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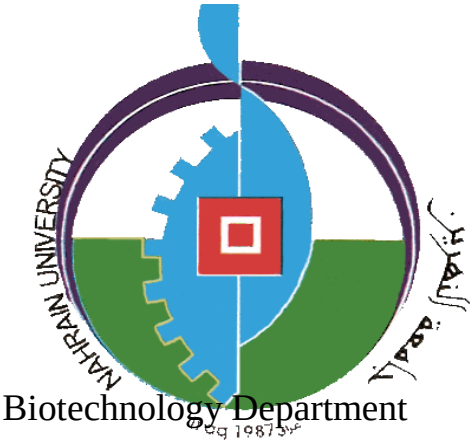

Biotechnology Department

Biochemical and Histopathollogical Study of *Moringa oleifera* Extract on the Fertility in Male Mice

A thesis

Submitted to the College of Science / Al- Nahrain University as a partial fulfillment of the requirements for the Degree of Master of Science in Biotechnology

By

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B.Sc. Biotechnology / College of Science / Al-Nahrain University (2016)

Supervised by

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Supervisor certification

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Dedication

To those who are the reason for my existence my father and mother

To the warm spring that floods my heart with love my uncle (*SHAMS)*, brothers, sisters, and daughter (*RIYAN*)

To memory of my Father

ENG. *BASHEER KHUDAER MOHAMED* 1962---2012

Acknowledgments

First of all things Praise to “Allah" who gave me the patient and strength to accomplish this work.

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I would gratefully like to express my gratitude and feelings of love to my mother, father, Uncle ***(Shams)***, brothers, sisters, my daughter ***(Riyan)*** and my best friends *Eslah Shaker, Atyaf adnan and Riyam Basim* for their encouragement, patience and the many sacrifices they presented during all stages of my study. To all these and other forgotten names who assist me in accomplishing this study, for all of them, I said “Thank you all”

Summary

This study was designed to find out the effect of *Moringa oleifera* leaf extracts on fertility of male albino mice. *M. oleifera* leaf extract was prepared by maceration of 50 gm of M. *oleifera* leaves with 70% methanol, then chemical detection of flavonoids, alkaloids, tannins, saponins, glycosides, terpenes and steroids were detected. Isolation of Flavonoid from these extracts. The use of thin layer chromatography technique helped in detection of Qurciten, Rutin and Luteoline of flavonoids extract; the presence of qurciten and rutin confirred by high performance liquid chromatography technique. Total flavonoid of *M. oleifera* leaf extracts was calculated by Spectrophotometric technique.

The 30 mice were divided equally into six groups, Group1:(negative control): mice treated with water, Group2 (positive control): mice treated with 0.36 mg/kg of Mesterolone (Proviron), Group3 (positive control): mice treated with 0.5 mg/kg of Ginseng drug (ginsavet) Group4: mice treated with 2.5 mg/ ml of extract (100mg/kg), Group 5: mice treated with 5 mg/ ml of extract (200 mg/kg), Group6: mice treated with 7.5 mg/ml of extract (300 mg/kg). The extract was administered orally for 15 days.

The effect of methanolic extract on the sperm including sperms concentration, percentage of viable sperms, percentage of morphologically abnormal sperms and an assay of serum testosterone were studied,.Then the histopathological sections of liver, kidney and testis were examined.

The results showed a significant increase ($p \leq 0.05$) in sperm concentration after 15 days of treatment with plant extract at doses 100

and 200mg/kg when compared with controls and with other group treatment at dose 300 mg/kg.

A significant increase ($p \leq 0.05$) in dead sperm was observed after treatment with plant extract at doses 300 mg/kg when compared with controls and with other treatments, (extract doses of 100 and 200mg/kg).

A significant increase ($p \leq 0.05$) Serum testosterone in mice treated with 100 and 200 mg/kg when compared with controls and with other group treated with plant extract at dose of 300 mg/kg. The pathological study of testis, Liver and kidney in controls and in mice treated with plant extract at doses 100 and 200 mg/kg show no significant changes, while at dose 300 mg/kg showing the presence of negative changes.

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List of abbreviations

Abbreviations	Full name
ELISA Enzyme	linked immunosorbent assay
FSH	Follicle Stimulating Hormone
g	gram
GnRH	Gonadotropin releasing hormone
HPLC	high performance liquid chromatography
kg	Kilogram
LH	Luteinizing hormone
mg	Milligram
M. oleifera	*Moringa oleifera*
nm	Nanometer
PBS	Phosphate buffered saline
RF	Retardation factor
ROS	Reactive oxygen species
rpm	rotation per minute
TLC	Thin Layer Chromatography
UV	Ultra violet
WHO	World Health Organization

Chapter One

Introduction and Literature Review

1. Introduction

There is a growing interest in alternative therapies and the therapeutic use of natural products, especially those derived from plants (Goldfrank *et al.*, 1982; Vulto and Smet, 1988; Mentz and Schenkel, 1989).

Infertility is an important issue for couples and male infertility disorders play a major role in approximately 50% of the cases. In the vast majority, male infertility is treatable and medical therapies are used to improve sperm production or as a preliminary therapy to boost production (Schiff *et al*., 2007). The testosterone estimate is useful in the management of male infertility (Zabul *et al*., 1994)

Medicinal plants and herbal medicine are one of the areas of investigation in which various drugs have been identified which affect fertility, both in a positive and a negative sense but some of which have side effects that are undesirable (Ahmad *et al.*, 1998). They are tested for the possible fertility regulatory functions in males or females (Bhatia *et al.*, 2010).

Herbal therapy can alleviate male infertility, irrespective of the etiology of such diseases (Anthony *et al.*, 2006). Some medicinal plants are extensively used as aphrodisiac to relieve sexual dysfunction or as fertility enhancing agents. They provide a boost of nutritional value thereby improving sexual performance and libido (Yakubu *et al*, 2007; Sumalatha *et al*., 2010).

M. oleifera is one of the best plants with a wide range of medicinal application (Vinodini, 2014). The antioxidants present in the leaves of the plant, acting in concert with the antioxidant system present in the epididymis further preserved and enhanced the process of spermatogenesis. Numerous studies now point to the elevation of a variety of detoxication and antioxidant enzymes as a result of treatment

with Moringa or with phytochemicals isolated from Moringa (Kumar, 2003). The main ingredient responsible for the *M. oleifera* ability to boost libido is the presence of flavonoids in this plant extract which has been implicated to have a role in altering androgen levels and may also be responsible for the enhanced male sexual behavior. (Padashetty and Mishra, 2007). Alkaloids can also cause facilitation of sexual behavior (Adimoelja, 2000).

1.1. This project aims to

1- Study histopathological effect of active compounds of *M. oleifera* extract on fertility in male mice.

2- Quantitative and qualitative estimation of the total flavonoids in dried leaves of *M. oleifera*

2. Literature Review

2.1. *Moringa Oleifera*

M. oleifera is one of the known species of family Moringaceae (Nadkarni, 1976; Ramachandran *et al*., 1980). It is a tropical plant known to be of medicinal values (Fahey, 2005; Paliwal *et al*., 2011). Different parts of the plant have been found to possess some medicinal properties such as treatment of ascites, rheumatism (Anwar *et al*., 2007), venomous bites (Mishra *et al*., 2009), enhancing cardiac function (Limaye *et al*., 1995), inflammation (Ezeamuzie *et al*., 1996), liver disease (Rao and Misra, 1998), cancer, hematological, hepatic and renal function (Mazumder *et al*., 1999).anti-hypertensive (flower and seed), hypolipidemic (flower), anti-inflammatory (root and flower), and anti ulcer (bark) (Paliwal *et al*., 2011; Anwar *et al*., 2007). The plant *M. oleifera* is widely used as food product (Krishnamurthy *et al*., 2015).

2.1.1. Common Names and Taxonomy

As a member of the Moringaceae family, *M. oleifera*. Also known as Horseradish based on the taste of leaves, or Drumstick tree based on the appearance of its immature seed pods. It is one of the most useful trees that found throughout the tropics of the world (Jahn, 1988). While less frequently referred to as 'The Tree of Life' or 'Miracle Tree' due to its economical importance and versatility (Abe, 2013). Or Ben Oil Tree (Luqman, 2012). It is one of 14 species in the genus Moringa, which is the only genus in the family Moringaceae, names derived from the Malayalam word 'muringo' from southern India (Icfre, 1995). Taxonomically, the plant is classified (Olson, 1999) as the following:

Kingdom: Plantae

Subkingdom : Tracheobionta

Super division : Spermatophyta

Division : Magnoliophyta

Class : Eudicots

Subclass : Rosids

Order : Brassicales

Family : Moringaceae

Genus : Moringa

Species: Oleifera

2.1.2 Plant Distribution

M. oleifera, native of the western and sub-Himalayan tracts, India, Pakistan, Asia, Africa and Arabia (Somali *et al*., 1984; Mughal *et al*., 1999) distributed in the Philippines, Cambodia, Central America, North and South America and the Caribbean Islands (Morton, 1991).

2.1.3. Plant Description

M. oleifera as shown in figure (1-1) is a small or medium sized tree ranging in height from 5 to 12m with an open, umbrella-shaped crown, straight trunk and with thick, soft, corky, deeply fissured bark. The tree produces a tuberous tap root. The evergreen or deciduous leaves (depending on climate) have leaflets 1 to 2 cm in diameter in. The flowers are white or cream colored. The fruits (pods) are initially light green, slim, eventually becoming dark green, firm and long, depending on the variety. Fully mature, dried seeds are round or triangular, the kernel being surrounded by a lightly wooded shell with three papery wings (Folkard, 2004). The leaves of *M. oleifera* are said to have a spicy taste (hot, radish, and pungent) which begets the nickname of this tree being Horse Radish Tree (Doerr, 2009).

Figure (1-1): *M. oleifera* tree (Hyde, 2015)

2.1.4. Chemical Constituent

M. oleifera leaves are an important source of flavonoids compounds (Yang, 2008) such as Myricetin, Quercetin and kaempferol (Prakash, 2007). It is also have the calcium equivalent to 4 times that of milk, the vitamin C content is 7 times that of oranges, while it's potassium is 3 times that of bananas, 3 times the iron of spinach, 4 times the amount of vitamin A in carrots (Kamal, 2008). It also contains tannins, alkaloids, saponins, reducing sugars, carbohydrates, eugenol and glycosides (Sato *et al.*, 2004; Cushine and Lamb, 2009). The leaves, especially young shoots are widely utilized as a good source of protein, vitamin E, β-carotene, amino acids and different polyphenolics (Luqman, 2012). Therefore, it is used as an alternative

source of nutritional supplements and growth promoters in different countries (Anwar, 2007). The edible Moringa leaves contain essential provitamins, including ascorbic acid, carotenoids (Lako *et al.*, 2007).

2.1.5. Uses in Folke Medicine

The flowers, leaves, and roots are used in folk remedies for tumors, the seed for abdominal tumors .The root for dropsy. Root juice is applied externally as counter-irritant. Leaves applied to sores, rubbed on the temples for headaches. Bark, leaves and roots are acrid and pungent, and are taken to promote digestion. Oil is somewhat dangerous if taken internally, but is applied externally for skin diseases. Roots are bitter, act as a tonic to the body and lungs, and are emmenagogue, expectorant, mild diuretic and stimulant in paralytic afflictions, epilepsy and hysteria (Hartwell, 1971).

2.2. The Reproductive System of male Mice

The male mouse reproductive tract is comprised of four main sections: the testes are responsible for producing sperm, the epididymis is where spermatozoa undergo maturation, the penis at which where urine and semen are released, and the ductus deferens at which the tube in which semen is transported to the penis figure (1-2) (Griffin, 2004).

In addition there are several accessory glands located along the male reproductive tract. Such as the prostate gland and the seminal vesicles that store and produce a clear, slightly alkaline fluid, that combines with sperm to form semen. The alkalinity aids in neutralizing the acidic fluids of the vaginal tract, therefore increasing the opportunities of sperm surviving. The prostate also helps regulate the flow of urine from the bladder to the penis. The mouse also has a preputial gland that is

responsible for releasing pheromones, which are chemicals that are used to attract a female mouse for mating (Cook, 1965).

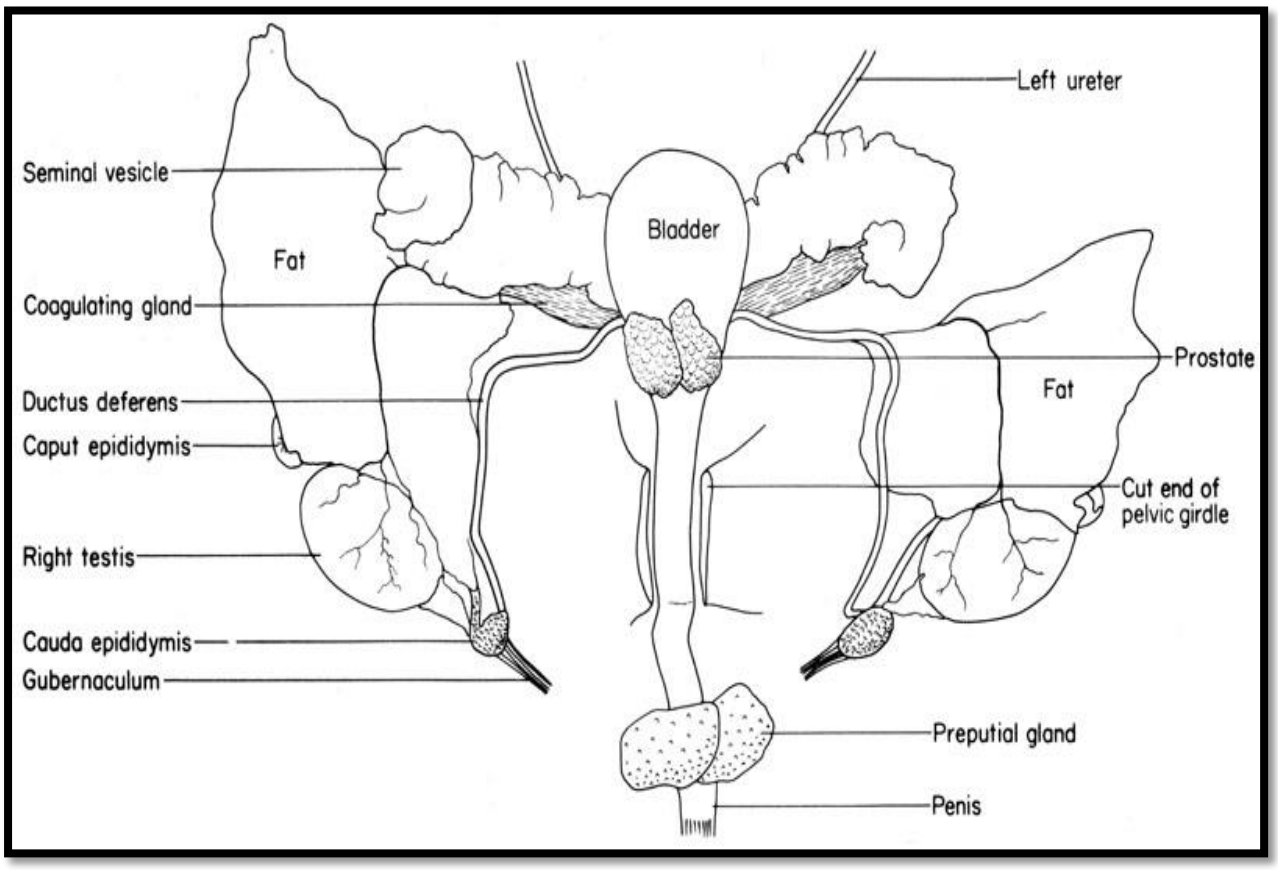


Figure (1-2): Overview of the male mouse reproductive tract (cook, 1965).

2.2.1. Spermatogenesis

Spermatogenesis is a multi-step process of germ cell development which occurs within the seminiferous tubules of the testes that determines male fertility.

The initial cells in this process are called spermatogonia, which yield primary spermatocytes by mitosis. The primary spermatocyte divides meiotically (Meiosis I) into two secondary spermatocytes; each secondary spermatocyte divides into two spermatids by Meiosis II. These develop into mature spermatozoa (sperm cells) Figure (1-3). Thus, the

primary spermatocyte gives rise to two cells that is the secondary spermatocytes, and these by their subdivision produce four spermatozoa (Ivell, 2007).

