INVESTIGATING EFFECTS OF AQUEOUS ROOT EXTRACT OF *Mondia whitei* ON SPERM FUNCTIONALITY

By

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A thesis submitted in conformity with the requirements for the degree of Master of Science in the Department of Medical Biosciences, University of the Western Cape

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DECLARATION

I declare that the *investigating effects of aqueous root extract of Mondia whitei on sperm functionality* 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.

MAUREEN BILINGA TENDWA

Signed: _____

Date: _____

DEDICATION

This thesis is dedicated with gratitude to my entire family for the sacrifices they have made to ensure I achieve my dreams.

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LIST OF ABBREVIATIONS

2H4MBZA	2-hydroxy-4-methobenzaldehyde
ALH	Amplitude of lateral head displacement
ALT	Alanine transaminase
ANOVA	One way analysis of variance
AR	Acrosome reaction
AST	Aspartate transaminase
ATP	Adenosine triphosphate
BCF	Beat cross frequency
BSA	Bovine serum albumin
Ca^{2+}	Calcium ions
cAMP	Cyclic monophosphate
CASA	Computer-aided sperm/semen analysis
CAT	Catalase
VCL	Curvilinear velocity
CTC	Chlorotetracyclin stain
DAG	Inositol-triphosphate diacyglycerol
DHE	Dihydroethidine
dH_2O	Distilled water
dUTP	Deoxyuridine triphosphate
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
FANCA	Fanconi anaemia
FSH	Follicle stimulating hormone
E & N	Eosin-Nigrosin
GACP	Good agricultural and collection practices

GnRH	Gonadotropin releasing hormone
GR	Glutathione reductase
GSH	Glutathione
GSR	Glutathione reductase
GSSH	Oxidized glutathione
GnRH	Hypothalamic gonadotropin releasing hormone
H_2O_2	Hydrogen peroxide
·OH	Hydroxide radicals
HTF	Human tubular fluid
HOCl	Hypochlorus acid
·OHCL	Hypochlorite radicals
ICSI	Intracytoplasmic sperm injection
ALH	Lateral head displacement
LH	Luteinizing hormone
LIN	Linearity
LPO	Lipid peroxidation
MMP	Mitochondrial membrane potential
N	Normality
n	Number of samples
Na_2CO_3	Sodium carbonate
NaCl	Sodium chloride
NO	Nitric oxide
O^{2-}	Superoxide anion
OS	Oxidative stress
OSS	Oxidative stress status
·ROOH	Peroxyl radicals

PLC	Tyrosine Kinase
PBS	Phosphate buffered saline
PIP_2	Phospatidyl-Inosital-biophosphate
PKA	Protein kinase
PUFA	Polyunsaturated fatty acids
$\cdot ROO^-$	Peroxyl radicals
ROS	Reactive oxygen species
RT	Room temperature
SOD	Superoxide dismutase
$\cdot O_2^-$	Superoxide anions
STR	Straightness
TDF	Testis determining factor
TdT	Terminal deoxynucleotidyl transferase
TUNEL	Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labelling
VAP	Average path velocity
VCL	Curvilinear velocity
VSL	Straight-line velocity
WOB	Wobble
WHO	World health organisation
Zn	Zinc
ZP	Zona pellucida

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ABSTRACT

Introduction

Mondia whitei commonly known as "White Ginger" is a highly acclaimed medicinal plant that is extensively used across Africa. *M. whitei* is used as treatment for sexual dysfunction and is considered to be an aphrodisiac by traditional medicine practitioners. Yet, scientific evidence to support these claims are minimal and those that are published possess ambiguity. To date, only one study reporting the *in vitro* effect of the aqueous rhizome extract of *M. whitei* on human sperm motility is available. Therefore, the aim of the study was to determine the *in vitro* effects of *M. whitei* in human sperm functions.

Materials and Methods

Roots of *Mondia whitei* obtained from the tropical Kakamega rain forest, located in the Western Province of Kenya, were cleaned and chopped into smaller segments. These pieces were oven-dried at 25^0C for 3 days and milled to form a powdery substance which was infused with hot (about 70^0C) distilled water for 1 hour. After cooling and filtration, the extract was frozen at -20^0C and subsequently freeze-dried. The dried extract was then stored at 4^0C in a closed container until experimentation.

A total of 60 semen samples were collected: 28 of them represented healthy sperm donors and 32 infertile patients. Among these subjects, oligozoospermic and asthenozoospermic semen samples were identified and analysed separately. Sperm were washed using human tubular fluid medium supplemented with bovine serum albumin (HTF-BSA) and incubated for 1 hour at 37^0C with different concentrations of *M. whitei* (0.0185, 0.185, 1.85, 18.5 and 185 μg/ml). A sample without *M. whitei* served as control. Sperm cell motility, vitality, reactive oxygen species (ROS) production, mitochondrial membrane potential (MMP), capacitation, acrosome reaction and DNA fragmentation were assessed.

Results

Total motility and the percentage of sperm with intact MMP showed significant dose-dependent increases in both groups (patient and donor), while, the percentages of progressively motile sperm only revealed significant increases in the patient group. Besides, the percentage of ROS-positive spermatozoa showed significant trend towards higher concentrations in the patient group only. Conversely, a trend towards reduced sperm DNA-fragmentation could be observed in the patient, but not the donor group. Similar tendencies were noted in oligozoospermic and asthenozoospermic, but not for normozoospermic subjects. Yet, sperm vitality, capacitation, acrosome reaction and kinematic parameters were not affected.

Conclusions

Phytochemicals present in *M. whitei* root extract maintains spermatozoa total motility, progressive motility and intact-MMP and DNA integrity. However, at therapeutic concentration (<1.85 µg/ml) it does not trigger sperm intrinsic superoxide production nor increase ROS by causing oxidative stress, that leads to DNA fragmentation.

KEYWORDS

Acrosome Reaction

Capacitation

Infertility

DNA fragmentation

Mitochondrial Membrane Potential (MMP)

Mondia whitei

Motility

Reactive Oxygen Species (ROS)

Spermatozoa

Viability

CHAPTER 1: INTRODUCTION

1.1 General insight

The great diversity and therapeutic potential of plants could be beneficial if substantial research into the traditional use of plants as medicine would be done. This study seeks to clarify the role of *Mondia whitei* in the traditional treatment of male infertility.

Human male fertility is a vital aspect of reproduction based on the ability of spermatozoa to fertilize and activate the egg and support early embryonic life. However, it has been considered lower than most animals (Jørgensen et al., 2001), with increasing infertility rates in many countries affecting one in six couples (Sharpe et al., 2003; Kamel, 2010). The issue of male infertility is multi-factorial with some men suffering from low fertility in spite of having adequate numbers of sperm with normal morphology and motility. However, the lack of knowledge in regard to the multi-factorial causes of male infertility has proved challenging on the rational approach towards development of effective therapies (Wu et al., 1989). According to Agarwal and his colleagues (2014), about 30,625,864 to 30,641,262 of the male population worldwide suffer from infertility based on the World Health Organisation (WHO) 2010 standards.

One of the first people to alert the world on the possibilities of declining male fertility is said to be Professor Niels Skakkeback (Dindyal, 2004). The Danish scientists' study showed that sperm count from healthy men had dropped by more than half in 50 years. This study was conducted at the Department of Growth and Development at Copenhagen University, between 1938 and 1992 and it involve 14,947 men (Carlsen et al., 1992). Despite the fact that Carsen's and colleagues findings caused a lot of debate (Swan et al., 2000) subsequent studies have confirmed these outcomes. For instance, an investigation done in Paris which involved 1350 sperm donors found a decrease in sperm count by approximately 2% each year over the past 23 years (Sharpe et al., 1995). Dindyal et al., (2004) recorded a 25% decline in sperm count over 20 years with an average of 100

million/ml recorded in 1950. These figures went down in 1970 and 1990 to 75 million/ml and 50 million/ml, respectively.

Discovering the complexity of spermatogenic development has been the centre core of many researchers over the past decades, for a clear understanding of what factors are involved during this process. This will enable the development of strategic measures to address challenges posed in issues related to human male infertility (Ruwanpura et al., 2010). On the other hand, medicinal plants have been extensively used, particularly in Third World and developing countries, in the treatment of male reproductive dysfunction despite the fact that little research has been done on them (Semwal et al., 2013). This shortcoming can be addressed through advancement in research which has provided a corner stone for standard analysis of sperm function.

This chapter outlines an understanding of the male reproductive system, endocrine regulations and formation of spermatozoa. It also focuses on the history of traditional herbalism in use of *M. whitei* to address issues of male infertility.

1.2 Human male reproductive system

1.2.1 An overview

The male reproductive system is a linkage of internal and external organs whose function is to produce, maintain, conduct and deliver viable sperm to fertilize an ovum (Rhodes, 1969; Sherwood, 2011; Scanlon and Sanders, 2014). This is made possible through spermatogenesis, endocrine and paracrine hormonal regulation of reproductive function i.e. (Gonadotrophin releasing hormone [GnRH], Follicle-stimulating hormone [FSH], Luteinizing hormone [LH], Inhibin and testosterone [androgen]) (Sofikitis et al., 2008; O'Shaughnessy, 2014) and deliverance of semen containing spermatozoa into the female reproductive tract through sexual intercourse. The organs of the male reproductive system include: testes (site for spermatogenesis and hormonal production); epididymis (storage of mature spermatozoa and site for maturation); vas deferens and accessory

glands (seminal vesicles, prostate, and bulbourethral gland) (Figure 1). The testis, accessory glands and epididymis are instrumental in the formation of the seminal fluid which serves as: a lubricant to the passage spermatozoa must progress through; media through which spermatozoa are transported; protecting agent of spermatozoa from the acidic environment of the female genital tract and source of energy for the sperm activities since it contains fructose (De Kretser et al., 1998). The external genitalia comprise of the scrotum (encloses the testes) and the penis (an erectile organ that houses the distal portion of the urethra) (Martini et al., 2015).

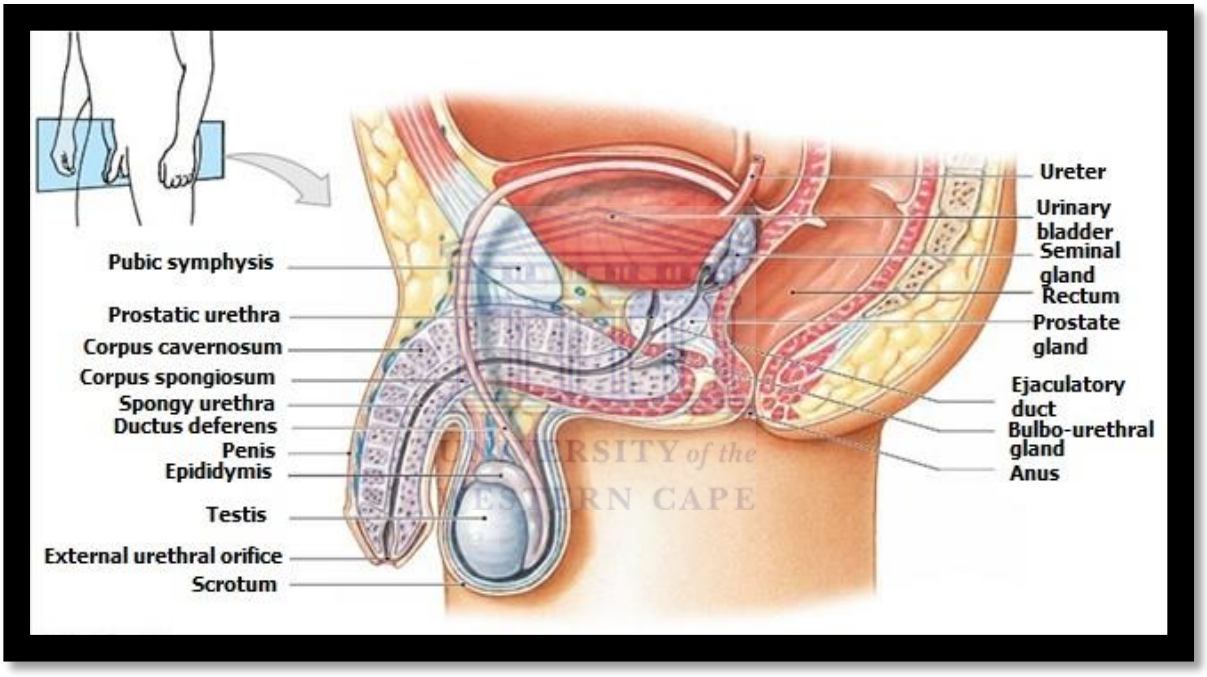

Figure 1: The Male Reproductive system - Sagittal section of the male reproductive organs showing the testes (site for spermatogenesis and hormonal production); epididymis (site for maturation and storage of spermatozoa) and accessory glands (seminal vesicles, prostate and bulbourethral gland) (Martini et al., 2015).

1.2.2 The testes

The development of the gonad is a unique embryological condition which can either become an ovary or testes (Gilbert, 2000). Though, formation of the testes will then depend on early activation of a gene localized in the Y-chromosome known as TDF (testis determining factor) (Hadley et al.,

1985). Basically, the testis determining Sry (Y-encoded gene) triggers the undifferentiated genital ridge also known as bio-potential gonad to develop into testes (Wilhelm et al., 2007).

Sertoli cells are the first cells to differentiate, therefore setting a trend of testes differentiation (Mackay, 2000). Direct testicular activities, organisation and coordination are thought to be prompted via a signal from the Sertoli cells (Cortes et al., 1987). Though the mechanisms involving the differentiation are poorly understood (Sharpe, 2010). Hardley et al. (1985) supposed that Sertoli cells surround the germ cells which have migrated into the gonad, followed by attraction of peritubular myoid cells encircling the Sertoli-germ cell complex, resulting in the formation of the seminiferous cord (Wilhelm et al., 2007). The cord undergoes elongation as Sertoli and germ cells proliferate, after it anchors itself at both ends in the rete testis. Concurrently, foetal Leydig cells differentiate and begin to produce testosterone (Quinn and Koopman, 2012). This differentiation takes place in the interstitial space between the seminiferous cords (Sharpe, 2010).

Follicle stimulating hormone (FSH) induces Sertoli cell proliferation in neonate producing a final number of cells that differentiate terminally during puberty (Ross and Capel, 2005). Later, Sertoli cells lose their proliferation ability (Tan et al., 2005; Macedo, 2012). Testosterone produced within the Leydig cells in the developing testis act to stimulate the Sertoli cells proliferation (Quinn and Koopman, 2012). Besides, the number of sperm cells produced in adulthood is determined by the number of Sertoli cells within the testis (Orth et al., 2000; Sharpe et al., 2003). Thus, only a given number of germ cells through their development into sperm cells can be supported by each Sertoli cell (Orth et al., 1988). Proliferation of Sertoli cells takes place during foetal life, post and neo-natal period and just before puberty (Ross and Capel, 2005). According to Plant and Marshall (2001), the importance of Sertoli cell proliferation and factors that regulate this process during the different periods of development may differ: for instance, during foetal and neonatal period; testosterone seems to play an important role whilst, FSH is of more significance during puberty. Additionally, proliferation of Sertoli cell during this period may also be affected by lifestyle and environmental factors at any of those stages (Rebourcet et al., 2016). In his review Sharpe (2010)

stated that this impact could directly lessen the quality of sperm production which may directly affect sperm count.

1.2.3 Testicular feature

The testes located in the scrotal sac are two oval organ (3 cm anterior to posterior, 2 cm wide and 4 cm long each) (Figure 2) (Marieb and Hoehn, 2007). They possesses two major functions, gametogenic and endocrine, of which, the endocrine function entails synthesis of male sex hormones while the gametogenic function involves production of hormone (Mortimer, 1994). Each testis is surrounded by two connective tissues, the outermost tissue found in the anterior lateral surfaces is the Tunica vaginalis, moving inwards is Tunica albuginea, which is a thick fibrous connective tissue that subdivides the testis into about 250 lobules. Each lobule contains 1 to 4 seminiferous tubules and interstitial connective tissues (Sherwood, 2011). According to Marieb (2007), the seminiferous tubule is made up of complex stratified epithelium containing two basic cell population known as the Sertoli cells and spermatogenic cells. Basically, Sertoli cells are physical support cells that do not undergo replication, while spermatogenic cells (stem cells) regularly replicate and differentiate into mature sperm as they migrate towards the lumen.

The interstitial connective tissue contain Leydig cells, that function in-producing and releasing testosterone (Wistuba et al., 2007). In the long run, the seminiferous tubules merge into larger set of tubules known as the rete testis. Ultimately, the rete testis drains into a larger tubule known as efferent ducts which in turn drains into the epididymis which is made up of head, body and tail. Lastly but not least, the tail dilates into ductus deferens (Figure 2) (Macedo, 2012; Martini et al., 2015). The ductus deferens continues up the spermatic cord into the body through the inguinal canal. Right and left ductus deferens proceeds cranially over the ureters behind the urinary bladder. As the duct passes behind the bladder, it has attached to its gland called the seminal vesicles. However, prior to this attachment, the ductus enlarges into the ampulla (site of sperm storage before ejaculation) (Marieb and Hoehn, 2007).

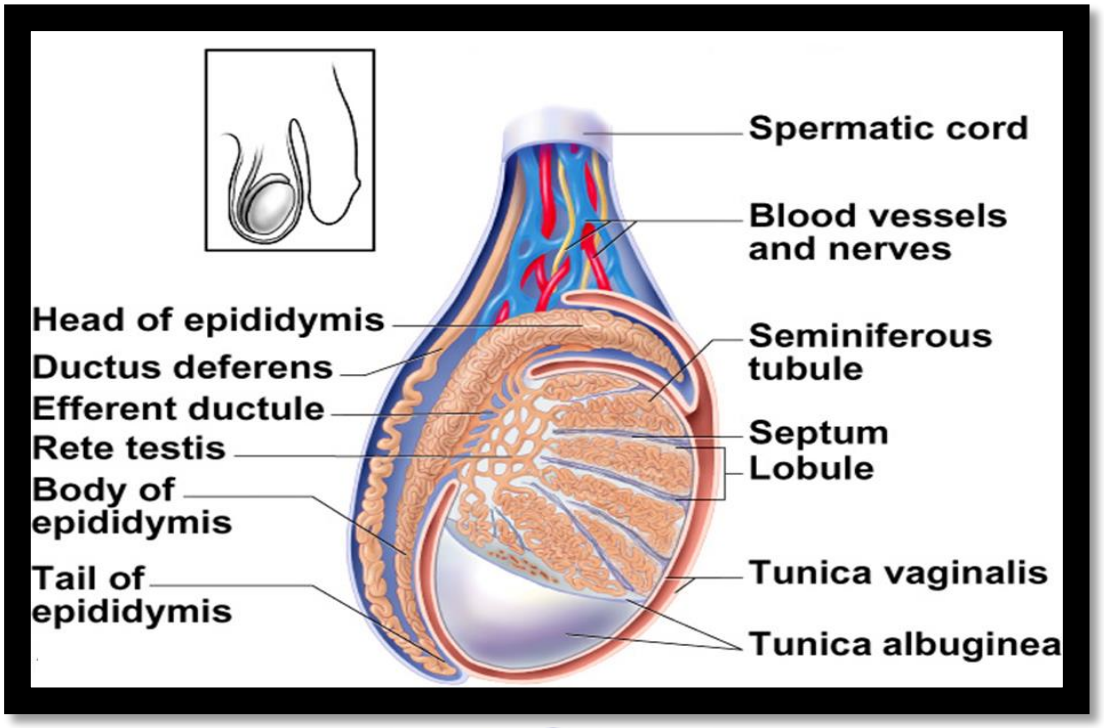

Figure 2: The testis: sagittal section of the testis showing the two tunics connective tissue; Epididymis (connection between testis and ductus deferens); ductus deferens and accessory glands (Marieb et al., 2007).

1.2.4 Spermatogenesis

One of the most important functions in life is the production of spermatozoa through spermatogenesis, which takes place in the male gonad (testis). The process is complex and needs highly organised tissues that are potently regulated by hormonal interaction, cell to cell communication and differential gene expression (Steger et al., 1998). In summary, spermatogenesis is an essential, complex biological process, leading to the formation of highly specialized mature spermatozoa within the testis through an organised cascade of events (Macedo, 2012).

During this process, spermatogenia undergo meiotic recombination, reduction of genome to haploid state and immense cellular modification that results in motile spermatozoa capable of crossing the female reproductive tract, enduring various biological assaults for viability and eventually fertilizing a mature oocyte to give rise to an embryo (Carrell, 2013). This process is a highly co-ordinated mechanism fundamental in ensuring constant sperm production, characterised

by mitotically inactive Sertoli and germ cells undergoing mitosis, meiosis and spermiogenesis (Steger et al., 1998; Hogarth and Griswold, 2010). The production of functional spermatozoa in the testes takes approximately 74 days and an additional 12 days for maturation of released sperm in the epididymis (Durairajanayagam et al., 2015), yielding about 100 million spermatozoa each day (Reijo et al., 1995). Spermatogenesis commences at puberty and continues until death (Clermont, 1963; Clermont and Antar, 1973) and is characterised by a series of events that originate at the basal compartment and ends at the apical compartment of the seminiferous tubule (Durairajanayagam et al., 2015).

1.2.4.1 Occurances in the basal compartment of the seminiferous tubule

This compartment marks the origin of spermatogenesis with spermatogonial renewal and proliferation via mitosis and differentiation; cell cycle progression from type B spermatogonia to preleptotene spermatocytes (Figure 3) (Cheng and Mruk, 2012). Firstly, stem cells called spermatogonia divide by mitosis to produce two daughter cells. Type A spermatogonia divide into type A_{pale} and type A_{dark}, although it is debatable how this cells are involved in proliferation activities (Steger et al., 1998). Spermatogonia are referred to as A_{pale} spermatogonia as soon as they enter the differential pathway (Hogarth and Griswold, 2010). Type A_{pale} spermatogonia undergo self-renewal to generate the stem cell pool based on their chromatin structure. In time, type A_{pale} spermatogonia will then develop into type B spermatogonia (precursor of sperm) and eventually proceed to preleptotene followed by leptotene primary spermatocytes (De Kretser et al., 1998; Hogarth and Griswold, 2010). In the human, undifferentiated A_{dark} spermatogenia have the capacity to become gametes but are not yet committed to the process. However, they do undergo mitosis to increase the testicular stem cell population and provide progenitor cells that undergo spermatogenesis (Meehan et al., 2000).

1.2.4.2 Occurrences in the ad-luminal compartment of the seminiferous tubule

A summary of activities in this compartment includes: 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 (Figure 3) (Cheng and Mruk, 2012). Meiotic events that enable maturation of spermatocytes encompass chromatin condensation in the nucleus, whereby spermatogenia enter the first meiotic division (prophase) is subdivided into: Preleptotene and Leptotene stages during which the chromatin condenses into visible chromosomes, followed by Zygotene and Pachytene stages which is characterised by pairing of homologous chromosomes to become primary spermatocyte. This is followed by the Diplotene stage in which each cell undergoes chromosomal crossing over. Hence, the formation of duplicates, of which 22 pairs are autosomal chromosomes, 2 X-chromosomes and 2 Y-chromosomes (Cheng and Mruk, 2012), takes place. These cells then undergo meiosis II, which is characterised by separation of sister chromatids into individual cells. The division of every spermatid leads to the formation of four haploid gametes (23 chromosomes) termed as round spermatids (Hogarth and Griswold, 2010).

1.2.4.3 Spermiogenesis: Spermiation and maturation in the epididymis

Spermiogenesis is a process by which haploid spermatids become specialized spermatozoa by the process of elongation and differentiation. There are four phases involved in this process namely Golgi phase, cap phase, acrosome phase and maturation phase (Steinberger, 1971). Through the four processes each round spermatid becomes fully compacted and develops into an elongated spermatid that undergoes spermiogenesis to form spermatozoa (Figure 4) (Durairajanayagam et al., 2015).

Lastly, spermiation, which involves the release of mature spermatozoa from the lumen of seminiferous tubule takes place (Hogarth and Griswold, 2010; Durairajanayagam et al., 2015).

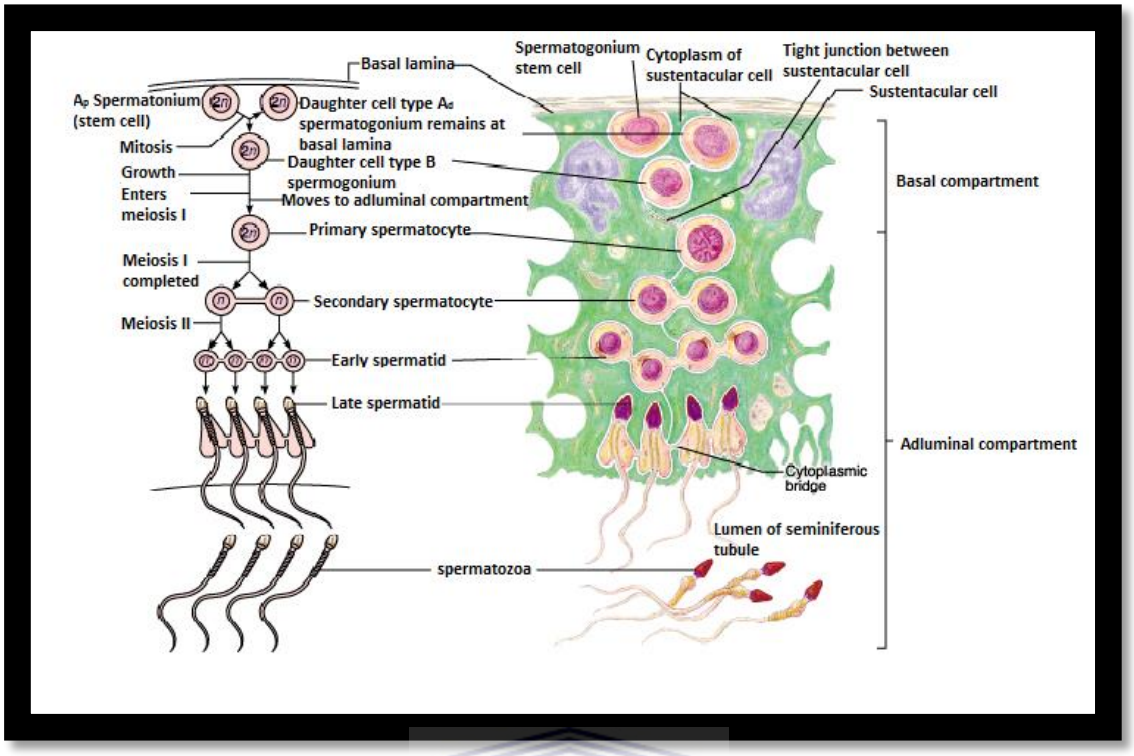

Figure 3: summary of 3 stages of spermatogenesis: spermatoginal phase taking place at the basal compartment of the seminiferous tubule. Spermatogenia proliferate by mitotic division to produce A_{dark} (Ad) spermatogonium which proceed to form stem cells and A_{pale} (Ap) spermatogonium which proceed through spermatogenesis to form primary spermatocytes: Spermatocyte and spermatid phase taking place at the adluminal compartment of the seminiferous tubule. Spermatocyte phase: primary spermatocyte enter meiosis I to form secondary spermatocyte which enters meiosis II and results in the formation of spermatids. Spermatid phase: spermatid becomes specialized and motile spermatozoa (Adopted from Pearson Edu, 2013).

From the lumen, spermatozoa leave the testis to the epididymis through the rete deferens; owing to the fact that the peritubular Myoid cells are able to conduct peristalsis necessary to move, at this stage sluggishly motile elongated testicular spermatozoa in the direction of the efferent duct to the epididymis (Wistuba et al., 2007). In the epididymis, spermatozoa released from the testis get subjected to additional physiological maturation to acquire motility and the potential to fertilize an oocyte (Figure4) (Hogarth and Griswold, 2010). This process involves several biological and morphological changes encompassing compacting of nuclear substances via, enzyme mediated oxidative cross-linking protamines (Fujii and Tsunoda, 2011), which are enabled by the epididymal

micro-environment that helps maintain osmolarity. Hence, stabilizes the sperm membrane (Mortimer, 1997).

1.2.5 Endocrine regulation of spermatogenesis

Normal function of the testes and reproductive functions are regulated by interaction of the highly complex endocrine hypothalamus-pituitary-gonadal axis. The onset of puberty in the human male is strongly characterised by the production of testosterone, which is influenced upon stimulation of luteinising hormone (LH) and Leydig cells (Wistuba et al., 2007; Knez, 2013). Endocrine regulation of spermatogenesis is then maintained through the relationship between gonado-trophins, steroid and testicular somatic cells (Sertoli and Leydig cells).

1.2.6 The male reproductive hormones

The hormonal reproductive axis in male consist of three main components: the hypothalamus, the pituitary and the testis (Hall and Guyton, 2011). This axis usually functions in a tightly controlled manner to produce hormones necessary for normal sexual development, sexual function and fertility (Wistuba et al., 2007). Hormones produced by glands in this axis include: hypothalamic gonadotropin releasing hormone (GnRH), pituitary gonadotropins [follicle stimulating hormone (FSH) and lutenising hormone (LH)] and testicular steroids (Rizzo, 2015). These hormones function in an integrative way to ensure normal development and regulation of the male reproductive system (Wistuba et al., 2007). Dysfunction in either the hypothalamus, pituitary or the testes may results in widespread repercussion such as ambiguous genitalia, delayed puberty and infertility (Hall and Guyton, 2011).

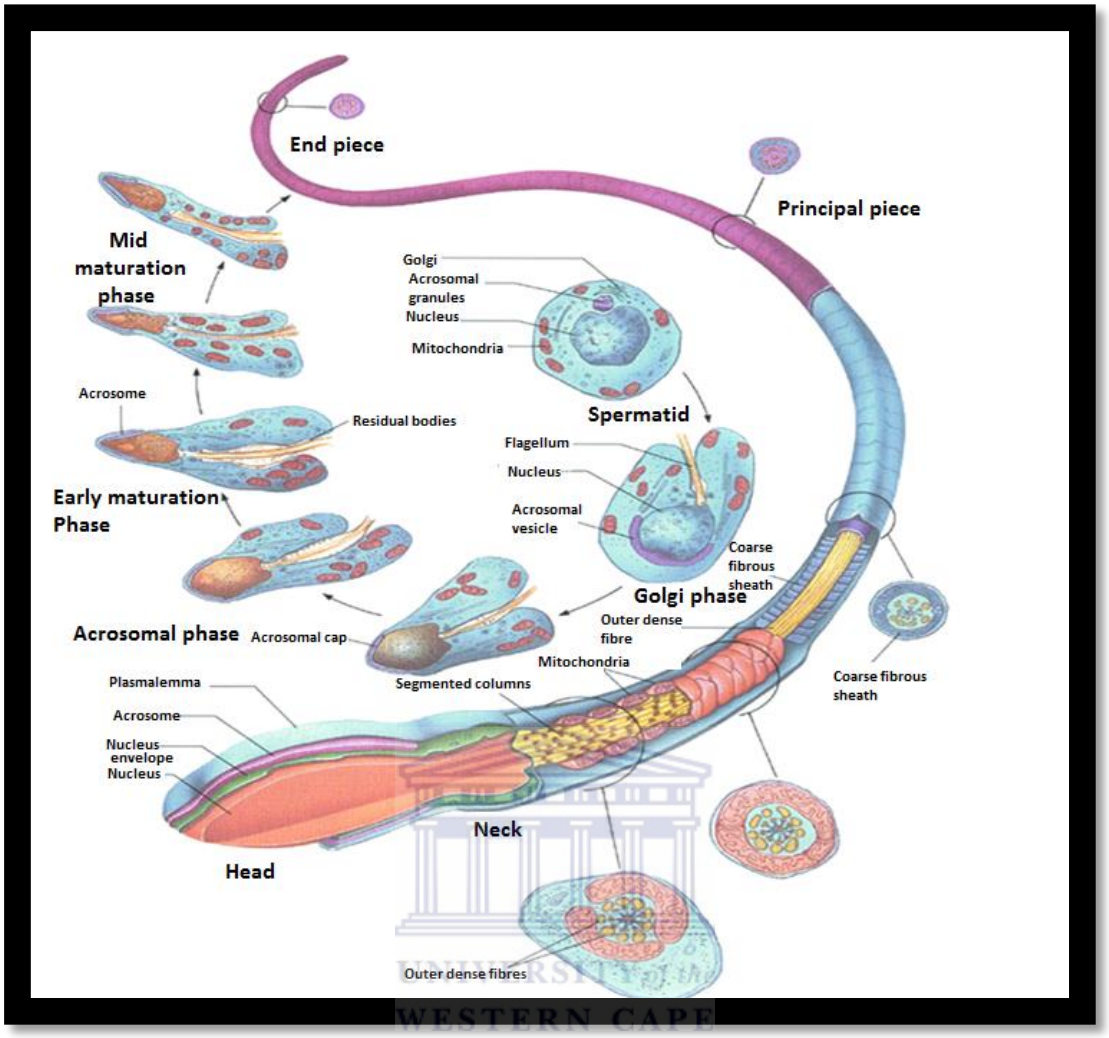

Figure 4: Illustration of a round spermatid undergoing the different phases of spermiogenesis which involves appearance of acrosome vesicles and flagella, growth of acrosome and flagella, shedding of excess cytoplasm to become specialized spermatozoa. (Pearson Edu, 2011)

1.2.6.1 Function of gonado-tropin-releasing hormone (GnRH)

GnRH is a neuro-hormone produced by specific neuro cells, whose main function is stimulation of the synthesis and secretion of LH and FSH by the anterior pituitary gland. The neuro cells are distributed throughout the hypothalamus and release hormones at its neuro-terminals (Meccariello et al., 2014). The preoptic area of the hypothalamus contains most of the GnRH neurons. GnRH is pulsatile and periodically occurring every 1-2 hours (Rizzo, 2015). The GnRH-containing neurons project to the median eminence where they release GnRH from their axon terminals into

the hypophyseal portal system. In the portal capillary, the GnRH travels to the anterior pituitary gland to influence the production of LH and FSH (Meccariello et al., 2014).

1.2.6.2 Function of gonado-tropins hormones (FSH and LH)

Gonado-tropin (LH and FSH) hormones are hetero-dimer glycoprotein hormones secreted from the anterior pituitary gland by gonado-tropic cells under the stimulation of GnRH (Pernasetti et al., 2001; Hall and Guyton, 2011). LH and FSH exert their effects on their respective target cells located chiefly in the testes. They do so mainly by activating the cyclic adenosine monophosphate second messenger which activates specific enzymes in the respective target cells (Wistuba et al., 2007).

LH stimulates the interstitial (Leydig) cells in the testis to stimulate testosterone hormone release; the amount of testosterone produced in male is thought to be approximately directly proportional to LH (Achard et al., 2009). According to Wistuba (2007), the main function of LH is determining secondary sexual characteristics by stimulating the secretion of testosterone. Guyton and Hall (2011) give more insight by saying the production of mature male gametes is also dependant on LH, which is a gonadotrope glycoprotein. It stimulates Leydig cells to produce testosterone (Achard et al., 2009). LH is increased in the perinatal period for a short period (7-13 weeks) before its activity is decreased again and remains slow until puberty (Kuiri-Hänninen et al., 2014). The levels are then elevated again at the onset of puberty. As a results, Leydig cells get stimulated to produce androgens again. Afterwards, this relationship is maintained through a feedback loop during male adulthood (Meccariello et al., 2014).

FSH in turn stimulates Sertoli cells in the seminiferous tubules of the testes to grow and secrete various spermatogenesis substances (Wistuba et al., 2007). At the same time, testosterone and dihydrotestosterone diffuse into the seminiferous tubule to act on spermatogenesis. In addition, evidence has shown that FSH is important in multiplication of spermatogonia supported by testosterone action for proliferation of the germ cell line and plays a major role for the renewal of

type-A spermatogonia (Simoni et al., 2008). Thus, both FSH and testosterone contribute to the final maturation of spermatozoa, and protect the germ cell line against apoptosis (Hall and Guyton, 2011).

1.2.6.3 Function of Inhibin B

Inhibin B is a glycoprotein which is synthesized by Sertoli cell through stimulation from FSH (Wistuba et al., 2007). The regulation of inhibin B is strongly associated with gonadotropins whose level decreases in males as they age (Ramaswamy and Plant, 2001). The onset of puberty activates the production of inhibin B (Meccariello et al., 2014). In early postnatal testis development, an increase of inhibin B levels is seen leading to down-regulation of the hypothalamic-pituitary-gonado axis which results in the changes in testosterone, LH and FSH levels (Wistuba et al., 2007). FSH action and Sertoli cell proliferation are associated with inhibin B activity during pre-pubertal testis formation (Simoni et al., 2008). Afterwards, the level of inhibin B declines which mirrors Sertoli cell density (Wistuba et al., 2007).

The major determinant of inhibin B in adulthood (after completion of spermatogenesis in puberty) is the germ cells (Ramaswamy and Plant, 2001). Rising androgen levels are thought to be the activating factor in germ cell proliferation which secondarily depends on FSH and has been associated with changes in Inhibin B levels (Meccariello et al., 2014). Thus, inhibin B levels are indicated in spermatogenic efficiency since the level directly correlates with sperm count (Wistuba et al., 2007).

The two main functions of heterodimeric gonadotropin FSH (glycoprotein), a major contributing factor in the regulation of spermatogenesis, in adulthood are: Regulation of inhibin B and determination of Sertoli cell number in immature testis. FSH influences maturation of Leydig cells (Simoni et al., 2008). All these processes are mediated via G-protein coupled transmembrane FSH receptors (Wistuba et al., 2007).

1.2.6.4 Function of Testosterone

Testosterone is the principle male steroid hormone from the androgen group (Simoni et al., 2008). In the male, testosterone is mainly secreted in the testes by interstitial Leydig cells. Small amounts are also secreted by the adrenal glands (Rizzo, 2015). The major function of testosterone in the testes is the maintenance of spermatogenesis via stimulation of Sertoli cells; its actions, are mediated through intracellular androgen receptor which acts as a transcription factor (Simoni et al., 2008). Testosterone produced by Leydig cells, passes into the Sertoli cells and binds to receptors. The combination of testosterone with the receptors is required for the Sertoli cells to function normally. In addition, testosterone is converted to two other steroids in the Sertoli cells: estrogen and dihydrotestosterone (Hall and Guyton, 2011).

The Sertoli cells also secrete a protein called androgen-binding protein into the seminiferous tubules (Rizzo, 2015). Testosterone and dihydrotestosterone bind to androgen-binding protein and are carried along with other secretions of the seminiferous tubules to the epididymis. Estradiol and dihydrotestosterone may be the active hormones that promote sperm cell formation (Hall and Guyton, 2011). According to Rizzo (2015), other functions of testosterone include: promotion of growth, differentiation, and function of accessory organs of reproduction; maintenance of normal reproductive function in the adult, stimulates transport and delivery of sperm and increases sexual drive (libido) in both men and women.

1.2.7 The male reproductive cells

1.2.7.1 Sertoli cells

These cells were first described in 1865 by Enrico Sertoli hence, origin of the name. He suggested they provided a number of nutrients for the germ cells within the seminal epithelium. Thereafter, many studies have stipulated the role of Sertoli cells in spermatogenesis which include: provision of nutrition at the differentiating sperms, formation of the blood-testes-barrier tight junctions, involvement in selective permeability hindering pathogens that may harm sperm cells and allowing the passing through of beneficial substances like testosterone, secretion of adequate fluid into the

lumen of the seminiferous tubule, which helps in sperm maturation towards the epididymis tubule and synthesis and metabolism of steroids (Dym and Raj, 1977; Wistuba et al., 2007; Sherwood, 2011).

1.2.7.2 Leydig cells

Leydig cells (interstitial endocrinocytes) are clusters of endocrine cells found between seminiferous tubules, whose function is derived from endocrine and paracrine inter-communication (Ge et al., 2009). These cells mainly synthesize and secret male sex hormones (androgens), the most important of which is testosterone (Wistuba et al., 2007). Endocrine stimulation by LH induces Leydig cells to secret testosterone. In turn, testosterone diffuses into the seminiferous tubule and drives spermatogenesis together with FSH (Ge et al., 2009). Therefore, making Leydig cell significant in spermatogenesis (Hall and Guyton, 2011).

1.2.8 Hypothalamus-pituitary-gonadal axis

1.2.8.1 Hypothalamus-pituitary-gonado axis initial stimulation

The hypothalamus-pituitary-gonadal axis has to be stimulated prior to the onset of puberty to initiate the sexual life (Wistuba et al., 2007). This process is initiated through the release of Kisspeptin from the arcuate nuleus of the brain by specialized neurons (kiss-1-neuron) to stimulate gonadotropin releasing hormone (GnRH). Hence, GnRH is released as a result of kiss-1-neuron stimulating GnRH neurons in the hypothalamus leading to expression of GPR54 receptors, which binds kiss-peptin via a G-protein cascade (Smith et al., 2006). In turn, the release of GnRH from the hypothalamus in pulsatile intervals of 60 to 120 minutes, initiates the release of FSH and LH. Receptors expressed by Sertoli cells bind FSH, thus acting to stimulate spermatogenesis whilst LH stimulates Leydig cells to produce testosterone in the testis (Wistuba et al., 2007; Sharpe, 2010). The initial release of Kiss-peptin initiates pubertal events in both male and female (Smith et al., 2006).

1.2.8.2 Hypothalamus-pituitary-gonadal axis function

Gonadotropin Releasing Hormone (GnRH) is released from the hypothalamus in pulse rate intervals every 90-120 minutes (Cheng and Mruk, 2012). The portal blood carries GnRH to the anterior pituitary glands, which contains the gonadotrope cells (receptors of GnRH) (George et al., 2011). Subsequently, the activation of GnRH receptors that is, a beta seven transmembrane G-protein-coupled receptor that stimulates the isoform of phosphoinositide phospholipase C, which goes on to mobilise protein kinase C takes place, resulting in activation of proteins involved the synthesis and secretion of LH and FSH (Wistuba et al., 2007). As a result of the stimulation by gonadotropins, steroidal hormones such as testosterone, estrogen and progesterone are consequently stimulated and released at target organs (testes in males and ovaries in females). These hormones are important for male and female secondary characteristics which include maintaining, supporting and ensuring reproduction, bone density and muscle mass (Ge et al., 2009; Hall and Guyton, 2011; Cheng and Mruk, 2012). However, when the testosterone level is at an increase, inhibin (synthesis in the Sertoli cells due to FSH) suppresses the effects of FSH locally in the testis, as well as FSH in the pituitary (Wistuba et al., 2007), by inhibiting the hypothalamus in releasing GnRH in a negative feedback (Figure 5). Thus, the system is kept in equilibrium.

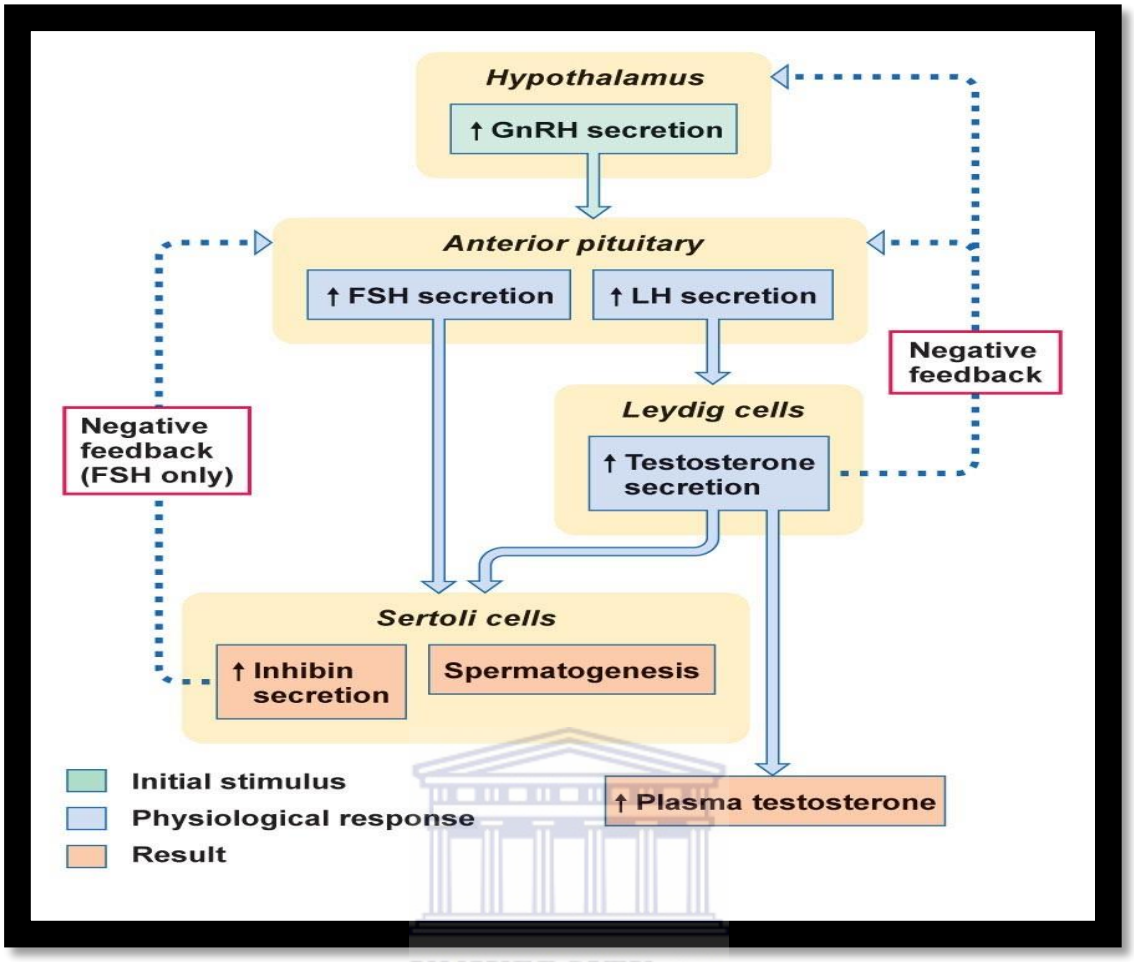

Figure 5: Illustration of hormonal regulation of male reproductive function: hypothalamus regulates activity of anterior pituitary (adenohypophysis), adenohypophysis synthesizes hormones (LH and FSH) that modulate activities of Sertoli and Leydig cells, Luteinizing Hormone (LH): stimulates testosterone production by Leydig cells, Follicle Stimulating Hormone (FSH): stimulates production of sperm in conjunction with testosterone by regulating the activity of Sertoli cells (Pearson Edu, 2011)

1.2.9 Sperm function parameters analysis

1.2.9.1 Motility

Sperm motility is an aspect of semen quality which is believed to be important in defining fertility in relation to achievement of pregnancy (Yanagimachi et al., 1976). In general, it gives a measure of integrity of the sperm axoneme and tail structure, metabolic machinery of the mitochondria and morphology (Pacey, 2006). Sperm movement is generated from the flagellum (tail structure) (Paoli et al., 2011) providing the propulsion for swimming, which is critical for sperm motility (Coetzee

et al., 1989; Pacey, 2006). Furthermore, sperm with good kinematic motility parameter are able to penetrate cervical mucus better (Aitken et al., 1991).

Studies have indicated sperm motility to be a predictive value for pregnancy rates, since reduced sperm motility may reduce the chances of fertilization of the ovum, an aspect common in infertile men (Coetzee et al., 1989; Henkel, 2005). Bartoov et al. (2002) found sperm motility to significantly correlate with fertilization *in vitro* and has been clinically used to predict pregnancy (Aboua et al., 2009). Likewise, studies have shown that motility can directly be correlated to mitochondrial membrane potential (Kasai et al., 2002), plasma membrane integrity (Aitken et al., 1991) and morphology (Kruger et al., 1987).

However, knowing the percentage of sperm motility could be convenient for standardizing experimental studies whose interest would be examining sperm movement in isolation (Hirano et al., 2001), but might not be suitable when tests are being performed for clinical purposes (Mortimer et al., 1986). Guzick et al. (2001) strongly support the fact that sperm motility provides useful information for diagnosing male infertility, but not in isolation as diagnostics of infertility. Hirano et al. (2001) and Mortimer (1994) in conjunction with their co-workers, contribute by stating that more detailed evaluation on sperm motility is a potentially valuable contribution in infertility diagnostics.

Determining sperm motility parameters has revolutionized over the years (Mortimer et al., 2015). These parameters have now been standardized with the aid of motion analysis systems. Currently, WHO (2010) guideline reference values have been adopted as a standard methodology for laboratories engaged in semen analysis. It recommends the use of motility grading system as follows (Hirano et al., 2001):

- Total motility: total spermatozoa moving actively (moving linearly or in a large circle, not considering speed) and all other patterns of motility with an absence of progression.

- Progressive motility: spermatozoa moving actively forward in a straight pattern.
- Curvilinear velocity (VCL): measurement of the speed of travel of the centroid of the sperm head over a given period of time, i.e. the local speed along the curvilinear path traced by the sperm head.
- Straight line velocity (VSL): straight line distance between the first and last centroid positions for a given period of time rather than the sum of all intermediate distances.
- Average path velocity (VAP): spatially averaged path that eliminates the wobble of the sperm head, while preserving the basic curvature of the path, i.e. average path velocity based on every 5th frame of VCL path.
- Linearity of forward progression (LIN): ratio of VSL to VCL, values range from 0 to 100 with a value of 100 representing cells swimming in a straight line pattern and expressed as percentage.
- Straightness (STR): linearity of the spatial average path, expressed as ratio of VSL to VAP.
- Beat cross frequency (BCF): timed average rate at which the curvilinear sperm trajectory crosses its average path trajectory.
- Hyperactivation: calculated capacitation of mature spermatozoa of all humans and functionally associated with sperm acrosome reaction.

1.2.9.2 Vitality

This parameter is an estimate of membrane integrity of the sperm cell (WHO, 2010). Sperm vitality assay testing is used to determine if the non-motile sperm are alive or dead and is indicated when sperm motility is less than 5% to 10% (Vasan, 2011). This in turn gives a reflection of accuracy of living non-motile spermatozoa. Ideally, the percentage of dead spermatozoa should be slightly excided by percentage of live spermatozoa (Björndahl et al., 2003). Sperm viability testing was introduced in 1984 by Jeyendran and his colleagues, they suggested non-motile viable sperm may

be used for intracytoplasmic sperm injection (ICSI) (Hossain et al., 1998). Sperm viability contributes to sperm-oocyte activation and fertilization and further development steps during embryogenesis. Therefore, selection of mature viable sperm for injection into the ooplasm is very important (Sati et al., 2015).

According to Anerao et al. (2010) the quality of sperm has been found to be more significant than the count. Poor motility has been associated with reduced viability of spermatozoa (Dhar et al., 1968; Savita and Huma, 2010). Moreover, the percentage of viable sperm has been highly correlated with spermatozoa with optimal functional mitochondria (Magistrini et al., 1997; Papaioannou et al., 1997). In addition, a study by De Lamirande and Gagnon (1992) found that reactive oxygen species (ROS) caused 10% - 20% decline in viability of human spermatozoa. Baumber et al. (2000) also found ROS to have caused a similar percentage decrease in equine sperm viability.

1.2.9.3 Reactive oxygen species (ROS)

ROS are highly reactive oxidizing agents and diffusible molecules that belong to the class of free-radicals and peroxides which are generated in cells as by-product of aerobic respiration and metabolism (Halliwell, 1996; Valko et al., 2007). The most common ROS that have effected human reproductive biology are free radicals i.e. molecules with a single, impaired electron in the outer orbits such as superoxide anions ($\cdot O_2^-$), hydroxide radical (·OH), peroxyl radicals (·ROOH) and hypochlorite radical (·OHCl.) (Sharma and Agarwal, 1996; de Lamirande et al., 1997a; Henkel et al., 2003). These unpaired electrons are extremely reactive with a half-life range of a nano-second to milli-second, hence, they practically react at the site of generation (Halliwell, 1989; Henkel, 2012). Resulting to oxidation of biomolecules such as unsaturated fatty acids, sulfhydryl proteins, nucleic acids and hyaluronic acid, and this oxidative damage is a key component of pathological processes such as inflammation, carcinogenesis and degenerative disease of ageing (Halliwell and Gutteridge, 1999). On the other hand, the most common non- radicals ROS are hydrogen peroxide (H_2O_2) or hypochlorous acid (HOCl) which are either oxidizing agents or easily converted into free radicals

(Petersen et al., 1980; Hamada et al., 2012). H_2O_2, is more stable, uncharged, with a higher oxidant potential, and can cross plasma membranes unlike the $\cdot O_2^-$ anion and other free radicals (Halliwell, 1989). Possibly, the most damaging quality of H_2O_2 is that, it has the potential of splitting into very dangerous ·OH radical when it accepts an electron from ferrous or cuprous ions in a reaction known as Fenton reaction (Nieschlag et al., 1997). Furthermore, H_2O_2 is the main secretory products of the leukocytes (Clark and Klebanoff, 1978), these cells are involved in fighting infection in the male genital tract (Aitken et al., 1994; Sharma and Agarwal, 1996; Moustafa et al., 2004; Henkel, 2005).

Therefore, ROS can either be produces extrinsically by leukocytes or intrinsically by the male germ cells (Barroso et al., 2000, Henkel, 2005, 2012). The location of the production plays a role in sperm function in that, high level of extrinsic ROS produced by leukocytes appear to rather impair sperm motility, while intrinsic ROS production seems to preferentially affect sperm DNA fragmentation (Henkel, 2005). In addition, Studies have also shown that supra-physiological production of ROS causes oxidative stress (imbalance between ROS production and scavenging activities of reactive oxygen species) which leads to sperm dysfunction (Figure 6) (Bansal and Bilaspuri, 2010) by causing damage to the plasma membrane (Alvarez et al., 1987; Iwasaki and Gagnon, 1992; Aitken et al., 1998), or damage to DNA through stimulation of strand break and other oxidative damage in human spermatozoa (Figure 6) (Hughes et al., 1996; Twigg et al., 1998a; Aitken and Baker, 2006). On the other hand, physiological production of ROS is essential for normal physiological function since it leads to the activation of the intracellular pathways responsible for sperm maturation, capacitation, hyperactivation, acrosome reaction, chemotactic process and fusion with female gamete (Vasan, 2011; Du Plessis et al., 2015). Antioxidants (ROS scavengers) such as superoxide dismutase (SOD), catalase (CAT), glutathione (GSH), and the glutathione peroxidase/ reductase system, maintain the scavenging activities in the gonads and seminal fluid (Alvarez et al., 1987; Halliwell, 1989).

