

**EFFECT OF *BASELLA ALBA* AND *HIBISCUS MACRANTHUS* ON  
TM4 SERTOLI CELL FUNCTIONS**

By

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## DECLARATION

I, the undersigned, hereby declare that “Effect of *Basella alba* and *Hibiscus macranthus* in TM4 Sertoli Cell Function” is my own work and has not previously in its entity, or in part, been submitted for any degree or examination in any other university. All the resources I have quoted have been indicated and acknowledged by complete references.

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## PUBLICATIONS

A part of the thesis was presented as poster at the following occasions:

- Science Faculty Research Open Day, 2008: “Effect of *Basella alba* on TM4 Sertoli cell function”.
- PSSA, Stellenbosch, 2009: Effect of *Basella alba* and *Hibiscus macranthus* on TM4 Sertoli cell functions”.
- Science Faculty Research Open Day, 2009: Effect of *Basella alba* and *Hibiscus macranthus* on TM4 Sertoli cell functions”.

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## **DEDICATION**

This work is dedicated to the Almighty God for His abundant grace and strength.

*“I can do all things through Christ who strengthens me”*

Phillipians 4:13



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## ABSTRACT

*Basella alba* (BA) and *Hibiscus macranthus* (HM) are used by traditional healers in Cameroon to treat male sexual fertility problems. Previous studies showed that *in vivo* administration of the leaf extracts of both plants caused a significant increase in rat seminal vesicle weight and spermatozoa numbers was accompanied by a significant increase in serum testosterone.

The aim of this study was to establish the effects of BA and HM extracts on Sertoli cell functions. TM4 cell line was used in this study as it exhibited properties similar to the Sertoli cells (Mather, 1982). Sertoli cell play a key role in spermatogenesis by regulating and supporting germ cell development. Therefore, any alterations in Sertoli cell physiology or structure may lead to impaired spermatogenesis, germ cell loss and male infertility. Developing germ cells in the seminiferous tubule require a constant supply of lactate and pyruvate (Jutte et al, 1981; 1982) and toxicant induced alterations in these nutrients have been shown to induce germ cell necrosis (Monsees et al., 2000).

TM4 Sertoli cells were cultured in DMEM/Ham F-12 (M) for one day and exposed to 0.01, 0.1, 1, 10, 100 µg/ml of BA and HM extracts, respectively, for four further days. The extracts were dissolved in 0.5 % DMSO in M, while 0.5 % and 2% DMSO in M were used as negative or positive controls, respectively, and 100mM ethanol as positive control where indicated.

Results obtained from the Sertoli cells exposed to BA extracts, showed that the plant extract had no significant effect on the cell viability but induced a significant concentration-dependent increase in lactate (19-67%) and pyruvate levels (39-102%) and a concentration-dependent decrease in the protein content (9-42%). The H&E histological study confirmed that the BA extract had no cytotoxic effect, as there were no changes in the morphology of the cell. Likewise, apoptotic study using DAPI showed no alteration in the nucleus when compared to the negative control.

The HM plant extract significantly enhanced mitochondrial dehydrogenase activity (7 fold) in the Sertoli cells but caused only slight alterations in the lactate and pyruvate levels. There was no effect seen in the protein content of the Sertoli cells. H&E and DAPI staining revealed that there were neither changes in the morphology of the cells nor any alteration regarding the mitotic and apoptotic indices. Thus, the HM extract did not have a cytotoxic effect on the cells.

This study demonstrated that the *Basella alba* methanol extract may enhance spermatogenesis as it stimulated the source of energy required for the development of germ cells without exerting a cytotoxic effect. The *Hibiscus macranthus* extract stimulated mitochondrial dehydrogenase activities and may thus trigger changes in Sertoli cell physiology. In summary, both plant extracts enhanced certain Sertoli cell functions and thus might explain the positive *in vivo* effects of the combined plant extracts on rat spermatogenesis observed by Moundipa et al. (1999).

**Keywords:** Medicinal plants, *Basella alba*, *Hibiscus macranthus*, TM4 cells, Lactate, Pyruvate, Cell viability, Protein content.

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## LIST OF ABBREVIATION

ABP	Androgen Binding Protein
ADP	Adenosinediphosphate
Apaf 1	Apoptosis protease activating factor 1
ATP	Adenosinetriphosphahate
BA	Basella alba
b-FGF	basic fibroblast growth factor
BTB	Blood Testis Barrier
Ca <sup>2+</sup>	Calcium
cAMP	cyclic Adenosine monophosphate
CDK1s	Cyclic dependent kinase inhibitors
Cl <sup>-</sup>	Chloride
CO <sub>2</sub>	Carbon dioxide
Cyt c	cytochrome c
d	Day
DAPI	4',-6-diamidino-2-phenylindole dihydrochloride
DBP	di-n-butylphthalate
DEHP	di-(2-ethylhexyl) phthalate
DHT	Dihydrotestosterone
DISC	death inducing signaling complex
DNA	Deoxyribonucleic acid
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbant assay
FADD	Fas associated death domain
FGF I	Fibroblast growth factor 1
FGF II	Fibroblast growth factor 2
FSH	Follicle Stimulating Hormone
g	Gram
GH	Growth hormone

GnRH	Gonadotropin Releasing Hormone
H <sup>+</sup>	Hydrogen
HCO <sub>3</sub> <sup>-</sup>	Bicarbonate
HCl	Hydrogen chloride
HM	Hibiscus macranthus
HPTA	Hypothalamic Pituitary Testicular Axis
H <sub>2</sub> O	Water
IGF-1	Insulin –like growth factor-1
IGF-2	Insulin –like growth factor-2
IL-1	Interleukin-1 $\alpha$
K <sup>+</sup>	Potassium
LH	Luteinizing hormone
mg	Milligram
Mg <sup>2+</sup>	Magnesium
mEq/l	Miliequivalent per litre
MIS	Mullerian inhibiting substance
mm	millimeter
ml	milliliter
mol/l	mole per liter
mmol/l	millimole per liter
MTP reader	Microtitre plate reader
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
Na <sup>+</sup>	Sodium
Na <sup>+</sup> -K <sup>-</sup>	Sodium Potassium
NF $\kappa$ B	nuclear factor kappa B
oPRL	ovine prolactin
PBS	Phosphate buffered saline
PKA	protein Kinase A
PRL	Prolactin
rABP	rat Androgen Binding Protein

SGF	Seminiferous growth factor
SGSGF	Sertoli cell secreted growth factor
SPARC	Secreted protein acidic and rich in cysteine
T	Testosterone
T3	3, 5, 3 <sup>1</sup> -triiodo-L-thyronine
TGF- $\alpha$	Transforming growth factor- $\alpha$
TGF- $\beta$	Transforming growth factor $\beta$
$\mu$ l	microlitre
XTT	2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5- carboxanilide inner salt



# CHAPTER ONE

## Introduction

### 1.1 Overview of the Male Reproductive System

The human male reproductive system consists of series of organs located outside the body and around the pelvic region of the male. It allows for the production and release of sperm, production of the sex hormones and the passage of urine to the exterior. The male reproductive system (figure 1) consists of the external genital organs (i.e. scrotum and penis) and the internal genital organs (includes testes, epididymides, ductus deferens, seminal vesicles, ejaculatory ducts, prostate and the bulbourethral glands).

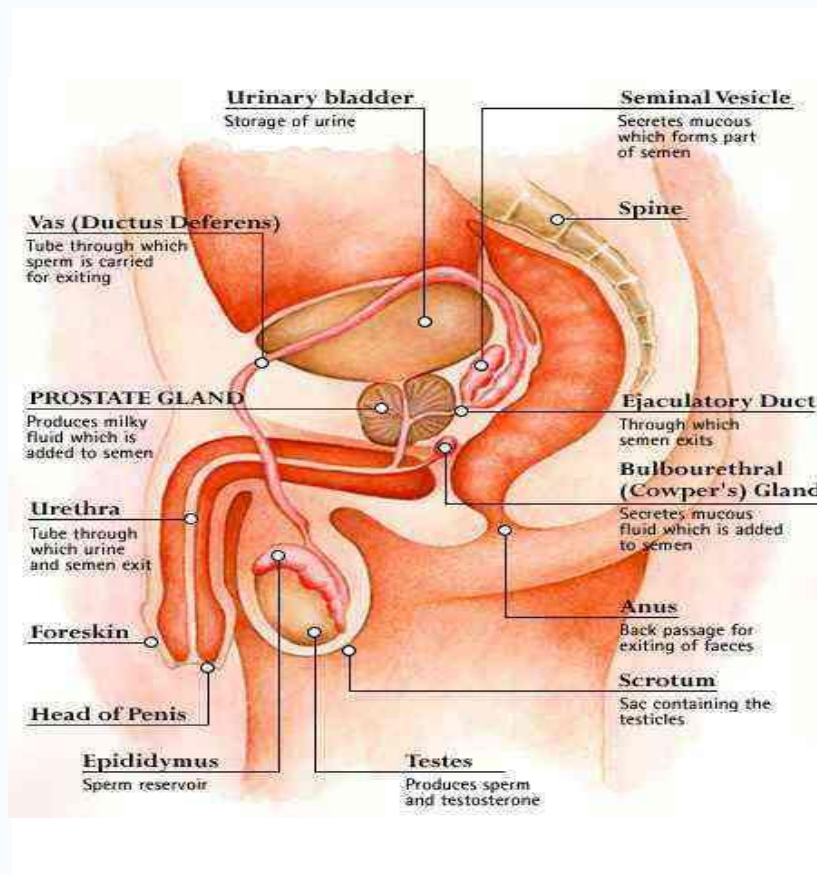


Figure 1: The male reproductive system ([http:// www.phoenix5.org/prostate](http://www.phoenix5.org/prostate) draw).

### ***1.1.1 Scrotum***

The scrotum is situated posteroinferior to the penis and inferior to the pubic symphysis (figure 1). It is a cutaneous fibromuscular sac encloses the testes and the spermatic cords. The scrotum consists of a heavily pigmented skin and dartos fascia (responsible for the wrinkled appearance of the scrotum). Contraction of the dartos muscle causes the skin of the scrotum to wrinkle, decreasing heat loss. This action contributes to the regulation of temperature in the testes for normal spermatogenesis to occur.

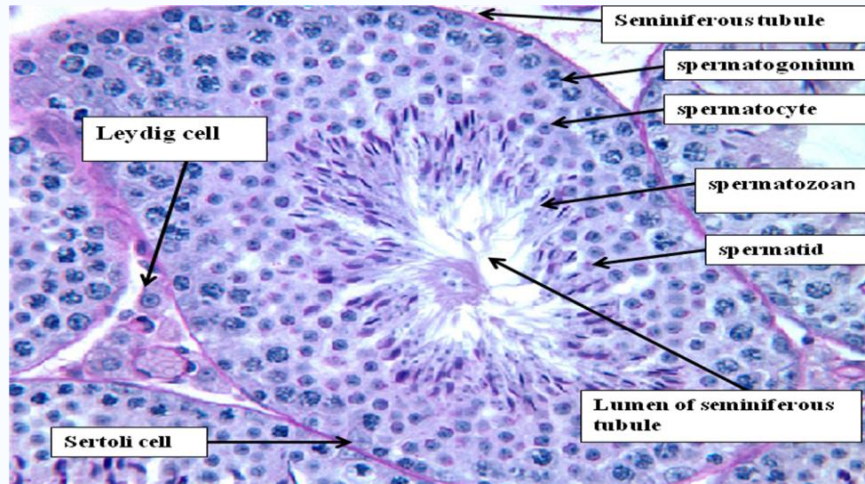
### ***1.1.2 Penis***

The penis is the male copulatory organ and common outlet for the passage of urine and semen (figure 1). It consists of the root, body and glans penis. It is composed of three cylindrical bodies of erectile cavernous tissue (corpora cavernosa and corpus spongiosum) enclosed by the tunica albuginea.

### ***1.1.3 Testes***

The testis is an oval organ suspended by the spermatic cord in the scrotum (figure 1). The testis consists of a right and left side separated by the median scrotal septum and measures about 5cm in length, 2.5cm in breadth and 3cm anteroposterior diameter (Sinnatamby, 1999). The germ and Sertoli cells are located within the seminiferous tubules while Leydig cells are located in the interstitial spaces between seminiferous tubules (Sinnatamby, 1999; figure 2).

The testes are composed of many duct systems, which include convoluted seminiferous tubules in which sperm cells formed are transported to the rete testis (a network of canals at the termination of the straight tubule). Efferent ductules transport the sperm cells from the rete testis to the epididymis where they are stored.



**Figure 2:** Diagram showing the cells present in the testis (<http://reproductivegenomics.jax.org>)

Development of the germ cells into mature spermatozoa occurs in the seminiferous tubules while Sertoli cells supply supportive and nutritive substances to germ cells and the production of testosterone is the function of Leydig cells located in interstitial spaces of the testes.

For optimal testicular temperature, several compensatory mechanisms take place. These include the lack of subcutaneous fat and rich sweat glands in the scrotum for efficient dissipation of heat, counter-current exchange of heat by the pampiniform plexus, actions of the dartos and cremaster muscles to lower and lift the testes. The cremaster muscle contract to provide more warmth by shortening the spermatic cord, which brings the testes close to the body. On the other hand, contraction of the dartos muscle results in wrinkling of the scrotum to decrease heat loss. When cool temperature is required, the cremaster muscle relaxes by lengthening the spermatic cord and lowering the testicle away from the warm body.



#### ***1.1.4 Epididymis***

The epididymis is formed by minute convolutions of the ducts of the epididymis and it is divided into three parts (head, body and tail; figure 1). The head of the epididymis is composed of lobules formed by the coiled ends of 12-14 efferent ductules while the body consists of the convulated duct of the epididymis and the tail is continuous with the ductus deferens (Moore & Dalley, 1999).

The epididymis lies on the posterolateral surface of the testis (figure 1) with the head attached to the upper pole by vasa efferentia and the tail to the lower pole of the testis by loose connective tissue. It is responsible for the storage and maturation of the sperm (Moore & Persuad, 1998).

#### ***1.1.5 Ductus deferens***

The ductus deferens also known as the vas deferens is a continuation of the duct of the epididymis at the tail of the epididymis (figure 1). It ascends the spermatic cord and passes through the inguinal canal, crossing over the external iliac vessels to enter the pelvis. It ends by joining the duct of the seminal vesicle to form the ejaculatory duct. During its course, the ductus deferens enlarges to form the ampulla of the ductus deferens where spermatozoa are store for a short while before transported to the ejaculatory duct (Moore & Dalley, 1999).

The proximal part of the ductus deferens absorbs fluid produced by the seminiferous tubules of the testes and the ductus itself contributes only a small volume of the seminal fluid (Sinnatamby, 1999).

#### ***1.1.6 Seminal vesicles***

The paired seminal vesicle is an elongated structure of approximately 5cm long (Moore & Dalley, 1999) that lies between the fundus of the bladder and the rectum (figure 1). They secrete a thick alkaline fluid that mixes with the sperm as they traverse the ejaculatory duct and urethra. The seminal vesicles are the source of the seminal fluid fructose and prostaglandins (Griffin & Ojeda, 1992). The pair of seminal vesicles

produces 60% of the total volume of the seminal fluid (Sinnatamby, 1999). They join the ductus deferens to form the ejaculatory ducts.

### ***1.1.7 Ejaculatory ducts***

Each ejaculatory duct is about 2.5cm long and arises by the union of the ductus deferens and the duct of the seminal vesicle (figure 1). The ejaculatory ducts converge to open on the seminal colliculus or just within the opening of the prostate utricle (Moore & Dalley, 1999). It acts as a passage for the semen to the urethra.

### ***1.1.8 Prostate***

The prostate is a firm, walnut sized, partly glandular, partly fibro muscular organ that lies beneath the bladder and above the urogenital diaphragm (figure 1). It is approximately 3cm long (Moore & Dalley, 1999) and penetrated by the proximal part of the urethra.

The prostate is the largest accessory gland of the male reproductive system and consist of a central and peripheral zone, accounting for about 25% and 75% of the glandular substances respectively.

The prostatic duct of the peripheral zone opens into the prostatic sinus and the ducts of the central zone open on the seminal colliculus around the orifices of the ejaculatory duct. The prostate produces about 30% of the total volume of the seminal fluid (Sinnatamby, 1999). The prostate is the source of seminal fluid spermine, citric acid, zinc and acid phosphatase (Griffin & Ojeda, 1992).

### ***1.1.9 Bulbourethral gland***

The bulbourethral gland also known as the Cowper's gland is a pea sized structure that lie posterolateral to the membranous part of the urethra (figure 1). The duct from each gland is about 2.5cm long, with a diameter of 1cm and opens into the bulb of the penile urethra. The glands provide a small contribution to the seminal fluid that enters the urethra during sexual arousal (Sinnatamby, 1999).

## *1.2 Spermatogenesis*

Spermatogenesis is the entire sequence of events that leads to the transformation of spermatozoa from the developing germ cells (spermatogonia) in the seminiferous tubules of the testes (figure 3). Spermatogonia formed during the foetal period remain dormant in the seminiferous tubule until puberty (Holstein et al., 2003). Spermatogenesis starts at puberty and usually continuous uninterruptedly until death with a slight decrease in the quantity of produced sperm with increase in age (Holstein et al., 2003; <http://en.wikipedia.org>). During spermatogenesis three stages can be distinguished and includes spermatocytogenesis, maturation of spermatocytes and spermiogenesis (Holstein et al., 2003)

Several types of spermatogonia can be distinguished by their position in the basal part of the germinal epithelium, their morphology and stain ability of nuclei: Type A and Type B spermatogonia (Clermont, 1966). At puberty, the Type A spermatogonium begins to proliferate through several mitotic divisions to form Type A spermatogonium and Type B spermatogonium. The Type A undergoes mitotic divisions to produce more spermatogonia while the Type B spermatogonia undergo gradual changes to become the primary spermatocytes up to the spermatids. This describes the process of spermatocytogenesis.

The maturation of spermatocytes involves changes in chromatin condensation in the nucleus after spermatogonial division, which starts at the leptotene stage of prophase in the basal compartment of the germinal epithelium (Holstein et al., 2003). Each spermatogonium undergoing differentiation gives rise to 16 primary spermatocytes (Griffin & Ojeda, 1992). The primary spermatocytes pass Sertoli cell barrier to reach the adluminal compartment and continue with the other prophase stages that is the zygotene stage, the pachytene and the diplotene stage. During prophase, there is reduplication of DNA, condensation of chromosomes, pairing of homologous chromosomes and crossing over of the chromosome. After this division, the primary spermatocytes become secondary spermatocytes and undergo no DNA replication but divide quickly to form the spermatids (Holstein et al., 2003)

In summary, each primary spermatocyte undergoes the first meiotic division (which is a reduction phase) to form two haploid secondary spermatocyte with 23 chromosome each. Secondary spermatocytes undergo a second mitotic division with each forming two haploid spermatids (23 chromosomes). In other words, from each primary spermatocyte, four haploid spermatids are formed and eventually four spermatozoa.

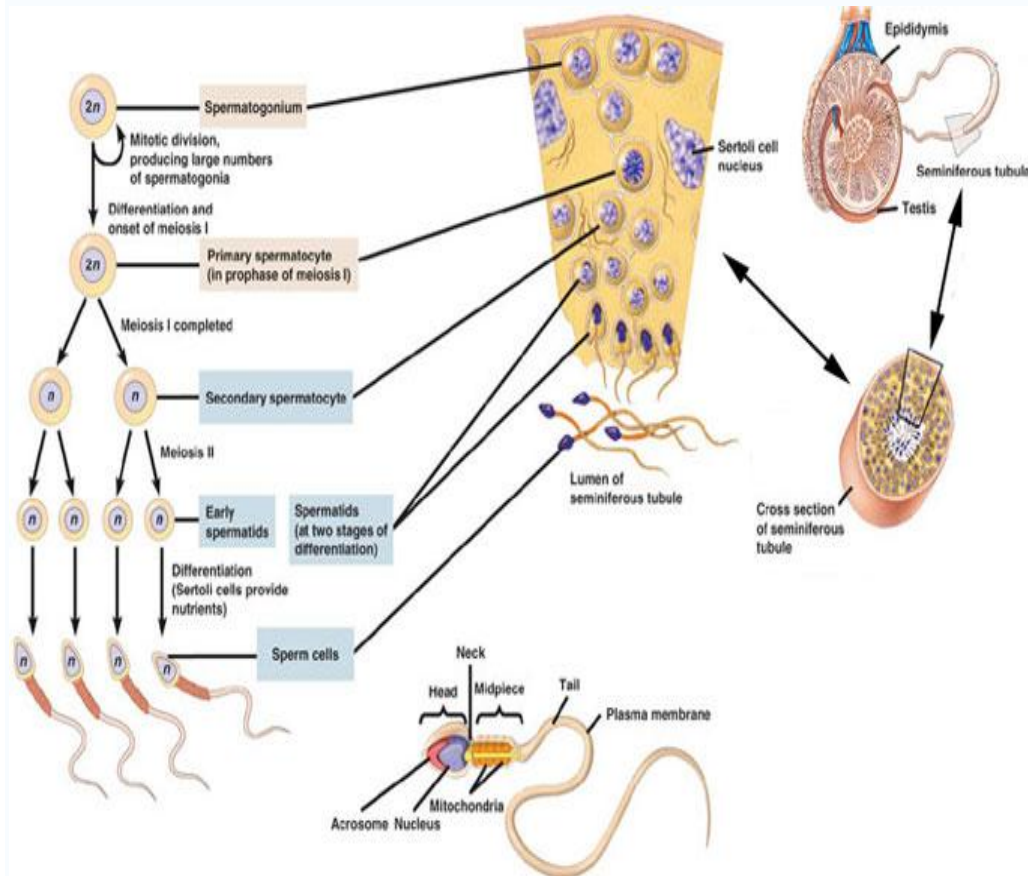
The spermatids undergo transformation into spermatozoa through a series of events in a process called spermiogenesis. Spermiogenesis involves the transformation of the round spermatids with a nucleus, Golgi apparatus, centriole and mitochondria into elongated spermatozoa. The nucleus of the spermatid forms the head, Golgi apparatus is forms acrosomic cap, the centriole forms the axial filament and annulus with some of the axial filament forming the tail and most of the cytoplasm of the spermatid, shed away with the cell membrane persisting as a covering for the spermatozoa (Moore & Persuad, 1998).

When spermiogenesis is complete, spermatozoa enter the lumina of the seminiferous tubules. This release of the spermatozoa into the lumen of seminiferous tubule is referred to as spermiation. In the human, it takes about sixty-four (64) days for the entire process of spermatogenesis to take place including the spermiogenesis (Moore & Dalley, 1999).

The process of spermatogenesis in the seminiferous tubule is regulated by both intratesticular and extratesticular factors. The intratesticular influence involves the Leydig cells in the intertubular space secreting testosterone, neuroendocrine substances and growth factors directed to neighboring Leydig cells, blood vessels, lamina priopia of the seminiferous tubule and Sertoli cells. These secretions are involved in maintenance of the Sertoli cells and cells of the peritubular tissue (Holstein et al., 2003). Different growth factors from the Sertoli cells also play a role in the regulation of spermatogenesis.

Extratesticular influence is derived from the hypothalamus and the pituitary gland whereby pulsatile secretions from gonadotropin releasing hormone (GnRH) of the hypothalamus initiates the release of Luteinizing hormone (LH) from the pituitary gland. As a result of this stimulation, testosterone is produced from the Leydig cell which not only influences the spermatogenesis in the seminiferous tubule but it is distributed

throughout the body and provide feedback to the pituitary gland that is related to the secretory activity of the Leydig cells (Holstein et al., 2003).



**Figure 3:** Diagram illustrating the different stages of spermatogenesis and site where it occurs ([http:// porpax.bio.miami.edu](http://porpax.bio.miami.edu))

### ***1.3 Morphology of the Sertoli cell***

The Sertoli cell, named after its discoverer, Enrico Sertoli (1842- 1910), is also known as the ramifying cell, sustentacular cell, supporting cell or nurse cell. It is a tall (75-100µm) simultaneously columnar and stellate cell with a base solidly attached to a basement membrane, an apex that reaches the tubular lumen and numerous lateral and apical veil-like processes that extends between and around every germinal cell (Russell & Griswold, 1993). Its nucleus occupies from 8% in human to 18% in mouse (Russell & Griswold, 1993) of its cell volume and it is irregularly shaped.

Sertoli cells of most species have a high degree of specialized function due to the unequal distribution of cell organelles (Russell & Griswold, 1993). This compartmentalization of the organelles or high concentration of organelles in different regions of its cytoplasm depicts the regional functioning of the Sertoli cell in relationship to the physiological function or secretion of the cell (Russell & Griswold, 1993). Mitochondria within the cytoplasm of Sertoli cells are numerous and scattered throughout the cells with its shape and appearance of their cristae characteristic of its species (Russell & Griswold, 1993).

The smooth endoplasmic reticulum predominates the rough endoplasmic reticulum in Sertoli cell of most mammalian species. This sparse number of rough endoplasmic reticulum suggests that Sertoli cells are primarily not a protein-secreting cell or has a relatively small capacity for protein synthesis but may be involved in steroid synthesis and/ or metabolism of lipids and steroids (Russell & Griswold, 1993).

The cytoplasm of Sertoli cells also contains the Golgi apparatus that is composed of multiple separate elements scattered throughout the basal cytoplasm. Also present are lipids in the form of lipid droplet in mammalian species (Lacy, 1962) with the size and appearance of the droplet varying with each species (Fawcett, 1975). Histological sections of the Sertoli cells show a higher number of lipid droplets with increasing age of the rat, indicating the biological clock of the testis (Holstein, 1999). Lysosomes scattered within the cytoplasm may be responsible for degradation of residual bodies after the release of sperm (Bozzola & Russell, 1992).

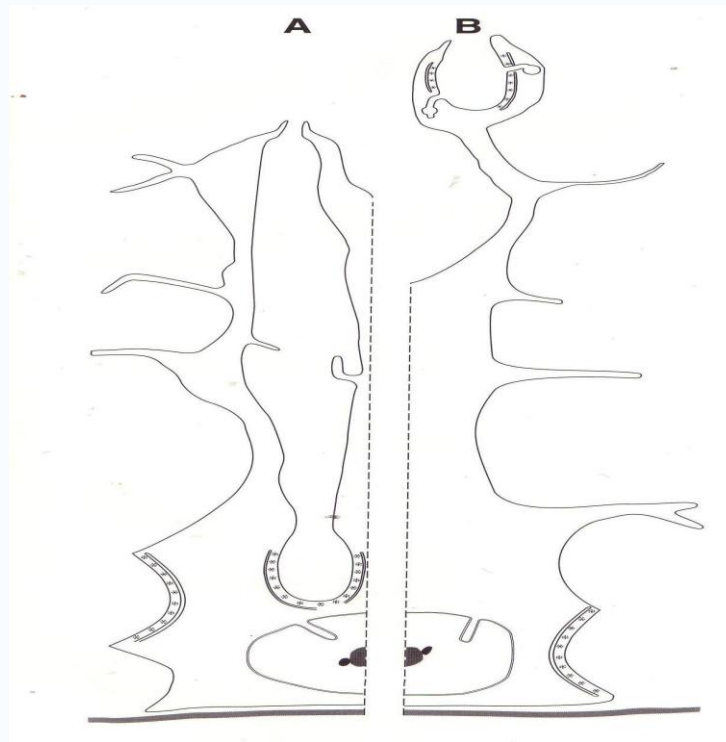
At the junction of the seminiferous tubule with the straight tubule called the transition zone, there is a gradual loss of germinal cells with only Sertoli cells and few spermatogonia present. Some variations exist in the Sertoli cells present in this zone such as the transition of the irregular columnar epithelium of the Sertoli cells to the low cuboidal epithelium of the straight tubule connecting to the rete testis (Hermo & Dworkin, 1988). The number of rough endoplasmic reticulum compared to smooth endoplasmic reticulum is more, with fewer mitochondria. Sertoli cells are also not centripetally orientated but slanted in the direction of the fluid flow (Russell & Griswold, 1993).

Sertoli cells undergo rapid morphological differentiation after the establishment of inter-Sertoli cell tight junctions called the blood-testis barrier. Which include large increase in cell size, extent of cytoplasmic processes between germ cells, pattern modification in the nuclear chromatin condensation and nuclear envelop infoldings and changes in the development of the cellular organelles (Jegou, 1992). Blood-testis barrier located at the basal lamina of the seminiferous tubules creates a physical barrier between the blood vessels of the interstitium and the adluminal compartment of the seminiferous tubules.

A close morphological association exists between the Sertoli cell and the developing germ cells at different stages of their development (figures 2 and 5). Morphometric analysis has shown that each Sertoli cell is associated with ~30-50 germ cells at each stage of the spermatogenic cycle in the epithelium (Cheng & Mruk, 2002).

Sertoli cells are classified as Type A and Type B based on their shape (figure 4) during the spermatogenic cycle. Type A Sertoli cells show deep crypts for elongated spermatids while the Type B cells show apical expansions with no crypt that contain elongated spermatids that are almost ready to be released (Wong & Russell, 1983; Russell et al., 1986) and support the movement of the elongated spermatid toward the lumen. The Type A Sertoli cell of the rat changes into a Type B during spermatogenic cycle (Russell & Griswold, 1993).

Three-dimensional aspects of the Sertoli cell of the Shiba goat that was investigated (Kurohmaru & Nishida, 1987) using a scanning electron microscopy depicts the apical part of some Sertoli cells to be expanded as spermatids that resided within the crypt of the cell emerged from its apical aspect and this corresponds to the Type A Sertoli cells. Other micrographs show that some Sertoli cells with an apical expansion are partially around the head of spermatids undergoing spermatogenesis indicating these to be the Type B Sertoli cells.



**Figure 4:** Structure of the Type A (A) and Type B (B) Sertoli cells. Type A Sertoli cells show deep crypts for elongated spermatids while Type B Sertoli cells have apical expansions that contains elongated spermatids ready for spermiation. (Russell & Griswold, 1993)



#### **1.4      *Physiology of the Sertoli cells***

Sertoli cells create an appropriate environment for germ cell proliferation and maturation (spermatogenesis). Studies show that the germ cells depend on the Sertoli cells for nutritional and structural support (Costa et al., 1998, Jegou, 1993, Russell & Griswold 1993). Tight junctions separating the germinal epithelium into an apical and basal compartment (see 1.5.4) connect the Sertoli cells. This tight junction forms the blood-testis barrier of the testis and developing germ cells pass this barrier to enter the adluminal compartment. In the rat, the entire process of germ cell development, except for the early phase of spermatogenesis from Type B spermatogonia to preleptotene and leptotene spermatocyte, is segregated from the systemic circulation because of the presence of the blood-testis barrier (BTB) near the basal lamina (Dym & Clermont 1970; Dym & Cavicchia, 1977; 1978, Russell & Peterson, 1985; Setchell, 1980; Setchell et al., 1969).

The biochemical activity of the postnatal rat Sertoli cells depicts a multiple and diverse functional capability with the time of onset and continuity varying with the stages of development of the Sertoli cells (table 1). This activity encloses a wide range of functions related to the prepubertal and pubertal testicular development. Many of them show age related changes (Russell & Griswold, 1993). Therefore, a disruption in the structure or function of the Sertoli cell would lead to impairment in spermatogenesis and may eventually cause male infertility (Monsees et al., 2000).

