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EFFECTS OF LIBYAN TRADITIONAL PLANTS ON THE REPRODUCTIVE SYSTEM OF MALE AND FEMALE RATS

by

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A dissertation submitted in fulfillment of the requirements for the degree of

Philosophiae Doctor (PhD)

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Faculty of Natural Sciences

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December 2015

KEY WORDS

Medicinal plants

Antioxidants

Radish seed (*Raphanus sativus Linn*)

Black seed (*Nigella sativa L*)

Flaxseed (*Linum usitatissimum*)

Date palm pollen (*Phoenix dactylifera L*)

Nutmeg (*Myristica fragrans*)

Radish seed (*Raphanus sativus Linn*)

Reproduction

Fertility

Sperm function

Hormones

DECLARATION

I, Abdalla Ramadan Elgenaidi, hereby declare that the dissertation “Effects of Libyan traditional plants on the reproductive system of male and female rats” hereby submitted by me for the PhD degree in Medical BioSciences at the University of the Western Cape has not been submitted previously at this or any other university, and are true record of the work carries out myself and all sources of information acknowledged.

Abdalla Ramadan Elgenaidi

Date.....

Signed.....

DEDICATION

I would like to dedicate this work to my late father, Ramadan Elgenaidi you were my source of inspiration and will always remember you for this. May the almighty God (Allah) bless you.

ACKNOWLEDGEMENTS

* First of all, thanks to God (Allah), the Lord of the universe, the most Merciful, who blessed me throughout my life and give me good health, patience and the strength to complete this study.
* I am endlessly grateful to my home country Libya for the financial support without which it would have been impossible to do this work.
* All my appreciation and admiration cannot be expressed in simple words to my supervisor, Professor TK Monsees, for accepting me as his PhD student; for his guidance, support and encouragement contributed towards the completion of my thesis.
* Special thanks to my co-supervisor, Professor R. Henkel, Head of Department of Medical Biosciences, for his support and encouragement.
* I am grateful to my lab colleagues, Dr CS Opuwari and Dr Nicole Sibuyi for their untiring support.
* Thanks very much to Dr Ahmed Hussein from the chemistry department, for training me in ethanolic plant extraction.
* Special thanks go to Dr Mervin Meyer from the Department of Biotechnology for giving me space in his laboratory.
* I am also grateful to Professor van der Horst and Dr. L. Maree for providing me with the relevant training in the use of the SCA software.
* I would like to sincerely thank my friends and colleagues, Emhemed Abukhttalla, Mohamed Elshowesh, Nicole Haines, Thomson Msiska, and Yesmein Alshibani.
* Last but not the least, many thanks should go to my lovely mother for being a pillar of my strength.
* To my dear wife, thanks for the support and patience you have given me during the years of my PhD studies. You were a great inspiration and may the almighty God (Allah) bless you abundantly.
* To the rest of my family members and friends, thank you for your support.

Abdalla R Elgenaidi

ABSTRACT

EFFECTS OF LIBYAN TRADITIONAL PLANTS ON THE REPRODUCTIVE SYSTEM OF MALE AND FEMALE RATS

In different parts of the world, medicinal plants have demonstrated a lot of health benefits to mankind and remains an important source for the discovery of new bio-active compounds. Libya is a typical example of a country where medicinal plants are widely used.

Plant extracts of five Libyan medicinal plants were used in this study to investigate their *in vivo* effects on spermatogenesis and steroidogenesis in male rats and on ovulation and fertility in female rats. The *In vitro* effects of these plant extracts were also investigated on TM3 Leydig cells and MCF 7 breast cancer cells.

A phyto-chemical analysis of the five Libyan medicinal plants (flaxseed, black seeds, radish seed, date palm pollen and nutmeg) was done. The results showed that date palm pollen had a higher antioxidant activity than all of the above mentioned plants. In addition to this, *Nigella sativa* was observed to possess high flavonol content as well as high antioxidant activity.

Male rats exposed to flaxseed, radish seeds and date palm pollen showed no significant alterations in body weight gain, whereas date palm pollen (240 mg/kg, $p < 0.05$) promoted an increase in body gain. This study also revealed a significant increase in the relative testicular weight of animals exposed to either flaxseed (300mg/kg) or date palm pollen (120mg/kg). In addition, the relative weights of the seminal vesicles of all treated groups showed significant increased values. The level of serum testosterone showed a significant increase after exposure to radish seed (80mg/kg) and a significant dose- dependent increase for date palm pollen when compared to control ($P< 0.05$). In contrast, flaxseed caused a dose-dependent significant ($p <0.01$) decrease in testosterone level at radish seed (300mg/Kg). All plant extracts caused a significant increase in sperm concentration. Sperm vitality significantly ($p < 0.05$) increased by radish seed (80mg/kg), flaxseed (300mg/kg)

and date palm pollen (120, 240mg/kg) respectively. Total progressive motility improved significantly at flaxseed (300 mg/kg) ($p < 0.001$) as well as date palm pollen ($p < 0.01$). Histological examination of the cross sections of the testis showed clear presence of all stages of spermatogenesis in all the treated groups. Rat epididymides showed normal morphological appearance and their lumen were filled with spermatozoa. The diameter of seminiferous tubules in male rats exposed to date palm pollen (120 and 240 mg/kg) was significantly higher ($p < 0.001$). The heights of the germ cell epithelia within the seminiferous tubules were also significantly increased in all treated groups. Liver and renal functions tests showed a significant decrease in Alanine transaminase (ALT) and creatinine in all treated groups ($p < 0.05$), and this demonstrates the lack of cytotoxic effects of date palm pollen, radish seed and flaxseed on the rats. However, these plant extracts produced a non-significant ($p > 0.05$) increase in Aspartate transaminase (AST) levels. Besides this, superoxide dismutase activity (SOD) in testis was increased significantly by radish seed (160 mg/kg), flaxseed (200 mg/kg) and date palm pollen (120 mg/kg). There was also improved catalase activity in testis of male rats exposed to radish seed and date palm pollen.

Regarding male sexual behavior, the time to reach the female and the mount frequency decreased significantly in male rats exposed to flaxseed (300 mg/kg) and date palm pollen (120 and 240 mg/kg; $p > 0.05$) thus, these plant extracts exhibit aphrodisiac properties. In addition, exposure of male rats to date palm pollen (120 mg/kg) produced a significant ($p < 0.01$) increase in the number of embryos in untreated female rats.

In the female rats, the body weight gain was not affected ($p > 0.05$). However, the relative uterus weights exposed to nutmeg (200 mg/kg) and date palm pollen (120 and 240 mg/kg) were significantly decreased ($p < 0.05$). In addition, the relative weights of ovaries after treatment with nutmeg (400 mg/kg) and black seed (400 mg/kg) showed significantly increased values ($p < 0.01$). Serum FSH was significantly increased ($p > 0.05$ or 0.01) when the female rats have been exposed to black seed (200 mg/kg), nutmeg (200 mg/kg) or date palm pollen (120 mg/kg). The LH level significantly ($p < 0.01$) decreased following exposure to black seed

(200 mg/kg), date palm pollen (120 mg/kg). On the other hand, serum LH concentration was significantly increased in female rats exposed nutmeg (400 mg/kg; $p > 0.05$). The creatinine activity in female rat serum in all treated groups was significantly decreased ($p < 0.05$). Whereas the higher dose of date palm pollen (240 mg/kg) caused only a non-significant decrease. ALT activity in serum of female rat exposed to either black seed (400 mg/kg) or date palm pollen (120 and 240 mg/kg) was shown to decrease significantly ($p < 0.05$). Histology of the reproductive organs, kidney and liver in the female rats showed no obvious alterations in any of the treated groups. In addition, the number of embryos in female rats significantly increased ($p < 0.01$; $p < 0.001$) following exposure of female rats to black seeds 400 and date palm pollen 240 mg/kg, respectively.

Incubation of TM3 Leydig cells with radish seeds for 24, 48 or 72 hours caused a significant ($p < 0.01$) decrease in mitochondrial dehydrogenase activity. Besides that, date palm pollen and flaxseed increased the mitochondrial dehydrogenases activity of TM3 Leydig cells. In addition, higher concentration of date palm pollen, nutmeg and black seed were cytotoxic to MCF7 breast cells. In testis slices testosterone secretion *in vitro* was significantly increased by flaxseed (500 µg/ml; $p > 0{\cdot}05$) and date palm pollen (500 µg/ml; $p > 0{\cdot}01$). MCf-7 cells treated with BS 10-50 µg/ml black seed and nutmeg 10-50µg/ml significantly increased cell proliferation. However, the treatment with date palm pollen produced only a weak estrogenic effect, which resulted in a concentration dependent significant increase as observed between 50-1000 µg/ml date palm pollen.

In conclusion, in this study, we observed that date palm pollen, radish seed and flaxseed increased libido as well as steroidogenesis and spermatogenesis, improved hepato and nephron-protective effects. In female rats, the plant extracts NM, BS and date palm pollen potentiated the production of gonadotropic hormones. In addition to this, at lower concentrations these medicinal plants promoted cell growth, whereas at higher concentrations they inhibited cell proliferation of MCF-7 breast cancer cells. The anti-oxidant effects of these plant extracts have been implicated for the above mention effects.

CONFERENCE PROCEEDINGS

- A.R Elgenaidi, T.K Monsees (2014): *In vivo* effects of traditional Libyan plants on the male rat reproductive system, 42th Congress of the Physiology Society of Southern Africa PSSA, 14.-17.09.2014, Durban, South Africa, abstract book p 23.

- A.R Elgenaidi, T.K Monsees (2014): The effects of ethanol extracts of Date palm pollen, Flaxseeds and Radish seeds on the male rat reproductive system, UWC Science Faculty Postgraduate Research Open Day, 28 October 2014, Bellville, South Africa, abstract book p 21.

- A.R Elgenaidi, T.K Monsees (2015): *Phoenix dactylifera, Linum usitatissimum*, and *Raphanus sativus* Significantly Improve Sexual Behavior and Fertility in Male Rats, UWC Science Faculty Postgraduate Research Open Day, 22 October 2014, Bellville, South Africa, abstract book p 34.

LIST OF ABBREVIATIONS

ABP	Androgen-binding protein
ALT	Alanine transaminase
AST	Aspartate transaminase
BCF	Beat cross frequency
BHT	Butylated hydroxytoluene bromide
BS	Black seed
BSA	Bovine serum albumin
BW	Body weight
Ca_2+	Calcium
CAT	Catalase
CBB	Coomassie Brilliant Blue G
CD	Charcoal-dextran
CO_2	Carbon dioxide
Cu	Copper
dH_2O	Distilled water
DHS	Demographic and Health Surveys
DHT	Dihydrotestosterone
DMACA	4-(Dimethylamino)-cinnamaldehyde
DMEM/F-12	Dulbecco's modified eagle's medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DPP	Date palm pollen
DPPH	2, 2-diphenyl-1-picrylhydrazy
EDTA	Ethylenediaminetetraacetic acid
F-C	Folin-Ciocalteaus reagent
FS	Flaxseed

FSH	Follicle stimulating hormone
GAE	Gallic acid equivalent
GC–MS	Gas chromatography-mass spectrometry
GnRH	Gonadotropin releasing hormone
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathione
GSSH	Oxidized glutathione
H+	Hydrogen ion
H_2O_2	Hydrogen peroxide
HCG	Human chorionic gonadotropin
HCl	Hydrochloric acid
hGH	Human growth hormone
HNO_2	Nitrous acid
HO_2•	Hydroperoxyl
HOCl	Hypochlorous acid
LH	Luteinizing hormone
LIN	Linearity
LOO•	Lipid peroxyl
LOOH	Lipid peroxide
LPO	Lipid peroxidation
M	Molar
MDA	Malondialdehyde
MF	Mount frequency
ML	Mount latency
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium
N	Normality
Na_2CO_3	Sodium carbonate

Na_2HPO_4	Disodium hydrogen phosphate
NaCl	Sodium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate
NaH_2PO_4	Anhydrous monobasic sodium phosphate
NaOH	Sodium hydroxide
nm	Nanometer
NM	Nutmeg
NO	Nitric oxide
NO_2•	Nitrogen dioxide
O_2	Oxygen
O_2-	Superoxide anion
O_3	Ozone
OH-	Hydroxyl radicals
ONOO-	Peroxynitrite anion
OSS	Oxidative stress status
PBS	Phosphate buffered saline
PI	Propidium iodide
RO_2•	Peroxyl
ROS	Reactive oxygen species
RS	Radish seed
RT	Room temperature
SCA	Sperm Class Analyzer
SDG	Secoisolariciresinol diglucoside
sec	Second
SOD	Superoxide dismutase
SST	Serum separator tube
TBHQ	Ter-butyl thymoquinone
TM	Traditional medicine

TPC	Total polyphenols activity
TPTZ	2,4,5-Tri (2-pyridyl)-S-triazine
TQ	Thymoquinone
v/v	Volume per volume
VAP	Average path velocity
VCL	Curvilinear velocity
VSL	Straight-line velocity
w/v	Weight per volume
WHO	World Health Organization
WOB	Wobble
Zn	Zinc

TABLE OF CONTENT

LIST OF FIGURES

The in vitro antioxidant assay performed on this plant reveals significant antioxidant potential compared with black seed, flaxseed, nutmeg, radish seed and date palm pollen as a standard butylated hydroxytoluene (BHT). DPPH

LIST OF TABLES

CHAPTER I

1 GENERAL INTRODUCTION

1.1 Medicinal plants

According to the World Health Organization (WHO 2005) report traditional medicine (TM) encampuses all medicines that fall under Chinese medicine, Indian ayurvola and Arabic unani medicine and to various forms of indigenous medicine. Historical circumstances and cultural beliefs have formed the basis for continued use of TM in Asia and Latin America. A good number of the Chinese (40%) and Africans (80%) use TM to meet their health care needs (WHO 2005). The use of herbs or plants to treat diseases in Arabic countries on a broader scale and Libya on a minor scale dates long time in history (Elmestiri, 2007; Kotb, 1985).

Culture, tradition, and religion affect many aspects of the people's lives including reproduction and fertility. The fragile balance between what the individual needs and what society finds to be normal can have a profound impact on personality, coping, wellbeing and sexual behavior. Furthermore, the cultural differences can impact the sex drive in an infertility context (Papreen et al., 2000). Childlessness is a big issue in many societies (Sundby, 1997). And because of this many couples seek medical help in order to solve this problem (Ikechebelu et al., 2003).

Scientific evidence suggests that around 80 % of Africans depend on traditional healthcare practitioners and medicinal plants for their daily healthcare needs (Johnson et al., 2007; McKay and Blumberg, 2007). Natural products have reduced pain, suffering and revolutionized the practices of medicine. In respect of this, more than 60% of permitted and pre-new drug application (NDA) candidates are either natural products or related to them. (Demain, 1999). In spite of obvious deficiency of scientific data for African herbal medicine's quality, safety and efficacy, they are still widely used (Johnson et al., 2007). To this extent pharmacological exploration of some African traditional medicinal plants has shown that many of

these medicinal plants possess therapeutic attributes which are safer than the current conventional medicines used for the treatment of erectile dysfunction and infertility. On the contrary, most of the conventional medicines are associated with serious side effects including headache, facial flushing, hypotension, myalgia and dyspepsia (Supuran et al., 2006).

Research on traditional medicinal plants has shown that their potential to improve male fertility is partially due to presence of antioxidants. These antioxidants have been noticed to improve several processes (spermatogenesis, steroidogenesis) of male reproductive function (Nantia et al., 2009). Medicinal plants are used either alone or as formulation of several plants to treat various forms of male sexual dysfunctions. In respect of this, a combination of plant formulations has been observed to treat idiopathic infertility (Agrawal and Kulkarni, 2003; Rama Devi et al., 2004; Tempest et al., 2005; Xu et al., 2003).

Plant-based traditional medicines have played an important role in treatment of many diseases and in health care for centuries. In developing countries, in addition to the great importance of using plants in health care, it is considered as the source for discovery of new drugs for human diseases. Plants are esteemed as the greatest source of natural antioxidants (Anwar and Przybylski, 2012; Assa and Widjanarko, 2014; Demiray et al., 2009). People in the rural areas are more dependent on the nature than in the urban areas. The natures fulfil their essential demands through agriculture, hunting, fishing and also collecting medicinal plants for health problems. People in the rural area live closer to nature, and therefore medicinal plants and flora in general are well known by them (Alhassan, 2012). In developed countries the extensive use of herbal medicine is due to its natural source and its relative safety (Gurib-Fakim, 2006).

Libyan historical studies demonstrate that the eastern Mediterranean coastal region of has a rich supply of medicinal herbs (El-Mokasabi, 2014). To this extent Libyans have used medicinal plants as part of folk medicine besides spices and food flavors. In Libya there are many medicinal plants and examples include marjoram, sage,

nigella, myrtle, thyme, absinthe, clove, oak, karkade, and rosemary (Kotb, 1985). However, little or no research has been done to investigate their effects on fertility. It is in this light that we would like to investigate the effects of date palm pollen, *Phoenix dactylifera* (DPP), Nutmug, *Myristica fragrans* (NM), black seed, *Nigella sativa* Linn (BS), *Raphanus sativus*, Radish seed (RS) and Flaxseed or *Linum usitatissimum* (FS).

1.2 Reproductive System

1.2.1 The male reproductive system

The male reproductive system consists of:

- The testes, a series of ducts, accessory glands, and supporting structures. The ducts include the epididymides, the ducta deferentia and urethra.
- Accessory glands include the seminal vesicles, the prostate gland, and the bulbourethral glands.
- Supporting structures include the scrotum and the penis (Risbridger and Taylor, 2006).

The testis is a complex organ that serves two crucial functions:

a. Synthesis and secretion of testosterone.
b. Production of a sufficient number of competent spermatozoa to attain fertility.

The testes are contained in a scrotum which is located outside the body. The testes consist of seminiferous tubules, in which the sperm cells are produced in a process called spermatogenesis. The interstitial space is the apartment where Leydig cells responsible for the production of testosterone are located. The epididymides are there to provide a site for storage, gain of motility and maturation of spermatozoa (de Kretser et al., 1998).

The ducta deferentia lead from the testes into the pelvis, where they join the ducts of the seminal vesicles to form the ampullae. Extensions of the ampullae, called the ejaculatory ducts, pass into the prostate and empty into the urethra within the

prostate. The urethra then passes through the penis to the outside of the body (Risbridger and Taylor, 2006).

The accessory glands, testes and epididymides help in the formation of seminal fluid which is responsible for the transportation of spermatozoa, lubrication of the passage ways for spermatozoa, energy provision to the spermatozoa through fructose and buffers the sperm from the low pH of the female vaginal tract (de Kretser et al., 1998).

1.2.2 Hormonal Control of Testicular Function

Due to the cyclic course of spermatogenesis, spermatogenesis takes place in individual seminiferous tubules whereas steroidogenesis takes place in interstitial compartment in between the seminiferous tubules. The function of the testes and therefore the function of seminiferous tubules and interstitial compartment is mainly controlled by the hypothalamus and the pituitary gland (endocrine gland) (Risbridger and Taylor, 2006). At puberty, hormonal control of the testicular function involves hypothalamic neurosecretory cells. These cells increase the secretion of gonadotropin-releasing hormone (GnRH) which in turn stimulates gonadotrophs in the anterior pituitary to secrete more of luteinizing hormone (LH) and follicle stimulating hormone (FSH) (Figure 1.1).

LH stimulates interstitial cells to secrete the hormone testosterone. Via negative feedback, testosterone suppresses secretion of LH by anterior pituitary gonadotrophs and suppresses secretion of GnRH by hypothalamic neurosecretory cells. In some target cells, such as those in the external genitalia and prostate, the enzyme 5alpha-reductase converts testosterone to an androgen called dihydrotestosterone (DHT). FSH acts indirectly via receptors located on Sertoli cells to stimulate spermatogenesis (Figure 1.1). FSH and testosterone act synergistically on the Sertoli cells to stimulate secretion of androgen-binding protein (ABP) into the lumen of the seminiferous tubules and into the interstitial fluid around the spermatogenic cells. Testosterone stimulates the final steps of

spermatogenesis in the seminiferous tubules. Once the degree of spermatogenesis required for male reproductive functions has been achieved, Sertoli cells release inhibin, a protein hormone that inhibits FSH secretion by the anterior pituitary (Figure 1.1). If spermatogenesis is proceeding too slowly, less inhibin is released, which permits more FSH secretion and an increased rate of spermatogenesis (Holdcraft and Braun, 2004).

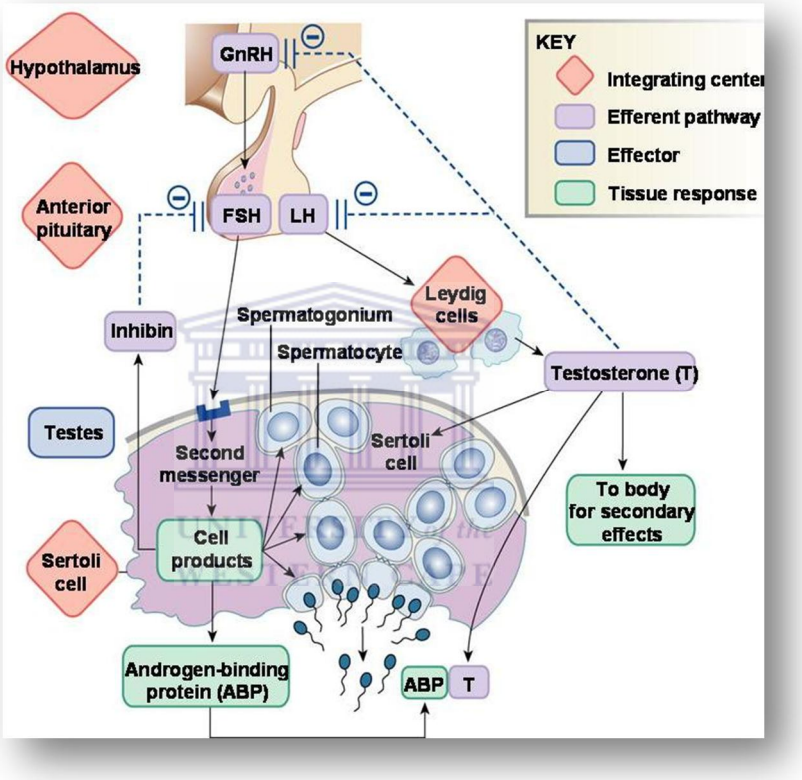

Figure 1.1: Hormones and negative feedback loops that control secretion of testosterone and spermatogenesis.

- The hypothalamus releases gonadotropin-releasing hormone (GnRH).
- GnRH stimulates the anterior pituitary to secrete FSH and LH. FSH causes sustentacular (Sertoli) cells to release androgen-binding protein (ABP).
- LH stimulates interstitial (Leydig) cells to release testosterone.
- ABP binding of testosterone enhances spermatogenesis.
- Feedback inhibition on the hypothalamus and pituitary results from rising levels of testosterone and increased production of inhibin.

Source: http://www.austincc.edu/apreview/PhysText/Reproductive.html# 10th May 2015

1.2.3 Sperm Cell Development

In most mammals, each spermatogenic cycle lasts around 9 to 12 days, whereas the total duration of spermatogenesis lasts nearly 40 to 54 days. Particularly in humans, the entire spermatogenic process is very long and lasts more than 74 days (Aslam et al., 1999).

Until puberty, the testes remain relatively simple and unchanged. During this time the interstitial cells are not prominent enough and the seminiferous tubules lack a lumen and are not yet functional. In boys 12–14 years of age, the interstitial cells increase in number and size, a lumen develops in each seminiferous tubule, and sperm cell production begins. The germ cells are the ones that divide and differentiate to form sperm cells during spermatogenesis. Also present the Sertoli cells that are responsible for the nourishment of the germ cells besides producing, together with the interstitial cells, a number of hormones such as androgens, estrogens, and inhibin. In between the Sertoli cells are tight junctions that form the blood-testis barrier which protects the sperm cells from being destroyed by the immune system. This barrier only develops at puberty (Vanputte et al., 2013).

Seminiferous tubules contain spermatogonia which divide by mitosis to produce more spermatogonia. Some of the daughter cells produced from these mitotic divisions remain as spermatogonia and continue to produce additional spermatogonia (Figure 1.2). Other daughter cells differentiate to form primary spermatocytes via meiosis. Each primary spermatocyte passes through the first meiotic division to become two secondary spermatocytes. Each secondary spermatocyte undergoes a second meiotic division to produce two even smaller cells called round spermatids (Figure 1.2). Each spermatid contains 23 pairs of homologous chromosomes of which 22 are autosomes and the remainder are either X or Y chromosome. During spermatogenesis, each spermatid develops a head, a mid-piece, and a tail (or flagellum) to become a spermatozoa (Holdcraft and Braun, 2004; Vanputte et al., 2013).

The head of the spermatozoa is unique in that it contains the nucleus whereas the mid- contains large numbers of mitochondria, which produce the ATP necessary for microtubule movement. The flagellum consists of microtubules that help in the swimming of the sperm. At the end of spermatogenesis, the developing sperm cells gather around the lumen of the seminiferous tubules, with their heads directed toward the surrounding Sertoli cells and their tails directed towards the center of the lumen (Vanputte et al., 2013).

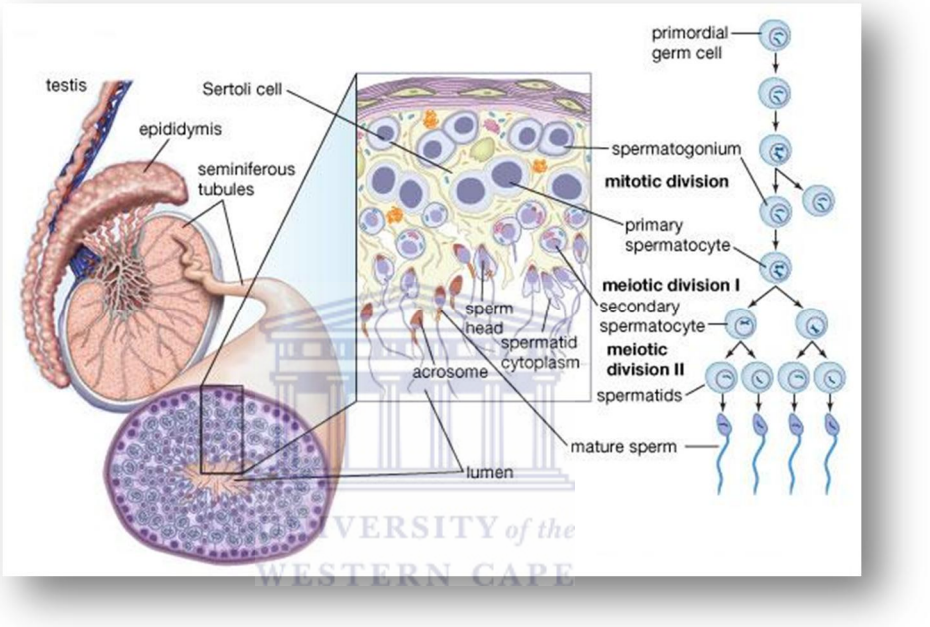

Figure 1.2: Seminiferous tubules and spermatogenesis

- A primary spermatocyte undergo meiosis I, forming two secondary spermatocytes.
- Secondary spermatocytes undergo meiosis II and their daughter cells are called spermatids. Spermatids are small round cells seen close to the lumen of the tubule.
- Spermatids only have 23 chromosomes and are said to be haploid (n chromosomal number).
- Gamete formation is by meiosis, in which the number of chromosomes is halved (from 2n to n). Thus, we say meiosis of spermatocytes forms spermatids.

Source: http://global.britannica.com/EBchecked/topic/559418/spermatogenesis# 10th May 2015

1.2.4 The female reproductive system

The female reproductive system consists of external and internal reproductive organs and mammary glands (Heikkil et al., 2001; Kalantaridou et al., 2004; Veras

et al., 2009). The internal reproductive organs are within the pelvis between the urinary bladder and the rectum (Aristophanous, 2010). They consist of the ovaries, the uterine tubes and the uterus. The vagina is on the midline with the ovaries on each side of the uterus. A group of ligaments holds the internal reproductive organs in place. The most conspicuous is the broad ligament, an extension of the peritoneum that spreads out on both sides of the uterus and attaches to the ovaries and uterine tubes. The external genitalia consists of the mons pubis, pubic hair, labia majora, labia minora, clitoris and vaginal opening (Vanputte et al., 2013).

1.2.5 Hormonal Regulation of the Female Reproductive Cycle

In women the gonadotropin-releasing hormone (GnRH) is secreted by the hypothalamus and controls the ovarian and uterine cycles (Figure 1.3). It stimulates the release of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) from the anterior pituitary (Christensen et al., 2012). FSH initiates follicular growth, while LH stimulates further development of the ovarian follicles. In addition, both FSH and LH stimulate the ovarian follicles to secrete estrogens. LH stimulates the theca cells of a developing follicle to produce androgens. Under the influence of FSH, the androgens are taken up by the granulosa cells of the follicle and are then converted into estrogens. At mid-cycle, LH triggers ovulation and then promotes formation of the corpus luteum, hence the name luteinizing hormone. Stimulated by LH, the corpus luteum produces and secretes estrogens, progesterone, relaxin, and inhibin (Christensen et al., 2012; Vanputte et al., 2013) Estrogens secreted by ovarian follicles have several important functions (Figure 1.3).

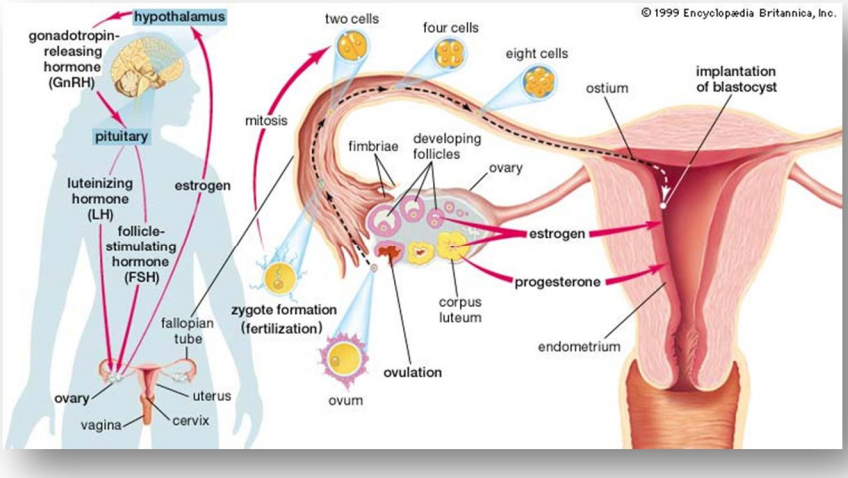

Figure 1.3: Hormonal regulation of the female reproductive cycle.

- GnRH stimulates the release of FSH and LH which stimulate follicle growth and maturation, and low-level estrogen release.
- Rising estrogen levels inhibit the release of FSH and LH and prod the pituitary to synthesize and accumulate these gonadotropins.
- Estrogen levels increase and high estrogen levels have a positive feedback effect on the pituitary, causing a sudden surge of LH.
- The LH spike stimulates the primary oocyte to complete meiosis I, and the secondary oocyte continues on to metaphase II.

Source:http://global.britannica.com/EBchecked/topic/478202/progesterone 10th May 2015

•They promote the development and maintenance of female reproductive structures, secondary sex characteristics, and the breasts.

• Increase protein anabolism, including the building of strong bones. In this regard, estrogens are synergistic with human growth hormone (hGH).

• Lower blood cholesterol level, as observed in women under age >50 these women have a much lower risk of coronary artery disease than men of comparable age.

• Moderate levels of estradiol, progesterone and androgen in the blood inhibit both the release of GnRH by the hypothalamus and secretion of LH and FSH by the anterior-pituitary. Progesterone, secreted mainly by cells of the corpus luteum, cooperates with estrogens to prepare and maintain the endometrium for implantation of a fertilized ovum and to prepare the mammary glands for milk secretion. High levels of progesterone also inhibit secretion of GnRH and LH. The

small quantity of relaxin produced by the corpus luteum during each monthly cycle relaxes the uterus by inhibiting contractions of the myometrium. During pregnancy, the placenta produces large quantities of relaxin which help in relaxing uterine smooth muscle, increase the flexibility of the pubic symphysis and dilate the uterine cervix during delivery of the baby. Inhibin is secreted by granulosa cells of growing follicles and by the corpus luteum after ovulation. It inhibits secretion of FSH and, to a lesser extent LH (Vanputte et al., 2013).

1.2.6 Oogenesis and Fertilization

The formation of female gametes begins in the fetus. In human, the fourth month of development, the ovaries contain 5 million oogonia. By the time the baby is born many of the oogonia are degenerated and the remaining ones begin to undergo meiotic division leading to the production of primary oocyte. From birth to puberty, the number of primary oocytes decreases to around 300,000 – 400,000. Only about 400 primary oocytes will complete development and give rise to the secondary oocytes that are eventually released from the ovaries (Sathananthan et al., 2006).

1.2.7 Ovulation

Ovulation is the release of a secondary oocyte from an ovary. Just before ovulation, the primary oocyte completes the first meiotic division to produce a secondary oocyte and a polar body. Unlike meiosis in males, cytoplasm is not split evenly between the two cells. The secondary oocyte contains much more of the cytoplasm. Present in the cytoplasm are organelles such as mitochondria and nutrients that increase the viability of the secondary oocyte (Figure 1.4). The polar body either degenerates or divides to form two polar bodies. Eventually, the polar bodies degenerate. The secondary oocyte begins the second meiotic division, but it stops in metaphase II. After ovulation, the secondary oocyte may be fertilized by a sperm cell (Vanputte et al., 2013).

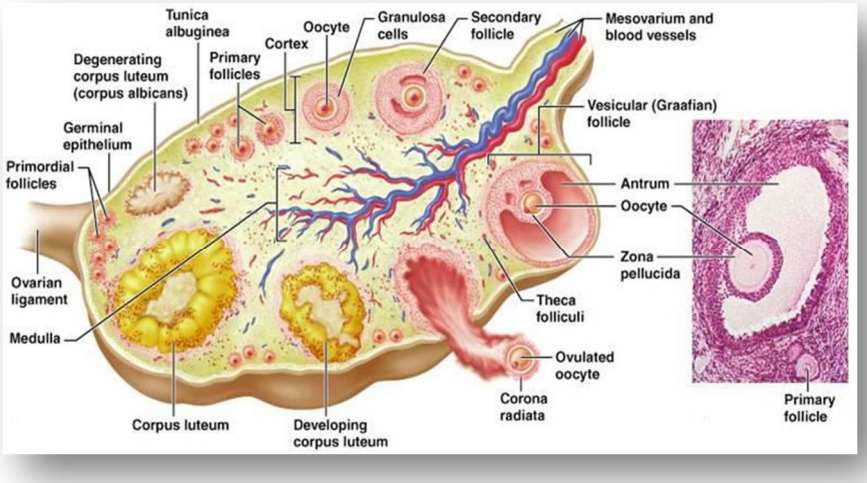

Figure 1.4: Cross section of ovary showed process of ovulation

Follicular Phase

- The primordial follicle, directed by the oocyte, becomes a primary follicle.
- The primary follicle becomes a secondary follicle.
- Ttheca folliculi and granulosa cells produce estrogens.
- The secondary follicle becomes a mature vesicular follicle (Graafian follicle).

Ovulation

- Occurs midcycle
- Occurs when the ovary wall ruptures and expels the secondary oocyte
- Mittelschmerz – a twinge of pain sometimes felt at ovulation
- 1-2% of ovulations release more than one secondary oocyte.

Source:http://www.austincc.edu/apreview/PhysText/Reproductive.html# **10th May 2015**

1.2.7.1 Reproductive cycle in rats.

Rats do not need the presence of males to induce ovulation. However there is some evidence that stimulation from male rodents can induce or indeed hasten ovulation. Rats have a 4-6 day cycle which consists of 4 stages – estrus, proestrus, diestrus I (a.k.a metestrus) and diestrus II (Nakatani et al., 1991).

Protestrus is defined by the presence of nucleated epithelial cells that are round. However, sometimes these cornify rapidly, especially in mice. Estrus (figure 1.5 & 1.6) is characterized by non-nucleated, cornified epithelial cells. Metestrus (or

Diestrus I) typically has a low cell number, often with a lot of cell debris. Diestrus II contains mostly lymphocytes (Nakatani et al., 1991).

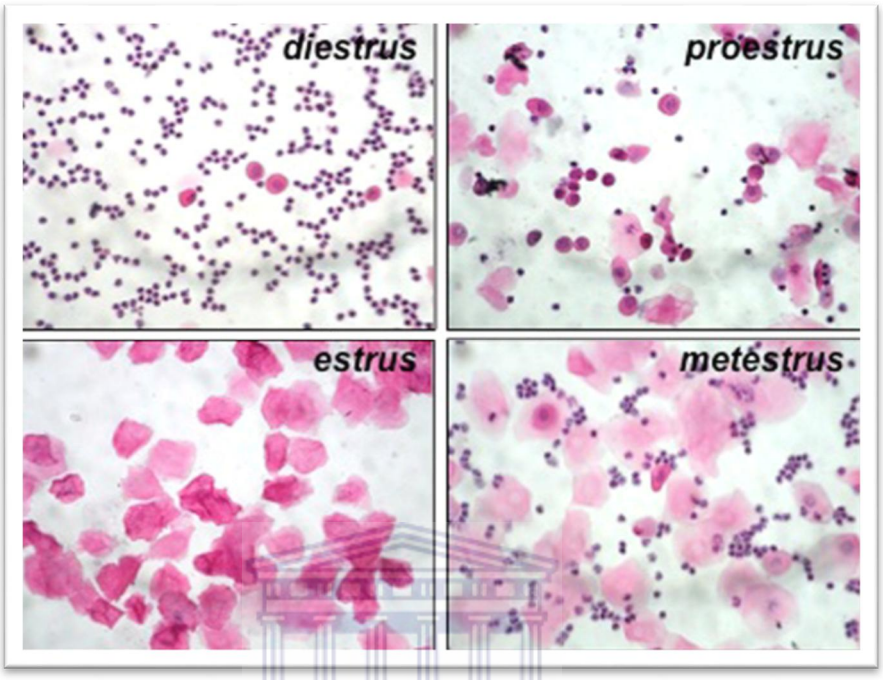

Figure 1.5: Representative pictures from hematoxylin/eosin-stained samples from vaginal lavage at each phase of the estrous cycle.

Source: Zenclussen et al., 2014

Stage	Ovary	Uterus	Vagina	Smear
Diestrus	Small follicles only are present with large corpora lutea from the previous ovulation. These secrete for only a very short time unless pregnancy or pseudopregnancy intervene.	Small and anaemic, low motility, lumen small and slit-like. Cells of the uterine mucosa columnar; polymorphonuclear leucocytes in stroma; endometrial glands collapsed, atrophic.	Epithelium thin, mitotic figures infrequent. Leucocytes abundant in stroma, migrate through the epithelium into vaginal lumen.	Stringy mucous in which are entangled many leucocytes and a few nucleated epithelial cells.
Proestrus	Some follicles grow rapidly.	Become more vascular, water content increases, organ distends. Contractility more pronounced. Epithelial cells become higher (continuing into estrus). Leucocytes disappear from mucosa. Endometrial glands hypertrophy.	Epithelium thickens, numerous mitoses in inner layers. Old layers of epithelium line the lumen. Leucocytes no longer migrate through the epithelium. Superficial epithelial cells slough off into lumen.	Largely small, round, nucleated epithelial cells, singly or in sheets. None to few leucoytes.
Estrus	Ovulation in the rat is spontaneous and occurs about 10 hours after the beginning of estrus. "Heat" (receptivity) lasts about 13 hours. Usually 10-20 eggs ovulated each time.	gains maximum vascularisation. Epithelial cells reach maximum development. No leucocytes.	Outer layer of epithelial cells become cornified and sloughed into the lumen. In early estrus these cells retain their nuclei, but in later stages no nuclei visible and the cells are irregular, flat, cornified plates. The skin around the vaginal orifice becomes swollen.	Contains hundreds of large cornified cells (squames) with degenerate nuclei. Towards the end of estrus the smear becomes "cheesy" - masses of adherent cornified cells.
Metestrus	Many corpora lutea, which secrete only for a very short time, and small follicles.	Epithelium continues vacuolar degeneration and replacement. Leucocytes in stroma. Decrease in size and vascularity.	Deeper layers of the estrous epithelium now line the lumen, the older, superficial layers having become cornified and sloughed off. Reduction of mitotic activity in epithelium. Leucocytes in stroma and migrating through the epithelium into the lumen.	Many leucocytes and a few cornified cells.

Figure 1.6: Estrus stages and cycle.

Source: https://embryology.med.unsw.edu.au/embryology/index.php/Mouse_Estrous_C 18th October 2015

1.2.8 Oogenesis and Follicular Development

Sobti (2008) describes oogenesis as a process that leads to the formation of a mature ovum (egg) from primordial germ cells. In brief, primordial germ cells migrate to the developing gonad to form oogonia (figure 1.7) which then undergoes mitotic division to form the primary oocyte. The primary oocyte mark the start of the first meiotic division in which DNA replication occurs with each chromosome having two chromatids. The division process is arrested at the prophase stage until the onset of sexual maturation at puberty. The division process start again at the onset of sexual maturation. The net effect is the formation of a secondary oocyte and its first polar body. This is followed by a second meiotic division and it leads to the formation of a haploid mature ovum and a second polar body. The mature ovum (egg) produced is then released at some point during ovulation.

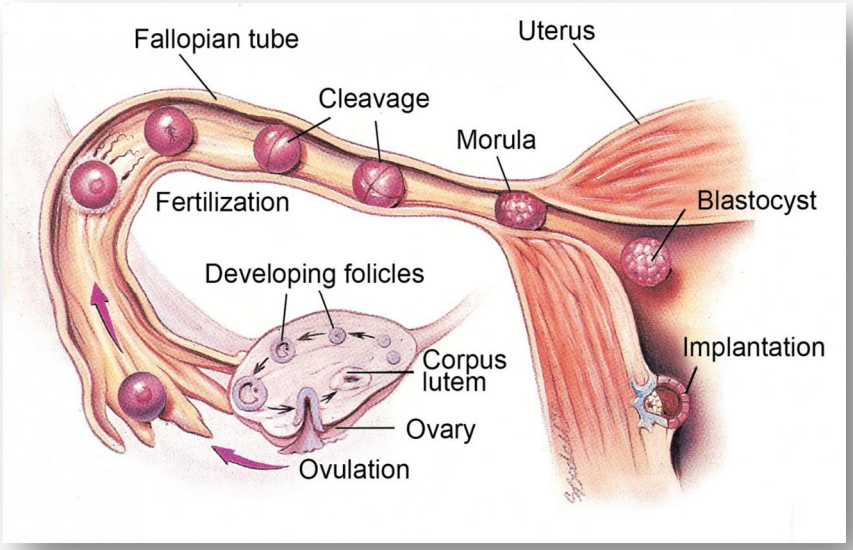

Figure 1.7: The processes of ovulation and fertilization.

- A zygote is the first cell of a new individual, the result of the fusion of DNA from sperm and egg.
- The zygote begins rapid mitotic cell divisions.
- The zygote stage is in the uterine tube, moving toward developmental stage from the start of cleavage until the ninth week.
- The embryo first undergoes division without growth the embryo enters the uterus at the 16-cell state and floats free in the uterus temporarily.
- Uterine secretions are used for nourishmen.

Source:https://online.science.psu.edu/sites/default/files/biol011/Fig-10-24-Ovulation.jpg
10th May 2015

1.2.9 Fertilization and Pregnancy

After sperm cells are ejaculated into the vagina during sexual intercourse, they are transported through the cervix, the body of the uterus, and the uterine tubes to the ampulla. The swimming ability of the sperm and muscle contractions of the uterus propel sperm cells through the female reproductive tract. During sexual intercourse, oxytocin is released from the posterior pituitary of the female, and the semen introduced into the vagina contains prostaglandins. Both of these hormones stimulate smooth muscle contractions in the uterus and uterine tubes. While passing through the vagina, uterus, and uterine tubes, the sperm cells undergo capacitation, the removal of proteins and the modification of glycoprotein's of the sperm cell

plasma membranes. After ovulation, local currents are produced by movements of the fimbriae, which surround the surface of the mature follicle just before ovulation occurs. These currents sweep the ovulated secondary oocyte from the peritoneal cavity into the uterine tube. A sperm cell usually encounters and fertilizes a secondary oocyte in the ampulla of the uterine tube. Fertilization can occur up to about 24 hours after ovulation. Some hours after fertilization, the nuclear materials of the haploid ovum and sperm unite. The diploid fertilized ovum is now called a zygote and begins to undergo cell divisions while moving toward the uterus. It arrives in the uterus 6 to 7 days after ovulation and implants at the fundus of the uterine cavity. After the first three months of pregnancy, the corpus luteum is no longer needed to maintain pregnancy (Vanputte et al., 2013).

CHAPTER II

2 LITERATURE REVIEW

2.1 Infertility

Infertility is “a disease of the reproductive system defined by the failure to achieve a clinical pregnancy after 12 months or more of regular unprotected sexual intercourse.”(Zegers-Hochschild et al., 2009). Clinically there are two types: primary and secondary infertility. When a woman is unable to ever bear a child, either due to the inability to become pregnant or the inability to carry a pregnancy to a live birth she would be classified as having primary infertility. (WHO, 2015). Following either a previous pregnancy or a previous ability to carry a pregnancy to a live birth, she would be classified as having secondary infertility. (WHO, 2015). A WHO evaluation of Demographic and Health Surveys (DHS) data (2004), estimated that more than 186 million ever-married women of reproductive age in developing countries were maintaining a "child wish”, translating into one in every four couples.

Infertility is a life crisis with invisible losses, and has many consequences. Childless couples often experience social stigma and isolation. Infertile couple’s identity, status and economic security are clearly threatened by infertility which consequently, is a foremost source of worry leading to lowered self-confidence and a sense of powerlessness (Alhassan, 2012). Studies have shown that in resource-poor countries, where children are highly valued for cultural and economic reasons, childlessness often creates massive problems for couples; especially for the women who are generally accused for the infertility (Alhassan, 2012). Because of the serious social and economic effect of childlessness on couples, they try to find out a variety of treatments though a treatment seeking pattern has not clearly emerged. Sometimes the infertile couple show a delay in the seeking of medical help which might be due to their fear of facing the final definite diagnosis of their problem, emotional stress or escaping from the discomforting tests that they would undergo

(Bunting and Boivin, 2007). Couples therefore seek varied traditional methods and religious practices (Alhassan, 2012; Gerais and Rushwan, 1992; Gerrits, 1997). In Africa and most of the developing world, forms of infertility treatment depend on many factors including the couple's socio-economic status, decision-making within the family, level of information and availability of treatment (Alhassan, 2012; Bunting and Boivin, 2007). Drug expensiveness sometimes results in cessation of treatment or resort to unqualified practitioners (Mehta and Kapadia, 2008). Male infertility is usually treated according to the underlying cause.

Incomplete development of the testis, reproductive system diseases for example orchitis, rise in scrotal temperature, immunological disorders, endocrine disturbances, lifestyle and environmental or nutritional factors have been considered as the main causes of male infertility (Mehraban et al., 2014). Normal hypothalamic-pituitary-testicular axes are needed for men fertility. So any defect in each of them can lead to infertility in men. Testicular abnormalities are found in about 30 to 40 percent of cases with male infertility. 10 to 20 percent are caused by a blockage in the pathway that sperm use to exit the testes during ejaculation, up to 2 % of causes are related to pituitary/hypothalamic abnormalities and up to 50 % of male infertility cases have unknown reasons (idiopathic) (Ikechebelu et al., 2003). So the previous mentioned causes of male infertility result in semen analysis categories which fall into azoospermia (no spermatoazoa), oligospermia (low sperm count) and asthenozoospermia (abnormal sperm morphology) or combinations thereof (Ikechebelu et al., 2003). Currently, the therapy for male infertility in the developed countries shows that, there has been a strong shift away from evaluating and treating the man and proceeding directly to expensive artificial reproductive technologies. In addition, there is the formal microsurgery to correct varicoceles or obstruction of the male reproductive ductal system. (Crimmel et al., 2001). Previous trials for medical therapy of male factor infertility have included hormonal therapy (GnRH agonists and antagonists, gonadotropins, anti-estrogens, testosterone, and aromatase inhibitors), antioxidant therapy, antibiotics, corticosteroids, methyl xanthene's, vitamins, minerals and amino acids (zinc and arginine), and angiotensin-converting enzyme inhibitors (Crimmel et al., 2001). Still no

medication is approved by the United States Food and Drug Administration for treatment of male infertility. This is partly due to a lack of adequate controlled studies for potential therapy (Alhassan, 2012).

Many men shy away from conventional therapies because of worries about side effects and lack of efficacy. Long-term satisfaction with current therapies ranges from 40% to 70% (Jarow et al., 1996). Nowadays many patients prefer alternative or complementary medicine which is becoming increasingly popular (Crimmel et al., 2001). This is confirmed by Eisenberg when he proved that there is an increase in total visits to alternative medicine practitioners by 47.3%, from 1990 to 1997 (Eisenberg et al., 1998). In Africa however, most people depend on plants and their extracts for the treatment of disorders including infertility (Gerais and Rushwan, 1992; Gerrits, 1997). High level of anxiety, depression and psychological stress clearly affect the infertile couples not only when they reach to the diagnosis of their infertility, but also when they undergo assisted reproduction techniques (Alhassan, 2012).

2.2 Ingredients of medicinal plants

Plants contain a wide range of antioxidants, such as flavonoids, anthocyanins, cartenoids, vitamins and endogenous metabolites (Choi et al., 2002). These plant-derived antioxidants have been shown to act as singlet and triplet oxygen quenchers, peroxide decomposers, enzyme inhibitors and synergists (Choi et al., 2002).

Anti-oxidants are naturally produced in the human body and decrease with age and with overproduction of free radicals (Goldfarb, 1993; Haddock, 1992). The biggest amount of antioxidant compounds are found in fruits, vegetables and medicinal herbs (Sies et al., 1992). Natural products, mainly obtained from dietary sources provide a large number of antioxidants. (Cody et al., 1985; Oluwaseun and Ganiyu, 2008). However, diminished antioxidant activity, increases ROS and results in oxidative stress which can cause damage to cellular lipids, proteins and DNA (Alvarez and Storey, 1984; Pasqualotto et al., 2008).

In the last decade the scientists discovered free radicals and their side effects (Abdollahi et al., 2004). These free radicals are formed in the body during normal metabolic processes. Aerobic and anaerobic interactions are involved in the oxidation of carbohydrates, fats and proteins which supply the body with energy and at the same time lead to generation of free radicals. Tissue damage is enhanced by overproduction of the free radicals. Unsaturated lipid molecules of cell membranes are particularly prone to free radical damage. Oxidative damage or free radical damage extends beyond cell membranes to include RNA, DNA, and protein enzymes. Many environmental factors like lead, pesticides, cadmium, ionizing radiation, alcohol, cigarette smoke, UV light and pollution stimulate free radical generation resulting in different complications and toxicity in the body (Halliwell and Aruoma, 1993; Halliwell, 2009; Kelvin J. A. Davies, 1992).

Cell damage by free radicals can be prevented by anti-oxidants which are substances that give protective effect and are capable of mopping up free radicals. Health problems such as: cancer, aging, heart diseases and gastric problems etc are mainly caused by free radicals which are toxic by-products of natural cell metabolism.

Figure 2.1, shows that any presence of infection or inflammation in both the male and female genital tracts will lead to an increase in the synthesis of reactive oxygen species (ROS). And due to decrease in the antioxidants, ROS can in turn cause direct damage to DNA, proteins and lipids or result in a state of oxidative stress. As a result of oxidative stress, lipid peroxidation (LPO) in sperm is increased which in turn causes diminished sperm function (motility, viability, capacitation and acrosome reaction) and the end result is decreased fertility. Chemokines can also lead to oxidative stress with subsequent reduced sperm function (Sikka, 1996).

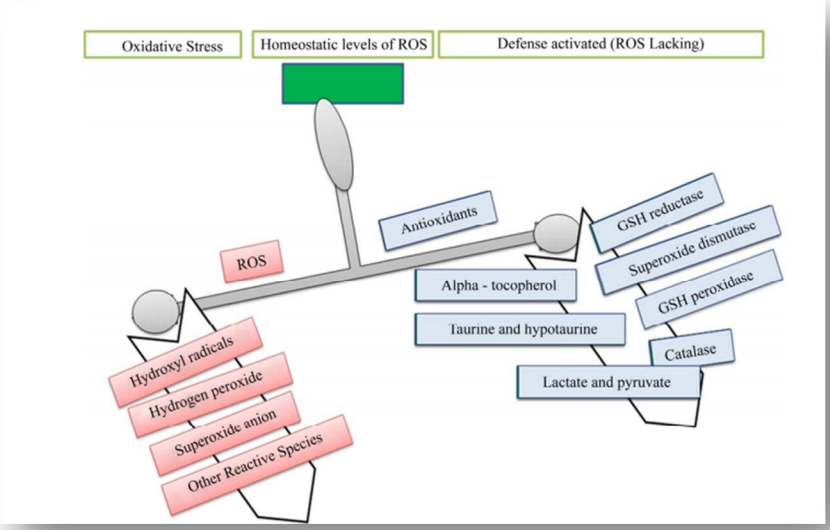

Figure 2.1: Imbalance between oxidant and antioxidant.

Source: Rahman et al., 2012

Oxidative damage liability have been suggested to be gender related as oxidative damage level in DNA was found to be greater in males than in females (Proteggente et al., 2002) In addition mitochondrial oxidant synthesis and oxidative damage to mitochondrial DNA was considerably lower in female rats compared to the male rats (Borrás et al., 2003; Sastre et al., 2006). In addition, the females might have intrinsically higher antioxidant capacity due to higher level of vitamin E and increased activity of glutathione peroxidase (GPx) and glutathione reductase (GR) (Chen et al., 1992; Yamamoto et al., 2002).

2.2.1 Free radicals, reactive oxygen and nitrogen species

A free radical is defined as a molecule or molecular fragment that contain one or more unpaired electrons in its outermost atomic or molecular orbital and are capable of independent existence (Gutteridge et al., 2011).

Reactive oxygen species (ROS) are highly reactive oxidizing agents and diffusible molecules that belong to the class of free-radicals that are generated in cells as a derivative of aerobic respiration and metabolism (Al-Gubory et al., 2010).

ROS and Reactive Nitrogen Species (RNS) includes radicals such as superoxide ($O_2^{\bullet -}$), hydroxyl (OH•), peroxyl (RO_2•), hydroperoxyl (HO_2•), alkoxyl (RO•), peroxyl (ROO•), nitric oxide (NO•), nitrogen dioxide (NO_2•) and lipid peroxyl (LOO•); and non-radicals like hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), ozone (O_3), singlet oxygen (O_2), peroxynitrate (ONOO−), nitrous acid (HNO2), dinitrogen trioxide (N2O3), and lipid peroxide (LOOH) (Aseni et al., 1987). These reactive oxygen species are produced as a normal consequence of biochemical procedures in the body and as a result of increased contact to environmental and/or dietary xenobiotics (Cook and Samman, 1996; Kumpulainen and Salonen, 1999; Pourmorad et al., 2006).