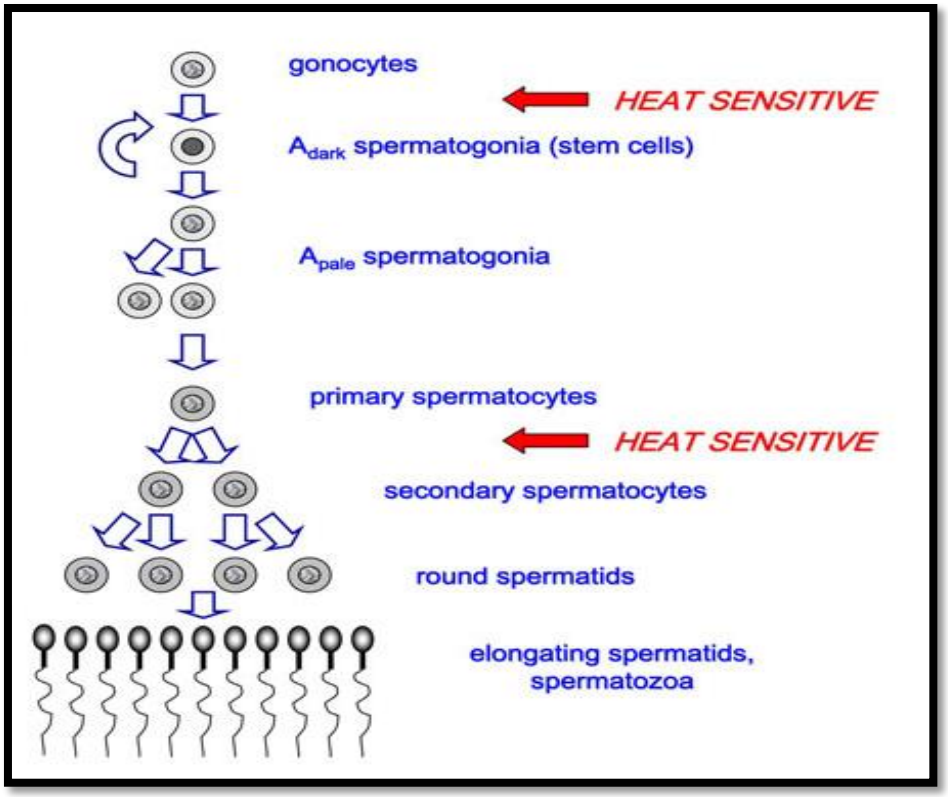


Figure (1-3): Diagram to illustrate spermatogenesis (Ivell, 2007).

2.2.2. Testosterone

Testosterone, a steroid hormone from the androgen group, is naturally produced in the body and is secreted from the Leydig cells of the testes in men (Mooradian, 1987) and from the theca cells of the ovaries in women, the zone reticularis of the adrenal cortex, and the skin In men (Zouboulis, 2004). Figure (1-4) shown the biosynthesis of testosterone from cholesterol (Orth, 2012)

Testosterone is one of the most important male hormone plays role in the development of reproductive tissues and promotion of

secondary sex characteristics such as muscle growth and strength, bone mass, and growth of body hair (Brooks, 1975). Testosterone production is significantly higher in men than in women, the hormone is important in the health and well-being (Laaksonen *et al*., 2004 and Shores, 2006).

Testosterone is generally measured as either free (unbound) or total (unbound and chemically bound) (Matsumoto, 2002).

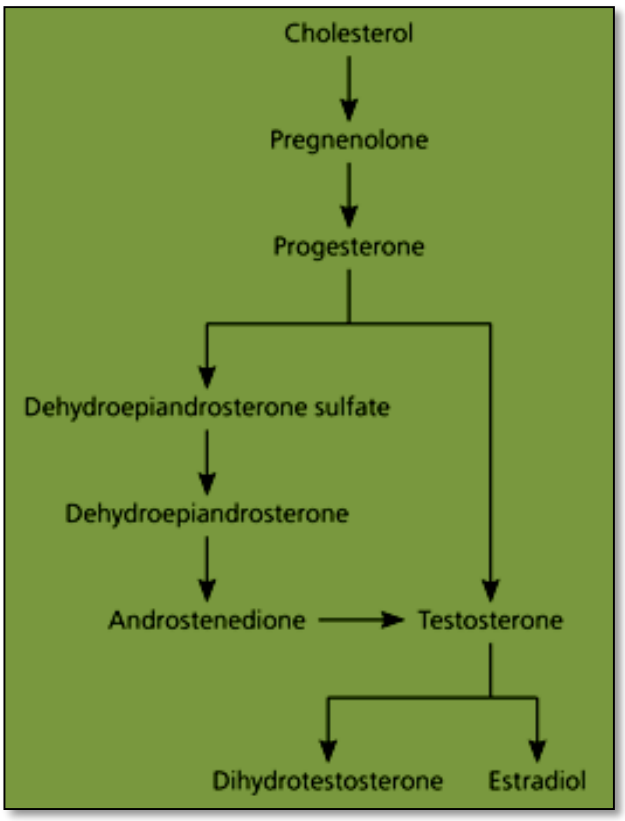


Figure (1-4): Biosynthesis of Testosterone from cholesterol (Orth, 2012)

2.2.3. Regulation of testosterone

The hypothalamus, the pituitary, and the testes form an integrated system that is responsible for the adequate secretion of male hormones and normal spermatogenesis. Figure (1-5) shown the regulation of male androgens (Layman, 2007). The endocrine components of the male reproductive system are integrated in a classic endocrine feedback loop. The testes require stimulation by the pituitary gonadotropins, luteinizing

hormone (LH) and follicle-stimulating hormone (FSH), which are secreted in response to hypothalamic gonadotropin releasing hormone (GnRH). The effect of LH and FSH on germ cell development is mediated by the androgen and FSH receptors that are present on Leydig and Sertoli cells, respectively. Whereas FSH acts directly on the germinal epithelium, LH stimulates the secretion of testosterone by Leydig cells. Testosterone stimulates sperm production and virilization, in addition to providing feedback to the hypothalamus and pituitary to regulate GnRH secretion. FSH stimulates Sertoli cells to support spermatogenesis and secrete inhibit B, which negatively regulates FSH secretion. The GnRH pulse generator is the main regulator of puberty and the production of GnRH starts early in fetal life. As a result, gonadotropin levels change drastically during fetal development, childhood (Layman, 2007)

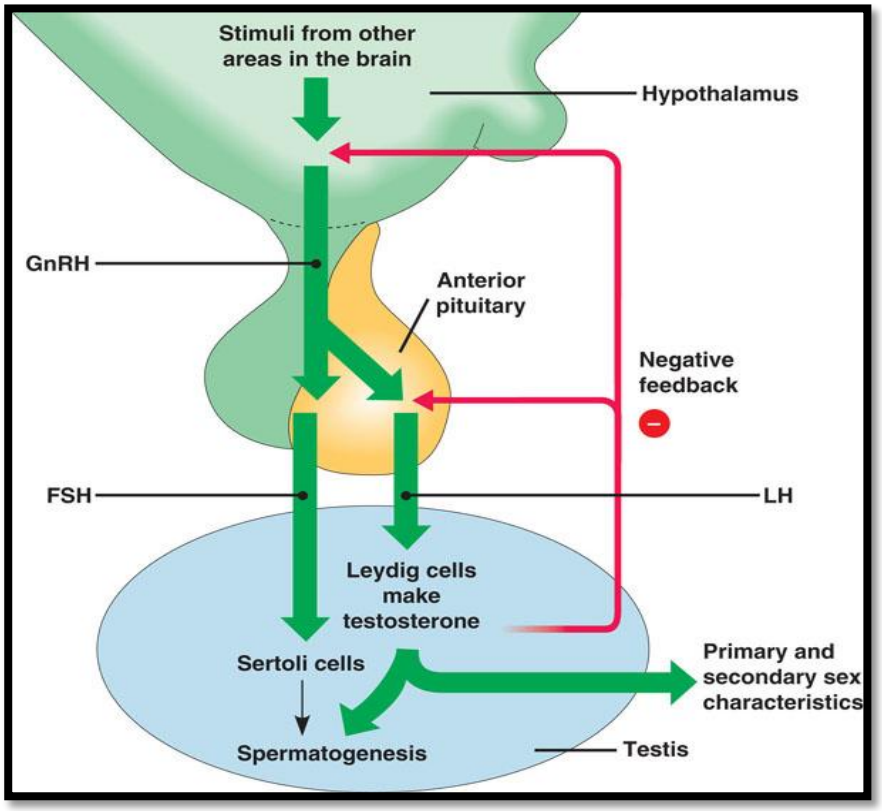


Figure (1-5): Regulation of male androgens (Sex hormones) (Layman, 2007)

2.3. Male infertility

Infertility is defined as the inability of a couple to conceive after at least 12 months of unprotected sexual intercourse. There are two types of Infertility, the first is the primary infertility that affecting about 15% of a couples and it is occur after the first pregnancy and the second type is the secondary infertility that affecting about 52% of a couples and it is occur directly after the married (Larsen, 2000). Globally, it has been estimated that approximately 20-25% of the infertility problems are attributable to the male partner (World Health Organization, 1997). Failure of pituitary gland to secret FSH and LH will result in disruption of testicular function leading to infertility. Testosterone, estradiol and inhibin control the secretion of gonadotrophins through feedback mechanism (Weinbauer, 1995). Semen analysis and hormone evaluation are essential parameters for fertility (Guyton, 1981). Oxygen is essential for animal life, Cells living under aerobic conditions constantly face the oxygen (O2) paradox – O2 is required to support life, but its metabolites such as free radicals or reactive oxygen species (ROS) can modify cell functions, endanger cell survival, or both. Male infertility may caused by oxidative stress that is the state in which an oxidant-generation system overcomes an antioxidant defense system, a process that is involved in many diseases, figure (1- 6) shown the factors contributing to oxidative stress-induced male infertility (Agarwal, 2002) excessive production of (ROS) can damage sperm, and ROS have been extensively studied as one of the mechanisms of infertility. Superoxide anion, hydroxyl radical and hydrogen peroxide are some of the major ROS present in seminal plasma (Agarwal, 2002). Reproductive ability in the male contains the production of semen containing normal spermatozoa with suitable concentration, together with the desire and ability to mate (Oyeyem, 2008). *M. oleifera* can enhance sexual activity in male mice and may have an opportunity to exert its effect depending on certain conditions, in various doses and

times (Watcho *et al*., 2001; Watcho *et al*., 2004) and in sexual condition of male animals (Gauthaman *et al*., 2002).

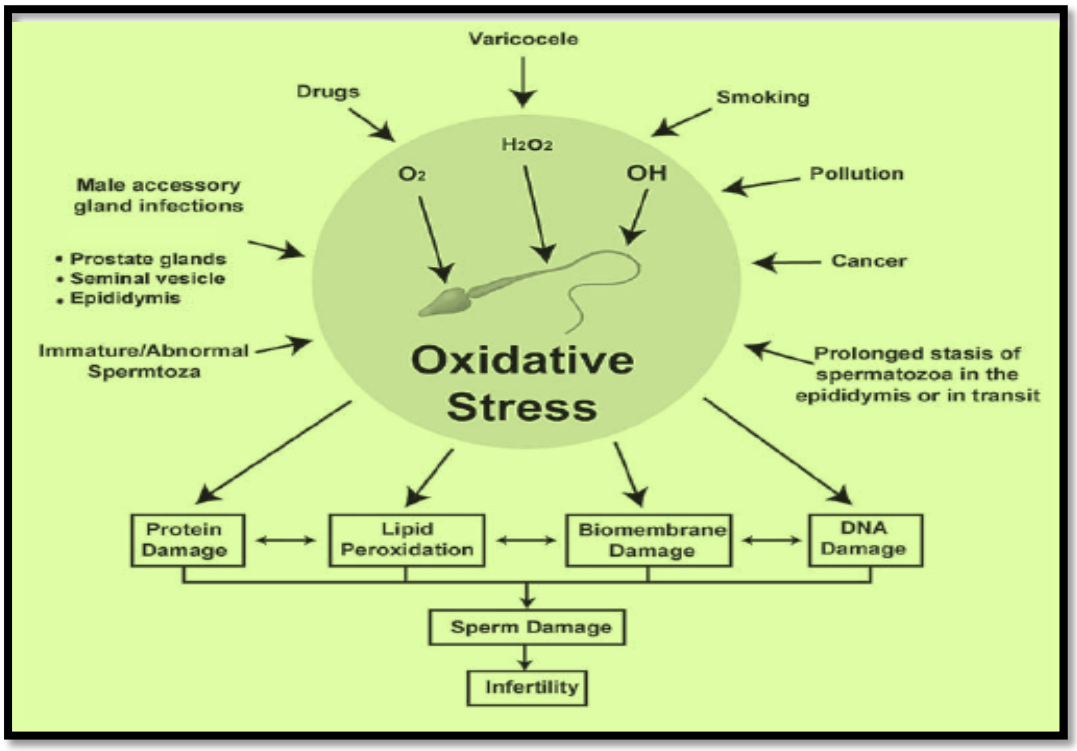


Figure (1-6): Factors contributing to oxidative stress-induced male infertility (Agarwal, 2002).

Chapter Two

Materials and Methods

2. Materials

2.1. Equipments and instruments

The following is the equipments and instruments used in the present study, listed with their Companies and origins.

Table (2-1): The equipments and instruments used in this study

Equipment	Company	Origin
Compound light microscope	Motic	Japan
Digital camera	Mercury	China
Disposable Petri-dishes	Sterilin	England
Disposable syringes	CMP	Turkey
Distilled water	GLF	Germany
Electric Balance	Sartorius	Germany
Electric blender	Sartorius	Germany
Electrical Oven	Memmert	Germany
Filter papers	Halzfeld	Germany
Gauzes	Halzfeld	Germany
Pyrex flasks different sizes	Terumo	Japan
Glass slides and cover slips	Sail Brand	China
HPLC Apparatus	waters/487	USA
Hemocytometer	Neubauer	Germany

Incubator	Memmert	Germany
Lyophilizer	Fisher	U.K
Micropipette (Different sizes)	Gilson	France
Micro centrifuge	Beckman	England
Oven	Osaw	India
Reflex Apparatus	Electrothermal	England
Refrigerator	Ariston	Japan
Rotary evaporator	Buchi	Switzerland.
Spectrophotometer	LABOMED.INc.	Japan
TLC Paper(silica gel Gf254 aluminum plates)	Barcelona	Spain
U.V. Light	Shighi	England

2.2. Chemicals and Reagents

The following chemicals and reagents used in this study are classified according to the manufactured company.

Table (2-2): The chemicals and reagents used in the present study

Chemical Material	**Company**	**Origin**
Acetic anhydride	BDH	England

AlCl3	Fluka	Switzerland
Chloroform.	BDH	England
Eosins stain	BDH	England
Ethanol	BDH	England
Ethyl acetate	BDH	England
Formalin	Analar	England
Formic acid	BDH	England
Ferric chloride (FeCl2)	Fluka	Switzerland
Ginsavit	Julphar	United Arab Emirates
Glacial acetic acid	BDH	England
Hydrochloric acid (HCl)	BDH	England
Kaempferol	Xian	China
Lead acetate	Leo	France
Methanol absolute	BDH	England

mercuric chloride (HgCl2)	Fluka	Switzerland
n-hexane	BDH	England
Petroleum ether	Fluka	Switzerland
Potassium iodide(KI)	Fluka	Switzerland
Potassium chloride(KCL)	Fluka	Switzerland
Potassium hydroxide(KOH)	BDH	England
Proviron	Bayer	Germany
Quercetin	Xian	China
Rutin	Xian	China
Sulphric acid	Analar	England
Sodium hydroxide (NaOH)	BDH	England
Sodium nitrite	Fluka	Switzerland
Xylene	BDH	England

2.3. Kit

The following kit used in this study:

Table (2-3): Testosterone kit.

Kit	Company	Origin
Testosterone Enzyme Immunoassay test kit	ICN	USA

2.4. Solutions preparation.

2.4.1. Phosphate buffer saline (PBS)

One tablet of PBS dissolved in 100 ml of distilled water.

2.4.2. Ferric chloride solution (1%)

The solution was prepared by dissolving 1g of ferric chloride in 100 ml of distilled water (Stahl, 1969).

