Effect of *Cissampelos capensis* rhizome extract on human sperm capacitation and acrosome reaction

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Magister Scientiae

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DECLARATION

I declare that the "**Effect of *Cissampelos capensis* rhizome extract on human sperm capacitation and acrosome reaction**" is my own work, that it has not been submitted for any degree or examination at any other university and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

……………………................... **………………….**

FULL NAME **DATE**

……………………………

SIGN

DEDICATION

This thesis is dedicated to my loving mother and my wife.

"In the name of Allah, Most Gracious, Most Merciful (1) Praise be to Allah, the Cherisher and Sustainers of the worlds (2) Most Gracious, Most Merciful (3) Master of the Day of Judgment (4) Thee do we worship, and Thine aid we seek (5) Show us the straight way (6) The way of those on whom Thou hast bestowed Thy Grace, those whose (portion) is not wrath, and who go not astray (7). True are the words of God."

Sûrat Al-Fâtihah (The Opening 1)

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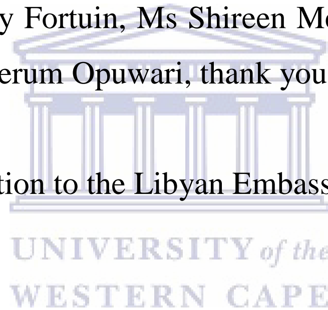

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KEY WORDS

Cissampelos capensis

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Motility

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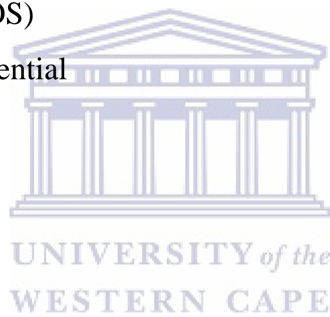
Capacitation

Reactive Oxygen Species (ROS)

Mitochondrial Membrane Potential

Apoptosis

DNA fragmentation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ABSTRACT

Introduction

Cissampelos capensis, is commonly known by the Afrikaans name "dawidjies" or "dawidjieswortel". *C. capensis* is the most important and best known medicinal plant of the family Menispermaceae used by the Khoisan and other rural people in the western regions of South Africa. Among numerous other ailments, it is traditionally taken to treat male fertility problems. Yet, no studies have investigated the effects of this plant or its extracts on human spermatozoa. The aim of study was to investigate the effects of *C. capensis* rhizome extracts on sperm function.

Materials and Methods

Fresh *Cissampelos capensis* rhizomes were collected from the Cape Nature Reserve near Belhar in the Western Cape, South Africa, during the summer season (February). The rhizomes were cleaned and chopped into smaller segments, these pieces were oven-dried at 25°C for approximately 3 days and milled to form a powdery substance which was infused with hot (about 70°C) distilled water for 1 hour. After cooling and filtration extract was frozen at -20°C until it was freeze-dried, the dried extract was then stored at 4°C in a closed container until experimentation.

This study includes two parts (part 1: investigation of the effect of *Cissampelos capensis* rhizomes extract CRE on sperm functions; part 2: investigation of CRE fractions with and without progesterone on sperm functions).

Part 1: A total of 77 semen samples was collected. Spermatozoa were washed with Human tubular fluid-Bovine serum albumin (HTF-BSA) medium and incubated with different concentrations of *C. capensis* (0, 0.05, 0.5, 5, 50, 200 µg/ml) for 1 hour at 37°C. Sperm motility, vitality, acrosome reaction, reactive oxygen species (ROS), capacitation, annexin V-binding, DNA fragmentation and mitochondrial membrane potential (Aψm) were determined.

Part 2: A total of 26 semen samples was collected. Spermatozoa were washed with HTF-BSA medium and incubated for 2 hours. Fractionation (F) of CRE with methanol was prepared as F1= 0% MeOH, F2= 30% MeOH, F3= 60% MeOH and F4= 100% MeOH in combination with 20 µg/ml progesterone (P4) or without P4 and incubated with different concentrations of *C. capensis* (0, 0.05, 0.5, 5, 50, 200 µg/ml) for 1.5 hour at 37°C. Sperm motility, reactive oxygen species (ROS), capacitation were determined.

Results

Part 1: While viability, annexin V-positivity and Aψm were not affected, the percentages of ROS-positive, TUNEL-positive, capacitated and hyperactivated spermatozoa increased significantly and dose-dependently.

Part 2: F1 yielded higher significance than F2, F3 and F4 for ROS, capacitation and hyperactivation. No effect was found for the other parameters.

Conclusion

It is concluded that the alkaloids present in the F1 of the extract of *C. capansis* rhizomes triggered sperm intrinsic superoxide production leading to sperm capacitation and acrosome reaction induced by P4.

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Chapter 1: Introduction

1.1 Male Reproductive System

The organs and structures of the male reproductive system provide men the ability to fertilise a women's ovum (egg) to generate offspring. Furthermore, the organs and structures which comprise the male reproductive system, include the testes, where spermatozoa are produced, the epididymis where the male germ cells mature and are stored, as well as the penis (Foley, 2001; Seeley et al., 2003). The penis has a single duct called the urethra; this releases both spermatozoa and urine. In addition, included in the male reproductive system are the accessory sex glands, which consist of the prostate gland, bulbourethral (Cowper's) glands and the seminal vesicles (Figure 1) (Greenspan and Gardner, 2001; Pearson Education, 2004). These glands excrete special fluids known as seminal fluid which supports, maintains and protects spermatozoa as they travel through the male reproductive tract and the female vagina.

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The hypothalamic-pituitary-gonadal axis (HPGA) is an important system in reproduction as it maintains hormonal homeostasis. It comprises of the hypothalamus, pituitary and gonads. Gonadotropin Releasing Hormone (GnRH) is released from the hypothalamus in pulse rate intervals every 90-120 minutes (Mruk and Cheng, 2010; Kopera et al., 2010) to stimulate the release of gonadotrophins, Follicular-stimulating hormone (FSH) and Luteinizing hormone (LH) from the anterior lobe of the pituitary. Subsequently, as a result of the stimulation by gonadotrophins, 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 (Greenspan and Baxter, 1994; Greenspan and Gardner, 2001).

The main male hormone testosterone causes the appearance of the male sexual characteristics, such as facial hair growth and other masculine features together with growth hormones, FSH and LH (Greenspan and Baxter, 1994; Greenspan and Gardner, 2001). Immature germ cells develop through several stages and

finally become mature sperm cells called spermatozoa. This development of spermatogenic stem cells into mature spermatozoa is called spermatogenesis (Norton et al., 1994; Hess and De Franca, 2008; Mathur and D'Cruz, 2011).

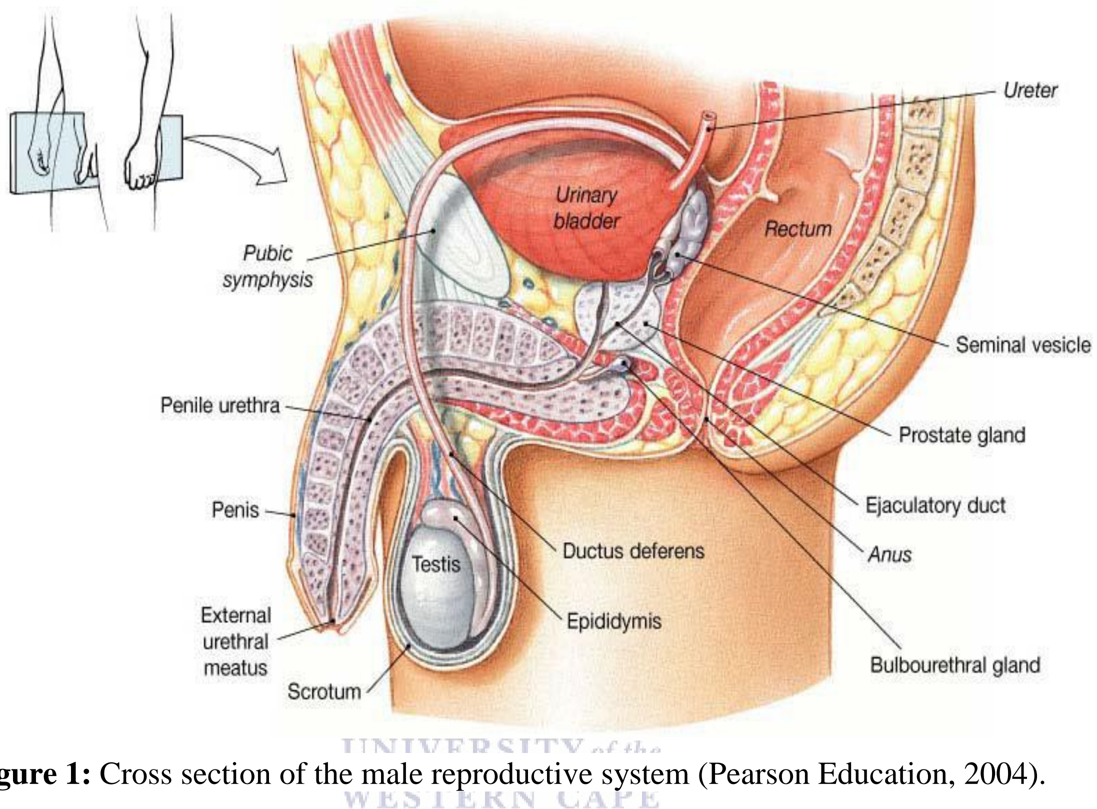

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Figure 1: Cross section of the male reproductive system (Pearson Education, 2004).

1.2 Spermatogenesis

Innate to their function to fertilize oocytes, spermatozoa ejaculated from the male reproductive tract have unique features in terms of structure and physiology. The most immature male germinal cell is the spermatogonium. It is the basic, self-renewing stem cell of the male germ cell line (Figure 2). The first mitotic division of spermatogonia are to increase their number with daughter cells, either proceeding into spermatogenesis, forming replacement stem cells, or degeneration (Bart et al., 2002). At the onset of puberty, large primary spermatocytes are formed by spermatogonia which pass through the blood testis barrier (BTB) formed by tight junctions of adjacent Sertoli cells (Kato et al., 2009). After an additional few days, these spermatogonia continue in division thereby forming more spermatocytes which eventually develops into spermatids that are eventually modified to become spermatozoa.

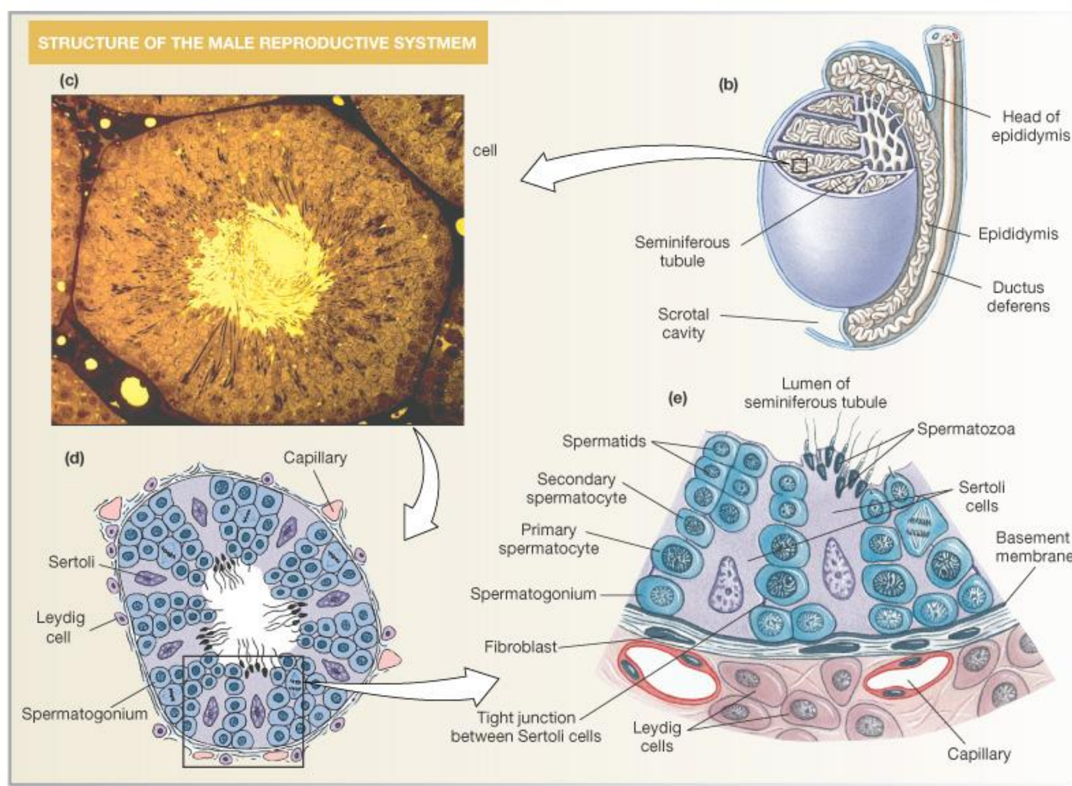

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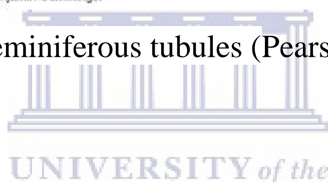
Figure 2: Illustration of seminiferous tubules (Pearson Education, 2004).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The spermatocytes are diploid (23 chromosomes in duplicate). During the meiotic change from the spermatocyte stage to the spermatid stage they emerge as haploid. Therefore, this implies that one set of 23 chromosomes will go to one spermatid and the other set to the second spermatid (Cheng and Mruk, 2002). The entire period of development from germinal cell to spermatozoa takes approximately 75 days in the human (Clermont and Trott, 1969; Clermont and Antar, 1973; Andersen Berg et al., 1990; Russell et al., 1990; Rosiepen et al., 1994, 1997).

The production of spermatozoa from the onset of puberty in human males is a continuous process resulting in up to 120 million mature spermatozoa per day (Clermont and Antar, 1973; Franca and Godinho, 2003). Each round spermatid still has to progress into an elongated spermatozoon (Figure 3) composed of a head, mid-piece and tail (Roosen-Runge, 1962; Cleremont, 1972; Hess, 1999), as spermatids still have epithelial characteristics. The condensed nucleus is found

within the head of the sperm cell and a thin cytoplasmic and cell membrane layer surrounding its surface (Okanlawon and Dym, 1996; Olofsson, 1999). At the anterior two thirds of the head on the outside is a thick cap called the acrosome that is formed mainly from the Golgi apparatus. This contains a number of enzymes which are essential for entry into the ovum allowing for fertilization.

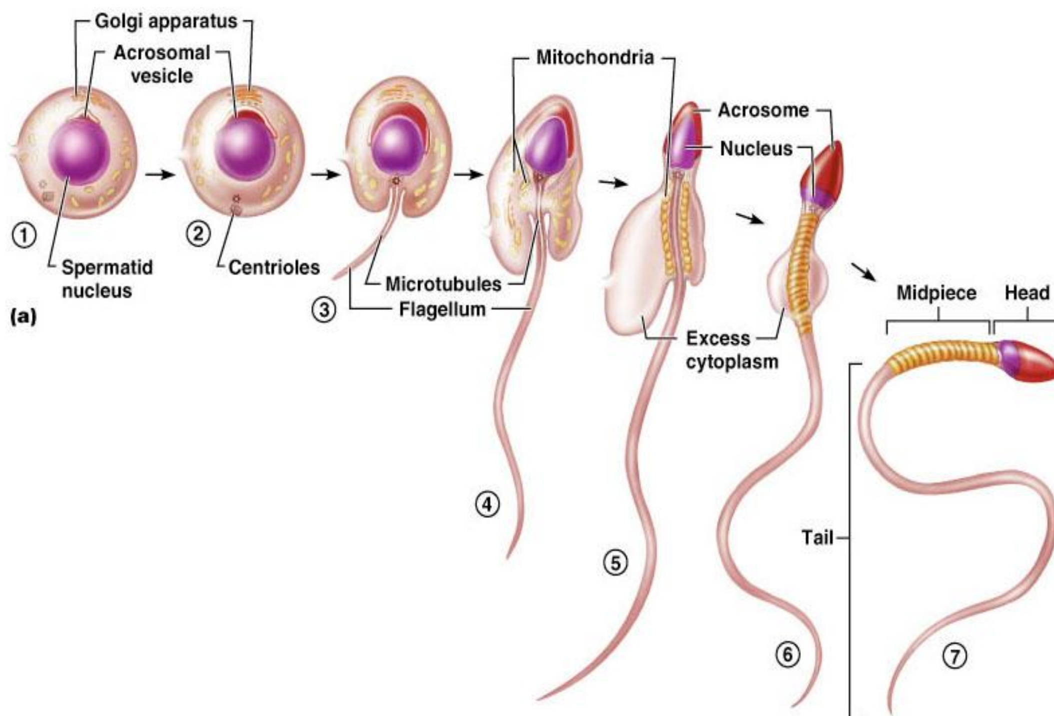

Figure 3: Spermiogenesis and spermatozoon structure (Pearson Education, 2004).

1.3 Infertility

About 15% of all couples, which amounts to 50-80 million couples at reproductive age globally and annually, have difficulties conceiving (WHO, 1992; Tielemans et al., 2002). In more than half of these cases the problem lies with the male partner (Hull et al., 1985; de Kretser and Baker, 1999). Infertility is described as the inability of a couple to induce pregnancy after one year of regular, unprotected intercourse during the fertility phase of the menstrual cycle (WHO, 1992; Nieschlag et al., 2000; Evers, 2002).

In Africa, within male dominated societies, infertility is considered as a female problem (Dyer et al., 2004). This often leaves the couple with overwhelming feelings of hopelessness, frustration and despair.

Assisted reproductive techniques (ART) such as *in vitro* fertilization (IVF), intracytoplasmic sperm injection (ICSI) to alleviate and treat male infertility are available in many countries. However, these methods are expensive and the surgery may cause added negative impact on enhancing male fertility (Gleicher and Barad, 2006). On other hand, in Africa, these techniques are not readily available or accessible mainly because they are unaffordable for the majority of people. Therefore, approximately 70% of patients are resorting to traditional or complementary medicine (Bannerman, 1993; Calixto, 2000). For this reason, many people with male fertility problems turn to traditional medicines.

1.4 Measures of Sperm Function Parameters

1.4.1 Motility

Sperm motility is a complex function, the understanding of which requires integration of cell biology with reproductive physiology, biochemistry, biophysics and clinical andrology. This parameter is part of the standard semen analysis according to WHO (WHO, 2010). Sperm motility is dependent on the sperm flagellum, which provides the propulsion for swimming (Coetzee et al., 1989; Shulman et al., 1998). Motility patterns of spermatozoa have been investigated throughout the years and expressed as different parameters. These parameters were often mistaken to be similar and described with the same characteristics until a consensus on these parameters was reached by the Automated Sperm Motility Analysis (Mortimer, 1990). With the aid of motion analysis systems, these parameters have now been standardized. The motility of each spermatozoon is graded as follows:

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

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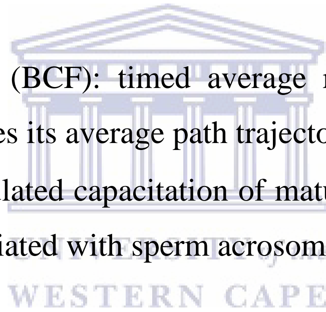
- Curvilinear velocity (VCL): measurement of the speed of travel of the centroid of the sperm head over a given period of time, i.e. the local speed along the curvilinear path traced by the sperm head.
- Straight line velocity (VSL): straight line distance between the first and last centroid positions 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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1.4.2 Mitochondrial membrane potential

The synthesis of metabolic energy in the form of adenosine triphosphate (ATP) via oxidative phosphorylation, fatty acid oxidation, regulation of reduction-oxidation and calcium signalling, and the control of apoptosis are all regulated in the mitochondria (Van Loo et al., 2002; Mattson et al., 2008). These organelles have inner and outer membranes that separate the matrix from the inter membrane space (IMS) and the IMS from the cytosol. The respiratory chain complexes of the electron transport chain and ATP synthesis occurs in the inner mitochondrial membrane (IMM) (Mattson et al., 2008). This inner membrane is a special structure folded into cristae.

Since sperm motility is one of the key factors for fertilizing ova (Ruiz-Pesini et al., 2000) and requires energy provided by the mitochondria. This relationship

can be seen by the significant correlations between motility and mitochondrial membrane potential (Kasai et al., 2002; Henkel et al., 2012).

1.4.3 DNA fragmentation

DNA fragmentation can be described as single and double DNA strand breaks within spermatozoa and can be associated with some male infertility cases (Irvine et al., 2000). Improper DNA packaging and chromatin condensation, ligation (McPherson and Longo, 1992; Sakkas et al., 1999, 2002) and apoptosis, oxidative stress (Agarwal et al., 2003; Henkel et al., 2003; 2005a) are only a few of the causes resulting from sperm DNA fragmentation/damage. Life style and environmental factors as causative parameters for this kind of DNA damage should also be taken into account (Fraga et al., 1996).

Spermatozoa with damage DNA are still capable of fertilizing oocytes (Twigg et al., 1998; Henkel et al., 2004) when using ART techniques as they escape the natural screening of DNA fragmented sperm in the female genital tract. This may lead to detrimental outcomes as embryos that developed through these methods of conception may reach full term and may present later in life with genetic disorders or even cancers (Aruoma, 1994; Aitken and Krausz, 2001; Gandini et al., 2004). Also, DNA-damaged spermatozoa can be associated with a reduction in fertilization ability, impaired pre-implantation development, miscarriage and morbidity in the offspring (Zini and Sigman, 2009; Aitken and De Iuliis, 2010; Avendaño and Oehninger, 2011).

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As the importance of DNA damage within spermatozoa has been established, the examination of this principle aspect is of equal importance. It is not only mandatory to understand the principles these different test systems are based on, but also to know which aspects of DNA damage these test system are measuring to distinguish between assays that measure potential DNA damage or real DNA damage for example the assay TdT (terminal deoxynucleotidyl transferase) mediated dUDP nick end labelling (TUNEL) (Gorczyca et al., 1993; Henkel et al., 2003; Henkel and Franken, 2011).

The TUNEL assay is the specific test to detect single and double-strand DNA breaks (Gorczyca et al., 1993; Henkel et al., 2007). It works by means of an enzymatically catalysed reaction using the template-independent terminal deoxynuleotidyl transferase (TdT) it incorporates biotinylated or fluorescinated dUTP to the 3'-OH ends of the DNA, which increase with the number of strand breaks (Shamsi et al., 2008). Its relevance in respect of sperm function as well as of fertilization and pregnancy has been proven repeatedly (Sun et al., 1997; Henkel et al., 2004). The TUNEL assay evaluates DNA fragmentation, which is a late stage of apoptosis. However, the assay cannot distinguish between apoptosis and necrotic cells and other parameter of apoptosis (Henkel et al., 2004).

1.4.4 Apoptosis

Cell death can be distinguished into two modes namely apoptosis and necrosis. In context, apoptosis is the "ability of a cell to self-destruct". In doing so, cells activate intrinsic cellular suicide signalling programs when they are defective, damaged or the renewal of cells are needed (Kerr et al., 1972; Wyllie et al., 1980). It can also be further distinguished on the basis of differences in morphological and biochemical characteristics and classified as early and late onset of cell death (Kerr et al., 1972). When cells undergo apoptosis, several morphological and biochemical changes occur such as cell shrinkage, surface membrane anamolies, apoptotic body formation, chromatin condensation and fragmentation of DNA (Fadok et al., 1992).

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The early onset of apoptosis may be characterized by plasma membrane alterations in which translocation of phosphatidyserine (PS) from the inner to the outer leaflet and phospholipid asymmetry occurs from the inner part to the outer layer of the plasma. This externalization of the PS aids in the detection process during apoptosis. In sperm, such cells with exposed PS can be identified by the fluorescence-labelled, Ca^{2+}-dependent phospholipid-binding protein Annexin-V (Vermes et al., 1995; Glander and Schaller, 1999). In combination with propidium iodide (PI) or 4',6-diamidino-2-phenylindole (DAPI), a simultaneous distinction between live and dead spermatozoa is possible (Pinkel et al., 1982; Garner, 2006; Kuželová and Chrenek, 2013).

1.4.5 Reactive oxygen species

During oxidative metabolism, unstable oxygen molecules are formed by any cell in their mitochondria as by-products which are called reactive oxygen species (ROS). Some of them are free radicals, i.e. molecules with a single, impaired electron. Examples of ROS are hydroxyl ions ($\cdot OH^-$) and superoxide ($\cdot O_2^-$) and non-free radicals are hydrogen peroxide and lipid peroxide (Petersen et al., 1980; Alaa Hamada et al., 2012). ROS are extremely reactive with half-life times in the nano-second to milli-second range. Other forms of highly reactive molecules also include reactive nitrogen species (RNS); some examples include nitrous oxide, nitroxyl ion, and peroxynitrite. ROS and RNS also exist within spermatozoa or externally the semen (Darley-Usmar et al., 1995; Sikka, 2001; Lee and Cheng, 2004; Pacher et al., 2007). The external sources of ROS within the male genital tract and semen may derive from leukocytes or immature/damaged spermatozoa (Henkel, 2012). At high levels, ROS also causes damage to lipids, proteins, carbohydrates and nucleic acid. The latter, contributing to poor DNA packaging and effecting the quality of sperm fertilizing potential and function (Aitken and West, 1990; Keating et al.,1997; Esfandiari et al., 2003). On the other hand, at low levels, ROS are beneficial and essential to stimulate sperm function such as capacitation (Leclerc et al., 1997), enhance zona pellucida binding (Aitken et al., 1998), and promote acrosome reaction and hyperactivation (De Lamirande et al., 1997). Biochemically, ROS trigger capacitation through the redox regulation of tyrosine phosphorylation (Leclerc et al., 1997; Aitken et al., 1998).

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1.4.6 Capacitation and acrosome reaction

Capacitation is a sequence of biochemical and physiological changes spermatozoa undergo when they exit the seminal plasma and pass through the female genital tract in order to be able to undergo acrosome reaction (Yanagimachi, 1994). The changes in this process include an increase in sperm plasma membrane fluidity which is due to cholesterol loss (Osterhoff et al., 1999), an increase in ionic influx by membrane hyperpolarization and an increase in the degree of protein

phosphorylation (Zeng et al., 1995; Tardif et al., 2001; Jha and Shivaji, 2002). These changes aid the sperm cells cause a change in the motility pattern to hyperactivation (Yanagimachi, 1994). Subsequently, sperm are enabled to undergo the acrosome reaction. These events are crucial for the spermatozoon to penetrate the zona pellucida and fuse with the oocyte plasma membrane (Yanagimachi, 1994).

The acrosome is an organelle that covers the anterior one-half to two-thirds of the sperm head by a membrane which resembles a cap-like structure (Figure 4). This organelle houses essential enzymes such as hyaluronidase and acrosin (Breitbart and Spungin, 1997). Morphologically, the release of these ezymes, exocytotically during fusions between the outer acrosomal and sperm plasma membrane is characterized as acrosome reaction (Breitbart and Spungin, 1997).

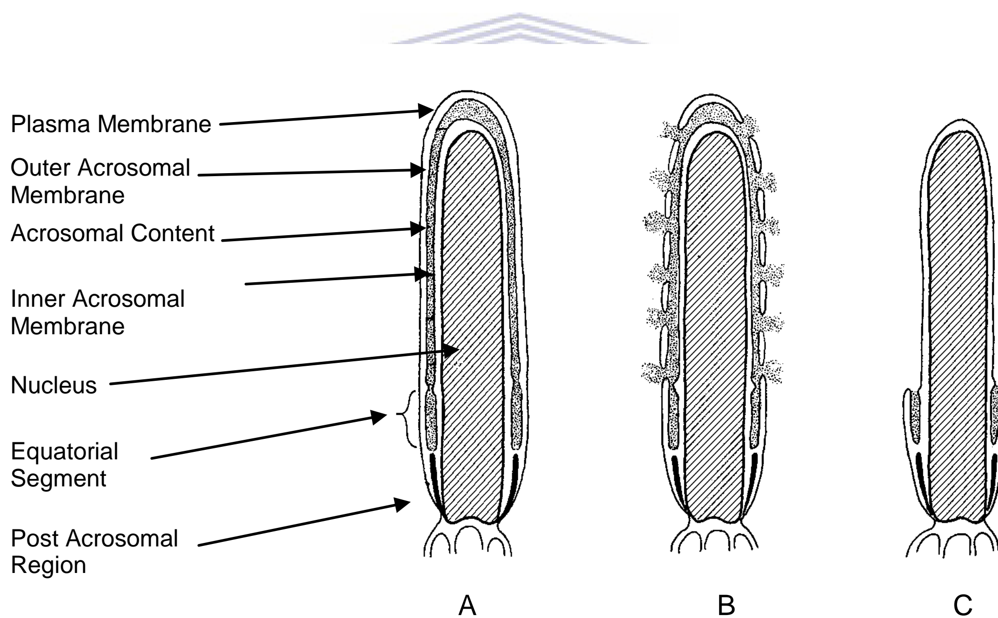

Figure 4: Shows the process of Acrosome reaction. (A) Cap-like structure: Fusion of outer acrosomal membrane with plasma membrane after stimulation. (B) Membrane vesicles with acrosomal content (acrosin) is released. (C) Inner acrosomal membrane forms outer surface in the area of the sperm head (Mann and Lutwak-Mann 1981).

Yet, in order for acrosome reaction (AR) to commence capacitation needs to be initiated. Normally, the acrosome reaction occurs only after physiologically and biochemically altered intracellular events within the spermatozoa has already taken place, under physiological conditions, the reaction is induced by follicular fluid, serum albumin, glycosaminoglycans or glycoproteins (G_1 protein) from the zona pellucida (ZP) (Yanagimachi, 1981; Suarez et al., 1986; Cross et al., 1988). In an *in vitro* set-up it is not easy to obtain the ZP; an alternative would be the steroid hormone, progesterone (Harper et al., 2006).

Progesterone arising from the HPGA in the female initiates AR similar to that of ZP by using the Ca^{+2} -influx pathway (Blackmore et al., 1990; Garcia and Meizel, 1999; Kirkman-Brown et al., 2000). This Ca^{2+} -influx is necessary for capacitation and successful fertilization in invertebrates for and its importance for mammalian fertilization was reported by Iwamatsu and Chang (1971).

