IN VITRO EFFECTS OF AQUEOUS EXTRACT OF *ASPALATHUS LINEARIS* (ROOIBOS) ON HUMAN SPERM CELLS

by

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DISSERTATION

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DECLARATION

I declare that the ***IN VITRO*** **EFFECTS OF AQUEOUS EXTRACT OF ROOIBOS TEA ON HUMAN SPERM CELLS** dissertation hereby submitted to the University of Limpopo, for the degree of Masters in Medical Sciences Chemical pathology has not previously been submitted by me for a degree at this or any other university; that it is my work in design and in execution, and that all material contained herein has been duly acknowledged.

Signed 

Date 12/April/2020

DEDICATION

To my father Mr Mulalo Godfrey Takalani and my mother Ms Shonisani Mercy Makhuvha.

To everyone battling with anxiety, "*If you can't fly then run, and if you can't run then walk, if you can't walk then crawl, but whatever you do you have to keep moving forward"* Martin Luther King Jr.

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- I would love to express my warm appreciation to my humble mother, Ms Shonisani Mercy Makhuvha for these words, “Ni to nanga u tambula kana udi phina”. Your wise words made me who I am today.
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- Last but not least, many thanks go to myself for believing that I can achieve anything in this world no matter the obstacles. I salute myself for having a courageous mind.

ABSTRACT

This study investigated the effect *of* rooibos tea on human sperm *in vitro.* Semen samples from 50 healthy men and 50 patients were treated with aqueous extracts of fermented rooibos (0 µg/ml, 0.10 µg/ml, 1.0 µg/ml, 10 µg/ml, 100 µg/ml) and unfermented rooibos (0 µg/ml, 0.15 µg/ml, 1.5 µg/ml, 15 µg/ml, 150 µg/ml) for one hour at 37ºC. Thereafter, sperm parameters were analysed. Exposure to unfermented rooibos had no effect on sperm motility, vitality and DNA fragmentation in both groups ($P>0.05$). However, this treatment significantly increased the production of reactive oxygen species, thus influencing the acrosome reaction, and demonstrated a decrease in mitochondrial membrane potential in the donor group ($P<0.05$). Treatment with fermented rooibos increased progressive motility in the donor group ($P<0.05$) but had no effect on sperm vitality, reactive oxygen species and the acrosome reaction in either group ($P>0.05$). A decrease in mitochondrial membrane potential and DNA fragmentation was observed in the donor group and the patient group ($P<0.05$). Rooibos tea maintains certain sperm functional parameters, and this may positively affect fertility rate.

KEY CONCEPTS:

Antioxidants; *Aspalathus linearis* (Rooibos)*;* Infertility; Medicinal plants; Reactive oxygen species; Sperm function

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RESEARCH PRESENTATIONS AND PAPERS

- Takalani, NB., Adefolaju, GA., Henkel, R. & Opuwari, CS. 2019. *In vitro* effects of *Aspalathus linearis* on human sperm cells. *Anatomical Society of Southern Africa, April 08, 2019, Pilanesberg, North West, South Africa.*
- Opuwari, CS., Takalani, NB., Adefolaju, GA. & Henkel, R. 2020. Effects of fermented rooibos on sperm from infertile men *in vitro. Experimental Biology, April 4-7, San Diego, California, USA.*
- Takalani, NB., Adefolaju, GA., Henkel, R. & Opuwari, CS. 2020. Effects of fermented rooibos on asthenozoospermia and oligozoospermia semen *in vitro*. *Anatomical Society of Southern Africa 48th Annual Conference, 19-22 August 2020*. Accepted.

TERMINOLOGY

The following terms are used as they are:

Antioxidant	An antioxidant refers to a substance that can trap free radicals before oxidative mutilation can occur (Canda, Oguntibeju & Marnewick, 2014). Antioxidants act as the first line of defence, scavenging or detoxifying the damaging effect caused by reactive oxygen species and oxidative stress (Walczak-Jedrzejowska, Wolski & Slowikowska-Hilczer, 2013).
Aspalathus linearis	*Aspalathus linearis* (rooibos) is a herbal plant that is found in the Cederberg district of the Western Cape in South Africa. *Aspalathus linearis* belongs to the family Fabaceae and comprises 278 different species (Andrews & Andrews, 2017).
Infertility	Infertility is clinically alluded to as the inability to conceive after 12 months of regular sexual intercourse without the use of contraceptives or any traditional medication (Vander Borght and Wyns, 2018).
Reactive oxygen species	Reactive oxygen species (ROS) are highly reactive oxygen derivatives with half-life times in the nano- to milli-second range. (Wu, 2015).
Spermatozoa	A spermatozoon refers to a fully developed motile sex cell that is capable of fertilising an egg cell naturally and that has a compact head and a flagellum for swimming (Malić Vončina, Golob, Ihan, Kopitar, Kolbezen & Zorn, 2016).

LIST OF ABBREVIATIONS

ALH	Amplitude of lateral head displacement
ANOVA	Analysis of variance
AR	Acrosome reaction
ATP	Adenosine triphosphate
BCF	Beat cross frequency
BSA	Bovine serum albumin
Ca^{2+}	Calcium ion
cAMP	cyclic monophosphate
CASA	Computer-aided sperm analysis
CAT	Catalase
DAG	Inositol-triphosphate diacyglycerol
DHE	Dihydroethidine
E & N	Eosin-Nigrosin
GP_X	glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathione
H_2O_2	Hydrogen peroxide
HTF	Human tubular fluid
IVF	*In-vitro* fertilisation
LIN	Linearity
LPO	Lipid peroxidation

MMP	Mitochondrial membrane potential
NaCl	Sodium chloride
NO	Nitric oxide
O^{2-}	Superoxide anion
OH	Hydroxyl radical
OS	Oxidative stress
PBS	Phosphate buffered saline
PKA	Protein kinase A
ROS	Reactive oxygen species
RT	Room temperature
SOD	Superoxide dismutase
STR	Straightness
TDF	Testis-determining factor
TUNEL	Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling
VAP	Average path velocity
VCL	Curvilinear velocity
VSL	Straight-line velocity
WHO	World Health Organization
WOB	Wobble

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CHAPTER ONE: INTRODUCTION AND BACKGROUND

1.1 INTRODUCTION

Infertility is clinically alluded to as the inability to conceive after one year of regular sexual intercourse without the use of contraceptives or any traditional medication (Vander Borght & Wyns, 2018). Infertility has been ranked as one of the major problems affecting human society. According to the World Health Organization WHO, (2004), approximately 10–15% of couples of reproductive age experience infertility of which 50% is reported to be caused by a male factor (Agarwal et al., 2019) A systematic review by Kumar and Singh (2015) assert that nearly 580 million individuals (5–8% of couples) experience the ill effects of infertility at some stage in their reproductive lives. Of these, almost 372 million people (186 million couples) live in developed and underdeveloped countries. In South Africa, 15–20% of couples of reproductive age are affected by infertility (Mascarenhas et al., 2012), for which the principal causes are tubal factor infertility (57%), male factor infertility (36%) and anovulation (29%) (Rizk, Agarwal & Sabanegh, 2019). The high rate of infertility has been associated with the high prevalence of sexually transmitted diseases, incomplete development of the testes, and hypothalamic and pituitary abnormalities (Tsevat, Wiesenfeld, Parks & Peipert, 2017).

According to the WHO (2010), semen analyses remain the true diagnosis before any fertility assumption can be made. Male infertility may be caused by azoospermia (no spermatozoa), oligozoospermia (low sperm count), asthenozoospermia (poor sperm motility) as well as teratospermia (abnormal sperm morphology) (Rowe, Comhaire, Hargreave & Mahmoud, 2000; Sharma, 2017). Other principal causes of infertility are erectile disfunction, sperm DNA damage and disruption in delicate processes such as spermatogenesis; these have been associated with reactive oxygen species (ROS) and oxidative stress (OS) (Direkvand-Moghadam, Delpisheh & Direkvand-Moghadam, 2015).

Although infertility is a global issue, its treatment is often positioned last on the health agenda and is seen as a luxury in life (Blank, 2019; Moudi, Piramie, Ghasemi & Ansari, 2019; Yilmaz & Kavak, 2019). Well-known treatments for infertility comprise assisted reproductive techniques, surgical repair of blocked fallopian tubes, induction of ovulation, and donor insemination (Cahill, 2019). Sadly, these are used less in underdeveloped African societies due to the medical costs and knowledge (Cahill, 2019). Barrenness in Africa has been progressively recognised (Agarwal, Mulgund, Hamada & Chyatte, 2015; Gerais & Rushwan, 1992; James et al., 2018). Epidemiological research has pointed out an increase in infertility from Western countries (reaching 20−40% in certain regions), and reference is made to an 'infertility belt' reaching Sub-Saharan Africa (Agarwal et al., 2015; Coşkun & Çavdar, 2018; Gerais & Rushwan, 1992; Larsen, 2000). Anthropological and sociological studies confirm the considerable suffering related to infertility due to the negative psychosocial consequences that include marital problems, physical and verbal abuse, and societal stigmatisation (Hasanpoor-Azghdy, Simbar & Vedadhir, 2014).

Recently, studies have placed more focus on women, probably due to the perception that in African society, females are responsible for infertility and as such are 'blamed' (Hasanpoor-Azghdy et al., 2014; Larsen, 2000; Mbemya et al., 2017). Only a few studies have focused on the male counterparts, and most of these studies were focused on assessing the aetiology and prevalence of male infertility. As a result, very little is known about men's experiences of infertility in Africa (Agarwal et al., 2015; Lock & Nguyen, 2018; Vigodner, 2011).

High levels of anxiety and depression affect infertile couples, not only when they reach the diagnosis stage of their infertility but also when they resort to traditional methods of treatment with fear of side effects and effectiveness (Akhtari, Bioos & Sohrabvand, 2015; Fine, 2018). Recently, the recognition of plants affecting human health and the rise in pharmacological studies have led to a preference for complementary techniques to combat infertility problems (Ahmadi Bashiri, Ghadiri-Anari & Nadjarzadeh, 2016). Alrowais and Alyousefi (2017) verified that overall, appointments with different traditional practitioners have increased by 47.3% from 1990 to date. In many African society, most

people depend on the biodiversity for treatment of different maladies and disorders, including male and female infertility (Bazrafshani, Khandani, Pardakhty, Tajadini & Pour Afshar, 2019). Tradition and religion influence numerous aspects of an individual's life, including reproduction (Vertovec & Rogers, 2018). The delicate harmony between what the individual needs and what society deems important profoundly affects personality, prosperity and sexual life (DeCapua & Wintergerst, 2016). In some cultural groups, pregnancy, motherhood and fatherhood represent a solid milestone that is highly admired and treasured (DeCapua & Wintergerst, 2016). Barrenness, specifically in African society, has been a major problem amongst couples, leading them to resort to traditional practices to find fertility clarification (Bandyopadyay & MacPherson, 2019).

The use of floras as treatment for various communicable and non-communicable diseases goes back several millennia and has significantly added to the development of pharmaceuticals, with about 25% of modern drugs being derived from plant parts (Fletcher, 2015). The rise in herbal medicinal plant preference for corporeal and emotional treatment within the modern age is mainly because herbal plants have more benefits, are less invasive and are less expensive than many modern approaches such as nuclear transfer, artificial insermination aswell as intra cytoplasmic injection (Lock & Nguyen, 2018).

Many plant extracts, portions or fragments isolated from these medicinal plants are used principally to combat different causes of male infertility such as lack of sexual desire, erectile dysfunction, absence of orgasm and different sperm aberrations (Malviya, Malviya & Jain, 2016; Singh, Dhole, Saravanan & Baske, 2017). It has been shown that traditional herbal extracts have a positive impact on sperm parameters due to the presence of antioxidants (Njila et al., 2019). Antioxidants found within herbal medicinal plant extracts are capable of improving fundamental processes of the male reproductive system such as spermatogenesis and steroidogenesis (Adewoyin et al., 2017). Different *in vitro*, *in vivo* and clinical research studies have ascertained the practical use of herbal plant extracts in the enhancement of male fertility parameters (Chikhoune et al., 2015; Lampiao, Krom & du Plessis, 2008; Mbemya et al., 2017). Although male infertility is

stressful, it is gratifying to learn that practicing healthy lifestyle changes such as exercising and following a balanced diet can enhance a man's sperm count (CST-S & PsyD, 2016). Another natural remedy reported to assist men with infertility problems is rooibos (Abuaniza, 2013).

Aspalathus linearis, commonly known as rooibos is a shrub that grows naturally in the Cederberg region of the Western Cape Province of South Africa (Sishi, 2018). *Aspalathus linearis* or *A. linearis* is manufactured as fermented and unfermented. Fermented rooibos tea is manufactured by fermentation of the plant propagules and leaves to obtain a red tea, while unfermented rooibos tea (green rooibos) keeps oxidation to a minimum and contains better protective antioxidant properties (Sishi, 2018). This herbal plant is regularly used to make a mild savory tisane equipped with polyphenol antioxidants, the absence of caffeine and very little tannins (Smith & Swart, 2016). Over the years, consumption of rooibos tea has increasingly gained recognition due to its reputation as a healthy herbal beverage (da Silva Pinto, 2013). Different flavonoids have been derived from rooibos and include flavonols, flavones and dihydrochalcones (Joubert & de Beer, 2014). A monomeric flavonoid, namely aspalathin, is found in rooibos tea. Aspalathin is a C-C linked dihydrochalcone glucoside (Smith & Swart, 2016). Several studies have reported that the antioxidant properties found in rooibos are beneficial in combating infertility (Wyk and Wink, 2018).

A study on the effects of fermented rooibos, unfermented rooibos, Chinese green tea, commercial rooibos and green tea supplements on rat sperm demonstrated that both sperm count and motility were significantly higher in rats treated with unfermented and fermented rooibos compared with the other groups (Awoniyi, Aboua, Marnewick & Brooks, 2012). It has also been demonstrated that the antioxidants in rooibos tea prevent OS, which causes deterioration at a cellular level on a variety of tissues from organs to sperm (Rahman, Huang, Zhu, Feng& Khan, 2018).

Baba and his associates (2009), demonstrated that rooibos tea significantly averted DNA damage and swelling through its anti-oxidative activity in rats. Fermented rooibos extract

was shown to protect testicular tissue against oxidative impairment through increasing the antioxidant defense mechanisms in rats while reducing lipid peroxidation (LPO) (Awoniyi, Aboua, Marnewick, du-Plesis & Brooks, 2011). In addition, Opuwari and Monsees (2014) revealed that unfermented rooibos significantly improved sperm concentration, viability and motility while fermented rooibos improved sperm vitality, capacitation and the acrosome reaction (AR) in rats. Although different studies have been conducted using animals, rooibos could potentially have the same positive effect on men experiencing infertility problems (Van Wyk & Wink, 2018).

1.2 RESEARCH PROBLEM

Aspalathus linearis (rooibos) is a herbal medicinal plant originally from South Africa's fynbos and well known for its medicinal effects in treating different medical conditions. Several *in-vivo* studies have demonstrated its beneficial effects in numerous cell lines. 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. As spermatozoa are exceptionally vulnerable towards ROS, rooibos tea may improve seminal parameters such as motility, vitality, hyperactivation and capacitation of the spermatozoa thereby heightening chances of a successful ferilization. The antioxidant properties of *A, linearis* contribute largely to the inhibition of free radicals that damage spermatozoa, owing to the significant amount of enzymatic and non-enzymatic antioxidants found in the rooibos plant. To date, no study has been conducted using rooibos tea to determine its effect on human sperm parameters. Therefore, this study aims to investigate the in vitro effects of *Aspalathus linearis* on human sperm cells.

1.3 PURPOSE OF THE STUDY

1.3.1 Research question

What are the *in vitro* effects of aqueous extract of rooibos tea on human sperm cells?

1.3.2 Aim of the study

The aim of the study was to investigate the *in vitro* effects of aqueous extract of rooibos tea on human sperm cells.

1.3.3 Objectives of the study

The objectives of the study were as follows:

1. Determine the effect of rooibos aqueous extract on sperm motility
2. Determine the effect rooibos aqueous extract on sperm vitality
3. Determine the effect of rooibos aqueous extract on capacitation and the acrosome reaction in spermatozoa
4. Determine the ability of rooibos aqueous extract to protect sperm cells against DNA fragmentation
5. Determine the effect of rooibos aqueous extract on sperm mitochondrial membrane potential
6. Determine the effect of rooibos aqueous extract on the production of reactive oxygen species in spermatozoa
7. Compare the effects of rooibos aqueous extract on fertile and infertile human sperm

CHAPTER TWO: LITERATURE REVIEW

2.1 MEDICINAL PLANT

Throughout human existence, humanity has relied on nature and its resources to cater for everyday needs such as food, shelter and clothing and has considered it a source of remedies to treat different ailments (Fieldhouse, 2013). For thousands of years, plants have been the only source of traditional medicine in various tribes before modern change (Fieldhouse, 2013). Evidence supports that plants have been used as treatments for 60 000 years; scripts focusing on traditional medicine date back 5 000 years in India and Egypt and at least 2 500 years in Greece and Central Asia (Solecki, 1975; Ang-Lee, Moss & Yuan, 2001). With reference to infertility, several medicinal plants have been widely used due to their antioxidant activity in the male reproductive system; these include *Alpinia galangal, Citrullus vulgaris, Danae racemosa, Apium graveolens* and *Cinnamomum zeylanicumo.* The above-mentioned plants are reported to have phytochemicals, which have a positive effect on sperm function and the process involved in enhancing sperm capability to fertilise the oocyte (Mazaheri, Shahdadi & Nazari Boron, 2014; Ahmed, 2016; Kooti et al., 2017).

Traditional medicinal plants consist of organic plant material that may undergo industrial modification for the treatment of different illnesses (Pan et al., 2014). More than 80% of people depend on traditional drugs, specifically plants, as their principal source of healthcare (Kumar, Jalaluddin, Rout, Mohanty & Dileep 2013). This statistic does not only include highly populated countries such China and India but also many parts of Sub-Saharan Africa (Kumar et al., 2013). Traditional medicine is regarded as the oldest cultural form of cure, and consultation is initiated by a traditional healer (Ekeopara & Ugoha, 2017). A traditional healer refers to a person who makes use of ancient techniques that are handed down from one healer to another to treat a patient suffering from one or more ailments (Agbor & Naidoo, 2016). Methods used by traditional healers include the use of roots and other plant propagules (Zuma, Wight, Rochat & Moshabela, 2016). Traditional medicine is a well-known form of complementary medicine used in Sub-

Saharan Africa (Zuma et al., 2016). Its popularity is ascribed to its affordability, availability and effortlessness compared with modernised healthcare services (Agbor & Naidoo, 2016). However, proof of its effectiveness remains uncertain (Chatfield, 2018).

Unorthodox fertility services are prevalent in Africa and are rendered by traditional medical professionals (James, Taidy-Leigh et al., 2018). Studies outside Africa have observed the use rate of complementary and traditional approaches by men and women seeking fertility clarifications (Mahroozade, Sohrabvand, Bios, Nazem & Nazari, 2016; James, Wardle, Steel & Adams, 2018; Vyas, Gamit & Rava, 2018). A study in Uganda reported a 76.2% commonness of natural medicine use amongst women looking for infertility treatment, while a Nigerian report revealed that more than two-thirds of infertile couples (69%) look for care through traditional medicinal practitioners (James et al., 2018). The WHO supports the use of therapeutic plants and welcomes specialists and researchers to explore the use of restorative plants as a wellspring of new medications (Sofowora, Ogunbodede & Onayade, 2013). Plant parts are widely used to treat different aspects of male infertility such as erectile dysfunction, sperm abnormalities, endocrine disorders, ejaculatory and relaxation dysfunction and the absence or loss of orgasm (Prasad, Shyma & Raghavendra, 2014). The biological effectiveness of natural herbals is well established in different animal and human studies (Pan et al., 2014).

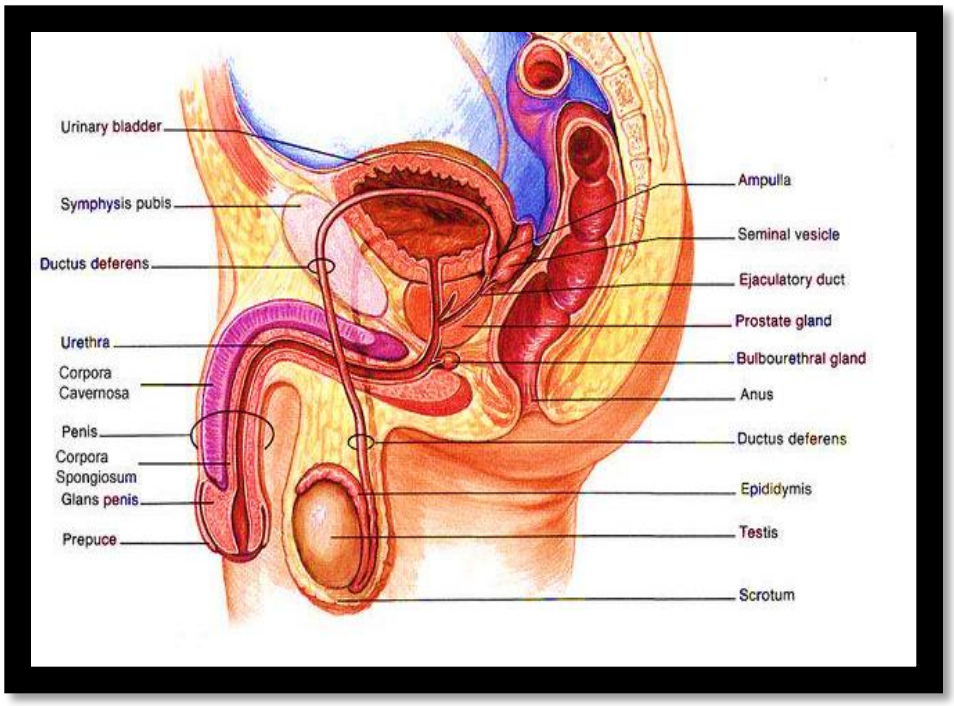
2.2 THE REPRODUCTIVE SYSTEM

2.2.1 The male reproductive system

The external and internal organs of the male reproductive system work together to produce quality sperm for fertilization of an oocyte in the female reproductive system (Sherwood, 2015). This is made possible via the process of spermatogenesis and endocrine and paracrine hormonal regulation of the reproductive function involving gonadotrophin releasing hormone, follicle stimulating hormone ,luteinising hormone, inhibin and testosterone (Chikhoune et al., 2015). The male reproductive organs consist

of the testicles, epididymis, vas deferens and the ejaculatory and accessory glands such as the seminal vesicles, prostate and bulbourethral glands (Figure 2.1) (Sherwood, 2015). Seminiferous tubules are located within the testes and produce sperm cells during spermatogenesis (Goossens & Tournaye, 2017). Additionally, the testes deliver the male sex hormone, testosterone, which is essential in the development of male sex organs (Esteves & Miyaoska, 2015). Sperm cells produced during spermatogenesis are transported to the epididymis where they develop and gain motility (Corradi, Corradi & Greene, 2016). The accessory glands contribute to the formation of seminal fluid, which has the following functions:

- Conveys the spermatozoa to the vagina
- Acts as a source of energy for the active sperm since it contains fructose
- Shields the spermatozoa against the acidic conditions within the vagina
- Initiates lubrication of the pathway through which spermatozoa must swim (Sherwood, 2015)



Sagittal view of the male reproductive organs showing the site for spermatogenesis and hormonal production (testicle), the site for maturation and storage of spermatozoa (epididymis), the ejaculatory duct that delivers sperm to the urethra and the accessory glands (seminal vesicles, prostate gland and bulbourethral gland)

Figure 1.1: The male reproductive system

Available at:

https://anatomy-medicine.com/the-male-reproductive-system/168-the-male-reproductive-system.html

2.2.2 Spermatogenesis

Spermatogenesis refers to a biological process that involves multiplication of spermatogonia, meiosis of spermatocytes and differentiation of spermatids to form gametes, which are capable of gaining motility and fertilizing an oocyte (de Kretser Loveland & O'Bryan, 2016). Spermatogenesis begins in the seminiferous tubules, which

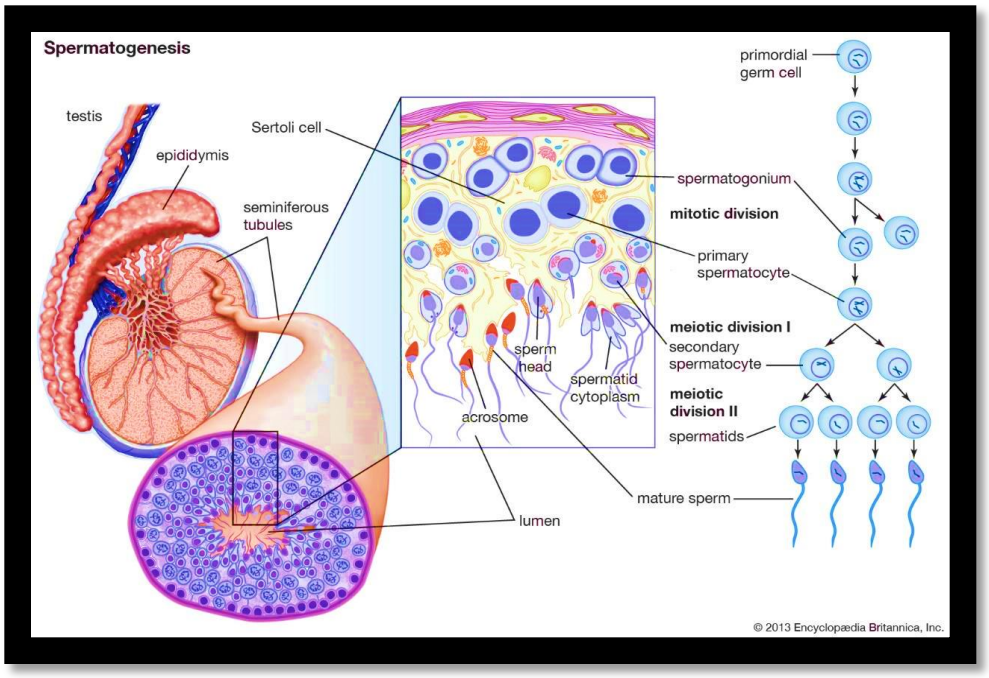
in humans are ~200 µm in diameter and ~600 metres in length, inhabiting ~60% of the testicle capacity (Szmelskyj, Aquilina & Szmelskyj, 2015). Sperm production takes place in the testicular seminiferous tubules consisting of peritubular tissue and seminiferous epithelium (Szmelskyj, Aquilina & Szmelskyj, 2015). As germ cells mature, they traverse the seminiferous epithelium, a procedure that involves restructuring of the Sertoli-germ cell junctions and the Sertoli-Sertoli cell junctions at the blood-testis barrier (Chen, Mruk, Xiao & Cheng, 2017). The blood-testis barrier is regarded as one of the most taut tissue barriers within the mammalian body and separates the seminiferous epithelium into two sections, the basal and luminal compartments (Chen et al., 2017). This barrier formed by the Sertoli cells is essential in preventing aggregates of systematic circulation and hormones from affecting the maturation of sperm and in preventing the immune system from recognizing sperm cells as destructive agents (Cole, 2016). Furthermore, the Sertoli cells are responsible for the following: development of the sperm cells by supplying the seminiferous epithelium with nutrition; conveyance of mature spermatids into the lumen of the seminiferous tubules; secretion of androgen-binding protein; and interaction with endocrine Leydig cells (Cole, 2016).

In simple terms, spermatogenesis is a fundamental, complex mammalian procedure that prompts the arrangement of exceptionally matured spermatozoa inside the testes through a series of events (Griswold, 2015). During this biological procedure, spermatogonia begin to replicate through the process of mitosis to form two identical cells (Sherwood, 2015). One of the identical cells is used to renew the pool of spermatogonia; these cells are referred to as Type A1 spermatogonia (Vigodner, 2011). The renewal of spermatogonia ensures the fertility of males throughout their entire reproductive age (Carmichael, 2011; Griswold, 2015). The remaining Type B spermatogonia eventually form mature sperm cells (Griswold, 2015). Type B spermatogonia divide several times by mitosis to form indistinguishable diploid cells known as spermatocytes and thereafter undergo meiosis while still connected by cytoplasmic bridges (Jan et al., 2017) see figure 2.2.

- Meiosis I – forms two haploid cells known as secondary spermatocytes
- Meiosis II – forms four haploid cells referred to as spermatids

The cytoplasmic bridges separate, leading to the discharge of the spermatids into the lumen of the seminiferous tubule in a process referred to as spermiation (Mäkelä & Toppari, 2017). The spermatids undergo spermiogenesis (differentiation and remodeling into mature spermatozoa) as they travel along the seminiferous tubules until they reach the epididymis where they are stored and undergo the last phases of development (Goossens & Tournaye, 2017; Mäkelä & Toppari, 2017).

In humans, the production of functional spermatozoa in the testicles takes approximately 74 days with an additional 12 days for maturation of released sperm in the epididymis. Spermatogenesis commences at puberty and continues until death (Griswold, 2015 Goossens & Tournaye, 2017). During sex, the developed sperm cells are released into the female reproductive tract where they undergo a process of conditioning called capacitation (Komeya, Sato & Ogawa, 2018).



Spermatogenesis is the origin and development of the sperm cells within the male reproductive organs, the testes. Sperm cells are produced within the testes in structures called seminiferous tubules. Once the sperm has matured, it is transported through the long seminiferous tubules and stored in the epididymis of the testes until it is ready to leave the male body (Encyclopedia Britannica, 2013)

Figure 1.2: The process of spermatogenesis

Avaialble at

https://www.britannica.com/science/spermatogenesis

2.3 INFERTILITY

For thousands of years, failure to conceive has been deemed a personal tragedy. Millions of couples worldwide have experienced infertility leading to social segregation, with the female being considered the main culprit (Stentz, Koelper, Barnhart, Sammel & Senapati, 2019). Becoming a parent (motherhood or fatherhood) is viewed as a cultural standard, an achievement that commands and draws respect in the circle of life within African societies (Vyas et al., 2018). Although infertility is primarily dealt with pathologically, the psychological and emotional effects are unbearable (Agarwal et al., 2015). When patients learn about their infertile status, they encounter emotions such as denial, anger, depression, rejection, guilt and feelings of insignificance (Mekonnen, 2017).These emotional manifestations are the psychological effects ofinfertility, which mature into what is often called 'crisis of infertility' (Agarwal et al., 2015; Mekonnen, 2017). In addition, marital problems, physical and verbal abuse, and social segregation have been reported to be associated with infertility (Whitehouse & Hollos, 2014).

The inability to conceive after one year of regular unprotected sexual intercourse without the use of a contraceptive is viewed as infertility, a disease of the reproductive system (Agarwal et al., 2019). Infertility affects approximately 15% of couples worldwide, which is about 48.5 million couples (Agarwal et al., 2015). Males are solely accountable for 20–30% of the infertility incidence and overall, females contribute to 50% of the cases (Agarwal et al., 2015). A summary of the incidences, prevalence and causes of infertility is presented in Figure 2.3. At the beginning of the 21st century, the WHO (2004) stated that approximately 60–80 million people worldwide suffered from infertility.

Male infertility is the inability of a male to impregnate a fertile female and is further subdivided into primary and secondary infertility (Kumar & Singh, 2015; Adewoyin et al., 2017). Primary infertility refers to couples who have not become pregnant after at least one year of having sex without using birth control methods. Secondary infertility refers to couples who have been able to get pregnant at least once but are now unable (for instance, loss of libido, erectile dysfunction, endocrine disorders, chronic diseases) is unable to achieve pregnancy any more (Benksim, Elkhoudri, Ait Addi, Baali & Cherkaoui, 2018). A systematic review, meta-analysis and population-based study by Agarwal and his associates (2015) reported that the global data on infertility attributed to male factors range from 20% to 70%, with Africa and Central and Eastern Europe having the highest prevalence rate (Agarwal et al., 2015; Kumar & Singh, 2015; Polis, Cox, Tunçalp, McLain & Thoma, 2017). On average, 10.1% of couples in Africa are affected by infertility (Kumar & Singh, 2015). King (2018) reported that the prevalence of infertility is escalating drastically across West Africa, Central Africa and the Middle East. Botha, Kruger, Van Der Merwe & Nosarka (2009) assessed the health status of a cohort of men residing in South Africa using the Tygerberg strict criteria. In the study, 32.4% of the men were diagnosed with subfertility, while 11.9% had sperm aberrations such as azoospermia, triple defect and double defect.

Idiopathic causes of male infertility account for 20% of its incidence and are associated with ROS and OS (Mayorga-Torres, Camargo, Cadavid, du Plessis & Cardona Maya, 2017). Production of ROS by human spermatozoa contributes significantly to the aetiology of poor sperm production and function (Alahmar, 2019). Oxidative stress triggered by an increased generation of ROS affects the normal integrity and functionality of the spermatozoa and may cause infertility (Agarwal et al., 2019). Increased levels of ROS result in poor sperm motility, morphology and vitality. Moreover, research has proved that increased levels of ROS cause DNA fragmentation, damage to mitochondrial membrane potential (MMP) and reduced sperm viability (Du Plessis, Agarwal, Halabi Tvrda, 2015;Adewoyin et al., 2017 & Agarwal et al., 2019). In addition, ROS diminish intracellular adenosine triphosphate (ATP), which prompts a decline in axonemal protein phosphorylation (Zorov, Juhaszova & Sollott, 2014).

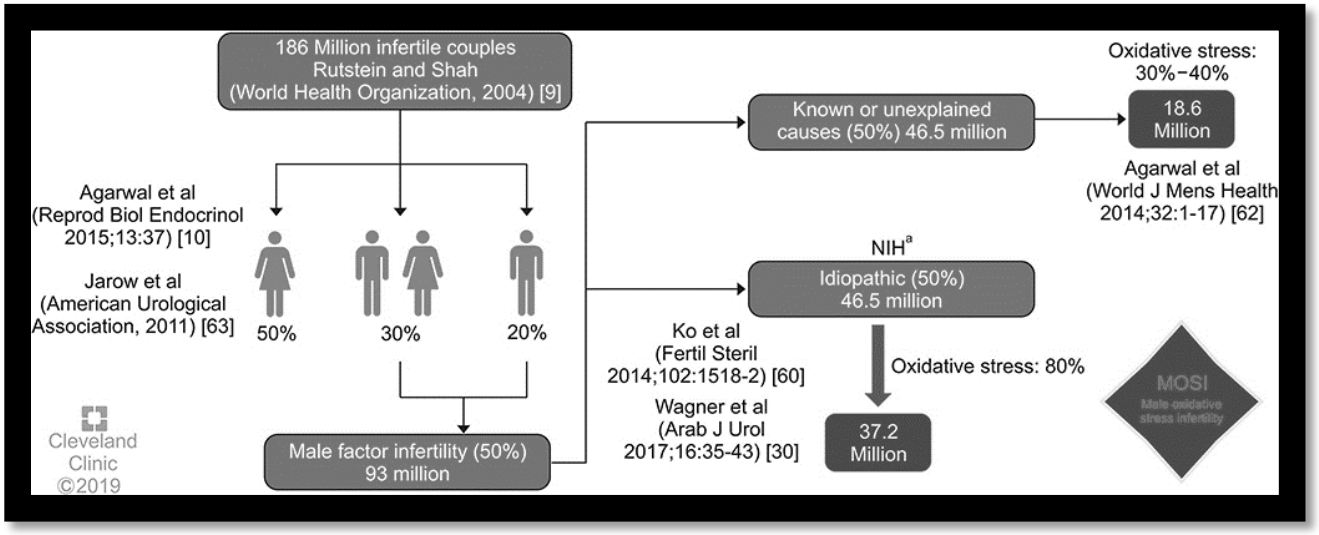


Figure 1.3: Summary of infertility statistics

Source: Agarwal et al., 2019

2.3.1 Sperm motility

Sperm motility is crucial in defining fertility because poor sperm motility (asthenozoospermia) negatively affects fertilisation (Pereira, Sá, Barros & Sousa, 2017). Sperm motility refers to the movement and swimming capacity of the spermatozoa and acts as an indicative measure of viability of the sperm tail, morphology, mitochondria and the sperm axoneme (Anifandis, Amiridis, Dafopoulos, Daponte & Dovolou, 2018). The motility of the sperm is controlled by the flagella, which are responsible for propelling the spermatozoa to the female reproductive tract (Lehti & Sironen, 2017). Following the release of sperm into the female reproductive tract, the spermatozoa strive to reach the oocyte, forming an asymmetrical flagellar movement known as hyperactivation to arrive at the oviduct (Lehti & Sironen, 2017). The flagellar movement is influenced by the use of ATP, a biochemical compound that provides energy (Skinner, Mannowetz, Lishko & Roan, 2019). Once ejaculated, the sperm are exposed to various conditions and need to adapt to increase their chances of reaching the ovum (Pereira et al., 2017). Furthermore,

different kinematic parameters (curvilinear velocity, rectilinear or straight-line velocity, average path velocity, linearity, straightness, beat cross frequency, wobble, amplitude of lateral head displacement and hyperactivation) work in association to enable the spermatozoa to penetrate the cervical mucus within the female reproductive tract (Valverde, Madrigal, Caldeira, Bompart, & Murga,2019).

Routine semen analysis provides important information concerning sperm motility, concentration, morphology and viability and indicates the chances of fertility (Fair & Romero-Aguirregomezcorta, 2019). Analysis of sperm motility is crucial in the diagnosis of male infertility (Creasy & Chapin, 2013)

2.3.2 DNA fragmentation and mitochondrial membrane potential

The integrity of the sperm DNA is crucial for successful fertilisation and full development of an embryo (Sedó, Bilinski, Lorenzi, Uriondo, & Noblía, 2017). Sperm DNA fragmentation is a term used to describe aberration in genetic material within the sperm, which in turn leads to male subfertility, *in-vitro* fertilisation (IVF) failure and sometimes, failed implantation (Evenson, 2016). In simple terms, DNA fragmentation is a term used to describe the breakage that occurs within the single- or double-strand structures before or after ejaculation, causing DNA obliteration (Lewis, 2013). Sperm DNA fragmentation affects the crucial processes and parts needed for successful fertilisation to occur such as IVF, conception, blastocyst development and correct transmission of genetic material (Lewis, 2015). Sperm DNA becomes vulnerable to mutilation if the chromatin network is not complete during the process of spermatogenesis when protamine replacement occurs in elongating spermatids (Evenson, 2016). Research has shown that infertile males have higher levels of sperm DNA damage and poorer sperm DNA integrity than fertile men and that the transmission of DNA-damaged spermatozoa heightens the risk of genetic complications in the progenies (Esteves, Gosálvez, López-Fernández, Núñez-Calonge & Caballero, 2015 ; Simon, Zini, Dyachenko, Ciampi & Carrell, 2017; Watson, 2015). Moreover, several studies have revealed that sperm DNA can be associated with poor seminal parameters such as reduced sperm motility, low concentration, reduced AR, reduced MMP and abnormal sperm morphology (Alahmar, 2019; Majzoub, Arafa, Mahdi,

Agarwal & Al Said,2018 ; Pourmasumi, Sabeti, Rahiminia, Mangoli, Tabibnejad & Talebi, 2017).

Other reported causes of DNA damage are associated with ROS and high levels of OS (Van Houten, Santa-Gonzalez & Camargo, 2018). Reactive oxygen species have a detrimental effect on sperm DNA, leading to the formation of 8-oxo-7, 8-dihydro-2′-deoxyguanosine, the chief oxidative product of sperm DNA that triggers DNA fragmentation and has mutagenic effects on the integrity of the DNA (Jeng, Pan, Chao & Lin, 2015). At higher levels, ROS cause OS damage (Wagner, Cheng & Ko, 2017). After gaining access into the sperm, the ROS target genetic materials, destroying mitochondrial DNA and inhibiting intracellular ATP production, which is essential for the transport of genetic materials (Agarwal, Cho, Esteves & Majzoub, 2017; Jeng, Pan, Chao & Lin, 2015; Zandieh, Vatannejad, Doosti, Zabihzadeh, & Haddadi, 2018). Without proper ATP production, both functionality and sperm motility are affected, and male infertility can occur as a result (Marchetti, Ballot, Jouy, Thomas & Marchetti, 2011). Furthermore, DNA damage exhibits increased levels of ROS, which result in the reduction of sperm MMP and are associated with failed IVF rates, while spermatozoa that display intact MMP are said to have an intact acrosome function with improved chances of fertilisation (Slowinska, Liszewska, Judycka, Konopka & Ciereszko, 2018).

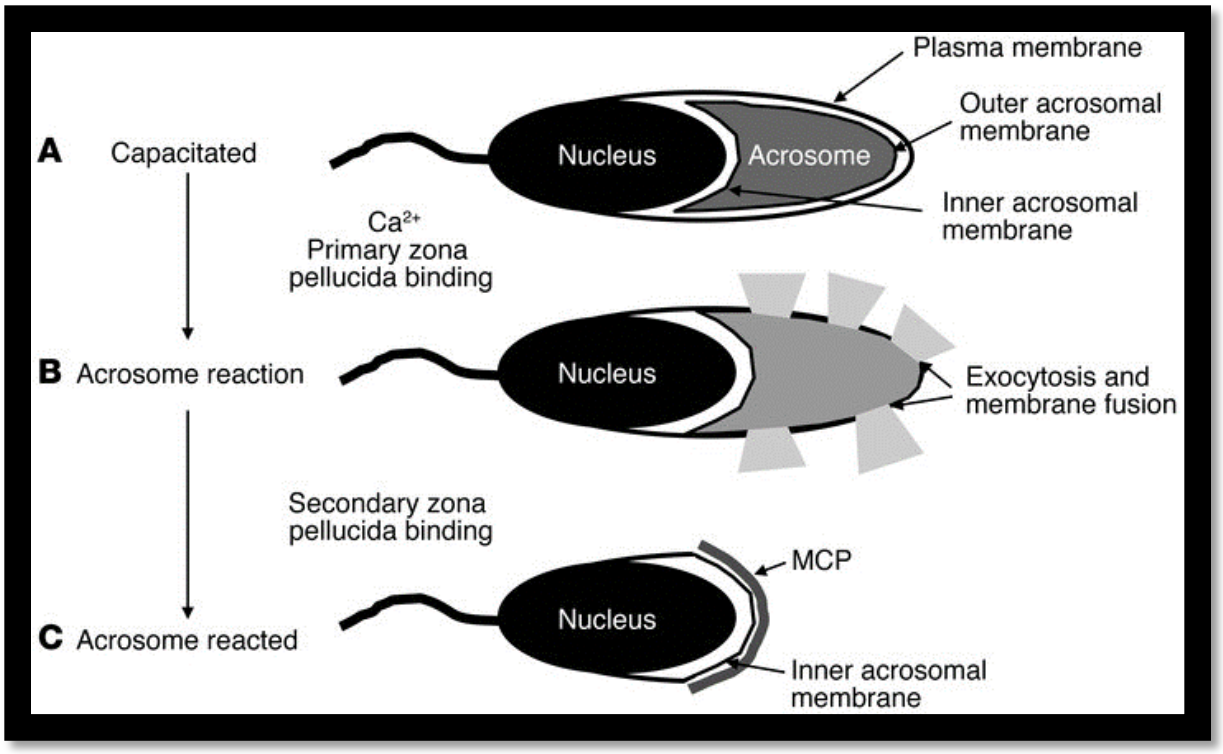
2.3.3 Capacitation and acrosome reaction

Biologically, ejaculated spermatozoa stay within the female reproductive tract for a few hours before attaining the ability to fertilise the oocyte. In humans, sperm must vacate the seminal fluid immediately after ejaculation and thereafter move to the fallopian tube (Suarez, 2016; Stical, Puga Molina, Buffone, Visconti & Krapf, 2016). As the spermatozoa vacate the ejaculate via the cervical mucus, they undergo several biochemical modifications known as capacitation (Stival et al., 2016) .

There are several biochemical and physiological variations of capacitation (Jin & Yang, 2016). Biochemical variations linked with the capacitation procedure comprise an efflux of cholesterol from the plasma membrane, resulting in a high level of membrane fluidity

and penetrability by bicarbonate and calcium ions, hyperpolarisation of the plasma membrane, changes in protein phosphorylation and protein kinase (PKA) activity, an increase in bicarbonate concentration and intracellular pH, and raised calcium ion (Ca^{2+}) and cyclic adenosine monophosphate (cAMP) levels (Jin & Yang, 2016). Capacitation is subdivided into two signalling processes, slow and fast events. These two processes occur amidst the channelling of spermatozoa within the female reproductive system (Kunkitti, Bergqvist, Sjunnesson & Axnér, 2015). The fast events encompass the stimulation of the energetic and asymmetric movement of the flagella, and these occur immediately after the spermatozoa exit the epididymis (Stival et al., 2016) . The fast event is dependent on PKA activation induced by the Ca^{2+} and HCO_3^- dependent soluble adenylyl cyclase (Francou, Girela, De Juan, Ten, Bernabeu & De Juan, 2017). It has been suggested that Ca^{2+} is transported into the cell by the sperm-specific Ca^{2+} channel (CatSper) and HCO_3^- by the Na^+/HCO_3^- cotransporter (Esteves & Miyaoska, 2015). The slow events involve variations in the array of motion (Stival et al., 2016). Protein tyrosine phosphorylation is another milestone of capacitation, occurring in the last stages of capacitation at different intervals of cAMP/PKA activation (Francou et al., 2017). The beginning of the slow events of capacitation is marked by the removal of cholesterol from the membrane by bovine serum albumin and the increase in its fluidity (Gupta, 2015).

