Investigations on the *in vitro* effects of aqueous *Eurycoma longifolia* Jack extract on male reproductive functions

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DECLARATION

I declare that the "**Investigations on the *in vitro* effects of aqueous *Eurycoma longifolia* Jack extract on male reproductive functions**" is my own work, that it has not been submitted for any degree or examination at any other university and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

……………………………..   …………………..
FULL NAME   **DATE**

…………………………….
SIGN

DEDICATION

This thesis is dedicated to my loving mother Patricia and to the Almighty Father.

"For all things were created by Him, and all things exist through Him and for

Him. To God be the glory for ever! Amen."

Romans 11: 36

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To the rest of my family and friends, thank you for your support and love.

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PUBLICATIONS

Part of this thesis has been published, submitted for publication or presented at conferences:

Papers:

1. Erasmus N, Solomon MC, Fortuin KA, Henkel RR (2012). Effect of *Eurycoma longifolia* Jack (Tongkat Ali) extract on human spermatozoa *in vitro*. Andrologia 44: 308-314.

2. Solomon MC, Erasmus N, Henkel RR (2013). *In vivo* effects of *Eurycoma longifolia* Jack (Tongkat Ali) extract on reproductive functions in the rat. Andrologia (submitted).
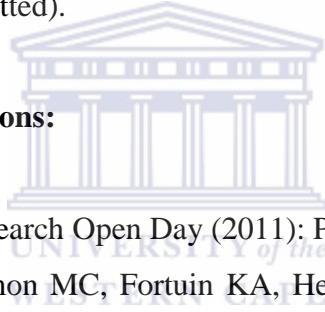
Abstracts and Presentations:

- UWC Science Research Open Day (2011): Poster Presentation
  Erasmus N, Solomon MC, Fortuin KA, Henkel RR: Effect of *Eurycoma longifolia* Jack (Tongkat Ali) extract on human spermatozoa *in vitro*, Bellville, South Africa; abstract book p. 33.

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  Erasmus N, Solomon MC, Fortuin KA, Henkel RR: Effect of aqueous extract of *Eurycoma longifolia* Jack (Tongkat Ali) on human spermatozoa *in vitro*. Stellenbosch, South Africa, abstract book p. 54.

- 40th Physiology Society of Southern Africa (2012): Oral Presentation
  Solomon M, Erasmus N, Henkel RR: The effect of *Eurycoma longifolia* (Tongkat Ali) on reproductive functions in the rat. Stellenbosch, South Africa, abstract book p. 53.

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- 7th European Congress of Andrology (2012): Oral Presentation
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Investigations on the *in vitro* effects of aqueous *Eurycoma longifolia* Jack extract on male reproductive functions

KEY WORDS

Eurycoma longifolia Jack (Tongkat Ali)

Spermatozoa

Motility

Viability

Acrosome Reaction

Reactive Oxygen Species (ROS)

Mitochondrial Membrane Potential

DNA fragmentation

TM3-Leydig cells

TM4-Sertoli cells

Cell Proliferation

Testosterone

Pyruvate

Cytotoxicity

ABSTRACT

Introduction

Eurycoma longifolia (Tongkat Ali; TA) is a Malaysian shrub used to treat various illnesses including male infertility. Considering that TA is also used to improve male fertility and no report regarding its safety has been published, this study investigated the effects of a patented, aqueous TA extract on various sperm and testicular functions.

Materials and Methods

This study encompasses two parts (part 1: on spermatozoa; part 2: on TM3-Leydig and TM4-Sertoli cells).

Part 1: Semen samples of 27 patients and 13 fertile donors were divided into two groups, washed and swim-up prepared spermatozoa, and incubated with different concentrations of TA (1, 10, 20, 100, 2000 µg/ml) for 1 hour at 37°C. A sample without addition of TA served as control. After incubation with TA, the following parameters were evaluated: viability (Eosin-Nigrosin test), total and progressive motility (CASA), acrosome reaction (triple stain technique), sperm production of reactive oxygen species (ROS; dihydroethidium test; DHE), sperm DNA fragmentation (TUNEL assay) and mitochondrial membrane potential ($\Delta\psi$m) (Depsipher kit).

Part 2: TM3-Leydig and TM4-Sertoli cells incubated with different concentrations of TA (0.4, 0.8, 1.6, 3.125, 6.25, 12.5, 25, 50 µg/ml) and control (without extract) for 48 and 96 hours. After incubation with TA, the following parameters were evaluated: viability (XTT), cell proliferation (protein assay), testosterone (testosterone ELISA test) and pyruvate (pyruvate assay).

Results

Part 1: For washed spermatozoa, significant dose-dependent trends were found for viability, total motility, acrosome reaction and sperm ROS production. However, these trends were only significant if the highest concentrations were included in the calculation. In the swim-up spermatozoa, ROS production of spermatozoa showed a biphasic relationship with its lowest percentage at 10 µg/ml, yet, no significance could be observed (P=0.9505). No influence of TA could be observed for sperm DNA fragmentation nor $\Delta\psi$m.

Part 2: The viability rates and protein production of TM3-Leydig and TM4-Sertoli cells at 48-hour exposure to TA showed increases whereas at 96-hour incubation periods viability and protein production declined especially as from concentration 25 µg/ml TA. Similar results could be seen for TM4-Sertoli cells pyruvate production. The testosterone production at 48-hour exposure marginally increased (P=0.0580) at the highest (50 µg/ml) concentration of TA. However, at 96-hour exposure to TA the testosterone production significantly (P=0.0065) increased. It is also apparent that after 96 hours the concentration of testosterone has increased [12 x 10^{-4} ng/ml] when compared to 48-hour exposure [6 x 10^{-7}ng/ml] of Tongkat Ali.

Conclusion

Part 1: Results indicate that the Tongkat Ali extract has no deleterious effects on sperm functions at therapeutically used concentrations (<2.5 µg/ml).

Part 2: The cytotoxic effect of TA are only presented at higher concentration from 25 µg/ml. TM3-Leydig cells appears to be more resilient than TM4-Sertoli cells in viability and protein production yet at prolonged periods of exposure it is detrimental. Testosterone production only increases after 96 hours exposure to TA.
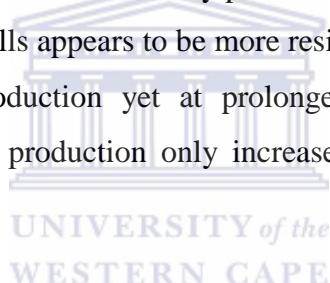
Chapter 1: Introduction

1.1 Overview of Male Reproductive System

The male reproductive system has three main functions: (i) hormonal regulation of reproductive functions consisting of endocrine function, production and release of hormones such as Gonadotropin releasing hormone (GnRH), Follicle-stimulating hormone (FSH), Luteinizing hormone (LH), testosterone (androgen) and inhibin; (ii) spermatogenesis, which is the formation of male gametes in the form of spermatozoa; and (iii) performance of the male sexual act in delivery of spermatozoa contained within semen into the female reproductive tract, which allows them to access the mature ova.

1.2 Production and maturation of spermatozoa in testes

The testes are located in the scrotum (Figure 1), a loose pouch of skin and muscles that acts not only as a holder, but also as a "climate control system" for the testes. For normal sperm development, the testes must be at a temperature slightly cooler (35ºC) than body temperature. Special muscles, dartos and cremaster, allow it to contract and relax, moving the testicles closer to the body for warmth or farther away from the body to cool the temperature (Ivell, 2007).

Each testis consists of about 250 lobules containing one to four seminiferous tubules in a human male adult and comprises 80 % of the mass of the testis (Figure 2). Between the tubules, there are small groups of hormone-producing Leydig cells. The tubular wall consists of a thin tunica propria; largely embody myoid cells, a basal membrane, and the germinal epithelium, which comprise of spermatogenic cells and Sertoli cells. The latter are cells with a complex function in the maturation of spermatozoa (Bart et al., 2002) as well as the growth and regulation of spermatogenesis.

Spermatogenesis is the process in which spermatozoa are produced and matured within the seminiferous tubules (Hess and De Franca, 2008; Mathur and D'Cruz, 2011).
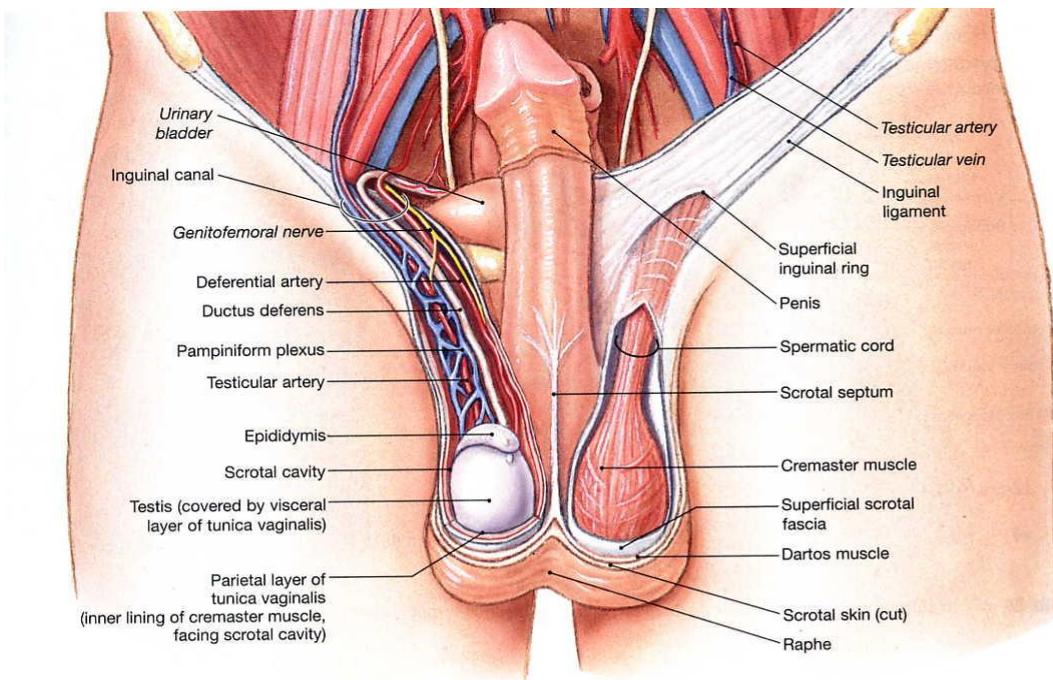
Figure 1: Anterior view of the male reproductive anatomy and structures indicating the specialized layers, blood vessel and muscles (Martini, 2004).
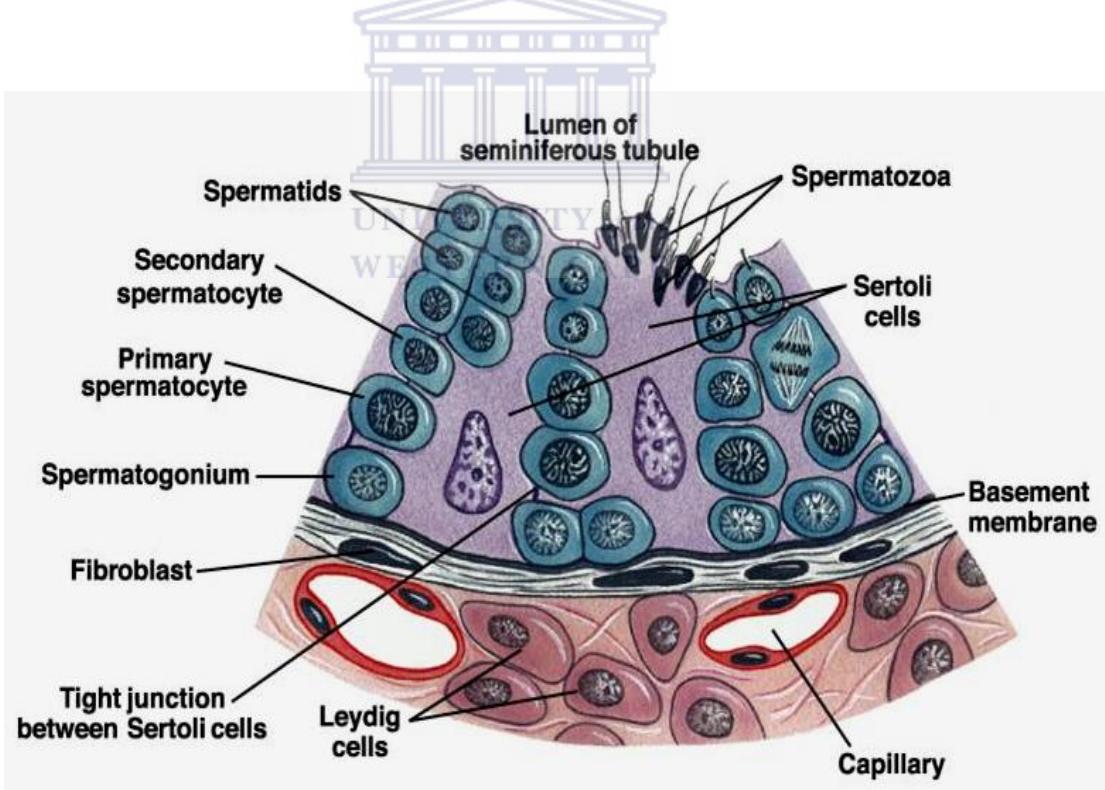
Figure 2: Cross section of seminiferous tubules illustrating the different stages a spermatozoon undergoes with the presence of Sertoli cells and Leydig cells (Pearson education 2004).

1.2.1 Spermatogenesis

Spermatogenesis is a series of cellular events involving the formation of mature sperm (spermatozoa) from germ cells (spermatogonia). The spermatogenic process takes place in the germinal epithelium, which is, separated into an apical and basal compartment by tight junctions formed between the Sertoli cells. These tight junctions form the blood-testes barrier (BTB) and function to protect developing germ cells against harmful agents and immunological influences (Kato et al., 2009). Protection is necessary because many agents can disturb the delicate process of meiotic cell division (Bart et al., 2002).

In the human, spermatogenesis takes approximately 75 days (Clermont and Trott, 1969; Clermont and Antar, 1973; Andersen Berg et al., 1990; Russell et al., 1990; Rosiepen et al., 1994, 1997; Franca and Godinho, 2003). It is a continuous process producing up to 120 million spermatozoa per day as from the onset of puberty in human males (Clermont and Trott, 1969; Clermont and Antar, 1973).

Cheng and Mruk (2012) summarized the spermatogenic process as follows: spermatogonial renewal and proliferation via mitosis and differentiation; cell cycle progression from type B spermatogonia to preleptotene spermatocytes in the basal compartment (Figure 3); cell cycle progression from zygotene and pachytene to diplotene spermatocytes , followed by meiosis I and II; development of round spermatids to spermatozoa via spermiogenesis; and spermiation (release of mature spermatozoa from germinal epithelium into the tubular lumen), which occurs in the apical compartment behind the BTB.

Spermatogonia, formed during the foetal period, remain dormant until pubescence (Bonomi et al., 2012). These spermatogonia lie next to the basement membrane of the seminiferous tubule. Spermatogonia can be classified into two types Type A (Type A pale and Type A dark) and Type B, which are determined by there shape of nuclei and chromatin patterns (Clermont, 1966).

Type A and Type B spermatogonia proliferate through mitotic divisions. Each of these cell types has a different fate. While Type A spermatogonia reproduce

themselves for the continuous production of a pool of these cells. Type B spermatogonia undergo further changes and develop into primary spermatocytes.
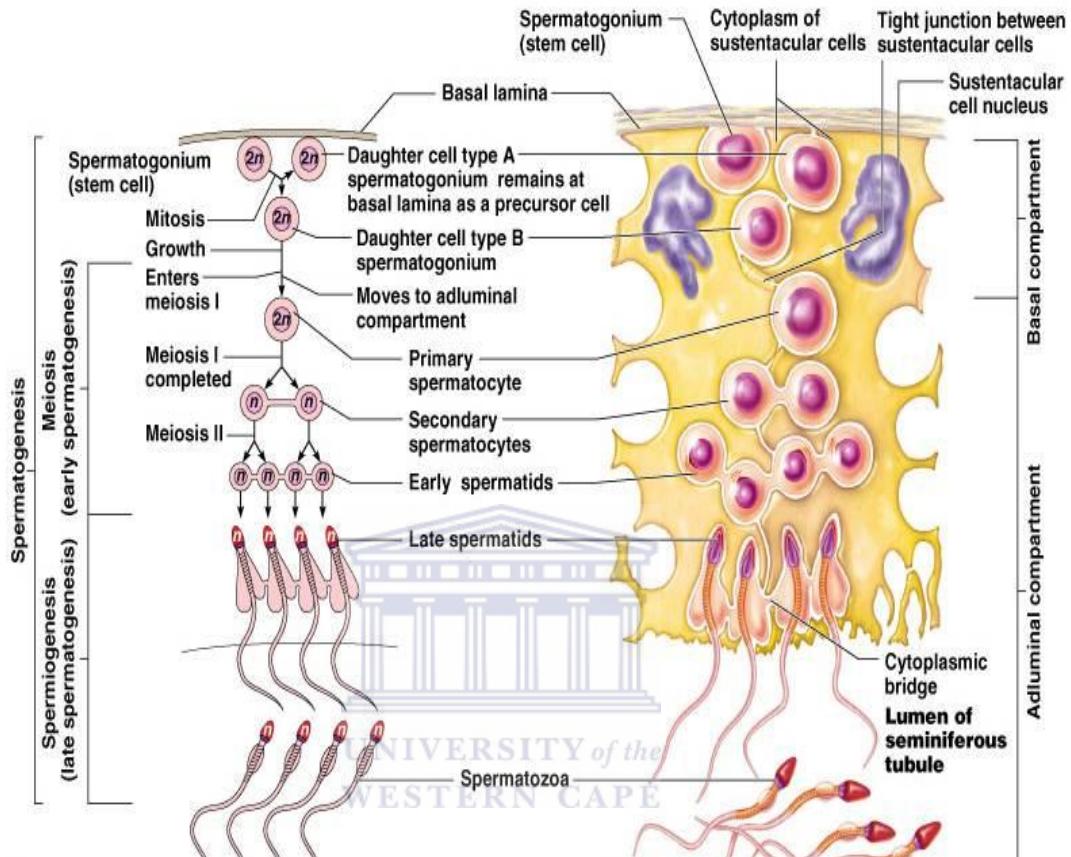
Figure 3: Diagrammatic illustration of spermatogenesis, displaying mitotic and meiotic divisions of the cell stages spermatozoa undergoes within the seminiferous tubules (Pearson education 2004).

The maturation of the spermatocytes entails changes in the chromatin condensation in the nucleus due to meiotic events. During meiosis, two divisions occur spermatogonia then enter the first meiotic division, the prophase, is subdivided into the following stages: Preleptotene and Leptotene stages characterized by chromatin condensation into visible chromosomes, Zygotene-Pachytene stage during which homologous chromosomes paired and become primary spermatocytes (Figure 4).

At this stage, each cell undergoes chromosomal crossing over (Diplotene stage) and has a duplicate set of 46 chromosomes comprising of 22 pairs of duplicate autosomal chromosomes, 2 X chromosomes and 2 Y-chromosomes. As the first meiotic division completes, the daughter Type B cells have become the secondary spermatocytes, which now have a haploid (23) number of chromosomes.

The secondary spermatocytes undergo the second meiotic division and result in smaller cells called spermatids, located in the adluminal compartment of the seminiferous tubules (Cheng and Mruk, 2002).

THREE PHASES:

A - Proliferation, renewal and differentiation of spermatogonia: dark (Ad), and pale (Ap) spermatogonia; B or type B differentiated spermatogonium.

B - Meiosis: involves primary and secondary spermatocytes; The steps of the prophase of the first meiotic division are Pl, preleptotene spermatocyte; L, Leptotene; Z, Zygotene; EP, early pachytene; MP, mid pachytene and LP, late pachytene.

C - Spermiogenesis: spermatids metamorphose into spermatozoa.
Other labels:
M, mitoses; G, Golgi apparatus; A, acrosome; C, centrioles; T, tail; m, mitochondria; RB, residual bodies.

Figure 4: Diagrammatic illustration of the three phases of spermatogenesis (Pearson education 2004).

In contrast, spermiogenesis is the process whereby elongation and differentiation of the spermatids produced from spermatogenesis occurs. The spermatids undergo 4 phases: Golgi phase, Cap phase, Acrosomal phase and Maturation phase (Stevens and Low 1997 refer to Figure 5). In brief, pre- acrosomal granules appear in the Golgi apparatus and fuse to form a membrane-bound acrosomal vesicle close to the nuclear membrane: The acrosomal vesicle changes shape to enclose

the anterior half of the nucleus and become the acrosomal cap (Moore and Persuad, 1998). Thereafter, the increasingly dense nucleus flattens and elongates. With the changed shape of the nucleus, the cytoplasm between the acrosomal cap and the anterior cell membrane migrates to the posterior part of the cell. Surplus cytoplasm from the neck and mid-piece regions are 'pinched off' and phagocytized by Sertoli cells. These, still immature spermatozoa, disconnect from the Sertoli cell surface and lie free in the seminiferous tubule lumen marking the end of spermiogenesis. This release of spermatozoa into the lumen is called spermiation (Moore and Dalley, 1999).

Furthermore, spermatogenesis is controlled by intratesticular end extratesticular factors. Intratesticular factors involve Leydig cells, which are located in the interstitial space of the testes, and Sertoli cells, which are located in the seminiferous tubules as mentioned before (Holstein et al., 2003). While, Leydig cells secrete testosterone and growth factors, which facilitate the maintenance of the Sertoli cells, Sertoli cells function in nursing germ cells (Griswold, 1995).
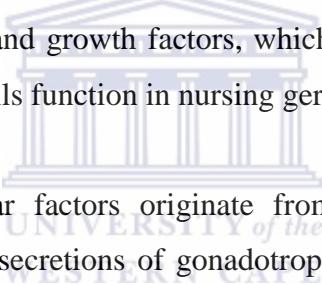
In addition, extratesticular factors originate from the hypothalamus and the pituitary gland. Pulsating secretions of gonadotropin-releasing hormone (GnRH) from the hypothalamus initiates the release of Luteinizing hormone (LH) from the anterior lobe of the pituitary gland. Follicular stimulating hormone is also secreted by the adenohypophysis. The intratesticular and extratesticular factors together make up the Hypothalamic–Pituitary–Gonadal axis (Mruk and Cheng, 2010; Kopera et al., 2010).
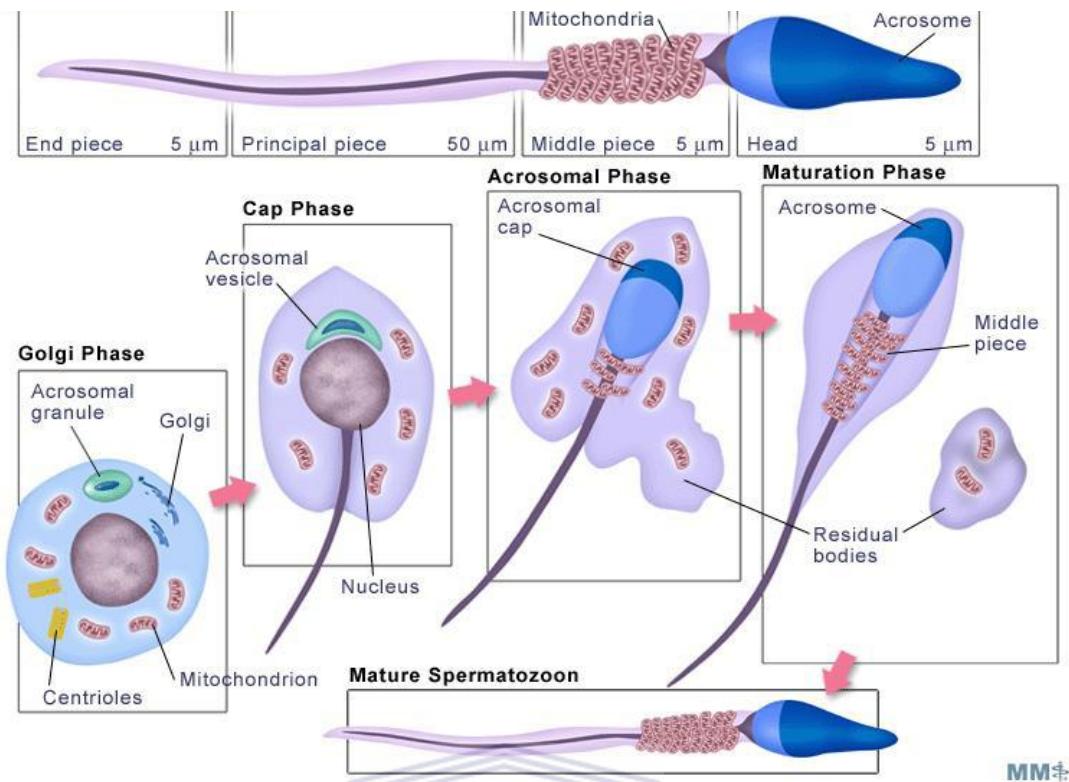
Figure 5: Spermiogenesis (Stevens and Lowe, 1997; Pearson Education, 2004).

1.2.2 Hypothalamic-Pituitary-Gonadal Axis

The Hypothalamic-Pituitary-Gonadal Axis (Figure 6) is comprised of the hypothalamus, anterior pituitary gland and the testes. The axis regulates by using both positive (amplifies signals) and negative feedback systems (restores homeostasis). The hypothalamus releases GnRH through pulse regulation and stimulates the anterior pituitary to release FSH and LH (Mruk et al., 2010; Kopera et al., 2010). In turn, FSH acts on the Sertoli cells where androgen-binding protein (ABP) is then synthesised and the process of spermatogenesis stimulated. ABP binds testosterone and hydroxytestosterone produced outside of the seminiferous tubule and is essential in maintaining the high concentrations of these hormones for germ cell maturation (Prante et al., 2008).

LH facilitates Leydig cells and stimulates their testosterone production, which consequently targets stimulation of Sertoli cells. When the testosterone level is at an increase, inhibin (synthesis in the Sertoli cells due to FSH) inhibits the hypothalamus in releasing GnRH in a negative feedback. Thus, the system is kept in equilibrium.

1.2.3 Physiology of Sertoli cells

Sertoli cells create an appropriate environment for germ cell maturation and proliferation and are therefore regarded as 'nursing cells'. Subsequently, germ cells depend on Sertoli cells for nutrition and support (Jeagou, 1993; Russell and Griswold, 1993; Costa et al., 1998).

In performing this aspect of Sertoli cell function, these cells provide substances such as carbohydrates, amino acids, lipids, metallic elements and vitamins to the germ cells (Russell and Griswold, 1993; Cheng and Mruk, 2002). One other function of Sertoli cells in its supporting role is the synthesis and release of several proteins, including ABP as well as the secretion of a potassium- and chloride-rich fluid into the lumen of seminiferous tubules (Jeagou, 1992).
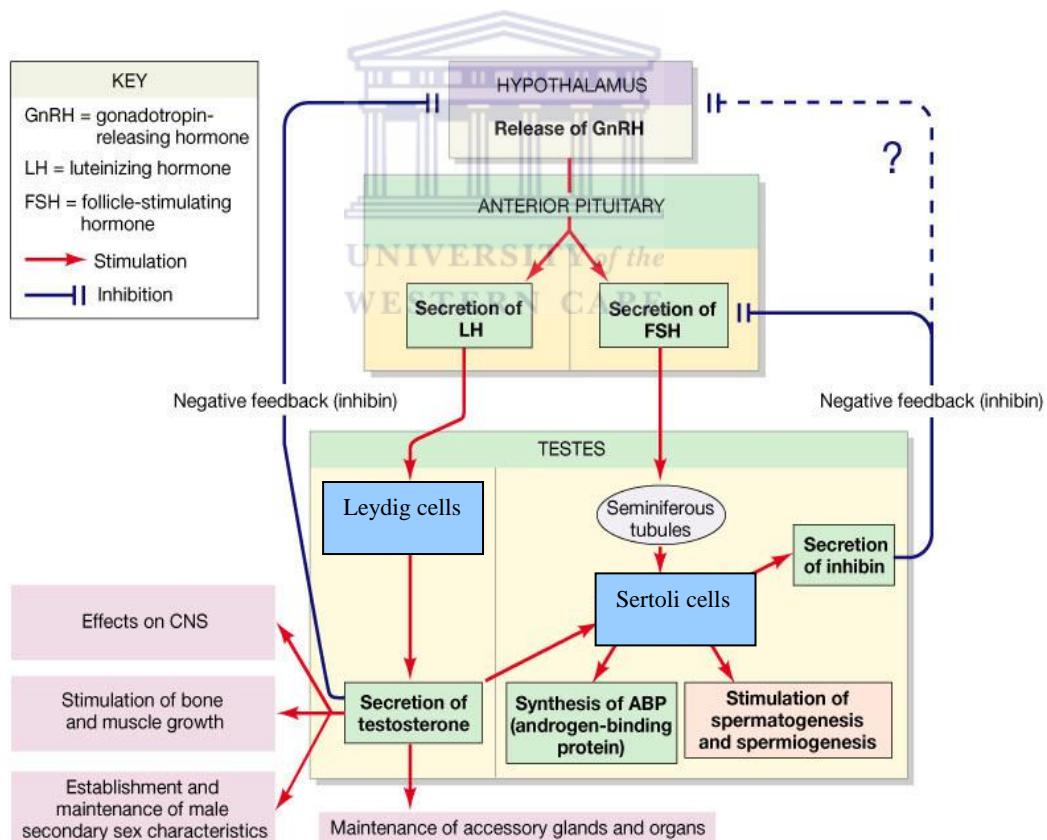
Figure 6: Modified diagrammatic illustration of hypothalamic-pituitary-gonadal axis (Pearson Education, 2004).