2.2.2 Free radical reactions

Once free radicals are formed, they start a chain of reactions which results in the production of a batch of free radicals that are involved in other reaction cycles. Free radical reactions steps are: (Manavalan and Ramasamy, 2001).

- Initiation step: radicals' formation.
- Propagation step: in which the free radical is reproduced repeatedly due to chain reactions.
- Termination step: radicals' destruction.

Generation and sources of free radicals: Internal and external substances can be involved in the formation of free radicals both inside and outside of the cell (Figure 2.1). Some sources of free radicals are as follows (Nagendrappa, 2005; Valko et al., 2006)**:**

- UV radiations, X-rays, gamma rays and microwave radiation.
- Metal-catalyzed reactions.
- Oxygen free radicals in the atmosphere.
- Inflammation initiates neutrophils and macrophages to produce ROS and RNS and neutrophils stimulated by exposure to microbes (Elberry et al., 2011)
- Mitochondria-catalyzed electron transport reactions.

- ROS formed from several sources like mitochondrial cytochrome oxidase, xanthine oxidases, and neutrophils and by lipid peroxidation.
- ROS generated by the metabolism of arachidonic acid, platelets, macrophages and smooth muscle cells.
- Interaction with chemicals, automobile exhausts fumes, cigarettes smoking, cigars, beedie.
- Burning of organic matter during cooking, forest fires, volcanic activities.
- Industrial effluents, excess chemicals, alcoholic intake, certain drugs, asbestos, certain pesticides and herbicides, some metal ions, fungal toxins and xenobiotic.

2.2.3 Antioxidants

Antioxidants are any substances that interrupt or prevent oxidative damage to a target molecule. Because of this antioxidants act as scavengers or detoxificants (ROS scavengers) which can stop cell and tissue injury. Whilst male infertility can be caused by improper balance between ROS generation and scavenging activities in human spermatozoa (Iwasaki and Gagnon, 1992), in females changes in physiological ROS will affect the following important processes: folliculogenesis, oocyte maturation, corpus luteum and uterine function, embryogenesis, embryogenic implantation and fetoplacental (Agarwal et al., 2008). Many pathological processes are found to affect these female reproductive processes if there was an imbalance between antioxidants and ROS production (Agarwal and Allamaneni, 2004; Agarwal et al., 2006). Antioxidants are both endogenous and exogenous in origin (Jacob, 1995) and include:

- Endogenous enzymatic antioxidants.
- Non enzymatic, metabolic and nutrient antioxidants.
- Metal binding proteins like ferritin, lactoferrin, albumin and ceruloplasmin.
- Phytoconstituents and phytonutrients.

The endogenous antioxidant defence systems in the body are classified into two groups such as enzymatic and non-enzymatic.

2.2.4 The enzymatic antioxidant defence system

In the gonads and seminal fluid, the detoxification activity is mostly caused by enzymatic antioxidants (ROS scavengers) such as superoxide dismutase (SOD), catalase (CAT) (Gutteridge et al., 2011; Jeulin et al., 1989; Nissen and Kreysel, 1983).

2.2.4.1 Superoxide dismutase (SOD)

This enzyme scavenges superoxide anions or radicals (O_2^-) to H_2O_2 and is considered as the first line of defense against ROS (Felton and Summers, 1995). There are three forms of SOD (Al-Gubory et al., 2010): copper- zinc containing SOD (SOD1), a dimeric protein located mainly in the cytoplasm. Manganese containing SOD (SOD2), a homotetrameric protein located in the mitochondria (Weisiger and Fridovich, 1973) and extracellular SOD (SOD_3) which is a copper and zinc containing tetrameric glycoprotein (Marklund, 1982; Marklund et al., 1982). So SOD safeguards spermatozoa from oxygen toxicity and lipid peroxidation (Alvarez and Storey, 1984). The disputation reaction is as follows:

$$2(O_2^-) + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$$

2.2.4.2 Glutathione peroxidase (GPx)

Glutathione peroxidase is present in the cytoplasm and the mitochondria of the cells and it is found in two forms: selenium - independent and selenium - dependent enzymes. GPX removes H_2O_2 by coupling its reduction to H_2O with oxidation of GSH. The reaction is the following:

$$H_2O_2 + 2GSH \xrightarrow{GPX} 2H_2O + GSSG$$

Glutathione, the thiol- containing tripeptide occurs in two forms: reduced (GSH) and oxidized (GSSH) form. Glutathione (GSH), is a powerful antioxidant of the overall cellular defensive mechanisms against ROS (Babich et al., 2011). It is

present within the cytosol of cells and is the major intracellular non- protein thiol compound. The main function of GSH is to scavenge free radicals and peroxide produced during normal cellular respiration that would otherwise cause oxidative damage to lipids, proteins, and nucleic acids (Schuck et al., 2008). Oxidative stress is characterised by depletion of intracellular GSH, which is mostly the case in cancer cells (Pelicano et al., 2004).

2.2.4.3 Catalase (CAT)

Catalase is found in peroxisomes (Chance et al., 1979) and catalyses the conversion of hydrogen peroxides to water and oxygen (Al-Gubory et al., 2010).

$$2H_2O_2 \xrightarrow{\text{Catalase}} 2H_2O + O_2$$

2.2.5 Non-enzymatic antioxidant system (dietary antioxidants)

The non-enzymatic defence system include vitamin E, vitamin C and reduced glutathione (GSH) (Harris, 1992; Jacob, 1995). Vitamins C and E are non-enzymatic which are less reactive. By trapping peroxyl and other reactive radicals they interrupt radical chain reactions (Ali et al., 1996; Johnson et al., 2003; Willcox et al., 2004).

2.2.5.1 Vitamin E

Vitamin E is a lipid soluble vitamin. Tocopherols is considered as the active form of vitamin E and is present in many forms: α-, β-, γ-, δ- tocopherols of which α- tocopherol is shown to be the most active homologous form. During lipid peroxidation, α- tocopherol reacts with ROS and produce lipid radicals which in turn protect the cell membrane from oxidation (Traber and Atkinson, 2007).

2.2.5.2 Vitamin C

Vitamin C (ascorbic acid) is a water soluble vitamin. It protects against DNA damage and scavenges ROS (Al-Gubory et al., 2010).

2.2.5.3 Metabolic antioxidants

Non-enzymatic antioxidants can also be divided into metabolic antioxidants and nutrient antioxidants (Al-Gubory et al., 2010). Metabolic antioxidants are the endogenous antioxidants, such as lipoid acid, glutathione, L-arginine, coenzyme Q10, melatonin, uric acid, bilirubin, metal-chelating proteins, transferrin etc. they are produced in the body (Ali et al., 1996; Dröge, 2002).

2.2.5.4 Nutrient antioxidants

Nutrient antioxidants are exogenous antioxidants, which cannot be produced in the body but needs to be provided through diet or supplements viz trace metals (selenium, manganese, zinc, copper, and ferrous), flavonoids, omega-3 and omega-6 fatty acids etc. They act as cofactors in the regulation of antioxidant enzymes or take place in the active site required for the functions of antioxidant enzymes (Bettger, 1993; Dashti et al., 1994). Vitamin E (α-tocopherol) and C are the non-enzymatic antioxidants which exist within a normal cell and can be supplied through diet (Tiwari, 2001).

2.2.5.5 Polyphenols

Vegetables, fruits and plant derived beverages such as tea, red wine, and extra virgin olive oil are the most common source of polyphenols (Al-Gubory et al., 2010). Flavonoids and phenolic acid are groups involved in phenolic structures (Al-Gubory et al., 2010). The flavonoids include catechin, resveratrol, quercitin, anthocyanins, hesperitin derivatives and phenolic acids (Al-Gubory et al., 2010). Because of their toxicity at high doses, they are needed in balanced amounts (Abu-

Darwish and Abu-Dieyeh, 2009; Michalak, 2006). Natural antioxidants can be primary or secondary antioxidants. Primary antioxidants are responsible for chain-breaking and can react directly with lipid radicals and change them into stable products. Secondary antioxidants are basically preventive antioxidants that lower the oxidation rate by different mechanisms (Touré and Xueming, 2010). Primary antioxidants most often act by giving a hydrogen atom, while secondary antioxidants may act by binding metal ions that catalyze oxidative processes via oxygen scavenging, UV radiation absorption, enzyme inhibition and hydrogen peroxide decomposition (Touré and Xueming, 2010).

Therefore antioxidants, synthesized in the body or supplied from outside like phytoconstituents, play important role to protect the body from free radical- induced injury (Sen and Chakraborty, 2010). As spermatozoa are vulnerable towards ROS induced lipid peroxidation, these antioxidants may play role in enhancing fertility through a likely increase in sperm motility and function (Sikka, 1996). Oxidative stress and free radical production will result if there are any imbalances between free radicals and antioxidant, and therefor sperm damage and infertility may result. This will occur due to high concentration of poly unsaturated fatty acids in the sperm head which make it easy target to the free radicals (Alvarez and Storey, 1995).

2.3 Black seed (*Nigella sativa L)*

Nigella sativa L., which is also known as black cumin or black seeds, belongs to the botanical family of Ranunculaceae (Kamal et al., 2010). The active ingredient of this plant is found in its seeds. This amazing plant has a magnificent history (Kamal et al., 2010; Muthu Kumara and Kwong Huat, 2001). It is mentioned by many religious and ethnic books. For example it is mentioned by Prophet Mohammed as universal healer that is the treatment for every ailment except the ageing and death. Also mentioned in the holy bible as the curative cumin and described as the melanthion of Hippocrates and Dioscordes (Tembhurne et al., 2014).

Nigella sativa is a bushy, self-branching plant with divided leaves and pale bluish or white flowers which reproduce a fruit capsule that consist of many white trigonal seeds. Once the fruit capsule has matured, it opens up and the seeds (Figure 2.2) contained within are exposed to the air becoming black in color (black seeds) that contain a considerable amount of oil (Chevallier, 2001). It is an annual herb grown over a great distance in different parts of the world (Ghafoor, 2007).

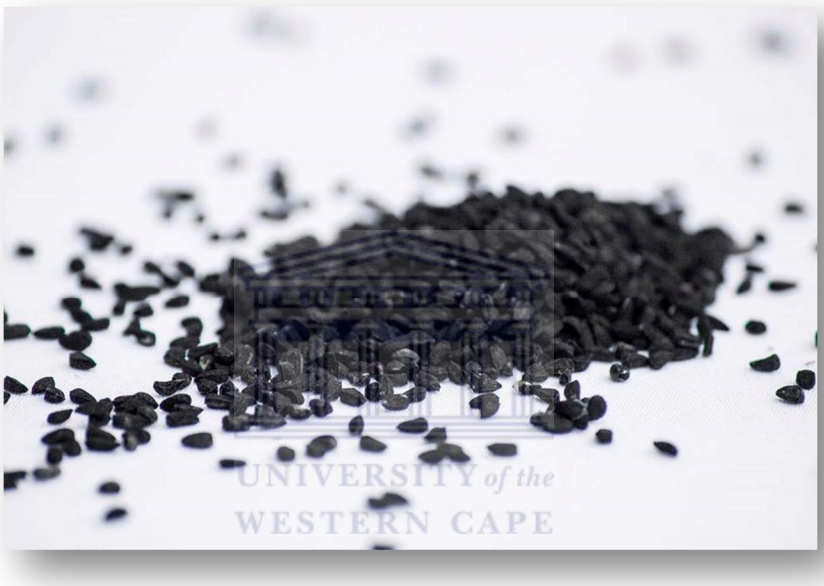

Figure 2.2: Images of Black seeds.
Picture taken by author 25th March 2015.

Nigella sativa beneficial effects has been investigated widely on the cardiovascular system (Tasawar et al., 2011) and the immune system where it includes immune potentiating, immune modulating and interferon-like activities (Alsaif, 2008). In addition, it contains also the potent anti-inflammatory mediators prostaglandins and leukotriens(Salem, 2005). *Nigella sativa* has beneficial effects on digestive system (Hassan et al., 2012) as well as homeostasis (Abdel-Sater, 2009; El Bagir, 2010; Tasawar et al., 2011). In the Middle East and India, *Nigella sativa* is used for the treatment of asthma, cough, bronchitis, headache, rheumatism, fever, influenza and eczema (Meziti et al., 2012). As a traditional medicine, *Nigella sativa* increases milk production, promotes menstruation in the female and has beneficial

role for the treatment of postmenopausal symptoms due to its oestrogen like effects (Saadat, 2011). *Nigella sativa* seeds have been shown to increase the weight of reproductive organs, sperm motility and concentration in cauda epidydimides and testicular ducts of the male albino rats. Spermatogenesis was found to be increased at primary and secondary spermatocyte level. It shows also an increase in the number of pregnant rats (Mohammad et al., 2009). Because of extended medicinal uses of *Nigella sativa,* it has undergone extensive phytochemical investigation.

Nigella sativa seeds contain 28 to 36 % fixed oil, proteins, alkaloid, saponin and 0.4 to 2.5 % essential oil. Unsaturated fatty acids, which make the main composition of fixed oil, include: arachidonic, eicosadienoic, linoleic and linolenic acid. The saturated fatty acid present in the oil are palmitic, stearic and myristic acid (Hajhashemi et al., 2004). Gas chromatography-mass spectrometry (GC–MS) analysis of the black seeds's essential oil revealed that the pharmacologically active constituents of volatile oil are thymoquinone (Figure 2.3B), dithymoquinone, thymol (Figure 2.3C) and thymohydroquinone. Dithymoquinone is the dimerised form of thymoquinone (Ghosheh et al., 1999; Hajhashemi et al., 2004).

The crystalline active principle, nigellone is the only constituent of the carbonyl fraction of the oil. The other constituents of the volatile oil of the seed are p-cymene carvacrol, t-anethole, 4-terpineol and longifoline. Also there are four alkaloids that have been reported as constituent of *Nigella sativa* seeds. Nigellicine (Figure 2.3D) and nigellidine have an indazole nucleus whereas nigellimine (Figure 2.3E) and N-oxide of nigellimine are isoquinolines (Atta-ur-Rahman et al., 1995). Recently, a triterpene saponin alfa herein was isolated from the seeds of *Nigella sativa.* α-heredin (Figure 2.3F) is known to have antitumor activity (Tembhurne et al., 2014). The ethanolic extract of the seeds contain three flavonoids namely quercetin and kaempferol 3- glucosyl (1 -2) galactosyl (1 -2) glusoside and quercitin –3-(6-ferulolyl glucosyl) (1 -2) galactosyl (1 -2) glucoside (Merfort et al., 1997). Other than those triglycosidequercetin 3-glucoside, kaempferol 3-glucoside and rutin were also isolated from the seeds of *Nigella sativa.* Nutritional components such as

carbohydrates, fats vitamins mineral elements and proteins including eight or nine essential amino acid are also found to be another ingredient of *Nigella sativa* seeds.

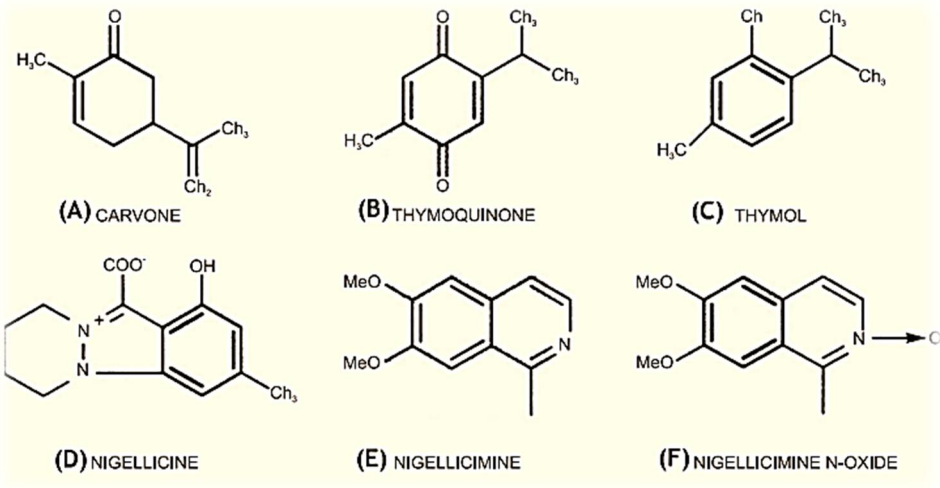

Figure 2.3: Chemical structure of some of the main potentially bioactive compounds in *Nigella sativa.*

A. Carvone is a member of a family of chemicals called terpenoids. Carvone is found naturally in many essential oils.
B. Thymoquinone is a phytochemical compound found in the plant Nigella sativa. It is also found in select cultivated Monarda fistulosaplants.
C. Thymol is a natural monoterpene phenol derivative of cymene, C10H14O,isomeric with carvacrol (also known as 2-isopropyl-5-methylphenol, IPMP).

D, E and F The alkaloids nigellicine, nigeglanine, and nigellidine are indazoles.

Source: (Paarakh, 2010).

Potassium, calcium, phosphorus and magnesium are the minerals that present dominantly in the seeds (Haron et al., 2014). The *Nigella sativa* seeds are also a source of iron (Salem and Hossain, 2000). Monosaccharides in the form of glucose rhamnose, xylose and arabinose are also found. The seeds contain carotene, which is converted by liver to vitamin A. In one study, the oral administration of aqueous extracts of the *Nigella sativa* seeds for 14 days has been shown to cause no toxicity symptoms in male Sprague Dawley rats (Tennekoona et al., 1991). The safety of consuming *Nigella sativa* seeds was also reported by Al-Homidan et al. (2002). Although several studies have reported the safety of consuming N. sativa seeds, one comprehensive study has shown that the plant is relatively unsafe if consumed in certain concentrations for prolonged periods of time (Zaoui et al., 2002).

The basis of many human diseases and conditions might be partially related to the endogenous generation of free radicals and reactive oxygen species (Lamirande & Gagnon 1992). So the antioxidant effect of *Nigella sativa* may explain some of its benefits in traditional medicine. Its essential oil, thymoquinone and other components like carvacrol, anethole and 4-terpineol displayed anti-oxidant effects through strengthening the oxidant detoxification system *in vitro*. The free radical scavenging effect of thymol, thymoquinone and dithymoquinone have been shown (Kruk et al., 2000). Thymoquinone and fixed oil of *Nigella sativa* were also found to inhibit non-enzymatic peroxidation in ox brain phospholipid liposomes (Houghton et al., 1995).

Also in non-enzymatic peroxidation in liposomes assay and deoxyribose degradation assay there were effective OH radical scavenging components (Miguel, 2010). The antioxidant effect of thymoquinone (TQ) and a synthetic structurally related ter-butyl thymoquinone (TBHQ) were examined *in vitro*. Interestingly, both TQ and TBHQ efficiently inhibited iron-dependant microsomal lipid peroxidation in a concentration dependent manner (Badary et al., 2003). Thymoquinone has also renal protective effect through its antioxidant activity (Ali and Blunden, 2003).

2.4 Flaxseed (*Linum usitatissimum*)

Flaxseed (*Linum usitatissimum)* (Figure 2.4), also known as linseeds, is a member of the Linaceae family. It is considered as functional food due to its nutritional value and also due to its beneficial effects on the human body as used in traditional medicine (Amaral, 2007). Flaxseed is native to west Asia and the Mediterranean (Berglund, 2002; Tarpila et al., 2005). It is also cultivated for linen fiber or for oil from its seeds, which is also called linseed oil. The oil forms about 40 % of the seeds (Anwar and Przybylski, 2012). The spherical fruit capsules consists of five compartments, each one contains two seeds. The seed have a smooth shiny surface, dark brown to yellow in color, and flat and oval in shape with a pointed tip (Thompson and Cunnane, 2003)

Figure 2.4: Images of Flaxseed.
Picture taken by author 25th March 2015..

The chemical composition of *Linum usitatissimum* revealed that it consists of 28% total dietary fibre, 20% protein, 41% fat, 3.4% ash, 7.7% moisture (Ganorkar and Jain, 2013), and lignans (Figure 2.5) which are phyto compounds that have chemical structures like oestrogen (Chilibeck and Cornish, 2008).

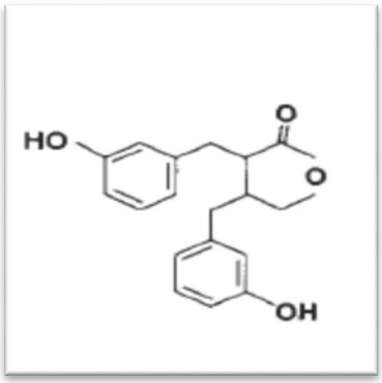

Figure 2.5: The basic structure of lignans.
Source: http://www.bcerc.org 15th May 2015.

Lignans are diphenolic compounds formed by the coupling of two coniferyl alcohol residues that are present in the plant cell wall (Touré and Xueming, 2010). Lignans found in flaxseed are Matairesinol, Isolariciresinol, Lariciresinol, and Pinoresinol.

The predominant lignin in flaxseed is Secoisolariciresinol diglucoside (SDG) with minor amount of pinoresinol and matairesinol (Meagher et al., 1999; Thompson et al., 1991). The intestinal flora act as converter of flaxseed lignans to the mammalian lignans enterolactone and enterodiol (Figure 2.6) (Bouaziz et al., 2010) which have stronger anti-oxidant function than Secoisolariciresinol diglucoside (Kitts et al., 1999; Touré and Xueming, 2010). Literature has reported the anti-oxidant activity of flaxseed a factor which may increase the use of flaxseed. It acts mainly as hydroxyl radical scavenges (Zanwar et al., 2011) Secoisolariciresinol diglucoside may provide health benefits in another way through induction of phase 2 proteins. Phase 2 enzymes are generally characterized by their ability to either encouraging the scavenging of oxidants or decreasing the possibility of oxidant development (Juurlink, 2001); thus, inducing phase 2 protein expression decreases oxidative stress. Enterolactone is also a phase 2 protein inducer (Wang et al., 1998).

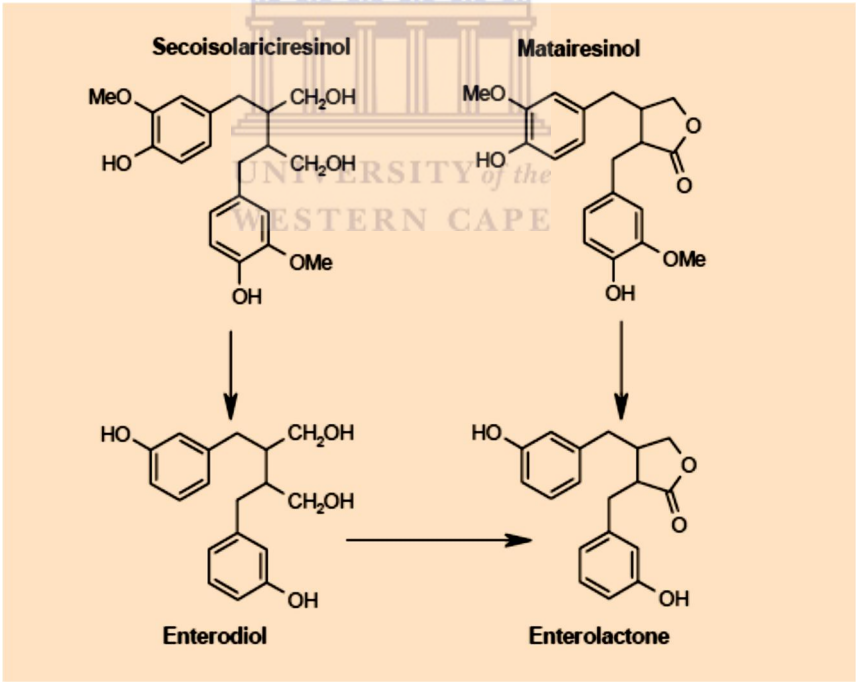

Figure 2.6: Conversion of mammalian lignan precursors SDG and matairesinol to enterodiol and enterolactone by the gut microflora.

Source: Johnsson, 2004.

Flaxseed phyto-compounds may act as oestrogen agonist or antagonist due to the possibility of its binding to oestrogen receptors according to the original oestrogen level. Lignans act as oestrogen antagonist in pre-menopausal women where the oestrogen concentration is normal. This is due to their ability to enhance the negative feedback loop, and therefore decrease oestrogen activity (Chilibeck and Cornish, 2008). The opposite usually occurs in the post-menopausal women where the oestrogen concentration is lower than that in the pre-menopausal women. In this situation the lignans will act as oestrogen agonist thus potentiate its action but to a level lower than the normal one (Serock et al., 2008).

Adding to estrogen receptors binding, phytoestrogens may act as endogenous estrogens concentration modulator through activation or inhibition of certain enzymes, such as sulfatase, 17b hydroxysteroid dehydrogenase, aromatase, and sulfotransferase. It might also affect the bioavailability of sex hormones by binding to or stimulating the synthesis of sex hormone binding globuline (Colli et al., 2012). In another study, lignans inhibited the binding of estrogen and testosterone to receptors on sex-binding globulin (Touré and Xueming, 2010). Further to this, enterodiol and enterolactone have been reported to relieve lower urinary tract symptoms in patients with benign prostatic hyperplasia through inhibition of the enzyme 5 a-reductase (Colli et al., 2012). So the lignans add many beneficial effects to flaxseed including controlling the menopausal symptoms; preventing osteoporosis and controlling hormone-dependent uterine and breast cancers by reducing the proliferative effect of endogenous oestrogen (Soares et al., 2010) and the ability to inhibit aromatase (Colli et al., 2012). In addition flaxseed decrease the incidence of prostate cancer and decrease rate of tumor growth (Anwar and Przybylski, 2012).

In men, the source of estrogens is mainly from testosterone conversion to estradiol catalyzed by the enzyme aromatase. It is proved that the flaxseed lignans competitively inhibit aromatase enzyme (Brooks and Thompson, 2005). 10% dietary flaxseed increased serum testosterone and estradiol levels and gave higher relative weights of the accessory sex gland, all prostate lobes, the seminal vesicle,

and the testes (Collins et al., 2003). Prenatally and/or postnatally exposure of rats to a flaxseed/flax meal decreased the prostate weight, and increased the serum LH and cauda epididymal sperm counts in the rat (Sprando et al., 2000).

Flaxseed oil is rich in polyunsaturated omega 3 fatty acids. It contains linoleic acid (LA) and 54-59% a-linolenic acid (18:3n-3, ALA), making flaxseed one of the richest sources of ALA (Retana-Márquez et al., 2012). Therefore flaxseed seems to protect against many cardiovascular diseases through lowering cholesterol and triglycerides levels and regulating the lipid profile (Riediger et al., 2008). Arginine, aspartic acid and glutamic acid are found in relatively high amount in flaxseed whereas lysine, methionine and cysteine are found in limited amount (Ganorkar and Jain, 2013). The soluble fibers in flaxseed are acting as blood sugar-regulating and cholesterol-lowering factor. The remaining two third of the fibers are insoluble ones which prevent constipation and act as laxatives (Goyal et al., 2014). Flaxseed can protect against colon cancer in multiple intestinal neoplasia in mice (Oikarinen et al., 2005) and in rats (Dwivedi et al., 2005). Flaxseed might also enhance bone development but not strength nor mineral density (Ward et al., 2001).

2.5 Date palm pollen (*Phoenix dactylifera L*)

Date palm pollen (*Phoenix dactylifera L)* (Figure 2.7), which is member of monocotyledon family Arecaceae (Phillipson, 2001), grows widely in the Arabian Peninsula, the Mediterranean, and North Africa countries, parts of India and hotter parts of the USA (Ahmed et al., 2008). Male gametocytes, which are known as pollen grains, are produced by stamens; part of the date palm male flower which is widely used as a tonic to improve male fertility (Soegaard and Dam, 2013).

DPP was used also by ancient Egyptian and Chinese in the treatments of many other diseases (Hassan 2011). Date palm pollen has antibacterial (Baltrušaityte et al., 2007), antifungal (Ozcan, 2004), antioxidant (Campos et al., 2003; Leja et al., 2007), anti-inflammatory (Choi, 2007; Hammed et al., 2012) and hepato-protective activities (Uzbekova et al., 2003).

Figure 2.7: Date palm pollen.
Picture taken by author 25th March 2015.

In comparison with bee pollen which is heterogeneous, impure, and of unknown source, DPP is homogeneous, pure, of a known source and easily standardized. There are many types of DPPs, all of them named (*Phoenix dactylifera* L. family palmae) and their chemical composition varies with the condition of handling and storage (Stanley and Linskens, 1974). DPPs are rich in biochemically and nutritionally important substances such as minerals, zinc, selenium, iron, cooper and cobalt, trace elements, a wide range of carbohydrates, organic acids, lipids including saturated and unsaturated fatty acids, sterols, nucleic acids, free amino acids, vitamins A, E, and C and over 100 kinds of enzymes and co-factors (Hassan 2011), for which they are regarded as good food resources (Metwaly et al., 2013). Saturated fatty acids in the date palm pollen include stearic and palmitic acid, while unsaturated fatty acids contain linoleic and oleic acids which could inhibit 5 -α reductase enzyme (Shariati and Esfandiar, 2008).

Phytochemical analysis of DPP revealed the presence of Triterpenes, saponins, α-amirin, esteron-like compounds, carbohydrates and\or glycosides (Amin et al., 1969; Desoukey and Bishr, 2012). Isolated rutin, five different flavonoid compounds and estradiol, estriol, and estrone were determined (Abbas and Ateya,

2011). Phenolic components, such as flavonoids, phenolic acids, and phenolic diterpenes are the main antioxidant content of date palm pollen (Bahmanpour et al., 2013).

DPP was reported to have gonad-stimulating activities (Soliman and Soliman, 1958), mainly due to the presence of esterone (Dostal et al., 1996). DPP contains also a growth hormone-like substance (Mahran et al., 1976). DPP and male palm flowers were traditionally regarded as aphrodisiacs and fertility enhancers (Khare CP, 2007) for both men and women in ancient Egypt. Estradiol and flavonoid components positively affect the sperm quality (Kostyuk et al., 2004; Vayalil, 2002). DPP might act primarily on the testes, although, its effect on the hypo-pituitary axis could not be ruled out (Abedi et al., 2012).

Rat sperm parameters such as the sperm count, motility and morphology have seen to be improved by the DPP (Adaay and Mattar, 2012). This improvement of sperm parameters occurred in concomitant with improvement of the DNA quality. In addition the number of interstitial cells (Leydig's cells) was also increased by DPP administration as well as the blood level of estradiol and testosterone. Estradiol and testosterone are found also at high concentrations in rat testis and seminal fluids after administration of DPP (Bahmanpour et al., 2006). Three different cell types, Sertoli, Leydig and germ cells are found to be responsible on estrogen synthesis in the male reproductive tract (Kostyuk et al., 2004). Estrogen regulates the reabsorption of luminal fluid in the head of the epididymis (Hess et al., 2001). Some studies suggested that estrogen may be involved in regulating the regeneration of spermatogonial stem cells, as male reproductive tissues contains estrogen receptors (Bahmanpour et al., 2006).

DPP contains gonadotropin stimulating substances and steroid precursors (Adaay and Mattar, 2012; Phillipson, 2001) which could enhance testosterone production. For this reason, they added DPP to animal mash to enhance growth, and observed an increase in the plasma testosterone level (Ali et al., 1999). Also a growth

hormone-like substance (Mahran et al., 1976) and its anabolic effect may be incorporated in this enhancement.

Testosterone is the hormone which controls spermatogenesis, maturation and motility of the epididymal spermatozoa and sexual desire (Cheesbrough, 1994). The use of DPP as treatment of male infertility could improve the semen parameters (Bahmanpour et al., 2006) including quality, sexual desire and rate of intercourse in the male rats and therefore increase the pregnancy rate(Jequir, 1998). DPP has preventive role against the chemotherapeutic-induced infertility in males (Al-Kharage and Rokaya, 1982). Cadmium induced testicular damage and subsequently induced infertility in the male rats can be reversed by therapeutic administration of DPPs (El-Neweshy et al., 2013). Similarly, date palm pollen intake reverses spermatotoxicity produced by mercury probably by activation of testicular, endocrine and antioxidant system (Saeed et al., 2015) and has also a protective effect on cisplatin-induced male infertility in rats (Abedi et al., 2014).

In the field of *in vitro* human sperm activation, Saad et al. (2012) showed that supplementation of the culture medium with 20% DPP resulted in improvement in the sperm motility. DPP alone or in combination with zinc sulfate capsules significantly increased the human serum LH, FSH and testosterone levels in concomitant with sperm count and motility. Sexual desire was also significantly increased (Al-Sanafi et al., 2006; Marbeen et al., 2005).

The aphrodisiac effect of the DPP extract may be due to the presence of alkaloids, saponins and flavonoids through different central and peripheral neural pathway. DPP might be used as sexual behavior stimulator and in the treatment of human male impotence (i.e. sexual arousal/erection disorders) and pre-mature ejaculation. Its action may be due to the effect on sexual arousal and dopamine release from nucleus accumbens (Abedi et al., 2014). Dopamine influences motor activity in mesolimbic tract and activates numerous behavior and genital reflexes (Gauthaman and Ganesan, 2008; Padashetty and Mishra, 2007).

Certain steroids, flavonoids, saponins, and lipid could also increase sexual behavior. These components might raise the level of luteinizing hormones (LH) which in turn stimulate the endogenous estrogen production (Yakubu et al., 2008, 2005). Erection occurs due to vasodilation of blood vessels of penis (Zarrindast et al., 2004). That could be induced by alkaloid components of DPP which have estrogenic properties (Mahran et al., 1976). In addition smooth muscle relaxation of corpus cavernosum of the penis is caused by saponin components of DPP which acts as nitric oxide (Abedi et al., 2014).

Oral administration of DPP leads to a rebalance of the damaging effects of lead acetate in female rats. This was demonstrated by a decrease in the atretic follicles and an increase in the development and maturation of follicles together with an increase in the fertility and pregnancy rate (Hammed et al., 2012). Administration of DPP as a source of antioxidant in the maturation medium- during the process of *in vitro* maturation of preantral follicles in mice - increased the follicular diameter and improve the *in vitro* maturation rate and survival rate of oocytes, and antrum formation rate (Farzaneh et al., 2015).

DPP is considered as safe herbal medicine (Rasekh et al., 2015). As antioxidant DPP lead to decrease the high elevation of urea and creatinine caused by lead acetate toxicity (Hammed, 2015).

2.6 Nutmeg (*Myristica fragrans*)

Nutmeg is the kernel of broadly oval seed of *Myristica fragrans* Houtt (Figure 2.8) which is a member of the (Myristicaceae) family. *Myristica fragrans* is a bushy evergreen tree 10 – 20 m high (Van Gils and Cox, 1994), indigenous to the Banda islands (Maluku or Moluccas islands) in Indonesia, formerly known as the Spice Islands of Indonesia. 75% of the world Nutmeg production is from Indonesia (Assa and Widjanarko, 2014). It is also cultivated in the Caribbean, Southeast Asia, the Pacific Islands, south India, Sri Lanka, Sumatra, Malaysia (Barceloux, 2008; Raghavan, 2006) North Australia and the Pacific Islands (Al-Jumaily and Al-

Amiry, 2012). It is widely used world-wide as both spicy flavouring (Tajuddin et al., 2005) and traditional medicine (Van Gils & Cox 1994; Harris 2003).

Figure 2.8: Nutmeg.
Picture taken by author 25th March 2015.

In traditional medicine, nutmeg and nutmeg oil have been used for the treatment of digestive system disorders like stomach cramps, nausea, diarrhoea, and flatulence. It is also used in the treatment of rheumatism, cholera, psychosis, and anxiety. In addition, it is widely popular as aphrodisiac and abortifacient (Barceloux, 2008). It is used in both Western and Chinese herbal medicine as muscle relaxant, sedatives, carminative, and anti-emetic, and also for kidney disorders and liver disease (Jacqueline, 2005). In Indochina, it is used to treat dysentery, anorexia, and colic. Malarial debility and rheumatism are also treated by nut Meg in Indonesia (Ozaki et al., 1989). Myristicafragrans seed oil is used also for blood pressure control (Grover et al., 2002), and acts as antithrombotic and hypolipidemic (Tajuddin et al., 2005). It has also been documented to have antioxidant property (Murcia et al., 2004), and nervous stimulant (Tajuddin et al., 2005). The effect of the oil of nutmeg on the fertility and induction of meiotic chromosome rearrangements in mice and their first generation have been studied (Agarwal et al., 2009).

Phytochemical investigations revealed that nutmeg contains a volatile oil, a fixed oil, proteins, fats, starch and mucilage (Janssens et al. 1990; Isogai et al. 1973). The essential oil comprises about 3.9 to 16.5% of the nut Meg kernel contents, on the

other hand it comprises about 6 to 26.1% of the nut meg mace contents (Maya et al., 2004). The kernel contents showed 30 – 55% oil and 45 – 60% solid matter, the volatile oil comprises about 5–15% of the nutmeg kernel, while the fixed oil accounts for 24 – 40% (Al-Jumaily and Al-Amiry, 2012). The fixed oil contains myristin and myristic acid. The volatile oil contains pinene, sabinene, camphene, myristicin, elemicin, isoelemicin, eugenol, isoeugenol, methoxyeugenol, safrole, dimeric phenylpropanoids, lignas and neolignas (Isogai et al., 1973). Eugenol, as major compound, acts as vasodilator (Criddle et al., 2003), muscle relaxant (Damiani et al., 2003), and maintains the activities of enzymes like superoxide dismutase, catalase, glutathione peroxidase, glutamine transferase and glucose-6-phosphate dehydrogenase and therefore inhibits lipid peroxidation (Kumaravelu et al., 1996).The ethanolic extract (50%) of nutmeg showed significant aphrodisiac effect in male mice in form of mounting frequency and mating performance which are markers for sexual function (Tajuddin et al., 2003). The aphrodisiac effects of myristicafragrans might be due to its nervous stimulating property (Tajuddin et al., 2005). Myristicin is a natural benzodioxole compound (phenylpropan derivative) (Jeong and Yun, 1995). Myristicin, or methoxysafrole, is the principal aromatic constituent of the volatile oil of nutmeg.

2.7 Radish seed (*Raphanus sativus Linn*)

Raphanus sativus Linn (Figure 2.9) Cruciferae family is also known as fejl in Arabic countries. It is annual, medium size herb, and well known during all previous historical periods. It was well known to the ancient Greek and ancient inhabitants of Egypt –even before the pyramids were built-, and was popular in ancient Rome as well. In Mediterranean area they use the roots, leaves, and seeds in cooking and treatments of many diseases.

Figure 2.9: Radish seed.
Picture taken by author 25th March 2015.

Raphanus sativus are used to treat gastrointestinal, biliary, hepatic, urinary, and respiratory disorders (Ghayur and Gilani, 2006). Also Mediterranean people use *Raphanus sativus* to treat cardiovascular diseases such as hypertension (Duke, 2002; Said, 2013). This hypotensive cardiovascular inhibitory effect is mediated by cholinergic receptors (Ghayur and Gilani, 2006). In addition, they use it as an antiurolithiatic (Vargas et al., 1999), anti-inflammatory and antibleeding (Nagar, 1993). *Raphanus sativus* protect against influenza (Prahoveanu and Eşanu, 1986), and has antimicrobial (Aboul Ela et al., 1996) and antioxidant agents (Lugasi et al., 1998). Phytochemical examination revealed that radish contains proteins, polyphenols, flavonoids, peroxidases, isoperoxidases (Vargas et al., 1999), alkaloids such as coumarins, saponin (El-Sayed, 2001), pyrrolidine, isoquinoline, phenethylamine and sulphuric compounds such as glucoparin, sinigrin (Vargas et al., 1999).

Radish seed also contains sulforaphene. These substances lower the uric acid level in the serum that are related to inflammation circulating markers and free radical reactions (Zaman, 2004). Raphanin, which is the other name of Sulforaphene (Schwartz, 1998), is very strong against gram positive and gram negative bacteria

such as streptococcus, Pyococcus, Pneumococcus and Escherichia coli. Raphanin also possess blastokolic (seed germination suppression) effect. The seeds of radish are also contain a broad-spectrum antibiotic, named machrolysin, which is specific against *Mycobacterium tuberculosis* (Singh and Singh, 2013).

Raphanus sativus extract protects against zearalenone induced testicular toxicity which might be due to its ability to the inhibit oxidative process by counteracting reactive oxygen species as well as its interaction with estrogen receptors that are occupied by the mycotoxin zearalenone (Ben Salah-Abbès et al., 2009). In order to improve the fertility and immunity of rabbits El-Tohamy et al. (2010) compared the fertility enhancing activity of Radish seed with those of rocket and black cumin. He found that Radish seed increase the sperm motility, decrease the abnormal sperm concentration, and increase the live sperm concentration.

The beneficial effect of *Raphanus sativus* is partly due to the presence of compounds named glucosinolates, which are hydrolyzed by myrosinase or by thioglucosidase activity of the intestinal microflora into isothiocyanates (Murillo and Mehta, 2001). The isothiocyanate has anti-microbial activity, anti-mutagenic, anti-carcinogenic and anti-atherosclerosis activity (Suh et al., 2006). Radish seed contain anthocyanins which are important group of dietary antioxidants that have many physiological functions. They protect living cells from oxidative damage leading to prevention of diseases (Matsufuji et al., 2003).

Through its direct scavenging activity *Raphanus sativus* extract was found to remove oxygen free radicals such as hydroxyl radical, singlet oxygen, hydrogen peroxide, peroxyl radical, and hypochlorous acid (Liu et al., 2005), as well as increase Superoxide dismutase activity (SOD) and Glutathione peroxidase (GPx) in testis (Kouadio et al., 2007). The red radish pigment (pelargodinin-3-sophoroside-5-glucoside) had almost the same antioxidative activity as butylated hydroxytoluene (BHT) at the same concentration (Xiaoling et al., 2001). Granules of radish root extract protected the cell membrane against lipid peroxidation in rats fed on fat-rich diet (Sipos et al., 2002).

Aims and objectives

This study aimed to investigate the effects of selected Libyan medicinal plant extracts *in vivo* on sexual behavior, spermatogenesis and steroidogenesis in male rats and on ovulation and fertility in female rats.

The aims of the study were to:

1. Investigate the flavonol and flavonoid content of medicinal plant extracts.
2. Assess the effects of the plant extracts on the reproductive system, liver and kidney functions of both male and female rats.
3. Investigate the effect of the medicinal plants on sexual behavior and reproductive performance of both male and female rats.
4. Determine the cytotoxicity of the plant extracts on TM3 Leydig and MCF7 breast cancer cell lines.

CHAPTER III

3 MATERIALS & METHODS

3.1 Chemicals and reagents

3.1.1 Merck (Johannesburg, South Africa)

- Dimethylsulfoxide (DMSO).
- Ethanol.
- Glacial acetic.
- Hydrochloric acid.
- Hydrogen peroxide.
- Phosphoric acid.
- Potassium dihydrogen phosphate.
- Potassium dihydrogen orthophosphate.
- Sodium pyruvate.

3.1.2 Sigma Aldrich (St Louis, MO, USA)

- Ammonium acetate.
- α- ketoglutarate.
- Bovine serum albumin.
- (+)-catechin.
- Coomassie.
- Brilliant blue G.
- Butylated hydroxytoluene BHT.
- Creatinine.
- 4- (Dimethylamino)-cinnamaldehyde (DMACA).
- 2, 4-dinitrophenylhydrazine.
- 2, 2-diphenyl-1-picyl-hydrazyl DPPH.
- Ethylene diamine tetraacetic acid.
- Folin-Ciocalteau reagents.
- Gallic acid.

- Glacial acetic acid.
- Human chorionic gonadotropin (HCG).
- 6-hydroxy-2, 5, 7, 8- tetramethylchroman-2-Carboxylic acid.
- L-alanine.
- L-aspartic acid,
- Methanol.
- Formaldehyde.
- Picric acid.
- Pyrogallol.
- Quercetin, sodium acetate.
- Sodium carbonate ($Na2CO_3$).
- Thiazolyl blue tetrazolium bromide (MTT).
- 2- thiobarbituric acid.
- Trichloroacetic acid.
- 2,4,5-Tri (2-pyridyl)-S-triazine (TPTZ)

3.1.3 Biochrom (Berlin, Germany)

- Fetal bovine serum.
- Horse serum.
- Dulbecco's modified Eagle's (DMEM/F-12 1x).

3.1.4 BM Scientific (Cape Town, South Africa)

- Sodium chloride.
- Sodium hydroxide.

3.1.5 Applichem (Darmstadt, Germany)

- Tris-hydrochloride

3.1.6 Oxoid (England)

- Phosphate buffered saline

3.2 Collection and preparation of plant samples

The plants (Black seed, Nutmeg, Radish seed, Flaxseed and date palm pollen) were obtained from the market in Misurata city in Libya. Except for Date Palm pollen flowers and seeds all the plants were first washed with tap water followed by distilled water before allowing them to air dry. The plants were then converted into powder using an electrical mill and stored in polyethylene bags at $4^{O}C$.

3.3 Extract preparation

The ethanolic extracts of black seed, nutmeg, radish seed, flaxseed and date palm pollen were prepared following the conventional protocols (Hassan et al., 2012): 10 g of plant powder was steeped in 100 ml 95% ethanol for three days, then filtrated through eight layered muslin cloth, followed by filter paper (Whatman No.1). Using a vacuum rotary evaporator, the ethanol filtrate was concentrated at a low temperature, under reduced pressure. This gave solid masses of ethanolic extracts for black seed (2.4 g), nutmeg (1.94 g), radish seed (1.4 g), flaxseed (0.7 g) and date palm pollen (1.8g) they were stored in sterile bottles at (4 °C).

For cell culture studies, 1 g of ethanolic extract was re-suspended in 1ml dimethyl sulfoxide. The suspension was then filtered using a 0.22µm sterile syringe filter and stored in sterile vials at (4 °C). Further dilutions in cell culture medium were made to prepare 10, 50, 100, 500, 1000 µg/ml of plant extracts.

3.4 Ethical Clearance

The study was committed to the principles of laboratory care developed by the Ethics Committee of University of the Western Cape, South Africa. Ethics registration no: 13/2/37.

3.5 Animals

A total of 252 rats (n = 126 male) and (n = 126 female) were used for this study in a complete randomized design. Sexually healthy male white Wister rats were bred

in the animal facility of the Department of Medical Bioscience, University of the Western Cape by mating rats for a period of seven days. At weaning (21 days), male pups were kept in separate cages. During mating, pregnancy and after birth all animals were acclimatized to the laboratory requirement (temperature 24 -27 °C and 12 hour light-dark cycle). The rats were allowed free access to a solid pellet diet and water ad libitum throughout the treatment period.

3.6 Experimental design and extract administration

Forty two male rats were randomly placed into 7 groups (A1-G1) with a population of 6 animals per group. Animals in group A, which served as the control received 0.1 ml of tap water per day in addition to the above mentioned water and diet (3.5). This negative group animals were force feed with water just to subject them to the same conditions (handling stress) as the treated groups. For force feeding a needle of 14 gauge, 3 inch long with a 4mm ball diameter was used. In order to prepare the working solution (doses); stock plant extracts were diluted with fresh tap water. Animals in groups B1, C1, D1, E1, F1 and G1 were treated with doses of (80, 160 mg/kg) radish seed, (200, 300 mg/kg) flaxseed and (120, 240 mg/kg) palm pollen of the ethanolic extract, respectively, with volume of 0.1 ml/rat. All administrations were done daily between 8:00 a.m and 9:00 a.m for 52 days.

The female rats were exposed (200, 400 mg/kg) black seed, (200, 400 mg/kg) nutmeg and (120, 240 mg/kg) palm pollen of the ethanolic extract, respectively, with a volume of 0.1 ml/rat and were randomly placed into 7 groups (A2-G2) with a population of 6 animals per group. Animals in group A, which served as the control received 0.1 ml of tap water (force feeding). All administrations were done daily between 8:00 a.m and 9:00 a.m for 21 days. Figure 3.1 gives an overview of the experimental procedure followed during the study.

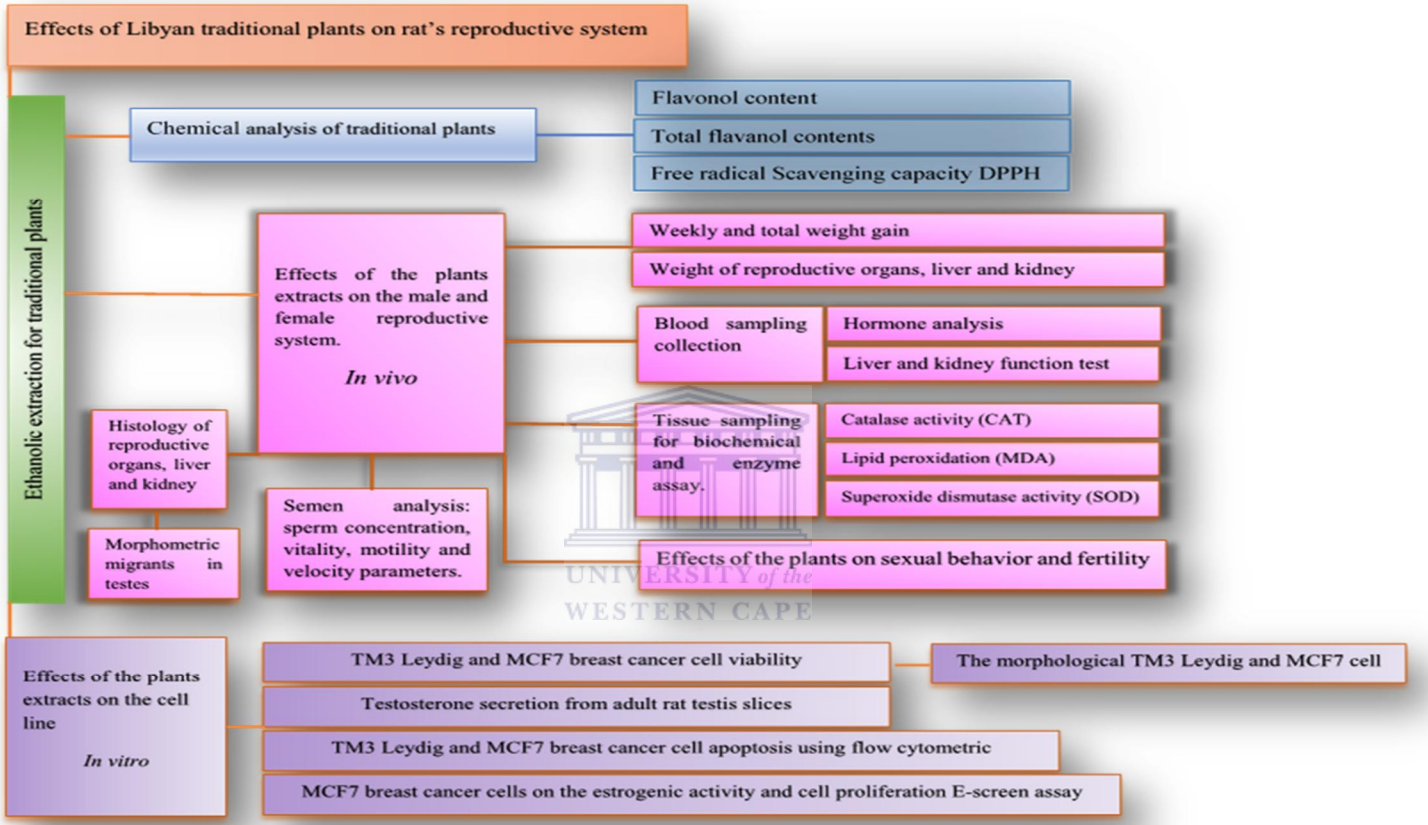

Figure 3.1: Methodological frame

3.7 Chemical analysis of *Linum usitatissimum, Raphanus sativus, Nigella sativa, Myristica fragrans* and *Phoenix dactylifera*

Ethanolic extracts prepared from black seed, nutmeg, radish seed, flaxseed and date palm pollen were collected randomly and stored at (- 20 °C) for later chemical analysis (Flavonol content, total flavanol contents and free radical Scavenging capacity).

3.7.1 Antioxidant activity screening free radical scavenging activity assay (DPPH method)

Free radicals scavenging activity was evaluated using L- ascorbic acid as standard antioxidant. The radical scavenging activity was measured using the stable radical DPPH according to the method described by Brand-Williams et al. (1995) with some modifications. For each plant 1mg/ml of ethanolic plants extract was diluted as follows: 0.2, 0.4, 0.6, 0.8, 1.0 mg/ml. Thereafter, DPPH solutions were also prepared by dissolving 6 mg of DPPH in 100 ml methanol. A mixture of 2 ml of DPPH solution and 1 ml of extract from each dilution was prepared. This mixture was vortexed and left to stand in the dark for 30 minutes. The absorbance was measured at 517 nm in a UV-VIS Spectrophotometer.

$$\text{Calculation \% Scavenging activity} = \left[\frac{(\text{Absorbance of control} - \text{Absorbance of sample})}{\text{Absorbance of control}} \times 100\right]$$

IC50 values, which represented the concentration of the extract that caused 50% neutralization of DPPH radicals, were calculated from the plot of inhibition percentages against concentration.

3.7.2 Determination of total polyphenols

Analysis of total polyphenols present in tea and herbal infusion was done using the Folin-Ciocalteaus reagent (F-C reagent; Singleton & Rossi, 1965) with gallic acid as standard. In short, six standards were prepared in triplicate by adding 0.1% Gallic

acid in dH_2O to final concentrations of 10, 20, 40, 60, 80 and 100 µg/mL (appendix A). Samples were diluted with dH_2O and prepared in triplicates (Appendix B). To the standards, plants extracts and blank, 10% F-C reagent and 7.5% sodium bicarbonate were added in a ratio 5: 4: 1. All samples were thoroughly mixed and incubated for 2 h at 37 ºC and read at 765 nm. A standard curve using Gallic acid was used to determine the amount of total polyphenols (Appendix C). The total polyphenols content of the tea or herbal infusion was expressed as mg Gallic acid equivalent (GAE)/ mL of plants extracts.

3.7.3 Determination of flavonols

The flavonol/flavones levels were evaluated according to Mazza et al. (1999) in which quercetin was used as a standard. Flavanol presence in plant extracts was evaluated using standards that were prepared in triplicate using quercetin stock in 95% ethanol to final concentrations of 1, 2, 4, 8, 16 and 32 mg/mL (Appendix D). These samples were further diluted and prepared in triplicates. To 50µL blank, standards or samples, 50µL 0.1% HCl in 95% EtOH and 900µL was added and incubated for 30 min at room temperate. Absorbance readings were taken at 360 nm and the amount of flavonol in the samples was calculated from the quercetin standard curve. Results obtained were expressed as mg Quercetin equivalent/ mL of plants extracts.

3.8 Effect of *Linum usitatissimum, Raphanus sativus, Nigella sativa, Myristica fragrans* and *Phoenix dactylifera* on the reproductive system, liver and kidney functions.

