The Effect of Libyan Date Palm Pollen and Flax Seed on General and Specific Properties of Testicular and Breast Cancer Cells

By

Yasmein Omran Alshibani

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

Magister Scientiae

Department of Medical Biosciences

Faculty of Natural Science

Supervisor: **Prof. Thomas K. Monsees**

May 2016

DECLARATION

I, Yasmein Omran Alshibani, hereby declare that "**The effects of Libyan Date Palm Pollen and Flax seed on General and Specific Properties of Testicular and Breast Cancer Cell Lines**" is my own work and has not been submitted for any degree or examination in this or any other university. All the resources that I have used have been indicated and fully acknowledged by complete references.

Full name..... Date.....

Signed.....

DEDICATION

This work is dedicated to my father ***OMRAN ALSHIBANI*** and my husband ***ESAM ELMKAHIL***. Thank you both for your endless support and care that you have provided.

ACKNOWLEDGEMENTS

Thank you ALLAH, the omnipotent, to give me the strength and ability to finish this work.

Say: 'Lord, grant me an entrance of sincerity and an exit of sincerity, and give me from yours a victorious power. '. Surat Al-Esra Aya (81) ‘Quran’.

My supervisor Professor T. K. Monsees: thank you for your guidance, patience, encouragement and understanding for the different situations that I passed through.

Many thanks go to Professor David Fisher and Dr. Mervin Meyer for allowing me to work in their laboratories.

Mr. Abdallah Elgenaidi: thank you sincerely for all of the things that you helped me with.

To my mother and my mother in law: no words can explain what you have done and do for me. Your kindness and love strengthen me in every step of my way and your Doaa opened all the closed doors.

Many thanks for everyone that gave a hand during my study: Fathya Alshibani, Shireen Mentor, Mustafa Dra, Fatima Alatraq, Habeeb and Nicole.

For my sisters, thank you for your insistence to finish my study and not to quit whatever the obstacles.

PUBLICATIONS

Part of the thesis “Effect of Libyan Date Palm Pollen Extract on the Function of TM4 Sertoli Cell Line” was presented as a poster at UWC Science Faculty Research Open Day (2015).

KEY WORDS

Medicinal plants

Infertility

Date Palm Pollen (DPP)

Flax seed

TM4 Sertoli cells

MCF-7 Breast cancer cells

Cytotoxicity

E-SCREEN assay

Inhibin - B hormone

LIST OF ABBREVIATIONS

Abbreviation	Full Name
ALA	Alfa linoleic acid
ALCs	Adult Leydig cells
AMH	Antimullarian hormone
ABP	Androgen binding protein
APH	Atypical prostatic hyperplasia
ANOVA	Analysis of variance
ALA	Alfa linolenic acid
ATCC	American Type Cell Culture
BTB	Blood testis barrier
Bcl2	B-cell lymphoma 2
CO2	Carbon dioxide
CD-FBS	Charcoal dextran stripped fetal bovine serum
cAMP	Cyclic adenosine monophosphate
CYP19	Aromatase enzyme
CYP11A1	Cholesterol side chain cleavage enzyme
CREM	cAMP-responsive element modulator
DMEM	Dulbecco's Modified Eagles Medium
DMSO	Dimethylsulphoxide
DPPH	2,2-diphenyl-1-picrylhydrazyl
DNA	Deoxyribonucleic acid
DHT	Dihydrotestosterone
DPP	Date palm pollen
ERE	Estrogen receptor element
ER	Estrogen receptor
EGF	Epidermal growth factor
EDTA	Ethyl Diamine Tetra Acetic acid

EIA	Enzyme immunoassay
FACS	Fluorescence-activated cell sorting flowcytometer
FSH	Follicular stimulating hormone
FGF	Fibroblast growth factor
Fig	Figure
FBS	Fetal bovine serum
GDNF	Glial cell derived neurotrophic factor
GGT	Gamma glutamyl transpeptidase enzyme
GPER	G protein coupled estrogen receptors
GnRH	Gonadotropin hormone-releasing hormone
HPLC	High-performance liquid chromatography
HSD17B3	17β-hydroxysteroid dehydrogenase type III
IGF-I	Insulin – like growth factor I
JAMs	Junctional adhesion molecules
LH	Luteinizing hormone
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
MTP	Microtiter plate reader
mRNA	Messenger ribonucleic acid
MAT	Matairesinol
MCF-7	Human breast adenocarcinoma cell line
NGF	Nerve growth factor
OD	Optic density
PBS	Phosphate Buffered Saline
PNA	P-nitroanilide
PCR	Polymerase chain reaction
R	Ohmic resistance
SCF	Stem cell factor
SD	Standard deviation
SDG	Secoisolariciresinol
StAR	Steroidogenic acute regulatory protein

TEER	Transepithelial electrical resistance
TJs	Tight junctions
TM4	Mouse Sertoli cell line
TNFα	Tumor necrosis factor-α
WHO	World health organization
ZO-1	Zonula occludens1

TABLE OF CONTENTS

LIST OF FIGURES

ABSTRACT

The Effects of Libyan Date Palm Pollen and Flax Seed on General and Specific Properties of Testicular and Breast Cancer Cell Lines

There is increasing concern worldwide by researchers with regards to the assessing of safety and therapeutic consumption of the plants used in traditional medicine. Date palm pollen (DPP) and flax seed have been used traditionally to improve fertility in Libya. DPP extracts have shown several reproductive beneficial effects. *In vivo*, studies have revealed the ability of DPP to increase sperm concentrations, ameliorate the testicular toxicity induced by cadmium and lead, raise testosterone, as well as LH and FSH hormone levels. Flax seed phytochemical analysis showed lots of valuable components such as lignans and α linolenic acid to which were attributed its positive health effects like antitumor, antioxidant and protective effects against coronary heart diseases. Moreover, flax lignans have both estrogenic and antiestrogenic potential. This study was aimed at testing the effects of Libyan DPP and flax seed on the Sertoli (TM4) cell line and human breast adenocarcinoma (MCF - 7) cell line.

Different concentrations (0.01, 0.1, 1, 10, 100 and 1000 μg/ml) of ethanolic extracts of DPP and flax seed, respectively, were used to assess the morphology of TM4 and MCF - 7 cells after 24 and 72 hours exposure. Mitochondrial dehydrogenase activity as a marker of cell viability was measured by MTT assay after 24 and 72 hours exposure. Apoptotic effects were assessed by flow cytometeric APO percentage assay. TM4 cell production of Inhibin - B hormone and GGT enzyme activity under the effects of DPP or flax seed was determined by use of ELISA kits. Transepithelial electrical resistance (TEER) assay were used to detect the effect of DPP or flax seed on TM4 cell monolayer integrity. Finally the plants potential phytoestrogenic activity was determined by use of E - SCREEN assay in MCF – 7 breast cancer cells.

Higher concentrations of DPP significantly increased the activity of mitochondrial dehydrogenase enzyme of TM4 cells after 24 hours associated with increasing cell number as detected in a microphotograph. Flax seed concentrations less than 100 µg/ml reduced TM4 cell viability but there were no morphological changes visible after 24 hours. MCF - 7 cells viability was reduced after 24 and 72 hours treatment with DPP and flax seed. DPP concentrations beyond 1 µg/ml significantly raised the TEER of TM4 monolayer over 72 hours while flax seed treatments caused a significant increase only after 72 hours of exposure. TM4 cells GGT activity increased significantly after exposure to higher concentrations of DPP and all flax seed concentrations. Significant stimulatory effects of all the concentrations of DPP or flax seed on TM4 inhibin - B hormone productions have been detected. Apoptotic studies showed no significant changes. E - SCREEN assay resulted in significant reduction in MCF - 7 proliferation rate under the effect of low concentrations of DPP or flax seed. Higher concentrations of the plant extracts, however, stated to increase MCF – 7 cell proliferation, this exerts weak estrogenic activities.

In conclusion, the main finding of this study is that DPP and flax seed showed stimulatory effects on TM4 cells proliferation. The resistivity of TM4 cells monolayer which reflect the integrity of blood – testis barrier (BTB) was also significantly increased as well as inhibin - B production and GGT enzyme activity. In addition DPP and flax seed respectively showed inhibitory effects on MCF - 7 cells viability. This study indicated that DPP or flax seed may enhance spermatogenesis through their stimulatory action on Sertoli cells. Moreover, both plants could reduce breast cancer cells viability. However, further investigations are required to elucidate the exact mechanisms behind these obtained findings.

Chapter I: Introduction

1.1 An overview of the male reproductive system

The male reproductive system comprises the testicles, epididymis, vas deferens, penis and accessory sex glands with different locations inside and outside the abdominal cavity. Its main function is the maintenance of a continuous spermatogenesis with occasional delivery of the viable semen with a high density of sperm cells (> 60 × 10^6/mL in 3 to 5 mL of semen) to the female reproductive tract. Spermatogenesis takes place within the convoluted seminiferous tubules of the testes followed by maturation of the sperm in the epididymis to be ejaculated through the vas deferens into the urethra in conjunction with seminal fluid from the prostate gland and seminal vesicles creating a suspension of sperm cells (semen) that ejaculate through the penile urethra (Koeppen *et al*., 2010; Mawhinney and Mariotti, 2013). At puberty, the brain alerts the hypothalamus to increase the secretion of gonadotrophin releasing hormone (GnRH) which further stimulates gonadotropic hormones (follicular stimulating hormone {FSH} and luteinizing hormone {LH}) production leading to testicular maturation and increased testosterone hormone production by Leydig cells (Stanfield, 2012).

Steroidogenesis is the process of testosterone synthesis in response to LH stimulation of plasma membrane receptors of Leydig cells. This leads to a number of reactions ended by conversion of pregnenolone to testosterone (Zirkin and Chen, 2000). Testosterone has numerous functions including development of accessory sex glands (prostate and seminal vesicle) and libido. It also increases muscular volume and strength, enhances long bone growth, stimulates protein synthesis by liver, and stimulates hair growth. In testicular tissue, Sertoli cells and Leydig cells, but not the sperm cells, are shown to express androgen receptors highlighting the importance of testosterone in the spermatogenesis process (Nieschlag *et al*., 2010).

1.2 Anatomy of the testes

Testes are ovoid organs, slightly flattened, located outside the body just below the penis and housed in the scrotum hanging at the inferior end of the spermatic cord. This location maintains the testicular temperature at about 2 degrees lower than the body temperature, which is crucial for optimal sperm development. Testes are divided by a scrotal septum and each testicle measures 4 cm in length, 2.5 cm in diameter and weighs 20 - 30 g in adult male. A continuation of the abdominal peritoneum covers the external surface of the testes in form of a dual layered serous sac (tunica vaginalis) with a small volume of serous fluid filling the gap between the visceral and parietal layers.

The tunica albuginea is a strong fibrous fascia that covers the testicle and reflected posteriorly forming the mediastinum testis which contains the rete testis. Extending from the inner surface of the capsule, there are several fibrous septa running radically and dividing the testicle into 250 - 300 lobules. Within each lobule there are numbers of (1 – 4) seminiferous tubules (tubuli seminiferi) with a highly coiled course. The tubules open into a network of channels called the rete testes from which a small ductule arises to connect the rete testes with the epididymis on the superior pole of the testes. Blood supply of the testes comes from the testicular arteries, which arise from the aorta. The pampiniform plexus is an anastomosis of venous vessels formed by the veins of the testicle and the epididymis. Thereafter it forms the testicular vein. On the left side the testicular vein drains into the renal vein but on the right side it drains in the inferior vena cava (Olivetti and Grazioli, 2010; Snell, 2011).

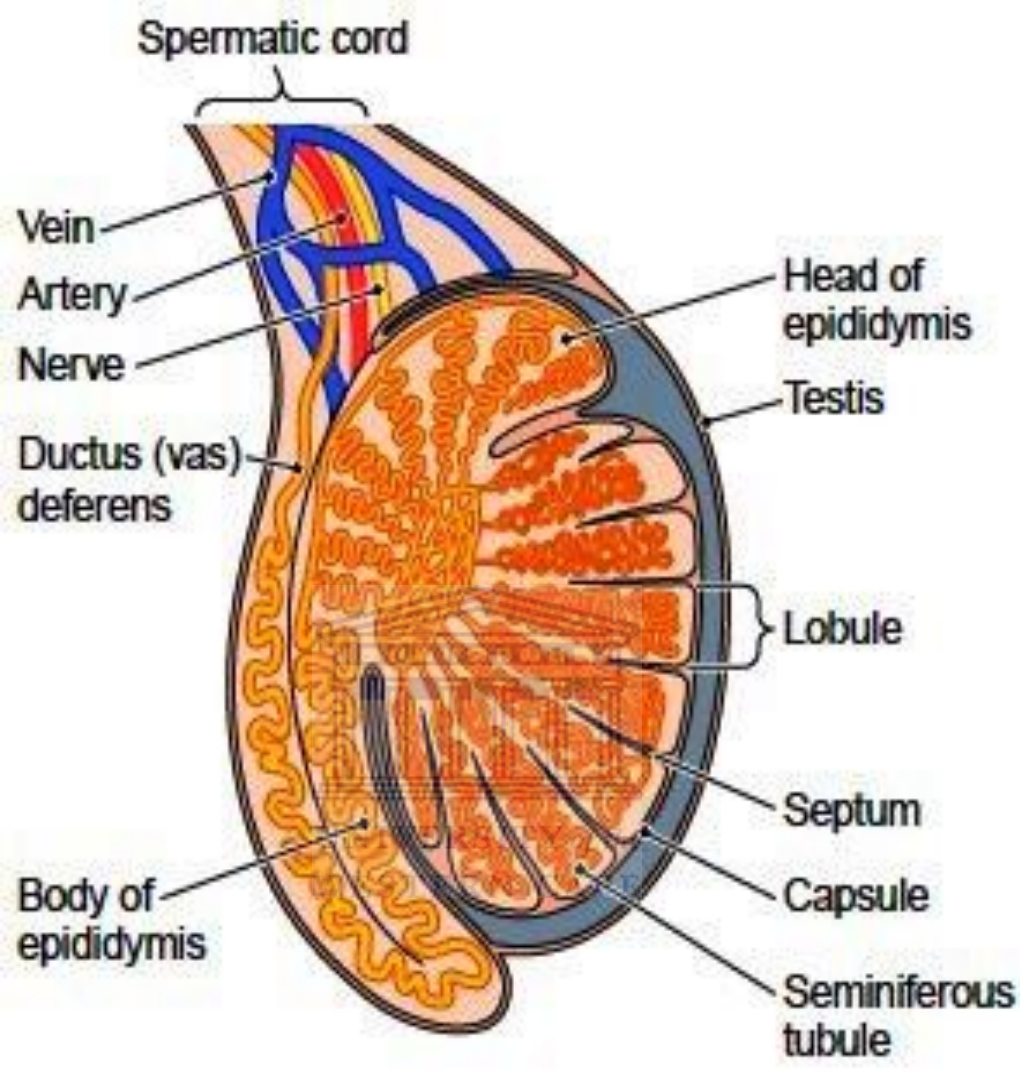

Figure 1.1: Longitudinal section of the testis showing seminiferous tubules and epididymis. (http://encyclopedia.lubopitko-bg.com/Male_Reproductive_System.html).

1.3 Histology of the testes

Two compartments can be distinguished within each lobule of the testes: Tubular compartment and peritubular compartment (interstitial compartment).

1.3.1 Seminiferous Tubules

Tubular compartment consists of the seminiferous tubules which are surrounded by connective tissue, flat myoid cells and a basement membrane. The epithelium is a complex stratified epithelium with two distinguishable cell populations: the supporting non - proliferating cells (Sertoli cells) and germ cells with different stages of maturation (Fig 1.2).

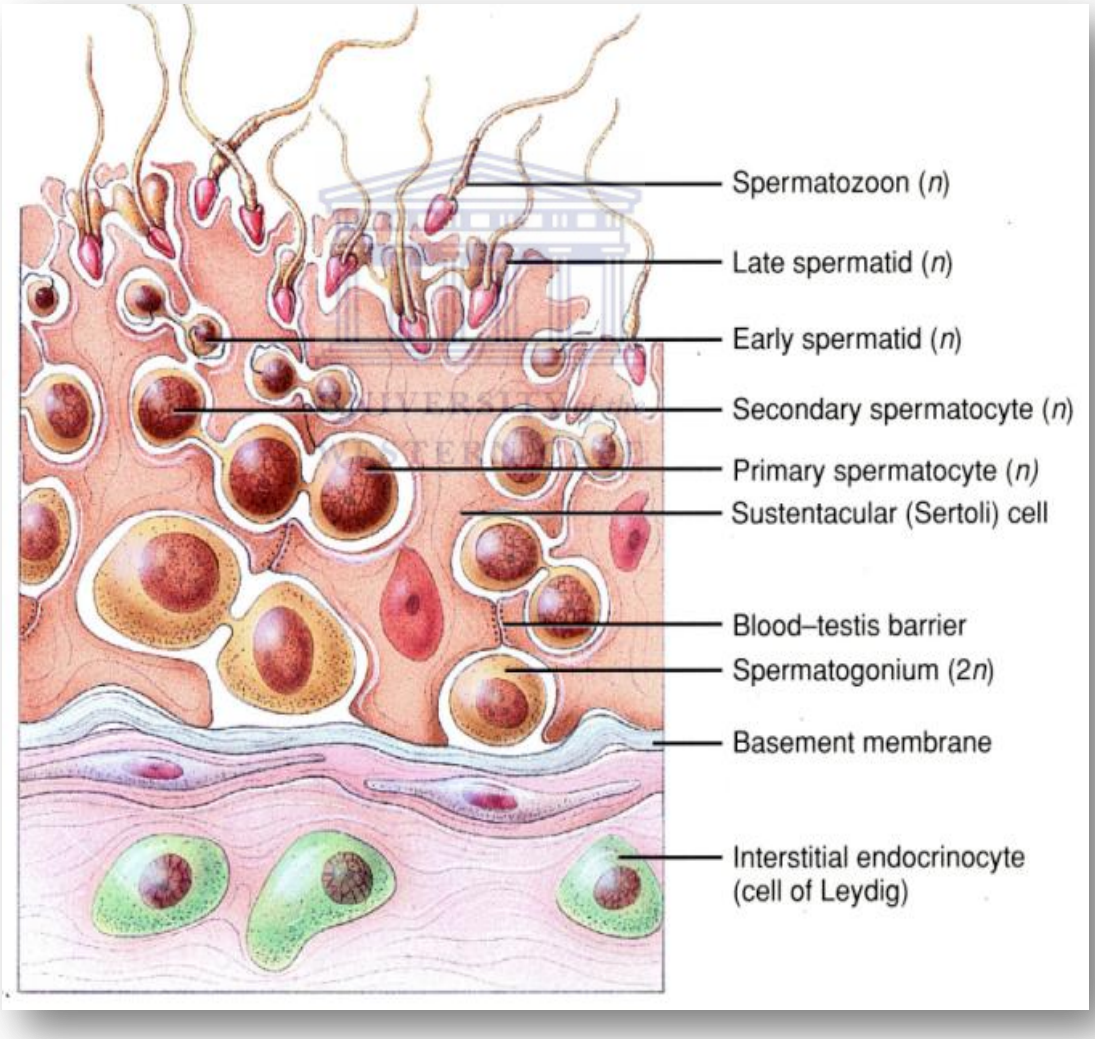

Figure 1.2: Cross section of the seminiferous tubule showing the histology of the tubules. (https://www.studyblue.com/notes/note/n/reproductive-system/deck/238286).

1.3.2 Spermatogenesis

Spermatogenesis is the developmental and differentiation process of the diploid spermatogonia cells to yield haploid spermatozoa (sperm cells). It begins at puberty and continues throughout the life (Meistrich and Hess, 2013).

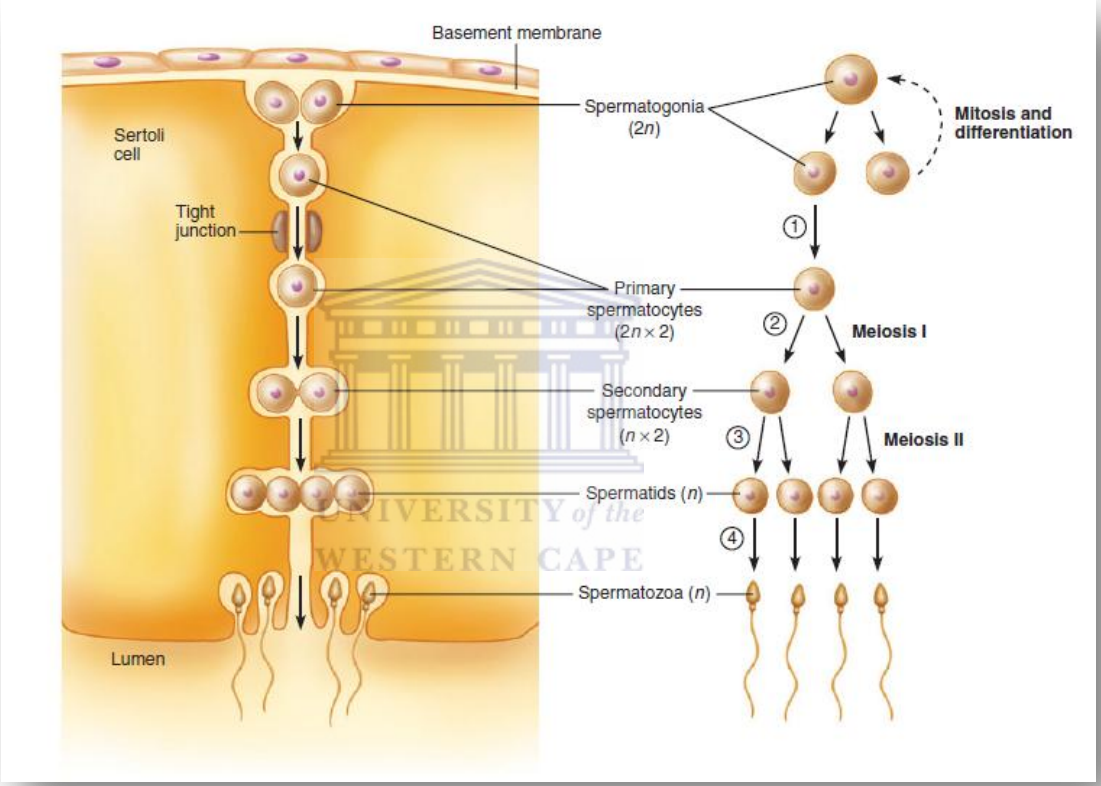

Figure 1.3: The process of development of the germ cells to spermatozoa. A single diploid (2n) primitive spermatogonium cell develops and differentiates to yield 4 haploid (n) spermatozoa (Stanfield, 2012).

Spermatogonia are the undifferentiated cells that are located at the bottom of the epithelium of the seminiferous tubules; they proliferate and differentiate in a process called spermatogoniogenesis. As they migrate up toward the luminal part they are named primary spermatocytes and start DNA synthesis yielding in 46 duplicated

chromosomes. The first meiotic division results in two secondary spermatocytes with duplicated 23 chromosomes which then further undergo the second meiotic division to yield haploid germ cells (spermatids) with one single set of 23 chromosomes. Thereafter, the spermatids are transformed to spermatozoa (sperm cells) by a process called spermiogenesis. Those are to be released from the germinal epithelium into the tubular lumen in another process named spermiation (Nieschlag *et al.*, 2010).

1.3.3 Sertoli cells

They are the essential somatic cells of the testis which supports spermatogenesis (Griswold, 1998). They were first described by Enrico Sertoli in 1865 as branched cells that related to the production of spermatozoa.

1.3.3.1 Structure of Sertoli cells

Sertoli cells are tall, columnar, polarized cells that set on the basement membrane and extend to the lumen of the seminiferous tubules with cytoplasmic expansions projected to surround various generations of the developing germ cells, forming complicated membrane associations by means of gap and tight junctions. As a result of this close contact, different names (mother cell, nurse cell and supporting cell) have been given to the Sertoli cell. The total surface area of the Sertoli cell varies between 12000 - 16000 μm^2 from which about 40 % is in close contact with the surface of the spermatids (Griswold, 2014; Skinner and Griswold, 2004).

The Sertoli cell has the ability to alter its form according to the stage of the cycle of the seminiferous epithelium, thereby two types of Sertoli cells are distinguished (Fig. 1.4), type A Sertoli cells with deep crypts of the cytoplasm formed by germ cells and type B which supports the moving elongated spermatids and have apical expansions.

The Sertoli cell nucleus has a unique appearance with membranous indentations and a prominent nucleolus. The cytoplasm has large numbers of mitochondria with the highest concentration in the body of the cell indicating the high metabolic activity of the cell. The morphological variation is a characteristic feature for Sertoli cell mitochondria which can be long, cup shaped, or even donut shaped. This characteristic can be used to distinguish Sertoli cells from the germ cells (Griswold, 2014).

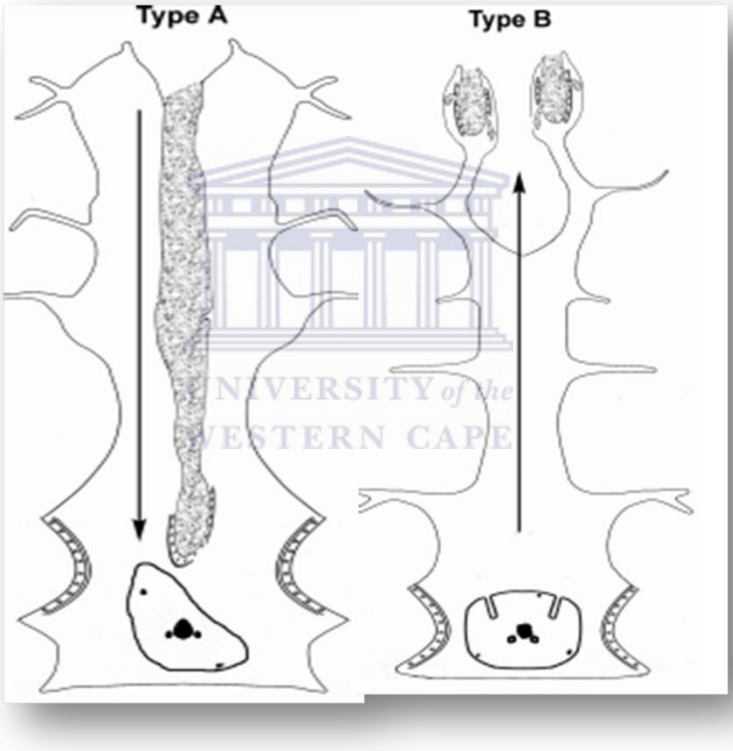

Figure 1.4: Type A and type B Sertoli cells (Griswold, 2014). Type A Sertoli cell with deep crypts of the cytoplasm formed by germ cells while type B has apical expansions which support the moving elongated spermatids.

In addition, the activity of the mitochondrial enzymes (dehydrogenase enzyme) has been used as a parameter to measure Sertoli cell viability (Monsees *et al.*, 2000).

Moreover, within the cytoplasm there are lysosomes and residual bodies scattered and about 0.3 % of testicular mass is phagocytosed daily (De Franca *et al*., 1995).

Ectoplasmic specializations are junctional structures between Sertoli - Sertoli or Sertoli - germ cells, composed of the plasma membrane of the two neighboring cells, bundles of actin filaments on the Sertoli cell cytoplasmic side situated between Sertoli cell plasma membrane and a thin cistern of smooth endoplasmic reticulum. The ectoplasmic specializations are located in two major sites, apical and basal. In basal regions they form with other junctions a belt like junction complexes between adjacent Sertoli cells (blood - testis barrier) while at the apical region they help the movement of developing spermatids towards the luminal side (Griswold, 2014; Skinner and Griswold, 2004).

1.3.3.2 Function of Sertoli cells

1. Providing structural support for sperm cells in all stages.
2. Guidance of sperm cells toward the lumen as they advance to later stages of spermatogenesis (spermiation).
3. Production and secretion of tubular fluid which maintains patency of the tubules as the blood - testis barrier prevents the reabsorption of the secreted intraluminal fluid which in turn creates a pressure that sustains the patency of the lumen. This fluid has a unique composition of a high concentration of potassium, but low sodium concentrations, bicarbonate, magnesium, chloride ions, inositol, glucose, carnitine, glycerophosphorylcholine, amino acids and several proteins (Nieschlag *et al.*, 2010).
4. Formation of blood - testis barrier (BTB): known also as Sertoli cell barrier. These are inter - Sertoli cell junctional complexes formed between adjacent Sertoli cells around the basal aspect of the seminiferous epithelium. Different forms of junctions can be seen within the BTB. These are tight junctions, adherens junctions, desmosome-like junctions, gap junctions and ectoplasmic

specialization. BTB divides the seminiferous epithelium into a basal compartment which contain the diploid spermatogonia and early stage primary spermatocytes and an adluminal compartment containing later stage primary spermatocytes and all the other stages of sperm cells (Koeppen *et al*., 2010; Tu'uhevaha *et al*., 2007). The tight junctions control the permeability throughout the barriers which prevents the bidirectional passage of molecules creating a specialized, immunologically safe environment for developing sperms. Actin, zonula occludens 1 (ZO - 1), occluding, claudin, espin, and gelsolin are proteins which are involved in the structure of the blood testes barrier. However, species variation of this structure has been observed (Skinner and Griswold, 2004). Blood testes barriers also create an apical and basal polarity within the cell by creating a boundary between the apical and basolateral domains of a cell which differ in protein and lipid composition. This leads to the creation and maintenance of epithelial (and endothelial) cell polarity (Cheng and Mruk, 2002). This polarity was maintained even when the Sertoli cells were cultured on a microporous substrate coated with an extracellular matrix with enhancement of Sertoli cell secretions (Ueda *et al*., 1988). The BTB undergoes restructuring when the preleptotene spermatocytes need to move apically during the developmental process. α Catenin and ZO - 1 proteins become disengaged and stop the physical interaction, although the disruption of protein – protein interactions within the occluding–ZO - 1 complex or the N–cadherin–α-catenin complex at the tight junction or basal ectoplasmic specializations will not show immediate disruption of the other and vice versa. Moreover, the BTB above the migrating preleptotene spermatocytes undergoes assembly and disassembly. The disassembled state provides enough time for new BTB to be assembled behind the moving spermatocytes. This prevents any disturbance of the immunological barrier during the seminiferous epithelial cycle of spermatogenesis (Cheng and Mruk, 2012). There are several theories which explain the events of disassembly/reassembly of the Sertoli cells tight junctions. To mention some;

first is the “zipper theory” which suggests that inter-Sertoli tight junctions (TJs) composed of fibrils that completely surround the basal domains of Sertoli cells, break down to adjust the passage of preleptotene or leptotene spermatocytes while new occluding zonules restructure behind the migrating preleptotene spermatocytes. Second is the “intermediate cellular compartment theory” which proposes the presence of a compartment occupied by germ cells in transit from the basal to the adluminal compartment. Third, the “stress theory” or “repetitive removal of membrane segments theory” suggests that the continuous upward migration of large numbers of germ cells creates a stress against the Sertoli cell occluding zonule. This may result in junction proliferation, changes in orientation, and disintegration in the fibrils composing the occluding zonule (Cheng and Mruk, 2002). An *in vitro* assay to assess the integrity of the tight junctions in the Sertoli cell culture models is available nowadays. This is based on the measurement of the transepithelial electrical resistance (TEER) of barrier forming cells grown on porous membranes by use of a simple device provided with two electrodes. The electrodes (positioned one on each side of the cell monolayer) provide a defined direct current voltage (U) against the cellular monolayer resulting in a current (I) which is measured and the ohmic resistance is generated (R where R = U/I) (Benson *et al*., 2013).

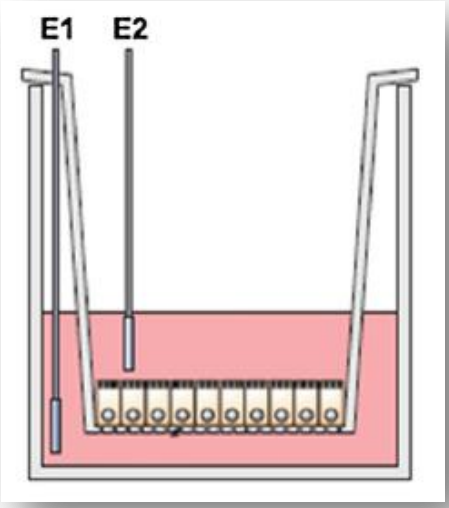

Figure 1.5 : Measurement of the TEER by use of the chopstick-like electrodes (E1,E2) (Benson *et al.*, 2013).The long electrode (E1) is placed in the well while the short electrode (E2) is placed in the filter but not touching the cell monolayer.

