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Physiology, Biochemistry and pharmacology Department

The effect of *Spirulina spp*. on the cardiovascular system aging induce by D-galactose on male rabbits

A thesis

Submitted to the Council of the College of Veterinary Medicine, University Of Karbala in Partial Fulfillment of the Requirements for the Degree of Master of Science in Veterinary Medicine/ Physiology

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1444 A.H **2022 A.D**

بسم ٱللّٰه الرَّحْمٰنِ الرَّحِيمِ

﴿ وَقُلْ رَبِّ زِدْنِي عِلْمًا﴾

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We certify that this thesis **(The effect of *Spirulina* spp. on the Cardiovascular system aging induce by D-galactose on Male Rabbits)** has been prepared by ***Hiba Aba Alkreem Kadhim*** under my supervision at the college of Veterinary Medicine,

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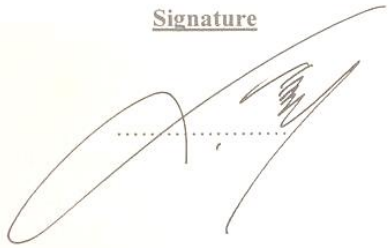

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I hereby declare that this thesis is my origin work except for equations and citations which have been fully acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at University of Kerbala or other institutions.

Hiba Aba Alkreem Kadhim

/ / 2022

DEDICATION

I present fragments of my humble research as a gift to my master, the Awaited Alimam Mahdi (may God bless him and grant him peace).

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

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

To my dear fiance Haider who supported me , In every step of the search. There are no words to express my thanks for you.

To My brothers, and sisters you are the bright moons, you are a blessing from the sky.

... Who was my partner in every …

To the flowers in my life …. my dear friends

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Hiba Abd Alkreem Kadhim

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List of abbreviations

Abbreviations	Meaning
8-OHdG	8-hydroxy-2-deoxyguanosine
AA	arachidonic acid
AF	atrial fibrillation
AGE s	advanced glycation end products
AGE s	advanced glycation end products
ALA	γ-linolenic acid
AMH	anti-mullerian hormone
BW	Body weight
CAT	catalase
cDNA	complementary DNA
CK-MB	creatine kinase-myocardial band
cTnI	Cardiac troponin I
CVS	Cardiovascular system
Cyclic GMP	Cyclic *guanosine monophosphate*
DGA	Diacylglycerol
d-gal	d-galactose
DHA	docosahexaenoic acid

DW	**distal water**
ECG	**Electrocardiogram**
EDHF	**endothelium-dependent hyperpolarizing factor**
EDRF	**endothelium-derived relaxing factor**
EDRF	**endotheliumdependent relaxation factor**
eNOS	**nitric oxide endothelial**
EPA	**eicosapentaenoic acid**
ERK	**extracellular signal-regulated kinase**
ET-1	**Endotheline 1-**
FFA	**free fatty acids**
FSH	**follicle stimulating hormone**
GLA	**gamma-linolenic acid**
GLA	**gamma-linolenic acid**
GLP-1	**glucagon-like peptide-1**
GPX	**glutathione peroxidase**
GPX-Se	**selenium-established glutathione peroxidase**

GR	**glutathione reductase**
GSH	**Glutathione**
GSH-Px	**Plasma glutathione peroxidase**
H2O2,	**hydrogen peroxide**
HDL-C	**high density lipoprotein-cholesterol**
HF	**Heart failure**
Inos	**nitric oxide inducible**
IP3	**inositol trisphosphate**
LA	**linolenic acid**
L-Arg	**L-arginine**
LH	**Luteinizing hormone**
MAPK	**mitogen-activated protein kinase**
MDA	**Malonydialdehyde**
MDA	**Malondialdehyde**
mRNA	**messenger ribonucleic acid**
MSG	**Sodium Glutamate**
NADPH	**nicotinamide adenine dinucleotide phosphate**
nNOS	**nitric oxide neuronal**
NO	**Nitric oxide**
NOS	**nitric oxide synthase**
NT	**Nitro-tyrosine**

O2-.	**superoxide radical**
OH-●	**Hydroxyl radical**
ONOO	**peroxynitrite ,**
PCOS	**polycystic ovary syndrome**
PCOS	**polycystic ovary syndrome**
PGE	**Prostaglandin**
PGI2	**prostacyclin**
PKC	**protein kinase C**
PLA2	**phospholipase A2**
PLD	**phospholipase D**
PUFAs	**Polyunsaturated fatty acids**
RAGE	**receptorfor advanced glycation end products**
RAGE	**Receptor for advanced glycation end products**
ROS	**reactive oxygen species**
Rpm	**Round per minut**
SDA	**stearidonic acid**
SOD	**Superoxide Dismutase**
WBCs	**white blood cells**

Abstract

The study is performed during the period from17 of December 2021 to 17 of January 2022 and it was designed to investigate the role of *Spirulina* on heart and aorta damage induced by D-galactose (D-gal) in male rabbits . Some cardiac biomarkers were estimated in the serum protein cardiac biomarkers Endothelin 1(ET1),cardiac troponine I(cTnI) and Nitric oxide (NO),some oxidant peroxynitrite (ONOO) and malonydialdehyde (MDA) and antioxidant glutathione (GSH) concentration and Electrocardiograph waves (P, QRS and T) and ST ,QT interval were monitored and histopathological examination of heart and aorta tissues were performed.

Twenty healthy adult male rabbits,weighting (1100 -1500 gm), randomly and equally divided into four groups(5/group), normal as control group GI ,animals were received 150 mg/kg BW S/C daily of D-galactose as GII, animals were intubated 500mg/kg BW orally of *Spirulina* as GIII while GIV group was 150 mg/kg BW daily S/C of D-galactose with 500mg/kg BW orally of *Spirulina* for four weeks. blood samples were collected by cardiac punctures technique at 4 weeks of experimental study to evaluate the possibility of recovery of heart and aorta tissue damage induced by D-galactose.

The result of our study indicates a significant ($p \leq 0.05$)increase in the serum levels of ONOO, MDA with a significant increase in the serum level of Cardiac troponin I and Endothelin-1 in animal group received D-galactose in addition the results revealed a significant ($p \leq 0.05$) decrease in the serum level concentration of GSH and NO.

The results of electrocardiogram records the current study showed the prolongation in the waves (P,QRS,T) and (ST,QT)intervals in the GII group compared to control and GIII,GIV groups.

Oral intubation of 500mg/kg B.W daily of *Spirulina* with D-galactose in group GIV .The results showed an improvement recovery in heart and aorta finding through a significant($p \leq 0.05$) decrease in the serum level of the ONOO and MDA conc. ,it also showed a significant increase($P \leq 0.05$) in the serum level of GSH and NO conc. compared to GI,GII groups.

The present study result showed a significant ($p \leq 0.05$)decrease in the serum Endothelin-1 and Troponin-I in GIII and GIV compared with another groups .The recovery role of the *Spirulina* in the electrocardiograph in the GIV showed a significant($p \leq 0.05$)decrease in the (P, QRS,T)waves and (ST,QT) intervals compared to GII group .

The histological change examination of heart and aorta in GII received D-gal showed necrosis , inflammation and infiltration of inflammatory cell, and congestion ,on the other hand thickening in aorta compared with other group and study shown protective role of *Spirulina* in heart and aorta in GIV.

In conclusion , our study was conducted the injuries induced by D-gal ,it should be avoided especially in heart and aorta disorder from excess D-gal which can be fortified with orally intubation of 500mg/kg B.W of *Spirulina* to recover its adverse effects .

Chapter one:Introduction

1.1 Introduction

Aging is a multifactorial process associated with physiological decline. There is a substantial amount of data supporting the positive relation between the process of aging and the progressive decline in antioxidant function combined with increased mitochondrial ROS(reactive oxygen species) generation and increased accumulation of oxidant products.**(Sauland Kosinsky, 2021)**

Metabolic therapy involves the administration of a substance normally occurring in the body in order to favorably influence metabolic reactions occurring within the cell , and it is an under –utilized method for the treatment of different heart diseases .This form of therapy differs fundamentally from standard cardiovascular pharmacological therapy by improving cellular energy production ,suppressing free radical generation metabolic therapy of the *Spirulina* is an under-utilised method for the treatment of different heart diseases. This form of therapy differs fundamentally from standard cardiovascular pharmacological therapy. It involves the administration of a substance normally occurring in the body in order to favourably influence metabolic reactions occurring within the cell, by improving cellular energy production and suppressing free radical generation **(Hadj *et al* .,2007).**

Spirulina has antioxidant activity that contain of the material such, as compound element, γ-linolenic acid, tocopherol, the phycocyanin β-carotene, and phenolic, *Spirulina* suspension his act as strong scavenging activities control against superoxide and hydrogen peroxide radicals **(Huang *et al*.,2021).**

The cardiovascular benefits of *Spirulina* use are described in many papers and several reports suggesting that *Spirulina* may have a beneficial effect in the prevention of cardiovascular diseases. Decreases in blood pressure and plasma lipid concentrations, especially triacylglycerols and low density lipoprotein-cholesterol have been demonstrated as a result of oral consumption of *Spirulina*. **(Abdelhay *et al* .,2019 ; Hajati*et al* .,2020 ;Atallah *et al* .,2021)**

Spirulina has also been shown to indirectly modify the total cholesterol and high density lipoprotein-cholesterol values **(Atallah *et al* .,2021).** A recent human trial validates the above referenced review in an open sample of the population. Results showed that total cholesterol and triacylglycerols were significantly decreased in the *Spirulina* group, and HDL-C levels saw a significant increase, while both systolic and diastolic blood pressure decreased **(Firdous *et al* .,2021).**

D-galactose (D-gal) is a monosaccharide that has the same chemical formula as glucose, i.e., $C_6H_{12}O_6$. It is similar to glucose in its structure, differing only in the position of one hydroxyl group, this difference, gives galactose different chemical and biochemical properties to glucose . **(Prajit *et al*., 2020).**

Galactose treatment have shown to induce oxidative stress in the heart tissues of animals by increasing oxidant and free radicals with decrease antioxidant **(Lei *et al*., 2016; Dehghani *et al*., 2018; Li *et al*., 2019; Xu *et al*., 2019).**

D-gal is metabolized to galactose-1-phosphate at normal concentration by D-galactokinase or galactose-1-phosphate uridyl-transferase, but not at high levels. Instead, at increased concentration, D-gal is converted to galactitol, which accumulates in cells and then induces osmotic stress and ROS, **(Umbayev *et al*., 2020)**

When an exogenous dose of D-galactose is given beyond normal concentration, this can induce aging effects in several organs by increasing oxidative stress, apoptosis and inflammation. **(Ullah *et al*., 2020; Li *et al*., 2020)**

Azman and Zakaria, (2019) first established astrocytes agingmodel using D-galactose. It was discovered that D-galactose treatment significantly decreased the levels of glutamine synthetase messenger ribonucleic acid (mRNA) and protein in the cultured senescent astrocytes, and they displayed less resistance to the glutamate-induced gliotoxicity. The study was further continued by **(Cao *et al*., 2019)** to investigate the similarity between D-galactose-induced and naturally occurring senescent astrocytes.The results showed that the age-related changes in mitochondrial energy metabolism, glycolysis activity,and glutamate-glutamine recycling in D-galactoseinduced senescent are not fully consistent with those innaturally occurring senescent astrocytes.

Aging induced by D-galactose increases cardiac senescence markers and ag-gravates the impairment of metabolic parameters, cardi- ac and mitochondrial function, and increases oxidative stress ,inflammation, apoptosis**(Bo-Htay *et al* .,2020)**.

Aim of study:

The study was designed to investigate the ameliorate role of *Spirulina* on the damage induced by D-galactose on the heart and aorta by measuring the following parameters:

1. Cardiac and blood vessels damage biomarker e.g. Cardiac troponin-I, Endothelin-1 and Nitric Oxide (NO).
2. Electrocardiographs recording (P,QRS and T waves with ST, QT intervals)
3. Serum biochemical parameters: Oxidant(ONOO, MDA) and antioxidant (GSH)
4. Histological examination on the heart and aorta tissue by use Eosin and hematoxin stain and Masson trichome stain.

Chapter Two: Review Of The Related Literature

2.Review of The Related Literature

2.1. *Spirulina* platensis

Spirulina is a multicellular, filamentous cyanobacterium. On microscopic observation it appears as blue-green filaments composed of cylindrical cells arranged in unbranched trichomes characterized by helical shape (Fig. 2.3).**(Jung, *et al* .,2019).** Its name derives from the nature of its filaments **(Jung, F *et al*., 2019).**

Figure (2.3)Typical form of *Spirulina* platensis. **(Jung, *et al* .,2019).**

Spirulina is considered the "food of the future" because it contains over fifty healthy nutrients, including vitamins, minerals and amino acids. Compared to other foods or by weight, *Spirulina* is considered one of the most nutritious foods in the world: rich in proteins containing all essential amino acids, including B vitamins, iron, magnesium, potassium and many other vitamins and minerals, as well as antioxidants. **(Prisa,2019).**

Spirulina has the advantage over other microalgae that, as a bacterium, it does not have a cell wall of cellulose, so no chemical or physical processing is required to be digested **(Sushma and Sharma,2021).**

Spirulina is a nutraceutical food supplement, but its additional possible health advantages created a lot of interest in recent years as a possible source of medicinal compounds and has been shown in multiple studies shows its therapeutic values, including antioxidant, immunomo-stimulatory, anti-inflammatory, anticancer, antiviral, and antibacterial properties **(Afkhami *et al*.,2021; Metekia *et al* .,2022)**

2.1.1 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 **(Rahim *et al* .,2021).** *Spirulina* suspension naturally grows in high-salt alkaline water reservoirs in subtropical and tropical areas including America, Mexico, Asian and Central Africa **(Selim *et al* .,2018).**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 **(Sleim *et al*.,2018).** 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 **(Mizera *et al*., 2019).***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 nontoxic in nature **(Nazari *et al* .,2021).**

2.1.2.The basic biochemical composition of *Spirulina*

Spirulina has high quality protein content (55–70 percent of the dry weight), which is more than other commonly used plant sources such as dry soybeans (35 percent), peanuts (25 percent) or grains (8–10%). The biochemical composition of *Spirulina* can be summarized as follows **(Jung *et al* ., 2021).**

1. **Protein:** *Spirulina* contains unusually high amounts of protein, between 55 and 70 percent of dry weight 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**.(Rahman, 2021).**

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) **(Jung *et al*.,2019).**
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 **(Safari *et al* .,2021).**
4. **Minerals:** *Spirulina* is a rich source of potassium, and also contains calcium, chromium, copper, iron, magnesium, manganese, phosphorus, selenium, sodium and zinc. **(Jung *et al*.,2019).**
5. **Photosynthetic pigments:** *Spirulina* contains many pigments including chlorophyll a, xanthophyll, betacarotene, echinenone, myxoxanthophyll, zeaxanthin, canthaxanthin, diatoxanthin, 3-hydroxyechinenone, betacryptoxanthin, oscillaxanthin, plus the phycobiliproteins c-phycocyanin and allophycocyanin. *Spirulina* is a complete food resource of Chlorophyll, Phycocyanin, and Carotenoids. It is also has an application as a natural dye in food industry, cosmetic and pharmaceutical industry **(Darwish *et al* .,2020).**
6. **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 **(Hayes *et al* ., 2020).**
7. **Carotenoids:** *Spirulina* extracts containing carotenes and various carotenoids are frequently used as natural coloring materials **(Guroy *et al* .,2022).** 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 **(Germoush *et al* .,2022).**

2.1.2.1.*Spirulina* pharmaceutical compounds

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**.(Abdelhay *et al* .,2019).**

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 (**ElSheekh *et al* ., 2021).**

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. (Hilaland Duygu, 2020)

Spirulina is a concentrated source of gamma-linolenic acid (GLA) which is a precursor of prostaglandin (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**(El-Moursy *et al* .,2019; Sahinand Akpinar Bayizit, 2020).**

2.1.3.*Spirulina* metabolism

Spirulina platensis 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 **(Raheem *et al*.,2018).** 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 **(Raheem *et al*.,2018).**

Moreover, *Spirulina* has been produced as a superior nutrient because of its high content of protein and carotenes **(Mendez *et al* .,2020),**and it is the most industrially cultivated microalgal species **(Dehghani *et al* ., 2018).** Its superior characteristics such as high pH tolerance and high salt tolerance **(Mendez *et al* .,2020).**

Metabolic simulation using a genome-scale metabolic model has been widely used for rational metabolic design for the improvement of targetmolecule production **(Doan *et al* .,2021; Kumari and Bharti,2022).**

2.2 D-galactose

Chemical formula :- C6H12O6

D-galactose is an aldohexose , a reducing sugar that occurs naturally in the body and in many foods such as milk, butter, cheese, yogurt, honey, beets, plums, cherries, figs, and celery **(Azman and Zakaria,2019).**

Galactose was first identified in milk by Louis Pasteur in 1856, who denominated it as ‘lactose’. Only later, it was named ‘galactose’ from the Greek word ‘galakt’which means ‘milk’. Like most sugars, occurs more frequently in nature in its D-configuration. For a healthy adult, the maximal recommended daily dose of galactose is 50 g and most of it can be metabolized and excreted from the body within about 8 hr after ingestion **(Zhao *et al* .,2021).**

However, at high levels, it can be converted into aldose and hydroperoxide under the catalysis of galactose oxidase, resulting in the generation of reactive oxygen species (ROS) **(Hakimizadeh *et al* ., 2021).**

D-galactose is a reducing sugar, and when it accumulates in the body, it can react with the free amines of amino acids in proteins and peptides to form a Schiff base, an unstable compound **(Farajdokht *et al* .,2021).**

The biological importance of galactose, however, goes beyond its importance as a nutrient and a metabolite **(Bo-Htay *et al*.,2020).**

Galactose appears to have been selected by evolutionary pressure to also exert a crucial structural role. Indeed, despite the fact that it differs from glucose in the configuration of the hydroxyl group at the carbon-4 position, galactose has a myriad of specific functional and structural roles in living organisms that cannot be exerted by glucose **(Szilagyi,2019)**.

2.2.1 Galactose metabolism

D-galactose metabolized by The Leloir pathway which named after Luis Federico Leloir, who first described it **(Delnoy *et al* .,2021).** In the first step, galactose mutarotase facilitates the conversion of β-Dgalactose to α-D-galactose since this is the active form in the pathway,next, αD-galactose is phosphorylated by galactokinase to galactose 1- phosphate. In the third step, D-galactose-1-phosphate transferase converts galactose 1- phosphate to UDP-galactose using UDP-glucose as the uridine diphosphate source.Finally UDP-galactose 4-epimerase recycles the UDP-galactose to UDP-glucose for the transferase reaction. Additionally, phosphoglucomutase converts the D-glucose 1- phosphate to D-glucose 6-phosphate **(Delnoy *et al* .,2021).**

2.2.2.Oxidative stress and D-galactose

The term oxidative-stress is that characterized imbalances by the development of reactive oxygen species (ROS) related free radicals and the antioxidant protection system. Hydroxyl radical (OH●-), superoxide radical (O2 .-), and nitric oxide (NO) are the most common reactive oxygen species. **(Delnoy *et al* .,2021).**

Oxidative stress is a common pathophysiological phenomenon that causes damage to cells by continuous releasing of ROS that largely originated from biotic and abiotic stressors exposure **(Singh *et al* .,2022).**

The common features of various reactive oxygen species are cellular damage building blocks or biomolecules such as lipids, DNA, proteins **(Mazraedoost *et al*.,2021; Taurone *et al* .,2022).**

Oxidative stress is one of the main factors responsible for organ damage through oxidative injury due to lipid peroxidation marker production, which has caused several diseased conditions including diabetes, cardiovascular dysfunction, iron overload, iron deficiency anemia, cancer, reproductive dysfunction, Al-Zheimers disease and aging **(Senoner and Dichtl, 2017; Ahmad and Ahsan, 2020; Panda *et al*., 2022).**

Experimentally induced oxidative stress include exposure to 0.5 - 1% H2O2 **(ALdoseri and Khudair, 2016),** methionine overload **(Al-bazii and Khudiar,2017)** , high fructose diet **(Khudair,2016),** acrylamide **(Khudiar and Hussein,2017),** and high cholesterol diet**(Fareed *et al* .,2019).** Recently, D-galactose **(Sun *et al*.,2020),** D-galactoseamine **(Li *et al*., 2019),** monosodium glutamate **(Rosa *et al*., 2018)** and lipopolysaccharid are regarded as oxidative stress promoters **(Eren *et al*., 2018)**.

Excess D-galactose is reduced by galactose reductase to form galactitol which can lead to osmotic stress. Additionally, high level of d -galactose can be oxidized by galactose oxidase to yield hydrogen peroxide; increased hydrogen peroxide causes a decrease in antioxidant enzymes (SOD) **(Sun *et al*.,2020).**

Furthermore, d -galactose can initiate non-enzymatic glycation reactions to form advanced glycation end products (AGE s) after weeks or months. When AGE s reacts with their receptors (RAGE), ROS production occurs through NADPH oxidase activation. H2O2, hydrogen peroxide; SOD superoxide dismutase; RAGE, receptorfor advanced glycation end products; ROS, reactive oxygen species **(Bo-Htay *et al*.,2020)** as show in figure (2.1)

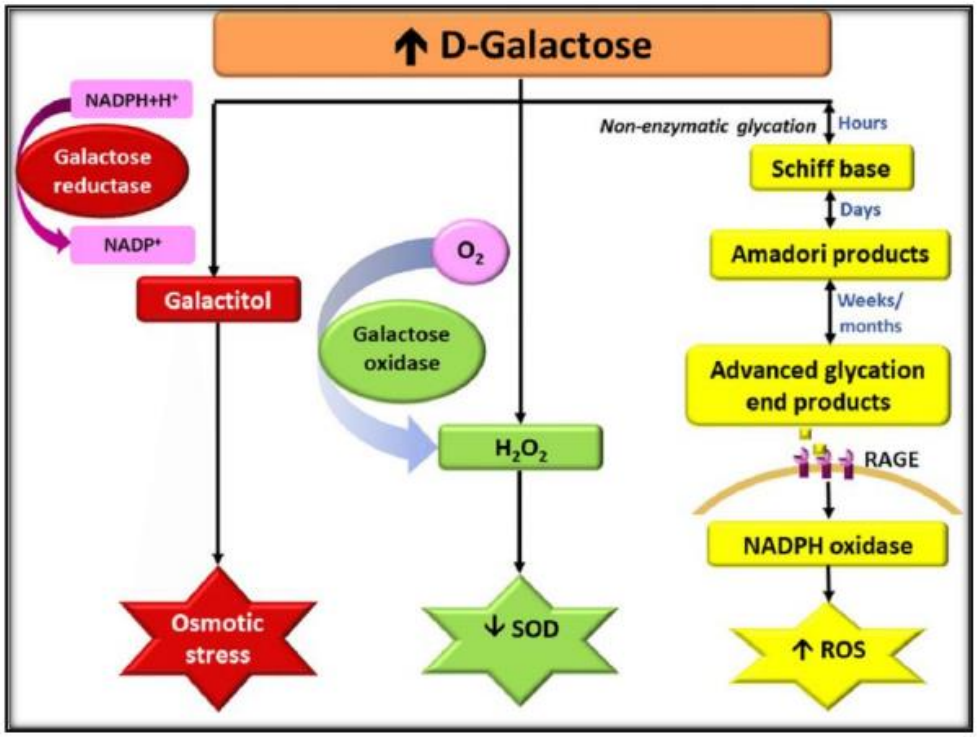


Figure (2.1): Induces oxidative stress by D-galactose. **(Bo-Htay *et al*.,2018)**

2.2.3D-galactose and aging

Aging is a process characterized by the accumulation of biological changes occurring gradually, leading to a person's functional decline over time **(Azmanand Zakaria , 2019).**

Generally, aging models can be classified into two categories: naturally aging model and accelerated aging model. Naturally aging model is time consuming and leads to a huge expense, therefore accelerated aging models are more preferred due to its easy application, shorter duration of study and higher survival rate of the animals throughout the experimental period **(Blat *et al* .,2021).**

There are various types of accelerated aging models which include hydroxyurea treatment and D-galactose-induced for in vitro studies and radiation-induced, jet lag-induced, senescence-accelerated prone mice, Klotho mouse, thymusremoved, and D-galactose-induced for in vivo studies **(Mahdi *et al* .,2021;Wang *et al*.,2020).**

Among these aging models, D-galactose-induced aging model is the most preferred due to its convenience, least side effects, and the higher survival rate throughout the experimental period (**Cebe *et al*., 2014**)

D-galactose-induced accelerated aging in various body organs : D-galactose-induced brain aging has been widely used and is established to be beneficial for aging studies. The administration of D-galactose into animals may induce brain aging similar in many ways to human brain aging, including cognitive deficits, mitochondrial dysfunction, neuronal degeneration and apoptosis, increased oxidative stress, and decreased ATP production **(Lee *et al* .,2020; Yooand Kim 2021; Cheng *et al* ., 2022**).