The male rats were sacrificed by CO_2 inhalation after a 52 day treatment period with the ethanolic extracts of Radish seed, flaxseed and date palm pollen. Sodium pentobarbital solution could not be use her because it was shown to interacts with acrosome reaction measurement (Jiménez et al., 2011). Weights measured at autopsy included body, left testis, left epididymis, seminal vesicles (including

seminal fluid and coagulating gland), prostate, kidneys and liver. These organs were fixed in Bouin's fixative solution for about a week.

Saturated picric acid	750 ml
Formaldehyde (37%)	250 ml
Glacial acetic acid	50 ml

The female rats were sacrificed after a 21 day treatment with ethanolic extracts of Black seed, Nutmeg and Palm pollen by subcutaneously injection with sodium pentobarbital solution (150-200 mg/kg). Vaginal smears were taken before autopsy as described by Marcondes et al. (2002). Weights measured at autopsy included body, uteri, ovaries, kidneys, and liver. These organs were fixed in 10 % neutral-buffered formalin solutions for about a week. The 10% neutral buffered formalin solution was prepared in the following way:

Formaldehyde (37 %)	100 ml
Distilled water	900 ml
Anhydrous monobasic sodium phosphate (NaH_2PO_4)	4.0 g
Disodium hydrogen phosphate (Na_2HPO_4)	6.5 g

3.8.1 Blood sample collection and storage

Blood samples were collected by cardiac puncture immediately after euthanization. Serum was collected using serum separator tube (SST Greiner Bio-One) and allowed to clot for two hours at room temperature before centrifugation for 15 minutes at 1000xg. Approximately 2ml serum was removed, aliquoted and stored at - 80^{o}C.

3.8.2 Serum testosterone concentration

For the quantification of serum testosterone, the Testosterone ELISA kit (DRG Instruments GmbH, Marburg, Germany) was used. This assay was performed according to the manufacturer's protocol. This involved pipetting 25µl of the standards, controls and treatment samples into the wells of the 96-well plate, provided with the kit. To these wells, 200µl of conjugate enzyme was added. The plate was then placed on a shaker and after one hour the contents were discarded followed by washing three times with the diluted washing buffer. Wash buffer was

prepared by dissolving 30ml buffer into 1170 ml distilled water. The plate was then tapped dry on tissue paper so that no wash buffer droplets or air bubbles were present in the wells. After this, 200µl of substrate at 37°C (tetra methyl benzidine) was added to the wells. The plate was then incubated for 15 min at room temperature; following which 100 µl of stop solution (0.5 M H2SO4) was added to end the enzymatic reaction. The DRG Instruments Microtiter Plate Reader was used to read the plate at 450 nm. Absorbance readings were used to produce a standard curve which was used to determine the concentrations of testosterone (Appendix E).

3.8.3 Follicle stimulating hormone (FSH)

Follicle stimulating hormone (FSH) levels in serum were determined using a rat specific FSH ELISA (CSB-E06869r, COSABIO, China). In respect of this, a blank well was set to which no solution was added. This was followed by the addition of 50 µl of Standard to six wells. The standards were done in duplicate. Besides this, 6 samples from the treatment groups were also added in replicates of six. Thereafter, 50µl of HRP-conjugate was added to each well (not to Blank well), followed by 50µl antibody to each well. After mixing, the plate was incubated for 1 hour at 37°C. Each well was then aspirated and washed. This process was repeated twice for a total of three washes. Each well was washed by filling it with 200µl Wash Buffer using a multi-channel pipette. The plate was then allowed to stand for 10 seconds to allow for the complete removal of liquid. In the last wash, any remaining Wash Buffer was removed by decanting. The plate was then inverted and blotted against clean paper towels. Further to this, 50 µl of Substrate A and 50 µl of Substrate B were then added to each well and mixed before incubating for 15 minutes at 37°C. The plate was kept away from drafts and other temperature fluctuations in the dark. Finally 50 µl of stop solution was added to each well. The plate was gently tapped to promote thorough mixing. The optical density was then determined using a microplate reader at 450 nm. A standard curve was used to deduce the level of FSH produced in serum and the results obtained were expressed

as mIU/ml. An average of the duplicate readings for each standard and samples were obtained and from which the average optical density of Blank was subtracted.

3.8.4 Luteinizing hormone (LH)

Luteinizing hormone (LH) levels in the serum were determined using a rat specific ELISA (CSB -E12654r, COSABIO, China). In brief, this assay employs the competitive inhibition enzyme immunoassay technique. In this technique, the microtiter plate provided is pre-coated with goat-anti-rabbit antibody. Standards or samples are then added to the appropriate microtiter plate wells with an antibody specific for LH and Horseradish Peroxidase (HRP) conjugated LH. The competitive inhibition reaction takes place between HRP labelled LH and unlabelled LH with the antibody. A substrate solution is added to the wells and the colour develops in opposite to the amount of LH in the sample. The colour development is stopped and the intensity of the colour is measured. In short, the assay involved the dispensation of 25 µL of standards (0, 10, 20, 40, 100 and 200 mIU/mL) and samples into appropriate wells. This was followed by the addition of 100 µL enzyme conjugate to each well, gently mixed for 10 sec and incubated for 30 min at RT. At the end of the incubation time, the wells were emptied using a multichannel pipette and then rinsed five times with 400 µL distilled water per well. Any remaining liquid was removed by tapping the plate gently on an absorbent paper. Thereafter, 100µL substrate solutions was added to each well and incubated for 10 min at RT. Finally 50µL of stop solution was added to each well. The absorbance was then read at 450 nm within 10 min of adding the stop solution. A standard curve was used to deduce the level of LH produced in serum and the results obtained were expressed as mIU/mL.

3.8.5 Creatinine

The serum level of creatinine of both male and female rats was determined according to Bartels et al. (1972). Basically, serum from the rats or standard solution (0, 15, 30, 60, 90, 120 and 150 mg/L) of creatinine in dH_2O was added to a working

solution in the ratio 1:10. The working solution was composed of alkaline picrate (0.1g picric acid in 50mL dH_2O and 50mL 0.4M NaOH at 4ºC). After 15 minutes of incubation at RT absorbance was read at 492nm. Thereafter, the activity of creatinine in serum was deducted from a standard curve and the results obtained were expressed as mg/L creatinine.

3.8.6 Alanine transaminase

Serum alanine transaminase (ALT) activity was determined according to Reitman and Frankel (1957). In principel, 15 µL of standard solution (containing 0.22 mg/mL sodium pyruvate, alanine transaminase substrate (0.89g alanine and 0.015g of α- ketoglutarate in 50 mL phosphate buffer) and potassium phosphate buffer (0.1M, pH 7.4) and 12.5 µL alanine transaminase substrate for blank and serum were placed in a 96 well plate and incubated at 37^0C for 5 min. After which, 2.5 µL of serum was added into the appropriate wells and incubated at 37ºC for 30 min. Then, 25 µL of 2, 4-dinitrophenylhydrazine (0.2%) in 37% HCl and dH_2O was added to each well, mixed and incubated at RT for 20 min. Following which, 250 µL NaOH (0.4M) was added to all wells, mixed and incubated at RT for 30 min. The absorbance readings were taken at 492 nm. The activity of ALT in serum was deducted from a standard curve and expressed as IU/L.

3.8.7 Aspartate transaminase

Serum aspartate transaminase (AST) activity was determined according to Reitman and Frankel, (1957). Basically, 15µL of standard solution (containing containing 0.22mg/mL sodium pyruvate, aspartate transaminase substrate (0.015g α-ketoglutarate, 1.33 g aspartic acid, 12.5mL 1N NaOH, dissolved and adjusted to pH 7.4 with 8.5% phosphoric acid) and potassium phosphate buffer (0.1M, pH 7.4) and 12.5µL aspartate transaminase substrate for blank and serum were placed in a 96 well plate. After 5min of incubation at 37^0C 2.5µL of serum was added into the appropriate wells and incubated again at 37^0C for 1 h. Thereafter, 25 µL of 2, 4-dinitrophenylhydrazine (0.2%) in 37% HCl and dH2O, was then added to each well,

mixed and incubated at RT for 20 min. Following which, 250 µL NaOH (0.4M) was added to all wells, mixed and incubated at RT for 30 min and absorbance readings were taken at 492 nm. Activity of AST in serum was deducted from a standard curve and the result obtained was expressed as IU/L.

3.8.8 Sperm count

Small pieces of cauda epididymis were cut and placed in a 1ml pre-warmed phosphate buffered saline (PBS) for 5 min at 37ºC. With the help of a Makler counting chamber (Sefi-Medical Instrument) sperm concentration was determined. This was achieved by placing diluted epididymal sperm (1:9) on the chamber; from which five rows were counted and its mean determined in millions per milliliter (10^6/ml).

3.8.9 Sperm vitality

This involved the release of spermatozoa from the cauda epididymis. This was achieved by immersing the cauda epididymis in a Petri dish containing 1mL of 1% BSA in DMEM/ Hams F-12 at 37ºC. Sperm viability was determined using the FluoVit viability assay (FluoVit Microptic, Spain) according to the manufacturer's protocol. In this assay 10 µl of sperm sample were put in an eppendorf tube. To this sample 1 µl Hoechst was added before incubating for 5min at 37ºC. Hoechst was used to identify live cells because of its ability to intercalate with DNA in the nucleus. This was followed by the addition of 1 µl Propidium iodide (PI) and incubation for another 5min at 37ºC. PI was used in order to identify sperms that were dead or had compromised cell membrane. A fluorescence microscope was used to count live (blue) and dead (red) cells either directly through the microscope or indirectly via photographs.

3.8.10 Sperm motility

In order to determine sperm motility the right epididymis was put in a Petri dish (35 x 10 mm) and immersed with 1 ml of 1 % BSA in DMEM/ Hams F12 at 37ºC.

The cauda epididymis was then transferred to a new Petri dish with 1 ml of 1 % BSA in DMEM/ Hams F-12. The cauda epididymis was cleaned of small blood vessels and fat under a stereo microscope. In order to let the sperms come out of the cauda epididymis tiny pieces of the cauda epididymis were prepared and were then transferred into another Petri dish containing 1 ml of 1 % BSA in DMEM/ Hams F-12 5 µl of this sperm cloud was collected, placed on a 5 µL standard count 4 chamber slide (Leja Products B.V., Nieuw Vennep, Netherlands) and motility determined using an Olympus microscope equipped with camera (SCA1024) and Sperm Class Analyzer software (SCA, Microptic, Barcelona, Spain). The following sperm parameters were determined: percentage of total motile sperm, percentage of progressive motile sperm, curvilinear velocity (VCL, µm/s), straight-line velocity (VSL, µm/s), average path velocity (VAP, µm/s), amplitude of lateral head displacement (ALH, µm), and linearity (LIN, %), Wobble (WOB, %), beat cross frequency (BCF, Hz) (Appendices I and G). Total motile sperm take into account the rapid progressive sperm (Type a), slow progressive motile sperm (Type b) and the non-progressive motile sperm (Type c). Progressive motile sperm covers the rapid progressive sperm (Type a) and slow progressive sperm (Type b). Sperm parameters such as VCL and BCF are indicators of sperm viability, while VAP, VSL, STR, and LIN are markers of sperm progression. Furthermore, STR and LIN also describe sperm swimming patterns (Duty et al., 2004).

3.8.11 Histopathology

Organs were harvested and preserved immediately. The preservation involved placing male and female reproductive organs in Bouin's fixative solution and the rest of the organs in formalin solution. Tissues were then dehydrated by immersing them in a series of increasing alcohol concentration before putting them in two changes of 100% alcohol for 2 hours each. The dehydrated tissues were then embedded in paraffin wax blocks (Table 3.2).

Table 3-1: 18 hour cycle of processing tissues

Steps	Solutions	Concentration	Time (hour)
1	ethanol	70%	2
2	ethanol	80 %	2
3	ethanol	90 %	2
4	ethanol 1	100%	2
5	ethanol 2	100%	2
6	Xylene1	-	2
7	Xylene 2	-	2
8	Wax bath 1	-	2
9	Wax bath 2	-	2

Tissue sections were later cut using a rotary microtome at 6µm thickness and mounted on labeled slides. After floatation and drying processes, the paraffin wax was melted by incubating at 60 °C for 10 minutes. Tissue sections were then de-paraffinized by immersing them in two changes of xylene for 5 minutes followed by washing in 100% alcohol and descending solutions of alcohol each for 3 minutes. Staining process started with immersion of tissue sections in Ehrlich's hematoxylin for 15 minutes and then counter stained with 1% aqueous eosin for 1 minute (Table 3.3). Excess stains were removed by washing with tap water following the normal procedures. Tissue sections were then dehydrated with ascending grades of alcohol ending with two washes with xylene. The structural organization of the sectioned tissues was later observed under a microscope using a low-power objective.

Table 3-2: Procedure for Haematoxylin and Eosin stain

Steps	Solutions	Concentration	Time (min)
1	Xylene 2x	-	5
2	ethanol 1	100%	5 each
3	Ethanol	90%	5
4	Ethanol	80 %	5
5	Haematoxylin	-	15
6	Rinsed in tap water	-	1
7	Scott's tap water	-	2
8	acid alcohol	1%	2
9	Eosin	-	1
10	Rinsed in tap water	-	1

11	Ethanol	80 %	2
12	Ethanol	90%	2
13	ethanol 1	100%	2
14	Xylene 2x	-	2 each

3.8.12 Tissue sampling for biochemical and enzyme assay

Immediately after sacrificing the rat, the right testes (male rat) and kidney and a part of the liver (both male and female) were cleaned of fat and stored at -80ºC. Testis (20% w/v), kidney (20% w/v) and liver (20% w/v) tissue was rapidly thawed and homogenized in 1mL ice-cold Tris-buffered saline (Tris HCl 20mM, NaCl 150mM, pH 7.4) using PRO 200 homogenizer (pro scientific Inc, Oxford, USA), followed by centrifugation (5000 x g, 30 min and 4ºC). The supernatant was collected afterwards and stored at (- 80ºC) for future use in antioxidant and biochemical assays.

3.8.12.1 Lipid peroxidation

Oxidative stress in cells and tissues can result in cell injury and in most cases is characterized by lipid peroxidation. However, malondialdehyde has been identified as a natural product of lipid peroxidation. Measurement of MDA using TBARS assay is the most commonly used method for screening and monitoring lipid peroxidation. In the present study, lipid peroxidation was determined by quantification of the thiobarbituric acid reacted substances (TBARS) such as malondialdehyde (MDA) according to Yagi (1984). In short, a ratio of 2:1 was used to mix TBA reagent (15% v/v trichloroacetic acid and 0.25N HCl) with the tissue supernatant. The mixture was heated for 15 min at 95ºC, allowed to cool and centrifuged (1000 g, 10 min). The supernatant was collected and absorbance measured at 532nm. The extinction coefficient of 1.56 $mol^{-1} \cdot cm^{-1}$ was used.

3.8.12.2 Superoxide dismutase activity

Total superoxide dismutase activity was evaluated by the method of pyrogallol autoxidation according to Marklund and Marklund (1974) with a slight modification (Ben Abdallah et al., 2009). Tissue supernatant (100 µl) was mixed with Tris- EDTA-HCl buffer (1.5 mL, pH 8.5) and pyrogallol (100 µL, 15 mM in dH2O) and incubated at 25ºC for 10 min. The reaction was determined by the addition of HCl (50 µL, 1N), and the activity measured at 440 nm. One unit was determined as the amount of enzyme that inhibited the oxidation of pyrogallol by 50 %. The result obtained was expressed as units.

3.8.12.3 Catalase activity

Catalase activity was measured as previously described by Aebi (1984). In brief, 100 µL of tissue supernatant was mixed with 400 µL of PBS (pH 7.4) and 500µL of 20 mM H_2O_2 and measured at 240 nm at 25ºC within 2 min. The extinction coefficient of 43.6 mol-1cm-1 for H_2O_2 was used for calculation. One unit of CAT is defined as the activity of the enzyme that catalysed the reduction of 1 µmol of H_2O_2 per units.

3.9 Effect of *Linum usitatissimum, Raphanus sativus, Nigella sativa, Myristica fragrans* and *Phoenix dactylifera* on the male sexual behavior and fertility

3.9.1 Sexual behavior

In order to assess the sexual behavior; male rats were placed in a quiet room. After 10 minutes of adaptation a stimulus-receptive female (female rat injected subcutaneously with 30 µg/mL estradiol benzoate and 500 µg/mL progesterone in olive oil, 48 h and 4h respectively before pairing) was gently introduced to the cage containing the male rat. The parameters below were monitored according to Ratnasooriya and Dharmasiri (2000) and Watcho et al. (2006): mount latency (ML),

the amount of time taken to have the first mount since the introduction of the female; mount frequency (MF), the number of mounts preceding ejaculation and number of mounts within 30 min. Besides this, the number of sniffs after 3 min of pairing was also recorded within 30 min.

3.9.2 Fertility test

On the morning after pairing female rats were observed for the presence of a vaginal plug; this is a kind of a gelatinous secretion that gets deposited into a female genital tract by the male. This secretion has been observed to harden and plug or glue the female genital tract together (Quammen, 1998). Besides this, we also looked for the presence of spermatozoa in the vaginal smear as described by Marcondes et al. (2002). The presence of vaginal plug or spermatozoa was regarded as day 0 of gestation.

In the absence of any of the above, vaginal smear testing was performed for 10 -12 days to determine pregnancy or pseudo-pregnancy (this is a false pregnancy in which the animal has all the characteristics of pregnancy with no foetus developing in the uterus. It is normally caused by extended diestrus) (James et al., 2007). After 20 days of gestation, laparotomy was done under light ether anaesthesia and the female rats were subsequently euthanized by cervical dislocation (Appendix H). The following reproductive parameters were calculated (Ratnasooriya and Dharmasiri, 2000; Watcho et al., 2006).

Index of libido	(number mated/number paired) x (100)
Fertility index	(number pregnant/ number paired) x (100)
Implantation index	(total number of implantation/ number mated) x (100)
Pre- implantation loss	[(number of corpora lutea- number of implants)/ number of corpora lutea] x (100)
Post- implantation loss	[(number of implants- number of viable implants)/ number of implants] x (100)
Quantal pregnancy	(number pregnant/number mated) x (100)

3.10 The *In vitro* effects of *Linum usitatissimum, Raphanus sativus, Nigella sativa, Myristica fragrans* and *Phoenix dactylifera* on cells viability, apoptosis and hormone activity

3.10.1 Cytotoxicity test

Viability of the TM3 (ATCC® CRL-1714™ Leydig cell) and MCF-7 (ATCC® HTB-22™, breast cancer cells) was measured using the MTT assay. MTT assay is dependent on the cellular reduction of yellow MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] by mitochondrial dehydrogenases of viable cells to a blue, insolube formazan product which can be measured spectrophotometrically.

In this assay, live cells have active mitochondria which generates NADH; a reducing agent that transfers electrons to MTT (Berridge and Tan, 1993). In this case, increased absorbance readings on a spectrophotometer represent the amount of mitochondrial dehydrogenase activity coming from cells that are alive whilst its decrease points towards loss of cell viability.

In order to determine the viability of the cells, 20000 cells/mL of TM3 or 25000 cells/mL MCF-7 breast cancer cells were grown in a 96 well plates for 24, 48 and 72 h. Ethanolic plant extracts of the black seed, radish seed, nutmeg, flaxseed and date palm pollen were lyophilized and reconstituted in DMEM/Ham's F12 medium (containing 2.5 % bovine serum and 5% horse serum for TM3 and 10% bovine serum for MCF7 breast cancer cells, respectively.

Cells were treated with 50, 100, 500 – 1000 µg/mL of each plan extract, DMEM/Ham's F12 medium for a further 24, 48 and 72 h. Thereafter, the supernatants were removed and cells were washed and covered with 200 µl DMEM/Ham's F12 medium. Following this, 20µL of MTT (1 mg/mL in PBS) was added to each well and incubated for 4 h at 37^0C.

Micrographs were taken and subsequently, the supernatant was removed by aspiration and the plate was tapped upside down on paper towels to remove any remaining fluid. 50 µl of DMSO was then added to each well to dissolve the precipitated dye. The plate was then agitated on a shaker for 15 min. Absorbance of the dye was measured with an ELISA reader (Thermo electron corporation, South Africa) at a wavelength of 560 nm with a reference wavelength of 750 nm.

The percent vitality was then calculated using following equation:

$$\text{Vitality (\%)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of contro}} \times 100$$

3.10.2 Testis slices

Sexually mature (90-day-old) Wistar rats were sacrificed by CO_2 asphyxiation. The testes were harvested and their capsule removed. The testes were sliced (mean weight per piece 45.5 ± 5.5 mg) and then placed in an eppendorf tube with 1.5 ml Dulbecco's modified Eagle's (DMEM:F12) culture medium containing 0.1% bovine serum albumin (BSA), 2.5 µl/ml penicillin streptomycin and kept under a humidified atmosphere with 5 % CO_2 at 37ºC. After preincubation for 30 min, the medium was replaced with medium containing plant extracts (500 or 1000 µg mL) or medium containing plant extracts plus 10 IU mL−1 hCG. Preliminary studies showed that hCG concentrations over (i.e. stimulated testosterone production) 1 IU mL−1 gave a maximal response. After 180 min incubation, testicular slices were removed from culture dishes and the supernants were frozen at -80°C for future testosterone ELISA assay.

3.10.3 E-screen assay

3.10.3.1 Removal of sex steroids by charcoal-dextran

Charcoal was washed twice with cold sterile water immediately before using. A 5 % charcoal-0.5 % dextran suspension was prepared. Charcoal-dextran (CD)

suspension aliquots of a volume similar to the serum aliquots to be processed were centrifuged at 2500 rpm for 10 min. Supernatants was aspirated and serum aliquots were mixed with the charcoal pellets. This charcoal-serum mixture was maintained in suspension by rolling at 4 cycles /min at 37oC for 1 hr. This suspension was centrifuged at 2000 g for 20 min. The supernatant was then filtered through a 0.45-pm filter. CD – treated sera were stored at -20°C until needed.

3.10.3.2 Proliferation assay of MCF-7 cells

The E-screen assay was carried out in MCF-7 cells according to Soto et al. (1995) with some modifications. The cells were seeded with normal growth medium in 96 well plates at a density of approximately $5x10^4$ cells /ml. After attachment of cells (24 h) growth medium was removed and cells were washed with PBS to remove all estrogenic compounds. Growth medium was replaced by phenol red free DMEM with similar supplements as growth medium but containing 5% CD-FBS and cells were allowed to grow for 48 h. CD-FBS was used to minimize the estrogenic and steroid activity of serum and to synchronize the cells in the G0/G1-phase of the cell cycle after 48 h growth. Exposure medium was refreshed and plant ethanolic extract was added to wells in triplicate at different concentrations. A positive (with E2, 1 nM estradiol-17β) and a solvent control (05% DMSO without E2 or plant ethanolic extract) were included on each plate. After 4 days of incubation exposure medium was removed and cells were trypsinised using 0.025% trypsin. Cell proliferation was measured using The Tali® Image Cytometer. In brief, the number and proportion of viable and dead cells was determined using the Tali™ viability Kit – Dead Cell Red (Cat. no. A10786). In the present study, the machine captured 20 fields each covering 0.233 µL of the sample (Appendix I). Subsequently, total cell count, number of live, dead and apoptotic cells was obtained in a form of tables and histograms. The fluorescence of cells was measured at an emission wavelength of 525 ±20 nm (green channel) and excitation wavelength of 530 nm /585 nm (red channel).

3.10.4 Apoptosis Assay

To quantify apoptosis, we used APOPercentage™ apoptosis assay (Biocolor Ltd, Belfast, Ireland). In this assay apoptotic cells stain red and their cell membrane undergoes through a membrane flip-flop event by which phosphatidylserine is translocated to outer leaflet. Phosphatidyl serine transmembrane movement results in the uptake of APOPercentage dye by apoptotic committed cells. Dye uptake continues until blebbing of the apoptotic committed cell occurs. No further dye can then enter the defunct cell and the dye that has accumulated within the cell is not released. Necrotic cells do not retain the dye. Prior to the start of the experiment blank sample, negative control and positive control (6% DMSO) were prepared in triplicate. Test samples consisted of cells exposed to 500 or 1000µg/ml BS, RS, FS, NM and DPP, respectively for 72h. In this thesis, A 24 well plate was used to culture 5 x 10^4 cells in 1000 µl culture medium. After attain 80% confluency the cells were washed with PBS. Thereafter, the cells were trypsinized and placed in 15ml centrifuge tubes. To each of these tubes 250µl of APOPercentage apoptosis dye was then added. Subsequently, the cells were then incubated at 37ºC with 5% CO_2 for 30 minutes. The cells were then washed in 4ml PBS to remove non-cell bound dye. The tube was then spun in a centrifuge to form a pellet. This was followed by the re-suspension of the cells in 250ul PBS and thereafter transferred into an Eppendorf cup. The samples were then taken to the BD 6 accurri Becton, (Dickinson and Company USA) FACS machine for the determination of apoptotic cells. On the FACS machine 10 000 events were read using the FL3 channel (red).

3.11 Statistical analysis

Statistical analysis was done using Medcalc statistical software (Version12.1.3.0, Mariakerke, Belgium). To test for normal distribution, Kolmogorov-Smirnoff test was done, followed by an independent sample t-test. If data was not normally distributed, a Mann-Whitney test was performed. Data was expressed as mean ± standard deviation and a p value of < 0.05 was considered statistically significant. Analysis of variance (ANOVA) was also performed between groups and a p value

< 0.05 was regarded to be statistically significant. TUKEY outlier detection test was also performed. The graphs were analyzed using Graph Pad Prism version 6.

CHAPTER IV

4 Result of the chemical analysis of *Linum usitatissimum, Raphanus sativus, Nigella sativa, Myristica fragrans* and *Phoenix dactylifera*

Samples of ethanolic extracts prepared from black seed (200 and 400 mg/kg BW), nutmeg (200 and 400 mg/kg BW), radish seed (80 and 160 mg/kg BW), flaxseed (200 and 300 mg/kg BW) and date palm pollen (120 and 240 mg/kg BW) were collected randomly during the time of force-feeding the rats and stored at – 20 C^{o} for later chemical analysis (Flavonol content, total flavanol contents and free radical Scavenging capacity).

4.1 Flavonol content

Figure 4.1 showed that the flavonol content was significantly higher in black seed (P<0.5) compared to nutmeg, radish seed, flax seed and date palm pollen. On the other hand, nutmeg and date palm pollen had significantly higher (P<0.5) flavonols compared to radish seed and flaxseed.

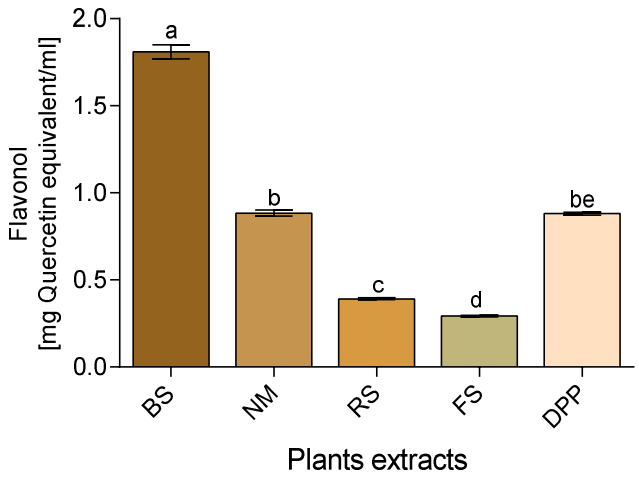

Figure 4.1: Flavonol content in *Linum usitatissimum, Raphanus sativus, Nigella sativa, Myristica fragrans* and *Phoenix dactylifera* Values are the means ± SD of 10 replicates.
Abbreviations: BS, Black seed; NM, Nutmeg; RS, Radish seed; FS, Flaxseed; DPP, Date palm pollen; Means on each bar followed by the same letter do not differ significantly. If the letter differs then $p < 0.05$.

4.2 Total flavonoid content

It can be clearly noticed that the total flavonoid content was highest in date palm pollen (Figure 4.2), compared to all other plant extracts (p<0.05). In contrast, black seed had the lowest total flavonoid content (p<0.05).

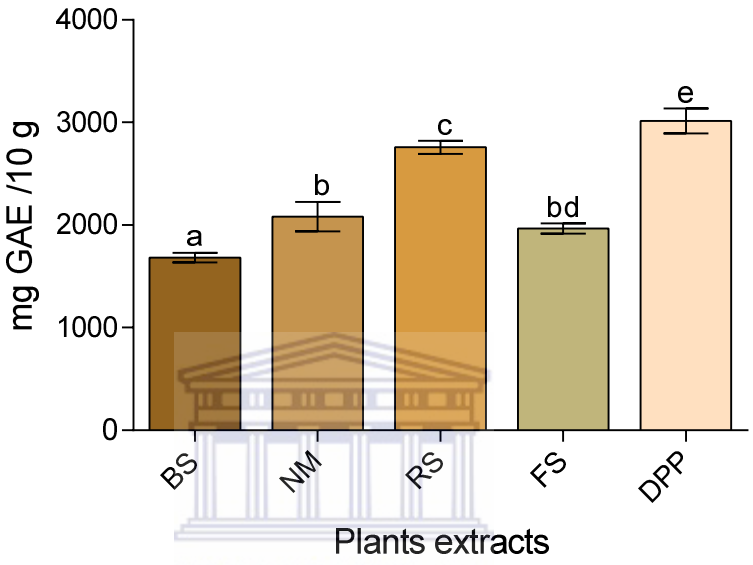

Figure 4.2: Total flavonoid content in *Linum usitatissimum, Raphanus sativus, Nigella sativa, Myristica fragrans* and *Phoenix dactylifera.*
Values are the means ± SD of 10 replicates. Abbreviations: BS, Black seed; NM, Nutmeg; RS, Radish seed; FS, Flaxseed; DPP, Date palm pollen; GAE, Gallic acid equivalent. Means on each bar followed by the same letter do not differ significantly. If the letter differs then $p < 0.05$.

4.3 Antioxidant activity by the DPPH method

The *in vitro* antioxidant assay performed on this plant reveals significant antioxidant potential compared with black seed, flaxseed, nutmeg, radish seed and date palm pollen as a standard butylated hydroxytoluene (BHT). DPPH radicals are widely used in the model system to investigate the scavenging activity of several natural phytocompounds. The result of IC50 valuesfor DPPH scavenging activities in this study indicates that the plants were potentially active. BS, NM, FS and DPP extract shows inhibition of (BS 58.48 %, NM 45.21%, RS 40.93 %, 40.71 % FS

and DPP 52.78 %) significantly (P>0.5) as compared to Butylated hydroxytoluene (BHT) which shows 83.22% (Figure 4.3).

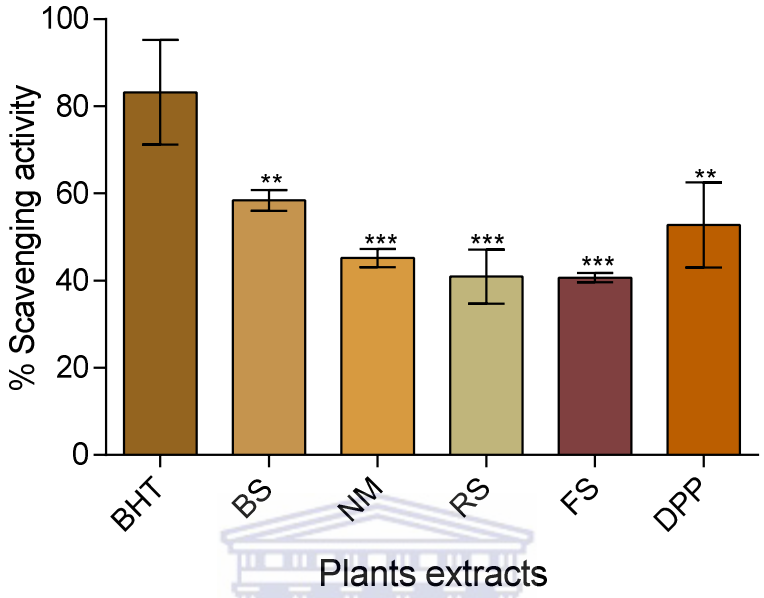

Figure 4.4: free radical Scavenging capacity in *Linum usitatissimum, Raphanus sativus, Nigella sativa, Myristica fragrans* and *Phoenix dactylifera.*
Values are the means ± SD of 10 replicates. Abbreviations: BS, Black seed; NM, Nutmeg; RS, Radish seed; FS, Flaxseed; DPP, Date palm pollen; **, $p < 0.01$; ***, $p < 0.001$ compared to Butylated hydroxytoluene (BHT) (paired t -Test analysis).

CHAPTER V

5 Result of the effect of *Linum usitatissimum, Raphanus sativus* and *Phoenix dactylifera* on the male reproductive system.

5.1 Weekly and total weight gain

Male rats force- fed with radish seed (80 and 160 mg/kg), flaxseed (200 and 300 mg/kg) and date palm pollen (120 and 240 mg/kg) for 52days. The weekly weight gain in the male rats treated with the ethanolic plant extracts showed no significant difference compared to the control (figure 5.1). In the same way, the total weight gain in the male rats also did not differ significantly compared to the treated groups after 52 days exposure to the ethanolic plant extracts. However, date palm pollen at 240 mg/kg showed a significant increase in weight gain (figure 5.2; $P < 0.05$). Also, flaxseed (300 mg/kg) and date palm pollen (120 mg/kg) showed a small but not significant ($P > 0.05$) rise in total body weight. On the other hand, both concentrations of Radish seed and flaxseed (200 mg/kg) showed a tendency of decreased weight RS (80 and 160 mg/kg) (paired T-Test analysis).

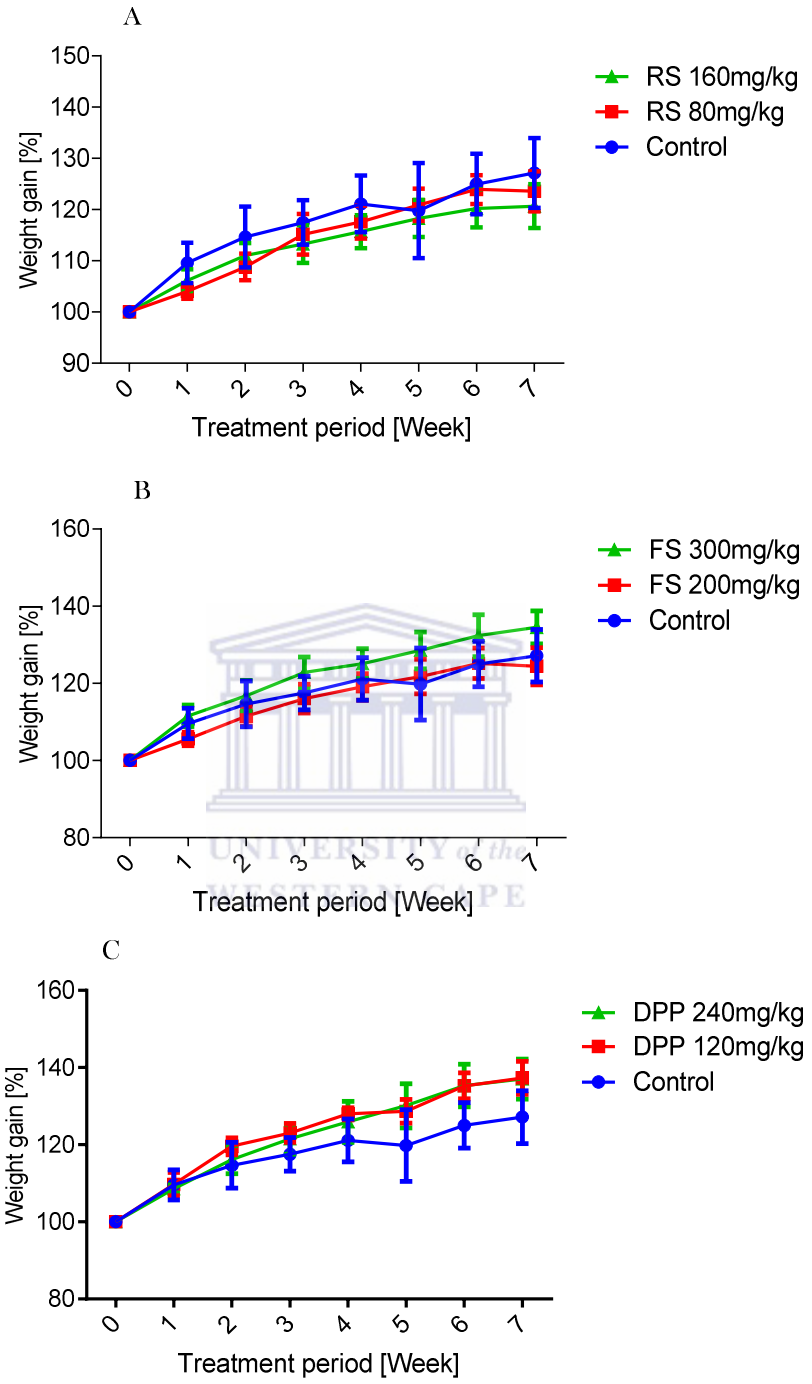

Figure 5.1: Effect of *Linum usitatissimum, Raphanus sativus* and *Phoenix dactylifera* on the weekly weight gain for the period of treatment in male rats.
Values represented are the mean ± SD of 6 animals per group. Abbreviations: A, Radish seed; B, flaxseed; C, date palm pollen.

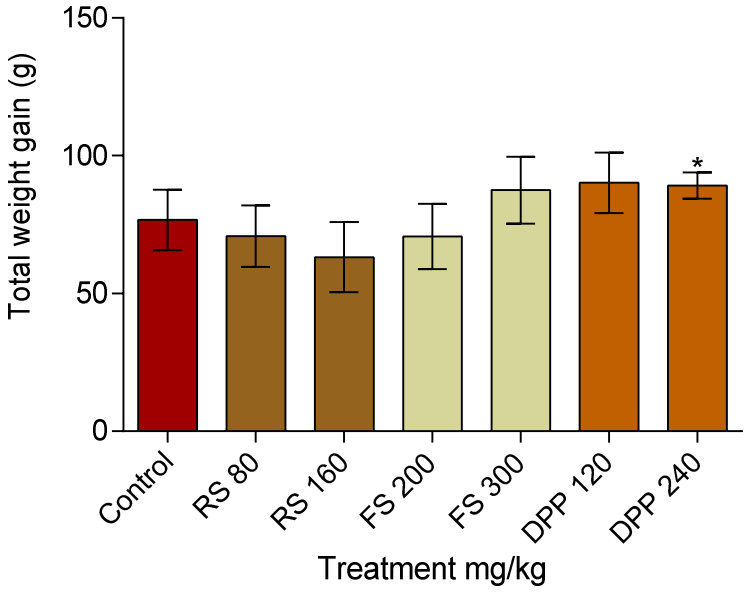

Figure 5.2: The effect of *Linum usitatissimum, Raphanus sativus* and *Phoenix dactylifera* on body weight gain in male rats.
Values represented are the mean ± SD of 6 animals per group. Abbreviations: RS, Radish seed; FS, Flaxseed; DPP, Date palm pollen; *, p<0.05 compared to control (paired T-Test analysis).

5.2 Reproductive organs, liver and kidney weight in male rats

Table 5.1 showed no significant difference in the relative weights of epididymis, prostate and kidney (p>0.05). However, relative testicular weights of animals exposed to either flaxseed (300 mg/kg) or palm pollen (120 mg/kg) showed significant increased values (p<0.01). Similarly, the absolute weight of the prostate in animals exposed to palm pollen 240 showed significant increased values (p > 0.05). In addition, the relative weights of the seminal vesicles in all treated groups showed significant increased values (p < 0.01). Also the absolute weight of seminal vesicles showed significant increased values (p < 0.01) by using radish seed (80 mg/kg) and the both concentration of flaxseed. The absolute weight of the livers showed significant increase in both Radish seed groups compared to the control, but showed a tendency to decrease in the both palm pollen groups. However, the groups exposed to radish seed (80 mg/kg) and date palm pollen (120 mg/kg) showed a significant increase in the liver relative weight (p < 0.01)

Table 5-1 :Weight [g] of reproductive organs, liver and kidney after 52 days treatment with control, Radish seed, Flaxseed and Date palm pollen in male rats

Treatment [mg/kg] Organ [g]	Control	RS 80	RS 160	FS 200	FS 300	DPP 120	DPP 240
Testis Absolute Relative	3.77 ±0.13 0.95 ±0.06	3.66 ±0.25 0.98 ±0.59	3.67 ±0.10 1.00 ±0.13	3.59 ±0.13 0.99 ±0.08	3.83 ±0.27 1.10 ±0.09**	3.67 ±0.45 1.10 ±0.06**	3.46 ±0.23 1.02 ±0.12
Epididymis Absolute Relative	2.16 ± 0.51 0.53 ± 0.11	1.63 ±0.18** 0.43 ±0.07	1.46 ±0.15* 0.41 ±0.03	1.68 ±0.37 0.46 ±0.10	1.53 ±0.27* 0.43 ±0.04	1.48 ±0.12** 0.44 ±0.05	1.32 ±0.15** 0.42 ±0.11
Seminal vesicles Absolute Relative	1.41 ±0.14 0.35 ±0.03	1.70 ±0.07* 0.45 ±0.02***	1.55 ±0.24 0.42 ±0.06***	1.70 ±0.31* 0.46 ±0.07**	1.64 ±0.22* 0.47 ±0.07**	1.54 ±0.23 0.47 ±0.09*	1.52 ±0.19 0.44 ±0.07*
Prostate Absolute Relative	0.70 ±0.14 0.17 ±0.03	0.63 ±0.14 0.17 ±0.03	0.70 ±0.03 0.18 ±0.004	0.56 ±0.19 0.15 ±0.05	0.54 ±0.11 0.16 ±0.04	0.59 ±0.24 0.18 ±0.07	0.57 ±0.06* 0.16 ±0.01
Liver Absolute Relative	17.61 ± 1.52 4.35 ± 0.39	18.96± 2.38* 5.06 ± 0.42*	16.73± 1.64* 4.54 ± 0.44	17.15 ± 2.15 4.73 ± 0.51	16.86 ± 1.70 4.81 ± 0.08	15.64 ± 1.38 4.73 ± 0.60*	14.48 ± 2.04 4.22 ± 0.49
Kidneys Absolute Relative	3.52 ± 0.05 0.89 ± 0.08	3.54 ±0.14 0.93 ±0.02	3.66 ±0.09* 1.00 ±0.13	3.21 ±0.36 0.88 ±0.05	3.23 ±0.29* 0.91 ±0.02	3.03 ±0.23* 0.91 ±0.08	2.88 ±0.38* 0.84 ±0.11

Values represented are the mean ± SD of 6 animals per group. Abbreviations: RS, Radish seed; FS, Flaxseed; DPP, Palm pollen *, $p<0.05$, ** $p<0.01$ compared to control. Relative organ weight = organ weight/final body weight * 100.

5.3 Endocrine hormones.

5.3.1 Testosterone

The level of serum testosterone produced by the male rats (Figure 5.3) showed a significant increase after exposure to Radish seed (80 mg/kg) and a significant dose dependent increase for date palm pollen when compared to control ($p < 0.05$). In contrast, there was a decrease in testosterone when FS was given at a dose of 300mg/kg.

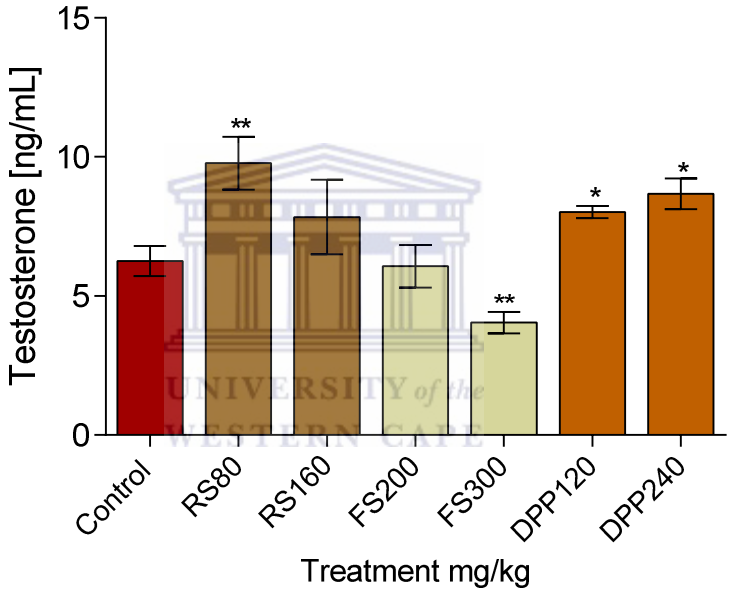

Figure 5.3: Effect of *Linum usitatissimum, Raphanus sativus* and *Phoenix dactylifera* on testosterone production.
Values represented are the mean ± SD of 6 animals per group. Abbreviations: RS, Radish seed; FS, Flaxseed; DPP, Date palm pollen; *, p<0.05; **, p<0.01 compared to control. (Paired T-Test analysis).

5.3.2 Follicle stimulating hormone (FSH)

All plant extracts caused a tendency to a dose-dependent decrease in FSH serum values. This decrease was significant after exposure to FS (300 mg/kg, $p < 0.01$) and DPP (120 mg/kg, $p < 0.05$; 240 mg/kg, $p < 0.01$) (Figure 5.4).

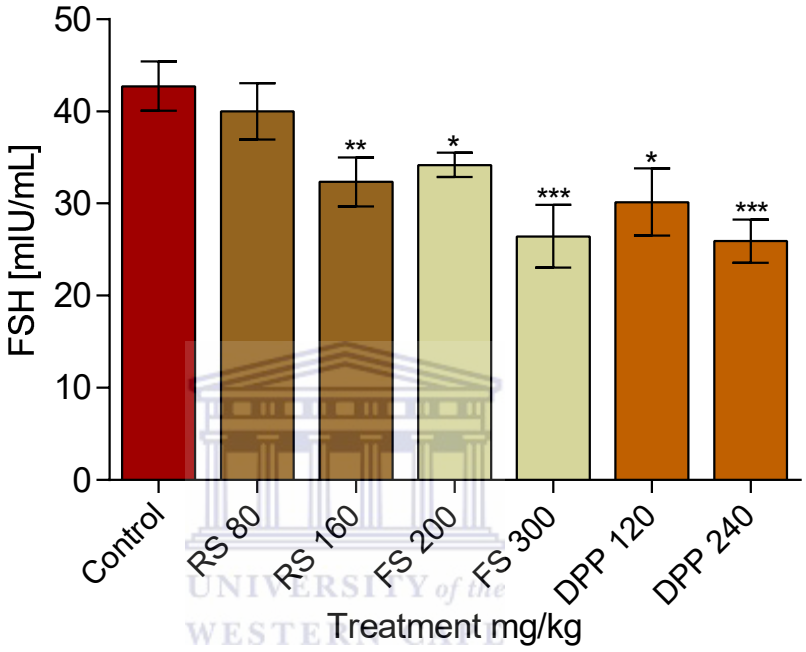

Figure 5.4: Effect of *Linum usitatissimum, Raphanus sativus* and *Phoenix dactylifera* on follicle stimulating hormone production.
Values represented are the mean ± SD of 6 animals per group. Abbreviations: RS, Radish seed; FS, Flaxseed; DPP, Date palm pollen; *, p<0.05; ***, p<0.001 compared to control. (Paired T-Test analysis)

5.3.3 Luteinizing hormone (LH)

Figure 5.5 shared no significant change in serum LH levels after exposure to the plant extracts. Although most plant extracts showed a tendency to slightly increased LH values.

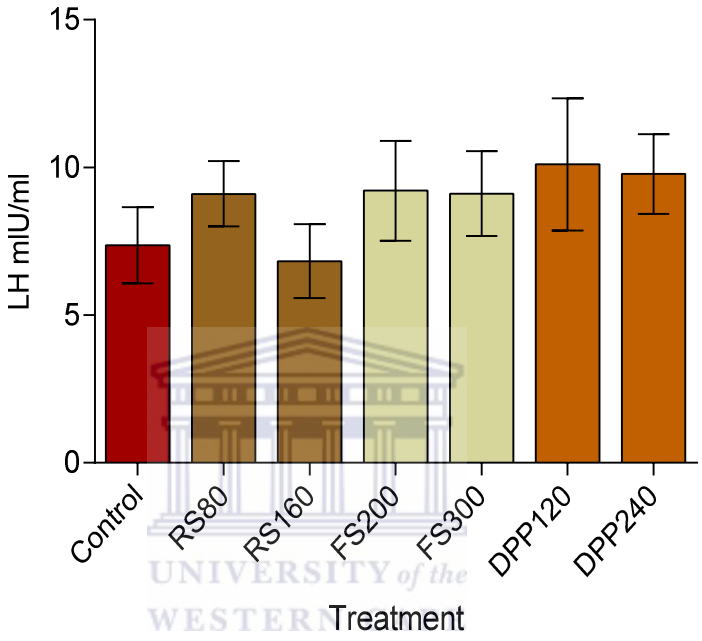

Figure 5.5: Serum levels of *Linum usitatissimum*, *Raphanus sativus* and *Phoenix dactylifera* on Luteinizing hormone production.
Values represented are the mean ± SD of 6 animals per group. Abbreviations: RS, Radish seed; FS, Flaxseed; DPP, Date palm pollen. (paired T-Test analysis).

5.4 Sperm concentration

RS and FS caused a significant dose-dependent increase in sperm concentration compared to the control group. In respect of this, lower concentration of RS and FS showed significant increased ($p < 0.05$) sperm concentration compared to the control. Treatment with higher doses of RS or FS (300 mg/kg) showed a high significant increased ($p < 0.01$) sperm concentration compared to the control. At the same time when DPP was given at a dose of 120 mg/kg or 240 mg/kg it induced a high significant increased ($p < 0.01$) sperm concentration compared to the control (Figure 5.6).

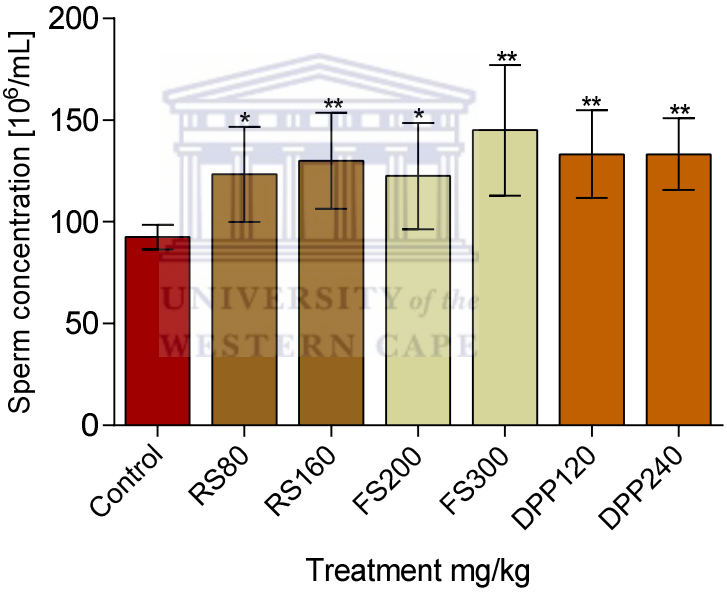

Figure 5.6: Effect of *Linum usitatissimum, Raphanus sativus* and *Phoenix dactylifera* on sperm concentration.
Values represented are the mean ± SD of 6 animals per group and at least 200 sperm per animal were analysed. Abbreviations: RS, Radish seed; FS, Flaxseed; DPP, Date palm pollen; *, $p<0.05$; **, $p<0.01$ compared to control.

5.5 Sperm vitality

Figure 5.7 presents that sperm vitality has become stronger in all treatment groups compared to the control. However, a significant increase was observed in sperm vitality by the groups exposed to radish seed (80 mg/kg, $p < 0.01$), flaxseed (300 mg/kg, $p < 0.05$) and date palm pollen (120,240 mg/kg, $p < 0.05$).

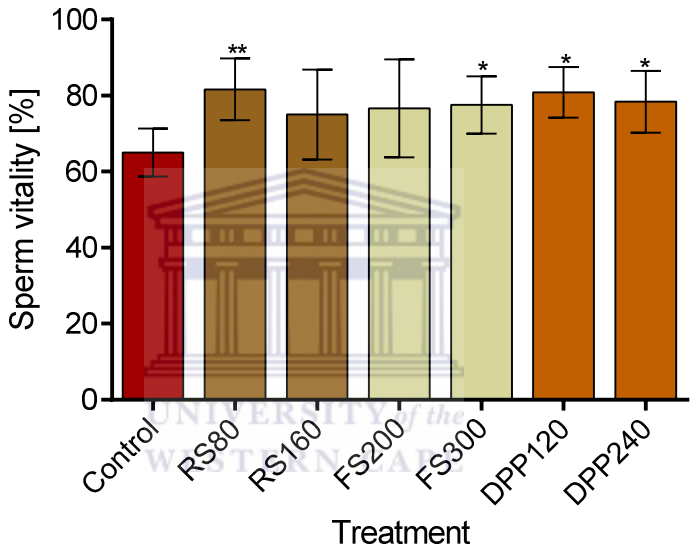

Figure 5.7: Effect of *Linum usitatissimum, Raphanus sativus* and *Phoenix dactylifera* on sperm vitality.
Values represented are the mean ± SD of 6 animals per group and at least 200 sperm per animal were analysed. Abbreviations: RS, Radish seed; FS, Flaxseed; DPP, Date palm pollen; *, $p<0.05$; **, $p<0.01$ compared to control.

5.6 Sperm motility

5.6.1 Progressive motility

Progressive motility (figure 5.8) improved significantly in the groups exposed to FS (300 mg/kg, $p < 0.001$) as well as DPP (120mg/kg, 240mg/kg, $p < 0.01$) compared with the control, while the RS (80, 160 mg/kg) and FS (200 mg/kg) showed no effect.

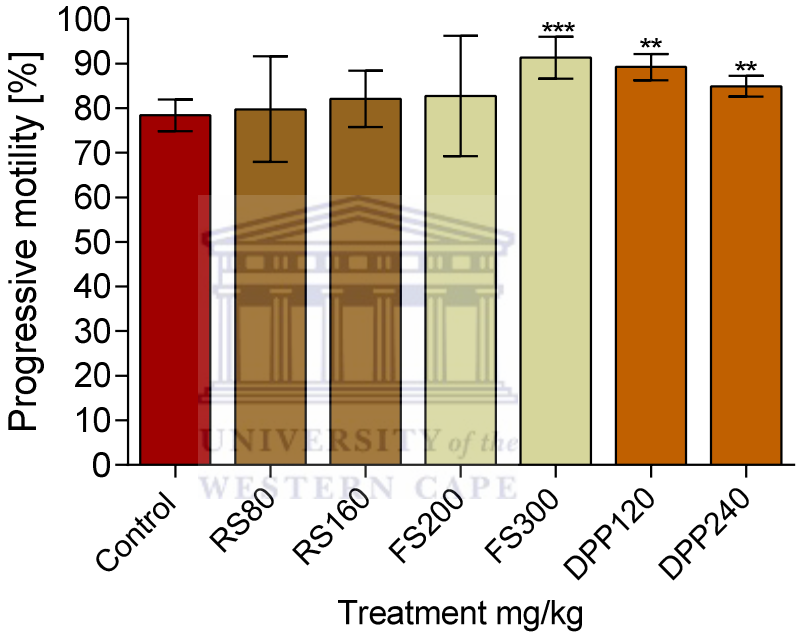

Figure 5.8: Effect of *Linum usitatissimum, Raphanus sativus* and *Phoenix dactylifera o*n sperm progressive motility.
Values represented are the mean ± SD of 6 animals per group after 52 day treatment and at least 200 sperm per animal were analysed. Abbreviations: RS, Radish seed; FS, Flaxseed; DPP, Date palm pollen; **, $p<0.01$; ***, $p<0.001$ compared to control.

