U. dioica* may be as a result of the presence of flavonoids, ascorbic acid, carotenoids, tannins and lignin's among the plant constituents (Gilani and Janbaz, 1995). These bioactive chemicals are known to target the anti-oxidation mechanisms of cell damage in the body by serving as free radical scavengers and therefore promoting hepatoprotection. Similar studies suggesting hepatoprotection using herbal

extracts from U. dioica have also been done (Konrad *et al.*, 2000; Thangapazham *et al.*, 2007).

5.4.3. Bone Marrow

Chronic kidney disease (CKD) causes bone loss, particularly in cortical bone, through formation of cortical pores which lead to skeletal fragility. Animal models of CKD have shown variability in the skeletal response to CKD (Metzger *et al.*, 2021). Not surprisingly, skeletal fragility is a common comorbidity of CKD with hip fracture incidence higher in CKD patients compared to those with normal kidney function (Kim *et al.*, 2016).

Irrespective of disease severity, CKD patients experience higher rates of post-fracture complications, hospitalization, and mortality than the non-CKD population (Tentori *et al.*, 2014; Kuo *et al.*, 2015; Kim *et al.*, 2016). Also in rats, both adenine-induced CKD and CKD induced by chronic nitric oxide inhibition result in more severe disease phenotype in male vs. female rats (Ogirima *et al.*, 2006; Fanelli *et al.*, 2017).

adenine (closest to mean of porosity), and adenine mice with the highest porosity. Male adenine mice had lower trabecular, lower trabecular thickness, higher trabecular separation and lower trabecular number (Metzger *et al.*, 2021). The primary finding of this study is that both male and female mice develop skeletal complications due to adenine-induced CKD, including high bone turnover, cortical thinning, and development of cortical porosity (Metzger *et al.*, 2021). As a result, if there is inflammatory or renal disease that blocks or diminish the response of the bone marrow.

Figure (4-9) histological section in bone marrow treated with adenine + *Urtica dioica* leaves powder, showed bone ameliorate architecture marrow cellularity, there is well distribution of megakaryocytes with well erythroid cells maturation.

Chapter Six

Conclusion and Recommendations

6. Conclusion and Recommendations

6.1. Conclusion

1- Induced CRD by adenine lead to disturbance in coagulation system, changes in blood parameters, disorder in kidney and liver function test (urea and creatinine) and cause harmful defect in the kidney liver and bone marrow.

2- *Urtica dioica* leaves ameliorates adenine-induced CRF in rats may include anti-oxidant and anti-inflammatory actions by reduction of the occurrence of the renal toxicity.

3- *Urtica dioica* is effective in significantly ameliorating some of the physiological and histopathological actions of CRF, that UD leaves powder can improve the Hb, PCV, RBC concentrations, serum iron, Frritin, FEP content in blood which can improve the concentrations of urea and creatinine

6.2. Recommendations

1- Further research into the lipophillic extracts of stinging nettle to discover the bioactive compound(s) responsible for the reported anti-inflammatory activity is required.

2- Anemia has complex pathways, and there are likely to be other important factors involved that were not measured.

3- The nephroprotective effect of UD is, at least in part, related to its antioxidant compounds and anti-inflammatory characteristics; however, exact pathways involved in this effect need further experimental and clinical studies.

4- It is worth looking into whether nettles should be included more frequently in people's diets to enhance health and prevent disease.

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APPENDIX

APPENDIX

Appendix (I): Estimation of Serum Urea Concentration

Procedure

Wave length: 578 nm

Wave length	340 nm
Optical path	1 cm
Sample reagent	1: 100
Reagent volume	I ml
Sample volume	10 µl
Reagent blank limited	Low 0.9 AU High 2.0 AU
Sensitivity	0.9 mg/dl (0.15 mol/L)
Linearity	300 mg/dl (49.8 mmol/L)

	Standard	Specimen
Reagent	1 ml	1 ml
Standard	10 µl	--------
Specimen	--------	10 µl

Mix, and after 30 second read the absorbance Al of standard or specimen. Exactly 1 minute later, read the absorbance A2 of standard or specimen.

Calculation

Δ A Specimen = A1 specimen –A2 specimen

Δ A standard = A1 standard –A2 standard

Serum urea concentration (mg/dl) = Δ A Specimen

_____

Δ A standard

Where n=50.0 mg/dl (8.33 mmol/L)

Appendix (II): Estimation of Serum Creatinine Concentration

Procedure

Pipette in well identified test tube	Blank	Standard	Sample
Distilled water	0.5	-------	-------
Standard 2 mg/dl	-------	0.5	-------
Trichloroactic acid 1.2 mol/L	0.5	0.5	-------
Supernatant	-------	-------	1 ml
Reagent mixture (picric acid +NaOH)	1 ml	1 ml	1 ml

Mix and let stand for 20 minute at 20-250 C measure the absorbance of specimen and standard against reagent blank at 246 nm.