2.4.3. Haematoxylin stain and Eosin stain

These stains solutions were prepared in the Department of pathology at Educational Laboratories of Baghdad Medical City. They were used to stain the histological sections of mice organs.

2.4.4. Potassium hydroxide solution:

Prepared by dissolving (50g) of potassium hydroxide in (100ml) of distilled water (Jaffer *et al*., 1983).

2.4.5. Mayer's reagent

Two solutions were prepared; the first one was prepared by dissolving 1.58 grams of mercuric chloride (HgCl2) in 60 ml of distilled water, while the second solution was prepared by dissolving 5 grams of potassium iodide (KI) in 10 ml of distilled water. Both solutions were mixed and the volume was made up to 100 ml with distilled water (Smolensk *et al.*, 1972).

2.4.6. Mesterolone (Proviron) dose preparation

A daily therapeutic dose of 25 mg of oral proviron tablet was used in this study. However, we calculated the human dose based on the physiological calculation for a 70 kg man, such that Proviron tablet was dissolved in distilled water to prepare a dose of 0.36 mg/kg administered to the animal (Shittu, 2009).

2.4.7. Ginseng drug (Ginsavit) dose preparation

A daily therapeutic dose of 40 mg of oral ginsavit capsule was used in this study. However, we calculated the human dose based on the physiological calculation for a 70 kg man, such that ginsavit capsule was dissolved in distilled water to prepare a dose of 0.5 mg/kg administered to the animal (Shittu, 2009).

2.5. Methods

Scheme (2-1) shown the Main steps of the research plan

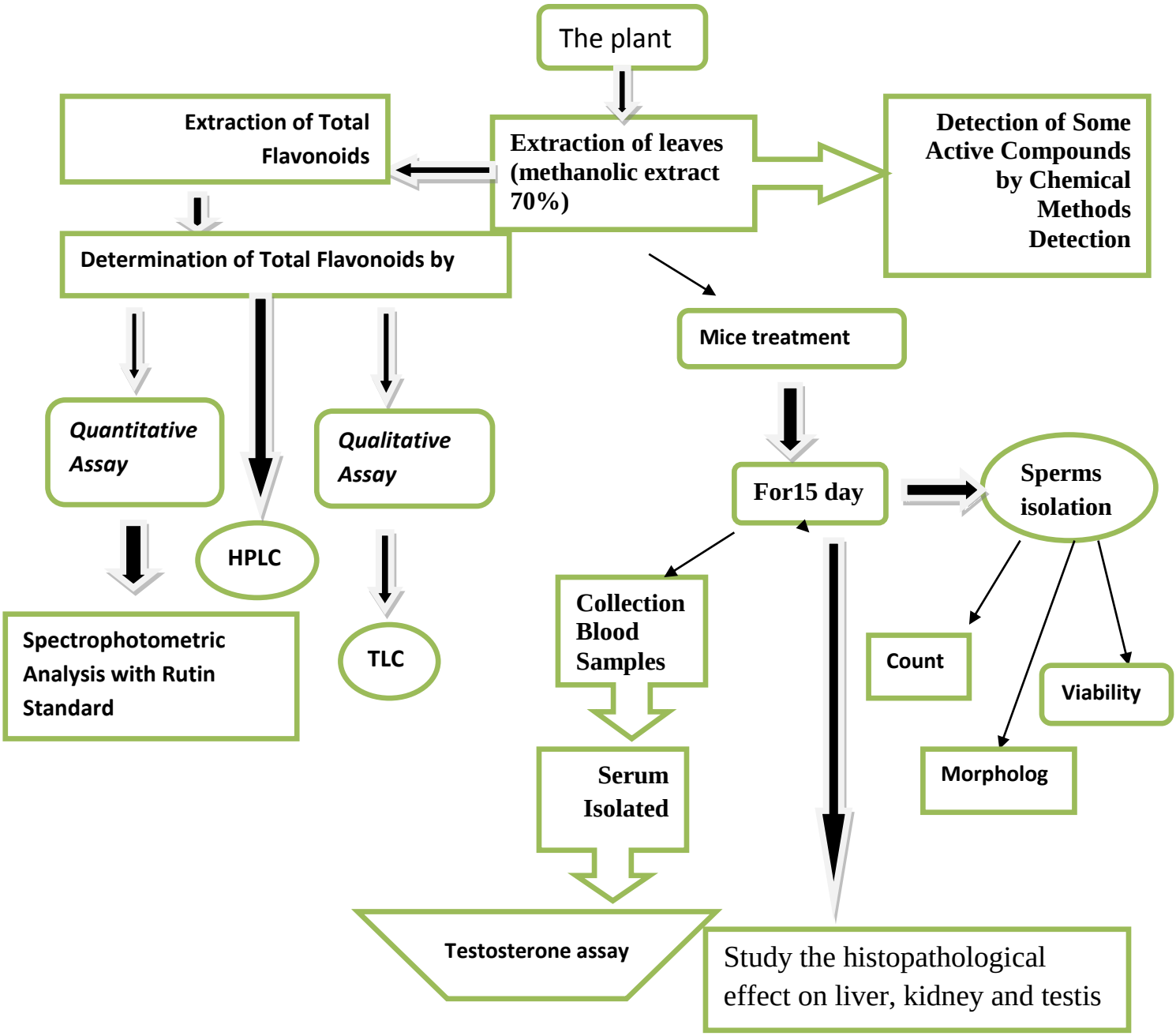
2.5.1. Plant Collection

M. oleifera was obtained from a local plantation in Baghdad and identified by Professor Dr. Ali Al- Mosawy (Department of Biology, College of Science. Baghdad University) the plant leaves were air dried at room temperature and stored until used.

2.5.2. Plant Extracts

The dried leaves was powdered using a blinder for 10 minutes, and then extracted with methanol (70%), 50 grams of the processed plant were extracted in 250 ml of the solvent and left in shaker incubator (40ºC) for 24hrs. Extract was then filtered with gauze followed by filter paper .The extract was then evaporated at (45ºC) using a rotary evaporator and the resultant crude extract was dried using lyophilizer. Dried extract was collected, weighed and kept in freeze at (-20ºC) until use (Arokiyaraj *et al.*, 2007). The weight of residue of *M. oleifera* leaf

extracts was 8g which represents 16% of the original leaves sample weight. The appearance of the residue was dark green in color.



Scheme (2-1): Main steps of the research plan

2.6. Detection of Some Active compounds in Plant Extract

2.6.1. Chemical Detection of Plant Extracts

1. Detection of Flavonoids

The detecting solution was prepared by mixing 10 ml of ethanol (50%) with 10 ml of potassium hydroxide (50%), and then 5 ml of this solution was added to 5 ml of the plant extract. The appearance of yellow color was an indicator of the presence of flavonoids (Jaffer *et al*., 1983).

2. Detection of Terpenes and Steroid

An aliquot of 1 ml of methanol extract was mixed with few drops of chloroform , then a drop of acetic anhydride and drop of concentrated sulphuric acid were added, brown precipitate appeared which representing the presence of terpenes, the appearance of dark blue color after few minutes would represent the presence of steroids (Al-Abid, 1985).

3. Detection of Tannins

An aliquot of (25ml) of methanolic extract was mixed with ferric chloride solution (FeCl2) (1%; w/v), the appearance of greenish-blue color was an evidence for the presence of tannins (Harbone, 1984).

4. Detection of Alkaloids

An aliquot of 10 ml of the plant extract was acidified by adding HCL,Test it by Mayer’s reagent and appearance of white precipitate indicates the presence of alkaloids (Trease and Evans, 1987).

5. Detection of Saponins

Saponins were detected by this method (Stahle, 1969):

- An aliquot of 5 ml of the plant extract was added to 1-3 ml of 3% ferric chloride solution, a white precipitate was developed indicating the presence of saponins.

6. Detection of Glycosides

About 3ml from the extract was hydrolyzed with HCl for 1-2 hours on water bath. Then the hydrolysate transferred to glass tube and heated with 7ml Benedict's reagent. The reagent contained blue copper(II) ions(Cu+2) which were reduced to copper(I)ions(Cu+1) in the presence of reducing sugar and heating, which precipitated as insoluble red copper(I) oxide (Harborne, 1998).

2.7. Extraction of Flavonoids

Two grams, from dried methanol extract was reflected for 8hr using 200 ml of 2M HCl solution. Filtered and the filtrate was cooled then transferred to a separator funnel. The aglycon moiety was extracted by 50 ml ethyl acetate for three times. The collected ethyl acetate layers were washed with distilled water to remove the excess acid, and then evaporated to dryness by rotary evaporator at 40ºC. The dried residue then re-dissolved in 50 ml 50% ethanol (Harborne, 1984)., the yielded residue was (2.52 g) of 50 g dried leaves.

2.8 Determination of Total Flavonoids by.

2.8.1 Quantitative Assay

At january in Biotechnology Research Center/Al-Nahrain University, Rutin standard stock solution was prepared in 50%ethanol (1mg/ml) from which serial dilutions were made to get rutin standard solutions with concentration of 0.2, 0.5, 1, 2.5 and 5mg/ml. Amount of 1ml was transferred from each standard solution and from the extracted flavonoid into a glass tubes, then 0.75 ml of 5% sodium nitrite solution was added and mixed well to be left to stand at room temperature for 5 minutes. To all tubes 1.5 ml of 10% $AlCl_3$ in 50% ethanol was added, shacked well and left to stand at room temperature for another 5 minutes.

At last 5ml of 1N NaOH solution was added to all tubes (Wang *et al.*, 2009).

The absorbance was read at 510nm, and a standard curve was plotted between the concentration and the absorbance, then the amount of total flavonoid was calculated as rutin from the equation of straight line that obtained from the plotted curve.

2.8.2 Qualitative Assay

At january in Biotechnology Research Center/Al-Nahrain University, Thin-layer chromatography (TLC) is a very commonly used technique because it is simple. It used to separate the extracted flavonoids into the components. Table (2-4) showing the solvent systems used as a mobile phase to select the most proper one that separate the extracted *Moringa oleifera* flavonoids efficiently.

Table (2-4): Solvent systems were used in this study as a mobile phase of TLC and their Ratios

Solvent System	Symbol	Ratio
Glacial acetic acid: *n*-Hexane: Ethyl acetate	a	1:6.2:2.8
Glacial acetic acid: Chloroform: Formic acid	b	0.7:8.8: 0.5
***n*-Butanol : Distilled water: Glacial acetic acid**	c	4:5:1

Standard solutions was prepared 0.1mg/ml in 50%ethanol from rutin, kaempferol, quercetin, luteolin, then mixed of standard solutions well and put one spot from each of sample (the extracted plant flavonoid) and standards on a thin layer chromatography (TLC) , TLC was activated at 100°C for 30 minutes in an oven and cooled at room

temperature before use. This equation used to calculate the Rf value (IUPAC, 2006).

$$\text{Rf Value} = \frac{\text{Distance from Baseline travelled by Solute}}{\text{Distance from Baseline travelled by Solvent}}$$

2.8.3. Detection of Flavonoids Compound by HPLC

At january in Ibn Sina Center / Ministry of Industry and Minerals, HPLC application for flavonoids standards rutin and quercetin and for flavonoid of the plant leaves extract which was used for qualitative detection of the flavonoids. The condition for detection of rutin and quercetin as follow:

Mobile phase: Methanol: Water (70:30)

Column: C18 (25cm)

Flow rate: 0.5ml/min.

Injected volume: 10µl.

Wave length: 280nm.

Instrument: waters/487 USA

2.9. Laboratory Animals

At November in Biotechnology Research Center/Al-Nahrain University, the laboratory animals used in this study were the 30 albino male mice they were supplied by the Drug Control Center, Ministry of Health, and their age at the start of the experiment was 6-8 weeks, and their weight was 20-25 grams. They were divided into 6 groups; each group was placed in a separate plastic cage. The cages were put in a room with optimal temperature (25C) the animals were given water and fed throughout the experimental work.

2.10. Experimental Design

The experiment was designed to evaluate the effect of three doses (100,200 and 300 mg/kg) of *M. oleifera* extract (methanol extract), as well as, proviron, ginseng (positive controls) and water (negative controls) on fertility. Therefore, the animals were divided into six groups (each group contains 5 mice). The extract was dissolved in distilled water to facilitate its oral administration to the mice. Then the mice were sacrificed after (15 day) of treatment.

Group1 **(negative control): The mice were treated with water.**

Group2 **(positive control): mice were treated with 0.36 mg/kg Mesterolone (Proviron).**

Group3 **(positive control): mice were treated with 0.4 mg /kg Ginseng drug(ginsavit).**

Group4**: mice were treated with 2.5 mg/ ml of extract (100 mg/kg).**

Group 5**: mice were treated with 5 mg/ ml of extract (200 mg/kg).**

Group6**: mice were treated with 7.5 mg/ ml of extract (300 mg/kg).**

2.11. Collection of Blood Samples and Determination of Testosterone levels

At the end of the experiment, blood was drawn from the heart directly by stab the heart (using syringe) to get the largest amount of blood and collected into microcentrifuge tubes. Blood samples were centrifuged at 5000 rpm for15min to get serum, then the serum had frozen (-20°C) in refrigerator until the testosterone assay. The testosterone concentration was determined using the Testosterone Enzyme Immunoassay kit.

2.12. Semen Preparation

Soon after killing mice and dissection, the epididymes and testes were removed for study the sperm concentration, morphological and viability.

2.12.1. Sperm viability and morphology

The epididymes minced with small scissors in Petri dish containing phosphate buffer saline (PBS) 1ml. A drop of semen suspension was mixed with a drop of eosin stain (1%) a thin smear of semen -eosin was put on the slide and then mixed by other slide which used to make a thin smear in a third slide and the third slide left to dry at room temperature, the slides was examined under light microscope at (40x). The dead sperms stain pink color while the live one is bright without color. Also the morphology of abnormal sperm was determined by this stain. The sperm viability was estimated according to the following equation (Hafez, 1987)

Percentage of dead sperm % = (NO. of dead sperm /total NO. of sperm) X 100

The percentage of sperm abnormality was estimated according to the following equation (Dale and Edler, 1997).

Abnormality % = (No. of abnormal sperms/ total NO. Of sperm) X 100

2.12.2. Sperm Concentration

The testes minced with small scissors in Petri dish containing 1ml of 0.9% normal saline with one drop of formalin. Sperm concentration was calculated according to the following steps:

- The sperm suspension prepared was pulled by RBC pipette till "0.5" mark, and then diluted with the diluting solution till "101" mark, so the dilution rate is 1:200

- The content of the pipette was mixed; the cover slip was placed over the hemocytometer.
- A small amount of the diluting solution containing sperms was placed at the edge of the cover slip and drawn by the capillary action under the cover slip.

The slide was placed under the microscope and the number of sperms was counted in five large squares. The number of sperm / ml was calculated using the following formula (Salisbury, 1961).

$$\text{No. of sperm} = \frac{N \times 200 \times 400 \times 1000}{80 \times 1/10}$$

N: number of sperms in the five squares.

200: inversion of the dilution rate.

400: inversion of the small square size.

80: number of small squares in the hemocytometer.

1/10: depth of the hemocytometer.

2.13. Histopathological Study

Mice were sacrificed by cervical dislocation (Installing the head, the scissors on the neck pulling the tail). Then dissected to get the kidney, liver and testes which were washed with distilled water before saving in 10% formalin. The procedure of Bancroft and Stevens (1982) was followed to prepare sections for histopathological examinations. Prepare in the histopathology Department at Educational Laboratories of Medical Baghdad City

The procedure is summarized as the following:

1-Washing: Samples were placed in ethanol 70% for 12h.

2-Dehydration: They were dehydrated with ascending concentrations (70, 80, 90 and 99%) of ethanol. With two hours for each concentration

3-Clearing: Samples were placed in xylene for two hours.

4-Infiltration: The samples were first placed in paraffin- xylene (1:1) for 30 min at 57-58 °C, and then in paraffin alone for 2 hrs at 60-70°C inside the oven.

5-Embedding: The samples were embedded in pure paraffin wax (melting Temperature 60-70 °C) and left to be solidified at room temperature.

6-Sectioning: The paraffin block was sectioned by a rotary microtome at a thickness of 5 microns, and then the sections were transferred to a slide covered with Mayer's albumin. The sections of tissues were placed in a water bath (35-40°C) for a few seconds.