These intracellular events mentioned above triggered by spermatozoa binding to the ZP and thyrosine kinase receptor and the interaction with G_1 protein (Ward and Kopf, 1993; Breitbart and Spungin, 1997) causing membrane depolarization allowing the influx of Ca^{+2} (Figure 5). This influx triggers activation of protein-C kinase (PKC) increase membrane fluidity (Baldi et al 1996), protein tyrosine phosphorylation (PTP) (Brucker and Lipford, 1995) an increase in cyclic adenosine monophosphate (cAMP) concentrations and protein phosphorylation (PP) (Baldi et al 1996; Breitbart and Spungin, 1997). In addition, it decreases the cholesterol/phospholipid relation of the plasma membrane and activates changes in swimming patterns to hyperactivated motility of the sperm. Increased levels, of thyrosine kinase trigger increases in capacitation which also leads to membrane fusion of acrosome reaction. Under stressed conditions, heat shock protein 90-alpha (HSP90α) which is an abundant cellular protein (Richter and Buchner, 2001; Picard, 2002) plays a significant role in the signal transduction pathways of a variety of cellular processes including spermatogenesis (Gruppi et al., 1991), induces the triggering of capacitation and the initiation of acrosome reaction (Richter and Buchner, 2001; Picard, 2002).

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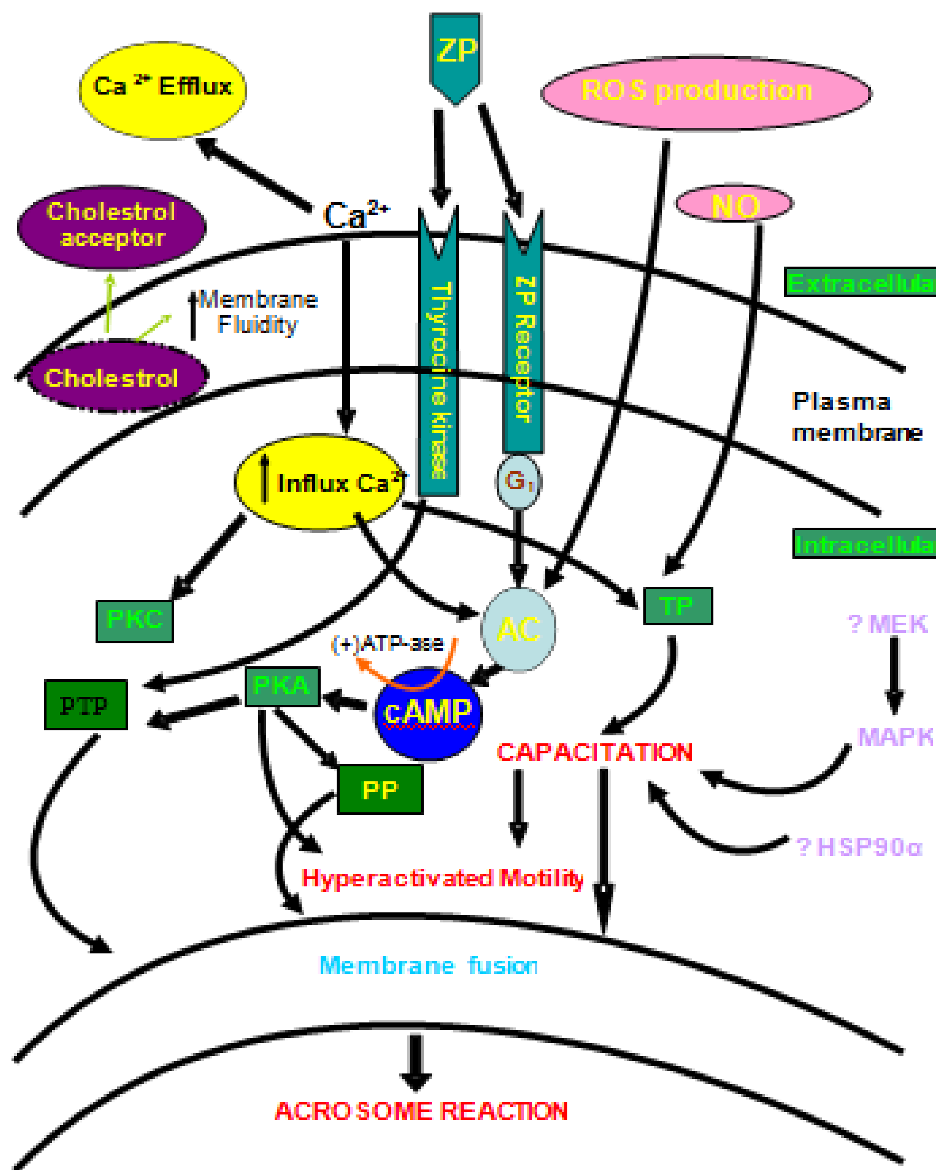

Figure 5: Hypothetical signal pathway of capacitation and acrosome reaction. The binding of zona protein (ZP) to the ZP and the Thyrocine Kinase receptor on the plasma membrane follows an reactive cascade of events. Increase of protein thyrocine phosphorylation (PTP); the G_1 protein relays the signal to the membrane-bound enzyme adenylate cyclase (AC) to increase the generation of cyclic adenosine monophosphate (cAMP). This triggers secondary messengers, protein kinase (PKA- which is cAMP dependant), Ca^{2+} and phospholipid kinase (PKC) to increase protein phospharylation. The increase of intracellular Ca^{2+} as a result of the ZP activation may be due to the membrane fluidity of cholesterol binding to extracellular acceptors. The activation of cAMP consequently results in more energy required and increases the (+) ATP-ase release. This in turn results in capacitation and allowing sperm hyperactivated motility to commence leading to membrane fusion and acrosome reaction. On the other hand an alternate pathway in the increase of nitro-oxide (NO) and reactive oxygen species (ROS) may also activate thyrocine phospharylation (TP) resulting in capacitation and ending in acrosome reaction. **HSP90α**: Heat Shock Protein 90α; **MAPK/MEK**: Mitogen Activated Protein Kinase (according to Brucker and Lipford, 1995; Baldi et al., 1996; Breitbart and Spungin, 1997).

1.5 Traditional Medicine

The World Health Organization (WHO) defines traditional medicine as "the knowledge, skills and practice of holistic care, recognized and accepted for its role in the maintenance of health and the treatment of diseases. It is based on indigenous theories handed down from generation to generation for its beliefs and experiences" (WHO, 2000).

Non-industrilized countries (third world and developing countries) have adopted this method of treatment as their basic health care system and in high demand as a new therapy worldwide (Bannerman, 1993; WHO, 2000; Calixto, 2000) in 60-80% of cultures such as Chinese, Arabic and African. Traditional medicine practices approaches illness in a holistic manner and is deeply rooted in a specific social-cultural context and varies between communities and therefore gives traditional medicine its diversity. The African continent, currently has changed its focus on widespread health-care application. This has highlighted and driven new research, investment and design of programmes into this field to improve the health care system in several developing countries such as India and China including the African countries (Yuan and Lin, 2000; Rukangira, 2001; Fouche et al., 2008; Ashidi et al., 2010).

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In Africa traditional healers play an important role in the health of millions of people. In South Africa, an estimated 27 million indigenous medicine consumers are located. Specifically, households in KwaZulu-Natal spend between 4% and 6% of their annual incomes on indigenous medicine and services (Mander, 1999).

Many medicinal herbs have been claimed to enhance sexual functioning and fertilizing potential in men. In Asia and Eastern Europe, *Tribulus terrestris* is a medicinal plant that has been used for centuries to increase sexual desire and enhance erection (Adimoelja, 2000). People have also been using plants such as *Panax ginseng* for the treatment of impotency and improving sexual stamina (Nocerino et al., 2000). Additionally, Tongkat Ali (*Eurycama longifolia*), a plant growing throughout South East Asia has also been used as an aphrodisiac to enhance testosterone levels and to treat erectile dysfunction by the countries natives (Tambi and Imran, 2010; Tambi et al., 2012).

The central Andes in South America, Gonzalez et al. (2003) has investigated three species of *Lepidium meyenni* (Maca) that have also been traditionally used as an aphrodisiac by the aboriginal folk to enhance male fertility for centuries. Research proved Maca to reduce spermatogenic damage (Gonzalez et al., 2005). With further investigation of one of the hypocotyls, Black Maca, the spermatogenic cycle in rats revealed that it affects sperm count as early as 1 day after beginning of treatment. Red Maca has an antagonistic effect on prostatic hyperplasia in adult mice (Gonzalez et al., 2006, 2008) and prostate zinc levels in rats with testosterone-induced prostatic hyperplasia (Gonzalez et al., 2012).

In West Africa, *Hibiscus macranthus* and *Basella alba* have been shown to significantly increase testosterone production (Moundipa et al., 2006). In Ghana, *Mondia whitei*, a known aphrodisiac has also revealed that aqueous administration enhances sperm total and progressive motility (Lampiao et al., 2008). Furthermore, the yombi tree (*Pausinystalia yohimbi*) is use to increase sexual arousal and dysfunction mainly due to its alkaloid *Yohimbine* as the active compound. Additionally, *Securidaca longipedunculata* (Polygalaceae) and *Fadogia agrestis* (Rubiaceae), also found in Africa, are used by traditional healers to treat erectile dysfunction (Meyer et al., 2008).

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1.6 *Cissampelos capensis*

Cissampelos capensis (Figure 6), is found in the Western Cape Region of South Africa and locally known by the Khoisan and rural folk by the Afrikaans name 'dawidjies' or 'dawidjieswortel' and Xhosa ‘mayisake’. It is the most commonly used medicinal plant species from the Menispermacae family (De Wet et al., 2011). The Menispermaceae family, is indigenous to Southern Africa, has seven genera which include *Cissampelos*. Their popular medicinal usages locally and throughout the world are due to its rich content of isoquinoline alkaloids (Table 1) (Barbosa-Filho, 2000; De Wet et al., 2011). The genus is represented in Southern Africa with four species, namely: *C. capensis, C. hirta* Klotzsch*, C. mucronata* A. Rich and *C. torulosa* E. Mey. ex Harv (De Wet and Van Wyk, 2008).

with twining stems, sprawling about 1 m tall, B) small silver grey leaves and C) Rhizomes when cut has star-like appearance (www.southrenafricanplants.net; www.ispot.org.za; Van Wyk et al., 2000).

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Table 1: The main components of which the *C. capensis* extract is composed as determined by HPLC in *C. capensis* leaves, stems and rhizomes (according to De Wet et al., 2011).

Isolated alkaloid	Type of the alkaloid	Plant part
Bulbocapnine	An aporphine alkaloid	Leaves
Dicentrine	An aporphine alkaloid	Leaves
Salutaridine	A morphinane alkaloid	Leaves
Cissacapine	Bisbenzyltetrahydroisoquinolin alkaloid	Stems Rhizome
Cycleanine	Bisbenzyltetrahydroisoquinolin alkaloid	Stems Rhizome
Insularine	Bisbenzyltetrahydroisoquinolin alkaloid	Stems
12-*O*-methylcurine	Bisbenzyltetrahydroisoquinolin alkaloid	Rhizome

Cissampelos capensis is a dioecious perennial sprawling or twining rambling shrub with thick, divergent branches and twining stems is the only endemic species in Southern Africa occurring in the winter rainfall region. Its leaves are alternate without hairs, ovate to heart-shaped, up to 2.5cm wide and 2.5cm long and thin petioles up to 3cm long. Interestingly, variations in the leaves arise between inland and coastal populations. Its xerophytic adaptations appear with small glaucous leaves and along the coast the leaves tend to be larger and less glaucous (De Wet el al., 2002; Van Wyk et al., 2002, 2009). Flowering occurs during February to May bearing fruit with small fleshy orange berries. The rhizomes are measured as and up to 2.5cm in diameter.

In Khoisan ethnomedicine, *Cissampelos capensis* is of special significance because of its variety in treatment applications (Van Wyk and Gericke, 2000; Van Wyk el al., 2002; De Wet and Van Wyk, 2008). The rhizomes are widely used as a blood purifier and a diuretic medicine; it is also applied to treat ailments such as fever, diabetes, stomach and skin cancer, cholera, syphilis, colic, bladder problems, snakebite, tuberculosis, menstrual problems, prevention of miscarriage and expelling the placenta (Watt and Breyer-Brandwijk, 1962; Smith, 1966; Rood, 1994; Van Wyk and Gericke, 2000; Von Koenen, 2001). Amongst these different usages, it is also said that the rhizome extract can be used to treat male fertility problems.

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In general, it is assumed that the medicinal therapeutic activity of the rhizomes is due to alkaloids such as the alkaloid bisbenzyltetrahydroisoquinoline, which is known to have anti-inflammatory effects, muscle relaxant and anti-carcinogenic activity (Van Wyk et al., 2002; De Wet, 2008; Van Wyk et al., 2009). However, no studies are available reporting the effect of *C. capensis* rhizomes extract on male reproduction function. Therefore, this study aimed at investigating the effects of an aqueous *C. capensis* extract on the functions of spermatozoa in humans.

1.7 Aim of the study

C. capensis is widely used as a medicinal plant but no studies are available reporting the effect of this plant on male sperm function. Thus, this study aimed at investigating the effect of an aqueous *C. capensis* rhizome extract on the following parameters:

- Capacitation
- Acrosome reaction
- Motility
- Vitality
- DNA fragmentation
- Mitochondrial Membrane Potential
- Sperm ROS production

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Chapter 2: Materials and Methods

2.1 Chemicals and Equipment

In this study, all chemicals used were, where possible, of analytical or *in vitro* culture standard.

BDH Biochemical, Poole, England supplied:

- Sodium Pyruvate

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 (Na2HPO4)
- Ethanol
- Magnesium sulphate heptahydrate ($MgSO_4$ * 7 H_2O)
- Potassuim chloride (KCl)
- Sodium bicarbonate ($NaHCO_3$)
- Xylene
- 40 % Formaldehyde

Knittel Gläser, Braunschweig, Germany, supplied:

- Superfrost slides
- Standard count 4 chamber slides (Leja®)
- Microscope slides

Lasec, Cape Town, South Africa, supplied:

- Filter paper (Munk Tell)

Merck, Wadeville Gauteng, South Africa, supplied:

- Glucose (anhydrous)

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- Hydrochloric acid (HCl)
- Hydroxymethyl amino methane (TRIS)
- Potassium diphosphate (KH_2PO_4)
- Sodium chloride (NaCl)
- Sodium hydroxide (NaOH)
- Triethanolamine hydrochloride
- 25% Glutaraldehyde

Millipore, Billerica, USA:

- Millipore distilling machine

Molecular Probes, Eugene, USA, supplied:

- Dihydroethidine (DHE)

Oxoid, Hampshire, England, supplied:

- Phosphate Buffered Saline (PBS)

Promega, Madison, USA, supplied:

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

Sigma-Aldrich, Steinheim, Germany, supplied:

- Annexin V
- Bismarck Brown Y (BBY)
- Bovine serum albumin (BSA) (analytical grade)
- Chlortetracycline (CTC)
- Cysteine
- Dimethylsulphoxide (DMSO)
- Eosin Y
- Glycerol
- Hoechst bid-benzimide 33258
- Mounting medium
- Methanol (MeOH)

- Nigrosin
- Phenol red (dye)
- Progesterone
- Rosè Bengal (RB)
- Supelco-Visiprep™ manifold
- Triton X-100
- Trypan Blue (TB)
- 1,4 diazabicyclo(2.2.2)octane (DABCO)
- polyvinylpyrrolidone (PVP40)
- paraformaldehyde
- 60 % Sodium lactate (Na-Lactate)

Trevigen, Gaithersburg, USA, supplied:

- DePsipher kit

Waters Corporation, Milford, USA:

- Oasis® Hydrophilic-Liphophilic Balance (HLB) 6cc cartridge

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Whatman, Madestone, England:

- Filter paper

Virtis, Warminste, USA:

- Virtis freeze drier

Centrifuges

Labortechnik, Wehingen, Germany, supplied:

- Hermle Z160M
- Hermle Z200A

Incubators

Lasec, Cape Town, South Africa, supplied:

- Series 2000

Microscopes

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

2.2 Media

2.2.1 Medium used for spermatozoa

For washing of spermatozoa, the medium Human Tubular Fluid (HTF) was prepared according to Quinn et al. (1985). This medium is described as the best for the preparation of spermatozoa as it allows for optimum working time and delays death (Quinn et al.,1985). HTF medium is composed of the following substances, which are similar to those found in the female fallopian tube milieu: 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), 20 mM HEPES and dissolved in distilled water. After the solution has dissolved completely, osmolarity was adjusted to 280 mOsmol/kg and 10 mg/ml bovine serum albumin (BSA) were added before working with the medium (HTF-BSA).

2.3 Study design

The effects of the extract on ejaculated human spermatozoa on the following parameters were investigated: Motility, viability, DNA fragmentation, mitochondrial membrane potential, acrosome reaction, capacitation, ROS production and apoptosis (Annexin V binding) (Figure 7).

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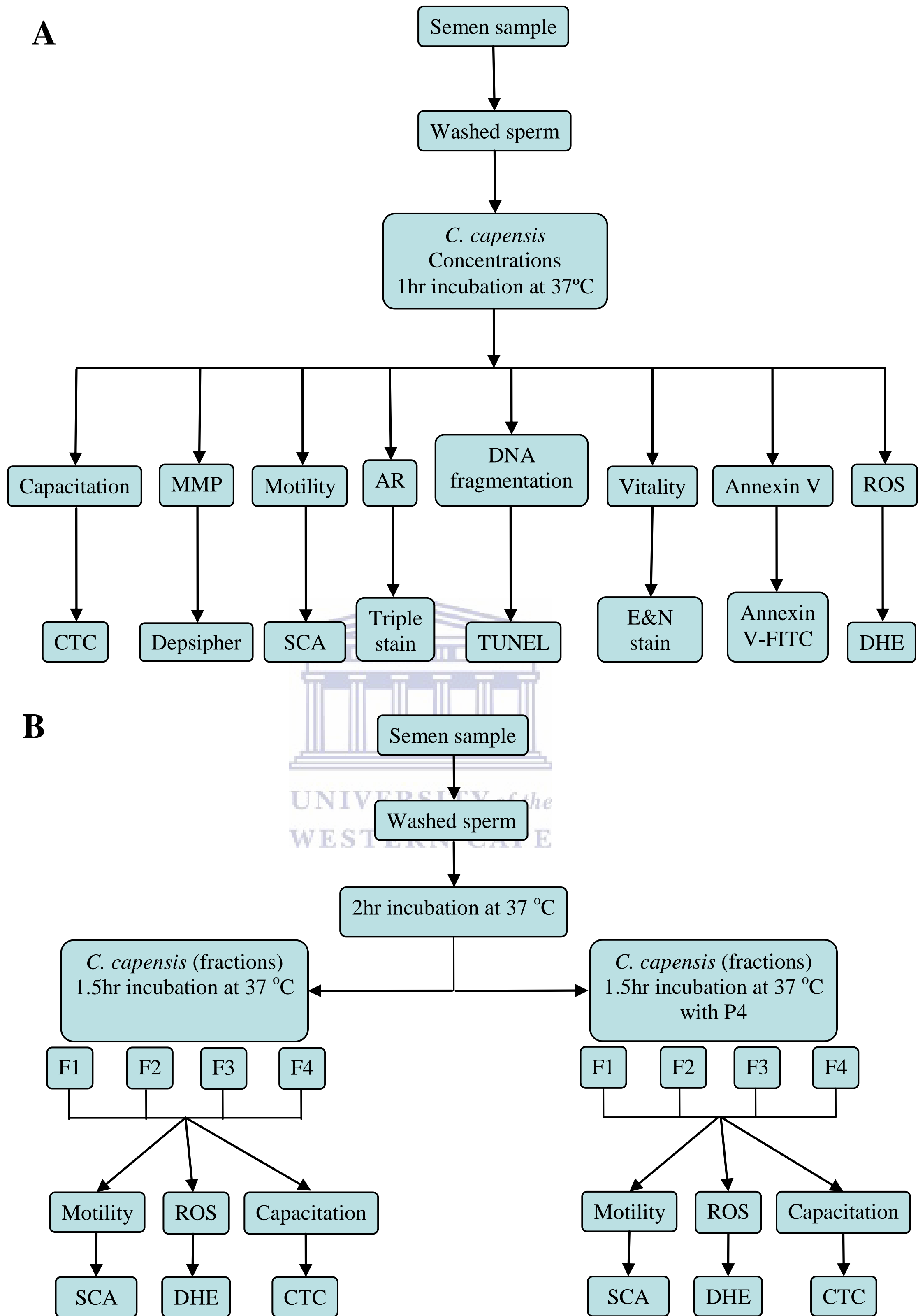
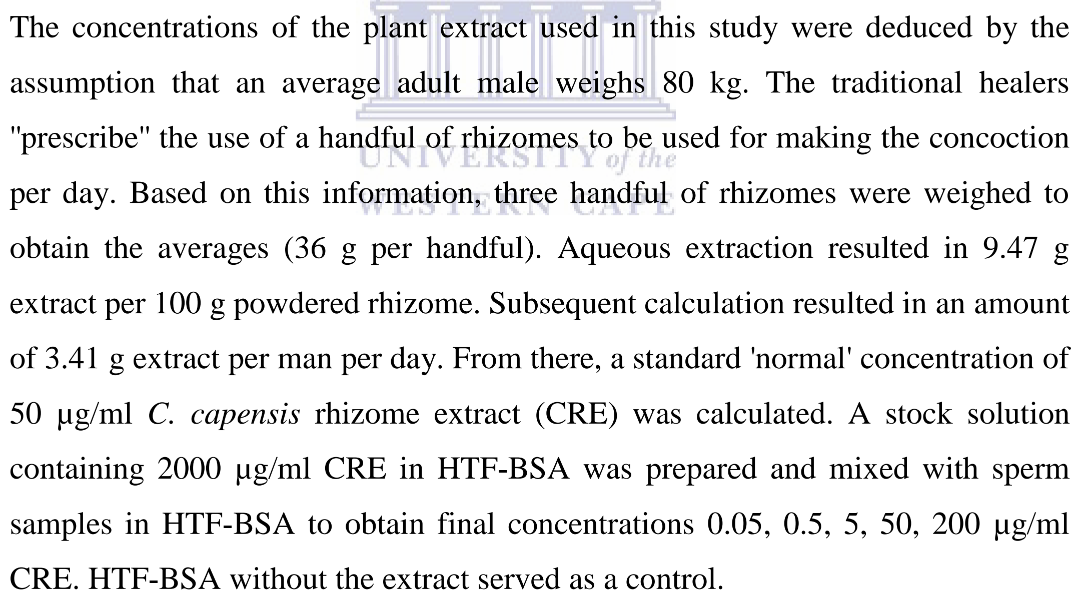


Figure 7: Study design for spermatozoa with *C. capensis* rhizomes extract incubation at different concentrations displaying investigative parameters and testing procedures. A) before fractionation and B) after fractionation with the addition of progesterone. CTC-chlortetracycline, SCA-Sperm Class Analyzer, E&N-Eosin-Nigrosin, AR-acrosome reaction, FITC-Fluorescein isothiocyanate, DHE-dihydroethidine, ROS-Reactive oxygen species, MMP-mitochondrial, AR-acrosome reaction, P4-progesteron and F-fractionation.

2.4 Collection and processing of *Cissampelos capensis* rhizomes

2.4.1 Collection and freeze drying of *Cissampelos capensis* rhizomes

Fresh *Cissampelos capensis* rhizomes (Figure 8) were collected from the Cape Nature Reserve near Belhar in the Western Cape, South Africa, during the summer season (February). In preparation for use in this project, the rhizomes were cleaned and chopped into smaller segments of approximately 2-3 cm of length. Then, these pieces were oven-dried at 25°C for approximately 3 days. Subsequently, segments were placed in a mill to form a powdery substance which was infused with hot (about 70°C) distilled water for 1 hour. After cooling and filtration through Whatman 1 filter paper, the filtered extract was frozen at -20°C until it was freeze-dried using a Virtis freeze-drier machine under the supervision of Mr. Liburne Cyster (Department Biodiversity and Conservation Biology, UWC). The dried extract was then stored at 4°C in a closed container until experimentation.



The concentrations of the plant extract used in this study were deduced by the assumption that an average adult male weighs 80 kg. The traditional healers "prescribe" the use of a handful of rhizomes to be used for making the concoction per day. Based on this information, three handful of rhizomes were weighed to obtain the averages (36 g per handful). Aqueous extraction resulted in 9.47 g extract per 100 g powdered rhizome. Subsequent calculation resulted in an amount of 3.41 g extract per man per day. From there, a standard 'normal' concentration of 50 µg/ml *C. capensis* rhizome extract (CRE) was calculated. A stock solution containing 2000 µg/ml CRE in HTF-BSA was prepared and mixed with sperm samples in HTF-BSA to obtain final concentrations 0.05, 0.5, 5, 50, 200 µg/ml CRE. HTF-BSA without the extract served as a control.

Figure 8: *Cissampelos capensis* rhizomes shown in its natural habitat at the Cape Flats nature reserve, University of the Western Cape.

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2.4.2 Fractionation of aqueous *Cissampelos capensis* rhizomes extract for *in vitro* experiments

Fractionating a crude extract of a plant in which the active compounds are unknown allows for insight into further investigation. Based on chemical or physical properties a plant extract is separated into sub-fractions to isolate and identify one or more active compounds which sets this technique apart from other separation techniques (Bertaux and Montmessin, 2001). Therefore, in order to further understand the effect the *C. capensis* rhizomes extract, fractionation was performed under the supervision of Prof. Patrick Bouic at the Synexa Life Sciences Laboratory in Milnerton, South Africa.

The fractionations were established with various concentrations of methanol (MeOH), namely; 0%, 30%, 60% and 100%. This technique separates the extract into hydrophobic or hydrophilic compounds. In accord, the fraction gradient is estimated as fraction 1 being most hydrophibic (0% MeOH) and fraction 4 as most hydropholic (100% MeOH). Freeze-dried fractions *C. capensis* rhizome extract was dissolved in double-distilled water (ddH_2O) which was dispensed from a Millipore distilling machine to a concentration of 0.1 g/ml (Figure 9).

Thereafter, a vacuum pump was connected to the Supelco-Visiprep™ manifold where a glass test tube was inserted for solvent collection. The Oasis® Hydrophilic-Liphophilic Balance (HLB) 6cc cartridge was mounted on 1 nipple (Figure 10) and a constant pressure was maintained at 7 kPa by controlling the nipple and pressure valves. Following, the activation of the bed with 4 ml methanol and calibrated with 4 ml purified water before 1 ml of sample was loaded. The test tube was then removed and replaced with one for F1 (0% MeOH) collection. After absorption of the extract into the bed, 4 ml of purified water was added to clean out the remaining debris. This process was continued for F2 to F4. For F2, the cartridge bed was loaded with 1 ml 30% MeOH, F3 60% MeOH and F4 100% MeOH. As a precaution when changing the tubes, the vacuum pump was turned off and the nipple valves opened.

After obtaining the completed fractions F1, F2 and F3 were frozen at -20 C^{o} and freeze-dried where as F4 due to the 100% MeOH had to undergo an evaporation step before freeze-drying procedure. The differences in the effects of *C. capensis* crude extract (0.05, 0.5, 5, 50, 200 µg/ml) on the male reproductive system, could subsequently be investigated with the 4 fractions, *in vitro*.

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Figure 9: The freeze-dried *C. capensis* rhizome diluted in ddH_2O at a concentration of 0.1 g/ml to be used in fractionation.

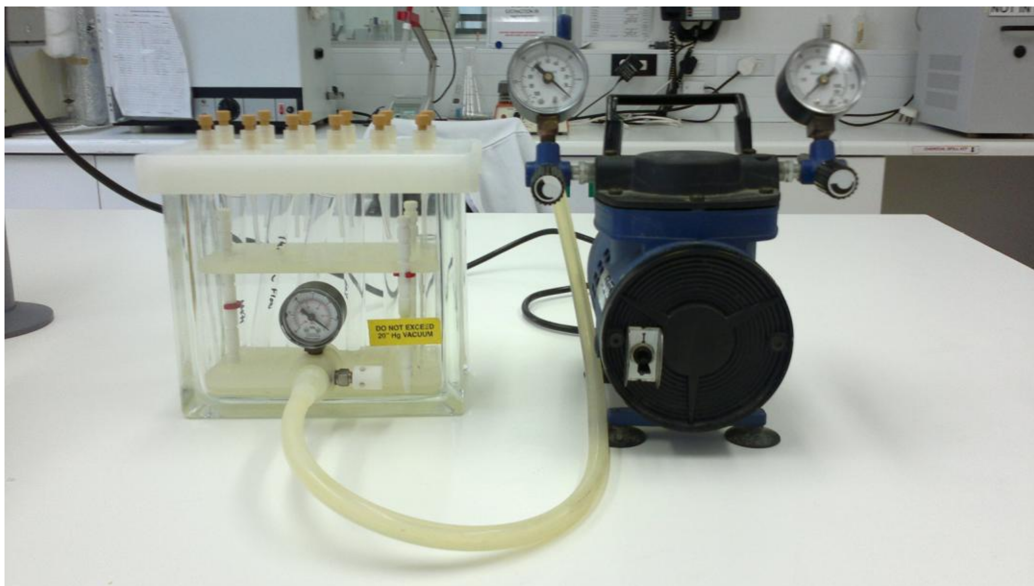

Figure 10: Oasis® HLB 6cc cartridge mounted on a Supelco-Visiprep™ manifold during pump vacuum of *C. capensis* fractionation.

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2.5 Investigation of sperm parameters *in vitro*

2.5.1 Preparation of spermatozoa *in vitro*

This study was ethically approval by the local Institutional Review Board and patients and sperm donors gave informed consent. A total of 103 semen samples were collected after 3-5 days abstinence from patients (n=61) attending the infertility clinic of Tygerberg Hospital, Tygerberg, South Africa, and Vincent Palotti Hospital, Pinelands, South Africa, respectively, as well as fertile sperm donors (n=42).

After liquefaction at room temperature, semen samples were diluted 1:5 with human tubular fluid medium (Quinn et al., 1985), supplemented with 1% bovine serum albumin (HTF-BSA) (280 mOsmol/kg) and centrifuged for 10 minutes at 500xg. The supernatant was discarded and the pellet resuspended in fresh HTF-BSA. Subsequently, sperm suspensions were incubated with CRE at different concentrations for 1 hour at 37°C (Figure 7A).