**Table 1:** Sertoli Cell Activity and Related Changes during Postnatal Development in the Rat

<b>Germ Cell Differentiation</b>	<b>Start of Spermatogenesis</b>	<b>Meiosis Begins</b>	<b>Pachytene Spermatocytes</b>	<b>Spermatids</b>
<b>Sertoli Cell Kinetics</b>	Number Increases Gradually Decline in Mitotic Activity		Cessation of Mitosis	Stable Population
<b>Blood-Testis Barrier</b>	Non-occlusive Sertoli Cell Junctions		Barrier Formation	Functional Barrier
<b>Tubular Lumen Development</b>	Seminiferous Cords Lack Lumen		Lumen Formation Established	
<b>Anti-Mullerian Hormone</b>	Declines from Peak Foetal Levels		Negligible to Undetectable	
<b>Androgen Binding Protein</b>		Production Begins	Marked Increase	Gradual Increase Plateau Adult Levels
<b>Androgen Receptors</b>			Increased Production	Further Increase
<b>Estrogen Synthesis</b>	Active Conversion from Testosterone		Rapid Decrease in Aromatase Activity	
<b>FSH Receptors</b>		Increase in Number and Activity		Sharp Decline in Sertoli Cell Responsiveness to FSH
<b>Inhibin Production</b>		Maximal Activity		Decrease with Advancing Age
<b>Growth Factors</b>				Increase in Sertoli Cell Secreted Growth Factor Production
<b>Other Biochemical Changes</b>			Increase in Total RNA Concentration, Protein Synthetic Activity, Polyamine Concentrations, $\gamma$ - Glutamyl Peptidase Activity	
<b>Days of Age</b>	<b>7</b>	<b>14</b>	<b>21</b>	<b>28</b> <b>35</b>

(Russell & Griswold, 1993)

### ***1.5 Functions of Sertoli cell***

The functions of Sertoli cells controlled by androgens and pituitary gonadotropin follicle-stimulating hormone are mostly associated with different stages of the developing germ cells (figure 5).

#### ***1.5.1 Sustentacular or supportive role***

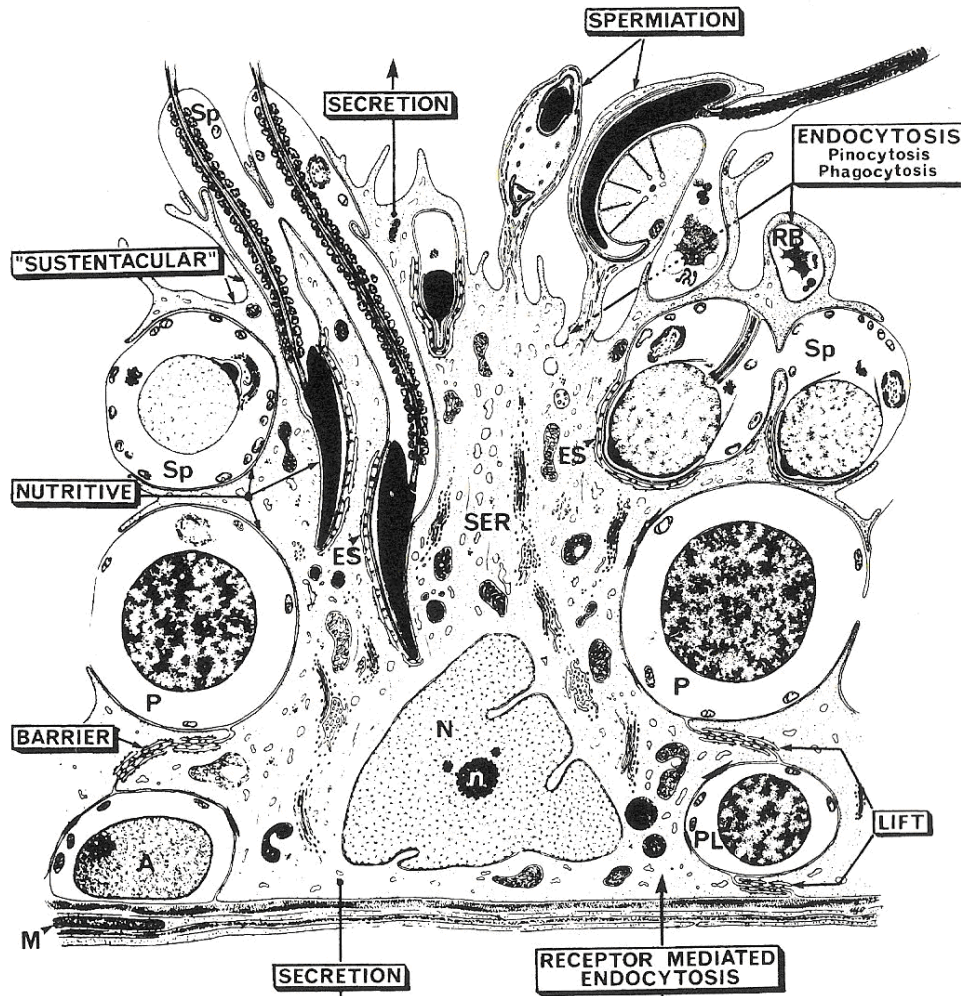
The Sertoli cell plays a sustentacular or supportive role by maintaining the positions of the differentiating germ cells (i.e. the spermatocytes and spermatids; figure 5) in the adluminal compartment of the seminiferous epithelium (Russell & Griswold, 1993). They carry out their supporting functions by creating an environment suitable for the attachment and development of the germ cells by providing the nutrients and hormones required by them.

#### ***1.5.2 Pinocytosis***

Pinocytosis is a fluid-phase endocytosis that takes place at the apex of the Sertoli cell. It results in the formation of lysosomes, which fuses with the phagosomes and contributes to their elimination from the seminiferous epithelium (Russell & Griswold, 1993).

#### ***1.5.3 Phagocytosis***

The Sertoli cells phagocytose or internalize and eliminate residual cytoplasmic bodies released from the spermatids at the time of spermiation during spermatogenesis (Russell & Griswold, 1993).



**Figure 5:** Diagram illustrating the relationship of a Sertoli cell with the various germ cells of the rat and its functions. N, Nucleus and n, nucleolus of a Sertoli cell; A, spermatogonium; PL, preleptotene spermatocyte; P, pachytene spermatocyte; Sp, spermatids; ES, ectoplasmic specialization; RB, residual body; M, myoid cell; SER, smooth endoplasmic reticulum (Russell & Griswold, 1993).

#### ***1.4.4 Formation of blood-testis barrier***

The blood-testis barrier (BTB) is a tight junction between Sertoli cells at the basal lamina of the seminiferous tubules (figures 6 and 7). It creates a physical barrier between the blood vessels of the interstitium and the adluminal compartment of the seminiferous tubules of the testes.

Time of formation of the BTB varies with mammalian species (Table 2), it is absent at birth and in early postnatal period. Establishment of the occlusive junction that restricts the flow of substances within the seminiferous tubule occurs during pre-pubertal development. The time of appearance and completion of the occlusive junction coincides with the appearance and differentiation of spermatocytes in the initial spermatogenic cycle (Russell & Griswold, 1993).

**Table 2:** Timing of Sertoli Cell Barrier Formation

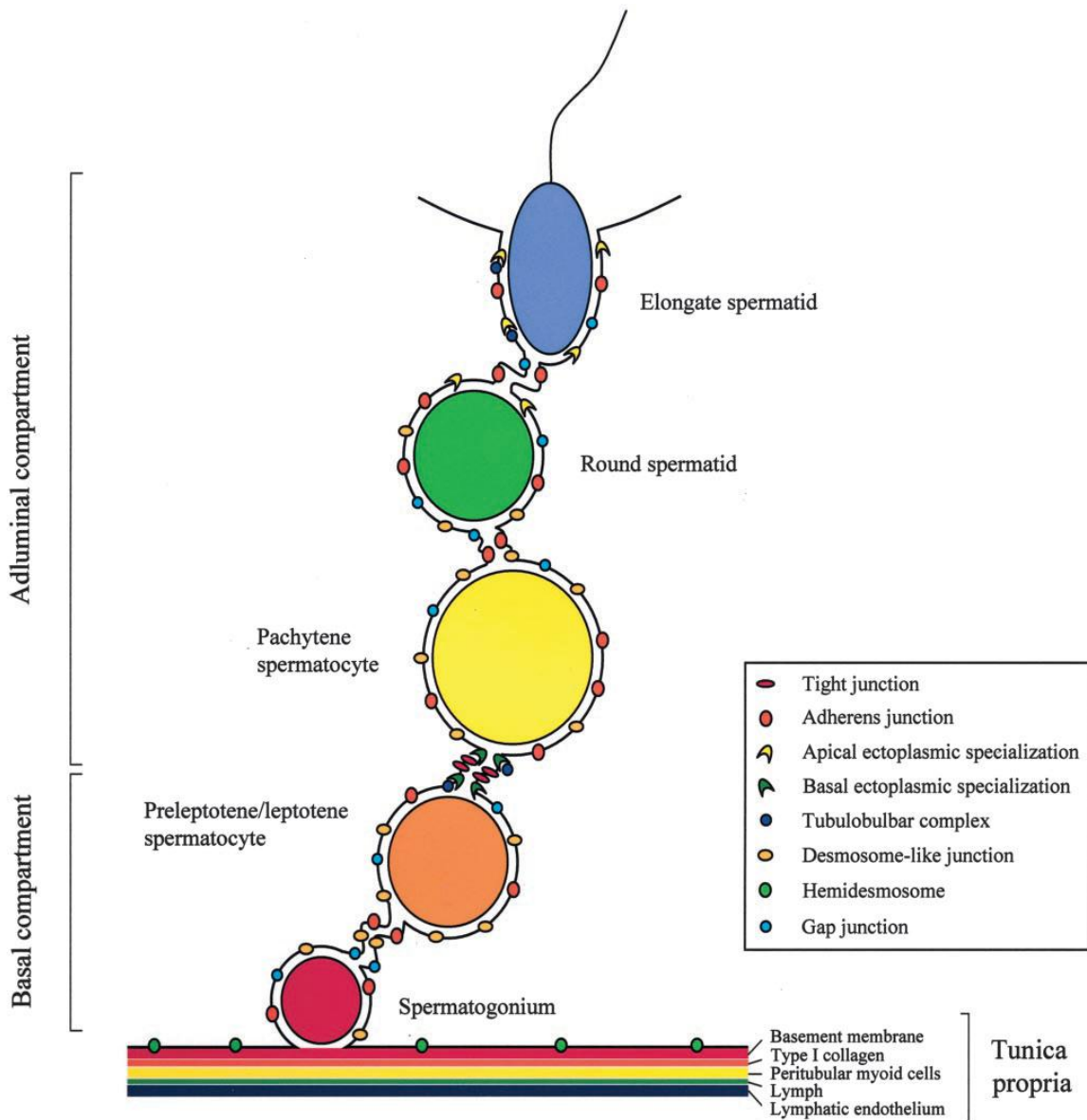
<b>Species</b>	<b>Age of Barrier Establishment</b>
Mouse	10-16 days
Rat	15-18 days
Hamster	20 days
Guinea Pig	15-21 days
Rabbit	10-11 weeks
Pig	17 weeks
Dog	20 weeks
Bull	24-28 weeks
Mink	32-34 weeks
Human	11-14 years

Based on electron microscopy, freeze fracture and trace exclusion studies (Russell & Griswold, 1993)

The blood-testis barrier plays an important role in compartmentalization by creating a barrier that restricts the diffusion of solutes through paracellular routes (Stevenson & Keon, 1998; Mitic & Anderson, 1998). It also creates an apical and basal polarity within the cell, which differ in protein and lipid composition. This in turn creates and maintains epithelial and endothelial cell polarity (Rodriguez-Boulan & Nelson, 1989). This role can be described as the fencing function of BTB.

Seminiferous tubule is separated into a basal and adluminal compartments by blood-testis barrier (Meng et al., 2005; figure 6). The basal compartment contains the spermatogonia and early spermatocytes while the adluminal compartment contains meiotic spermatocytes and spermatids at various stages of spermiogenesis (figure 6). Apart from the early stage of development of the germ cells from Type B spermatogonia to the preleptotene and leptotene spermatocyte, the BTB segregates the entire process of germ cell development from the systemic circulation (Cheng & Mruk, 2002). The inter-Sertoli cell junctions (BTB) (Jegou, 1992) prevent direct access to substances from the extratubular compartment to the adluminal compartment of the seminiferous tubule. Because of the segregation, the developing germ cells rely on the Sertoli cells for the supply of the needed nutrients and biological factors.

The BTB creates an immunological barrier that sequesters antigenic determinants residing on germ cell surfaces from the systemic circulation. This barrier also excludes the entry of circulating immunoglobulins and lymphocytes into the adluminal compartment (Setchell, 1980). Thus, the blood-testis barrier plays a crucial role in normal spermatogenesis by shielding developing germ cells from blood-borne mutagenic substances and by preventing autoimmune reactions against sperm cells from occurring (Mann et al., 2003).



**Figure 6:** Diagram illustrating the compartment formed by the blood-testis barrier (Mruk & Cheng, 2004)

#### ***1.4.5 Disassembly and Reassembly***

In order to prevent disruption in the microenvironment, the tight junctions disassemble (breakdown) and reassemble (reconstitutes) in a coordinated manner at the tight junctions of the blood-testis barrier. To enable the movement of the early spermatocytes (preleptotene and leptotene spermatocytes) from the basal compartment to the adluminal compartment of the seminiferous epithelium for further development (Dym & Cavicchia, 1977; Russell, 1977; figure 7) while the spermatogonia remain attached to the basal compartment.

Four theories were proposed for the regulation of the disassembly and reassembly of the Sertoli cell tight junction. They include the following:

- (a) “Zipper” theory: This proposes that tight junctions (TJs) at the basal domains of the Sertoli cells break down to accommodate the passage of preleptotene or leptotene spermatocytes across the BTB while forming new TJ fibrils under the migrating preleptotene and leptotene spermatocytes (Pelletier & Byers, 1992). This theory has limitations in that there are no *in vivo* studies showing leakage of tracers into the tubular lumen even for a short period and it does not explain what triggers the dissociation and association of TJs fibrils to facilitate the upward movement of preleptotene and leptotene spermatocytes across the BTB (Cheng & Mruk, 2002).
- (b) Intermediate cellular compartment theory: Russell (1977) observed the presence of a compartment occupied by the germ cells in transit from basal to adluminal compartment of the seminiferous epithelium. The limitation to this theory is the fact that only one occluding zonule per Sertoli cell at any one time was found in subsequent morphological studies (Pelletier & Byers, 1992). However, a defined intermediate compartment in seminiferous epithelium of mature boar had been shown of which, the BTB must “open” for the migration of germ cells without disrupting its integrity (Yazama, 2008).

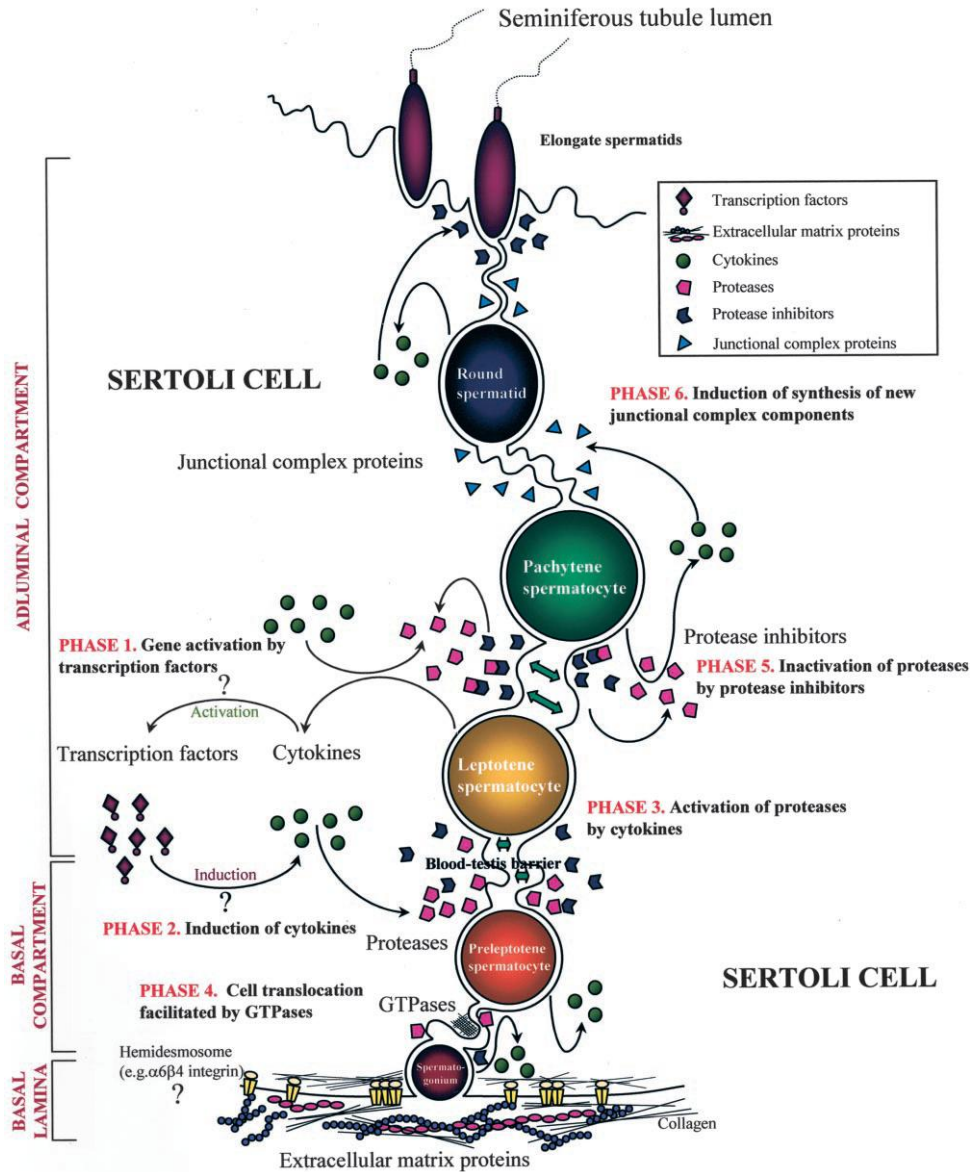
Wang & Cheng, (2007) showed that during migration of germ cells in the seminiferous epithelium, cell adhesion molecules (such as junctional adhesion



molecules (JAM)/coxsackie and adenosine receptors (CAR)/nectin) provide transient adhesion between Sertoli and germ cells through homophilic and heterophilic interactions.

- (c) Stress/repetitive removal of membrane segment theory: proposed a continuous upward movement of a large number of germ cell caused stress on Sertoli cell TJs leading to changes in orientation, disintegration and proliferation TJ fibrils (Pelletier & Byers, 1992). This did not explain what triggered and facilitated the upward movement of the germ cells and how stress induced changes in junction orientation and breakage in the TJs fibrils (Cheng & Mruk, 2002).
- (d) Junction restructuring theory: this theory proposed that the germ cell movement consist of intermittent phases of cell junction disassembly and assembly. This allowed for timely passage of germ cells from one site to another in the epithelium (Mruk & Cheng, 2000). For example, tight junctions comprised of transmembrane proteins, such as occludin and claudin (Tsukita & Furuse, 1999) must gradually disassemble (figure 7) one fibril at a time to allow for the passage of preleptotene/leptotene spermatocytes across the blood-testis barrier (Mruk & Cheng, 2004).

It was hypothesized that the germ cell movement required a number of factors such as cytokines, proteases, protease inhibitors, protein kinases, protein phosphatases, junctional complex and extracellular matrix proteins, and GTPases (figure 7). However, disassembled junctions must quickly reassemble to maintain the integrity of the seminiferous epithelium (Mruk & Cheng, 2004).



**Figure 7:** The cascade of events leading to the migration of germ cells from the basal to adluminal compartment of the seminiferous epithelium (phases 1–6). This hypothesis explains that for the migration of germ cells from the basal to the adluminal compartment of the seminiferous tubule a series of factors such as cytokines, proteases, protease inhibitors, protein kinases, protein phosphatases, junctional complex and extracellular matrix proteins, and GTPases must be involved (Mruk & Cheng, 2004).

#### ***1.4.6 Spermiation***

It entails the release of the late spermatids from the Sertoli cell's crypts, migration of the spermatids from the depth of the seminiferous epithelium toward the lumen. Followed by retention of the encapsulated heads of spermatids along the surface of seminiferous epithelium by the Sertoli cell processes with disengagement of the heads of spermatids from the Sertoli cell apical processes and phagocytosis of residual bodies that detach from the spermatozoa (Russell & Griswold, 1993).

#### ***1.4.7 Nutrition***

The Sertoli cells provide nutritive substances such as sugars, amino acids, lipids, metallic elements, vitamins for the development of the germinal cells necessary for their development (Russell & Griswold, 1993; Mruk & Cheng, 2004).

Spermatocytes and spermatids in the adluminal compartment depend on the Sertoli cells for the supply of nutritive substances necessary for their adequate development, after the migration of the leptotene and preleptotene spermatocytes from the basal to adluminal compartment. Since the blood testis barrier segregates the developing germ cells from the systemic circulation, the developing germ cells rely on the Sertoli cells for their nutritive supply.

#### ***1.4.8 Secretions***

The Sertoli cells are secretory cells (Mruk & Cheng, 2004) which secrete proteins (Table 3), seminiferous tubule fluid, peptides and steroids (Jegou, 1992). Glycoproteins make up 15% of the proteins synthesized and secreted by Sertoli cells (Griswold, 1988).

**Table 3:** Proteins secreted by the Sertoli cells

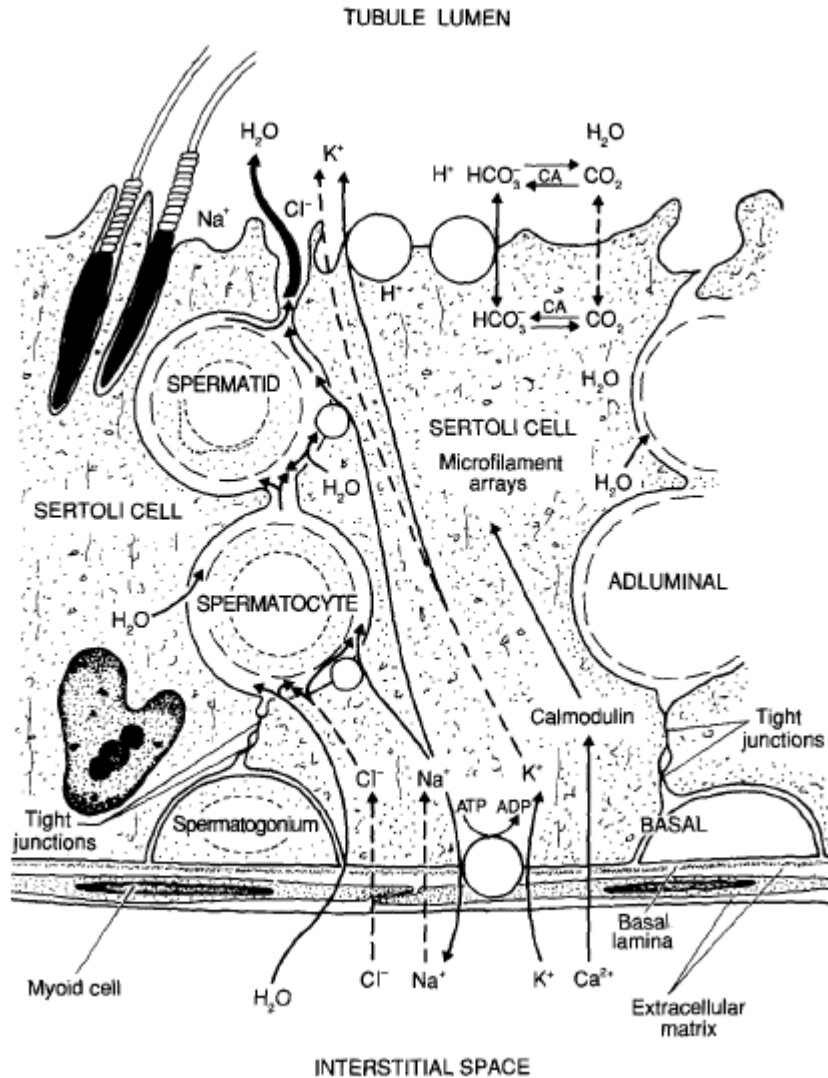
<b>Category and Protein</b>	<b>Other Names</b>	<b>Functions</b>
<b><i>Transport and Bioprotection</i></b>		
Transferrin	none	iron transport
Ceruloplasmin	none	copper transport
Saposin	SGP-1, testibumin	binds glycol-sphingolipids to aid in metabolism
SGP-2	Clusterin, TRPM SP 40; 40, Apo J S45; S35	lipid transport
Androgen Binding Protein	ABP	transport of T and DHT
SPARC (Secreted Protein Acidic and Rich in Cysteine)	osteonectin	calcium binding protein?
IGF Binding Proteins		IGF transport
Riboflavin Binding Protein		Riboflavin transport
<b><i>Proteases and Protease inhibitors</i></b>		
Plasminogen Activator		protease
Cyclic Protein-2	cathepsin L	protease inhibitor
Cystatin		protease inhibitor
$\alpha_2$ -Macroglobulin		protease inhibitor
Type IV Collagenase		protease
Metalloproteinases		proteases
<b><i>Basement membrane</i></b>		
Collagen IV		
Laminin		
Proteoglycans		
<b><i>Hormones/Growth Factors</i></b>		
Mullerian Inhibiting Substance	MIS	inhibition of Mullerian duct development
Inhibin		inhibition of FSH release
Insulin-like Growth Factors	somatomedins A and C, IGF	growth factors
Prodynorphins		
Interleukin-1 $\alpha$	IL-1	mitogen
Transforming Growth Factor $\alpha$ and $\beta$	TGF- $\beta$ TGF- $\alpha$	
Basic Fibroblast Growth Factor	b-FGF	growth factor
<b><i>(unpurified or incompletely characterized factors with hormone-like or growth factor activity)</i></b>		
Sertoli Cell Secreted Growth Factor	SCSGF	growth factor
Seminiferous Growth Factor	SGF	growth factor
Leydig Cell Stimulatory Activity		
<b><i>Other uncharacterized</i></b>		
Testin		

Russell &amp; Griswold, 1993

The glycoproteins include transport proteins (e.g. transferrin, ceruloplasmin, androgen binding protein etc), proteases and protease inhibitors, basement membranes (collagen IV, proteoglycans, laminin) and growth factors or paracrine factors. Other substances secreted by Sertoli cells includes energy substrates, water, ions and many other substances both at their apical and basal poles required to carry out their functions (Russell & Griswold, 1993; Mruk & Cheng, 2004).

Secretion of seminiferous tubule fluid is essential for the supply of nutrients to germ cells, spermatozoa and transport of chemical substances from the apical to basal portion of the seminiferous tubule (Jegou, 1992). Composition of the fluid showed that potassium level (110mEq/l) was ten times higher in tubule than in the plasma. Likewise, the presence of very low protein concentration in the tubules compared to the plasma with smaller differences in sodium (35 mEq/l less than the plasma) and chloride concentrations (Waites & Gladwell, 1982; Setchell & Brooks, 1988; Tuck, et al., 1970; Levine & Marsh, 1971).

Sertoli cells are responsible for the fluid secretions in the seminiferous tubule (Tuck et al., 1970). The tight junctions between the basal parts of the Sertoli cells could be involved in the pumping of potassium and bicarbonate that are transported to the intercellular canaliculi resulting in a hypertonic content. The hypertonicity induced an influx of water along the canalicular wall to produce an isotonic fluid down the lumen of the seminiferous tubule (Jegou, 1992; figure 8). Fisher (2002) on a contrary view, stated that the seminiferous tubule was solely responsible for the production of its luminal fluid and injection of oil into seminiferous tubules (Tuck et al., 1970) would have damaged the apical membranes of Sertoli cells resulting in the different ionic composition between primary and free flow fluids.



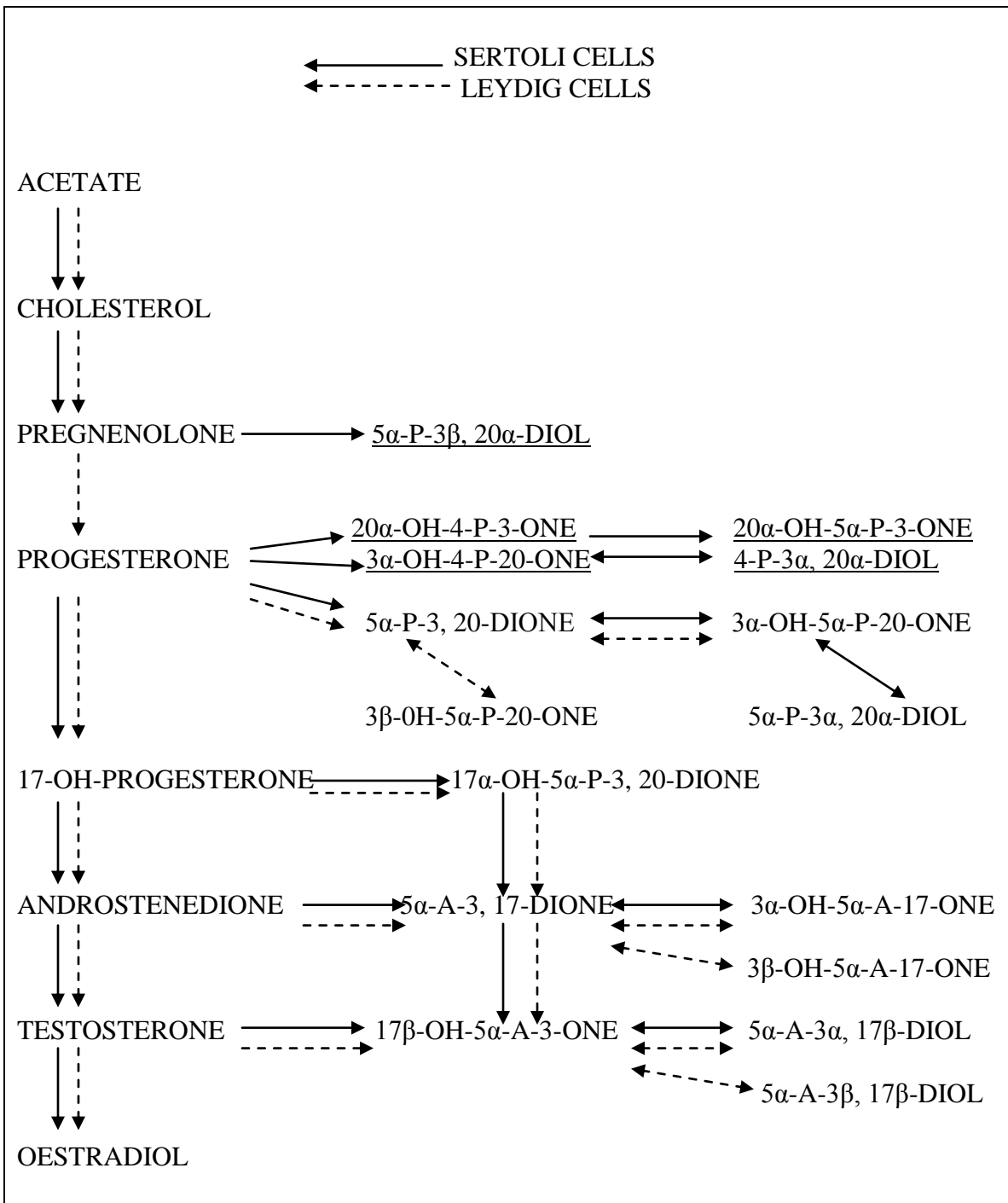
**Figure 8:** Schematic representation of the possible mechanism of elaboration of the seminiferous tubule fluid (Fawcett, 1975; Waites & Gladwell, 1982)

The Sertoli cell also has a marked capacity to synthesize and metabolize steroids (such as 5 $\alpha$ -P-3 $\beta$ , 20 $\alpha$ -DIOL; 20 $\alpha$ -OH-4-P-3-ONE; 3 $\alpha$ -OH-4-P-20-ONE; 17 $\beta$ -OH-5 $\alpha$ -A-3-ONE) of which some are not produced by the Leydig cells (Wiebe et al., 1987; Figure 9) and this could be due to the presence of a well-developed smooth endoplasmic reticulum in the cytoplasm of the Sertoli cells (Fawcett, 1975).