Following capacitation, the sperm undergoes AR (Figure 2.4). During fertilisation, the capacitated sperm fuse with the cumulus oophrous of the egg, which then binds with the zona pellucida (ZP) with its plasma membrane intact (Kunkitti et al., 2015). After binding with the ZP, the spermatozoa undergo an exocytotic procedure known as the AR (Kunkitti et al., 2015). This event is a prerequisite for conception because it allows passage of the spermatozoa via the ZP and its subsequent fusion with the egg oolemma (Megnagi, Finkelstein, Shabtay & Breitbart, 2015). The subsequent procedures include the ability to produce hyperactivated motility and exhibit chemotactic behaviour and the ability to fertilise an oocyte (Jin & Yang, 2016; Subramoniam, 2017).



(A) The acrosome reaction of capacitated spermatozoa occurs after binding to the zona pellucida. (B) Upon this event, the outer acrosomal membrane fuses with the plasma membrane, releasing the acrosome contents. (C) MCP is expressed on the IAM and thus is exposed following the acrosome reaction.

Figure 1.4: Schematic representation of the acrosome reaction of human spermatozoa

Source: Riley-Vargas, Lanzendorf & Atkinson, 2005

2.3.4 Reactive oxygen species and oxidative stress

Reactive oxygen species refers to the by-products of oxygen-free radicals produced by both endogenous and exogenous processes (Wu, 2015). The production of ROS by human spermatozoa contributes significantly to the aetiology of poor sperm production and function (Bui, Sharma, Henkel & Agarwal, 2018). In semen analysis, measurement of ROS production in human spermatozoa is a prerequisite for any fertility diagnosis (Wagner et al., 2017). Commonly known ROS affecting the male reproductive system include peroxyl (ROO−), hydroxyl (OH^-) radicals, superoxide (O^{2-}) anions and H_2O_2

(Homa, Vessey, Perez-Miranda, Riyait & Agarwal, 2015). Although they are not recognised as ROS, nitrogen compounds such as nitric oxide (NO) and peroxynitrite anion ($ONOO^-$) also appear to play a crucial role in oxidation-reduction reactions in infertility; these are then referred to as nitrogen reactive species (Homa et al., 2015).

The principal ROS that is excessively generated by human spermatozoa is O^{2-}, which is a common by-product of oxidative phosphorylation during cell respiration in the mitochondria (Agarwal et al., 2017). The superoxide anion (O^{2-}) reacts with itself in a dismutation reaction to synthesise hydrogen peroxide (H_2O_2) and an oxygen atom (Homa, Vessey, Perez-Miranda, Riyait & Agarwal, 2015). The O^{2-} can synthesise the hydroxyl radical (OH) through the metal-catalysed Haber-Weiss reaction or the Fenton reaction, whereby ferrous particles act as reducing agents in the genesis of the OH from H_2O_2 (Zorov et al., 2014). A highly reactive OH is responsible for the initiation of the LPO process, which consequently leads to loss of sperm function (Durairajanayagam, 2019). Nitric oxide is synthesised in a redox-reaction between L-arginine and oxygen, which forms L-citrulline as a by-product (Durairajanayagam, 2019). This reaction is initiated by the reduced form of nicotinamide adenine dinucleotide phosphate and catalysed by the enzyme nitric oxide synthase (Meccariello & Chianese, 2018). Nitric oxide reacts with the O^{2-} to generate a profoundly harmful peroxynitrite anion (De Jonge, 2018).

The reaction between O^{2-} and NO occurs multiple times faster than the dismutation of superoxide catalysed by the enzyme superoxide dismutase (SOD) (Bartesaghi & Radi, 2018). As the NO concentration reaches the nanomolar interval and estimated SOD concentration in the tissue, competition results between NO and SOD for the expulsion of O^{2-} by synthesising peroxynitrite (Doshi, Sharma & Agarwal, 2015). Aggregation of reactive nitrogen species (RNS) adds to a condition of nitrosative pressure, which is harmful to the male reproductive system (Drzeżdżon, Jacewicz & Chmurzyński, 2018). Furthermore, free radicals trigger the oxidation of biomolecules that include sulfhydryl, protein, nucleic acids and unsaturated fatty acids (Phaniendra, Jestadi & Periyasamy, 2015). Oxidative damage is the principal cause of inflammation, carcinogenesis and other pathological processes (Roychoudhury, Agarwal, Virk & Cho, 2017).

Oxidative stress can emerge intrinsically from the sperm itself and from an assortment of conditions that are known to affect a male's potential to achieve pregnancy (Hosen, Islam, Begum, Kabir & Howlader, 2015). These conditions can extend from inherent variations to the norm of natural exposures (Hosen et al., 2015). Although ROS are extremely reactive compounds that contribute significantly to the oxidative damage of cells including spermatozoa, they are also important for normal sperm function (Agarwal et al., 2019). In physiological terms, ROS are important for the normal functioning of delicate semen parameters such as sperm capacitation, hyperactivation, AR and fertilization of the oocyte (Bui et al., 2018). In supra-physiological terms, extrinsic ROS are extremely detrimental to human sperm motility, while intrinsic ROS affect the DNA fragmentation of the sperm (Sabeti Pourmasumi, Rahiminia, Akyash & Talebi, 2016). See Figure 2.5.

Studies have revealed that sperm DNA damage may cause embryonic death and health complications in progeny (Agarwal, Mulgund et al., 2014; Ko, Sabanegh & Agarwal, 2014). The absence of a cytoplasmic defence system causes spermatozoa to become more vulnerable to OS (Aitken, Gibb, Baker, Drevet & Gharagozloo, 2016). The potential impact of OS as a major contributor to sperm damage was raised after observing plummeting motility of human spermatozoa treated with high levels of oxygen tension (MacLeod, 1943, cited in Aitken et al., 2016). Furthermore, Aitken (2017) demonstrated a significant correlation between LPO and OS as a major contributor to the aetiologies of infertility in males. Numerous research studies have indeed confirmed the association between ROS, loss of motility and DNA damage (Agarwal, Mulgund et al., 2014; Wagner et al., 2017; Zandieh et al., 2018). Spermatozoa are extremely sensitive to oxidative damage by LPO due to the high content of polyunsaturated fatty acids such as docosahexaenoic acid in their membranes (Van Tran, Malla, Kumar & Tyagi, 2017). Additionally, ROS reduce intracellular ATP, which triggers a decrease in axonemal protein phosphorylation (Van Tran et al., 2017). The disfigurement in the membrane caused by LPO induced by ROS is demonstrated by an elevated level of the by-product malondialdehyde in the semen (Agarwal, Virk, Ong & du Plessis, 2014). Membrane LPO has been associated with midpiece morphological defects affecting the MMP of the sperm (Agarwal, Virk et al., 2014).

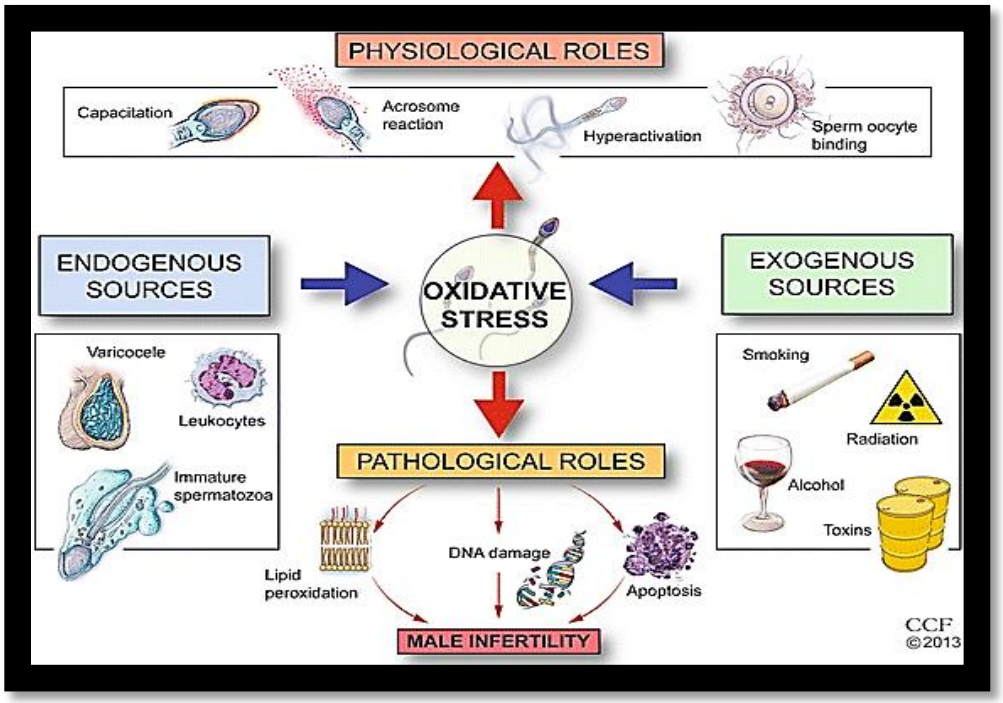


Figure 1.5: Scheme signifying the interacting mechanisms in the role of oxidative stress affecting sperm function and fertility.

Source: Themes, 2017

2.4 ANTIOXIDANTS

An antioxidant refers to a substance that can trap free radicals before oxidative mutilation can occur (Canda et al., 2014). Antioxidants act as the first line of defence, scavenging or detoxifying the damaging effect caused by ROS and OS (Walczak-Jedrzejowska et al., 2013). Antioxidants can be further classified into two utilitarian categories, enzymatic and non-enzymatic antioxidants and can be characterised by their water or lipid dissolvability (Zhang, Shen, Zhu & Xu, 2015). An increased level of ROS has the ability to deplete the total antioxidant activity, which in turn causes OS, triggering lipid, protein and DNA damage and thereby ravaging sperm cell integrity (Walczak-Jedrzejowska et al., 2013). Consequently, an antioxidant defence mechanism against the damaging effects of ROS is produced in the seminal fluids (Agarwal, Virk et al., 2014). Whether enzymatic or

non-enzymatic, the antioxidant defence mechanism maintains an equilibrium between the production and the metabolism of ROS and OS (Martin-Hidalgo, Bragado, Batista, Oliveira & Alves, 2019). Non-enzymatic antioxidants include vitamins C and E, hypotaurine, taurine, L-carnitine and lycopene (Martin-Hidalgo et al., 2019). The enzymatic antioxidants include SOD, catalases, glutathione peroxidase (GP_X), glucose-6-phosphate dehydrogenase and glutathione (GSH) (Tan, Norhaizan, Liew, Sulaiman & Rahman, 2018). This antioxidant defence system contributes largely to the scavenging of ROS productions, thereby plummeting chances of abnormal spermatogenesis (Martin-Hidalgo et al., 2019). Studies have demonstrated that dietary antioxidants have the ability to improve semen quality and improve the chances of successful assisted reproductive techniques in infertile patients (Collins & Rossi, 2015).

Spermatozoa are defenceless against ROS due to the intrinsic absence of intracellular antioxidant enzymes; therefore, total body antioxidant capacity becomes more significant in protecting sperm cells against the damaging effect of ROS (Ko et al., 2014). Antioxidants are perceived to be the most vital defence agents against free radicals and are essential to revive various sperm parameters (Tan et al., 2018). The endogenous application of enzymatic antioxidants increases the chances of fertility through reviving sperm motility, viability, hyperactivation and AR (Martin-Hidalgo et al., 2019). In gonads and seminal fluid, the scavenging process is regulated by antioxidants such as SOD, catalase (CAT), GSH and the glutathione peroxidase-reductase system (Dare, Oyeniyi & Olaniyan, 2014).

In the scope of human reproduction, seminal plasma is said to be the most abundant source of the antioxidants α-tocopherol, vitamin C, uric acid, CAT and SOD (Dare et al., 2014). Studies have validated that GPx and SOD can be found in the epididymis, while the GPx pair and SOD are situated within the cytoplasm of the midpiece of the sperm cell (Atukeren, 2018). However, the level of antioxidants in spermatozoa are not adequate to offer complete protection against the scavenging activities of ROS (Atukeren, 2018). Furthermore, infertile males have been shown to demonstrate an absence or lower levels of antioxidants such as GSH in their seminal plasma than fertile males (Martin-Hidalgo et al., 2019). However, it is also important to note that seminal plasma supplemented with

antioxidants is generally castoff during the cryopreservation procedure (Kurutas, 2016). *In vitro* and *in vivo* studies have been conducted using antioxidants to lessen the lethal effects of ROS and LPO on the physiological function of spermatozoa (Gadella & Luna, 2014). Studies revealed that oral prescriptions or food with antioxidants such as GSH, cysteine, vitamin E and vitamin C have positive effects on semen quality in humans (Gharagozloo & Aitken, 2011), in boar (Bathgate, 2011), in bulls (Büyükleblebici, Tuncer, Bucak, Eken & Sarıözkan, 2014), in stallions (Gibb, Butler, Morris, Maxwell & Grupen, 2013) and in dogs (Koziorowska-Gilun & Strzeżek, 2011). However, oral supplementation with vitamin E did not improve the human sperm quality in fresh semen (Zhandi, Ansari, Roknabadi, Shahneh & Sharafi, 2017). Thus, the effect of antioxidants on sperm quality is still inconsistent and necessitates further investigation (Ahmadi et al., 2016).

2.5 NATURAL ANTIOXIDANTS

Natural antioxidants are produced in the human body through metabolic processes or are derived from natural sources (Adewoyin et al., 2017). Their mode of action depends on their physical and chemical properties and components of bioactivity (Rahman et al., 2018). Natural antioxidants can be classified into two categories, enzymatic antioxidants and non-enzymatic antioxidants (Adewoyin et al., 2017).

2.5.1 Enzymatic antioxidant defence system

An enzymatic antioxidant refers to an antioxidant defence system that is responsible for detoxifying ROS within the seminal fluids and the gonads (Ahmed, 2016). These antioxidants include SOD, GPx and CAT (Ighodaro & Akinloye, 2018).

2.5.1.1 Glutathione peroxide

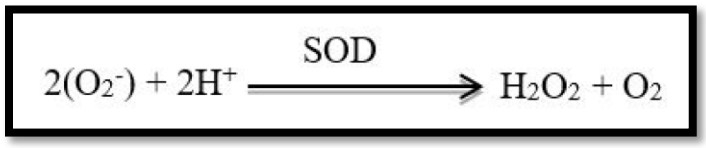
Glutathione peroxidase is a selenium-containing antioxidative enzyme that is found in the cytoplasm and the mitochondria of the cell (Ighodaro & Akinloye, 2018). Glutathione peroxide exists in two forms: selenium-independent enzymes and selenium-dependent enzymes (Ighodaro & Akinloye, 2018). Glutathione peroxide confiscates $2H_2O$ by coupling its reduction to HO with oxidation of GSH (Ahmadi et al., 2016). The reaction is as follows:

Glutathione, a thiol-containing tripeptide, is found in two forms: reduced (GSH) and oxidised (GSSG) (Ulrich & Jakob, 2019). Glutathione is said to be a potent antioxidant in the overall cellular defence mechanism against the ravaging activity of ROS (Kurutas, 2016). Glutathione scavenges the free radicals and peroxides produced during normal cellular respiration that cause oxidative damage to lipids, proteins and nucleic acids(Jat and Nahar, 2017). Additionally, GPx protects the mammalian spermatozoa against oxidative and sperm DNA damage (Aitken & Koppers, 2011).

2.5.1.2 Superoxide dismutase

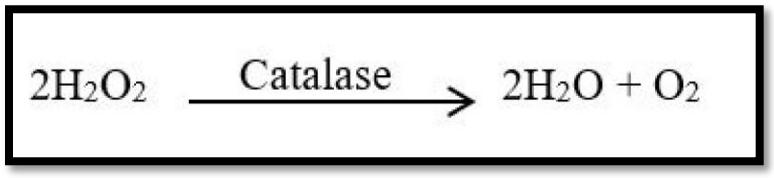
Superoxide dismutase acts as a first-line defence mechanism against OS in living cells (Ighodaro & Akinloye, 2018). First-line defence mechanisms play a crucial role in controlling the formation of ROS (Lee et al., 2018). Superoxide dismutase is present in the prostate gland and seminal vesicles (Wang, Branicky, Noë & Hekimi, 2018). Moreover, due to the scavenging property of SOD, this enzyme is said to work efficiently with CAT and GPx (Durairajanayagam, 2019). Superoxide dismutase exists in three forms: SOD-1, SOD-2 and SOD-3 (Mao, Yuan, Lv, GAO & Yin, 2019). The first intracellular form, SOD-1, is situated in the cytoplasm and contains copper and zinc in the active centre (Mao et al., 2019). The second intracellular form, SOD-2, is found within the

mitochondria, with manganese in the active centre (Mao et al., 2019). Lastly, the third form, SOD-3, is extracellular. This form functions in the extracellular space and is associated with the surface polysaccharide 2 (Lubrano & Balzan, 2015). Furthermore, Ighodaro and Akinloye (2018) demonstrated that SOD maintains the physiology of the sperm by reducing the formation of ROS and free radicals. The dismutation reaction is as follows:



2.5.1.3 Catalase

Catalase is an enzymatic antioxidant present in humans and rabbits. It functions efficiently with other biological enzymatic antioxidants such as SOD (Lubrano & Balzan, 2015). While SOD constitutes the first-line defence mechanism by converting O_2 to $2H_2O$, CAT is the $2H_2O$ detoxifier (Ighodaro & Akinloye, 2018). The reaction is as follows:



2.5.2 Non-enzymatic antioxidant

Non-enzymatic antioxidants are nourishing antioxidants and include vitamin C and vitamin E (Puthur, 2016). Non-enzymatic antioxidants play a crucial role in maintaining sperm physiology fertilisation (Ulrich & Jakob, 2019). *In vivo* studies have shown that non-enzymatic antioxidants play a crucial role in maintaining sperm physiology (Amidi, Pazhohan, Shabani Nashtaei, Khodarahmian & Nekoonam, 2016; Khazaei & Aghaz,

2017; Smith & Swart, 2016). In addition, a well-balanced diet is a key element in enhancing male fertility potential (Puthur, 2016).

2.5.2.1 Vitamin C and vitamin E

Vitamin C is regarded as the most vital water-dissolvable antioxidant existing in extracellular liquids (Smith & Swart, 2016). Vitamin C is found in fruits such as kiwi, papaya, mango and strawberries (de Oliveira, Rosa, Simões-Ambrósio, Jordao & Deminice, 2019). The antioxidant property of vitamin C is capable of hindering the formation of ROS such as H_2O_2, singlet oxygen and 0^{2-} (Yadav & Sharma, 2016). In addition, vitamin C inhibits the damaging effect of ROS before LPO is activated, thereby protecting the sperm against sperm DNA damage. This is achieved by reducing the oxidised tocopherol and shielding the human sperm cells from oxidative harm by invalidating the impact of hydroxyl (Adewoyin et al., 2017). Studies have proved that semen with excess ROS have low levels of vitamin C (Jat & Nahar, 2017). Vitamin E is the principal lipid-soluble antioxidant and is equipped with a shielding layer of unsaturated fat (Raederstorff, Wyss, Calder, Weber & Eggersdorfer, 2015). Vitamin E consists of tocopherols and tocotrienols. The tocopherols found in vitamin E are classified as α, β, γ (Raederstorff et al., 2015). Sources of vitamin E include wheat germ, avocados, palm oil and vegetable oil (Gan, Chan, Yang, Li &, Zhang, 2019). During LPO, ROS reacts with α-tocopherol to form lipid radicals that are responsible for protecting the cell membrane against oxidation (Gan et al., 2019). A combination of both vitamin C and vitamin E was shown to shield spermatozoa against DNA fragmentation and peroxidative attack (Mao et al., 2019).

2.5.2.2 Carotenoids

Carotenoids are non-enzymatic antioxidants found in both fruits and vegetables. Because of their pigment property in plants, carotenoids are crucial in the process of photosynthesis and light regulation (Saini, Nile & Park, 2015). Studies have also

demonstrated that carotenoids can treat OS and suppress the formation of ROS (Metcalfe & Alonso-Alvarez, 2010). The most important compound found in the carotenoids family is lycopene (Saini et al., 2015). Studies have demonstrated high concentrations of lycopene within the testes, thus maintaining the process of spermatogenesis due to its antioxidant properties (Jat & Nahar, 2017; Lubrano & Balzan, 2015; Ulrich & Jakob, 2019). Studies have also demonstrated that lycopene is capable of improving sperm motility (Agarwal, Virk et al., 2014; Ahmadi et al., 2016; Majzoub et al., 2018).

2.6 HISTORY OF ROOIBOS

The history of rooibos known as red bush or red tea is established in the Cederberg district of the Western Cape in South Africa. Rooibos is a highly variable shrub or shrublet up to 2 m high, Its young branches are often reddish. The leaves are green and needle-like, 15-60 mm long and up to about 1 mm thick (Van Wyk & Wink, 2018). They are without stalks and stipules and may be densely clustered. The yellow flowers, which appear in spring to early summer, are solitary or arranged in dense groups at the tips of branches (Van Wyk & Wink, 2018).The fruit is a small lance-shaped pod usually containing one or two hard seeds.The Khoisan, the indigenous Bushmen of the district, have been gathering the leaves from the rooibos plant for hundreds of years (Van Wyk & Wink, 2018). The leaves are used as natural solutions for different ailments and are cherished for their flavourful taste (Van Wyk & Wink, 2018). The history of rooibos tea ended with the diminishing of the Khoisan clans but fortunately, a botanist named Carl Humberg rediscovered the leaves in 1772 and progressively resuscitated enthusiasm for the tea across the boarder (Ross, 2008). In 1904, Benjamin Ginsberg, a Russian migrant with connections to tea production began showcasing the beverage as a ‘Mountain Tea’, a herbal option to black tea (Patrickson, Malgas & Oettle, 2008). During World War II when bringing in tea from Asian nations was almost inconceivable, rooibos tea became a popular option and was transported nationally and internationally (Morton, 1983). However, rooibos retailers encountered difficulties because of the high cost of rooibos seeds due to the scarcity of the plant (Morton, 1983). Given the high cost value, rooibos

tea was thus difficult to purchase until the late 1960s when the rooibos tea history took an unexpected turn (Flack, 2018). In 1968, Annique Theron (a South African woman) distributed a book on the astonishing medical advantages of rooibos tea (Gorelik, 2017). *In vitro* and *in vivo* research studies have demonstrated the beneficial effects of rooibos (Kwak, Han & Bae, 2015; Mabhida et al., 2018; Sasaki, Nishida & Shimada, 2018); however, more scientific research is needed to validate the claim. Interest in rooibos tea has increased by more than 50% in the past few years (Flack, 2018). Rooibos tea is now the most famous tea in African, Asian and European nations (Barham & Sylvander, 2011).

2.6.1 ROOIBOS PLANT

Rooibos plant belongs to kingdom *plantae*,*Fabaceae* family,oder of *Fabales,genus Aspalathus linearis and species name A.linearis*, this plant comprises 278 different species (Andrews & Andrews, 2017). The rooibos tea, is a bush-like leguminous plant (Figure 2.6) that is prevalent in the Cederberg Mountains and neighbouring regions in the Western Cape province of South Africa (Ajuwon, Marnewick & Davids, 2015). Rooibos is cultivated in this region as a herbal tea for commercial purposes (Ajuwon, Marnewick & Davids, 2015). The rooibos plant has a solid taproot measuring approximately two metres, dark rose coloured branches and bright green, needle-like leaves (Ajuwon et al., 2015). Rooibos can be categorised further into four types, scarlet, grey, black and red-brown (Stander, Van Wyk, Taylor & Long, 2017). The scarlet type, can further be categorised into the Cederberg categories of Nortier and the Cedarberg type (wild growing) (Stander et al., 2017). The grey and black types are not processed because of their unpleasant flavour and the quality of the tea harvested (Nash & Ward, 2016).With reference to the past, rooibos was discovered by the Cape Khoi clan and has been used for approximately 230 years (Human, 2017). Commercially, rooibos production has grown from 524 tonnes in 2003 to 10 600 tonnes at present (Human, 2017). Rooibos tea is perceived to have numerous health benefits such as improving skin, treating hair, controlling mental conditions, aiding diabetes and obesity, and improving libido and other chronic and infectious diseases (Malongane, McGaw & Mudau, 2017; Sanderson et al., 2014; Sasaki et al., 2018; Smith & Swart, 2016).

A: leaves of *Aspalathus linearis*

B: flowers of *Aspathus linearis*

Figure 1.6: Rooibos plant

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https://www.inaturalist.org/photos/15225302

Aspalathus linearis could be used as fermented or unfermented (Figure 2.7).Villaño and colleagues (2010) demonstrated that unfermented rooibos contains high levels of antioxidants compared with fermented rooibos. Using different assays to determine the phenolic and flavanone content in rooibos plant (Nash and Ward, 2016). Barek and colleagues (2015) discovered that the total phenolic and flavanone content was higher in the unfermented extract than in the fermented extract after both long and short infusion times (10–20 minutes and 5–15 minutes respectively). In addition, the unfermented rooibos (green rooibos) extracts contained higher quantities of aspalathin than the fermented rooibos (Smith and Swart, 2018). Research studies indicate that aspalathin glycosides constitute about 4-12% of unfermented rooibos. Approximately 43% of the total antioxidant activity in green rooibos (aqueous extracts) is triggered by aspalathin (Joubert et al., 2005).

Figure 1.7: Portraits of (A) fermented and (B) unfermented rooibos

Available at

(A): https://www.eldon.com.my/r101; (B): https://www.ambeans.com/products/green-rooibos-loose-leaf-tea

2.6.2 Active ingredients and chemical constituents

Rooibos tea is a caffeine-free drink that has a low concentration of tannins and is said to be beneficial for pregnant women, infants and people who are caffeine sensitive (Joubert, Jolley, Koch, Muller, Van der Rijst & de Beer, 2016). The increase in rooibos preference is due to its taste, affordability and health benefits (Joubert et al., 2016). Some of the most important compounds found within rooibos include cyclic dihydrochalcone (aspalalinin), flavonols (quercetin, isoquercetin, hyperoside, rutin and quercetin-3-0-β-D-robinoside), monomeric flavan-3-ol (+)-catechin), dihydrochalcone (aspalathin and notofagin), oligomeric flavan-3-ol (procyanidin B3), luteolin-7-0-glucoside and chrysoeriol), flavanones (dihydro-orientin, dihydro-iso-orientin and hemiphlorin) and flavones (orientin, iso-oorientin, vitexin, isovitexin, luteolin) (Canda et al., 2014; Van der Merwe, de Beer, Joubert & Gelderblom, 2015). See Figure 2.8.

Rooibos is unique in its monomeric flavonoid composition; it contains two unique compounds, aspalathin and aspalalinin (Ajuwon et al., 2015). Aspalathin is a C-linked dihydrochalcone glucoside and aspalalinin is a cyclic dihydrochalcone (Ajuwon et al., 2015). The chemical assembly of aspalathin is distinctive due to the deficiency of the O-glycosidic bond, which attaches a sole sugar moiety to an infinite amount of flavonoids (Canda et al., 2014). Compared with fermented rooibos, unfermented rooibos possesses a higher level of aspalathin (Canda et al., 2014; Kwak et al., 2015).

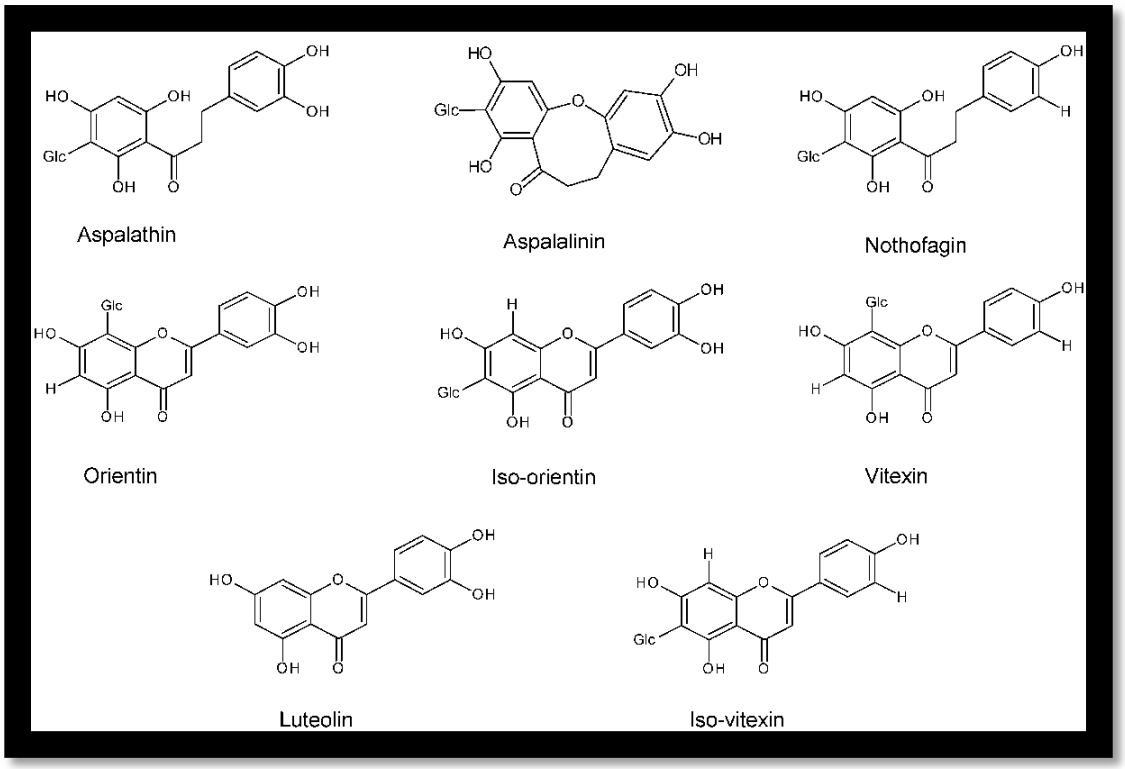


Figure 1.8: Major phenolic compounds found in rooibos

Source: Ajuwon et al., 2015

2.6.3 Antioxidant activities of rooibos on male reproduction

Antioxidants such as polyphenols, flavonoids and phenolic acids are capable of scavenging free radicals, which then hinder the oxidative cellular damage (Nash & Ward, 2016). In another study by Gadow, Joubert & Hansmann, (1997) ; Marnewick,

Gelderblom, & Joubert, (2000), the antioxidant activity within rooibos was determined using the α, α-diphenyl-β-picrylhydrazyl (DPPH) free radical scavenging method, which revealed unfermented rooibos to have the highest level of antioxidant when compared with fermented and semi-fermented rooibos (unfermented rooibos > fermented rooibos > semifermented rooibos). The concentration of soluble solids in unfermented rooibos was higher than the fermented extract; however, the concentration of solids in the unfermented tea was still lower than that in the black and green teas (Gadow et al., 1997; Marnewick et al., 2000). Marnewick and his colleagues (2000) also demonstrated that unfermented rooibos contains a high amount of polyphenols, flavonoids and nonflavonoids in comparison with fermented rooibos extract. Furthermore, Barek and colleagues (2015) mentioned that the total phenolic and flavanone content of rooibos was higher in the unfermented extract than the fermented extract.

An *in vivo* study in rats determined that unfermented rooibos significantly improved sperm concentration, viability and motility while fermented rooibos improved sperm vitality (Awoniyi et al., 2012). In this *in vivo* study, Awoniyi et al. (2012) also demonstrated that unfermented and fermented rooibos extracts improved sperm quality, protected the sperm cells against induced OS and increased antioxidant enzymes. However, consumption of rooibos may result in an early AR of the spermatozoa, which may impair fertility chances (Opuwari & Monsees, 2014). Although acrosin disrupted rats were shown to be fertile with reduced litter size, their spermatozoa possessed equal ability to penetrate the ZP, the dispersal of cumulus oophorus cells was, however, shown to be slower (Isotani et al., 2017).

In an *in vivo* study, Awoniyi et al. (2011) investigated the modulation of OS by the ethnic herbal tea, rooibos, a Chinese green tea, commercial rooibos and green tea supplements in rat testicular tissue. Their results showed that both fermented rooibos and green tea extracts are capable of protecting testicular tissue against oxidative impairment through the increment of antioxidant defence mechanisms in rats together with plummeting LPO. Noh and colleague (2012) investigated the effect of CRS-10 (dandelion and rooibos extract complex) on andropause symptoms. It was concluded that daily consumption of

CRS-10 improved the quality of life amongst ageing male respondents. The results unveiled the potential of CRS-10 as a harmless and effective natural substance for combating andropause symptoms (Noh et al., 2012).

CHAPTER THREE: RESEARCH METHODOLOGY

3.1 INTRODUCTION

The purpose of Chapter Three is to describe the methodology that was used in the study. This chapter includes details of the research methods, research design, sampling, inclusion criteria, study site, data collection, ethical considerations and data analysis.

3.2 RESEARCH METHOD

This study employed a quantitative and experimental design.

3.2.1 Experimental design

Experimental research design is research carried out with a scientific approach in which a number of variables are kept constant while another set of variables is considered the subject being measured by the experimental procedure (Opie & Brown, 2019). Experimental design is useful when scientists do not have adequate data to motivate and substantiate an idea or conclusion (Johnson & Christensen, 2019). Under such circumstances, several tests need to be conducted to validate the proposed idea. This study is experimental since various concentrations of rooibos were exposed to human sperm to test the effects of rooibos on seminal parameters according to the objectives of the study.

3.2.2 Quantitative design

The term ‘health research’, also known as ‘medical examination’ refers to a research procedure that is carried out to learn more about human health. Health research also aims to find better ways to prevent and treat different illnesses and conditions. Quantitative health research is characterized as a systematic examination of a series of

events by accumulating quantifiable information and performing measurable, numerical or computational procedures (Green & Thorogood, 2018). Quantitative research in health sciences strives to produce useful, factual and reliable data sets. After the effects of administration of a drug or extract have been verified on a sample population, the statistical record of the pragmatic results will deliver objective outcomes that can be generalized to a larger population (Goertzen, 2017). The statistical approaches related to quantitative research are suitable for maximizing dependent variables based on the independents, thus transforming into a competence for recognizing and applying mediations that may broaden the quality and quantity of life for a patient (Devi, 2017). This study recruited 50 infertile patients screened and confirmed to be infertile by Obstetrics and gynaecology department within Tygerberg Hospital and Vincent Pallotti u Hospital and 50 screened donors recruited by the medical bioscience, andrology laboratory within the Western University to determine different seminal parameters.

3.3 RESEARCH EXPERIMENTAL DESIGN

3.3.1 Research setting

The study was conducted in the Department of Medical Biosciences, University of the Western Cape, Western Cape Province, South Africa. The Western Cape, known as *Wes-Kaap*, is a South African province situated on the south-western coast of the country covering 129 449 km^2 (Otto, 2018). In 2017, the Western Cape Province was ranked as the third-most populated province with 6.5 million inhabitants (Otto, 2018). Semen samples used for the experiment were obtained from two reputable hospitals situated in the Western Cape, namely Tygerberg Hospital and Vincent Pallotti Hospital (Figure 3.1). Tygerberg Hospital is a well-known academic hospital situated in Bellville, Cape Town (Miseer, Mann & Davis, 2019). This academic hospital opened in 1976 and is considered the second-largest operating hospital in the Western Cape. Tygerberg Hospital is in partnership with the Health Science Faculty of Stellenbosch University as a teaching

health facility for academic and research purposes. Vincent Pallotti Hospital, regarded the most-established reputable hospital with clinical excellence, is situated in Pinelands, Cape Town and deals with assisted reproduction at its Aevitas Fertility Clinic for couples seeking fertility solutions (McMillan, 2016).

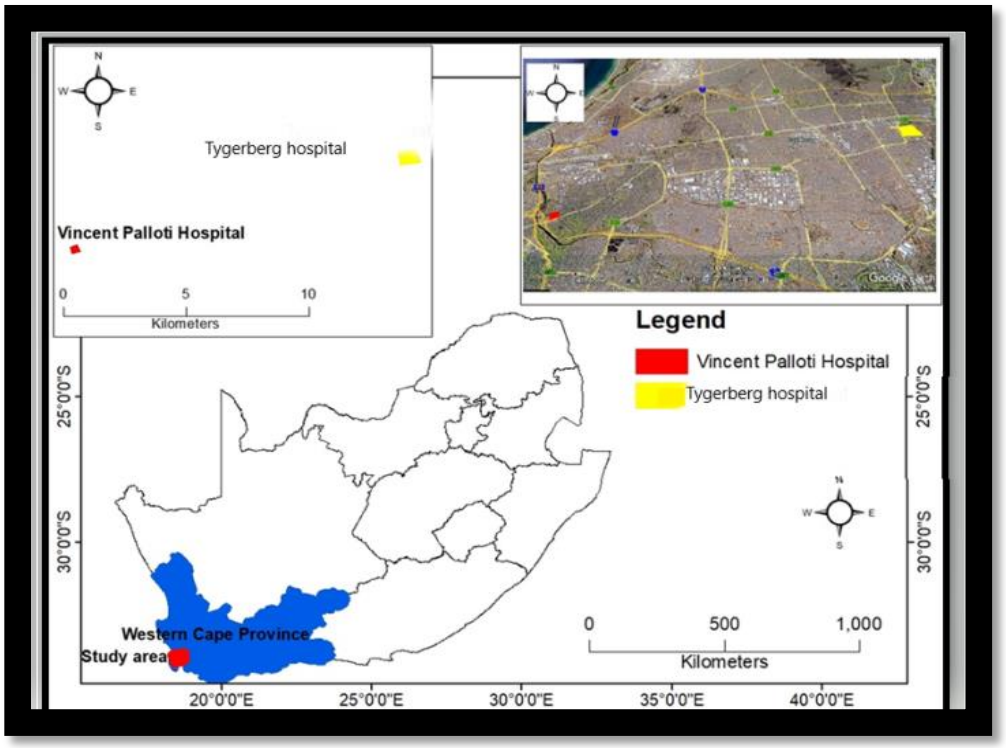


Figure 2.1: South African map showing the study area

3.3.2 Sampling

3.3.2.1 Population

Population sampling refers to the method of selecting a subset of subjects that are representative of an entire population (Fain, 2017). The target population in this study comprised infertile males attending infertility clinics at either Tygerberg Hospital or Vincent Pallotti Hospital and donors within the Western Cape province. Human semen

was obtained from 100 men, encompassing 50 healthy volunteers and 50 infertile patients between the ages of 18 years and 45 years from both the donor and the patient groups. Semen samples with a concentration of ≤15 million sperm/ml (oligozoospermia) or <32% progressive motility or <40% sperm/ml total motility (asthenozoospermia) (WHO, 2010) were identified and investigated separately. The former were taking part in a semen donor programme at the Andrology Research Laboratory in the Department of Medical Biosciences, University of the Western Cape. The latter were attending either the Reproductive Biology Unit, Department of Obstetrics and Gynaecology, Stellenbosch University at Tygerberg Hospital or the Vincent Pallotti Hospital for assisted reproductive treatment or fertility problems (Shalaweh, Bouic, Weitz & Henkel, 2015). The sample was obtained by convenience sampling. Convenience sampling referes to non-probability sampling that includes samples being drawn from that part of the population that is close to hand.

3.3.2.2 Sampling method

The Cochran formula was used to determine the sample size required for this study (Cochran, 1977).

$$n_0 = \frac{Z^2 pq}{e^2}$$

Where:

- **n_0** is the sample size
- **Z** is the Z value for the desired level of confidence
- **p** is the estimated proportion of the population that has the attribute in question
- **q** is 1-p
- **e** is the desired level of precision (margin of error)

$$n_0 = \frac{(1.65)^2 (0.25) (1-0.25)}{(0.1)^2}$$

$$n_0 = 50$$

3.3.2.3 Source and preparation of aqueous extraxt of rooibos tea

Dried rooibos (Five RosesTM) was purchased from a retail store (Limpopo, South Africa). Aqueous extract (2%) were prepared by infusing 20 g of fermented rooibos tea or 20 g of unfermented rooibos tea each, in 1 litre distilled water for five minutes with continuous stirring. The teas were initially filtered through a layer of cheesecloth, followed by a Whatman No. 4 filter paper and thereafter a Whatman No (Whatman, Madestone, England). 1 filter paper. The filtrates were stored in a light-limiting cold room. This limited the effect of light exposure on the polyphenols in the tea since light exposure is said to cause a decrease in polyphenol levels (Qian, Liu, Deng, Miao & Cai, 2016). Thereafter, the filtrates were freeze dried and stored in a cool (4^{o}C) and dry place until ready to use. The freeze-dried extracts were subsequently reconstituted in human tubular fluid (HTF) (Quinn, Kerin, & Warnes, 1985), supplemented with 1% bovine serum albumin (Sigma Aldrich ,St Louis, MO, USA) (HTF-BSA) to final concentrations of 0.1 µg/mL, 1.0 µg/mL, 10 µg/mL, 100 µg/mL and 0.15 µg/mL, 1.5 µg/mL, 15 µg/mL, 150 µg/mL for fermented and unfermented rooibos respectively and administered as per the recommended daily dose for an 80 kg individual.

Summary calculations of rooibos daily dosage for an 80 kg person

- ❖ Rooibos recommended dose = 6 cups/day
- ❖ 1 cup ≙ 150 mL
- ❖ Human weight = 80 kg
- ❖ Average extract yield of fermented rooibos = 0.85 g/L
- ❖ Average extract yield of unfermented rooibos = 1.34 g/L

Fermented

$\frac{0.85g}{1000ml} = \frac{x}{150ml}$

= 0.1275g x 6 cups/day

= 0.76 g

$= \frac{0.76g}{80\ 000ml} = \frac{x}{ml}$

= 0.0000095g/ml

= 0.0095 µg/ml

= 10 µg/ml

= (0.1, 1.0, 10, 100 µg/ml)

Unfermented

$= \frac{1.34g}{1000ml} = \frac{x}{150ml}$

= 0.201g x 6 cups/day

= 1.206g

$= \frac{1.206g}{80\ 000ml} = \frac{x}{ml}$

= 0.0000150g/ml

= 0.015 µg/ml

= (0.15, 1.5, 15, 150 µg/ml)

Inclusion and exclusion criteria

Before establishing eligibility for the study through inclusion and exclusion criteria, each semen sample was screened to determine the total sperm concentration, total motility and progressive motility.

- Inclusion criteria

Semen samples were collected from men aged between 18 years and 45 years from both the donor and the patient groups. Semen samples with a concentration of ≤15 million sperm/ml (oligozoospermia) or <32% progressive motility or <40% sperm/ml total motility (asthenozoospermia) (WHO, 2010) were identified and investigated separately.

- Exclusion criteria

Semen samples indicating azoospermia and semen samples with low volume (<1 ml) were excluded irrespective of the sperm concentration.The first 10 samples were discarded as bio-hazardous waste.

3.4 DATA COLLECTION

3.4.1 Source and preparation of human sample

Informed consent (appendices 1–3) was obtained from the infertile patients and screened donors. Human semen was collected from unproven healthy donors (n=50) and patients (n=50). The former were taking part in a semen donor programme at the Andrology Research Laboratory in the Department of Medical Biosciences, University of the Western Cape. The latter were attending either the Reproductive Biology Unit, Department of Obstetrics and Gynaecology, Stellenbosch University at Tygerberg Hospital or the Vincent Pallotti Hospital for assisted reproductive treatment or fertility problems. Semen samples from both donors and infertile patients were collected by masturbation into sterile vials following three to five days of sexual abstinence peding availability of the donor or patient. Thereafter, the available semen samples were incubated at 37ºC for 10–20 minutes to allow liquefaction within the first hour of acquisition pending availability. Thereafter, 2 µl of liquefied semen was placed in a warmed Leja slide and sperm

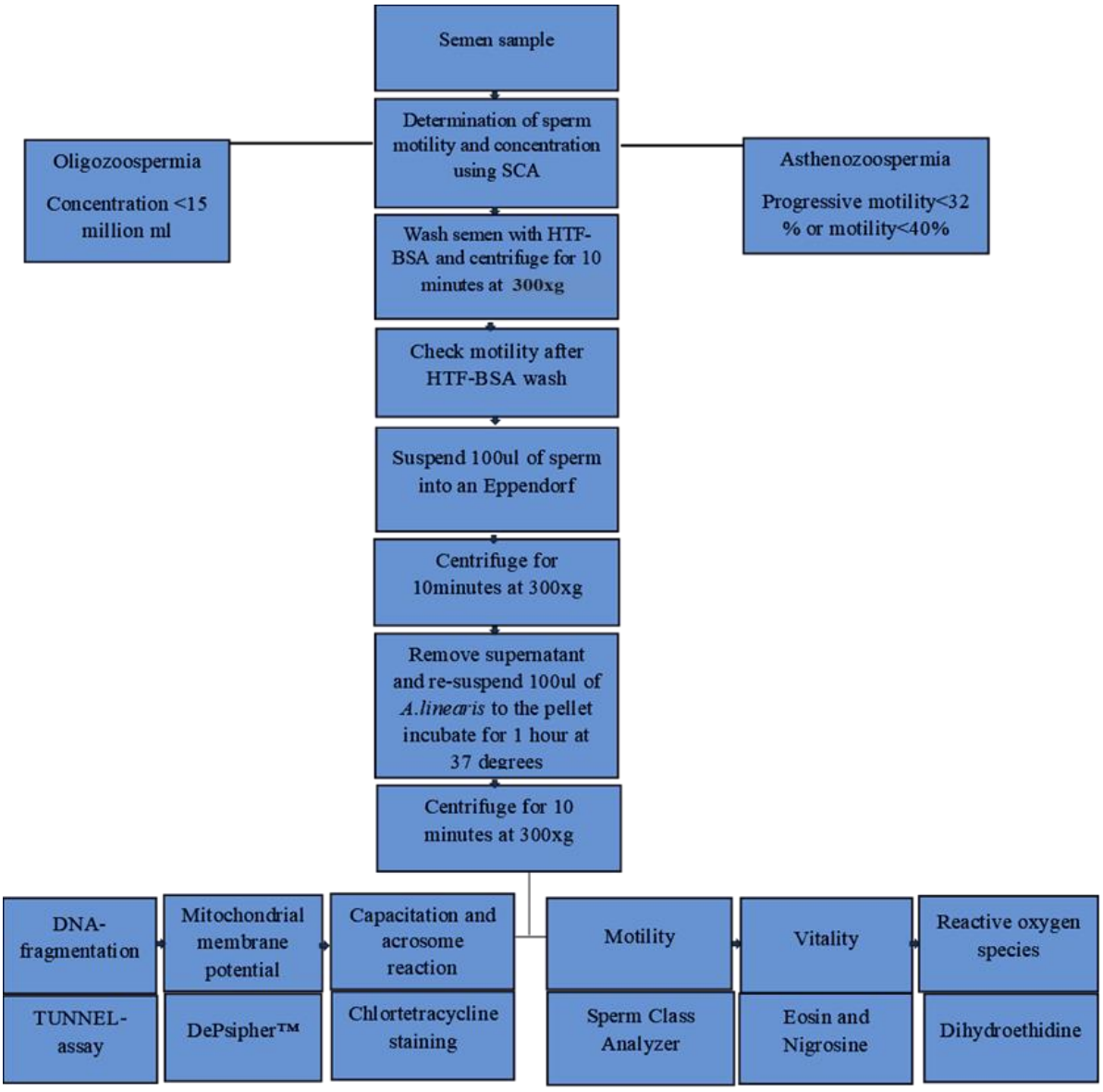
concentration and motility were determined using the Sperm Class Analyzer (Microptic, Barcelona, Spain).