A study conducted by Auzanneau et al. (2003) indicates that the large amount of lactate secreted by Sertoli cells plays an important role in the maintenance of an acidic microenvironment within the lumen seminiferous tubule (Robinson and Fritz, 1981; Riera et al., 2002). Furthermore, production of lactate by Sertoli cells is an effect due to the regulation of glucose transport and lactate dehydrogenase (LDH) including its activity (Riera et al., 2002). Thus, the microenvironment is essential for the survival of germ cells and for the proliferation of spermatogonia as it utilizes the lactate as an energy substrate (Hamamah and Gatti, 1998; Mauduit et al., 1999; Auzanneau et al., 2003).

In an earlier study, Grootegoed et al. (1984) used this theory to determine the toxicity within Sertoli cells by investigating pyruvate and lactate production. Furthermore, Pyruvate is metabolic converted either into lactate or to acetyl-CoA (Equation 1A). The reaction is catalyzed by the enzyme lactate dehydrogenase (LDH) (Equation 1B). Lactate is thus a "waste product", since it has no metabolic fate other than being reconverted into pyruvate (Erkkila et al., 2002). Therefore, the accumulation of lactate predicts the toxicity within the cell (Robinson and Fritz, 1981).
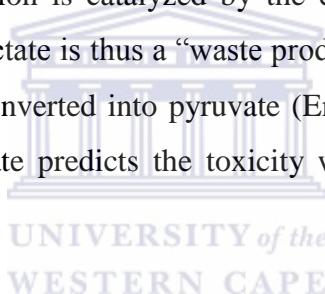
Follicular stimulating hormone (extratesticular factor) released from the pituitary gland acts directly onto the Sertoli cells (Norton et al., 1994) which stimulates the synthesis of ABP and inhibin. ABP is secreted into the lumen of the seminiferous tubules near the developing spermatogonia and maintains the upkeep of high testosterone levels as ABP binds this androgen (Stevens and Lowe, 1997).

Inhibin is secreted into the seminiferous tubular fluid and the interstitial fluid of the testes and inhibits the secretion of FSH from the anterior pituitary gland (Skinner and Griswold, 2004). Inhibin causes a negative feedback on the anterior pituitary to exert an inhibitory effect on LH secretions and in turn reduces the testosterone levels (Cummings and Bremner, 1994).

(A)

Glucose $\longrightarrow$ Glucose-6-PO$_4$

Lactate $\longleftarrow$ Pyruvate

Acetyl- CoA

(B)

Equation 1: Pyruvate +NADH + H$^+$ $\Leftarrow$(LDH)$\Rightarrow$ Lactate +NAD$^+$
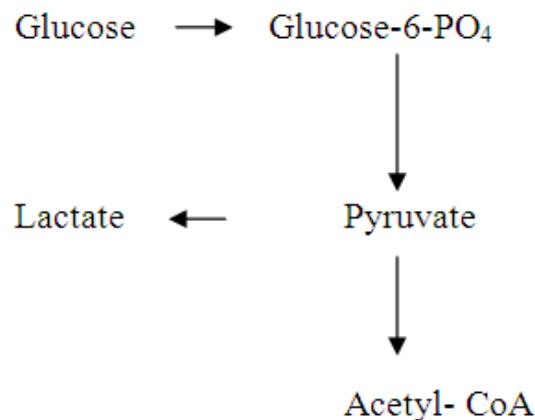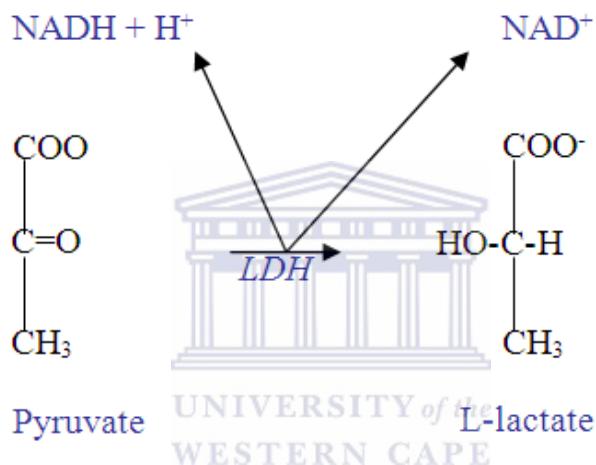
Glucose (Carbohydrates) converted to Glucose-6-PO$_4$ to form pyruvate, which has two fates due to further metabolic activities, acetyl-CoA and Lactate (A). The enzyme LDH facilitates the conversion of pyruvate to lactate (B).

1.2.4 Physiology of Leydig cells

Leydig cells are the primary sites of steroidogenesis in the testis (Foley, 2001). Luteinizing hormone produced by the anterior pituitary gland as a result of the stimulation of GnRH from the hypothalamus acts directly on to Leydig cells. When Leydig cells are stimulated, they synthesize and release testosterone (Norton et al., 1994). Testosterone is the main male steroid hormone and is important for spermatogenesis and male sexual characteristics (Hess and De Franca, 2008; Mathur and D'Cruz, 2011) such as increased bone and muscle mass, erythropoietin and potency/libido. Thus, a deficiency would alter these in a

negative way. However, testosterone also inhibits the release of GnRH and LH in a negative feedback via the anterior-pituitary (Bonomi et al., 2012). Therefore, the production of testosterone needs to be regulated. Moreover, a low testosterone level indicates an age-associated decline in testicular function leading to hypogonadism (Tambi et al., 2012). Impaired Leydig cell function therefore leads to a lower synthesis of LH levels and libido (Winters et al., 1984 and 1997; De Slypere et al., 1987; Vermulen, 2000).

1.3 Infertility

Infertility can be described as the failure of a couple to induce pregnancy after one year of regular, unprotected intercourse during the fertile phase of the menstrual cycle (Nieschlag et al., 2000; Evers, 2002). Feng (2003) and Roberts (1998) reported that infertility affects about 15 % of couples seeking clinical treatment to have children. Globally, the number of infertile couples amount to 50-80 million annually (WHO, 1992). Impaired fertility of the male partner is the cause or the contribution of up to two-thirds of all couples unable to conceive spontaneously (Nieschlag et al., 2000).
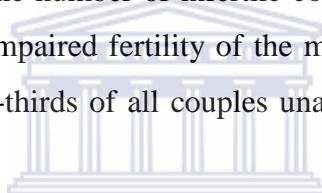
Since a conservative treatment of male infertility is not possible in most cases, this leaves 50% of the cases classified as idiopathic (Sherins, 1995). Consequently, the therapeutic options focus on assisted reproduction (ART), such as *in vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI). The latter of which is most invasive, even causing numerous problems and serious concerns (Halliday, 2012). Yet, if ART is inevitable, it is essential to investigate the causes of the infertility and to understand its mechanisms.

Normally, the investigation of male infertility starts with a routine semen analyses, in specific, the semen quality examined by means of sperm concentration (Calvo et al., 1994), motility (Ron-el et al., 1991; Robinson et al., 1994) and morphology (Kruger et al., 1986; Menkveld et al., 1990). However, many men who demonstrate normal parameters on standard semen analysis remain unable to induce pregnancy (Hull et al., 1985; WHO, 1992; Baker, 1994). Lewis (2007) reported when determining male infertility these parameters, strictly followed by WHO diagnostics over the past two decades are insufficient. In more

recent years, this procedure was complemented by functional parameters (Henkel et al., 2005a; Aziz et al., 2007; Abu et al., 2012), which aimed at determining the sperm cells' functional capacity.

Fertilization is regarded as a multifactorial process (Amann, 1989; Henkel et al., 2005a). Therefore, it is essential that all facets of the male fertility aspect be thoroughly investigated in this regard. Thus, it is imperative that functional parameters such as capacitation (Sukcharoen et al., 1995; Ho et al., 2001) and the ability to undergo stimulated acrosome reaction (Figure 7) (Chang 1951; Austin 1952; Henkel et al., 1993) be integrated into the routine fertility analyses (Franken and Oehninger, 2012).

Recently, these parameters were complemented by the determination of DNA damage, sperm reactive oxygen species (ROS) and mitochondrial membrane potential (MMP) as abnormalities and have, in the past been shown to be predictive of infertility (Henkel and Franken, 2011). Particularly, sperm DNA damage may contribute to embryonic death (Seli et al., 2004) or the offspring suffering from health complications (Twigg et al., 1998; Aitken and Krausz, 2001).

1.4 Investigative Functional Parameters of Spermatozoa
1.4.1 Motility

Motility of spermatozoa is as a predictive value for pregnancy rates as low sperm motility may reduce the chances of fertilization of the ovum, which is common in infertile men (Coetzee et al., 1989; Shulman et al., 1998; Henkel et al., 2005a). Thus, it is important to accurately record the motility content of a semen sample. A recent review conducted by Franken and Oehninger (2012) on the assessment of semen analysis and sperm function, highlighted the importance of changes in the analysis of semen as described in the new World Health Organization manual (WHO, 2010). The authors indicated that the 2010 WHO manual recommend the use of a simple system for grading motility. This system therefore distinguishes spermatozoa with progressive or non-progressive motility from those that are immotile.

The motility of each spermatozoon is graded as follows:

- progressive motility: spermatozoa moving actively, either linearly or in a large circle, regardless of speed;

- non-progressive motility: all other patterns of motility with an absence of progression, i.e., swimming in small circles, the flagellar force hardly displacing the head, or when only a flagellar beat can be observed;

- Immotility: no movement.

Furthermore, extensive research concludes that motility can directly be correlated to mitochondrial membrane potential (Kasai et al., 2002), plasma membrane integrity, which is reportedly affected by ROS production (Aitken et al., 1991) and morphology (Kruger et al., 1986; Katz et al., 1990; Zavos and Centola, 1992; Menkveld et al., 1996).

1.4.2 Reactive oxygen species (ROS), DNA and mitochondrial membrane damage

As in any other living cell, in spermatozoa, energy is largely aerobically produced by means of enzymatically controlled mitochondrial oxidative phosphorylation and oxidation of hydrogen (Henkel, 2012). Thereby, ROS are by-products of the oxygen free radicals due to the oxidative process of energy generation within the spermatozoa (Henkel, 2012). Although ROS are energy-rich, highly reactive compounds that contribute to the oxidative damage of cells including spermatozoa, (Henkel, 2012) ROS are also considered necessary for normal sperm function (Aitken and West, 1990; Plante et al., 1994; Esfandiari et al., 2003; Aitken and Baker, 2006) as they trigger important functions such as capacitation (De Lamirande et al., 1997).

Sperm exhibiting excess residual cytoplasm due to poor sperm and morphology immaturity are deemed to generate excessive amounts of ROS. This could be self-damaging (Gomez et al., 1996; Aitken et al., 1997; Aziz et al., 2004) especially to the sperm DNA and also leads to poor fertilizing potential (Keating et al., 1997). These damages by ROS include the induction of oxidative damage to lipids, nucleic acid, proteins and carbohydrates. Apparently, it contributes to 30 % of the

cases of male infertility (Iwasaki and Gagnon, 1992; Ochsendorf et al., 1994; Shekarriz et al., 1995) aging, cancer and many other diseases (Aruoma, 1994). ROS have a high oxidative potential (Halliwell and Gutteridge, 1989) and very short half-life time in the nanosecond (10^{-9} s) (·OH; hydroxyl radicals) to millisecond range (10^{-3} s) ($·O_2^-$; superoxide anion). Therefore, these radicals react at the site of generation, which explain the oxidative damage to lipids and nucleic acids. The location of the production (extrinsic by leukocytes or intrinsic by the male germ cells) also plays a role as extrinsic ROS produced by leukocytes appear to rather impair sperm motility, while intrinsic ROS production seems to preferentially affect sperm DNA fragmentation (Henkel et al., 2005b).

Considering the vulnerability of spermatozoa to oxidative stress, due to exceptionally high content of polyunsaturated fatty acids (Alvarez et al., 1987; Zalata et al., 1998), the male germ cells have to be protected by relevant scavengers against these oxidants right from the site of sperm production, the testes, as developing spermatozoa during spermiogenesis have very limited ability to repair DNA. Here, the Sertoli cells provide for protection (Den Boer et al., 1990) but may not be sufficient and the investigation of excessive ROS is necessary to determine exact cause of impaired male fertility.

1.4.3 Acrosome Reaction

Acrosome reaction (AR) is the exocytotic release of the acrosomal contents and predictive of fertilization *in vitro* (Cummins et al., 1991; Henkel et al., 1993) and is essential for sperm penetration of the zona pellucida (ZP) (Bleil and Wassarman, 1983; Wassarman, 1999, Kopf and Gerton, 1991). The penetration of the ZP depends on the enzyme, acrosin contained in the acrosome of the spermatozoa in humans (Henkel et al., 1993). Subsequently, these enzymes are released during the AR by fusion of the acrosomal and plasma membranes (Figure 8). The interaction of the ZP glycoprotein, ZP3, and a sperm receptor (Bleil and Wassarman, 1983; Yanagimachi, 1981) initiates the AR reaction and fusion. Essentially, only acrosome reacted spermatozoa can penetrate the ZP (Koehler et al., 1982).

(A)

The Acrosome Reaction
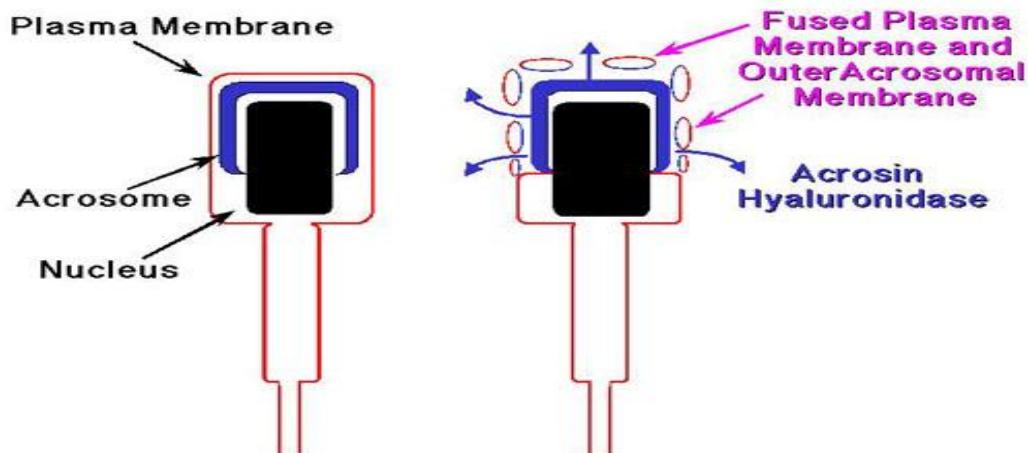
(B)

Sperm Interaction with the Zona
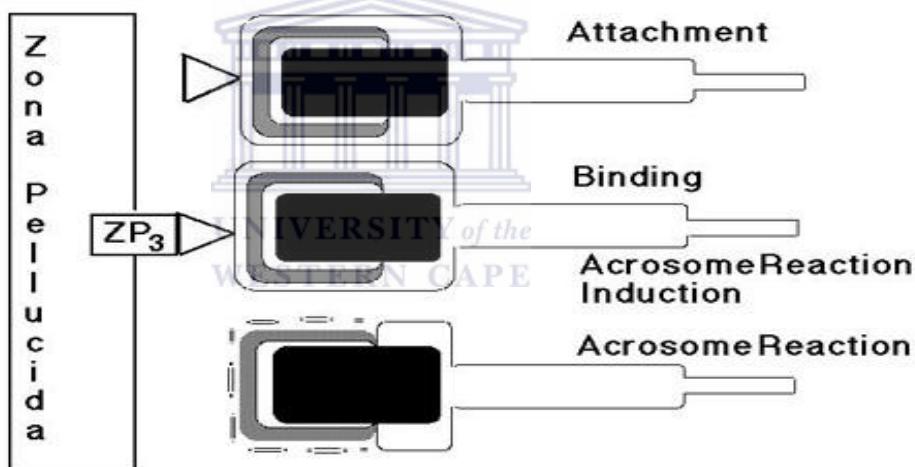
Figure 7: A) Schematic representation of the acrosome reaction. The acrosomal membranes underlying the plasma membrane overlying the nucleus. The fusion of sperm plasma membrane and the acrosomal membrane allow the exposure of acrosomal contents, enzymes such as acrosin. B) The event is triggered by the interaction of a ZP glycoprotein, ZP3, and a sperm receptor (www.ansci.wisc.edu).

1.5 Investigative history of traditional herbal medicine safety and efficacy

The World Health Organization (WHO, 1978) defines traditional medicine as the sum of all knowledge and practices, whether explicable or not, used in diagnosing, preventing and eliminating physical, mental or societal imbalances. It relies exclusively on practical experience and observation handed down from generation to generation, whether verbally or in writing. This definition is equivalent to the South African Traditional Health Practitioners Act (Act No.35 of 2004) which is "to provide for a regulatory framework to endure the efficacy, safety and quality of traditional health care services".

It is also estimated by the World Health Organization that 60-80 % of the population of non-industrialized countries rely on traditional health care for their basic health care needs, either on its own or in conjunction with modern medical care. Thus, the demand for traditional medicine is high in many countries, particularly in third world or developing countries (Bannerman, 1993). During the past decade, there has been an increased global interest in the practice of traditional healers and their use of medicinal plants to treat illnesses (Akerele, 1994). Due to its accessibility and affordability, medicinal plants are an important aspect, especially for poorer people in third world or emerging countries (Farnsworth, 1994).

The majority of research into safety and efficacy of medicinal plants are mainly performed *in vitro* or based on animal models and only a very limited number of studies on humans, probably due to negative clinical trial results, drug interactions or toxic reactions have been conducted (Pittler et al., 2000; Ernst, 2002; Ameh et al., 2011). Considering that, traditional remedies are normally applied as aqueous concoctions of the plant material with countless different compounds dissolved, sometimes even with synergistic or antagonistic actions, it is generally very difficult to identify the actual active ingredient of an herbal medication (Low and Tan, 2007). In addition, the medicinal properties may relate to the soil and environmental factors and thus have inconsistent results (Low and Tan, 2007).

Current literature suggests that traditional healers should become the subject of intense research with a view to establish new ways of strengthening collaboration between modern health care providers and the traditional healers (Kaido et al., 1997; Izzo and Ernst, 2001; Ameh et al., 2011).

1.6 Male infertility and herbal medicine

Infertility is a personal, societal and economic problem as previously defined (Low and Tan, 2007). It is a public health concern as it affects people indiscriminately, and about 25 % of couples will seek assistance with reproduction at some point during their relationship (Chandra, 1994). Kamal et al. (2003) and Wang (1999) stated that the available methods of fertility regulation have become inadequate to meet changing needs of couples due to different geographical, cultural and religious background around the world. Even though the review was conducted with regard to plants and male fertility regulation in contraceptive approaches, the same could be argued concerning enhancing male fertility for the growing demand.
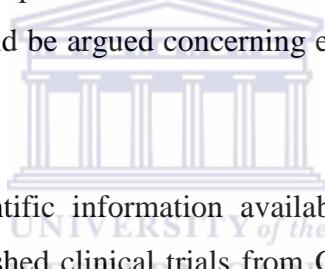
With regard to the scientific information available on the efficacy of herbal medicine, over 450 published clinical trials from China on diseases like asthma, cancer, diabetes mellitus and heart disease exist, but the number of published trials on fertility is estimated to be only 50-60 (Tang et al., 1999; Yuan and Lin, 2000; Pach et al., 2002). Although numerous medicinal plants have been characterized biochemically, only very few plants traditionally used to treat male fertility problems have been investigated. Therefore, due to the growing interest in traditional medicinal plants have furthered developments in scientific research to incorporate the medicinal properties of plants in the treatment of male factor infertility across the world has furthered.

In the Peruvian central Andes, Gonzalez et al. (2003) performed a study on the hypocotyls of three species of *Lepidium meyenni* (Maca) that are used as an aphrodisiac by indigenous people to enhance male fertility. It could further be shown that Maca reduces spermatogenic damage induced by a single dose of malathion in mice (Gonzalez et al., 2005). Additionally, in 2006 and 2008, the effect of Black Maca on one spermatogenic cycle in rats as well as the

antagonistic effect of Red Maca on prostatic hyperplasia in adult mice (Gonzalez et al., 2006, 2008) and the effect of Red Maca on prostate zinc levels in rats with testosterone-induced prostatic hyperplasia (Gonzalez et al., 2012).

In China and India, *Tribulus terrestris* (devil's weed) is a medicinal plant that has been used for centuries to increase sexual desire and enhance erection (Adimoelja, 2000). People have also been using plants such as *Panax ginseng* (Asian ginseng) for the treatment of impotency and improving sexual stamina (Nocerino et al., 2000). In West Africa, a study on plant extracts of *Hibiscus macranthus* and *Basella alba* (Malabar spinach) showed that these extracts enhance testosterone production (Moundipa et al., 2005, 2006). The bark of the Yohimbe tree *(Pausinystalia yohimbe)* contains a chemical called yohimbine, which is used to treat sexual dysfunction and enhances libido (Carey and Johnson, 1996). An *in vitro* study on *Mondia whitei,* which is used as an aphrodisiac in Ghana, has shown that an aqueous administration *in vitro* enhanced total motility and progressive motility (Lampiao et al., 2008).
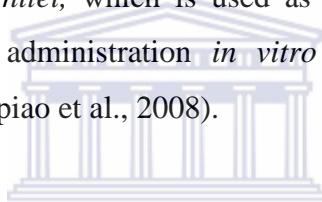
1.7 *Eurycoma longifolia* Jack

In Malaysia, *Eurycoma longifolia* Jack (Figure 8), a plant from the family of Simaroubaceae, more commonly known as Tongkat Ali (TA; means literally: "Ali's walking stick") is used for similar purposes (Jaganath and Ng, 2000; Bhat and Karim 2010). It so named, as the roots are long, slender and twisting. It is the most popular folk medicine in South East Asia including Myanmar, Indochina, Thailand, Laos, Cambodia (Darise et al., 1982). *E. longifolia* is identified as 'Pasak Bumi' or 'Bedara Pahit' in Indonesia and 'Ian-don' in Thailand (Thoi and Suong, 1970; Darise et al., 1982; Kuo et al., 2004). In Vietnam, *E. longifolia* is named 'Cay ba binh', translated as 'a tree which cures hundreds of diseases' (Choo and Chan, 2006). The Malaysian sub-species of *E. longifolia*, is commonly found as an under storey in the lowland forests at up to 500 m above sea level (Kuo et al., 2004).

The process of consuming the root also follows the traditional way, wherein the roots are cut into smaller portions, boiled, and consumed as a tea but produces bitterness (Bhat and Karim, 2010) mainly due to quassinoid compound presence

(Council of Europe 1991). Therefore, traditional healers suggest mixing of the extract (chipped roots) with honey, sugar syrup or dates for reducing the bitterness (Bhat and Karim 2010). However, a few experienced traditional healers suggest that 'the more the bitterness' – the 'better' is the efficacy.

A review by Bhat and Karim (2010) on the ethnobotany and pharmacology of TA reported that almost all parts of the Tongkat Ali plant are traditionally used for therapeutic purposes. In the past, treatment with Tongkat Ali has been non-specific, rather as a tonic or a supplement for general well-being (Bhat and Karim, 2010). The plant extract is traditionally used for its antimalarial, aphrodisiac and anti-pyretic activities (Chan et al., 1995; Kuo et al., 2004). Furthermore, scientific studies have shown that Tongkat Ali extracts exhibit antiulcer (Tada et al., 1991), cytotoxic (Zakaria et al., 2009) and even mutagenic properties (Abd Razak et al., 2007). This could be evident as the roots contain a variety of chemical compounds including alkaloids, quassinoids, diterpenoids, eurycomoside, eurycolactone and laurycolactone (Morita et al., 1993; Ang et al., 2002; Bedir et al., 2003; Miyake et al., 2010). Apart from these compounds, a eurypeptide with aphrodisiac properties has also been identified (Ali and Saad, 1993).
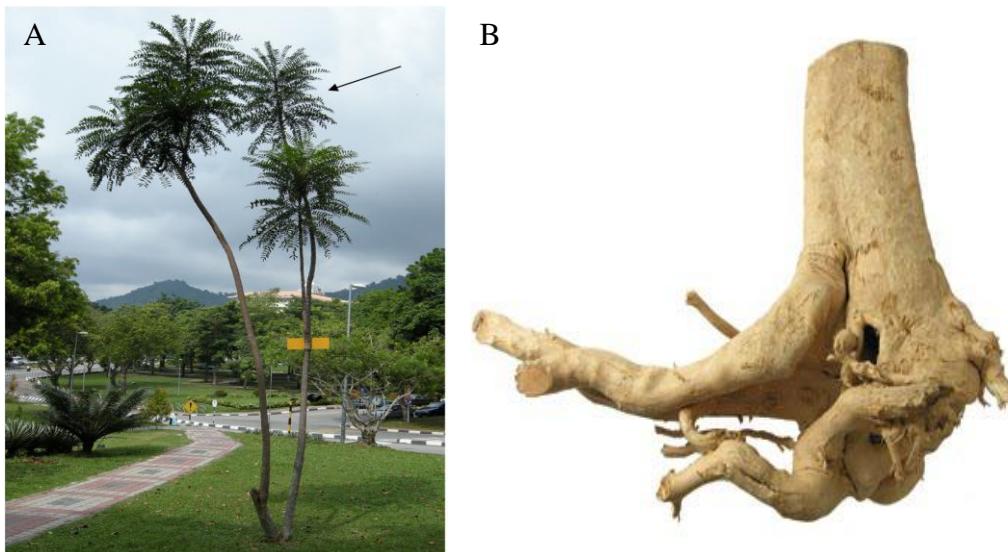
Figure 8: A) *Eurycoma longifolia* tree in its natural habitat in Malaysia indicated by the arrow B) twisting roots of Tongkat Ali (Bhat and Karim, 2010; www.tongkatalisideeffects.com/tongkat-ali-review).

1. 8 Aim of study

TA is widely used but no study is available investigating the safety of its use, nor the effects on human sperm and testicular cells.

Therefore, this study aimed at:

- The cytotoxic effects of reproductive functions in regard to fertility and offspring namely: Motility, Viability, Acrosome reaction, DNA damage, Mitochondrial Membrane Potential and ROS production;
- Providing clarity on the effects and safety of TA *in vitro* on spermatozoa, Sertoli and Leydig cells;
- The release of hormonal response and cytotoxic effect on Sertoli and Leydig cells;
- The mechanism of action of TA on the Hypothalamic-pituitary-gonal axis.

Chapter 2: Materials and methods

2.1 Chemicals and Equipment

In this study, all chemicals used, where possible, were of analytical or cell culture grade.

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

- TM3-Leydig and TM4-Sertoli cells

BDH Biochemical, Poole, England supplied:

- Sodium Pyruvate (Na-Pyruvate)

Bio-Rad, Hercules CA, USA supplied:

- DC protein assay reagent A and reagent B

Corning incorporated, Mexico City, USA supplied:

- 24- and 96-well plates
- Tissue culture flasks (25 cm^2)
- Test tubes (15 cm and 50 cm)
- Eppendorf vials

DRG instruments GmbH, Marburg, Germany supplied:

- Testosterone ELISA kit

Gibco Invitrogen, Karlsruhe, Germany supplied:

- Dulbecco's Modified Eagles Medium + GlutaMAX^{TM} (DMEM)
- F-12 Ham's nutrient mixture
- 2.5% Fetal Bovine Serum (FBS)
- 5% Horse serum

Kimix chemicals, Eppingdust, South Africa supplied:

- Calcium Chloride dihydrate ($CaCl_2 * 2 H_2O$)
- Disodiumphosphate ($NaHPO_4$)
- Ethanol

- Magnesium Sulphate heptahydrate (MgSO$_4$ * 7 H$_2$O)
- Potassuim Chloride (KCl)
- Sodium Bicarbonate (NaHCO$_3$)
- Xylene
- 40 % Formaldehyde

Knittel Gläser, Braunschweig, Germany supplied:

- Superfrost slides

Lasec, Cape Town, South Africa supplied:

- Filter paper (240mm thick, Munk Tell)

Merck, Wadeville Gauteng, South Africa supplied:

- Glucose (anhydrous)
- Hydrochloric acid (HCl)
- Hydroxymethyl Aminomethane (TRIS)
- Potassium diphospate (KH$_2$PO$_4$)
- Sodium Chloride (NaCl)
- Sodium Hydroxide (NaOH)
- Triethanolamine hydrochloride
- 25% Gluteraldehyde
- 0.25% Trypsin/Ethyl Diamine Tetra Acetic acid (EDTA)

Molecular Probes, Eugene, USA supplied:

- Dihydroethidine (DHE)

Oxoid, Hampshire, England supplied:

- Phosphate Buffered Saline (PBS)

Promega, Madison, USA supplied:

- TUNEL kit (terminal deoxynucleotide transferase mediated dUTP nick-end labelling)

Roche, Mannheim, Germany supplied:

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

Sigma-Aldrich, Steinheim, Germany supplied:

- Bismarck Brown Y (BBY)
- Bovine serum albumin (BSA) [analytical grade)
- Dimethylsulphoxide (DMSO)
- Eosin Y
- Glycerol
- Human chorionic gonadotropin hormone (hCG)
- N-(2-hydroxyethyl)piperazine-N-2-ethanesulfonic acid (HEPES)
- Human Serum albumin (HSA) (analytical grade)
- Lactate dehydrogenase (LDH)
- Mounting medium
- Nigrosin
- Phenol red (dye)
- Penicillin
- Rosè Bengal (RB)
- Sodium dodecylsulphate (SDS)
- Streptomycin
- Triton X-100
- Trypan Blue (TB)
- 60 % Sodium Lactate (Na-Lactate)

Trevigen, Gaithersburg, USA supplied:

- DePsipher kit

Centrifuges

Labortechnik, Wehingen, Germany supplied:

- Hermle Z160M
- Hermle Z200A

Incubators

Lasec, Cape Town, South Africa supplied:

- Series 2000

Nuaire, Playmouth MN, USA supplied:

- Autoflaw CO_2 Air-jacketed

Laminar Flow

Nuve, Ankara, Turkey supplied:

- LN Series

Microscopes

Zeiss, Oberkochen, Germany supplied:

- Epifluorescence

Zeiss, Cape Town, South Africa supplied:

- Photomicroscope III

Plate shaker

Greiner Bio-One, Frickenhausen, Germany supplied:

- 96-well flat bottom

Plate reader

Thermo Scientific, Kanagawa, Japan supplied:

- 96-well ELISA (Enzyme-linked immunosorbant assay)

Spectrophotometer

Beckman, Fullerton CA, USA supplied:

- Model DU 640

Sperm Class Analyzer (SCA)

Microptic S.L., Barcelona, Spain supplied:

- Version 4.1.0.1

2.2 Media

2.2.1 Medium used for spermatozoa

Human tubular fluid medium (HTF) was used for spermatozoa and prepared according to Quinn et al. (1985). This medium is said to mimic the female fallopian tube milieu, thus delaying cell death to perform all testing parameters in an adequate time frame. The medium is made up of the following substances: 101.60 mM NaCl, 4.69 mM KCl, 2.04 mM $CaCl_2$ * 2 H_2O, 0.02 mM $MgSO_4$ * 7 H_2O, 0.37 mM KH_2PO_4, Phenol red (dye indicator), 25 mM $NaHCO_3$, 2.78 mM Glucose (anhydrous), 0.33 mM Na-Pyruvate, 21.40 mM Na-Lactate (60 % syrup), Penicillin, Streptomycin, 20 mM HEPES and dissolved in distilled water. After the solution has thoroughly dissolved, osmolarity was adjusted to 280 mOsmol/kg and 10 mg/ml HSA (HTF-HSA) added before working with the medium.