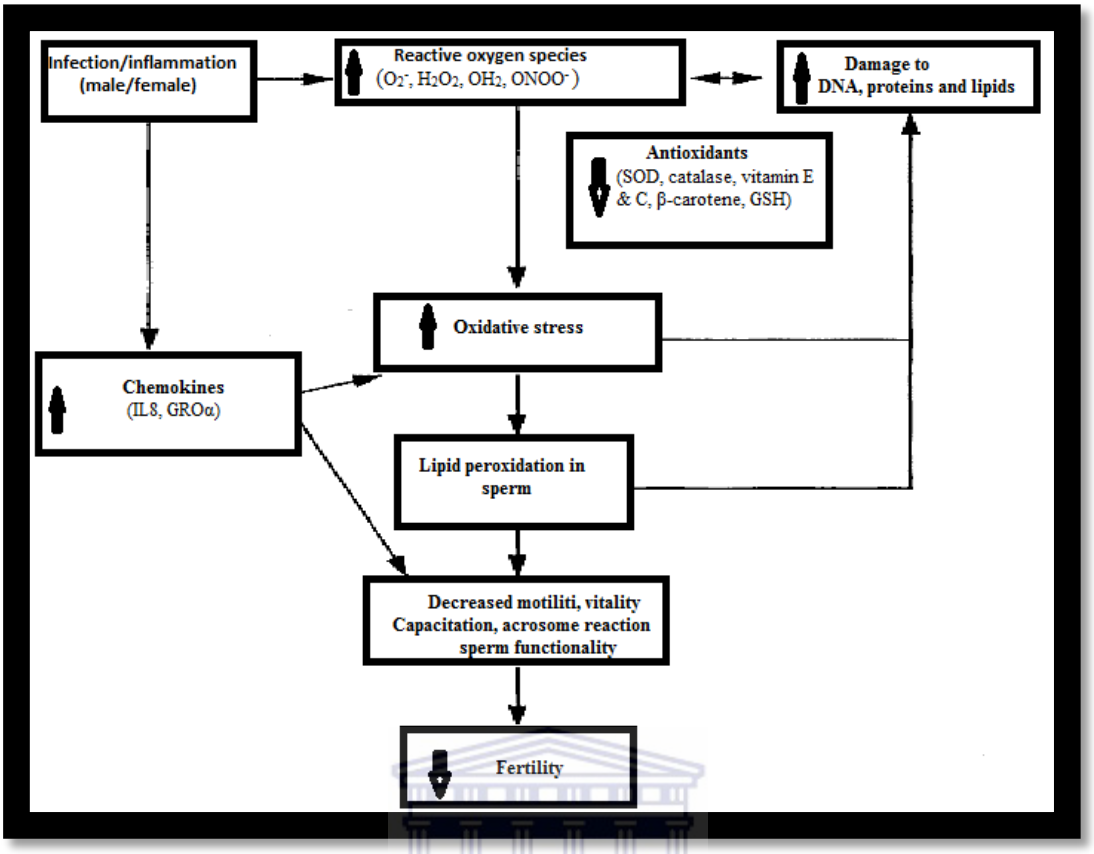

Figure 6: Structure suggesting interacting mechanisms in the role of oxidative stress and antioxidants affecting sperm function and fertility (SOD: antioxidants superoxide dismutase, GSH: glutathione reductase, IL8: interleukin 8, GROα: growth regulated protein alpha) (Adopted from (Lector, 1996).

1.2.9.4 DNA fragmentation

High levels of compatible and complex chromosomes capable of decondensation are qualities of fertile spermatozoa. Any form of chromatin mutation or DNA fragmentation may affect reproductive outcome (Agarwal and Said, 2003). Therefore, accurate transmission of paternal genetic information is attributed to sperm DNA integrity (Agarwal and Said, 2003; Franken and Oehninger, 2012). DNA fragmentation is the breakage that occurs on single or double-strand structures of spermatozoa DNA either, prior to or post-ejaculation causing DNA destruction (Rappa et al., 2016). Various factors can induce DNA damage in spermatozoa including: strand breakage that might occur in the process of spermatogenesis, endogenous endonucleases induced DNA fragmentation, chromatin condensation, oxidative stress post testicular DNA fragmentation

prompted by passage through the male reproductive tract, incidence of apoptosis during the process of spermatogenesis and environmental factors such as air pollution and smoking. Nonetheless, several studies have linked ROS production to all causes of sperm DNA damage (Lopes et al., 1998b; Sakkas et al., 1999; Agarwal and Said, 2003; Wang et al., 2003; Henkel, 2005).

Furthermore, studies have shown negative effects of DNA fragmentation for instance, high levels of sperm DNA damage have been directly correlated to poor seminal parameters such as reduced sperm concentration, motility or abnormal morphology (Irvine et al., 2000; Saleh et al., 2002), and reduced acrosome reaction (Ozmen et al., 2007). Additionally, more investigations have affirmed the correlation of DNA fragmentation with oxidative stress and impaired sperm function (Lopes et al., 1998a) which has negative influence on *in vitro* fertilization rate (Aitken et al., 1998; Henkel et al., 2003), and intrauterine insemination (IUI) (Duran et al., 2002).

Spermatozoa from fertile men have been found to have less DNA damage than those from fertile men (Zini and Libman, 2006). Besides, Barroso et al. (2000) reported that nuclear defects like abnormal chromatin structure, aneuploidy, chromosome microdeletion and DNA strand break have been observed in spermatozoa from infertile men.

1.2.9.5 Mitochondrial membrane potential (MMP)

The sperm cells vital biological function is the transfer of male genome to female oocyte, a process which requires energy necessary for supporting sperm motility and active protein modifications such as phosphorylation (Miki, 2006) and, mitochondria function to produce this energy in the form of adenosine triphosphate (ATP) (Donnelly et al., 2000). Through the formation of an electronic potential across the outer and inner mitochondrial membrane during oxidative phosphorylation. This is also necessary for the import of mitochondrial proteins, calcium homeostasis and metabolite transport (Bourgeron, 2000). Therefore, the mitochondrial membrane potential (MMP) is the parameter that best indicates mitochondrial function and is a reflection of mitochondrial energy in form of ATP (Donnelly et al., 2000).

Abnormalities in the structure of the sperm mitochondria (mid-piece area) had been linked to decreased sperm motility (Mann and Lutwak-Mann, 1981) and male infertility (Miki, 2006), thus highlighting the importance of this functional component (Mann and Lutwak-Mann, 1981; Turner, 2005). According to Mundy et al. (1995), sperms from asthenozoospermic males (individuals producing sperm with less than 40% total motility) were found to have smaller mid-pieces and a lower number of mitochondrial gyres. Therefore, leading to the reduction in enzymatic activities of the electron transport chain linked to the reduced sperm motility in asthenozoospermic patients (Folgerø et al., 1993).

In addition, MMP has been found to correlate with other sperm functions making its measurement a reliable indicator of sperm quality (Barroso et al., 2000; Donnelly et al., 2000; Marchetti et al., 2002; Marchetti et al., 2004). Spermatozoa that portray a reduced MMP contain increased level of ROS (Wang et al., 2003) and are associated with decreased *in vitro* fertilization rates (Marchetti et al., 2002). Similarly, spermatozoa that display intact MMP in most cases have intact acrosome function and great fertilization capacity. In addition they have normal morphology and motility (Grunewald et al., 2008).

Once the MMP is compromised, permeable transition pores (multi-component protein that regulates oxidative phosphorylation) are formed (Chang et al., 2002). They then aggregate in the mitochondrial membrane and induce cell death as a result of turning into a non-specific channel (Grimm and Brdiczka, 2007). The second mechanism in which the MMP will be compromised is through the induction of membrane permeability through various coordination of Bcl-protein (Chipuk et al., 2006), a process associated with caspases activation (Miki, 2006). Agarwal et al. (2004) stated that several andrological pathologies like immunological infertility, reduced sperm fertilization capability and varicocele have been associated with caspase activities. In terms of ejaculated sperm, caspase activity has been associated with low concentration of sperm cells, reduced motility, low level of fertilization rates, phosphatidylserine externalization of plasma

membrane leading to disrupted functionality (Marchetti et al., 2002; Cayli et al., 2004; Said et al., 2004; Grunewald et al., 2008). Nonetheless, compromised mitochondrial function leads to decreased mitochondrial membrane function which in turn affects the DNA integrity and is also accompanied by ROS production (Kim et al., 2013).

1.2.9.6 Capacitation and acrosome reaction

1.2.9.6.1 Capacitation

Capacitation is described as the biochemical and structural changes that spermatozoa undergo in the female reproductive tract in order to be able to execute acrosome reaction and fertilize an oocyte (Yanagimachi et al., 1976; Vasan, 2011; Ickowicz et al., 2012). These changes are comparatively associated with hyperactivation and acrosome reaction (de Lamirande et al., 1997a). In the human, this process takes about 4-5 hours with a gradual increase of the calcium ion (Ca^{2+}) concentration, while acrosome reaction takes place within minutes with a large influx of Ca^{2+} (Stock and Fraser, 1989). Capacitation as stated by de Lamirande et al. (1997b) is triggered by the increase in cAMP level influenced by influx of Ca^{2+}, which leads to increased membrane fluidity due to altered lipid composition (cholesterol loss) (Figure 7) (Go and Wolf, 1985) and changes in enzyme activities (phosphorylation of tyrosine residues in proteins) (Visconti et al., 1995), also changes in the patterns of spermatozoon motility to hyperactivated motility (Yanagimachi, 1970). There after spermatozoa can proceed to acrosome reaction (Go and Wolf, 1985).

1.2.9.6.2 Acrosome reaction

The acrosome is a cap-like membrane structure organelle that covers the anterior one-half to two thirds of the sperm head (Figure 8). The discharge of hydrolytic enzymes (hyaluronidase and acrosin) (Vigil et al., 2011) from the vesicle in the acrosome of a sperm cell during fusion between the outer acrosomal and sperm plasma membrane is termed as acrosome reaction (Riley-Vargas et al., 2005). During this fusion, small lipid cumuli are generated from both membranes, stabilising each other until they become independent units (Vigil et al., 2011). The membrane housing the

acrosome enzyme loses continuity and stability leading to the release of acrosome content to the external medium (Go and Wolf, 1985).

Furthermore, Wassarman (1987) found that the zona pellucida (ZP) is composed of three glycoproteins namely ZP1, ZP2 and ZP3. Multiple subunits of ZP2 and ZP3, form filament that are bound together by ZP1. When sperm binds to ZP3 it causes a change in Ca^{2+} (Ickowicz et al., 2012) and Na^{+} (Wassarman, 1987) flux across the plasmalemma which results in the acrosome reaction (Meilzel, 1984). The binding of the ZP to the plasma membrane can take place through two different receptors (Ickowicz et al., 2012), either by G_i coupled receptors which leads to phospholipase C (PLC) β_i activation or by tyrosine kinase (TK) receptor coupled by (PLC)γ (Huneau et al., 1994).

In case ZP binds to G_i-coupled receptors, it leads to the regulation of adenylyl cyclase (AC) (Ickowicz et al., 2012), hence, elevation of increased cyclic monophosphate cAMP and activation of protein kinase (PKA) (Breitbart and Spungin, 1997). Subsequently, PKA will activate Ca^{2+}-channels leading to more calcium moving from the anterior of the acrosome to cytosol (Ickowicz et al., 2012). The rise in Ca^{2+} level triggers activation of protein-kinase-c (PKC), which will lead to further opening of Ca^{2+}-channel, thus increased Ca^{2+} level (Huneau et al., 1994) (Figure 9). This leads to increased membrane fluidity (Lee et al., 1987), protein tyrosine phosphorylation (PTP) (Ickowicz et al., 2012), increased cAMP concentration (Huneau et al., 1994) with protein phosphorylation (Breitbart and Spungin, 1997). The Gi and TK can also activate NA^{+}/H^{+} pump leading to alkalization of the cytosol (Ickowicz et al., 2012). According to Benoff et al. (1996), capacitation and acrosome reaction are key parameters for assessing sperm fertilization capacity. In support Schuffner et al. (2002) states sperm acrosome reaction and hyperactivation are prerequisites to fertilization. Though, only hyperactivated sperm that move along a curved tract have been found to have the ability of penetrating the zona pellucida as opposed to those that are moving in a rectilinear manner (Schuffner et al., 2002).

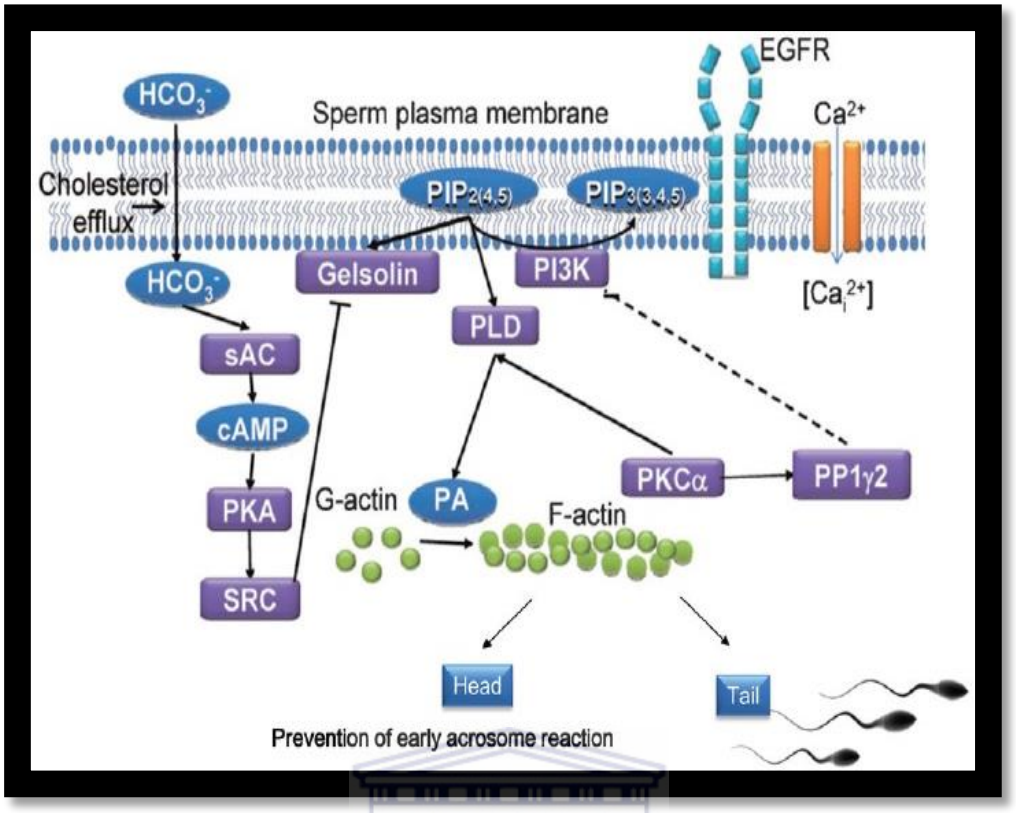

Figure 7: A model describing the biochemical cascade in sperm capacitation: intracellular bicarbonate (HCO_3^-) activates soluble adenyl cyclase (sAC) to generate cyclic adenosine monophosphate (cAMP) leading to protein kinase A (PKA) activation and cholesterol efflux from the sperm plasma membrane which further stimulate the HCO_3^-/sAC/cAMP/PKA cascade. PKA activates Src tyrone kinases to phosphorylate/inactivate phosphotidyl-Inisitol-triphosphate (PIP2) bound gelsolin. PIP2 is a cofactor for phospholipase D (PLD) activation and this activation is stimulated by protein kinase C α (PKCα), leading to phosphatidylcholine hydrolysis and production of phosphatidic acid (PA) which mediates the conversion of G-actin to F-actin. Thus, activation of PLD and prevention of F-actin dispersion by inhibiting gelsolin, allows F-actin formation. F-actin in the head prevents immature acrosome reaction and in the tail F-actin regulates sperm motility including (hyperactivation) HA motility (Adopted from Ickowicz et al., 2012).

Therefore, spermatozoa depicting the necessary motility parameters may achieve capacitation necessary to cross the oocyte barrier, bind to the zona pellucida and finally complete the acrosome reaction to initiate pregnancy (Bakalczuk et al., 2016). As a result, clarification of cellular mechanism inducing acrosome reaction is very significant to the clinical evaluation of sperm functionality (Benoff et al., 1997). Since, human spermatozoa which are incapable of undergoing acrosome reaction will not fertilize an oocyte following conventional insemination in vitro (Overstreet et al., 1980; Benoff et al., 1997).

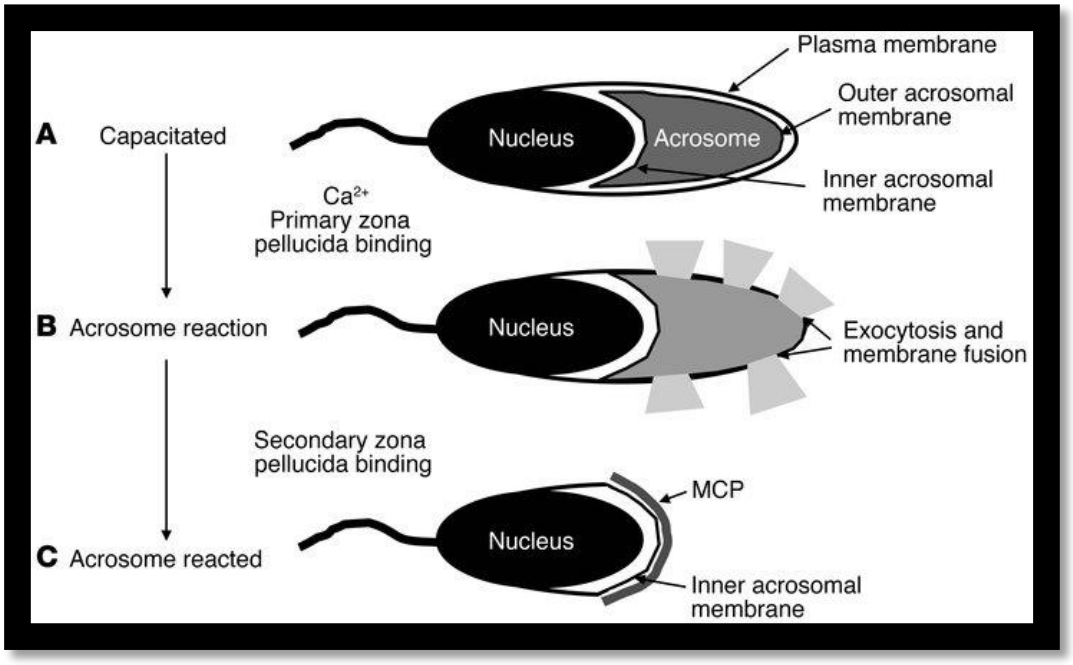

Figure 8: Representation of acrosome reaction of human spermatozoa. A-capacitated spermatozoa binds to zona pellucida and causes acrosome reaction, B-fusion of outer membrane with plasma membrane, releasing acrosomal contents (acrosin) and C-Inner acrosomal membrane exposed to form outer surface of sperm head (Riley-Vargas et al., 2005).

1.3 Infertility

Infertility is defined as the failure of a couple to become pregnant after one year of regular, unprotected sexual intercourse (Saxena and Rastogi, 2015). Worldwide, about 15-20% of couples are affected by infertility during their reproductive life, with approximately 7.5-10% of male of reproductive age group being infertile (Miyamoto et al., 2011; Belloc et al., 2014; Saxena and Rastogi, 2015). According to (Ollero et al., 2001), one in every six couples of reproductive age presents with infertility, with 50% of the causes being classified as idiopathic. Pei et al. (2005) also, states that approximately 50% of infertility is as a result of male infertility. Approximately 4.8 million couples worldwide are infertile with the highest rate reported in Eastern Europe and Africa (Figure 10) (Agarwal et al., 2015), although compiling absolute statistics on occurrences of male infertility in some countries is difficulty as a result of the stigmas associated with it due to cultural and religious differences (Said et al., 2004).

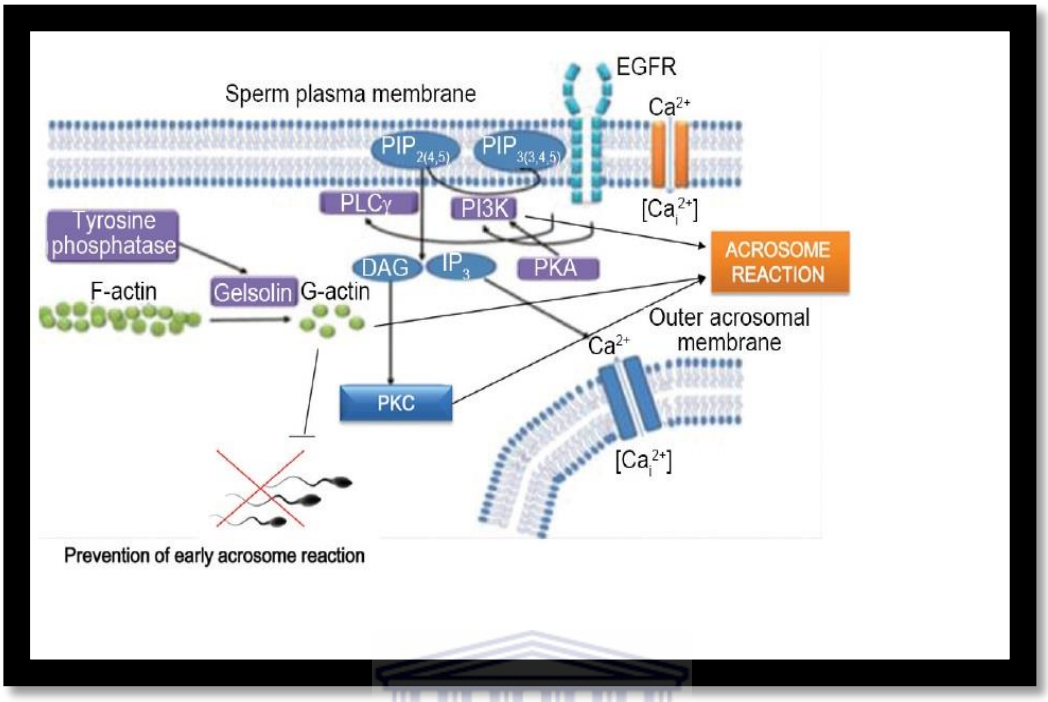

Figure 9: A model describing the biochemical cascade that leads to the acrosome reaction (AR): binding of capacitated spermatozoa to the oocyte zona pellucida causes a fast and elevated increase in intra-spermatozoal calcium ion (Ca^{2+}) concentration. As a result, active phospholipase C (PLC) catalyses, phosphatidyl-Inositol-biophosphate (PIP_2) hydrolysis to produce Inositol-triphosphate (IP3) and diacylglycerol (DAG) and the release of bound p-gelsolin. The p-gelsolin undergoes dephosphorylation/ activation by tyrosine phosphatase leading to conversion of F-actin to G-actin. IP3 activates Ca^{2+} channel in the outer acrosomal membrane which reduces intra-acrosomal Ca^{2+} leading to the activation of Ca^{2+}- dependent-Ca^{2+}channel in the plasma membrane which causes further increase in intracellular Ca^{2+} which together with DAG activate protein kinase C (PKC) which mediates the acrosome reaction. Also, PKA-dependent PI3K activation occurs towards the end of the capacitation process, involves in the mechanism leading to the AR (Adopted from Ickowicz et al 2012).

For instance, in Africa and Middle East men do not undergo fertility tests in most cases because, female counterparts take the blame for being infertile in many occasions (Agarwal et al., 2015). The culture protects and retains the infertile mens' masculine identity in the society by letting in a brother or relative of the infertile man to impregnate their wife (Agarwal et al., 2015). This prevents them from ridicule from other community members. For instance, in Uganda amongst the Banyakore ethnic group a direct translation of terms used to describe an infertile man would be, "a

person without legs" or "one trampled by a goat" (Kamatenesi-Mugisha and Oryem-Origa, 2005). Other communities will refer to them as "worthless" (Agarwal et al., 2015). These men are not supposed to be given any position of responsibilities or leadership since they are regarded as abnormal. They are socially excluded from society and muted when they make a contribution, hence reducing their self-esteem (Kamatenesi-Mugisha and Oryem-Origa, 2005). Psychologically, infertile men will try to have affairs with other women to test the viability of their manhood which could put them at risk of getting sexually transmitted infections (Kamatenesi-Mugisha and Oryem-Origa, 2005).

Reproductive healthcare has been regarded as the second most prevalent core problem on the African continent (Kamatenesi-Mugisha and Oryem-Origa, 2005). A wide range of disorders can be associated with male factor infertility ranging from genetic defects, endocrine disorders, abnormal spermatogenesis, azoospermia, oligozoospermia, idiopathic factors, asthenozoospermia, teratozoospermia, oligoasthenozoospermia, oligoasthenoteratozoospermia, varicocele, chromosome defects, systemic disorders affecting fertility (Kartageners syndrome, fanconi anaemia (FANCA), myotonic dystrophy, sickle cell anaemia and β-Thalassemia) and idiopathic causes. A great number of unresolved idiopathic infertility cases in human male might be attributed to the complexity of the spermatogenic process, which might result to mutation of different genes (Matzuk and Lamb, 2008; Benkhalifa et al., 2014).

Technology advancement in the past two decades has seen the development of assisted reproductive technology (ART) (Ola, 2012) and intracytoplasmic sperm injection (ICSI) (Wang et al., 2003), as some of the approaches that can be used to address other causes of male infertility (Merchant et al., 2011). Despite the technology advancement, it has been challenging to address the poorly understood genetic, environmental and idiopathic causes of male infertility (Santi et al., 2015). Medical treatments of unexplained male infertility have been proven not of much benefit (Dabaja and Schlegel, 2014), resulting to the seeking of alternative form of treatment like the use of ethno-medicinal plants like *Mondia whitei* (Watcho et al., 2007).

Figure 10: World map showing studied cases of male factor infertility percentage per area (Asia, Africa, America, Europe and Oceania) as illustrated by Agwaral et al. (2015).

1.4 Botanical medicine

Traditional medicine (TM) also known as complementary and alternative medicine (CAM) is classified as the oldest health care system (Abdullahi, 2011). According to the (WHO 2000), TM "is the sum total of skills, knowledge and practices based on theories, beliefs and experiences indigenous to different cultures, whether applicable or not, used in maintenance of health, as well as prevention, diagnosis and improvement or treatment of physical and mental illness". In addition, the WHO (2000) stated that a traditional healer is, "a person who is recognized by the community where she or he lives as someone competent to provide health care by using, plants, animals and minerals substances and other methods based on social, cultural and religious practices".

Taking Sub-Saharan Africa into account, one traditional healer attends to approximately 500 patients in the population. This shows that contemporary ethno-botanical medicine continues being

a centre core of thousands of South Africans and Africans at large, with an estimated 80% of the population in use of plant medicine (Ernst, 2000; WHO, 2002). Putting across the important role TM plays in the lives of African people and their potential to serve as a crucial component of comprehensive health care strategy, this was the main, if not the only, form of healthcare for millions of African people before the introduction of cosmopolitan medicine (Abdullahi, 2011). In countries like Ethiopia, 90% of the population use herbal remedies as primary health care (Mahomoodally, 2013) and 70-80% of Ugandans rely on traditional healers for basic healthcare, with a number of ailments ranging from erectile dysfunction and sexual importance being treated traditionally for ages (Kamatenesi-Mugisha and Oryem-Origa, 2005).

Medicinal plants have been used for centuries in treatment of diseases and body system dysfunction and there contribution to the development of pharmaceuticals cannot be ignored since 25% of modern medicine is derived from plants (Nantia et al., 2009). According to Mahomoodally (2013) modern comprehensive medicine has its roots in TM with information on important cures passed from generation to generation through verbal communication.

Increasing public interest and awareness in Natural Medicine has led pharmaceutical industries and researchers to pay more attention on medicinal plants (Benzie and Wachtel-Galor, 2011). The paradigm shift from Western to herbal medicine, may partly be due to the fact that synthetic drugs have always shown adverse reaction and other undesirable side effects, especially seen from incorrect use. Hence, the belief those natural products are safe because they are more harmonious with biological system (Rates, 2001; Erasto et al., 2005; Vandebroek and Balick, 2012).

Plant medicine not only provides the body with nutrients but also improves body function. Researchers have been encouraged and urged by WHO to evaluate the use of medicinal plants as sources of treatment, since they are easily available, accessible and affordable (Nantia et al., 2009). According to (Erasto et al., 2005), the low income group and indigenous Africans living in the rural areas may not access modern medicine. On the other hand, African traditional medicine

(indigenous) herbs are easily accessible and cost effective (Erasto et al., 2005). In fact, cultural and economic reasons have been linked to the use of medicinal plants prompting the WHO to encourage African member states to promote and integrate traditional medicinal practices in the health care system (Muhammad and Awaisu, 2008).

Van Wyk et al. (1997) states that South Africa has got a diverse and rich flora which forms part of the medicinal wealth that ensures the Primary health care and livelihood of millions of its citizen is addressed. Hence, the past decade has prompted increased interest and reorganisation of indigenous medicinal plants of South Africa. These plant species are regarded as precious and highly valued, considering the rate at which the vegetation is getting depleted in this part of the world. There is a need to document and verify the precious knowledge in regards to medicinal plants. This will then result in better health services being provided to South Africa and Africa at large and enable integration of valued indigenous knowledge in herbal medicine with modern knowledge and technology (Mander et al., 2007). In a laudable effort to make ethno-botanical medicine scientific, there is a paradigm shift to evidence based medicine that is why this study is focused on giving scientific investigation on the biological activities associated with *Mondia whitei* to validate the traditional use in treatment of male infertility.

1.5 *Mondia whitei*

1.5.1 Description

The genus Mondia of the Apocynaceae family is a woody, robust and vigorous aromatic perennial plant that grows from a large tuberous root stock. It has large heart-shaped opposite leaves and produces reddish, purple flowers borne in branched inflorescences (Figure 11) (Iwu, 2014). The species that are distributed in the Southern part of Africa flower from October to March, while those in the Northern part of Africa flower from May to August (Aremu et al., 2011). The roots spread laterally beneath the soil surface which enables easy harvesting. They taste like ginger or

liquorice and have an aroma reminding one of vanilla. Initially, the taste is bitter then becomes sweeter with time. The large oval follicle fruits (75–100 × 44 mm) are almost woody and contain approximately (180-320) seeds (Van Wyk, 2011).

The name Mondia is derived from the Zulu name umondi/mundi, while the species name whitei was in honour of the collector Mr. A. S. Whitei (Hutchings et al., 1996; Aremu et al., 2011). The common name include: white ginger, tonic root (English); mudondo (Angola); mudondo, bondo, molo busio (Cissongo- Central African Republic); nbondo, kimbiolongwa, kumba (ngwaka-Democratic Rebuplic of Congo); ubasangbwandiya, gatimba (Lugware-Democratic Republic of Congo); lacadje (Fula-Guinea Bissau); mkobela (Luhya-Kenya); citambalo (Malawi); Umondi, mundi, mindi (Zulu-South Africa); sedando omutano (Uganda) and Mungurawu (Shona-Zimbabwe) (Gelfand et al., 1985; Crouch et al., 1998; Aremu et al., 2011; Watcho et al., 2013).

1.5.2 Geographical location

The plant is indigenous to South, Central, East and West Africa (Hutchings et al., 1996). In South Africa, the plant is found mostly on the coast and midlands in KwaZulu-Natal, Limpopo province, extending to Swaziland and Zimbabwe. The distribution stretches to North and Southern parts of Sudan and West as far as Senegal, favouring tropical and sub-tropical areas. The medicinal plant specific location in West Africa include: Nigeria, Guinea, and Ghana; Central Africa: Cameroon; East Africa: Kenya, Uganda and Tanzania; Southern Africa: Malawi, Zimbabwe, Angola, Mozambique, South Africa (Figure 12) (Aremu et al., 2011).

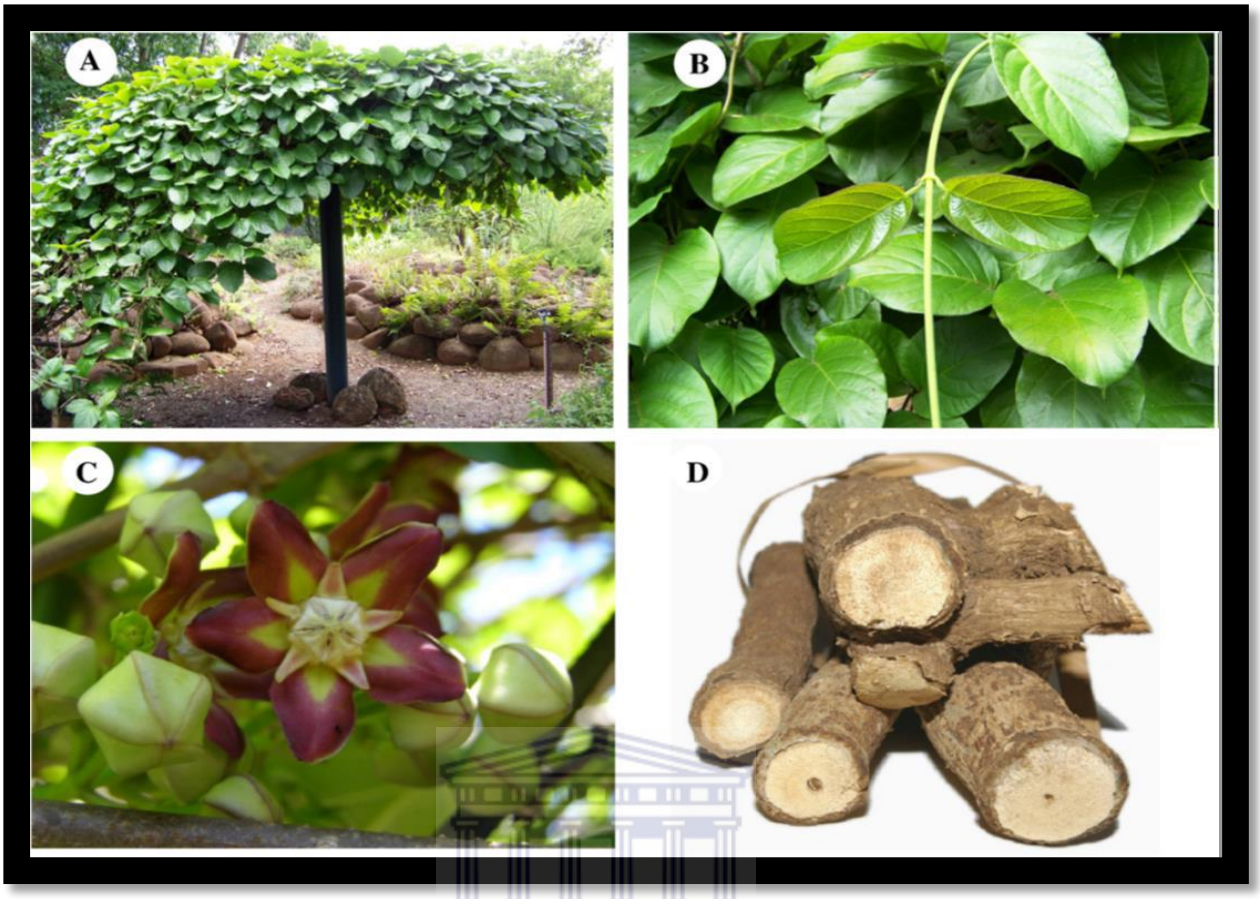

Figure 11: *Mondia whitei:* A- creeping climber with leafless basal portion of stem, the leaves appear in a canopy on top of a well-supporting iron bar; B-twining stems with large leaves (100-300 x 50-150mm) opposite, with deeply notched heart shaped base and stalk (30-55mmlong); C-young buds and fully opened branched inflorescences flowers with reddish purple corolla lobes about 14mm long with green margin; and-mature roots (Aremu et al., 2011).

1.5.3 Phytochemistry of *Mondia whitei*

To date, only a few studies exist on the isolation of bioactive compounds from *M. whitei*. The most common and well-known compound isolated from *M. whitei* is 2-hydroxy-4-methoxybenzaldehyde (Figure 13; no. 5), a potent tyrosinase inhibitor and an isomer of vanillin (Kubo and Kinst-Hori, 1999b). This compound has also been isolated from *M. whitei* by earlier researchers (Koorbanally et al., 2000; Oketch-Rabah, 2012). In addition, Koorbanally et al. (2000) reported for the first time the isolation of isovanillin (Figure 13; no. 6) previously thought to be a synthetic compound found in *M. whitei* (Koorbanally et al., 2000). Subsequently, a study performed by Patnam et al. (2005) reported the isolation and structural elucidation of an unusual chlorinated coumarinolignan, 5-

chloropropacin (Figure 13:, no. 3), along with eight known compounds including propacin (Figure 13; no. 1) and its 5′-methoxylated analogue by means of NMR and mass spectral analysis (Patnam et al., 2005). This was the first report of the isolation of coumarins and coumarinolignan (Figure 13; no.2) as well as chlorinated coumarinolignan from the genus *Mondia*.

Figure 12: Illustrate the distribution areas of *M. whitei*, from West Africa (Guinea, Ghana and Nigeria), Central Africa (Cameroon, Gabon and DR. Congo), East Africa (Kenya, Uganda and Tanzania) and Southern Africa (South Africa, Malawi, Angola, Mozambique, and Zimbabwe) (Venter et al., 2009).

Nutritional analysis indicated that Mondia is rich in minerals and vitamins. The yields of the different nutrients from roots and leaves, respectively, are as follows (mg/g): potassium (11.34, 32.05); sodium (5.61, 24); magnesium (1.40, 2.83); calcium (3.08, 8.25); iron (0.20, 0.43); zinc (0.03, 0.07); copper (0.003, 0.06); manganese (0.64, 0.05); cadmium and lead (trace, trace); crude protein (4.35, 21.8); beta-carotene (0.004, 0.022); thiamine (0.78, 3.70); niacin (0.52, 8.15);

riboflavin (0.62, 2.45); fructose (0.008, 0.015); xylose (9.17, 18.70); and glucose (2.40, 9.0) (Iwu, 2014).

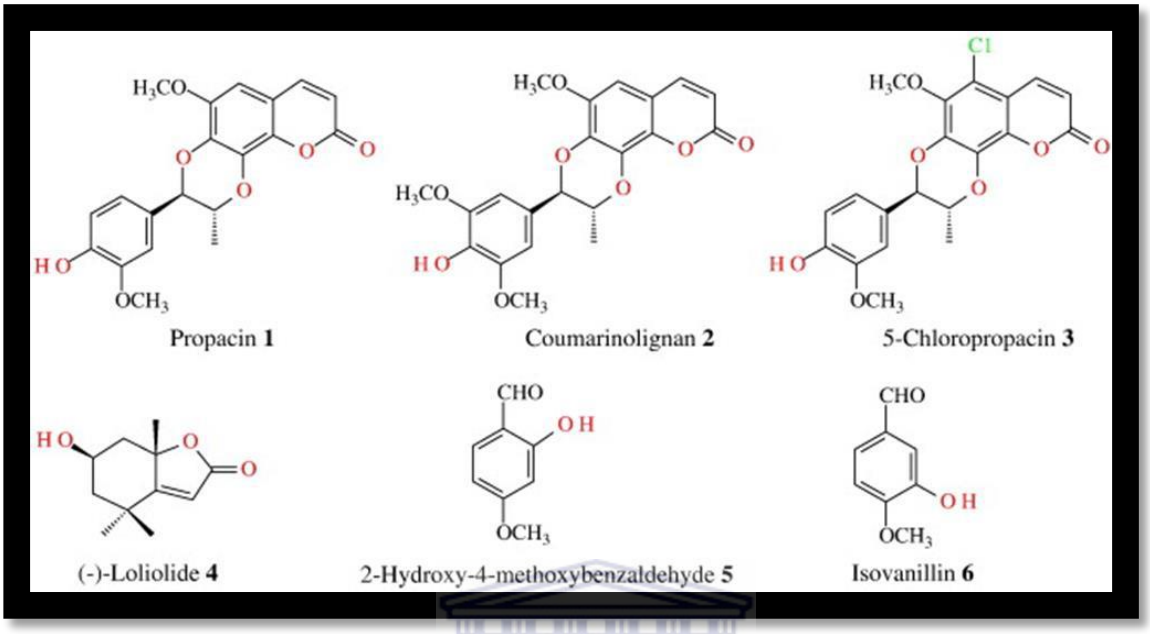

Figure 13: Chemical structures of various compounds isolated from *M. whitei*: 1: Propacin, 2: Coumarinolignan, 3: 5-Chloropropacin, 4: (-)-Loliolide, 5: 2-Hydroxy-4-methoxybenzaldehyde 6: Isovanillin (Aremu et al., 2011).

The conduction of bioassays led to the isolation of a monoterpene lactone (Figure 13; no. 4) (-)-loliolide which displayed affinity to a transporter, serotonin (5-Hydroxytryptamine) (SERT), in a binding assay (Neergaard et al., 2010). Qualitative phytochemical analysis of the ethanoic extract of *M. whitei* indicated the presence of reducing sugars and triterpenes. However, other essential phytochemical groups such as alkaloids, flavonoids, phenolic and saponins were not detected (Quasie et al., 2010). Subsequently, the presence of phenolic, flavonoids and tannins were detected *in vivo*. The authors also reported anti-oxidant activity by assessing the high hydroxyl radical scavenging activity (Bouba et al., 2010). Some scholars suggest that the presence of a wide range of plant secondary metabolites such as phenolic, flavonoids and tannins in plants have a direct correlation to the exhibited biological activities. An investigation by Moyo et al. (2010) attributed anti-oxidant bioactivity to the presence of phenolic compounds in the plant, to some of the observed

bioactivities due to the secondary metabolites detected in the extract (Moyo et al., 2010). Aremu et al. (2011) critically reviewed the phytochemical composition of *M. whitei* roots and leaves and found high levels of disparities in some parameters such as the values for calcium and magnesium which remains a major concern (Aremu et al., 2011). Thus, the attributing factors that play a vital role in the plant physiology and sensitivity of equipment used should be taken into account when investigating the effect of a phytochemical, such as the collection localities, seasonal variation and all factors that could influence the properties of the extract and investigation.

1.5.4 Ethno-botanical use

Historically, in folklore medicine across Africa, Mondia has been used in addressing several ailments (Quasie et al., 2010). Most ethnic groups use Mondia to treat gastrointestinal conditions such as mild laxative, appetite stimulation and for abdominal pains; alleviate nausea, treatment of fever, bilharzias' fit in children and stress/ tension in adults and several sexual dysfunctions (Aremu et al., 2011). Documents show that the root decoction has been used to induce labour in Uganda, treat malaria infection in Nigeria and Benin (Gelfand et al., 1985), anthelmintic infections in Nigeria and male infertility in Cameroon (Iwu, 2014). In Kenya, Mondia is used for many ailments e.g. ringworms, skin diseases and tension (Aremu et al., 2011), while in Cameroon it is used to treat urinary tract infection, jaundice, headache, diarrhoea and impotence (Watcho et al., 2007). Furthermore, Ghana traditional practitioners have used Mondia in management of erectile dysfunction and low sperm count (Martey and He, 2010). Yet, the Bondeni use the roots for body pain as well as the Maasai, while in Zimbabwe the roots are generally used for abdominal pain, constipation, poor appetite, schistosomiasis and as an aphrodisiac. The Shambala use the unspecified parts for fits in children, heartburn and impotence. Additionally, the Haya use it for uterine stimulation during child birth (Gelfand et al., 1985). The roots were chewed by the Zulu to relieve flatulence, indigestion, stomach ache and stimulate appetite (Hilton-Taylor, 1996).

1.5.5 *In vitro* studies and *in vivo* studies done on *M. whitei*

The roots of Mondia are often chewed by men as a tonic and aphrodisiac, and for treatment of sexual asthenia, erectile dysfunction and increase sperm production (Martey and He, 2010). Few studies carried out to investigate the effect of this plant extract on male reproductive functions have given conflicting results (Lampiao et al., 2008; Martey and He, 2010; Watcho et al., 2006; 2007; 2013). The root extract of Mondia was found to have contraceptive properties as well as properties associated with enhancing male fertility.

1.5.5.1 *In vitro* studies

In vitro study on the effect of aqueous extract of *M. whitei* on the motility and progressive motility of spermatozoa on a time dependant manner, showed no significant difference between the control and the treated group at 30, 60 and 90 minutes after incubation (Lampiao et al., 2008). A statistical difference was seen after 120 minutes of incubation with highest concentration being significantly higher than the control group.

1.5.5.2 *In vivo* studies

Watcho et al. (2001) conducted an *in vivo* study on the effect induced by long term admission of a hexane extract of dried root of *M. whitei* at doses of 500 and 1000mg/Kg on body organ weights, biochemical, haematological and physiological responses of vas deferens to norepinephrine parameters of rats treated for 30 days findings, gave evidence of reversible antispermatogenic effects of *M. whitei.*

In 2006, Watcho et al. Conducted another study on the long term investigation of a hexane extract of Mondia on doses of 500 and 1000mg/Kg on body organ weight and found that the relative weight of caput epididymis, ventral prostate and seminal vesicle were increased (Watcho et al., 2006). In 2004, he did more studies using both hexane extract of Mondia in spinal rat showed effect of fictive ejaculation, which appeared not to be mediated through dopamine pathway, hence justifying ethno-medicinal use of the plant (Watcho et al., 2013).

1.6 Aim of the study

M. whitei is a widely used medicinal plant across Africa in traditional medicine for treatment of sexual dysfunction yet very minimal scientific evidence to support the therapy exist. One the other hand, evidence from the existing studies possess ambiguity. To date, only one study (Lampiao et al., 2008) on effect of aqueous root extract of *M. whitei* on human sperm motility has been documented. However, it only focused on one sperm parameter (total motility and progressive motility) which only gives a fraction but not comprehensive information on sperm functionality. Secondly, none of the studies took into account the use of traditional therapeutic doses. Therefore, based on the traditional extraction methods, mode of application and therapeutic dosage, this study is aimed at investigating the effect of aqueous root extract of *M. whitei* on:

- Ability to enhance human sperm function by looking at motility and kinematic parameters associated with motility, capacitation, acrosome reaction and mitochondrial membrane potential.
- Human sperm cell cytotoxicity by assessing the vitality, DNA-damage and reactive oxygen species.
- The optimum dosage form that boost human sperm functionality.
- Provide clarity on the safety of *in vitro* use of Mondia in human sperm.

CHAPTER 2: MATERIALS AND METHODS

2.1 Chemicals and Equipment

In this study, all chemicals used were of highest possible quality of analytical or *in vitro* culture standard, and were purchased from the following companies:

Corning Incorporated, New York, USA, supplied:

- Test tubes (15 ml and 50 ml)
- Eppendorf vials (1.5 ml)

Kimix Chemicals, Eppingdust, South Africa, supplied:

- Calcium chloride dihydrate ($CaCl_2 * 2\ H_2O$)
- Disodiumphosphate (Na_2HPO_4)
- Ethanol
- Magnesium sulphate heptahydrate ($MgSO_4 * 7\ H_2O$)
- Potassium chloride (KCl)
- Sodium bicarbonate ($NaHCO_3$)

Knittel Gläser, Braunschweig, Germany, supplied:

- Superfrost slides
- Microscope slides

Lasec, Cape Town, South Africa, supplied:

- Filter paper (Munk Tell)

Merck, Wadeville Gauteng, South Africa, supplied:

- Glucose (anhydrous)
- Hydrochloric acid (HCl)

- Hydroxymethyl amino methane (TRIS)
- Potassium monophosphate (KH_2PO_4)
- Sodium chloride (NaCl)
- Sodium hydroxide (NaOH)

Millipore, Billerica, USA:

- Millipore distilling machine

Trevigen, Gaithersburg, USA, supplied:

- DePsipher kit

Whatman, Madestone, England:

- Filter paper 1

Centrifuges

Labortechnik, Wehingen, Germany, supplied:

- Hermle Z160M
- Hermle Z200A

Incubators

Lasec, Cape Town, South Africa, supplied:

- Series 2000

Leja, Nieuw-Vennep, Netherlands. Supplied:

- 8 chambers Leja slides

Freeze dryer

Virtis, Prague, Czech Republic, supplied:

- Sentry 2.0

Microscopes

Nikon Instruments Inc., Tokyo, Japan:

- Eclipse i50

Zeiss, Oberkochen, Germany, supplied:

- Epifluorescence

Zeiss, Cape Town, South Africa, supplied:

- Photomicroscope III

Sperm Class Analyzer (SCA)

Microptic S.L., Barcelona, Spain, supplied:

- Version 4.1.0.1
- Version 5.1 Evolution

Osmometer

Wescor, Inc, Logan, Utah, USA, supplied:

- Model 5100C

2.2 Study design

This study was approved by the Research Committee of the Faculty of Science, University of Western Cape, and ethical clearance was obtained by relevant Institutional Review Boards.

For this *in-vitro* study, different sperm parameters were investigated on samples either obtained from healthy donors or patients after incubation for 1 hour with different concentration of aqueous root extract of *Mondia whitei*. The following parameters were investigated: motility, vitality, DNA fragmentation, mitochondrial membrane potential, acrosome reaction, ROS production and capacitation. Subsequently, on WHO (2010) criteria; semen samples with concentration ≤ 15million sperm/ml (oligoozospermia) or progressive motility < 32%/ or total motility < 40% sperm/ml motility (asthenozoospermia) from both donors and patient group were identified and the effect of the Mondia extract on the different parameters was investigated.

2.3 Media

2.3.1 Medium used for spermatozoa

Quinn et al. (1985) described the Human Tubular Fluid (HTF) medium as optimal for the preparation of spermatozoa as it resembles the ionic composition of human tubular fluid and therefore, delays cell death and allows optimum time to execute all testing parameter. Hence, HTF was the preferred medium for washing of spermatozoa in this study. The basic composition of the HTF medium consists of the following substances which mimics those found in the female fallopian tube: 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 was fully dissolved, osmolarity was adjusted to 280 mOsmol/kg. Finally, 10 mg/ml bovine serum albumin (BSA) were added before working with the medium (HTF-BSA). The medium was sterilized by filtration through 0.22 µm filter (Merck Millipore, Tullagreen Carrigtwohills, Ireland).

The experimental set up of the study was as follows:

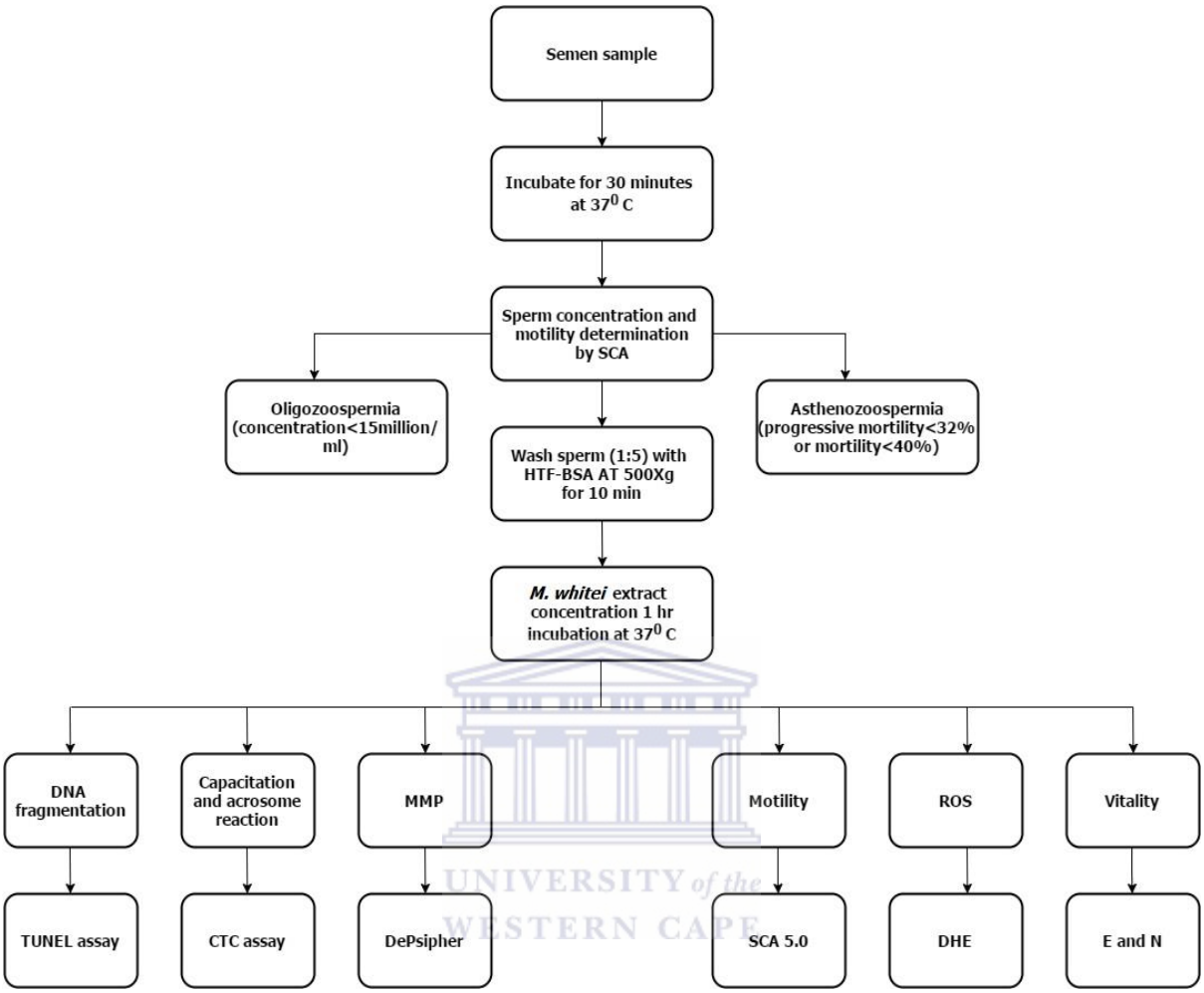

Figure 14: The study design analysed spermatozoa functionality, after incubation with different concentration of *M.whitei* (0.0, 0.0185, 0.185, 1.85, 18.5, and 185) µg/ml root extract displaying investigative parameters and testing procedures. CTC-chlortetracycline, SCA-Sperm Class Analyser, E+N-Eosin-Nigrosin, DHE-dihydroethidine, ROS-Reactive oxygen species, MMP-Mitochondrial membrane potential.

2.4 Plant material

2.4.1 Collection and identification of plant material

The roots of *Mondia whitei* were obtained from Kakamega rain forest located in the Western Province of Kenya on 23rd April, 2015. The plant was identified by Paul Ambani a local ethno-botanist. WHO (2003) guidelines of good agricultural and collection practices (GACP) for medicinal plants were observed. The roots of Mondia were identified and harvested under the best possible conditions, ecological non-destructive methods of collection were employed. Only some of the lateral roots were located and collected. The root specimen were washed, dried and deposited at the University of Western Cape (UWC), Bellville, South Africa, Department of Medical Biosciences. In the laboratory, the roots were equally rewashed and cut into smaller pieces of approximately 1-2 centimetres and dried for a week at 33^0C. Afterwards, the roots were ground into fine uniform powder.

2.4.2 Extract preparation

To prepare a hot aqueous root of Mondia, 1000 ml of distilled water were heated to about 75^0C and 100 grams of powdered extract were then infused into water and allowed to stand at room temperature for one hour before it got filtered. The filtrate was swirled in liquid nitrogen for about 10 minutes to accelerate the freezing process. Alternatively, the extract was frozen at -20^0C for 20 hours. To yield a water soluble extract, the frozen sample was freeze-dried for 4 days under the guidance of Mr. Lilburn Cyster, Department of Biodiversity and Conservation Biology (UWC), using a Vertis freeze drier.

Traditional healers generally prescribed a handful of Mondia roots per daily intake. Based on this, an average of three handful of Mondia roots were weighed (54.5g). The average yield obtained after 50g of Mondia roots extract was dissolved in 1 litre of water was 15.58g that is 31% weight to weight (w/w) yield. Subsequent calculations were based on the fact that the average man weighs about 80kg and would consume 14.8g of the extract per day.