5.6.2 Total motility

The total motility (figure 5.9) was significantly enhanced in the groups exposed to FS (300 mg/kg) as well as DPP (120,240mg/kg, $p < 0.01$) compared with the control, while the other treatment groups showed a tendency to increased values ($p > 0.05$) compared with the control.

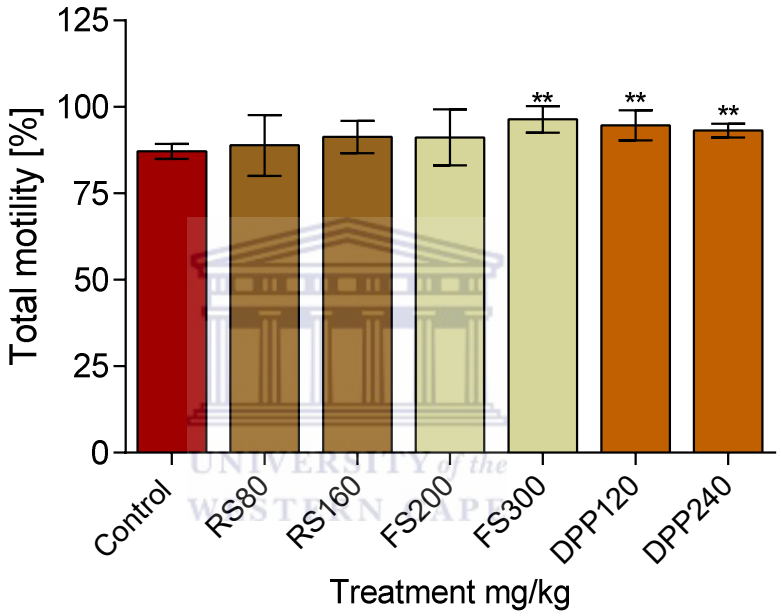

Figure 5.9: Effect of *Linum usitatissimum, Raphanus sativus* and *Phoenix dactylifera* on total sperm motility.
Values represented are the mean ± SD of 6 animals per group after 52 day treatment and at least 200 sperm per animal were analysed. Abbreviations: RS, Radish seed; FS, Flaxseed; DPP, Date palm pollen; **, $p<0.01$ compared to control.

5.6.3 Total static

The figure 5.10 compares the total percentage of static sperm, it showed that flaxseed (300 mg/kg) and date palm pollen (120 and 240 mg/kg) significantly decreased the percentage of total static spermatozoa ($p < 0.05$) while radish seed (80 and 160 mg/kg) and flaxseed (200 mg/kg) resulted in a non-significant decrease ($p > 0.05$) compared with the control group.

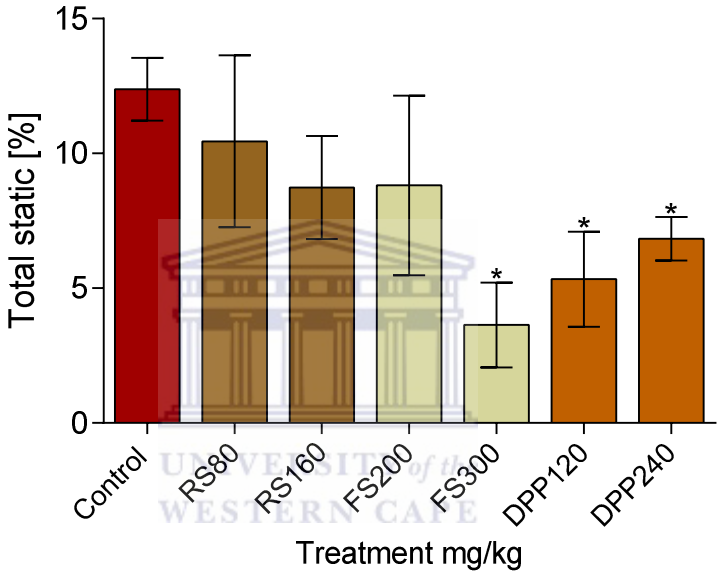

Figure 5.10: Effect of *Linum usitatissimum, Raphanus sativus* and *Phoenix dactylifera* on total sperm static.
Values represented are the mean ± SD of 6 animals per group and at least 200 sperm per animal were analysed. Abbreviations: RS, Radish seed; FS, Flaxseed; DPP, Date palm pollen; *, p<0.05 compared to control.

5.7 Sperm velocity parameters (VCL, VSL, VAP, ALH, LIN, WOB and STR)

Figures 5.12 to 5.15 show that the sperm velocity parameters VSL, VAP, LIN and WOB were not significantly different from the control; however VSL and VAP showed a susceptibility to increased values when compared to the control (p>0.05),

on the other hand LIN and WOB displayed on a non-significant decrease in all treatment groups (p>0.05) compared with the control group.

The curvilinear velocity (VCL; Figure 5.11) was shown to have a tendency to improved values in all treatment groups (p>0.05). In addition, the VCL value after treatment with date palm pollen (240 mg/kg) was significantly higher than the control ($p < 0.05$).

As can be seen from figure 5.16 a dose-dependent lateral head displacement (ALH) was significantly higher in animals exposed to radish seed (160 mg/kg) and flaxseed (300 mg/kg) ($p < 0.05$) and palm pollen (240 mg/kg) ($p < 0.01$) compared to the control.

Beat cross frequency (BCF) on rat sperm figure 5.18 was significantly enhanced in the groups exposed to radish seed (80 mg/kg) and flaxseed (300 mg/kg) ($p < 0.05$), while the other treatment groups showed a tendency to increased values ($p > 0.05$) compared with the control.

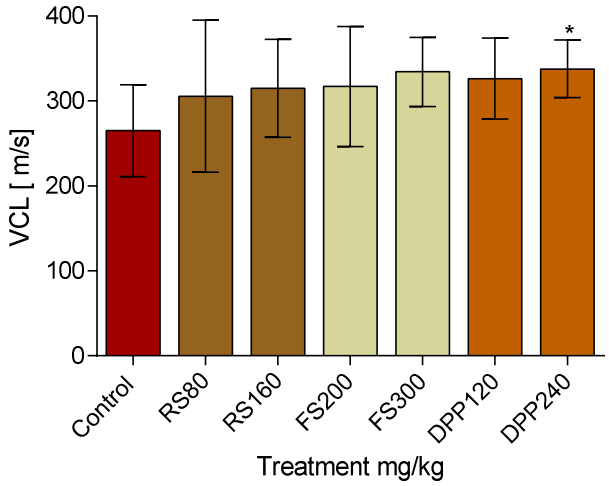

Figure 5.11: Effect of *Linum usitatissimum, Raphanus sativus* and *Phoenix dactylifera* on curvilinear velocity (VCL).
Values represented are the mean ± SD of 6 animals per group and at least 200 sperm per animal were analyzed. Abbreviations: RS, Radish seed; FS, Flaxseed; DPP, Date palm pollen; *, p<0.05 compared to control.

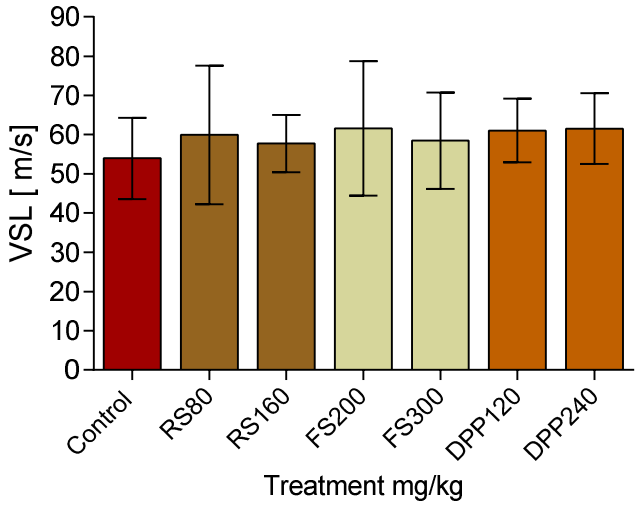

Figure 5.12: Effect *of Linum usitatissimum, Raphanus sativus* and *Phoenix dactylifera* on straight line velocity (VSL).
Values represented are the mean ± SD of 6 animals per group and at least 200 sperm per animal were analyzed. Abbreviations: RS, Radish seed; FS, Flaxseed; DPP, Date palm pollen.

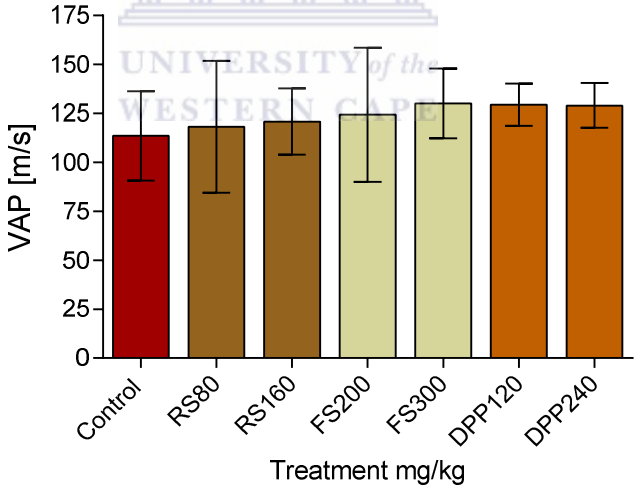

Figure 5.13: Effect of *Linum usitatissimum, Raphanus sativus* and *Phoenix dactylifera* on average path velocity VAP.
Values represented are the mean ± SD of 6 animals per group and at least 200 sperm per animal were analyzed. Abbreviations: RS, Radish seed; FS, Flaxseed; DPP, Date palm pollen.

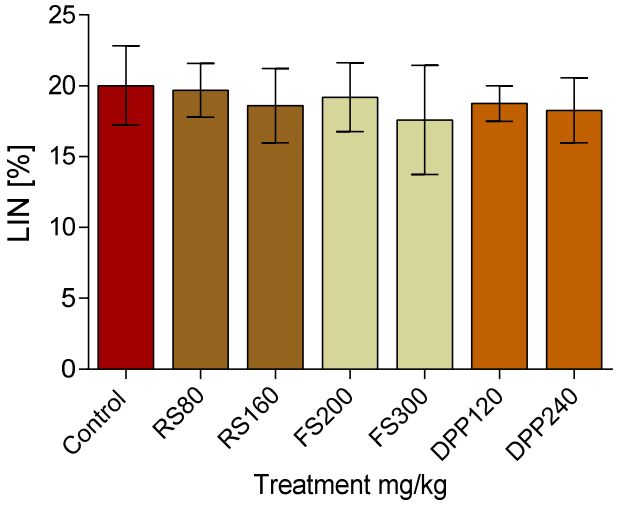

Figure 5.14: Effect *of Linum usitatissimum, Raphanus sativus* and *Phoenix dactylifera* on linearity (LIN) on rat sperm.
Values represented are the mean ± SD of 6 animals per group and at least 200 sperm per animal were analyzed. Abbreviations: RS, Radish seed; FS, Flaxseed; DPP, Date palm pollen.

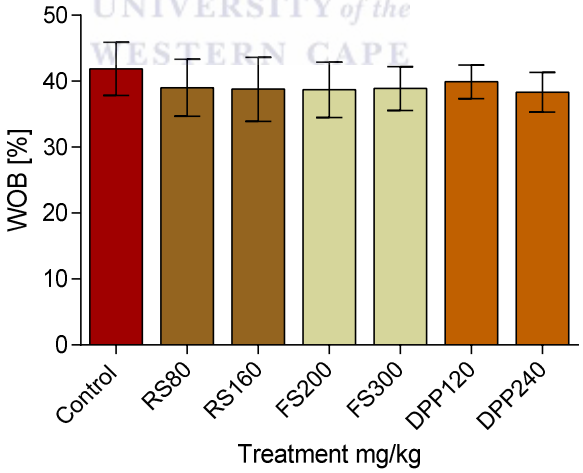

Figure 5.15: Effect of *Linum usitatissimum, Raphanus sativus* and *Phoenix dactylifera* on wobble (WOB) on rat sperm.
Values represented are the mean ± SD of 6 animals per group and at least 200 sperm per animal were analyzed. Abbreviations: RS, Radish seed; FS, Flaxseed; DPP, Date palm pollen.

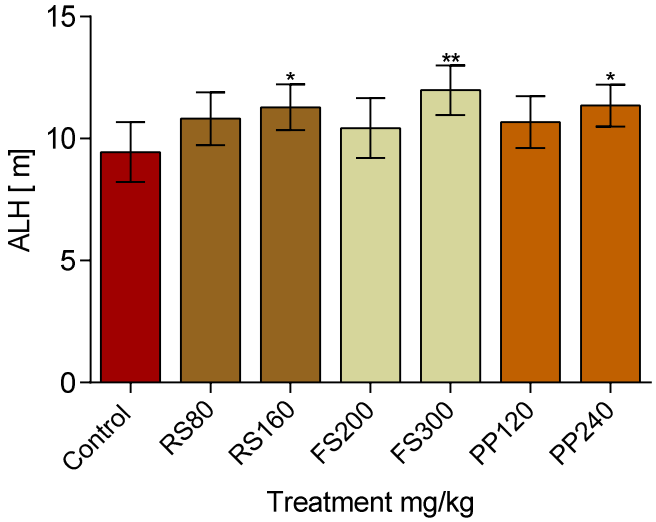

Figure 5.16: Effect *of Linum usitatissimum, Raphanus sativus* and *Phoenix dactylifera* on amplitude of lateral head displacement (ALH). Values represented are the mean ± SD of 6 animals per group and at least 200 sperm per animal were analyzed. Abbreviations: RS, Radish seed; FS, Flaxseed; DPP, Date palm pollen; *, p<0.05; **, p<0.01 compared to control.

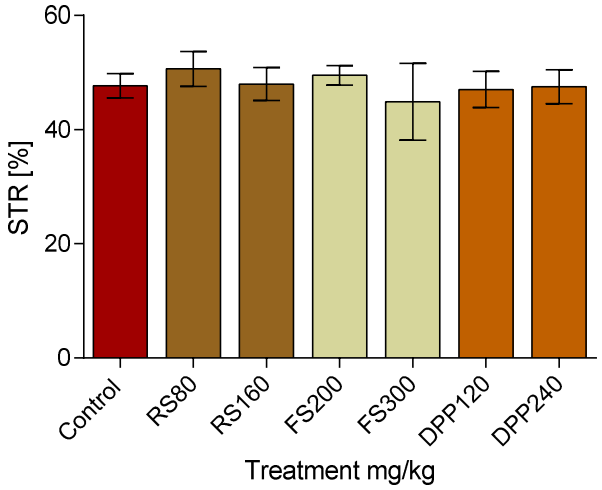

Figure 5.17: Effect of *Linum usitatissimum, Raphanus sativus* and *Phoenix dactylifera* on linear is on straightness (STR) on rat sperm. Values represented are the mean ± SD of 6 animals per group and at least 200 sperm per animal were analyzed. Abbreviations: RS, Radish seed; FS, Flaxseed; DPP, Date palm pollen.

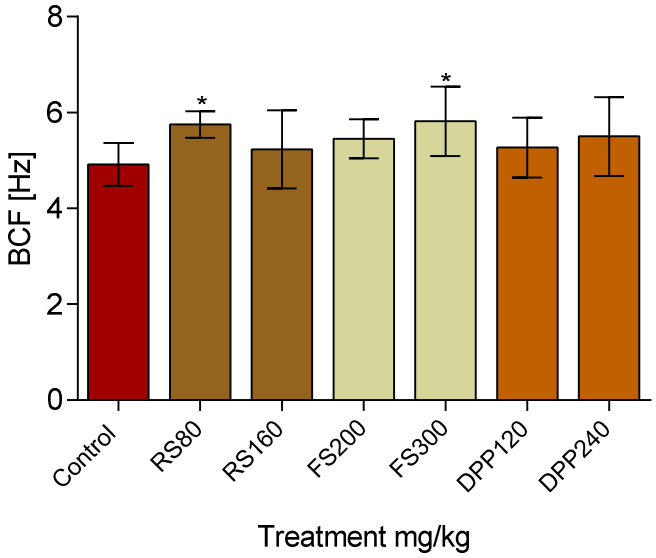

Figure 5.18: Effect of *Linum usitatissimum, Raphanus sativus* and *Phoenix dactylifera* on beat cross frequency (BCF) on rat sperm. Values represented are the mean ± SD of 6 animals per group and at least 200 sperm per animal were analyzed. Abbreviations: RS, Radish seed; FS, Flaxseed; DPP, Date palm pollen; *, $p<0.05$ compared to control.

5.8 Creatinine activity

It can be clearly noticed that the activity of serum creatinine in male rats exposed to all treatments was decreased compared to the control group (figure 5.19). This decrease was significant in rats exposed to radish seed (160 mg/kg) flaxseed (200 and 300 mg/kg) and date palm pollen (240 mg/kg).

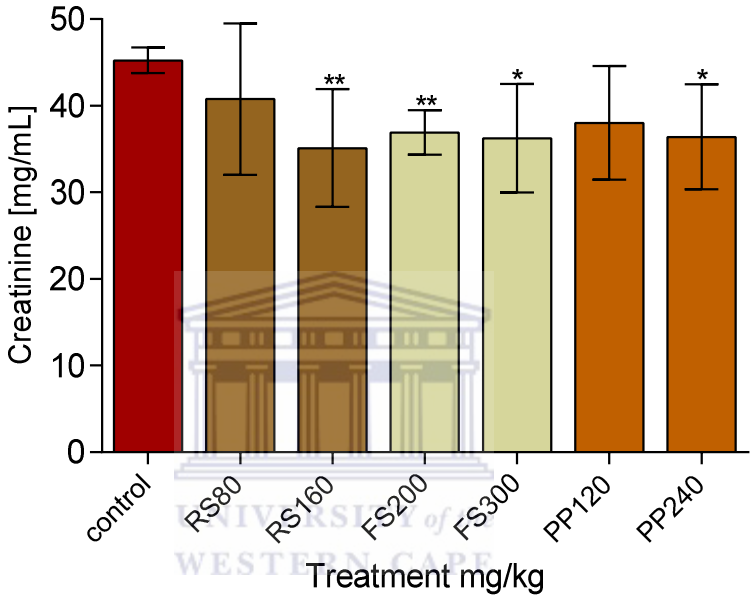

Figure 5.19: Effect of *Linum usitatissimum, Raphanus sativus* and *Phoenix dactylifera* on creatinine activity in male rat serum.
Values represented are the mean ± SD of 6 animals per group. Abbreviations: RS, Radish seed; FS, Flaxseed; DPP, Date palm pollen; *, $p<0.05$; **, $p<0.01$ compared to control.

5.9 Alanine transaminase activity (ALT)

Figure 5.20 showed that the serum alanine transaminase (ALT) activity in the male rats decreased significantly in all treatments (p<0.05) except in the flaxseed (300 mg/kg) treated groups.

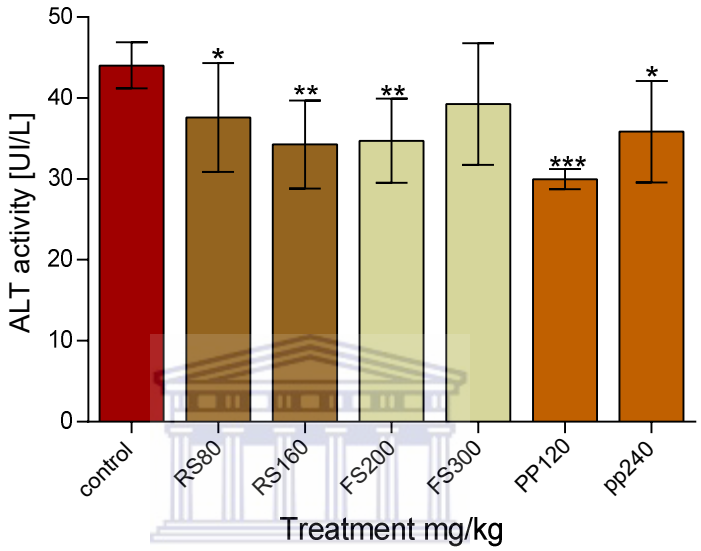

Figure 5.20: Effect of *Linum usitatissimum, Raphanus sativus* and *Phoenix dactylifera* on Alanine transaminase (ALT) activity in male rat serum.
Values represented are the mean ± SEM of 6 animals per group. Abbreviations: RS, Radish seed; FS, Flaxseed; DPP, Date palm pollen; *, p< 0.05; **, p<0.01; ***, p<0.001 compared to control.

5.10 Aspartate transaminase activity (AST)

Figure 5.21 showed that serum level of aspartate transaminase (AST) activity was not significantly increased in the male rats that have been treated with radish seed (80 and 160 mg/kg) and flaxseed (200 and 300 mg/kg) (p < 0.05). On the other hand AST dropped non- significantly in male rat exposed to date palm pollen (p > 0.05) compared to control group.

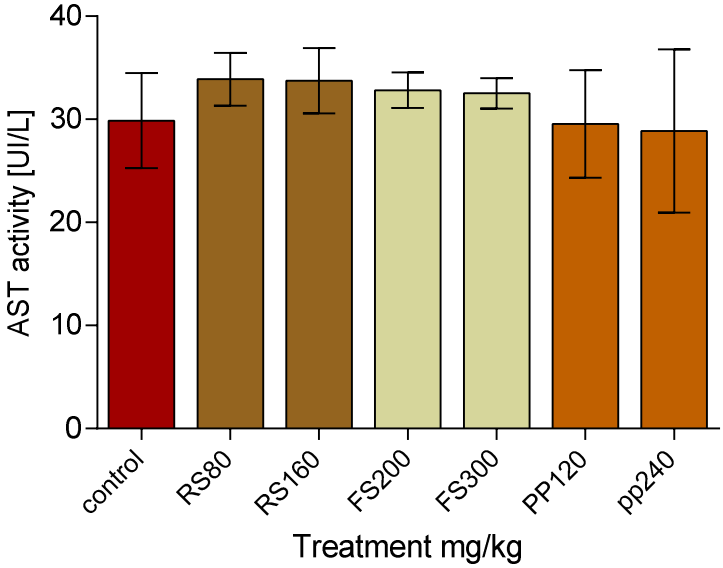

Figure 5.21: Effect of *Linum usitatissimum, Raphanus sativus* and *Phoenix dactylifera* on Aspartate transaminase (AST) activity in male rat serum.
Values represented are the mean ± SD of 6 animals per group. Abbreviations: RS, Radish seed; FS, Flaxseed; DPP, Date palm pollen.

5.11 Biochemical assays on testes, liver and kidney

5.11.1 Catalase activity (CAT)

Figure 5.22 showed an increase in the catalase activity in testis of male rats that was significant after exposure to radish seed (160 mg/kg) ($p > 0.05$) and date palm pollen (240 mg/kg; $p > 0.01$) compared to the control group. On the other hand exposure to flaxseed (200 and 300 mg/kg) showed a non- significant drop in CAT activity ($p > 0.05$) compared to control group.

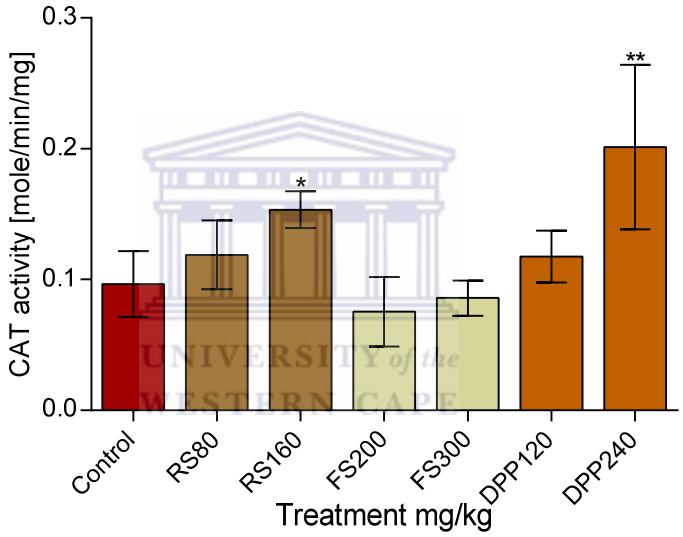

Figure 5.22: Effect of *Linum usitatissimum, Raphanus sativus* and *Phoenix dactylifera* on catalase activity in the male rat testis.
Values represented are the mean ± SD of 6 animals per group. Abbreviations: RS, Radish seed; FS, Flaxseed; DPP, Date palm pollen; *, $p< 0.05$; **, $p<0.01$ compared to control.

In the liver of rats exposed to radish seed (160 mg/kg) a significant increased CAT activity ($p < 0.05$; figure 5.23) was observed. Flaxseed (300 mg/kg) and date palm pollen (120 and 240 mg/kg) showed non-significantly enhanced CAT levels in the liver ($p > 0.05$). On the other hand, non- significant decreased CAT levels were noticed after treatment with radish seed (80 mg/kg) and flaxseed (200 mg/kg; $p > 0.05$) compared to the control.

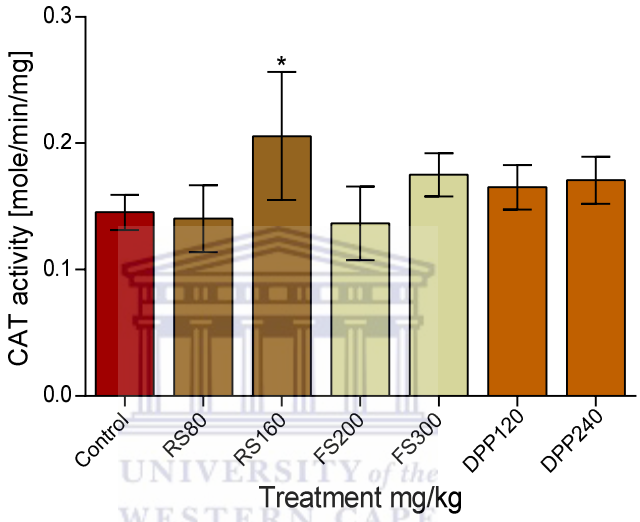

Figure 5.23: Effect of *Linum usitatissimum, Raphanus sativus* and *Phoenix dactylifera* on catalase activity in the male rat liver.
Values represented are the mean ± SD of 6 animals per group. Abbreviations: RS, Radish seed; FS, Flaxseed; DPP, Date palm pollen; *, $p < 0.05$ compared to control.

CAT activity levels in male rat kidney exposed to the plant extracts showed no significant changes ($p > 0.05$; figure 5.24) compared to control group. However, flaxseed (both concentrations) and palm pollen (120 mg/kg) caused a slight, but non-significant increase in CAT activity.

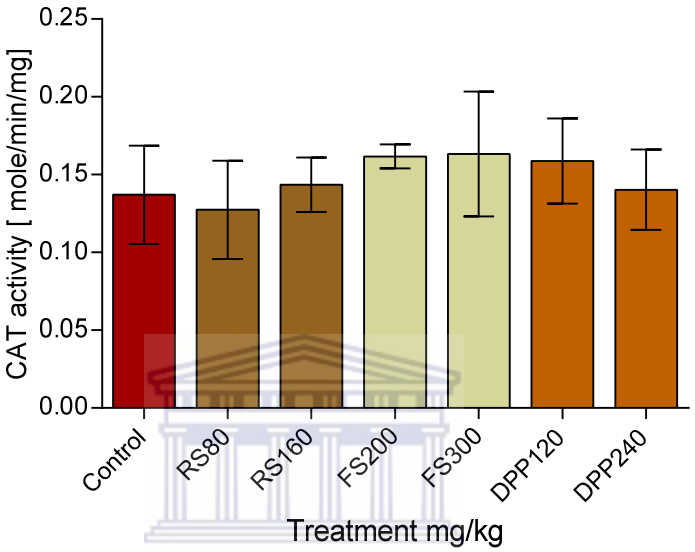

Figure 5.24: Effect of *Linum usitatissimum, Raphanus sativus* and *Phoenix dactylifera* on catalase activity in the male rat kidney.
Values represented are the mean ± SD of 6 animals per group. Abbreviations: RS, Radish seed; FS, Flaxseed; DPP, Date palm pollen; *, p<0.05 compared to control.

5.11.2 Superoxide dismutase activity (SOD)

In general, treatment with the plant extracts caused an increase in testicular SOD activity (figure 5.25). The rise was significant for RS (160 mg/kg), FS (200 mg/kg) and DPP (120 mg/kg).

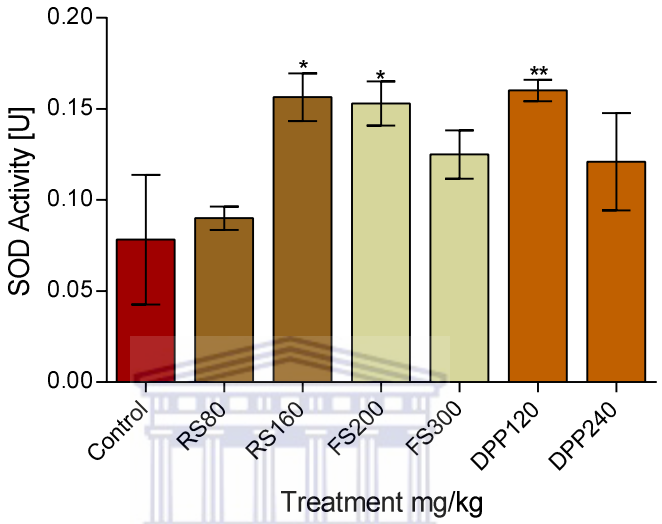

Figure 5.25: Effect of *Linum usitatissimum, Raphanus sativus* and *Phoenix dactylifera* on superoxide dismutase (SOD) activity in the male rat testis. Values represented are the mean ± SD of 6 animals per group. Abbreviations: RS, Radish seed; FS, Flaxseed; DPP, Date palm pollen; *, $p< 0.05$; **, $p<0.01$ compared to control.

In the livers of male rats exposed to radish seed (80 and 160 mg/kg) flaxseed (300 mg/kg) and date palm pollen (120 mg/kg) SOD activity was significantly increased ($p < 0.05$; figure 5.26). On the other hand, exposure to flaxseed (200 mg/kg) and date palm pollen (240 mg/kg) caused SOD levels that were similar to the control ($p > 0.05$).

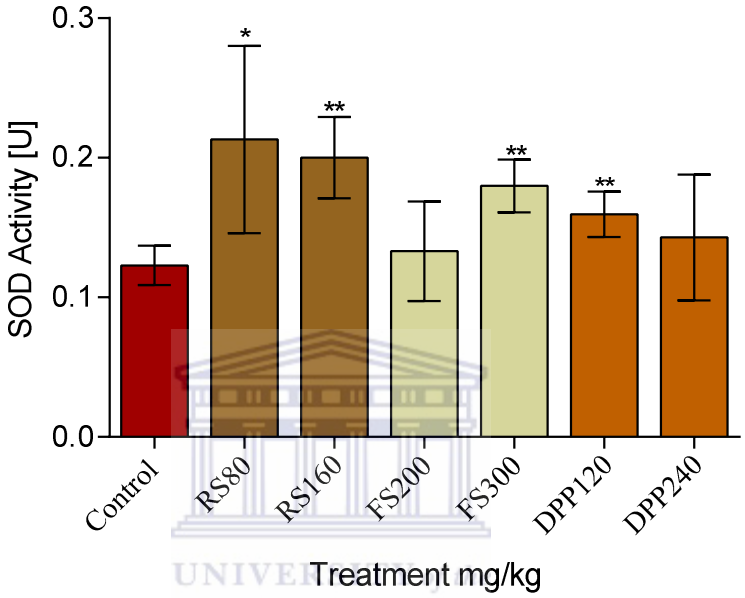

Figure 5.26: Effect of *Linum usitatissimum, Raphanus sativus* and *Phoenix dactylifera* on superoxide dismutase (SOD) activity in the male rat liver.
Values represented are the mean ± SD of 6 animals per group. Abbreviations: RS, Radish seed; FS, Flaxseed; DPP, Date palm pollen; *, $p<0.05$; **, $p<0.01$ compared to control.

As can be seen from figure 5.27 there was a significant increase in superoxide dismutase (SOD) activity in male rat kidney in groups exposed to radish seed (160 mg/kg; $p < 0.05$) compared to the control. SOD activity showed no significant changes in other treatment groups ($p > 0.05$). However, date palm pollen (240 mg/kg) caused a significant decrease ($p < 0.05$) in SOD activity compared to the control group.

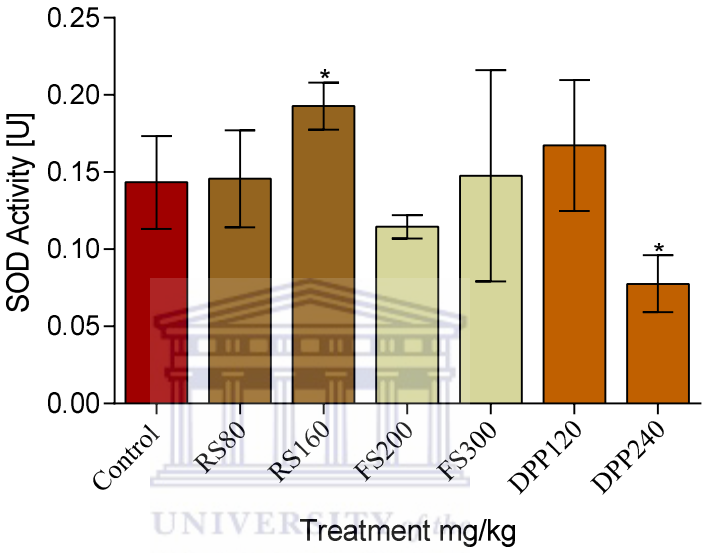

Figure 5.27: Effect of *Linum usitatissimum, Raphanus sativus* and *Phoenix dactylifera* on superoxide dismutase (SOD) activity in the male rat kidney.
Values represented are the mean ± SD of 6 animals per group. Abbreviations: RS, Radish seed; FS, Flaxseed; DPP, Date palm pollen; *, $p<0.05$compared to control.

5.11.3 Lipid peroxidation

From figure 5.28, shown below, male rats exposed to either flaxseed (300 mg/kg) or date palm pollen 120 and 240 mg/kg) showed a significant increase in the level of lipid peroxidation (LPO) measured as thiobarbituric acid reactive substances (TBARS) in the testis compared to the controls ($p < 0.05$). On the other hand, radish seed (80 and 160 mg/kg) caused no changes ($p > 0.05$). Flaxseed (200 mg/kg) tends to decrease the level of TBARS ($p > 0.05$) when compared to the control.

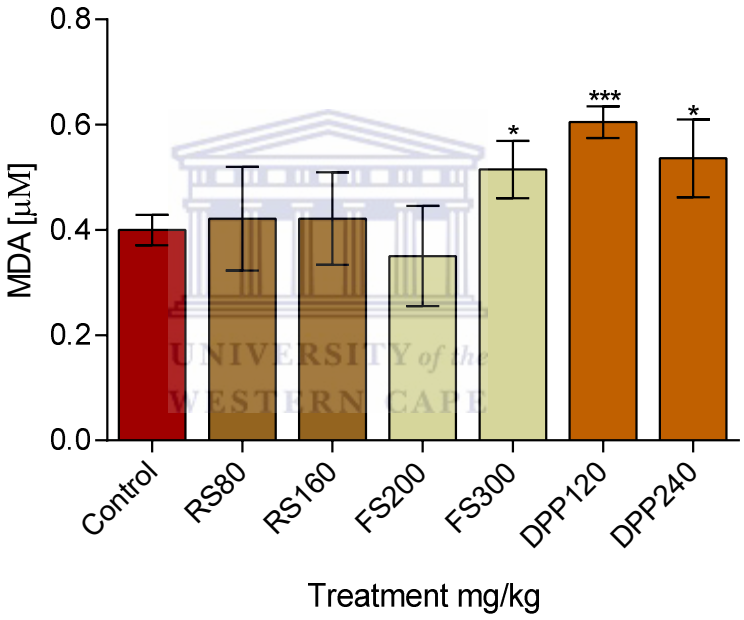

Figure 5.28: Effect of *Linum usitatissimum, Raphanus sativus* and *Phoenix dactylifera* on lipid peroxidation (MDA) in male rat testis.
Values represented are the mean ± SD of 6 animals per group. Abbreviations: RS, Radish seed; FS, Flaxseed; DPP, Date palm pollen; *, $p<0.05$; ***, $p<0.001$ compared to control.

Lipid peroxidation levels in the liver of male rats exposed to either radish seed (80 mg/kg) or date palm pollen (240 mg/kg) showed no significant differences compared to the control ($p < 0.05$; figure 5.29).In contrast, a tendency to decreased values was observed in the groups exposed to radish seed (160 mg/kg), flaxseed (200 and 300 mg/kg) and date palm pollen (120 mg/kg; $p > 0.05$).

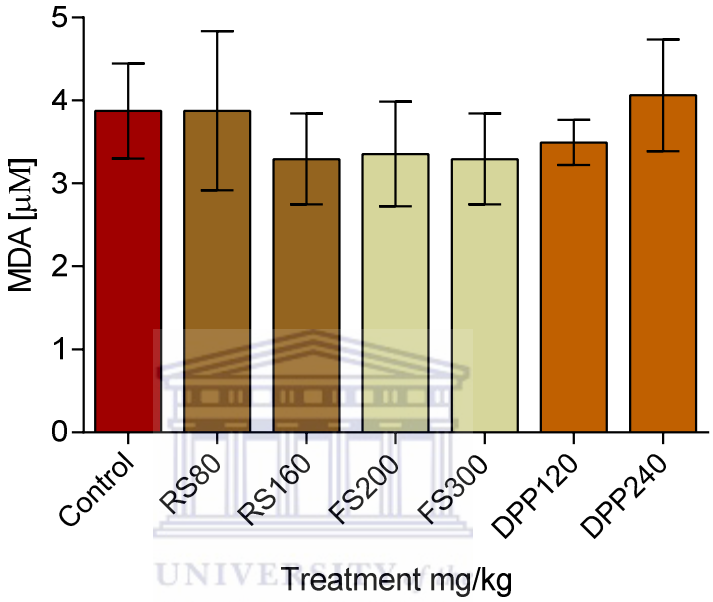

Figure 5.29: Effect of *Linum usitatissimum, Raphanus sativus* and *Phoenix dactylifera* on lipid peroxidation (MDA) in male rat livers.
Values represented are the mean ± SD of 6 animals per group. Abbreviations: RS, Radish seed; FS, Flaxseed; DPP, Date palm pollen.

In the kidneys of male rats exposed to radish seed (80 mg/kg), flaxseed (200 and 300 mg/kg) and date palm pollen (120 mg/kg) the level of MDA showed no significant difference to the control ($p < 0.05$; figure 5.30). On the other hand, radish seed (160 mg/kg) displayed a tendency to decreased values ($p > 0.05$) compared to control group.

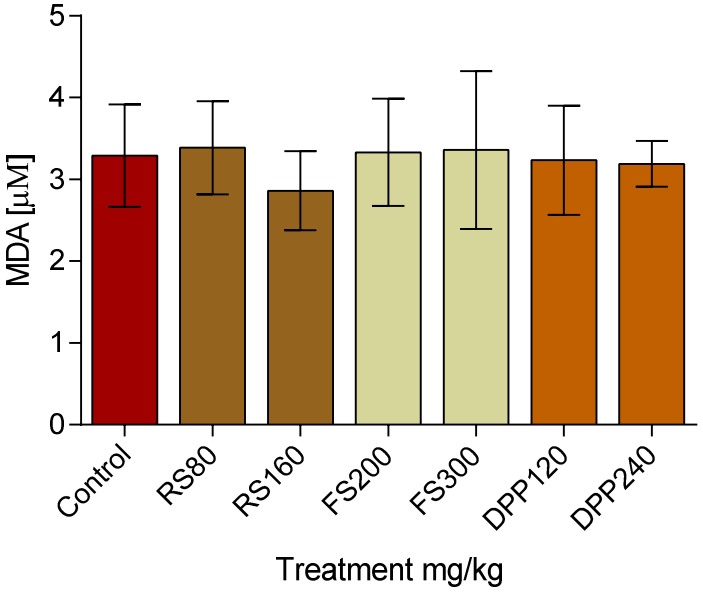

Figure 5.30: Effect of *Linum usitatissimum, Raphanus sativus* and *Phoenix dactylifera* on lipid peroxidation (MDA) in male rat kidneys.
Values represented are the mean ± SD of 6 animals per group. Abbreviations: RS, Radish seed; FS, Flaxseed; DPP, Date palm pollen.

5.12 Histology of reproductive organs, kidney and liver in male rats

Histological examination of the cross sections of the testis in rats exposed to radish seed (80 and 160 mg/kg), flaxseed (200 and 300 mg/kg) and date palm pollen (120 and 240 mg/kg) (Figures 5.31, 5.32 and 5.33) showed clear presence of all stages of spermatogenesis in all the treated groups. Besides this, there was a possible increased presence of spermatozoa in the seminiferous tubule lumen of treated rats.

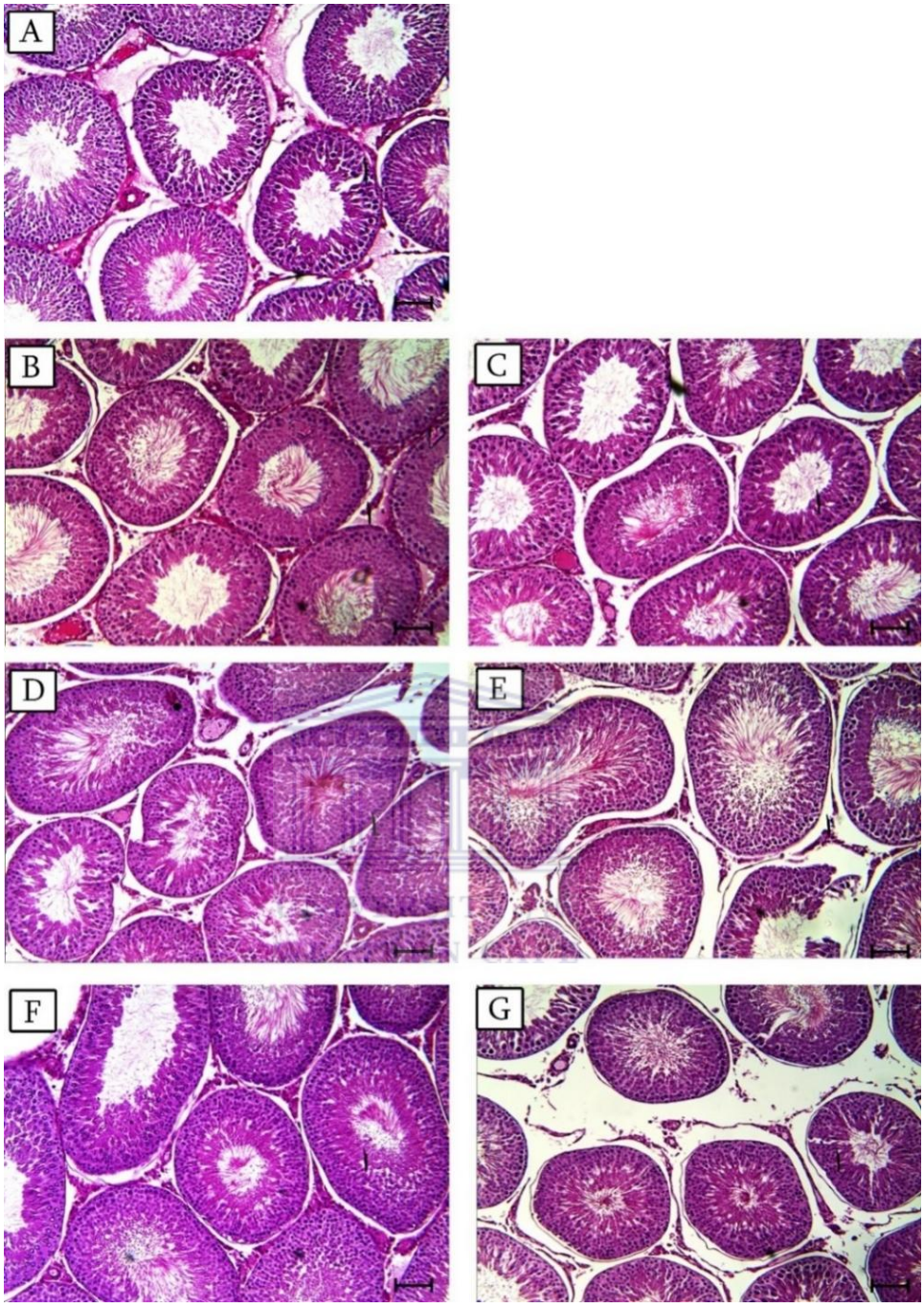

Figure 5.31: Photomicrograph of the male rat testis exposed to *Linum usitatissimum, Raphanus sativus* and *Phoenix dactylifera* for 52 days using H&E staining.
A; control, B; Radish seed (80mg/kg), C; Radish seed (160mg/kg), D; Flaxseed (200mg/kg), E; Flaxseed (300mg/kg), F; Date palm pollen (120mg/kg), G; Date palm pollen (240mg/kg). Bar = 100µm

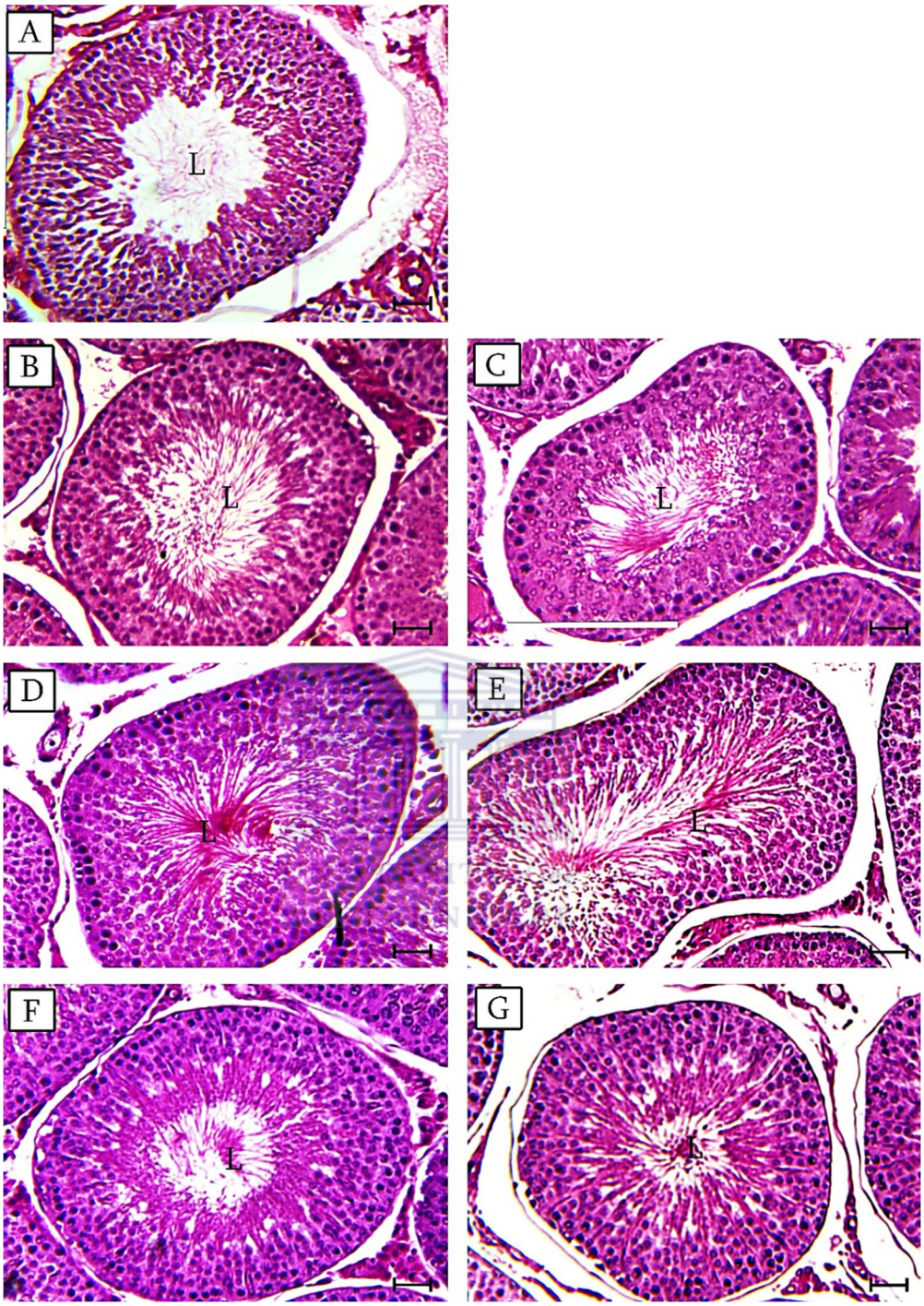

Figure 5.32: Photomicrograph of the male rat testis exposed to *Linum usitatissimum*, *Raphanus sativus* and *Phoenix dactylifera* for 52 days using H&E staining.
A; control, B; Radish seed (80 mg/kg), C; Radish seed (160 mg/kg), D; Flaxseed (200 mg/kg), E; Flaxseed (300 mg/kg), F; Date palm pollen (120 mg/kg), G; Date palm pollen (240 mg/kg), L; lumen of seminiferous tubule. Bar = 50 µm.

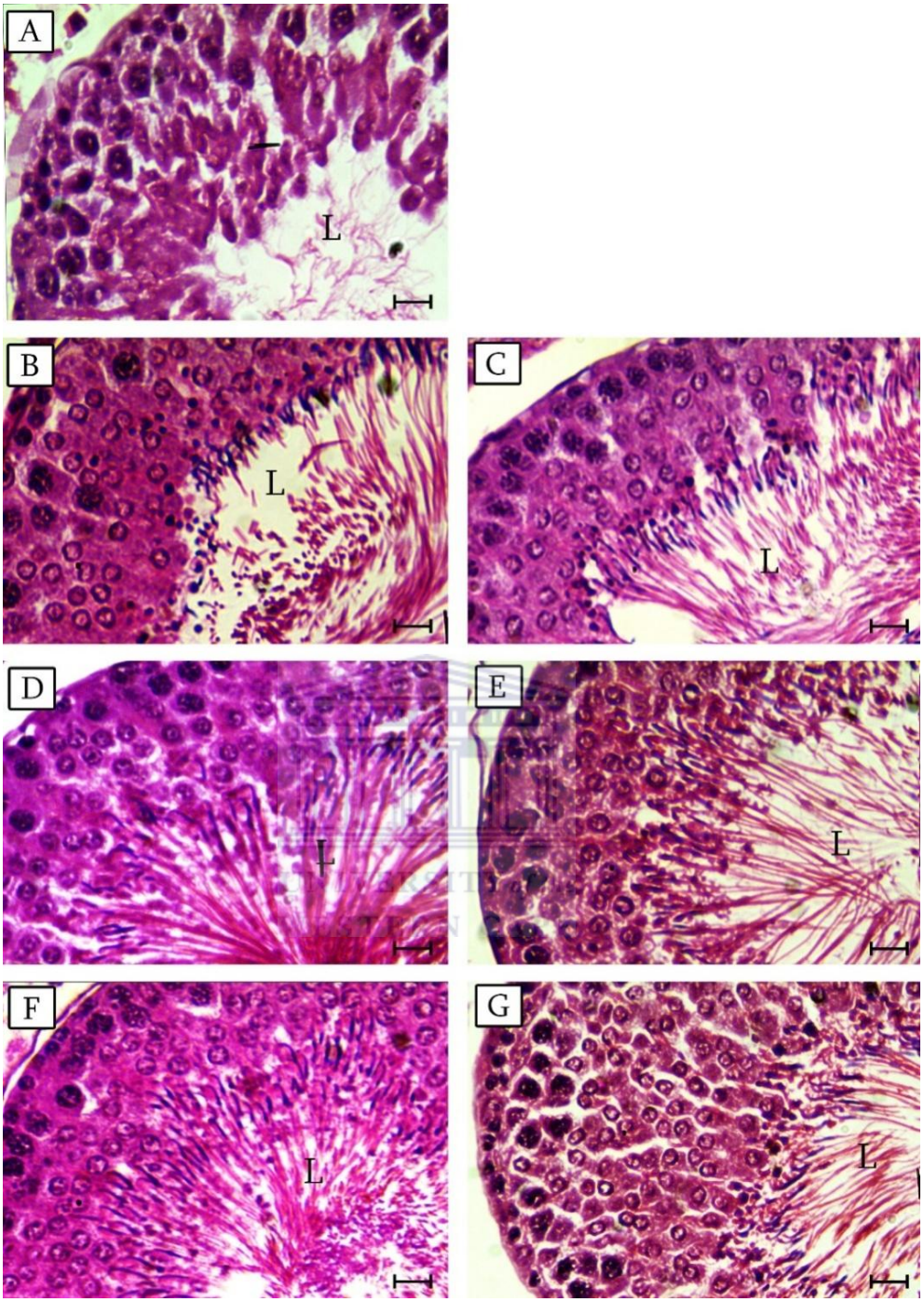

Figure 5.33: Photomicrograph of the male rat testis exposed to *Linum usitatissimum, Raphanus sativus* and *Phoenix dactylifera* for 52 days using H&E staining.
A; control, B; Radish seed (80 mg/kg), C; Radish seed (160 mg/kg), D; Flaxseed (200 mg/kg), E; Flaxseed (300 mg/kg), F; Date palm pollen (120 mg/kg), G; Date palm pollen (240 mg/kg), L; lumen of seminiferous tubule. Bar = 20 µm.

Rat epididymis (cauda and caput) light microscopic examination of the H&E sections showed normal histological structure of regular caput and cauda epididymal tubules (figures; 5.34, 5.35). Both, the cauda and caput epithelia of the epididymides, have a normal morphological appearance and contained abundant spermatozoa in their individual lumen in all treatment groups as compared to the control. However, there seems to be an increase in spermatozoa in the lumen of epididymi (figures 5.34) exposed to flaxseed (300 mg/kg) and date palm pollen (120 mg/kg) compared to control group.