2.2.2 Medium used for cell culture

The growth medium used to culture TM3-Leydig and TM4-Sertoli cells contains the following media at a ratio: 50 % Dulbecco's Modified Eagles Medium + GlutaMAX^{TM} [+ 4.5g/L D-glucose + pyruvate] (DMEM), 50 % F-12 Ham's nutrient mixture [+L glutamine], 2.5 % Fetal Bovine Serum (FBS), 5 % Horse serum and supplemented with penicillin and streptomycin.

2.3 Study design

This study has two parts of *in vitro* experimental set-ups namely; spermatozoa (Figure 9A) and cell culture investigation (Figure 9B). This study received ethical clearance by the Ethics Committee at the Faculty of Science, University of the Western Cape, and the Review Board at the University of Stellenbosch.

2.4 Plant extract

A patented (Patent number: WO0217946), standardized, water-soluble extract of the root of *Eurycoma longifolia* (Tongkat Ali; TA Physta^{TM}) was supplied by Biotropics Berhad; Kuala Lumpur, Malaysia.

Part 1
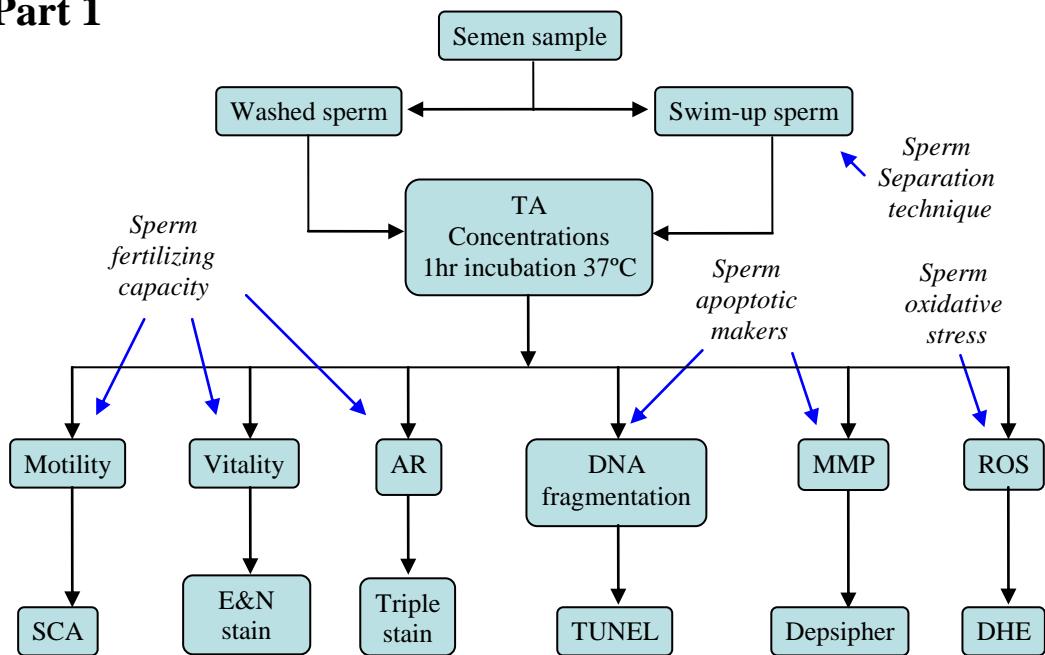
Part 2
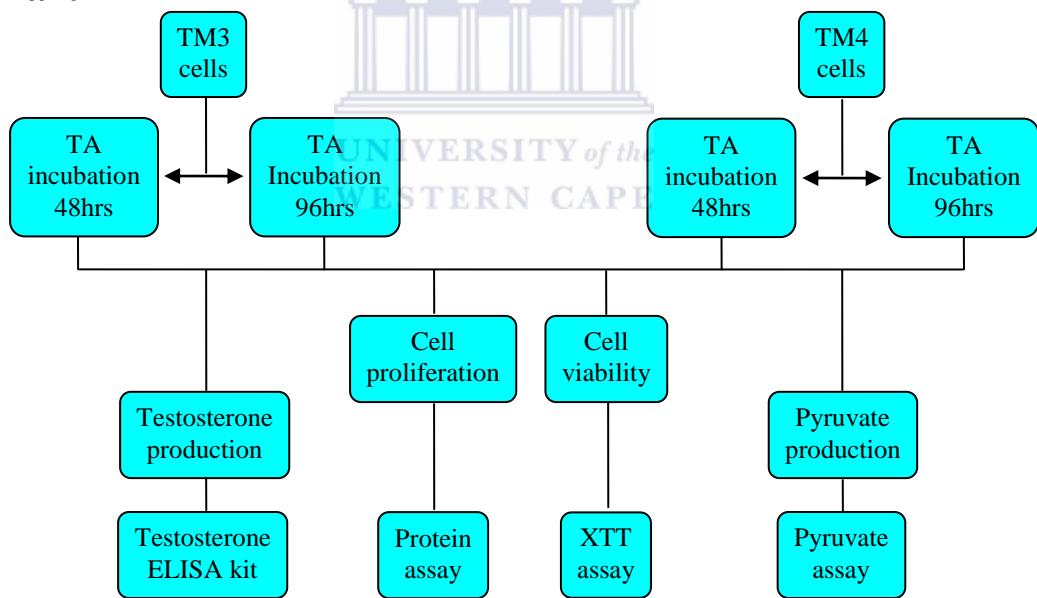
Figure 9: Part 1) Study design for spermatozoa and Part 2) TM3-Leydig and TM4-Sertoli cells, with TA (Tongkat Ali) incubation at different concentrations displaying investigative parameters and testing procedures. ROS: Reactive oxygen species, MMP: mitochondrial membrane potential, E&N: Eosin-Nigrosin, AR- acrosome reaction, SCA-Sperm Class Analyser.

2.5 Investigation of sperm parameters *in vitro*

2.5.1 Concentrations of TA used for incubations for spermatozoa

A stock solution containing 5 mg TA/100 µl of HTF-HSA was prepared. Final concentrations were established of 1 µg/ml, 10 µg/ml, 20 µg/ml, 100 µg/ml, 2000 µg/ml and a control sample without TA extract. These concentrations were used throughout the investigation with spermatozoa.

2.5.2 Preparation of spermatozoa *in vitro*

A total of 40 semen samples were obtained from donors and patients attending the Reproductive Biology Unit in the Department of Obstetrics and Gynaecology, University of Stellenbosch at the Tygerberg Academic Hospital, Tygerberg, South Africa; and Vincent Palotti Hospital, Pinelands, South Africa, for fertility problems or assisted reproduction treatment. The samples were collected by masturbation into sterile plastic cups following 2 to 4 days of sexual abstinence.
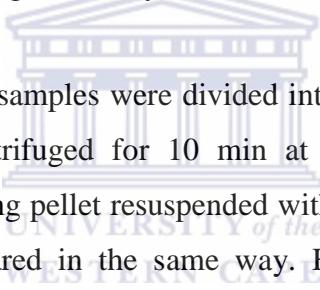
After liquefaction, semen samples were divided into two aliquots and diluted 1:5 with HTF-HSA and centrifuged for 10 min at 500xg. The supernatant was discarded and the remaining pellet resuspended with fresh HTF-HSA (wash). The second aliquot was prepared in the same way. However, the pellet was then overlaid with 500 µl fresh HTF-HSA and incubated for 1 hour at 37°C to let motile spermatozoa swim up (swim-up). Subsequently, both samples (wash and swim-up) were incubated with TA extract at the different concentrations for 1 hour at 37°C.

2.5.3 Determination of sperm motility

Motility of spermatozoa is as a predictive parameter for pregnancy as low sperm motility may reduce the chances of sperm fertilizing the ovum (Coetzee et al., 1989; Shulman et al., 1998; Henkel et al., 2005a). Therefore, it is important to accurately determine the motility of a semen sample.

Sperm motility in both washed and swim-up samples was measured with the Motility/Concentration module of the Sperm Class Analyzer (SCA). An aliquot of 10 µl of the sperm suspension was put on a glass slide covered with a cover slip and the motility of at least 100 sperm were analysed according to WHO criteria set by the SCA system with a Zeiss Photomicroscope III and a 100X oil immersion objective. The eight different kinematic parameters (Maree et al., 2011) which were analyzed are summarized in Table 1.

Table 1: The different sperm kinematic parameters recorded with SCA

Parameter	Unit	Description
Motility	%	Total motility
Concentration	$\times 10^6$/ml	Number of spermatozoa
VCL (Curvilinear velocity)	µm/s	Curvilinear velocity along actual swimming path
VSL (Straight-line velocity)	µm/s	Straight-line velocity along shortest path from start to end point
VAP (Average path velocity)	µm/s	Average path velocity based on every 5th frame of VCL path
LIN (Linearity)	%	Linearity of a curvilinear path, expressed as VSL/VCL
STR (Straightness)	%	Straightness, expressed as VSL/VAP
ALH (Amplitude of lateral head)	µm/s	Amplitude of lateral head displacement

2.5.4 Determination of sperm viability

The assessment of sperm viability is one of the basic elements of semen analysis and distinguishes between immotile dead sperm and immotile live sperm (Björndahl et al., 2003).

Viability was determined by using the Eosin-Nigrosin staining technique (WHO, 2010). The stain was prepared by dissolving 0.67 g of Eosin Y and 0.9 g of NaCl in 100 ml of distilled water with gentle heating. Subsequently, 10 g of Nigrosin

was added to the Eosin solution and brought to boil. The solution was allowed to cool at room temperature and was then filtered through filter paper of 0.5 mm thickness to remove coarse and gelatinous precipitates. The filtrate was stored in a dark glass bottle until use.

To stain, 50 µl of the sperm suspension were added to 50 µl of the Eosin-Nigrosin in an Eppendorf vial. A smear was made on a glass slide and left to air dry. Slides were then viewed with a 100X oil immersion objective using a light microscope. At least 200 spermatozoa were counted and the percentage of live sperm was then calculated. Dead sperm appear pink/purple and live sperm white.
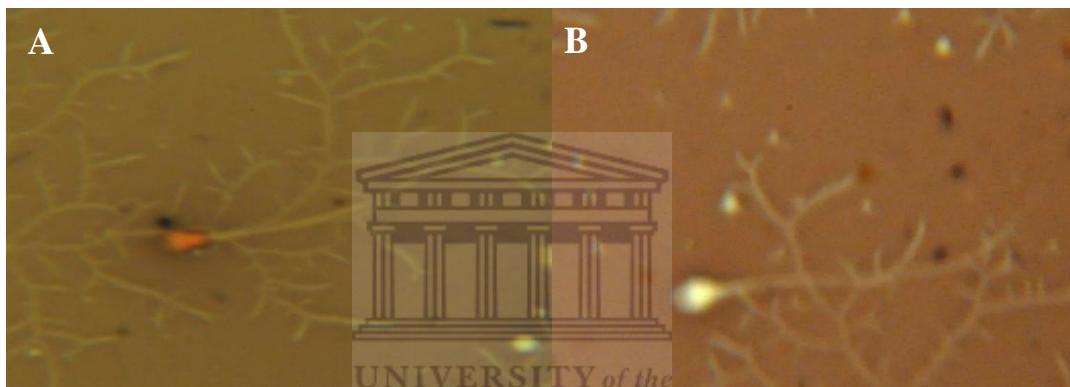
Figure 10: Eosin-Nigrosin stain A) Dead spermatozoa stained pink and B) live spermatozoa appear white (magnification X1000).

2.5.5 Determination of sperm acrosome reaction

Acrosome reaction (AR) is the exocytotic release of the acrosomal contents and predictive of fertilization *in vitro* (Cummins et al., 1991; Henkel et al., 1993) and is essential for sperm penetration of the zona pellucida (ZP) (Wassarman et al., 1983).

Acrosome reaction was determined by using the Triple staining technique according to Talbot and Chacon (1981) following the protocol of Henkel et al. (1993). The Triple stain consists of Trypan Blue (TB), Rosè Bengal (RB) and Bismarck Brown Y (BB Y), where gluteraldehyde was used as fixative. Each of which were prepared as follows:

Trypan Blue stain (determination of dead cells):

Dissolve 2 % TB in cold HTF then filtered with filter paper to remove all undissolved granules. The solution was then stored until use.

Rosè Bengal stain (outlines the intact acrosomal cap):

Dissolve 0.8 % RB in 0.1 M Tris added to 250 ml of distilled water. The pH was adjusted to 5.6 with concentrated HCl and continuing with 2 N HCl as required. The solution was then stored in a dark bottle as it is light sensitive (storage up to ½ week).

Bismarck Brown Y stain (determination of live cells):

Dissolve 0.8 % BB Y in distilled water and pH was adjusted to 1.8 with HCl. The solution was then stored in a dark bottle (storage up to 2 weeks).

Glutaraldehyde solution (fixative):

Mix 3 % glutaraldehyde to HTF and stored (until required).

The samples, after incubation with TA, were aliquoted to 100 µl then incubated for 2 hours at 37°C. Following incubation 100 µl of 2 % Trypan blue were added and incubated for a further 15 min at 37°C. Subsequently, 1 ml HTF (without HSA) was added and centrifuged for at 300xg 10 min as a washing step, the supernatant was discarded and the pellet fixed with 200 µl of 3 % glutaraldehyde and incubated for 20 min at 37°C.

Afterwards, samples were then diluted with 1 ml HTF, centrifuged at 300xg for 10 min, supernatant discarded and a smear was made with 10 µl of the pellet and air dried. Following the air dry process, slides were stained with 0.8 % Bismarck brown for 5 min in water bath at 40°C then washed 3 times with distilled water and counter stained with 0.8 % Rosé Bengal for 1 hour at room temperature. Slides were then washed 3 times with tap water and immersed in 100 % ethanol and removed to air dry.

Subsequent to staining procedure, the slides were viewed with a 100X oil immersion objective using a light microscope. Analyses of at least 200 sperm were performed for live reacted and live non reacted acrosomal sperm and a percentage of live reacted sperm was calculated (Figure 10).
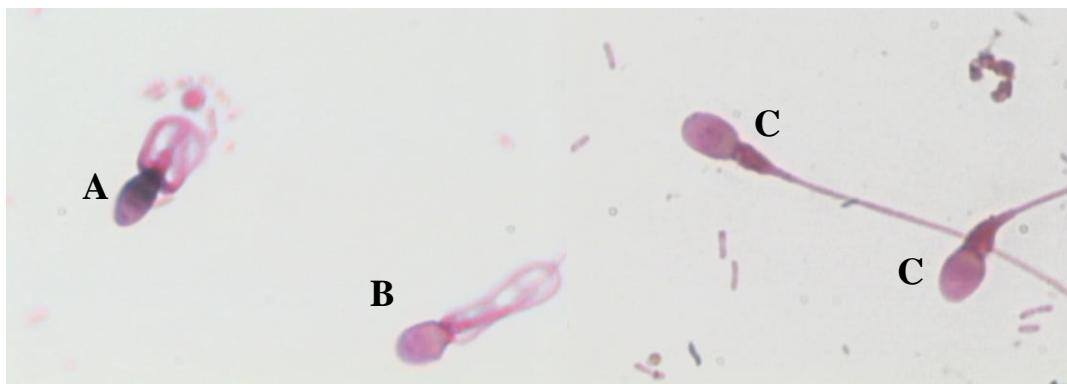
Figure 10: Triple stain display in sperm acrosomal reaction; A) dead reacted, B) live reacted, C) live non-reacted (magnification X1000).

2.5.6 Determination of sperm ROS production

The sperm ROS production was determined according to Henkel et al. (2003) using dihydroethidine (DHE) fluorescing probe and can be visualized as sperm that produced excessive ROS.

Sperm samples were washed with HTF-HSA and aliquoted. A stock solution of 20 µM DHE in PBS at pH 7.4 was prepared. Sperm samples of 100 µl were centrifuged for 10 min at 500xg, supernatant discarded and resuspended in 100 µl PBS and 20 µl of DHE stock added and incubated for 15 min at 37°C. Thereafter, 10 µl of each sample concentration was viewed on a slide covered by a cover slip under oil immersion using an epifluorescence microscope (Zeiss). At least 200 spermatozoa counted and the percentage of red fluorescing sperm (Figure 11), indicating excess ROS production, was calculated.
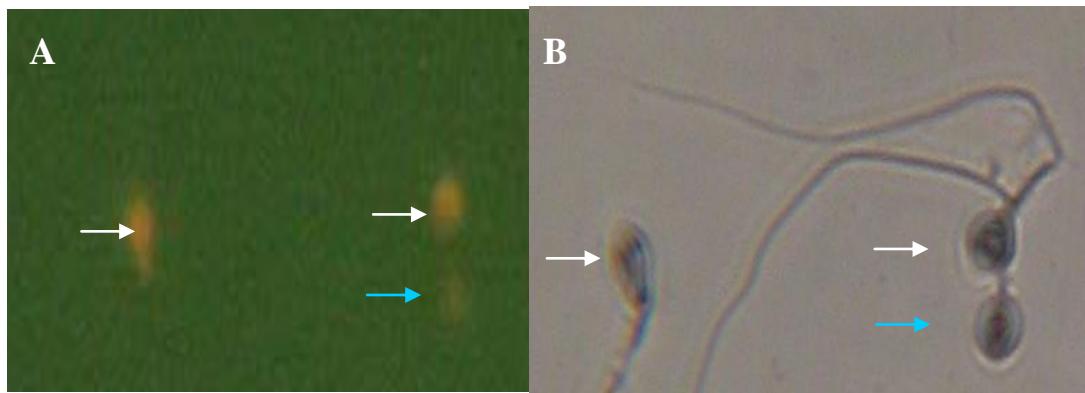
Figure 11: A) Spermatozoa after staining with DHE; ROS-positive sperm (white arrows) fluoresced orange/red, and B) ROS -negative sperm (blue arrows) (X1000 magnification).

2.5.7 Determination of the sperm mitochondrial membrane potential (Δψm)

During mitochondrial respiration within spermatozoa energy produced and stored as an electrochemical gradient across the mitochondrial membrane. This accumulation of energy in healthy cells creates a mitochondrial trans-membrane potential, called delta-psi (Δψm). Disruption of mitochondrial membrane potential (MMP/Δψm) induces depolarization of the trans-membrane potential causing the onset of apoptosis in spermatozoa and loss of oxidative phosphorylation (Ly et al., 2003). Accordingly, calculation of the mitochondrial membrane potential provides and indication of mitochondrial functionality (Donnelly et al., 2000).

The percentage of spermatozoa with intact mitochondrial membrane potential was determined by means of the lipophilic cationic dye 5,5'6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide. The protocol provided by the manufacturer was modified as follows: Reaction buffer was diluted with distilled water 1:10 and 20 µl Stabilizer was added per milliliter buffer with 1 µl of DePsipher dye. Finally, this solution was added to 500 µl prepared Reaction buffer and vortexed thoroughly and finally centrifuged for 1 min at 10,000xg.

Following the preparation of the DePsipher solution, only 50 µl of the supernatant were used to re-suspend the pellet of 100 µl sperm suspension. Spermatozoa were then incubated for 20 min at 37°C. After the incubation period, 10 µl of each sample was viewed with 488 nm excitation and 590 emission filters with at X1000 magnification with an oil immersion objective using an epifluorescence microscope (Zeiss).

At least 200 spermatozoa were evaluated. Spermatozoa exhibiting a green fluorescence within their mid pieces were regarded as having disturbed $\Delta\psi$m (Figure 12), while those spermatozoa showing red fluorescence were regarded as having intact $\Delta\psi$m. The percentage of sperm with intact $\Delta\psi$m was calculated.
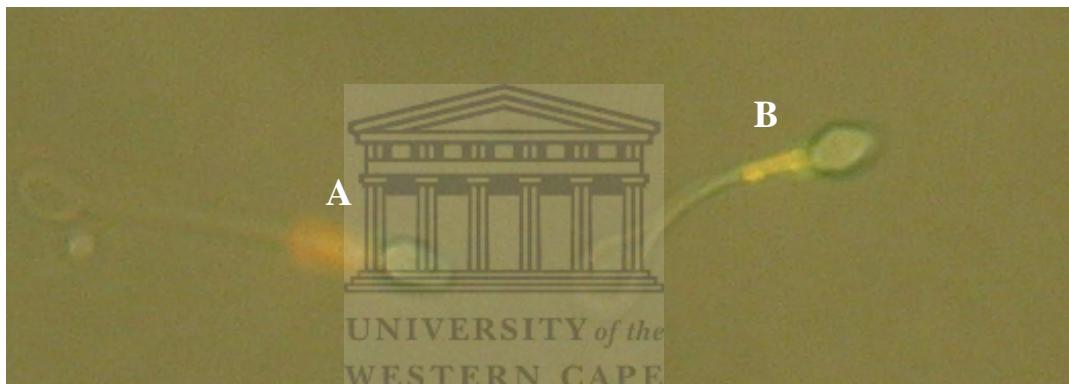
Figure 12: A) intact mitochondrial membrane potential fluorescing orange in the mid-piece of spermatozoa and B) disrupted mitochondrial membrane potential fluorescing green (X1000 Magnification).

2.5.8 Determination of sperm DNA-fragmentation

The TUNEL assay was used according to Henkel et al. (2004). The assay is used as it detects DNA single and double strand breaks (Gorczyca et al., 1993) in an enzymatic reaction by labelling the free 3'-OH and the modified nucleotides with terminal deoxynucleotidyl transferase.

After incubation, samples were centrifuged at 300xg for 10 min and the pellet was resuspended with 100 µl PBS. Thereafter, a smear was made using 100 µl of sperm suspension on Superfrost slides. After air drying, slides were fixed with freshly prepared 4 % methanol-free formaldehyde in PBS for 25 min at 4°C and subsequently washed in PBS for 5 min at room temperature. Thereafter, slides

were permeabilized in 0.2 % triton X-100 in PBS for 5 min and washed with fresh PBS twice for 5 min. Excess liquid was removed by tapping. After the permeabilization, samples were equilibrated with 100 µl of equilibrium buffer for 10 min. Hereafter, 20 µl of TdT incubation buffer (containing; 45 µl Equilibrium buffer, 5 µl Nucleotide mix and 1 µl TdT enzyme) were then added to each slide and covered with plastic cover slips and incubated in a dark humidified chamber for 1 hour at 37°C.

Following, cover slips were carefully removed and slides were then immersed in diluted 2x SSC solution in a Coplin jar for 15 min at room temperature to terminate the reaction. Thereafter, slides were washed twice in distilled water for 5 min at room temperature. Excess water was carefully removed and slides were analyzed using an epifluorescence microscope (Zeiss) at 400X magnification with an oil immersion objective.

At least 200 spermatozoa were counted. Spermatozoa that fluoresced brightly were counted as TUNEL-positive (Figure 13) indicating DNA fragmentation has occurred within the sperm nucleus and a percentage was calculated.
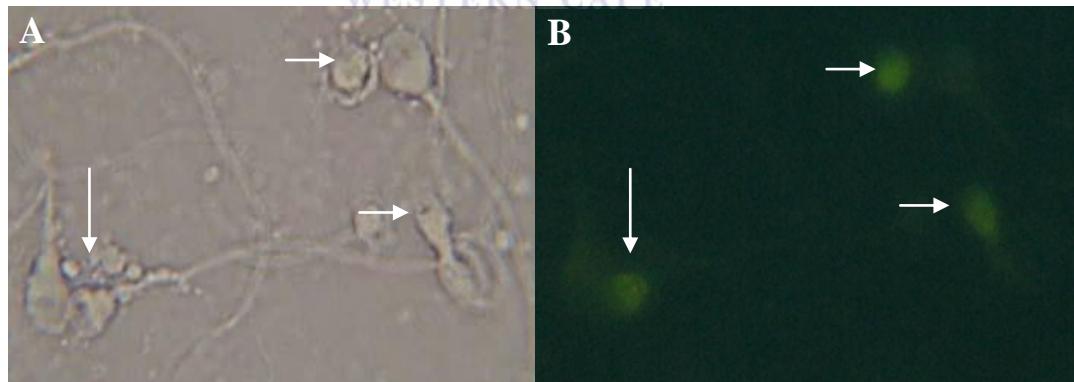
Figure 13: A) Sperm with nuclear DNA damage B) TUNEL-positive sperm fluoresced bright green (white arrows) after TUNEL assay (400X magnification).

2.6 Investigation of cell culture *in vitro*

2.6.1 Concentrations of TA used for incubations for TM3-Leydig and TM4-Sertoli cells

TM3-Leydig and TM4-Sertoli cells were used and final concentrations namely; 0.4 µg/ml, 0.8 µg/ml, 1.6 µg/ml, 3.125 µg/ml, 6.25 µg/ml, 12.5 µg/ml, 25 µg/ml, 50 µg/ml, and control (without extract) were prepared of the extract with growth medium. Preparations were performed under sterile working conditions in laminar flow and sterilized with 70 % alcohol, aliquoted in 50 ml sterile test tubes and frozen at 4°C till further use.

2.6.2 Preparation of TM3-Leydig and TM4-Sertoli cells *in vitro*

Two cell lines, TM3-Leydig cells and TM4-Sertoli cells were used in this study. TM3-Leydig cells were used as they produced testosterone and TM4-Sertoli cells in the presence of Follicular stimulating hormone (FSH) increases the cyclic Adenosine Monophosphate (cAMP) production and thereby leading to pyruvate production, as functional parameter.
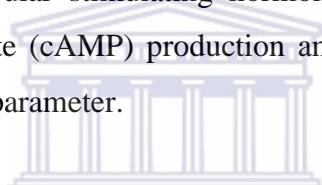
TM3-Leydig and TM4-Sertoli cells were acquired from ATCC. The cells were obtained from BALB/c mice testis age 11 to 13 days old and culturing instructions followed. A 1 ml vial of frozen cells was thawed in a water bath at 37°C and sterilized with 70 % ethanol. Thereafter, 1 ml of the cells was transferred into a sterile 25 cm^2 tissue culturing flask with growth medium.

Following this, the flask was incubated at 37°C and 5 % CO_2 for 24 hours. After the 24 hours incubation period the medium was changed and the cells were allowed to grow to a 70 to 80 % confluence. Thereafter, cells were allowed to grow for 48 hours before the depleted media was removed and replaced with fresh growth medium. This was performed for both TM3-Leydig and TM4-Sertoli cell lines under sterile working conditions.

2.6.3 Sub-culturing of cells

Sub-culturing is conducted to prevent cell death due to over growth of the cells and thereby depleting nutrients provided within the growth medium. After cells have grown to 70 to 80 % confluency, depleted medium was removed from the

flask by pipette and cells were washed with 3 ml of PBS. Thereafter, 1 ml of 0.25 % (w/v) Trypsin 0.53 mM EDTA (detachment agent; lyses cells) was added and allowed to stand for a 5 min under visual control. To inactivate Trypsin, 1 ml of growth media as added to the flask.

Following the detachment process, 1 ml of the detached cells were transferred into a new tissue culture flask with fresh growth medium with supplements as mentioned previously and passage recorded. Recording the passage gives an indication to track the age of the cells. Fresh growth medium was in turn added to the first flask as well and both incubated for further growth.

2.6.4 Freezing of cells

Freezing of cells was performed to store cells until needed. When the cells reached 70 to 80 % confluency the same procedure was followed as with sub-culturing. Except after the cells detached and trypsin was inactivated, the cells were transferred into a test tube whereby it is centrifuged at 727xg for 5 min. The supernatant was removed and 1 ml growth medium plus 10 % DMSO (freezing medium) added. Thereafter, cells were resuspended and transferred into a cryovial. Following, cells within the cryovial were allowed to cool down in a step gradient manner over night at room temperature, then -80°C freezer followed by -196°C in liquid Nitrogen.

2.6.5 Plating/Seeding of cells

As cells reached 70 to 80 % confluency, the medium removed and the flask was washed twice with 1 ml PBS to remove floating or dead cells. Thereafter, 1 ml of trypsin was added to the flask and allowed to detach. To inactivate trypsin, 1 ml of growth media was added to the flask and cells resuspended. Moreover, cells were transferred into a sterile test tube and centrifuged at 727xg for 5 min.

The supernatant was removed and 1 ml fresh growth medium was added and cells resuspended by careful aspiration with pipette. An Eppendorf vial was used and 20 µl of 2 % Trypan blue (dead cell stain indicator), 15 µl PBS and 5 µl (if small pellet) or 10 µl (if big pellet) of cells were added.

To determine the amount of cells needed to perform the experiment a total cell count was conducted: 10 µl of the suspension were transferred onto a haemocytometer counting chamber and viewed under the microscope. The total cell count result was calculated (Equation 2) according to the formula below:

Equation 2: <u>Number of cells needed</u>

Total number of cells counted X 1000 = volume of cells required (µl)

Therefore, for TM3-Leydig cells 4000cells/ml for 48 hours experiments and 8000cells/ml for 96 hours experiments per 96-well plates proved optimum. For TM4-Sertoli cells 4000cells/ml for 48 hours and 2000cells/ml for 96 hours experiments were used per 24-well plates.