3.4.2 Preparation of human tubular fluid medium

Quinn et al., (1985) described the synthetic HTF Medium as essential for the preparation and washing of spermatozoa because its ionic composition resembles that of natural HTF and thus delays cell death and enables optimum time for carrying out all sperm parameters. The synthetic HTF Medium was the preferred medium for washing spermatozoa in this study. The HTF Medium consists of the following substances, some of which mimic those found in the female fallopian tube: 101.60 mM NaCl, 4.69 mM KCl, 2.04 mM $CaCl_2$,$2H_2O$, 0.02 mM $MgSO_4$,$7H_2O$, 0.37 mM KH_2PO_4, phenol red (dye indicator), 25 mM $NaHCO_3$, 2.78 mM glucose (anhydrous), 0.33 mM sodium pyruvate, 21.40 mM sodium lactate (60% syrup), penicillin, streptomycin, 20 mM HEPES. The substances were dissolved in distilled water and once fully dissolved, osmolarity was adjusted to 280 mOsmol/kg. Finally, 10 mg/ml bovine serum albumin (BSA) was added before working with the medium (HTF-BSA). The medium was sterilised by filtration through a 0.22 µm filter (Merck Millipore, Tullagreen, Carrigtwohill, Ireland).

3.4.3 Experimental procedure for semen analysis.

Calculations were done to guarantee that uniform measurements of semen sample (7.5 million/ml) and HTF concentrations were maintained throughout the experimental study. The semen samples were diluted with HTF-BSA and centrifuged for 10 minutes at 300 xg (Labortechnik, Wehingen, Germany). The supernatants were discarded, and the pellets were re-suspended in HTF-BSA. The suspensions of spermatozoa in the HTF-BSA were incubated with different concentrations of fermented rooibos (0.10, 1.0, 10, 100 µg/ml) and unfermented rooibos (0.15, 1.5, 15, 150 µg/ml) for one hour at 37 ºC (Figure 3.2).



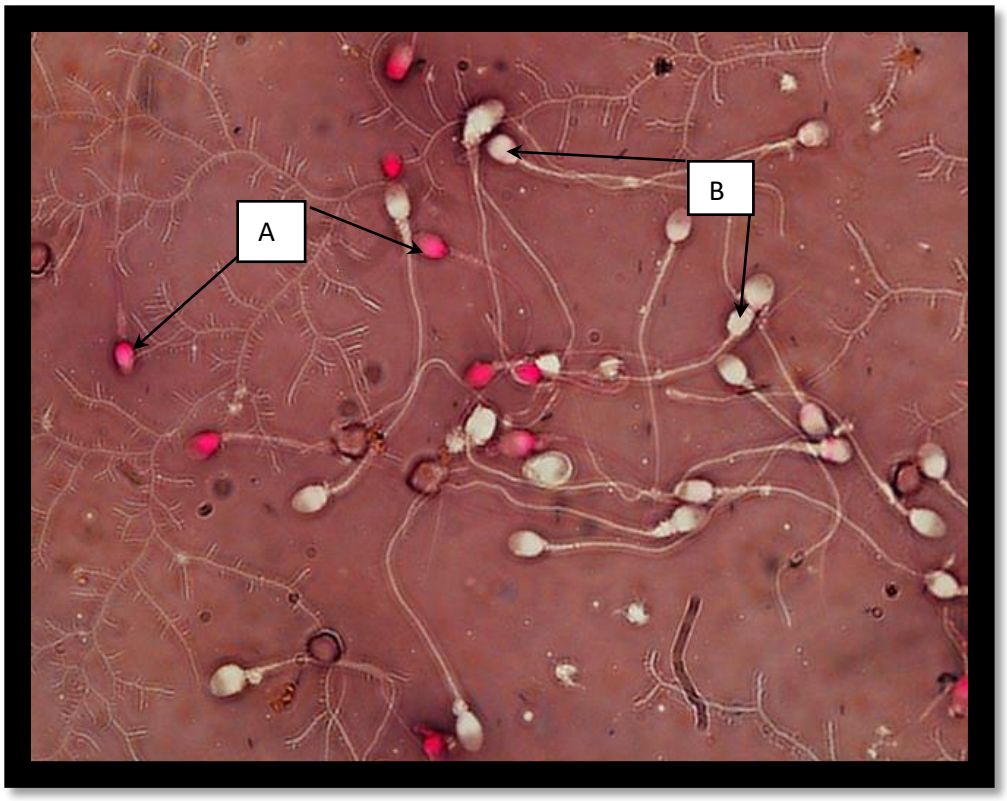
Study design for analyses of spermatozoa functionality, after incubation with different concentration of aqueous extract of fermented (0, 0.10, 1.0, 10, 100 µg/ml) and unfermented (0, 0.15, 1.5, 15, 150 µg/ml) rooibos extract. Abbreviations: CTC-chlortetracycline, SCA-Sperm Class Analyser, E+N-Eosin-Nigrosin, DHE-dihydroethidine, ROS-Reactive oxygen species, MMP-Mitochondrial membrane potential.

Figure 2.2: Experimental study design.

3.4.3.1 Determination of sperm vitality

The one-step eosin-nigrosin staining technique was used to determine viability according to the 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, 10g of nigrosin was added, and the solution was brought to the boil. The solution was filtered through filter paper in order to remove coarse and gelatinous precipitates. The filtered solution was stored in a dark glass bottle at room temperature (RT) until use.

After the one-hour incubation period with the different concentrations of rooibos tea at 37^{o}C, 50 µl of the sperm suspension was mixed with 50 µl of the eosin-nigrosin stain (1:1) in an Eppendorf vial. A smear was made on a glass slide and left to air dry. Slides were then viewed using a light microscope with a 100x oil immersion objective in the bright field. A total of 200 spermatozoa were counted, and the percentage of live sperm was calculated. Dead sperm appeared pink and live sperm white (Figure 3.3).



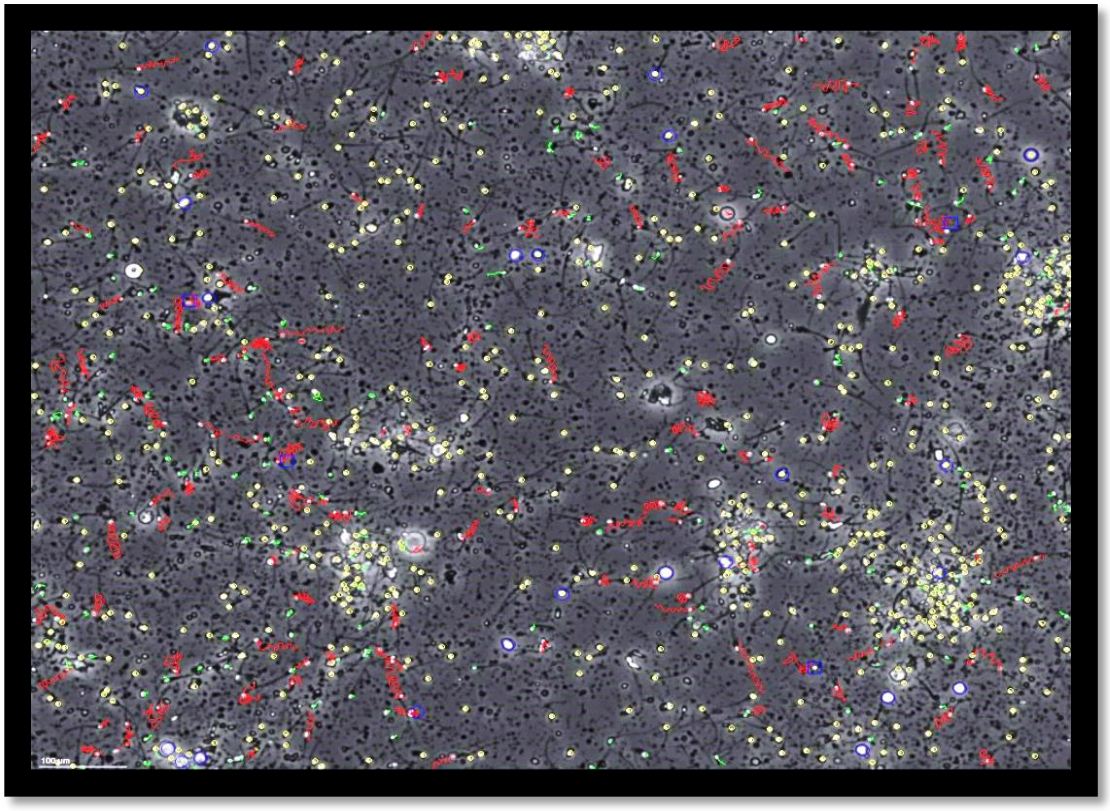
(A): Dead spermatozoa are stained pink; (B): live spermatozoa appear white/unstained (1000x magnification)

Figure 2.3: One-step eosin-nigrosin stain of human spermatozoa

3.4.3.2 Determination of sperm motility

Sperm motility was determined by means of Sperm Class Analyser® (SCA) version 5.0 computer-aided sperm analysis system version 4.1.0.1 (Microscopic S.L. Barcelona, Spain). After one-hour exposure with the different concentrations of rooibos tea at 37ºC, 2 µl of each sperm suspension was placed on a Leja slide. Motility of at least 200 spermatozoa was analysed at 37ºC according to the criteria set by the WHO (2010) with a Nikon Microscope (Nikon Instruments Inc., Tokyo, Japan) at 100x Phase Contrast 1. The following sperm kinematic parameters were analysed: total motility, progressive motility, immobility, velocity curve line (VCL), straight-line velocity (VSL), velocity average path (VAP), linearity (LIN), straightness (STR), beat cross frequency (BCF), amplitude of

lateral head displacement (ALH), wobble (WOB) and hyperactivation (Figure 3.4 and Table 3.1).



Red and green represent progressive motile sperm; blue represents non-progressive motile sperm; yellow represents immotile sperm

Figure 2.4: Determination of sperm concentration and motility

Table 2.1: Kinematic parameters observed using SCA software

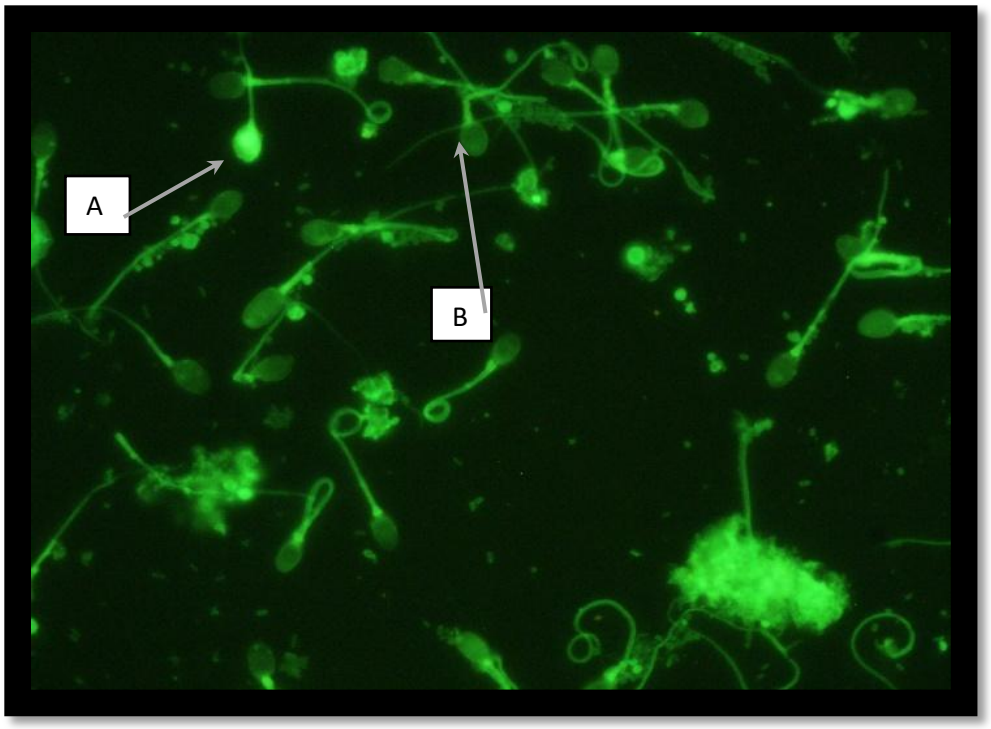
SPERM PARAMETER	MEASUREMENT OF MOVEMENT
Total motility (%)	Spermatozoa showing movement
Progressive motility (%)	Spermatozoa moving either linearly or in a circle regardless of speed
Velocity curve line (VCL) (µm)	Time-averaged velocity of a sperm head along its actual curvilinear path
Velocity straight line (VSL) (µm)	Time-average velocity of a sperm head along the straight line between its first detected position and its last
Velocity average path (VAP) (µm)	Time-average velocity of a sperm head along its average path
Linearity (LIN) (%)	Linearity of the curvilinear path = VSL/VCL
Straightness (STR) (%)	Linearity of the average path = VSL/VAP
Beat across frequency (BCF) (Hz)	Magnitude of lateral displacement of a sperm head about its average path
Amplitude of lateral displacement (ALH) µm	Magnitude of lateral displacement of a sperm head about its average path
Wobble (WOB) (%)	Measure of oscillation of the actual path about the average path = VAP/VCL
Hyperactivation (%)	Distinctive motility acquired by mature spermatozoa to enable fertilisation of an intact ovum

3.4.3.3 Sperm DNA fragmentation

Measurement of DNA fragmentation was conducted using the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) assay (Henkel, 2005) .The

TUNEL assay measures DNA damage directly by detection of single and double DNA strand breaks (Henkel, 2017). These DNA strand breaks are detected in an enzymatic reaction by labelling the free 3'-OH and the modified nucleotides with terminal deoxynucleotidyl transferase.

After incubation with the different concentrations of rooibos aqueous extracts at 37°C, 100 µl sperm suspension was added to 100 µl phosphate buffered saline (PBS) (Oxoid Ltd., Hampshire, England) and subjected to centrifugation at 300 xg (Labortechnik, Wehingen, Germany) for 10 minutes. The resultant pellets were re-suspended and wet smears were made on a StarFrost™ slide (Knittel Gläser, Braunschweig, Germany) and left to air dry at RT. The slides were then fixed in 4% formaldehyde in PBS (pH 7.4) for 25 minutes at 4°C. After fixation, the slides were washed in PBS for five minutes at RT and permeabilised in 0.2% Triton™ X-100 (Sigma-Aldrich, St. Louis) in PBS for five minutes at RT. Thereafter, the slides were rinsed twice in PBS for five minutes at RT, and 100 µl of equilibration buffer was added to each slide and allowed to equilibrate for 10 minutes. Thereafter, 20 µl of TUNEL reagent (DeadEnd™, Promega, Madison, WI, USA) was added to each slide and cooled with a plastic cover slip (Promega). After incubation for 60 minutes at 37°C in a humidified chamber protected from light, the reaction was halted by immersion in 2× SSC (Promega) for 15 minutes. Slides were then washed in PBS three times, and more than 200 randomly selected sperm were immediately analysed using a fluorescence microscope (Zeiss, Oberkochen, Germany) with a 488 nm excitation filter and a 510–530 nm emission filter at 400x magnification with an oil immersion objective. Sperm with normal DNA showed only slight background staining (TUNEL-negative), while sperm with fragmented DNA exhibited a bright green fluorescence (TUNEL-positive). At least 200 spermatozoa were counted, and TUNEL-positive sperm were recorded as a percentage of the total number of sperm per field (Figure 3.5).



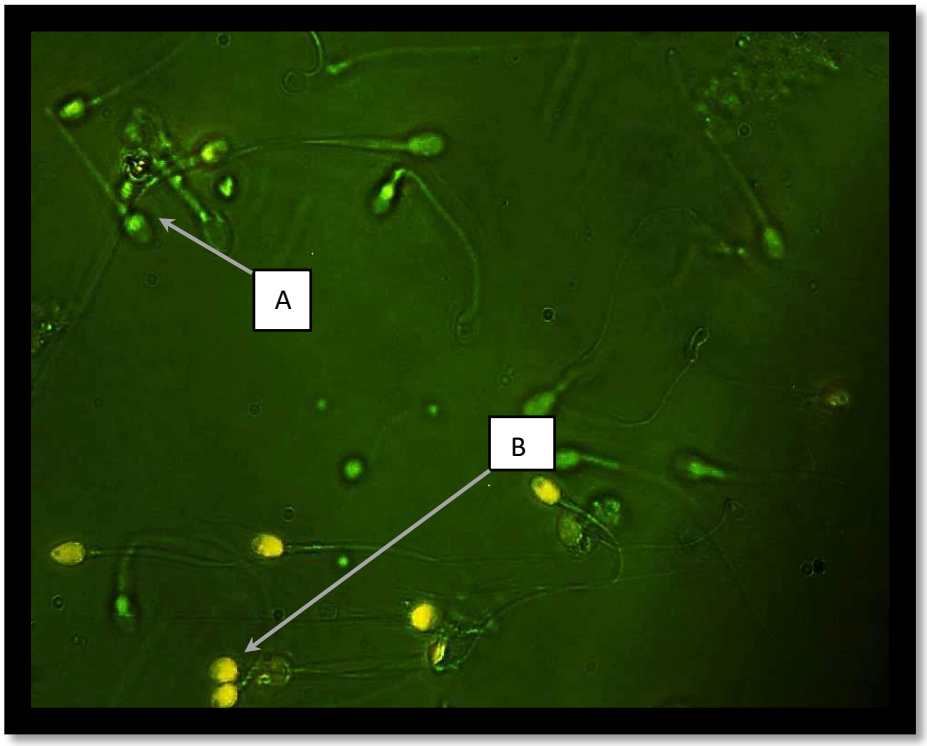
(A): Sperm with fragmented DNA exhibited a bright colour (TUNEL-positive); (B): Sperm with normal DNA showed only a slight background staining (TUNEL-negative)

Figure 2.5: Determination of DNA fragmentation using TUNEL assay

3.4.3.4 Determination of reaction oxygen species in spermatozoa

Reactive oxygen species production was determined using dihydroethidine (DHE) as a fluorescing probe according to Henkel et al. (2005). A stock solution was prepared using 20 µM DHE in PBS, with the pH adjusted to 7.4. After incubation of sperm samples with the aqueous extracts of rooibos at the different concentrations for one hour at 37ºC, aliquots of 100 µl of spermatozoa were centrifuged for 10 minutes at 500 xg (Labortechnik, Wehingen, Germany). Thereafter, the supernatants were discarded, and the samples were re-suspended in 100 µl PBS and 20 µl DHE stock and re-incubated for a further 15 minutes 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). Bright orange fluorescing sperm indicated excessive ROS production while a white colour indicted

absence of ROS (Fgure 3.6). A total of 200 spermatozoa were analysed and result represented as the percentage of ROS-positive spermatozoa.



(A): ROS-negative spermatozoa did not fluoresce; (B) ROS-positive spermatozoa fluoresced bright orange (1000x magnification)

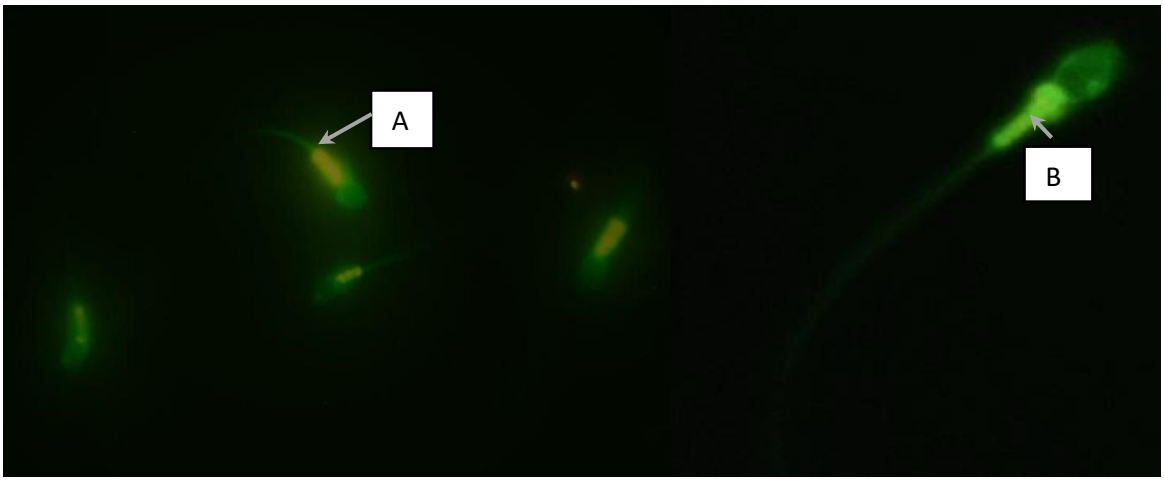
Figure 2.6: Human spermatozoa after staining with dihydroethidine

3.4.3.5 Determination of mitochondrial membrane potential in spermatozoa

Intact MMP in sperm was determined using a lipophilic cationic dye (DePsipher™, Trevigen, Minneapolis, USA). The following modification was implemented from the protocol provided by the manufacturer. The reaction buffer was diluted with pre-warmed distilled water (1:10) at 30^{o}C, and 20 µl of stabiliser was added per millilitre of buffer with 1 µl of DePsipher dye. Thereafter, the solution was added to 500 µl of prepared reaction buffer, vortexed thoroughly and centrifuged for one minute at 300 xg (Labortechnik,

Wehingen, Germany). Finally, the supernatant was transferred into a test tube for immediate use.

After incubation with rooibos aqueous extracts for one hour at 37 °C, 100 µl of each concentration was diluted with PBS (1:1) and subjected to centrifugation for 10 minutes at 300 xg (Labortechnik, Wehingen, Germany). The pellets were then re-suspended with 50–200 µl of DePsipher™ and incubated at 37°C for 20 minutes protected from light. After incubation, the sperm suspensions were centrifuged for 10 minutes at 300 xg (Labortechnik, Wehingen, Germany) and the pellet was re-suspended with a reaction buffer. Immediately thereafter, spermatozoa were observed using a fluorescence microscope with a 488 nm excitation filter (Zeiss) at 400x magnification. Sperm showing intense red/orange fluorescence were considered healthy with intact MMP (590 nm emission filter). In sperm with disrupted MMP, the monomer dye fluoresced green (530 nm emission filter), and these spermatozoa were generally classified as dying cells (Figure 3.6). A total of 200 spermatozoa were counted and the number with intact MMP was recorded as a percentage of the total number of sperm counted.

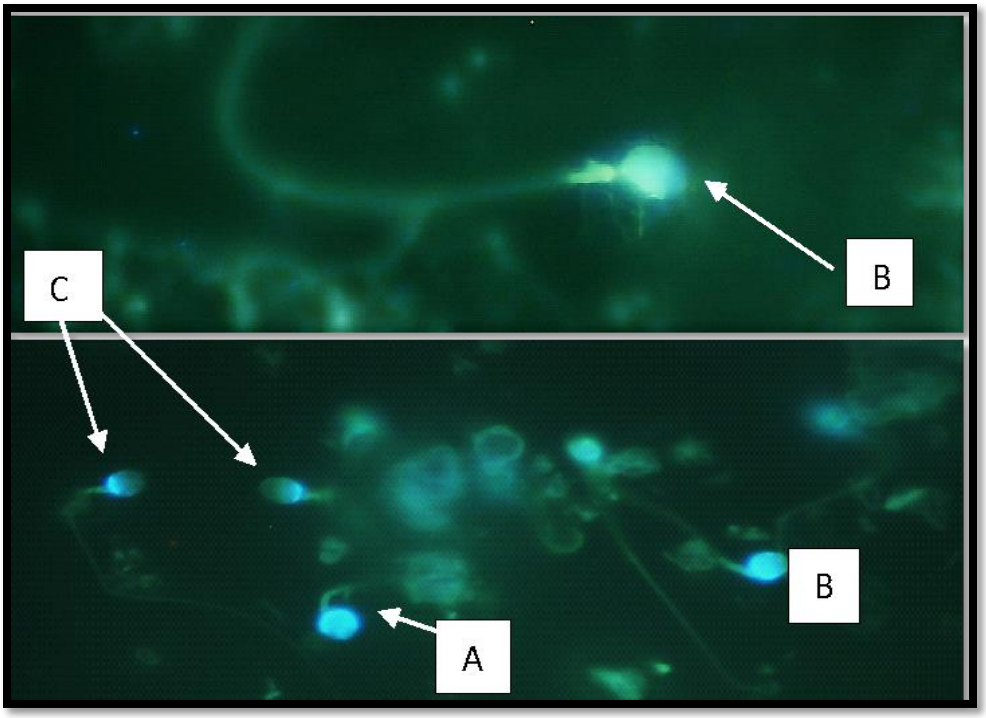


(A): Spermatozoa with intact Δψm show red fluorescence; (B) Spermatozoa with disrupted Δψm show green fluorescence (1000x magnification)

Figure 2.7: Determination of MMP using DePsipher™

3.4.3.6 Determination of capacitation and acrosome reaction in spermatozoa

The chlorotetracyclin (CTC) fluorescence assay protocol as stipulated by Green et al. (1996) was used in assessing the state of capacitation and AR. A stock staining solution of Hoechst 33258 (100 mg/mL) was prepared in distilled water. This solution can be stored at 4ºC for one month. Before use, the stock solution was diluted 1:1000 in HTF and then with sperm suspensions in HTF-BSA (1:100). Thereafter, the samples were incubated at RT for two minutes before being washed by centrifugation with 4 ml of 2% polyvinylpyrrolidone (PVP40) in HTF at 900 xg for five minutes. The CTC solution (pH 7.8) was prepared on the day of use and contained 750 µM CTC in a buffer of 130 mM NaCl, 5 mM cysteine in 20 mM Tris-HCl. The bottle containing the solution was kept wrapped in foil at 4ºC until use. The Hoechst-treated spermatozoa (45µl) were mixed with an equal volume of CTC solution and 8 µl of 12.5% w/v paraformaldehyde in 0.5 M Tris-HCl (pH 7.4) was added. Subsequently, 10 µl of the suspension was placed on a slide, and one drop of 0.22 M 1.4 diazabicyclo (2.2.2) octane (DABCO) dissolved in glycerol and 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). 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 3.8). At all three stages, bright fluorescence on the midpiece could be seen.



(A) Uncapacitated acrosome-intact sperm – entire sperm head shows a bright fluorescence with or without a brighter equatorial band; (B) capacitated acrosome-intact sperm – part of the sperm head fluoresces brightly but post-acrosome region does not; (C) capacitated acrosome-reacted sperm is non-fluorescent and appears with or without a fluorescent, post-acrosomal region (1000x magnification)

Figure 2.8: Chlortetracycline staining of human sperm

3.5 ETHICAL CONSIDERATIONS

3.5.1 Ethical clearance and approval

Ethical clearance was obtained from Turfloop Research Ethics Committee (TREC) of the University of Limpopo (TREC/46/2019:PG) (Appendix A) and the Biomedical Research Ethics Committee (BMREC) of the University of the Western Cape (BM18/3/17) (Appendix B). Permission to access the health facilities was obtained from Vincent Pallotti Hospital (Appendix C) and Tygerberg Hospital (Appendix D)

3.5.2 Protection from psychological harm

The adverse risks linked with this research study were minimal; the subject donated sperm through masturbation after three to five days of sexual abstinence. The obtained semen was exposed to different concentrations of rooibos and thereafter, semen parameters were investigated. The psychological aspect in this research was addressed by explaining the procedure to the donor in private before obtaining donor consent to participate in the study. Each donor was given an information sheet that explained the procedure in detail. A counsellor within the proximity of the laboratory was available should the donor become traumatised by the outcomes of basic semen analysis.

3.5.3 Disposal of waste

At the end of the experiment, all leftover samples were discarded as biohazardous waste into red bins, which were then stored in biohazard boxes. These boxes were collected by the waste management company that serviced the University of the Western Cape.

3.5.4 Anonymity and confidentiality

No patient or donor names were disclosed in the recording of data or disclosed to anyone in the dissemination of the results. Donor codes were given to the respective donors as a way of protecting their identity in the semen donation process. Consent forms were presented to the subjects in three different languages, English, Afrikaans and Xhosa (Appendix E, F, G) to avoid language barriers.

3.6 DATA ANALYSES

Data were analysed using Graph Pad Prism (version. 8.3.1, Graph Pad Software Inc.; California). To test for normality, the D'Agostino-Pearson omnibus test was used. One-way analysis of variance (ANOVA) and Tukey's test were used for normally distributed data, while the Kruskal-Wallis test and Dunnetts' multiple test were employed for non-parametric analysis of comparisons of group means. Statistical significance was considered at $P<0.05$.

3.6.1 Reliability, validity and bias

3.6.1.1 Reliability

Reliability is the degree to which a test is free from measurement errors, and a reliable test is observed when it can be used by different researchers under stable conditions with consistent results (Polgar & Thomas,2013). Reliability of the results was ensured by minimising human errors, by following all experimental steps accurately and where possible, by repeating the experiment several times. Infertile and fertile patients were diagnosed by doctors or well-trained experts to ensure reliability of the results.

3.6.1.2 Validity

Validity determines whether the research truly measures what it is intended to measure or how truthful the research results are (Johnson & Christensen, 2019) .To ensure validity of data, the number of infertile males was the same as the number of fertile males at the commencement of the experiment. In addition, the study did not deviate from its objectives. Validity was assured by using the correct assay and the correct equipment for this *in vitro* study.

3.6.1.3 Quality assurance

To avoid any form of microbial contamination, respective donors together with researchers worked in a sterile environment. Researchers handled all the required experimental tools correctly, wore gloves and followed laboratory rules to ensure the safety of everyone involved in the experiment**.**

3.6.1.4 Bias

According to Johnson and Christensen (2019), bias is an act of deviation from data collection, data analysis and data interpretation, which can lead to incorrect conclusions being drawn from the data. Bias in this study could not be avoided since convenience sampling was used. Sampling bias in terms of data collection could not be prevented since only donors and patients who were willing to donate their semen were involved in the research.

CHAPTER FOUR: RESULTS

4.1 *IN VITRO* EFFECTS OF AQUEOUS EXTRACT OF ROOIBOS ON HUMAN SPERM FUNCTIONALITY

Informed consent was obtained from the respective donors. Human semen was obtained from 100 (n=100) men, encompassing 50 healthy volunteers and 50 infertile patients. The former were taking part in a semen donor programme at the Andrology Research Laboratory in the Department of Medical Biosciences, University of the Western Cape. The latter were attending either the Reproductive Biology Unit, Department of Obstetrics and Gynaecology, Stellenbosch University at Tygerberg Hospital or the Vincent Pallotti Hospital for assisted reproductive treatment or fertility problems. Data were analysed using Graph Pad Prism (Graph Pad Software Inc; California) version. 8.3.1 and presented descriptively by demonstrating the effect of rooibos on the donor, patient and combined groups. The patient group was further subdivided into two groups, oligozoospermia and asthenozoospermia.

4.2 SUMMARY STATISTICS OF PARAMETERS MEASURED

Standard semen parameters (sperm concentration, progressive motility, total motility, volume and viscosity) are displayed in Table 4.1. The means of all the semen parameters were above the minimum reference values according to the WHO (2010).

Table 3.1: Summary statistics of parameters measured in this study

Summary statistics	Group	Concentration ($x10^6$/ml)	Progressive motility (%)	Total motility (%)	Volume (ml)	Viscosity (sec)
Number of values	Donor	50	50	50	50	50
	Patient	50	50	50	50	50
Minimum	Donor	3.74	0.500	11.8	0.50	10.0
	Patient	5.36	0.99	11.8	1.00	4.00
Median	Donor	54.2	23.8	45.4	2.25	20.0
	Patient	37.0	33.2	52.3	1.50	9.50
Maximum	Donor	133	68.4	90.9	5.00	27.0
	Patient	210	80.5	95.2	4.00	19.0
Mean	Donor	60.8	25.0	48.5	2.50	19.2
	Patient	44.4	37.4	53.8	1.72	9.79
Std. Deviation	Donor	32.0	20.2	19.9	1.17	4.97
	Patient	36.6	21.1	25.3	0.81	2.89
Std. Error of Mean	Donor	4.52	2.86	2.82	0.16	0.70
	Patient	5.17	2.98	3.57	0.11	0.40
Coefficient of Variation	Donor	52.61%	80.83%	41.15%	46.95%	25.82%
	Patient	82.39%	56.47%	46.95%	47.61%	29.50%

4.3 EFFECT OF UNFERMENTED AND FERMENTED ROOIBOS ON HEALTHY DONORS, INFERTILE PATIENTS AND COMBINED GROUP

A total of 100 semen samples were included in the study: 50 healthy donors and infertile patients were treated with fermented rooibos (0 µg/ml, 0.10 µg/ml, 1.0 µg/ml, 10 µg/ml, 100 µg/ml) and 50 healthy donors and infertile patients were treated with unfermented rooibos (0 µg/ml, 0.15 µg/ml, 1.5 µg/ml, 15 µg/ml, 150 µg/ml). The summary of the findings are tabulated in Table 4.2 and Table 4.3. Furthermore, oligozoospermia (unfermented: n=7; fermented: n=3) and asthenozoospermia (unfermented: n=16; fermented: n=11). Semen samples were identified and analysed separately. Samples

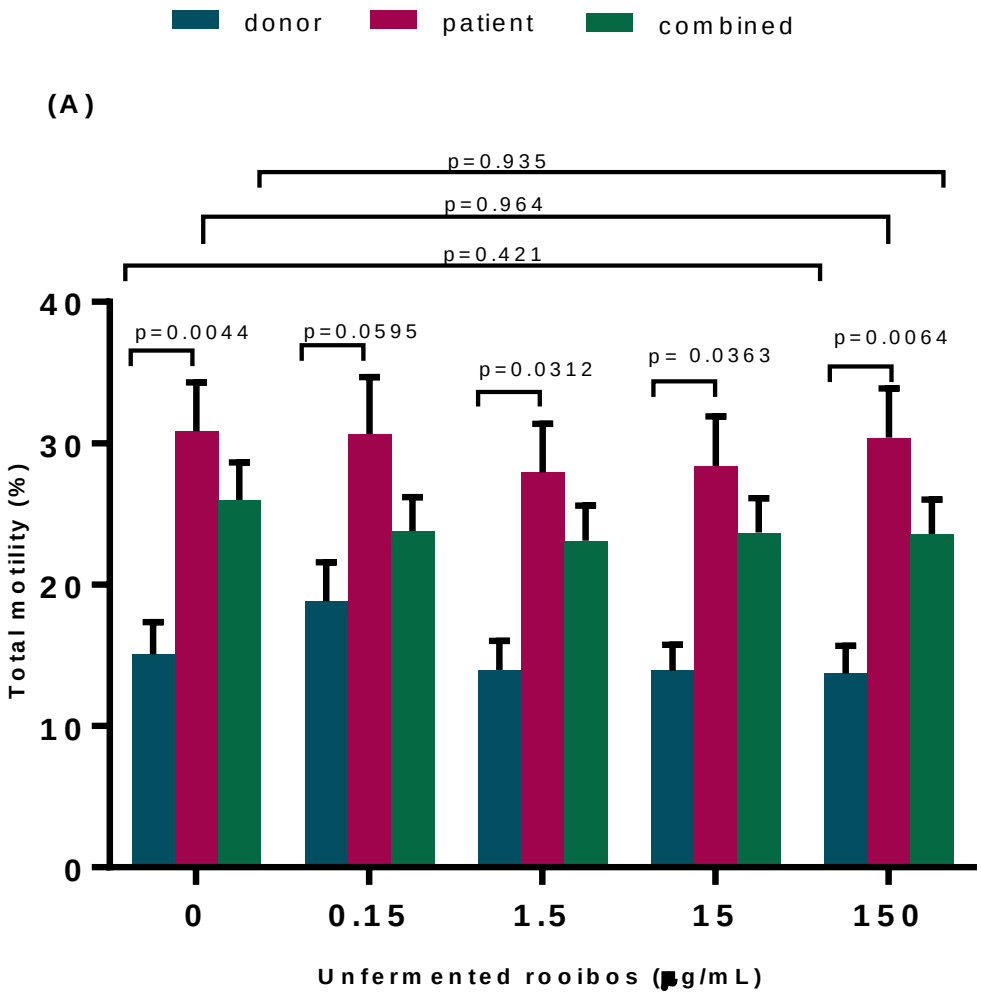
from six patients were excluded because they were azoospermic or only dead spermatozoa were detected. The cut-off value defining male was as follows: inability to conceive after one year of unprotected sex, oligozoospermia (concentration <15 million/ml) and asthenozoospermia (progressive motility <32% or total motility <40%) (WHO, 2010).

4.3.1 Sperm motility and kinematic parameter

Table 4.2 demonstrates that after one-hour incubation of spermatozoa with increasing concentrations of unfermented rooibos (0 µg/mL, 0.15 µg/mL, 1.5 µg/mL, 15 µg/mL, 150 µg/mL), no significant differences in the sperm velocity parameters (VAP, VCL, VSL, ALH, BCF, LIN, STR, WOB and hyperactivation) were observed in the donor, patient and combined groups (P>0.05).

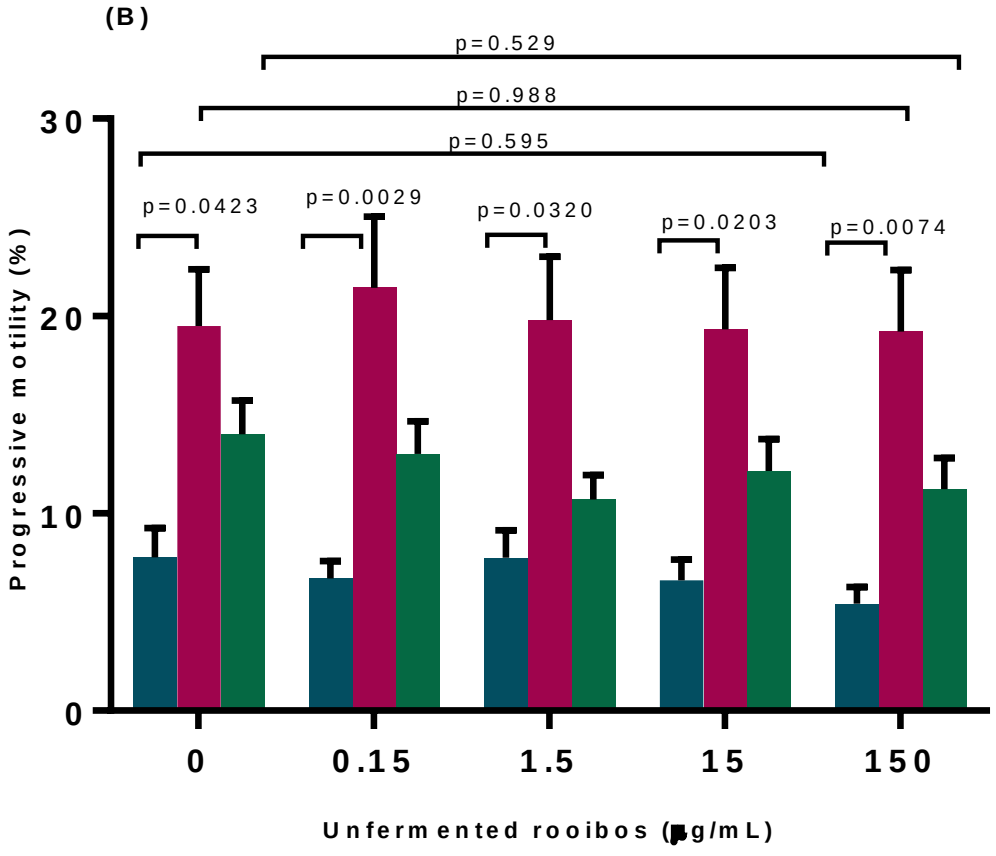
Total remained unchanged in the donor, patient and combined groups following exposure to aqueous extracts of unfermented rooibos (donors: P=0.421; patients: P=0.964; combined: P=935) (Figure 4.1a). The repeated measures ANOVA revealed no trends in the groups (donors: P=0.318; patients: P=0.676; combined: P=0.519). In addition, the progressive motility of spermatozoa in all groups was not affected by treatment with unfermented rooibos for one hour (donors: P=0.595; patients: P=0.988; combined: P=0.529) (Figure 4.1b), and no trend was observed in any of the groups (repeated measures ANOVA donors: P=0.453; patients: P=0.804; combined: P=0.124). Regarding non-progressive motility, no significant differences or trends in the donor, patient and combined groups that were exposed to unfermented rooibos were shown (donors: P=0.452; patients: P=0.731; combined: P=0.195 and repeated measures ANOVA donors: P=0.231; patients: P=0.303; combined: P=0.063) (Figure 4.1c). Lastly, regarding the percentage of immotile sperm, no significant differences in the donor, patient and combined groups that were exposed to unfermented rooibos were indicated (donors: P=0.437; patients: P=0.987; combined: P=0.459) (Figure 4.1d). Repeated measures ANOVA also showed no trend in the treated groups (donors: P=0.354; patients: P=0.913; combined: P=0.459). Furthermore, two-way ANOVA multiple comparison revealed a

significant ($p<0.05$) treatment effects on concentration 0 µg/mL, 0.15 µg/mL, 1.5 µg/mL, 15 µg/ml, 150 µg/mL in progressive motility and immotile sperm of patient and donors, while total motility only revealed significant treatment effect on concentration 0 µg/ml, 1.5 µg/mL, 15 µg/mL, and 150 µg/mL. Also two way ANOVA multiple comparison revelad no significant treatment effect on non progressive sperms in donors and patients 9 ($p>0.05$) (See figure 4.1 a, b, c, d).