Sertoli cells secrete lactate and pyruvate required by the developing germ cells in the adluminal compartment of the seminiferous tubules (Jutte et al., 1981). Deficient supply

of lactate and pyruvate will lead to germ cell necrosis and testicular atrophy. On the other hand, very high concentrations of lactate may disturb spermatogenesis because of the concomitant secretion of hydrogen ions. This will lead to a drop in pH in the immediate surrounding of the germ cells and may affect their development (Monsees et al., 1998). *In vitro* exposure of Sertoli cells to a number of reproductive toxicants e.g. phthalate esters (Williams & Foster, 1989), nitrobenzene (Allenby et al., 1990), gossypol (Monsees et al., 1998) shows a dose-dependent increase in lactate secretion.

Exposure of rat primary Sertoli cell culture for 24 hour to gossypol (3-6  $\mu\text{M}$ ) significantly enhances the secretion of lactate but reduces the secretion of inhibin without affecting the cell viability. However, higher concentration (9-15  $\mu\text{M}$ ) of gossypol shows a significant decrease of lactate and inhibin, viability and mitochondrial function (Monsees et al., 1998). Thus, an increase in lactate production is often a sensitive and specific response of Sertoli cells to toxin exposure (Monsees et al., 2000).



**Figure 9:** Pathways of steroids biosynthesis and metabolism observed in the Sertoli cells (solid arrows) and Leydig cells (dashed arrows). Metabolites unique to the Sertoli cells are underlined. (Wiebe et al., 1987)



## ***1.6 Regulation of Sertoli cell***

### ***1.6.1 Endocrine regulation***

The hypothalamus or pituitary gland releases hormones into the bloodstream to reach its target- Sertoli cells. Communication among the hypothalamus, pituitary gland and the testis forms the hypothalamic-pituitary-testicular axis.

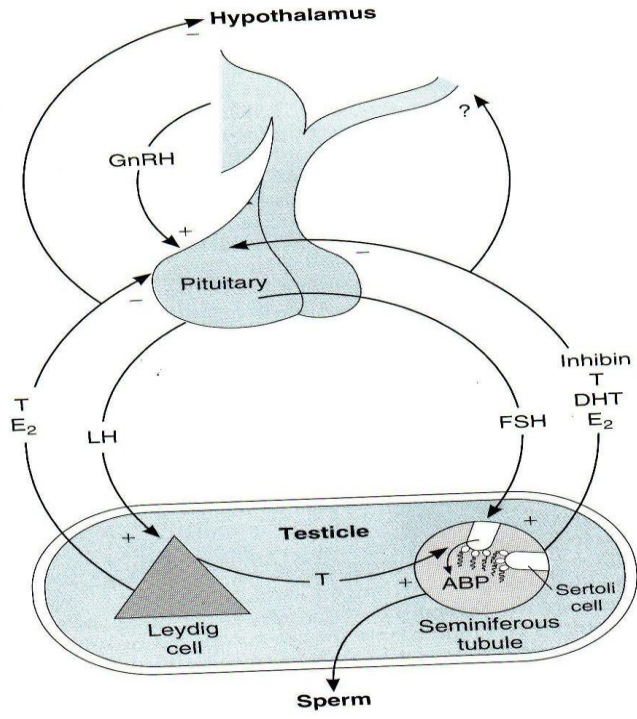
Hypothalamic-pituitary-testicular axis (HPTA; figure 10) is a homeostatic system that is responsible for maintaining, supporting and ensuring reproduction, bone density, muscle mass and other vital physiological and psychological processes. HPTA in summary regulates spermatogenesis and testosterone production.

As shown in figure 10, the hypothalamus synthesizes Gonadotropin – releasing hormone (GnRH) and secretes it into the hypothalamo-hypophysial portal blood in pulses every 90- 120 minutes (Greenspan & Gardner, 2001; Griffin et al., 1992; Greenspan & Baxter, 1994). In the anterior pituitary gland, GnRH binds to the gonadotrophes and stimulates the release of Luteinizing hormone (LH) and to a lesser extent, Follicle Stimulating Hormone (FSH) into the general circulation (Greenspan & Baxter, 1994).

Leydig cells have specific membrane receptors which bind to LH. Binding of the Leydig cells to the membrane receptors leads to the activation of adenylyl cyclase and generation of cAMP/ protein kinase and the steroidogenic acute regulatory protein (Warrel et al., 2005) resulting in the synthesis and secretion of androgens (figure 10).

Secretion of LH from the anterior pituitary is regulated through a negative feedback mechanism (figure 10) in that, increased level of androgens inhibits the secretion of LH from the anterior pituitary through a direct action on the pituitary and an inhibitory effect at the hypothalamic level.

The hypothalamo-pituitary-testicular axis in turn is regulated by the negative feedback of hydrophobic (e.g. steroids i.e. testosterone, estradiol and dihydrotestosterone) and hydrophilic hormones (such as inhibin and activin).



**Figure 10:** Diagram showing the hypothalamic-pituitary-testicular axis. (GnRH, gonadotropic-releasing hormone; LH, luteinizing hormone; FSH, follicle-stimulating hormone; T, testosterone; DHT, dihydrotestosterone; ABP, androgen-binding protein; E<sub>2</sub>, estradiol; +, positive influence; -, negative influence; (Greenspan & Gardner, 2001)

**1.6.1.1 Follicle-Stimulating Hormone**

FSH is the major endocrine hormone that regulates the functions of the Sertoli cells. In most species, including man, Sertoli cells replicate only during foetal, neonatal and prepubertal life mainly under the influence of follicle-stimulating hormone (FSH) and proliferation stops with the maturation of Sertoli cells at the onset of puberty (Pellinemi et al., 1993).

The Sertoli cell being the primary target for FSH action in the mammalian testis, has a significant number of FSH receptors and its binding to FSH stimulates the production of androgen binding protein (ABP) which allows a high concentration of testosterone to be maintained (Tindall et al., 1981). ABP is bidirectionally transported into the blood (20%) and seminiferous tubule fluid (80%; Sharpe, 1988). The portion of ABP transported into the seminiferous tubule travels with spermatozoa into the epididymis (Heindel & Treinen,

1989). Distribution of ABP in the testis or transport to the epididymis, or both factors, may play an important role in the completion of spermiogenesis (Pogach et al., 1993).

Occupancy of the receptors depends on stimulation of adenylyl cyclase and decrease in the activity of a cytoplasmic  $\text{Ca}^{2+}$ -sensitive cAMP phosphodiesterase (Tindall et al., 1981). Humans who lack a functional FSH receptor develop smaller testes than do normal males. These men also exhibit disturbed spermatogenesis (oligozoopermia and teratozoopermia), although they remain fertile (Tapanainen et al., 1997). Likewise, a reduction in Sertoli cell number was observed in the testes of mice lacking this receptor (Dierich et al., 1998).

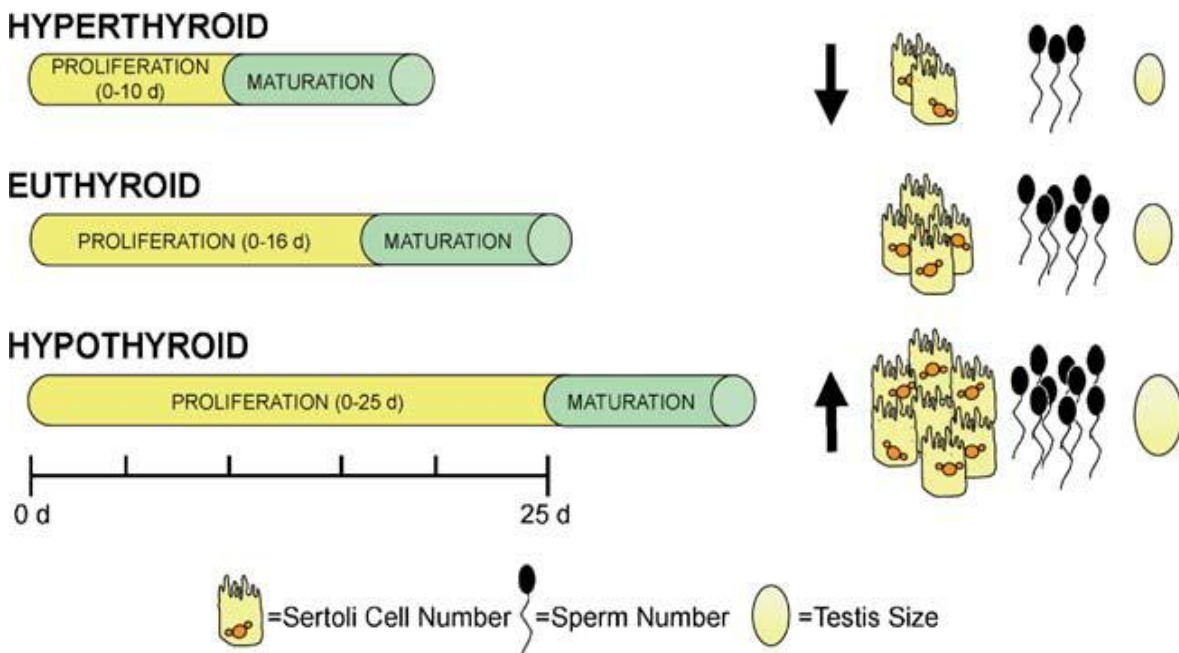
The molecular mechanism of action of FSH was best recognized by the stimulation of adenylyl cyclase/ cAMP pathway through the activation of a G protein (Meroni et al., 2002). In addition to the adenylyl cyclase/ cAMP pathway, other signaling events such as increased intracellular  $\text{Ca}^{2+}$  levels (Grasso & Reichert, 1989; Gorczynska & Handelsman, 1991), activation of phospholipase  $\text{A}_2$  (Jannini et al., 1994) and nuclear factor kappa B (NF $\kappa$ B) translocation (Delfino & Walker, 1998) have been demonstrated for the mechanism of action of FSH.

Stimulation of adenylyl cyclase and cAMP phosphodiesterase increases intracellular accumulation of cAMP, activates cAMP-dependent protein kinase and phosphorylates a variety of protein substrates (Tindall et al., 1981). Elevation of cAMP level leads to the activation of protein kinase A (PKA; Meroni et al., 2002).

Exposure of animals to exogenous estrogens during the replicative stage (foetal, neonatal and prepubertal period) of the Sertoli cells before its maturation may lead to the suppression of FSH secretion by the pituitary gland, resulting in a decrease in the rate of proliferation of the Sertoli cells (Sharpe et al., 1995).

### 1.6.1.2 Thyroid Hormone and Thyroid-Stimulating Hormone

Thyroid hormone is important for the growth and development of several tissues. Reports show that adult testes are not responsive to thyroid hormone (Barker & Klitgaard, 1952; Oppenheimer et al., 1974). However, an important role of triiodothyronine in testicular function has been reported (Petersen & Söder, 2006; Holsberger & Cooke, 2005). Hypothyroidism inhibits the differentiation of the Sertoli cell (figure 11) by prolonging the phase of the Sertoli cell proliferation, thereby elevating the Sertoli cell number, testis size and sperm count (van Haastert et al., 1992; 1993, Cooke & Miesami, 1991) while hyperthyroidism shortens the phase of proliferation of the Sertoli cells.



**Figure 11:** Effect of Thyroid hormone on the developing testis (Holsberger & Cooke, 2005)

In summary, high levels of 3,5,3'- triiodo-L-thyronine (T3) enhance differentiation and give rise to smaller testes and a lower sperm count (Petersen & Söder, 2006) by terminating Sertoli cell proliferation and stimulating the functional maturation of these cells (Holsberger & Cooke, 2005).

Studies have indicated two cyclic dependent kinase inhibitors (CDKIs), p27Kip1 and p21Cip1, to be involved in the proliferation of Sertoli cells. The expression of p27Kip1 is inversely correlated with Sertoli cell proliferation, with low p27Kip1 levels in rapidly proliferating neonatal Sertoli cells and high p27Kip1 expression in post mitotic adult Sertoli cells (Beumer et al., 1999). p27Kip1 and p21Cip1 may be normally induced by T3, and the increased levels of these CDKIs may be a major factor in terminating Sertoli cell proliferation during development (Holsberger & Cooke, 2005). T3 stimulates the expression of p21Cip1, which suggest that this CDKI is also involved in the process by which T3 turns off Sertoli cell proliferation during development (Buzzard et al., 2003)

#### ***1.6.1.3 Prolactin***

Prolactin (PRL) receptors are expressed in Sertoli cells, although the role of prolactin still appears to be controversial (Guillaumot & Benahmed, 1999). However, reports show hyperprolactinemia leads to male hypogonadism (Bartke, 1980). PRL affects gonadal function through its interaction with LH and FSH biosynthesis (Bartke et al., 1977). Sertoli cells have been shown to be potential targets for prolactin action in the porcine testis and ovine PRL significantly increases FSH binding to Sertoli cells in a dose-dependent manner (Guillaumot et al., 1996).

#### ***1.6.1.4 Growth Hormone***

Sertoli cells possess both Growth Hormone (GH) and Insulin-like Growth Factor-I (IGF-1) receptors and produce IGF-I (Gomez et al., 1998; Tres et al, 1986; Lobie et al, 1990). Growth hormone may play an important role in the proliferation of the Sertoli cells, in that, administration of GH to boars resulted in enhanced Sertoli cell size and maturation (Swanlund et al, 1995) while a reduction in testicular size is seen in GH- deficient and GH- resistant animals (Bartlett et al, 1990; Zhou et al, 1997).

### ***1.6.2 Paracrine/ autocrine regulation***

Paracrine regulation occurs when surrounding cells such as the Leydig cells, peritubular myoid cells or developing myoid cells secrete molecules that regulate Sertoli cells. Autocrine regulation occurs when autocrine factors produced by Sertoli cell acts on itself (Heindell & Treinen, 1989).

#### ***1.6.2.1 Testosterone***

Androgens are very important in the regulation of spermatogenesis with testosterone being the core androgen that regulates spermatogenesis in the testis, mostly during the maturation of spermatids. Leydig cells, peritubular cell and Sertoli cells in the testis express androgen receptors.

The levels of androgen receptor are highest in stage VII of the spermatogenic cycle and this stage is thought to be the most regulated by and sensitive to testosterone (Kerr et al., 1993). Testosterone increases ABP activity *in vitro* in isolated Sertoli cell cultures (Louis & Fritz, 1977).

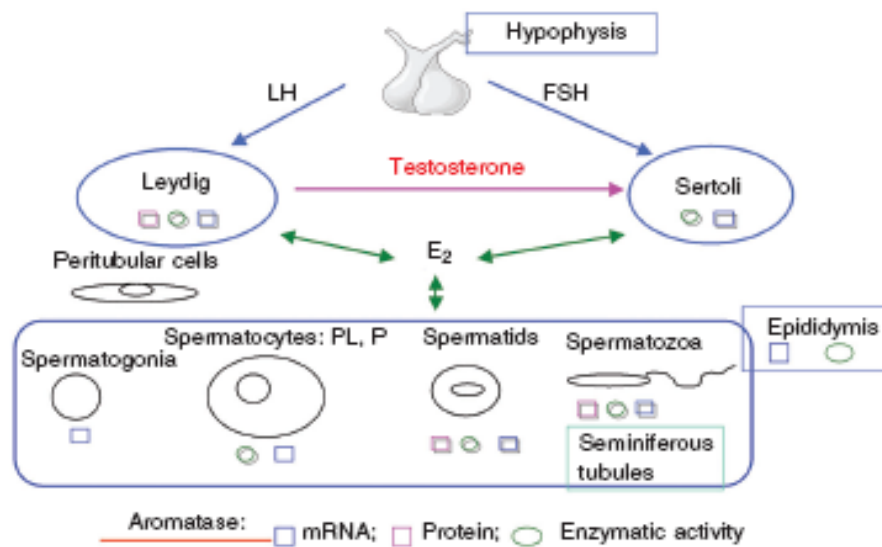
Androgens elevate intracellular  $\text{Ca}^{2+}$  concentration in freshly isolated, suspension cultures of Sertoli cells within seconds of stimulation (Gorczyńska & Handelsman, 1995).

Testosterone maintains the binding competency of Sertoli cells to the spermatid but cannot induce or restore this structural/functional characteristic of the cell and the competency of the Sertoli cell binding is necessary for maximal spermiogenesis by maximizing the binding of spermatid to Sertoli cells at stage VIII of the spermatogenic cycle (Muffly et al., 1993). Germ cell development stopped at spermatocyte or early spermatid stage when the androgen receptors were removed from the Sertoli cells (Chang et al., 2004; De Gendt et al., 2004), this shows the relevance of the androgen receptors in maintaining normal spermatogenesis.

### 1.6.2.2 Estrogen

Aromatase catalyses the conversion of testosterone to estradiol and shown to be present in developing germ cells, Leydig and Sertoli cells and epididymal cells (figure 12: Carreau et al., 2007). Sertoli cells express aromatase during the prepubertal period for the aromatization of androgens to estrogen with p450 aromatase enzyme complex (Dorrington & Armstrong, 1975; Dorrington et al., 1978).

FSH stimulate the activity of aromatase in prepubertal Sertoli cells (Tsai-Morris et al., 1985; Mallea et al., 1987; Dorrington & Armstrong, 1975; Dorrington et al., 1978). However, as Sertoli cells differentiate in response to FSH, the expression of aromatase reduces leading to a negligible amount of estrogen been secreted by the cells (Dorrington et al., 1978).



**Figure 12:** Localization of aromatase in adult testicular cells. PL= Preleptotene, P=Pachytene, E<sub>2</sub>= Estradiol (Carreau et al., 2007)

### ***1.6.2.3 P Mod-S***

Peritubular cells in the seminiferous tubules produce proteins that modulate the functions of Sertoli cells (P Mod-S). P Mod-S stimulates Sertoli cells to produce androgen binding protein and testicular transferri (Skinner & Fritz, 1985).

A study by Hutson & Stocco, (1981), showed a positive correlation between the number of peritubular cells and the efficiency of androgen-binding hormone secretion in culture. P Mod-S under the control of androgen inhibits the induction of aromatase activity in Sertoli cells (Verhoeven & Cailleau, 1988).

### ***1.6.2.4 Growth factors***

A number of receptors for growth factors such as fibroblast growth factors-1 and -2 (FGF-1, FGF-2), insulin-like growth factors I and II (IGF-I, IGF-II), transforming growth factor-  $\alpha$  (TGF- $\alpha$ ), activin A and epidermal growth factor (EGF) have been found in the testis with the Sertoli cells having receptors for most of them (Koike & Noumura 1995; Caussanel et al., 1996; Cancilla et al., 2000, Cupp & Skinner, 2001; Handelsman et al., 1985; Table 4) .

Growth factors such as FGF-2, IGF-I, IGF-II, TGF-  $\alpha$ , EGF, activin A have a stimulatory effect on Sertoli cells (Koike & Noumura 1995; Caussanel et al., 1996; Cancilla et al., 2000, Cupp & Skinner, 2001; Handelsman et al., 1985 ). FGF-2 increases the number of Sertoli cell number in culture in a time and dose-dependent manner (van Dissel-Emiliani et al., 1996).

IGF-1 stimulates DNA synthesis (Borland et al., 1984), increases transferrin and lactate production in immature Sertoli cell (Oonk et al., 1989; Skinner & Griswold, 1983). The presence of BTB prevents the interstitial fluid-derived IGF-1 from directly affecting sequestered germ cells. Hence, Sertoli cell production of IGF-1 controls the proliferation of the germinal cells (Russell & Griswold, 1993). IGF-11 receptors are present in both Sertoli cells and germinal cells (O' Brien et al., 1989). IGF-11 stimulates the



differentiation of Sertoli cells by cross reacting with IGF-1 receptors (Borland et al., 1984).

Sertoli cells and peritubular cells but not germinal cells express TGF- $\alpha$  which stimulate DNA synthesis and cell division in peritubular cells but not Sertoli cells (Skinner et al., 1989) which makes the role of TGF- $\alpha$  in Sertoli cell- germ cell interaction to be unclear. EGF share similar protein structure as TGF- $\alpha$  and hence acts on same receptor to stimulate cell growth (Carpenter, 1987). EGF had been shown to be involved in the maintenance of spermatogenesis (Stastny & Cohen, 1972).

TGF-  $\beta$  produced by Sertoli cells is modulated by gonadotropin (Skinner & Moses, 1989; Benahmed et al., 1989). It acts as a growth inhibitor in the testis (table 4) and different sub-types of TGF-  $\beta$  (TGF-  $\beta$ 1, TGF-  $\beta$ 2, TGF-  $\beta$ 3) are produced in mammals with Sertoli cells expressing all three forms of the sub-types (Mullaney & Skinner, 1993).

Seminiferous Growth Factor (SGF) (Bellve & Feig, 1984) stimulates growth in transformed TM4 Sertoli cells, TM3 Leydig cells and 6-day-old mouse Sertoli cells. SGF induces the proliferation of TM4 cells and the effect of SGF is inhibited by 1 $\mu$ M-1nM retinoic acid but not retinol or retinyl acetate (Braunhut et al., 1990).

**Table 4: Regulatory Agents Produced by Sertoli Cells**

<b>Secretory Product</b>	<b>Proposed Site Action</b>	<b>Potential Function</b>
<b>Growth Factors</b>		
IGF-1	Sertoli/germinal/peritubular/ Leydig	metabolism growth
TGF- $\alpha$	peritubular/?germinal/?Sertoli	growth stimulation
TGF- $\beta$	peritubular/?germinal/?Sertoli	growth inhibition/ cellular differentiation
IL-1	?germinal	growth regulation
FGF	Sertoli/?germinal	growth stimulation
<b>Other Regulatory Agents</b>		
Inhibin	Leydig/pituitary	alter steroidogenesis/ regulate FSH
MIS	foetal gonad	promote gonadal development
LHRH-like factor	Leydig	steroidogenesis

? denotes speculated site of action (Russell & Griswold, 1993)

#### **1.6.2.5 Inhibin B**

Sertoli cells produce and secrete inhibin B into the circulation in response to FSH stimulation. Inhibin B inhibits the secretion of FSH from the anterior pituitary gland and the circulating FSH from the pituitary and other inhibins and activins from the Leydig cells (Skinner & Griswold, 2004) influence the regulation of inhibin secretion. The level of circulating inhibin B and FSH changes in dynamic pattern from birth to adulthood depicting their changing relationship between both hormones. There is a positive correlation between both hormones during early puberty, which possibly reflects the role of the FSH in stimulating the proliferation of the Sertoli cells and at advanced puberty and adulthood, there is a negative correlation between them depicting the role of inhibin B as a suppressing the level of FSH (Skinner & Griswold, 2004). The level of inhibin B is high during early postnatal life but there is a decline in its level until puberty where it increases (Petersen & Söder, 2006).

### ***1.7 Sertoli cells Toxicants***

The major function of the Sertoli cell is to create an environment suitable for germ cell development and maturation. Therefore, an alteration in the function of the Sertoli cell may lead to germ cell loss and infertility. Sertoli cells have been shown to be adversely affected on exposure to several reproductive toxicants. The basal part of the Sertoli cell is readily available for blood- borne toxicants.

Several toxicants like cisplatin (Wallace et al., 1989, Russell et al., 1980), gossypol (Monsees et al., 1998), phthalate esters (Thomas et al., 1978) and alkyl phenols (De Jager et al., 1998), hexane, hexanone and dinitrobenzene (Chapin et al., 1983; Williams & Foster, 1989), lindane and DDT (Hodgson & Levi, 1996; Dalsenter et al., 1996), lead and cadmium (Janecki et al., 1992; Alexander et al., 1996; Robins et al., 1997), nitro-aromatics (Foster et al., 1986), 1- (2,4-dichlorobenzyl)-1H-indazole-3-carboxylic acid (Buthala & Lobl, 1979) are known to target Sertoli cells, leading to the destruction of their morphology or impairment of functions.

The mechanism of action of the toxicants on Sertoli cells includes disruption of the blood testis barrier, alteration of germ cell attachment, germ cell loss, insufficient apical cytoskeletal support, metabolic insult, defects in microtubule-dependent transport, alteration in receptor and second messenger and disruption of seminiferous epithelium (Russell & Griswold, 1993).

Exposure of Sertoli cells to glycerol damages the blood testis barrier and thus affects spermatogenesis (Cheng & Mruk, 2002). Intratesticular administration of glycerol leads to a long-term aspermatogenesis in rats but does not have an effect on the Leydig cell steroidogenesis, serum FSH, Luteinizing hormone (LH), testosterone level and secondary sexual characteristics (Wiebe & Barr, 1984). Di-(2-ethylhexyl) phthalate (DEHP) is a toxicant that affects Sertoli cell membrane by inhibiting FSH-mediated signal transduction, which reduces Sertoli cell proliferative activity (Heindell & Chapin, 1989). Cisplatin disrupts the blood-testis barrier upon acute administration (Pogach et al., 1989).

The testicular manifestations of the exposure of toxicant to the Sertoli cells includes vacuolation, apical sloughing and shedding, germ cell necrosis, decreased seminiferous tubule fluid secretion, changes in distribution, quantity or biochemical properties of testicular components, interstitial release of Sertoli cell proteins (Russell & Griswold, 1993).

### ***1.8 TM4 Sertoli cells***

TM4 Sertoli cells are cell lines derived from the primary culture of Sertoli cell enriched preparation from normal testes of 11-13 day old prepubertal Balb/c mice (Mather, 1980). It has a number of characteristics similar to Sertoli cells, which include responsiveness to Follicle-Stimulating Hormone, and lack of response to Luteinizing Hormone. It also has enzyme and receptor expression pattern similar to primary Sertoli cell preparation (Mather et al, 1982).

TM4 Sertoli cells are cultured using the DMEM/ F-12 mixture supplemented with 2.5% fetal calf serum and 5% horse serum and incubated at 37<sup>0</sup>C in an incubator with 5% CO<sub>2</sub> air atmosphere.

Calcitonin enhanced the formation of cAMP in cultured TM4 cells and also stimulated the secretion of rat androgen-binding protein (rABP) by primary cultures of Sertoli cells (Nakhla et al., 1989). Nakhla et al. (1989) also showed that increased cAMP in Sertoli cells was associated with a stimulated secretion of rABP. This indicates that calcitonin is another hormone that stimulates both cAMP accumulation and rABP secretion.

## ***1.9 Medicinal plants and male reproduction***

### ***1.9.1 Effect of medicinal plants on fertility***

Infertility is defined as the failure to conceive after one year of regular unprotected sexual intercourse. Approximately 15% of couples who attempt to conceive within the first year fail to do so (WHO, 1991). The cause of infertility in about 50% of affected couples is a result of male factor (Shefi & Turek, 2006). Male infertility symptoms involve different aspects such as libido dysfunction, sexual asthenia, erectile dysfunction, loss of orgasm, sperm abnormality, ejaculatory and relaxation dysfunctions (Nantia et al., 2009).

The use of extracts, fractions and isolated compounds from several medicinal plants have been shown to be useful in the treatment of symptoms associated with male infertility (Mallick et al., 2007; Zheng et al., 2000; Gonzales et al., 2002; Rubio et al., 2006). Several medicinal plants have been used in the study of their effects on the male reproduction. These plants have either antifertility or anti-infertility effect on the male reproductive system (Zhang et al., 1989; Mallick et al., 2007; Al-Qarawi, 2005; Monsees et al., 1998).

A positive effect on libido (sexual desire) is seen in rats exposed to extracts of *Lepidium meyenii* (Zheng et al., 2000), *Tribulus tesselatta* (Gauthaman et al., 2002) and *Panax ginseng* (Murphy et al., 1998). On the other hand, an increase in erectile function was observed in rats exposed to different doses of extracts from *Ruta chalepensis* (Al-Qarawi, 2005), *Lycium barbarum* fruit (Luo et al., 2006), MTEC (a formulated herbal drug which consist of aqueous- methanol extracts of *Musa paradisiacal*, *Tamarindus indica*, *Eugenia jambolana* and *Coccina indica*), *Massularia acuminata* and *Satureja khuzestanica* essential oil; Mallick et al., 2007).

Several other plant extracts have also been shown to enhance sperm qualitative and quantitative parameters. For instance, *Nigella sativa* oil (Bashandy, 2006) increased seminal vesicle weight, plasma testosterone level, sperm count and motility and decreased sperm abnormality. *Lepidium meyenii* (Maca; Gonzales et al., 2002; Rubio et

al., 2006) activated the onset and progression of spermatogenesis while the juice of pomegranate (*Punica granatum* L.; Türk et al., 2008) increased the epididymal sperm concentration, sperm motility, diameter of the seminiferous tubule, and spermatogenic cell density and decreased the number of abnormal sperm.

On the other hand, gossypol, a constituent of cotton plant seed (*Gossypium* sp.; Jones, 1991) is known to be a potent antifertility agent in mammals (Zhang et al., 1989) with testicular cells been a target (Monsees et al, 1998). Gossypol reduced the cell viability, lactate production and inhibin at higher concentration but increased the lactate production at low concentrations in Sertoli cells (Monsees et al., 1998).

Mali et al. (2002) showed that 50% ethanol extract of *Martynia annua* root decreased testicular and epididymal sperm count, serum testosterone and LH levels, weight of the testes, epididymides, seminal vesicles and ventral prostate and caused lesion on the seminiferous tubules. Prolonged administration of triptolide (an isolated compound from the Chinese medicinal plant, *Tripterigium wilfordii* Hook f.) significantly reduced the cauda epididymal sperm count, completely stopped sperm motility, leaving the seminiferous epithelium with a single layer of cells consisting of Sertoli cells and a few spermatogonia in some animals (Huynh et al., 2000). These effects would possibly have an effect on spermatogenesis.

### ***1.9.2 Effects of Basella alba and Hibiscus macranthus on male reproduction***

*Basella alba* (figure 13) is a fast growing, soft- stemmed, perennial plant found in the tropics. It is about 10m in length, thick and semi- succulent with a mild flavor and mucilaginous texture. In Africa, the mucilaginous cooked shoots are commonly used as vegetable leaves (Grubben & Denton, 2004). *Basella alba* is rich in vitamin A, vitamin C, iron, and calcium and also a rich source of chlorophyll. The mucilage type of the plant is a rich source of soluble fiber and thought to remove mucus and toxins from the body.

*Basella alba* has been shown to be useful as astringent for the treatment of diarrhoea and as laxatives (Larkcom et al., 1991, Phillips & Rix, 1995), as an antidote to poisons, as

diuretic, febrifuge, and also to alleviate labour (Duke & Ayensu, 1985), as rubefacient and also applied to swellings, used to treat boils and catarrh (Manandhar, 2000).

