EFFECT OF TEA AND HERBAL INFUSIONS ON MAMMALIAN REPRODUCTION AND FERTILITY

By

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KEY WORDS

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ABSTRACT

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Camellia sinensis (tea) and *Aspalathus linearis* (rooibos) may improve reproductive function owing to their antioxidant properties. To test this hypothesis, male and female rats were given 2% and 5% green tea (Gt), black tea (Bt), unfermented rooibos (Ur) or fermented rooibos (Fr) as sole source of drinking for 52 and 21 days respectively. Control rats received tap water. In addition, TM3 Leydig cells were exposed to 0.025, 0.05, 0.1 and 0.5 % aqueous extracts of green tea, black tea, unfermented and fermented rooibos for 24h.

In vitro analysis of tea and the herbal infusion revealed the phenolic property and antioxidant capacity (FRAP) in the order Gt > Bt > Ur > Fr. *Camellia sinensis* and *Aspalathus linearis* revealed no significant effect on serum antioxidant capacity ($p > 0.05$) and lipid peroxidation (MDA) in the kidney or liver in both male and female rats and in the testes of the male rats ($p > 0.05$). In addition, the antioxidant levels were maintained in the testes, liver and kidneys in both the male and female rats.

In the male rats, no significant alterations were observed in body weight gain, liver and reproductive organs weight, and serum testosterone ($p > 0.05$). Only, 5% green tea significantly increased testosterone level ($p < 0.05$). Seminiferous tubules displayed complete spermatogenesis with abundant sperm in the lumen in all treated groups. However, a significant decrease in diameter and germinal

epithelial height of these tubules were observed ($p < 0.05$). In the epididymides, epithelial height of caput region showed a significant increase ($p < 0.01$), while the cauda region was increased by *Camellia sinensis* but decreased by *Aspalathus linearis*. Sperm concentration improved significantly by green tea and unfermented rooibos ($p < 0.05$), while black tea and fermented rooibos produced a non significant effect ($p > 0.05$). Sperm viability was enhanced in all treatment groups ($p < 0.05$). Furthermore, green tea, black tea and unfermented rooibos significantly improved the motility of rat sperm ($p < 0.05$); fermented rooibos tended to improve it ($p > 0.05$). In addition, green tea, black tea and fermented rooibos enhanced acrosome reaction ($p < 0.05$). Creatinine activity was significantly higher in rats treated with black tea, unfermented rooibos or fermented rooibos ($p < 0.05$), green tea tended to increase it ($p > 0.05$) reflecting the significant increased kidney weight in the treatment groups at high concentrations. Liver markers, ALT and AST, decreased significantly in all treated groups ($p < 0.05$), except in 5% fermented rooibos where a significant increase in AST level was observed ($p < 0.01$).

In the female rats, the body weight gain, and reproductive organs weight was not affected ($p > 0.05$). However, 5% fermented rooibos reduced the ovarian weight ($p < 0.05$), while 5% unfermented rooibos significantly increased the uterine weight ($p < 0.05$). Liver weight increased significantly by black tea and unfermented rooibos ($p < 0.05$) while the kidney weight increased significantly by 5% black tea ($p < 0.05$). No significant effect was observed in the level of FSH produced, on the other hand, *Camellia sinensis* significantly lowered the level of LH ($p < 0.05$), while *Aspalathus linearis* had no effect ($p > 0.05$). Creatinine activity was enhanced significantly only by 5% fermented rooibos ($p < 0.05$). Liver markers, ALT and AST were reduced in most treated groups except in fermented rooibos where an increase was observed. In addition, histological sections revealed no obvious alteration in the ovaries, uteri, kidneys and liver of all treated female rats.

Camellia sinensis and *Aspalathus linearis* significantly reduced the level of testosterone produced in TM3 Leydig cells under stimulated conditions *in vitro* ($p < 0.05$). Furthermore, both plants maintained the viability and morphology of the cells. However, at 0.5% of either plant extracts, a significant decrease in the viability ($p < 0.05$) and altered morphology of the TM3 Leydig cells was observed.

In conclusion, *Camellia sinensis* and *Aspalathus linearis* significantly improved certain sperm function which might be attributed to their high level of antioxidant activity. However, the prolonged exposure of both plant extracts might result in subtle structural changes in the male reproductive system and impair kidney function. In addition, fermented rooibos at high concentration may also impair the functions of the liver. *In vitro*, both plants were shown to possess anti-androgenic property on TM3 Leydig cells. Furthermore, both *Camellia sinensis* and *Aspalathus linearis* may be classified as weak phytoestrogens due to the changes in the weight of the uterus and ovaries observed.

May, 2013

DECLARATION

I declare that *Effect of tea and herbal infusions on mammalian reproduction and fertility* is my own work, that it has not been submitted for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

Full name..... Date.....

Signed.....

DEDICATION

This work is dedicated to my husband (Dr. MA Opuwari) for giving me such an incredible support and motivation.

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LIST OF ABBREVIATIONS

Δ^5-3β-HSD	Δ^5 3β - hydroxysteroid dehydrogenase
17β-HSD	17β - hydroxysteroid dehydrogenase
ADI	average daily intake
AE	Acrosome exocytosis
AEAC	Ascorbic acid equivalent antioxidant capacity
ALH	Amplitude of lateral head displacement
ALT	Alanine transaminase
AOA	Antioxidant activity
AR	Acrosome reaction
AST	Aspartate transaminase
BCF	Beat cross frequency
BSA	Bovine serum albumin
Bt	Black tea
BW	Body weight
Ca^{2+}	Calcium
CAT	Catalase
CBB	Coomassie Brilliant Blue G
CO_2	Carbon dioxide
Cr	Chromium
Cu	Copper
d	day
dH_2O	Distilled water
DMACA	4-(Dimethylamino)-cinnamaldehyde
DMEM/F-12	Dulbecco's modified eagle's medium

DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DPPH	2, 2-diphenyl-1-picrylhydrazyl
DTND	5, 5'-dithiobis-(2-nitrobenzoic acid or Ellman's reagent
EC	Epicatechin
ECG	Epicatechin gallate
EDTA	Ethylenediaminetetraacetic acid
EGC	Epigallocatechin
EGCG	Epigallocatechins-3-gallate
F-C reagent	Folin-Ciocalteaus reagent
Fe	Iron
Fe^{2+}	Iron (III) ion
Fe^{3+}	Iron (III) ion
FeIII-TPTZ	FeIII-tripyridyltriazine
FeII-TPTZ	FeII- tripyridyltriazine
Fr	Fermented rooibos
FRAP	Ferric reducing antioxidant power
FSH	Follicle stimulating hormone
g	gram
GAE	Gallic acid equivalent
GnRH	Gonadotropin releasing hormone
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathione
GSR	Glutathione reductase
GSSH	Oxidized glutathione

Gt	Green tea
h	hour
H^+	Hydrogen ion
H_2O_2	Hydrogen peroxide
HCG	Human chorionic gonadotropin
HCl	Hydrochloric acid
LH	Luteinizing hormone
LIN	Linearity
LPO	Lipid peroxidation
M	Molar
MDA	Malondialdehyde
MF	mount frequency
Mn	Manganese
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
N	Normality
n	number of samples
Na_2CO_3	Sodium carbonate
Na_2HPO_4	Disodium hydrogen phosphate
NaCl	Sodium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate
NaH_2PO_4	Anhydrous monobasic sodium phosphate
NaOH	Sodium hydroxide
nm	nanometer
NO	Nitric oxide
NO^-	Nitric oxide
O_2^-	Superoxide anion

O_2	Oxygen
^oC	degree Celsius
OH^-	Hydroxyl radicals
$ONOO^-$	Peroxynitrite anion
OSS	Oxidative stress status
PBS	Phosphate buffered saline
PUFA	Polyunsaturated fatty acids
ROO^-	Peroxyl radicals
ROS	Reactive oxygen species
RT	room temperature
Se	Selenium
sec	second
SNARE	Soluble NSF Attachment Protein Receptor
SOD	Superoxide dismutase
SS	soluble solid
TF_1	Theaflavins
TF_2A	Theaflavins-3-gallate
TF_2B	Theaflavins-3´-gallate
TF_3	Theaflavins-3, 3´-gallate
TPC	Total polyphenols activity
TPTZ	2, 4, 5-Tri (2-pyridyl)-S-triazine
TRH	Thyroid releasing hormone
UI/L	international unit per liter
Ur	Unfermented rooibos
v/v	volume per volume
VAP	Average path velocity

VCL	Curvilinear velocity
VSL	Straight-line velocity
w/v	Weight per volume
WOB	Wobble
Zn	Zinc

CHAPTER ONE

GENERAL INTRODUCTION

Approximately 25% of couples, who attempt to conceive, fail to do so within the first year of unprotected regular sexual intercourse (Dohle et al., 2005). One of the causes of male infertility has been associated with excessive reactive oxygen species (ROS) production by abnormal spermatozoa (Sikka, 1996; Dohle et al., 2005). Imbalances between antioxidants and ROS production has been associated with the initiation and development of pathological processes affecting female reproductive processes (Agarwal & Allamaneni, 2004; Agarwal et al., 2006).

Camellia sinensis (tea) and herbal tea (tisane) such as A*spalathus linearis* are commonly used as beverages and have gained commercial success (Joubert et al., 2008; Figueiroa et al., 2009). These plants contain polyphenols such as epigallocatechins-3-gallate (EGCG), theaflavins, aspalathin and flavanone among others, as their active compounds and possess strong antioxidant activity (Modder & Amarakoon, 2002; Bramati et al., 2003; Joubert et al., 2008; Figueiroa et al., 2009).

Green tea and EGCG significantly decreased serum level of testosterone, luteinizing hormone, estradiol and the weights of androgen/ estrogen sensitive organs (Sayama et al., 1996; Kao et al., 2000; Zheng et al., 2004; Chandra et al., 2011). However, Satoh et al. (2002) showed green tea to increase the levels of luteinizing hormone and testosterone. Black tea increased serum level of testosterone and prolonged ejaculatory latency (Ratnasooriya & Fernando, 2008). Rooibos, increased body weight and egg production in quail hens but decreased weight in male birds (Jurani et al., 2008); however, it resulted in increased sperm motility and concentration in male rats (Awoniyi et al., 2012).

The aim of this study was to explore the effects of *Camellia sinensis* and *Aspalathus linearis*, on different aspects of male and female reproductive functions and fertility based on the conflicting and few data on *Camellia sinensis* and *Aspalathus linearis* that were available at the start of this thesis.

Objective

The aim of this study was to investigate the effects of green and black tea (*Camellia sinensis*) and herbal infusions of indigenous South African Rooibos (*Aspalathus linearis*) on mammalian reproduction, fertility and the physiology of the male and female reproductive organs.

In particular, the following questions shall be answered:

Can *Camellia sinensis* and *Aspalathus linearis*, or components of their plant extracts

- Interfere with spermatogenesis and sperm functions, i.e. alter sperm counts, motility or physiology?
- Have a protective effect on sperm by reducing reactive oxygen species (ROS) level?
- Alter the histology of the female or male genital tract?
- Alter general and/or specific functions of cells involved in the reproductive process?
- Alter serum levels of relevant hormones such as testosterone, Luteinizing hormone (LH) or Follicle stimulating hormone (FSH)?
- Affect mating behaviour and number of offspring?

Aqueous tea extracts shall be used in different concentrations. The number of samples to be used per group will be six (n=6). Statistical analysis of data

acquired shall be done using MedCalc statistical programme. A p value < 0.05 will be considered significant and bar or line graphs will be used for graphical representations.

This thesis shall be drawn up in nine chapters. Chapter 1 shall give a general introduction to the topic. Chapter 2 will give an in depth information on literature reviews relevant to the research topic. Chapter 3 shall describe the materials and methods employed in all areas of this research. Chapters 4- 8 shall describe the results obtained based on the different experiments performed and each experiment shall have its chapter. Thus, chapter 4 present the results on the chemical analysis of the teas (antioxidant properties). Chapter 5 shall delve into the results obtained on the effect of the *Aspalathus linearis* and *Camellia sinensis* on the male reproductive system, liver and kidney functions. Chapter 6 shall describe the results obtained on the effect of *Aspalathus linearis* and *Camellia sinensis* on the female reproductive system, liver and kidney function. Chapter 7 shall describe the effect of *Aspalathus linearis* and *Camellia sinensis* on male sexual behaviour and fertility. Chapter 8 shall throw more light on the results obtained on the in vitro effects of *Aspalathus linearis* and *Camellia sinensis* on TM3 cell line. Chapter 9 gives an in depth discussion on the results obtained from all chapters in line with previous literature, with conclusion and future recommendations.

CHAPTER TWO

LITERATURE REVIEW

2.1 INFERTILITY

Infertility can be defined as the inability to conceive after 1 year of regular unprotected sex in women less than 35 years not using contraceptives and six months in women older than 35 years (Poland et al., 1985; Infertility, 1991; Practice Committee of the American Society for Reproductive Medicine, 2008). Approximately 15% of couples who attempt to conceive fail to do so (Poland et al., 1985; Infertility, 1991). The cause of infertility is attributed to 30% in male, 35% in female, 20% in both male and female, and 15 % is regarded as unknown or idiopathic (Tielemans et al., 2002).

Depending on the course of treatment, the cause of male infertility may be defined as either surgical (congenital anatomic anomalies, varicocele, erectile dysfunction and obstruction of the ductal system in the reproductive tract) or medical (may include immunological conditions like antisperm antibodies, infectious disease linked to anatomical obstruction, endocrinopathy, exposure to gonadotoxin and systemic illness, spermatogenic dysfunction and cryptorchidism (Meacham et al., 2007). Decreased semen quality is a primary cause of male infertility and it is characterized by low sperm motility and viability (Banihani et al., 2011). Male infertility may be associated with low sperm production (oligoozoospermia), poor sperm motility (asthenozoospermia) or abnormal sperm morphology (teratozoospermia) or a combination of all three (oligoasthenoteratozoospermia) (Guzick et al., 2001). Idiopathic causes of male infertility have been associated with excessive generation of reactive oxygen species (ROS) by spermatozoa and contaminating leukocytes associated with genitourinary tract inflammation (Sikka, 2002). The mammalian spermatozoa have membranes that are rich in polyunsaturated fatty acids (PUFA), exhibit no capacity for membrane repairs and are able to generate a significant amount of ROS, mainly the superoxide anion and

hydrogen peroxide. That makes them prone to oxygen induced damage mediated by lipid peroxidation (an indicator of oxidative stress which involves the extraction of electrons from cell membranes by free radicals) which would result in the production of membrane destruction product like malondialdehyde (MDA) (Jones et al., 1979; Aitken & Clarkson, 1987; Sheweita et al., 2005; Babich et al., 2011). However, physiologic levels of ROS are considered important mediators of normal sperm functions such as sperm hyperactivation, capacitation and acrosome reaction (de Lamirande & Gagnon, 1995; Griveau et al., 1995).

The most common causes of female infertility include ovulatory disorders, endometriosis, pelvic adhesions, tubal blockage or damage, and hyperprolactinemia (Unuane et al., 2011). Hypothyroidism has been associated with reproductive disorders ranging from abnormal sexual development to menstrual irregularities and infertility based on several interactions between thyroid hormone and the female reproductive system (Poppe & Velkeniers, 2004). Hyperprolactemia occurs as a result of an increased production of thyroid releasing hormone (TRH) and has been implicated in ovulatory dysfunction (Longcope et al., 1990). Hypothyroidism also interferes with the normal physiological pulsatile gonadotropin releasing hormone (GnRH) secretion, which is a pre-requisite for normal follicular development and ovulation (Poppe & Velkeniers, 2004). In addition, gonadal dysfunction may occur as a result of insufficient availability of thyroid hormone, as it mediates the FSH and LH/HCG receptor induction and progesterone secretion (Maruo et al., 1991).

2.2 OXIDATIVE STRESS AND REACTIVE OXYGEN SPECIES

Oxidative stress is a condition used to describe increased cellular damage induced by oxygen and oxygen-derived oxidants commonly referred to as reactive oxygen species (ROS) (Sikka et al., 1995). ROS are ubiquitous, highly reactive oxidizing agents and diffusible molecules that belong to the class of free-radicals that are

generated in cells as by-product of aerobic respiration and metabolism (Al-Gubory et al., 2010). The most common ROS that play a role in reproduction and fertilization include superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), peroxyl radicals (ROO^-), hydroxyl radicals (OH^-), nitric oxide (NO^-) and peroxynitrite anion ($ONOO^-$) (Sikka, 1996).

Spermatozoa produce ROS mostly from normal metabolic activity such as superoxide anion (O_2^-) (Alvarez et al., 1987) which dismutates to hydrogen peroxide (H_2O_2) either spontaneously or enzymatically (Halliwell & Gutteridge, 1989). O_2^- is not very harmful in itself due to its low reactivity and short life, however, reaction with its target can result in the production of toxic species such as thiol radicals (Halliwell & Gutteridge, 1989). H_2O_2 on the other hand, is more stable, uncharged, with a higher oxidant potential, and can cross cell membranes (Halliwell & Gutteridge, 1989).

In gonads and seminal fluid, the scavenging activity is maintained by antioxidants (ROS scavengers) such as superoxide dismutase (SOD), catalase (CAT), glutathione (GSH), and the glutathione peroxidase/ reductase system (Nissen & Kreysel, 1983; Alvarez et al., 1987; Alvarez & Storey, 1989; Jeulin et al., 1989; Halliwell & Glutteridge, 1989). In order to create a healthy environment for fertilization, there has to be a proper balance referred to as oxidative stress status (OSS) between ROS generation and scavenging activities as well as the right timing for ROS production, as cellular damage may result in an improper balance between ROS generation and scavenging activities (Sikka et al., 1995; de Lamirande et al., 1997; Figures 2.1 and 2.2). Improper balance between ROS generation and scavenging activities in human spermatozoa has been shown to be related to male infertility (Iwasaki & Gagnon, 1992).

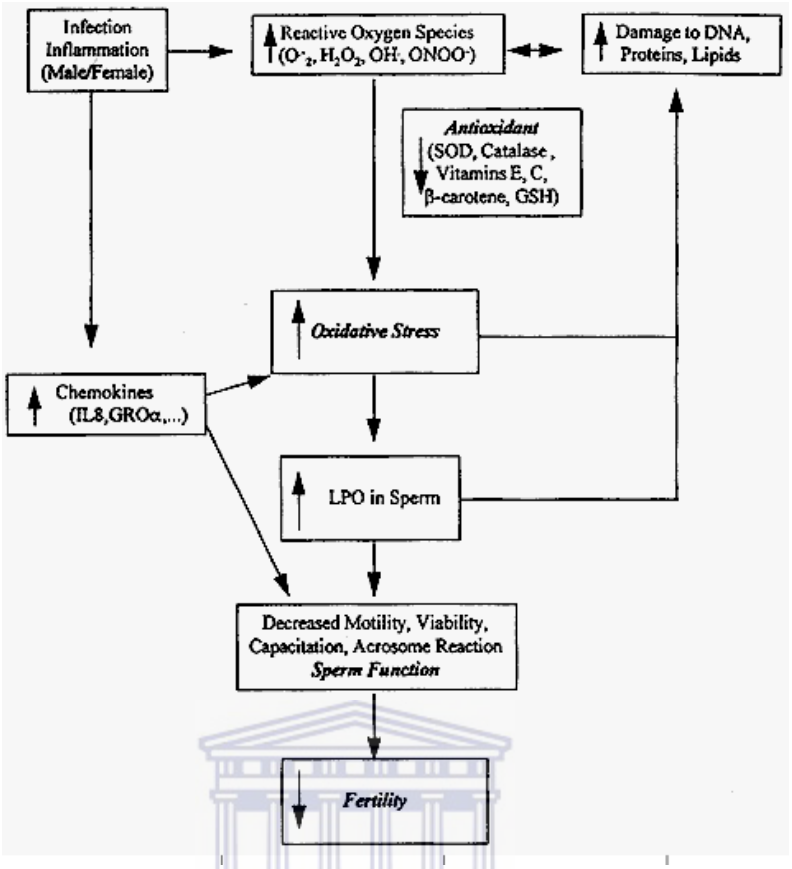


Figure 2.1: Scheme suggesting interacting mechanisms in the role of oxidative stress and antioxidants affecting sperm function and fertility (Sikka, 1996). SOD, superoxide dismutase; GSH, glutathione; LPO, lipid peroxidation.

A physiological level of ROS is required to play a regulatory role in folliculogenesis, oocyte maturation, corpus luteum and uterine function, embryogenesis, embryogenic implantation and fetoplacental development through various signaling transduction pathways (Agarwal et al., 2008). Imbalances between antioxidants and ROS production has been associated with the initiation and development of pathological processes affecting female reproductive processes (Agarwal & Allamaneni, 2004; Agarwal et al., 2006). An increased ROS level has also been associated with decreased sperm motility and increased motile sperm with decreased ROS level (Iwasaki & Gagnon, 1992). In addition, numerous leukocytes in semen samples were observed in severe male infertility cases (Wolff & Anderson, 1988; Aitken et al., 1992).

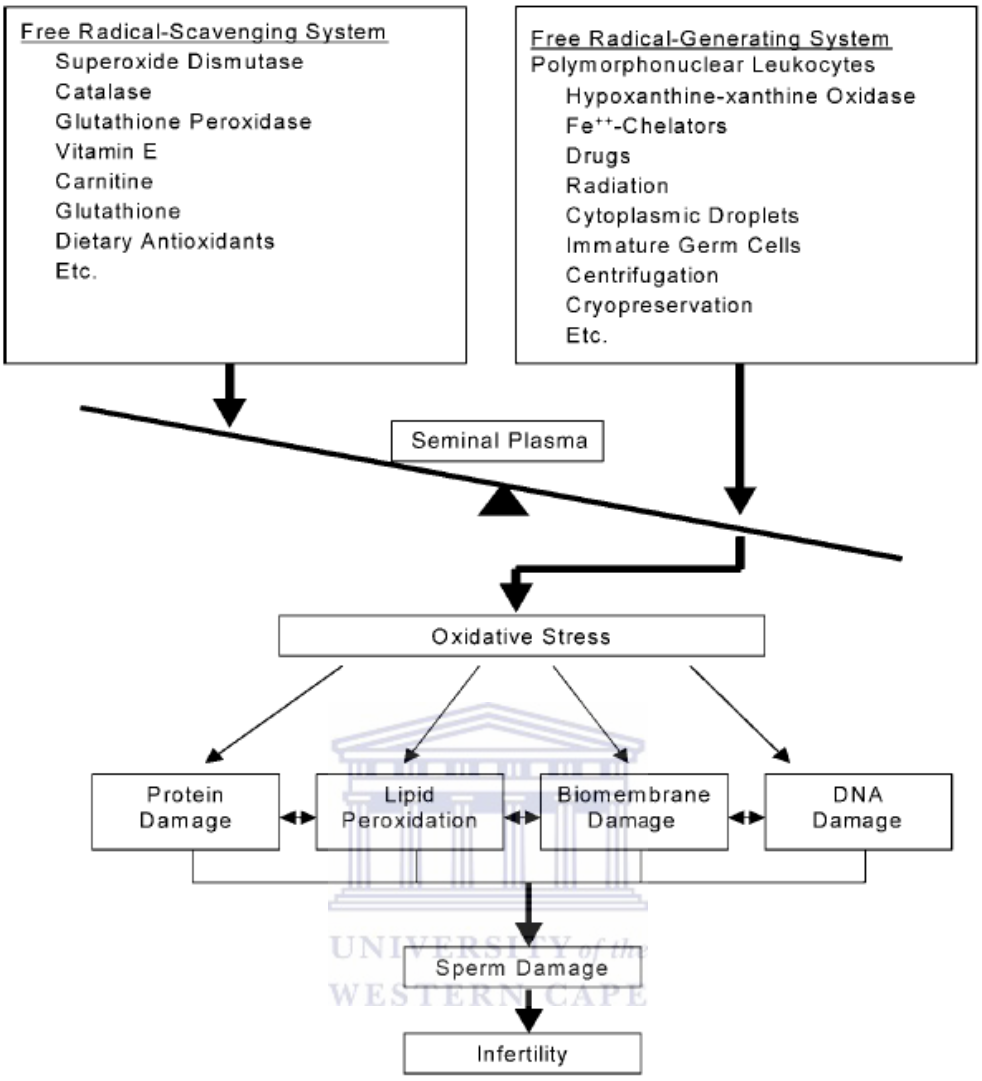


Figure 2.2: Reactive oxygen species generating and scavenging systems in seminal plasma responsible for oxidative stress and their role in infertility (Sikka, 2004).