7-Staining: The slides were first placed in xylene for 15-20 min, then it was put in descending concentrations (100, 90, 80 and 70%) of ethanol (2 min for each concentration) and finally put in tap water. After that, the slide was stained with haematoxylin for 5-15 min and then washed with tap water for 5 min. Then, the slide was placed in acidic alcohol for a few seconds and washed with distilled water. After washing, the slide was placed in eosin stain for 10-15 seconds, and then in ascending concentrations (70, 80, 90 and 99%) of ethanol (two minutes for each concentration). Finally the slide was cleared with xylene for 10 min.

8-Mounting: The slide was mounted with Canada balsam and covered with a cover slip. Then, the slide was examined microscopically to inspect the histopathological changes.

2.14. Statistical Analysis

The Statistical Analysis System- SAS (2012) program was used to regression and conclusion of difference factors in study parameters. Least significant difference –LSD test was used to significant compare between means in this study.

Results and Discussion

3. Plant Extracts

3.1. Active Compounds Detected in the Plant Extract

In this study the bioactive compounds of methanolic extract of *M. oleifera* leaves were determined. Chemical detections of active compounds in the leaves of *M. oleifera* crude methanolic extract were shown in table (3-1).

Table (3-1): Chemical detection of some active compounds in *M. oleifera* methanolic extract.

active compounds	Reagents	References	Indication	The results
Alkaloids	Mayer's reagent	(Trease and Evans, 1987)	White precipitate.	+
Flavonoids	Ethanol with KOH	(Jaffer *et al*., 1983)	yellow color	+
Glycosides	Benedict reagent	(Harborne, 1998)	Red precipitate	+
Steroids	chloroform, acetic anhydride, sulphuric acid	(Al-Abid, 1985)	dark blue color	+
Saponins	Shaking Extract ferric chloride	(Stahle, 1969)	foam white Precipitate	+
Tannins	Ferric chloride	(Harbone, 1984)	Greenish-blue color	+
Terpenes	chloroform, acetic anhydride, sulphuric acid	(Al-Abid, 1985)	brown precipitate	+

Note: + indicates the presence of the active compound

In this study chemical detections of secondary metabolites in the leaves of *M. oleifera* methanolic extract showed presence of flavonoids, alkaloids, glycosides, saponins, tannins, terpenes and steroids.

Previous pharmacological and chemical studies on *M. oliefera* leaves extract have indicated the presence of several active compounds,

including tannins, alkaloids, saponins, carbohydrates, and glycosides (Sato *et al*., 2004; Cushine and Lamb, 2009).

Many natural compounds have been isolated from *M. oleifera* leaves including Glycosides containing isothiocyanates, malonates and flavonoids (Faizi *et al*, 1994; Bennett *et al*, 2003; Miean *et al*, 2001). Flavonoids compounds (Yang, 2008) such as myricetin, quercetin and kaempferol (Prakash, 2007).

3.2. Quantitative and Qualitative of Total Flavonoids

3.2.1. Quantitative Assay

Results in Table (3-2) and Figure (3-1) indicate that total flavonoids in one gram of *M, oliefera* dried leaves was 22.5 mg/g represent 2.25% (w/w) de termined as rutin according to straight line equation figure (3-1).

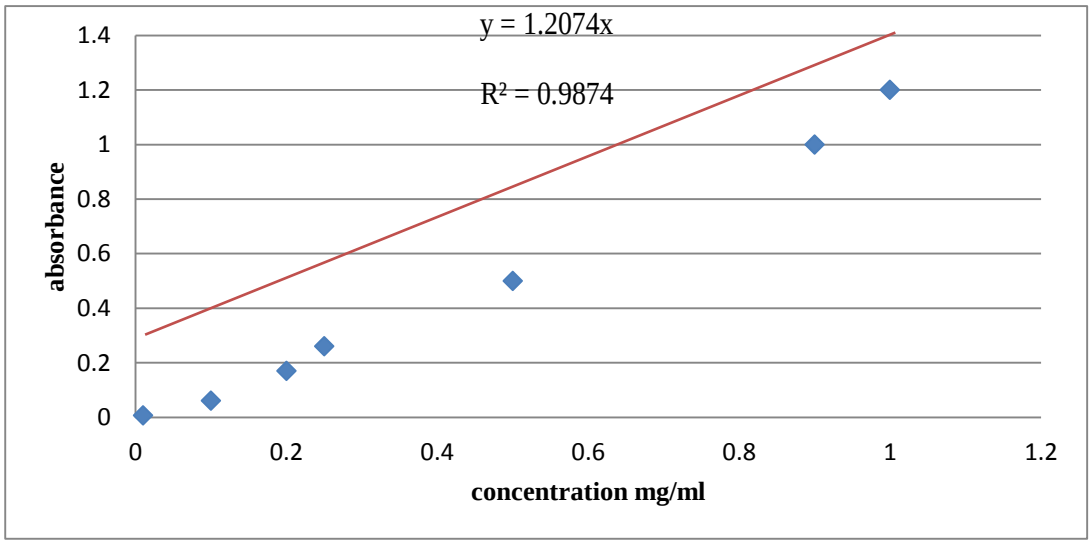


Figure (3-1): Standard curve for rutin as determined spectrophotometrically at 510 nm.

Table (3-2): Spectrophotometric analysis of *M. oleifera* total flavonoids and rutin standard solutions.

Solutions	Concentration (mg/ml)	Absorbance (at 510nm)
Rutin standard solutions	0.01	0.006
	0.1	0.062
	0.2	0.172
	0.25	0.266
	0.5	0.55
	1	1.263
The extracted solution	0.90	1.08666

The quantitative phytochemical screening of *M. oleifera* leaves extract revealed that the plant contains 1.643 % flavonoids, 0.148 % alkaloids (Julia, 2008).

3.2.2. Qualitative Assay.

The results in Figure (3-2) showed that TLC for plant extract indicated the presence of Rutin, Quareciten and Luteoline. They are the main flavonoids found in the plant that has the fertility activity on male mice. Flavonoids were determined by virtue of comparison with Rf (Retardation factor) values of the standards. Rf values of the standards are given in Table (3-3). Table (3-3) showing that mobile phase (b) was the best one because it gave good separation of the flavonoids, By comparison with (a) and (c) mobile phases that used in this study. Previous studies indicated the presence of flavonoids in *M. oleifera* leaves in particular, quercetin and kaempferol glycosides that broken down to yield the natural antioxidant flavonoids, querciten and kaempferol (Miean *et al*, 2001; Bennett *et al*, 2003).

Table (3-3): Detection of Flavonoids in *M. oleifera* methanolic extract by TLC.

Flavonoids	mobile phase (b)	RF Values of standard	Number of sample spots	RF Values of sample	Test
Querciten	Glacial acetic acid: Chloroform: Formic acid	0.07	4	0.07	Under UV light
				0.09	
Rutin		0.09		0.11	
Luteoline		0.12		0.2	

Figure (3-2): TLC chromatography for the mobile phase (b). *M. oliefera* flavonoids extract (F), Luteoline standard (L), Querciten standard (Q), Rutin standard (R) .

3.2.3. HPLC Analysis

HPLC analysis of the methanolic extract for *M. oleifera* obtained from dried leaves flavonoid extract indicated the presence of:

A. Quarecetin, with retention time (4.612) minutes, figure (3-3) in comparison with quarecetin standard (4.693) as figure (3-4).
B. Rutin, with retention time (3.486) minutes, figure (3-3) in comparison with Rutin standard (3.388) as figure (3-5).

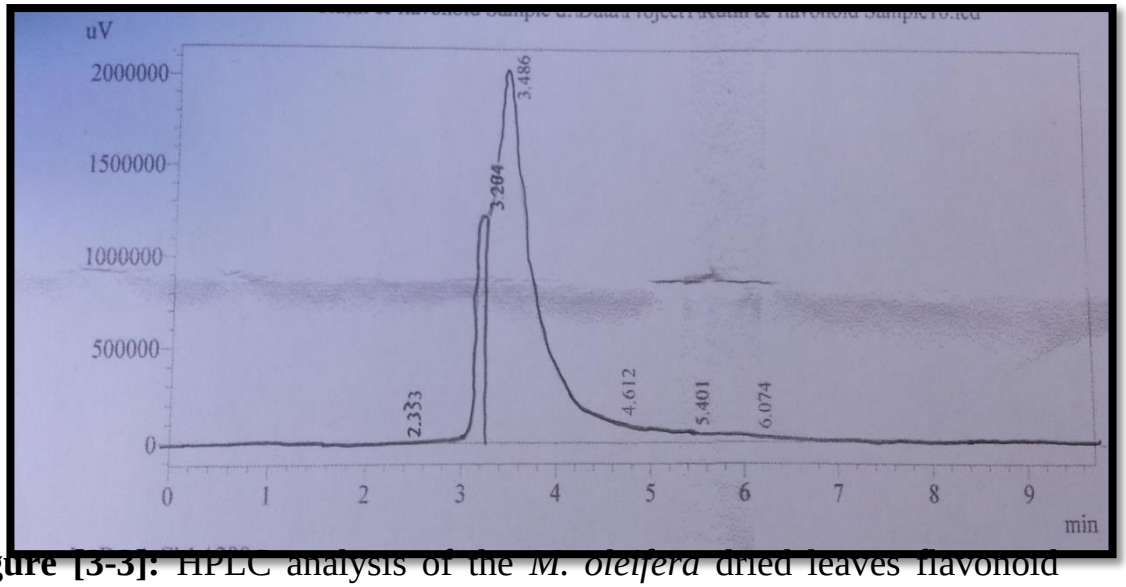


Figure [3-3]: HPLC analysis of the *M. oleifera* dried leaves flavonoid extract.

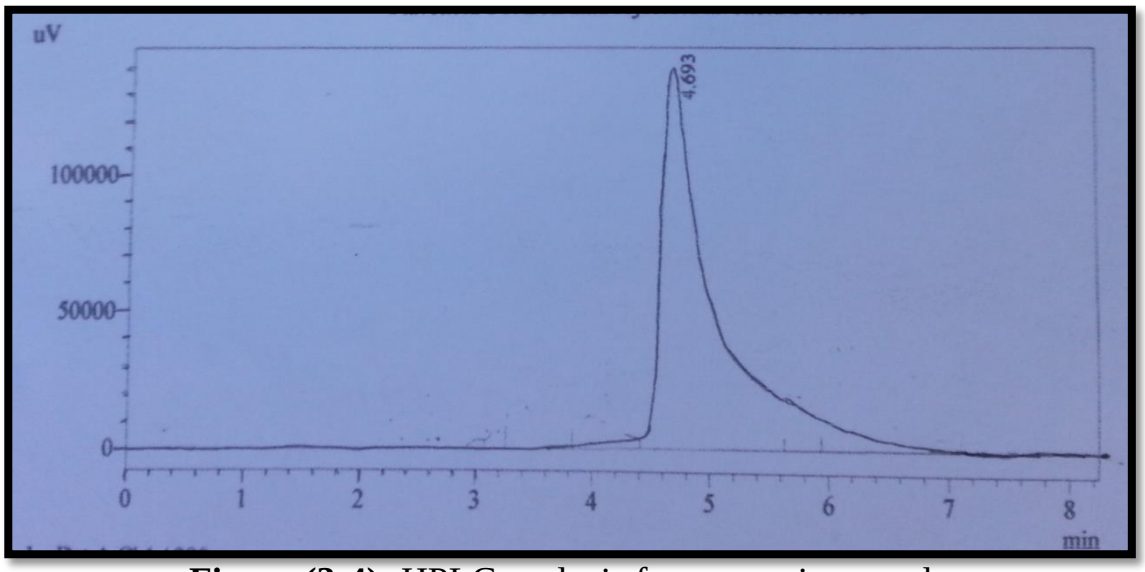


Figure (3-4): HPLC analysis for qurecetine standa

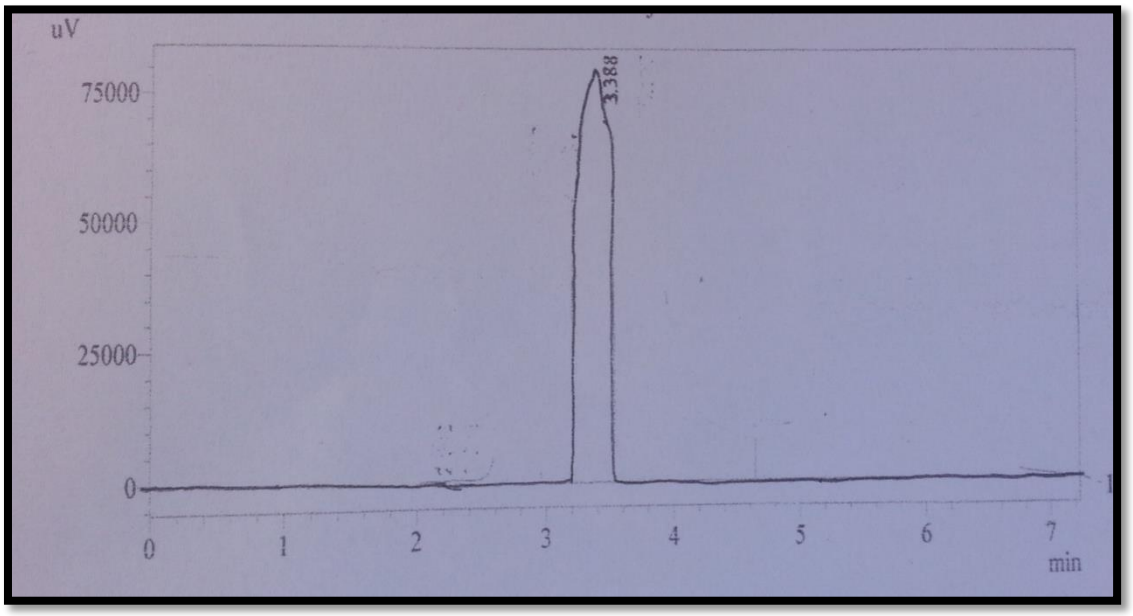


Figure (3-5): HPLC analysis for Rutin standard

3.3. Testosterone Assay (Testosterone serum concentration after 15 days)

Results in table (3-4) indicated a significant increase ($p \leq 0.05$) in serum testosterone concentration after 15 days in mice treated with *M. oleifera* extract compared with negative and positive controls-treated mice. Serum testosterone in *M. oleifera* treated mice with 100 and 200 mg/kg were elevated to (2.022 ± 0.16 2.056 ± 0.17 ng/ml) while in negative control, water treatment mice was (0.818 ± 0.03 ng/ml) and in positive control, mesterolone and gensing drug treatment mice (0.962 ± 0.01 1.012 ± 0.02 ng/ml). The levels of testosterone in all treated groups were measured (Figure 3-6). Testosterone concentrations were elevated in doses (100 and 200) mg/kg compared with the dose (300) mg/kg. So there is a significant increase ($p \leq 0.05$) in serum testosterone concentration as compared with the dose 300 mg/kg.

Table (3-4): Effect of *M. oleifera* methanolic extracts (100,200 and 300 mg/kg) for 15 days on serum testosterone concentration in mice**Different letters (A, B): Significant difference ($p \leq 0.05$) between means of column

Mice Groups		Testosterone concentration ng/ml x (10) Mean ± SD
Plant extract doses	100 (mg/kg)	2.022 ± 0.16 A
	200 (mg/kg)	2.056 ± 0.17 A
	300 (mg/kg)	0.742 ± 0.05 B
Positive controls	Ginseng drug	0.962 ± 0.01B
	Mesterolone	1.012 ± 0.02 B
Negative control	water	0.818 ± 0.03B
LSD value		0.292 **
** (P<0.05).		