Following for the second part of investigation, after washing sperm were incubated for two hours allowing for capacitation. Consequently, sperm suspensions were incubated with fractions of CRE (F1, F2, F3 and F4) at different concentrations

(0.05, 0.5, 5, 50, 200 µg/ml) and incubated for 1.5 hours at 37°C with or without Progesterone (P4). Progesterone was used to stimulate acrosome reaction *in vitro* as it is similar to the response produced by ZP (Blackmore et al., 1990; Garcia and Meizel, 1999; Kirkman-Brown et al., 2000). To prepare, P4 was dissolved in dimethylsulphoxide (DMSO), stock solution of P4 was prepared by diluting the thawed stock solution 1:10 in DMSO and waiting 30 minute before adding to the sperm suspension. Sperm suspensions were treated with 20 µg/ml P4 stock solution according to Liu et al. (2008). (Figure 7B).

2.5.2 Determination of sperm motility

Sperm motility plays an important role in fertilization as it is a good predictive parameter for pregnancy (Coetzee et al., 1989; Shulman et al.,1998). Low sperm motility may reduce the chances of sperm fertilizing the ovum and high sperm motility increases that probability (Henkel et al., 2005b).

Sperm motility was measured with the Motility/Concentration module of the Sperm Class Analyzer version 4.1.0.1. An aliquot of 10 µl of the sperm suspension was put on a glass slide covered with a cover slip and the motility of at least 100 sperm were analysed according to WHO criteria set by the SCA system with a Zeiss Photomicroscope III and a 100X oil immersion objective. The different kinematic parameters (Maree et al., 2011) which were analyzed were: total motility, progressive motility, velocity curve line (VCL), velocity straight line (VSL), velocity average path (VAP), linearity (LIN), straightness (STR), beat cross frequency (BCF), and hyperactivation (Table 2).

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Table 2: The different sperm kinematic parameters recorded with SCA

Parameter	Description	Unit
Total motility	Non-progressive motile and progressive motile	%
Progressive motility	Progressive motility	%
Concentration	Number of spermatozoa	$\times 10^6$/ml
VCL (Curvilinear velocity)	VCL is the local speed along the curvilinear path traced by the sperm head.	µm/s
VSL (Straight line velocity)	Straight-line velocity along shortest path from start to end point	µm/s
VAP (Average path velocity)	Average path velocity based on every 5th frame of VCL path	µm/s
LIN (Linearity)	LIN is reported as a ratio of VSL to VCL, expressed as a percentage	%
STR (Straightness)	Straightness, expressed as VSL/VAP	%
BCF (Beat Cross Frequency)	The time-average rate at which the Curvilinear sperm trajectory crosses its average path trajectory	Hz
Hyperactivation	Mature spermatozoa of all mammalian must complete a series of membrane and metabolic changes before they can fertilize an intact ovum, this process has been termed capacitation and is functionally associated with sperm acrosome reaction and acquisition of a distinctive type of motility called hyperactivation	%

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2.5.3 Determination of sperm viability

One of the most basic and essential methods used to identify dead from live sperm is the viability stain (Mortimer et al., 1990; Björndahl et al., 2004).

The eosin-nigrosin staining technique was used to determined viability according to WHO (2010). The staining solution was prepared by dissolving 0.67 g of Eosin Y and 0.9 g of NaCl in 100 ml of distilled water with gentle heating. Thereafter, 10 g of nigrosin was added to the eosin solution and brought to a boil. To remove coarse and gelatinous precipitates the solution was filtered through filter paper and subsequently, the filtrate was stored in a dark glass bottle at room temperature until use.

To stain, a ratio of 1:1 was used where 50 µl of the sperm suspension were added to 50 µl of the Eosin-Nigrosin 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 pink/purple and live sperm white (Figure 11).

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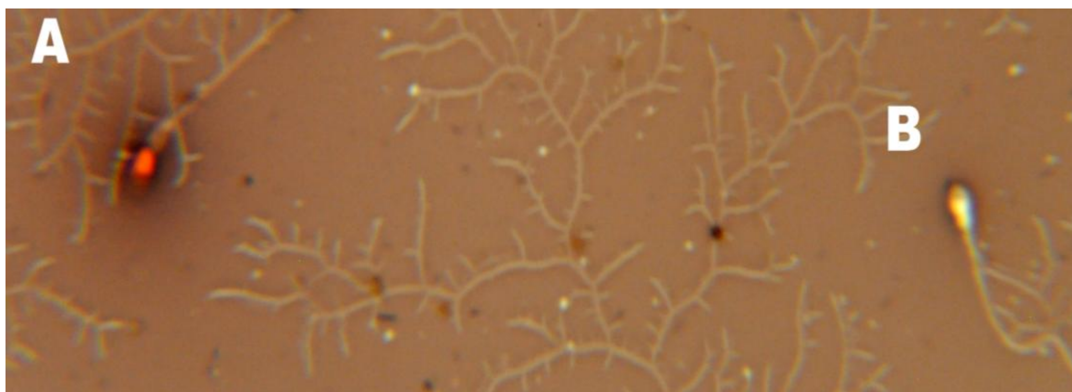

Figure 11: Eosin-Nigrosin stain of human spermatozoa, A) Dead spermatozoa stained pink; B) live spermatozoa appear white (magnification X1000).

2.5.4 Chlortetracycline fluorescence assay

Capacitation is a collective term for changes that a spermatozoon undergoes when it leaves the seminal plasma and comes into contact with the female reproductive tract. These changes include reorganization of membrane proteins,

metabolism of membrane phospholipids, a reduction in membrane cholesterol levels and sperm hyperactivation (Austin, 1952; Chang, 1951; Yanagimachi, 1994). The capacitation state of the spermatozoa was assessed using the chlorotetracyclin (CTC) fluorescence assay method as described previously (Green et al., 1996).

A 100 mg/ml stock solution of Hoechst 33258 was made up in distilled water and stored at 4°C for up to 1 month. Before use, this stock solution was diluted 1:1000 in HTF and then further 1:100 with sperm suspension 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 was prepared on the day of use and contained 750 µM CTC, and a buffer of 130 mM NaCl, 5 mM cysteine in 20 mM Tris-HCl. The pH was adjusted to pH 7.8. This solution was kept wrapped in foil at 4°C 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 12). At all three stages bright fluorescence on the midpiece could be seen.

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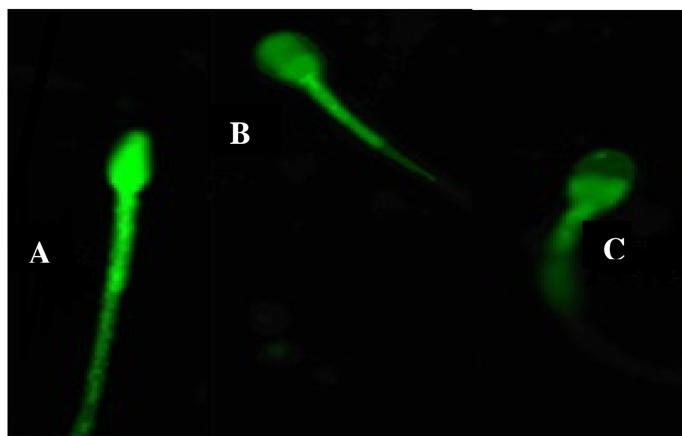

Figure 12: Chlortetracycline staining of human spermatozoa. The following staining patterns can be seen; A) whole sperm head shows bright fluorescence, with or without a brighter equatorial band; this is indicative of uncapacitated acrosome-intact spermatozoa. B) the acrosomal region of the sperm head fluoresce brightly but the post-acrosomal region does not; this denotes capacitated acrosome-intact spermatozoa. C) the acrosomal region of the sperm head is non-fluorescent with or without a fluorescent, post-acrosomal region; this indicates capacitated acrosome-reacted spermatozoa.

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2.5.5 Determination of sperm acrosome reaction

Acrosome reaction (AR) is a predictive fertilization parameter *in vitro* (Cummins et al., 1991; Henkel et al., 1993). The importance of acrosome reaction is in the penetration of the ZP as it is dependent on the release of the enzymes present within the sperm acrosome. Acrosome reaction was determined by using the triple staining technique according to Talbot and Chacon (1981) following the protocol of Henkel et al. (1993). The triple stain consists of Trypan blue (TB) for determination of dead cells, Rose Bengal (RB) for outlining the intact acrosomal cap and Bismarck brown Y (BB Y) for determination of live cells, whereas glutaraldehyde was used as fixative the solutions were prepared as follows:

To prepare TB; 2% trypan blue was dissolved in cold HTF then filtered with filter paper to remove all undissolved granules. The solution was then stored in a dark glass bottle at room temperature until use (storage up to 1 month). Subsequently, RB was prepared by dissolving 0.8% RB and 0.1 M Tris in 250 ml of distilled water. The pH was adjusted to 5.6 with concentrated HCl and continuing with 2 N HCl as required. The solution was then stored in a dark bottle as it is light sensitive at room temperature (storage up to ½ week). Simultaneously, 0.8% BB Y; was dissolved in distilled water and the pH adjusted to 1.8 with HCl. The solution was then stored in a dark bottle at room temperature (storage up to

2 weeks). Lastly, 3 % glutaraldehyde was prepared by using HTF and stored (until required).

Semen samples were diluted 1:5 with HTF-BSA and centrifuged for 10 minutes at 500xg. The supernatant was discarded, the pellet resuspended with fresh HTF-BSA and after incubation with *C. capensis* rhizomes extract, samples were aliquoted to 100 µl then incubated for 2 hours at 37°C. After incubation, 100 µl of 2% Trypan blue were added and incubated for a further 15 min at 37°C. Thereafter, samples were washed with 1 ml HTF (without BSA) and centrifuged for 10 minutes at 300xg.

The supernatant was discarded, 200 µl of 3% glutaraldehyde were used to fix the pellet and incubated for 20 minutes at 37°C. Afterwards, 1 ml of HTF (without BSA) was added to samples and centrifuged at 300xg for 10 min. Thereafter, the supernatant was discarded and 10 µl of the pellet was used to prepare a smear and left to air dry. Following, slides were stained with 0.8% BB for 5 min in a water bath at 40°C and washed 3 times with distilled water. For the counter stain, 0.8% RB was used and slides were immersed for 1 hour at room temperature. Slides were then washed 3 times with tap water and immersed in 100% ethanol and xylene to remove water. Finally, slides were air dried.

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After staining the slides were viewed with a 100X oil immersion objective using a light microscope. Analysis of at least 200 sperm was performed for live reacted (LR); live non-reacted (LNR); dead reacted (DR); and dead non-reacted (DNR) (Figure 13).

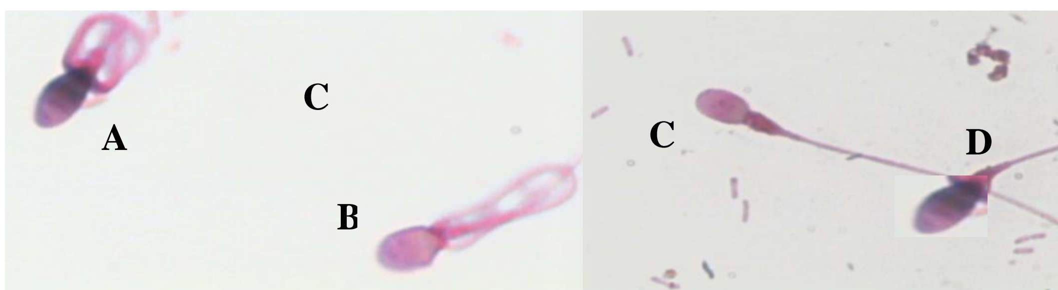


Figure 13: Triple stain display in sperm acrosomal reaction A) dead reacted, B) live reacted, C) live non-reacted and D) dead non-reacted.

2.5.6 Determination of reactive oxygen species (ROS) producing sperm

To determine ROS production using dihydroethidine (DHE) as fluorescing probe the protocol according to Henkel et al. (2003) was followed of DHE. A stock solution was prepared by using 20 µM DHE in PBS at pH 7.4. After incubation of sperm samples with *C. capensis* rhizome extract for 1hr at 37°C, 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 was 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). Red fluorescing sperm, indicate excessive ROS production. The percentage of the sperm was calculated from at least 200 spermatozoa (Figure 14).

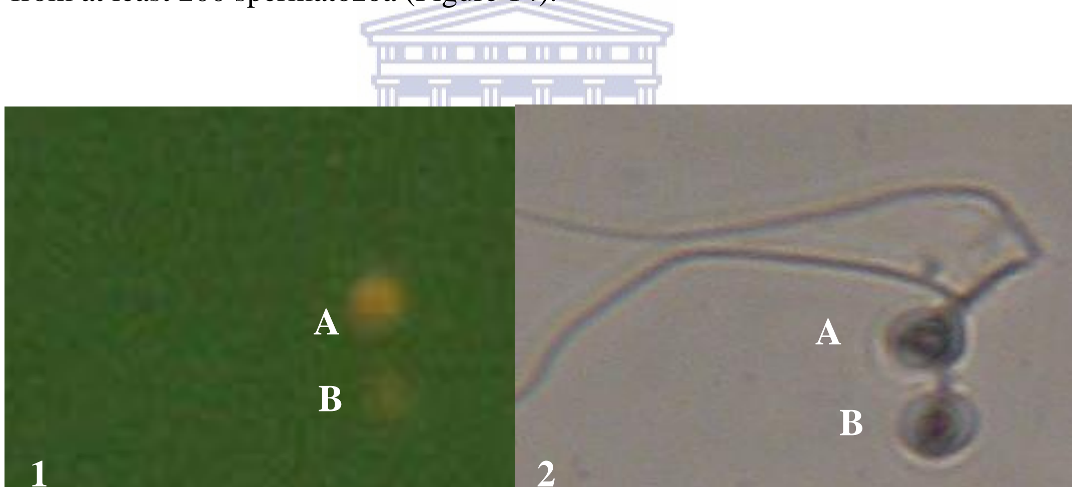


Figure 14: 1: Spermatozoa after staining with DHE; ROS-positive sperm (A) fluoresced orange/red, and 2: ROS -negative sperm (B).

2.5.7 Determination of the sperm mitochondrial membrane potential (Δψm)

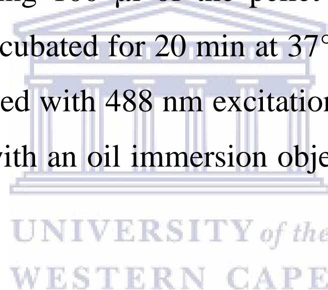
The energy produced within spermatozoa during mitochondrial respiration is stored as an electrochemical gradient across the mitochondrial membrane. Provided that the cell is healthy, this creates a mitochondrial trans-membrane potential, called delta-psi (Δψm).

In unhealthy cells the mitochondrial membrane potential (MMP/Δψm) induces depolarization of the trans-membrane potential causing the onset of apoptosis in

spermatozoa and loss of oxidative phosphorylation. Accordingly, calculation of the mitochondrial membrane potential (MMP) provides an indication of mitochondrial functionality (Ly et al., 2003).

Intact mitochondrial membrane potential was determined by means of 5,5'6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide which is a lipophilic cationic dye. The protocol provided by the manufacturer was modified as follows: The reaction buffer was diluted with distilled water 1:10 and 20 µl stabilizer was added per milliliter buffer with 1 µl of DePsipher dye. This solution was added to 500 µl prepared reaction buffer, vortexed thoroughly and centrifuged for 1 min at 10,000xg. The remaining supernatant was transferred into a test tube ready for immediate use.

Subsequently, after washing 100 µl of the pellet was resuspended in 50 µl of DePsipher solution and incubated for 20 min at 37°C. After this incubation, 10 µl of each sample were viewed with 488 nm excitation and 590 emission filters with at X1000 magnification with an oil immersion objective using an epifluorescence microscope (Zeiss).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Spermatozoa exhibiting a green fluorescence within their mid pieces were regarded as having disturbed $\Delta\psi m$ (Figure 15), while those spermatozoa showing red fluorescence were regarded as having intact $\Delta\psi m$. The percentage of sperm with intact $\Delta\psi m$ was calculated from at least 200 spermatozoa.

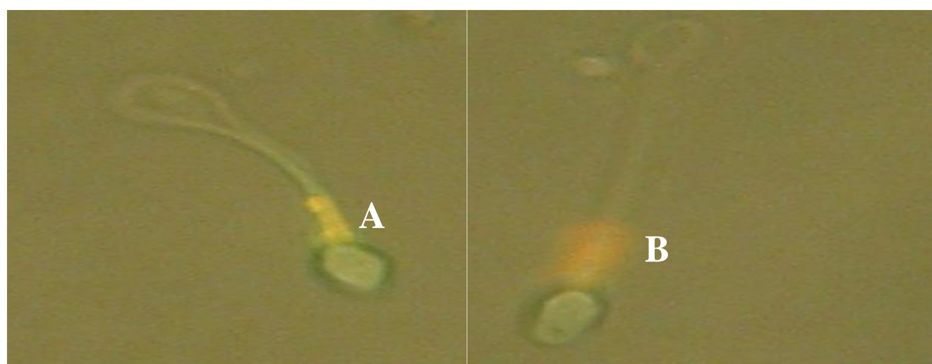

Figure 15: DePsipher™ stain, A) sperm with disrupted $\Delta\psi m$ had a green/yellow fluorescence and B) sperm with intact $\Delta\psi m$ showed red fluorescence.

2.5.8 Determination of sperm DNA-fragmentation

The DNA fragmentation of spermatozoa, according to Henkel et al. (2004) was determined by using the TUNEL (Terminal deoxynucleotide transferase-mediated dUTP nick-end labelling) assay. The suitability and reproducibility of the assay is accompanied by the adequate detection of single and double DNA strand breaks (Gorczyca et al., 1993).

Spermatozoa were incubated with different concentration of *C. capensis* rhizome extract for 1 hour at 37°C. Samples of 100 µl aliquots were centrifuged for 10 min at 300xg, the supernatant discarded and the pellet was resuspended with 100 µl PBS. A smear was made on Superfrost slides and air dried. After air drying, slides were fixed a freshly prepared 4% methanol-free formaldehyde in PBS for 25 min at 4°C. Slides were then washed in PBS for 5 min at room temperature, permeabilized with 0.2% triton X-100 in PBS for 5 min and washed with fresh PBS twice for 5 min. Excess liquid was removed from the slides by gentle tapping. Thereafter, 100 µl of equilibrium buffer were added for 10 min at room temperature. Then, TdT incubation buffer was prepared which consist of 45 µl equilibrium buffer, 5 µl nucleotide mix and 1 µl TdT enzyme. Of this TdT buffer, 20 µl were then added to each slide and covered with plastic cover slips and incubated in a dark humidified chamber for 1 hour at 37°C.

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To terminate the reaction, cover slips were carefully removed and slides were then immersed in diluted 2x SSC solution in a Coplin jar for 15 min at room temperature. Thereafter, slides were washed twice in distilled water for 5 min at room temperature and excess water was cautiously removed and slides were analyzed immediately.

An epifluorescence microscope (Zeiss) at 400X magnification with an oil immersion objective was used. Spermatozoa that fluoresced brightly were counted as TUNEL-positive (Figure 16) indicating DNA fragmentation has occurred within the sperm nucleus and a percentage was calculated from at least 200 sperm.

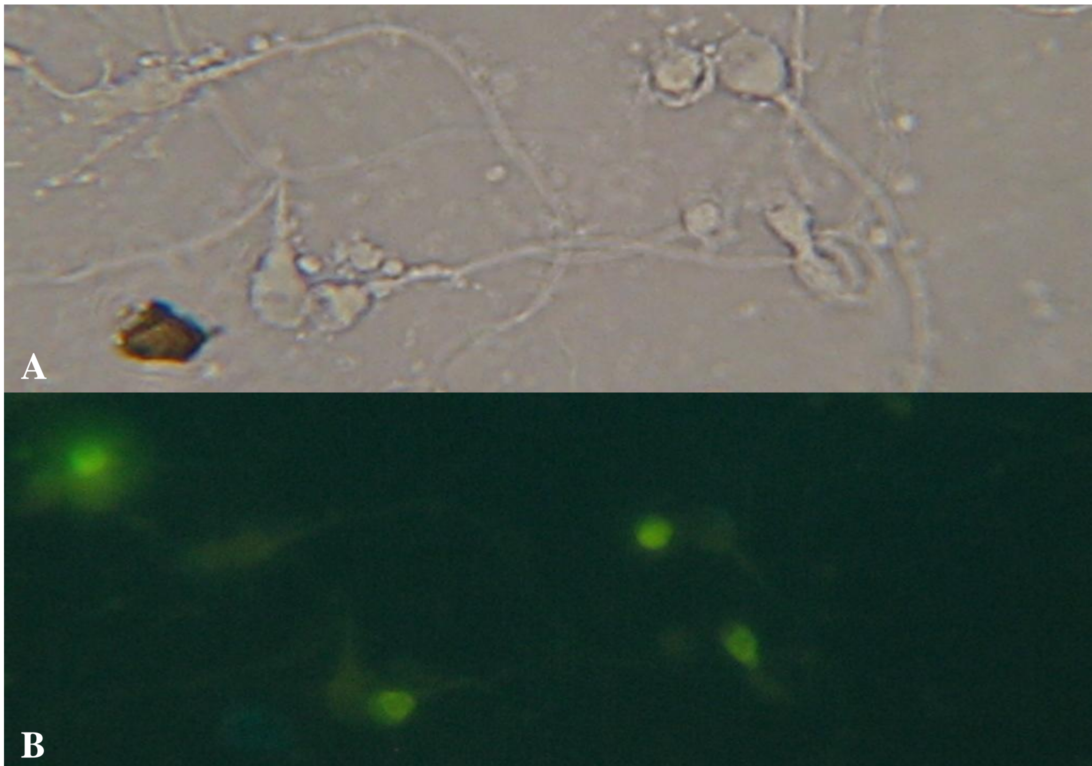

Figure 16: A) Sperm with nuclear DNA damage B) TUNEL-positive sperm fluoresced bright green after TUNEL assay

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2.5.9 Determination of apoptosis (Annexin V-binding)

Apoptotic sperm cells were indentified using the Annexin V-FITC Apoptosis Detection Kit (Sigma-Aldrich). Externalization of phosphatidylserine (PS) from the inner to the outer leaflet of the plasma membrane is an early step in the apoptotic process. Annexin V is a calcium-dependent phospholipid-binding protein with a very high affinity for PS (Vermes et al., 1995; Glander and Schaller, 1999). To differentiate apoptotic from necrotic spermatozoa, the sperm nuclei were stained with 4',6- diamidino-2-phenylindole dihyrochloride (DAPI). The procedure was conducted according to the protocol recommended by the manufacturer.

After incubation of spermatozoa with different concentrations of *C. capensis* rhizomes extract, samples were centrifuged for 10 min at 300xg and the supernatant was discarded. The pellet was resuspended with binding buffer (10mM Hepes/NaOH, 140mM NaCl, 2.5mM $CaCl_2$). Subsequently, 5 µl Annexin V-FITC were added with 195 µl of sperm suspension, mixed and incubated for

10 min at room temperature. After incubation, 10 µl of DAPI (5 µg/ml) were added. The sample was then centrifuged for 5 min at 500xg and resuspended in 190 µl binding buffer. Thereafter, smears were made on slides and one drop of 0.22 M 1,4diazabicyclo(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 a fluorescent microscope (Zeiss). A total of 200 spermatozoa were randomly assessed per slide in at least five fields. Spermatozoa were identified as follows:

1. Annexin (-) DAPI (-); Live, non-apoptotic (No fluorescence)
2. Annexin (+) DAPI (-); Live, apoptotic (green membrane and the nucleus not stained)
3. Annexin (+) DAPI (+); Dead, apoptotic (green membrane and blue nucleus)
4. Annexin (-) DAPI (+); Dead, non-apoptotic (only blue nucleus) (Figure 17).

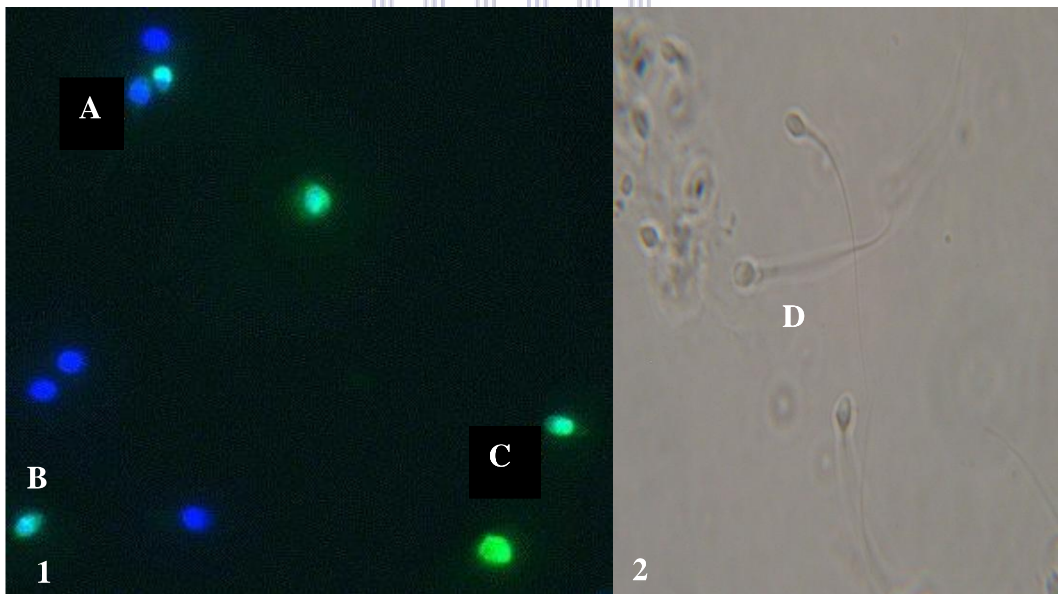

Figure 17: Annexin V-FITC Apoptosis to externalization of phosphatidylserine (PS); 1: A) Annexin (-) DAPI (+), B) Annexin (+) DAPI (+), C) Annexin (+) DAPI (-) and 2: D) Annexin (-) DAPI (-); Live, non-apoptotic (No fluorescence).

2.6 Statistical analysis

All statistical calculations were performed using the MedCalc Statistical Software (Version 12.3.0; MedCalc Software, Mariakerke, Belgium). After testing for normal distribution by means of the Kolmogorov-Smirnov test, appropriate tests (Spearman rank correlation, ANOVA, repeated measures analysis and independent t-test) were used for further analysis. Data were expressed as mean±SD or SEM. A P-value $P<0.05$ was regarded as significant.

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Chapter 3: Results

3.1 Fractionation of *C. capensis* crude extract: Yields

After collection and drying *C. capensis* rhizomes were cut into pieces and milled into powder. Hereafter, an infusion was prepared, cooled and frozen for freeze drying. Following the freeze-drying process, the powder obtained amounted to a yield of 9.47%. The powder (*C. capensis* crude extract) was then fractionated with 0% MeOH (F1), 30% MeOH (F2), 60% MeOH (F3) and 100% MeOH (F4) (Figure 18). All fractions obtained were similar to the crude extract in colour and no other variations were observed. The average yields as calculated from the yield of the crude extract obtained for all fractions were established as: F1= 25.7%, F2= 21.6%, F3= 24.3% and F4= 28.4%.

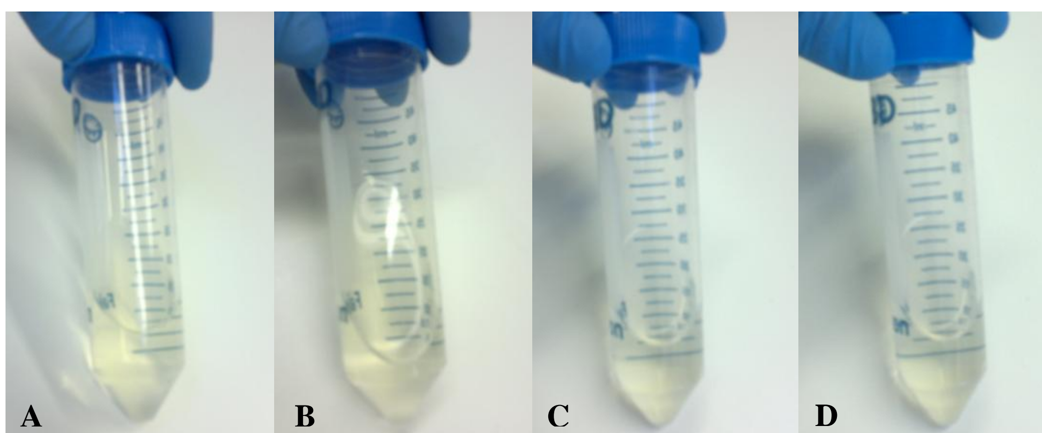

Figure 18: Different fractions of *C.capensis* A) F1= 0% MeOH, B) F2= 30% MeOH, C) F3= 60% MeOH and D) F4= 100% MeOH.

3.2 Effects of crude *C. capensis* rhizome extract on human spermatozoa *in vitro*

Treatment of ejaculated human spermatozoa from 77 semen samples (45 patients and 32 healthy sperm donors) with different concentrations of the *C. capensis* rhizome extract (0.05 µg/ml, 0.5 µg/ml, 5 µg/ml, 50 µg/ml and 200 µg/ml) was conducted for washed spermatozoa.

3.2.1 Effect of crude *C. capensis* rhizome extract on sperm motility

Summary results of the motility parameters analyzed are shown in Table 3. Although total and progressive motility were not affected by increasing concentrations of *C. capensis* rhizomes extract, significant, dose-dependent effects on various motion parameters were obvious.

While the values for VAP (Figure 19), VSL (Figure 20) and linearity (Figure 21) decreased with increasing concentrations of the extract, the percentage of hyperactivated sperm (Figure 22) and the beat cross frequency (Figure 23) increased significantly (Table 2). The VCL of the sperm cells and the straightness of the motion path remained unchanged.

Table 3: Summary statistics of sperm motility parameters. While total and progressive motility remained unchanged, parameters that are associated with sperm hyperactivation changed significantly.