From figure 2.1, presence of infection or inflammation in the male or female reproductive tract results in the increased production of ROS or chemokines. The former causes damage to DNA, proteins and lipids or can lead to a state of oxidative stress due to the decreased level of antioxidants. Oxidative stress would in turn lead to increased lipid peroxidation (LPO) in sperm which causes decreased sperm function (motility, viability, capacitation and acrosome reaction) and the result is decreased fertility. The presence of infection or inflammation in

the male or female reproductive tract results in increased level of chemokines which would also lead to oxidative stress or decreased sperm function.

Susceptibility to oxidative damage have been postulated to be gender related as the level of oxidative damage in DNA was found to be higher in males than in females (Proteggente et al., 2002). Another study also showed that mitochondrial oxidant production and oxidative damage to mitochondrial DNA was significantly lower in female rats compared to their male counterparts (Sastre et al., 2002; Borras et al., 2003). In addition, higher level of vitamin E and increased activity of glutathione peroxidase (GPx) and glutathione reductase (GR) was found in females, suggesting that the females may have intrinsically higher antioxidant capacity (Chen et al., 1992; Yamamoto et al., 2002).

2.3 ANTIOXIDANTS

Antioxidants scavenge and suppress the formation of ROS and lipid peroxidation (Sikka, 1996). However, elevated ROS, depleted total antioxidant activity or both can result in oxidative stress such as damage to cellular lipids, proteins and DNA (Alvarez & Storey, 1984; Pasqualotto et al., 2008). The antioxidant defense system includes enzymatic and non enzymatic (dietary) components. Enzymatic antioxidants includes glutathione (GSH), glutathione peroxidase (GPx), catalase (CAT), superoxide dismutase (SOD), while the non-enzymatic antioxidants may be from endogenous or exogenous sources and includes uric acid, bilurubin, vitamins E (α-tocopherol) and C, carotenoids and polyphenols, among others (Halliwell, 1995; Huang et al., 2005). These play a vital role in scavenging or suppressing free radicals, hence the application of these antioxidants could enhance fertility through a likely increase in sperm motility and function (Sikka, 1996).

Antioxidants play a role in protecting the gonads by maintaining a balance between ROS generation and scavenging activities (Sikka, 1996). An imbalance between the free radical- scavenging system and the free radical- generating system in seminal plasma as seen in figure 2.2 would result in oxidative stress and eventually infertility. Spermatozoa are susceptible to lipid peroxidation as a result of high concentration of polyunsaturated fatty acid in their plasma membrane (Alvarez & Storey, 1995). Application of these antioxidants can improve sperm motility and function (Sikka, 1996). Antioxidant activity according to 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging methods was shown to decrease in the order: green > unfermented rooibos > fermented rooibos > semifermented rooibos> black > oolong, while the β-Carotene bleaching method showed a decrease in the order: green > black > fermented rooibos > oolong > unfermented rooibos > semifermented rooibos (von Gadow et al., 1997). Furthermore, potent antioxidants can also display prooxidant effects which could lead to the oxidative damage of cellular components (Heim et al., 2002; Galati & O'Brien, 2004). Depending on the oxidative status within a cell, the antioxidant/prooxidant properties of flavonoids may be important to determine the fate of a cell, of which the biological response could either be beneficial or deleterious (Klaunig & Kamendulis, 2004).

2.3.1 Enzymatic antioxidant system

2.3.1.1 Superoxide Dismutase (SOD)

SOD controls cellular superoxide anions (O_2^-) by catalyzing the dismutation of O_2^- to form hydrogen peroxide (H_2O_2) and oxygen (Felton & Summers, 1995; Korsloot et al., 2004). SOD is found in three forms (Al-Gubory et al., 2010): copper- zinc containing SOD (SOD1), a dimeric protein located mainly in the cytoplasm. Manganese containing SOD (SOD2), a homotetrameric protein located in the mitochondria (Weisiger & Fridovich, 1973) and extracellular SOD (SOD3)

which is a copper (Cu) and zinc (Zn) containing tetrameric glycoprotein (Marklund, 1982; Marklund et al., 1982).

SOD protects spermatozoa against oxygen toxicity and lipid peroxidation (Alvarez et al., 1987). The dismutation reaction of SODs may be represented as follows:

$$2(O_2^-) + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2 \quad (2.1)$$

2.3.1.2 Catalase

Catalase is found within the peroxisomes (Chance et al., 1979) and catalyses the conversion of hydrogen peroxide to water and oxygen. The control of H_2O_2 production is important for the regulation of ROS propagation (Al-Gubory et al., 2010).

$$2H_2O_2 \xrightarrow{Catalase} 2H_2O + O_2 \quad (2.2)$$

2.3.1.3 Glutathione peroxidase (GPXs)

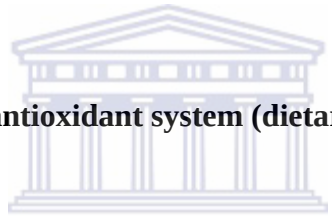
GPXs are found mainly in the cytoplasm and mitochondria and occur in two forms: selenium (Se) - independent and selenium - dependent enzymes. They catalyze the conversion of hydrogen peroxide to water as seen below:

$$H_2O_2 + 2GSH \xrightarrow{GPX} 2H_2O + GSSG \quad (2.3)$$

Glutathione, the thiol- containing tripeptide occurs in two forms: reduced (GSH) and oxidized (GSSH) form. GSH, the reduced glutathione, is an important component of the overall cellular defensive mechanisms against ROS (Babich et al., 2011). The main function of GSH is to scavenge free radicals and peroxide produced during normal cellular respiration, which would otherwise cause

oxidative damage to lipids, proteins, and nucleic acids (Schuck et al., 2008). A depletion of intracellular GSH is a hallmark indicator of oxidative stress, which is mostly the case in cancer cells (Pelicano et al., 2004). Normal cells are less susceptible to the cytotoxic damage by pro-oxidant polyphenols as a result of their ability to maintain a proper intracellular redox status with their antioxidant enzymes and sufficient supply of reduced GSH (Lu et al., 2000; Yamamoto et al., 2004; Chan et al., 2006).

GSSG, the oxidized form of glutathione is reduced to GSH with NADPH as its reducing agent. This reaction is catalyzed by glutathione reductase (GSR) (Chance et al., 1979).



2.3.2 Non-enzymatic antioxidant system (dietary antioxidants)

2.3.2.1 Vitamins

These are among the major source of dietary antioxidants that directly scavenge ROS (Sies & Stahl, 1995; Rock et al., 1996; Johnson et al., 2003). Vitamin E, a family of α-, β-, γ-, δ- tocopherols, of which α- tocopherol is shown to be the most active homologous form. It protects the cell membrane from oxidation during lipid peroxidation by reacting with ROS and lipid radicals produced (Traber & Atkinson, 2007). Vitamin C (ascorbic acid) which is a water soluble vitamin scavenges ROS and protects against DNA damage (Al-Gubory et al., 2010).

2.3.2.2 Trace elements

Trace elements such as Mn, Se, Zn, Cu and Fe, form part of the active site required for the functions of antioxidant enzymes or acts as cofactors in the regulation of antioxidant enzymes (Bettger, 1993; Dashti et al, 1995). However, they are required in balanced proportions, as most of them are toxic at high doses (Michalak, 2006; Abu-Darwish et al., 2009).

2.3.2.3 Polyphenols

Polyphenols occur naturally in vegetables, fruits and plant derived beverages such as tea, red wine, and extra virgin olive oil (Al-Gubory et al., 2010). It is characterized by the presence of several groups involved in phenolic structures such as flavonoids and phenolic acid (Al-Gubory et al., 2010). Commonly occurring flavonoids include catechin, resveratrol, quercitin, anthocyanins, hesperitin derivatives and phenolic acids (Al-Gubory et al., 2010).

2.4 CAMELLIA SINENSIS

Camellia sinensis (commonly referred to as tea) is grown in about 30 countries worldwide (Harold & Graham, 1992). Tea is produced from the buds and leaves of *C. sinensis* and consumed by two-third of the world's population (Harold & Graham, 1992). Tea is the mostly consumed beverage in the world besides water (Muktar & Ahmad, 2000).

Camellia sinensis is an evergreen shrub or a tree located in South East Asia and extensively cultivated in tropical countries like Sri Lanka, India, java, China, Japan, Bangladesh, Indonesia, Kenya and Turkey (Modder & Amarakoon, 2002). There are two varieties of tea, *C. sinensis* var. *sinensis* (China tea) which is grown in China, Japan and Taiwan, while *C. sinensis.*var.*assamica* (Assam tea) is predominantly found in south and south east Asia including Malaysia and Australia (Adiwinata et al., 1989; Caffin et al., 2004).

Tea is manufactured in three different forms: Green tea, black tea and oolong (figure 2.3). Of the tea produced worldwide, 78% is black tea, 20% is green tea, and 2% is oolong tea (Graham, 1999). Related to dry substance mass, fresh tea leaves contains on the average: 36% polyphenolic compounds, 25% carbohydrates, 15% proteins, 6.5% lignin, 5% ash, 4% amino acids, 2% lipids,

1.5% organic acids, 0.5% chlorophyll as well as carotenoids and volatile substances constituting less than 0.1% (Harold & Graham, 1992).

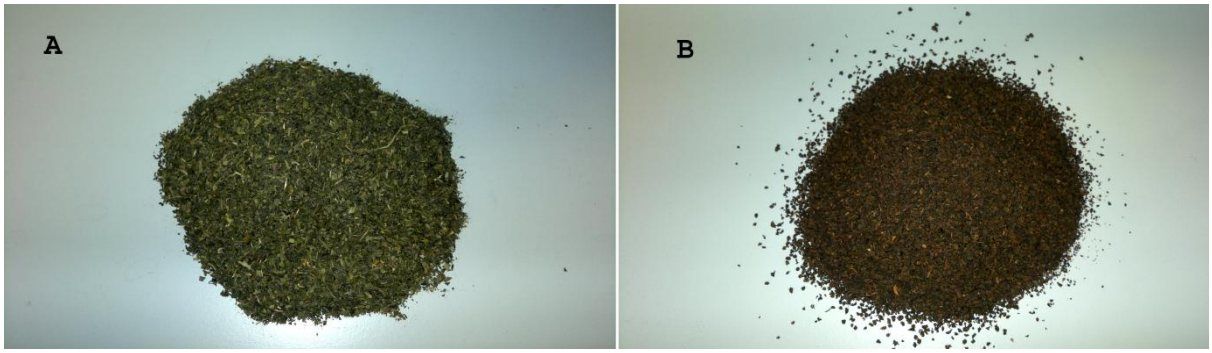


Figure 2.3: Images of green (A) and black tea (B) leaves

Among the polyphenols (figure 2.4), are a group of compounds commonly referred to as catechins that include most frequently epigallocatechin gallate (EGCG), epigallocatechin (EGC), epicatechin gallate (ECG), epicatechin (EC) and catechin, and in small amounts: gallocatechin, epigallocatechin digallate, 3-methylepicatechin gallate, catechin gallate, and gallocatechin gallate (Lunder 1989; Harold & Graham, 1992). The content of catechins present varies depending on the species, climate, season, horticultural practices, and leaf age of the tea; with green tea having a higher amount than oolong and black tea (Cabrera et al., 2003; Harold & Graham, 1992; Lin et al., 2003; Yen & Chen, 1995). Tea flavonols are regarded as the biologically active compounds of tea (Yang et al., 2000), with EGCG and ECG having the highest radical scavenging property (Henning et al., 2003). In addition, Chen et al. (2003) reported that young tea leaves contained more caffeine than EGCG and ECG than mature leaves. Accordingly, Lin et al. (2003) showed that old leaves contained less caffeine with more EGCG, EGC, EC and catechins than young leaves.

2.4.1 Antioxidative properties and bioavailability

Total polyphenol content was shown to be significantly higher in green tea than black tea (Yokozawa et al., 1998; Atoui et al., 2005; Yao et al., 2006). Green and black tea produced from var. *assamica* was shown to have higher polyphenols content than those produced from var. *sinensis* (Harbowy & Balentine, 1997). The amount of EGCG and total catechin was shown to be higher in green tea followed by oolong and black tea (Yen & Chen, 1995; Cabrera et al., 2003; Lin et al., 2003). Total polyphenols activity (TPC), antioxidant activity (AOA), ferric reducing antioxidant power (FRAP) and ascorbic acid equivalent antioxidant capacity (AEAC) was also shown to be higher in green tea than in black tea (Chan et al., 2007). This implies that green tea has higher antioxidant activity than black tea (Lee et al., 2002). However, Leung et al. (2001) argues that both teas have equal antioxidant activity as theaflavins produced during the fermentation process are equally effective antioxidants as catechins in green tea.

Lee et al. (2002) and Henning et al. (2004) showed that after the intake of green tea, a low bioavailability in plasma was observed with EGCG having the least amount present compared to EGC and EC. In plasma, green tea EGCG was found in its free form, while EGC and EC were mostly in their conjugated (methylated, sulfated and glucuronide) form with the mean peak plasma EGCG being 0.17μM after the intake of about 2 cups (Yang et al., 1998; Lee et al., 2002; Unno et al., 2005). Bioavailability of black tea is also low (Wiseman et al., 2001), as a maximum concentration of theaflavins found in blood plasma was 1ng/mL in two individuals who consumed 700 mg theaflavins, corresponding to about 30 cups of black tea, after 2h consumption (Mulder et al., 2001).

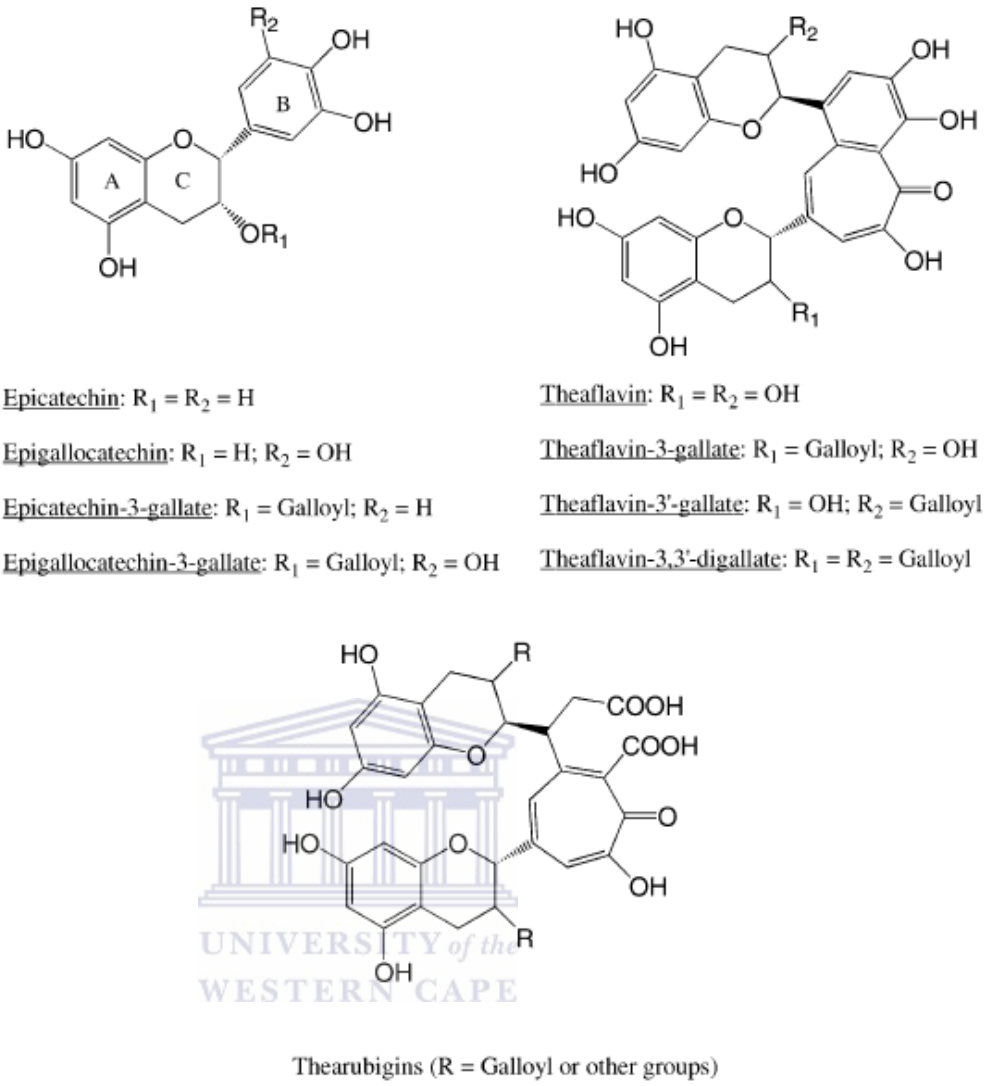


Figure 2.4: Major tea polyphenols (Lambert & Yang, 2003).

2.4.2 Green tea

Green tea is prepared by dehydration of leaves of *Camellia sinensis* which does not lead to oxidation of polyphenol constituents and as a result contains high level of monomeric polyphenols from catechins (Dufresne & Farnworth, 2001). During its manufacturing process of green tea, young tea leaves are picked up, rolled and steamed (Japan) or panned (China). Both processes prevent the oxidation of catechins by polyphenol oxidase (Graham, 1999). This way, it retains its original

colour and almost all its original polyphenol content (Chan et al., 2007). It contains 30- 42% catechins by dry weight (Lambert & Yang, 2003). Green tea polyphenols (catechin) was shown to exhibit both antioxidant and prooxidant properties (Leung et al., 2001; Azam et al., 2004; Babich et al., 2006).

Green tea is rich in polyphones (catechins and Gallic acid) and also contains carotenoids, tocopherols, ascorbic acid and minerals (Cr, Mn, Se, or Zn) (Devaliya et al., 2011). Consumption of green tea has been shown to be associated with reducing the incidence of chronic pathologies associated with oxidative stress such as cancer or cardiovascular diseases (Hollman et al., 1999).

Population-based studies have shown that green tea helps protect against cancer as incidence of cancer are low in countries where the people consume it regularly (Devaliya et al., 2011). Several studies have shown that green tea inhibits the onset or growth of cancer in different organs such as lung (Ohno et al., 1995; Setiawan et al., 2001), skin (Picard, 1996; Katiyar et al., 2000), stomach (Borrelli et al., 2004), bladder (Ohno et al., 1985), oesophagus (Gao et al., 1994).

Green tea reduced inflammation associated with inflammatory bowel disease (i.e Crohn's disease and ulcerative colitis) (Setiawan et al., 2001) and also exerts antimicrobial activity (Toda et al., 1989; Fukai et al., 1991). Studies show that green tea may boost metabolism, burn fat and control body weight (Kovacs et al., 2004; Westerterp-Plantenga et al., 2005).

In vivo studies revealed that green tea and EGCG significantly decreased serum level of testosterone, luteinizing hormone, estradiol and the weights of androgen/ estrogen sensitive organs (Sayama et al., 1996; Kao et al., 2000; Zheng et al., 2004; Chandra et al., 2011). However, Satoh et al. (2002) showed green tea to

increase the levels of luteinizing hormone and testosterone. Recently, Chandra et al. (2011) demonstrated that the intake of green tea extract (1.25%, 2.5% and 5%) in male rats for 26 days altered the morphology and histology of the testis and accessory glands, inhibiting spermatogenesis with preferential loss of matured and elongated spermatids, significantly decreasing sperm count and the serum level of testosterone, inhibited the activities of testicular Δ^5 3β and 17β - hydroxysteroid dehydrogenase (Δ^5 -3β-HSD and 17β-HSD respectively), while also increasing luteinizing hormone (LH) and follicle stimulating hormone (FSH) plasma levels.

2.4.3 Black tea

Black tea is prepared by fermentation of *Camellia sinensis* leaves which leads to oxidation and contains multimeric polyphenols (Haslam, 2003). During its production, the rolled up leaves are allowed to undergo oxidation by the activation of polyphenol oxidases, for 1½ to 2 h before air drying, and this process is referred to as fermentation. The fermentation process results in the formation of bisflavonols, theafllavins, and thearubigins with other ooligomers (Harbowy & Balentine, 1997; Lee et al., 2002; Wright et al., 2002). The mean percentages of components of solid extracts in black tea contains catechins (10–12%), theaflavins (3–6%), thearubigins (12–18%), flavonols (6–8%), phenolic acids and depsides (10–12%), amino acids (13–15%), methylxanthines (8–11%), carbohydrates (15%), proteins (1%), mineral matter (10%), and volatiles (<0.1%) (Harold & Graham, 1992).

Quinones which are the substrates of consecutive transformations are produced during the first stage of tea production (Owuor, 1986), whereby resulting catechin quinone reacts with each other in different ways (Lambert & Yang, 2003). For instance, theaflavins, - a dimeric catechin is formed when the quinone derived from a simple catechins or its gallate reacts with a quinone derived from a gallocatechin or its gallate (Lambert & Yang, 2003). There are various forms of

theaflavins (figure 4): theaflavins (TF_1), theaflavins-3-gallate (TF_2A), theaflavins-3′-gallate (TF_2B) and theaflavins-3, 3′-gallate (TF_3) (Davies et al., 1999). A distinctive element in the structure of theaflavins is the seven- member benzotropolone, other benzotropolone compound found in black tea include theaflavic acid and theaflagallin (Collier et al., 1973; Nonaka et al., 1983:1986; Davies et al., 1999). Thearubigins (figure 2.4) are formed from catechins during the fermentation process of black tea production (Haslam, 2003). Flavonols in black tea include myricetin, quercitin, kaempferol, and rutin occurring mainly as glycosides (Rice- Evans & Muller, 1997).