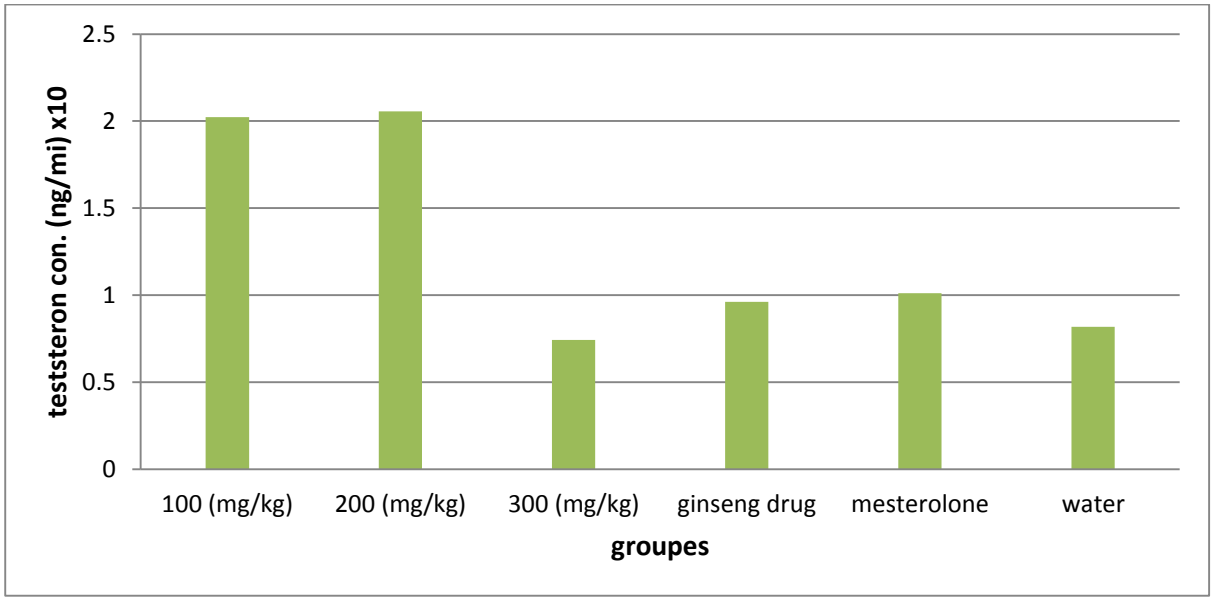


Figure (3-6): Serum testosterone concentration in mice after 15 days

The plant contained flavonoids compounds such as qureceine and rutin. Qurecetine could increase serum testosterone levels in male that has been found to improve the action of sex hormone (LH) .This hormone stimulates male testicles to produce greater levels of testosterone, which in turn helps increased sexual drive (Maz, 2004). Androgenic effect is attributable to testosterone levels in blood; *M. oleifera* leaf extracts have a role in testosterone secretion confer best availability of hormone to gonads. The testes, epididymis and other reproductive organs are structurally and physiologically dependent upon the testosterone (Amini and Kamkar, 2005).

3.4.1. Sperms Concentration

The results of sperms concentration in table (3-5) and Figure (3-7) showing a significant increase ($p \leq 0.05$) in sperms concentration after treatment with the plant extract at doses 100 and 200 mg/kg (201.40 ± 4.82 and 203.20 ± 4.94 sperm/ml) when compared with negative control, water (65.60 ± 2.62 sperm/ml), positive controls, mesterolone and gensing drug (151.80 ± 2.26 and 65.60 ± 2.62 sperm/ml) and also when compared with other group treated with plant extract at dose 300 mg/kg (65.00 ± 1.70 sperm/ml). The plant extract contained many active compounds especially flavonoids that contributed in an increasing sperms concentration. The mechanism for increased sperms concentration may be because the presence of quercetin which increased the numbers of spermatogonial cells by reduceing the oxidative damage in the testes (Mi and Zhang, 2005; Chandel *et al*., 2008). Other study found that quercetin increases the testosterone level so that quercetin led to boost sperm quality and fertility (Taepongsorat, 2008).

Table (3-5): Effect of *M. oleifera* leaves methanolic extract (100, 200 and 300 mg/kg) for 15 days on sperms concentration, percentage of morphologically, abnormal sperms and percentage of sperm viability in mice.

Groups		Sperm count ($x 10^6$) Mean ± SD	Dead sperms % Mean ± SD	Abnormal sperm % Mean ± SD
Plant extract doses	100 (mg/kg)	201.40 ± 4.82 A	13.40 ± 0.81 E	12.40 ± 1.28 D
	200 (mg/kg)	203.20 ± 4.94 A	12.80 ± 0.96 E	13.20 ± 1.11 DC
	300 (mg/kg)	65.00 ± 1.70 D	42.80 ± 1.85 A	38.00 ± 2.19 A
Positive controls	Ginseng drug	117.40 ± 0.93 C	24.60 ± 1.16 C	16.80 ± 0.92 BC
	mesterolone	151.80 ± 2.26 B	17.60 ± 0.87 D	16.80 ± 1.02 BC
negative controls	Water	65.60 ± 2.62 D	31.00 ± 1.05 B	19.80 ± 0.58 B
LSD value		9.492 **	3.424 **	3.753 **
** (P<0.05).				

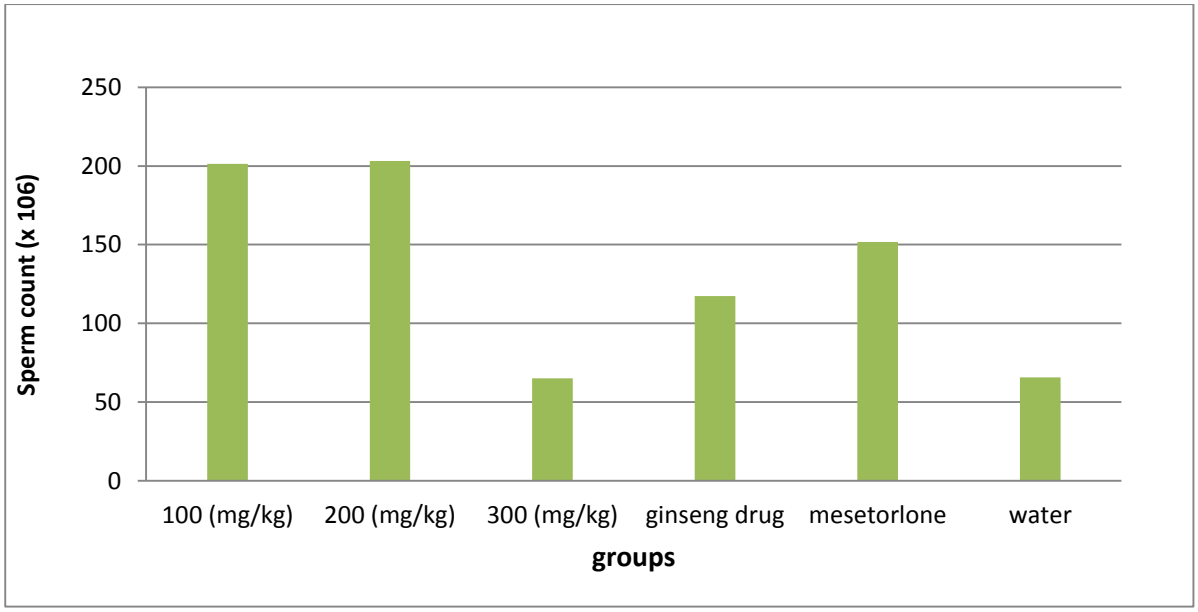
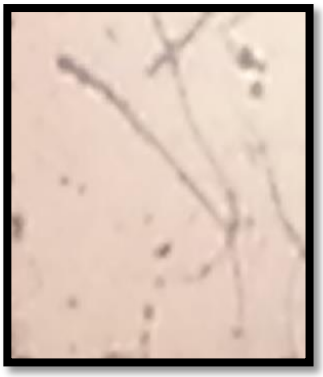


Figure (3-7): Sperm count in male mice treated with leaves methanolic extract of *M. oleifera* for 15 days

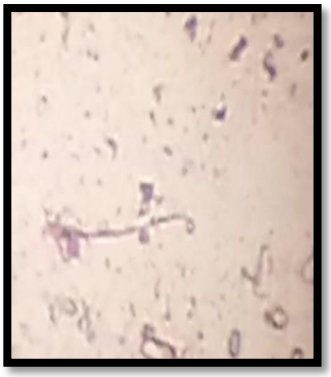
3.4.2. Morphologically Abnormal Sperms

Morphological analysis of sperms is an important aspect in the assessment of sperm functions (Katz *et al*.,1982).Results in table (3-5) and Figure (3-9) revealed a significant decrease ($p \leq 0.05$) in percentage of morphologically abnormal sperms after treatment with plant extract at doses 100 and 200 mg/kg (12.40 ± 1.28, 13.20 ± 1.11) when compared with negative controls (water treatment) (19.80 ± 0.58) and positive control (mesetorlone and ginseng drug) (16.80 ± 1.02, 16.80 ± 0.92) respectively and also when compared with other groups treated with plant extract at doses 300mg/kg (38.00 ± 2.19) morphological abnormalities of sperms were observed as in figure (3-8).

A- Terminally coiled tail tail sperm



B- Small head sperm



C- Short

D- Normal sperm

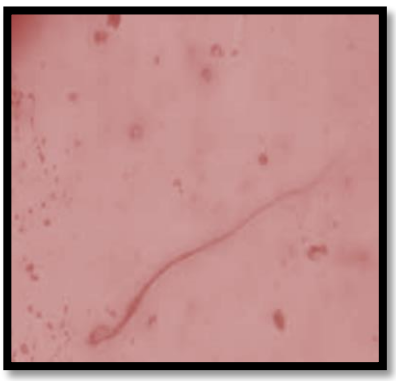


Figure (3-8): Effects of *M. oleifera* extract at doses 300 mg/kg on sperm morphology in mice.

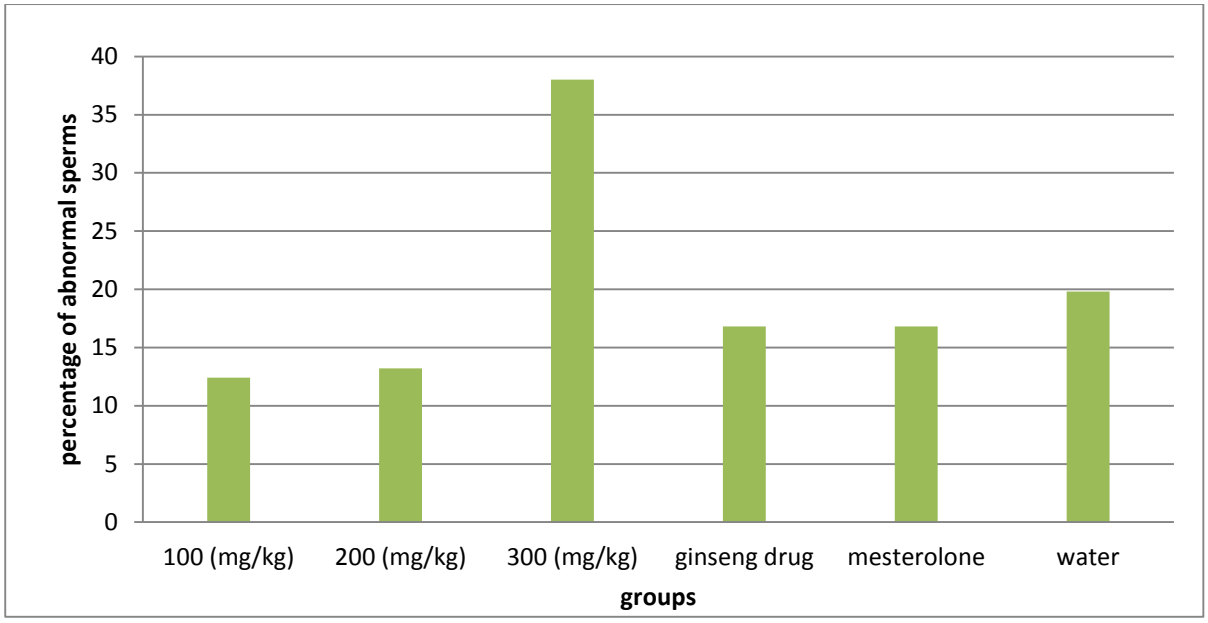


Figure (3-9): Morphologically abnormal sperms in male mice treated with leaves methanolic extract of *M. oleifera* for 15 days.

The results showed that after 15 days treatment with plant extract at dose (300 mg/kg). The plant extract affects on morphological abnormalities of sperms on the basis of a dose-dependent. The activity of plant extract can be referred to the flavonoids and also other compounds that act as antioxidant (Vongsak *et al*., 2013). These compounds protected the plasma membrane of the sperm against the influence of oxidative stress. The group treated with dose300 mg/kg showed a significant increase ($p \leq 0.05$) in percentage of morphologically abnormal sperms when compared with other groups treated with doses 100 and 200 mg/kg. The results showed that low doses were less effective on sperms morphology than high doses; this could be explained that high dose (300 mg/kg) was toxic and caused reduction in testosterone levels (Ajibade, 2013).

3.4.3. Sperms viability

Results in table (3-5) and Figure (3-10) clarified a significant decrease ($p \leq 0.05$) in percentage of dead sperms after treatment with

plant extract at doses 100 and 200 mg/kg, the percentage of dead sperms (13.40 ± 0.81, 12.80 ± 0.96) respectively when compared with negative controls (water treatment) (31.00 ± 1.05) and positive control (mesterolone and ginseng drug) (17.60 ± 0.87, 24.60 ± 1.16) respectively and also when compared with other group treated with plant extract at doses 300 mg/kg (42.80 ± 1.85). The group treated with dose 300 mg/kg showed a significant increase ($p \leq 0.05$) in percentage of dead sperms when compared with other groups treated with doses 100 and 200 mg/kg. The results showed that low doses were more effective on sperms viability than high doses; this could be explained that high dose (300 mg/kg) may be toxic. Flavonoids, like Rutin has shown a significant stimulating effects on sperm parameters like sperm count, sperm morphology and sperm viability, these results were in agreement with other studies of flavonoids effect on male reproductive system. Flavonoids including (quercetin) showed effect on the function of prostate (Shuk-mei ho, 1993).

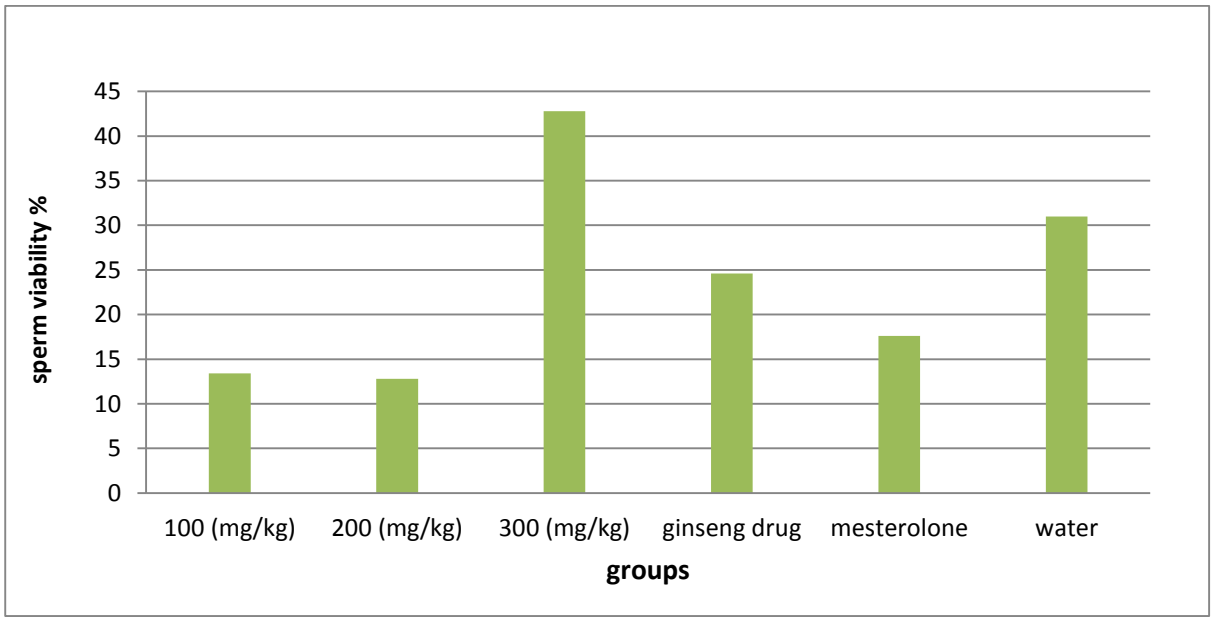


Figure (3-10): Sperm viability in male mice treated with methanolic extract of *M. oleifera* for 15 days.

