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Physiology, Biochemistry and Pharmacology Department

Evaluation of the Protective Effects of *Arthrosporia Platensis* (spirulina) Against Induced Chronic Renal Failure by Adenine in Male Rats Rattus norvegicus

A THESIS

Submitted to the Council of the College of Veterinary Medicine, University of Kerbala in Partial Fulfillment of the Requirement for the Degree of Master of Science in Veterinary Medicine / Physiology

By

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Azhar Mohammad Hassan

DEDICATION

To the one who strives to comfort me and make me happy ….. my father.

To my loves, merciful, Candle that light my way ..... my mother

My wife, when I look at you I wonder if I did something really good to get such a marvelous gift from God. You have been a blessing in my life and this is just to say I love you..

All the love, respect and appreciation to my family who stood with me and helped me.

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Waleed

Summery

The present study aims to evoluate the role of spirulina in reduction the ability of adenine induces renal failure and its complication in male rats. The study conducted at College of Veterinary Medicine /University of Kerbala during period from December 2019 to April 2020 . Thirty two adult male rats used in the present study and divided into four groups , first group consider as control group , the second group received adenine (100 mg / kg BW) intraperitoneal , while the third group receive adenine (100 mg / kg/BW) intraperitoneal combined with spirulina suspension (750 mg /kg/BW) and the fourth group received just spirulina suspension (750 mg /kg/BW) orally also with feed , at the end experiment blood was collected to assessment kidneys function (KIM-1,urea and creatinine), and the liver function parameters (AST and ALT),lipid profile(cholesterol ,triglyceride ,LDLand HDL).Antioxidant parameters (SOD,GSH,MAD and NO) and hematological parameter (Red blood cells, hemoglobin, PCV, platelets and WBC). kidney and liver isolated to study of histological changes. Results showed significance ($p \leq 0.05$) increases in kidney function test and results showed significance increase ($p \leq 0.05$) of lipid profile parameters in (cholesterol, triglyceride and low density lipoprotein) in the second group which administrated adenine while high density lipoprotein was decreased in the same group in the comparison with other groups. the results showed significance ($p \leq 0.05$) decrease in the GSH, SOD and increase in the MAD and NO in the second group in comparison with other groups. Results showed significance ($p \leq 0.05$) decrease in RBC, Hb, PCV and platelets in the second group in comparison with other groups while WBC was increased in the second group in compared to other groups. The results in the third group which received the spirulina suspension co_adminstrated

with adenine as antioxidant showed significance enhancement in the most of parameters which studied to return nearlly to the control group levels.

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Abbreviations	Meaning
ARF	Acute Renal Failure
ALT	Alanine aminotransferase
AST	Aspartate Aminotransferase
CVD	Cardio Vascular Diseases
CKD	Chronic Kidney Disease
CRF	Chronic Renal Failure
GSH	Glutathione
HDL	High density lipoprotein
LDL	Low density lipoprotein
MAD	Malondialdehyde
NO	Nitric oxide
$^{1}O2$	Single oxygen
SOD	Superoxide dismutase
µ/L	Unit per litter

Chapter one
Introduction

1-Introduction

Kidneys are efficient organs that represent the main control system to maintain homeostasis of the body. They are affected by various chemicals and drugs that may do effect the functions (Maliakel *et al.*, 2008).

Kidney disease is one of the reasons that lead to the reduction quality of individual life in now days (Chertow *et al.*, 2005). The kidneys of human are mainly participatory in dilution and concentrating various materials and chemical factors that may reach a rise concentration and turn into toxics (Loh and cohen, 2009).Renal failure (RF) is considered pathologically deadly because of serious hormonal and metabolic disturbances (Go *et al.*, 2004). Whereas some models of animal renal failure are used to evaluate the pathogenic damage of organs pathogenesis (Bellomo *et al.*, 2004). Chronic renal failure (CRF) results from irreversible and progressive damage of wide numbers of functioning nephrons. Serious clinical signs often do not happen until the number of functional nephrons falls to at least 70 to 75 per cent below normal. In fact, relatively normal blood concentrations of most electrolytes and normal body fluid volumes can still be maintained until the number of functioning nephrons decreases below 20 to 25 per cent of normal (Guyton and Hall, 2006). CRF is usually prevalent and adversely influences human health, life span and raises costs to health-care systems in throughout the world. Renal toxicities, glomerulonephritis, vasculopathies and diabetic nephropathy may promote oxidative conditions, increase susceptibility of acute renal failure (Nasri *et al*., 2014). The free radicals one of causes that lead to kidney failure. Oxidative stress is known as a main pathological

process in renal failure which activates various proinflammatory cytokines and growth factors, finally leads to glomerulosclerosis, tubulointerstitial fibrosis, tubular cells apoptosis and senescence, as well as deactivated cellular regenerative pathways (Dehghan Shahreza, F. 2017). Spirulina are indicated to filamentous free-floating microalgae with spiral characteristics of its filaments. It is officially called Arthrosporia *sp* powder, relationship to the category of cyanobacteria with feature of photosynthetic ability (Sapp2005 and Komarek *et al*.,2016). Spirulina suspension have anti-oxidant properties and scavenge the free radicals' duo to its have 1,1-diphenyl-2-picrlhydrayl (DPPH) in hepatic failure induced rats (Gabr *et al.*, 2020).

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The Spirulina suspension has antioxidant activity that contain of the material such as compound element, γ-linolenic acid, tocopherol, the phycocyanin β-carotene, and phenolic, (Chopra and Bishnoi, 2008). Spirulina suspension his act as strong scavenging activities control against superoxide and hydrogen peroxide radicals (Huang *et al.,*2007). Spirulina have protective action against, the oxidatives stress induced by lead acetates in the kidney and liver rats (Ponce Canchihuamanet *et al.*, 2010).

Aims of study

The study aims to investigate the spirulina powder antioxidant activity and its probable enhancement effects against adenine-induced chronic renal failure, via following objectives:

Introduction

1.Induceing chronic renal failure by adenine.

2. Evaluate effect of adenine powder and spirulina suspension on kidney and liver function.

3.Evaluate effect of adenine powder and spirulina powder suspension on anti-oxidant status.

4.Evaluate effect of adenine powder and spirulina powder suspension on lipid profile and blood picture .

Chapter two
Review of literature

2.1. Kidney

The renal system is composed of the kidneys, the ureters, bladder in addition to urethra. The kidneys are located in both sides of the body in the retroperitoneum area. The kidney is composed of two layers the cortex is the outer layer that contains nephron parts; glomeruli, proximal tubules, cortical portions of loops of Henle, distal tubules and cortical collecting ducts (Lynelle and Carima, 2011). The nephron is the functional and structural unit of the kidney. It consist of glomerulus, Bowman capsule, proximal convoluted tubule, loop of Henle, distal convoluted tubule and collecting ducts (Lynelle *et al.*, 2011).

Nephron is classified according to its location within kidney mass into two types, cortical nephrons (short loop nephrons) have their glomeruli located in the outer cortex, and juxtamedullary nephrons(long loop nephrons) deeply embedded in the cortex, just near the medulla (Jennette *et al.*, 2007). Eighty five percent of nephrons is cortical their functions are excretory and regulatory, while15% of nephrons are juxtamedullary and their functions are concentration and dilution of urine (Lynelle *et al.*, 2011).

2.1.1. Renal system functions

The renal system functions included (Niemczyk, *et al.*,2012; Eaton and Pooler, 2009; Sahay, *et al*., 2012).

1.Plasma osmolarity regulation due to it has a direct controls the amount of water and ions that excretes which influence on the total blood volume and that have an impact on blood pressure .

2. Regulation of (pH) together with the lungs.

3.Filtration of the blood from waste product and foreign materials such as nitrogenous waste.

4.Secretion of hormones such as renin which act as the stimulator to aldosterone from cortex in adrenal gland , this hormone enhance reabsorption of sodium in kidney

5.Secretion of erythropoietin hormone which responsible for production of erythrocytes in the bone marrow and vitamin D.

6.Regulation of blood electrolytes such as sodium, potassium, calcium magnesium, chloride, bicarbonate, and phosphates.

2.2. Renal failure

Renal failure is a status of kidney in which it failed to eliminate and concentrate metabolic west products from the blood, hemostasis the fluids, electrolytes, and regulate the pH in the extracellular fluids. There are various reasons may lead to kidney illness; systemic disease, and/or urinary tract defects unconnected to kidney (Kasper *et al*., 2005). The kidney has substantial and major function in excretion of chemicals and drugs. So the renal failure may lead to reservation, of these compounds, which may accumulate gradually to toxic levels (Finn and Porter, 2003; Ferguson *et al.*, 2008).

2.2.1. Acute Renal Failure

Acute renal failure(ARF) is the sudden interruption of kidney function resulted from obstruction, decreased of the circulation, or disease in

the renal tissue. with the treatment can usually be reversible. Else it can progress to form end-stage renal disease (ESRD) or chronic renal failure CRF (Ferrone, 2003; Nitescu, 2007). ARF is a rapid decline in function of kidney related with changing in urinary output, azotemia, and disruption of biochemical homeostasis. In severity, it can be characterized by increasing the levels of blood urea (B.U) and serum creatinine and oliguria due to the sudden deterioration of kidney function through hours to days (Lattanzio and Kopyt, 2008).

Acute renal failure is possible to become reversible if the inducing factors can be corrected or removed before permanent renal damage has done. ARF is one of the most common threat that patients in intensive care units are suffering from it also with a mortality (Thadhani *et al*.,1996, Albright 2001and Singri *et al*.,2003). The mortality rate from ARF has not altered substantially from the 1960 (Molitoris *et al*.,2000; and Singri *et al*.,2003), because ARF is being in high ratio usually in older persons than before, and it is frequently associated with other life-threatening cases, like trauma, shock, and sepsis .In Acute renal failure, the glomerular filtration rate is decline , that lead to decrease excretion of nitrogenous wastes, disturbance of fluid and electrolyte balance(Hilton, 2006) .

2.2.2. Chronic Renal Failure

Chronic kidney disease (CRD) is a serious global health problem, and is now considered a key determinant of the poor health outcomes of major no communicable diseases (Couser *et al*.,2011). Several factors influence the onset and progression of this CRD, such as obesity, hypertension and diabetes mellitus. After these factors, there is index of a pathophysiological role for

inflammation and oxidative stress in Chronic CRD and its complications (Himmelfarb 2004). Inflammation and oxidative stress are prominent features of Chronic kidney disease and its complications in humans (Cachofeiro *et al*.,2008). Increased oxygen radical formation was found in CRD, in the presence of a reduced antioxidant agents (Morena *et al* .,2005**)**. Moreover, signs of inflammation and oxidative stress are increased, malondialdehyde content and peroxidation, or C-reactive protein and IL-6 (Zilmer *et al***.,**2001). Inflammation and oxidative stress are also major mediators of the disease, exerting similar effects in the surgically-induced chronic renal failure model in rats (Korish *et al*.,2010). Patients with CRD have high plasma concentrations of inflammatory mediators (such as creactive protein, tumor necrosis factor. Chronic renal fail patients combine common atherogenic risk factors such as age, diabetes mellitus, hypertension, smoking and dyslipidaemia with factors more specifically related to the uraemic state such as dyslipoproteinaemia.

2.3. Induction of chronic renal failure (CRF)

There are many methods in creations of chronic kidneys failure diseases, including paracetamol, the introduction of kidney failure as well as by gentamicin used to induce renal failure as well as the adenine used to induce renal failure and others mistrials.

2.4. Adenine induction of chronic renal failure

Adenine used to stimulate chronic renal failure in rats male the adenine is given injection intraperitoneally by different dosage for four weeks,(Al Za 'abi *et al.*, 2015). 2 ,8dihydroxy adenine and Adenine are released in the urine and lower soluble of 2 , 8-dihydroxy adenine leads to it precipitations in the kidneys tubules of the nephron. (Bertram *et al*., 2010and Bollée *et al*., 2012). When give the adenine by the oral that causes occlusion of renal tubules relation which daily secretion nitrogenous substances causes biochemical and physiological status resembling Chronic kidney disease in humans .(Nasir *et al.*, 2012and Ali *et al.*, 2010). The administration of adenine intraperitoneally can be consideration an replacement valedictorian prototype to oral adeninepowder for the induce of Chronic kidneys diseases the advantages of this prototype are that adenine powder direct enters the systemic circulations by passing any possiblely local (intestinal) direct physical interaction with any enteral ameliorating agent.. (Ali *et al*., 2014). It also is more practical, convenient and accurate (Al Za'abi *et al*., 2015).

2.5. Adenine

Adenine is derived from the nucleotide inosine monophosphate. It produced on a pre-existing ribose phosphate via a specific pathway by using substrates from glycine, glutamine, and aspartic acid (amino acids). Also, fusion with the enzyme tetrahydrofolate (Wang *et al*., 2002). It is a nitrogen hetero cycles. It presents at very low level in blood, and excess adenine is transformed to 2,8-dihydroxyadenine (DHA) when oxidized by xanthine dehydrogenase in the kidney. Adenine produces adenosine, a nucleoside, by linking with ribose, and then forms adenosine triphosphate (ATP), a

nucleotide, by adding three phosphate groups to adenosine. ATP is used in cell metabolism as one of the most important methods of transferring chemical energy between chemical reactions, maintaining energy balance (Ereciñska and Wilson, 1982). In addition, adenosine is a critical signaling molecule that is induced under ischemic and hypoxic conditions (Fredholm,2007). Thus, the increased adenosine in the kidney indicated renal lesions due to ischemia or hypoxia, and decreased level of adenosine in the kidney may result from increased cell apoptosis, functional destruction and ATP depletion (Tang *et al*., 2015). The low solubility of 2,8-dihydroxyadenine makes its precipitation in kidney especially in nephron tubules (Yokozawa *et al*., 1986).

Waste compounds excretion from kidney are stopped by obstruction of renal tubular due to 2,8- dihydroxyadenine. This, in turn, will lead to elevation of creatinine and urea nitrogen concentration in the blood (Zhao *et al*., 2013). Adenine endogenously produced as a by-product of the polyamine pathway and is cleared by adenine phosphoribosyl transferase. When functional APRT is not found ,adenine becomes significant substrate for xanthine dehydrogenase (XDH), which in turn oxidizes adenine into 2,8-dihydroxyadenine(DHA) (Engle *et al*.,1996;and Stockelman *et al*., 1998). When adenine presents in high amounts in mammalian metabolism ,it will be a substantial substrate for xanthine dehydrogenase, that has the ability to oxidize adenine to form 2,8dihydroxyadenine (DHA) by an intermediate as 8-hydroxy adenine (Stockelman *et al*., 1998 and Benedetto *et al*., 2001). Animal model of CRF induced by an adenine-rich diet was firstly described by Yokozawa et al., (1986). Adenine and 2,8-dihydroxy adenine are released in the urine because the low solubility of 2,8dihydroxy adenine leads to it

precipitation in the kidney tubules of the nephron (Bertram *et al.*, 2010; Bollée *et al*., 2012).

2.5.1 Mechanism action of adenine

Adenine is a nitrogen hetero cycles that finally metabolite to uric acid. It is efficiently scavenger adenine phosphoribosy1-transferase and is found at very little concentration in circulation (Engle *et al*.,1996). When adenine presents in high amounts in mammalian metabolism, it will be a substantial substrate for xanthine dehydrogenase, that has the ability to oxidize adenine to form 2,8dihydroxyadenine (DHA) by an intermediate as 8-hydroxy adenine (Stockelman et al., 1998 and Benedetto *et al*., 2001).

Animal model of CRF induced by an adenine-rich diet was firstly described by Yokozawa *et al*., (1986). Adenine and 2,8-dihydroxy adenine are released in the urine because the low solubility of 2,8dihydroxy adenine leads to it precipitation in the kidney tubules of the nephron (Bertram *et al.*, 2010; Bollée *et al*., 2012). A adenine produces abnormalities in metabolism of mammalian that likely resemble chronic renal insufficiency in humans. Nitrogen compounds excretion are stopped by renal tubular occlusion due to 2,8-dihydroxyadenine precipitation which result in the accumulation of urea and creatinine in the blood and leading to elevation of various guanidine compounds(Yokozawa *et al*.,1986).

2.6. Kidney injury molecule -1 (KIM -1)

Kidney injury molecule -1 KIM-1 is a type I transmembrane glycoprotein which is not found in normal kidney. The elevation in releasing of this protein was found at highly levels on the proximal tubule cells specially on the apical

membrane after nephrotoxic or ischemic injury (Guo *et al*., 2012). In rats it is widely induced in the tubules after ischaemic or toxic injury (Ichimura *et al*., 1998, Ichimura *et al*., 2004). In rat model study proved that KIM-1 as an excellent biomarker of kidney injury which is better than creatinine in serum to predict injury of proximal tubule (Ichimura *et al*., 1998). KIM-1 also named TIM-1 (T Cell immunoglobulin and mucin domain containing protein-1) (Zhang *et al*., 2008; Schiffl and Lang, 2012;Charlton *et al*., 2014). Studies showed that the ectodomain of KIM-1 is shed and acts as an urinary biomarker, and others viewed that KIM-1 is a predict marker in mammalian and its expression to kidney injury/ toxicity was highly sensitive and specific (Ichimura *et al*., 1998; Vaidya *et al*., 2008; Han *et al*., 2009). According to the Food and Drug Administration (FDA) and European Medicines Agency (EMEA), KIM-1 has been considered as potent biomarker to detect kidney injury induced by drug, in preclinical studies related with kidney toxicity in rats, and on a step by step basis for the translation of the drugs that potentially nephrotoxic to first in human studies (Bonventre *et al*., 2010). In some of preclinical rat toxicology study, the agreement viewed that urinary KIM-1 was not only better than the common markers such as blood urea nitrogen(BUN), serum creatinine(SCr), urinary neutrophil gelatinase-associated lipocalin (NGAL) for detecting renal injury, but KIM -1 was highest performing biomarker of all those that have been tested in the study (Vaidya *et al*., 2010). In patients with clear cell-type renal cell carcinoma (RCC), KIM-1 was found at high levels (Han *et al*., 2005). Renal cell carcinoma, resemble injury of kidney tubular, is related with differentiation of proximal tubule cell.The Critical Path Institute's Preventive Safety Testing Consortium (PSTC) showed that KIM-1 in humans as an early predict biomarker for assessment acute kidney tubular toxicity in preclinical studies and the FDA and EMEA

also promoted its use for safety assessment on a case-bycase basis in clinical trials (Vaidya *et al*., 2008). KIM-1 gives the ability to the epithelial cells to identify and phagocytosis the dead cells that are found in the post-ischaemic kidney and share in the damaging the lumen of tubule that characterizes acute renal failure. That done due to KIM-1 is a phosphatidylserine receptor which identify apoptotic cells directing them to lysosomes. KIM-1 also acts as a receptor for oxidized lipoproteins and it very good at recognizing apoptotic cell 'eat me' signals. (Savill and Fadok ,2000; Ichimura *et al*., 2008). It also have the ability to the facilitate apoptotic debris clearance from the tubular lumen, KIM-1 has potent role in restricting the autoimmune response to injury because it is known in many systems that phagocytosis of apoptotic bodies. Acute protective responses, however, not necessarily translate to chronic effects of KIM-1 expression, a clinically relevant issue (Vaidya *et al*., 2008) and others. Have showed that many chronic renal failure cases release KIM-1 protein in their proximal tubules (Imarah, 2017and Van Timmeren *et al*., 2007).

2.7. Anemia related with chronic renal failure

Anemia is an early sign and a common and severe complication of chronic kidney disease, especially in the end-stage renal disease. In general, renal anemia occurs mainly due to the absolute deficiency of erythropoietin. Anemia in CRD is typically normocytic, normochromic, and hypoproliferative.Anemia of chronic kidney disease is a multifactorial process due to relative erythropoietin deficiency, uremic-induced inhibitors of erythropoiesis, shortened erythrocyte survival, and disordered iron homeostasis (Babitt and Lin 2012). Anemia of renal disease is common and is chiefly due to a decrease in erythropoietin production and It is necessary to

investigate other treatable causes of anemia in renal failure patients and anemia of renal disease is associated with adverse cardiac events, heart failure, myocardial infarction and death. Erythropoietin levels are not indicative of anemia in renal failure patients. One should target a hemoglobin level of no more than 11.5 g/d anemia is generally defined as hemoglobin of less than 13.0 g/dL in men and less than 12.0 g/dL in premenopausal women. Anemia ofchronic kidney disease is a form of normocytic normochromic, hypoproliferative anemia. Among other complications of CKD, it is frequently associated with poor outcomes in chronic renal failure and increases mortality.(Collins and Ebben et al ., 2001). The anemia of chronic renal failure probably has more clinical significance than was previously recognized. Of the almost 100,000 patients on maintenance dialysis in the United States, almost all are anemic; only about 3% have a normal hematocrit (Charles *et al.*, 2007).

