College of science

Department of Botany and Microbiology

Application of *Spirulina* to enhance Liver's Functions: Effects and Safety

Prepared by:

Wahida Haza'a Al qahtani

Supervised by:

Prof. Ali Abdullah Al-Homaidan

Prof. Ammar Cherkess Alrikabi

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Department of Botany and Microbiology

Approval Sheet

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By

Wahida Haza'a Al-Qahtani

This thesis has been examined on 25/4/2016 and approved by:

Committee members:

Professor Ali A. Al-Homaidan	supervisor.....
Professor Ali H. Bahkali	member .....
Professor Ibrahim A. Arif	member.....
Professor Ahmed A. Al-Qahtani	member.....
Professor Abdullah S. Al-Homida	member.....

بِسۡمِ ٱللَّهِ ٱلرَّحۡمَٰنِ ٱلرَّحِيمِ

قال الله تعالى في كتابه الكريم:

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آل عمران : 18

إهداء

إليكم اخوتي أحبتي ....

إلى قلوب كانت صغيرة، أخذت اسقيها من دمع عيني حرقة وشوقا.

ومن دم قلبي لهفة وحبا، حتى كبرت وصار قلبي يرقص فرحا حين لقياها.

إلى قلوب ماتزال صغيرة، أسقيها من دمي وروحي وتسقيني من ثغرها البريء باسمة ومتفائلة وحانية.

إليكم أولادي صوت فرحتي بل سر قوتي....

إليك أبي " يا حبيبي أنت" مت جسدا وعشت روحا ذائبة في روحي....

إليك أمي " يا حبيبتي أنت" نهر العطاء الذي لا ينتهي.

أنت ما أنت **!!!** "أنت أم لا كالأمهات "....

إليك نفسي...

من الحياة تألمت وتعلمت ...

إليك وحيـــــــــــــــده....

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Table of Contents

List of Tables

List of Figures

List of Abbreviations

Abbreviation	Meaning
AIN	The American Institute of Nutrition
ALP	Alkaline phosphatase
AOAC	Association of official analytical chemistry
APAP	Acetaminophen
ARASCO	Arabia Agricultural Service Company
AST	Aspartate aminotransferase
ASYBUF	Assay buffer
ASYREAG	Reaction solution
BHT	Butylated hydroxytoluene
CAT	Catalase
CDNB	Chloro-2, 4-dinitrobenzene
D-GalN	D-galactosamine
DNPH	Dinitrophenylhydrazine
DPI	Diphenyleneiodonium
DTNB	5, 5¢-dithiobis 2-nitrobenzoic acid
ESA	European Space Agency
GGT	γ-glutamyl transpeptidase
GOT	Glutamate oxaloacetoacetate transaminase
GPT	Glutamate pyruvate transaminase
GPX	Glutathione peroxidase
GR	Glutathione reductase
GSH	Reduced glutathione
GST	Glutathione transferase
H & E	Hematoxylin and eosin
HDL	High-density lipoprotein
HIV-1	Human immunodeficiency virus-1
IFN-γ	Interferon-γ
Ig	Immunoglobulin
IIMSAM	The Intergovernmental Institution for the use of

	Micro-algae '*Spirulina*' Against Malnutrition
IL-4	Interleukin-4
LDL	Low optical density
LOD	Low-density lipoprotein
NADPH	Nicotinamide adenine dinucleotide phosphate – oxidase
NASA	National Aeronautics and Space Administration
NK	Natural Killer
PER	Peroxide substrate solution
PNA	p-nitroaniline
Ppm	Part per million
ROS	Reactive oxygen species
SOD	Superoxide dismutase
SPSS	Statistical Package for Social Sciences
t-Bu-OOH	Tert-butyl hydroperoxide
TNB	5-thio- 2-nitrobenzoic acid
TP	Total protein
XOD	Xanthine Oxidase

Abstract

Liver is one of the most important organs in the human body as it is responsible for remediation of toxic substances that enters the body. In addition, it helps in the fat metabolism. The liver may be exposed to different infections and/or diseases; such as cirrhosis and hepatitis, all diseases adversely affect the liver functions; and therefore, cause many other health problems. There is a massive need for constant, reliable and safe medications to overcome these health problems without affecting any other parts of the human body.

Spirulina is a member of Cyanobacteria. It has very high nutritional value. Moreover, many studies investigated the beneficial effects of *Spirulina* including good effects antioxidant, antiviral, and anticancer. *Spirulina*, also, lowers the harmful Low density lipoprotein (LDL) cholesterol ratio in the blood and increases the High density lipoprotein (HDL) cholesterol. The most important part is that there are many studies approved the safety of *Spirulina* as a supplementary diet for humans.

S. platensis was cultured at the Department of Botany and Microbiology, College of Science, King Saud University, the *Spirulina* powder was prepared by Association of official analytical chemistry AOAC (1990) method. The powder was analyzed to examine its mineral content and the aqueous extract was used in the study implementation.

Thirty-six Wister Albino male Rates were used and randomly divided into 6 groups. Group A as control with no treatments, group B received D-GalN only, group C received 0.5% Butylated hydroxytoluene (BHT) as an antioxidant and D-GalN, group D received 3% *Spirulina* and D-GalN, group E received 6% *Spirulina* and D-GalN and group F received 9% *Spirulina* and D-GalN. The rats were made to fast for 24 hours before starting the study. All

the rats were fed with free water and food during the study. BHT and *Spirulina* were added to the diet according to the stated concentrations. All the groups (except group A) received the D-GalN through intraperitoneal IP injection with concentration of 300 mg/kg body weight at the 7^{th} day from the start of the experiment. After 24 hours of the D-GalN application, rats were anaesthetized by IP injection of Ketamine (100mg/Kg body weight) and xylazine (16mg/Kg body weight) .Blood was collected from heart directly before scarifying, and serum was separated and stored at -80 °C freezer for further analysis. Liver was taken and preserved in 10 % buffered formalin (pH 7.4) to be used in histological examination. Total protein level, albumin level, cholesterol level and oxidative stress enzymes activities were analyzed in blood serum. Moreover, liver function enzyme activities and lipid profile level were measured. Rat Liver were examined histologically to observe the damage caused by the application of D-GalN.

Analysis of *Spirulina* powder showed that the proteins and carbohydrates represented the major constitutes of the powder with percentages of 40.77% and 35.44%, respectively. It, also, had high concentration of iron (294.26 ppm) and manganese (51.78 ppm).

The application of D-GalN caused severe toxicological effects represented by significant increases ($P \leq 0.05$) in the levels of the examined enzymes Alkaline phosphatase ALP, Aspartate aminotransferase AST, Glutamate oxaloacetoacetate transaminase GOT, Glutamate pyruvate transaminase GPT and γ-glutamyl transpeptidase GGT and cholesterol. On the other hand, it caused significant decrease ($P \leq 0.05$) in total protein and albumin level. The addition of BHT alleviated the harmful effects of D-GalN through decreasing the levels of ALP, AST, GOT, GPT, GGT and cholesterol, in addition increasing total protein and albumin levels compared to groups B.

Different concentrations of *Spirulina* added to the rats' diet showed a protective effect compared to BHT, against D-GalN toxicological effects. The levels of ALP, AST, GOT, GPT , GGT and cholesterol were lower in rats that received *Spirulina* comparing to group B. Increasing the *Spirulina* concentration caused more reductions in these enzymes and cholesterol concentrations in blood serum or liver. These levels of enzymes and cholesterol were almost identical in the groups received 9% *Spirulina* and BHT which provide an evidence for the protective effects of *Spirulina* for the liver. Moreover, *Spirulina* increased the levels of total protein and albumin compared to group B. *Spirulina* showed good effects on the antioxidant activity of the studied enzymes. The application of D-GalN caused significant reduction in the activities of SuperOxide dismutase (SOD), Glutathione peroxidase (GPX), catalase (CAT), Glutathione (GSH) and Glutathione-s-transferase (GST). However, the addition of BHT to the diet system improved the activity of those enzymes as compared to groups A with no treatment and group B received D-GalN only. The lowest concentration of *Spirulina* (3%) slightly improved the activity of these enzymes compared to groups A and B. The more the concentration of *Spirulina* added, the better the activity of these enzymes was. Group F received 9% *Spirulina* showed almost identical values for these enzymes with the group C that received BHT only. SOD activity in group F was 0.28 U/L (significantly increased compared to group B 0.13 U/L) and 0.32 U/L in group C (significantly increased compared to group B 0.13 U/L). Similarly, 9% of *Spirulina* resulted in 35 U/L of GPX which was very close to that of BHT (37 U/L), and this was about 3.9 fold more when compared to group B (9 U/L) and about 2.2 fold more than group A (16U/L). Meanwhile, CAT activity in group B was 0.8 U/L and improved with the addition of BHT and 9% *Spirulina* to 2.6 U/L and 2.2 U/L, respectively. GSH

activity was also improved from 7 U/L in group B to 16.5 U/L in group C and about 14.5 U/L groups E and F (6% and 9% *Spirulina* with no significant difference between the two groups). All the applied concentrations of *Spirulina* improved the activity of GST when compared to group B (33 U/L) to be around 47 U/L which was very closes that of BHT (48 U/L). The results obtained from the histological examination confirmed the other obtained results. The treatment with D-GalN caused fatty degeneration, necrosis, and apoptosis and the inflammatory cells were scattered around the congested blood vessels. However, the application of BHT improved the hepatocyte architecture in different areas around the central veins and portal tracts. Similarly, *Spirulina* application resulted in improvement in hepatocytes and showed normal structure in different zones of liver compared to group B, especially at the concentration of 6% and 9%. The results obtained in this study clearly approved the antioxidant and hepatoprotective effects of *Spirulina* addition to the diet. They also provided evidences that *Spirulina* is safe to be used as a supplementary diet, cake, Nourishing condiment or any other synthetic shape of dietary.

Chapter One

Introduction

1. Chapter one:

1.1.Introduction:

According to the United Nations World Health Organization *Spirulina* is “the best for tomorrow,” and since then it has got popularity recently as a healthy food supplement (Anita, et. al. 2010). *Spirulina* have many benefits for many body systems, especially immune system, and several toxicological studies have revealed that *Spirulina* is safe for human use (Salazar et al., 1996; Belay, 2002). Thus *Spirulina* has now become one of the substances that are listed by the US Food and Drug Administration under the category generally recognized as Safe “GRAS” (Tarantino, 2003).

Today *Spirulina* can be found in health food stores and as a dietary supplement in many forms as healthy drinks or tablets.(Colla, LM; Bertolin, TE; Costa, JA (2003). "Fatty acids profile of Spirulina platensis grown under different temperatures and nitrogen concentrations.". Zeitschrift für Naturforschung C 59 (1-2): 55–9

Several experimental studies have advocated that *Spirulina* has antiviral, anticancer, and immune-supporter properties (Mathew et al., 1995; Hirahashi et al., 2002) and is considered as a rich source of protein and vitamins supplement in human diets without any notable side-effects owing to its high (up to 70%) content of protein, besides vitamins especially B12 and pro-vitamin A (β-carotenes), and minerals especially iron (Karkos et al., 2011;Kapoor and Mehta, 1993;Simpore et al., 2006). It is also rich in tocopherols, phenolic acids, and γ-linolenic acid. Moreover, it lacks cellulose cell walls and therefore it can be very easily digested (Dillon et al., 1995). These facts support that *Spirulina* have very important ingredients that protect human body from diseases that spread all over the world now days.

Cyanobacteria have been used as dietary supplements, without showing any significant side-effects, for several years (Kay, 1991), and *Spirulina* is cultivated all over the world as a dietary supplement as well as a lunch food, and is available as tablet, flake or in powder form. It is also used as a fertilizer supplement in the aquaculture, aquarium and poultry feed (Vonshak, 1997).

Spirulina is a microscopic filamentous cyanobacterium commercially cultivated for food use. The name '*Spirulina*' indicates the filamentous nature and the spiral or helical shape. This name also is used to refer the dried biomass of *Arthrospira platensis* and defined as an oxygenic photosynthetic bacterium found in fresh and marine waters, all over the world. *Spirulina* grows only in alkaline lakes with an extremely high pH and in large outdoor ponds under controlled conditions. However it is relatively easy to cultivate. However, there are only a few areas worldwide that have the ideal sunny climate for production of this alga (Al-Homaidan, 2002).

Malnutrition is a major health and social problem from which many people are suffering, especially children. It affects almost 800 million people, 20% of all in the developing countries. It is associated with about half of all children's death worldwide (Kumar et al, 2002). Studies show that Saudi Arabia has large number of people suffering from malnutrition (Mohammad et al, 2010).

Malnutrition is a condition that results from eating a diet in which nutrients are not enough or are too much such that it causes health problems (Henri Josserand et al, 2008). Nutrients include a lot of vitamins and minerals that support the body systems. People who suffer from malnutrition are those people who incur diseases such as cancers, toxicity etc. Deficiency of essential nutrients are responsible for changes in immunity that manifests as changes in

production of T-cells, secretory IgA antibody response, cytokines and NK-cell activity.

Liver, the most important organ which correlates with the biochemical activities in the human body, has great capacity to detoxify toxic substances, and it's the organ in which useful compounds are synthesized. Therefore any injury in the liver inflicted by hepatotoxic agents or by radiation affects the overall normal functions of body (Al-Kahtani, 2004). Earlier studies on *Spirulina* suggested evidences for the benefits of using *Spirulina* as a desirable diet in the experimental animals after induction with chemicals that caused common diseases. Further many studies suggested that *Spirulina* may modulate the immune system by complementing the nutritional deficiencies (Hirahashi et al., 2002). *Spirulina* was suggested as a powerful stimulant for the immune system, based on the results obtained with experimental animals, which showed increase in the phagocytic and natural killer activities when compared with normal (sham) animals (Qureshi and Ali, 1996).

Spirulina was also was observed to inhibit several types of cancer inducted in experimental animals (Mohan et al., 2006; Roy et al., 2007). In vitro and animal studies have suggested that *Spirulina* possesses antiviral effects (Hernandez-Corona et al., 2002; Shih et al., 2003). Moreover, hypocholesterolemic effects have been reported in some animal studies (Nagaoka et al., 2005). Several reports have indicated that *Spirulina* has an antitoxic effect against many toxicants including Mercury (Sharma et al., 2007), D-galactosamine D-galN, and Acetaminophen APAP (Lu et al., 2010), and Copper toxicity (James et al., 2009).

Because of C-phycocyanin (C-PC) one of the major biliproteins of *Spirulina* with antioxidant and radical scavenging properties. C-PC, a selective cyclooxygenase-2 inhibitor, induces apoptosis in lipopolysaccharide-

stimulated RAW 264.7 macrophages. It is also known to exhibit anti-inflammatory and anticancer properties (Reddy et al., 2003) besides the possible antiviral properties. The active component of the water extracts of *S. platensis* is a sulfated polysaccharide, calcium *Spirulina* (Ca-Sp). Hayashi et al. (1996) stated that Ca-Sp inhibits the *in vitro* replication of several enveloped viruses including Herpes simplex type I, human cytomegalovirus, measles and mumps virus, influenza A virus and human immunodeficiency virus-1 virus (HIV-1). However, little knowledge is available regarding the protective effect of *Spirulina* against liver injuries (Lu et al., 2010). Lu et al. (2010) found that dietary *Spirulina* could alleviate D-GalN and APAP-induced liver injuries in rats significantly.

In this context, the present study presented in this thesis aims at investigating the beneficial protective effects of *S. platensis* against liver injuries induced by D-GalN and evaluate the generally considered concept that '*Spirulina* is safe for human use'.

1.2. Objectives of the present study

The main objective of this research is to examine the beneficial protective effects of *Spirulina* against liver injuries induced by (D-GalN) and ensuring safety to human health using animal model studies. Accordingly the present study includes the following specific objectives:

- Cultivation of locally cultivated *Spirulina* and analysis of its proximate content.
- Analyse the activities of (GOT), (GPT) and (AST) in blood serum of Wister albino rats' models.
- Analyse the activities of antioxidant enzymes: (SOD), (CAT), (GSH), (GPX) and (GST) in rats following *Spirulina* diet as compared to control diet rats.
- Analyse the activities of (GGT) and (ALP) in liver tissues.
- Evaluate the level of damage occurred in liver tissues in rats following *Spirulina* diet as compared to control diet rats.
- Assess the beneficial effects attributing to increase of liver efficiency and safety of using *Spirulina* as diet for human.

Chapter Two

Literature review

2. Chapter two: Literature review:

2.1. Overview of *Spirulina*:

Spirulina is cyanobacterium in the shape of a spiral coil, live in both sea and fresh water. It belongs to the class of cyanobacteria, and has the capability of conducting photosynthesis (Sapp 2005; Komárek and Hauer2009). Although cyanobacteria are not related to any of the various eukaryotic algae, they are often referred to as 'algae' because of their existence in aquatic environment and their capability to conduct photosynthesis (Schopf 2012). Earlier classification placed *Spirulina* in the plant kingdom since it has plant pigments and ability to conduct photosynthesis. However, now it is included in the kingdom of bacteria based on the better understanding of its genetics, physiology, and biochemical properties (Vonshak, 1997). '*Spirulina*' consumed as human and animal food is primarily produced from two species of cyanobacteria namely *Spirulina platensis* and *Spirulina maxima.*

Spirulina grow naturally in water reservoirs which is highly alkaline - salt water in tropical and subtropical areas including America, Mexico, East Asia and Central Africa (Vonshak, 1997; Gershwin and Belay, 2008). Although a large number of *SPIROLINA* species are known, only three species of *Spirulina*، namely *S. platensis*, *S. maxima* and *S. fusiformis* were intensively investigated since these species are edible with high nutritional as well as potential therapeutic values (Khan et al. 2005; Karkos et al 2008).

Earlier studies mainly focused on the nutritional value of *Spirulina* as a food source. It has been reported that, as early as over 400 years ago, *Spirulina* was consumed as food by the Mayas, Toltecs and Kanembu in Mexico during the Aztec civilization (Ciferriand and Tiboni 1985). *Spirulina* growing in the Lake Texcoco were harvested, dried and used to make

Spirulina cake as food. It has also been consumed in Central Africa over centuries by the Chadians. *Spirulina* harvested from the Lake Kossorom in Chad was used to make cake or broths as meals and also sold in the market (Abdulqader 2000). The nutritional value of *Spirulina* is well recognized, since it has high protein content (60–70% by dry weight) and is rich in vitamins, minerals, essential fatty acids and other nutrients (Vonshak, 1997; Gershwin and Belay 2008).

In the middle of 70's the Intergovernmental Institution for the use of Micro-algae '*Spirulina'* Against Malnutrition (IIMSAM) was launched and IIMSAM aimed to fight against starvation and malnutrition in the world by promoting *Spirulina* as high nutritional food because of its unusual high nutritional values (Habib et al, 2008). Further, *Spirulina* was recommended by both National Aeronautics and Space Administration (NASA) and the European Space Agency (ESA) as one of the primary foods during long-term space missions.