Proven catechins in black tea have been placed in hierarchy based on their antioxidative properties: epigallocatechin≡ epigallocatechin gallate >>epicatechin gallate = epicatechin > catechin (Dreoosti, 1996; Rice-Evans et al., 1996; Ahmad et al., 2000). Besides the catechins, theaflavins have been shown to have a higher antioxidative property than EGCG, which is the strongest antioxidant among the catechins (Miller et al., 1996; Leung et al., 2001).

Black tea was shown to be anticarcinogenic and antimutagenic (Schuck et al., 2008). The health benefit of black tea may be attributed to its polyphenols (Gardner et al., 2007) with theaflavins, one of its components, considered the most effective anticarcinogenic compound (Schuck et al., 2008). Other health benefit of black tea includes potent diuretic activity (Ratnasooriya et al., 2006), reduced incidence of cardiovascular diseases (Sesso et al., 1999). In vivo study, showed that black tea possess marked aphrodisiac activity and caused elevated serum testosterone male rats (Ratnasooriya & Fernando, 2008).

Antioxidative properties of black tea include preventing generation of free radicals and inhibiting lipid peroxidation through interaction between its

polyphenol and transition metal (Rice-Evans et al., 1997; Lunder, 1989) and increasing the activity of antioxidants in organisms (Luczaj & Skrzydlewska, 2005). Despite its proven antioxidative properties, prooxidative properties of catechins and theaflavins have being demonstrated (Decker, 1997; Oikawa et al., 2003; Babich et al., 2005; 2006). The antioxidant/prooxidant behavior of tea polyphenolics are dependent upon several factors, such as metal-reducing potential, chelating behavior, pH, and solubility (Decker, 1997).

2.5 ASPALATHUS LINEARIS

Aspalathus linearis commonly known as rooibos (also referred to as *A. contaminate, A. corymbosus, Borbonia pinifolia or Psoralea linearis*) grows naturally in the Cederberg area in the western parts of the Western Cape Province of South Africa for its commercial use as herbal tea or tisane (Dahlgren, 1968; Mckay & Blumberg, 2007). Rooibos is manufactured in two different forms: unfermented and fermented rooibos (figure 2.5). Unfermented rooibos contains higher level of antioxidants compared to the fermented (von Gadow et al., 1997). Higher temperature and water-to-leaf ratio, and longer extraction time was shown to enhance the extraction of soluble solids and polyphenols from rooibos (Joubert 1988b; Joubert & Hansmann 1990; Joubert, 1990b). Marnewick et al. (2000) found in 2% (w/v) solutions of rooibos, a significant higher amount of total polyphenols, Flavonoids, and non-flavonoids in unfermented rooibos than in fermented rooibos with no significant difference in soluble solids. However, a significant difference in total polyphenols and soluble solids was observed in unfermented and fermented rooibos (Standley et al., 2001). These differences in findings may be attributed to the enzymatic and chemical modifications during fermentation and to processing methods i.e. sun drying or controlled drying (Joubert, 1996; Standley et al., 2001).

The major compounds (figure 2.6) present in rooibos include dihydrochalcone (aspalathin and notofagin), cyclic dihydrochalcone (aspalalinin), flavonol

(quercetin, isoquercetin, hyperoside, rutin and quercetin-3-0-β-D-robinoside), monomeric flavan-3-ol ((+)-catechin), oligomeric flavan-3-ol (procyanidin B3), flavonone (dihydro-orientin, dihydro-iso-orientin and hemiphlorin), flavones (orientin, iso-oorientin, vitexin, isovitexin, luteolin, luteolin-7-0-glucoside and chrysoeriol) (Joubert et al., 2008). Rooibos contains very small amount of (+) catechin (Marais, 1996) and aspalathin is the major flavonoid in unfermented rooibos (Joubert, 1996) of which the later is mostly oxidized to flavonones during fermentation (Joubert 1996; Marais, 1996).

Aspalathus linearis was shown to possess anticarcinogenic and antimutagenic properties based on its antioxidant polyphenols activities with unfermented rooibos having a higher protective effect than fermented rooibos (Marnewick et al., 2000). Other health benefits associated with A*spalathus linearis* includes hepatoprotective effect, phytoestrogenic activity, antispasmodic effects, antihaemolytic effect, anti-aging properties, anti-microbial and antiviral effects, vasodilatory effects, modulation of lipid profile amongst others (Joubert et al., 2008; Marnewick et al., 2009; 2011).

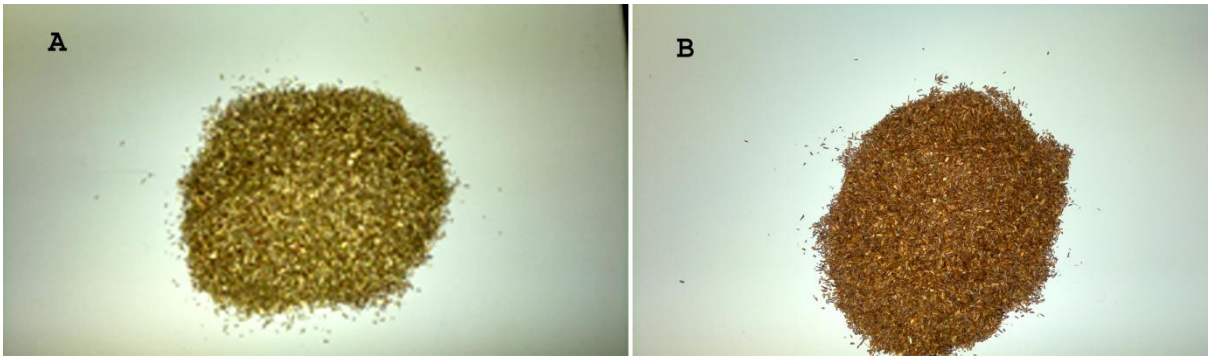


Figure 2.5: Images of unfermented rooibos (A) and fermented rooibos (B).

2.5.1 Antioxidative property and bioavailability

Antioxidant activity in rooibos determined according to DPPH radical scavenging method showed unfermented rooibos to have the higher amount (unfermented rooibos> fermented rooibos > semifermented rooibos) (von Gadow et al., 1997). Soluble solids in unfermented rooibos were also shown to be significantly higher than in fermented rooibos, however, it contained less soluble solids compared to green and black tea (Black tea ≥ green tea> unfermented rooibos > fermented rooibos) (von Gadow et al., 1997; Marnewick et al., 2000). Marnewick et al. (2000) also showed that unfermented rooibos had a significantly higher amount of total polyphenols, flavonoids and non flavonoids present compared to fermented rooibos.

Rooibos possess a very low bioavailability and reach the blood circulation in low amounts (Breiter et al., 2011). Flavonoids from rooibos were shown to be absorbed to a very low extent with only trace amounts detectable in urine samples and no detectable amount of metabolite present in blood, for up to 24 h after its consumption in pigs and human (Kreuz et al., 2008; Stalmach et al., 2009; Courts & Williamson, 2009). In an animal study, three metabolites of aspalathin were detected in urine sample after exposure to rooibos (Kreuz et al., 2008). On the other hand, Stalmach et al. (2009), detected four metabolites of aspalathin while Breiter et al. (2011), identified seven different metabolites derived from aspalathin and nothofagin with two unchanged components (aspalathin and nothofagin) in urine sample of humans exposed to rooibos. Small amounts of unchanged flavonoids (e.g. aspalathin and orientin) were identified in the plasma of volunteered men exposed to unfermented rooibos (Breiter et al., 2011).

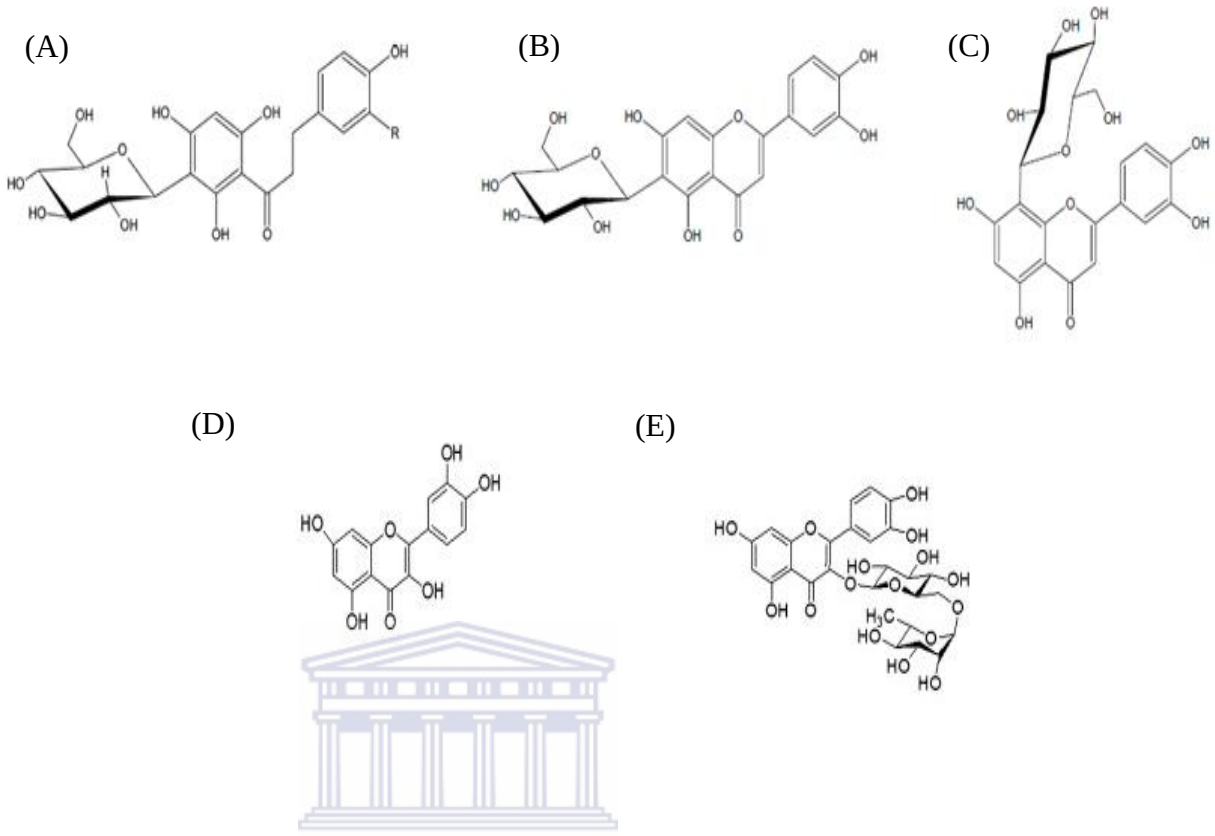


Figure 2.6: Major phenolic compounds present in *Aspalathus linearis.*

Structure of (A) dihydrochalcones (aspalathin (R=OH) and nothofagin (R=H)); (B, C) flavones (Iso- orientin and orientin respectively); (D, E) flavonols (quercitin and rutin repectively (reproduced with modification from Joubert et al., 2003: 2005; Abid et al., 2012).

2.6 REPRODUCTIVE SYSTEM

The reproductive system can be divided into the male and female reproductive system.

2.6.1 The male reproductive system

The male reproductive system consists of the testes, epididymis, vas deferens and accessory glands (seminal vesicles, prostate, and bulbourethral gland in the human). The testis consists of the seminiferous tubule, in which the sperm cells are produced in a process called spermatogenesis, and the interstitial space, where the Leydig cells responsible for the production of testosterone (figure 2.7) are found. Spermatozoa are transported into the epididymides, where they become mature, gain motility and are stored. Spermatozoa are also stored in the ampulla of the ductus deferens, where they are transported through peristaltic movement into the urethra (Ductus deferens, 2013). The accessory glands, testes and epididymis contribute to the formation of the seminal fluid whose functions include the following:

- Vehicle for the transport of spermatozoa
- Lubrication of the passage through which spermatozoa must travel
- Protects the sperm from the acidic environment in the female genital tract
- Contains fructose, a source of energy for the active sperm.

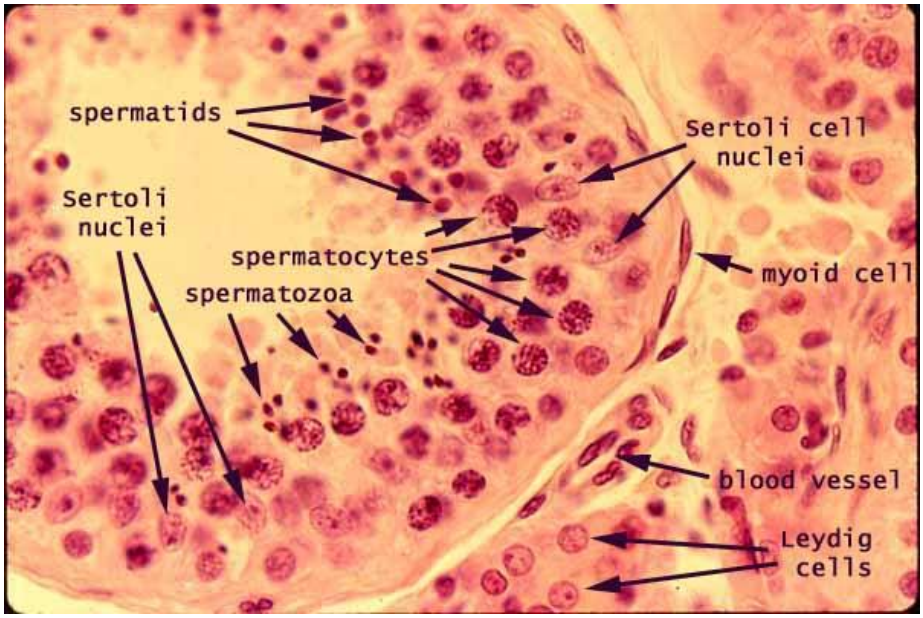


Figure 2.7: Cross section of the seminiferous tubule in testes
http://www.siumed.edu/~dking2/erg/RE028b.htm

2.6.1.1 Spermatogenesis

Spermatogenesis can be defined as a biological process that involves the transformation of immature germ cells into mature spermatozoa over a period of time in the seminiferous tubule of the testes (figure 2.8) (Hess, 1999). It can be divided into three phases:

- Proliferation
- Meiosis
- Differentiation

Proliferation can be described as the process in which the germ cells (spermatogonia) located at the base of the seminiferous tubule proliferate by mitotic division and multiply repeatedly to replenish the germinal epithelium. In meiosis (reduction- division), the germ cell (spermatocytes) increases its DNA contents, divides twice to produce four haploid germ cells (spermatids). Finally, in the last stage, the haploid germ calls undergo a process termed spermiogenesis (differentiation), whereby:

- There is elongation of the nucleus and chromatin condensation
- The Golgi apparatus produces lysosomal-like granules over the nucleus for the formation of the acrosomal cap which contains hydrolytic enzymes and is required for sperm-egg interaction and fertilization
- The cell forms a long tail with the mitochondria in the proximal region and loses its excess cytoplasm, which is eventually phagocytised by the Sertoli cells (Hess, 1999).

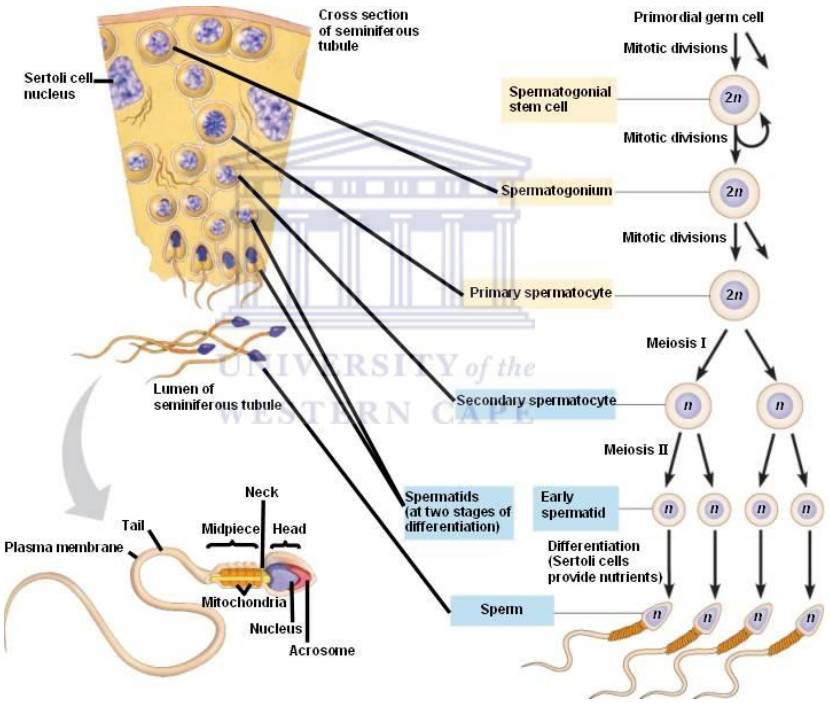


Figure 2.8: Spermatogenesis

(http://bio1152.nicerweb.com/Locked/media/ch46/spermatogenesis.html)

2.6.2 The female reproductive system

The female reproductive system consists of the ovaries, uterine tubes, uterus and vagina. The ovaries are responsible for the production of ovum (egg) and hormones (oestradiol and progesterone). The process of production of an ovum is referred to as oogenesis. An oogonium that is formed from the germinal epithelium grows into a primary oocyte, enclosed within a Graafian follicle and undergoes its first meiotic division into the secondary division shortly before being shed from the ovary (Sobti, 2008). The Graffian follicle develops into a corpus luteum after ovulation. The uterine tube receives the oocyte and provides a site for fertilization. Upon, fertilization, the egg becomes implanted in the uterus, where the embryo develops until child birth (Sobti, 2008).

2.6.2.1 Oogenesis

Oogenesis can be defined as the production of a mature ovum (egg) from primordial germ cells (Sobti, 2008). It involves the migration of primordial germ cells to the developing gonad to form oogonia (figure 2.9). It then undergoes mitotic division to form primary oocyte and begins its first meiotic division in which DNA replication occurs with each chromosome having two chromatids. This division is then arrested at the prophase stage until the onset of sexual maturation at puberty. It then proceeds at the onset of sexual maturation under the influence of hormones forming a secondary oocyte and its first polar body. The second meiotic division occurs, leading to the formation of a haploid mature ovum and a second polar body. The mature ovum (egg) produced is then released at some point during ovulation (Sobti, 2008).

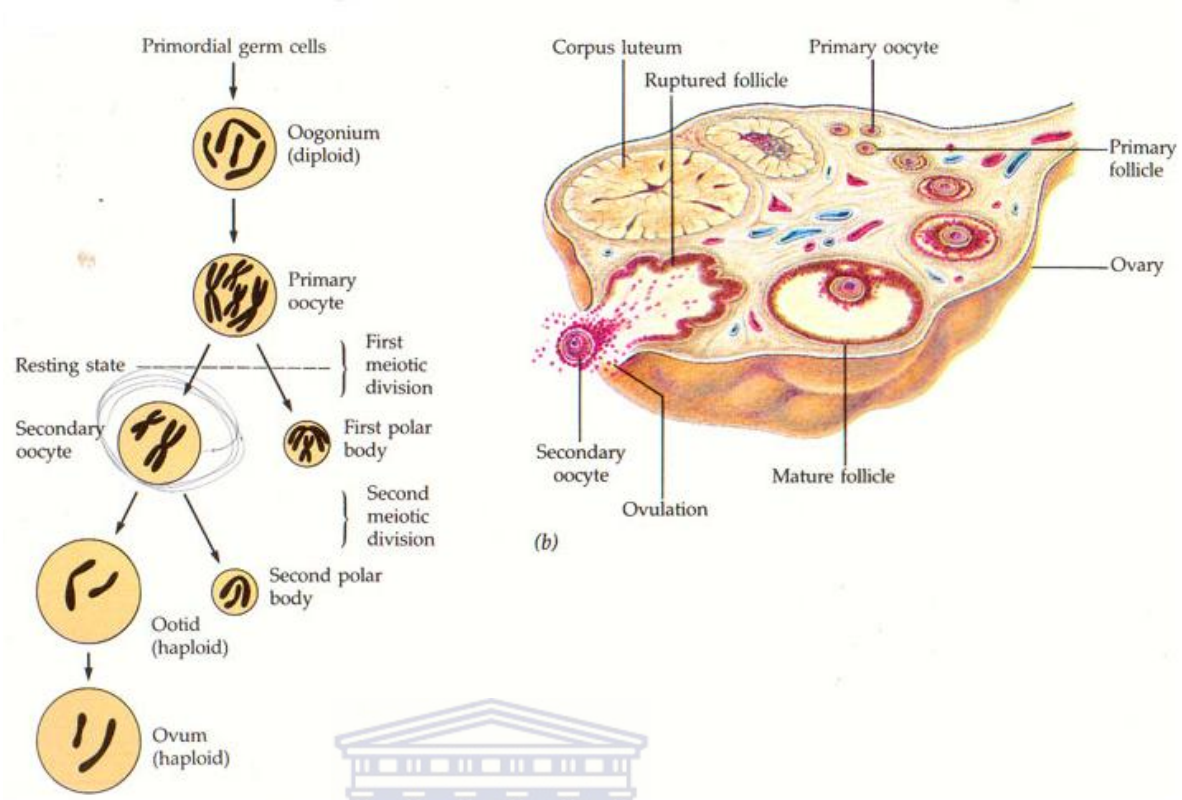


Figure 2.9: Oogenesis

(http://ldysinger.stjohnsem.edu/ThM_599d_Beg/02_Biology/02_spermat-oogen.htm)

2.6.2.2 Phytoestrogens and the female reproductive tract

Natural estrogens are involved in the development and function of the reproductive tracts in male and female, neuroendocrine tissues, bone and mammary gland (Sonnenschein et al., 1998). Environmental ostrogens are derived mainly from phytoestrogens or synthetic oestrogens (Burton & Wells, 2002). Phytoestrogens are naturally occurring phytochemicals present in plants and their products, are structurally and functionally similar to isoflavones (17β-oestradiol) or synthetic oestrogens such as diethylstilboestrol (lignins). Examples of

isoflavone include coumestrol, genistein, diadzen and equol (Aldercreutz et al., 1982; Axelson et al., 1982; Whitten et al., 1995; Boué et al., 2000).

According to Whitten et al. (1995) and Sonneschein et al. (1998), phytoestrogens exert their biological activity by:

- mimicking the action of endogenous estrogens
- acting as oestrogen antagonists
- altering the pattern of synthesis and metabolism of endogenous hormone
- modifying hormone receptor values

However, in the absence of oestrogens, isoflavones have a weakly oestrogenic effect, and may exhibit an antiestrogenic property in their presence (Cline et al., 1998).