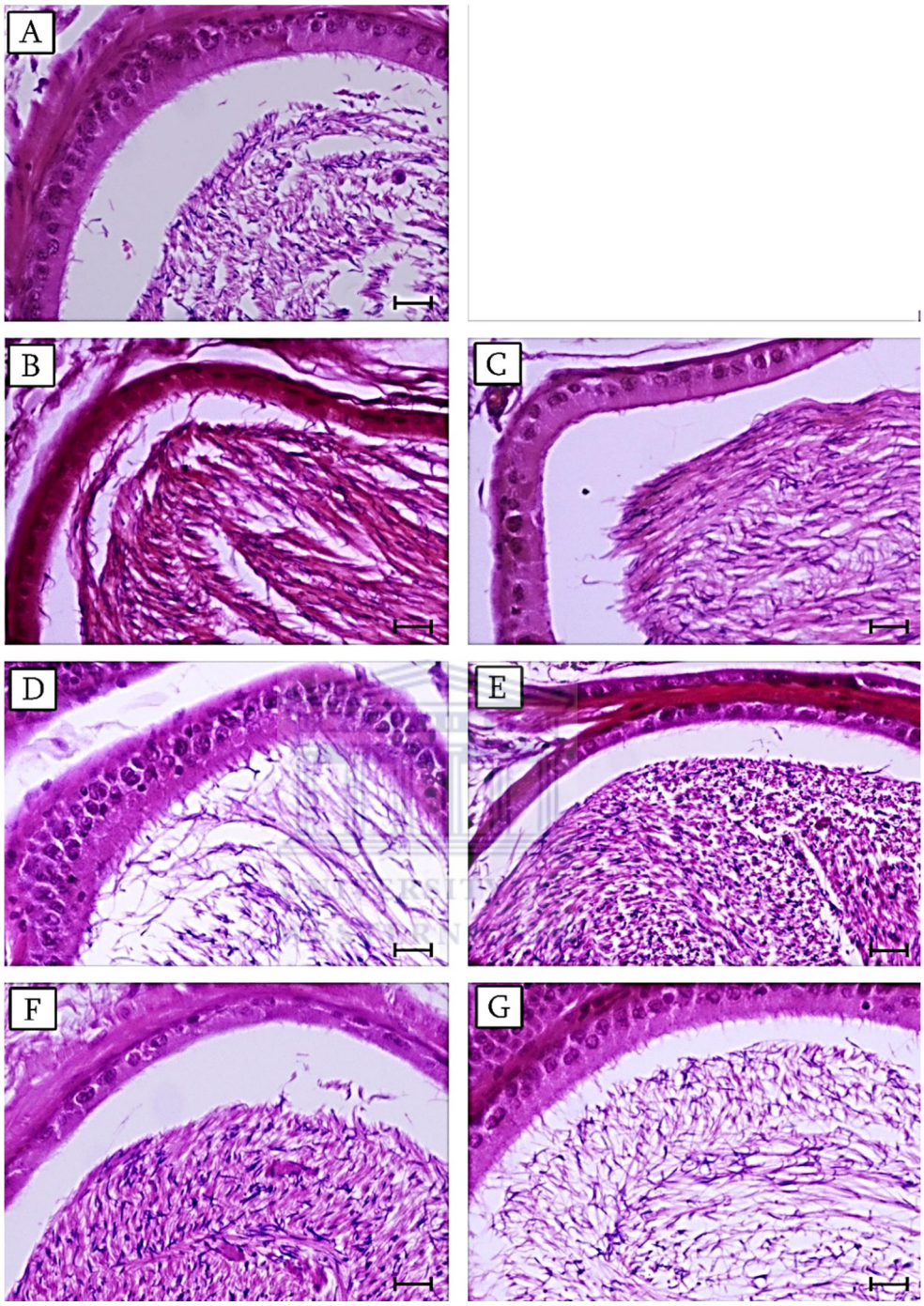

Figure 5.34: Morphology of the male rat epididymis CAUDA exposed to *Linum usitatissimum, Raphanus sativus* and *Phoenix dactylifera* for 52 days using H&E staining. A; control, B; Radish seed (80 mg/kg), C; Radish seed (160 mg/kg), D; Flaxseed (200 mg/kg), E; Flaxseed (300 mg/kg), F; Date palm pollen (120 mg/kg), G; Date palm pollen (240 mg/kg). Bar = 20 µm

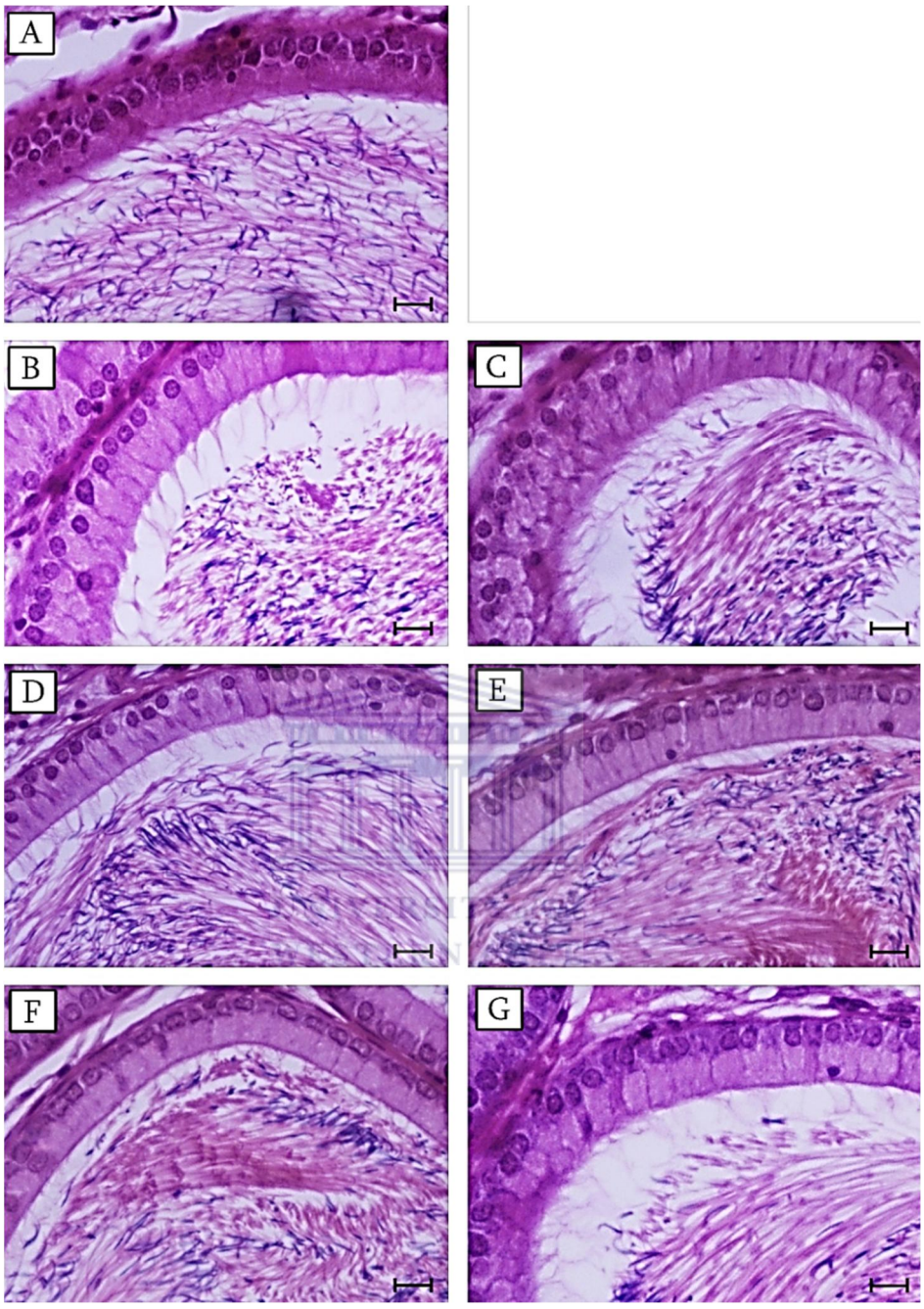

Figure 5.35: Morphology of the male rat epididymis CAPUT exposed to *Linum usitatissimum, Raphanus sativus* and *Phoenix dactylifera* for 52 days using H&E staining. A; control, B; Radish seed (80 mg/kg), C; Radish seed (160 mg/kg), D; Flaxseed (200 mg/kg), E; Flaxseed (300 mg/kg), F; Date palm pollen (120 mg/kg), G; Date palm pollen (240 mg/kg). Bar = 20µm.

Hepatic morphology (figure 5.36), as evaluated by light microscopy, showed a normal histological structure in all treated rats as well as in the control.

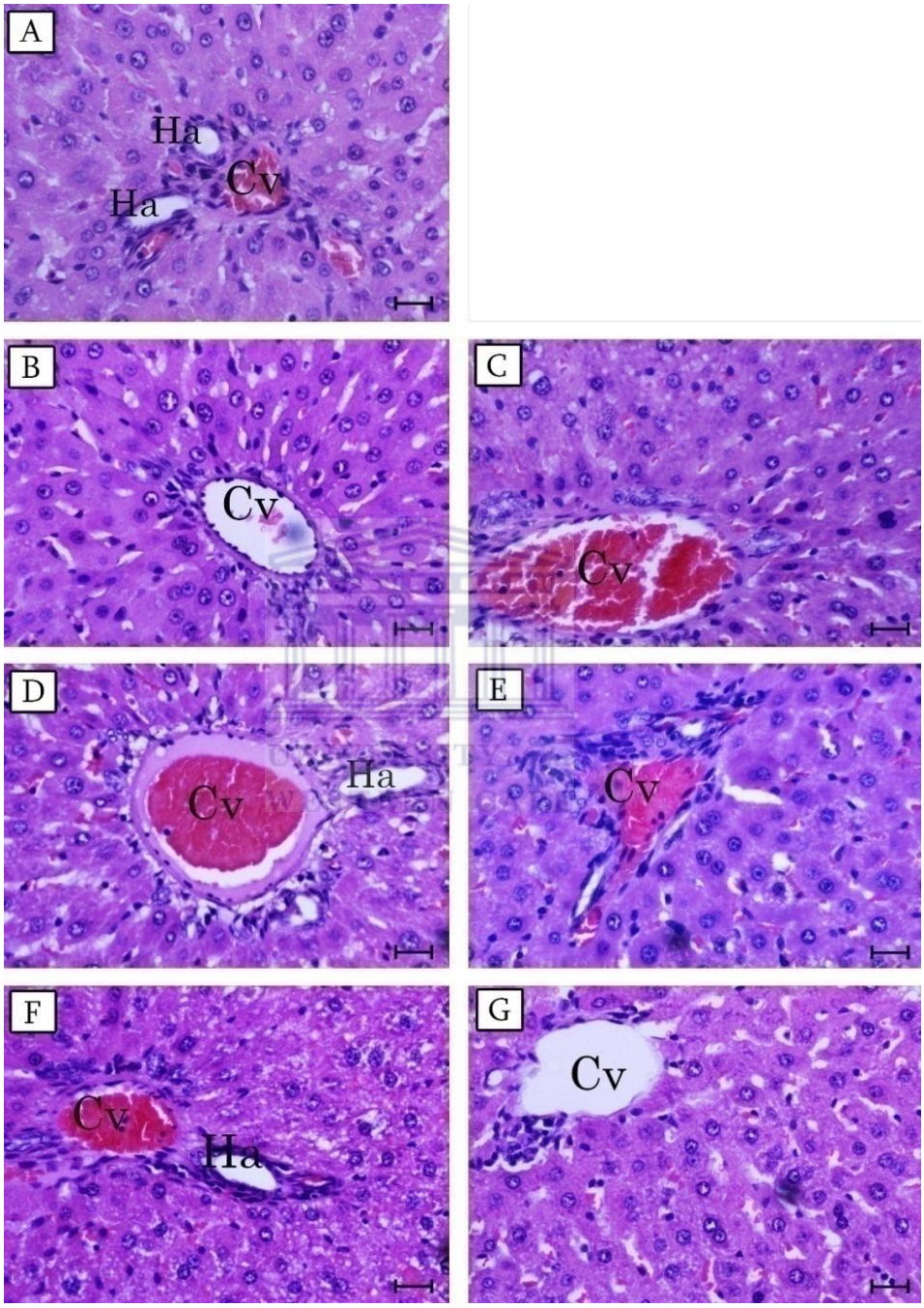

Figure 5.36: Morphology of the male rat liver exposed to *Linum usitatissimum, Raphanus sativus* and *Phoenix dactylifera* for 52 days using H&E staining.
A; control, B; Radish see (80 mg/kg), C; Radish seed (160 mg/kg), D; Flaxseed (200 mg/kg), E; Flaxseed (300 mg/kg), F; Date palm pollen (120 mg/kg), G; Date palm pollen (240 mg/kg), Cv; central vein; Ha; hepatic artery. Bar = 20 µm

Using H&E staining, histological examination of the cross sections of the rat's kidney (figure 5.37) showed a normal structure in all treated rats as well as in the control.

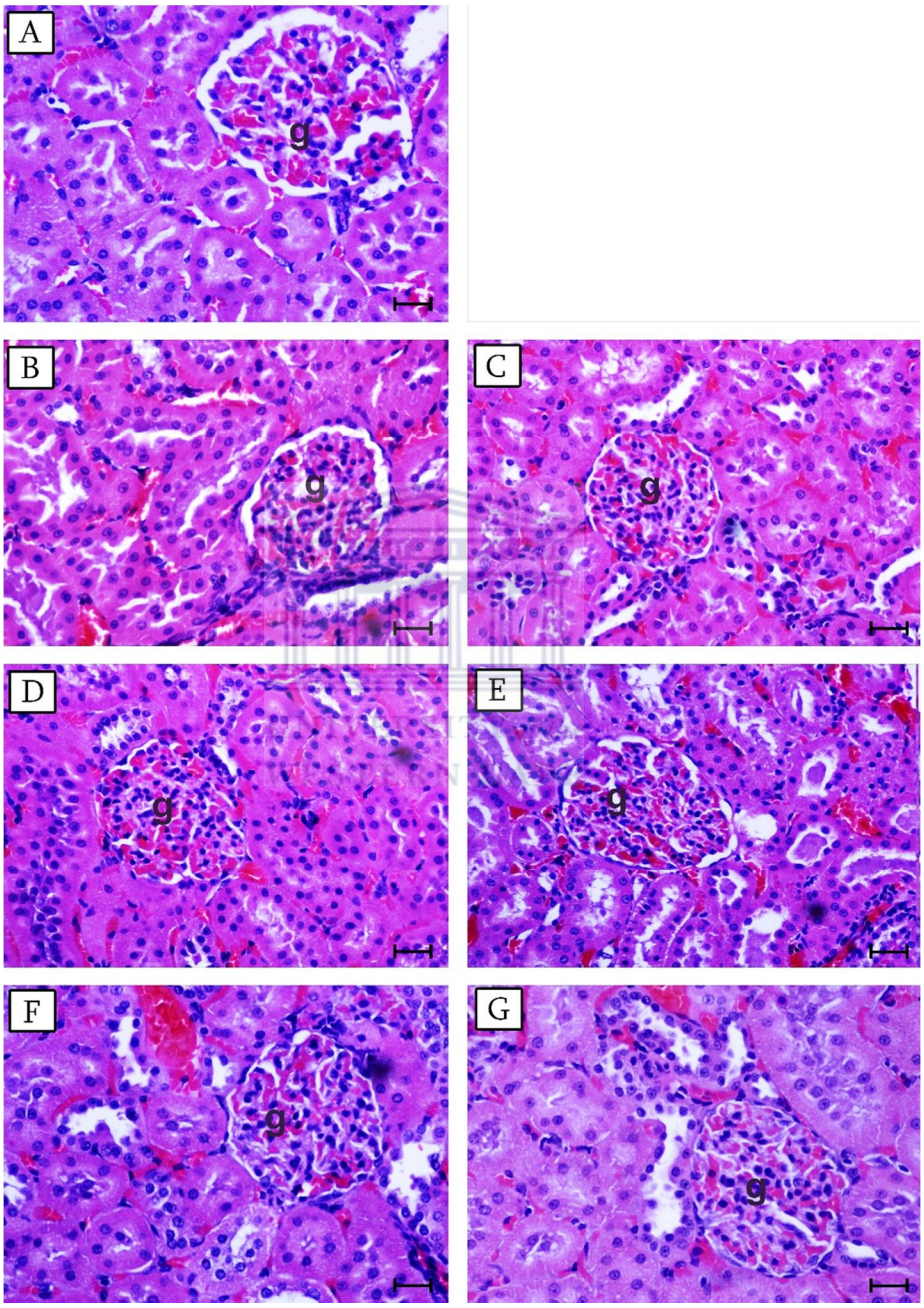

Figure 5.37: Morphology of the male rat kidney exposed to *Linum usitatissimum, Raphanus sativus* and *Phoenix dactylifera* for 52 days using H&E staining.
A; control, B; Radish see 80n mg/kg, C; Radish see 160 mg/kg, D; Flaxseed 200 mg/kg, E; Flaxseed 300 mg/kg, F; Date palm pollen 120 mg/kg, G; Date palm pollen 240 mg/kg, g; glomerulus; Bar = 100 µm.

5.13 Morphometric measurement in testis

As follows from the figure 5.38, the diameter of seminiferous tubules in male rats exposed to date palm pollen (120 and 240 mg/kg) was significantly higher than the control group (p<0.001). On the other hand, radish seed and flaxseed showed values similar to control group. Also, the heights of the germ cell epithelium within the seminiferous tubules (figure 5.39) were significantly increased in all treated groups compared to the control.

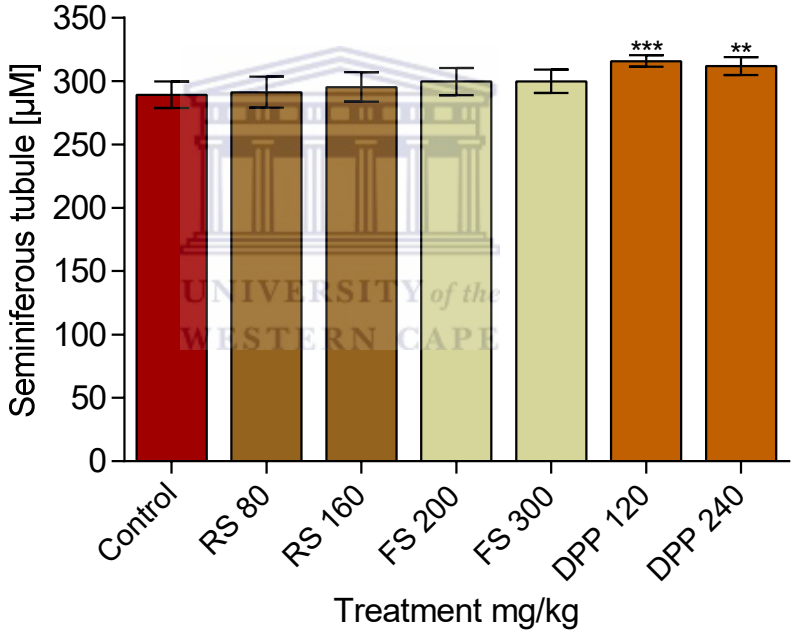

Figure 5.38: Effect of *Linum usitatissimum, Raphanus sativus* and *Phoenix dactylifera* on the diameter of seminiferous tubules in the testes.
Values represented are the mean ± SD of 6 animals per group. Abbreviations: RS, Radish seed; FS, Flaxseed; DPP, Date palm pollen; **, p<0.01; ***, p<0.001 compared to control.

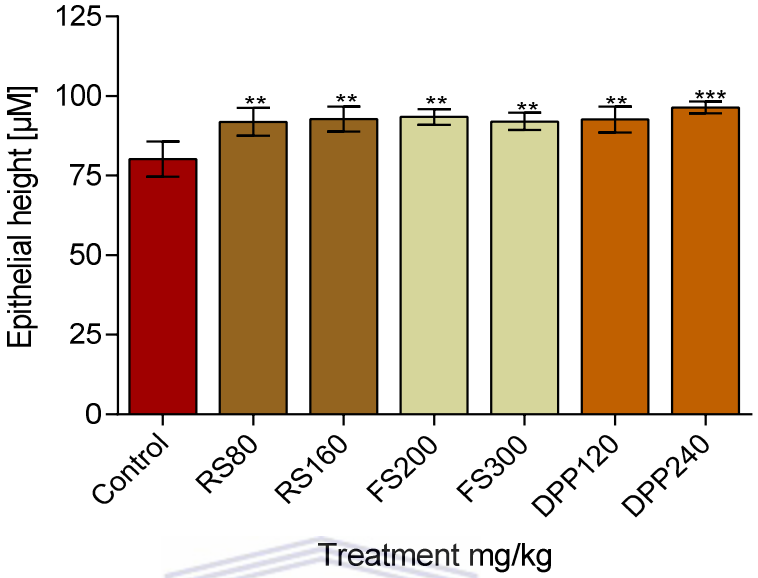

Figure 5.39: Effect of *Linum usitatissimum, Raphanus sativus* and *Phoenix dactylifera* on the epithelial heights of seminiferous tubules in the testes. Values represented are the mean ± SD of 6 animals per group. Abbreviations: RS, Radish seed; FS, Flaxseed; DPP, Date palm pollen; **, $p<0.01$; ***, $p<0.001$ compared to control.

CHAPTER VI

6 Result of the effect of *Nigella sativa*, *Myristica fragrans* and *Phoenix dactylifera* on the female reproductive system

6.1 Body weight gain

Black seed (BS), nutmeg (NM) and date palm pollen (DPP) were tested at various concentrations, BS (200 mg/kg), BS (300 mg/kg), NM (200 mg/kg), NM (400 mg/kg), and DPP (120 mg/kg), DPP (240 mg/kg) on female rat via force feeding for 21 days. The weekly weight gain in the female rats treated with the ethanolic plant extracts showed no significant difference ($P < 0.05$) compared to the control (Fig 6.1).

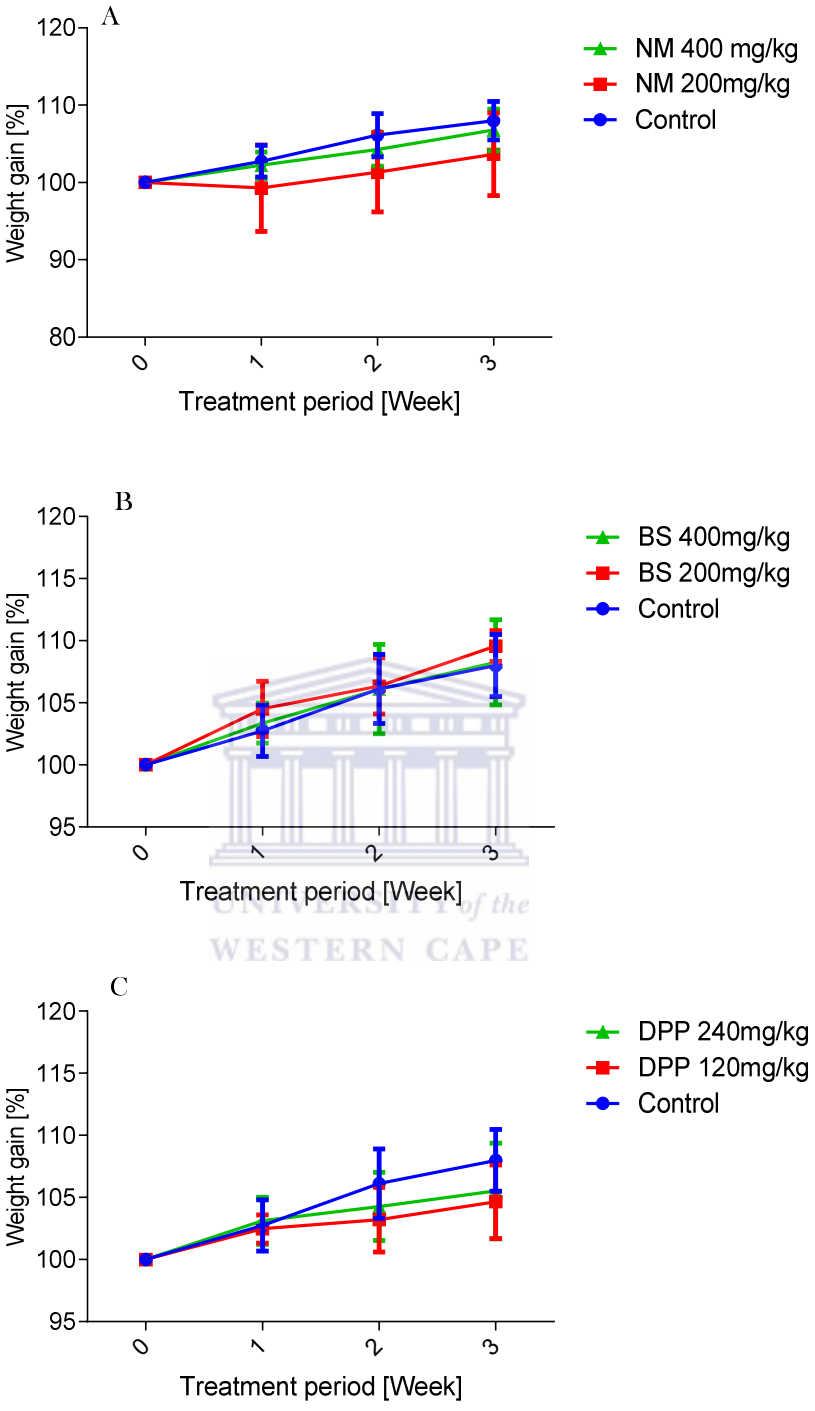

Figure 6.1: Effect of *Nigella sativa, Myristica fragrans* and *Phoenix dactylifera* on the weekly weight gain in the female rats.
Values represented are the mean ± SD of 6 animals per group. Abbreviations: A, Nutmeg; B, Black seed; C, Date palm pollen.

The total weight gain in the female rats showed a non-significant increase after treatment with black seed (200 and 400 mg/kg; $p < 0.05$) compared to the treated groups after 21 days. However, NM and DPP showed a tendency of a lower ($p > 0.05$) total weight gain compared to control group.

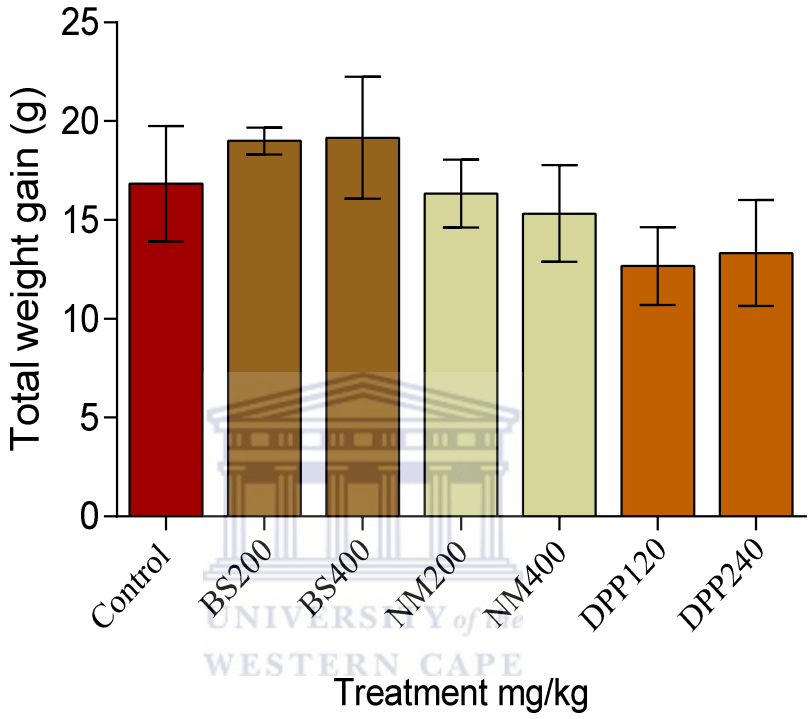

Figure 6.2: Effect of *Nigella sativa*, *Myristica fragrans* and *Phoenix dactylifera* on the total weight gain in the female rats.
Values represented are the mean ± SD of 6 animals per group. Abbreviations: BS, Black seed; NM, Nutmeg; DPP, Date palm pollen.

6.2 Reproductive organs, liver and kidney weights in female rats

Table 6.1 showed no significant differences in most of the absolute and relative weights of reproductive organs (uterus and ovaries) as well as liver and kidney (p>0.05). However, the relative uterus weights of animals exposed to nutmeg (200 mg/kg) and date palm pollen (120 and 240 mg/kg) showed significantly decreased values ($p < 0.05$). Similarly, the absolute weight of the uterus in animals exposed to nutmeg (200 mg/kg), black seed (400 mg/kg) and palm pollen (120 mg/kg) showed significantly decreased values ($p<0.05$) compared to control. In addition, the relative weights of ovaries after treatment with nutmeg (400 mg/kg) and black seed (400 mg/Kg) showed significantly increased values ($p < 0.01$). Also, the absolute weight of ovaries showed significant increased values ($p < 0.05$) after exposure to nutmeg (400 mg/kg). Absolute and relative weights of liver showed no significant changes in all treated groups ($p < 0.05$) compared to the control. However, there was a tendency to lower absolute and relative kidneys weights in all treated groups ($p > 0.05$).

Table 6-1: Weight [g] of reproductive organs, liver and kidney after 21 days treatment with Control, Black seeds, Nutmeg and Date palm pollen in female rats

Treatment Organ	Control	NM 200	NM 400	BS 200	BS 400	DPP 120	DPP 240
Uteri							
Absolute	0.71 ± 0.097	0.51 ± 0.14*	0.69 ± 0.13	0.54 ± 0.13	0.54 ± 0.10*	0.50 ± 0.06**	0.62 ± 0.12
Relative	0.299 ± 0.04	0.21 ± 0.04*	0.31 ± 0.06	0.25 ± 0.05	0.24 ± 0.04	0.20 ± 0.02**	0.23 ± 0.05**
Ovaries							
Absolute	0.127 ± 0.006	0.127 ± 0.006	0.140 ± 0.012*	0.120 ± 0.014	0.137 ± 0.010	0.127 ± 0.018	0.125 ± 0.009
Relative	0.052 ± 0.002	0.050 ± 0.003	0.06 ± 0.006**	0.055 ± 0.007	0.062 ± 0.005**	0.051± 0.005	0.046 ± 0.006
Liver							
Absolute	8.00 ±0.42	8.87 ±0.83	9.15 ±1.45	8.39 ±0.93	9.63 ±1.95	8.63 ±0.96	8.81 ±0.52
Relative	3.36 ±0.24	3.75 ±0.35	3.80 ±0.68	3.51 ±0.33	3.81 ±0.65	3.75 ±0.39	3.78 ±0.22
Kidneys							
Absolute	1.94 ±0.17	1.82 ±0.09	1.83 ±0.20	1.61 ±0.09	1.93 ±0.24	1.67 ±0.07	1.85 ±0.22
Relative	0.81 ±0.08	0.77 ±0.06	0.76 ±0.08	0.68 ±0.04	0.77 ±0.07	0.73 ±0.01	0.79 ±0.07

Values represented are the mean ± SD of 6 animals per group. Abbreviations: BS, Black seeds; NM, Nutmeg; DPP, Palm pollen; *, $p<0.05$; ** $p<0.01$, compared to control. Relative organ weight= organ weight/final body weight * 100.

6.3 Follicle stimulating hormone and luteinizing hormone

6.3.1 Follicle stimulating hormone

As shown in Fig. 6.3, the serum FSH was significantly increased (P>0.05 or 0.01) when the female rats have been exposed to black seed (200 mg/kg), nutmeg (200 mg/kg) or date palm pollen (120 mg/kg). These are always the lower doses of the plant extracts tested. Interestingly, the higher doses caused a decline of serum FSH to levels slightly above (BS and DPP) or similar (NM) to the control.

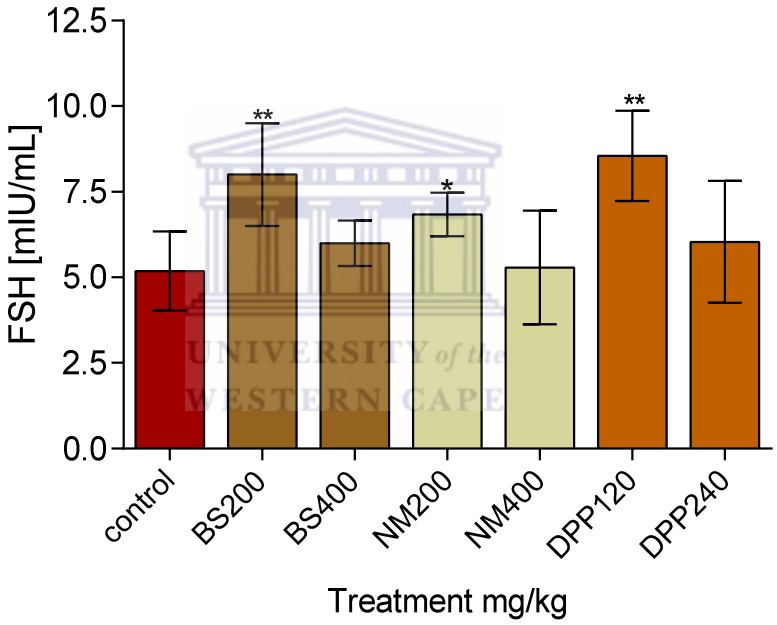

Figure 6.3: Effect of *Nigella sativa, Myristica fragrans* and *Phoenix dactylifera* on the serum follicle stimulating hormone (FSH) in the female rats.
Values represented are the mean ± SD of 6 animals per group. Abbreviations: BS, Black seed; NM, Nutmeg; DPP, Date palm pollen,*, p<0.05; ** p<0.01, compared to control.

6.3.2 Luteinizing hormone

As follows from figure 6.4, the serum luteinizing hormone (LH) level showed a significant decrease when female rats were exposed to black seed (200 mg/kg), date palm pollen (120 mg/kg) or date palm pollen (240 mg/kg; $P < 0.01$) compared to control. On the other hand, serum LH concentration was significantly increased in female rats exposed nutmeg (400 mg/kg; $P > 0.05$) compared to control. The higher dosages of nutmeg (400 mg/kg) or Black seed (400 mg/kg) caused no significant alteration.

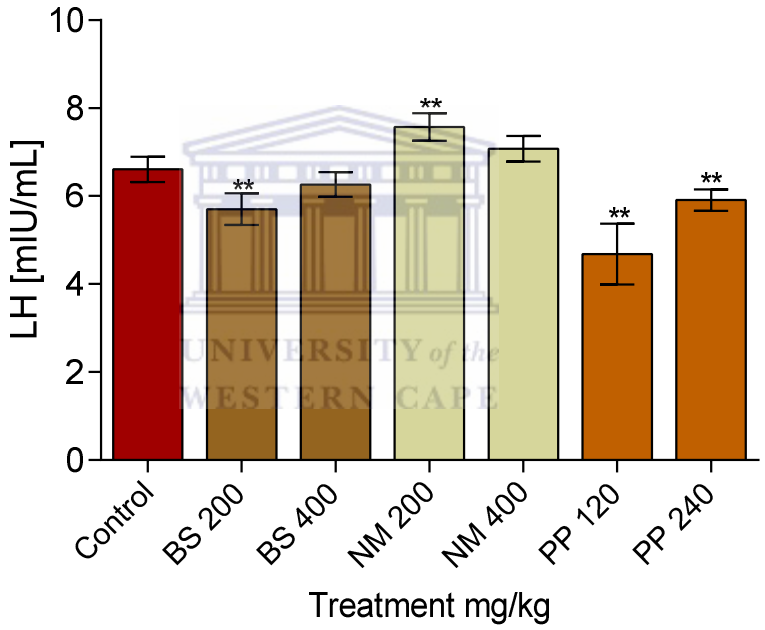

Figure 6.4: Effect of *Nigella sativa, Myristica fragrans* and *Phoenix dactylifera* on the serum Luteinizing hormone (LH) in the female rats.
Values represented are the mean ± SD of 6 animals per group. Abbreviations: BS, Black seed; NM, Nutmeg; DPP, Date palm pollen,*, P<0.05; ** P<0.01, compared to control.

6.4 Creatinine activity in serum activity

As shown in figure 6.5, the creatinine activity in female rat serum in all treated groups was significantly decreased compared to the control ($p < 0.05$). Only BS and NM caused a dose-dependent significant decrease. In contrast, the lower dose of DPP (120 mg/kg) lowered serum creatinine activity significantly, whereas the higher dose (240 mg/kg) caused only a non-significant decrease.

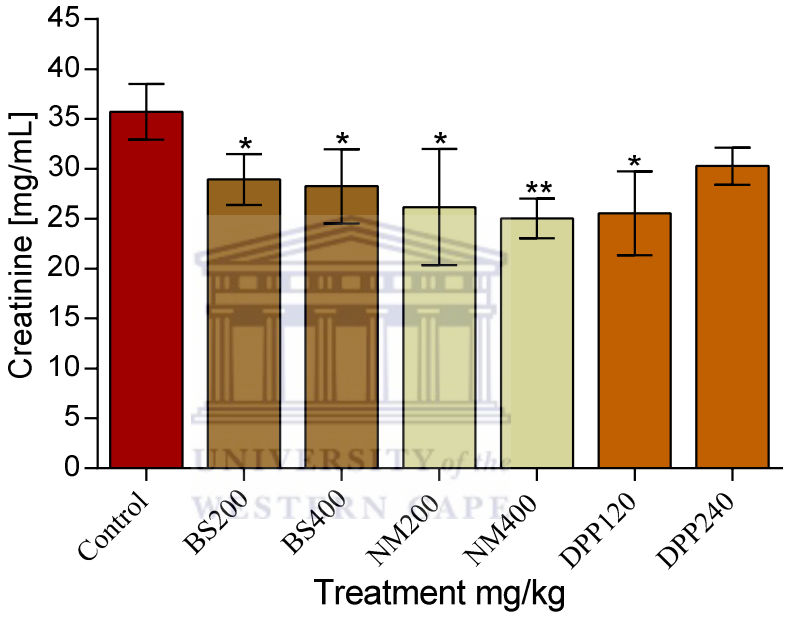

Figure 6.5: Effect of *Nigella sativa, Myristica fragrans* and *Phoenix dactylifera* on the Creatinine activity in the female rats.
Values represented are the mean ± SD of 6 animals per group. Abbreviations: BS, Black seed; NM, Nutmeg; DPP, Date palm pollen,*, p<0.05; ** p<0.01, compared to control.

6.5 Alanine transaminase activity (ALT)

Alanine transaminase (ALT) activity in serum of female rat (Figure 6.6) exposed to either black seed (400 mg/kg) or date palm pollen (120 and 240 mg/kg) was shown to decrease significantly ($p < 0.05$) compared to control group. Also, a tendency to a dose-dependent, non-significant decrease in ALT activity was observed in the groups exposed to either black seed (200 mg/kg) or nutmeg (200 and 400 mg/kg; $p < 0.05$).

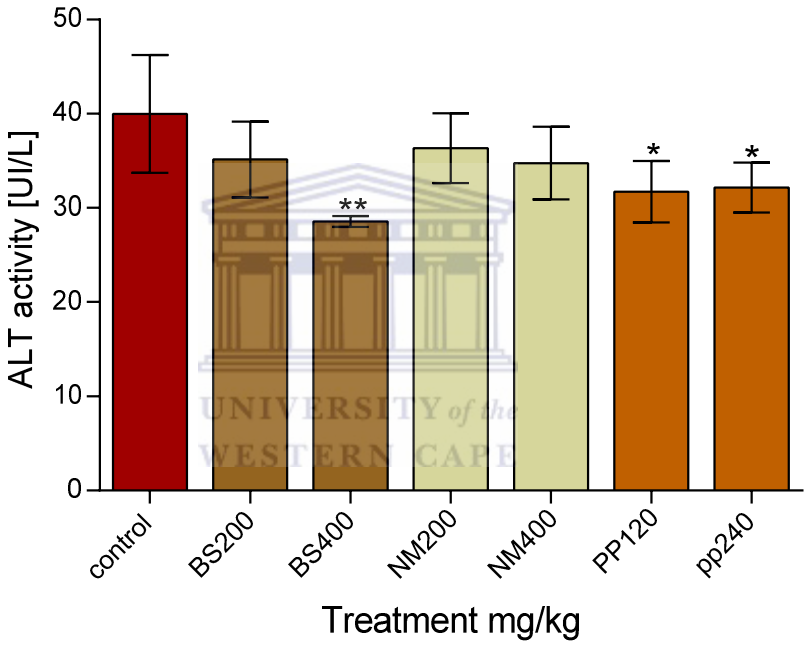

Figure 6.6: Effect of *Nigella sativa, Myristica fragrans* and *Phoenix dactylifera* on the alanine transaminase (ALT) activity in serum of female rat.
Values represented are the mean ± SD of 6 animals per group. Abbreviations: BS, Black seed; NM, Nutmeg; DPP, Date palm pollen, *, p<0.05; ** p<0.01, compared to control.

6.6 Aspartate transaminase activity (AST) in serum

As follows from the figure 6.7, the aspartate transaminase (AST) activity in serum of female rats exposed to black seed showed no significant difference compared to the control ($p>0.05$). In addition, serum (AST) activity displayed a non- significant decrease by nutmeg (200 mg/kg) or date palm pollen (120 and 240 mg/kg; $p < 0.05$). In contrast AST activity showed a significant decrease in rats exposed to nutmeg (200 mg/kg) ($p > 0.05$) compared to control.

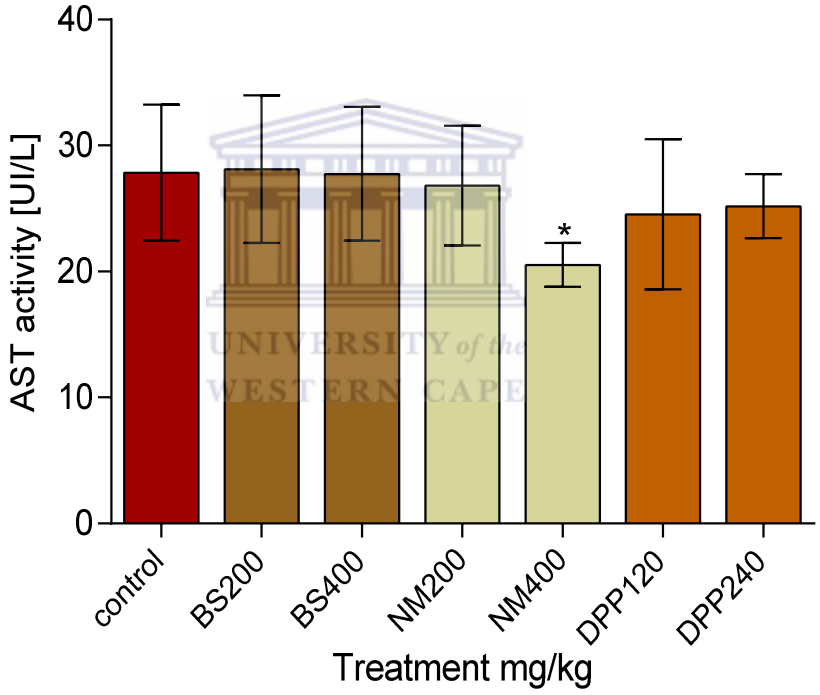

Figure 6.7: Effect of *Nigella sativa, Myristica fragrans* and *Phoenix dactylifera* on the aspartate transaminase (AST) activity in serum of female rat.
Values represented are the mean ± SD of 6 animals per group. Abbreviations: BS, Black seed; NM, Nutmeg; DPP, Date palm pollen, *, $p<0.05$, compared to control.

6.7 Biochemical assays in liver and kidney

6.7.1 Catalase activity (CAT)

Catalase activity (CAT) levels in the liver of female rats exposed to either nutmeg (200 and 400 mg/kg) showed a significant dose-dependent decrease compared to the control ($p < 0.05$; figure 6.8). However, black seed (400 mg/kg) and date palm pollen (120 mg/kg) showed only a non-significantly decreased CAT levels in the liver ($p > 0.05$). On the other hand non- significant increased CAT levels were observed after treatment with black seed (200 mg/kg) and date palm pollen (240 mg/kg; $p > 0.05$).

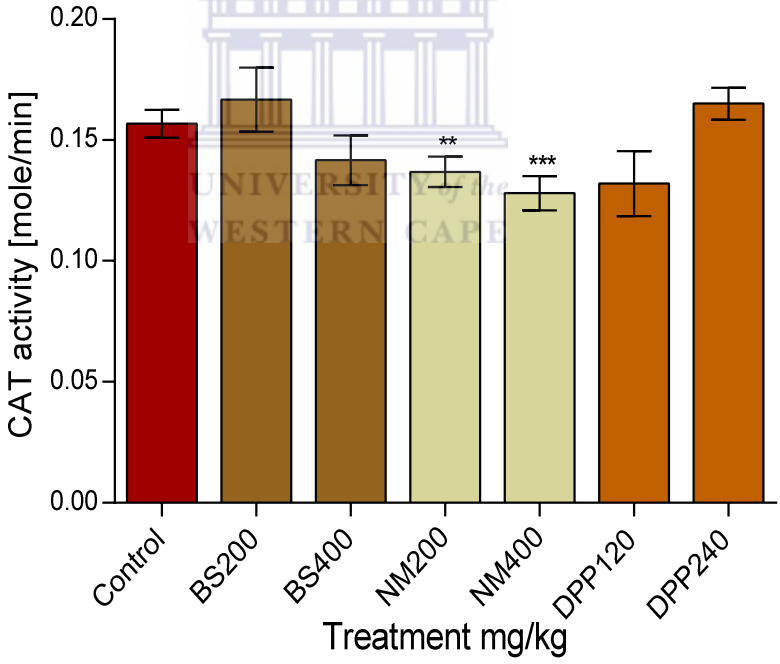

Figure 6.8: Effect of *Nigella sativa, Myristica fragrans* and *Phoenix dactylifera* on the catalase activity (CAT) levels in the liver of female rats. Values represented are the mean ± SD of 6 animals per group. Abbreviations: BS, Black seed; NM, Nutmeg; DPP, Date palm pollen, **, p<0.01, ***, p<0.001compared to control.

Overall, catalase activity (CAT) in kidneys of female rats exposed to the plant extracts did not change compared to control levels (figure 6.9).

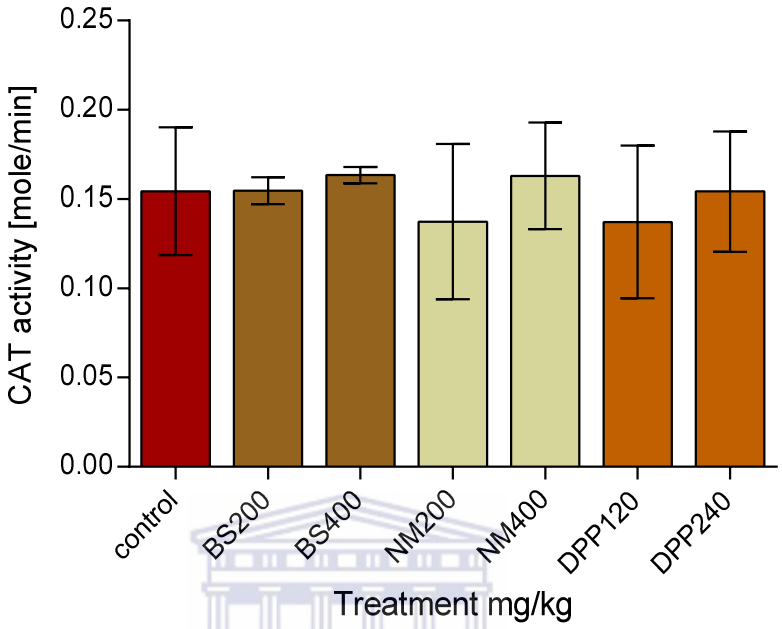

Figure 6.9: Effect of *Nigella sativa, Myristica fragrans* and *Phoenix dactylifera* on the catalase activity (CAT) levels in the kidney of female rats. Values represented are the mean ± SD of 6 animals per group. Abbreviations: BS, Black seed; NM, Nutmeg; DPP, Date palm pollen.

6.7.2 Superoxide dismutase activity (SOD)

Overall, SOD activity in the female rat liver was unchanged after treatment with plant extracts when compared to the control. However the activity of superoxide dismutase (SOD) showed a slight, non-significant increase after treatment with black seed (200 mg/kg) and date palm pollen (120 mg/kg and 240 mg/kg; $p > 0.05$, figure 6.10).

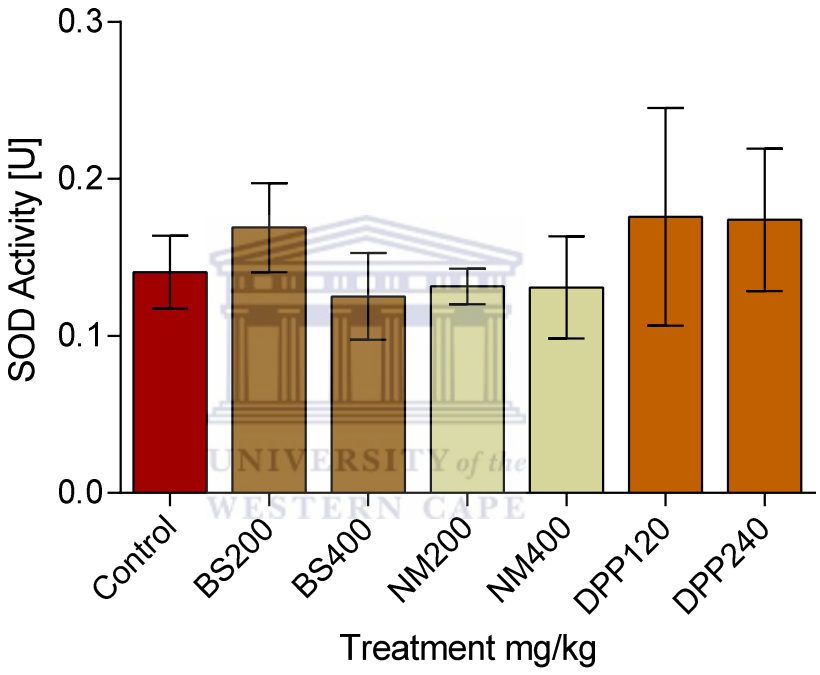

Figure 6.10: Effect of *Nigella sativa, Myristica fragrans* and *Phoenix dactylifera* on the Superoxide dismutase activity (SOD) levels in the kidney of female rats. Values represented are the mean ± SD of 6 animals per group. Abbreviations: BS, Black seed; NM, Nutmeg; DPP, Date palm pollen.

Similarly, SOD activities in kidneys of the treated rats did not differ significantly from control values. However, there was a trend to slightly increased SOD enzyme activities for the lower dosage of each plant extract tested (figure 6.11).

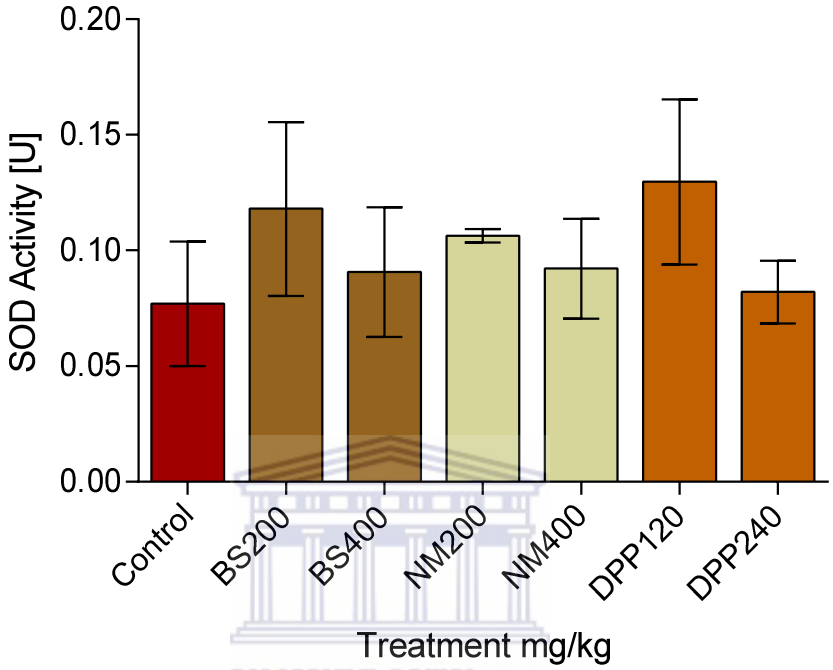

Figure 6.11: Effect of *Nigella sativa, Myristica fragrans* and *Phoenix dactylifera* on the Superoxide dismutase activity (SOD) levels in the kidney of female rats.
Values represented are the mean ± SD of 6 animals per group. Abbreviations: BS, Black seed; NM, Nutmeg; DPP, Date palm pollen.

6.7.3 Lipid peroxidation (MDA)

It was noted that there were no significant changes in the level of lipid peroxidation (MDA) in female rats treated with black seed, nutmeg and date palm pollen (figure 6.12). However, NP, DPP and BS (400 mg/kg) showed a trend to higher MDA levels.

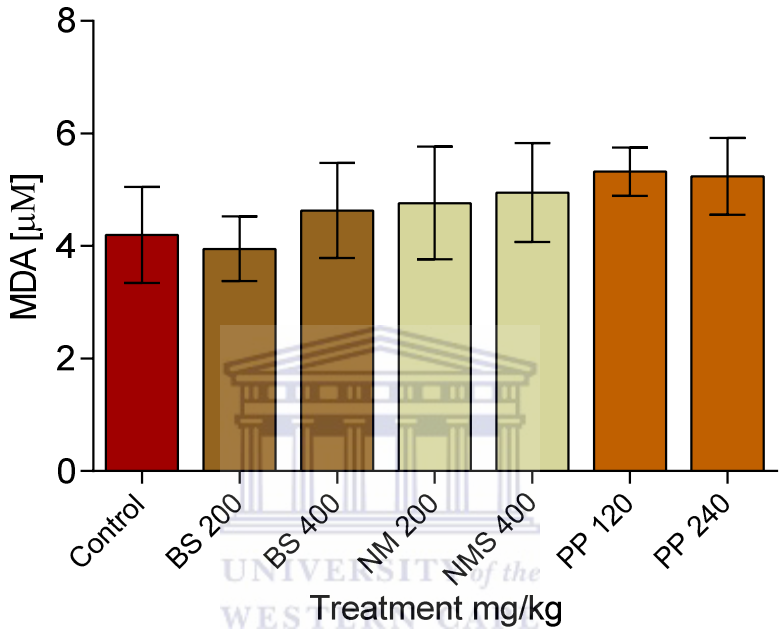

Figure 6.12: Effect of *Nigella sativa, Myristica fragrans* and *Phoenix dactylifera* on the lipid peroxidation (MDA) levels in the liver of female rats.
Values represented are the mean ± SD of 6 animals per group. Abbreviations: BS, Black seed; NM, Nutmeg; DPP, Date palm pollen.

Significantly, elevated lipid peroxidation (MDA) levels were observed in the female rats kidney treated with ethanolic extract of nutmeg (400 mg/kg) and date palm pollen (120 and 240 mg/kg; $P < 0.05$). But lipid peroxidation (MDA) levels increased non- significantly ($P > 0.05$), after treatment with 200 and 400 mg/kg of black seed and nutmeg (200 mg/kg) (Figure 6.13).