Great efforts and extensive investigations have been made during the 1980's on the development of nutraceuticals (a food containing health-giving additives and having medicinal benefit) or functional food for preventing and or managing various diseases. *Spirulina* has become one of such nutraceutical food with diverse beneficial effects on an array of disease conditions. It has been reported that consumption of *Spirulina* as diet supplement has health benefits in preventing or managing hypercholesterolemia، hyperglycerolemia, certain inflammatory diseases, allergies, cancer, environmental toxicant and drug-induced toxicities, viral infections, cardiovascular diseases, diabetes and other metabolic disease among others (Khan et al, 2005; Karkos et al, 2008; Kulshreshtha 2008). Specific emphasize was given to the beneficial effects including potential of *Spirulina* on cardiovascular diseases with highlights on

Spirulina's hypolipidemic, antioxidant and anti-inflammatory activities in preclinical and clinical studies.

2.2. Biology of *Spirulina*

Spirulina are free-floating filamentous cyanobacteria that have a cylindrical, multicellular trichomes characterics in an open left-hand helix. It is naturally found in tropical and subtropical lakes with high pH and high concentrations of carbonate and bicarbonate. *S. platensis* occurs in Africa, East Asia and South America, whereas *S. maxima* are confined to Central America. *S. maxima* and *S. platensis* species were classified under the genus *Spirulina* (Vonshak 1997).

2.3. Classification of *Spirulina*

Taxonomical position of *Spirulina* is as follows

Domain: Bacteria

Kingdom: Eubacteria

Division: *Cyanobacteria*

Order: *Oscillatoriales*

Family: Oscillatoriaceae

Genus: *Spirulina*

Species: *S.abbreviate, S.agilis, S.agilissima, S.albida, S.ardissoni, S.baltica, S.bayannurensis, S.breviarticulata, S.cabrerae, S.caldaria, S.cavanillesiana, S.condensate, S.corakiana, S.flavovirens, S.funiformis, S.gessneri, S.gomontiana, S.gomonti, S.gordiana, S.gracilis, S.innatans, S.labyrinthiformis, S.laxa, S.laxissima, S.legitima, S.magnifica, S.major, S.margaritae, S.mariae, S.massartii, S.maxima, S.miniata, S.minima, S.mukdensis, S.nodosa, S.nordstedtii, S.okensis, S.oscillarioides, S.platensis, S.princeps, S.pseudotenuissima, S.robusta, S.rosea, S.schroederi,*

S.sigmoidea, S.socialis, S.spirulinoides, S.subsalsa, S.subtilissima, S.supersalsa, S.tenerrima, S.tenuior, S.tenuis, S.tenuissima, S.thermalis, S.turfosa, S.versicolor, S.weissii

Fig 2.1. Short *Spirulina* strands

2.4. History of *Spirulina*

Consumption of *Spirulina* as food was recorded by Bernal Diaz del Castillo, a member of Hernan Cortez's troops, who reported in 1521 (the first historical record of *Spirulina* consumption). *Spirulina maxima* (*A. maxima*) was harvested from Lake Texcoco, then dried, and sold for human consumption in a Tenochtitlan markets (today Mexico City). *Spirulina* was a food source for the Aztecs and other Mesoamericans until the 16th century (Diaz Del Castillo, 1928; Osborne et al 2005).

An illustration from the Florentine Codex show that by skimming the surface of lakes with ropes the Aztecs harvested *Spirulina*, and dried the algae into square cakes which was consumed as a nourishing condiment. The Aztecs called it 'Tecuitlatl', meaning stone's excrement.

Spirulina was found in abundance at Lake Texcoco by French researchers in the 1960s, but there was no reference for its use as a daily food source after the 16th century (Ciferri, 1983). In early 1970's large-scale production plant of *Spirulina* was established by Sosa Texcoco (Abdulqader 2000). In the 1961 short story 'The Voice of the Dolphins'; Leo Szilard postulated the development of algae-based food supplements (which he called "Amruss"). *Spirulina* may have an even longer history in Chad, as far back as the 9th century Kanem Empire. Nevertheless it is still in daily use today, dried into cakes called dihé, which are used to make broths for meals, and also sold in markets. In Chad the *Spirulina* is harvested from small lakes and ponds around lakes (Abdulqader, 2000).

2.5. History of *Spirulina* in Saudi Arabia:

In 1999, the interest in *Spirulina* was started in Saudi Arabia by the Agricultural Service Company (ARASCO) when it established a small farm of this microalga (Al-Homaidan, 2002). Dry powders were examined for its effect on chicken macrophage phagocytic function and nitrite production (Al-Batshan et al, 2001). A few articles on the effect of temperature, pH, and salinity on the growth and protein content of two species of *Spirulina* isolated in Saudi Arabia (Al-Homaidan et al, 2005); effect of heavy metals and uranium on chlorophyll, DNA, protein content and ultrastructure of the cyanobacterium, *S. platensis* (Sabbagh, 2006); and heavy metal levels in Saudi Arabian *Spirulina* (Al-Homaidan, 2006) were reported from Saudi Arabia.

2.6. Cultivation of *Spirulina*:

Spirulina is cultivated around the world, and it is used as a human dietary supplement, as well as a whole food. It is available in many forms like tablet, flake, and powder. It is also used as a feed supplement in the aquaculture, aquarium, and poultry industries (Allaby, 1992). *Spirulina* production is mostly done through large scale cultivation in open-channel raceway ponds using paddle-wheels to agitate the water. Commercial production of *Spirulina* is being done in the United States, Thailand, India, Taiwan, China, Pakistan, Burma and Chile (Babadzhanov et al 2004).

Spirulina grow up in medium with alkaline pH around 8.5, and at a temperature around 30^{o}C (86^{o}F). They have the ability to produce their own food without the need for source of organic carbon. In addition, *Spirulina* have an ensemble of nutrients to grow up in an aquarium home or pond. A simple nutrient feed for growing *Spirulina* includes: Baking Soda - $NaHCO_3$ - 16 g/L = 60.56 g/gal Potassium Nitrate - KNO_3 - 2 g/L = 7.57 g/gal, Sodium Chloride - NaCl - 1 g/L = 3.78 g/gal, Potassium Phosphate - KH_2PO_4 - 0.1 g/L = .378 g/gal, Iron Sulphate–and $FeSO_4 * 7H_2O$ - 0.01 g/L = .0378 g/gal. *Spirulina* have been tested to grow in human urine at 1:180 parts and it was successfully grown (Babadzhanov et al 2004). After 7days, 97% of NH_4+-N, 96.5% of total phosphorus (TP) and 85–98% of urea in the urine (ca. 120-diluted) were removed by the microalgae under autotrophic culture (30^{o}C) (Chang et al 2013).

2.7. *Spirulina* Photosynthetic pigments content:

Spirulina have many pigments including chlorophyll a, xanthophyll, beta-carotene, echinenone, myxoxanthophyll, zeaxanthin, canthaxanthin, diatoxanthin, 3'-hydroxyechinenone, beta-cryptoxanthin and oscillaxanthin, besides phycobiliproteins c, phycocyanin and allophycocyanin (Vonshak 1997).

2.8. Physiology of Liver:

The liver plays an important central function in human physiology which is in fact a dual function: Primarily liver is the most important metabolic organ and secondly it is the largest exocrine gland producing the bile and bile salt, which is essential for fat metabolism.

The most important function of the liver, as metabolic organ, is the constant detoxication of the blood. Without it, the whole organism would not be able to survive even one day in the presence of oxidant reagents. A massive amount of 1.5 liters of blood passes through the liver every single minute. This is the reason for its reddish-brown color. Liver cells are responsible for production; storage and allocation of a great number of essential nutrients, and some waste products are converted into serviceable elements, other harmful ones to excrete. At the same time the liver produces vitamin D and vitamin A and B12. Briefly, liver function as a producer of essential substances (e.g. glucose and proteins), clotting factors in the blood, in the breakdown of fats and other harmful substances, and in the removal of toxic substances (Beichel, 2005).

2.9. Anatomy of the Liver:

Liver is the biggest organ among all human inner organs and is located in the upper right side of the abdomen, where it is protected by cage ribs. The weight of standard human liver is around 1.5 kg. The most common liver shape is triangular and found in around 65% of the cases. Sometimes liver with concave region or indentations is also noted. Other common shapes include rectangular (12%), the policeman cap type (14%), spherical (3%) and others that are not similar to any of these groups (Rauber et all, 1988). Because of its soft and fragile consistence, the shape of the liver is subject to changes due to movements of the body and breathing, as well as its location. The liver is roughly held in its position by the vascular system. In addition, one part is adhered to the diaphragm. The whole liver shape consists of two main parts, the right (bigger) lobe (lobus dexter) and the left lobe (lobus sinister) (Rauber et al, 1988).

2.10. Antioxidant:

The antioxidant is a molecule that inhibits the oxidation of molecules due to oxidizing reagents. Oxidation is a chemical reaction that transfers electrons or hydrogen from a substance to an oxidizing agent. Oxidation reactions can produce free radicals. In turn, these radicals can start chain reactions. When the chain reaction occurs in a cell, it can cause damage or death to the cell. Antioxidants terminate these chain reactions by removing free radical intermediates, and inhibit other oxidation reactions. They do this by being oxidized themselves, so antioxidants are often reducing agents such as thiols, ascorbic acid, or polyphenol (Sies and Helmut, 1997).

Although oxidation reactions are pivotal for life, they can also be damaging. Plants and animals maintain complex systems of multiple types of antioxidants, such as glutathione, vitamin C, vitamin A, and vitamin E as well

as enzymes such as catalase, superoxide dismutase, and various peroxidases. Insufficient levels of antioxidants or inhibition of the antioxidant enzymes cause oxidative stress and may damage or kill cells (Jha et al, 1995).

Oxidative stress cause damage to cell structure and cell function due to overly reactive oxygen-containing molecules and chronic excessive inflammation. Oxidative stress seems to play a significant role in many human diseases, including cancers (Baillie et al 2009). The use of antioxidants in pharmacology is intensively studied, particularly in treatments for stroke and neurodegenerative diseases. For these reasons, oxidative stress can be considered to be both the cause and the consequence of some diseases (Bjelakovic et al 2007).

2.10.1. Classification of antioxidant:

Antioxidants are classified into two types, depending on their solubility in water (hydrophilic) or in lipids (lipophilic). Generally water-soluble antioxidants react with oxidants in the cell cytosol and the blood plasma, while lipid-soluble antioxidants protect cell membranes from lipid peroxidation polyphenol (Sies and Helmut,1997). These compounds may be synthesized in the body or gained from the diet (Vertuani et al, 2004). The different antioxidants are present at a wide range of concentrations in body fluids and tissues, such as glutathione or ubiquinone mostly present within cells, while others such as uric acid are more evenly distributed. Some antioxidants are only found in a few organisms and these compounds can be important in pathogens and can be virulence factors (Miller and Britigan, 1997).

2.10.1.1. Glutathione:

Glutathione is a cysteine-containing peptide found in most forms of aerobic life responsible for free radical mechanism of lipid peroxidation. It is

not required in the diet and is instead synthesized in cells from its constituent amino acids (Evelson et al, 2001).

2.10.1.2. Melatonin:

Melatonin is a powerful antioxidant, and it easily crosses cell membranes and the blood–brain barrier. It forms several stable end-products upon reacting with free radicals. It has been referred to as a terminal (or suicidal) antioxidant (Tan et al, 2000).

2.11. Antioxidant compounds:

In recent years, there has been an explosive interest in the use of antioxidant nutritional supplements (Gigante et al, 2007; Simpore et al, 2006). Epidemiological evidence indicated that intake of vitamins, minerals، and food ingredient may help to protect the body against heart disease, cancer, and the aging process, and antioxidants may have a protective effect in preventing these diseases or lessening their severity (Wu et al, 2005; Marcason, 2007). Several activities of the antioxidants are mediated by inhibition of reactive oxygen species which are generated during the oxidative explosion. Thus, the usefulness of antioxidants in protecting cellular components against oxidative stress is well established.

2.12. Liver diseases:

2.12.1. Fascioliasis:

Fascioliasis is a parasitic infection in liver caused by a Liver fluke of the *Fasciola* genus, mostly *Fasciola hepatica* (Mas-Coma, 2005).

2.12.2. Hepatitis:

Hepatitis is a condition of ‘Inflammation’ in liver caused mainly by viruses (viral hepatitis) but also by some liver toxins (Longo 2012).

2.12.3. Alcoholic Liver disease:

Alcoholic liver disease is a condition referred to any hepatic appearance of alcohol overconsumption, including fatty liver disease, alcoholic hepatitis, and cirrhosis. Analogous terms such as "drug-induced" or "toxic" liver disease are also used to refer to the range of disorders caused by various drugs and environmental chemicals (O'Shea et al, 2010).

2.12.4. Fatty Liver disease (Hepatic Steatosis):

Fatty liver disease (hepatic steatosis) is a reversible condition where large vacuoles of triglyceridef at accumulate in liver cells. Non-alcoholic fatty liver disease is a spectrum of disease associated with obesity and metabolic syndrome, among other causes. Fatty liver may lead to inflammatory disease, eventually and cirrhosis (Cotran et al, 1998).

2.12.5. Cirrhosis:

Cirrhosis is the formation of fibrous tissue (fibrosis) in the place of liver cells that have died due to a variety of causes, including viral hepatitis, alcohol overconsumption, and other forms of liver toxicity. Cirrhosis causes chronic liver failure (Li et al, 1999).

2.12.6. Primary Liver Cancer:

Primary liver cancer is most commonly manifested as Hepatocellular Carcinoma and Cholangiocarcinoma. Rarer forms include Angiosarcoma and Hemangiosarcomaof the liver (Ahmed et al, 2008).

2.12.7. Primary Biliary Cirrhosis:

Primary biliary cirrhosis is a serious autoimmune disease of the bile capillaries (Hirschfield and Gershwin, 2013).

2.12.8. Primary Sclerosing Cholangitis:

Primary sclerosing cholangitis is a serious chronic inflammatory disease of the bile duct, which is believed to be autoimmune in origin (Sleisenger, 2006).

2.12.9. Centrilobular Necrosis of Liver:

Centrilobular necrosis of liver can be caused by leakage of enteric toxins into circulation. Salmonella toxins in ileum have been shown to cause severe damage to liver hepatic cells (Giallourakis et al, 2002).

2.12.10. Budd– Chiari Syndrome:

Budd–Chiari syndrome is the clinical picture caused by occlusion of the hepatic vein, which in some cases may lead to Cirrhosis (Rajani et al, 2009).

2.12.11. Hereditary diseases:

Hereditary diseases that cause damage to the liver include hemochromatosis, involving accumulation of iron in the body, and Wilson's disease, which causes the body to retain copper. Liver damage is also a clinical feature of alpha 1-antitrypsin deficiencyand glycogen storage disease type II (YashRoy, 2000).

2.12.12. Transthyretin-related hereditary amyloidosis:

In Transthyretin-related hereditary amyloidosis the liver produces a mutated transthyretin protein which has severe neurodegenerative and/or cardiopathic effects. Liver transplantation can provide a curative treatment option (Ando and Ueda 2008).

2.12.13. Gilbert's syndrome:

Gilbert's syndrome is a genetic disorder of Bilirubin metabolism found in about 5% of the population, and can cause mild jaundice (Dugdale2013). There are also many pediatric liver diseases including biliary atresiaalpha-1 (Dugdale 2013).

2.13. *Spirulina* keeps the Liver healthy:

Spirulina help the liver to do the protective role to the human body and attributes beneficial effect on the liver. There are several evidences available in literatures which are presented below:

2.14. Liver Enzymes:

A study on rats with insulin resistance, induced by fructose, reported that *Spirulina* diet at low doses (0.33g/kg in rats) could be correlated with decreased serum (GOT) and serum (GPT) levels in serum, and their increase in levels indicated hepato-cellular necrosis and membrane damage, signaling less liver cell damage (Jarouliya et al , 2012). Reductions in GOT and GPT have also been noted in humans, but after consumption of 2 or 4 g of *Spirulina* for 3 months and both were not statistically significant (p value >0.05) Another study on rats recorded increased levels of the liver (ALP), (AST) and (ALT) on Cisplatin injections, and treatment of the animals with a combination of *Spirulina* (1g/kg) and Vitamin C (500mg/kg). The effect of the treatment resulted in the normal regulation of liver enzymes.

2.15. Fibrosis:

Studies on the effect of *Spirulina* on nicotinamide adenine dinucleotide phosphate – oxidase (NADPH) inhibition, indicated inhibition of proliferation of stellate cells besides serving as a therapeutic alternative in instances of liver fibrosis (McCarty, 2009). This hypothesis is based on suppression of stellate cell proliferation by activation of the ERb receptor (via one of the Soy IsoflavonesGenistein (Liu et al, 2002) and in part estrogen itself (Itagaki et al, 2005) working vicariously through suppressing NADPH oxidase activity. DPI (Chemical that inhibits NADPH activity) has also been shown to reduce stellate cell proliferation (Bataller et al, 2003; Adachi et al, 2005).

2.16. Evidence-Based Human Applications

2.16.1. *Spirulina* and Chronic Fatigue:

Spirulina has been promoted as “the food of the future" with “exceptional constituents" that contribute to high energy levels. A few of these constituents such as polysaccharides (Rhamnose and Glycogen) and essential fat (GLA) are absorbed easily by human cells and help in energy release. *Spirulina* increases healthy lactobacillus in the intestine, enabling the production of Vitamin B6 that also helps in energy release. Despite this promotion, the only available placebo-controlled randomized trial showed that the scores of fatigue were not significantly different between *Spirulina AND* placebo. *Spirulina* administered at a dose of 3 g day−1 did not ameliorate fatigue more than the placebo in any of the four subjects and possibly it has no effect on chronic fatigue (Baicus and Baicus 2007).

2.16.2. Allergy, Rhinitis, and Immunomodulation:

Spirulina has been well documented in literature to exhibit anti-inflammatory properties by inhibiting the release of histamine from mast cells (Yang et al, 1997; Kim et al, 1998). In another randomized, double-blind placebo-controlled trial study individuals with allergic rhinitis were fed daily, either with placebo or *Spirulina* for 12 weeks (Mao, 2005). Peripheral blood mononuclear cells were isolated before and after the *Spirulina* feeding and levels of cytokines (interleukin-4 (IL-4), interferon-γ (IFN-γ) and interleukin-2), which are important in regulating immunoglobulin (Ig) E-mediated allergy, were measured. The study showed that high dose of *Spirulina* significantly reduced IL-4 levels by 32%, demonstrating the protective effects of this microalga toward allergic rhinitis. Ishii et al.(1999) studied the influence of

Spirulina on Ig A levels in human saliva and demonstrated that it enhances Ig A production, suggesting a pivotal role of microalga in mucosal immunity.

A Japanese team identified the molecular mechanism of the human immune capacity of *Spirulina* by analyzing blood cells of volunteers with pre- and post-oral administration of hot water extract of *S. platensis*. IFN-γ production and Natural Killer (NK) cell damage were increased after administration of the microalga extracts to male volunteers (Hirahashi, 2002).