Calculation

(A of Specimen)

Creatinine (mg/dl) = → 2 (Standard Concentration)

(A of Standard)

Appendix (III): Complete Blood Count

Procedure

The sample is collected drawing the blood into a tube containing an anticoagulant typically (EDTA) to stop it from clotting The testing is typically performed by an automated analyzer Analysis begins when a well mixed blood sample is placed on a rack in the analyzer. The instrument utilizes flow cells, photometers and apertures to analyze different elements in the blood. On board the analyzer, the sample is diluted and aspirated into at least two different channels, one of which is used to count red blood cells and platelets, the other to count white blood cells. Additional channels may be used for differential white blood cell counts and specialized measurements of platelets.

Calculation

Blood cell counting occurs by flow cytometry, in which a very small amount of the specimen is aspirated, diluted and passed through an aperture and a flow cell. Sensors count and identify the number of cells passing through the aperture using two main principles: electrical impedance and light scattering Impedance-based cell counting operates on the Coulter principle, which measures the drop in current as cells pass through an aperture to count cells and calculate their sizes. Because red blood cells, white blood cells and platelets have different average sizes, this technique allows the three types of cells to be differentiated. Light scattering

techniques direct a laser at individual cells and determine cellular size and complexity by measuring the amount of light scattered at different angles. Forward scatter, which refers to light scattered between 0 and 10 degrees of the beam's axis, correlates with cellular size, while side scatter (light scattered at a 90-degree angle) correlates with cellular complexity. White blood cells, red blood cells and platelets, as well as individual types of white blood cells, can be distinguished based on light scattering characteristics.

Appendix (IV): Estimation of Rat Serum Ferritin (FE) ELISA Kit

Assay Procedure

1. Add standard: Set Standard wells, testing sample wells. Add standard 50μl to standard well.

2. Add sample: Set blank wells separately (blank comparison wells don’t add sample and HRP-conjugate reagent; other each step operation is same).

testing sample well, add sample dilution 40μl to testing sample well, then add testing sample 10μl (sample final dilution is 5-fold), add sample to wells, don't touch the well wall as far as possible, and gently mix.

3. Add enzyme: Add HRP-Conjugate reagent 100μl to each well, except blank well.

4. Incubate: After closing plate with closure plate membrane, incubate for 60 min at 37℃.

5.Configurate liquid: 20-fold wash solution diluted 20-fold with distilled water and reserve

6.Washing: Uncover Closure plate membrane, discard Liquid, dry by swing, add washing buffer to every well, still for 30 s then drain, repeat 5 times, dry by pat.

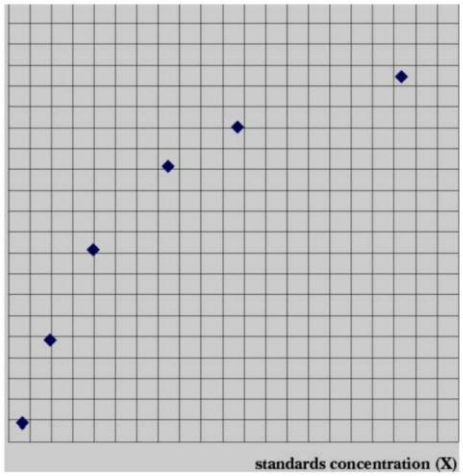
7. Color: Add chromogen solution a 50ul and chromogen solution B to each well, evade the light preservation for 15 min at 37℃.

8. Stop the reaction: Add Stop Solution 50μl to each well, Stop the reaction (the blue color change to yellow color).

9. Assay: Take blank well as zero, read absorbance at 450 nm after adding stop solution and within 15 min.

Calculate

Take the standard density as the horizontal, the OD value for the vertical, draw the standard curve on graph paper, find out the corresponding density according to the sample OD value by the Sample curve, multiplied by the dilution multiple, or calculate the straight line regression equation of the standard curve with the standard density and the OD value, with the sample OD value in the equation calculate the sample density.



This chartis for reference only

Assay Range

1.875 ng/mL - 60 ng/mL

Appendix (V): Estimation of Rat Free Erythrocyte Protoporphyrin (FEP) ELISA Kit.