VAP: velocity average path; VCL: velocity curvilinear; VSL: velocity straight line; H: hyperactivation; BCF: beat cross frequency; LIN: linearity; STR: straightness

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	Control	0.05 µg/ml	0.5 µg/ml	5 µg/ml	50 µg/ml	200 µg/ml	ANOVA (P value)
Total Motility (%)	52.33±19.13	47.47±17.38	51.95±18.51	54.86±20.45	50.14±17.59	51.90±18.68	0.296
Progressive motility (%)	21.39±12.45	19.53±9.84	19.39±8.89	20.62±9.80	19.98±9.89	18.97±8.70	0.736
VAP (µm/s)	62.71±20.30	59.56±19.70	58.87±19.06	58.39±17.95	56.03±19.11	50.56±14.27	0.005
VCL (µm/s)	89.38±21.19	88.13±20.91	87.12±22.48	88.91±21.58	88.72±23.01	85.83±21.15	0.935
VSL (µm/s)	52.07±20.15	48.13±18.36	47.71±17.70	47.69±19.02	44.90±17.96	41.35±16.05	0.002
H (%)	1.54±0.71	1.60±0.76	1.64±0.75	1.68±0.98	1.81±0.78	2.04±0.85	0.004
BCF (Hz)	19.27±2.36	20.10±4.02	19.81±3.46	20.38±2.93	20.62±3.06	22.00±3.12	<0.001
LIN	55.60±10.20	54.25±11.83	53.80±11.59	53.18±11.71	50.36±11.83	46.61±8.90	<0.001
STR	76.52±8.56	76.11±9.66	75.26±11.20	76.97±8.37	75.84±8.60	74.42±8.35	0.629

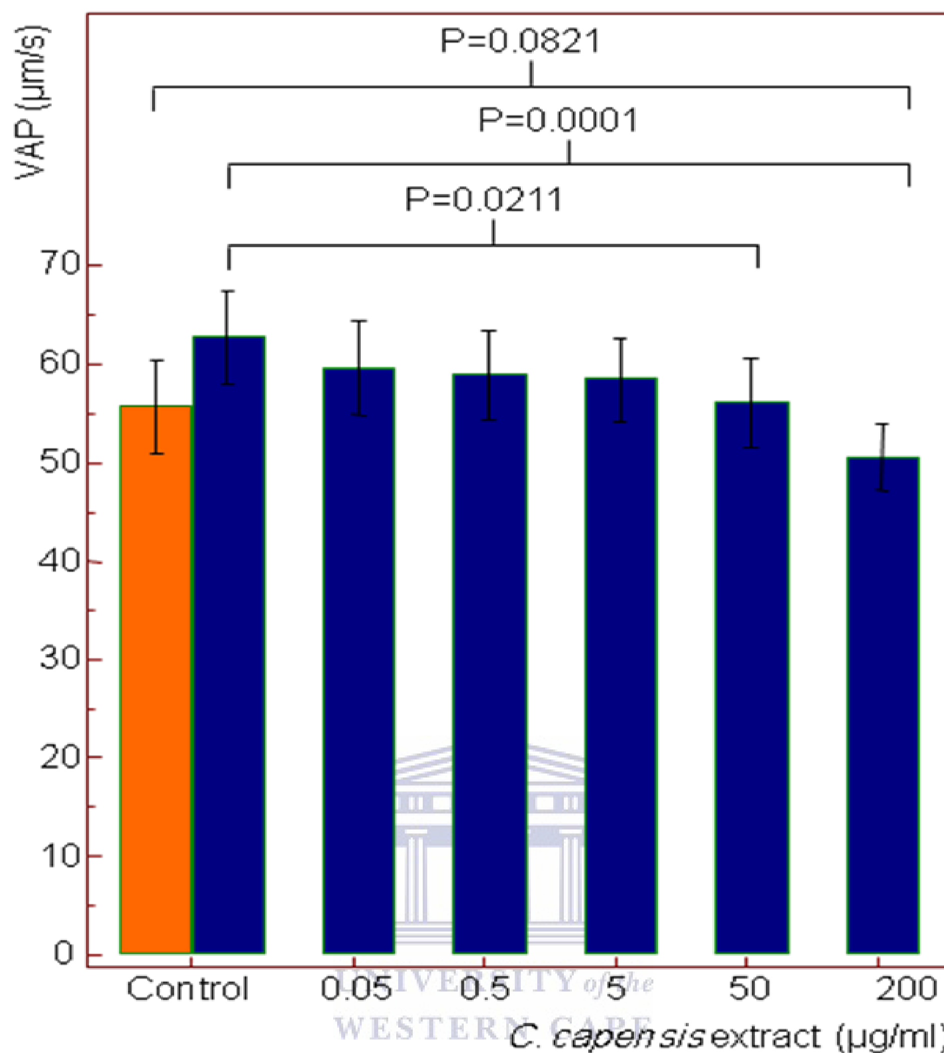

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Figure 19: The effect of different concentrations of *C. capensis* extract (0.05, 0.5, 5, 50 and 200 μg/ml) on VAP *in vitro*. The orange bar represent before incubation, whereas blues bar represent after 1 hour incubation. Values shown as mean±SEM (n=70).

When compared with the control before incubation, sperm incubated at a *C. capensis* concentration of 200 μg/ml showed a marginal significant decrease (P=0.0821) of VAP. In contrast, when compared with the control after 1hour incubation in 200 μg *C. capensis* /ml, this difference was highly significant (P=0.0001). Values started becoming significant (P=0.0211) from a *C. capensis* concentration of 50 μg/ml. ANOVA (P=0.005) as well as repeated measures analysis revealed significant (P<0.0001) trends.

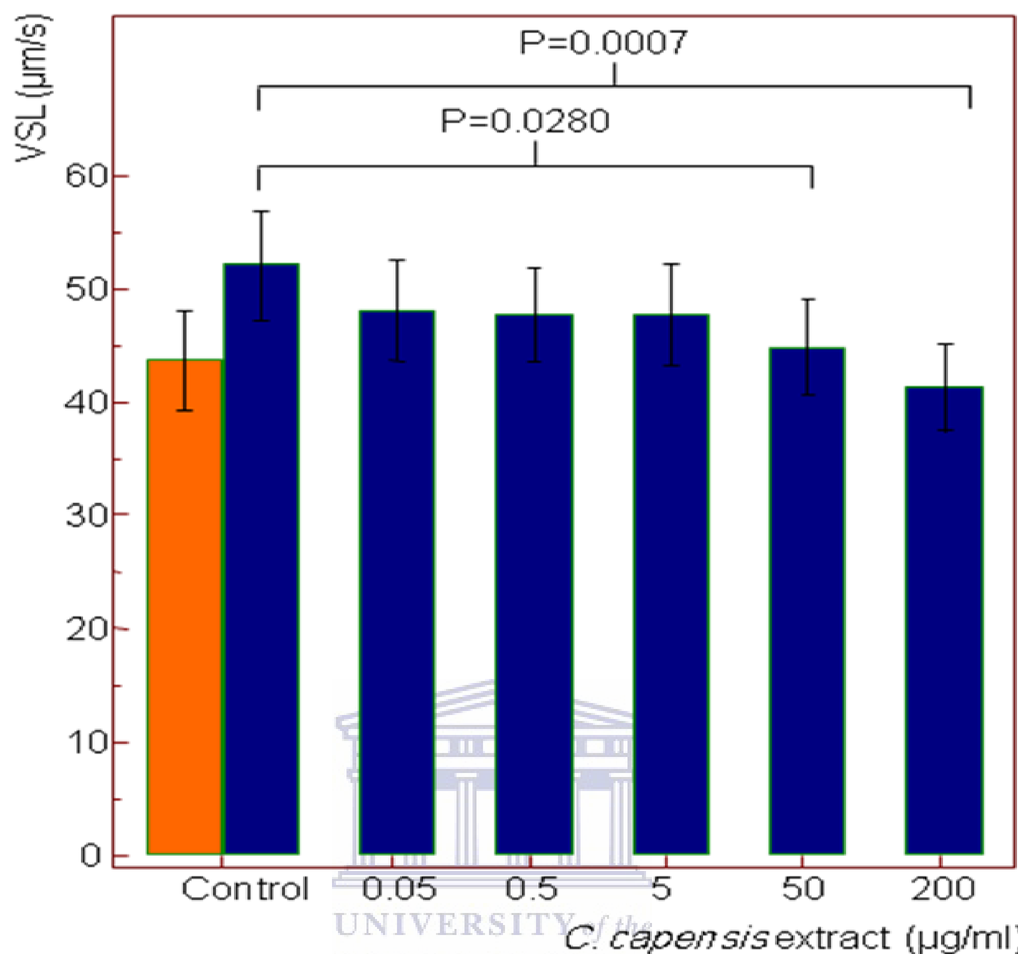

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Figure 20: The effect of different concentrations of crude *C. capensis* extract (0.05, 0.5, 5, 50 and 200 µg/ml) on straight line velocity (VSL) *in vitro*. The orange bar represents before incubation, whereas blue bars represent after 1 hour incubation. Values shown as mean±SEM (n=70).

When compared with the control after incubation, sperm incubated at a *C. capensis* concentration of 200 µg/ml showed a significant decrease (P=0.0007) as well as at 50 µg/ml showed a significant decrease (P=0.0280) of VCL. ANOVA (P=0.020) as well as repeated measures analysis revealed significant (P<0.0001) trends.

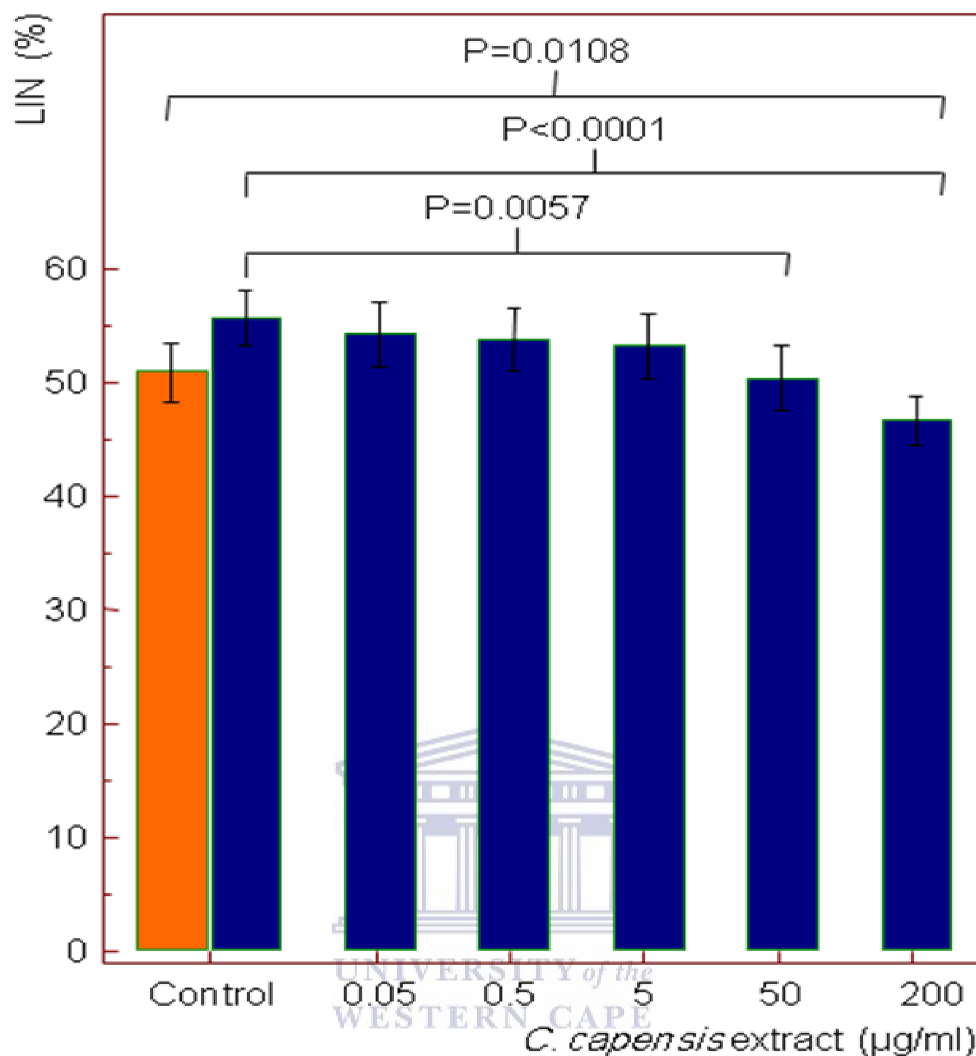

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Figure 21: The effect of different concentrations of crude *C. capensis* extract (0.05, 0.5, 5, 50 and 200 µg/ml) on linearity (LIN) *in vitro*. The orange bar represents before incubation, whereas the blue bar represent after 1 hour incubation. Values shown as mean±SEM (n=70).

When compared with the control before incubation, sperm incubated at a *C. capensis* concentration of 200 µg/ml showed a marginal significant decrease (P=0.0108) of LIN. In contrast, when compared with the control after 1hour incubation in 200 µg *C. capensis* /ml, this difference was highly significant (P<0.0001). Values started becoming significant (P=0.0057) from a *C .capensis* concentration of 50 µg/ml. ANOVA (P<0.001) as well as repeated measures analysis revealed significant (P<0.0001) trends.

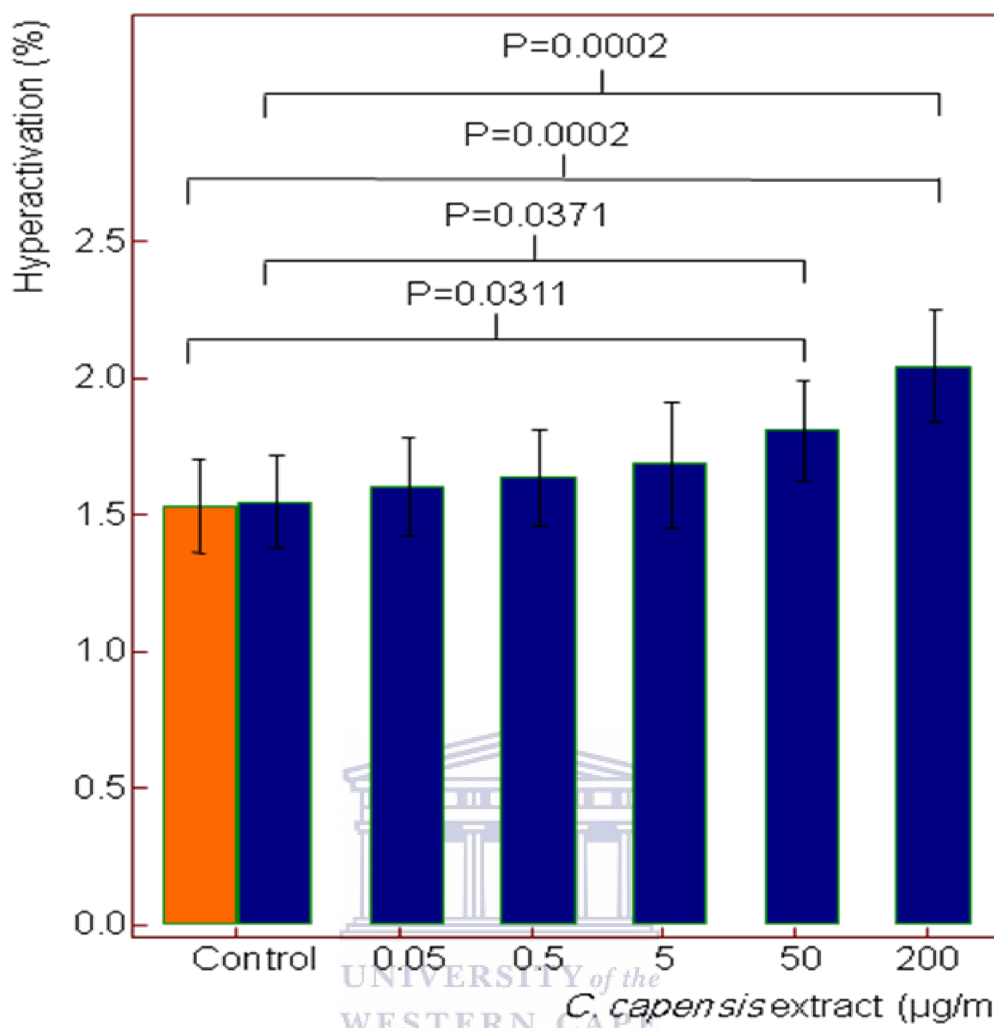

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Figure 22: The effect of different concentrations of crude *C. capensis* extract (0.05, 0.5, 5, 50 and 200 µg/ml) on hyperactivated *in vitro*. The orange bar represents before incubation, whereas the blue bars represent after 1 hour incubation. Values shown as mean±SEM (n=70).

When compared with the control before incubation, sperm incubated at a *C. capensis* concentration of 50 µg/ml showed a significant increase (P=0.0311) as well as significant increase (P=0.0371) when compared with the control after 1hour incubation. Also, when compared with the control before and after 1hour incubation (same effect) in 200 µg *C. capensis* /ml, a highly significant increase (P=0.0002). ANOVA (P=0.004) as well as repeated measures analysis revealed significant (P<0.0001) trends.

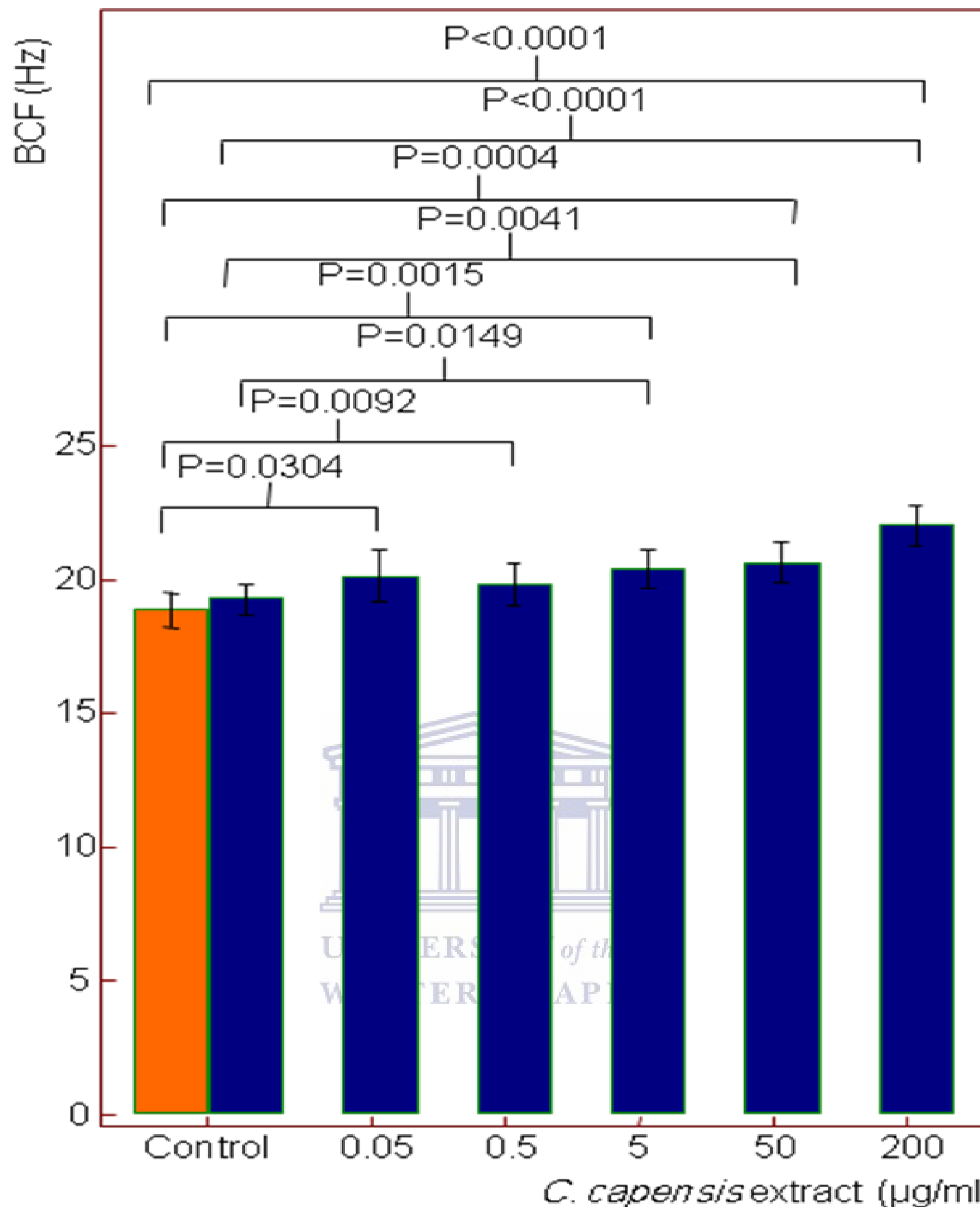

Figure 23: The effect of different concentrations of crude *C. capensis* extract (0.05, 0.5, 5, 50 and 200 μg/ml) on beast cross frequency (BCF) *in vitro*. The orange bar represents before incubation, whereas blue bars represent after 1 hour incubation. Values shown as mean±SEM (n=70).

When compared with the control before incubation, sperm incubated at a *C. capensis* concentration of 0.05 μg/ml showed a significant increase (P=0.0304), as well as (P=0.0092) of 0.5 μg/ml, (P=0.0015) of 5 μg/ml, (P=0.0004) of 50 μg/ml and (P<0.0001) of 200 μg/ml of BCF. Furthermore, when compared with the control after 1hour incubation in 200 μg *C. capensis* /ml, this was highly significant increase (P<0.0001), as well as (P=0.0149) of 5 μg/ml and (P=0.0041)

of 50 µg/ml. ANOVA (P<0.001) as well as repeated measures analysis revealed significant (P<0.0001) trends.

In Table 4, 5 and 6, all correlations of the hyperactivation, progressive and total motility with other motility parameters that were investigated in the study are summarized.

Table 4: Correlations of the hyperactivation with total and progressive motility; VAP: velocity average path; VCL: velocity curvilinear; VSL: velocity straight line

	N	r	P-value
Total Motility (%)	689	0.249	<0.0001
Progressive motility (%)	689	0.181	<0.0001
VAP (µm/s)	689	0.114	0.0027
VCL (µm/s)	689	0.158	<0.0001
VSL (µm/s)	688	0.066	0.0819

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Table 5: Correlations of the progressive motility with total motility; STR: straightness; VAP: velocity average path; VCL: velocity curvilinear; VSL: velocity straight line

	N	r	P-value
Total Motility (%)	690	0.577	<0.0001
STR (%)	690	0.367	<0.0001
VAP (µm/s)	690	0.737	<0.0001
VCL (µm/s)	690	0.627	<0.0001
VSL (µm/s)	689	0.683	<0.0001

Table 6: Correlations of the total motility with VAP: velocity average path; VCL: velocity curvilinear; VSL: velocity straight line

	N	r	P-value
VAP (µm/s)	690	0.272	<0.0001
VCL (µm/s)	690	0.207	<0.0001
VSL (µm/s)	689	0.316	<0.0001

3.2.2 Effect of crude *C. capensis* rhizome extract on sperm viability and mitochondrial membrane potential

After exposing ejaculated human spermatozoa to different concentrations of *C. capensis* rhizome extract, the results for viability as determined in the triple stain did not reveal any difference to the control. Although, results obtained after AVOVA were not significant (P=0.664), an ANOVA trend analysis after repeated measures analysis revealed a significant trend (P=0.0019) (Figure 24).

For the mitochondrial membrane potential (Δψm), no effect of the *C. capensis* rhizome extract was found (ANOVA: P=0.864) (Figure 25).

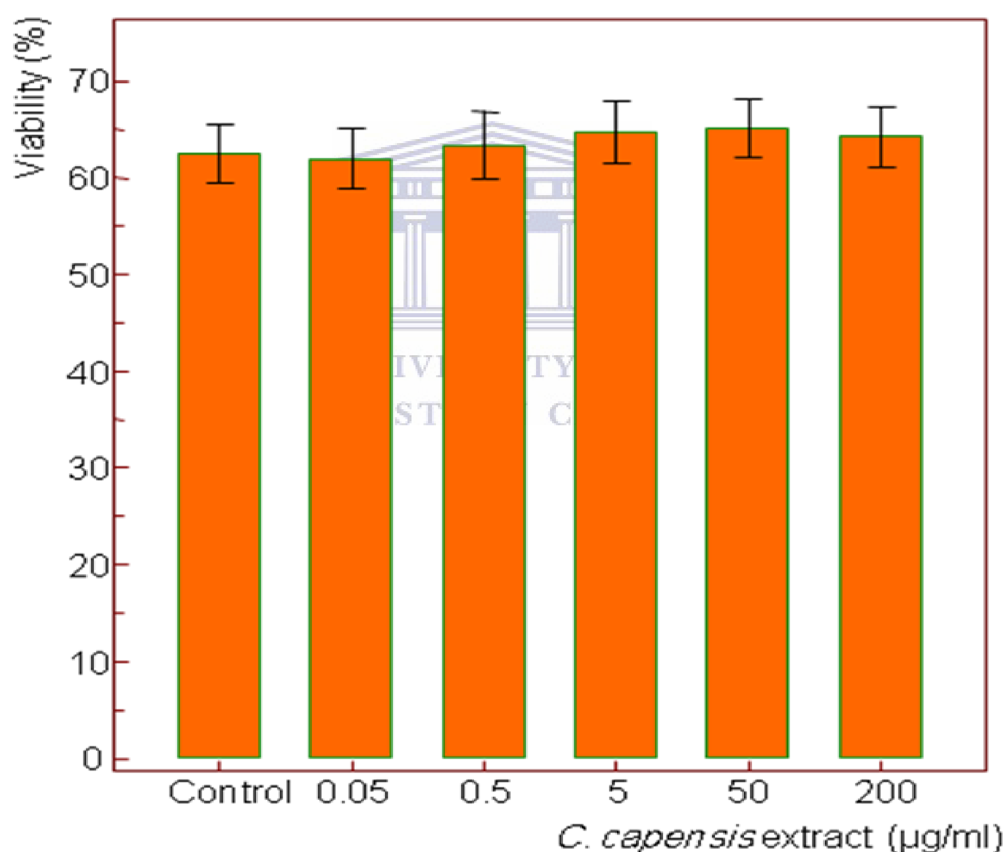

Figure 24: The effect of different concentrations of crude *C. capensis* extract (0.05, 0.5, 5, 50 and 200 µg/ml) on viability as determined by means of the trypan blue incorporation in the triple stain *in vitro*. Values shown as mean±SEM (n=70). No significant difference was found when compared concentrations with control, as well as the result obtained after ANOVA in not significant (P=0.664). However, a trend analysis after repeated measures analysis revealed a significant trend (P=0.0019).

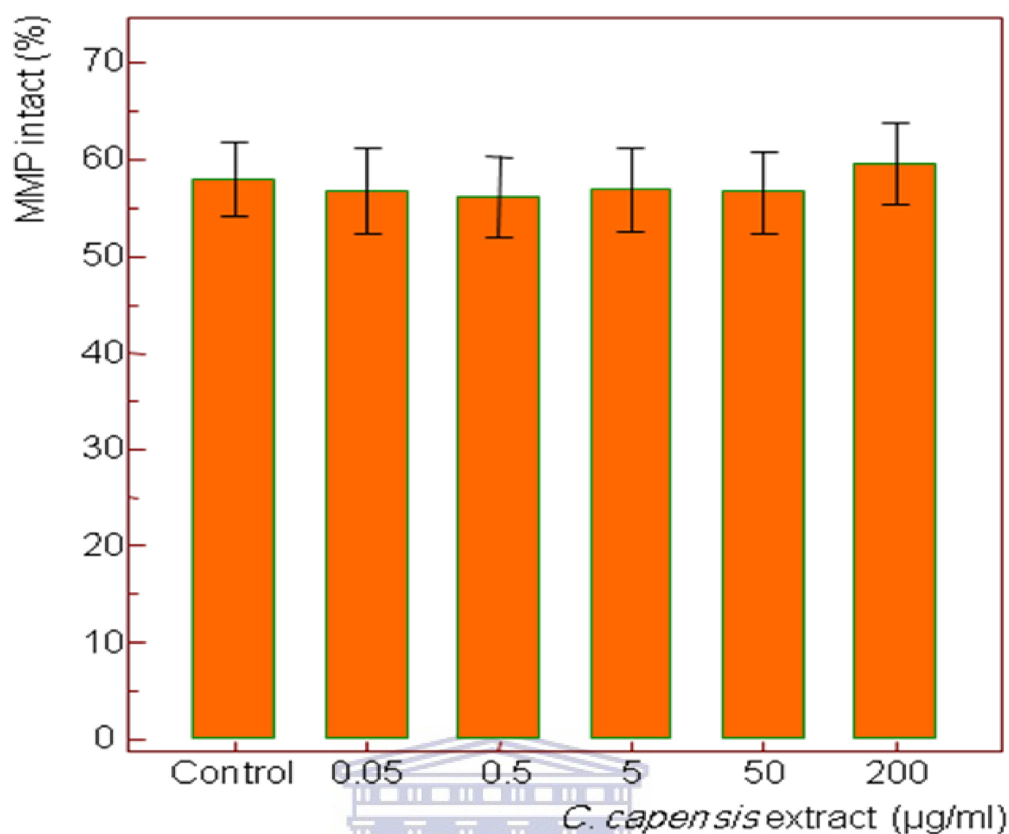

Figure 25: The effect of different concentrations of crude *C. capensis* extract (0.05, 0.5, 5, 50 and 200 µg/ml) on sperm intact-mitochondrial membrane potential *in vitro*. Statistical difference shown as P<0.05. Values shown as mean±SEM (n=40). No significant difference was found when compared concentrations with control, as well as the result obtained after ANOVA in not significant (P=0.864) and a trend analysis after repeated measures (P=0.7168). No significance can be seen.

3.2.3 Effect of *C. capensis* rhizome extract on sperm DNA fragmentation

Increasing concentrations of the extract resulted in a dose-dependent, highly significant (ANOVA: P<0.001) increase in the percentage of spermatozoa with DNA fragmentation. Even the lowest concentration (0.05 µg/ml) used caused a significant (P=0.0113) increase.

Both, ANOVA and ANOVA trend analysis revealed a clear (P<0.0001) dose dependant effect (Figure 26).