From previous studies, phytoestrogens were shown to exhibit uterotrophic effect (increased uterine weight) and reduced ovarian weight, which may involve retention of uterine fluid in the lumen and hyperplasia of the endometrium (El Samannoudy et al., 1980; Whitten et al., 1992). For example, neonatal rats exposed to either coumestrol or genistein had increased uterine wet and dry weight until day 10 after post natal life (Medlock et al., 1995a: 1995b; Santell et al., 1997). Furthermore, phytoestrogens like coumestrol and diethylstibestrol were shown to induce a significant long term abnormalities in the reproductive tract that included vaginal cyst, persistent vaginal cornification, endometrial squamous metaplasia, absence of *corpora lutea*, increased ceroid deposition in the ovaries and haemorrhagic follicles, decreased number of Grafiann follicle and increased number of atretic follicle in mice (Burroughs et al., 1990b; Murthy et al., 1997). Whitten et al. (1993), showed that these animal with persistent cornification of the vaginal epithelial cells, indicates persistent estrous stage, failed to respond to priming of oestrogen followed by progesterone with a LH surge at the expected time. This implies that exposure of phytoestrogens during the neonatal period

could affect the hypothalamo- hypophysial- ovarian axis (Burroughs et al., 1985: 1990a; 1990b). In the adult mice, intraperitoneal administration of benzene extract from flowers of *H rosa sinensis* induced irregular oestrous cycle with a dose dependent prolonged estrus and metestrus stage (Murthy et al., 1997). Resveratrol, a phytoestrogen naturally found in grape with a structure similar to diethylstilbestrol decreased body weight, disrupted oestrous cyclicity, increased ovarian weight in intact female rats (Henry & Witt, 2002).

In the humans, phytoestrogens have been shown to exert an effect on the menstrual cycle; however, these findings are inconsistent (Whitten & Naftolin, 1998). For instance, Cassidy et al. (1994) revealed that a diet containing 60mg soy protein delays the onset of menstruation and prolongs the follicular phase of the cycle, suppressing the mid cycle of LH and FSH peak plasma concentration, increasing plasma concentrations of oestradiol and decreasing the concentration of cholesterol in the follicular phase. This is indicative of the antiestrogenic effect of phytoestrogens in premenopausal women. On the other hand, Duncan et al. (1999) illustrated that a diet rich in soy isoflavone reduced plasma estrone concentration but had no impact on the length of the menstrual cycle, follicular or luteal phases. The effect of phytoestrogens in the female genital tract may be dependent on the age and duration of exposure (Burton & Wells, 2002).

2.6.3 Fertilization

Fertilization can be defined as the fusion of gametes to produce a new organism; in humans it involves the fusion of an ovum with the sperm leading to the formation of an embryo. For fertilization to occur, the spermatozoa undergo capacitation in the female reproductive tract, after which they undergo two calcium (Ca^{2+}) dependent physiological processes, namely an acrosome reaction and hyperactivation (Yanagimachi, 1994).

Capacitation is a post-ejaculatory modification of the sperm surface, which is species-specific and time-dependent (Van Kooij et al., 1986; Fraser, 1984). Sperm membranes are rich in polyunsaturated fatty acids (PUFA) and high amounts of PUFA provides the sperm cells with the structural fluidity required to engage in capacitation (Aitken, 1997). It involves the mobilization and/or removal of certain surface components from the plasma membrane of the spermatozoa such as glycoproteins, decapacitation factor, acrosome-stabilizing factor, and acrosin inhibitor (Mansour et al., 2008). Bicarbonate induces capacitative changes (Boatman & Robbins, 1991; Gadella & Harrison, 2000; Flesch et al., 2001). A high level of bicarbonate induces activation of adenylate cyclase- a key event in sperm capacitation, in the lumen of the oviduct (Harrison et al., 1996).

The acrosome reaction (AR), also known as acrosome exocytosis (AE), is a terminal morphologic alteration of spermatozoon, and a synchronized and highly regulated process that is required for fertilization (Knobil & Neill, 1994). This results in the inner acrosomal membrane being the limiting membrane of the anterior sperm head, releasing the content of acrosome such as hydrolytic enzymes, and as a result the sperm head fuse with the oocyte membrane, leading to fertilization (Yanagimachi, 1994; Tollner et al., 2000; Kumi-Diaka & Townsend, 2003). Ca^{2+} is an essential mediator of acrosome reaction (Gonźalez-Martínez et al., 2001), and an efflux of Ca^{2+} from within the acrosome is required in the presence of high cytosolic Ca^{2+} (De Blas et al., 2002: 2005). As with other secretory events, sperm uses the SNARE (SNAP (Soluble NSF Attachment Protein) Receptor) fusion machinery and regulatory components (Tomes et al., 2005) to fuse with the oocyte.

The acrosome reaction can occur spontaneously when ejaculated or epididymal spermatozoa are incubated at 37^{o}C over a period of time in a defined tissue culture medium containing bovine serum albumin (BSA) (Fenichel et al., 1991: 1995; Yanagimachi, 1994; Mansour et al., 2008; Bendahmane et al., 2002). Albumin is a major component of the female reproductive system and it is thought to act as an acceptor of cholesterol or phospholipids during *in vivo* or *in vitro* capacitation (Bendahmane et al., 2002). BSA facilitates capacitation by the efflux of cholesterol or phospholipids from the plasma membrane of spermatozoa, leading to increased fluidity and permeability of sperm membrane. Thus, a transflux of ions occur, which triggers the acrosome reaction (Davies et al., 1979; Tulsiani et al., 1998; Visconti et al., 1999; Abou- Haila & Tulsiani, 2000).

Hyperactivation can be described as the distinct qualitative change in sperm motility when the progressively moving spermatozoa become extremely vigorous and less progressive and exhibit large amplitudes of head displacement (Yanagimachi, 1970). Hyperactivated motility is thought to facilitate the penetration of the zona pellucida or play a role in the transport of spermatozoa in the uterine tube (Yanagimachi, 1994).

Excessive production of ROS have been shown to cause sperm pathology, while a low and controlled concentration of ROS plays important roles in sperm physiology and in the acquisition of sperm fertility (de Lamirande et al., 1997). O_2^- and H_2O_2 exposed to human spermatozoa increased the development of hyperactivation and capacitation (de Lamirande & Gagnon, 1993a). SOD prevented hyperactivation and capacitation in human sperm (de Lamirande & Gagnon, 1993). CAT added to human sperm incubated in B_2 Menezo medium reduced both hyperactivation and the A23187-induced acrosomal reaction, without affecting the percentage of motile or viable cells (Griveau et al., 1994). In

addition, NO inhibited human sperm motility at high concentration, however, low concentration of NO resulted in increased capacitation without affecting motility (Zini et al., 1995).

As shown in figure 2.10, fertile spermatozoa produce very low amount of ROS in their basal state. However, when incubated under capacitating conditions, superoxide production (O_2^-) is stimulated and depending on the condition, O_2^- and H_2O_2 are formed by the dismutation of O_2^- which can induce sperm capacitation. In addition, human sperm capacitation is also enhanced by nitric oxide (NO) (de Lamirande et al., 1997). Induction of the acrosome reaction in capacitated spermatozoa further stimulates production of O_2^- which causes the release of unesterified fatty acids from the plasma membrane of these cells and H_2O_2 seems to be involved in the acrosome reaction (de Lamirande et al., 1997). Finally, pre-treatment of mouse spermatozoa with ferrous iron and ascorbic acid caused a significant increase in lipid peroxidation and an improved rate of fertilization which may be due to an increased binding of spermatozoa to the mouse zona pellucida (de Lamirande et al., 1997).

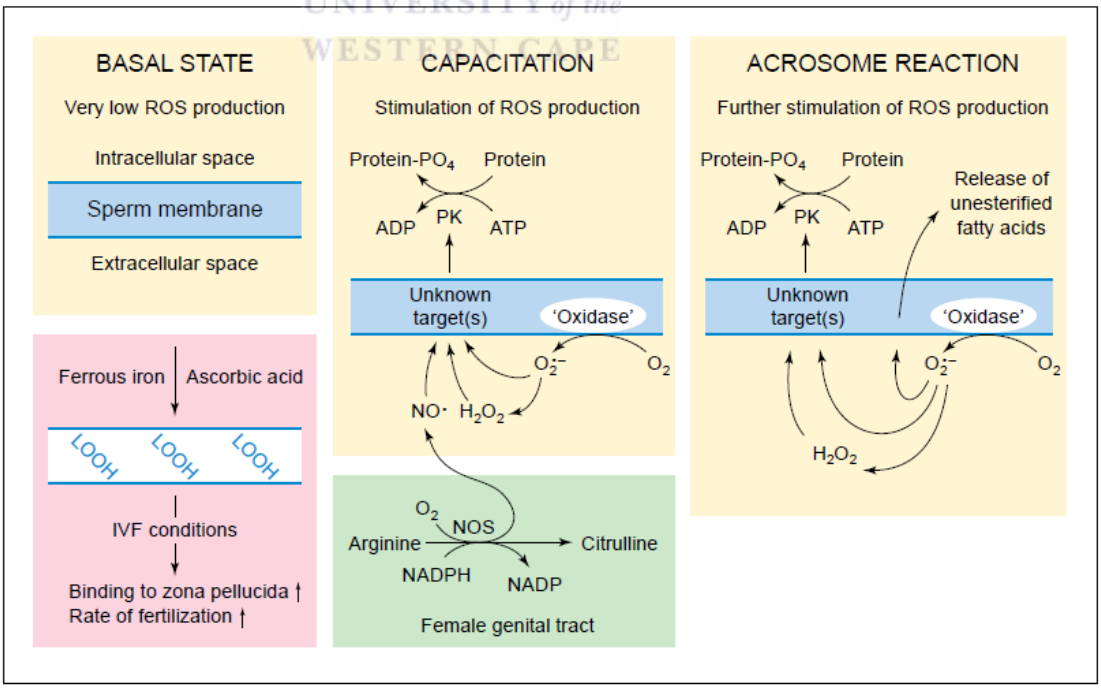


Figure 2.10: Schematic representation of the involvement of reactive oxygen species (ROS) in sperm capacitation and acrosome reaction (reproduced from de Lamirande et al., 1997).

2.6.4 Regulation of the reproductive system

The secretion of testosterone is regulated through a negative feedback loop that involves the hypothalamus and anterior pituitary gland (figure 2.11). A low level of testosterone stimulates the release of gonadotropin releasing hormone (GnRH) from the hypothalamus to the anterior pituitary gland via the portal system. The release of GnRH stimulates the release of luteinizing hormone (LH) and/or follicle stimulating hormone (FSH) by the anterior pituitary. High levels of testosterone result in the inhibition of the hypothalamus and anterior pituitary, thereby decreasing the secretion of GnRH and LH (Rhodes & Tanner, 2003).

LH stimulates the production of testosterone from the Leydig cells of the testes in male, but induces ovulation in the female. FSH, together with testosterone, stimulates the spermatogenesis in the seminiferous tubule in the male. In the female, FSH controls the development and maturation of the follicle. Inhibin, a protein secreted by the Sertoli cells in the testes, inhibits the secretion of FSH by the anterior pituitary gland, hence inhibiting spermatogenesis (Sobti, 2008). Oestrogen stimulates the maturation of the uterus and vagina, development of the female secondary sexual characteristics and triggers the onset of the menstrual cycle (Sobti, 2008). Progesterone, produced mainly in the corpus luteum increases the secretory activity and vascularisation of the uterus and decreases its contractility. In synergy with oestrogen, progesterone plays a role in implantation of the placenta, the maintenance of the foetus and the development of the mammary glands (Sobti, 2008).

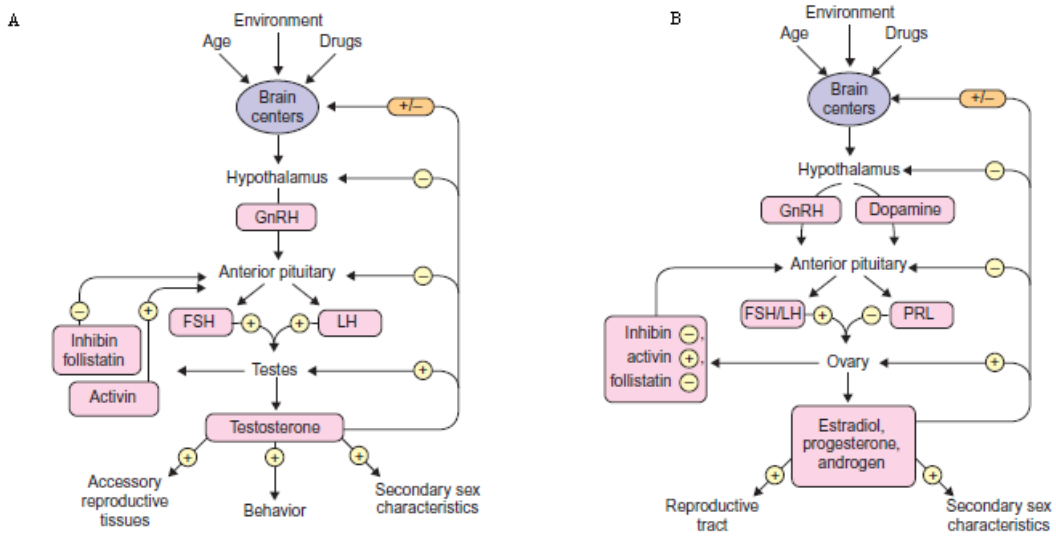


Figure 2.11: Regulation of the male (A) and female (B) reproductive system. Plus indicates positive and minus indicates negative feedback regulation (reproduced from Rhodes & Tanner, 2003).

2.6.5 Oestrous cycle

The reproductive cycle of the female rat is referred to as the oestrous cycle and is characterized based on the cell types observed in the vaginal smears as proestrus (nucleated epithelial cells), estrus (anucleated epithelial cells), metestrus (same proportion among leukocytes, cornified and nucleated epithelial cells) and diestrus (mainly leukocytes) with mean length of cycle of 4 or 5 days (Evans & Long, 1922; figure 2.12). Ovulation occurs at the beginning of proestrus until the end of estrus (Young et al., 1941; Schwartz, 1964).

There are fluctuations in the level of hormone in the female reproductive system during the oestrous cycle. For instance, the level of prolactin, LH and FSH remain low during the oestrous cycle and increase in the afternoon of proestrus. Estradiol begins to increase at metestrus reaching its peak during proestrus and returns to

the baseline at estrus. Progesterone increases during metestrus and diestrus and decreases afterward and then reaches its second peak toward the end of proestrus (Smith et al., 1975; Spornitz et al., 1999).

The varying amounts of sex hormones, particularly estradiol-17b and progesterone during the different stages of the oestrous cycle induce cyclic changes in the cell morphology and histology of the uterine epithelium and ovaries of the rat (Spornitz et al., 1994). At diestrus, the uterus is small and inactive with a slit-like lumen lined with low cuboidal or columnar epithelium showing occasional degenerate cells. While in the ovaries, the corpora lutea attains maximum size with vacuoles at the centre and the presence of early formation of fibrous tissue (Westwood, 2008). During proestrous, the endometrium is lined by tall cuboidal or columnar epithelium with frequent mitosis and little or no epithelial cell degeneration of the gland or epithelium. The vasculature of the endometrium becomes prominent, the lumen becomes dilated, and there is the presence of edema in the stroma. In the ovaries, degeneration of the corpora lutea occurs with marked presence of central fibrous tissue formation (Westwood, 2008). In estrus, there is cellular degeneration in the gland and epithelial lining of the uterus, loss of mitotic activity and leucocyte infiltration, while the dilation of the lumen may persist until late estrus. At this stage the ovaries, present degenerated corpora lutea and sometimes newly formed corpora lutea with central fluid filled cavity, which is devoid of fibrous tissue formation (Westwood, 2008). Lastly, during metestrus, there is continued vacuolar degeneration in the epithelium of the uterus with marked returned of mitotic activity. While in the ovaries, the *corpora lutea* contains central fluid filled cavity devoid of fibrous tissue formation (Westwood, 2008).

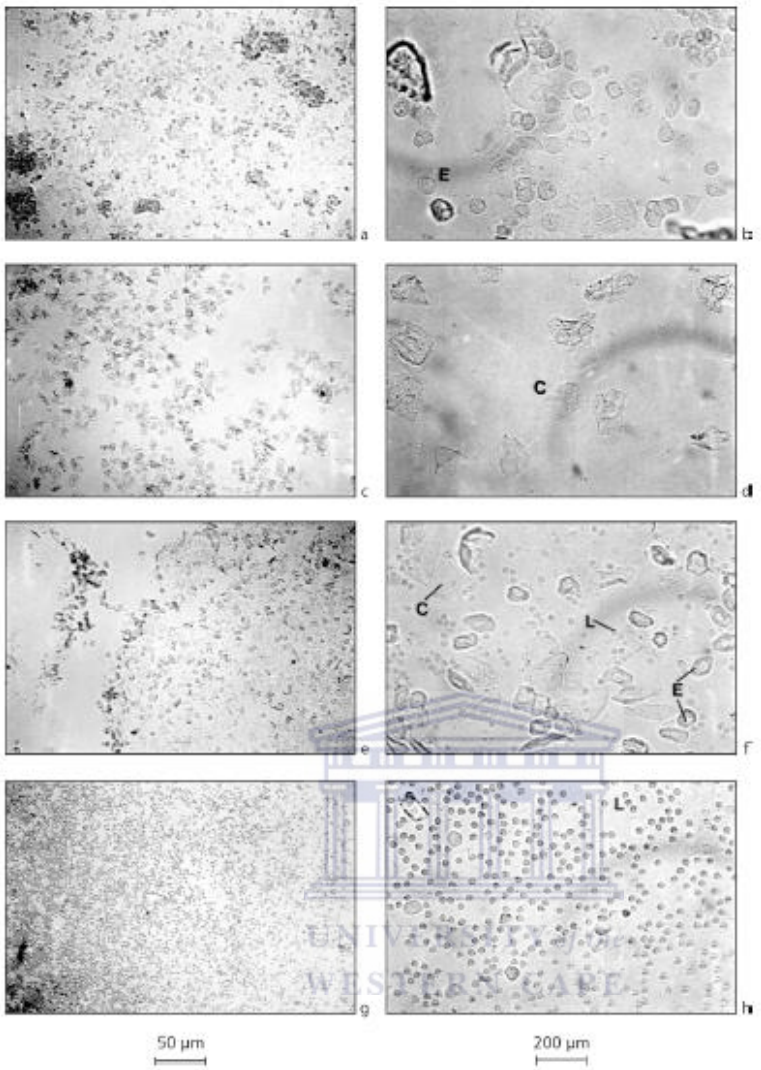


Figure 2.12: The oestrous cycle from vaginal smear of female rats at proestrus (a, b), estrus (c, d), metestrus (e, f) and diestrus (g, h). Leukocytes (L), epithelial (E) and cornified (C) cells are indicated (reproduced from Marcondes et al., 2002).

CHAPTER THREE

MATERIALS & METHODS

3.1 CHEMICALS AND REAGENTS

Dimethylsulfoxide (DMSO), ethanol, glacial acetic and hydrochloric acid, hydrogen peroxide, phosphoric acid, potassium dihydrogen phosphate, potassium dihydrogen orthophosphate, sodium pyruvate where purchased from Merck Chemicals (Johannesburg, South Africa). Ammonium acetate, α- ketoglutarate, bovine serum albumin, (+)-catechin, coomassie brilliant blue G, creatinine, 4-(Dimethylamino)-cinnamaldehyde (DMACA), 2,4-dinitrophenylhydrazine, Ethylenediaminetetraacetic acid, Folin-Ciocalteau reagents, Gallic acid, glacial acetic acid, human chorionic gonadotropin (HCG), 6-hydroxy-2,5,7,8-tetramethylchroman-2-Carboxylic acid, Iron(III) chloride, L-alanine, L-aspartic acid, methanol, p-formaldehyde, picric acid, pyrogallol, quercitin, sodium acetate, sodium carbonate (Na_2CO_3), thiazolyl blue tetrazolium bromide (MTT), 2-thiobarbituric acid, trichloroacetic acid, 2,4,5-Tri (2-pyridyl)-S-triazine (TPTZ), were obtained from Sigma Aldrich (St. Louis, MO, USA). Fetal bovine serum, Horse serum, Dulbecco's modified eagle's medium (DMEM/F-12 1x) (Biochrom, Berlin, Germany), sodium chloride and sodium hydroxide were purchased from BM Scientific (Cape Town, South Africa). Tris-hydrochloride was purchased from applichem (Ottoweg, Germany) and phosphate buffered saline (Oxoid, England).

3.2 PLANT MATERIAL

Green tea (unfermented; *Camellia sinensis*: Five roses) and black tea (fermented; *Camellia sinensis*: Five roses) were purchased from a retail store in Cape Town. Unfermented and fermented rooibos (*Aspalathus linearis*) was a gift from Rooibos Ltd (Clanwilliam, South Africa).

3.3 PREPARATION OF TEA

Freshly boiled tap water was added to concentrations of 2g/100ml and 5g/100ml for unfermented and fermented rooibos, green and black tea for 5 min. Afterwards, it was filtered through a cheese cloth and Whatman's filter paper (no 4 and 1 respectively) using a vacuum system. The aqueous extracts were allowed to cool to room temperature and fed to rats *ad libitum*. Fresh tea was prepared every second day.

3.4 ANIMALS

Male and female white Wistar rats were bred in the animal facility of the Medical Bioscience department, University of the Western Cape by mating rats for a period of seven days. At weaning (21 days), male and female pups were kept in separate cages. During the mating, pregnancy and post-partum all animals were allowed free access to standard rat chow and tap water *ad libitum* and were housed under standard conditions in a room at temperature of 21–24 ^{o}C with constant 12 h light/dark cycle. All experiments conducted in this study received ethical clearance by the ethical committee in the University of the Western Cape (registration No: 11/7/40).

3.5 TREATMENT PROTOCOL

The animals (male and female) were randomly divided into nine different groups, with six animals assigned to each group (n=6) and 3 animal per treatment group were housed in separate cages. Each group received the following treatment *ad libitum* using a water bottle:

- Group one received tap water
- Group two received 2% green tea
- Group three received 5 % green tea
- Group four received 2 % black tea
- Group five received 5% black tea
- Group six received 2% unfermented rooibos
- Group seven received 5 % unfermented rooibos
- Group eight received 2 % fermented rooibos
- Group nine received 5% fermented rooibos

3.6 CHEMICAL ANALYSIS OF TEA AND HERBAL INFUSION

During the period of treatment, at least 3 samples (2% and 5%) of tea and herbal infusions prepared were randomly collected at different time points and stored at -20^{o}C for further use.

3.6.1 Determination of soluble solids

Glass vials were kept in a drying oven (120ºC) overnight, placed in a desiccator and weighed afterwards. Each tea and herbal infusion sample (1 mL) in triplicate was then placed in the vials, dried overnight (120ºC), allowed to cool in a desiccator and weighed again. Results obtained were expressed as mg/mL of tea or herbal infusion.