A double-blind, placebo-controlled study from Turkey evaluated the effectiveness and tolerability of *Spirulina* for treating patients with allergic rhinitis. It was observed that *Spirulina* consumption significantly improved the symptoms and physical findings compared with placebo ($P < .001$), including nasal discharge, sneezing, nasal congestion and itching (Cingi et al, 2008).

It is well understood fact that deficiency of nutrients is responsible for changes in immunity, which manifests as changes in production of T-cells, secretory IgA antibody response, cytokines and NK-cell activity. In that context the above mentioned studies suggested that *Spirulina* may modulate the immune system by its role in covering nutritional deficiencies.

2.16.3. Antiviral Applications:

In-Vitro Studies

There are no *in-vivo* studies providing strong evidence supporting the possible antiviral properties of *Spirulina*. The active component of the water extracts of *S. platensis*is is a sulfated polysaccharide, calcium spirulan (Ca-Sp). According to Hayashi et al, (1996) Ca-Sp inhibits the in-vitro replication of several enveloped viruses including Herpes simplex type I, human cytomegalovirus, measles and mumps virus, influenza A virus and human immunodeficiency virus-1 virus (HIV-1). Another *in-vitro* study showed that an aqueous extract of *S. platensis* inhibited HIV-1 replication in human T-

cells, peripheral blood mononuclear cells and Langerhan cells (Ayehunie, 1998). The advantage of using herbs and algal products with proven antiviral properties in fighting certain viruses is that they can be used through immune modulation even when the infection is established. Of course, these promising effects need to be studied further in animal models and humans before any definitive conclusions are drawn.

2.16.4. Cholesterol-Lowering Effects and Effects on Diabetes:

Cardiovascular disease remains the number one cause of death in developed countries, despite increased awareness, and high cholesterol is one of the most important risk factors in atherosclerosis (Nakaya et al, 1988). In the first human study, 4.2 g day−1 of *Spirulina* was given to 15 male volunteers. Although there was no significant increase in high-density lipoprotein (HDL) levels, they observed a significant reduction of low-density lipoprotein (LDL) cholesterol after 8 weeks of treatment. The atherogenic effect also declined significantly in the above group (Nakaya et al, 1988). Ramamoorthy and Premakumari (1996) administered *SPIRULINA* supplements in ischemic heart disease patients and found a significant reduction in blood cholesterol, triglycerides and LDL cholesterol and an increase in HDL cholesterol. According to them more research is needed before *Spirulina* can be recommended to lower cholesterol levels although its role as a natural food supplement in combating hyper lipidaemia, in combination with other therapeutic options, should not be overlooked. Mani et al (2002) also observed a significant reduction in LDL : HDL ratio in 15 diabetic patients who were given *Spirulina* diet. However, this study was inconclusive and more in depth studies are needed before *Spirulina* can be recommended in diabetes.

2.16.5. Anticancer Effects:

It has been argued that the combined antioxidant and immune modulation characteristics of *Spirulina* may have a possible mechanism of tumor destruction and hence play a role in cancer prevention. In spite of many animal and *in-vitro* studies available there has been only one trial with human subjects which focused specifically at the effects of *Spirulina* on oral carcinogenesis, in particular leukoplakia (Mathew 1995). The study conducted by Mathew et al (1995) on a cohort of 77 patients originated from previous trials on hamsters that showed tumor regression after topical application or intake of *Spirulina* extract (Shklar1987 and Schwartz 1988). 45% of their study on cohort showed complete regression of leukoplakia after taking *Spirulina* supplements for 1 year. They also noted that there was no rise in the serum concentration of retina l β-carotene despite supplementation and concluded that other constituents within *Spirulina* may have been responsible for the anticancer effects.

2.16.6. Chronic Arsenic Poisoning:

Millions of people in Bangladesh, India, Taiwan and Chile are known to consume high concentration of arsenic through drinking water and are at risk of chronic arsenic poisoning for which there is no specific treatment. A placebo-controlled, double-blind study was conducted to evaluate the effectiveness of *Spirulina* extract plus zinc in the treatment of chronic arsenic poisoning (Misbahuddin, 2006). Forty-one patients with chronic arsenic poisoning were randomly treated by either placebo (17 patients) or *Spirulina* extract (250 mg) plus zinc (2 mg) (24 patients) twice daily for 16 weeks. Each patient was supplied with arsenic-safe drinking water by installing a locally made water filter at household level. Effectiveness of *Spirulina* extract plus zinc was evaluated by comparing changes in skin manifestations (clinical

scores) and arsenic contents in urine and hair, between the placebo- and *Spirulina* extract plus zinc-treated groups. Results showed that consumption of *Spirulina* extract plus zinc, twice daily, for 16 weeks may be useful for the treatment of chronic arsenic poisoning with melanosis and keratosis.

2.16.7. Antioxidant Effects:

To date there are no *in-vivo* human studies on possible antioxidant effects of *Spirulina*. C-phycocyanin (C-PC) is one of the major biliproteins of *Spirulina* with antioxidant and radical scavenging properties. C-PC, a selective cyclooxygenase-2 inhibitor, induces apoptosis in lipopolysaccharide-stimulated RAW 264.7 macrophages. It is also known to exhibit anti-inflammatory and anticancer properties (Reddy et al 2003).

2.17. General quality and safety assurance for *Spirulina*:

In 1970's, *Spirulina* underwent extensive safety studies with animals and fish. Independent feeding tests in France, Mexico and Japan showed no undesirable results and no toxic side effects on humans, rats, pigs, chickens, fish and oysters. Many independent rat feeding trials were conducted in Japan and no negative effects at all were found for acute or chronic toxicity or reproduction. The re-introduction of *Spirulina* as a health food for human consumption in the late 1970s and the beginning of the 1980s was associated with many controversial claims which attribute to *Spirulina* a role of a 'magic agent' that could do almost everything, from curing specific cancer to antibiotic and antiviral activity. Most claims were never backed up by detailed scientific and medical research. Nevertheless, one cannot ignore the fact that more than 70 per cent of the current *Spirulina* market is for human consumption, mainly as health food (Vonshak, 2002, Shimamatsu, 2004, Henrikson, 2010). So, there are established national and international quality standards for *Spirulina*

products recently, cyanobacterial toxins have become a major issue in public health due to the increased occurrence of toxic cyanobacterial blooms. These toxic blooms contain algae that produce hepatotoxins called microcystins (Carmichael, 1994). *Spirulina* companies like Earthrise Farms have already developed methods for the determination of these toxins and actually certify each lot of their product to be toxin free. *Spirulina* does not normally contain microcystins but contamination of outdoor culture by other cyanobacteria is a possibility.

Chapter Three

Materials and Methods

3. Chapter three: Materials and Methods:

3.1. Cultivation of *Spirulina*

Food grade *Spirulina* powder was prepared using *Spirulina platensis* cultured at the Department of Botany and Microbiology, College of Science, King Saud University The microalga *S. platensis* was obtained from the culture collection of algae at the University of Texas at Austin, USA, (UTEX NO. LB 2340). It was propagated in the laboratory according to the methods described by Vonshak (1997) and Al-Homaidan (2002). The strain was cultivated using Zarrouk medium (Zarrouk, 1966). Outdoor cultures were carried out according to the methodology described for central Saudi Arabia (Al-Homaidan, 2002). Harvesting was done by filtration through nylon filters (150–200 mesh). After harvesting, *S. platensis* was rinsed with deionized water and dried overnight in an oven at 80 ^{o}C. The dried biomass was ground well and sieved using a standard metal sieve (100 lm pores) and stored in a desiccator to avoid moisture absorption.

Fig 3.1 *Spirulina platensis* cultured under the supervision of Prof. Ali Al-Homaidan.

3.2. Preparation of *Spirulina* powder

The aqueous extract of *Spirulina* was prepared according to the method described by Ciferri (1999). In brief, 10 g of *Spirulina* powder was weighed accurately in a beaker, added with 100 mL of water, and the mixture was stirred with a magnetic stirrer. The mixture was heated cautiously in a steam bath for ١min, sonicated to shrinkage on the surface of cells to facilitate the release of the protein phycocyanin, cooled at 4 ^{o}C, and kept in a refrigerator overnight.

3.3. Chemical analysis of *Spirulina* Powder

The chemical composition and nutritional value analysis of the dried samples of *Spirulina* (powder) were conducted by following Association of official analytical chemistry AOAC (1990) methods in the Saudi Commercial Laboratories, IDAC Quality & safety, (Adel and Saleh, 2014)

3.4. Experimental animals:

All animal experiments were done using Wister Albino male Rat at the King Saud University- Pharmacy College – Animal Care Center during the period (24 May 2014 – 7 Jun 2014).

Thirty six Wister albino rats, 5-6 weeks old, weighing about120-180 g were individually housed in single rat cage (Technoplast co.) prepared with Sawdust bedding and fed with free water / food on day-night 12 hours cycle. The animals were distributed in cages one week before the experiment for Acclimatization and were fed with the standard diet based on the American Institute of Nutrition (AIN)-93G formula (Reeves et al., 1993) (Table 3.1).

All experimental procedures and protocols in this study including euthanasia were conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals, Institute for Laboratory Animal Research (NIH Publications No. 80-23; 1996) as well as the

Ethical Guidelines of the Experimental Animal Care Center, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia.

Table 3.1. The American Institute of Nutrition (AIN)-93G formula (Reeves et al, 1993).

	Ingredient	**Amount in 35 gm**
1	Calcium Carbonate 40.0% Ca	5.0 gm Ca
2	Potassium Phosphate, Monobasic 28.7% K 22.8% P	1.56 gm P
3	Potassium Citrate 1 H2O 36.2% K	3.6 gm K
4	Potassium Sulfate 44.9% K 18.4% S	0.3 gm S
5	Magnesium Oxide 60.3 Mg	0.5 gm Mg
6	Sodium Chloride 39.3% Na 60.7% Cl	1.0gm Na 1.6 gm Cl
7	Cupric Carbonate 57.5% Cu	6.0 mg Cu
8	Potassium Iodate 59.3% I	0.2 mg I
9	Ferric Citrate 17.4 17.4% Fe	37 mg Fe
10	Manganese Carbonate 47 8% Mn	10.5 mg Mn

11	Sodium Selenate 41.8% Se	0.2 mg Se
12	Zinc Carbonate 25.1% Zn	30 mg Zn
13	Chromium K Sulfate 12 H2O 10.4% Cr	0.0009625 gm Cr
14	Ammonium Molybdate 4 H2O 54.3% Mo	0.0001511 gm Mo
15	Sodium Silicate 9 H2O 9.89% Si	1.45 Si/1000 g
16	Lithium Chloride	0.0174 Li Cl/1000g
17	Boric Acid	0.0815 BA/1000g
18	Sodium Fluoride 45.2% F	0.00100 gm F
19	Nickel Carbonate	0.0318 NC/1000g
20	Ammonium Vanadate	0.0066
21	Sucrose	221.026

3.5. Experimental design:

Thirty six rats were randomly divided into 6 groups consisting of six animals each. All animals were made to fast 24 h before the commencement of the study. The groups were as follows

1. Group A: ‘Control’ Group- This group included normal rats which received no treatment.
2. Group B: This group included rats with acute hepatotoxicity induced with single dose (300 mg/kg body weight by IP) of (D-galN).
3. Group C: This group included rats with acute hepatotoxicity that received treatment with 0.5% BHT mixed with diet.
4. Group D: This group included rats with acute hepatotoxicity that received treatment with 3% *Spirulina* mixed with diet.
5. Group E: This group included rats with acute hepatotoxicity that received treatment with 6% *Spirulina* mixed with diet.
6. Group F: This group included rats with acute hepatotoxicity that received treatment with 9% *Spirulina* mixed with diet.

Fig 3.2. Experimental design of Wister Albino rats groups.

3.6. Drugs and Chemicals

3.6.1. D-galactosamine (D-GaIN):

(D-GaIN) induces Acute hepatotoxicity, which is good experimental model based on its capacity to reduce the intracellular pool of uridine monophosphate in hepatocytes (Muntane 2000; Fouad 2004). D-GaIN obtained from (Sigma,China), and was IP injected all groups to induce hepatotoxicity except group A (control).

3.6.2. Butylated hydroxytoluene (BHT):

(BHT) has been shown to protect experimental animals against various hepatotoxins such as allyl alcohol (Hunason, 1972) since. BHT was observed to prevent chemically induced tumours or acute toxic effects of some chemicals. Hence, BHT was used in the present study.

BHT (obtained from (LOBAL Chemie, Mumbai- India) which is a widely used phenolic antioxidant, was chosen as the diet supplement (0.5 %) at the expense of maize starch for the group C.

3.7. Acute hepatotoxicity induction:

Acute hepatotoxicity in rats was induced by IP injection of D-GalN at a dose of 300mg/kg body weight on day 7, while the rats in 'Control group A' were injected with the same volume of 0.9% saline alone (Lu et al., 2010). Diets were withheld for 4 h before and after D-GalN administration (8 h in total). After 24 h D-GalN was injected.

3.8.Blood samples collection:

Blood after one week of treatment animal was dissected under Ketamine (100 mg/Kg) and Xylazine (16 mg/Kg) by IP injection, Blood was collected directly from heart and transferred to plan tube and centrifuged after complete clotting to separate the serum, serum was stored in 1ml Eppendorf tube and under -80 ^{o}C freezer.

Liver was taken in tube containing 10 % buffered formalin (pH 7.4) and used for histopathology test under microscope after embedding the same in paraffin wax. Sections with a size of 4 μm thickness were made, stained with hematoxylin and eosin (H & E), and examined for morphological evidence of liver injury.

Enzymatic Analysis.

3.9. Analysis of Enzymes, antioxidants and cholesterol

Enzymes including (SOD), (GPX), (CAT), (GST), (AST), (GSH)), (GOT), and(GPT) activities were anylysed in blood serum; (ALP),and (GGT) activities were anylysed in liver; Cholesterol levels, Total protein (TP) and albumin levels were anylysed in blood serum.

All were analysed as detailed below.

3.9.1. Superoxide dismutase (SOD):

Superoxide dismutase (SOD) was analysed using diagnostic kit obtained from OxiSelect™. Protocol provided by the vendor was strictly followed for the assay.

Assay Principle:

Superoxide anions (O_2-) are generated by a Xanthine/Xanthine Oxidase (XOD) system, and then detected with a Chromagen Solution. However, in the presence of SOD, these superoxide anion concentrations are reduced, yielding less colorimetric signal.

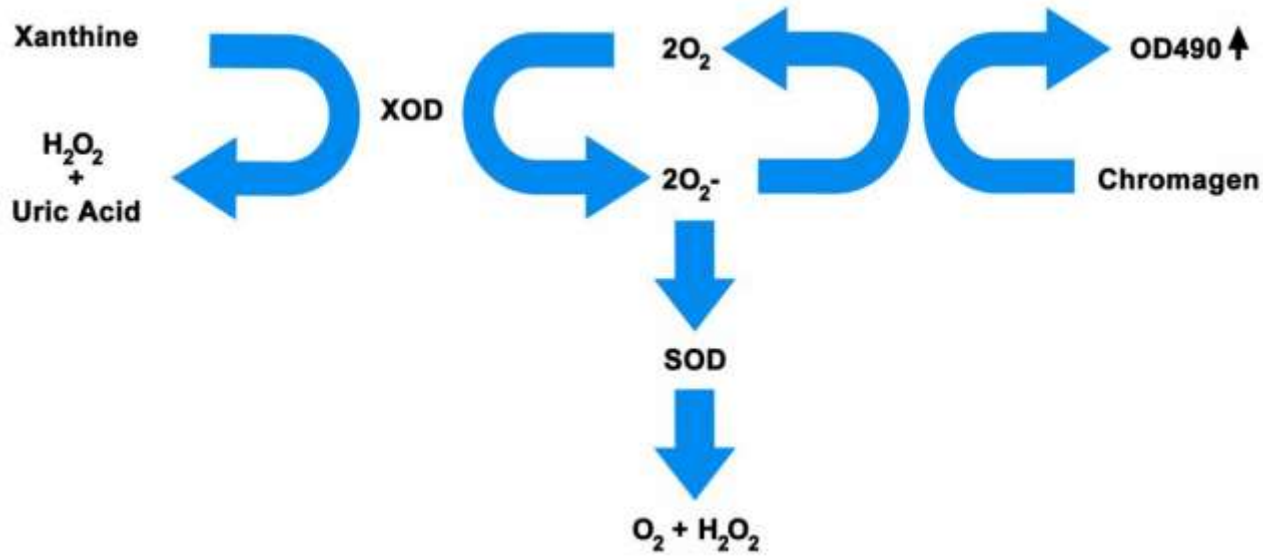

Fig 3.3: Principle of Superoxide dismutase assay:

Assay Protocol

1. Prepared serum including a blank in a 96-well microtiter plate according to the details given below. Pre-incubation time was allowed whenever inhibitor was used.

Sample	**Blank**	**Component**
X µL	0 µL	SOD Sample
Y µL	0 µL	Inhibitor (optional)
5 µL	5 µL	Xanthine Solution
5 µL	5 µL	Chromagen Solution
10 µL	10 µL	10X SOD Assay Buffer
70-(X+Y) µL	70 µL	DI Water
90 µL	90 µL	Total

3.9.2. Glutathione peroxidase (GPx):

Glutathione peroxidase (GPx) was analysed using diagnostic kit obtained from ALPCO US. Protocol provided by the vendor was strictly followed for the assay.

Assay principle:

This kit uses an indirect determination method. It is based on the oxidation of glutathione (GSH) to oxidized glutathione (GSSG) catalyzed by GPx, which is then coupled to the recycling of GSSG back to GSH utilizing glutathione reductase (GR) and NADPH (β-Nicotinamide Adenine Dinucleotide Phosphate, reduced).

The decrease in NADPH absorbance was measured at 340 nm in UV-Visible spectrophotometer (APEL PD.303UV) during the oxidation of NADPH to NADP+ which is indicative of GPx activity, since GPx is the rate limiting factor of the coupled reactions.

$$\text{R-OOH} + 2\ \text{GSH} \xrightarrow{\text{GPx}} \text{R-OH} + \text{GSSG} + H_2O$$

$$\text{GSSG} + \text{NADPH} + H^+ \xrightarrow{\text{GR}} 2\ \text{GSH} + \text{NADP}^+$$

GPx is glutathione peroxidase, GR is Glutathione reductase, and R-OOH is organic peroxide.

The reaction was performed at 25 ^{o}C and pH 8.0, and was started by adding organic peroxide, tert-butyl hydroperoxide (t-Bu-OOH).

(This substrate is suitable for the assay since its spontaneous reaction with GSH is low and it is not metabolized by catalase. The reaction with tert-butyl hydroperoxide measures the amount of selenium-containing glutathione peroxidase activity present in the sample. If the presence of non-Se enzymes is suspected, cumene hydroperoxide can be used as the substrate at a concentration of 0.25–1.0 mM. This will measure the total GPx (Se and non-Se enzymes) activity. The difference between the activities is observed with cumene hydroperoxide activity and the tert-butyl hydroperoxide is the non-Se glutathione peroxidase activity).