A mixture of both *Basella alba* and *Hibiscus macranthus* (figure 14) is used by traditional healers in the Western Province of Cameroon to cure male sexual asthenia and virility (Moundipa et al., 2005).

Aqueous extract obtained from the mixture of fresh or dried leaves of *Basella alba* and *Hibiscus macranthus* increased testosterone production in adult male rat (Moundipa et al., 1999). The presence of abundant spermatozoa in the lumen of the seminiferous tubules with a high production of testosterone was observed in the rats exposed to the extracts (Moundipa et al., 1999).

A number of studies were performed to analyze the mechanism of action of these plant extracts using different extract of organic solvent such as hexane, methylene chloride and methanol (Moundipa et al., 2005). Exposure of methanol extracts of *Basella alba* to bull or rat Leydig cells caused a significant concentration dependent increase in testosterone production after 12 hours incubation (Moundipa et al., 2005). In contrast, *Hibiscus macranthus* extract caused a slight increase at 10 µg/ml and a decrease at higher concentration of the extract (100µg/ml) in testosterone production compared to the control (Moundipa et al., 2005). This indicated *Basella alba* to be the plant responsible for the production of testosterone as testosterone production increased in a concentration-dependant manner with the extract.

In a study aimed at finding an *in vitro* system for the measurement of the androgenic effects of different extracts of *Hibiscus macranthus* and *Basella alba*, testes slices were put in culture tubes containing 0.5ml of DMEM/ Ham F12 medium. The synthesis and secretion of testosterone was significantly enhanced (Moundipa et al., 2006).



**Figure 13:** *Basella alba* (Property of Prof P.F. Moundipa)



**Figure 14:** *Hibiscus macranthus* (Property of Prof P.F. Moundipa)



### ***1.10 Apoptosis***

Apoptosis first described by Kerr et al. (1972) is a programmed cell death that plays an important role in the development and maintenance of tissue homeostasis (Prindull, 1995) by maintaining cell numbers and cellular positioning within comprising of different cell compartment (Jin & El-Deiry, 2005). It also plays an important role in removing damaged, infected or neoplastic cells (Kannan & Jain, 2000).

A line has to be drawn for optimal function of apoptosis, as too little or too much apoptosis may lead to detrimental biological consequences (Nagata, 1996) such as developmental defects, autoimmune disease, neuro-degenerative diseases or cancer (Thompson, 1995).

Characteristics of a cell undergoing apoptosis include cell membrane blebbing, cell shrinkage, chromatin condensation and DNA fragmentation. The apoptotic cell is eventually engulfed by macrophages or neighboring cells (Savill & Fadok, 2000).

Apoptosis as a form of cell death differ distinctively from necrosis. Necrosis is a non-physiologic cell death and involves clumping of chromatin onto ill-defined masses, swelling of organelles, rupture of the cell membrane and disintegration of the cell (Kerr & Harmon, 1991).

#### ***1.10.1 Apoptotic Pathways***

Apoptosis is triggered by various factors such as the receptor- mediated signals, withdrawal of growth factors, anti-tumor drugs and damage to DNA (Kannan & Jain, 2000). Depending on the factor that triggers the cell death programme, an extrinsic or intrinsic apoptotic pathway is activated.

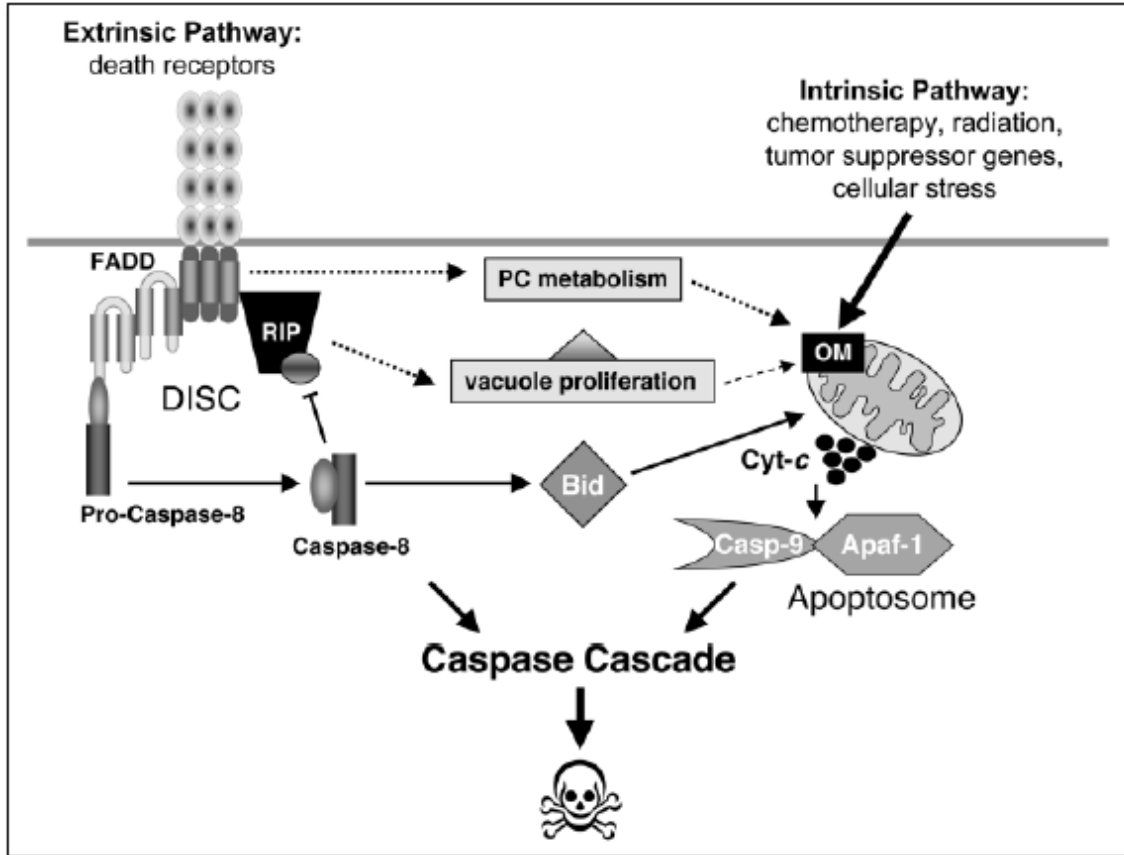
##### ***1.10.1.1 Extrinsic pathway***

The extrinsic pathway is activated when apoptotic stimuli comprising of extrinsic signals such as the binding of death inducing ligands (TNF, FasL and TRAIL) to the receptors on the cell surface (Jin & El-Deiry, 2005). When the cytokine ligands bind to the members

of TNF $\alpha$  receptor super- family called the death receptors (Fas, TNF receptor, TRAIL receptors). This leads to the oligomerization of the receptors and the recruitment of Fas Associated Death Domain (FADD) and caspase 8 and the formation of death inducing signaling complex (DISC). Auto activation of caspase 8 results in the activation of effector caspases (such as caspases -3, -6, -7) which functions as downstream effectors of the cell death programme (Ashkenazi & Dixit, 1998; figure 15).

#### ***1.10.1.2 Intrinsic pathway***

The intrinsic pathway, which is mediated by diverse apoptotic stimuli such as DNA damage, induced by irradiation or chemicals, deprivation of growth factors or oxidative stress (Jin Z& El-Deiry, 2005) and converges at the mitochondria (Green et al., 2004). This leads to the release of cytochrome c (cyt c) from the mitochondria to the cytoplasm. The cytoplasmic cyt c binds to the apoptosis protease activating factor 1 (Apaf-1) and procaspase 9. This results in the formation of an intracellular DISC-like complex known as apoptosome in which caspase-9 is activated leading to the activation of caspase -3, which initiates apoptosis (Shi, 2002; figure 15).



**Figure 15:** Pathway for apoptosis (Khosravi-Far & Esposti, 2004)

### ***1.11 Aim of this study***

*Basella alba* and *Hibiscus macranthus* extracts may enhance spermatogenesis *in vivo*, and therefore potential effects on Sertoli cells is possible. All of the parameters below have been shown previously by different authors (Monsees et al, 1998; Jutte et al., 1983) to be suitable markers to monitor the effect of substances on Sertoli cell physiology.

Thus, this research work is aimed at investigating the effect of the plant extracts, *Basella alba* and *Hibiscus macranthus* on TM4 Sertoli cell functions based on the following parameters:

- Determination of cell viability or cytotoxicity
- Determination of biochemical secretion where the production of lactate, pyruvate, protein content and inhibin will be examined
- Morphological studies using haematoxylin and eosin staining and DAPI staining techniques

## CHAPTER TWO

### Materials and Methods

#### 2.1 *Chemicals*

##### 2.1.1 *Gibco, Germany supplied:*

- Dulbecco's Modified Eagle's medium + GlutaMAX™-1(DMEM; 31966)
- F-12 Ham nutrient mixture (21765)
- 2.5 % Foetal bovine serum (10500)
- HEPES (15630)
- 5 % Horse serum (26050)
- Sodium bicarbonate (25080)
- Sodium pyruvate (11360)
- 0.25 % Trypsin/Ethyl Diamine Tetra Acetic acid (EDTA; 25200)

##### 2.1.2 *Sigma-Aldrich, Germany supplied:*

- Bovine albumin serum (A1470)
- $\beta$ -Nicotinamide adenine dinucleotide ( $\beta$ -NAD; 43410)
- $\beta$ -Reduced disodium salt hydrate ( $\beta$ -NADH; N8129)
- Bouin's solution
- Dimethylsulphoxide (DMSO; D2438)
- 4',-6-Diamidino-2-phenylindole dihydrochloride (DAPI)
- Glutamic pyruvate transaminase (G-9880)
- Glycerol
- L- Lactic- dehydrogenase Type II (L-2500)
- Poly-L-Lysin solution Thiazoyl blue tetrazolium bromide (MTT)
- Sodium-L-Lactate
- Triton x-100
- Trypan Blue

**2.1.3 Merck, South Africa supplied:**

- DPX Mounting medium
- EDTA disodium salt
- L- glutamate
- Potassium diphosphate ( $\text{KH}_2\text{PO}_4$ )
- Sodium chloride
- Sodium Hydroxide
- Triethanolamine hydrochloride

**2.1.4 BDH Biochemical, England supplied:**

- Sodium pyruvate

**2.1.5 Roche, Germany supplied:**

- XTT [2,3-Bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt] ready to use solution.

**2.1.6 Oxoid, England supplied:**

- Phosphate Buffered Saline (PBS)

**2.1.7 Bio- Rad supplied:**

- Dc protein assay reagent A and B

**2.1.8 Kimix, South Africa supplied:**

- Disodiumphosphate ( $\text{Na}_2\text{HPO}_4$ )

**2.1.9 Corning, South Africa supplied:**

- 24 and 96 well plates
- Pipettes

**2.1.10 Lasec, Germany supplied:**

- Cover slip

**2.1.11 Grenier Bio-one, Germany supplied:**

- Tissue Culture dishes

**2.1.12 Saarchem, South Africa supplied:**

- Dimethylsulphoxide (DMSO)

## **2.2 Plant extracts:**

- *Basella alba*
- *Hibiscus macranthus*

were provided by Prof. Paul Moundipa, University of Yaounde 1, Cameroon.

## **2.3 Cell line**

TM4 cells were acquired from American Type Cell Culture (CRL-1715, USA). The cell line was used as it has a number of characteristics similar to Sertoli cells, which include responsiveness to Follicle-Stimulating Hormone, and also lacks responsiveness to Luteinizing Hormone. It also has enzyme and receptor expression pattern similar to primary Sertoli cell preparation (Mather et al, 1980; 1982).

## **2.4 Preparation of plant extracts**

Air-dried leaves of both *Hibiscus macranthus* (0.5kg) and *Basella alba* (0.5kg) obtained from Batoufam (Western Province, Cameroon) were powdered in a mill. Following which, the powder of each plant was successively subjected to hexane (500ml for 5 hours, three times), methylene chloride (500ml for 5 hours, three times) and methanol (500ml for 6 hours, three times) extraction by maceration under gentle agitation in a glass vessel for 48 hours at room temperature. The methanol extracts of both *Hibiscus macranthus* and *Basella alba* were concentrated under vacuum for dryness and the greenish viscous residues obtained were tested (Moundipa et al., 2005). A stock solution of 100 µg/ml of each extract dissolved in negative control (containing 0.5% dimethylsulphoxide (DMSO) in complete culture medium) was prepared, filtered and stored at 4°C in a sterile condition. In order to prepare 0.01, 0.1, 1 and 10 µg/ml final concentrations of the extract, stock solution of 100 µg/ml extract was diluted with negative control through a serial dilution.

## **2.5 *TM4 Sertoli cell culture***

### **2.5.1 *TM4 Cells Culturing***

TM4 Sertoli cells were acquired from American Type Culture Collection (ATCC) and prepared according to the instructions given by the ATCC. The vial containing frozen cells was thawed by gentle agitation in a 37°C water bath and sterilized by spraying with 70% ethanol. Following that, 1ml of the cells was transferred to a 25cm<sup>2</sup> tissue culture flask of 60ml capacity, containing 5ml of Dulbecco's Modified Eagle's medium [+ 4.5g/L D-glucose + pyruvate] / F12 Ham nutrient mixture [+ L-glutamine] supplemented with 2.5% fetal calf serum and 5% horse serum and incubated at 37°C in an incubator in a 5% CO<sub>2</sub> air atmosphere for 24 hours. The medium was changed after 24 hours of resuscitation. The cells were allowed to grow to 70-80% confluency for sub culturing in order to have sufficient cells with the medium changed after 48 hours.

### **2.5.2 *Sub cultivation of cells***

At 70-80% confluency of cells, the cell lines were sub cultured to prevent cell death. This was done by removing the spent medium with a pipette, and then the cells were washed shortly, twice with about 3ml PBS without Ca<sup>2+</sup>/Mg<sup>2+</sup>. After which, 1ml trypsin/EDTA was added to the flask, shortly rinsed over the cell layer and removed thereafter. The cells were incubated for about 5 minutes at 37°C.

The detachment process was examined under the microscope until all the cells became rounded and detached from the surface of the flask. The remaining trypsin was inactivated and the cells suspended by adding 1ml of the complete medium to the flask. And then, 1ml of the cell suspension were transferred into another flask containing 5ml fresh medium and 5ml of the complete medium was added to the previous flask and incubated for further growth.



### ***2.5.3 Freezing of cells***

At 70-80 % confluence of cells, the cells were frozen away following the same procedure as the sub culturing except that after the cells are detached, 1ml of the complete culture medium is added to the flask containing the cells. The detached cells with the medium were transferred into a centrifuge tube and centrifuged at 727 x g for 5 minutes. After which, the supernatant was removed and 1ml complete medium with 10% DMSO (freezing medium) was added to it and aspirated and transferred into a cryo vial, slowly cooled down overnight in a Styrofoam box in other for the cells to gradually adjust to the decreased temperature and then stored at -80°C.

### ***2.5.4 Plating of cells***

At 70-80% confluency of cells are sub cultured by removing the spent medium with a pipette and the cells washed twice with about 3ml PBS without Ca<sup>2+</sup>/Mg<sup>2+</sup>. Into a flask, 1ml trypsin/EDTA was added, shortly rinsed over the cell layer and removed thereafter. The cells were incubated for about 5 minutes and examined under the microscope until all the cells were detached from the surface of the flask and suspended.

To inactivate trypsin, 1 ml of the culture medium was added to the flask and the suspended cells were transferred into a centrifuge tube and centrifuged at 727 x g for 5 minutes. Thereafter, the medium was removed from the tube and 1ml fresh medium was then added and aspirated.

Into an eppendorf cup was added 20 µl of trypan blue, 15 µl PBS and 5 µl cell suspensions was added giving us a dilution factor of 8. Following that, 10 µl of the cell suspension was then transferred to a haemocytometer counting chamber in other to count the total number of cells. Once the total number total number was known, the number of cells needed was calculated using the formula below:

$$\frac{\text{Number of cells needed}}{\text{Total number of cells counted}} * 1000 = \text{volume of cell required } (\mu\text{l})$$

In preliminary experiments, an optimal initial cell concentration was established (figures 18; 19). This was done by plating 400, 800 and 1200 cells/200µl in 96 well plates with each containing either the complete culture medium, 1% DMSO or 2% DMSO for five days. On the fifth day, each concentration of cells was exposed to either 100 µl or 50 µl of XTT reagent. Thus, 1ml of 4000 cells/ml or 200 µl of 5000 cells/ml (= 1000 cells/well) were plated in 24 or 96 well plates respectively for 1 day in complete culture medium. The next day, the medium was removed and the adherent cells exposed to the five different concentrations (0.01, 0.1, 1, 10, 100 µg plant extract/ml final concentration in complete culture medium) of either *Basella alba* or *Hibiscus macranthus*, negative control (0.5% DMSO in complete culture medium), and positive control (2% DMSO in complete culture medium) for another four days.

Thereafter, supernatants from the 24 well plates were collected from each well to determine the concentration of lactate, pyruvate and inhibin B (Monsees et al., 2000) and the cells are used to determine the protein content. Cell viability was determined in a further set of experiment in 96 well plates using either the XTT or MTT assay with the ELISA reader (Thermo electron corporation, South Africa).

The experiments were repeated at least two times in triplicates and the result reported here represents one set of experiment from two different plates with four individual wells for lactate, pyruvate, inhibin and protein. Three different plates with six individual wells were used for cell viability (MTT/XTT).

## **2.6 Determination of cell viability**

To examine the viability of the TM4 Sertoli cells, both XTT and MTT assays were performed in separate experiments for comparison purpose. MTT assay was performed as it is cheaper and economical than the ready to use XTT. Further more, the XTT reagent is soluble and does not require dissolving of the converted dye as it is the case with the MTT. The result reported here represents the mean±SD of one set of experiment from three different plates with six individual wells per parameter.

### 2.6.1 XTT Assay

The viability of the cells was measured using the XTT [2, 3-Bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt] colorimetric assay. Active cells are able to reduce the tetrazolium salt XTT to orange coloured compounds of formazan, which is water-soluble. The number of metabolic active cells depicts the activity of the enzyme and this will be reflected in the concentration of the dye formed.

The XTT ready to use solution was prepared according to the manufacturer's instruction by mixing 0.02ml electron coupling reagent with 1ml XTT labeling reagent on the day of the assay.

To determine the viability of the cells, 5000 cells/ml (i.e. 1000 cells/200 µl culture medium) were cultured in a 96 well plate for 24 hour. The next day, the medium was removed and the adherent cells were exposed to five different concentrations of either plant extract (0.01, 0.1, 1, 10, 100 µg/ml), negative control (0.5% DMSO in complete culture medium) and positive control (2% DMSO in complete culture medium) for another 4 days. Thereafter, 100 µl of ready to use XTT solution was added to each well and incubated for 2 hours at 37°C. The cells were measured at 450nm (XTT) and 620nm (background) using an ELISA reader (Thermo electron corporation, South Africa).

The optical density (OD) was then calculated by subtracting the absorbance of the background wavelength from the absorbance of the test wavelength (XTT). The results derived were expressed as 100% of the control using equation 1.

$$\text{Percent Vitality (\%)} = \frac{\text{OD (Test)}}{\text{OD (Control)}} * 100 \dots\dots\dots \text{(Equation 1)}$$

### 2.6.2 MTT Assay

Viability of the cells was also measured using the MTT [Thiazoyl blue tetrazolium bromide] assay. MTT assay is dependent on the cellular reduction of MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] by the mitochondrial dehydrogenase of viable cells to a blue formazan product which can be measured spectrophotometrically.

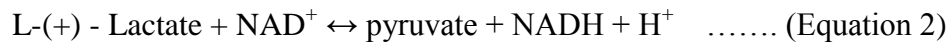
In order to determine the viability of the cells, 5000 cells/ml (i.e. 1000 cells/200  $\mu$ l culture medium) were cultured in a 96 well plate for 24 hours. The next day, the medium was removed and the adherent cells were exposed to the five different concentrations (0.01, 0.1, 1, 10, 100  $\mu$ g/ml) of the extracts, control (0.5% DMSO in complete culture medium) and positive control (2% DMSO in complete culture medium) for 4 days.

MTT stock solution was prepared by dissolving 1mg MTT/ ml PBS. The dissolving process was facilitated by rotating for approximately 1 h at room temperature. MTT solution was filtered using a syringe filter of 0.1 $\mu$ m. A volume of 20  $\mu$ l was added to each well plate containing 200  $\mu$ l of the extracts (i.e. making one-tenth of the culture volume) and incubated for 3 hours at 37°C. Thereafter, the supernatant was removed and 100  $\mu$ l of DMSO was added to each well to dissolve the precipitated dye.

Absorbance of the dye was measured with an ELISA reader (Thermo electron corporation, South Africa) at a wavelength of 540nm with a reference wavelength of 690nm. The optical density (OD) was calculated by subtracting the absorbance of the reference wavelength from the absorbance of the test wavelength. Percent vital cells compared to the control were calculated as stated above in equation 1.

## 2.7 Determination of lactate

Lactate is determined by the reaction (equation 2) involving its oxidation by nicotinamide- adenine dinucleotide (NAD) to pyruvate. This reaction is catalyzed by L-lactate dehydrogenase (L-LDH). The amount of NADH formed is stoichiometric with the amount of L-lactic acid and the increase in NADH is determined by its absorbance at 340 nm. This equilibrium reaction usually favours the right hand side except where pyruvate is trapped by glutamate-pyruvate transaminase (GPT) in the presence of L-glutamate does it go to the left.



After 4 days exposure of the cells to the different concentration of plant extracts and controls, aliquots of the supernatant were collected from each well plate and heated for 5 minutes at 95°C to stop enzymatic activities. Aliquots were allowed to cool and then stored at -20°C for up to 2 weeks.

Glutamate buffer was prepared by dissolving 56.75g L-(+)-glutamate in 380ml NaOH (1mol/L) and filled up to 500 ml with distilled water. The neutral glutamate solution stored in aliquots of 20 ml was frozen at -20°C until when required. On the day of lactate determination, 9.4 ml NaOH (1mol/L) was added to 20 ml of the neutral glutamate solution with final concentration 0.52 mol/L at pH of 8.9.  $\beta$ -NAD solution was prepared by first weighing 40mg  $\beta$ -NAD in Eppendorf cup with 2ml distilled water added to it, to make a final concentration of 30mmol/L.

To determine the lactate concentration (Noll, 1984), 80  $\mu$ l supernatant from each well was added to 700  $\mu$ l distilled water, 250  $\mu$ l glutamate buffer, 30  $\mu$ l  $\beta$ -nicotinamide-adenine dinucleotide and glutamate-pyruvate transaminase (10  $\mu$ l, 21 units/ml) was added, mixed thoroughly, incubated at room temperature and absorption was measured after 10 minutes (E10 value) using a spectrophotometer at 340nm. Lactate dehydrogenase (11  $\mu$ l, 45units/ml) was added, mixed thoroughly and incubated for 2 hours at room temperature. Thereafter, the absorption was measured again (E120 value) at 340nm.

For evaluation, the difference (D) between the values of E10 and E120 were calculated and their concentration derived using a lactate standard curve.

### ***2.7.1 Lactate Standard Curve***

The lactate standard curve was performed by first making a stock solution by dissolving 4.93mg of sodium-L-lactate in 10ml distilled water, after which a serial dilution was performed to obtain concentrations of 2, 4, 8, 12 and 16µg/ml by diluting the stock solution with distilled water (dH<sub>2</sub>O). That is:

2 µg/ml= 5 µl stock solution + 775 µl dH<sub>2</sub>O

4 µg/ml= 10 µl stock solution + 770 µl dH<sub>2</sub>O

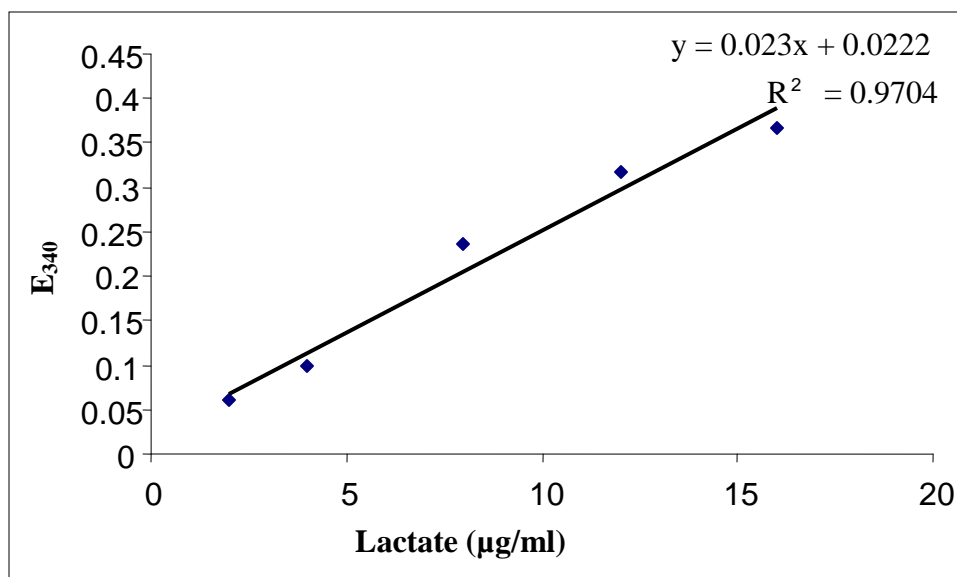
8 µg/ml= 20 µl stock solution + 760 µl dH<sub>2</sub>O

12 µg/ml= 30 µl stock solution + 750 µl dH<sub>2</sub>O

16 µg/ml= 40 µl stock solution + 740 µl dH<sub>2</sub>O

From each standard concentration (2, 4, 8, 12, 16 µg/ml), 780 µl was taken and added to 250 µl glutamate buffer, 30 µl β-nicotinamide-adenine dinucleotide, after which, glutamate-pyruvate transaminase (10 µl, 21 units/ml) was added, mixed thoroughly, incubated at room temperature and absorption was measured after 10 minutes (E10 value) using a spectrophotometer at 340nm. Lactate dehydrogenase (11 µl, 45units/ml) was added, mixed thoroughly and incubated for 2 hours at room temperature. Thereafter, the absorption was measured again (E120 value) at 340nm.

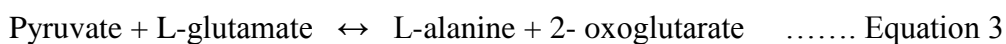
For evaluation, the difference (D) between the values of E10 and E120 were calculated and used to plot the graph (figure 16) for which the concentration of the lactate produced by TM4 cell exposed to the respective plant extracts were derived from.



**Figure 16:** Lactate Standard Curve

### 2.8 Determination of pyruvate

Pyruvate is also determined by the principle below (equation 3) with glutamate-pyruvate transaminase acting as the catalyzing agent:



For this reaction to take place, pyruvate is trapped by glutamate-pyruvate transaminase (GPT) in the presence of L-glutamate.

To prepare triethanolamine buffer, 23.3g triethanolamine hydrochloride was dissolved in 200ml distilled water and 0.47g EDTA was also added and adjusted to a pH of 7.6 with 20ml 2N NaOH. Distilled water was added to get a final volume of 250ml. NADH+H<sup>+</sup> solution was prepared by dissolving it in distilled water (5mg/ml; 7mmol/L). Lactate dehydrogenase was diluted 1:10 (i.e. 100µl LDH + 900µl buffer) with potassium phosphate buffer before performing the assay. To prepare 10mmol/L potassium phosphate buffer, 142mg disodiumphosphate (Na<sub>2</sub>HPO<sub>4</sub>) and 27mg potassium

diphosphate ( $\text{KH}_2\text{PO}_4$ ) were dissolved in 100ml glycerol. pH was titrated to 7.5 with 0.5M NaOH.

Into 20  $\mu\text{l}$   $\text{NADH}+\text{H}^+$  solution and 600  $\mu\text{l}$  triethanolamine buffer (0.5M, PH 7.6, 5mM EDTA), 200  $\mu\text{l}$  of cell culture supernatant was added, mixed thoroughly and incubated for 10 minutes (E10 value) and measured at 340nm using a spectrophotometer. Lactate dehydrogenase (10  $\mu\text{l}$ , 45units/ml) was added, mixed thoroughly and incubated for 1 hour (E60 value) and the mixture was again measured at 340nm (Czok & Lamprecht, 1974).

For evaluation, the difference (D) between the values of E10 and E60 was calculated and their concentrations derived from a pyruvate standard curve (figure 17).

### ***2.8.1 Pyruvate standard curve***

The pyruvate standard curve was performed by first making a stock solution by dissolving 11mg of sodium pyruvate in 100ml triethanolamine buffer, after which a serial dilution was performed to obtain concentrations of 1.4, 2.8, 5.5 and 11  $\mu\text{g}/\text{ml}$  by diluting the stock solution with triethanolamine buffer (TE buffer). That is:

1.4  $\mu\text{g}/\text{ml}$  = 12.5  $\mu\text{l}$  stock solution + 187.5  $\mu\text{l}$  TE buffer

2.8  $\mu\text{g}/\text{ml}$  = 25  $\mu\text{l}$  stock solution + 175  $\mu\text{l}$  TE buffer

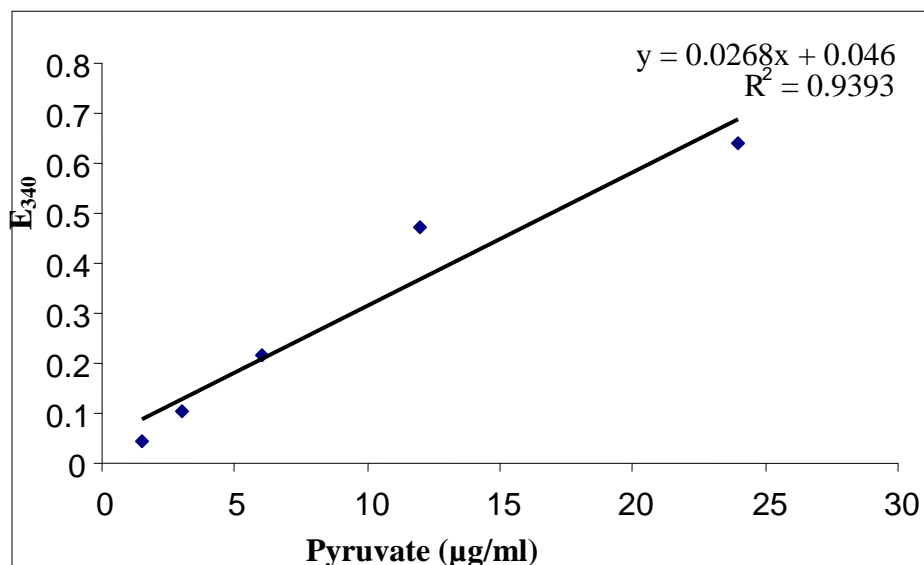
5.5  $\mu\text{g}/\text{ml}$  = 50  $\mu\text{l}$  stock solution + 150  $\mu\text{l}$  TE buffer

11  $\mu\text{g}/\text{ml}$  = 100  $\mu\text{l}$  stock solution + 100  $\mu\text{l}$  TE buffer

Into 20  $\mu\text{l}$   $\text{NADH}^+\text{H}^+$  solution and 600  $\mu\text{l}$  triethanolamine buffer (0.5M, PH 7.6, 5mM EDTA), 200  $\mu\text{l}$  of each pyruvate standard was added, mixed thoroughly and incubated for 10 minutes (E10 value) and measured at 340nm using a spectrophotometer. Lactate dehydrogenase (10  $\mu\text{l}$ , 45units/ml) was added, mixed thoroughly and incubated for 1 hour (E60 value) and the mixture was again measured at 340nm.