2.8 .Oxidative stress effect on chronic renal failure

Oxidative stress (OS), defined as disturbances in the pro-/antioxidant balance, is harmful to cells due to the excessive generation of highly reactive oxygen (ROS) and reactive species nitrogen (RNS). All so oxidative stress defines an imbalance between formation of reactive oxygen species (ROS) and antioxidative defiance mechanisms. When the balance is not disturbed, OS has a role in physiological adaptations and signal transduction. However, an excessive amount of ROS and RNS result in the oxidation of biological molecules such as lipids, proteins, and DNA. Oxidative stress has been reported in kidney disease, due to both antioxidant depletions as well as increased ROS production. The kidney is a highly metabolic organ, rich in oxidation reactions in mitochondria, which makes it vulnerable to damage

caused by OS, and several studies have shown that OS can accelerate kidney disease progression. Also, in patients at advanced stages of chronic kidney disease , increased OS is associated with complications such as hypertension, atherosclerosis, inflammation, and anemia. In this review, we aim to describe OS and its influence on chronic kidney disease progression and its complications. The potential role of various antioxidants and pharmacological agents, which may represent potential therapeutic targets to reduce OS in both pediatric and adult chronic kidney disease patients (Daenen et al.,2019). Oxidative Stress in Serum and Renal Tissue of Rats with Chronic Renal Failure. oxidative stress is one common feature that increase the incidence and mortality of CRD patients. Chronic kidney disease -related oxidative stress (Gao *et al* .,2019). The generate oxidative stress It is generally accepted that ROS such as hydrogen peroxide (H2O2) or hypochlorous acid (HOCl), and free radicals such as superoxide (O2−), hydroxyl radical (OH·), and nitric oxide (NO·), are continuously formed in vivo (Halliwell 1993). Thus, detection of ROS per se does not yet define oxidative stress; however, in a situation where antioxidative defence mechanisms are attenuated, it is the imbalance between formation of ROS and defence mechanisms that creates oxidative stress. Renal sources for ROS are activated macrophages, vascular cells and various glomerular cells (Ichikawa and Kiyama *et al*.,1994). The balance between formation of ROS and antioxidative defence mechanisms depends on the activity of enzymes such as superoxide dismutases (SOD), catalase, NO-synthase, and, as emphasized in the study (Klemm et al.,2001).Glutathione peroxidase this balance, however, is rather fragile, difficult to predict, and strongly dependent on environmental conditions (Halliwell 1993). AS illustrated in Figure (2-1) for example, once O2− is formed, the activity of SOD will transform it to H2O2. H2O2, in the presence

of sufficient catalase activity, will be converted to harmless H2O and O2. However, too much SOD relative to H2O2-removing catalase can be deleterious, giving rise to the formation of the highly reactive hydroxyl radical in the presence of metal ions such as Fe2+ or Cu2+ (Fenton reaction) On the other hand, when there is too little SOD activity, OH· also can be produced from O2−via the haber weiss reaction. ROS can be formed from vascular and glomerular cells including fibroblasts, from leucocytes, and from renal interstitial cells (Halliwell 1999). Different cellular enzymes, including mitochondrial oxidases, lipoxygenase, cyclooxygenase, myeloperoxidase, NADPH oxidase, xanthine oxidase, and in the case of L-arginine or tetrahydrobiopterin depletion, NO-synthase have been identified as cellular sources of ROS formation. The biological effects of oxidative stress Formation of ROS is part of the unspecific defence system of an organism against, for example, bacteria and other microbes. However, ROS may also affect cells of the host organism, in particular at sites of inflammation. The latter plays a role in a variety of renal diseases, such as glomerulonephritis, acute or progressive renal failure, or tubulointerstitial nephritis (Ichikawa and Kiyama et al.,1994). The capable of enhancing generation of ROS via stimulation of NADH/ NADPH-dependent oxidases in endothelial cells, smooth muscle cells, juxtaglomerular cells, and mesangial cells (Galle *et al*., 1995).

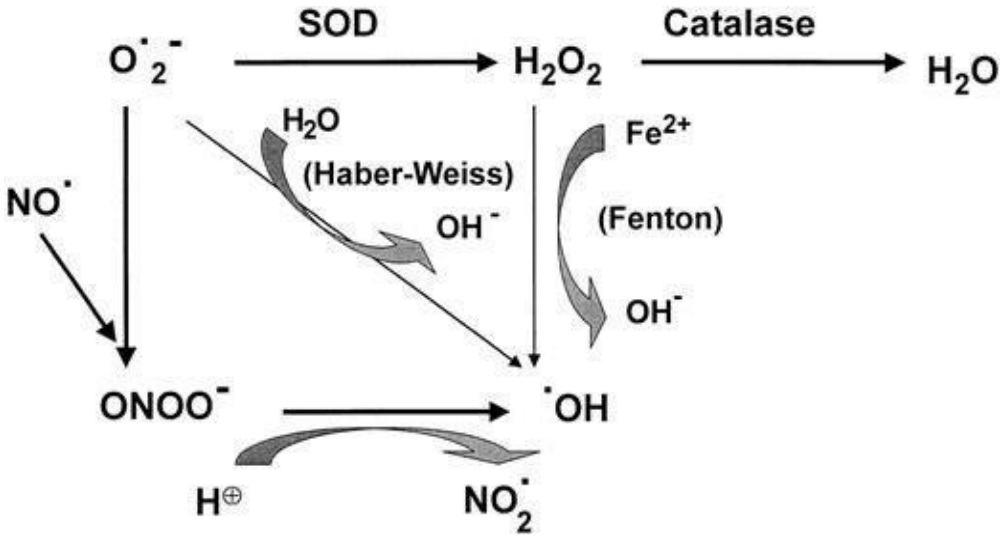


Figure. (2-1) Formation of reactive oxygen species (Klemm *et al*.,2001).

The antioxidant studies in uremia as mentioned above, studies using antioxidant treatment to prevent vascular and other diseases revealed equivocal effects in non-renal patients (Klemm, 2001and Kushi,1996). Since renal patients live under particularly pro-oxidative conditions(Galle,2001).question arises whether there may be particular benefit of antioxidant treatment for this subset of patients. Unfortunately, only a few antioxidant intervention studies with clinical endpoints have been published referring to renal patients.

2.9. Spirulina powder compounds

Spirulina are ubiquitous, spirally coiled or filamentous prokaryotic cyanobacteria and possess significant morphological similarity figures (2-2) and (2-2) (Henrikson, 2009). The main characteristics of Spirulina are the loosely coiled trichomes of varying width with cross-walls, which are visible in light microscopy These related strains are differentiated traditionally based on their morphology helix type, distribution of pores in the cell wall, visibility of septa under light microscopy, diameter and fragmentation type of trichomes

(Sili, and Vonshak 2012). The cyanobacterium Spirulina platensis as a potential source of proteins and pharmaceuticals is commercially cultivated. Phytoplankton comprises organisms such as diatoms, dinoflagellates, green and yellow-brown flagellates, and blue-green algae (Matos, 2019).

Figure (2-2) Spirulina in natural shape of Spirulina (Henrikson; 2009) .

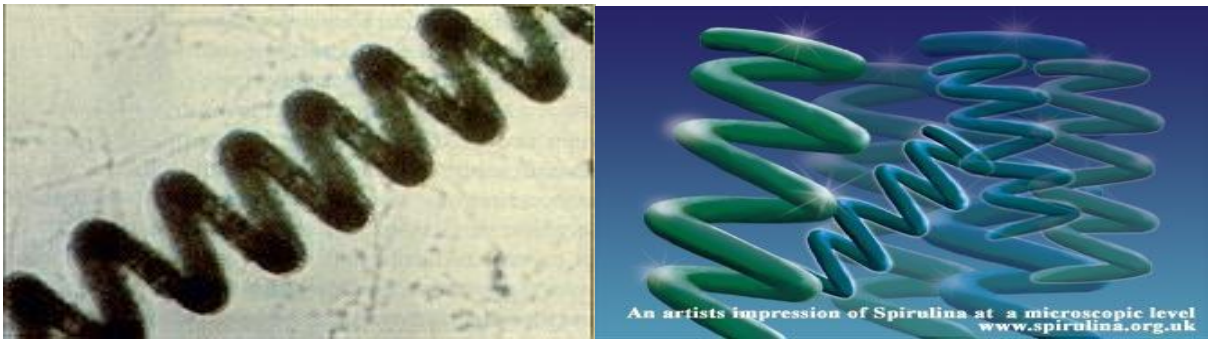


Figure (2-3) Spiral shaped of Spirulina (Henrikson; 2009).

As photosynthetic organisms, these groups play a key role in the productivity of oceans and constitute the basis of the marine food chain. Among several alga genera, Spirulina suspension and Chlorella deserve special attention due to their importance as human food and there in *vitro* and/or in *vivo* antioxidant potential.These algae can be extensively grown to obtain a protein rich material of alimentary use such as (foodstuff for diet complementation) or industrial use as (blue pigments, emulsifiers, thickening

and gelling agent) (Habib et al., 2008). The chemical composition of Spirulina indicates that it has a high nutritional value due to a wide range of essential nutrients, such as vitamins, minerals and proteins (Dillon and Phan 1993). Spirulina could increase carotenoid content and pigment (Lu *et al*.,2002). Spirulina suspension is one of the cyanobacterium more frequently found in tropical and salty waters with alkaline pH. Its growth rate in culture is higher than those observed for any other vegetal species and it is comparable to yeasts and bacteria (Richmond, 1988). Spirulina's life cycle allows that its biomass doubles each.

2.9.1. History of spirulina

Spirulina suspension was initially classified in the plant kingdom because of its richness in plant pigments as well as its ability of photosynthesis. It was later placed in the bacteria kingdom based on new understanding on its genetics, physiology and biochemical properties (Vonshak, *et al*.,1996). Spirulina suspension naturally grows in high-salt alkaline water reservoirs in subtropical and tropical areas including America, Mexico, Asian and Central Africa (Ismail *et al* .,2015). The nutritional value of *Spirulina* is well recognized with its unusual high protein content (60–70% by dry weight) and its richness in vitamins, minerals, essential fatty acids and other nutrients (Ismail *et al*.,2015;Alam *et al* .,2013). Recent studies suggest that *Spirulina,* a unicellular blue-greenalga may have a variety of health benefits and therapeutic properties and is also capable of acting as an antioxidant and anti-inflammatory agent (Asghari *et al.,* 2016). Spirulina is also used for health food, feed and forthe biochemical products since 1980s. In fact, spirulina is the most concentrated and nutritious whole food known to science, moreover Spirulina has got no side effects and is non-toxic in nature

(Alam et al .,2013). Spirulina suspension is free-floating filamentous microalgae growing in alkaline water bodies. As early as over 400 years ago, *Spirulina* was eaten as food by the Mayas, Toltec's and Kanembu in Mexico during the Aztec civilization. Spirulina is a well-known source of valuable food supplements, such as proteins, vitamins, amino acids, minerals, fatty acids, etc. It is widely used in human and animal nutrition, as well as in the cosmetic industry. Both in vivo and in vitro trials have shown effective and promising results in the treatment of certain cancers and allergies, anemia, hepatotoxicity, viral infection, vascular diseases, radiation protection, and obesity. The antioxidant activities of spirulina were demonstrated in a large number of preclinical study. Antioxidants in preventing many human diseases. Moreover, of this study spirulina can be used as a source of antioxidants. Analysis Analysis of the nutritional properties of spirulina showed first and foremost an exceptionally high protein content, of the order of 60–70 percent of its dry weight; it also showed the excellent quality of its proteins (balanced essential amino acid content). This first data was enough to launch many research projects for industrial purposes in the 1970s, because micro-organisms (yeast, chlorella, spirulina, some bacteria and moulds) seemed at that time to be the most direct route to inexpensive proteins – the iconic "single cell proteins. (Habib.,*et al*.,2008).

2.9.2. Spirulina as a feed supplement for humans

Spirulina is an alga containing avaluable combination of substances, including those practically absent in common food. Energetic value of 10 g dried Spirulina powder –29 kcal: fat –0.7 g, protein –5.7 g, carbohydrate –2.4 g, vitamin A –57 IU, vitamin B1 –0.24 mg (thiamine), B2(riboflavin) –0.38 mg, B3(nicotinamide) –1.3 mg, B6(pyridoxine) –0.4 mg, B9(folic acid) –

9.4 mg, vitamin C –1 mg, vitamin E –0.5 mg, natrium –104 mg, potassium –136 mg, calcium –12 mg, magnesium –20 mg, iron –2.8 mg, zinc –0.2 mg (Peciukoniene *et al*.,2001) .Dry Spirulina has 60-70 % protein, 10-20 % carbohydrate, 9-14 % fat, 4 % nucleic acid,4-6 % minerals. Spirulina is a source of β-carotene and iron(Kay;1991). Research shows it to stimulate the immune system, build both red and white blood cells and assist detoxification (Lisheng,1991). Spirulina also is a best balanced highly efficient dietary supplement, which satisfies the demands of all systems of the organism and, what is most important, improves the condition of its immune system and is a source of easily available iron(Gleeson and Bishop 2000, Gleeson 2001). The Spirulina enhances the haemopoietic system and increases resistance to hypoxia (Nieman, Pedersen 1999 ;Maranesi *et al*., 1984). productivity an order of magnitude Spirulina has a high protein and vitamin content. 20g Dried Spirulina provides the full RDA of vitamin B12, 70% RDA for thiamine, 50% RDA for riboflavin, and 12 % for niacin. Other favourable nutritional attributes of Spirulina include the high content of p-carotene(provitamin A) and essential unsaturated fatty acids (Richmond, 1988). Aside from the nutritional properties of Spirulina, reports have also been made on the therapeutic properties associated with this cyanobacterium (Richmond, 1988). Clinical trials have shown that spirulina can serve as a supplementary

cure for many diseases. As well as improving immunological function Spirulina also is used for health food, feed and for the biochemical products since 1980s (Richmond, 1988).

Immune system for human the Academy of Chinese Military Medical Sciences showed that spirulina could effectively improve the survival rate of

mice after exposure to a lethal dose of radiation, prolong their survival time, and improve their immunity and activity of superoxide dismutase (SOD). Some hospitals in Kunming city, Yunan Province, have adopted spirulina as an auxiliary medicine, which proved to be effective in lowering blood lipid, combating fatigue and increasing the level of immunoglobulin A (IgA) and immunoglobulin M (IgM). Phycocyanin of *Spirulina platensis* inhibits the growth of human leukemia K562 cells when supplemented with diet (Liu *et al*., 2000).

supplement Nutritional Spirulina is rich in high quality protein, vitamins, minerals and many biologically active substances (Holman , Malau 2013). Its cell wall consists of polysaccharide which has a digestibility of 86 percent, and could be easily absorbed by the human body. There are different categories of spirulina food where pills and capsules made from dry spirulina are important. The Wuhan Botanical Institute China has collaborated investigating the effect of oral intake of spirulina pill on the physical status of athletes (Li, Qi 1997). The results showed that female athletes showing an increase in their haemochrome level, whereas the male athletes did not show any apparent increase after taking 10 g spirulina pills per day for four weeks. The lung capacity of juvenile weight-lifting and *Jujutsu* athletes was improved. The spirulina pill had no effect on blood pressure. In Vietnam, first culture of spirulina was conducted in 1980s (Nguyen *et al*., 1980). Mass culture of spirulina was started in 1990s (Kim;1990). *Spirulina platensis* powder is used as a health food tablet under the brand name “Linavina” and “Pirulamin” in Vietnam. Another canned product named as “Lactogil” is used to enhance milk secretion in mothers showing a decrease in lactation. Good results have been obtained by treating children suffering from serious

malnutrition diseases with spirulina powder at Thuanhai Hospital, Viet Nam. Spirulina contains high concentrations of essential polyunsaturated fats (PUFAs). Spirulina platensis contains up to 2 percent of dry biomass of γ-linolenic acid (GLA), which is synthesized through direct desaturation of linoleic acid. Numerous studies showed that GLA and subsequent PGE1 deficiency may figure in many degenerative diseases. The few known sources of GLA include human milk and spirulina, and oil extracts of the evening primrose plant, blackcurrant and borage seeds; 10 g of spirulina provide over 100 mg of GLA (more than two capsules of evening primrose oil) which can help cure arthritis, heart disease, obesity and zinc deficiency (Henrikson; 1989). Furthermore, a study conducted by the Department of Internal Medicine of Tokai University, Japan, on 30 male employees with high cholesterol, mild hypertension and hyperlipidemia concluded that spirulina lowered serum cholesterol, triglyceride and LDL. Group A consumed 4.20 g of spirulina daily for eight weeks, and total cholesterol dropped within four weeks from 244 to 233 mg. Group B consumed spirulina for four weeks, then stopped; serum cholesterol decreased, but then returned to the initial level. Food source when the algal cells or filaments of spirulina are transformed into powder it can provide the basis for a variety of food products, such as soups, sauces, pasta, snack foods, instant drinks and other recipes. Attempts have been made by Proteus, a marketing company mainly associated with Earthrise Farms in the United States of America, to incorporate spirulina into a variety of food products such as granola bars and various kinds of pasta (Vonshak, 1990). Spirulina powder is also an ingredient of an orange-flavoured chewable wafer and other types of candy, of protein flours (10 percent spirulina added to soybean or to milk-egg powders), and of Pastalina, a green soy-whole wheat noodle. The preparation of fermented foods such as cheese, yogurt and tofu,

offered many new possibilities to the use of spirulina. Furthermore, extraction methods could provide a decoloured spirulina powder (yellow-white) which is odourless and tasteless, and thus suitable for widespread use.

2.9.3.1. Spirulina chemical composition

The basic biochemical composition of spirulina can be summarized as follows :

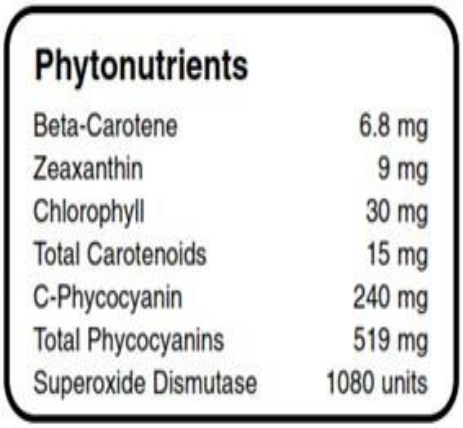
1. Protein: Spirulina contains unusually high amounts of protein, between 55 and 70 percent of dry weight (Phang *et al*., 2000). It is a complete protein, containing all essential amino acids, though with reduced amounts of methionine, cystine, and lysine, as compared to standard proteins such as that from meat, eggs or milk, however superior to all standard plant protein, such as that from legumes.

2.Essential fatty acids: Spirulina has a high amount of polyunsaturated fatty acids (PUFAs), 1.5–2.0 percent of 5–6 percent total lipid. In particular spirulina is rich in γ-linolenic acid (36 percent of total PUFAs), and also provides γ-linolenic acid (ALA), linoleic acid (LA, 36 percent of total), stearidonic acid (SDA), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and arachidonic acid (AA) (Phang *et al*., 2000).

3. Vitamins: Spirulina contains vitamin B1 (thiamine), B2 (riboflavin), B3 (nicotinamide), B6 (pyridoxine), B9 (folic acid), B12 (cyanocobalamin), vitamin C, vitamin D and vitamin E(Phang *et al*., 2000).

4. Minerals: Spirulina is a rich source of potassium, and also contains calcium, chromium, copper, iron, magnesium, manganese, phosphorus, selenium, sodium and zinc.