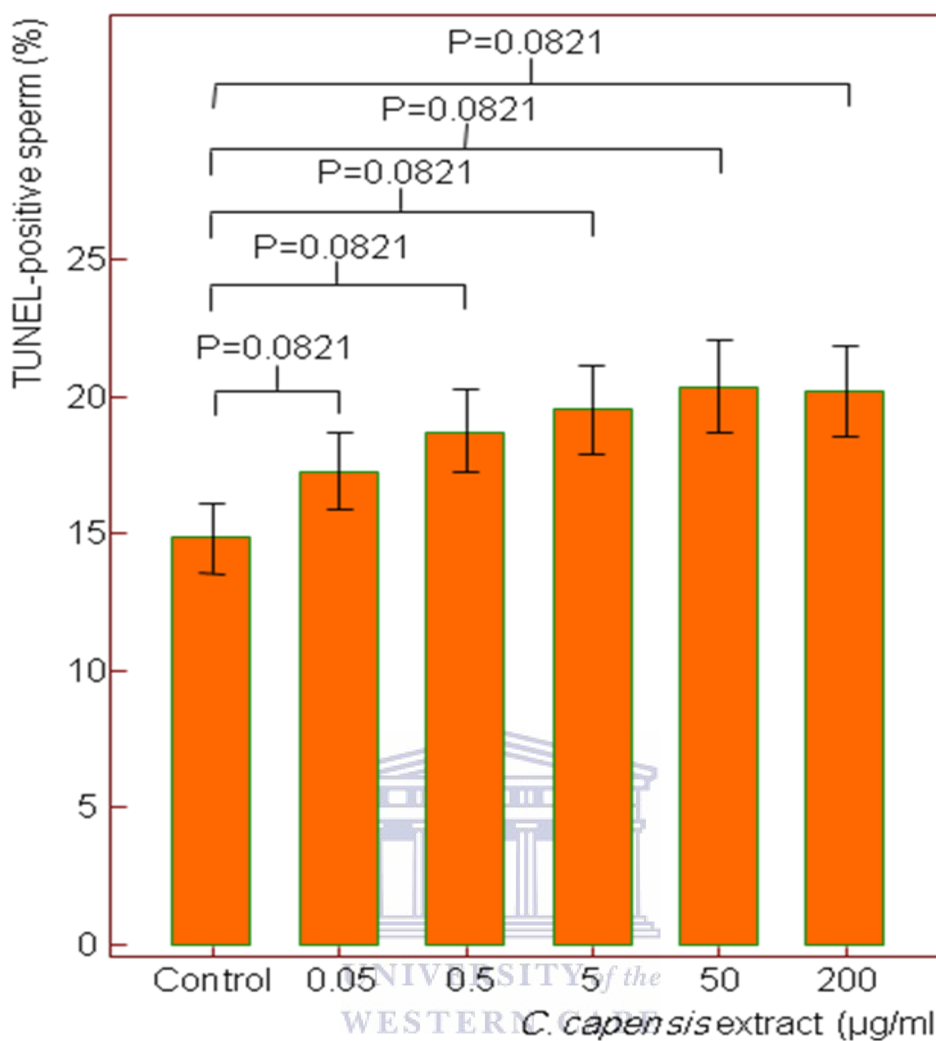

Figure 26: The effect of different concentrations of crude *C. capensis* extract (0.05, 0.5, 5, 50 and 200 µg/ml) on sperm DNA fragmentation *in vitro*. Values shown as mean±SEM (n=70).

A significant increase in spermatozoa with DNA damage as from 0.05 µg/ml (P=0.0133), 0.5 µg/ml (P=0.001) and highly significant at 5, 50 and 200 µg/ml (P<0.0001) can be seen. Both, ANOVA and ANOVA repeated measures analysis revealed a significant (P<0.0001) trend.

3.2.4 Effect of *C. capensis* rhizome extract on sperm reactive oxygen species

A significant positive trend (ANOVA: P<0.001) towards higher percentages of ROS-positive spermatozoa was found at higher concentrations of *C. capensis* rhizome extract (Figure 27). In direct comparison, the control differed significantly from the incubation with 0.5 µg/ml (P=0.0309), 5 µg/ml (P=0.0015) and 50 µg/ml (P=0.0001) and highly significant (P<0.0001) at 200 µg/ml. An ANOVA trend analysis after repeated measures analysis revealed a significant trend (P<0.0001).

As expected, a significant negative relationship between the sperm ROS production (r= -0.194; P=0.0064) and the total sperm motility was observed. Furthermore, a significant positive correlation (r=0.263; P=0.0004) with the percentage of DNA-damaged spermatozoa as determined by means of the TUNEL assay was observed. All other parameters investigated in this study showed no significant relationship.

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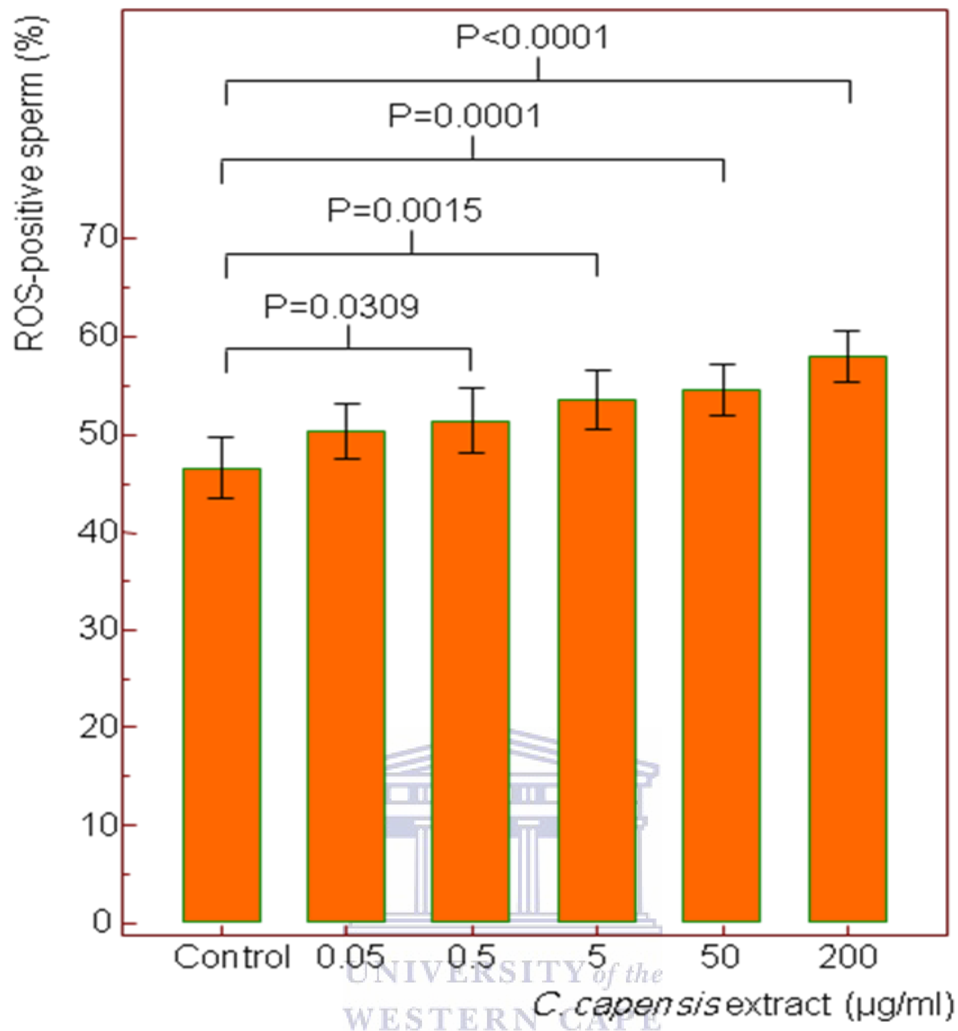

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Figure 27: The effect of different concentrations of crude *C. capensis* extract (0.05, 0.5, 5, 50 and 200 µg/ml) on sperm ROS production *in vitro*. Values shown as mean±SEM (n=40).

Effect of different concentrations of *C. capensis* extract on the percentage of ROS-positive spermatozoa. A significant, dose-dependent increase (ANOVA: P<0.001) towards higher percentages of ROS-positive sperm cells can be seen at higher concentrations of the *C. capensis* extract. The control differs significantly different from the incubation with 200 µg/ml (P<0.0001).

3.2.5 Effect of crude *C. capensis* rhizome extract on sperm apoptosis

With regard to apoptosis is terms of the externalization of PS as determined by means of annexin V-binding, the treatment of spermatozoa with increasing concentrations of *C. capensis* rhizome extract did not result in any effect (Table 7). ANOVA analysis also did not show any significance, neither for the percentage of annexin V-pos spermatozoa (P=0.741) (Figure 28), nor for the percentage of dead sperm as determined with DAPI (P=0.986) (Figure 29).

Table 7: Summary statistics of the sperm apoptosis parameters after incubation with increasing concentrations of *C. capensis* extract. No effect can be seen.

	Control	0.05 µg/ml	0.5 µg/ml	5 µg/ml	50 µg/ml	200 µg/ml	ANOVA (P value)
Normal, non-apoptotic sperm (Annexin V (-) / DAPI (-) (%)	53.81±8.17	53.47±7.99	53.67±6.22	52.58±6.94	53.53±7.29	53.83±6.89	0.981
Sperm in early apoptosis (Annexin V (+) / DAPI (-) (%)	13.67±2.91	13.52±2.57	13.00±1.55	13.47±2.22	13.39±2.43	13.36±2.61	0.905
Sperm in late apoptosis (Annexin V (+) / DAPI (+) (%)	23.19±5.01	22.94±4.54	22.92±4.11	24.11±4.33	23.28±4.94	22.44±4.72	0.762
Necrotic sperm (Annexin V (-) / DAPI (+) (%)	9.39±4.16	10.00±3.60	10.44±3.70	9.92±3.10	9.81±3.66	10.19±4.20	0.909

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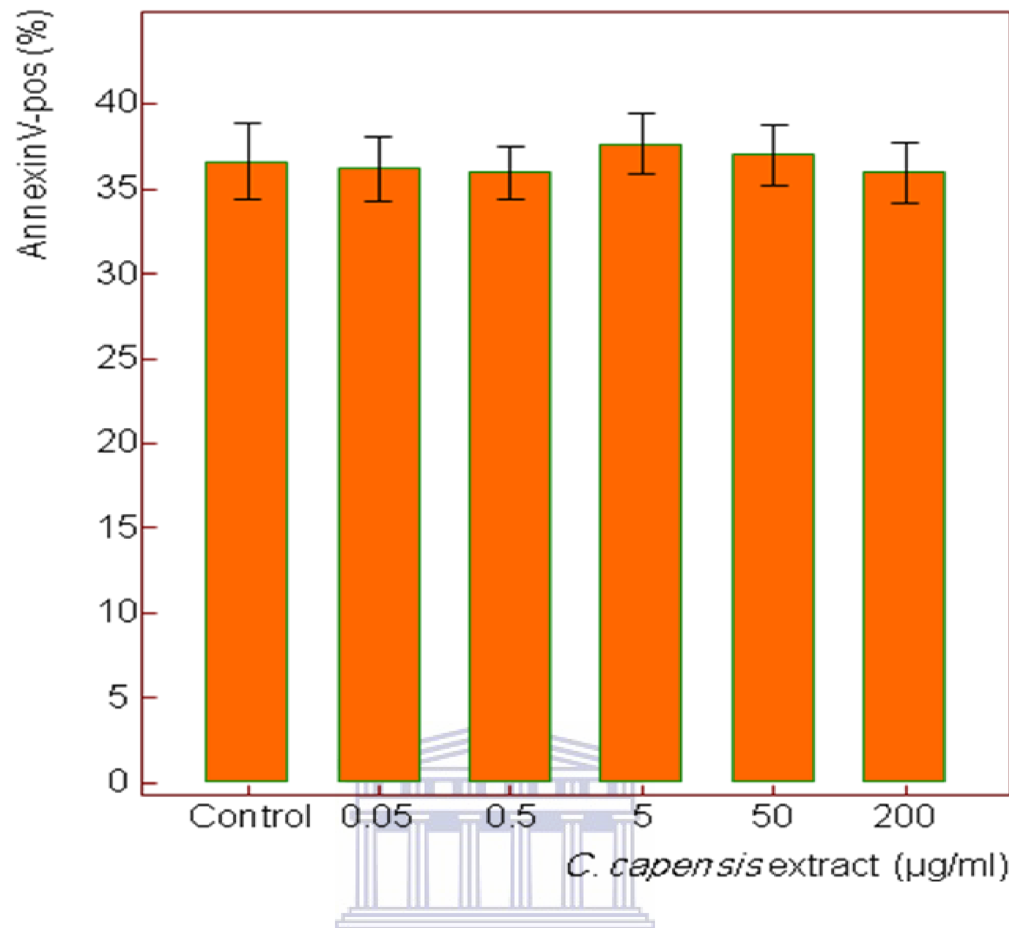

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Figure 28: The effect of different concentrations of crude *C. capensis* extract (0.05, 0.5, 5, 50 and 200 µg/ml) on sperm apoptosis (Annexin V-positive) *in vitro*. Values shown as mean±SEM (n=36). No significance can be seen.

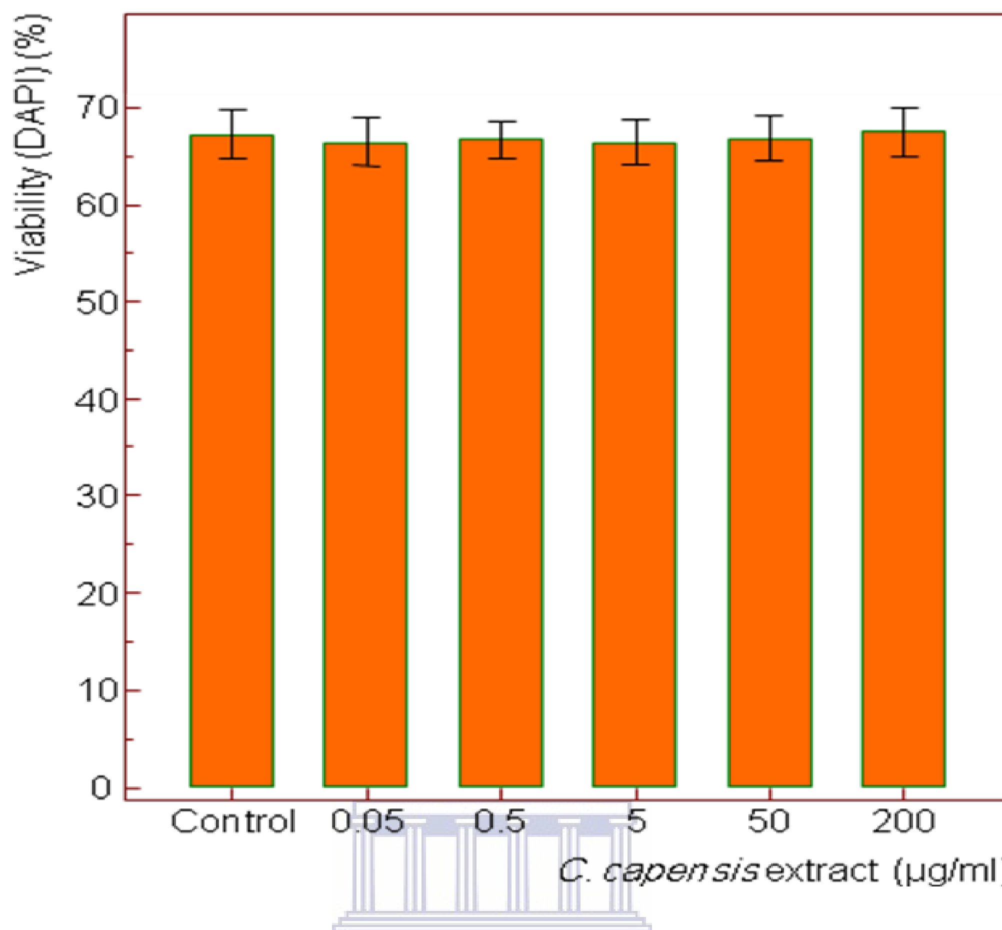

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Figure 29: The effect of different concentrations of crude *C. capensis* extract (0.05, 0.5, 5, 50 and 200 µg/ml) on sperm (viability DAPI-positive) as determined by means of DAPI incorporation *in vitro*. Values shown as mean±SEM (n=36). No significant difference was found at all concentrations.

3.2.6 Effect of crude *C. capensis* rhizome extract on sperm capacitation and acrosome reaction as determined by CTC stain

Sperm capacitation and acrosome reaction was determined by means of the CTC assay (Table 8). A significant negative trend (ANOVA: P<0.001) towards higher percentages of uncapacitated, acrosome-intact spermatozoa was found at higher concentrations of extract.

A significant, positive trend (ANOVA: P<0.001) towards higher percentages of capacitated spermatozoa was found. Values higher than 5µg/ml (P=0.0001)

differed significantly from the control. An ANOVA trend analysis after repeated measures analysis revealed a significant trend (P<0.0001) (Figure 30).

Furthermore, a significant positive trend (ANOVA: P<0.001) towards higher percentages of capacitated, acrosome intact spermatozoa was found at higher concentrations of extract (Figure 31). In contrast, acrosome reaction of capacitated spermatozoa (Figure 32) appeared to reveal only a marginal increase, while the difference between the control and the highest concentration (200 µg/ml) of the extract used is only marginally significant (P=0.0718). The result obtained after ANOVA is not significant (P=0.229). Yet, a trend analysis after repeated measures analysis reveals a significant trend (P=0.003).

Highly significant strong positive relationships between the percentage of capacitated, acrosome-reacted sperm as determined by CTC stain and progressive motility (r=0.456; P=<0.0001) were observed. Obviously, the percentage of capacitated, acrosome-reacted sperm was highly significantly and positively correlated with the total motility spermatozoa (r=0.316; P<0.0001). Furthermore, a significant, positive correlation (r=0.170; P=0.0110) with sperm ROS production was found. All other parameters investigated in this study showed no significant relationship.

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Table 8: Summary statistics of the sperm capacitation and acrosome reaction as determined by CTC stain after incubation with increasing concentrations of *C. capensis* extract.

	Control	0.05 µg/ml	0.5 µg/ml	5 µg/ml	50 µg/ml	200 µg/ml	ANOVA (P value)
Capacitated sperm (%)	32.05±7.97	32.91±6.82	33.21±6.30	35.02±6.66	37.64±7.97	40.08±8.97	< 0.001
Capacitated, acrosome-intact spem (%)	22.10±5.82	22.02±5.09	22.48±5.66	23.45±5.76	25.81±7.11	28.43±8.70	< 0.001
Capacitated, acrosome-reacted sperm(%)	9.94±4.02	10.89±3.69	10.40±3.04	11.29±3.91	11.83±3.92	11.64±3.99	0.229
Uncapacitated, acrosome-intact sperm (%)	68.00±7.89	66.91±6.98	66.97±6.47	65.08±6.62	62.24±8.06	59.81±9.82	< 0.001

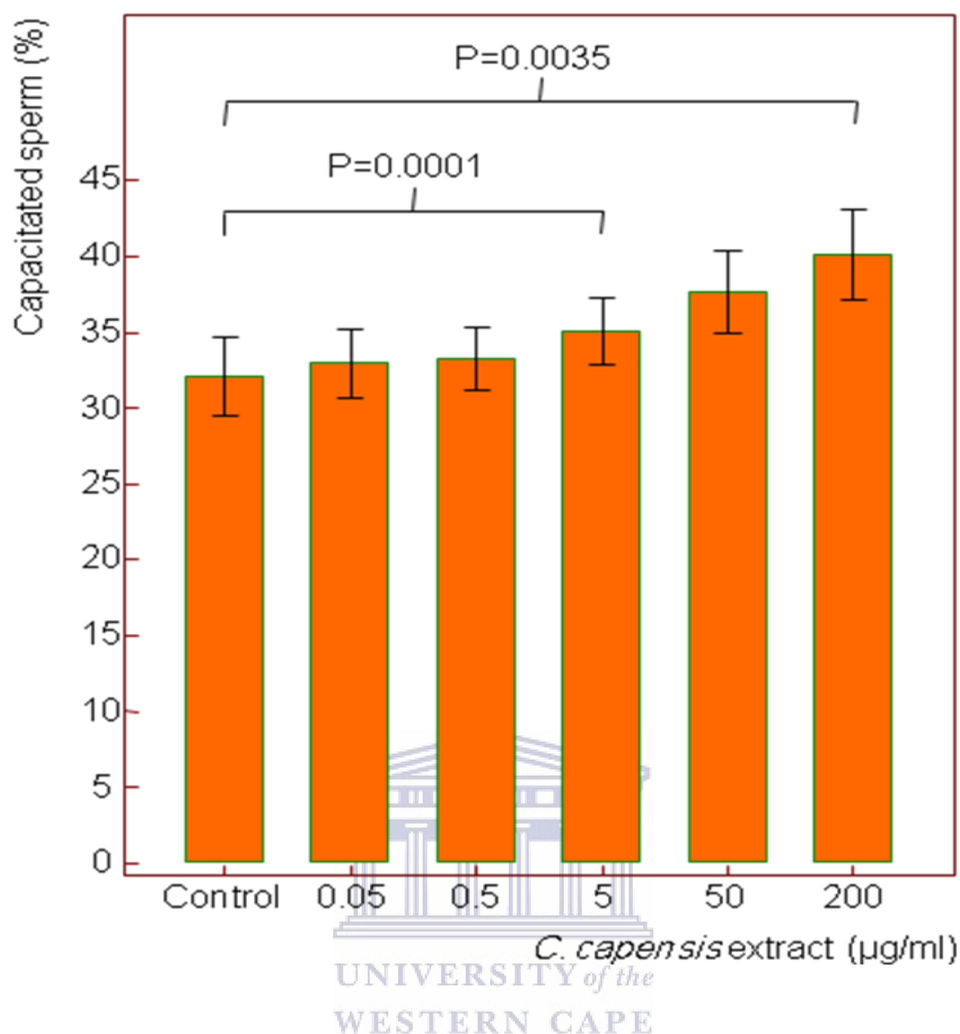

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Figure 30: The effect of different concentrations of crude *C. capensis* extract (0.05, 0.5, 5, 50 and 200 µg/ml) on capacitated spermatozoa *in vitro*. Values shown as mean±SEM (n=37).

When compared with the control after incubation 1houre, sperm incubated at a *C. capensis* concentration of 5 µg/ml showed a significant increase (P=0.0001) as well as (P=0.0035) of 200 µg/ml. ANOVA (P<0.001) as well as repeated measures analysis revealed significant (P<0.0001) trends.

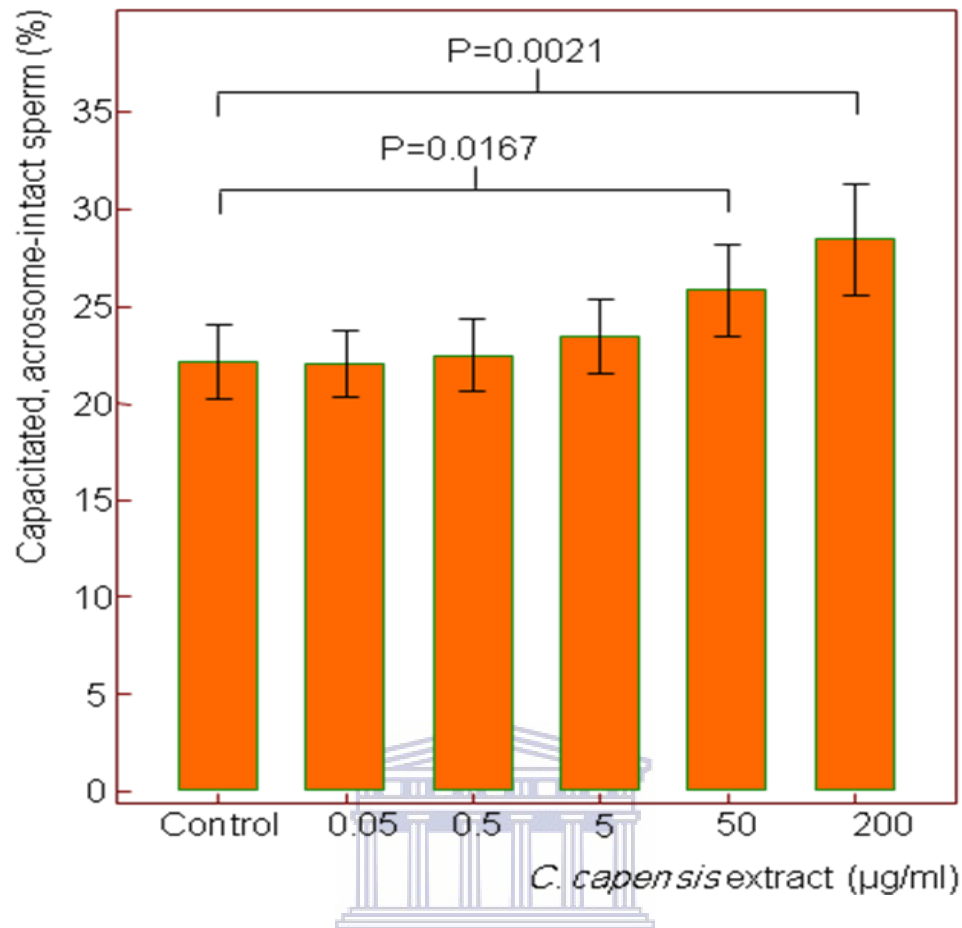

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Figure 31: The effect of different concentrations of crude *C. capensis* extract (0.05, 0.5, 5, 50 and 200 µg/ml) on capacitated, acrosome-intact spermatozoa *in vitro*. Values shown as mean±SEM (n=37).

When compared with the control after incubation 1houre, sperm incubated at a *C. capensis* concentration of 50 µg/ml showed a significant increase (P=0.0167) as well as (P=0.0021) of 200 µg/ml. ANOVA (P<0.001) as well as repeated measures analysis revealed significant (P=0.0012) trends.

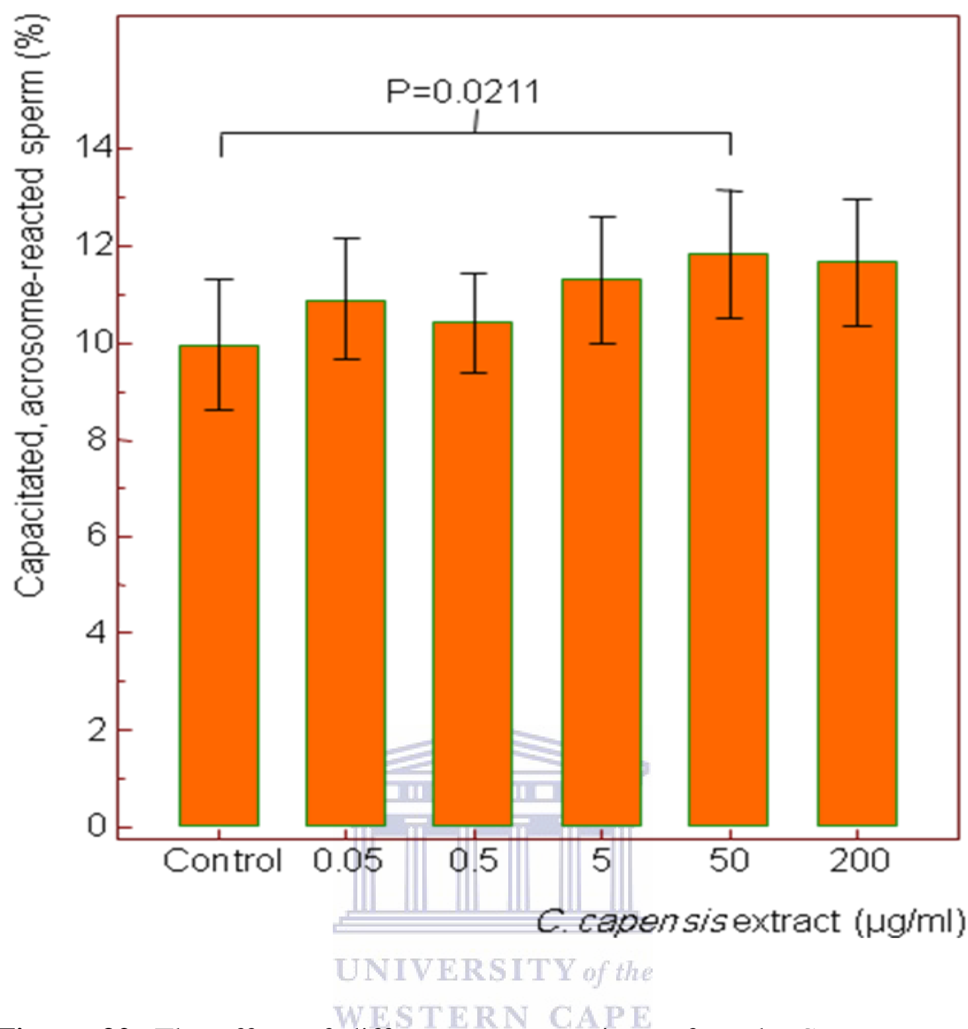

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Figure 32: The effect of different concentrations of crude *C. capensis* extract (0.05, 0.5, 5, 50 and 200 µg/ml) on capacitated, acrosome-reacted spermatozoa *in vitro*. Values shown as mean±SEM (n=37).

Increasing concentrations of *C. capensis* extract seem to have a marginal direct effect (ANOVA: P=0.229) on acrosome reaction since the control does not significantly (P=0.0718) differ from the incubation with 200 µg/ml. In contrast, a significant increase when compared with the control (P=0.0337) at 50 µg/ml. Yet, a repeated measures analysis reveals a trend (P=0.0030).

3.2.7 Effect of crude *C. capensis* rhizome extract on sperm acrosome reaction as determined by triple stain

Summary data on acrosome reaction as determined by means of the triple stain are shown in Table 8. Although there is a marginal (P=0.082) decline after incubation with 50 µg/ml of the extract as compared with the control, no effect of the extract on the percentage of dead, acrosome-reacted spermatozoa is obvious (ANOVA: P=0.121). For live, non-reacted sperm, the treatment of spermatozoa with increasing concentrations of *C. capensis* rhizomes extract also did not result in any effect.

There was also no difference between the control and the highest concentration used (P=0.9846) for live acrosome-reacted sperm (ANOVA: P=0.850) (Figure 33). Nevertheless, a increase in the percentage of dead acrosome reacted sperm could be seen from the control to a concentration of 50 µg/ml (P=0.0212). Yet, a significant trend (ANOVA: P=0.015) could be found.