One unit of glutathione peroxidase is defined as the activity that causes the formation of 1.0 mmol of NADP+ from NADPH per minute at pH 8.0 and 25^{o}C in a coupled reaction in the presence of reduced glutathione, glutathione reductase, and tert-butyl hydroperoxide.

Assay protocol:

1. 20 µl of sample or ASYBUF (Assay buffer) for the blank was added in the corresponding well.
2. Then 200 µl of ASYREAG (reaction solution) was added in each well.
3. The micro titer plate reader was set up and measured the absorbance at 340 nm over a time period of 8 minutes; at constant temperature of 25^{o}C.
4. Enough of PER (peroxide substrate solution) was prepared in a pipetting tray.
5. Then 20 µl of PER (peroxide substrate solution) Added to each well. 8-channel pipette was used to minimize time delays.
6. Measurement was performed.

3.9.3. Catalase (CAT):

Catalase (CAT) was analysed using diagnostic kit obtained from Sigma-Aldrich. Protocol provided by the vendor was strictly followed for the assay.

Principle of Assay:

Catalase is able to decompose hydrogen peroxide by two different reaction pathways. In the first, known as the "catalytic" pathway, 2 molecules of hydrogen peroxide are converted to water and oxygen (catalytic activity):

$$\text{Protein-Fe}^{3}\text{+ + } H_2O_2 \longrightarrow \text{Protein-Fe}^{3}\text{+ -OOH (Primary Complex) + } H_2$$

$$\text{Protein-Fe}^{3}\text{+ -OOH + } H_2O_2 \longrightarrow \text{Protein-Fe}^{3}\text{+ -OH + } H_2O + O_2$$

$$\text{The overall reaction gives: 2 } H_2O_2 \longrightarrow 2\ H_2O + O_2$$

The primary complex can also decompose by another pathway (peroxidatic decomposition):

$$2\ \text{Protein-Fe}^{3}\text{+ -OOH + } AH_2 \longrightarrow \text{Protein-Fe}^{3}\text{+ -OH + H2O + A}$$

Where, AH2 is an internal or external donor of hydrogen. Low molecular weight alcohols can serve as electron donors. The catalytic pathway is predominant when the hydrogen peroxide concentration is greater than 0.1 mM and the peroxidatic pathway is dominant when the hydrogen peroxide concentration is less than 0.1mM or the substrate is alkyl peroxide.

Assay protocol:

1. The sample was Prepared as a protocol and appropriate volume (1 ml) of the sample was transferred to a micro centrifuge tube.
2. 75–100 ml of 1´ Assay Buffer was added to the micro centrifuge tube.
3. The reaction was started by the addition of 25 ml of the Colorimetric assay substrate solution.
4. Contents were mixed by inversion followed by incubation for 1–5 minutes.

5. Then added with 900 ml of the Stop solution and the tube were inverted.
6. Then removed a 10 ml aliquot of the catalase enzymatic reaction mixture and transferred to another micro centrifuge tube. Added 1 ml of the Color Reagent and mixed by inversion.

The mixture was maintained for at room temperature 15 minutes, for color development. Then the absorbance was measured at 520 nm in a UV-Visible spectrophotometer.

3.9.4. Glutathine S-Transferase (GST):

Glutathine S-Transferase (GST) was analyzed using diagnostic kit obtained from Sigma-Aldrich. Protocol provided by the vendor was strictly followed for the assay.

Assay principle:

GST catalyzes the conjugation of L-glutathione to Chloro-2, 4-dinitrobenzene (CDNB) through the thiol group of the glutathione.

$$GSH + CDNB \xrightarrow{GST} \text{GS-DNB Conjugate} + HCl$$

The reaction product, GS-DNB Conjugate, absorbs at 340 nm. The rate of increase in the absorption is directly proportional to the GST activity in the sample.

Assay protocol:

1. 10-ml reaction master mix were Prepared, which was sufficient for 10 assays when a 1 ml quartz cuvette was used. The solution was freshly prepared for each assay series and used within 60 minutes of preparation.
2. 1 ml of the substrate solution was Transferred to a quartz cuvette and read the Blank absorbance at 340 nm in a UV-Visible APEL PD.303

UV spectrophotometer on a kinetic program: which read every 30 seconds over a period of 5 minutes after a lag time of 1 minute

3. 2-50 ml of GST sample/ 2 ml of the GST control were added, provided with the kit, directly to the quartz cuvette containing up to 1 ml substrate solution. Mixed by covering the cuvette with a parafilm and inverting several times.

3.9.5. Aspartate aminotransferase (AST):

Aspartate aminotransferase (AST) was analyzed using diagnostic kit obtained from XpressBio co. Protocol provided by the vendor was strictly followed for the assay.

Assay principle:

The Enzymatic Assay Kit uses a coupled enzymatic reaction scheme: aspartate and α-ketoglutarate are first converted to glutamate and oxaloacetate which is converted by malate dehydrogenase to make malate and NAD+. The conversion of the NADH chromophore to NAD^+ product, measured at 340 nm, is proportional to the level of AST enzyme in the sample. The absorbance of each well at 340 nm is measured using a plate reader. The concentration of AST in each sample is then directly determined from the change in absorbance at 340 nm within 5 minutes time. Dilutions of the AST Control, included in the kit, are used to construct a standard curve to calibrate the assay and confirm assay linearity.

Assay protocol:

1. 10 µL of each sample or standard (in duplicate) were Added to the microplate wells using a multichannel pipet
2. 240 µL of Reagent Mix were Added to the wells.

3. Immediately the absorbance of each sample were measured at 340. Exactly 5 min later, measured absorbance again.

3.9.6. Glutamate oxaloacetoacetate transaminase (GOT):

Glutamate oxaloacetoacetate transaminase (GOT); was analyzed using diagnostic kit obtained from - SPINREACT

Assay Principle:

The glutamic transaminase enzymes, serum glutamic oxalacetic (GOT) and serum glutamic pyruvic (GPT), catalyse the transfers of the amino group of glutamic acid to oxalacetic acid and pyruvic acid in reversible reactions. The transaminase activity is proportional to the amount of oxalate or pyruvate formed over a definite period of time and is measured by a reaction with 2,4-Dinitrophenylhydrazine (DNPH) in alkaline.

Assay Protocol

Aliquots of 0.5 mL of GOT substrate taken in vials were mixed with aliquots of 100 µl of standard and samples separately and incubated for 30 min .at 37 ^{o} C. Then aliquots of 0.5 ml of color developer was added to the reaction mixture, mixed the contents and then incubated for 20 min at room temperature. Then 5 ml aliquots of NAOH 0.4 N was added to the contents, mixed well and incubated at room temperature for 5 min. At the end of incubation the absorbance was measured against water blank at 505 nm

Calculation was done as mentioned below:

Concentration of sample GOT = (OD sample / OD standard) × concentration of standard.

3.9.7. Glutamate pyruvate transaminase (GPT):

Glutamate pyruvate transaminase (GPT); was analyzed using diagnostic kit obtained from MYBIOSOURCE, UK.

The micro titer plate provided in this kit has been pre-coated with an antibody specific to GPT. Standards or samples are then added to the appropriate

micro titer plate wells with a biotin-conjugated antibody preparation specific for GPT and Avid in conjugated to Horseradish Peroxidase (HRP) is added to each micro plate well and incubated. Then a TMB (3,3',5,5' tetramethyl-benzidine) substrate solution is added to each well. Only those wells that contain GPT, biotin-conjugated antibody and enzyme-conjugated Avid in will exhibit a change in color. The enzyme-substrate reaction is terminated by the addition of a sulphuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450 nm ± 2 nm. The concentration of GPT in the samples is then determined by comparing the O.D. of the samples to the standard curve.

1. 100µl of Standard, Blank, or Sample were added well. Cover with the adhesive strip. Incubate for 2 hours at 37°C.
2. The liquid Remove of each well, don’t wash.
3. 100µl of Biotin-antibody working solution were added to each well. Incubate for 1 hour at 37°C. Biotin-antibody working solution may appear cloudy. Warm up to room temperature and mix gently until solution appears uniform.
4. Each well were Aspirate and wash, repeating the process three times for a total of three washes. Wash: Fill each well with Wash Buffer (200µl) and let it stand for 2 minutes, then remove the liquid by flicking the plate over a sink. The remaining drops are removed by patting the plate on a paper towel. Complete removal of liquid at each step is essential to good performance.
5. 100µl of HRP-avidin working solution were added to each well. Cover the microtiter plate with a new adhesive strip. Incubate for 1 hour at 37°C.
6. The aspiration and wash were repeat five times as step 4.

7. 90µl of TMB Substrate were add to each well. Incubate for 10-30 minutes at 37°C. Keeping the plate away from drafts and other temperature fluctuations in the dark.
8. 50µl of Stop Solution were add to each well when the first four wells containing the highest concentration of standards develop obvious blue color. If color change does not appear uniform, gently tap the plate to ensure thorough mixing.
9. The optical density of each well determine within 30 minutes, using a microplate reader set to 450 nm.

3.9.8. Alkaline phosphatase (ALP);

Alkaline phosphatase (ALP); (Liver) - was analyzed using BioAssay Systems' QuantiChromTM Alkaline Phosphatase Assay Kit.

Alkaline phosphatase (ALP) catalyzes the hydrolysis of phosphate esters in an alkaline environment, resulting in the formation of an organic radical and Inorganic phosphate. In mammals, this enzyme is found mainly in the liver and bones. Marked increase in serum ALP levels, a disease known as hyperalkalinephosphatasemia, has been associated with malignant biliary obstruction, primary biliary cirrhosis, primary sclerosing cholangitis, hepatic lymphoma and sarcoidosis. Simple, direct and automation-ready procedures for measuring ALP activity in serum are becoming popular in Research and Drug Discovery. BioAssay Systems' QuantiChromTM Alkaline Phosphatase Assay Kit is designed to measure ALP activity directly in biological samples without pretreatment. The improved method utilizes p nitrophenyl phosphate that is hydrolyzed by ALP into a yellow colored product (maximal absorbance at 405nm). The rate of the reaction is directly proportional to the enzyme activity.

1. 50 µL samples were added into 1-cm cuvettes.
2. 950 µL Working Solution were added to samples. Mix briefly.
3. OD were Readed at 405nm shortly after the mixing, and again after 4 min.
4. Calculation: ALP activity of the sample

Note:

- If sample ALP activity exceeds 800 IU/L, dilute samples in saline and repeat the assay, multiply the result by the dilution factor.
- Incubation can be prolonged for samples with low ALP activity.

Calculation:

Concentration of sample GOT = (OD sample / OD standard) × concentration of standard.

3.9.9. Gamma-glutamyl transferase (GGT):

Gamma-glutamyl transferase (GGT) was analysed using diagnostic kit obtained from XpressBio Co. Protocol provided by the vendor was strictly followed for the assay.

Assay principle:

The γ-Glutamyl Transferase (GGT) Enzymatic Assay Kit uses an enzymatic reaction to measure enzyme levels in serum. The assay measures the cleavage of a specific GGT substrate (γ-glutamyl-p-ntiroanilide) by the enzyme. The production of the p-nitroaniline (pNA) product, measured at 405 nm, is proportional to the level of GGT enzyme in the sample. The absorbance of each sample well at 405 nm is measured using a plate reader. The concentration of GGT in each sample is then directly determined from the change in absorbance at 405 nm within 10 minutes. Dilutions of the pNA Control, included in the kit, are used to construct a standard curve to calibrate the assay and confirm assay linearity.

Assay protocol:

1. Assay components were warmed up to room temperature before use.
2. 10 µL of each sample or standard (in duplicate) were added to the microplate wells using a multichannel pipet
3. 240 µL of GGT Reagent Mix were added to the wells.
4. Immediately the absorbance of each sample were measured at 405 nm in UV-Visible APEL PD.303 UV spectrophotometer. Exactly 10 minutes later, measured the absorbance again.
5. For each point, determined the increase in absorbance per 10 minute time interval by subtracting the absorbance at the initial time point from the absorbance at the 10 minute time point.

3.9.10. Antioxidant-Glutathione (GSH):

Antioxidant-Glutathione (GSH): was analysed using diagnostic kit obtained from Sigma-Aldrich. Protocol provided by the vendor was strictly followed for the assay.

Assay principle:

The biological sample is first deproteinized with the 5% 5-Sulfosalicylic Acid Solution, centrifuged to remove the precipitated protein, and then assayed for glutathione. The measurement of GSH uses a kinetic assay in which catalytic amounts (nmoles) of GSH cause a continuous reduction of 5, 5¢-dithiobis(2-nitrobenzoic acid) (DTNB) to TNB and the GSSG formed is recycled by glutathione reductase and NADPH. The GSSG present also react to give a positive value in this reaction.

1. $2\ GSH + DTNB \longrightarrow GSSG + 2\ TNB$
2. $GSSG + NADPH + H \xrightarrow{+\text{Glutathione reductase}} 2\ GSH + NADP^{+}$

The combined reaction:

$$DTNB + H^{+} + NADPH \xrightarrow{\text{Glutathione reductase GSSG/GSH}} 2\ TNB + NADP^{+}$$

The reaction rate is proportional to the concentration of glutathione up to 2 mM. The yellow product, 5-thio- 2-nitrobenzoic acid (TNB) is measured spectrophotometrically at 412 nm. The assay uses a standard curve of reduced glutathione to determine the amount of glutathione in the biological sample.

Assay protocol:

1. A plate reader was set to 412 nm with kinetic read at 1 minute intervals for 5 minutes.
2. The reaction scheme was set up according to 'Perform every test in duplicate'.
3. The first 2 wells contained only 10 ml of the 5% 5-Sulfosalicylic Acid Solution as a reagent blank. Added duplicate 10 ml samples of the prepared Glutathione Standard Solutions into separate wells of the plate. Added varying volumes of the unknown sample in duplicate into separate wells (up to 10 ml sample). The final volume of the unknown sample was made up to 10 ml with 5% SSA Solution.
4. 150 ml of the Working Mixture were added to each well with a multichannel pipette and mixed by pipetting up and down.
5. Incubated for 5 minutes at room temperature and then added 50 ml of the diluted NADPH Solution with a multichannel pipette. Mixed by pipetting up and down.
6. Using the plate reader measured the absorbance in each well. Subtracted the reagent blank value from every measurement.

3.9.11. Cholesterol:

Cholesterol was analysed using diagnostic kit obtained from CELL BIOLABS Inc. Protocol provided by the vendor was strictly followed for the assay.

Assay principle:

Cell Biolabs' Total Cholesterol Assay Kit measures the total cholesterol within serum, plasma, lysate, or tissue samples. The assay is based on the enzyme driven reaction that quantifies both cholesterol esters and free cholesterol. Cholesterol esters are hydrolyzed via cholesterol esterase into cholesterol, which is then oxidized by cholesterol oxidase into the ketone cholest-4-en-3-one plus hydrogen peroxide. The hydrogen peroxide is then detected with a highly specific colorimetric probe. Horseradish peroxidase catalyzes the reaction between the probe and hydrogen peroxide, which bind in a 1:1 ratio. Samples are compared to a known concentration of cholesterol standard in a 96-well microtiter plate format. Samples and standards are incubated for 45 minutes and then read with a standard 96-well colorimetric plate reader.

Assay protocol:

Each cholesterol standard and sample was assayed in triplicate. A freshly prepared standard curve was used each time the assay was performed.

1. 50 µL of the diluted cholesterol standards/ samples were added to a 96-well microtiter plate.
2. 50 µL of the prepared Cholesterol Reaction Reagent were added to each well and mixed the well contents thoroughly.
3. The plate wells were covered to protect the reaction from light. Incubated the plate for 45 minutes at 37 ^{o}C.
4. Read the plate were read with a spectrophotometric microplate reader in the 540-570 nm range.

5. Calculated the concentration of cholesterol within samples by comparing the sample absorbance values to the cholesterol standard curve.

3.9.12 Total Protein (TP):

Total protein concentration was determined by BioAssay Systems' QuantiChromTM protein assay kit. This Kit is based on an improved pyrogallol red-molybdate protein dye-binding assay. The color intensity at 600nm is directly proportional to the total protein concentration in the sample.

Assay protocol:

Aliquots of 20 µL dH_2O, 20 µL Protein Standard (bovine serum albumin and 20 µL of Samples were pipetted out into separate cuvettes.
To the aliquots taken in each cuvette 1 mL of the Reagent was added and mixed thoroughly. The Mix was then incubated for 10 min. and measured the absorbance at OD 600 nm in a UV-Visible spectrophotometer.

Calculation:

Total protein concentration of a Sample was calculated as
(Total Protein)= × n × 100 (mg/dL) OD sample – OD H_2O OD standard – OD H_2O where OD sample, OD standard and OD H_2O are the optical density values of the Sample, the Standard and the H_2O (blank), respectively. Whenever calculated protein concentrations were higher than 200 mg/dL, Samples were diluted in water and repeated the assay. Results obtained were multiplied by the dilution factor n.

3.9.13 Albumin :

Albumin was analyzed using diagnostic kit 'The Rat Albumin ELISA kit' obtained from Alpha Diagnostic.

The Rat Albumin ELISA kit is based on the binding of rat albumin in samples to two antibodies, one immobilized on the microtiter wells, and the other conjugated to horseradish peroxidase (H R P) enzyme. After a washing step, chromogenic substrate was added and color was developed by the enzymatic reaction of H R P on the TMB (3, 3', 5, 5'-Tetramethylbenzidine) substrate, which is directly proportional to the amount of albumin present in the sample. Stopping Solution were added to terminate the reaction, and absorbance at 450nm was then measured using an ELISA microtiter well reader. The concentrations of albumin in samples and control were calculated from a curve of standards containing known concentrations of albumin.

3.10 Histology:

Effect of *Spirulina* diet on liver was monitored by observing the damage occurred in liver tissues in rats by histological examination of liver of rats fed with *Spirulina* diet as well as control diet.

Liver samples were processed by standard histopathological techniques (Hunason, 1972).

- Processing Procedure

PRNCIPLE:

The VIP Tissue Processor (Sakura Tissue-Tek VIP5) were used to the preparation of tissues for subsequent sectioning on a microtome (from paraffin

blocks).the purpose was to replace the water in the tissue with paraffin wax. Paraffin wax supported the tissue for thin sectioning step, as it is hard enough to be a solid, but soft enough to section.

Tissue must be adequately fixed prior to tissue processing (although the processing schedule incorporate a fixation component to ensure that fixation is adequate.)

Processing were enhanced by the application of vacuum, pressure and heat (heat must be used with discretion.) These components have been adjusted for every step of the processing cycle.

The VIP processor is an example of a ‘closed’ tissue processing system. Closed processors give better results than ‘open’ systems. There is no evaporation of solutions or solvents, much better programming control and the ability to apply heat, vacuum and pressure.

Processing times vary depending on size of the tissue therefore different programs are used (to accommodate the largest tissue blocks to be processed on that particular processing run.)