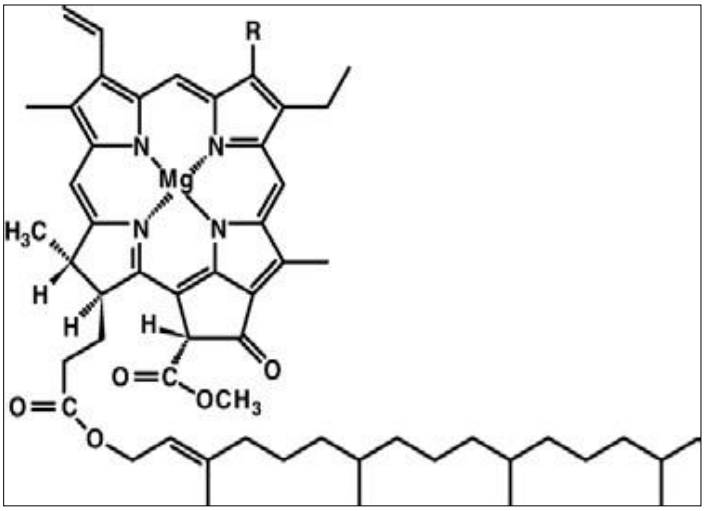
5. Photosynthetic pigments: Spirulina contains many pigments including chlorophyll a, xanthophyll, betacarotene, echinenone, myxoxanthophyll, zeaxanthin, canthaxanthin, diatoxanthin, 3-hydroxyechinenone, beta-cryptoxanthin, oscillaxanthin, plus the phycobiliproteins c-phycocyanin and allophycocyanin. *Spirulina* is a complete food resource of Chlorophyll, Phycocyanin, & Carotenoids. It is also has an application as a natural dye in food industry, cosmetic and pharmaceutical industry (Hosseiniand Mohamadamini,20015).



Figurer (2.4). Contain of pigment in one-gram *Spirulina* (Ferruzzi , Blakeslee,2007)

2.9.3.1.1. Chlorophyll

Chlorophyll is an essential compound in many everyday products. It is used not only as an additive in pharmaceutical and cosmetic products but also as a natural food coloring agent. Additionally, it has antioxidant and antimutagenic properties (Fig.2- 4) (Ferruzzi , Blakeslee2007).



Figurer.(2.5). ***Chlorophyll of structure(Krinsky and Johhnson,2005).***

Chlorophyll has been found to accelerate wound healing by more than 25% in some studies. Since chlorophyll stimulates tissue growth; it prevents the advancement of bacteria and speeds up the wound healing process. Chlorophyll is similar in chemical structure to hemoglobin and, as such, is predicted to stimulate tissue growth in a similar fashion through the facilitation of a rapid carbon dioxide and oxygen interchange. Because of this property, chlorophyll is used not only in the treatment of ulcers and oral sepsis but also in proctology (Krinsky and Johhnson,2005). Chlorophyll derivatives such as pheophorbide *b* and pheophytin *b* have always been known as strong antioxidants. However, these derivatives exist in very low concentrations in fruits and vegetables. The most significant activity of chlorophyll derivatives in the prevention of cancer is the trapping of mutagens in the gastrointestinal tract. Furthermore, the ability of both natural and commercial chlorophyll derivatives to act as photosensitizers have enabled their utilization as effective agents in photodynamictherapy of cancer. in *vitro* and in *vivo* consistent with the prevention of cancer including antioxidant activity, antimutagenic

activity, modulation of xenobiotic metabolizing enzymes, and induction of apoptotic events in cancer cell lines (Krinsky and Johhnson,2005).

2.9.3.1.2. Carotenoids

Spirulina extracts containing carotenes and various carotenoids (Fig. 6) are frequently used as natural coloring materials (Moorhead et al .,1993). Carotenoids are vitally important antioxidants. Numerous studies have indicated that people whose diets contain a lot of foods rich in carotenoids lower their risk of developing various types of cancer (Khan *et al* .,2005).

2.9.3.2.Spirulina pharmaceutical compounds

1. Pharmaceutical compounds containing Spirulina as the active ingredient induce accelerated cicatrization of wounds. Also spirulina and it's enzymatic hydrolysates promote skin metabolism, and prevents the formation of scar tissue and iodine present in Spirulina is the same type as that found in thyroid glands, and feeding with Spirulina has been found to result in growth stimulation. Furthermore Phycocyanin is an accessory blue pigment in spirulina, the concentration of which is controlled by the prevailing lighting conditions. When given orally to laboratory mice, phycocyanin resulted in a significant decrease in death when the mice were exposed to liver tumour cells. It is thought that phycocyanin generally stimulates the immune system, which may explain the higher lymphocyte activity found in the experimental group (Richmond,1988). In additional the intake of p-carotene (provitamin A) has been linked to a reduction in cancer risks. The high p-carotene level in spirulina suggests that it too may decrease certain cancer risks when ingested in appropriate amounts. Spirulina is a concentrated source of gamma-linolenic acid (GLA) which is a precursor of prostaglandin EI (PGE). PGE is involved

in many essential tasks in the body, including the regulation of blood pressure, cholesterol synthesis, inflammation and cell proliferation. Studies have shown that GLA (and subsequently PGE) may aid in the combatting of arthritis, heart disease, obesity and zinc deficiency. GLA deficiency has also been linked to alcoholism, manic depression. spirulina uses for antioxidant that have material working antioxidant(Richmond, 1988).

2.9.3.3 .Spirulina metabolism

Spirulina platensisafilamentousnon-N_2-fixing cyanobacterium, is promising for use as a biomass feedstock because of its capability to accumulate a large amount of glycogen, which is an excellent feedstock for biofuel production (Aikawa *et al*.,2013)(Ueno *et al*.,1998) . Under optimal light intensity and nitrate concentration, the intracellular glycogen content was reported to be up to 70% of the dry cell weight ina platensis (Aikawa *et al*.,2012 and Aoyama *et al*.,1996). Moreover, A. platensis has been produced as a superior nutrient because of its high content of protein and carotenes (Vonshak *et al*.,2002). and it is the most industrially cultivated microalgal species (Pulz *et al.,* 2004). Its superior characteristics such as high pH tolerance and high salt tolerance (Vonshak *et al.*,2002).could prevent contamination by other organisms in outdoor cultivation. Metabolic simulation using a genome-scale metabolic model has been widely used for rational metabolic design for the improvement of target molecule production (Tokuyama *et al*., 2014and Lee *et al.,* 2007). A genome-scale metabolic model is an in silico metabolic model that includes most of the metabolic reactions and metabolites of the target strain (Feist *et al.*,2008). Flux balance analysis (FBA) enables the simulation of the metabolic flux distribution in the whole metabolic reactions of the metabolic model with the following two

assumptions: steady-state metabolism and maximization of cell growth (Orth *et al.*,2010). This method can be applied to analyze the effects of gene manipulation and culture conditions on the metabolic flux distribution, and various studies have reported successful improvement of target production (Tokuyama *et al*., 2014 and Lee *et al*.,2007). In cyanobacteria, several genome-scale metabolic models have been constructed, such as those for Synechocystis sp.PCC 6803 (Yoshikawa Ket al.,2010), Synechococcus sp. PCC 7002 (Vu *et al.*,2013)and Cyanothece sp. ATCC 51142 (Segrè *et al*., 2012). thus providing a detailed understanding of cyanobacterial metabolism. In the case of A. platensis, two small metabolic models were constructed that include 22 metabolic reactions (Meechai et al., 2004).and 121 metabolic reactions(Cogne *et al.*,2003).Agenome-scale metabolic model of A. platensis C1 (PCC9438) including 875 reactions was constructed, and metabolic phenotypes and essential genes under various culture conditions, such as autotrophic and mixotrophic conditions, were analyzed(Klanchui *et al.*,2012). However, metabolic simulations to identify the candidate genes to be manipulated for improvement of target production have not been conducted. Spirulina platensis NIES-39 and developed metabolic engineering strategies to improve production of valuable materials, such as glycogen and ethanol. A. platensis NIES-39 is a well-studied A. platensis strain, as the whole genome sequence(Fujisawa *et al*.,2010), glycogen production (Aikawa *et al*.,2013), and metabolome (Hasunuma *et al*.,2013).Have been analyzed. After construction of the metabolic model, simulation results using the constructed model were evaluated by comparison with the experimental results with regard to growth rates and growth capabilities on various organic substrates. Moreover,

metabolic engineering strategies to improve autotrophic production of glycogen and ethanol were developed using the metabolic simulation.

2.9.3.4. Spirulina effects on kidney and liver functions

Spirulina study have found effective effect on kidney function through the exploration of kidney failure either by induced or by gentamicin or by cisplatin or by diclofenac or by adenine after the induction of kidney failure has been noticed and there is a clearer rise in urea and creatinine levels as well as it has been observed Almost a loss of kidney function, and after were given spirulina, when a clear and significant observation was made in urea and also creatinine, they were improved, as well as urea absorption, and the experiment was carried out on rats after renal failure was introduced and samples were taken, and after spirulina was given, selected samples were taken. A comparison between them and notice a reduction in urea and creatinine and noting the work of kidneys at a very large rate (Kuhad *et al.*,2006). After given spirulina suspension improved the enzymes liver.

2.9.3. 5. Spirulina effect on lipid profile

Spirulina effect on Many animal and human studies that have repeatedly reported ability to the decease lipid in animal and human (Ismail, Hossain;2015; Park and Kim, 2006). Reported that the plasma levels of triglyceride, total cholesterol, and LDL-cholesterol in Korean elderly people were significantly decreased after spirulina supplementation. the spirulina increase and balance the HDL. Spirulina effect on the ALT and AST that appearance effect after intake the spirulina decrease in levels. Spirulina enhance from function liver and spirulina effect on lipid. The A significant decrease was observed after the treatment by spirulina for 30days the levels

of CHO, TRI, and LDL . The serum level of HDL was increased significantly as compared to the control rats (Sheekh and Hamad *et al.*,2014). The spirulina effect on liver fibrosis is a chronic liver disease that will further develop to cirrhosis if severe damage continues to form. A potential treatment for liver attributed to inhibit activated hepatic stellate cell (HSC) proliferation and, subsequently, to induce HSC apoptosis. It has been reported that antioxidants are able to inhibit the proliferation of HSCs. In this study, the aqueous extract of Spirulina was chosen as the source of antioxidant to investigate the inhibitory effect on the proliferation of HSC (Farooq *et al.*, 2006).

2.9.3.6. Antioxidants effect of spirulina

Effect of spirulina intervention at (500 × 2) mg, (500 × 4) mg doses on the serum content of oxidants, antioxidants, cholesterol, TG, and HDL and on the activity of burst enzymes in COPD patients. Spirulina is a potent mixture of antioxidants and most of Spirulina's health benefits are associated (Ismail,2015). with its antioxidant pigments. These are carotenoids (mixture of carotenes and xanthophylls), chlorophyll and the unique blue pigment phycocyanin. A little of its usage in medicine has been established by numerous studies still more of its hidden properties are yet to be explored. Some of its properties such as anti oxidant, anti inflammatory, anti cancer, anti aging (prevents cell death), Drug delivery system.(Choopani;2016). An external food source of GLA such as spirulina, therefore, plays a crucial role in regulating the cholesterol levels (Ismail,2015). Regulation of a variety of basic biochemical functions in the body including the regulation of cholesterol synthesis. Requires the prostaglandin PGE1 (Ismail; 2015).Spirulina suspension plays an important role in increasing the following enzymes, namely, SOD, and GSH, they were increased significantly after intake of

spirulina for 30 days (Ismail;2015). The spirulina that have specific material act on antioxidant the C-phycocyanin Phycocyanin is one of the key ingredients that make Spirulina such a wonderful Super food, and a vital difference between Spirulina and other green foods like chlorella, wheat grass and barley (Subhashini, *et al* .,2005). The Japanese have found that phycocyanin protects the liver and the kidneys during detoxification, as well as activating the immune system. *Spirulina* contains up to 2,000 IU/g dry weight of β-carotene (Krinsky and Johhnson,2005)..Eating foods rich in antioxidants such as carotenoids, phycocyanin, superoxide dismutase and vitamins C and E is another great way to help prevent cancer (Devasagayam *et al* .,2010).

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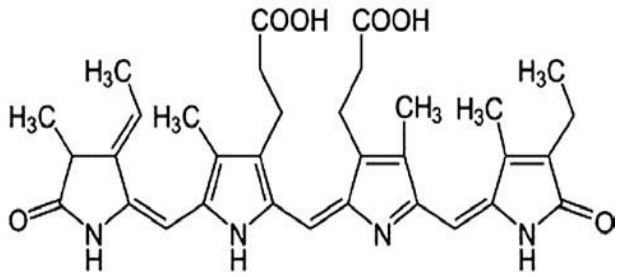

Figure (2.6) Chemical structure of C-phycocyanin (Devasagayam *et al* .,2010).

2.9.3.7. Spirulina effects on hematopoietic

Studies have proven that spirulina has a significant and important role on the blood components in terms of maintaining blood components from diseases and also treating them in the event of a defect in the blood components. During the experiment, spirulina was given to people after taking blood samples,

which was considered a measure of the experiment before and after spirulina. Spirulina suspension was given to people twice a day, and after two weeks of giving the dose, blood samples were taken. It was found that spirulina had a major effect on the blood components on red blood cells. It had a significant effect, where an increase in red blood cells was observed compared to the scale. An abnormality of the number of white blood cells, as well as improvement in platelet count, as well as it was observed that hemoclopene became an increase, and from this we come out that people who were given spirulina had an increase in oxygen transport, as it was observed improve and increase the immune system, as well as preserving forms of blood components and after that Spirulina continued to be administered and results subsequently noted because the spirulina contain vitamins that are very important to the haemopoietic and they are vitamins(folic acid, thiamine, pyridoxine, iron, B12 and protein(Milasius *et al.*,2009).

Chapter three
Materials and Methods

3.1. Materials

3.1.1. The experiment protocol

The study was performed in the animal's house of College of University of Karbala at Veterinary Medicine.

Thirty two adults' male's rats used in the present study and divided into four groups as following:-

1. Frist group animal of this group were received 0.25 ml of dimethyl sulfoxide (DMSO) orally.

2. Second group animals of this group were injection with adenine intraperitonealy (100mg/kg) for 30 days to induce renal failure.

3. Third group animal of this group were co-administrated adenine (100mg/kg) intraperitoneal and spirulina suspension (750 mg/ kg) orally for 30 days.

4. Fourth group rats in this group were treated with spirulina suspension (750 mg /kg) orally for 30 days.

3.1.2 .Chemicals and instruments

3.1.2.1. All laboratory suppliers and chemicals were used in this study are listed in table (3-1).

No.	Chemicals	Origin and Company
1	Adenine powder	Sigma Aldrich Company(USA).
2	ALT(GPT) Colorimetric. Kit	SPECTRUMcompany, Germany
3	AST(GOT) Colorimetric Kit	SPECTRUMcompany, Germany
4	Chloroform	Noorbrok, England
5	Creatinine Colorimetric Kit	SPECTRUM company, Egypt.
6	Dimethylsulfoxide(DMSO)	LOBA Chemie
7	Ethanol	Merk, Germany
8	Formalin10%	TEDIA company. USA
9	Glutathione(GSH)	Elabscience Biotechnology/ China
10	Kidney injury molecule 1 ELISA Kit	Elabscience Biotechnology/ South korea
11	Paraffin wax	Merck, Germany
12	Superoxide dismutase	Elabscience Biotechnology/ Germany
13	Lipid profile	Germany
14	Urea kit	SPECTRUcompany, Germany
15	Xylole	Scharlau, Spain
16	Nitric oxide	Germany

3.1.2.2. The instrument used in this study with their suppliers and sources are shown in table (3-2)

No.	Instruments	Suppliers and sours
1	Analytical Sensitive Balance	Sartorius / Germany
2	Balance for animals	Shimadu company - Japan
3	Centrifuge	Hettich Roto fix11/ Japan
4	Digital camera	ToupCam/ China
5	Dissection set	China
6	EDTA tube	Germany
7	Electric grinder	China
8	ELISA reader	bioKIT/ south korea
9	ELISA washer	bioKIT/ south korea
10	Eppendorf tubes	China
11	Freezer	Hitachi / Japan
12	Gel tubes	Germany
13	Incubator	BINDER / Germany
14	Insulin Syringes	Italy
15	Light microscope	Lieca / China
17	Micropipette 100-1000 µl	Germany
18	Micropipette 1-100 µl	Germany
19	Mince machine	HITACHI/Japan
20	Optical microscope with a Tablet PC	OPTICA/ Italy
21	Rotary Macrotome	Germany
22	Auto heamo analyzer	GENEX X CHEM-S1/ USA
23	Spectrophotometer	Sesil, England
24	Sterile Syringes (1, 3 and 5 ml)	China

25	Test tube	China

3.2 Methods

3.2.1. Prepersion of adenine and spirulina suspension

Adenine was obtained from Sigma Aldrich Company(USA)and dissolved in DMSO 100mg/1ml.

3.2.2. Spirulina (*Arthrosporia sp*) suspension.

Company of spirulina turkey (ASSOS). Spirulina powder was suspended with DMSO (750mg/1ml) (Yener *et al*.,2013).

3.3. Experimental animals

The rats remained in the animal's houses for adaptation to the laboratory environment for two weeks before they were used.

Experiment was done by using 32 healthy adult male rats (Rattus norvegicus) weighting 200 - 250 gm obtained from the animal's house in College of Science, at Nahrain –University. The animals were placed in the animal's house of Collage of Veterinary Medicine, Karbala-University, with standard environment situations temperature (22-28 C°) and 12-hour light cycle dark.

3.4. Collection of blood and tissue sample:

At the end of experiment the male rats before being sacrificed were anaesthetized by putting them in a closed jar containing cottons sucked with chloroform, then. Puncture of heart was done by using a disposable syringe 10 ml and 6 ml of blood was gently drawn slowly. One ml of blood collected in heparinized tube for hematological parameters were measured as soon as possible. Then parts of blood place in test tube containing gel which leaves the serum at room temperature for 30 minutes and then is used for centrifugation at 3000 rpm to separate the concentration for 15 minutes and is placed in Eppendorf tubes which are kept in a freezers at-20 C°, after heart

puncture and blood collections, the abdominal cavity was opened and kidney and liver were excised then placed in formalin (10%) as fixatives for histological preparations

3.5. Parameters of study

1-Kidney function tests: serum urea, Creatinine and KIM

2-Liver function tests: AST and ALT

3-Antioxidant status in blood (GSH, SOD ,NO and MDA).

4-Hematolgical parameters (RBCs, WBCs, Hb, platelets and PCV).

5-Lipid profile (LDL, HDL, CHOL and TG).

3.6. Experimental design

Thirty two adult's male's rats was used in the presented study and divided into four groups as following.

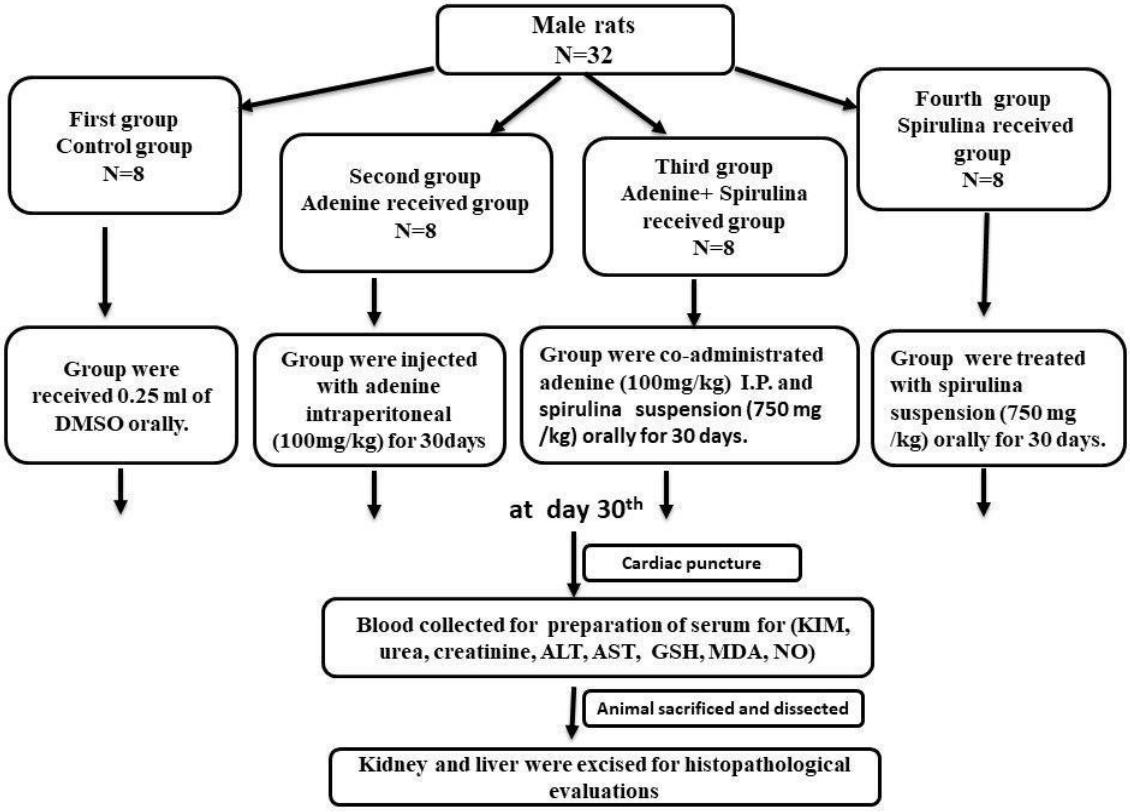

Figure (3-2): Represented experimental design.