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Table 9: Summary statistics of the sperm acrosome reaction as determined by triple stain after incubation with increasing concentrations of crude *C. capensis* extract. live reacted (LR); live non-reacted (LNR); dead reacted (DR); and dead non-reacted (DNR)

	Control	0.05 µg/ml	0.5 µg/ml	5 µg/ml	50 µg/ml	200 µg/ml	ANOVA (P value)
DNR (%)	28.15±7.18	29.02±12.74	25.20±9.35	26.45±8.36	23.75±7.32	27.42±9.59	0.121
DR (%)	13.17±3.56	15.02±3.79	13.92±3.53	12.62±3.34	15.55±5.30	14.82±5.35	0.015
LNR (%)	42.15±7.14	41.07±7.81	44.20±10.43	45.15±9.60	44.32±11.24	41.05±9.16	0.200
LR (%)	16.62±6.77	16.92±7.21	16.67±8.29	15.22±5.10	17.17±8.60	17.20±7.53	0.850

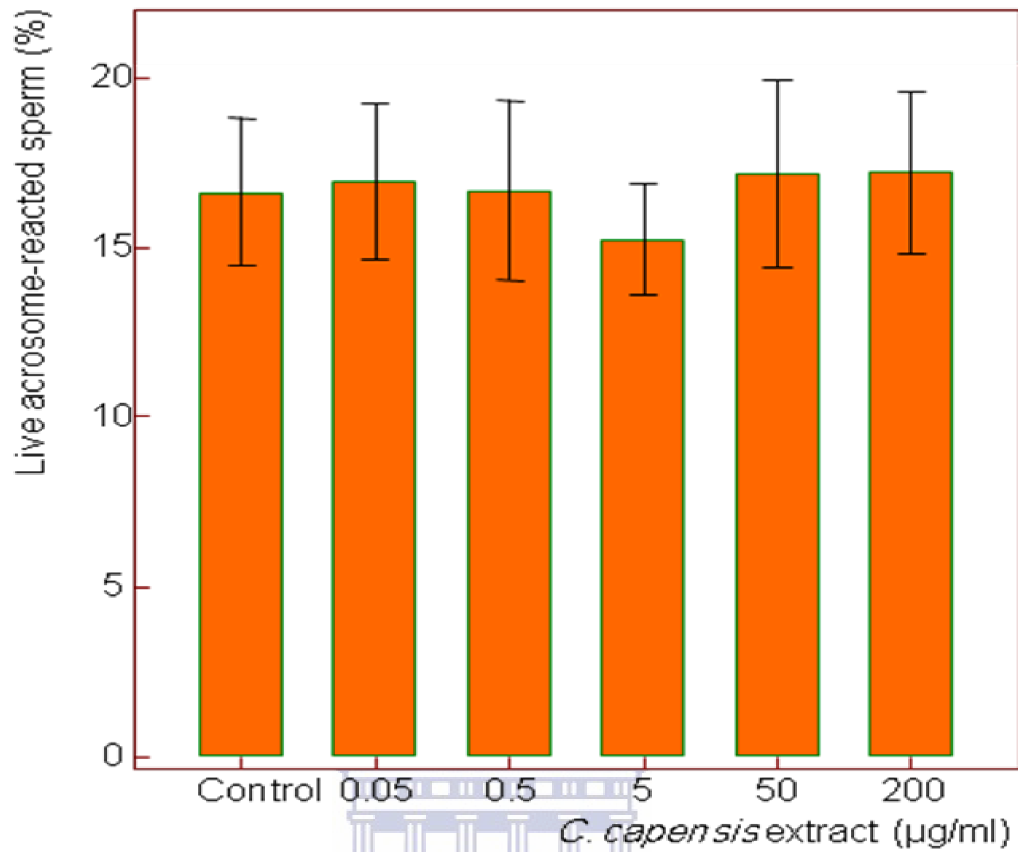

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Figure 33: The effect of different concentrations of crude *C. capensis* extract (0.005, 0.5, 50 and 200 µg/ml) on live, acrosome- reacted spermatozoa *in vitro*. Values shown as mean±SEM (n=40).

Increasing concentrations of the extract do not have an effect (ANOVA: P=0.850) on acrosome reaction. The control does not differ (P=0.9846) from the incubation with 200 µg/ml. Also, no effect can be seen after a trend analysis after repeated measures (P=0.8117).

3.3 Effects fractions of *C. capensis* extract on human spermatozoa *in vitro*

Treatment of ejaculated human spermatozoa from 26 semen samples (17 patients and 9 healthy sperm donors) with different concentrations of the fractions of the *C. capensis* extract (0.05 µg/ml, 0.5 µg/ml, 5 µg/ml, 50 µg/ml and 200 µg/ml) was conducted for washed spermatozoa.

3.3.1 Effect fractions of *C. capensis* extract on sperm motility

Summary results of the motility parameters analyzed for fraction 1 without progesterone (P4) are shown in Table 9. Although progressive motility, velocity curvilinear and straightness were not affected by increasing concentrations of fraction 1 (F1), significant, dose-dependent effects on various motion parameters were obvious. While values for VAP, VSL and linearity decreased with increasing concentrations of the F1 extract, the percentage of hyperactivated sperm and the beat cross frequency increased significantly (Table 10). Also, summary results of the motility parameters analyzed for F1 with P4 are shown in Table 11. While progressive motility and straightness were not affected by increasing concentrations of F1, the values for VAP, VSL, VCL and linearity decreased with increasing concentrations of the F1 extract. The percentage of hyperactivated sperm and the beat cross frequency increased significantly

The summary result for the F2 fraction without P4 on motility parameters are shown in Table 12. Although total and progressive motility and straightness were not affected by increasing concentrations of F2, significant dose-dependent effects on various motion parameters were obvious. While the values for VAP, VCL, VSL and linearity decreased with increasing concentrations of the F2 extract, the percentage of hyperactivated sperm and the beat cross frequency increased significantly (Table 12).

For the F2 fraction with P4, summary results of the motility parameters are shown in Table 13. Although progressive motility, VAP, VSL and linearity were not affected by increasing concentrations of F2, the values total motility and VCL decreased with increasing concentrations of the F2 extract. In contract, the percentage of hyperactivated sperm, the beat cross frequency and straightness increased significantly (Table 13).

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Table 10: Summary statistics of sperm motility parameters (Fraction 1 without progesterone). While progressive motility, velocity curvilinear and straightness remained unchanged, total motility and parameters that are associated with sperm hyperactivation changed significantly. VAP: velocity average path; VCL: velocity curvilinear; VSL: velocity straight line; H: hyperactivation; BCF: beat cross frequency; LIN: linearity; STR: straightness

	Control	0.05 µg/ml	0.5 µg/ml	5 µg/ml	50 µg/ml	200 µg/ml	ANOVA (P value)
Total Motility (%)	44.16±5.04	45.40±4.58	45.20±4.97	46.50±3.89	48.04±3.64	49.92±2.14	<0.001
Progressive motility (%)	14.04±3.91	14.73±2.88	14.26±2.44	13.40±2.62	13.90±2.33	14.38±2.25	0.752
VAP (µm/s)	63.05±3.30	59.10±3.64	59.74±2.79	58.50±3.74	55.69±6.42	51.09±3.07	<0.001
VCL (µm/s)	87.93±4.39	84.38±11.03	82.94±9.66	86.89±3.74	85.79±3.84	83.73±2.77	0.154
VSL (µm/s)	53.32±4.90	53.31±4.50	50.57±5.06	48.06±10.01	45.81±5.67	41.18±3.75	<0.001
H (%)	1.11±0.25	1.26±0.34	1.52±0.37	1.51±0.33	1.76±0.24	1.97±0.29	<0.001
BCF (Hz)	16.23±2.41	17.31±1.89	17.98±1.66	18.28±1.83	19.52±1.68	19.95±1.93	<0.001
LIN	54.30±2.42	52.31±2.76	50.82±4.38	49.00±5.05	47.14±4.23	43.32±2.32	<0.001
STR	73.77±3.17	72.33±2.80	72.58±2.38	71.05±6.16	71.97±2.54	71.52±3.85	0.272

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Table 11: Summary statistics of sperm motility parameters (Fraction 1 with progesterone). While progressive motility and straightness remained unchanged, total motility and parameters that are associated with sperm hyperactivation changed significantly. VAP: velocity average path; VCL: velocity curvilinear; VSL: velocity straight line; H: hyperactivation; BCF: beat cross frequency; LIN: linearity; STR: straightness

	Control	0.05 µg/ml	0.5 µg/ml	5 µg/ml	50 µg/ml	200 µg/ml	ANOVA (P value)
Total Motility (%)	46.68±4.20	48.33±4.25	48.37±4.37	48.72±4.11	50.28±4.59	52.58±2.67	<0.001
Progressive motility (%)	17.42±4.58	18.05±4.24	17.24±3.10	16.14±3.22	16.85±2.15	16.32±2.00	0.472
VAP (µm/s)	64.81±3.27	61.85±2.88	60.59±4.01	59.02±2.92	59.10±3.53	53.33±2.34	<0.001
VCL (µm/s)	92.00±2.27	91.05±2.88	89.69±3.48	90.61±3.48	90.00±4.11	85.99±3.07	<0.001
VSL (µm/s)	54.19±7.10	52.59±6.58	51.45±6.29	49.95±6.68	47.51±6.99	42.72±3.86	<0.001
H (%)	1.28±0.17	1.46±0.34	1.77±0.32	1.84±0.29	1.98±0.16	2.22±0.29	<0.001
BCF (Hz)	18.10±2.12	19.09±1.92	19.71±1.46	20.53±1.37	21.30±1.34	22.10±1.89	<0.001
LIN	57.97±1.61	55.52±2.64	52.92±3.78	51.34±4.35	49.95±2.47	47.45±4.31	<0.001
STR	75.96±2.68	75.14±2.04	74.39±2.03	74.21±2.45	74.04±3.00	73.69±3.77	0.107

Table 12: Summary statistics of sperm motility parameters (Fraction 2 without progesterone). While total and progressive motility and straightness remained unchanged, the parameters that are associated with sperm hyperactivation changed significantly. VAP: velocity average path; VCL: velocity curvilinear; VSL: velocity straight line; H: hyperactivation; BCF: beat cross frequency; LIN: linearity; STR: straightness

	Control	0.05 µg/ml	0.5 µg/ml	5 µg/ml	50 µg/ml	200 µg/ml	ANOVA (P value)
Total Motility (%)	43.42±4.28	44.02±3.70	44.84±3.56	44.90±3.89	45.59±2.49	45.18±2.90	0.407
Progressive motility (%)	13.69±3.35	14.33±2.74	14.53±2.41	14.42±2.79	15.25±2.74	15.29±246	0.448
VAP (µm/s)	62.93±2.80	58.73±3.99	58.03±3.86	56.27±3.95	55.71±3.81	56.43±3.66	<0.001
VCL (µm/s)	87.67±4.08	85.78±3.34	84.70±2.61	83.37±2.85	83.49±1.89	84.09±2.81	<0.001
VSL (µm/s)	52.79±5.16	49.69±4.77	48.77±4.85	46.89±4.85	47.95±4.83	46.31±4.94	0.001
H (%)	1.08±0.17	1.36±0.33	1.43±0.36	1.52±0.27	1.44±0.22	1.42±0.22	<0.001
BCF (Hz)	16.50±1.99	18.09±1.66	18.79±1.67	19.10±1.99	19.37±2.28	19.15±2.05	<0.001
LIN	54.29±2.42	51.52±3.30	50.34±2.85	50.01±2.92	49.65±3.26	49.25±3.73	<0.001
STR	74.00±2.39	73.35±2.21	72.22±1.76	71.97±2.05	71.85±2.15	72.74±1.91	0.0.19

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Table 13: Summary statistics of sperm motility parameters (Fraction 2 with progesterone). Sperm hyperactivation, straightness, velocity straight line and beat cross frequency changed significantly, while total and progressive motility, linearity, velocity straight line and velocity average path remained unchanged. VAP: velocity average path; VCL: velocity curvilinear; VSL: velocity straight line; H: hyperactivation; BCF: beat cross frequency; LIN: linearity; STR: straightness

	Control	0.05 µg/ml	0.5 µg/ml	5 µg/ml	50 µg/ml	200 µg/ml	ANOVA (P value)
Total Motility (%)	46.67±4.21	45.19±3.44	43.91±3.35	44.17±3.08	43.48±3.74	43.70±3.53	0.051
Progressive motility (%)	16.32±4.11	16.07±3.33	15.86±2.93	15.79±2.91	15.09±3.08	15.66±2.73	0.888
VAP (µm/s)	64.51±3.05	62.94±2.45	62.41±2.04	62.60±2.44	62.36±2.58	62.74±2.26	0.072
VCL (µm/s)	92.24±2.32	90.38±1.51	90.70±2.62	91.19±1.67	91.36±1.58	91.61±1.28	0.040
VSL (µm/s)	54.25±5.63	52.71±5.17	51.54±5.96	51.83±4.17	51.95±4.23	51.85±4..09	0.530
H (%)	1.24±0.14	1.28±0.10	1.36±0.17	1.32±0.15	1.44±0.22	1.41±0.19	0.002
BCF (Hz)	17.80±1.84	18.48±1.70	18.62±2.49	19.34±2.79	20.01±2.03	20.42±2.35	0.002
LIN	57.78±1.65	58.23±2.54	58.00±3.24	57.68±3.61	58.87±6.87	59.11±6.68	0.893
STR	76.56±2.50	79.32±1.99	80.62±1.41	79.95±1.33	78.55±5.84	80.28±2.63	<0.001

The summary results for the F3 fraction without P4 on motility parameters are shown in Table 14. While total and progressive motility and straightness were not affected by increasing concentrations of F3, significant dose-dependent effects on various motion parameters were obvious, the values for VAP, VCL, VSL and linearity decreased with increasing concentrations of the F3 extract, the percentage of hyperactivated sperm and the beat cross frequency increased significantly (Table 14).

For the F3 fraction with P4, summary results of the motility parameters are shown in Table 15. Although progressive motility, VAP, VSL and linearity were not affected by increasing concentrations of F3, the values total motility and VCL decreased with increasing concentrations of the F3 extract, the percentage of hyperactivated sperm, the beat cross frequency and straightness increased significantly (Table 15).

The summary results for the F4 fraction without P4 on motility parameters are shown in Table 16. While total and progressive motility were not affected by increasing concentrations of F4, significant dose-dependent effects on various motion parameters were obvious, the values for VAP, VCL, VSL and linearity decreased with increasing concentrations of the F4 extract, the percentage of hyperactivated sperm, the beat cross frequency and straightness increased significantly (Table 16).

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For the F4 fraction with P4, summary results of the motility parameters are shown in Table 17. Although progressive motility, VCL, and linearity were not affected by increasing concentrations of F4, the values total motility, VAP and VSL decreased with increasing concentrations of the F4 extract, the percentage of hyperactivated sperm, the beat cross frequency and straightness increased significantly (Table 17).

Table 14: Summary statistics of sperm motility parameters (Fraction 3 without Progesterone). While total and progressive motility and straightness remained unchanged, the parameters that are associated with sperm hyperactivation changed significantly. VAP: velocity average path; VCL: velocity curvilinear; VSL: velocity straight line; H: hyperactivation; BCF: beat cross frequency; LIN: linearity; STR: straightness

	Control	0.05 µg/ml	0.5 µg/ml	5 µg/ml	50 µg/ml	200 µg/ml	ANOVA (P value)
Total Motility (%)	41.27±4.22	42.05±3.65	43.77±4.06	43.55±3.75	44.32±2.51	43.29±2.30	0.064
Progressive motility (%)	12.41±3.06	13.06±2.35	13.60±1.79	12.93±1.93	13.75±2.28	13.93±1.83	0.262
VAP (µm/s)	64.30±1.94	60.24±1.28	60.24±1.52	59.00±1.86	58.47±1.44	59.10±1.80	<0.001
VCL (µm/s)	85.14±3.09	84.41±2.88	83.79±1.69	82.40±1.67	82.85±1.99	82.87±2.26	0.002
VSL (µm/s)	49.93±2.32	47.24±2.72	45.71±3.23	44.13±3.55	44.39±3.27	43.49±3.57	<0.001
H (%)	1.06±0.22	1.22±0.15	1.29±0.16	1.36±0.13	1.41±0.12	1.28±0.06	<0.001
BCF (Hz)	17.31±1.38	17.62±1.43	17.71±2.15	17.67±3.03	18.78±2.06	19.28±2.54	0.032
LIN	54.74±2.44	52.58±2.86	50.94±2.23	51.13±2.50	50.98±2.71	51.70±2.00	<0.001
STR	73.38±2.32	72.23±1.86	72.19±2.16	71.68±2.25	72.31±2.41	73.41±2.18	0.080

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Table 15: Summary statistics of sperm motility parameters (Fraction 3 with Progesterone). While progressive motility, velocity average path, velocity straight line and linearity remained unchanged, the total motility and parameters that are associated with sperm hyperactivation changed significantly. VAP: velocity average path; VCL: velocity curvilinear; VSL: velocity straight line; H: hyperactivation; BCF: beat cross frequency; LIN: linearity; STR: straightness

	Control	0.05 µg/ml	0.5 µg/ml	5 µg/ml	50 µg/ml	200 µg/ml	ANOVA (P value)
Total Motility (%)	43.78±3.08	43.11±2.22	441.79±1.83	42.72±1.39	41.56±1.23	41.80±1.20	0.002
Progressive motility (%)	16.60±4.69	16.35±4.24	16.38±3.75	16.49±3.31	14.94±3.81	15.82±3.28	0.759
VAP (µm/s)	65.08±3.10	63.38±2.40	63.04±2.56	63.47±3.08	63.83±2.74	63.31±2.54	0.227
VCL (µm/s)	92.34±2.32	90.56±1.19	90.58±1.07	91.81±2.00	91.77±0.98	91.74±1.11	0.001
VSL (µm/s)	51.95±6.27	50.76±5.94	50.46±7.72	51.33±5.12	51.55±5.26	51.30±4.77	0.975
H (%)	1.22±0.17	1.26±0.12	1.32±0.13	1.32±0.14	1.51±0.28	1.46±0.22	<0.001
BCF (Hz)	17.31±1.38	17.62±1.43	17.71±2.15	17.67±3.03	18.78±2.06	19.28±2.54	0.032
LIN	57.28±1.68	57.35±2.93	57.60±3.85	57.29±3.61	57.00±4.33	57.12±4.26	0.9971
STR	75.38±2.53	78.47±1.78	80.34±2.0.87	79.73±1.54	76.70±7.55	79.02±2.29	<0.001

Table 16: Summary statistics of sperm motility parameters (Fraction 4 without progesterone). While total and progressive motility remained unchanged, the parameters that are associated with sperm hyperactivation changed significantly. VAP: velocity average path; VCL: velocity curvilinear; VSL: velocity straight line; H: hyperactivation; BCF: beat cross frequency; LIN: linearity; STR: straightness

	Control	0.05 μg/ml	0.5 μg/ml	5 μg/ml	50 μg/ml	200 μg/ml	ANOVA (P value)
Total Motility (%)	45.57±3.02	45.99±2.34	45.91±2.53	46.26±3.53	46.86±1.63	47.08±1.99	0.417
Progressive motility (%)	14.98±3.10	15.61±2.46	15.47±2.57	15.92±2.68	16.76±2.25	16.66±2.20	0.202
VAP (μm/s)	61.57±2.84	57.22±5.04	55.83±4.15	53.55±3.49	52.95±3.32	53.77±2.93	<0.001
VCL (μm/s)	90.21±3.20	87.15±3.18	85.62±3.01	84.34±3.39	84.14±1.53	85.32±2.74	<0.001
VSL (μm/s)	55.65±5.55	52.14±5.05	51.84±4.11	49.65±4.28	51.52±3.02	49.14±4.39	<0.001
H (%)	1.11±0.10	1.40±0.30	1.38±0.31	1.61±0.25	1.47±0.29	1.55±0.21	<0.001
BCF (Hz)	17.14±2.46	18.92±1.43	19.54±1.60	20.01±1.60	20.75±1.07	20.25±0.71	<0.001
LIN	53.84±2.31	50.46±3.35	49.74±3.23	48.90±2.86	48.32±3.19	46.80±3.35	<0.001
STR	74.63±2.29	72.47±2.50	72.26±1.23	72.26±1.77	71.40±1.75	72.07±1.26	<0.001

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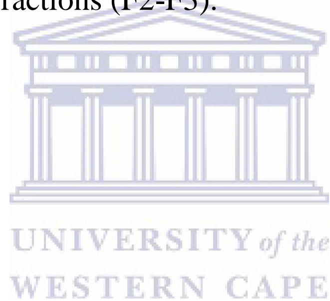
Table 17: Summary statistics of sperm motility parameters (Fraction 4 with progesterone). While progressive motility, velocity curvilinear and linearity remained unchanged, total motility and parameters that are associated with sperm hyperactivation changed significantly. VAP: velocity average path; VCL: velocity curvilinear; VSL: velocity straight line; H: hyperactivation; BCF: beat cross frequency; LIN: linearity; STR: straightness

	Control	0.05 μg/ml	0.5 μg/ml	5 μg/ml	50 μg/ml	200 μg/ml	ANOVA (P value)
Total Motility (%)	49.57±2.88	47.28±3.10	46.03±3.10	45.62±3.56	45.41±4.33	45.61±3.98	0.002
Progressive motility (%)	16.05±3.40	15.80±2.03	15.35±1.60	15.09±2.21	15.24±2.11	15.50±2.01	0.782
VAP (μm/s)	63.95±2.89	62.51±2.42	61.79±0.98	61.73±0.91	60.90±1.13	62.17±1.77	<0.001
VCL (μm/s)	92.14±2.31	90.21±1.76	90.83±3.54	90.58±0.87	90.96±1.92	91.48±1.43	0.077
VSL (μm/s)	56.55±3.61	54.67±3.17	52.63±3.00	52.33±2.84	52.35±2.78	52.41±3.16	<0.001
H (%)	1.27±0.09	1.31±0.08	1.41±0.20	1.33±0.16	1.38±0.10	1.37±0.15	0.025
BCF (Hz)	18.29±2.12	19.34±1.48	19.53±2.46	21.02±0.74	21.24±0.89	21.57±1.37	<0.001
LIN	58.29±1.46	59.12±1.65	58.41±2.42	58.08±3.55	60.75±8.26	61.11±7.92	0.248
STR	77.74±1.79	80.17±1.79	80.90±1.75	80.18±1.03	80.40±1.98	81.55±2.29	<0.001

Furthermore, when comparing fractions with P4 and without P4 (Figure 34A and B) on VAP, fraction 1 showed a significant decrease in a dose-dependent manner. No effect was found in the other fractions (F2-F3). For VSL when comparing fractions with P4 and without P4 (Figure 35A and B), fraction 1 showed a significant decrease in a dose-dependent manner; no effect was found in the other fractions (F2-F3).

When comparing fractions with P4 and without P4 (Figure 36A and B) on linearity, fraction 1 and 4 showed a significant decrease in a dose-dependent manner; no effect was found in the fraction F2 and F3.

Moreover, comparing fractions with P4 and without P4 (Figure 37A and B) on hyperactivation, fraction 1 showed a significant increase at higher concentrations; no effect was found in the other fractions (F2-F3).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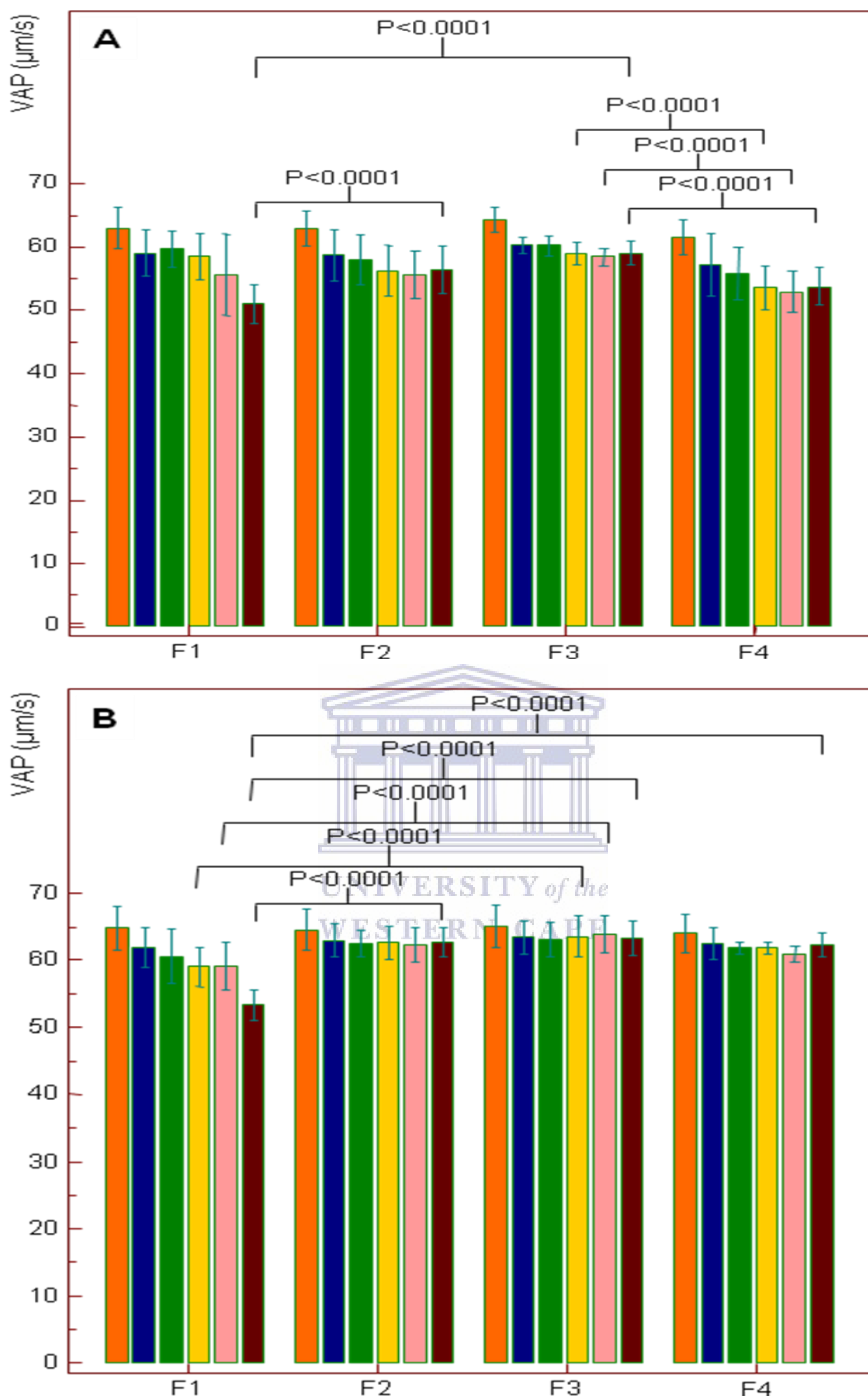

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Figure 34: The effect of *C. capensis* methanolic fractions on velocity average path (VAP) *in vitro*. The orange bar represents 0 µg/ml, blue bar 0.05 µg/ml, green bar 0.5 µg/ml, yellow bar 5 µg/ml, pink bar 50 µg/ml and maroon bar 200 µg/ml.

A) without P4; B) with P4. Values shown as mean±SEM (n=26).

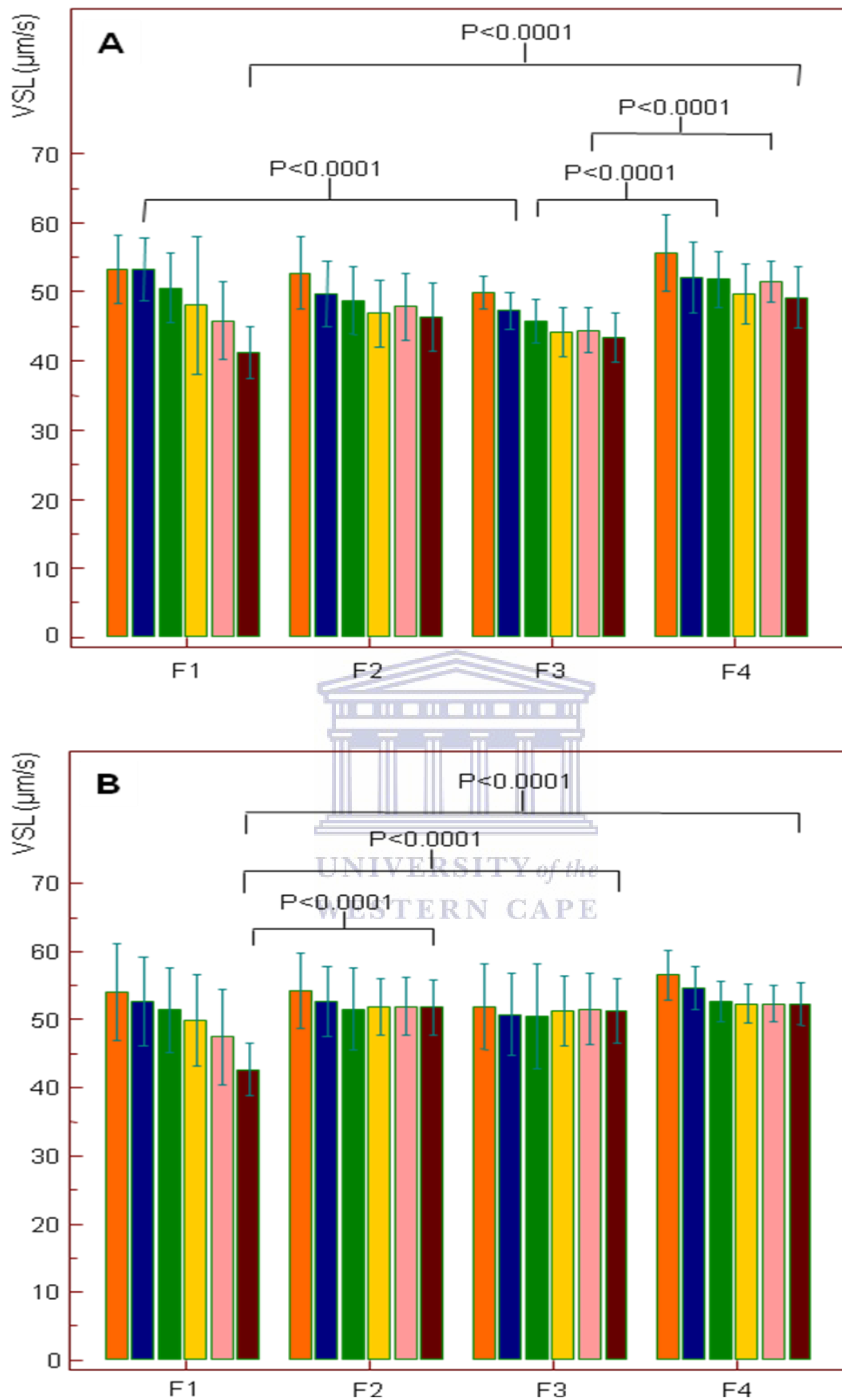

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Figure 35: The effect of *C. capensis* methanolic fractions on velocity straight line (VSL) *in vitro*. The orange bar represents 0 µg/ml, blue bar 0.05 µg/ml, green bar 0.5 µg/ml, yellow bar 5 µg/ml, pink bar 50 µg/ml and maroon bar 200 µg/ml.
A) without P4; B) with P4. Values shown as mean±SEM (n=26).