After plating the cells and adding growth medium to the wells for the experiment, the cells were incubated at 37°C and 5 % CO_2 for 24 hours to acclimatise and attach. Thereafter, TA in 200 µl of growth medium was added to final concentrations of 0.4 µg/ml, 0.8 µg/ml, 1.6 µg/ml, 3.125 µg/ml, 6.25 µg/ml, 12.5 µg/ml, 25 µg/ml, 50 µg/ml, and control (without extract) for both TM3-Leydig and TM4-Sertoli cells.

2.6.6 Determination of cell viability of TM3-Leydig and TM4-Sertoli cells

A test for cell viability is performed to determine the percentage of living cells or any stress caused on cells. Viability was tested for both TM3-Leydig and TM4-Sertoli cells for 48 and 96 hours using the XTT assay. XTT is a colorimetric assay for the non-radioactive quantification of cell proliferation and viability. The assay is based on the cleavage of the yellow tetrazolium salt XTT to form an orange formazan dye by metabolic active cells (Scudiero et al., 1988) and thus can be quantified using ELISA reader.

After the cells were seeded in a 96-well plate and acclimatised for 24 hours, the medium was removed and replaced by adding the different Tongkat Ali concentrations and incubated for 48 hours and 96 hours, respectively. On the termination day of the experiment, 0.02 ml electron coupling reagent (provided by kit) and 1 ml XTT labelling reagent were mixed together. One hundred µl of the

XTT mixture were added to each well and incubated for 4 hours at 37°C with each plate covered with aluminium foil as the test is light sensitive. Following the incubation period, each plate was read using an ELISA reader. The absorbance was read at 450 nm wave length. The optical density was then calculated.

2.6.7 Determination of protein concentration in TM3-Leydig and TM4-Sertoli cells

Total protein content of the cells is used as reference or base value for the measurement of biochemical parameters. Protein determination is performed by using BIO-RAD Protein assay, which is based on the modified method of Lowry et al. (1951). In this process, the dye binds unspecifically to amino groups of any protein within the sample.

After the 48 hours and 96 hours of incubation with the different concentrations of TA and control without TA, the protein contents were determined for cell proliferation. The medium of each well was removed and 200 µl lysis reagent consisting of 0.5 M NaOH + 0.1 % SDS were added. Following the addition of the reagent, the plates were incubated at room temperature for 30 min on a plate shaker on a gentle setting. Next, 20 µl of the lysate was transferred into another 96-well plate in duplicate and 25 µl of reagent A and 200 µl of reagent B were added and incubated for 30 min at room temperature. The plate was read by an ELISA reader at the wavelength of 690 nm and compared to a standard curve which was established by using BSA.

2.6.7.1 BSA standard curve

A stock solution of 1400 µg/ml of BSA was prepared by dissolving it in 1 ml of the lysis reagent and further diluted into concentrations blank (only lysis reagent), 200, 600 and 1000 µg/ml (Table 2). Following, a standard curve was calculated and the results compared to determine the effects of the different TA concentrations for both TM3-Leydig and TM4-Sertoli cells (Figure 14).

Table 2: BSA concentration dilutions for Standard curve

BSA Stock solution (µl)	Lysis reagent (µl)	BSA Concentration (µg/ml)
100	40	1000
100	133	600
100	600	200
0	600	0 (blank)
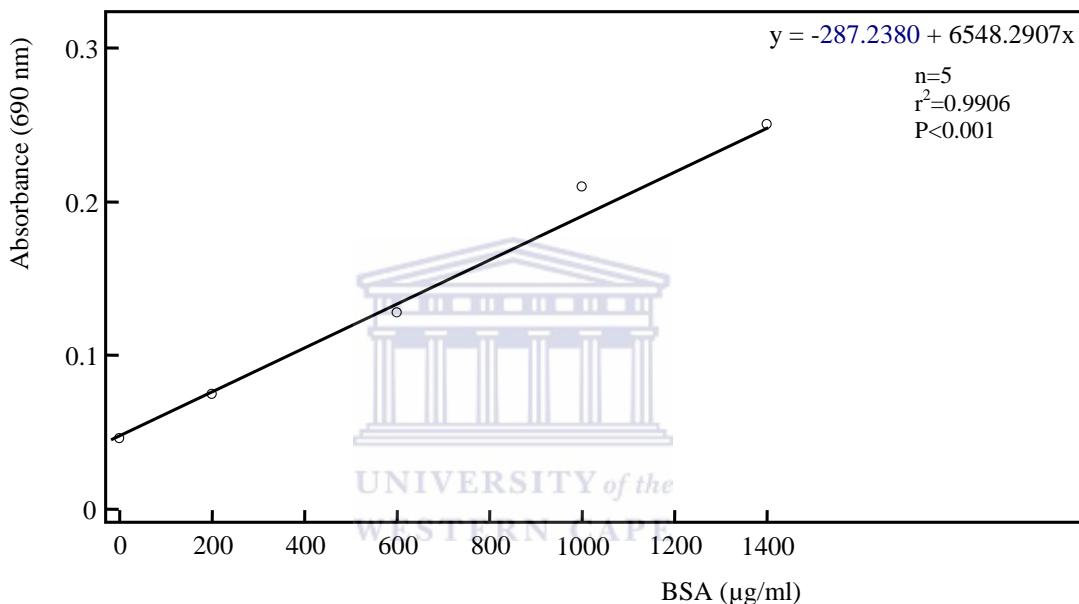
Figure 14: BSA Standard Curve graph

2.6.8 Determination of testosterone production in TM3-Leydig cells

Testosterone is an anabolic steroid produced by Leydig cells in the testes. TM3-Leydig cells are unable to produce testosterone without human chorionic gonadotropin (hCG) and thus it is added to trigger the production of testosterone.

A stock solution was made of hCG and 5 µl/ml was added to each concentration of TA. After TM3-Leydig cells were exposed to TA at periods of 48 hours and 96 hours, respectively testosterone production was tested by following the manufacturer's instructions of the Testosterone ELISA kit. All necessary solutions and antibody-coated microplate were provided in the kit.

Microtiter plate:

A 96-well microtiter plate which is coated with monoclonal (mouse) anti-testosterone antibody.

Standard:

Standard vials of 1 ml each with concentrations 0, 0.2, 0.5, 1, 2, 6, 16 ng/ml.

Enzyme conjugate:

Testosterone conjugated to horseradish peroxidase, 1 vial 25 ml.

Substrate solution:

Containing tetramethylbenzidine (TMB), 1 vial 25 ml.

Stop solution:

Containing 0.5 M H_2SO_4, 1 vial 14 ml.

Wash solution:

To prepare the wash solution 30 ml of the 40x concentrated solution were added to 1170 ml of deionized water to a final volume of 1200 ml (2 weeks storage).

The testing procedure required 25 µl sample to be transferred into the 96-well microtiter plate with the addition of 200 µl Enzyme Conjugate mixed thoroughly for 10 sec and incubated for 1 hour at room temperature. After the incubation period, the contents were shaken out briskly and rinsed 3 times with 400 µl of diluted wash solution and striked sharply on an absorbent paper towel to remove excess solution. Following this, 200 µl of Substrate solution were added to each well and incubated for 5 min at room temperature upon which 100 µl of Stop solution were added to stop the enzymatic process.

The absorbance was determined at 450 nm with an ELISA plate reader and the concentration of testosterone production was determined using Equation 3 (48 hours) and 4 (96 hours).

Equation 3: y = (6.0333) + (-16.3948) LOG(x)

Equation 4: y = (8.5668) + (-15.9414) LOG(x)

2.6.9 Determination of pyruvate in TM4-Sertoli cells

Pyruvate is determined by the principle whereby pyruvate is trapped by glutamate-pyruvate transminase (GPT) in the presence of L-glutamate. The concentration of pyruvate was determined by preparing the following solutions;

Triethanolamine buffer (TE buffer):

Triethanolamine hydrochloride 23.3 g and 0.47 g EDTA were dissolved in 200 ml of distilled water. Afterwards, the pH was adjusted to pH 7.6 by the slow addition of 20 ml 2 N NaOH following the addition of distilled water to the final volume of 250 ml.

$NADH + H^+$ solution:

Prepared by adding 5 mg/ml of $NADH + H^+$ to distilled water.

Potassium phosphate buffer:

This solution was prepared by weighing 142 mg Na_2HPO_4 and 27 mg KH_2PO_4 then dissolved in 100 ml of glycerol. Afterwards, the pH adjusted to pH 7.5 with 0.5 M NaOH.

Lactate dehydrogenase (LDH):

Prepared by adding 100 µl of LDH to 900 µl triethanolamine buffer on the day of testing.

After incubation with TA at 48 hours and 96 hours, respectively, 200 µl of the supernatant medium was transferred into an Eppendorf vial and heated for 5 min at 95ºC (inactivation of enzymes) and frozen at -18ºC until day of testing. To determine the pyruvate concentration, samples were then thawed at room temperature, 600 µl TE buffer and 20 µl $NADH+H^+$ solution added and thoroughly mixed. Following, samples were incubated at room temperature for 10 min (E10) and measured at 340 nm using a spectrophotometer. After the 10 min incubation 10 µl of LDH was added and incubated at room temperature for

1 hour (E60) and measured again at 340 nm. The difference (D) between E10 and E60 was calculated and compared to the pyruvate standard curve.

2.6.9.1 Pyruvate standard curve

Pyruvate standard curve (Figure 15) was determined by preparing a stock solution of 11 mg of sodium pyruvate in 100 ml of TE buffer. The standard curve was calculated using concentrations namely; 1.4 µg/ml, 2.8 µg/ml, 5.5 µg/ml and 11 µg/ml (Table 3) and pyruvate concentrations established.

Table 3: Concentrations of Pyruvate Standard curve

Pyruvate concentration (mg/ml)	Pyruvate stock solution (µl)	TE buffer (µl)
1.4	12.5	187.5
2.8	25	175
5.5	50	150
11	100	100

The standard curve plot shows:
$$y = (-0.2503) + (0.05962) x + (-0.00001694) x^2$$
n=6
$r^2=0.9961$
P<0.001
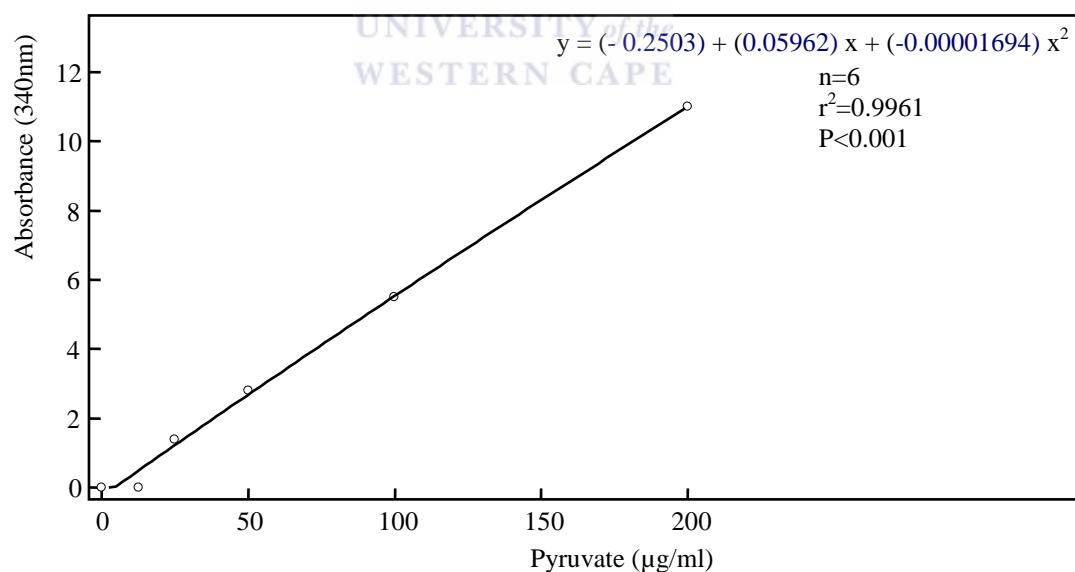
Figure 15: Standard curve for Pyruvate.

2.7 Statistical analysis

All statistical calculations were performed using the MedCalc statistical software (Version 12.3; Mariakerke, Belgium). After testing for normal distribution by means of the Kolmogorov-Smirnov test, appropriate statistical tests, either parametric (Pearson correlation, repeated measures ANOVA, paired samples (t-test) or non-parametric (Spearman Rank correlation, Wilcoxon test) were performed. Tukey tests were carried out to detect outliers. Data were expressed as mean±SD or SEM. A P-value of $P<0.05$ was considered significant.

Chapter 3: Results

3.1 Effects of Tongkat Ali extract on human spermatozoa *in vitro*

Treatment of ejaculated human spermatozoa from 40 semen samples (27 patients and 13 healthy sperm donors) with different concentrations (1 µg/ml, 10 µg/ml, 20 µg/ml, 100 µg/ml and 2000 µg/ml) of the Tongkat Ali (TA) extract was conducted for washed spermatozoa as well as for spermatozoa prepared by means of the swim-up technique. The following parameters were tested: total motility (%), progressive motility (%), viability (%), live acrosome reacted sperm (AR) (%), sperm production of reactive oxygen species (ROS) (%), DNA fragmentation (%) and intact mitochondrial membrane potential (%).

3.1.1 Effect of Tongkat Ali on sperm motility

Although a marked decrease in total motility at the highest concentration (2000 µg/ml) was found for both washed and swim-up spermatozoa when compared to the control after incubation with TA, only a marginal significance (P=0.0554) was found for washed spermatozoa. For samples, which were just washed, a significant trend (ANOVA trend analysis: P=0.0452) to lower total motility rates could be observed towards higher concentrations of TA, yet no trend was found for swim-up spermatozoa (ANOVA trend analysis: P=0.4600) (Figure 16A and B).

For progressively motile spermatozoa no trend was observed for washed (ANOVA trend analysis: P=0.1420) and swim-up separated germ cells (ANOVA trend analysis: P=0.0878). Moreover, although the rates were lower for washed (P=0.1650) and swim-up spermatozoa (P=0.0770) at the highest concentration used, no significant decline in progressive motility was found (Figure 17A and B).
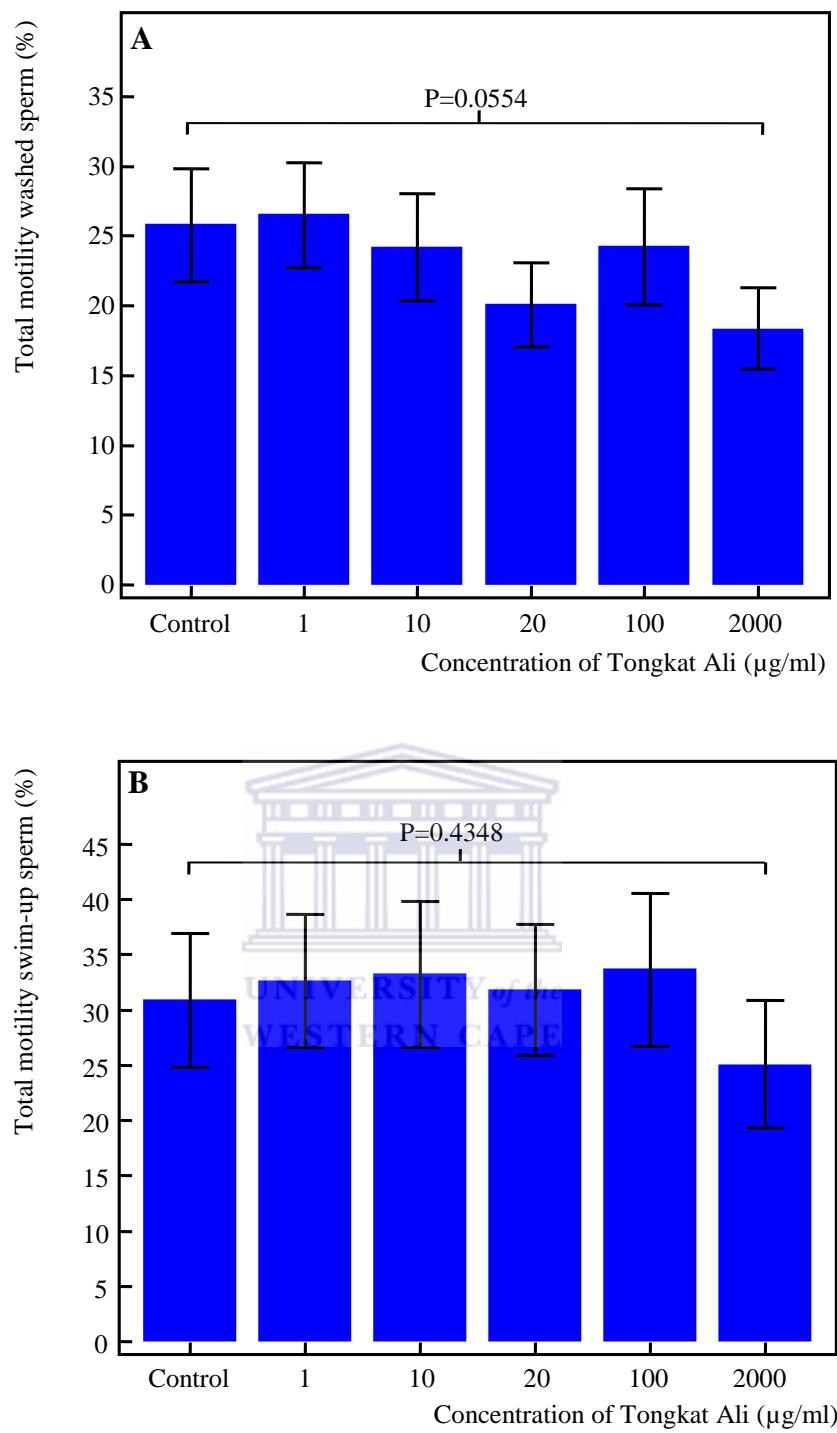
Figure 16: The effect of different concentrations of TA (1, 10, 20, 100 and 2000 μg/ml) on total sperm motility *in vitro*. Statistical difference shown as P<0.05.

A: Effect of different concentrations of TA on washed sperm total motility. Values shown as SEM (n=38). A marginal significant decrease (P=0.0554) at 2000 μg/ml with a significant trend (ANOVA trend analysis P=0.0452) toward lower motility rates can be observed.

B: Effect of different concentrations of TA on swim-up sperm total motility. Values shown as mean±SD (n=18). Although a marked decrease is observed at 2000 μg/ml no significance was observed nor any trend (ANOVA trend analysis: P=0.4600).
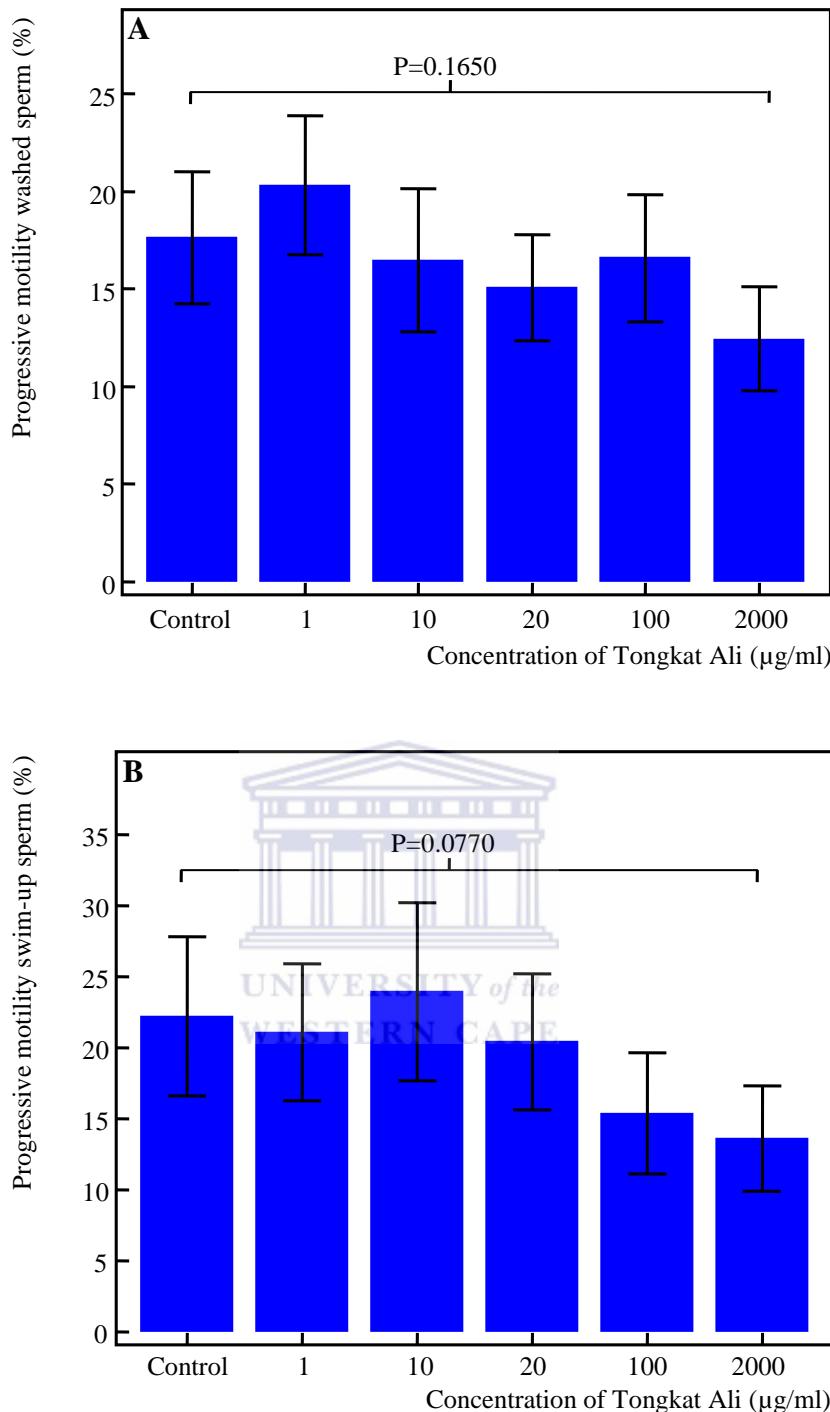
Figure 17: The effect of different concentrations of TA (1, 10, 20, 100 and 2000 μg/ml) on progressive sperm motility *in vitro*. Statistical difference shown as P<0.05.

A: Effect of different concentrations of TA on progressive motility in washed sperm. Values shown as mean±SD (n=38). No significance was observed (P=0.1650) although a marked decrease is seen at 2000 μg/ml. No trend was found (ANOVA trend analysis: P=01420).

B: Effect of different concentrations of TA on progressive motility in swim-up sperm. Values shown as mean±SD (n=18). No significance was observed (P=0.0770) although a marked decrease is seen at 2000 μg/ml. No trend was found (ANOVA trend analysis: P=0.0878).

3.1.2 Effect of Tongkat Ali on sperm viability

After exposing to different concentrations of TA, washed sperm samples showed a significant dose-dependent trend (ANOVA trend analysis: P=0.0059) towards lower viability rates were observed at higher concentrations of TA (Figure 18A). Nevertheless, only the highest concentration (2000 µg/ml) used differed significantly (P=0.0284) from the control, while the difference at 100 µg/ml TA was marginally significant (P=0.0744). In contrast, results for the swim-up spermatozoa neither revealed any difference to the control, nor any trend (ANOVA trend analysis: P=0.4219) (Figure 18B).

3.1.3 Effect of Tongkat Ali on sperm acrosome reaction

While increasing concentrations of TA resulted in a dose-dependant, highly significant (ANOVA trend analysis: P<0.0001) increase in the percentage of live acrosome reacted spermatozoa in washed samples with significant levels starting as from 20 µg/ml TA (Figure 19A), swim-up separated spermatozoa did not show any response to the treatment (Figure 19B).

3.1.4 Effect of Tongkat Ali on sperm reactive oxygen species

A significant positive trend (ANOVA trend analysis: P=0.0080) towards higher percentages of ROS-positive spermatozoa was found at higher concentrations of TA for washed spermatozoa (Figure 20A). In direct comparison, the control differed significantly from the incubation with 20 µg/ml (P=0.0353) and 2000 µg/ml (P=0.0028). For the swim-up spermatozoa, a biphasic relationship was observed. A decrease in the percentage of ROS-positive sperm could be seen from the control to a concentration of 10 µg/ml TA (P=0.1272) followed by an increase from to 2000 µg/ml TA (P=0.1142). Yet, no significant trend (ANOVA Trend analysis: P=0.0580) could be found (Figure 20B).
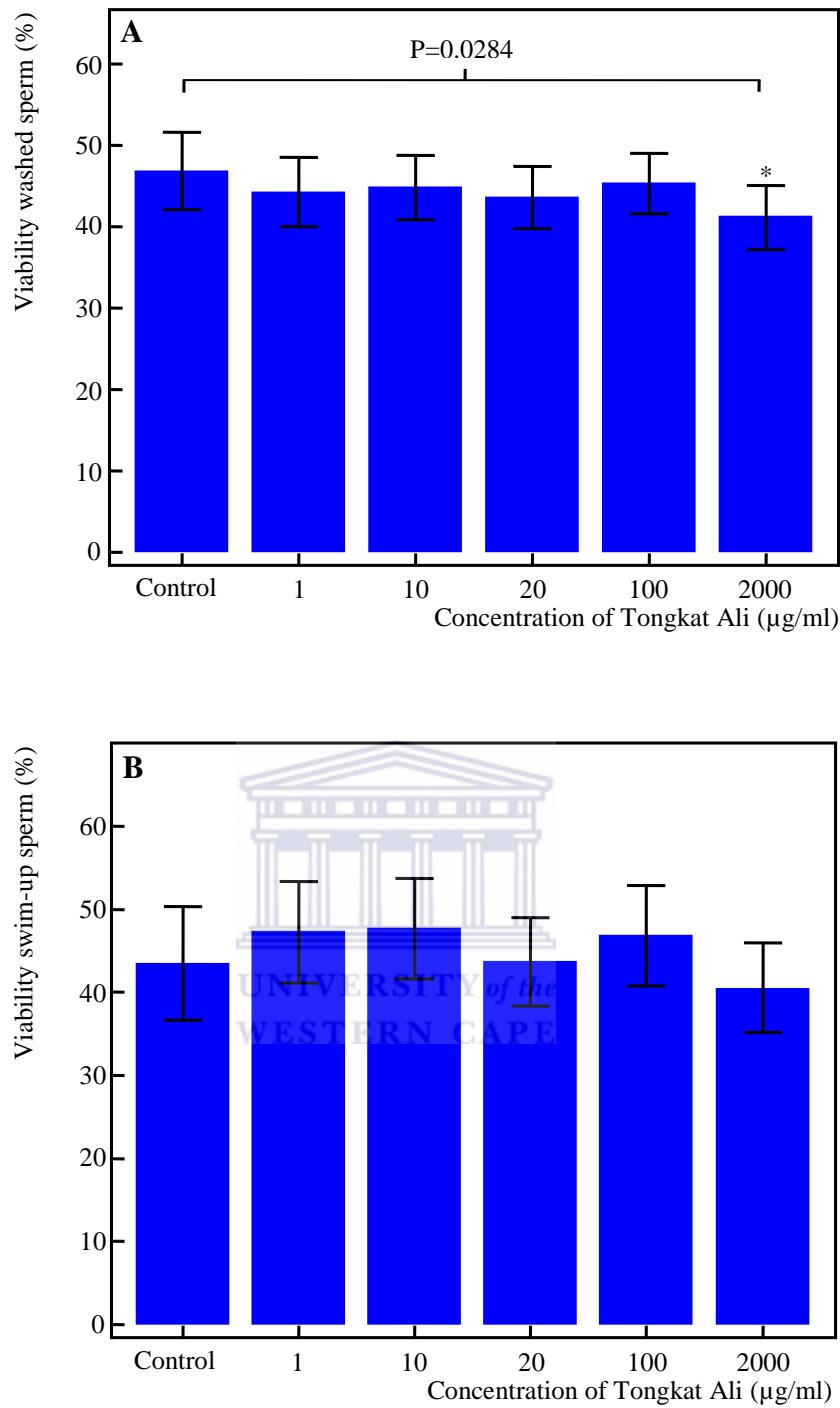
Figure 18: The effect of different concentrations of TA (1, 10, 20, 100 and 2000 μg/ml) on sperm viability *in vitro*. Statistical difference shown as P<0.05 [*].