3.7. Estimation Rat Elisa Kit Kidneys injury molecule-1(KIM-1) Appendix I

Purpose

The procedure was done according to the instructions of the manufacture of ELIZA Kit -Elabscience biotechnology/ china. Our Rat Kidneys injury molecules1 (KIM-1) ELISA kit is to assay Kim-1 levels in serum illustrated in Appendix **l.**

3.7.1. Serum urea estimation: Urea concentration in serum was determined by using a special kit (SPECTRUM- Urea Kit, Germany- IFUFCC40), by using device (Spectrophotometer Sesil, England) and procedure illustrated in appendix II

3.7.2. 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)

3.8. Estimation of serum lipid profile illustrated in Appendix III

Cholesterol concentration was measured by using Cormay cholesterol kit produced by PZ CORMAY S.A. company. oxidation and after enzymatic hydrolysis

, the cholesterol is determined in the presence of phenol and peroxidase,

4-aminoantipyrine and the hydrogen peroxide forming quinoneimine the indicator

(Fasce, 1982). Appendix IV

3.9. Estimation of Antioxidant Enzyme illustrated in Appendix IV

3.9.1. Estimation of serum superoxide dismutase (SOD) concentration

3.9.2. Estimation of serum malondialdehyde (MDA).

estimated Malondialdehyhe was by Thiobarbituric acid (TBA) assay method of (Muslih *et al* .,2002)

3.9.3. Estimation of serum NO synthase .

Endothelial NO synthase (ENOs) was used by Nitric Oxide Synthase 3, Endothelial ELISA Kit by Bioassay Technology Laboratory by method (Breddt and Snyder , 1994) ,

3.9.4. Estimation Glutathione (GSH).

Serum glutathione concentration has been measured by using the Ellmans reagent method previously used by (Alzamely *et al*.,2001) .

3.10. Hematological Parameters

The hematological parameters were done in Laboratory of Research and Studies / College of Veterinary Medicine / Central Research Laboratory University of Kerbala by using VET hematological auto analyzer (count 60) made in Genex company. This instrument used two reagents only (Dilute and Lyse) and Maintenance reagent (Probe cleanser only) and it has a picture mechanical inside with thermal paper . The hematological parameters estimated by the instrument were (RBC, WBC, Plat, PCV, Hb,)

3.10.1 Evaluate of histological changes Technique (E & H) stain illustrated in Appendix Vl.

The kidneys, liver 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.

3.11. Statistical analysis

The data were analyzed by using one-way analysis of variance (ANOVA) and significant difference between groups was of the level ($P \leq 0,05$) according to least significant difference (LSD)(version 29,SPSS,in UAS 2010).

Chapters four

Results

4. Results

4.1 The Effect of spirulina suspension on kidney and liver function indices in male rats with treated adenine induction chronic renal failure (CRF)

4.1.1 Kidney injury molecule KIM-1 of concentration

KIM-1 level is very valuable biomarker in detect renal failure. Table (4-1) appearance significant increase in KIM -1 levels in group of rat treated with adenine for 30 days to induce chronic renal failure compare with control and spirulina treated groups. Spirulina suspension respired group three of the present experiment lead to reduced harmful effects of adenine significantly ($p \leq 0.05$) but KIM-1 level was still higher than that observed in control and spirulina alone groups.

4.1.2. Urea concentration

Levels of serum urea were significantly ($p \leq 0.05$) increased in adenine group in comparison with control group as appear in table (4-1). Rats that administered spirulina powder suspension co-administered adenine injection, showed significant ($p \leq 0.05$) decrease in urea concentration in compare with the group adenine but it's still significantly higher ($p \leq 0.05$) than that recorded in control and spirulina alone groups (table 4-1).

4.1.3. Creatinine concentration.

Analysis of variance revealed significantly increased (P≤ 0.05) in creatinine concentration in rat treated with adenine in comparison with control and other treated groups as shown in table (4-1). On the other hand, spirulina powder suspension caused significant decrease (P≤ 0.05) in creatinine levels when administered with adenine and alone but don't reach to its value recorded in control group.

4.1.4. Aspartate amino transferase concentration (AST)

Adenine group showed significant elevation (P≤0.05) in AST concentration compare with group control, while group that treated with adenine+ spirulina powder suspension showed significant decrease (P≤ 0.05) in AST activity in comparison with adenine group. spirulina suspension when administrated alone was no significant (P≤ 0.05) comparison with group control. (4-1).

4.1.5. Alanine Transferase concentration (ALT)

As in AST, ALT activity was shown significant increases(P≤ 0.05) in the adenine group compare other group control and with treated groups. While male rats received adenine plus spirulina powder suspension revealed significant redaction (P≤ 0.05) in ALT in compare with adenine group. ALT value in control group still lowest than that receded in third group but it was resemble to that registered in spirulina alone.

Results

Table (4-1) The Effect of spirulina powder on kidney and liver function parameters in male rats with induced chronic renal failure.

Parameters / Groups	KIM mg/dl	UEAR mg/dl	Creatinine mg/dl	AST u/ml	ALT u/ml
Contol	C 116.04 ± 1.998	C 21.33 ± 0.881	C 0.26 ± 0.043	C 95.16 ± 33.408	C 45.50 ± 2.77014
Adenine	|A 395.27 ± 18.78	A 71.16 ± 4.26	A 3.49 ± 0.20	A 325.83 ± 40.21	A 102.83 ± 13.961
Spirulina and adenine	B 175.86 ± 8,96	B 37.16 ± 4.57	B 1.81 ± 0.20	B 145.16 ± 51.077	B 68.33 ± 7.99
Spirulina	C 106.10 ± 1.99	C 26.50 ± 4.20	B 1.47 ± 0.09376	C 94.16 ± 46.70	C 54.16 ± 5.47
LSD	12.03	6.23	0.46	46.8	12.3

N=8

Different letters represent significant difference

($P \leq 0.05$) means ± SD

4.2 Effect of adenine and spirulina powder on some lipid profile parameters of male rats

Table (4-2) in which the Cholesterol, TG and LDL level were significant higher ($P \leq 0.05$). In the male rats group of adenine injected intraperitonealy with adenine in comparison with control and other treated groups. Spirulina powder suspension caused significant ($P \leq 0.05$) decline in TG Cholesterol and Low density Lipid level in the rats concurrently treated with adenine in compare with rats received adenine alone. In contrast HDL level significantly ($P \leq 0.05$) decreased male rats of adenine group in comparison with other studied groups and spirulina caused significant increased ($p \leq 0.05$) in HDL level when gavage alone and with adenine in comparison with the group of adenine.

Table (4-2) The Effect spirulina powder on biomarker of lipid profile in male rats with induced chronic renal failure.

Parameters / Groups	Chole (Mg/dl)	Triglycerides (Mg/dl)	LDL (Mg/dl)	HDL (Mg/dl)
Contol	C 115.16 ± 17.96	C 46.36 ± 3.59	B 110.06 ± 8.33	BC 31.16 ± 2.023
Adenine	A 217.16 ± 13.58	A 175.00 ± 11.80	A 198.16 ± 10.06	C 17.50 ± 2.5
Spirulina and adenine	B 171.66 ± 14.63	A 167.50 ± 12.15	B 107.50 ± 6.91	B 23.09 ± 2.89
Spirulina	C 138.33 ± 10.39	B 73.83 ± 4.96	C 68.00 ± 5.30	A 42.11 ± 2.023
LSD	24.52	17.37	15.41	10.08

N=8

Different letters represent significantly difference

($P \leq 0.05$) means ± SD

4.3. Effect of adenine and spirulina suspension on some serum antioxidant activity of male rats with adenine induced chronic renal failure.

4.3.1. Estimation Glutathione (GSH) concentration

Adenine group show significant decrease ($p \leq 0.05$) in kidney homogenate GSH activity in comparison with control and spirulina groups. Administration of spirulina powder suspension with adenine and alone showed increase GSH activity in comparative with the group of adenine and it became close to group control (table 4-3).

4.3.2. Estimation of Superoxide dismutase(SOD) concentration

SOD significantly decrease ($p \leq 0.05$) in adenine group in comparison with control group as shown in table (4-3). Spirulina powder + adenine group showed significant increase SOD count in comparative with the group of chronic renal failure and leads to elevated it but don't reach to the result shown in spirulina powder and control groups.

4.3.3. Estimation of serum NO Synthase.

Nitric oxide concentration was significantly increase ($p \leq 0.05$) in adenine group in comparison with control group as shown in table (4-3). Enhancement effect of spirulina powder was appeared when administered with adenine via decrease NO level in comparative with the group adenine alone and NO concentration in spirulina alone group was close to control group (table 4-3).

4.3.4. Estimation of Serum Malondialdehyde (MDA).

According to result of MDA which show same pattern of NO .It was significantly increase ($p \leq 0.05$) in adenine group in comparison with control and other treated groups as shown in table (4-3). Spirulina powder +adenine

group showed decrease MDA concentration in comparative with the group of adenine

Table (4-3) The Effect of spirulina suspension on serum Antioxidant activity of male rats with induced chronic renal failure.

Parameters / Groups	GSH Ng/ml	SOD Ng/ml	MAD µM/l	NO. µM/l
Contol	A 9.82 ± 1.13	A 41.43 ± 8.12	C 82.44 ± 12.21	C 170.15 ± 27.16
Adenine	C 6.27 ± 1.08	C 24.83 ± 4.53	A 118.61 ± 18.13	A 195.27 ± 11.92
Spirulina and adenine	B 8.19 ± 0.21	B 34.64 ± 3.68	B 100.23 ± 22.09	B 181.42 ± 13.12
Spirulina	A 10.16 ± 1.23	A 38.90 ± 4.51	C 79.86 ± 19.65	C 171.83 ± 10.21
LSD	1.21	4.11	13,74	9.18

N=8

Different letters represent significantly difference

(P≤ 0,05) means ± SD

4.4. Adenine and spirulina suspension effect of biomarker blood indices of male rat

The count of RBC, Hb and PCV means were showed a significantly decreased ($p \leq 0.05$) in group of adenine – induced renal failure in comparsion with control and other treated groups. Whereas, spirulina caused to reduce effects of adenine resulting in rise the means of above mentioned parameters to reach that of control group as appear in table (4-4). Platelet count showed significant decrease in adenine group when compared to group control and when injected by adenine+ spirulina powder group increase platelet in compared to the group adenine.

According to platelets count, adenine group. The result revealed presence significant decline ($p \leq 0.05$) in platelets count of rats administered adenine in comparison with control and spirulina groups

Table (4-4) in which, the WBC count was showed exerted significantly increases ($P \leq 0.05$)

In the group of male rats injected intraperitoneal with adenine in comparison with other of control and groups. In group treated with spirulina showed a significant decrease ($P \leq 0.05$) in WBCs value in comparison with control group.

Results

Table (4-4) the effect spirulina suspension on biomarker of blood in male rats with induction chronic renal failure.

parameter / Groups	Red blood cell $\times 10^6$cell/mm^3	hemoglobin g/dl	PCV %	platelets $\times 10^3$ cell/mm^3	WBC $\times 10^3$ cell/mm^3
Contol	AB 5.20 ± 0.147	AB 11.74 ± 0.987	A 42.1 ± 2.97	A 28.89 ± 3.17	C 2.42 ± 0.26
Adenine	C 2.91 ± 0.073	C 5.13 ± 0.742	B 32.40 ± 2.62	B 19.73 ± 2.61	A 11.22 ± 0.37
Spirulina and adenine	B 4.69 ± 0.105	B 9.89 ± 0.611	A 40.02 ± 1.38	A 25.70 ± 2.72	B 6.79 ± 0.78
Spirulina	A 6.42 ± 0.060	A 12.68 ± 0.385	A 43.28 ± 1.30	A 27.01 ± 5.81	C 3.40 ± 0.38
LSD	1.23	1.97	6.74	5.28	1.72

N=8

Differential number represent significant difference

($P \leq 0.05$) means ± SD

4.5. Histopathological changes evolution of included by spirulina suspension

4.5.1. Kidney

In the figures (4-1) and (4-2) showed the histological section of kidney of control group that showed the appearance normal architecture and absence fibrosis. Figure (4-3). The histopathology section obtained from the kidneys of adenine-treated rats, that causes presence of significant inflammation, hemorrhage and fibrosis. In the second group of male rats administrated by adenine stain (H&E) exhibited tubular necrosis swelling with necrotic material. Include dilatation of large number of tubules, mixed inflammatory cells infiltration of the interstitial focal tubular atrophy figure (4-4). The kidney of the group which treated with spirulina suspension and adenine powder improved in compared with other of the group kidney treated with adenine alone in stain (H&E).figures(4-5)and(4-6)

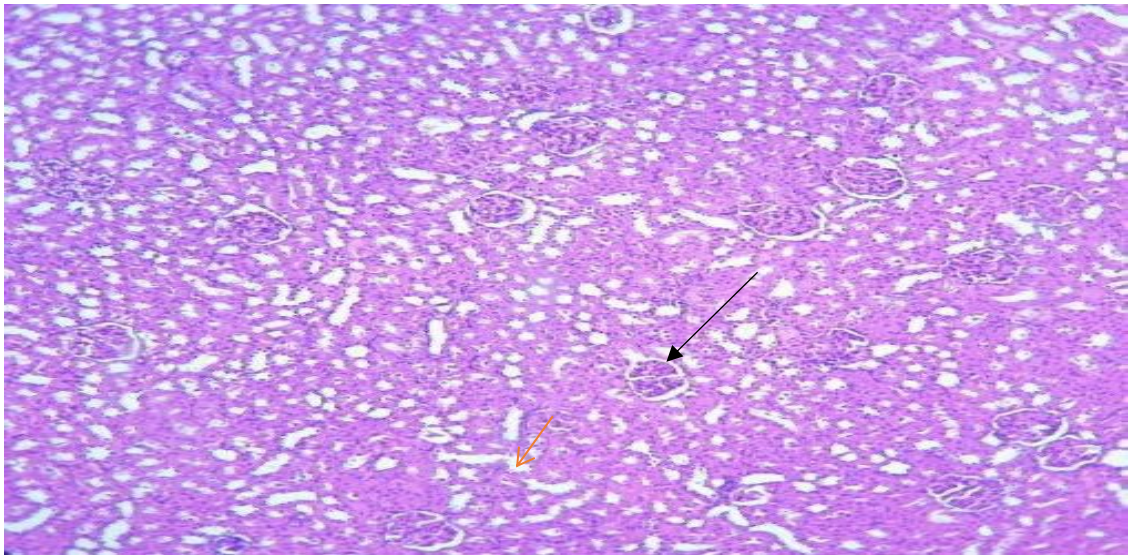

Figure (4-1) kidney section of control group male rats shows normal glomerulus (black arrow) and renal tubules (red arrow) (X100).

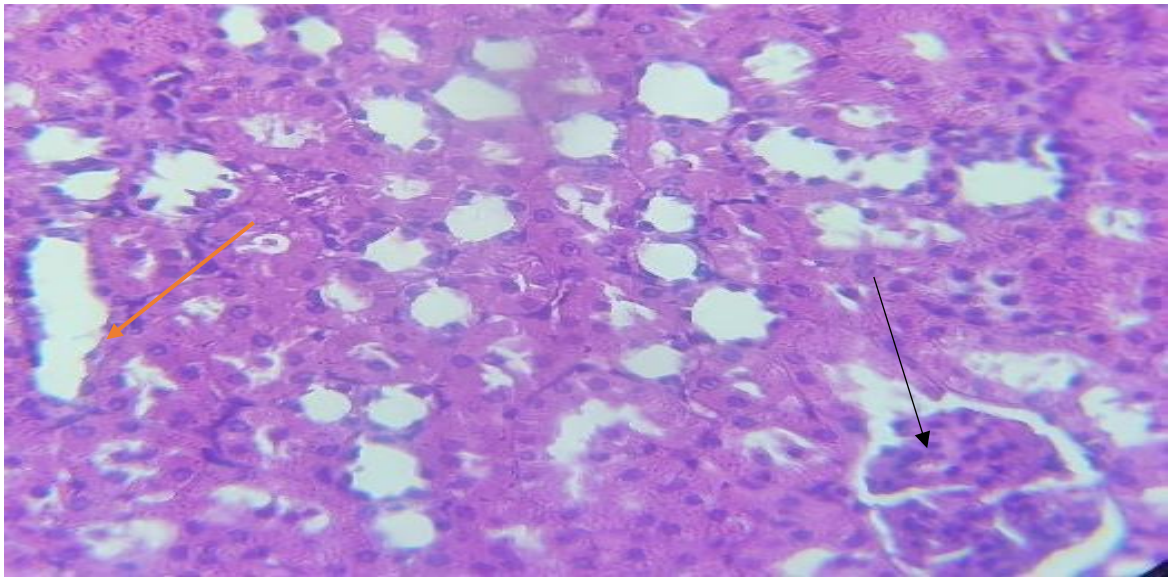

Figure (4-2) kidney section of group control male rats are shows normal glomerulus (black arrow) and renal tubules (red arrow**) (X400).**

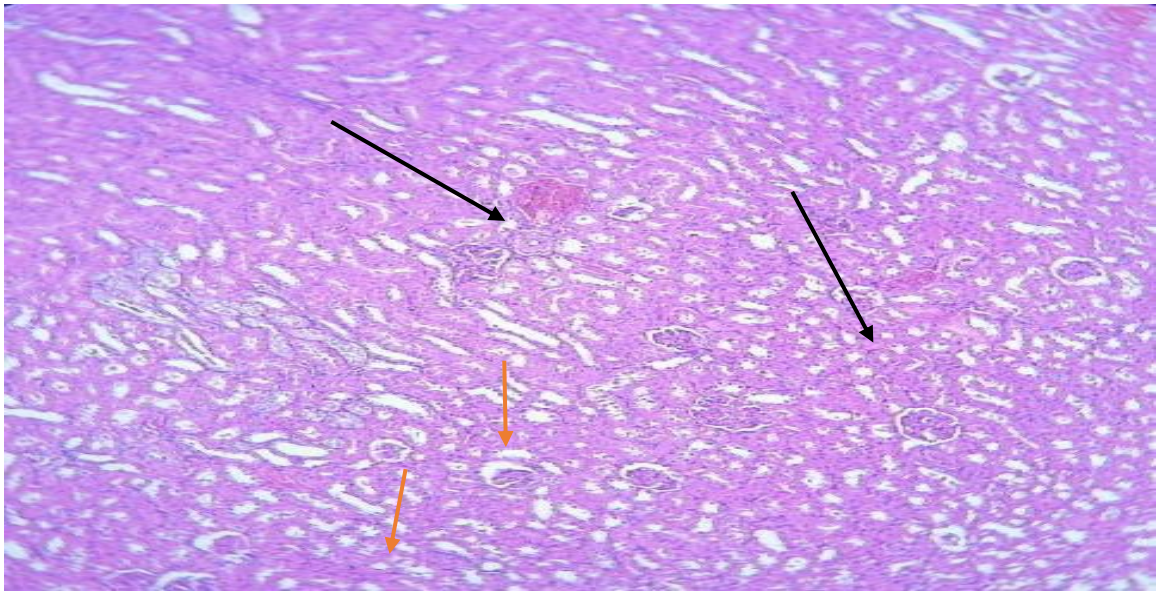

Figure (4-3) the kidney when injected by adenine group that are causes hemorrhage (black arrow) atrophy of capillary tubule (red arrow). Show stain (H&E). (X100)

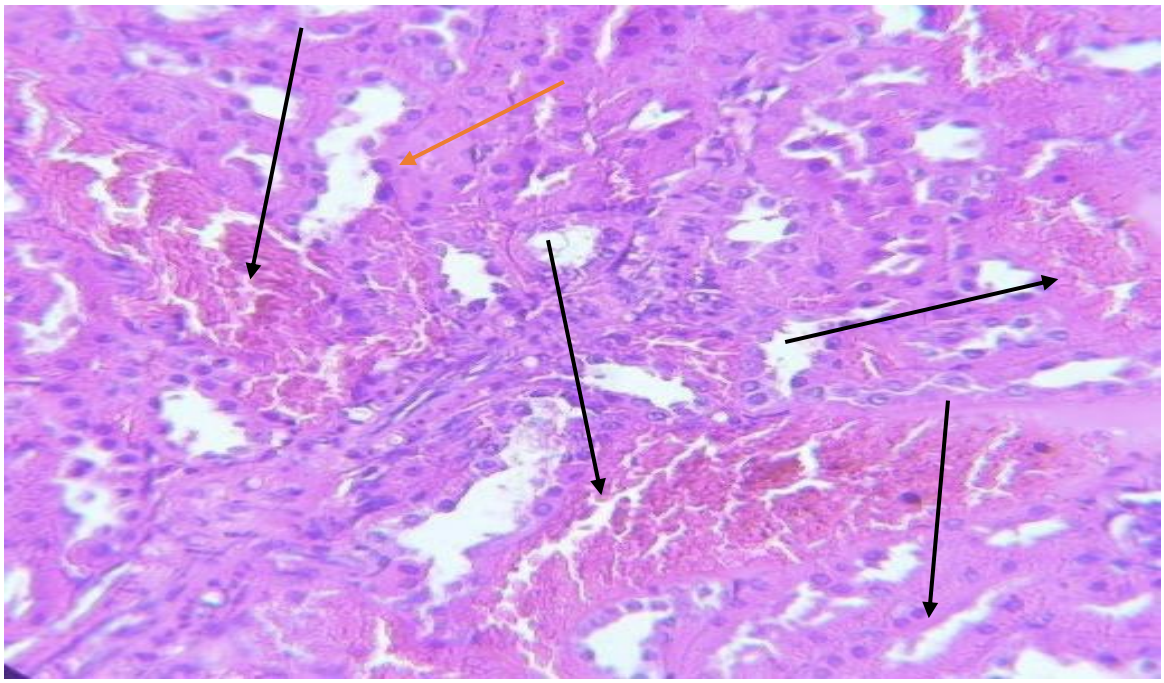

Figure (4-4) kidney showed significantly increase in change when treated male rats by adenine alone hemorrhage (black arrow) and dilatation of tubules lumen (red arrow). Showed stain (H&E). (X400).