Processing occurs by diffusion in 3 distinct stages:

•Dehydration: The removal water. Alcohol is the most common dehydrant. Graded (increasing strength) alcohols are used to prevent excessive hardening.

•Clearing: Generally, dehydrant and infiltrant are not sufficiently miscible to promote proper or adequate processing. This is an intermediary step and utilizes a solvent miscible with dehydrant and the infiltrant. Xylene is the most common clearing agent. It also makes tissue translucent and dissolves fat.

•Infiltration: Paraffin wax is the most common infiltrant. It must be heated to make it molten, usually to approximately 60-63°C (a few degrees above the melting point of the paraffin wax). If the paraffin wax is too hot, the plastic

polymers will be damaged. In addition, exposing tissue to excessive heat may cause physical damage and subsequent staining difficulties.

- Embedding Procedure:

After tissue cassettes are removed from the tissue processors, embedding center is used to prepare paraffin blocks for sectioning on a microtome. An appropriate size mold is selected, filled with paraffin and the tissue oriented according to policy. The plastic tissue-processing cassette is subsequently placed on top of the mold and the paraffin is left to harden. Once cool, the paraffin block can be removed from the mold for sectioning.

1. Press Light key to turn on work light.

2.After the tissue cassettes have been transferred from the tissue processor to heated chamber of the embedding center; using heated forceps, remove one cassette from the paraffin bath and place on either right or left hot plate(depending on your work flow direction.) then remove the cassette's cover.

3. One base mold that would best fit the tissue in the cassette have been selected and removed from heated chamber, (be sure that there will be a gap of at least several millimeters around the tissue once it is placed in the mold.) place under the paraffin dispenser.

4. While holding base mold under paraffin dispenser, press fingerplate to dispense only enough paraffin to half-fill base mold.

5. Base mold were Placed on hot plate under paraffin dispenser

6. By Using forceps, tissue were placed from cassette into base mold (the tissue may need to be removed the tissue paper if it was wrapped prior to processing). If tissue do not sink to bottom of mold, use forceps to lightly push

tissue down (into proper position) on the bottom of base mold, Care must be taken to ensure that all of the tissue is transferred to the mold.

7. The base mold were moved to the cold spot. Bottom of the base mold rapidly cools and a thin layer of paraffin solidifies, if the tissue is not correctly positioned, move base back to hot plate; then use forceps to reposition tissue, Return base mold to cold spot. Repeat this procedure as necessary until tissue are properly oriented. Never allow paraffin to completely solidify during this process.

8. One cassette placed over properly positioned tissue in base mold. Embedded tissue adhere to cassette.

9. The base mold were moved to the hot plate under paraffin dispenser.

10. While holding the cassette under paraffin dispenser (and above center hot plate), fingerplate were pressed to dispense paraffin into base mold until the cassette is filled with paraffin. Do not overfill base mold.

11. Base mold placed containing embedded tissue onto cooling plate, The surface of the cooling plate will usually be covered with a light layer of frost (depending on ambient humidity). This enhances heat exchange between the warmed base mold and the cooling plate.

12. The process were repeated until all tissue cassettes have been embedded

13. Insured the paraffin block is completely solidified befor remove paraffin block from base mold.

14. the excess paraffin from the edges of the plastic cassette were Pare on place in numerical order with the other cassettes so that the number of blocks can be compared to the data noted on the daily log sheet.

- Sectioning:

PROCEDURE FOR CUTTING PARAFFIN SECTIONS- STEPWISE

1. All materials assemble setup water bath. The water bath should be heated to approximately 45°C (check with spirit thermometer).

1. The temperature too high may cause cracking artifact due to rapid expansion of the sections, particularly lymphoid tissue.

2. Before inserting the blade into the microtome, the hand wheel was locked.

3. The blade was inserted.

4. The knife-block back was moved to within a few millimeters of the face of the paraffin block. The knife- edge must not touch the face of the paraffin block. Tighten the knife-block clamping lever to fix the knife- block in position.

5. Hand wheel to being facing the paraffin block; the hand wheel and then were turned when the block is above the knife-edge the trimming wheel a turned short distance. These two movements were repeated until the paraffin block beings to trim. The first cut will indicate whether the paraffin block needs to be adjusted in the object clamp to give a fuller face to the tissue block. It is not necessary to carry out whole rotation with the hand wheel. Particularly with small fragment of tissue, it is better to move the hand wheel only up and down through a short distance.

6. Faced blocks are placed on a tray of ice to chill. This makes the wax harder which in turn holds the tissue firmer, which allows for thinner sections.

7. Paraffin sections and manipulate were cut onto the water bath using forceps, probe or brush.

- Staining Procedure:

PRINCIPLE:

The Hematoxylin & Eosin stain is the most used stain in Histology. It is the basis of most diagnosis and the starting point for all investigations.

Hematoxylin stains the nucleus blue-purple. Eosin stains the cytoplasm and connective tissue elements pink to red. Together these two stains make the best general morphological stain in use.

SOLUTIONS:

1. GILL'S HEMATOXYLIN
2. EOSIN SOLUTION

PROCESDURE – STEPWISE:

1. Deparaffinize section in tow changes of xylene was take 3 minutes to each.
2. Hydrate through two changes of absolute alcohol, two changes of 95% alcohol and one change of 70% alcohol were obtained.
3. Slide was rinsed in tap water.
4. Stain in Gill's hematoxylin for was incubate for two minutes.
5. Slide was thoroughly rinsed in tap water.
6. Slide was dipped in 70% alcohol, 10 to 20 dips.
7. Slide stain in Eosin was incubate for two minutes.
8. Slide was Rinse in water 1 dip.
9. Slide was dehydrated in 95% alcohol, two changes, 100% alcohol, and two changes.
10. Slides was Placed in xylene for cover slipping, and examined for morphological evidence of liver injury under microscope.

3.11. Statistical analysis

All statistical analysis were conducted using the Statistical Package for Social Sciences (SPSS), ANOVA one way between groups analysis of variance and post hoc comparisons using the Tukey HSD test indicates the mean score for groups. To see the effect of treatment significant or not at (person value $\leq$ 0.05)

Chapter Four

Results

4. Chapter four: Results

4.1. *Spirulina* Analysis

Data obtained for the proximate analyses carried out for the *S. platensis* locally cultivated in the laboratory (**Fig 4.1**) and the commercially procured samples (**Fig 4.2**) showed that both samples have almost an identical composition of the various significant constituents. From the data presented in **Table 4.1** it may be seen that except for the crude protein and total carbohydrates all other variables including moisture content, total fat, ash content, and crude fiber recorded identical values. Whereas crude protein recorded a meagre 0.81 % difference among the values recorded for the cultivated sample (40.44%) and the commercial sample (40.77%). Similarly total carbohydrates recorded a least value of 0.92 % difference among the values recorded for the cultivated sample (35.44%) and the commercial sample (35.77%). Cultivated samples recorded marginally less values for crude protein and more for total carbohydrates than the commercial sample. While cultivated sample recorded relatively less crude protein and more total carbohydrates, commercial samples recorded higher crude protein and less total carbohydrates (**Table 4.1**).

Variables phosphorus, potassium and sodium also recorded insignificant difference in levels of their content with respect to their different source. Thus, phosphorus was noted to be slightly higher (1.00%) in cultivated sample compared to that in the commercial one (0.98%). Whereas, both potassium (1.26%) and sodium (2.98%) were less in their content in cultivated samples when compared to the potassium(1.28%) and sodium (3.03%) content in the commercial samples (**Table 4.1**).

Similarly data presented in **Table 4.1** and in **Fig 4.3** for the analyses of various minerals in the cultivated (**Fig 4.3**) and commercially procured (**Fig**

4.4) *Spirulina* samples indicated that manganese, copper, iron, zinc, chromium and boron contents were slightly higher in levels in the commercial sample of *Spirulina* compared to that in locally cultivated sample. Whereas molybdenum, selenium and beta carotene showed slightly higher levels of content in locally cultivated *Spirulina* compared to that in commercial samples. Nevertheless it was also noted that the difference in the levels of content of minerals was very marginal and insignificant.

Table 4.1. Constituents of *Spirulina* powder obtained from locally cultivated sample (Sample A) and from a commercial source (Sample B)

Variables	METHOD REF.	LOD	SAMPLE A	SAMPLE B	UNIT
MOISTURE	AOAC-930.15	7.72		7.72	%
CRUDE PROTEIN	AOAC-2001.11	0.10	40.44	40.77	%
TOTAL FAT	AOAC-2003.06	0.26		0.26	%
ASH	AOAC-978.10	15.19		15.19	%
CRUDE FIBER	AOAC-942.05	0.62		0.62	%
TOTAL CARBOHYDRATES	CALCULATION	--	35.77	35.44	%
PHOSPHORUS	AOAC-991.25	0.0000100	1.00	0. 98	%
POTASSIUM	AOAC-975.03	0.0000020	1.26	1.28	%
SODIUM	AOAC-975.03	0.0000040	2.98	3.03	%
MANGANESE	AOAC-975.03	0.027	51.78	53.34	Ppm

COPPER	AOAC-975.03	0.02	11.82	12.14	Ppm
IRON	AOAC-975.03	0.036	292.00	294.26	Ppm
ZINC	AOAC-975.03	0.02	40.30	41.35	Ppm
MOLYBDENUM	AOAC-975.03	0.0005	0.31	0.29	Ppm
CHROMIUM	AOAC-975.03	0.001	1.54	1.57	Ppm
SELENIUM	AOAC-975.03	0.0001	1.55	1.54	Ppm
BORON	AOAC-975.03	0.001	34.83	35.45	Ppm
BETA CAROTENE	HPLC LUNN	0.200	33. 68	33.17	Ppm

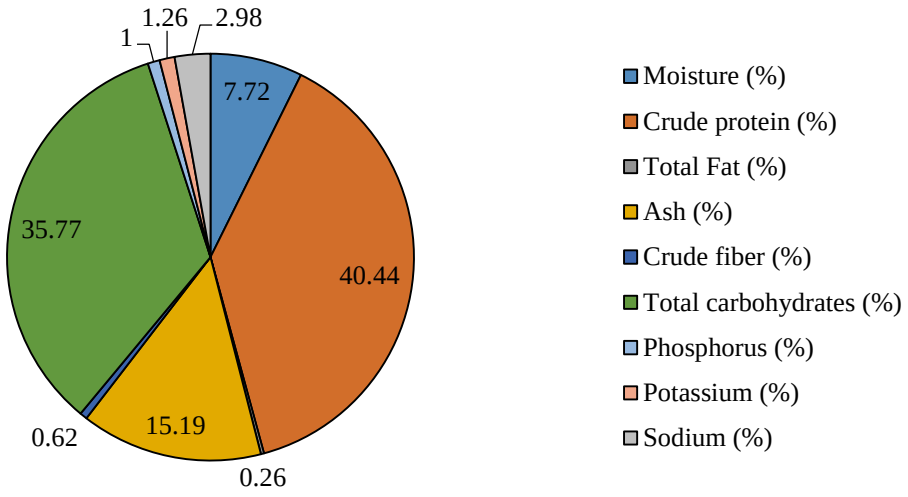

Fig 4.1. Percentage composition of various constitutes of *Spirulina* powder obtained from locally cultivated sample.

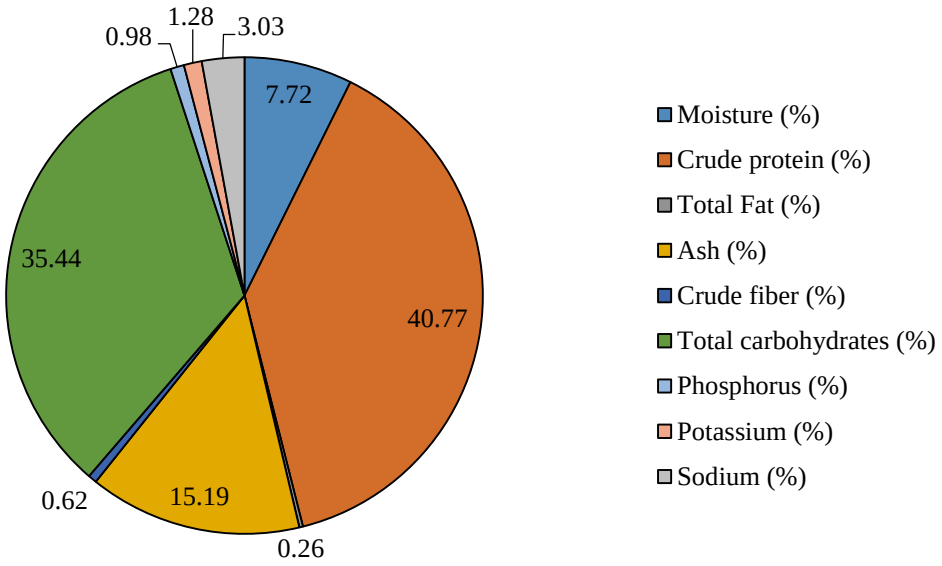

Fig 4.2. Percentage composition of various constitutes of *Spirulina* powder obtained from a commercial source.

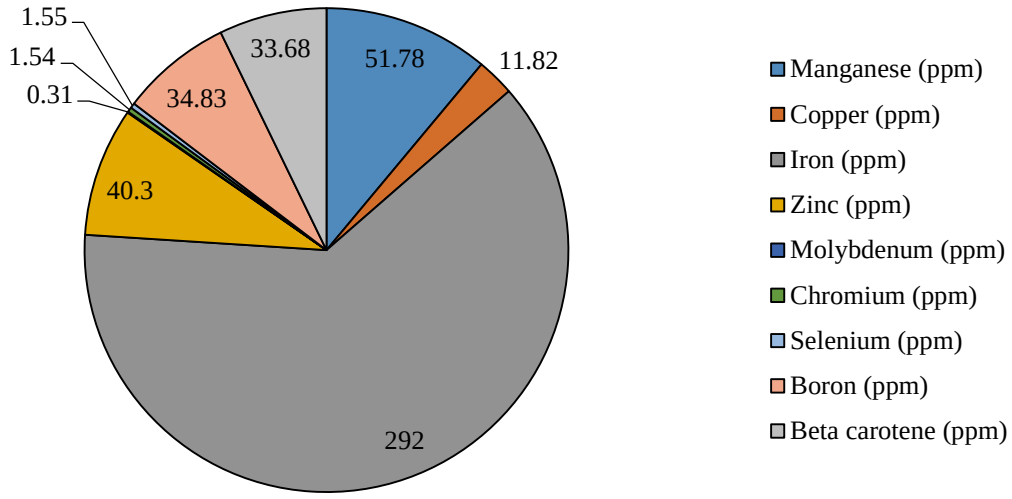

Fig 4.3. Mineral contents (ppm) of *Spirulina* powder obtained from locally cultivated sample.

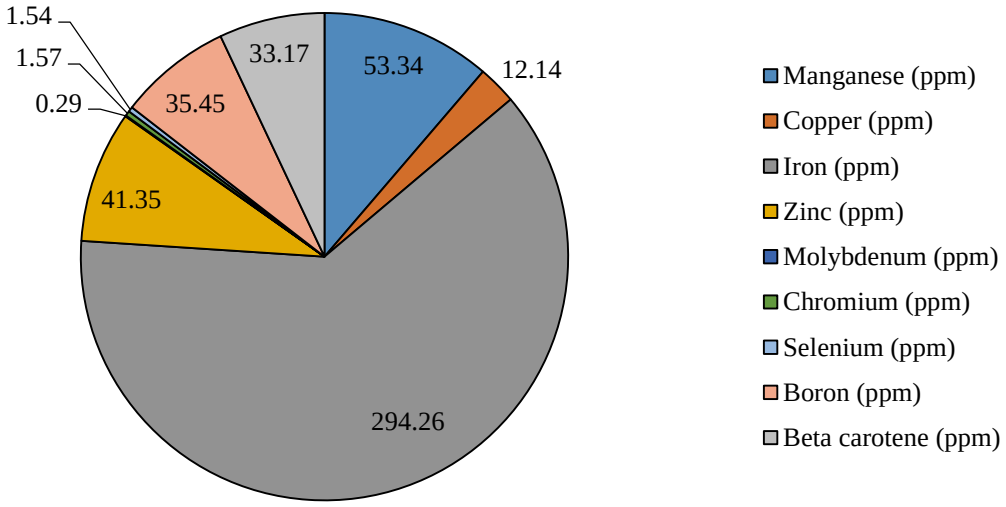

Fig 4.4. Mineral contents (ppm) of *Spirulina* powder obtained from a commercial source.

4.2. Biochemical Analysis of Enzymes Activity

No mortality was observed in the experimental animals during the study period. The results of this study presented in **Table 4.2** revealed that D-GalN induced severe toxicological effects as evidenced by the significant increases in alkaline phosphatase (ALP) (from 117.17±6.74 U/Lto 918.67±7.06 U/L), aspartate aminotransferase (AST) (from 115.33±18.54 U/Lto510.00±53.86 U/L), glutamate oxaloacetoacetate transaminase (GOT) (from 121.67±21.29 U/L to 473.50±20.13 U/L), glutamate pyruvate transaminase (GPT) (from 95.67±7.87 U/L to 353.33±13.85 U/L), γ-glutamyl transpeptidase (GGT) (from 4.43±0.48 U/L to 13.97±1.62 U/L) and cholesterol (from 70.50±4.42 mg/dL to 323.67±20.88 mg/dL) along with significant decreases in total protein (TP) (from 8.47±1.24 mg/dL to 6.18±0.75 mg/dL)and albumin (from 5.70±0.65 mg/dL to 4.62±0.44 mg/dL).

Whereas, treatment with BHT resulted in a significant decrease in the levels of ALP (from 918.67±7.06 U/L to 516.00±22.71 U/L), AST (from 510.00±53.86 U/L to 201.50±7.58 U/L), GOT (from 473.50±20.13 U/L to 198.17±9.87 U/L), GPT (from 353.33±13.85 U/L to 130.50±2.88 U/L), GGT (from 13.97±1.62 U/L to 6.60±0.25 U/L) and cholesterol (from 323.67±20.88 mg/dL to 117.67±9.93 mg/dL) when compared to the levels recorded with that group treated with D-GalN. It was observed that BHT treated group which was previously treated with D-GalN could result in the rapid reduction in the level of ALP (44%), AST (61%), GOT (58%), GPT (63%), GGT (53%) and cholesterol (64%). Similarly, there was a significant increase in the levels of TP (from 6.18±0.75 mg/dL to 7.27±1.07 mg/dL) and albumin (from 4.62±0.44 mg/dL to 5.03±0.52 mg/dL) compared to that group treated with D-GalN. However, the levels of the enzymes were relatively higher than the levels recorded for the control group except in the case of total protein and albumin

which were less than that of the control group. These observations indicated that BHT treatment has partially reversed the effect of D-GalN and attributed protection against the toxic effect of D-GalN.