1. Prepare all reagents: Before starting assay procedure. It is recommended that all standards and samples be added in duplicate to the Microelisa Stripplate.

2. Add standard: Set Standard wells, testing sample wells. Add standard 50 µl to standard well.

3. Add Sample: Add testing sample 10 µl then add Sample Diluent 40ul to testing sample well; blank well doesn't add anything.

4. Add 100µl of HRP-conjugate reagent to each well, cover with an adhesive strip and incubate for 60 minutes at 37°C.

5. Aspirate each well and wash, repeating the process four times for a total of five washes. Wash by filling each well with wash solution (400ul) using a squirt bottle, manifold dispenser or auto washer. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining wash solution by aspirating or decanting. Invert the plate and blot it against clean paper towels.

6. Add chromogen solution A 50µl and chromogen solution B 50ul to each well. Gently mix and incubate for 15 minutes at 37°C. protect from light.

7. Add 50 µl stop solution to each well. The color in the wells should change from blue to yellow. If the color in the wells is green or the color change does not appear uniform, gently tap the plate to ensure thorough mixing.

8. Read the Optical Density (O.D.) at 450 nm using a microliter plate reader within 15 minutes.

Calculation

1. This standard curve is used to determine the amount in an unknown sample. The standard curve is generated by plotting the average O.D. (450 nm) obtained for each of the six standard concentrations on the vertical (Y) axis versus the corresponding concentration on the horizontal (X) axis.

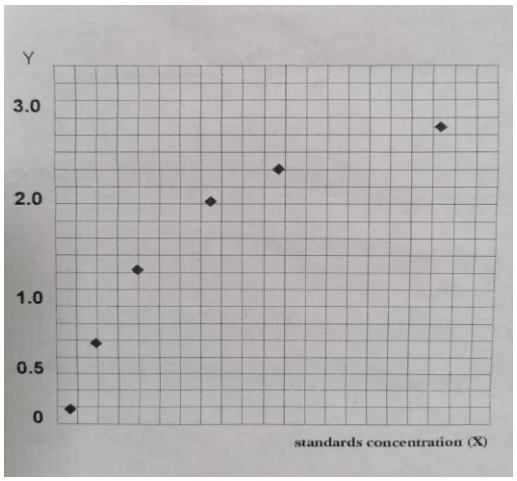
2. First, calculate the mean O.D. value for each standard and sample. All O.D. values, are subtracted by the mean value of the zero standard before result interpretation. Construct the standard curve using graph paper or statistical software.

3. To determine the amount in each sample, first locate the O.D. value on the Y-axis and extend a horizontal line to the standard curve. At the point of intersection, draw a vertical line to the X-axis and read the corresponding concentration.

4. Any variation in operator, pipetting and washing technique, incubation time or temperature, and kit age can cause variation in result. Each user should obtain their own standard curve.

5. The sensitivity by this assay is 0.1 ng/ml.

6. Standard curve



Appendix (VI): Estimation of Rat Serum Hepcidin (Hepcidin) ELISA Kit

Assay Procedure

1. Add standard: Set standard wells, testing sample wells. Add standard 50 µl to standard well.

2. Add sample: Set blank wells separately (blank comparison wells don't add sample and HRP-conjugate reagent, other each step operation is same), testing sample well. add Sample dilution 40µl to testing sample well, then add testing sample 10µl (sample final dilution is 5-fold), add sample to wells,

don't touch the well wall as far as possible, and gently mix.

3. Add enzyme: Add HRP-conjugate reagent 100 µl to each well, except blank well.

4. Incubate: After closing plate with Closure plate membrane, incubate for 60

min at 37℃.

5. Configurator liquid: 20-fold wash solution diluted 20-fold with distilled water and reserve.

6.washing: Uncover Closure plate membrane, discard Liquid, dry by swing, add washing buffer to every well, still for 30s then drain, repeat 5 times, dry by pat.