3.5. Histopathological effects on liver, kidney and testes of mice

Different Histopathological changes were observed in liver, kidney and testes in all groups of mice. normal appearance of hepatocytes was shown in Liver section of mice treated with water as negative control (figure 3-11) and Liver section of mice treated with mesterolone and ginseng drug as positive controls (figures 3-12, 3-13). Mice treated with plant extract at doses 100 and 200 mg/kg showed normal appearance of hepatocyte cells (figures 3-14, 3-15) while mice treated with high dose 300 mg/kg of plant extract showed the presence of degenerated hepatocyte cells ,inflammatory cells infiltration and necrosis (figure 3-16).

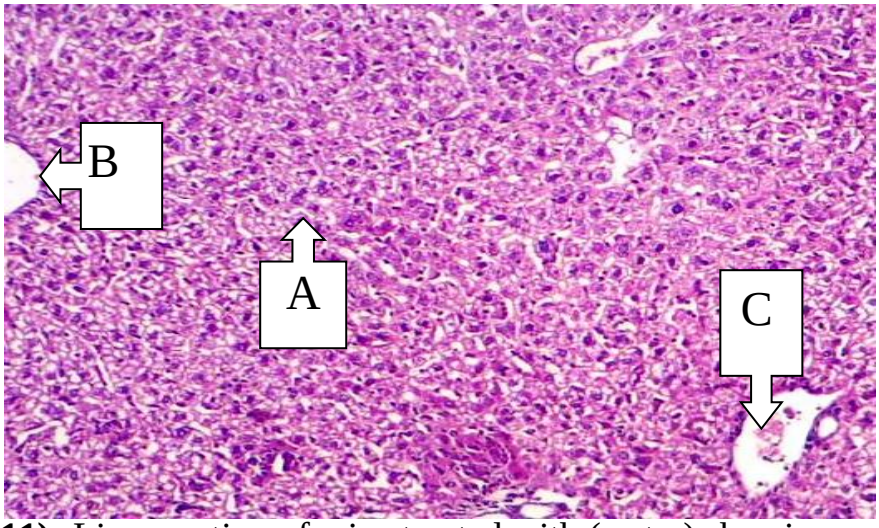


Figure (3-11): Liver section of mice treated with (water) showing normal appearance of hepatocyte cells (A) central vein (B) portal area (C) (H and E; X100).

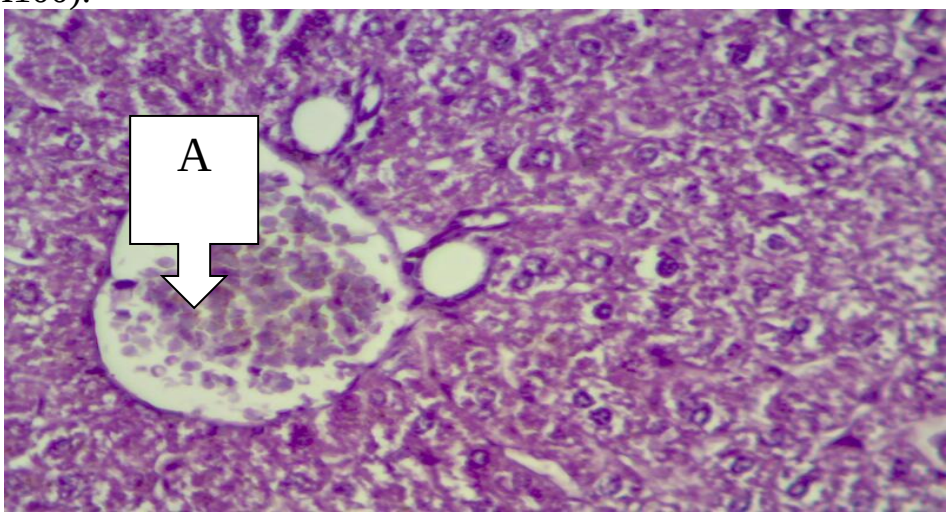


Figure (3-12): Liver section of mice treated with (mesterolone) showing normal appearance of hepatocyte cells, Portal area (A) (H and E; X400).

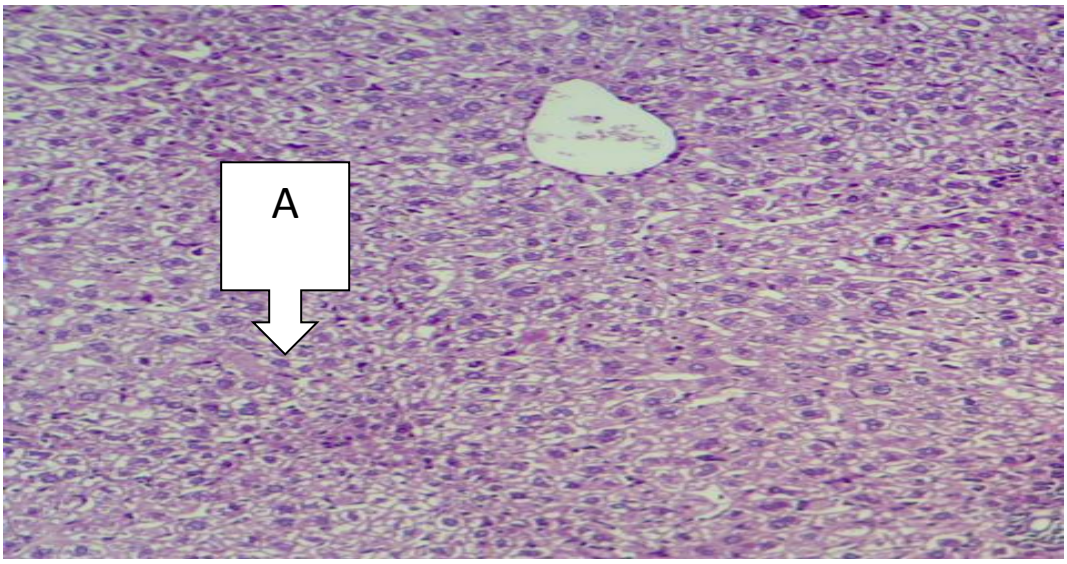


Figure (3-13): Liver section of mice treated with (ginseng drug) showing normal appearance of hepatocyte cells (A) (H and E; X200).

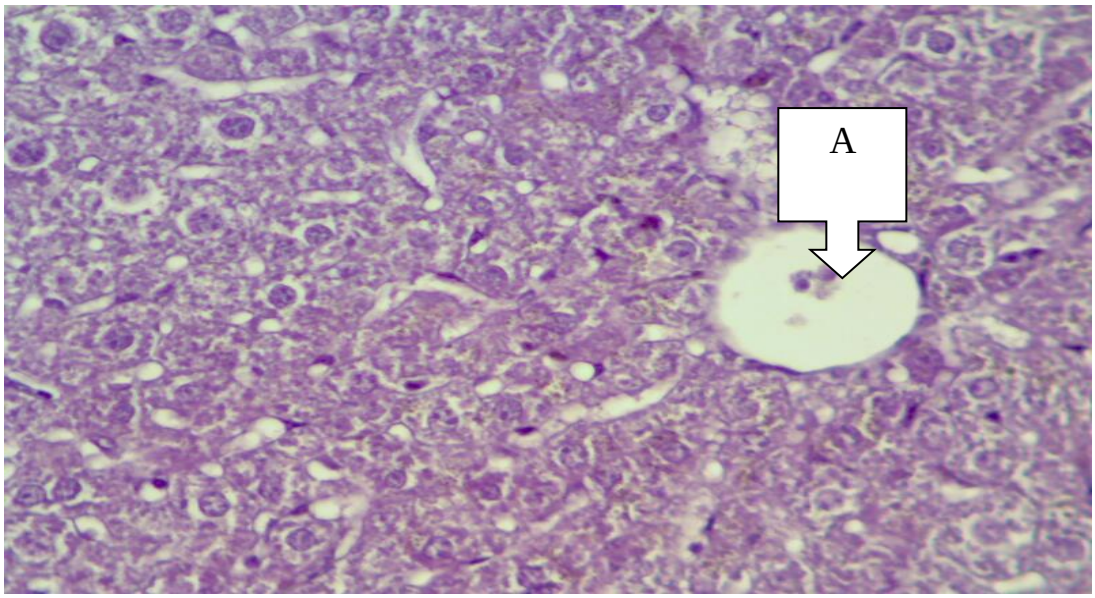


Figure (3-14): Liver section of mice treated with plant extract at dose 100 mg/kg showing normal appearance of hepatocyte cells, central vein (A) (H and E; X400).

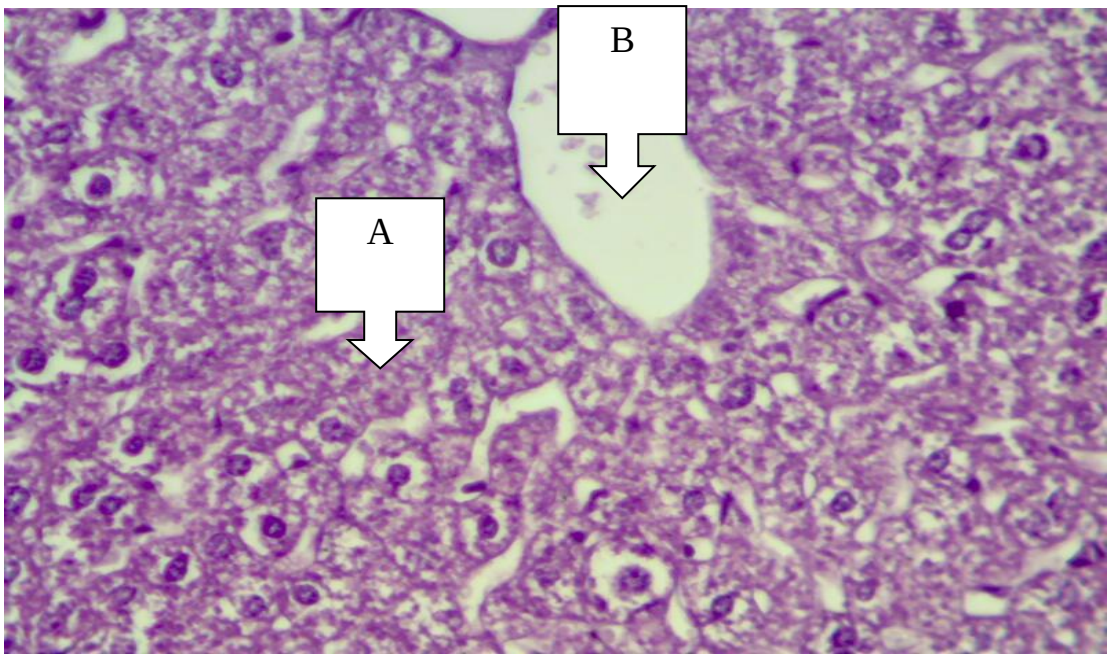


Figure (3-15): Liver section of mice treated with plant extract at dose 200 mg/kg showing normal appearance of hepatocyte cells (A) central vein (B) (H and E; X400).

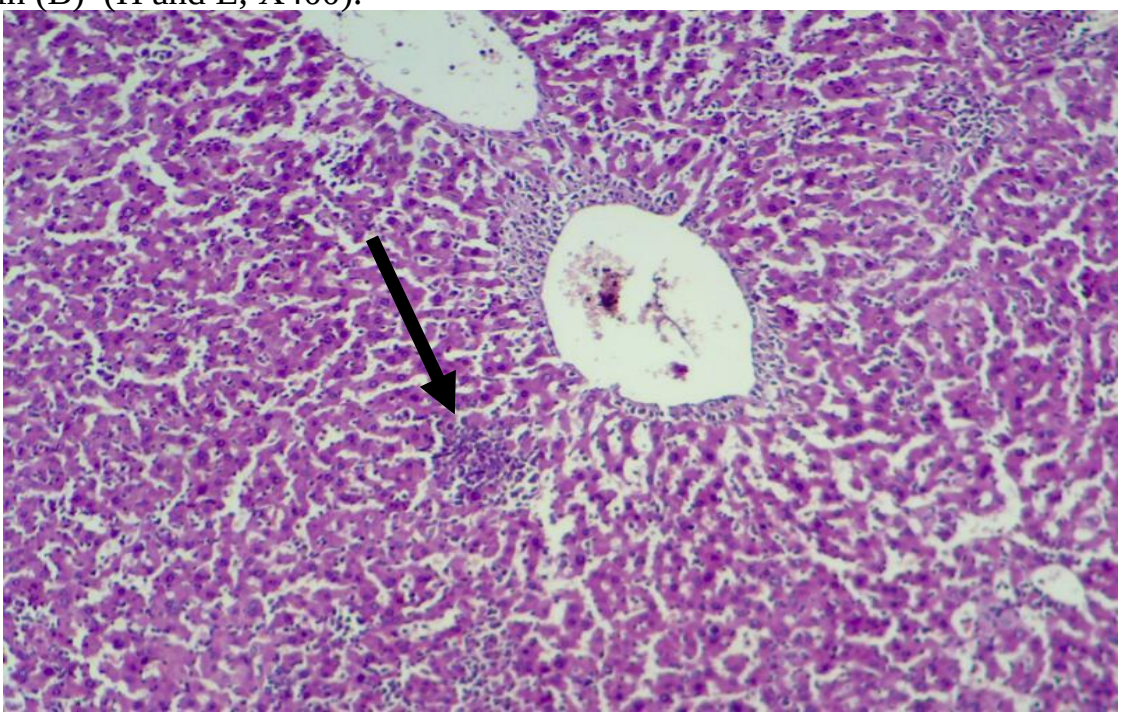


Figure (3-16): Liver section of mice treated with plant extract at dose 300 mg/kg showing dispersed, degenerative changes and necrosis of a hepatocyte (A) with inflammatory cells infiltration, (H and E; X200).

Kidney section of mice treated with water as negative control showed normal appearance of epithelial cells, glomerulus, distal tubules and proximal tubule (figures 3-17).

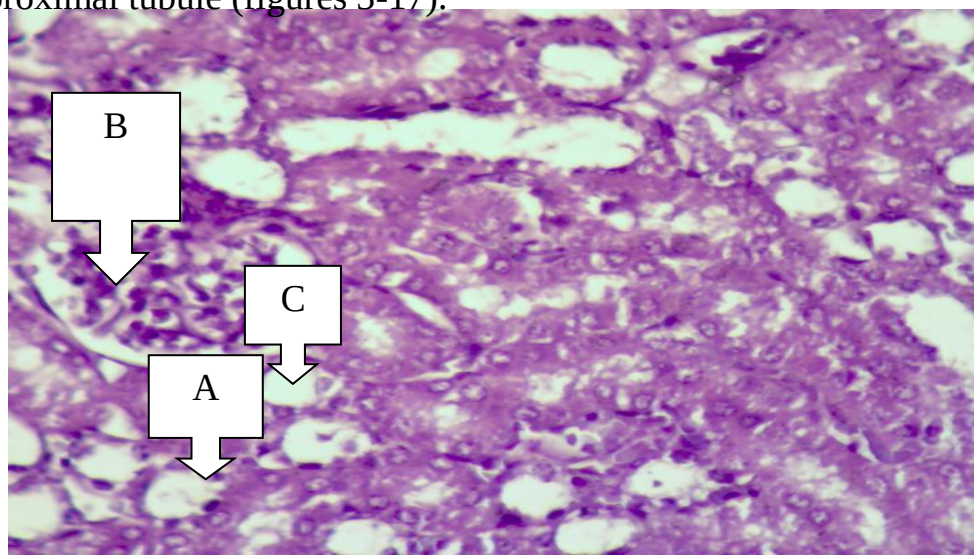


Figure (3-17): Kidney section of mice treated with (negative control, water) showing normal appearance (A) represent the distal convoluted tubules, (B) represent the glomerulus and (C) proximal convoluted tubule(H and E ;400X).