5. Phagocytosis: Sertoli cells are responsible for removal of residual bodies and degenerating germ cells from the seminiferous epithelium as well as engulfment of apoptotic spermatogenic cells (Shiratsuchi, 2013; Guraya, 2012). They transport the phagocytosed elements from the luminal part to more peripheral position. A Sertoli cell retains its phagocytic function even *in vitro* in cell culture (Guraya, 2012).
6. Secretory function: Sertoli cells secrete glycoproteins which can be categorized into: (A) the facilitators of the transport of ions and hormones such as androgen binding protein (ABP), transferrin and ceruloplasmin. ABP maintains a high concentration of androgen within the adluminal compartment, the lumens of the seminiferous tubules and the proximal parts of the male reproductive tracts (Koeppen *et al.*, 2010). (B) Proteases and proteases inhibitors which have a role in tissue remodeling during spermiation or movement of preleptotene spermatocytes. (C) Structural components of the basement membrane between the Sertoli and the peritubular cells.

Furthermore the Sertoli cells secrete about 60 different cytokines and hormones belonging to the transforming growth factor (TGFβ) family such as antimullarian hormone (AMH), glycoproteins, stem cell factor (SCF), glial cell line derived neurotrophic factor (GDNF) and Inhibin. The AMH plays an important role during the developmental period as it enhances the regression of the embryonic mullarian duct (Koeppen *et al.*, 2010; Giovagnoli *et al.*, 2014; Sofikitis *et al.*, 2008). Inhibin hormone is a heterodimer protein hormone related to transforming growth factor β family, composed of α and β subunits. Depending on the type of β subunit (βA or βB) inhibin - A or inhibin - B is categorized. While inhibin - B is the physiologically important form of inhibin in the male, serum inhibin - A levels are undetectable. Inhibin production is induced by follicular stimulating hormone, which then negatively feeds back on gonadotropes to inhibit follicular stimulating hormone (FSH) production. Thus, inhibin keeps FSH levels within a set point (Koeppen *et al.*, 2010; Pierik, 1998)

7. Expression of the enzyme CYP19 (aromatase) which is responsible for the conversion of testosterone to the potent estrogen estradiol -17 which enhance the spermatogenesis in humans (Koeppen *et al.*, 2010).
8. Transduction of signals from FSH and testosterone hormones as they are the only cells in the seminiferous tubules that possess the FSH receptors and testosterone receptors, representing the major targets of the ultimate hormonal signals that regulate spermatogenesis (Walker and Cheng, 2005).
9. Production of gamma glutamyl transpeptidase enzyme (GGT) which is a heterodimeric integral membrane glycoprotein present on the outer cell surface. GGT is responsible for the transfer of a glutamyl group between peptides and amino acids and is involved in the metabolism of glutathione. It is used as a marker for Sertoli cells (Caston and Sanborn, 1988; Lu and Steinberger, 1977; Palladino *et al.*, 1994).

1.3.4 Interstitial compartment

In human testes, the interstitial compartment represents about 12 – 15 % of the total testicular volume while in rodents it represents 2.6 % where 10 – 20 % of it is occupied by Leydig cells (Nieschlag *et al.*, 2010).

1.3.4.1 Leydig cells

Franz Leydig was the first one who described the Leydig cells in 1850. Adult Leydig cells (ALCs) have the ability to synthesize cholesterol *de novo*, as well as to acquire it through lipoprotein receptors, which is stored as cholesterol esters to be converted to testosterone. The synthesis of testosterone is mediated successively by steroidogenic acute regulatory protein (StAR), cholesterol side chain cleavage P450 (CYP11A1), 3β-hydroxysteroid dehydrogenase (type II in humans and type I in mice), 17α-hydroxylase/17, 20-lyase P450 (CYP17A1), and 17β-hydroxysteroid dehydrogenase type III (HSD17B3) (Mawhinney and Mariotti, 2013; Teerds and Huhtaniemi, 2015).

1.4 Hormonal control of testicular function

1.4.1 Gonadotropin hormone-releasing hormone (GnRH)

GnRH is a decapeptide which is produced by the hypothalamus and carried by the hypothalamic-hypophysial portal system to the gonadotropic cells of the anterior pituitary leading to the release of both FSH and LH. GnRH secretion occurs in pulses either every hour or once a day with a very short half - life (< 10 minutes) and is mostly retained and degraded in the pituitary gland immediately after secretion by several peptidase systems (Amory and Bremner, 2001; Nieschlag *et al.*, 2010).

Gonadal hormones can decrease gonadotropin release both by decreasing the GnRH release from the hypothalamus and by affecting the ability of the GnRH to stimulate

gonadotropin secretion from the pituitary itself. Testosterone is the main hormone which controls GnRH secretion in man; it inhibits gonadotropin secretion via negative feedback both at the hypothalamic and pituitary level. Testosterone and its metabolite dihydrotestosterone (DHT) act mainly at the hypothalamic level by decreasing the frequency of GnRH pulsatile secretion (Amory and Bremner, 2001; Nieschlag *et al*., 2010).

1.4.2 Gonadotropins

Luteinizing hormone (LH) and follicular stimulating hormone (FSH) are glycoprotein hormones produced by the pituitary gland and control the function of the gonads. Binding of LH or FSH to their receptors leads to activation of adenylate cyclase, increase in the intracellular cAMP and activation of protein kinase mediated protein phosphorylation (Amory and Bremner, 2001; Nieschlag *et al*., 2010).

LH promotes Leydig cells growth and proliferation. It binds to LH receptors on Leydig cells leading to hydrolysis of cholesterol esters and expression of steroidogenic active regulatory protein (StAR). This in turn stimulates steroidogenesis. In addition, it increases the expression of steroidogenic enzyme genes and lipoprotein receptors. Testosterone and its metabolites, dihydrotestosterone (DHT) and Estradiol-17β have a negative feedback action on LH production (Koeppen *et al.*, 2010).

FSH binds to Sertoli cells and spermatogonial membrane FSH receptors. It initiates the spermatogenesis at puberty and is also required to maintain quantitatively normal levels of spermatogenesis in adulthood. Moreover, it enhances the growth of the seminiferous tubules during development; therefore it is mainly important for determining testicular volume and size. In addition, it stimulates the production of Inhibin hormone which on the other hand feeds back negatively on the gonadotropes suppressing the FSH production (Amory and Bremner, 2001; Koeppen *et al*., 2010).

1.4.3 Steroid hormones

Testosterone is the essential local regulator of spermatogenesis with 80 fold increase of the intratesticular concentration relative to human male serum (Nieschlag *et al.*, 2010). The consequences of low intratesticular concentrations of testosterone include apoptotic death of germ cells and loss of the adhesion of the round spermatids to Sertoli cells. Bcl-2- modifying factor is involved in the process of germ cell death associated with reduced intratesticular testosterone concentrations (Sofikitis *et al.*, 2008)

Testosterone and its metabolites, DHT and estradiol-17β have a negative feedback action on the hypothalamic neurons and pituitary gonadotropes which leads to inhibition of GnRH and LH production. Moreover, they inhibit the expression of LH and GnRH receptors (Koeppen *et al.*, 2010).

Estradiol, which is formed by aromatization of testosterone either peripherally or centrally, has a direct effect on spermatogenesis regulation via estrogen receptors. Presence of estrogen receptors α (ER1); Estrogen receptors β (ER2) and G protein coupled estrogen receptors (GPER) in all testicular tissues support this finding (Chimento *et al*., 2014). Moreover, the requirement of estrogen-dependent ERα signaling during neonatal period to guarantee adult spermatogenesis and fertility has been established (O'Shaughnessy, 2014).

1.4.4 Growth factors

A number of growth factors play important roles in the local regulation of testicular function. Among those are the transforming growth factor (TGF) - α and - β**,** Inhibin, Activin, nerve growth factor (NGF), insulin - like growth factor I (IGF - I), fibroblast growth factor (FGF) and epidermal growth factor (EGF).

While Activin has a stimulatory effect on spermatogenesis, Inhibin has inhibitory action. Inhibin serum concentrations are interrelated with spermatogenic activity, testis size and sperm production. Therefore it is used as an indicator of local spermatogenic defects. Modulation of the function of Leydig cells under effects of Inhibin and Activin has also been detected (Nieschlag *et al.*, 2010; Stanfield, 2012). EGF has stimulatory effects on the steroidogenic activity of human testes while IGF-I has a stimulatory effect on DNA synthesis in mitotic germ cells. Testicular size showed enlargement in patients after administration of IGF-I (Nieschlag *et al.*, 2010).

1.5 Female breast

1.5.1 An overview of the human female breast

The human female breast is considered as an accessory reproductive organ. It remains undeveloped until puberty. Breast tissues are developed in response to estrogen and progesterone hormones stimulation. Developed female breasts are located on the anterior chest wall extending from the second rib to the sixth rib and laterally breast tissues extend to the axilla forming the axillary tail. Breasts are attached to the underlying pectoralis muscle by connective tissue. Each mature female breast is formed of a pigmented areola surrounding a central nipple. About 16 – 20 openings of lactiferous tubules are located in each nipple. Histologically, the breast is divided radically by fibrous tissue bands into lobes. Within each lobe there is a number of lobules. Each lobule is composed of alveoli that lead to a lactiferous duct. In between the lobules there is adipose tissue (Fig. 1.6). Physiologically, the main function of breasts is related to milk formation and production (Wylie, 2005).

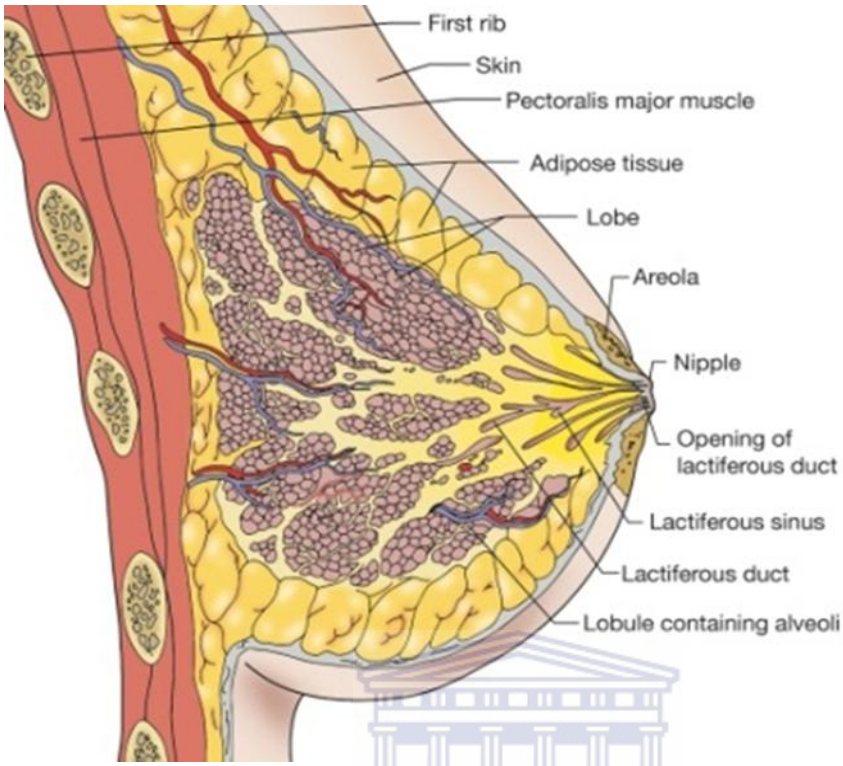

Figure 1.6: Sagittal section showing the human female breast structure. (http://www.genesiscare.co.uk/breast-cancer).

1.5.2 Breast cancer

Breast cancer is the second leading cause of cancer death of women after lung cancer and the most commonly diagnosed cancer next to skin cancer in United States with a peak incidence at 45 - 55 years of age. Epidemiological studies of cancer incidence and deaths performed in eastern regions of Libya have shown that breast cancer was responsible for 14.8 % of female breast cancer deaths amongst 1367 cancer deaths (El Mistiri *et al*., 2015). However, breast cancer occurs in men as well, although with 100 - fold less frequency (DeSantis *et al*., 2014; Moongkarndi *et al*., 2004). The American Public Health Association has noticed several factors like obesity, age over 30 years at first child or nulliparity as a predisposition for breast cancer. In addition, dietary factors like consumption of fat are associated with breast cancer etiology (Wiseman, 2000).

1.5.3 MCF-7 cell line

Human breast adenocarcinoma (MCF - 7) cells were first obtained from pleural effusion of a 69 years old patient with metastatic breast cancer in Detroit and then developed by Dr. Herbert Soule at Michigan Cancer Foundation (MCF). Worldwide MCF - 7 cells are used as a standard model in many laboratories. The cells express estrogen receptors (Levenson and Jordan, 1997). It has been suggested that MCF - 7 cells estrogen receptor α is responsible for cell proliferation while estrogen receptor β antagonize this proliferation effect (Mense *et al*., 2008). The presence of ER α in MCF - 7 cell line and its proliferative response to estradiol stimulation makes it a good screening tool for the effects of xenoestrogens via the E – SCREEN assay (Brooks *et al*., 1973).

1.6 Medicinal plants

There are increasing concerns worldwide by researchers to assess safety, therapeutic consumption and the constitutions of plants used in traditional medicine. Traditional medicine which is an expression for any "non-western" medical practice, is a vital part of health care and can be found in every country (Alsabri *et al*., 2012; Fabricant and Farnsworth, 2001; WHO, 2013). Recently, people around the world have shown more interest in the utilization of herbal medicine to treat simple but also complicated ailments. They believe in herbal medicine safety in comparison with western medicine (Johnson *et al*., 2007; Pan *et al*., 2014).

1.6.1 Medicinal plants and infertility

Infertility is a global health problem that can be defined as the inability to conceive after a year or more of regular unprotected sexual intercourse. International estimation of infertility has shown that 72 million people are affected all around the world. In developing countries, 9 % of couples are affected (Asemota and Klatsky,

2015). Male factors are responsible for 40 % of couple infertility (Mohammadi *et al*., 2013). Although accurate data representing the rates of male infertility worldwide is deficient, Agarwal *et al.* have concluded in their study that 30 million men worldwide are infertile; Africa and Eastern Europe have the highest rates (Agarwal *et al*., 2015). Many factors can lead to male infertility such as the incomplete development of testis, increased testicular temperature, immunological problems, endocrine disturbances and environmental and nutritional factors. Disturbances in spermatogenesis, steroidogenesis and the hormonal regulation of testicular cells are the main causal factors (Edjenguele *et al*., 2014; Mehraban *et al*., 2014).

To treat male infertility, people trust traditional medicine which is based on herbal mixtures because they cost less physically and emotionally. They are also less invasive than other treatment options. The effectiveness of the medicinal plants in improvement of male fertility can be attributed to different mechanisms including enhancement of antioxidant enzymes production and scavenging of free radicals, increase of testosterone levels and improvement of libido disorders. They can have a direct effect on the cavernous muscle, affect the levels of the hormones responsible for spermatogenesis control (LH and FSH) and may improve sperm motility and numbers by increasing the expression of cAMP-responsive element modulator (CREM) mRNA and CREM protein (Mehraban *et al*., 2014; Mohammadi *et al*., 2013).

1.6.2 Medicinal plants and breast cancer

Therapeutic practices that are followed to treat cancer such as chemotherapy, surgery and radiation are not fully effective compared to the high incidence or low survival rate of most cancers. Therefore, obtaining a new approach to treat breast cancer remains one of the challenges in cancer research (Moongkarndi *et al.*, 2004). Wherefore, several bioactive dietary composites are used as anticancer agents to treat breast cancer such as Resveratrol (a phytoalexin from grapes) and Epigallocatechin

Gallate (a polyphenolic compound found in green tea). They produce their effect either by regulation of antioxidant enzyme activities or by induction of apoptosis (Bonofiglio *et al*., 2015).

Phytoestrogens are plant derivatives with chemical structure similar to estrogens. They are found in several plants like date palm pollen, flax seed, soy beans, grapes, pumpkin seed, red clover, peas and beans. Among these phytoestrogens are lignans, isoflavonoids and coumarins. Several studies have reported the ability of these compounds to reduce the risk of hormone - dependent cancers like breast cancer. That may explain the lower incidence of breast cancer among Asian populations who consume large amounts of soy products. Phytoestrogens are able to bind to estrogen receptors (ER) leading to either estrogenic or antiestrogenic effects depending on the presence of endogenous estrogen.

The mechanisms that are responsible for producing anticarcinogenic effects of the phytoestrogens include free radicals scavenging, inhibition of aromatase, competing with estradiol to bind nuclear estrogen receptors, angiogenesis inhibition and inhibition of tyrosine kinases (Mehraban *et al.*, 2014; Sprando *et al.*, 2000; Szewczyk *et al.*, 2014; Waldschläger *et al.*, 2005).

Several compounds have shown stimulatory effects on the growth of MCF - 7 cells. Estradiol, enterolactone and equol (mammalian lignans) caused a marked increase in the proliferation of MCF - 7 cells when cultured in phenol red free media (as the phenol red itself has estrogenic stimulatory effects) (Welshons *et al*., 1987). Similarly, flax leaf extract showed a significant enhancement of MCF - 7 cells mitochondrial dehydrogenase activity measured by MTT assay *in vitro* and 50 µg/ml flax root extract showed a significant increase in MCF - 7 cells proliferation in the same study (Szewczyk *et al*., 2014).

1.6.3 Date Palm Pollen

Palm trees belonging to Arecaceae family have a wide distribution in many areas including the Middle East and North Africa. They have nutritional, medicinal, socio-economic and ecological importance. Extracts from date palm fruit were reported to have anti - fungal, antiviral, antibacterial, anti – inflammatory and anti - apoptotic activities. In addition, extracts from different parts of the palm tree like the pits, date fruit and the edible kernels have been reported to possess aphrodisiac effects and increased the testosterone hormone concentration in rats (Abbas and Ateya, 2011; Bastway *et al*., 2010; Daoud *et al*., 2015; Elberry *et al*., 2011).

Flowers of male date palm tree produce a powder material called Date Palm Pollen (DPP) (Fig. 1.7) which has been widely used as a gonadal stimulant and enhancer of fertility in both males and females (Daoud *et al.*, 2015; Elberry *et al.*, 2011). Moreover, the histopathological features of male Wistar rats prostate with induced atypical prostatic hyperplasia (APH) were improved after oral administration of DPP suspension and DPP extract (Elberry *et al.*, 2011). In addition, oral feeding of female albino rats with DPP for 6 weeks resulted in significant increase in blood levels of LH and FSH hormones in comparison with non-treated rats while feeding them lead acetate showed a significant reduction of those hormones. In the same study a combined feeding of female rats with lead acetate and DPP led to no changes in the levels of the mentioned hormones (Hammed *et al*., 2012) suggesting an ameliorating effect of DPP on heavy metal induced toxicity.

Figure 1.7: Date Palm Pollen. (https://maher-shop.com/img/cms/Palm).

Similarly, DPP showed a therapeutic potency on rat testicular toxicity induced by cadmium and a protective effect on sperm parameters against the experimentally induced adverse effects of electromagnetic field in rats (Baharara *et al*., 2015; El-Neweshy *et al*., 2013). Furthermore, DPP showed a significant increase in male albino rat's sexual behavior parameters such as libido index, mounting and intromitted ejaculation compared to the untreated control animals (Abedi *et al*., 2014). Besides, several *in vivo* studies have reported the androgenic effects of DPP and its ability to increase serum testosterone levels in rats (Arfat *et al*., 2014; Bahmanpour *et al*., 2006; Mehraban *et al*., 2014). Likewise DPP administration to male rabbits resulted in a significant increase in testicular weight and sperm concentration in comparison with the non- treated group (Faleh and Sawad, 2006). In addition, *in vitro* cultivation of granulosa cells and oocytes from female mice in the media supplied with DPP resulted in a significant increase in the rate of follicle

growth and maturation (Abdollahi *et al*., 2015). Moreover, *in vitro* assessment of DPP effect on mouse spermatogonia and Sertoli cell viability showed no cytotoxic effects (Mahaldashtian *et al*., 2015). Many components like alkaloids, steroids, flavonoids, vitamins and tannins have been revealed by phytochemical analysis of DPP (El-Azim *et al*., 2015).

1.6.4 Flax seed

Flax is a herb that belongs to the Linaceae Family. It is native to Mediterranean regions and West Asia. Canada is the main producer with 38 % of total world production. The flax seeds (Fig. 1.8) are flat and oval with soft shiny surface and variable colors either dark brown or yellow and have a nutty crispy taste (Ganorkar and Jain, 2013). It has been reported that the utilization of flax seed led to many biological effects like lowering blood cholesterol, laxative effects and control of blood glucose level. Moreover, regular consumption of flax seed have shown to produce therapeutic effects on several chronic illnesses such as heart diseases, diabetes, stroke, obesity, Alzheimer and cancer (Bekal *et al.*, 2015).

Flax seeds contain many constituents like soluble fibers, α - linolenic acid (ALA), poly unsaturated fatty acids in form of omega - 3, and lignans. It has been reported that flax seeds are the richest dietary source of lignans amongst which are secoisolariciresinol diglucoside, pinoresinol, lariciresinol and matairesinol. Flax lignans have been attributed with numerous positive health effects like antitumor, antioxidant and protective effects against coronary heart diseases. Moreover, flax lignans have both estrogenic and antiestrogenic potential (Adolphe *et al*., 2010; Krajčová *et al*., 2009).

Figure 1.8: Flax seeds. (https://en.wikipedia.org/wiki/Flax).

In the human intestine, flax seed lignans are converted by microbiota to enterodiol and enterolactone. *In vitro*, mouse mammary gland epithelial cells that were transfected with a 3xERE-Luc reporter gene (HC11-ERE cells) were used to test the estrogenicity of enterolactone. Enterolactone caused a significant enhancement of reporter gene expression via both estrogen receptors (α and β) (Penttinen *et al*., 2007). Moreover, it was detected that the flax seed was able to regress the growth of MCF - 7 human breast cancer xenograft in ovariectomized athymic mice with low estrogen levels and counteract the stimulatory growing effect of soybean on the same experimental model (Saarinen *et al*., 2006). On the contrary, the flax seed metabolites, enterolactone and enterodiol, showed stimulatory effects on the growth of *in vitro* cultured MCF - 7 cells (Welshons *et al.*, 1987).

Furthermore, flax seed and flax seed meal showed no effect on the fertility or fetal development when rat dams were fed 40 % and 26 % respectively, while hormone related effects in form of lengthening of estrous cycle and changes in reproductive development of offspring were detected when rats were fed flax seed or flax seed meal during gestation, lactation and post lactation (Collins *et al.*, 2003). In addition, adding flax seed to the rabbit diet has revealed improvement in sperm quality (Mourvaki *et al*., 2010).

1.7 Apoptosis

Apoptosis is a physiological event of programmed cell death which is mandatory to maintain adult tissues homeostasis and eradicate cells which are damaged (by diseases or injurious agents) or no longer required. It can be detected at any stage of the life since development up to aging and its disturbance is associated with many pathological conditions like neurodegenerative diseases, ischemic injury, autoimmune ailments and cancer (Ashkenazi, 2008; Elmore, 2007).

In comparison with necrosis, apoptosis is an energy consuming controlled process, which might involve single cell or cluster of cells where the cell is an active participant. On the other hand, necrosis is a passive energy-independent death of (usually) group of cells. In spite of the different mechanisms, both necrosis and apoptosis represent morphologic manifestation of shared biochemical system known as Apoptosis-Necrosis Continuum (Jain *et al*., 2014; Zeiss, 2003).

Several stimulants, either physiological or pathological, are responsible for induction of apoptosis. This is followed by the execution phase during which the cell loses its contact with neighboring cells and then undergoes many morphological changes like shrinkage, blebbing of the cellular membrane, condensation of the chromatin and fragmentation of the nucleus (Elmore, 2007; Hengartner, 2000). These characteristic features are the consequence of many biochemical events involved in the activation

of proteolytic enzymes called caspases which are responsible for destruction of the cellular cytoskeleton and metabolic proteins (Zeiss, 2003). The elimination phase includes the engulfment of cells that had undergone apoptosis by phagocytes; therefore no inflammatory reaction is involved (Jain *et al.*, 2014).

Phosphatidylserine is a phospholipid that is physiologically present at the inner leaflet of the plasma membrane. Externalization of phosphatidylserine from the inner to the outer leaflet is one of the initial marks of apoptosis which is used as a marker in apoptosis detection *in vitro* as it can be detected by use of annexin V or APOPercentage assays (Badmus *et al*., 2015; Zeiss, 2003; Ziegler and Groscurth, 2004).

1.8 Objectives of this study

People in Libya throughout the time trust plants as a source of folk medicine although further studies are required to assess the validity of the plant folk - medicinal use (El-Mokasabi, 2014). Therefore two herbs from Libya (DPP and flax seed) are used in this study to detect their effects on male reproduction by use of a mouse Sertoli cell line (TM4) and on breast cancer by use of the human breast adenocarcinoma cell line (MCF - 7).

This study aimed at:

- Assessment of cytotoxicity of DPP and flax seed ethanolic extracts on mouse Sertoli cell line (TM4) and human breast cancer (MCF - 7) cell line.

- Determination of potential apoptotic effects of DPP and flax seed ethanolic extracts on mouse Sertoli cell line (TM4) and human breast cancer (MCF - 7) cell lines.

- Assessment of the effects of DPP and flax seed ethanolic extracts on the integrity of the tight junctions between adjacent TM4 cells by electro-biological study (TEER) assay.

- Determination of the effect of DPP and flax seed ethanolic extracts on the activity of gamma glutamyl transpeptidase enzyme (GGT) produced by mouse Sertoli cell line (TM4).

- Determination of the effect of DPP and flax seed ethanolic extracts on the production of Inhibin - B hormone by mouse Sertoli cell line (TM4).

- Determination of the estrogenicity of DPP and flax seed ethanolic extracts by use of MCF - 7 cells via the E - SCREEN assay.

Chapter II: Materials and methods

2.1 Materials

2.1.1 Chemicals and equipment

American Type Cell Culture (ATCC), Manassas, USA supply:

- Human breast cancer (MCF- 7) cell line (ATCC® HTB22™).
- Mouse Sertoli (TM4) cell line (ATCC® CRL – 1715™).

Becton Dickinson (BD) Biosciences Pharmingen, San Diego, USA supply:

- FACScan instrument (BD Accuri™ C6 flowcytometer).

Biocolor Ltd. (Carrickfergus, Northern Ireland) supply:

- APOPercentage dye.

BMG Labtech supply:

- Microplate reader.

Corning, South Africa supply:

- Cell culture plates (12, 24, 96 wells).
- Pipettes (5, 10, 25 ml).
- Tissue culture flasks.
- Test tubes (15 ml and 50 ml).

- Cell culture spatula.

Gibco - thermo scientific, Germany supply:

- Donor equine serum (Cat No SH30074.03).
- Dulbecco's Modified Eagles Medium/F – 12 with phenol red (Cat No SH30023.01).
- Dulbecco's Modified Eagles Medium/F – 12 without phenol red (Cat No SH30272.01).
- Fetal bovine serum (Cat No SV301600.03).
- 0.25 % Trypsin / Ethyl Diamine Tetra Acetic acid (EDTA) (Cat No SH30236.02).

Lasec, Cape Town, South Africa supply:

- Inverted Microscope System.

Merck, Millipore Ltd, USA supply:

- Milicell® 24–well tissue culture inserts.
- Milicell® - ERS resistance system.

Oxoid, England supply:

- Phosphate Buffered Saline (PBS).

Saarchem, South Africa:

- Dimethylsulphoxide (DMSO).

Sigma-Aldrich, Germany supply:

- Dimethylsulphoxide (DMSO).
- Thiazolyl blue tetrazolium bromide (MTT).
- β Estradiol.
- Charcoal (activated).
- Dextran from Leuconostoc spp (mol wt 450,000 – 650,000).
- Trypan blue.

Sigma-Aldrich, USA supply:

- Inhibin- B EIA kit. (Cat No RAB0325)
- Gamma glutamyl transferase (GGT) activity colorimetric assay kit. (Cat No MAK089)

Whatman, Madestone, England supply:

- Filter paper. (Whatman No.1).

2.2 Methods

2.2.1 Preparation of ethanolic extract of the plants

DPP and flax seeds were brought from a local shop in Libya. Flax seeds were cleaned with water and then both herbs were allowed air - drying at room temperature for 48 hours before being grinded using an electrical - mill. To prepare the ethanolic extract, 10 g powder of each plant was measured and immersed with 100 ml 95 % ethanol in a glass flask, then covered with parafilm and kept steeped at room temperature. After

three days the solutions were filtered through eight layered muslin cloth, followed by filter paper (Whatman No.1).

To yield the semisolid ethanolic filtrates, the extracts were allowed to rotate under reduced pressure in a vacuum rotary evaporator resulting in 1.8 g DPP and 0.7 g flax seed extract. After that 1 g of each extract was dissolved in 1 mL dimethyl sulfoxide, then filtered using (0.22 μm) sterile syringe filter and stored in sterile vials at 4 °C.

Stock solutions of DPP and flax seed of 5000 μg/ml concentration were prepared with DMEM medium under the lamina flow and complete sterile conditions and stored in – 20 °C till use. Further concentrations (0.01, 0.1, 1, 10, 100, 1000 μg/ml) were prepared with DMEM medium freshly each time for cell treatments under the lamina flow and complete sterile conditions.

2.2.2 Phosphate buffered saline (PBS)

One tablet of PBS was dissolved in 100 ml distilled water and left to be completely dissolved by the use of magnetic shaker. The PBS solution was sterilized by autoclaving then stored in the fridge.

2.2.3 Cell culture media

Dulbecco's Modified Eagles Medium/F – 12 Hams serum 1:1 supplied with L - glutamine and HEPES buffer was used to culture both cell lines (TM4 and MCF-7 cells). For TM4 cells, DMEM medium was further supplied with 2.5 % fetal bovine serum and 5 % horse serum, while the medium for MCF - 7 was supplied with 5 % fetal bovine serum. All the manipulation of the media was done under the lamina flow and complete sterile conditions. Media supplied with nutrients and sera was referred as complete growth media.

2.2.4 Freezing medium

Freezing medium is the medium that was used to store cells at a very low temperature and prevent the formation of ice crystals. 10 % sterile DMSO was added to complete culture medium and stored at – 20 °C until use.

2.2.5 TM4 and MCF - 7 cell lines

Mouse Sertoli cells (TM4) and human breast adenocarcinoma cells (MCF - 7) were obtained from ATCC. A 1 ml vial of frozen cells was thawed in a water bath at 37 °C and sterilized with 70 % ethanol. Thereafter, 1 ml of the cells was transferred into a sterile 25 cm^2 tissue culturing flask with proper complete growth medium under the lamina flow and complete sterile conditions. Then the flask was sprayed with 70 % ethanol and kept in the incubator at 37 °C and 5 % CO2. After 24 hours the medium was replaced with a fresh one and the cells were incubated until 70 – 80 % confluence reached with changing the medium every second or third day when it showed signs of depletion. The protocol was followed for both cell lines.

2.2.5.1 Sub - culturing of the cells

When the cells reached 80 % confluence they were sub- cultured. First the depleted medium was discarded and the cells washed with 2 ml warm sterile PBS which was then discarded. The cells were detached enzymatically by adding 1 ml of 0.25 % Trypsin / EDTA and allowed to stand for 5 - 15 minutes in the incubator under visual control until 80 - 90 % of the cells were round and detached. To inactivate trypsin, 2 ml of warm complete growth medium was added to the flask. Thereafter 1 ml of the cells were transferred into new tissue culture flasks with fresh warm complete growth medium and 1 ml was left in the original flask with complete culture medium. All flasks were labeled carefully with the cell name and passage number, sprayed with

70 % ethanol and incubated at 37 °C and 5 % CO2 for further growth. The same steps were followed for both cell lines.

2.2.5.2 Cryopreservation of the cells

70 - 80 % confluent flasks were used to freeze the cells. This protocol follows the same steps as in the sub- culturing processes until the step of stopping the action of the trypsin. Then the contents of the flask were transferred to a 15 ml sterile test tube and the cells were pelleted by centrifugation at 150 x g for 5 minutes. Thereafter the supernatant was discarded and 1 ml of freezing medium added and the cells were resuspended by gentle pipetting up and down several times and then transferred into a cryovial. First the cells were kept overnight in a Styrofoam box in a -80 °C freezer to allow slow cooling down and then stored at -196 °C in liquid Nitrogen. The same steps were followed for both cell lines.

2.2.5.3 Counting of the cells

Cells were counted every time before seeding in plates to perform the experiments. Using a 70 - 80 % confluent flask the same protocol for sub-culturing was followed. After stopping of trypsin action, the contents of the flask were transferred to a 15 ml sterile tube and pelleted by centrifugation at 150 x g for 5 minutes. Thereafter the supernatant was discarded and the cells resuspended in 1 ml complete culture medium. In an Eppendorf vial a volume of 10 µl cell suspension was transferred and mixed with 10 µl of Trypan blue dye which stained only dead cells. 10 µl of this suspension was transferred to the haemocytometer counting chamber and visualized under the photomicroscope where the number of cells in each quadrant was counted and then the total number of the cells in the flask was determined according to the following equation

Equation 1 $$\frac{\text{summation of cells number in the 4 quadrants}}{4} * 2 * 10^4 \text{ cell/ml}$$

The number of cells needed was calculated using the formula below

Equation 2 $$\frac{\text{number of cells needed}}{\text{number of cells counted}} * 1000 = \text{volume required in } \mu l$$

The same steps were followed for the both cell lines with exception of the media which were proper for each cell line.