Among the three different concentrations of *Spirulina* powder (3%, 6%, and 9%) used to treat rats previously treated with D-GalN, the group that was treated with 9% recorded maximal effect compared to 3% and 6% (**Table 4.2**). Further, it was noted that the effect of *Spirulina* concentration was evident by the progressive increase in effect along with increase in concentration as testified by the variation in the levels of the enzymes tested and in cholesterol content. The results conclusively indicated that 9% *Spirulina* powder is effective compared to lesser concentrations of 6% and 3 %. However it was also noted that there was significant decrease in TP and albumin levels as compared to control group. Further it was also noted that for *Spirulina* concentrations of 6% and 9% the levels of total protein and albumin were almost identical indicating that increase in concentrations beyond 6% is not required unlike with other variables.

Treatment with *Spirulina* diets at 9% resulted in a significant decrease in the levels of ALP (from 918.67±7.06 U/L to 570.50±37.73^{c} U/L), AST (from 510.00±53.86 U/L to 209.67±14.08^{b} U/L), GOT (from 473.50±20.13 U/L to 235.50±33.92^{c} U/L), GPT (from 353.33±13.85 U/L to 155.17±26.90^{c} U/L), GGT (from 13.97±1.62 U/L to 7.75±0.63^{c} U/L) and cholesterol (from 323.67±20.88 mg/dL to 195.50±4.04^{c} mg/dL) when compared to the levels recorded with that group treated with D-GalN.

It was observed that *Spirulina* (9%) treated group which was previously treated with D-GalN could result in the rapid reduction in the level of ALP (38%), AST (59%), GOT (50%), GPT (56%), GGT (45%) and cholesterol (40%). Similarly, there was a significant increase in the levels of TP (from

6.18±0.75 mg/dL to 7.20±0.91 mg/dL) and albumin (from 4.62±0.44 mg/dL to 5.03±0.60 mg/dL) compared to that group treated with D-GalN. However the levels of the enzymes were relatively higher than the levels recorded for the control group except in the case of total protein and albumin which were less than that of the control group. These observations indicated that *SPIRULINA* (9%) treatment has reversed the effect of D-GalN and attributed protection against the toxic effect of D-GalN.

It was observed that *Spirulina* (9%) treated group, which was previously treated with D-GalN, could also record very good protective effect identical to the effect of BHT observed in the present study. Thus it was observed that the percent reduction in the levels of ALP was **38%** compared to **44%** with BHT (Difference of 6%), AST**59%** compared to **61%** with BHT (Difference of 2%),, GOT **50**% compared to **58**% with BHT(Difference of 8%), GPT (**56**% compared to **63**% with BHT (Difference of 7%), GGT**45**% compared to **53**% with BHT(Difference of 8%), and cholesterol **40**% compared to **64**% with BHT (Difference of 24%). It could be seen that the difference in terms of percent reductions were less than **10%** between BHT and *Spirulina* in all the cases except in the case of cholesterol where it was **24**%. Whereas in the case of total protein and albumin 9% *Spirulina* recorded almost identical effect as it was recorded with BHT.

Table 4.2. Effect of different concentrations of *Spirulina* on serum and liver biochemical parameters in control rats and rats treated with D-GalN.

Parameters	**Groups**					
	Control	**D-GalN**	**BHT**	***SPIRULINA* 3%**	***SPIRULINA* 6%**	***SPIRULINA* 9%**
ALP (U/L liver)	117.17 ± 6.74^{a}	918.67 ± 7.06^{e}	516.00 ± 22.71^{b}	693.67 ± 44.04^{d}	597.00 ± 44.92^{c}	570.50 ± 37.73^{c}
AST (U/L serum)	115.33 ± 18.54^{a}	510.00 ± 53.86^{e}	201.50 ± 7.58^{b}	370.50 ± 16.93^{d}	290.67 ± 31.24^{c}	209.67 ± 14.08^{b}
GOT (U/L serum)	121.67 ± 21.29^{a}	473.50 ± 20.13^{f}	198.17 ± 9.87^{b}	353.50 ± 9.77^{e}	302.17 ± 4.88^{d}	235.50 ± 33.92^{c}
GPT (U/L serum)	95.67 ± 7.87^{a}	353.33 ± 13.85^{f}	130.50 ± 2.88^{b}	238.33 ± 13.34^{e}	206.50 ± 7.40^{d}	155.17 ± 26.90^{c}
GGT (U/L liver)	4.43 ± 0.48^{a}	13.97 ± 1.62^{f}	6.60 ± 0.25^{b}	11.42 ± 0.59^{e}	9.62 ± 0.46^{d}	7.75 ± 0.63^{c}
Cholesterol (mg/dL)	70.50 ± 4.42^{a}	323.67 ± 20.88^{f}	117.67 ± 9.93^{b}	257.83 ± 5.34^{e}	217.17 ± 4.36^{d}	195.50 ± 4.04^{c}
TP (mg/dL)	8.47 ± 1.24^{a}	6.18 ± 0.75^{b}	7.27 ± 1.07^{b}	6.30 ± 0.67^{b}	7.18 ± 0.93^{b}	7.20 ± 0.91^{b}
Albumin (mg/dL)	5.70 ± 0.65^{a}	4.62 ± 0.44^{b}	5.03 ± 0.52^{b}	4.80 ± 0.41^{b}	5.00 ± 0.43^{b}	5.03 ± 0.60^{b}

Alkaline phosphatase ALP, Aspartate aminotransferase AST, Glutamate oxaloacetoacetate transaminase GOT, Glutamate pyruvate transaminase GPT, γ-glutamyl transpeptidase GGT, Cholesterol and Total protein TP.

Values with different letters are significantly different ($P \leq 0.05$).

4.3. Antioxidant Enzymes

4.3.1. Superoxide dismutase

Results obtained for the analysis of antioxidant activity of (SOD) is presented in **Fig 4.5**. From the data obtained it was observed that D-GalN treatment led to a rapid and significant reduction in SOD from 0.20U/L (control group) to 0.13U/l when compared to control. However the toxic effect of D-GalN could be effectively reversed by the subsequent treatment of the group with BHT. Thus it was noted that BHT treatment resulted in SOD levels of 0.32U/L compared to 0.13U/L recorded with D-GalN. These results indicated that the SOD levels were increased almost 2.5 times more when compared to D-GalN treatment and about 1.6 times more than control group (0.20U/L). Further when *Spirulina* powder was used as diet at three different concentrations for the D-GalN treated group it was observed that *Spirulina*, at all the three tested concentrations, could not only effectively reverse the effect of D-GalN but also could boost SOD levels compared to control group (0.13U/L). Among the three concentrations 9% of *Spirulina* could result in 0.28U/L of SOD which was very close to that of BHT (0.32U/L) and about 2.15 fold more when compared to that of D-GalN treatment. These observations indicated not only protective effect of *Spirulina* against toxicity but also enabled beneficial activity in terms of enhanced antioxidant activity of SOD in liver. Further there was no significant difference between *Spirulina* 6% and *SPIRULINA*9% groups.

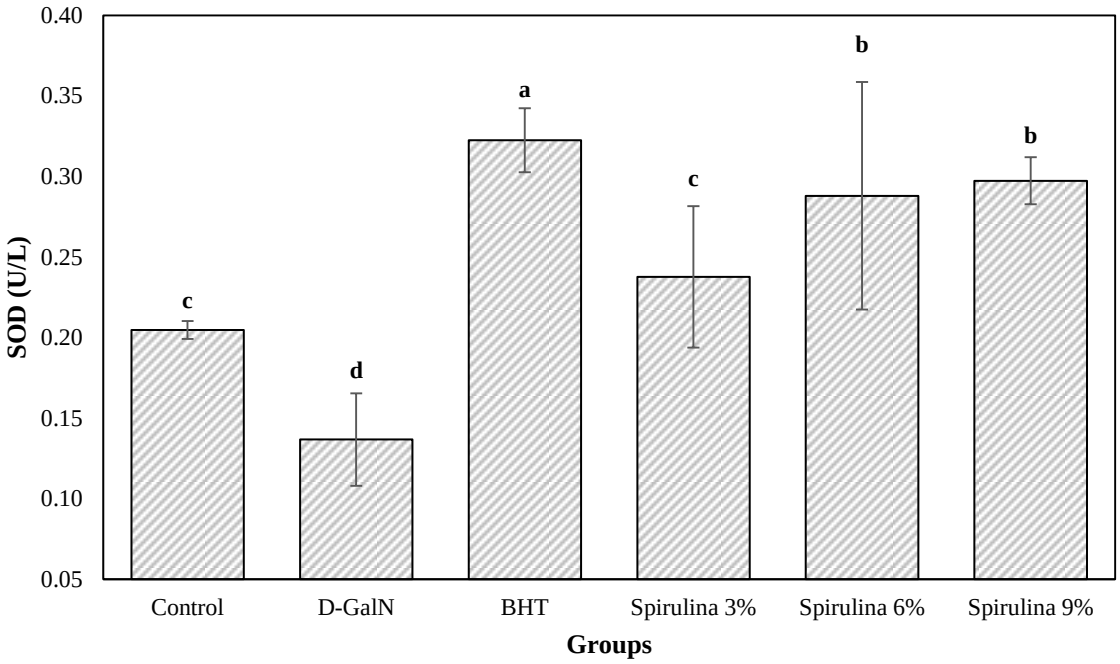

Fig 4.5. Effect of different concentrations of *Spirulina* on superoxide dismutase (SOD) activity in rats treated with D-GalN.

Bars with different letters are significantly different ($P \leq 0.05$).

4.3.2. Glutathione peroxidase (GPX)

Results obtained for the analysis of antioxidant activity of (GPx) is presented in **Fig 4.6**.The antioxidant activity of glutathione peroxidase (GPX) was reduced significantly in the rats administrated with D-GalN. Thus the levels of GPx showed significant reduction in GPx from 16U/L (control group) to 9U/l when compared to control. But supplementation with BHT or *Spirulina* diets increased its activity significantly (**Fig 4.6**). Thus it was noted that BHT treatment resulted in GPx levels of 37U/L compared to 9U/L recorded with D-GalN. These results indicated that the GPx levels were increased almost 4.1 times more when compared to D-GalN treatment and about 2.3 times more than control group (16U/L). Further when *Spirulina* powder was used as diet at three different

concentrations for the D-GalN treated group it was observed that *Spirulina*, at all the three tested concentrations, could not only effectively reverse the effect of D-GalN but also could boost GPx levels compared to control group (9U/L). While the GPX antioxidant activities were significantly increased in all the *Spirulina* -treated groups compared with those in the corresponding control groups, treatment with the 9% *Spirulina* diet showed more potent capability in increasing the GPX levels compared with the 3% and 6% *Spirulina* diet. Among the three concentrations, 9% of *Spirulina* could result in 35U/L of GPx which was very close to that of BHT (37U/L) and about 3.9 fold more when compared to that of D-GalN treatment and about 2.2 fold more than that of control (16U/L). These observations indicated not only protective effect of *Spirulina* against toxicity but also enabled beneficial activity in terms of enhanced antioxidant activity of GPx in liver.

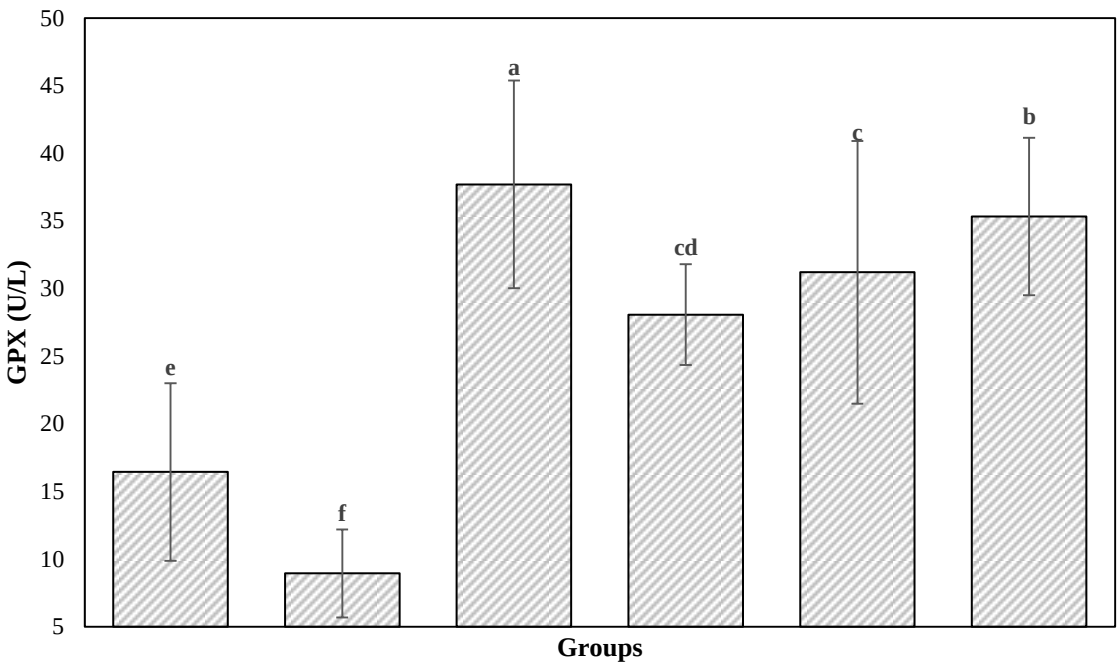

Fig 4.6. Effect of different concentrations of *Spirulina* on glutathione peroxidase (GPX) activity in rats treated with D-GalN.
Bars with different letters are significantly different ($P \leq 0.05$).

4.3.3. Catalase (CAT)

Results obtained for the analysis of antioxidant activity of (CAT) is presented in **Fig 4.7.** From the data obtained it was observed that D-GalN treatment led to a rapid and significant reduction in CAT from 1.5 U/L (control group) to 0.8 U/l when compared to control. However the toxic effect of D-GalN could be effectively reversed by the subsequent Oral administration of BHT which led to increased antioxidant activity of catalase (CAT) as compared to D-GalN and control groups (**Fig 4.7**). Thus it was noted that BHT treatment resulted in CAT levels of 2.6 U/L compared to 0.8 U/L recorded with D-GalN. These results indicated that the CAT levels were increased almost 3.25 times more when

compared to D-GalN treatment and about 1.7 times more than the control group (1.5 U/L). Further when *Spirulina* powder was used as diet at three different concentrations for the D-GalN treated group it was observed that *Spirulina*, at all the three tested concentrations, could not only effectively reverse the effect of D-GalN but also could boost CAT levels compared to control group (0.8 U/L). Among the three concentrations 9% of *Spirulina* could result in 2.2 U/L of CAT which was very close to that of BHT(2.6 U/L) and about 2.75 fold more when compared to that of D-GalN treatment and about 1.46 fold more than that of control(1.5 U/L). Further there was no significant difference between *Spirulina* 6% and *Spirulina* 9% groups. It was also noted that there was no significant difference between using BHT, *SPIRULINA*6% and *SPIRULINA*9% on the activity of CAT. Application of *Spirulina* with concentration of 3% did not appear to have any beneficial effect on the antioxidant activity of CAT since there was no difference between *SPIRULINA*3% and control groups. These observations indicated not only protective effect of *Spirulina* against toxicity but also enabled beneficial activity in terms of enhanced antioxidant activity of CAT in liver.

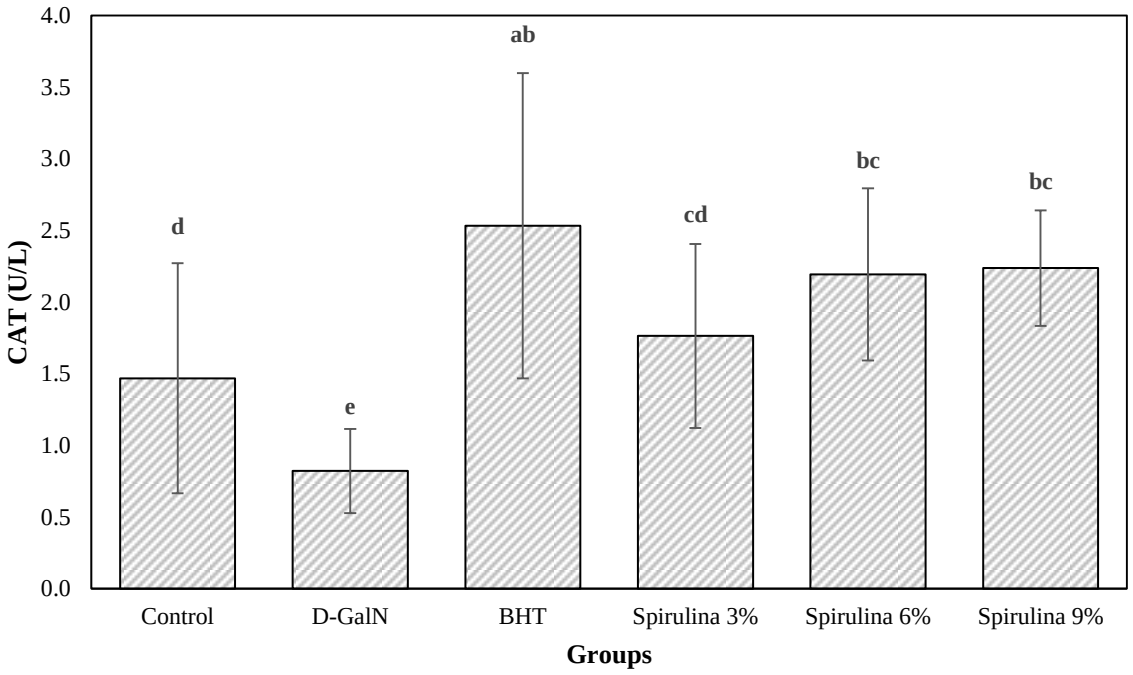

Fig 4.7. Effect of different concentrations of *Spirulina* on catalase (CAT) activity in rats treated with D-GalN.

Bars with different letters are significantly different ($P \leq 0.05$).

4.3.4. Glutathione (GSH)

From the data presented in **Fig 4.8** for the analysis of antioxidant activity of (GSH) it was observed that D-GalN treatment led to a rapid reduction in GSH from 8 U/L (control group) to 7 U/l when compared to control. Nevertheless, the oral administration of BHT and *Spirulina* led to increase in the levels of GSH effectively reversing the effect of D-GalN treatment. Thus it was noted that BHT treatment resulted in GSH levels of 16.5 U/L compared to 7 U/L recorded with D-GalN. These results indicated that the GSH levels were increased almost 2.36 times more when compared to D-GalN treatment and about 2.06 times more than control group (8 U/L). Further when *Spirulina* powder was used as diet at three different concentrations for the D-GalN treated group it was observed that

Spirulina, at all the three tested concentrations, could not only effectively reverse the effect of D-GalN but also could boost GSH levels compared to control group (8 U/L). Increasing the *Spirulina* concentration from 6% to 9% did not show a positive effect on the level of GSH activity. Among the three concentrations 9% of *Spirulina* could result in 14.5 U/L of GSH which was very close to that of BHT(16.5 U/L) and about 2.07 fold more when compared to that of D-GalN treatment and about 1.8 fold more than that of control (8 U/L). Further there was no significant difference between *Spirulina* 6% and *Spirulina* 9% groups. These observations indicated not only protective effect of *Spirulina* against toxicity similar to BHT but also enabled beneficial activity in terms of enhanced antioxidant activity of GSH in liver.