A: Effect of TA on washed spermatozoa values shown as SEM (n=32). A significant difference (P=0.0284) is observed at 2000 μg/ml with significant trend (ANOVA trend analysis: P=0.0059) toward higher concentrations shows lower viability rates.
B: Effect of TA on swim-up spermatozoa vales shown as mean±SD (n=22). No significant difference was found nor any trend (ANOVA trend analysis: P=0.4219).
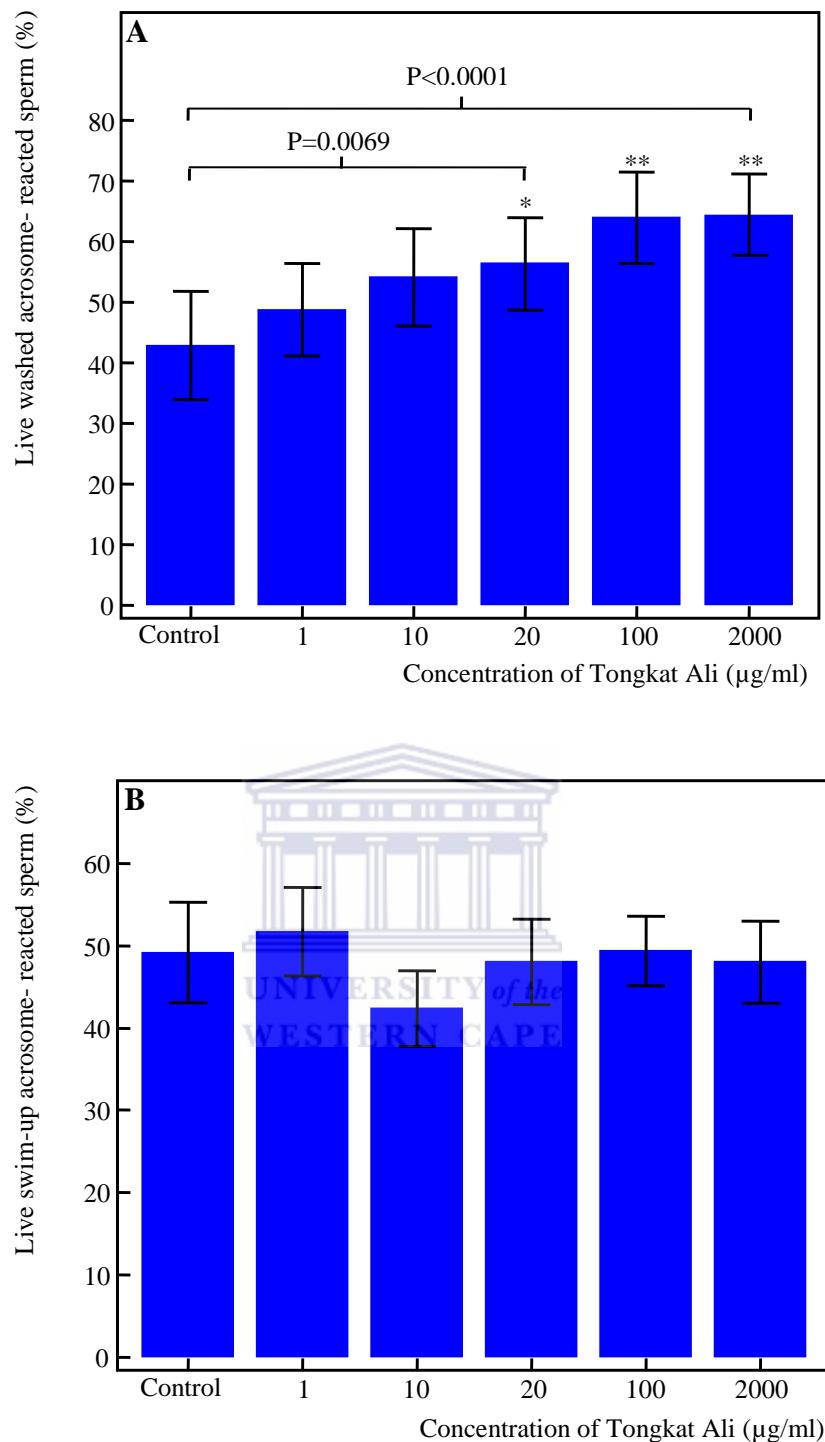
Figure 19: The effect of different concentrations of TA (1, 10, 20, 100 and 2000 μg/ml) on live reacted sperm *in vitro*. Statistical difference shown as P<0.05 [*] and highly significant P<0.0001 [**].

A: Effect of different concentrations of TA on the percentage of live washed acrosome-reacted spermatozoa. Values shown at mean±SD (n=28). A significant increase in acrosome reaction as from 20 μg/ml (P=0.0069) and highly significant at 100 μg/ml and 2000 μg/ml TA (P<0.0001). A highly significant trend (ANOVA trend analysis: P<0.0001) towards higher acrosome reaction rates is observed at higher concentrations of TA.

B: Effect of different concentrations of TA on the percentage of live swim-up acrosome-reacted spermatozoa after swim-up values shown as mean±SD (n=22). No significant difference can be seen nor any trend (ANOVA trend analysis: P=0.8792).
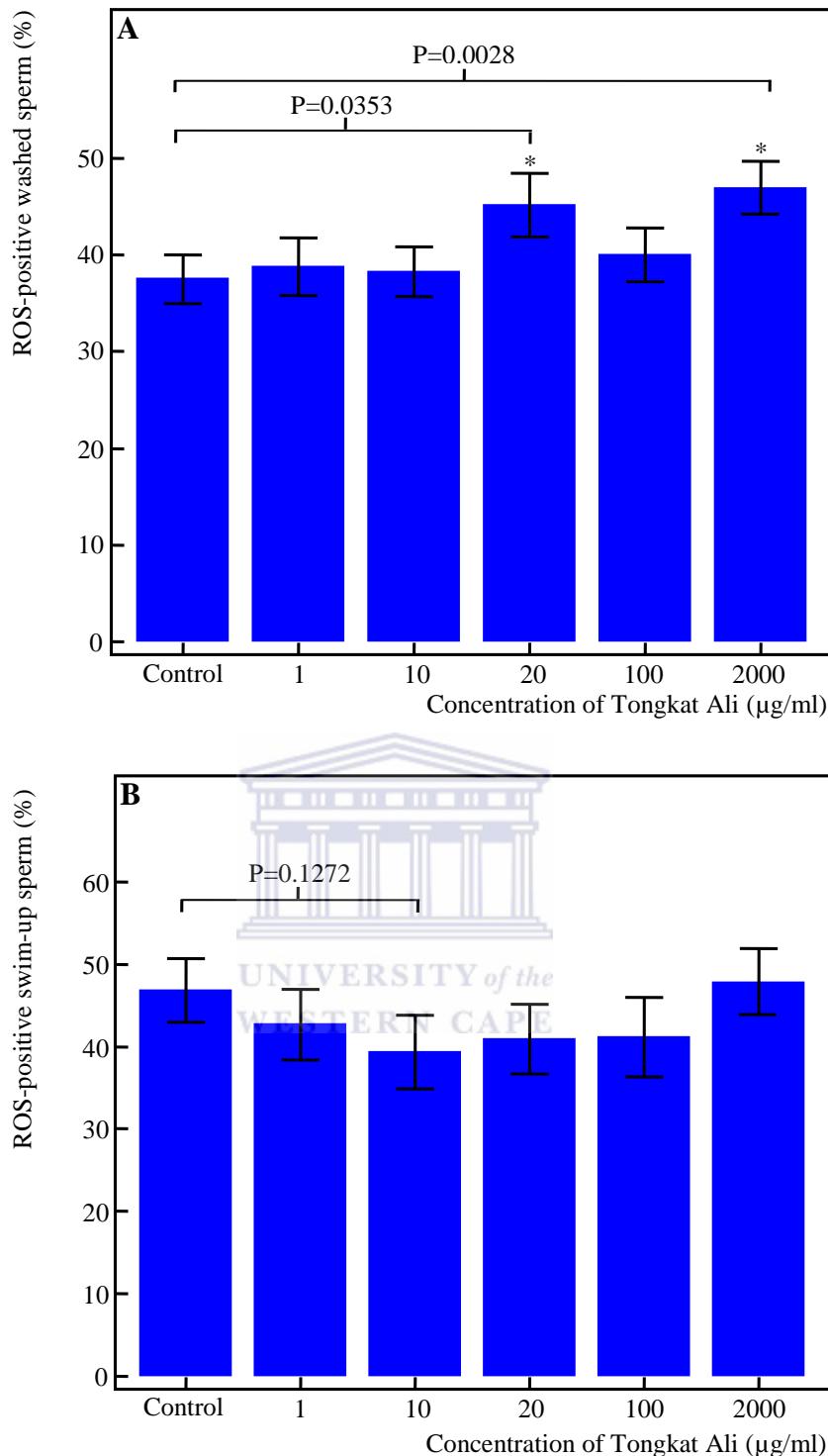
Figure 20: The Effect of different concentrations of TA (1, 10, 20, 100 and 2000 μg/ml) on sperm ROS production *in vitro*. Values shown as mean±SD with Statistical difference shown as P<0.05 [*].

**A**: Effect of TA on the percentage of ROS-positive washed spermatozoa (n=38). A significant trend (ANOVA trend analysis: P=0.008) towards higher percentages of ROS-positive spermatozoa can be seen at higher concentrations of TA with a significant difference (P=0.0028) at 2000 μg/ml.
B: Effect of TA on the percentage of ROS-positive swim-up spermatozoa (n=22). A biphasic, but no significant relationship (P=0.1272) with the lowest percentage of ROS-positive sperm at 10 μg/ml TA extract nor any trend (ANOVA trend analysis: P=0.9505) is observed.

3.1.5 Effect of Tongkat Ali on sperm mitochondrial membrane potential and DNA fragmentation

With regard to mitochondrial membrane potential, the treatment of washed spermatozoa with increasing concentrations of TA did not result in any affect (Figure 21).

Also, no detrimental effect of the TA extract on sperm DNA fragmentation was found neither for washed (ANOVA trend analysis: P=0.9171) (Figure 22A), nor for swim-up sperm (ANOVA trend analysis: P=0.7057) (Figure 22B). Nevertheless, a marked increase at a concentration of 100 µg/ml in the washed sperm set up was found (P=0.3585). However, this appeared to be only within the statistical variation.
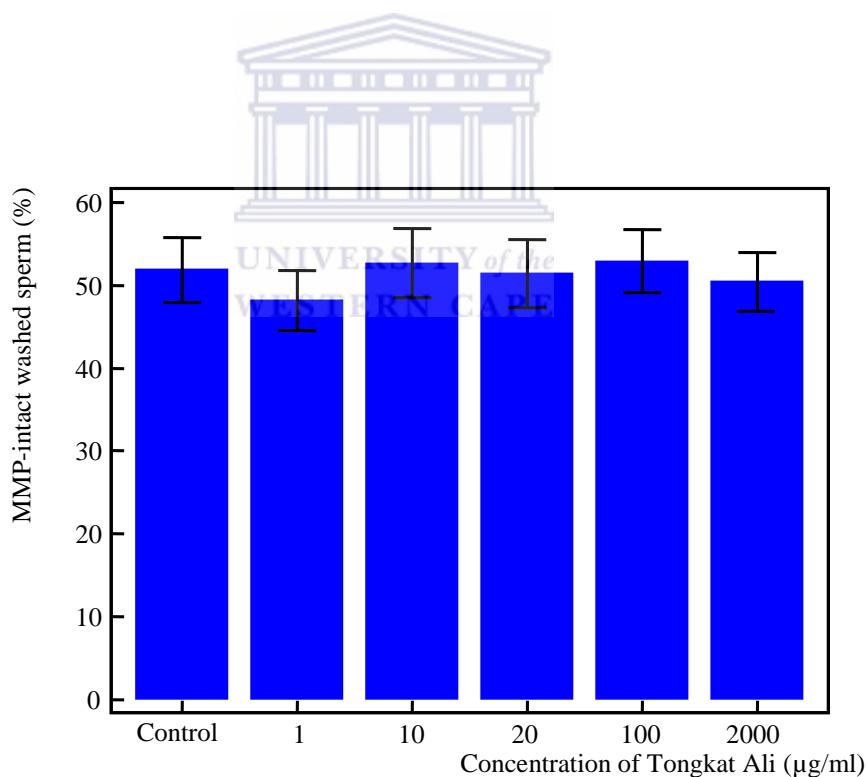
Figure 21: Effect of different concentrations of TA (1, 10, 20, 100 and 2000 µg/ml) on intact-mitochondrial membrane potential for washed sperm *in vitro*. Statistical difference shown as P<0.05. Values shown as mean±SD (n=40). No significance can be seen.
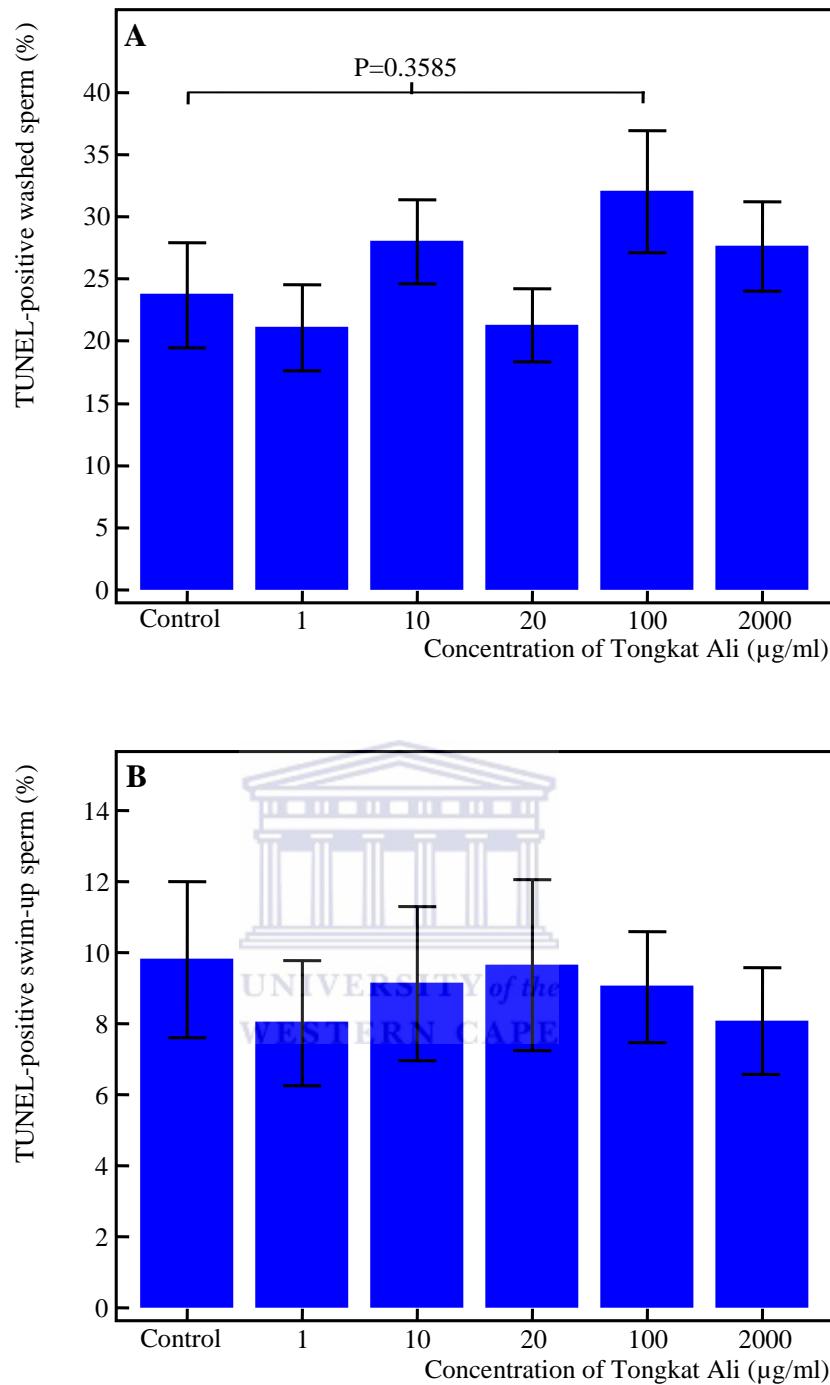
Figure 22: The Effect of different concentrations of TA (1, 10, 20, 100 and 2000 µg/ml) on DNA fragmented sperm *in vitro*. Statistical difference shown as P<0.05.

A: Effect of TA on DNA fragmented TUNEL-positive washed sperm. Values shown as mean±SD (n=25). Although a marked increase at 100 µg TA extract/ml can be observed no significant difference (P=0.3585) nor any trend (ANOVA trend analysis: P=0.9171) is seen.
B: Effect of TA on DNA fragmented TUNEL-positive swim-up sperm. Values shown as mean±SD (n=23). No significant difference is observed nor any trend (ANOVA trend analysis: P=0.7057).

3.2 Effects of Tongkat Ali on TM3-Leydig and TM4-Sertoli cells *in vitro*

All cells were cultured in complete DMEM-F12 media under 5 % CO_2 and 95 % air at a temperature of $37^{\circ}C$ until 70-80 % confluency in culture flasks are reached.

TM3-Leydig cells were seeded at concentrations of 8000 cells/ml (for 48 hours exposure to the extracts) and 4000 cells/ml (for 96 hours exposure to the extracts) into 96-well plates. After 24 hours incubation, the TM3-Leydig cells were exposed to TA at 0.4, 0.8, 1.6, 3.125, 6.25, 12.5, 25 and 50 µg/ml. The following parameters were tested: viability, protein and testosterone concentration.

Similarly, TM4-Sertoli cells were seeded at concentrations of 4000 cells/ml (for 48 hours exposure to the extracts) and 2000 cells/ml (for 96 hours exposure to the extracts) into 24-well plates. After incubation for 24 hours, TM4-Sertoli cells were exposed to 0.4, 0.8, 1.6, 3.125, 6.25, 12.5, 25 and 50 µg/ml of TA. The following parameters were tested: viability, protein and pyruvate concentration.
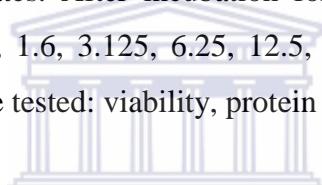
3.2.1 Effect of TA on TM3-Leydig cell viability

After TM3-Leydig cells were incubated with the different concentrations of TA for 48 hours, a steady, yet not significant (ANOVA trend analysis: P=0.1088) increase in viability rates between 19 % and 43 % as compared to the control was found (Figure 23A). Although an increase in viability rates of 43 % was observed at a concentration of 50 µg/ml TA, this increase was not significant (P=0.0833).

In TM3-Leydig cells that were incubated for 96 hours with TA at different concentrations, no significant difference was found at all concentrations, even though at 25 µg/ml a marked decrease of 13 % (P=0.0600) was seen. Yet, a dose dependency (ANOVA trend analysis: P= 0.0104) towards decreased viability percentage was observed (Figure 23B).

When comparing the viability rates of the 48-hour and 96-hour incubation periods with TA extract, at 48-hours exposure all concentrations produced an increase in viability (19-43 %), where as at 96-hours exposure a lesser increase in viability

(3-9 %) was observed. However at higher concentrations a decline in viability rates was seen 25 µg/ml (-13 %) and 50 µg/ml (-8 %) which indicates cell death.
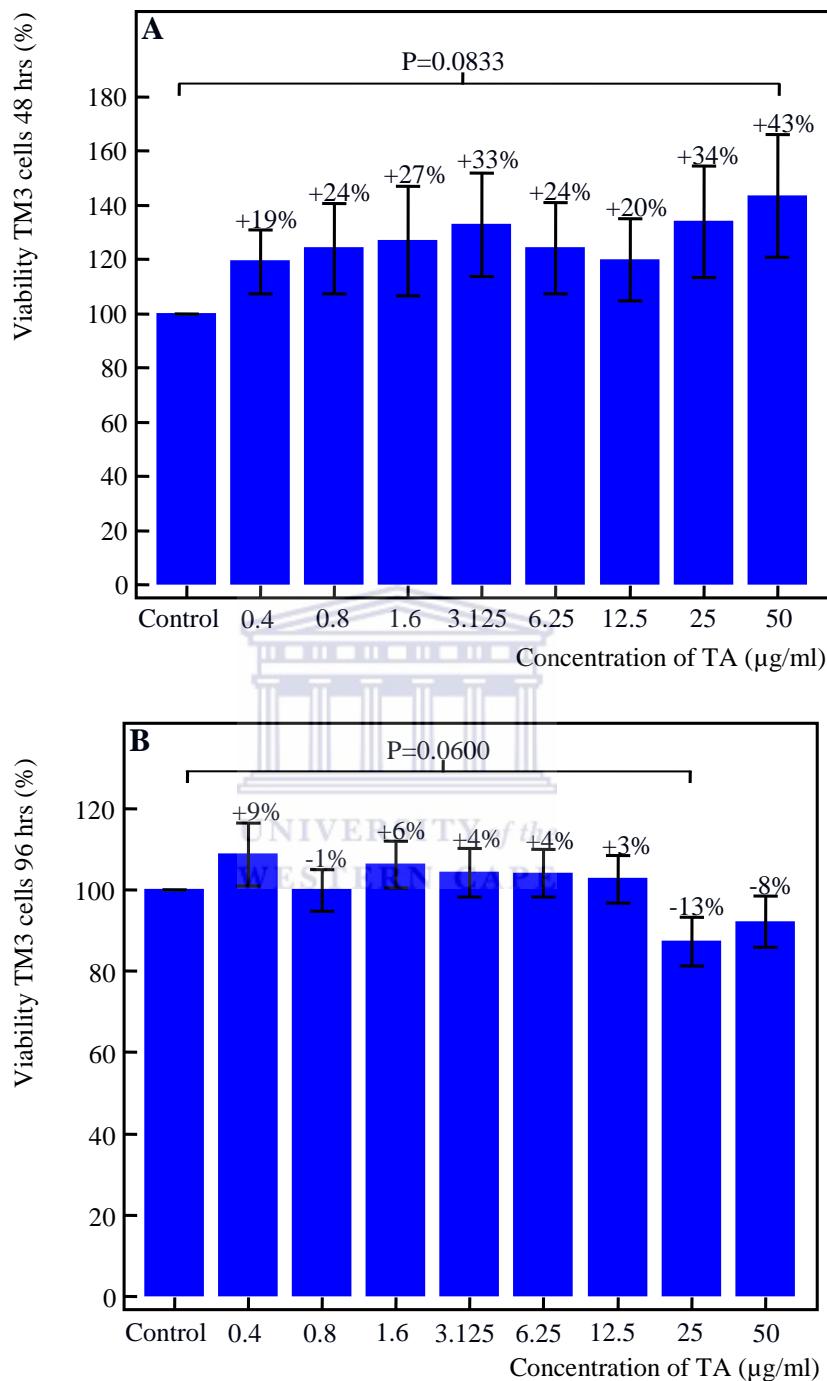
Figure 23: The effect of TA (0.4, 0.8, 1.6, 3.125, 6.25, 12.5, 25 and 50 µg/ml) on TM3-Leydig cells viability *in vitro*. Values are shown as SEM (n=12), increase [+] or decrease [-] and statistical significance at P < 0.05 [*].
A: Effect of TA on TM3-Leydig cell viability after 48 hours exposure. No significant difference can be seen. Even an increase of 43 % at highest concentration 50 µg/ml (P=0.0833) no trend (ANOVA trend analysis: P=0.1088) is observed.
B: Effect of TA on TM3-Leydig cell viability after 96 hours exposure. Although a marked decrease of 13 % at 25 µg/ml (P=0.0600) shows no significant difference however a significant trend (ANOVA trend analysis: P=0.0104) in a decrease viability was found.

3.2.2 Effect of TA on TM4-Sertoli cell viability

After TM4-Sertoli cells were incubated with different concentrations of TA for 48 hours, all concentrations showed a significant increase in viability rates when compared to the control. While a rapid increase in viability is seen at lower doses a steady significant decline could be seen from 46 % (0.8 µg/ml) to 11 % (50 µg/ml) at higher doses (ANOVA trend analysis: P=0.0001) (Figure 24A).

After TM4-Sertoli cells were incubated for 96 hours with different concentrations of TA, a biphasic relationship is seen with slow significant increase in viability from 3 % (0.4 µg/ml) to 35 % (6.25 µg/ml) (ANOVA trend analysis: P=0.0009) followed by a significant decrease to 27 % (ANOVA trend analysis: P<0.0001) at higher doses of TA when compared to the control (Figure 24B).

When comparing 48-hour to 96-hour exposure periods, at 48 hours of TA exposure all concentrations showed an increase in viability whereas in 96 hours incubation only significant increases are noted at 12 % (3.125 µg/ml) and 35 % (6.25 µg/ml) with a significant decline in viability rates at higher (50 µg/ml) doses (P=0.0004). The decrease in viability at 25 µg/ml and 50 µg/ml indicates cell death.
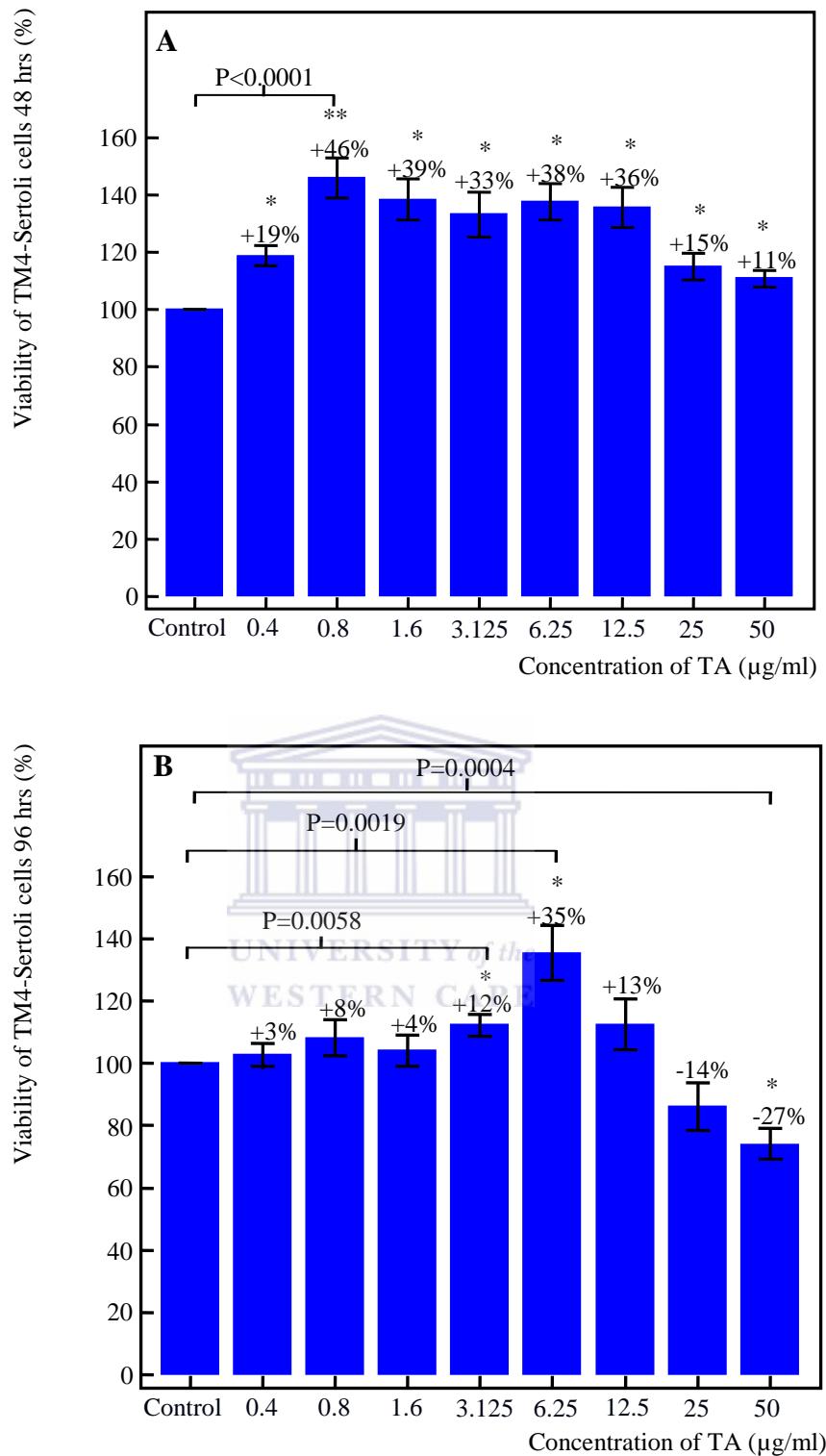
Figure 24: The effect of TA (0.4, 0.8, 1.6, 3.125, 6.25, 12.5, 25 and 50 µg/ml) on TM4-Sertoli cells viability *in vitro*. Values are shown as SEM (n=8), increase [+] or decrease [-] with statistical significance established at P < 0.05 [*] and highly significant P<0.0001 [**].

A: Effect of TA on TM4-Sertoli cells viability after 48 hours exposure. All concentrations show significant increases in viability. A significant trend (ANOVA trend analysis: P=0.0001) could be found.
B: Effect of TA on TM4-Sertoli cell viability after 96 hours exposure. Significant increases of 12 % and 35 % in viability are seen. Yet at concentration 50 µg/ml a 27 % significant decrease (P=0.0004) is observed. No significant trend (ANOVA trend analysis: P=0.1077).

3.2.3 Effect of TA on TM3-Leydig cell protein concentration

After TM3-Leydig cells were incubated for 48 hours with TA, the protein concentration only increased slightly with no significant trend (ANOVA trend analysis: P=0.5212) (Figure 25A). Furthermore, the percentage change in an increase of protein was not more than 2 % to 14 % when directly compared to the control, yet no significant trend (ANOVA trend analysis: P=0.7800) could be observed (Figure 25B).

After TM3-Leydig cells were incubated for 96 hours with TA, significant decreases in protein concentration are seen at all other concentrations of TA. Only an increase in protein concentration was seen at 1.6 µg/ml with no significant difference (P=0.2127). However, a significant trend towards decreased protein concentration (ANOVA trend analysis: P=0.0025) at higher values were found (Figure 26A). Additionally, significant decreases in percentage change of protein concentration were found from 19 % to 38 % when compared to the control. Furthermore, a significant trend toward decreased protein percentage (ANOVA trend analysis: P=0.0006) was also found (Figure 26B).