2.2.6 MTT assay

2.2.6.1 Preparation of MTT solution

5 mg MTT powder was dissolved in 5 ml sterile PBS in a 15 ml tube, then wrapped in aluminum foil and rotated for 1 hour. After being filtered under lamina flow with a 0.22 μm syringe filter the solution was kept in a fridge to be used within two weeks' time.

2.2.6.2 MTT assay procedure

MTT assay is based on the reduction of yellow MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] by the mitochondrial dehydrogenase enzyme of viable cells to blue formazan crystals.

For the TM4 cells, cells were seeded at a density of 3000 cells/ well in a 96 well plate for 24 hours and 1500/ well for 72 hours. While for MCF - 7 cells, 5000/well was seeded for 24 hours and 1000/well for 72 hours in 96 well plates. Cells were allowed to attach and grow for 24 hours in complete culture medium to be treated the next day with six concentrations (0.01, 0.1, 1, 10, 100, 1000 μg/ml) of the ethanolic extracts

of each of DPP and flax seed, six wells per each concentration. Another six wells were used for untreated negative control and six wells for 8 % DMSO as positive control.

After incubation for 24 hours or 72 hours the media were discarded from the plates and the cells washed with PBS (100 µl / well) and then 200 µl of fresh complete culture media was added for each well and 20 µl of MTT solution was added for each well, thereafter the plate was kept in the incubator.

After 3 hours the supernatant was discarded. The plate was tapped upside down on paper towels to remove all remaining fluid and 100 µl of DMSO was added for each well to dissolve the blue formazan and the absorbance was measured with a microplate (MTP) reader at a wavelength of 540 nm with a reference wavelength of 690 nm to negate the effect of cell debris and precipitated proteins which may be produced by the dissolving process. The optical density was calculated by subtracting the absorbance of the reference wavelength from the absorbance of the test wavelength. The viability of the cells, which reflects the metabolically active cells that were able to reduce the MTT salt by mitochondrial dehydrogenase enzyme, was measured as a percentage of the negative control according to the following equation

Equation 3 $$\text{percentage of viable cells} = \frac{(\text{OD}) \text{ of treated cells}}{(\text{OD}) \text{ of untreated cells}} * 100$$

The effect of 0.1 % DMSO (the final DMSO concentration in the highest concentrations of plant extracts) on the viability of MCF-7 breast cancer and TM4 Sertoli cells were tested in a preliminary assay. The serial dilution of the plant extracts were led to further dilution of the DMSO vehicle.

2.2.7 Study of the morphological changes

Cells that were seeded for MTT assay were also used to study the morphological changes yielded by the plant extracts. Cells were observed under the inverted system microscope and microphotographs were taken after 24 hours and 72 hours exposure.

2.2.8 APOPercentage assay

APOPercentage assay using a FACScan (Becton Dickson, USA) was used to detect apoptosis in cultured cells. The APOPercentage dye detects cellular changes occurring during early apoptosis which include a disturbance of the cytoplasmic membrane phospholipids resulted in externalization of inner plasma membrane component phosphatidylserine and loss of asymmetric composition of the plasma membrane (Meyer *et al*., 2008).

APOPercentage dye enters the cell once the phosphatidylserine residues are externalized to the outer membrane surface and the uptake continues until blebbing of the cell occurs. Apoptotic cells can be quantified by flowcytometry or visualized under transmission light microscope. In this study flowcytometry was used to quantify apoptosis.

Cells were seeded in 24 well plates in a density of 5 x 10^4 cells / well in complete culture media and incubated for 24 hours before treatment with 0.1, 10 and 1000 µg/ml of DPP or flax seed ethanolic extracts with preservation of three wells per plate untreated and another three with 6 % DMSO as a positive control. It has been reported that DMSO induced apoptosis in human leukemic HL-60 cell line. Besides, it was also able to induce apoptotic degeneration of rat CNS tissues. In the same study there was an elevation in activated caspase-3 positive neurons with morphological features of apoptosis. It has been suggested that the mechanism by which DMSO induced apoptosis is similar to that produced by ethanol since cellular

degenerative effects produced by DMSO or ethanol were overcome by depolarizing effects of potassium chloride (Hanslick *et al*., 2009; Hong and Wang, 1995).

The plates were incubated for another 24 hours. Thereafter the supernatant of each well was transferred to a 15 ml centrifuge tube (collecting all floating cells). Then the cells were washed with PBS (300 µl / well) twice and the PBS was also collected in the same tube. Then 500 µl / well of trypsin were added and the cells allowed to detach under visual control, again all the contents of the well was transferred to the corresponding tube.

APOPercentage dye was diluted in complete culture medium (1: 160 v/v) and 100 µl of the dye was added to the tubes and incubated for 30 minutes at 37 °C with 5 % CO2. After the incubation, 500 µl PBS were added to each tube and the cells resuspended carefully then centrifuged for 5 min at 3000 rpm. The cell pellet obtained was resuspended in 500 µl PBS and transferred to an eppendorf cup and analyzed on a FACScan instrument (BD Biosciences Pharmingen, San Diego, CA, USA) provided with a 488 nm argon laser as a light source and the dye was recorded at 670 nm using filter 3 (FL3). At least 10,000 cells per sample were acquired (Badmus *et al*., 2015; Meyer *et al*., 2008).

2.2.9 Transepithelial electrical resistance across TM4 cell monolayer (TEER assay)

Measurement of transepithelial electrical resistance across a cell monolayer is an indicator of the integrity and permeability of this monolayer (Hulkower and Herber, 2011).

The dual chamber system composed of the transwell filters with Sertoli cells growing on them was used as *in vitro* model of the blood - testes barrier (BTB) as the cells

were in contact with media at both apical and basolateral surfaces which represents the tubular and the interstitial fluids respectively (Hu *et al*., 2014).

The Milicell® 24 – well tissue culture inserts with 12 mm size, 0.6 cm^2 effective surface area and 0.45 μm pore size were used in this assay. The filters were placed in the wells of 24 well plates inside the laminar flow hood and complete sterile conditions by the use of sterile forceps care full to avoid touching the membrane.

A 70 - 80 % confluent flask was trypsinated and the cells counted as mentioned previously, thereafter seeded at a density of $3x10^5$ / well with complete growth culture medium with great care not to trap air bubbles between Sertoli cells which cause unsteadiness of TEER records (Chung *et al*., 2001). 400 μl medium was pipetted inside the insert and 600 μl outside the filter, blank filters without cells were included in the plate also, then incubated in 37 °C with 5 % CO2 for 24 hours.

After 24 hours the media were discarded cautiously not to damage the membrane and the cells were exposed in quadruplicate to different concentrations (0.01, 0.1, 1, 10, 100 and 1000 μg/ml) of ethanolic extracts of DPP or flax seed which have been prepared freshly on the same day of treatment. The cells were once more incubated for 24 hours.

TEER was measured by means of a Milicell® Volt – Ohm meter. First the plates were left inside the lamina flow for 20 minutes to obtain room temperature. Meanwhile the electrodes were immersed in 70 % ethanol for 15 minutes followed by immersion in PBS for another 15 minutes to stabilize the electrode interface.

The Milicell® Volt – Ohm meter was sat on R mode then switched on. Thereafter the electrodes were placed inside the well in a position where the short one was inside the filter while the long electrode was touching the bottom of the well outside the filter. Then the resistance was recorded. The resistance was recorded after 24, 48, and 72

hours of exposure, and the media were changed every morning and replaced with the same treatments every time.

All the measurements were normalized to a blank well (transwell insert + medium without cells) and then multiplied by the cell growth area according to the following equation:

Equation 4 $$TEER\ (\Omega cm^2) = (R_{sample} - R_{blank}) \times A$$

Where R sample = resistance readings of treated groups, R blank = resistance readings of unseeded filter with medium only and A = effective surface area of the filter = 0.6 cm^2.

2.2.10 Determination of TM4 gamma glutamyl transpeptidase activity (GGT assay)

GGT is a membrane bound protein that is involved in the transportation of a gamma glutamyl group to other peptides and has been used as a marker of Sertoli cells (Lipshultz *et al*., 1982). GGT activity was determined by the use of GGT colorimetric assay kit (cat number MAK089) which is based on the measurement of the chromogen P- nitroanilide (PNA) that is produced by transfer of γ – glutamyle group from L – γ – Glutamyle – p – nitroanilide under the effect of GGT enzyme. One unit of GGT is the amount of enzyme that will create 1 mmole of pNA per minute at 37 °C.

A 70 - 80 % confluent flask of TM4 cells was trypsinated and the cells counted as mentioned previously and seeded in a density of $5x10^4$ / well in sterile 24 well plate under complete aseptic technique and incubated for 24 hours. Next day they were exposed to freshly prepared different concentrations of DPP or flax seed extracts and kept for 72 hours in the incubator.

2.2.10.1 GGT assay procedure

The manufacturer's assay protocol was followed and numbers of reagents were prepared before the conduction of the assay. First the substrate was reconstituted with 10 ml assay buffer in 15 ml sterile test tube, mixed well, wrapped with aluminum foil and kept in an ice box. Next 100 µl distilled water was added to the positive control vial, mixed well, wrapped with foil and kept in the ice box.

Thereafter, in a 96 well plate, the prewarmed solution was added in different volumes (0, 4, 8, 12, 16 and 20 µl), ran in duplicate to generate 0, 8, 16, 24, 32, and 40 nmol / well standards after the volume was adjusted to 100 µl in each well with the assay buffer.

The supernatant of the TM4 cells was discarded and the cells were homogenized by use of a spatula and the ice cold assay buffer, and then centrifuged at 13000 x g for 10 minutes. Thereafter 10 µl of each of the untreated cells (negative control), the positive control (supplied with the kit) and the test samples were added to the wells and brought to 100 µl with the assay buffer and the plate kept in the incubator. After 3 minutes the initial measurement was read with MTP reader at 418 nm wave length followed by reading of the plate every 5 min until the highest reading exceeded the reading of highest standard was recorded.

In aim to measure the PNA concentration yielded, the change of measurement was first calculated by subtracting the initial reading from final reading for each sample then the values were determined according to the standard curve.

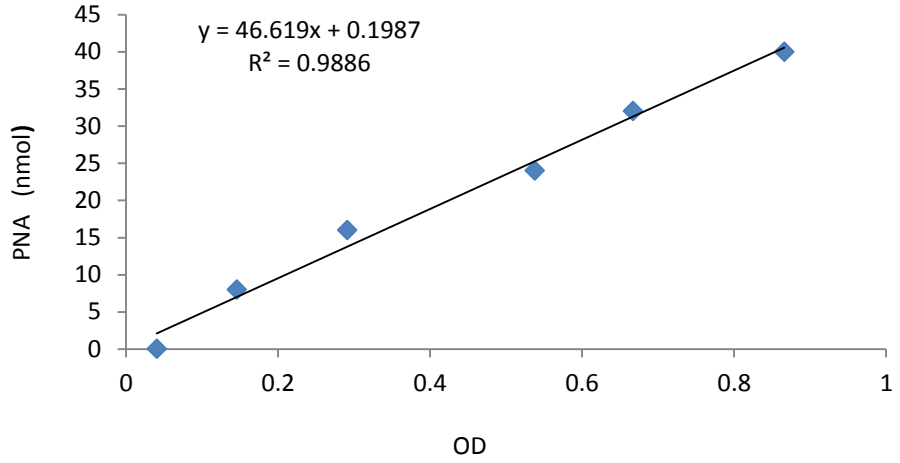

Figure 2.1: Standard curve for the determination of P - nitroanilide (PNA). The standard curve shows a linear relationship between the PNA concentrations and the optical density.

The activity of the GGT enzyme was measured according to the following equation

Equation 5

$$\text{GGT activity} = \frac{B}{\text{Reaction time x V}}$$

Whereas the B = amount of PNA (nmol), reaction time = final time – initial time in minutes and V = the volume of the sample in ml, thereafter the GGT activity was expressed as nmol/min/ml = milliunit/ml.

2.2.11 Determination of TM4 inhibin - B production

To determine the effect of DPP and flax seed on TM4 production of inhibin - B, the cells were seeded in 24 well plates at density of 5 x 10^4 cell / well and incubated overnight in the 37 °C with 5 % CO2 incubator. Next day different concentrations (0.01, 0.1, 1, 10, 100, 1000 µg/ml) of both herbs were prepared on the same day and cells were treated and incubated for 72 hours.

2.2.11.1 Inhibin - B assay procedure

In this study the measurement of TM4 cells production of inhibin - B hormone under the effect of plants extract was assessed by use of inhibin - B EIA kit (cat number RAB0325). The kit is specific for (human, mouse and rat) with a detection limit of 34.6 pg/ml of inhibin – B hormone.

Numbers of reagents were prepared before the assay was conducted. First 40 ml deionized water was added to 10 ml assay diluent B to generate 5 fold dilution which was used to dilute the anti-inhibin - B detection antibody up to 100 fold by mixing the tube gently up and down. Thereafter 100 µl of the diluted anti-inhibin antibodies was added to each well of the 96 microplate supplied with the kit and pre-coated with anti-rabbit secondary antibody, then the plate was covered with a plastic cover supplied with the kit and kept overnight on the orbital shaker at 4 °C.

Further preparation of the other reagents was carried out during incubation time of the plate with the anti-inhibin antibodies taking care that all the reagents were kept in an ice box during the preparation procedure. The biotinylated inhibin - B vial was centrifuged briefly and 5 µl of it was mixed gently with 5 ml of 1 x assay diluent B. This solution was used as a diluent to prepare the inhibin standards

Inhibin – B standard stock solution (10000 pg/ml) was prepared in a 1.5 ml tube by adding 8 µl of standard inhibin - B peptide (which was briefly centrifuged before being used) to 792 µl of the solution mentioned previously as a diluent for standards, and the tube was labeled as 10000 pg/ml. Subsequently a series of dilutions was performed to generate the further concentrations of the standards including transfer of 50 µl of the stock to another tube containing 450 µl of the solution mentioned previously as a diluent for standards and mixed thoroughly. This step was repeated for the other concentrations (1000, 100, 10, 1 pg/ml), whereas the zero standard tube contained only 450 µl of 100 pg/ml biotinylated inhibin - B.

A 10 fold dilution of biotinylated inhibin - B was prepared by mixing 2 µl of it with 18 µl assay diluent B, from which 2 µl was added to the positive control vial (supplied with the kit), mixed and another volume of 101 µl of 1 x assay diluent B was added. The samples were prepared by adding 247 µl of the supernatant of each sample to 2.5 µl of 10 fold diluted biotinylated inhibin - B. The 20 x wash concentrate was brought to room temperature before it was diluted to 1 x with deionized water.

The next day the solution of the anti-inhibin antibody was discarded and the plate washed 4 times with 300 µl of 1 x wash solution with great caution to remove the fluid completely each time by blotting the plate against paper towel. Then 100 µl of each standard, positive control, negative control, samples and assay diluent (as a blank) were added to the corresponding well and the plate was covered again with the plastic cover and kept overnight on the orbital shaker at 4 °C. After 24 hour the streptavidin vial was centrifuged first then diluted with 1 x assay diluent to generate 400 fold dilution. After that the contents of the 96 well plate were discarded, the plate was washed as previously explained. Then 100 µl of the prepared streptavidin solution was added to each well and the plate was incubated on the orbital shaker for 45 minutes and the supernatant discarded, washed again followed by adding of 100 µl of the substrate reagent with incubation on the shaker in a dark room for 30 minutes.

The last step was adding of 50 µl of stop solution to each well and the absorbance was read immediately at 450 nm. To plot the standard curve the percentage of absorbance (B/B_0) was calculated first according to the following equation

Equation 6

$$B/B_0 = \frac{\text{B-blank OD}}{\text{B zero -blank OD}}$$

Where the B = optic density of the sample or standard and B_0 = optic density of zero standard. The values of the inhibin - B hormone in the samples were interpolated from the standard curve by use of Graph pad prism statistic software.

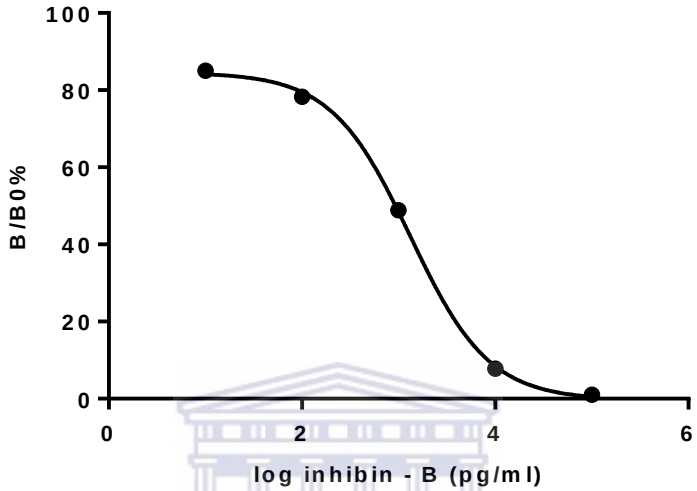

Figure 2.2: Four parameter logistic regression standard curve for the determination of inhibin - B hormone. The curve was created by Graph pad prism software. $R^2 = 0.99$

2.2.12 Assessment of the estrogenic effect of the DPP and flax seed (E - SCREEN assay

The comparison of the proliferation of MCF - 7 cells before and after treatment with materials that supposed to have estrogenic effect or 17 β estradiol hormone as a positive control is the concept of the E - SCREEN assay (Soto *et al*., 1995).

2.2.12.1 Preparation of charcoal stripped fetal bovine serum (CD - FBS)

The protocol of Dang and Lowik (2005) was followed to remove the steroid hormones from the FBS that was used in the E - SCREEN assay. First 250 ml of FBS were inactivated by keeping it for 45 minutes in the water bath at 56 °C, and then a solution of 0.25 g dextran dissolved in 1000 ml Tris buffer (pH 8) was prepared. In

addition, 2.5 g charcoal was further added to the previous solution which then was kept stirring at 4 °C for 24 hours and then centrifuged at 1000 x g. Then the supernatant was discarded and the dextran - coated charcoal pellets was mixed with the preheated FBS and incubated at 45 °C for 45 minutes. Thereafter, the sera were centrifuged to remove the charcoal and then filtered with syringe filter units (0.22 µm) and kept at – 20 °C until use.

2.2.12.2 E - SCREEN assay

MCF - 7 cells were seeded with DMEM growth medium supplied with 5 % FBS in 96 well plates at a density of $5x10^3$/well and incubated in 5 % CO_2, 95 % air and 37 °C incubator. Next day the media were discarded and the cells washed with PBS and allowed to grow for another 48 hours in phenol red free DMEM media supplied with 5 % CD - FBS. Furthermore, the MCF - 7 cells were treated with different concentrations (0.01, 0.1, 1, 10, 100, 1000 µg/ml) of DPP or flax seed ethanolic extracts. In addition, 1 nM estradiol was used as a positive control and the cells were incubated in 5 % CO_2, 95 % O_2 and 37 °C incubator.

After 4 days, the proliferations of the MCF - 7 cells were assessed by use of MTT following the same protocol as described previously.

2.2.13 Statistical analysis

All the statistical calculations were done using Graph pad prism software (GraphPad Prism 6.03, GraphPad Software Incorporation, San Diego, USA). Kolmogorov-Smirnoff test was used to test the normal distribution of the data and the ANOVA-trend analysis followed by Tukey test was performed. Data were expressed as mean ± SD and a p value < 0.05 was considered to be statistically significant. All experiments were repeated at least three times.

Chapter III: Results

3.1 Effect of plants ethanolic extracts on cell morphology

With the aim to study any morphological changes that might occur by exposure of TM4 and MCF - 7 cells to the plants ethanolic extracts, microphotographs were taken by x100 and x200 magnifications via an inverted microscope after 24 hours and 72 hours exposure time, respectively. DMSO at 8% concentration was used to induce cytotoxic morphological features. As there was no significant difference between x20 magnified micrographs after 24 hours and 72 hours exposure, therefore only the micrographs after 24 hours were represented.

3.1.1 Effect of Date Palm Pollen (DPP) on TM4 cell morphology

Microphotographs of TM4 cells that were treated with different concentrations of DPP extract (0.01 - 1000 µg/ml) for 1 or 3 days showed no morphological changes (Fig. 3.1 3.2 and 3.3). On the contrary, cells showed a marked increase in numbers especially those treated with high concentrations of DPP (100 - 1000 µg/ml) compared to negative control cells. Cells which were treated with 8% DMSO showed shrinkage and obvious reduction in number.

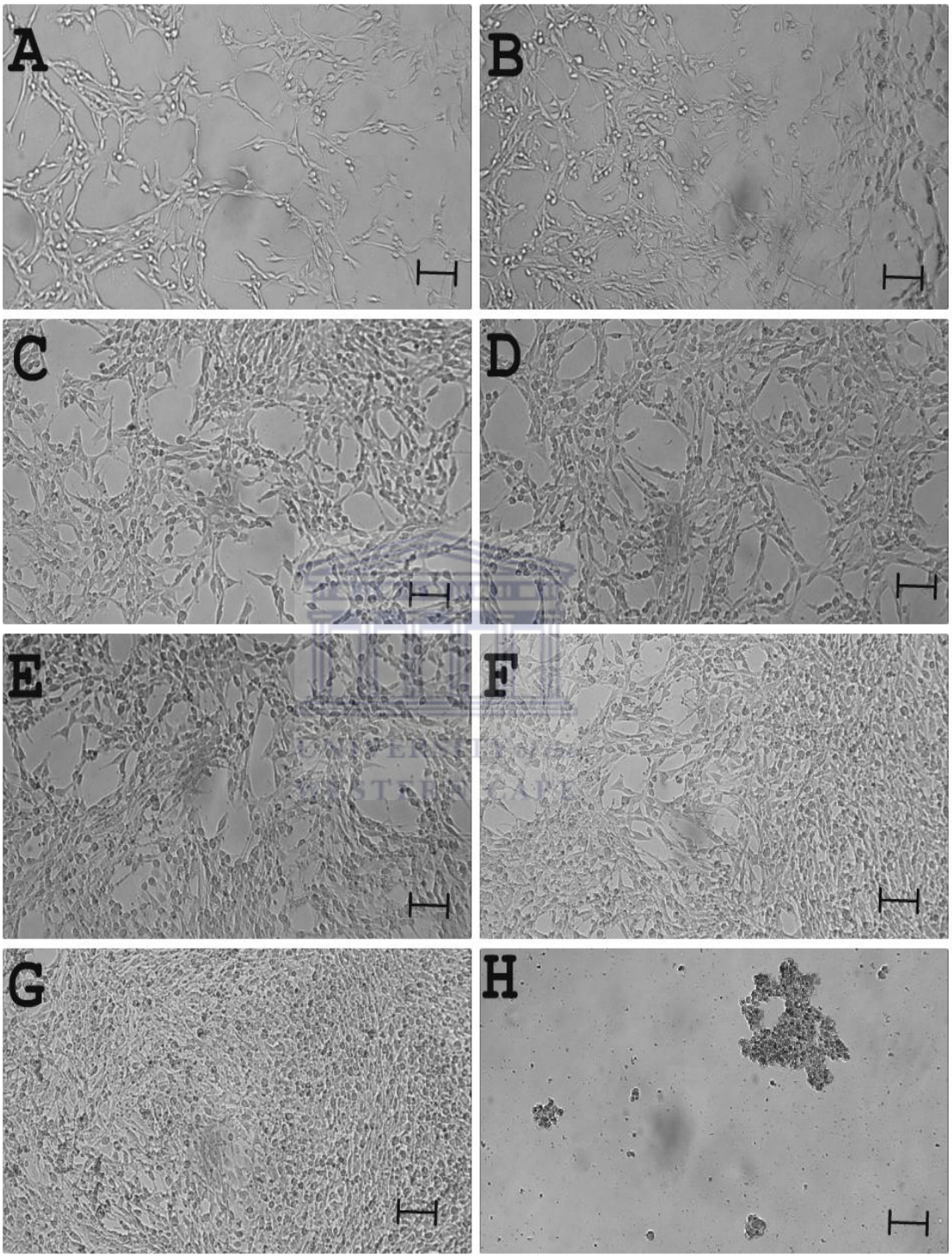

Figure 3.1: Micrographs showing the effect of DPP on TM4 cells after 24 hours at 100 x magnification. Abbreviations: A = untreated cells, B = 0.01 µg/ml, C = 0.1 µg/ml, D = 1 µg/ml, E = 10 µg/ml, F = 100 µg/ml, G = 1000 µg/ml, H = 8 % DMSO. Bar = 50 µm.

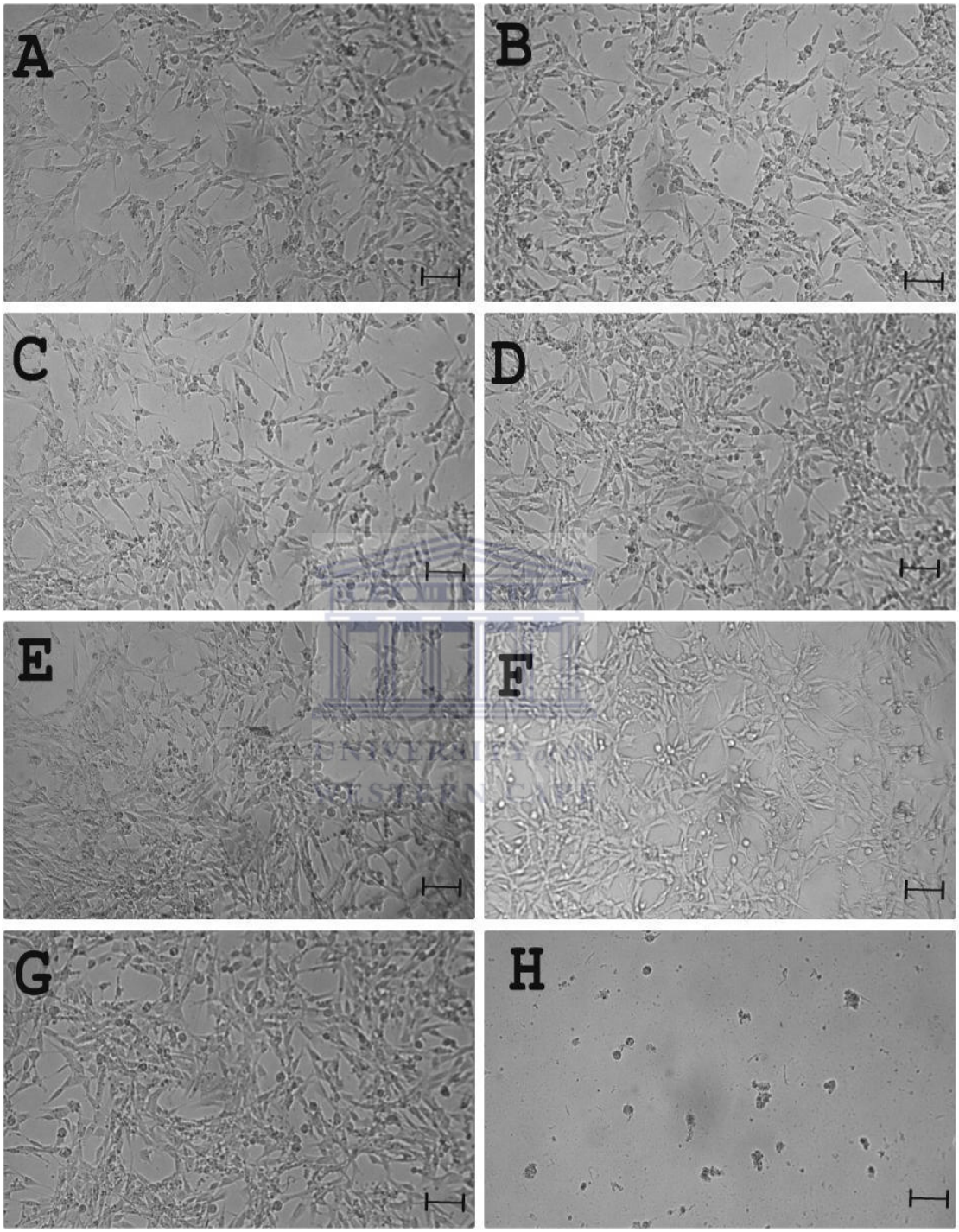

Figure 3.2: Micrographs showing the effect of DPP on TM4 cells after 72 hours at 100 x magnification. Abbreviations: A = untreated cells, B = 0.01 µg/ml, C = 0.1 µg/ml, D = 1 µg/ml, E = 10 µg/ml, F = 100 µg/ml, G = 1000 µg/ml, H = 8 % DMSO. Bar = 50 µm.

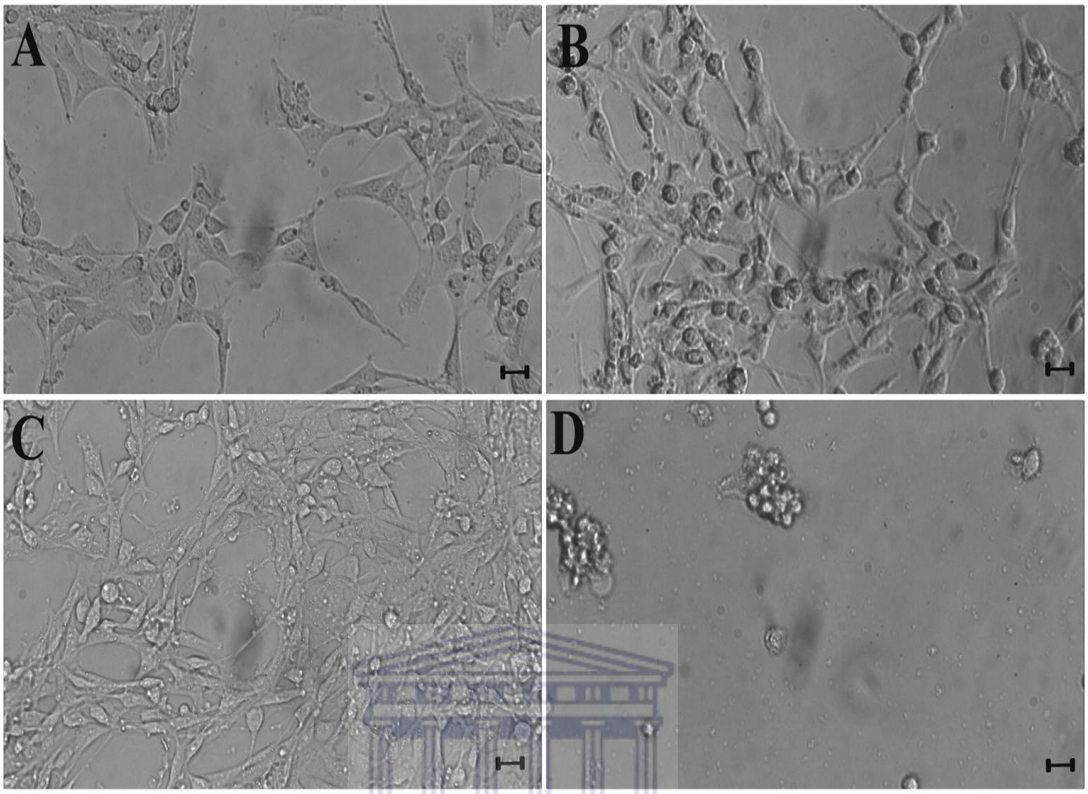

Figure 3.3: Morphology of TM4 cells after 24 hours DPP exposure at 200 x magnification. There is no morphological changes between DPP treated groups (B, C) and untreated control cells (A). DMSO treated cells appear clumped and markedly reduced in number. Abbreviations: A = untreated cells, B = 0.1 µg/ml, C = 1000 µg/ml, D = 8 % DMSO. Bar = 40 µm.

3.1.2 Effect of flax seed on TM4 cell morphology

Treating the TM4 cells with different concentrations (0.01 – 1000 µg/ml) of flax seed extract resulted in no changes in the morphology of the cells after 1 or 3 days in relation to untreated cells as can be seen in Fig. 3.4, 3.5 and 3.6. On the other hand, the cells displayed a marked increase in their numbers compared to untreated cells while the group which was treated with 8 % DMSO displayed marked reduction in the number of cells and also showed morphological changes in form of shrinkage and rounding of the cells.