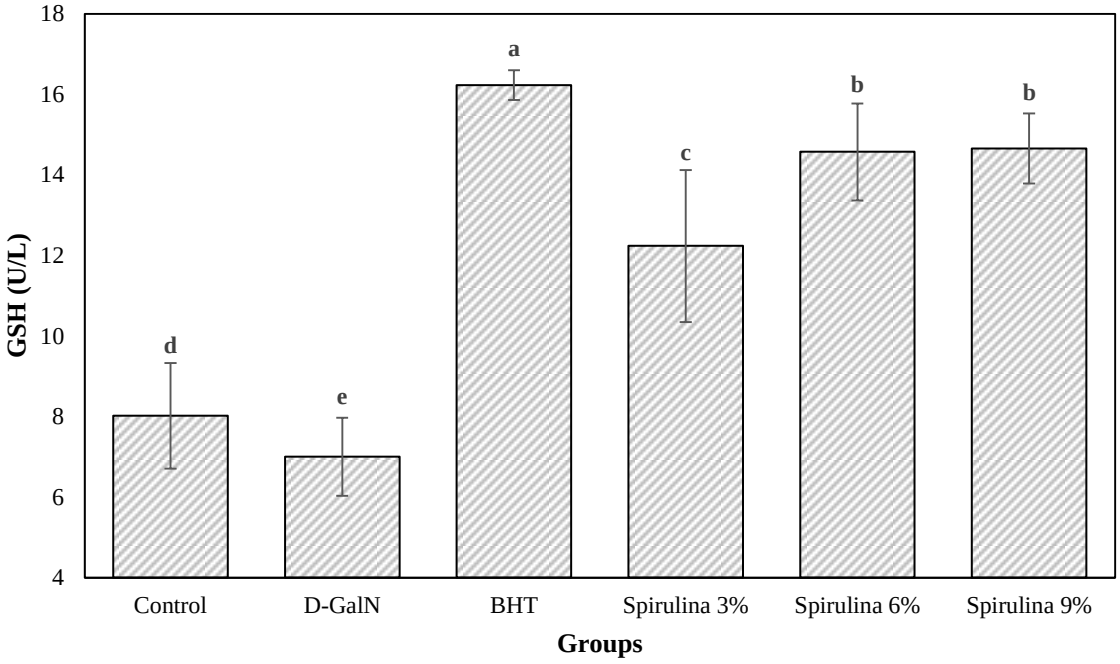

Fig 4.8 Effect of different concentrations of *Spirulina* on reduced glutathione (GSH) activity in rats treated with D-GalN.

Bars with different letters are significantly different ($P \leq 0.05$).

4.3.5. Glutathione transferase (GST)

Injecting the experimental animals with D-GalN in the absence of BHT or *Spirulina* caused a significant reduction in the antioxidant activity of (GST) as compared to the negative control group (Group that was not treated with d-GalN). Results obtained for the analysis of antioxidant activity of the glutathione transferase (GST) is presented in **Fig 4.9**. From the data obtained it was observed that D-GalN treatment led to a rapid and significant reduction in GST from 44 U/L (control group) to 33 U/l when compared to control. However the toxic effect of D-GalN could be effectively reversed by the subsequent treatment of the group with BHT. Thus it was noted that BHT treatment resulted in GST levels of 48 U/L compared to 33 U/L recorded with D-GalN. These results indicated that the GST levels were increased almost 1.45 times more when compared to D-GalN treatment and about 1.09 times more than control group (44 U/L). Further when *Spirulina* powder was used as diet at three different concentrations for the D-GalN treated group it was observed that *Spirulina*, at all the three tested concentrations, could not only effectively reverse the effect of D-GalN but also could boost GST levels compared to control group (44 U/L). Among the three concentrations 9% of *Spirulina* could result in 47 U/L of GST which was very close to that of BHT(48 U/L) and about 1.42 fold more when compared to that of D-GalN treatment and about 1.07 fold more than that of control (44 U/L). Further there was no significant difference between *Spirulina* 6% and *Spirulina* 9% groups as well between *Spirulina* treated and BHT treated groups. However, different concentrations of *Spirulina* appeared to have no beneficial effect on the activity of GST as compared to control group, although *Spirulina* enhanced the levels of GSt compared to D-GalN group. These observations indicated not only protective effect of *Spirulina* against toxicity but also enabled beneficial activity in terms of enhanced antioxidant activity of GST in liver identical to BHT.

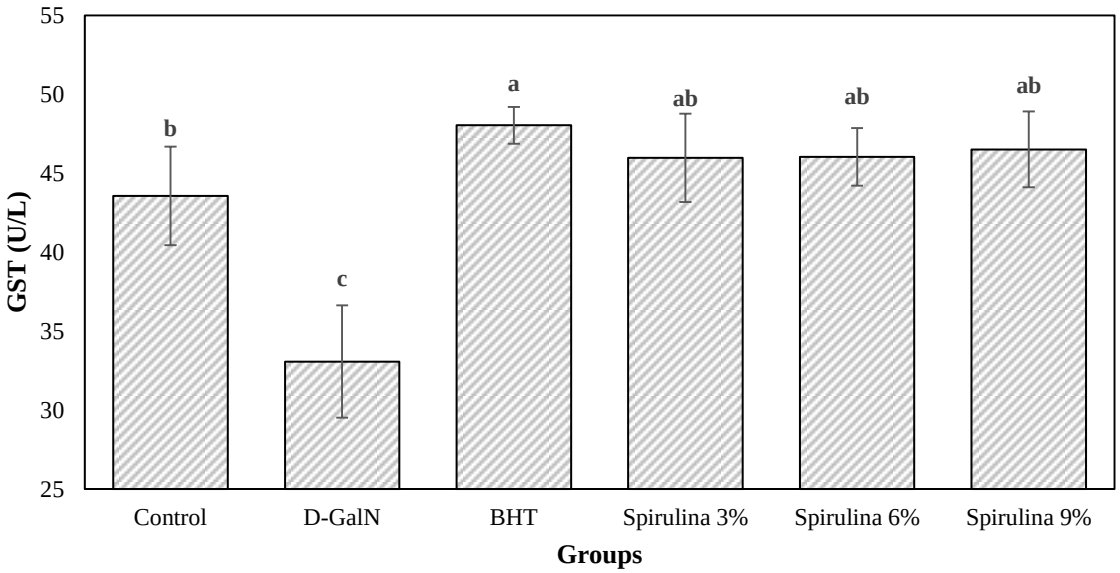

Fig 4.9. Effect of different concentrations of *Spirulina* on glutathione transferase (GST) activity in rats treated with D-GalN.

Bars with different letters are significantly different ($P \leq 0.05$).

4.4. Histologic Examination

The biochemical results were confirmed by the histological examination of liver tissues. The livers of animals treated with D-GalN showed fatty degeneration, necrosis, and apoptosis and the inflammatory cells were scattered around the congested blood vessels (**Fig 4.10A**). The livers of animals treated with D-GalN plus BHT showed marked improvement in hepatocyte architecture in different areas around the central veins and portal tracts (**Fig 4.10B**). The liver sections of animals treated with *Spirulina* 3% showed that most hepatocytes had a normal structure in different zones **(Fig 4.10C).** The liver sections of animals treated with *Spirulina* 6% and 9% showed more improvement in hepatocytes and had a normal structure in different zones than the liver sections of animals treated with *Spirulina* 3% (Fig 4.10D). These results strongly indicated the protective effect of *Spirulina* on the liver tissue against toxic substances

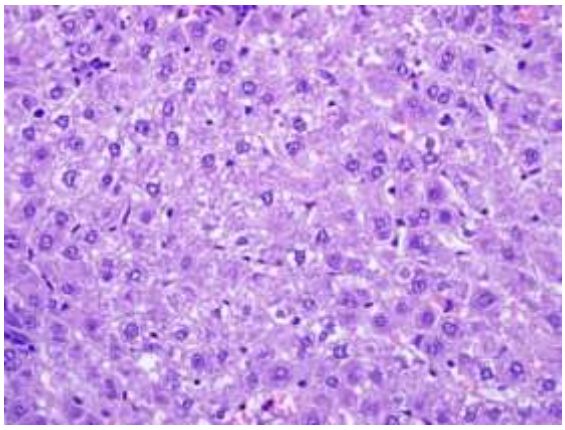

Fig 4.10. A (H&E X400)

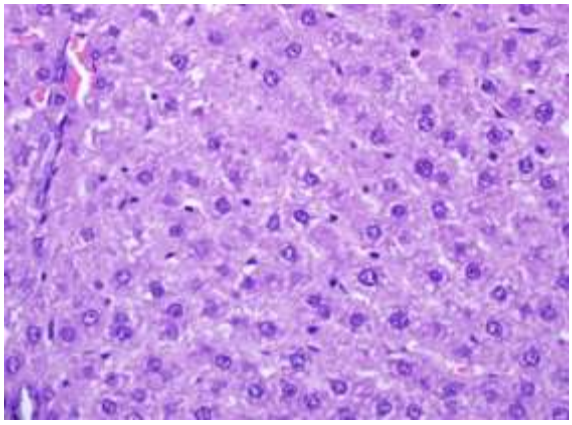

Fig 4.10. B (H&E X400)

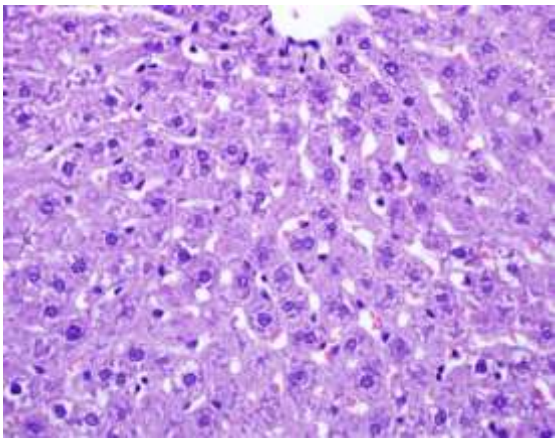

Fig 4.10. C (H&E X400)

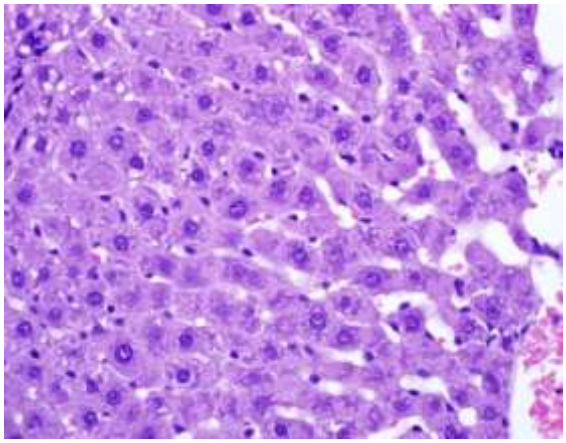

Fig 4.10. D (H&E X400)

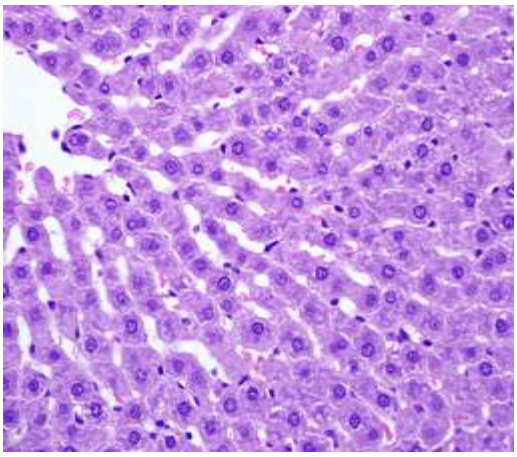

Fig 4.10. E (H&E X400)

Fig 4.10 (A-D). Photomicrographs of liver sections; (A) In rats treated with D-GIN showed vacuolar degeneration, and necrosis, (B) In rats treated with D-GalN plus BHT moderate improvement in hepatocyte architecture, (C) In rats treated with D-GIN plus *SPIRULINA*3%, most liver cells have normal structure, and (D) In rats treated with D-GIN and *SPIRULINA*6% and 9% most liver cells have normal structure.(E) In rats with no treatment. H&E- hematoxylin and eosin.

Chapter Five

Discussion

5. Chapter five: Discussion

Protection of foods and humans against oxidative damage caused by free radicals such as hydroxyl, peroxyl, and superoxide radicals has drawn great attention in recent times and consequently the potential antioxidant and anticancer properties of different natural products such as *Spirulina* is extensively explored (Namiki, 1990). Some studies indicated a positive correlation of an increased dietary intake of natural antioxidants with decreased coronary heart disease and cancer mortality and a longer life expectancy (Halliwell, 2007; Rios et al., 2009) and Oxidative and inflammatory pathways have been observed to play important roles in D-galactosamine (D-GalN)-induced hepatitis model (Keppler et al., 1968).

In the present study effect of (D-GalN) in the rats was studied by monitoring the different metabolic enzymes associated with liver injuries. No mortality was observed in the experimental animals during the study period. The results of this study revealed that D-GalN induced severe toxicological effects evidenced by the significant increases in (ALP), (AST) (GOT), (GPT),(GGT)and cholesterol levels along with significant decreases in (TP) level and albumin level in the rat administrated with D-GalN.

Whereas, treatment with BHT resulted in a significant decrease in the levels of ALP, AST, GOT, GPT, GGT and cholesterol compared to the levels recorded with the group treated with D-GalN. It was observed that BHT treated group, which was previously treated with D-GalN, could result in rapid reductions in the level of ALP (44%), AST (61%), GOT (58%), GPT (63%), GGT (53%) and cholesterol (64%). Similarly, there was a significant increase in the levels of TP level and albumin level compared to that group treated with D-GalN. These observations indicated that BHT treatment has reversed the effect of D-GalN and attributed protection against the toxic effect of D-GalN. In a similar manner the animals treated with D-GalN on subsequent treatment by supplementation with

Spirulina (6 or 9%) in the diets, also led to significant reversal in the levels of the same enzymes by suppression of negative effect indicating a beneficial dietary role in food.

Treatment with *Spirulina* diets at 9% level resulted in a significant decrease in the levels of ALP, AST, GOT, GPT, GGT and cholesterol compared to the levels recorded with that group treated with D-GalN. It was observed that *Spirulina* (9%) treated group could result in the rapid reduction in the levels of ALP (38%), AST (59%), GOT (50%), GPT (56%), GGT (45%) and cholesterol (40%). Further it was observed that *Spirulina* (9%) treated group, could record very good protective effect identical to the effect of BHT observed in the present study. Thus it was observed that the percent reduction in the levels of ALP was **38%** compared to **44%** with BHT (Difference of 6%), AST-**59%** compared to **61%** with BHT (Difference of 2%), GOT- **50**% compared to **58**% with BHT (Difference of 8%), GPT (**56**% compared to **63**% with BHT (Difference of 7%), GGT-**45**% compared to **53**% with BHT(Difference of 8%), and cholesterol **40**% compared to **64**% with BHT (Difference of 24%). It could be seen that the difference in terms of percent reductions were less than **10%** between BHT and *Spirulina* in all the cases except in the case of cholesterol where it was **24**%. Whereas in the case of total protein and albumin 9% *Spirulina* recorded almost identical effect as it was recorded with BHT. Thus the results of the present study strongly suggest that *Spirulina* could provide a significant protection against D-GalN-induced liver injuries.

Serum levels of these enzymes are very sensitive markers employed in the diagnosis of liver diseases. The increase of ALP, AST, GOT, GPT, GGT and cholesterol levels in the D-GalN-intoxicated rat indicated D-GalN-induced oxidative stress and lipid peroxidation. These results also indicate degenerative changes and hypofunction of the liver (Abdel-Wahhab et al., 2007). Further these results clearly showed that D-GalN has a harmful and stressful influence on the

hepatic tissue. Treatment with dietary *Spirulina* significantly decreased the serum AST, GOT and GPT levels compared to D-GalN only group, suggesting that *Spirulina* might scavenge reactive oxygen species generated from D-GalN intoxication and hence prevents hepatic cellular AST, GOT and GPT from leaking into the blood (Miyake, 1979). The reduction of liver enzyme parameter, ALP was significant and showed as a specific marker of liver injury due to toxic drugs, alcohol and virus. The protective effect of *Spirulina* might be possibly an outcome of stabilization of plasma membrane thereby preserving the structural integrity of cell as well as the repair of hepatic tissue damage caused by D-GalN.

It was reported earlier that the alcohol extract of *Spirulina* inhibited lipid peroxidation more significantly than the chemical antioxidants like α-tocopherol and β-carotene (Miranda et al., 1998). Whereas treatment with *Spirulina* led to improvement in the biochemical parameters studied, and in the histological conditions of the liver in D-GalN-treated rats. Previous studies have indicated that some active constituents of *Spirulina* have strong antioxidant activity and provoke a free radical scavenging enzyme system.

Spirulina is considered as a valuable additional food source of some macro- and micro-nutrients including high-quality protein, iron, γ-linolenic fatty acids, carotenoids, and vitamins (Weber et al., 2003). Moreover, the protective role of *Spirulina* may be attributed to the presence of β-carotene (Mazo et al., 2004), vitamins C and E (Seshadri et al., 1991), enzyme SOD, selenium, and brilliant-blue polypeptide pigment phycocyanin (Henrikson, 1989; Mathew et al., 1995). Luxia et al. (1996) reported that β-carotene in *Spirulina* may decrease cell damage, especially the damage to DNA molecules, thus playing a role in the repair of the regeneration process of damaged liver cells. β-Carotene in *Spirulina* may also scavenge free radicals generated by D-GalN. It has been established that carotenoids from microalgae exert their action against liver injury by lipid

peroxidation, either through decreased production of free radical derivatives or due to the antioxidant activity of the protective agent itself (Kuriakose and Kurup, 2010). Phycocyanin (major pigment of cyanobacteria) significantly reduced the hepatotoxicity caused by D-GalN which induces the formation of free radicals. The hepatoprotective effect of phycocyanin was therefore attributed to the inhibition of reaction involved in the formation of reactive metabolites and possibly due to its radical scavenging activity.

Human body has an effective mechanism to prevent and neutralize the free radical induced damage. This is accomplished by a set of endogenous antioxidant enzymes SOD, GPX, CAT, GSH and GST. SOD removes superoxide radical by converting it into H2O2 which is rapidly converted to water by CAT or GPX. GPX reduces lipid hydroperoxides to alcohols. Therefore, any alteration in the activity of these enzymes may result in a number of deleterious effects due to accumulation of superoxide radicals and hydrogen peroxide. Oxidative stress which through a series of events deregulates the cellular functions leading to various pathological conditions results when the balance between (ROS) production and antioxidant defenses is lost. Any compound, natural or synthetic, with antioxidant properties may contribute towards the partial or total alleviation of this type of damage. In the present study, decline in the level of antioxidant enzymes SOD, GPX, CAT, GSH and GST observed in D-GalN treated rat is a clear manifestation of excessive formation of free radicals and activation of lipid peroxidation system resulting in tissue damage. The significant increase ($P \leq 0.05$) in the concentration of these constituents in liver tissues of *Spirulina* and BHT treated animals indicate antioxidant effect of *Spirulina*. It was reported that *Dunaliellasalina*, a green marine algae was observed to have the ability to protect against oxidative stress, in animal models under in-vivo conditions (Kuriakose and Kurup, 2010).