When comparing the 48-hour to 96-hour exposure, at 48 hours a slight increase in protein concentration is seen whereas at 96 hours a general decrease is noted with the only exception at a concentration of 1.6 µg/ml, showing an increase of 23 %.
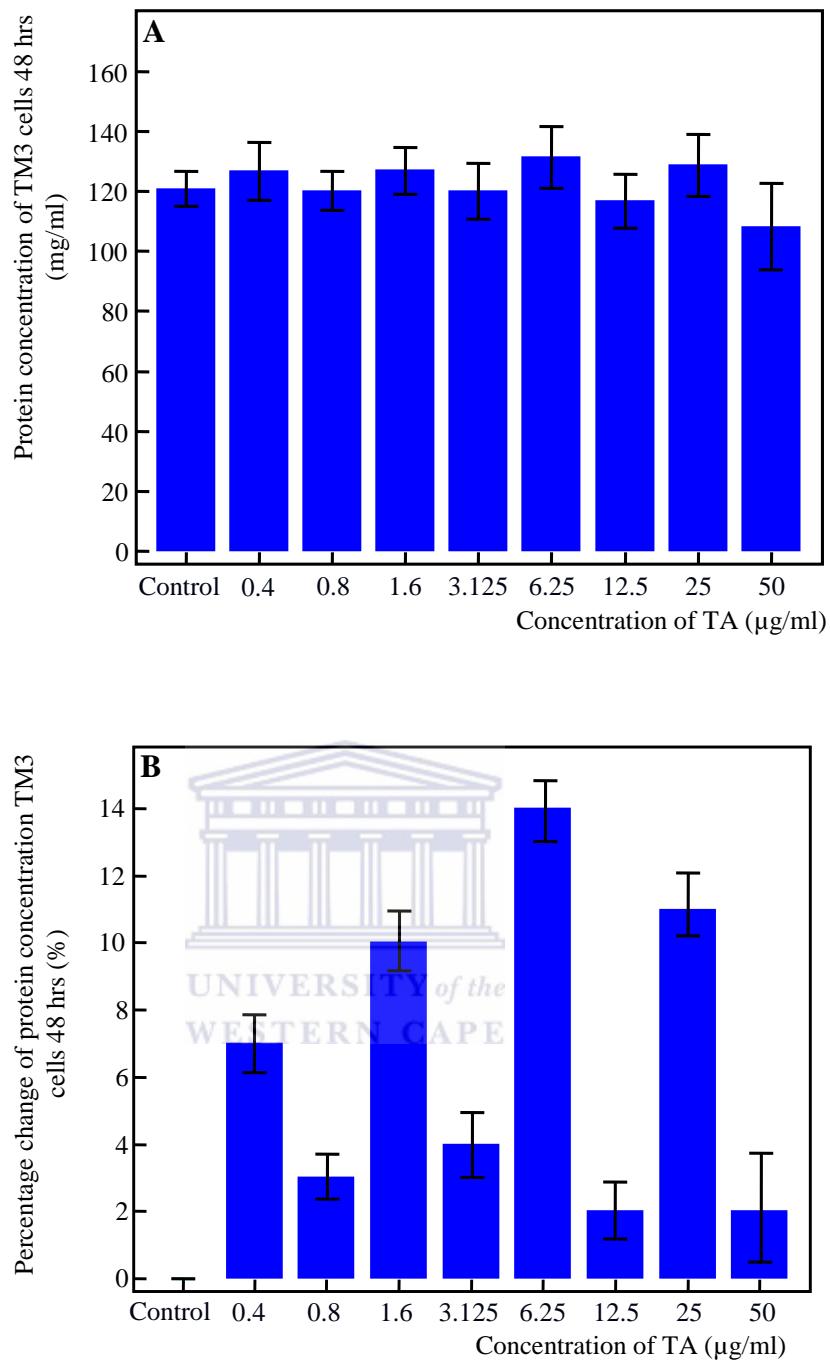
Figure 25: The effect of TA (0.4, 0.8, 1.6, 3.125, 6.25, 12.5, 25 and 50 µg/ml) on TM3-Leydig cells protein concentration *in vitro*. Values are shown as SEM (n=12) and statistical significance at $P < 0.05$ [*].

A: Effect of TA on TM3-Leydig cell protein concentration after 48 hours exposure neither significant difference nor any trend is observed.
B: Effect of TA on TM3-Leydig cells percentage change of protein concentration after 48 hours exposure. Although all concentrations show an increase in protein percentage with 14 % as the highest neither significant difference nor any trend is observed.
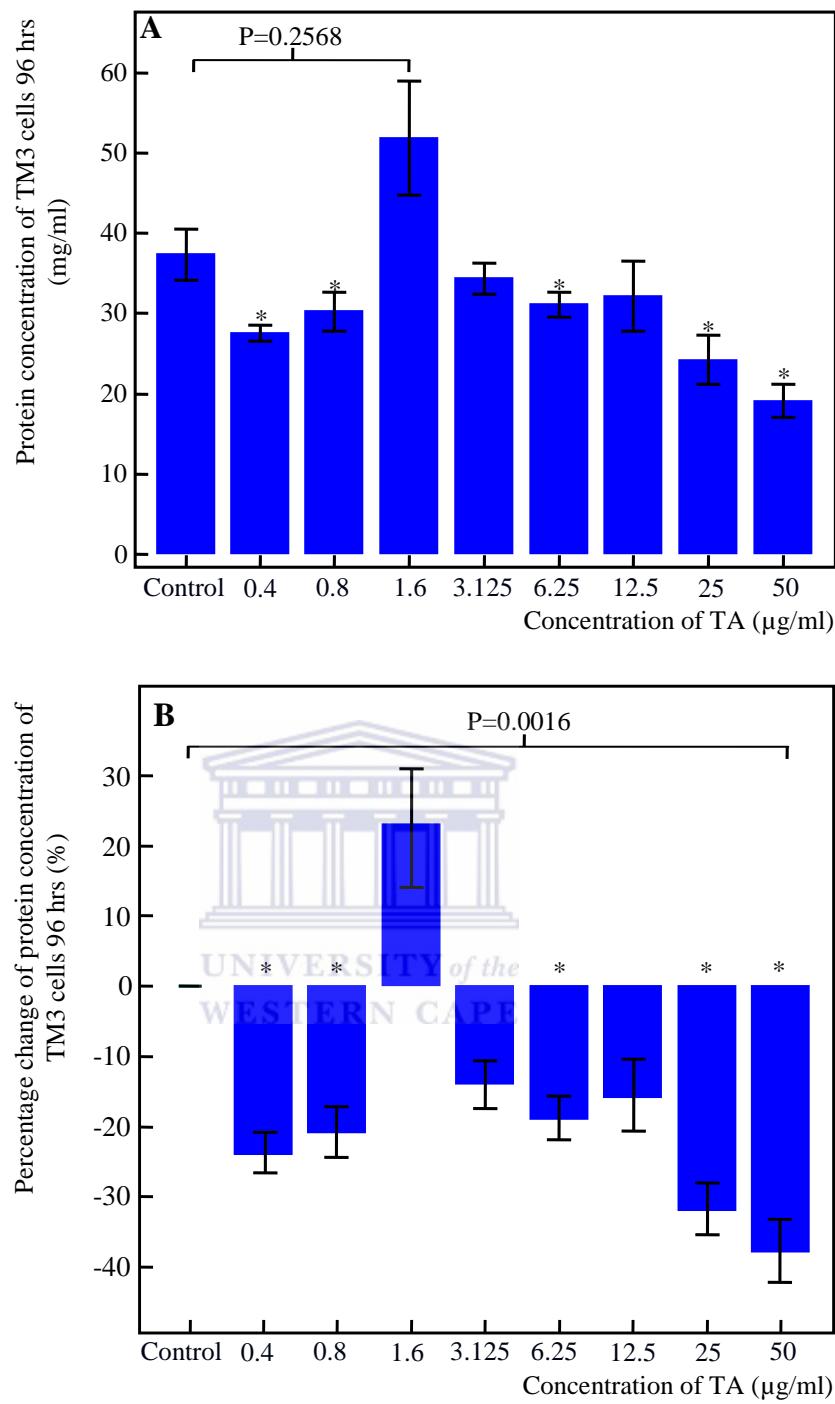
Figure 26: The effect of TA (0.4, 0.8, 1.6, 3.125, 6.25, 12.5, 25 and 50 µg/ml) on TM3-Leydig cells protein concentration *in vitro*. Values are shown as SEM (n=12), increase [+] or decrease [-] and statistical significance at P < 0.05 [*].

A: Effect of TA on TM3-Leydig cells protein concentration after 96 hours exposure. All concentrations show a decrease in concentration yet only at 1.6 µg/ml, a non-significant increase is observed. A significant trend (ANOVA trend analysis P=0.0025) in decreased protein concentration was found.
B: Effect of TA on TM3-Leydig cells percentage change of protein concentration after 96 hours exposure. Although a marked increase of 23 % is noted no significant difference is seen. Significant decrease in protein percentage is observed as from 19 % to 38 % with a significant trend (ANOVA trend analysis: P=0.0006).

3.2.4 Effect of TA on TM4-Sertoli cell protein concentration

After TM4-Sertoli cells were incubated for 48 hours with different concentrations of TA, at 12.5 µg/ml TA the highest increase in protein concentration is shown. However, only a marginal significant difference (P=0.0593) could be found when compared to the control (Figure 27A). Conversely, two marked non-significant decreases at 3.125 µg/ml and 50 µg/ml (ANOVA trend analysis: P=0.0872) was observed. Moreover, protein percentage change revealed a non-significant increase between 8 % and 25 %. No trend (ANOVA trend analysis: P=0.1152) could be found (Figure 27B).

Subsequently, in TM4-Sertoli cells exposed for 96 hours with TA, no significant difference was shown even at the highest concentration of 50 µg/ml (P=0.0804) when compared to the control. Nonetheless, a marginal dose-dependant significant decline (ANOVA trend analysis: P=0.0527) in the protein concentration could be found (Figure 28A). Furthermore, percentage change in protein validates the results found for actual concentrations. A significant decrease in protein percentage between 11 % and 50 %, (ANOVA trend analysis: P=0.0251) toward higher concentrations was found (Figure 28B).

Upon comparing the 48-hour to the 96-hour exposure, at 96 hours a general decrease in protein is shown and only a single 6 % increase at the dosage 0.8 µg/ml is observed. This is in contrast to the slight increase in protein at all concentration of TA for 48 hours.
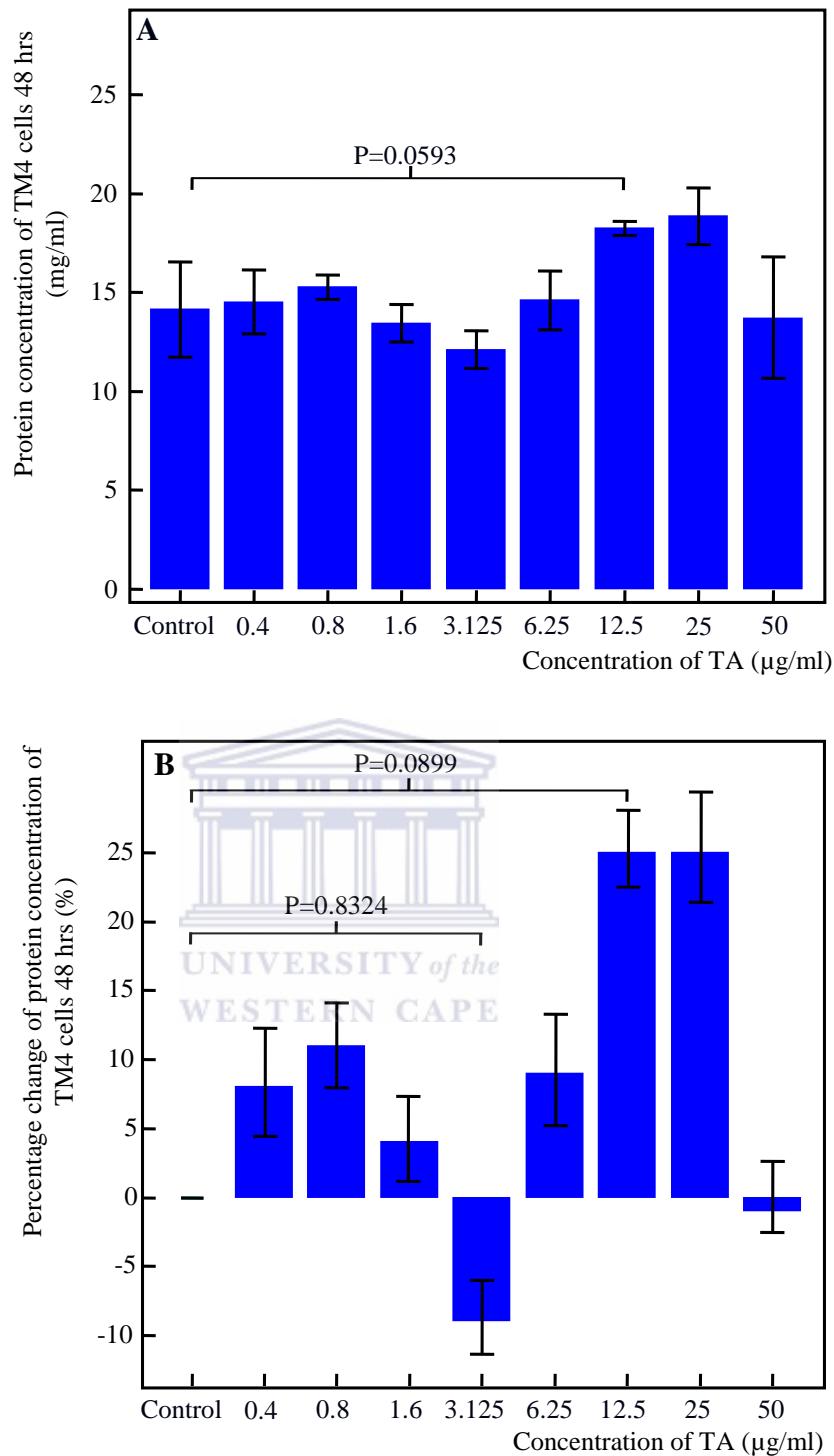
Figure 27: The effect of TA (0.4, 0.8, 1.6, 3.125, 6.25, 12.5, 25 and 50 µg/ml) on TM4-Sertoli cells protein concentration *in vitro*. Values are shown as SEM (n=8), increase [+] or decrease [-] and statistical significance at P < 0.05 [*].

A: Effect of TA on TM4-Sertoli cells protein concentration after 48 hours exposure, a marginal increase (P=0.0593) is observed at 12.5 µg/ml with no significant trend (ANOVA trend analysis: P=0.0872).

B: Effect of TA on TM4-Sertoli cells percentage change of protein concentration after 48 hours exposure, no significant difference nor any trend is observed.
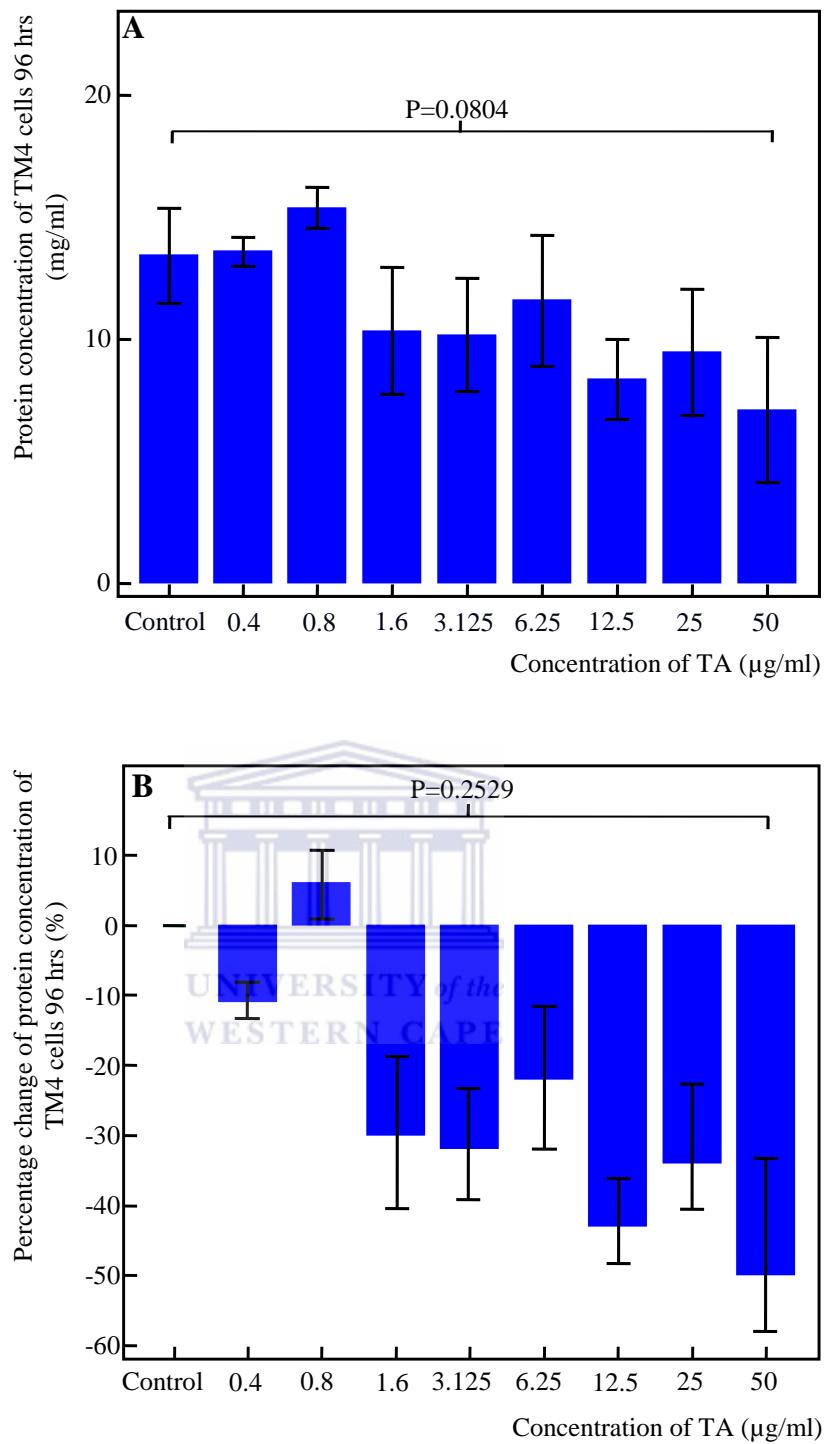
Figure 28: The effect of TA (0.4, 0.8, 1.6, 3.125, 6.25, 12.5, 25 and 50 μg/ml) on the protein concentration of TM4-Sertoli cells *in vitro.* Values are shown as SEM (n=8), increase [+] or decrease [-] and statistical significance at P < 0.05 [*].

A: TM4-Sertoli cells protein concentration after 96 hours exposure. No significant difference is observed even though a marked decrease can be seen at the highest concentration, however, a significant trend (ANOVA trend analysis: P=0.0251) in decreasing protein content is observed.
B: TM4-Sertoli cells percentage change in protein after 96 hours exposure. Although a marked decrease of 50 % is seen at the highest concentration no significant difference (P=0.0804) is shown yet a borderline significant trend (ANOVA trend analysis: P=0.0527) in decreased protein percentage is observed.

3.2.5 Effects of TA on testosterone production in TM3-Leydig cells

TM3-Leydig cell media were analyzed for testosterone production and the cells lysed to ascertain the measurement of protein production. Testosterone concentrations obtained were subsequently compared and normalized to protein content in each well to obtain the amount of testosterone (ng/mg protein) produced.

After TM3-Leydig cells were exposed for 48 hours to TA, the concentration of testosterone appears rather constant (Figure 29A). When comparing the different concentrations of TA to the control, only a marginal difference ($P=0.0580$) could be seen at the highest concentration (50 µg/ml), yet a significant trend towards higher doses of TA (ANOVA trend analysis: $P=0.0145$) was found. Additionally, the percentage change in testosterone concentrations only showed an increase of 1-4 % with decreases observed at concentrations 0.4 µg/ml (2 %) and 6.25 µg/ml (1 %) TA with no significance (Figure 29B). Nevertheless, a trend could be observed (ANOVA trend analysis: $P=0.0162$).
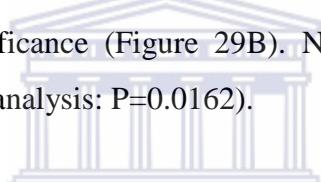
Furthermore, when normalizing the testosterone production to the protein concentration in the well, a decrease can be seen as from the lowest dose 0.4 µg/ml ($P=0.0377$) to 6.25 µg/ml TA ($P=0.0499$) compared to the control (Figure 30A). The percentage change of testosterone showed similar results (Figure 30B) with significant decreases of 22 % ($P=0.0107$) and 31 % ($P=0.0282$). Yet, no trend was found in both, the testosterone production normalized to the protein concentration in the well (ANOVA trend analysis: $P= 0.9776$) and its percentage change (ANOVA trend analysis: $P=0.8936$).

After TM3-Leydig cells were incubated for 96 hours with TA, the testosterone concentration in the wells only showed a significant difference ($P=0.0065$) at the highest (50 µg/ml) concentration of TA compared to the control (Figure 31A). Yet, no trend (ANOVA trend analysis: $P=0.1156$) was observed. Additionally, no change in the percentage testosterone production could be seen. However, a significant trend was found (ANOVA trend analysis: $P=0.0249$) (Figure 31B).

Furthermore, when normalizing the testosterone production to the protein concentration in the well, a peak is observed at the highest (50 µg/ml) concentration of TA but no difference (P=0.1010) was found. Yet, at the lowest (0.4 µg/ml) concentration of TA a significant increase in testosterone production (P=0.0224) and percentage change of testosterone (P=0.0291) was revealed compared to the control (Figure 32A and B). Additionally, the percentage change of testosterone production normalized to the protein concentration and shows an increase (Figure 32B) between 2-146 % yet no trend was observed (ANOVA trend analysis: P=0.1261).

When comparing the testosterone concentration after 48-hour and 96-hour exposure periods both displayed no fluctuation. However, at 96-hour incubation the concentration proved to be higher than at 48-hour incubation, 12×10^{-4} ng/ml and 6×10^{-5} ng/ml, respectively (Figure 29A and Figure 31A). Moreover, when comparing the testosterone production normalized to the protein concentration for percentage change, at 48-hours exposure to TA no increase was shown in contrast to 96-hours exposure (Figure 30B and Figure 32B).
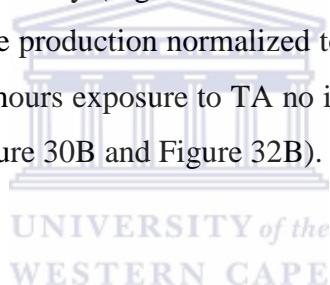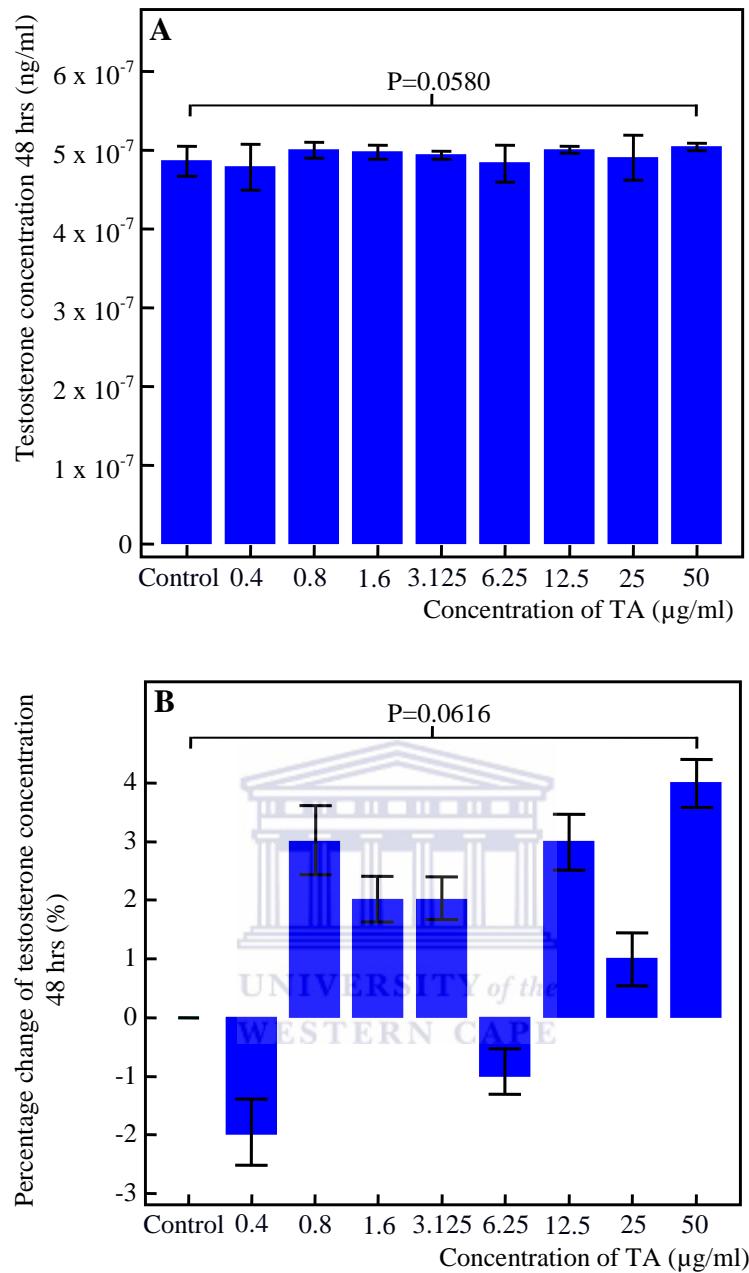
Figure 29: The effect of TA (0.4, 0.8, 1.6, 3.125, 6.25, 12.5, 25 and 50 µg/ml) on TM3-Leydig cells testosterone concentration *in vitro*. Values are shown as SEM (n=12), increase [+] or decrease [-] and statistical significance at P < 0.05 [*].

A: Effect on TM3-Leydig cells testosterone concentration for 48 hours. A marginal statistical difference (P=0.0580) could be seen at the highest concentration (50µg/ml), yet a significant trend (ANOVA trend analysis: P=0.0145) was found.

B: Effect on TM3-Leydig cells percentage change of testosterone concentration for 48 hours, shows an increase of 1-4 %, with decreases observed at concentrations 0.4 µg/ml (2 %) and 6.25 µg/ml (1 %) TA with no significance, however, a trend could be found (ANOVA trend analysis: P=0.0162).
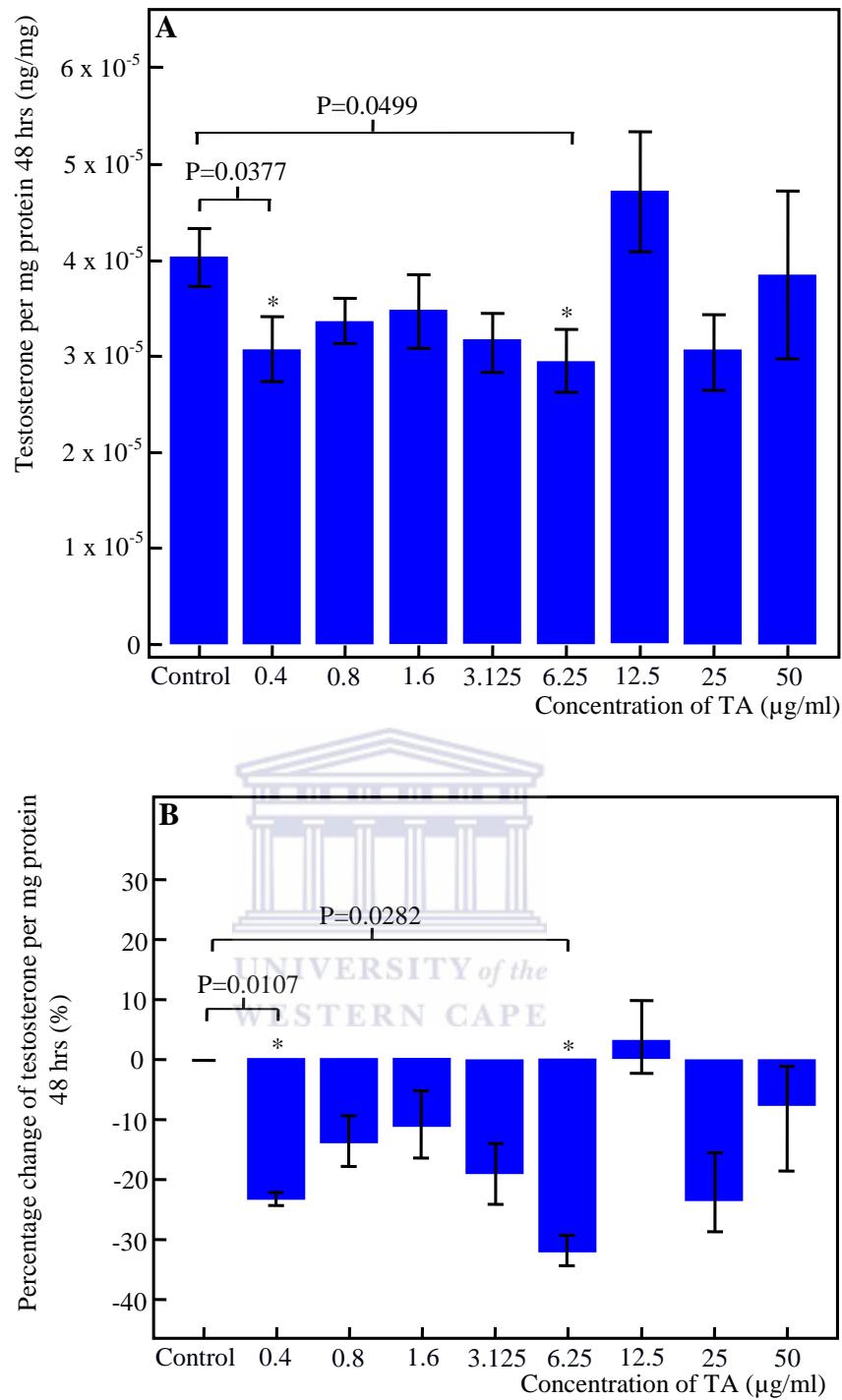
Figure 30: The effect of TA (0.4, 0.8, 1.6, 3.125, 6.25, 12.5, 25 and 50 µg/ml) on testosterone production normalized to protein concentration of TM3-Leydig cells *in vitro*. Values are shown as SEM (n=12), increase [+] or decrease [-] and statistical significance at P < 0.05 [*].