Summary calculation of the average dosage a person should take per day:

In order to obtain yield of 15.58g, 50g of Mondia was dissolved in 1000cm^3 of water. Therefore, a handful (54.50g) of Mondia roots gave an equivalent of 14.8g.

Equation

- $\frac{54.50 \times 15.58}{50}$ = 14.8 g intake per person in a day.

Extract concentration (g/L) = (14.8 ÷80,000)

The standard 'normal' concentration in a person would then be 1.85 µg/ml of *M. whitei* root extract. A stock solution containing 185 µg/ml Mondia extract in HTF-BSA was prepared and serial dilutions with a final concentration range of (0.0185, 0.185, 1.85, 18.5, 185 µg/ml) were obtained to accommodate both supra and hypo-physiological ranges. HTF-BSA without Mondia root extract served as a control (0 µg/ml).

2.5 *In vitro* investigation of spermatozoa

2.5.1 Sample collection

Human semen samples were obtained from 60 men: 28 of them were healthy volunteers taking part in a semen donor programme at the Andrology Research Laboratory in the Department of Medical Biosciences, University of the Western Cape. The other 32 samples were obtained from 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 Palloti Hospital, Pinelands, South Africa, for assisted reproductive treatment or fertility problems. Samples were collected by masturbation into sterile plastic containers following 3-5 days of sexual abstinence. The sample collection procedure was in accordance with ethical guidelines

of the University of Western Cape. The Helsinki Declaration governing research on humans was also adhered to (WHO, 2003).

2.5.2 Preparation of spermatozoa *in vitro*

2.5.2.1 Determination of sperm concentration

Semen samples were incubated at 37ºC for 10-20 minutes to allow liquefaction within the first one hour of acquisition. Thereafter, 2µl of liquefied semen were placed in a pre-warmed (37^0C) 8 chamber Leja slide (Leja, Nieuw-Vennep, Netherlands) and sperm concentration was determined using computer aided semen analysis (CASA).

2.5.2.2 Washing of spermatozoa

Based on the concentration of spermatozoa in the sample, calculations were done to ensure that a standard amount of spermatozoa was maintained throughout the study. Thereafter, the semen was diluted 1:5 with HTF-BSA and centrifuged for 10 minutes at 500xg. The supernatant was discarded and the pellet re-suspended in HTF-BSA. Suspensions of spermatozoa in HTF-BSA were subsequently incubated with the different concentrations of *M. whitei* aqueous root extract for 1 hour at 37ºC.

2.5.3 Determination of sperm vitality

Several dye exclusion techniques have been developed to assess sperm vitality, but the 1-step eosin-nigrosin (E&N) technique is recommended by the WHO (2010), and is established as the Andrology standard in assessing sperm cell vitality (Cooper et al., 2002; Björndahl et al., 2004; WHO, 2010). The E&N test is used to determine if non-motile sperm are alive or dead and is indicated when sperm motility is less than 5% - 10%. The stain does not stain live sperm, therefore live sperm will not absorb the dye (Figure 15) (Vasan, 2011).

The 1-step eosin-nigrosin staining technique was used to determine viability according to the WHO (2010). The staining solution was prepared by dissolving 0.67g of Eosin Y and 0.9g of NaCl in 100

ml of distilled water with gentle heating. Thereafter, 10g of nigrosin were added and brought to boil. The solution was filtered through filter paper in order to remove coarse and gelatinous precipitates. The filtered solution was stored in a dark glass bottle at room temperature until use.

To stain, 50 µl of the sperm suspension (after 1 hour incubation with different concentration of *M. whitei* at 37^0C), were mixed with 50 µl of the Eosin-Nigrosin stain in an Eppendorf vial. A smear was then made on a glass slide and left to air dry. Slides were then viewed with a 100X oil immersion objective in the bright field using a light microscope. A total of 200 spermatozoa were counted and the percentage of live sperm was then calculated. Dead sperm appear red/purple and live sperm white.

Figure 15: 1 – Step eosin-nigrosin stain of human spermatozoa. Dead spermatozoa stained pink (A) live spermatozoa appear white/unstained (B) (X1000 magnification)

2.5.4 Determination of sperm motility

Sperm cell motility was measured using the motility module of Sperm Class Analyzer 5.0 (SCA Evolution, MICROPTIC S.L Barcelona, Spain) (Figure 16). 8-chamber Leja slides were employed and filled with 2µl of each sperm suspension after 1 hour incubation with different concentrations of *M. whitei* at 37^0C. Motility of at least 360 spermatozoa was analysed at 37ºC according to the

criteria set by the WHO (2010) with a Nikon Microscope (Nikon Instruments Inc., Americas) at 100X PH1. The following different kinematic parameters were analysed: total motility, progressive motility, velocity curve line (VCL), velocity straight line (VSL), velocity average path (VAP), linearity (LIN), straightness (STR), beat cross frequency (BCF), amplitude of lateral head displacement (ALH), wobble (WOB) and hyperactivation (Table 1) .

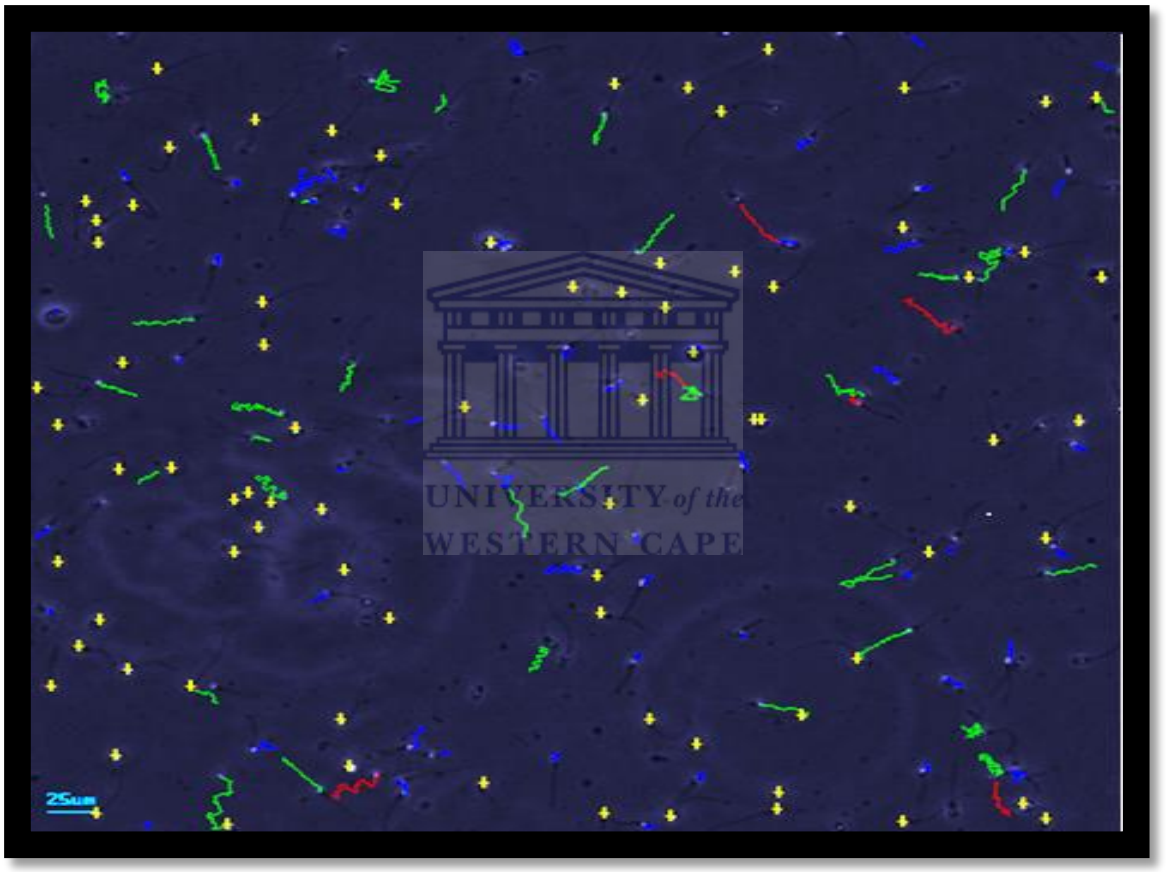

Figure 16: Determination of sperm concentration and motility by means of Sperm Class Analyser® (SCA) 5.0 analysis. CASA system version 4.1.0.1 (Microscopic S.L. Barcelona, Spain). This Figure indicates motility: Red and green comprised progressive motile sperm, blue representing non-progressive motile sperm and yellow representing immotile sperm.

Table 1: Different sperm kinematic parameters measured by the SCA® system

Parameter and units	Description of parameters	Measurement of movements
Total motility (%)	Progressive + non-progressive motile	Spermatozoa showing movement
Progressive motile (%)	Rapid + slow progressive motile	Spermatozoa with moving actively: either linearly or in a circle regardless of speed
Immotile (%)	Static	No movement
LIN (%)	Linearity	Linearity of the curvilinear path = VSL/VCL
STR (%)	Straightness	Linearity of the average path = VSL/VAP
ALH (μm)	Amplitude of lateral head displacement	Magnitude of lateral displacement of a sperm head about its average path
BCF (Hz)	Beat across frequency	Magnitude of lateral displacement of a sperm head about its average path
Hyperactivation (%)	Hyperactivation	Distinctive motility acquired by mature spermatozoa to enable fertilization of an intact ovum
WOB (%)	Wobble	Measure of oscillation of the actual path about the average path=VAP/VCL
VSL (μm/s)	Straight-line velocity	Time-averaged velocity of a sperm head along the straight line between its first detected position and its last
VAP (μm/s)	Average path velocity	Time-averaged velocity of a sperm head along its average path
VCL (μm/s)	Curvilinear velocity	Time-averaged velocity of a sperm head along its actual curvilinear path

2.5.5 Determination of production of reactive oxygen species (ROS) in spermatozoa

ROS production was determined using dihydroethidine (DHE) as fluorescing probe according to Henkel et al. (2003). A stock solution was prepared by using 20 µM DHE in PBS; the pH was adjusted to 7.4. After incubation of sperm samples with *M. whitei* aqueous root extract at the different concentrations for 1hr at 37ºC, an aliquot of 100 µl of spermatozoa was centrifuged for 10 min at 500xg. After the supernatant was discarded, samples were resuspended in 100 µl PBS and 20 µl DHE stock and then incubated for 15 min at 37°C.

Following this incubation period, 10 µl of each sample were viewed on a slide covered by a cover slip under oil immersion using an epifluorescence microscope with 488 nm excitation and 590 emission filters (Zeiss). Bright orange fluorescing sperm indicate excessive ROS production. The percentage of ROS-positive sperm was calculated from at least 200 spermatozoa (Figure 17).

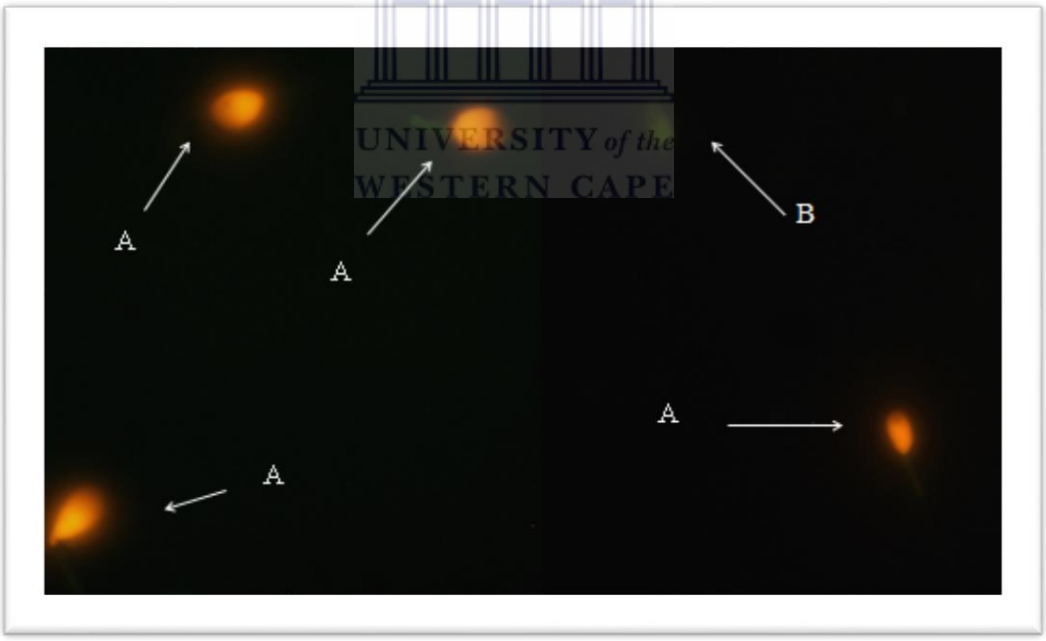

Figure 17: Human spermatozoa after staining with DHE. ROS positive spermatozoa fluorescing bright orange (A); ROS negative spermatozoa did not fluoresces (B) (X1000 magnification)

2.5.6 Determination of sperm mitochondrial membrane potential (MMP)

Intact mitochondrial membrane potential (MMP) in sperm was determined using a lipophilic cationic dye (DePsipher™, Trevigen, Minneapolis, USA). The following modification was implemented from the protocol provided by the manufacturer: The reaction buffer was diluted with pre-warmed distilled water (37^0C) 1:10 and 20 µl stabilizer were added per milliliter buffer with 1 µl of DePsipher dye. Thereafter, the solution was added to 500 µl prepared reaction buffer, vortexed thoroughly and centrifuged for 1 min at 10,000xg. Finally, the supernatant was transferred into a test tube ready to be used, immediately.

After incubation with Mondia aqueous root extract for 1 hour at 37^0C, 100µl of each concentration were diluted with PBS (1:1) and subjected to centrifugation for 10 minutes at 300Xg. The pellets were then re-suspended with 50-200 µl of DePsipher™ and incubated at 37°C for 20 minutes protected from light. After incubation, the sperm suspensions were centrifuged and the pellet was re-suspended with a reaction buffer. Spermatozoa were then immediately observed using a fluorescence microscope with a 488 nm excitation filter (Zeiss) at ×400 magnification. Sperm showing intense red/orange fluorescence were considered healthy with intact MMP (590 nm emission filter). In sperm with disrupted MMP, the monomer dye fluoresced green (530 nm emission filter) and they were generally classified as dying cells (Figure 18). A total of 200 spermatozoa were counted and the number with intact MMP, was recorded as a percentage of the total number of sperm counted.

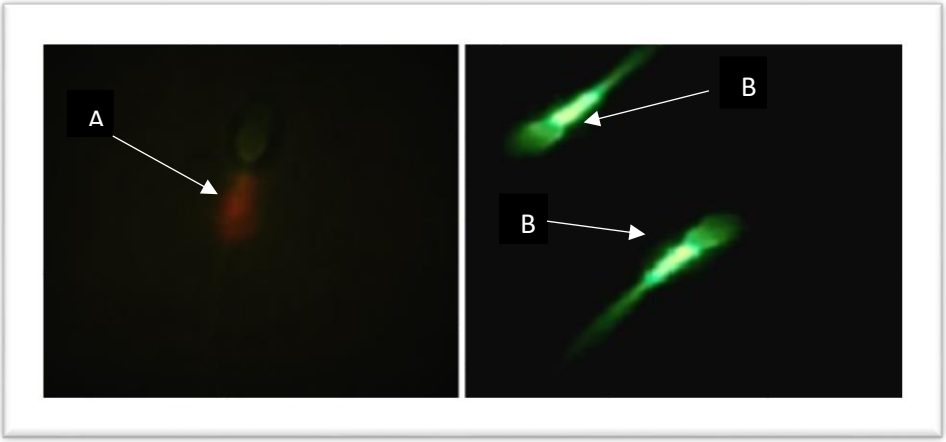

Figure 18: Determination of MMP using DePsipher™. Spermatozoa with intact Δψm showing red fluorescence (A), while those with disrupted Δψm have a green fluorescence (B). (X1000 magnification)

2.5.7 Determination of DNA fragmentation in spermatozoa

DNA fragmentation was measured by using the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) assay (Henkel et al., 2003). The TUNEL assay measures DNA damage directly by detection of single and double DNA strand breaks (Henkel, 2007). These DNA strand breaks are detected in an enzymatic reaction by labelling the free 3'-OH and the modified nucleotides with terminal deoxynucleotidyl transferase.

After incubation with *M. whitei* aqueous root extract at different concentrations at 37^0C, 100 µl sperm suspension were added to 100 µl of phosphate buffered saline (PBS) (Oxoid Ltd., Hampshire, England) and subjected to centrifugation at 300Xg for 10 minutes. The resultant pellets were re-suspended, wet smears were made on a StarFrost™ slide (Knittel Gläser, Braunschweig, Germany) and left to air-dry at room temperature (RT). Slides were then fixed in 4% formaldehyde in PBS (pH 7.4) for 25 minutes at 4°C. After fixation, slides were washed in PBS for 5 minutes at RT and permeabilized in 0.2% Triton™ X-100 (Sigma) in PBS for 5 minutes at RT. Subsequently, slides were rinsed twice in PBS for 5 minutes at RT and 100 µL of equilibration buffer were added to each slide and allowed to equilibrate for 10 minutes. After this, 20 µl of TUNEL reagent (DeadEnd™, Promega, Madison, WI, USA) were added to each slide and cooled with a plastic

cover slip (Promega). Following incubation for 60 minutes at 37°C in a humidified chamber protected from light, the reaction was terminated by immersion in 2×SSC (Promega) for 15 minutes. Slides were then washed in PBS three times and more than 200 randomly selected sperm were immediately analysed using a fluorescence microscope (Zeiss, Oberkochen, Germany), with a 488 nm excitation filter and a 510-530 nm emission filter at ×400 magnification with an oil immersion objective. Sperm with normal DNA only showed a slight background staining (TUNEL-negative), while those with fragmented DNA exhibited a bright green fluorescence (TUNEL-positive). At least 200 spermatozoa were counted and TUNEL-positive sperm were recorded as a percentage of the total number of sperm per field (Figure 19).

Figure 19: Human sperm with nuclear DNA damage. TUNEL-positive sperm fluorescent bright green (A) after TUNEL assay. TUNEL-negative sperm (B) showed background staining only. (×1000)

2.5.8 Determination of capacitation and acrosome reaction in spermatozoa

The chlorotetracyclin (CTC) fluorescence assay protocol as stipulated according to Green et al. (1996), was used in assessing the state of capacitation and acrosome reaction. Distilled water was used to make 100 mg/ml stock solution of Hoechst 33258, then it was stored at 4ºC for up to 1 month. Before use, this stock solution was diluted 1:1000 in HTF and then further 1:100 with sperm suspensions in HTF-BSA. Thereafter, samples were incubated at room temperature for 2 minutes before being washed by centrifugation through 4 ml of 2% polyvinylpyrrolidone (PVP40) in HTF at 900xg for 5 minutes.

The CTC solution (pH 7.8) was prepared on the day of use and contained 750 µM CTC in a buffer of 130 mM NaCl, 5 mM cysteine in 20 mM Tris-HCl. This solution was kept wrapped in foil at 4ºC until use. Hoechst-treated spermatozoa (45µl) were mixed with an equal volume of the CTC solution and 8 µl of 12.5% w/v paraformaldehyde in 0.5 M Tris-HCl (pH 7.4) were added. Subsequently, 10 µl of the this suspension were placed on a slide and one drop of 0.22 M 1.4 diazabicyclo(2.2.2)octane (DABCO) dissolved in glycerol: PBS (9:1) was mixed in carefully to retard fading of the fluorescence. Slides were then viewed with a 100x oil immersion objective using fluorescent microscope (Zeiss). In each sample, 200 live cells (Hoechst-negative) cells were assessed for CTC staining patterns as follows: uniform fluorescence over the entire head (characteristic of non-capacitated), acrosome-intact cells); fluorescence-free band in the post-acrosomal region (characteristic of capacitated, acrosome-intact cells); and dull or absent fluorescence over the sperm head (characteristic of capacitated, acrosome-reacted cells) (Figure 20). At all three stages bright fluorescence on the mid-piece could be seen.

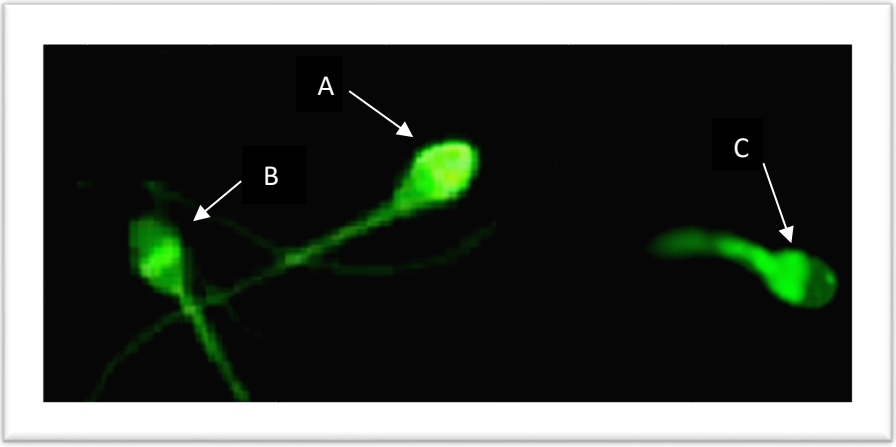

Figure 20: Chlortetracycline staining of human sperm. Uncapacitated acrosome intact sperm (A) entire sperm head shows a bright fluorescence with or without a brighter equatorial band; capacitated acrosome-intact sperm (B) part of the sperm head fluoresce brightly but the sperm head post-acrosome region does not; capacitated acrosome-reacted sperm (C) is non-fluorescent with or without a fluorescent, post-acrosomal region. (X1000).

2.6 Statistical analysis

The MedCalc® statistical software (version 15.8; MedCalc Software, Mariakerke, Belgium) was used for statistical analyses. Normal distribution was tested by means of the D'Agostino-Pearson test (D'agostino and Pearson, 1973). Afterwards, either parametric (Pearson correlation, repeated measure ANOVA), or non-parametric (Spearman Rank correlation) tests were employed accordingly. Data were expressed as mean±SD or SEM. A P-value of less than ($P<0.05$) was regarded as significant.

CHAPTER THREE: RESULTS

3.1 Effect of aqueous root extract of *M. whitei* on human spermatozoa functionality *in vitro*

Different concentrations of aqueous root extract of *M. whitei* (0 µg/ml, 0.0185 µg/ml, 0.185 µg/ml, 1.85 µg/ml, 18.5 µg/ml, 185 µg/ml) were used for the treatment of washed human spermatozoa from 60 semen samples. Results were captured after 1 hour incubation with *M. whitei* root extract at the above concentrations to indicate the extract effects on different sperm parameters.

3.1.1 Summary statistics of parameters measured in this study

A total of 60 semen samples have been included in the study: 28 of them represented healthy sperm donors; the other 32 represented infertile patients attending the fertility clinics mentioned above. The summary of the findings are tabulated in tables 2 and 3. Furthermore, oligozoospermic and asthenozoospermic semen samples were identified and analysed separately. Samples from seven patients were excluded because they were azoospermic or only dead spermatozoa were detected. Four samples from healthy participants were classified as patient samples because of bacteriospermia and reduced sperm concentration. The cut-off value defining male infertility were as follows: time for pregnancy of partner (≥ 1 year), oligozoospermia (concentration < 15 million/ml) and asthenozoospermic (progressive motility < 32% or total motility < 40%) (WHO, 2010; Marchetti et al., 2013; Simon et al., 2013).

Following D'Agostino–Pearson reference values test for normality, sperm parameters were measured as either Mean±SD or Median±range. Moreover, as any other common biological phenomenon, data from most parameters showed a high standard deviation.

3.1.2 Summary of sperm kinematic parameters of donor, patient and combined (donors and patients) group

Whereas most motion parameter values in the donor group remained higher than in the patient group after 1 hour incubation of spermatozoa with increasing concentration of the *M. whitei* root extract (Table 2 and 3), there were no significant differences (one-way ANOVA analysis) nor a trend (repeated measures ANOVA analysis) in all the groups (donors, patients and combined: donors and patients).

More so, as expected the patients showed significantly lower values for VAP than the donors. However, there were no changes nor a trend between different concentrations in VAP in all groups (Figure 21). This is most obvious in the controls and at low concentrations of *M. whitei* root extract. In contrast, at the highest *M. whitei* concentrations, no statistical significance could be observed, although the patient and combined group follow the same pattern as in the other concentration groups (one way ANOVA: donors; P=0.699, patients; P=0.105, donors and patients; P=0.804).

In addition, when compared to the control after 1 hour incubation, spermatozoa at the highest (185 µg/ml) concentration of *M. whitei* roots extract showed an increase (P=0.0195) in VCL in the patient group, as well as, the combined (patient and donor) (P=0.0022) group (Figure 22). Though, no trend was observed in the donor and combined (donor and patient) group (repeated measure ANOVA: donor P=0.595 and combined P=1.000). Yet, there was a significant trend (repeated measure ANOVA P=0.024) in sperm VCL for the patient group.

Table 2: Summary statistics of sperm kinematic motility parameters, following incubation with increasing concentrations of *M. whitei* aqueous root extract for 1 hour.

Kinematic Parameters	Group	Control	0.0185 µg /ml	0.185 µg /ml	1.85 µg /ml	18.5 µg /ml	185 µg /ml
VAP (µm s^{-1})	**Donors***	36.0 ±10.79	38.47 ±11.16	37.59±11.34	37.44 ±10.28	37.06 ±11.23	35.16 ±9.11
		36.95(20.7-89)	41.2(14.7-67.3)	39.7(16-61.4)	39.4(14-58.4)	38(10.4-68.2)	36.4(18.3-54)
	Patients	27.88 ±9.78	28.93 ±7.57	29.08 ±9.47	28.20 ±10.47	30.50 ±8.34	31.63±10.81
		25.1(14-48.8)	29.7(12.9-45)	28.3(16.1-57.1)	27.4(7-54.6)	30(15.4-45.0)	30(12.9-57.1)
VCL (µm s^{-1})	**Donors***	66.09± 15.96	71.33 ±13.11	70.08 ±15.10	70.25 ± 13.34	70.26 ±14.79	69.87± 12.05
		66.9(37-94.7)	70.9(41-92.8)	69.3(42.1-96)	72.3(39.6-91)	71(35.7-95.4)	67(50-91.3)
	Patients	54.33 ±10.27	56.85 ±8.22	57.12 ±10.75	53.60 ±13.29	58.88 ±10.86	60.66 ±10.43
		54.9(34-76.2)	59(41.7-70.8)	57.2(37.4-81)	56(25.5-79.5)	58.8(32.2-82)	59.4(41-81.1)
VSL (µm s^{-1})	**Donors**	27.24± 10.95	30.38 ±12.65	29.26 ±14.30	28.30 ±10.84	28.30 ±12.02	28.00± 12.9
		22.6(6.4-50.9)	24.8(6.7-61)	26.4(8.2-71.9)	27.5(6.5-53)	28.2(5.4-61.8)	25.4(10-66.2)
	Patients*	20.62 ±9.25	20.89 ±9.06	17.37 ±6.31	20.26 ±10.48	21.98 ±9.69	23.58 ±10.92
		17.5(8-40.2)	19.6(6.7-41.7)	17.5(8.2-28.1)	18.5(4.8-49)	20.2(9.8-48)	22.1(6.7-49)
ALH (µm)	**Donors***	2.26 ±0.46	2.33 ±0.29	2.34 ±0.32	2.30 ±0.37	2.4 ±0.39	2.21 ±0.43
		2.2(1-3.5)	2.3(1.4-2.9)	2.3(1.8-3.3)	2.3(1.2-2.9)	2.3(1.7-3.1)	2.1(1.1-3.1)
	Patients	1.75 ± 0.67	1.98 ± 0.74	1.95± 0.67	1.85 ± 0.71	2.02± 0.4	2.09 ± 0.53
		1.9(0-29)	2.1(0.5-3.5)	2.1(0-2.7)	2(0-2.7)	2.1(1.0-2.8)	2(0.7-3.3)

Data comprise of donors (n=28) and patients (n=32) which is expressed as mean±SD and median (range). An asterix (*) indicate data with normal distribution. VAP: velocity average path; VCL: velocity curvilinear; VSL: velocity straight line; ALH= average lateral head displacement; SD; standard deviation.

Donors: ANOVA P-value (VAP: P= 0.941; VCL: P= 0.850; VSL: P= 0.954; ALH: P= 0.622)

Patients: ANOVA P-value (VAP: P= 0.105; VCL: P= 0.213; VSL: P= 0.160, ALH: P= 0.364)

Table 3: Summary statistics of sperm kinematic motility parameters, following incubation with increasing concentration of *M. whitei* root extract for 1 hour.

Kinematic Parameters	Group	Control	0.0185 µg /ml	0.185 µg /ml	1.85 µg /ml	18.5 µg /ml	185 µg /ml
Hyperactivation (%)	**Donors**	3.98 ±2.83	4.79 ±2.93	4.08 ±3.16	4.32 ± 2.54	4.37 ±3.24	4.73 ±3.34
		30(2-11.4)	4.8(0-10.6)	3.1(0.3-11.7)	3.9(0.8-10)	3.2(0.2-11.2)	4.9(0.6-13)
	Patients	1.35 ±1.28	1.08 ±0.97	1.55 ±1.71	1.83 ±2.07	2.14 ±3.57	2.40 ±2.21
		1.1(0.0-5.7)	1.1(0-4.7)	1.1(0-5.7)	1.3(0-7.0)	1.3(0.0-18.6)	1.5(0-9.1)
LIN (%)	**Donors**	37.71± 11.09	37.06 ± 10.5	35.69 ±11.75	35.84 ± 10.79	35.39 ±11.33	35.11 ±10.67
		36.3(16-60.7)	36.6(19-67.3)	35.7(18.8-66)	35.5(5-58.5)	33.5(17-60)	33.9(8.8-56)
	Patients*	35.51± 10.67	34.79 ± 10.53	33.57 ±10.45	37.32 ± 12.22	38.90 ±11.18	37.47 ±13.84
		34.9(20-57.4)	33.1(11-61.3)	34.8(11-61.3)	37.3(18.9-62)	37.8(16.6-61)	36.2(16-61.3)
STR (%)	**Donors**	68.97 ±12.97	69.16 ±12.09	67.32 ±12.68	68.08 ±12.93	67.34 ±12.65	66.28± 12.36
		12.9(20.7-80)	70.6(8.6-91.2)	66.4(25.7-67.3)	66.5(9.7-91)	68.5(28.1-90.6)	68.6(26.8-84.6)
	Patients	68.10 ±10.14	68.88 ±10.87	68.08 ±12.63	68.97 ±11.03	72.09 ±10.31	70.25 ±12.33
		68.4(9.8-85.4)	71.3(48.8-85.5)	69.2(29-87.1)	68.1(49.8-85.1)	71.6(46.6-85.8)	72.3(47.4-85.8)
WOB (%)	**Donors**	51.37 ± 9.57	50.91 ± 9.76	50.90 ±10.22	50.53 ±9.11	49.91 ±10.56	49.86 ±9.21
		51.2(21-68.3)	51.6(19-71.8)	50.2(51.9-71.8)	50.3(25-67.8)	50.2(18.4-71)	49.2(22-67.5)
	Patients*	49.10 ±7.95	50.09 ±8.15	49.40 ±7.62	49.83 ±9.50	51.49 ±9.22	51.72 ±11.15
		48.6(32-66.7)	49.5(30.7-66.8)	49.8(36.5-70.4)	50.2(27.3-71)	51.9(30.7-71)	51.9(30.7-71)
BCF (HZ)	**Donors**	13.79 ±2.68	13.70 ±2.28	13.37 ±2.64	13.99 ±3.08	13.67 ±2.90	13.35 ±2.57
		36.3(16-60.7)	36.6(19-67.3)	35.69(18.8-66)	33.5(5.1-58)	33.5(17-60)	33.9(8.8-55)
	Patients	11.07 ±5.02	11.81 ±5.15	11.31 ±4.54	10.39 ±4.77	11.82 ±4.25	11.94 ±4.01
		12.2(0-5.7)	13(0-23.3)	12.2(0-18.8)	22.8(0-17.8)	13.3(1.9-17)	12.7(3.6-18)

Data comprise of donors (n=28) and patients (n=32) which is expressed as mean±SD and median (range). An asterix (*) indicate data with normal distribution. BCF; beat across frequency, LIN; linearity, STR; straightness, WOB; wobble, SD; standard deviation.
Donors ANOVA P-value (Hyperactivation: P= 0.441; BCF: P= 0.937; LIN: P= 0.928; STR: P= 0.921; WOB: P= 0.990)
Patients ANOVA P-value (Hyperactivation: P= 0.611; BCF: P=0.789; LIN: P= 0.611; STR: P= 0.636; WOB: P= 0.820

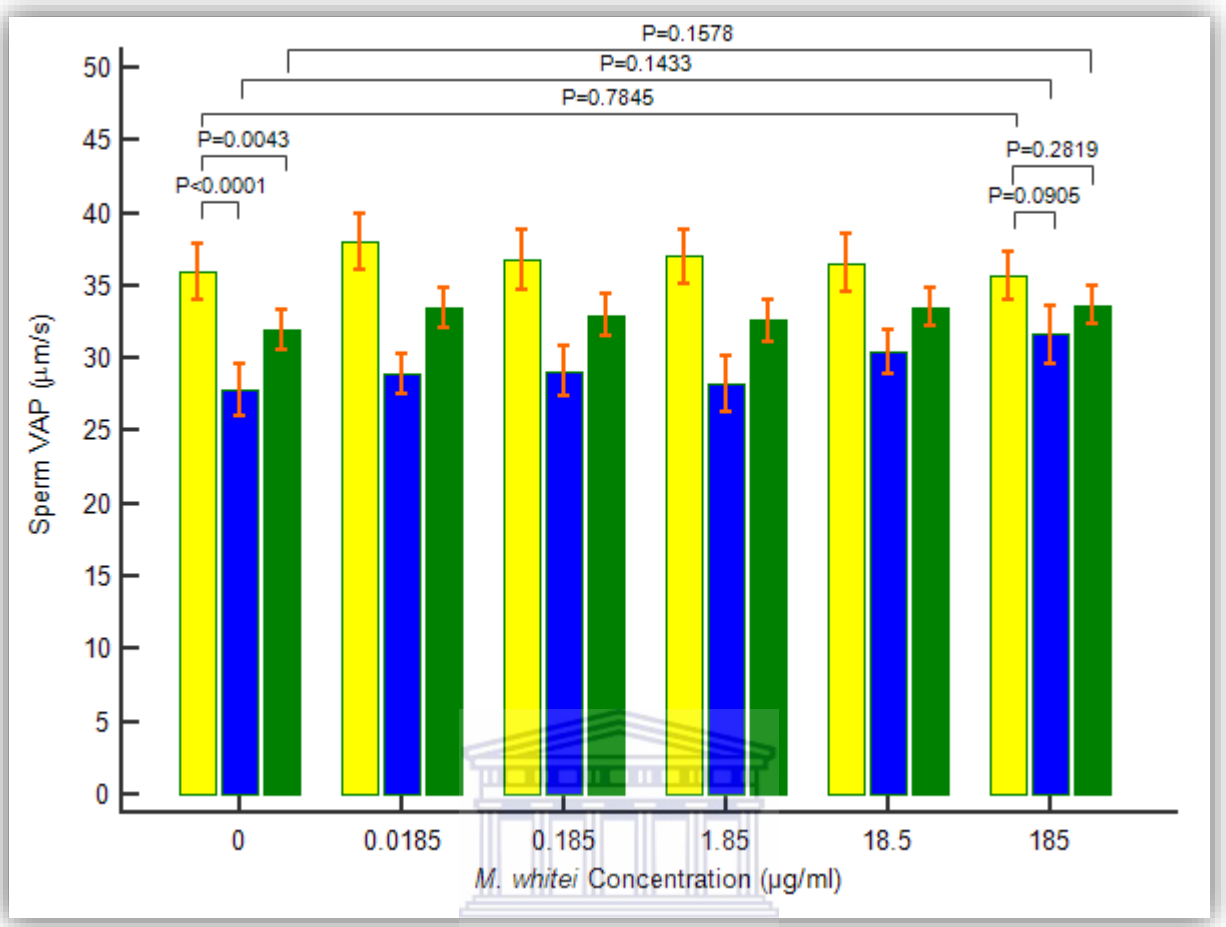

Figure 21: Effect of different concentrations of *M. whitei* root extract (0.0185, 0.185, 1.85, 18.5 and 185 µg/ml) on velocity average path (VAP) of washed spermatozoa *in vitro*. Yellow represents: Healthy donors (n=28); Blue: Patients (n=32) and Green: Combined group of healthy donors and patients (n=60). Values shown as mean±SEM. No significant difference; one-way ANOVA: (donors; P=0.699, patients; P=0.105, combined; P=0.804) nor trend can be seen in all groups: repeated measure ANOVA: (donors; P=0.966, patients; P=0.135, combined; P=0.398).

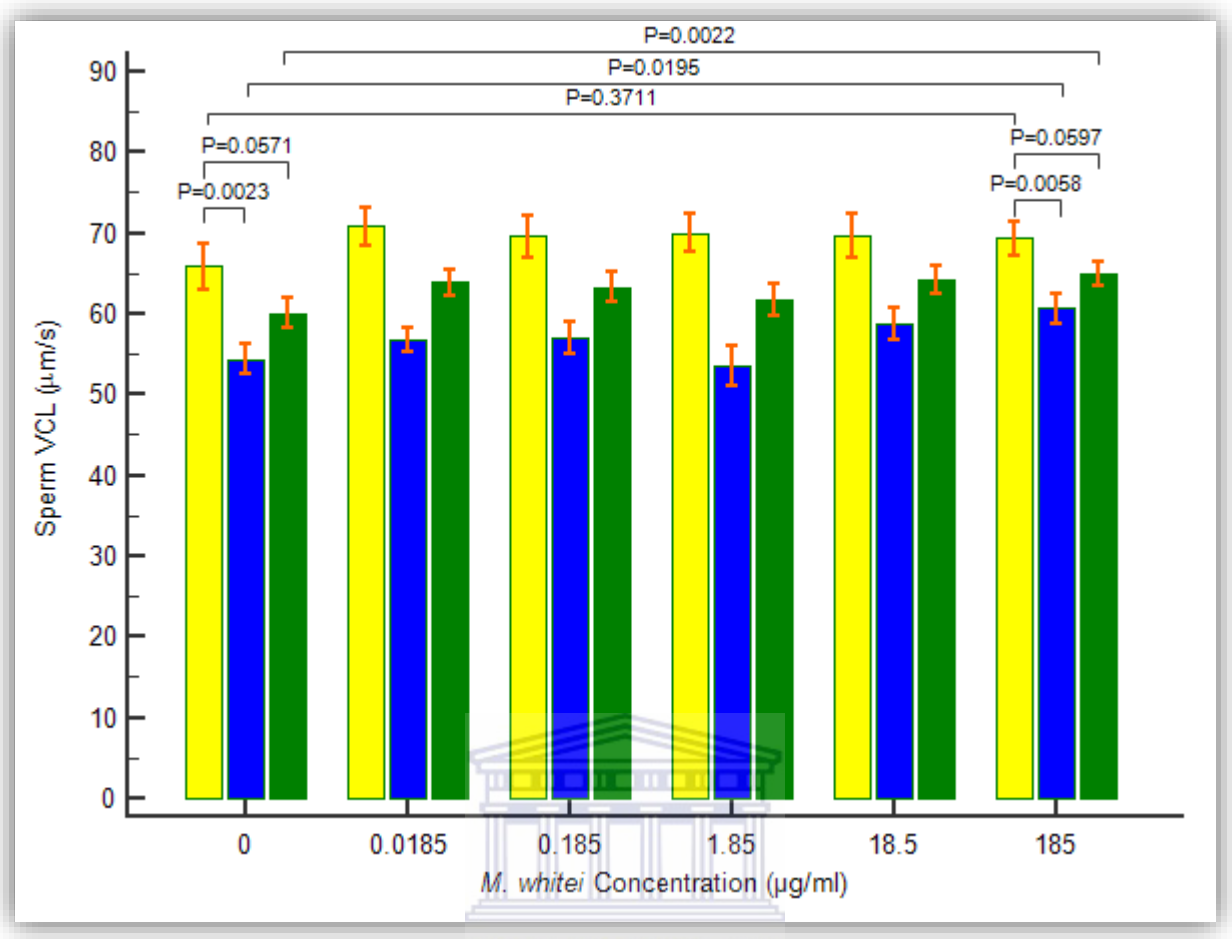

Figure 22: Effect of different concentrations of *M. whitei* root extract (0.0185, 0.185, 1.85, 18.5 and 185 µg/ml) on velocity curvilinear (VCL) of washed spermatozoa *in vitro*. Yellow represents: healthy donors (n=28); Blue: patients (n=32) and Green: combined group of healthy donors and patients (n=60). Values shown as mean±SEM. An increase in VCL is seen between the control and the highest concentration in patient (P=0.0195) and combined (P=0.0022) group, with a significant trend (repeated measure ANOVA: P=0.024) seen on the patient group. Yet, no trend is observed in the donor and the combined group**:** **r**epeated measure ANOVA (donors; P=0.595, donors and patients; P=1.000). No significant change was seen in all groups: one-way ANOVA (donors; P=0.850, patients; P=0.213, combined; P=0.531)

Apart from the combined (donor and patient) group which portrayed a noticeable significant (P=0.0375) increase on VSL at 185 µg/ml when compared to the control (Figure 23), there was no significant difference between different concentrations of *M. whitei* root extract nor trend on VSL across all groups. This is more apparent in the controls and at lower concentrations. Besides, at highest concentrations of *M. whitei*, no statistical differences could be seen in all groups: one-way ANOVA analysis: (donor; P=0.936, patients; P=0.160, donor and patient; P=0.535).

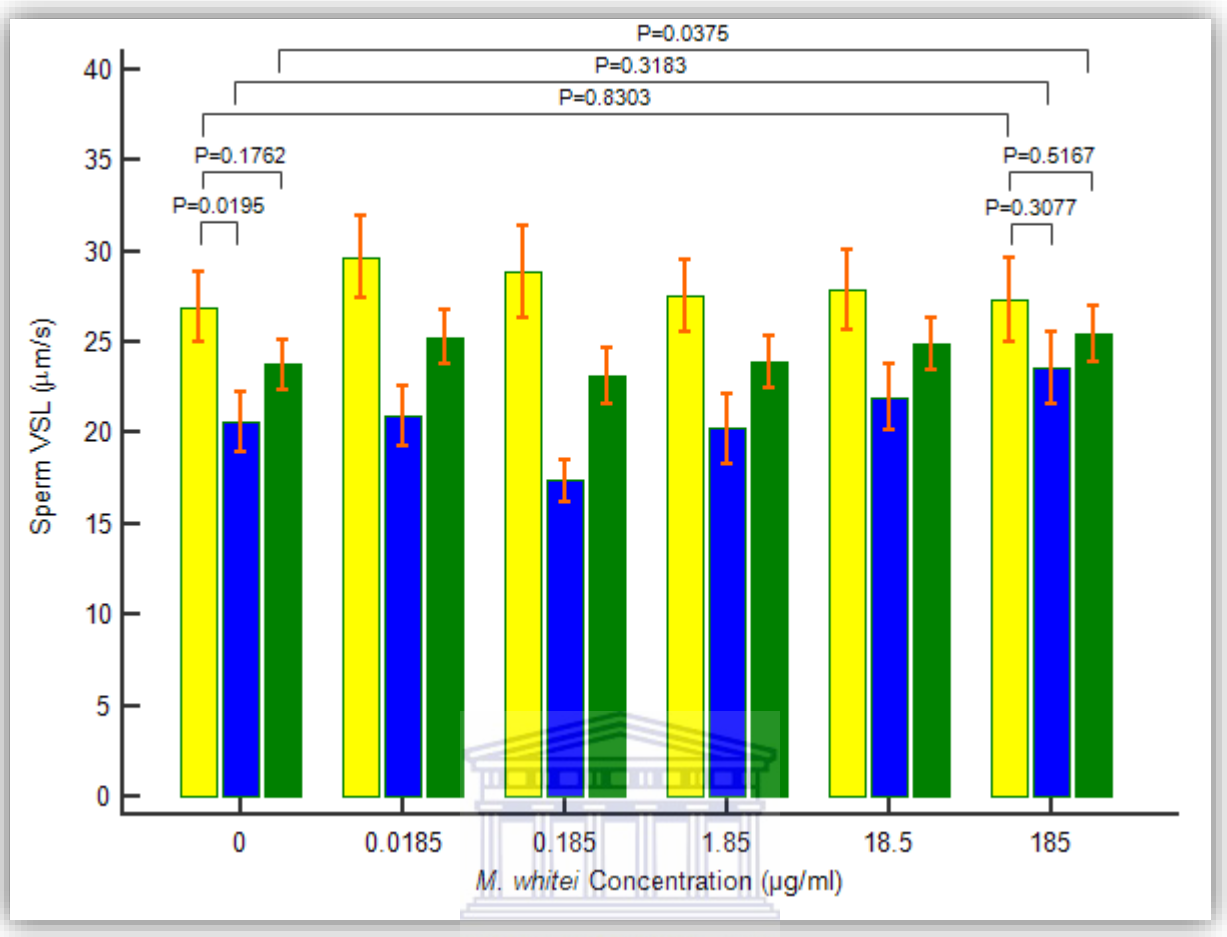

Figure 23: Effect of different concentrations of *M. whitei* (0.0185, 0.185, 1.85, 18.5 and 185) µg/ml on velocity straight line (VSL) of washed spermatozoa *in vitro*. Yellow represents: healthy donors (n=30); Blue: patients (n=30) and Green: combined group of healthy donors and patients (n=60). Values shown as mean±SEM. No changes were found at all concentrations: One-way ANOVA analysis (donor; P=0.936, patients; P=0.160, donor and patient; P=0.535).

Following 1 hour incubation of spermatozoa with different concentrations of *M. whitei* root extract, a marginal significant (P=0.0438) increase in ALH was observed when the controls in the patient group were compared with the highest concentration (185 µg/ml) (Figure 24). However, there were no changes between different concentrations of *M.whitei* nor a trend in ALH across all groups, especially in the control and at low concentration. In contrast, no changes could be observed at the highest concentration of *M. whitei* too.

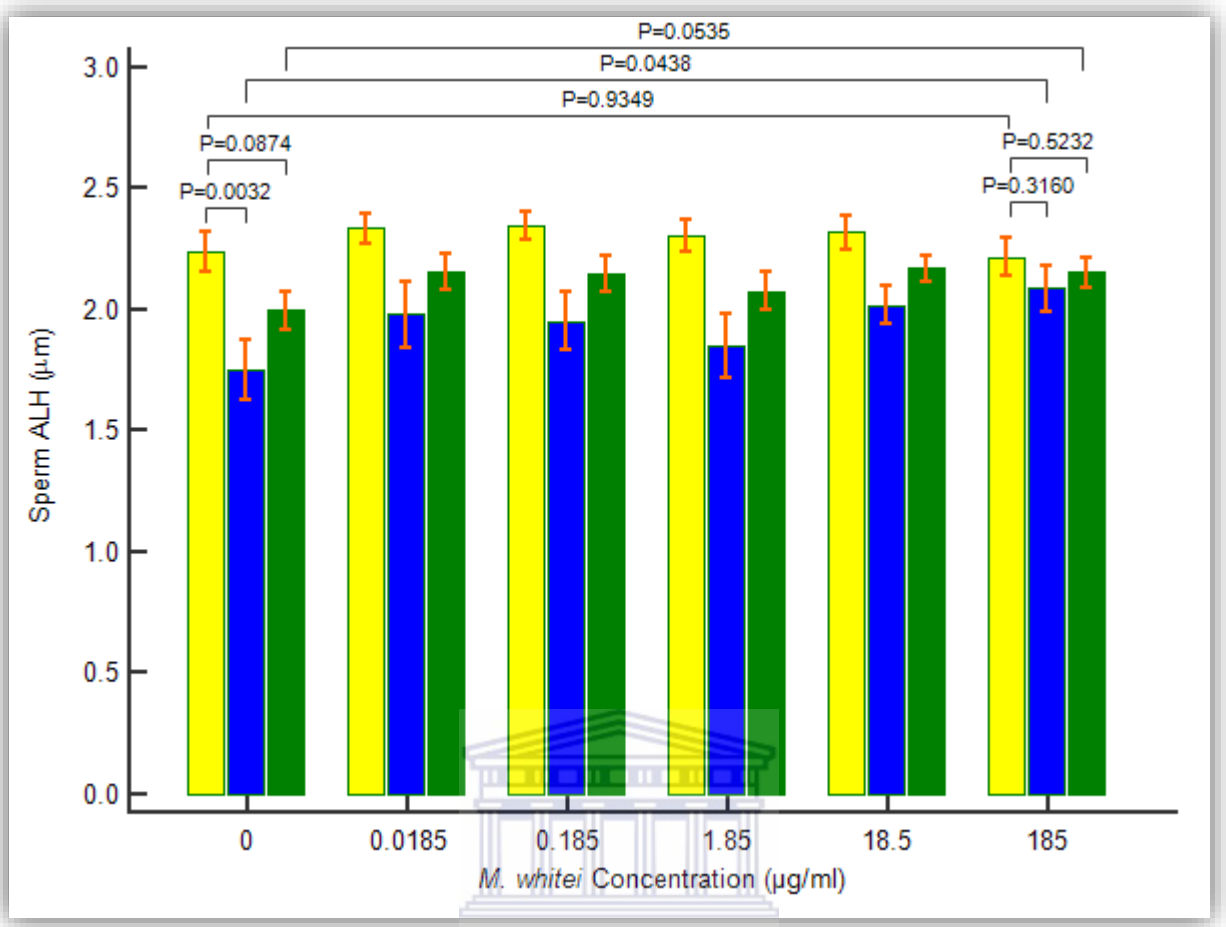

Figure 24: Effect of different concentrations of *M. whitei* (0.0185, 0.185, 1.85, 18.5 and 185 µg/ml) on the average lateral head displacement (ALH) of washed spermatozoa *in vitro*. Yellow represents: healthy donors (n=28); Blue: patients (n=32) and Green: Combined group of healthy donors and patients (n=60). Values shown as mean±SEM. No statistical significant differences nor trend is seen: one-way ANOVA (donors P=0.483; patients P=0.478; combined P=0.391) and repeated measure ANOVA (donors P=0.777; patients P=0.118; combined P=0.440). However, a significant (P=0.0438) increase of ALH from the control to the highest (185 µg/ml) concentration is seen on the patients group.

As expected, the donors showed significantly higher values of hyperactivated spermatozoa than the patients. Nonetheless, there was a significant (P=0.0301) increase in the percentage of hyperactiavated spermatozoa observed in the combined (donor and patient) group at 185 µg/ml when compared to the control, while no significant increase was reflected in the patient group as well as the donor group (Figure 25). Further analysis by one-way ANOVA (donor: P=0.824; patient: P=0.314, combined: P=0.666) and repeated measure ANOVA (donor: P=0.762, patient: P=0.059, combined: P=0.130) showed no changes nor trend in all groups.

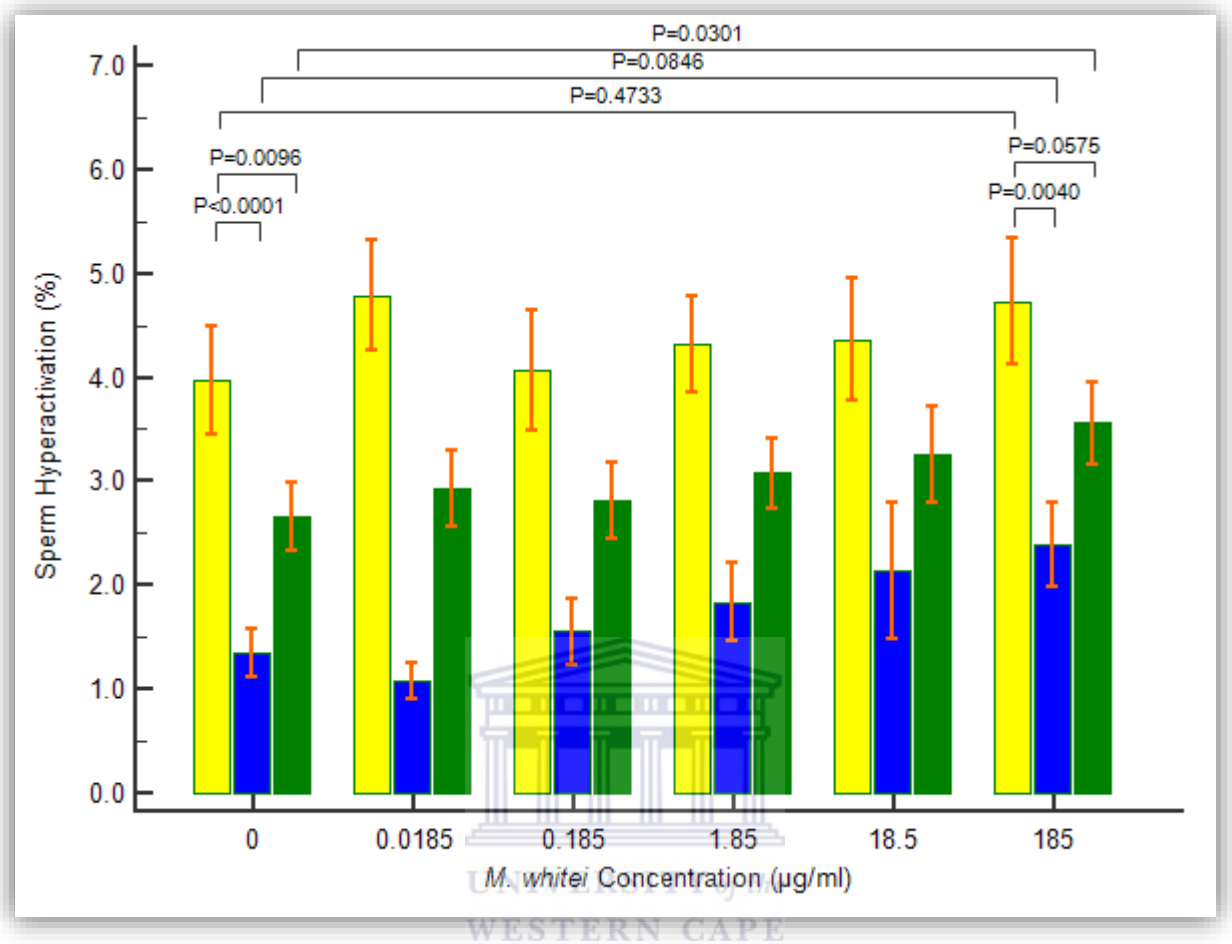

Figure 25: Effect of different concentrations of *M. whitei* (0.0185, 0.185, 1.85, 18.5 and 185µg/ml) on hyperactivated washed spermatozoa *in vitro*. Yellow represents: healthy donors (n=28); Blue: patients (n=32) and Green: combined group of healthy donors and patients (n=60). Values shown as mean±SEM. A significant (P=0.0301) increase in the percentage of hyperactiavated spermatozoa for the combined (donor and patient) group is observed at 185 µg/ml when compared to the control. No changes nor trend can be seen in all the groups.