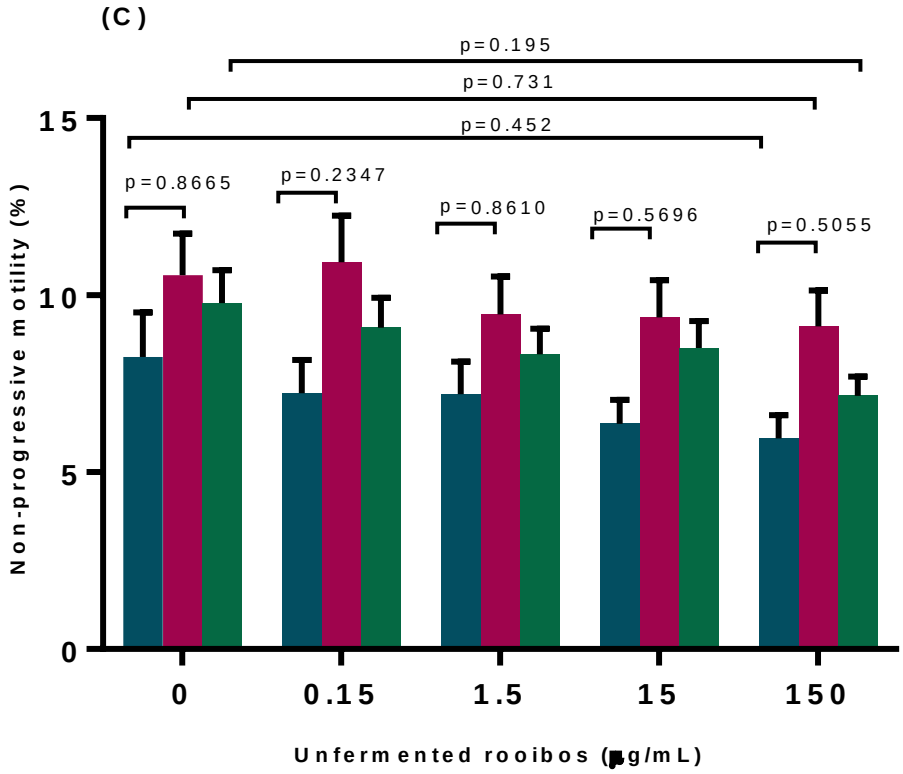
Values represented are the mean ± SEM after one-hour incubation with various concentrations of unfermented rooibos. (Colour code: Blue = healthy donor; Pink = infertile patient; Green = patients and donors)

Figure 3.1: Effect of unfermented rooibos on human sperm – (a) total motility; (b) progressive motility; (c) non-progressive motility; (d) immotile sperm



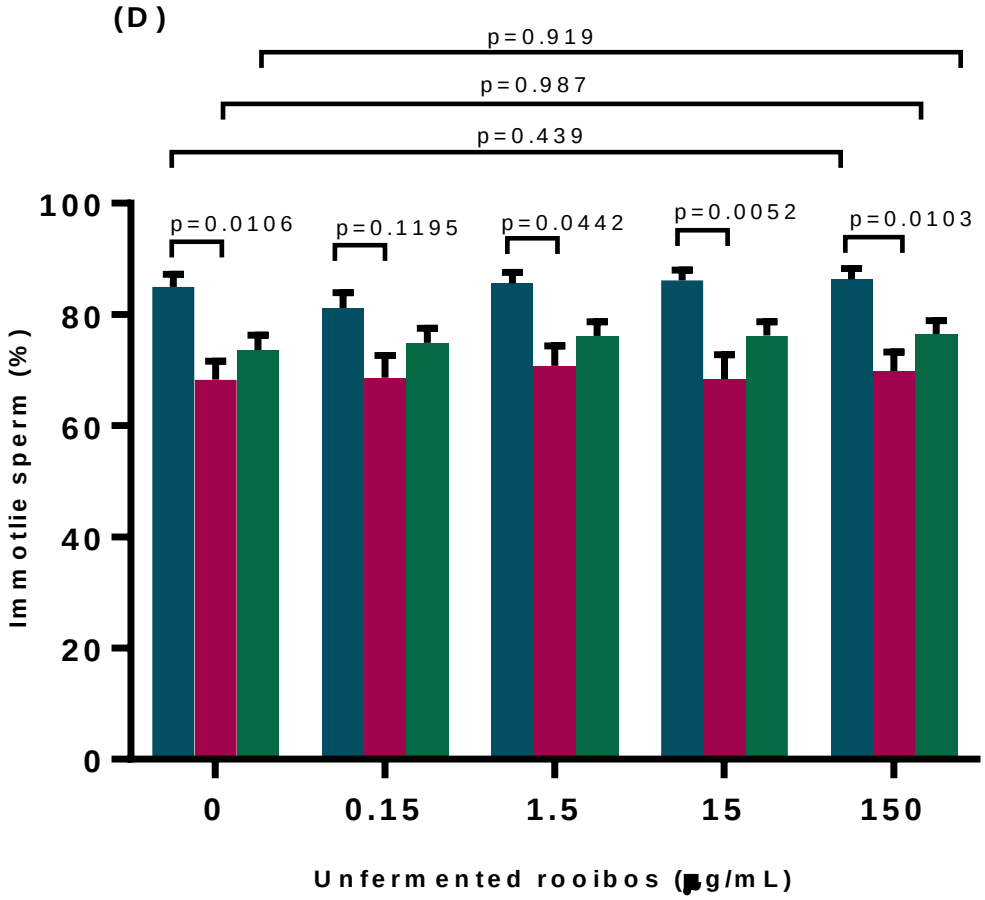
Values represented are the mean ± SEM after one-hour incubation with various concentrations of unfermented rooibos. (Colour code: Blue = healthy donor; Pink = infertile patient; Green = patients and donors)

Figure 4.1b: Effect of unfermented rooibos on human sperm – progressive motility



Values represented are the mean ± SEM after one-hour incubation with various concentrations of unfermented rooibos. (Colour code: Blue = healthy donor; Pink = infertile patient; Green = patients and donors)

Figure 4.1c: Effect of unfermented rooibos on human sperm – non-progressive motility



Values represented are the mean ± SEM after one-hour incubation with various concentrations of unfermented rooibos. (Colour code: Blue = healthy donor; Pink = infertile patient; Green = patients and donors)

Figure 4.1d: Effect of unfermented rooibos on human sperm – immotility

Table 3.2: Summary statistics of sperm kinematic motility parameters

Kinematic parameter	Group	Aqueous extract of unfermented rooibos (µg/mL)					P value
		0	0.15	1.5	15	150	
VAP (µm s^{-1})	Donor	16.8±1.53	18.1±1.98	18±2.21	16.9±1.53	19.3±2.07	0.87
	Patient	21.4±2.04	21.4±1.42	22.9±2.17	22.9±2.17	24.9±2.34	0.76
	Combined	19.1±1.31	20.4±1.40	20.3±1.57	19.1±1.29	22.1±1.60	0.56
VCL (µm s^{-1})	Donor	40.8±2.78	44.2±3.45	45.9±4.40	42.0±2.63	44.8±3.21	0.82
	Patient	53.0±3.37	56.5±3.22	56.6±4.43	56.4±3.50	55.8±3.30	0.94
	Combined	48.8±2.55	50.4±2.49	51.3±3.18	49.2±2.40	50.3±2.41	0.96
VSL (µm s^{-1})	Donor	24.6±2.07	24.7±2.11	24.7±2.41	23.4±1.62	26.0±2.11	0.93
	Combined	27.3±1.52	28.0±1.48	28.6±1.84	27.6±1.56	29.8±1.66	0.82
	Patient	30.1±2.14	31.4±1.88	30.9±2.10	31.7±2.43	33.6±3.37	0.84
ALH (µm)	Donor	1.9±0.04	2.0±0.08	2.06±0.89	1.94±0.05	2.01±0.08	0.57
	Patient	2.24±0.06	2.43±0.10	2.43±0.10	2.47±0.11	2.35±0.11	0.52
	combined	2.10±0.05	2.21±0.075	2.24±0.07	2.17±0.06	2.09±0.05	0.33
Hyperactivation (%)	Donor	0	0	0	0	0	0
	Patient	0.08±0.04	0.02±0.09	0.31±0.10	0.28±0.10	0	0
	Combined	0	0	0	0	0	0
LIN (%)	Donor	60.1±1.85	61.2±2.08	61.2±2.49	61.5±2.78	63.3±3.03	0.92
	Patient	61.6±2.08	64.5±2.12	66.4±2.11	61.5±2.39	64.1±1.96	0.42
	Combined	60.8±1.38	62.9±1.49	63.8±1.65	61.5±1.81	63.7±1.79	0.61
STR (%)	Donor	35.6±1.92	36.8±2.05	35.0±2.17	38.1±2.57	38.6±2.53	0.74
	Patient	36.6±1.93	39.7±2.36	40.9±2.37	36.1±2.43	39.5±1.88	0.47
	Combined	36.1±1.35	38.2±1.56	37.9±1.65	37.1±1.76	39.1±1.49	0.72
WOB (%)	Donor	58.8±1.58	54.7±1.65	52.4±2.07	55.5±1.92	56.4±1.81	0.62
	Patient	54.4±1.47	56.6±1.79	56.5±1.74	54.8±1.82	56.8±1.65	0.56

	Combined	54.5±1.07	55.7±1.22	54.5±1.37	54.6±1.31	56.6±1.21	0.68
BCF (HZ)	**Donor**	4.64±0.56	5.07±0.54	4.98±0.54	4.68±0.43	5.35±0.47	0.87
	Patient	5.77±0.48	6.63±0.42	6.91±0.56	5.93±0.42	6.14±0.36	0.34
	Combined	5.22±0.37	5.86±0.35	6.14±0.44	5.73±0.42	5.73±0.30	0.85

VAP: Average path velocity; VCL: Curvilinear velocity; VSL: Straight-line velocity; ALH: Amplitude of lateral head displacement; LIN: Linearity; STR: Straightness; WOB: Wobble; BCF: Beat cross frequency. An asterix (*) indicate data with significant p-vlaue

Values represented are the mean ± SEM of donor (n=25), patient (n=25) and combined (n=50) sperm exposed to the various concentrations of unfermented rooibos.

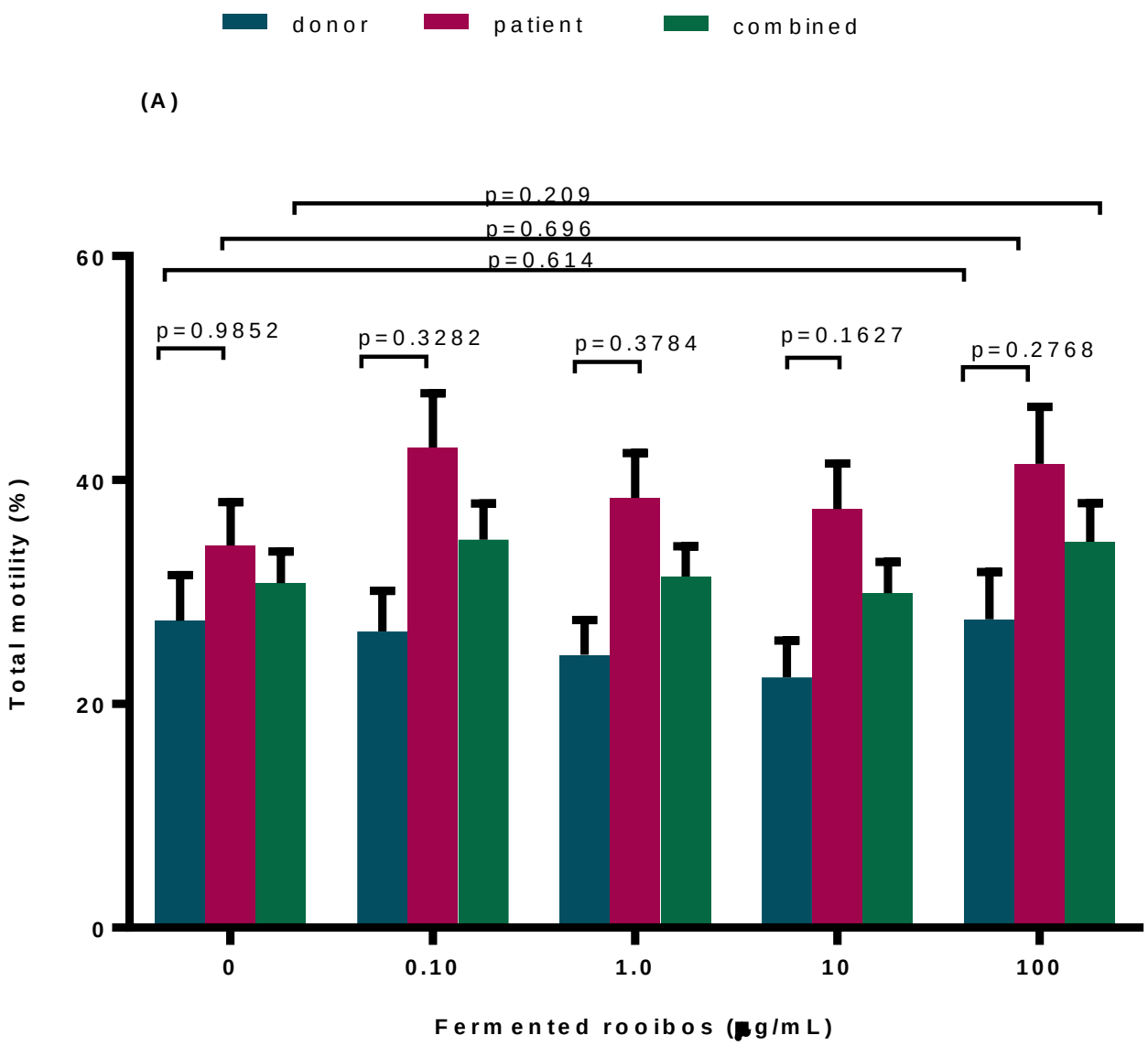
Similarly, no significant effect was observed in the sperm velocity parameters following the exposure of both the donor and the patient spermatozoa to increasing concentrations of fermented rooibos (0 µg/ml, 0.10 µg/ml, 1.0 µg/ml, 10 µg/ml, 100 µg/ml) for one hour (P>0.05). See Table 4.3.

Figures 4.2a to 4.2d demonstrate the effects of fermented rooibos on sperm motility, progressive motility, non-progressive motility and immotility. As shown in Figure 4.2a, total motility remained unchanged in the donor, patient and combined groups that were exposed to the various concentrations of aqueous extracts of fermented rooibos (0 µg/mL, 0.10 µg/mL, 1.0 µg/mL, 10 µg/mL, 100 µg/mL). The results are as follows: One-way ANOVA (donors: P=0.696; patients: P=0.614; combined: P=0.708) and repeated measures ANOVA (donors: P=0.351; patients: P=0.240; combined: P=0.209).

Also, progressive motility showed no significantly change with fermented rooibos extract (donors: P=0.721). The patient and combined groups, however, showed no significant difference when compared with the control group (patients: P=0.625; combined: P=0.238). See Figure 4.2b.

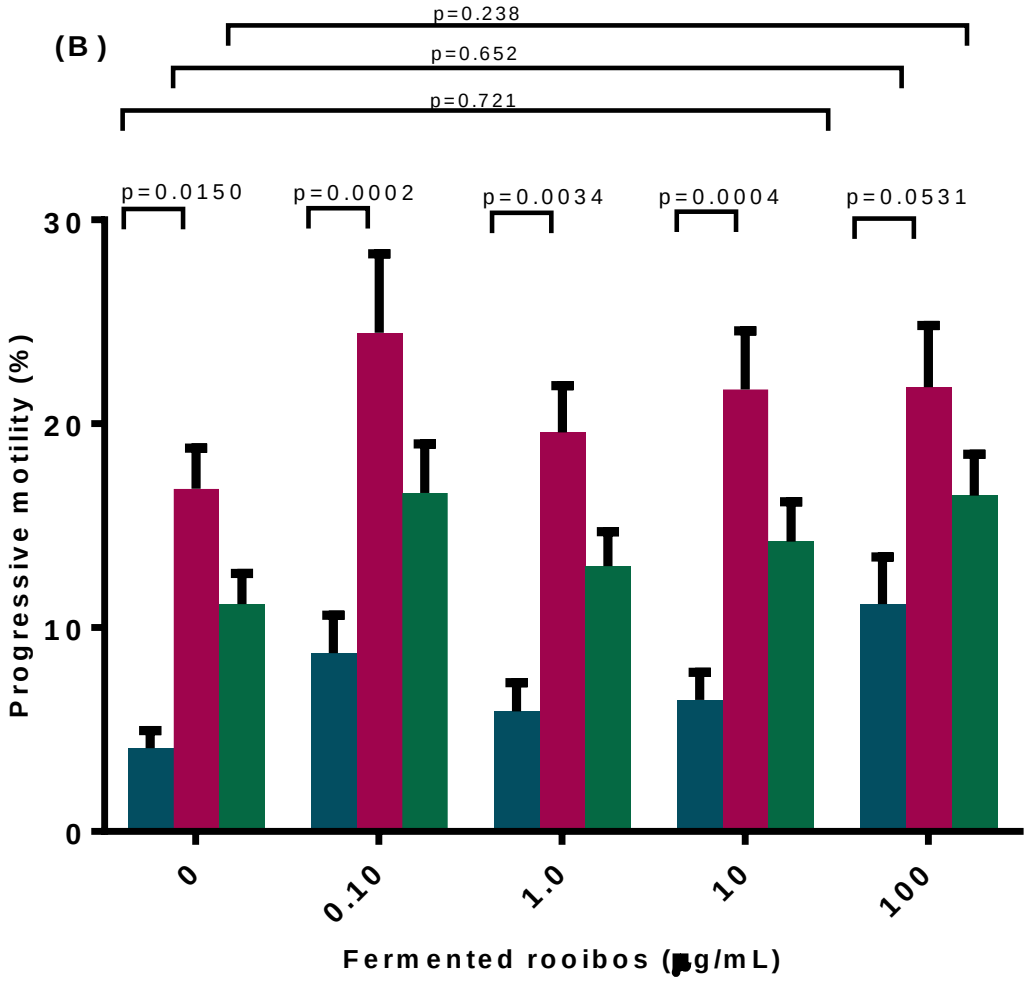
Regarding non-progressive motility, there was no significant difference in the donor, patient and combined groups that were exposed to fermented rooibos (donors: P=0.729; patients: P=0.251; combined: P=0.733). See Figure 4.2c. However, a significant trend of a decrease in non-progressive motility was seen in all groups: (repeated measures ANOVA donors: P=0.006; patients: P=0.040; combined: P=0.035). Lastly, no significant

difference regarding immotile sperm was shown in the donor, patient and combined groups that were exposed to fermented rooibos (donors: P=0.586; patients: P=0.1381: combined: P=0.983).Furthermore two-way ANOVA multiple comparision showed a significant treatment effects in progressive motility of donor and patients after one hour of incubation with 0 µg/mL, 0.10 µg/mL, 1.0 µg/mL, 10 µg/mL, 100 µg/mL of fermented rooibos.On the other hand two way ANOVA revealed a significant treatment effect in concentration 0.10 µg/mL,10 µg/mL and 100 µg/mL of fermented rooibos in immotile sperm of donors and patients ($p<0.05$).More so two-way ANOVA multiple comparison showed no significant treatment effect ($p>0.05$) on total motility and non progressive motility between donors and patients.



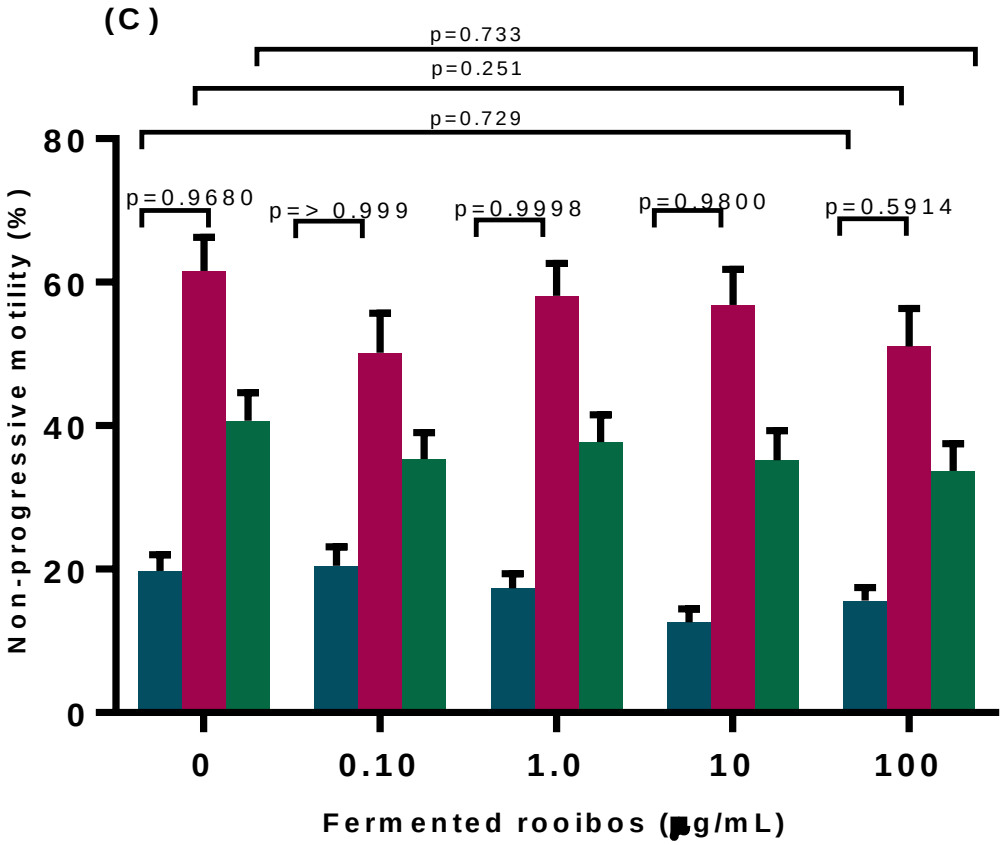
Values represented are the mean ± SEM after one-hour incubation with various concentrations of fermented rooibos. (Colour code: Blue = healthy donor; Pink = infertile patient; Green = combined)

Figure 3.2: Effect of fermented rooibos on human sperm – (a) total motility; (b) progressive motility; (c) non-progressive motility; (d) immotile sperm.



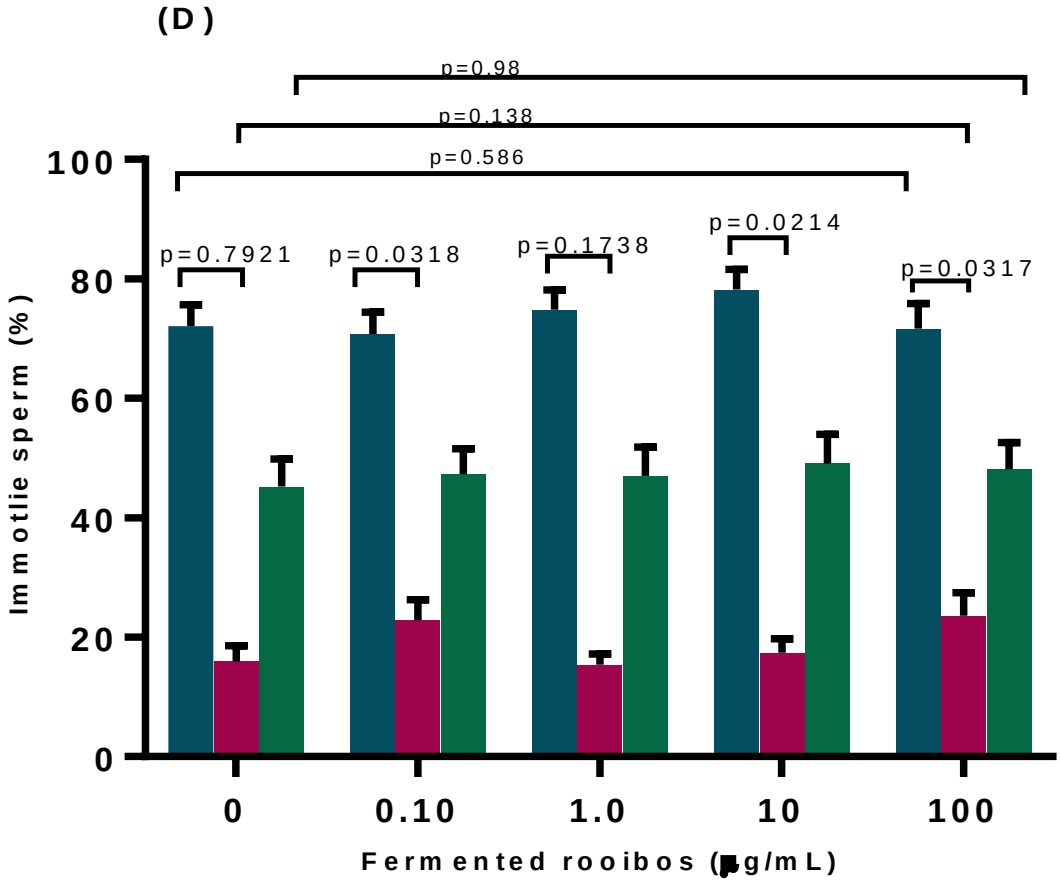
Values represented are the mean ± SEM after one-hour incubation with various concentrations of fermented rooibos. (Colour code: Blue = healthy donor; Pink = infertile patient; Green = combined)

Figure 4.2b: Effect of fermented rooibos on human sperm – progressive motility



Values represented are the mean ± SEM after one-hour incubation with various concentrations of fermented rooibos. (Colour code: Blue = healthy donor; Pink = infertile patient; Green = combined)

Figure 4.2c: Effect of fermented rooibos on human sperm – non-progressive motility



Values represented are the mean ± SEM after one-hour incubation with various concentrations of fermented rooibos. (Colour code: Blue = healthy donor; Pink = infertile patient; Green = combined)

Figure 4.2d: Effect of fermented rooibos on human sperm – immotility

Table 3.3: Summary statistics of sperm kinematic motility parameters: fermented rooibos

Kinematic parameter	Group	Aqueous extract of fermented rooibos (µg /ml)					P value
		0	0.10	1.0	10	100	
VAP(µms)	Donor	25.4±1.96	23.6 ±1.81	23.9±1.83	27.7±2.18	25.3±1.85	0.74
	Patient	23.0±2.17	18.9±1.49	23.4±1.77	24.6±1.78	24.4±2.12	0.23
	Combined	24.2±1.46	22.4±1.27	23.7±1.26	26.1±1.41	24.8±1.39	0.39
VCL(µms-1)	Donor	62.2±2.54	65.9±3.46	64.2±2.65	71.9±3.84	69.4±2.97	0.32
	Patient	49.9±3.04	48.7±3.04	50.3±2.50	51.2±2.28	52.5±2.83	0.89
	Combined	59.0±2.21	57.3±2.58	57.4±2.06	61.6±2.66	60.8±2.37	0.51
VSL(µms-1)	Donor	34.8±1.96	35.0±2.24	33.1±2.16	37.5±2.40	36.9±2.30	0.64
	Patients	30.6±2.18	29.1±2.25	31.2±1.73	31.9±1.71	32.7±2.02	0.75
	Combine	32.7±1.48	32.0±1.63	32.2±1.38	34.7±1.51	34.8±1.56	0.52
ALH (µm)	Donor	2.07±0.07	2.13±0.87	2.13±0.06	2.28±0.10	2.24±0.09	0.36
	Patient	2.26±0.08	2.13±0.07	2.21±0.55	2.15±0.06	2.24±0.65	0.77
Hyperactivation (%)	Donor	0.22±0.08	0.11±0.05	0.27±0.11	0.50±0.16	0.28±0.09	0.13
	Patient	0.10±0.04	0.11±0.46	0.10±0.04	0.22±0.08	0.29±0.0.9	0.16
	Combined	8.86±1.59	0.10±0.03	0.19±0.06	0.30±0.08	0.24±0.05	0.45
LIN (%)	Donor	63.9±2.54	67.1±1.82	62.8±1.99	64.3±3.01	61.5±2.72	0.58
	Patient	62.7±2.09	62.5±1.85	62.8±1.98	63.9±1.92	61.8±2.11	0.96
	Combined	63.3±1.63	64.8±1.33	62.8±1.39	64.1±1.77	61.7±1.70	0.68
STR (%)	Donor	39.3±1.99	38.6±1.64	35.5±1.64	35.5±1.84	36.5±1.64	0.43
	Patient	38.8±2.22	37.6±1.93	38.9±1.74	40.9±1.87	39.4±2.06	0.82
	Combined	39.1±1.47	38.1±1.26	37.2±1.28	40.1±1.32	37.9±1.32	0.56
WOB (%)	Donor	55.3±1.47	54.2±54.2	54.0±1.65	54.4±1.63	52.7±1.36	0.83

	Patient	57.3±1.69	56.2±1.62	57.4±1.23	58.8±1.37	58.5±1.35	0.82
	Combined	56.3±1.12	55.2±1.15	55.7±1.05	56.6±1.10	55.6±1.03	0.90
BCF (HZ)	**Donor**	18.4±0.73	18.0±0.83	16.6±0.86	18.7±0.81	16.8±0.91	0.26
	Patient	5.57±0.39	6.86±0.86	5.10±0.38	5.92±0.33	5.92±0.38	0.16
	Combined	12.5±1.05	12.5±0.99	11.3±0.98	12.7±1.09	11.8±0.96	0.87

VAP: Average path velocity; VCL: Curvilinear velocity; VSL: Straight-line velocity; ALH: Amplitude of lateral head displacement; LIN: Linearity; STR: Straightness; WOB: Wobble; BCF: Beat cross frequency. An asterix (*) indicate data with significant p-vlaue

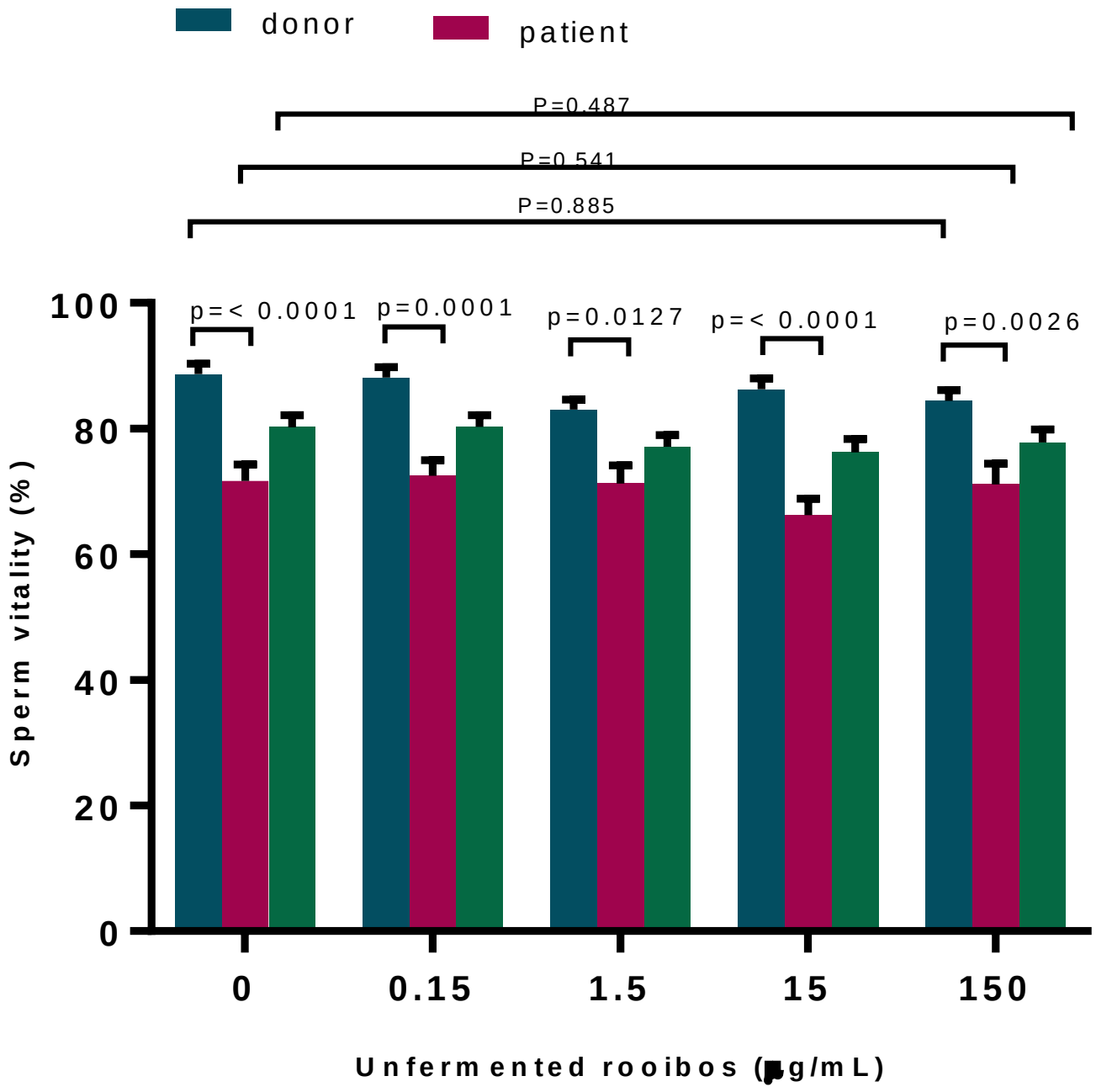
Values represented are the mean ± SEM of donor (n=25), patient (n=25) and combined (n=50) sperm exposed to the various concentrations of fermented rooibos.

4.3.2 Sperm vitality

Incubation of human sperm with increasing concentrations of aqueous extracts of unfermented rooibos caused no significant change in the percentage of total live sperm in the donor, patient, and combined groups (donors: P=0.885; patients: P=0.541; combined: P=0.487) (Figure 4.3). Repeated measures ANOVA also showed no significant trends (donors: P=0.100; patients: P=0.405; combined: P=0.312). Further analysis showed a significant treatment effects between donors and patients after 1 hour of incubation with unfermented rooibos in all concentrations(two-way ANOVA:P<0.05). See figure 4.3

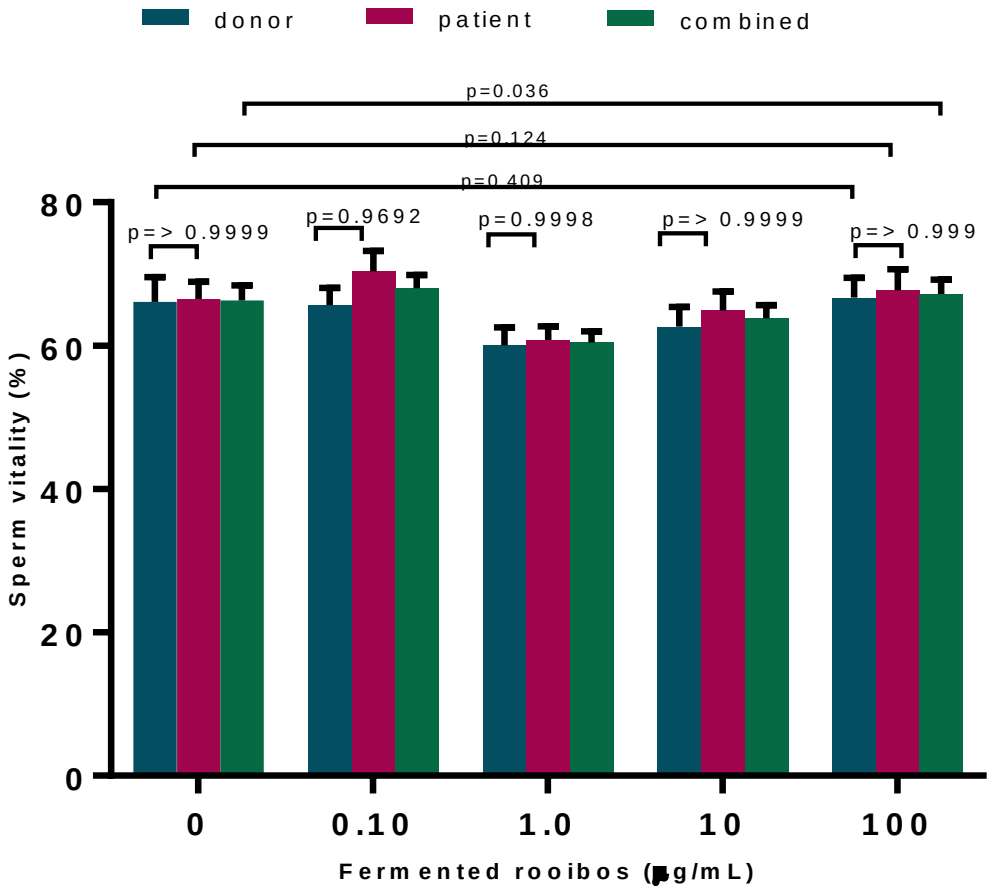
However, exposure of human sperm to the respective concentrations of fermented rooibos for one hour significantly decreased the percentage of live sperm in the combined group while the sperm of the donor and patient groups revealed no significant difference when compared with the control group (donors: P=0.409; patients: P=0.124; combined: P=0.036). Repeated measures ANOVA, however, demonstrated no significant effect in the groups (donors: P=0.186; patients: P=0.109; combined: P=0.012). Further analysis showed no significant treatment effects on percentage of live sperm between donors and

patients after 1 hour of incubation with fermented rooibos in all concentrations (two-way ANOVA:P>0.05). See Figure 4.4.



Values represented are the mean ± SEM after one-hour incubation with various concentrations of unfermented rooibos. (Colour code: Blue = healthy donor; Pink = infertile patient; Green = combined)

Figure 3.3: Effect of unfermented rooibos on human sperm vitality *in vitro*



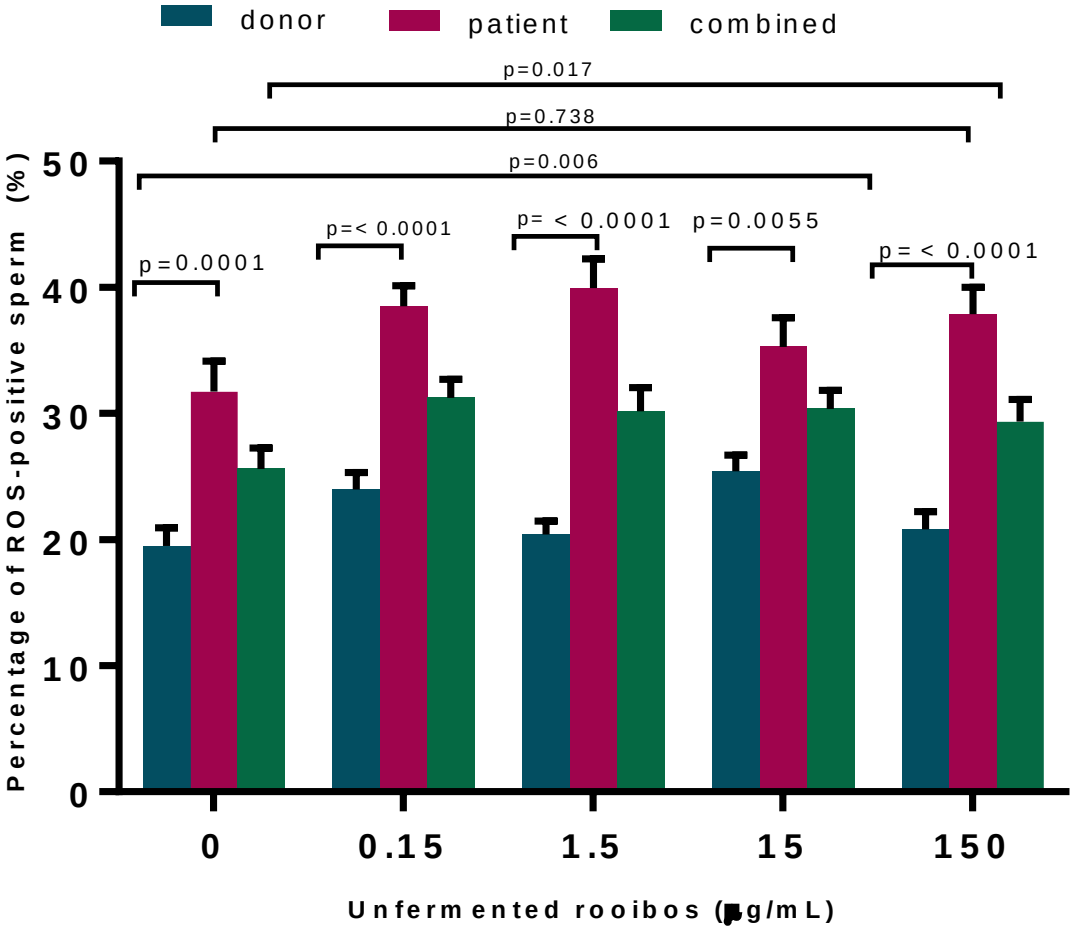
Values represented are the mean ± SEM after one-hour incubation with various concentrations of fermented rooibos. (Colour code: Blue = healthy donor; Pink = infertile patient; Green = combined).

Figure 3.4: Effect of fermented rooibos on human sperm vitality *in vitro*

4.3.3 Reactive oxygen species in human sperm

Incubation of human sperm with a concentration of 15 µg/mL of unfermented rooibos extract for one hour significantly increased the percentage of ROS-positive sperm in the donor group (One-way ANOVA donors: P=0.006). Repeated measures ANOVA also showed a significant trend towards an increase in ROS-positive sperm in the donor group (repeated measures ANOVA donors: P=0.012). However, the patient and combined groups showed no significant difference compared with the control group (One-way ANOVA patients: P=0.783; combined: P=0.140), but a repeated measures ANOVA

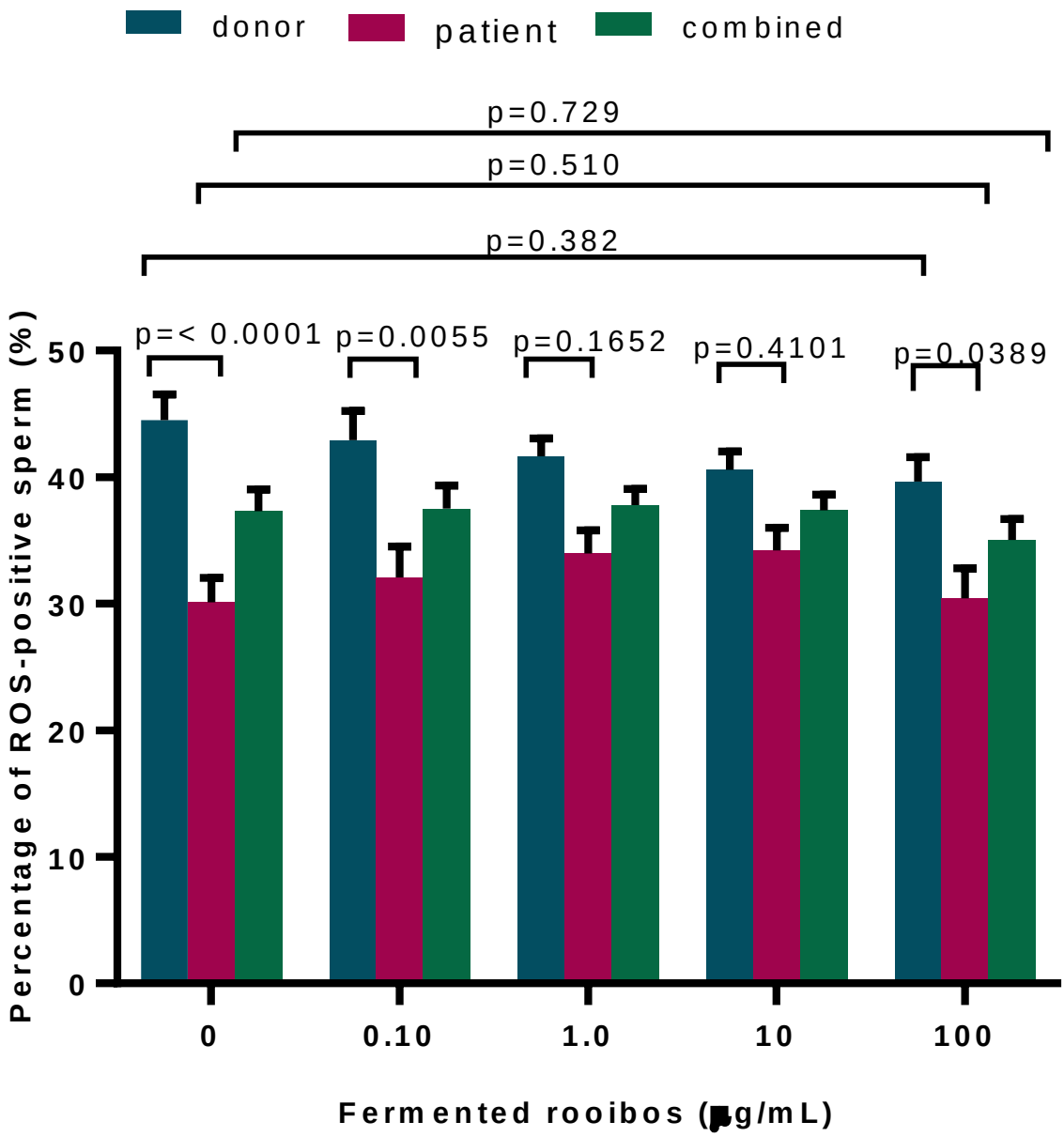
revealed a trend towards an increase in ROS-positive sperm in the patient and combined groups (patients: P=0.06; combined: P=0.017). More so incubation with unfermented rooibos showed a significant treatment effects on percentage of ROS_positive sperms between donors and patients after 1 hour of incubation with all concentrations (0 µg/mL,0.15 µg/mL,1.5 µg/mL,15 µg/mL,150 µg/mL) of unferemented rooibos (two-way ANOVA:P>0.05). See figure 4.5.



Values represented are the mean ± SEM after one-hour incubation with various concentrations of unfermented rooibos. (Colour code: Blue = healthy donor; Pink = infertile patient; Green = combined)

Figure 3.5: Effect of unfermented rooibos on reactive oxygen species in human sperm *in vitro*

However, incubation of human sperm with increasing concentrations of aqueous extracts of fermented rooibos caused no significant differences or trends in the percentage of ROS-positive sperm in the donor, patient and combined groups. (One-way ANOVA donors: P=0.382; patients: P=0.510; combined: P=0.729 and repeated measures ANOVA donors: P=0.362; patients: P=0.337; combined: P=0.538). More so two-way ANOVA revelead a significant treatment effect on ROS-positive sperm in concentration as high as 0.10 µg/mL and 100 µg/mL between donors and patients, see Figure 4.6.