A: The effect of TA on TM3-Leydig cells testosterone per mg protein after 48 hours exposure. A decrease can be seen as from the lowest dose 0.4 µg/ml (P=0.0377) to 6.25 µg/ml TA (P=0.0499). Yet, no trend was found (ANOVA trend analysis: P= 0.9776).
B: The effect of TA on TM3-Leydig cells percentage change of testosterone per mg protein after 48 hours exposure. Significant decreases of 22 % and 31 % are shown however no trend could be found (ANOVA trend analysis: P=0.8936).
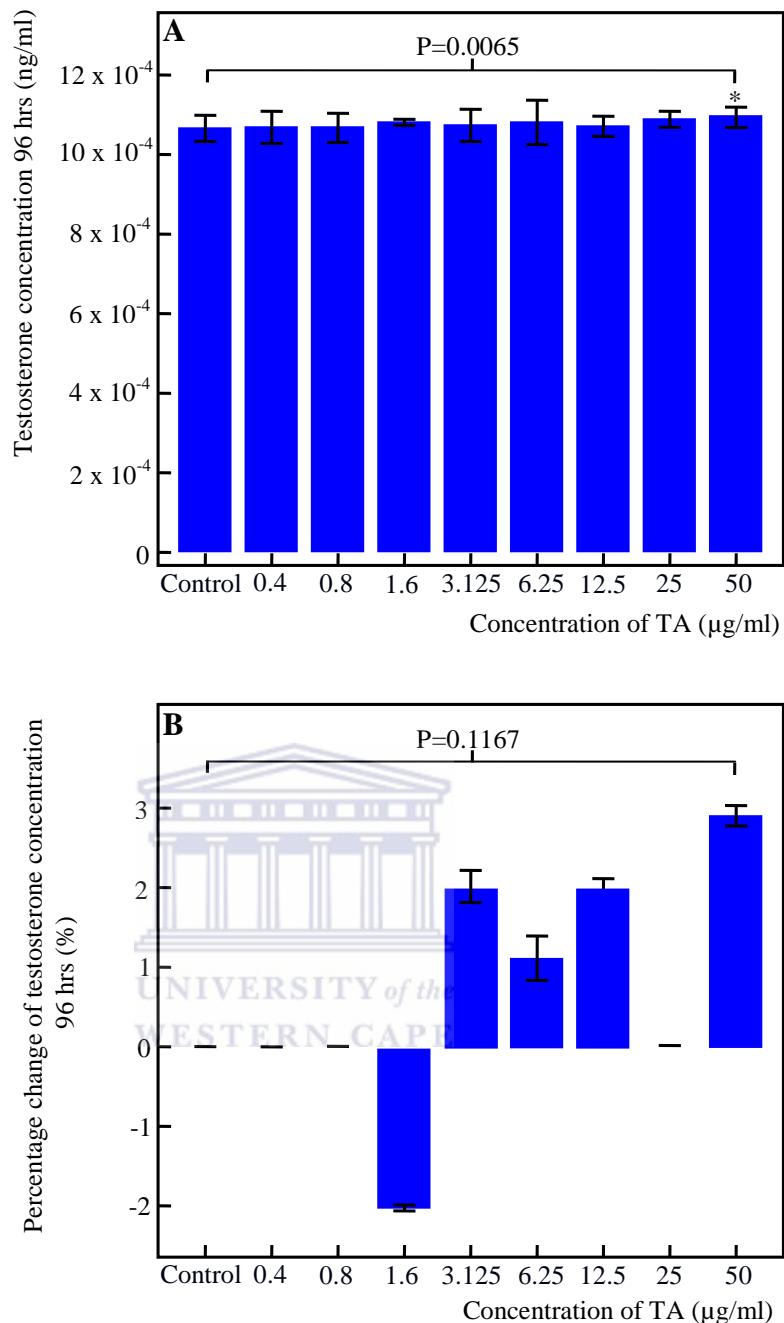
Figure 31: The effect of TA (0.4, 0.8, 1.6, 3.125, 6.25, 12.5, 25 and 50 μg/ml) on testosterone concentration in TM3-Leydig cells *in vitro*. Values are shown as SEM (n=12), increase [+] or decrease [-] and statistical significance at $P < 0.05$ [*].

A: The effect of TA on TM3-Leydig cells testosterone concentration for 96 hours exposure. Only showed significant difference (P=0.0065) at the highest (50 μg/ml) concentration of TA, with no trend (ANOVA trend analysis: P=0.1156).

B: The effect of TA on TM3-Leydig cells percentage change of testosterone concentration for 96 hours exposure. No significant change with 1-3 % is shown. Although a decrease is seen at concentration 1.6 μg/ml TA of 2 %, no significant difference (P=0.4774) is observed, however, a significant trend was found (ANOVA trend analysis: P=0.0249).
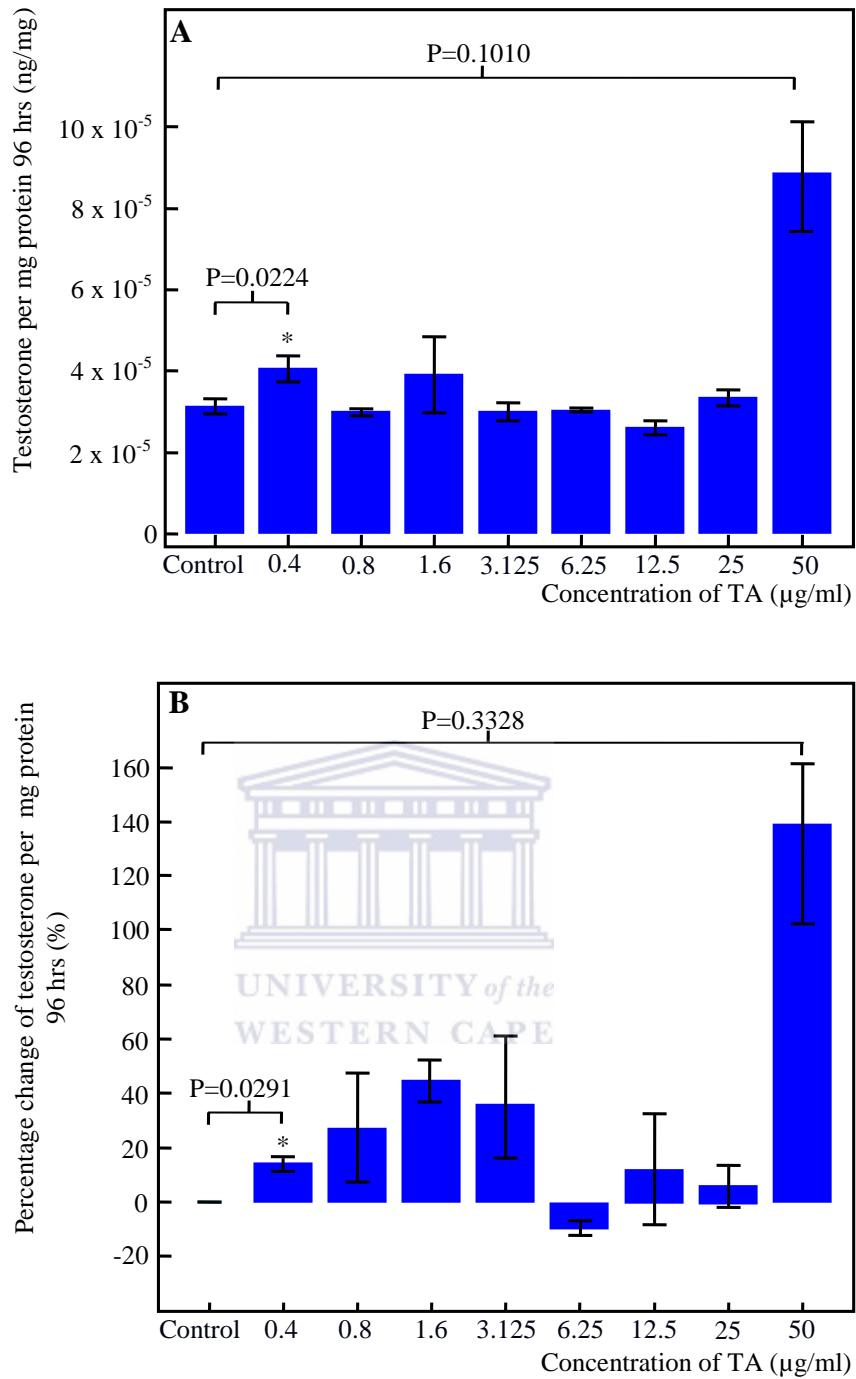
Figure 32: The effect of TA (0.4, 0.8, 1.6, 3.125, 6.25, 12.5, 25 and 50 μg/ml) on testosterone concentration of TM3-Leydig cells *in vitro*. Values are shown as SEM (n=12), increase [+] or decrease [-] and statistical significance at P < 0.05 [*].

A: The effect of TA on TM3-Leydig cells testosterone normalized to protein concentration after 96 hours exposure. Although a peak is observed at the highest (50 μg/ml) concentration no significant difference (P=0.1010) is shown, only at the lowest (0.4 μg/ml) concentration of TA (P=0.0224). However, no trend (ANOVA trend analysis: P=0.1261) was found.
B: The effect of TA on TM3-Leydig cells percentage change of testosterone per mg protein after 96 hours exposure. The percentage change shows an increase between 2-146 % yet no significant difference could be found, except at the lowest (0.4 μg/ml) concentration (P=0.0219). No trend (ANOVA trend analysis: P=0.4430) could be found.

3.2.6 Effects of TA on TM4-Sertoli cell pyruvate production

The pyruvate concentration was determined by using the supernatant medium. Data obtained were subsequently compared to the protein content in the well.

After TM4-Sertoli cells were exposed to TA for 48 hours, a biphasic relationship could be observed (Figure 33A). At lower doses no significance could be found. However, from 3.125 µg/ml TA (P=0.0002) to 25 µg/ml TA (P=0.0260) significant increases in pyruvate concentration were observed when directly compared to the control. The highest concentration (50 µg/ml) of TA, shows a marginal significant decline (P=0.0541) when compared to the control, yet when compared to concentration 25 µg/ml TA a significant decrease (P=0.0168) could be observed. However, no trend (ANOVA trend analysis: P=0.1188) could be found. Additionally, in percentage change of pyruvate production, a significant increase of 42-72 % can be seen (Figure 33B) at higher doses of TA when compared to the control. Yet, at the highest concentration a significant decline (P=0.0006) of 55 % is observed. No trend (ANOVA trend analysis: P=0.1062) could be found.
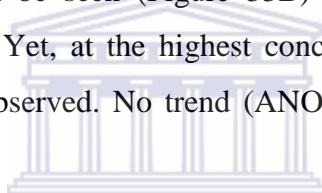
Furthermore, when pyruvate production were normalized to the protein content in the well, similar results could be seen (Figure 34A) as for the pyruvate concentration. Significant increases could be observed as from 6.25 µg/ml (P=0.0099) to 25 µg/ml TA (P=0.0177) when compared to the control. A non-significant decrease is observed at 50 µg/ml TA (P=0.1379). Nevertheless, a significant trend (ANOVA trend analysis: P=0.0243) in a dose-dependant manner was found. For percentage change of pyruvate production normalized to protein content, significant increases could be seen (Figure 34B) at 12.5 µg/ml (P=0.0412) and 25 µg/ml TA (P=0.0194) of 136 and 89 %, respectively with a significant trend (ANOVA trend analysis: P=0.0416) in a dose-dependant manner.

TM4-Sertoli cells that were incubated for 96 hours with TA, all concentrations showed a significant difference (Figure 35A) compared to the control and an even higher significance at 0.4 µg/ml and 6.25 µg/ml TA (P<0.0001) with significant decreases in pyruvate at 12.5 µg/ml (P=0.0001) and 25 µg/ml TA (P=0.0001) were observed. Nonetheless, a trend (ANOVA trend analysis: P=0.0004) in a dose dependant manner was found. Additionally, a significant decrease in percentage change in pyruvate production is seen at all concentrations of TA (Figure 35B) of 16 % (P=0.0079) to 105 % (P<0.0001) with a significant trend found (ANOVA trend analysis: P=0.0024).

Also, when pyruvate production was normalized to the protein content in the well, significant differences at all concentrations of TA could be seen except at the highest (50 µg/ml) concentration (P=0.1021) when compared to the control (Figure 36A). However, no trend (ANOVA trend analysis: P=0.2813) was found. Additionally, for the percentage change in pyruvate production when normalized to the protein content in the well, significant decreases can be seen at all concentrations. A significant trend (ANOVA trend analysis: P=0.0212) could also be found (Figure 36B). The percentage change of pyruvate production normalized to the protein concentration ranged from 68 to 115 %, compared to all other concentrations of TA. The highest (50 µg/ml) dose produced a lesser decline at 45 % (P=0.0289).

When comparing the percentage changes of pyruvate production normalized to the protein concentration for 48-hour and 96-hour exposure periods, after 48 hours of incubation, pyruvate concentrations showed increases between 5 % and 72 % and only a decrease at the highest concentration of TA. In contrast, the 96-hour exposure showed a decrease at all concentrations. This could be due to cell death at higher concentrations and longer exposure periods to TA.
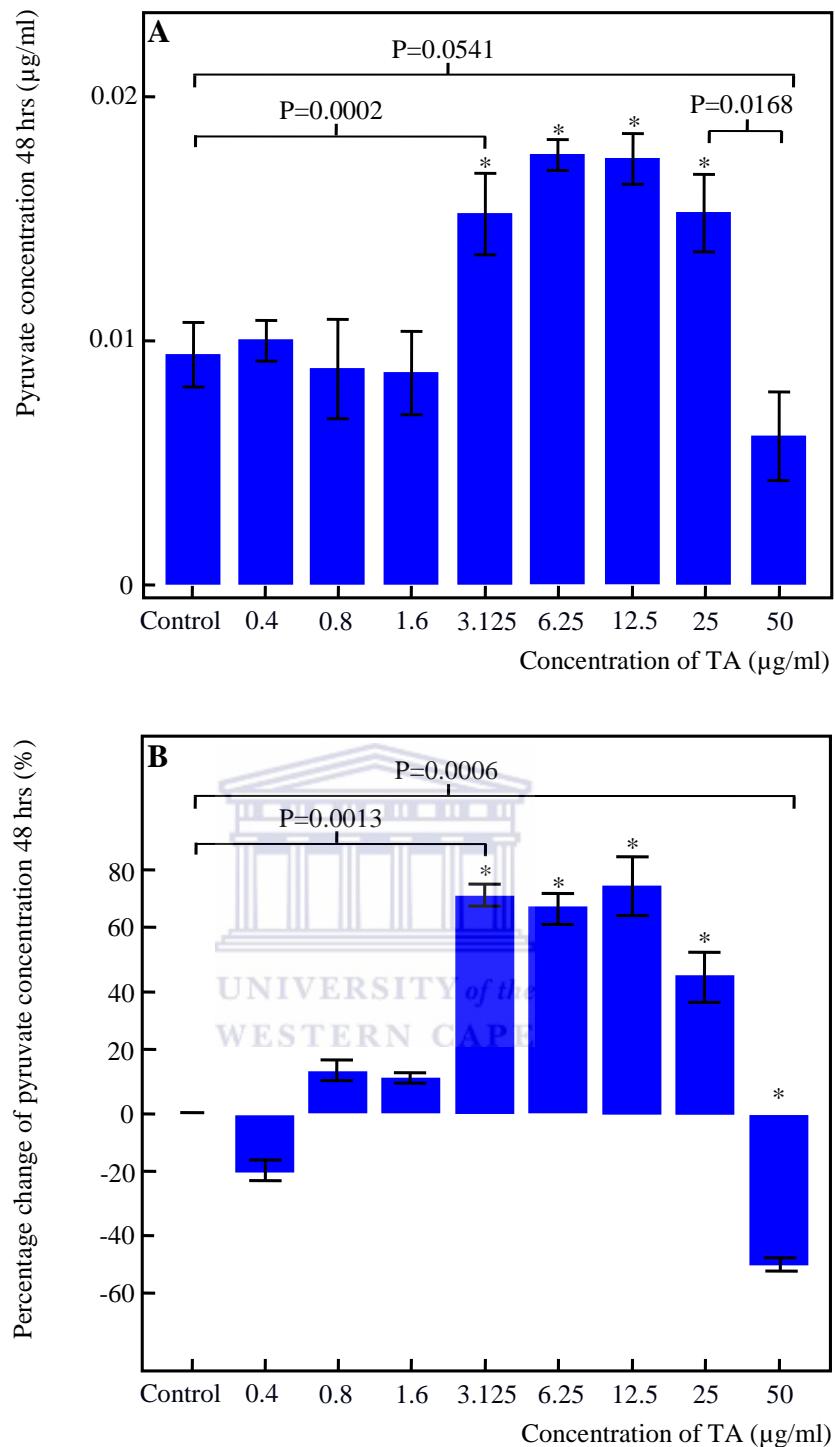
Figure 33: The effect of TA (0.4, 0.8, 1.6, 3.125, 6.25, 12.5, 25 and 50 µg/ml) on TM4-Sertoli cells pyruvate concentration *in vitro*. Values are shown as SEM (n=8) and statistical significance at P < 0.05 [*].

A: The effect of TA on TM4-Sertoli cells pyruvate concentration after 48 hours exposure. At concentration 3.125 µg/ml (P=0.0002) to 25 µg/ml (P=0.0260) significant increases are seen. At 50 µg/ml only shows a marginal significant decline (P=0.0541). No trend is found (ANOVA trend analysis: P=0.1188).

B: The effect of TA on TM4-Sertoli cells percentage change of pyruvate concentration after 48 hours exposure. A significant increase of 42-72 % in pyruvate can be seen at higher doses of TA. Yet, at the highest concentration a significant decline (P=0.0006) of 55 % is observed, however, no trend (ANOVA trend analysis: P=0.1062) could be found.
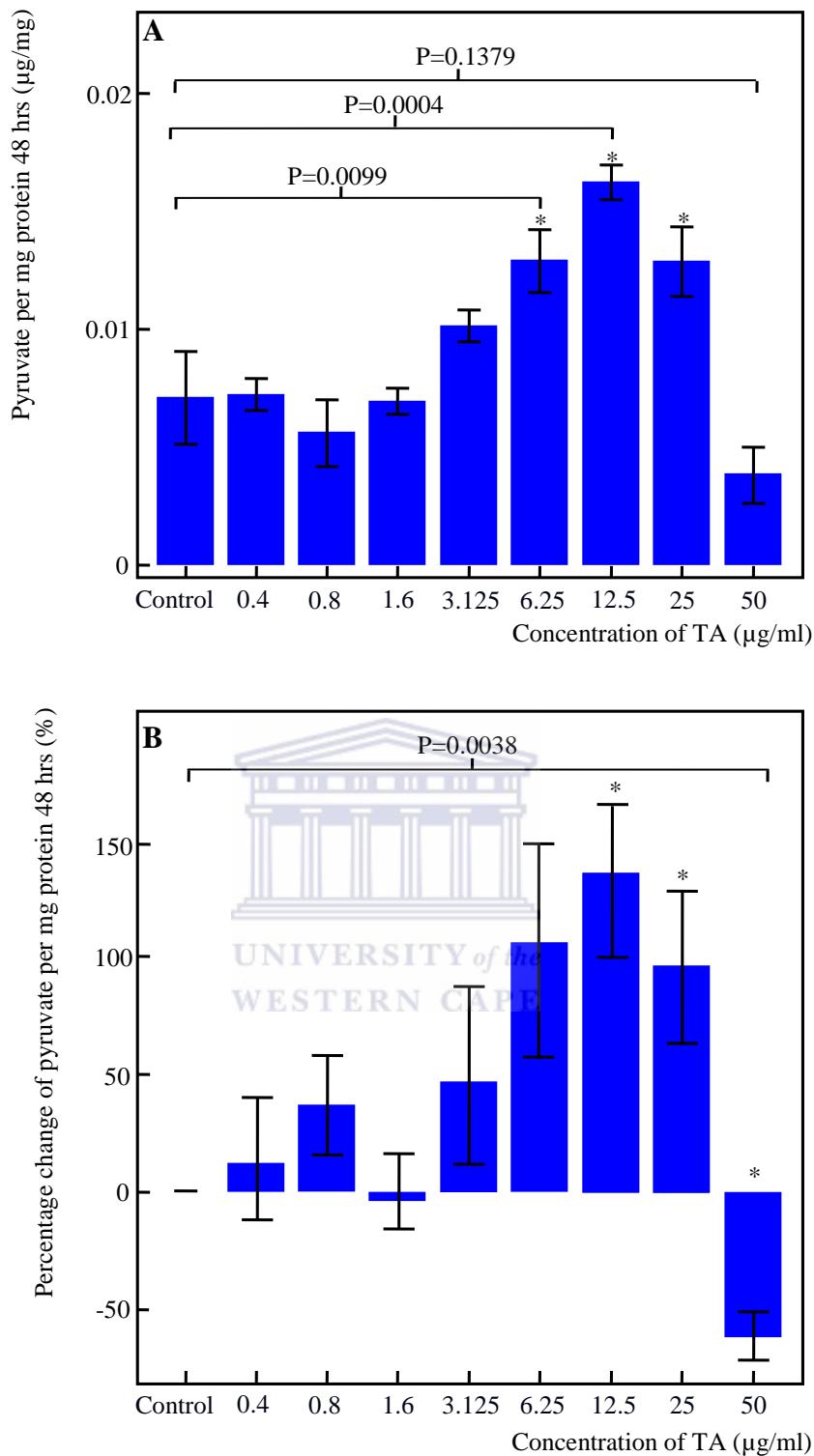
Figure 34: The effect of TA (0.4, 0.8, 1.6, 3.125, 6.25, 12.5, 25 and 50 µg/ml) on TM4-Sertoli cells pyruvate-protein concentration *in vitro*. Values are shown as SEM (n=8) and statistical significance at P < 0.05 [*].

A: The effect of TA on TM4-Sertoli cells pyruvate-protein concentration after 48 hours exposure. Significant increases can be observed as from 6.25 µg/ml (P=0.0099) to 25 µg/ml TA (P=0.0177). A significant trend (ANOVA trend analysis: P=0.0243) could also be found.
B: The effect of TA on TM4-Sertoli cells percentage change of pyruvate per mg protein after 48 hours exposure. Significant increases can be seen at 12.5 µg/ml (P=0.0412) and 25 µg/ml TA (P=0.0194) of 136 and 89 %. A significant trend observed (ANOVA trend analysis: P=0.0416).
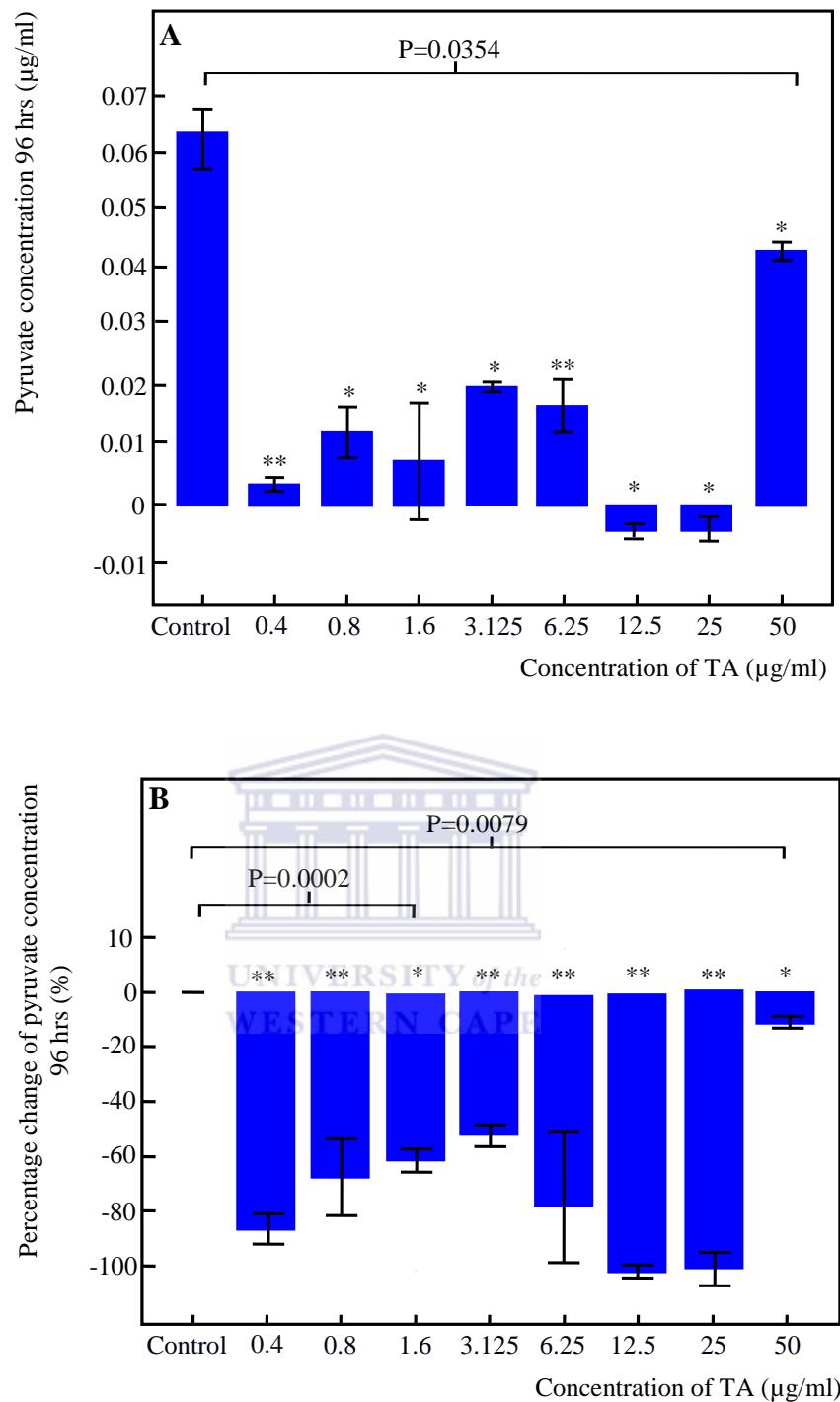
Figure 35: The effect of TA (0.4, 0.8, 1.6, 3.125, 6.25, 12.5, 25 and 50 µg/ml) on TM4-Sertoli cells pyruvate concentration *in vitro.* Values are shown as SEM (n=8) and statistical significance at P < 0.05 [*] and highly significance [**].

A: The effect of TA on TM4-Sertoli cells pyruvate concentration after 96 hours exposure. All concentrations showed significant difference and even higher significance at 0.4 µg/ml as wells as 6.25 µg/ml TA (P<0.0001) with significant decreases in pyruvate at 12.5 µg/ml (P=0.0001) and 25 µg/ml TA (P=0.0001). Nonetheless, a trend (ANOVA trend analysis: P=0.0004) was found.
B: The effect of TA on TM4-Sertoli cells percentage change of pyruvate concentration after 96 hours exposure. A significant decrease in pyruvate percentage is seen at all concentrations of between 16 % (P=0.0079) to 105 % (P<0.0001) with a significant trend found (ANOVA trend analysis: P=0.0024).
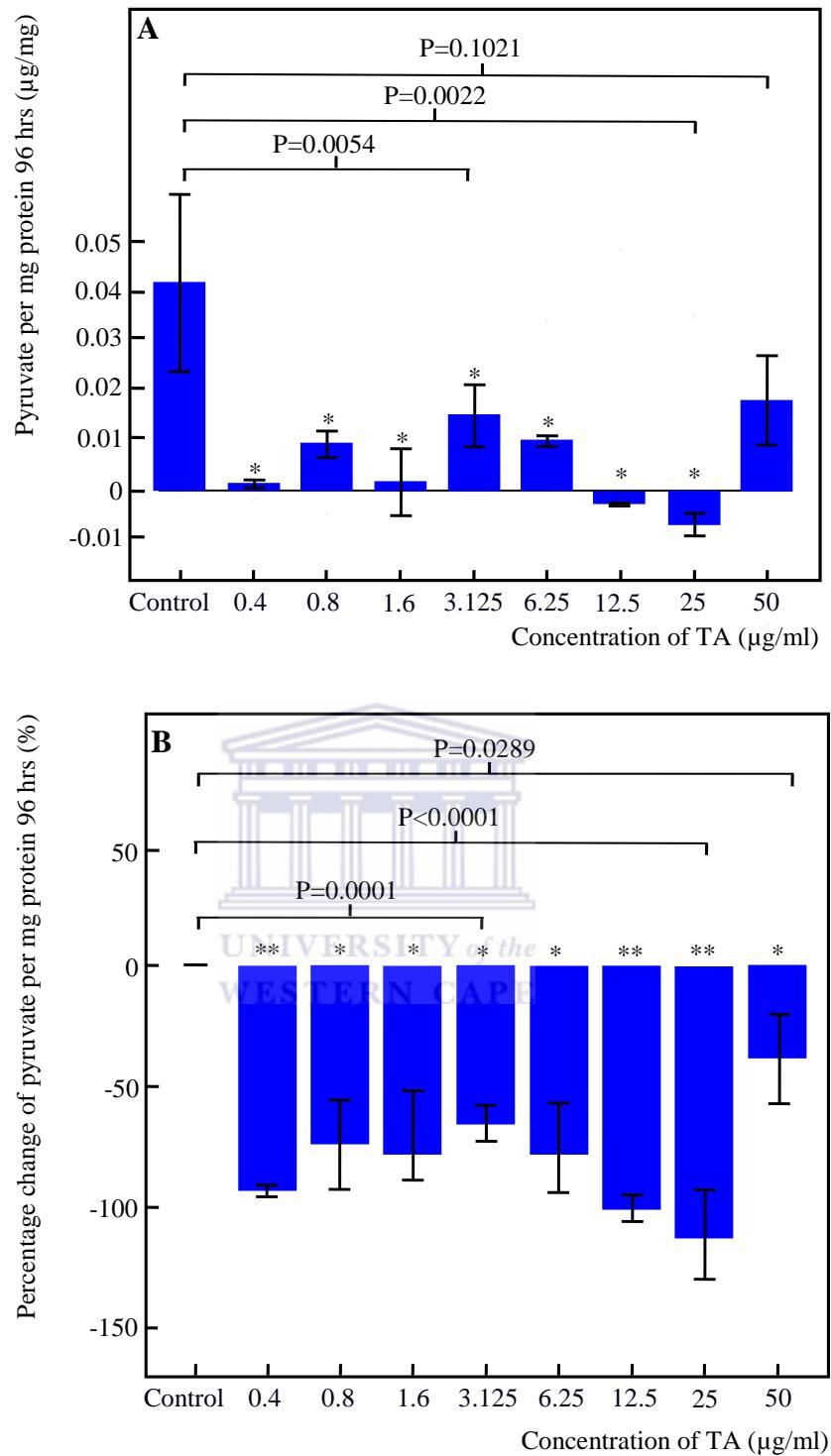
Figure 36: The effect of TA (0.4, 0.8, 1.6, 3.125, 6.25, 12.5, 25 and 50 µg/ml) on TM4-Sertoli cells pyruvate-protein concentration *in vitro*. Values are shown as SEM (n=8) and statistical significance at P < 0.05 [*] and highly significance [**].