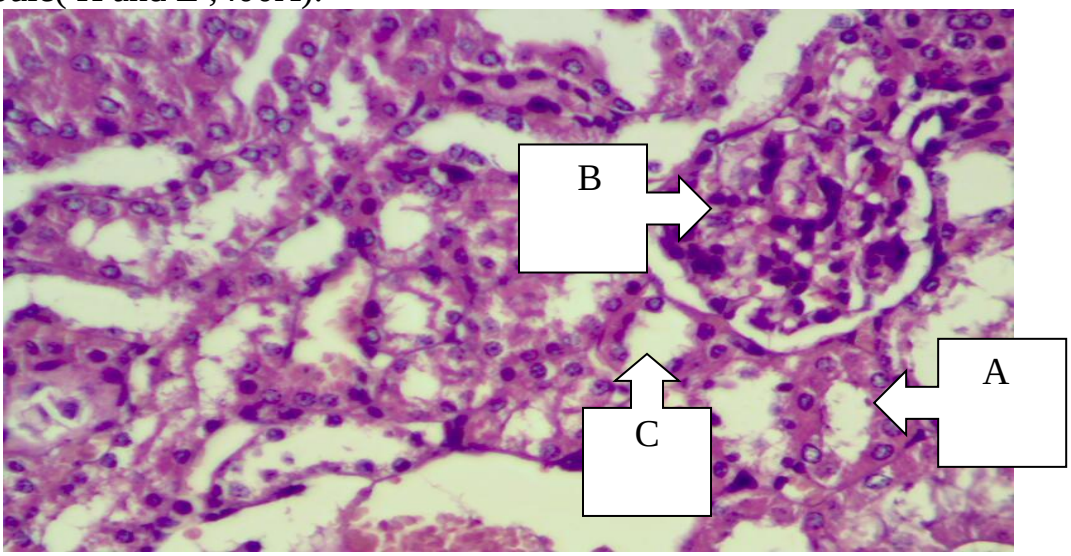


Figure (3-18): Kidney section of mice treated with (positive control, proviron) showing normal appearance (A) represent the distal convoluted tubules, (B) represent the glomerulus and (C) proximal convoluted tubule(H and E ;400X).

Kidney section of mice treated with mesterolone and ginseng drug (positive controls) showed the presence of normal appearance of epithelial cells, distal tubules and proximal tubule (figures 3-18, 3-19).

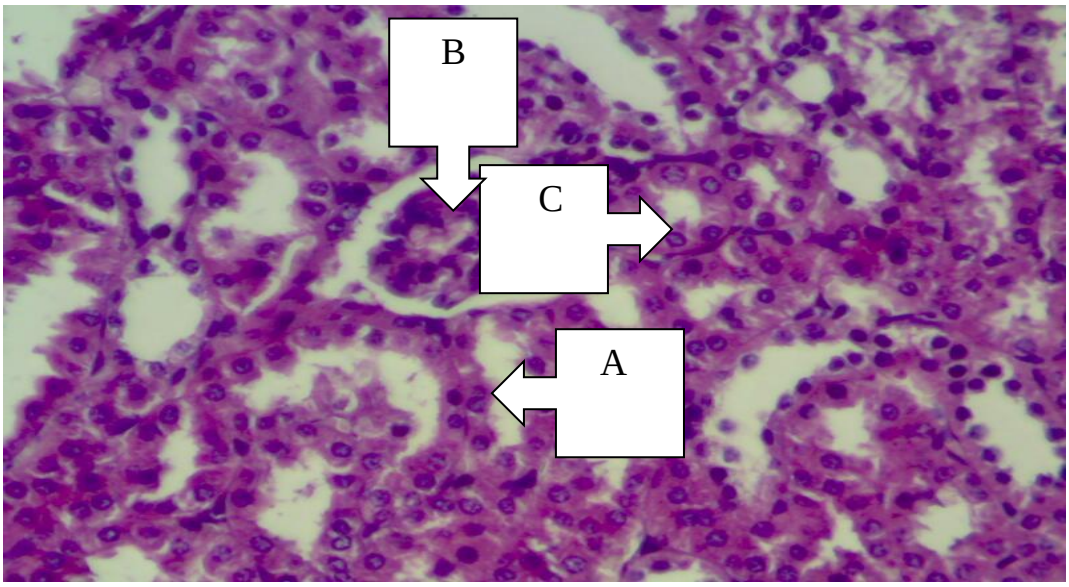


Figure (3-19): Kidney section of mice treated with (positive control, ginseng extract) showing normal appearance (A) represent the distal convoluted tubules, (B) represent the glomerulus and (C) proximal convoluted tubule(H and E ;400X).

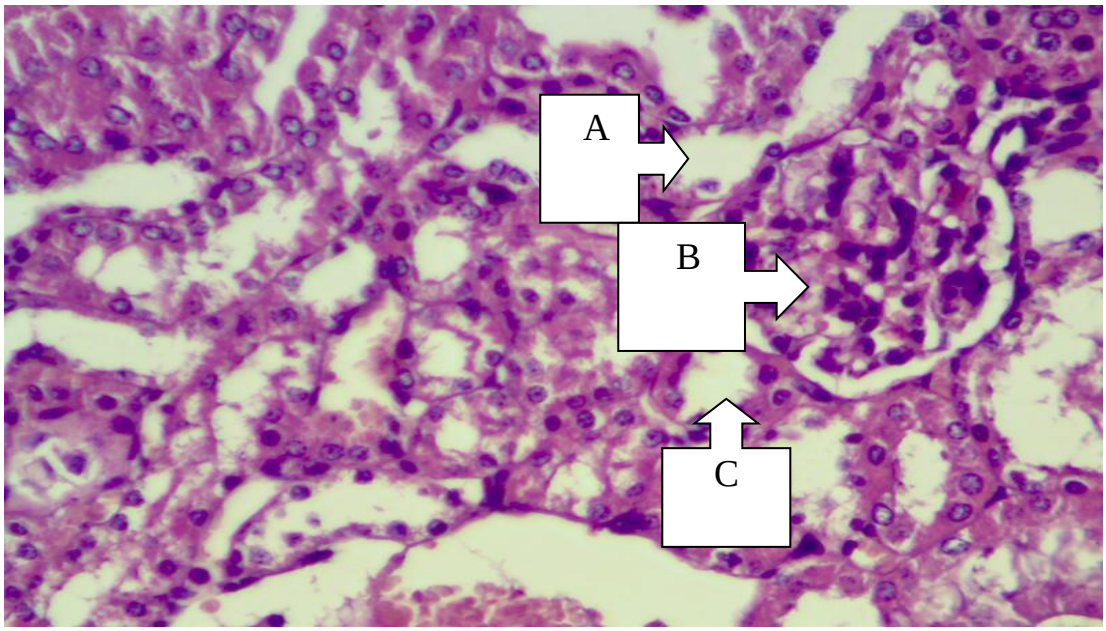


Figure (3-20): Kidney section of mice treated with plant extract at dose 100 mg/kg showing normal appearance, (A) represent the distal convoluted tubules, (B) represent the glomerulus and (C) proximal convoluted tubules of nephrons (H and E ;400X).

Mice treated with plant extract at doses 100 and 200 mg/kg showed the presence of normal appearance of a kidney section (figure 3-20, 3-21), while mice treated with high dose 300 mg/kg) of the plant extract showed tubular cell necrosis (figures 3-22).

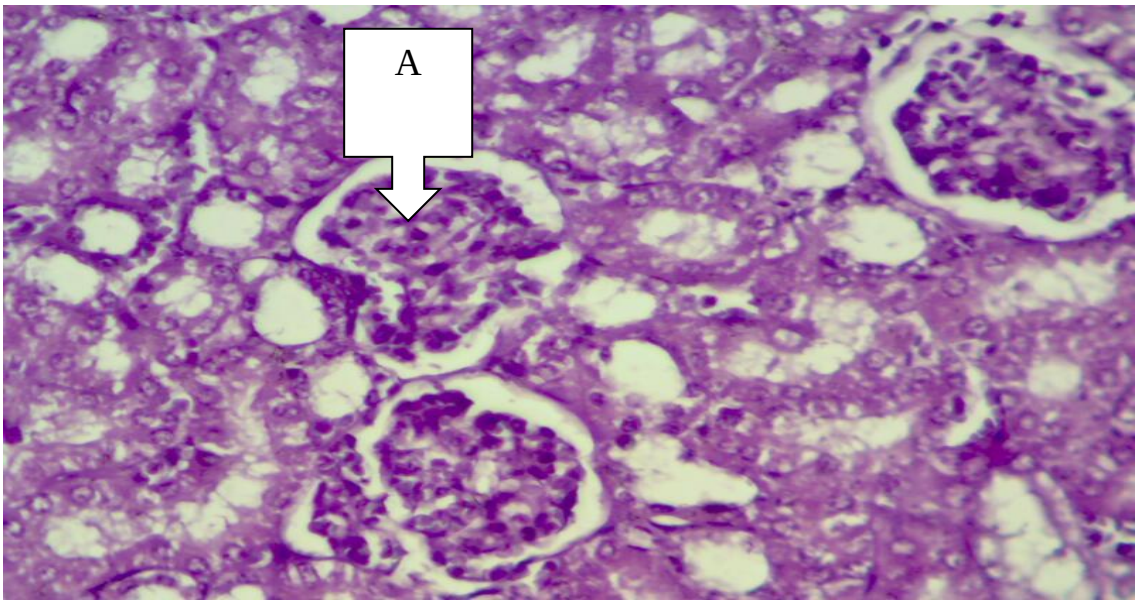


Figure (3-21): Kidney section of mice treated with plant extract at dose 200 mg/kg showing normal appearance, (A) represent the glomerulus (H and E; 400X).

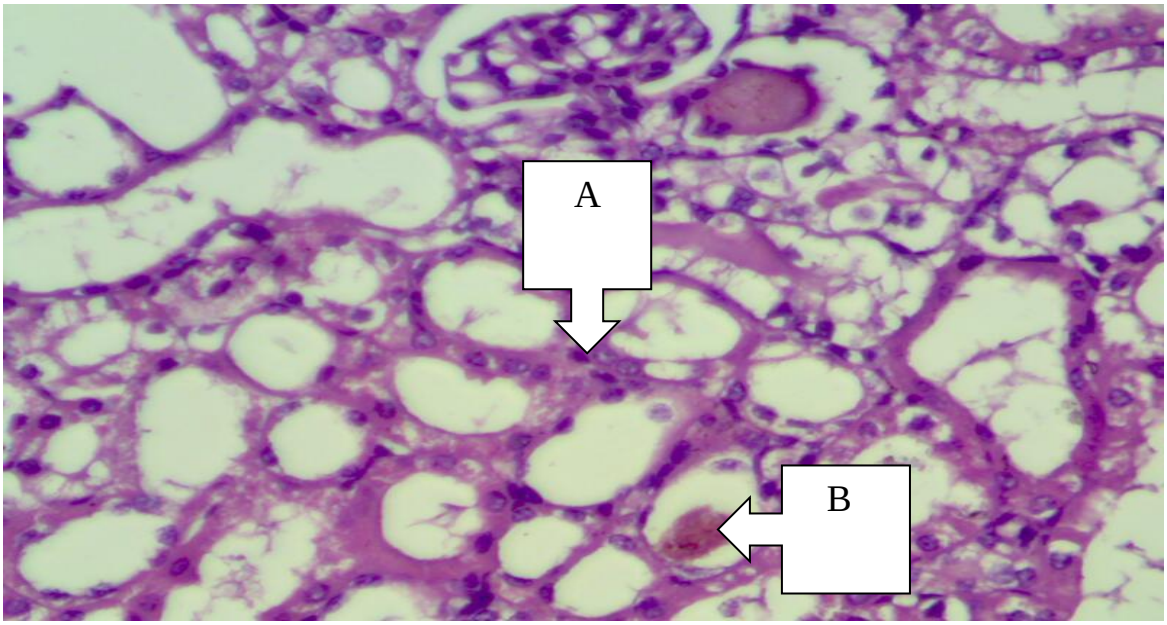


Figure (3-22): Kidney section of mice treated with plant extract at dose 300 mg/kg showing degenerative and apoptosis of renal tubules epithelia (A) with presence of hyaline cast (B) (H and E; 400X).

Testis section of mice treated with water, negative control showed normal appearance of seminferous tubules lumen, spermatogonia, leydig cells, sperms and seminferous tubules (figure 3-23). Testis section of mice treated with mesterolone and ginseng drug, positive control showed the presence of normal appearance of a seminferous tubules lumen, spermatogonia, leydig cells, sperms and seminferous tubules (figures 3-24, 3-25).

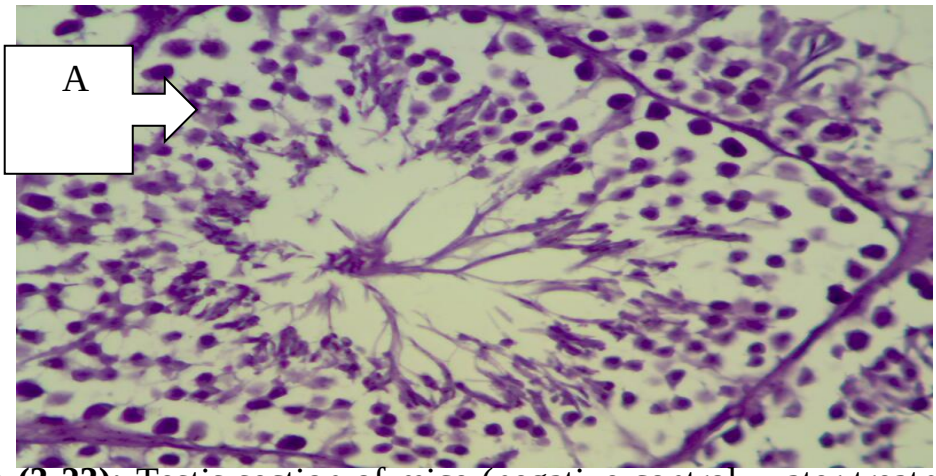


Figure (3-23): Testis section of mice (negative control, water treatment) Showing normal appearance of spermatogonia cells (A) (H and E; 200X)

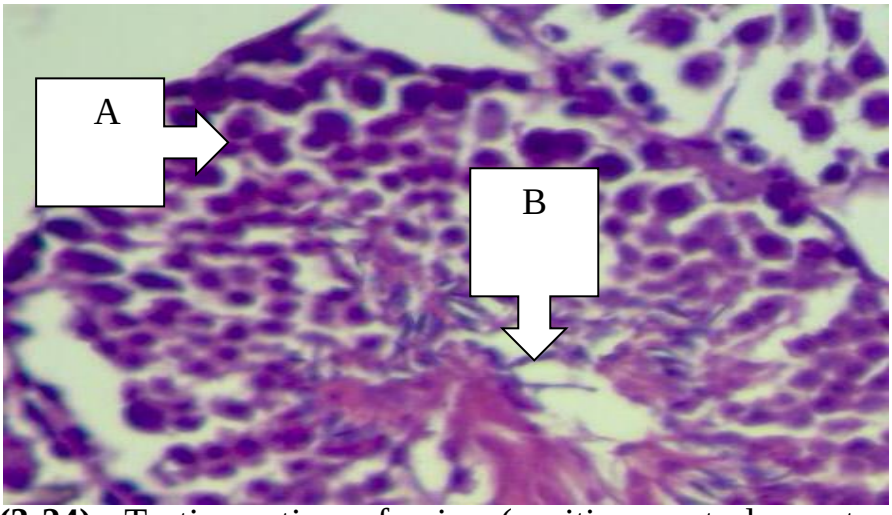


Figure (3-24): Testis section of mice (positive control, mesterolone) Showing normal maturation of spermatogonia cells (A) with presence of sperm (B) (H and E; 400X).