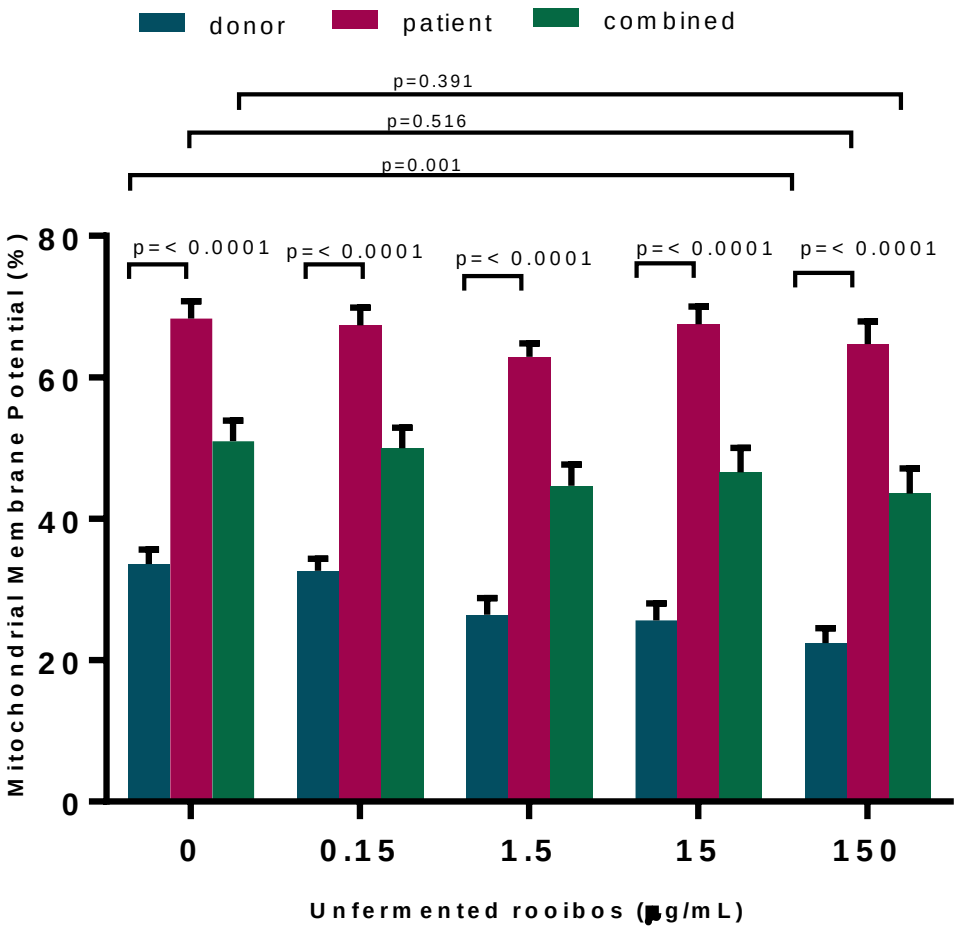


Values represented are the mean ± SEM after one-hour incubation with various concentrations of fermented rooibos. (Colour code: Blue = healthy donor; Pink = infertile patient; Green = combined)

Figure 3.6: Effect of fermented rooibos on reactive oxygen species in human sperm *in vitro*

4.3.4 Mitochondrial membrane potential in human sperm

Higher concentrations of unfermented rooibos (15 µg/mL and 150 µg/mL) caused a significant decrease in the percentage of spermatozoa with intact MMP in the donor group ($P<0.05$). See Figure 4.7. The repeated measures ANOVA with increasing concentrations of unfermented rooibos also showed a negative trend in the percentage of spermatozoa with intact MMP in the donor group (donors: $P=0.0023$). However, the combined and the patient groups showed no significant difference in the percentage of spermatozoa with intact MMP (One-way ANOVA patients: $P=0.516$; donors: $P=0.001$; combined: $P=0.391$). Repeated measures ANOVA unveiled a decrease in spermatozoa with intact MMP in the combined group, with no trend observed in the patient group (patients: $P=0.434$; combined: $P=0.005$). More so two-way ANOVA revelead a significant treatment effect ($p<0.05$) on percentage of sperms with intact mitochondria in all concentration (0 µg/ml, 0.15 µg/mL, 1.5 µg/mL, 15 µg/mL, 150 µg/mL) of unfermented rooibos in donors and patients, see Figure 4.6.

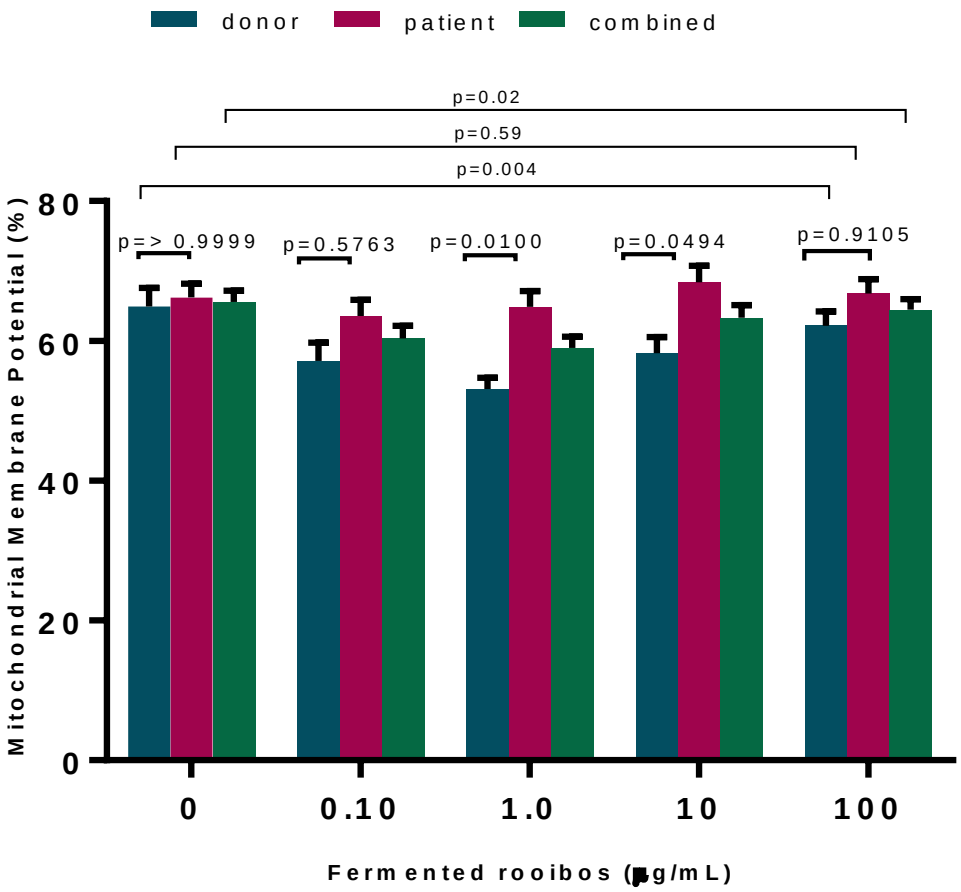


Values represented are the mean ± SEM after one-hour incubation with various concentrations of unfermented rooibos. (Colour code: Blue = healthy donor; Pink = infertile patient; Green = combined)

Figure 3.7: Effect of unfermented rooibos on intact mitochondrial membrane potential

Incubation with increasing concentrations of fermented rooibos showed a significant decrease in the percentage of spermatozoa with intact MMP in the donor and combined groups (donors: P=0.004; combined: P=0.027). See Figure 4.8. The repeated measures ANOVA revealed a significant dose-dependent decrease in intact MMP in the donor and combined groups (donors: P=0.001; combined: P=0.009). However, neither a significant difference nor a trend was seen in the percentage of spermatozoa with intact MMP in the patient group treated with fermented rooibos (One-way ANOVA patients: P=0.597; repeated measures ANOVA patients: P=0.534). Also, two-way ANOVA revelead a significant treatment effect ($p<0.05$) on percentage of sperms with intact mitochondria in

concentration as high as (1.0 µg/mL, 10 µg/mL) of fermented rooibos in donors and patients.See Figure 4.8.



Values represented are the mean ± SEM after one-hour incubation with various concentrations of fermented rooibos. (Colour code: Blue = healthy donor; Pink = infertile patient; Green = combined)

Figure 3.8: Effect of fermented rooibos on intact mitochondrial membrane potential

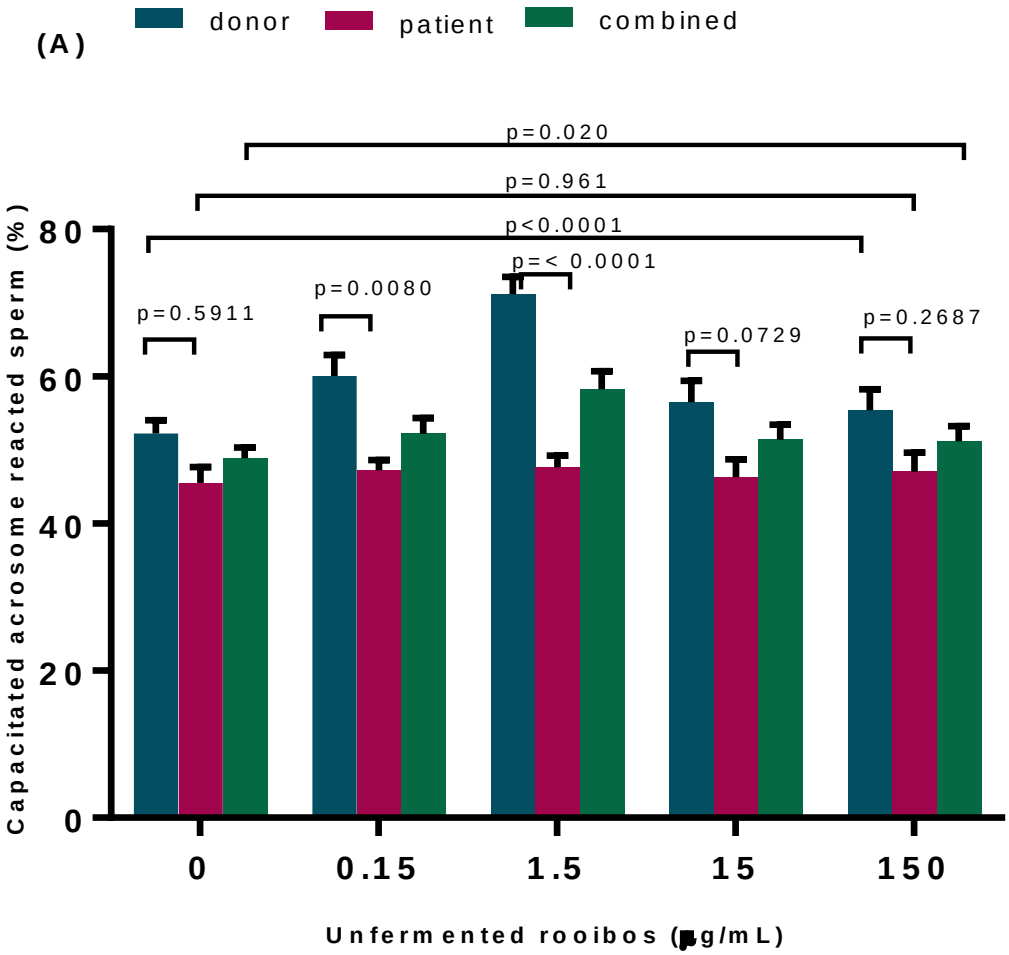
4.3.5 Capacitation and acrosome reaction

Exposure of human sperm to aqueous extract of unfermented rooibos significantly significant increase in the percentage of capacitated and acrosome-reacted spermatozoa in the donor and the combined groups was only observed at the 1.5 µg/ml concentration (donors: P<0.0001; combined: P=0.020). More so, two-way ANOVA revealed a significant treatment effect (P<0.05) in acrosome reacted sperm of donors and patients in

concentration as high as 0.15 µg/mL and 1.5 µg/Ml of unfermented rooibos. See Figure 4.9a.

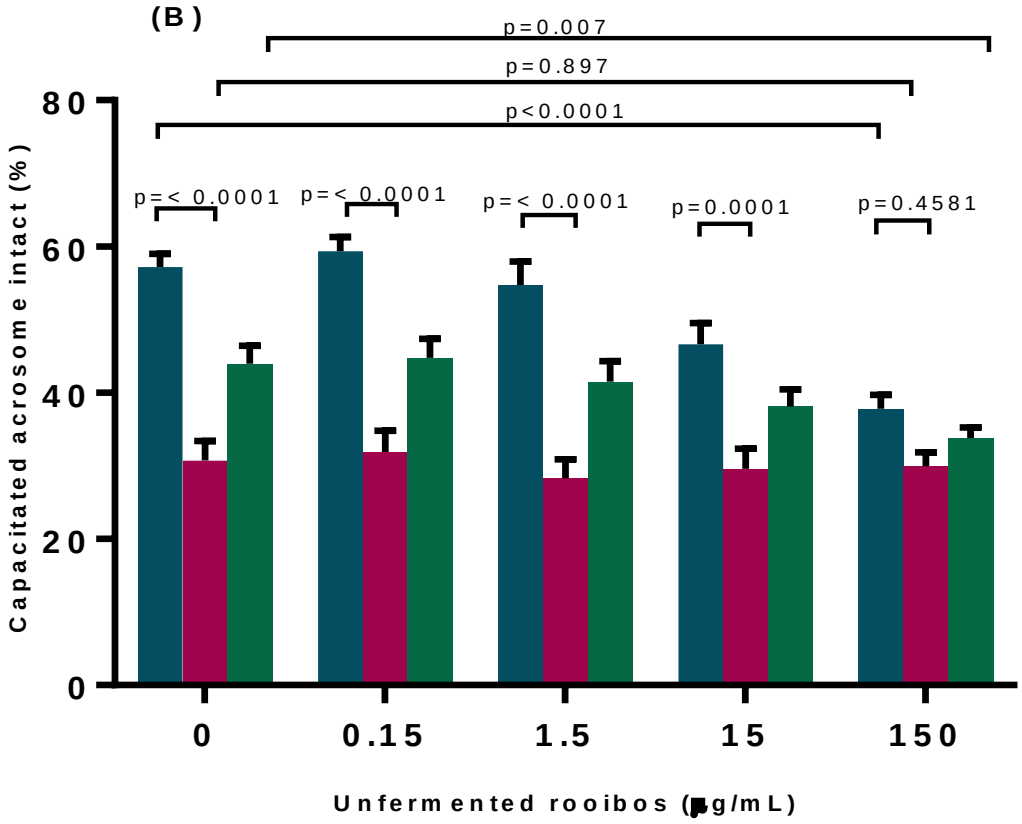
In addition exposure of human sperm to aqueous extract of unfermented rooibos concentration 150 µg/ml aqueous extract of unfermented rooibos significantly decreased the percentage of capacitated and acrosome-intact spermatozoa in the donor and the combined groups compared with the control group (donors: $P<0.0001$; combined: $P=0.007$). See Figure 4.9b. No significant effect was observed in the patient group ($P=0.897$). The repeated measures ANOVA showed a significant decreasing trend in value in the donor and the combined groups (donors: $P<0.001$; combined: $P=0.0002$) but not in the patient group (patients: $P=0.714$).Also, two-way ANOVA revealed a significant treatment effect ($P<0.05$) in capacitated acrosome intact sperm of donors and patients in concentration as high as 0 µg/mL,0.15 µg/mL,1.5 µg/mL and15µg/Ml of unfermented rooibos,no significant effect was seen in concentration 150 µg/mL ($P>0.05$) of unfermented rooibos. See Figure 4.9b.

Lastly, the percentage of uncapacitated, acrosome-intact spermatozoa decreased significantly at the 150 µg/ml concentration of unfermented rooibos extract in both the donor and the combined groups (donors: $P<0.0001$; combined: $P<0.0001$), while the patient group was unaffected (patients: $P=0.623$).Also, two-way ANOVA revealed a significant treatment effect ($P<0.05$) in uncapacitated acrosome intact sperm of donors and patients in concentration as high as 0 µg/mL, and 15µg/Ml of unfermented rooibos,no significant effect was seen in concentration 0.15 µg/mL,1.5 µg/mL, and150 µg/mL ($P>0.05$) of unfermented rooibos See Figure 4.9c.



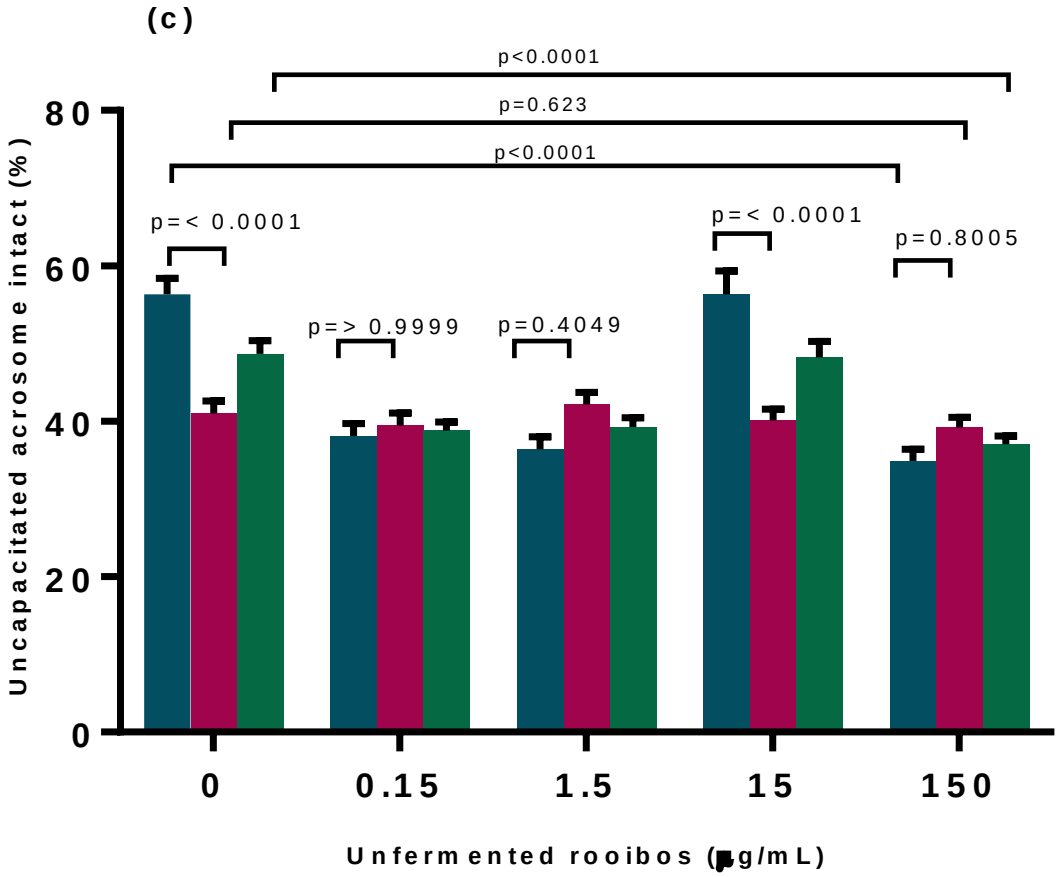
Values represented are the mean ± SEM after one-hour incubation with various concentrations of unfermented rooibos. (Colour code: Blue = healthy donor; Pink = infertile patient; Green = combined)

Figure 3.9: Effect of unfermented rooibos on capacitated and (a) acrosome-reacted; (b) capacitated acrosome intact ; (c) uncapacitated acrosome intact spermatozoa.



Values represented are the mean ± SEM after one-hour incubation with various concentrations of unfermented rooibos. (Colour code: Blue = healthy donor; Pink = infertile patient; Green = combined)

Figure 4.9b: Effect of unfermented rooibos on capacitated and acrosome-intact spermatozoa



Values represented are the mean ± SEM after one-hour incubation with various concentrations of unfermented rooibos. (Colour code: Blue = healthy donor; Pink = infertile patient; Green = combined)

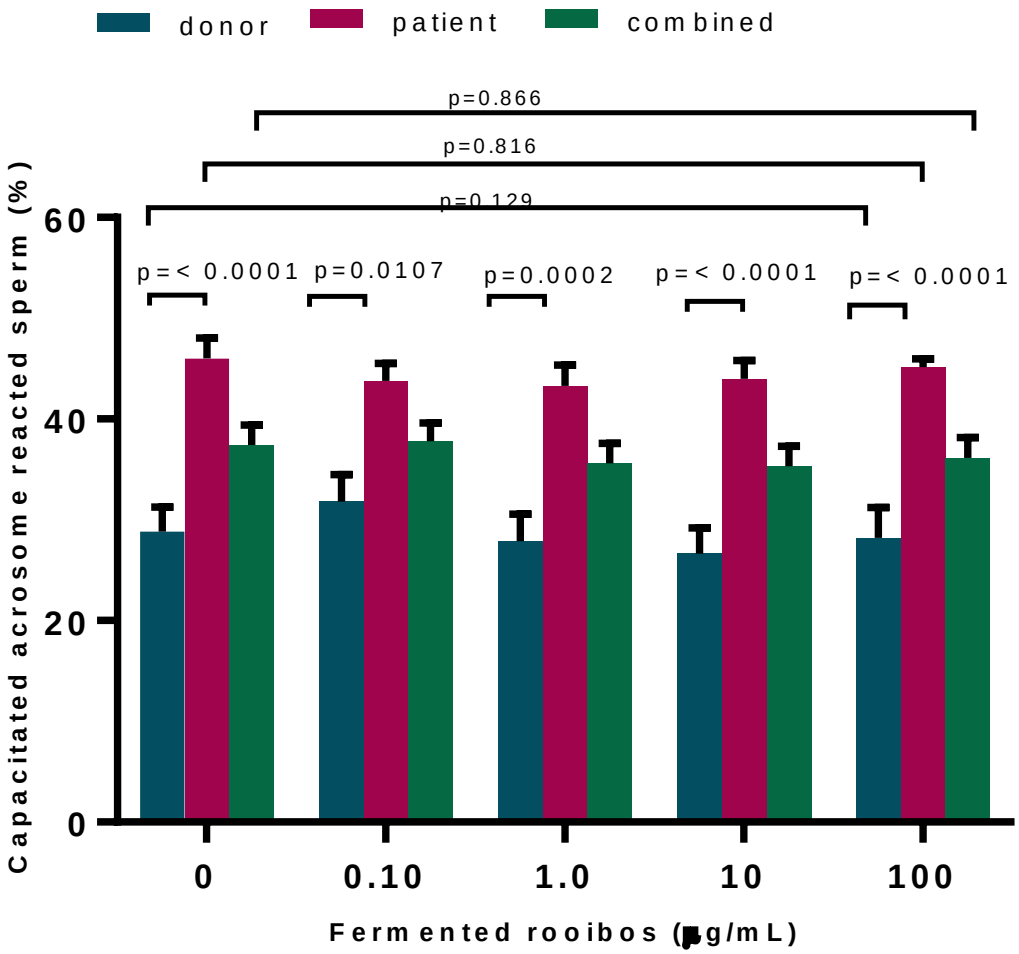
Figure 4.9c: Effect of unfermented rooibos on uncapacitated and acrosome-intact spermatozoa

On the contrary, incubation of human sperm with increasing concentrations of aqueous extracts of fermented rooibos had no significant effects on the percentage of capacitated, acrosome-reacted sperm in the donor, patient and combined groups (donors: P=0.129; patients: P=0.816; combined: P=0.866). See Figure 4.10a. In addition, the repeated measures ANOVA showed no trends in the percentage of capacitated, acrosome-reacted spermatozoa in the groups (donors: P=0.419; patients: P=0.617; combined: P=0.496). Further analysis revealed significant treatment effects on capacitated acrosome reacted

sperm of donors and patients in all concentration (0 µg/mL,0.10 µg/mL,1.0 µg/mL,10 µg/mL,100 µg/mL) of fermented rooibos (two-way ANOVA: $P<0.05$). See Figure 4.10a.

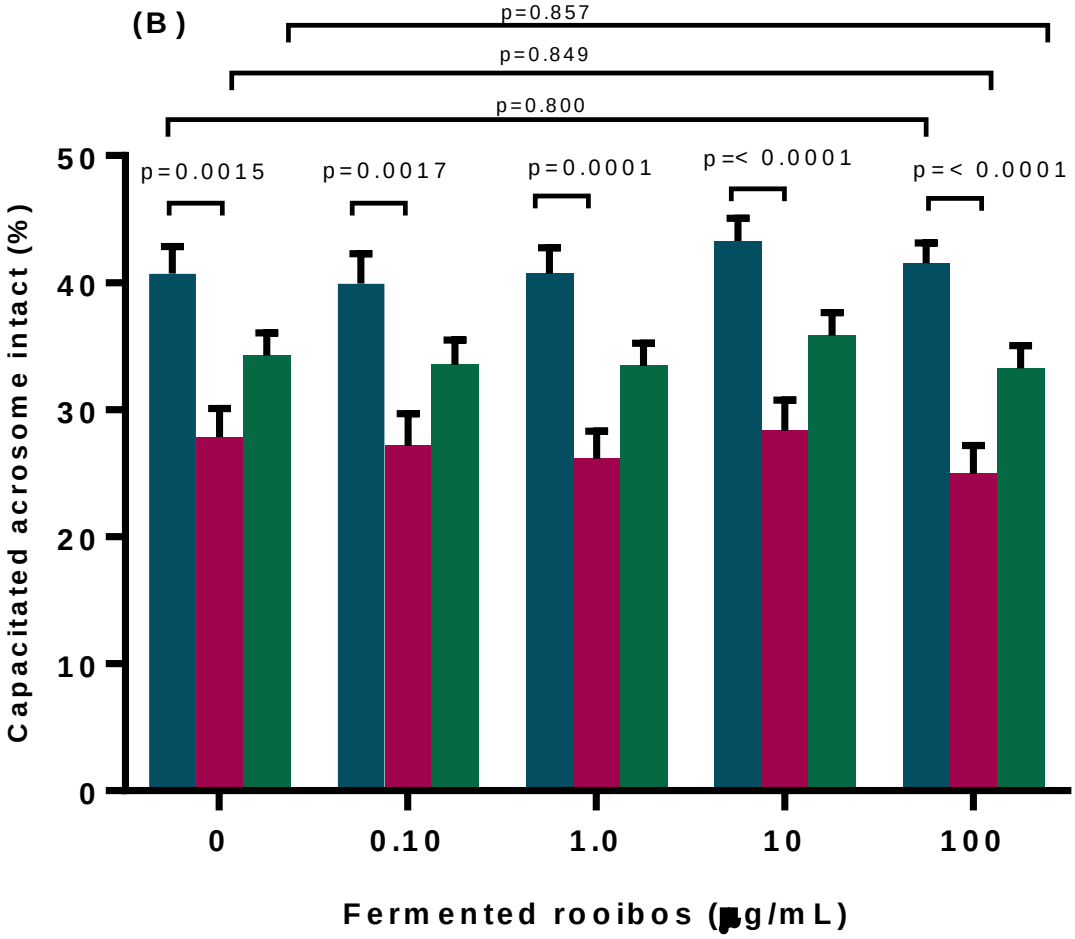
Moreover, no significant differences or trends were observed in the percentage of capacitated, acrosome-intact spermatozoa in the donor, patient or combined group (One-way ANOVA donors: $P=0.800$; patients: $P=0.849$; combined: $P=0.857$ and repeated measures ANOVA donors: $P=0.789$; patients: $P=0.702$; combined: $P=0.658$). Further analysis showed a significant treatment effects on capacitated acrosome intact sperm of donors and patients in all concentration 0 µg/mL,0.10 µg/mL,1.0 µg/mL,10 µg/mL,100 µg/mL of fermented rooibos (two-way ANOVA: $P<0.05$) See Figure 4.10b.

Furthermore, fermented rooibos had no significant effect on the percentage of uncapacitated, acrosome-intact sperm (One-way ANOVA donors: $P=0.516$; patients: $P=0.305$; combined: $P=0.857$ and repeated measures ANOVA donors: $P=0.516$; patients: $P=0.172$; combined: $P=0.366$).Incubation with fermented rooibos revealed a significant treatment effect (two-way ANOVA:$P<0.05$) in the percentage of uncapacitated acrosome intact sperm in all concentrations (0.10 µg/mL,1.0 µg/mL,10 µg/mL,100 µg/mL)of fermented rooibos,while the control showed no significant effect in two groups (two-way ANOVA:$P>0.05$). See Figure 4.10c.



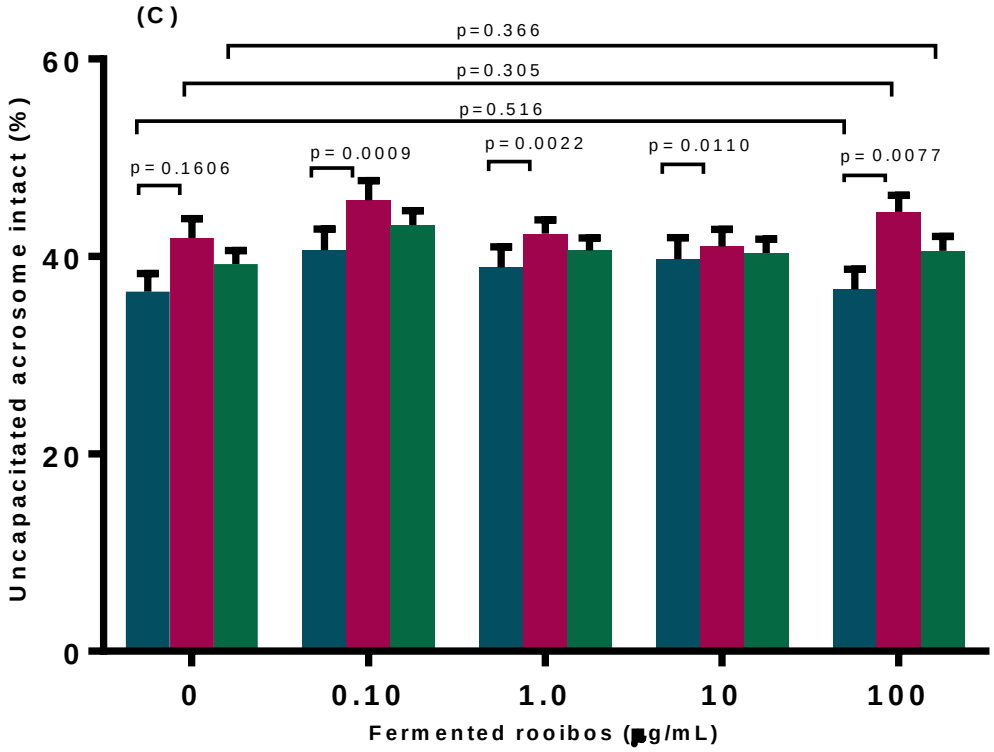
Values represented are the mean ± SEM after one-hour incubation with various concentrations of fermented rooibos. (Colour code: Blue = healthy donor; Pink = infertile patient; Green = combined)

Figure 3.10: Effect of fermented rooibos on (a) acrosome-reacted; (b) capacitated acrosome intact ; (c) uncapacitated acrosome reacted spermatozoa.



Values represented are the mean ± SEM after one-hour incubation with various concentrations of fermented rooibos. (Colour code: Blue = healthy donor; Pink = infertile patient; Green = combined)

Figure 4.10b: Effect of fermented rooibos on capacitated and acrosome-intact spermatozoa



Values represented are the mean ± SEM after one-hour incubation with various concentrations of fermented rooibos. (Colour code: Blue = healthy donor; Pink = infertile patient; Green = combined)

Figure 4.10c: Effect of fermented rooibos on uncapacitated, acrosome-intact spermatozoa

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

Table 4.4 demonstrates the correlations between the percentage of ROS-positive spermatozoa and the sperm functional parameters of intact MMP, DNA fragmentation (TUNEL) and capacitated acrosome-reacted spermatozoa following treatment with unfermented rooibos. The percentage of spermatozoa with intact MMP showed no significant correlation in the donor group (r=0.02, P=0.81), While the patient group (r=-0.32, P=0.002) showed a significant correlation between ROS and MMP, Also the combined group showed a weak significant positive correlation (r=0.47, P=0.001). No significant correlation between the percentage of ROS-positive and the percentage of sperm with acrosome-reacted sperm was observed in the donor group (r=0.06; P=0.94)

and the patient group (r=-0.10, P=0.27). However, a weak negative correlation between acrosome-reacted sperm and ROS-positive sperm was shown in the combined group (r=-0.32, P=0.001). No significant correlation was observed between TUNEL-positive sperm and ROS-positive sperm in the donor group (r=0.004, P=0.96), the patient group (r=-0.02, P=0.78).However the combined group(r=0.20, P=0.009) showed a weak significant negative correlation between percentage of ROS positive sperm with DNA fragmentated sperm.

Table 3.4: Correlation of percentage of ROS-positive sperm with sperm functional parameters in unfermented rooibos

PARAMETER	Donor		Patient		Combined	
	r	P	r	P	r	P
MMP-intact (%)	0.02	0.81	-0.32	0.0002*	0.47	0.001*
TUNEL (%)	0.004	0.96	-0,02	0.78	0.20	0.009
Capacitated, Acrosome-reacted (%)	-0.06	0.94	-0.10	0.27	-0.32	0.001*

MMP: Mitochondrial membrane potential; TUNEL: Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labelling. An asterix (*) indicate data with significant correlation.

Table 4.5 portrays the correlations between the percentage of ROS-positive spermatozoa and the sperm functional parameters of intact MMP, DNA fragmentation (TUNEL) and capacitated acrosome-reacted spermatozoa following the treatment with fermented rooibos. No significant correlation was demonstrated between the percentages of ROS-positive spermatozoa and spermatozoa with intact MMP in the patient group (r=-0.07; P=0.46), donor group (r=0.07, P=0.45).Also the combined group showed a weak significant negative correlation between ROS positive sperm with sperm that possess an intact MMP (r=-0.22, P=0.003). In addition, no significant correlation was observed between the percentage of ROS-positive spermatozoa and the percentage of TUNEL-positive sperm in the combined group (r=0.11, P=0.08), the donor group (r=-0.10, P=0.25) and the patient group (r=0.06, P=0.0.48). Furthermore, regarding the acrosome-reacted sperm, no significant correlation was seen in the donor group (r=-0.01, P=0.95), the

patient (r=0.15, P=0.10) while the combined group showed a weak signicant negative correlation (r=-0.25, P=0.002).

Table 3.5: Correlation of ROS-positive sperm with sperm functional parameters in fermented rooibos

PARAMETER	Donor		Patient		Combined	
	R	P	r	P	R	P
MMP-intact (%)	-0.07	0.46	0.07	0.45	-0.22	0.003*
TUNEL (%)	-0.10	0.25	0.06	0.48	0.11	0.08
Capacitated, Acrosome-reacted (%)	-0.01	0.95	0.15	0.10	-0.25	0.001*

MMP: Mitochondrial membrane potential; TUNEL: Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labelling. An asterix (*) indicate data with significant correlation

Table 4.6 highlights the correlations between the percentage of total motility of spermatozoa and the sperm functional parameters of intact MMP, progressive motility and capacitated acrosome-reacted spermatozoa following treatment with unfermented rooibos. A strong significant positive correlation was observed between total motility and progressive motility in the donor group (r=0.79, P=0.0001), patient group (r=0.86, P=0.0001) and the combined group (r=0.86, P=0.001). No significant correlation was observed between percentage of total motility and intact MMP spermatozoa in the donor group (r=0.06, P=0.53), the patient group (r=0.02, P=0.82) while the the combined group revealed a weak signicant positive correlation (r=0.42, P=0.001). Similarly, no significant correlation was seen between acrosome-reacted sperm and total motility sperm in the donor group (r=0.12, P=0.18), the patient group (r=-0.14, P=0.10) with the combined group showing a weak significant negative correlation (r=-0.24, P=0.0002).

Table 3.6: Correlation of total motility with sperm functional parameters in unfermented rooibos

PARAMETER	Donor		Patient		Combined	
	r	p	r	p	r	P
MMP-intact (%)	0.06	0.53	0.02	0.82	0.42	0.001*
Progressive motility	0.79	0.0001*	0.86	0.0001*	0.86	0.001*
Capacitated, Acrosome-reacted (%)	0.12	0.18	-0.14	0.10	-0.24	0.0002*

MMP: Mitochondrial membrane potential. An asterix (*) indicate data with significant correlation

Table 4.7 shows correlations between the percentage of total motility and the sperm functional parameters of progressive motility, intact MMP and capacitated, acrosome-reacted spermatozoa following treatment with fermented rooibos.The percentage of progressively motile spermatozoa showed a significant positive significant correlation with total motility in the donor group (r=0.64, P=0.001), the patient group (r=0.71, P=0.001) and the combined group (r=0.79, P=0.001). In addition, the percentage of spermatozoa with intact MMP showed no significant correlation with the percentage of total motile spermatozoa in the donor group (r=0.09, P=0.30), and the combined group (r=0.05, P=0.36) while the patient group showed a negative weak significant correlation (r=-0.18, P=0.04). Lastly, the percentage of capacitated, acrosome-reacted cells also showed a significant weak positive correlation with the percentage of total motile spermatozoa in the donor group (r=0.26, P=0.002), the patient group (r=0.38, P=0.001) and the combined group (r=0.34, P=0.001).

Table 3.7: Correlation of total motility with sperm functional parameters in fermented rooibos

PARAMETER	Donor		Patient		Combined	
	r	P	r	P	r	P
MMP-intact (%)	0.09	0.30	-0.18	0.04*	0.05	0.36
Progressive motility	0.64	0.001*	0.71	0.001*	0.79	0.001*
Capacitated, Acrosome-reacted (%)	0.26	0.002*	0.38	0.001*	0.34	0.001*

MMP: Mitochondrial membrane potential.An asterix (*) indicate data with significant correlation

Table 3.8: Correlation of total motility with sperm functional parameters in fermented rooibos

The relationships between the percentage of hyperactivated, ROS-positive spermatozoa and capacitated, acrosome-reacted spermatozoa following the treatment with fermented rooibos are shown in Table 4.8. The percentage of ROS-positive spermatozoa revealed no significant correlation with hyperactivated sperm in the donor group (r=-0.04, P=0.63), the patient group (r=0.15, P=0.83) and the combined group (r=-0.03, P=0.63). Furthermore, no significant relationship was observed between the hyperactivated spermatozoa and the percentage of acrosome-reacted sperm in the donor group (r=0.15, P=0.08), the patient group (r=0.20. P=0.73) and the combined group (r=-0.06, P=0.36)

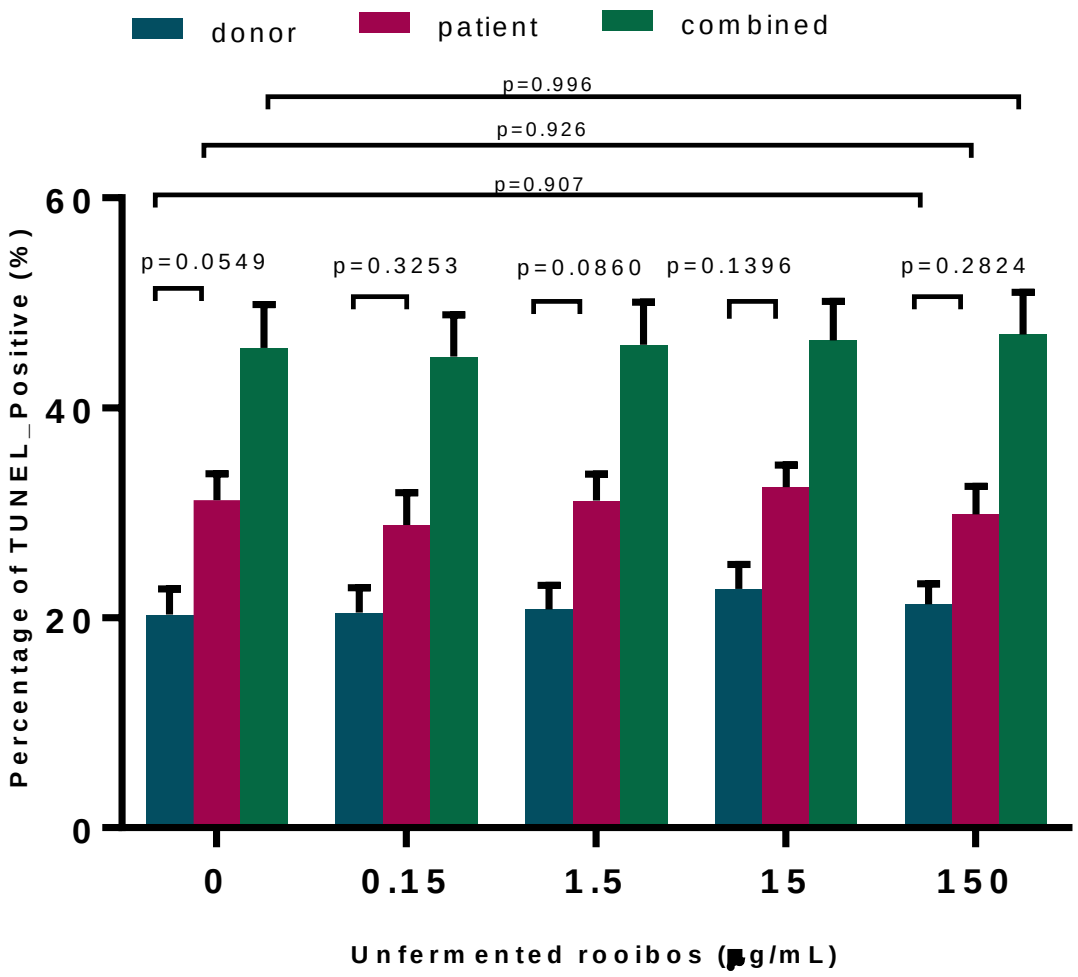
Table 3.8: Correlation of hyperactivation with sperm functional parameters for fermented rooibos

PARAMETER	**Donor**		**Patient**		**Combined**	
	r	P	r	P	r	P
ROS-positive (%)	-0.04	0.63	0.15	0.83	0.03	0.63
Capacitated, **Acrosome-reacted (%)**	0.15	0.08	0.20	0.73	-0.06	0.36

ROS: Reactive oxygen species**.** An asterix (*) indicate data with significant correlation

4.3.7 Sperm DNA fragmentation

Exposure of human sperm to increasing concentrations of aqueous extracts of unfermented rooibos had no significant effects on the percentage of TUNEL-positive sperm in the donor, patient and combined groups (donors: P=0.909; patients: P=0.926; combined: P=0.996) (Figure 4.11). Similarly, no significant trends were observed with the repeated measures ANOVA (donors: P=0.832; patients: P=0.894; combined: P=0.907).Futhermore, treatment of sperm cells with different concentrations of unfermented rooibos did not have a significant treatment effect in percentage of DNA-fragmented sperm in donors and patients (two-way ANOVA: P>0.05).See figure 4.11.

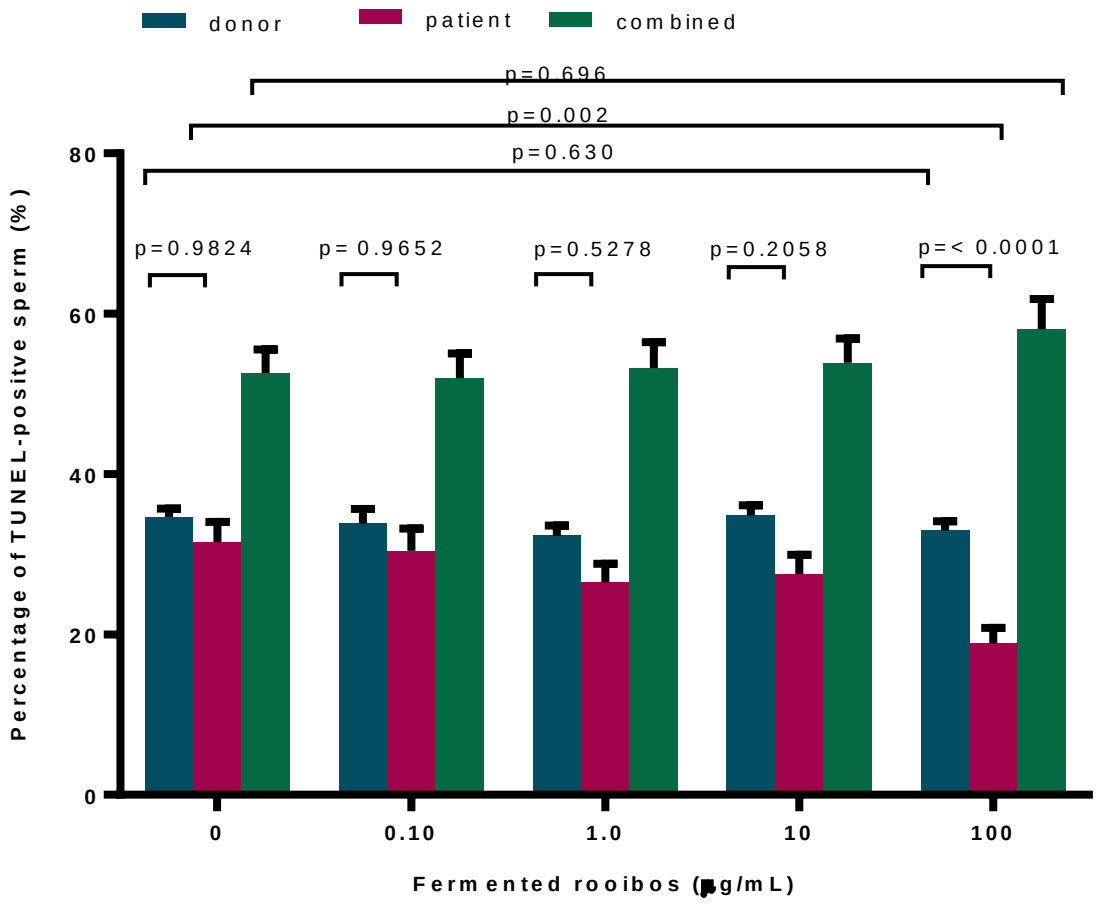


Values represented are the mean ± SEM after one-hour incubation with various concentrations of unfermented rooibos. (Colour code: Blue = healthy donor; Pink = infertile patient; Green = combined)

Figure 3.11: Effect of unfermented rooibos on human sperm DNA fragmentation

Following exposure of human sperm to the respective concentrations of fermented rooibos for one hour, a significant decrease in the percentage of TUNEL-positive sperm was observed at the 100 µg/mL concentration in the patient group (P=0.002). However, no significant difference was observed in the donor and combined groups when compared with the control group (donors: P=0.630; combined: P=0.696) (Figure 4.12). The repeated measures ANOVA demonstrated a dose-dependent decrease in the percentage of TUNEL-positive sperm in the patient group (P=0.0007). Although the repeated measures ANOVA revealed no trend in the donor group, the combined group portrayed a significant

increase in TUNEL-positive sperm in a dose-dependent manner (donors: P=0.0.595; combined: P=0.027).Also, treatment of sperm cells with different concentrations of fermented rooibos had a significant treatment effect (two-way ANOVA: P<0.05) in percentage of DNA-fragmented sperm in donors and patients in concentration as high as 100 µg/ml. While other concentrations of fermented rooibos had no significant treatment effect in the two groups (two-way ANOVA: P>0.05).See figure 4.12.