While the primary effects of age on the lungs are increased in alveolar size and reduced elastic recoil which may facilitates airway closure and increased in residual volume **(Rysz *et al* .,2021),**D-galactose treatment was proven to cause modification of lung elastic constitution **(Blat *et al* .,2021)** , also caused lung injury such as inflammatory infiltration and alveolar wall destruction **(Pan *et al* .,2021).**

On the other hand kidney are particularly affected by age. With age, functional renal mass, renal blood flow, and glomerular filtration rate decreases, with the occurrence of glomerulosclerosis, tubular atrophy, and interstitial fibrosis **(Wei *et al* .,2020)**

While in Male reproductive system aging is notable for reductions in testicular secretion of testosterone, increase in LH and FSH secretions, structural changes in the testis, penis, and accessory sexual glands, and alterations in sexual function, spermatogenesis, and fertility **(Singh *et al* .,2021),** also female reproductive system is affected by age, ovarian aging would cause reductions in plasma anti-mullerian hormone (AMH) level, D-galactose administration increased the AMH level approximately four-fold higher than the control abnormally high AMH levels are detected in ovarian cancer and polycystic ovary syndrome (PCOS) patients,high total

testosterone level and abnormal estrous cycle were also observed following D-galactose administration **(Dunn ,2021).**

On other hand integumentary system aging ,skin aging is a progressive loss of skin tissue, characterized by significant decrease in the thickness of dermis, less cell layers, and increase in accumulation of subcutaneous fat **(da Silva *et al* ., 2021).**

Also liver functions tend to decline gradually with aging mainly due to the attack of ROS. Since D-galactose is mainly metabolized in the liver, excess of D-galactose in the body may significantly affect the liver.D-galactose treatment have been shown to induce oxidative stress in the liver by increasing nitric oxide(NO), Malondialdehyde (MDA), and 8-hydroxy-2-deoxyguanosine (8-OHdG) and decreasing superoxide dismutase (SOD), catalase (CAT) , glutathione peroxidase (GPX) and total antioxidant capacity in liver tissues **(Lei *et al*., 2016; Chen *et al*., 2018; Margotta *et al*., 2018; Prajit *et al*. ,2020; Xu *et al*., 2019**)

Heart Aging increases the risk of cardiovascular diseases, associated with excess ROS and oxidative stress,D-galactose treatment have shown to induce oxidative stress in the heart tissues of animals by increasing MDA and nitric oxide (NO) and decreasing antioxidant enzymes such as SOD, CAT, glutathione peroxidase (GSH-Px), NOS, and total antioxidant capacity **(Dehghani *et al*. 2018; Azman and Zakaria, 2019; Xu *et al*.2019; Li *et al* ., 2021).**

Aside from oxidative stress,D-galactose caused a severe disarrangement in cardiac architecture ,the cardiomyocytes of the Dgalactose-treated animals were disordered with large interstitial spaces between the cells and higher number of apoptotic cardiomyocytes **(Wang *et al* .,2020),**in conjunction to that, the heart tissues of the Dgalactose-treated animals showed cardiac muscle fiber plumping, fuzzy structure and twisted shortening, significantly widened interval, and obvious capillary vessel of myocardial interstitial congestion **(Rusu *et al* ., 2020).**

Other C.V.S aging pathways ,several studies have shown that Mono Sodium Glutamate (MSG) is extensively metabolized by the intestinal enterocytes. that only small fractions of luminally administered glutamate are absorbed into the mesenteric venous blood. dietary glutamate is extensively metabolized by the intestine and that

oxidation to CO2 is a major metabolic fate. The oxidation to CO2 is a major metabolic fate of enteral glutamate even when the dietary intake fed is 3-4 fold higher than normal **(Brant,2021)** ,MSG causes many heart diseases such as Myocardial infaction ,atherosclerosis **(Abd-Elkareem *et al* .,2021**),also Klotho deficiency causes cardiac aging via impairing the Nrf2-GR pathway, it's caused heart function declined ,as evidenced by decreases in fractional shortening, ejection fraction, and cardiac output in addition heart size and weight, cardiomyocyte size and cardiac fibrosis were increased (**Chen *et al* .,2021**).

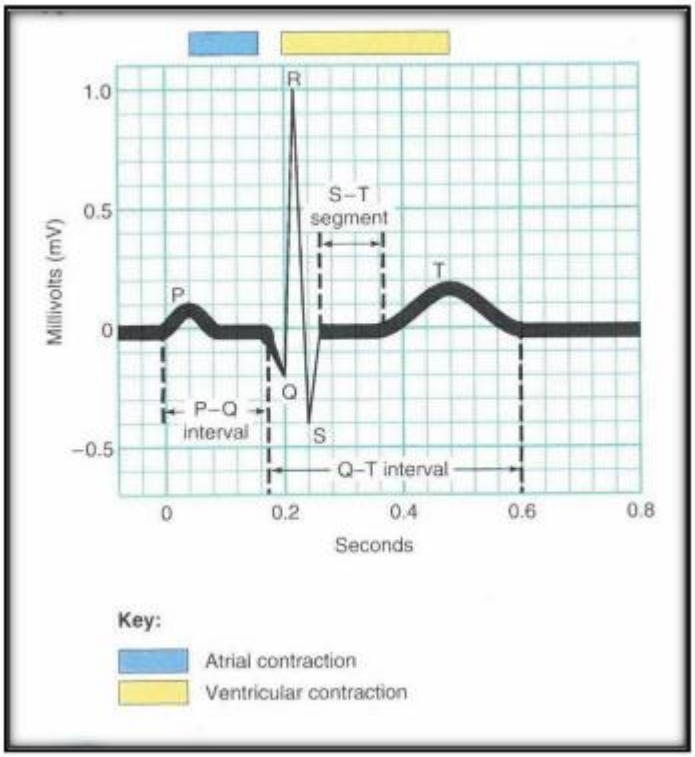
2.3 The Electrocardiograph (ECG)

Electrocardiography is a simple and practical diagnostic test in rabbits with suspected or confirmed cardiac disease. An electrocardiogram (ECG) is critical to diagnosing and managing arrhythmias or syncope. The ECG may also be a helpful addition to the cardiac database. However, one should not use an ECG to assess or detect chamber enlargement or hypertrophy. In most rabbits, the ECG can be recorded with the rabbit awake and under only minimal restraint **(Orcuttand Malakoff, 2020).**

Electrocardiography allows the electrical activity of the heart to be detected and recorded as an electrocardiogram (ECG) by applying an electrical stimulus to stimulate the cardiac muscle cells to coordinate heart contraction (systole) and relaxation (diastole). The electrical activity and, therefore, the ECG is affected by , heart size, electrical pathways through the heart and conduction through the heart and to the skin surface **(difazio *et al* ., 2021).**

The trace is used to detect,abnormal heart rate and rhythm,enlargement of the heart , electrical disturbance of the heart ,the presence of poor conduction between the heart and the skin (eg, fluid around the heart). **(Orcutt and Malakoff,2020).**

The electrocardiogram (ECG) shows waves that reflects the atrial and ventricular polarization and despolarization. After depolarisation of the atria (P wave on an ECG), the atria contract compressing blood in the atrial chambers and push residual blood out into the ventricles. This signifies the last part of the ventricular resting phase (diastole) and the blood within the ventricles is referred to as the end diastolic volume (EDV). The atria then relax and then the electrical impulse is transmitted to the



ventricles, which undergo depolarisation (QRS wave on an ECG). T wave refers to ventricular repolarization.**(Barros, 2019).**

Figure (2.2) Electrocardiograph wave and cardiac cycle events. **(Barros ,2019).**

2.4.Cardiac troponin

One of the most common causes of death is ischemic heart disease. Because of the high mortality and morbidity of myocardial infarction due to ischemic heart disease, rapid and reliable diagnosis is vital in clinical analysis **(Liu *et al*.,2016)**

Examinations and clinical findings used in the diagnosis of the disease do not always give reliable results, because of these reasons, proteins with increased amounts in the blood circulation due to cardiac injury can be used as biomarkers for early diagnosis. Cardiac troponins, the specific biomarker for myocardial tissue, consists of cardiac troponin I (cTnI) and troponin T (cTnT). For myocardial infarction, cardiac troponin I and troponin T are more specific and sensitive than other cardiac biomarker such as creatine-kinase and myoglobin **(Shah *et al*.,2018).**

One of the commonly used cardio specific biomarkers is cardiac troponin I (cTnI) **(Karimi *et al* .,2019 ; Cimen *et al*.,2020).**

Cardiac troponin I (cTnI) is a protein consisting of 210 amino acids with isoelectric point (pI) of 9.87 **(Cimen *et al* .,2020).** Cardiac troponin I (cTnI) used as acute myocardial infarction in the early diagnostic. The significant range of cardiac troponin I (cTnI) is 0.001–0.1 ng/mL in the blood **(Tager *et al* .,2019).**

The level of cTnI concentration is around 0.001 ng/mL in normal patients. The level of cTnI concentration is around 0.1 ng/mL in acute heart failure **(Fathil *et al* .,2015).**

2.5.Endothelin-1

Endothelin-1 (ET-1) is the main cardiac endothelin produced by cardiomyocytes, endothelial cells, and cardiac fibroblasts. It is interesting to note that ETA receptors represent most of the Endothelin receptors present on cardiomyocytes. The endothelin system in the heart seems to affect inotropy and chronotropy, but also mediate cardiac hypertrophy and remodeling in congestive heart failure through its mitogenic properties **(Stockelman *et al*.,2021).**

Endothelin-1 (ET1) is a 21-residue vasoconstrictive peptide , Endothelin peptides are resulted in multiple tissues, and they represent as modulators of vascular tone, cell proliferation and hormone production . Endothelin-1 (ET1) is concerning to endothelial dysfunction, and other cells are also able of output the ET-1, such as vascular smooth muscle cells , mesangial cells of the kidney **(Sharma *et al* .,2021).**

The endothelin system participates in both physiology and pathology of the cardiovascular system , although it is important to note that the extent of this contribution is both species- and vascular beddependent. The endothelin system has a role in the regulation of basal vascular tone and altered expression/activity of ET-1 could contribute to the development of diseases such as hypertension, atherosclerosis, and vasospasm after subarachnoid hemorrhage , of three endothelin isoforms only ET-1 is produced constitutively by endothelial cells and, therefore, it plays the most important role in regulating vascular function. Interestingly, under inflammatory

conditions vascular smooth muscle cells have the capacity to generate ET-1 **(Giusto,2018; Abeysingheand Roulston,2018).**

A number of stimuli, including thrombin, insulin, cyclosporine, epinephrine, angiotensin II, cortisol, inflammatory mediators, hypoxia and vascular shear stress have been shown to increase ET-1 levels**(Pathak *et al* ., 2021) .**

Endothelin-1 is secreted mainly on the basal side of the endothelial cells to act on ETA receptors on the underlying smooth muscles cells, as well as on ETB receptors on endothelial and on some smooth muscle cells. The endothelin produced vasodilatation when administered to anaesthetized, spontaneously hypertensive . This transient depression of blood pressure was explained via the production of NO and prostacyclin by the stimulation of ETB receptors located in endothelial cells . The subsequent vasoconstritive response is mediated by the action of ET-1 on ETA and ETB receptors on vascular smooth muscle cells **(Lamanna, 2020).**

2.6.Nitric oxide

Nitric oxide (NO) is a gaseous biological mediator that accounts for the vasodilator activity of endothelium-derived relaxing factor (EDRF) , a nonprostaglandin vasorelaxant substance first described in the endothelial cells , it is generated from the guanidino-nitrogen of L-arginine yielding citrulline , and plays a prominent role in controlling a variety of functions in the cardiovascular,immune, reproductive, and nervous systems **(Alrafiah,2021; Marletta,2021).**

Nitric oxide (NO) has an important role in myometrial function showing that NO generated by nitric oxide synthase (NOS) from arginine has a relaxant effect on the myometrium and that this relaxation is specifically blocked by inhibitors of NOS, Nitric oxide production is catalyzed by three major isoforms of NO synthase (NOS), neuronal (nNOS), inducible (iNOS), and endothelial (eNOS) enzymes. The nNOS and eNOS are considered to be constitutively expressed and activated by calcium entry into cells, whereas iNOS is calcium-independent, and its synthesis is induced in inflammatory and other cell types by stimuli such as endotoxin and proinflammatory cytokines. Although cDNAs for the respective proteins are found almost in all mammalian cells, under physiological conditions,eNOS is the major NOS isoform

expressed in the endothelial cells . In contrast, inflammation and cell damage are often associated with the expression of iNOS **(Brito *et al* ., 2018;Komorowskiand Hewlings,2019; Choi *et al* .,2019**).

In the heart, both, eNOS and iNOS, have been involved in signalling pathways that modulate the contractile properties of cardiac myocytes. The eNOS isoform is expressed within the heart in the endothelium both of the endocardium and of the coronary vasculature, in cardiac myocytes, and in specialized cardiac conduction tissue and its activity seems to be regulated by the contractile state of the heart . In contrast, iNOS expression is induced by cytokines in cardiac myocytes, endocardial endothelium, infiltrating inflammatory cells, vascular smooth muscle, fibroblast, and microvascular endothelium **(Mosqueira *et al* ., 2021).**

Nitric oxide is generated and released from the endothelial cells both under basal and agonist stimulated conditions. Shear stress and pulsatile flow are major stimuli that cause release of NO under basal conditions . In the cardiovascular system, NO not only causes vessel relaxation, but also inhibits platelet adhesion and aggregation, smooth muscle cell proliferation, monocyte adhesion,expression of different adhesion molecules and ET-1 production . The effects of NO on myocardial functions are still a matter of extensive investigation. There is now evidence showing that the basal endogenous NO production supports myocardial contractility and heart rate, whereas the expression of iNOS has been reported to have cardiodepressive actions because of the negative inotropic effects of NO at high concentrations**(Lee *et al* .,2020; Huang *et al* .,2021).**

Chapter Three: Methodology

3. Methodology

3.1. Materials

3.1.1. Instruments and Equipment:

All the devices utilized as a part of this study are summarized in Table 3.1.

Table 3.1. Apparatus and equipment with their manufactures.

No.	Apparatus and Equipment	Company	and Origin
1.	Anatomicalset(Scissors, Forceps, Scalpel)	Chemo lab	China
2.	Balance	Denver	Germany
3.	Beakers (100, 250, 500, 1000)	Chemo lab	India
4.	Centrifuge	Hettich	Germany
5.	Colony flask	Chemo lab	India
6.	Cotton	India	Entrepreneur
7.	Digital balance	Denver	Germany
8.	Digital camera	Canon	China
9.	Electrocardiography	Biocare	USA
10.	ELIZA printer	Epson	Japan
11.	ELIZA reader	Biotek	USA
12.	Eppendorf's tubes	Chemo lab	India
13.	Filter paper	Chemo lab	India
14.	Gel tube	Chemo lab	India
15.	Incubator	Lab tech	Korea
16.	Insulin syringe	Eldawlia	Egypt
17.	Light Microscope	Olympus	Japan
18.	Micropipettes(different volumes)	Dragonmed	China
19.	Microscope with camera	Olympus	Japan
20.	Microtome	Leica RM	USA
21.	Pipette tips (10 – 1000) µl volume	Chemo lab	China
22.	Refrigerator	Denka	japan
23.	Sensitive balance	Sartorius lab	Germany
24.	Slide and cover slip	Chemo lab	China
25.	Spectrophotometer	EMCLAB	Germany
26.	Surgical gloves	Chemo lab	China
27.	Syringe (1 ml, 5 ml)	Chemo lab	China
28.	Test tubes	Chemo lab	China
29.	Vortex	Sturat	United Kingdom
30.	Water bath	Labtech	Korea

3. 1.2. Chemicals and Kits All the chemicals and the standard kits used in this study are shown in Table 3.2.

Table 3.2: Chemicals and Kits with their suppliers.

	Chemicals	Origin	and Company
1.	Cardiac troponin ELIZA Kit	China	Gunagzhou
2.	D-galactose	India	Thomas Baker
3.	Dimethylsulfoxide(DMSO)	Chemie	LOBA
4.	Endothelin-1 ELIZA kit	China	Bioassay
5.	Eosine-hematoxylin stain	India	Himedia Lab
6.	Ethanol	Germany	Merck
7.	Formalin10%	USA	TEDIA company.
8.	Nitric oxide ElIZA KIT	USA	MyBiosource
9.	Paraffin wax	Germany	Merk
10.	Peroxnitrite ELIZA KIT	USA	MyBiosource
11.	*Spirulina*	USA	DXN
12.	Xylole	Spain	Scharlau
13.	Glutathione ELIZA KIT	China	Elabscience Biotechnology
14.	rabbit MDA ELIZA KIT	USA	MyBiosource

3.2. Experimental Animals

The study is performed during the period from17 of December 2021 to 17 of January 2022 .Twenty healthy adult male rabbits,weighting (1100 -1500 gm), were obtained .They were placed in the animal house of Veterinary Medicine College of Kerbala University, temperature was maintained at (21-25) C̊ the air of the room was changed continuous by using ventilation vacuum and with light /dark cycle 12: 12 h/day.

3.2.1. The experiment design

Twenty male rabbits were divided randomly into four groups, (5/group).

1. GI rabbits were recived distal water (D.W) (as control) for 4 weeks
2. GII rabbits were received (150mg/kg BW SC) of D-galactose dissolved by D.W for 4 weeks according to method reported by **Bo-Htay *et al* . (2020).**
3. GIII rabbits were received (500 mg/kg BW orally) of *Spirulina* dissolved with D.W for 4 weeks according to method reported by**Abdel-Daim *et al* . (2020).**
4. GIV rabbits were received (150 mg/kg BW SC) of D-galactose combined with (500mg/kg BW orally) of *Spirulina* for 4 weeks

3.2.2 Blood collection

Animals were anesthetized by injection of (90mg/kg) Ketamine and (40mg/kg) Xylazine .Blood samples were collected at 4 weeks of study of experiment via heart puncture technique was done by using a 10 ml disposable syringe and 10 ml of blood was drawn slowly and gently . Ten ml of blood collected in gel test tubes (for serum preparation) which leaves for 30 minutes in room temperature and then used for getting serum by centrifugation at 3000 rpm for 15 minutes to separate serum and put in Eppendorf tubes which kept at freezer in -20C . Eppendorf tube which kept at freezer in -20C(Amin and Ahlfors, 2008) . The abdominal lumen was opened and heart and aorta were removed and then placed in formalin (10%) as fixative for histological preparation

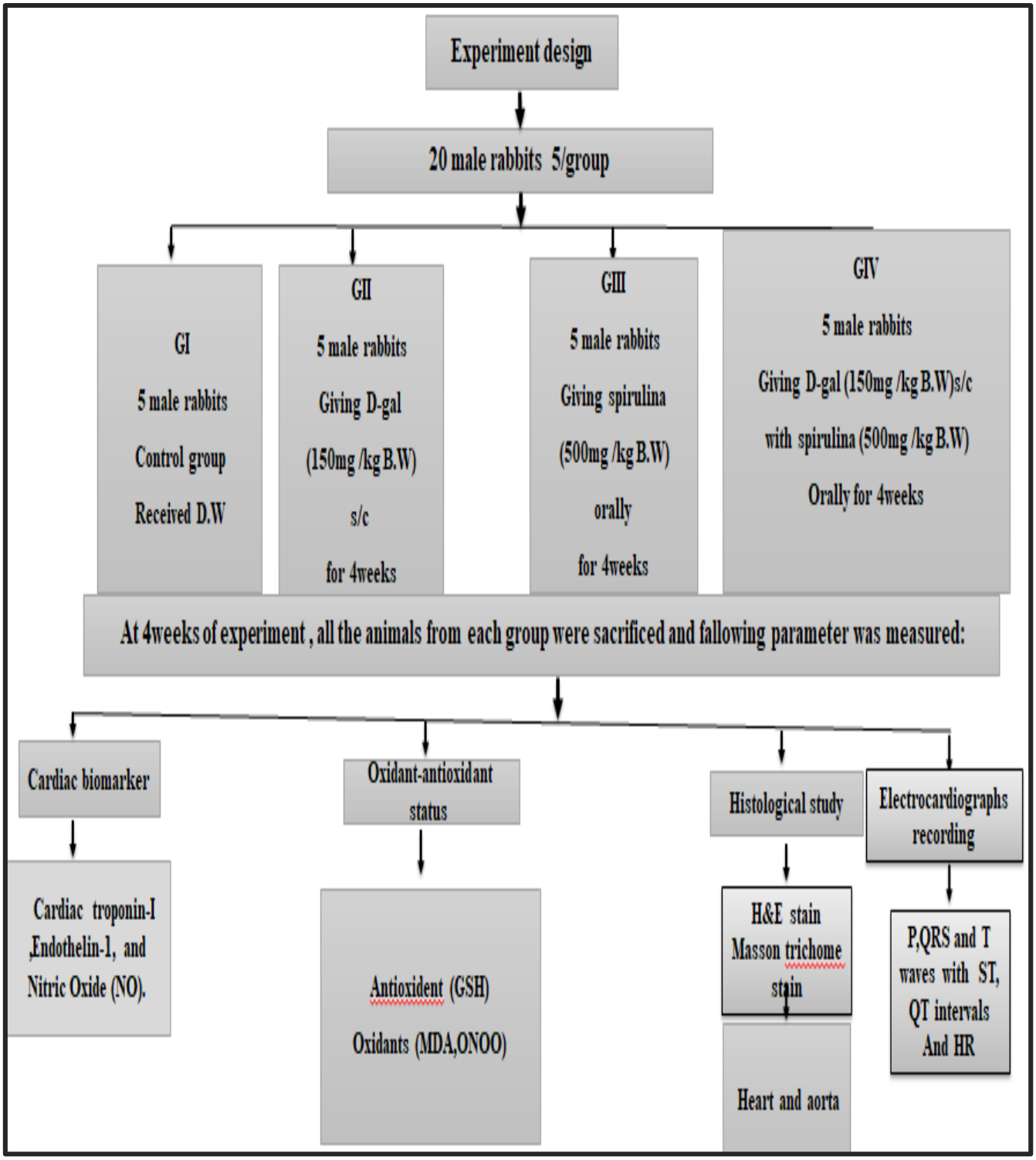


Figure (3.1): Represented experimental design .

3.3. Electrocardiograph

3.3.1.Electrocardiogram recording

- Place the rabbit in sternal recumbency on a table or an assistant's lap
- Attach standard crocodile clip electrodes(with the teeth bent outwards or sawn off to reduce discomfort) to a fold of skin just proximal to both elbows and midway between the stifles and hocks
- Apply surgical spirit to the skin and electrodes to enhance conduction
- Place ECG cables , with the red cable on the right forelimb , the yellow cable on the left forelimb, the green cable on the left hindlimb and the black cable (earth electrode) on the right hindlimb
- After a brief period of acclimatisation (minimum 10 seconds), take ECG recordings **(Orcutt and Malakoff,2020),**Figure (3-2).

The electrodes were fixed calmly and accurately after calming the animal repeated the process multiple times before taking readings in order to tame the animal on the device and to keep the animal calm and not to get a state of fear and panic and after the connection leaves the animal for 10 to 15 minute after making sure that the animal was quiet .The measurement was done on the basis of the method of mono-lead which was called (Einthoven Triangle) ,the reading on a method on the amplification force 10Mm/Mv and the electrical voltage 25Mm/s and speed ½ second .

The measurement of the ECG was done without anesthesia , where we note in the diagram of the heart on the special graphic paper which is divided into large squares divided into small squares each large square contains five small squares and be read horizontally and vertically .The vertical –axis voltage of the reading represent the electrical voltage .The small square is equal to(1Mm=0.1Mv) . The horizontal shape (Horizontal axis-time) on the reading. The small square is equal in milliseconds 1Mm=0.04second. The speed of the paper is equal and the p wave represent depolarization in the atria . AS the atria muscle are less thick and smaller than the ventricular muscles ,the device cant capture the electric waves of their propagation and repolarization and

amplify it through the amplifier , the QRS complex wave represent the depolarization of the ventricular muscle .The T wave represent the repolarization of the ventricular muscle which is a state of rest and relaxation.

Figure(3.2) represents how to connect the electrodes of electrical diagram of the male rabbits ,represent the red electrode (right arm) and yellow electrode (left hand) and electrode green (left leg) while black electrode represent ground connector (right leg) **(Orcutt and Malakoff,2020).**

3.4.Biochemical parameter :

3.4.1.Estimation of cardiac markers

3.4.1.1.Estimation of serum cardiac troponin I(ng/ml) .

Cardiac troponin I(cTnI) was measured by kit produced by Guangzhou, P.R.China .company **Adams, (1994)** ,as show Appendix I.

3.4.1.2. Estimation of serum Endothein-1 (ng/l)

Endothelin 1 (ET-1) was measured by Rabbit Endothelin 1 Enzyme-Linked Immunosorbent Assay (ELISA) kit by Bioassay Technology Laboratory by method of **Goldie , (2000)** ,as Shown appendix II.