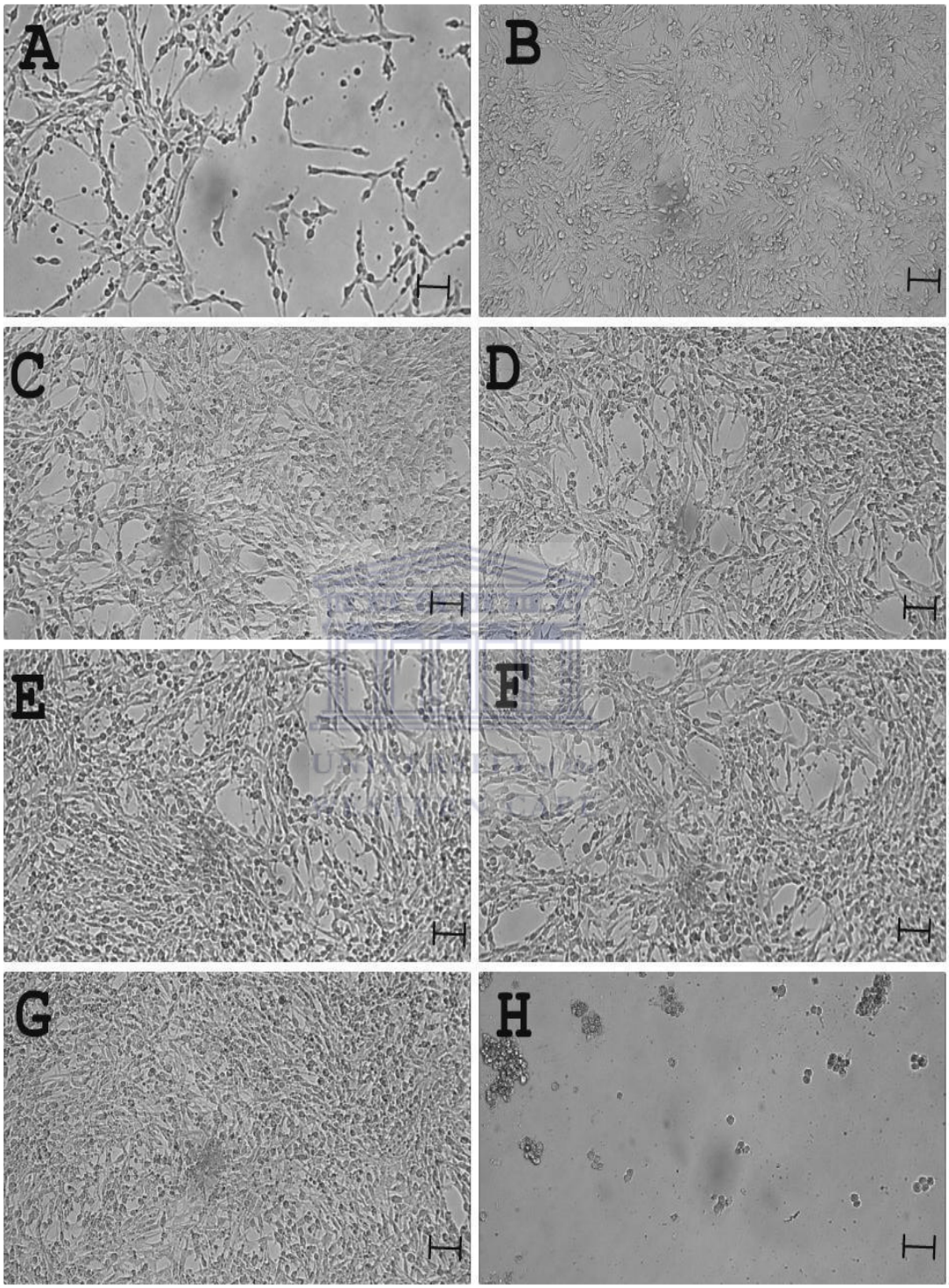

Figure 3.4: Micrographs showing the effect of flax seed on TM4 cells after 24 hours at 100 x magnification. Abbreviations: A = untreated cells, B = 0.01 µg/ml, C = 0.1 µg/ml, D = 1 µg/ml, E = 10 µg/ml, F = 100 µg/ml, G = 1000 µg/ml, H = 8 % DMSO. Bar = 50 µm.

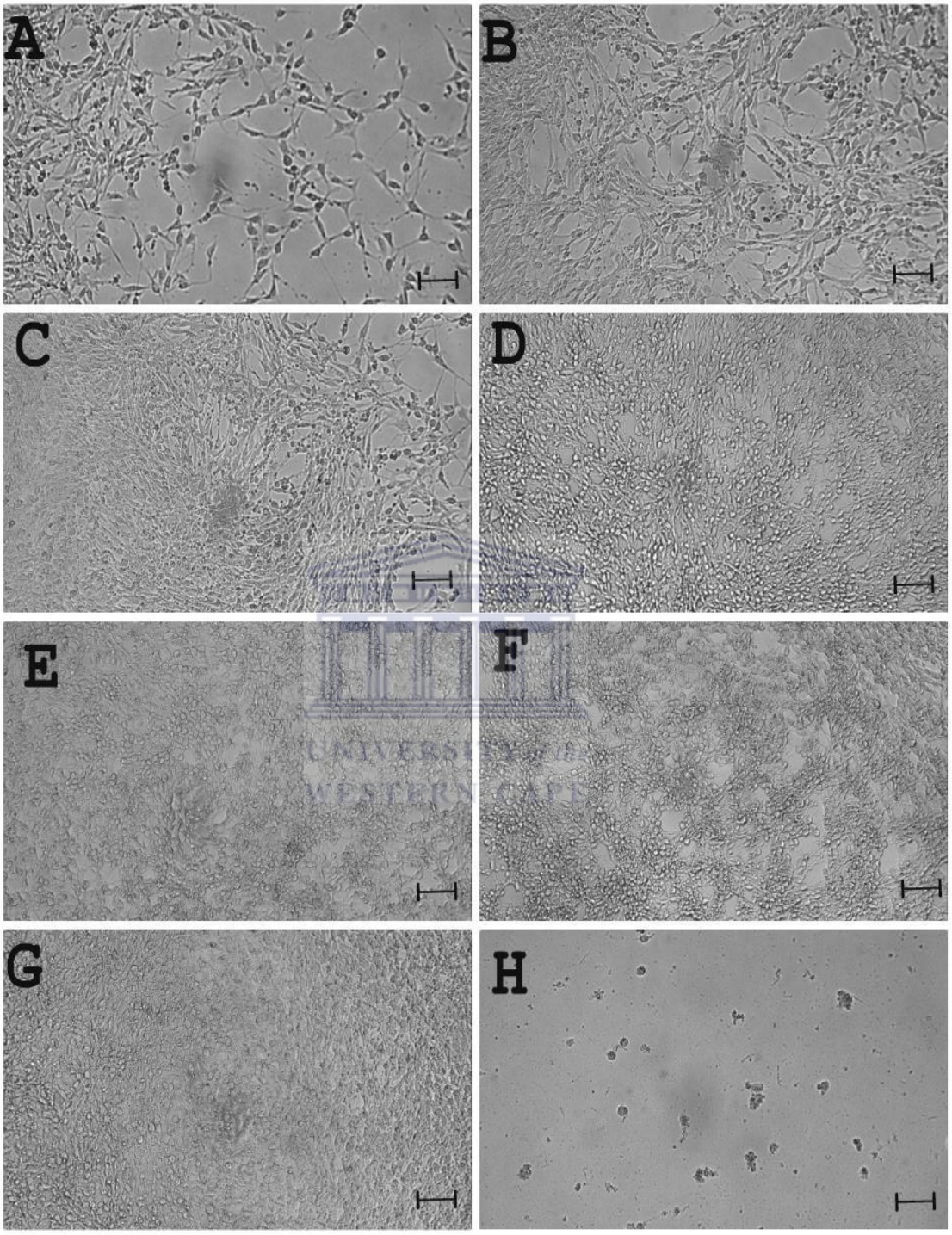

Figure 3.5: Micrographs showing the effect of flax seed on TM4 cells after 72 hours at 100 x magnification. Abbreviations: A = untreated cells, B = 0.01 µg/ml, C = 0.1 µg/ml, D = 1 µg/ml, E = 10 µg/ml, F = 100 µg/ml, G = 1000 µg/ml, H = 8 % DMSO. Bar = 50 µm.

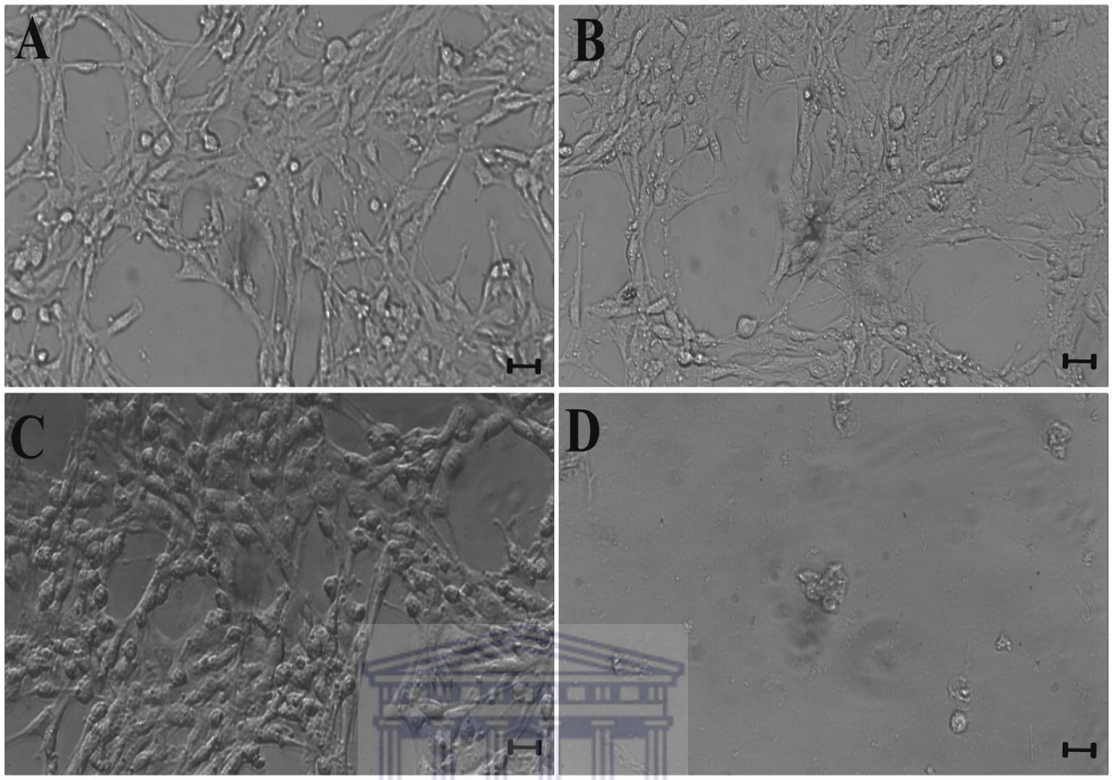

Figure 3.6: Morphology of TM4 cells after 24 hours flax seed exposure at 200 x magnification. There is no morphological changes between flax seed treated groups (B, C) and untreated control cells (A). DMSO treated cells appear clumped and markedly reduced in number. Abbreviations: A = untreated cells, B = 0.1 µg/ml, C = 1000 µg/ml, D = 8 % DMSO. Bar = 40 µm

3.1.3 Effect of DPP on MCF - 7 cell morphology

When compared to negative control group, all the DPP treated groups of MCF - 7 cells for 1 or 3 days showed no morphological changes (Fig. 3.7, 3.8 and 3.9). However, cells seem to display reduction in the number specifically at higher concentrations (1000 µg/ml). Moreover the MCF - 7 cells which were exposed to 8 % DMSO revealed shrinkage and a decrease in cell numbers.

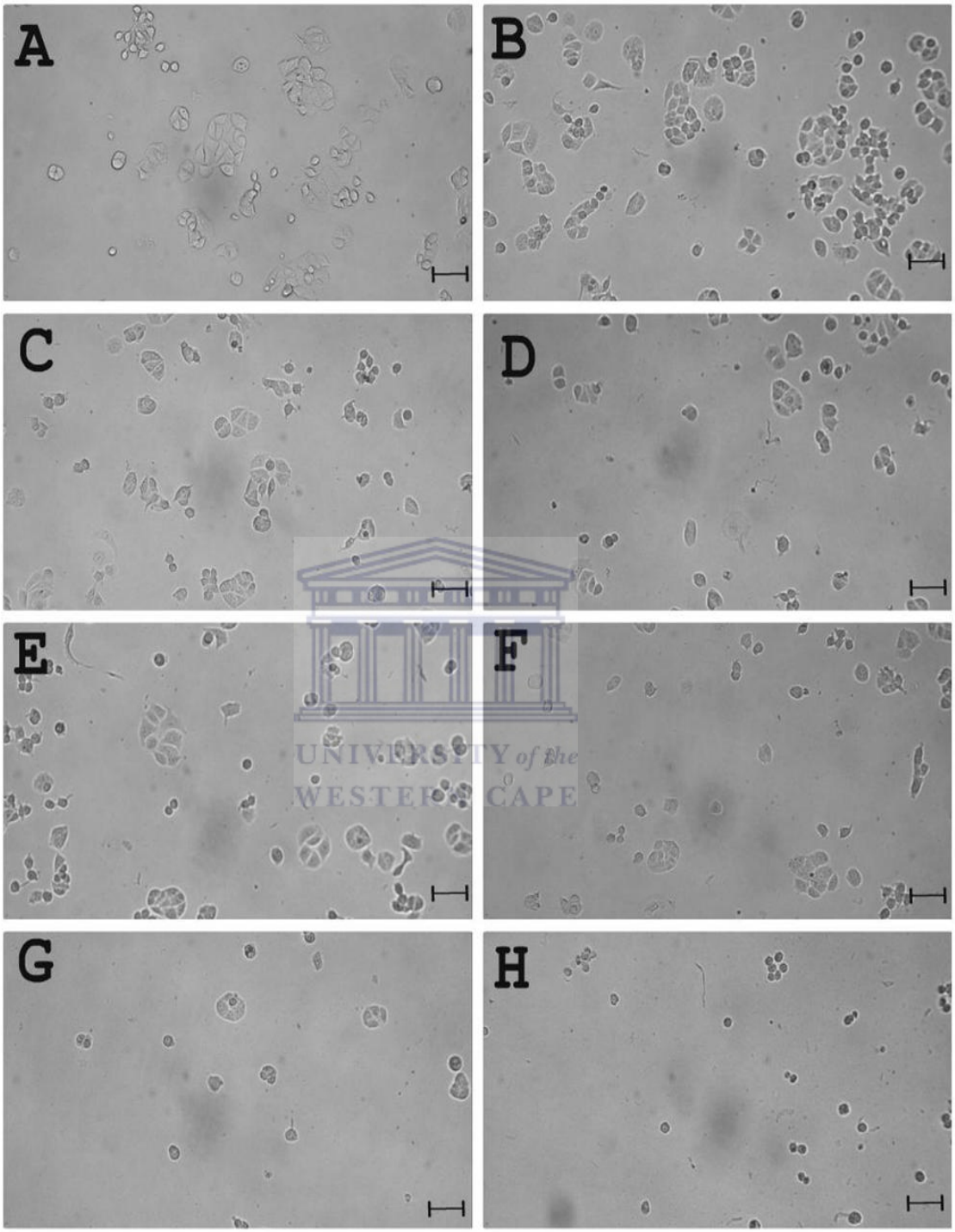

Figure 3.7: Micrographs showing the effect of DPP on MCF-7 cells after 24 hours at 100 x magnification. Abbreviations: A = untreated cells, B = 0.01 µg/ml, C = 0.1 µg/ml, D = 1 µg/ml, E = 10 µg/ml, F = 100 µg/ml, G = 1000 µg/ml, H = 8 % DMSO. Bar = 50 µm.

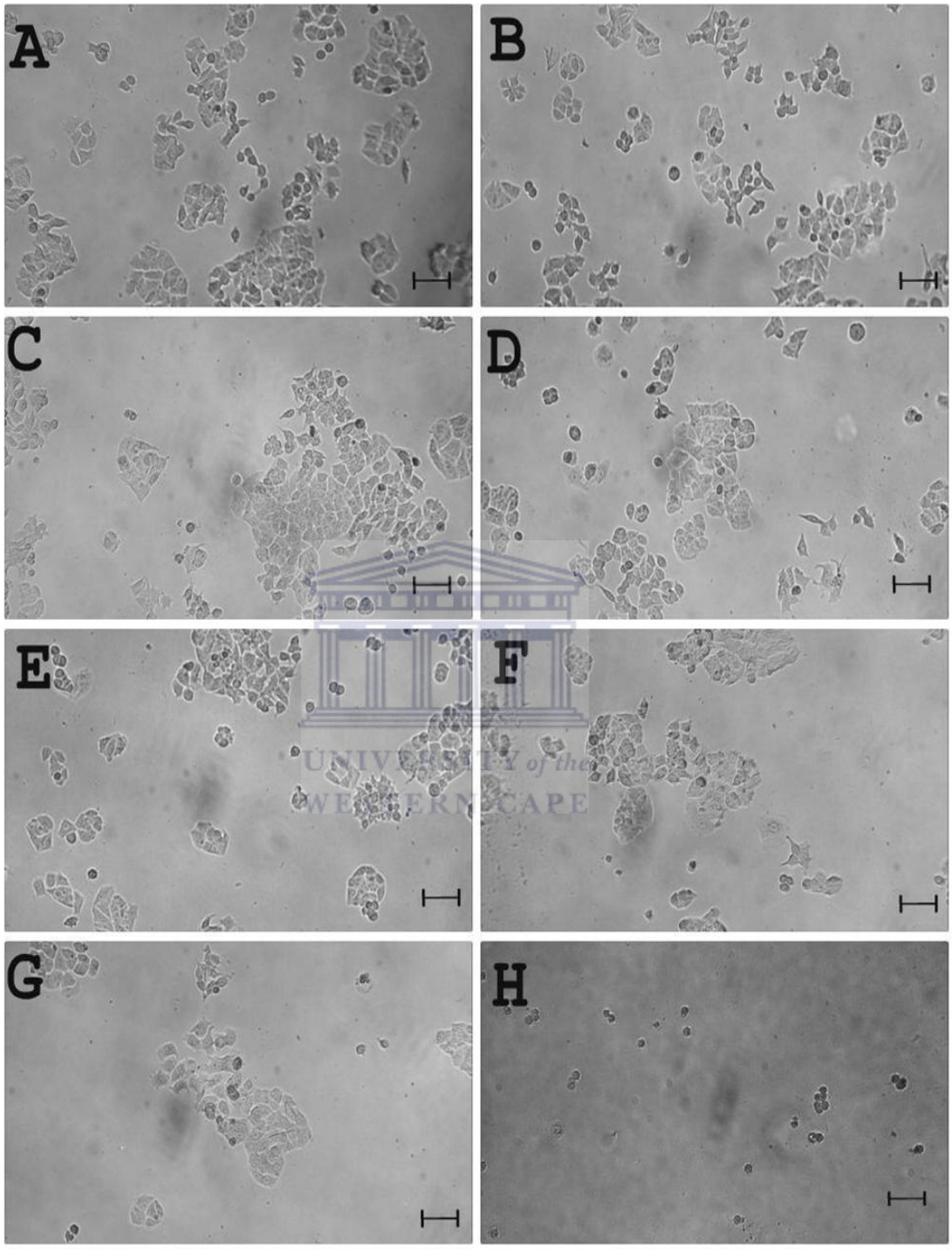

Figure 3.8: Micrographs showing the effect of DPP on MCF-7 cells after 72 hours at 100 x magnification. Abbreviations: A = untreated cells, B = 0.01 µg/ml, C = 0.1 µg/ml, D = 1 µg/ml, E = 10 µg/ml, F = 100 µg/ml, G = 1000 µg/ml, H = 8 % DMSO. Bar = 50 µm.

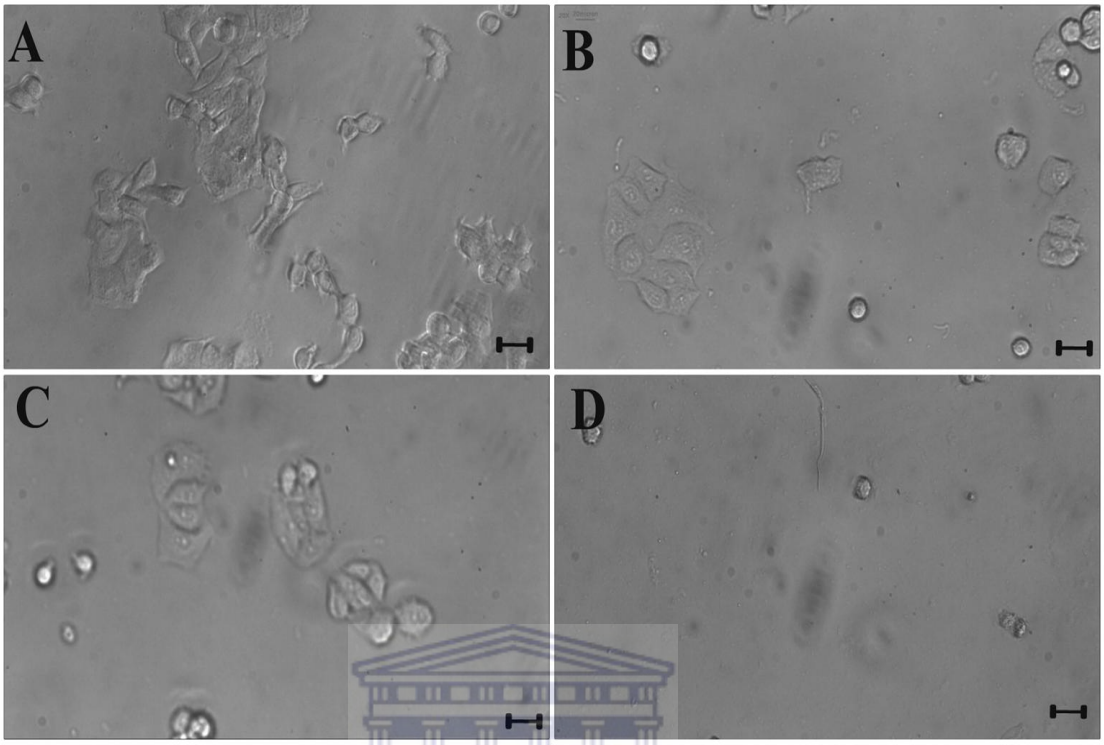

Figure 3.9: Morphology of MCF-7 cells after 24 hours DPP exposure at 200 x magnification. There is no morphological changes between DPP treated groups (B, C) and untreated control cells (A). DMSO treated cells markedly reduced in number. Abbreviations: A = untreated cells, B = 0.1 µg/ml, C = 1000 µg/ml, D = 8 % DMSO. Bar = 40 µm.

3.1.4 Effect of flax seed on MCF-7 cell morphology

As could be seen in Fig. 3.10, 3.11and 3.12, MCF - 7 cells that were exposed to different concentrations (0.01 - 1000 µg/ml) of flax seed showed no morphological alterations when compared to untreated cells, although a decline in cell number was detected at higher concentrations (1000 µg/ml). Again 8 % DMSO showed reduction in cell numbers and shrinkage of the cells.

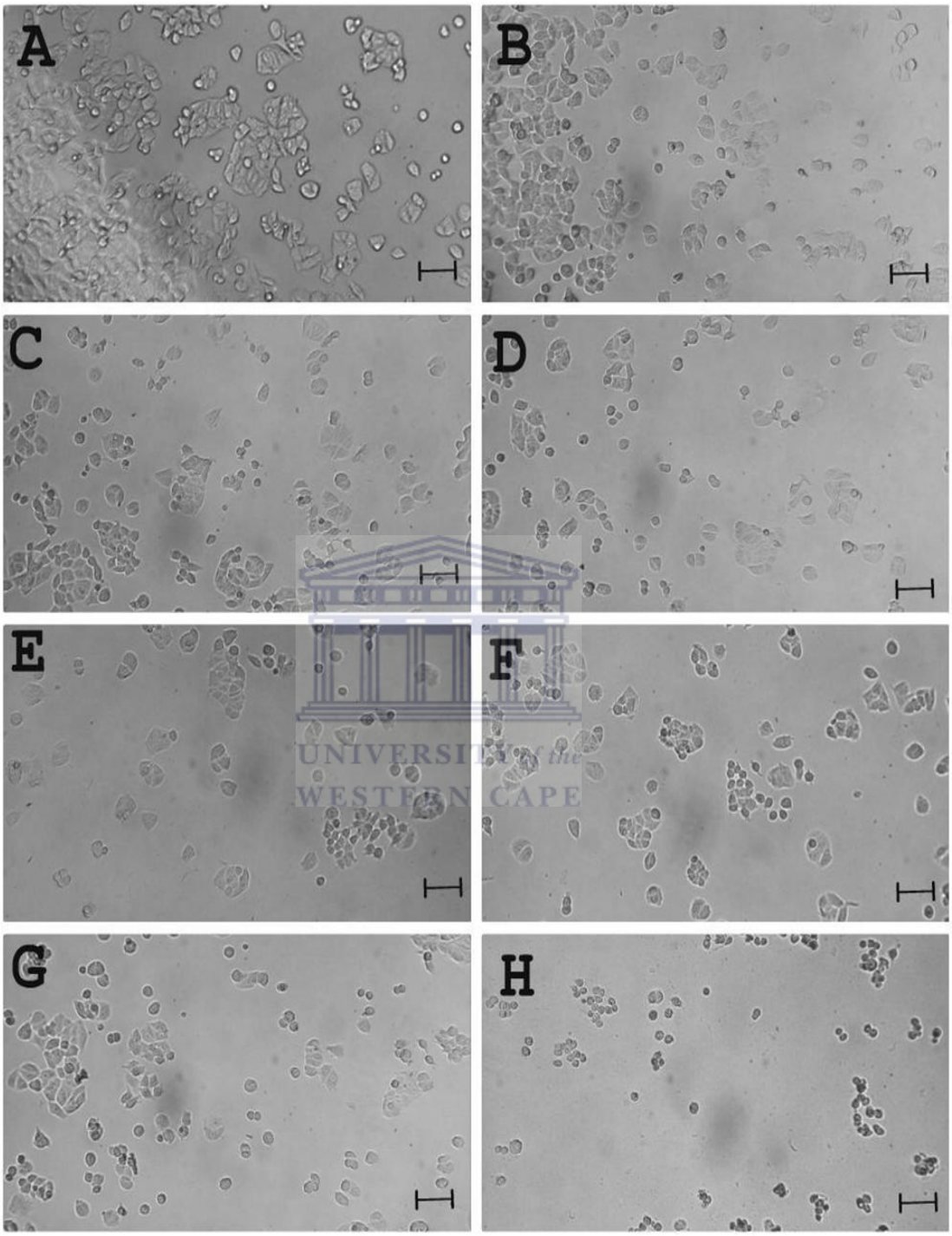

Figure 3.10: Micrographs showing the effect of flax seed on MCF-7 cells after 24 hours at 100 x magnification. Abbreviations: A = untreated cells, B = 0.01 µg/ml, C = 0.1 µg/ml, D = 1 µg/ml, E = 10 µg/ml, F = 100 µg/ml, G = 1000 µg/ml, H = 8 % DMSO. Bar = 50 µm.

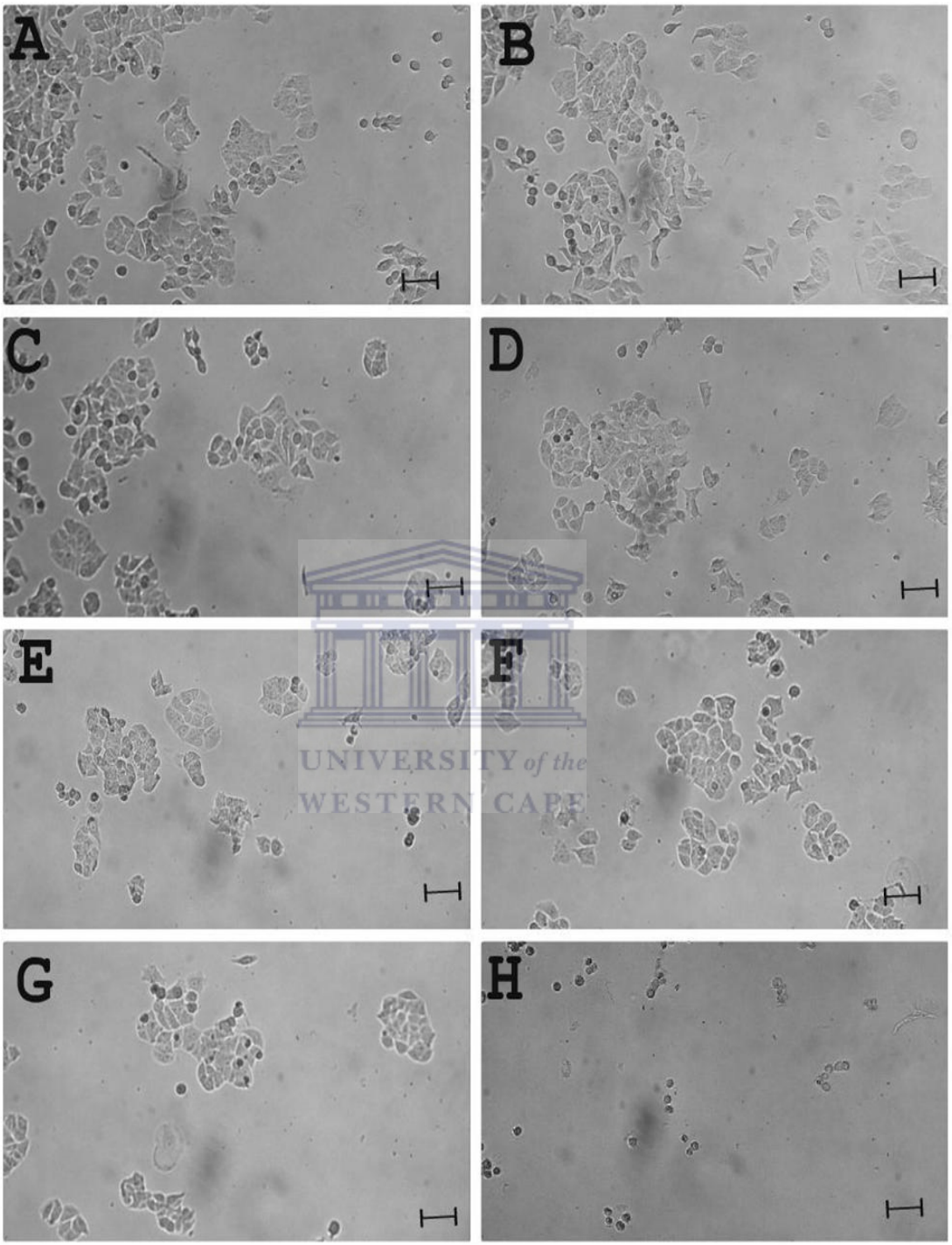

Figure 3.11: Micrographs showing the effect of flax seed on MCF-7 cells after 72 hours at 100 x magnification. Abbreviations: A = untreated cells, B = 0.01 µg/ml, C = 0.1 µg/ml, D = 1 µg/ml, E = 10 µg/ml, F = 100 µg/ml, G = 1000 µg/ml, H = 8 % DMSO. Bar = 50 µm.

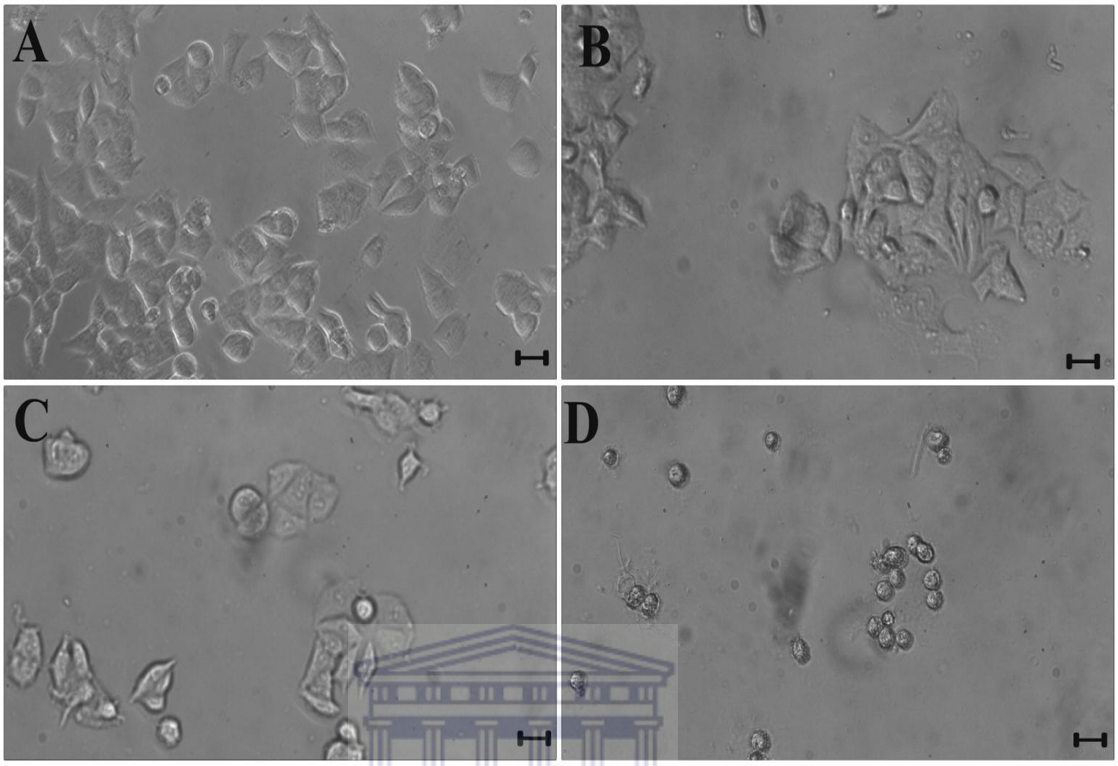

Figure 3.12: Morphology of MCF-7 cells after 24 hours flax seed exposure at 200 x magnification. There are no morphological changes between flax seed treated groups (B, C) and untreated control cells (A). DMSO treated cells markedly reduced in number. Abbreviations: A = untreated cells, B = 0.1 µg/ml, C = 1000 µg/ml, D = 8 % DMSO. Bar = 40 µm

3.2 Study of mitochondrial dehydrogenase enzyme activity by means of MTT assay

In order to test a potential cytotoxic effect of the DPP and flax seed extracts on the viability of TM4 and MCF - 7 cell lines, the cells were allowed to grow for 24 hours in a cell culture medium. Then they were exposed to six concentrations of each of the plant extracts (0.01 - 1000 µg/ml) for 1 or 3 days. The safety of 0.1 % DMSO on the viability of MCF-7 and TM4 Sertoli cells were conducted in a preliminary assay. It exhibited no cytotoxic effects on both used cell lines (Adefolaju *et al*, 2015).