On the other hand, there were no changes in the linearity of sperm movement between different concentrations of *M. whitei* root extract for all the groups (Figure 26). This is apparent in the controls and at low concentrations, more so the highest concentrations of *M. whitei* showed no changes too. (One-way ANOVA: donor: P=0.917, patient: P=0.611, combined: P=0.697) nor trend (repeated measure ANOVA: donors: P=0.291, patients: P=0.204, donors and patients: P=0.808) for all groups.

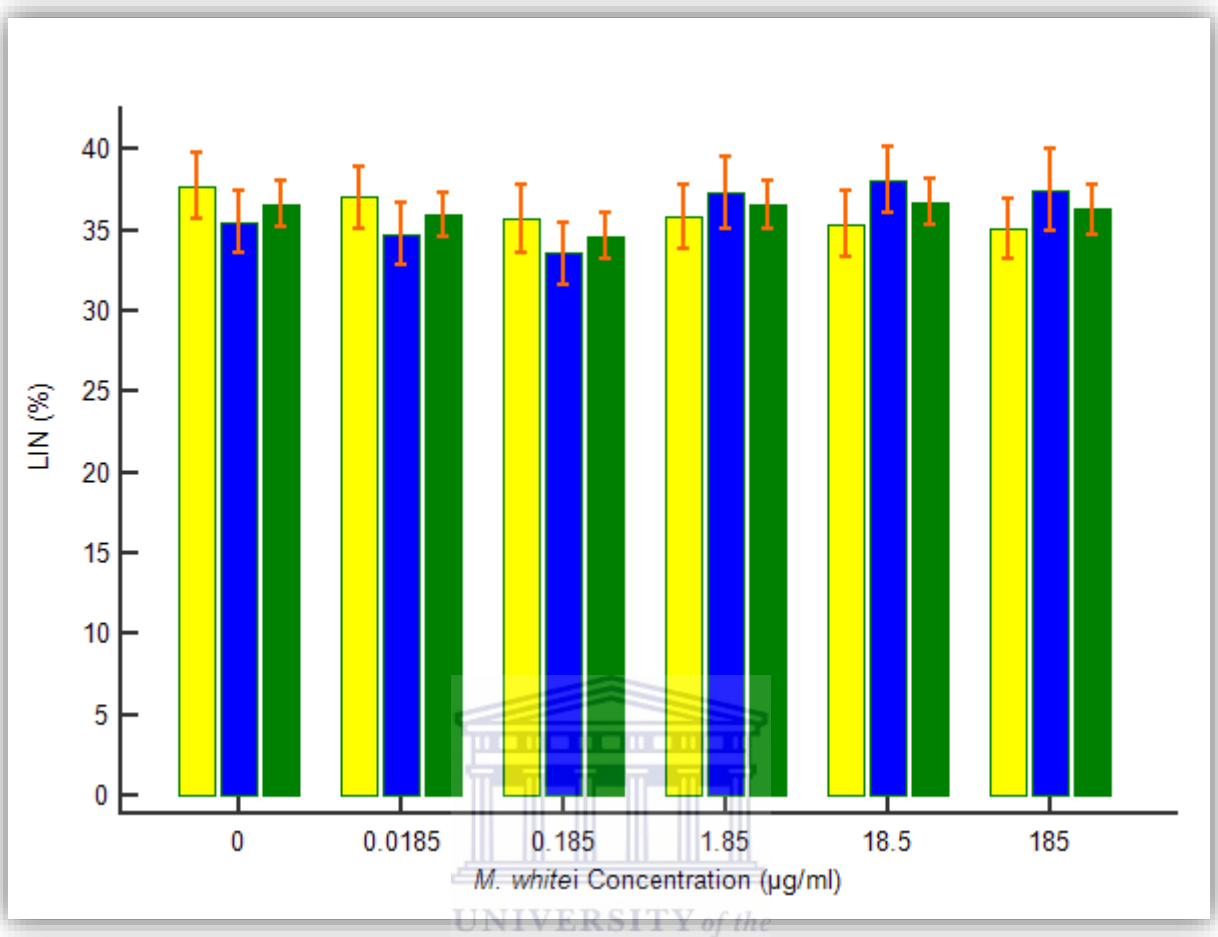

Figure 26: Effect of different concentrations of *M. whitei* (0.0185, 0.185, 1.85, 18.5 and 185 µg/ml) on linearity (LIN) of washed spermatozoa *in vitro*. Yellow represents: healthy donors (n=28); Blue: patients (n=32) and Green: combined group of healthy donors and patients (n=60). Values shown as mean±SEM. No significant difference was found at all concentrations.

Percentage straightness remained the same in all groups with increasing concentration of *M.whitei*. Hence, no significant changes (one-way ANOVA: donor: P=0.925, patient: P=0.636, donor and patient: P=0.824) could be observed (Figure 27).

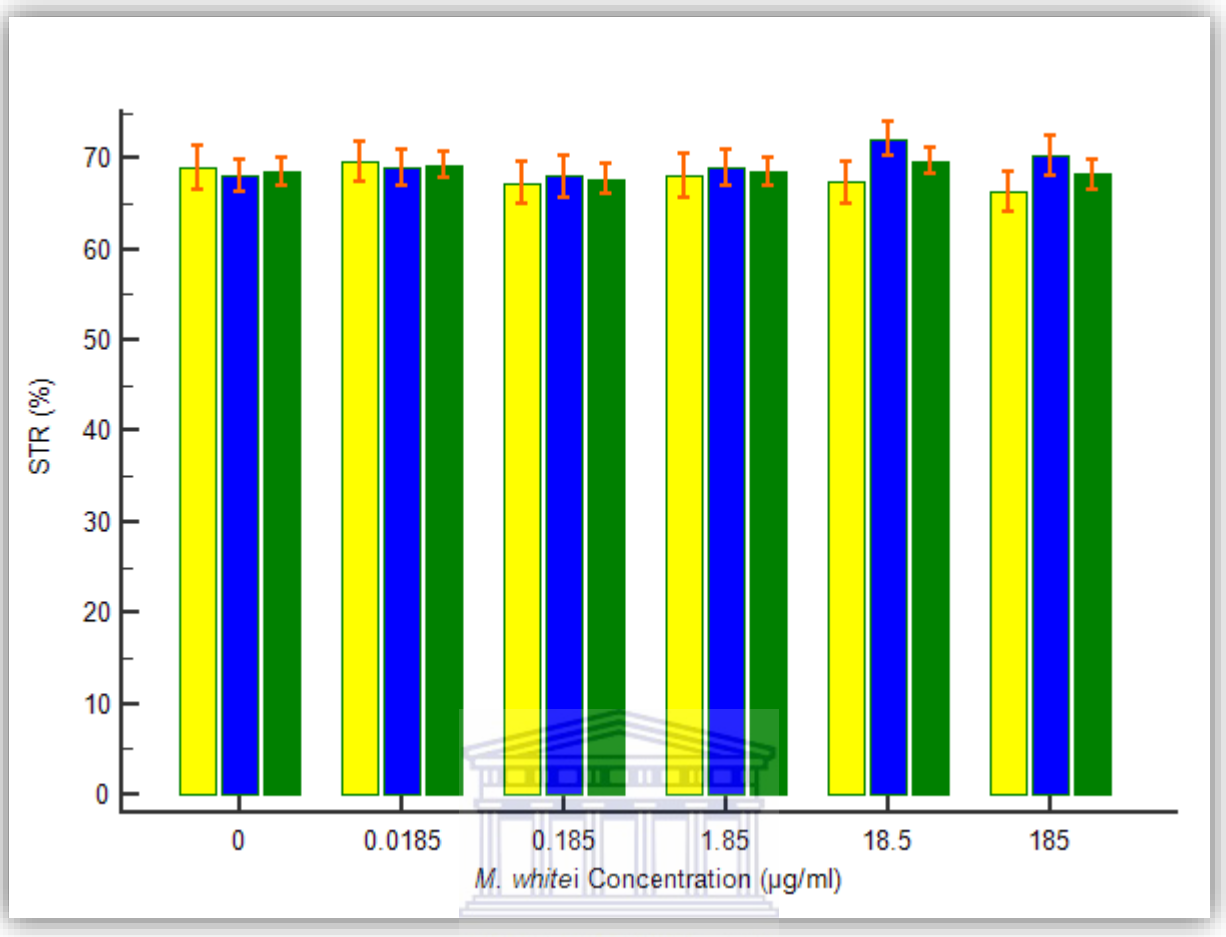

Figure 27: Effect of different concentrations of *M. whitei* (0.0185, 0.185, 1.85, 18.5 and 185 µg/ml) on percentage straightness (STR) of washed spermatozoa *in vitro*. Yellow represents: healthy donors (n=28); Blue: patients (n=32) and Green: combined group of healthy donors and patients (n=60). Values shown as mean±SEM. No significant difference was found at all concentrations.

Figure 28 displays the results for the treatment of spermatozoa with increasing concentrations of *M. whitei*, whereby there were no changes in the percentage of BCF at different concentrations across all groups in comparison to the control. Besides, further analysis with the use of one-way ANOVA: (donors: P=0.936, patients: P=0.636, donors and patients: P= 0.860) revealed no significant change in all the groups.

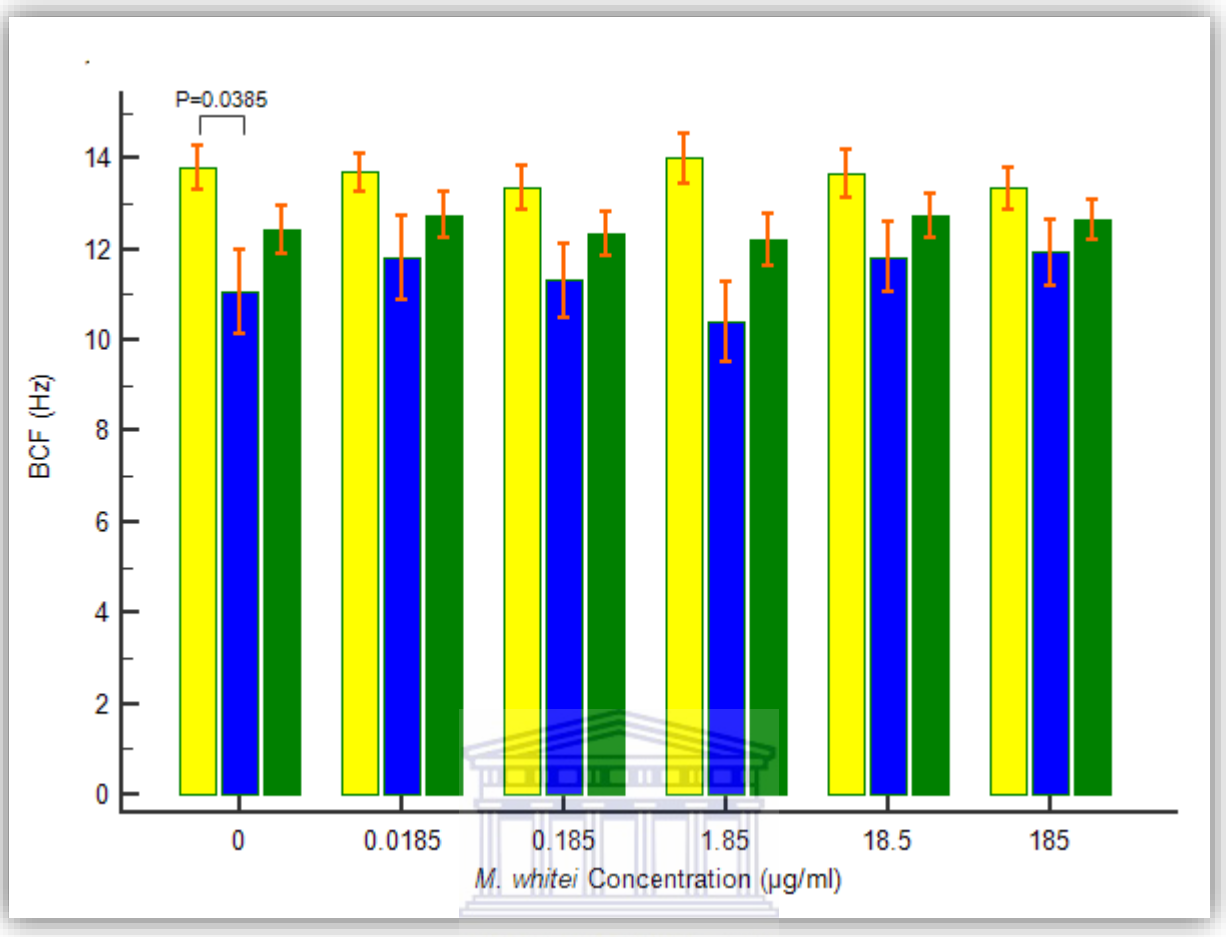

Figure 28: Effect of different concentrations of *M. whitei* (0.0185, 0.185, 1.85, 18.5 and 185 µg/ml) on beat across frequency (BCF) of washed spermatozoa *in vitro*. Yellow represents: healthy donors (n=28); Blue: patients (n=32) and Green: combined group of healthy donors and patients (n = 60). Values shown as mean±SEM. No changes nor trend was found at all concentrations.

The percentage wobble remained the same with increasing concentration of *M. whitei* root extract. Hence no changes nor trend were seen (one-way ANOVA: donors P=0.989, patients P=0.820, donors and patients P=0.960) (Figure 29).

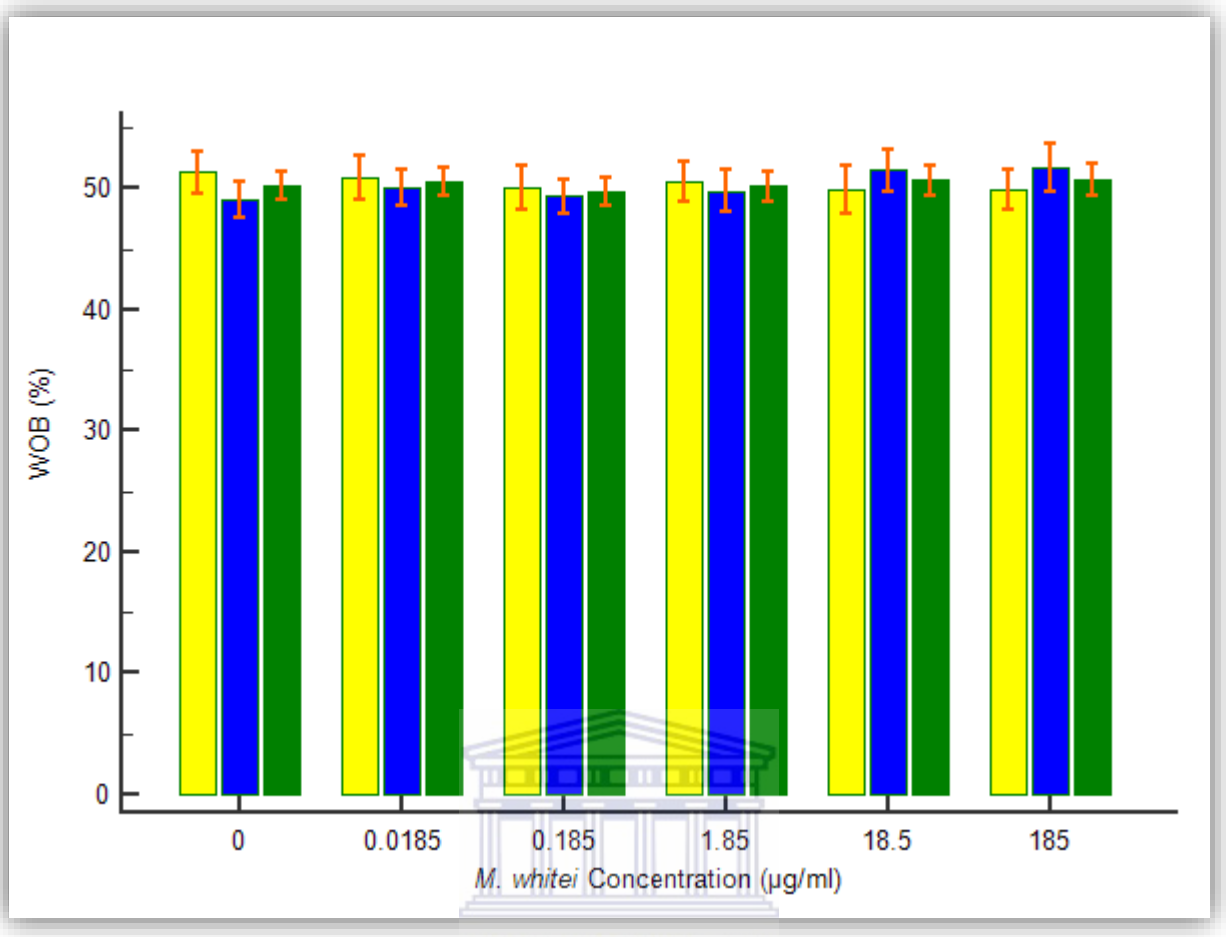

Figure 29: Effect of different concentrations of *M. whitei* (0.0185, 0.185, 1.85, 18.5 and 185 µg/ml) on wobble of washed spermatozoa *in vitro*. Yellow represents: healthy donors (n=28); Blue: patients (n=32) and Green: combined group of healthy donors and patients (n=60). Values shown as mean±SEM. No significant difference was found at all concentrations.

3.1.3 Effect of *M. whitei* aqueous root extract in sperm motility

Figures 30 and 31 display total motility and progressive motility for the three groups (donors, patients and combined group: donors and patients) respectively, after one hour of incubation at different concentrations of *M. whitei* extract. Despite the fact that there was a highly significant increase in total motility for the three groups: donor (P=0.0064), patient (P=0.0001) and the combined (donor and patient) (P<0.0001) group; no trend (repeated measure ANOVA: P=0.144) could be observed over all concentrations in the donor group. On the contrary, a highly significant (one-way ANOVA: P=0.0001) dose-dependent increase in motility to as low as 0.185 µg/ml extract (P=0.0392) was seen in the patient group (Figure 30). In

addition, the combined (donor and patient) group portrayed a highly significant trend (one-way ANOVA: P=0.0009; repeated measure ANOVA: P=0.0003) in percentage of total motile spermatozoa.

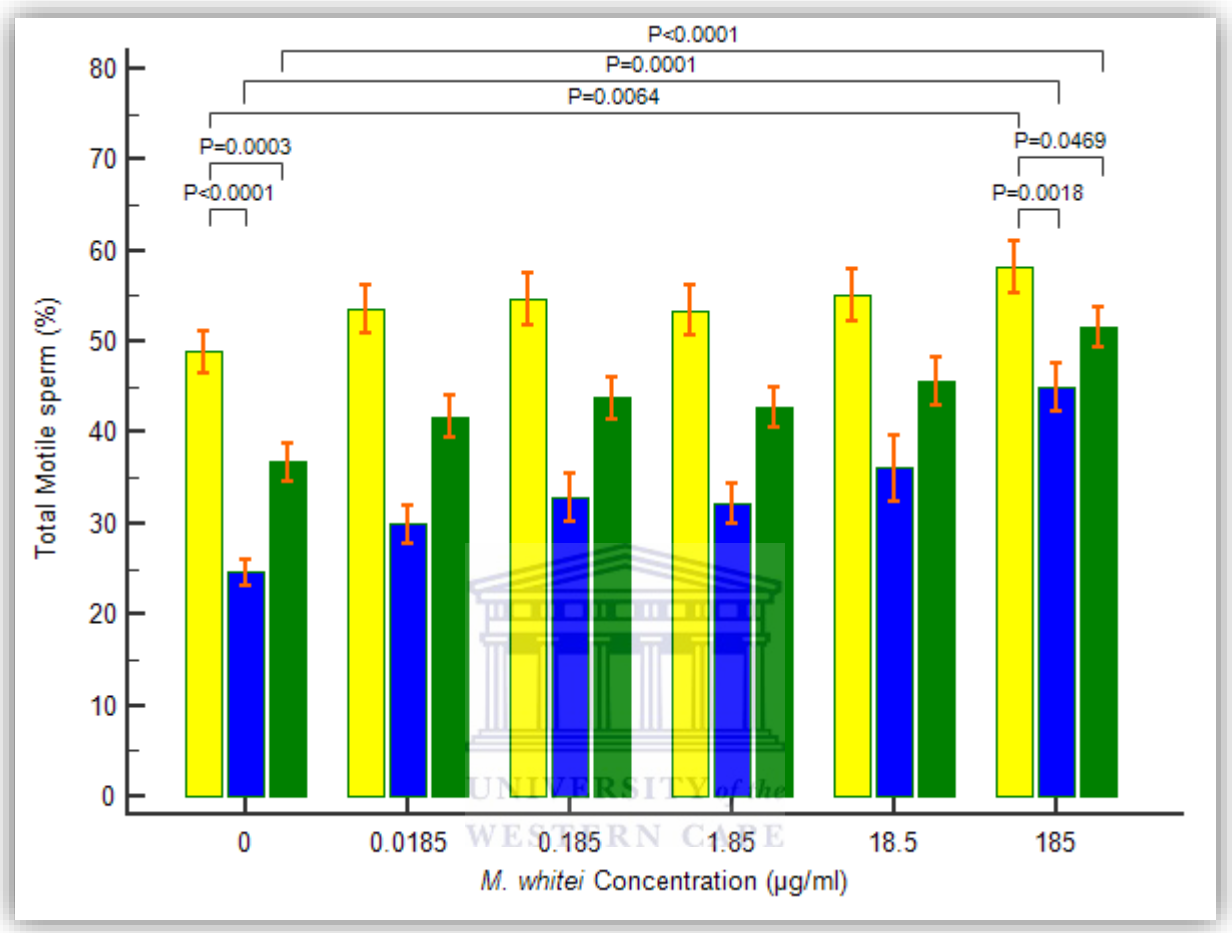

Figure 30: Effect of different concentrations of *M. whitei* root extract (0.0185, 0.185, 1.85, 18.5 and 185 µg/ml) on total motility of washed spermatozoa *in vitro*. Yellow represents: healthy donors (n=28); Blue: patients (n=32) and Green: combined group of healthy donors and patients (n=60).Values are shown as mean±SEM. No significant difference can be seen nor any trend in the donors group. A significant (repeated measure ANOVA: P=0.0001) dose-dependent increase to as low as the concentrations of 0.185µg/ml (P=0.0392) is seen on the patient group, whilst, the combined group shows an increase in percentage of motile sperm (P<0.0001) at 185 µg/ml when compared to the control with significant trend (repeated measure ANOVA: P = 0.0009).

Nonetheless, there was no trend for progressive motility in the donor (repeated measure ANOVA: P=0.3618) as well as the combined group (repeated measure ANOVA: P=0.0624); even so, the combined group portrayed a highly significant (P=0.0008) increase in progressive motility when the highest

concentration (185 µg/ml) was compared to the control, whereas the donor group showed no statistical (P=0.2252) difference. The patient group showed a highly significant (one-way ANOVA P=0.0002) dose-dependent increase on progressive motility when the highest (185 µg/ml) concentration was compared to the control (Figure 31). Similarly, further analysis using the repeated measures ANOVA revealed a highly significant (P=0.0068) positive trend between control and the highest concentration (185 µg/ml).

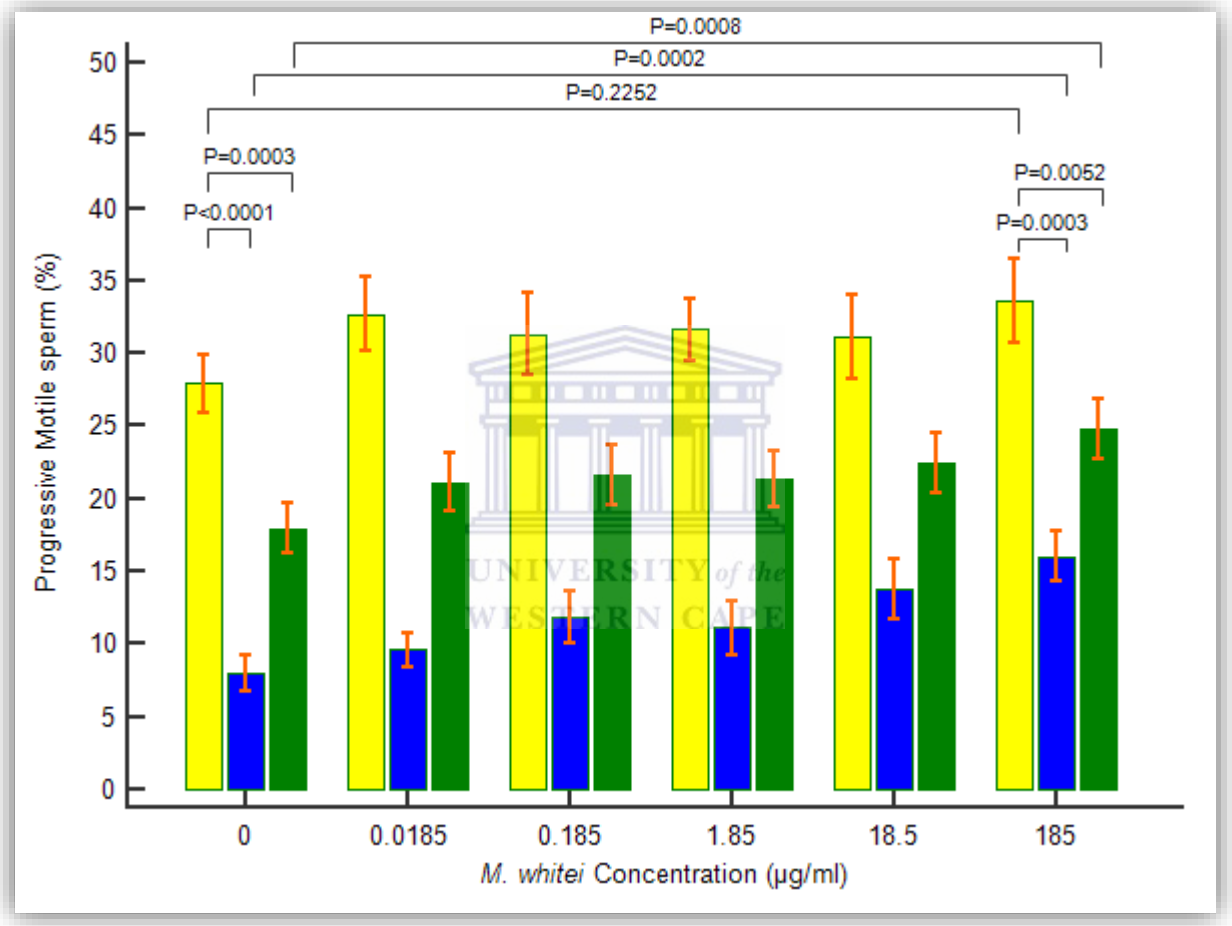

Figure 31: Effect of different concentrations of *M. whitei* root extract (0.0185, 0.185, 1.85, 18.5 and 185 µg/ml) on progressive motility of washed spermatozoa *in vitro*. Yellow represents: healthy donors (n=28); Blue: patients (n=32) and Green: combined group of healthy donors and patients (n=60). Values are shown as mean±SEM. No significant difference can be seen nor any trend in the donors group. A strong significant (one-way ANOVA P=0.0002) dose-dependent increase and trend (repeated measure ANOVA: P=0.0068) is seen on the patient group. An increase (P=0.0008) in percentage of total motile sperm is observed at 185 µg/ml when compared to the control with no significant trend (repeated measure ANOVA: P=0.0624) on the combined group.

3.1.4 Effect of *M. whitei* aqueous root extract on sperm vitality

Following the exposure of different concentrations of *M. whitei* root extract to washed spermatozoa, patients' sperm showed generally lower vitality as compared to the donors (Figure 32). However, the donor group (P=0.1102), the patient group (P=0.2579) and the combined (donor and patient) group (P=0.0684) showed no change in sperm vitality when various concentrations were compared to the control. Nevertheless, the results acquired after one-way ANOVA analysis were not significant in any group. Further analysis with repeated measure ANOVA revealed a marginal significant (P=0.0401) trend for the donor group. Yet, there was no trend for the patient (P=0.319) and the combined (P=0.187) group.

3.1.5 Effect of *M. whitei* aqueous root extract on sperm mitochondrial membrane potential (MMP)

Treatment of washed spermatozoa with increasing concentrations of *M. whitei* root extract depicted a highly significant dose-dependent increase in the percentage of spermatozoa with intact MMP across all groups: one-way ANOVA (patient; P<0.0001, combined (donor and patient); P<0.0001 and donor; P=0.0007) (Figure 33). More so, comparison between the control and the highest concentration (185µg/ml) also showed a significantly higher increase in percentage of spermatozoa with intact mitochondrial membrane potential in all groups: patient group (P<0.0001); donors and patient group (P<0.0001) and donor group (P=0.0001). Equally, a highly significant positive trend was seen across all groups: repeated measure ANOVA (donors: P=0.0001, patients: P<0.0001, combined (donors and patients: P<0.0001).

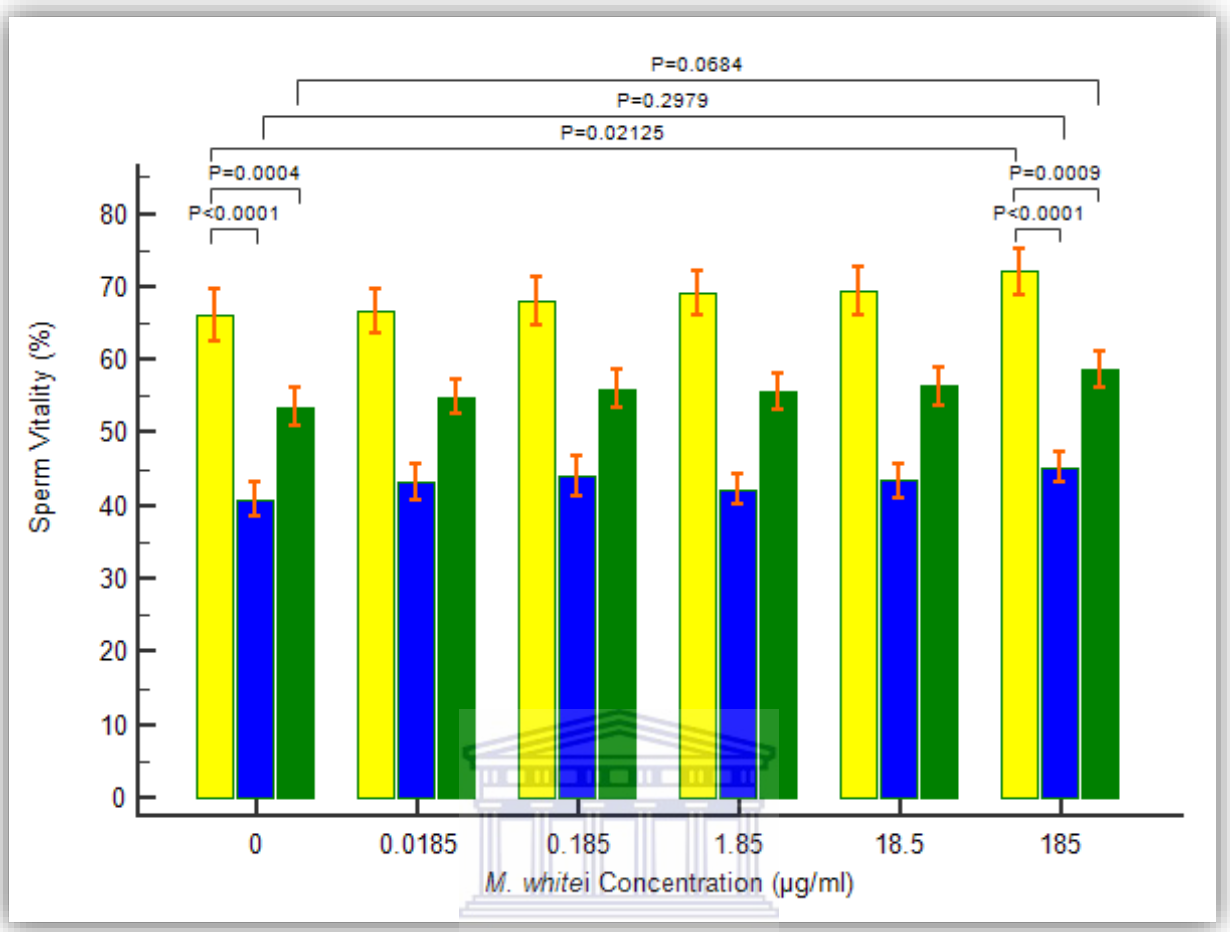

Figure 32: Effect of different concentrations of *M. whitei* (0.0185, 0.185, 1.85, 18.5 and 185 µg/ml) on vitality of washed spermatozoa *in vitro*. Yellow represents: healthy donors (n=28); Blue: patients (n=32) and Green: combined group of healthy donors and patients (n=60). Values shown as mean±SEM. No significant difference: one-way ANOVA (donors; P=0.797, patients P=0.914, donors & patients; P=0.853) nor trend can be seen in patients and combined group: repeated measure ANOVA (patients: P=0.3188, combined: P=0.1868), however, a significant positive trend is seen in the donors group (P=0.0401).

3.1.6 Effect of *M. whitei* aqueous root extract in sperm DNA fragmentation

In the patient group, a dose-dependent declining (repeated measure ANOVA P=0.004) trend for the percentage of spermatozoa with DNA fragmentation was observed at higher concentrations. The direct comparison showed a significantly (P=0.0245) lower value at 18.5 µg/ml, along with a further marked, significantly (P=0.0112) reduced value at the highest concentration of 185 µg/ml as compared to the control (Figure 34). On the other hand, the donor group showed no change (P=0.6858) when the control was compared to the highest concentration (185 µg/ml). Furthermore, one-way ANOVA revealed no significant

change in all groups [one-way ANOVA: donor P=0.764, patient P=0.058 and combined (patients and donors) P=0.223].

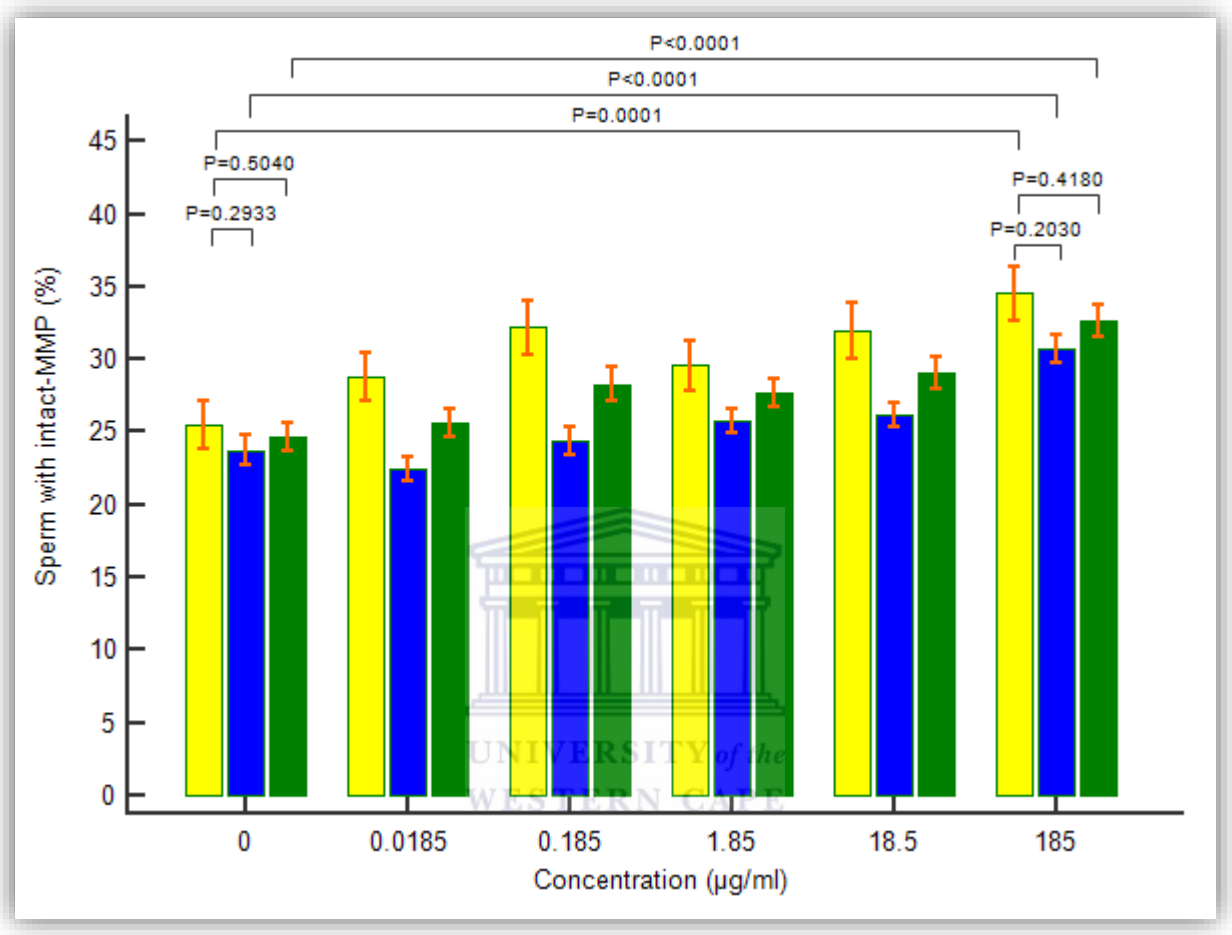

Figure 33: Effect of different concentrations of *M. whitei* (0.0185, 0.185, 1.85, 18.5 and 185 µg/ml) on the percentage of sperm with intact mitochondrial membrane potential *in vitro*. Yellow represents: healthy donors (n=28); Blue: patients (n=32) and Green: combined group of healthy donors and patients (n=60). Values shown as mean±SEM. A highly significant dose-dependent percentage increase in MMP positive spermatozoa is seen across all groups: One-way ANOVA (donors; P=0.0007, patients; P<0.0001, combined; P<0.0001). Similarly, a highly significant positive trend of MMP positive spermatozoa percentage is seen across all groups: repeated measure ANOVA (donor: P=0.0001, patient: P<0.0001, combined: P<0.0001).

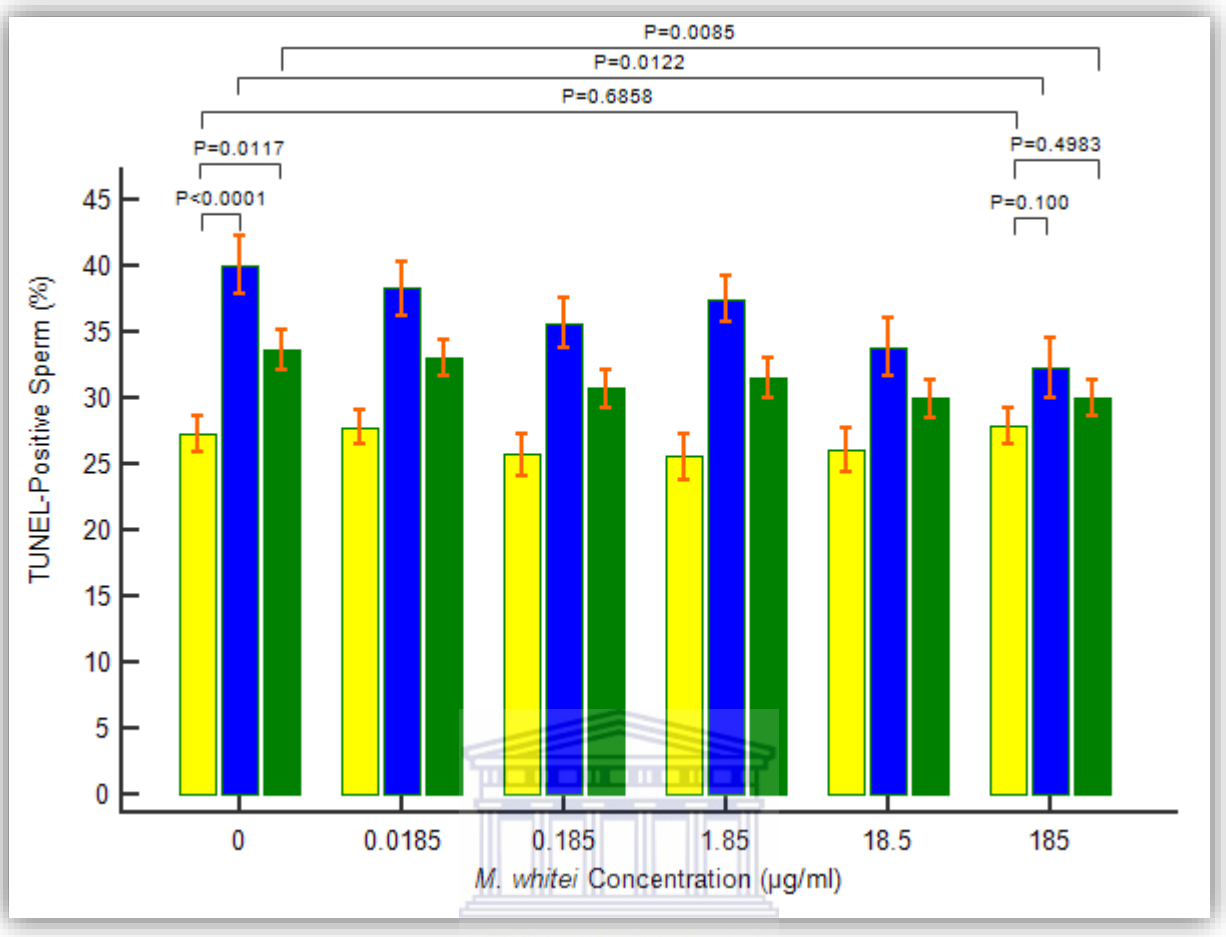

Figure 34: Effect of different concentrations of *M. whitei* (0.0185, 0.185, 1.85, 18.5 and 185 µg/ml) on DNA fragmentation *in vitro*. Yellow represents: healthy donors (n=28); Blue: patients (n=32) and Green: combined group of healthy donors and patients (n=60).Values shown as mean±SEM. Sperm showing a significant (P=0.0112) decrease in percentage of DNA fragmented sperm in the patient group, as well as the combined group (P=0.0085) when the control is compared to the highest (185 µg/ml) concentrations. No significant difference (one-way ANOVA: donors P=0.0764, patients P=0.058, combined P=0.223) nor trend (repeated measure ANOVA: donors P=0.661) is seen on the mentioned group. However a significant trend is seen on the patient and combined group (repeated measure ANOVA: patients P=0.004 and combined P=0.019).

3.1.7 Effect of *M. whitei* aqueous root extract in reactive oxygen species

Incubation with increasing concentration of *M. whitei* root extract showed a significant trend (ANOVA trend analysis: patients: P=0.0005, combined: P=0.0005) towards increase percentage of ROS-positive spermatozoa (Figure 35). In fact, concentrations as low as 0.185 µg/ml showed a significant (P=0.0076) increase in the percentage of ROS-positive spermatozoa in comparison to the control in the patient group. However, no change, nor trend was observed in the donor group (one-way ANOVA: P=0.1782 and

repeated measures ANOVA: P=0.661), even though there was a significant difference between the control and the highest concentration (P = 0.0009).

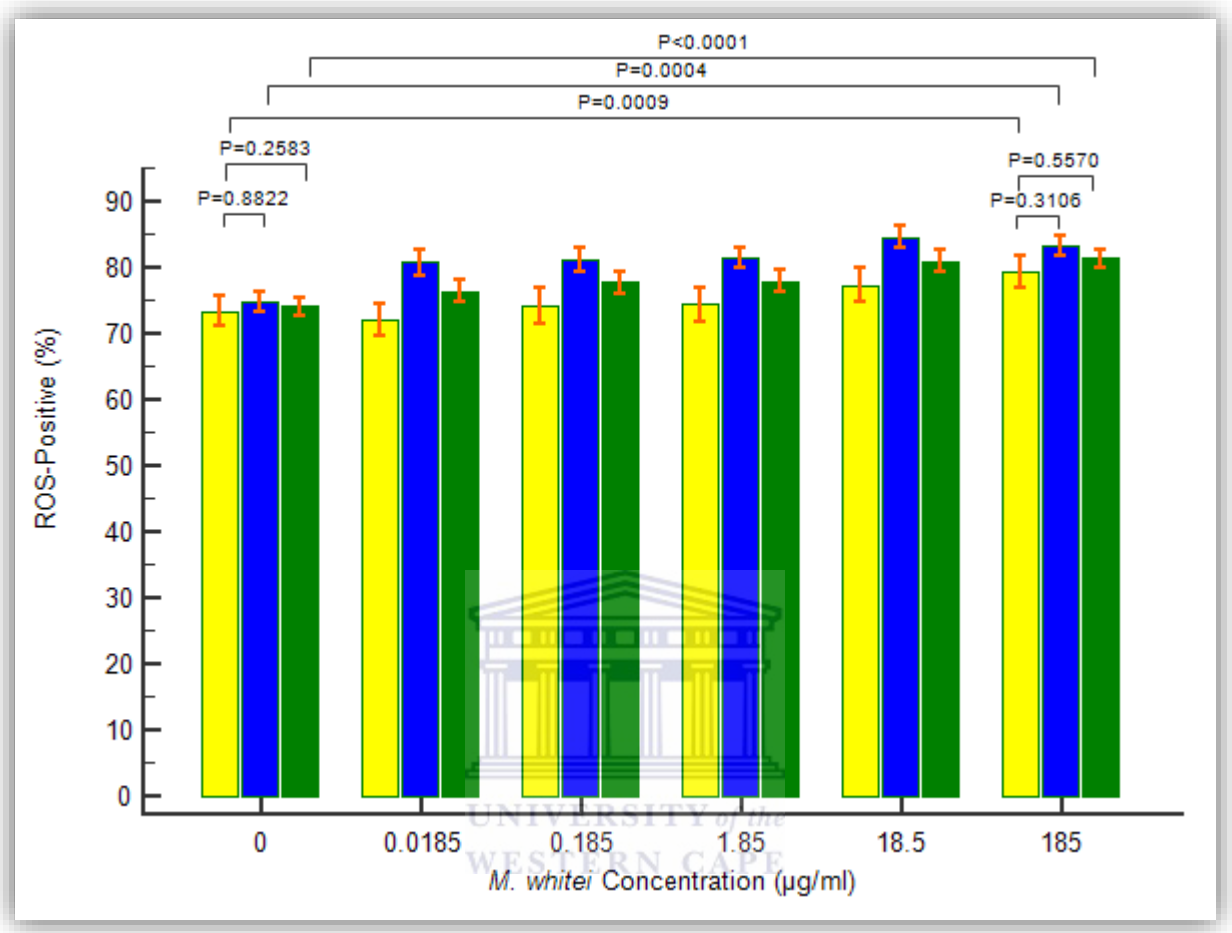

Figure 35: Effect of different concentrations of *M. whitei* (0.0185, 0.185, 1.85, 18.5 and 185 µg/ml) on the percentage ROS-positive washed spermatozoa *in vitro*. Yellow represents: healthy donors (n=28); Blue: patients (n=32) and Green: combined group of healthy donors and patients (n=60). Values shown as mean±SEM. A significant trend (repeated measure ANOVA: patients: P=0.0005, combined: P=0.0005) towards increases percentage of ROS-positive spermatozoa is seen in both groups. Essentially, an increase in the percentage ROS-positive spermatozoa is seen: (donors P=0.0009, patient P=0.0004, combined P<0.0001) when the control of each group is compared to the respective highest concentration (185 µg/ml).

3.1.8 Effect of aqueous extract of *M. whitei* at different concentration on capacitation and acrosome reaction of human sperm samples.

The chlortetracycline fluorescence (CTC) assay (Table 4) shows no change in the percentage of capacitated and acrosome-reacted sperm across all groups (donors, patients, donors and patients).

As expected, the values for capacitated spermatozoa were significantly higher in the donor group than the patient and the combined (patient and donor) group (Figure 36). Further analysis using one-way ANOVA showed no effect across all groups: (one-way ANOVA: donors P= 0.432, patients P=0.452 and combined [donors and patients] P=0.832). The same applied to repeated measure ANOVA whereby no trend was seen across all groups: (repeated measure ANOVA: donors P=0.370, patients P=0.084 and combined P=0.204).

The CTC assay revealed no changes in the percentage of acrosome-reacted sperm (Figure 37) in all groups: (one-way ANOVA: donors P=0.9578, patients P=0.9068, combined [donors and patients] P=0.9384), similarly no trend was seen in all the groups: repeated measure ANOVA (donors P=0.735, patients P=0.917 and combined P=0.822).

Table 4: Summary statistics of the percentage of capacitated and capacitated acrosome-reacted spermatozoa as detected by chlortetracycline fluorescence (CTC) assay after 1 hour incubation with increasing concentrations of *M. whitei* root extract.

Concentration (µg/ml)	**Capacitated (%)**			**Capacitated acrosome reacted sperm (%)**		
	Donors	**Patients**	**Donors + patients**	**Donors**	**Patients**	**Donors + patients**
Control	76.77±8.87	41.83±15.69	59.30±21.68	24.50±8.39	12.43±7.14	18.47±9.8
0.0185	76.50±10.43	45.80±15.01	61.15±20.10	27.03±9.56	13.70±8.87	20.37±11.35
0.185	73.83±10.19	48.30±16.06	61.06±18.54	23.50±7.74	13.63±6.60	18.57±8.68
1.85	75.97±10.98	49.46±16.97	62.72±19.48	23.30±9.19	13.58±7.40	18.43±9.62
18.5	76.37±10.97	47.50±17.09	61.93±20.26	24.93±7.76	12.77±5.80	18.85±9.16
185	78.80±11.90	49.93±19.7	64.37±21.73	24.23±7.39	12.83±7.27	18.53±9.26
One-way ANOVA P-value	0.641	0.452	0.832	0.568	0.974	0.877

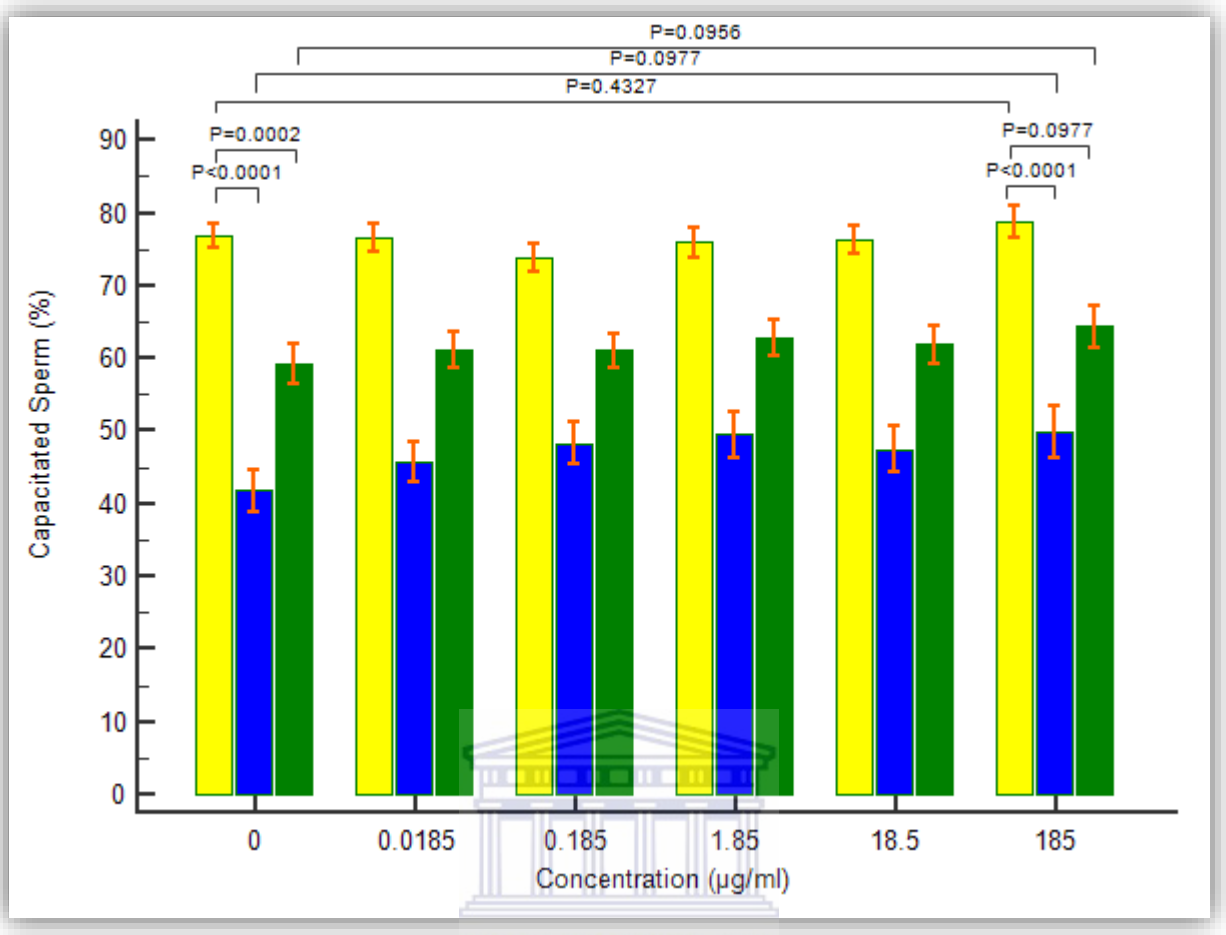

Figure 36: Effect of different concentrations of *M. whitei* (0.0185, 0.185, 1.85, 18.5 and 185 µg/ml) on the percentage of capacitated spermatozoa *in vitro*. Yellow represents: healthy donors (n=28); Blue: patients (n=32) and Green: combined group of healthy donors and patients (n=60). Values shown as mean±SEM. No significant difference nor trend is seen in all groups: one-way ANOVA (donors; P= 0.432, patients; P=0.452 and combined [donors and patients] P=0.832) and repeated measure ANOVA (donors; P=0.370, patients; P=0.084 and combined P=0.204).