3.4.1.3. Estimation of serum Nitric oxide (NO) µM/L

Nitric oxide (NO) was measured by method of **Chang *et al* . (1998)** , as Shown appendix III.

3.4.3. Estimation of serum oxidant

3.4.3.1. Estimation of serum peroxynitrate (ONOO) µM/L

Peroxynitrate was measured by method of **Vanuffelen , (1998)** , as Shown appendix IV .

3.4.3.2. Estimation of Serum Malondialdehyde (MDA):µM/l

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

3.4.4 .Estimation of serum reduced Glutathione (mg/dl)

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

3.5. Histological study

For histopathological study of rabbit were anaesthetized and sacrificed by withdrawal of blood from heart .immediately after death the heart and aorta with its main branches were excised , blotted open longitudinally and preserved in 10% neutral formalin buffer solution till the preparation of histological section several tissue section were prepared according to **Mescher , (2010),** as Shown appendix XI .

3.6. Statistical Analysis

Data was analyzed as one-way ANOVA using the general linear model (GLM) procedure to SPSS 22.0 software (Corp, 2011). Four treatment means were separated using a “protected” Duncan`s analysis in level ($p<0.05$).

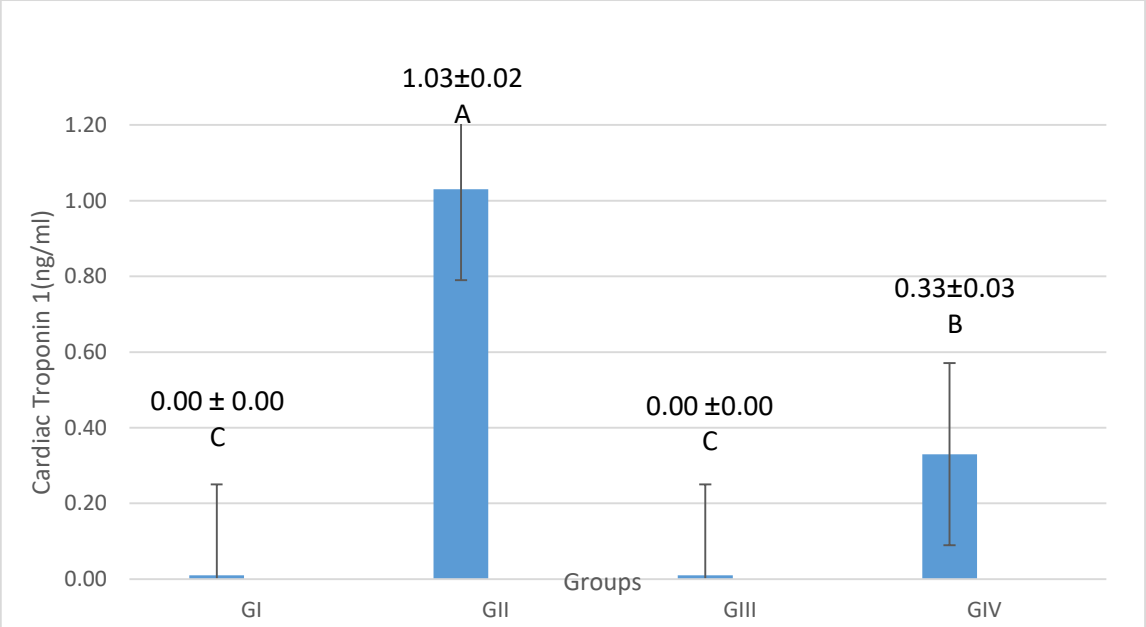
Chapter four: Results and Analysis

4.Results and Analysis

4.1 Effect of *Spirulina* on proteins cardiac biomarkers (Cardiac troponin I , Endothelin 1, Nitric Oxide) on D-galactose treated male rabbits

4.1.1.Cardiac troponin 1

The main value of serum Cardiac troponin 1 show a significant ($p \leq 0.05$) increases in GII group when compared with GI, GIII and GIV groups. On the other hand the main value of serum Cardiac troponin 1 show a significant ($p \leq 0.05$) decrease in GIV group when compared with GI and GIII. While there is no significant ($p > 0.05$) differences between GI and GIII groups,figure (4.1) .

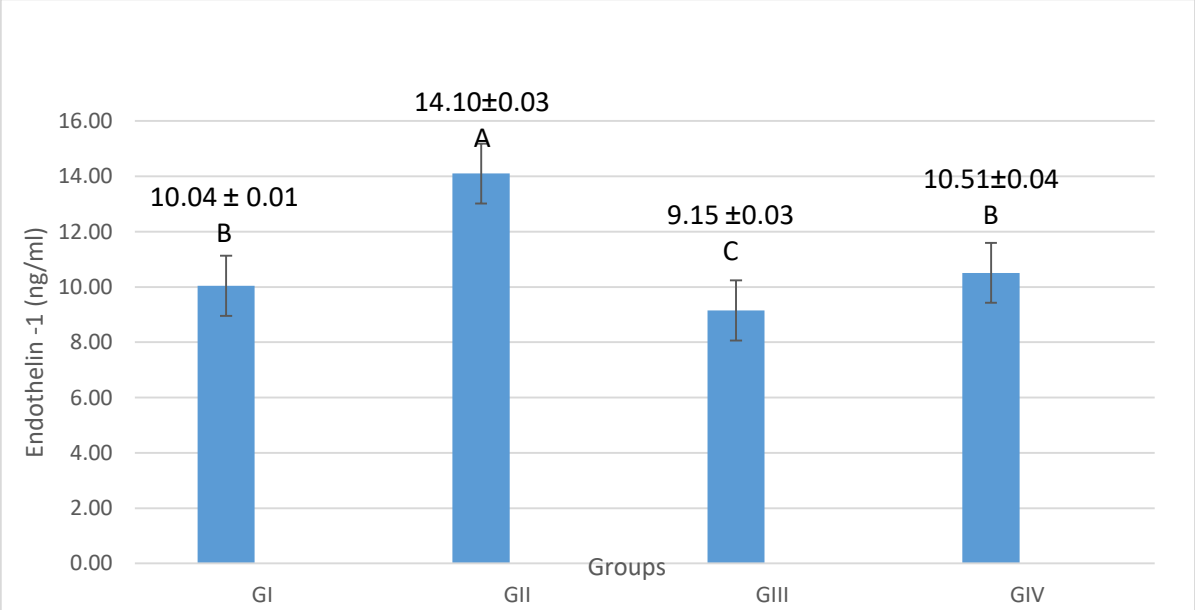


Value are expressed as mean ±SE n=5/group , significant differences ($p \leq 0.05$)The different letters refer to significant difference between difference groups GI =control, GII =D-gal 150 mg /kg /day, GIII=*Spirulina* 500 mg /kg /day, GIV =D-gal+*Spirulina*

Figure (4.1) Effect of daily oral intubation of *Spirulina* for 4 weeks on serum Cardiac Troponin 1(ng/ml) concentration of D-galactose treated male rabbits .

4.1.2Endothelin -1

The main value of serum Endothelin -1 show a significant ($p \leq 0.05$) increases in GII group when compared with GI, GIII and GIV groups. Beside a significantly ($p \leq 0.05$) decrease in the main value of serum Cardiac troponin 1 in GIII group when compared with G1 and G IV group. While there is no significant ($p > 0.05$)differences between G I and G IV groups ,figure (4.2).

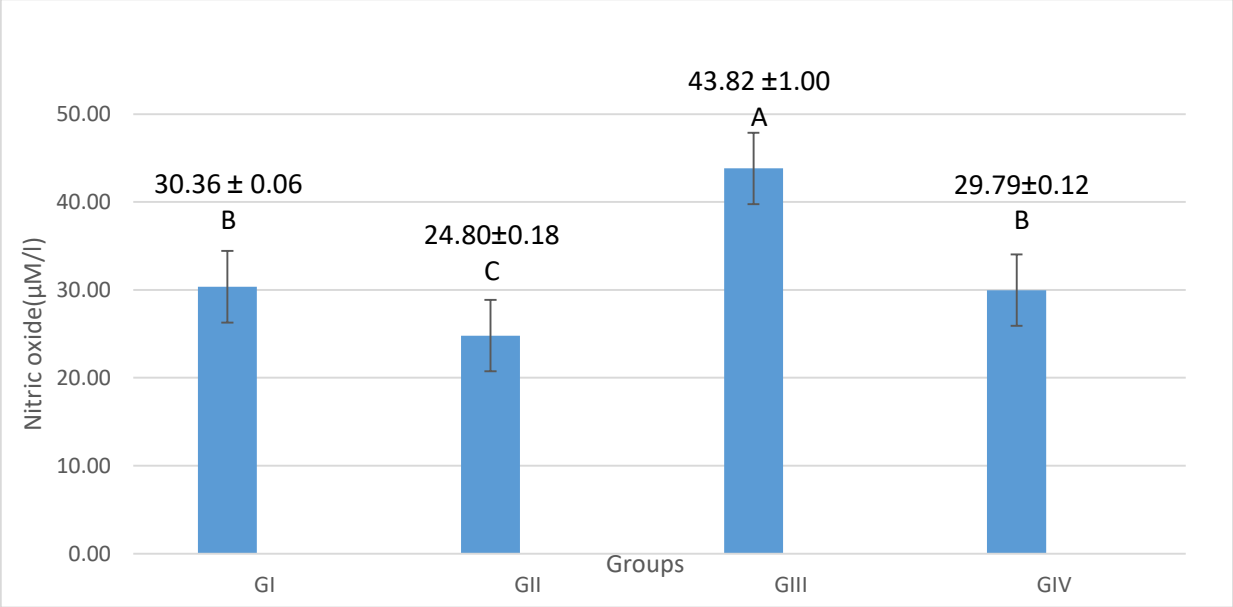


Value are expressed as mean ±SE n=5/group , significant differences ($p \leq 0.05$)The different letters refer to significant difference between difference groups GI =control, GII =D-gal 150 mg /kg /day, GIII=*Spirulina* 500 mg /kg /day, GIV =D-gal+*Spirulina*

Figure (4.2) Effect of daily oral intubation of *Spirulina* for 4 weeks on serum Endothelin -1 (ng/ml) concentration of D-galactose treated male rabbits

4.1.3Nitric oxide

The main value of serum nitric oxide show a significant ($p \leq 0.05$) decrease in GII group when compared with GI, GIII and GIV groups. Beside a significant ($p \leq 0.05$) increases in the main value of serum Nitric oxide in GIII group when compared with G I , G II and G IV group. While there is no significant ($p > 0.05$) differences between GI and GIV groups, figure (4.3)



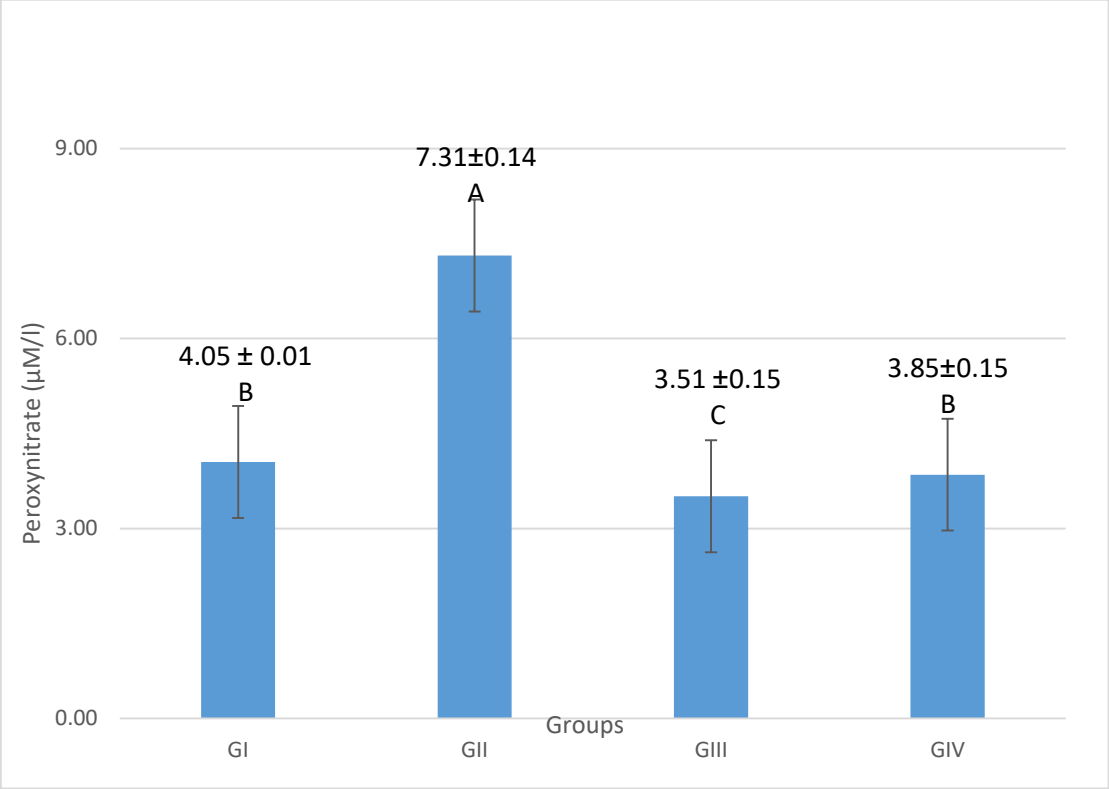
Value are expressed as mean ±SE n=5/group , significant differences ($p \leq 0.05$)The different letters refer to significant difference between difference groups GI =control, GII =D-gal 150 mg /kg /day, GIII=*Spirulina* 500 mg /kg /day, GIV =D-gal+*Spirulina*

Figure (4.3) Effect of daily oral intubation of *Spirulina* for 4 weeks on serum Nitric oxide(µM/l) concentration of D-galactose treated male rabbits

4.2 Effect of *Spirulina* on serum oxidant (ONOO,MDA) concentration and serum antioxidant (GSH)in D-galactose treated male rabbits

4.2.1 Peroxynitrate (ONOO)

The main value of serum ONOO show a significant (p≤0.05) increase in GII group when compared with GI, GIII and GIV groups. Beside a significant (p≤0.05) decreases in the main value of serum ONOO in GIII group when compared with G I and G IV group. While there is no significant (p>0.05) differences between GI and GIV groups, figure (4.4)

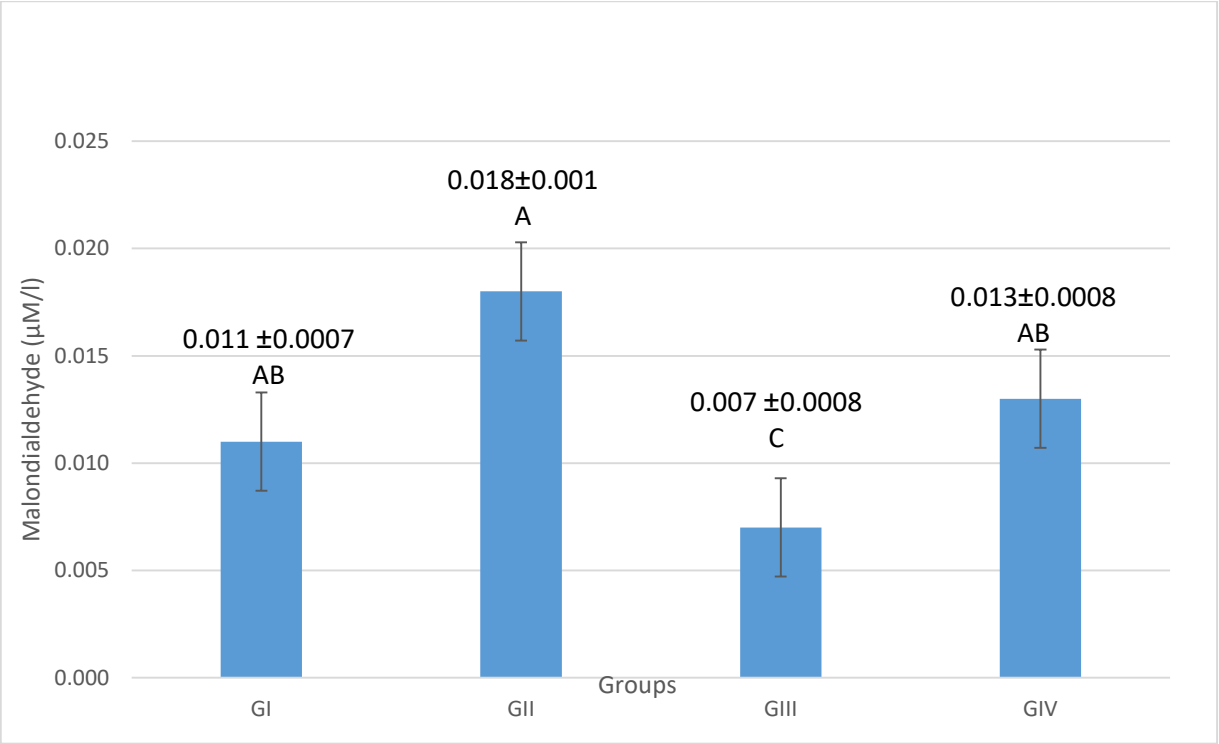


Value are expressed as mean ±SE n=5/group , significant differences (p≤0.05)The different letters refer to significant difference between difference groups GI =control, GII =D-gal 150 mg /kg /day, GIII=*Spirulina* 500 mg /kg /day, GIV =D-gal+*Spirulina*

Figure (4.4) Effect of daily oral intubation of *Spirulina* for 4 weeks on serum Peroxynitrate (µM/l) concentration of D-galactose treated male rabbits

4.2.2Malondialdehyde (MDA)

The main value of serum MDA show a significant ($p \leq 0.05$) increase in G II group when compared with GI, GIII and GIV groups. Beside a significantly decreases in the main value of serum Malondialdehyde in G III group when compared with G I and G IV group. While there is no significant ($p > 0.05$) differences between G I and G IV groups, figure (4.5)

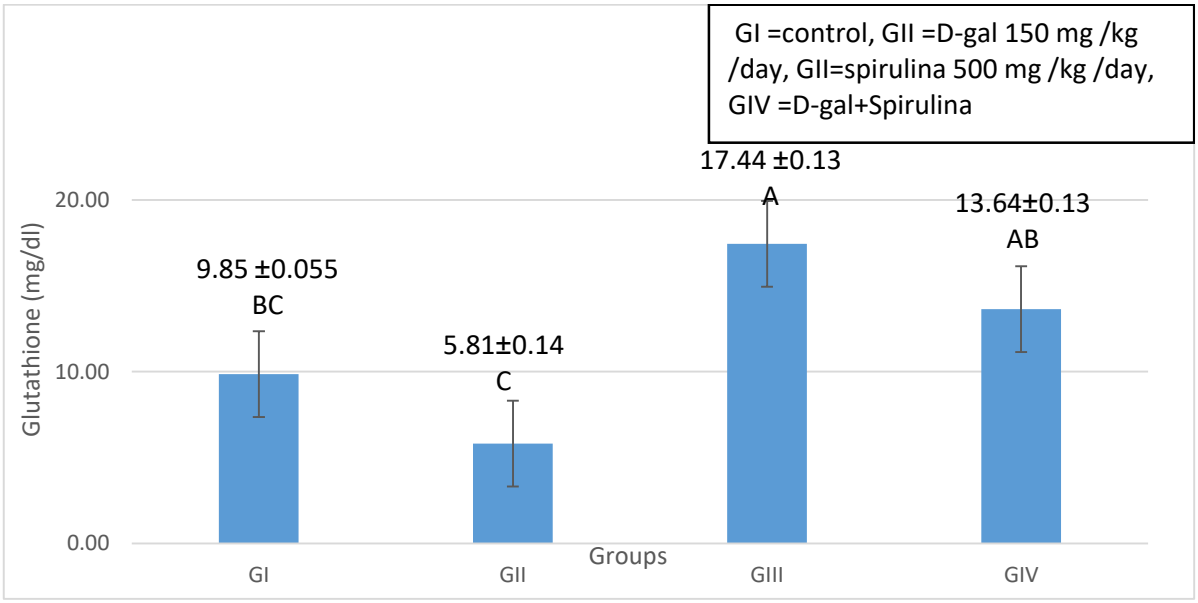


Value are expressed as mean ±SE n=5/group , significant differences ($p \leq 0.05$)The different letters refer to significant difference between difference groups GI =control, GII =D-gal 150 mg /kg /day, GIII=*Spirulina* 500 mg /kg /day, GIV =D-gal+*Spirulina*

Figure (4.5) Effect of daily oral intubation of *Spirulina* for 4 weeks on serum Malondialdehyde (µM/l) concentration of D-galactose treated male rabbits

4.2.3Glutathione (GSH)

The main value of serum GSH show a significant ($p \leq 0.05$) decrease in GII group when compared with GI, GIII and GIV groups. Beside a significantly increases in the main value of serum Glutathione in GIII group when compared with other groups, figure (4.6)



Value are expressed as mean ±SE n=5/group , significant differences ($p \leq 0.05$)The different letters refer to significant difference between difference groups GI =control, GII =D-gal 150 mg /kg /day, GIII=*Spirulina* 500 mg /kg /day, GIV =D-gal+*Spirulina*

Figure (4.6) Effect of daily oral intubation of *Spirulina* for 4 weeks on serum Glutathione (mg/dl) concentration of D-galactose treated male rabbits

4.3 Protective role of *Spirulina* in Electrocardiograph (ECG) in D-galactose treated male rabbits.

4.3.1.Protective role of *Spirulina* in Electrocardiograph (ECG) waves in D-galactose treated male rabbits.

There was no significant (P<0.05) difference in wave (P,ORS, T) in the mean value of lead II ,between experimental groups (GI,GIV) as shown in table (4.1).

There was a significant increase (p<0.05) in the mean value of waves (P,QRS,T)in the lead II was shown in the end of the experiment and detected after 4 weeks in GII received D-gal compare to GIII received *Spirulina* and GIV while combined of D-gal and *Spirulina* treated rabbits and caused significant decrease(p<0.05) in compared to GIl. Also the same table showed A significant decrease(p<0.05)in the mean value of HR in the GII compared to another groups(GI,GIII,GIV).and shown a significant increase (p-0.05) in mean value of (ST.QT) intervals in the GII compared to another groups(Gl, GIII and GIV),Tables (4.1);(4.2)

Table (4.1)Effect of daily oral intubation of *Spirulina* for 4 weeks on Electrocardiograph (ECG) waves and heart rate of D-galactose treated male rabbits

Groups / Wave	GI (Control)	GII (D-galactose)	GIII (*Spirulina*)	GIV (D-gal +*Spirulina*)
QRS (s)	**4.17 ± 0.007 B**	**5.48 ±0.017 A**	**4.03 ±0.013 C**	**5.03±0.013 B**
QRS (mv)	**0.02 ± 0.001 C**	**0.04±0.004 A**	**0.03±0.002 B**	**0.02 ±0.001 C**
P (s)	**1.17 ± 0.004 C**	**1.32 ±0.007 A**	**1.14±0.009 B**	**1.17 ±0.003 C**
P(mv)	**0.02 ± 0.005 C**	**0.04 ±0.00 A**	**0.03 ±0.004 B**	**0.02 ±0.002 C**
T(s)	**2.65 ± 0.009 AB**	**3.01 ±0.006 A**	**2.15 ±0.013 B**	**2.02±0.009 C**
T(mv)	**0.07 ± 0.002 B**	**0.09 ±0.003 A**	**0.07 ±0.005 B**	**0.04±0.004 C**
HR (b/m)	**249.66 ± 1.22 BC**	**207.34 ±0.52 C**	**252.16 ±0.07 AB**	**255.04±0.01 A**

Value are expressed as mean ±SE n=5/group , significant differences ($p \leq 0.05$)The different letters refer to significant difference between difference groups GI =control, GII =D-gal 150 mg /kg /day, GIII=*Spirulina* 500 mg /kg /day, GIV =D-gal+*Spirulina*

Table (4.2)Effect of daily oral intubation of *Spirulina* for 4 weeks on Electrocardiograph (ECG) intervals concentration of D-galactose treated male rabbits

Groups / **Interval**	**GI (Control)**	**GII (D-galactose)**	**GIII (*Spirulina*)**	**GIV (D-gal +*Spirulina*)**
ST(s)	**0.11 ±0.001 B**	**0.13±0.002 A**	**0.10 ±0.001 C**	**0.11±0.001 B**
QT(s)	**0.118 ± 0.000 B**	**0.147 ±0.001 A**	**0.117 ±0.000 B**	**0.116±0.013 B**

Value are expressed as mean ±SE n=5/group , significant differences ($p \leq 0.05$)The different letters refer to significant difference between difference groups GI =control, GII =D-gal 150 mg /kg /day, GIII=*Spirulina* 500 mg /kg /day, GIV =D-gal+*Spirulina*

4.3 .2Protective role of *Spirulina* in Electrocardiograph (ECG) intervals in D-galactose treated male rabbits.