3.2.1 Effect of DPP on TM4 cells mitochondrial dehydrogenase activity

TM4 cells incubated with the different concentrations of DPP for 24 hours displayed no significant changes in the mitochondrial dehydrogenase enzyme activities (Fig. 3.13) when cells were treated with low concentrations (0.01 - 10 µg/ml). Higher concentrations, however, expressed significantly increased activity of the mitochondrial dehydrogenase enzyme, compared to control cells (p values of 0.0002 - 0.0006 for 100 -1000 µg/ml concentrations). A highly significant reduction in the enzyme activity ($p < 0.0001$) was caused by the positive control (8 % DMSO).

After 72 hours exposure, all the concentrations yielded a slight but significant reduction in the activity of the mitochondrial enzyme at 100 - 1000 µg/ml, ($p = 0.02$ - 0.04) respectively, compared to control cells (Fig. 3.14). However, 8 % DMSO treated cells were still showing a more obvious reduction in the viability in compare to untreated cells, ($p < 0.0001$).

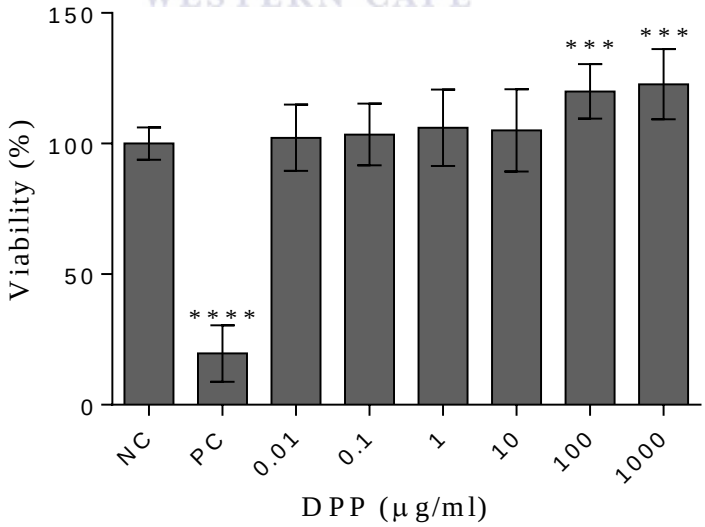

Figure 3.13: Viability of TM4 cells treated with DPP (0.01-1000) µg/ml after 24 hours. Values represented as the mean ± SD (n = 6). *** indicates p value ≤ 0.001 and **** = p value ≤ 0.0001 compared to NC group. Abbreviations: NC = untreated cells, PC = 8 % DMSO treated cells.

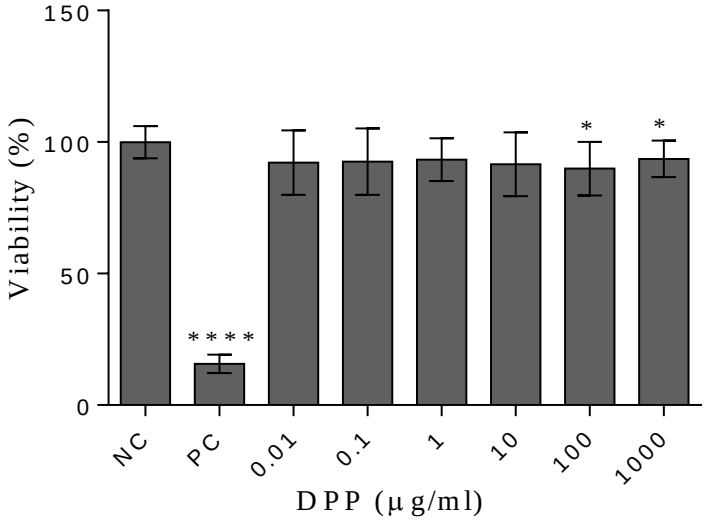

Figure 3.14: Viability of TM4 cells treated with DPP (0.01-1000) µg/ml after 72 hours. Values represented as the mean ± SD (n = 6). * indicates p value ≤ 0.05 and **** = p value ≤ 0.0001 compared to NC group. Abbreviations: NC = untreated cells, PC = 8 % DMSO treated cells.

3.2.2 Effect of flax seed on TM4 cells mitochondrial dehydrogenase activity

The activity of the mitochondrial dehydrogenase of TM4 cells after treatment with flax seed for 24 hours displayed (Fig. 3.15) a slight, but significant, reduction in the groups treated with 0.01, 0.1, 1, 10 µg/ml, ($p = 0.02, 0.03, 0.01, 0.04$) correspondingly. Yet the reduction showed in the groups treated with higher concentrations (100 - 1000 µg/ml) of flax seed was not significant compared to control group. A significant decrease was observed in 8 % DMSO treated group, ($p < 0.0001$).

The groups exposed to flax seed for 72 hours (Fig. 3.16) showed a marginal decline in the activity of mitochondrial dehydrogenase enzyme. However this reduction was only significant for the groups treated with higher concentrations (100 - 1000 µg/ml), ($p = 0.03 - 0.04$). 8 % DMSO treated group showed a highly significant reduction in the viability compared to untreated cells, ($p < 0.0001$).

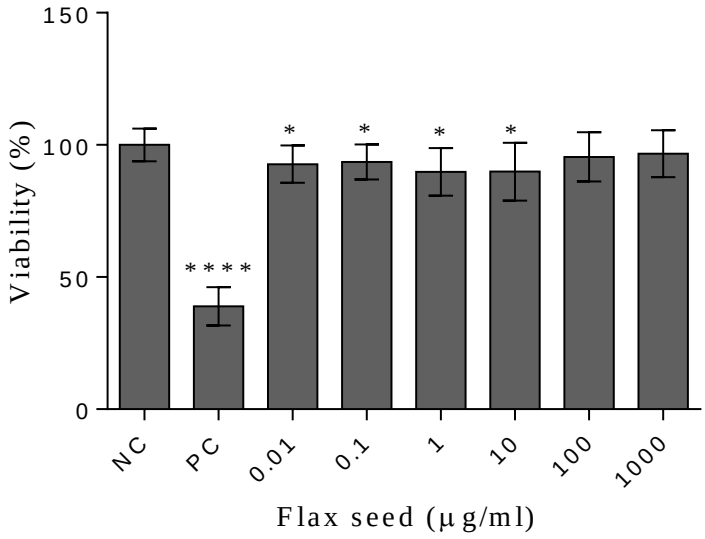

Figure 3.15: Viability of TM4 cells treated with flax seed (0.01-1000 µg/ml) after 24 hours. Values represented as the mean ± SD (n = 6). * indicates p value ≤ 0.05 and **** = p value ≤ 0.0001 compared to NC group. Abbreviations: NC = untreated cells, PC = 8 % DMSO treated cells.

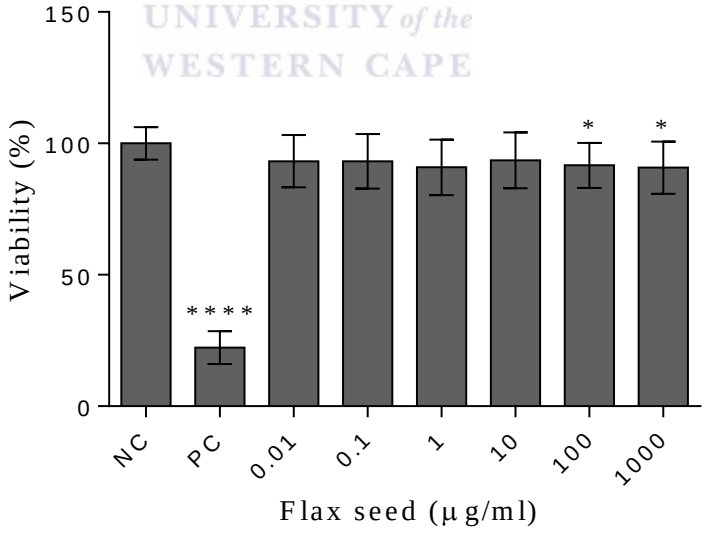

Figure 3.16: Viability of TM4 cells treated with flax seed (0.01-1000 µg/ml) after 72 hours. Values represented as the mean ± SD (n = 6). * indicates p value ≤ 0.05 and **** = p value ≤ 0.0001 compared to NC group. Abbreviations: NC = untreated cells, PC = 8 % DMSO treated cells.

3.2.3 Effect of DPP on MCF - 7 cells mitochondrial dehydrogenase activity

The treatment of MCF - 7 cells with DPP for 24 hours resulted in decreased activity of their mitochondrial dehydrogenases in a concentration- dependent manner (Fig. 3.17). While all the concentrations showed significant decrease, the higher concentrations (100 - 1000 µg/ml) expressed the most significant reduction, ($p = 0.0002$ and < 0.0001 respectively).

A similar effect of a concentration - dependent decline of the mitochondrial dehydrogenase enzyme activity was detected after 72 hours exposure of MCF - 7 cells to DPP in comparison to untreated group (Fig. 3.18). However, low concentrations (0.01 - 0.1 µg/ml) showed no significant decrease, while at 1 - 10 µg/ml significant reduction in the viability, ($p = 0.006$ - 0.001) was detected. Yet the highest decrease of the dehydrogenases activity ($p = 0.0002$) was observed at 100 - 1000 µg/ml.

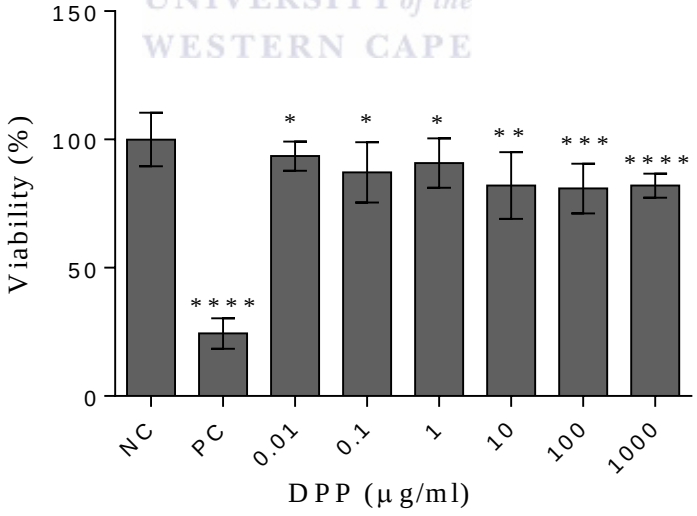

Figure 3.17: Viability of MCF-7 cells treated with DPP (0.01-1000 µg/ml) after 24 hours. Values represented as the mean ± SD (n = 6). * indicates p value ≤ 0.05, ** = p value ≤ 0.01, *** = p value ≤ 0.001 and **** = p value ≤ 0.0001 compared to NC group. Abbreviations: NC = untreated cells, PC = 8 % DMSO treated cells.

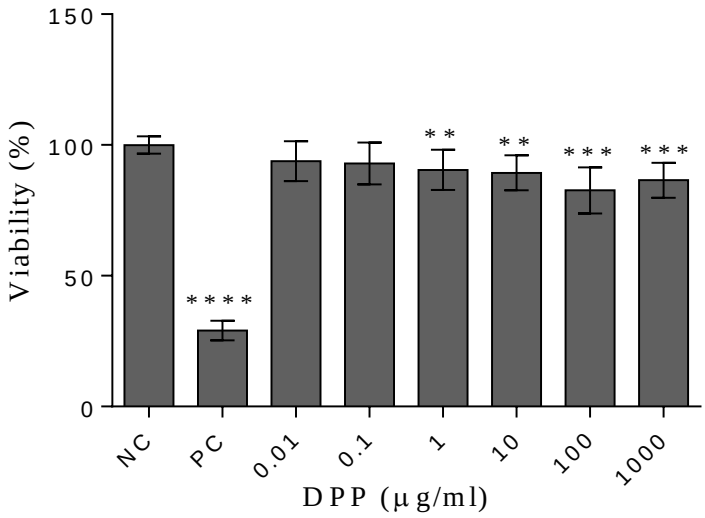

Figure 3.18: Viability of MCF-7 cells treated with DPP (0.01-1000 µg/ml) after 72 hours. Values represented as the mean ± SD (n = 6). ** indicates p value ≤ 0.01, *** = p value ≤ 0.001 and **** = p value ≤ 0.0001 compared to NC group. Abbreviations: NC = untreated cells, PC = 8 % DMSO treated cells.

3.2.4 Effect of flax seed on MCF-7 cells mitochondrial dehydrogenase activity

All the concentrations of flax seed extract showed slightly decreased activity of the mitochondrial dehydrogenase of MCF-7 cells after 24 hours exposure time when compared to control group (Fig. 3.19), although this reduction was not significant at 0.01 - 0.1 µg/ml, a significantly higher decrease in the enzyme activity occurred at higher concentrations (1, 10, 100 and 1000 µg/ml) with the following p values in the same order (0.03, 0.0005, 0.0004 and < 0.0001) indicating a concentration - dependent decline in cell viability.

After 72 hours exposure, the enzyme activity still displayed the same manner of a concentration - dependent decline (Fig. 3.20). Yet only 100 - 1000 µg/ml flax treated groups expressed significant reduction, p = (0.02 - 0.001) respectively compared to control group.

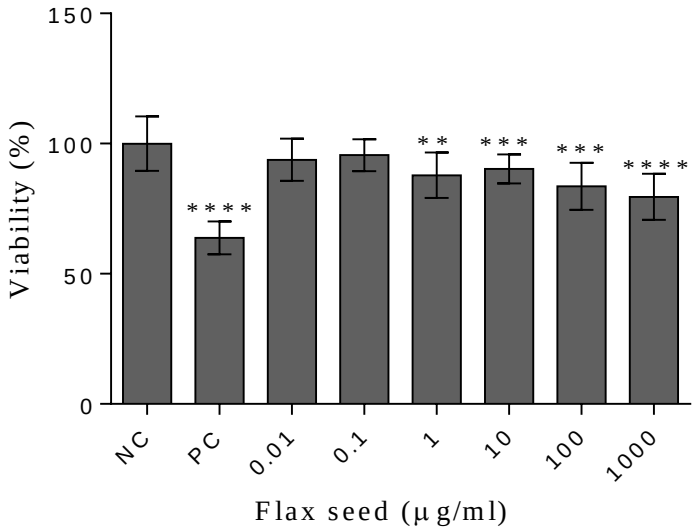

Figure 3.19: Viability of MCF-7 cells treated with flax seed (0.01 - 1000 µg/ml) after 24 hours exposure. Values represented as the mean ± SD (n = 6). ** indicates p value ≤ 0.01, *** = p value ≤ 0.001 and **** = p value ≤ 0.0001 compared to NC group. Abbreviations: NC = untreated cells, PC = 8 % DMSO treated cells.

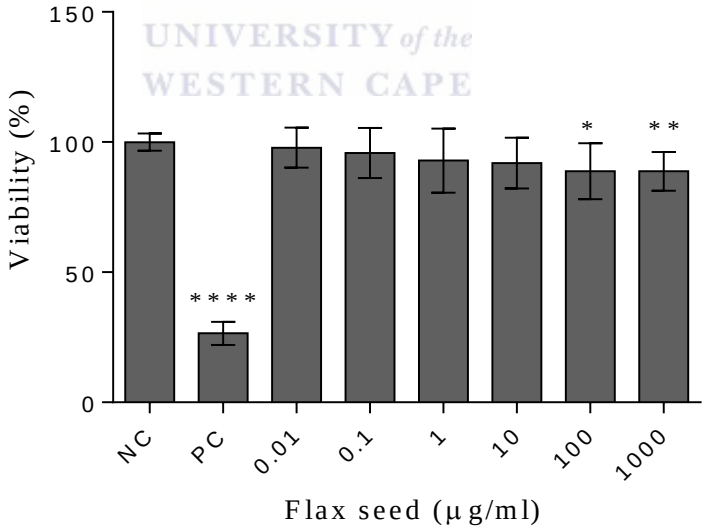

Figure 3.20: Viability of MCF-7 cells treated with flax seed (0.01 - 1000 µg/ml) after 72 hours exposure. Values represented as the mean ± SD (n = 6). * indicates p value ≤ 0.05, ** = p value ≤ 0.01and **** = p value ≤ 0.000 compared to NC group 1. Abbreviations: NC = untreated cells, PC = 8 % DMSO treated cells.

3.3 Quantification of apoptosis by using APOPercentage™ flowcytometry assay

Flowcytometry quantitative measurement of apoptotic cells were used to test the apoptotic effect of DPP and flax seed on TM4 and MCF-7 cells after 24 hours exposure. In the present study DMSO was used to induce apoptosis (Hanslick *et al*., 2009; Hong and Wang, 1995).

3.3.1 Influence of DPP on apoptosis in TM4 Sertoli cells

The APOPercentage assay showed a strong tendency to increase in the apoptotic cells percentage after 24 hours exposure with 0.1 and 1000 µg/ml DPP (Fig. 3.21.and 3.22) which peaked significantly ($p = 0.02$) after exposure to 10 µg/ml of DPP. Treatment with 6 % DMSO significantly increased the percentage of apoptotic cells ($p = 0.02$) compared to the untreated group.

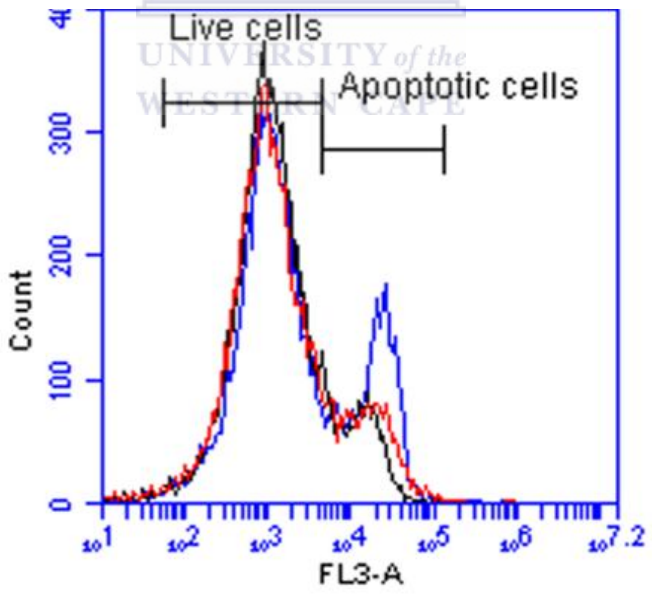

Figure 3.21: Flow cytometer analysis of influence of DPP on apoptosis in TM4 Sertoli cells. Overlay histogram showing live cells to the left and apoptotic cells to the right. The black histogram represents untreated cells, blue histogram represents 6 % DMSO and the red histogram represents 1000 µg/ml of DPP.

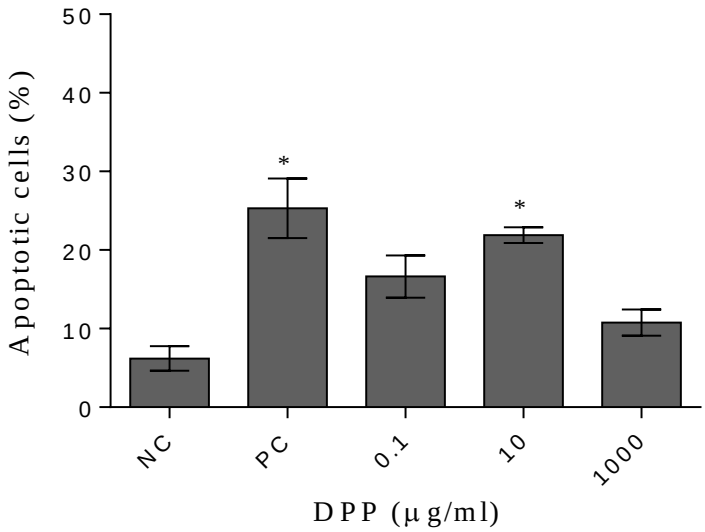

Figure 3.22: Quantification of apoptosis in TM4 cells treated with DPP (0.1, 10, 1000 µg/ml) after 24 hours exposure, values represented as mean ± SD (n = 6). Abbreviations: NC = untreated cells, PC = 6 % DMSO treated cells, * = p value ≤ 0.05 compared to NC group.

3.3.2 Influence of flax seed on apoptosis in TM4 Sertoli cells

TM4 cells that were exposed to different concentrations of flax seed displayed a tendency to increase in the apoptotic cells percentage at groups treated with 0.1 and 10 µg/ml (Fig. 3.23 and 3.24). On the contrary, a higher concentration of 1000 µg/ml caused a tendency to decrease in the apoptotic cells percentage. The positive control caused a significant three – fold rise in apoptotic cells.

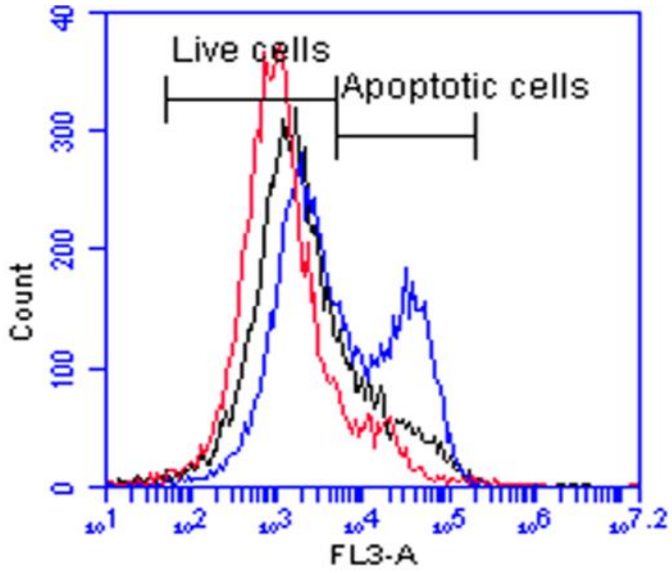

Figure 3.23: Flow cytometer analysis of influence of flax seed on apoptosis in TM4 Sertoli cells. Overlay histogram showing live cells to the left and apoptotic cells to the right. The black histogram represents untreated cells, blue histogram represents 6 % DMSO and the red histogram represents 1000 µg/ml of flax seed.

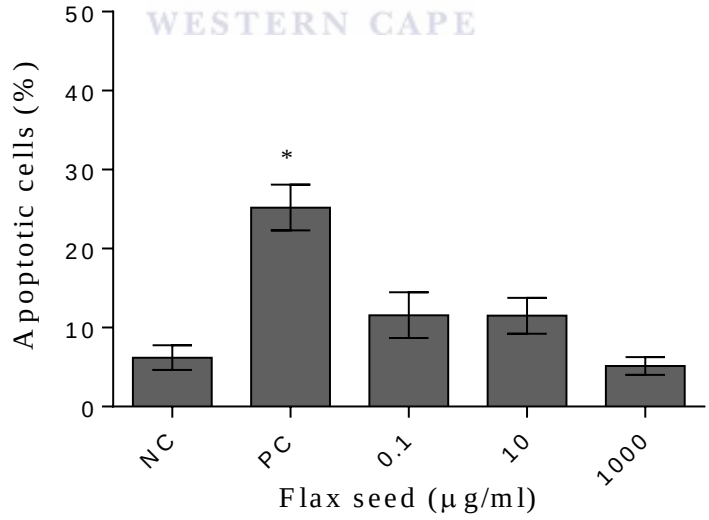

Figure 3.24: Quantification of apoptosis in TM4 cells treated with flax seed (0.1, 10, 1000 µg/ml) after 24 hours exposure, values represented as mean ± SD (n = 6). Abbreviations: NC = untreated cells, PC = 6 % DMSO treated cells, * = p value ≤ 0.05 compared to NC group.

3.3.3 Influence of DPP on apoptosis in MCF - 7 breast cancer cell

The APOPercentage assay revealed a significant increase in apoptotic cells ($p = 0.01$) among the positive control group. However DPP at 0.1 and 1000 µg/ml raised the percentage of apoptotic cells without significance, while 10 µg/ml of DPP showed a strong tendency to decrease in the apoptotic cells percentage compared to the negative control group of MCF-7 cells (Fig. 3.25 and 3.26).

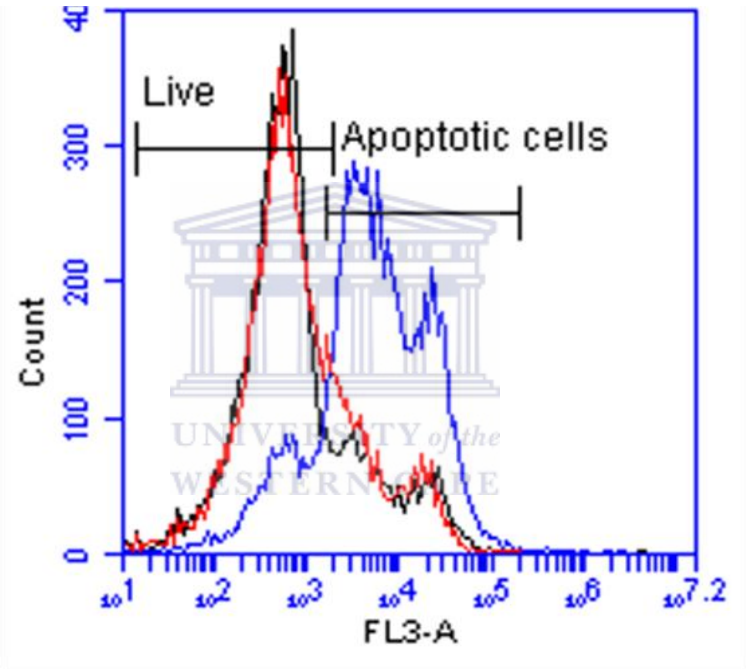

Figure 3.25: Flow cytometer analysis of influence of DPP on apoptosis in MCF-7 cells. Overlay histogram showing live cells to the left and apoptotic cells to the right. The black histogram represents untreated cells, blue histogram represents 6 % DMSO and the red histogram represents 1000 µg/ml of DPP.

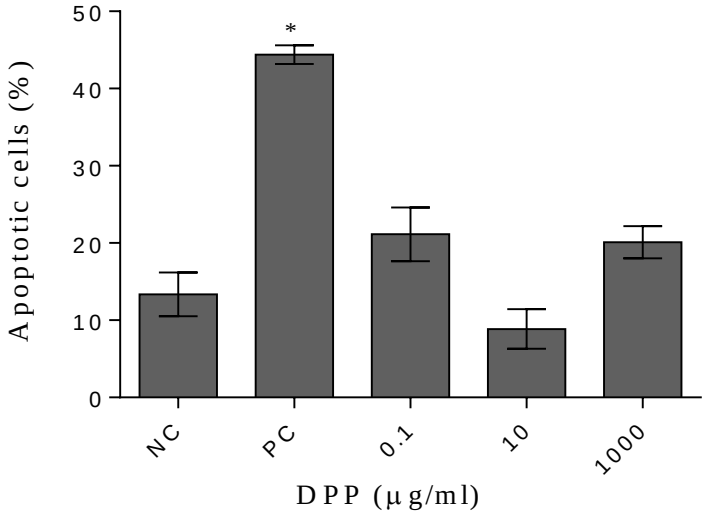

Figure 3.26: Quantification of apoptosis in MCF-7 cells treated with DPP (0.1, 10, 1000 µg/ml) after 24 hours exposure, values represented as mean ± SD (n = 6). Abbreviations: NC = untreated cells, PC = 6 % DMSO treated cells, * = p value ≤ 0.05 compared to NC group.

3.3.4 Influence of flax seed on apoptosis in MCF - 7 breast cancer cell

Flax seed treatments for 24 hours led to slight increasing and decreasing changes in the apoptotic cells percentage of MCF - 7 cells (Fig. 3.27 and 3.28). A concentration - dependent increase was displayed firstly at 0.1 and 10 µg/ml followed by a reduction at 1000 µg/ml; yet, all the changes were insignificant in comparison with negative control. However a significant increase could be seen in the positive control group (p = 0.01).

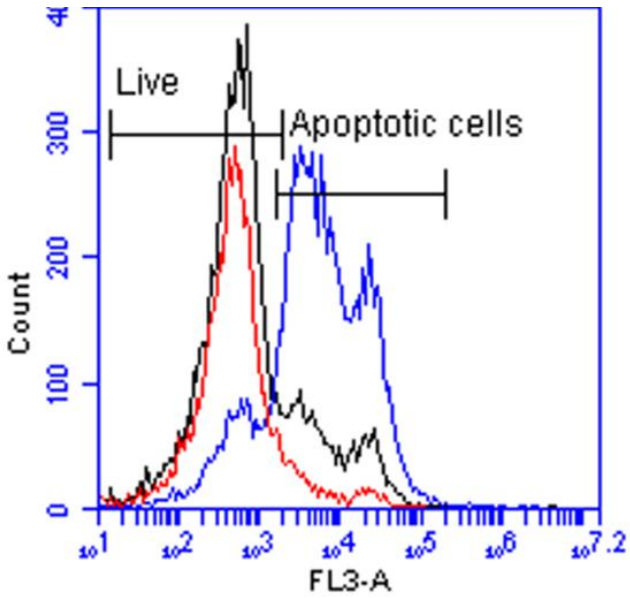

Figure 3.27: Flow cytometer analysis of influence of flax seed on apoptosis in MCF-7 cells. Overlay histogram showing live cells to the left and apoptotic cells to the right. The black histogram represents untreated cells, blue histogram represents 6 % DMSO and the red histogram represents 1000 µg/ml of flax seed

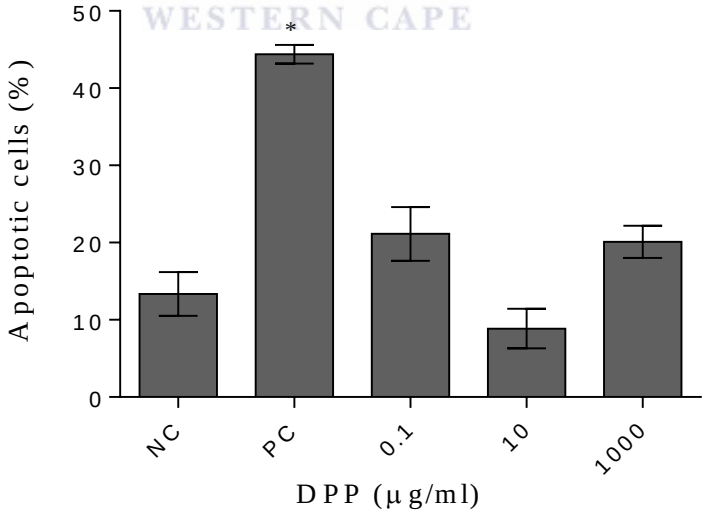

Figure 3.28: Quantification of apoptosis in MCF-7 cells treated with flax (0.1, 10, 1000 µg/ml) after 24 hours exposure, values represented as mean ± SD (n = 6). Abbreviations: NC = untreated cells, PC = 6 % DMSO treated cells, * = p value ≤ 0.05 compared to NC group.

3.4 Transepithelial electrical resistance Study (TEER assay)

To assess the integrity of the tight junctions between adjacent TM4 Sertoli cells the trans-epithelial electrical resistance was measured across the cell monolayers using the electrical resistance system. When TM4 cells were incubated with the plant extracts for 72 hours and the TEER measured daily, the following results were obtained.