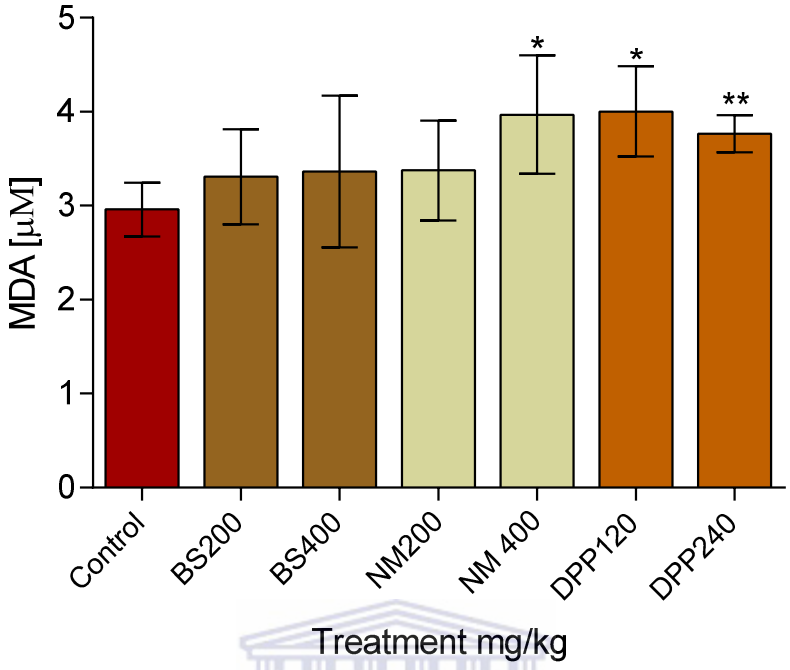

Figure 6.13: Effect of *Nigella sativa, Myristica fragrans* and *Phoenix dactylifera* on the lipid peroxidation (MDA) levels in the kidney of female rats.
Values represented are the mean ± SD of 6 animals per group. Abbreviations: BS, Black seed; NM, Nutmeg; DPP, Date palm pollen.*, p <0.05; ** p<0.01, compared to control.

6.8 Histology of the reproductive organs, kidney and liver in the female rats

Figure 6.14 showed the morphology of female rat ovaries exposed to black seed, nutmeg and date palm pollen for 21 days using H&E staining. The ovaries contained follicles at all stages of development and had a normal appearance of corpus luteum (CL) with marked central fibrous tissue formation in all treated groups which was comparable to the control group. In addition, there was no significant sign of edema, cystic follicles, or retained oocytes.

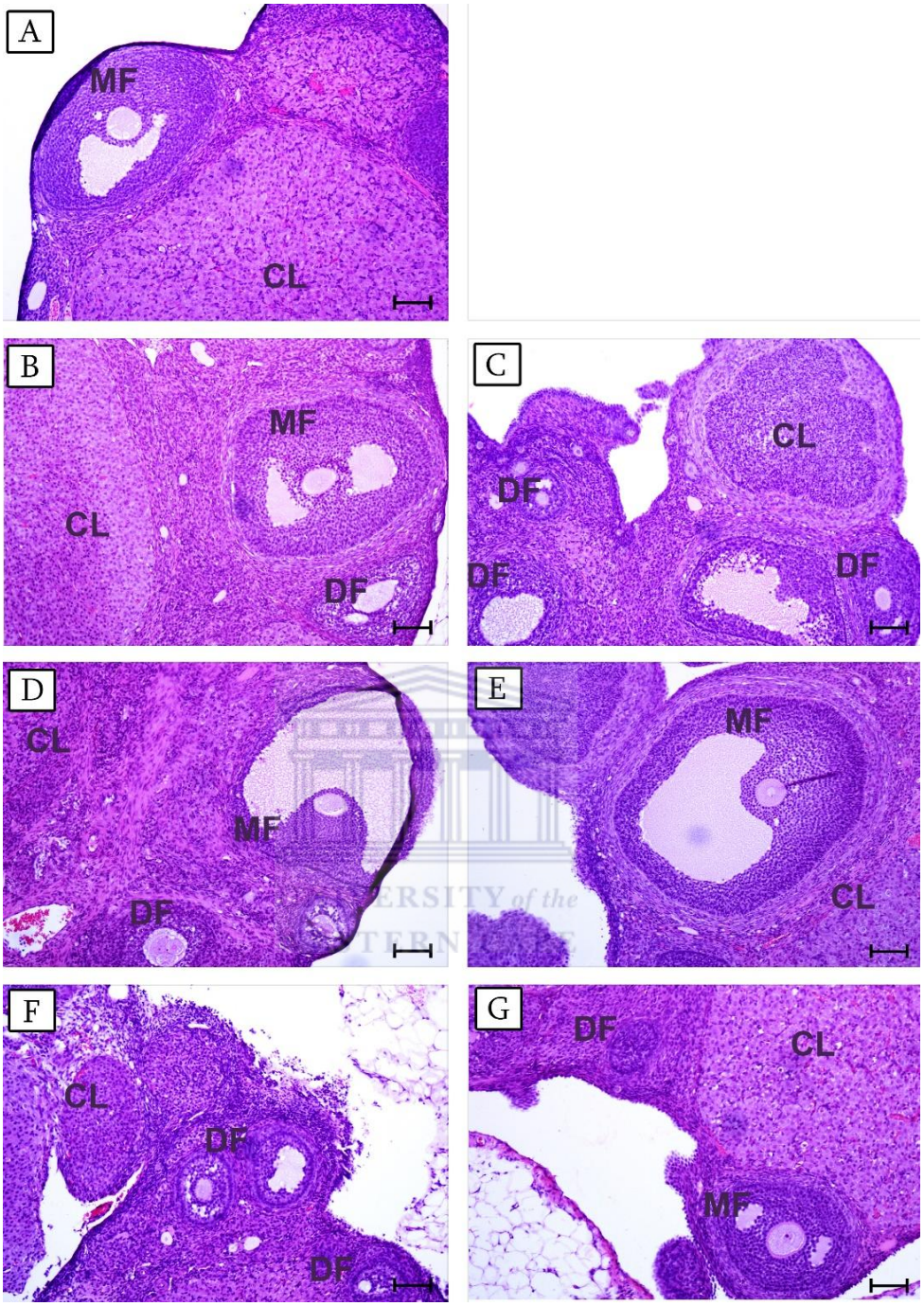

Figure 6.14: Morphology of the female rat ovaries exposed to *Nigella sativa, Myristica fragrans and Phoenix dactylifera* for 21 days using H&E staining.
A; Control, B; Black seed (200 mg/kg), C; Black seed (400 mg/kg), D; Nutmeg (200 mg/kg), E; Nutmeg (400 mg/kg), F; Date palm pollen (120 mg/kg), G; Date palm pollen (240 mg/kg), CL, corpus luteum; DF, developing follicle; MF, mature follicle; Bar = 50µm.

Exposure of female rats to BS, NM and DPP after 21 days the histology of the uterus (figure 6.15) showed normal morphology in the endometrium with the presence of endometrial glands, myometrium and perimetrium.

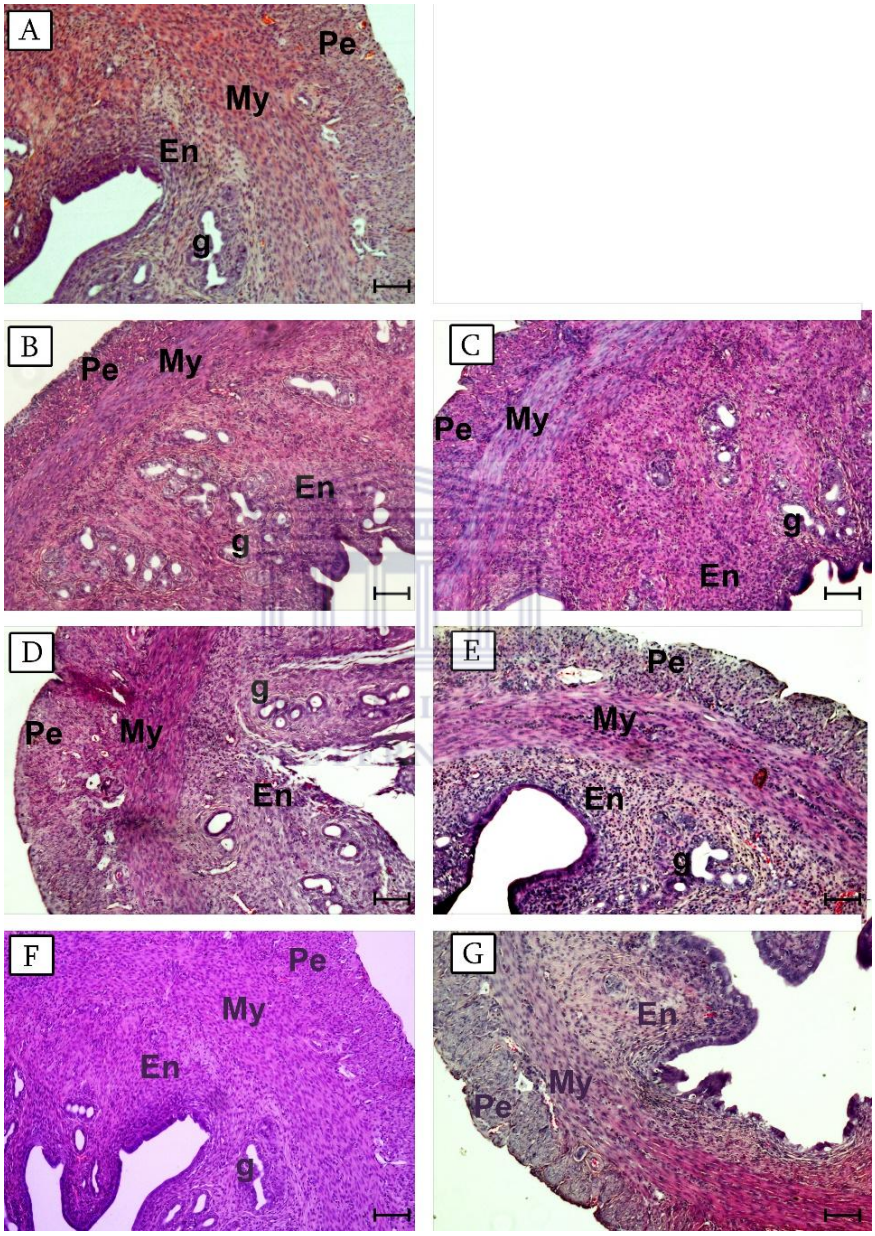

Figure 6.15: Morphology of the female rat uterus exposed to *Nigella sativa, Myristica fragrans and Phoenix dactylifera* for 21 days using H&E staining.
A; Control, B; Black seed (200 mg/kg), C; Black seed (400 mg/kg), D; Nutmeg (200 mg/kg), E; Nutmeg (400 mg/kg), F; Date palm pollen (120 mg/kg), G; Date palm pollen (240 mg/kg), Pe, perimetrium; My, myometrium; En, endometrium; g, endometrial gland; Bar = 50µm.

Hepatic morphology, as evaluated by light microscopy, showed a normal histological structure in control and treated animals (figure 6.16).

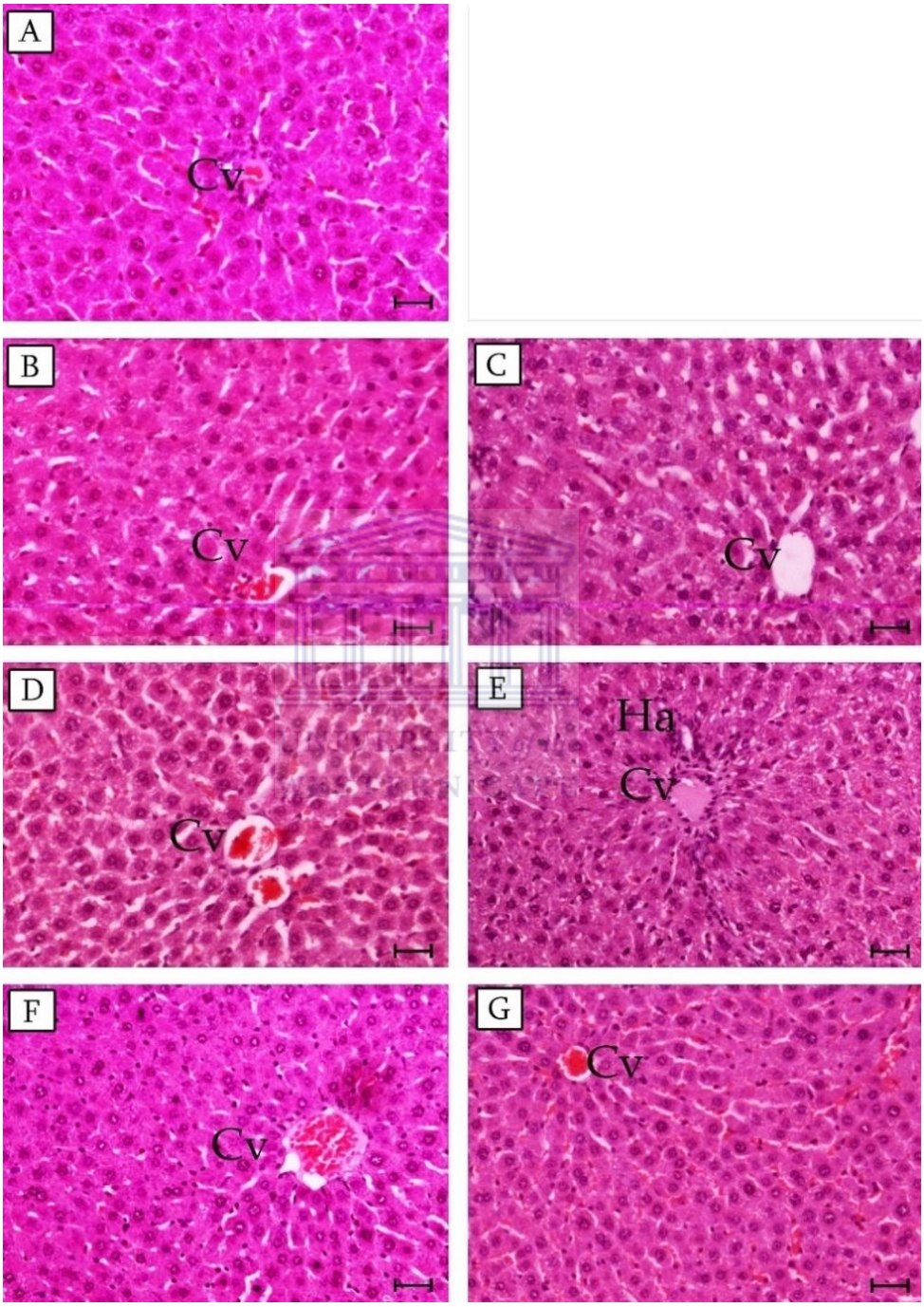

Figure 6.16 : Morphology of the female rat liver exposed to *Nigella sativa, Myristica fragrans and Phoenix dactylifera* for 21 days using H&E staining.
A; Control, B; Black seed (200 mg/kg), C; Black seed (400 mg/kg), D; Nutmeg (200 mg/kg), E; Nutmeg (400 mg/kg), F; Date palm pollen (120 mg/kg), G; Date palm pollen (240 mg/kg), Cv, central vein; Ha, hepatic artery; Bar = 20µm.

Using H&E staining, histological examination of the cross sections of the rat's kidney (figure 6.17) showed a normal structure in all treated rats as well as in the control.

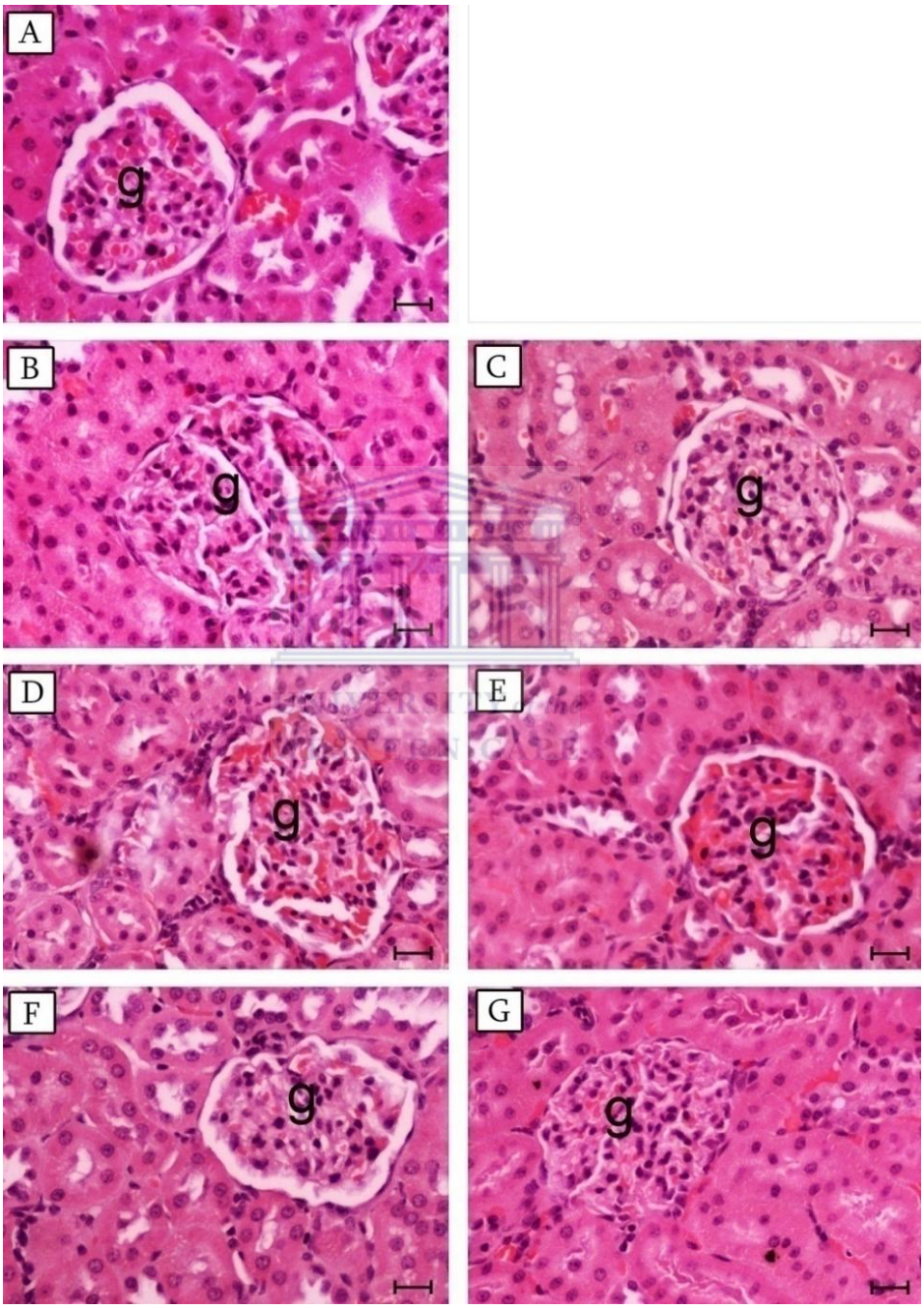

Figure 6.17: Morphology of the female rat kidney exposed to *Nigella sativa, Myristica fragrans and Phoenix dactylifera* for 21 days using H&E staining.
A; Control, B; Black seed (200 mg/kg), C; Black seed (400 mg/kg), D; Nutmeg (200 mg/kg), E; Nutmeg (400 mg/kg), F; Date palm pollen (120 mg/kg), G; Date palm pollen (240 mg/kg), g, glomerulus; Bar = 100µm

CHAPTER VII

7 Effect of *Linum usitatissimum, Raphanus sativus* and *Phoenix dactylifera* on the male sexual behavior and fertility

7.1 Male sexual behavior

The number of sniffs by male rats exposed to radish seed (160 mg/kg) and flaxseed (200 mg/kg) increased non-significantly ($p > 0.05$) while male rats exposed to flaxseed (300 mg/kg) showed similar sniff number to the control ($p > 0.05$). In contrast, radish seed (80 mg/kg) and both concentrations of date palm pollen caused a non-significant decrease ($p > 0.05$; figure 7.1).

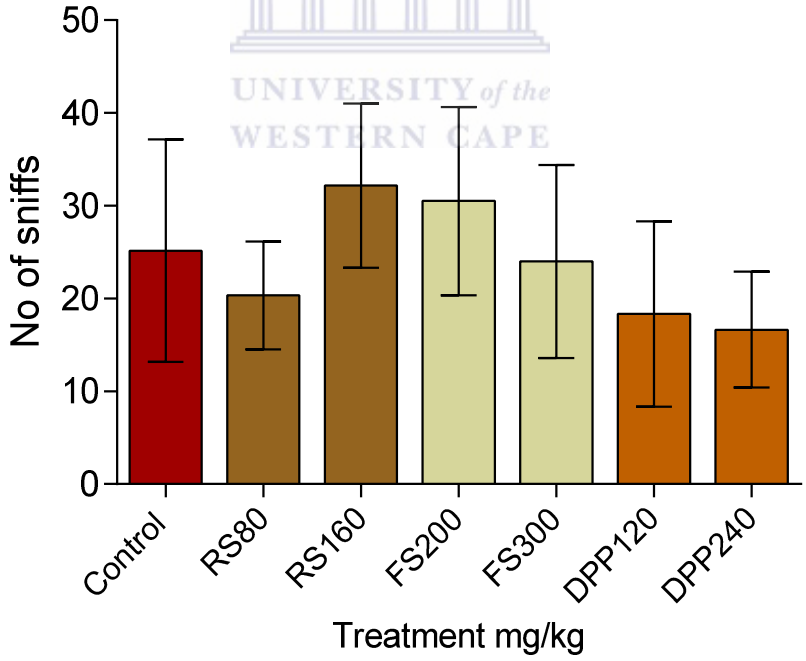

Figure 7.1: No of sniffs in male rats exposed to *Linum usitatissimum, Raphanus sativus* and *Phoenix dactylifera*.
Values represented are the mean ± SD of 6 animals per group. Abbreviations: RS, Radish seed; FS, Flaxseed; DPP, Date palm pollen.

The time to reach the female (figure 7.2) was dose-dependently shorter after treatment with all of the plant extracts. A significant decrease was observed in male rats exposed to flaxseed (300 mg/kg) and date palm pollen (120 and 240 mg/kg; $p < 0.05$) compared to the control.

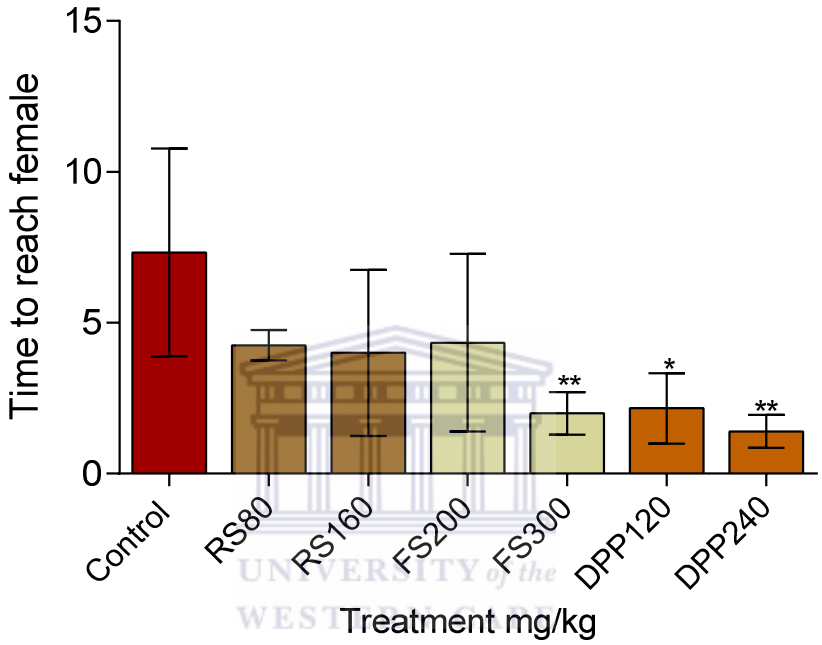

Figure 7.2: Time to reach female after exposure to *Linum usitatissimum, Raphanus sativus* and *Phoenix dactylifera*.
Values represented are the mean ± SD of 6 animals per group. Abbreviations: RS, Radish seed; FS, Flaxseed; DPP, Date palm pollen; *, p< 0.05; **, p<0.01 compared to control.

Mount frequency (figure 7.3) was significantly higher in a dose-dependent manner after exposure to flaxseed (300 mg/kg) and both concentration of date palm pollen ($p < 0.05$). But it showed no significant difference in the groups exposed to radish seed or 200 mg/kg of flaxseed ($p > 0.05$) compared to the control.

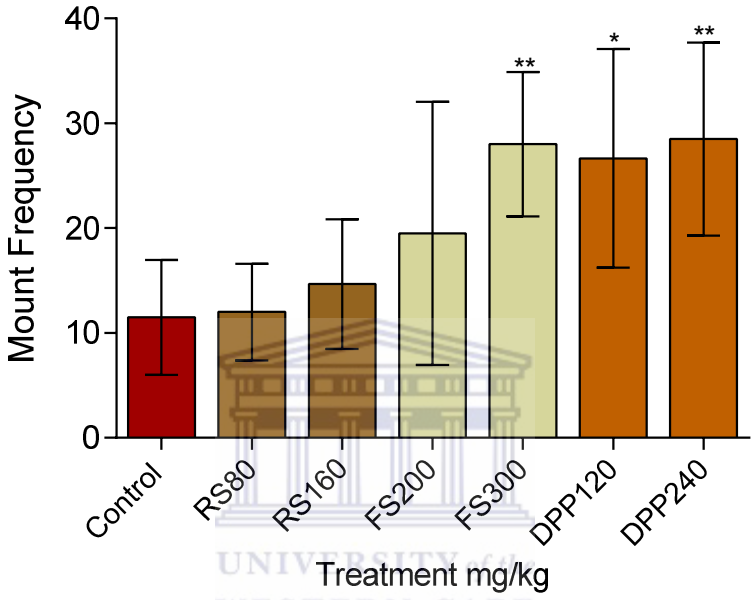

Figure 7.3: Mount frequency of male exposure to *Linum usitatissimum, Raphanus sativus* and *Phoenix dactylifera*.
Values represented are the mean ± SD of 6 animals per group. Abbreviations: RS, Radish seed; FS, Flaxseed; DPP, Date palm pollen; *, $p < 0.05$; **, $p<0.01$ compared to control.

Mount latency showed a non- significant decrease in the groups treated with radish seed (80 mg/kg), flaxseed (300 mg/kg) and date palm pollen ($p > 0.05$) compared to the control (figure 7.4). On the other hand, groups exposed to radish seed (160 mg/kg) and especially flaxseed (200 mg/kg) displayed trend to an increased mount latency.

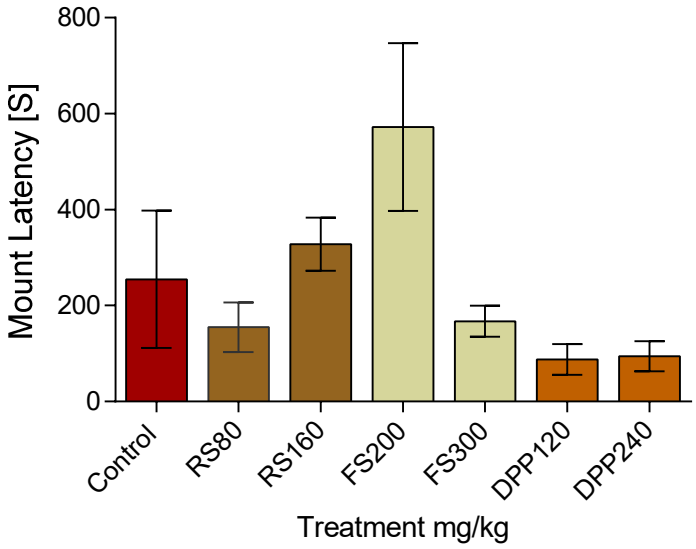

Figure 7.4: Mount latency of male exposure to *Linum usitatissimum, Raphanus sativus* and *Phoenix dactylifera*.
Values represented are the mean ± SD of 6 animals per group. Abbreviations: RS, Radish seed; FS, Flaxseed; DPP, Date palm pollen.

7.2 Fertility and reproductive performance of male rats exposed to *Linum usitatissimum, Raphanus sativus* and *Phoenix dactylifera*

The effects on fertility and reproductive performance of male rats exposed to radish seed, flaxseed and palm pollen are summarized in table 7.1. The female rats used to investigate this parameter were untreated. The observed maximum effect on index of libido in male rats exposed to radish seed, flaxseed and date palm pollen was comparable to the control group. Quantal pregnancy (percentage of pregnant female rats per number mated), implantation index (percentage of total implantation obtained per number mated) and fertility index (percentage of pregnant female rats per number paired) was increased by radish seed (160 mg/kg), both concentrations of flaxseed and date palm pollen, while decreased by radish seed (80 mg/kg). Pre-implantation loss (% of pregnancy loss before implantation) and post implantation loss (% of pregnancy loss after implantation) were decreased in all treated groups except radish seed (80 mg/kg) which showed increased values compared to control group.

Exposure of male rats to DPP 120 produced a significant ($p < 0.01$) increase in the number of embryos in untreated female rats compared to the untreated control group (Figure 7.5).

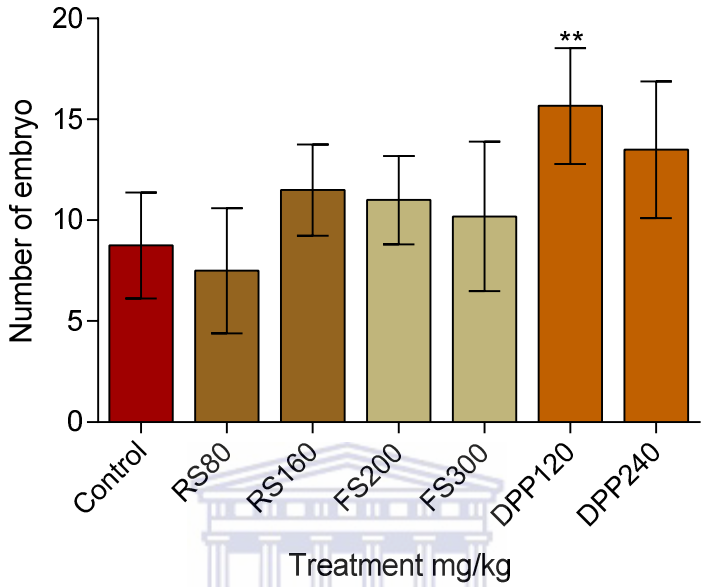

Figure 7.5 Number of embryos following exposure of male rats to *Linum usitatissimum, Raphanus sativus* and *Phoenix dactylifera*. Values represented are the mean ± SD of 6 animals per group. Abbreviations: RS, Radish seed; FS, Flaxseed; DPP, Date palm pollen; *, $p < 0.05$; **, $p<0.01$compared to control.

Table 7-1: Fertility and reproductive performance of male rats exposed to Radish seed, Flaxseed and Palm pollen.

Parameters %	Control	RS80 mg/kg	RS160 mg/kg	FS200 mg/kg	FS300 mg/kg	DPP120 mg/kg	DPP240 mg/kg
Index of libido	100	100	100	100	100	100	100
Quantal pregnancy	50 (3/6)	50 (3/6)	100 (6/6)	100 (6/6)	83.3 (5/6)	100 (6/6)	100 (6/6)
Implantation index	683.33	533.33	1150	1100	850	1300	1285.66
Pre-implantation loss	49.59	59.89	28.5	28.53	36.92	25.85	26.02
Post-implantation loss	88.39	87.17	59.52	54.59	76.86	56.53	49.96
Fertility index	50	50	100	100	83.3	100	100

Abbreviations: RS, Radish seed, FS, Flaxseed; DPP, Palm pollen.

7.3 Effect of *Nigella sativa, Myristica fragrans* and *Phoenix dactylifera* on the female fertility and reproductive performance

Table 7.2, shows the effects of fertility and reproductive performance of female rats exposed to black seeds, nutmeg and palm pollen. It was observed that the index of libido in all female treated rats was comparable to the control group. Male rats used to investigate this parameter were untreated. On the other hand, quantal pregnancy (percentage of pregnant female rats per number mated), implantation index (percentage of total implantation obtained per number mated) and fertility index (percentage of pregnant female rats per number paired) was increased by both concentration of black seed, nutmeg (200 mg/kg) and date palm pollen (240 mg/kg). But values were similar to control after exposure to nutmeg (400 mg/kg) and date palm pollen (120 mg/kg) respectively. In contrast, pre-implantation loss (% of pregnancy loss before implantation) and post implantation loss (% of pregnancy loss after implantation) were decreased in all treated groups compared to control group.

Exposure of female rats to black seeds or date palm pollen produced more embryos compared to the control. This increase was significant at the high dose of these plant extracts. NM caused a non-significant increase in embryo numbers (Figure 7.6).

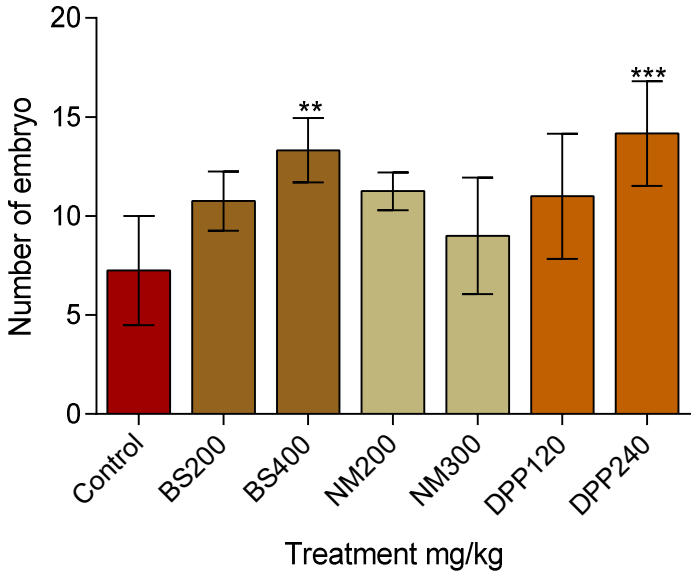

Figure 7.6: Number of embryo in the female rats exposed to *Nigella sativa, Myristica fragrans* and *Phoenix dactylifera*.
Values represented are the mean ± SD of 6 animals per group. Abbreviations: RS, Radish seed; FS, Flaxseed; DPP, Date palm pollen; **, p< 0.01; ***, p<0.001compared to control,

Table 7-2: Fertility and reproductive performance of female rats exposed to Black seeds, Nutmeg and Palm pollen.

Index of libido	100	100	100	100	100	100	100
Quantal pregnancy	50 (3/6)	66.66 (4/6)	100 (6/6)	66.66 (4/6)	50 (3/6)	50 (3/6)	100 (6/6)
Implantation index	500	716.66	1316.6	750	516.66	583.33	1383.33
Pre-implantation loss	59.45	57	39.23	39.18	47.45	45.65	38.51
Post-implantation loss	13.33	4.65	3.79	6.66	9.67	4.85	4.81
Fertility index	50	66.66	100	66.66	50	50	100

Abbreviations: BS, Black seeds, NM, Nutmeg; DPP, Palm pollen.

CHAPTER VII

8 Result of the *in vitro* studies.

8.1 Effect of *Linum usitatissimum, Raphanus sativus* and *Phoenix dactylifera* on TM3 Leydig cells viability

The MTT assay was used to investigate the cytotoxic effects of FS, RS and DPP on TM3 Leydig cells. In respect of this, the activity of mitochondrial dehydrogenase of TM3 Leydig cells treated with the above plant extracts was obtained as absorbance readings on a spectrophotometer. As shown below, the activity of mitochondrial dehydrogenase (absorbance readings) in some of the treated samples was higher than the negative control. These absorbance readings do not imply that the cells are 'healthier' but rather indicate the cells' response to reactive oxygen species (ROS) induced stress and indeed increased energy production coming from the mitochondria to withstand the stress. In other words, the increased absorbance readings represent the amount of mitochondrial dehydrogenase activity coming from cells that are alive whilst its decrease points towards a decrease in mitochondrial dehydrogenase activity and loss of cell viability.

Figure 8.1A, showed a significant decrease ($p < 0.01$) in mitochondrial dehydrogenase activity of the cells for all exposure times 24, 48 and 72 h and all four concentrations tested when the normal TM3 cell line was treated with radish seed compared with untreated control group. Cell viability was reduced to 60% and 50% after 72 hours by 500 and 1000 mg/ml radish seed extract respectively.

Figure 8.1B, displays a significant decrease in mitochondrial dehydrogenase activity in cells exposed to flaxseed (500 and 1000 µg/ml) after 24h ($p < 0.01$). Also it shows a significant decrease after exposure time of 48h by flaxseed (50, 100 and 500 µg/ml) compared with untreated control group ($p < 0.01$). Interestingly, no significant changes were observed after exposure to FS for 72 h, except at the highest concentration.

The activity of mitochondrial dehydrogenase in TM3 cells exposed to date palm pollen showed a significant increase by 50 and 100 µg/ml ($p < 0.01$) for exposure times 24 and 72h compared with untreated control group. However, at 500 and 1000 µg/ml, there was only a non- significant increase ($p > 0.05$). In addition, there were significant decreases ($p < 0.05$) for exposure time 48h within all concentrations except at 1000 µg/ml, which showed no significant difference.

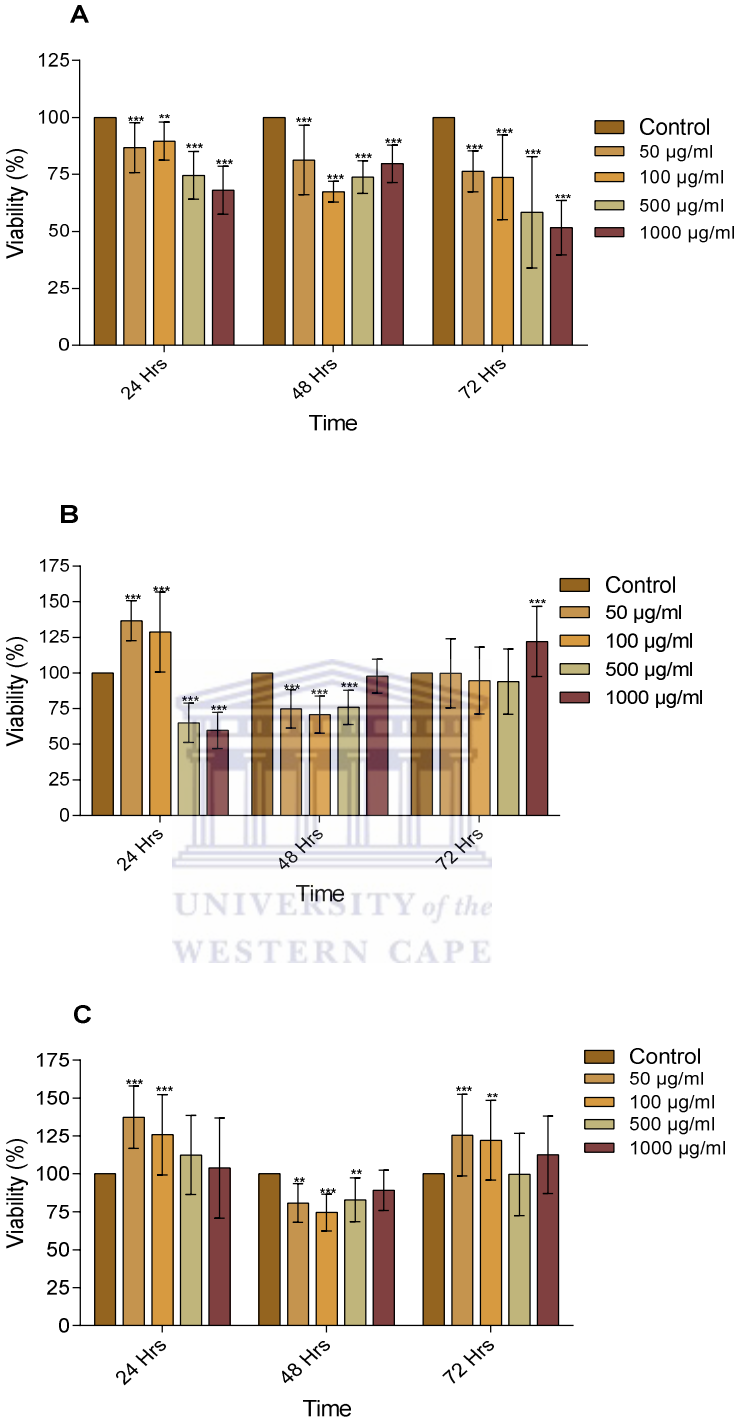

Figure 8.1: Viability of TM3 Leydig cells exposed to *Linum usitatissimum, Raphanus sativus* and *Phoenix dactylifera.*
Values represented are the mean ± SD of six determinations repeated in three independent experiments. Abbreviations: (A) radish seed; (B) flaxseed; (C) date palm pollen; *, $p<0.05$, **, $p<0.01$; ***, $p<0.001$compared to the control.

8.2 Effects of *Linum usitatissimum, Raphanus sativus* and *Phoenix dactylifera* on TM3 Leydig cells

The morphological appearance of TM3 cells under the phase contrast microscope, showed that, the control group revealed the presence of normal dividing cells, while cells exposed to radish seed at concentration 50, 100, 500 and 1000 µg/ml for 72h showed characteristic changes in the morphology of the cells which included cell shrinkage and irregular shape (figures 8.2 B2 – B4 - 8.3 B2 – B4). Similar to radish seed, the cells exposed to 500 and 1000 µg/ml of nutmeg (figures 8.2 C3 – C4 - 8.3 C3 – C4) and to 1000 µg/ml of date palm pollen, showed the same cell shrinkage and irregular shape (figures 8.2 D4 - 8.3 D4). On the other hand, TM3 Lydige cells treated with lower concentration of nutmeg and date palm pollen 50, 100, 500 µg/ml showed normal appearance of dividing cells which is similar to the control group (figures 8.2 C, D - 8.3 C, D).

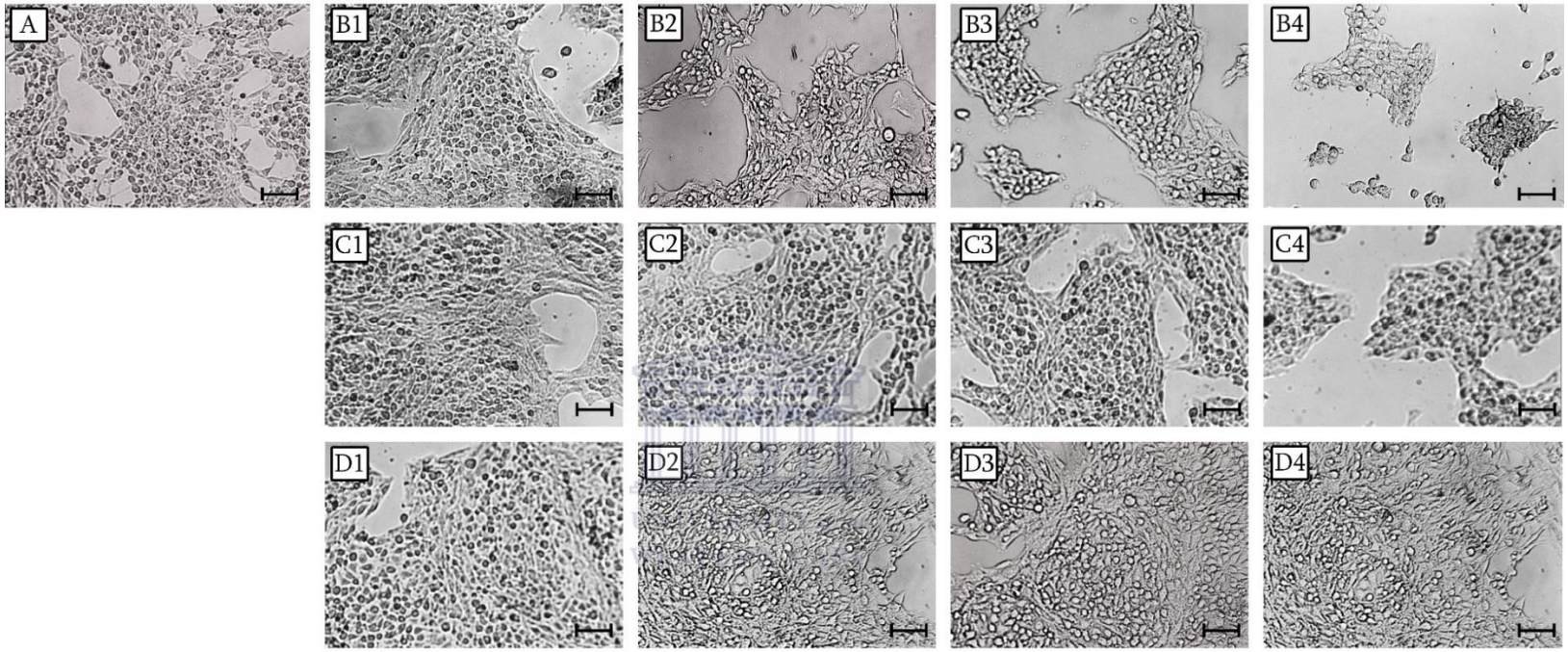

Figure 8.2: Morphology of TM3 Leydig cells exposed to *Linum usitatissimum, Raphanus sativus* and *Phoenix dactylifera* after 72 h.
Abbreviations: (A) control (B) Radish seed (C)Flaxseed (D) Date palm pollen (1) 50 µg/ml (2) 100 µg/ml (3) 500 µg/ml (4) 1000 µg/ml; Bar = 50 µm.

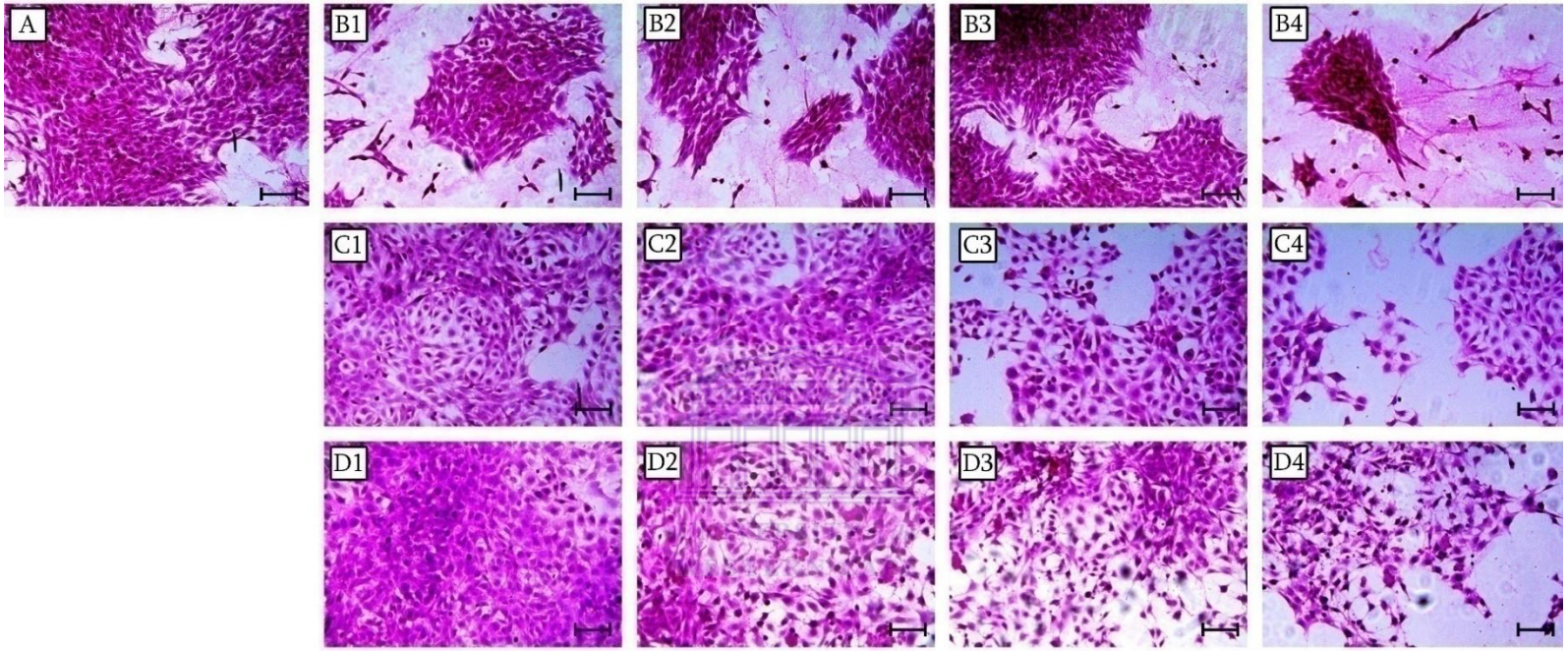

Figure 8.3: Morphology of TM3 Leydig cells exposed to *Linum usitatissimum, Raphanus sativus* and *Phoenix dactylifera*, after 72h using H&E staining.
Abbreviations: (A) control (B) Radish seed (C) Flaxseed (D) Date palm pollen (1) 50 µg/ml (2) 100 µg/ml (3) 500 µg/ml (4) 1000 µg/ml; Bar = 50 µm.

8.3 Detection of apoptotic TM3 Leydig cells after exposure to *Linum usitatissimum, Raphanus sativus* and *Phoenix dactylifera* for 72h

Flow cytometric analysis (figure 8.4) revealed a significant increased amount of apoptotic TM3 cells (86.93 %) in positive control (DMSO 6 %) in comparison with the negative control sample. In addition, radish seed (1000 µg/ml), flaxseed (1000 µg/ml) and date palm pollen (1000 µg/ml) induced non-significant increase in the amount of apoptotic cells ($P > 0.05$) compared with the negative control group. However, the lower concentration of (100 µg/ml) of all the plant extracts did not change the apoptotic status of the Leydig cells.

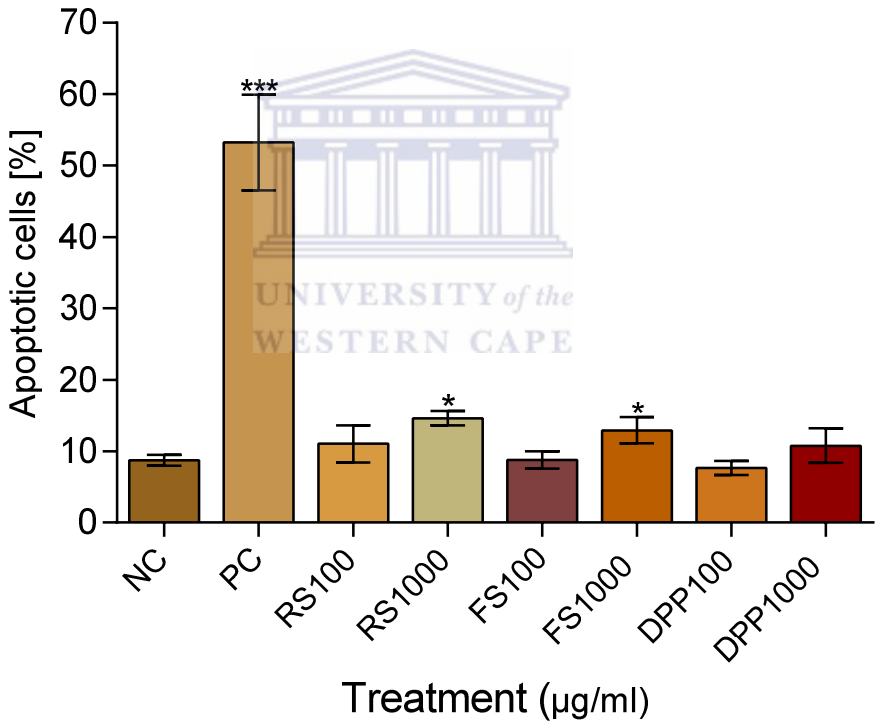

Figure 8.4: Percentage of apoptotic TM3 Leydig cells exposed to *Linum usitatissimum, Raphanus sativus* and *Phoenix dactylifera.*
Values represented are the mean ± SD of six determinations repeated. Cells were stained with Annexin V-FITC using Apoptosis Detection kit and analyzed by flow cytometry. Abbreviations: NC, negative untreated control; PC, positive control; RS, radish seed; FS, flaxseed; DPP, date palm pollen; *, $p < 0.05$, **, $p < 0.01$; ***, $p < 0.001$ compared to the untreated control.

The flow cytometry results below showed the apoptotic effect of date palm pollen, on the TM3 Leydig cells. In this thesis, date palm pollen (1000 µg/ml) increased the number of live cells (88.66 %) compared to untreated cells (90.32 %). In summary, the percentage of apoptotic cells observed in this thesis, was not statistically significant ($p > 0.05$) compared to the negative control (Figure 8.5).

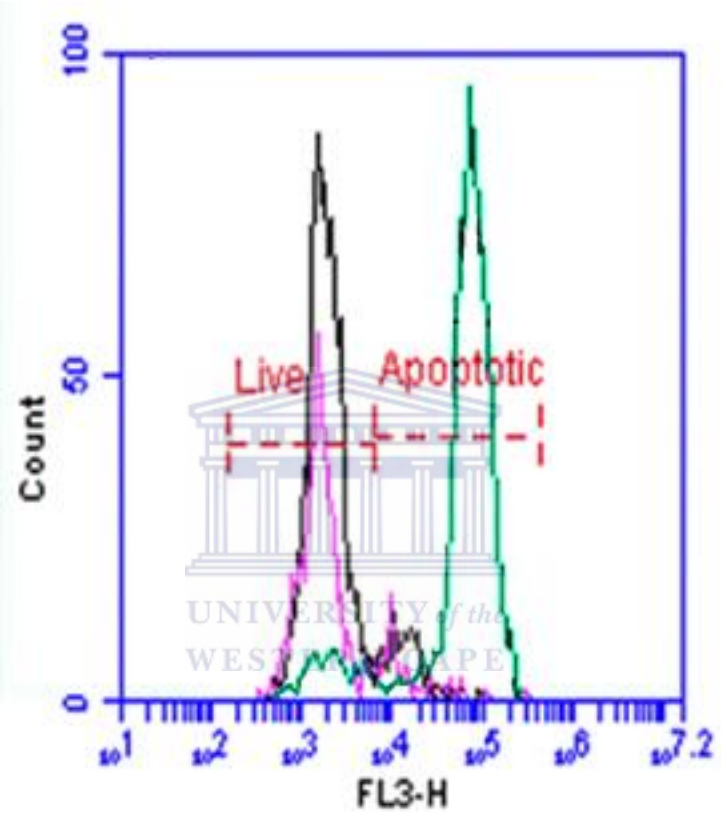

Figure 8.5: Induction of apoptosis in TM3 Leydig cells following exposure to *Phoenix dactylifera* using flow cytometry.
Abbreviations: Black line represent negative control; Purple line represent date palm pollen; Green line represent positive control (DMSO 6 %) X axis shows the relative fluorescence on a logarithmic scale, and the Y axis represent cell number.