A: The effect of TA on TM4-Sertoli cells pyruvate per mg protein after 96 hours exposure. No significance could be seen at the highest (50 µg/ml) concentration (P=0.1021). No trend (ANOVA trend analysis: P=0.2813) was found.
B: The effect of TA on TM4-Sertoli cells percentage change of pyruvate per mg protein after 96 hours exposure. Significant decreases can be seen at all concentrations with a significant trend found (ANOVA trend analysis: P=0.0212).

Chapter 4: Discussion

Traditional medicine, although rooted in a specific socio-cultural context, does not significantly impact Western medicine as it remains a non-standard health service, depending on the countries socio-economic status, that is included but not always recognized by government (WHO, 2002; Payyappallimana, 2009). This may be as a result of the pharmacological properties of medicinal plants and herbal extracts not being fully understood. Medicinal plants contain phytochemicals and numerous chemical compounds which can be used in pharmacology by isolating the active compounds to generate new medicines and provide alternative healing methods (WHO, 2002). Even though approximately 25 % of prescriptions from clinicians contain active ingredients extracted from a plant source, little is known about these traditional approaches to combat diseases in Western medicine (Castleman, 1995).
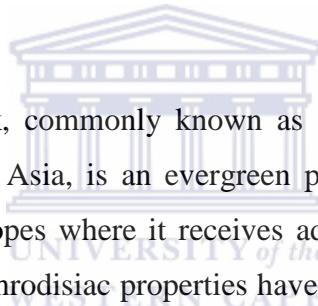
Eurycoma longifolia Jack, commonly known as Tongkat Ali in Malaysia, and indigenous to South-East Asia, is an evergreen plant (Bhat and Kharim, 2010) growing on the jungle slopes where it receives adequate shade and water (Bhat and Kharim, 2010). Its aphrodisiac properties have been shown to increase sexual motivation in rat models (Ang and Sim, 1997) and it has shown increased serum testosterone levels in treated rats (Zanoli et al., 2009; Solomon et al., 2013 submitted). This may be as a result of the root source of the plants containing eurypeptides, which exert and enhance their effects by the biosynthesis of various androgens. Studies by Ali and Saad (1993) have revealed that the eurypeptide apparently works by activating CYP17 (17 α-hyroxylase/17, 20 lyase) enzyme to enhance the metabolism of pregnenolone and 17-OH-pregnenolone to yield more dehydroepiandrosterone (DHEA) as well as the metabolism of progesterone and 17-OH-progesterone to 4-androstendione and to testosterone.

Considering that the extract is traditionally used to treat sexual dysfunction such as erectile dysfunction and to enhance libido (Low and Tan, 2007), this is the first study aimed at investigating the *in vitro* effects of a patented, water-soluble extract of Tongkat Ali on human sperm parameters testing the safety and efficacy of the treatment with this extract.

4.1 Determination of suitable *in vitro* incubation concentrations with an aqueous extract of *Eurycoma longifolia* (Tongkat Ali; TA) and subsequent testing for potential cytotoxicity

4.1.1 Incubation concentrations of TA for spermatozoa

The calculation of the concentrations (1, 10, 20, 100, 2000 μg/ml) of TA was based on previous studies by Tambi and Imran (2010) and Tambi et al. (2012) where 200 mg extract per individual were given, daily. Assuming that an average male weighs about 80 kg, the concentration of TA administered is 2.5 μg/ml *in vivo.* Thus, the concentrations used for the functional parameters of spermatozoa were deemed to be in a tolerable range.

4.1.2 Incubation concentrations of TA for cell culture

Boik (1996) described the plant screening program of the US NCI (United States National Cancer Institute) on cytotoxic activity of a crude extract to be cytotoxic (*in vitro),* when the concentration of the extract causes 50 % of the cells to die in KB cells (Human mouth epidermal carcinoma) is less than 20 μg/ml, after incubation between 48-72 hours. Zakaria et al. (2009) used this description as a rough guideline to establish the cytotoxicity of TA on 4 malignant and 2 non-malignant cancer cell lines (Human hepatocellular carcinoma cells-HepG2, Human melanoma cells-HM3KO, Ovarian cancer cells-CaOV3, Human epithelial cancer cells [Henrietta Lacks]-HeLa, non-malignant-CCD11114sk and Chang's liver cells) at concentrations from 0.2 μg/ml to 100 μg/ml. In the current study, concentrations of 0.4, 0.8, 1.6, 3.125, 6.25, 12.5, 25 and 50 μg/ml of TA were used as it arrays within the range based on the previous study mentioned.

4.2 Effects of TA on functional parameters of spermatozoa

4.2.1 Effects of TA on spermatozoa viability

One of the most basic and essential semen analysis techniques is the assessment of the viability of spermatozoa. Whilst investigating the cells viability one can establish the amount of dead/ live cells and employ this method as a cytotoxic marker. In the current study, the Eosin-Nigrosin technique was used, as spermatozoa with structurally intact cell membranes will not take up the stain (Motimer et al., 1990; Bjorndahl et al., 2003, 2004) and thus would provide

insight to the effects of Tongkat Ali on the viability of washed and swim-up spermatozoa.

When observing the effect of TA on the viability of washed sperm, a decrease in the amount of viable germ cells was found at the highest (2000 µg/ml) concentration of TA (P=0.0284) and a significant trend (ANOVA trend analysis: P=0.0059) in dose dependency. In contrast, this was not evident for the swim-up spermatozoa investigation (ANOVA trend analysis: P=0.4219). Taking into consideration that the washed fraction of spermatozoa does not exclude leukocytes and sperm cells that were already dead or dying upon staining. This may be a reason for the decrease in viability compared to the swim-up fraction, which only consist of the most viable germ cells.

Currently, there are no studies pertaining to TA on the viability of human spermatozoa. Consequently, the results obtained from the recent investigation can not be compared to confirm or contradict the findings observed. However, the numerous investigations of the cytotoxic effect of TA on cancer cells (Kardono et al., 1991; Tada et al., 1991; Kuo et al., 2004; Cheah and Azimahtol, 2004; Nurhanan et al., 2005; Zakaria et al., 2009; Nurkhasanah et al., 2009) and the exhibition of many different compounds within TA (Darise et al., 1982, 1983; Morita et al., 1993; Ang et al., 2002; Bedir et al., 2003) may explain the decrease in viability at higher concentrations. One of the cytotoxic activities of TA as an anticancer agent is membrane blebbing causing loss of membrane integrity leading to cell death.

Therefore, it could be hypothesized that TA may have caused membrane damage of the spermatozoa which caused the decrease in cell viability. Nevertheless, it could be safely stated that Tongkat Ali is not harmful at doses lower than 100 µg/ml TA *in vitro*, as the mechanisms of the action of TA compounds are still unknown.

4.2.2 Effect of TA on sperm motility

A study performed by Tambi and Imran (2010) on idiopathic male infertility with TA treatment proved an improvement in seminal parameters which included sperm concentration, semen volume and sperm morphology. Although these parameters would assist prediction of sperm motility in the current study, the results obtained could not fully substantiate the results obtained by the previous study. In the recent study, both washed and swim-up spermatozoa, a marked decline was observed in the total and progressive motility. However, a significant trend (ANOVA trend analysis: P=0.0452) to lower total motility rates could only be observed towards higher concentrations of TA in the washed sample set. Small sample size and the inclusion of normo-, oligo- and/or asthenozoospermia patients could be possible causes for this result. Therefore, TA may not be the only cause of the lowered motility rates.

4.2.3 Effect of TA on sperm acrosome reaction

Acrosome reaction has been directly related to the fertility rates in males as only acrosome-reacted spermatozoa can penetrate the zona pellucida (Koehler et al., 1982; Henkel et al., 1993). Also, capacitation is essential for normal acrosome reaction (Yanagimachi, 1981). In the current study, the percentage of live acrosome reacted spermatozoa was calculated. In the washed sample set, a clear significant increase in acrosomal reacted spermatozoa towards higher concentrations was evident from the low dose of 20 µg/ml TA (P=0.0069).

Considering, that acrosomal reaction is a morphological and functional parameter of spermatozoa, it can be directly correlated to the cells ability to bind to the zona pellucida (Menkveld et al., 1991, 1996; Lui and Baker, 1992; Franken and Oehninger, 2012). By considering the importance of the ability of the acrosome to undergo reaction it can be used as a positive predictor in the diagnostic and therapeutically suitable technique for couples evaluated for ART (Patrat et al., 2002).

Furthermore, Franken et al. (1997) showed a strong relationship between normal sperm morphology and the inducibility of the acrosome reaction. A number of studies have also concluded that sperm sensitivity to acrosome reaction inducers

was reduced in infertile patients (Krausz et al., 1995, 1996). With this knowledge, in the present study, theoretically it can be assumed that TA compounds may act similarly to non-physiological inducers such as Calcium-Ionophor by interacting with the acrosomal membranes calcium channel gate, leading to calcium influx and the initiation of acrosome reaction (Yanagimachi 1981; Roldan and Harrison, 1989; De Lamirande et al., 1997; Lui and Baker, 1998).

On the other hand, the increase in acrosomal reaction can only be observed in the wash sperm fraction and not for the swim-up. It could be argued that the centrifugation process during the washing stage could possibly have contributed to the artificial loss of the acrosome.

4.2.4 Effect of TA on sperm ROS production

Previous studies have shown that in normal spermatozoa, low levels of ROS are, in fact, essential and beneficial to the cell function and have been shown to stimulate sperm capacitation (Leclerc et al., 1997), enhance zona pellucida binding (Aitken et al., 1989) and promote acrosome reaction and hyperactivation (De Lamirande et al., 1993, 1997, 2008). By contrast, excessive levels of ROS from sources such as leucocytes (extrinsic) or spermatozoa (intrinsic) are harmful and lead to lipid peroxidation of the sperm plasma membrane, thus compromising sperm functions including DNA integrity (Agarwal et al., 2003; Agarwal and Said, 2005; Henkel et al., 2005b; Aitken and Baker, 2006).

In the current study, ROS-positive spermatozoa were more prevalent at higher unphysiological concentrations (2000 μg/ml) of Tongkat Ali in both, washed and swim-up method. However only significant increases in ROS was observed for washed spermatozoa (P=0.0028) with a positive trend (ANOVA trend analysis: P=0.0080) towards higher percentages of ROS-positive spermatozoa at higher concentrations of TA and only marginal (ANOVA trend analysis: P=0.0580) in the swim-up spermatozoa.

In recent human trials performed by Tambi (2002, 2003, 2005, and 2006) Tongkat Ali proved having anti-stress and anti-aging effects. Shuid et al. (2011) suggested that this may be due to TA's ability to normalise growth hormone and at the same time its high content level of superoxide dismutase (SOD) enables it to scavenge superoxide-free radicals and inhibit lipid peroxidation.

Additionally, plant phenolic compounds have been reported to possess antioxidant activity by reducing the damaging properties of reactive oxygen species (ROS) (Sawa et al., 1999; Yussof and Iwansyah, 2011). Antioxidant supplementation is thought to improve male reproductive functions (Kruger and Coetzee, 1999; Lenzi et al., 2003; Kefer et al., 2009). Since, TA extract had no effect on the washed and swim-up spermatozoa; this may indicate that the compounds contained, such as phenolics, could contribute to the improvement in sperm concentration and sperm morphology, supporting the claim of human health benefits (Zhang and Hamauzu, 2004).

4.2.5 Effect of TA on sperm mitochondrial membrane potential (Δψm) and DNA fragmentation

The disruption of sperm mitochondrial membrane potential is associated with the early stage of apoptosis which may lead to DNA fragmentation (Kroemer et al., 1997). In the present study, the extract had no effect on the Δψm of the spermatozoa. This observation may imply that although TA has cytotoxic compounds it does not impair the Δψm nor cause any disruption to the mitochondria of spermatozoa and its mechanism of action relays at a different site of target. In addition, as previously concluded in the current study, the extract had no effect on the motility, and as Δψm is directly associated to motility of spermatozoa (Kasai et al., 2002; Henkel et al., 2012) and it may confirm the theory that the TA's cytotoxic effect has a different site of target other than the mitochondrial membrane.

Furthermore, DNA fragmentation was investigated as an apoptotic marker additionally to facilitate the results observed by the extract on the Δψm. DNA fragmentation is therefore an important factor to take into consideration as this functional parameter plays an imperative role in advanced reproductive techniques. Some of these techniques of assisted reproduction, namely intracytoplasmic sperm injection (ICSI) even allow spermatozoa to bypass the natural selection barrier (Twigg et al., 1998), which may result in babies born with birth defects, chromosomal abnormalities or predisposition to cancers (Kurinczuk and Bower, 1997).

In this study, no effect of the TA extract on sperm DNA fragmentation could be observed in both sample methods and the recorded lower DNA fragmentation levels after swim-up preparation of the spermatozoa have to be regarded as an effect of the sperm preparation process (Younglai et al., 2001; Jackson et al., 2010). Therefore, the extract can be said to have no deleterious effects on DNA fragmentation of spermatozoa *in vitro*.
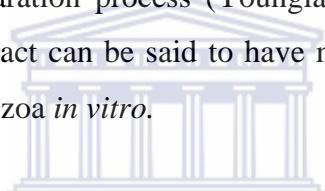
In addition, ejaculated, healthy human spermatozoa are apparently incapable of initiating apoptosis (Lachaud et al., 2004) although some parts of the apoptotic pathways seem to be activated (Aitken and Koppers, 2011). As from the elongated spermatid stage, male germ cells do not display the characteristic morphological changes of apoptosis, although DNA fragmentation can be determined and a translocation of phosphatidylserine has taken place in some cells, which might be due to the initiation of chromatin condensation and morphogenesis of spermatozoa (Blanco-Rodriguez and Martinez-Garcia, 1999).

Thus, the cytotoxic effects of TA compounds described by Kuo et al. (2004) and Nurhanan et al. (2005) appears not to be mediated by the induction of apoptosis as it has been described for eurycomanone, particularly for cancerous liver cells by up-regulation of p53 and pro-apoptotic Bax in the apoptosis pathway (Zakaria et al., 2009) with the induction of DNA fragmentation in the end.

4.3 Effects of TA on functional parameters of TM3-Leydig and TM4-Sertoli cells

4.3.1 Cytotoxic effects of TA on TM3-Leydig and TM4-Sertoli cells viability and cell proliferation

The XTT assay was used to determine the cytotoxicity of Tongkat Ali on TM3-Leydig and TM4-Sertoli cells. The biochemical procedure is based on the activity of mitochondrial dehydrogenase, which is only active in viable cells (Scudiero et al., 1988).

In the present study, a contrast in viability occurred at 48 hour exposure to TA between TM3-Leydig and TM4-Sertoli cells. A peak in viability was reached at the highest (50 μg/ml) concentration of TA in the TM3-Leydig cells whereas in TM4-Sertoli cells its peak was attained at a lower dose of 0.8 μg/ml TA. In addition, an increased mitochondrial activity at only 48 hours exposure would indicate that both cell lines are in fact stressed, with TM3-Leydig cells being more resilient. Moreover, at 96-hour exposure, both TM3-Leydig and TM4-Sertoli cells revealed a remarkable decrease in viability as concentrations of TA increased and cellular death is clearly evident at 25 and 50 μg/ml TA.
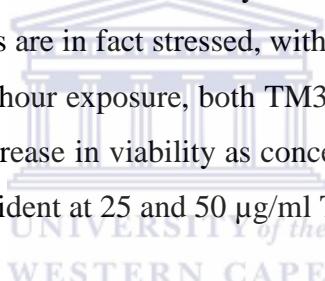
As mitochondrial dehydrogenase are only active in viable cells, the results obtained in the present study suggests, Tongkat Ali having cytotoxic effects on the cell at higher concentrations. This outcome concurs with the study performed by Nurkhasanah et al. (2009) on anticancer claims of eurycomanone. The cytotoxicity of eurycomanone as an identified anticancer compound has been well established (Kardono et al., 1991; Tada et al., 1991; Kuo et al., 2004; Cheah and Azimahtol, 2004), killing 50 % of cells at less than 20 μg/ml, reducing viability and proliferation significantly making it a potent antiproliferative agent (Wall et al., 1987; Boik, 1996).

A couple of theoretical reasons could explain the mechanism of Tongkat Ali's effects on TM3-Leydig and TM4-Sertoli cells in the current study. Firstly, if the cells are stressed and dying under the cytotoxic effects of TA, they cannot perform their specific roles adequately, as seen in the observation of cell protein concentration decreasing in both cell lines at 96-hour exposure.

In TM3-Leydig cells, the concentration of protein decreased from 140 to 50 mg protein/ml and in TM4-Sertoli cells from 20 x 10^{-2} to 15 x 10^{-2} mg protein/ml. This may coincide with the results found in the investigations previously performed by Zakaria et al. (2009) and Nurkhasanah et al. (2009) proving that eurycomanone causes obvious morphological changes, including the loss of adhesion, rounding, sporadical distribution, decreased cell size and membrane blebbing.

Secondly, in humans, during the first months of postnatal life, the Hypothalamic-Pituitary-Gonadal axis is activated (Burger et al., 1991; Andersson et al., 1998a and b) and GnRH is released through pulse regulation (Mruk and Cheng, 2010; Kopera et al., 2010). The mechanism and the functional significance of this early activation are still unclear (Andersson and Skakkebaek, 2001). Yet, this early stimulation of the testis with GnRH is important for the Sertoli cell proliferation before the onset of puberty (Cortes et al., 1987) and almost certainly for Leydig and germ cells. The latter occur only after the onset of puberty (Müller and Skakkebaek, 1984; Andersson et al., 1998a; Main et al., 2000). As it is known, the function of Sertoli cells are to nursing germ cells and synthesize androgen binding protein (ABP), and Leydig cells produce testosterone which binds to ABP (Prante et al., 2008). If TA affects any of these tasks via the Hypothalamic-Pituitary-Gonal axis, it may lead to the disruption in the process of germ cell maturation in juveniles, further causing small testes size, low spermatozoa and testosterone production.

4.3.2 Effect of TA on testosterone production in TM3-Leydig cells

Schiavi et al. (1990) reported an association between testosterone and libido. It was concluded that men with high levels of testosterone display higher intensity of sexual activity and desire, than men with lower levels of testosterone. With these said plants extracts which are reported aphrodisiacs can be assumed to increase testosterone levels. Although there are many plant extracts reportedly having aphrodisiac properties and improve male sexual functions, only few have been scientifically proven to increase testosterone levels (Patel et al., 2011).

As mentioned previously, *Tribulus terrestris* (devil's weed), *Panax ginseng* (Asian ginseng), *Pausinystalia yohimbe* (Yohimbe tree) and *Hibiscus macranthus* and *Basella alba* (Malabar spinach) are scientifically alleged to either increase sexual desire and sexual dysfunction, enhance erection, impotency or improve sexual stamina (Carey and Johnson, 1996; Adimoelja, 2000; Nocerino et al., 2000). However, only *Hibiscus macranthus* and *Basella alba* are reported to increase testosterone production as well (Moundipa et al., 2005; 2006).

Alongside, *Hibiscus macranthus* and *Basella alba*, Tongkat Ali has also been reported to exhibit aphrodisiac properties and increase sexual motivation and performance (Ang and Sim, 1997; 1998a; 1998b; Ang and Ngai, 2001; Zanoli et al., 2009). Recently, a few reports on its ability to increase serum testosterone levels have been published (Chan et al., 2009; Zanoli et al., 2009; Tambi et al., 2012; Henkel et al., unpublished). Furthermore, upon isolation of different compounds of the root extract, a bioactive peptide 4.3 kDa was identified as the potential phytoandrogen and labelled eurypeptides (Ali and Saad, 1993; Sambandan et al., 2006; Asiah et al., 2007). This bioactive complex is supposed to enhance the effect on the biosynthesis of androgens (Ali and Saad, 1993).

The actual biochemical mechanism for testosterone-enhancing effect of TA is still not known. Although, it is believed that, the bioactive eurypeptides facilitates the activation of CYP17 enzyme (17 α-hyroxylase/17, 20 lyase). This enzyme metabolically cascades into the increased conversion of pregnenolone and 17-OH-pregnenolone to yield more dehydroepiandrosterone (DHEA). It also impedes the metabolism of progesterone and 17-OH-progesterone to 4-androstendione and testosterone (Ali and Saad, 1993).

In the present study, testosterone production by TM3-Leydig cells was investigated after the exposure of Tongkat Ali at different concentrations for 48 and 96 hours. The testosterone production after the 48-hour exposure period marginally increased (P=0.0580) whereas after 96 hours of exposure the testosterone production significantly (P=0.0065) increased at the highest (50 μg/ml) concentration of TA when compared to the control. Additionally, it is also apparent that after 96 hours the concentration of testosterone has increased

[12 x 10^{-4} ng/ml] when compared to 48-hour exposure [6 x 10^{-7} ng/ml] of Tongkat Ali.

In the current observation, it appears that Leydig cells need at least 96 hours exposure to TA to physiologically adjust and produce testosterone. Theoretically, Tongkat Ali can therefore be said to work on Leydig cells directly *in vitro*. However, a possible enhancing effect that TA may have on the Hypothalamic-Pituitary-Gonadal axis can at this point not be ruled out.

4.3.4 Effect of TA on pyruvate production in TM4-Sertoli cells

Boussouar and Benahmed (2004) stated 'energy production in most cells requires catabolism of glucose to pyruvate by the enzymes of the glycolytic pathway'. Sertoli cells catabolize glucose to produce lactate and pyruvate as energy substrates for germ cell survival and maturation by the stimulation of FSH (Jutte et al., 1981; Grootegoed et al., 1985; Boussouar and Benahmed, 2004). Toxins are also believed to target Sertoli cells (Monsees et al., 2000). Therefore, the investigation of the effects of any plant extract is essential. Additionally, Boussouar and Benahmed (2004) also suggested that an increase in metabolism within the Sertoli cell may be a defence mechanism by the release of lactate or an indication of cell toxicity.

As previously mentioned pyruvate is metabolically converted to lactate as a waste product (Erkkila et al., 2002). In the present study, pyruvate was investigated by exposing TM4-Sertoli cells to TA for 48 hours and 96 hours. At 48-hour at lower doses, TA showed no effect. Additionally, the optimum doses, 3.125 µg/ml (P=0.0002) to 25 µg/ml (P=0.0260) displays a stimulatory effect of TA with significant increases in the pyruvate production. This may suggest an increase of cell proliferation or the effect of toxicity leading to stress within the cell. However, the latter suggestion may be more suitable since at the highest dose (50 µg/ml TA) a marginal decrease (P=0.0541) with a significant decline of 55 % in pyruvate production was observed. Subsequently, this decline in pyruvate production of 6 % to 105 % was further displayed at 96-hour exposure for all concentrations of TA.

Theoretically, when comparing the pyruvate production to the viability of the Sertoli cells, the results obtained logically indicates cell death. In effect, as the Sertoli cells are perceived to be dying, no metabolic activity is evident and therefore no pyruvate would be produced. This observation is extremely vital as Sertoli cells are essential in the process of spermatogenesis and germ cell maturation. Any damage to these cells may lead to smaller testes, low sperm counts or azoospermia, decrease in hormonal stimulation and disruption of the blood-testis barrier (BTB) which all lead to male fertility (Monsees et al., 2000; Aitken et al., 2004; Wong and Cheng, 2011; Sharpe, 2010).

Since the interest in plants and their effects on male fertility has been growing, the screening for antifertility agents within these plants has also been accumulating (Kamal et al., 2003). Numerous plants that have been screened are reported to enhance male reproductive functions, yet it may also hinder testicular functions (Ogbuewu et al., 2011). Kamal et al. (2003), D'Cruz et al. (2010) and Ogbuewu et al. (2011) extensively reviewed several plants with known anti-fertility effects. These include: *Embelia ribes* berries showing spermicidal activity by inhibition of sperm count and activity of enzymes of energy metabolism and alters testicular histology (Purandare et al., 1979; Agarwal et al., 1986); *Azadirachta indica* (neem) affects the structure and function of testis by damaging the seminiferous tubules, resulting in the loosening of germinal epithelium, degeneration of germ cells and the derangement of germ cell types (Choudhary et al., 1990; Shaikh et al., 1993; Joshi et al 1996; D'Cruz et al., 2010); Crude ripe paw paw (*Carica papaya*) seeds caused degeneration of the germinal epithelium and germ cells (Udoh and Kehinde, 1999); *Abrus precatorious* causes testicular degeneration characterized by reduced number of cells in the epithelium along with reduction in the number of sperm cells (Adedapo et al., 2007); *Ocimum sanctum* demonstrates detrimental effects on the ultra structure of the testis (Lohiya et al., 2008; Manivannan et al., 2009); *Thespesia populnea* exhibits effects by enlargement of the Sertoli cells and reducing germ cell attachment (Krishnamoorthy and Vaithinathan, 2003).

Taking these investigations into consideration, the mechanism of Tongkat Ali's cytotoxicity of the effects on Sertoli cells can only be speculated as anti-

proliferatory and cell membrane damage *in vitro*. The current study did not address the effects of TA on morphology of the cells or the hormonal system that may include the Hypothalamic-Pituitary-Gonadal axis, nor possible effects on the prostate including prostate cancer. Such investigations should be carried out in future to obtain a full representation of the actions of TA in living organisms.

4.4 Conclusion and further outlook

In conclusion, from the results of this study, it can safely be assumed that the extract has no deleterious effects on functional sperm parameters at therapeutically used concentrations (<2.5 µg/ml) supporting *in vivo* data by Tambi (2006). At lower dosages, TA may even be beneficial as an antioxidant as observed with ROS. Only, TA concentrations higher than 20 µg/ml displayed detrimental effects on acrosome reaction and sperm viability in washed spermatozoa. Additionally, *in vivo*, the female genital tract has different sites of natural sperm selection where defective sperm are eliminated (Henkel, 2012) and therefore the results obtained in the washed fraction may differ as observed in the swim-up method. The concentration of 20 µg/ml would reflect a dosage of 2000 µg/ml as it is about100 times more *in vivo*. Taking the metabolic activity of the liver into account, one can assume that the concentration that will show cytotoxic effects is even higher (Satayavivad et al., 1998).

The cytotoxic effects of TA are only presented at higher concentration from 25 µg/ml. TM3-Leydig cells appears to be more resilient than TM4-Sertoli cells in viability and protein production, yet at prolonged periods of exposure it is detrimental. Tongkat Ali acts directly on the cell as prolonged exposure revealed an increase in testosterone production in TM3-Leydig cells. However, the Hypothalamic-Pituitary-Gonadal axis and markers such as LH, FSH and inhibin should be investigated in future as the enhancing effects of TA could not be currently shown. Further *in vivo* studies are underway to investigate the safety of TA for the use of treating male infertility.

Tongkat Ali is suitable for possible treatment to enhancement testosterone levels to reduce aging male symptoms. It can also be used therapeutically as a natural antioxidant.

Chapter 5: References

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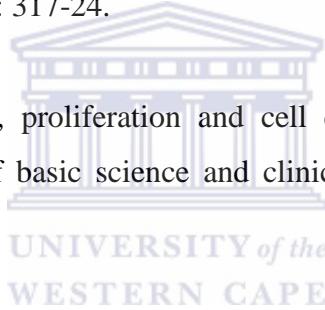
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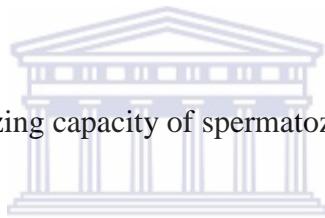
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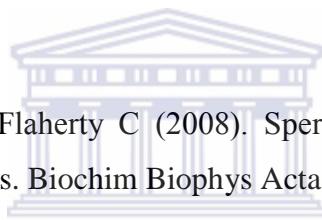
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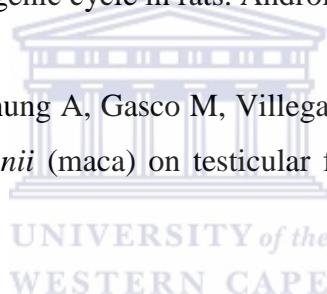
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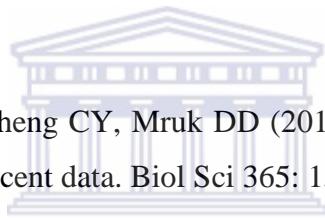
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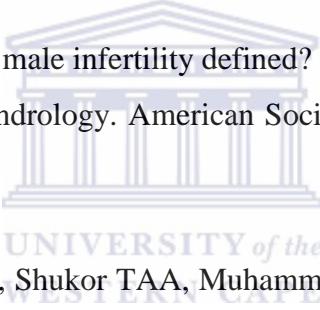
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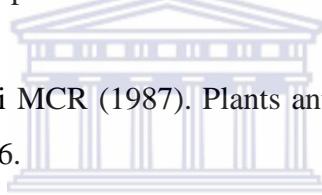
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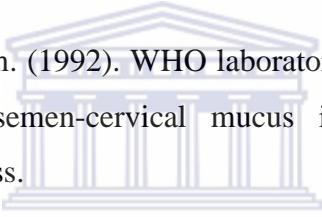
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