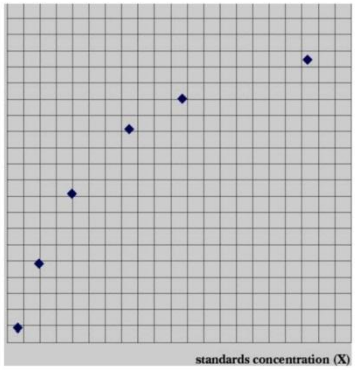
7. Color: Add chromogen solution A 50 μl and chromogen solution B to each well, evade the light preservation for 15 min at 37℃

8. Stop the reaction: Add Stop Solution 50μl to each well, Stop the reaction (the blue color change to yellow color).

9. Assay: Take blank well as zero, read absorbance at 450nm after adding stop solution and within 15 min.

Calculate

Take the standard density as the horizontal, the OD value for the vertical, draw the standard curve on graph paper, find out the corresponding density according to the sample OD value by the sample curve, multiplied by the dilution multiple, or calculate the straight line regression equation of the standard curve with the standard density and the OD value, with the sample OD value in the equation, calculate the sample density



This chartis for reference only

Assay Range

3.75 ng/mL - 120 ng/m

Appendix (VII): Histological Study

Histological Technique (E&H) Stain

The kidney, liver and bone of each animal were quickly removed and rapidly weighed then prepared for histological study according to Mescher method, (2010) with aid of the light microscope as the following steps:

*** Fixation**

The specimen fixated in the formalin 10% for 24–48 hours. In front of the bone marrow sample placed in a Bouin's solution.

*** Washing and Dehydration**

After fixation the specimens washed with water to remove the fixative in order to avoid the interaction between the fixative and staining materials used later. By dehydration the water had been completely extracted from fragments by bathing them successively in a graded series of ethanol and water (70%, 80%, 90%, and 100% ethanol).

*** Clearing**

Bathing the dehydrated fragments in solvent (Xylene) for 30–60 minutes, this step was repeated 3 times. As the tissues clearing, they generally became transparent.

*** Infiltration and Embedding**

Once the tissue fragments were impregnated with the solvent, they were placed in melted paraffin in an oven, typically at 52 ℃. The heat causes the solvent to evaporate, and the space within the tissues becomes filled with paraffin

*** Sectioning**

After holds from the oven, the specimen let at room temperature to be solid and removed from their containers in order to sectioning they were put in the rotary microtome and were sliced by the microtome, a steel blade into sections 5 micrometers thick. The sections were floated on water bath (50–55 ^{o}C), then transferred into glass slides coated with Mayers albumin as adhesive substance and left to dry.

*** Staining**

The histological sections of the studied organs were stained with Hematoxylin-Eosin stain.

Staining Procedure

1. Deparaffinize sections, 2 changes of xylene, 10 minutes each.

2. Re-hydrate in 2 changes of absolute alcohol, 5 minutes each.

3. 95% alcohol for 2 minutes and 70% alcohol for 2 minutes.

4. Wash briefly in distilled water.

5. Stain in Harris hematoxylin solution for 8 minutes.

6. Wash in running tap water for 5 minutes.

7. Differentiate in 1% acid alcohol for 30 seconds.

8. Wash running tap water for 1 minute.

9. Bluing in 0.2% Ammonia water or saturated Lithium Carbonate solution for 30 seconds to 1 minute.

10. Wash in running tap water for 5 minutes.

11. Rinse in 95% alcohol, 10 dips.

12. Counterstain in Eosin-Phloxine solution for 30 seconds to 1 minute.

13. Dehydrate through 95% alcohol, 2 changes of absolute alcohol, 5 minutes each.

14. Clear in 2 changes of Xylene, 5 minutes each.

15. Mount with Xylene based mounting medium Trichrome stain (Modified masson's) procedure.

The procedure was done according to Trichrome Stain Kit (Modified Masson's) Scy Tek Laboratories, Inc./ USA.

Procedure

1. Mordant in Bouins solution, microwave 1 minute, allow to strand 15 minute

2. Wash in running tap water to remove the picric acid, 5 minutes.

3. Weigerts working hematoxylin,10 minute.

4. Blue in running tap water for 5 minute, rinse in distilled water.

5. Biebrich scarlet for 5 minute

6. Rinse in distilled water

7. Phosphotungistic/phosphomolyboic acid for 10 minute, discard solution

8. Transfer directly into Anilline blue for 5 minutes.

9. Rinse in distilled water.

10. 1% Acetic acid for 1minute, discard solution, rinse in distilled water.

11. Dehydrate, clear, and coverslip.

Conventional method: Mordant in Bouins solution, 60 C° for 1 hour.

Bouin's solution, is a compound fixative used in histology. It was invented by French Biologist Pol Bouin and is composed of picric acid, acetic acid and formaldehyde in an aqueous solution. It is a good fixative when tissue structure with a soft and delicate texture must be preserved. The acetic acid in this fixative lyses red blood cells and dissolves small iron and calcium deposits in tissue. The acetic acid in this fixative lyses red blood cells and dissolves small iron and calcium deposits in tissue.