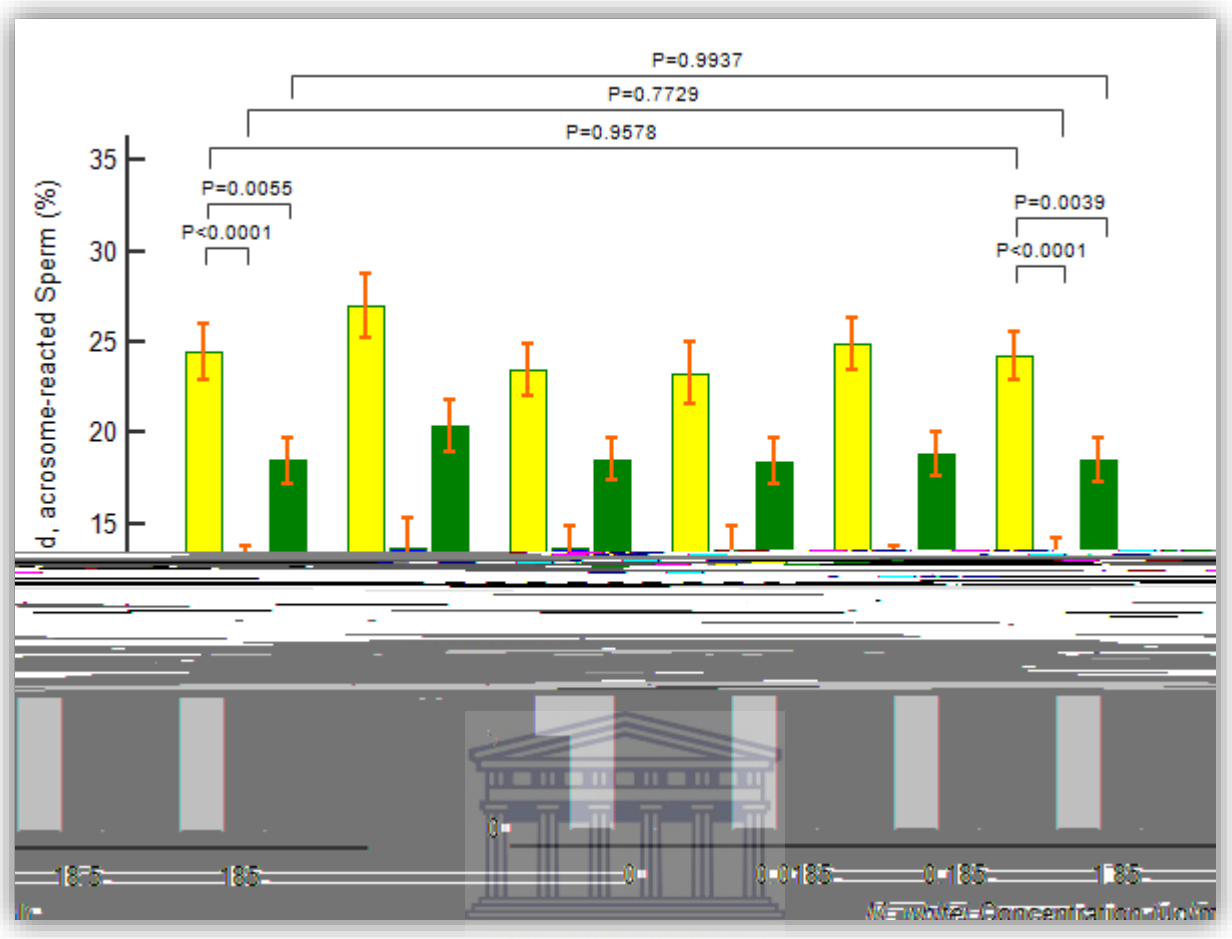

Figure 37: Effect of different concentrations of *M. whitei* (0.0185, 0.185, 1.85, 18.5 and 185 µg/ml) on the percentage of capacitated, acrosome-reacted spermatozoa *in vitro*. Yellow represents: healthy donors (n=28); Blue: patients (n=32) and Green: combined group of healthy donors and patients (n=60). Values shown as mean±SEM. No significant change nor trend can be seen: one-way ANOVA: (donors P=0.9578, patients P=0.9068, combined P=0.9384) and repeated measure ANOVA: (donors P=0.735, patients P=0.917 and combined P=0.822).

3.1.9 Correlations of various sperm parameters within the donor, patient and combined (donor and patient) groups

As illustrated in Table 5, a strong positive relationship of the percentage of total motile spermatozoa with progressively motile spermatozoa in the donors group (r=0.6760; P<0.0001), patients group (r=0.7715; P<0.0001) and the combined (donors and patients) group (r=0.8049; P<0.0001) were observed. Similarly, hyperactivated spermatozoa from the donors group (r=0.4607; P<0.0001), patients group (r=0.6268; P<0.0001) and combined (donors and patients) group (r=0.6407; P<0.0001) were significantly, positively

correlated with total motility. Moreover, the percentages of capacitated, acrosome-reacted sperm from the patients group (r=0.3312; P<0.0001) and percentages with intact-MMP (r=0.2425; P=0.0009) also portrayed a significant, weak positive correlation; however, the donors and the combined group showed no significant relationship.

Table 5: Correlation of total motility with sperm functional parameters.

PARAMETER	**Donors**		**Patients**		**Patients + Donors**	
	r	P	r	P	r	P
Intact-MMP (%)	-0.0726	< 0.3327	0.2452	0.0009	0.1927	0.0002
Hyperactivation (%)	0.4606	< 0.0001	0.6268	< 0.0001	0.6407	< 0.0001
Progressive-motility (%)	0.6760	< 0.0001	0.7715	< 0.0001	0.8049	< 0.0001
Capacitated, acrosome-reacted (%)	0.1095	0.1435	-0.1308	0.0800	0.3317	< 0.0001

Table 6 portrays the correlations between the percentage of ROS-positive spermoatozoa and the following sperm functional parameters: Intact mitochondrial membrane potential (MMP), DNA-fragmentation (TUNEL) and capacitated acrosome-reacted spermatozoa. Interestingly, the percentage of DNA-fragmented spermatozoa (r=-0.3096; P<0.0001) in the patient group was significantly, negatively correlated with the percentage of ROS-positive spermatozoa as opposed to the donor group (r=0.2023; P=0.0063). In the combined group, no (r=0.0641; P=0.2529) correlation was seen. Regardless of there being no significant correlation between the percentage of ROS-positive and the percentage of sperm with intact-MMP in the patient group (r=0.7886; P=0.2926), there was a significant weak negative correlation with the combined (donors and patients) group (r=-0.1622; P=0.0020) as well as, a weak significant positive correlation on the donors group (r=0.1696; P=0.0023). The percentage of ROS-positive spermatozoa did not relate to the percentage of capacitated acrosome-reacted spermatozoa in all groups: donor (r=0.0285; P=0.7092), patients (r=0.0787; P=0.2975) and donors and patients (r= - 0.0831; P=0.1152).

Table 6: Correlation of the percentage of ROS-positive sperm with sperm functional parameters

PARAMETERS	Donors		Patients		Patients + Donors	
	r	P	r	P	r	P
Intact-MMP (%)	0.1696	0.0023	0.7886	0.2926	-0.1622	0.0020
TUNEL (%)	0.2023	0.0063	-0.3096	< 0.0001	0.0641	0.2529
Capacitated, acrosome-reacted (%)	0.0285	0.7092	0.0787	0.2975	- 0.0831	0.1152

The relationships between the percentage of hyperactivated, ROS-positive and capacitated acrosome-reacted spermatozoa as shown in Table 7 revealed no significant relationship in the donor group and the combined group for the percentage of ROS-positive spermatozoa and capacitated, acrosome-reacted spermatozoa. However, the patients group showed a significant positive correlation (r=0.2664, P<0.0001) for the percentage of capacitated, acrosome-reacted sperm. Same tendency was reflected for the percentage of ROS-positive, only the patients group had a weak correlation (r=0.152, P=0.0421) with hyperactivated spermatozoa.

Table 7: Correlation of the percentage of hyperactivated sperm with sperm functional parameters

PARAMETERS	Donors		Patients		Patients + Donors	
	r	P	r	P	r	P
ROS-positive sperm (%)	0.105	0.1602	0.152	0.0421	-0.002	0.9667
Capacitated, acrosome-reacted (%)	- 0.0952	0.2026	0.2664	< 0.0001	- 0.0543	0.4730

3.2.1 Effect of aqueous root extract of *M. whitei* on total and progressive motility in oligozoospermic and normozoospermic sperm samples

Exposure of ejaculated washed spermatozoa to the root extract of *M. whitei* caused a highly significant (P=0.0013), dose-dependent increase in total motility in the oligozoospermic (one-way ANOVA: P=0.012) group (Figure 38). On the other hand, a significant (P=0.0064) increase in total motility was also observed when the control was compared to the highest concentration (185 µg/ml) but no significant effect (one-way ANOVA P=0.144) was seen in the normozoospermic group. In addition, repeated measure ANOVA showed a trend in both groups: repeated measure ANOVA (oligozoospermia P=0.002; normozoospermia P=0.0111) with a higher increase in the oligozoospermic group. Clearly, the difference between both groups at the highest concentration used (185µg/ml) was insignificant (p=0.2364) whereas, the difference was significant (P=0.0023) in the control.

After 1 hour treatment of washed spermatozoa with increasing concentration of *M. whitei*, no change nor trend was observed for progressive motility in both groups: One-way ANOVA (oligozoospermia P=0.3178; normozoospermia P=0.869) and repeated measure ANOVA (oligozoospermia P=0.055; normozoospermia P=0.3618) (Figure 39).

3.2.2 Effect of aqueous root extract of *M. whitei* on sperm kinematic parameters of oligozoospermic and normozoospermic group.

As expected, the mean values of the kinematic parameters in the normozoospermic group were higher than the olizoospermic group (Table 8). However, no change was seen in all the kinematic parameters from both groups.

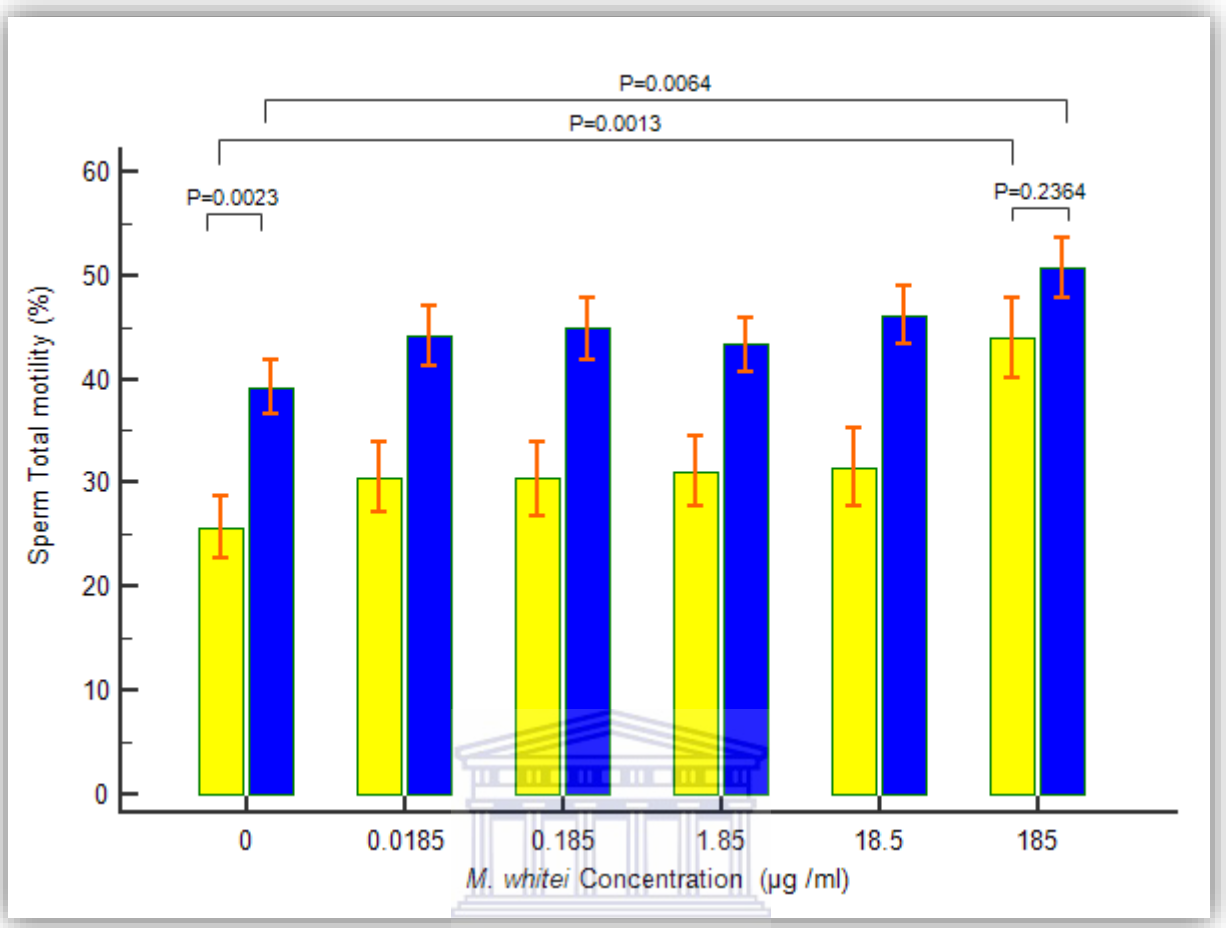

Figure 38: Effect of different concentrations of *M. whitei* (0.0185, 0.185, 1.85, 18.5 and 185 µg/ml) on the percentage of total motility of washed spermatozoa *in vitro*. Yellow represents: oligozoospermic (concentration <15 million/ml) group (n=19); Blue: normozoospermic (concentration >15 million/ml) group (n=28).Values shown as mean±SEM. A significant dose-dependent increase in sperm percentage total motility is seen in the oligozoospermia group (one-way ANOVA: P=0.012) but not on the normozoospermia group (one-way ANOVA: P=0.144). Virtually, an increase in (oligozoospermic: P=0.0013 and normozoospermic: P= 0.0064) is seen at highest concentration (185 µg/ml) when compared to the control in both groups. Repeated measure ANOVA is significant (oligozoospermic P=0.002, normozoospermic P=0.0111).

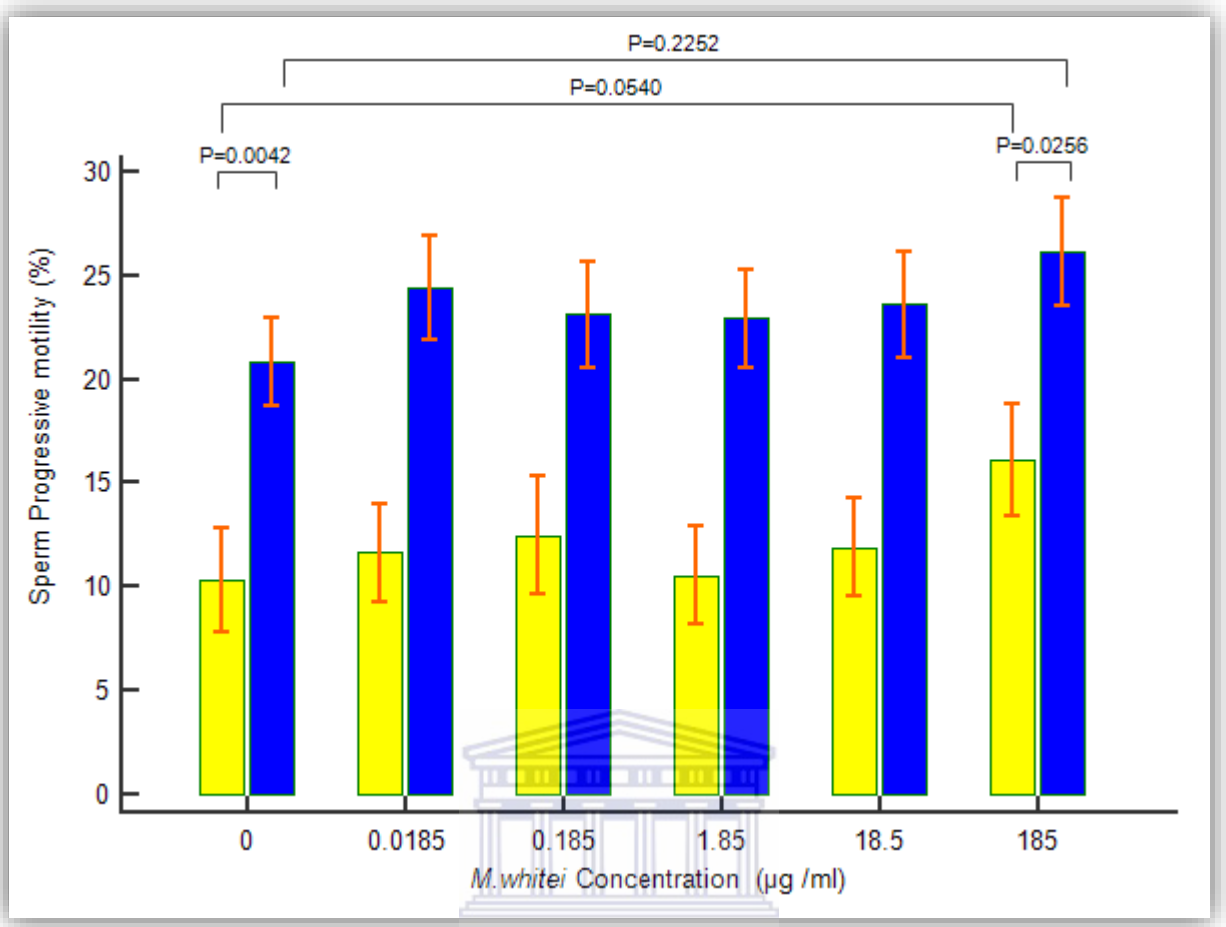

Figure 39: Effect of different concentrations of *M. whitei* (0.0185, 0.185, 1.85, 18.5 and 185µg/ml) on the percentage of progressive motility of washed spermatozoa *in vitro*. Yellow represents: oligozoospermic (concentration <15 million/ml) group (n=19); Blue: normozoospermic (concentration >15 million/ml) group (n=28). Values shown as mean±SEM. One-way ANOVA is not significant (oligozoospermic P=0.3178, normozoospermic P=0.869), as well as, repeated measure ANOVA (oligozoospermic P=0.055; normozoospermic P=0.361) in both group.

Table 8: Summary statistics of sperm kinematic motility parameters, following incubation with increasing concentrations of *M. whitei* root extract for 1 hour.

Kinematic Parameters	Group	Control	0.0185 µg /ml	0.185 µg /ml	1.85 µg /ml	18.5 µg /ml	185 µg /ml
VAP (µm s^{-1})	**Oligozoospermic**	22.81±7.34	31.0±6.55	27.34±5.41	25.14±9.25	30.04±8.56	29.95±7.54
	Normozoospermic	36.57±10.79	38.47±11.16	37.59±11.34	37.44±10.28	37.06±11.23	36.16±9.11
VCL (µm s^{-1})	**Oligozoospermic**	53.9±9.88	58.5±8.36	43.37±17.39	51.28±13.38	57.3±10.48	59.55±8.87
	Normozoospermic	66.09±15.96	71.33±13.11	70.08±15.10	70.25±13.34	70.26±14.79	69.87±12.05
VSL (µm s^{-1})	**Oligozoospermic**	18.03±6.79	22.03±7.54	19.89±20.7	17.18±8.23	22.16±8.87	21.88±8.36
	Normozoospermic	27.24±10.95	30.38±12.65	29.26±14.30	28.30±10.84	28.3±12.30	28.20±12.87
ALH (µm)	**Oligozoospermic**	1.69±0.69	2.06±0.67	1.89±0.72	1.88±0.73	1.99±0.33	2.01±0.54
	Normozoospermic	2.26±0.46	2.3±0.29	2.40±0.32	2.27±0.37	2.40±0.39	2.17±0.43
Hyperactivation (%)	**Oligozoospermic**	1.44±1.14	1.79±2.06	1.97±2.39	1.97±2.39	1.4±1.63	2.33±2.1
	Normozoospermic	4.30±2.92	4.7±2.98	4.1±3.26	4.2±2.57	4.4±3.3	4.7±3.50
LIN (%)	**Oligozoospermic**	32.59±8.37	36.08±10.38	33.95±8.86	40.2±12.3	40.2±12.3	36.02±12.63
	Normozoospermic	38.18±11.35	37.6±10.6	36.1±12.0	36.2±11.10	365.6±11.57	35.5±10.3
STR (%)	**Oligozoospermic**	66.52±8.24	67.72±10.82	69.92±12.33	66.86±9.19	47.36±8.05	68.4±12.71
	Normozoospermic	69.20±13.40	70.10±12.4	67.3±13.1	68.01±13.4	67.3±13.0	66.5±12.70
WOB (%)	**Oligozoospermic**	48.80±6.64	50.18±6.8	49.87±5.81	47.15±8.42	53.96±8.42	51.22±9.77
	Normozoospermic	51.70±9.80	51.22±10.00	50.48±10.52	50.80±9.40	50.10±10.90	50.1±9.50
BCF (HZ)	**Oligozoospermic**	10.16±5.25	12.12±5.28	11.46±4.37	9.57±4.42	12.01±4.34	11.68±4.10
	Normozoospermic	13.70±02.80	13.70±2.40	13.40±2.70	14.10±3.10	13.60±09.00	13.40±2.60

Data comprises of oligozoospermic (concentration<15 million/ml) group (n=19) and normozoospermic (concentration>15 million/ml) group (n=28) and is expressed as mean±SD. VAP: velocity average path; VCL: velocity curvilinear; VSL: velocity straight line; ALH: average lateral head displacement; hyperactivation; BCF: beat across frequency; LIN: linearity; STR: straightness; WOB: wobble; SD: standard deviation.

Oligozoospermic ANOVA P-value (VAP: P=0.053; VCL: P= 0.058; VSL: P=0.181; ALH: P=0.585; hyperactivation: P=0.763; BCF: P=0.320; LIN: P=0.297; STR: P=0.085; WOB: P=0.148)

Normozoospermic ANOVA P-value (VAP: P=0.941; VCL: P=0.850; VSL: P=0.954; ALH: P=0.662; hyperactivation: P=0.441; BCF: P=0.937; LIN: P=0.928; STR: P=0.921; WOB=0.990)

Different concentrations of *M. whitei* did not cause any effect on velocity average path (VAP) for spermatozoa in both groups: (repeated measure ANOVA: oligozoospermic; P=0.289 and normozoospermic; P=0.972) (Figure 40); with one-way ANOVA P=0.053 in the oligozoospermic group and P=0.975 in the normozoospermic group. However, the velocity curvilinear (VCL) of spermatozoa significantly (P=0.0008) increased from the control to concentration at 185 µg/ml on the oligozoospermic group (Figure 41). Yet, further analysis of VCL using one-way ANOVA and repeated measure ANOVA showed no changes nor a trend in both groups: one-way ANOVA (oligozoospermia P=0.058 and normozoospermia P=0.869) and repeated measure ANOVA (oligozoospermia P=0.412 and normozoospermia P=0.394).

3.2.3. Effect of aqueous root extract of *M. whitei* on sperm vitality and mitochondrial membrane potential

As expected, the sperm vitality values in normozoospermic group were significantly higher than those in the oligozoospermic group (Figure 42), with no dose-dependent effect in both groups: repeated measure ANOVA (oligozoospermia P=0.339, normozoospermia P=0.151), as well as, one-way ANOVA (oligozoospermia P=0.937, normozoospermia P=0.797).

Treatment of washed spermatozoa with increasing concentration of *M. whitei* root extract resulted in a dose-dependent increase in the percentage of spermatozoa with intact mitochondrial membrane potential in both groups: One-way ANOVA (oligozoospermia P=0.0001 and normozoospermia P=0.0007), as well as repeated measure ANOVA for a trend analysis (oligozoospermia P=0.0007 and normozoospermia P=0.0001) (Figure 43). In fact, a highly significant increase was seen at 185 µg/ml (oligozoospermia P=0.0027 and normozoospermia P=0.0001) when compared to the controls in both groups.

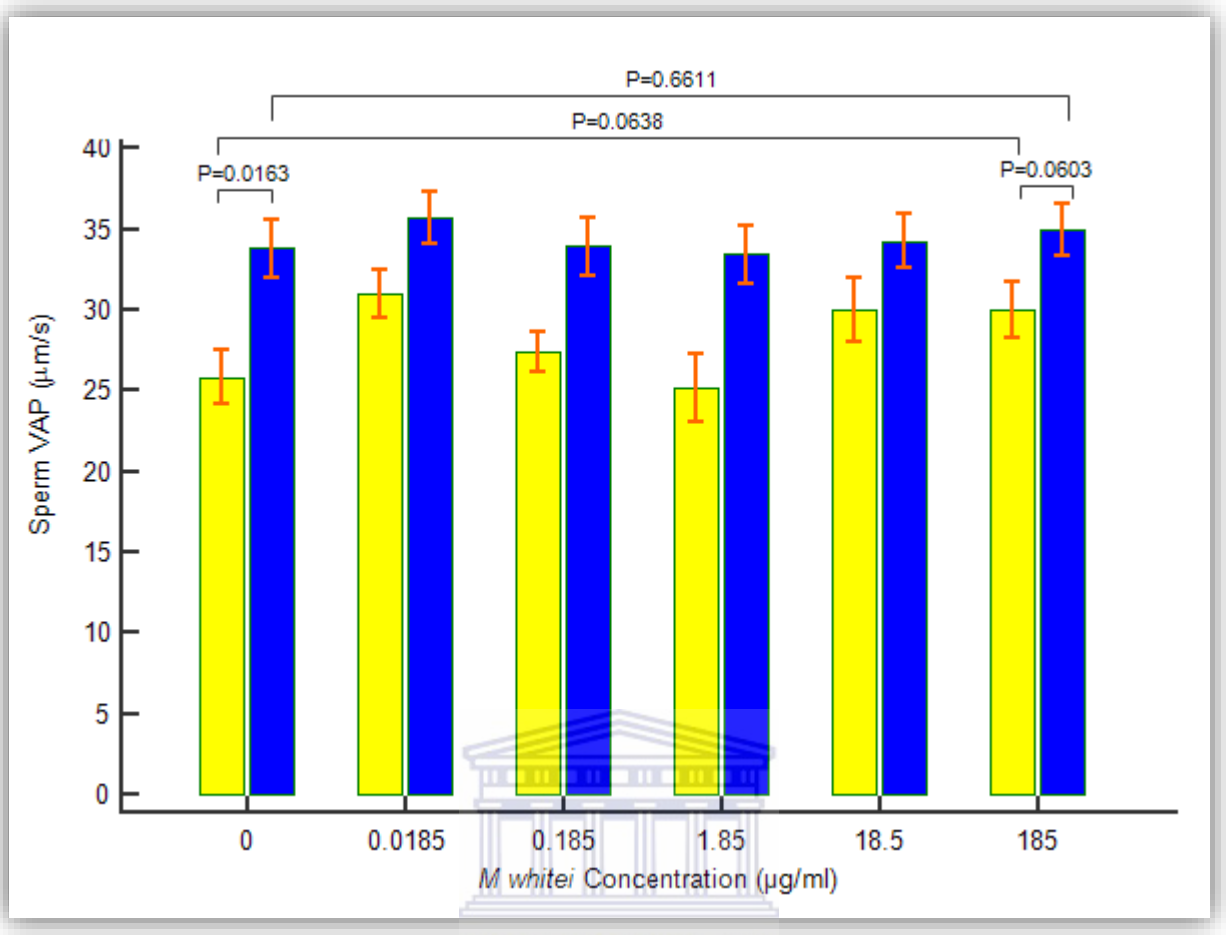

Figure 40: Effect of different concentrations of *M. whitei* (0.0185, 0.185, 1.85, 18.5 and 185 µg/ml) on velocity average path (VAP) of washed spermatozoa *in vitro*. Yellow represents: oligozoospermic (concentration <15 million/ml) group (n = 19); Blue: normozoospermic (concentration >15 million/ml) group (n = 28). Values shown as mean±SEM. No effect nor trend is seen in both groups: one-way ANOVA (oligozoospermia P=0.053 and normozoospermia P=0.975) and repeated measure ANOVA (oligozoospermia P=0.289 and normozoospermia P=0.972).

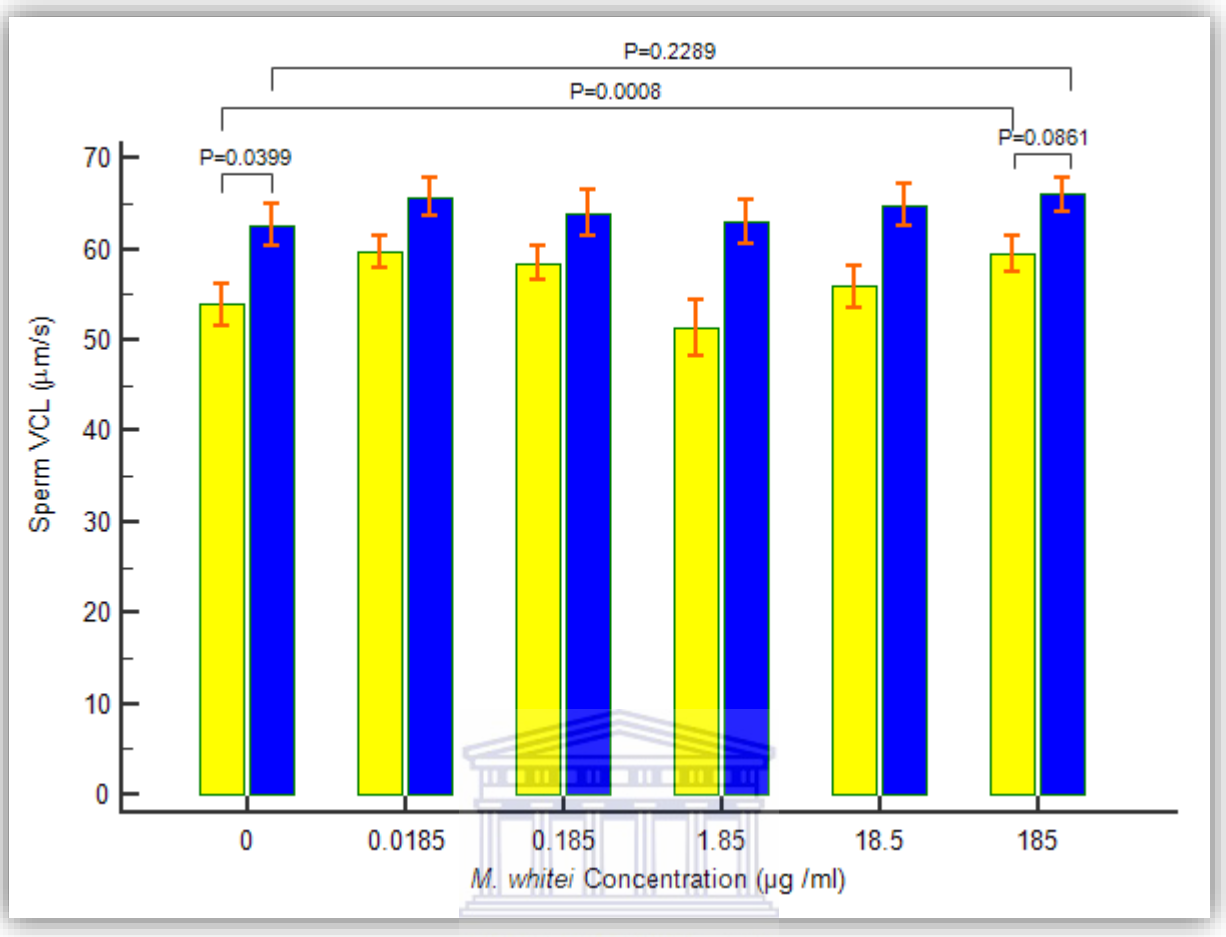

Figure 41: Effect of different concentrations of *M. whitei* (0.0185, 0.185, 1.85, 18.5 and 185 µg/ml) on velocity curvilinear (VCL) of washed spermatozoa *in vitro*. Yellow represents: oligozoospermic (concentration <15 million/ml) group (n = 19); Blue: normozoospermic (concentration >15 million/ml) group (n = 28).Values shown as mean±SEM. Significant (P=0.0008) increase in sperm VCL is seen from the control to concentrations at 185 µg/ml. One-way ANOVA and repeated measure ANOVA show no effect nor trend in both groups: one-way ANOVA (oligozoospermia P= 0.058 and normozoospermia P=0.869) and repeated measure ANOVA (oligozoospermia P=0.412 and normozoospermia P=0.349).

3.2.4 Effect of aqueous root extract of *M. whitei* on the percentage of ROS-positive spermatozoa

After incubation with different concentrations of *M. whitei* for 1 hour, repeated measure ANOVA showed a significant positive trend in the percentage of ROS-positive sperm in both groups (oligozoospermia P=0.0013 and normozoospemia P=0.0015) (Figure 44). Yet, one-way ANOVA showed no changes in both groups: (oligozoospermia: P=0.096 and normozoospermia: P=0.178). In direct comparison, the percentage of ROS-positive spermatozoa in the control was significantly lower than in those incubated with 185 µg/ml in the oligozoospermic (P=0.0172) as well as normozoospermic subjects (P=0.0009).

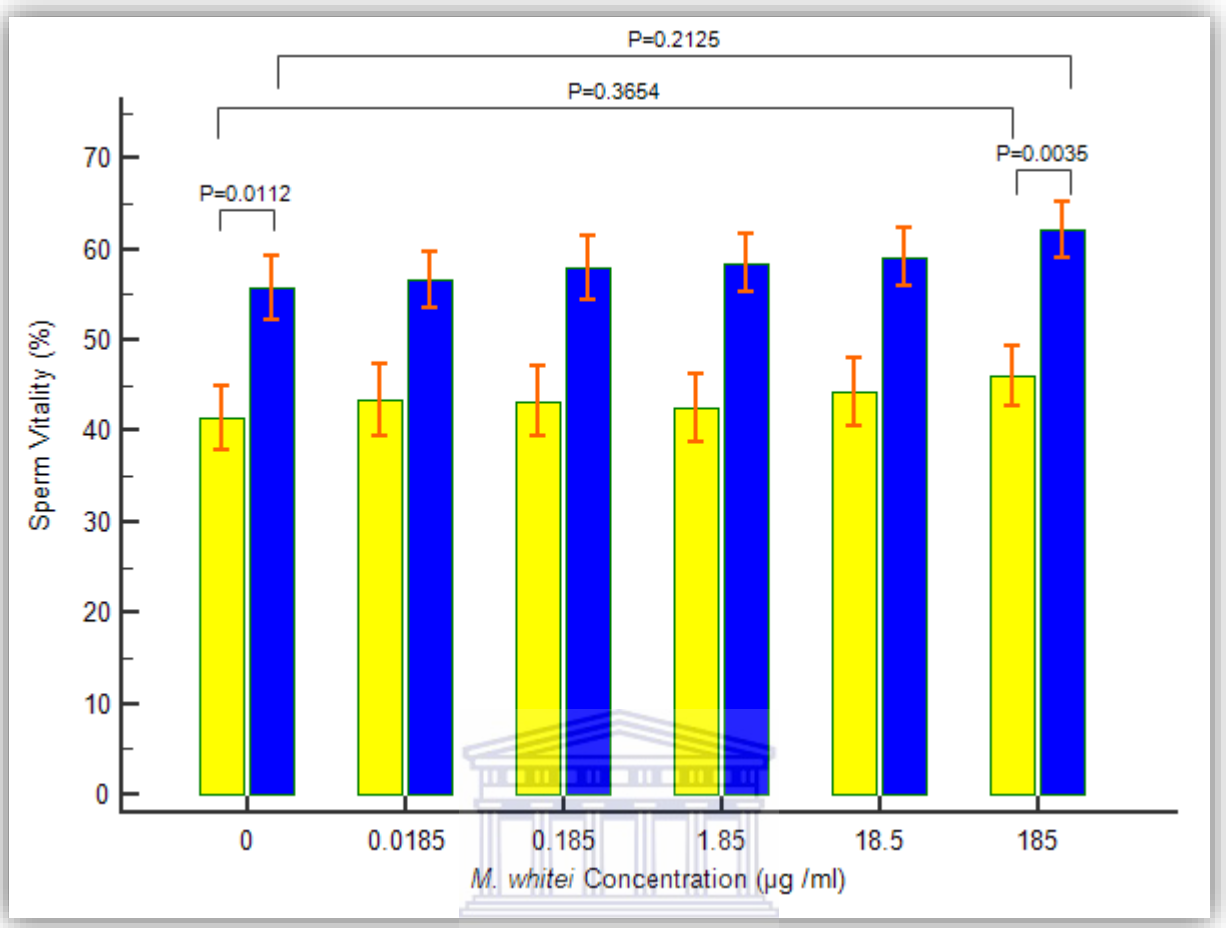

Figure 42: Effect of different concentrations of *M. whitei* (0.0185, 0.185, 1.85, 18.5 and 185µg/ml) on the percentage of vitality of washed spermatozoa *in vitro*. Yellow represents: oligozoospermic (concentration <15 million/ml) group (n=19); Blue: normozoospermic (concentration >15 million/ml) group (n=28). Values shown as mean±SEM. There is no dose-dependent effect in vitality in both groups: the repeated measure ANOVA (oligozoospermia P=0.339 and normozoospermia P=0.151) and one-way ANOVA (oligozoospermia P=0.937 and normozoospermia P=0.797).

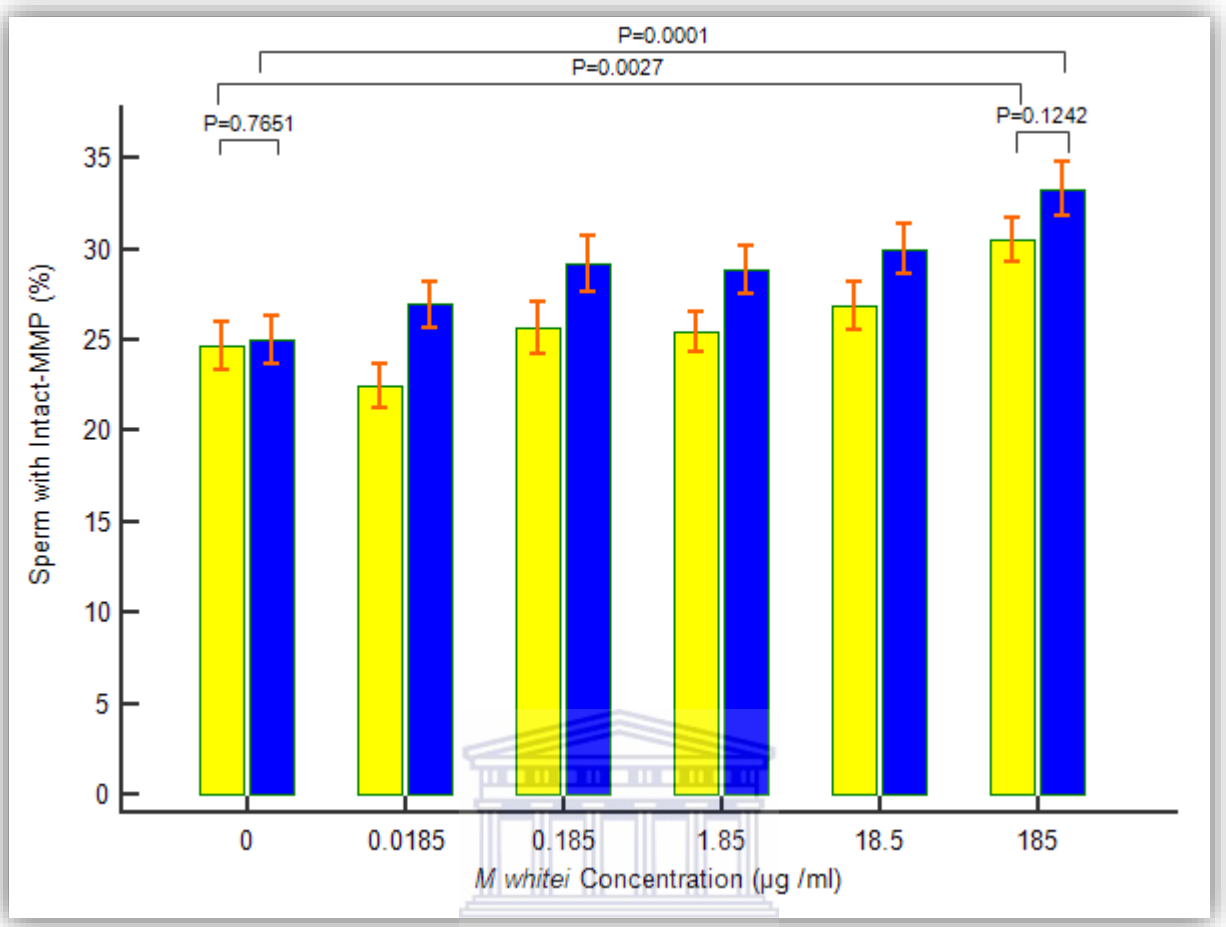

Figure 43: Effect of different concentrations of *M. whitei* (0.0185, 0.185, 1.85, 18.5 and 185 µg/ml) on the percentage of intact mitochondrial membrane potential (MMP) of washed spermatozoa *in vitro*. Yellow represents: oligozoospermic (concentration <15 million/ml) group (n = 19); Blue: normozoospermic (concentration >15 million/ml) group (n = 28). Values shown as mean±SEM. A highly significant dose-dependent increase (one-way ANOVA: oligozoospermia P=0.0001 and normozoospermia P=0.0007) and trend (repeated measure ANOVA: oligozoospermia P=0.0007 and normozoospermia P=0.0001) is seen in both groups. In fact, a highly significant increase is seen in the oligozoospermic (P=0.0027) and normozoospermic (P=0.0001) groups when their controls are compared to their highest concentrations (185 µg/ml).

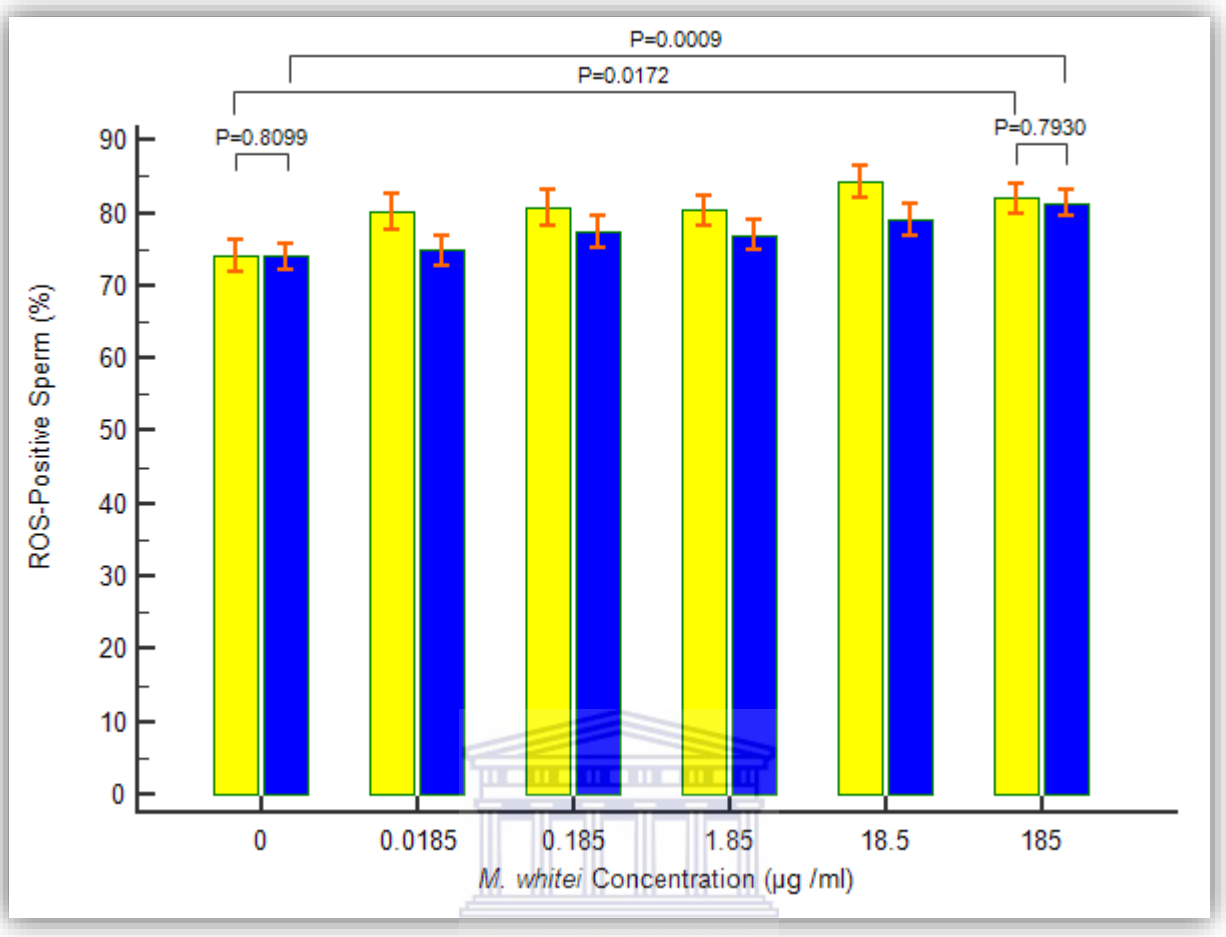

Figure 44: Effect of different concentrations of *M. whitei* (0.0185, 0.185, 1.85, 18.5 and 185 µg/ml) on the percentage of ROS positive washed spermatozoa *in vitro*. Yellow represents: oligozoospermic (concentration <15 million/ml) group (n=19); Blue: normozoospermic (concentration >15 million/ml) group (n=28). Values shown as mean±SEM. A significant trend is seen in both groups (repeated measure ANOVA: oligozoospermia P=0.0013 and normozoospermia P=0.0015). No significant change is seen (one-way ANOVA: oligozoospermia P=0.144 and normozoospermia). However, a significant increase is seen from the groups controls to concentrations at 185µg/ml (oligozoospermia P=0.0172 and normozoospermia P=0.0009).

3.2.5 Effect of aqueous root extract of *M. whitei* in DNA fragmentation

One-way ANOVA did not show any effect of *M. whitei* on DNA-fragmentation of washed spermatozoa from both groups (oligozoospermia P=0.144 and normozoospermia P=0.764) (Figure 45). However, the oligozoospermic group showed a significant negative trend (repeated measure ANOVA P=0.0048), while no trend could be established in the normozoospermic group (repeated measure ANOVA P=0.435). In direct comparison a significant (P=0.0245) decrease in the percentage of DNA-fragmented spermatozoa

from the control to the highest concentration (185 µg/ml) used was found in the oligozoospermic group. Thus, the extract only showed an effect on the sperm from the oligozoospermic group.

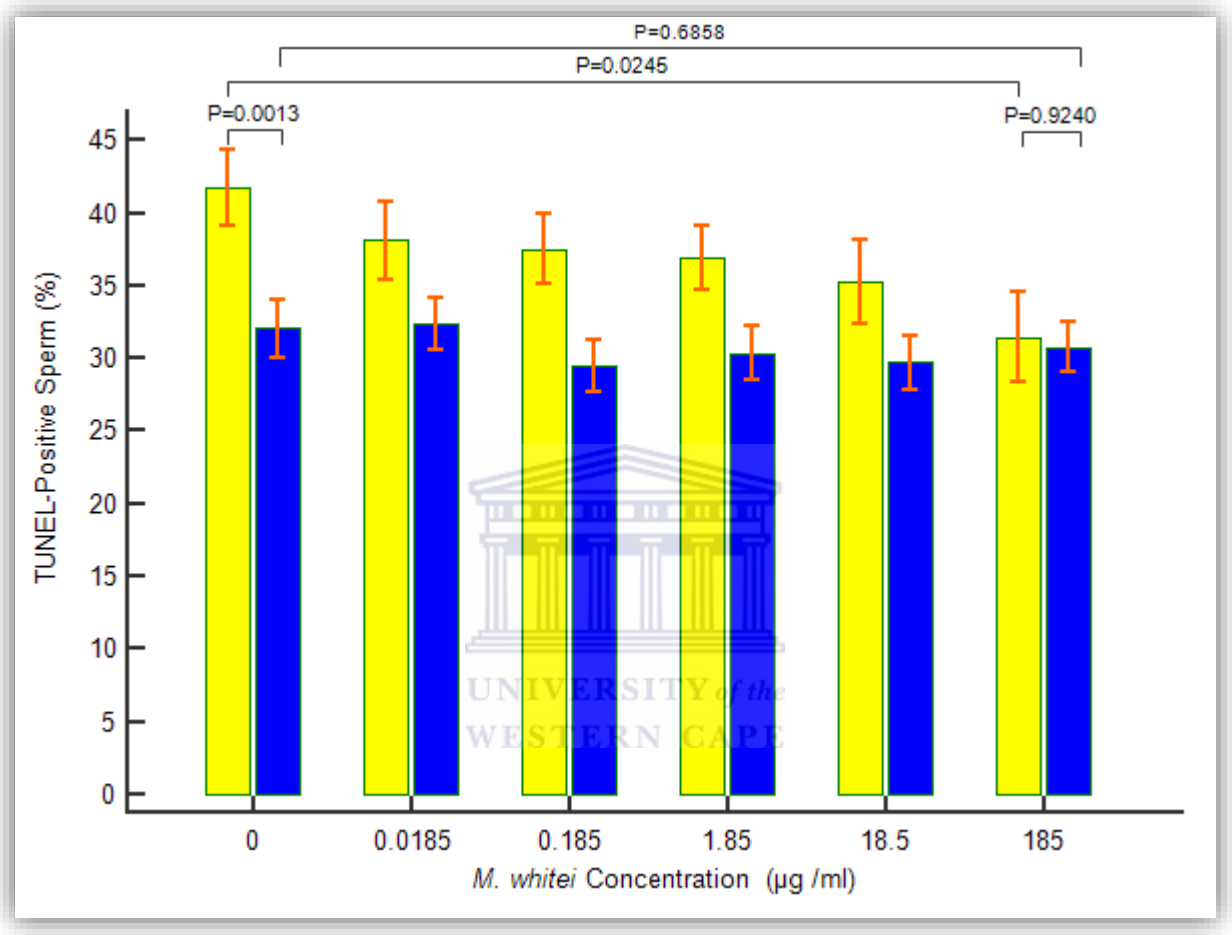

Figure 45: Effect of different concentrations of *M. whitei* (0.0185, 0.185, 1.85, 18.5 and 185 µg/ml) on the percentage of DNA fragmentation of washed spermatozoa *in vitro*. Yellow represents: oligozoospermic (concentration <15 million/ml) group (n=19); Blue: normozoospermic (concentration >15 million/ml) group (n=28). Values shown as mean±SEM. At 185 µg/ml, there is a significant (P=0.0245) decrease on the percentage of DNA-fragmented cells in comparison to the control in the oligozoospermic group. However, one-way ANOVA is not significant in both groups (oligozoospermia P=0.144 and normozoospermia P=0.764). While, the repeated measure ANOVA is significant (P=0.0048) in the oligozoospermic group but not significant (P=0.435) in the normozoospermic group.

3.2.6 Effect of aqueous root extract of *M. whitei* in sperm capacitation and acrosome reaction

Despite the fact that the values for normozoospermic subjects were obviously higher than those for oligozoospermic subjects (Figure 46 and 47), the chlortetracycline fluorescence (CTC) assay (Table 9) revealed no effect of the extract on the percentage of capacitated and acrosome-reacted sperm in both groups (oligozoospermic and normozoospermic). In addition, trend analysis using repeated measure ANOVA was not significant for both the percentage of capacitated (oligozoospermia P=0.909 and normozoospermia P=0.370) and capacitated, acrosome-reacted sperm (oligozoospermia P=0.551 and normozoospermia P=0.731) from both groups. Similarly, the one-way ANOVA revealed no effect on capacitated (oligozoospermia P=0.6804 and normozoospermia P=0.806) and capacitated acrosome-reacted spermatozoa from both groups (oligozoospermia P=0.986 and normozoospermia P=0.856).

Table 9: Summary statistics of the sperm capacitation and acrosome reaction on oligozoospermic (concentration <15 million/ml) and normozoospermic (concentration >15 million/ml) sperm samples as detected by CTC stain after 1 hour incubation with increasing concentrations of *M. whitei* root extract.

Concentration (µg/ml)	Capacitated sperm (%)		Capacitated-acrosome reacted sperm (%)	
	Oligozoospermia	Normozoospermia	Oligozoospermia	Normozoospermia
Control	46.95±17.81	63.87±21.55	16.47±9.06	20.30±10.16
0.0185	49.42±16.10	65.78±19.28	16.10±6.76	22.68±11.36
0.185	49.37±16.52	62.78±19.35	15.10±7.39	20.07±8.89
1.85	53.58±17.42	65.63±20.02	15.32±5.86	19.66±10.10
18.5	52.37±16.35	65.67±19.43	14.89±8.59	20.30±9.81
185	55.37±20.94	68.20±21.34	14.47±8.59	20.34±9.50
One-way ANOVA P-value	0.699	0.885	0.263	0.786

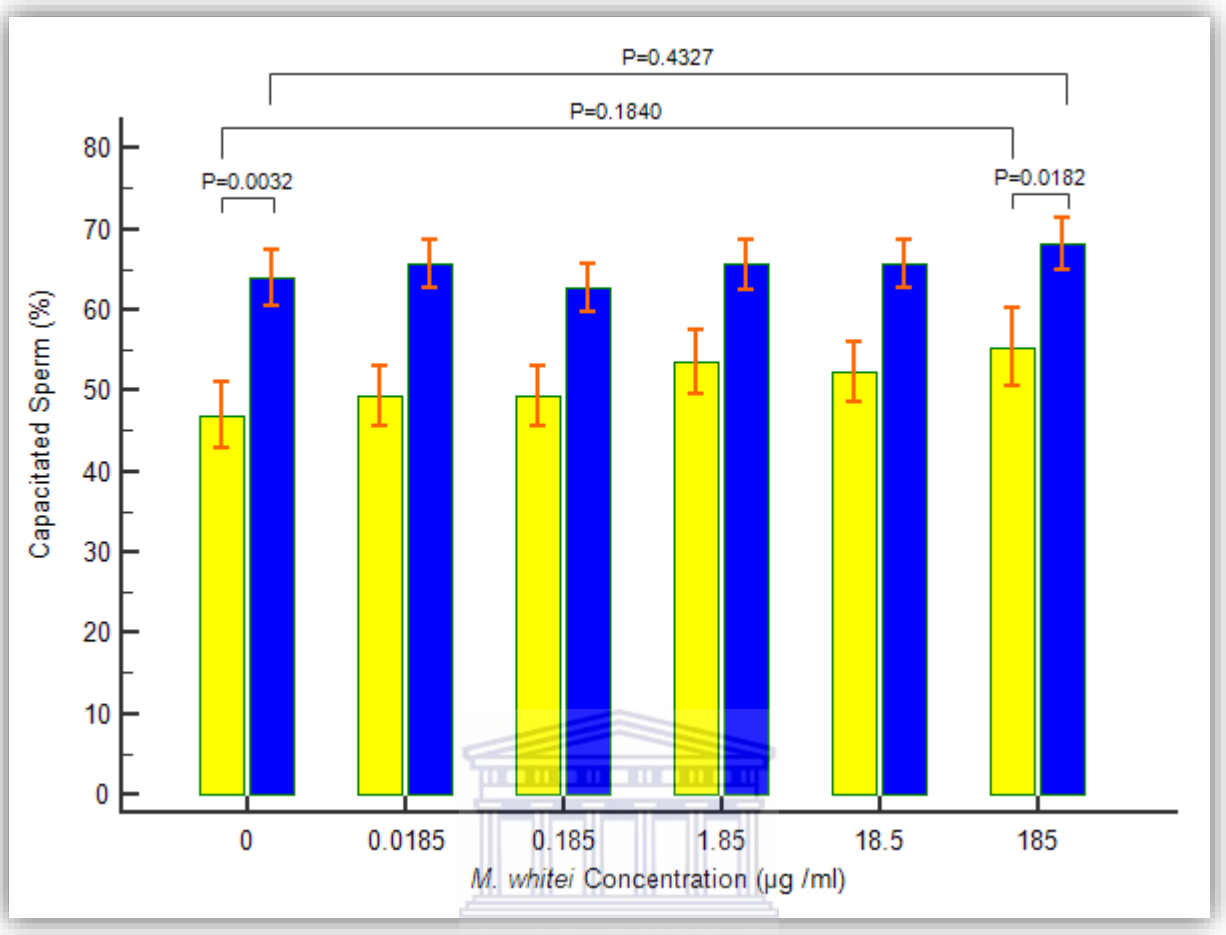

Figure 46: Effect of different concentrations of *M. whitei* (0.0185, 0.185, 1.85, 18.5 and 185 µg/ml) on the percentage of capacitated washed spermatozoa *in vitro*. Yellow represents: oligozoospermic (concentration <15 million/ml) group (n=19); Blue: normozoospermic (concentration >15 million/ml) group (n=28). Values shown as mean±SEM. There is no dose-dependent effect in the percentage of capacitated spermatozoa in both groups: repeated measure ANOVA (oligozoospermia; P=0.909 and normozoospermia; P=0.370) and one-way ANOVA (oligozoospermia; P=0.680 and normozoospermia; P=0.432).