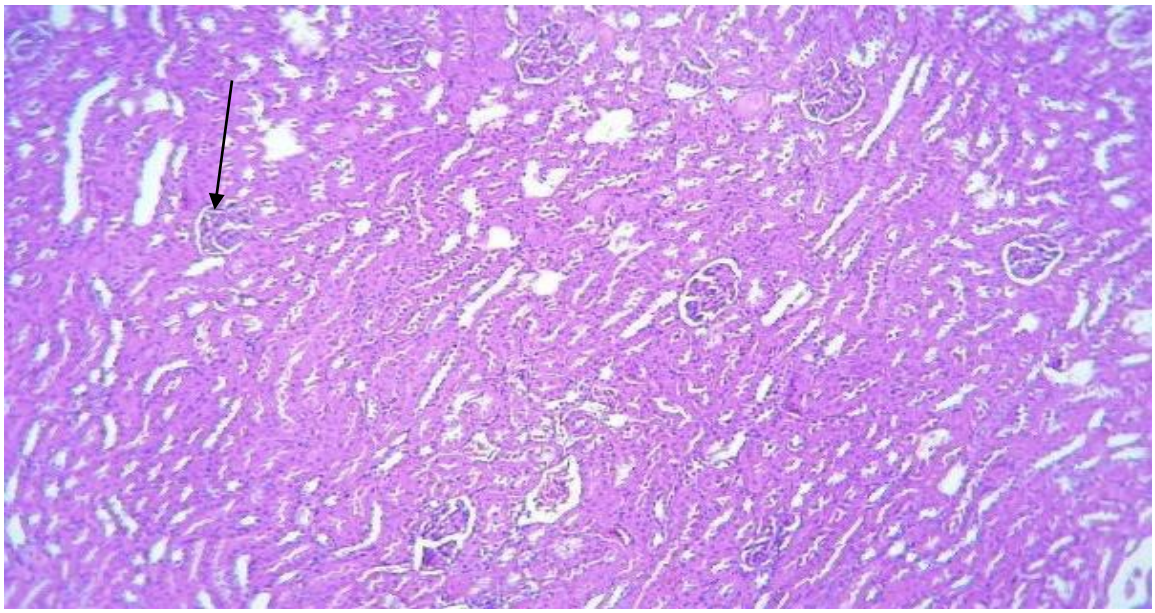

Figure (4-5), showed in Light micrograph of histological changes in kidney male rats when treated by adenine +spirulina suspension that enhancement to normal glomerular and showed black arrow stain (H&E).(X100).

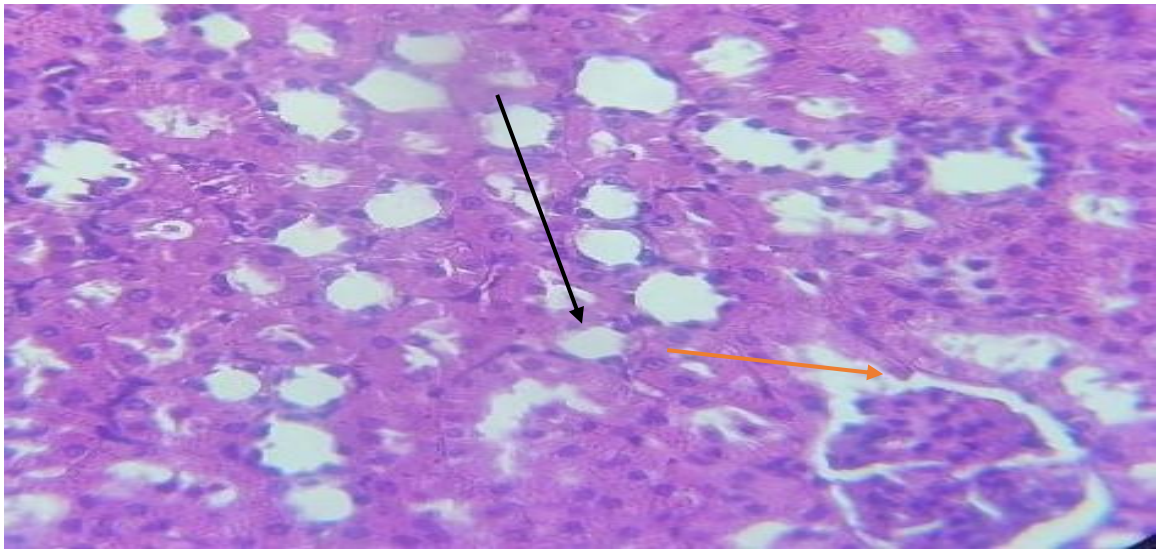

Figure (4-6), photomicrograph of kidney in male rats group treatment by adenine +spirulina suspension repair and return normal renal tubules (black arrow) and stop bleeding (red arrow) when comparison with group of adenine alone showed stain (H&E).(X400).

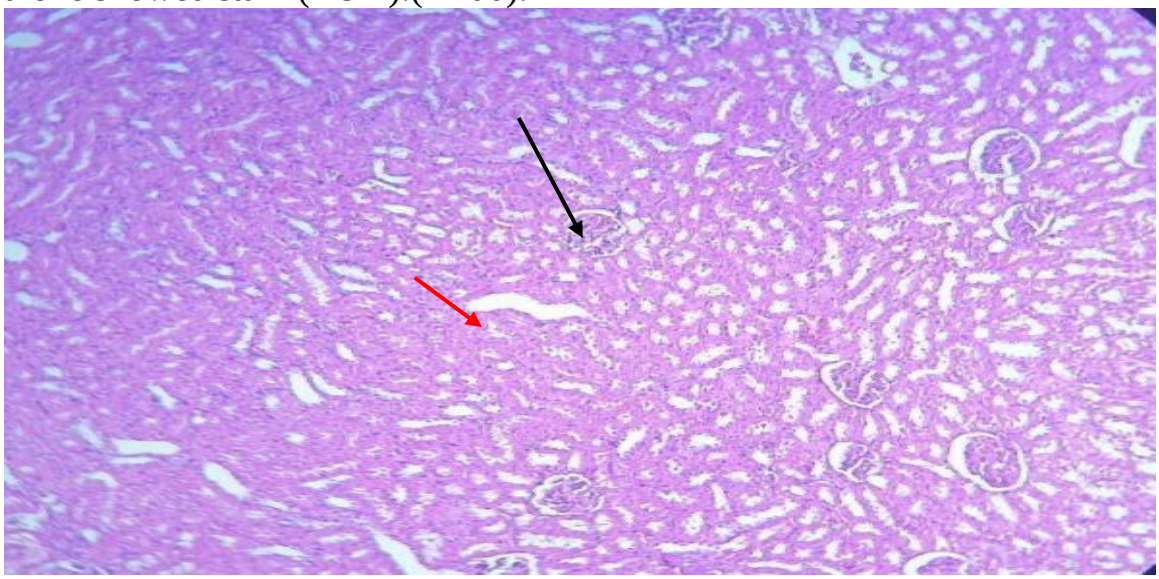

Figure (4-7) all most of architecture improved and still the cells of kidney normal when treated by spirulina suspension alone the male kidney of group spirulina suspension male rats are normal glomerulus (black arrow) and granular tubules (red arrow) (H&E).(X100).

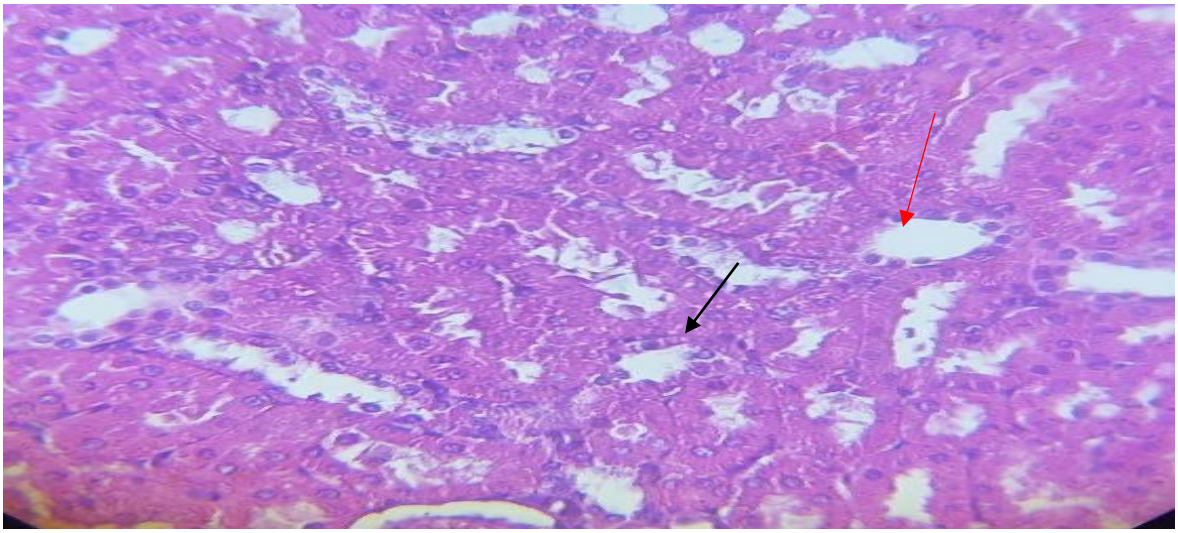

Figure (4-8) kidney section of group spirulina suspension male rats are normal glomerulus (black arrow) and tubules granular (red arrow) (X400).

4.5.2.Liver

Liver tissue from the control groups (figure 4-9,4-10) stained with (H&E) shows normal portal areas show either no inflammatory infiltrates and parenchymal tissue with no lobular inflammation. The liver tissue treated with adenine shows infiltration of the liver tissue with inflammatory cells (lobular inflammation), The portal areas show mild to moderate round cell inflammatory infiltrate (Figure 4-11,4-12). Histopathological sections in groups that induced chronic renal failure when treated with spirulina suspension powder + adenine powder (Figure 4-12,4-13). It has shown return normal architecture of center vein, normal hepatocyte surrounded the central vein. Spirulina suspension group enhancement (figure 4-14,4-15)

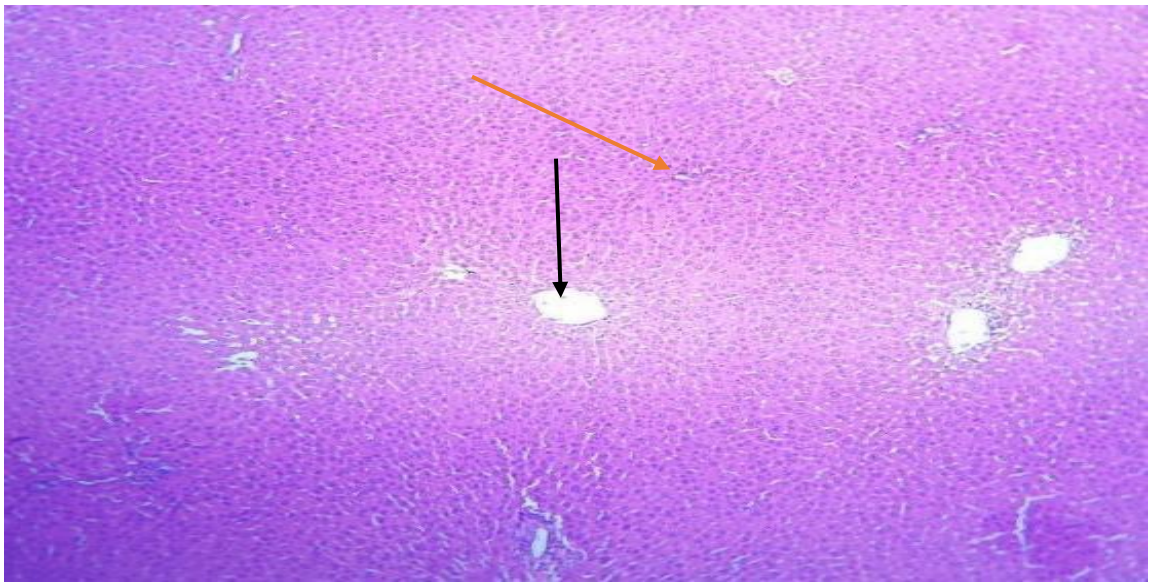

Figure (4-9) normal all cells such as hepatocytes thin (red arrow) and central vein thick (black arrow). Showed stain (H&E).(X100).

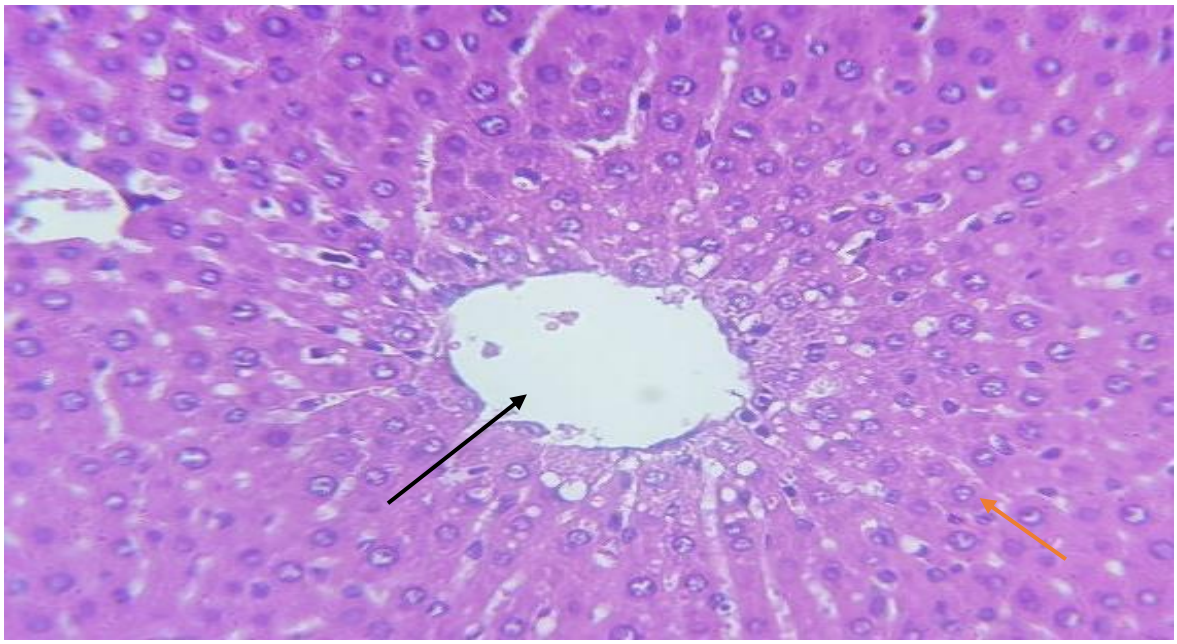

Figure (4-10), Liver in male rats of control group appear central vein (black arrow) and normal hepatocytes (red arrow) arranged in an irradiation manner showed stain (H&E).(X40).

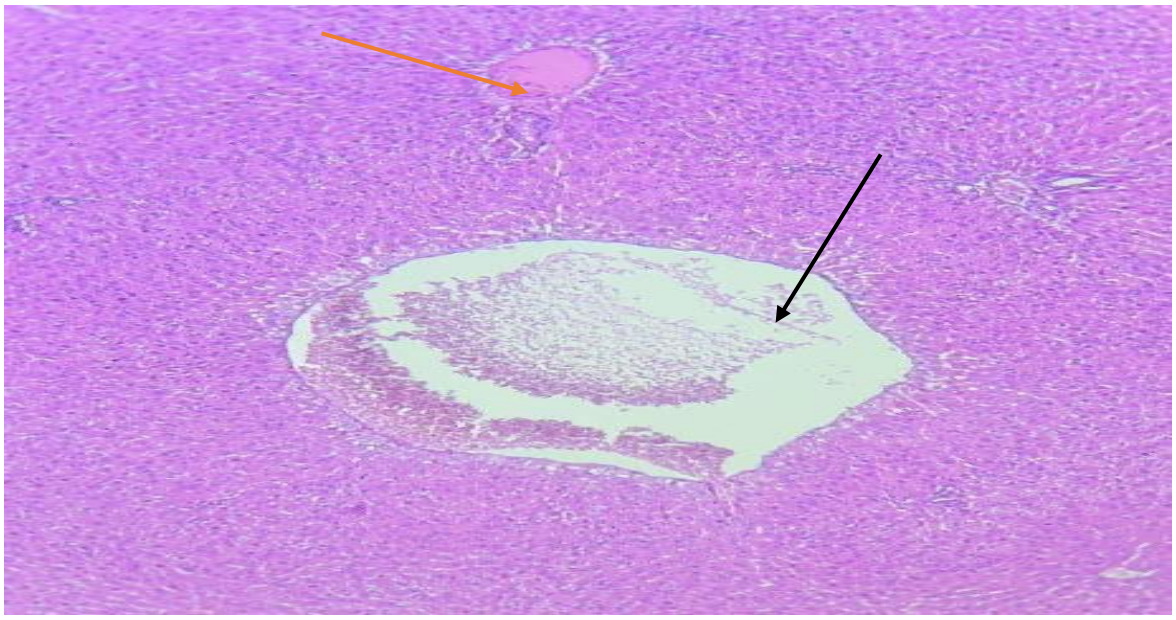

Figure (4-11) liver in rats male section that appear when treated with adenine group showed bleeding(black arrow) and congestion(red arrow) that showed stain (H&E).(X100).