For evaluation, the difference (D) between the values of E10 and E60 was calculated and used to plot against the standard (figure 17).





**Figure 17:** Pyruvate Standard Curve

### ***2.9 Determination of protein content***

Medium was removed after the four days of exposure to the extracts and the cells were dissolved in 200 µl lysis reagent (0.5M NaOH/0.1% Sodium-dodecylsulphate (SDS)) by incubating at room temperature for 30 minutes and gently shaking. To determine the protein content, 20 µl of the lysate were transferred in duplicate to a 96 well plate. After which, 25 µl of reagent A and 200 µl of reagent B were added to each well and incubated for another 30 minutes at room temperature (Bio-rad assay; Lowry et al., 1951) and the absorption measured with an MTP-Reader at  $\lambda=690\text{nm}$ . The protein content was determined using albumin bovine serum as standard (figure 18).

The result derived from this process represented the amount of protein per ml. However, the amount of protein obtained was divided by 5 in order to obtain the actual amount of protein per well, as 200µl of lysis buffer was used to dissolve the cells. This was then compared to the respective concentrations of lactate and pyruvate for each well.

### 2.9.1 Bovine serum albumin standard curve

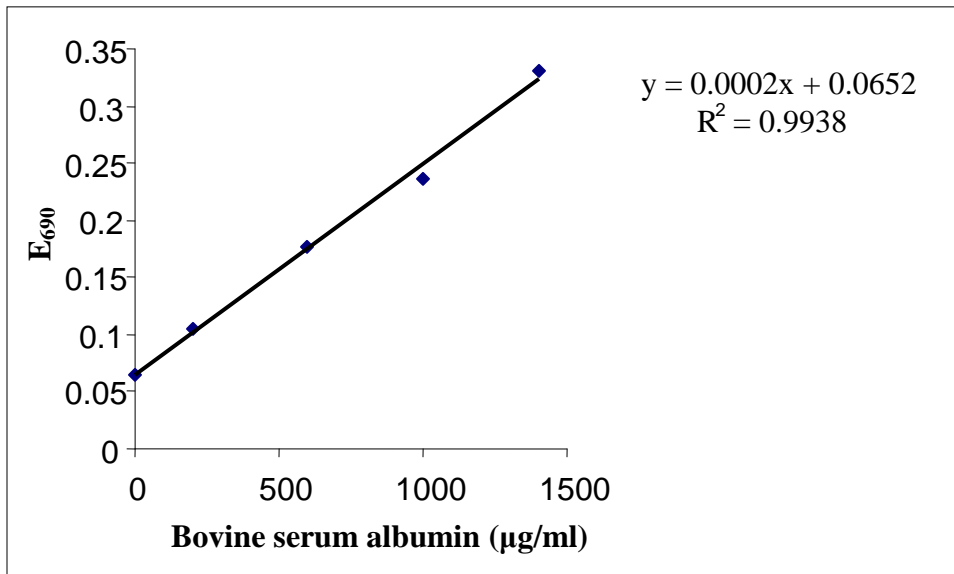
A stock solution of 1400 µg/ml albumin bovine serum was prepared by dissolving it in lysis solution. The stock solution was further diluted to make a concentration of 1000, 600 and 200µg/ml. That is;

1000 µg/ml = 100 µl stock solution + 40 µl lysis reagent

600 µg/ml = 100 µl stock solution + 133 µl lysis reagent

200 µg/ml = 100 µl stock solution + 600 µl lysis reagent

From each standard concentration, 20 µl was taken and placed in duplicate in a 96 well plate and a blank containing only lysis reagent. Into each well, 25 µl of reagent A and 200 µl of reagent B was added and incubated for another 30 minutes at room temperature (Bio-rad assay; Lowry et al., 1951) and the absorption measured with an MTP-Reader at  $\lambda=690\text{nm}$ .



**Figure 18:** Bovine serum albumin standard curve.

## **2.10 Determination of inhibin B**

In order to determine the effect of *Basella alba* and *Hibiscus macranthus* on TM4 Sertoli cell inhibin B production, 4000 cells/ml were incubated for 4 days after a 1 day pre-incubation. At the end of the exposure time, the supernatant was transferred into an Eppendorf vial, heated for 5 minutes at 95°C to inactivate the enzymes, and kept frozen in -20°C. The supernatant was sent to Klinik fuer Dermatologie, Universitaetsklinikum, Jena, Germany for analysis.

Before the assay, SDS was added to all the spent media of the cells exposed to different concentrations of either *Basella alba* or *Hibiscus macranthus* extract and inhibin B standards and heated at 100°C. They were then treated with hydrogen peroxide. These treatments enhance the specificity and sensitivity of the ELISA. The samples were then placed in the wells of microtitre plate coated with monoclonal antibody to the inhibin  $\beta$ -B subunits, so that the antigens in the samples would bind to the antibodies through its  $\beta$ -B subunits. This process will result in the formation of a red product of which the colour intensity is proportional to the concentration of inhibin present in the sample. The detection limit of this assay was 15.3 pgml<sup>-1</sup>.

## **2.11 Morphological Studies**

### **2.11.1 Haematoxylin and eosin staining**

TM4 Sertoli cells were plated on cover slips (22x22mm) in tissue culture dishes (35x10mm) for 1 day. The cells were then exposed to 1, 10, 100  $\mu$ g/ml and 0.01, 0.1, 1, 10 100  $\mu$ g/ml of the extracts for 1 day and 4 days respectively, negative and positive controls (0.5% and 2% DMSO in complete culture medium respectively) after 1 day pre-incubation. For 1 day and 4 days exposures, 10000 cells/ml and 4000 cells/ml were used respectively.

The cover slips were first cleaned with acid alcohol (1% HCl, 70% Ethanol), coated with Poly-L-Lysin (1:9 in distilled water), kept over night and sterilized under the UV light for 15 minutes before use.

To study the morphology of the cells, haematoxylin and eosin staining technique (Kiernan, 1990) was used. Cover slips were first removed from the tissue culture dishes and placed in a cover slip holder. Cells on each cover slip were fixed with Bouin's fixatives for 30 minutes, left in 70% ethanol for 20 minutes and rinsed with tap water. The cells were then stained with haematoxylin for 20 minutes and rinse with running tap water for 2 minutes in order for the stain to be developed. They were then rinsed with 70% ethanol, stained with 1% eosin for 2 minutes, and rinsed again for 5 minutes twice in 70%, 96% and 100 % ethanol and xylene before mounting on the slide and viewed under the microscope (Zeiss, Oberkochen, Germany).

### ***2.11.2 Mitotic Index***

TM4 Sertoli cells were plated on cover slips (22x22mm) in tissue culture dishes (35x10mm) for 1 day. The cells were then exposed to 1, 10, 100 µg/ml and 0.01, 0.1, 1, 10 100 µg/ml of the extracts for 1 day and 4 days respectively, negative and positive controls (0.5% and 2% DMSO or 100mM ethanol in complete culture medium respectively) after the 1 day pre-incubation. For 1 day and 4 days exposures, 10000 cells/ml and 4000 cells/ml were used respectively.

The cover slips were treated and the cells were fixed and stained using H&E (Kiernan, 1990) as stated in 2.11.1. The cells were viewed and counted under the microscope (Zeiss, Germany).

The mitotic index was obtained using equation 4 below and a total of 6 fields were viewed and the mean ± SD expressed as %.

$$\frac{\text{Number of dividing cells}}{\text{Total number of cells in the field of view}} = \text{mitotic index} \quad \text{-----} \quad \text{equation 4}$$

## ***2.12 Nuclear studies***

### ***2.12.1 DAPI Staining***

After 1 day pre-incubation of TM4 Sertoli cells, the cells were exposed to 1, 10, 100 µg/ml for 1 day and 0.01, 0.1, 1, 10 100 µg/ml of the extracts for 4 days in another set of experiment. Negative and positive controls of 0.5% DMSO and 100mM ethanol in culture medium respectively were also used. Jang et al., (2002) showed that 100mM ethanol induced apoptosis in TM3 mouse Leydig cells. The cells were studied for the presence of apoptotic bodies.

The cells were plated on cover slips (22x22mm) in tissue culture dishes (35x10mm). The cover slips were treated as stated in 2.11.1. For 1 day and 4 days exposure, 10000 cells/ml and 4000cells/ml were used respectively.

For apoptotic study, the cover slip were first removed from the tissue culture dishes and place in a cover slip holder and washed with PBS. Thereafter, the cells were fixed by the use of 4% formaldehyde for 30 minutes at room temperature followed by permeabilization with 0.5 % Triton X-100 for 5 minutes at room temperature and rinsed with PBS and the excess drained from the cover slips. The nuclei were then visualized by incubating with DAPI (1µg/ml) for 30 minutes at room temperature. The cover slips were mounted on a microscope slide with DPX as a mounting medium and viewed with the fluorescence microscope (Zeiss, Oberkochen, Germany).

### ***2.12.2 Apoptotic count***

After 1 day pre-incubation of TM4 Sertoli cells, the cells were exposed to 1, 10, 100 µg/ml and 0.01, 0.1, 1, 10 100 µg/ml of the extracts for 1 and 4 days respectively with negative and positive controls as 0.5% DMSO and 100mM ethanol in culture medium respectively.

The cover slips were treated and the cells were fixed and stained as stated in 2.11.1. The cells were then viewed and counted under the microscope (Zeiss).

To determine the number of apoptotic cells present, 200 cells were counted (equation 5) and the percentage of apoptotic bodies present was calculated.

$$\frac{\text{Number of apoptotic bodies}}{200 \text{ cells}} * 100 = \text{apoptotic index} \text{ ----- Equation 5}$$

### **2.13 Statistical analysis**

Data were expressed in mean±SD and statistical differences were determined by student's t-test. Statistical analysis was done using the MedCalc statistical software (v9.3.0.0). Data were first tested for normal distribution using Kolmogorov–Smirnov test. For normal distribution, independent student *t*-test was used to compare the test and control. The results are considered significant when  $P < 0.05$ . Experiments were repeated at least two times with similar findings. One representative set of data was shown.

## CHAPTER THREE

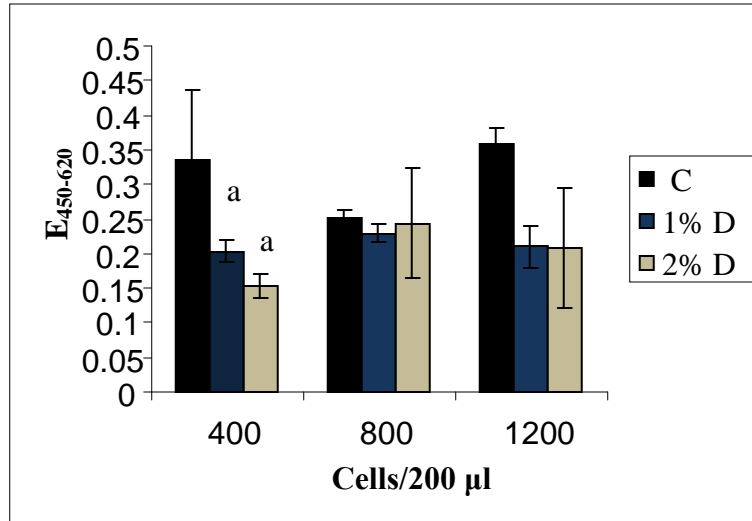
### Results

#### *3.1 Establishment of optimal cell density*

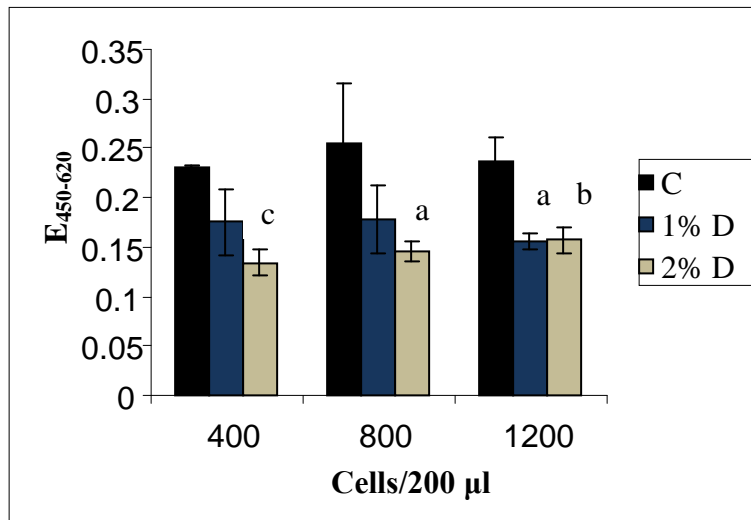
To determine the optimal density of cells to be used in this study, 400, 800 and 1200 cells/ 200  $\mu$ l were incubated for five days with either the complete culture medium, 1% DMSO or 2% DMSO in 96 well plates. DMSO is known to be cytotoxic at concentrations of 1% or above. Thus, DMSO served here as a positive control to check the accuracy of the XTT or MTT assay. On the day of the assay, either 50 or 100  $\mu$ l of the XTT reagent was used to test for the viability of the cells. The recommended concentration ratio of the XTT solution to culture medium is 1:2 which corresponded to 100  $\mu$ l. To save costs, we also analyzed if it was possible to reduce the amount of the XTT reagent by 50% (i.e. using 50  $\mu$ l) without affecting the accuracy of this assay.

The activity of mitochondrial dehydrogenases increased roughly with the number of cells plated, showing a plateau between 800 to 1200 cells (figures 19 and 20). Compared to the complete culture medium, 1% and 2% DMSO reduced the activity of the mitochondrial dehydrogenase, thus demonstrating a significant cytotoxic effect. The graphs also showed that using half (figure 20) of the XTT reagent volume did not reduce the sensitivity of the assay.

Therefore, an optimal density of 1000 cells/ 200  $\mu$ l (i.e. 5000 cells/ml) was established as the optimal cell density, whereas 2% DMSO was found optimal as the positive control and 100  $\mu$ l of XTT reagent as sufficient and effective for the purpose of cytotoxicity study.



**Figure 19:** Effect of different cell densities on the activity of mitochondrial dehydrogenases (100µl XTT reagent). Result depicted as mean  $\pm$  SD (n=6). C indicated control with 100µl XTT; 1% D indicated 1% DMSO with 100µl XTT; 2% D indicated 2% DMSO with 100µl XTT; <sup>a</sup> indicated significant difference from the respective control at  $P < 0.05$ .



**Figure 20:** Effect of different cell densities on the activity of mitochondrial dehydrogenases (50µl XTT reagent). Result depicted as mean  $\pm$  SD (n=4). C indicated control with 50µl XTT; 1% D indicated 1% DMSO with 50µl XTT; 2% D indicated 2% DMSO with 50µl XTT; <sup>a</sup> indicated significant difference from the respective control at  $P < 0.05$ ; <sup>b</sup> indicated as  $P < 0.01$ ; <sup>c</sup> indicated  $P < 0.001$ .



### **3.2 Effect of plant extracts on TM4 Sertoli cell viability**

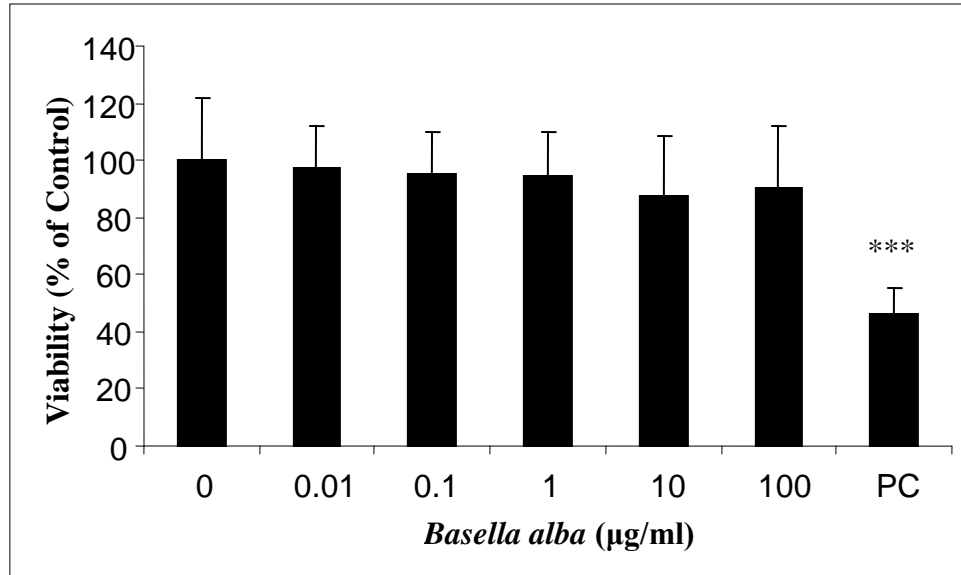
#### **3.2.1 Effect of *Basella alba* extract on TM4 cell viability**

In order to examine the viability of the cells, 1000 cells/200µl (i.e 5000 cells/ml) were pre-incubated for 24 hours and exposed to five different concentrations (0.01 -100 µg/ml) of *Basella alba* for another four days. The cells were then tested for viability using 2, 3-Bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt (XTT reagent) and in a different set of experiments, thiazoyl blue tetrazolium bromide (MTT reagent). The values derived were expressed as 100 % of the control.

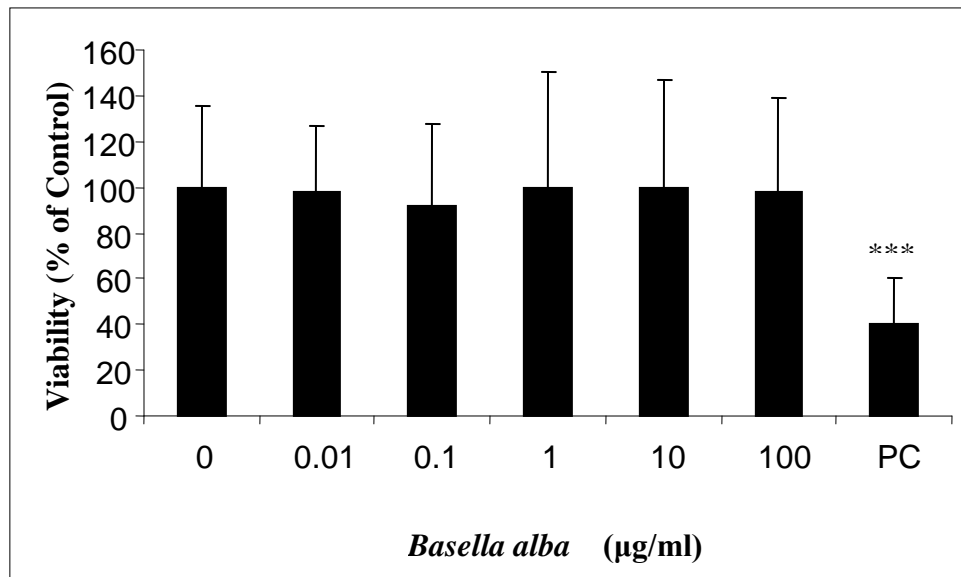
By using the XTT assay, TM4 Sertoli cells exposed to *Basella alba* (figure 21) showed no significant difference compared to the negative control ( $P>0.05$ ). However, at higher concentrations (10-100 µg/ml) of the plant extract there was a slight decrease in the activity of the mitochondrial dehydrogenase activities compared to the lower concentrations (13.7 % decrease at 10 µg/ml and 9.7 % decline at 100 µg/ml). The result also showed that the activity of the mitochondrial dehydrogenase in the positive control was significantly reduced by 50% compared to the negative control ( $P< 0.001$ ; figure 21).

The use of MTT assay also showed that there was no significant difference in the exposure of TM4 Sertoli cells to the plant extract compared to negative the control (figure 22,  $P>0.05$ ). The activity of mitochondrial dehydrogenase in the cells exposed to the positive control was also reduced by 50% as compared to the control ( $P<0.001$ ; figure 22).

Thus, it appears that the *Basella alba* methanol extract had no effect on the viability of the TM4 Sertoli cells when using mitochondrial dehydrogenase activity as the marker.



**Figure 21:** Effect of *Basella alba* on TM4 Sertoli cell viability. The cells were cultured in DMEM/Ham 12 medium for 24h and exposed to *Basella alba* extracts for 4 days. Cell viability was determined by XTT assay. Result depicted as mean  $\pm$  SD (n=18). PC, Positive control (2% DMSO in culture medium); \*\*\* indicated significant difference from the respective control at  $P < 0.001$ .



**Figure 22:** Effect of *Basella alba* on TM4 Sertoli cell viability. The cells were cultured in DMEM/Ham 12 medium for 24h and exposed to *Basella alba* extracts for 4 days. Cell viability was determined by MTT assay. Result depicted as mean  $\pm$  SD (n=18). PC, Positive control (2% DMSO in culture medium); \*\*\* indicated significant difference from the respective control at  $P < 0.001$ .

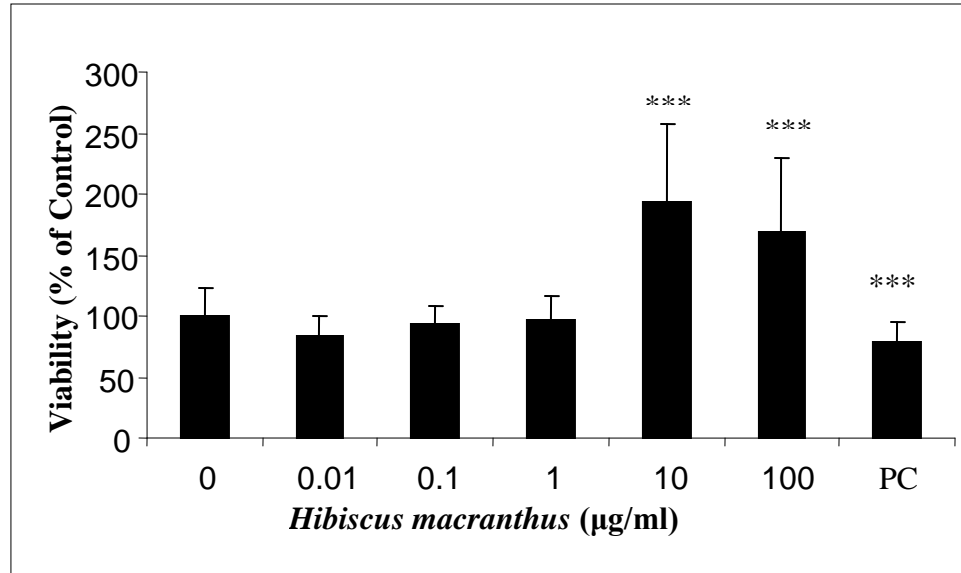
### 3.2.2 Effect of *Hibiscus macranthus* extract on TM4 Sertoli cell viability

To determine the viability of the TM4 cells, 5000 cells/ml (i.e 1000 cells/200  $\mu$ l) were incubated for 24 hours and then exposed to the five different concentrations (0.01-100  $\mu$ g/ml) of *Hibiscus macranthus* for four days. Thereafter, the cells were tested for viability using 2,3-Bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt (XTT reagent) and thiazoyl blue tetrazolium bromide (MTT reagent). The values derived were expressed as 100 % of the control.

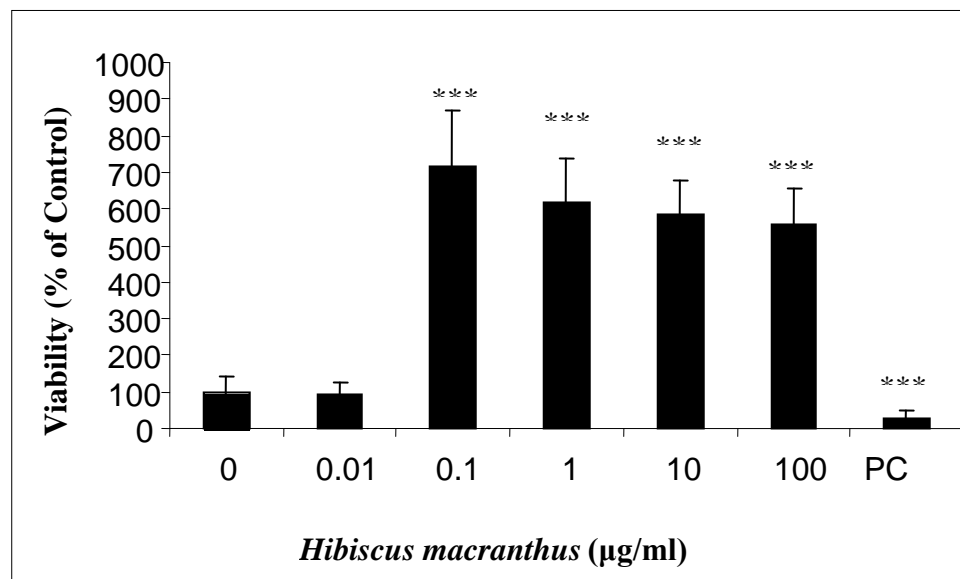
Figure 23 shows that the use of XTT to determine the viability of the Sertoli cells exposed to *Hibiscus macranthus* indicated no significant difference for the lower concentrations ( $P>0.05$ ; 0.01–1  $\mu$ g/ml). However, a significant increase was seen at higher concentrations ( $P<0.001$ ; 10-100  $\mu$ g/ml). The results showed an increase of 100% in the activities of the enzyme with 10-100  $\mu$ g/ml of the plant extract compared to the negative control.

The MTT assay revealed that the lowest concentration (0.01  $\mu$ g/ml) of the extract had no significant effect on the viability of the cells ( $P>0.05$ ). However, a significant and sharp increase in the activities of the mitochondrial dehydrogenases was seen from 0.1-100  $\mu$ g/ml ( $P< 0.001$ ; figure 24) after exposure to the *Hibiscus macranthus* extract. This rise seems to decrease slightly with increasing concentrations of the extract. There was an increase in mitochondrial dehydrogenase activities about 7 fold in the TM4 Sertoli cells exposed to 0.1  $\mu$ g/ml, 6 fold in 1  $\mu$ g/ml, 5 fold in 10 and 100  $\mu$ g/ml of *Hibiscus macranthus* methanol extract.

In summary, the viability assays showed that at higher concentrations of *Hibiscus macranthus* extract, the mitochondrial dehydrogenase activities of TM4 cells were significantly increased.



**Figure 23:** Effect of *Hibiscus macranthus* on Sertoli cell viability. The cells were cultured in DMEM/Ham 12 medium for 24h and exposed to *Hibiscus macranthus* extracts for 4 days. Cell viability was determined by XTT assay. Result depicted as mean  $\pm$  SD (n=18). PC, Positive control (2% DMSO in culture medium); \*\*\* indicated significant difference from the respective negative control at  $P < 0.001$ .



**Figure 24:** Effect of *Hibiscus macranthus* on Sertoli cell viability. The cells were cultured in DMEM/Ham 12 medium for 24h and exposed to *Hibiscus macranthus* extracts for 4 days. Cell viability was determined by MTT assay. Result depicted as mean  $\pm$  SD (n=18). PC, Positive control (2% DMSO in culture medium); \*\*\* indicated significant difference from the respective control at  $P < 0.001$ .

### **3.3 Effect of plant extracts on TM4 Sertoli cell lactate production**

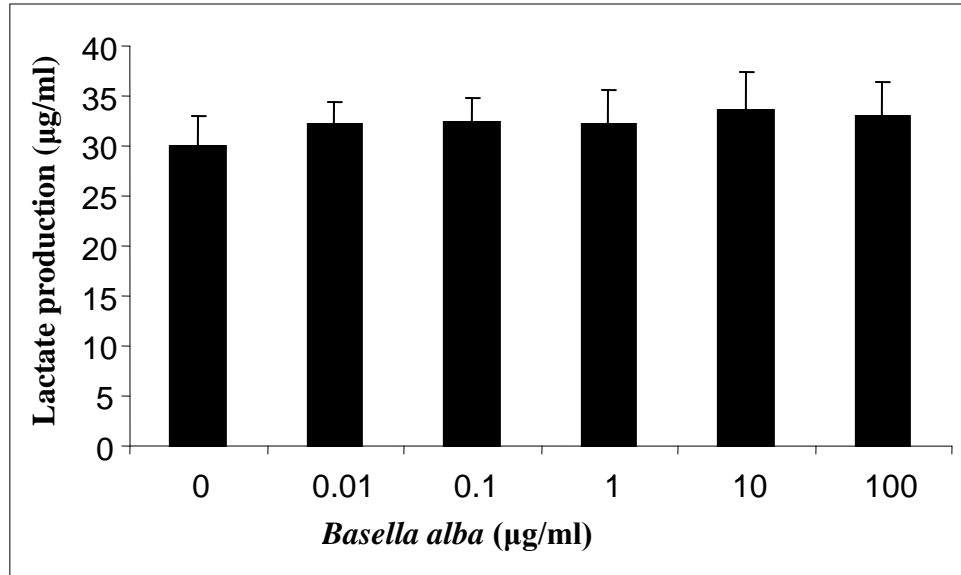
#### **3.3.1 Effect of *Basella alba* extract on TM4 Sertoli cell lactate production**

To determine the effect of *Basella alba* extract on the lactate production of TM4 Sertoli cells, TM4 cells were incubated for 24h with the culture medium. Following which, the cells were exposed to the five different concentrations of *Basella alba* and incubated for another four days. Spent medium was then used to determine the lactate concentration ( $\mu\text{g/ml}$ ). Lactate concentrations derived were also normalized to the protein content in each well to get the amount of lactate ( $\mu\text{g/mg}$  protein, Table 5) that was produced.

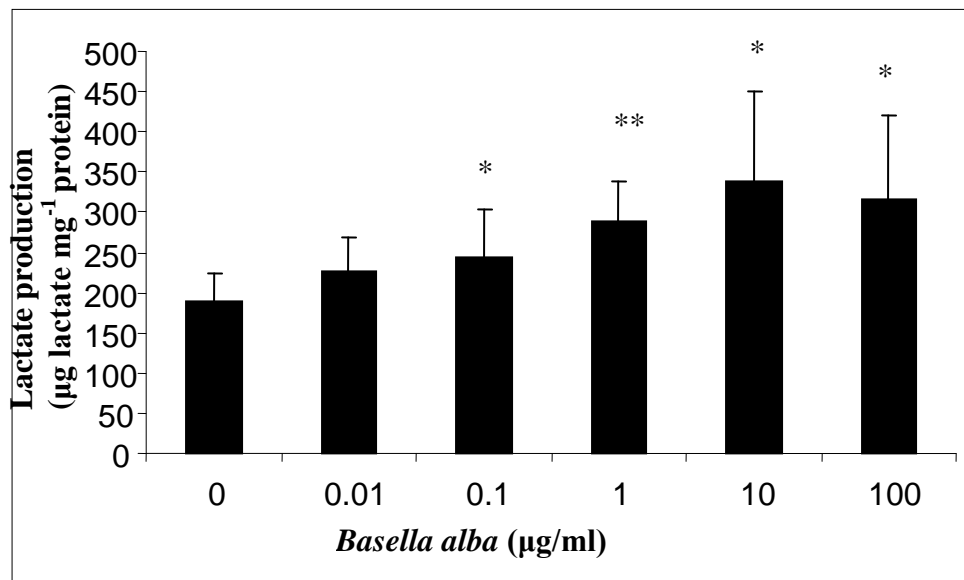
Figure 25 shows no significant difference in lactate production ( $\mu\text{g/ml}$ ) compared to the negative control ( $P>0.05$ ) after 4 days exposure to the extract of *Basella alba*.