3.4.1 Effect of DPP on Sertoli TM4 cells transepithelial electrical resistance

After 24 hours there was a slight tendency to drop in the TEER of TM4 cells monolayers when exposed to low concentrations of DPP (0.01 - 0.1 µg/ml). This was followed by a slight increase within the group treated with 1 µg/ml with no significance when compared to control group (Fig. 3.29). On contrast, groups which were exposed to higher concentrations (10 - 1000 µg/ml) displayed a highly significant increase in the resistance ($p < 0.0001$). At 48 hours TEER showed no change within 0.01 µg/ml treated group in relative to the control group. Thereafter increased resistance recorded when the cells exposed to higher concentrations with high significance at concentrations between 10 – 1000 µg/ml ($p < 0.0001$). The low concentrations of DPP showed no significant changes after 72 hours as yet, while higher treatments (1 - 1000 µg/ml) revealed the significant increase ($p < 0.006$) in compare to control group.

In comparison with 24 hours, treatment of TM4 monolayer with higher concentrations of DPP (10 – 1000 µg/ml) resulted in significant decrease ($p < 0.0001$) in TEER after 48 hours followed by increase after 72 hours but still significantly ($p = 0.007$-0.001) lower than the resistance after 24 hours.

Thus, treatment of TM4 Sertoli cells with higher concentrations of DPP resulted in a concentration - dependent increase in the electrical resistance over the three days of exposure in comparison with untreated groups.

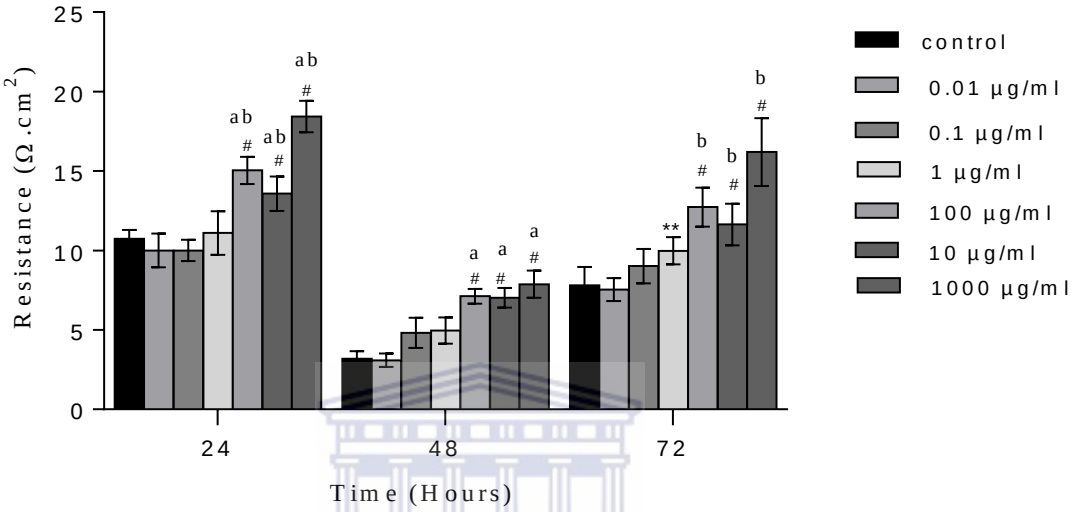

Figure 3.29: Effect of DPP on TEER of TM4 cells over 3 days. Values are represented as mean ± SD (n = 4). # indicates significant difference ($p \leq 0.0001$) compared to control group. Bars with same superscript(s) are significantly different ($p \leq 0.05$) in comparison with the same concentration from 24 hours exposure.

3.4.2 Effect of flax seed on Sertoli TM4 cells Transepithelial electrical resistance

The changes of TEER of TM4 cells exposed to flax seed for 24 hours ranged between decline (0.01 µg/ml) and increase at concentrations from 0.1 - 1000 µg/ml relative to control group, although none of those changes were significant with control.

After 48 hours, the resistance of TM4 cells monolayer which was treated with flax seed showed no significant changes when compared to control group. Yet, a statistical significant rise was detected at 1000 µg/ml ($p = 0.001$) (Fig. 3.30).

A concentration - dependent rise in the TEER could be seen among the TM4 cells monolayer incubated with flax seed for 72 hours in comparison to control group. Beyond 0.01 µg/ml all the treatments recorded significant increases reaching a peak at 100 - 1000 µg/ml ($p < 0.0001$).

In comparison with 24 hours, treatment of TM4 cells monolayer with flax seed concentrations (0.01 – 100 µg/ml) resulted in a tendency to reduction in TEER after 48 hours , however, 1000 µg/ml of flax seed showed a tendency to increase. After 72 hours flax seed concentrations higher than 0.1 µg/ml led to higher TEER records in comparison with 24 hour treatments with only significance at 1000 µg/ml ($p = 0.04$).

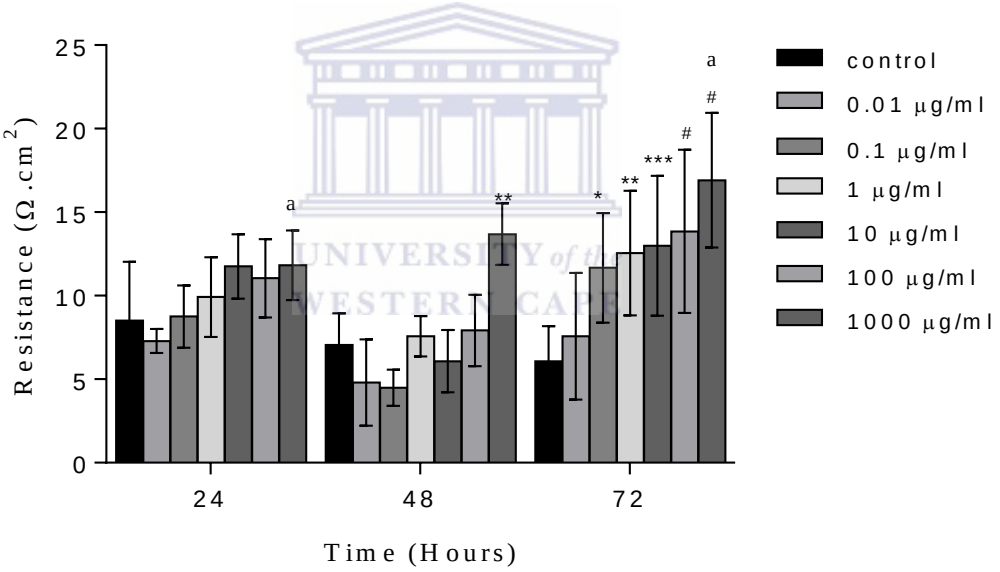

Figure 3.30: Effect of flax seed on TEER of TM4 cells over 3 days. Values represented as mean ± SD (n = 4). * indicates $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$ and # = $p \leq 0.0001$ in comparison with control. Bars with same superscript are significantly different ($p \leq 0.05$) in comparison with the same concentration from 24 hours exposure.

3.5 TM4 Sertoli cell Gamma glutamyl transpeptidase (GGT) enzyme activity

To demonstrate the effect of DPP and flax seed extracts on the activity of GGT enzyme, TM4 cells were exposed to different concentrations (0.01, 0.1, 1, 10, 100 and 1000 µg/ml) of the plant extracts for 72 hours and the GGT enzyme activity was measured according to the manufacturer protocol.

3.5.1 Effect of DPP on TM4 Sertoli cell GGT enzyme activity

The activity of GGT enzyme showed no significant changes among the groups treated with lower concentrations of DPP. However, as the concentrations of DPP increased (100 - 1000 µg/ml), a significantly higher activity of GGT was observed ($p \leq 0.01$). The activity of GGT revealed a high significant rise ($p = 0.0007$) among positive control (supplied with the kit) compared to negative control (Fig. 3.31).

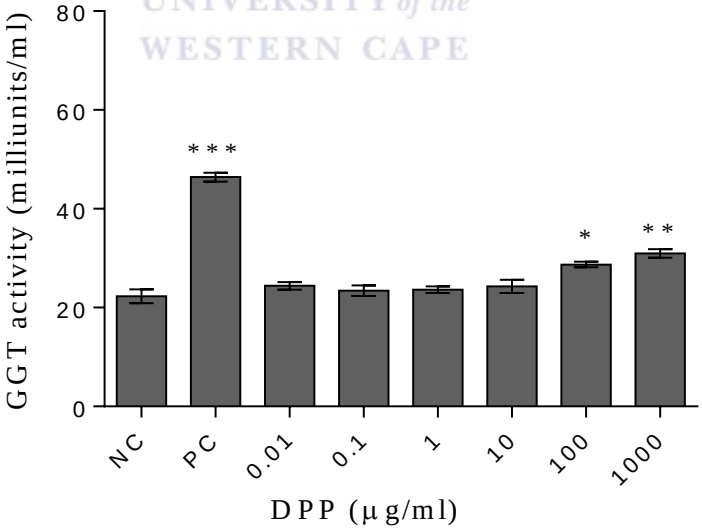

Figure 3.31: Effect of DPP (0.01-1000 µg/ml) on TM4 GGT enzyme activity after 72 hours of exposure. Data represented as mean ± SD (n = 4). Abbreviations: NC = untreated cells, PC = positive control, * = p value < 0.05, ** = p value ≤ 0.01, *** = p value ≤ 0.001 compared to NC group, GGT = gamma glutamyl transpeptidase enzyme.

3.5.2 Effect of flax seed on TM4 Sertoli cell GGT enzyme activity

All groups of TM4 cells exposed to different concentrations of flax seed extract showed (Fig. 3.32) a significant increase in the activity of GGT enzyme relative to negative control cells after 72 hours exposure time ($p < 0.003$).

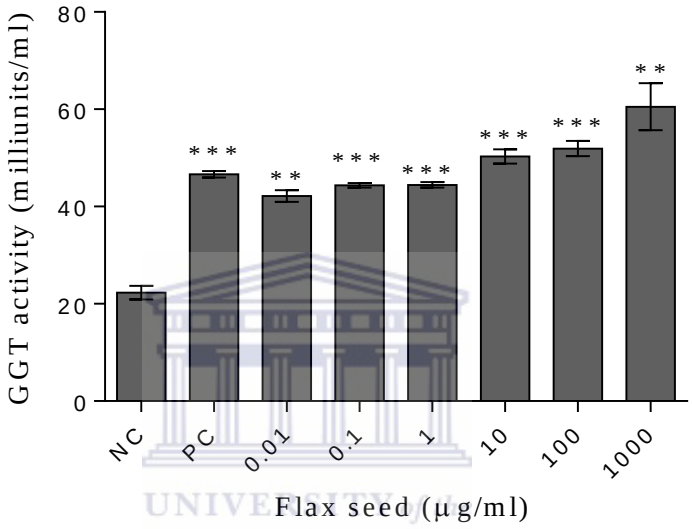

Figure 3.32: Effect of flax seed (0.01-1000 µg/ml) on TM4 GGT enzyme activity after 72 hours of exposure. Data represented as mean ± SD (n = 4). Abbreviations: NC = untreated cells, PC = positive control, ** = p value ≤ 0.01, *** = p value ≤ 0.001 compared to NC group, GGT = gamma glutamyl transpeptidase enzyme.

3.6 TM4 Sertoli cell Inhibin - B hormone production

To study the effect of DPP and flax seed extracts on mouse Sertoli cells inhibin - B production, TM4 cells were kept for 72 hours with different concentrations of the plant extracts (0.01 – 1000 µg/ml), after that the supernatant was used freshly to analyze the inhibin contents according to the protocol of the kit.

3.6.1 Effect of DPP on TM4 Sertoli cell Inhibin - B production

Compared to untreated cells, all the concentrations of DPP caused a significant concentration - dependent increase in the production of Inhibin - B with the p value ranging between 0.01 - 0.0002 for 0.01 - 1000 µg/ml DPP, respectively (Fig. 3.33). The positive control (supplied with the kit) showed very high values of Inhibin - B hormone (9737.65 pg/ml), thus we did not display these values in Fig. 3.33 and.3.34.

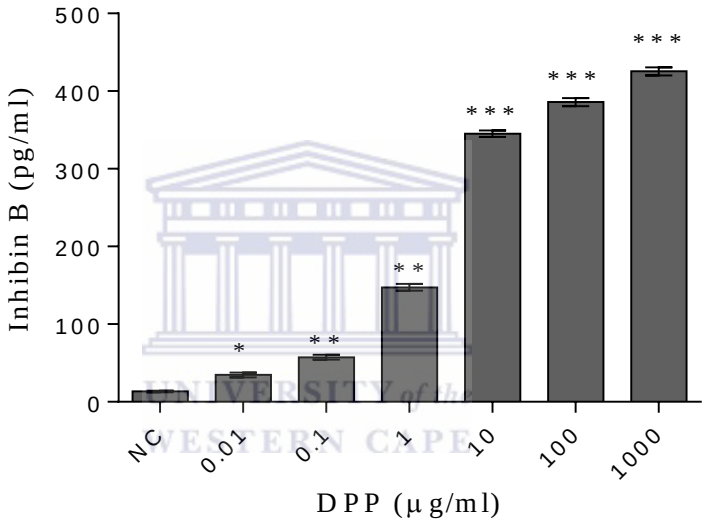

Figure 3.33: Effect of DPP (0.01-1000 µg/ml) on TM4 inhibin production after 72 hours. Data represented as mean ± SD (n = 4). Abbreviations: NC = untreated cells, * = p value ≤ 0.05, ** = p value ≤ 0.01, *** = p value ≤ 0.001 compared to NC group. The positive control showed very high records of (9737.65 pg/ml), and is thus not included in the figure

3.6.2 Effect of flax seed on TM4 Sertoli cell Inhibin - B production

A concentration - dependent manner toward significantly increased production of inhibin hormone was detected when mouse Sertoli cells were treated with different concentrations of flax seed ethanolic extract for 72 hours; with exception of the lowest concentration (0.01µg/ml) which displayed no changes compared to untreated

cells (Fig. 3.34). The positive control recorded the highest reading of inhibin hormone (9737.65 pg/ml), (not shown in Fig. 3.33 and 3.34).

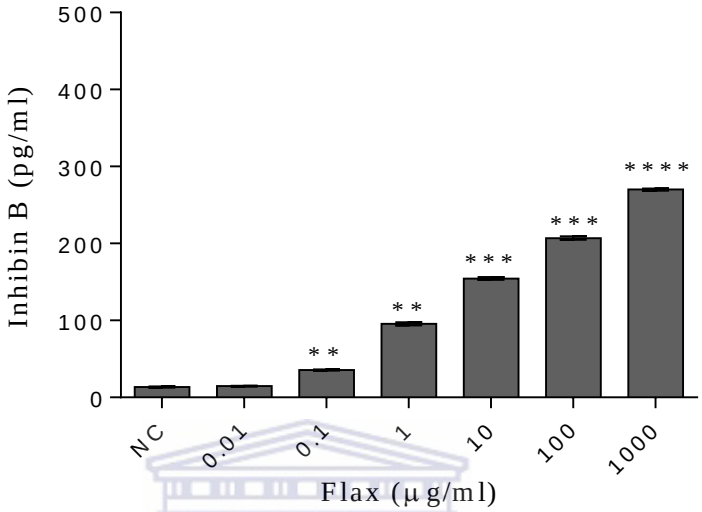

Figure 3.34: Effect of flax seed (0.01-1000 µg/ml) on TM4 inhibin production after 72 hours. Data represented as mean ± SD (n = 4). Abbreviations: NC = untreated cells, ** = p value ≤ 0.01, *** = p value ≤ 0.001 and **** = p value ≤ 0.0001 compared to NC group. The positive control showed very high record of (9737.65 pg/ml) and is thus not included in the figure.

3.7 Quantitative assessment of estrogenicity of plant ethanolic extracts by using E - SCREEN assay

This quantitative assay is based on comparing the cell numbers of MCF-7 cells that were treated with different concentrations of DPP and Flax seed with the untreated (negative control) cells and estradiol treated (positive control) cells. The (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reagent was used to assess the proliferation of MCF-7 cells after 6 days treatment with extracts or 1 nM estradiol. As the MTT assay measures the activities of mitochondrial dehydrogenases in living cells, this assay reflects also the numbers of cells. In contrast to the usual MTT assay, media used in the E – SCREEN assay was free of phenol red (which displays weak estrogenic activity) and all sera were stripped of steroids (such as

estrogen and androgen) by charcoal – dextran treatment. Also, the incubation time was 6 days instead of 1 or 3 days.

3.7.1 Assessment of the estrogenicity of DPP by mean of E - SCREEN assay

17β - Estradiol (1 nM) treatment of MCF - 7 cells yielded the highest significant proliferative effect when compared to untreated cells (p = 0.0002). All lower concentrations of DPP (0.01 – 10 µg/ml) produced a decline in the proliferation percentage of MCF – 7 cells with significancies ranged between p = 0.002-0.0001 (Fig. 3.35). However, mitochondrial dehydrogenase activities started to rise after exposure to DPP concentrations of 100 and 1000 µg/ml in comparison with lower concentrations.

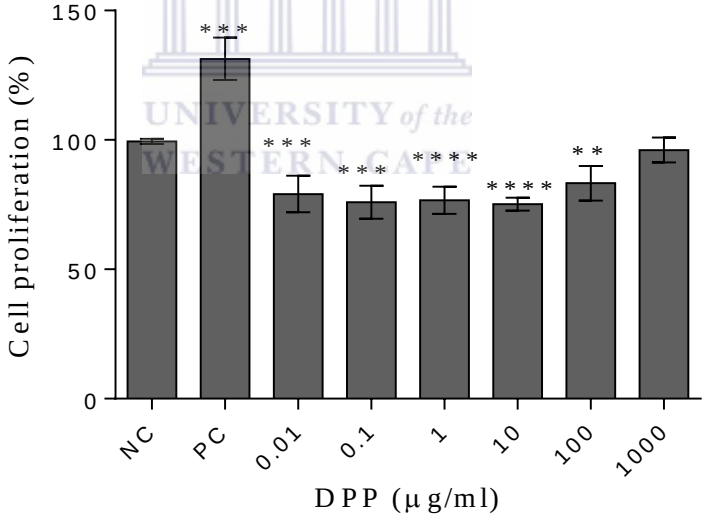

Figure 3.35: Estrogenic effect of DPP (0.01 – 1000 µg/ml) using E- SCREEN assay after 144 hours. MTT was used to measure the proliferation of MCF- 7 cells. Data represented as mean ± SD (n = 8). Abbreviations: NC = negative control, PC = positive control treated with 1 nM estradiol. * Indicates p value ≤ 0.05, ** = p value ≤ 0.01, *** = p value ≤ 0.001 and **** = p value ≤ 0.0001 compared to NC group.

3.7.2 Assessment of the estrogenicity of flax seed by mean of E - SCREEN assay

The estrogenic effect of flax seed was measured by E- SCREEN assay for 144 hours. All concentrations revealed a significant decrease in the proliferation of MCF-7 cells (Fig. 3.36) with the highest significance among groups exposed to 0.1- 1 µg/ml where the $p \leq 0.0001$. However, mitochondrial dehydrogenase activities started to rise after exposure to flax seed concentrations of 100 and 1000 µg/ml. Again, 17 - β estradiol showed a marked increase in MCF-7 proliferation ($p = 0.0001$).

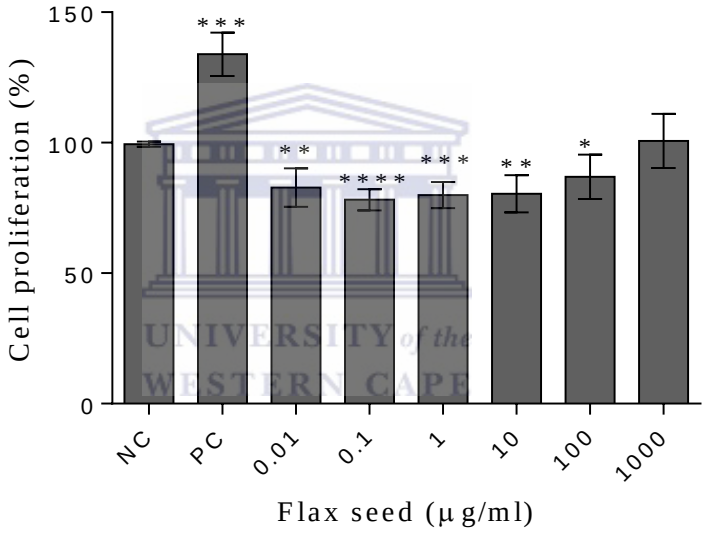

Figure 3.36: Estrogenic effect of flax seed (0.01 – 1000 µg/ml) using E- SCREEN assay after 144 hours. MTT was used to measure the proliferation of MCF- 7 cells. Data represented as mean ± SD (n = 8). Abbreviations: NC= negative control, PC= positive control treated with 1 nM estradiol. * indicates p value ≤ 0.05, ** = p value ≤ 0.01, *** = p value ≤ 0.001 and **** = p value ≤ 0.0001 compared to NC group.

Chapter IV: Discussion

Male infertility is responsible for 40 % of the infertility problems within couples according to WHO estimations. The causes vary from genetic disorders, genital duct obstruction, varicocele, reduced sperm production, low semen quality parameters, to problems with erection. In order to treat these problems, people rely either on modern (western) medicine or herbal (traditional) medicine (Mohammadi *et al*., 2013).

The utilization of herbs, herbal materials, herbal formulations, and herbal products that contain parts of plants to treat illnesses is defined as herbal medicine or phytomedicine where either the whole plant or any of its parts could be used in herbal therapy. The use of medicinal plants has been recorded since the Middle Paleolithic age about 60,000 years ago (Fabricant and Farnsworth, 2001). The number of people who have chosen the herbal medicine as a choice to improve their health has been increased in the recent era (Fabricant and Farnsworth, 2001; Pan *et al*., 2014).

Among the medicinal plants that are in use to treat male infertility are Date palm pollen (DPP) and flax seed. DPP was consumed by the early Egyptians and ancient Chinese for fertility enhancement (El-Kashlan *et al*., 2015). Moreover, phytochemical studies of DPP and flax seed revealed the presence of valuable constituents. Flavonoids, estradiol and estrone have been detected in DPP. Similarly flavonoids, glycerides, saponins and alkaloids were detected in flax seed. In addition flax seed also has antioxidant properties (Abbas and Ateya, 2011; Bekal *et al*., 2015).

Several *in vivo* studies have reported the androgenic effects of DPP and its ability to increase serum testosterone levels in rats (Arfat *et al*., 2014; Bahmanpour *et al*., 2006; Mehraban *et al*., 2014). Moreover, DPP administration to male rabbits resulted in significant increase in testicular weight and sperm concentration in comparison with the non- treated group (Faleh and Sawad, 2006). In addition, *in vitro* cultivation of granulosa cells and oocytes from female mice in the media supplemented with

DPP resulted in significant increase in the rate of follicle growth and maturation (Abdollahi *et al*., 2015).

4.1 Effects of ethanolic plants extracts on TM4 Sertoli cell line

4.1.1 TM4 Sertoli cell viability and morphology

Development of germ cells occurs due to an intimate relationship with Sertoli cells extending from the base to the apex of the Sertoli cells. Therefore Sertoli cells provide support and play an important function in spermatogenesis. Sertoli cells respond to FSH proliferative effect so they might be used as an indicator of the proliferative effects of plant extracts (Osibote *et al.*, 2011) with potential gonadotrophic properties.

MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) salt is reduced by the mitochondrial dehydrogenase of viable cells to form blue formazan crystals and the amount of formazan produced is proportional to the number of living cells present in the cell culture (Mosmann, 1983). Sertoli cell viability is often assessed by use of mitochondrial dehydrogenase activity (Monsees *et al*., 2000). However, as the MTT assay measures the activity of a mitochondrial enzyme, it also reflects changes in mitochondrial activity due to cell stress or toxicant exposure. Thus it is important to cross – check the actual cell numbers in microphotographs, to decide if a toxic effect or a proliferative effect (i. e. more cells and thus more mitochondrial activity) is the cause for any changes in MTT reduction compared to the control.

Treatment of Sertoli cells with ethanolic extract of DPP for 24 hours showed a significant increase in mitochondrial dehydrogenase activity at higher concentrations (100 – 1000 μg/ml) (Fig. 3.13). This increase in the mitochondrial enzyme activity was associated with a marked increase in the proliferation of the cells at the

corresponding concentrations as can be seen in the microphotographs (Fig. 3.1 and 3.3).

The growth of the TM4 cell line is under control of FSH hormone (Mather, 1980). It also has been reported that DPP showed gonadotrophic activity as it caused improvement of sperm motility, count and morphology when adult male rats were fed aqueous suspension of DPP for 35 days (Bahmanpour *et al*., 2006). Thus, the mechanism involved in the DPP enhancing effect on the proliferation of TM4 cells might be due to gonadotrophin activity or steroid constituents present in the DPP. In addition, real time PCR studies of rat primary Sertoli cell cultures detected the presence of both estrogen receptors ER - α and ER - β. Also these cells proliferated significantly when estradiol was added to the medium (Lucas *et al.*, 2008). It has been further proved that DPP contains estrogenic compounds in form of estriol, estradiol and estrone (Abbas and Ateya, 2011). Therefore DPP might also induce Sertoli cells proliferation through the estrogen receptor pathway.

Other mechanism might also be behind the proliferation enhancement effect of DPP on Sertoli cells. The antioxidant property of DPP is well established in the literature: 2,2-diphenyl-1-picrylhydrazyl (DPPH) and β carotene studies of Tunisian DPP acetone extract have shown potent antioxidant activities with the best DPPH scavenging activity at 46.56 ± 0.28 µg/ml (Daoud *et al.*, 2015). Furthermore, *in vitro* cultivation of preantral follicles isolated from 2 - 3 week old female mice in the presence of 10, 20, 30 or 40 µg/ml of DPP extract has shown improvement in the maturation rates with significant survival rate of the follicles on day 12 of culture at 20 µg/ml of DPP. Those finding were attributed to the antioxidant activity of DPP (Abdollahi *et al.*, 2015). Moreover, *in vivo* studies have demonstrated that administration of 40 mg/kg DPP to adult Wistar male rats ameliorated reproductive organs toxicity induced by cadmium and attenuated cadmium deleterious effects on spermatogenesis (El-Neweshy *et al.*, 2013). In addition, exposure of male Albino rats to carbofuran has led to a reduction in the weight of testes and increased

morphological sperm abnormalities. In the same study, co-administration of DPP at 60 mg/kg for 70 days with the tested dose of carbofuran has shown a significant increase in sperm numbers and motility and a decrease in spermatozoa morphological abnormality compared to the carbofuran group (Kobeasy *et al.*, 2015).

On the contrary, incubation of the TM4 cells with DPP for 72 hours resulted in a slight but significant decrease of the dehydrogenase enzyme activity at higher concentrations (100 – 1000 μg/ml) (Fig. 3.14). This might be explained by the hypothesis that the prolonged exposure to the plant extract reduces its proliferation enhancement effects and rather caused a mild toxic effect when compared to the short time exposure (Osibote *et al*., 2011).

TM4 cells that were treated with different concentrations of flax seed (0.01, 0.1, 1, 10 μg/ml) have displayed (Fig. 3.15) a small but significant decrease in the mitochondrial dehydrogenase activity ($p \leq 0.04$) associated with marked increase in the cell numbers (Fig. 3.4 and 3.6) after 24 hours exposure. While 72 hours treatment (Fig. 3.16) resulted in no significant changes in the enzyme activity except a slight but significant decrease at higher concentrations of 100 – 1000 μg/ml ($p = 0.03$ and 0.04 respectively). The microphotographs also did not show morphological changes in TM4 cells treated with flax seed. These findings indicate a non-cytotoxic effect of flax seed on Sertoli cells which is in agreement with previous *in vivo* and *in vitro* studies. The morphometric studies of testicular structure of the rats exposed to flax seed (20 or 40 %) or flax meal (13 or 26 %) showed normal unaltered structure of testicular tissues compared to untreated rats (Sprando *et al*., 2000). In addition, a main lignan component of flax seed, Secoisolariciresinol (SDG) (Westcott and Muir, 2003) showed no significant *in vitro* effect on lymphocyte cells proliferation when these cells were incubated with (10 – 100 μM) SDG *in vitro* (Rhee and Brunt, 2008).

In conclusion, exposure of TM4 Sertoli cells to either DPP or flax seed ethanolic extracts showed proliferative effects as can be seen in the microphotographs.

However, the small significant reduction in TM4 mitochondrial dehydrogenase enzyme activity which was seen after 72 hours exposure to DPP or flax seed was not associated with any morphological changes in comparison with control group.

4.1.2 Influence of ethanolic plants extracts on apoptosis in TM4 Sertoli cell line

In eukaryotes, apoptosis is an important biological event where the cells induce the intrinsic death cascade when exposed to different damaging effects aiming to maintain the internal environment balance (Kumi-Diaka *et al.*, 1998). In the testis spontaneous apoptosis is responsible for removal of 75% of the germ cells (Allan *et al.*, 1992).

Treatment of Sertoli TM4 cells with ethanolic extract of DPP has revealed no major alterations in apoptotic cells percentage in comparison with untreated cells after 24 hours exposure (Fig. 3.21 and 3.22), with exception of a significant elevation at 10 µg/ml of DPP. This is in agreement with the non - cytotoxic and proliferative effects that has been detected in the microphotographs and obtained by the MTT assay in the current study after 24 hours treatment of TM4 cells with higher concentrations of DPP.

The literature has reported a controversy in DPP apoptotic activity. Some *in vivo* and *in vitro* studies have suggested that the DPP has antiapoptotic activity. DPP increased the concentration and the percentage of human spermatozoa with normal shape *in vitro* when the culture medium was supplied with 5 mg/ml DPP (Al-dujaily *et al.*, 2012). In addition, *in vivo* exposure of adult male Wistar rats to 150 mg/kg of DPP for 56 days has revealed a reduction in the overexpression of certain apoptosis markers (caspase-3 and Fas-L) induced by thyroxin and propylthiouracil to normal levels in rat testicular tissues (El-Kashlan *et al.*, 2015). In contrast, Elberry *et al* (2011) has suggested that DPP has apoptotic activity as it reduced the induced hyperplastic picture of prostatic acini in male rats.

Treatment of TM4 cells with ethanolic extract of flax seed showed a non - significant increase in the percentage of apoptotic cells compared to untreated cells, but a decrease at the highest concentration (Fig. 3.23 and 3.24). The antiapoptotic activity of flax seed through potential downregulation of the genes implicated in apoptosis has been studied in animal models where the feeding of mice 10 % flax seed meal has led to a reduction in the expression of the main genes of ubiquitin - proteasome pathway in lung tissue. In this pathway (Fig. 4.1), several enzymes (ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2), and ubiquitin ligases (E3)) act consecutively to transfer a covalent bond with ubiquitin from one enzyme to the next. The final step is to bind a target protein with the ubiquitin providing it to ubiquitin - proteasome system to be eliminated (Bonifacino and Weissman, 1998). Flax seed downregulation of the genes in this pathway has indicated lower incidence of apoptosis (Dukes *et al*., 2012). Thus, the results obtained in this study might be attributed to the same mechanisms. In this study, treatment of TM4 Sertoli cells with 6 % DMSO showed a significant increase in apoptotic cells percentage. It has been reported that DMSO induced apoptosis in human leukemic HL-60 cell line. Besides, it was also able to induce apoptotic degeneration of rat CNS tissues. In the same study there was an elevation in activated caspase-3 positive neurons with morphological features of apoptosis. It has been suggested that the mechanism by which DMSO induced apoptosis is similar to that produced by ethanol since the cellular degenerative effect produced by DMSO or ethanol could be overcome by depolarizing effects of potassium chloride (Hanslick *et al*., 2009; Hong and Wang, 1995).

In conclusion, most of the ethanolic extracts of DPP or flax seed did not induce significant changes of apoptotic cell percentage of Sertoli cells after 24 hours exposure. This supports the proliferative effect detected in the microphotographs and the non -cytotoxic effects on TM4 Sertoli cells in this thesis which were assessed by MTT test at the same time frame.