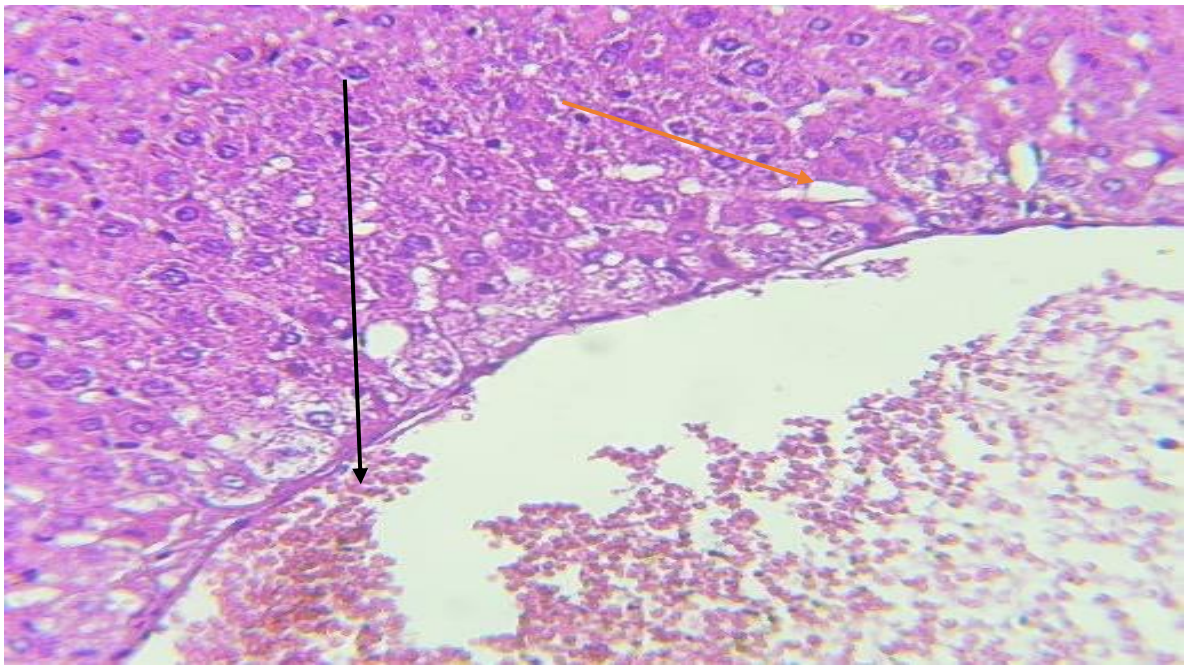

Figure (4-12) liver in microscopic showed of histological changes in liver rats male group treated with adenine alone that caused hemorrhage(black arrow)coagulated degeneration of hepatocyte loss irradiation architecture(red arrow) showed stain (H&E).(X400).

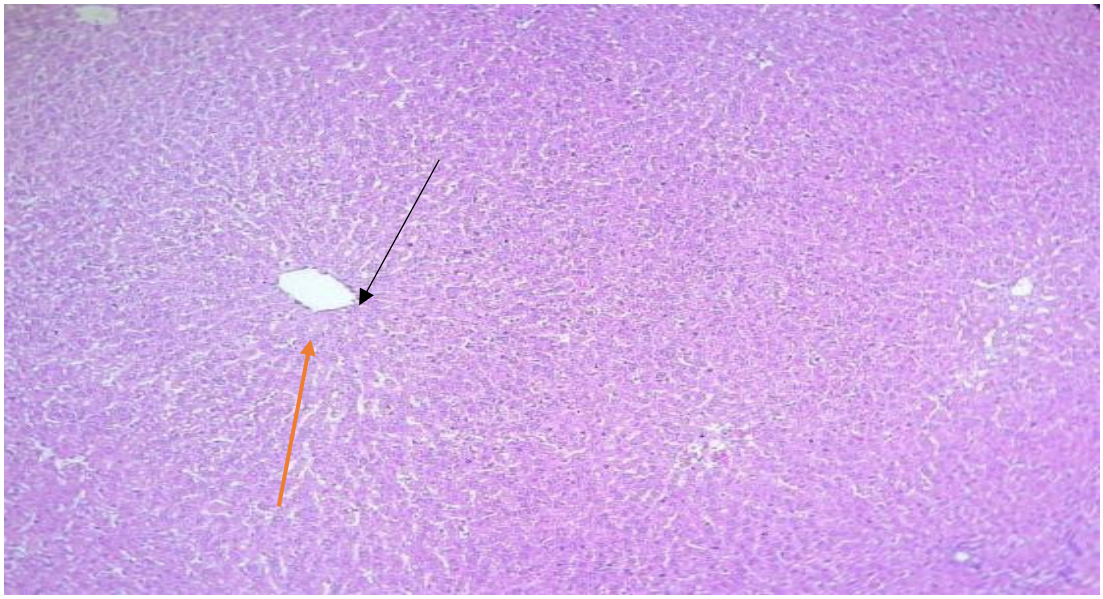

Figure (4-13), spirulina suspension + adenine treated in this section of liver showed return to same normal of the histological, It has architecture of center vein (black arrow), normal hepatocyte (red arrow) surrounded the central vein. showed stain (H&E). (X100).

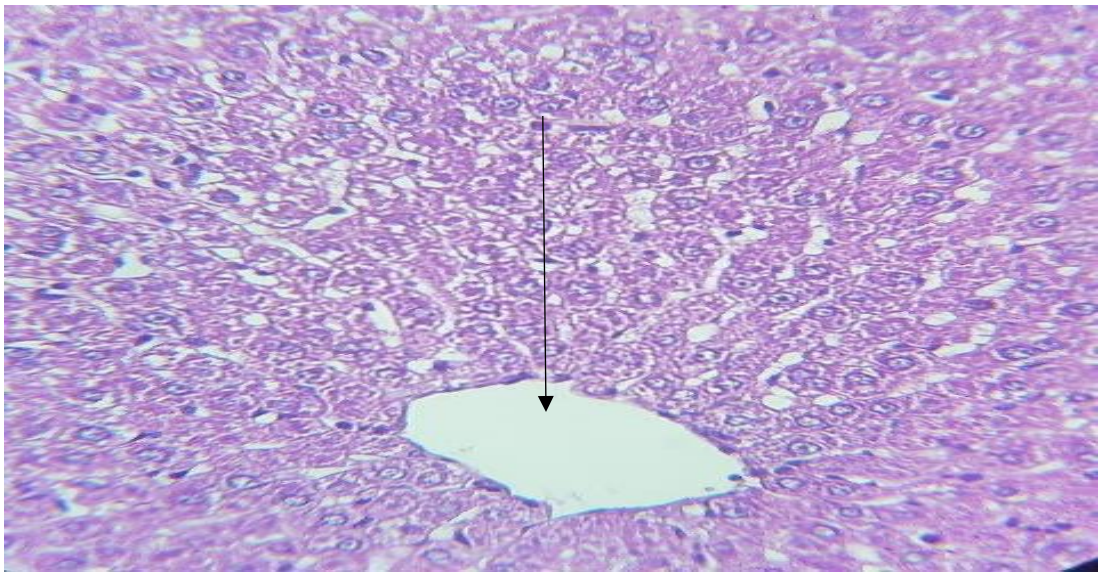

Figure (4-14), Aphotomicrograph of section in spirulina suspension+asdenine treated liver adult male rats of center vein (black arrow)different study group treatment with adenine alone showed stain (H&E) ,(X400)

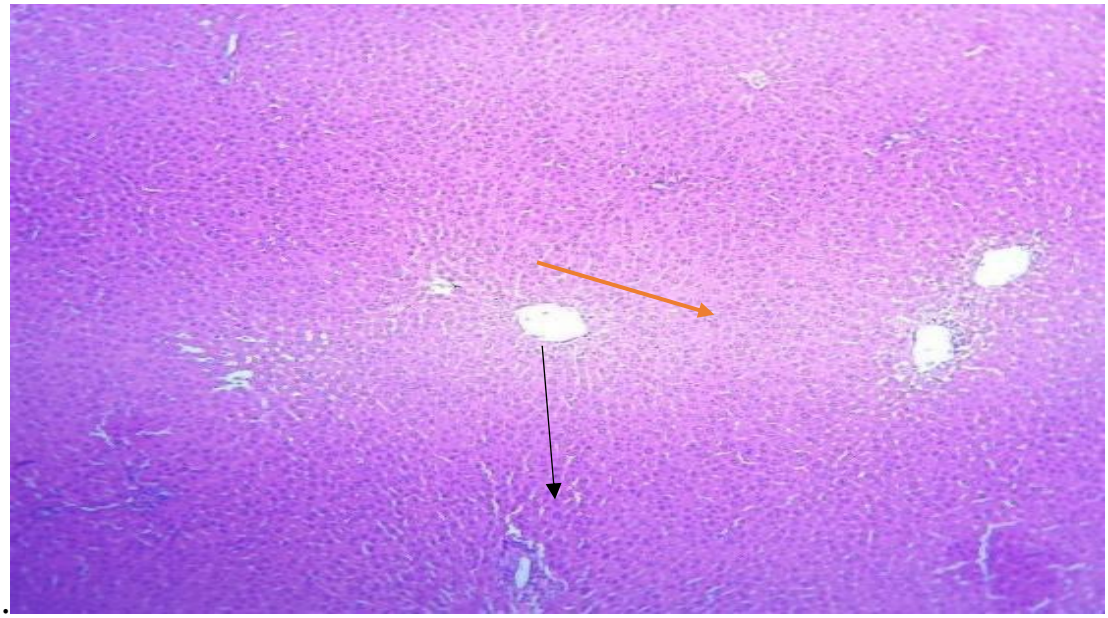

Figure (4-15), A photomicrograph of section in liver adult male rats of different study group treatment with adenine alone and spirulina +adenine showed improved the hepatocytes (red arrow) will arranged normal in radiating and center vein (black arrow)showed stain (H&E),(X100).

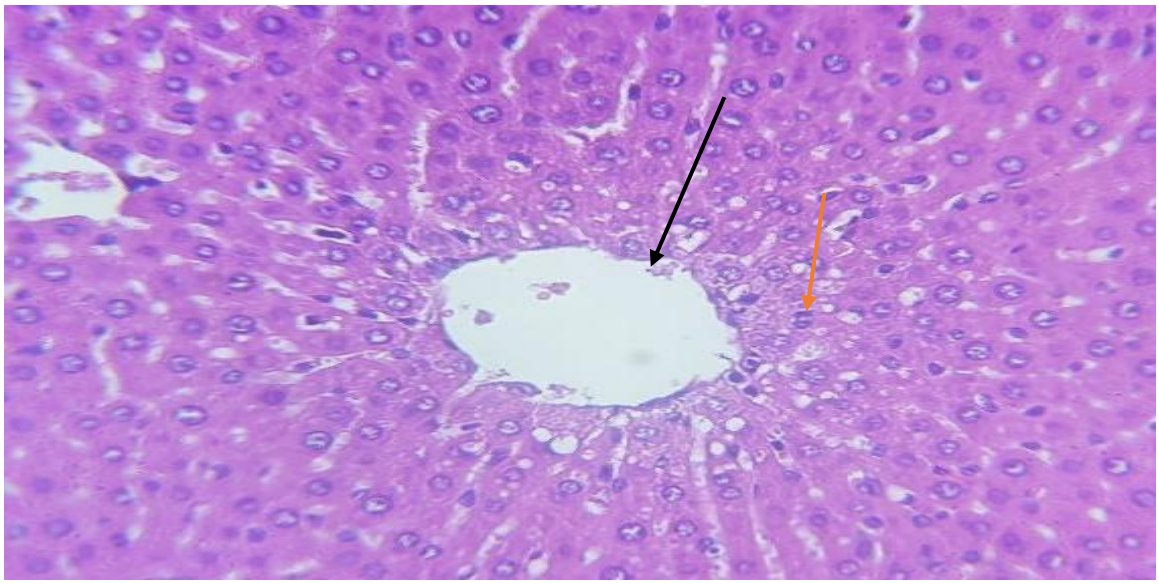

Figure (4-16) liver A photomicrograph of histological in male rats spirulina alone improved and normal all cells such as hepatocytes thin (red arrow) and central vein thick (black arrow) arranged in an irradiation manner showed stain (H&E),(X400).

Chapter five

Discussion

Discussion

5-1. Adenine induced chronic renal failure

Induce of chronic renal failure by adenine in the current study was confirmed according significant elevation of KIM-1, urea and creatinine (4-1) in addion to histopathological changes that occurred in rat kidney treated with adenine powder .In other the results of the presents was in accepted with together previous studies (Alqutb ,(2019),Al Za'abi *et al.*, (2015) and Hassan, 2018).

5.2. Kidney function biomarker KIM-1, urea and creatinine

A transmembrane protein with 90-kDa molecular whight called KIM-1 is overexpressed found in proximal tubules o f in rat and has an important role in repaire epithelial layer of renal tubule in rat kidney with ischemic injury (Han *et al*., 2002 ; Huo *et al*., 2010).Under natural condition, KIM-1 undetectable in urine (Ichimura *et al*., 1998), but it is found in high value after tubular damage (Bonventre, 2009).KIM-1 considered a remarkable biomarker for acute renal tubular kidney damage (Liangos *et al.*, 2007). In other result adenine causes a significant elevation of the level of KIM-1in serum. The existing study accepted with other previous studies (Imarah, 2017; Al Za'abi *et al*., 2015: Alqutb and Hassan, 2018). Aguiar et al.,(2015) who found rise of KIM-1 concentration in induction CRF in male rats also O'Seaghdha *et al* ., (2013) and Driver et al., (2014), who reported elevation KIM-1 in patients with CRF in humans by comparison with control groups. This elevation of KIM-1 may be due to missing its polarity to be production directly into the interstitial fluid where it, can reenter into the circulations (Sabbisetti *et al*., 2014). Another possible reason is the physiological stress of renal dysfunctions kidney causes to no-renal cell expressing Kidney Injury Molecule- 1 (KIM-1) to higher its level in circulation. Another possibility is with renal dysfunction, scavenging of KIM-1 from the circulation is less

efficient. Patients with the lower glomerular filtration rate have the elevation plasma KIM-1 concentrations （Miao *et al*., 2017). Urea in chronic renal disease the significantly elevation concentration of urea may be referred to disorder the weakness in distribute of proximal convoluted tubules function and transport function of epithelial cells in accumulation tubules (Ledoux and Gowda, 2008). Level of urea showed was significantly elevation in adenine group in compared with control group showed in (4-1).

Significantly elevation of urea level in group indices chronic kidney disease by compare with control group and in other much with result reported with (Al Za'abi *et a l*., 2015; Zhang *et al* ., 2015 and Imarah, 2017). The secretion of nitrogenous compounds in adenine treatment rats is curbed by renal tubular closing, because of the formation of 2,8 –dihydroxyadenine crystals, leading to collecting of various guanidine compounds such as (gaunidinosuric acid and methylguanidine) and urea nitrogen in blood (Ali *et al*., 2014 and Imarah, 2017). Creatinine is a nitrogenous organic acid which is formation of ATP necessary for the production of energy to skeletal muscle followed by its transference to creatinine is measurement of its serum level and secrete in the urine and, is the most inductor commonly used sign of function renal (Zhang *et al.,* 2015). If the level of the urea and creatinine become higher the diffuse impairment of proximal convoluted tubules function ,in collecting tubules and this may index disturbed transportations function of cells epithelial (Ledoux, a nd Gowda, 2008), some present in other studies have shown that the changes in urea levels ,and serum creatinine, may be other causes to the necrotic changes in renal parenchyma (Pier, 2004and 1987 ,Fung , Clark). Creatinine level significantly rise ($p \leq 0.05$) in adenine group by comparison with the control group in the present study as shown in table (4-1) this data accepted with (Ali et al ., 2013 and La et al., 2018). In rat, that present

significant elevated ($p \leq 0.0`5$) creatinine inplasma levels by compare with control group, the current study also agreed with study by Imarah, (2017) who found a rise in serum creatinine of induced chronic renal disease in adult male rats.

Long lasting intake of adenine suspension had been documented, induces the secretion of nitrogen compound by blocking kidney renal tubules. It creates metabolic defects similar to chronic kidney failure `CRF` in humans. During metabolism mammalians, it is an important substrate ($p\leq 0,05$) for xanthine dehydrogenase when adenine was found excessive. Adenine powder transferat to `2, 8 dihydroxyadenine.This enzyme can oxidize DHA adenine and DHA precipitate in renal tubules could be have low solubility (Miller and Ormord, 1980; Yokozawa *et al*., 1986 and Shuvy *et al*., 2011). It lead to disturbance in filtration and finally lead to increase serum creatinine level. (Imarah, 2017). Significant elevation ($p \leq 0.0`5$). In the level of the creatinine in the serum might have been due to its higher emission from the muscles and-or decline secretion from the kidneys (Yilmaz *et al*., 2018).

On another hand, co-administration of spirulina powder with adenine in the current study caused a significant enhancement of the renal function indicators. This result in coordinate with that reported by Gargouri et al., (2018) who found that spirulina protected neonate rat from leads-induced nephrotoxicity via its antioxidant properties. This enhancement in KIM, urea and creatinine might be attributed to ability of phycocyanin (a biliproteins pigment found in spirulina components) to the accelerated regeneration of tubular malfunction that caused by adenine or due to a diuretic activity of the phycocyanin (Farooq *et al*., 2004). In addition, Abdel-Daim (2014) reported that the presence potassium in alga which in turn possess a diuretic effect.

Beside, spirulina alga rich in flavonoids that can demonstrate the increase of diuresis because flavonoids cause polyuria (Yuliana *et al.*, 2009).

5.3. Biochemical indices (AST and ALT)

Aspartate transaminase and alanine aminotransferase are two enzymes of the most reliable marker of hepatocellular injury or necrosis. Their is level significantly elevation in a various of liver disorders. The two alanine aminotransferase is thought to be more specific for hepatic injury because it is found manly in liver cytosole and in lower concentrations elsewhere (Giboneyi, 2005). The results show significant elevation ($p \leq 0.05$) in Aspartate transaminaes and alanine aminotransferase activities, found in male rats treated by adenine (100 mg/ kg /BW) intraperitoneally compare with control group (4-1). These results were matched with results obtained by (Al Za'abi *et al.*, 2015).Induction chronic renal disease in ratsmale .

Adenine powder is' 2, 8-dihydroxyadenine and its metabolite DHA, have lower solubility and precipitates in the tubules renal causing to their is occlusions and the develops of uremia (Nasir *et al.*, 2012). Moreover, adenine powder has a tendency for causeing inflammatory reactions and several oxidative ,occur in tissues renal this could inspire an elevation in several the oxidative and inflammatory markers (Waring,(2011); Baumgarten ; Gehr, 2011 ; Moonie, 2011;Ali *et al.*, 2013; Ali *et al.*, 2014 ;).The rise in the oxidative derivative of deoxyguanosine, 8OHdG, one of the main oxidative DNA components, the adenine powder treated groups often display oxidative stress within the cells. Such oxidative biomarkers may have a high systemic toxicity that could cause damage to many other organs such as the heart and liver. (Astor *et al.*, 2012 and Fraga *et al.*, 1990). Alanine aminotransferase`ALT`and Aspartate transaminase (AST) found in liver

significant elevation Compared to the control, adenine powder treated groups indicate a degree of liver damage that may be caused by oxidative and inflammatory adenine reactions according to (Imarah 2017and Amacher, 2002; Lacour *et al*., 2005).

Additionally rats that co administrated Spirulina with adenine exhibited significant ($P < 0.05$) reduced in the activity Aspartate transaminase AST and alanine aminotransferase ALT serum enzymes.