Male rabbits' electrocardiograph intervals of GI (Control) revealed no significant differences in GI. The length of the period between the systolic ventricular and diastolic which represents the QT interval equals 0.12 milliseconds as a result of the distance between the R-R interval being 0.24 milliseconds. The P-R interval ratio represents the time required for the electrical signal interval from the atrium to the SA node and is equal to 0.08 millisecond.

As shown in figure, the value of the electrical wave QRS complex representing ventricular depolarization equals 0.06 millisecond Appendix (VII).

Electrocardiograph of GII received D-gal in male rabbits, showed significant prolongation of (P, QRS,T), alteration of ST,QT intervals shown in appendix (VIII), and decrease in heart rate compared to other groups (GI,GIII,GIV).

While Electrocardiograph GIII received*Spirulina* demonstrating nonsignificant prolongation of waves (P,QRS,T) in GIII and nonsignificant prolongation of HR compared to GII as shown in appendix (IX).

On the other hand Electrocardiograms of GIV combined of D-gal and *Spirulina* treated rabbits revealed no significant change in prolongation waves (P,QRS,T) and no significant change in (ST, QT) intervals, as shown in appendix (X).

4.4.Histological study

4.4.1.Histomicrometry examination

In the control group of our study, we used both of the histology stains(H&E and Masson trichome). The elastic artery seems to have three layers: the tunic intima, which is made up of endothelium, basal lamina, subendothelial connective tissue, smooth muscle cells, and the first layer, the internal elastic lamina. The tunic medium had a second layer of collagen fibers that were thicker and had more fenestrated elastic laminae and less smooth muscle. The third layer of connective tissue is called the tunic adventitia. It is made up of collagen, elastic fibers, and vaso vasorum. Figure(4.7,4.8)

Aortic artery histomorphology in rabbits treated with D-galactose GII was examined in comparison to those of controls and other groups. showed irregular and necrosis (pyknosis)nuclei of endothelial cells in the intimal surface, loss of squamous cells with regular tunica aortic media, dearranged or deterioration of elastic fiber architecture and moderate to sever necrosis of smooth muscles fibers in the tunica media, and sever mononuclear inflammatory cells infiltration with comparatively thickness on the adventitia . Figure(4.9,4.10).

On the other hand, we found that the intima, media, and serosa layers of the aortic wall in *Spirulinal*-treated rabbit groups looked like normal microscopic structures, semi normal tunica intima endothelia, and significant regular wide tunica media with arranged elastic and collagen fibers with outer area of tunica adventitia Figure(4.11;4.12).

A microscopic examination of the aortas wall of rabbits administered D-galactose and *Spirulina*l reveals that their architecture is near look like to that of the control groups, when compared to the D-galactose-treated rabbits group, and markedly alleviated the damage D-gal disorientation in aortic tissue, by improving histological picture of artery layers ,depletion of tunica media by decrease collagen fibers and return dearranged elastic lamina so, mild desquamations necrosis of smooth muscles with slight vacuolation and less or absent blood vessel congestion and less mononuclear inflammatory infiltrations cells. Figure(4.13; 4.14).

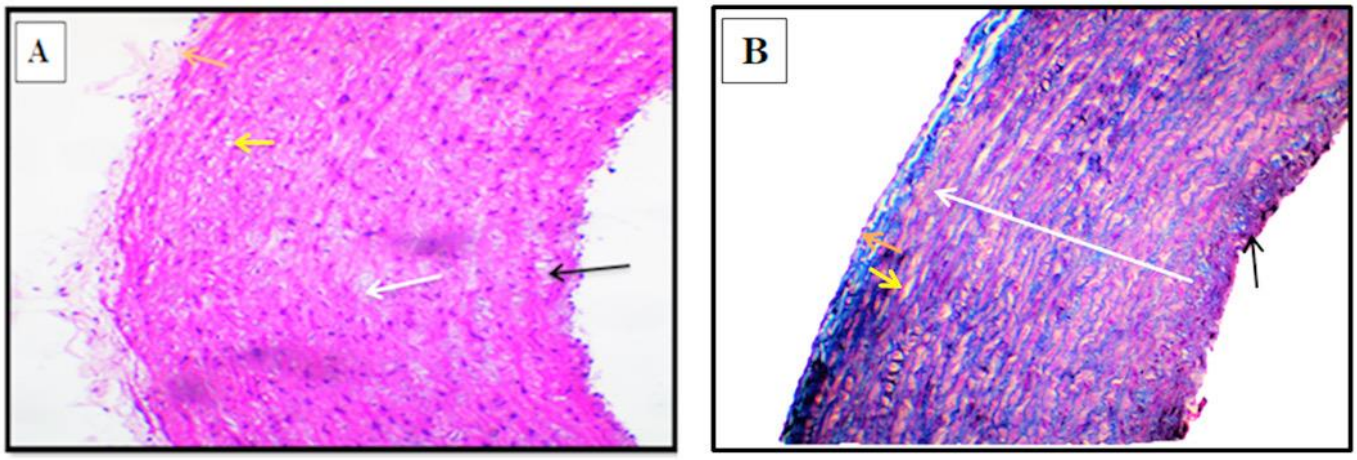
Finding results in the heart muscles study which was consist from the basic histological structures tissues. Inner endocardium layer with purkinje fibers and myocardium layer made up myocardial fibers with markedly central nuclei and connective tissue contain blood vessels were all visible in photomicrograph of section cardiac tissues taken from a rabbit that was assigned to the control group in the study. Figure (4.15,4.16)

On the other side of analysis histological photograph of the D-gal treated animals heart tissues showed had lost its normal architectural appearance, had an abnormally thickened endocardium and an increase in Purkinje fibers with severe fatty tissue precipitation. It also showed that myocardial fibers had become disorganized, with remarkable pyknotic nuclei, indicating myofiber necrosis . Inflammatory cells infiltration with sever congested blood vessels . Figure (4.17,4.18).

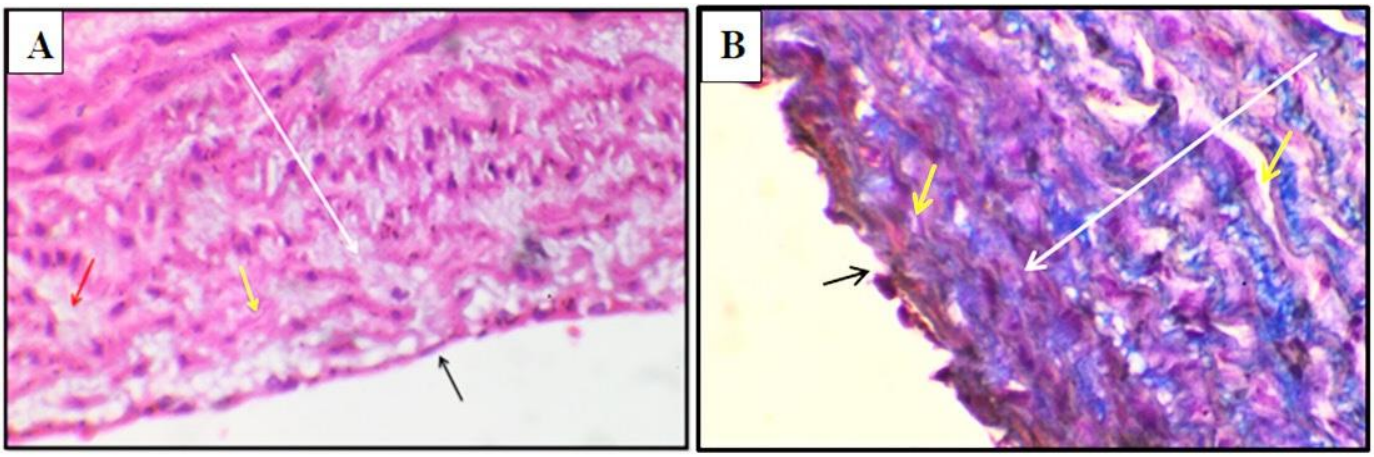
The cardiac histomicrometry section of rabbit groups were given *Spirulina* showed normal architectural features in an endothelia of endocardium, straitening and arrangement fibers of myocardium layer with their large significant nuclei. Figure (4.19,4.20).

While these findings were observed in heart tissue sections from animals treated with (antioxidant) *Spirulina* and (oxidative) D-galactose. Mild thickened

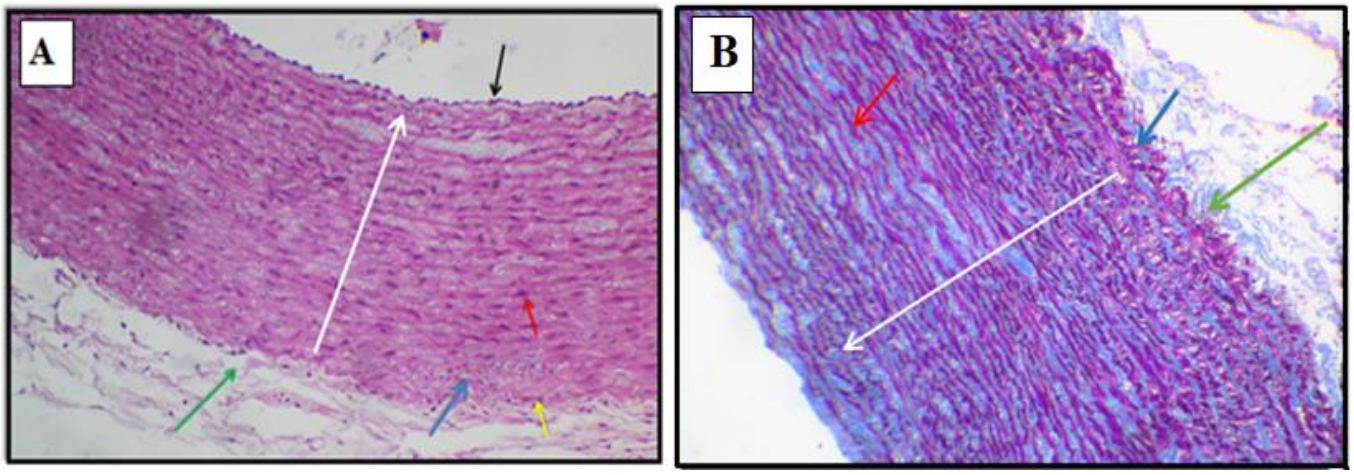
endocardium, significant arrangement of myocardial fibers in the myocardium indicating their reversible to normal arrangement, slight necrotic changes in some myofibers represented by pyknotic nuclei with decreased collagen fibers in some regions and accumulation others, and mild to moderate congestion in blood vessels . figure (4.21,4.22).



Figure(4.7) Photomicrograph of Aorta section from a control group animal showed , normal histological architecture of aortic wall, normal tunica intima(black arrow) endothelia , significant regular wide tunica media(white arrow)with arranged elastic fibers (yellow arrow), and narrow ,outer area of tunica adventitia (orange arrow). (A-H and E, B- Masson's Trichome ,10X).



Figure(4.8) Photomicrograph of Aorta section from a control group animal showed , normal histological architecture of aortic wall, normal tunica intima(black arrow) endothelia , significant regular and eosinophilic stained tunica media(white arrow)with arranged elastic fibers (yellow arrow) ,remarkable smooth muscle fibers which revealed in deep red to purple color (green arrow), collagen fibers (red arrow) . (A-H and E**;** B-Masson's Trichome, 40X).



Figure(4.9) Photomicrograph of Aorta section from D galactose treated group animal showed, necrosis of tunica intima endothelia(nuclear pyknosis) (black arrow) , regular tunica media(white arrow)with de arranged elastic laminae (yellow arrow) , moderate to sever necrosis of smooth muscle fibers(red arrow), sever Mononuclear inflammatory cells infiltration(blue arrow),and relatively thickness on the tunica adventitia (green arrow). (A-H and E,B- Masson's Trichome, 10X).

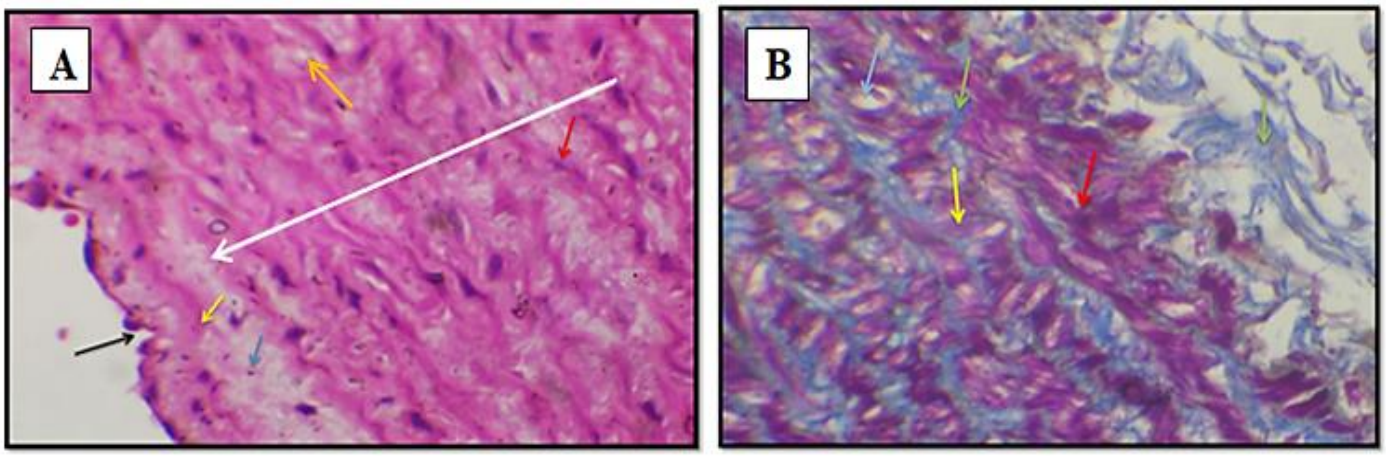
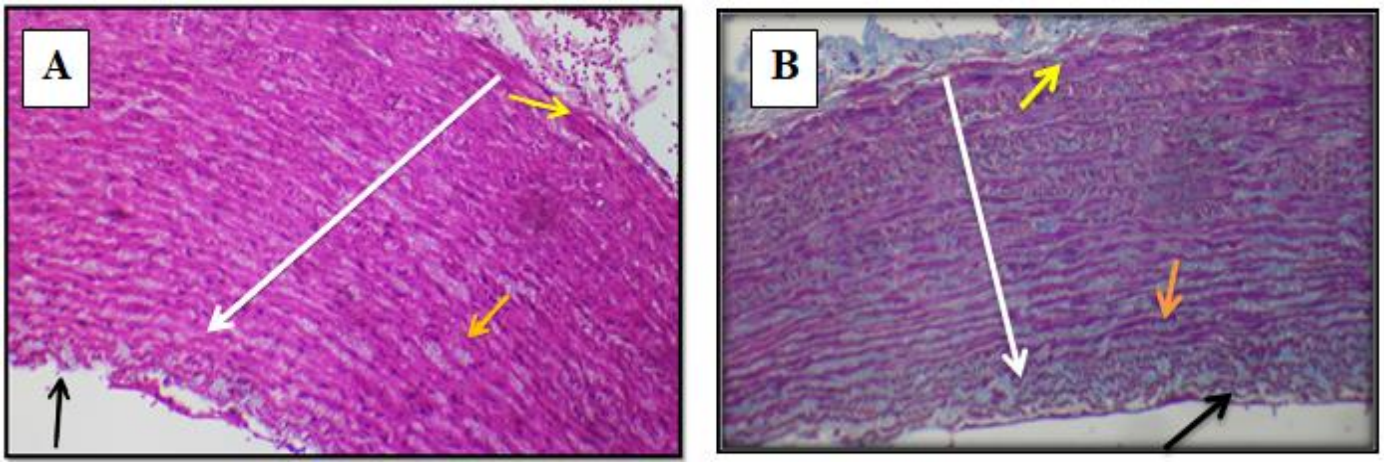
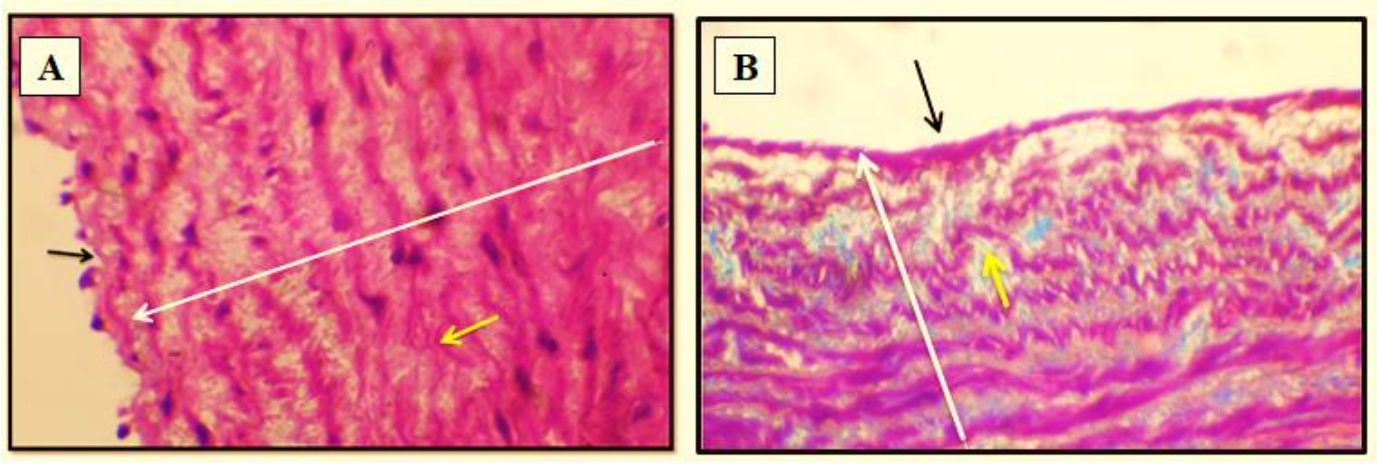


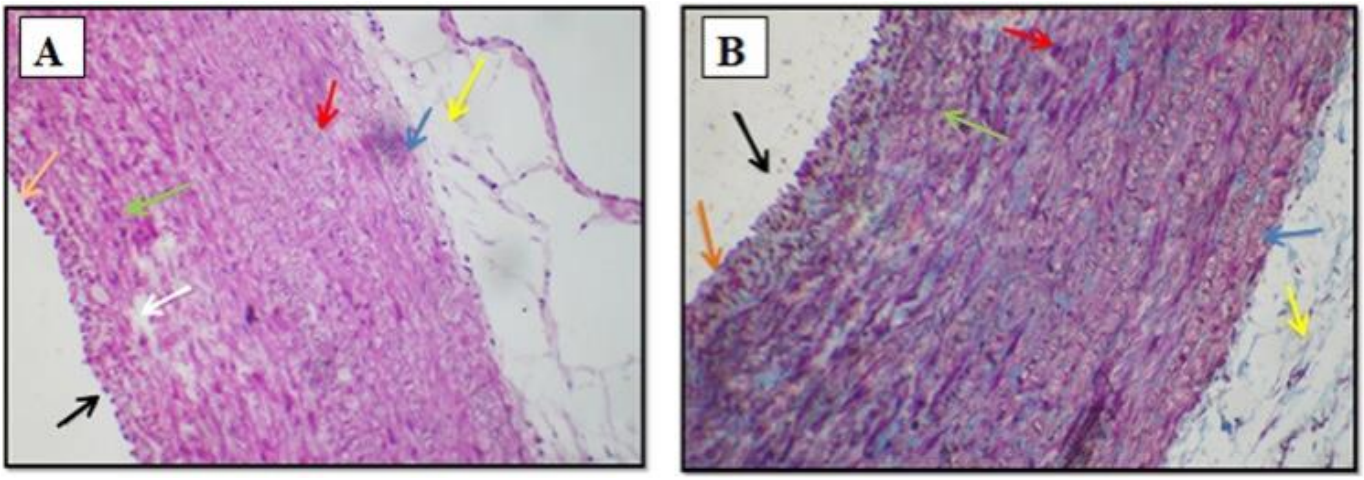
Figure (4.10) Photomicrograph of Aorta section from D galactose treated group animal showed, necrosis of tunica intima endothelia(nuclear pyknosis) with loss of endothelia in some areas (black arrow) , regular tunica media(white arrow)with dearranged elastic lamina (yellow arrow) , moderate to sever necrosis of smooth muscle fibers (red arrow) and slight vacuolation (orange arrow) , with deep blue stained collagen fibers (green arrow). Mononuclear inflammatory cells infiltration(blue arrow) . (A-H and E,B- Masson's Trichome, 40X).



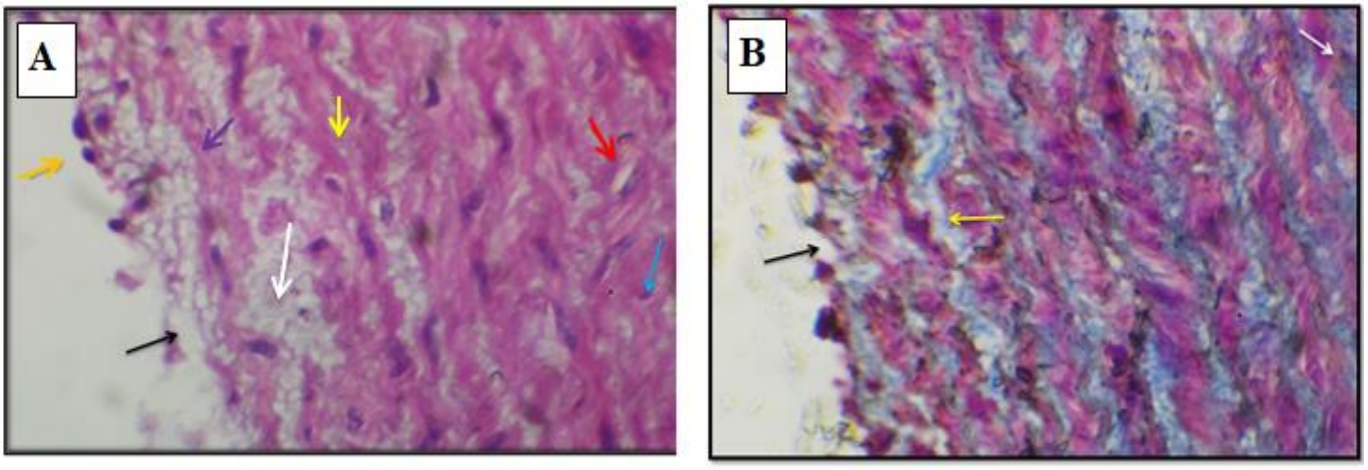
Figure(4.11) Photomicrograph of Aorta section from *Spirulina*l treated group animal look like normal histological architecture of aortic wall, normal tunica intima(black arrow) endothelia , significant regular wide tunica media(white arrow)with arranged elastic fibers (orange arrow), and narrow ,outer area of tunica adventitia (yellow arrow). (A-H and E ,B-Masson's Trichome , 10X).



Figure(4.12) Photomicrograph of Aorta section from *Spirulina* treated group animal revealed near to normal histological structure of aortic wall, seminormal tunica intima(black arrow) endothelia , significant regular tunica media(white arrow) and significant arranged elastic laminae (yellow arrow) parallel with blue collagen fibers in Masson's Trichome stain and white on HandE stain (A-H and E ,B-Masson's Trichome, 40X).



Figure(4.13) Photomicrograph of Aorta section from *Spirulina* and D galactose treated group revealed ,focal necrosis (desquamation)of tunica intima endothelia (black arrow), other areas showed normal endothelia (orange arrow) , areas of depletion in tunica media with decreased collagen fibers in some regions (green arrow) and accumulation others (white arrow), mild necrosis of smooth muscle fibers (red arrow) , less Mononuclear inflammatory cells infiltration(blue arrow), decreased collagen fibers in tunica adventitia (yellow arrow). (A-H and E ,B-Masson's Trichome, 10X).



Figure(4.14) Photomicrograph of Aorta section from *Spirulina* and D galactose treated group revealed,focal necrosis (desquamation) of tunica intima endothelia (black arrow) ,some other areas reveal normality in endothelial tissue (Orange arrow) , areas of depletion in tunica media(white arrow)with decreased collagen fibers (yellow arrow) , mild necrosis of smooth muscle fibers with slight vacuolation (red arrow) less Mononuclear inflammatory cells infiltration(blue arrow), dearranged elastic fibers (purple arrow) . (A-H and E ,B-Masson's Trichome, 40X).