Values represented are the mean ± SEM after one-hour incubation with various concentrations of fermented rooibos. (Colour code: Blue = healthy donor; Pink = infertile patient; Green = combined)

Figure 3.12: Effect of fermented rooibos on sperm DNA fragmentation

4.4 EFFECT OF ROOIBOS ON OLIGOZOOSPERMIA AND ASTHENOZOOSPERMIA GROUPS

4.4.1 Sperm motility and kinematic parameters in oligozoospermia and asthenozoospermia groups

Incubation of human sperm with increasing concentrations of aqueous extracts of unfermented rooibos for one hour had no effect on the sperm velocity parameters in both the oligozoospermia group and the asthenozoospermia group ($P>0.05$; Table 4.9). Similarly, the extract of unfermented rooibos had no significant effect on the percentage of total motility in the oligozoospermia and asthenozoospermia groups (oligozoospermia: $P=0.713$; asthenozoospermia: $P=0.962$) (Figure 4.13a). The repeated measures ANOVA also showed no significant trend in the percentage of total motility (oligozoospermia: $P=0.238$; asthenozoospermia: $P=0.658$). Also ,no significant treatment effects was seen on the percentage of total motility of sperm in two groups after 1 hour of incubation with unfermented rooibos in all concentrations (two-way ANOVA:$P>0.05$).

In addition, there was no significant difference or trend observed in total progressive motility in either the oligozoospermia group or the asthenozoospermia group (One-way ANOVA oligozoospermia: $P=0.573$; asthenozoospermia: $P=0.988$ and repeated measures ANOVA oligozoospermia: $P=0.157$; asthenozoospermia: $P=0.809$) (Figure 4.13b).More so a significant treatment effects was seen on the percentage of sperms with progressive motility in two groups after 1 hour of incubation with concentration 0.15 µg/mL unfermented rooibos (two-way ANOVA:$P<0.05$).No effect was seen on other concentrations (0 µg/mL,1.5 µg/mL,15 µg/mL,150 µg/mL) of unfermented rooibos(Figure 4.13b).

Furthermore, unfermented rooibos had no significant effect on the non-progressive motility in either group (One-way ANOVA oligozoospermia: $P=0.373$; asthenozoospermia; $P=0.667$ and repeated measures ANOVA oligozoospermia: $P=0.374$; asthenozoospermia: $P=0.302$). Also ,no significant treatment effects was seen on the percentage of non-progressive motility of sperm in two groups after 1 hour of

incubation with unfermented rooibos in all concentrations (two-way ANOVA:P>0.05) (Figure 4.13c).

Lastly, unfermented rooibos indicated no trend and had no significant effect on the percentage of immotile spermatozoa in either group (One-way ANOVA oligozoospermia: P=0.398; asthenozoospermia: P=0.966 and repeated measures ANOVA oligozoospermia: P=0.076; asthenozoospermia: P=0.831). More so ,no significant treatment effects was seen on the percentage of immotile sperm in two groups after 1 hour of incubation with unfermented rooibos in all concentrations (two-way ANOVA:P>0.05) (Figure 4.13d).

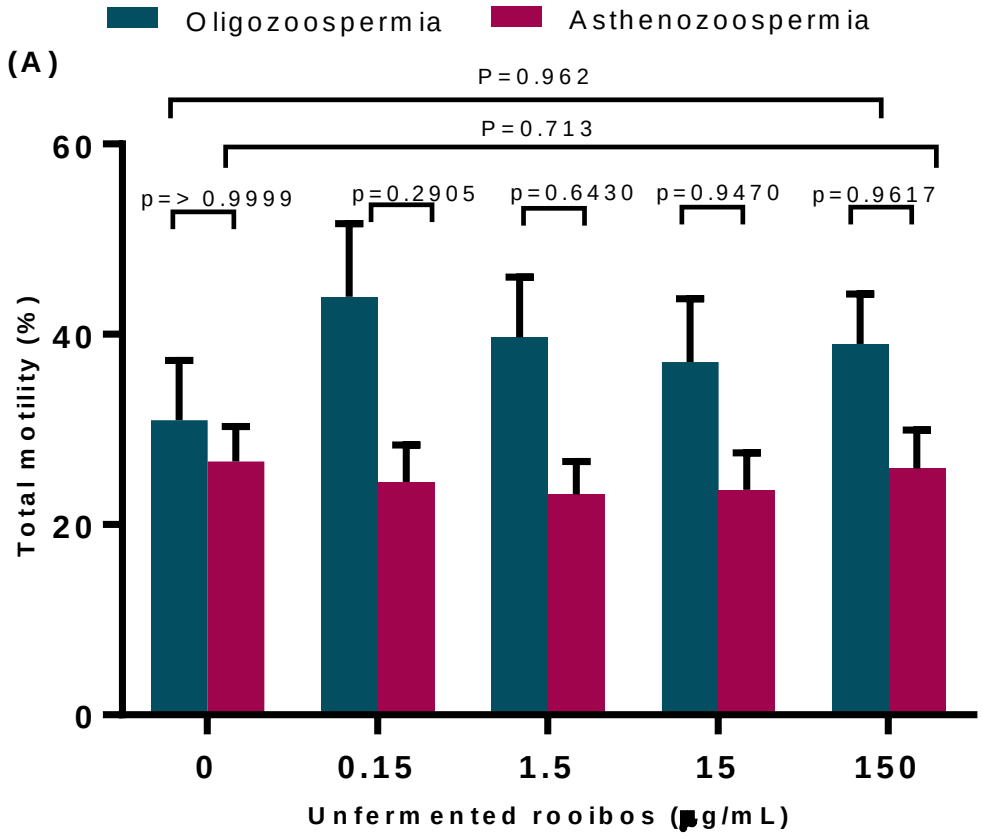
Table 3.9: Summary statistics of sperm kinematic motility parameters for unfermented rooibos

Kinematic parameter	Group	Aqueous extract of unfermented rooibos (µg /mL)					P-value
		0	0.15	1.5	15	150	
VAP (µm s^{-1})	Oligozoospermia	29.1±4.96	35.7±7.57	34.1±6.98	28.6±6.57	33.2±5.35	0.91
	Asthenozoospermia	18.8±2.03	20.1±1.72	19.6±1.78	18.1±2.28	21.7±2.47	0.76
VCL (µm s^{-1})	Oligozoospermia	62.1±7.29	66.7±7.17	64.3±7.64	60.9±6.91	64.7±6.81	0.98
	Asthenozoospermia	50.0±4.05	54.6±3.73	57.2±5.31	53.3±4.73	53.1±4.22	0.84
VSL (µm s^{-1})	Oligozoospermia	30.8±3.80	36.1±4.39	36.0±6.17	31.8±4.27	35.2±4.27	0.89
	Asthenozoospermia	29.9±3.04	29.7±1.99	30.9±2.89	30.7±3.37	33.5±3.32	0.90
ALH (µm)	Oligozoospermia	2.76±0.28	2.82±0.23	2.71±0.23	2.71±0.0.2	2.65±0.248	0.99
	Asthenozoospermia	2.11±0.06	2.21±0.05	2.42±0.13	2.33±0.13	2.09±0.07	0.10
Hyperactivation (%)	Oligozoospermia	0.429±0.2	0.929±0.4	0.786±0.2	0.643±0.3	0.571±0.2	0.83
	Asthenozoospermia	0	0.231±0.1	0.333±0.1	0.321±0.1	0	0.06
LIN (%)	Oligozoospermia	61.8±4.35	67.8±2.95	67.3±4.37	58.7±4.21	67.6±3.28	0.34
	Asthenozoospermia	59.2±2.68	60.6±2.79	62.3±2.68	58.0±2.76	64.1±2.70	0.79
STR (%)	Oligozoospermia	35.9±3.67	42.7±3.41	41.8±4.87	34.9±3.98	41.0±2.71	0.47
	Asthenozoospermia	32.4±2.56	35.6±3.03	36.6±2.95	32.3±2.99	37.5±2.68	0.72
WOB (%)	Oligozoospermia	52.5±2.55	59.5±2.86	56.6±3.58	54.7±2.78	57.3±2.10	0.47

	Asthenozoospermia	53.1±2.00	53.8±2.30	53.8±2.22	51.2±2.40	56.1±2.44	0.66
BCF (HZ)	**Oligozoospermia**	6.21±0.63	6.89±0.51	7.02±0.54	6.11±0.81	6.64±0.39	0.76
	Asthenozoospermia	6.07±1.01	6.36±0.62	6.24±1.23	6.31±1.03	8.64±1.36	0.31

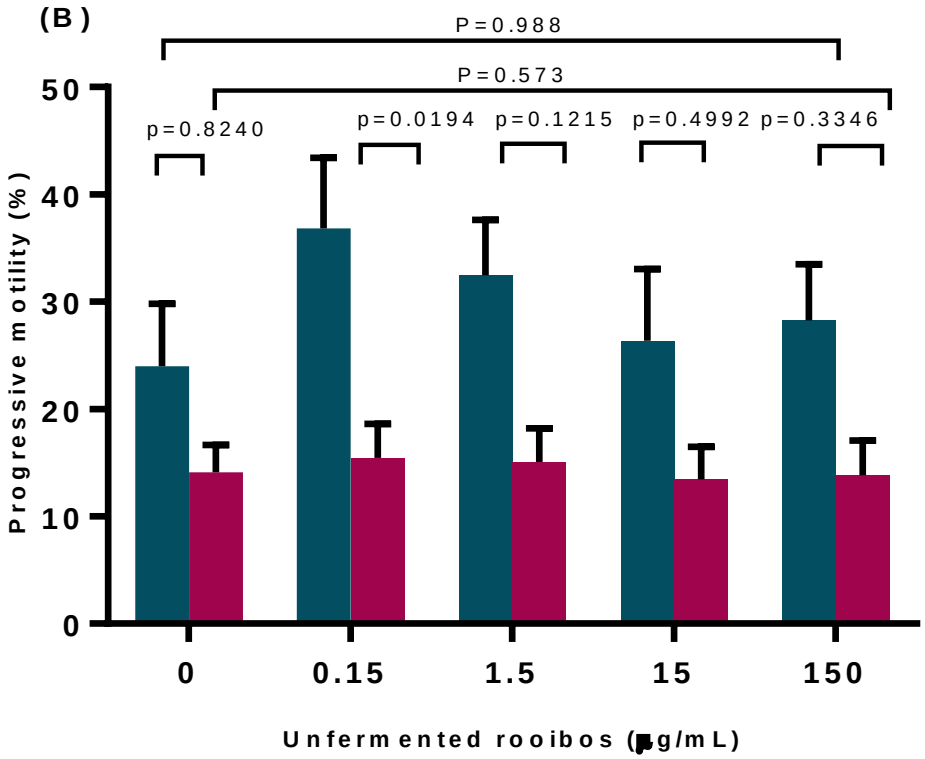
VAP: Average path velocity; VCL: Curvilinear velocity; VSL: Straight-line velocity; ALH: Amplitude of lateral head displacement; LIN: Linearity; STR: Straightness; WOB: Wobble; BCF: Beat cross frequency. An asterix (*) indicate data with significant p-vlaue

Values represented are the mean ± SEM of oligozoospermia (n=7) and asthenozoospermia (n=16) sperm exposed to the various concentrations of unfermented rooibos.



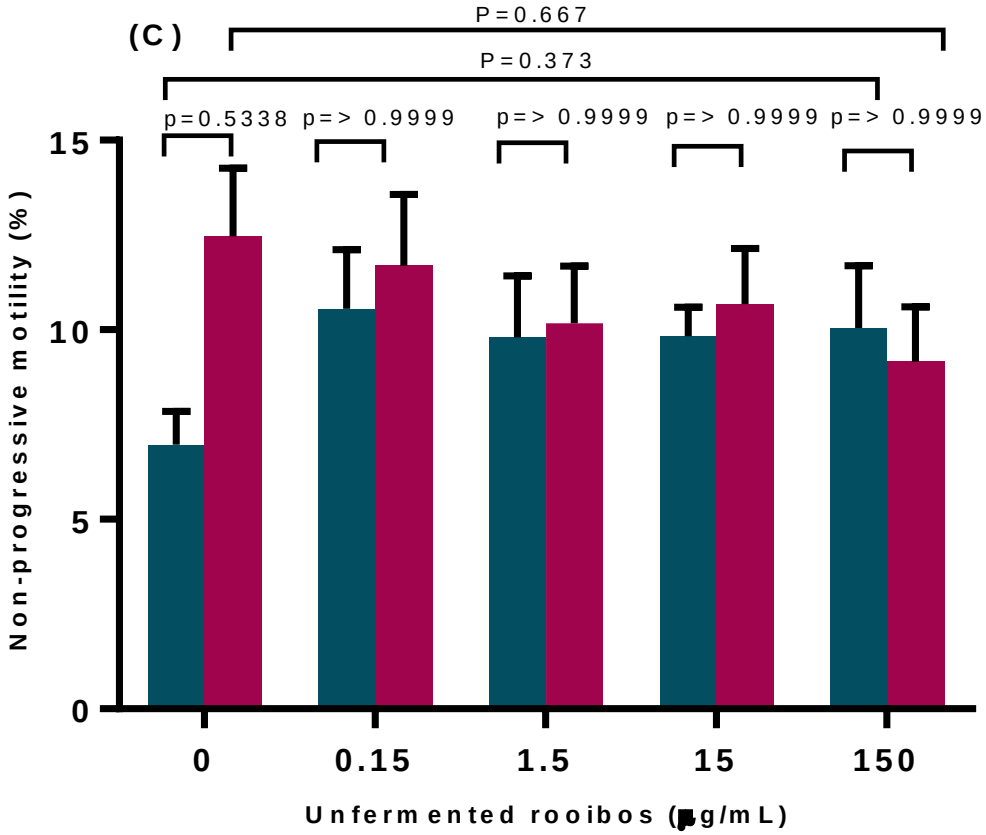
Values represented are the mean ± SEM after one-hour incubation with various concentrations of unfermented rooibos. (Colour code: Blue = oligozoospermia; Pink = asthenozoospermia)

Figure 3.13: Effect of unfermented rooibos on human sperm – (a) total motility; (b) progressive motility; (c) non progressive motility; (d) immotile spermatozoa



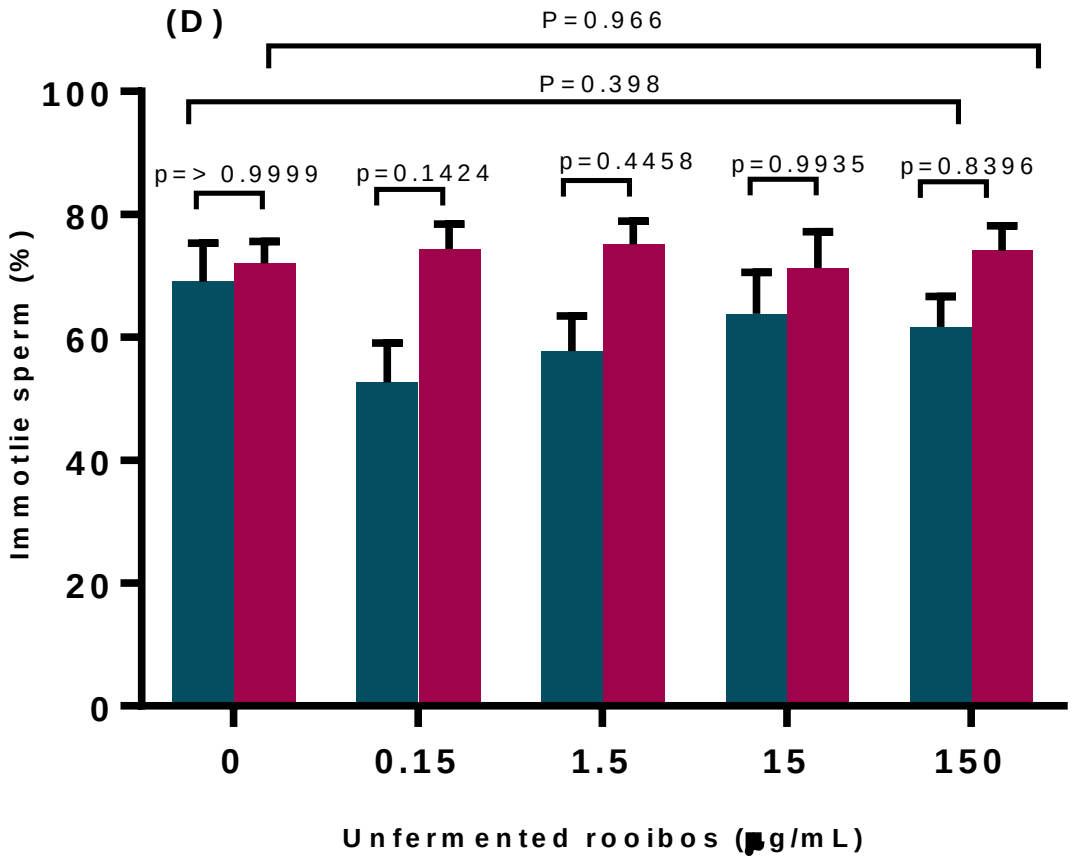
Values represented are the mean ± SEM after one-hour incubation with various concentrations of unfermented rooibos. (Colour code: Blue = oligozoospermia; Pink = asthenozoospermia)

Figure 4.13b: Effect of unfermented rooibos on human sperm – progressive motility



Values represented are the mean ± SEM after one-hour incubation with various concentrations of unfermented rooibos. (Colour code: Blue = oligozoospermia; Pink = asthenozoospermia)

Figure 4.13c: Effect of unfermented rooibos on human sperm – non-progressive motility



Values represented are the mean ± SEM after one-hour incubation with various concentrations of unfermented rooibos. (Colour code: Blue = oligozoospermia; Pink = asthenozoospermia)

Figure 4.13d: Effect of unfermented rooibos on human sperm – immotility

Exposure of human sperm to increasing concentrations of aqueous extracts of fermented rooibos had no significant effects on the sperm velocity parameters in the oligozoospermia and asthenozoospermia groups (P>0.05; Table 4.10). Similarly, fermented rooibos did not affect the percentage of total motility in either the oligozoospermia group or the asthenozoospermia group (One-way ANOVA oligozoospermia: P=0.785; asthenozoospermia: P=0.218 and repeated measures ANOVA oligozoospermia: P=0.383; asthenozoospermia: P=0.164) (Figure 4.14a). More so ,no significant treatment effects was seen on the percentage of total motility in two

groups after 1 hour of incubation with fermented rooibos in all concentrations (two-way ANOVA:P>0.05)

In addition, no significant difference or trend in total progressive motility was demonstrated in either the oligozoospermia sample or the asthenozoospermia sample (One-way ANOVA oligozoospermia: P=0.981; asthenozoospermia: P=0.729 and repeated measures ANOVA oligozoospermia: P=0.597; asthenozoospermia: P=0.314).Just as total motility ,no significant treatment effect was seen on percentage of progressive motile sperm between two group in all concentration of fermented rooibos (two-way ANOVA:P>0.05) (Figure 4.14b).

Furthermore, fermented rooibos had no significant effects on non-progressive motility in the oligozoospermia and asthenozoospermia groups (One-way ANOVA oligozoospermia: P=0.842; asthenozoospermia: P=0.321 and repeated measures ANOVA oligozoospermia: P=0.487; asthenozoospermia: P=0.262). However, a two-way ANOVA showed a significant treatment effect in two groups on the percentage of sperms with non progressive motility in all concentrations of fermented rooibos (two-way ANOVA: P>0.05) (Figure 4.14c).

Lastly, fermented rooibos revealed no trends and caused no significant changes in the percentage of immotile spermatozoa in both the oligozoospermia and asthenozoospermia groups (One-way ANOVA oligozoospermia: P=0.196; asthenozoospermia: P=0.554 and repeated measures ANOVA oligozoospermia: P=0.452; asthenozoospermia: P=0.479).More so, two-way ANOVA revealed a significant treatment effect on immotile sperm on concentration 0µg /mL,0.10µg /mL and 100µg /mL of fermented rooibos in two groups (Figure 4.14d).

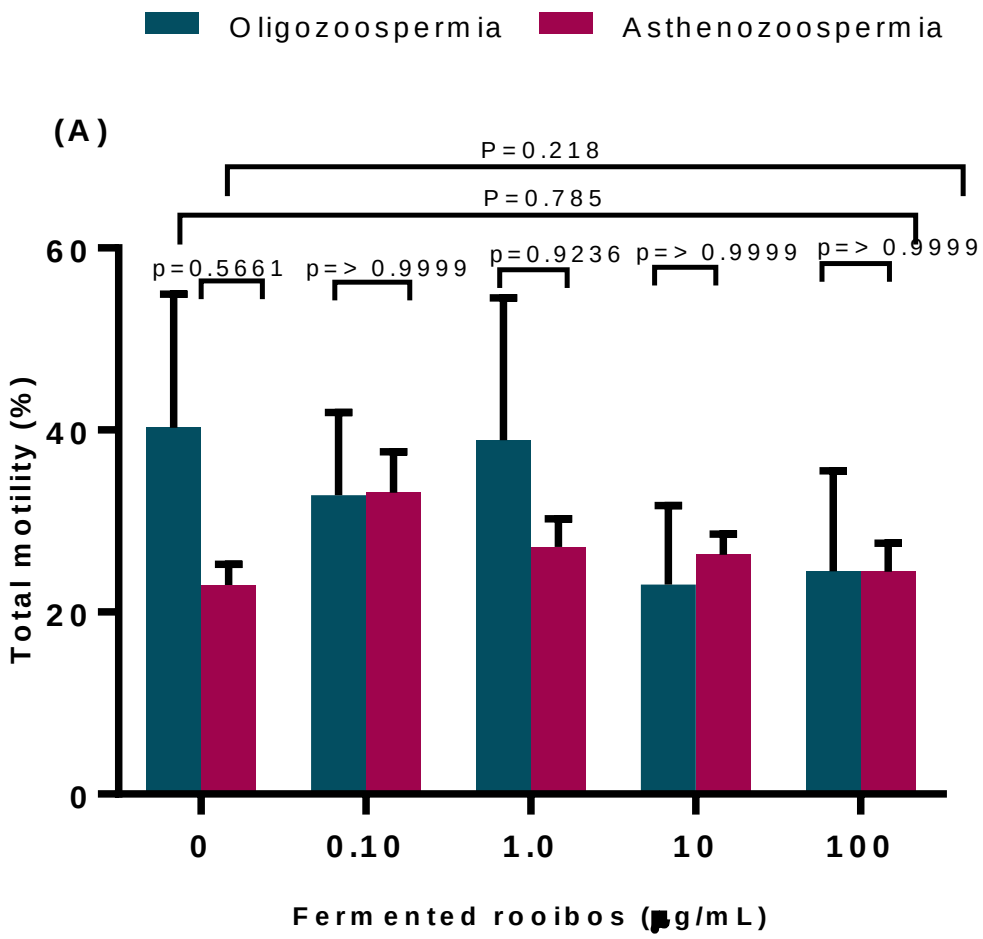
Table 3.10: Summary statistics of sperm kinematic motility parameters for fermented rooibos

Kinematic parameter	Group	Aqueous extract of fermented rooibos (µg/mL)					P-value
		0	0.10	1.0	10	100	
VAP (µm s-1)	Asthenozoospermia	27.4±4.02	19.9±2.79	25.4±3.38	25.1±3.31	25.5±3.48	0.68
	Oligozoospermia	18.3±3.23	16.6±1.45	23.0±7.25	22.7±2.70	32.0±10.4	0.45
VCL (µm s-1)	Asthenozoospermia	52.5±5.03	50.8±5.77	50.6±4.14	49.1±4.12	50.6±4.40	0.99
	Oligozoospermia	51.2±8.09	46.0±7.20	40.5±2.03	52.0±7.14	61.2±12.5	0.60
VSL (µm s-1)	Asthenozoospermia	33.8±3.8	31.6±4.26	32.1±3.16	31.0±3.12	32.0±3.27	0.98
	Oligozoospermia	32.0±10.4	27.0±3.66	24.7±2.62	30.2±7.16	38.7±9.33	0.53
ALH (µm)	Asthenozoospermia	67.3±3.71	67.5±2.60	65.2±3.30	66.3±3.31	64.7±3.04	0.98
	Oligozoospermia	2.05±0.11	1.92±0.08	2.26±0.14	1.93±0.19	2.14±0.07	0.35
Hyperactivation (%)	Asthenozoospermia	0.045±0.05	0.18±0.07	0.05±0.05	0.04±0.04	0.136±0.13	0.63
	Oligozoospermia	0	0	0.167±0.16	0.67±0.16	0	0.58
LIN (%)	Asthenozoospermia	67.3±3.71	67.5±2.60	65.2±3.30	66.3±3.31	64.7±3.04	0.96
	Oligozoospermia	57.8±2.22	55.9±2.76	63.3±8.13	62.3±3.60	65.9±8.88	0.73

STR (%)	**Asthenozoospermia**	43.6±3.49	42.0±3.19	42.3±2.90	33.1±3.18	41.8±3.06	0.99
	Oligozoospermia	31.0±3.87	30.5±1.70	34.2±2.76	37.5±2.30	43.0±9.99	0.44
WOB (%)	**Asthenozoospermia**	59.6±2.30	58.1±2.30	59.7±1.79	59.0±2.36	57.1±1.85	0.98
	Oligozoospermia	50.6±2.94	51.7±0.95	50.7±1.28	54.6±0.60	57.6±1.19	0.59
BCF (HZ)	**Asthenozoospermia**	5.54±0.63	7.42±1.32	5.57±0.665	5.61±0.65	6.49±0.66	0.43
	Oligozoospermia	5.34±1.27	4.82±0.35	4.47±0.370	4.94±0.14	4.70±1.09	0.94

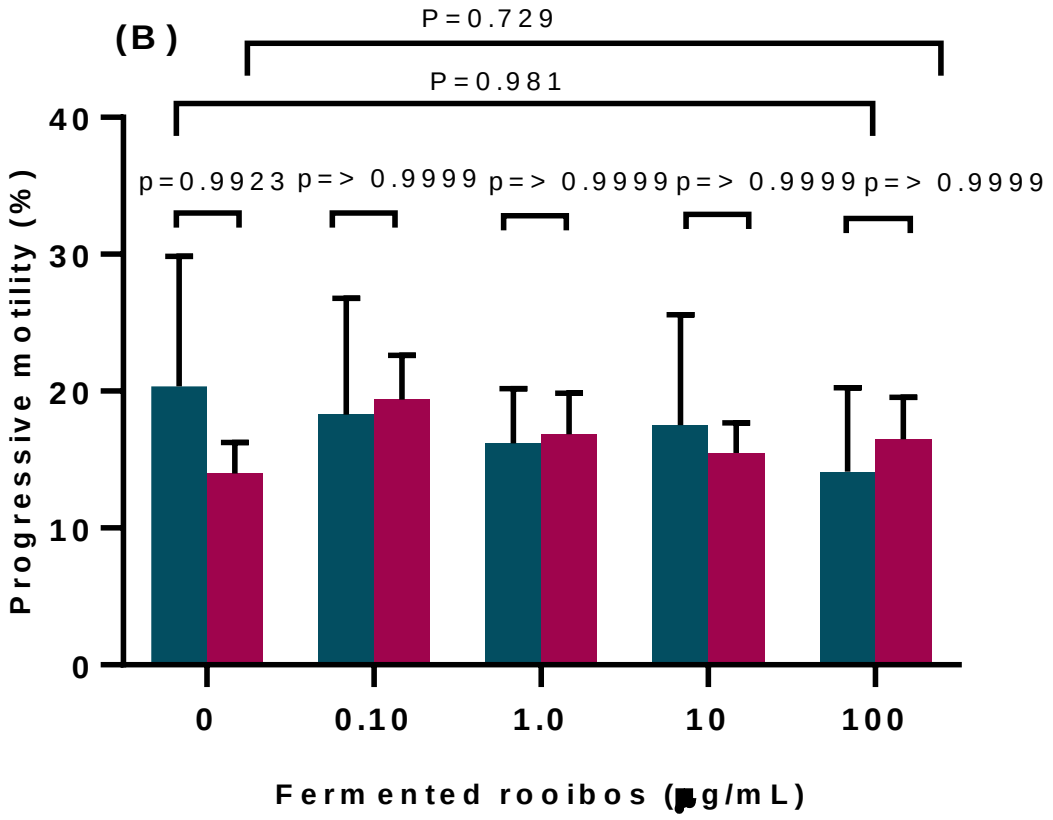
VAP: Average path velocity; VCL: Curvilinear velocity; VSL: Straight-line velocity; ALH: Amplitude of lateral head displacement; LIN: Linearity; STR: Straightness; WOB: Wobble; BCF: Beat cross frequency. An asterix (*) indicate data with significant p-vlaue

Values represented are the mean ± SEM of oligozoospermia (n=3) and asthenozoospermia (n=11) sperm exposed to the various concentrations of fermented rooibos.



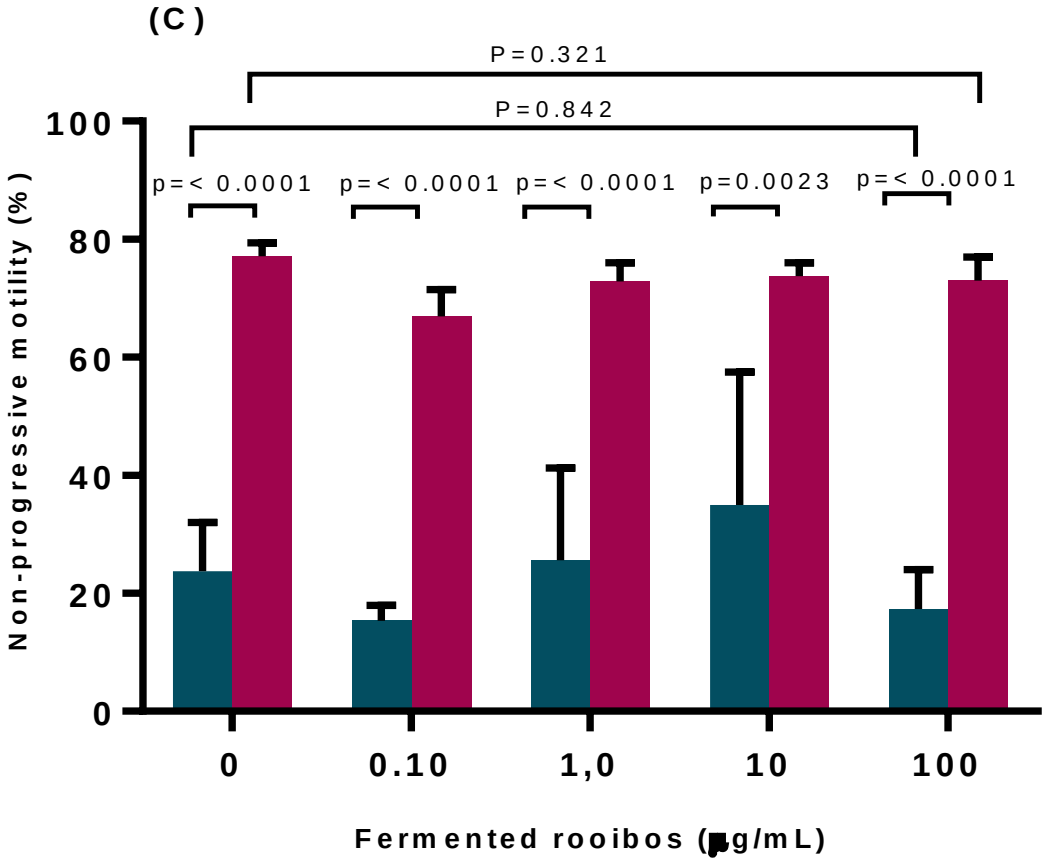
Values represented are the mean ± SEM after one-hour incubation with various concentrations of fermented rooibos. (Colour code: Blue = oligozoospermia; Pink = asthenozoospermia)

Figure 3.14: Effect of fermented rooibos on human sperm – (a) total motility; (b) progressive motility ;(c) non progressive motility; (d) immotile spermatozoa.



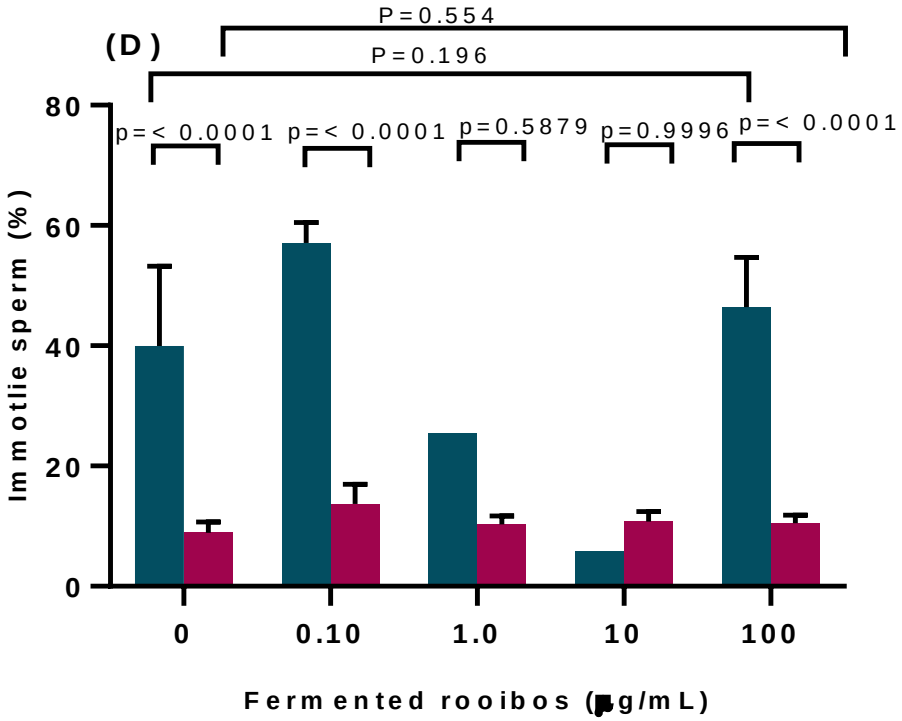
Values represented are the mean ± SEM after one-hour incubation with various concentrations of fermented rooibos. (Colour code: Blue = oligozoospermia; Pink = asthenozoospermia)

Figure 4.14b: Effect of fermented rooibos on human sperm – progressive motility



Values represented are the mean ± SEM after one-hour incubation with various concentrations of fermented rooibos. (Colour code: Blue = oligozoospermia; Pink = asthenozoospermia)

Figure 4.14c: Effect of fermented rooibos on human sperm – non-progressive motility

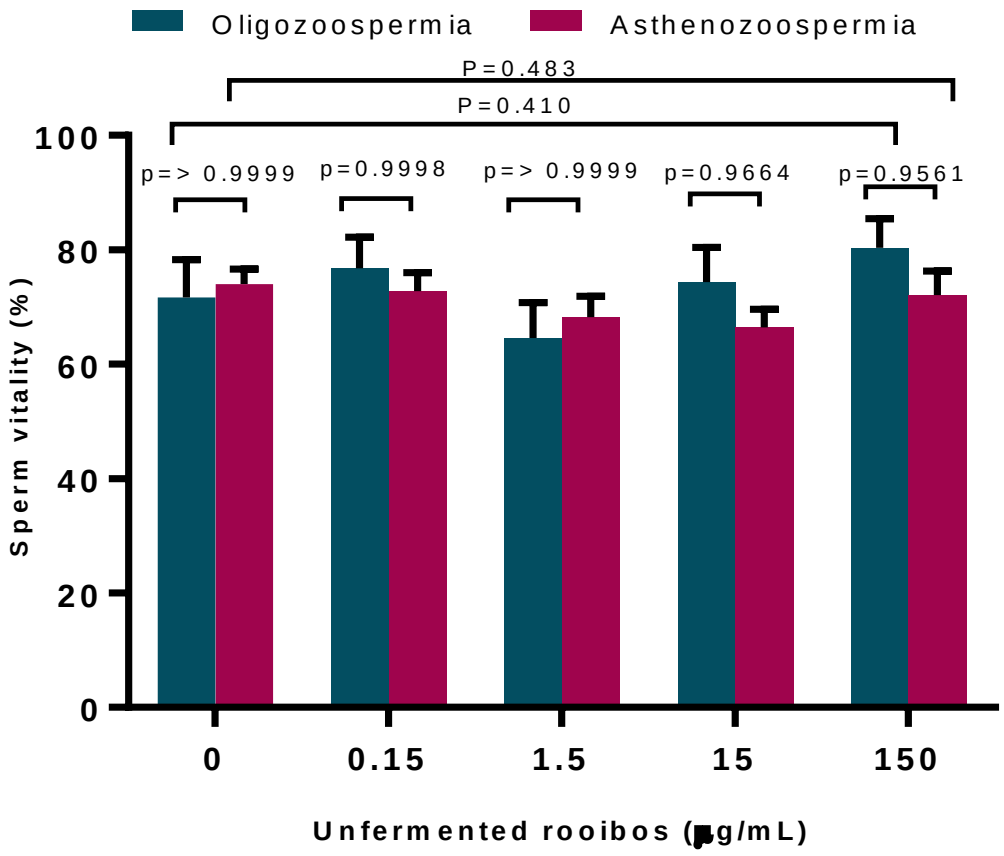


Values represented are the mean ± SEM after one-hour incubation with various concentrations of fermented rooibos. (Colour code: Blue = oligozoospermia; Pink = asthenozoospermia)

Figure 4.14d: Effect of fermented rooibos on human sperm – immotility

4.4.2 Sperm vitality

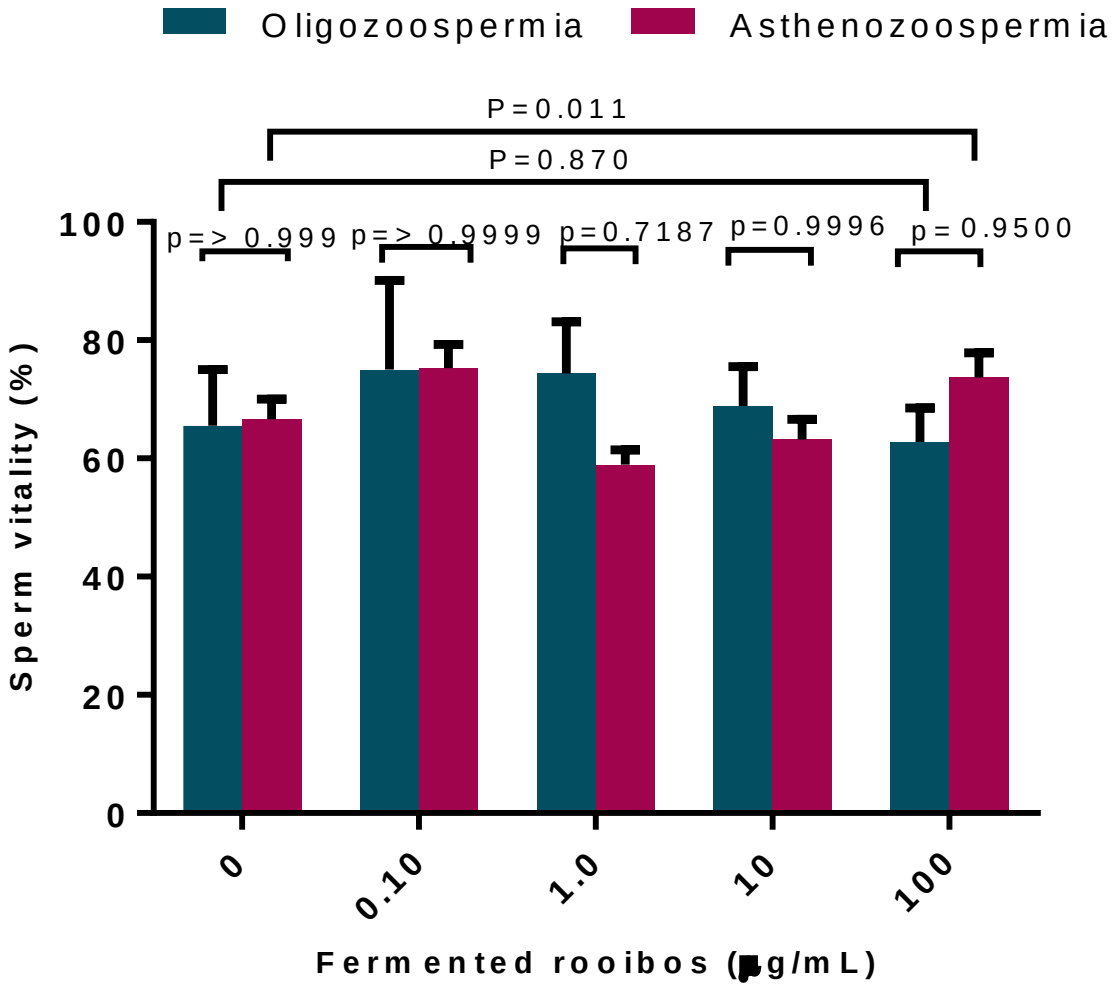
Incubation of human sperm with increasing concentrations of aqueous extracts of unfermented rooibos had no significant effects on the percentage of live sperm in the oligozoospermia and asthenozoospermia groups (oligozoospermia: P=0.410; asthenozoospermia: P=0.483). Further analysis showed no significant treatment effects on percentage of live sperm in two groups after 1 hour of incubation with unfermented rooibos in all concentrations (two-way ANOVA:P>0.05) (Figure 4.15).



Values represented are the mean ± SEM after one-hour incubation with various concentrations of unfermented rooibos. (Colour code: Blue = oligozoospermia; Pink = asthenozoospermia)

Figure 3.15: Effect of unfermented rooibos on human sperm vitality in oligozoospermia and asthenozoospermia samples

However, exposure of human sperm to the respective concentrations of fermented rooibos for one hour caused a significant increase in concentration 0.10 µg/mL in the percentage of live sperm in the asthenozoospermia group (P=0.011) (Figure 4.16). No significant change was seen in the oligozoospermia group (P=0.87) and no trend was observed (repeated measures ANOVA: P=0.734). Also, no significant treatment effects on percentage of live sperm in two groups after 1 hour of incubation with fermented rooibos in all concentrations (two-way ANOVA :P>0.05) (Figure 4.16).



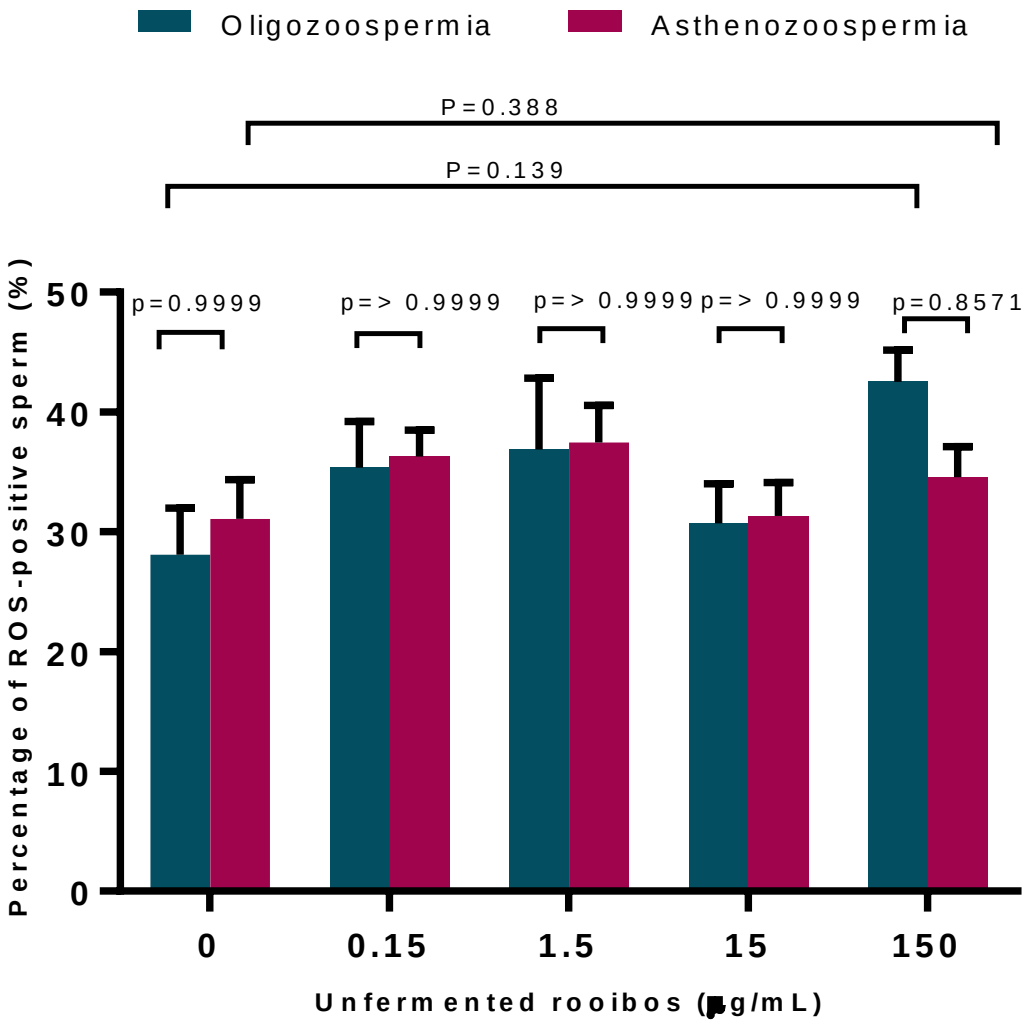
Values represented are the mean ± SEM after one-hour incubation with various concentrations of fermented rooibos. (Colour code: Blue = oligozoospermia; Pink = asthenozoospermia)

Figure 3.16: Effect of fermented rooibos on human sperm vitality in oligozoospermia and asthenozoospermia samples

4.4.3 Reactive oxygen species in the human sperm

Incubation of human sperm with increasing concentrations of aqueous extracts of unfermented rooibos for one hour caused no change in the percentage of ROS-positive sperm in the oligozoospermia group (P=0.139) and the asthenozoospermia group (P=0.338). Further analysis showed no significant treatment effects on percentage of

ROS-positive sperm in two groups after 1 hour of incubation with ufermented rooibos in all concentrations (two-way ANOVA:P>0.05) (Figure 4.17).