3.6.2 Determination of total polyphenols

Analysis of total polyphenols present in tea and herbal infusion was done using the Folin-Ciocalteaus reagent (F-C reagent; Singleton & Rossi, 1965) with Gallic acid as standard. In brief, six standards were prepared in triplicate by adding 0.1% Gallic acid in dH_2O to final concentrations of 10, 20, 40, 60, 80 and 100 (µg/mL) (appendix A). Samples were diluted with dH_2O and prepared in triplicates (appendix B). To the standards, tea and tisane samples and blank, 10% F-C reagent and 7.5% Sodium bicarbonate were added in a ratio 5: 4: 1. All samples were thoroughly mixed and incubated for 2 h at 37 ºC and read at 765 nm. A standard curve using Gallic acid as standard was used to determine the amount of total polyphenols (appendix C).The total polyphenols content of the tea or herbal infusion was expressed as mg Gallic acid equivalent (GAE)/ mL of tea or herbal infusion.

3.6.3 Determination of flavonols

The flavonol/flavones content according to Mazza et al. (1999) was determined using quercitin as standard. In order to determine the presence of flavonols in the respective teas and herbal infusion, standards were prepared in triplicate using quercetin stock in 95% ethanol to final concentrations of 1, 2, 4, 8, 16 and 32 mg/mL (appendix D). Likewise, samples were further diluted (appendix E) and prepared in triplicates. To 50 µL blank, standards or samples, 50 µL 0.1% HCl in 95% EtOH and 900 µL was added and incubated for 30 min. Absorbance was read at 360 nm and the amount of flavonol in the samples was derived from a standard curve using quercetin as the standard (appendix F). Results obtained were expressed as mg Quercetin equivalent/ mL of tea or herbal infusion.

3.6.4 Determination of flavanol

Flavanol/proanthocyanidin was determined with (+) catechin as standard (McMurrough & McDowell, 1978). The standards were prepared in triplicate to a final concentration of 5, 10, 20, 30 and 40 µg/ mL by mixing catechin stock solution with methanol (appendix G). Also, the respective tea or herbal infusion samples were prepared in triplicate with a further dilution (appendix G). Briefly, 1000 µL 4-(Dimethylamino) - cinnamaldehyde (DMACA) was added to 200 µL blank, standard and tea samples. Standards were incubated for 2 min and tea and tisane samples at different times (green and black tea (5 min); unfermented and fermented rooibos (15 min) based on the maximum absorption obtained. Absorbance was read at 640 nm. The amount of catechin present in the samples was deduced from a standard curve (appendix I) and results obtained were expressed as mg Catechin equivalent/ mL of tea or herbal infusion.

3.6.5 Determination of ferric reducing antioxidant power (FRAP)

Antioxidant activities in the teas were measured using ferric reducing antioxidant power (FRAP) using Trolox as standard (Benzie and Strain, 1996). FRAP measures the ability of compounds to act as electron donor. A lower level of reduction potential indicates that less energy is required for electron donation resulting in higher antioxidant activity. This method measures the ability of a compound to reduce Fe^{3+} (oxidant in system) to produce $Fe^{2+.}$ The FRAP assay uses antioxidants as reductants in a redox-linked colorimetric method, where there is a reduction of FeIII-TPTZ to FeII-TPTZ (FeIII-tripyridyltriazine complex to FeII-tripyridyltriazine) by biological antioxidants. FeII-TPTZ has an intense indigo/blue colour. Briefly, five standards where prepared in triplicate mixing TROLOX (5mM) and methanol to a final concentration of 100, 150, 200, 400 and 600 µM (appendix J). Teas and the herbal infusions were diluted with dH_20 (1:24). To 10µL standard, tea and herbal infusion samples and blank, 300 µL FRAP reagent (10:1:1; 300mM sodium acetate buffer: 10mM TPTZ: 20mM

$FeCl_3$) and 30 µL was added, vortexed, incubated in at 37ºC for 4 min and absorbance read at 593 nm with a micro plate reader. The level of FRAP in serum was deduced using a standard curve (appendix K) and the result obtained was expressed as µM Trolox/ mL of tea or herbal infusion.

3.7 EFFECT OF CAMELLIA SINENSIS AND ASPALATHUS LINEARIS AND REPRODUCTION, LIVER AND KIDNEY FUNCTIONS

Sexually mature virgin male Wistar rats (63 day-old; 6 animals per group) weighing 200- 250g received either 2% or 5 % of green tea, black tea, unfermented rooibos or fermented rooibos for 52 days.

Sexually mature virgin female Wistar rats (84 day-old; 6 animals per group) weighing 180- 230g received the respective treatments for 21 days. For 10-12 days before treatment, vaginal smears were taken every morning and estrous cycle of female rats was determined microscopically according to Marcondes et al. (2002) and only the normal cycling ones were used for the purpose of this study. Treatment began on day of diestrous and terminated as they enter pro-estrous.

On a daily basis the amount of fluid taken was recorded and rat body weight was determined weekly. Based on the amount of soluble solids present in each tea, a percent soluble solid (% SS) and an average daily intake (ADI) of fluid or polyphenols (soluble solid/total polyphenols/flavonol/flavanol) expressed as mg/100g BW was calculated:

% SS = 100/ soluble solids of corresponding tea [mg/mL] * total polyphenols/flavonol/flavanol of corresponding tea [mg/mL] (3.1)

ADI= average fluid intake during 52 days [mL] /100 * % SS (3.2)

At the end of treatment, animals were sacrificed using CO_2. Final body and organ weights were taken into account. Blood was obtained through cardiac puncture for hormonal and biochemical assays. Left cauda epididymis was excised and used for the determination of sperm concentration, vitality, motility and acrosome reaction. Testis, right epididymis, seminal vesicles and prostate (male rats), uterus and ovaries (female rats), liver and kidney were fixed in appropriate fixatives for histological purposes. Left testis (male rats), liver and kidney were frozen at -80^{o}C for biochemical assays.

3.7.1 Tissue histology

The male rats were sacrificed after a 52 day treatment with the tea or herbal infusion by CO_2 inhalation. Weights measured at autopsy included body, left testis, left epididymis, seminal vesicles (including seminal fluid and coagulating gland), prostate, kidneys, liver, and stomach. These organs were fixed in Bouin's solution for about a week.

The Bouin's solution was prepared in the following way:

- saturated aqueous picric acid 375 mL
- formaldehyde (37%) 125 mL
- glacial acetic acid 25 mL

After fixation, the tissues were cut in small sections and placed in properly labelled cassettes. After which, they were dehydrated through a series of graded ethanol, cleared in xylene and infiltrated in wax in an automated Leica TP 1020 tissue processor (Leica Biosystems, Germany) in an 18 hour cycle as shown in table 3.1.

Table 3.1: 18 hour cycle of processing tissues

Steps	Solutions	Time (hour)
1	70% ethanol	2
2	80 % ethanol	2
3	90 % ethanol	2
4	100% ethanol 1	2
5	100% ethanol 2	2
6	Xylene 1	2
7	Xylene 2	2
8	Wax bath 1	2
9	Wax bath 2	2

At the end of the 18 hour cycle, the tissues were embedded in paraffin wax and cut into sections of 6-µm. Following that, they were placed on a staining rack and put in a hot air oven at 60^{o}C for approximately 30 min to further fix the tissues and to melt the wax and then stained with haematoxylin (1 g haematoxylin, 50 g potassium alum, 0.2 g sodium iodate, 1 L dH_20, 50 g choral hydrate and 1 g citric

acid) and eosin (1% eosin, 1% phloxine and distilled water (2:1:3)), as shown in table 3.2 and mounted for histopathological examination.

Table 3.2: Procedure for Haematoxylin and Eosin stain

Steps	**Solutions**	**Time (min)**
1	Xylene (x2)	5 each
2	100% ethanol (x2)	5 each
3	90% ethanol	5
4	80% ethanol	5
5	Haematoxylin	15
6	Rinsed in tap water	1
7	Scott's tap water	2
8	1% acid alcohol	2
9	Eosin	1
10	Rinsed in tap water	1
11	80% ethanol	2
12	90% ethanol	2
13	100% ethanol	2
14	Xylene (x2)	2 each

Testis diameter and the epithelial height of seminiferous tubule were measured at 100 x magnification. At least 30 tubular profiles (five fields) were randomly chosen and measured per animal. In addition, the number of seminiferous tubules per field was counted to a total of 10 fields per animal. Epithelial heights of 10 cauda and caput epididymis each were also measured.

The female rats were sacrificed after a 21 day treatment with respective tea and herbal infusion on day of reaching pro-oestrous by CO_2 inhalation. Vaginal smears were taken before autopsy as described by Marcondes et al. (2002) to

adjust for the oestrous cycle. Weights measured at autopsy included body, uteri, ovaries, kidneys, and liver. These organs were fixed in 10 % neutral-buffered formalin solutions for about a week.

The 10% neutral buffered formalin solution was prepared in the following way:

- Formaldehyde (37%) 100 mL
- Distilled water 900 mL
- Anhydrous monobasic sodium phosphate (NaH_2PO_4) 4.0 g
- Disodium hydrogen phosphate (Na_2HPO_4) 6.5 g

The tissues were then dehydrated through a series of graded ethanol immersions, cleared in xylene, infiltrated in the automated tissue processor as described earlier. And subsequently embedded in paraffin wax, cut into sections of 6-µm and stained with haematoxylin and eosin (Table 3.2) for histopathological examination.

3.7.2 Blood Collection

At the end of treatment, animals were sacrificed by CO_2 inhalation, blood samples were collected by cardiac puncture, and allowed to clot for 30 min. Clear serum was separated by centrifugation (3000 x *g*, 15 min) and stored at -80^{o}C for further use.

3.7.2.1 Testosterone

The level of testosterone from serum obtained from the male rats exposed to the different teas was quantified using an ELISA (EIA 1559, DRG Instruments GmBH, Marburg, Germany). In brief, 25 µL of testosterone standards (0, 0.2, 0.5, 1, 2, 6 and 16 ng/ mL) and samples were dispensed into appropriate wells.

Afterwards, 200 µL enzyme conjugate was added to each well, mixed thoroughly for 10 sec and incubated for 60 min at RT. At the end of the incubation time, the contents were briskly shaken out and rinsed three times with 400 µL diluted wash solution per well. Residual droplets were removed by striking the wells sharply on absorbent paper. Then, 200 µL substrate solution was added to each well and incubated for 15 min at RT and the reaction was put to an end with the addition of 100 µL stop solution to each well and absorbance read at 450 nm within 10 min of adding the stop solution. The sensitivity of the DRG ELISA was found to be 0.083 ng/mL. A standard curve was used to deduce the level of testosterone produced in serum (appendix L) and was the result obtained was expressed as ng/mL.

3.7.2.2 Follicle stimulating hormone

The level of follicle stimulating hormone (FSH) in serum obtained from the female rats exposed to the different teas was quantified using an ELISA following an extraction method (EIA 1785, DRG Instruments GmBH, Marburg, Germany).

To be in line with the detection range of this assay (0.86 – 100 mIU/mL), FSH in serum had to be enriched by a factor of 3.75. Firstly, 1 mL diethylether was added to 100 µl serum, mixed twice (20 sec) using a vortex and centrifuged (3800 x *g*, 5 min). Samples were then frozen at -80^{o}C and the liquid organic supernatant was decanted (ether fraction). Afterwards, the ether fraction was allowed to evaporate at RT under a hood to a volume of 100 µL, and the tube rinsed with 150µL ether and vortexed briefly. The ether fraction was again allowed to evaporate to a volume of 20 µL and reconstituted with 75 µL steroid free human serum (zero standards). Ether fractions were again vortexed briefly and centrifuged (3800 x *g*, 3 min).

The ether fraction was then used to determine the FSH level according to the manufacturer's instruction. In brief, 50 µL of standards (0, 5, 15, 50, 100 and 200 mIU/mL) and samples were dispensed into appropriate wells. Afterwards, 100 µL enzyme conjugate was added to each well, mixed thoroughly for 30 sec and incubated for 45 min at RT. At the end of the incubation time, the contents were briskly shaken out and rinsed five times with 400 µL distilled water per well. Residual droplets were removed by striking the wells sharply on absorbent paper. Then, 100 µL substrate solutions was added to each well, gently mixed for 10 sec and incubated for 20 min at RT in the dark. The reaction was put to an end by the addition of 100 µL stop solution to each well, gently mixed for 30 sec and absorbance read at 450 nm within 10 min of adding the stop solution. A standard curve was used to deduce the level of FSH produced in serum (appendix M) and the result obtained was expressed as mIU/mL.

3.7.2.3 Luteinizing hormone

The level of luteinizing hormone (LH) in serum obtained from the female rats exposed to the different teas was quantified using an ELISA (EIA 1289, DRG Instruments GmBH, Marburg, Germany).

In order to be in line with the detection range of this assay (1.27 – 200 mIU/mL); LH in serum had to be enriched as was described earlier in section 3.7.2.2. The ether fraction was then used to determine the LH level according to the manufacturer's instruction. In brief, 25 µL of standards (0, 10, 20, 40, 100 and 200 mIU/mL) and samples were dispensed into appropriate wells. Afterwards, 100 µL enzyme conjugate was added to each well, mixed thoroughly for 10 sec and incubated for 30 min at RT. At the end of the incubation time, the contents were briskly shaken out and rinsed five times with 400 µL distilled water per well. Residual droplets were removed by striking the wells sharply on absorbent paper.

Then, 100 µL substrate solution was added to each well and incubated for 10 min at RT and the reaction was put to an end by the addition of 50 µL stop solution to each well and absorbance read at 450 nm within 10 min of adding the stop solution. A standard curve was used to deduce the level of LH produced in serum (appendix N) and the results obtained were expressed as mIU/mL.

3.7.2.4 Creatinine

Serum level of creatinine was determined according to Bartel et al. (1972). In brief, serum (from male or female rats) or standard solution (0, 15, 30, 60, 90, 120 and 150 mg/L of creatinine in dH_2O) was added to a working solution in the ratio 1:10. Working solution consisted of alkaline picrate (0.1g picric acid in 50mL dH_2O and 50mL 0.4M NaOH at 4^oC). Absorbance was read at 492 nm after 15 min of incubation at RT. Activity of creatinine in serum was deducted from a standard curve (appendix O) and the results obtained were expressed as mg/L.

3.7.2.5 Alanine transaminase

Serum level of alanine transaminase (ALT) was determined according to Reitman & Frankel (1957). 15 µL of standard solution (containing 0.22 mg/mL sodium pyruvate, alanine transaminase substrate (0.89g alanine and 0.015g of α-ketoglutarate in 50 mL phosphate buffer) and potassium phosphate buffer (0.1M, pH 7.4) and 12.5 µL alanine transaminase substrate for blank and serum were placed in a 96 well plate and incubated at 37^oC for 5 min. After which, 2.5 µL of serum was added into the appropriate wells and incubated at 37^oC for 30 min. Then, 25 µL of 2, 4-dinitrophenylhydrazine (0.2%) in 37% HCl and dH_2O was added to each well, mixed and incubated at RT for 20 min. Following which, 250 µL NaOH (0.4M) was added to all wells, mixed and incubated at RT for 30 min and absorption was read at 492 nm. Activity of ALT in serum was deducted from a standard curve (appendix P) and expressed as IU/L.

3.7.2.6 Aspartate transaminase

Serum level of aspartate transaminase (AST) was determined according to Reitman & Frankel. (1957). 15 µL of standard solution (containing containing 0.22 mg/mL sodium pyruvate, aspartate transaminase substrate (0.015g α-ketoglutarate, 1.33 g aspartic acid, 12.5 mL 1N NaOH, dissolved and adjusted to pH 7.4 with 8.5% phosphoric acid) and potassium phosphate buffer (0.1M, pH 7.4) and 12.5 µL aspartate transaminase substrate for blank and serum were placed in a 96 well plate and incubated at 37^{o}C for 5 min. After which, 2.5 µL of serum was added into the appropriate wells and incubated at 37^{o}C for 1 h. Then, 25 µL of 2, 4-dinitrophenylhydrazine (0.2%) in 37% HCl and dH_2O, was added to each well, mixed and incubated at RT for 20 min. Following which, 250 µL NaOH (0.4M) was added to all wells, mixed and incubated at RT for 30 min and absorption was read at 492 nm. Activity of AST in serum was deducted from a standard curve (appendix Q) and and the result obtained was expressed as IU/L.

3.7.2.7 Ferric reducing antioxidant power (FRAP)

Antioxidant activities in the sera were measured using ferric reducing antioxidant power (FRAP) using Trolox as standard (Benzie and Strain, 1996). To 10µL standard, serum and blank, 300 µL FRAP reagent (10:1:1- 300mM sodium acetate buffer: 10mM TPTZ: 20mM FeCl3) and 30 µL was added, vortex, incubated in at 37ºC for 4 min and absorbance read at 593 nm with a micro plate reader. The level of FRAP in serum was deduced using a standard curve (appendix K) and the result obtained was expressed as µM Trolox/ mL of serum.

3.7.3 Tissue sampling for biochemical and enzyme assay

Immediately after sacrificing the rat, right testes (male rat) and kidney and a part of liver (both male and female) were cleaned of fat and stored at -80^{o}C. Testis, kidney (20% w/v), and liver (20% w/v) tissue was rapidly thawed and homogenized in 1mL ice-cold Tris- buffered saline (Tris-HCl 20mM, NaCl 150Mm, pH 7.4) using PRO 200 homogenizer (proscientific Inc, Oxford, USA), followed by centrifugation (5000 x *g*, 30 min and 4^{o}C). The supernatant was collected afterwards and stored at -80^{o}C and was used for antioxidant and biochemical assays.

3.7.3.1 Lipid peroxidation

Lipid peroxidation was assessed by a specific spectrophotometric assay that measures the thiobarbituric acid reacted substances (TBARS) such as the malondialdehyde (MDA) according to Yagi (1984). In brief, TBA reagent (15% v/v trichloroacetic acid and 0.25N HCl) was mixed with the supernatant in a ratio 2:1. The mixture was heated for 15 min at 95^{o}C, allowed to cool and centrifuged (1000 g, 10 min). The supernatant was collected and absorbance measured at 532 nm. The extinction coefficient of 1.56 mol^{-1}l cm^{-1} was used. The MDA concentration was expressed as nanomolar of MDA per milligram of protein.

3.7.3.2 Glutathione

Glutathione level was evaluated according to Ellman (1959). In principle, 5, 5'-dithiobis-(2-nitrobenzoic acid) (DTNB or Ellman's reagent) reacts with –SH groups of glutathione to give a yellow complex which is absorbed at 412 nm. In brief, 3 mL of Ellman's reagent (4.96mg in 250mL potassium phosphate buffer (0.1M, pH 6.5) was added to 20 µL of supernatant, homogenized and incubated for 15 min. Absorbance was measured at 412 nm against blank. Extinction

coefficient of 13, 600 mol^{-1} cm^{-1} was used for calculation and result obtained was expressed millimolar thiol per milligram of protein.

3.7.3.3 Superoxide dismutase activity

Total superoxide dismutase activity was determined by the method of pyrogallol autoxidation according to Marklund & Marklund (1974) with a slight modification (Abdallah et al., 2009). Supernatant (100 µl) was mixed with Tris-EDTA-HCl buffer (1.5mL, pH 8.5) and pyrogallol (100 µL, 15mM in dH_2O) and incubated at 25^oC for 10 min. The reaction was determined by the addition of HCl (50µL, 1N), and the activity measured at 440 nm. One unit was determined as the amount of enzyme that inhibited the oxidation of pyrogallol by 50%. The result obtained was expressed as units per milligram of protein.

3.7.3.4 Catalase activity

Catalase activity was measured as previously described by Aebi (1984). 100 µL of supernatant was mixed with 400 µL of PBS (pH 7.4) and 500 µL of 20mM H_2O_2 and measured at 240nm at 25^oC within 2 min. The extinction coefficient of 43.6 $mol^{-1}cm^{-1}$ for H_2O_2 was used for calculation. One unit of CAT is defined as the activity of the enzyme that catalysed the reduction of 1 µmol of H_2O_2 per min per milligram of protein.

3.7.3.5 Protein determination

Protein determination was performed according to Lowry et al. (1951) with bovine albumin serum as standard. 25 µL and 200 µL of reagent A and B (Bio-rad laboratory, Hercules, USA) respectively was added to 20 µL supernatant, and incubated for 30 min. Absorbance was measured at 630nm. The amount of

protein in samples was deduced from a standard curve using bovine serum albumin as standard (appendix R).

3.7.4 Sperm concentration

Freshly isolated cauda epididymis was cut in small pieces and placed in a 1 mL pre-warmed phosphate buffered saline (PBS) for 5 min at 37^{o}C. Sperm concentration was determined using Markler counting chamber (Sefi-Medical Instrument, Haifa, Israel). Diluted Epididymal sperm (1:9) was placed on the chamber and five rows were counted and an average was calculated in millions per milliliter (10^6/mL).

3.7.5 Sperm vitality

Freshly isolated cauda epididymis was placed in a Petri dish containing 1mL of 1% BSA in DMEM/ Hams F-12 at 37^{o}C and the sperm allowed to "swim out". From this sperm cloud, sperm viability was determined using the fluorescent Duo vital kit following the manufacturer's instruction (Microptic, Barcelona, Spain). In brief, 1 µL each of the fluorochrome solution red and fluorochrome solution green was added to 5 µL of sperm sample on a clean slide. Solutions were mixed and covered with a cover slip. Afterwards, 100 sperm were analyzed under a fluorescence microscope. Sperm fluorescing "green" or "red" were scored as alive or dead respectively (appendix S).

3.7.6 Sperm motility

Immediately after sacrificing the rat, the right epididymis was placed in a Petri dish (35 x 10 mm) containing 1ml of 1% BSA in DMEM/ Hams F-12 at 37^{o}C. The cauda epididymis was transferred to a new Petri dish with 1ml of same medium and cleaned of small blood vessels and fat under a stereo microscope. A

tiny piece of the cauda epididymis was placed in another Petri dish containing 1ml of same medium and the sperm allowed to "swim out". 5µl of this sperm cloud was collected, placed on a 5µL standard count 4 chamber slide (Leja Products B.V., Nieuw Vennep, Netherlands) and motility determined using Sperm Class Analyzer (Microptic, Barcelona, Spain). The following sperm parameters were determined: percentage of total motile sperm, percentage of progressive motile sperm, curvilinear velocity (VCL, µm/s), straight-line velocity (VSL, µm/s), average path velocity (VAP, µm/s), amplitude of lateral head displacement (ALH, µm), and linearity (LIN, %), Wobble (WOB, %), beat cross frequency (BCF, Hz) (appendices T and U). Total motile sperm take into account the rapid progressive sperm (Type a), slow progressive motile sperm (Type b) and the non-progressive motile sperm (Type c). Progressive motile sperm covers the rapid progressive sperm (Type a) and slow progressive sperm (Type b). Sperm parameters such as VCL and BCF are indicators of sperm viability, while VAP, VSL, STR, and LIN are markers of sperm progression. Furthermore, STR and LIN also describe sperm swimming patterns (Schettgen et al., 2002; Duty et al., 2004).