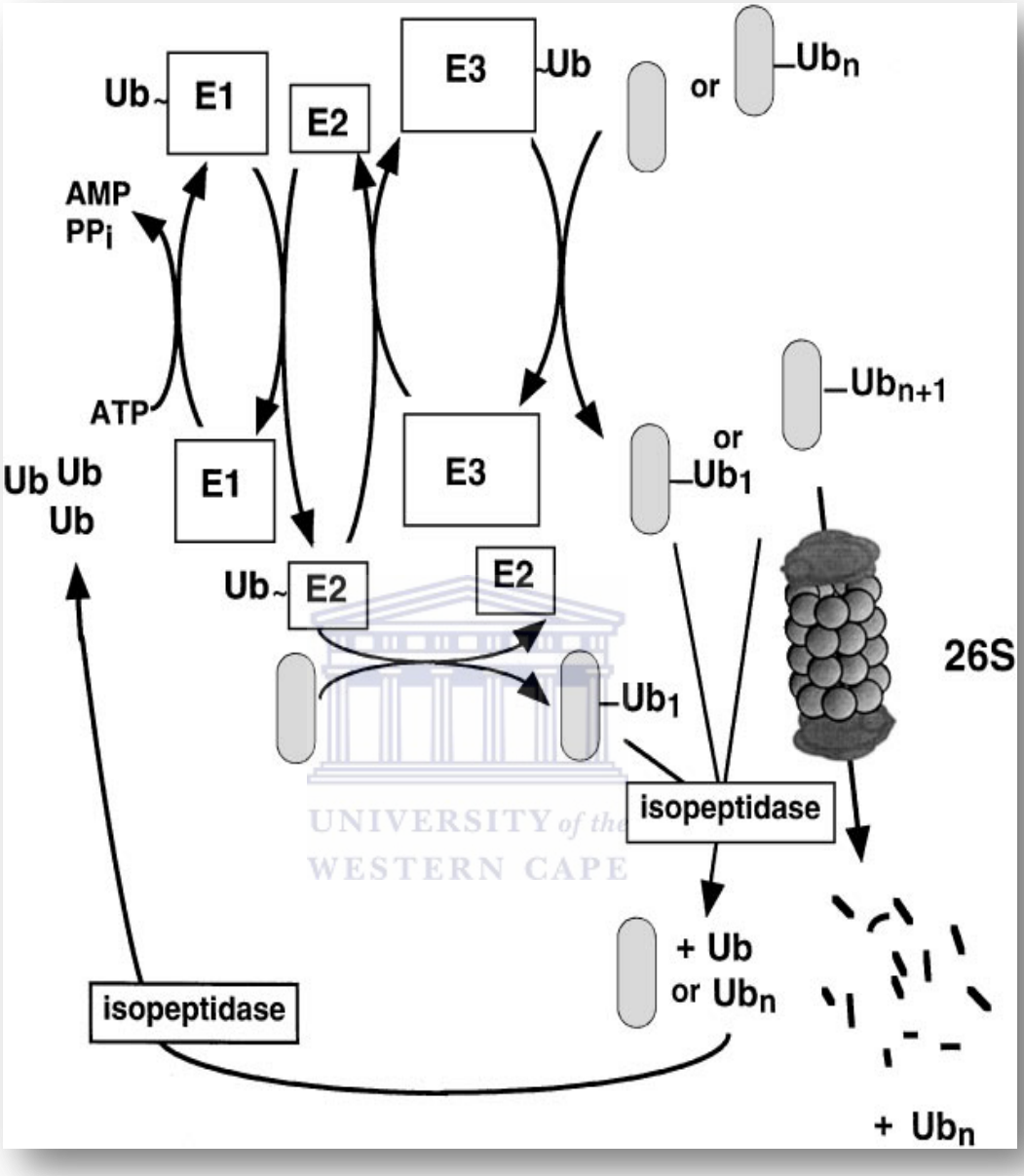

Figure 4.1: The ubiquitin-proteasome pathway (Bonifacino and Weissman, 1998). Ubiquitination of a substrate (Shaded object) leads to its degradation via 26S proteasomes. Abbreviations: E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme), E3 (ubiquitin-protein ligase), Ub (ubiquitin).

4.1.3 Transepithelial electrical resistance study

Adjacent Sertoli cells are able to adhere via tight junctions to form the blood testis barrier (BTB) which is accountable for closing the inter - cellular space in epithelial cellular sheets and generating two different fluid compartments with establishment of apical and basolateral membrane territories (Gye, 2003). These barriers can be affected by external stimuli as they form a dynamic equilibrium and their integrity can be quantified *in vitro* directly by measurement of transepithelial electrical resistance (TEER) (Benson *et al.*, 2013). Therefore measurement of the resistivity of Sertoli cells monolayers by means of TEER is an assessment of the function of the blood testis barrier (Carette *et al.*, 2013).

Treatment of Sertoli (TM4) cells with ethanolic extract of DPP has led to a significant increase in the electrical resistance of the epithelial cells monolayer (Fig. 3.29) in a concentration-dependent manner at higher concentrations throughout the experimental timeframe (24 – 72 hours). This increase in TEER values reflects establishment and tightness of the tight junctions (Brown *et al*., 2007). Although further research is required to detect the exact mechanism by which DPP has significantly elevated TEER, different theories can be proposed. In this thesis, DPP has shown a significant increase in the proliferation of TM4 Sertoli cells which means more established tight junctions, and thus subsequently high transepithelial electrical resistance records.

Moreover, it is well established in the literature that oxidative stress leads to disruption in the cellular junctions between adjacent epithelial and endothelial cells (Pointis *et al.*, 2011). Reactive oxygen species have shown ability to interrupt the cellular junctions between Sertoli-Sertoli cells through the phosphatidylinositol 3-kinase (PI3K)/c-Src tyrosine kinase/focal adhesion kinase (FAK) signaling pathway. Oxidative stress causes an activation of PI3K through a translocation of regulatory unit (p85) from the cytoplasm to the plasma membrane. This leads to further

stimulation of protein kinase (c-Src) which in testis is mainly found at the BTB and the ectoplasmic specialization. Stimulated c-Src causes phosphorylation of junction proteins and subsequently a translocation of these proteins from junctional side to the cytoplasm. The final result of this pathway is interruption of tight and adherens junctions and disturbance of cell adhesion (Wong and Cheng, 2011). In addition, activation of c-Src tyrosine kinase triggers endocytosis and degradation of adherens junction proteins like N-cadherin which leads to disturbance in epithelial and endothelial junctions. Moreover, activated c-Src stimulates ubiquitination of E-cadherin junctional protein exposing it to internalization and degradation (Inumaru *et al.*, 2009).

DPP has many antioxidant components in form of flavonoids, polyphenol and carotenoids (Daoud *et al.*, 2015). Therefore, the increase in the TEER readings and tightness of BTB model of the Sertoli cells monolayer could be further attributed to neutralization of the free radicals by this antioxidant power. Furthermore, several specific proteins are involved in the formation of the Sertoli cells tight junctions such as occludin, claudins, zonula occludens (ZO) and junction adhesion molecules (JAMs) (Fig. 4.2) (Tu'uhevaha *et al.*, 2007; Pointis *et al.*, 2011). In an experimental study using rat primary Sertoli cells cultured on Matrigel coated bicameral chambers, treatments with testosterone at 28 μg/ml or FSH at 2.35 IU/ml have showed a significant increase in TEER and expression of claudin - 11 mRNA (Tu'uhevaha *et al.*, 2007). We assume that the significant increase in the TEER readings caused by DPP treatments might be triggered by the same or similar mechanisms.

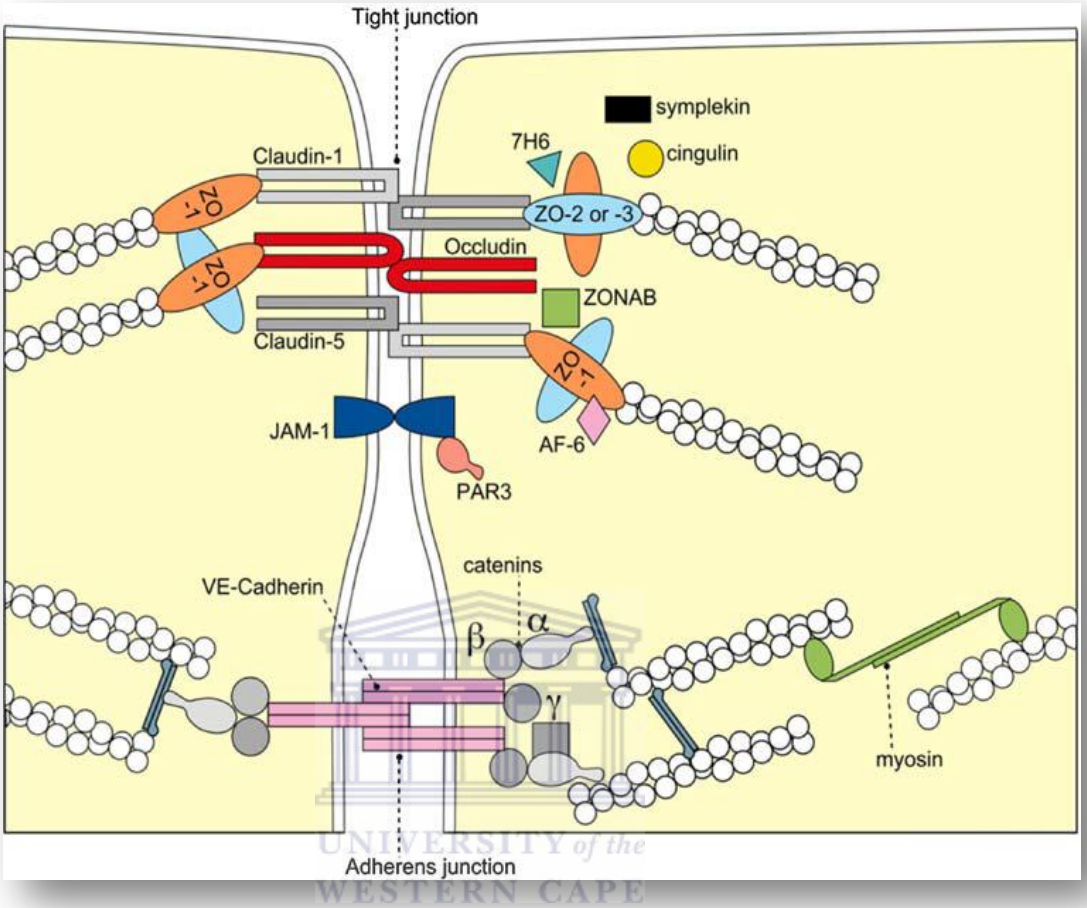

Figure 4.2: Diagram of junctional proteins arrangement at the blood testis barrier (BTB) (Förster, 2008).Tight junction is formed of claudin, occludin, ZO and JAM proteins.

Consistently the hormone-mediated consequences and gonadotropin-like effects of DPP have been proposed in the literature. Administration of 40 mg/kg DPP by adult male rats has shown many therapeutic effects on the harmful reproductive response produced by cadmium. Particularly DPP improved spermatogenesis (El-Neweshy *et al.*, 2013). Cadmium has many adverse effects on Sertoli cells and the BTB. It causes over production of free radicals and subsequently oxidative stress damage and necrosis of testicular tissues. These testicular damaging effects of cadmium lead to a reduction in testosterone production and deterioration in semen quality parameters in form of low number of spermatozoa (Farag *et al.*, 2016). Cadmium is also able to

interrupt TJ-associated microfilaments and destruction of BTB in rats. Moreover, cadmium has shown a capability to reduce the expression of tight junction occludin protein in cultured rat Sertoli cells (Chung and Cheng, 2001). Thus, in this study, another mechanism could be behind the effect of DPP on increasing the tightness of BTB *in vitro* model. We suggest that DPP might have a stimulatory effect on the expression of junctional proteins associated with BTB.

Although TEER values showed significant increase at higher treatments of DPP, it was difficult to maintain high levels of TEER after three days of culturing TM4 cells on HA filters which might be because of lack of supportive Matrigel coating of the filters. The same reason was proposed by Gye (2003) as the *in vitro* culturing of Sertoli cells on Matrigel-untreated HA filter inserts could not maintain high level of TEER for more than 4 days.

A significant concentration - dependent increase in the electrical resistance of the TM4 cells monolayer has been detected after 72 hours treatment with different concentrations of flax seed ethanolic extract. This was statistically not significant after 24 hours and only significant at 1000 µg/ ml after 48 hours (Fig. 3.30). The antioxidant activity of flax seed might be the reason of this significant increase in the resistivity of the epithelium monolayers as it has been reported that the flax seeds are potent antioxidants where their antioxidant effect has exceeded the antioxidant activity of the standard elagic acid (Bekal *et al.*, 2015).

The present results concluded that DPP and flax seed ethanolic extracts are beneficial in establishing and tightening BTB tight junctions. Thus they might influence spermatogenesis through this effect.

4.1.4 Effect of ethanolic plants extracts on TM4 gamma glutamyl transpeptidase (GGT) activity

GGT is a heterodimeric integral membrane glycoprotein present on the outer surface of Sertoli cells. GGT is responsible for the transfer of a glutamyle group between peptides and amino acids and is involved in the metabolism of glutathione. It is used as a marker for Sertoli cells (Caston and Sanborn, 1988; Lu and Steinberger, 1977; Palladino *et al.*, 1994). Moreover, it has been suggested that the reduction in Sertoli GGT production is an indicator of Sertoli cell dysfunction (Nurudeen and Ajiboye, 2012).

Incubation of Sertoli (TM4) cells with different concentrations of DPP or flax seed ethanolic extract for 72 hours (Fig. 3.31 and 3.32) stimulated the activity of GGT enzyme significantly at higher concentrations (100 – 1000 μg/ml) compared with negative control levels.

GGT has a significant role in the metabolism of glutathione which is an antioxidant with powerful scavenging activity that protects the cells against the harmful effects of free radicals and oxidative stress. It is has been reported that Sertoli cells have high levels of reduced glutathione (GSH) (Bauche *et al*., 1994) which is required to eliminate the powerful oxidant hydrogen peroxide (H_2O_2). Glutathione peroxidase (GPx) converts hydrogen peroxide into O_2 and water. In the same reaction the reduced glutathione (GSH) is oxidized to form oxidized glutathione (GSSG). Regeneration of GSH from GSSG is catalyzed by glutathione reductase (Pham-Huy *et al.*, 2008). The GGT enzyme provides Sertoli cells with amino acids which are required to synthesize the reduced glutathione. It breaks the γ- glutamyl bond of extracellular glutathione, releasing the cysteinyl-glycine dipeptide. Cysteine is an important element to create the reduced glutathione (Palladino *et al.*, 1994).

It is well known that the functions of Sertoli cell are under control of FSH, androgens (testosterone) and multiple polypeptide factors produced locally such as transforming growth factor-β (TGFβ), insulin-like growth factor-I (IGF-I) and tumor necrosis factor-α (TNFα) (Schteingart *et al.,* 1999). Specifically, addition of 100 ng/ml of FSH to the media containing isolated rat Sertoli cells has resulted in a significant increase in GGT production. Also a significant increase of GGT production was obtained by adding basic fibroblast growth factor in concentrations beyond 10 ng/ml (Schteingart *et al.*, 1999). Furthermore, cultured human Sertoli cells have responded to FSH at 0.05 – 0.5 µg/ml and dibutyryl cAMP at a concentration of 0.1 µg/ml with a significant increase in GGT production (Lipshultz *et al.*, 1982).

As it has been suggested that DPP exerts gonadotropic effects (Bahmanpour *et al*., 2006), we assume that the increase in the TM4 Sertoli cell GGT activity after exposure to DPP might be mediated through its gonadotropic – like action. Many *in vivo* and few *in vitro* studies on the effect of DPP on spermatogenesis enhancement have been reported. DPP at 0.06, 0.25 and 0.62 mg/mL concentrations has showed an ability to maintain spermatogonial cells and Sertoli cells in cultures up to 2 weeks with no cytotoxic effects despite the difficulty to keep spermatogonial cells in culture (Mahaldashtian *et al.*, 2015). In addition, administration of 5 and 25 ml/kg DPP by male rabbits for 8 weeks has resulted in enhancement of spermatogenesis with significant increase in primary, secondary spermatocytes and round spermatids (Faleh and Sawad, 2006).

Besides, it could be that the marked stimulating effect of DPP and flax seed on the proliferation of TM4 Sertoli cells is behind the significant increase in GGT enzyme activity. More Sertoli cells are associated with additional secretory function.

In conclusion DPP or flax seed ethanolic extracts has significantly increased the activity of TM4 Sertoli cell GGT enzyme activity at higher concentrations which

might indicate their potential ability to improve glutathione mediated oxidative defense mechanisms.

4.1.5 Effect of ethanolic plants extracts on TM4 inhibin – B production

Inhibin is a glycoprotein hormone consisting of two chains α and β. Depending on the type of β subunit (βA or βB) inhibin is categorized into two subgroups: A and B. Inhibin - B is the only form of inhibin that has been detected in human male serum and it is produced by Sertoli cells. Inhibin production is induced by follicular stimulating hormone (FSH) which is produced by the pituitary gland. Inhibin then negatively feeds back on the pituitary gland to control (inhibit) FSH production. Thus, inhibin keeps FSH serum levels within a set level (Berne *et al.*, 2008; Pierik, 1998). Inhibin - B has been regarded as a specific marker for Sertoli cells to check a regular function of the gonads (Ramaswamy *et al.*, 2003). Inhibin – B -produced by Sertoli cells into the circulation- works on the pituitary gland to reduce FSH production and the amount of inhibin - B produced, is dependent on Sertoli cell activity. It has been detected that serum inhibin levels in healthy fertile men was 187 +/- 28 pg/ml, but it was significantly less in infertile men with different causes of infertility ranged between 10 – 45 pg/ml (Anawalt *et al*., 1996). In rats, plasma inhibin B levels range between 90 – 100 pg/ml (Pfaff *et al*., 2013). High inhibin - B serum levels are associated with good sperm production (Afzalzadeh *et al.*, 2015).

Treatment of TM4 cells with different concentrations of DPP or flax seed for 72 hours has shown a major and significant concentration - dependent increase in inhibin - B production (Fig. 3.33 and 3.34). To our knowledge the current study is the first to show the effect of ethanolic extract of DPP or flax seed on inhibin - B production by TM4 Sertoli cell line.

In this research, the exact mechanisms that lead to significant elevations of inhibin - B levels are still unknown. However, we might refer this to the phytoestrogenic

properties of both DPP and flax seed which has been well established and is mentioned previously in this thesis. Moreover, and by use of LC-MS/MS methods, flax seed from different cultivars has shown high levels of major phytoestrogens in form of secoisolariciresinol (SECO) and matairesinol (MAT) (Krajčová *et al.,* 2009). It has been reported that addition of estradiol at 0.01 – 0.1 ng/ml concentrations in the presence or absence of FSH to primary Sertoli cell cultures from immature rats has led to a significant increase in inhibin - B levels in a dose - dependent manner. Our finding supports this suggestion (Depuydt *et al.,* 1999).

In conclusion, stimulatory effect of each of DPP and flax seed on Sertoli TM4 cell production of inhibin - B hormone suggests that they have supportive effect on spermatogenesis.

4.2 Effect of ethanolic plants extracts on MCF - 7 cell line

4.2.1 MCF - 7 cell line viability and morphology

The estrogen receptor – positive human mammary adenocarcinoma cell line (MCF - 7) is used widely as endocrine responsive experimental model (Clarke *et al*., 2001). The present results of the MTT assay showed (Fig. 3.17 and 3.18) a small but significant concentration – dependent decrease in the viability of the MCF - 7 cells treated with ethanolic extract of DPP for 24 hours. Toxic effects of DPP starts at all concentrations after one day but did not get more severe at day 3. This might indicate that the proliferative effect of DPP overcomes its toxic effect. This view is supported by the microphotographs (Fig. 3.7, 3.8 and 3.9), which showed considerably more cell numbers after 72 hours.

Phytoestrogens are herbal phenolic compounds that demonstrate a structure similar to mammalian estrogen (17β - estradiol). They have different affinity for estrogen receptors (ERα and ERβ) (Sirtori *et al*., 2005). Activation of ERα leads to

proliferative effects whereas binding to ERβ leads to inhibition of proliferation. Therefore, final effects of phytoestrogens on certain cell type depend on distinctive expression of ERα and ERβ (Mense *et al*., 2008). The phytoestrogens constituents of DPP are well established in the literature as discussed before. In this thesis we assume that short time exposure (24 hours) of MCF-7 cells to DPP might activate ERβ leading to slight inhibition of proliferation. On the contrary longer time exposure (72 hours) proliferative effect might be mediated through ERα.

In addition, it could be that DPP ethanolic extract has a slightly toxic effect on MCF - 7 cell line which started appearing after short time exposure, but the phytoestrogenic effect of DPP might overcome its toxic effect after prolonged exposure.

Flax seed extract produced a concentration - dependent reduction in mitochondrial dehydrogenase enzyme activity of MCF - 7 cells measured by MTT assay after 24 hours (Fig. 3.19). After 72 hours no changes of dehydrogenase enzyme activity can be seen except a slight significant reduction at high concentrations (100 – 1000 µg/ml) (Fig. 3.20). This reduction was not associated with any changes regarding the morphology and proliferation of MCF - 7 cells as can be seen in microphotographs (Fig. 3.10, 3.11 and 3.12). The mechanisms by which flax seeds modulate the inhibition and proliferation of MCF-7 cells are not understood yet. In this study, flax seeds might produce its effects through estrogen receptors (the same mechanism discussed for DPP) as they are a rich source of phytoestrogens (lignans and secoisolariciresinol diglucoside). In addition it could be slightly toxic to MCF – 7 cell line but this effect was overcome by prolonged exposure.

In conclusion, both ethanolic extracts of DPP and flax seed have shown slight cytotoxic effects on MCF - 7 cell line after 24 hours exposure. However, the toxic effects did not get more severe after longtime exposure where the proliferation of the cells enhanced after 72 hours.

4.2.2 Influence of ethanolic plants extracts on apoptosis in breast cancer (MCF-7) cells

Apoptosis is a physiological route of programmed cell death which when deregulated will lead to generation of many undesirable effects like cancer pathogenesis. It plays a crucial role in tissue homeostasis (Ashkenazi, 2008).

Exposure of MCF-7 cells to the ethanolic extract of DPP for 24 hours resulted in no significant changes in the percentage of the apoptotic cells between the negative control group and DPP treated groups (Fig. 3.25 and 3.26). In summation with the other results in this study we suggest that the moderate reducing effect of DPP on MCF-7 cell proliferation at 1000 µg/ml after 24 hours is not mediated via apoptosis. This is further supported by the proliferative effect of DPP on MCF-7 cells that was obtained after 72 hours by the corresponding concentration in this research.

Flax seed ethanolic extract has changed the percentage of the apoptotic breast cancer cells without reaching significance (Fig. 3.27 and 3.28). At lower concentrations (0.1 - 10 µg/ml) flax seed has caused a rise in apoptotic cells percentage which then decreased insignificantly at 1000 µg/ml after 24 hours exposure. Again, there is a controversy in the literature reports of apoptotic/antiapoptotic activity of flax seed. *In vivo* apoptotic effects of flax seed has been reported by Thompson *et al.*, 2005 where apoptotic studies on biopsies from postmenopausal women with breast cancer who ate 25 g flax seed daily between the time of diagnosis up to surgical intervention has revealed a significant increase in the apoptotic index where the median reached to 30.7% in comparison to placebo groups. This was associated with decreased expression of receptor tyrosine - protein kinase erbB-2. Binding of protein (erbB-2) to erbB receptors leads to increase in the signaling potency of these receptors resulting in uncontrolled proliferation of cells and tumor formation (Tan and Yo, 2007). On the other hand the antiapoptotic activity of flax seed and its role in ubiquitination genes regulation in lung tissue has been discussed previously in this

thesis. The present results are in agreement with the antiapoptotic effects of flax seeds at higher concentrations.

According to the present results we concluded that neither DPP nor flax seed has significantly changed the percentage of apoptotic cell percentage of MCF-7 cells.

4.2.3 Assessment of ethanolic plants extracts estrogenicity by E - SCREEN assay

The E - SCREEN assay is a reliable method to assess the estrogenicity of compounds quantitatively by use of proliferation measurement of estrogen receptor - positive MCF-7 cells (Soto *et al.*, 1995). MCF - 7 cells have been shown to express both estrogen receptors ERα and ERβ, although levels of mRNA for ERβ were much lower than ERα (Boersma and Mosselman, 2000).

Binding of estrogen agonist or estrogen antagonists to ERs leads to dimerization and activation of these receptors followed by numbers of events like induction of proliferation or binding to the Estrogen Response Element (ERE) (Boersma and Mosselman, 2000). The ligand binding domain of ERα and ERβ was shown to admit large number of compounds. These are predominantly compounds whose structure includes a phenol ring with hydrophobic regions (Brzozowski *et al.*, 1997). From this point of view it was mandatory in the E - SCREEN assay to use a medium without phenol red and to eliminate all steroidogenic compounds from the serum. This was achieved in this study by treating the serum with charcoal and dextran.

As discussed previously in this thesis, phytoestrogens demonstrate a structure similar to mammalian estrogen 17β - estradiol (Sirtori *et al.*, 2005). Both DPP and flax seed are regarded as good sources of phytoestrogens. In addition, by use of Folin-Ciocalteu method DPP from Tunisian origins has revealed a presence of eight phenolic compounds classified into phenolic acids and flavonoids. Those are gallic acid, catechin, caffeic acid, epicatechin, vanillic acid, coumarin, quercitin and rutin

(Daoud *et al.*, 2015). Moreover, in an *in vivo* study to determine the estrogenic activity of DPP, female immature rats that were treated with 500 mg/kg body weight of date seed extracts or estradiol (2 mg/kg) for 10 days showed a significant rise in the plasma level of estrogen compared to control group (Ammar *et al.*, 2009). Likewise, flax seed from different cultivars possess high levels of major phytoestrogens in form of secoisolariciresinol (SECO) and matairesinol (MAT) by use of LC-MS/MS method) (Krajčová *et al.*, 2009).

Several compounds possess estrogenic activity, it has been reported that phenol red is able to bind to the estrogen receptor of MCF-7 cells with low affinity (0.001% that of estradiol) (Berthois *et al*, 1986). In addition, DPP also was mentioned as a lower estrogenic compound relative to 17 – β estradiol. 17 – β estradiol led to 97 % increase in female rat plasma estrogen level while DPP caused only 46 % increase (Ammar *et al*., 2009).

Results of the E–SCREEN assay in this project (Fig. 3.35 and 3.36) after incubation of MCF - 7 cells for 6 days with different concentrations of DPP or flax seeds in phenol red free medium supplied with charcoal stripped fetal bovine serum (CD-FBS), has detected that lower concentrations of DPP and flax seed (0.01 – 10 µg/ml) caused a significant drop in mitochondrial dehydrogenase activity and are thus cytotoxic. At higher concentrations (100 - 1000 µg/ml), however, mitochondrial dehydrogenase enzyme activity started to rise. This suggests that at these higher concentrations the proliferative (i.e. estrogenic effect of DPP and flax seed) becomes more pronounced than the cytotoxic effect. Thus, DPP and flax seed extracts can be regarded to contain weak estrogenic activity as can be seen in figure 4.3. On the other hand 1 nM estradiol was able to produce a significant increase in MCF – 7 proliferation which indicates the validity of the assay protocol (Mense *et al.*, 2008).

Although the E – screen test in this thesis has revealed a significant reduction in the viability of MCF-7 cells that was measured by MTT after 6 days incubation with

lower concentrations of DPP or flax seed ethanolic extracts, the microphotographs (Fig. 3.8 and 3.11) of MCF-7 cells from the proliferation assay did show that MCF-7 cells were proliferating after 3 days exposure to DPP and flax seed. This difference could be attributed to the different serum components that were used in the medium. In the proliferative assay the cells were treated with complete serum (non - stripped). Whereas the serum that used in the E – screen assay was treated with charcoal – dextran. In addition the phenol red component of the medium also might cause this discrepancy. E-screen assay depends on eliminating the estrogenic effect of phenol red so estrogenic effect of plant extract more likely to be seen.

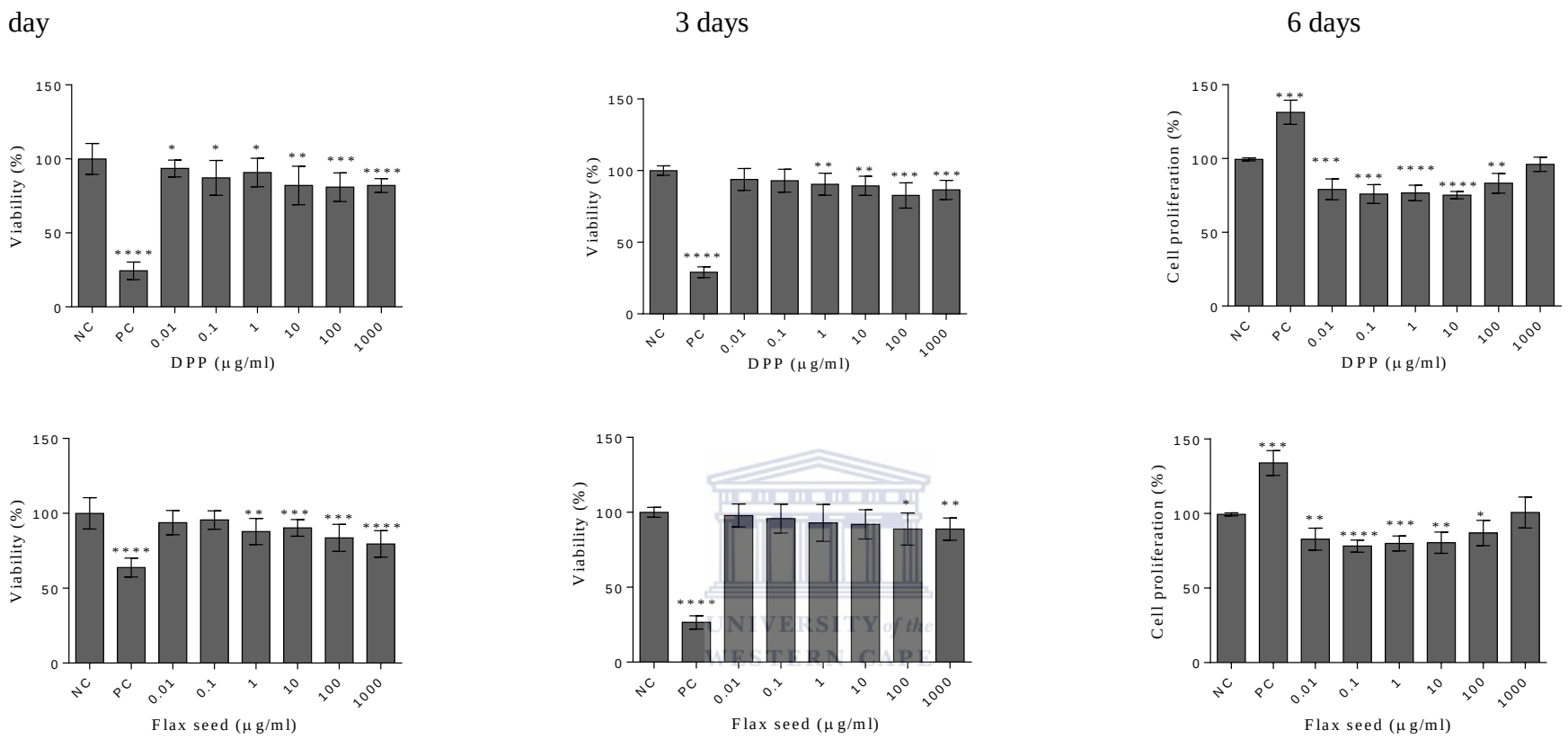

Figure 4.3: Comparison between MTT results from the cytotoxicity assay (1 day and 3 days exposure of MCF-7 cells to ethanolic plants extracts) and E-SCREEN assay (6 days exposure of MCF-7 cells to ethanolic plants extracts). A weak estrogenic effect could be seen after prolonged exposure (6 days) of MCF-7 cells to higher concentrations (100-1000 µg/ml) of DPP and flax seed.* indicates p value ≤ 0.05, ** = p value ≤ 0.01, *** = p value ≤ 0.001 and **** = p value ≤ 0.0001 compared to NC.

4.3 Conclusion and further outlook

In conclusion, results obtained from this research indicated that neither DPP nor flax seed ethanolic extracts have major deleterious effects on Sertoli (TM4) cell viability, proliferation and function in the *in vitro* culture system. At 0.01-1000 µg/ml concentrations both ethanolic plant extracts caused a significant increase in the proliferation of TM4 cells after 24 hours incubation. On the other hand, both herbal extracts showed reduction in the viability of human breast cancer (MCF - 7) cells after 24 hours which, however, did not did not deteriorate further 72 hours incubation because then the estrogenic effect seems to stimulate cell proliferation.

Measurement of the electrical resistance of Sertoli cells monolayers by means of TEER assay is an assessment of the function of the blood testis barrier (Carette *et al*., 2013). DPP at higher concentrations raised the resistance of Sertoli cells monolayer measured by TEER assay significantly over 72 hours which indicate the benefit of DPP on the maintenance of BTB integrity in the *in vitro* model. Flax seed also showed a significant increase in TEER in a concentration - dependent manner after 72 hours. Additionally, both herbal ethanolic extracts exhibited an increase in the secretory function of TM4 Sertoli cells. Inhibin B hormone production was significantly enhanced in a concentration - dependent manner. Moreover, TM4 cells GGT enzyme activity was increased under the effects of both plants extracts indicating high activity of the Sertoli cells.

The phytoestrogenic activity of both herbs is well established in the literature (Abbas and Ateya, 2011; Bekal *et al*., 2015) and was confirmed in this study. However, we found that lower concentrations of DPP and flax seed were slightly cytotoxic after 6 days of exposure. With 100 and 1000 µg/ml, the estrogenic effect of these compounds was detected which led to a rise in cell proliferation.