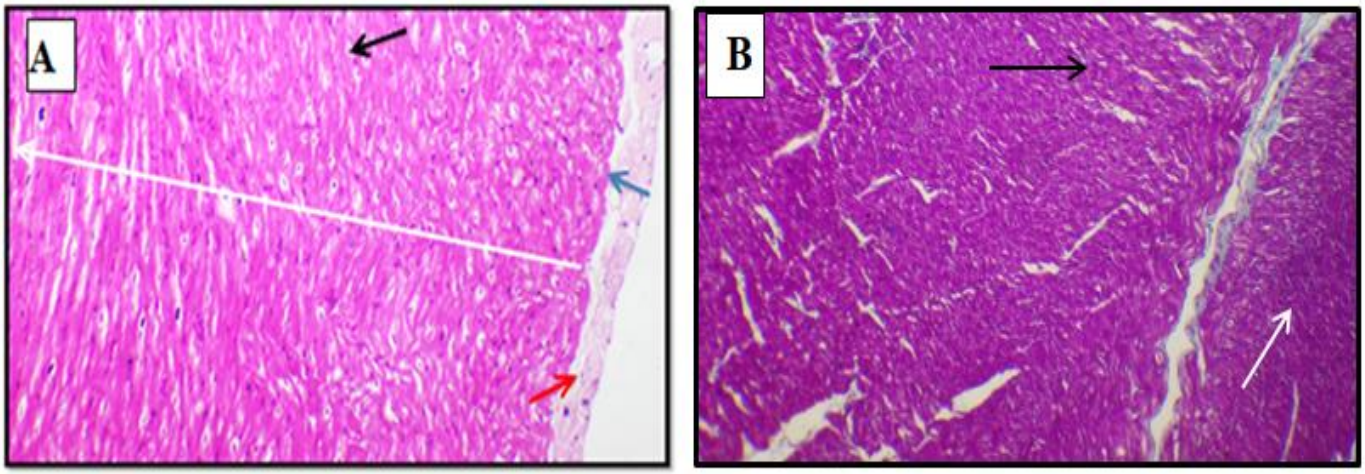


Figure (4.15) Photomicrograph of heart tissue section from control group animal , showed the normal structure of heart muscle , normal endocardium (blue arrow)with Purkinje fibers (red arrow), significant normal myocardial fibers in the myocardium (white arrow) with remarkable nuclei (black arrow). (A-H and E ,B-Masson's Trichome, 10X).

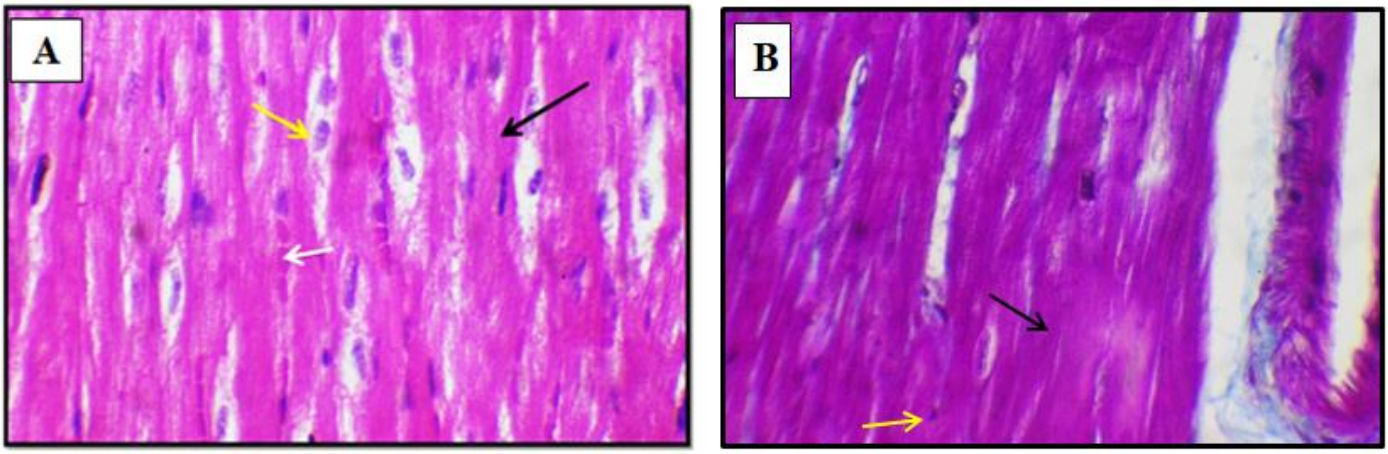


Figure (4.16) Photomicrograph of heart tissue section from control group animal , showed the normal structure of myocardium , significant normal longitudinal myocardial fibers (black arrow) with remarkable large nuclei (yellow arrow) , between the myofibers there is connective tissue containing blood vessels (white arrow). (A-H and E ,B-Masson's Trichome, 40X).

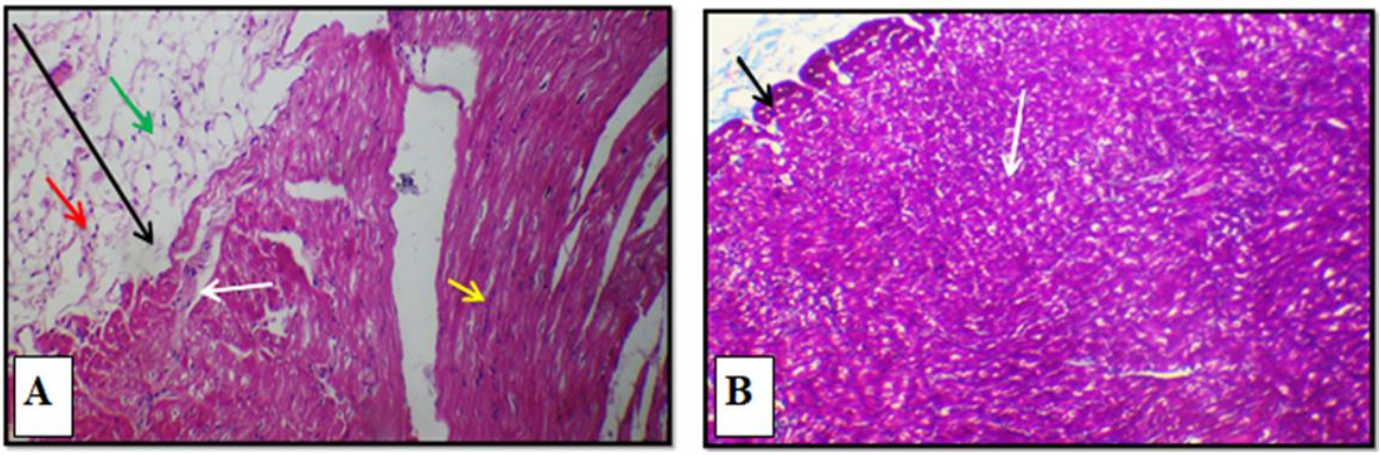


Figure (4.17) Photomicrograph of heart tissue section from a D galactose treated group animal , revealed the loss of the normal architectural appearance of heart muscle , significant thickened endocardium (black arrow)with proliferation of Purkinje fibers (red arrow) and present of sever fatty tissue precipitation (green arrow) , significant disarrangement myocardial fibers in the myocardium (white arrow) with remarkable pyknotic nuclei demonstrates myofiber necrosis (yellow arrow). (A-H and E ,B-Masson's Trichome, 10X).

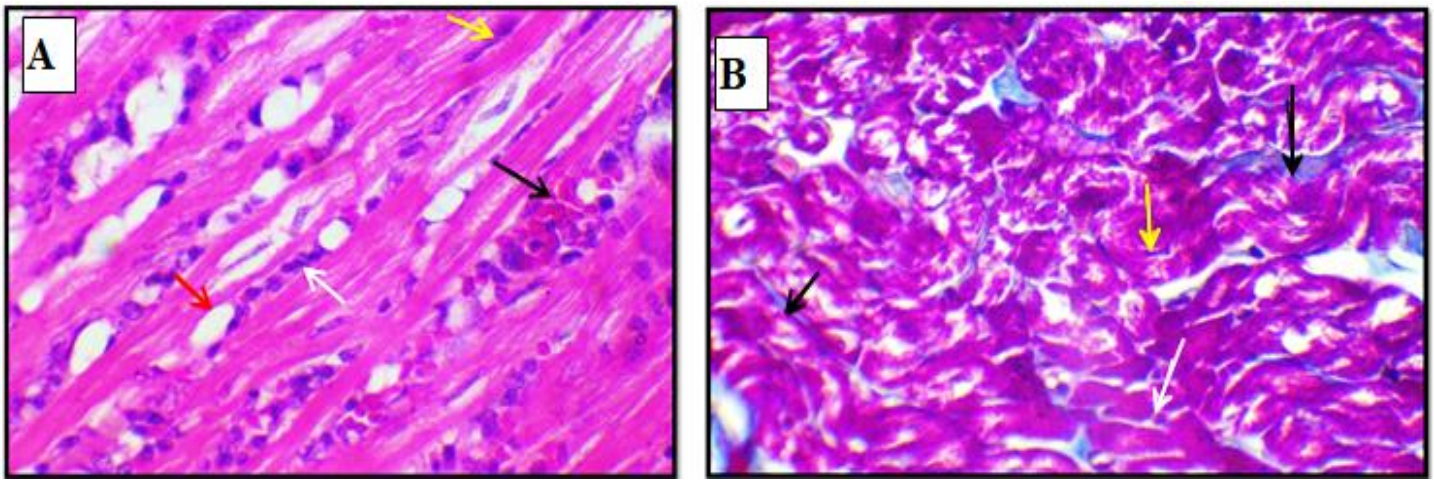


Figure (4.18) Photomicrograph of heart tissue section from a D galactose treated group animals , revealed the loss of the normal architectural appearance of heart muscle , inflammatory cells infiltration (white arrow), proliferation of Purkinje fibers with sever congested blood vessels (black arrow) and present of sever fatty tissue infiltration (orang arrow) , with remarkable pyknotic nuclei demonstrates myofiber necrosis (yellow arrow). (A-H and E ,B-Masson's Trichome, 40X).

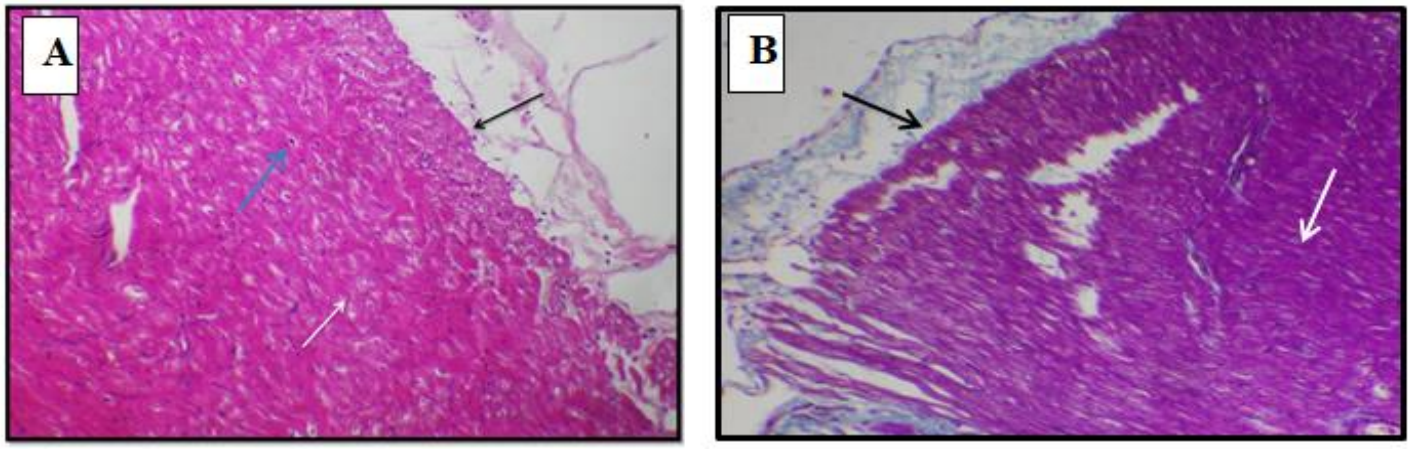


Figure (4.19) Photomicrograph of heart tissue section from *Spirulina* treated group animals , revealed normal architectural appearance of heart muscle, endocardium (black arrow), significant arrangement of myocardial fibers in the myocardium (white arrow),with remarkable nuclei (blue arrow). (A-H and E ,B-Masson's Trichome, 10X).

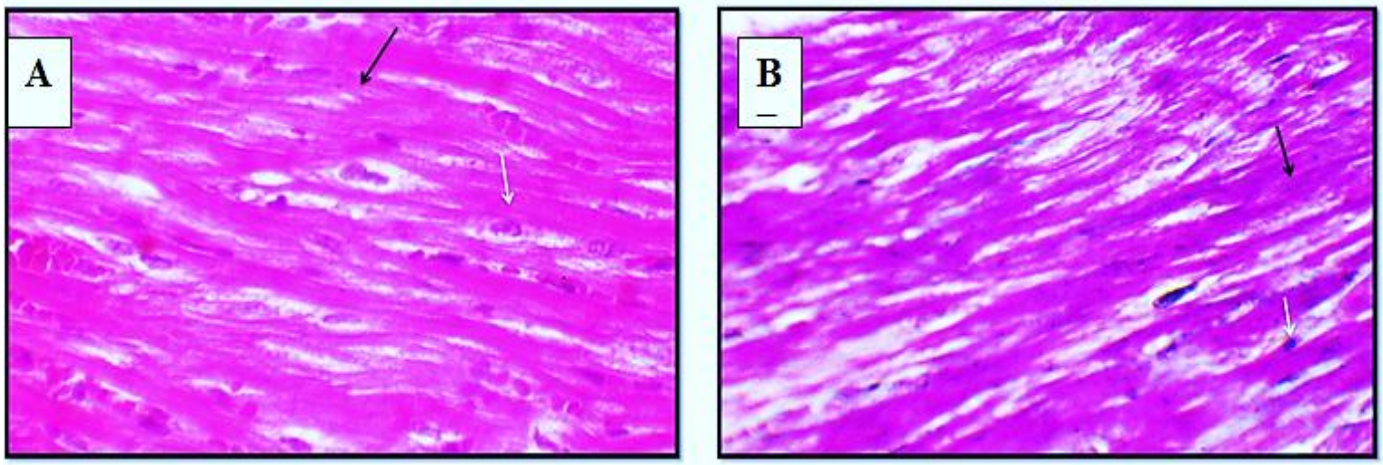


Figure (4.20) Photomicrograph of heart tissue section from *Spirulina* treated group animals , revealed the normal architectural appearance of heart muscle , normal straitening and arrangement of myofibers (black arrow), with their large significant nuclei (white arrow). (A-H and E ,B-Masson's Trichome, 40X).

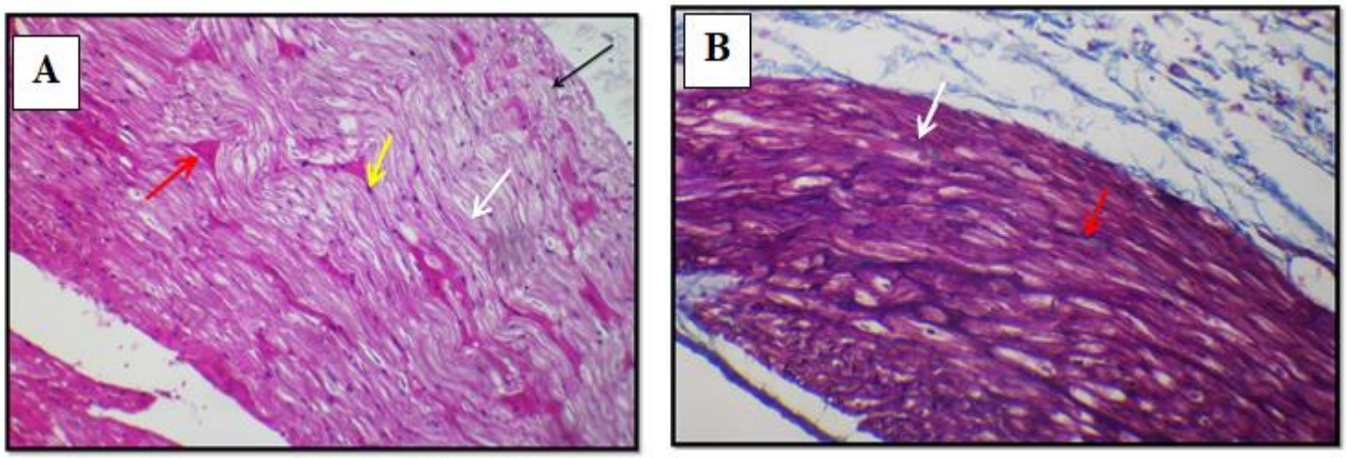


Figure (4.21) Photomicrograph of heart tissue section from *Spirulina* and Dgalactose treated group animal , showed mild thickened endocardium (black arrow), significant arrangement of myocardial fibers in the myocardium indicates their reversible to normal arrangement (white arrow) with slight necrotic changes in their in some myofibers represented by pyknotic nuclei (yellow arrow) , mild to moderate congestion in blood vessels (red arrow). (A-H and E ,B-Masson's Trichome, 10X).

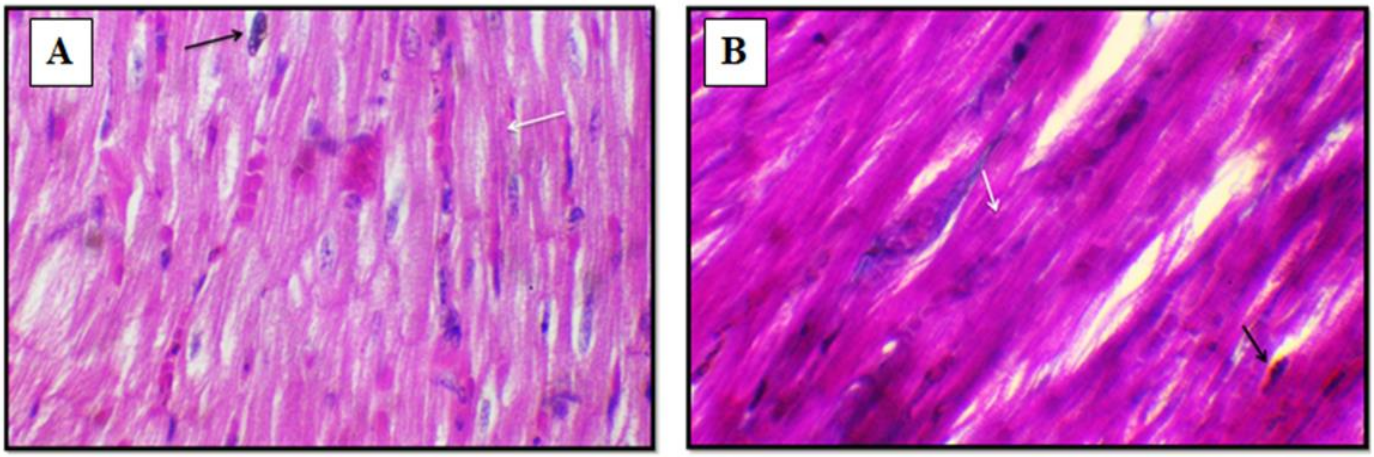


Figure (4.22) Photomicrograph of heart tissue section from *Spirulina* and Dgalactose treated group animal , showed significant arrangement of myocardial fibers in the myocardium indicates their reversible to normal arrangement (white arrow) with slight necrotic changes in their in some myofibers represented by pyknotic nuclei (black arrow). (A-H and E ,B-Masson's Trichome, 40X).

Chapter five:Discussion

5.Discussion

The present study showed that oral intubation of 500mg/kg B.W daily in 4weeks of *Spirulina* could offered from cardioprotective against cardiac and aortic damage induced by excess of s/c administration of D-gal on male rabbits .

5.1Effect of *Spirulina* on the cardiac biomarkers (Troponin I , Endothelin -1 , Nitric oxide) on the damage induced by D-galctose in male rabbits .

5.1.1 Cardiac troponin I

Cardiac troponins (cTnI) are highly specific and sensitive laboratory markers of myocardial insult and are considered as the gold standard for biochemical detection of myocardial injury In addition cardiac troponin were proposed as marker of cardiac cell death, it's a protein was now widely used and established as the guideline recommended marker in order to assist in the diagnosis of acute myocardial infarction. (**Boeddinghaus *et al* .,2018; Jaffe *et al*., 2021 ; Kui *et al*., 2021)**

The current study show a significant ($P \leq 0.05$) increase in G II group when compared to other group as showed in figure (4.1) and this result agreement with **(Sui and Gao, 2014;Ma *et al* .,2021; Hong *et al* .,2021).**

Increased serum cTnI levels in the D-galactose treated rabbits marked elevations in serum creatine kinase-myocardial band (CK-MB) activity were registered,this may be referred to concentrations of diagnostic markers of myocardial damage that are released into the extracellular fluid once myocardial cells are damaged. This is important because elevated cTnI levels can predict risk of myocardial ischemia in the stress subjected patients cardiac cell death and subsequent infarction **(Chaulin, 2021; Chaulin and Duplyakov, 2021; Pourali *et al* .,2021).**

When the heart is subjected to oxidative stress, the membrane can become porous or even break, allowing cytosolic enzymes to leak into the blood stream and elevate their serum levels. This proves that the elevated cTnI levels in the D-galactose group were due to this reason **(Wassie *et al* .,2021).**

For this reason, cTnI is one of the most sensitive and specific blood markers for identifying myocardial injury when other forms of muscle damage are present (**Khazaal *et al* .,2022).** In the 3-10 hours following cardiac injury, troponins are detectable in the bloodstream due to the release of cTnI from cardiomyocytes **(Cascella *et al* .,2022).**

Myofibrillar proteins such as troponin I, C and T modulate interaction between actin and myosin through calcium in the myocardium **(Hou *et al* ., 2022).** Among them, cardiac troponin I (cTnI) is considered as high accurate specific biomarker of myocardium damage **(Xin *et al* ., 2021).**

Following, enhancement of cardiomyocytes permeability and damage, cTnI releases into blood as a marker for the diagnosis of heart muscle damage and positive correlation has been identified between blood concentrations of cTnI and myocardial damage **(Mishra *et al*., 2018)**

On the other side cardiac troponin I dropped significantly (P≤0.05) in the group GIII and GIV that recived *Spirulina*. The study's results are the same as those of **(Alwaleed *et al*., 2021).**

The levels of cTnI generated by D-galactose were dramatically reduced due to the presence of *Spirulina*. Rabbits given *Spirulina* had their cTnI levels return to normal.In *Spirulina*, antioxidants like -carotene and Cphycocyanin may play a role in this.

These research shows that *Spirulina* can prevent oxidative stress in cardiac myocytes **(Abdul-Adel *et al*., 2019; Jaeschke *et al*., 2021 ; Abdel-Moneim *et al*., 2022).**

Spirulina's non-enzymatic antioxidants and antioxidant enzymes are responsible for scavenging free radicals, which in turn boosts immunological function, protects against disease, and benefits health. For these reasons, *Spirulina* has been heralded as a "superfood" for its purported ability to combat diseases, slow the effects of aging, protect against cancer, and otherwise enhance health **(Grosshagauer *et al*., 2020).**

5.1.2 Endothelin-1

The current study show a significant (P≤0.05) increase in the mean value of Endothelin-1 in G II group when compared to other group as showed in figure (4.2) and this result agreement with **(Evans *et al* .,2000; Friedman *et al* .,2014)**

Key players in the control of vascular tone include endothelin-1, oxygen-derived free radicals, and nitric oxide. Paracrine-autocrine substance endothelin-1 promotes prolonged vaso-constriction, controls cell development and proliferation, and communicates with thrombin, vasopressin, and angiotensin II. Recent research has linked endothelial dysfunction in hypertension and atherosclerosis to oxygen-derived free radicals like superoxide anions (02), hydroxyl radicals (OH*), and hydrogen peroxide (H2O2).Endothelin-1 (ET-1) is released by endothelial cells, and the

levels of this protein in the blood increase by a factor of two to three in patients with heart failure, regardless of the underlying cause **(Genovesi *et al* .,2021;Biasucci *et al* .,2021**).

Endothelin-1 (ET-1) is a vasoconstrictor that is made by endothelial cells. It is the natural opposite of nitric oxide, which widens blood vessels. ET-1 helps keep the tone of the blood vessels and controls cell growth by turning on the ETA and ETB receptors. Shear stress and other physical factors or stimuli Secretion is increased by thrombin, epinephrine, angiotensin II, growth factors, cytokines, and free radicals of ET-1. By contrast, mediators like nitric oxide (NO), Cyclic guanosine monophosphate cyclic GMP, atrial natriuretic peptide, and prostacyclin reduce the amount of ET-1 that is made by the body. So, the effects of the ET-1 are carefully studied under normal conditions.regulated by stopping or starting the release of ET-1 from endothelium**(Camarda *et al* .,2022; Mekuria *et al* .,2021)**.