3.7.7 Assessment of acrosome reaction

The status of the sperm acrosome was assessed as described by Miller and Larson, 1999. In brief, following the sperm preparation for sperm motility, 100 µl sperm sample from epididymis of rats exposed to different teas and herbal infusions were fixed with 4% p-formaldehyde in PBS (500 µL) at pH 7.4 for 5 min, centrifuged (3000 x *g*, 3 min). Sperm cells were resuspended with 10mM ammonium acetate (100-150 µL) at pH 9.0, smeared on glass slides and air dried. Cells were stained with 0.22% Coomassie Brilliant Blue G (CBB) solution prepared in 50% (v/v) methanol and 10% (v/v) glacial acetic acid for 2 min at room temperature. Stained cells were washed with distilled water to remove excess dye, air dried, and covered with a cover slip over a drop of glycerol, and observed under a bright field microscope at 1000X magnification. Spermatozoa with intact acrosome showed blue stain over the sperm head (acrosomal cap

present) while spermatozoa undergoing or undergone acrosomal reaction showed partial or no blue colour over the sperm head (acrosomal cap absent) (appendix V). 200 spermatozoa were scored in duplicate and the percentage of spermatozoa that had undergone acrosomal reaction was calculated.

3.8 EFFECT OF CAMELLIA SINENSIS AND ASPALATHUS LINEARIS ON MALE SEXUAL BEHAVIOUR AND FERTILITY

Sexually mature inexperienced male Wistar rats (63 day-old; 6 animals per group, weighing 180- 270g) were treated with the respective tea extract for 52 days with control having water as sole source of drinking. At the end of treatment, each male was paired with one receptive female rat (1:1) or each male was paired with two receptive female rats (1:2) in half of each male treatment group (n=3). The female rats (sexually mature inexperienced female Wistar rats (115 day-old, weighing 170- 260g) were made receptive using 30 µg/mL estradiol benzoate and 500 µg/mL progesterone in olive oil subcutaneously, 48 h and 4h respectively before pairing.

3.8.1 Sexual behaviour

The sexual behaviour of male rats was tested in a quiet room after a 10 minutes adaptation period. A stimulus- receptive female (female rat injected with 30 µg/mL estradiol benzoate and 500 µg/mL progesterone in olive oil subcutaneously (appendix W), 48 h and 4h respectively before pairing) was gently placed in the cage with the male rat. The following parameters were monitored according to Ratnasooriya and Fernando (2008) and Watcho et al. (2007): mount latency (ML), the time elapsed from the introduction of the female into the cage until the first mount; mount frequency (MF), the number of mounts preceding ejaculation and number of mounts. In addition, the number of snifs after 3 min of pairing was also taken into account.

3.8.2 Fertility test

The female rats were observed on the following morning after pairing for the presence of vaginal plug (a gelatinous secretion deposited by a male into a female genital tract and later hardens into a plug or glues the tract together) (David Quammen, 1998) or spermatozoa in the vaginal smear as described by Marcondes et al. (2002). The presence of vaginal plug or spermatozoa was regarded as day 0 of gestation. If absent, vaginal smear was performed for 10 -12 days (appendix X) to determine pregnancy or pseudo-pregnancy (it is a state in the animal appears pregnant, however, there is no foetus developing in the uterus and is usually caused by extended diestrus) (pseudopregnancy, 2004). On day 20 of gestation, the female rats underwent laparotomy under light ether anaesthesia and were euthanized by cervical dislocation (appendix Y). The following reproductive parameters were calculated (Watcho et al., 2007; Ratnasooriya and Fernando, 2008):

Index of libido = (number mated/number paired) * 100 (3.3)

Quantal pregnancy = (number pregnant/number mated) *100 (3.4)

Implantation index = (total number of implantation/ number mated) * 100 (3.5)

Pre- implantation loss = [(number of corpora lutea- number of implants)/ number of corpora lutea] * 100 (3.6)

Post- implantation loss = [(number of implants- number of viable implants)/ number of implants] *100 (3.7)

Fertility index = (number pregnant/ number paired) *100 (3.8)

3.9 *IN VITRO* EFFECT OF CAMELLIA SINENSIS AND ASPALATHUS LINEARIS ON TM3 CELL VIABILITY AND TESTOSTERONE PRODUCTION

3.9.1 Cytotoxicity test

25000 cells/ mL of TM3 cells (ATCC, Leydig cells derived from 11-13d mouse testes) were seeded in a 96 well plates for 24 h. 5% aqueous extract of the teas and herbal infusions were lyophilized and reconstituted in DMEM/Ham's F12 medium (containing 2.5 % bovine serum and 5% horse serum). Then the cells were exposed to 250, 500, 1000 or 5000 μg/mL of either green tea, black tea, unfermented rooibos or fermented rooibos, DMEM/Ham's F12 medium (non - stimulated or stimulated control) in the presence or absence of HCG (6 mIU) or DMEM/Ham's F12 medium containing 4% DMSO (positive control) for a further 24 h. Following that, supernatants were removed, washed and replaced with DMEM/Ham's F12 medium. 20 μL of MTT (1mg/mL in PBS) was added to each well and incubated for 4 h. Afterwards, supernatant was removed and converted dye was dissolved in 50 μL DMSO per well, agitated with a shaker for 10 min and read at wavelength of 560nm with a background subtraction of 750nm. The percent vitality was then calculated using equation 3.9;

$$\% \text{ vitality} = \frac{\text{Extinction (test)}}{\text{Extinction (control)}} \times 100 \qquad (3.9)$$

3.9.2 Testosterone

25000 cells/ mL of TM3 cells were seeded in a 96 well plates for 24 h, then exposed to 0.025, 0.05, 0.1 or 0.5 % of either green tea, black tea, unfermented rooibos or fermented rooibos, DMEM/Ham's F12 medium (non- stimulated or stimulated control) in the presence or absence of HCG (6 mIU) and DMEM/Ham's F12 medium containing 4% DMSO (positive control) for a further

24 h. The supernatant was collected and stored at -20^{o}C for the determination of the production of testosterone using the ELISA (EIA 1559, DRG Instruments GmBH, Marburg, Germany) as described by the manufacturer (see 3.7.2.1).

3.10 Statistical analysis

Statistical analysis was done using Medcalc statistical software (Version12.1.3.0, Mariakerke, Belgium). To test for normal distribution, Kolmogorov-Smirnoff test was done, followed by independent sample t-test. If data was not normally distributed, Mann-Whitney test would be performed. Data was expressed as mean ± standard deviation and a p value of < 0.05 was considered statistically significant. ANOVA- trend analysis was also performed between groups and a p value < 0.05 was regarded to be statistically significant. Tukey outlier detector's test was also performed.

CHAPTER FOUR

RESULTS OF THE CHEMICAL ANALYSIS OF TEA AND HERBAL INFUSION

Aqueous extracts prepared from *Camellia sinensis* (2% and 5%; Green tea and black tea) and *Aspalathus linearis* (2% and 5% ; unfermented and fermented rooibos) during the period of exposure to male and female rats were collected randomly and stored at – 20^{o}C for chemical analysis (soluble solids, total polyphenols, flavonol and flavanol content and the ferric reducing antioxidant power (FRAP).

4.1 SOLUBLE SOLIDS

Figure 4.1 showed that the soluble solid content in *Camellia sinensis* and *Aspalathus linearis* was in the following decreasing order: Gt ≥ Bt > Ur > Fr (p < 0.05) in both 2% and 5% of the tea and herbal infusion. A concentration-dependent increase in the amount soluble solids per type of tea was also observed (p < 0.05).

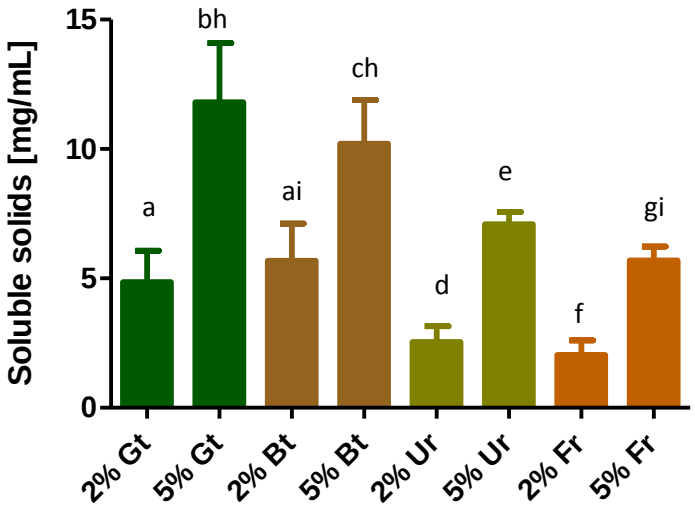


Figure 4.1: Soluble solids content of *Camellia sinensis* and *Aspalathus linearis*

Values are the means ± SD of 12 replicates. Abbreviations: Gt, Green tea; Bt, Black tea; Ur, Unfermented rooibos; Fr, Fermented rooibos. Means on each bar followed by the same letter do not differ significantly. If the letter differs, then $p < 0.05$

4.2 TOTAL POLYPHENOLS

Figure 4.2 showed that total polyphenols content was highest in green tea compared to black tea and herbal infusions ($p < 0.05$). In the 2% range of the teas, the following was observed; Gt ≥ Bt > Ur > Fr ($p < 0.05$) while in 5% of the teas and herbal infusions, the amount of total polyphenols in decreasing order was observed as Gt > Bt > Ur > Fr ($p < 0.05$). A concentration-dependent increase was also observed per type ($p < 0.05$).

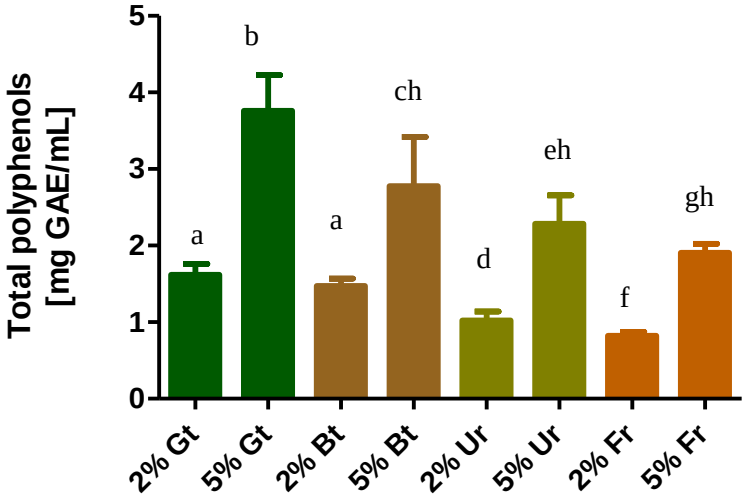


Figure 4.2: Total polyphenols in *Camellia sinensis* and *Aspalathus linearis*

Values are the means ± SD of 12 replicates. Abbreviations: Gt, Green tea; Bt, Black tea; Ur, Unfermented rooibos; Fr, Fermented rooibos; GAE, Gallic acid equivalent. Means on each bar followed by the same letter do not differ significantly. If the letter differs, then $p < 0.05$

4.3 FLAVONOL CONTENT

Flavonol content in tea and herbal infusion in figure 4.3 was shown to be present in the following decreasing order: Fr > Bt > Ur ≥ Gt in the 2% range ($p < 0.05$). However, 5% concentrations of tea and herbal infusion revealed the flavonol content to be as follows: Fr ≥ Bt > Ur > Gt ($p < 0.05$). In addition, a significant concentration- dependent increase was observed in each type ($p < 0.05$).

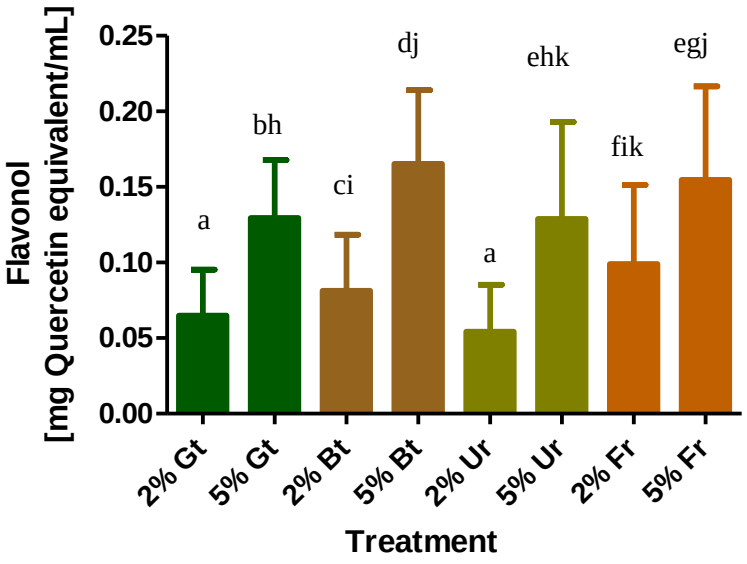


Figure 4.3: Flavonol content of *Camellia sinensis* and *Aspalathus linearis*

Values are the means ± SD of 12 replicates. Abbreviations: Gt, Green tea; Bt, Black tea; Ur, Unfermented rooibos; Fr, Fermented rooibos. Means on each bar followed by the same letter do not differ significantly. If the letter differs, then $p < 0.05$

4.4 FLAVANOL

Flavanol content of tea and herbal infusion as shown in figure 4.4 showed that green tea has significant higher amount of catechin compared to black tea and the herbal infusions ($p < 0.05$). Result shown can be summarized as follows: 2% range of the teas and herbal infusions in a decreasing order, Gt > Bt ≥Ur > Fr ($p < 0.05$) was observed. In addition, 5% concentrations of the teas and tisanes also showed green tea to have the highest amount of catechin compared to the rest in the order: Gt > Bt > Ur > Fr ($p < 0.05$). A significant concentration- dependent increase in flavanol content was also observed in each type ($p < 0.05$).

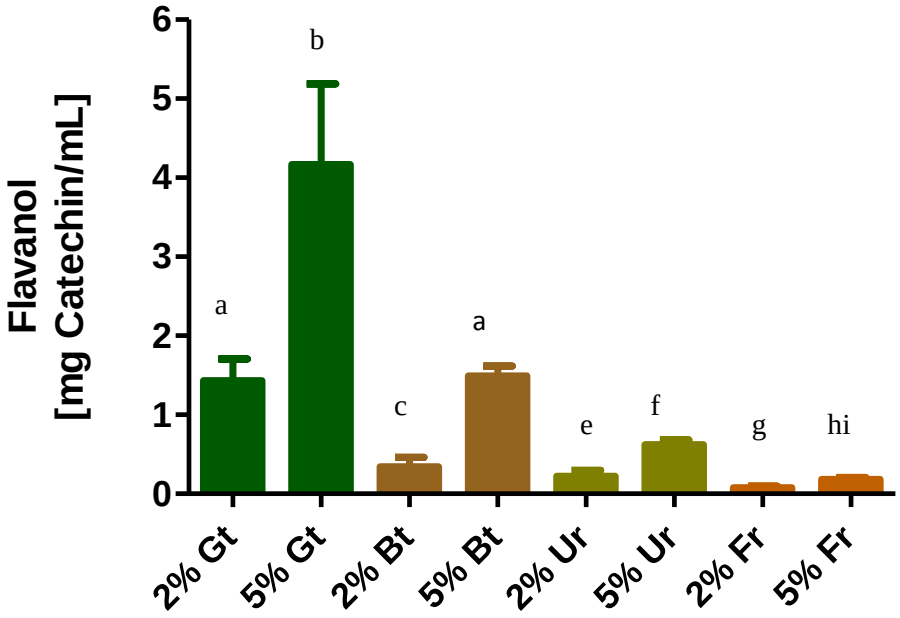


Figure 4.4: Flavanol content of *Camellia sinensis* and *Aspalathus linearis*
Values are the means ± SD of 12 replicates. Abbreviations: Gt, Green tea; Bt, Black tea; Ur, Unfermented rooibos; Fr, Fermented rooibos. Means on each bar followed by the same letter do not differ significantly. If the letter differs, then $p < 0.05$

4.5 FERRIC REDUCING ANTIOXIDANT POWER (FRAP)

FRAP, which measures the ability of compounds to act as electron donor, was shown to be significantly higher in green tea than black tea and the herbal infusions in the order: Gt > Bt ≥Ur > Fr ($p < 0.05$) in both 2% and 5 % groups. A significant concentration- dependent increase was also observed in each type of tea ($p < 0.05$) as shown in figure 4.5.

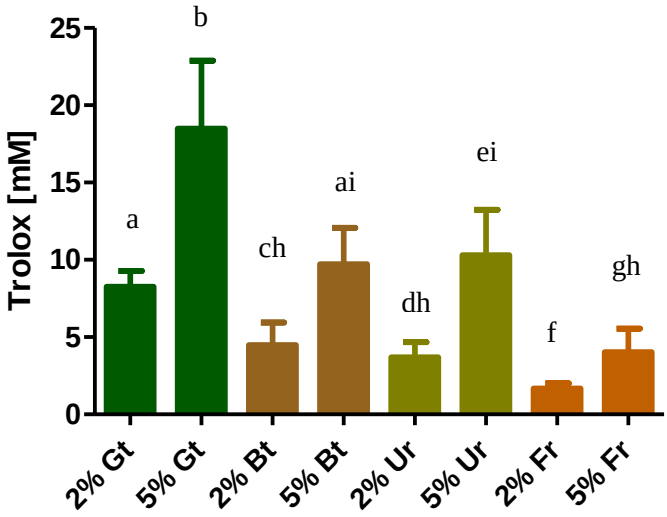


Figure 4.5: Ferric reducing antioxidant power (FRAP) in *Camellia sinensis* and *Aspalathus linearis*

Values are the means ± SD of 12 replicates. Abbreviations: Gt, Green tea; Bt, Black tea; Ur, Unfermented rooibos; Fr, Fermented rooibos. Means on each bar followed by the same letter do not differ significantly. If the letter differs, then $P < 0.05$

CHAPTER FIVE

RESULTS OF THE EFFECT OF CAMELLIA SINENSIS AND ASPALATHUS LINEARIS ON MALE REPRODUCTION, LIVER AND KIDNEY FUNCTIONS

5.1 AVERAGE DAILY INTAKE OF FLUID AND EXTRACT PARAMETERS

During the first week (week 1; table 5.1) of exposure to *Camellia sinensis* and *Aspalathus linearis*, fluid intake was significantly reduced in most treatment groups compared to the controls ($p < 0.05$). However, as time progressed (weeks 2-8), fluid intake in all treatment groups was more or less similar to the control ($p > 0.05$). On overall, fluid intake in male rats exposed to different concentrations (2 % or 5 %) of either *Camellia sinensis* or *Aspalathus linearis*, did not differ with the control group ($p > 0.05$). However, the groups treated with either 5% unfermented rooibos or 5 % black tea showed that there was significant decline in fluid intake when compared to the controls ($p < 0.05$).

Table 5.2, showed that the average daily intake (ADI) of soluble solids in animals exposed to 2% concentration of all teas was similar between green tea and black tea, but higher than unfermented and fermented rooibos in that order (Gt = Bt > Ur > Fr; $p < 0.05$). In the 5 % range of tea and herbal infusion, however, the ADI of soluble solids taken by the animals showed that green tea was significantly higher than black tea, unfermented rooibos and fermented rooibos in that order: Gt > Bt > Ur = Fr; $p < 0.05$). A significant dose-dependent increase was also observed in each type (5% Gt > 2% Gt; 5 % Bt > 2% Bt; 5 % Ur > 2 % Ur; 5% Fr > 2% Fr; $p < 0.05$).

Total polyphenols soluble solids (% SS; table 5.2) intake was significantly higher in the *Aspalathus linearis* than *Camellia sinensis* in the order: unfermented

rooibos ≥ fermented rooibos > green tea ≥ black tea ($p < 0.05$). However, the ADI of total polyphenols was significantly higher in *Camellia sinensis* than *Aspalathus linearis* ($p < 0.05$), and the order observed was green tea ≥ black tea > unfermented rooibos > fermented rooibos.

The flavonol content of fermented and unfermented rooibos soluble solids (% SS; table 5.2) was significantly higher ($p < 0.05$) than green and black tea, which did not differ significantly (Fr > Ur> Gt ≥ Bt; $p > 0.05$). In addition, ADI was similar in most group but significantly higher ($p < 0.05$) in fermented rooibos than others, followed by unfermented rooibos, black tea and green tea.

Flavanol content of *Camellia sinensis* soluble solids (% SS; table 5.2) was significantly higher than *Aspalathus linearis* ($p < 0.05$). Green tea had the highest amount, followed by black tea, unfermented and fermented rooibos (Gt > Bt ≥ Ur > Fr). Likewise, ADI intake of flavanol was significantly higher in green tea, followed by black tea, unfermented and fermented rooibos ($p < 0.05$).

Table 5.1: Average daily intake of fluid (mL/100g BW) over 52 days in male rats

Treatment	Mean fluid intake over 8 weeks period (mL/100g BW)								Mean Fluid intake (mL/100g BW)
	Wk (1)	Wk (2)	Wk (3)	Wk (4)	Wk (5)	Wk (6)	Wk (7)	Wk (8)	
Control	19.0±2.0[a]	15.5±2.0[a]	14.2±1.9 [a]	13.6±1.2[a]	11.5±0.5[a]	11.9±1.2[a]	11.4±0.9[a]	10.4±0.8[a]	13.4±0.6[a]
2% Gt	15.6±1.3[b]	14.2±0.5[a]	12.8±1.9[a]	13.2±0.9[a]	12.8±0.8[b]	11.5±0.8[a]	11.5±0.4[a]	11.6±1.2[a]	12.9±0.5[a]
5% Gt	16.4±1.5[b]	13.0±2.2[a]	12.2±2.4[a]	12.9±0.6[a]	12.3±0.7[b]	11.1±1.4[a]	10.3±1.4[a]	11.0±1.5[a]	12.4±0.6[a]
2% Bt	17.6±4.1[a]	13.6±1.7[a]	13.8±1.5[a]	12.2±1.1[a]	11.5±0.9[a]	11.2±1.0[a]	9.8±1.3[b]	9.8±1.0[a]	12.5±1.0[a]
5% Bt	14.7±2.7[b]	13.8±1.4[a]	12.0±1.2[a]	11.8±1.2[b]	10.8±1.5[a]	10.8±0.9[a]	9.8±0.9[b]	9.5±1.5[a]	11.6±0.6[b]
2% Ur	14.2±1.2[b]	13.0±2.8[a]	13.1±1.5[a]	13.0±1.9[a]	11.5±0.9[a]	11.2±1.9[a]	10.3±0.7[b]	11.0±0.7[a]	12.2±0.8[a]
5% Ur	13.1±1.8[b]	12.7±3.0[a]	12.0±1.9[a]	11.7±0.9[b]	10.1±0.5[b]	9.9±1.2[b]	9.3±0.8[b]	9.5±0.4[b]	11.0±0.9[b]
2% Fr	16.0±2.4[b]	14.2±2.6[a]	14.2±1.8[a]	12.6±1.4[a]	11.5±1.1[a]	12.0±1.6[a]	10.0±0.9[b]	10.7±1.1[a]	12.7±0.6[a]
5% Fr	14.3±1.5[b]	14.6±2.6[a]	13.3±2.1[a]	12.8±2.1[a]	12.7±2.1[a]	12.3±1.7[a]	11.0±1.9[a]	13.2±3.0[a]	13.0±0.5[a]

Values represented are the mean ± SD of 6 animals per group. Abbreviations: C, control; Gt, green tea; Bt, black tea; Ur, unfermented rooibos; Fr, fermented rooibos; Wk, week. Means in the column followed by the same letter do not differ significantly. If the letter differs, then $p < 0.05$ compared to the control.