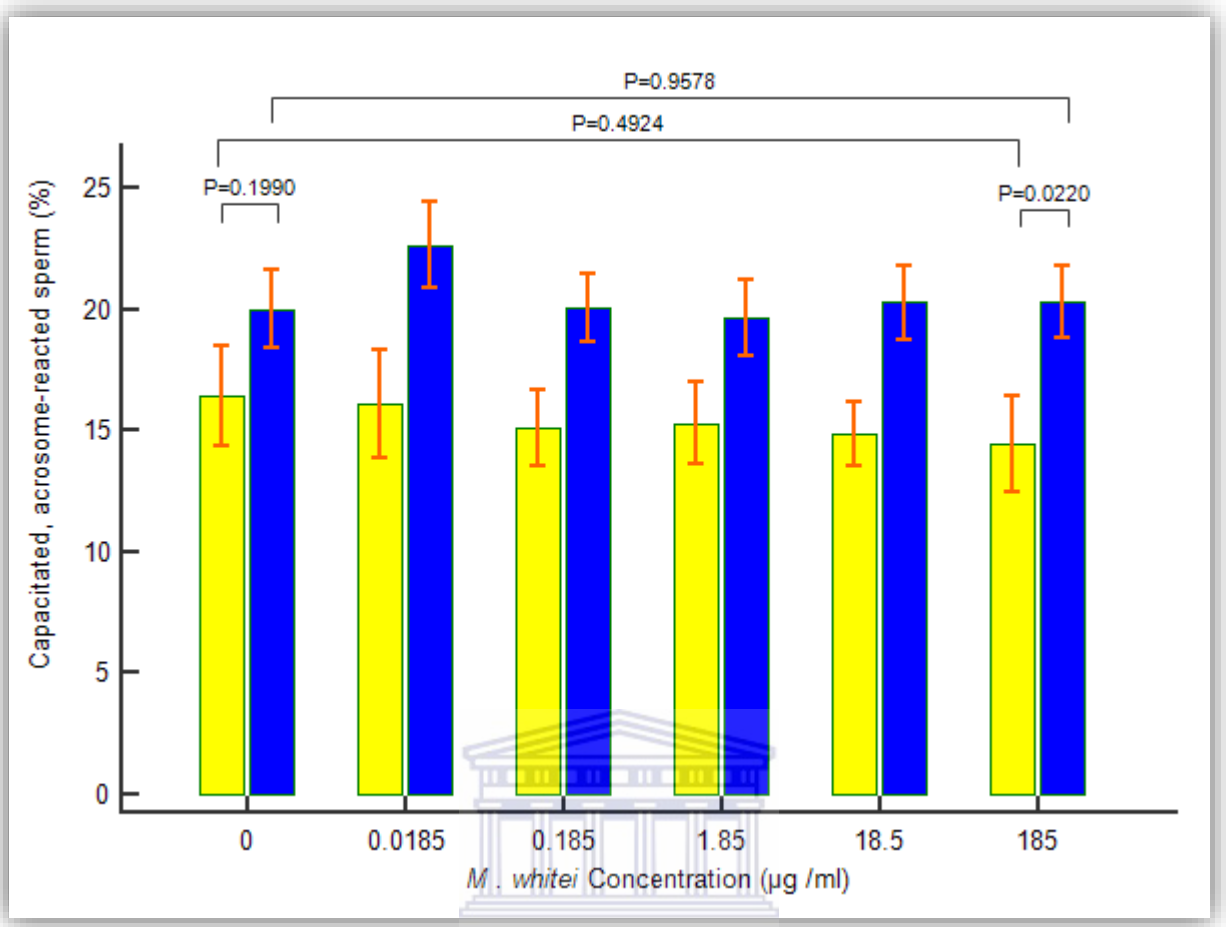

Figure 47: Effect of different concentrations of *M. whitei* (0.0185, 0.185, 1.85, 18.5 and 185 µg/ml) on the percentage of capacitated acrosome-reacted washed spermatozoa *in vitro*. Yellow represents: oligozoospermic (concentration <15 million/ml) group (n=19); Blue: normozoospermic (concentration >15 million/ml) group (n=28). Values shown as mean±SEM. There is no dose-dependent effect nor trend seen in the percentage of acrosome-reacted spermatozoa in both groups: repeated measure ANOVA (oligozoospermia P=0.551 and normozoospermia P=0.731), as well as, one-way ANOVA (oligozoospermia P=0.986 and normozoospermia P=0.856)

3.2.7 Correlations of various sperm parameters within oligozoospermic and normozoospermic groups

Correlations between the percentage of total motility and the sperm functional parameters hyperactivation, progressive motility, intact-MMP and capacitated, acrosome-reacted spermatozoa revealed that total motilility spermatozoa had a significant strong positive relationship with the percentage of hyperactivated spermatozoa in both groups: oligozoospermia (r=0.647, P<0.0001) and normozoospermia (r=0.592, P<0.0001) (Table 10). Similarly, the percentage of progressively motile spermatozoa strongly significantly

correlated with the percentage of total motile spermatozoa in both groups: oligozoospermia (r=0.768, P<0.0001) and normozoospermia (r=0.795, P<0.0001). The percentage of spermatozoa with intact-MMP showed a weak but positive and significant correlation with the percentage of total motile spermatozoa in the oligozoospermic (r=0.343, P=0.002) group, but with no significant relationship in the normozoospermic group. On the other hand, the percentage of capacitated, acrosome-reacted cells was significantly weakly positively correlated with the percentage of total motile spermatozoa in the oligozoospermic (r=0.378, P<0.0001) group with no relationship in the normozoospermic subjects.

Table 10: Correlation of total motility with sperm functional parameters.

PARAMETERS	**Oligozoospermia**		**Normozoospermia**	
	r	P	r	P
Hyperactivation (%)	0.647	<0.0001	0.592	<0.0001
Progressive motility (%)	0.768	<0.0001	0.795	<0.0001
Intact-MMP (%)	0.343	0.0002	0.117	0.0666
Capacitated, acrosome-reacted (%)	0.378	<0.0001	0.086	0.3657

Table 11 shows the correlation between percentages of ROS-positive spermatozoa with the following sperm functional parameters: the percentages of spermatozoa with intact-MMP, DNA-fragmentation and capacitated, acrosome-reacted sperm. The percentage of ROS-positive spermatozoa had a significant weak negative association with percentage of spermatozoa with intact-MMP in the normozoospermic (r=-0.195, P=0.0022) but no relationship with the percentage of spermatozoa with intact-MMP in the oligozoospermic group. However, the percentage of ROS-positive spermatozoa had a significant weak negative relationship with the percentage of spermatozoa with DNA fragmentation in the oligozoospermic (r=0.243, P=0.0091), but no relationship with percentage of DNA-fragmented spermatozoa in the normozoospermic group. The same applied for the percentage of capacitated, acrosome-reacted spermatozoa where both groups had no significant relationship with ROS.

Table 11: Correlation of the percentage of ROS-positive sperm with sperm functional parameters.

PARAMETERS	Oligozoospermia		Normozoospermia	
	r	P	r	P
Intact-MMP (%)	0.078	0.4068	-0.195	0.0022
TUNEL (%)	-0.243	0.0091	0.111	0.0834
Capacitated, acrosome-reacted (%)	0.027	0.7749	0.008	0.9014

The relationship between the percentage of hyperactivated, ROS-positive and capacitated acrosome-reacted spermatozoa as shown in Table 12 revealed a strong relationship of the percentage of hypearctivated spermatozoa with the percentage of ROS-positive spermatozoa in oligozoospermic (r=0.678, P<0.0001) subjects but not normozoospermic (r=0.626, P<0.0569) subjects (Table 13), whereas, the percentage of sperm with intact-MMP had a significant weak relationship with the percentage of hyperactivation spermatozoa in the oligozoospermic (r=0.321, P=0.0006) group, with no significant correlation in the normozoospermic group.

Table 12: Correlation of the percentage of hyperactivated sperm with sperm functional parameters

PARAMETERS	Oligozoospermia		Normozoospermia	
	r	P	r	P
ROS-positive (%)	0.678	<0.0001	0.626	0.569
Intact-MMP (%)	0.321	0.0006	0.056	0.3830

3.3.1 Effect of aqueous root extract of *M. whitei* on total and progressive motility in asthenozoospermic and normozoospermic samples

Computer-assisted sperm analysis (CASA) revealed a dose-dependent increase and trend in total sperm motility after exposure to increasing concentrations of the extract in the asthenozoospermic group: (one-way ANOVA: P=0.024); (repeated measure ANOVA: P=0.0047). Essentially, total motility in asthenozoospermic samples significantly (P=0.0010) increased from the control to 185 µg/ml extract (Figure 48). In contrast, in the normozoospermic samples no change (one-way ANOVA P=0.197) was found despite the fact that, repeated measures ANOVA revealed a significant (P=0.0047) and positive trend between the control and the highest concentration (185 µg/ml) used.

More so, there was a significant increase in the percentage of progressively motile sperm from the control in the asthenozoospermic (P=0.0149) as well as normozoospermic (P=0.0001) groups to the highest concentration (185 µg/ml) (Figure 49). On further analysis, one-way ANOVA depicted no changes in both groups (one-way ANOVA: asthenozoospermia P=0.203 and normozoospermic P=0.498). In addition, repeated measures ANOVA also revealed no trend between the control and 185 µg/ml in both groups (repeated measure ANOVA: asthenozoospermia P=0.576 and normozoospermia P=0.249).

3.3.2 Effect of aqueous root extract of *M. whitei* on sperm kinematic parameters

As anticipated, the mean values for the various parameters in the normozoospermic subjects were generally higher than those in asthenozoospermic subjects (Table 13). No change nor any trend could be established by one-way ANOVA and repeated measure ANOVA, respectively

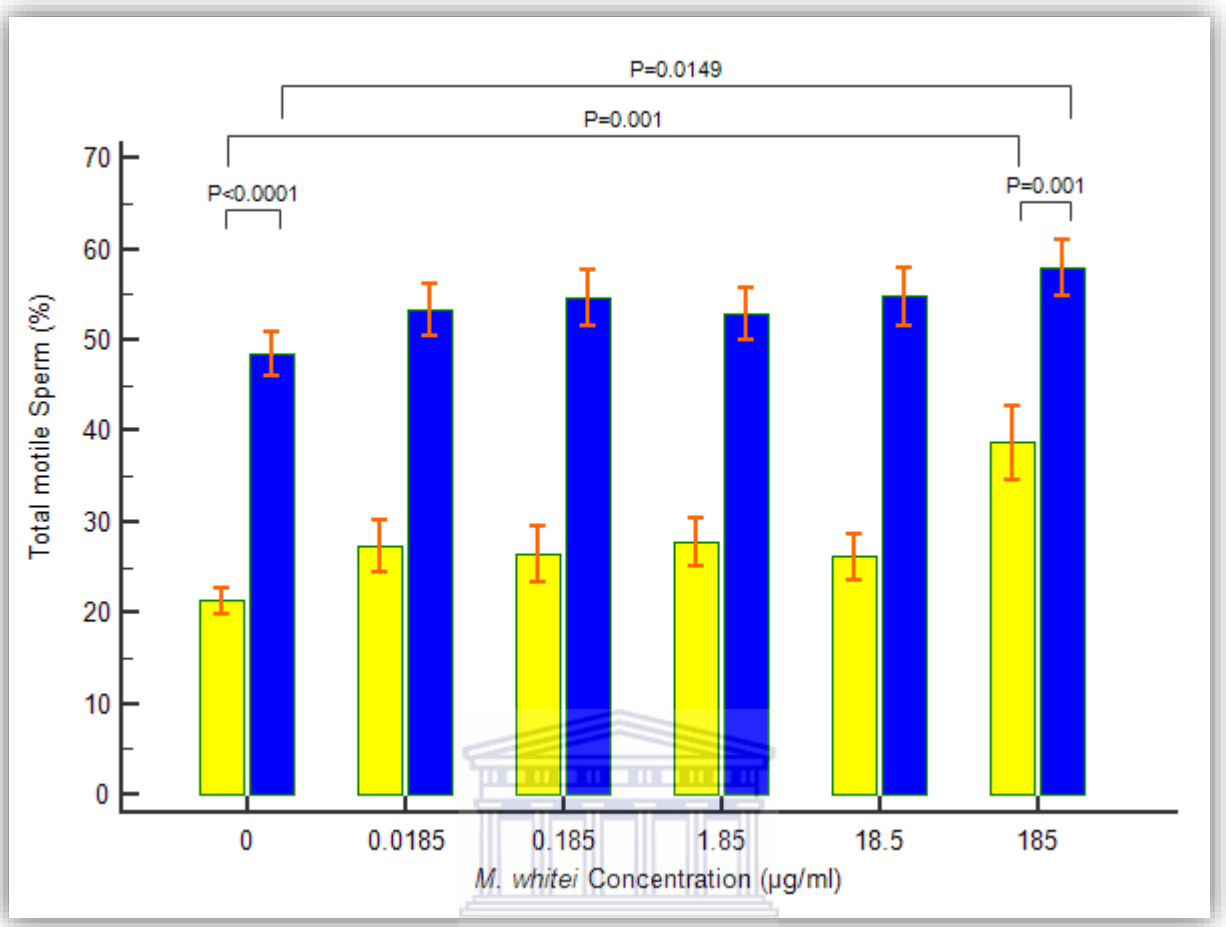

Figure 48: Effect of different concentrations of *M. whitei* (0.0185, 0.185, 1.85, 18.5 and 185 µg/ml) on the percentage of total motility of washed spermatozoa *in vitro*. Yellow represents: asthenozoospermic (progressive motility <32% or total motility <40%) group (n=20); Blue: normozoospermic (progressive motility >32% or total motility >40%) group (n=28). Values shown as mean±SEM. A significant (P=0.0010) dose-dependent increase in progressive motility is seen in asthenozoospermic (one-way ANOVA P=0.024). In addition, repeated measure ANOVA show significant trend in both groups (asthenozoospermia P=0.004 and normozoospermia P=0.023). However, one-way ANOVA show no changes in normozoospermia (one-way ANOVA P=0.197).

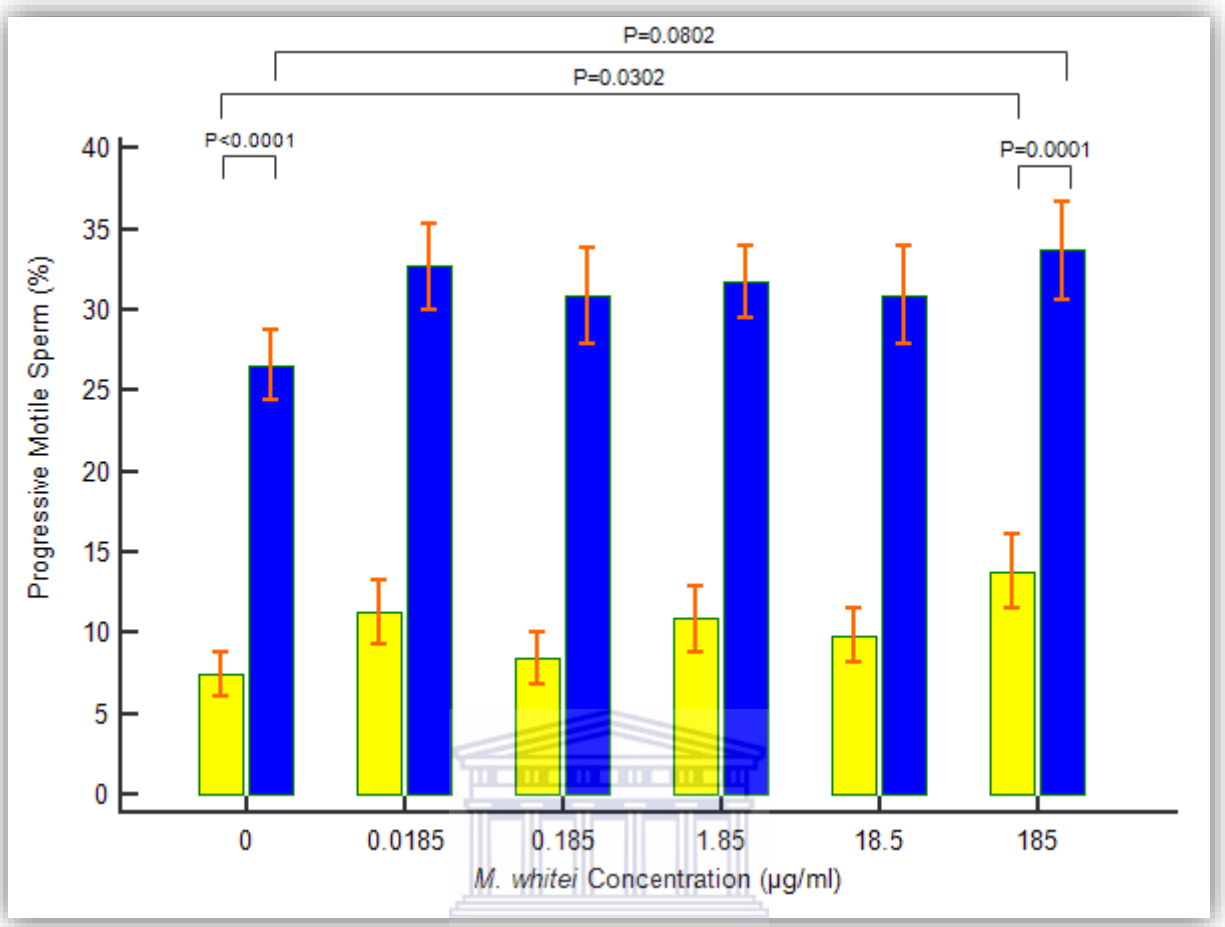

Figure 49: Effect of different concentrations of *M. whitei* (0.0185, 0.185, 1.85, 18.5 and 185 µg/ml) on the percentage of progressive motility of washed spermatozoa *in vitro*. Yellow represents: asthenozoospermic (progressive motility <32% or total motility <40%) group (n=20); Blue: normozoospermic (progressive motility >32% or total motility >40%) group (n=28). Values shown as mean±SEM. A significant increase in the percentage of progressive motile sperm is seen between the control and 185 µg/ml is both (asthenozoospermic P=0.0302 and normozoospermic P=0.082) groups. However, there is no changes (one-way ANOVA: asthenozoospermia P=0.203 and normozoospermia P=0.498) and trend (repeated measure ANOVA: asthenozoospermia P=0.576 and normozoospermia P=0.249) seen in both group.

Table 13: Summary statistics of sperm kinematic motility parameters, following incubation with increasing concentration of *M. whitei* for 1hour.

Kinematic Parameters	Group	Control	0.0185 µg /ml	0.185 µg /ml	1.85 µg /ml	18.5 µg /ml	185 µg /ml
VAP (µm s^{-1})	**Asthenozoospermic**	27.80±11.423	30.37±10.56	26.88±7.38	27.29±11.99	29.37±8.18	31.32±11.22
	Normozoospermic	36.57±10.79	37.47±11.16	37.59±11.34	37.44±10.28	37.06±11.23	36.16±9.11
VCL (µm s^{-1})	**Asthenozoospermic**	53.17±11.89	58.9±11.25	52.91±9.03	55.03±13.58	70.25±13.34	61.61±11.37
	Normozoospermic	66.09±15.96	71.31±13.11	70.08±15.10	70.25±13.34	70.26±14.79	68.87±12.05
VSL (µm s^{-1})	**Asthenozoospermic**	22.04±11.29	20.86±10.97	17.45±6.95	18.83±10.73	20.9±7.73	24.16±11.18
	Normozoospermic	27.24±10.95	30.38±12.65	29.26±14.30	28.39±10.84	28.30±12.29	28.0±12.29
ALH (µm)	**Asthenozoospermic**	1.68±0.65	2.06±0.68	1.89±0.72	1.88±0.67	1.99±0.44	2.01±0.61
	Normozoospermic	2.26±0.46	2.3±0.29	2.38±0.32	2.072±0.37	2.40±0.39	2.20±0.43
Hyperactivation (%)	**Asthenozoospermic**	1.54±1.42	1.69±0.83	1.97±1.75	1.67±1.74	1.3±1.52	2.4±2.6
	Normozoospermic	4.01±2.92	4.67±2.98	4.10±3.26	4.23±2.57	4.41±3.29	4.60±3.52
LIN (%)	**Asthenozoospermic**	34.79±12.30	35.90±11.18	33.25±7.76	35.52±13.43	35.59±11.7	36.5±13.99
	Normozoospermic	38.18±11.35	37.63±10.555	36.10±11.96	37.29±11.74	36.28±11.14	35.55±10.28
STR (%)	**Asthenozoospermic**	65.42±10.43	71.62±11.32	67.42±10.63	66.36±10.89	71.36±8.05	69.4±12.88
	Normozoospermic	69.21±12.43	70.1±12.41	70.90±12.97	68.10±13.39	67.32±12.89	66.49±12.70
WOB (%)	**Asthenozoospermic**	47.82±8.7	51.83±9.7	48.47±6.18	48.15±10.62	51.60±9.02	50.62±10.27
	Normozoospermic	51.67±9.80	51.23±10.01	50.54±10.50	50.81±9.49	50.14±910.94	50.09±9.54
BCF (HZ)	**Asthenozoospermic**	11.00±4.65	11.12±5.77	10.46±3.47	14.57±3.10	10.81±4.93	11.78±3.67
	Normozoospermic	13.74±2.75	13.66±2.43	13.44±2.75	14.42±3.13	13.60±92.96	13.42±5.59

Data comprises of asthenozoospermic group (n=20) and normozoospermic group (n=28) and is expressed as mean±SD. BCF; beat across frequency, LIN; linearity, STR; straightness, ALH; average lateral head displacement, BCF; beat across frequency, LIN; linearity, STR; straightness, wobble (WOB), SD; standard deviation.

Asthenozoospermic ANOVA P-value (VAP: P=0.743; VCL: P=0.205; VSL: P=0.619; ALH: P=0.270; hyperactivation: P=0.335; LIN: P=0.343; STR: P=0.503; WOB: P=0.651; BCF: P=0.920).

Normozoospermic ANOVA P-value (VAP: P=0.941, VCL: P=0.850, VSL: P=0.954; ALH: P=0.662; hyperactivation: P=0.441, LIN: P=0.928, STR: =0.921; WOB: P=0.990, BCF=0.937).

3.3.3 Effect of aqueous root extract of *M. whitei* on vitality and mitochondrial membrane potential (MMP)

Exposure to the extract revealed no effect on sperm vitality in both groups (one-way ANOVA: asthenozoospermic P=0.983 and normozoospermic: P=0.596) (Figure 50). Similarly, no trend was observed in both groups (repeated measure ANOVA: asthenozoospermic P=0.601 and normozoospermic P=0.076).

With regard to the mitochondrial membrane potential, there was a highly significant (one-way ANOVA: asthenozoospermic: P=0.0004 and normozoospermic: P=0.031) dose-dependent increase in the percentage of sperm with intact MMP seen in the both groups (Figure 51). Virtually, there was a significant increase in asthenozoospermic: P=0.0008 and normozoospermic: P=0.0016 group when their controls were compared to the highest (185 µg/ml) concentrations. When analysed further, repeated measure ANOVA showed a significant trend from the control to 185 µg/ml (repeated measure ANOVA: asthenozoospermic P=0.0002 and normozoospermic P=0.002).

3.3.4 Effect of aqueous root extract of *M. whitei* on the percentage of ROS-positive sperm

Treatment of sperm with increasing concentrations of *M. whitei* showed a significant positive trend towards higher values by means of repeated measure ANOVA (asthenozoospermic: P=0.011 and normozoospermic: P=0.015) for the percentage of ROS-positive sperm in both groups (Figure 52), but with one-way ANOVA no change could be observed (asthenozoospermic: P=0.091 and normozoospermic: P=0.1784). More so, there was a dose-dependent increase in the percentage of ROS-positive sperm from the control to the highest concentrations (185 µg/ml) in the asthenozoospermic (P=0.0050) as well as the normozoospermic group (P=0.0251).

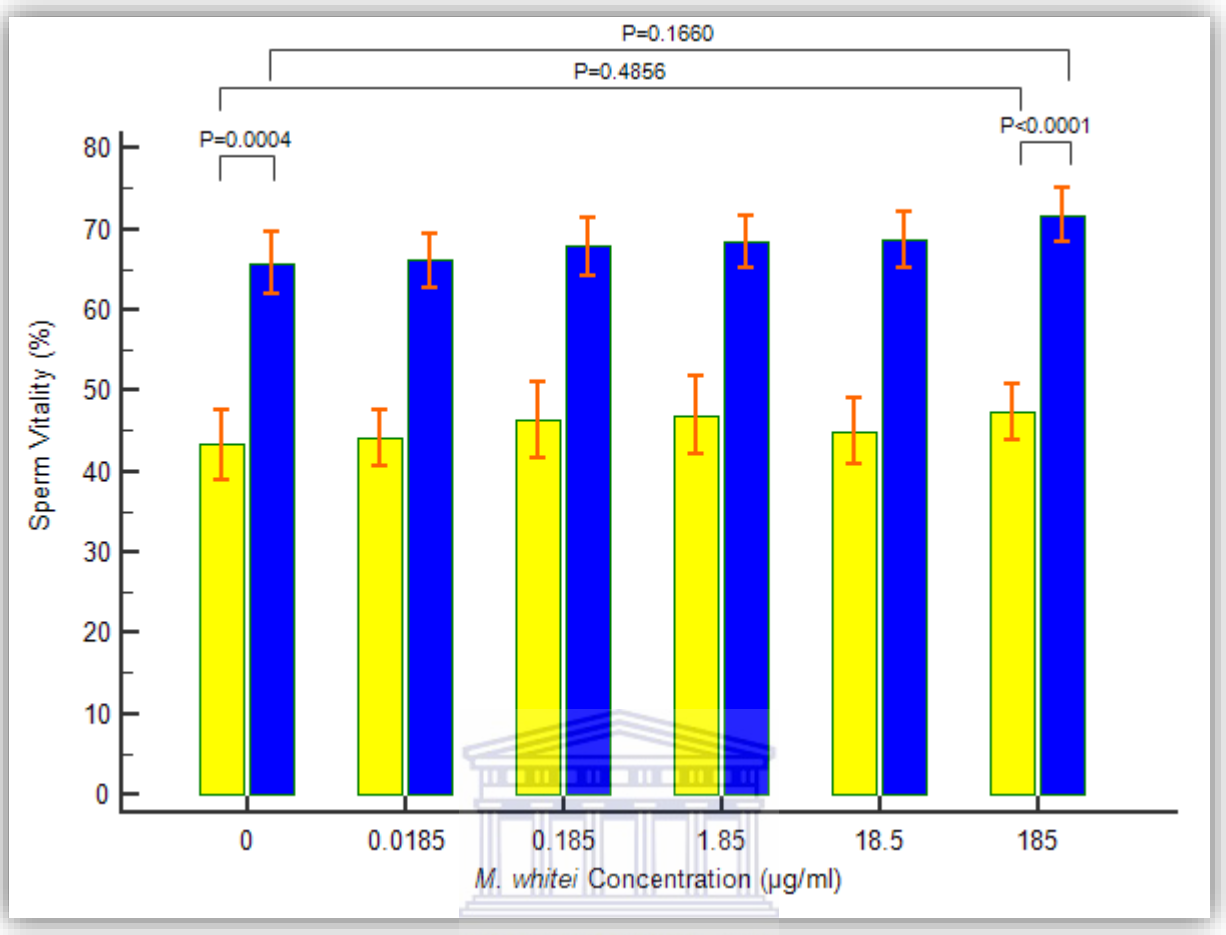

Figure 50: Effect of different concentrations of *M. whitei* (0.0185, 0.185, 1.85, 18.5 and 185 µg/ml) on the percentage of vitality of washed spermatozoa *in vitro*. Yellow represents: asthenozoospermic (progressive motility<32% or total motility<40%) group (n=20); Blue: normozoospermic (progressive motility>32% or total motility>40%) group (n=28). No dose-dependent effect (one-way ANOVA: asthenozoospermic P=0.983 and normozoospermic: P=0.596) nor trend (repeated measure ANOVA: asthenozoospermic P=0.601 and normozoospermic P=0.076) is seen in both groups.

3.3.5 Effect of aqueous root extract of *M. whitei* in the percentage of DNA-fragmentation

There was no effect of the extract on the percentage of DNA-fragmented sperm in both groups (one-way ANOVA: asthenozoospermic P=0.725 and normozoospermic P=0.764; repeated measure ANOVA: asthenozoospermic: P=0.297 and normozoospermic: P=0.435) (Figure 53).

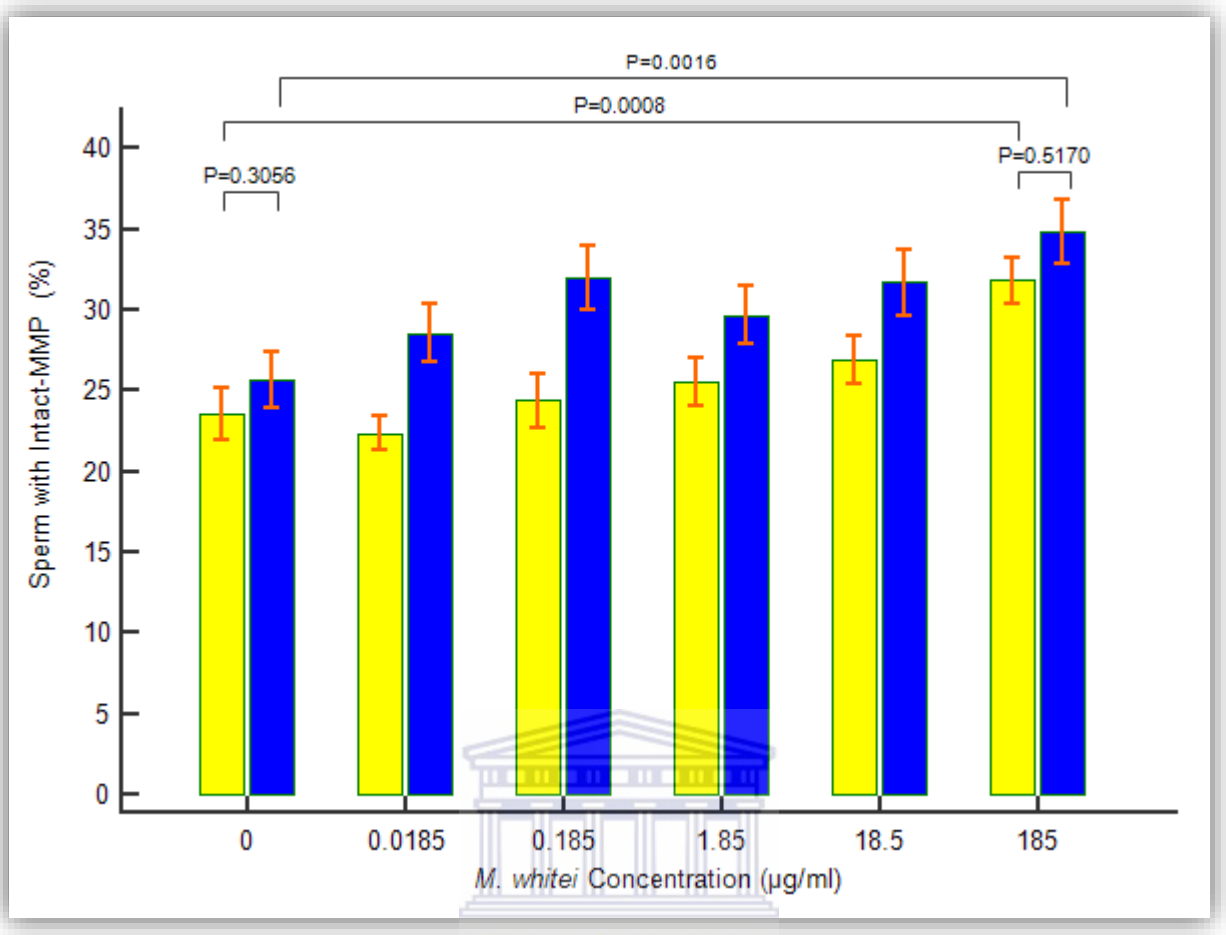

Figure 51: Effect of different concentrations of *M. whitei* (0.0185, 0.185, 1.85, 18.5 and 185 µg/ml) on the percentage of washed spermatozoa with intact-MMP *in vitro*. Yellow represents: asthenozoospermic (progressive motility<32% or total motility<40%) group (n=20); Blue: normozoospermic (progressive motility>32% or total motility>40%) group (n=28). Values shown as mean±SEM. A highly significant dose-dependent increase (one-way ANOVA: asthenozoospermia P=0.0004 and normozoospermia P=0.031) and trend (repeated measure ANOVA: asthenozoospermia P=0.0002 and normozoospermia P=0.002) is seen in both groups.

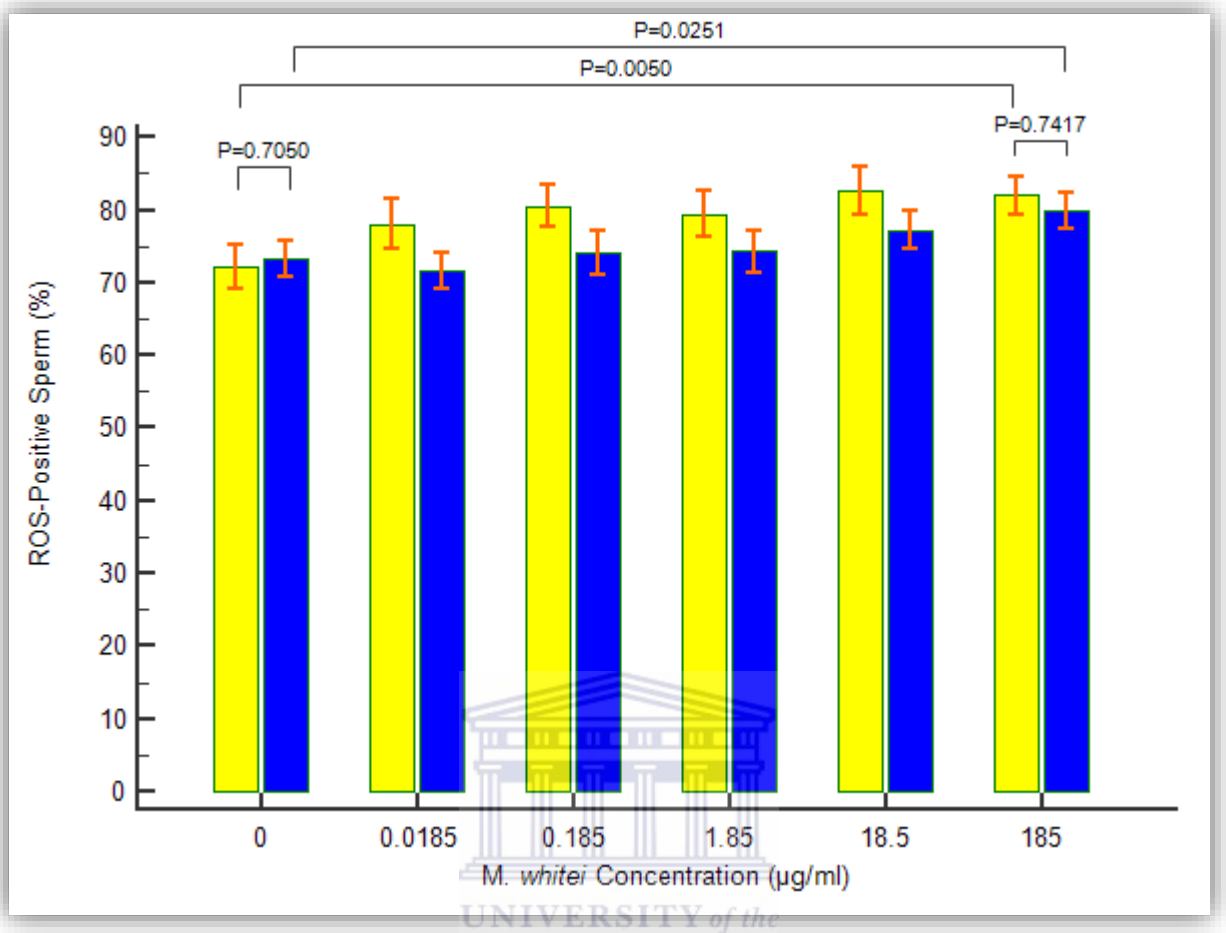

Figure 52: Effect of different concentrations of *M. whitei* (0.0185, 0.185, 1.85, 18.5 and 185 µg/ml) on the percentage of ROS-positive washed spermatozoa *in vitro*. Yellow represents: asthenozoospermic (progressive motility<32% or total motility<40%) group (n=20); Blue: normozoospermic (progressive motility>32% or total motility>40%) group (n=28). Values shown as mean±SEM. A significant trend (repeated measure ANOVA: asthenozoospermic P=0.011 and normozoospermic P=0.015), but no change (one-way ANOVA: asthenozoospermic: P=0.091 and normozoospermic: P=0.1784) in the percentage of ROS-positive sperm is seen in both groups (Figure 38). More so, there is an increase in the percentage of ROS-positive sperm from the control to the highest concentrations (185 µg/ml) in the asthenozoospermic (P=0.0050) group as well as the normozoospermic (P=0.0251).

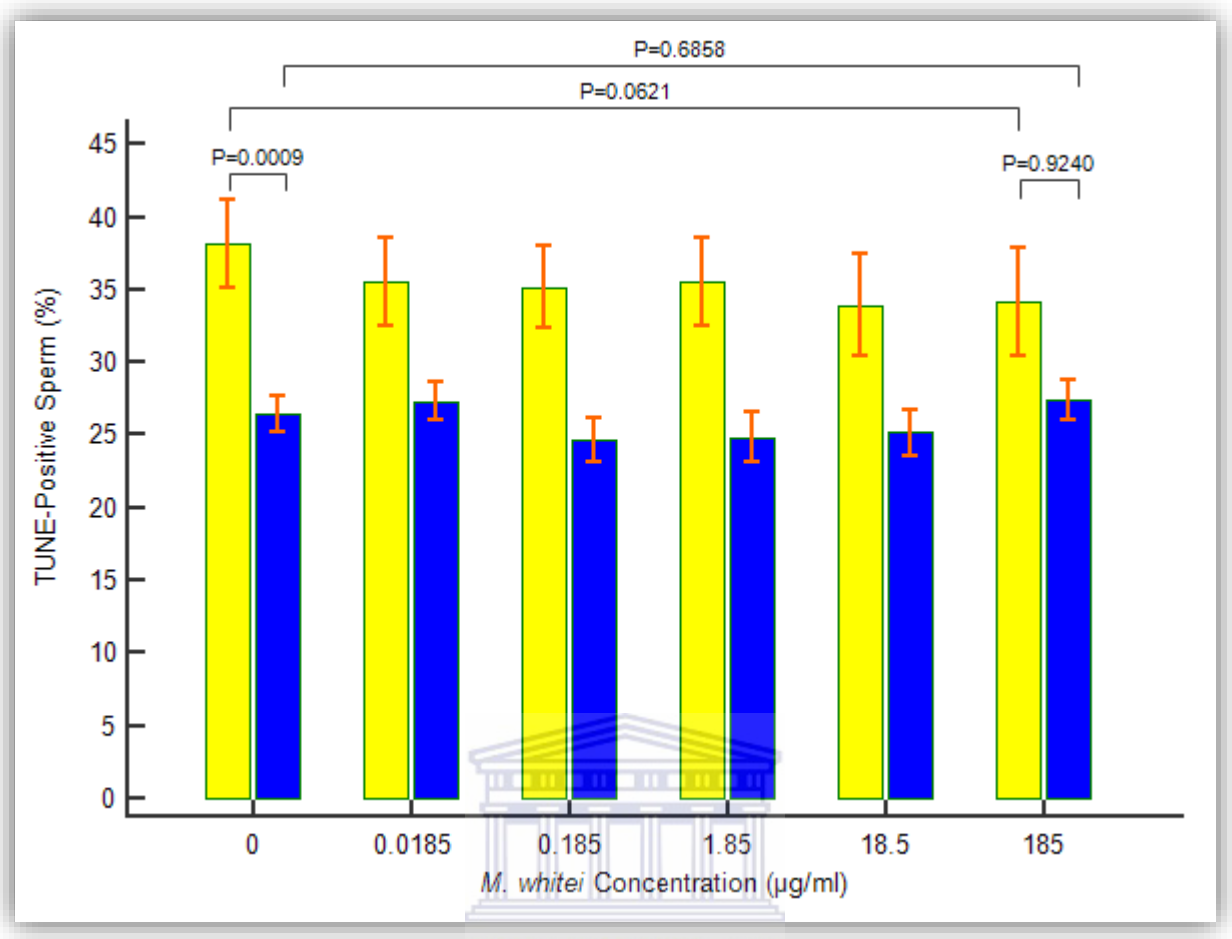

Figure 53: Effect of different concentrations of *M. whitei* (0.0185, 0.185, 1.85, 18.5 and 185 µg/ml) on the percentage of DNA fragmentation of washed spermatozoa *in vitro*. Yellow represents: asthenozoospermic (progressive motility<32% or total motility<40%) group (n=20); Blue: normozoospermic (progressive motility>32% or total motility>40%) group (n=28). Values shown as mean±SEM. There is no dose-dependent effect in the percentage of DNA fragmented sperm in both groups (one-way ANOVA: asthenozoospermic P=0.725 and normozoospermic P=0.764) nor trend (repeated measure ANOVA: asthenozoospermic: P=0.297 and normozoospermic: P=0.435).

3.3.6 Effect of *M. whitei* on sperm capacitation and acrosome reaction in asthenozoospermic and normozoospermic samples

The chlortetracycline fluorescence (CTC) assay (Table 16) revealed no changes in the percentage of capacitated and capacitated, acrosome-reacted spermatozoa in both groups (asthenozoospermic and normozoospermic) after exposure to increasing concentrations of the *M. whitei* root extract; no trend could be determined on capacitation (repeated measure ANOVA: asthenozoospermia: P=0.129 and

normozoospermia: P=0.370) and the percentage of capacitated acrosome-reacted sperm (repeated measure ANOVA (asthenozoospermia: P=0.898 and normozoospermia: P=0.731) (Figure 54 and 55). Similarly, one-way ANOVA revealed no effect on capacitated (asthenozoospermia: P=0.757 and normozoospermia: P=0.806) and capacitated, acrosome-reacted sperm (asthenozoospermia: P=0.548 and normozoospermia: P=0.856) in both groups.

Table 14: Summary statistics of the sperm capacitation and acrosome reaction in asthenozoospermic sperm samples as detected by CTC stain after 1 hour incubation with increasing concentrations of *M. whitei* root extract.

Concentration (µg/ml)	Capacitated (%)		Capacitated acrosome reacted sperm (%)	
	Asthenozoospermia	Normozoospermia	Asthenozoospermia	Normozoospermia
Control	47.69±18.97	63.87±21.55	15.93±9.12	20.30±10.16
0.0185	50.19±18.47	65.78±19.28	16.38±11.09	22.68±11.36
0.185	49.75±15.19	62.78±19.35	15.31±5.79	20.07±8.89
1.85	53.50±18.49	65.63±20.02	15.81±10.44	19.66±10.10
18.5	54.69±18.83	65.67±19.43	14.56±7.78	20.30±9.81
185	56.25±23.57	68.20±21.34	15.81±11.06	20.34±9.50
One-way ANOVA P-value	0.699	0.885	0.263	0.786

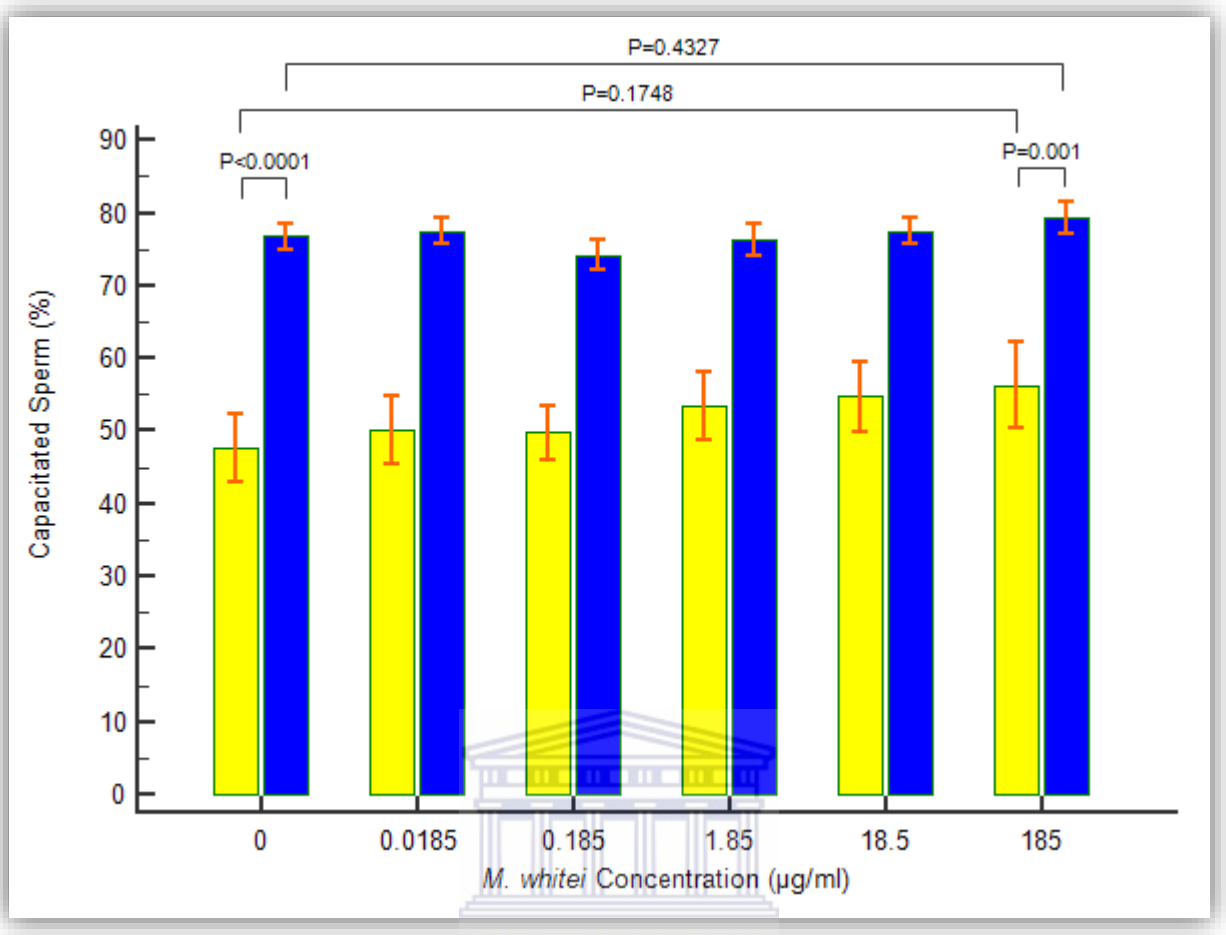

Figure 54: Effect of different concentrations of *M. whitei* (0.0185, 0.185, 1.85, 18.5 and 185 µg/ml) on the percentage of capacitated washed spermatozoa *in vitro*. Yellow represents: asthenozoospermic (progressive motility<32% or total motility<40%) group (n=20); Blue: normozoospermic (progressive motility>32% or total motility >40%) group (n=28). Values shown as mean±SEM. No trend (repeated measure ANOVA: asthenozoospermia P=0.129 and normozoospermia P=0.370) nor dose-dependent change (one-way ANOVA: asthenozoospermia P=0.757 and normozoospermia P=0.432) is seen in both groups.

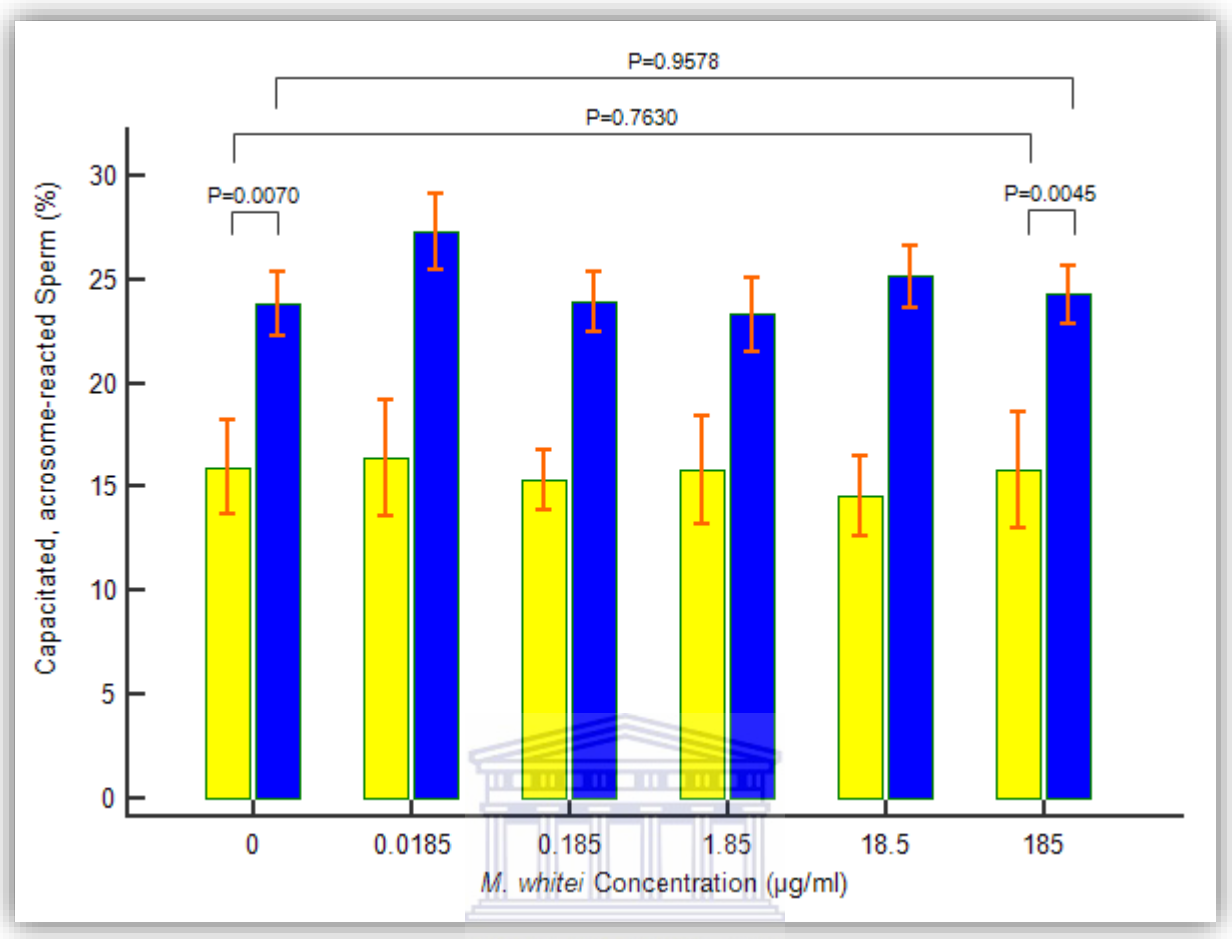

Figure 55: Effect of different concentrations of *M. whitei* (0.0185, 0.185, 1.85, 18.5 and 185 µg/ml) on the percentage of capacitated acrosome-reacted spermatozoa *in vitro*. Yellow represents: oligozoospermic (progressive motility<32% or total motility<40%) group (n=20); Blue: normozoospermic (progressive motility>32% or total motility>40%) group (n=28). Values shown as mean±SEM. No dose-dependent effect (one-way analysis: asthenozoospermia: P=0.548 and normozoospermia: P=0.806) nor trend **(**repeated measure ANOVA: asthenozoospermia: P=0.898 and normozoospermia: P=0.731) is seen in both groups.

3.3.7 Correlations of various sperm parameters within the asthenozoospermic and normozoospermic group

As expected, total motility strongly and significantly positively correlated with hyperactivation (r=0.671; P<0.0001) and progressive motility (r=0.733; P<0.0001) in the asthenozoospermic group as well as normozoospermic group (hyperactivation: r=0.464; P<0.0001; progressive motility (r=0.685; P<0.0001) (Table 15).

Table 15: Correlation of total motility with sperm functional parameters.

PARAMETERS	Asthenozoospermia		Normozoospermia	
	r	P	r	P
Hyperactivation (%)	0.671	<0.0001	0.464	<0.0001
Progressive motility (%)	0.733	<0.0001	0.685	<0.0001
Intact-MMP (%)	0.018	0.0399	0.086	0.0666
Capacitated, acrosome-reacted (%)	0.146	0.3675	0.487	0.5372

The percentage of ROS-positive sperm weakly but significantly positively correlated with the percentage of intact-MMP sperm (r=0.172, P=0.0260) in the asthenozoospermic subjects as well as normozoospermic (r=0.017, P=0.0460). Contrary, the percentage of ROS-positive spermatozoa weakly but significantly negatively correlated with the percentage of TUNEL-positive sperm (r=-0.034, P=0.0073) in the asthenozoospermic subjects (figure 16). However, there was no statistical significant relationship between the percentage of ROS-positive and capacitated, acrosome-reacted sperm in both groups. In addition, the percentage of spermatozoa with intact MMP had no relationship with percentage ROS-positive sperm in the normozoospermic subjects, neither did the percentage of TUNEL-positive sperm have a relationship with percentage ROS-positive sperm in the asthenozoospermic group.

Table 16: Correlation of the percentage of ROS-positive sperm with sperm functional parameters

PARAMETERS	Asthenozoospermia		Normozoospermia	
	r	P	r	P
Intact-MMP (%)	0.172	0.0260	0.017	0.0460
TUNEL (%)	-0.034	0.0073	0.206	0.7431
Capacitated, acrosome-reacted (%)	0.001	0.9997	0.032	0.6861

Table 17 portrays the relationship between hyperactivated spermatozoa, the percentage of progressive motility of spermatozoa and the percentage of sperm with intact MMP. A strong significant positive relationship was observed between hyperactivated spermatozoa and progressive motility from both groups (asthenozoospermic: r=0.513; P<0.0001 and normozoospermic: r=0.502, P<0.0001). Also, there was a weak significant (r=0.321; P=0.0006) relationship between the percentage of hyperactivated spermatozoa and the percentage of intact-MMP sperm in the asthenozoospermic subjects. Yet, there was no relationship in the normozoospermic subjects.