However, when the lactate concentration per well ( $\mu\text{g}$ ) was compared with their respective protein content (mg) as in figure 26, it showed a concentration-dependent increase in the lactate concentration with a plateau at 100  $\mu\text{g/ml}$ . At 0.01  $\mu\text{g/ml}$  there was no significant difference, however, 0.1  $\mu\text{g/ml}$  to 100  $\mu\text{g/ml}$  showed a significant difference compared to the negative control ( $P<0.05$ ).

This demonstrated that the *Basella alba* methanol extract significantly increased the production of lactate in the TM4 Sertoli cells. Using normalized values (i.e. negative control set to 100%), Table 5 shows a significant increase in the lactate production ranging from 29 to 79% after exposure to 0.1 -10  $\mu\text{g/ml}$  of the extract.



**Figure 25:** Effect of *Basella alba* on TM4 Sertoli cell lactate accumulation. The cells were cultured in DMEM/Ham 12 medium for 24h and exposed to *Basella alba* extracts for 4 days. Result depicted as mean  $\pm$  SD (n=8).



**Figure 26:** Effect of *Basella alba* on Sertoli cell lactate accumulation normalized to total protein content. The cells were cultured in DMEM/Ham 12 medium for 24h and exposed to *Basella alba* extracts for 4 days. Result depicted as mean  $\pm$  SD (n=8). \*\* indicated significant differences from the respective negative control values at  $P < 0.01$ ; \* indicated  $P < 0.05$ .

### 3.3.2 Effect of *Hibiscus macranthus* extract on TM4 Sertoli cell lactate production

After 24 hour incubation of the TM4 cells with the culture medium, the cells were exposed to the five different concentrations of *Hibiscus macranthus* and incubated for four days. The medium was then used to determine the lactate concentration ( $\mu\text{g/ml}$ ; figure 27), whereas the remaining cells were lysed to measure the total protein content. The lactate concentrations derived were normalized to protein content in each well to get the amount of lactate ( $\mu\text{g/mg}$  protein; figure 28; table 5) that was produced.

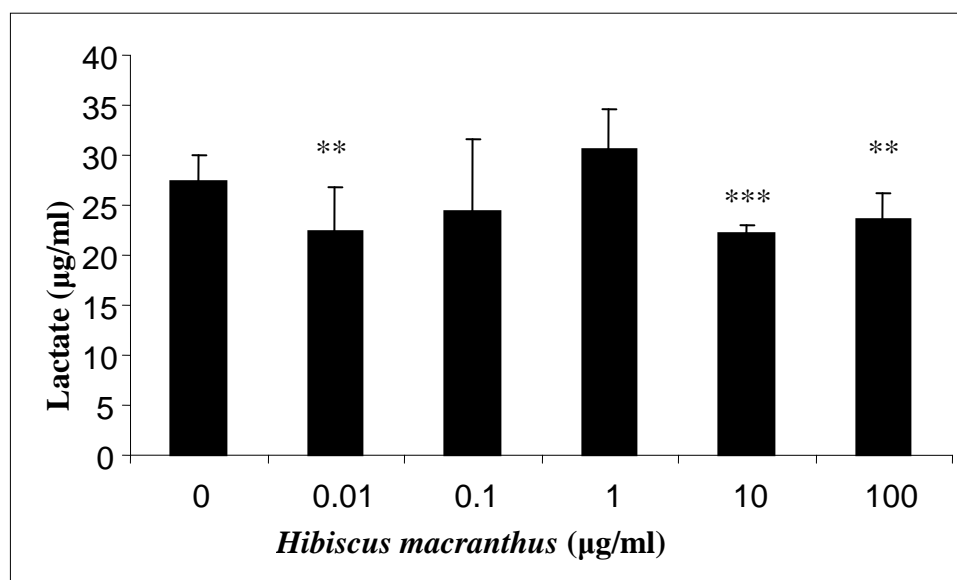
Figure 27 shows a significant decrease in the production of lactate ( $P < 0.01$ ) from 10-100  $\mu\text{g/ml}$  and at 0.01  $\mu\text{g/ml}$  of *Hibiscus macranthus* extract. However, when the amount of lactate produced was compared to the protein content per well (i.e. to the actual number of cells in the respective well), it showed that there was no significant difference ( $P > 0.05$ ) between the different concentration of the plant extract and the negative control. Only at the lowest concentration tested, a significant decrease in lactate production was observed (figure 28;  $P < 0.01$ ).

In summary, the results (table 5) demonstrated that the *Basella alba* methanol extract significantly increased the production of lactate in TM4 Sertoli cells in a concentration-dependant manner. In contrast, the effect of *Hibiscus macranthus* was much less pronounced and not significant.

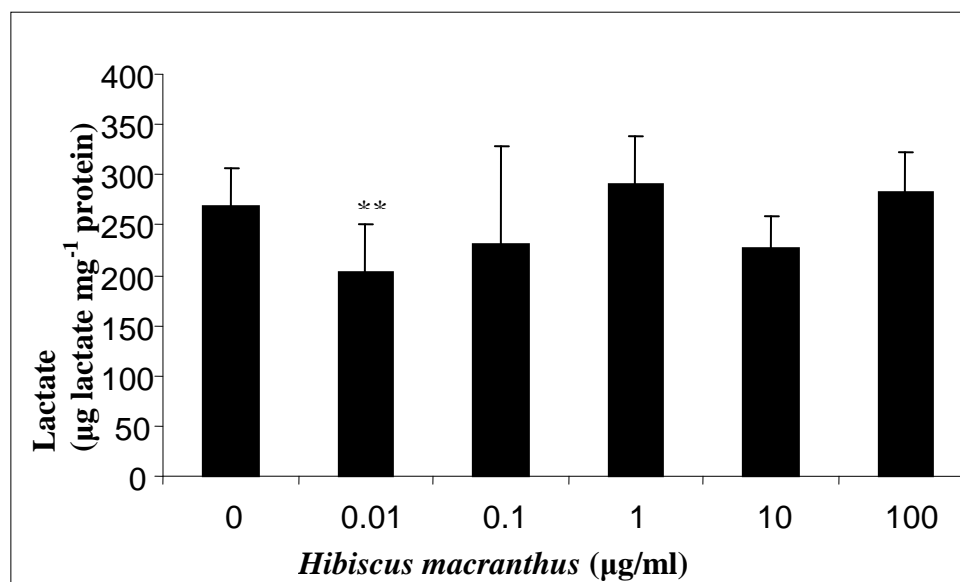
**Table 5:** Effect of *Basella alba* and *Hibiscus macranthus* on lactate production

( $\mu\text{g/ml}$ ) extract	0	0.01	0.1	1	10	100
<i>Basella alba</i>	100 $\pm$ 17.8	119.8 $\pm$ 22.5	129.3 $\pm$ 30.7*	151.9 $\pm$ 27.5**	179.3 $\pm$ 59.1*	167.3 $\pm$ 54.8*
<i>Hibiscus macranthus</i>	100 $\pm$ 13.7	75.5 $\pm$ 18**	85.5 $\pm$ 36.7	108.3 $\pm$ 17.3	84.7 $\pm$ 11.7	104.9 $\pm$ 15.1

**Note:** Result represented as mean  $\pm$  SD (n=8). The values ( $\mu\text{g/mg}$  protein) were normalized with the control set to 100%. \*\* indicated significant differences from the respective negative control values at  $P < 0.01$ ; \* indicated  $P < 0.05$ .



**Figure 27:** Effect of *Hibiscus macranthus* on lactate accumulation. The cells were cultured in DMEM/Ham 12 medium for 24h and exposed to *Hibiscus macranthus* extracts for 4 days. Result depicted as mean  $\pm$  SD (n=8). \*\*\* indicated significant differences from the respective negative control values at  $P<0.001$ ; \*\* indicated  $P<0.01$ .



**Figure 28:** Effect of *Hibiscus macranthus* on lactate accumulation normalized to protein content. The cells were cultured in DMEM/Ham 12 medium for 24h and exposed to *Hibiscus macranthus* extracts for 4 days. Result depicted as mean  $\pm$  SD (n=8). \*\* indicated significant differences from the respective control values at  $P<0.01$ .



### 3.4 Effect of plant extracts on TM4 Sertoli cell pyruvate production

#### 3.4.1 Effect of *Basella alba* extract on TM4 cell pyruvate production

In order to ascertain the effect of *Basella alba* on TM4 cell pyruvate production, the cells were incubated for 24h with culture medium and then exposed to five different concentrations (0.01-100 µg/ml) of *Basella alba* for another four days. The spent medium was then used to determine the pyruvate concentration (µg/ml), whereas the remaining cells were lysed to measure the total protein content. Pyruvate concentrations derived were then compared normalized to protein content in each well to get the amount of pyruvate (µg/mg protein, table 6) that was produced.

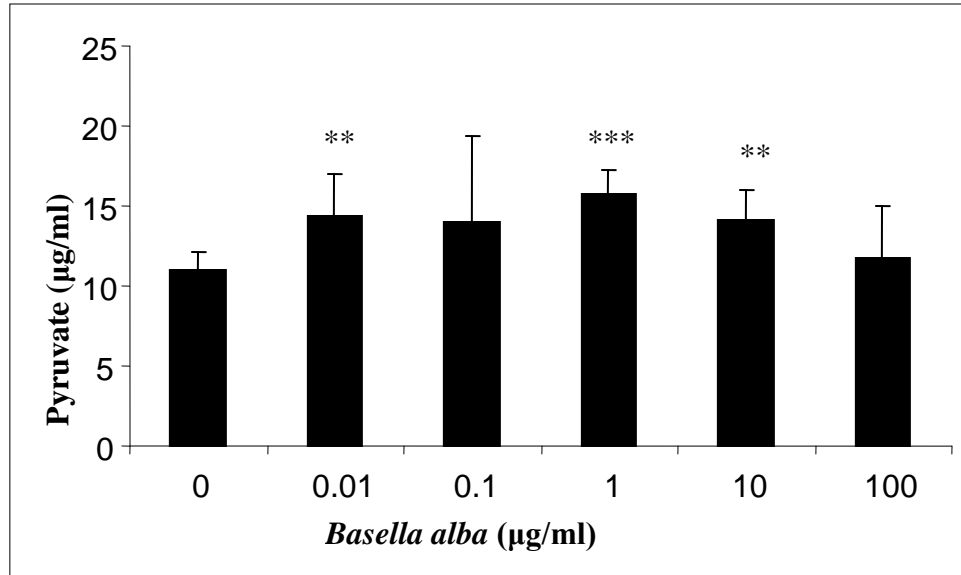
Pyruvate production (µg/ml) from figure 29 shows a significant increase, that peaked around 1 µg/ml, when compared to the negative control ( $P<0.001$ ). However, at 100 µg/ml of the plant extract there is no significant difference compared to the control.

After normalizing the pyruvate production (µg/ml) to the protein content to get the actual amount of pyruvate (µg/mg protein) produced, a significant concentration-dependent increase was observed (figure 30). For better comparison with the *Hibiscus macranthus* extract, the data were also normalized by setting the negative control value to 100%. This reflected the ‘natural’ production of pyruvate by TM4 Sertoli cells. Table 6 also indicates a huge rise in pyruvate production of 39 – 131% and 102% after exposure to 0.01 to 10 µg/ml and 100 µg/ml of *Basella alba* extract, respectively.

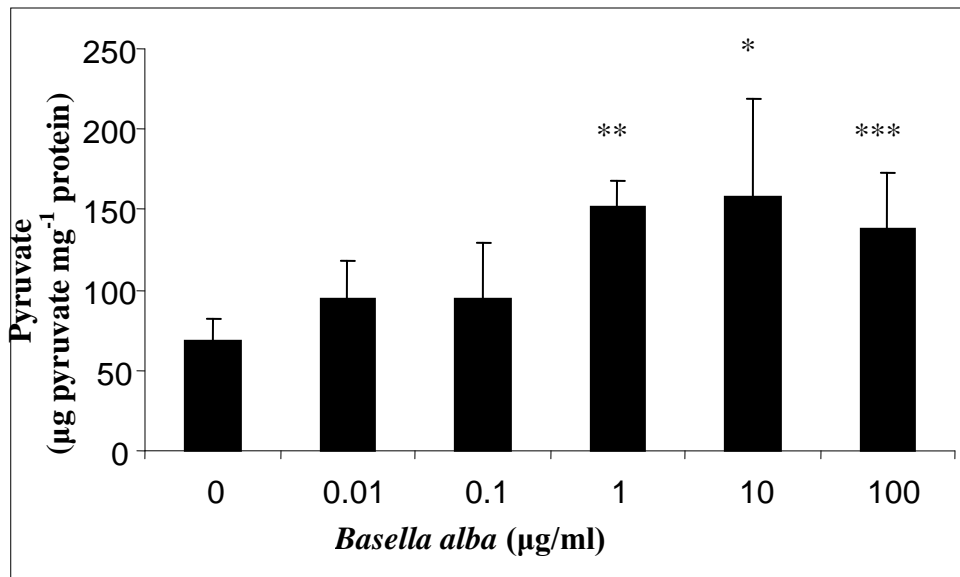
**Table 6:** Effect of *Basella alba* and *Hibiscus macranthus* on pyruvate production

(µg/ml) extract	0	0.01	0.1	1	10	100
<i>Basella alba</i>	100±20.1	139±35	138.2±52.1	222±24.1**	231.4±88.8*	202.9±50.7***
<i>Hibiscus macranthus</i>	100±18.6	97.7±17.8	109.7±18	105.8±31.5	112.6±15.6	148.2±10.4***

**Note:** Result represented as mean ± SD (n=8). The values (µg/mg protein) were normalized with the control set to 100%. \*\*\* indicated significant differences from the respective control values at  $P<0.001$ ; \*\* indicated  $P<0.01$ ; \* indicated  $P<0.05$ .



**Figure 29:** Effect of *Basella alba* on Sertoli cell pyruvate accumulation. The cells were cultured in DMEM/Ham 12 medium for 24h and exposed to *Basella alba* extracts for 4 days. Result depicted as mean  $\pm$  SD (n=8). \*\*\* indicated significant differences from the respective control values at  $P<0.001$ ; \*\* indicated  $P<0.01$ .



**Figure 30:** Effect of *Basella alba* on Sertoli cell pyruvate production normalized to protein content. The cells were cultured in DMEM/Ham 12 medium for 24h and exposed to *Basella alba* extract for 4 days. Result depicted as mean  $\pm$  SD (n=8). \*\*\* indicated significant differences from the respective control values at  $P<0.001$ ; \*\* indicated  $P<0.01$ ; \* indicated  $P<0.05$ .

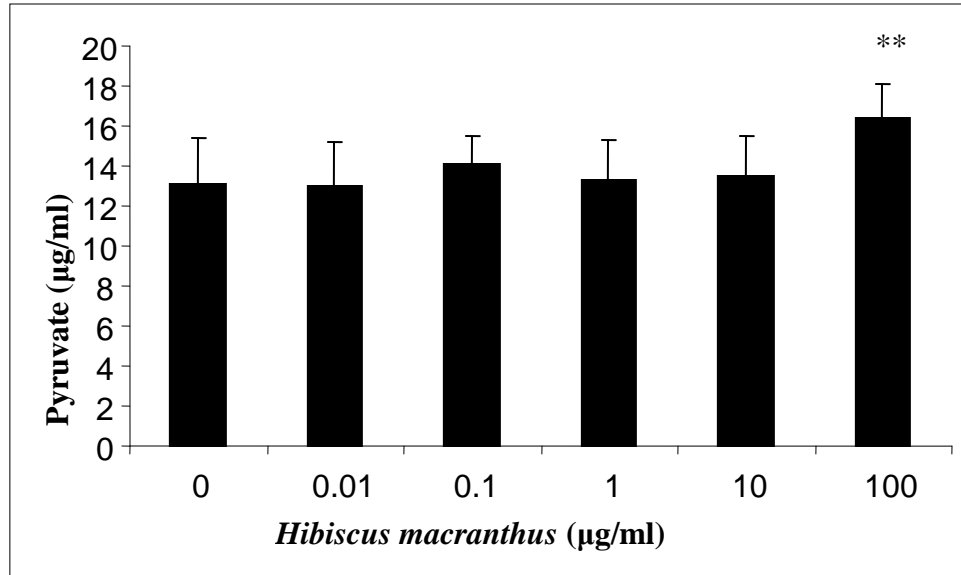
#### 3.4.2 Effect of *Hibiscus macranthus* extract on TM4 Sertoli cell pyruvate production

To examine the production of pyruvate in TM4 Sertoli cells exposed to *Hibiscus macranthus* extract, TM4 cells were incubated for 24h with the culture medium. Following which, they were exposed to five different concentrations (0.01-100 µg/ml) of *Hibiscus macranthus* and incubated for another four days. The spent medium was then used to determine pyruvate concentration (µg/ml). Pyruvate concentrations derived were then normalized to the protein content in each well to get the amount of pyruvate (µg/mg protein; table 6) that was produced.

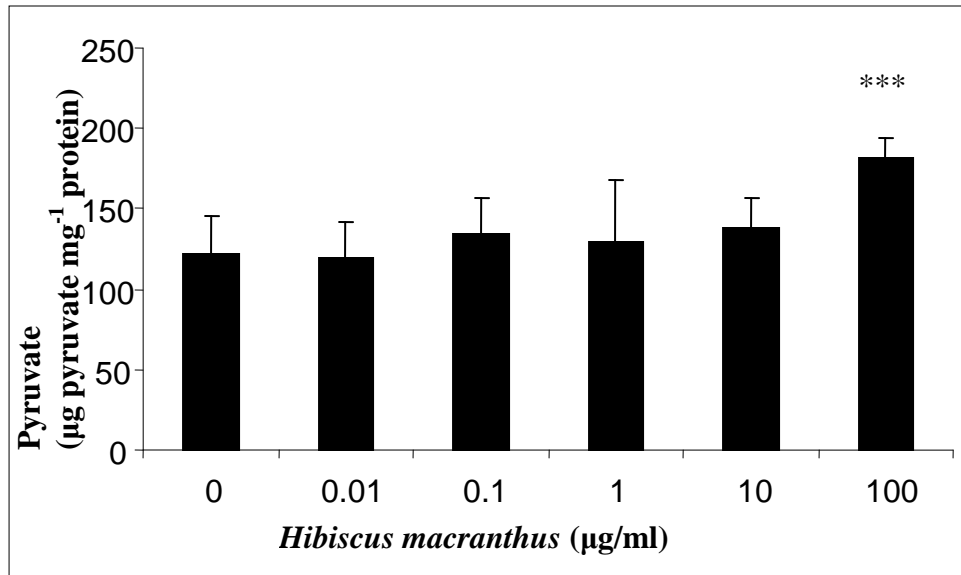
TM4 Sertoli cells exposed to lower concentrations (0.01-10 µg/ml) of the *Hibiscus macranthus* extract showed no significant difference ( $P>0.05$ ) compared to the negative control. However, at 100 µg/ml there is a small but significant increase ( $P<0.01$ ; figure 31). The normalization to the amount of pyruvate produced per protein content (µg/mg protein) also showed no significant effect for the plant extract compared to the control except at the highest concentration where there was an increase in the amount of pyruvate produced ( $P<0.001$ ; figure 32).

This showed that *Hibiscus macranthus* methanol extract up to 10 µg/ml did not have any significant effect on the production of pyruvate in TM4 Sertoli cells. Only the highest plant extract concentration showed a slight but significant increase in pyruvate secretion ( $P<0.001$ ; 19% increase; table 6; figure 32).

Taken together, the results demonstrated that the *Basella alba* methanol extract significantly increased the production of pyruvate in TM4 Sertoli cells in a concentration-dependant manner. In contrast, the effect of *Hibiscus macranthus* was much less pronounced and only significant for the highest concentration investigated.



**Figure 31:** Effect of *Hibiscus macranthus* on pyruvate accumulation. The cells were cultured in DMEM/Ham 12 medium for 24h and exposed to *Hibiscus macranthus* extract for 4 days. Result depicted as mean  $\pm$  SD (n=8). \*\* indicated significant differences from the respective control values at  $P < 0.01$ .



**Figure 32:** Effect of *Hibiscus macranthus* on pyruvate accumulation normalized to protein content. The cells were cultured in DMEM/Ham 12 medium for 24h and exposed to *Hibiscus macranthus* extract for 4 days. Result depicted as mean  $\pm$  SD (n=8). \*\*\* indicated significant differences from the respective control values at  $P < 0.001$ .

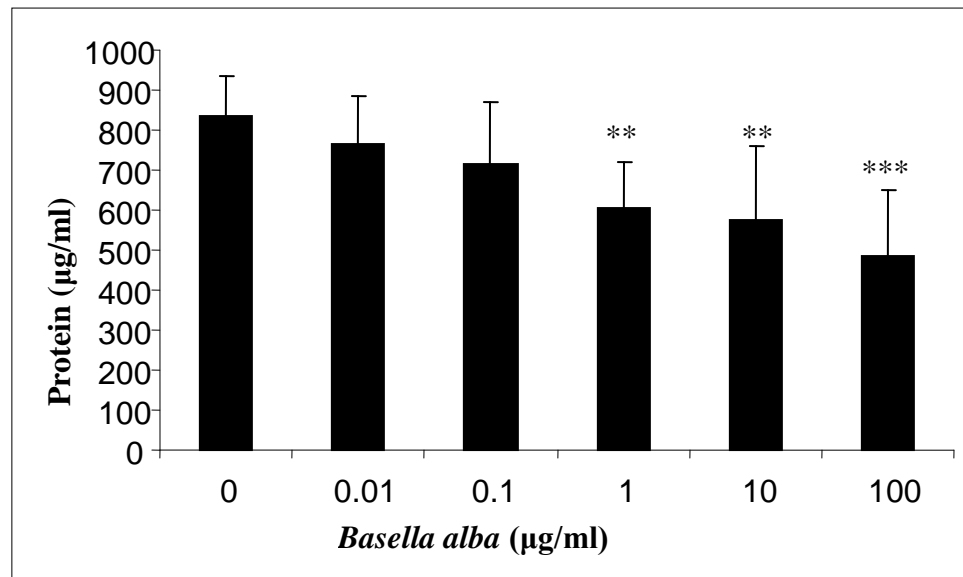
### 3.5 Effect of plant extract on TM4 Sertoli cell protein content

#### 3.5.1 Effect of *Basella alba* extract on TM4 Sertoli cell protein content

To determine the total protein content of TM4 cells exposed to *Basella alba*, 4000 cells/ml were incubated for 24 hour with the culture medium and then exposed to five concentrations of *Basella alba* for another four days. At the end of the incubation period, the cells were analyzed for total protein content using bovine serum albumin as a standard (figure 18).

Figure 33 shows a concentration dependent decrease in the protein content of Sertoli cells after four days exposure to *Basella alba* methanol extract. The lower concentrations of plant extract showed no significant difference compared to the negative control while the higher concentration (1- 100  $\mu\text{g/ml}$ ) caused a significant decrease (16-27% from 1 -100  $\mu\text{g/ml}$ ) in total protein content ( $P < 0.01$ ).

Thus, higher concentrations of the *Basella alba* methanol extract significantly decreased the amount of protein in TM4 Sertoli cells.



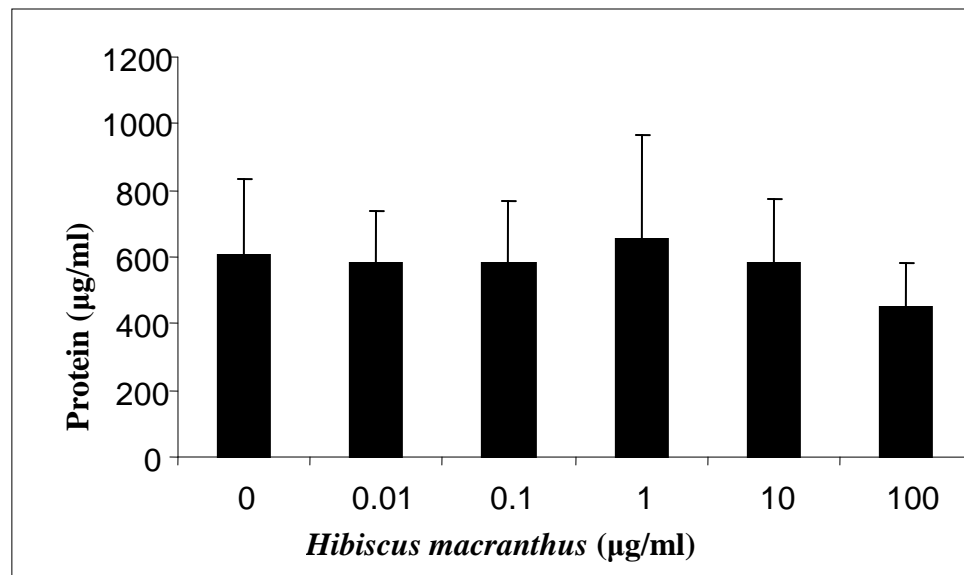
**Figure 33:** Effect of *Basella alba* on Sertoli cell protein content.

The cells were cultured in DMEM/Ham 12 medium for 24h and then exposed to *Basella alba* extract for 4 days. Result depicted as mean  $\pm$  SD (n=8). \*\*\* indicated significant differences from the respective negative control values at  $P < 0.001$ ; \*\* indicated as  $P < 0.01$ .

### 3.5.2 Effect of *Hibiscus macranthus* extract on TM4 Sertoli cell protein content

To determine the total protein content in TM4 cells exposed to *Hibiscus macranthus* extract, 4000 cells/ml were incubated for 24 hour with the culture medium and afterwards exposed to five concentrations (0.01- 100  $\mu\text{g/ml}$ ) of *Hibiscus macranthus* for four days. At the end of the incubation period, the cells were analyzed for protein content using bovine serum albumin as a standard (figure 18).

Figure 34 shows that after four days exposure of *Hibiscus macranthus* extract to TM4 Sertoli cells there was no significant effect on the protein content ( $P>0.05$ ).



**Figure 34:** Effect of *Hibiscus macranthus* on Sertoli cell protein content. The cells were cultured in DMEM/Ham 12 medium for 24h and then exposed to *Hibiscus macranthus* extract for 4 days. Result depicted as mean  $\pm$  SD (n=8).

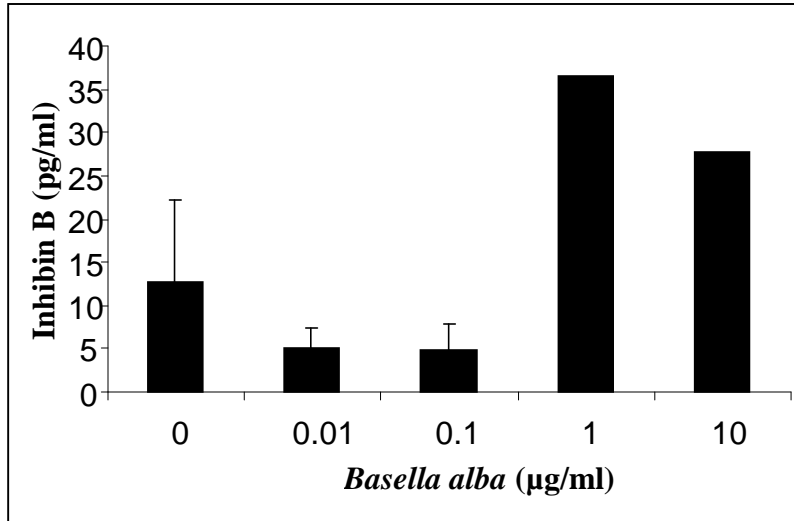
### **3.6 Effect of plant extracts on TM4 Sertoli cell inhibin B production**

#### **3.6.1 Effect of *Basella alba* on TM4 Sertoli cell inhibin B production**

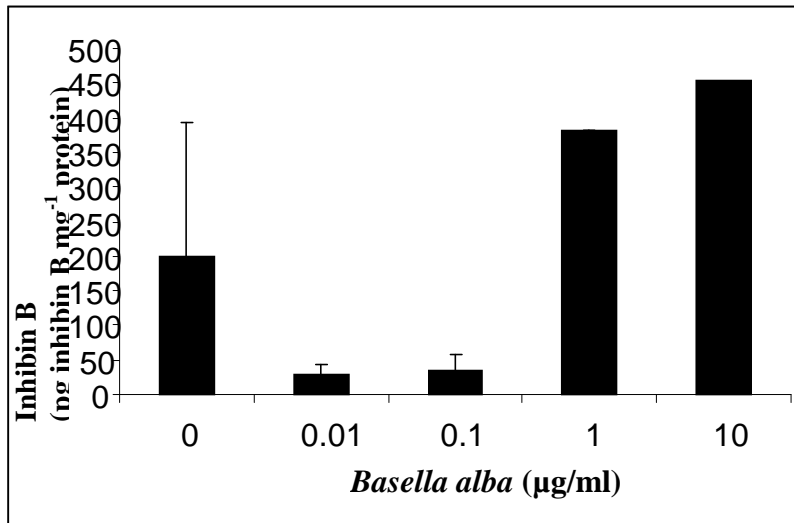
The cells were pre-incubated for 24h with the complete culture medium following which, they were exposed to the five different concentrations (0.01 -100 µg/ml) of *Basella alba* and incubated for another four days. Medium was then used to determine inhibin concentration (pg/ml; figure 35), whereas the cell were lysed to measure the total protein content. Inhibin B concentrations derived were then normalized to protein content in each well to obtain the amount of inhibin B (pg/mg protein; figure 36) that was produced. Unfortunately, the concentration of many samples was below the detection limit of 15.3 pg/ml inhibin-B and thus the amount of inhibin produced in TM4 cells exposed to 100 µg/ml of the *Basella alba* extract was not detected. The medium concentrations of the extracts (1-10 µg/ml) were represented in the results as n=1. Therefore, the data presented should be regarded as preliminary results that ought to be repeated after optimization of the assay procedure.

That said, Figure 35, shows a non-significant drop in inhibin B levels at low concentrations ( $P>0.05$ ). However, at higher concentrations of *Basella alba* (1-10 µg/ml;  $P>0.05$ ) an increase in the production of inhibin was noticed. The normalization to the amount of inhibin B (pg) per protein (mg) showed a very similar picture. Again, there was a rise in the production of inhibin after exposure to 1-10 µg/ml of the plant extract (figure 36).

This result indicated that higher concentrations of the *Basella alba* methanol extract may increase the production of inhibin B.



**Figure 35:** Effect of *Basella alba* on inhibin B production. The cells were cultured in DMEM/Ham 12 medium for 24h and exposed to *Basella alba* extract for 4 days. Result depicted as mean  $\pm$  SD (n=4). 1-10  $\mu\text{g/ml}$  of the extract represented as n=1.