The results of the study revealed the therapeutic role of the Spirulina against hepatotoxins caused by adenine. The results of the present study are consistent together with the results reported by Hassanen et al., (2015) who demonstrated a reduced activity of AST and ALT as a result of spirulina administration against toxic activity of doxorubicin. Moreover, Luxia *et al.* (1996) mention that β-carotene of spirulina may reduce cell damage, in particle that related to damage of DNA molecules, thus possess the ability to repair of regeneration process of injured liver cells

5.4 .Antioxidant indices (SOD, GSH and NO) and MDA

Superoxide dismutase (SOD) is actually the first detoxifying enzymes in the cell and therefore the most effective antioxidants. It is considered as the endogenous antioxidant enzyme is essential, which acts as first line part protection mechanism against reactive oxygen species ROS (Fridovich, 1995 and Dringen *et al.*, 2005). Concentration of SOD and GSH in plasma is significantly redaction ($P \leq 0.05$) in adenine powder group in comparison to controlgroup as shown (4-5). Adenine powder DHA and its metabolite, they precipitate the renal tubule that have poor solubility in cause their occlusion and uremia production (Wyngaarden and Dunn, 1957 and Nasir *et al*., 2012). The elevation in the oxidant derivative of 8-OHdG deoxyguanosine, which is

one of the main oxidant DNA items, in the adenine treated groups. It also indicates oxidative stress inside the cells, such biomarkers are oxidative on time as told before previously, can have potential systemic toxicity leading to damage of some other internal organs, such as, the liver and heart (Amacher, 2002; Lacour *et al*., 2005 and Imarah, 2017), High toxic agent producing a variety of ROSs, contain nitric oxide (NO.), single oxygen (1O2) ,hydrogen peroxide (H2O2), superoxide (O2.-), peroxyl radical (ROO.)and dimethylarsinics peroxyl radicals (CH3)2AsOO).(Pi *et al*., 2003 ; Rin *et al* . , and 1995 Shi *et al*., 2004). Such substances can inhibition antioxidants enzyme, especially GSH-dependents enzyme like this glutathione peroxidase (GSH- `Px), glutathione -transferases GSH and GSH reductase, by binding to their sulfhydryl SH groups , (Schiller *et a*l . , 1977 and Waalkes e t al ., 2004). Toxic element leads to increase in the lipid per oxidations (Monteiro *et al.,* 1991). Significant low in the activity SOD of tissue (Nehru and Dua, (1997), and Tripathi *et al.,* 2001 and). So the adenine as a toxic element may cause same event mentioned above. Nitric oxide concentration was significantly elevation ($p \leq 0.0`5$) in adenine powder group in comparison with control group as shown in tables (4-4) and According to result of MDA which show same pattern of NO. It was significantly increase ($p \leq 0.05$) in adenine powder group in comparison with control as shown in table (4-4). BY Chen et al., (2008). This study showed that adenine induced chronic renal failure driven redox imbalances, as shown by elevated levels of NO and MDA, in addition to decline of GSH levels and SOD activity.ROS-induced lipid peroxidation formed MDA which considered as important a biomarkers of oxidative stress. Increased levels of MDA have demonstrated damage to the renal tissue and altered membrane function, (Baba *et al.,* 2016 and Brakati *et al.,* 2019). NO is a cellular mediator

involved in different physiological roles. Improved response to NO adenine treatment may be causes stimulation of nitrosative stress responses, as a result of upregulation of No s 2, the rate limit enzyme in NO synthesis (Owumi and Dim, 2019). CO-administration of spirulina suspension significantly returned these enzymatic activities near the normal values in control group. The spirulina contain C-phycocyanin which possess a potential antioxidant activity and scavenging free radicals such as hydroxyl radicals and superoxide. The protection of C-phycocyanin against adenine demonstrated the radical scavenging activity and its inhibitory effect on lipid peroxidation chain reaction that mentioned by Abdel-Daim *et al.*, (2013). In addition Karadeniz et al., (2009) revealed that Pre-treatment with Spirulina platenesis might cause reducing the cadmium's harmful effect and the antioxidant features of spirulina may be via the reduction of NO and MDA as well as the elevation of SOD and GSH levels. Spirulina suspension can effectively improve CRF antioxidation effect in male rats and decreases free radical dandification and preserves the kidneys.

5.5. The effect of adenine on lipid profile (LDL, HDL,TG and chol).

Analysis of variance in the found study revealed a significant increase in cholesterol, TG and LDL level in the group of male rats injected intraperitonealy with adenine in comparison with control and other treated groups and our results in agreement with that reported by Ghelani et al., (2019). Elevation of LDL in serum of rat with adinine induced chronic renal failure, may be resulted from the downregulation of LDL receptors in responses to chronic renal failure (Liang and Vaziri,1996) **.** According total cholesterol increment in the present study could be occur as a result of the acceleration biosynthesis of cholesterol throughout the up-

regulation of enzyme called HMG-CoA reeducates (Liang *et al.*, 2005 and Vaziri and Liang,1995) other explanation to rise cholesterol levels is due to a relative decrease of elimination of cholesterol via liver due to down-regulation of enzyme called cholesterol 7 α-hydroxylase (CYP7A1) in animals undergoing chronic kidney disease (Pandak et al .,1994 and Pahl et al., 1998)

Elevation triglyceride in the blood of chronic kidney disease patients are most common among lipid abnormalities (Vaziri and Moradi ,2006; Attman and Samuelsson 2009; and Kwan *et al.*, 2007). Hypertriglyceridemia in rats male together with adenine powder induced Chronic renal insufficiency in the present study may be due to the disturbance in regulation of different rerated enzymes such as lipoprotein lipase and hepatic lipase, apolipoproteins and receptor involved in metabolism of triglycerides (Tsimihodimos et al., 2011)(Vaziri and Liang 1996)` reported that the deficiency of lipoprotein lipase activities in the adipose tissue, skeletal muscle as well as in myocardium may involve to the elevation of triglyceride in chronic kidney disease. In addition, expression and activity of hepatic lipase protein is also reduced in rats with chronic kidney disease (Jin *et al.*, 2013,Liang and 1997 and Vaziri).

Furthermore, in the found study, rats male with induced renal failure exhibit a decreased in HDL levels compared to control and other treated groups. Decrease of plasma HDL concentration is mostly related with chronic kidney disease because imparied maturation of cholesterol(chol) ester poor HDL - 3 to cholesterol (chol) rich HDL-2 (Ghelani *et al.*, 2019). The other possible reson dor reduction of HDL may be related to an enzyme named lecithin–cholesteryl acyltransferase which play important role in HDL maturation ((Pandak *et al*.,1994).

Treatment with spirulina along that showed decreases significantly in serum of LDL,Cholest and Trigly when comparative with group adenine . The HDL level significantly increase when comparative with group adenine in this study reported with other study(Bhat *et al*.,2020).

The hypolipaemic ability of spirulina was also reported in mice with alloxan-induced diabetes represented by reduce triacylglycerol and LDL as well as increase in HDL levels (Rodríguez-Hernández. *et al*.,2001). The enhancement of lipid profile could be occurs secondarily to activation of AMP-activated protein kinase signalling pathway which lead to downregulates the gene expression that involved in synthesis of lipid such as 3-hydroxy-3-methyl glutaryl coenzyme A reductase, Sterol regulatory transcription element binding factor-1c, and acetyl CoA carboxylase (DiNicolantonio *et al*., 2020). Moreover, spirulina has ability to change alter microbiota of gut to lowering effects of lipid via increase count of Study have revealed an increase in abundance of Prevotella, which in turn increases metabolism of bile leading to reduce lipid levels in the blood. Firmicutes are another group of bacteria which have ability to reduce serum LDL concentrations, which improved with spirulina supplementation. (Li *et al.*, 2018)

5.6. The effect of adenine on some hematological parameters in male rat

The adenine induced CRF in rats showed a significant decrease ($P \leq 0.05$in red blood cells (RBC), haemoglobin levels (Hb) and haematocrits (PCV) this result agrees with (Hamada *et al*., 2008) decrease in platelet count agrees with study accepted by(Malyszko *et al*., 1996 and elevated WBC count according to study by (Bash *et al*., 2009 and Habib *et al*., 2017). All these result compared with control group. Anemia related to inflammatory

disorders, such as kidney damage is involved with rapid decrement in renal function associated with decline production of erythropoietin, the main hormone of erythropoiesis (Nangaku, and Eckardt, 2006). However, erythropoietin resistance or decrease responsively to it in presence of inflammatory mediators may demonstrate the inability to reach and maintain the normal blood indices levels. Inflamed kidney may be in efficient to produce adequate erythropoietin. This finding is in agreement with (Nangaku, and Eckardt,2006) who reported that the primary cause is deficiency of erythropoietin in spite of different mechanisms by which the pathogenesis of renal anemia is various. In addition, marker and inflammatory mediators, such as C -reactive protein, hepcidins and IL-6, were found to antagonize the responses to (EPO) (Kalantar-Zadeh et al., 2003).

Treatment with spirulina along with adenine ameliorated blood indices to the normal concentrations. The beneficial effects of spirulina supplementation on blood parameters may be due to its protein richness in addition to presence fatty acids, vitamins, essential amino acids, minerals, carotenoids and other antioxidant chemicals (Sánchez *et al.,* 2003).

5.7 Histopathological change

Histopathological section in kidney of male rats from adenine group stained (H and E) showed numerous changes such as interstitial inflammation , inter tubular inflammation and in addition to the fibrosis .

Adenine and its metabolite, DHA (2-8-Diahydroxy adenine), are of lower precipitate and solubility in the kidney tubules they lead to their occlusion and the creation of uremia, (Nasir *et al*., 2012). Adenine also has the ability to induce many oxidative and inflammatory reactions in the renal tissue. This can cause certain oxidizing and inflammatory markers to increase (Baumgarten and Gehr , 2011).

Discussion

In this study when treated by spirulina alone present improved in liver tissue if comparative with group adenine alone and adenine +spirulina powder ,in other result reported (Elzawahry et al,.2016).

Histopathological section from rat liver of rats treated by adenine which is stained by (H and E stain) showed an infiltration of the liver tissue with inflammatory cells (lobular inflammation). The portal areas show mild to moderate round cell inflammatory infiltrates in comparison with control group that showed normal architecture and histology. This probably indicated some tissue damage in the liver of adenine treated rats, which showed variance severity with results obtained by (Feere *et al*., 2015). In addition, hepatic histology showed inflammatory infiltration of the portal and hepatic area suggesting some degree of tissue damage. However, we are not certain whether the degree and the extent of this damage explain the discrepancy in this results agreement with (Feere *et al*., 2015).This change in liver may be due to indirectly chronic renal failure or directly by toxic effect of adenine on hepatic cell, this finding is support by elevation in the activity of liver enzyme as mention(4-4). The present study showed the protective effect of spirulina powder against liver damage tissue, inflammatory infiltration and improved of liver enzyme, our results in consistent with results of Elzawahry et al (2016) and Amin et al (2006). Spirulina resulting in regressed these structur alterations in the liver and kidney might be due to involvements of ROS in mediating these histological changes. A positive effects was discovered on kidney function when adult rats were treatment together with adenine +spirulina suspension. The protective effect of spirulina could be resulting from antioxidant effect of Phycocyanin pigment, one of the elements components of spirulina.

Chapter Six

Conclusions and Recommendations

Conclusions

6.1.Conclusions

From the present study one can conclude that:

1. Adenine able to induce chronic renal failure at dose 100mg /kg bw according to increases in KIM, urea and creatinine and histological change in kidney and liver that occurs in the present study.

2. Adenine also cussed systemic oxidative stress represent by increases in MDA as well as caused disorder in blood picture, lipids profile and liver function enzymes.

3. Co-administrated of spirulina exhibit incremented in all studies parameter and reduce the harmful effects of adenine.

6.2. Recommendations.

1. Conduit study to investigate therapeutic effect of spirulina in chronic renal failure.

2. Conduct study to evaluate different doses of spirulina against chronic renal failure.

3. Evaluate antioxidant actively of spirulina in reproductive system of male and female.

4. Investigate protective role of spirulina in cardiovascular disorder.

5. Assessment the role of spirulina as sliming supplement.

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Appendix l
Principle of KIM-1

This ELISA kit uses Sandwich-ELISA as the method. The Microelisa stripplate provided in this kit has been pre-coated with an antibody specific to Kim-1. Standards or samples are added to the appropriate Microelisa stripplate wells and combined to the specific antibody. Then a Horseradish Peroxidase (HRP)- conjugated antibody specific for Kim-1 is added to each Microelisa stripplate well and incubated. Free components are washed away. The TMB substrate solution is added to each well. Only those wells that contain Kim-1 and HRP conjugated Kim-1 antibody will appear blue in color and then turn yellow after the addition of the stop solution. The optical density (OD) is measured spectrophotometrically at a wavelength of 450 nm. The OD value is proportional to the concentration of Kim-1. You can calculate the concentration of Kim-1 in the samples by comparing the OD of the samples to the standard curve.

Materials provided with the kit

	Materials provided with the kit	96 determinations	Storage
1	User manual	1	R.T.
2	Closure plate membrane	2	R.T.
3	Sealed bags	1	R.T.
4	Microelisa stripplate	1	2-8℃
5	Standard：1080 pg/ml	0.5ml×1 bottle	2-8℃
6	Standard diluent	1.5ml×1 bottle	2-8℃
7	HRP-Conjugate reagent	6ml×1 bottle	2-8℃
8	Sample diluent	6ml×1 bottle	2-8℃
9	Chromogen Solution A	6ml×1 bottle	2-8℃
10	Chromogen Solution B	6ml×1 bottle	2-8℃
11	Stop Solution	6ml×1 bottle	2-8℃
12	Wash Solution	20ml (30X)×1bottle	2-8℃

Sample preparation

1. Serum preparation

After collection of the whole blood, allow the blood to clot by leaving it undisturbed at room temperature. This usually takes 10-20 minutes. Remove the clot by centrifuging at 2,000-3,000 rpm for 20 minutes. If precipitates appear during reservation, the sample should be centrifugated again.

Procedure

1. Dilution of Standards

Ten wells are set for standards in a Microelisa stripplate. In Well 1 and Well 2, 100µl Standard solution and 50µl Standard Dilution buffer are added and mixed well. In Well 3 and Well 4, 100µl solution from Well 1 and Well 2 are added respectively. Then 50µl Standard Dilution buffer are added and mixed well. 50µl solution is discarded from Well 3

and Well 4. In Well 5 and Well 6, 50µl solution from Well 3 and Well 4 are added respectively. Then 50µl Standard Dilution buffer are added and mixed well. In Well 7 and Well 8, 50µl solution from Well 5 and Well 6 are added respectively. Then 50µl Standard Dilution buffer are added and mixed well. In Well 9 and Well 10, 50µl solution from Well 7 and Well 8 are added respectively. Then 50µl Standard Dilution buffer are added and mixed well. 50µl solution is discarded from Well 9 and Well 10. After dilution, the total volume in all the wells are 50µl and the concentrations are 720 pg/ml，480 pg/ml，240 pg/ml，120 pg/ml and 60 pg/ml, respectively.

1080 pg/ml 720 pg/ml 480 pg/ml 240 pg/ml 120 pg/ml 60 pg/ml

2. In the Microelisa stripplate, leave a well empty as blank control. In sample wells, 40µl Sample dilution buffer and 10µl sample are added

(dilution factor is 5). Samples should be loaded onto the bottom without touching the well wall. Mix well with gentle shaking.

3. Incubation: incubate 30 min at 37℃ after sealed with Closure plate membrane.

4. Dilution: dilute the concentrated washing buffer with distilled water (30 times for 96T and 20 times for 48T).

5. Washing: carefully peel off Closure plate membrane, aspirate and refill with the wash solution. Discard the wash solution after resting for 30 seconds. Repeat the washing procedure for 5 times.

6. Add 50 μl HRP-Conjugate reagent to each well except the blank control well.

7. Incubation as described in Step 3.

8. Washing as described in Step 5.

9. Coloring: Add 50 μl Chromogen Solution A and 50 μl Chromogen Solution B to each well, mix with gently shaking and incubate at 37℃ for 15 minutes. Please avoid light during coloring.

10. Termination: add 50 μl stop solution to each well to terminate the reaction. The color in the well should change from blue to yellow.

11. Read absorbance O.D. at 450nm using a Microtiter Plate Reader. The OD value of the blank control well is set as zero. Assay should be carried out within 15 minutes after adding stop solution.

Calculation of Results

Known concentrations of Rat Kim-1Standard and its corresponding reading OD is plotted on the log scale (x-axis) and the log scale (y-axis) respectively. The concentration of Rat Kim-1 in sample is determined by plotting the sample's O.D. on the Y-axis. The original concentration is calculated by multiplying the dilution factor

Appendix II

Serum urea estimation: Urea concentration in serum was determined by using a special kit (SPECTRUM- Urea Kit, Egypt- IFUFCC40), by using device (Spectrophotometer Sesil, England).

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.

$$2NH_4 + 2\ \alpha\text{-KG} + 2NAD + H_2O \xrightarrow{\text{GLDH}} 2NADH_2 + \text{L-Glutamate}$$

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 578nm.

Appendix

Reagent

Reagent standared urea (st)	50 mg/ml	8.33 mmol/L
Reagent Tris Buffer	*PH 8.5*	*50 mmol/L*
α-Ketoglutarate	-------	10 mmol/L
GLDH	-------	8.0 K U/L
Urea	-------	50 K U/L
Sodium azide	------	8.0 mmol/L
NADH	------	> 0.20 mmol/L
Sodium azide	-------	8 mmol/L

Procedure

Wave length: 578nm

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

Procedure

Mix, and after 30 seconds read the absorbance A1 of the 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

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

$$\text{Serum urea concentration (mg/dl)} = \frac{\Delta \text{ A specimen}}{\Delta \text{ Astandard}} \times n$$

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

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)

Appendix

Principle.

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

Creatinine + picrate ⟶ NaOH Yellow-red complex

Reagent

Procedure

Let stand reagent and specimen at room temperature

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

Mix and let stand for 20 minutes at 20-250C.measure the absorbance of specimen and standard against reagent blank at 546nm.

Calculation:

$$\text{Creatinine (mg/dl)} = \frac{\text{(A of Specimen)}}{\text{(A of standard)}} \longrightarrow 2 \text{ (standard concentration)}$$

Appendix III

Serum Aspartate aminotransferase Activity (AST) concentration

Serum Aspartate aminotransferase activity (AST) is determined by using a special kit (SPECTRUM AST – kit, Egypt- IFUFCC22), by using device (Spectrophotometer Sesil, England)

Principle

Colorimetric determination of AST activity is obtained according to the following reactions:

AST: Aspartate + α keto glutarate $\overset{\text{AST}}{\rightleftharpoons}$ oxaloacetate + glutamate

The reaction

The oxaloacetate formed is measured in its derivative form, 2,4-Di nitrophenylhydrazone .

Reagents:

Reagent 1 AST	Phosphate buffer pH7.5 L-Aspartate 2-Oxoglutarate Sodium Hydroxide Sodium Azide	100 mmol / l 100 mmol / l 5 mmol / l 140mmol/l 12 mmol/l
Reagent2 Color Reagent	2.4dinitroph enylhydrazin e HCL	2 mmol /l 8.4 %

Appendix

Procedure Wave length: 546 nm (530 – 550 nmZero adjustment: reagent blank: Pipette into test tubes:

Reagent	**Reagent blank**	**Sample**
Reagent(Buffer)	0.5ml	0.5ml
Sample	-------	100µl
Distilled water	100µl	------

Mix and incubate for exactly 30 minutes at 370

Reagent 2	**0.5ml**	**0.5ml**

Mix and incubate for exactly 20 minutes at 20-250C̊

Sodium hydroxide	**5ml**	**5ml**

Mix and measure absorbance of specimen against reagent blank at 546nm after 5 minutes.

Appendix

Calculation

Obtain the AST activity from the following table.

Absorbance	Value of AST U/L	Absorbance	Value of AST U/L
0.020	7	0.100	36
0.030	10	0.110	41
0.040	13	0.120	47
0.050	16	0.130	52
0.060	19	0.140	59
0.0170	23	0.150	67
0.080	27	0.160	76
0.090	31	0.170	89

Linearity:

The assay is linear up to 89 U/L. if the absorbance exceeds 0.170 at 546 nm, sample should be diluted 1+9 using sodium chloride and repeat the assay (result x10).

Serum Alanine aminotransferase Activity(ALT)

Appendix

Determination

Serum Alanine aminotransferase activity ALT is determined by using a special kit (SPECTRUM ALT – kit, Egypt- IFUFCC25), by using device (Spectrophotometer Sesil, England).

Principle

Colorimetric determination of ALT activity is obtained according to the following reactions:

$$\text{ALT : Alanine + } \alpha \text{ keto glutarate} \xrightleftharpoons{\text{ALT}} \text{pyruvate + glutamate}$$

The pyruvate formed is measured in its derivative form, 2,4dinitrophenylhydrazone

Reagents

Reagent 1 ALT	Phosphate buffer pH7.5 D-Alanine 2-Oxoglutarate Sodium Azide	100 mmol /l 200 mmol /l 6 mmol /l 12 mmol/l
Reagent 2 Color reagent	2.4dinitrophenylhydrazine	2 mmol /l

Procedure

Wave length: 546 nm (530 – 550 nm) adjustment: reagent blank:

Pipette into test tubes:

Reagent	Reagent blank	Sampl e
Reagent(Buffer	0.5ml	0.5ml
Sample	-------	100µl
Distilled water	100µl	------

Mix and incubate for exactly 30 minutes at 370C

Reagent 2	**0.5ml**	**0.5ml**

Mix and incubate for exactly 20 minutes at 20-250C

Sodium ydroxide	**5ml**	**5ml**

Mix and measure absorbance of specimen against reagent blank at 546nm after 5 minutes.

Calculation

Obtain the ALT activity from the following table

Absorbance	Value of ALT U/L	Absorbance	Value of ALT U/L
0.025	4	0.275	48
0.050	8	0.300	52
0.075	12	0.325	57
0.100	17	0.350	62
0.125	21	0.375	67
0.150	25	0.400	72
0.175	29	0.425	77
0.200	34	0.450	83
0.225	39	0.475	88
0.250	43	0.500	94

Linearity: the assay is linear up to 94 U/L .if the absorbance exceeds 0.5 at 546nm , sample should be diluted 1+9 using sodium chloride and repeat the assay (result x 10).