Endothelial dysfunction is one of the first signs that something is wrong with the blood vessels. Changed the way it works endothelium can be caused by both a decrease in the bioavailability of NO and an increase in the synthesis, release, or activity of ET1. If the body doesn't make enough vasodilators or vasoconstrictors, blood vessels may get too big or too small. cause problems with the way blood flows through the body.Since improper regulation of the endothelin system is a major cause of several heart diseases,The ETA and ETB receptors are good therapeutic targets for diseases that are linked to high levels of ET1. ET receptor antagonists may be able to change the course of a disease because they can protect the integrity of the endothelium when the endothelin system is too active **(Said *et al* .,2019; Mustapha *et al* ., 2021;NG *et al* .,2022)**

The findings of a previous study suggest that oxidative stress, in the form of that caused by D-gal, leads to an increase in the synthesis of the large endothelin-1 protein. Oxidative stress could be shown to increase endothelin-1-promoter activity as well as pre-proendothelin-1 mRNA synthesis. **(Rathod *et al*.,2022).**

An autocrine redox-sensitive mechanism that regulates ET-1 production has been described more recently ET-1-stimulated expression of the vascular NADPH oxidase leads to increased production of reactive oxygen species including superoxide anion, which in turn results in increased synthesis of preproendothelin-1 mRNA and protein, as well as ET receptors ET-1 stimulates the expression of the vascular NADPH oxidase. Therefore, numerous pathways mediated by a variety of distinct mediators converge to boost ET-1 production and release.**(Momot *et al* .,2022)**

Cardiomyocytes, fibroblasts, hepatocytes, neurons, and vascular smooth muscle cells all express ETA receptors, which have a higher affinity for ET-1 than for ET-2 and ET-3 . The activation of

phospholipase C (PLC) by the binding of ET-1 to ETA receptors results in the production of the second messengers inositol trisphosphate (IP3) and diacylglycerol (DAG), both of which can promote intracellular calcium release and protein kinase C (PKC) activation The stimulation of phospholipase D (PLD)-mediated DAG synthesis, phospholipase A2 (PLA2)-induced arachidonic acid release, and activation of the mitogen-activated protein kinase (MAPK) cascade are additional signaling pathways that depend on ETA receptors These signaling pathways have a role in the long-term regulation of cell proliferation, adhesion, and migration in the heart and vasculature as well as the short-term modulation of vascular smooth muscle tone **(Cobos-Segarra *et al*.,2018; Cameron *et al* .,2019; Zhang and Xu,2020).**

The study demonstrates that both endogenous and exogenous ROS enhance ET-1 production by poorly controlled diabetic rat glomeruli and that ROS scavengers suppress ET-1 production both in vitro and in vivo**(Liu *et al*., 2020).**

High glucose also regulates ET-1 receptor levels downward in cultured rat mesangial cells. Therefore hyper glycemia may increase the synthesis of ET-1, which may play some role in the pathogenesis of diabetic nephropathy **(Garcia-Herreros *et al* .,2020)**.

While there is growing evidence that superoxide production are mediated by NADPH oxidase can induce increases in ET-1. **(Kostov, 2021).**

The same figure revealed a significant decrease in Endothelin-1 in GIII and GIV in comparison with GII. The result of the study agreement with **(Martinez *et al* .,2018)**

Spirulina has been also found to be effective in the treatment of various oxidative stress models **(Mahmoud *et al*., 2021 ; Hassaan *et al* .,2021).** It also commonly found in dietary components which inhibits oxidative stress by scavenging free radicals **(Li, 2022).**

Oxidative stress plays a key role in the development of many cardiovascular diseases ,including atherosclerosis , hypertension and heart failure (Paravicini and Touyz ,2008)it has been found that *Spirulina* prevented eNOS and neuronal NOs but decreased inducible NOs , Meanwhile , it is decreased expression levels of ET-1 **(Abdel-Daim *et al* ., 2020).**

Therefor *Spirulina*, are anti-oxidants, anti-inflammatory, and anti-tumor. They also boost the immune system and protect organs from damage. Autotrophic *Spirulina*l is the only place you can get GLA, which has as much as 500 times as much as human milk **(Han *et al*.,2021;Trotta *et al* .,2022).** Linoleic acid and gamma-linolenic acid (GLA) are important in the treatment of heart

disease because they are important parts of mitochondrial phospholipids **(Machowiec *et al* .,2021;Majewski *et al* .,2022).**

5.1.3 Nitric Oxide

Nitric oxide, often known as NO, is an endogenous regulatory molecule that plays a role in a diverse range of physiological activities across a number of organ systems. This straightforward molecule with only two atoms serves as a messenger in a wide variety of cellular processes, some of the most important of which include neurotransmission, the control of blood pressure, and immunomodulation. It has also been suggested that NO plays a number of important physiological roles in the cardiovascular system, including the following: In addition to regulating cardiac contractility and platelet aggregation, nitric oxide (NO) is an important factor in determining the baseline tone of the vasculature. NO is produced by many different kinds of cells in the body, including endothelial cells, macrophages, and nervous cells **(Aramide *et al* .,2021; Adams *et al* .,2022) .**

These three types of cells express high levels of the enzymes responsible for producing NO: endothelial NO synthase (eNOS), inducible NO synthase (iNOS), and neuronal NO synthase (nNOS), respectively. Through a complicated oxygen-dependent five-electron transfer mechanism, these NOS systems constantly catalyze intracellular L-arginine (L-Arg), which results in the production of equimolar quantities of NO and L-citrulline. L-Arg is the precursor to the formation of nitric oxide and controls its production in both healthy and pathological settings (**Bo-Htay *et al* .,2020**).

Since NO is an important endothelial-derived vasodilator and cardioprotector, low NO levels can also contribute to the tissue damage caused by D-gal. Most of the time, a sign of higher oxidative pressure is less nitric oxide (NO) production, which can also make NO less effective at protecting the heart**(Bo-Htay *et al* .,2020).**

The current study show a significant decrease in the mean of **Nitric Oxide** in G II group as shown in figure(4 .3). The study's results are the same as those of **(Bo-Htay *et al*.,2021).**

It's noteworthy to note that D-gal-accelerated ageing results in a drop in endogenous NO levels as well. Low NO levels may contribute to D-gal-induced tissue injury because NO is an essential endothelial-derived vasodilator and cardioprotector. NO, a gaseous signaling molecule, is essential for a wide range of cellular and systemic processes. This gaseous molecule may have several

physiological and pathological roles because of its activities on similar molecular targets, according to recent investigations.**(Wu *et al*.,2017)**

D-galactose treatment have shown to induce oxidative stress in the heart tissues of animals by increasing MDA and nitric oxide (NO) and decreasing antioxidant enzymes such as SOD, CAT, glutathione peroxidase (GSH-Px), NOS, and total antioxidant capacity (**Lei *et al*. 2016; Dehghani *et al*. 2018; Xu *et al*., 2019).**

D-gal showed enhanced ROS liberation along with decreased NO production because NO-dependent relaxation and NO production were decreased in oxidative stress condition . Although decreased endothelium-dependent relaxation and NO production were found, eNOS protein expression was increased. These results are in agreementment with another report showing that in type 2 diabetes, This indicates that the decreased NO- dependent relaxation in D-gal rats is not due to decreased expression of eNOS but instead is related to the decreased ability of the enzyme to produce NO **(Qian *et al* .,2018; Lee and Im, 2021).**

NO is considered the main mediator of endothelium-dependent relaxation . prostacyclin PGI2 and endothelium-dependent hyperpolarizing factor(EDHF) are also important regulators of vascular activity ,particularly in resistance vessel , although the mechanism of vasodilation induced by the different endotheliumdependent relaxation factor (EDRF) differs , each individual mediators possesses. The capacity to interact with component involve in the synthesis /activation of other mediators and control the activity . there is clear evidence the EDRFs work cooperatively in a complex but integrated manner to maintain homeostasis to the vasculatures. NO may initiate the expression of antioxidant enzymes and nitrosative stress resistance genes . NO enhances the antioxidant potency of GSH by forming Snitrosoglutathione that is about 100 fold more potent than that of GSH **(Han *et al* .,2018;Zhao *et al* .,2019)**

Compared to GII, GIII and GIV getting *Spirulina* had a significant increase in NO mean level. The study's results are the same as those of other studies **(Brito *et al* .,2018;de *et al* .,2019;Mohiti *et al*., 2021).**

In vitro, *Spirulina* made the endothelium make and release more nitric oxide and the vasoconstricting agent prostanoid, which is made of cyclooxygenase. Together with as phycocyanin, the ingredients in *Spirulina* can boost the production of endothelial nitric oxide synthase over time. This makes nitric oxide more bioavailable **(Diniz *et al*.,2020).**

Spirulina improves the redox states of plasma and endothelium-dependent vasodilation . *Spirulina* also induce phosphorylation in human umbilical vascular endothelial cells and the human monocytic leukemia cell line-1 **(Aladaileh *et al*.,2020).**

Studies suggest that improved endothelial function due to *Spirulina* is at least partially attributed to recoupling of eNOS and increased NO bioavailabilityThe antioxidant effect of NO it acts through its effects on vascular smooth muscle, by improving microcirculation and maintaining of organ blood flow and blood pressure regulation **(Brito *et al* .,2018; Carrizzo *et al* .,2019)** .

Spirulina can scavenge a number of free radicals in hydrophilic and lipophilic environments and found to be capable of regenerating many endogenous antioxidants in the body **(Albtoosh *et al* .,2022).**

On the other hand, the antioxidant activity of recombinant phycocyanin protein is β-carotene, the other main ingredient of *Spirulina* with strong antioxidant properties, causes beneficial effects against oxidative stress status through inhibition of iNOS expression, nitric oxide (NO) production and singlet oxygen-mediated lipid peroxidation **(Naeini *et al* .,2021)**

5. 2. Effect of *Spirulina* on some serum oxidant (ONOO , MDA) and antioxidant(GSH) on the damage induced by D-galactos in male rabbits.

5.2.1. Serum Peroxynitrite (ONOO)

The present study showed a significant increase in the mean level of Peroxynitrite (ONOO) in G II as compared with other groups as showed in figure (4.4) and this result agreement with**(Zheng,2020; Rahman *et al* ., 2022)**

D-galactos treatment has been shown to cause oxidative stress in animal heart tissues, oxidative stress is when the balance between the generation of reactive oxygen species and the antioxidant mechanism is disrupted, a precursor of various reactive oxygen species, including hydrogen peroxide, hydroxyl radical, and peroxynitrite which plays a critical role in vascular oxidative stress **(Pizzino *et al* .,2017;Takaishi *et al*., 2021).**

The superoxide dismutation catalyzed by an enzyme such as superoxide dismutase produces hydrogen peroxide, resulting in hydroxyl radical via the Fenton reaction and ferrous iron in

biological systems .The interaction of superoxide with nitric oxide leads to peroxynitrite production, and thus,causes vascular dysfunction **(Patik *et al* .,2021; Lahiri *et al* .,2021)**.

D-gal causes oxidative stress which is appears by an important increase in rates of lipid peroxidation, Peroxynitrate itself is also strong oxidant and can react with electron rich groups**(Zheng, 2020).** Peroxynitrate can also be formed through superoxide react NO to formed Peroxynitrate . Increased glutamate release is sustained activation of glutamate receptors ,and increased accumulation of calcium (Ca+2). There is direct evidence that activation of glutamate receptors and the Ca+2 influx induces the formation of reactive oxygen species, superoxide anion and hydrogen peroxide **(Ajayi *et al* .,2021)**.

Oxidative stress initiates lipid peroxidation cascades that leads to the damage of highly vulnerable cell membranes during the first few days after injury . ONOO has been shown to trigger apoptosis in the cardiomyocytes, as well as endothelial and the vascular smooth muscle cell, induce decrease in spontaneous contractions of the cardiomyocytes and cause irreversible inhibition of mitochondrial respiratory chain **(Ziegler *et al*., 2020).**

Damage to proteins caused by free radicals leads to changes in amino acid residues, cross-linking of side chains, and fragmentation,Different RNS, like peroxynitrite, attack free/protein-bound tyrosines to make free/protein-bound NT. This may help us understand how autoimmune diseases start and develop. The formation of Nitro-tyrosine (NT) is a specific protein modification caused by peroxynitrite. Finding NT in proteins is used as a biomarker for peroxynitrite activity in the body. Peroxynitrite and other reactive nitrogen species can also oxidize and nitrate the tyrosine and tryptophan residues in proteins. This is called "nitrosative stress." Protein damage caused by oxidative stress can be caused by direct oxidation of protein side-chains by ROS and/or RNS, adducts of secondary products of oxidation of sugars (glycoxidation) or polyunsaturated fatty acids (lipid peroxidation). ROS formation, antioxidant levels, and the ability to use proteolysis to get rid of oxidized proteins all play a role in how much oxidized protein builds up. Nitration of tyrosine residues can drastically change the structure and function of proteins. This suggests that protein nitration may be fundamentally linked to oxidative cell damage and may be able to predict it **(Flores-Tamez *et al*.,2019;Feng *et al* .,2022).**

Peroxynitrate inhibits NF B activation in cardiac and endothelial cell lines caused by inflammatory stimuli and activates extracellular signal-regulated kinase (ERK), a mitogen-activated protein kinase (MAPK) associated with hypertrophic and antiapoptotic response in the heart. In addition, it stimulates or inhibits platelet aggregation depending on the environment, induces the

upregulation of adhesion molecule in endothelial cells, disrupts endothelial glycocalyx, and may increase the adhesion of neutrophils to endothelium through complex interactions with various cell signaling pathways (**Boccellino *et al*., 2018**).

Compared to the other treated groups, which shows that the serum ONOO- level in the GIII group has significant decrease. *Spirulina* has a lot of active ingredients, like phycocyanin and beta-carotene, which work well as antioxidants and anti-inflammatories **(Deng and Chow, 2010; Ragusa *et al* .,2021).**

Therefor it was found that phycocyanin was an antioxidant and a pain reliever. **(Abaza *et al* .,2021, Omar *et al* .,2022).** Phycocyanin can get rid of free radicals like alkoxyl, hydroxyl, and peroxyl radicals. It can also stop the production of nitrite and stop the expression of an enzyme called inducible nitric oxide synthase (iNOS) (**Ibrahim *et al*.,2021;Royand Pabbi,2022;Li *et al*.,2022)** .

Cyanobacteria *Spirulina*, which was consumed as food in some traditional cultures and is now used as a nutraceutical supplement, has been shown to have powerful antioxidant effects in studies conducted on rodents and to be protective in a wide variety of rodent models of health disorders, particularly those that are caused by oxidant stress. These findings have been gleaned from research conducted on rodents. This has been traced back to the fact that *Spirulina* contains an unusually high amount of the protein known as phycocyanin. Phycocyanin carries a chromophore known as phycocyanobilin (PhyCB) that is capable of harvesting light energy. This light energy is then used by the organism to drive the production of ATP .(As a result, it serves a purpose that is comparable to that of chlorophyll.) PhyCB is a metabolite of bilirubin's biosynthetic precursor biliverdin, and it can act as a substrate for biliverdin reductase within cells. Biliverdin reductase then transforms it to phycocyanorubin, a substance that is extremely similar to bilirubin in terms of its chemical structure . In point of fact, there is evidence to suggest that phycocyanorubin possesses the same potential to block NADPH oxidase complexes as bilirubin . In mouse research, the potential of orally administered phycocyanin, entire *Spirulina*, or free PhyCB to produce substantial antioxidant effects is satisfactorily explained by this finding **(El Arab *et al* .,2019; McCartyand Lerner,2020; McCarty *et al* .,2021).**

Spirulina has free radical scavenger properties and direct antioxidant effects on the recycling of other cellular antioxidants. *Spirulina* has been also found to be effective in the treatment of various oxidative stress models, such as ischemia-reperfusion, diabetes, improve levels of blood lipids, suppress oxidation, reduce blood pressure and lower blood sugar **(Han *et al* .,2021) .**

5.2.2 Malonydialdehyde (MDA)

Our result shown a significant increase in mad level in D-gal group when compared to other group as showen in figure (4-5) ,and this data agreementment with **(Qu *et al* ,.2016; Mahdi *et al* .,2021)**

MDA is a molecule that is produced as a by product of lipid peroxidation and is included in the group of chemicals that react with thiobarbituric acid. The level of MDA was utilized as a measure of lipid peroxidation **(Othman *et al* ., 2018).** MDA can spread to distant cellular structures, where it can cause further cellular damage, including damage to DNA. The oxidative process known as lipid peroxidation generally takes place at low levels in all of a living thing's cells and tissues. According to **(Renu *et al*., 2021),** under normal circumstances, a range of antioxidant systems are responsible for controlling the peroxidative process. MDA is a type of lipid peroxide that is produced when oxidation occurs. Additionally, the level of MDA in vivo directly indicates the amount of oxidation that has occurred **(Zhu *et al*., 2019).**

In the current investigation, MDA was found to be responsible for a reduction in the serum levels of the antioxidant enzymes SOD, GSH-Px, and CAT. According to the findings of certain studies, superoxide radicals are able to suppress CAT activity, and the increased H2O2 that results from suppressing CAT activity may also suppress SOD function **(Altuntas *et al*.,2002).** It's possible that an increase in the creation of free radicals is to blame for the rise in MDA levels rather than an improvement in the scavenging system's capacity to get rid of those radicals blood MDA and a decrease in GSH levels, these findings are in keeping with a number of laboratory investigations that revealed a modification in the antioxidants status of various tissues as a consequence of an increase in the lipid peroxidation that occurs in these tissues after oxidative stress was induced. **(AL-Zubaidi *et al* ., 2021; Mahdi et al .,2021)**

There are a great deal of anti-oxidant defense systems that are enzymatic [superoxide] substances that are nonenzymatic [such as ascorbic acid, glutathione, and -tocopherol] and enzymatic [such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx)] can fight free radicals. keep the right balance between the production of ROS and the protection against harm provided by ROS **(Hayat *et al* .,2012).**

The fact that there was an increase in MDA levels in older and more mature rats that had been treated with D-gal was followed by activity on the GPx that was unchanged, thus leads to the conclusion that is either a limited response of GPx to increasing pro-oxidant circumstances or none

at all, a shift in the activity of GPx is the root cause of the elevated MDA levels. **(HadziPetrushev *et al*.,2015).**

Malondialdehyde is a measurement of the amount of lipid peroxidation that has occurred in the tissues regarded as one of the most significant indicators of oxidative stress, which can alter various organs **(Ramiah *et al*.,2019).** The enhancement of it by D-galactose in the ongoing study demonstrated that D-gal had caused oxidative damage to the cells. Several studies imply a strong damage to mitochondria and formation of reactive oxygen species, primarily hydrogen peroxide in cells **(Al-Kurdy,2020).**

The present study showed that D-gal increase serum MDA level as result of oxidative stress which is manifested by significant increase in lipid peroxidation and reduce in GSH,MDA is evidence of lipedperoxidtion it is final products of polyunsaturated fatty acids peroxidation in the cells. An increase in free radicals causes overproduction of MDA **(HadziPetrushev *et al*.,2015).**

Reactive oxygen species readily attack the polyunsaturated fatty acids of the fatty acid membrane, initiating a self-propagating chain reaction .The destruction of membrane lipids and the end-products of such lipid peroxidation reactions are especially dangerous for the viability of cells , even tissues. Enzymatic (catalase, superoxide dismutase). Since lipid peroxidation is a self-propagating chain-reaction, the initial oxidation of only a few lipid molecules can result in significant tissue damage **(Mylonas and Kouretas 1999; Jurcau and Ardelean,2022).**

D-gal causes oxidative stress which is manifested by significant increase in levels of lipid peroxidation as evidenced by increased levels of MDA and by the decrease superoxide dismutase and reduced glutathione, glutathione peroxidase and glutathione S-transferase **(Li *et al* .,2019).** MDA is an evidence of lipid peroxidation promote by iron overload . This study revealed that D-gal supplementation generates free radicals and a depletion of anti-oxidants in the thymus and spleen a precursor to the pathogenesis of many diseases **(Khedr *et al*.,2022).**

They present study showed the D-gal causes oxidative stress which is demonstrated by important increase in the levels of lipid peroxidation as shown by increased levels of MDA in heart **(Mas-Bargues *et al* .,2021)**

CO-administration of *Spirulina* suspension brought these enzyme activities back to normal levels in the control group by a significant amount. C-phycocyanin, which is found in *Spirulina*, has the potential to act as an antioxidant and get rid of free radicals like hydroxyl radicals and superoxide. **(Abdel-Daim *et al*.,2020)** said that C-ability phycocyanin's to protect against adenine

showed that it can get rid of free radicals and slow down the chain reaction of lipid peroxidation. *Spirulina*'s antioxidant properties might work by lowering MDA and raising GSH levels **(El-Tantawy,2016)**.

Spirulina reduces the amount of the hydroxyl radical and it also scavenges the peroxide and the superoxide radical . *Spirulina* can scavenge a number of free radicals in different environments .It is found to be capable of regenerating many endogenous antioxidants in the body **(Hidayati *et al*.,2020) .**

The protective role of *Spirulina*a on lipid peroxidation status ,it may be attributed to the bioactivity of *Spirulina* to directly react with oxidation ,as well as its ability to interfere with the oxidation processes in the lipid and aqueous cellular compartment **(Chaouachi *et al* ., 2022).**

On administration of *Spirulina* the level of lipid peroxidation in plasma liver and brain was significantly decreased so after administration of *Spirulina* the antioxidant was primarily accumulated in the liver, heart and skeletal muscle after which it efficiently crosses the blood brain barrier to accumulate in several brain regions **(Gargouri *et al* .,2018).**

5.2.3 Glutathione (GSH)

According to the results of our research there was a significant decrease in the mean value of GSH concentration in group G II compared to the other groups. This final findings is regular with **(Aquilano *et al*.,2014; Kwon *et al*., 2019; Heil *et al* .,2020 ; Mahdi *et al*., 2021)** .

Reduced glutathione is an important anti-oxidant that helps neutralize the damage caused by ROS ,glutathione can stop the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS), which contribute to the onset of cardiovascular disease. Some of the specifics covered include: reactive oxygen species (ROS), reactive nitrogen species (RNS), and antioxidant defenses; reduced glutathione (GSH) and its role as an antioxidant; GSH's role in preventing cardiovascular disease; and the relationship between reactive species production and the onset of cardiovascular disease. GSH is directly involved in reducing the harmful effects of both the disease itself and the oxidative effects of ROS and RNS, so understanding the relationship between these two factors will shed light on how to best design therapeutic strategies to help restore normal physiological conditions in patients suffering from a variety of cardiovascular diseases. **(Bajic *et al* ., 2019;Omidkhoda *et al*.,2020; Matuz-Mares *et al* .,2021).**

D-galactose induced ROS production within the body, which can be defined as D-gal increasing oxidative pressure and ROS formation, as well as blocking cycteine absorption and production, resulting in reduced GSH stages and an increase in ROS**(Firdaus,2022).**

In a study involving isolated rabbits organ exogenous GSH shown to protect organ damage **(Guo *et al* .,2022)**furthermore, the GSH perfusion medium resulted in an increase in the intracellular level in both damaged and healthy organ by entering the directly or indirectly into the cells **(Yoon *et al* ., 2018).**

Glutathione , an endogen antioxidants reduced in the oxidative status as signalizing of oxidative stresses , reflecting the redox commensuration between oxidation and antioxidations .Various oxidants and antioxidants have been additive effect on oxidative status study appeared that administrated D-gal lead to decreased cardiac reduced glutathione (GSH) level in the serum **(Li *et al* .,2019).**

Excessive ROS that could be produce by D-gal has detrimental effect on body organ including LPO and DNA damage **(Yuan *et al*.,2019).** In support of this finding, decreased levels of endogenous antioxidant enzymes such as SOD and Gsh-Px **(Zhang *et al*.,2022).** and reduced GSH levels were demonstrated in D-galactose-treated rats. Evidence shows that D-galactose administration increased expression of oxidative stress and decreased expression of antioxidants**(Li *et al*.,2021)** .Intracellular ROS production and propagation are controlled by highly complex and integrated antioxidant systems. Mammalian cells have evolved a variety of interrelated enzymatic antioxidant mechanisms which enable them to cope with oxidative environments **(Al-Gubory and Garrel ,2022).**

D-galactose enhanced ROS levels, which increased serum GSH ,under oxidative stress, the cellular antioxidants capacity is not counterbalancing the oxidative damage induced by free radicals and environmental toxins **(Jeremy *et al*., 2019).** SOD, CAT, and G-Prx may boost antioxidant defenses. SOD converts superoxide radicals to hydrogen peroxide, which CAT and G-Prx reduce to water **(Wang *et al*., 2020; Hou *et al*., 2022).** D-gal is a body-natural vitamin **(Margotta *et al*.,2018).** As D-gal is injected into rodents, its quantity in cells becomes too high for galactose oxidase to convert it to aldose and hydrogen peroxide, causing superoxide anions **(Wang *et al* .,2020).** Oxidation in the body produces free radicals beyond the body's scavenging capacity, leading to lipid peroxidation **(Zhou *et al* .,2021).** The final decomposition products (such as MDA) can combine with proteins, nucleic acids, phospholipids, and other substances, destroying the

chemical structure of intracellular life substances and disrupting cell function**(Margotta *et al*.,2018).** They can also damage normal tissue cells and affect normal osmotic pressure, leading to metabolic disorders of vital organs and death **(Guo *et al* .,2022).** Pathophysiological changes during natural and D-gal-induced aging are related to ROS and oxidative stress, and rats of different ages react differently to D-gal **(Tavanai and Mohammadkhani 2017).** Pro-oxidant/antioxidant equilibrium is reflected by antioxidant enzyme activity and lipid peroxidation **(Sun *et al*., 2020)** found that D-galactose promoted organ death.