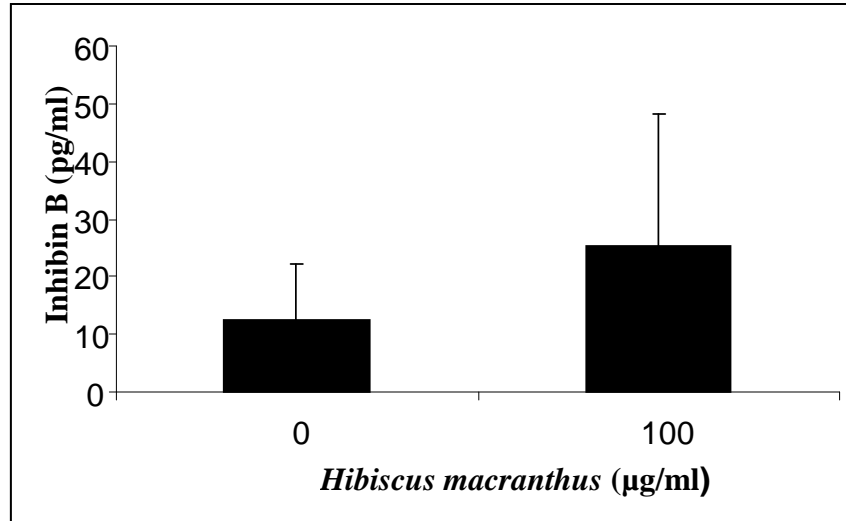
**Figure 36:** Effect of *Basella alba* on inhibin B production normalized to protein content. The cells were cultured in DMEM/Ham 12 medium for 24h and exposed to *Basella alba* extract for 4 days. Result depicted as mean  $\pm$  SD (n=4). 1-10  $\mu\text{g/ml}$  of the extract represented as n=1.



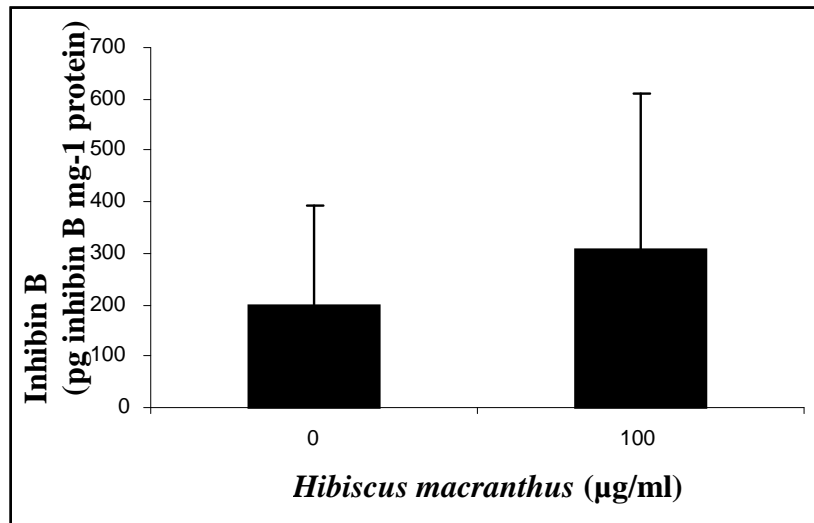
### **3.6.2. Effect of *Hibiscus macranthus* extract on TM4 Sertoli cell inhibin B production**

The cells were pre-incubated for 24h with the complete culture medium following which, they were exposed to the five different concentrations (0.01 -100 µg/ml) of *Hibiscus macranthus* and incubated for another four days. Medium was then used to determine inhibin concentration (pg/ml; figure 37), whereas the cell were lysed to measure the total protein content. Inhibin B concentrations derived were then normalized to protein content in each well to obtain the amount of inhibin B (pg/mg protein; figure 38) that was produced. Unfortunately, the concentration of many samples was below the detection limit of 15.3 pg/ml inhibin B and thus the amount of inhibin produced in TM4 cells exposed to 100 µg/ml of *Hibiscus macranthus* extract was only detected. Therefore, the data presented should be regarded as preliminary results that ought to be repeated after optimization of the assay procedure.

Compared to the negative control, figure 37, showed no significant difference in the production of inhibin B ( $P > 0.05$ ). The amount of inhibin B (pg) per protein (mg) also showed no significant difference (figure 38). However, both figures indicated that 100 µg/ml *Hibiscus macranthus* methanol extract may cause a slight increase in the production of inhibin B.



**Figure 37:** Effect of *Hibiscus macranthus* on Sertoli cell inhibin B production. The cells were cultured in DMEM/Ham 12 medium for 24h and exposed to *Hibiscus macranthus* extract for 4 days. Result represented as mean  $\pm$  SD (n= 6).



**Figure 38:** Effect of *Hibiscus macranthus* on Sertoli cell inhibin B production normalized to protein content. The cells were cultured in DMEM/Ham 12 medium for 24h and exposed to *Hibiscus macranthus* extract for 4 days. Result represented as mean  $\pm$  SD (n=6).

### **3.7 Effect of plant extracts on TM4 Sertoli cell morphology**

#### **3.7.1 Effect of *Basella alba* extract on TM4 Sertoli cell morphology**

##### **3.7.1.1 Morphology**

To study the effect of the different plant extracts on the morphology of the TM4 Sertoli cells, they were exposed to *Basella alba* (0.01- 100 µg/ml) for either 1 or 4 days after 1 day of pre-incubation and stained with H&E thereafter. Figures 39 and 40 shows that their morphology were not affected by the plant extract. The cells exposed to the different concentration of *Basella alba* extract show intact nucleus, prominent nucleoli and the presence of dividing cells with intact cell membranes. However, the nuclei of the cells exposed to the different concentrations of the plant extract showed more prominent nucleoli compared to the control.

##### **3.7.1.2 Mitotic Index**

After staining the cells with H&E, the number of dividing cells which represented different phases of mitotic cell division (prophase, anaphase, metaphase and telophase) were quantified per field of view, in order to determine the effect of *Basella alba* (0.01- 100 µg/ml) on TM4 Sertoli proliferation. Tables 7 and 8 showed that the different concentrations of *Basella alba* did not significantly ( $P>0.05$ ) alter the mitotic index after 1 or 4 days of exposure. Jang et al. (2002) showed that 100mM ethanol induced apoptosis in TM3 mouse Leydig cells. However, TM4 cell proliferation after 1 day exposure to 100mM ethanol showed a no significant reduction in its proliferation (Table 7). In contrast, exposure to 2% DMSO in complete culture medium caused a significant decrease ( $P<0.05$ ) after 4 days of exposure. However, it did not significantly affect the cell proliferation after 1 day of incubation.

### **3.7.2 Effect of *Hibiscus macranthus* on TM4 Sertoli cell morphology**

#### **3.7.2.1 Morphology**

In order to study the morphology of the TM4 Sertoli cells, they were exposed to *Hibiscus macranthus* for either 1 or 4 days after a 1 day pre-incubation and stained with H&E. From figures 41 and 42, the different concentrations of *Hibiscus macranthus* did not alter the normal morphology of the cells as there were intact cell membranes and the nuclei contain prominent nucleoli.

#### **3.7.2.2 Mitotic Index**

After staining the cells with H&E, the effect of different concentrations of *Hibiscus macranthus* (0.01- 100 µg/ml) on TM4 Sertoli was quantified in order to determine its effect on the cell proliferation. Tables 7 and 8 showed that the different concentrations of *Hibiscus macranthus* did not significantly ( $P>0.05$ ) have an effect on the mitotic index after 1 or 4 days exposure. 100mM ethanol, also did not significantly affect the cell proliferation after 1 or 4 days exposure of the plant extract. However, 2% DMSO in complete culture medium did not significantly affect the cell proliferation after 1 day incubation but did significantly decrease the cell proliferation after 4 days exposure ( $P<0.05$ ).

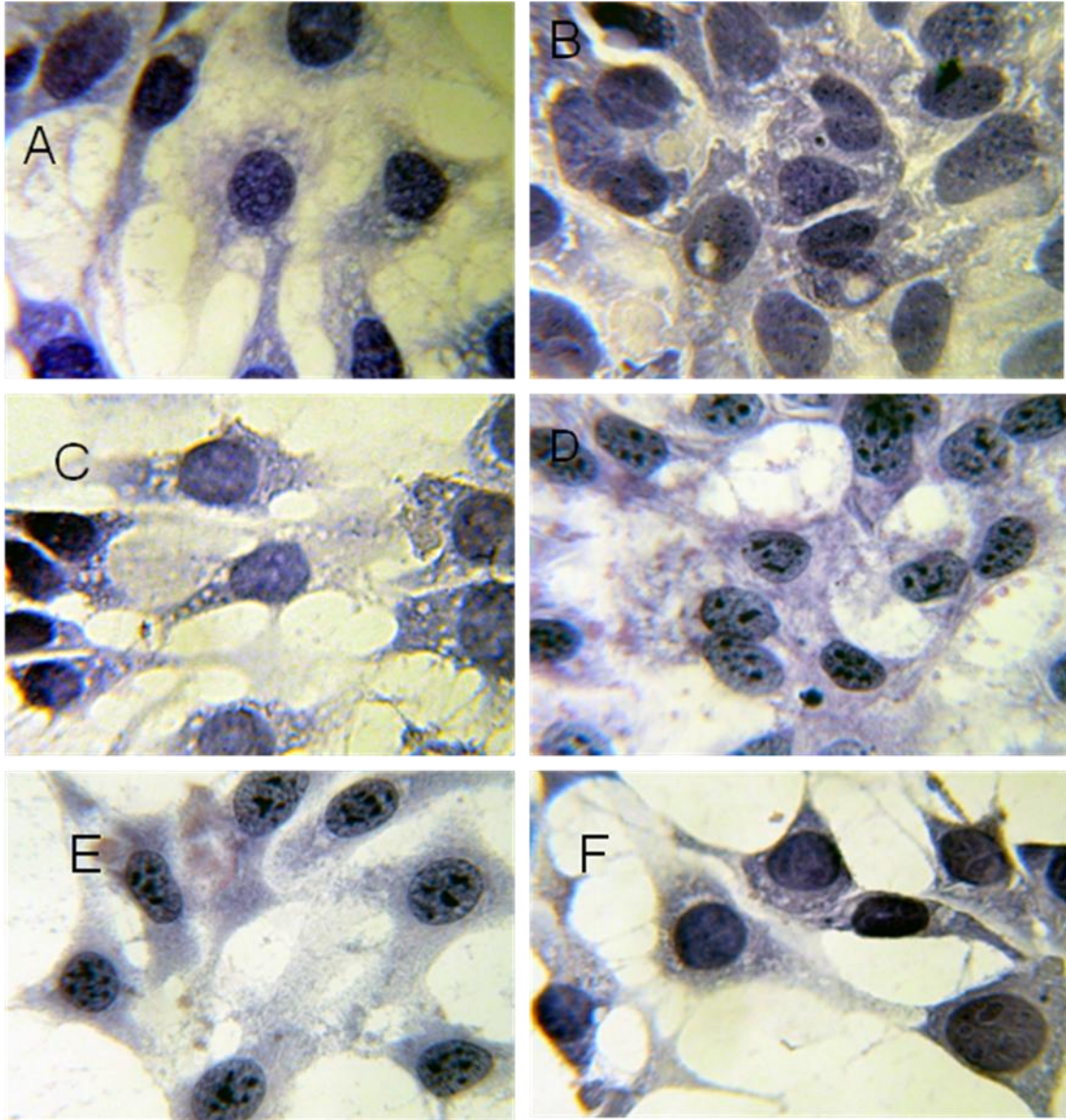


Figure 39: Effect of *Basella alba* on TM4 Sertoli cell morphology (H&E staining) after 1 day exposure. A) Control B) 1 µg/ml C) 10 µg/ml D) 100 µg/ml E) 2% DMSO F) 100mM ethanol. The cells in A-F show prominent nucleoli in the nucleus.



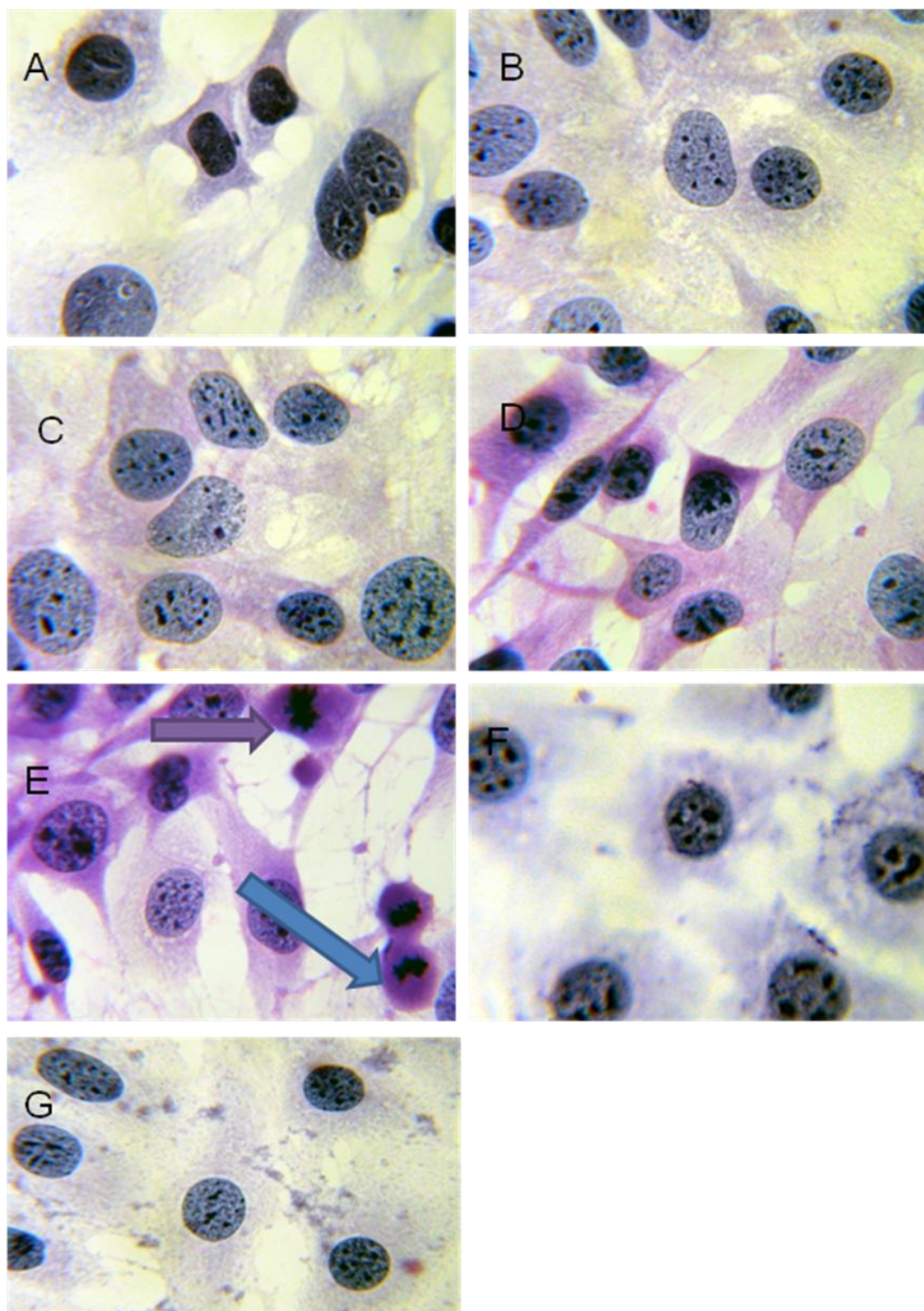


Figure 40: Effect of *Basella alba* on TM4 Sertoli cell morphology (H&E staining) after 4 days exposure. A) Control B) 0.01 µg/ml C) 0.1 µg/ml D) 1 µg/ml E) 10 µg/ml F) 100 µg/ml (G) 2% DMSO. Cells in A-G show no changes in their morphological appearance and prominent nucleoli in the nuclei were observed; blue arrow in E shows a dividing cell in anaphase; purple arrow in E shows a cell in metaphase stage.

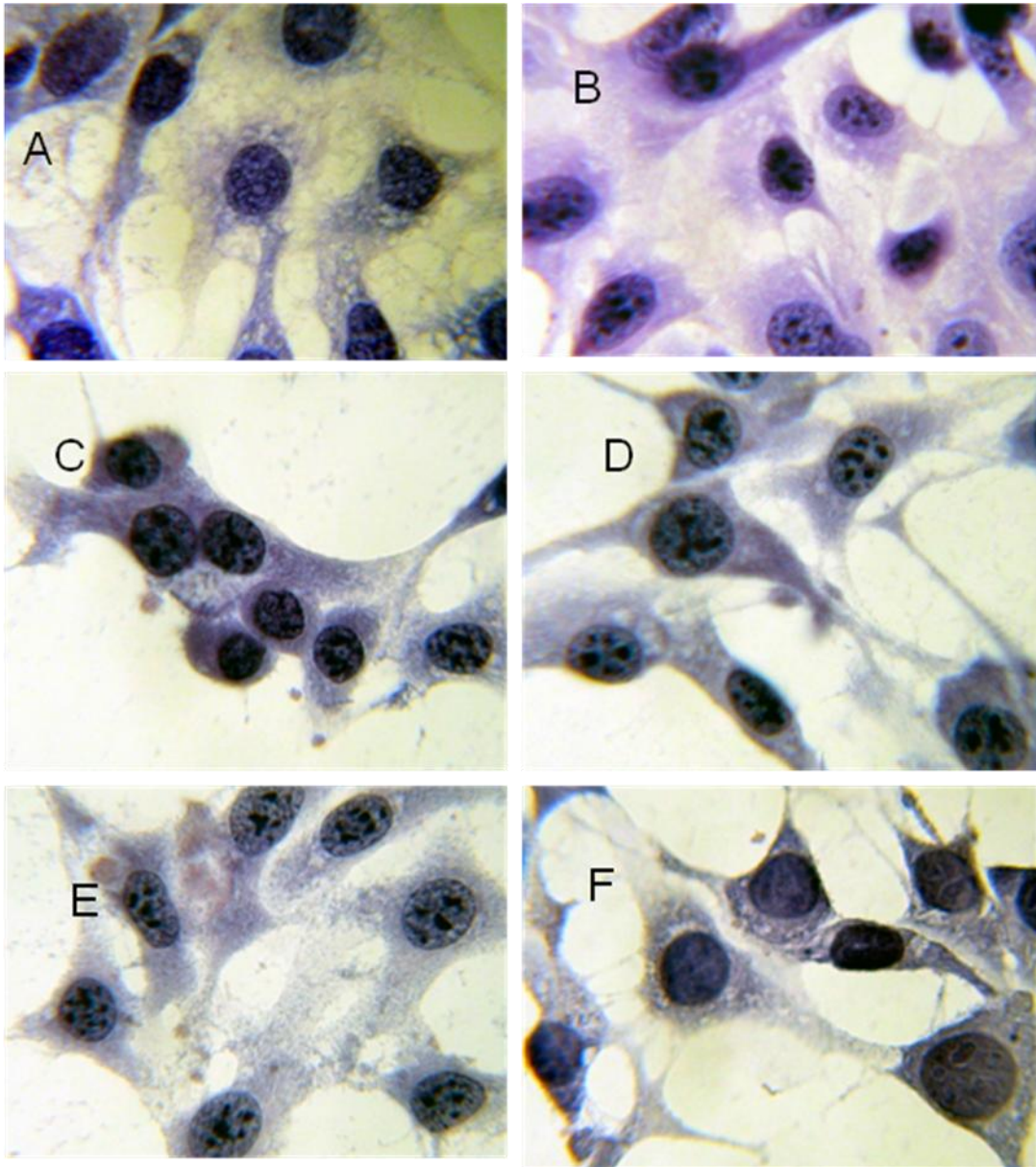


Figure 41: Effect of *Hibiscus macranthus* on TM4 Sertoli cell morphology (H&E staining) after 1 day exposure. A) Control B) 1  $\mu\text{g}/\text{ml}$  C) 10  $\mu\text{g}/\text{ml}$  D) 100  $\mu\text{g}/\text{ml}$  E) 2% DMSO F) 100mM ethanol. The cells in A-F show no changes in morphological appearance and prominent nucleoli observed in the nuclei.



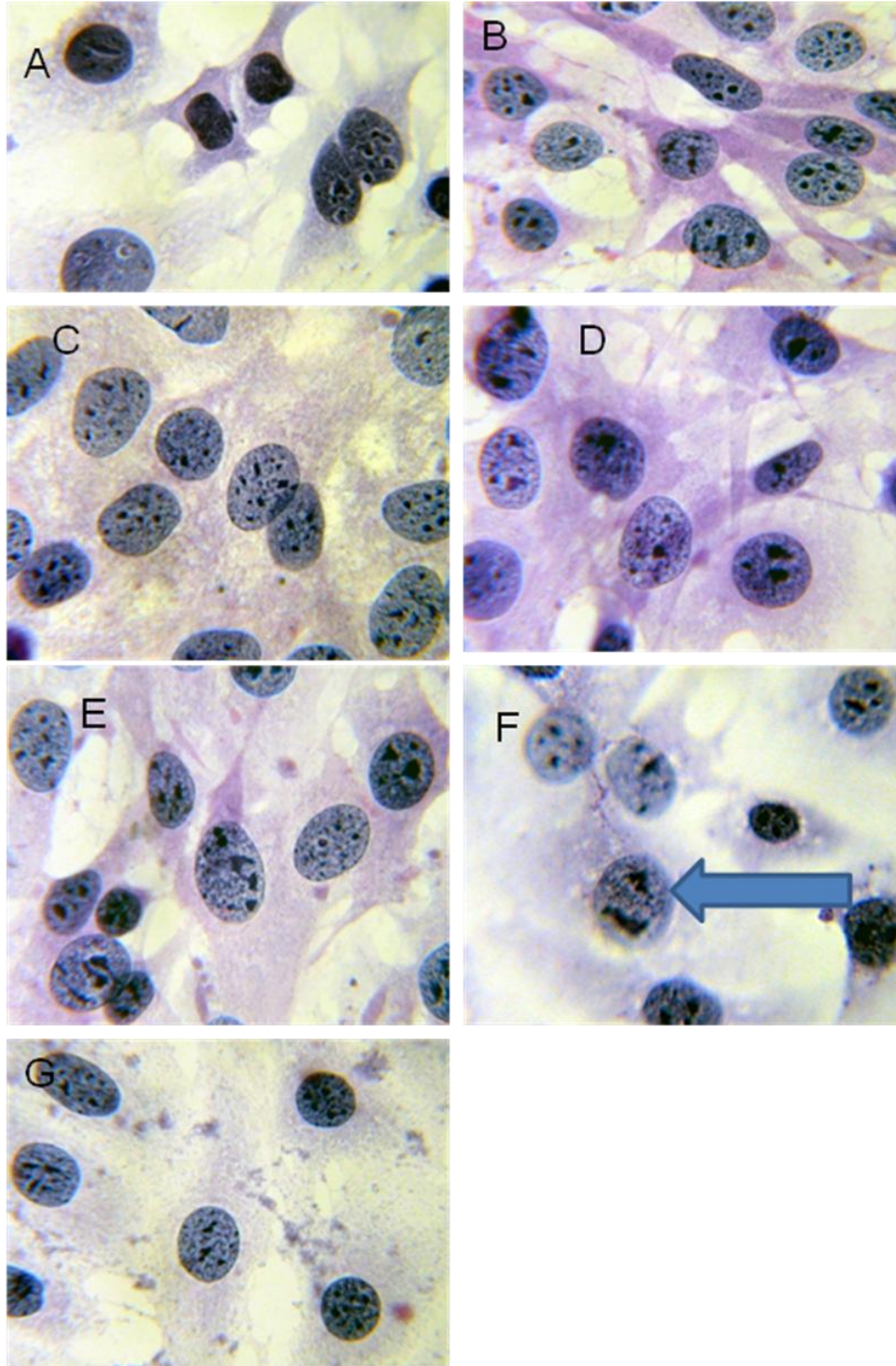


Figure 42: Effect of *Hibiscus macranthus* on TM4 Sertoli cell morphology (H&E staining) after 4 days exposure. A) Control B) 0.01 µg/ml C) 0.1 µg/ml D) 1 µg/ml E) 10 µg/ml F) 100 µg/ml (G) 2% DMSO. Cells in A-G show no changes in morphological appearance and had prominent nucleoli in the nuclei. Blue arrow in F shows a cell in early anaphase.



**Table 7:** Effect of *Basella alba* and *Hibiscus macranthus* on mitotic index after 1 day exposure

<b>Concentrations</b>						
<b>Extracts</b>	<b>0</b>	<b>1</b>	<b>10</b>	<b>100</b>	<b>Ethanol</b>	<b>DMSO</b>
<i>Basella alba</i>	10.3±4.1	8.6±4.8	11.3±8.5	6.3±2.5	7.1±4.1	4.4±5.6
<i>Hibiscus macranthus</i>	10.3±4.1	6.7±3.7	10±11.5	6.7±3.2	7.1±4.1	4.4±5.6

**Note:** Result was depicted as mean ± SD (n=6). Ethanol, 100mM in complete culture medium; DMSO, 2% in complete culture medium. No significant difference between respective concentrations of the plant extracts and control was observed.

**Table 8:** Effect of *Basella alba* and *Hibiscus macranthus* on mitotic index after 4 days exposure

<b>Concentrations</b>							
<b>Extracts</b>	<b>0</b>	<b>0.01</b>	<b>0.1</b>	<b>1</b>	<b>10</b>	<b>100</b>	<b>DMSO</b>
<i>Basella alba</i>	7.5±5.3	3.6±3	5.6±3.2	11.9±9	7.3±6.3	4±3.3	0.72±1.8*
<i>Hibiscus macranthus</i>	7.5±5.3	8.1±4.9	6.2±3.8	5.6±4.5	4.1±4.8	4.6±3	0.72±1.8*

**Note:** Result depicted as mean ± SD (n=6). DMSO, 2% in complete culture medium; \* indicated significant difference from the respective control values at  $P < 0.05$ .

### ***3.8 Effect of plant extract on TM4 Sertoli cell nuclear morphology***

#### ***3.8.1 Effect of *Basella alba* on TM4 Sertoli cell nuclear morphology***

##### ***3.8.1.1 Nuclear morphology***

In order to study the nuclear morphology of the TM4 Sertoli cells, they were exposed to different concentration of *Basella alba* (0.01-100 µg/ml) for either 1 or 4 days after a 1 day pre-incubation and stained with DAPI. From figures 43 and 44, the different concentrations of *Basella alba* did not alter the nuclear morphology of the cells, as there were very rare number of cells undergoing apoptosis.

##### ***3.8.1.2 Apoptotic Index***

TM4 Sertoli cell exposed to the different concentrations (0.01-100 µg/ml) for either one or 4 days were quantified to determine the effect of the plant extract on apoptosis. The characteristic features of a cell undergoing apoptosis which includes cell membrane blebbing, cell shrinkage, chromatin condensation and DNA fragmentation where used for this purpose. The results show that the percentage of apoptotic cells was less than 5% as it was very rare to find a cell undergoing apoptosis.

### ***3.9 Effect of *Hibiscus macranthus* on TM4 Sertoli cell nuclear morphology***

#### ***3.9.1 Nuclear morphology***

In order to study the nuclear morphology of the TM4 Sertoli cells, they were exposed to different concentration of *Hibiscus macranthus* (0.01-100 µg/ml) for either 1 or 4 days after a 1 day pre-incubation and stained with H&E staining. From figures 45 and 46, the different concentrations of *Hibiscus macranthus* did not alter the nuclear morphology of the cells, as there were no apoptotic bodies present.

#### ***3.9.2 Apoptotic index***

TM4 Sertoli cell exposed to the different concentrations (0.01-100 µg/ml) for either 1 or 4 days were quantified to determine the effect of the plant extract on apoptosis. The characteristic features of a cell undergoing apoptosis which includes cell membrane blebbing, cell shrinkage, chromatin condensation and DNA fragmentation where used for

this purpose. Result showed that the percentage of apoptotic cells was less than 5% of the cell population, as it was very rare to find a cell undergoing apoptosis.