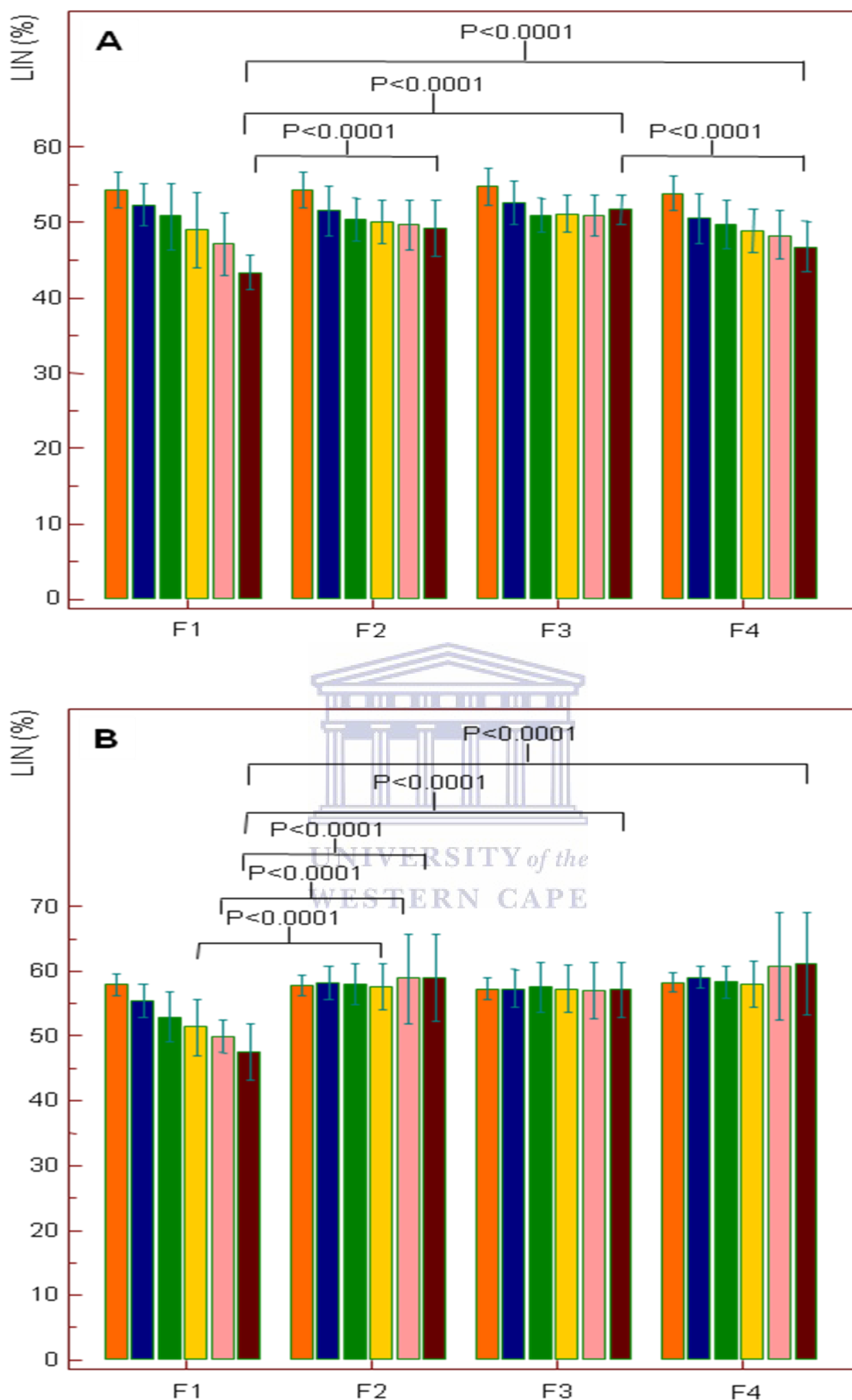

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Figure 36: The effect of *C. capensis* methanolic fractions on linearity (LIN) *in vitro*. The orange bar represents 0 µg/ml, blue bar 0.05 µg/ml, green bar 0.5 µg/ml, yellow bar 5 µg/ml, pink bar 50 µg/ml and maroon bar 200 µg/ml.

A) without P4; B) with P4. Values shown as mean±SEM (n=26).

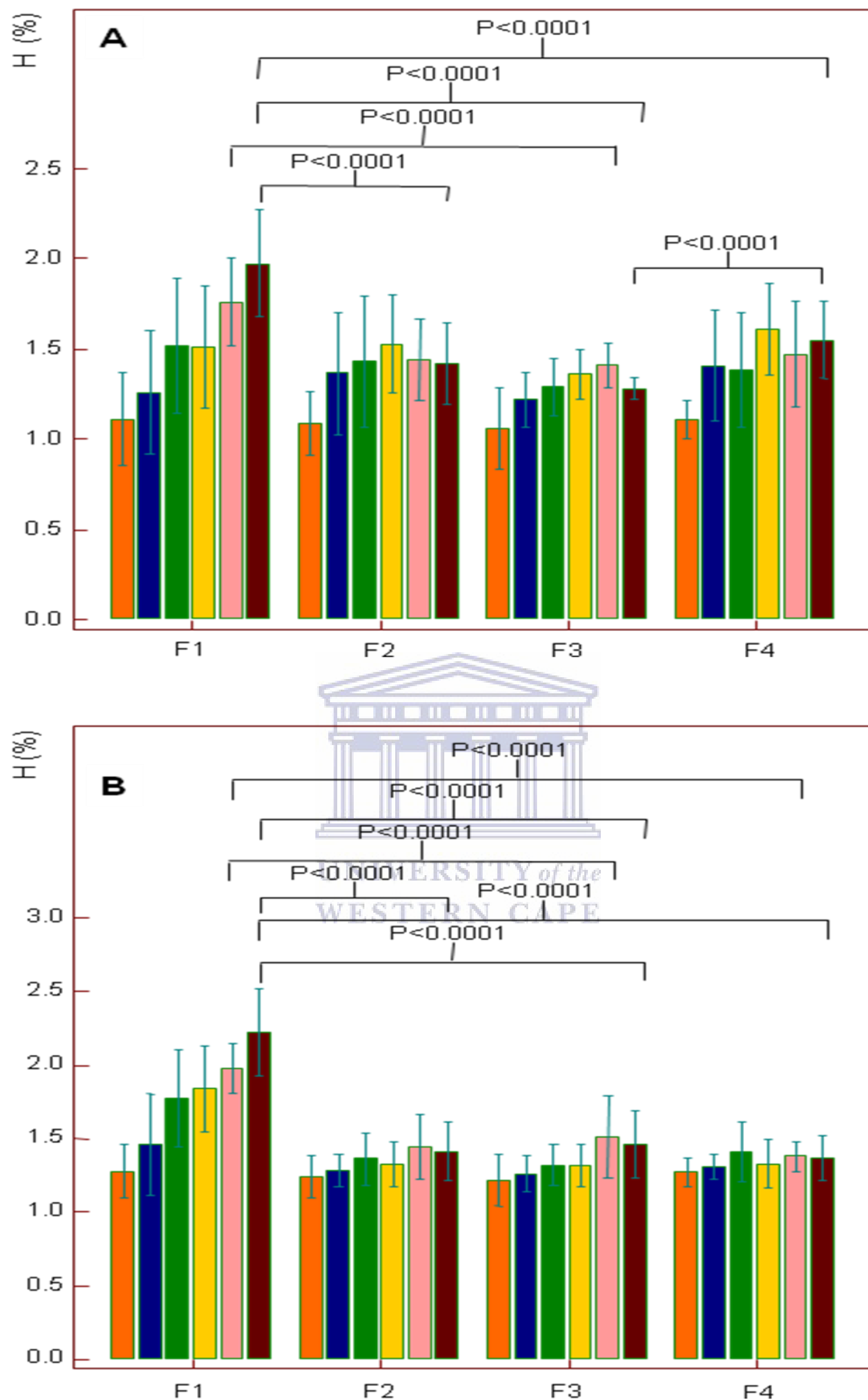

Figure 37: The effect of *C. capensis* methanolic fractions on hyperactivation (H) *in vitro*. The orange bar represents 0 µg/ml, blue bar 0.05 µg/ml, green bar 0.5 µg/ml, yellow bar 5 µg/ml, pink bar 50 µg/ml and maroon bar 200 µg/ml.

A) without P4; B) with P4. Values shown as mean±SEM (n=26).

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3.3.2 Effect fractions of *C. capensis* extract on sperm reactive oxygen species (ROS)

Summary results of the percentage of ROS-positive spermatozoa for the fractions without P4 are shown in Table 18. Although F2 and F3 remained unchanged with increasing concentrations of the *C. capensis* extract, F1 and F4 changed significantly with increasing concentrations of the *C. capensis* crude extract.

Furthermore, summary results of the percentage of ROS-positive spermatozoa for the fractions with P4 are shown in Table 19. Although F2 and F3 remained unchanged with increasing concentrations of the *C. capensis* extract, F1 and F4 changed significantly with increasing concentrations of the *C. capensis* extract.

When comparing fractions with P4 and without P4 (Figure 38A and B) on ROS-positive spermatozoa, fraction 1 showed a significant increase in a dose-dependent manner; no effect was found in the other fractions (F2-F3).

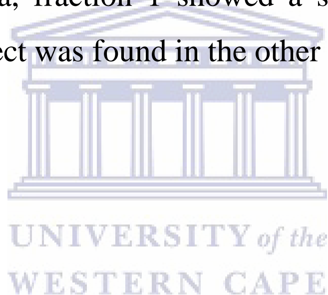
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Table 18: Summary statistics of the percentage of ROS-positive spermatozoa after incubation with increasing concentrations of fractions 1, 2, 3 and 4 of *C. capensis* extract (without progesterone). While fractions 2 and 3 remained unchanged with increasing concentrations of the *C. capensis* extract, fractions 1 and 4 changed significantly with increasing concentrations of the *C. capensis* crude extract. ROS: reactive oxygen species; F: fraction

	Control	0.05 µg/ml	0.5 µg/ml	5 µg/ml	50 µg/ml	200 µg/ml	ANOVA (P value)
ROS-F1 (%)	40.35±4.19	42.60±4.12	43.95±4.75	53.30±4.48	50.45±3.26	53.80±2.68	<0.001
ROS-F2 (%)	41.60±3.84	43.25±3.91	43.70±3.64	43.55±3.85	44.10±3.33	44.90±3.35	0.120
ROS-F3 (%)	43.10±4.41	45.30±4.05	45.60±3.81	45.30±4.63	45.60±3.73	46.80±3.36	0.120
ROS-F4 (%)	40.10±2.31	41.20±2.33	41.80±2.09	41.80±1.36	42.60±1.90	43.00±1.89	<0.001

Table 19: Summary statistics of the percentage of ROS-positive spermatozoa after incubation with increasing concentrations of fractions 1, 2, 3 and 4 of *C. capensis* extract (with progesterone). While fractions 2 and 3 remained unchanged with increasing concentrations of the *C. capensis* crude extract, fractions 1 and 4 changed significantly with increasing concentrations of the *C. capensis* extract. ROS: reactive oxygen species; F: fraction

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	Control	0.05 µg/ml	0.5 µg/ml	5 µg/ml	50 µg/ml	200 µg/ml	ANOVA (P value)
ROS-F1 (%)	44.50±3.73	49.35±4.78	50.65±4.63	53.25±4.21	55.45±3.26	56.45±3.88	<0.001
ROS-F2 (%)	45.60±4.34	46.70±4.13	45.95±3.66	46.70±3.81	47.95±4.29	48.65±4.22	0.156
ROS-F3 (%)	46.50±3.34	45.60±3.43	45.40±3.18	44.10±2.44	45.00±3.14	45.80±3.91	0.307
ROS-F4 (%)	44.70±4.98	47.80±4.44	46.50±4.00	49.30±2.97	50.90±2.95	51.50±1.79	<0.001

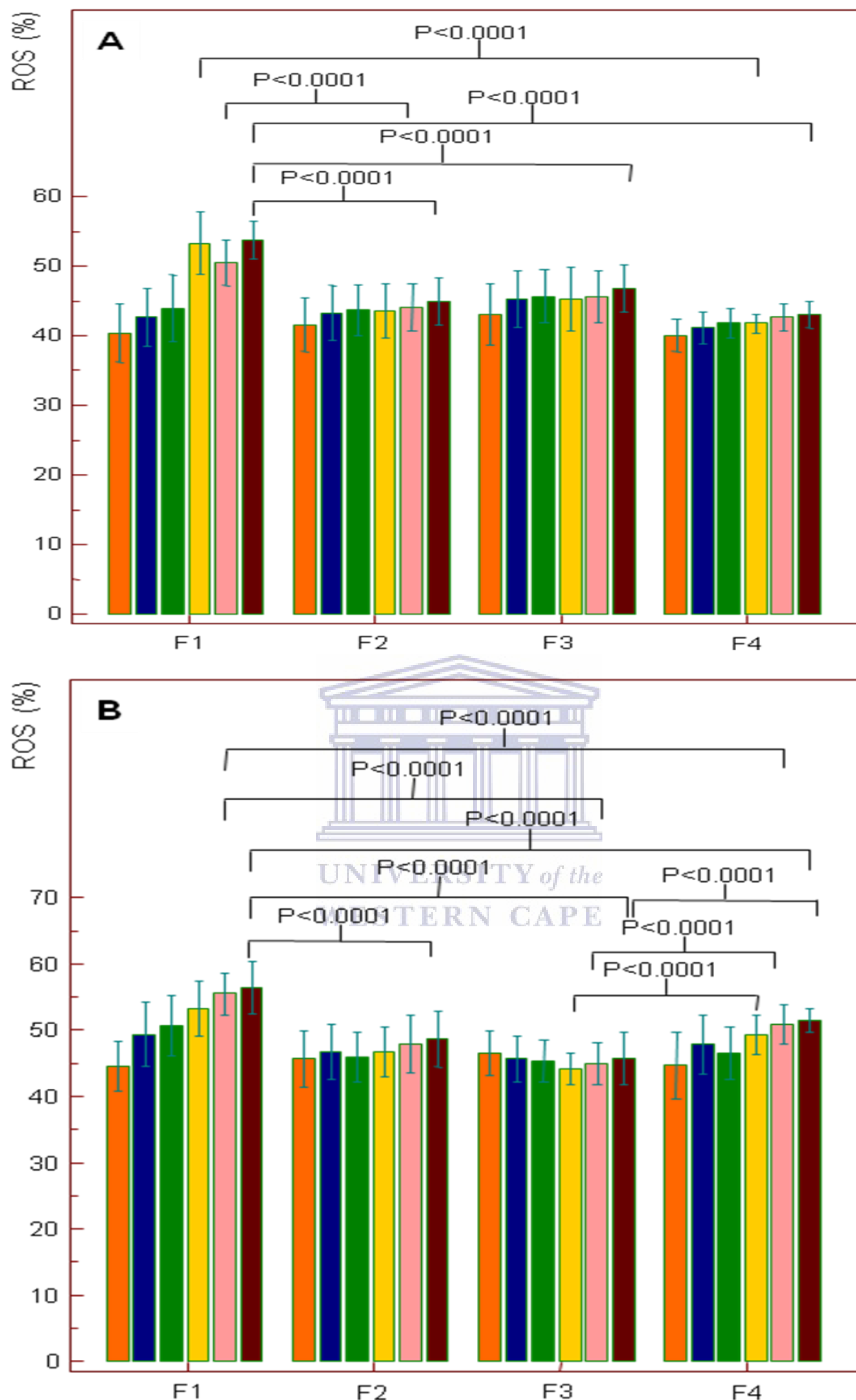

Figure 38: The effect of *C. capensis* methanolic fractions on reactive oxygen species (ROS) *in vitro*. The orange bar represents 0 µg/ml, blue bar 0.05 µg/ml, green bar 0.5 µg/ml, yellow bar 5 µg/ml, pink bar 50 µg/ml and maroon bar 200 µg/ml.

A) without P4; B) with P4. Values shown as mean±SEM (n=26).

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3.3.3 Effect fractions of *C. capensis* crude extract on sperm capacitation and acrosome reaction as determined by CTC stain

Summary results of the sperm capacitation and acrosome reaction as determined by means of the CTC assay, analyzed for fraction 1 without progesterone (P4) are shown in Table 20. A significant positive trend (ANOVA: $P<0.001$) towards higher percentages of capacitated, acrosome-intact and capacitated spermatozoa was found at higher concentrations. Also, summary results for fraction 1 with P4 are shown in Table 21. A significant positive trend (ANOVA: $P<0.001$) towards higher percentages of capacitated, acrosome-intact, capacitated, acrosome-reacted and capacitated spermatozoa was found at higher concentrations.

The summary results for the fraction 2 without P4 are shown in Table 22. A significant positive trend (ANOVA: $P<0.001$) towards higher percentages of capacitated, acrosome-intact and capacitated spermatozoa was found at higher concentrations. For the fraction 2 with P4, summary results are shown in Table 23. A significant positive trend (ANOVA: $P<0.001$) towards higher percentages of capacitated, acrosome-reacted and capacitated spermatozoa was found at higher concentrations.

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The summary results for the fraction 3 without P4 are shown in Table 24. A significant positive trend (ANOVA: $P<0.001$) towards higher percentages of capacitated, acrosome-intact and spermatozoa was found at higher concentrations. For the fraction 3 with P4, summary results are shown in Table 25. A significant positive trend (ANOVA: 0.016) towards higher percentages of capacitated spermatozoa was found at higher concentrations and a significant positive trend (ANOVA: 0.008) towards higher percentages of capacitated, acrosome-reacted spermatozoa was found at higher concentrations.

The summary results for the fraction 4 without P4 are shown in Table 26. A significant positive trend (ANOVA: $P<0.001$) towards higher percentages of capacitated, acrosome-intact; reacted and capacitated spermatozoa was found at higher concentrations. For the fraction 4 with P4, summary results are shown in Table 27. A significant positive trend (ANOVA: $P<0.001$) towards higher

percentages of capacitated, acrosome-intact; reacted and capacitated spermatozoa was found at higher concentrations.

Furthermore, when comparing fractions with and without P4 (Figure 39A and B) on capacitated spermatozoa, fraction 1 showed a significant increase in a dose-dependent manner; no effect was found in the other fractions (F2-F3),

When comparing fractions with P4 and without P4 (Figure 40A and B) on capacitated, acrosome-intact spermatozoa, fraction 1 showed a significant increase in a dose-dependent manner, no effect was found in the fractions (F2-F4). Also, when comparing fractions with P4 and without P4 (Figure 41A and B) on capacitated, acrosome-reacted spermatozoa, fraction 1 showed a significant increase in a dose-dependent manner; no effect was found in the fractions (F2-F4).

Moreover, progesterone stimulation caused dose-dependent increase in the inducibility (induced AR - spontaneous AR) of acrosome reaction as shown for the percentage of capacitated, acrosome-reacted spermatozoa (ΔAR) (Figure 42). While all concentrations for fractions 2, 3 and 4 similarly caused an initial increase and then a decrease, fraction 1 resulted in a constant dose-depentent increase, which was much bigger than for the other fractions. When comparing fractions on capacitated delta acrosome-reacted (Figure 42), fraction 1 showed a highly significant increase in a dose-dependent manner.

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Table 20: Summary statistics of the sperm capacitation and acrosome reaction as determined by CTC stain after incubation with increasing concentrations of *C. capensis* crude extract for fraction 1 without progesterone.

	Control	0.05 µg/ml	0.5 µg/ml	5 µg/ml	50 µg/ml	200 µg/ml	ANOVA (P value)
Capacitated sperm (%)	18.90±2.90	21.70±2.99	24.70±2.71	26.45±4.29	28.90±4.31	34.20±5.52	< 0.001
Capacitated, acrosome-intact spem (%)	12.80±3.05	13.95±2.81	15.55±2.60	17.30±2.57	18.40±3.23	21.30±5.02	< 0.001
Capacitated, acrosome-reacted sperm(%)	7.55±1.43	8.85±1.26	9.70±1.49	10.75±1.71	12.00±1.77	10.10±3.52	0.009
Uncapacitated, acrosome-intact sperm (%)	81.10+±2.90	79.60±4.50	75.35±2.70	73.05±3.81	71.30±4.49	66.05±5.20	0.178

Table 21: Summary statistics of the sperm capacitation and acrosome reaction as determined by CTC stain after incubation with increasing concentrations of *C. capensis* extract crude extract for fraction 1 with progesterone.

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	Control	0.05 µg/ml	0.5 µg/ml	5 µg/ml	50 µg/ml	200 µg/ml	ANOVA (P value)
Capacitated sperm (%)	25.68±2.72	28.70±3.04	31.40±2.70	32.70±3.09	33.30±3.07	40.35±6.30	< 0.001
Capacitated, acrosome-intact spem (%)	14.35±2.88	14.65±2.34	18.65±2.43	18.85±2.20	20.05±2.70	25.60±6.15	< 0.001
Capacitated, acrosome-reacted sperm(%)	12.00±2.80	13.20±2.44	13.10±2.46	13.20±2.70	13.00±2.02	15.00±1.89	<0.001
Uncapacitated, acrosome-intact sperm (%)	64.45±6.70	62.15±5.62	59.60±4.61	60.85±6.04	62.60±6.24	62.15±5.87	< 0.001

Table 22: Summary statistics of the sperm capacitation and acrosome reaction as determined by CTC stain after incubation with increasing concentrations of *C. capensis* extract crude extract for fraction 2 without progesterone .

	Control	0.05 µg/ml	0.5 µg/ml	5 µg/ml	50 µg/ml	200 µg/ml	ANOVA (P value)
Capacitated sperm (%)	19.15±2.94	22.35±3.89	23.15±3.88	24.15±4.25	25.30±4.60	24.80±3.69	< 0.001
Capacitated, acrosome-intact spem (%)	11.90±2.31	14.50±2.76	15.20±2.91	16.30±2.86	16.65±2.75	16.40±2.64	< 0.001
Capacitated, acrosome-reacted sperm(%)	7.00±1.29	7.20±1.79	7.95±2.06	7.85±2.03	8.65±2.36	8.40±1.81	0.127
Uncapacitated, acrosome-intact sperm (%)	81.10±2.90	77.70±3.92	76.85±3.88	75.85±4.25	74.75±4.59	75.00±4.70	< 0.001

Table 23: Summary statistics of the sperm capacitation and acrosome reaction as determined by CTC stain after incubation with increasing concentrations of *C. capensis* extract crude extract for fraction 2 with progesterone.

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	Control	0.05 µg/ml	0.5 µg/ml	5 µg/ml	50 µg/ml	200 µg/ml	ANOVA (P value)
Capacitated sperm (%)	32.00±2.10	26.85±2.10	26.55±2.74	27.50±3.83	27.60±2.94	28.00±3.71	< 0.001
Capacitated, acrosome-intact spem (%)	13.85±2.30	15.05±1.90	15.00±2.33	15.15±2.18	15.65±2.45	16.25±2.71	0.046
Capacitated, acrosome-reacted sperm(%)	9.15±0.98	11.35±1.95	11.95±1.98	11.40±2.03	12.15±2.13	11.55±2.01	<0.001
Uncapacitated, acrosome-intact sperm (%)	65.70±5.50	73.20±2.04	73.45±2.30	72.35±2.85	72.35±2.87	72.95±4.67	< 0.001

Table 24: Summary statistics of the sperm capacitation and acrosome reaction as determined by CTC stain after incubation with increasing concentrations of *C. capensis* extract crude extract for fraction 3 without progesterone .

	Control	0.05 µg/ml	0.5 µg/ml	5 µg/ml	50 µg/ml	200 µg/ml	ANOVA (P value)
Capacitated sperm (%)	18.50±3.15	21.10±3.95	21.60±3.92	22.90±4.75	24.20±5.26	23.30±3.84	0.001
Capacitated, acrosome-intact spem (%)	11.70±2.05	14.10±2.35	14.50±2.21	15.60±2.89	16.30±2.67	15.30±2.15	< 0.001
Capacitated, acrosome-reacted sperm(%)	6.80±1.43	7.00±2.05	7.10±2.40	7.30±2.51	7.90±2.95	8.00±2.10	0.468
Uncapacitated, acrosome-intact sperm (%)	81.50±3.15	78.90±3.95	78.40±3.92	77.10±4.75	75.90±5.24	77.30±4.98	0.003

Table 25: Summary statistics of the sperm capacitation and acrosome reaction as determined by CTC stain after incubation with increasing concentrations of *C. capensis* extract crude extract for fraction 3 with progesterone.

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	Control	0.05 µg/ml	0.5 µg/ml	5 µg/ml	50 µg/ml	200 µg/ml	ANOVA (P value)
Capacitated sperm (%)	23.20±1.82	25.90±1.74	25.70±3.04	26.20±4.39	25.70±2.47	25.40±2.72	0.016
Capacitated, acrosome-intact spem (%)	14.00±2.51	14.50±2.25	14.30±2.79	14.20±2.14	14.40±2.16	15.30±2.97	0.660
Capacitated, acrosome-reacted sperm(%)	9.20±1.00	11.40±2.16	11.60±2.43	11.10±2.44	11.40±2.52	10.70±2.20	0.008
Uncapacitated, acrosome-intact sperm (%)	65.90±4.77	74.10±1.74	74.30±2.20	73.70±2.43	74.20±2.37	74.50±2.56	< 0.001

Table 26: Summary statistics of the sperm capacitation and acrosome reaction as determined by CTC stain after incubation with increasing concentrations of *C. capensis* extract crude extract for fraction 4 without progesterone.

	Control	0.05 µg/ml	0.5 µg/ml	5 µg/ml	50 µg/ml	200 µg/ml	ANOVA (P value)
Capacitated sperm (%)	19.80±2.54	23.60±3.37	24.70±3.11	25.40±3.21	26.40±3.47	26.30±3.46	< 0.001
Capacitated, acrosome-intact spem (%)	12.10±2.53	14.90±3.05	15.90±3.32	17.00±2.55	17.00±2.79	17.50±2.60	< 0.001
Capacitated, acrosome-reacted sperm(%)	7.20±1.10	8.60±0.94	8.80±1.10	8.40±1.14	9.40±1.14	8.80±1.36	<0.001
Uncapacitated, acrosome-intact sperm (%)	80.70±2.55	76.50±3.47	75.30±3.11	74.60±3.21	73.60±3.47	72.70±2.86	< 0.001

Table 27: Summary statistics of the sperm capacitation and acrosome reaction as determined by CTC stain after incubation with increasing concentrations of *C. capensis* extract crude extract for fraction 4 with progesterone.

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	Control	0.05 µg/ml	0.5 µg/ml	5 µg/ml	50 µg/ml	200 µg/ml	ANOVA (P value)
Capacitated sperm (%)	22.80±2.33	27.80±1.98	27.40±2.06	28.80±2.54	29.50±1.90	30.60±2.43	< 0.001
Capacitated, acrosome-intact spem (%)	13.70±2.05	15.60±1.23	15.70±1.45	16.10±1.74	16.90±2.02	17.20±1.98	< 0.001
Capacitated, acrosome-reacted sperm(%)	9.10±0.69	11.30±1.71	12.30±1.30	11.70±1.45	12.90±1.25	12.40±1.31	<0.001
Uncapacitated, acrosome-intact sperm (%)	65.50±6.14	72.30±1.89	72.60±2.06	71.00±2.55	70.50±1.90	71.40±5.66	< 0.001

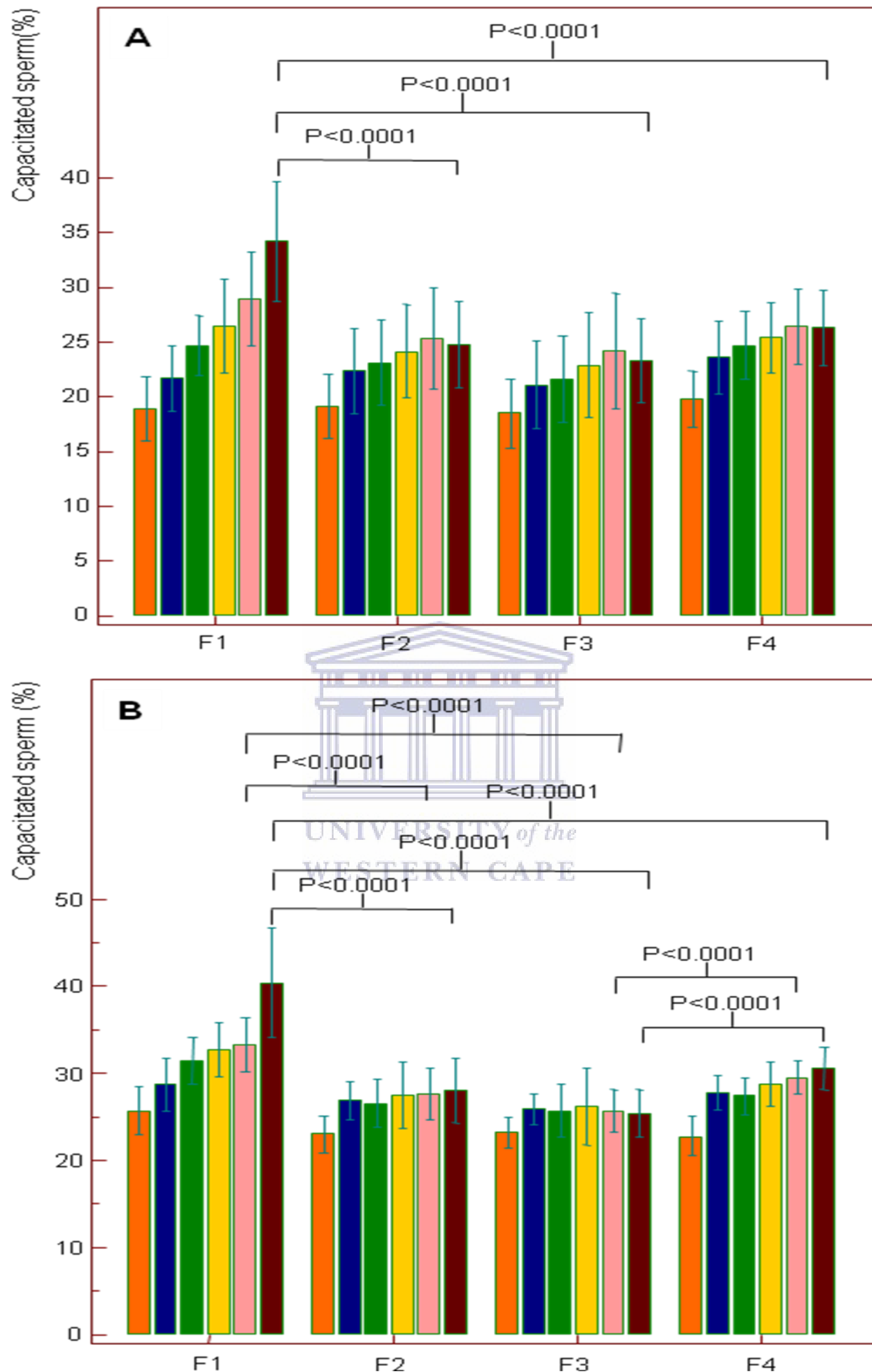

Figure 39: The effect of *C. capensis* methanolic fractions on capacitated spermatozoa *in vitro*. The orange bar represents 0 µg/ml, blue bar 0.05 µg/ml, green bar 0.5 µg/ml, yellow bar 5 µg/ml, pink bar 50 µg/ml and maroon bar 200 µg/ml. A) without P4; B) with P4. Values shown as mean±SEM (n=26).