In agreementment with other studies **(Abdel-Daim *et al*., 2018 ; Pawar *et al*., 2020 ; Zeng *et al*., 2020 ; Abd El-Hady *et al*., 2022)** we found that CO-administration of *Spirulina* suspension dramatically restored glutathione concentration to within normal range in GII and GIV when compared with GII

Ability of *Spirulina* platensis to reduce lipid peroxidation, as well as its effects on the activities of SOD, CAT, glutathione peroxidase (GPx), and glutathione reductase (GR), when iron-induced oxidative stress was applied to SH-SY5Y neuroblastoma cells. The levels of reduced glutathione in these cells were enhanced by *Spirulina* platensis, which also maintained the activity of the cellular antioxidant enzymes total GPx, GPx-Se, and GR. According to **(Koohkan *et al*., 2022),** there is a possibility that the antioxidant capacity of *Spirulina* platensis could be increased when the organism is subjected to greater environmental stress.

For instance, **(Khan *et al*., 2022),** investigated whether or not the cultivation of cells in medium that was supplemented with varying concentrations of hydrogen peroxide could result in an increase in the amounts of certain bioactive compounds found in *Spirulina* platensis. This was done in order to test the hypothesis that the amount of these compounds could be increased (H2O2). They identified a significant connection between increased H2O2 concentrations and increasing levels of cellular lipophilic antioxidants (total carotenoids and -tocopherol) and hydrophilic antioxidants. This was true for both lipophilic and hydrophilic antioxidants (glutathione and ascorbic acid).

The addition of *Spirulina* significantly restored those enzymatic sports to near-ordinary stages with inside the manage organization. *Spirulina* comprises C-phycocyanin, which has antioxidant interest and might scavenge loose radicals like hydroxyl radicals and superoxide. C- phycocyanin safety's towards D-gal confirmed radical scavenging interest in addition to an inhibitory influence at the lipid peroxidation chain reaction, as referred to through way of means of **(Abdel-Daim *et al*., 2020).**

Spirulina therapy increased glutathione stages and shielded the interest of cell antioxidant enzymes which includes glutathione peroxidase (GPX), selenium-established glutathione peroxidase (GPX-Se), and oxidized glutathione reductase (GR). *Spirulina*'s antioxidant potential was confirmed by these data. According to a new in vitro study **(Pawar *et al*., 2020).**

Spirulina was found to be effective against cardiovascular diseases, owing to their inhibitory effect on the production of reactive oxygen species (ROS), therefore , it is urgent to widely screen the bio-active components of *Spirulina* with special biological functions **(Zeng *et al* ., 2020)** .

The antioxidant activities of *Spirulina* may be attributed to the presence of potent antioxidant components including betacarotene, vitamin C, vitamin E, zeaxanthin, diatoxanthin, echinenone, xanthophyll and phycocyanin, which can protect against oxidative damage **(Abd El-Hady *et al* ., 2022).**

4.3. Protective role of *Spirulina* in Electrocardiograph (ECG) in D-galactose treated male rabbits.

The electrocardiogram (ECG) is one of the standard technologies used to monitor and analyze cardiac function; therefore, the electrocardiogram tracings were recorded for the purpose of analyzing heart rate and rhythm problems. The ECG pattern that is associated with age-related cardiac dysfunction underwent substantial changes, and there was also a large increase in the levels of cardiac biomarkers in the serum, as well as oxidative stress and inflammation in the heart **(Heil *et al* .,2020**).

According to the findings of the research, giving D-gal to male rabbits for a period of four weeks led to a considerable increase in the prolongation of (P,QRS,T) waves as well as (ST,QT) intervals. These findings are consistent with those of another study carried out by **(El-Baz *et al*.,2019)**.

Recent findings from a number of studies suggest that dietary D-gal is associated with the development of a wide variety of diseases, as well as the induction of changes to the cardiovascular system that can lead to sudden death. These changes include increases in blood pressure and arrhythmias, including ventricular fibrillation. In addition to the recognized effects it has on metabolism, D-gal is a molecule that, according to the findings of some research, may play a role in the development of certain disorders such as diabetes and cardiovascular disease **(Azman and Zakaria,2019).**

Due to the fact that the exposure of the enteroendocrine cell line to dietary doses of D-gal lowers glucagon-like peptide-1 (GLP-1) secretion and reduces beta cell mass, the risk of developing cardiovascular illnesses and neuropathies is increased **(Koepsell,2020).**

An electrocardiogram, also known as a time-voltage graph, depicts the electrical activity of the heart and offers a wealth of information despite taking up just a small amount of physical space. Electrical impulses can be produced by cardiac muscle activity as well. Muscle contraction, which is what produces the pulse, typically occurs after electrical activity in the heart. The measurement of the time of electrical conduction and the voltage involved typically indicates the function of the heart, with different parts of the electrocardiogram complex representing the various stages of conduction. This is true even though there are a variety of factors that can have an effect on cardiac function. P waves, PR intervals, Q waves, QRS complexes, ST segments, and T waves are the components that make up the typical sinus rhythm complex. Because of the potential for these parameters to be altered in patients, this study focused on analyzing the P wave, PR interval, Q wave, and QRS complex **(Ji *et al* .,2019).**

Damage to the cell membrane and an increase in the membrane's permeability are both caused by reactive oxygen species (ROS), which in turn leads to an influx of extracellular calcium. Over time, the damage leads to an accumulation of calcium inside the cells, which results in abnormal cardiac contractions, apoptosis, and other damaging effects. attenuated galactosemia-induced elevation of oxidative stress and blocked the reductions of myocardial (Na,K)-ATPase and calcium ATPases **(Sharmila *et al* .,2018)**.

It is well recognized that cardiac dysfunction caused by diabetes is accompanied by alterations in both the Na+/K+-ATPase and Ca2+-ATPase activities **(Onikanni *et al* .,2022)** Ca2+- ATPase activity was shown to be reduced in diabetic rats in the current investigation, and an accompanying rise in total calcium levels was seen, as was the case in the earlier study **(Tuchina and Tuchin, 2022).**

In diabetic cardiomyopathy and other complications of diabetes mellitus, an impaired calcium homeostasis was documented **(Liu and Dudley , 2020).** Ca2+-ATPase is the primary active calcium transport protein that is responsible for the maintenance of normal intracellular calcium levels in a wide variety of cell types. It does this by ensuring that calcium levels remain within the usual range. It was shown that abnormal Ca2+-ATPase activity as well as intracellular calcium levels were essential processes responsible for the cardiac dysfunction that type 1 diabetic animals had **(Wu *et al* .,2021)**

The osmotic action of high glucose (cell shrinkage), which has been shown to activate G proteins, most likely through a stretch receptor, which in turn stimulates calcium channels may be an explanation for the elevated intracellular concentration of calcium **(Garg *et al* .,2019)**.

The findings of the current investigation demonstrated a statistically significant connection between the quantity of galactose in the blood and the incidence of abnormal ECG readings in the group that had been exposed to D-galactose. The heart is an organ that has a high demand for oxygen, is very sensitive to oxidative reactions, and is easily damaged by oxidative stress**(Spoelstra-de Man *et al* .,2018)**.

The production of reactive oxygen species (ROS), the induction of cell autophagy, the damage to endothelial cells, and the causation of heart hypertrophy are all caused by D-galagtose-induced oxidative stress **(Lee *et al*.,2020)**.

In addition, reactive oxygen species have the ability to cause damage to mitochondria, trigger the release of pro-apoptotic proteins, activate the downstream proteins of the caspase family, and initiate cell apoptosis, all of which contribute to the destruction of cardiac cells **(Checa and Aran,. 2020)**.

Excessive reactive oxygen species (ROS) can cause myocardial cell hypertrophy, apoptosis, necrosis, and fibrosis, which can eventually lead to excitation–contraction coupling dysfunction, arrhythmia, and myocardial remodeling. Long-term exposure to ROS can also cause myocardial cell necrosis,these are some of the most major contributors to disorders of the cardiovascular system**(Liu *et al* .,2020)**.

The intake of D-gal, has been associated to cause atrial fibrillation (AF) .Nevertheless, studies have failed to proof a connection between D-gal and those symptoms, including the association of D-gal with AF, a common cardiac arrhythmia, in self-reported D-gal-sensitive patients **(Wei *et al* .,2021).**

Several subtypes of galactose receptors are extensively and variably expressed in diverse structures, and each have its own particular distribution **(Juliano,2016)**.

The outcome of an electrocardiograph showing atrial fibrillation generated by D-gal in male rabbits due to the discovery of many galactose receptors in the skin epidermis, the heart's electrical conduction, and the heart itself **(Selvakumar *et al* .,2016).**

D-gal are an exotoxin of the cardiac, and there is a detectable change in heart rate or rhythm after being given D-gal. It is possible that these receptors are involved in significant cardiac functions (such as contraction, rhythm, and coronary circulation), and as a result, they may be implicated in the pathobiology of cardiac diseases . **(Paz,2019).**

Thus , diets contain excesses of D-gal alter atrial depolarization or the conduction of electrical stimuli from the atria to the ventricle **(Friedrichs *et al* .,2012;Tram *et al* .,2021)**.

ECG changes have been observed that show an elevation of ST-segments in the D-gal group, as demonstrated by the current study. This may be the result of myocardial necrosis that was triggered by D-gal **(Pan *et al* .,2021),** while treatment with *Spirulina* may have helped to improve the ST-segment interval, which is suggestive of the cell membrane-protecting function of *Spirulina* **(Puchkova *et al* .,2020)**.

The main clinical cardiomyopathy is a higher incidence of cardiac arrhythmias, including ventricular fibrillation, and a higher occurrence of sudden death because of alterations in ventricular repolarization. These alterations may cause changes in cardiovascular physiology and structure, which can be recorded by ECG **(Ji *et al* .,2019) .**

The QRS complex is the component of the sinus rhythm complexes that indicates ventricular depolarization,because ventricular depolarization requires a substantial voltage, the QRS complex is the greatest component of the sinus rhythm complexes **(Signal,2020)**.

The His bundle, bundle branches, hemi-branches, and Purkinje fibers make up the specific conduction system that guarantees impulses move quickly from the AV node to the ventricular muscle**(Ji *et al* .,2019).**

The duration of the QT interval, which indicates the total amount of time required for ventricular depolarization and repolarization, is calculated by starting at the beginning of the QT interval and continuing until the end of the T wave **(Gokalp and Ozbeyaz, 2022).**

The QT interval should be significantly shorter than the R-R interval that came before it. In patients with prolonged QT intervals, there is a delayed repolarization, which can lead to tachydysrhythmias and even abrupt cardiac death **(Rowlett and Villa,2020).**

Both a prolonged QRS complex and a prolonged QT interval imply that there has been a prolonging of the events that take place between ventricular depolarization and repolarization **(Hung *et al* .,2022).**

Exposure of D-gal may, in point of fact, be directly responsible for some of these irregularities in the heart's rhythm. The consumption of a food that contained D-gal led to hydro electrolytic modifications, which caused changes in the QRS complexes of the animals and, as a consequence, in the QT and QTc intervals of those animals **(Krayukhina *et al* .,2008; Wu *et al* .,2010)**.

In point of fact, such changes in electrocardiographic patterns could be explained by polyuria, which is brought on by osmotic diuresis as a consequence of a rise in glycemia **(Rusu *et al*.,2020**).

This excessive urinary loss causes a decrease in potassium and other electrolytes, which may be responsible for the increase in the membrane repolarization period represented by a prolonged QT interval ,additionally, this decrease in potassium and other electrolytes causes a decrease in the body's overall fluid balance**(Ji *et al* .,2019)**.

Alterations in voltage-dependent potassium ion channels are another potential cause of the lengthened QT interval. Not only because of the direct effects of [K+], but also because the cellular balances of [K+], [Na+], and [Ca2+] are interlinked through [Na+]-[K+] ATPase and [Na+]-[Ca2+], lower and higher values of serum [K+] can have electrophysiological effects that commonly promote cardiacarrhythmias **(Mubagwa,2020).**

This is not solely because of the direct effects of [K+], but Insufficient levels of extracellular potassium result in a hyperpolarization of the resting membrane potential, which paradoxically results in an increase in the excitability of cardiomyocytes**(Skogestad, and Aronsen, 2018).**

This effect is attributed to an increased number of accessible [Na+] channels and a reduced ability of IK1 to generate outward current that protects against membrane depolarization ,[Na+] channels are ion channels that allow sodium ions to pass through them **(Bhuyanand Chakraborti,2019)**.

Ca2+ overload has been recorded in beating hearts that were perfused with low levels of potassium, according to studies. According to the findings of one study, the [Ca2+]- induced[K+] channel was active in hearts that had been subjected to low [K+] (which corresponds to clinical hypokalemia), but it was not active in hearts that had been exposed to normal [K+] **(Zhou *et al*., 2021).**

Spirulina has been proven to protect against lipid peroxidation by directly scavenging free radicals, boosting the activities of catalase, and superoxide dismutase in the body **(Chaouachi *et al* .,2022)**.

Spirulina is a good source of the phycobilliproteins and Phycocyanin is one of them with many beneficial effects being a nutraceutical as well as therapeutic agent **(Agrawal *et al*., 2021).**

Phycocyanin is reported to have potent therapeutic regimen in having strong anticancer, antidiabetic, hepatoprotective and neuroprotective effects to list a few and that to be principally due to its strong antioxidant activity **(Agrawal *et al*., 2021).** It scavenges free radicals, protects against lipid peroxidation and has been studied for its anti-cancer effect on malignant solid tumors **(Jiang *et al*., 2017).**

Phycocyanin is documented to protect against diabetic as well as cisplatininduced nephrotoxicity by inhibiting oxidative stress and activation of antioxidant enzymes **(kim *et al* .,2019).** Protective effects of phycocyanin on ischemia/reperfusion cardiac dysfunctions and liver injuries have also been demonstrated through its potent antioxidant profile along with antiapoptotic activities **(Gdara *et al*. 2018).**

Study suggested that Phycocyanin is a promising source of natural antioxidants that could have great importance as nutritional as well as therapeutic supplement in preventing or slowing the progress of ageing and age associated oxidative stress related degenerative disorders (**Matos *et al* .,2017)**.

In the research that we conducted, treatment with *Spirulina* did not demonstrate any meaningful therapeutic effect in terms of the rates of AF recurrence or cardioversion **(Sharma *et al* .,2012).** Blockers of the Na+ channel cause an increase in the width of the QRS complex **(Harrigan and Brady ,1999).** It's possible that the QRS complexes in some instances will take on the pattern of well-known bundle branch blocks **(Corradi *et al*.,2019).**

Na+ channel blocking drugs, on the other hand, have the potential to influence cardiac pacemaker cells. Bradycardia may develop when the pacemaker cells of the heart, which are dependent on the influx of sodium ions, depolarize more slowly than normal. The combination of a wide QRS complex and bradycardia is an ominous sign in [Na+] channel blocker poisoning caused by anticholinergic and sympathomimetic drugs. This may indicate that the Na+ channel blockade is so profound that tachycardia does not occur, despite clinical muscarinic antagonism or adrenergic agonism **(Rushton and Holstege,2020).**

In comparison with the other groups, the HR of rabbits who were given D-gal exhibited a substantial drop in HR in GII (GI,GIII,GIV). These data lend credence to the findings of **(El-baz *et al* .,2019).**

After the administration of D-gal, it was reported that the HR dropped significantly, and after four weeks, it had returned to its previous steady state. This decrease in HR might be partially explained by less time spent being physically active. The decreased bradycardic reflex in D-gal-treated rabbits may have been the result of a decrease in the vagal influence on the heart. This reduction may have suggested deficits in the vagal reserve that is employed during heart rate responses elicited by baroreceptors. In the event that this activity is reduced, bradycardia will also be reduced in severity. Therefore, the reduction in the bradycardic response may have been caused by a reduction in the sinus node's reactivity to parasympathetic stimulation **(Schauerte *et al*.,2001).**

An increase in cardiac oxidative stress and inflammatory mediators were found to be associated with age-related cardiac dysfunction. This dysfunction was also associated with dramatic alterations in the ECG pattern and a prominent increase in the levels of blood cardiac biomarkers. In addition, the histological analysis of the heart indicated significant alterations in the architecture of the cardiac tissue. The ECG pattern revealed an irregular rhythm of heartbeats, a depressed ST height, negative T waves, and elevated PR and QRS intervals in comparison to normal rats, all of which are indicators of myocardial illness . Additionally, the rats' heartbeats exhibited a negative T wave pattern. In addition, a substantial rise in heart rate was observed alongside an increase in the serum levels of cardiac function-relevant indicators such as HS, CK-MK, and LDH, as well as a decrease in the myocardial GLUT-4 content as compared to the group that served as the control. Previous research has revealed that age-related alterations in impulse propagation may be associated to aberrations in the pattern of ventricular activation. These findings were found to be significant enough to warrant further investigation (**Jianu *et al* .,2020)**.

Recent research , has revealed that changes in the intervals between heartbeats that occur naturally with aging may be connected with fibrosis and hypertrophy. In line with the findings of earlier research, the production of age-related cardiac dysfunction by D-gal demonstrated a significant increase in the levels of IL-6, iNOS, SOD, and NF-B in the hearts of the test subjects. An imbalance between the formation of reactive oxygen species (ROS) on the one hand and antioxidant activities and NO bioactivity on the other is widely believed to be a contributor to the aging process . In addition, proinflammatory signaling in an aging cardiovascular system is mediated by inflammatory cytokines like IL-6, which play a role in the process. Greater levels of IL-6 are found in old myocardium; this leads to increased myocardial injury as well as matrix remodeling , which includes collagen deposition. It has been demonstrated in the past that an impaired level of cardiomyocyte relaxation is related with an increased level of cardiac NF-kB

activity, which is connected with an enhanced level of myocardial activity in older rats **(El-Baz *et al* .,2019; Jianu *et al* .,2020)**.

Changed the cardiac activity, as evidenced by the elongation of the ventricular repolarization time (Q-T interval), as well as the rise in heart rate. In addition, D-gal-induced cardiotoxicity in rats results in an increase in action potential duration in isolated myocytes, which in turn causes K* currents to be altered. The renin-angiotensin system is disrupted and altered by D-gal, which also causes an increase in sympathetic activity. In addition to this, it is possible that it is responsible for the lengthening of OT as well as vasoconstriction by an increase in the cytosolic calcium content brought about by a reduction in the Na'/K'-ATPase activity brought about by a suppression of NO production. It is possible that an increase in the amounts of circulating free fatty acids (FFA), which occurred as a result of D-gal-induced cardiopathology, also led to abnormalities in the Q-T interval. In healthy patients, FFA exposure has the potential to cause endothelial dysfunction and to lengthen the Q-T interval. It has been noted in earlier observations,. Indeed, high glucose concentrations in the perfusion medium of isolated working rat hearts elicited a considerable lengthening of the QT interval. This finding supports the hypothesis that QT prolongation is caused by glucose **(Hu *et al* .,2022; Warnock,2022; Yang *et al* .,2022)**.

5.4 Histological Changes of the heart and aorta

Studies have shown that the aortic tunica medial thickening and microscopic structures found in the control rabbit groups are consistent with findings published by other researchers **(Heil et al .,2020; Wang et al .,2021).**

The outcomes examination aortic wall in our research histological changes agreement with explanation authors' they indicated effect D-gal. D-galactose is one of the chemicals that contribute to toxicity in the cardiovascular system, and it's induce histological changes of aorta, such as structure damage, necrosis of tunica intima endothelia, Mononuclear inflammatory cells infiltration, and necrosis of smooth muscle fibers **(Dai *et al* ., 2018; Dehghani *et al* ., 2019; Wang *et al* ., 2020).** The previously studies in mice have demonstrated that D-gal can induce aortic injury, including oxidative stress, inflammation, endothelial dysfunction and elastic fiber disorganization, which can lead to aortic injuries **(Wang *et al*., 2020).**

Furthermore some of these major effect of D-gal are formation of collagen bridges, and decrease in the elastin/collagen ratio .The most described event involving the elastic fibers in atherosclerosis is the alteration of the elastin/collagen ratio, due to elastic fiber degradation under the atheroma plaque by elevated elastase - type protease levels **(Armaini and Imelda, 2021).** What was stated in the research results are consistent with what the our results in D-gal rabbit group indicated regarding the interpretation of their research.

From another point of view to the opinions of other researchers, the vascular structural and functional alterations during aging is associated with critical modifications of the vascular wall such as endothelial dysfunction and changes arterial thickness and stiffness. Endothelial dysfunction includes reduced vasodilator and antithrombotic properties, with increased oxidative stress and inflammatory cytokines, increasing the risk of atherosclerosis and thrombosis. Furthermore, the endothelial barrier becomes porous and vascular smooth muscle cells migrate to sub endothelial spaces and deposit extracellular matrix proteins resulting in the thickening of the tunica adventitia. Central arterial stiffness is related to the loss of elastic fibers and the increase of collagen accumulation in the vessel wall, which deteriorates vascular functionality. Endothelial dysfunction and arterial stiffness are mediators connected closely in vascular dysfunction during aging. If the artery is more rigid, greater will be the exposure of the endothelium to hemodynamic load, promoting endothelial activation, inflammation, and oxidative damage**.(Dai *et al* .,2018 ;Lee *et al* .,2020 ; Zhang *et al* .,2021)** , these similar what we've discovered via our research in the rabbit groups received D-gal we've observed outcomes under line examination of vascular slide of elastic artery by light microscope that clarified desquamation (focal necrosis) of tunica intima endothelia, furthermore we can saw the tunica media was dearranged elastic fibers 'with piling up collagen fibers present in several areas and depletion others when compared with control rabbit groups, and sever mononuclear inflammatory cells infiltration. We also notice relatively thickness in adventitia tunica.

But at the other hand, we showed that all three layers of the aorta in *Spirulina* treated rabbit groups (the intima, the media, and the serosa) had normal microscopical details structures when compared with d-gal treated rabbit groups. This is related with the ameliorate action of *Spirulina*, which is why it has antioxidant, anti-inflammatory, anti-neoplastic, and anti-proliferative properties, as reported by**(Afkhami *et al* .,2021; Metekia *et al* .,2022)**.

Antioxidant therapies have been shown to attenuate aging-induced changes through endothelial dysfunction and changes in the extracellular arterial matrix that cause central arterial stiffness and mention phycocyanin present in *Spirulina*stands out because of its high antioxidant capacity and scavenging of free radicals due to its stability **(Li ,2022; Han *et al* .,2021).** As well during the mechanism of action it's to involve the release of nitric oxide, reducing oxidative stress. they found in the Previous study the supplementation with powder of *Spirulina*, *Spirulina* has the ability to modulate both in chronic vascular tone and lipid peroxidation in the aorta of Wistar rats, *Spirulina*is quite effective for the prevention of endothelial dysfunction, but the other parts of *Spirulina*l, like carotenoids, chlorophylls, and vasoactive peptides, work together to keep cardiovascular problems from occur, probably stop all heart problems from happening. Also found that the C-phycocyanin is linked to vascular endothelial modulation**(Atallah *et al* ., 2021 ; Elbialy *et al* .,2021 ; Chaouachi *et al* .,2022)** . These results authors similar our results of histological section stained large artery examined of D-gal and *Spirulina*l supplementations rabbit groups of intimal layer endothelia reveal normality and focal necrosis in others areas , with more arranged and organization of lamina fibers of tunica media, as well mild necrosis in smooth muscles and few inflammatory cells in filtrations' this refer minimal microscopic architectural abnormalities' demonstrates that the study was accurate in its assertion that *Spirulina* had a protective effect on histological changes that induce by D-gal.