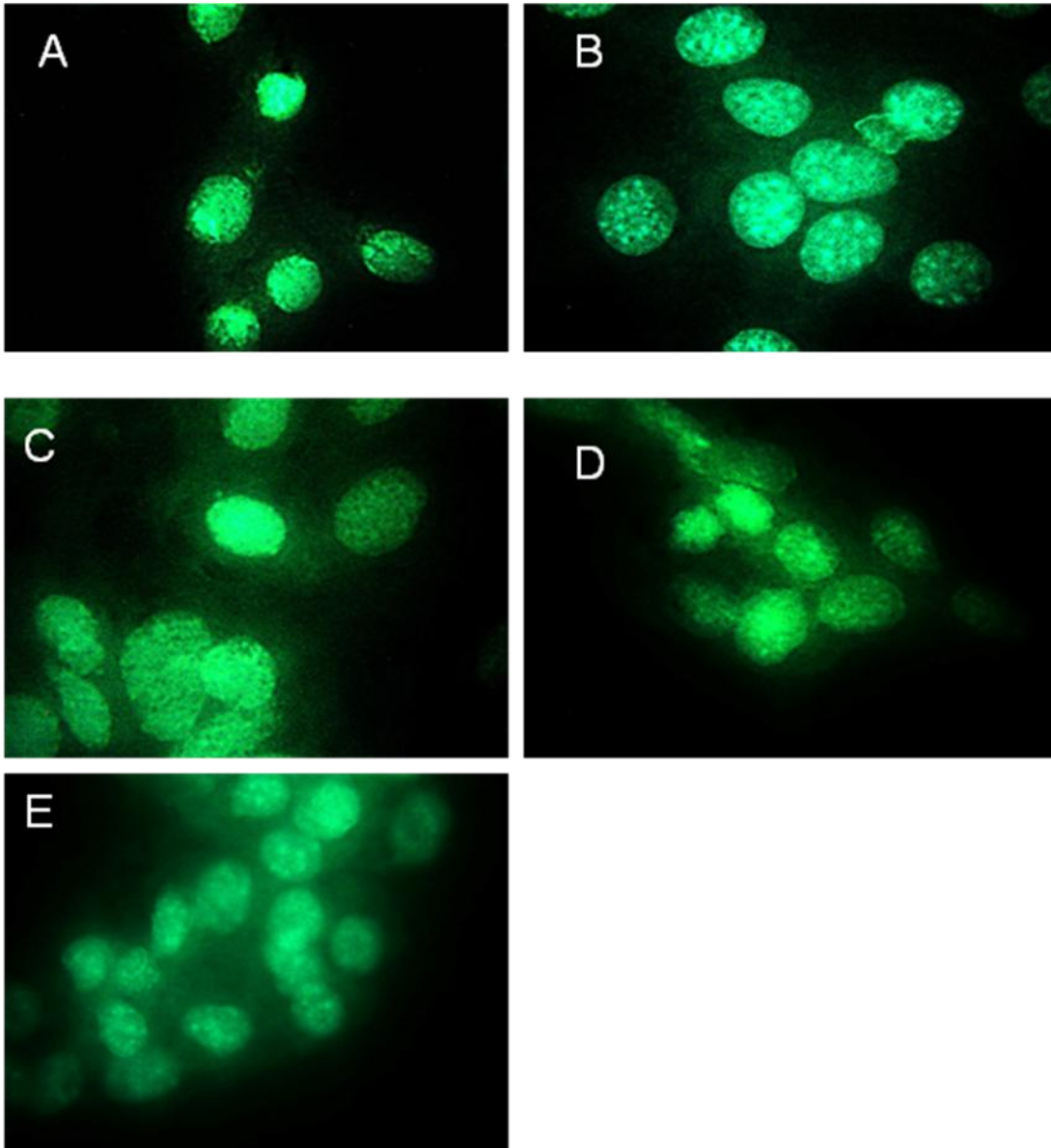


Figure 43: Effect of *Basella alba* on TM4 Sertoli cells nuclear morphology (DAPI staining) after 1 day exposure. A) Control B) 1 µg/ml C) 10 µg/ml D) 100 µg/ml E) 100mM ethanol. Cells in A-E show no cell undergoing apoptosis. Magnification X1000

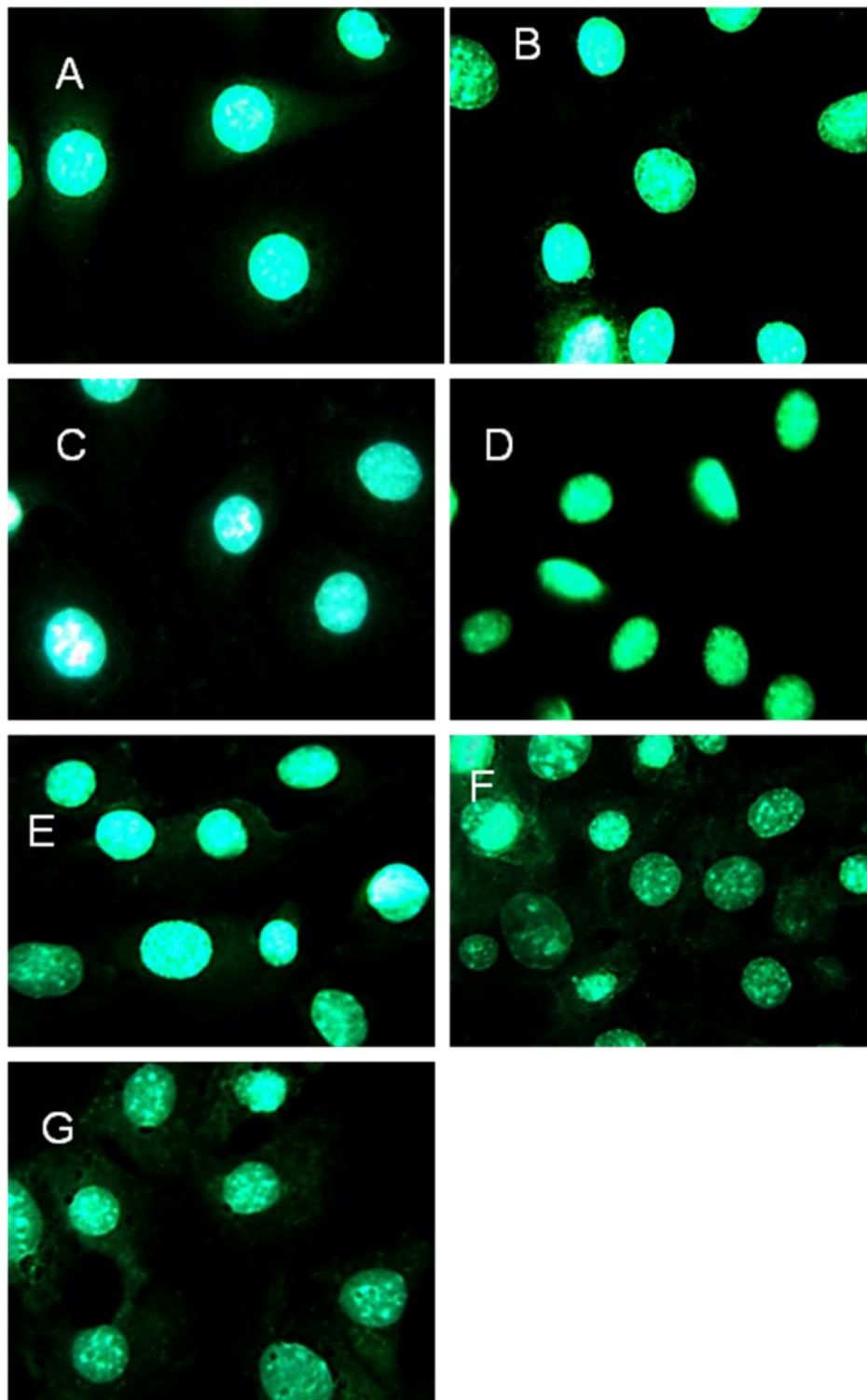


Figure 44: Effect of *Basella alba* on TM4 Sertoli cells nuclear morphology (DAPI staining) after 4 days exposure. A) Control B) 0.01  $\mu\text{g/ml}$  C) 0.1  $\mu\text{g/ml}$  D) 1  $\mu\text{g/ml}$  E) 10  $\mu\text{g/ml}$  F) 100  $\mu\text{g/ml}$  (G) 100mM ethanol. Cells in A-G show no cell undergoing apoptosis. Magnification X1000

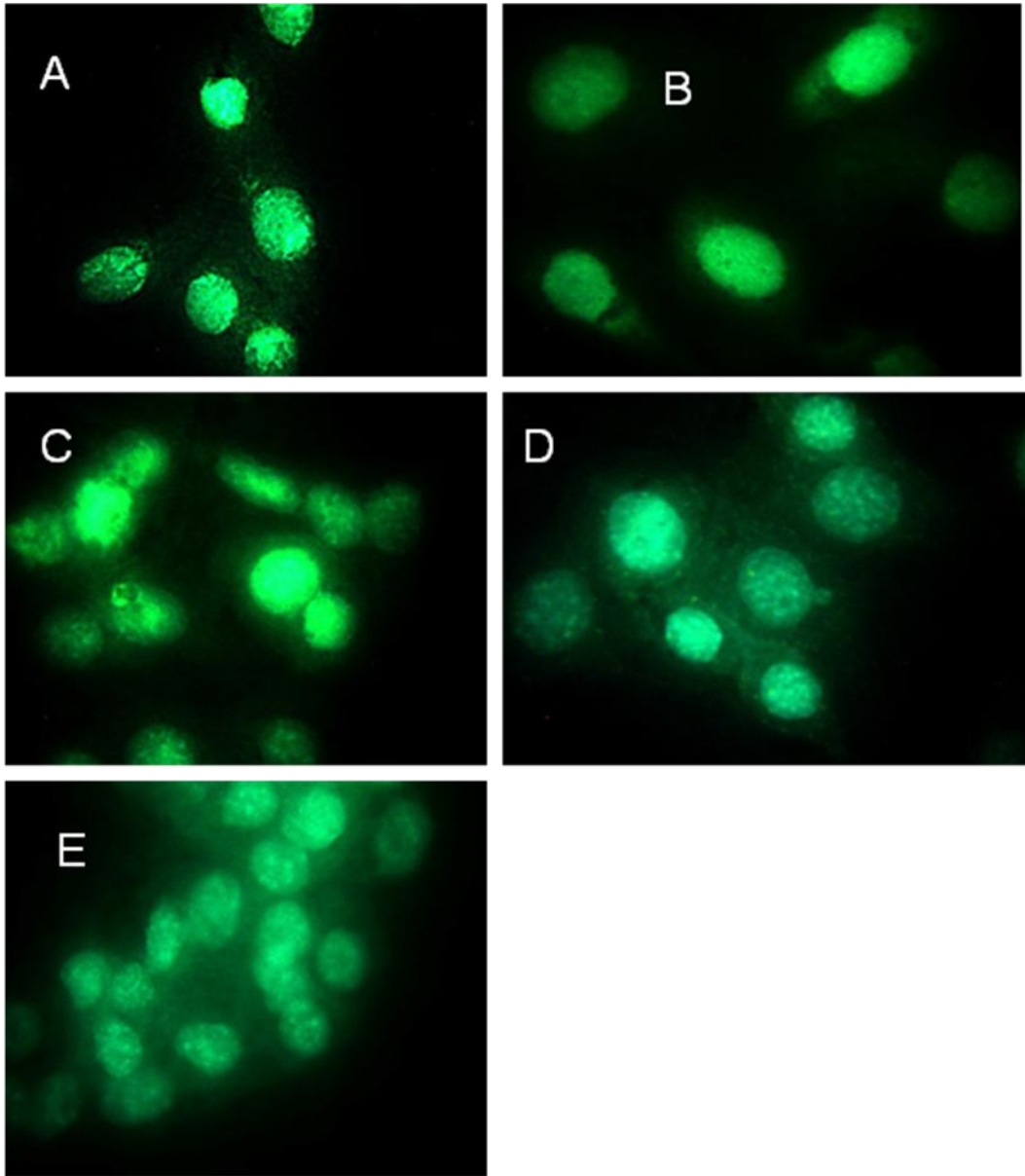


Figure 45: Effect of *Hibiscus macranthus* on TM4 Sertoli cells nuclear morphology (DAPI staining) after 1 day exposure. A) Control B) 1 µg/ml C) 10 µg/ml D) 100 µg/ml E) 100mM ethanol. Cells in A-E show no cell undergoing apoptosis. Magnification X1000

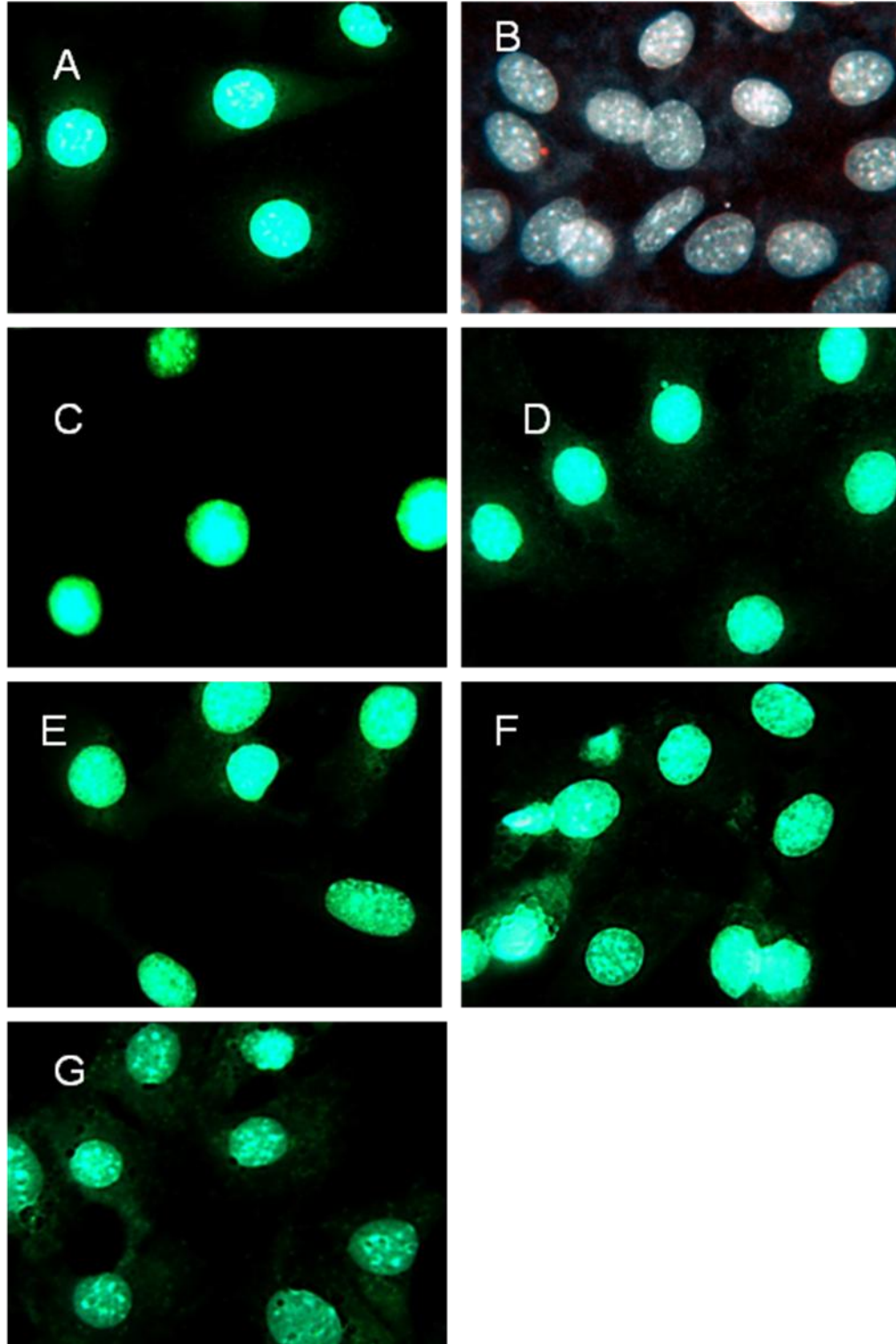


Figure 46: Effect of *Hibiscus macranthus* on TM4 Sertoli cells nuclear morphology (DAPI staining) after 4 days exposure. A) Control B) 0.01 µg/ml C) 0.1 µg/ml D) 1 µg/ml E) 10 µg/ml F) 100 µg/ml (G) 100mM ethanol. Cells in A-G show no cell undergoing apoptosis. Magnification X1000

## CHAPTER FOUR

### Discussion

In a previous study, the mixture of fresh or dried *Basella alba* and *Hibiscus macranthus* extracts given to mature male rats caused a significant increase in their body weight, seminal vesicles, serum level of testosterone as well as high activity of prostatic acid phosphatase (Moundipa et al., 1999). Adult rat and bull Leydig cells exposed to either *Basella alba* or *Hibiscus macranthus* showed *Basella alba* to possess androgenic properties as it increased testosterone levels while *Hibiscus macranthus* showed no androgenic effect but inhibited testosterone production at higher concentrations (Moundipa et al., 2005).

#### ***4.1 Effect of Basella alba and Hibiscus macranthus in TM4 Sertoli cells***

##### ***4.1.1 Cell viability***

Mitochondrial dehydrogenase activity is often used to measure Sertoli cell viability (Monsees et al., 2000) and Monsees et al. (1998) suggested the mitochondria of the Sertoli cell to be a possible target for toxicants.

*Basella alba* did not show any significant effect on the activity of the mitochondrial dehydrogenase in the Sertoli cells compared to the negative control. Thus, vitality of Sertoli cells was not influenced by *Basella alba* (figures 21, 22). Since MTT or XTT are reduced by the mitochondrial dehydrogenases, the activities of the enzymes are reflected and this showed that the said extract did not affect the enzymes activity.

The mitochondrion is the power house of a cell and where there is no influence of *Basella alba* on the mitochondrial enzymes, it thus imply that the enzyme activities in the cells may not be affected.

On the other hand, exposure of the Sertoli cells to low concentration of *Hibiscus macranthus* had no effect on the activity of the mitochondrial dehydrogenase of the Sertoli cells. However, at higher concentration there is a marked increase of more than 1

fold in the activity of the mitochondrial dehydrogenase which indicates that this plant extract may target the mitochondria of the Sertoli cell (figures 23, 24).

Compared to the XTT assay, the MTT assay seems here to be more sensitive in detecting the metabolic activity of the Sertoli cells. Using the MTT assay, cells exposed to *Hibiscus macranthus* showed a significant increase in the activity of the mitochondrial dehydrogenases from 0.1 µg/ml onwards, while the XTT assay showed the significant rise starting from 10 µg/ml of the plant extract. However, this difference in the sensitivity of both assays may be also a result of different passage of cells used.

The number of Sertoli cells per cm<sup>3</sup> of the testis ranges from 24-41million in rabbits and humans, respectively (Russell et al., 1990). An increase in the activity of mitochondrial dehydrogenase may result in increased cell proliferation, which could increase the interaction between the Sertoli cells and the developing germs cells. However, the mitotic index of the TM4 Sertoli cells exposed to both plant extract did not reveal any significant difference (Tables 7 and 8) compared to the negative control. This indicated that the observed enhanced conversion of the MTT/XTT dye was related to significantly higher mitochondrial dehydrogenase activities rather than increasing cell numbers.

The developing germ cells in the adluminal compartment of the seminiferous tubule rely on the Sertoli cells for nutritional and structural supports (Jegou, 1993; Russell & Griswold, 1993; Costa et al., 1998). Thus, an increased interaction between Sertoli cells and the germ cells provides the required support of the Sertoli cells to the germ cells and enhances spermatogenesis. Hence, *Hibiscus macranthus* may enhance the development of the germ cells through an increased interaction between the developing germ cells and the Sertoli cells.

An increased activity of the mitochondrial dehydrogenases could as well indicate a cellular stress reaction (Monsees et al., 2000) as seen in the Sertoli cells exposed to *Hibiscus macranthus*.



#### ***4.1.2 Lactate production***

The Sertoli cells play supportive role in the development of the germ cells and secrete among other substances lactate as stated in chapter 1.4.8. Isolated mammalian spermatogenic cells do not efficiently utilize glucose (Jutte et al., 1981; 1982; Mita & Hall, 1982). The Sertoli cells metabolize glucose to lactate and pyruvate, which then can be used as an effective energy source for the survival of the germ cells (Robinson & Fritz, 1981; Jutte et al., 1982; 1983). Lactate and pyruvate are secreted at a high rate and are required by the pachytene spermatocytes and round spermatids to carry out their energy requiring processes (Jutte et al., 1981, Mita & Hall, 1982).

In order to determine the actual biochemical activity in the cells, normalization of lactate production to the protein content is crucial. As the total protein content depicts the number of cell present. Doing so, this study showed that the amount of lactate accumulated as normalized to the protein content by the TM4 cells exposed to *Basella alba* was significantly increased (figure 26; table 5). Hence, *Basella alba* enhanced the production of lactate which is the preferred energy of the germ cells.

Sertoli cells metabolize glucose and excrete lactate and pyruvate in a molar ratio of 4-5: 1 (Jutte et al., 1983; Vivarelli et al., 1984) or 10: 1 (Le Gac et al., 1983). Under the control of the endocrine system primarily the FSH, insulin and insulin growth factor-1 convert glucose to lactate for the survival of the germ cells (Boussouar & Benahmed, 2004). However, the reason why Sertoli cells preferentially export lactate rather than pyruvate to the germ cells is not known.

Determination of lactate released from cultured Sertoli cells is a measure of increased cell metabolism, cell cytotoxicity or both (Boussouar & Benahmed, 2004). A study showed that pharmacological deprivation of lactate resulted in a decrease in the viability of male germ cells (Trejo et al., 1995). An excessive production of lactate may be toxic to the developing germ cells and spermatogenesis. The production of lactate during glycolysis produces also protons resulting in an acidification of the external environment of the

cells. This acidification could lead to germ cell death or reduce germ cell attachment (Monsees et al., 1998). The increased production of lactate by the cells exposed to *Basella alba* may be therefore also indicative of being toxic to the germ cells.

However, the highest production of lactate by the TM4 cells exposed to *Basella alba* is about 1 fold which is much less compared to gossypol (a polyphenol naturally occurring in cotton seed) which is a known toxicant to the developing germ cells (Monsees et al., 1998). Gossypol (3-6 $\mu$ M) significantly enhanced the secretion of lactate, decreased the viability of the Sertoli cells after 24 hours (Monsees et al., 1998) and caused cytoplasm vacuolization of the Sertoli cell (Zhuang et al., 1983).

The production of lactate and pyruvate in Sertoli cells is used as a specific sensitive marker of *in vitro* toxicity. Both 1,3- dinitrobenzene (1,3-DNB;  $10^{-6}$ - $10^{-4}$  M) and mono-(2-ethylhexyl) phthalate (MEHP;  $10^{-8}$ - $10^{-4}$  M) have been shown to increase the level of lactate by three to four folds in a dose related manner within 24 hour exposure to the Sertoli cells (Williams & Foster, 1998). Nitrobenzene ( $10^{-3}$  M) also increased the secretion of lactate and caused a gross morphological change in the Sertoli cells. Likewise, four isomers of dinitrotoluene (DNT) increased the level of lactate with increasing concentration of DNT. At 100  $\mu$ M, 3, 4- DNT caused morphological changes with extensive vacuolation of the Sertoli cells (Reader & Foster, 1990).

In this thesis, the *Basella alba* methanol extract caused a significant increase in lactate production. However, no changes in the morphology (figures 39, 40) and viability of the Sertoli cells were detected. Thus, the *Basella alba* extract can be described as a non toxic substance to the Sertoli cells when compared to the effects produced by the known toxicants mentioned above.

On the contrary, Sertoli cells exposed to *Hibiscus macranthus* revealed a significant decrease in the production of lactate at 0.01  $\mu$ g/ml (figure 28, table 5). In summary, *Hibiscus macranthus* did not have any major effect on the production of lactate in Sertoli

cells, whereas *Basella alba* led to a huge and significant increase in the secretion of this carbohydrate.

#### **4.1.3 Pyruvate production**

Systemic glucose is taken up by the Sertoli cells through the specific glucose transporter GLUT1 and processed into lactate during glycolysis (Boussouar & Benahmed, 2004). Lactate leaves the Sertoli cells by monocarboxylate transporter MCT1, is taken up by the meiotic germ cells through their specific monocarboxylate transporters MCT 2 and is then oxidized to pyruvate (Boussouar & Benahmed, 2004).

Like lactate, pyruvate is a major product of spent medium of Sertoli cells. Pyruvate and lactate production by the Sertoli cells are markedly stimulated by FSH (Jutte et al., 1983). Irrespective of the presence of lactate, pyruvate supports high rate of protein synthesis in isolated spermatocytes and spermatids during short term incubation (Jutte et al., 1983). In this study, pyruvate accumulation was significantly increased in the cells exposed to *Basella alba* (figure 30; table 6). An elevated level of pyruvate provides the developing germ cell with the required energy, hence spermatogenesis may be enhanced.

The production of pyruvate and lactate by Sertoli cells and the ratio of pyruvate to lactate may be dependent on conditions that have effect on cellular  $\text{NAD}^+/\text{NADH}$  ratio through the modulation of processes such as glucose utilization and citric acid activity (Jutte et al., 1983).

In contrast, exposure of the Sertoli cells to *Hibiscus macranthus* showed no significant effect on the production of pyruvate except at 100  $\mu\text{g}/\text{ml}$  of the extract where there was a significant increase (figure 31). Likewise, when compared to the protein content, pyruvate showed no significant effect but at 100  $\mu\text{g}/\text{ml}$ , where there was an increase (figure 32; table 6).

Isomers of dinitrotoluene (DNT) increased the level of pyruvate production in a dose related manner with extensive vacuolization of the Sertoli cell (Reader & Foster, 1990). Nitrobenzene also increased the secretion of pyruvate and caused a significant alteration in the morphology of the Sertoli cells (Allenby et al., 1990). The testicular manifestations of the exposure of toxicant to the Sertoli cells includes vacuolization, apical sloughing and shedding of germ cells, germ cell necrosis, decreased seminiferous tubule fluid secretion, changes in distribution, quantity or biochemical properties of testicular components, interstitial release of Sertoli cell proteins (Russell & Griswold, 1993).

In this study, *Basella alba* methanol extract, which increased the production of pyruvate in a concentration dependent manner did not bring about the testicular morphological manifestations mentioned above. Thus, it can be classified as non-toxic to the Sertoli cells.

*Hibiscus macranthus* methanol extract can also be said to be non-cytotoxic to the Sertoli cell as the morphology of the Sertoli cell was not altered even though the level of pyruvate at 100µg/ml was significantly increased.

#### **4.1.4 Inhibin B**

Sertoli cells produce and secrete inhibin into the circulation, which regulates the secretion of FSH by the anterior pituitary hormone through a negative feedback loop (De Krester & Robertson, 1989; Guyton & Hall, 2000). Inhibin presented as two forms and includes inhibin A and inhibin B. Inhibin B was shown to be the important physiological molecular form in men (Illingworth et al., 1996) and rats (Sharpe et al., 1999).

Inhibin is composed of two dissimilar disulphide-linked subunits ( $\alpha$  and  $\beta$  subunits). The  $\beta$ -subunits consist of  $\beta$ A and  $\beta$ B forms. The  $\alpha$ -subunit is linked to either  $\beta$ A or  $\beta$ B subunit to form inhibin A or inhibin B respectively (De Krester & McFarlane, 1996).

In this study, the TM4 Sertoli cells exposed to the 0.01- 10 µg/ml of *Basella alba* showed no significant difference compared to the control ( $P>0.05$ ; figures 35, 36). However, at 1-

10µg/ml, the production was increased compared to the lower concentrations ( $P>0.05$ ; figures 35, 36).

TM4 Sertoli cells exposed to 100 µg/ml of *Hibiscus macranthus* also showed an increased production of inhibin B. However, the increase in inhibin B production was not significant ( $P>0.05$ ; figures 37, 38). The cells exposed to the extract of *Hibiscus macranthus* (0.01-10 µg/ml) showed no detectable production of inhibin B (figures 37, 38).

In this study, 1ml of the various concentrations of either *Basella alba* or *Hibiscus macranthus* extracts was used to incubate the cells for 4 days. As a result, the inhibin production of most samples was below the detection limit. In the future, a smaller medium volume will be used, which should increase the concentration of inhibin. Also, an increased number of cells per incubation time might be helpful to optimize the sensitivity this assay.

Both *Basella alba* and *Hibiscus macranthus* methanol extract may increase the production of inhibin B as shown in figures 35 to 38. However, due to the experimental difficulties, this should be regarded as a preliminary study. Therefore, a further investigation into the effect of both plant extracts in the production of inhibin B is recommended. A repeat of the experiment was not possible due to time- and the financial involvements of sending the samples again to the Klinik Fuer Dermatologie Universitaetsklinikum Jena, Germany for analysis.

#### **4.1.5 Morphological studies**

The results obtained from TM4 Sertoli cells exposed to *Basella alba* methanol extract showed that the total mitotic index was not significantly affected (tables 7 and 8) after 1 or 4 days of exposure. H&E staining also showed that there was no alteration in the cell structure (figures 39, 40).

Likewise, the total mitotic index of the cells exposed to *Hibiscus macranthus* methanol extract was not significantly influenced (tables 7 and 8). From figures 41 & 42, the morphology of the TM4 Sertoli cells was also not affected.

The Sertoli cell is presented with a nucleus with different shapes, a relative homogenous nucleoplasm and a tripartite nucleolus. This study showed that the mitotic indices of TM4 Sertoli cells exposed to the positive controls, 2% DMSO or 100 mM ethanol, were not significantly affected after 1 day of exposure. This may indicate that the exposure time was too short to induce any visible morphological effect. However, there was a significant decrease in the mitotic index after 4 days of exposure to DMSO. This showed the cytotoxic effect of DMSO at this concentration and confirmed also the results obtained by the XTT and MTT assays.

#### **4.1.6 Nuclear studies**

Apoptosis is a biological process that plays an important role in maintaining normal development and tissue homeostasis. A cell undergoing apoptosis is characterized morphological changes, which includes cell membrane blebbing, cell shrinkage, chromatin condensation and DNA fragmentation (Savill & Fadok, 2000; Kerr et al., 1972). In the testis, 75% of the germ cells are reduced by spontaneous apoptosis (Allan et al., 1992). However, excessive or inadequate apoptosis of the testicular cells will result in abnormal spermatogenesis or testicular tumors (Lin et al., 1997).

To maintain homeostasis in the adult human body, approximately 10 billion cells are produced daily to replace the cells dying as a result of apoptosis (Renehan et al., 2001)

with the number of cells increasing significantly with increased apoptosis during normal development, aging or diseases (Susan, 2007).

The results obtained from the TM4 Sertoli cells exposed to *Basella alba* showed mainly a normal appearance of the nucleus with no cell membrane blebbing, cell shrinkage or chromatin condensation (figures 43, 44). The number of cells undergoing apoptosis was very small, well below the 5% range. This is comparable to the apoptosis rate in the negative control. Also, exposure of the *Hibiscus macranthus* methanol extract to the TM4 Sertoli cells did not indicate an excessive presence of apoptotic bodies or cells undergoing apoptosis (figures 45, 46).

Ethanol was used to induce apoptosis in Leydig cell cultures (Jang et al., 2002). However, this thesis showed that TM4 Sertoli cells exposed to 100mM ethanol for 4 days did not have elevated numbers of cells undergoing apoptosis.

Assessment of the cell viability through MTT and XTT assay showed no cytotoxic effect of either *Basella alba* or *Hibiscus macranthus* methanol extracts on TM4 Sertoli cells. In addition, both extracts showed no characteristic changes in the morphology of the TM4 Sertoli cells. Hence, these results demonstrated that both *Basella alba* and *Hibiscus macranthus* methanol extracts did not exhibit a cytotoxic effect on the TM4 Sertoli cell. In addition, apoptotic bodies, which are critical morphological criteria for apoptosis, were very rare in the Sertoli cells exposed to the plant extracts.

## 4.2 Conclusion

In this study, the *in vitro* effect of methanol extracts of both *Basella alba* and *Hibiscus macranthus* were investigated on the TM4 Sertoli cell line. The main outcome is summarized in the following table:

**Table 9:** Summary of the most important findings of this investigation

Objectives	Outcome
<p><b>Cytotoxicity study</b></p> <ul style="list-style-type: none"> <li>To determine the effect of <i>Basella alba</i> and <i>Hibiscus macranthus</i> on Sertoli cell viability</li> </ul>	<ul style="list-style-type: none"> <li><i>Basella alba</i> had no significant effect on the cell viability</li> <li><i>Hibiscus macranthus</i> significantly enhanced mitochondrial dehydrogenase activities of Sertoli cells at higher concentrations.</li> </ul>
<p><b>Biochemical Secretion</b></p> <ul style="list-style-type: none"> <li>To determine the effect of both plant extracts on the secretions of Sertoli cells using lactate, pyruvate, protein content and inhibin.</li> </ul>	<ul style="list-style-type: none"> <li><i>Basella alba</i> significantly increased the production of lactate and pyruvate. However, there was a significant decrease in the protein content at higher concentration of the extract. It may also increase the secretion of inhibin at higher concentration.</li> <li><i>Hibiscus macranthus</i> had no significant effect on the production of lactate, protein content and pyruvate. Only at the highest concentration of the plant extract the production of pyruvate was significantly increased. <i>Hibiscus</i></li> </ul>



	<i>macranthus</i> extract may also increase production of inhibin.
<p><b>Morphological studies</b></p> <ul style="list-style-type: none"> <li>To determine the effect of both plant extracts on the morphology of the cell and the nuclei using H&amp;E and DAPI staining techniques.</li> </ul>	<ul style="list-style-type: none"> <li>The cell morphology was not altered by <i>Basella alba</i>. It also had no significant effect on the mitotic and apoptotic indices of the cell.</li> <li><i>Hibiscus macranthus</i> did not alter the morphology of the cells and had no significant effect on the apoptotic and mitotic indices.</li> </ul>

In summary, regarding the Sertoli cell, both plant extracts are not cytotoxic with respect of the methods and period investigated in this study. This supports the results obtained from previous *in vivo* studies (Moundipa et al., 1999; 2005) which showed no general toxic effect.

Previous studies have indicated the androgenic effect of *Basella alba* in increasing testosterone production in rat and bull Leydig cells while *Hibiscus macranthus* showed no androgenic effect (Moundipa et al., 1999; 2005). This thesis indicated that both *Basella alba* (carbohydrates, inhibin) and *Hibiscus macranthus* (mitochondria, inhibin) may have an overlapping effect in enhancing the functions of the Sertoli cell. This in turn might explain the positive *in vivo* effect of the combined plant extracts on rat spermatogenesis (Moundipa et al., 1999).

Sertoli cells plays a crucial role in spermatogenesis of which its importance cannot be overemphasized. As *Basella alba* and *Hibiscus macranthus* plant extracts enhanced the functions of the Sertoli cell through stimulating its secretions pattern, spermatogenesis in turn may be improved.

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