In summary, both DPP and flax seed have beneficial effects on TM4 Sertoli cells and thus may enhance spermatogenesis by increasing Sertoli cell support to germ cells and have cytotoxic effects on breast cancer (MCF - 7) cells at lower concentrations. Further

investigations are required to detect the exact mechanisms by which DPP and flax seed exhibited their effects on both cell lines.

Chapter V: References

Abbas, F. A., Ateya, A. M. (2011). Estradiol, Esteriol, Estrone and Novel Flavonoids from Date Palm Pollen. *Aust J Basic Appl Sci*, *5*(8), 606-14.

Abdollahi, F. S., Baharara, J., Nejad-Shahrokhabadi, K., Namvar, F., Amini, E. (2015). Effect of *Phoenix Dactylifera* Pollen Grain on Maturation of Preantral Follicles in NMRI Mice. *Journal of HerbMed Pharmacology*, *4*(3), 93-97.

Abedi, A., Karimian, S. M., Parviz, M., Mohammadi, P., Roudsari, H. R. S. (2014). Effect of Aqueous Extract of *Phoenix Dactylifera* Pollen on Dopamine System of Nucleus Accumbens in Male Rats. *Neuroscience & Medicine*, *5*, 49-59.

Adefolaju, G. A., Theron, K. E., Hosie, M. J. (2015). BAX/BCL-2 mRNA and protein expression in human breast MCF-7 cells exposed to drug vehicles-methanol and dimethyl sulfoxide (DMSO) for 24 hrs. *Nigerian medical journal: journal of the Nigeria Medical Association*, *56*(3), 169.

Adolphe, J. L., Whiting, S. J., Juurlink, B. H., Thorpe, L. U., Alcorn, J. (2010). Health Effects with Consumption of The flax Lignan Secoisolariciresinol Diglucoside. *British Journal of Nutrition*, *103*(07), 929-938.

Afzalzadeh, M. R., Ahangarpour, A., Amirzargar, A., Varnamkhasti, M. K., Ganjalidarani, H. (2015). The Effect of *Vitis Vinifera L*. Juice on Serum Levels of Inhibin B, Sperm Count in Adult Male Rats. *The world journal of men's health*, *33*(2), 109-116.

Agarwal, A., Mulgund, A., Hamada, A., Chyatte, M. R. (2015). A unique View on Male Infertility Around The globe. *Reprod Biol and Endocrinol*, *13*(37), 1-9

Al-dujaily, S. S., Al-shahery, N. J., Zabbon, A. A. (2012). Effect of *Phoenix Dactylifera* Pollen on *in Vitro* Sperm Activation of Infertile Men Saad. *Al-Mustansiriyah J Sci*, *23*(6), 27-34.

Allan, D. J., Harmon, B. V., Roberts, S. A. (1992). Spermatogonial apoptosis has three morphologically recognizable phases and shows no circadian rhythm during normal spermatogenesis in the rat. *Cell proliferation*,*25*(3), 241-250.

Alsabri, S. G., Zetrini, A. E., Ermeli, N. B., Mohamed, S. B., Bensaber, S. M., Hermann,

A., Gbaj, A. (2012). Study of eight medicinal plants for antioxidant activities. *J. Chem. Pharm. Res*, *4*(8), 4028-4031.

Ammar, N. M., Al-Okbi, S. Y., Mohamed, D. A., El-Kassem, L. T. A. (2009). Antioxidant and Estrogen like Activity of the Seed of *Phoenix dactylifera L*. Palm growing in Egyptian Oases. *Report and Opinion, 1*(3), 1-8.

Amory, J. K., Bremner, W. (2001). Endocrine regulation of testicular function in men: implications for contraceptive development. *Molecular and cellular endocrinology*, *182*(2), 175-179.

Anawalt, B. D., Bebb, R. A., Matsumoto, A. M., Groome, N. P., Illingworth, P. J., McNeilly, A. S., and Bremner, W. J. (1996). Serum inhibin B levels reflect Sertoli cell function in normal men and men with testicular dysfunction. *The Journal of Clinical Endocrinology & Metabolism*, *81*(9), 3341-3345.

Arfat, Y., Mahmood, N., Ahmad, M., Tayyab, M., Zhao, F., Li, D. J., Zhihao, C., Yin, C., Shang, P., Qian, A. R. (2014). Effect of date palm pollen on serum testosterone and intra-testicular environment in male albino rats. *African Journal of Pharmacy and Pharmacology*, *8*(31), 793-800.

Asemota, O. A., Klatsky, P. (2015). Access to infertility care in the developing world: the family promotion gap. *Semin Reprod Med*, *33*(1), 17-22.

Ashkenazi, A. (2008). Targeting the extrinsic apoptosis pathway in cancer. *Cytokine & growth factor reviews*, *19*(3), 325-331.

Badmus, J. A., Ekpo, O. E., Hussein, A. A., Meyer, M., Hiss, D. C. (2015). Antiproliferative and apoptosis induction potential of the methanolic leaf extract of Holarrhena floribunda (G. Don). *Evidence-Based Complementary and Alternative Medicine*, *2015*, 1-11.

Baharara, J., Amini, E., Salek-Abdollahi, F., Nikdel, N., Asadi-Samani, M. (2015). Protective effect of date palm pollen (*Phoenix dactylifera*) on sperm parameters and sexual hormones in male NMRI mice exposed to low frequency electromagnetic field (50 Hz). *Journal of HerbMed Pharmacology*,*4*(3), 75-80.

Bahmanpour, S., Talaei, T., Vojdani, Z., Panjehshahin, M. R., Poostpasand, A., Zareei, S., Ghaeminia, M. (2006). Effect of *Phoenix dactylifera* pollen on sperm

parameters and reproductive system of adult male rats. *Iranian Journal of medical sciences*, *31*(4), 208-212.

Bastway A, M., Hasona, N. A., Selemain, A. H. (2008). Protective effects of extract from dates (*Phoenix dactylifera L*.) and ascorbic acid on thioacetamide-induced hepatotoxicity in rats. *Iranian Journal of Pharmaceutical Research*, *7*(3), 193-201.

Bauché, F., Fouchard, M. H., Jégou, B. (1994). Antioxidant system in rat testicular cells. *FEBS letters*, *349*(3), 392-396.

Bekal, M., Kumari, S., Sharmila, K. P. (2015). Preliminary Phytochemical Screening of Flaxseed and Assessment of It's *In Vitro* Antioxidant Activity. *World Journal of Pharmacy and Pharmaceutical Sciences*, *4*(8), 952-958 .

Benson, K., Cramer, S., Galla, H. J. (2013). Impedance-based cell monitoring: barrier properties and beyond. *Fluids Barriers CNS*, *10*(5), 1-11.

Berthois, Y., Katzenellenbogen, J. A., and Katzenellenbogen, B. S. (1986). Phenol red in tissue culture media is a weak estrogen: implications concerning the study of estrogen-responsive cells in culture. *Proceedings of the National Academy of Sciences*, *83*(8), 2496-2500.

Boersma, C., Mosselman, S. (2000). Estrogen Receptors alpha and beeta: two Receptors of a Kind?. *Current medicinal chemistry*, *7*(5), 561-576.

Bonifacino, J. S., Weissman, A. M. (1998). Ubiquitin and the control of protein fate in the secretory and endocytic pathways. *Annual review of cell and developmental biology*, *14*(1), 19-57.

Bonofiglio, D., Giordano, C., De Amicis, F., Lanzino, M., Andò, S. (2015). Natural Products as Promising Antitumoral Agents in Breast Cancer: Mechanisms of Action and Molecular Targets. *Mini reviews in medicinal chemistry*, *16*(8), 596-604

Brooks, S. C., Locke, E. R., Soule, H. D. (1973). Estrogen receptor in a human cell line (MCF-7) from breast carcinoma. *Journal of Biological Chemistry*, *248*(10), 6251-6253.

Brown, R. C., Morris, A. P., O'Neil, R. G. (2007). Tight junction protein expression and barrier properties of immortalized mouse brain microvessel endothelial cells. *Brain research*, *1130*(1), 17-30.

Brzozowski, A. M., Pike, A. C., Dauter, Z., Hubbard, R. E., Bonn, T., Engström, O., Ohman, L., Greene, G. L., Gustafsson, J. A., Carlquist, M. (1997). Molecular basis of agonism and antagonism in the oestrogen receptor. *Nature*, *389*(6652), 753-758.

Carette, D., Perrard, M. H., Prisant, N., Gilleron, J., Pointis, G., Segretain, D., Durand, P. (2013). Hexavalent chromium at low concentration alters Sertoli cell barrier and connexin 43 gap junction but not claudin-11 and N-cadherin in the rat seminiferous tubule culture model. *Toxicology and applied pharmacology*, *268*(1), 27-36.

Caston, L. A., Sanborn, B. M. (1988). Regulation of testicular and Sertoli cell gamma-glutamyl transpeptidase by follicle-stimulating hormone. *Biology of reproduction*, *38*(1), 109-113.

Cheng, C. Y., Mruk, D. D. (2002). Cell junction dynamics in the testis: Sertoli-germ cell interactions and male contraceptive development. *Physiological reviews*, *82*(4), 825-874.

Cheng, C. Y., Mruk, D. D. (2012). The blood-testis barrier and its implications for male contraception. *Pharmacological reviews*, *64*(1), 16-64.

Chimento, A., Sirianni, R., Casaburi, I., Pezzi, V. (2014). Role of estrogen receptors and G protein-coupled estrogen receptor in regulation of hypothalamus–pituitary–testis axis and spermatogenesis. *Frontiers in Endocrinology*, *5*, 1-8.

Chung, N. P., Cheng, C. Y. (2001). Is Cadmium Chloride-Induced Inter-Sertoli Tight Junction Permeability Barrier Disruption a Suitable *in Vitro* Model to Study the Events of Junction Disassembly during Spermatogenesis in the Rat Testis?. *Endocrinology*, *142*(5), 1878-1888.

Chung, N. P., Mruk, D., Mo, M. Y., Lee, W. M., Cheng, C. Y. (2001). A 22-amino acid synthetic peptide corresponding to the second extracellular loop of rat occludin perturbs the blood-testis barrier and disrupts spermatogenesis reversibly *in vivo*. *Biology of reproduction*, *65*(5), 1340-1351.

Clarke, R., Leonessa, F., Welch, J. N., Skaar, T. C. (2001). Cellular and molecular pharmacology of antiestrogen action and resistance. Pharmacological reviews, 53(1), 25-71.

Collins, T. F., Sprando, R. L., Black, T. N., Olejnik, N., Wiesenfeld, P. W., Babu, U. S.,

Bryant, M., Flynn,T. J., Ruggles, D. I. (2003). Effects of flaxseed and defatted flaxseed meal on reproduction and development in rats. *Food and chemical toxicology*, *41*(6), 819-834.

Cross section of the seminiferous tubule. In: (https://www.studyblue.com/notes/note/n/reproductivesystem/deck/238286). Accessed on 5th Nov, 2015.

Dang, Z. C., Lowik, C. W. G. M. (2005). Removal of serum factors by charcoal treatment promotes adipogenesis via a MAPK-dependent pathway. *Molecular and cellular biochemistry*, *268*(1-2), 159-167.

Date palm pollen. In: https://maher-shop.com/img/cms/Palm. accessed on 20th Des, 2015.

Daoud, A., Malika, D., Bakari, S., Hfaiedh, N., Mnafgui, K., Kadri, A., Gharsallah, N. (2015). Assessment of polyphenol composition, antioxidant and antimicrobial properties of various extracts of Date Palm Pollen (DPP) from two Tunisian cultivars. *Arabian Journal of Chemistry*, 1-12.

De Franca, L. R., Ye, S. J., Ying, L., Sandberg, M., Russell, L. D. (1995). Morphometry of rat germ cells during spermatogenesis. *The Anatomical Record*, *241*(2), 181-204.

Depuydt, C. E., Mahmoud, A. M., Dhooge, W. S., Schoonjans, F. A., Comhaire, F. H. (1999). Hormonal regulation of inhibin B secretion by immature rat sertoli cells *in vitro*: possible use as a bioassay for estrogen detection. *Journal of andrology*, *20*(1), 54-62.

De Santis, C., Ma, J., Bryan, L., Jemal, A. (2014). Breast cancer statistics, 2013. *CA: a cancer journal for clinicians*, *64*(1), 52-62.

Dukes, F., Kanterakis, S., Lee, J., Pietrofesa, R., Andersen, E. S., Arguiri, E. Tyagi, S., Showe, L., Amrani, Y., Christofidou-Solomidou, M. (2012). Gene expression profiling of flaxseed in mouse lung tissues-modulation of toxicologically relevant genes. *BMC complementary and alternative medicine*, *12*(1), 1-11.

Edjenguele, S. N. B., Nantia, E. A., Manfo, P. T., Vidari, G., Fewou, P. M., Monsees, T. K. (2014). Effects of Methanol Fractions of Basella alba L. (*Basellaceae*) on TM3 Leydig cells steroidogenesis. *Journal of Biological and Chemical Research*, *31*(2),

912–922.

El-Azim, M. H. A., El-Mesalamy, A. M., Yassin, F. A., Khalil, S. A. (2015). Identification Phenolic and Biological Activities of Methanolic Extract of Date Palm Pollen (*Phoenix Dactylifera*). *Journal of Microbial & Biochemical Technology*, *7*(1), 47-50

Elberry, A. A., Mufti, S. T., Al-Maghrabi, J. A., Abdel-Sattar, E. A., Ashour, O. M., Ghareib, S. A., Mosli, H. A. (2011). Anti-inflammatory and antiproliferative activities of date palm pollen (*Phoenix dactylifera*) on experimentally-induced atypical prostatic hyperplasia in rats. *Journal of Inflammation*, *8*(40), 1-13.

El-Kashlan, A. M., Nooh, M. M., Hassan, W. A., Rizk, S. M. (2015). Therapeutic Potential of Date Palm Pollen for Testicular Dysfunction Induced by Thyroid Disorders in Male Rats. *PLOS one*, *10*(10), 1-23.

El Mistiri, M., Salati, M., Marcheselli, L., Attia, A., Habil, S., Alhomri, F., Spika, D., Allemani, C. and Federico, M. (2015). Cancer incidence, mortality, and survival in Eastern Libya: updated report from the Benghazi Cancer Registry. *Annals of epidemiology*, *25*(8), 564-568.

El-Mokasabi, F. M. (2014). The state of the art of traditional herbal medicine in the eastern mediterranean coastal region of Libya. *Middle-East Journal of Scientific Research*, *21*(4), 575-582.

Elmore, S. (2007). Apoptosis: a review of programmed cell death.*Toxicologic pathology*, *35*(4), 495-516.

El-Neweshy, M. S., El-Maddawy, Z. K., El-Sayed, Y. S. (2013). Therapeutic effects of date palm (*Phoenix dactylifera L.*) pollen extract on cadmium-induced testicular toxicity. *Andrologia*, *45*(6), 369-378.

Fabricant, D. S., Farnsworth, N. R. (2001). The value of plants used in traditional medicine for drug discovery. *Environmental health perspectives*, *109*(Suppl 1), 69-75

Faleh, B. H., Sawad, A. A. (2006). Effect of Date Palm Pollen Grains Extracts (*Phoenix dactylifera L*) on Spermatogenic Activity of Male Rabbits. *J Al-Basrah Res*, *5*(1-2), 1–10.

Farag, M. R., EL-Aziz, R. A., Ali, H. A., Ahmed, S. A. (2016). Evaluating the ameliorative efficacy of *Spirulina platensis* on spermatogenesis and steroidogenesis in cadmium-intoxicated rats. *Environmental Science and Pollution Research*, *23*(3), 2454-2466.

Flax seed. In: https://en.wikipedia.org/wiki/Flax. accessed on 15th Des, 2015.

Förster, C. (2008). Tight junctions and the modulation of barrier function in disease. *Histochemistry and cell biology*, *130*(1), 55-70.

Ganorkar, P. M., Jain, R. K. (2013). Flaxseed–a nutritional punch. *International Food Research Journal*, *20*(2), 519-525.

Giovagnoli, S., Mancuso, F., Vannini, S., Calvitti, M., Piroddi, M., Pietrella, D., Arota, L., Falabella, G., Galli, F., Moretti, M., Neri, L. M., Bodo, M., Capitani, S., Cameron, D. F., Ricci, M., Lucas, G., Calafiore, R. (2014). Microparticle-loaded Neonatal Porcine Sertoli Cells for Cell-based Therapeutic and Drug Delivery System. *Journal of Controlled Release*, *192*, 249-261.

Griswold, M. D. (Ed.). (2014). Sertoli Cell Biology. Academic press. Second edition.

Griswold, M. D. (1998). The central role of Sertoli cells in spermatogenesis. *Seminars in Cell & Developmental Biology*, *9*(4), 411-416.

Guraya, S. S. (2012). Biology of spermatogenesis and spermatozoa in mammals. Springer Science and Business Media. Second edition.

Gye, M. C. (2003). Changes in the expression of claudins and transepithelial electrical resistance of mouse Sertoli cells by Leydig cell coculture. *International journal of andrology*, *26*(5), 271-278.

Hammed, M. S., Arrak, J. K., Al-Khafaji, N. J., Hassan, A. A. (2012). Effect of Date palm pollen suspension on ovarian function and fertility in adult female rats exposed to lead acetate. *Divala Journal of Medicine*, *3*(1), 90-96.

Hanslick, J. L., Lau, K., Noguchi, K. K., Olney, J. W., Zorumski, C. F., Mennerick, S., and Farber, N. B. (2009). Dimethyl sulfoxide (DMSO) produces widespread apoptosis in the developing central nervous system. Neurobiology of Disease, 34(1), 1–10.

Hengartner, M. O. (2000). The biochemistry of apoptosis. *Nature*, *407*(6805), 770-776.

Hong, S., Wang, Y. (1995). Apoptosis of human leukemic HL-60 cells induced to differentiate by treatment with RA or DMSO. Cell Research, 5, 181–186.

Hu, Y., Wang, R., Xiang, Z., Qian, W., Han, X., Li, D. (2014). Mixture effects of nonylphenol and di-n-butyl phthalate (monobutyl phthalate) on the tight junctions between Sertoli cells in male rats *in vitro* and *in vivo*. *Experimental and Toxicologic Pathology*, *66*(9), 445-454.

Hulkower, K. I., Herber, R. L. (2011). Cell migration and invasion assays as tools for drug discovery. *Pharmaceutics*, *3*(1), 107-124.

Inumaru, J., Nagano, O., Tanihar, H., Umezawa, k., Niwa, S.,Takahashi, E., Ishimoto, T., Nakamura, S., Suzuki, Y., Saya, H. (2009). Molecular mechanisms regulating dissociation of cell–cell junction of epithelial cells by oxidative stress. *Genes to Cells*, *14*(6), 703-716.

Jain, M., Kasetty, S., Khan, S., Desai, A. (2014). An Insight to Apoptosis. *Journal of Research and Practice in Dentistry*, *2014*(2014), 1–12.

Johnson, Q., Syce, J., Nell, H., Rudeen, K., Folk, W. R. (2007). A randomized, double-blind, placebo-controlled trial of Lessertia frutescens in healthy adults. *PLOS Clinical Trial*, *2*(4), 1-7.

Kobeasy, M., El-Naggara, A., Abdallah, A. A. (2015). A novel methods for protective role against reproductive toxicity of carbofuren in male rats using palm pollen grains and vanadyl(II) folate as a new compound. *Journal of Chemical and Pharmaceutical Research*, *7*(4), 1142–1148.

Koeppen, B. M., Stanton, B. A. (2010). Berne & Levy Physiology. Mosby Elsevier. Sixth edition.

Krajčova, A., Schulzová, V., Hajslova, J., Bjelková, M. (2009). Lignans in flaxseed. *Czech Journal of Food Sciences*, *27*, 252-255.

Kumi-Diaka, J., Rodriguez, R., Goudaze, G. (1998). Influence of genistein (4′, 5, 7-trihydroxyisoflavone) on the growth and proliferation of testicular cell lines. *Biology of the Cell*, *90*(4), 349-354.

Levenson, A. S., Jordan, V. C. (1997). MCF-7: the first hormone-responsive breast cancer cell line. *Cancer research*, *57*(15), 3071-3078.

Lipshultz, L. I., Murthy, L., Tindall, D. J. (1982). Characterization of Human Sertoli Cells *in Vitro*. *The Journal of Clinical Endocrinology & Metabolism*, *55*(2), 228-237.

Longitudinal section of the testis. In: http://encyclopedia.lubopitko-bg.com/Male_Reproductive_System.html. Accessed on 23rd Nov, 2015.

Lu, C., Steinberger, A. (1977). Gamma-glutamyl transpeptidase activity in the developing rat testis. Enzyme localization in isolated cell types. *Biology of reproduction*, *17*(1), 84-88.

Lucas, T. F., Siu, E. R., Esteves, C. A., Monteiro, H. P., Oliveira, C. A., Porto, C. S., Lazari, M. F. M. (2008). 17 beta-estradiol induces the translocation of the estrogen receptors ESR1 and ESR2 to the cell membrane, MAPK3/1 phosphorylation and proliferation of cultured immature rat Sertoli cells. *Biology of reproduction*, *78*(1), 101-114.

Mahaldashtian, M., Makoolati, Z., Ghorbanian, M. T., Naghdi, M., Kouhpayeh, S. A. (2015). *In vitro* cytotoxicity effects of date palm (*Phoenix dactylifera L.*) pollen on neonate mouse spermatogonial stem cells. *Natural product research*, *29*(6), 578-581.

Mather, J. P. (1980). Establishment and characterization of two distinct mouse testicular epithelial cell lines. *Biology of reproduction*, *23*(1), 243-252.

Mawhinney, M., Mariotti, A. (2013). Physiology, pathology and pharmacology of the male reproductive system. *Periodontology 2000*, *61*(1), 232-251.

Mehraban, F., Jafari, M., Akbartabar Toori, M., Sadeghi, H., Joodi, B., Mostafazade, M., Sadeghi, H. (2014). Effects of date palm pollen (*Phoenix dactylifera L.*) and Astragalus ovinus on sperm parameters and sex hormones in adult male rats. *Iranian journal of reproductive medicine*, *12*(10), 705-712.

Meistrich, M. L., Hess, R. A. (2013). Assessment of spermatogenesis through staging of seminiferous tubules. *Spermatogenesis: Methods and Protocols*, *927*, 299-307.

Mense, S. M., Hei, T. K., Ganju, R. K., Bhat, H. K. (2008). Phytoestrogens and breast cancer prevention: possible mechanisms of action. *Environmental health perspectives*, *116*(4), 426-433.

Meyer, M., Essack, M., Kanyanda, S., Rees, J. (2008). A low-cost flow cytometric assay for the detection and quantification of apoptosis using an anionic halogenated fluorescein dye. *Biotechniques*, *45*(3), 317-320.

Mohammadi, F., Nikzad, H., Taherian, A., Amini Mahabadi, J., Salehi, M. (2013). Effects of herbal medicine on male infertility. *Anatomical Sciences Journal*, *10*(4), 3-16.

Monsees, T. K., Franz, M., Gebhardt, S., Winterstein, U., Schill, W. B., Hayatpour, J. (2000). Sertoli cells as a target for reproductive hazards. *Andrologia*, *32*(4-5), 239-246.

Moongkarndi, P., Kosem, N., Kaslungka, S., Luanratana, O., Pongpan, N., Neungton, N. (2004). Antiproliferation, antioxidation and induction of apoptosis by Garcinia mangostana (mangosteen) on SKBR3 human breast cancer cell line. *Journal of ethnopharmacology*, *90*(1), 161-166.

Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of immunological methods*, *65*(1-2), 55-63.

Mourvaki, E., Cardinali, R., Dal Bosco, A., Corazzi, L., Castellini, C. (2010). Effects of flaxseed dietary supplementation on sperm quality and on lipid composition of sperm subfractions and prostatic granules in rabbit.*Theriogenology*, *73*(5), 629-637.

Nieschlag, E., Behre, H. M., Nieschlag, S. (2010). Andrology: Male reproductive health and dysfunction. Springer Science and Business Media. Third edition.

Nurudeen, Q. O., Ajiboye, T. O. (2012). Aqueous root extract of Lecaniodiscus cupanioides restores the alterations in testicular parameters of sexually impaired male rats. *Asian Pacific Journal of Reproduction*, *1*(2), 120-124.

O'Shaughnessy, P. J. (2014). Hormonal control of germ cell development and spermatogenesis. *Seminars in cell & developmental biology*, *29*(2014), 55-65.

Olivetti, L., Grazioli, L. (Eds.). (2010). Imaging of urogenital diseases: a color atlas. Springer Science and Business Media.

Osibote, E., Noah, N., Sadik, O., McGee, D., Ogunlesi, M. (2011). Electrochemical sensors, MTT and immunofluorescence assays for monitoring the proliferation

effects of cissus populnea extracts on Sertoli cells. *Reproductive Biology and Endocrinology*, *9*(1), 65.

Palladino, M. A., Laperche, Y., Hinton, B. T. (1994). Multiple forms of gamma-glutamyl transpeptidase messenger ribonucleic acid are expressed in the adult rat testis and epididymis. *Biology of reproduction*, *50*(2), 320-328.

Pan, S. Y., Litscher, G., Gao, S. H., Zhou, S. F., Yu, Z. L., Chen, H. Q., Zhang, S. F., Tang, M. K., Sun, J. N., Ko, K. M. (2014). Historical perspective of traditional indigenous medical practices: the current renaissance and conservation of herbal resources. *Evidence-Based Complementary and Alternative Medicine*, *2014*, 1-20.

Penttinen, P., Jaehrling, J., Damdimopoulos, A.E., Inzunza, J., Lemmen, J.G., van der Saag, P., Pettersson, K., Gauglitz, G., Mäkelä, S. and Pongratz, I. (2007). Diet-derived polyphenol metabolite enterolactone is a tissue-specific estrogen receptor activator. *Endocrinology*, *148*(10), 4875-4886.

Pfaff, T., Rhodes, J., Bergmann, M., and Weinbauer, G. F. (2013). Inhibin B as a marker of Sertoli cell damage and spermatogenic disturbance in the rat. *Birth Defects Research Part B: Developmental and Reproductive Toxicology*, *98*(1), 91-103.

Pierik, F. H., Vreeburg, J. T., Stijnen, T., De Jong, F. H., Weber, R. F. (1998). Serum inhibin B as a marker of spermatogenesis. *The Journal of Clinical Endocrinology & Metabolism*, *83*(9), 3110-3114.

Pham-Huy, L. A., He, H., Pham-Huy, C. (2008). Free radicals, antioxidants in disease and health. *Int J Biomed Sci*, *4*(2), 89-96.

Pointis, G., Gilleron, J., Carette, D., Segretain, D. (2011). Testicular connexin 43, a precocious molecular target for the effect of environmental toxicants on male fertility. *Spermatogenesis*, *1*(4), 303-317.

Ramaswamy, S., Marshall, G. R., Pohl, C. R., Friedman, R. L., Plant, T. M. (2003). Inhibitory and stimulatory regulation of testicular inhibin B secretion by luteinizing hormone and follicle-stimulating hormone, respectively, in the rhesus monkey (Macaca mulatta). *Endocrinology*, *144*(4), 1175-1185.

Rhee, Y., Brunt, A. (2008). Effects of flaxseed lignan on *in vitro* mitogen-stimulated T cell proliferation. *Journal of Applied Research*, *8*(3), 208-216.

Saarinen, N. M., Power, K., Chen, J., Thompson, L. U. (2006). Flaxseed attenuates the tumor growth stimulating effect of soy protein in ovariectomized athymic mice with MCF-7 human breast cancer xenografts. *International journal of cancer*, *119*(4), 925-931.

Sagittal section showing the position and the structure of female breast. In: http://www.genesiscare.co.uk/breast-cancer. Accessed on 22nd of April 2016.

Schteingart, H. F., Meroni, S. B., Cánepa, D. F., Pellizzari, E. H., Cigorraga, S. B. (1999). Effects of basic fibroblast growth factor and nerve growth factor on lactate production, gamma-glutamyl transpeptidase and aromatase activities in cultured Sertoli cells. *European journal of endocrinology*, *141*(5), 539-545.

Shiratsuchi, A., Osada, Y., Nakanishi, Y. (2013). Differences in the mode of phagocytosis of bacteria between macrophages and testicular Sertoli cells. *Drug discoveries & therapeutics*, *7*(2), 73-77.

Sirtori, C. R., Arnoldi, A., Johnson, S. K. (2005). Phytoestrogens: end of a tale?. *Annals of medicine*, *37*(6), 423-438.

Skinner, M. K., Griswold, M. D. (Eds.). (2004). Sertoli cell biology. Academic press.

Snell, R. S. (2011). Clinical anatomy by regions. Lippincott Williams and Wilkins. Ninth edition

Sofikitis, N., Giotitsas, N., Tsounapi, P., Baltogiannis, D., Giannakis, D., Pardalidis, N. (2008). Hormonal regulation of spermatogenesis and spermiogenesis. *The Journal of steroid biochemistry and molecular biology*,*109*(3), 323-330.

Soto, A. M., Sonnenschein, C., Chung, K. L., Fernandez, M. F., Olea, N., Serrano, F. O. (1995). The E-SCREEN assay as a tool to identify estrogens: an update on estrogenic environmental pollutants. *Environmental health perspectives*, *103*(Suppl 7), 113-122.

Sprando, R. L., Collins, T. F. X., Wiesenfeld, P., Babu, U. S., Rees, C., Black, T., Olejnik, N., Rorie, J. (2000). Testing the potential of flaxseed to affect spermatogenesis: morphometry. *Food and chemical toxicology*, *38*(10), 887-892.

Stanfield, C. L. (2012). Principles of Human Physiology. Pearson Education. Fifth edition

Szewczyk, M., Abarzua, S., Schlichting, A., Nebe, B., Piechulla, B., Briese, V., Richter, D. U. (2014). Effects of extracts from Linum usitatissimum on cell vitality, proliferation and cytotoxicity in human breast cancer cell lines. *Journal of Medicinal Plants Research*, *8*(5), 237–245.

Tan, M., and Yu, D. (2007). Molecular mechanisms of erbB2-mediated breast cancer chemoresistance. In *Breast Cancer Chemosensitivity*, 119-129. Springer New York.

Teerds, K. J., and Huhtaniemi, I. T. (2015). Morphological and functional maturation of Leydig cells: from rodent models to primates. *Human reproduction update*, 1-19.

Thompson, L. U., Chen, J. M., Li, T., Strasser-weippl, K., Goss, P. E. (2005). Dietary Flaxseed Alters Tumor Biological Markers in Postmenopausal Breast Cancer. *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research*, *11*(10), 3828–3835.

Tu'uhevaha, J., Sluka, P., Foo, C. F., Stanton, P. G. (2007). Claudin-11 expression and localisation is regulated by androgens in rat Sertoli cells *in vitro*. *Reproduction*, *133*(6), 1169-1179.

Ueda, H., Tres, L. L., Kierszenbaum, A. L. (1988). Culture patterns and sorting of rat Sertoli cell secretory proteins. *Journal of Cell Science*, *89*(2), 175–188.

Waldschläger, J., Bergemann, C., Ruth, W., Effmert, U., Jeschke, U., Richter, D. U., Kragle, U., Piechulla, B., Briese, V. (2005). Flax-seed extracts with phytoestrogenic effects on a hormone receptor-positive tumour cell line. *Anticancer research*, *25*(3A), 1817-1822.

Walker, W. H., Cheng, J. (2005). FSH and testosterone signaling in Sertoli cells. *Reproduction*, *130*(1), 15-28.

Welshons, W. V., Murphy, C. S., Koch, R., Calaf, G., Jordan, V. C. (1987). Stimulation of breast cancer cells*in vitro* by the environmental estrogen enterolactone and the phytoestrogen equol. *Breast cancer research and treatment*, *10*(2), 169-175.

Westcott, N. D., Muir, A. D. (2003). Flax seed lignan in disease prevention and health promotion. *Phytochemistry Reviews*, *2*(3), 401-417.

WHO Traditional Medicine Strategy 2014-2023. 2013. Available at: http://apps.who.int/iris/bitstream/10665/92455/1/9789241506090_eng.pdf.

Accessed on 5th Oct, 2015.

Wiseman, R. A. (2000). Breast cancer hypothesis: a single cause for the majority of cases. *Journal of epidemiology and community health*, *54*(11), 851-858.

Wong, E. W., Cheng, C. Y. (2011). Impacts of environmental toxicants on male reproductive dysfunction. *Trends in pharmacological sciences*, *32*(5), 290-299.

Wylie, L. (2005). Essential anatomy and physiology in maternity care. Elsevier Health Sciences. Second edition.

Zeiss, C. J. (2003). The apoptosis-necrosis continuum: insights from genetically altered mice. *Veterinary Pathology Online*, *40*(5), 481-495.

Ziegler, U., Groscurth, P. (2004). Morphological features of cell death. *Physiology*, *19*(3), 124-128.

Zirkin, B. R., Chen, H. (2000). Regulation of Leydig cell steroidogenic function during aging. *Biology of reproduction*, *63*(4), 977-981.