The relation between the hepatic tissue damage and elevation of the relevant serum enzymes was well documented by(Dhu et al.2004). According to Matsuo et al., (1989) the observed increase in the activities of serum AST of D-GalN intoxicated rats is likely due to lipid peroxidation of biomembranes which cause leakage of cellular components. The present observations on histopathological alterations in the liver induced by D-GalN concur with the earlier observations made by early investigators. Further these results are also in agreement with Lu et al. (2010), who mentioned that D-GalN intoxication produced significant hepatic damage as evidenced by increase in the leakage of AST. The increase in plasma level of AST may be perhaps due to increased synthesis of AST and amplified biliary pressure (Kumar et al., 2005).

D-GalN led to a significant decrease in plasma protein level which could happen due to inhibition of amino acid transporters (Brookes and Kristt, 1989) or RNA synthesis (Sarafian and Verity, 1983).

In this present study, cholesterol concentrations of different *Spirulina* groups showed a significant reduction as compared with D-GalN group. Colla et al. (2008) had reported the hypocholesterolemic effect of *Spirulina* in rabbits fed on diet enriched with cholesterol. It was observed that *Spirulina* decreased plasma cholesterol level suggesting a protective effect on the cardiovascular system (Ray, 1991). The hypo-triglyceridemic effect of *Spirulina* may be due to its effect on increase in the activity of lipase (Iwata et al., 1990). The presence of antioxidant compounds like phycocyanin and β-carotene, γ-linolenic acid and sulfated polysaccharide in *Spirulina* could have led to the decrease in plasma lipids levels. Seo et al. (2004) reported that β-carotene reduced the elevation of cholesterol and triglycerides of diabetic rats and Kim et al. (2004) found that feeding of rats with γ-linolenic acid rich oil lowers plasma triacylglycerol and inhibited hepatic fatty acid synthesis which may result in a hypolipidemic effect. Nagaoka et al. (2005)

suggested that phycocyanin caused hypocholesterolemic activity in rats. They hypothesized that phycocyanin binds to bile acids in the jejunum, and this binding affects the micellar solubility of cholesterol and then suppresses cholesterol absorption. Whereas, Godard et al., (2009) reported that both sulfated polysaccharides and γ-linolenic acid showed hypolipidemic effect

The results obtained in this study revealed that the *Spirulina* with concentration of 6% and 9% has nearly the same effect as BHT. The protective effects of BHT may be attributed to its antioxidant activity due to its phenolic antioxidant active substances. Conversely, the protective effects of *Spirulina* against D-GalN-induced liver toxicity may be attributed to its antioxidant and free radical scavenging activities due to its higher contents of proteins, lipids, and carbohydrates, elements such as zinc, magnesium, manganese, selenium, and some vitamins including β-carotene, riboflavin, cyanocobalamin, α-tocopherol, and α-linoleic acid. None of these constituents have been reported in literature to be harmful for human. Krishnakumari et al. (1981) reported that *S.platensis* when in pure form and administered to rats orally up to the dosage of 800 mg/kg of body weight, did not exert any toxic action, and the long-term dietary supplementation of *Spirulina*, up to 5%, may be consumed without evident toxic side-effects (Yang et al., 2011). Becker and Venkataraman (1986) demonstrated that *Spirulina* represents an unobjectionable source of unconventional protein of good quality and short-term feeding tests with rats that did not reveal any negative symptoms which would impair the utilization of *Spirulina* as food or feed. In the present study, *Spirulina* did not cause any reduction in body weight gain of rats and no deaths or clinical signs of toxicity. These results are in consistent with the report of Salazar et al. (1996).

The results obtained from the histological examination confirmed the other obtained results. The treatment with D-GalN caused fatty degeneration, necrosis,

and apoptosis and the inflammatory cells were scattered around the congested blood vessels. However, the application of BHT improved the hepatocyte architecture in different areas around the central veins and portal tracts. Similarly, Spirulina application resulted in improvement in hepatocytes and showed normal structure in different zones of liver compared to group B, especially at the concentration of 6% and 9%. The results obtained in this study clearly approved the antioxidant and hepatoprotective effects of Spirulina addition to the diet. They also provided evidences that Spirulina is safe to be used for human as a supplementary diet .

Overall assessment of the results obtained in the present study very clearly indicated the positive effect of *Spirulina*, when used as diet, on the safety and protection of liver from injuries caused by toxicants. Hence it can be very well advocated as an ideal diet for protection against any liver injuries.

Recommendations

Liver suffers from many diseases during the life period. Some dietary supplements protect the liver against these diseases. *Spirulina* had shown to be an effective hepatoprotective dietary supplement. In the present investigation, *Spirulina platensis* greatly alleviated the harmful effects caused by injection with D-GalN. It showed a great antioxidant and hepatoprotective effects through:

1. Increasing the activity of ALP, AST, GOT, GPT, GGT and decreasing the level of cholesterol.
2. Increasing levels of total protein and albumin.
3. Enhancing the antioxidant activity of SOD, GPX, CAT, GSH and GST.
4. Improving the hepatocyte architecture.

These beneficial effects of *Spirulina* were generally accelerated via increasing the concentration of *Spirulina* added to the diet. This study concludes that adding *Spirulina* to the dietary system could enhance liver functions through protect it against injuries induced by oxidative substances such as D-GalN.

Based on the findings of the current study, the researcher recommends the followings:

1. Increase the awareness in Saud Arabia, about the beneficial effects of using *Spirulina platensis* as a dietary supplement.
2. Introducing *Spirulina* as an antioxidant and hepatoprotective factor against liver injuries induced by different chemicals, particularly D-GalN.
3. Aware people about the concept that '*Spirulina* is safe" for human use, without any other side effects.
4. Further studies are needed to deeply examine the mechanisms of *Spirulina* positive effects on liver and the overall health.
5. *Spirulina* could have other positive effects, such as being anti-cancer factor, which should be examined and investigated more.

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apoptosis، كما انتشرت الخلايا الالتهابية حول الأوعية الدموية المحتقنة. ومع ذلك، فقد أدت إضافة مادة BHT إلى تحسن البناء التركيبي لأنسجة الكبد في العديد من المناطق حول الأوعية المركزية. وبالمثل، فقد أدت إضافة السبيرولينا إلى تحسن في البناء التركيبي للكبد وأظهرت الأنسجة بشكل طبيعي في مناطق كثيرة من الكبد مقارنة بالمجموعة (ب) وبصفة خاصة عند استخدام التركيزين ٦% و٩%. وتبرهن النتائج المتحصل عليه في هذه الدراسة بشكل واضح على التأثيرات المضادة للأكسدة والوقائية للكبد للسبيرولينا عند إضافتها للنظام الغذائي. كما أن تلك النتائج توفر دليل قوي على كون استخدام السبيرولينا كمكمل غذائي هو أمر آمن ولا ضرر منه.

في المجموعات التي أضيف مستخلص السبيرولينا لنظامها الغذائي مقارنة بالمجموعة (ب). ولقد أدى زيادة تركيز السبيرولينا المضافة للنظام الغذائي، إلى المزيد من النقص في تركيز هذه الإنزيمات و مستوى الكوليسترول سواء في مصل الدم أو أنسجة الكبد. ولقد كان تركيزهذه الإنزيمات ومستوى الكوليسترول متماثل تقريباً في المجموعتين اللتين تلقتا السبيرولينا بتركيز ٩% أو مادة BHT، وهو ما يوفر دليل قوي على التأثيرات الوقائية للسبيرولينا على الكبد. وعلاوة على ذلك، فإن إضافة السبيرولينا أدت إلى زيادة مستويات البروتين الكلي والألبيومين مقارنة بالمجموعة (ب). وأظهرت السبيرولينا تأثيراً إيجابياً على النشاط المضاد للأكسدة للإنزيمات التي تمت دراستها في هذه التجربة. فقد ادى الحقن بمادة D-GalN إلى نقص كبير في نشاط إنزيمات SOD, GPX, CAT, GSH, GST. إلا أن إضافة مادة BHT للنظام الغذائي حسن من نشاط هذه الإنزيمات عند المقارنة بالمجموعة الضابطة (أ) والتي لم تتلق أي معاملة أو المجموعة (ب) التي تم حقنها بمادة D-GalN فقط. كما أدى التركيز الأقل من السبيرولينا (٣%) إلى تحسن طفيف في نشاط تلك الإنزيمات عند المقارنة بالمجموعتين (أ) و(ب). وكلما زاد تركيز السبيرولينا المضاف للنظام الغذائي، كلما زاد نشاط هذه الإنزيمات في مصل الدم وأنسجة الكبد. فقد أظهرت المجموعة التي تلقت السبيرولينا بتركيز ٩% (المجموعة و) قيم لنشاط هذه الإنزيمات مماثلة تقريباً للقيم التي أظهرتها المجموعة التي تلقت مادة BHT (المجموعة ج). فكان نشاط إنزيم SOD في المجموعة (و) هو ٠.٢٨ وحدة (حوالي ٢.١٥ مرة أكثر من المجموعة (ب) "١٣ وحدة") وكان حوالي ٠.٣٢ وحدة في المجموعة (ج) (حوالي ٢.٥ مرة أكثر من المجموعة ب). وبالمثل أدى تركيز ٩% من السبيرولينا إلى نشاط إنزيم GPX بمعدل ٣٥ وحدة والذي كان قريباً جداً للقيمة التي وجدت عند إضافة مادة BHT (٣٧ وحدة)، وقد كان ذلك يعادل ٣.٩ أضعاف للمجموعة ب (٩ وحدات)، ويعادل ٢.٢ ضعفاً للمجموعة أ (١٦ وحدة). وفي نفس الوقت، كان نشاط إنزيم CAT يعادل ٠.٨ وحدة وتحسن بإضافة مادة BHT أو السبيرولينا بتركيز ٩% ليصبح ٢.٦ وحدة و٢.٢ وحدة، على التوالي. كما تحسن أيضاً نشاط إنزيم GSH من ٧ وحدات في المجموعة (ب) إلى ١٦.٥ وحدة في المجموعة (ج) وحوالي ١٤.٥ وحدة في المجموعتين (هـ) و(و) بدون أي فروقات ذات دلالة إحصائية بين هاتين المجموعتين. ولقد أدت كل التركيزات المضافة من السبيرولينا إلى تحسن نشاط إنزيم GST مقارنة بالمجموعة (ب) والتي كان نشاط الإنزيم فيها ٣٣ وحدة ليصبح ٤٧ وحدة وهو ما كان قريباً جداً لنشاط الإنزيم في المجموعة (ج) بقيمة ٤٨ وحدة. وقد أكدت النتائج المتحصل عليها من الفحص النسيجي للأنسجة الكبد النتائج السابق ذكرها. فقد أدى الحقن بمادة D-GalN إلى تدهور دهني fatty degeneration، وموت الخلايا necrosis، وموت الخلايا المبرمج

٥. المجموعة (هـ) تم إضافة المستخلص المائي السبيرولينا بنسبة ٦% إلى نظامها الغذائي.

٦. المجموعة (و) تم إضافة المستخلص المائي السبيرولينا بنسبة ٩% إلى نظامها الغذائي.

وتم تصويم الجرذان لمدة ٢٤ ساعة قبل بدء التجربة ومعاملاتها المختلفة. كما تم إتاحة الغذاء والماء بشكل مستمر للجرذان طوال فترة التجربة. وتم حقن كل المجموعات (ما عدا المجموعة أ) حقنة Intraperitoneal بمادة D-GalN بتركيز ٣٠٠مج/كجم من وزن الجسم خلال اليوم السابع من تاريخ بدء وبعد ٢٤ ساعة من الحقن بمادة D-GalN تم تخدير الجرذان عن طريق حقنهIntraperitoneal بمادة الكيتامين Ketamine بتركيز ١٠٠ مجم/ كجم من وزن الجسم و مادة الزايليزينXylazin بتركيز ١٦ مجم/ كجم من وزن الجسم ، ثم قتلها. تم جمع الدم بعد قتل الجرذان مباشرة، ثم تم فصل المصل serum وتخزينه في المجمد على درجة حرارة -٨٠ °م لحين إجراء التحليلات عليه. كما تم أخذ الكبد وحفظه في محلول فورمالين منظم ذو درجة حموضة pH ٧.٤ وذلك لاستخدامه في الفحص النسيجي (الهستولوجي). تم قياس مستوى البروتين الكلي والألبيومين وبعض نشاط الإنزيمات (سوبر أكسيد ديسميوتياز SOD، جلوتاثيون بيروكسيدياز GPX، كتالياز CAT، جلوتاثيون ترانسفيريياز GST، أسبارات أمينوترانسفيرياز AST، جاوتامات أوكسالوأسيتات ترانسأمينياز GOT، جلوتامات بيروفيت ترانسأمينياز GPT، وجلوتاثيون GSH) في مصل الدم. كما تم قياس نشاط ألكالاين فوسفاتياز ALP، وألفا-جلوتاميل ترانسفيرياز GGT في أنسجة الكبد. كما تم فحص أنسجة كبد الجرذان لقياس مدى الضرر المستحث من قبل مادة D-GalN.

أو ضح تحليل مسحوق السبيرولينا أن البروتين والكربوهيدرات (السكريات) هما المكونين الأساسيين لها حيث شكلا نسبة ٤٠.٧٧% و٣٥.٤٤% على التوالي. كما احتوى مسحوق السبيرولينا على نسب عالية من الحديد (٢٩٤.٢٦ جزء في المليون) والماغنسيوم (٥١.٧٨ جزء في المليون). بشكل عام، أدى الحقن بمادة D-GalN إلى تأثيرات سامة كبيرة تمثلت في الزيادة المضطردة لنسبة الإنزيمات ALP, AST, GOT, GPT, GGT وكذلك مستوى الكوليسترول. وعلى الجانب الآخر، أدى الحقن بهذه المادة إلى انخفاض معنوي ($P \leq 0.05$) في البروتين الكلي والألبومين. ولقد أدت إضافة مادة BHT إلى النظام الغذائي للجرذان إلى تخفيف الآثار الضارة لمادة D-GalN من خلال تقليل نسبة الإنزيمات ALP, AST, GOT, GPT, GGT ومستوى الكوليسترول، وكذلك زيادة مستوى البروتين الكلي والألبومين وذلك عند مقارنتها بالمجموعة (ب). كما أظهرت التركيزات المختلفة من مستخلص السبيرولينا المضافة للنظام الغذائي للجرذان تأثيرات إيجابية مماثلة لتأثيرات مادة BHT ضد التأثيرات السامة لمادة D-GalN. فقد كان نشاط الإنزيمات السابق ذكرها ومستوى الكوليسترول منخفضه

الملخص العربي

يعتبر الكبد واحداً من أهم أعضاء الجسم البشري بشكل عام، حيث أنه المسئول عن التخلص من السموم التي تدخل الجسم يومياً. كما أنه يساعد في المسارات و أيض الدهون. ونظراً لطبيعة وظائفه الحيوية، يتعرض الكبد إلى العديد من الممرضات والأمراض مثل التليف والالتهاب الفيروسي وما إلى ذلك. فتؤثر هذه الأمراض بشكل سلبي على وظائف الكبد في الجسم؛ وبالتالي تؤدي إلى العديد من المشكلات الصحية. ومن هنا كان هناك حاجة ماسة إلى علاجات ثابتة وآمنة ويمكن الاعتماد عليها من أجل التغلب على هذه المشكلات والأمراض، وذلك من دون التأثير سلباً على أي أعضاء أخرى في الجسد.

إن السبيرولينا هو واحد من مجموعة الـ Cyanobacteria والتي تمتلك قيمة غذائية كبيرة. وعلاوة على ذلك، فقد أشارت العديد من الدراسات إلى التأثيرات الإيجابية للسبيرولينا بما في ذلك تأثيراتها المضادة للأكسدة والمضادة للفيروسات وكذلك المضادة للسرطان في الخلايا. كما أنها تعمل على خفض مستوى البروتين الدهني منخفض الكثافة LDL Cholesterol ورفع نسبة البروتين الدهني عالي الكثافة HDL Cholesterol. والأهم من ذلك أن العديد من تلك الدراسات أشارت إلى كون استخدامها في النظام الغذائي آمناً بالنسبة للإنسان.

وبناء على تلك المعلومات المتوفرة، تهدف هذه الدراسة إلى دراسة التأثيرات الإيجابية للنوع *Spirulina platensis* (والذي تم جمعه من البيئة المحلية في المملكة العربية السعودية، واستزراعه في قسم النبات والأحياء الدقيقة بكلية العلوم، جامعة الملك سعود) في الوقاية من الأضرار التي تحدث للكبد نتيجة تناول مركب الدي-جالاكتوزأمين D-Galactosamine (D-GalN)، بالإضافة إلى تقييم مدى أمان استخدام السبيرولينا في النظام الغذائي للإنسان.

ومن أجل الوصول إلى هذه الأهداف تم استخدام السبيرولينا المزروعة لتجهيز مسحوق يمكن استخدامه في نظام غذائي. ومن ثم تم تحليل جزء من هذا المسحوق كيميائياً لمعرفة محتواه من العناصر الغذائية، وتم استخدام المستخلص المائي لهذا المسحوق في تنفيذ التجربة. تم استخدام ست وثلاثون ذكر جرذ Wister albino rats وتم تقسيمهم عشوائياً إلى ست مجموعات كل منها تتكون من ست٦ فئران:

١. المجموعة (أ) مثلت المجموعة الضابطة ولم تتلق أي معاملة.
٢. المجموعة (ب) تم حقنها بمادة D-GalN فقط.
٣. المجموعة (ج) تم إضافة مادة بتيوليتد هيدوكسي تولوين Butylated hydroxytoluene (BHT) بتركيز ٠.٥% إلى نظامها الغذائي.
٤. المجموعة (د) تم إضافة المستخلص المائي السبيرولينا بنسبة ٣% إلى نظامها الغذائي.

جامعة الملك سعود

كلية العلوم

قسم النبات والأحياء الدقيقة

استخدام السبيرولينا لتحسين وظائف الكبد: التأثيرات والأمان

قدمت هذه الرسالة استكمالاً لمتطلبات الحصول على درجة الدكتوراه في قسم النبات والأحياء الدقيقة، كلية العلوم

جامعة الملك سعود

إعداد الطالبة

وحيدة هزاع القحطاني

إشراف

المشرف الرئيس: أ.د. علي عبد الله الحميدان

المشرف المساعد: أ.د. عمار شركيس الركابي

١٤٣٦ – ١٤٣٧ هـ