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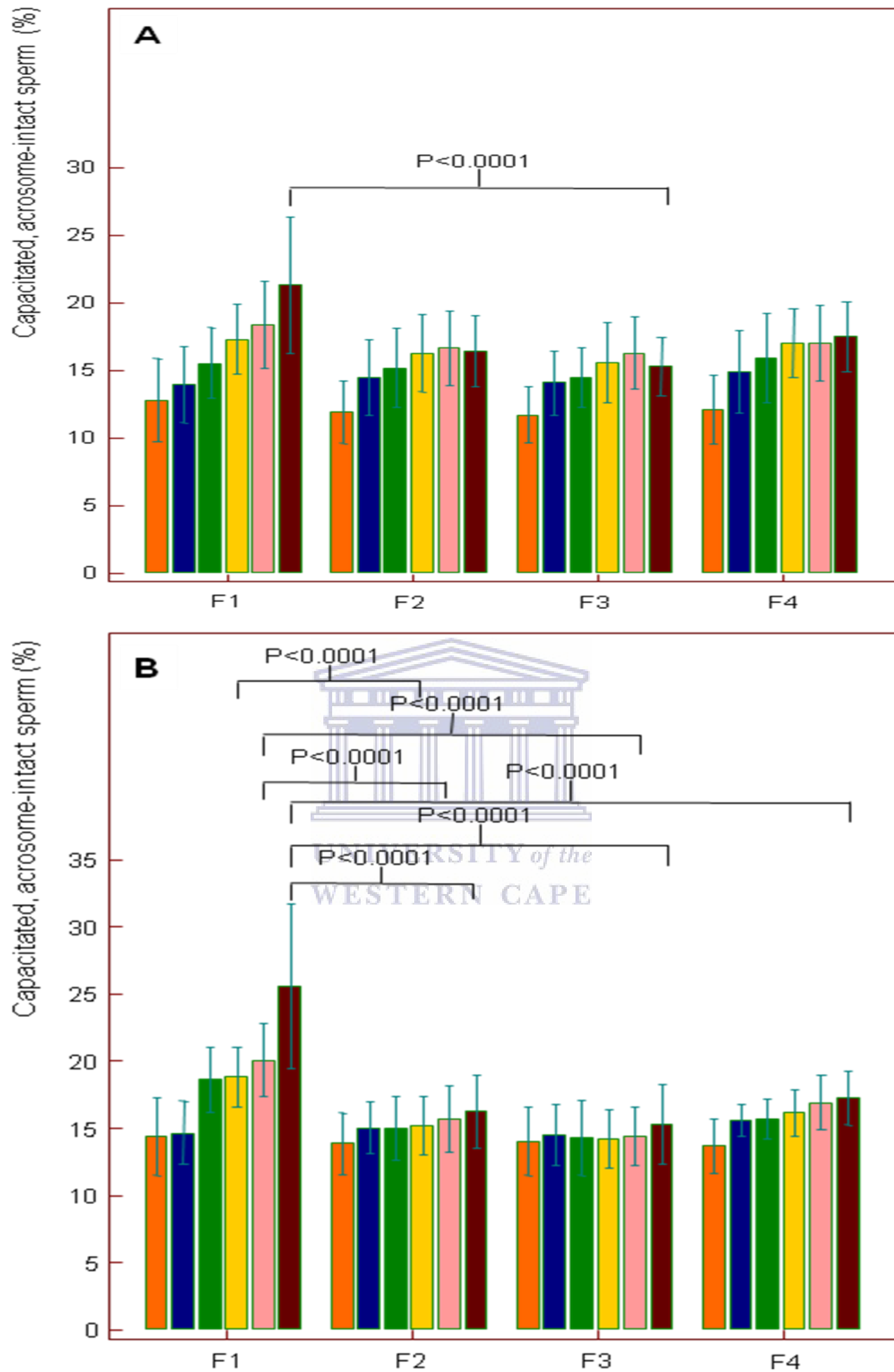

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Figure 40: The effect of *C. capensis* methanolic fractions on capacitated, acrosome-intact spermatozoa *in vitro*. The orange bar represents 0 µg/ml, blue bar 0.05 µg/ml, green bar 0.5 µg/ml, yellow bar 5 µg/ml, pink bar 50 µg/ml and maroon bar 200 µg/ml.

A) without P4; B) with P4. Values shown as mean±SEM (n=26).

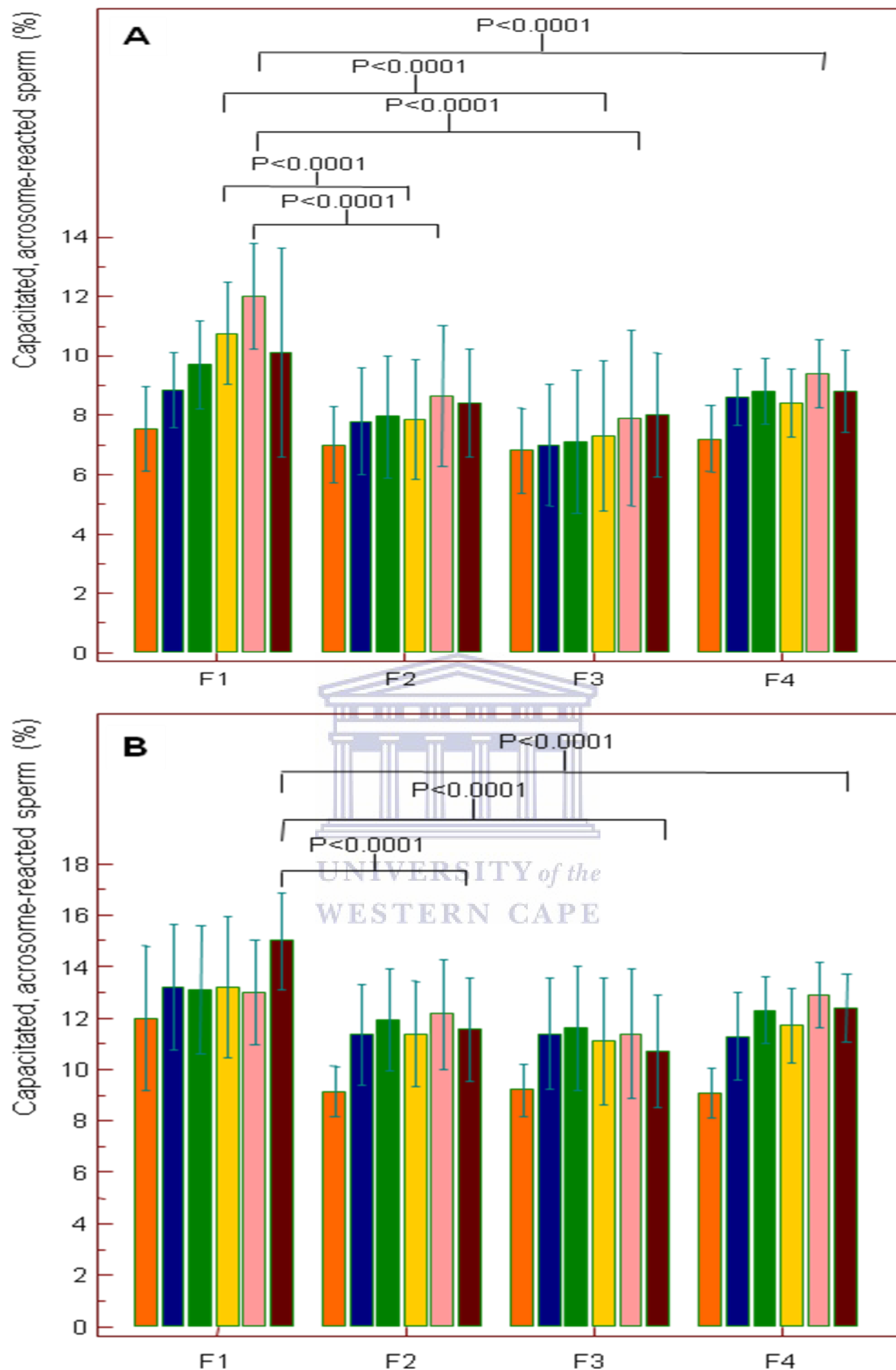

Figure 41: The effect of *C. capensis* methanolic fractions on capacitated, acrosome-reacted spermatozoa *in vitro*. The orange bar represents 0 µg/ml, blue bar 0.05 µg/ml, green bar 0.5 µg/ml, yellow bar 5 µg/ml, pink bar 50 µg/ml and maroon bar 200 µg/ml.

A) without P4; B) with P4. Values shown as mean±SEM (n=26).

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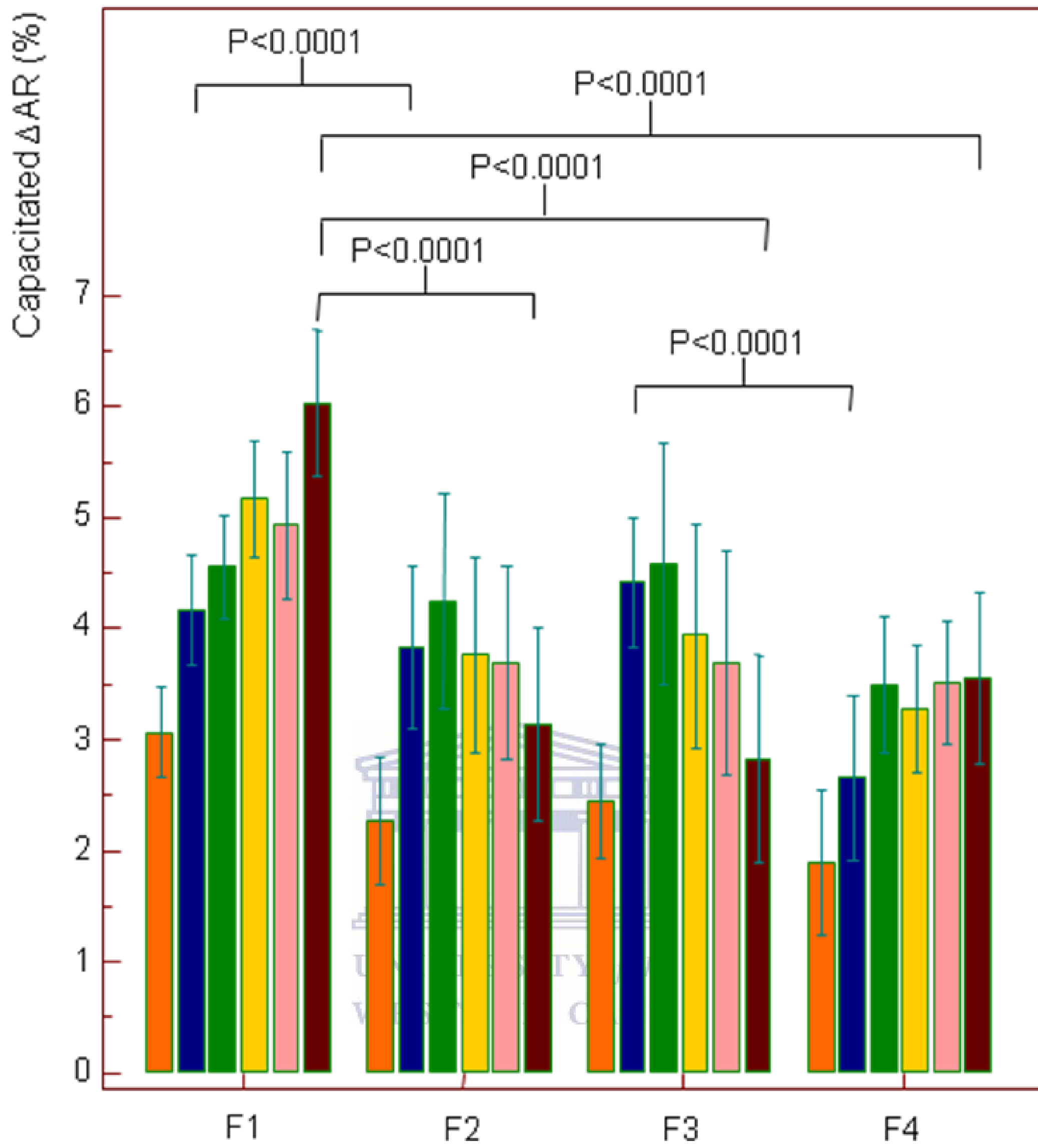

Figure 42: The effect of *C. capensis* methanolic fractions on capacitated delta acrosome-reacted (ΔAR) spermatozoa *in vitro*. The orange bar represents 0 µg/ml, blue bar 0.05 µg/ml, green bar 0.5 µg/ml, yellow bar 5 µg/ml, pink bar 50 µg/ml and maroon bar 200 µg/ml. Values shown as mean±SEM (n=29).

When compared fraction 1 with the control at the higher concentrations showed a significant increase (P<0.0001). In contrast, highly significant decrease (P<0.001) when compared fraction 1 with fraction 2 were found at 0.05 and 200 µg/ml.

Both, fraction 3 and 4 when compared with the fraction 1 only highly significant decrease (P<0.001) was found at 200 µg/ml. When comparing fraction 3 with fraction 4, a highly significant decrease (P<0.001) was found at 200 µg/ml.

Chapter 4: Discussion

The use of herbal remedies to treat ailments is as old as the humankind itself. Several diverse lines of evidence indicate that herbal medicines assimilate the oldest and most widespread form of medication (Vogel, 1991). Furthermore, the regulation and legislation of herbal medicines and traditional healing differs from country to country. In South Africa, the traditional health care service is regulated by the Traditional Health Practitioners Act No. 22 of 2007, which provides for a regulatory framework and ensures the efficacy, safety and quality of the treatment. The high frequency of use of traditional remedies is due to the socio-cultural and socio-economical context in such countries. Yet, this type of medicine has barely been studied scientifically (Castleman, 1997; Halberstein, 2005).

In spite of the great progress in modern Western medicine, plants or plant-based active substances are still used as a contribution in health care, even in Western medicine. It is estimated that an average of 25% of modern medicines originated directly or indirectly from medicinal plants (De Smet, 1997), sometimes even without the clinician knowing (Castleman, 1997). Reportedly, for anti-cancer and anti-infective drugs, this proportion is at over 60% (Cragg et al., 1997). Herbal products are widely distributed and are sold in pharmacies as well as supermarkets. Theses herbal products contain phytochemicals and other chemical compounds found in medical plants that are used to produce specific concentrations of pharmacological active compounds (Castleman, 1997).

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Since the *Cissampelos capensis* rhizome extract (CRE) is used by traditional healers to treat male reproductive problems and no studies are available that investigated the effects of CRE on human spermatozoa, this study is the first one throwing some light on the effects of this extract on ejaculated human spermatozoa *in vitro*.

This plant is the only endemic species in its family found in South Africa that grows during the winter season. The medicinal value of the rhizomes is mainly attributed to their alkaloid content, thus it is available throughout the year. While the leaves contain mainly three alkaloids namely bulbocapnine, dicentrine and salutaridine with anti-microbial, anti-bacterial, anti-fungal and anti-inflammatory activity, respectively, the rhizomes contain mainly bisbenzyltetrahydroisoquinoline alkaloids, with cissacapine, 12-O-methylcurine and cycleanine as main alkaloids with average alkaloid yields of 15.3%, 35.9% and 46.3%, respectively (De Wet et al., 2011).

4.1 Determination of suitable *in vitro* incubation concentrations with an aqueous extract of *Cissampelos capensis* rhizomes extract

4.1.1 Incubation concentrations of CRE for spermatozoa

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The concentrations of the plant extract used in this study were deduced by the assumption that an average male weighs 80 kg. The traditional healers "prescribe" the use of a handful of rhizomes to be used for making the concoction per day. From there, a standard 'normal' concentration of 50 µg/ml *C. capensis* rhizome extract (CRE) was calculated and a stock solution containing 2000 µg/ml CRE in HTF-BSA was prepared and mixed with sperm samples in HTF-BSA to obtain final concentrations 0.05, 0.5, 5, 50, 200 µg/ml CRE. HTF-BSA without the extract served as a control. These concentrations were used throughout the experiment and was deemed non-toxic and safe as the *in vivo* concentrations would be assumed to be much higher as the calculated prescribed amount per man per day used is 3.41 g of the extract.

For the second part of this study, CRE was fractioned with methanol (F1= 0% MeOH, F2= 30% MeOH, F3= 60% MeOH and F4= 100% MeOH) in combination with 20 µg/ml progesterone to induce acrosome reaction or without this hormone, applying the same final concentrations of 0.05, 0.5, 5, 50, 200 µg/ml CRE. HTF-BSA without the extract served as a control

4.2 Effects of CRE on functional parameters of spermatozoa

4.2.1 Effects of CRE on spermatozoa viability

In the current study, the assessment of viability of the spermatozoa was used as parameter to investigate possible cytotoxic effects of CRE on human spermatozoa as this parameter has not been tested before. No direct cytotoxic effect at any concentrations used was found when comparing each to the control. Thus, the compounds in the extract did not show any cytotoxic effect on spermatozoa with *in vitro*.

4.2.2 Effect of CRE on sperm motility and hyperactivation

As sperm motility plays an important role in fertilization and is a good predictive parameter for pregnancy (Coetzee et al., 1989; Shulman et al., 1998), low sperm motility may reduce the chances of sperm fertilizing the ovum and high sperm motility increases that probability (Henkel et al., 2005b).

In the current study, treatment of sperm with CRE showed no effect on sperm total and progressive motility. However, a significant and dose-dependent increase in sperm hyperactivation was shown and indicated by all relevant parameters that characterize hyperactivation (decreased VAP, VSL and LIN; increased BCF) before and after fractionation. Thus, CRE caused a change in the motility pattern, at least in a small percentage of spermatozoa. Hyperactivation is one of the physiological changes that are associated with the process of capacitation, which can be regarded as a preparatory step of the male germ cell for acrosome reaction (Chang, 1951; Austin, 1952). The question arises whether this stimulation of hyperactivation is caused directly by CRE or if this event is mediated perhaps by reactive oxygen species (ROS) as ROS physiologically function as trigger of capacitation and hyperactivation (Aitken and West, 1990; De Lamirande et al., 1997; Aitken et al., 1998; Aitken and De Iuliis, 2010).

After fractionation of CRE and incubation of spermatozoa with or without the addition of P4 to induce acrosome reaction, hyperactivation still showed an increase. Although the F2-, F3- and F4-fractions revealed a significant increase, the F1-fraction produced even higher levels and significance. However, when observing the results, as hyperactivation increased so too has the effect of CRE on ROS with no significant correlation.

4.2.3 Effect of CRE on sperm ROS production

As reported by O'Flaherty et al. (2006a), low levels of ROS are essential for normal sperm function in terms of the initiation of capacitation and acrosome reaction as well as the relevant triggering and modulation of protein tyrosine phosphorylation involved in these events (O'Flaherty et al., 2006b). On the other hand, elevated ROS levels have repeatedly been associated with male infertility (Aitken et al., 1998; Agarwal et al., 2003; Henkel et al., 2004) including loss of sperm motility and DNA damage. In the current study, dihydroethidium (DHE) was used to detect ROS in terms of intercellular superoxide as in previous investigations it has been shown to be a good detection system (Henkel et al., 2005a; de Lamirande and O'Flaherty, 2008; Mupfiga et al., 2013) and frequently used for the highly sensitive detection of superoxide in mitochondria of living cells (Whiteman et al., 2009).

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In the present study, intercellular superoxide concentrations increased after incubation of sperm with both the crude extract of CRE and fractionated extract in a dose-dependent manner. Although, all fractions showed significantly increased values, the F1-fraction produced the highest significant increase in ROS. This effect can be attributed to the alkaloid content of the *C. capensis* rhizomes. Alkaloids can exhibit various effects on cells ranging from suppressing intracellular ROS-production (Zhao et al., 2012), antiproliferative effects (Slunska et al., 2010), loss of mitochondrial membrane potential with intracellular ROS imbalance (Chiu et al., 2012) to antioxidative effects (Jung et al., 2009). For ergot alkaloids, a significant inhibitory effect on calcium ionophore-induced

acrosome reaction was shown *in vivo* on stallion sperm (Fayrer-Hosken et al., 2012). In this study, seeing that superoxide ($\cdot O_2^-$) was detected and is triggered by CRE, one can deduce that CRE is stimulating intracellular $\cdot O_2^-$ production, which subsequently triggered capacitation and caused sperm DNA fragmentation (Henkel et.al., 2005a). Therefore, even for the increase in sperm hyperactivation and all the relative changes in various motility parameters, this intercellular increase$\cdot O_2^-$ might be responsible. Nevertheless, in the light of the relatively low correlation coefficient between the percentage of ROS-positive spermatozoa and hyperactivation, other factors might have either contributed to the induction of hyperactivation, or prevented a more distinct relationship. In the light of both, the crude and fractionated extract comprise of several compounds, this idea has to be investigated further.

4.2.4 Effect of CRE on sperm capacitation and acrosome reaction

Capacitation is essential for normal acrosome reaction to occur (Yanagimachi, 1981, 1994) as only acrosome-reacted spermatozoa can penetrate the zona pellucida (Koehler et al., 1982). The percentage of acrosome-reacted sperm as well as the ability of sperm to induce acrosome reaction (inducibility) can also be directly related to the fertility rates in males as a positive predictor in diagnostic and therapeutic techniques for ART (Cummins et al., 1991; Henkel et al., 1993).

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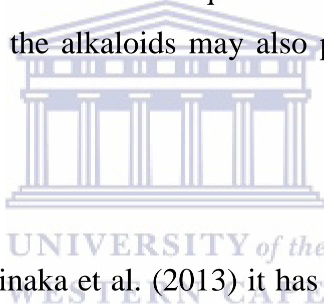
In the current study, the percentage of capacitated spermatozoa and capacitated acrosome-reacted cells (ANOVA trend analysis: P=0.0030) as determined by the CTC stain showed significant and dose-dependent increases (ANOVA trend analysis: P<0.0001) toward higher concentrations of CRE. On the other hand, the percentage of live-reacted spermatozoa determined by triple stain did not reveal a significant result (P=0.850). This apparent discrepancy would have to be explained by possible methodological differences in the detection of acrosome reaction. It would also be supported by the fact that although there was a marginal decline after incubation with 5µg/ml CRE, no trend could be established.

On the other hand, one could assume that the stimulus by CRE via an increased production of $\cdot O_2^-$ would not be sufficient to induce acrosome reaction and may need additional stimulation. This assumption could be supported by the fact that bisbenzyltetrahydroisoquinoline alkaloids including 12-O-methylcurine and cycleanine have been described as potent Ca-antagonists (Martinez et al., 1998; Guedes et al., 2002), thus inhibiting a Ca^{2+}- influx, which has been described as a trigger of acrosome reaction (Storey et al., 1992). Thus, the alkaloids might have stimulated capacitation but inhibited the initiation of acrosome reaction. This inhibition of calcium channels could be the reason why acrosome reaction did not or only marginally stimulated although capacitation was significantly triggered in the present study. Yet, by the inclusion of P4 to induce acrosome reaction showed that this trigger is still necessary, and can then lead to acrosome reaction. Although an additional stimulation is required when incubated with CRE other mechanisms of action by the alkaloids may also play a role in the induction of acrosome reaction.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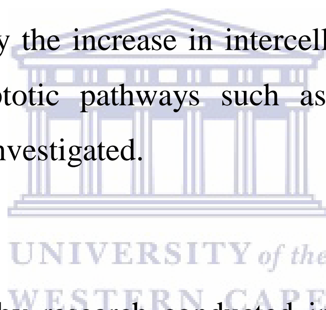
In a recent report by Haginaka et al. (2013) it has been shown that biscoclaurine alkaloids including cycleanine, which is a compound of the *C. capensis* rhizomes, interacts with the middle domain of heat shock protein 90α (HSP90α) suggesting inhibitory functions of these alkaloids on this highly conserved molecular chaperone protein. HSP90α is an abundant cellular protein, which is induced under stress conditions (Richter and Buchner, 2001; Picard, 2002). With regard to the triggering of capacitation and initiation of acrosome reaction, one also has to consider that HSP90 plays a significant role in the signal transduction pathways of a variety of cellular processes including spermatogenesis (Gruppi et al., 1991). With regard to capacitation and acrosome reaction of spermatozoa, Hou et al. (2008) showed that geldonamycin a specific HSP90 inhibitor, promoted NO production and thereby acrosome reaction in the boar.

4.2.5 Effect of CRE on sperm mitochondrial membrane potential (Δψm), sperm DNA-fragmentation and Annexin V-binding

The current research on CRE has no prior results on sperm functions. It is therefore important to analyze the cytotoxic or possible DNA damage effect that it may have on spermatozoa. Thus, advanced techniques such as Δψm and Annexin V-binding were employed as early apoptotic markers relating to the membrane of the cell (Vermes et al., 1995; Kroemer et al.,1997) and DNA-fragmentation (TUNEL assay) a late apoptotic marker.

In this study, no effect on Annexin V-binding or Δψm was observed. However, an increase in DNA-fragmentation could be found. This may indicate that the DNA damage may be caused by the increase in intercellular ROS stimulated by CRE. Nevertheless, other apoptotic pathways such as caspase 3/6, Bax, Bcl, for example, still need to be investigated.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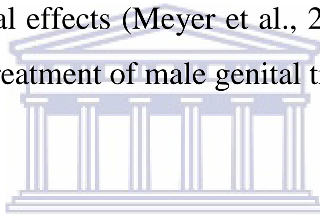
In addition, it is known by research conducted in the past that healthy human spermatozoa are incapable of actively initiating apoptosis (Lachaud et al., 2004; Aitken and Koppers, 2011) as at the spermatid stage the Sertoli cells would have aborted damaged cells before their release. Nevertheless, some of the male germ cells that were earmarked for apoptosis escape the elimination process (Sakkas et al., 1999). This hypothesis has been called 'abortive apoptosis'. On the other hand, there are also topological hinderance as the mitochondria, which are essential to apoptosis, are located in the mid-piece of the flagellum and are only connected with the sperm head via the very tiny neck-piece (Aitken and Koppers, 2011). Apparently, this does not prevent sperm from activating key enzymes such as caspase 3/7 for apoptosis (Mupfiga et al., 2013).

4.3 Conclusion and further outlook

In conclusion, this investigation on the effect of *Cissampelos capensis* rhizome extract shed light on the potential action of the alkaloid compounds of the extract on sperm function *in vitro*. It does not directly improve male fertility/sperm functions *in vitro* in terms of a possible treatment, but rather modulates sperm functional parameters as seen *in vitro*. Therefore, from this study, no direct conclusion on the *in vivo* effect of CRE in terms of a rational treatment of male fertility problems can be made. The medicinal effect of treatment with *C. capensis* possibly rather focuses on erectile dysfunction such as the extract of the bark of the yombi tree (*Pausinystalia yohimbi*), which is used to increase sexual arousal and dysfunction mainly due to its alkaloid *Yohimbine* as the active compound. Considering that the alkaloid content of the *C. capensis* rhizomes has also been shown to have antibacterial effects (Meyer et al., 2008), it might also be possible that CRE is useful in the treatment of male genital tract infections.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With regard to using the extract as a treatment for male reproductive problems, further research is necessary to establish how this extract works. Also, the active compounds still need to be identified and characterized to understand the mechanism of action. Further studies may also elucidate the direct action of these alkaloids on sperm function as well as the therapeutic use and safety of this herbal extract for the treatment of male reproductive functions.

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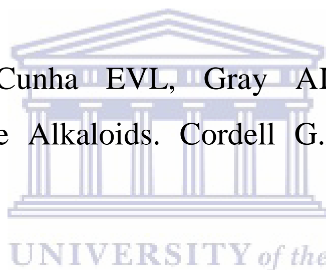
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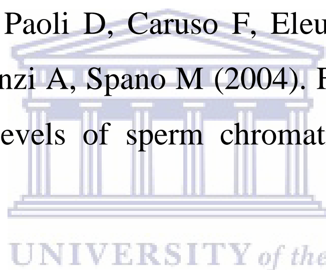

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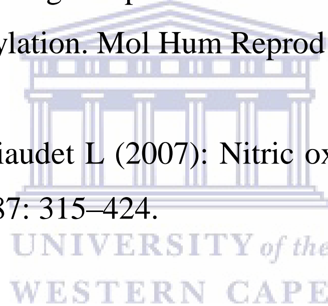
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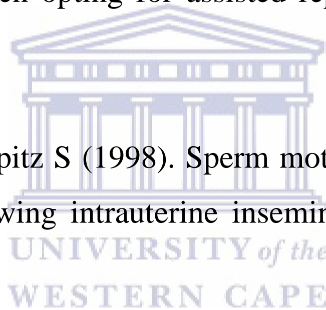
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