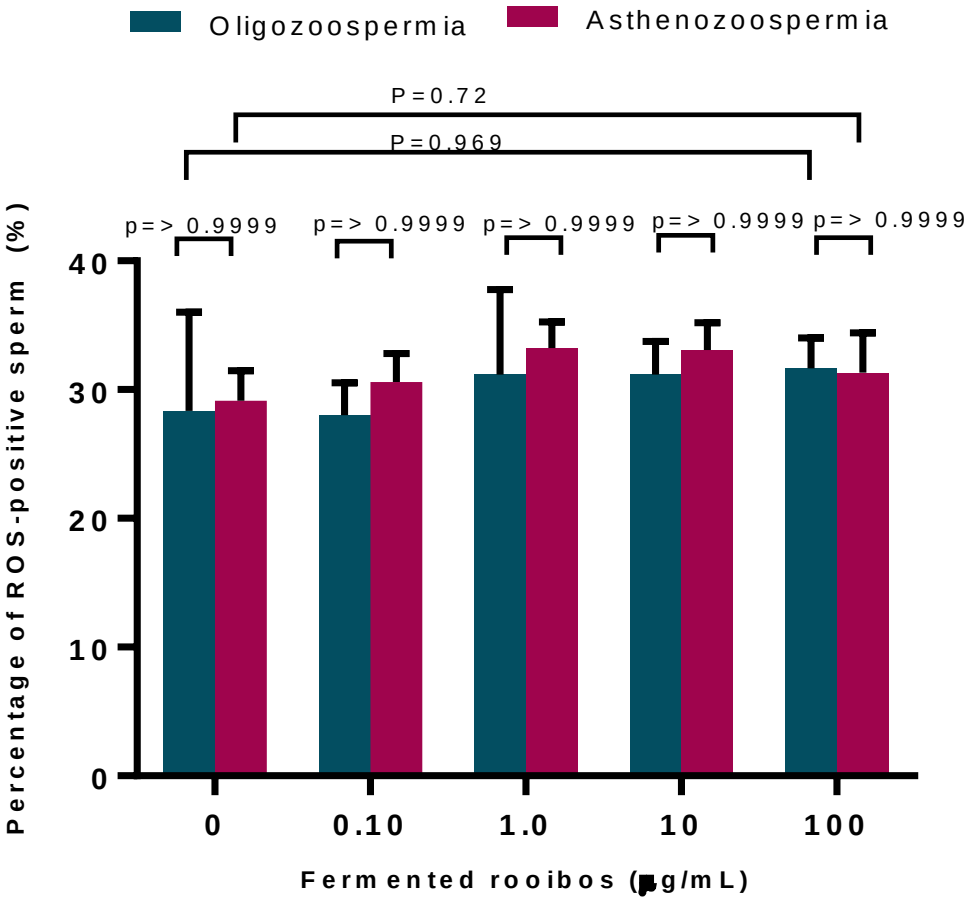


Values represented are the mean ± SEM after one-hour incubation with various concentrations of unfermented rooibos. (Colour code: Blue = oligozoospermia; Pink = asthenozoospermia)

Figure 3.17: Effect of unfermented rooibos on sperm reactive oxygen species in oligozoospermia and asthenozoospermia samples

Following the incubation of human sperm with the respective concentrations of fermented rooibos for one hour, a significant increase in the percentage of ROS-positive sperm in the asthenozoospermia group was observed at 100 µg/mL (P<0.0001; Figure 4.18). However, no significant differences in the percentages of ROS-positive sperm were

observed in the oligozoospermic samples (P=0.969; Figure 4.18). Furthermore two-way ANOVA showed no significant treatment effects on percentage of ROS-positive sperm in two groups after 1 hour of incubation with fermented rooibos in all concentrations (two-way ANOVA:P>0.05)

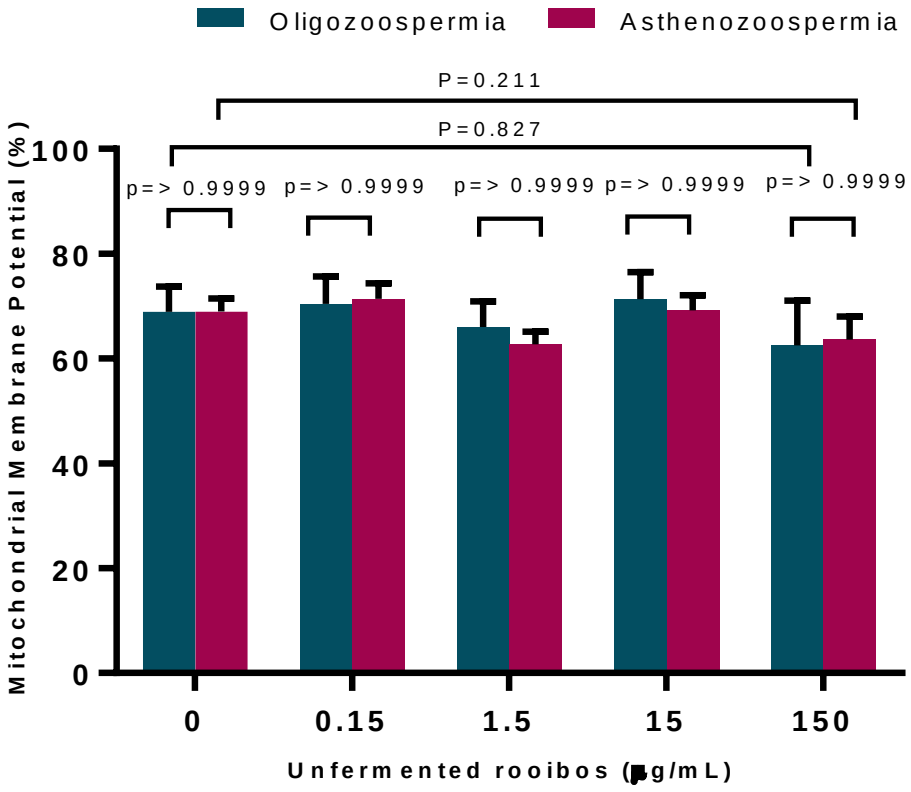


Values represented are the mean ± SEM after one-hour incubation with various concentrations of fermented rooibos. (Colour code: Blue = oligozoospermia; Pink = asthenozoospermia)

Figure 3.18: Effect of fermented rooibos on sperm reactive oxygen species in oligozoospermia and asthenozoospermia samples

4.4.4 Mitochondrial membrane potential in the human sperm

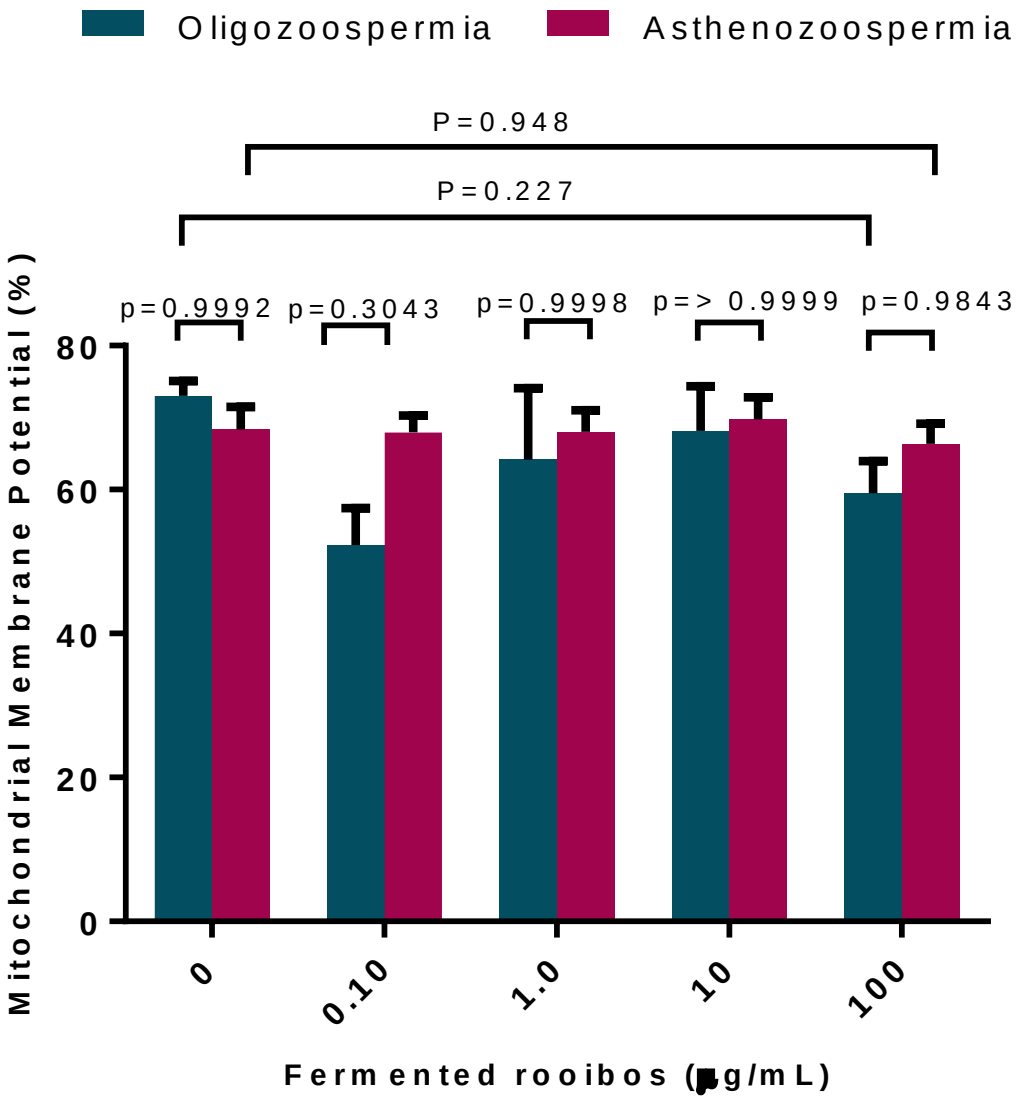
Exposure of human sperm to increasing concentrations of aqueous extracts of unfermented rooibos caused no significant change in the percentage of sperm with intact MMP in both the oligozoospermia group (P=0.827) and the asthenozoospermia group (P=0.221) (Figure 4.19). Similarly, no trend in the percentage of sperm with intact MMP in either the oligozoospermia group or the asthenozoospermia group was observed (repeated measures ANOVA oligozoospermia: P=0.660; asthenozoospermia: P=0.167). More so, two-way ANOVA showed no significant treatment effects on percentage of sperms with intact mitochondria in two groups after 1 hour of incubation with unfermented rooibos in all concentrations (two-way ANOVA:P>0.05).



Values represented are the mean ± SEM after one-hour incubation with various concentrations of unfermented rooibos. (Colour code: Blue = oligozoospermia; Pink = asthenozoospermia)

Figure 3.19: Effect of unfermented rooibos on intact mitochondrial membrane potential

As in the case of unfermented rooibos, the fermented rooibos extract caused no significant change in the percentage of sperm with intact MMP in either the oligozoospermia group (P=0.227) or the asthenozoospermia group (P=0.948) (Figure 4.20). The repeated measures ANOVA also showed no trend in the percentage of sperm with intact MMP in either group (oligozoospermia: P=0.2076; asthenozoospermia: P=0.852).Two-way ANOVA also showed no significant treatment effects on percentage of sperms with intact mitochondria in two groups after 1 hour of incubation with unfermented rooibos in all concentrations (two-way ANOVA:P>0.05)



Values represented are the mean ± SEM after one-hour incubation with various concentrations of fermented rooibos. (Colour code: Blue = oligozoospermia; Pink = asthenozoospermia)

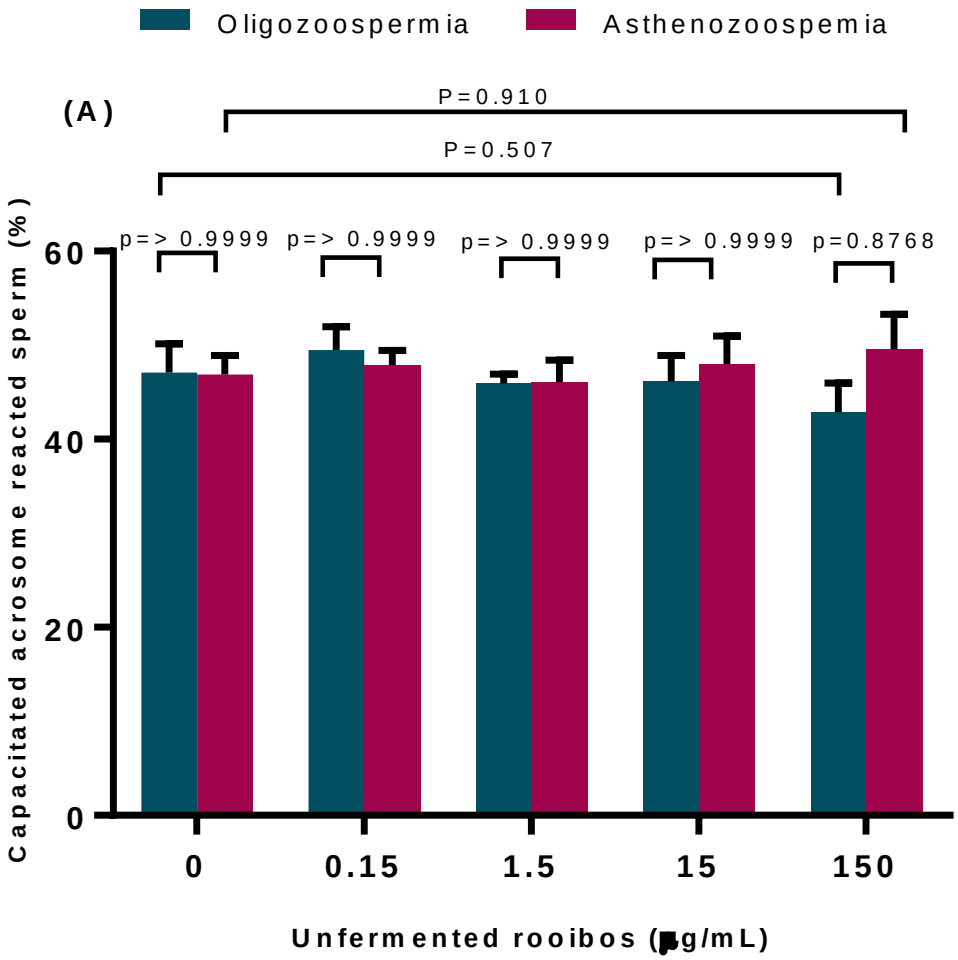
Figure 3.20: Effect of fermented rooibos on intact mitochondrial membrane potential

4.4.5 Capacitation and acrosome reaction

Incubation of human sperm with increasing concentrations of aqueous extracts of unfermented rooibos showed no significant effects in the percentage of capacitated, acrosome-reacted sperm in both the oligozoospermia group (P=0.507) and the asthenozoospermia group (P=0.910) (Figure 4.21a). Further analysis from two-way ANOVA showed no significant treatment effects on percentage of capacitated acrosome reacted sperm in two groups after 1 hour of incubation with ufermented rooibos in all concentrations (two-way ANOVA:P>0.05)

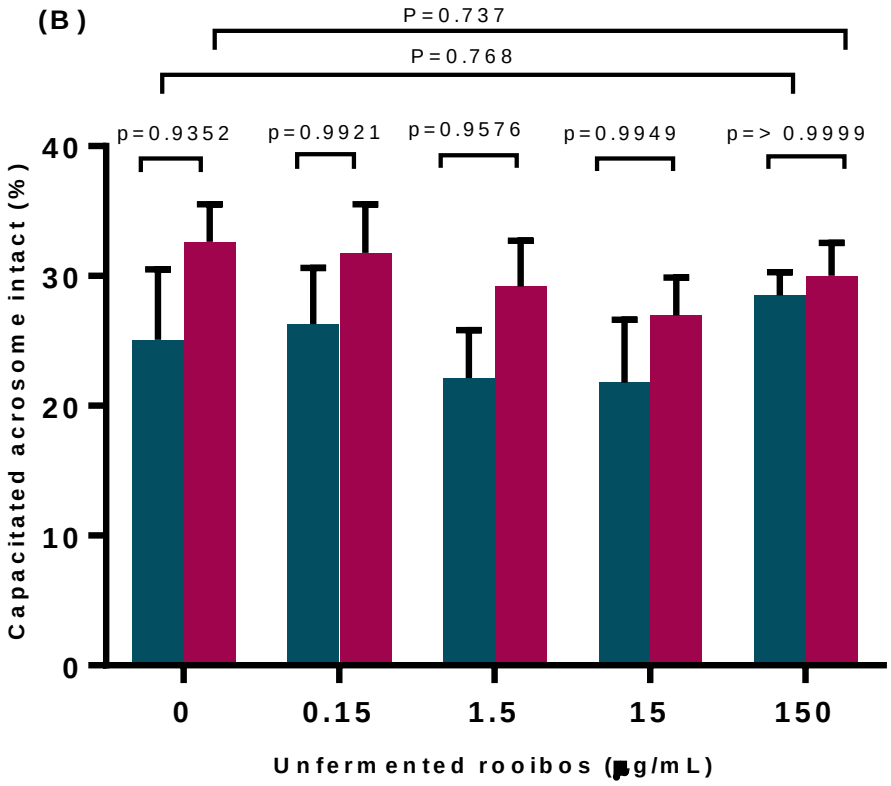
In addition, no significant difference was observed in the percentage of capacitated, acrosome-intact sperm in the oligozoospermia group (P=0.768) or the asthenozoospermia group (P=0.737).Also, two-way ANOVA showed no significant treatment effects on percentage of capacitated acrosome intact sperm in two groups after 1 hour of incubation with ufermented rooibos in all concentrations (two-way ANOVA: P>0.05) (Figure 4.21b).

It is noteworthy that unfermented rooibos caused a significant decrease in uncapacitated, acrosome-intact sperm on concentration 0.15 µg/mL in the subjects with asthenozoospermia while that of the subjects with oligozoospermia remained unchanged (One-way ANOVA oligozoospermia: P=0.949; asthenozoospermia: P=0.005) and repeated measures ANOVA oligozoospermia: P=0.853; asthenozoospermia: P=0.001). More so two-way ANOVA showed no significant treatment effects on percentage of uncapacitated acrosome intact sperm in two groups after 1 hour of incubation with ufermented rooibos in all concentrations (two-way ANOVA:P>0.05) (Figure 4.21c).



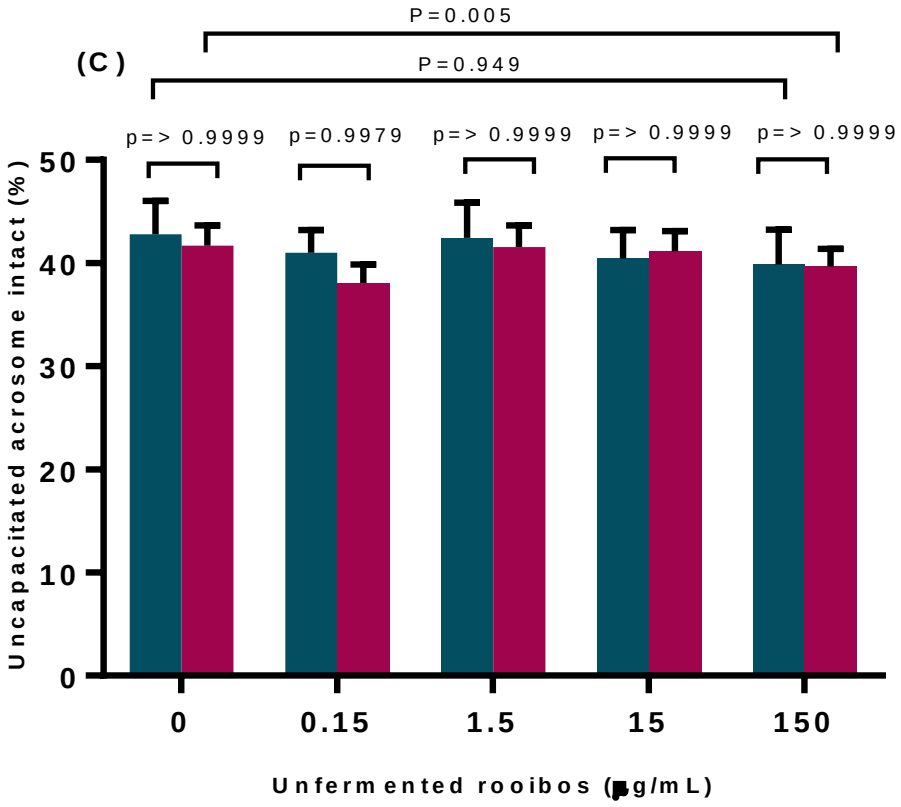
Values represented are the mean ± SEM after one-hour incubation with various concentrations of unfermented rooibos. (Colour code: Blue = oligozoospermia; Pink = asthenozoospermia)

Figure 3.21: Effect of unfermented rooibos on human sperm – (a) capacitation and acrosome-reaction; (b) capacitated acrosome intact; (c) uncapacitated acrosome intact spermatozoa.



Values represented are the mean ± SEM after one-hour incubation with various concentrations of unfermented rooibos. (Colour code: Blue = oligozoospermia; Pink = asthenozoospermia)

Figure 4.21b: Effect of unfermented rooibos on human sperm capacitation and acrosome reaction – capacitated and acrosome-intact



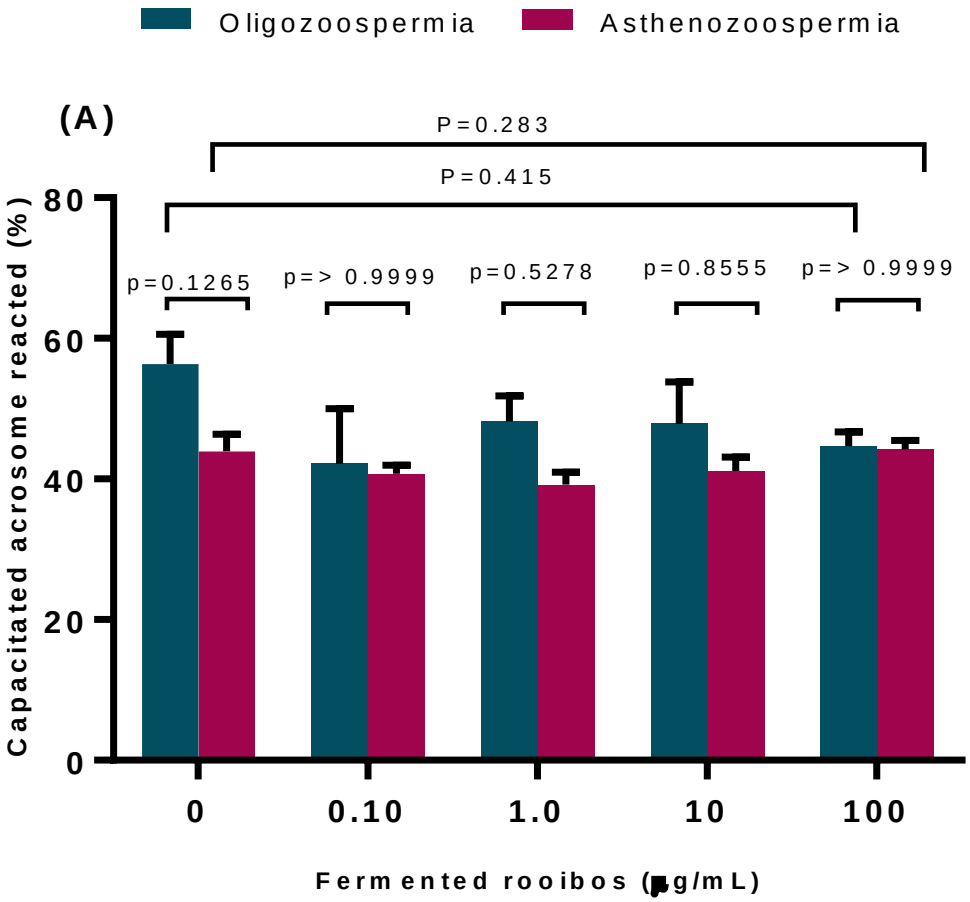
Values represented are the mean ± SEM after one-hour incubation with various concentrations of unfermented rooibos. (Colour code: Blue = oligozoospermia; Pink = asthenozoospermia)

Figure 4.21c: Effect of unfermented rooibos on human sperm capacitation and acrosome reaction – uncapacitated and acrosome-intact

As occurred with unfermented rooibos, incubation of human sperm with increasing concentrations of aqueous extracts of fermented rooibos demonstrated no significant effects in the percentage of capacitated, acrosome-reacted spermatozoa in both the oligozoospermia group (P=0.4155) and the asthenozoospermia group (P=0.238) (Figure 4.22a). The repeated measures ANOVA also showed no significant trend in the percentage of capacitated, acrosome-reacted spermatozoa in both groups (oligozoospermia: P=0.2438; asthenozoospermia: P=0.124). Also no significant treatment effects was seen on the percentage of capacitated acrosome reacted sperm in two groups after 1 hour of incubation with fermented rooibos in all concentrations (two-way ANOVA:P>0.05)

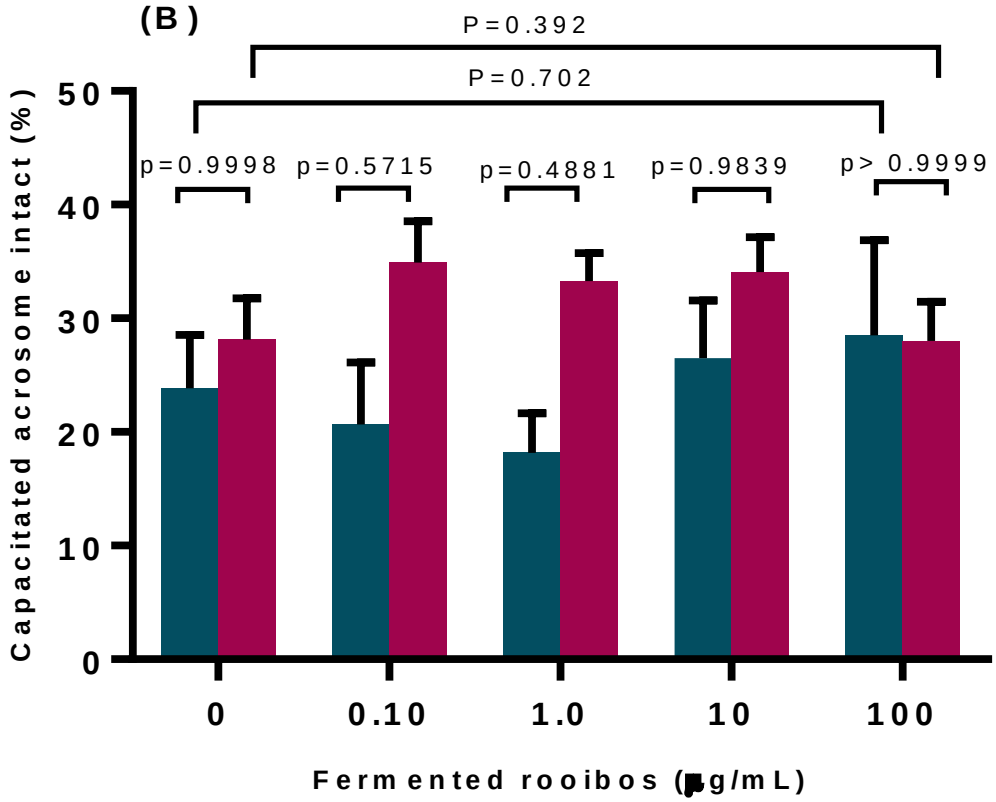
In addition, fermented rooibos caused no significant change and no trend was observed in the percentage of capacitated, acrosome-intact spermatozoa in the oligozoospermia group or the asthenozoospermia group (One-way ANOVA oligozoospermia: P=0.7021; asthenozoospermia: P=0.392 and repeated measures ANOVA oligozoospermia: P=0.612; asthenozoospermia: P=0.089). Just as above no significant treatment effects was seen on the percentage of capacitated acrosome intact sperm in two groups after 1 hour of incubation with fermented rooibos in all concentrations (two-way ANOVA:P>0.05) (Figure 4.22b).

Lastly, fermented rooibos had no significant effects on the percentage of uncapacitated, acrosome-intact spermatozoa in both groups (One-way ANOVA oligozoospermia: P=0.376; asthenozoospermia: P=0.989 and repeated measures ANOVA oligozoospermia: P=0.3119; asthenozoospermia: P=0.951). More so,no significant treatment effects was seen on the percentage of uncapacitated acrosome intact sperm in two groups after 1 hour of incubation with fermented rooibos in all concentrations (two-way ANOVA:P>0.05) (Figure 4.22c).



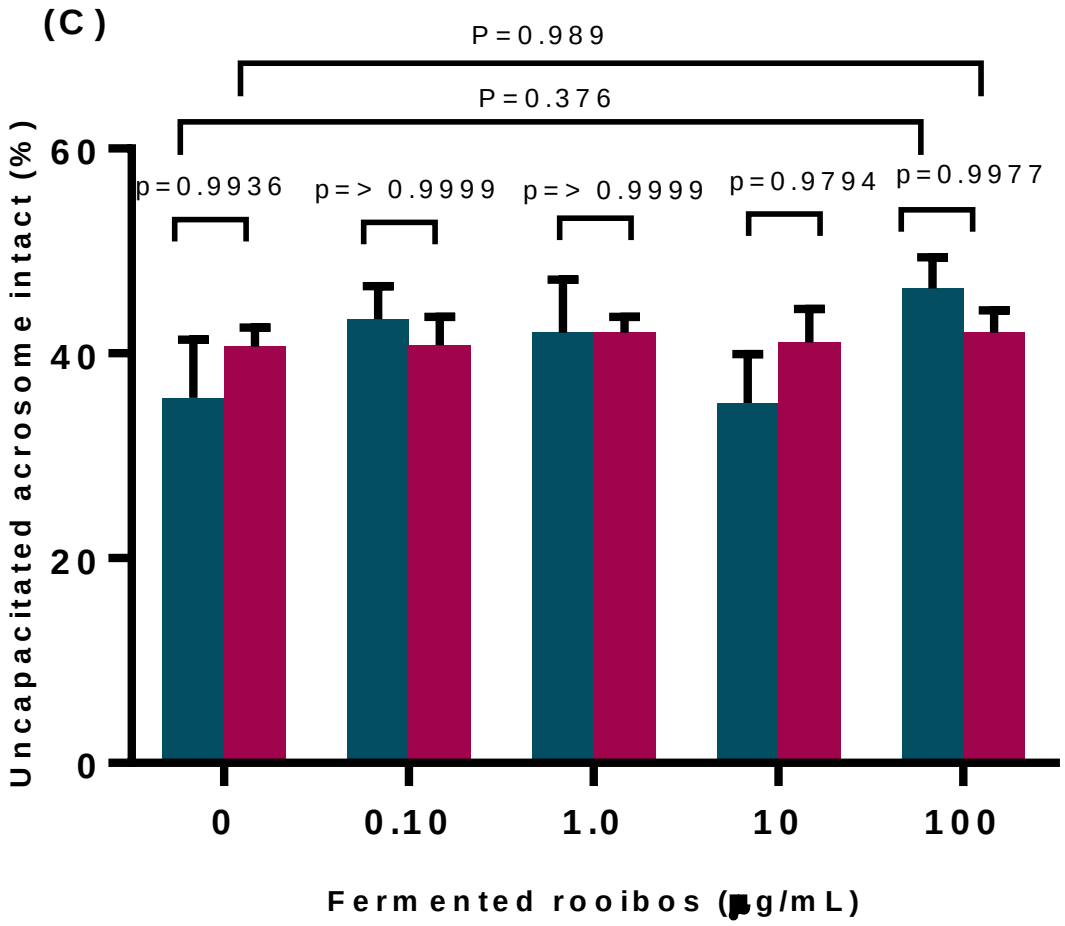
Values represented are the mean ± SEM after one-hour incubation with various concentrations of fermented rooibos. (Colour code: Blue = oligozoospermia; Pink = asthenozoospermia)

Figure 3.22: Effect of fermented rooibos on human sperm– (a) capacitation and acrosome-reaction; (b) capacitated acrosome intact; (c) uncapacitated acrosome intact spermatozoa.



Values represented are the mean ± SEM after one-hour incubation with various concentrations of fermented rooibos. (Colour code: Blue = oligozoospermia; Pink = asthenozoospermia)

Figure 4.22b: Effect of fermented rooibos on human sperm capacitation and acrosome reaction – capacitated and acrosome-intact



Values represented are the mean ± SEM after one-hour incubation with various concentrations of fermented rooibos. (Colour code: Blue = oligozoospermia; Pink = asthenozoospermia)

Figure 4.22c: Effect of fermented rooibos on human sperm capacitation and acrosome reaction – uncapacitated and acrosome-intact

4.4.6 Correlations of various sperm parameters within oligozoospermia and asthenozoospermia groups

Table 4.11 displays the correlations between the percentages of ROS-positive spermatozoa and the sperm functional parameters of MMP, DNA fragmentation (TUNEL) and capacitated, acrosome-reacted spermatozoa following exposure to aqueous extracts

of unfermented rooibos. The ROS-positive spermatozoa demonstrated no significant correlation with the percentage of intact MMP spermatozoa in the oligozoospermia group (r=-0.18, P=0.28) while the asthenozoospermia group showed a weak significant negative correlation (r=-0.22, P=0.04). Also no significant correlation was observed between the ROS-positive spermatozoa and the TUNEL-positive sperm in the oligozoospermia group (r=-0.10, P=0.55) and the asthenozoospermia group (r=-0.13, P=0.23). Furthermore, no significant correlation was observed between the ROS-positive spermatozoa and capacitated, acrosome-reacted sperm in the oligozoospermia group (r=-0.08, P=0.62) and the asthenozoospermia group (r=0.15, P=-0.16).

Table 3.11: Correlation between percentage of ROS-positive sperm and sperm functional parameters with unfermented extract

PARAMETER	Oligozoospermia		Asthenozoospermia	
	r	P	r	P
MMP-intact (%)	-0.18	0.28	-0.22	0.04*
TUNEL (%)	-0.10	0.55	-0.13	0,23
Capacitated, Acrosome-reacted (%)	-0.08	0.62	0.15	-0.16

MMP: Mitochondrial membrane potential; TUNEL: Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labelling. An asterix (*) indicate data with significant correlation

Table 4.12 presents the correlations following exposure to fermented rooibos aqueous extracts between the percentages of ROS-positive spermatozoa and the percentages of the follwing sperm functional parameters: spermatozoa with intact MMP, DNA fragmentation and capacitated, acrosome-reacted sperm. The percentage of ROS-positive sperm revealed no significant correlation with the percentage of spermatozoa with intact MMP for the oligozoospermia group (r=0.05, P=0.09) and the asthenozoospermia group (r=0.10, P=0.95). Similarly, the percentage of TUNEL-positive sperm did not indicate a significant relationship with the percentage of ROS-positive sperm in the oligozoospermia group (r=-0.41, P=0.50) and the asthenozoospermia group (r=-0.50, P=0.45). Furthermore, no statistical significant correlation was observed

between the percentage of ROS-positive and the percentage of capacitated, acrosome-reacted sperm in both the oligozoospermia group (r=0.05, P=0.99) and the asthenozoospermia group (r=-0.50, P=0.45).

Table 3.12: Correlation between ROS-positive sperm and sperm functional parameters with fermented rooibos

PARAMETER	Oligozoospermia		Asthenozoospermia	
	r	P	r	P
MMP-intact (%)	0.05	0.09	0.100	0.95
TUNEL (%)	-0.41	0.50	-0.50	0.45
Capacitated, Acrosome-reacted (%)	0.05	0.99	-0.50	0.45

MMP: Mitochondrial membrane potential; TUNEL: Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labelling. An asterix (*) indicate data with significant correlation

Correlations between the percentage of total motility and the sperm functional parameters of hyperactivation, progressive motility, intact MMP and capacitated, acrosome-reacted spermatozoa following treatment with aqueous extracts of unfermented rooibos are demonstrated in Table 4.13. Total motility indicated showed a weak significant positive correlation with the percentage of progressive motility spermatozoa in the oligozoospermia group (r=0.45, P=0.005) while the asthenozoospermia group showed a strog significant positive correlation (r=0.91, P=0.001). The percentage of acrosome-reacted spermatozoa showed no significant correlation with the percentage of total motile spermatozoa in the oligozoospermia group (r=0.08, P=0.62) and asthenozoospermia group (r=-0.16, P=0.13). Moreover, the percentage of hyperactivated spermatozoa showed no significant correlation with the percentage of total motile spermatozoa in the oligozoospermia group (r=0.14,P=0.39) while the asthenozoospermia group showed a weak signifact positive correlation (r=0.42, P=0.004). In addition, the percentage of intact-MMP spermatozoa showed no significant relationship with the percentage of total motile spermatozoa in both the oligozoospermia group (r=-0.18, P=0.28) and the asthenozoospermia group (r=0.03, P=0.73).

Table 3.13: Correlations between the percentage of total motility and the sperm functional parameters with unfermented rooibos

PARAMETER	Oligozoospermia		Asthenozoospermia	
	r	P	r	P
MMP-intact (%)	-0.18	0.28	0.03	0.73
Progressive motility (%)	0.45	0.005*	0.91	0.0001*
Capacitated, Acrosome-reacted (%)	-0.08	0.62	-0.16	0.13
Hyperactivation (%)	0.14	0.39	0.42	0.004*

MMP: Mitochondrial membrane potential. An asterix (*) indicate data with significant correlation

Correlations between the percentage of total motility and the sperm functional parameters of hyperactivation, progressive motility, intact MMP and capacitated, acrosome-reacted spermatozoa were observed following the treatment of spermatozoa from oligozoospermia and asthenozoospermia patients with fermented rooibos. Table 4.14 shows that total motility demonstrated no significant correlation with the percentage of progressive motility spermatozoa in both the oligozoospermia group (r=0.50, P=0.45) and the asthenozoospermia group (r=0.90, P<0.08). Furthermore, acrosome-reacted sperm showed no significant relationship with total motility in both the asthenozoospermia group (r=-0.80, P=0.13) and the oligozoospermia group (r=0.60, P=0.35). Moreover, total motility had no significant correlation with hyperactivated spermatozoa in the oligozoospermia group (r=-0.89, P=0.33) and the asthenozoospermia group (r=0.61, P=0.30). Lastly, no significant relationship was seen between total motility and intact MMP in both the oligozoospermia group (r=0.07, P=0.91) and the asthenozoospermia group (r=0.60, P=0.35).

Table 3.14: Correlations between the percentage of total motility and the sperm functional parameters with fermented rooibos

PARAMETER	Oligozoospermia		Asthenozoospermia	
	r	P	r	P
MMP-intact (%)	0.30	0.68	0.07	0.91
Progressive motility (%)	0.50	0.45	0.90	0.08
Capacitated, Acrosome-reacted (%)	0.60	0.35	-0.80	0.13
Hyperactivation (%)	-0.89	0.33	0.61	0.30

MMP: Mitochondrial membrane potential. An asterix (*) indicate data with significant correlation.

The relationships between the percentage of hyperactivated, ROS-positive and capacitated, acrosome-reacted spermatozoa following the treatment of unfermed rooibos spermatozoa from the oligozoospermia and asthenozoospermia samples are shown in Table 4.15. No significant correlations were observed between the percentage of hyperactivated spermatozoa and the percentage of ROS-positive spermatozoa in the oligozoospermia group (r=0.14, P=0.39) and the asthenozoospermia group (r=0.10, P=0.38) (Table 4.15). In addition, the percentage of acrosome-reacted sperm had no relationship with hyperactivated spermatozoa in either the oligozoospermia group (r=0.05, P=0.74) or the asthenozoospermia group (r=-0.23, P=0.06).

Table 3.15: The relationships between percentages of hyperactivated sperm and functional parameters with unfermented rooibos

PARAMETER	Oligozoospermia		Asthenozoospermia	
	r	P	r	P
ROS-positive (%)	0.14	0.39	0.10	0.38
Capacitated acrosome-reacted (%)	0.05	0.74	-0.23	0.06

ROS: Reactive oxygen species. An asterix (*) indicate data with significant correlation

The relationships between the percentage of hyperactivated, ROS-positive spermatozoa and capacitated, acrosome-reacted spermatozoa from the oligozoospermia and asthenozoospermia patients after treatment with aqueous extracts of fermented rooibos are shown in Table 4.16. No significant correlation was observed between the percentage of hyperactivated spermatozoa and the percentage of ROS-positive spermatozoa in the asthenozoospermia group (r=-0.05, P=0.99) or the oligozoospermia group (r=0.70, P=0.66). Furthermore, the percentage of acrosome-reacted sperm had no significant relationship with the percentage of hyperactivated spermatozoa in the oligozoospermia group (r=0, P=0.99) and the asthenozoospermia group (r=-0.15, P=0.83).