Table 5.2: Average daily intake of antioxidant parameters of the male rats receiving the respective tea extract during 52 days treatment

Samples	Fluid Intake		Soluble Solids [ss]		Total Polyphenols		Flavonol		Flavanol	
	ADI [ml]	ADI [mg/100g BW]	[mg/ml]	ADI [mg/100g BW]	[%SS]	ADI [mg/100g BW]	[%SS]	ADI [mg/100g BW]	[%SS]	ADI [mg/100g BW]
Control	41.14±2.46^{a}	13.43±0.58^{a}	-	-	-	-	-	-	-	-
2% Gt	42.23±1.39^{a}	12.88±0.47^{a}	4.86±1.21^{a}	62.62±2.31^{a}	33.31±2.88^{a}	20.86±1.80^{a}	0.28±0.07^{a}	0.17±0.04^{a}	29.48±5.68^{a}	18.46±3.56^{a}
5% Gt	40.72±0.12^{a}	12.41±0.60^{a}	11.81±2.28^{bh}	146.56±7.48^{b}	31.84±3.96^{a}	46.68±5.81^{b}	0.09±0.01^{b}	0.13±0.02^{ae}	35.29±8.62^{a}	51.73±12.64^{b}
2% Bt	43.04±5.19^{a}	12.45±1.04^{a}	5.68±1.44^{ai}	70.72±5.90^{ah}	25.93±1.66^{be}	18.34±1.18^{a}	0.27±0.08^{a}	0.19±0.06^{a}	6.07±2.11^{bh}	4.30±1.48^{c}
5% Bt	34.49±2.19^{b}	11.63±0.56^{b}	10.21±1.69^{ch}	118.74±5.78^{c}	27.22±6.25^{ae}	32.32±7.42^{cg}	0.16±0.01^{c}	0.19±0.02^{ad}	14.62±1.21^{c}	17.37±1.44^{a}
2% Ur	37.09±6.22^{a}	12.16±0.74^{a}	2.55±0.60^{d}	31.03±1.88^{d}	40.06±4.69^{cfg}	12.43±1.46^{d}	0.80±0.16^{d}	0.25±0.05^{ad}	8.82±2.89^{dhi}	2.74±0.90^{dh}
5% Ur	34.20±1.38^{b}	11.04±0.80^{b}	7.10±0.47^{e}	78.38±6.18^{eh}	32.16±5.25^{af}	25.21±4.12^{ag}	0.30±0.09^{a}	0.24±0.07^{ad}	8.73±0.85^{ehi}	6.85±0.67^{e}
2% Fr	41.36±2.76^{a}	12.65±0.62^{a}	2.05±0.55^{f}	25.93±1.27^{f}	40.10±2.41^{dg}	10.40±0.63^{e}	2.02±0.35^{e}	0.52±0.09^{b}	3.78±1.01^{fhj}	0.98±0.26^{f}
5% Fr	39.42±2.73^{a}	13.01±0.50^{a}	5.69±0.54^{gi}	74.03±2.84^{gh}	33.47±2.07^{ac}	24.78±1.53^{fg}	0.49±0.05^{f}	0.36±0.04^{c}	3.21±0.44^{gj}	2.37±0.33^{gh}

Values represented are the mean ± SD of 6 animals per group. Abbreviations: C, control; Gt, green tea; Bt, black tea; Ur, unfermented rooibos; Fr, fermented rooibos; Wk, week; % SS= 100/ soluble solid of corresponding tea [mg/mL] * total polyphenols/flavonol/flavanol of corresponding tea[mg/mL]; ADI= average fluid intake during 52 days [mL] /100 * % SS. Means in the column followed by the same letter do not differ significantly. If the letter differs, then $p < 0.05$

5.2 BODY WEIGHT GAIN

The weekly weight gain in the male rats treated with the teas and herbal infusions showed no significant difference compared to the control (figure 5.1; $p > 0.05$). Likewise, the total weight gain in the male rats also did not differ significantly compared to the treated groups after 52 days exposure to the tea or herbal infusion (figure 5.2; $p > 0.05$). However, green tea showed an increased tendency in weight gain in a dose- dependent manner ($p > 0.05$; ANOVA- trend analysis: P= 0.2619). Black tea, on the other hand, was shown to have an dose- dependent decrease effect on the weight gain in animals exposed to it (ANOVA- trend analysis: P=0.0170). In addition, weight gain in rats exposed to unfermented rooibos, showed an increased tendency in a dose- dependent manner (ANOVA- trend analysis: P= 0.6796) while fermented rooibos group showed a tendency to decrease weight gain in an dose-dependent manner (ANOVA- trend analysis: P= 0.0472).

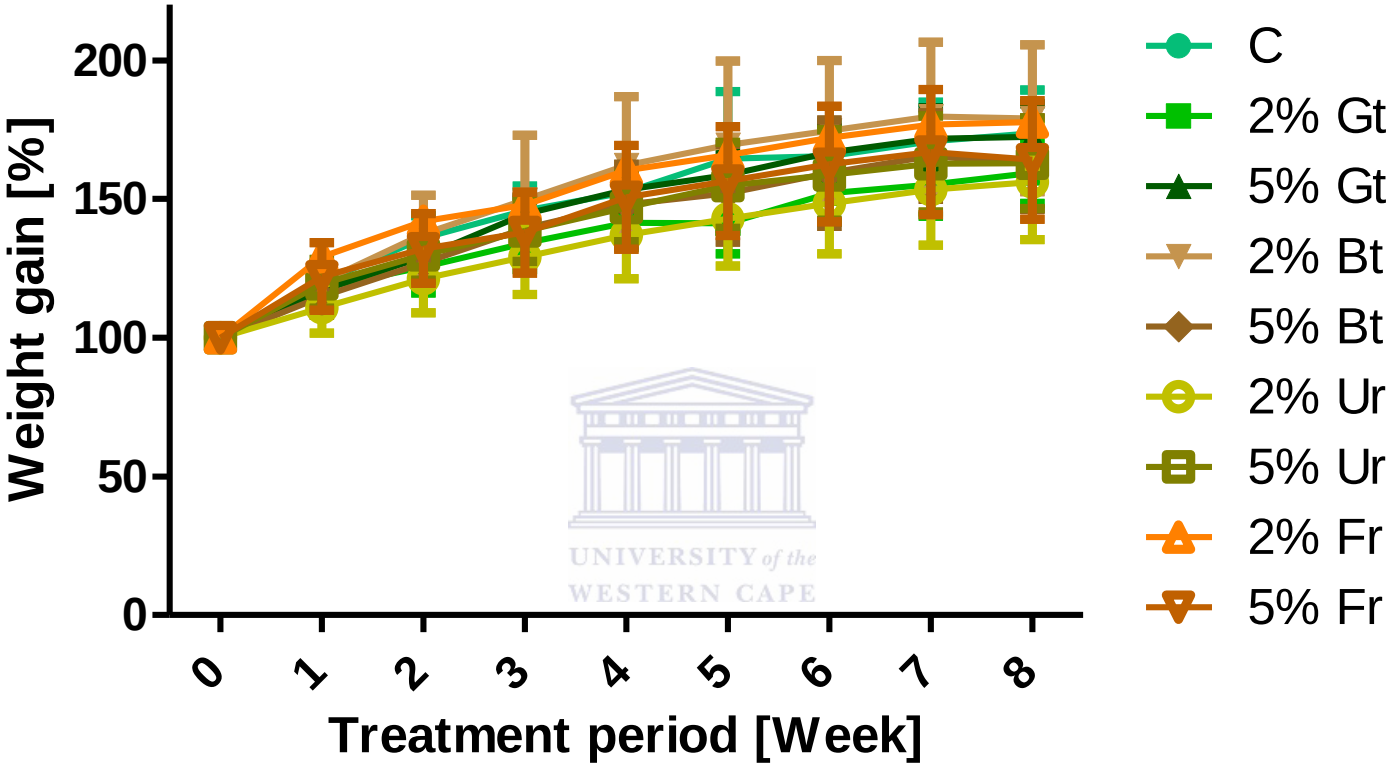


Figure 5.1: Weekly weight gain for the period of treatment in male rats. Values represented are the mean ± SD of 6 animals per group. Abbreviations: C, control; Gt, green tea; Bt, black tea; Ur, unfermented rooibos; Fr, fermented rooibos.

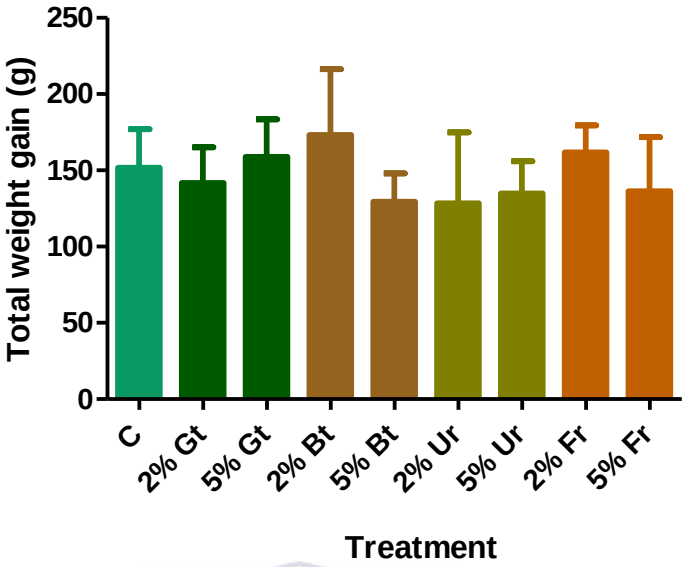


Figure 5.2: Effect of *Camellia sinensis* and *Aspalathus linearis* on body weight gain in male rats. Values represented are the mean ± SD of 6 animals per group. Abbreviations: C, control; Gt, green tea; Bt, black tea; Ur, unfermented rooibos; Fr, fermented rooibos.

5.3 REPRODUCTIVE ORGANS, LIVER AND KIDNEY WEIGHT IN MALE RATS

Table 5.3 showed no significant difference in the absolute and relative weights of reproductive organs (testes, epididymis, seminal vesicles and prostate) and liver ($p > 0.05$). However, absolute testes weights of animals exposed to either *Camellia sinensis* or *Aspalathus linearis* showed a tendency to increased values ($p > 0.05$). Similarly, the relative weight of testes in animals exposed to unfermented and fermented rooibos showed a tendency to increased values. In contrast, green and black tea was more or less similar to the control values ($p > 0.05$). In addition, the absolute and relative weights of the epididymis in all treated groups displayed a tendency to lower values ($p > 0.05$). Absolute weight of kidney did not differ significantly in most treated groups compared to the control, but showed a tendency to increase (table 5.3; $p > 0.05$). However, the groups exposed to 5% green tea, 2% black tea and 5% unfermented rooibos showed a significant increase in the absolute weight of the kidneys ($p < 0.05$). The relative weight of kidneys did not differ in the 2% range of all teas (table; $p > 0.05$), while a significant increase was observed in all groups exposed to the higher concentration (5%) of tea and tisane when compared to the controls ($p < 0.05$).

Table 5.3: Weight [g] of reproductive organs, liver and kidney after 52 days treatment with *Camellia sinensis* and *Aspalathus linearis* in male rats

Organ/ Treatment	Control	2% Gt	5% Gt	2% Bt	5% Bt	2% Ur	5% Ur	2% Fr	5% Fr
Testis									
absolute	1.68±0.15	1.82±0.15	1.74±0.05	1.88±0.17	1.43±0.45	1.73±0.11	1.72±0.11	1.80±0.08	1.80±0.11
relative	0.47±0.05	0.48±0.04	0.46±0.04	0.47±0.05	0.42±0.12	0.49±0.04	0.48±0.05	0.48±0.00	0.51±0.05
Epididymis									
absolute	0.65±0.09	0.64±0.05	0.54±0.16	0.66±0.08	0.56±0.14	0.59±0.06	0.57±0.06	0.59±0.03	0.58±0.03
relative	0.18±0.02	0.17±0.01	0.14±0.04	0.17±0.02	0.16±0.04	0.17±0.02	0.16±0.02	0.16±0.01	0.16±0.01
Seminal vesicles									
absolute	1.46±0.07	1.46±0.08	1.45±0.07	1.53±0.18	1.42±0.11	1.39±0.25	1.40±0.25	1.43±0.21	1.49±0.22
relative	0.41±0.03	0.39±0.05	0.38±0.03	0.38±0.03	0.42±0.06	0.39±0.06	0.39±0.07	0.38±0.05	0.42±0.06
Prostate									
absolute	0.47±0.11	0.48±0.10	0.50±0.14	0.42±0.13	0.49±0.11	0.48±0.08	0.56±0.09	0.52±0.12	0.54±0.12
relative	0.13±0.03	0.13±0.03	0.13±0.04	0.10±0.03	0.15±0.03	0.13±0.01	0.16±0.03	0.14±0.03	0.15±0.03
Liver									
absolute	16.54±2.14	17.00±1.01	17.53±1.97	18.27±3.13	15.03±1.31	15.88±1.91	16.75±1.66	16.88±1.08	15.05±1.82
relative	4.57±0.34	4.48±0.37	4.62±0.25	4.54±0.35	4.41±0.21	4.43±0.13	4.72±0.45	4.52±0.34	4.81±0.74
Kidneys									
absolute	3.07±0.35	3.31±0.18	3.50±0.19*	3.62±0.42*	3.43±0.30	3.13±0.28	3.40±0.08*	3.13±0.17	3.30±0.46
relative	0.85±0.05	0.87±0.05	0.93±0.05*	0.90±0.03	1.00±0.05*	0.88±0.06	0.96±0.04*	0.84±0.06	0.92±0.05*

Values represented are the mean ± SD of 6 animals per group. Abbreviations: C, control; Gt, green tea; Bt, black tea; Ur, unfermented rooibos; Fr, fermented rooibos; *, $p < 0.05$ compared to control. Relative organ weight= organ weight/final body weight * 100.

5.4 TESTOSTERONE

Figure 5.3, showed a dose dependent increase in the level of serum testosterone produced by the male rats exposed to green tea. However, this increment was only significant in the 5% Gt group ($p < 0.05$; ANOVA- trend analysis: P= 0.0272). Black tea had no significant effect in the level of testosterone produced, however a tendency to a lower values was observed ($p > 0.05$). Likewise, unfermented rooibos, revealed no significant effect on testosterone production but tend to decrease its level in a dose- dependent manner ($p > 0.05$; ANOVA- trend analysis: P= 0.0313). In like manner, fermented rooibos showed no significant effect but also presented a tendency to reduced values in a dose- dependent manner ($p > 0.05$; ANOVA- trend analysis: P= 0.0599).

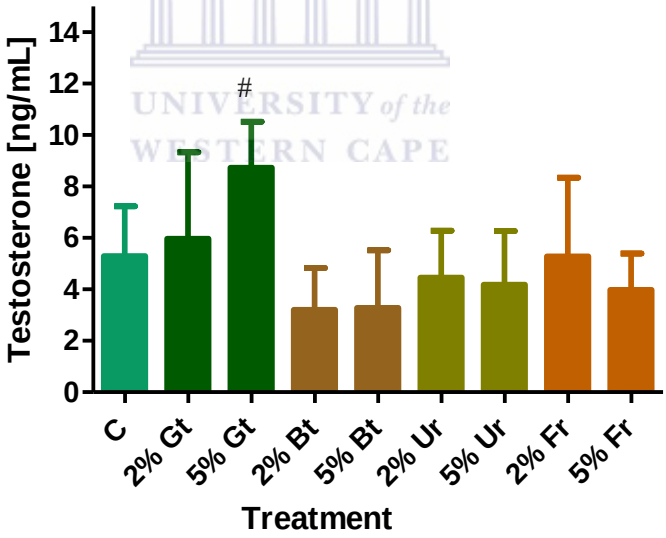


Figure 5.3: Effect of *Camellia sinensis* and *Aspalathus linearis* on testosterone production.

Values represented are the mean ± SD of 6 animals per group. Abbreviations: C, control; Gt, green tea; Bt, black tea; Ur, unfermented rooibos; Fr, fermented rooibos; $^{\#}$, $p < 0.01$ compared to control.

5.5 SPERM CONCENTRATION

Sperm concentration in figure 5.4 was shown to increase significantly in groups exposed to either green tea or unfermented rooibos ($p < 0.05$) compared to the control. However, there was no significant difference in the groups exposed to either black tea or fermented rooibos ($p > 0.05$). A trend to increasing values was observed in 2% black tea, 2% and 5 % fermented rooibos as 27.1%, 47.5 % and 20.8 % respectively while a decreasing trend in value was observed in the group exposed to 5% black tea (-13.8%). In addition, a dose- dependent decrease in sperm concentration was observed in green tea, black tea, unfermented and fermented rooibos (ANOVA- trend analysis: P= 0.7910; 0.0778; 0.0030; and 0.5393) respectively. But, as said above, the sperm concentration was still higher than in the control

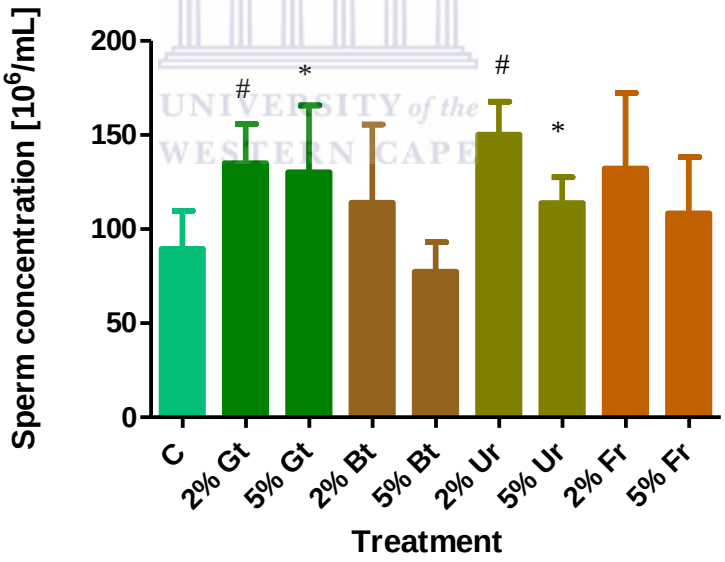


Figure 5.4: Effect of *Camellia sinensis* and *Aspalathus linearis* on rat sperm concentration.

Values represented are the mean ± SD of 6 animals per group and at least 200 sperm per animal were analysed. Abbreviations: C, control; Gt, green tea; Bt, black tea; Ur, unfermented rooibos; Fr, fermented rooibos; *, $p < 0.05$; $^{\#}$, $p < 0.01$ compared to control.

5.6 SPERM VITALITY

Figure 5.5 showed that sperm vitality was significantly enhanced in all treatment groups compared to the control ($p < 0.05$). 2% and 5 % green tea was shown to increase sperm vitality by 40 % and 54.8 % respectively in a dose- dependent manner (ANOVA- trend analysis: P= 0.0793). A 48.8 % and 44.4 % increase was observed in the group exposed to 2% and 5 % black tea. Unfermented rooibos on the other hand, increased sperm vitality by 23.3 % and 40 % in the 2% and 5 % groups respectively in a dose- dependent manner (ANOVA- trend analysis: P= 0.0705). In addition, a 41.7 % and 39.6 % increase was observed in the groups exposed to 2% and 5% fermented rooibos respectively.

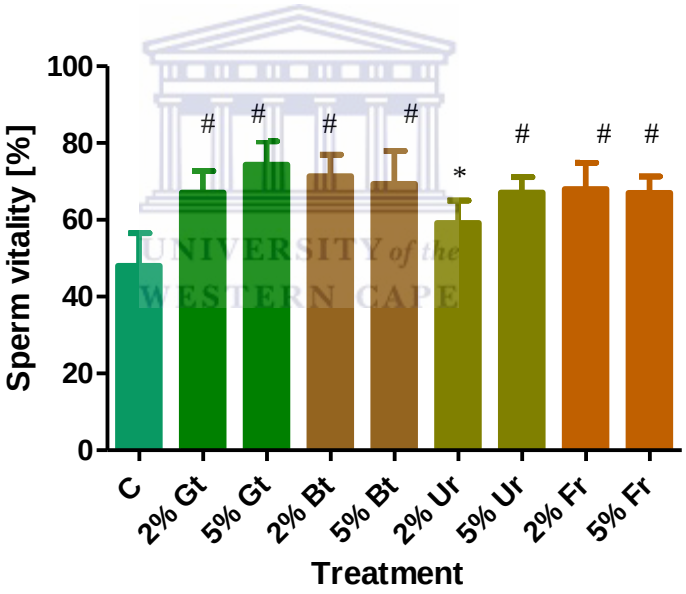


Figure 5.5: Effect of *Camellia sinensis* and *Aspalathus linearis* on rat sperm vitality.

Values represented are the mean ± SD of 6 animals per group and at least 200 sperm per animal were analysed. Abbreviations: C, control; Gt, green tea; Bt, black tea; Ur, unfermented rooibos; Fr, fermented rooibos; *, $p < 0.05$; $^{\#}$, $p < 0.01$ compared to control.

5.7 SPERM MOTILITY

Total progressive motility (figure 5.6) was significantly enhanced in the groups exposed to 2% Bt, 2% and 5 % unfermented rooibos ($p < 0.05$), while the other treatment groups showed a tendency to increased values ($p > 0.05$). Green tea (2% and 5%) improved total progressive motility by 12.4 % and 3.7 % respectively while black tea (2% and 5 %) augmented it by 15.2 % and 10.9 % respectively. Unfermented rooibos increased the percentage of total progressive motility by 14.8 % and 17.6 % in 2% and 5% respectively. It was also amplified by 11.8 % in both 2% and 5% fermented rooibos.

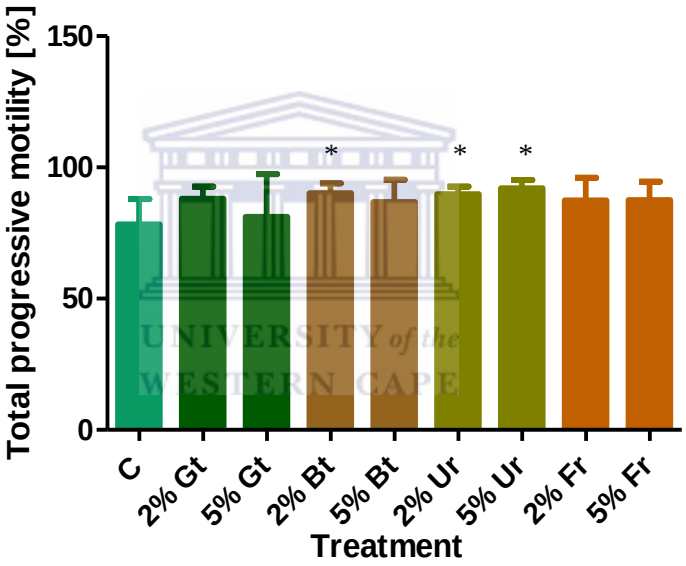


Figure 5.6: Effect of *Camellia sinensis* and *Aspalathus linearis* on rat sperm progressive motility.