الخلاصـــــة

أجريت هذه الدراسة لتقييم بعض عوامل التخثر والعوامل الكيموحيوية والتغيرات النسيجية للكبد والكلية ونخاع العظام في ذكور الجرذان البالغة والمعالجة بالأدنين لأستحداث فشل كلوي مزمن بالإضافة الى معرفة الدور الوقائي المحتمل لأوراق عشبة نبات القريص والتأثير العلاجي على فقر الدم الناجم من الفشل الكلوي المزمن. أجريت التجربة باستعمال ٤٥ من ذكور الجرذان البالغة، حيث قسمت هذه الجرذان الى خمسة مجاميع بصوره عشوائية ومتساوية لمدة ٧ أسابيع وكانت المجاميع كما يلي: -

المجموعة الأولى الضابطة حقنت حيواناتها داخل البريتون بمادة ثنائي مثيل السلفوكيد (DMSO) لمدة ٤ أسابيع وكانت تغذيتها على النظام الغذائي العادي، المجموعة الثانية الضابطة أعطيت مسحوق أوراق القريص بجرعة ٤% ممزوجا بالعلف لمدة ٤ أسابيع، المجموعة الثالثة تم حقنها بمادة الأدنين في البريتون بجرعة ١٠٠مجم/كجم من وزن الجسم لمدة ٤ أسابيع لأحداث الفشل الكلوي، المجموعة الرابعة حقنت بمادة الأدنين بجرعة ١٠٠مجم/كجم من وزن الجسم لمدة ٤ أسابيع لأحداث الفشل الكلوي ومن بعدها تم إعطاء مسحوق أوراق القريص بجرعة تراوحت ٤% ممزوجا بالعلف لمدة ٣ أسابيع، المجموعة الخامسة حقنت بمادة الأدنين بجرعة ١٠٠مجم/كجم من وزن الجسم لأحداث الفشل الكلوي وتم إعطاء مسحوق أوراق القريص بجرعة ٤% لمدة ٤ أسابيع في نفس الوقت.

أظهرت النتائج وجود ارتفاع معنوي في اليوريا في الدم، الكرياتنين في ($p\leq0.01$) وعدد كريات الدم البيضاء في FEP الدم، بروفيرين خلايا الدم الحمراء الحرة، المجموعة الثالثة المعالجة بالأدنين بالإضافة الى فريتين المصل وهبسدين المصل في عدد كريات الدم الحمراء وخلايا الدم المضغوطة ($p\leq0.01$) انخفاض معنوي

في الصفائح الدموية ومصل الحديد وخضاب الدم في مجموعة الأدنين المجموعة الثالثة بالمقارنة مع المجموعات الأخرى، بعد إعطاء مسحوق أوراق القريص نلاحظ وجود في اليوريا والكرياتنين والهبسدين والفريتين وعدد كريات الدم البيضاء($p\leq0.01$) انخفاض كبير في المجموعة المعالجة و FEPوبروفيرين خلايا الدم الحمراء الحرة في المجموعة الخامسة، في عدد كريات الدم الحمراء وخلايا الدم ($p\leq0.01$) وزيادة ذات دلاله إحصائية في خلايا الدم المضغوطة وخضاب الدم والصفائح الدموية ومصل الحديد في المجموعة الرابعة والخامسة بالمقارنة مع المجموعة الثالثة.

تظهر التغيرات النسيجية في الكلى والكبد ونخاع العظام أن المجموعة المعالجة بالأدينين قد تعرضت للضمور والتنكس خاصةً الأنابيب الكلوية التي تحتوي على ترسبات الأدنين البلورية بالإضافة إلى الارتشاح الالتهابي لخلايا مقارنةً بمجموعة السيطرة ولكن بعد إعطاء أوراق القريص تأثرت الانسجة وأصبحت قادرة على الرجوع الى شكلها الطبيعي ولكن لا يتم الشفاء تماما.

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة كربلاء/كلية الطب البيطري
فرع الفسلجة والكيمياء الحياتية والأدوية

التأثير العلاجي والوقائي لأوراق نبات القريص ضد استحداث الفشل الكلوي المزمن وفقر الدم المتزامن في ذكور الجرذان

رسالة مقدمة الى

مجلس كلية الطب البيطري

وهي جزء من متطلبات نيل درجة الماجستير في علوم الطب البيطري/الفسلجة

من قبل
فاطمة رسول جاسم
بكالوريوس طب وجراحة بيطرية كلية الطب البيطري/ جامعة كربلاء

بإشراف
الأستاذ المساعد الدكتور
ميادة صاحب حسن

١٤٤٣هـ **٢٠٢١م**