8.4 Effects *of Linum usitatissimum, Raphanus sativus* and *Phoenix dactylifera* on testosterone secretion from adult rat testis slices

As shown in figure 8.6, the testicular testosterone secretion *(testis slices)* was significantly increased by flaxseed (500 µg/ml; $P > 0{\cdot}05$) and date palm pollen (500 µg/ml; $P > 0{\cdot}01$) compared to the negative control group DPP (51.98 %) and FS (51.14 %). However, the other treated groups showed no significant difference ($P < 0{\cdot}05$) compared to negative and positive control groups.

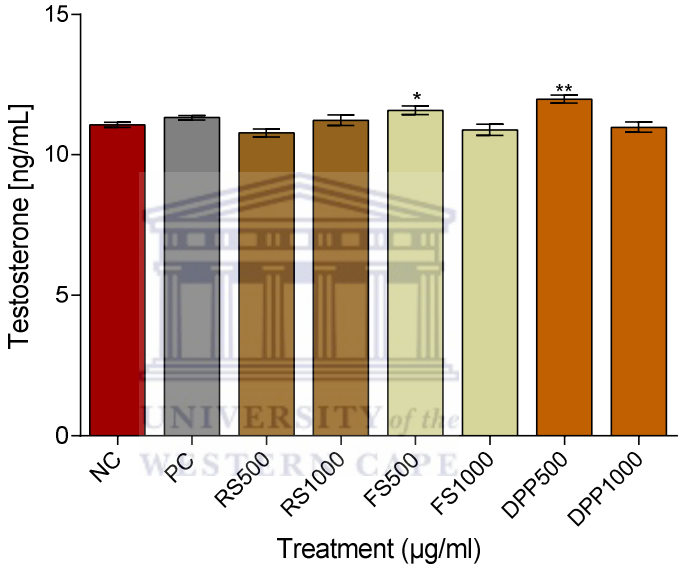

Figure 8.6: Determination of testosterone production in adult rat testis slices exposed to *Linum usitatissimum, Raphanus sativus and Phoenix dactylifera* in the presence or absence of HCG.
Values represented are the mean ± SD of 6 determinations repeated. Abbreviations: (NC) Negative control; (PC) Positive control; (RS500) Radish seed 500 µg/ml; (RS1000) Radish seed 1000 µg/ml; (FS 500), Flaxseed 500 µg/ml; (FS 1000), Flaxseed 1000 µg/ml; (DPP 500), Date palm pollen 500 µg/ml; (DPP 1000), Date palm pollen 1000 µg/ml; *, $p<0.05$; **, $p<0.01$, when compared with the negative control.

8.5 Effect of *Nigella sativa, Myristica fragrans* and *Phoenix dactylifera* on the MCF7 breast cancer cells viability

As already mentioned above, the MTT assay was used to investigate the cytotoxic effects of BS, NM and DPP on MCF-7 breast cancer cells. Similar to the MTT assay for TM3 Leydig cells above, higher mitochondrial dehydrogenase activity in some of the treated samples than the control does not mean that the cells are 'healthier' but rather indicate the cells' response to reactive oxygen species (ROS) induced stress and indeed increased energy production coming from the mitochondria to withstand the stress. In other words, the increased absorbance readings represent the amount of mitochondrial dehydrogenase activity coming from cells that are alive whilst its decrease points towards a decrease in mitochondrial dehydrogenase activity and above all; loss of cell viability.

According to the data shown in figure 8.7A, there is no significant difference ($p > 0.05$) in mitochondrial dehydrogenase activity for exposure times 24 and 48 h within the four concentrations when this cell line was treated with black seed compared with untreated control group. In contrast, the data showed a significant decrease in the activity of mitochondrial dehydrogenase after exposure to 72 h with any of the concentrations of black seed ($p < 0.05$).

The activity of mitochondrial dehydrogenase in MCF7 breast cancer cells exposed to nutmeg (figure 8.7B) was shown to be significantly decreased by various concentrations 50 and 100 µg/ml ($p < 0.01$) for exposure times 24 h. However, at 500 and 1000 µg/ml there was no significant decrease ($p > 0.05$) compared to the negative control. In addition, there are significant decreases ($p < 0.05$) after exposure for 72h with 500 and 1000 µg/ml concentrations of nutmeg extract. At 48h, nutmeg showed similar values to the control except for 1000 µg/ml that showed non-significant increase ($p > 0.05$).

Figure 8.7C, displays a significant concentration-dependent increase in mitochondrial dehydrogenase activity in MCF7 cells exposed to date palm pollen

(500 and 1000 µg/ml) after 24h of exposure ($p < 0.01$), also the result showed non-significant increase at concentrations 50 and 100 µg/ml compared with untreated control group ($p > 0.05$). In addition, all concentrations at exposure time 48h showed a non-significant increase ($p > 0.05$) compared to the negative control. Thereafter, at 72h exposure time the data, showed a concentration-dependent decrease in cell viability which became significant at the highest DPP concentration (100 µg/ml).

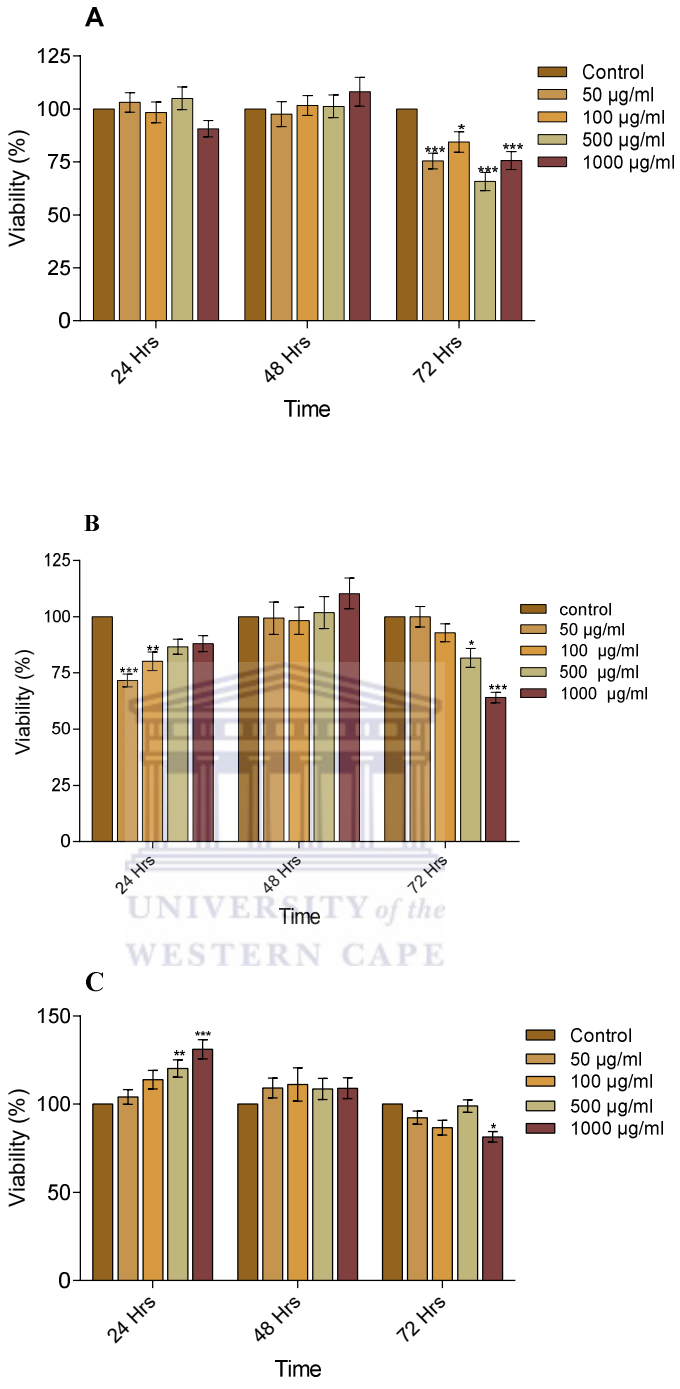

Figure 8.7: Viability of MCF7 breast cancer cells exposed to *Nigella sativa, Myristica fragrans* and *Phoenix dactylifera.*
Values represented are the mean ± SD of 6 determinations repeated in three independent experiments. Abbreviations: (A) black seed; (B) nutmeg; (C) date palm pollen; *, $p<0.05$, **, $p<0.01$; ***, $p<0.001$compared to the control.

8.6 Effects of *Nigella sativa, Myristica fragrans* and *Phoenix dactylifera* on the MCF7 breast cancer cells

The morphological appearance of MCF7 breast cancer cells after exposure to BS, NM and DPP for 72h was studied by light microscopy without (Figure 8.8) or with H & E staining (Figure 8.9). Under the phase contrast microscope, examination of untreated MCF7 breast cancer cells showed the presence of normal cell division, while cells exposed to the higher concentration of black seed, nutmeg and date palm pollen showed characteristic changes in their morphology which included cell shrinkage and irregular shape (figures 8.8-9 B4, C4 – D4). However, from figure 8.8 and 8.9, the morphology of MCF7 breast cancer cells exposed to black seed, nutmeg and date palm pollen, showed normal appearance of dividing cells at concentrations between 50 and 500 µg/ml.

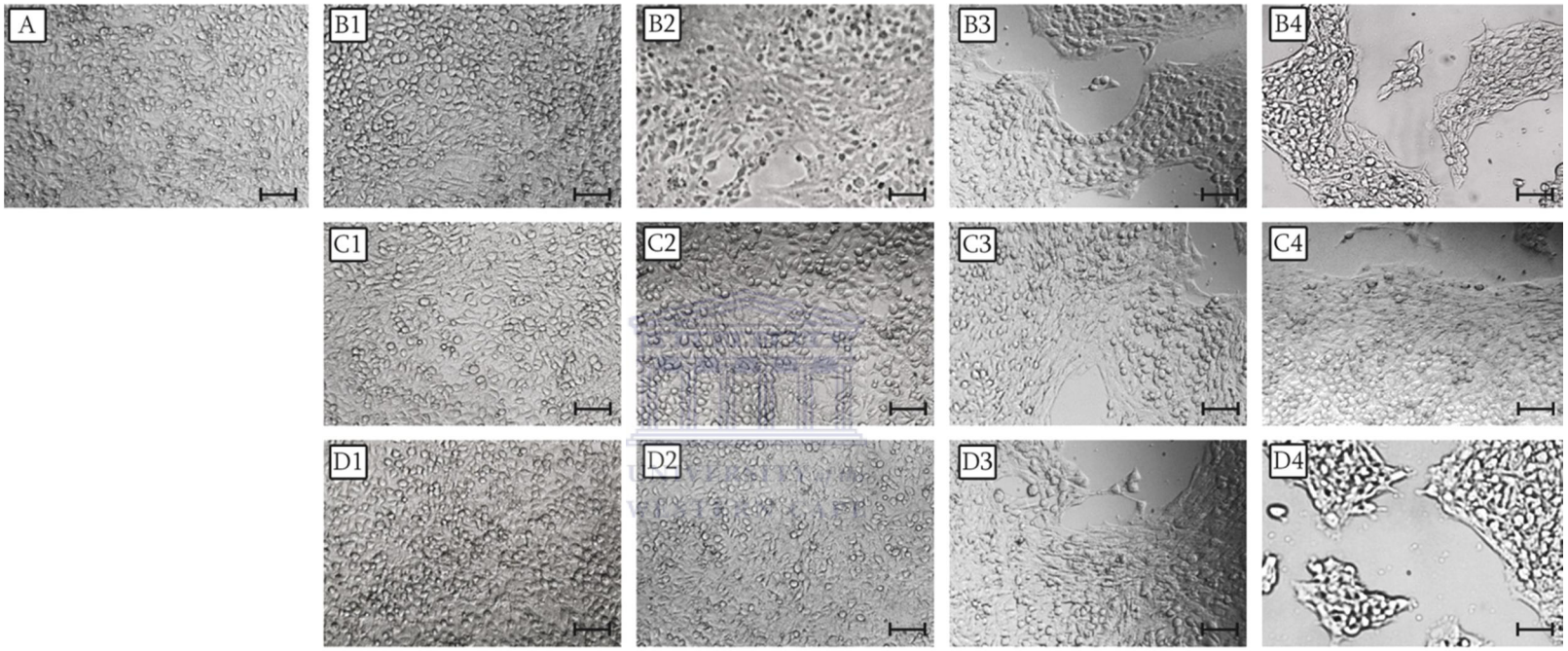

Figure 8.8 : Morphology of MCF7 breast cancer cells exposed to *Nigella sativa, Myristica fragrans* and *Phoenix dactylifera,* after 72h.
Abbreviations: (A) control (B) Black seed (C) Nutmeg (D) Date palm pollen (1) 50 µg/ml (2) 100 µg/ml (3) 500 µg/ml (4) 1000 µg/ml; Bar = 50 µm.

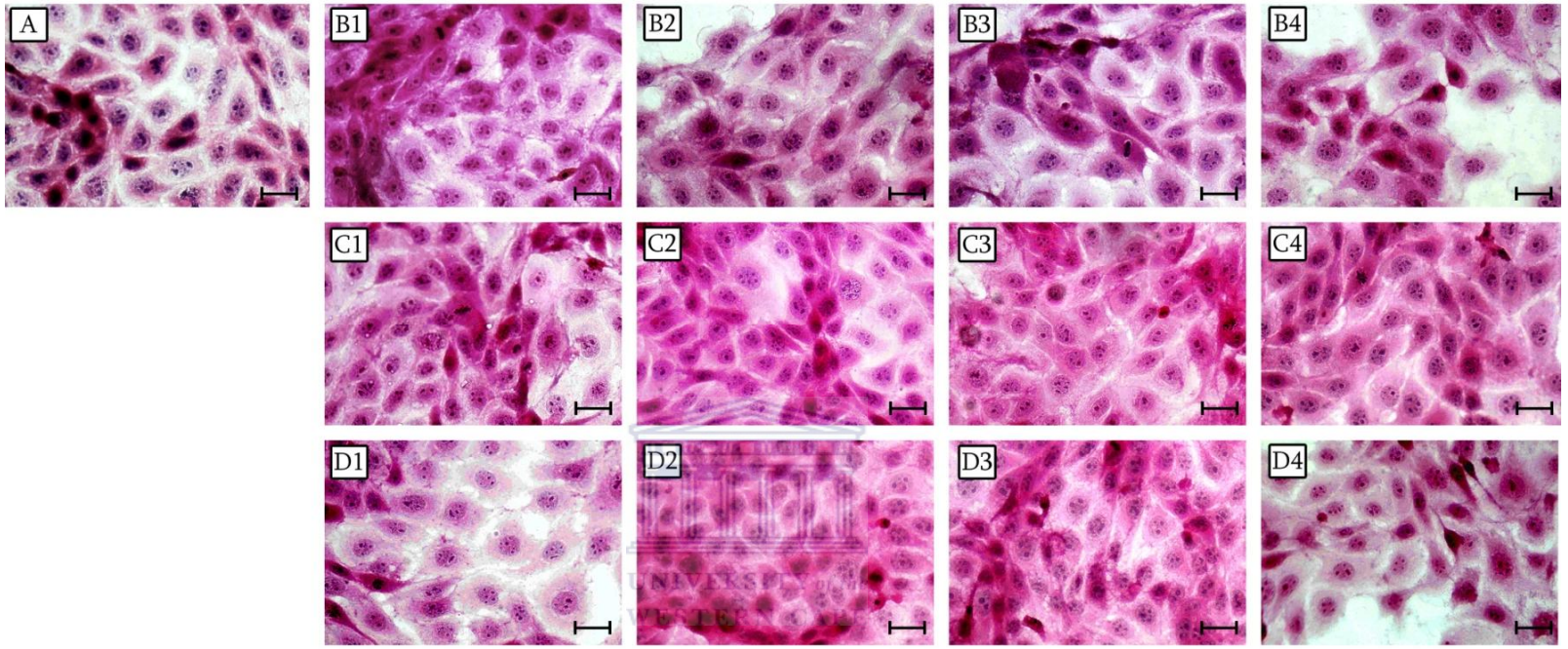

Figure 8.9: Morphology of MCF 7 breast cancer cells exposed to *Nigella sativa, Myristica fragrans* and *Phoenix dactylifera*, after 72 h using H&E staining.
Abbreviations: (A) control (B) Black seed (C) Nutmeg (D) Date palm pollen (1) 50 µg/ml(2)100 µg/ml (3) 500 µg/ml (4) 1000 µg/ml; Bar = 50µm

8.7 Detection of apoptotic MCF7 cells after exposure to *Nigella sativa, Myristica fragrans* and *Phoenix dactylifera*.

Apoptosis assay was performed to observe the effect of *Nigella sativa, Myristica fragrans* and *Phoenix dactylifera* on MCF-7 cell death. Therefore, apoptotic or necrotic cells determined by flow cytometry to observe the effect of changed concentration NC, negative untreated control; PC, positive control; BS, Black seed; NM, nutmeg; DPP, date palm pollen.

Apoptosis assay of MCF-7 cells showed the black seed (1000 µg/ml) increase in the number of apoptotic cells in positive control 6% DMSO, respectively when compared with negative control sample ($p < 0.001$) (Figure 8.10). In addition, Number of apoptotic cells slightly increased apoptotic MCF-7 cells by 1000 µg/ml (nutmeg and date palm pollen) to (15.4 % and 13.36 %) compare the negative control (5.8%).

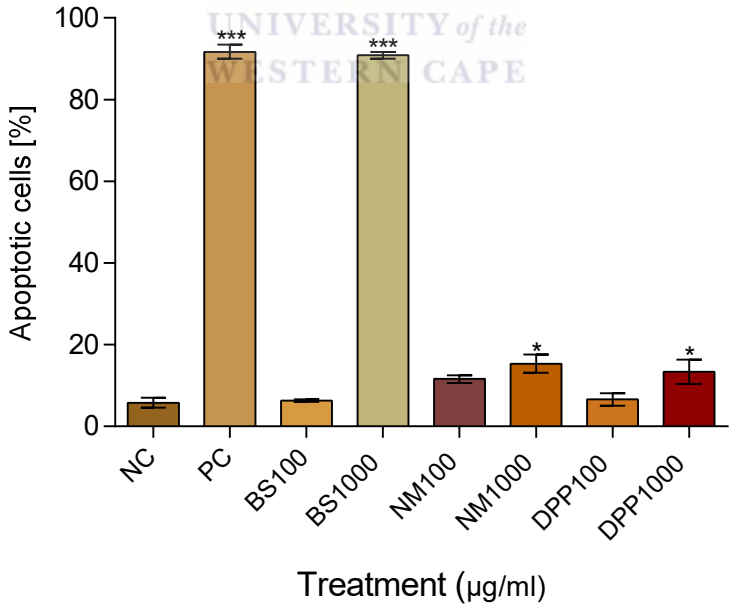

Figure 8.10: Apoptosis Induction of MCF7 breast cancer cells with *Nigella sativa, Myristica fragrans* and *Phoenix dactylifera*.
Values represented are the mean ± SD of 6 determinations repeated. Cells were stained with Annexin V-FITC using Apoptosis Detection kit and analyzed by flow cytometry. Abbreviations: NC, negative untreated control; PC, positive control; BS, Black seed; NM, nutmeg; DPP, date palm pollen; *, $p<0.05$; ***, $p<0.001$ compared to the untreated control.

Apoptosis assay results showed the effect black seed (1000 µg/ml) in MCF-7 breast cancer cells that there increased in number of apoptotic cells (90.8%) blue line compared to the negative control (5.8 %) pink line (Figure 8.11).

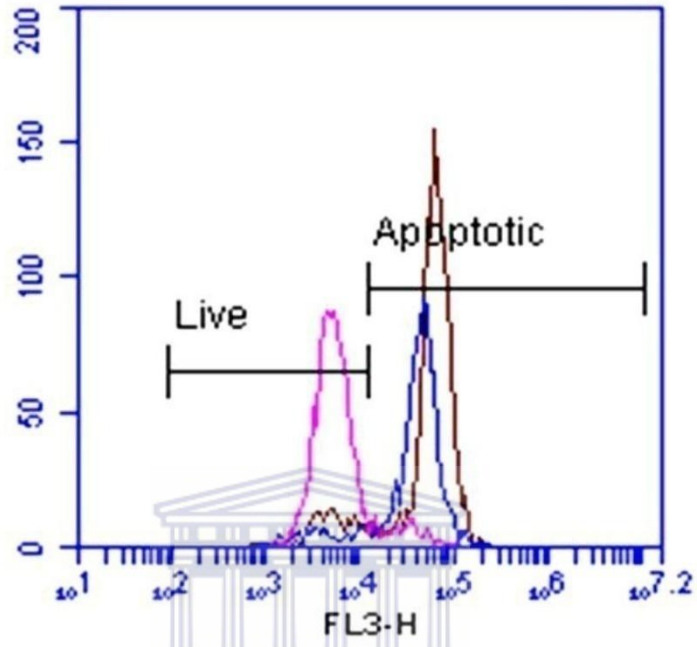

Figure 8.11: : Induction of apoptosis in TM3 Leydig cells following exposure to *Nigella sativa* using flow cytometry
Abbreviations: negative untreated control (pink line), Black seed (blue line), positive control DMSO 6% (brown line). X axis shows the relative fluorescence on a logarithmic scale, and the Y axis is cell number.

8.8 Effect of *Nigella sativa, Myristica fragrans* and *Phoenix dactylifera* in the MCF7 breast cancer cells on the estrogenic activity and cell proliferation

The E-SCREEN assay measures the estrogenic activity of a substance via its effect on cell proliferation in MCF-7 breast cancer cells which possess estrogen receptors. This assay is performed in media devoid of phenol red and the sera are stripped of any steroid compounds to exclude any estrogenic activities coming from the background. Under these conditions, BS and NM showed significant estrogenic activities.

The positive control, MCF 7 breast cancer cells treated with 17β-estradiol (1nM) showed significant (p<0.001) increase in cell proliferation (figures 8.12A, B and C)

compared with the negative control group. However, MCF-7 cells treated with BS initially produced a concentration dependent decrease in cell proliferation compared to the positive control. However, when compared to the negative control, cell proliferation increased significantly following treatment with 10 or 50µg/ml BS and thereafter significantly decreased between 100-1000 mg/ml (fig 8.12A). NM 10-50 µg/ml significantly increased cell proliferation followed by a concentration-dependent significant decrease compared to the negative control. Treatment with DPP produced only a weak estrogenic effect which resulted in a concentration dependent significant increase between 50-1000 µg/ml DPP.

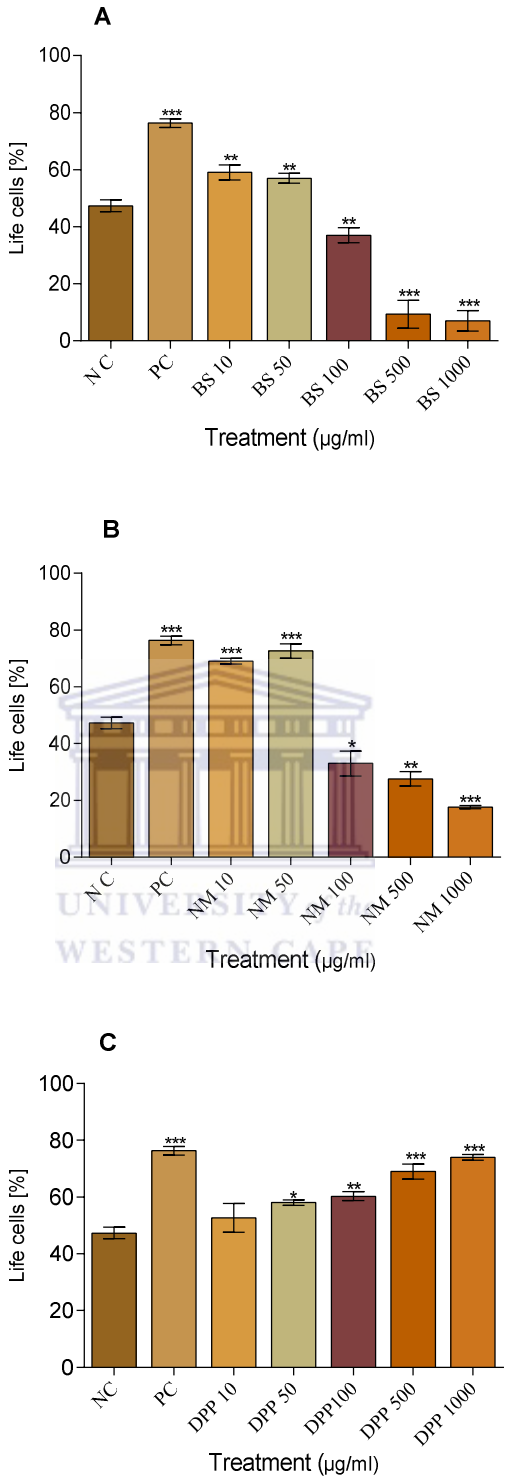

Figure 8.12 Estrogenic activity of MCF7 breast cancer cells exposed to *Nigella sativa, Myristica fragrans* and *Phoenix dactylifera*.
Values represented are the mean ± SD of 6 determinations repeated. Abbreviations: (A) black seed; (B) nutmeg; (C) date palm pollen; (10 – 1000 µg/ml); *, $p < 0.05$, **, $p<0.01$; ***, $p<0.001$compared to the control.

CHAPTER IX
DISCUSSION

9.1 Antioxidant capacity of *Linum usitatissimum, Raphanus sativus*, *Nigella sativa, Myristica fragrans* and *Phoenix dactylifera*

Flavonoids, anthocyanins, carotenoids, dietary glutathionine, vitamins and endogenous metabolites have been observed to possess antioxidant properties (Choi et al., 2002; Hertog et al., 1992; Larson, 1988). Lack of homeostasis between pro-oxidants and anti-oxidants can result in poor functioning of the male and female reproductive systems (Agarwal and Allamaneni, 2004; Agarwal et al., 2006; de Lamirande et al., 1997; Sikka, 1996). Monitoring changes in the levels of free radicals, the antioxidant activity or the formation of metabolic products by the liver can be used to assess the antioxidant activity of plant polyphenols (Decker et al., 2005). One objective of this thesis was to determine the antioxidant activity and total polyphenols content of flaxseed, radish seed, black seed, nutmeg and date palm pollen ethanolic extract with 2, 2-diphenyl-1-picrylhydrazyl (DPPH).

Previous studies by Basch et al. (2007) reported high antioxidant activity of flaxseed. In respect of this, several studies have shown positive health benefits of flaxseed in the management of human diseases (Ganorkar and Jain, 2013). In another study *Raphanus sativus* was identified as a potent antioxidant with high free radical scavenging activity (Beevi et al., 2010). High polyphenolic content has been attributed for its potent antioxidant and radical scavenging activity. A further analysis identified the presence of coumarin, saponins, flavonoids and anthocyanins in radish seeds (*Raphanus sativus*) (El-Sayed, 2001). It for this reason, it has been suggested as a potential source of natural antioxidants.

Nigella sativa was observed to possess high flavonol content as well as high antioxidant activity with DPPH. In addition to this, it contained the essential oil, thymoquinone and other components like carvacrol, anethole and 4-terpineol

(Tembhurne et al., 2014). The antioxidant action of *Nigella sativa* may explain its claimed usefulness in folk medicine.

A phyto-chemical analysis of date palm pollen identified estrone, α- amirin, triterpenoidal saponins and flavonoids as its main antioxidants (Hassan et al., 2012; Mahran et al., 1976). As a result of the high differences in alkaloid composition, a comparative analysis of antioxidant levels of Libyan traditional plants (flaxseed, black seeds, radish seed, date palm pollen and nutmeg) was done in this thesis. The results showed that date palm pollen had a higher antioxidant activity than all of the above mentioned plants. However the values obtained were significantly lower than those of butylated hydroxytoluene (BHT) which was the reference standard. These results are in agreement with what has been reported by Daoud et al. (2015) that all tested date palm pollen extracts exhibit significant phenolic and flavonoid content and display good antioxidant activities.

9.2 Effects of *Linum usitatissimum*, *Raphanus sativus* and *Phoenix dactylifera* on the male reproductive system

Male rats were exposed to flaxseed, radish seeds and date palm pollen for 52 days as the duration of spermatogenesis in rat takes about 52 days (Nakai et al., 2004). In this thesis, the weekly body weight gain in all treatment groups was similar to the control and increased progressively throughout the study period. In the same way, the total weight gain of the male rats also did not differ significantly after 52 days exposure to the ethanolic plant extract. The exception was DPP (240mg/kg), which showed an increase in weight gain. This agrees with the study carried out by Bahmanpour et al. (2006) in which they observed the effect of DPP on sperm parameters and reproductive system of adult male rat after 35 days of treatments.

Besides this, this thesis also revealed a significant increase in the relative testicular weight of animals exposed to either flaxseed (300 mg/kg) or date palm pollen (120 mg/kg). Similarly, the absolute weight of the prostate of animals exposed to date palm pollen (240 mg/kg) showed significant increased values. In addition, the

relative weights of the seminal vesicles of all treated groups showed significant increased values. Similarly, Yasir et al. (2014) also observed significant increases in body weight, testes weight and testosterone levels following exposure of adult male rats to DPP for 35 days. This effect was suggested to occur due to the presence of gonadotropin like substances in the DPP (Azooz et al., 2001).

In this thesis, whilst DPP is having its effects via the hypothalamic – pituitary-gonadal axis; we also observe DPP influencing the growth hormone / Insulin growth factor axis (GH/IGF-1). In line with this, treatment of male rats with 120 or 240 mg/kg DPP stimulated increased body mass compared to the control group (fig 5.2). Concomitantly, we observed increased testosterone levels (fig 5.3). So far no literature has reported the effect of DPP on body weight gain. However, some previous studies have reported the effect of testosterone on the development of lean body mass. Similarly, in this thesis we have reported increased testosterone levels following treatment of male rats with DPP. In line with this, testosterone has been reported to prime skeletal muscles for growth by increasing the net protein synthesis in the fasted state (Ferrando et al., 2002).

Reports from previous studies have shown increased testosterone levels within or near the physiological range producing increased muscle anabolism, lean body mass (LBM) and muscle strength similar to supra-physiological administration. These effects have been reported to occur concomitantly with increased androgen receptor (AR) and insulin growth factor 1(IGF-I) expression in skeletal muscle (Ferrando et al., 2002).

Besides this, growth hormone (GH) has been reported to possess anabolic role for supra-physiological levels of systemic GH or IGF-I in skeletal muscle of healthy individuals. In this regard, growth hormone (GH) and insulin-like growth factor I (IGF-I) have been reported to regulate body size (anabolic effects) in growing animals whilst its role in adults is less clear (Ferrando et al., 2002).

Further to this, GH binding results in dimerization of two GH receptors and intracellular signaling involves the Janus kinase and the signal transducers and

activators of transcription (Stat) pathway (Smit et al., 1996). GH stimulates the synthesis of IGF-I in the liver. GH administration causes rapid up-regulation of IGF-I mRNA and protein in the liver (Mathews et al., 1986). The effects of IGF-I are mediated mainly by the type 1 IGF receptor (IGFR1), which has tyrosine kinase activity and signals through the phosphatidylinositol 3 kinase (PI3K)/AKT pathway (Pandini et al., 2002).

Results of the present thesis (Table 9.1), showed a significant decrease in serum FSH levels in male rats exposed to FS (300 mg/kg), DPP (120 mg/kg) and DPP (240 mg/kg) relative to the negative control. On the contrary, there was a statistically non-significant increase in serum luteinizing hormone levels of rats treated with radish seed (80 mg/kg), flaxseed (200-300 mg/kg) and date palm pollen (120-240 mg/kg), respectively. In addition to this, the present thesis observed a statistically significant increase in serum testosterone levels following exposure of male rats to (80 mg/kg) of radish seeds and date palm pollen relative to the negative control. These results are in agreement with those obtained by Mehraban et al. (2014) following treatment with DPP (120 and 240 mg/kg) for 35 days. Similarly, twenty-five infertile men treated with 500 mg of DPP in capsules twice a day for 3 months showed significantly increased levels of testosterone (Mehraban et al., 2014).

Table 9-1: Effects of *Linum usitatissimum, Raphanus sativus* and *Phoenix dactylifera* on the male reproductive system

Plant extract	Endocrine hormones			Sperm concentration	Sperm vitality	Sperm motility		
	LH	FSH	Testosterone			progressive motility	total motility	static
RS 80	↗	↘	↑	↑	↑	=	↗	↘
RS 160	↘	↓	↗	↑	↗	↗	↗	↘
FS 200	↗	↓	↘	↑	↗	↗	↗	↘
FS 300	↗	↓	↓	↑	↑	↑	↑	↓
DPP 120	↗	↓	↑	↑	↑	↑	↑	↓
DPP 240	↗	↓	↑	↑	↑	↑	↑	↓

Abbreviations: RS, Radish seed; FS, Flaxseed; DPP, Date palm pollen; ↑, significant increase ↓, significant decreased; ↘, non- significant decreased, ↗, non- significant increase; = , equals to control.

As observed by Abedi et al. (2012) the positive increase in estradiol and testosterone level was observed in rats treated with a dose 140 mg/kg of DPP. This led to a maximum sexual aggression of the male rats and this was close to our dose of DPP (120 mg/kg) that significantly enhanced the testosterone level. Whilst the above mentioned plants are promoting sexual behaviour, a previous *in vivo* study using *Raphanus sativus* extract were observed to protect against Zearalenone (ZEN) induced reproductive toxicity, oxidative stress and mutagenic alterations in male BALB/c mice. Here, RS is negatively affecting testosterone levels and the reproductive system. A combined treatment of *Raphanus sativus* (15 mg/kg b.w) and ZEN (40 mg/kg b.w) on male mice for 28 days improved the negative effects of ZEN on the testes by increasing the testosterone levels (Ben Salah-Abbès et al., 2009).

In this thesis, flaxseed increased LH. In line with this, flaxseed has been reported to contain polyunsaturated fatty acids, particularly ALA, the essential omega-3 fatty acid, and linoleic acid (LA), the essential omega-6 fatty acid, protein, dietary fiber, lignan, specifically Secoisolariciresinol diglucoside (SDG) (Ganorkar and Jain, 2013). In the anterior pituitary gland linoleic acid has been reported to up-regulate the LHß mRNA expression and in turn increased LH levels. In support of this, is a previous study using real time PCR in which treatment of LHßT2 cells with linoleic acid induced an increase in LHß messenger RNA levels (Garrel et al., 2011).

In addition to this, studies have shown the presence of long fatty acid chain (LFAC) receptor GPR120 on rat pituitary cells. These receptors when treated with their agonist GW9508 have been observed to promote increased LH levels. Mechanically, the binding of the ligand to the GnRH receptor induces the activation of the mitogen activated protein kinase (MAPK) pathway. This can occur via the ERK1/2, JNK, and p38 pathways. Besides this, the MAPK pathways have also been reported to occur via protein kinase C (PKC) or in association with Raf and calmodulin in lipid rafts (Thackray et al., 2010).

The above mechanism has also been corroborated by Garrel et al. 2011 who reported that the binding of a ligand, GnRH to its receptor of the G protein-coupled receptor (GPCR) family in the presence of phospholipase Cß results in the opening of several signaling pathways. This is followed by the release of gonadotropin via exocytosis and at the same time up – regulate the levels of intracellular Ca^{2+}. Significant increase in intracellular Ca^{2+} levels in turn activates nitric oxide synthase type I (NOS1) cascade (NOS1/NO/soluble guanylate cyclase). This far, GnRH has been observed to activate several protein kinase C (PKC) isoforms, such as the novel PKC ε and - θ isoforms which couple the GnRH receptor (GnRH-R) to the cAMP pathway. Protein Kinase C (PKC), cyclic adenoside monophosphate (cAMP) and extracellular receptor kinase (ERK) pathways all contribute to GnRH-stimulated transcription of gonadotropin genes (Garrel et al., 2011).

In addition to this, FS enhanced sperm concentration, motility and vitality. In a previous investigation flaxseed was used as an alternative to hormone replacement therapy and cardiovascular risk reduction (Lemay et al. 2002; Hallund et al. 2006). Flaxseed consumption during lactation did not produce any observable effect on sexual maturation, development of reproductive organs, and 17b-estradiol in the female offspring (Soares et al., 2010).

However, no change in prostate morphology, serum testosterone and 17b-estradiol concentration was observed in male offspring in different reports (Cardozo et al., 2010; Ward et al., 2001). Reports from another study indicate that enhanced serum testosterone and estradiol levels besides an increase in relative weights of the accessory sex gland, prostate lobes, the seminal vesicle, and the testes (Collins et al., 2003).

Male infertility is commonly due to deficiencies in the semen which are mainly characterized by low sperm motility and viability (Banihani et al., 2012). In respect of this, low sperm production (oligoozoospermia), poor sperm motility (asthenozoospermia) or abnormal sperm morphology (teratozoospermia) or a combination of all the three (oligoasthenoteratozoospermia) (Guzick et al., 2001)

describe characteristics of an infertile male. In this thesis, exposure of rats to radish seeds (160 mg/kg), flaxseeds (300 mg/kg) and palm pollen (120 or 240 mg/kg) significantly increased sperm concentration relative to the control. This is consistent with the results of a previous study by Mehraban et al., (2014) which showed that the sperm count was significantly increased following administration of (120 and 240 mg/kg) doses of DPP. Similarly, administration of *Raphanus sativus* extract significantly increased the epididymal sperm number (Ben Salah-Abbès et al., 2009).

Further to this, positive results on the male reproductive system were observed following exposure of rats to flaxseed meal prenatally and/or postnatally. There was a decrease in the prostate weight, as well as an increase in the serum levels of LH and caudal epididymal sperm counts (Sprando et al., 2000).

In the present thesis, sperm vitality was high in all treatment groups and significantly increased in the groups exposed to radish seeds (80 mg/kg), flaxseeds (300 mg/kg) and date palm pollen (120,240 mg/kg) compared to the control. Sperm total motility and sperm progressive motility was significantly enhanced in the groups exposed to flaxseeds (300 mg/kg) and date palm pollen (120,240 mg/kg).

Present in flaxseed are polyunsaturated fatty acids such as omega 3 fatty acids and alpha linolenic acid. Omega-3 fatty acids, in particular docosahexaenoic acid (DHA), are important for sperm membrane integrity, sperm motility and viability, as well as, cold sensitivity (Gholami et al., 2011). Current evidences suggest that DHA may increase the flexibility and compressibility of the sperm tail and hence, improve the ability of the lipid bilayer to tolerate the stress of flagellar movement (Speake and Surai, 2003).

Previous studies have reported a decrease in the percentage of motile sperm following two week exposure to heat stress. In this study, feeding an omega-3 enriched nutriceutical improved the motion characteristics of fresh sperm assessed by CASA. Total motility, progressive motility and average path velocity of

neutraceutical-fed bulls were significantly higher than the control group (Gholami et al., 2011).

Flaxseeds besides containing omega – 3 fatty acids also contain alpha linolenic acid which can be converted into ecosapantaenoic acid and docosahexaenoic acid. The latter has been observed to induce acrosome reaction and fusogenic capacity of the spermatozoa (Comhaire and Mahmoud, 2003).

Further to this, lignans present in flaxseed possess antioxidant properties. Because of this, flaxseed has been shown to suppress tumor promoter induced hydrogen peroxide and superoxide anion formation. In addition to this, flaxseed also enhances the activity of antioxidant enzymes such as catalase, glutathione peroxide, glutathione reductase and superoxide dismutase. This protected sperms against apoptosis and DNA damage (Rajesha et al., 2006).

In line with this, exposure to *Raphanus sativus* extract in male mice increased the epididymal sperm number and the percentage of total motile spermatozoa (Mehraban et al., 2014). On the contrary, as noted by El-Neweshy et al. (2013) sperm motility was significantly impaired in Cd-treated rats however it significantly improved following DPP supplementation, although not to the control level. However, it was insignificantly altered in DPP-alone-treated rats. To the best of our knowledge, this thesis reports for the first time the ability of flaxseed to increase sperm concentration, vitality and motility.

In this thesis, fluid resorption effects of estradiol might be the cause of the weight gain observed in epididymis, testes and seminal vesicles which is due to presence of phytoestrogen as a steroidal component of DPP. These in turn may have influenced the sperm parameters (Hess et al., 1997).

The data presented here demonstrate that the sperm mobility phenotype can be attributed to specific sperm velocity parameters of individual sperm as determined by sperm class analyzer software (SCA). Sperm velocity parameters (VCL, VSL

and VAP) directly measure the motion of spermatozoa (Horimoto et al., 2000). In this thesis, sperm velocity parameters, which included VCL, VSL, VAP, ALH, LIN, STR, WOB and BCF, showed susceptibility to increased VSL and VAP values when compared to the control. In addition, VCL values in the groups exposed to the date palm pollen (240 mg/kg) were significantly increased relative to the controls. There was a significant increase in amplitude of lateral head displacement (ALH) in groups exposed to radish seeds (160 mg/kg), flaxseeds (300 mg/kg) and palm pollen (240 mg/kg). Beat cross frequency (BCF) was also significantly enhanced in the groups exposed to radish seeds (80 mg/kg) and flaxseed (300 mg/kg), while the other treatment groups showed a tendency to increased values compared with the control.

Alkaloids of *DPP* also showed estrogenic properties (Abedi et al., 2014) and they seem to regulate the renewal of spermatogenic cells and male reproductive tissues that possess oestrogen receptors (Hassan et al., 2012). Egyptian DPP was reported to have a high content of gonadotropin-like substances and estrogenic materials that have been shown to improve male fertility (El-Kashlan et al., 2015). DPP extracts have been observed to have stimulatory effect on the pituitary gland leading to increased LH release besides Leydig cell enhancement of 3β-HSD and 17β-HSD activities. A positive relationship has been previously demonstrated between the seminiferous tubule diameter and spermatogenic activity of the testis (Sinha Hikim et al., 1988).

In this thesis, the histological examination of the cross sections of the testis in rats exposed to radish seed, flaxseed and date palm pollen showed an increase in diameter of seminiferous tubules besides improved spermatogenesis relative to the control group. Using morphometric measurements of the epithelial heights of the seminiferous tubules in the testes of male rats exposed to date palm pollen, the epithelium cells was significantly higher compared to the control group. Similar to our findings, Mehraban et al. (2014) concluded that the suspension of DPP in distilled water may act as a fertility agent by increasing the diameter of seminiferous tubules in treated rats. This thesis, reports a novel observation, that radish seed and

flaxseed increase the diameter of seminiferous tubules and potentiate spermatogenesis.

The glomerular filtration rate (GFR) is measured by serum creatinine concentration, which is the index of renal function (Perrone et al., 1992). In this thesis, a significant decrease in the level of serum creatinine was observed in male rats exposed to all treatments compared to the control group (Table 9.2). Further to this, there was a non-significant increase in the relative weight of kidneys of male rats exposed to the radish seeds, flaxseed and date palm pollen. Another study on rats showed a significant decrease in creatinine levels in groups exposed to date palm pollen compared to control group (Hammed, 2015). These effects have been attributed to the filtration promotion effects of DPP and its ability to increase the efficacy of both kidneys (Mallhi et al., 2014).

Table 9-2: Effects of *Linum usitatissimum, Raphanus sativus* and *Phoenix dactylifera* on the male kidneys and liver function.

Plant extract	Testis			Liver			Kidney			ALT	AST	Creatinine
	CAT	SOD	LPO	CAT	SOD	LPO	CAT	SOD	LPO			
RS 80	↗	↗	↗	=	↑	=	↘	=	=	↓	↗	↓
RS 160	↑	↑	↗	↑	↑	↘	↗	↑	↘	↘	↗	↘
FS 200	↘	↑	↘	↘	↗	↘	↗	↘	=	↓	↗	↓
FS 300	↘	↗	↑	↗	↑	↘	↗	=	=	↓	↗	↓
DPP 120	↗	↑	↑	↗	↑	↘	↗	↗	=	↘	=	↘
DPP 240	↑	↗	↑	↗	↗	↗	=	↓	=	↓	=	↓

Abbreviations: RS, Radish seed; FS, Flaxseed; DPP, Date palm pollen; ↑, significant increase ↓, significant decreased; ↘, non- significant decreased, ↗, non- significant increase; = , equals to control.

Besides improving spermatogenesis, the above mentioned Libyan traditional plants reversed the adverse effects induced by different compounds. For example, mice treated with Zearalenone (ZEN) resulted in renal failure which was characterised by a significant increased activity of alkaline phosphatase (AP) and gamma glutamyl transferase (GGT) followed by a significant decrease in creatinine, total

protein, triglyceride and albumin. This renal failure was reversed by ω-3 polyunsaturated fatty acids found in fish oil (Westberg et al., 1989). In respect of this, flaxseed is also an excellent source of the ω-3 fatty acid and α-linolenic acid (ALA). In line with this, flaxseed and flax oil slowed the decline in renal function and reduced glomerular injury in a rat model with renal failure. Besides this, the flaxseed and flax oil modulated blood pressure, plasma lipids and urinary prostaglandins of this rat model to basal physiological levels (Ingram et al., 1995).

Acute or chronic liver injury eventually results in an increase in serum concentrations of aminotransferases. AST and ALT are enzymes that catalyze the transfer of α-amino groups from aspartate and alanine to the α-keto group of ketoglutaric acid to generate oxalacetic and pyruvic acids respectively, which are important citric acid cycle contributors (Giannini, 2005). The results of this thesis showed that the serum alanine transaminase (ALT) activity in the male rats decreased significantly in all the treatments and the serum level of aspartate transaminase (AST) activity did not increase significantly in the male rats treated with radish seed and flaxseed. On the other hand, ALT dropped insignificantly in male rat exposed to date palm pollen. Previous studies confirm that the reported decrease in the levels of ALT, AST and ALP in rats exposed to a protective dose of (150 mg/kg B.W) date palm pollen for 42 days. This treatment caused an improvement and enhancement of liver function against carbon tetrachloride induced harmful effects. (Araak and Abdulhussein, 2012). Many studies have collaborated the positive effects of flaxseed or its components on liver and kidney functions in various pathological conditions. For example, flaxseed oil significantly reduced the AST and ALT enzyme levels and reversed the non-alcoholic fatty liver in hamsters along with acute and chronic arthritis in albino rat models (Kaithwas and Majumdar, 2010; Yang et al., 2009). On the same way, flaxseed oil has been shown to lower the AST and ALT in radiation induced hepatotoxicity in mice (Bhatia et al., 2007).

Using light microscopy studies, Hammed (2015**)** established date palm pollen's ability to counteract the toxic effect of lead acetate associated with improvement of

renal histology. Besides this, rats exposed to DPP suspension developed normal hepatic lobular architectures with central veins and radiating hepatic cords (Araak and Abdulhussein, 2012).

Free radical-induced oxidative damage to spermatozoa has recently received considerable attention for its role in reducing sperm function and causing infertility. Factors that protect from such effects are, therefore, of great clinical importance. In the present thesis, exposure of male rats to radish seed (160 mg/kg), flaxseed (200 mg/kg) and date palm pollen (120 mg/kg) significantly increased superoxide dismutase activity (SOD). There was also improved catalase activity in testis of male rats exposed to radish seed and date palm pollen. El-Neweshy et al. (2013) noted that administration of DPP exhibited an antioxidant effect, as evidenced by improved GSH and restored LPO in the testes of Cd-treated rats.

Several studies on rats have shown the free radical scavenging abilities of *Raphanus sativus* extract. In respect of this, *Raphanus sativus* eliminates oxygen free radicals such as hydroxyl radical, singlet oxygen, hydrogen peroxide, peroxyl radical, and hypochlorous acid through its direct scavenging activity (Ben Salah-Abbès et al., 2009; Liu et al., 2005) leading to an increase in SOD and GPx in the testis (Ben Salah-Abbès et al., 2009).

In this thesis, we report that DPP, RS and FS possess therapeutic effects to improve male fertility. In brief, these medicinal plants enhanced LH levels and serum testosterone levels. The antioxidant capacities of these plants have been implicated in the scavenging of free radicals and then subsequently provide phyto-protection against reproductive toxicity and above all; improved sperm parameters.

9.3 *In vivo* effects of *Nigella sativa, Myristica fragrans* and *Phoenix dactylifera* on the female reproductive, kidneys and liver function

In this thesis, female rats were exposed to *Nigella sativa, Myristica fragrans* and *Phoenix dactylifera* for 21 d. This treatment period was chosen based on the duration of the oestrous cycle (4-5 days) in the female rat and was also used in previous studies (Nakatani et al., 1991).

Exposure of female rats to *Nigella sativa, Myristica fragrans* and *Phoenix dactylifera* did not alter the weight gain during the period of treatment. In the present thesis, rats treated with nutmeg (400 mg/kg) and black seed (400 mg/kg) showed significant increased values in the relative weights of the ovaries. Reports from a previous study describe the observed change in terms of ovary to body weight ratio (Arak and Assi, 2011) and point further to say that it occurred as a result of an increase in gonadotropins hormones (LH and FSH) levels which induced structural and histological changes with respect to ovarian weight, diameter and the numbers of follicles (Froment et al., 2002).

In this thesis (Table 9.3), there was a significant increase in FSH levels in female rats exposed to black seed and date palm pollen. On the other hand, LH levels decreased significantly in the same groups. In response to the rising levels of estrogen a pronounced peak in LH occurred during the proestrus phase of the estrous cycle of the female rats, which eventually results in ovulation. Any alterations in LH level could potentially reduce or delay ovulation, resulting in reduced litter size (Tyler and Gorski, 1980).

From the results of previous studies, *Nigella sativa* has been reported to elevate LH and FSH level (Arak and Assi, 2011). This elevation has been attributed to the active constituents of *Nigella sativa* that potentiate the hypothalamus or the pituitary glands to release and secrete gonadotropin hormone (Arak and Assi, 2011). Reports also indicate that DPP contains cholesterol, rutin, carotenoids, as well as estrone which are known to exhibit gonadotrophin activity in rats and may

be responsible for the increase in FSH and LH. Further to this, oral administration of DPP leads to amelioration of the damaging effects of lead acetate in female rats. This was demonstrated by a decrease in the atretic follicles and an increase in the development and maturation of follicles together with an increase in the fertility and pregnancy rate (Hammed et al., 2012).

On the other hand, to the best our knowledge we report for the first time that female rats exposed to nutmeg (400 mg/kg) showed significantly increased LH and FSH.

Results from a previous investigation showed that DPP contains flavonoids, rutins, carotenoids and vitamins that work as antioxidants (Baliga et al., 2011; Dasgupta and Klein, 2014; El Arem et al., 2014). Besides this, DPP is involved in the maintenance of urea concentration and decrease of creatinine levels (Rahmani et al., 2014). Similarly, results from the present thesis, showed a decreased creatinine activity in female rat serum treated with black seeds compared to the control. This has also been confirmed by previous results on nephron protective activity of *Nigella sativa* seed oil in nephrotoxicity induced by cisplatin and gentamycin (Tembhurne et al., 2014). The reason for the protective action is not certain but may be related to the antioxidant action of the drug and the fact that the neprotoxic drug may induce its effects via generation of free radicals (El Daly, 1998).

Nutmeg essential oils are powerful antioxidants (Dorman et al., 2000). In this thesis, the creatinine in female rat serum treated with nutmeg (200 and 400 mg/kg) was significantly decreased compared to the control. On the other hand, a previous study revealed that administration of nutmeg may have some deleterious effects on the renal cortex of adult Wister rats in both sexes at higher doses (1000 mg/kg) and may have an effect on kidney metabolic function (Alalwani, 2013). However, in our thesis the histological sections of the kidneys revealed no obvious morphological alteration as well as no change in kidneys weights.

ALT and AST are liver function markers (Huang et al., 2006). These liver enzymes catalyze transfer of alpha- amino group aspartate and alanine to the alpha-

ketoglutaric acid. Whereas ALT is primarily localized to the liver, AST is present in a wide variety of tissue, including heart, skeletal, kidney, brain, and liver. AST is present in both the mitochondria and cytosol of hepatocytes, but ALT is found only in the cytosol.

In the female rats, absolute and relative weights of liver demonstrated no significant increase following exposure to black seeds, nutmeg or date palm pollen compared to the control. In respect of this, Alanine transaminase (ALT) activity in serum of female rats exposed to either black seed or date palm pollen was shown to decrease significantly. In addition to this, serum (AST) activity showed no significant decrease. These results agree with those reported by Araak and Abdulhussein (2012) who concluded that date palm pollen protected the liver as demonstrated by stable AST and ALT levels following carbon tetrachloride induced harmful effects.

Table 9-3: *In vivo* effects of *Nigella sativa, Myristica fragrans* and *Phoenix dactylifera* on the female reproductive, kidneys and liver function

Plant extract	Endocrine hormones		Liver			Kidney			ALT	AST	Creatinine
	LH	FSH	CAT	SOD	LPO	CAT	SOD	LPO			
BS 200	↓	↗	↗	↗	↘	=	↗	↗	↘	=	↓
BS 400	↘	↑	↘	↘	↗	↗	↗	↗	↓	=	↓
NM 200	↑	↗	↓	↘	↗	↘	↗	↗	↘	↘	↓
NM 400	↗	=	↓	↘	↗	↗	↗	↑	↘	↓	↓
DPP 120	↓	↑	↘	↗	↗	↘	↗	↑	↓	↘	↓
DPP 240	↓	↗	↗	↗	↗	=	↗	↑	↓	↘	↘

Abbreviations: BS, Black seed; NM, Nutmeg; DPP, Date palm pollen; ↑, significant increase ↓, significant decreased; ↘, non- significant decreased, ↗, non- significant increase; = , equals to control.

Furthermore, the above results are similar to the ones that were obtained in a study that evaluated the hepatotoxicity of *Nigella sativa*. The result showed that a dose of (1 g/kg) *Nigella sativa* produced no significant change in serum alanine aminotransferase and aspartate aminotransferase between the treated and non-

treatment groups in rat (Dollah et al., 2013). Moreover, Dollah et al. (2013) observed no toxic effect on liver function of *Nigella sativa* at different doses for 4 weeks period.

Oxidative stress is responsible for most of the liver dysfunction and decreases the ability of the cells to fight against the injury (Mollazadeh and Hosseinzadeh, 2014). The liver is very sensitive to oxidative stress and damages caused by free radicals. Preventing the production of free radicals, scavenging free radicals or enhancement in antioxidant defence can minimize the adverse effects. (Mollazadeh and Hosseinzadeh, 2014).

Nigella sativa has been reported to contain thymoquinone which can inhibit iron-dependent lipid peroxidation in a concentration-dependent manner (Nagi and Mansour, 2000). It has also a potent O_2^- scavenger activity (Badary et al., 2003). With this characteristic, thymoquinone can decrease oxidative stress and increase antioxidant defense in the body. Thymoquinone can inhibit the expression of iNOS, that participates in oxidative stress and can increase the expression of antioxidant enzymes such as GSHPx and SOD (Danladi et al., 2013).

In this thesis, we report that NM, BS and DPP possess medicinal properties that are responsible for nephroprotection, hepatoprotectiion and improve many parameters of the female reproductive system through the potentiation of gonadotrophic hormones.

9.4 *In vivo* Effects of *Phoenix dactylifera, Linum usitatissimum* and *Raphanus sativus* on male rat sexual behavior and fertility outcome.