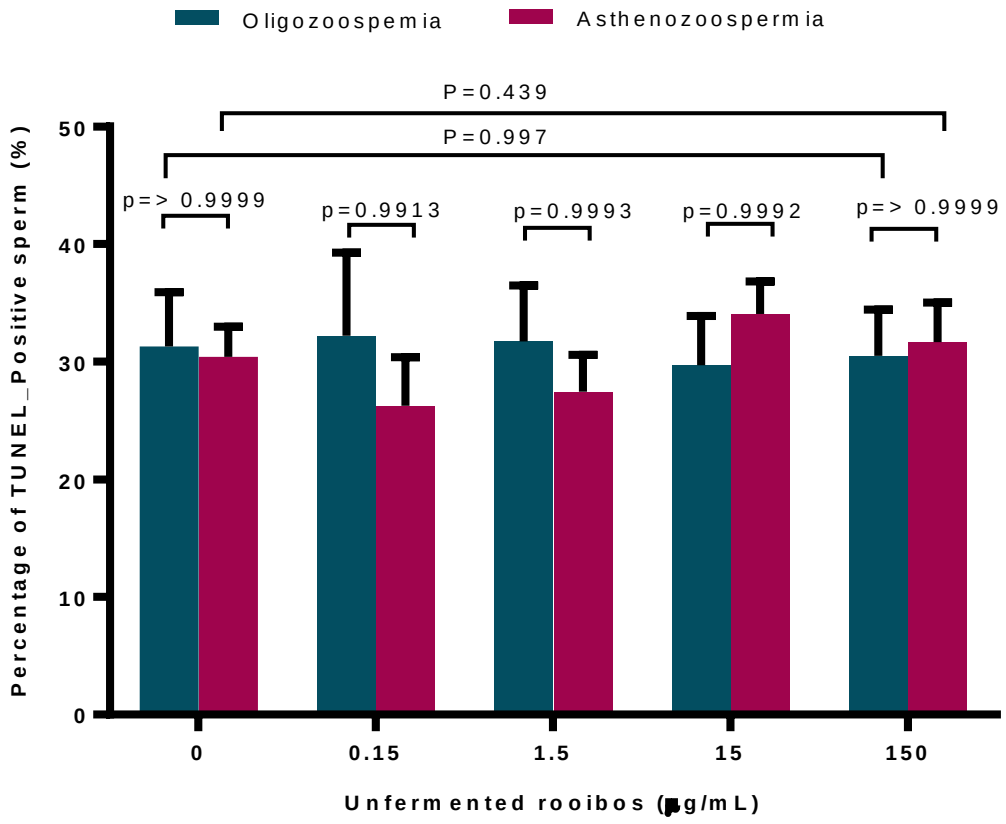
Table 3.16: The relationships between percentages of hyperactivated sperm and functional parameters with fermented rooibos

PARAMETER	Oligozoospermia		Asthenozoospermia	
	r	P	R	P
ROS-positive (%)	0.70	0.66	-0.05	>0.99
Capacitated acrosome-reacted (%)	0	>0.99	-0.15	0.83

ROS: Reactive oxygen species. An asterix (*) indicate data with significant correlation

4.4.7 Sperm DNA fragmentation

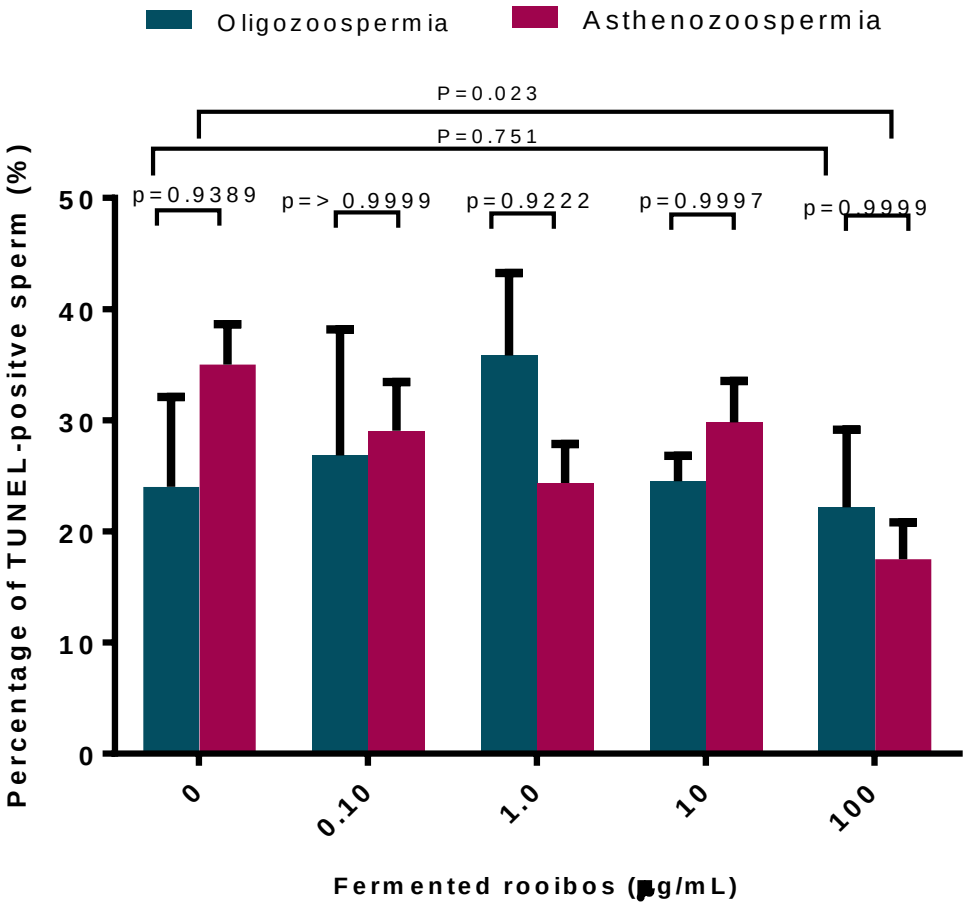
Exposure of human sperm to increasing concentrations of aqueous extracts of unfermented rooibos had no significant effects on the percentage of TUNEL-positive sperm in the oligozoospermia group (P=0.997) or the asthenozoospermia group (P=0.439). Similarly, the repeated measures ANOVA demonstrated no trend in the percentage of TUNEL-positive sperm in either group (oligozoospermia: P=0.967; asthenozoospermia: P=0.403) (Figure 4.23). Also ,no significant treatment effects was seen on the percentage of TUNEL-positive sperm in two groups after 1 hour of incubation with unfermented rooibos in all concentrations (two-way ANOVA:P>0.05)



Values represented are the mean ± SEM after one-hour of incubation with various concentrations of unfermented rooibos. (Colour code: Blue = oligozoospermia; Pink = asthenozoospermia)

Figure 3.23: Effect of unfermented rooibos on DNA fragmentation of sperm

Exposure of human sperm to the respective concentrations of fermented rooibos for one hour showed a significant reduction in the percentage of TUNEL-positive sperm in the asthenozoospermia group at a concentration of 100 µg/mL (P=0.023) (Figure 4.24). The trend of a decreased percentage of TUNEL-positive sperm in the asthenozoospermia group following exposure to fermented rooibos was also observed (repeated measures ANOVA: P=0.030). However, no significant difference was observed in the spermatozoa of the oligozoospermia group after exposure to fermented rooibos (P=0.751; Figure 4.24). Likewise, no trend was revealed by the repeated measures ANOVA in the oligozoospermia group exposed to fermented rooibos (P=0.655). As above ,no significant treatment effects was seen on the percentage of TUNEL-positive sperm in two groups after 1 hour of incubation with fermented rooibos in all concentrations (two-way ANOVA:P>0.05)



Values represented are the mean ± SEM after one-hour incubation with various concentrations of fermented rooibos. (Colour code: Blue = oligozoospermia; Pink = asthenozoospermia)

Figure 3.24: Effect of fermented rooibos on DNA fragmentation of sperm

CHAPTER FIVE: DISCUSSION

Humanity has always faithfully relied on nature and its resources to cater for its everyday needs and to provide remedies for the different diseases (Fieldhouse, 2013). Plants are the only source of traditional medicine, which has existed for millennia in different tribes (Fieldhouse, 2013). Rules and regulations for traditional medicine and traditional healing vary worldwide (Alostad, Steinke & Schafheutle, 2018). Traditional healthcare in South Africa is governed by the Traditional Health Practitioners Act, No. 22 of 2007, which emphasises the regulatory framework and ensures the effectiveness, safety and excellence of the treatment (Duvenhage & Louw, 2016). Furthermore, the WHO (2004) revealed that more than 80% of people globally depend on traditional medication, specifically plants, as their principal source of healthcare (Kumar et al., 2013). Reasons for the popularity of traditional medicine are ascribed to its affordability, availability and little effort compared with modernised healthcare services (Agbor & Naidoo, 2016).

Despite great improvement in modern synthetic medication, plant propagules and active substances from plant parts are still used in the manufacture of various drugs for healthcare services in Western medication (Ahmed, 2016). It is approximated that 25% of modern medicines are derived directly from medicinal plants with slight modifications (Atanasov et al., 2015). Currently, herbal medicinal products are sold worldwide in pharmacies and formal and informal supermarkets (Ang-Lee et al., 2001; James et al., 2018) . These naturally derived products possess antioxidants and chemical compounds that are used to manufacture precise concentrations of pharmacological compounds (Prasad et al., 2014). As indicated in the current study, different *in vitro, in vivo* and clinical research studies have indeed ascertained the practical use of various herbal extracts in the enhancement of male fertility parameters (Chikhoune et al., 2015; Lampiao et al., 2008; Mbemya et al., 2017).

Rooibos is used by traditional healers to treat different ailments; however, there are no scientific studies available that demonstrate the effects of rooibos on human

spermatozoa. Due to the antioxidant activity of rooibos, this study is the first *in vitro* study to reveal the effects of this extract on spermatozoa from fertile and infertile humans.

An *in vivo* study by Opuwari and Monses (2014) on the effect of rooibos on the reproductive function of male rats reported that treatment with unfermented rooibos significantly improved sperm concentration, vitality and motility. Fermented rooibos significantly improved sperm vitality but triggered a significant increase in spontaneous AR whereas unfermented rooibos did not (Opuwari & Monsees, 2014). The authors further indicated that prolonged exposure of male rats to rooibos tea may cause subtle aberrations in the male reproductive system that could result in infertility (Opuwari & Monsees, 2014). It should be mentioned that the study design had no questionable discrepancy, making the findings and conclusion noteworthy. Moreover, the therapeutic concentration and dose were taken into consideration, making the findings more valid. Using rooibos and palm oil on Wister rats, Ayeleso, Oguntibeju, Aboua and Brooks (2014) demonstrated that administration of both extracts exhibited no lethal effects on sperm motility parameters but rather showed some enhancement on progressive motility, WOB and LIN. Lastly, using boar sperm, Ros-Santaella and Pintus (2017) reported that fermented rooibos had a positive effect on several motility parameters, including progressive motility.

In the present study, total motility remained unchanged in the donor, patient and combined groups aswell as oligozoospermic and asthenozospermic group treated with fermented and unfermented rooibos. It can thus be concluded that rooibos tea has no considerable effect on sperm motility parameters of sperm *in vitro*.

Sperm velocity parameters are often rendered a prerequisite of sperm motion measurement (Giaretta, Munerato, Yeste, Galeati & Spinaci, 2017). In this study, the sperm velocity parameters of VCL, VSL, VAP, ALH, LIN, STR, WOB, BCF and hyperactivation in all treatment groups showed no significant change when compared with the control group. Nonetheless, a tendency towards increased values was observed. Sperm velocity parameters have been reported to have a significant relationship with sperm fertilising capacity (Boryshpolets, Kowalski, Dietrich, Dzyuba & Ciereszko, 2013;

Collodel et al., 2007). Sperm VCL and BCF have been reported to be important markers of sperm vitality, while VAP, VSL, STR and LIN are indicators of sperm movement, with STR and LIN being associated with the control of swimming arrays (Fair & Romero-Aguirregomezcorta, 2019; King, 2018). Also, antioxidants found in rooibos tea could enhance the fertilising capability of the sperm by affecting the motion and motility parameters. However, previous studies have demonstrated that although sperm motility is an indicative diagnostic measure of infertility (Jodar, Soler-Ventura & Oliva, 2017; Malm, Rylander, Giwercman & Haugen, 2019; Zhao, Diao, Ni, Hu & Yu, 2011), sperm motility alone cannot be used as a sole diagnostic indicator because various traits of spermatozoa are required to ensure successful fertilisation of an oocyte (Munoz-Cueto, Mañanós-Sánchez & Sánchez-Vázquez, 2019). Other parameters necessary for successful fertilisation include oxidation-antioxidant balance (Güvenç, Cellat, Gökçek, Yavaş & Özsoy, 2019; Nenkova, Petrov & Alexandrova, 2017), capacitation and AR (Xu, Guo, Zhu & Fan, 2018), normal sperm mitochondrial membrane function (Moraes & Meyers, 2018) and integrity of the sperm DNA (Henkel, 2005).

Opuwari and Monses (2014) demonstrated that fermented rooibos significantly improved sperm vitality in male rats. Results from this study showed that sperm vitality remained unchanged in the donor and patient groups treated with fermented rooibos, while those of the combined group decreased significantly when compared with the control group. It is noteworthy that the asthenozoospermia subjects showed an increase in the percentage of live spermatozoa but no change was seen in the oligozoospermia patients in the fermented rooibos group. Regarding the improvement in vitality in the asthenozoospermia samples, a systematic review by Majzoub and Agarwal (2018) concluded that in all 26 studies that reported on infertility cases, there was a significant positive effect of antioxidant therapy on basic semen parameters, advanced sperm function, results of artificial reproductive therapy and live-birth rate. This was due to the presence of vitamin E, vitamin C, carnitines, N-acetyl cysteine, co-enzyme Q10, zinc, selenium, folic acid and lycopene. Considering the above findings, it can be hypothesised that the antioxidant composition of fermented and unfermented rooibos may be useful in infertile patients, especially those who fall under the asthenozoospermia criteria. Nevertheless, exposure

to unfermented rooibos produced no change in sperm vitality in all groups, including the asthenozoospermia and oligozoospermia groups.

Furthermore, in this study, the integrity of the mitochondrial membrane within the spermatozoa of fertile donors, infertile patients and the combined group was examined and correlated with other functional parameters such as the presence of ROS in the human ejaculate. Reactive oxygen species play a crucial role in the pathologic physiology of sperm malfunction (Agarwal, Mulgund et al., 2014; Du Plessis et al., 2015; Simon, Haj-Yehia & Levi-Schaffer, 2000), and seminal OS status could be an important prognostic tool in the treatment of infertility (Güvenç et al., 2019; Nenkova et al., 2017). Infertile males have been found to have a higher level of ROS than fertile males, with the principal sources being immature spermatozoa and contaminating leucocytes (Durairajanayagam, 2019; Gilbert, 2002; Simon et al., 2000; Yadav & Sharma, 2016). A negative correlation between excessive ROS production and poor semen quality has been reported in several infertility cases globally (Du Plessis et al., 2015; Yadav & Sharma, 2016). Abnormal spermatozoa containing cytoplasmic retentions are also regarded as principal sources of ROS since they preserve a surplus of cytoplasmic enzymes that are involved in glucose metabolism such as glucose-6-phosphate dehydrogenase (Atukeren, 2018; Lee, Dutta, Maharjan, Liu & Lim ,2018; Meccariello & Chianese, 2018). Moreover, spermatozoa produce ROS through the mitochondrial electron transport chain during cellular respiration, with approximately 2% of expended oxygen being transformed into the O^{2-}, which often renders spermatozoa vulnerable to peroxidative and oxidative attack (Wagner et al., 2017). Furthermore, ROS generation can be increased by external lifestyle factors that contribute significantly to oxidative stress (Agarwa et al., 2014). Nonetheless, sperm cells are covered in a seminal milieu dominated by enzymatic antioxidants such as SOD, CAT, GPx and GSH in addition to non-enzymatic antioxidants that include vitamins C, E and B, carotenoids and carnitine (Tan et al., 2018). However, a balance between ROS and antioxidants is required since both oxidative and antioxidative stress are detrimental to sperm. Thus, an individual's OS levels should be accurately determined before prescribing supplementary antioxidants (Agarwal, Virk et al., 2014; Tan et al., 2018). In this study, exposure to unfermented rooibos showed a significant increase in levels of ROS and an increase in sperm with disrupted MMP in the donor group. This may have

been caused by defective sperm in the human ejaculate that associated with an increased level of ROS, thereby disrupting both the inner and the outer mitochondrial membranes (Agnihotri et al., 2016). In addition, the increased level of ROS may be due to increased OS triggered by an imbalance between ROS and the antioxidants in fermented and unfermented rooibos. Reasons for the higher levels of ROS production may include an increase in superoxide anion concentration and increased mitochondrial damage in addition to exogenous factors such as environmental pollution, nutrition, chronic inflammation, and psychological and emotional stress.This study further showed a significant correlation between ROS and MMP in both fermented and unfermented groups, studies have revealed that the correlation between mitochondrial and sperm morphology provides useful data on infertility diagnosis since sperm with morphological defects produce high levels of ROS (Fraczek et al., 2012; Liu et al., 2018).

Exposure to higher concentrations of fermented rooibos caused a significant decrease in the percentage of spermatozoa with intact MMP in both the donor and the combined group, while no change was seen in the patient group. No statistical change was seen in the level of ROS production in any of the groups treated with the fermented extract. Further analysis showed no statistical significance in ROS production and the MMP of patients with asthenozoospermia and oligozoospermia after treatment with both fermented and unfermented extract.More so a significant treatment effect was seen on the percentage of ROS-positive sperm and sperms with intact mitochondria treated with fermented and unfermented rooibos of patients and donors. Regarding the decrease in the integrity of the mitochondrial membranes of the sperm, rooibos tea caused this negative effect, altering the permeability and function of the mitochondrial membranes. This observation could imply that rooibos contains cytotoxic compounds that could impair the mitochondrial membrane; this is supported by the observation of a decrease in sperm vitality after exposure to fermented extract in the donor and combined groups. Nonetheless, the above ROS-MMP relationship could be associated with two mutually interconnected occurrences: ROS initiating damage to the mitochondrial membrane and the damaged mitochondrial membrane causing increased ROS production.

It has been reported in previous studies that H_2O_2 inhibits sperm motility, ATP levels and MMP (Engel, Springsguth & Grunewald, 2018). However, it was noted that low levels of H_2O_2 do not have a negative effect on the MMP of the spermatozoa (Fraczek et al., 2012; Meccariello & Chianese, 2018; Moraes & Meyers, 2018). This may demonstrate that physiological level of ROS does not affect the health of the mitochondrial membrane. Mitochondrial dysfunction has been shown to increase the production of ROS (Fraczek et al., 2012; Simon et al., 2000; Yadav & Sharma, 2016).

Changes in the MMP may occur during the primary phase of apoptosis (Wang & Youle, 2009). It has also been asserted that mitochondrial membrane damage is a crucial initiator of apoptosis in germ cells in the human testis (Agnihotri et al., 2016; Wang & Youle, 2009). This implies that if the apoptosis process within the germ cells is initiated but not finalised, spermatozoa with impaired mitochondria will be produced (Almeida et al., 2013). This apoptotic process is referred to as abortive apoptosis (Almeida et al., 2013). Abortive apoptosis is known to take place during sperm production or in men with abnormal semen parameters (Agnihotri et al., 2016; Meccariello & Chianese, 2018). This interrelationship may be due to a self-perpetuating cycle of impaired mitochondria producing ROS, thus damaging the mitochondrial membrane (Zorov et al., 2014). Although no significant correlation was observed between ROS-positive sperm and intact MMP in this study, it is well known that excessive ROS damages both the internal and external mitochondrial membranes, resulting in a discharge of cytochrome c, a protein known to activate caspases while inducing cell death (Zorov et al., 2014). Moreover, exposure ROS to the mitochondrial membranes trigger the release of an apoptosis-inducing factor that interacts with DNA directly and leads to DNA damage (Agarwal, Mulgund et al., 2014; Aitken & Koppers, 2011; Bui et al., 2018). A possible explanation for the lack of a significant correlation between the ROS and MMP in this study is that ROS can be generated through two pathways, that is, intrinsically by factors that include the spermatozoa themselves and extrinsically by leukocytes (Aitken & Koppers, 2011; Atukeren, 2018; Henkel, 2005). While spermatozoa have been shown to produce low amounts of ROS, leukocytes within semen ejaculate are the principal sources of ROS (Atukeren, 2018).

The success rate of fertilisation and proper growth of a human embryo relies on the health of the sperm (Castillo, Jodar & Oliva, 2018). Sperm DNA is the most important genomic material, and it is highly condensed and compacted within the sperm cell to prevent damage of any sort (Agarwal, Virk et al., 2014; Aitken & Koppers, 2011; Esteves et al., 2015). The DNA is wrapped around the histone proteins, which are gradually replaced by highly basic protamines for effective condensation of the sperm DNA, making the spermatozoa transcriptionally and translationally inactive (Jeng et al., 2015; Panner Selvam & Agarwal, 2018). During this procedure, the double-stranded DNA is subjected to torsional stress (Panner Selvam & Agarwal, 2018). Therefore, nicks and breaks occur within the DNA and recovery must take place for the proper array of chromatin networks (Panner Selvam & Agarwal, 2018;Ribas-Maynou and Benet, 2019). Failure to repair the DNA structure, the accumulative effect due to limited chromatin protamination results in DNA damage (Aitken & Koppers, 2011; Jeng et al., 2015; Ribas-Maynou & Benet, 2019).

Another reported cause of DNA damage is the production of ROS by the immature sperm cell (Jeng et al., 2015). Damage of spermatozoa by ROS occurs through epididymal transit, damaging the DNA of spermatozoa (Esteves et al., 2015). In addition, activation of the endonuclease or sperm caspases can cause DNA damage (Esteves et al., 2015). Sperm cells that pose high protamination and poor chromatin packing are more vulnerable to ROS attack (Esteves et al., 2015). Furthermore, sperm DNA fragmentation occurs due to poor disulphide cross-links within mature spermatozoa usually as a result of modification in the chromatin packaging (Esteves et al., 2015), and spermatozoa with reduced levels of disulphide cross-linking are susceptible to DNA damage (Jeng et al., 2015). Moreover, extrinsic and intrinsic apoptosis pathways are activated in the spermatozoa through ongoing exposure to high levels of ROS and RNS (Di Meo, Reed, Venditti & Victor, 2016). Activation of pro-apoptotic factors by ROS results in leakage of cytochrome c from the mitochondrial membrane, which in turn activates the intrinsic caspase cascade and results in sperm DNA damage. Furthermore, ROS cause damage to DNA through modification of all bases, synthesis of base-free sites, frame shifts, elimination of DNA cross-links and chromosomal assortment (Esteves et al., 2015). Oxidative stress is also associated with a high prevalence of single-strand and double-strand DNA breaks. Reactive oxygen species have been reported to trigger gene

mutations that include polymorphism and point mutation, resulting in low semen quality (Di Meo, Reed, Venditti & Victor, 2016). Other mechanisms such as DNA base-pair oxidation and denaturation may be involved. However, when DNA damage is minimal, spermatozoa undergo self-repair. The oocyte is also capable of repairing the damaged DNA of spermatozoa. But if the damage is widespread, embryo and apoptosis fragmentation can take place (Agarwal, Mulgund et al., 2014; Aitken & Koppers, 2011; Bui et al., 2018). In addition, DNA damage within the Y chromosome causes gene obliteration in the Y chromosome of the offspring, resulting in infertility (Di Meo et al., 2016).

In this study, exposure of human sperm to increasing concentrations of aqueous extracts of unfermented rooibos had no significant effect on the percentage of TUNEL-positive sperm in the donor, patient and combined groups. Further analyses also showed that incubation of human sperm with increasing concentrations of aqueous extracts of unfermented rooibos had no significant effect on the percentage of TUNEL-positive sperm in the group with oligozoospermia and asthenozoospermia. On the contrary, fermented rooibos significantly decreased the percentage of TUNEL-positive sperm in the patient group in a dose-dependent manner. However, no change was seen in the donor and combined groups when compared with the control group. Also, higher concentrations of fermented rooibos significantly decreased the percentage of TUNEL-positive sperm in the asthenozoospermia group, with no significant change observed in the oligozoospermia samples. Moreover, no significant correlation was found between ROS-positive spermatozoa and DNA fragmented sperm in any of the groups and subgroups treated with fermented and unfermented rooibos. Data from this study lends some evidence to studies that demonstrated that TUNEL positivity and ROS may not always have a relationship. This suggests that DNA fragmentation may solely be responsible for cell death (Elsamanoudy, Abdalla, Hassanien & Gaballah, 2016) and that ROS may sometimes directly damage the nuclear DNA within the spermatozoa (Schulte, Ohl, Sigman & Smith, 2010).

Biologically ejaculated spermatozoa stay within the female reproductive tract for a few hours before attaining the ability to fertilise the oocyte (Suarez, 2016). In humans, sperm

vacate the seminal fluid immediately after ejaculation and enter the fallopian tubes where they succeed in fertilising an egg cell. The newly fertilised cell is referred to as a zygote (Suarez, 2016). The zygote then moves down the fallopian tube and into the uterus (Suarez & Pacey, 2006; Xu et al., 2018). As the spermatozoa move out the ejaculate via cervical mucus, they encounter numerous biochemical changes such as capacitation (Jin & Yang, 2016). Capacitation is a prerequisite for the normal AR of spermatozoa, which must occur in order for the sperm cell to infiltrate the ZP (Ickowicz, Finkelstein & Breitbart, 2012; Xu et al., 2018). The AR has been reported to correlate with fertility cases in males since only acrosome-reacted spermatozoa can penetrate the ZP (de Lamirande, Leclerc & Gagnon, 1997; Kunkitti et al., 2015; Xu et al., 2018). Moreover, spermatozoa must go through the process of hyperactivation, which is crucial for the fertilisation process (Xu et al., 2018). Hyperactivation is regarded as the most important stage of capacitation; it is important for sperm to be able to separate from the isthmic reservoir within the female reproductive system and accelerate towards the ampulla of the fallopian tube to fertilise the egg cell (Jin & Yang, 2016; Lehti % Sironen, 2017).Hyperactivation involves several modifications within the sperm flagellar beat that cause an increase in flagellar bend amplitude and result in a force that cause attraction between the epithelium found in the fallopian tube and the spermatozoa (Francou et al., 2017; Xu et al., 2018).

This study supports the finding of Opuwari and Monsees (2014) that consumption of *A. linearis* may result in early AR of the spermatozoa, possibly impairing fertility chances. Additionally, Isotani et al. (2017) demonstrated that although acrosin-disrupted rats were shown to be fertile with reduced litter size and their spermatozoa possessed an equal ability to penetrate the ZP, the dispersal of cumulus oophorous cells was shown to be slower.

Furthermore, a weak significant negative correlation was seen between ROS-positive sperm and acrosome-reacted sperm in the combined group treated with unfermented rooibos. The physiological level of ROS is a prerequisite for sustaining critical redox-sensitive biochemical processes such as capacitation and hyperactivation without which fertilisation will not take place (Ickowicz et al., 2012). Furthermore, high level of ROS affects delicate processes that are important to achieve fertilisation, including

normal spermatogenesis and several sperm functions such as motility, capacitation, AR, motility, egg penetration and decondensation of the sperm head (Francou et al., 2017). In addition, H_2O_2 seems to be involved in the AR, and induction of the AR in capacitated spermatozoa further promotes the synthesis of O^{2-}, which triggers the release of unesterified fatty acids from the plasma membrane of the sperm cells (de Lamirande & Gagnon, 1995). Reactive oxygen specires are directly involved in the biochemical pathways that regulate capacitation and AR (Durairajanayagam, 2019). Premature AR in spermatozoa renders the sperm cells incapable of fertilisation (Francou et al., 2017) . However, the mode of action of ROS-mediated hyperactivation, capacitation and AR is not fully understood, more study need to be done to understand the mode of action of ROS in hyperactivation of sperm.

Research has also demonstrated a strong association between ROS production and capacitation, which is significant since they occur in the female reproductive tract (de Lamirande et al., 1997; Jin & Yang, 2016). Furthermore, spermatozoa synthesise extreme levels of ROS from the time of their release from the seminal fluid and just before the final assent in the fallopian tube to the site of conception (Francou et al., 2017; Kunkitti et al., 2015). Under these conditions, exposure of the sperm cell to ROS will be short-lived and serves to finish its preparation for pending fertilisation (Liu et al., 2018). Under circumstances where a sperm cell fails to accomplish fertilisation, the self-perpetuating ROS generation will in turn, result in a state of OS and over-capacitation (Di Meo et al., 2016; Jin & Yang, 2016). Eventually, the lipid aldehydes produced as a result of ROS-initiated peroxidation will induce free radical production from the mitochondria, forcing the sperm to initiate cell death (Ickowicz et al., 2012). Nevertheless, sperm acquisition of fertilisation ability depends on a low and controlled concentration of ROS (Homa et al., 2015). Premature AR in spermatozoa renders them incapable of fertilisation. Moreover, exogenous supplementation of O^{2-} induces human spermatozoa capacitation, hyperactivation and AR (Francou et al., 2017; Jin & Yang, 2016).

5.1 CONCLUSION AND RECOMMENDATIONS

Little is known about the *in vitro* antioxidant activity of rooibos tea on human sperm functional parameters. To the best of the researcher's knowledge, this is the first study investigating the effects of an aqueous rooibos tea on human sperm functions. However, other *in vivo* studies report on the antioxidant activity of *A. linearis* on improvement of sperm parameters in rats (Ajuwon et al., 2015; Canda et al., 2014; Opuwari & Monsees, 2014; Ros-Santaella & Pintus, 2017). From the findings of the current study, it could be concluded that rooibos tea maintains specific sperm functions due to its antioxidant activity. It appeared that exposure to extracts of fermented rooibos provided significant antioxidative protection against DNA fragmentation in the spermatozoa of the patient group. Further analyses also proved that the antioxidant within fermented rooibos offered significant protection to sperm DNA and vitality in patients with asthenozoospermia. However, no changes were observed in the patients with oligozoospermia. Conversely, in a dose-dependent manner, unfermented rooibos significantly triggered spontaneous AR, increased the level of ROS and disrupted MMP in the human sperm, thus indicating a possible impairing effect on sperm fertilising capability.

Considering the subtle functional changes observed, the researcher recommends further research to explore the mode of action and establish the fertilising capability of rooibos tea and its antioxidative effect following induction of OS. Furthermore, isolated active compounds from the extract may be employed to identify and explore beneficial compounds for the optimal functioning of the male reproductive system. Moreover, additional studies are proposed to elucidate the direct action of these antioxidants on sperm function and the therapeutic use and safety of this herbal plant in the treatment of the male reproductive system.

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APPENDICES

APPENDIX A: UNIVERSITY OF LIMPOPO PROJECT APPROVAL

University of Limpopo
Department of Research Administration and Development
Private Bag X1106, Sovenga, 0727, South Africa
Tel: (015) 268 3935, Fax: (015) 268 2306, Email: anastasia.ngobe@ul.ac.za

TURFLOOP RESEARCH ETHICS COMMITTEE

ETHICS CLEARANCE CERTIFICATE

MEETING: 06 March 2019

PROJECT NUMBER: TREC/46/2019: PG

PROJECT:

Title:	In vitro effects of aspalathus linearis on human sperm.
Researcher:	NB Takalani
Supervisor:	Dr CS Opuwari
Co-Supervisor/s:	Dr G.A Adefolaju
	Prof R Henkel
School:	Health Care Sciences
Degree:	Masters of Sciences in Medical Science

PROF P MASOKO
CHAIRPERSON: TURFLOOP RESEARCH ETHICS COMMITTEE

The Turfloop Research Ethics Committee (TREC) is registered with the National Health Research Ethics Council, Registration Number: REC-0310111-031

Note:
i) **This Ethics Clearance Certificate will be valid for one (1) year, as from the abovementioned date. Application for annual renewal (or annual review) need to be received by TREC one month before lapse of this period.**
ii) **Should any departure be contemplated from the research procedure as approved, the researcher(s) must re-submit the protocol to the committee, together with the Application for Amendment form.**
iii) **PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES.**

Finding solutions for Africa

APPENDIX B: UNIVERSITY OF THE WESTERN CAPE PROJECT APPROVAL

UNIVERSITY of the WESTERN CAPE

OFFICE OF THE DIRECTOR: RESEARCH
RESEARCH AND INNOVATION DIVISION

Private Bag X17, Bellville 7535
South Africa
T: +27 21 959 4111 /2948
F: +27 21 959 3170
E: research-ethics@uwc.ac.za
www.uwc.ac.za

11 May2018

Dr C Opuwari
Medical Biosciences
Faculty ofNaturalSciences

Ethics Reference NumberBM18/3/17

Project Title: In vitro effects of *AspalathusLinearis* and *Camellia sinesis*on the human sperm functionality

Approval Period: 10May2018 – 10 May 2019

I hereby certify that the Biomedical Science Research Ethics Committee of the University of the Western Cape approved the scientificmethodology and ethics of the above mentioned research project.

Any amendments, extension or other modifications to the protocol must be submitted to the Ethics Committee for approval.

Please remember to submit a progress report in good time for annual renewal.

The Committeemust be informed of any serious adverse event and/or termination of the study.

Ms Patricia Josias
Research Ethics Committee Officer
University of the Western Cape

PROVISIONAIREC NUMBER-130416050

FROM HOPE TO ACTION THROUGH KNOWLEDGE.

APPENDIX C: PERMISSION FOR SAMPLE COLLECTION AT VINCENT PALLOTTI HOSPITAL

Aevitas
Fertility Clinic • Cape Town

Dr Gerhard Hanekom *[MBChB, MMed (O&G), FCOG(SA), Cert Reproductive Medicine(SA)]* • **Dr Victor Hulme** *[MBChB, MMed (O&G)]*
• **Prof Thinus Kruger** *[MBChB, MPharMed, MMed (O&G), FCOG (SA), FRCOG (London), MD/PhD]*
• **Prof Igno Siebert** *[MBChB, MMed (O&G), LKOG/FCOG (SA), PhD*

11 September 2019

Ethics Reference: TREC/46/2019:PG

TITLE: In vitro effects of aqueous leaf extract of Aspalathus linearis on human sperm

Dear Ms. Takalani

PERMISSION TO CONDUCT YOUR RESEARCH AT AEVITAS CLINIC FROM 6.3.2019 TO 6.3.2020

1. In accordance with the Provisional Research Policy and Tygerberg Hospital Notice No 40/2009, permission is hereby granted for you to conduct the abovementioned research here at Aevitas Clinic.

Prof T I Siebert

Centre of Excellence in Fertility, Endometriosis & Endoscopic Surgery
Start Your Family with People Who Care

Drs Aevitas PTY
PR No: 1605925 VAT: 4160266
Life Vincent Pallotti Hospital Park Rd
Pinelands, 7405, South Af
Tel: +27 21 531 6999 Fax: +27 21 531 7
E-mail: info@aevitas.c
Website: www.aevitas.c

APPENDIX D: PERMISSION FOR SAMPLE COLLECTION AT TYGERBERG HOSPITAL

Western Cape Government
Health

TYGERBERG HOSPITAL
REFERENCE:
Research Projects
ENQUIRIES: **Dr GG Marinus**
TELEPHONE:**021 938 5752**

PROJECT NUMBER: TREC/46/2019: PG - Renewal

TITLE: **In Vitro effects of *Aspalathus Linearis* and *Camellia sinesis* on the human sperm functionality.**

Dear NB Takalani [Supervisor: Dr CS Opuwari]

PERMISSION TO CONDUCT YOUR RESEARCH AT TYGERBERG HOSPITAL.

1. In accordance with the Provincial Research Policy and Tygerberg Hospital Notice No 40/2009, permission is hereby granted for you to conduct the above-mentioned research here at Tygerberg Hospital.

2. Researchers, in accessing Provincial health facilities, are expressing consent to provide the Department with an electronic copy of the final feedback within six months of completion of research. This can be submitted to the Provincial Research Co-Ordinator (Health.Research@westerncape.gov.za).

DR GG MARINUS
MANAGER: MEDICAL SERVICES

DR D ERASMUS
CHIEF EXECUTIVE OFFICER
Date: 10 June 2019

Administration Building, Francie van Zijl Avenue, Parow, 7500 Private Bag X3, Tygerberg, 7505
tel: +27 21 938-6267 fax: +27 2 938-4890 www.capegateway.gov.za

APPENDIX E: INFORMED CONSENT FORM (ENGLISH)

In vitro effects of Aspalathus linearis on the Human sperm

Information sheet and consent to participate in Research

To whom it may concern,

You have been requested to consider participating in a scientific study on male sperm functions and medicinal plants. The aim of this study is to investigate the effects of *Aspalathus linearis* and *Camellia sinensis* on male reproductive functions using human sperm.

After collection of your semen samples masturbation, it will be exposed to various concentrations of the aqueous extract of *Aspalathus linearis* and *Camellia sinensis* and the following objectives will be answered:

Can the aqueous leaf extract of *Aspalathus linearis* and *Camellia sinensis* or its components?

- Alter semen parameters such as sperm concentration, motility, and vitality?
- Alter sperm fertilising capacity such as acrosome reaction, capacitation, and mitochondrial membrane potential?
- Have a protective function on sperm by reducing DNA fragmentation, and reactive oxygen species (ROS) production?
- Improve semen parameters in infertile men?

For those participating in the assisted reproductive treatment, after your semen has been processed for the treatment you are scheduled for, a small portion of the semen sample (<100 µl) will be taken for these additional tests. This procedure will under no circumstance affect the assisted reproduction treatment or outcome as only the left over samples shall be used. This will allow us determine the possible effects of *Aspalathus linearis* and *Camellia sinensis* on human sperm, as to whether it can improve male fertility or not.

The current study will not involve any additional medical risk, discomfort or cost. Participation in this scientific study is completely voluntary and participants are allowed to withdraw at any point in time until the data will be submitted for publication without any consequences. Withdrawal from the study should be done in a formally addressed email. In order to protect confidentiality of the participants, a unique number will be assigned to each sample and will contain no personal information of the volunteers involved in the study. All data will be confidential and available to the scientific researchers involved as well as to the participants if requested. The samples will be disposed of in the correct medical procedure by incineration once the data is captured.

The study has been ethically received by the UWC Biomedical Research Ethics Committee

(Approval number: _____)

In the event of any problems or concerns, additional information can be obtained from the doctor or scientific investigator directly by email:

Consent

I have been informed about the study titled "*In vitro* effects of *Aspalathus linearis* on the Human sperm" by the scientific investigator.

I understand the purpose and procedure of the study.

I have been given an opportunity to ask questions about the study and have had answers to my satisfaction.

I declare that my participation in this study is entirely voluntary and that I may withdraw at any point in time until the data will be submitted for publication without any consequences and without affecting any treatment or care that I am entitled to.

If I have any further questions or query related to the study, I understand that I may contact the researchers.

If I have any questions or concerns about my rights as a study participant, or if I am concerned about any aspect of the study or researcher, then I may contact:

_____ _____

Signature of Participant Date

_____ _____

Signature of Investigator Date

APPENDIX F: INFORMED CONSENT FORM (AFRIKAANS)

DEELNAME INGELIGTE TOESTEMMING VORM

***In vitro* effekte van *Aspalathus linearis* op die Menslike spermselle**

Inligtingsblad en toestemming om deel te neem aan Navorsing

Aan wie dit mag gaan,

U is versoek om te oorweeg om aan 'n wetenskaplike studie deel te neem oor manlike spermfunksies en medisinale plante. Die doel van hierdie studie is om die effekte van *Aspalathus linearis* op manlike voortplantingsfunksies te ondersoek deur menslike sperma te gebruik.

Na die insameling van u semenmonstermasturbasie sal dit blootgestel word aan verskillende konsentrasies van die waterige uittreksel van *Aspalathus linearis* en die volgende doelwitte sal ondersoek word :

- Om die effek van *A. linearis* op spermmotiliteit te bepaal.
- Om die effek *A. linearis* op sperm vitaliteit te bepaal.
- Om die effek van *A. linearis* op kapasitasie en akrosome reaksie in spermatozoa te bepaal.
- Om die vermoë van *A. linearis* te bepaal om spermselle teen DNA-fragmentering te beskerm.
- Om die effek van *A. linearis* op sperm mitochondriale membraanpotensiaal te bepaal.
- Om die effek van *A. linearis* op die produksie van reaktiewe suurstofspesies in spermatozoa te bepaal.
- Om vas te stel of *A. linearis* vrugbaarheid kan verbeter.

Vir diegene wat deelneem aan die bykomende voortplanting, nadat u sperma verwerk is vir die behandeling waarvoor u beplan word, sal 'n klein gedeelte van die semenmonster geneem word vir hierdie addisionele toetse. Hierdie prosedure sal onder geen omstandighede die geassisteerde voortplantingsbehandeling of uitslag beïnvloed nie

aangesien slegs die oorblywende monsters gebruik sal word. Dit sal ons toelaat om die moontlike effekte van *Aspalathus linearis* op menslike sperma te bepaal, of dit manlike vrugbaarheid kan verbeter of nie.

Die huidige studie sal nie enige bykomende mediese risiko, ongemak of koste insluit nie. Deelname aan hierdie wetenskaplike studie is heeltemal vrywillig en deelnemers mag op enige tydstip terugtrek totdat die data sonder enige gevolge vir publikasie ingedien sal word. Onttrekking uit die studie moet gedoen word in 'n formele aangespreek e-pos. Ten einde die vertroulikheid van die deelnemers te beskerm, sal 'n unieke nommer aan elke monster toegeken word en sal geen persoonlike inligting van die vrywilligers wat by die studie betrokke is, bevat nie. Alle data sal vertroulik en beskikbaar wees vir die betrokke wetenskaplike navorsers sowel as die deelnemers indien dit aangevra word. Die monsters sal deur die verbranding in die korrekte mediese prosedure weggedoen word sodra die data gevang is.

Die studie is eties ontvang deur die UWC Biomediese Navorsingsetiekkomitee

(Goedkeuringsnommer: _____)

In geval van enige probleme of probleme, kan addisionele inligting direk per e-pos by die dokter of wetenskaplike ondersoeker verkry word.

toestemming

Ek is op die hoogte van die studie getiteld "*In vitro* effekte van *Aspalathus linearis* op die menslike sperm" deur die wetenskaplike ondersoeker.

Ek verstaan die doel en prosedure van die studie.

Ek het die geleentheid gekry om vrae oor die studie te vra en antwoorde tot my bevrediging gehad.

Ek verklaar dat my deelname aan hierdie studie heeltemal vrywillig is en dat ek op enige tydstip kan terugtrek totdat die data sonder enige gevolge vir publikasie voorgelê sal word sonder om enige behandeling of sorg waarvoor ek geregtig is, te beïnvloed.

As ek verdere vrae of navrae het wat verband hou met die studie, verstaan ek dat ek die navorsers kan kontak.

As ek enige vrae of kommer het oor my regte as studieleier, of as ek bekommerd is oor enige aspek van die studie of navorser, kan ek kontak:

_____ _____

Handtekening van Deelnemer datum

_____ _____

Handtekening van Ondersoeker datum

APPENDIX G: INFORMED CONSENT FORM (ISIXHOSA)

INKQUBO YOMSEBENZISWANO OQHUBILEYO

Imiphumo ye- *in vitro* ye- *Aspalathus linearis* kwiiselingi zesininzi zesintu

Iphepha leenkcukacha kunye nemvume yokuthatha inxaxheba kuPhando

Kulowo ibhekisele kuye,

Uceliwe ukuba uthathe inxaxheba ekutheni uthathe inxaxheba kwisifundo sezenzululwazi kwimisebenzi yesilisa kunye nemithi yezityalo. Injongo yale sifundo kukuphanda iimpembelelo ze- *Aspalathus linearis* kwimisebenzi yokuzala yoluntu isebenzisa isisu somntu.

Emva kokuqokelelwa kweesampula zakho zeesisampuli, ziza kubonakala kwiindawo ezahlukahlukeneyo ze- *Aspalathus linearis* kunye neenjongo ezilandelayo ziya kuphandwa :

- Ukucacisa umphumo we- *A. linearis* kwi-sperm motility.
- Ukuqaphela umphumo *A. ulungelelaniso lobunzima* besilisa.
- Ukumisela umphumo we- *A. linearis* kwi-capacitory kunye ne-acrosome reaction in spermatozoa.
- Ukuqaphela ikhono lika- *A. linearis* ukukhusela i-cell spells malunga nokuhlukana kwe-DNA.
- Ukuqaphela umphumo we- *A. linearis* kwi-membrane ye-sperm mitochondrial.
- Ukumisela umphumo we- *A. linearis* kwimveliso yeentsholongwane ze-oksijeni esebenzayo kwi-spermatozoa.
- Ukuchonga ukuba i- *A. linearis* inokuphucula ukuzala.

Kulabo bathatha inxaxheba ekuncedeni unyango lokuzala, emva kokuba umlenze wakho uphuculwa unyango oye ucwangciselwe kuyo, is sm isahlulo sonke sesampuli yesondlo siya kuthathwa ngenxa yezi mvavanyo ezongeziweyo. Le nkqubo ayiyi kuphazamiseka unyango okanye uphuhliso oluza kuncedisa nje kuphela ukuba kusetyenziswe iisampuli

ezisele. Oku kuya kuvumela ukuba siqonde imiphumo ekhoyo ye- *Aspalathus linearis* kwi-sperm yabantu, nokuba ingaba yenze ngcono ukuzala komntu okanye cha.

Uphononongo lwangoku aluyi kubandakanywa nayiphi na ingozi yonyango engaphezulu, ingakhathazeki okanye iindleko. Ukuthatha inxaxheba kulolu cwaningo lwezenzululwazi ngokuzithandela kwaye abathathi-nxaxheba bavunyelwe ukuhoxisa naliphi na ixesha ngexesha lokuba idatha ingeniswe ukupapashwa ngaphandle kwemiphumo. Ukurhoxiswa kweso sifundo kufuneka kwenziwe kwikhompyutha echongiweyo. Ukuze ukhusele imfihlo yabathathi-nxaxheba, inombolo ekhethekileyo iya kubelwa isampula nganye kwaye ayiyi kuqulethelwa ngolwazi oluqulethwe ngamavavolontiya abandakanyekayo kwisifundo. Yonke idatha iya kuba yimfihlo kwaye ifumaneke kubaphandi bezesayensi ababandakanyekayo kunye nabachaphazelekayo ukuba bayacelwa. Iisampuli ziya kulahlwa kwinkqubo efanelekileyo yezocwangco ngokutshatyalaliswa xa idatha ithathwa.

Uphononongo luye lwafunyanwa ngokomthetho yiKomidi yeeNkcazo zoBuchule be-UWC

(Inombolo yemvume: _____)

Xa kwenzeka nayiphi na ingxaki okanye iinkxalabo, ulwazi olongezelelweyo lunokufumaneka kwigqirha okanye uphando loosayensi ngokuchanekileyo nge-imeyile:

Mvume

Ndixelelwe ngophando oluthi "Iimpembelelo ze-*In vitro* ze- *Aspalathus linearis* kunye kwi-Human sperm".

Ndiyayiqonda injongo nenkqubo yesifundo.

Ndinike ithuba lokuba ndibuze imibuzo malunga nokufunda kwaye ndifumene iimpendulo kwaneliseko lwam.

Ndiyaxela ukuba inxaxheba yam kulesi sifundo iphela ngokuzithandela kwaye ndiyakrhoxisa nanini na ixesha ngexesha le data lingafakwa ngencwadi ngaphandle kwayo nayiphi na imiphumo kwaye ngaphandle kokuchaphazela nayiphi na inyango okanye ukunakekelwa kwam.

Ukuba ndinemibuzo eminye okanye umbuzo ophathelene nesifundo, ndiyaqonda ukuba ndidibana nabaphandi.

Ukuba ngaba nayiphi na imibuzo okanye ukuxhalabisa ngamalungelo am nje njengomfundi othabatha inxaxheba, okanye ukuba ndixhalabele nayiphi na into yesifundo okanye umphandi, ngoko ndiyaqhagamshelana nayo:

_____ _____

U tyikityo lwaBathathi-nxaxheba Umhla

_____ _____

Isayinwe oMphandi Umhla