The microscopic architecture of the cardiac muscles in the control rabbit groups have been proven to be compatible with previous published findings **(Heil *et al* .,2020; Eroglu *et al* .,2022).**

The authors **(Lei *et al*., 2016; Chen *et al*., 2018 ; Triposkiadis *et al* ., 2019)** were evaluate the effect induced of D-galactose-treated animals, the morphological disarrangement in cardiac architecture with disordered large interstitial spaces between the cells and higher number of apoptotic cardiomyocytes, furthermore, obvious capillary vessel of myocardial interstitial congestion and functional changes in the heart due to aging explain the high rates of heart failure (HF) in old age . The mechanisms involved behind these changes include high levels of oxidative stress and inflammation, high rate of apoptosis, loss of regenerative capacity of cardiac progenitor cells, hypertrophy of remaining cardiomyocytes, loss in mitochondrial health, and unbalance of calcium homeostasis. Events are highly connected with heart failure HF Structural changes were observed regarding the reduction in the number of cardiomyocytes associated with their hypertrophy, collagen accumulation with disordered fibroblast arrangement, and its metabolites, and functionally ejection fraction .These assortment of cardiac tissue of rabbit groups that are supplementing D-gal revealed destroyed and disarrangement myocardial fibers in the myocardium

with sever fatty tissue precipitation also, we can see notable thickened endocardium and vascular congestion (**Chen *et al*., 2018;Kolstad *et al* .,2018; Azman and Zakaria, 2019; Triposkiadis *et al* .,2019; Li-Zhen *et al*., 2021)**.

However on the other hand, we demonstrated that the heart muscle in *Spirulina*-treated rabbit groups (the had the typical architectural appearance of heart muscle, normal straightening, and normal arrangement of myofibers, when contrasted with the heart muscle in other treated rabbit groups. Seeing that *Spirulina* has an ameliorating effect, which is why it possesses antioxidant, anti-inflammatory, anti-neoplastic, and anti-proliferative characteristics, as was described by **(Afkhami *et al* .,2021; Metekia *et al* .,2022)**

Finding out the treatment of *Spirulina* ameliorated the cardiotoxic effects and protected the heart from oxidative stress-induced damage. The protective effect of *Spirulina* could be due to the presence of antioxidants such as β-carotene and C- phycocyanin . The result of this study indicates that *Spirulina* can protect the cardiac myocytes against oxidative stress induced by DOX and confirms the earlier findings showing that induction of oxidative stress and lipid peroxidation are among the basic mechanisms responsible for the cardiotoxicity **(Jadaun *et al* .,2018;Zar and Ahmadi,2021; Albtoosh *et al*., 2022).** Furthermore, one of other studies in rabbits demonstrates that *Spirulina* the histological changes were suggesting protection from cellular damage by D-gal. **(liu *et al*., 2016;Qiu *et al*., 2022).** The changes demonstrated microscopically were similar to our results observed of use antioxidants *Spirulina* mixed with D-gal treated rabbits' group.

Chapter six: Conclusions and Recommendations

6. Conclusions and Recommendations

6.1.Conclusions

From the results and discussion obtained from our study, we can conclude the following-:

1. Subcutaneous injection of 150 mg /kg B.W of D-gal for 4 weeks caused significant elevates in the serum cardiac troponin I , Endothelin-1, MDA, and ONOO with depression in serum GSH and NO.
2. Ameliorate effects of the oral intubation of 500mg/kg B.W daily of *Spirulina* for 4weeks by the significant decrease in the serum cardiac troponin I , Endothelin-I and significant elevation in the GSH and NO.
3. The recovery role of the *Spirulina* in the electrocardiograph in GIII and GIV groups showed a significant decrease in the P,QRS and T waves compared with group received excessive supplementation of D-gal for 4 weeks .
4. Histopathological changes through the formation of marked atherosclerotic and cardiac damage is noticed in GII group with protective role of the *Spirulina* in the heart and aorta tissue damage .

6.2.Recommendations.

1. Expermintal study to investigate therapeutic effect of *Spirulina* in Congestive Heart Failure - CHF
2. Conduct study to evaluate different doses of *Spirulina* against heart failure.
3. Evaluate antioxidant actively of *Spirulina* in reproductive system of male and female.
4. Investigate protective role of *Spirulina* in CNS disorder.

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Appendix

Appendix

Appendix I

Cardiac troponin

Wondfo one step Troponin I whole Blood/Serum / Plasma Test is a rapid immunochrommatographic direct binding test for the visual detection of Troponin I antigen in human whole blood,serum and plasma specimens as an aid in the diagnosis of acute myocardial infarction (AMI) .Wondofo One Step Troponin 1 Whole Blood /Serum/Plasma Test uses double antibody sandwich method.

When the specimen is aadded into the sample well it is absorbed into the device by capillary action , mixes with the antibody –dye conjugate , and flows across the pre-coated membrane.

When the Troponin 1 level in the specimen is at or above the detection limit of of the test , Troponin I bound to the antibody-dye conjugate are captured by

Troponin I antibody immobilized in the Test Region (T) of the device , and this produced a colored test band that indicates appositive result.

When the Troponin I level in the specimen is Zero or below the detection limit of the test , there is not a visible colored band in the in the Test Region (T) of the device . this indicate a negative result.

To serve as a procedure control, a colored line will appear at the control region (C) ,if the test has been performed properly.

Test Procedure

Allow the device and specimen to equilibrate to room temperature (10C -30C) 1.Remove a testing device from the foil pouch by tearing at the notch and place it on a level surface.

2. For Veipuncture Whole Blood testing : Holding a sample dropper vertically , add 3-4 drops (about 80-100ml)of Whole blood specimen into the sample well(with an arrow marked) of the test cassette. For Fingerstick Whole Blood testing: Fill the capillary tube and transfer approximately 80-100ml of finger stick whole blood specimen into the sample well.

Appendix

For serum /plasma testing: Holding a sample dropper vertically , add 3-4 drops (about 80-100ml)of serum or plasma to the sample well.

2.Wait for 15 minutes and read results . Do not read results after 30 minutes .

AppendixII

Endothelin 1

This Kit is an Enzyme-Linked Immunosorbent Assay (ELIZA) . The plate has been pre-coated with Rabbit ET-1 present in the sample is added and binds to antibodies coated on the wells. And then biotinylated Rabbit ET-1 Antibody is added and binds to Et-1 in the sample. Then Streptavidin –HRP is added and binds to the Biotinylated ET_1 antibody. After incubation un bound Streptavidin-HRP is washed away during a washing step. Substrate solution is then added and color develops in proportion to the to the amount of Rabbit ET-1 . The reaction is terminated by addition of acidic stop solution and absorbance is measured at 450nm

Assay procedure

1. Prepare all reagents, standard solutions and samples as instructed. Bring all reagents to room temperature before use . The assay is performed at room temperature.

2. Determine the number of strips required for the assay . Insert the strips in the frames for use. The unused strips should be stored st 2-8 C̊.

3. Add 50µ1 standard well.. Note: Don't add antibody to standard well because the standard solution contains biotinylated antibody.

4. Add 40µ1 sample to sample wells and then add 10µ1 anti-ET-1 antibody to sample sample wells, then add 50µ1 streptavidin-HRPto sample wells and standard wells (Not blank control well). Mix well. Cover the plate with a sealer . Incubate 60 minutes at 37 C̊.

5. Remove The sealer and wash the plate 5 times with wash buffer . Soak wells with at least 0.35 ml wash buffer for 30 seconds to 1 minute for each wash . For automated washing , aspirate all wells and wash 5 times with wash buffer , ,overfilling wells with wash buffer . Blot the plate onto paper towels or other absorbent material

6. Add 50µ1 substrate solution A to each well and then add 50 µ1 substrate solution b to each well . incubate plate covered with anew sealer for 10 minutes at 37 C̊in the dark .

7. Add 50µ1 stop Solution to each well, the blue color will change into yellow immediately. 8. Determine the optical density (OD value) of each well immediately using a microplate reader set to a 450 nm within after adding the stop solution.

Appendix III

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

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 125 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.

Appendix IV

Peroxynitrate

5. Reagent Preparation

Briefly centrifuge small vials at low speed prior to opening.

5.1 Peroxynitrite Sensor Green stock solution (500X) Add 20 µL of DMSO into the vial of Peroxynitrite Sensor Green and mix well to make 500X stock solution.

Δ Note 20 µL of Peroxynitrite Sensor Green (500X) is enough for 1 plate.

Δ Note Unused Peroxynitrite Sensor Green (500X) can bealiquoted and stored at -20ºC in tightly sealed tubes. Avoid light and freeze-thaw cycles.

Appendix

5.2 Peroxynitrite Sensor Green working solution (10X) Add 10 µL of 500X DMSO reconstituted Peroxynitrite Sensor Green (from step 5.1) into 500 µL of Assay Buffer and mix well.

Δ Note The working solution is not stable; prepare it as needed before use.

7. Assay Procedure

- Equilibrate all materials and prepared reagents to room temperature just prior to use and gently agitate.
- Assay all standards, controls and samples in duplicate.

7.1 Run the peroxynitrite assay:

EITHER

1. Add 10 µL/well (96-well plate), or 2.5 µL/well (384-well plate) of Peroxynitrite Sensor Green working solution (from Step 5.2) in 90 µL (96-well plate) or 22.5 µL (384-well plate) cell culture per well in the cell plate.

Δ Note It is not necessary to wash cells before staining. It's recommended to stain the cells in full medium.

2. Co-incubate cells with Peroxynitrite Sensor Green (10X) and test compounds in your growth medium / buffer of choice at 37ºC for the desired period of time, protected from light.

Δ Note It is recommended to stain the cells in full medium. However, if the test compounds are serum sensitive, growth medium and serum factors can be aspirated away before staining. Resuspend cells in 1X Hank's salt solution and 20 130 mM Hepes buffer or the buffer of your choice after aspiration. Alternatively, cells can be stained in serum-free media. **Δ Note:** By way of example, we co-incubated RAW 264.7 macrophage cells with 50-200 µM SIN-1 Peroxynitrite Sensor Green in full medium at 37ºC for 1 hour to induce peroxynitrite. **OR**

3. Pre-stain cells Peroxynitrite Sensor Green at 37ºC for 1 hour in your growth medium / buffer of choice protected from light (as in 7.1.1) but in the absence of test compounds.

4. Remove the cell medium, then treat the cells with test compounds in the growth medium / buffer of your choice (without Peroxynitrite Sensor Green) at 37ºC for the desired period of time. ab233468 Peroxynitrite Assay Kit (Cell-based) 8

BOTH

5. Measure the fluorescence using a microplate reader at Ex/Em=490/530 nm or a fluorescence microscope using the FITC filter

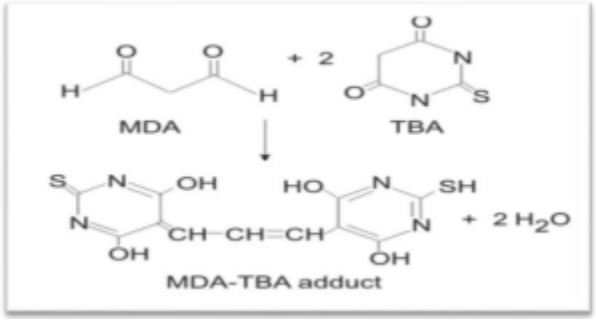
Appendix V

Estimation of Serum Malondialdehyde (MDA):

Malondialdehyhe was estimated by Thiobarbituric acid (TBA) assay method of Buege and 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.

Appendix

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 TCATBA-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

Appendix VI

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

Appendix

• 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.

Appendix

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 134 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.

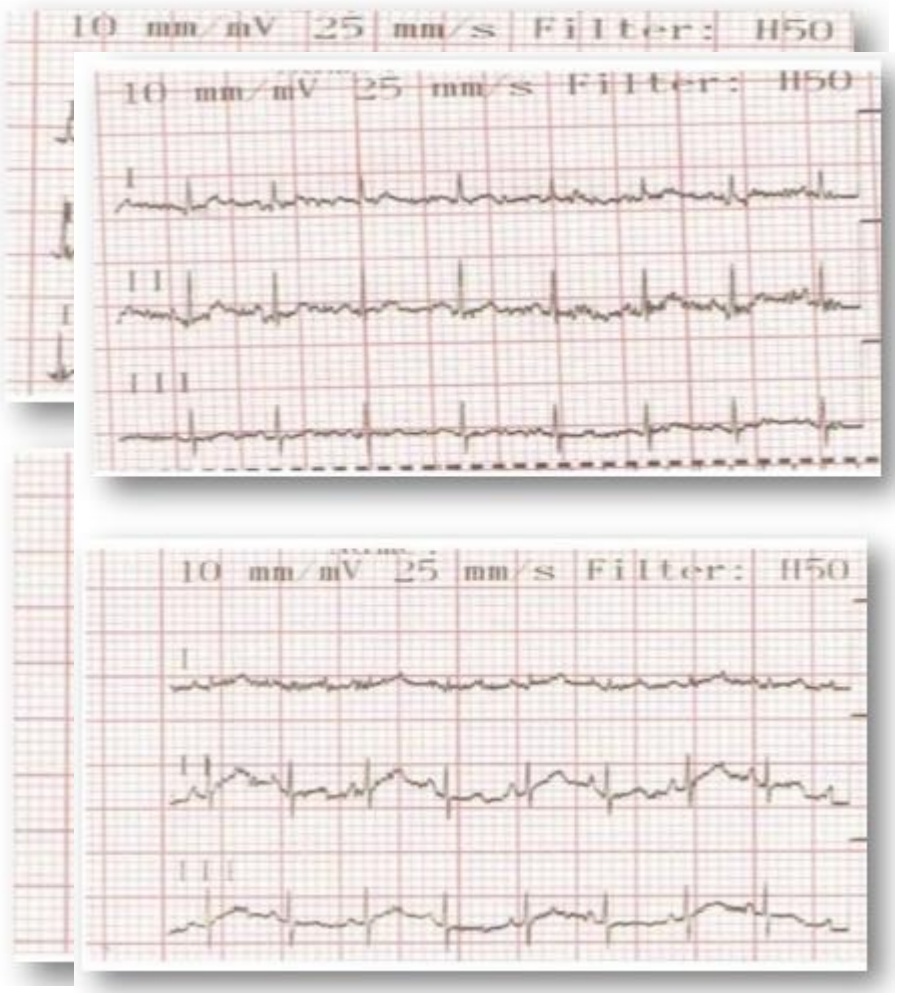
8. Cover the plate with aluminum foil or incubate plate in dark on shaker until absorbance is checked. For kinetic method absorbance at 0 minute is also recorded 9. Glutathione concentration can be determined by endpoint method or by kinetic method

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

Appendix

Appendix VII : Electrocardiograms of GI (control)**.**

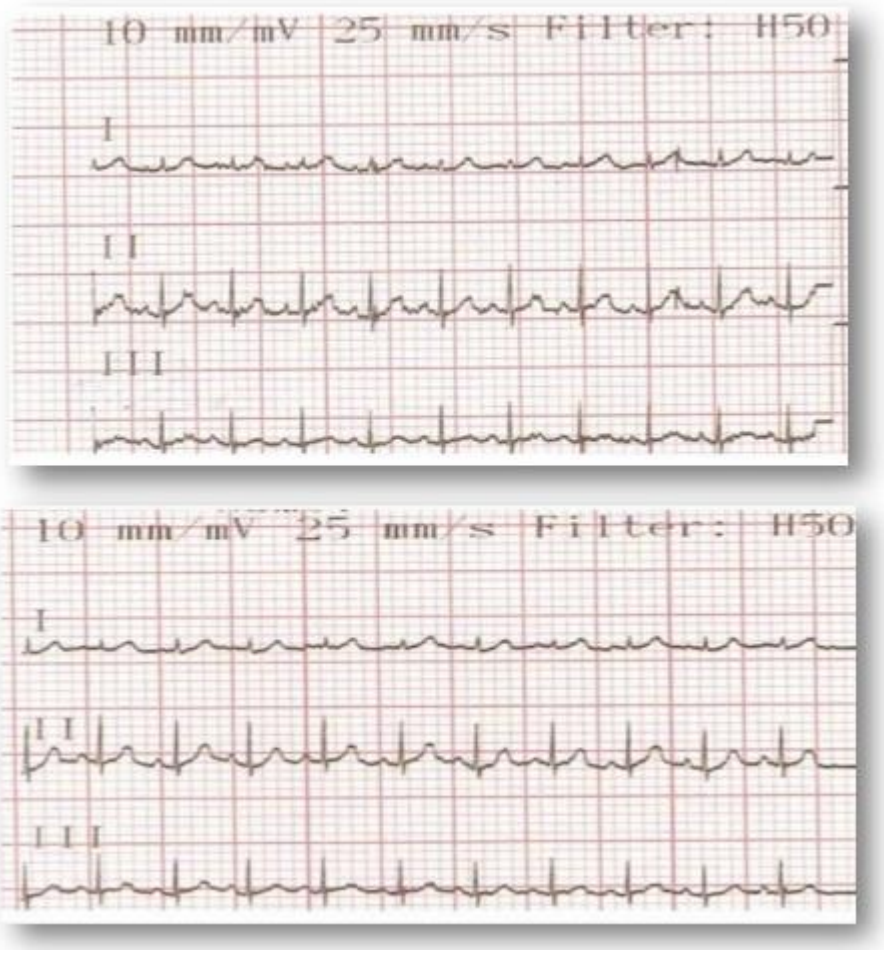


Appendix

Appendix VIII: Electrocardiograms of GII received D-gal in male rabbits

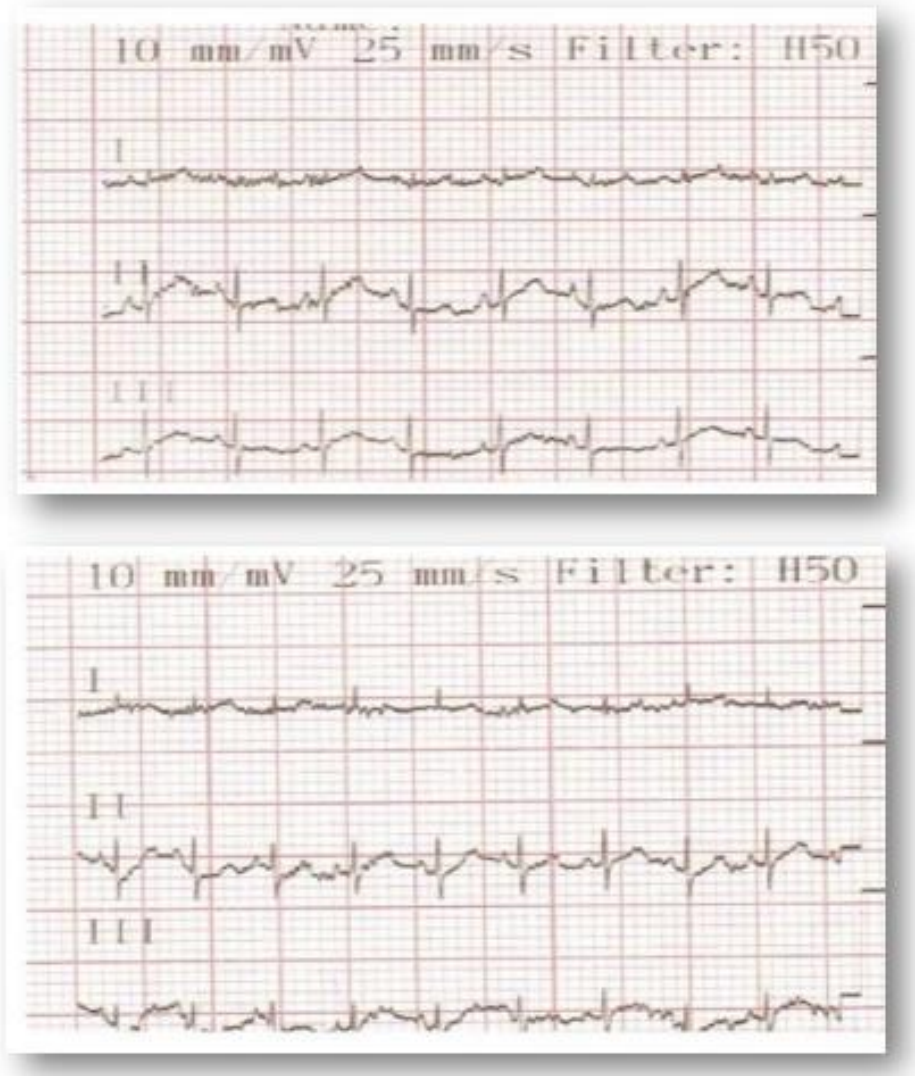
Appendix IX : Electrocardiograms of GIII recessived *Spirulina* in male rabbits.

Appendix



Appendix X : Electrocardiograms of GIV combined of D-gal and *Spirulina* treated rabbits

Appendix



Appendix XI

Histological study

Histological Technique(E & H) stain

Appendix

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 C. The heat causes the solvent to evaporate, and the space within the tissues becomes filled with paraffin

*** 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.

Appendix

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 ℃for 1 hour.

الخلاصة

تم إجراء الدراسة خلال الفترة من 17 ديسمبر 2021 إلى 17 يناير 2022 وتم تصميمها للتحقق من دور طحلب السبيرولينا في تلف القلب والشريان الابهر الناجم عن الديكالكتوز في ذكور الأرانب. تم تقدير بعض المؤشرات الحيوية القلبية في مصل الدم ، على سبيل المثال1 Endothelin .(، تروبونين I و (NO ، بعض المواد المؤكسدة ONOO)و (MDA ومضادات الأكسدة (GSH (وموجات تخطيط القلب P) ، QRSو (T و ST و QT الفاصل الزمني تم رصدهما وفحص نسيج القلب والابهر.

قسمت عشرون (20) ذكر ارنب إلى أربع مجموعات (5 / مجموعة) ، لم يتم إعطاء المجموعة الأولى أي مادة لمدة 4 اسابيع وعملت كمجموعة تحكم (G1) ، في المجموعة الثانية تم حقن المجموعة تحت الجلد بجرعة 150 مجم / كجم من وزن الجسم (G2 D-galactose) ، وتم تجريع ارانب المجموعة الثالثة ب السبايرولينا بنسبة 500 مجم / كجم من وزن الجسم (G3) والمجموعة الرابعة (G4)) بمزيج من D-galactose و *Spirulina* لمدة أربعة أسابيع. تم جمع عينات الدم باستخدام تقنية ثقب القلب في 4 أسابيع من الدراسة التجريبية .

تشير نتيجة دراستنا إلى زيادة معنوية ($p\leq0.05$) في مستويات مصل ONOO و MDA مع زيادة معنوية في مستوى مصل القلب من تروبونين القلب I و Endothelin-1 في المجموعة التي تلقت D-galactose بالإضافة إلى النتائج كشفت انخفاض معنوي ($p\leq0.05$)في مستوى تركيز GSH و NO في الدم.

نتائج تسجيل مخطط كهربية القلب أظهرت الدراسة الحالية استطالة الموجات (P ، QRS ، T)والفواصل الزمنية(ST، QT)في مجموعة GII مقارنة بمجموعات GI و GIII و.GIV

التجريع بمقدار 500 ملجم / كجم من وزن الجسم يوميًا من سبيرولينا في GIII جنبًا إلى جنب مع ديكالاكتوز في مجموعة .GIV أظهرت النتائج تحسنًا في انتعاش القلب والشريان الابهر من خلال انخفاض كبير ($p\leq0.05$) في مستوى مصل ONOO و MDA ، أظهر أيضًا زيادة معنوية ($P\leq0.05$) في مستوى مصل GSH و .NO conc مقارنة بمجموعات GI و.GII

أظهرت نتائجنا انخفاضًا معنويًا ($p\leq0.05$) في مصل Endothelin-1 و Troponin-I في GIII و GIV مقارنةً بـمجموعات أخرى. أظهر دور الوقائي للسبيرولينا في تخطيط القلب في GIII و GIV انخفاضًا كبيرًا ($p\leq0.05$)في موجات (P ، QRS، T)و فترات (ST ، QT)مقارنة بمجموعة.GII

أظهر فحص التغير النسيجي للقلب والشريان الابهر في GII الذي تم تلقيه بـ D-gal أن القلب أظهر نخر والتهاب وتليف وتسلل إلى الخلايا الالتهابية ونزيف واحتقان ، ومن ناحية أخرى سماكة في الشريان الابهر مقارنة بالمجموعة الأخرى وأظهرت الدراسة الدور الوقائي لـ سبيرولينا في القلب والشريان الابهر في.GIV

في الختام ، أجريت دراستنا على الإصابات الناجمة عن D-gal ، ويجب تجنبها خاصةً في القلب واضطراب الشريان الابهر من زيادة D-gal والتي يمكن تحصينها بالتجريع بمقدار 500 مجم / كجم من وزن الجسم من سبيرولينا للتقليل من آثارها الضارة.

الخلاصة

جمهورية العراق

وزارة التعليم العالي والبحث العلمي

جامعة كربلاء/كلية الطب البيطري

فرع الفسلجة والكيمياء الحياتية والادوية

تاثير السبايرولينا ضد شيخوخة الجهاز القلبي الوعائي المستحدثة بواسطة الديكالكتوز في ذكور الأرانب

رسالة

مقدمة إلى مجلس كلية الطب البيطري / جامعة كربلاء كجزء من متطلبات نيل درجة الماجستير في اختصاص علوم الطب البيطري / فرع الفسلجة

كتبت بواسطة

هبه عبد الكريم كاظم

بأشراف

أ.د. وفاق جبوري البازي

أ.م.د. منى حسين حسن

2022 م **1444 هـ**