In the present thesis (Table 9.4), the time taken for male rats to reach the female was shorter in rats exposed to flaxseed and date palm pollen. As reported by Abedi et al. (2012), some medicinal plants have been observed to possess aphrodisiac effects and most of these effects have been observed to occur through vasodilation, generation of nitric oxide, elevation of androgens and gonadotropins. Sexual

behavior and erection are very much dependent on androgen levels. In this case, DPP and RS promoted an increased production of androgens in the male rats which in turn might have promoted an enhanced penile erectile response (Giuliano et al., 1993; Mills et al., 1998). To this extent, an up-ward regulation in serum testosterone concentration by plant extracts might have promoted the improved sexual behavior observed through time taken to reach the female (Bahmanpour et al., 2006). Clinical data obtained in a previous study observed that slight increases in testosterone levels result in significant increased sexual desire and libido. This clinical observation reinforce the idea that plant extracts may promote an increase in serum testosterone level which in turn is responsible for enhanced sexual behaviour in animals (Majewska, 1995).

Table 9-4: *In vivo* Effects of *Phoenix dactylifera, Linum usitatissimum* and *Raphanus sativus* on male rat sexual behavior and fertility outcome.

Plant extract	No of sniffs	Time to reach female	Mount frequency	Mount latency	Number of embryos
RS 80	↘	↘	↗	↘	↘
RS 160	↗	↘	↗	↘	↗
FS 200	↗	↘	↗	↗	↗
FS 300	↗	↓	↑	↗	↗
DPP 120	↘	↓	↑	↘	↑
DPP 240	↘	↓	↑	↘	↗

Abbreviations: RS, Radish seed; FS, Flaxseed; DPP, Date palm pollen; ↑, significant increase ↓, significant decreased; ↘, non- significant decreased, ↗, non- significant increase; = , equals to control.

DPP and FS improve the mount latency, index of libido, fertility index, implantation index and quantal pregnancy. As mentioned earlier (Table 9.1), this present study showed that DPP and radish seeds increased plasma level of testosterone and luteinizing hormone LH.

These findings support the use of DPP and FS as medicinal plants for the treatment of premature ejaculation in traditional medicine. Increased MF and IF in treated rats

(indicating the sexual motivation and efficiency of erection and penile orientation) and an increase in libido might be the result of the increase in several hormones that are secreted from the pituitary (Yakubu et al., 2008; Yakubu et al., 2005).

Besides this, *Phoenix dactylifera*, date palm pollen, was reported to enhance the orientation of males towards females by increasing mounting frequency and ano-genital investigatory behavior. The improved sexual behavior was accompanied by higher releases of dopamine. In respect of this, *Phoenix dactylifera* acted as a dopamine agonist and can be used as an aphrodisiac that leads to further increases in dopamine release. Above all, DPP influence sexual arousal and performance. As such, DPP may be used as an aphrodisiac to solve sexual problems such as pre-mature ejaculation and impotency (Abedi et al., 2012).

In a previous study, Mehraban et al., 2014 reported an increase in LH and testosterone concentration following treatment of male rats with 120 mg/kg of *DPP*. The implication of this result was that DPP had a stimulatory effect on the hypothalamic-pituitary axis of male rats. Present in DPP are steroids, flavonoids, saponins, and lipids (Yakubu et al., 2008). For example, saponins promote an increase in endogenous testosterone levels by raising the level of leutinizing hormones (LH). In line with this, any increase in LH by the pituitary gland promotes a direct increase in the levels of testosterone (Gauthaman et al., 2002). In another previous study, treatment of twenty-five infertile men with a capsule containing 500 mg DPP pollen powder, significantly, increased the FSH serum, in addition to LH and testosterone levels (Marah et al., 2005)

Flaxseed contains polyunsaturated fatty acids, particularly ALA, the essential omega-3 fatty acid, and linoleic acid (LA), the essential omega-6 fatty acid, protein, dietary fiber, lignan, specifically Secoisolariciresinol diglucoside (SDG) (Ganorkar and Jain, 2013). In the anterior pituitary gland linoleic acid has been reported to up-regulate the LHß mRNA expression and in turn increased LH levels. This effect has previously been observed in both immortalized LßT2 gonadotrope cell line and rat primary cell cultures (Garrel et al., 2011).

Subsequently, lutensing hormone (LH) binds to receptors in the membranes of Leydig cells, and stimulates the secretion of testosterone (Papadopoulos, 2010). Testosterone production, involves blood-borne LH binding to a G-protein coupled receptor. This results in the activation of adenylate cyclase (AC) that converts ATP to cAMP, a major messenger of lutenising hormone action in Leydig cells. cAMP subsequently activates cAMP - dependent protein kinase. This promotes the transportation of free cholesterol into the mitochondria where it amplifies the effect of LH and cAMP. Imported cholesterol is metabolised to pregnenolone by the cytochrome P450 side chain cleavage (CYP11A1). Pregnenolone is subsequently metabolized in the smooth endoplasmic reticulum by a series of enzymes (CYP17, 3ß- hydroxysteroid dehydrogenase and 17 hydroxysteroid dehydrogenase to form testosterone (Papadopoulos, 2010).

Besides increasing lutenising hormone and serum testosterone levels, DPP also increased significantly the sperm count and motility. Sexual desire was also significantly enhanced. In line with this, wives of treated men got pregnant during the treatment period (Marbeen et al., 2005). This result is similar to what has been reported by this thesis; exposure of male rats to DPP 120 produced a significant increase in the number of embryos in untreated female rats compared to the untreated group.

In the present study, increased testosterone and LH levels reflect the positive influence of DPP on the pituitary gland; which promotes increased LH release and on Leydig cell and enhances 3β-hydroxysteriod dehydrogenase (3β-HSD) and 17β-hydroxysteriod dehydrogenase (17β-HSD) activities. Besides this, DPP contain Zinc, an element that plays a very crucial role in the synthesis of testosterone (El-Kashlan et al., 2015). We can also possibly say that DPP using its antioxidant properties potentiated leydig cells to up – regulate the production of testosterone by protecting Leydig cells and sertoli cells against apoptosis and DNA damage (Bahmanpour et al., 2006).

In addition to this, the palmitic acid and stearic acid present in DPP inhibited 5 alpha reductase enzyme. Because of this, less testosterone was converted to dihydrotestesterone, thereby subsequently increasing testosterone levels (Bahmanpour et al., 2006).

Further to this, DPP has antioxidant properties which might have protected leydig cells, sertoli cells, against apoptosis and DNA damage. In addition to this, it is also possible that estrogen content of DPP may have also have contributed to the up-regulation of testosterone and LH (Bahmanpour et al., 2006).

In this thesis, we report for the first time that RS has positive effects on male sexual behavior and fertility. In respect of this, RS enhanced the mount frequency and number of embryos in untreated females. Besides this, there was a slight decrease in the time taken to reach the female and also in mount latency.

9.5 *In vivo* Effects of *Phoenix dactylifera, Nigella sativa, Myristica fragrans* and on the female fertility and reproductive performance

Based on the results, it can be concluded that quantal pregnancy, implantation index and fertility index was increased by the higher concentrations of black seed, nutmeg and date palm pollen. In contrast, pre-implantation loss and post implantation loss were decreased in all treated groups compared to the control group. Also, the exposure of female rats to BS (400 mg/kg) and DPP (240 mg/kg) produced a significant increase in the number of embryos.

Similar to our finding, a previous study showed that female rats treated by *Nigella sativa* increased the number of implantations and the number of viable foetuses. The *Nigella sativa* also enhanced the number and the diameter of Graffian follicles (Mukhallad et al., 2009).

In addition to this, a previous study done by Gabr et al. (2014) reported an improvement in the percentage of mating success, fertility success and male fertility

index in male rats medicated with cisplatin and DPP (400 mg/kg) compared to cisplatin-control rats. In addition, implantation and embryo development was ameliorated by supplementing the culture medium with thymoquinone. Thymoquinone, a main component of *Nigella sativa*, when added to a culture media increased the developmental competency and improved the resistance of cultured mouse embryos to ROS (Saheera et al., 2014).

9.6 *In vitro* effect of *Linum usitatissimum, Raphanus sativus* and *Phoenix dactylifera* on the TM3 Leydig cells

This thesis would like to report that it is for the first time that FS, RS and DPP extracts have been used on TM3 Leydig cells. To begin with, mouse BALB/c testis is the prime source for TM3 Leydig cell line. These cells are found in the interstitial spaces of the seminiferous tubules and are responsible for the production and secretion of androgens (Shen et al., 2012). In the present study, MTT assay was used to determine the cytotoxic effects of *Linum usitatissimum, Raphanus sativus* and *Phoenix dactylifera* on TM3 Leydig cells in a concentration- and time dependent manner.

In this thesis, lower concentrations of *Linum usitatissimum* extract promoted mitochondrial dehydrogenase activity of TM3 Leydig cells in the first 24hrs. This cell viability was inhibited after 48 hrs and subsequently recovered after 72hrs.

The increased mitochondrial activity, which indicates cell viability, demonstrated by TM3 Leydig cells at 24 hrs has been attributed to the higher energy-consuming activities cells display before death. Moorghen et al. (1998) reported that the observed increased mitochondrial dehydrogenase activity is a mechanism that is adopted by cells to compensate for an increased apoptotic rate.

In this thesis (Table 9.5), TM3 Leydig cell exposure to date palm pollen for 24 hrs promoted mitochondrial dehydrogenase activity, however, at 48hrs the cell viability appeared to be inhibited and subsequently the cells recovered after being exposed

for 72 hours. In support of this observation, is a previous study in which date palm pollen, *Phoenix dactylifera* was observed not to have any cytotoxic effects on spermatogonial stem cells and Sertoli cells (Mahaldashtian et al., 2015). The non-cytotoxic effects demonstrated by DPP on TM3 Leydig cells has been attributed to the ability of DPP to block oxidative free radicals, prevent DNA damage, and neutralize inflammatory reactions (El-Kashlan et al., 2015). In line with this, a study done (Hassan et al., 2012) reported the protective effects of DPP against testicular damage caused by cadmium toxicity in male rats. Besides this, another previous study, revealed the preventive effects DPP possesses against adverse side effects of chemotherapy induced infertility in male subjects. In respect of this, *Phoenix dactylifera* pollen has been reported to contain various types of phytochemicals and nutrients such as carotenoids, flavonoids and phytosterols which are responsible for prevention of oxidative free radicals, DNA damage and neutralization of inflammatory reactions (Yasir et al., 2014). In addition to this, the above mentioned protective effects of DPP against cytotoxicity may be attributed to the antioxidant action of the extract through direct scavenging of ROS or interfering with free radical generation (Abdollahi et al., 2015).

In order to determine the cytotoxic effects of *Linum usitatissimum, Raphanus sativus* and *Phoenix dactylifera* on TM3 Leydig cells, the morphological appearance of TM3 Leydig cells after exposure to RS, FS and DPP extract for 72h was studied by light microscopy with Haematoxylin and Eosin staining. Photographs of cells treated with lower concentrations of RS and FS showed normal dividing TM3 Leydig cells with a cell density that was comparable to the untreated cells. However, at higher concentrations the cell density appeared to decrease in a concentration and time-dependent manner showing that FS and RS were cytotoxic to TM3 Leydig cells at higher concentration. In a similar manner, both low and higher concentrations of DPP did not show any changes in TM3 Leydig cell density. All the photographs showed cell numbers and morphology that were comparable to the untreated tissues.

Table 9-5: *In vitro* effect of *Linum usitatissimum, Raphanus sativus* and *Phoenix dactylifera* in the TM3 Leydig cells

Plant extract	Activity of mitochondrial dehydrogenase			Apoptotic	Testosterone secretion
	24H	48H	72H		
RS 50 µg/ml	↓	↓	↓	ND	ND
RS 100 µg/ml	↓	↓	↓	=	ND
RS 500 µg/ml	↓	↓	↓	ND	↘
RS 1000 µg/ml	↓	↓	↓	↑	=
FS 50 µg/ml	↑	↓	=	ND	ND
FS 100 µg/ml	↑	↓	=	↗	ND
FS 500 µg/ml	↓	↓	=	ND	↑
FS 1000 µg/ml	↓	=	↑	↑	↘
DPP 50 µg/ml	↑	↓	↑	ND	ND
DPP 100 µg/ml	↑	↓	↑	↘	ND
DPP 500 µg/ml	↗	↓	=	ND	↑
DPP 1000 µg/ml	↗	↘	↗	↗	↘

Abbreviations: RS, Radish seed; FS, Flaxseed; DPP, Date palm pollen; ↑, significant increase ↓, significant decreased; ↘, non- significant decreased, ↗, non- significant increase; = , equals to control; ND, not determined. Apoptotic = Externalisation of phosphatidylserine plus binding of annexin V.

Flow cytometry studies were done to assess the apoptotic effect of 1000 µg/ml DPP on TM 3 cells following 72 hour incubation. In this assay annexin V was used to quantify the number of cells whose membranes might have undergone through flip-flop process in which the phosphatidylserine (PS) is translocated to the outer part

of the cell membrane. The exposed PS attracts the annexin V and cells loaded with this labelled fluorophore were read on FL1 (530BP) and FL2 (585BP) channels. In this thesis, lower concentrations of DPP (100 µg/ml) promoted no significant decrease in the percentage of apoptotic whereas higher concentrations of DPP (1000 µg/ml) induced no significant increased percentage of apoptotic cells compared to the negative control. These results agree with the MTT assay done in this thesis, which showed that DPP has no cytotoxic effects on TM3 Leydig cells. However, previous studies have described the Fas ligand (FasL or CD95L) death pathway as a possible regulator of germ cell apoptosis in the rat testis especially under stressful conditions such as hormone deprivation (Rizk et al., 2014). Further to this, apoptosis in rat TM3 Leydig cells is characterized by over expression of pro-apoptotic markers such as caspase-3 and Fas-L. In agreement with this, co-administration of DPP extracts with L-thyroxine (L-T4) or propylthiouracil (PTU), reduced the induced over-expression of pro-apoptotic markers to normal level (El-Kashlan et al., 2015).

The process of apoptosis is regulated by the expression of several proteins. Two important proteins involved in apoptotic cell death are members of the bcl-2 family and a class of cysteine proteases known as caspases. The bcl-2 family can be classified into two functionally distinct groups: anti-apoptotic and pro-apoptotic. Bcl-2, an anti-apoptotic protein prevent cell death while bax, a pro-apoptotic protein promote cell death (Kim et al., 2005). It is being suggested that the flaxseed and radish seed used in this thesis, might have contributed to the potentiation of pro-apoptotic proteins whilst at the same time inhibiting the anti-apoptotic members of the bcl-2 family of TM3 Leydig cells. Further to this, DPP might have promoted the activation of executors of apoptosis; caspase-3 a member of the caspase family responsible for the proteolytic cleavage of many proteins. This can be a possible mechanism by which FS and RS might have induce apoptosis in TM3 Leydig cells.

In this thesis, incubation of radish seeds with TM 3 Leydig cells for 24, 48 or 72 hours caused a drop in cell viability of TM3 leydig cells. This effect was observed in three separate experiments. This indicated the reduced ability of TM3 Leydig

cells to reduce tetrazolium salt to formazin and it thus reflect a decrease in the viability of TM3 Leydig cells. This effect is unusual for plant extracts with antioxidant properties. Although in this thesis radish seed were cytotoxic to TM3 Leydig cells; a previous study by Hassan et al. (2011) demonstrated the ability of radish seed to reverse the genotoxicity and cytotoxicity induced by benzo (a) pyrene (BaP) in rat testes. However, *in vivo* results observed an increase in spermatogenesis and vitality. This *in vivo* result contradicts the *in vitro* result obtained in this thesis indicating that RS is cytotoxic to TM3 Leydig cells. Furthermore previous studies, reported that, rats treated with radish oils demonstrated significantly reduced genotoxic effects of BaP by decreasing the number of DNA strand breaks and the level of 8-hydroxy-2' -deoxyguanosine (8-OHdG) in germ cells (testis and sperm). Furthermore, the protective role of radish may be due to the higher content of isothiocyanate, kaempherol glycosides and tryptophan compounds which have the ability to scavenge free radical and enhance the DNA repair system or DNA synthesis. Besides this, Ben Salah-Abbès et al. (2009) reported that isothiocyanates in radish can increase the antioxidant status and lower oxidative damage and free radical generation.

In this thesis, apoptotic analysis of TM3 Leydig cells using flow cytometry following treatment with RS showed an enhanced significant increase in the number of apoptotic cells at 1000 μg/ml radish seed. In a previous study, *Raphanus sativus* root extract also induced cell death both in p53 proficient and p53 deficient cell lines through induction of apoptotic signaling pathway regardless of the p53 status of cells. The molecular mechanisms underlying, *Raphanus sativus* induced apoptosis may involve interactions among Bcl_2 family genes, as evidenced by up-regulation of pro-apoptotic genes and down-regulation of anti-apoptotic genes along with activation of Caspase-3 (Beevi et al., 2010).

In this thesis, lower concentration of DPP and FS promoted cell viability of TM3 Leydig cells. The protective effect of these plant extracts came probably from their ability to scavenge free radicals. However, RS proved to be cytotoxic.

9.7 *In vivo* and *ex vivo* effects of *Linum usitatissimum*, *Raphanus sativus* and *Phoenix dactylifera* on gonadotrophic hormones

The results of the present thesis showed that serum testosterone level increases significantly in date palm pollen and radish seed treated rats as compared with untreated rats. This thesis also reports that, treatment of rats with FS results in a significant decrease in serum testosterone levels. So far no literature has reported the effect of FS and RS on TM3 Leydig cells. However, reports from a previous study, indicated that L-Carnitine (LC) promotes an increased production of serum testosterone, FSH and LH in male moshtohor rabbits. Briefly, carnitine is a vitamin like and amino acid like substances which in a synthetic form exists as a D or L isomer. L-carnitine (LC) is the isomer which is physiologically active. In this regard, it promotes lipid oxidation by transporting long fatty acid chains into the mitochondria where they undergo β-oxidation (Bieber, 1988). The β-oxidation of L carnitine results in the production of metabolic energy in the form of ATP. Further to this, studies have shown the presence of abundant smooth endoplasmic reticulum, golgi complexes and numerous mitochondria in the cytoplasm of the Leydig cells (Bhat et al., 2010). Further to this, most of the enzymes involved in the synthesis of testosterone are located in the smooth endoplasmic reticulum and mitochondria of interstitial cells (Austin and Short, 1985). The increased availability of these structures together with increased amount of energy (ATP) and enzymes involved in testosterone synthesis result in increased testosterone and steroidal hormone levels in blood stream (Abdel-Hamed et al., 2014). In the present thesis, *in vivo* exposure of TM3 Leydig cells to DPP and RS seem to promote increased ATP production through lipid oxidation. DPP and RS might have facilitated the transportation of long fatty acid chains into mitochondria where they are involved in energy generation processes. At the same time DPP and RS might have promoted the removal of short-chain and medium-chain fatty acids from mitochondria that accumulate as a result of normal and abnormal metabolism.

FS have been reported to contain phytoestrogens such as lignans and secoisolariciresinol Diglycoside (SDG) (Saggar et al., 2010).

Reports from a previous short-term study on the effects of dietary phytoestrogens on regulatory behaviours, prostate weight, prostate 5alpha-reductase enzyme activity, reproductive hormone levels, and testicular steroidogenic acute regulatory peptide (StAR) levels in adult Sprague-Dawley rats indicated that animals fed on either a phytoestrogen-rich diet containing approximately 600 µg/g isoflavones (as determined by HPLC) or a phytoestrogen-free diet for 5 weeks results in a decrease in body and prostate weights. However, there was no significant change in prostate 5alpha-reductase enzyme activity. Further to this, plasma testosterone and androstenedione levels significantly decrease in treated rats compared to the untreated groups. However, there were no significant differences in plasma LH or estradiol levels between the diet groups. Testicular StAR levels were not significantly different between the treated groups compared to the untreated. These results showed that dietary phytoestrogens induce high plasma isoflavone levels over a relatively short period and significantly alter body and prostate weight and plasma androgen hormone levels without affecting gonadotropin or testicular StAR levels. In short this study showed that phytoestrogens have protective effects against benign prostatic hyperplasia and prostate cancer. The phytoestrogens are able to do this through male reproductive endocrinology where they are able to mimic mammalian estrogens (Weber et al., 2001).

The *Nigella sativa* oil treatment led to significant increase in LH and FSH levels which may be due to the direct effect of oil on hypothalamus which in turn increases Gonadotropic Releasing Hormone (GnRH). Furthermore, fatty acids can stimulate GnRH-dependent pathways that initiate changes in gonads function. The positive increased effect of estrogen and progesterone concentration in treated groups is maybe attributed to the contents of the *Nigella sativa* oil especially thymoquinone that enter in building of cholesterol which is important source of cholesterol esters that may have a role in estrogen and progesterone synthesis (Farooq and Ayfaa, 2011). The increase in testosterone level in treated groups may be due to the effects of Ns oil to stimulate the activity of 17ß-hydroxysteriod dehydrogenase the most

important key enzyme in the testosterone synthesis pathway (Gromadzka-Ostrowska et al., 2002).

In the present thesis, DPP and RS increased serum testosterone levels both *in vivo* and testis slices. At the same time FS stimulated increased testosterone levels in testis slices. These plant extracts seem to possess the ability to stimulate the activity of 17ß-hydroxysteriod dehydrogenase, a factor that results in increased testosterone synthesis. In another study, exposure of male rats to 10% flaxseed resulted in an increase in serum testosterone and estradiol levels besides higher relative sex organ weights and prostate cell proliferation. In a similar manner, lifetime exposure to 5% flaxseed decreased the adult relative prostate weight and cell proliferation. Whilst flaxseed protected against prostatic disease, it did not affect the sex hormone levels. However, its effects on reproduction were depended on the dose and timing of exposure (Tou et al., 1999).

In men, the source of estrogens is mainly from testosterone conversion to estradiol catalyzed by the enzyme aromatase. It is proven that the flaxseed lignans competitively inhibit aromatase enzyme (Brooks and Thompson, 2005). 10 % dietary flaxseed increased serum testosterone and estradiol levels and gave higher relative weights of the accessory sex gland, all prostate lobes, the seminal vesicle, and the testes (Collins et al., 2003). However, a study reported that prenatally and/or postnatally exposure of rats to a flaxseed/flax meal decreased the prostatic weight, and increased the serum LH and cauda epididymal sperm counts in the rat (Sprando et al., 2000).

DPP was reported to have gonad-stimulating activities (Soliman and Soliman, 1958), mainly due to the presence of esterone (Dostal et al., 1996). In respect of this, DPP has been reported to act primarily on the testes, although, its effect on the hypothalmis-pituitary axis could not be ruled out (Abedi et al., 2012).

In addition the number of interstitial cells (Leydig's cells) was also increased by DPP administration as well as the blood level of estradiol and testosterone. Estradiol

and testosterone are found also at high concentrations in rat testis and seminal fluids after administration of DPP (Bahmanpour et al., 2006). Three different cell types, Sertoli, Leydig and germ cells are found to be responsible on estrogen synthesis in the male reproductive tract (Kostyuk et al., 2004). DPP contains gonadotropin stimulating substances and steroid precursors which could enhance testosterone production. For this reason, DPP supplemented to animal mash enhance growth besides increasing the plasma testosterone level (Ali et al., 1999).

In a study done by Kobeasy et al. (2015) a significant decrease in the level of serum testosterone in methomyl-toxicated rats **was** reported. However, treatment of the rats with a combination of DDP and carbofuran significantly increased the testosterone level in serum of the treated rats. This far, DPP extracts have been reported to contain estrogenic compounds and oestrones–gonad stimulating compound that can improve male infertility. These components can increase sexual behaviour and stimulate endogenous testosterone levels by raising the level of luteinizing hormones (LH). In respect of this, date palm seed powder is also used in some traditional medicines to improve male infertility. Basically, DPP has been reported to possess antioxidant characteristic and estrogenic properties, which are important in solving the infertility problems in males which come about because of low concentration of estradiol or elevated level of ROS (Hassan et al., 2012).

9.8 Effect of *Nigella sativa, Myristica fragrans* and *Phoenix dactylifera* on the MCF7 breast cancer cells

In this Thesis, ethanolic extracts of *Nigella sativa, Myristica fragrans* and *Phoenix dactylifera* reduced cell viability of MCF-7 cells following 72h exposure.

In this Thesis (Table 9.6), MTT proliferation assay showed that ethanolic extracts of nutmeg was cytotoxic to MCF-7 cells when exposed for 72h. In a previous study, the addition of various concentrations of essential oil of *Myristica fragrans* in the MCF-7 breast cancer cell line also inhibited cell growth (Helen et al., 2011) In addition to this, Vidhya and Niranjali Devaraj (2011) investigated eugenol, a main

compound of nutmeg for its anticancer effect in human breast cancer cells (MCF-7). From the study eugenol was reported to inhibit the MCF-7 breast cancer cell growth both in concentration and time dependent manner. The cytotoxic activity of the nutmeg essential oil has been suggested to come from the presence of potential anti-cancer compounds such as myristicin, limonene, eugenol and terpinen- 4-ol.

Quantification of apoptotic cells using flow cytometry and annexin V showed a 15% increase in the percentage of MCF-7 apoptotic cells after treatment with NM (1000 mg/kg) for 72 hours compared to the untreated cells. In a previous study, eugenol-treated MCF 7 cells showed cell shrinkage, membrane blebbing and apoptotic body formation; the cardinal points of apoptotic death of MCF-7 cells (Helen et al., 2011).

In a previous study, thymoquinone (TQ), a main component of *Nigella sativa* was observed to inhibit the growth of human chronic myeloid leukemia cells KBM-5. This inhibited growth of cancer cells has been reported to occur through inactivation of the tumor-necrosis factor-kappa beta (NF-κB) (Sethi et al., 2008). In respect of this, thymoquinone, has been implicated for the *Nigella sativa* seeds anti-cancer effects. A report by Mbarek et al. (2007) collaborate the above observation to the extent that *Nigella sativa* seed extracts and its active compounds thymoquinone (TQ) and dithymoquinone have demonstrated cytotoxic activity against several types of tumors besides preventing tumor growth in mice. Further to this, *Nigella sativa* has been observed to induce telomere attrition and apoptosis (Gurung et al., 2010). Mbarek et al. (2007) also reports the presence of terpenes in *Nigella sativa*. The ethyl acetate extract has also been shown to possess anti-cancer effects (Mbarek et al., 200). In this Thesis, 1000 µg/ml of *Nigella sativa* induced an enhanced rate of apoptotic death. Whilst the mechanisms by which *Nigella sativa* induces apoptotic deaths in MCF-7 cells have not yet been established, reports indicate that TQ triggered apoptotic cell death in human colorectal cells through the induction of G1 phase arrest of the cell cycle (Arafa et al., 2011). Further to this, the induction of apoptosis by TQ was associated with an increase in mRNA expression of the p53 target gene, p21WAF1, and a significant inhibition of anti-

apoptotic Bcl-2 protein (Arafa et al., 2011). The mechanism of apoptotic deaths explained above might also be the mechanisms by which the *Nigella sativa* has induced apoptotic deaths in this thesis.

In this Thesis, lower concentrations of BS and NM extracts promoted proliferation MCF7 breast cancer cell whereas higher concentrations were cytotoxic to MCF7 breast cancer cells. In a previous study, Paruthiyil et al. (2004) reported that estradiol increases cell proliferation and causes tumor formation of MCF-7 cells expressing only ER alpha. In the present thesis it seems that lower concentrations of NM and BS seem to potentiate the ER alpha hence the observed increase in cell proliferation. However, higher concentrations of the two plant extracts, BS and NM proved to be cytotoxic. In a previous study, the presence of ER beta in MCF-7 cells inhibited cell proliferation *in vitro* and prevented tumor formation in a mouse xenograft model in response to estradiol. The main active compound of nutmug, eugenol has been reported to inhibit proliferation of different tumor cell lines in a dose and time dependent manner (Piaru et al., 2012).

The cytotoxic effects have been reported to occur because of the presence of the ER beta. In line with the present thesis, higher concentrations of BS and NM inhibited cell proliferation of the MCF7 breast cancer cells. The above result makes us suggest that NM and BS are responsible for the activation of the ER beta. Further to this, the ER beta receptor has been reported to inhibit cell proliferation via induction of G (2) cell cycle arrest. Basically, ER beta inhibits c-myc, cyclin D1 and cyclin A gene transcription. This results in the up-regulation of the expression of p21 (Cip1) and p27 (Kip1).

In summary, lower concentrations of the plant extracts used in this thesis potentiated the ER alpha; thus promote cell proliferation and tumor formation. On the other hand higher concentrations potentiated the ER beta thereby inhibiting cell growth of MCF-7 cells. In other words, ER alpha and ER beta have different affinities for estrogenic compounds present in BS and NM. That is these compounds might have a higher binding affinity for ER alpha hence low concentration of

estrogenic compound can bind to ER alpha but not ER beta. When the concentration increases, those compounds can also bind to ER beta which may have a lower binding affinity. The rat estrogen receptor (ER) dissociation constant (Kd) = 0.1 nM for ER alpha protein whereas 0.4 nM for ER beta protein (Kuiper et al., 1997).

Table 9-6: Effect of *Nigella sativa, Myristica fragrans* and *Phoenix dactylifera* on the MCF7 breast cancer cells

Plant extract	Activity of mitochondrial dehydrogenase			Apoptotic	Estrogenic activity
	24H	48H	72H		
BS 10µg/ml	ND	ND	ND	ND	↑
BS 50µg/ml	↗	↘	↓	ND	↑
BS 100µg/m	=	=	↓	=	↓
BS 500µg/m	↗	=	↓	ND	↓
BS 1000µg/m	↘	↗	↓	↑	↓
NM 10µg/ml	ND	ND	ND	ND	↑
NM 50µg/ml	↓	=	=	ND	↑
NM 100µg/m	↓	=	↘	=	↓
NM 500µg/m	↘	↗	↓	ND	↓
NM 1000µg/m	↘	↗	↓	↑	↓
DPP 10µg/ml	ND	ND	ND	ND	↗
DPP 50µg/ml	↗	↗	↘	ND	↑
DPP 100µg/m	↗	↗	↘	↗	↑
DPP 500µg/m	↑	↗	=	ND	↑
DPP 1000µg/m	↑	↗	↓	↑	↑

Abbreviations: BS, Black seed; NM, Nutmeg; DPP, Date palm pollen; ↑, significant increase ↓, significant decreased; ↘, non- significant decreased, ↗, non- significant increase; = , equals to control; ND, not determined. Estrogenic activity = able to bind to estrogen receptor. Apoptotic = Externalisation of phosphatidylserine plus binding of annexin V.

From previous studies, extracts of fruits, pits and edible kernels showed improvement of vital activities and increased the hormonal concentration in rats. Phytochemical studies of DPP have reported the presence of estrone, α-amirin, triterpenoidal saponins and flavonoids. Also present are gonadotrophic substances such as estrone, estradiol and estriol (Hassan et al., 2012).

Previous work on other cell lines showed that, *dactyfera polifera* through its constituent D-glucan showed antitumor activity against the growth of Sarcoma-180 solid tumors implanted in mice. D-glucan demonstrated a dose-dependent anticancer activity with an optimum activity at a dose of 1 mg/kg in tumour. However, the exact mechanism of its action have not yet been established (Ishurd and Kennedy, 2005). Studies by Vayalil (2002) have reported that date fruit extract possess anti-mutagenic properties in the Ames mutagenicity assay. In this assay, date fruit extract demonstrated a concentration-dependent inhibition of benzo (a) pyrene-induced mutagenecity on Salmonella tester strains TA-98 and TA-100 with metabolic activation. The date components proanthocyanidins, anthocyanins, selenium, β-carotene and phenolic acids were reported to be responsible for the observed antimutagenic effects (Baliga et al., 2011).

Further to this, date palm has been reported to possess anti-mutagenic activity through its ability to inhibit phase I enzymes such as CYP450 and enhance the activity of phase II enzyme. An experimental study showed that the antigenotoxicity of date pits is due to its ability to scavenge the alkyl radical or inhibit the aromatase activity of cytochrome P-450 or blocking the reaction between methane diazonium ion and DNA (Ishurd and Kennedy, 2005).

9.9 Conclusion

This study has shown that date palm pollen, radish seed and flaxseed possess therapeutic effects that can improve male fertility. Several studies have reported the antioxidant capacities of date palm pollen, radish seed and flaxseed. This antioxidant capacity has been implicated in the scavenging of free radicals which promotes increased gonadotropin and testosterone production. Consequently, this

results in improved steroidogenesis and spermatogenesis. Further to this, these medicinal plants provide phyto-protection against reproductive toxicity, cancer and enhance sperm parameters. However, normal physiological functions require a balance between antioxidant levels and ROS.

In addition to this, the present thesis demonstrated that, flaxseed and radish seed appear to be novel therapies for a dysfunctional male reproductive system. In support of this, the medicinal plants increased sperm production, enhanced sexual behavior and libido. This was witnessed by the increased mount latency and number of latency. Further to this, flaxseed produced a sharp decrease in the time taken to reach the female besides improving the mount frequency. On the other hand, the positive effect of DPP on male libido is well known since decades hence it was used here as a positive control.

This present thesis also reports that black seed, nutmeg and date palm pollen possess medicinal properties that can enhance female reproductive functions and fertility. These medicinal plants are also responsible for nephroprotection, hepatoprotection and can possible improve a dysfunctional female reproductive system through the potentiation of gonadotrophic hormones.

Further to this, the radish seed and flaxseed seems to enhance TM3 Ledyig cells function *in vitro*. In respect of this, spermatogenesis and testosterone secretion were greatly improved. Of note, was the estrogenic activity of BS and NM extracts that promoted MCF7 breast cancer cells proliferation at low concentration but cytotoxic at higher concentration. At this point, further research needs to be done in order to assess the clinical use of medicinal plants.

9.10 Future Perspective

Further studies are still needed to clear up the fertility outcome in both the male and female, as well as the chemical investigation of the date palm pollen compounds and there effects on the male and female reproductive systems.

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APPENDICES

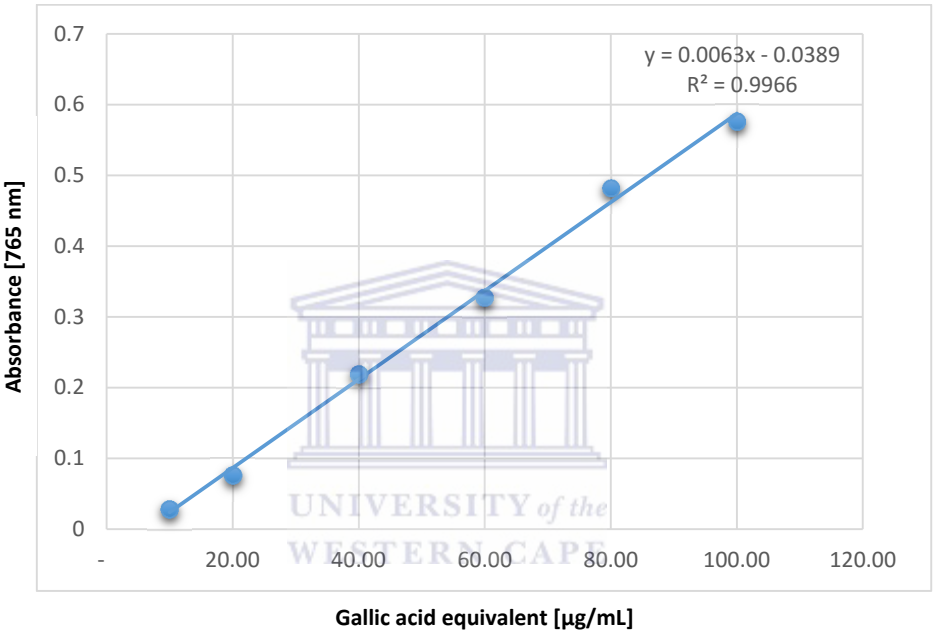
Appendix A: Gallic acid standard preparation for total polyphenol determination.

Standard solution	Volume of 0.1% Gallic acid (µL)	Volume of dH_2O	Final volume (ml)	Final dilution (µg/ml)
S1	16.7	1.65	1.67	10
S2	35	1.63	1.67	20
S3	66.7	1.6	1.67	40
S4	100	1.57	1.67	60
S5	133.3	1.53	1.67	80
S6	166.7	1.5	1.67	100

Appendix B: Preparation of Plant extracts concentrations using the suggested dilutions for total polyphenols determination.

Standard solution	Dilution	Plant extracts (µl)	Solvent (µl)	Total volume (ml)
Plant extracts	25 x	40	960	1
	45 x	22	978	1
	65 x	15.4	984.6	1

Appendix C: Standard curve for the determination of total polyphenols

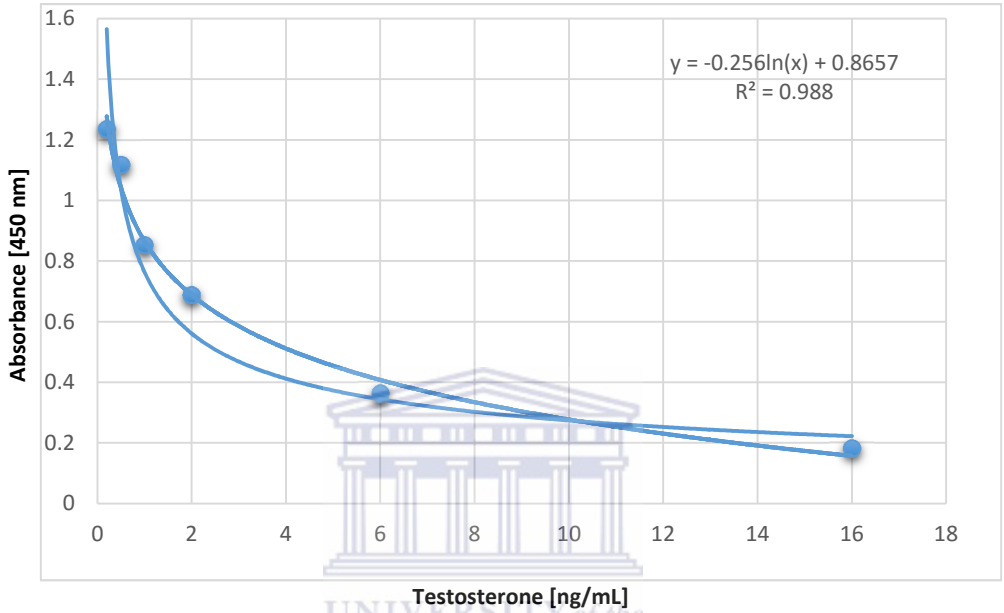


Appendix D: Quercitin standard for determination of flavonol

Test tubes	Quercitin stock (µL)	95% EtOH (µL)	Quercitin EtOH (mg/mL)	Final volume (mL)
Blank	-	1000	-	1
S1	200	800	8	1
S2	100	900	4	1
S3	50	950	2	1

S4	25	975	1	1

Appendix E: Standard curve for the determination of testosterone production



Appendix F: Definition of sperm parameters*

Parameter	Abbreviation	Definition	Unit
Total motility	-	Percentage motility of spermatozoa swimming with a VCL > 20 µm/s and VSL > 9 µm/s and the rest were regarded as immotile	%
Progressive motility	-	Percentage motility on only spermatozoa swimming in a progressively forward direction with a LIN> 30%, VAP > 20 µm/s and VSL > 30 µm/s	%
Curvilinear velocity	VCL	Time- average velocity of sperm head along its actual path	µm/s
Straight line velocity	VSL	Time- average velocity of sperm head projected along straight line between its first and last detected position	µm/s

Average path velocity	VAP	Time- average velocity of sperm head projected along its average spatial trajectory	µm/s
Linearity	LIN	Ratio of projected length to total length of curvilinear trajectory; LIN= VSL/VCL	%
Wobble	WOB	Expression of the degree of oscillation of the curvilinear path about its spatial average path; WOB = VAP/VCL	%
Amplitude of lateral head displacement	ALH	Maximum amplitude of lateral distance of the sperm head trajectory about its spatial average path	µm
Straightness	STR	Expression of the straightness of average path; STR = VSL/VAP	%
Beat cross frequency	BCF	Frequency of sperm head crossing the sperm average path in either direction	Hz

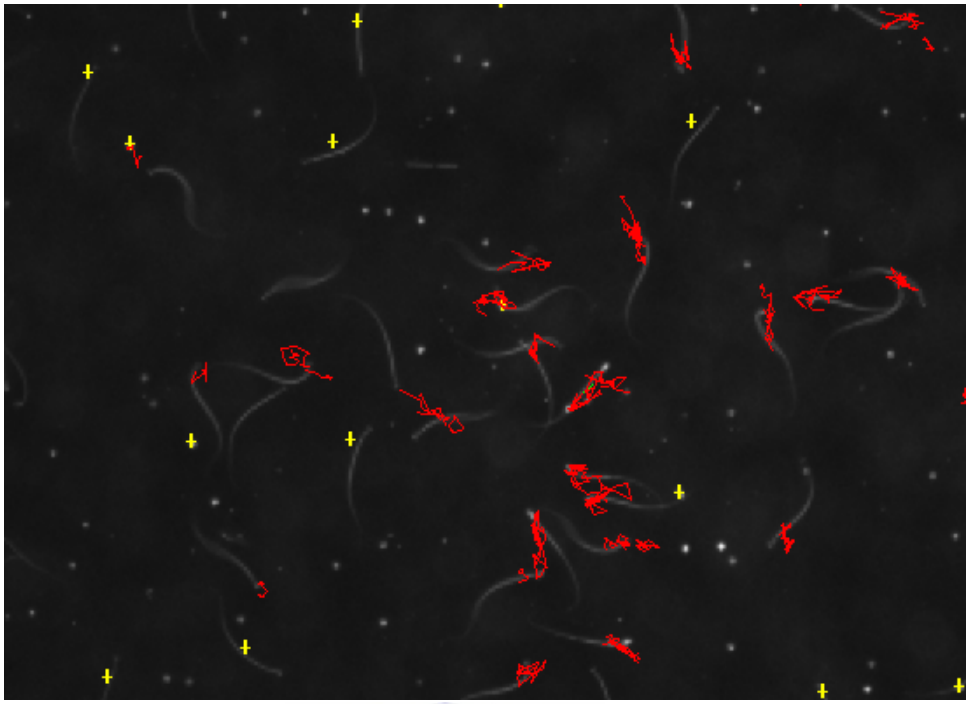
*From van der Horst et al. (1999) with slight modification.

Appendix G: Random representation of motility of rat sperm in the various treatment groups.

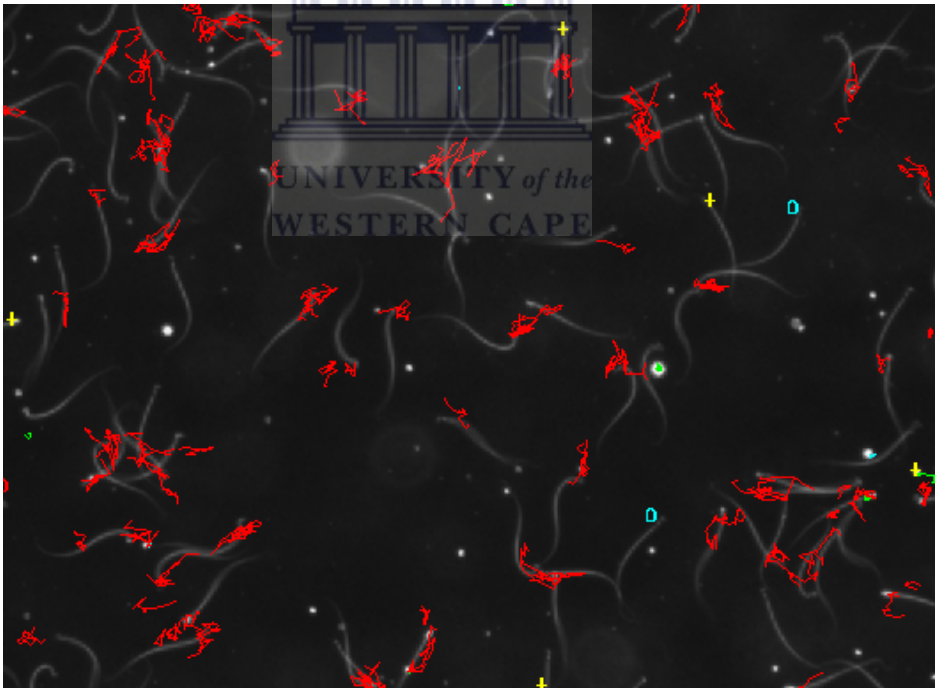
Sperm Class Analyzer (SCA) - Automatic system of sperm analysis by computer

Colour legend

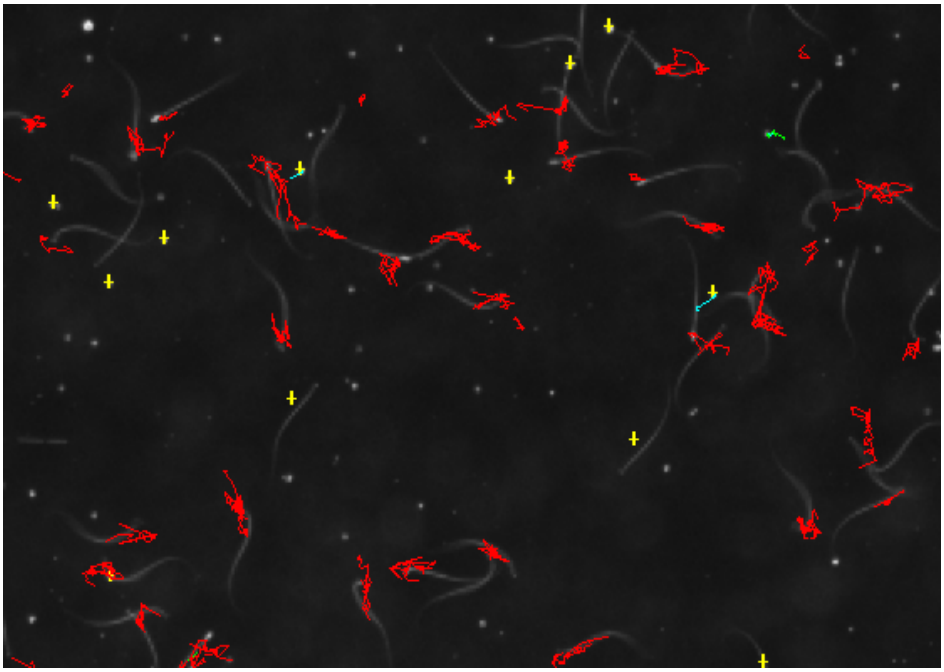
* Rapid progressive motility (type a)	* Non-progressive motility (type c)
* Slow progressive motility (type b)	* Immotile



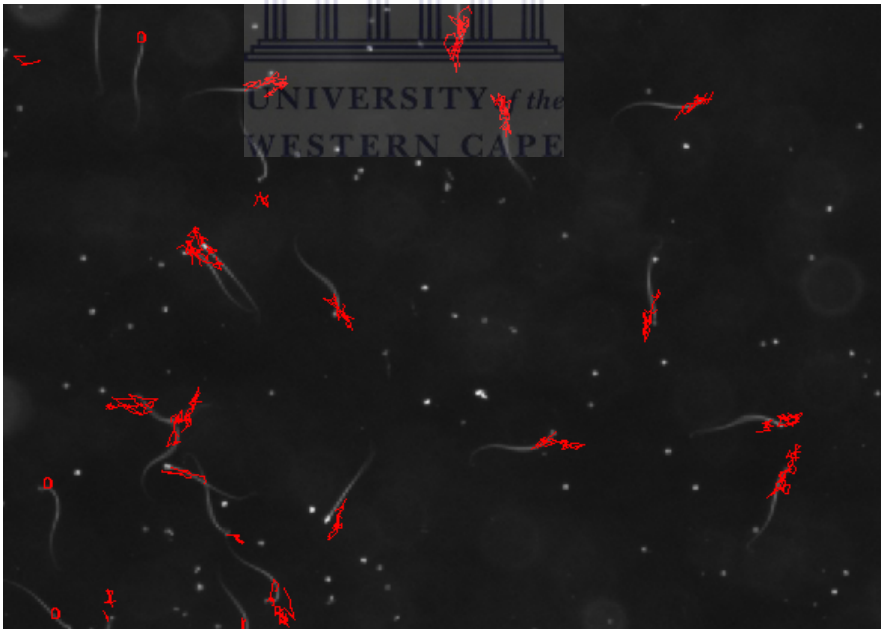
Control



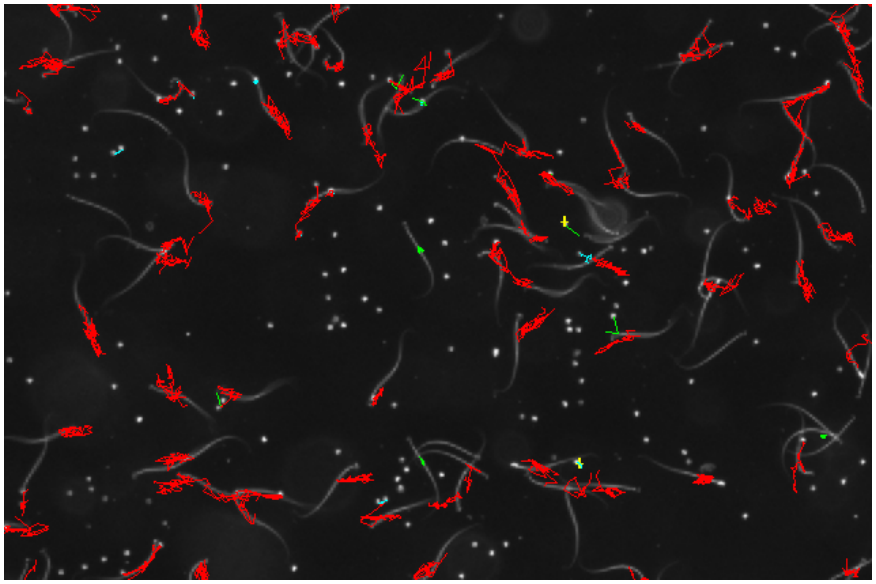
Radish seed 80 mg/kg



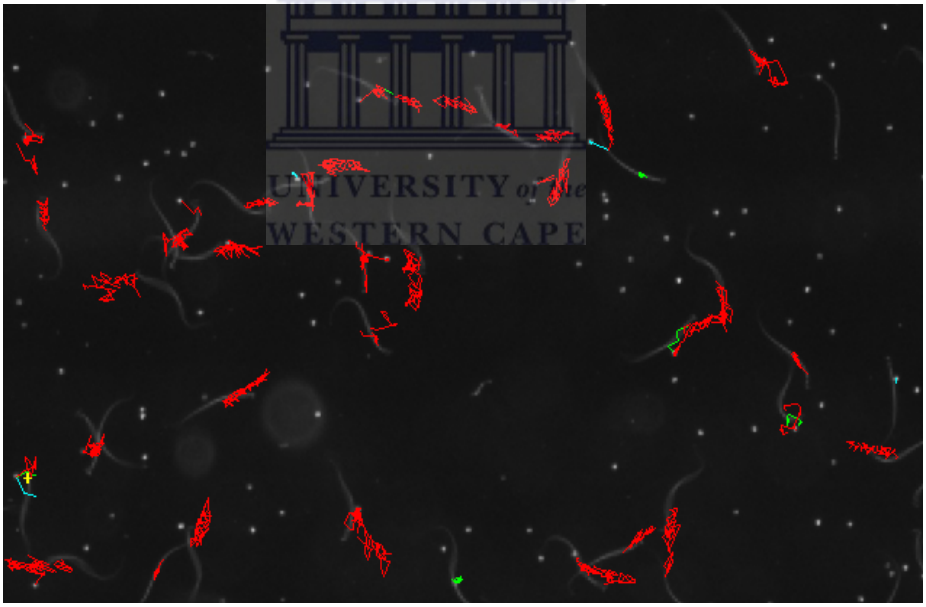
Radish seed 160 mg/kg



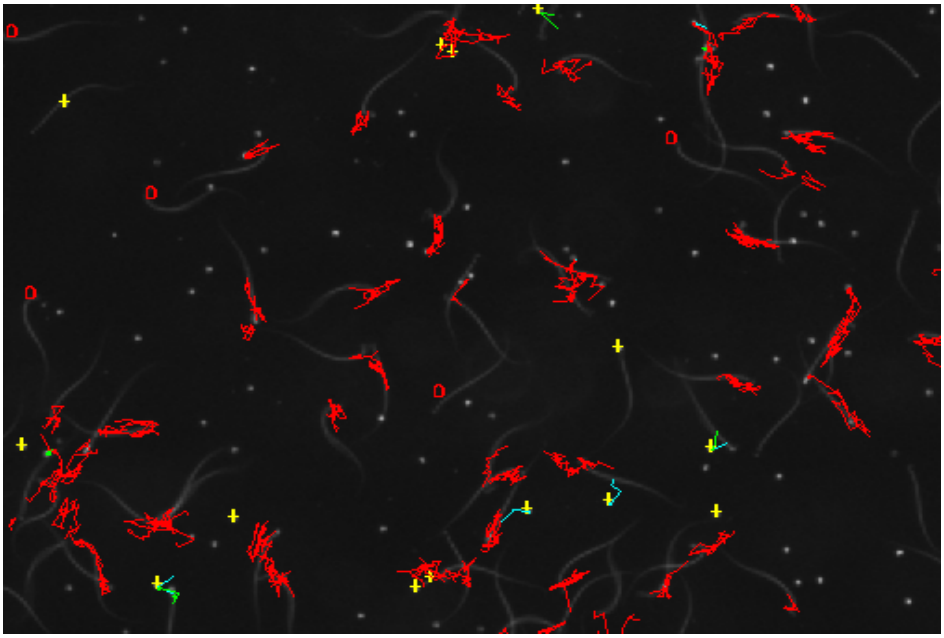
Flaxseed 200 mg/kg



Flaxseed 300 mg/kg

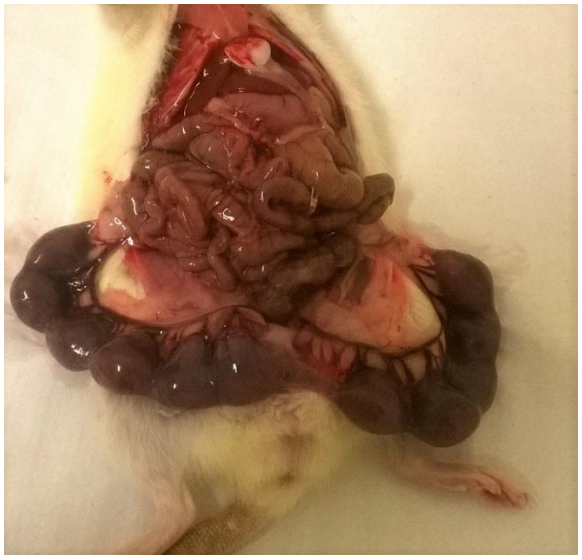


Date palm pollen 120 mg/kg



Date palm pollen 240 mg/kg

Appendix H: Dissected pregnant female rat



Appendix J: Tali® Image Cytometer