Table 17: Correlation of the percentage of hyperactivated sperm with sperm functional parameters

PARAMETERS	**Asthenozoospermia**		**Normozoospermia**	
	r	P	r	P
Progressive motility (%)	0.678	<0.0001	0.626	<0.0001
Intact-MMP (%)	0.321	0.0006	0.056	0.3830

CHAPTER 4: DISCUSSION

Reproductive health care has been regarded as one of the most prevalent problems on the African continent (Kamatenesi-Mugisha and Oryem-Origa, 2005). With increasing awareness of its detrimental effects, studies have recorded high prevalence rates of male infertility of up to 20-40% in certain regions of Africa labelled as `Infertility belt', which is a stretch across sub-Saharan Africa (Cates et al., 1985; Leke et al., 1993; Larsen et al., 2000; Dyer et al., 2004). A multitude of causes (Walczak-Jedrzejowska et al., 2013) render infertility difficult for a rational treatment plan. In addition, poorly defined pathological processes complicate the diagnosis and treatment even further (Howards, 1995). Due to traditional beliefs and economic reasons, many South Africans have resorted to using traditional medicines for the treatment of a range of health problems, especially where Western Medicine has fallen short (Digby, 2014).

Traditional Medicine is termed as the oldest and possibly the most popular therapeutic system (Mahomoodally, 2013), and has been regarded as a growing industry in South Africa. Herbal medicines contribute an estimated R 29 billion to the South African national economy with an equivalent of 27 million consumers, with the traditional healers being a driving force in this market (Mander et al., 2007; Moeng and Potgieter, 2011). In order to ensure the safety, efficacy and quality of the treatment rendered, the Traditional Health Care Service in South Africa is regulated by the Traditional Health Practitioner Act No. 22 of 2007 (Pefile, 2005).

As a result of growing interest in traditional medicine, there has been a number of local and international initiatives which undertake to explore the botanical resources of South Africa with the aim of screening indigenous plants for pharmacologically active compounds (Rybicki et al., 2012). For example, the South African Department of Health through the National Reference Centre of Health for African Traditional Medicine, in conjunction with Council for Scientific Research and Medicine Control Council are involved in various research programs aimed at developing new medicines by

analysing medicinal plants (Ngcobo et al., 2012). Furthermore, studies are centred on new plausible biologic technologies like phytochemical analysis, microarray methodology, metabolomic assessment and pharmacogenesis of these plants (Firenzuoli and Gori, 2007). In a previous study, Gericke (2011) agreed that significant research and development opportunities exist to discover novel and useful biological activities for South African medicinal plants. For that reason, South African research institutions focus more on phyto-pharmacological studies using indigenous plants with the aim to discovery a novel phyto-products for new markets (Hughes and Mbamalu, 2015).

According to De Smet (1997), approximately 25% of modern medicines or their active ingredients are derived from higher plants. Farnsworth and Soejarto (1985) indicated that 95 plants species have been listed as the source of 121 clinically useful prescriptions derived from medicinal plants. Phytochemicals found in these indigenous medicinal plants act individually, catalytically as well as synergistically; providing stability from the combined effect that is more beneficial than individual constituents in terms of improving health (Mahomoodally, 2013). Moreover, in conventional medicine, these active phytochemicals from medicinal plants are extremely purified and the standardized forms are used directly as drugs or therapeutic agents, as starting material for synthesis of new drugs or as a tool for drug development and testing (Calixto, 2000).

As a result of the renaissance in scientific exploration of medicinal plants with therapeutic actions (Vaidya, 2014), there has been an increasing trend in validating traditional medicine claims from scientific studies especially for plants which have been used to treat diseases (Brookes and Katsoulis, 2006). A good example is *M. whitei*, a plant of the Apocynaceae family, which is indigenous to South, Central, East and West Africa. Its roots are used in the treatment of male infertility (Aremu et al., 2011).

4.1 Previous studies on *M. whitei*

4.1.1 Previous *in vivo* and *in vitro* studies on *M. whitei* root extract

The primary medicinal use of *M. whitei* is based on the aphrodisiac properties of the extract and its use as a treatment for male infertility (Lampiao et al., 2008; Martey and He, 2010). Besides, studies by Watcho et al. (2001) initially suggested that the aqueous root extract of *M. whitei* has contraceptive properties. This claim was based on the observed inhibition of spermatogenesis and reduced fertility *in vivo*. This research group further evaluated the *in vivo* androgenic activity of the same extract and found an increase in serum and intra-testicular testosterone levels after chronic exposure to *M. whitei* aqueous root extract (Watcho et al., 2004). In 2006, Watcho et al. decided to use hexane, methylene chloride and methanol as solvent for the extraction of *M. whitei* roots and investigated the *in vivo* and *in vitro* androgenic activity and found that the methanolic extract had reversible androgenic effects and potentiated the actions of norepinephrine on the vas deferens in rats, while the hexane fraction had significant inhibitory effects against KCl-and adrenaline-induced contractions in isolated deferent ducts *in vitro*. Moreover, in 2007, Watcho et al. found that the extracts caused an increase in the number of intromissions and erectile frequencies of inexperienced male rats. From the above mentioned studies, it was deduced that the extract displays both contraceptive properties as well as properties associated with enhancing male fertility.

In Ghana, the *in vivo* aphrodisiac activities of the ethanoic root extract of *M. whitei* was investigated (Quasie et al., 2010). The study showed not only an increase in cyclic guanosine monophosphate (cGMP), but also improved and sustained erection which was associated with the activation or stimulation of nitric oxide synthase (NOS) activity (Quasie et al., 2010). These authors linked the mechanism of action to the phytochemicals found in the root extract which was presumed to either stimulate or activate NOS, which would result in the elevation of nitric oxide (NO) and cGMP and the

subsequent relaxation of vas the deferens and corpus cavernosum causing vasodilation which increased blood inflow into penile tissue resulting to erection (Quasie et al., 2010).

The aphrodisiac effects of *M. whitei* have also been investigated in combination with *Ekerbegia capensis* (cape ash), *Aloe exelsa* (noble aloe) and *Cucurbita pepo* (pumpkin seed) (Gundidza et al., 2009). Results showed a dose-dependent increase in sexual arousability (decreased mounting latency), copulatory efficiency (decreased intercoupulatory interval) and improved sexual sensation (increased in neuromotor activity) for the rats treated with *M. whitei*, *E. capensis*, *Aloe exelsa* and *C. pepo* seeds. The study displayed significant aphrodisiac properties with regards to sexual behaviour.

In spite of *Mondia whitei* root extract being used in traditional medicine for the treatment of male infertility, there appears to be a lack of research/information on its effect on human spermatozoa. Only one *in vitro* study (Lampiao et al., 2008) investigated the effects of an aqueous extract of *M. whitei* on human sperm total and progressive motility as the only parameter. Therefore, this is the first study to shed light on the *in vitro* effects of aqueous root extract of Mondia on different aspects of human sperm functionality by the use of traditional-based therapeutic concentrations, hence, assisting in the validation of the safety and efficacy of the traditional use of Mondia. Since, scientific studies into the biological properties of the extract and its phytochemical analysis have shed light on the plant traditional usage to treat male infertility, yet various observable ambiguities exist.

4.1.2 Phytochemical studies on *M. whitei* root extract

Mondia whitei belongs to the botanical family Apocynaceae, a plant family which generally contains a high loads of alkaloids (Bunel et al., 2014). Therefore, Bunel and colleagues hypothesized a possibility of presence of alkaloid in Mondia. These alkaloids were presumed to be responsible for the reported biological properties. Further investigation by these authors confirmed the presence of alkaloids known as 2-hydroxy-4-methoxybenzaldehyde (2H4MBZA) from Mondia root extract, which

was found to react with neurotransmitters to form adducts of dopamine γ-aminobutyric acid (GABA), norepinephrine and serotonin. These findings might give an explanation for the alleged aphrodisiac, tonic and anti-depression activities of the Mondia root extract (Bunel et al., 2014). An additional study indicated the 2H4MBZA alkaloid as a potent tyrosinase inhibitor (tyrosinase enzyme is responsible for melanin formation) (Batubara et al., 2010). The benzaldehyde group in 2H4MBZA was found to be a mixed type inhibitor for the oxidation of L-3-4-dihydroxyphenylalanine (L-DOPA) by forming Schiff base with primary amino group in the enzyme (Kubo and Kinst-Hori, 1999a). This could infer possible antioxidant properties from 2H4MBZA compound found in Mondia extract.

Furthermore, Foreman and Wernicke (1990) found dopamine and norepinephrine to have been implicated in regulation of sexual function. Additionally, Shishkina et al. (2001) stated that norepinephrine transmission seem to have a positive effect on sexual function, and it has been generally accepted as the principle neurotransmitter to control penile flaccidity and detumescence (Foreman and Wernicke, 1990). However, serotonin (5-HT) receptors have been found to possess both facilitatory and inhibitory effects on sexual function depending on the receptor sub-type and location (Fernández-Guasti et al., 1992; de Castilhos et al., 2006). On the other hand, dopaminergic neurons activation is secondary to nitric oxide synthase activation, which leads to the production of nitric oxide. In turn, nitric oxide activates oxytocinergic neurons in the paraventricular area which lead to the release of oxytocin in the extra-hypothalamic brain area and the spinal cord to induce erection (Melis and Argiolas, 1995).

In contrast, GABA receptor stimulation may produce different outcomes (Argiolas and Melis, 2002). Depending on the brain areas in which they act, $GABA_A$ and $GABA_B$ stimulation may either cause excitatory or inhibitory effects on penile erection and yawning (Argiolas and Melis, 2002). To facilitate penile erection dopamine, acts on D_1 or D_2 receptors on GABA neurons within the central nervous system, whereby activation of D_1 receptors initiates penile erection but decreases the number of

seminal emission while activation of D_2 inhibits sexual activity by suppressing erection but then favouring seminal emission (Steers, 2002; Nutsch et al., 2016).

In their previous study Watcho et al. (2012) found that two compounds (β-sitosterol and a mixture α- and β-amyrin acetate) in the hexane extract of Mondia significantly increased the mounting frequency, penile erection and ejaculatory latency of sexually naïve albino male rats. α-and β-amyrin acetate are triterpenes whereas β-sitosterol is a steroid compound. These compounds are thought to induce changes at the level of neurotransmission by modulating neuro-transmitters on the target cells (Sertoli and Leydig cells) or by increasing androgen levels (Kumar et al., 2000) via binding to hormone receptors or enzymes that metabolize hormones in the intermediary steroidal pathway (Baker, 1995). Therefore, increasing testosterone, dihydrotestosterone and dehydroepiandrosterone (Gauthaman and Ganesan, 2008). Mondia root extract has been found to contain phenolic and flavonoid compounds (Bouba et al., 2010; Gakunga et al., 2013), as well as vitamin E (Sharma and Agarwal, 1996; Van Wyk, 2011), all of which possess radical scavenging activities (Moyo et al., 2010), hence, generally protecting spermatozoa from oxidative stress caused by free radicals which may lead to protein oxidation, DNA damage, and lipid peroxidation (Halliwell, 1996).

4.2 Traditionally used concentration of root extract of *M. whitei*

Generally, traditional healers will prescribe a handful of dried roots of *M. whitei* to prepare a concoction for treatment of patients. This study focused on the principle of traditional healers' preparation to obtain an effective therapeutic concentrations of concoctions. Based on the assumption that an average man weights about 80kg, an average (14.80g) of three handful of *M. whitei* roots was obtained to establish the amount of aqueous root extract a patient would ingest per day. Thereafter, an effective therapeutic concentration of 1.85 µg/ml *M. whitei* aqueous root extract was calculated, and a stock solution containing 2000 µg/ml *M. whitei* in HTF-BSA was prepared. To account for both supra and hypo-physiological effects of the *M. whitei* root extract, a scale ranging from 0.0185-185 µg/ml

was used. Therefore, the concentrations used for the functional parameters of spermatozoa were considered to be in a tolerable range. Nonetheless, none of the previous studies attempted to take the prescribed therapeutic concentration used by the traditional healers into account, which could be a possible reason for conflicting results.

4.3 Effect of aqueous root extract of *M. whitei* on sperm vitality

Vitality is a reflection of the proportion of live, immotile, membrane-intact spermatozoa determined by the evaluation of cellular and membrane integrity (Björndahl et al., 2003; Klimowicz-Bodys et al., 2012; Moskovtsev and Librach, 2013). In the present study, the valuation of sperm viability was used as factor to investigate possible cytotoxic effects of the aqueous root extract of *M. whitei* on human spermatozoa.

Results from this study show that the extract had no significant direct toxic effect on sperm in all groups analysed (donor, patient, oligozoospermia, asthenozoospermia, and normozoospermia). Therefore, *M. whitei* extract does not show acute sperm cell cytotoxicity on human spermatozoa *in vitro*. Currently, there are no published studies that have investigated the effect of *M. whitei* on sperm cell vitality.

However, other studies have indicated that plant extracts might either increase, have no effect or decrease cell viability depending on the plant's chemical composition. For instance, plants containing antioxidants such as flavonoids and vitamins for example *Vernonia amygdalina* (Bitter leaf) have been found to possess modulating function therefore, they act to maintain sperm viability (Iwalewa et al., 2005). On the other hand, certain alkaloids found in some plant extract have been implicated in reduced sperm viability. The postulated mechanism of action of such alkaloids involves releasing metabolites which bind to cell molecules and cross link DNA causing cytotoxicity (Saalu et al., 2010).

Furthermore, analysis of *in vitro* effects of aqueous root extract of *Astragulus membranaceus* (Huảng qỉ) and *Eleutherococcocus senticosus* (Siberian ginseng) on human sperm were found to exhibit

significantly increased viability (Liu et al., 2004). The hypothesized mechanism of action of *Astragalus membranaceus* could be attributed to the chemical composition which consists of isoflavonoids, saponins, triterpene glycosides, polysaccharides, amino acids and trace elements (Ma et al., 2002). Additionally, the *in vivo* effect of these phytochemicals on type 1 and 2 diabetic rats have exhibited protection of pancreatic beta cells from autoimmune cell death, by immunomodulation of several inflammation and apoptotic cytokines, enzymes and proteins (Agyemang et al., 2013). Furthermore, the *in vivo* effects of flavonoids found in Astragalus have also been shown to poses antioxidant and cellular protection properties by exerting cellular antioxidant effects, scavenging free radicals, protection against lipid peroxidation hence protecting the cell against hypoxic challenges. Furthermore, the polysaccharides were linked to increase the protein synthesis and RNA activity in other tissues, equally, increase enzyme activities responsible for specific regulation of cellular metabolic activities (Agyemang et al., 2013). Therefore, it could be concluded that the combination of secondary metabolites found in Astragalus were not cytotoxic to spermatozoa but, might have acted synergistically to maintain sperm viability.

Conversely, *in vitro* effect of aqueous root extract of *Aconitum carmichaelli* (Monkshood), *Polyporus squamosus* (Zhu ling) (Liu et al., 2004) and *Cissampelos capensis* (Dawidjies) (Shalaweh et al., 2015) were found to have no effect on sperm viability; whereas, *in vitro* effect of aqueous extract of *Aegles mermelos* (Bael) and *Allium sativum* (garlic) have been exhibited to decrease the vitality of ejaculated human spermatozoa (Remya et al., 2009). The proposed active ingredient in Allium is allitridum which might act by causing sperm membrane disintegration on human ejaculated spermatozoa (Ogbuewu et al., 2011).

4.4 Effect of aqueous root extract of *M. whitei* on sperm motility

Sperm motility has been found to significantly correlate with fertilization (Kasai et al., 2002). Generally, it gives a measure of the integrity of the sperm axone and tail structure not forgetting the metabolic machinery of the mitochondria; rendering it as the best criterion to demonstrate the male fertilization capacity (Karpuz et al., 2007).

An *in vitro* study performed by Lampiao et al. (2008) on the effects of aqueous root extract of *M. whitei* on human sperm total and progressive motility reported that Mondia significantly enhanced total motility as well as progressive motility in a time-dependent manner. Hence, these authors concluded that the results from their study may open the way for the use of *Mondia whitei* especially in men affected with asthenozoospermia. However, the study design had some discrepancy making the conclusion questionable. Since, limited sperm functional parameters were investigated precisely total motility and progressive motility. In addition, the number of sperm donors used in this study was not stated and the rationale behind the usage of concentration ranges of 5, 10, 20 and 50 µL/mL was not substantiated, clearly the traditional therapeutic concentration was not taken into consideration. Taking everything into account, the results exhibited an increase in sperm motility after two hours incubation when the controls were compared to some of the concentration, there was no significant differences in total motility as well as progressive motility among the *Mondia whitei* treated groups at all time points.

In the recent study, treatment of sperm with aqueous root extract of *M. whitei* significantly increased total ($P<0.0001$) and progressive motility ($P=0.0002$) in patients' spermatozoa with a positive trend towards higher percentages of total motile spermatozoa (ANOVA trend analysis: $P=0.0001$) and progressive motile spermatozoa (ANOVA trend analysis: $P=0.0068$) at higher concentrations of Mondia. The donor (normozoospermic) group showed a slight increase in total motility (ANOVA trend analysis: $P=0.144$) as well as progressive motility (ANOVA trend analysis: $P=0.3618$). However, this did not reach statistical significance. Moreover, it can be deduced that aqueous root extract of

Mondia showed physiological effects on total and progressive motility especially in the patient group. However, the question arises whether these physiological effects can have a biological impact since there is an increase of about 25 % of the total motile sperm from the control to the highest concentration (185 µg/ml) in patients. The same question applies to the results obtained from the study conducted by Lampiao et al. (2008), which renders the conclusion obtained from their study stating that *Mondia whitei* significantly enhanced total motility as well as progressive motility in a time-dependent manner questionable.

Furthermore, various study on plant extracts have shown different outcome on sperm motility which is said to be predominately dependent on energy produced from the mitochondrial compartment of the sperm mid-piece (Ruiz-Pesini et al., 1998). For instance, different concentration of aqueous root extract of *Cissampelos capensis* (dawidjies) exhibited no effect on the sperm total and progressive motility. However, hyperactivation and beat across frequency increased with increase in concentration (Shalaweh et al., 2015). In a similar study, treatment of sperm with aqueous extract of *Erycoma longifolia* (Tongkat ali) showed a significant dose-dependent trend in total motility (Erasmus et al., 2012) a property which has been attributed to its phytochemical which includes; alkaloids, quassinoids, quassinoid diterpenoids, eurycomoside, eurycolactone and laurycolactone. Additionally, a different study also revealed that polyphenol-rich grape pomace extract maintained the percentage of total and progressive motility of spermatozoa compared to the control after 2 hours of sperm incubation. The proposed mechanism of action was attributed to the polyphenol-rich grape pomace extract free radicals scavenging capacity, rendering protection to spermatozoa from lipid peroxidation which leads to loss of motility (Harat et al., 2008).

Other investigations have revealed spermicidal properties of plant extract since they led to reduced human sperm motility like *Azadirachta indica* (Neem) (Khillare and Shrivastav, 2003), *Carica papaya* (Paw paw) (Lohiya et al., 2000), *Ruta gravoelens* (Rue) (Harat et al., 2008). In addition, a study by

Nath and colleagues (2013) revealed that highest (300mg/mL) concentration of *Ricinus communis* (Castor oil) extract led to 100% reduction in rapid progressive motility within 60 seconds and 100% immotility within 30 minutes of incubation. The spermicidal capacity of castor oil is attributed to ricinoleic acid which has been revealed as major compound in this plant (Raji et al., 2006).

Nonetheless, former studies have stated that as much as sperm motility provides useful information for diagnosing male infertility, it cannot be used in isolation as diagnostic parameter of infertility (Guzick et al., 2001) since numerous additional characteristics of spermatozoa are required to ensure successful fertilization with an oocyte (Aitken and Baker, 2006). These parameters include oxidation-antioxidant balance (Henkel, 2005), capacitation and acrosome reaction (Aitken, 2006), normal sperm mitochondrial membrane function (Marchetti et al., 2002) and DNA integrity of the sperm (Henkel et al., 2010).

4.5 Effect of aqueous root extract of *M. whitei* on intact-mitochondrial membrane potential of the sperm

Mitochondrial membrane potential is the parameter that best indicates mitochondrial function and is a reflection of mitochondrial energy production in form of adenosine triphosphate (ATP) (Donnelly et al., 2000). ATP can either be produced through oxidative phosphorylation which takes place in the mitochondria's intermediate tract or through glycolysis which occurs in the main tract (Piomboni et al., 2012). However, ATP production is termed to be more efficient in mitochondrial respiration than in glycolysis (Turner, 2003). The chemical energy (ATP) produced through both aerobic and anaerobic respiration can be used for sustaining sperm motility (Turner, 2003). Espinoza et al. (2009) termed mitochondrial membrane integrity to be a reflection of sperm quality, thus, rendering its evaluation highly recommendable to complement routine sperm analysis.

Besides, Paoli et al. (2011) suggested that sperm motility is dependent on the functional integrity of the mitochondria. This was well reflected in their study whereby the sperm total motility was positively correlated to intact sperm mitochondrial membrane potential. Furthermore, this relationship was confirmed in the current study, in which there was a significant positive association ($r=0.2452$; $P<0.0009$) between total motile and spermatozoa with intact MMP in the patient group. Further analysis of the patients sub-group that is oligozoospermic ($r=0.343$; $P<0.0002$) and asthenozoospermic ($r=0.018$; $P<0.0399$), also showed significant positive association. These findings support early results where there was improved sperm motility, and the MMP findings which revealed maintained MMP in the patient group and the subsequent sub-groups (oligozoospermic and asthenozoospermic). The improved percentage of sperm motility in relation to the percentage of intact MMP could be attributed to the maintained mitochondrial membrane potential, thus, enabling the breaking down of the reducing sugars (Quasie et al., 2010) found in *M. whitei* root extract through oxidative phosphorylation and glycolysis, hence, providing ATP energy fundamental in rendering the sperm motile since it assists in flagella movement (Troiano et al., 1998).

Most importantly, mitochondrial membrane potential has been termed as the most sensitive test of determining sperm quality (Marchetti et al., 2002) not forgetting its alteration has been associated with male infertility (Bourgeron, 2000). In the present study, further observation on the effect of *M. whitei* root extract on MMP showed that there was maintained mitochondrial membrane potential state of spermatozoa across all groups: one-way ANOVA analysis: donor ($P=0.0007$), patient ($P<0.0001$), oligozoospermic ($P=0.0001$), asthenozoospemic ($P=0.0004$) and normozoospermic ($P=0.0007$) in a dose-dependent manner. Therefore, the extract did not have a negative effect on the mitochondria hence, the mitochondrial membrane permeability and function was not altered. These observations may imply that Mondia does not contain cytotoxic compounds that may impair the mitochondrial

membrane nor cause any disruption to the mitochondria and supports the observation that the extract has no effect on viability.

Additionally, a study by Rossato et al. (2005) which evaluated the effect of cannabinoids and endocannabinoids components of *Cannabis sativa* (Marijuana) on mitochondrial function in human sperm, revealed that these compounds inhibited sperm mitochondrial activities in a dose-dependent manner. Hypothetically, cannabinoids are thought to interfere with mitochondrial electron transport which could be via depletion of NADH as well as interference with mitochondrial permeability transition pore complex. Similarly, *in vitro* effect of extract of *Typha capensis* (bulrush) was found to decrease mitochondrial membrane potential of spermatozoa (Henkel et al., 2009). On the other hand, aqueous extract of medicinal plants like *Ruta graveolus* (Rue) have been found to immobilize sperm motility without impairing sperm mitochondrial membrane potential and viability (Harat et al., 2008). Furthermore, Propolis (different plant products collected by bees) which can be described as bee glue containing complex chemical composition mainly flavonoids, was found to enhance the activity of mitochondrial respiratory complexes II and IV. However, the mechanism of action did not affect the coupling of the electron transport to ATP synthesis and mitochondrial membrane potential in permeabilized human spermatozoa *in vitro* (Cedikova et al., 2014). Whereas Ginsenoside plant-derived glycoside from *Panax ginseng*, has been shown to maintain mitochondrial membrane potential by preventing lipopolysaccharide stimulated cytokine production, sepsis and inflammation (Shen et al., 2007). Also, it has been reported to have anti-apoptotic and antioxidant properties (Hwang and Kim, 2013).

4.6 Effect of aqueous root extract of *M. whitei* on sperm DNA-fragmentation

Precise transmission of the paternal genomic information has been credited to sperm DNA integrity which has been identified to play a major role in male fertility as elevated sperm DNA fragmentation has been linked to reduced fertility levels (Hamamah et al., 1997; Agarwal and Said, 2003; Henkel et

al., 2004; Shamsi et al., 2011; Robinson et al., 2012). Additionally, spermatozoa from fertile men have been found to have less DNA fragmentation than those from infertile men (Zini and Libman, 2006).

The present study has no prior outcomes on DNA fragmentation on Mondia extract treated spermatozoa. On the whole, the results showed a trend (ANOVA trend analysis: P=0.004) towards a lower percentage of DNA-fragmented spermatozoa with increasing *M. whitei* extract concentration, especially in the patient, oligozoospermic and asthenozoospermic group (ANOVA trend analysis: patient, P=0.004; oligozoospermic, P=0.005, asthenozoospermic, P=0.0295). However, no significant effect was seen in the donor (normozoosermic) group. It may be hypothesized that washing of spermatozoa leads to the removal of seminal plasma which in turn gets rid of enzymes such as catalase and superoxide dismutases which scavenge for ROS (Shekarriz et al., 1995). Besides centrifugation increasing ROS generation in the already compromised patients' sperm. However, introduction of antioxidants from Mondia (phenols, flavonoids, Zinc and vitamin E) might have scavenged free radicals (Rahman, 2007) resulting in maintained DNA status of the spermatozoa.

Further analysis of the results in this study showed an inverse relationship between the percentage of sperm with DNA-fragmentation and percentage of ROS-positive spermatozoa in the patient (r=-0.3096; P<0.0001), oligozoospermic (r=-0.243; P=0.0091) and asthenozoospermic group (r=-0.034; P=0.0073). These results led to the hypothesis that the antioxidants (phenols, flavonoids, 2-hydroxy-4-methoxybenzaldehyde, vitamin E and zinc) found in Mondia extract could have acted by regulating the production of reactive oxygen species (ROS) hence, preventing oxidative stress (OS) since, antioxidants have previously been shown to decrease oxidative stress by directly scavenging ROS or by inhibiting cell proliferation secondary to the protein synthesis (Lobo et al., 2010).

Nevertheless, the imbalance of free radicals from excessive production of ROS and limited antioxidant defense results in OS (Doshi et al., 2012), which leads to sperm membrane and DNA damage (Agarwal

et al., 2006). Ordinarily, both enzymatic and non-enzymatic antioxidants scavenge free radical species and protect the body from over-exposure to oxidative stress (Doshi et al., 2012). In this case, Mondia roots have been found to contain non-enzymatic antioxidant such as phenols, flavonoids and vitamin E (Gopalakrishnan and Starlin, 2013). According to Moyo et al. (2010) secondary metabolites of phenolic and flavonoid compounds have been attributed for their antioxidant activities.

In addition, Bouba et al. (2010) reported anti-oxidant activity by assessing the high hydroxyl radical scavenging activity as a result of *in vivo* detection of phenolics, flavonoids and tannins. The roots also contain vitamins A, D, K, E and trace elements such as zinc, iron and calcium (Van Wyk, 2011). Vitamin E refers to a set of eight related tocopherols and tocotrienols fat soluble vitamins possessing antioxidant properties (Herrera and Barbas, 2001). This set of vitamins especially α-tocopherols, which is the most bioavailable vitamin E, protects membranes from oxidation by reacting with lipid radicals in a lipid chain peroxidation reaction hence, removing free radicals intermediates bringing the propagated reaction to an end (Brigelius-Flohe and Traber, 1999). Likewise, Lector (1996) found that vitamin E acts by protecting the spermatozoa against endogenous oxidative DNA and membrane damage, though, this vitamin is said to have minimal effects in improving the post-thaw sperm parameters (Lector, 1996). However, addition of vitamin E to cryoprotectants benefited post-thaw measurements such as motility and improved membrane integrity of bovine spermatozoa (Uysal et al., 2007). Besides, numerous studies have suggested that vitamin E supplementation can provide protection against sperm DNA damage (Lopes et al., 1998a; Russo, 2006). On the other hand, zinc and 2-hydroxy-4-methoxybenzaldehyde are an antioxidant that may regulate ROS production (Wang et al., 2003; Balasundaram et al., 2014).

Erasmus et al. (2012) showed that the extract of *Eurycoma longifolia* (Tongkat ali) containing alkaloids, quassinoids, quassinoid diterpenoids, eurycomoside, eurycolactone and laurycolactone did not have deleterious effect on sperm DNA integrity. Whereas, extract of *Cissampelos capensis*

(dawidjie) consisting of alkaloids such as bisbenzyltetrahydroisoquino group were reported to have led to DNA fragmentation of spermatozoa (Shalaweh et al., 2015). Additionally, other antioxidants may act by reducing lipid derived free-radicals with detrimental effects on sperm lipid peroxidation (Sharma and Agarwal, 1996). Therefore, it can be hypothesized that antioxidants found in the root extract of Mondia might have helped in the maintenance of DNA integrity of spermatozoa in the present study.

4.7 Effect of aqueous root extract of *M. whitei* on intrinsic reactive oxygen species production

As much as elevated amounts of reactive oxygen species (ROS) play an important role in the pathophysiology of sperm dysfunction (Aitken et al., 1998; Agarwal and Said, 2003), including impairment to the normal spermatozoa by inducing lipid peroxidation and DNA damage. Decreased (physiological) level have been found to be beneficial to spermatozoa (Agarwal and Said, 2003; Henkel, 2005; Aitken and Baker, 2006). For instance, preceding studies have shown that low levels of ROS production are essential and beneficial to normal sperm function like stimulation of sperm capacitation (O'Flaherty et al., 2006), enhancement zona pellucida binding (Aitken et al., 1989b; de Lamirande and Cagnon, 1993) and promotion of acrosome reaction and hyperactivation (de Lamirande and O'Flaherty, 2008).

Nevertheless, ROS can be produced either by intrinsic factors such as spermatozoa themselves or extrinsic sources such as leukocytes (Agarwal and Said, 2003; Henkel, 2005). Whereas spermatozoa have been shown to produce minor sources of ROS, leukocytes almost present in every ejaculate are the predominant derivatives of ROS. In the present study, dihydroethidium (DHE), which reacts with intracellular superoxide was used in the detection of ROS (de Lamirande et al., 1997a; Loft et al., 2003; Henkel, 2005; Aitken and Baker, 2006; Mupfiga et al., 2013; Shalaweh et al., 2015).

When investigating the effect of *M. whitei* extract on ROS production, the patient and combined group showed a higher tendency towards percentage of ROS-positive spermatozoa at higher concentrations

(repeated measure ANOVA: patients: P=0.005; combined: P=0.0015). Further analysis of the patient group revealed a significant trend in both groups (repeated measure ANOVA: oligozoospermic: P=0.001; asthenozoospermic: P=0.001). However, there was no change in the normozoospermic group (repeated measure ANOVA: P=0.661). Previous studies have demonstrated that ROS production by spermatozoa of oligozoospermic and asthenozoopermic patients is greater than that generated by spermatozoa of fertile donors (Aitken et al., 1998; Venkatesh et al., 2009), as sperm from patients exhibit defective sperm function which was associated with generating increased level of ROS (Aitken and Baker, 2013).

Currently, there are no studies that investigated the effect of *M. whitei* on intrinsic ROS production. The percentage of ROS-positive spermatozoa significantly and negatively correlated with the percentage of DNA-fragmented spermatozoa in the patient (r=-0.3096; P<0.0001), oligozoospermic (r=-0.243; P=0.0091) and asthenozoospermic (r=-0.034; P=0.0073) group as opposed to the donors (r=0.2023; P=0.0063). These results show that Mondia extract might have prevented the production of intrinsic ROS which have been found to positively correlate with DNA fragmentation (Henkel, 2005).

According to Wang et al. (2003), excessive ROS production is a possible inducer of apoptosis in human sperm; a process characterised by DNA fragmentation. Additionally, increased free radicals cause oxidative stress which leads to activation of apoptosis that results from induced cytochrome c and caspaces 9 and 3. Hence, reducing the high frequency of single- and double stranded DNA break in spermatozoa (Aitken and Koppers, 2011).

Excessive ROS disrupt both, the inner and outer mitochondrial membrane, which results in release of cytochrome c, a protein which activates caspases and induces apoptosis. Basically, the exposure of ROS to mitochondrial releases apoptosis-inducing factor, interacts with DNA directly and leads to DNA fragmentation (Zeitoun and Al-Damegh, 2014). Non-enzymatic antioxidants found in *Mondia*

whitei (phenolic, flavonoids, vitamin E, 2-hydroxy-4-methoxybenzaldehyde alkaloid and zinc) might have prevented oxidative stress by scavenging free radicals, thus, maintenance of DNA integrity. According to Degåspari and Waszczynskyj (2004), phenolic and flavonoid compounds exert antioxidants role due to their oxidoreduction properties, hence, neutralize ROS. Other studies have demonstrated that flavonoids scavenge free radicals such as 1,1-diphenyl-2-picrylhydrazyl (·DPPH), 2,2'-Azino-bis (3-ethyl-benzthiazoline-sulphonic acid) ($ABTS^{\cdot}$), superoxide ($\cdot O_2^-$), ($\cdot OH$) and peroxyl ($LOO\cdot$) radicals (Siddhuraju and Becker, 2003; Stagos et al., 2007).

Previous studied have reported vitamin E as a primary membrane protector in spermatozoa (Yousef et al., 2003) and is believed to be the initial defence mechanism against the peroxidation of polyunsaturated fatty acids (Liebler, 1993). Furthermore, studies have reported the ability of enzymatic antioxidants like vitamin E, to reduce ROS production (Foote, 1967; Bascetta et al., 1983; O'Flaherty et al., 1997). Hence, leading to the hypothesis that phytochemicals found in the root extract *M. whitei* inhibit the action of sperm intrinsic superoxide production that leads to DNA fragmentation and does not increase ROS by causing oxidative stress at therapeutic concentration (1.85µg/ml).

The scavenging ability of antioxidants helps neutralize the deleterious effects of free radical production (Askari et al., 1994; Dalvit et al., 1998; Bansal and Bilaspuri, 2010) due to their ability of eliminating oxidative stress (Miller et al., 1993; Kumar and Mahmood, 2001). In addition, enzymatic antioxidants such as superoxide dismutase (SOD), catalase (CAT), glutathione (GSH), and the glutathione peroxidase/ reductase system maintain the scavenging activities in the gonads and seminal fluid (Alvarez et al., 1987; Aprioku, 2013).

In order to create a healthy environment for fertilization, a proper balance referred to as oxidative stress status (OSS) between ROS generation and scavenging activities as well as the right timing for ROS production has to be maintained (Maneesh and Jayalekshmi, 2006; Cocuzza et al., 2007). Furthermore,

maintaining the balance between physiological generation of ROS in the spermatozoa and scavenging activities, as well as, their surrounding environment is of utmost importance (de Lamirande et al., 1997a). Physiological amounts of ROS are necessary for initiating capacitation (O'Flaherty et al., 1999), acrosome reaction (Villegas et al., 2003), hyperactivation of sperm motility (de Lamirande and Cagnon, 1993) and binding to zona pellucida and fusion with the oocyte (Sicherle et al., 2011). Contrary, excessive amounts of ROS have pathological effects on spermatozoa ranging from declined motility (Iwasaki and Gagnon, 1992), reduced sperm concentration (Aitken et al., 1991), sperm nuclear DNA damage (Twigg et al., 1998b) and apoptosis, hence, reduced fertilization (Sakkas et al., 1999).

As part of their physiological function mature spermatozoa are frequently exposed to ROS (either produced by the spermatozoa themselves or by seminal leukocytes) which causes oxidation. On the other hand, these sperm are also exposed to reductive status as a protection mechanism from antioxidants. Therefore, an equilibrium in oxidative status is paramount for normal physiological function of the spermatozoa (Henkel, 2011). Excess ROS production leads to oxidative stress (OS) which is an imbalance between oxidation and reduction. Therefore, limiting the proper functioning of mammalian spermatozoa by causing lipid peroxidation, formation of protein adducts and induction of oxidative DNA damage (Aitken et al., 2012). Though, a certain concentration of ROS is needed to trigger vital physiological reaction necessary in regulating hyperactivation and the ability of a spermatozoa to undergo acrosome reaction (Aitken et al., 2012). Then again, excessive antioxidants in the reproductive system results to reductive status which is not physiological and may induce cancer, rendering the treatment of patient with antioxidant debatable. Furthermore, there are no precise values of amount of oxidants or antioxidants to state when the redox equilibrium is reached. This dilemma is known as the oxidative paradox. For this reason, patients' semen should be analysed for ROS level and antioxidants capacity to ensure the fine balance between oxidants and antioxidants is maintained (Henkel, 2011).

4.8 Effect of aqueous root extract of *M. whitei* extract on capacitation, hyperactivation and acrosome reaction

Capacitation refers to the physiological changes spermatozoa must undergo in order to be able to penetrate and fertilize an egg (Overstreet and Bedford, 1974). Therefore, capacitation is the prerequisite for normal acrosome reaction (Yanagimachi, 1994) for sperm to penetrate the zona pellucida (Koehler et al., 1982). Acrosome reaction has been directly related to the fertility rates in males since only acrosome-reacted spermatozoa can penetrate the zona pellucida (Koehler et al., 1982).

Presently, there are no studies that investigated the effect of *M. whitei* on sperm capacitation and acrosome reaction. Though, in the current study, treatment of the sperm with the aqueous root extract of *M. whitei* did not result in any significant change nor trend in the percentage of capacitated spermatozoa and capacitated, acrosome-reacted cells across all groups as determined by the CTC stain. Since, the aqueous root extract of *M. whitei* does not trigger intrinsic superoxide production, capacitation is not triggered. Physiological ROS production is beneficial and essential to normal sperm function like stimulation of sperm capacitation, promote acrosome reaction and hyperactivation and enhances zona pellucida binding (Aitken et al., 1989a; de Lamirande and Cagnon, 1993; O'Flaherty et al., 2006; de Lamirande and O'Flaherty, 2008).

Nevertheless, sperm acquisition of fertilization ability depends on low and controlled concentration of ROS (de Lamirande and Gagnon, 1995). de Lamirande et al. (1997a) demonstrated that exogenous addition of superoxide anion induced human spermatozoa hyperactivation, capacitation and acrosome reaction. However, the mechanism of action of ROS mediated hyperactivation, capacitation and acrosome reaction are not fully understood (de Lamirande and Gagnon, 1995; Du Plessis et al., 2015). Though, the two processes that are believed to be responsible for physiological activation of adenyl cyclase, which is thought to play a role in human sperm capacitation are generation of ROS and calcium influx (de Lamirande et al., 1997a). On the other hand, physiological amounts of ROS production are

thought to be responsible in hyperactivation through a probable complex multiple influence and interaction (de Lamirande et al., 1997a). Yet, induction of acrosome reaction collectively depends on ROS generation, calcium influx, increase in adenyl cyclase activity and protein phosphorylation (Yanagimachi, 1994; Aitken et al., 1995; Leclerc and Kopf, 1995). Contrary, studies have indicated that ROS scavenges for superoxide anion and catalase prevented the effect of ROS (de Lamirande and Cagnon, 1993). Leading to the hypothesis that antioxidants (phenols, flavonoids, vitamin E, 2-hydroxy-4-methoxybenzaldehyde alkaloid and zinc) found in Mondia extract might have scavenged intrinsic superoxide anions hence, preventing hyperactivation, capacitation and acrosome reaction.

Furthermore, seminal plasma has been found to contain a high concentration of zinc as a membrane stabilizing compound, and is therefore considered a decapacitating factor (Andrews et al., 1994). A preliminary study by de Lamirande and Cagnon (1997) indicated that 50 µM of zinc decreased hyperactivation and capacitation on human spermatozoa. Yet, the percentage of sperm motility and vitality was not affected. Additionally, Riffo et al. (1992) observed that an addition of 100 µM of zinc to the incubation medium prevented human sperm capacitation and acrosome reaction. Hence, the assumption that the zinc content in Mondia might have led to the reduction of sperm hyperactivated motility and capacitation. Additionally, Mondia extract contains 2-hydroxy-4-methoxybenzaldehyde alkaloids, which have been found to possess free radical scavenging activities (Martey and He, 2010). Similarly, it may act through the same mechanism as the other mentioned antioxidant in Mondia thus, preventing hyperactivation, capacitation and acrosome reaction. Nonetheless, more studies need to be done on physiological effect of 2-hydroxy-4-methoxybenzaldehyde alkaloids on human sperm cells.

4.9 Conclusion

Previous studies have shown that Mondia root extract contains several phytochemical which are presumed to be responsible for the biological activities. These phytochemicals include antioxidants (phenols, flavonoids, vitamin E, 2-hydroxy-4-methoxybenzaldehyde alkaloids, and zinc) (Moyo et al.,

2010) of which free radical scavenging activities might have prevented oxidative stress by protecting the sperm from lipid peroxidation and consequently, maintained essential sperm characteristics such as motility, intact mitochondrial membrane potential and DNA damage (Halliwell, 1989). This was confirmed in the present study where sperm motility, intact MMP and DNA integrity were maintained. The presence of reducing sugars (Quasie et al., 2010) might have also influenced motility by providing energy in form of ATP through oxidative phosphorylation that takes place in a proper functioning mitochondria. In contrast, antioxidants scavenging activities might have compromised positive role of ROS on initiating hyperactivated motility, acrosome reaction and capacitation. According to (de Lamirande et al., 1997b) mild oxidative stress promote the fertilizing potential by enhancing hyperactivation, acrosome reaction and capacitation, through increased tyrosine phosphorylation. However, there is a thin line between physiological amount of ROS needed to facilitate the process of fertilization and pathological amount of ROS that is detrimental to the spermatozoa. Therefore, for proper functioning of the spermatozoa the threshold should be maintained. Having this in mind, it can be hypothesized increased deoxidation from antioxidant found in Mondia extract (phenolic, flavonoic, vitamin E, 2-hydroxy-4-methoxybenzaldehyde alkaloids, and zinc) inhibited sperm hyperactivated motility, capacitation and acrosome reaction.

The investigation of the effect of the aqueous root extract of *Mondia whitei* comprehensively sheds lights on the potential action on human male spermatozoa *in vitro*. Therefore, it can safely be assumed that the extract has no direct toxic effects on functional sperm parameters and does not trigger intrinsic ROS production at therapeutically used concentrations (<1.85 µg/ml). Since, phytochemicals found in Mondia do not increase ROS by causing oxidative stress the total motility, progressive motility, MMP and DNA integrity is maintained. In addition, results from the *in vitro* investigation suggest that the extract from Mondia may be beneficial especially to male patients (oligozoospermic and

asthenozoospermic) in terms of modulating sperm function but not as a possible treatment, since, it does not directly improve male fertility/sperm functions *in vitro.*

Moreover, previous *in vivo* studies have shown *Mondia whitei* extract to act via different mechanism as well to influence fertility. Therefore, in the quest to find solutions surrounding the issues of male infertility, many factors must be considered including a proper understanding of the hypothalamus-pituitary-gonadal axis and how it influences the Sertoli, Leydig and germ cells not forgetting the influence on FSH and LH. Additionally, basic knowledge about physiological and biochemical process involved in spermiogenesis will enable proper understanding of mechanism involved in abnormal sperm function. Besides, Mondia extract could have effect at any of the above point to influence fertility.

Therefore, continuous research on novel medicinal plants frequently used for treatments but lacking scientific validation should be encouraged, and more specific investigation should be done on Mondia root extract for a clear understanding on its mechanism of action. Furthermore, the traditional aspect of the mode of administration and preparation of the plant material must not be ignored.

CHAPTER 5: REFERENCES

Abdullahi, A. A., 2011. Trends and challenges of traditional medicine in Africa. African Journal of Traditional, Complementary and Alternative Medicines 8, 115-123.

Aboua, Y.G., du Plessis, S.S., Reichgelt, P., Brooks, N., 2009. The in vitro effects of superoxide, some commercially available antioxidants and red palm oil on sperm motility. Asian Journal Andrology 11, 695-702.

Achard, C., Courtillot, C., Lahuna, O., Méduri, G., Soufir, J.-C., Lière, P., Bachelot, A., Benyounes, H., Schumacher, M., Kuttenn, F., 2009. Normal spermatogenesis in a man with mutant luteinizing hormone. New England Journal of Medicine 361, 1856-1863.

Agarwal, A., Mulgund, A., Alshahrani, S., Assidi, M., Abuzenadah, A.M., Sharma, R., Sabanegh, E., 2014. Reactive oxygen species and sperm DNA damage in infertile men presenting with low level leukocytospermia. Reproductive Biology and Endocrinology 12, 126-136.

Agarwal, A., Mulgund, A., Hamada, A., Chyatte, M.R., 2015. A unique view on male infertility around the globe. Reproductive Biology and Endocrinology 13, 37-46.

Agarwal, A., Nallella, K.P., Allamaneni, S.S., Said, T.M., 2004. Role of antioxidants in treatment of male infertility: an overview of the literature. Reproductive biomedicine online 8, 616-627.

Agarwal, A., Said, T.M., 2003. Role of sperm chromatin abnormalities and DNA damage in male infertility. Human Reproduction Update 9, 331-345.

Agarwal, A., Sharma, R.K., Nallella, K.P., Thomas, A.J., Alvarez, J.G., Sikka, S.C., 2006. Reactive oxygen species as an independent marker of male factor infertility. Fertility and sterility 86, 878-885.

Agyemang, K., Han, L., Liu, E., Zhang, Y., Wang, T., Gao, X., 2013. Recent advances in Astragalus membranaceus anti-diabetic research: Pharmacological effects of its phytochemical constituents. Evidence-Based Complementary and Alternative Medicine 2013, 1-9.

Aitken, R., 2006. Sperm function tests and fertility. International journal of andrology 29, 69-75.

Aitken, R., Irvine, D., Wu, F., 1991. Prospective analysis of sperm-oocyte fusion and reactive oxygen species generation as criteria for the diagnosis of infertility. American journal of obstetrics and gynecology 164, 542-551.

Aitken, R., Paterson, M., Fisher, H., Buckingham, D., Van Duin, M., 1995. Redox regulation of tyrosine phosphorylation in human spermatozoa and its role in the control of human sperm function. Journal of Cell Science 108, 2017-2025.

Aitken, R.J., Baker, M.A., 2006. Oxidative stress, sperm survival and fertility control. Molecular and cellular endocrinology 250, 66-69.

Aitken, R.J., Baker, M.A., 2013. Causes and consequences of apoptosis in spermatozoa; contributions to infertility and impacts on development. International Journal of Developmental Biology 57, 265-272.

Aitken, R.J., Clarkson, J.S., Fishel, S., 1989a. Generation of reactive oxygen species, lipid peroxidation, and human sperm function. Biology of reproduction 41, 183-197.

Aitken, R.J., Clarkson, J.S., Hargreave, T.B., Irvine, D.S., Wu, F., 1989b. Analysis of the relationship between defective sperm function and the generation of reactive oxygen species in cases of oligozoospermia. J Androl 10, 214-220.

Aitken, R.J., Gordon, E., Harkiss, D., Twigg, J.P., Milne, P., Jennings, Z., Irvine, D.S., 1998. Relative impact of oxidative stress on the functional competence and genomic integrity of human spermatozoa. Biology of Reproduction 59, 1037-1046.

Aitken, R.J., Jones, K.T., Robertson, S.A., 2012. Reactive oxygen species and sperm function—in sickness and in health. Journal of andrology 33, 1096-1106.

Aitken, R.J., Koppers, A.J., 2011. Apoptosis and DNA damage in human spermatozoa. Asian Journal of Andrology 13, 36-42.

Aitken, R.J., West, K., Buckingham, D., 1994. Leukocytic infiltration into the human ejaculate and its association with semen quality, oxidative stress, and sperm function. Journal of andrology 15, 343-352.

Alvarez, J.G., Touchstone, J.C., Blasco, L., Storey, B.T., 1987. Spontaneous lipid peroxidation and production of hydrogen peroxide and superoxide in human spermatozoa. Superoxide dismutase as major enzyme protectant against oxygen toxicity. Journal of andrology 8, 338-348.

Andrews, J.C., Nolan, J.P., Hammerstedt, R.H., Bavister, B.D., 1994. Role of zinc during hamster sperm capacitation. Biology of reproduction 51, 1238-1247.

Anerao, A., Sharma, R., Rathore Mansee, G.A., 2010. Studies on human sperm motility and viability when treatment with rock salt. Journal of Pathology Research 1, 1-10.

Aprioku, J.S., 2013. Pharmacology of free radicals and the impact of reactive oxygen species on the testis. Journal of reproduction & infertility 14, 158-167.

Aremu, A., Cheesman, L., Finnie, J., Van Staden, J., 2011. Mondia whitei (Apocynaceae): A review of its biological activities, conservation strategies and economic potential. South African Journal of Botany 77, 960-971.

Argiolas, A., Melis, M., 2002. The neurophysiology of the sexual cycle. Journal of endocrinological investigation 26, 20-22.

Askari, H., Check, J., Peymer, N., Bollendorf, A., 1994. Effect of natural antioxidants tocopherol and ascorbic acids in maintenance of sperm activity during freeze-thaw process. Archives of andrology 33, 11-15.

Bakalczuk, G., Wdowiak, A., Lewicka, M., Sulima, M., Perovic, S., Anton, J., Kielak, M.A., 2016. Induction effectiveness of acrosome reaction in prepared human spermatozoa assessed using the CD46 surface antigen. European Journal of Medical Technology 1, 10-22.

Baker, M.E., 1995. Endocrine activity of plant-derived compounds: an evolutionary perspective. Experimental Biology and Medicine 208, 131-138.

Balasundaram, J., Selvaraj, S., Cv, C., 2014. Studies on phytochemical screening, antioxidant activity and extraction of active compound (swertiamarin) from leaf extract of enicostemma littorale. Asian Journal of Pharmaceutical and Clinical Research 146, 240-244.

Bansal, A.K., Bilaspuri, G., 2010. Impacts of oxidative stress and antioxidants on semen functions. Veterinary medicine international 137, 1-7.

Barroso, G., Morshedi, M., Oehninger, S., 2000. Analysis of DNA fragmentation, plasma membrane translocation of phosphatidylserine and oxidative stress in human spermatozoa. Human reproduction 15, 1338-1344.

Bartoov, B., Berkovitz, A., Eltes, F., Kogosowski, A., Menezo, Y., Barak, Y., 2002. Real-time fine morphology of motile human sperm cells is associated with IVF-ICSI outcome. Journal of Andrology 23, 1-8.

Bascetta, E., Gunstone, F.D., Walton, J.C., 1983. Electron spin resonance study of the role of vitamin E and vitamin C in the inhibition of fatty acid oxidation in a model membrane. Chemistry and physics of lipids 33, 207-210.

Batubara, I., Darusman, L., Mitsunaga, T., Rahminiwati, M., Djauhari, E., 2010. Potency of Indonesian medicinal plants as tyrosinase inhibitor and antioxidant agent. Journal of Biological sciences 10, 138-144.

Baumber, J., Ball, B.A., Gravance, C.G., Medina, V., Davies-Morel, M.C., 2000. The effect of reactive oxygen species on equine sperm motility, viability, acrosomal integrity, mitochondrial membrane potential and membrane lipid peroxidation. Journal of andrology 21, 895-902.

Belloc, S., Hazout, A., Zini, A., Merviel, P., Cabry, R., Chahine, H., Copin, H., Benkhalifa, M., 2014. How to overcome male infertility after 40: Influence of paternal age on fertility. Maturitas 78, 22-29.

Benkhalifa, M., Montjean, D., Belloc, S., Dalleac, A., Ducasse, M., Boyer, P., Merviel, P., Copin, H., 2014. Emerging molecular methods for male infertility investigation. Expert review of molecular diagnostics 14, 37-45.

Benoff, S., Barcia, M., Hurley, I.R., Cooper, G.W., Mandel, F.S., Heyner, S., Garside, W.T., Gilbert, B.R., Hershlag, A., 1996. Andrology: Classification of male factor infertility relevant to in-vitro fertilization insemination strategies using mannose ligands, acrosome status and anti-cytoskeletal antibodies. Human reproduction 11, 1905-1918.

Benoff, S., Hurley, I.R., Mandel, F.S., Cooper, G.W., Hershlag, A., 1997. Induction of the human sperm acrosome reaction with mannose-containing neoglycoprotein ligands. Molecular human reproduction 3, 827-837.

Benzie, I.F., Wachtel-Galor, S., 2011. Herbal medicine: biomolecular and clinical aspects. CRC Press.

Björndahl, L., Söderlund, I., Johansson, S., Mohammadieh, M., Pourian, M.R., Kvist, U., 2004. Why the WHO Recommendations for Eosin-Nigrosin Staining Techniques for Human Sperm Vitality Assessment Must Change. Journal of andrology 25, 671-678.

Björndahl, L., Söderlund, I., Kvist, U., 2003. Evaluation of the one-step eosin-nigrosin staining technique for human sperm vitality assessment. Human reproduction 18, 813-816.

Bouba, A., Njintang, Y., Scher, J., Mbofung, C., 2010. Phenolic compounds and radical scavenging potential of twenty Cameroonian spices. Agriculture and Biology Journal of North America 1, 213-224.

Bourgeron, T., 2000. Mitochondrial function and male infertility, The Genetic Basis of Male Infertility, Springer 28, 187-210.

Breitbart, H., Spungin, B., 1997. The biochemistry of the acrosome reaction. Molecular Human Reproduction 3, 195-202.

Brigelius-Flohe, R., Traber, M.G., 1999. Vitamin E: function and metabolism. The FASEB Journal 13, 1145-1155.

Brookes, K., Katsoulis, L., 2006. Bioactive components of Rhoicissus tridentata: a pregnancy-related traditional medicine. South African journal of science 10, 331-339.

Bunel, V., Hamel, M., Duez, P., Stevigny, C., 2014. Artifactual generation of an alkaloid in the course of Mondia whitei (Hook. f.) Skeels roots extraction: A clue to endogenous-formed bioactive compounds? Phytochemistry Letters 10, 101-106.

Calixto, J., 2000. Efficacy, safety, quality control, marketing and regulatory guidelines for herbal medicines (phytotherapeutic agents). Brazilian Journal of Medical and Biological Research 33, 179-189.

Carlsen, E., Giwercman, A., Keiding, N., Skakkebæk, N.E., 1992. Evidence for decreasing quality of semen during past 50 years. British medical journal 305, 609-613.

Carrell, D.T., 2013. Paternal influences on human reproductive success. Cambridge University Press.

Cates, W., Farley, T.M., Rowe, P.J., 1985. Worldwide patterns of infertility: is Africa different? The Lancet 326, 596-598.

Cayli, S., Sakkas, D., Vigue, L., Demir, R., Huszar, G., 2004. Cellular maturity and apoptosis in human sperm: creatine kinase, caspase-3 and Bcl-XL levels in mature and diminished maturity sperm. Molecular Human Reproduction 10, 365-372.

Cedikova, M., Miklikova, M., Stachova, L., Grundmanova, M., Tuma, Z., Vetvicka, V., Zech, N., Kralickova, M., Kuncova, J., 2014. Effects of the czech propolis on sperm mitochondrial function. Evidence-Based Complementary and Alternative Medicine 2014, 1-10.