Appendix IV

lipid profile test

Estimation of serum cholesterol concentration:

Principle:

Easter of cholesterol+H2O $Chol.\ estease$ Cholesterol + Fatty acids

Cholesterol +O2 $Chol.\ oxidase$ Cholest-4-en-one+H2O2

H2O+4-Aminophenazone + phenol $peroxidase$ Quinonimine

Reagent:

Reagent (1) Buffer solution: pipes PH 6.9 mmol/L, phenol 26 mmol/L

Reagent (2) vial of enzyme: cholesterol oxidase 300 U/L, peroxidase 1250 U/L, cholesterol esterase 300 U/L, 4-aminophenazone 0.4 mmol/L

Reagent (3): cholesterol standard 200 mg/dl

1. Manual procedure: Cholesterol concentration in serum samples was measured according to the following

a. Reagent and serum samples were brought to room temperature

b. Serum sample, blank and standard were treated as follow:

c. Tube contents were mixed and left to stand for 5 minutes at 37°C before reading

d. the absorbance of the standard was measured and sample was read via spectrophotometer at wavelength 505 nm against the blank

Tubes	Blank		Standard		Sample
Cholesterol standard (s)	-		10 ml		-
Sample	-		-		10 ml
Working Reagent	1ml		1ml		1ml

Calculation:

Result were calculated according to the following equation:Total Cholesterol concentration = (O.D sample)/ (O.D/ standard)× nn = 200 mg/dl

Estimation of serum triglyceride concentration (mg/dl):

Principle:

Triglyceride $\xrightarrow{lipoprotein\ lipase}$ Glycerol + fatty acid

Glycerol + ATP $\xrightarrow{Glycerol\ kinase,++}$ Glycerol-3-phosphate+ADP

Glycerol-3-P+O_2 $\xrightarrow{3-G-P-oxidase}$ Dihydroxyacetone ne-p+H_2O

H2O2+4-Aminophenazone+p+Chlorophenol$\xrightarrow{peroxidase}$ Quinonimine+ H_2O

Reagent:

Reagent (1) buffer solution: pipes buffer PH 7.2, 50 mm0l/L, p- chlorophenol 2 mmol/L

Reagent (2) Enzyme: lipoprotein lipase 150 000 U/I, glycerol kinase 800 U/U/I, glycerol-3-phosphate oxidase 4000 U/I, peroxidase 440 U/I, 4-aminophenazone 0.7 mmol/L, ATP 0.3 mmol/L.

Reagent (3) triglyceride Standard (S): Glycerol 200mg/dl.

Procedure:

Triglyceride concentration in serum samples was measured according to the

following:

a. Wave length/filter. 505nm (Hg546nm)/green

b. Temperature 37°C/R. T

c. Light path 1 cm

Appendix

Pipette into clean dry test tubes labelled as Blank (B), standard (S), and Test(T). Mix well and incubated at 37 °C for 5 min or at R. T (25 °C) for 15min. measure the absorbance of the standard

Calculation:

Results were calculated according to the following equation:

Triglyceride concentration mg/dl = (O.D sample) / (O.D standard) × n = 200 mg/dl

Additive sequence	Blank	Standard	Test
Working reagent	1.0	1.0	1.0
Distilled water	0.01	-	-
Triglyceride	-	0.01	-
standard			
Sample	-	-	0.01

Estimation of serum HDL-Cholesterol concentration (mg/dl):

Principle:

Cholesterol esters + H2O $Chol.esterase$ Cholesterol + fatty acid

Cholesterol +½O2 + H2O $Chol.oxidase$ Cholestenone + H2O2

2 H2O2 + 4-Aminoantipyrine + DCFS $peroxidase$ Quinoneimine + 4H2O

Reagent

Reagent (1) Good's buffer (pH 6.6)100 mmol/l, cholesterol esterase 1400 U/l, cholesterol oxidase 800 U/l, catalase 600 kU/l, N-(2-hydroxy-3-sulfopropyl)-3,5-dimethoxyaniline (HDAOS) 0.6 mmol/l

Reagent (2) Good's buffer (pH 7.0) 100 mmol/l peroxidase 3 kU/l 4–aminoantipyrine (4-AA) 4 mmol/L

Tubes	Blank	Standard	Sample
Distilled water	50 ml	-	-
Cholesterol standard (S)	-	50 ml	-
Sample supernatant	-	-	50 ml
Reagent	1.0ml	0ml	1ml

Procedure:

HDL-Cholesterol concentration in serum sample was measured according to the following steps: serum sample 40 – 60 mg/dl 1.04 1.55mmol/l, wavelength 600 nm,

temperature 37°C CORMAY HDL DIRECT is intended for automated analysers.

a. Reagent (A, B) and serum sample were brought to room temperature.

b. Serum sample, blank and standard were treated as followed:

c. 0.2 ml of sample was mixed with 0.5 ml of reagent (A) in centrifuge tube and let stand for 10 minute at room temperature.

d. Centrifuged at a minimum of 4000 r.p.m. for 10 minutes.

e. The temperature was collected carefully.

f. Sample supernatant, blank, standard and reagent (B)were treated as follows:

g. Tubes contents were mixed thoroughly and incubated for 10 minute at 37^{o} C.

h. the absorbance (A) of the standard was measured and sample was read via spectrophotometerat wave length 500 nm against the blank.

Calculation: results were calculated according to the following equation:

HDL-cholesterol concentration in the sample (mg/dl) = (Absorbance of the sample/Absorbance of standard) × concentration of standard × sample dilution factor (1.7).

Estimation of serum LDL-Cholesterol concentration (mg/dl):

Principle:

Cholesterol ester $\xrightarrow{chol.esterase}$ chol. + fatty acid

Cholesterol + O_2 $\xrightarrow{Chol.oxidase}$ chol. H_2O_2

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

Reagent:

Reagent (1) Good's buffer (pH 7,0) 50 mmol/l, cholesterol esterase 600 U/l, cholesterol oxidase 500 U/l, catalase 1200 kU/l, ascorbate oxidase 3 kU/l, TOOS [N-Ethyl-N-(2-hydroxy-3-sulfopropyl)-3-methylaniline] 2.0 mmol/l

Reagent (2) Good's buffer (pH 7,0) 50 mmol/l, peroxidase 5 kU/l, 4–aminoantipyrine (4-AA) 4 mmol/l.

Procedure:

wavelength 600 nm, temperature 37°C, CORMAY LDL DIRECT is intended for automated analysers. serum/plasma < 100 mg/dl < 2.59 mmol/l.

As LDL cholesterol is affected by a number of factors such as smoking, exercise, hormones, age and sex, each laboratory should establish its own reference ranges for local population.

Appendix V

Estimation of serum Superoxide dismutase (SOD) concentration

The procedure was done according to the instructions of the manufacture of ELIZA Kit -Elabscience biotechnology/ china.

Test principle

This ELISA kit uses Competitive-ELISA as the method. The microtiter plate provided in this kit has been pre-coated with SOD1. During the

reaction, SOD1 in the sample or standard competes with a fixed amount of SOD1 on the solid phase supporter for sites on the Biotinylated Detection Ab specific to SOD1. Excess conjugate and unbound sample or standard are washed from the plate, and Avidin conjugated to Horseradish Peroxidase (HRP) is added to each microplate well and incubated. Then a Tetramethylbenzidine (TMB) substrate solution is added to each well. The enzyme-substrate reaction is terminated by the addition of a sulphuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450 nm ± 2 nm. The concentration of SOD1 in the samples is then determined by comparing the OD of the samples to the standard curve.

Assay procedure

Bring all reagents and samples to room temperature before use. Centrifuge the sample again after thawing before the assay. All the reagents should be mixed thoroughly by gently swirling before pipetting. Avoid foaming**.** It's recommended that all samples and standards be assayed in duplicate.

Nitric Oxide

PROCEDURES Preparations. Dilute 20x ZnSO4 and 30x NaOH to 1-fold wi ater. The final concentrations are 75 mM ZnSO4 and 55 mM NaOH, respectively. Dilute Activation Buffer by mixing 1 volume of 3 x Activation Buffer with 2 volumes of distilled water. All diluted solutions can be stored at 4°C for 12 months

Appendix

No	Premix + H_2O + Gly Buffer	Vol (µL)	Nitrite (µM)
1	150µL + 0µL + 50 µL	200	50
2	120µL + 30µL + 50 µL	200	40
3	90µL + 60µL + 50 µL	200	30
4	60µL + 90µL + 50 µL	200	20
5	45µL + 105µL + 50 µL	200	15
6	30µL + 120µL + 50 µL	200	10
7	15µL + 135µL + 50 µL	200	5
8	0µL + 150µL + 50 µL	200	0

.

Procedure using 96-well plate:

1. Standards. Prepare 600 µL 50 µM Premix by mixing 30 µL 1.0 mM Standard and 570 µL distilled water. Dilute standard in centrifuge tubes as shown below. Add 50 µL Glycine Buffer per tube. Transfer 00 µL diluted standards into wells of a clear-bottom 96-well plate. 1

2. Deproteination is required for serum, plasma and other proteinaceous samples. Mix 100 µL sample with 80 µL 75 mM ZnSO4 in 1.5-mL tubes. If precipitation occurs, centrifuge 5 min at 14,000 rpm. Transfer supernatant to a clean tube containing 120 µL 55 mM NaOH. Pellet protein precipitates again (dilution factor n = 3). Transfer 210 µL supernatant and mix with 70 µL Glycine Buffer in a 1.5-mL centrifuge tube. If solution remains clear in these steps, deproteination is not required. Directly transfer 210 µL sample (dilution factor n = 1) and mix with 70 µL Glycine Buffer in a 1.5-mL centrifuge tube.

3. Activation of Cd. The number of Cd granules to be used is 3 x the number of samples. Transfer Cd granules in a 50-mL centrifuge tube. Wash Cd three times with water. Remove residual water with a pipet. Add 200 µL diluted 1 x Activation Buffer per granule and incubate 5 min at room temperature. Swirl tube intermittently. Wash three times with water. Activated Cd should be used

within 20 min. Note: cadmium is a toxic and expensive metal. Avoid direct contact (wear gloves). About 150 Cd granules are provided that are sufficient for about 50 samples. Used Cd granules should be stored or washed in 0.1 N HCl for at least 5 min. Washed 3 times with water and regenerated using the same activation procedure. Cd granules can be regenerated and used 7 times without loss of activity.

4. Nitrate Reduction. Dry the activated Cd granules on a filter paper (e.g. Kimwipes® EX-L). Add three Cd granules per sample (Step 2) and shake tubes intermittently. Incubate 15 min at room temperature. Transfer 2 x 100 µL samples (duplicate) into wells of the 96-well plate.

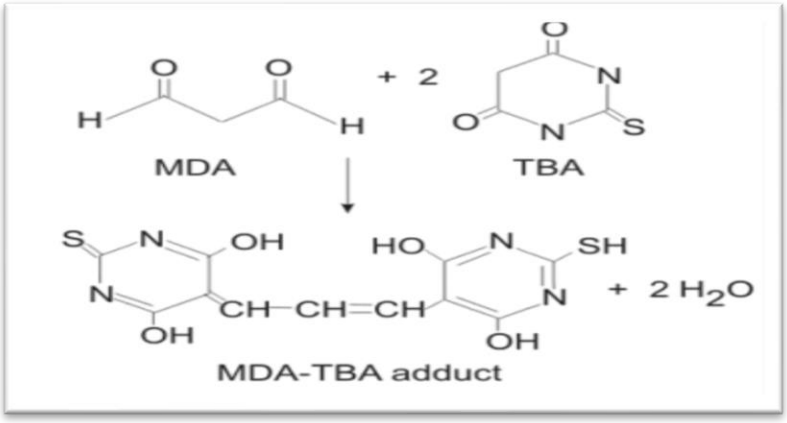
5. Assay. Add 50 µL Reagent A to all wells and tap plate lightly to mix. Add 50 µL Reagent B and mix. Incubate 5 min at room temperature. Read OD at 500-570nm (peak 540 nm). Signal is stable for > 60 min.

Estimation of Serum Malondialdehyde (MDA):

Malondialdehyhe was estimated by Thiobarbituric acid (TBA) assay method of Buege & Aust, 1978 on spectrophotometer .

Principle:

This method quantifies lipid peroxides by measuring aldehyde breakdown products of lipid peroxidation. Basic principle of the method is the reaction of one molecule of malondialdehyde and two molecules of thiobarbituric acid to form a red MDA-TBA complex which can be measure at 535 nm.



Stock TCA – TBA – HCl Reagent:

It was prepared by dissolving 15% W/V trichloroacetic acid and 0.375% W/V thiobarbituric acid and 0.25N HCl to make 100 ml (2.1 ml of concentrated HCl in 100 ml). This solution was mildly heated to assist in the dissolution of TBA. Dissolved 15 gm TCA and 0.375 mg thiobarbituric acid in 0.25 N HCl and volume was made up to 100 ml with 0.25 N HCl.

Procedure:

To 0.4 ml of serum, 0.6 ml TCA-TBA-HCl reagents were added. It was mixed well and kept in boiling water bath for 10 minutes. After cooling 1.0 ml freshly prepared 1N NaOH solution was added to eliminate centrifugation. This absorbance of pink colour was measured at 535 nm against blank which contained distilled water in place of serum. In blank 0.4 ml distilled water and

0.6 ml TCA-TBA-HCl reagent was mixed and boiled. Blank was always taken.

Calculation:

extinction coefficient of MDA at 535 nm is = 1.56 × 105

MDA concentration = χ / 0.0624 nmol / ml.

Glutathione(GSH)

Working Assay Mixture preparation

Immediately prior to use ,prepare the working Assay mixture as shown in table

Table 1 preperation is suitable for 48 reaction (100ml/well)

Reagents	Volume
Glutathione Assay Buffer [1X]	5 ml
Glutathione Reductase	8.7 µl
NADPH solution	10 µl

Note NADPH is light sensitive , therefore make the assay buffer in a brown vial or cover the vial with albuminum foil.

Ellman's Working Solution preparation

• Immediately prior to use, add 65 µl Ellman's Reagent stock solution to 2.5 ml of 1X

Glutathione Assay Buffer to make a working solution. You require 50 µl working

solution/ well.

NOTE: Ellman's Reagent is light sensitive, therefore make the working solution in abrown vial or cover the vial with aluminum foil

Assay protocol

1. Make dilutions of GSSG in microcentrifuge vials using 400 µM GSSG stock to

achieve final concentration of 1 µM, 0.8 µM, 0.6 µM, 0.4 µM, 0.2 µM and 0.1 µM

in 1X Glutathione Assay Buffer.

Prepare 1 µM stock of Oxidized Glutathione Standard by adding 2.5 µl of 400 µM

Oxidized Glutathione Standard solution to 1 ml of 1 X Glutathione Assay Buffer

and mix well.

Tube	Oxidized Glutathione Standard [1µM]	1 X Glutathione Assay Buffer	Final Concentration of Oxidized Glutathione (µM)
A	-	200 µl	0
B	20 µl	180 µl	0.1
C	40 µl	160 µl	0.2
D	80 µl	120 µl	0.4
E	120 µl	80 µl	0.6
F	160 µl	40 µl	0.8
G	200 µl	-	1

NOTE: The detection limit of this assay falls in 0.1-2.5 µM for GSSG and 0.2-5 µM for GSH, so different standard curves can be used.

NOTE: The standards should have the same concentration of Deproteination Reagent as the samples to ensure accurate estimations

2. Aliquot 50 µl GSSG standards into the wells performing in at least duplicate. (See format below).

3. Dilute the 5% Deproteination Solution in the samples to <0.5% by diluting the samples 1:10 with 1X Glutathione Assay Buffer.

NOTE: Lower than 0.5% Deproteination Reagent in sample is acceptable for the assay, however >0.5% Deproteination Reagent is not recommended as it may interfere with the assay. The standards should have the same concentration of Deproteination Reagent as the samples to ensure accurate estimations.

4. Aliquot 50 µl samples into the wells performing in at least duplicate. (See format below).

5. Add 100 µl of freshly prepared working assay mixture per well..

6. Incubate the plate at room temperature for 5 minutes.

7. Rapidly add 50 µl of freshly prepared Ellman's Reagent working stock solution per
well and mix several times by pipetting up and down.

8. Cover the plate with aluminum foil or incubate plate in dark on shaker until absorbance is checked. For kinetic method absorbance at 0 minute is also recorded

9. Glutathione concentration can be determined by endpoint method or by kinetic method

End point method: Read the plate at 405-415 nm, 25 minutes after addition of Ellman's Reagent.

Kinetic method: Read the plate at 405-415 nm at 5 minutes interval after addition of substrate for 30 minutes

AppendixVl

Histological study

Histological Technique(E & H) stain

The Heart and aorta 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.

* 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 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

Appendix

* Sectioning

After holdes 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.

Trichrome stain (modified masson's) procedure

The procedure was done according to Trichrome Stain Kit
(Modified Masson's) ScyTek Laboratories, Inc./ U.S.A.

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 5minute , 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 C̊ for 1 hour.

الخلاصة

تهدف الدراسة الحالية الى تقيم دور السبايرولينا في تقليل مضاعفات الفشل الكلوي المزمن الذي تم استحداثه بواسطة الأدنيين في ذكور الجرذان هذه الدراسة جرت في كلية الطب البيطري جامعة كربلاء خلال الفترة من)كانون الاول 2019 الى شهر نيسان 2020(.تم خلال هذه التجربة استخدام اثنان وثلاثون من ذكور الجرذان البالغة حيث تم تقسيمها الى اربعة مجاميع المجموعة الاولى تعتبر مجموعة السيطرة بينما المجموعة الثانية اعطيت الأدنيين)100ملجم/كجم(عن طريق حقنه تحت الجلد. اما المجموعة الثالثة تلقت الأدنيين)100ملجم/كجم(من خلال حقنه تحت الجلد ايضا مع مسحوق السبايرولينا عن طريق الفم)750 ملجم/كجم(. جرعت المجموعة الرابعة مسحوق السبايرولينا فقط ايضا عن طريق الفم .وفي نهاية التجربة تم اخذ عينات من الدم وفحصها لقياس

وظائف الكلى (kIM−1 , urea , creatinine) وتم اختبار وظائف الكبد (AST,ALT) وايضا فحص خصائص الدهون(cholesterol, triglycesrol, LDL , HDL). وتم قياس مضادات الأكسدة)SOD, GSH,MAD,ON(. وتم فحص خلايا الدم الحمر وخلايا الدم البيض والصفائح الدموية. تم اخذ مقطع

نسيجي من الكلى والكبد لدراسة التغيرات النسيجية/ اظهرت النتائج انخفاض معنوي ($P\geq 0.0`5$) في عدد كريات الدم الحمراء والهيموغلوبين والصفائح الدموية

في المجموعة الثانية مقارنة بالمجموعات الاخرى. بينما زادت خلايا الدم البيض بالمجموعة الثانية مقارنة بالمجموعات الاخرى. اظهرت النتائج زيادة معنويه

($P\geq 0.0`5$)في الكوليسترول والدهون الثلاثية والبروتين الدهني منخفض الكثافة في المجموعة الثانية التي تناولت الأدنيين بينما انخفض البروتين الدهني عالي

الكثافة في نفس المجموعة مقارنة مع المجموعات الاخرى. اظهرت النتائج انخفاض معنوي($0.0`5\geq P$) في المجموعة الثانية مقارنة مع المجموعة

الثانيه (GSH,MAI,ON) . اظهرت النتائج في المجموعة الثالثة التي تناولت مسحوق السبايرولينا مع الأدنيين زيادة معنوية في معظم المتغيرات التي تم دراستها وعودة كافة المتغيرات بالقرب من مجموعة السيطرة

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

تقيم التأثيرات الوقائية لسبايرولينا ضد الفشل الكلوي المزمن الذي تم استحداثه بواسطه بالادنيين في ذكور الجرذان

رسالة مقدمة الى

مجلس كلية الطب البيطري– جامعة كربلاء وهي جزء من متطلبات نيل درجة ماجستير علوم في الطب البيطري / الفسلجة

من قبل

وليد خالد يونس البهادلي

بكالوريوس طب وجراحة بيطرية – كلية الطب البيطري - جامعة كربلاء 2017

بأشراف أ.م. د عايد حميد حسن الموسوي

كلية الطب البيطري – جامعة كربلاء

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