Mice treated with plant extract at dose 100 mg/kg showed the presence of normal appearance of seminferous tubules lumen, spermatogonia, sperms, seminferous tubules and increased in leydig cells

(figure 3-26), while mice treated with plant extract at dose 200 mg/kg showed the presence of an increased in the number of germ layers and the presence of large numbers of mature sperms in the lumen and an increase in the numbers of leydig cells and sperms (figure 3-27)

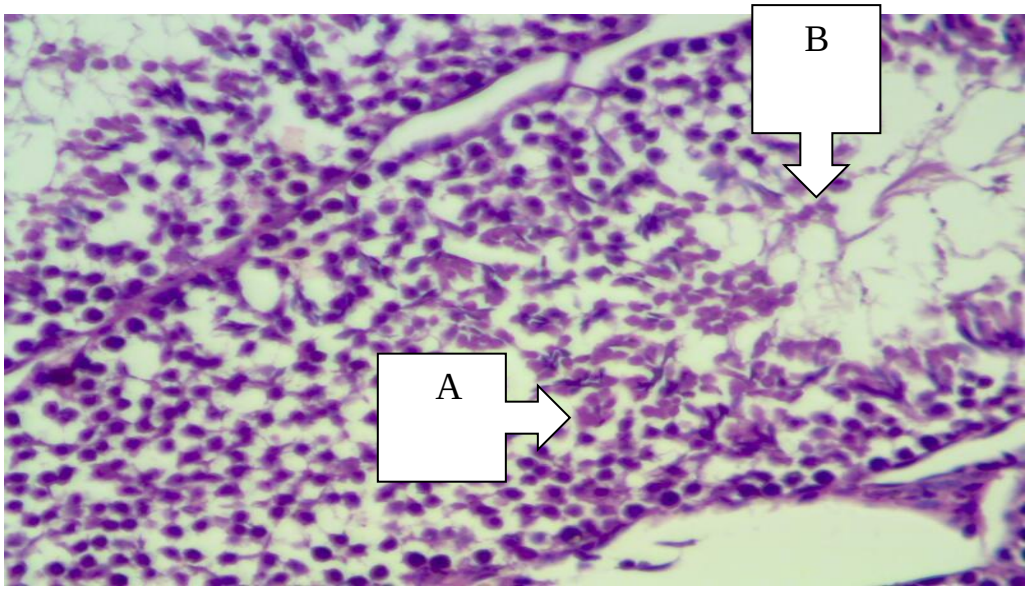


Figure (3-25): Testis section of mice (positive control, ginseng drug) showing normal maturation of spermatogonia cells (A) with presence of sperm (B) (H and E; 200X).

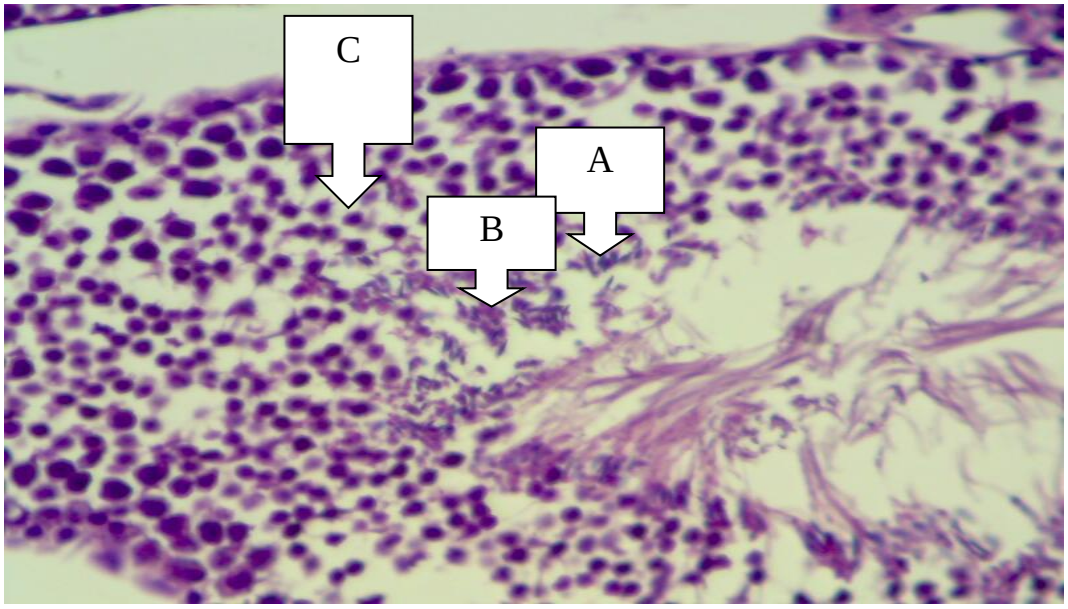


Figure (3-26): section of testis in mice treated with plant extract at dose 100 mg/kg showing well developed structure of somniferous tubules which consist of numerous of sperms (A) spermatid (B) spermatogonia (C) (H and E ;400X).

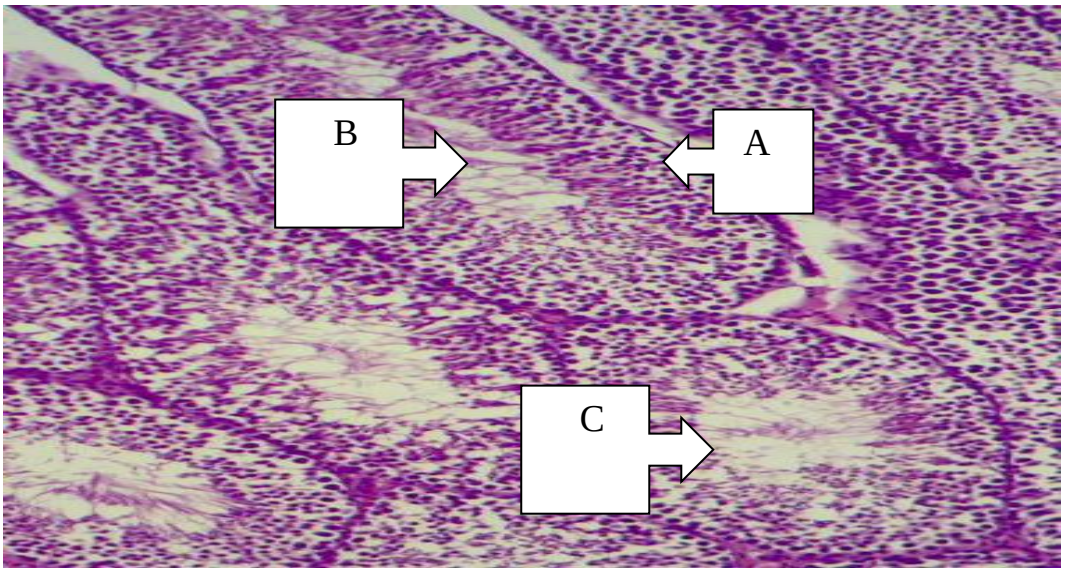


Figure (3-27): section of testis in mice treated with plant extract at dose 200 mg/kg showing well developed structure of somniferous tubules (A) which consist of numerous of sperms (B) spermatogonia (C) (H and E ;200X).

Mice treated with high dose 300 mg/kg of plant extract showed the presence of necrosis occurs in germ cell layers with affect in the sperms inside the lumen (figure 3-28).

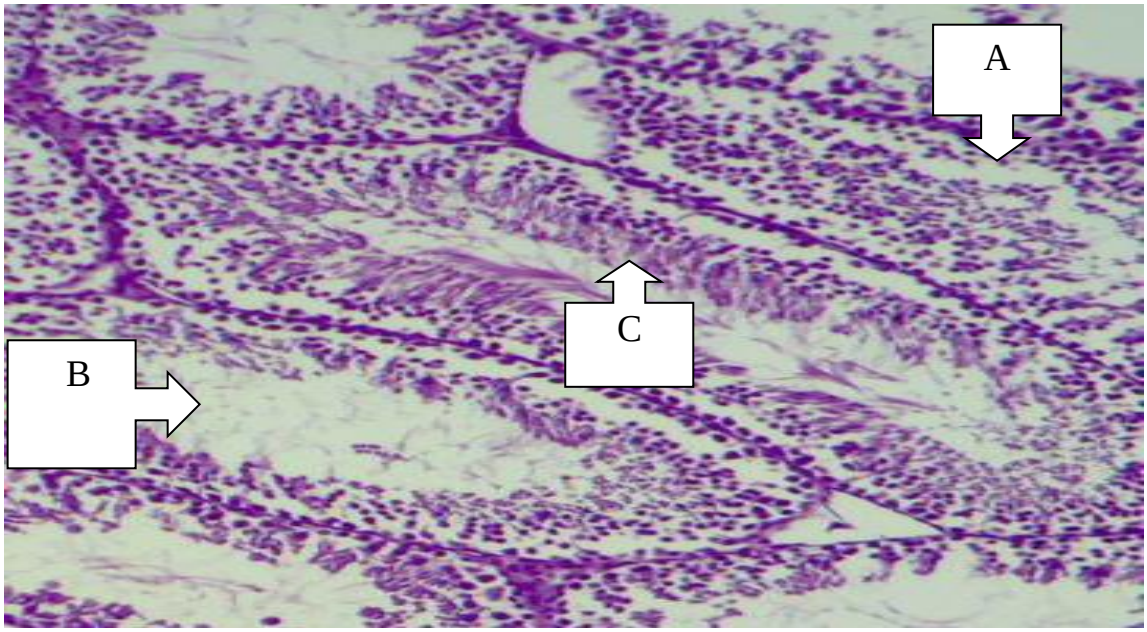


Figure (3-28): section of testis in mice treated with plant extract at dose 300 mg/kg showing immaturation of spermatogonia cell (A) with affect

in the sperms inside the lumen (B) with necrosis debris (C) (H and E ;400X).

The pathological study of testis, Liver and kidney in controls show no significant changes because the mice don't exposure to any arm matter and in mice treated with plant extract at doses 100 and 200 mg/kg also show no significant changes these two doses were very active and causing an increase in serum testosterone level and increase in viability, morphology and count of sperms, this is because the plant extract contained active compound such as flavonoids (Yang, 2008), that act as antioxidant (Mi and Zhang, 2005; Chandel *et al*., 2008), fight all oxidative damage that causes damaging to cells. Also the plant extract have nutrient that used as a tonic for the body (Kamal, 2008). While the plant extract at dose 300 mg/kg showing the presence of negative changes this may because the plant extract at high dose have opposite effect and toxic if used for prolonged periods (Oyagbemi *et al.*, 2013).

M. oleifera had anti-nephrotoxic effect (Paliwal *et al*., 2011). *M. oleifera* leaf extracts produce no harmful effect on the kidney of mice even in cases of chronic administration (Ezejindu *et al.*, 2014). *M. oleifera* leaf extracts have potency in protecting the liver from chemical toxicity and damage (Buraimoh, 2011). However the using of high dose has opposite effect and toxic if used for prolonged periods caused hepatic and kidney damage (Oyagbemi *et al*., 2013).

Chapter four

Conclusions and Recommendations

4.1. Conclusions

1- Leaves of *M. oleifera* were rich with flavonoid, total estimated flavonoid 22.5 mg\g represent 2.25% of the total extract.

2- Different classes of active compounds were detected in *M. oleifera* leaves methanolic extract including alkaloids, flavonoids, saponins terpenes, steroids glycosides, and tannins are considered to be responsible for it's effect on.

3-Active compounds of methanolic extract of *M. oleifera* leaves can increase testosterone level in serum after 15 days of oral ingestion. The plant extract caused increase in sperms count, viability at two doses 100 and 200 mg/kg when compared with the controls. So *M. oleifera* can enhance fertility in male mice however, it may also cause certain toxicities if it is used at high dose for a long period of time.

4- Some variation were observed in testes due to effect of *M. oleifera* in treated mice including an increasing in leydig cells and an increasing in the number of germ layers

4.2. Recommendations

1- Purification of different active compounds of *M. oleifera* leaves.

2- Qualitative and quantitative study of different active compounds presents in *M. oleifera* leaves.

3- Further studies on the effects of *M. oleifera* on different body hormones, adrenal gland and male reproductive system.

4- Study different pharmacological effects of plant extract including cytotoxic effect.

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الخلاصة

صممت هذه الدراسة إلى معرفة تأثير مستخلص أوراق شجرة البان على خصوبة ذكور الفئران البيض. أعد مستخلص أوراق شجرة البان بعملية النقع في محلول 70% الميثانول، بعدها تم الكشف الكيميائي عن المركبات الفلافونويد، القلويدات والتانين، الصابونين، جليكوسيدات، تربين والستوريدات. تم عزل الفلافينويد من مستخلص اوراق شجرة البان. ساعد استخدام تقنية الكروماتوكرافي الطبقة الرقيقة في الكشف عن وجود الكوارستين, الروتين واليوتيولين من الفلافينويد المعزول من المستخلص وقد عززت هذه النتائج عن طريق استخدام تقنية الكروماتوكرافي السائل عالي الجودة في الكشف عن وجود الكوارستين و الروتين من الفلافينويد المعزول من المستخلص. تم حساب كمية الفلافينويد الكلية في مستخلص اوراق شجرة البان باستخدام تقنية التحليل الطيفي. تم توزيع 30 من الفئران البيضاء وزنها (20-25غرام) بالتساوي إلى ستة مجاميع، المجموعة الاولى : (السيطرة السلبية) الفئران التي عولجت بالماء, المجموعة الثانية: (السيطرة الايجابية) الفئران التي عولجت مع 0.36 ملغم/كغم من عقار الميسترلون, المجموعة الثالثة: (السيطرة الايجابية) الفئران التي عولجت مع 0.5 ملغم/كغم من عقار جنسنك, المجموعة الرابعة: الفئران التي عولجت مع 2.5 ملغم / مل من مستخلص اوراق الشجر الميثانولي (100 ملغم/كغم), المجموعة الخامسة: الفئران التي عولجت مع 5 ملغم / مل من مستخلص اوراق الشجر الميثانولي (200 ملغم/كغم), المجموعة السادسة: الفئران التي عولجت مع 7.5 ملغم / مل من مستخلص اوراق الشجر الميثانولي (300ملغم/كغم), كان يعطى هذا المستخلص عن طريق الفم لمدة 15 يوما.

وتم دراسة تاثير المستخلص الميثانولي على الحيوانات المنوية للفئران بما في ذلك تركيز الحيوانات المنوية، نسبة حيوية الحيوانات المنوية ونسبة الحيوانات المنوية المشوهة، بالاضافة الى فحص تركيز هرمون التستوستيرون في الدم، بعدها اجريت دراسة مرضية نسيجية للكبد والكلى والخصى.

واظهرت النتائج زيادة ذات مغزى ($P \leq 0.0\ 5$) في تركيز الحيوانات المنوية بعد العلاج في الفئران التي عولجت بالمستخلص النباتي وبجرعات 100 و200 ملغم /كغم مقارنة بالسيطرة السلبية والايجابية و ايضا بالمقارنة مع المجموعة الاخرى التي عولجت مع المستخلص و بجرعة 300 ملغم/ كغم.

وأظهرت النتائج أن زيادة ذات مغزى ($P \leq 0.05$) في الحيوانات المنوية الميتة بعد العلاج مع المستخلص النباتي بجرعة 300 ملغم / كغم مقارنة بالسيطرة السلبية والايجابية و ايضا بالمقارنة مع المجموعات الاخرى التي عولجت بالمستخلص النباتي وبجرعات 100 و200 ملغم / كغم.

زيادة ذات مغزى ($P \leq 0.05$) في هرمون التستوستيرون في مصل الفئران التي عولجت بالمستخلص النباتي وبجرعات 100 و 200 ملغم / كغم بالمقارنة مع السيطرة السلبية والايجابية وأيضا بالمقارنة مع مجموعة أخرى التي عولجت بالمستخلص وبجرعة 300 ملغم / كغم.

كما اجريت دراسة مرضية للانسجة الكبد, الكلى والخصى في الفئران التي عولجت بالمستخلص النباتي وبجرعات 100 و 200 ملغم / كغم لم تظهر تغيرات مرضية اضافة الى السيطرة السلبية والايجابية لجميع الانسجة ، بينما في جرعة 300 ملغم / كغم تبين وجود تغييرات سلبية .

بسم الله الرحمن الرحيم

{يَهَبُ لِمَنْ يَشَاء إِنَاثًا وَيَهَبُ لِمَن يَشَاء الذُّكُورَ **(49)** أَوْ يُزَوِّجُهُمْ ذُكْرَانًا وَإِنَاثًا وَيَجْعَلُ مَن يَشَاء عَقِيمًا إِنَّهُ عَلِيمٌ قَدِيرٌ **(50)**}

صدق الله العظيم

سورة الشورى الايات (49-50)

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة النهرين/كلية العلوم
قسم التقانة الاحيائية

دراسة كيموحيوية ونسيجية مرضية لمستخلص اوراق شجرة البان
على خصوبة ذكور الفئران البيضاء

رسالة

مقدمة الى كلية العلوم - جامعة النهرين وهي جزء من متطلبات نيل درجة الماجستير في علوم التقانة الاحيائية

من قبل

نهى بشير خضير
بكالوريوس تقانة احيائية (2013)

بإشرف

ا.د. نبيل خلف العاني

جماد الاخر-1437 اذار-2016