Chang, L., Putcha, G., Deshmukh, M., Johnson, E., 2002. Mitochondrial involvement in the point of no return in neuronal apoptosis. Biochimie 84, 223-231.

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

Chipuk, J., Bouchier-Hayes, L., Green, D., 2006. Mitochondrial outer membrane permeabilization during apoptosis: the innocent bystander scenario. Cell Death & Differentiation 13, 1396-1402.

Clark, R.A., Klebanoff, S.J., 1978. Role of the classical and alternative complement pathways in chemotaxis and opsonization: studies of human serum deficient in C4. The Journal of Immunology 120, 1102-1108.

Clermont, Y., 1963. The cycle of the seminiferous epithelium in man. American Journal of Anatomy 112, 35-51.

Clermont, Y., Antar, M., 1973. Duration of the cycle of the seminiferous epithelium and the spermatogonial renewal in the monkey Macaca arctoides. American Journal of Anatomy 136, 153-165.

Cocuzza, M., Sikka, S.C., Athayde, K.S., Agarwal, A., 2007. Clinical relevance of oxidative stress and sperm chromatin damage in male infertility: an evidence based analysis. International brazilian journal of urology 33, 603-621.

Coetzee, K., Kruger, T., Menkveld, R., Swanson, R., Lombard, C., Acosta, A., 1989. Usefulness of sperm penetration assay in fertility predictions. Archives of andrology 23, 207-212.

Cooper, T., Björndahl, L., Vreeburg, J., Nieschlag, E., 2002. Semen analysis and external quality control schemes for semen analysis need global standardization. International journal of andrology 25, 306-311.

Cortes, D., Müller, J., Skakkebaek, N., 1987. Proliferation of Sertoli cells during development of the human testis assessed by stereological methods. International journal of andrology 10, 589-596.

Crouch, N., Nichols, G., Hutchings, A., 1998. Umondi: the versatile herb of Africa. Custos May, 24-25.

D'Agostino, R., Pearson, E.S., 1973. Tests for departure from normality. Empirical results for the distributions of b2 and√ b1. Biometrika 60, 613-622.

Dabaja, A.A., Schlegel, P.N., 2014. Medical treatment of male infertility. Translational Andrology and Urology 3, 9-16.

Dalvit, G., Cetica, P., Beconi, M., 1998. Effect of α-tocopherol and ascorbic acid on bovine in vitro fertilization. Theriogenology 49, 619-627.

de Castilhos, J., Marcuzzo, S., Forti, C.D., Frey, R.M., Stein, D., Achaval, M., Rasia-Filho, A.A., 2006. Further studies on the rat posterodorsal medial amygdala: dendritic spine density and effect of 8-OH-DPAT microinjection on male sexual behavior. Brain research bulletin 69, 131-139.

De Kretser, D., Loveland, K., Meinhardt, A., Simorangkir, D., Wreford, N., 1998. Spermatogenesis. Human Reproduction 13, 1-8.

de Lamirande, E., Cagnon, C., 1993. Human sperm hyperactivation and capacitation as parts of an oxidative process. Free Radical Biology and Medicine 14, 157-166.

De Lamirande, E., Gagnon, C., 1992. Reactive oxygen species and human spermatozoa: I. Effects on the motility of intact spermatozoa and on sperm axonemes. Journal of andrology 13, 368-368.

de Lamirande, E., Gagnon, C., 1995. Capacitation-associated production of superoxide anion by human spermatozoa. Free Radical Biology and Medicine 18, 487-495.

de Lamirande, E., Jiang, H., Zini, A., Kodama, H., Gagnon, C., 1997a. Reactive oxygen species and sperm physiology. Reviews of reproduction 2, 48-54.

de Lamirande, E., Leclerc, P., Gagnon, C., 1997b. Capacitation as a regulatory event that primes spermatozoa for the acrosome reaction and fertilization. Molecular Human Reproduction 3, 175-194.

de Lamirande, E., O'Flaherty, C., 2008. Sperm activation: role of reactive oxygen species and kinases. Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics 1784, 106-115.

De Smet, P.A., 1997. The role of plant-derived drugs and herbal medicines in healthcare. Drugs 54, 801-840.

Degåspari, C.H., Waszczynskyj, N., 2004. Propriedades antioxidantes de compostos fenólicos. Visão acadêmica 5, 33-40.

Dhar, M., Dhar, M., Dhawan, B., Mehrotra, B., Ray, C., 1968. Screening of Indian plants for biological activity: I. Indian journal of experimental biology 6, 232-247.

Digby, A., 2014. 8 South Africa's mixed economy of healthcare. Healthcare in Private and Public from the Early Modern Period to 2000. Routledge publishers, New York, U.S.A.

Dindyal, S., 2004. The sperm count has been decreasing steadily for many years in Western industrialised countries: Is there an endocrine basis for this decrease. The Internet Journal of Urology 2, 1-6.

Donnelly, E.T., O'Connell, M., McClure, N., Lewis, S.E., 2000. Differences in nuclear DNA fragmentation and mitochondrial integrity of semen and prepared human spermatozoa. Human Reproduction 15, 1552-1561.

Doshi, S.B., Khullar, K., Sharma, R.K., Agarwal, A., 2012. Role of reactive nitrogen species in male infertility. Reproductive Biology and Endocrinology 10, 109-120.

Du Plessis, S.S., Agarwal, A., Halabi, J., Tvrda, E., 2015. Contemporary evidence on the physiological role of reactive oxygen species in human sperm function. Journal of assisted reproduction and genetics 32, 509-520.

Durairajanayagam, D., Agarwal, A., Ong, C., 2015. Causes, effects and molecular mechanisms of testicular heat stress. Reproductive biomedicine online 30, 14-27.

Duran, E.H., Morshedi, M., Taylor, S., Oehninger, S., 2002. Sperm DNA quality predicts intrauterine insemination outcome: a prospective cohort study. Human Reproduction 17, 3122-3128.

Dyer, S.J., Abrahams, N., Mokoena, N., Van der Spuy, Z., 2004. 'You are a man because you have children': experiences, reproductive health knowledge and treatment-seeking behaviour among men suffering from couple infertility in South Africa. Human Reproduction 19, 960-967.

Dym, M., Raj, H.M., 1977. Response of adult rat Sertoli cells and Leydig cells to depletion of luteinizing hormone and testosterone. Biology of reproduction 17, 676-696.

Erasmus, N., Solomon, M., Fortuin, K., Henkel, R., 2012. Effect of Eurycoma longifolia Jack (Tongkat ali) extract on human spermatozoa in vitro. Andrologia 44, 308-314.

Erasto, P., Adebola, P., Grierson, D., Afolayan, A., 2005. An ethnobotanical study of plants used for the treatment of diabetes in the Eastern Cape Province, South Africa. African Journal of Biotechnology 4, 1458-1460.

Ernst, E., 2000. Prevalence of use of complementary/alternative medicine: a systematic review. Bulletin of the World Health Organization 78, 258-266.

Espinoza, J., Schulz, M., Sánchez, R., Villegas, J., 2009. Integrity of mitochondrial membrane potential reflects human sperm quality. Andrologia 41, 51-54.

Farnsworth, N.R., Soejarto, D.D., 1985. Potential consequence of plant extinction in the United States on the current and future availability of prescription drugs. Economic botany 39, 231-240.

Fernández-Guasti, A., Escalante, A.L., Ahlenius, S., Hillegaart, V., Larsson, K., 1992. Stimulation of 5-HT 1A and 5-HT 1B receptors in brain regions and its effects on male rat sexual behaviour. European journal of pharmacology 210, 121-129.

Firenzuoli, F., Gori, L., 2007. Herbal medicine today: clinical and research issues. Evidence-Based Complementary and Alternative Medicine 4, 37-40.

Folgerø, T., Bertheussen, K., Lindal, S., Torbergsen, T., Øian, P., 1993. Andrology: Mitochondrial disease and reduced sperm motility. Human reproduction 8, 1863-1868.

Foote, R., 1967. Influence of light and agitation on bovine spermatozoa stored with protective agents. Journal of dairy science 50, 1468-1474.

Foreman, M., Wernicke, J., 1990. Approaches for the development of oral drug therapies for erectile dysfunction, Seminars in urology 8, 107-112.

Franken, D.R., Oehninger, S., 2012. Semen analysis and sperm function testing. Asian Journal Andrology 14, 6-13.

Fujii, J., Tsunoda, S., 2011. Redox regulation of fertilisation and the spermatogenic process. Asian Journal Andrology 13, 420-423.

Gakunga, N., Sembajwe, L., John, K., Patrick, V., 2013. Phytochemical screening and antidiarrheal activity of ethanolic fresh root bark extract of Mondia whitei in albino rats. Journal Pharmacy Science Innovotion 2, 1-6.

Gauthaman, K., Ganesan, A.P., 2008. The hormonal effects of Tribulus terrestris and its role in the management of male erectile dysfunction–an evaluation using primates, rabbit and rat. Phytomedicine 15, 44-54.

Ge, R., Chen, G., Hardy, M.P., 2009. The role of the Leydig cell in spermatogenic function, Molecular Mechanisms in Spermatogenesis, Springer 379, 255-269.

Gelfand, M., Mavi, S., Drummond, R., Ndemera, B., 1985. The traditional medical practitioner in Zimbabwe: his principles of practice and pharmacopoeia. Mambo Press Gweru.

George, J.W., Dille, E.A., Heckert, L.L., 2011. Current concepts of follicle-stimulating hormone receptor gene regulation. Biology of reproduction 84, 7-17.

Gericke, N., 2011. Muthi to medicine. South African Journal of Botany 77, 850-856.

Gilbert, S.F., 2000. Developmental biology: The anatomical tradition. Saunders, Philadelphia, U.S.A.

Go, K., Wolf, D., 1985. Albumin-mediated changes in sperm sterol content during capacitation. Biology of reproduction 32, 145-153.

Gopalakrishnan, V.K., Starlin, T., 2013. Enzymatic and non-enzymatic antioxidant properties of Tylophora pauciflora wight and arn.–an in vitro study. Asian Journal of Pharmaceutical and Clinical Research 6, 68-71.

Green, C., Cockle, S., Watson, P., Fraser, L., 1996. A possible mechanism of action for fertilization promoting peptide, a TRH-related tripeptide that promotes capacitation and fertilizing ability in mammalian spermatozoa. Molecular reproduction and development 45, 244-252.

Grimm, S., Brdiczka, D., 2007. The permeability transition pore in cell death. Apoptosis 12, 841-855.

Grunewald, S., Said, T., Paasch, U., Glander, H.J., Agarwal, A., 2008. Relationship between sperm apoptosis signalling and oocyte penetration capacity. International journal of andrology 31, 325-330.

Gundidza, G., Mmbengwa, V., Magwa, M., Ramalivhana, N., Mukwevho, N., Ndaradzi, W., Samie, A., 2009. Aphrodisiac properties of some Zimbabwean medicinal plants formulations. African Journal of Biotechnology 8, 7164-7169.

Guzick, D.S., Overstreet, J.W., Factor-Litvak, P., Brazil, C.K., Nakajima, S.T., Coutifaris, C., Carson, S.A., Cisneros, P., Steinkampf, M.P., Hill, J.A., 2001. Sperm morphology, motility, and concentration in fertile and infertile men. New England Journal of Medicine 345, 1388-1393.

Hadley, M.A., Byers, S.W., Suárez-Quian, C.A., Kleinman, H.K., Dym, M., 1985. Extracellular matrix regulates Sertoli cell differentiation, testicular cord formation, and germ cell development in vitro. The Journal of cell biology 101, 1511-1522.

Hall, J.E., Guyton, A.C., 2011. Textbook of medical physiology. Saunders London.

Halliwell, B., 1989. Free radicals, reactive oxygen species and human disease: a critical evaluation with special reference to atherosclerosis. British journal of experimental pathology 70, 737-748.

Halliwell, B., 1996. Antioxidants in human health and disease. Annual review of nutrition 16, 33-50.

Halliwell, B., Gutteridge, J., 1999. The chemistry of free radicals and related 'reactive species'. Free radicals in biology and medicine 17, 3-16.

Hamada, A., Esteves, S.C., Nizza, M., Agarwal, A., 2012. Unexplained male infertility: diagnosis and management. International Brazilian journal of urolology 38, 576-594.

Hamamah, S., Fignon, A., Lansac, J., 1997. The effect of male factors in repeated spontaneous abortion: lesson from in-vitro fertilization and intracytoplasmic sperm injection. Human reproduction update 3, 393-400.

Harat, Z.N., Sadeghi, M.R., Sadeghipour, H.R., Kamalinejad, M., Eshraghian, M.R., 2008. Immobilization effect of Ruta graveolens L. on human sperm: a new hope for male contraception. Journal of ethnopharmacology 115, 36-41.

Henkel, R., 2005. The impact of oxidants on sperm function. Andrologia 37, 205-206.

Henkel, R., 2007. DNA fragmentation and its influence on fertilization and pregnancy outcome. Male Infertility: Diagnosis and Treatment. London: Informa 2007, 277-290.

Henkel, R., 2012. ROS and semen quality, Studies on Men's Health and Fertility, Springer 2016, 301-323.

Henkel, R., Fransman, W., Hipler, U., Schreiber, G., 2009. Typha capensis extracts decrease ROS production and affect human sperm functions. African Journal of Traditional, Complementary and Alternative medicines (AJTCAM) 6, 438-439.

Henkel, R., Hajimohammad, M., Stalf, T., Hoogendijk, C., Mehnert, C., Menkveld, R., Gips, H., Schill, W.-B., Kruger, T.F., 2004. Influence of deoxyribonucleic acid damage on fertilization and pregnancy. Fertility and sterility 81, 965-972.

Henkel, R., Hoogendijk, C., Bouic, P., Kruger, T., 2010. TUNEL assay and SCSA determine different aspects of sperm DNA damage. Andrologia 42, 305-313.

Henkel, R., Kierspel, E., Hajimohammad, M., Stalf, T., Hoogendijk, C., Mehnert, C., Menkveld, R., Schill, W.-B., Kruger, T.F., 2003. DNA fragmentation of spermatozoa and assisted reproduction technology. Reproductive biomedicine online 7, 477-484.

Henkel, R.R., 2011. Leukocytes and oxidative stress: dilemma for sperm function and male fertility. Asian J Androl 13, 43-52.

Herrera, E., Barbas, C., 2001. Vitamin E: action, metabolism and perspectives. Journal of physiology and biochemistry 57, 43-56.

Hilton-Taylor, C., 1996. Red data list of Southern African plants. National Botanical Institute. South Africa.

Hirano, Y., Shibahara, H., Obara, H., Suzuki, T., Takamizawa, S., Yamaguchi, C., Tsunoda, H., Sato, I., 2001. Andrology: Relationships between sperm motility characteristics assessed by the computer-

aided sperm analysis (CASA) and fertilization rates in vitro. Journal of assisted reproduction and genetics 18, 215-220.

Hogarth, C.A., Griswold, M.D., 2010. The key role of vitamin A in spermatogenesis. The Journal of clinical investigation 120, 956-962.

Hossain, A., Rizk, B., Barik, S., Huff, C., Thorneycroft, I., 1998. Time course of hypo-osmotic swellings of human spermatozoa: evidence of ordered transition between swelling subtypes. Human Reproduction 13, 1578-1583.

Howards, S.S., 1995. Treatment of male infertility. New England Journal of Medicine 332, 312-317.

Hughes, C.M., Lewis, S.E., McKelvey-Martin, V.J., Thompson, W., 1996. A comparison of baseline and induced DNA damage in human spermatozoa from fertile and infertile men, using a modified comet assay. Molecular human reproduction 2, 613-619.

Hughes, G.D., Mbamalu, O.N., 2015. Integrative medicine in resource constrained communities in South Africa: Embracing indigenous knowledge and traditional (herbal) medicine practice. Revista da ABPN 7, 214-235.

Huneau, D., Crozet, N., Ahmed-Ali, M., 1994. Estrous sheep serum as a potent agent for ovine IVF: effect on cholesterol efflux from spermatozoa and the acrosome reaction. Theriogenology 42, 1017-1028.

Hutchings, A., Scott, A.H., Lewis, G., Cunningham, A.B., 1996. Zulu medicinal plants: an inventory. University of Kwazulu Natal Press.

Hwang, Y.J., Kim, D.Y., 2013. Effects of ginsenoside-Rg1 on post-thawed miniature pig sperm motility, mitochondria activity, and membrane integrity. Journal of Embryo Transfer 28, 63-71.

Ickowicz, D., Finkelstein, M., Breitbart, H., 2012. Mechanism of sperm capacitation and the acrosome reaction: role of protein kinases. Asian Journal Andrology 14, 816-821.

Irvine, D.S., Twigg, J.P., Gordon, E.L., Fulton, N., Milne, P.A., Aitken, R., 2000. DNA integrity in human spermatozoa: relationships with semen quality. Journal of andrology 21, 33-44.

Iwalewa, E., Adewunmi, C., Omisore, N., Adebanji, O., Azike, C., Adigun, A., Adesina, O., Olowoyo, O., 2005. Pro-and antioxidant effects and cytoprotective potentials of nine edible vegetables in southwest Nigeria. Journal of medicinal food 8, 539-544.

Iwasaki, A., Gagnon, C., 1992. Formation of reactive oxygen species in spermatozoa of infertile patients. Fertility and sterility 57, 409-416.

Iwu, M.M., 2014. Handbook of African medicinal plants. CRC press. Nigeria.

Jørgensen, N., Andersen, A.-G., Eustache, F., Irvine, D.S., Suominen, J., Petersen, J.H., Andersen, A.N., Auger, J., Cawood, E.H., Horte, A., 2001. Regional differences in semen quality in Europe. Human Reproduction 16, 1012-1019.

Kamatenesi-Mugisha, M., Oryem-Origa, H., 2005. Traditional herbal remedies used in the management of sexual impotence and erectile dysfunction in western Uganda. African Health Sciences 5, 40-49.

Kamel, R.M., 2010. Management of the infertile couple: an evidence-based protocol. Reproductive Biology and Endocrinology 8, 21.

Karpuz, V., Göktürk, A., Koyutürk, M., 2007. The effects of sperm morphology and motility on the outcomes of intracytoplasmic sperm injection. International Journal of Technical Research and Applications 2, 2230-2242.

Kasai, T., Ogawa, K., Mizuno, K., Nagai, S., Uchida, Y., Ohta, S., Fujie, M., Suzuki, K., Hirata, S., Hoshi, K., 2002. Relationship between sperm mitochondrial membrane potential, sperm motility, and fertility potential. Asian journal of andrology 4, 97-104.

Khillare, B., Shrivastav, T., 2003. Spermicidal activity of Azadirachta indica (neem) leaf extract. Contraception 68, 225-229.

Kim, S., Agca, C., Agca, Y., 2013. Effects of various physical stress factors on mitochondrial function and reactive oxygen species in rat spermatozoa. Reproduction, Fertility and Development 25, 1051-1064.

Klimowicz-Bodys, M., Batkowski, F., Ochrem, A., Savič, M., 2012. Comparison of assessment of pigeon sperm viability by contrast-phase microscope (eosin-nigrosin staining) and flow cytometry (SYBR-14/propidium iodide (PI) staining)[evaluation of pigeon sperm viability]. Theriogenology 77, 628-635.

Knez, J., 2013. Endocrine-disrupting chemicals and male reproductive health. Reproductive biomedicine online 26, 440-448.

Koehler, J.K., Decurtis, I., Stenchever, M.A., Smith, D., 1982. Interaction of human sperm with zona-free hamster eggs: A freeze-fracture study. Gamete Research 6, 371-386.

Koorbanally, N.A., Mulholland, D.A., Crouch, N.R., 2000. Isolation of isovanillin from aromatic roots of the medicinal African liane, Mondia whitei. Journal of herbs, spices & medicinal plants 7, 37-43.

Kruger, T.F., Acosta, A.A., Simmons, K.F., Swanson, R.J., Matta, J.F., Veeck, L.L., Morshedi, M., Brugo, S., 1987. New method of evaluating sperm morphology with predictive value for human in vitro fertilization. Urology 30, 248-251.

Kubo, I., Kinst-Hori, I., 1999a. 2-Hydroxy-4-methoxybenzaldehyde: a potent tyrosinase inhibitor from African medicinal plants. Planta Medica 65, 019-022.

Kubo, I., Kinst-Hori, I., 1999b. 2-Hydroxy-4-methoxybenzaldehyde: a potent tyrosinase inhibitor from African medicinal plants. Planta Medica 65, 19-22.

Kuiri-Hänninen, T., Sankilampi, U., Dunkel, L., 2014. Activation of the hypothalamic-pituitary-gonadal axis in infancy: minipuberty. Hormone Research in Paediatrics 82, 73-80.

Kumar, H., Mahmood, S., 2001. The use of fast acting antioxidants for the reduction of cow placental retention and subsequent endometritis. The Indian Journal of Animal Sciences 71.

Kumar, P.S., Subramoniam, A., Pushpangadan, P., 2000. Aphrodisiac activity of Vanda tessellata (Roxb.) Hook exdon extract in male mice. Indian Journal of pharmacology 32, 300-304.

Lampiao, F., Krom, D., Plessis, S.S.d., 2008. The in vitro effects of Mondia whitei on human sperm motility parameters. Phytotherapy Research 22, 1272-1273.

Larsen, L., Scheike, T., Jensen, T.K., Bonde, J.P., Ernst, E., Hjollund, N.H., Zhou, Y., Skakkebæk, N.E., Giwercman, A., Team, D.F.P.P.S., 2000. Computer-assisted semen analysis parameters as predictors for fertility of men from the general population. Human Reproduction 15, 1562-1567.

Leclerc, P., Kopf, G.S., 1995. Mouse sperm adenylyl cyclase: general properties and regulation by the zona pellucida. Biology of reproduction 52, 1227-1233.

Lector, C., 1996. Oxidative stress and role of antioxidants in normal and abnormal sperm function. Frontiers in Bioscience 1, e78-86.

Lee, M.A., Trucco, G.S., Bechtol, K.B., Wummer, N., Kopf, G.S., Blasco, L., Storey, B.T., 1987. Capacitation and acrosome reactions in human spermatozoa monitored by a chlortetracycline fluorescence assay. Fertility and sterility 48, 649-658.

Leke, R.J., Oduma, J.A., Bassol-Mayagoitia, S., Bacha, A.M., Grigor, K.M., 1993. Regional and geographical variations in infertility: effects of environmental, cultural, and socioeconomic factors. Environmental health perspectives 101, 73.

Liebler, D.C., 1993. The role of metabolism in the antioxidant function of vitamin E. Critical reviews in toxicology 23, 147-169.

Liu, J., Liang, P., Yin, C., Wang, T., Li, H., Li, Y., Ye, Z., 2004. Effects of several Chinese herbal aqueous extracts on human sperm motility in vitro. Andrologia 36, 78-83.

Lobo, V., Patil, A., Phatak, A., Chandra, N., 2010. Free radicals, antioxidants and functional foods: Impact on human health. Pharmacognosy reviews 4, 118.

Loft, S., Kold-Jensen, T., Hjollund, N.H., Giwercman, A., Gyllemborg, J., Ernst, E., Olsen, J., Scheike, T., Poulsen, H.E., Bonde, J.P., 2003. Oxidative DNA damage in human sperm influences time to pregnancy. Human Reproduction 18, 1265-1272.

Lohiya, N.K., Kothari, L.K., Manivannan, B., Mishra, P.K., Pathak, N., 2000. Human sperm immobilization effect of Carica papaya seed extracts: an in vitro study. Asian journal of andrology 2, 103-110.

Lopes, S., Jurisicova, A., Sun, J.-G., Casper, R.F., 1998a. Reactive oxygen species: potential cause for DNA fragmentation in human spermatozoa. Human Reproduction 13, 896-900.

Lopes, S., Sun, J.-G., Jurisicova, A., Meriano, J., Casper, R.F., 1998b. Sperm deoxyribonucleic acid fragmentation is increased in poor-quality semen samples and correlates with failed fertilization in intracytoplasmic sperm injection. Fertility and sterility 69, 528-532.

Ma, X.Q., Shi, Q., Duan, J., Dong, T.T., Tsim, K.W., 2002. Chemical analysis of Radix Astragali (Huangqi) in China: a comparison with its adulterants and seasonal variations. Journal of agricultural and food chemistry 50, 4861-4866.

Macedo, C.C., 2012. Uso do sistema de cultivo bidimensional e tridimensional para diferenciação de células-tronco espermatogoniais e indução da espermatogênese in vitro em murinos. Universidade estadual paulista. UNESP.

Mackay, S., 2000. Gonadal development in mammals at the cellular and molecular levels. International review of cytology 200, 47-99.

Magistrini, M., Guitton, E., Levern, Y., Nicolle, J.C., Vidament, M., Kerboeuf, D., Palmer, E., 1997. New staining methods for sperm evaluation estimated by microscopy and flow cytometry. Theriogenology 48, 1229-1235.

Mahomoodally, M.F., 2013. Traditional medicines in Africa: an appraisal of ten potent African medicinal plants. Evidence-Based Complementary and Alternative Medicine 2013, 1-14.

Mander, M., Ntuli, L., Diederichs, N., Mavundla, K., 2007. Economics of the traditional medicine trade in South Africa: health care delivery. South African health review 111, 189-196.

Maneesh, M., Jayalekshmi, H., 2006. Role of reactive oxygen species and antioxidants on pathophysiology of male reproduction. Indian Journal of Clinical Biochemistry 21, 80-89.

Mann, T., Lutwak-Mann, C., 1981. Biochemistry of Spermatozoa: Chemical and Functional Correlations in Ejaculated Semen, Andrological Aspect, Male Reproductive Function and Semen, Springer 14, 195-268.

Marchetti, C., Jouy, N., Leroy-Martin, B., Defossez, A., Formstecher, P., Marchetti, P., 2004. Comparison of four fluorochromes for the detection of the inner mitochondrial membrane potential in human spermatozoa and their correlation with sperm motility. Human reproduction 19, 2267-2276.

Marchetti, C., Obert, G., Deffosez, A., Formstecher, P., Marchetti, P., 2002. Study of mitochondrial membrane potential, reactive oxygen species, DNA fragmentation and cell viability by flow cytometry in human sperm. Human Reproduction 17, 1257-1265.

Marieb, E.N., Hoehn, K., 2007. Human anatomy & physiology. Pearson Education. Springer, U.S.A.

Martey, O., He, X., 2010. Possible mode of action of Mondia whitei: an aphrodisiac used in the management of erectile dysfunction. Journal of Pharmacology and Toxicology 5, 460-468.

Martini, F.H., Nath, J.L., Bartholomew, E.F., 2015. Fundamentals of Anatomy and Physiology. 2005, Benjamin Cummings.

Matzuk, M.M., Lamb, D.J., 2008. The biology of infertility: research advances and clinical challenges. Nature medicine 14, 1197-1213.

Meccariello, R., Fasano, S., Pierantoni, R., Cobellis, G., 2014. Modulators of hypothalamic–pituitary–gonadal axis for the control of spermatogenesis and sperm quality in vertebrates. Frontiers in endocrinology 5, 135-147.

Meehan, T., Schlatt, S., O'Bryan, M.K., de Kretser, D.M., Loveland, K.L., 2000. Regulation of germ cell and Sertoli cell development by activin, follistatin, and FSH. Developmental biology 220, 225-237.

Meilzel, S., 1984. The importance of hydrolytic enzymes to an exocytotic event, the mammalian sperm acrosome reaction. Biological Reviews 59, 125-157.

Melis, M.R., Argiolas, A., 1995. Dopamine and sexual behavior. Neuroscience & Biobehavioral Reviews 19, 19-38.

Merchant, R., Gandhi, G., Allahbadia, G.N., 2011. In vitro fertilization/intracytoplasmic sperm injection for male infertility. Indian Journal of Urology 27, 121.

Miki, K., 2006. Energy metabolism and sperm function. Society of Reproduction and Fertility supplement 65, 309-325.

Miller, J., Brzezinska-Slebodzinska, E., Madsen, F., 1993. Oxidative stress, antioxidants, and animal function. Journal of dairy science 76, 2812-2823.

Miyamoto, T., Tsujimura, A., Miyagawa, Y., Koh, E., Namiki, M., Sengoku, K., 2011. Male infertility and its causes in human. Advances in urology 2012, 10-17.

Moeng, E., Potgieter, M., 2011. The trade of medicinal plants by muthi shops and street vendors in the Limpopo Province, South Africa. Journal of Medicinal Plants Research 5, 558-564.

Mortimer, D., 1994. Practical laboratory andrology. Oxford University Press on Demand. Ney York, U.S.A.

Mortimer, D., Pandya, I., Sawers, R., 1986. Relationship between human sperm motility characteristics and sperm penetration into human cervical mucus in vitro. Journal of reproduction and fertility 78, 93-102.

Mortimer, S.T., 1997. A critical review of the physiological importance and analysis of sperm movement in mammals. Human reproduction update 3, 403-439.

Mortimer, S.T., van der Horst, G., Mortimer, D., 2015. The future of computer-aided sperm analysis. Asian journal of andrology 17, 545-553.

Moskovtsev, S.I., Librach, C.L., 2013. Methods of sperm vitality assessment. Spermatogenesis: Methods and Protocols 927, 13-19.

Moustafa, M.H., Sharma, R.K., Thornton, J., Mascha, E., Abdel-Hafez, M.A., Thomas, A.J., Agarwal, A., 2004. Relationship between ROS production, apoptosis and DNA denaturation in spermatozoa from patients examined for infertility. Human Reproduction 19, 129-138.

Moyo, M., Ndhlala, A.R., Finnie, J.F., Van Staden, J., 2010. Phenolic composition, antioxidant and acetylcholinesterase inhibitory activities of Sclerocarya birrea and Harpephyllum caffrum (Anacardiaceae) extracts. Food Chemistry 123, 69-76.

Muhammad, B.Y., Awaisu, A., 2008. The need for enhancement of research, development, and commercialization of natural medicinal products in Nigeria: Lessons from the Malaysian experience. African Journal of Traditional, Complementary, and Alternative Medicines 5, 120-130.

Mundy, A., Ryder, T., Edmonds, D., 1995. Asthenozoospermia and the human sperm mid-piece. Human Reproduction 10, 116-119.

Mupfiga, C., Fisher, D., Kruger, T., Henkel, R., 2013. The relationship between seminal leukocytes, oxidative status in the ejaculate, and apoptotic markers in human spermatozoa. Systems biology in reproductive medicine 59, 304-311.

Nantia, E., Moundipa, P., Monsees, T., Carreau, S., 2009. Medicinal plants as potential male anti-infertility agents: a review. Andrologie 19, 148-158.

Neergaard, J.S., Rasmussen, H.B., Stafford, G.I., Van Staden, J., Jäger, A.K., 2010. Serotonin transporter affinity of (−)-loliolide, a monoterpene lactone from Mondia whitei. South African Journal of Botany 76, 593-596.

Ngcobo, M., Nkala, B., Moodley, I., Gqaleni, N., 2012. Recommendations for the development of regulatory guidelines for registration of traditional medicines in South Africa. African Journal of Traditional, Complementary and Alternative Medicines 9, 59-66.

Nieschlag, E., Behre, H.M., Nieschlag, S., 1997. Andrology. Springer. U.S.A.

Nutsch, V.L., Will, R.G., Robison, C.L., Martz, J.R., Tobiansky, D.J., Dominguez, J.M., 2016. Colocalization of Mating-Induced Fos and D2-Like Dopamine Receptors in the Medial Preoptic Area: Influence of Sexual Experience. Frontiers in behavioral neuroscience 10, 75-89.

O'Flaherty, C., Beconi, M., Beorlegui, N., 1997. Effect of natural antioxidants, superoxide dismutase and hydrogen peroxide on capacitation of frozen-thawed bull spermatozoa. Andrologia 29, 269-275.

O'Flaherty, C., Beorlegui, N., Beconi, M., 1999. Reactive oxygen species requirements for bovine sperm capacitation and acrosome reaction. Theriogenology 52, 289-301.

O'Flaherty, C., de Lamirande, E., Gagnon, C., 2006. Positive role of reactive oxygen species in mammalian sperm capacitation: triggering and modulation of phosphorylation events. Free Radical Biology and Medicine 41, 528-540.

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

Ogbuewu, I.P., Unamba-Oparah, I.C., Odoemenam, V.U., Etuk, I.F., Okoli, I.C., 2011. The potentiality of medicinal plants as the source of new contraceptive principles in males. North American journal of medical sciences 3, 255-265.

Oketch-Rabah, H.A., 2012. Mondia whitei, a medicinal plant from Africa with aphrodisiac and antidepressant properties: a review. Journal of dietary supplements 9, 272-284.

Ola, T.M., 2012. Assisted Reproductive Technology in Nigeria: Flawed or Favored? International Journal of Social Science and Humanity 2, 331-343.

Ollero, M., Gil-Guzman, E., Lopez, M.C., Sharma, R.K., Agarwal, A., Larson, K., Evenson, D., Thomas, A.J., Alvarez, J.G., 2001. Characterization of subsets of human spermatozoa at different stages of maturation: implications in the diagnosis and treatment of male infertility. Human Reproduction 16, 1912-1921.

Orth, J.M., GUNSALUS, G.L., LAMPERTI, A.A., 1988. Evidence From Sertoli Cell-Depleted Rats Indicates That Spermatid Number in Adults Depends on Numbers of Sertoli Cells Produced During Perinatal Development*. Endocrinology 122, 787-794.

Orth, J.M., Jester, W.F., Li, L.-H., Laslett, A.L., 2000. Gonocyte-Sertoli cell interactions during development of the neonatal rodent testis. Current topics in developmental biology 50, 103-124.

Overstreet, J., Bedford, J., 1974. Importance of sperm capacitation for gamete contact in the rabbit. Journal of reproduction and fertility 39, 393-398.

Overstreet, J.W., Yanagimachi, R., Katz, D.F., Hayashi, K., Hanson, F.W., 1980. Penetration of human spermatozoa into the human zona pellucida and the zona-free hamster egg: a study of fertile donors and infertile patients. Fertility and sterility 33, 534-542.

Ozmen, B., Caglar, G., Koster, F., Schopper, B., Diedrich, K., Al-Hasani, S., 2007. Relationship between sperm DNA damage, induced acrosome reaction and viability in ICSI patients. Reproductive biomedicine online 15, 208-214.

Pacey, A., 2006. Is quality assurance in semen analysis still really necessary? A view from the andrology laboratory. Human reproduction 21, 1105-1109.

Paoli, D., Gallo, M., Rizzo, F., Baldi, E., Francavilla, S., Lenzi, A., Lombardo, F., Gandini, L., 2011. Mitochondrial membrane potential profile and its correlation with increasing sperm motility. Fertility and sterility 95, 2315-2319.

Papaioannou, K., Murphy, R., Monks, R., Hynes, N., Ryan, M., Boland, M., Roche, J., 1997. Assessment of viability and mitochondrial function of equine spermatozoa using double staining and flow cytometry. Theriogenology 48, 299-312.

Patnam, R., Kadali, S.S., Koumaglo, K.H., Roy, R., 2005. A chlorinated coumarinolignan from the African medicinal plant, Mondia whitei. Phytochemistry 66, 683-686.

Pefile, S., 2005. South African legislation on traditional medicine. Science and Development Network. World health organisation, bulletin.

Pei, J., Strehler, E., Noss, U., Abt, M., Piomboni, P., Baccetti, B., Sterzik, K., 2005. Quantitative evaluation of spermatozoa ultrastructure after acupuncture treatment for idiopathic male infertility. Fertility and sterility 84, 141-147.

Pernasetti, F., Vasilyev, V.V., Rosenberg, S.B., Bailey, J.S., Huang, H.-J., Miller, W.L., Mellon, P.L., 2001. Cell-Specific Transcriptional Regulation of Follicle-Stimulating Hormone-β by Activin and Gonadotropin-Releasing Hormone in the LβT2 Pituitary Gonadotrope Cell Model 1. Endocrinology 142, 2284-2295.

Petersen, B.H., Lammel, C.J., Stites, D.P., Brooks, G.F., 1980. Human seminal plasma inhibition of complement. The Journal of laboratory and clinical medicine 96, 582-591.

Piomboni, P., Focarelli, R., Stendardi, A., Ferramosca, A., Zara, V., 2012. The role of mitochondria in energy production for human sperm motility. International journal of andrology 35, 109-124.

Plant, T.M., Marshall, G.R., 2001. The functional significance of FSH in spermatogenesis and the control of its secretion in male primates. Endocrine Reviews 22, 764-786.

Quasie, O., Martey, O.N.-K., Nyarko, A.K., Gbewonyo, W., Okine, L., 2010. Modulation of penile erection in rabbits by Mondia whitei: possible mechanism of action. African Journal of Traditional, Complementary and Alternative Medicines 7, 241-252.

Quinn, A., Koopman, P., 2012. The molecular genetics of sex determination and sex reversal in mammals, Seminars in reproductive medicine 30, 351-363.

Quinn, P., Kerin, J., Warnes, G., 1985. Improved pregnancy rate in human in vitro fertilization with the use of a medium based on the composition of human tubal fluid. Fertility and sterility 44, 493-498.

Rahman, K., 2007. Studies on free radicals, antioxidants, and co-factors. Clinical interventions in aging 2, 219-230.

Raji, Y., Oloyo, A.K., Morakinyo, A.O., 2006. Effect of methanol extract of Ricinus communis seed on reproduction of male rats. Asian journal of Andrology 8, 115-121.

Ramaswamy, S., Plant, T., 2001. Operation of the follicle-stimulating hormone (FSH)–inhibin B feedback loop in the control of primate spermatogenesis. Molecular and cellular endocrinology 180, 93-101.

Rappa, K.L., Rodriguez, H.F., Hakkarainen, G.C., Anchan, R.M., Mutter, G.L., Asghar, W., 2016. Sperm processing for advanced reproductive technologies: Where are we today? Biotechnology advances.

Rates, S.M.K., 2001. Plants as source of drugs. Toxicon 39, 603-613.

Rebourcet, D., Wu, J., Cruickshanks, L., Smith, S.E., Milne, L., Fernando, A., Wallace, R.J., Gray, C.D., Hadoke, P.W., Mitchell, R.T., 2016. Sertoli cells modulate testicular vascular network development, structure and function to influence circulating testosterone concentrations in adult male mice. Endocrinology, 32, 2016-1156.

Reijo, R., Lee, T.-Y., Salo, P., Alagappan, R., Brown, L.G., Rosenberg, M., Rozen, S., Jaffe, T., Straus, D., Hovatta, O., 1995. Diverse spermatogenic defects in humans caused by Y chromosome deletions encompassing a novel RNA-binding protein gene. Nature genetics 10, 383-393.

Remya, M., Sharma, R., Shoaib, H., Asad, R.J.U., Swati, S., 2009. In vitro effect of Aegle marmelos on human sperm motility. Journal of Medicinal Plants Research 3, 1137-1139.

Rhodes, P., 1969. Reproductive physiology for medical students. Churchill. London.

Riffo, M., Leiva, S., Astudillo, J., 1992. Effect of zinc on human sperm motility and the acrosome reaction. International journal of andrology 15, 229-237.

Riley-Vargas, R.C., Lanzendorf, S., Atkinson, J.P., 2005. Targeted and restricted complement activation on acrosome-reacted spermatozoa. The Journal of clinical investigation 115, 1241-1249.

Rizzo, D., 2015. Fundamentals of anatomy and physiology. Cengage Learning. New York.

Robinson, L., Gallos, I.D., Conner, S.J., Rajkhowa, M., Miller, D., Lewis, S., Kirkman-Brown, J., Coomarasamy, A., 2012. The effect of sperm DNA fragmentation on miscarriage rates: a systematic review and meta-analysis. Human Reproduction 10, 261-273.

Ross, A.J., Capel, B., 2005. Signaling at the crossroads of gonad development. Trends in Endocrinology & Metabolism 16, 19-25.

Rossato, M., Ion Popa, F., Ferigo, M., Clari, G., Foresta, C., 2005. Human sperm express cannabinoid receptor Cb1, the activation of which inhibits motility, acrosome reaction, and mitochondrial function. The Journal of Clinical Endocrinology & Metabolism 90, 984-991.

Ruiz-Pesini, E., Diez, C., Lapeña, A.C., Pérez-Martos, A., Montoya, J., Alvarez, E., Arenas, J., López-Pérez, M.J., 1998. Correlation of sperm motility with mitochondrial enzymatic activities. Clinical Chemistry 44, 1616-1620.

Russo, E.B., 2006. 11 The Solution to the Medicinal Cannabis Problem. Ethical issues in chronic pain management 4, 245-259.

Ruwanpura, S.M., McLachlan, R.I., Meachem, S.J., 2010. Hormonal regulation of male germ cell development. Journal of Endocrinology 205, 117-131.

Rybicki, E.P., Chikwamba, R., Koch, M., Rhodes, J.I., Groenewald, J.-H., 2012. Plant-made therapeutics: an emerging platform in South Africa. Biotechnology advances 30, 449-459.

Saalu, L., Kpela, T., Benebo, A., Oyewopo, A., Anifowope, E., Oguntola, J., 2010. The Dose-Dependent Testiculoprotective and Testiculotoxic Potentials of Telfairia occidentalis Hook f. Leaves Extract in Rat. International Journal of Applied Research in Natural Products 3, 27-38.

Said, T.M., Paasch, U., Glander, H.J., Agarwal, A., 2004. Role of caspases in male infertility. Human Reproduction Update 10, 39-51.

Sakkas, D., Mariethoz, E., Manicardi, G., Bizzaro, D., Bianchi, P.G., Bianchi, U., 1999. Origin of DNA damage in ejaculated human spermatozoa. Reviews of reproduction 4, 31-37.

Saleh, R.A., Agarwal, A., Nelson, D.R., Nada, E.A., El-Tonsy, M.H., Alvarez, J.G., Thomas, A.J., Sharma, R.K., 2002. Increased sperm nuclear DNA damage in normozoospermic infertile men: a prospective study. Fertility and sterility 78, 313-318.

Santi, D., Granata, A., Simoni, M., 2015. FSH treatment of male idiopathic infertility improves pregnancy rate: a meta-analysis. Endocrine connections 4, R46-R58.

Sati, L., Bennett, D., Janes, M., Huszar, G., 2015. Next day determination of ejaculatory sperm motility after overnight shipment of semen to remote locations. Journal of assisted reproduction and genetics 32, 117-125.

Savita, D., Huma, A., 2010. Antioxidant Potential some medicinal plants of Central India. Journal of Cancer Therapy 1, 87-90.

Saxena, A., Rastogi, A., 2015. Impairment of Nanog3. Stem Cell Dysregulation Associate with Male Infertility in Human. Hereditary Genet 4, 2161-2172.

Scanlon, V.C., Sanders, T., 2014. Essentials of anatomy and physiology. FA Davis. U.S.A.

Schuffner, A.A., Bastiaan, H.S., Duran, H.E., Lin, Z.-Y., Morshedi, M., Franken, D.R., Oehninger, S., 2002. Zona pellucida-induced acrosome reaction in human sperm: dependency on activation of pertussis toxin-sensitive Gi protein and extracellular calcium, and priming effect of progesterone and follicular fluid. Molecular human reproduction 8, 722-727.

Semwal, A., Kumar, R., Singh, R., Raina, G.S., Singh, K., Rohila, A., Gupta, G., Chhabra, S., Jain, S., Gupta, V., 2013. Nature's aphrodisiacs-A review of current scientific literature. International Journal of Recent Advances Pharmaceutical Research 3, 1-20.

Shalaweh, S., Erasmus, N., Weitz, F., Henkel, R., 2015. Effect of Cissampelos capensis rhizome extract on human spermatozoa in vitro. Andrologia 47, 318-327.

Shamsi, M.B., Imam, S.N., Dada, R., 2011. Sperm DNA integrity assays: diagnostic and prognostic challenges and implications in management of infertility. Journal of assisted reproduction and genetics 28, 1073-1085.

Sharma, R.K., Agarwal, A., 1996. Role of reactive oxygen species in male infertility. Urology 48, 835-850.

Sharpe, R.M., 2010. Environmental/lifestyle effects on spermatogenesis. Philosophical Transactions of the Royal Society of London B: Biological Sciences 365, 1697-1712.

Sharpe, R.M., Fisher, J.S., Millar, M.M., Jobling, S., Sumpter, J.P., 1995. Gestational and lactational exposure of rats to xenoestrogens results in reduced testicular size and sperm production. Environmental Health Perspectives 103, 1136-1147.

Sharpe, R.M., McKinnell, C., Kivlin, C., Fisher, J.S., 2003. Proliferation and functional maturation of Sertoli cells, and their relevance to disorders of testis function in adulthood. Reproduction 125, 769-784.

Shekarriz, M., Thomas, A.J., Agarwal, A., 1995. Incidence and level of seminal reactive oxygen species in normal men. Urology 45, 103-107.

Shen, L., LI, C., LIU, Y., 2007. Protective effect of ginsenoside Rg1 on glutamate-induced lung injury4. Acta pharmacologica sinica 28, 392-397.

Sherwood, L., 2011. Fundamentals of human physiology. Cengage Learning. U.S.A.

Shishkina, G.T., Kalinina, T.S., Sournina, N.Y., Dygalo, N.N., 2001. Effects of antisense to the α2A-adrenoceptors administered into the region of the locus ceruleus on behaviors in plus-maze and sexual behavior tests in sham-operated and castrated male rats. The Journal of Neuroscience 21, 726-731.

Sicherle, C., Maia, M., Bicudo, S.D., Rodello, L., Azevedo, H., 2011. Lipid peroxidation and generation of hydrogen peroxide in frozen-thawed ram semen supplemented with catalase or Trolox. Small Ruminant Research 95, 144-149.

Siddhuraju, P., Becker, K., 2003. Antioxidant properties of various solvent extracts of total phenolic constituents from three different agroclimatic origins of drumstick tree (Moringa oleifera Lam.) leaves. Journal of agricultural and food chemistry 51, 2144-2155.

Simoni, M., Weinbauer, G., Gromoll, J., Nieschlag, E., 2008. Role of FSH in male gonadal function.

Smith, J.T., Clifton, D.K., Steiner, R.A., 2006. Regulation of the neuroendocrine reproductive axis by kisspeptin-GPR54 signaling. Reproduction 131, 623-630.

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

Stagos, D., Spanou, C., Margariti, M., Stathopoulos, C., Mamuris, Z., Kazantzoglou, G., Magiatis, P., Kouretas, D., 2007. Cytogenetic effects of grape extracts (Vitis vinifera) and polyphenols on mitomycin C-induced sister chromatid exchanges (SCEs) in human blood lymphocytes. Journal of agricultural and food chemistry 55, 5246-5252.

Steers, W.D., 2002. Pharmacologic treatment of erectile dysfunction. Reviews in urology 4, S17-S25.

Steger, K., Aleithe, I., Behre, H., Bergmann, M., 1998. The proliferation of spermatogonia in normal and pathological human seminiferous epithelium: an immunohistochemical study using monoclonal antibodies against Ki-67 protein and proliferating cell nuclear antigen. Molecular human reproduction 4, 227-233.

Steinberger, E., 1971. Hormonal control of mammalian spermatogenesis. Physiological Reviews 51, 1-22.

Stock, C.E., Fraser, L.R., 1989. Divalent cations, capacitation and the acrosome reaction in human spermatozoa. Journal of reproduction and fertility 87, 463-478.

Swan, S.H., Elkin, E.P., Fenster, L., 2000. The question of declining sperm density revisited: an analysis of 101 studies published 1934-1996. Environmental health perspectives 108, 961-980.

Tan, K.A., De Gendt, K., Atanassova, N., Walker, M., Sharpe, R.M., Saunders, P.T., Denolet, E., Verhoeven, G., 2005. The role of androgens in Sertoli cell proliferation and functional maturation: studies in mice with total or Sertoli cell-selective ablation of the androgen receptor. Endocrinology 146, 2674-2683.

Troiano, L., Granata, A.R., Cossarizza, A., Kalashnikova, G., Bianchi, R., Pini, G., Tropea, F., Carani, C., Franceschi, C., 1998. Mitochondrial membrane potential and DNA stainability in human sperm cells: a flow cytometry analysis with implications for male infertility. Experimental cell research 241, 384-393.

Turner, R.M., 2003. Tales from the tail: what do we really know about sperm motility? Journal of Andrology 24, 790-803.

Turner, R.M., 2005. Moving to the beat: a review of mammalian sperm motility regulation. Reproduction, Fertility and Development 18, 25-38.

Twigg, J., Irvine, D., Aitken, R., 1998a. Oxidative damage to DNA in human spermatozoa does not preclude pronucleus formation at intracytoplasmic sperm injection. Human Reproduction 13, 1864-1871.

Twigg, J., Irvine, D.S., Houston, P., Fulton, N., Michael, L., Aitken, R.J., 1998b. Iatrogenic DNA damage induced in human spermatozoa during sperm preparation: protective significance of seminal plasma. Molecular Human Reproduction 4, 439-445.

Uysal, O., Bucak, M., Yavas, I., Varisli, O., 2007. Effect of various antioxidants on the quality of frozen-thawed bull semen. Journal of Animaland Vetinary Advances 6, 1362-1366.

Vaidya, A.D., 2014. Reverse Pharmacology-A paradigm shift for drug discovery and development. Curr Res Drug Discovery 1, 39-44.

Valko, M., Leibfritz, D., Moncol, J., Cronin, M.T., Mazur, M., Telser, J., 2007. Free radicals and antioxidants in normal physiological functions and human disease. The international journal of biochemistry & cell biology 39, 44-84.

Van Wyk, B.-E., 2011. The potential of South African plants in the development of new medicinal products. South African Journal of Botany 77, 812-829.

Van Wyk, B.-E., Oudtshoorn, B.v., Gericke, N., 1997. Medicinal Plants of South Africa. Briza.

Vandebroek, I., Balick, M.J., 2012. Globalization and loss of plant knowledge: challenging the paradigm. PloS one 7, e37643.

Vasan, S., 2011. Semen analysis and sperm function tests: How much to test? Indian journal of urology: IJU: journal of the Urological Society of India 27, 41-52.

Venkatesh, S., Deecaraman, M., Kumar, R., Shamsi, M., Dada, R., 2009. Role of reactive oxygen species in the pathogenesis of mitochondrial DNA (mtDNA) mutations in male infertility. Indian J Med Res 129, 127-137.

Venter, H., Verhoeven, R., Bruyns, P., 2009. Morphology and taxonomy of Mondia (Apocynaceae: Periplocoideae). South African Journal of Botany 75, 456-465.

Vigil, P., Orellana, R.F., Cortés, M.E., 2011. Modulation of spermatozoon acrosome reaction. Biological research 44, 151-159.

Villegas, J., Kehr, K., Soto, L., Henkel, R., Miska, W., Sanchez, R., 2003. Reactive oxygen species induce reversible capacitation in human spermatozoa. Andrologia 35, 227-232.

Visconti, P.E., Moore, G.D., Bailey, J.L., Leclerc, P., Connors, S.A., Pan, D., Olds-Clarke, P., Kopf, G.S., 1995. Capacitation of mouse spermatozoa. II. Protein tyrosine phosphorylation and capacitation are regulated by a cAMP-dependent pathway. Development 121, 1139-1150.

Walczak-Jedrzejowska, R., Wolski, J.K., Slowikowska-Hilczer, J., 2013. The role of oxidative stress and antioxidants in male fertility. Center of European Journal of Urology 66, 60-67.

Wang, X., Sharma, R.K., Sikka, S.C., Thomas, A.J., Falcone, T., Agarwal, A., 2003. Oxidative stress is associated with increased apoptosis leading to spermatozoa DNA damage in patients with male factor infertility. Fertility and sterility 80, 531-535.

Wassarman, P.M., 1987. The biology and chemistry of fertilization. Science 235, 553-560.

Watcho, P., Defo, P.B.D., Wankeu-Nya, M., Carro-Juarez, M., Nguelefack, T., Kamanyi, A., 2013. Mondia whitei (Periplocaceae) prevents and Guibourtia tessmannii (Caesalpiniaceae) facilitates fictive ejaculation in spinal male rats. BMC complementary and alternative medicine 13, 1-16.

Watcho, P., Djoukeng, C., Zelefack, F., Nguelefack, T., Ngouela, S., Kamtchouing, P., Tsamo, E., Kamanyi, A., 2007. Relaxant effect of Mondia whitei extracts on isolated guinea pig corpus cavernosum. Pharmacologyonline 2, 44-52.

Watcho, P., Fotsing, D., Zelefack, F., Nguelefack, T., Kamtchouing, P., Tsamo, E., Kamanyi, A., 2006. Effects of Mondia whitei extracts on the contractile responses of isolated rat vas deferens to potassium chloride and adrenaline. Indian journal of pharmacology 38, 33-46.

Watcho, P., Kamtchouing, P., Sokeng, S., Moundipa, P., Tantchou, J., Essame, J., Koueta, N., 2001. Reversible antispermatogenic and antifertility activities of Mondia whitei L. in male albino rat. Phytotherapy research 15, 26-29.

Watcho, P., Kamtchouing, P., Sokeng, S.D., Moundipa, P.F., Tantchou, J., Essame, J.L., Koueta, N., 2004. Androgenic effect of Mondia whitei roots in male rats. Asian journal of andrology 6, 269-272.

Watcho, P., Zelefack, F., Ngouela, S., Nguelefack, T.B., Kamtchouing, P., Tsamo, E., Kamanyi, A., 2012. Enhancement of erectile function of sexually naïve rats by β–sitosterol and α–β–amyrin acetate isolated from the hexane extract of Mondia whitei. Asian Pacific Journal of Tropical Biomedicine 2, S1266-S1269.

WHO, 2002. WHO traditional medicine strategy 2002-2005.

WHO, 2010. WHO laboratory manual for the examination and processing of human semen.

Wilhelm, D., Palmer, S., Koopman, P., 2007. Sex determination and gonadal development in mammals. Physiological reviews 87, 1-28.

Wistuba, J., Stukenborg, J.-B., Luetjens, C.M., 2007. Mammalian spermatogenesis. Func Dev Embryol 1, 99-117.

Wu, F., Aitken, R., Ferguson, A., 1989. Inflammatory bowel disease and male infertility: effects of sulfasalazine and 5-aminosalicylic acid on sperm-fertilizing capacity and reactive oxygen species generation. Fertility and sterility 52, 842-845.

Yanagimachi, R., 1970. The movement of golden hamster spermatozoa before and after capacitation. Journal of reproduction and fertility 23, 193-196.

Yanagimachi, R., 1994. Mammalian fertilization. The physiology of reproduction 1, 189-317.

Yanagimachi, R., Yanagimachi, H., Rogers, B., 1976. The use of zona-free animal ova as a test-system for the assessment of the fertilizing capacity of human spermatozoa. Biology of Reproduction 15, 471-476.

Yousef, M., Abdallah, G., Kamel, K., 2003. Effect of ascorbic acid and vitamin E supplementation on semen quality and biochemical parameters of male rabbits. Animal reproduction science 76, 99-111.

Zeitoun, M.M., Al-Damegh, M.A., 2014. Effect of Nonenzymatic Antioxidants on Sperm Motility and Survival Relative to Free Radicals and Antioxidant Enzymes of Chilled-Stored Ram Semen. Open Journal of Animal Sciences 5, 50-62.

Zini, A., Libman, J., 2006. Sperm DNA damage: clinical significance in the era of assisted reproduction. Canadian Medical Association Journal 175, 495-500.