Values are represented as mean ± SD after 52 day treatment. Number of rat per group = 6; at least 200 spermatozoa per animal were analyzed. Abbreviations: C, control; Gt, green tea; Bt, black tea; Ur, unfermented rooibos; Fr, fermented rooibos; * $p < 0.05$, compared with the control group.

Total motility (figure 5.7) improved significantly by 2% green tea, 2% and 5 % black tea, and 2% and 5% unfermented rooibos ($p < 0.05$) while fermented rooibos showed a tendency to increased values ($p > 0.05$). Green tea enhanced

total motility by 7.2 % and 3.0 % in 2 % and 5 % while black tea increased it by 11.3 % and 9.4% in 2% and 5 % respectively. An 8.4 %, 10.4 % 6.8 % and 5.8 % improvement was observed in the groups exposed to 2% and 5 % unfermented or fermented rooibos respectively. In addition, green tea, black tea and unfermented rooibos scaused a significant decline in the percentage of total static spermatozoa ($p < 0.05$) while fermented rooibos resulted in a non significant decrease ($p > 0.05$).

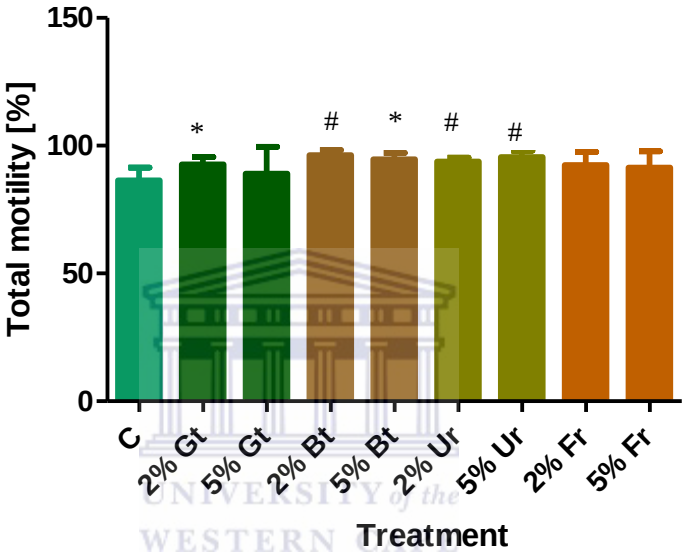


Figure 5.7: Effect of *Camellia sinensis* and *Aspalathus linearis* on rat sperm total motility.

Values are represented as mean ± SD after 52 day treatment. Number of rat per group = 6; at least 200 spermatozoa per animal were analyzed. Abbreviations: Abbreviations: C, control; Gt, green tea; Bt, black tea; Ur, unfermented rooibos; Fr, fermented rooibos; * $p < 0.05$, $^{\#}$ $p < 0.01$, compared with the control group.

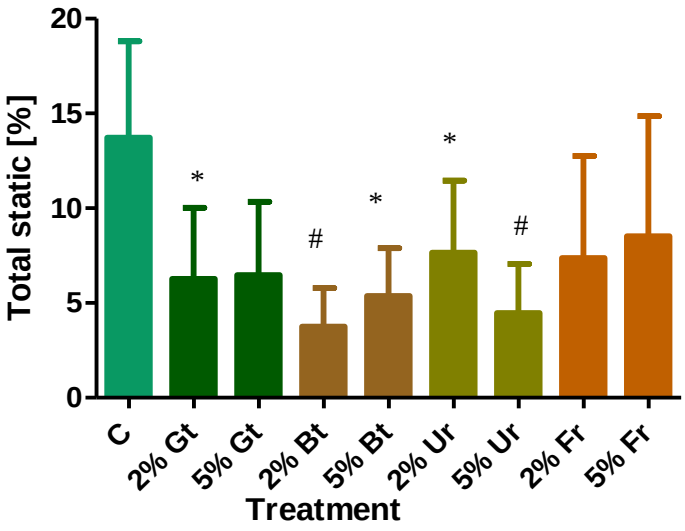


Figure 5.8: Effect of *Camellia sinensis* and *Aspalathus linearis* on total static sperm in rat.

Values are represented as mean ± SD after 52 day treatment. Number of rat per group = 6; at least 200 spermatozoa per animal were analyzed. Abbreviations: Abbreviations: C, control; Gt, green tea; Bt, black tea; Ur, unfermented rooibos; Fr, fermented rooibos; * $p < 0.05$, $^{\#}$ $p < 0.01$, compared with the control group.

Sperm velocity parameters (VCL, VSL, VAP, ALH and LIN; figures 5.8- 5.16) were not significantly different from the control; however, they showed a tendency to increased values when compared to the control ($p > 0.05$). STR was shown to also have a trend to improved values in all treatment groups except 5% unfermented rooibos ($p > 0.05$). WOB was shown to also have a tendency to improved values in most treatment group ($p > 0.05$). In addition, BCF values in the treatment groups were not significantly different from the controls ($p > 0.05$).

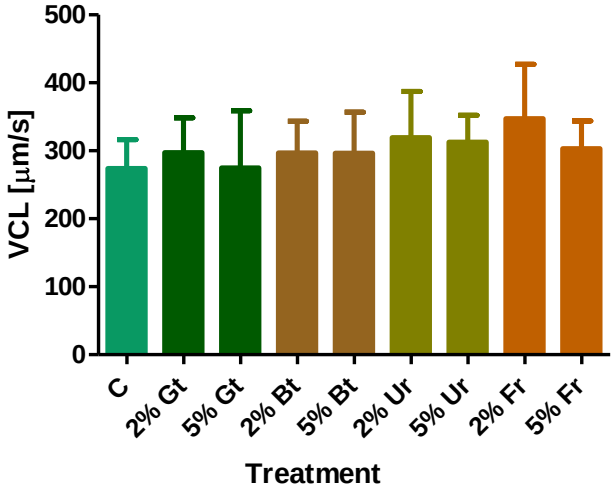


Figure 5.9: Effect of *Camellia sinensis* and *Aspalathus linearis* on curvilinear velocity (VCL) on rat sperm.

Values are represented as mean ± SD after 52 day treatment. Number of rat per group = 6; at least 200 spermatozoa per animal were analyzed. Abbreviations: Abbreviations: C, control; Gt, green tea; Bt, black tea; Ur, unfermented rooibos; Fr, fermented rooibos.

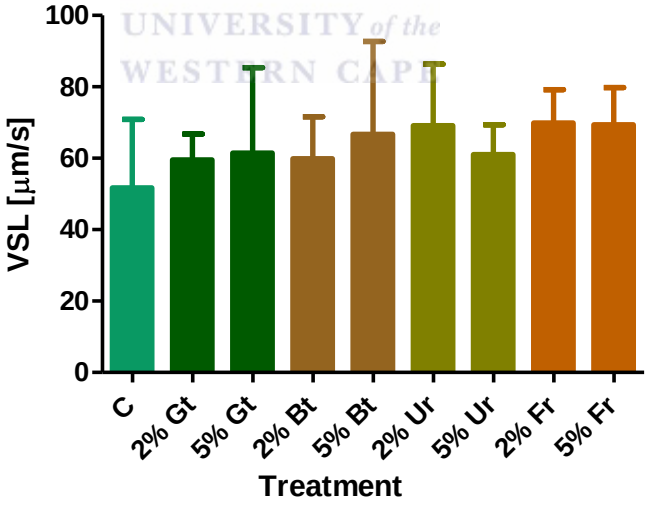


Figure 5.10: Effect of *Camellia sinensis* and *Aspalathus linearis* on straight line velocity (VSL) on rat sperm.

Values are represented as mean ± SD after 52 day treatment. Number of rat per group = 6; at least 200 spermatozoa per animal was analyzed. Abbreviations: Abbreviations: C, control; Gt, green tea; Bt, black tea; Ur, unfermented rooibos; Fr, fermented rooibos.

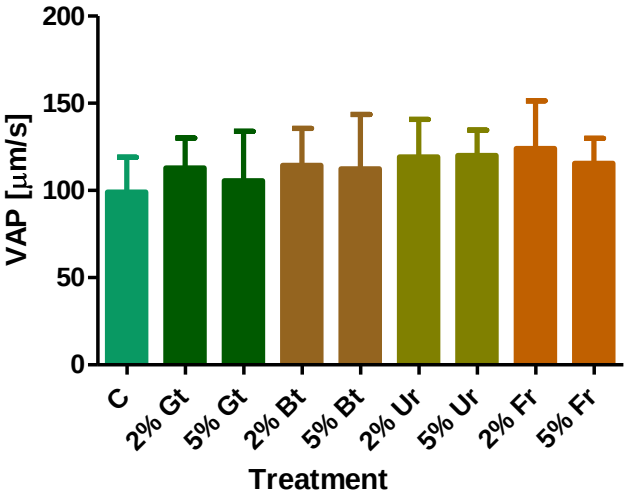


Figure 5.11: Effect of *Camellia sinensis* and *Aspalathus linearis* on average path velocity (VAP) on rat sperm.

Values are represented as mean ± SD after 52 day treatment. Number of rat per group = 6; at least 200 spermatozoa per animal were analyzed. Abbreviations: Abbreviations: C, control; Gt, green tea; Bt, black tea; Ur, unfermented rooibos; Fr, fermented rooibos.

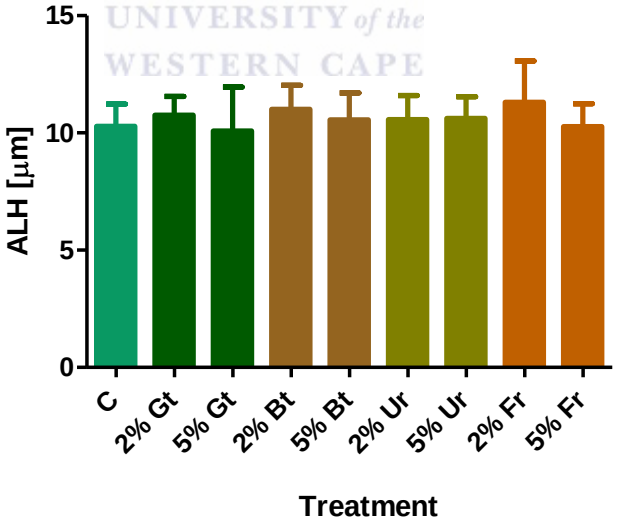


Figure 5.12: Effect of *Camellia sinensis* and *Aspalathus linearis* on amplitude of lateral head displacement (ALH) on rat sperm.

Values are represented as mean ± SD after 52 day treatment. Number of rat per group = 6; at least 200 spermatozoa per animal were analyzed. Abbreviations: Abbreviations: C, control; Gt, green tea; Bt, black tea; Ur, unfermented rooibos; Fr, fermented rooibos.

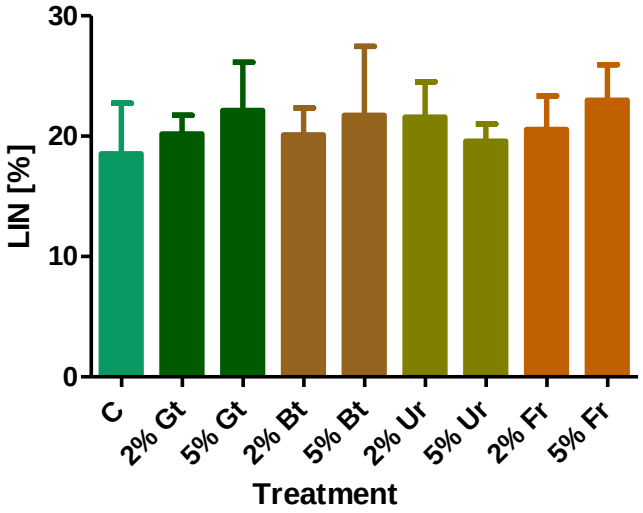


Figure 5.13: Effect of *Camellia sinensis* and *Aspalathus linearis* on linearity (LIN) on rat sperm.

Values are represented as mean ± SD after 52 day treatment. Number of rat per group = 6; at least 200 spermatozoa per animal were analyzed. Abbreviations: Abbreviations: C, control; Gt, green tea; Bt, black tea; Ur, unfermented rooibos; Fr, fermented rooibos.

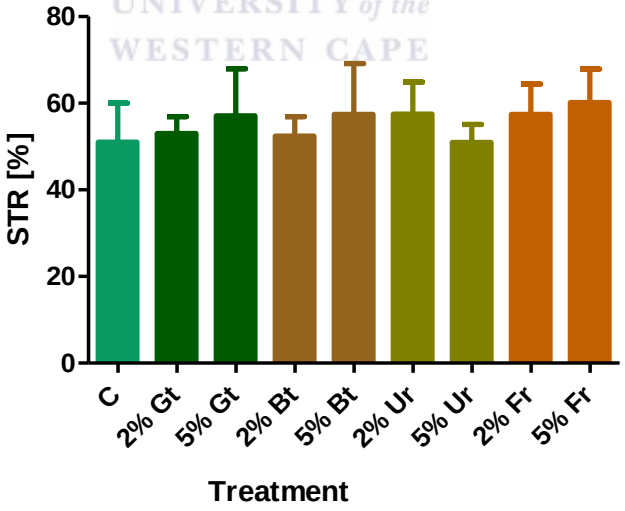


Figure 5.14: Effect of *Camellia sinensis* and *Aspalathus linearis* on straightness (STR) on rat sperm.

Values are represented as mean ± SD after 52 day treatment. Number of rat per group = 6; at least 200 spermatozoa per animal were analyzed. Abbreviations: Abbreviations: C, control; Gt, green tea; Bt, black tea; Ur, unfermented rooibos; Fr, fermented rooibos.

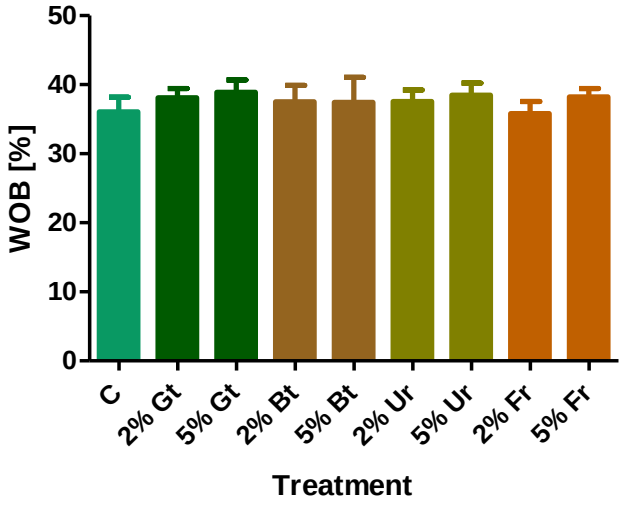


Figure 5.15: Effect of *Camellia sinensis* and *Aspalathus linearis* on wobble (WOB) on rat sperm.

Values are represented as mean ± SD after 52 day treatment. Number of rat per group = 6; at least 200 spermatozoa per animal were analyzed. Abbreviations: Abbreviations: C, control; Gt, green tea; Bt, black tea; Ur, unfermented rooibos; Fr, fermented rooibos.

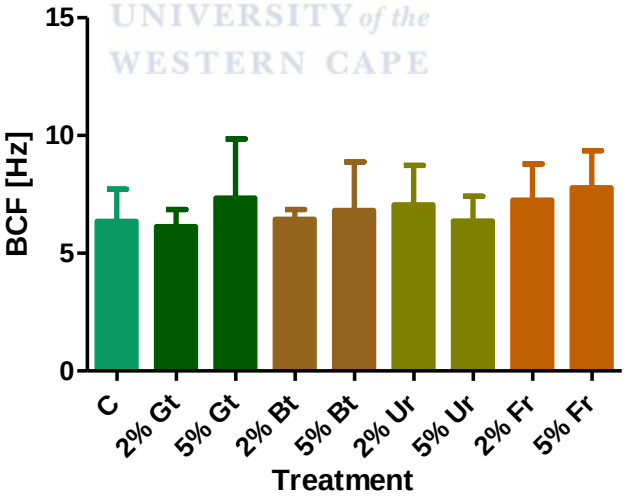


Figure 5.16: Effect of *Camellia sinensis* and *Aspalathus linearis* on beat cross frequency (BCF) on rat sperm.

Values are represented as mean ± SD after 52 day treatment. Number of rat per group = 6; at least 200 spermatozoa per animal were analyzed. Abbreviations: Abbreviations: C, control; Gt, green tea; Bt, black tea; Ur, unfermented rooibos; Fr,fermentedrooibos.

5.8 SPONTANEOUS ACROSOME REACTION

Male Wistar rats exposed to 2% and 5 % green tea, 5% black tea and 2 % fermented rooibos were shown to significantly increase the percentage of spontaneous acrosome reaction ($p < 0.05$; figure 5.17). The rest of the treatment group were not significantly different from the control ($p > 0.05$). However, there was a 9.4 %, 24.5 %, and 24. 5 % and 49.1 % increase observed in rats exposed to 2% black tea, 2% and 5 % unfermented rooibos and 5 % fermented rooibos respectively.

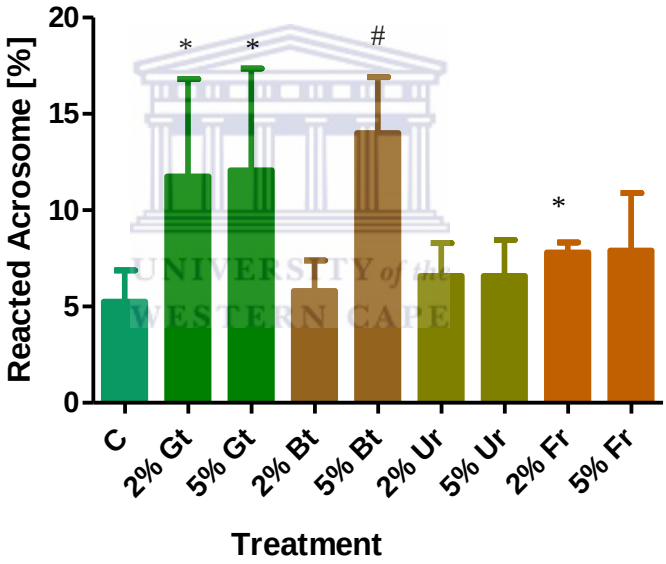


Figure 5.17: Effect of C*amellia sinensis* and *Aspalathus linearis* on spontaneous reacted acrosome in male rats after 52 days exposure.

Values represented are the means ± SD of 6 animals per group and at least 200 spermatozoa per animal were analyzed. Abbreviations: C, control; Gt, green tea; Bt, black tea; Ur, unfermented rooibos; Fr, fermented rooibos; *, $p < 0.05$; $^{\#}$, $p < 0.01$, compared to control.

5.9 CREATININE ACTIVITY

The activity of serum creatinine in male rats exposed to green tea showed no significant difference compared to the control ($p > 0.05$; figure 5.18). 2% black tea significantly increased the activity of creatinine ($p < 0.05$) while 5% black tea did not differ significantly with the control ($p > 0.05$), however, a dose- dependent decrease was observed (ANOVA- trend analysis: P= 0.0391). All rooibos teas investigated stimulates serum creatinine activity ($p < 0.05$), with exception to 5% unfermented rooibos that presented no significant effect ($p > 0.05$).

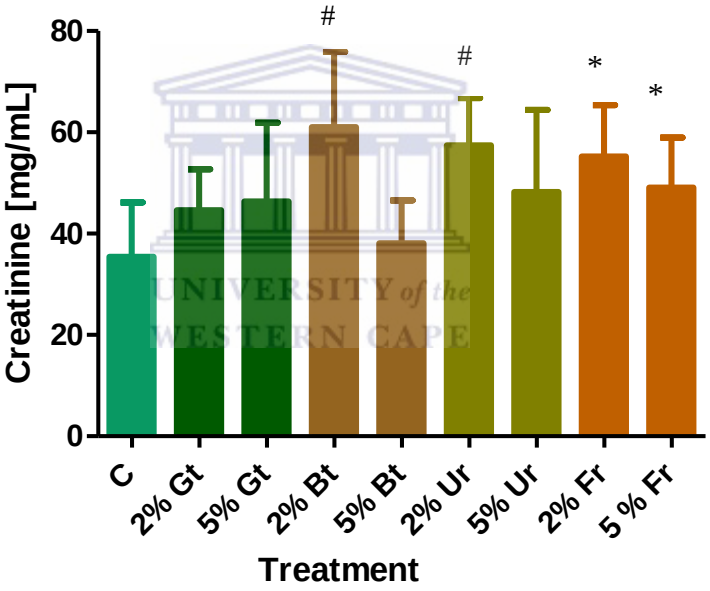


Figure 5.18: Effect of *Camellia sinensis* and *Aspalathus linearis* on creatinine activity in male rat serum.

Values represented are the mean ± SD of 6 animals per group. Abbreviations: C, control; Gt, green tea; Bt, black tea; Ur, unfermented rooibos; Fr, fermented rooibos; *, $p < 0.05$; $^{\#}$, $p < 0.01$ compared to control.

5.10 ALANINE TRANSAMINASE ACTIVITY (ALT)

Serum alanine transaminase (ALT) activity decreased significantly in all treatment groups ($p < 0.05$) excluding 5% fermented rooibos ($p > 0.05$) (figure 5.19). A dose- dependent decrease was observed in the groups exposed to green tea (ANOVA- trend analysis: P= 0.1430), black tea (ANOVA- trend analysis: P= 0.9429), and unfermented rooibos (ANOVA- trend analysis: P= 0.1859).

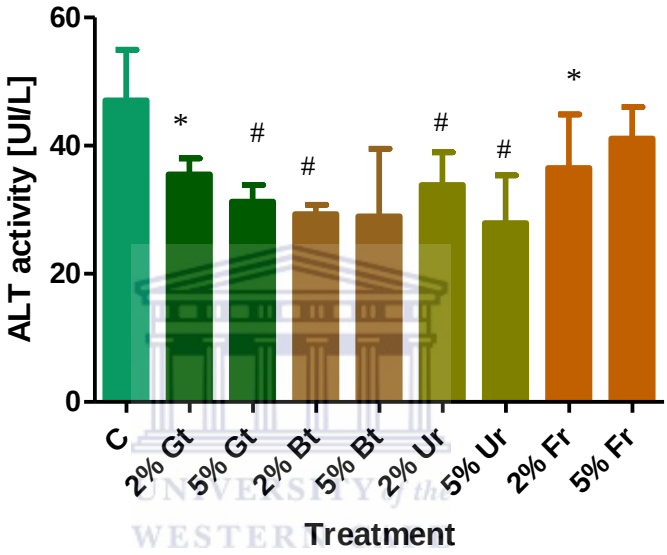


Figure 5.19: Effect of *Camellia sinensis* and *Aspalathus linearis* on Alanine transaminase (ALT) activity in male rat serum.

Values represented are the mean ± SD of 6 animals per group. Abbreviations: C, control; Gt, green tea; Bt, black tea; Ur, unfermented rooibos; Fr, fermented rooibos; *, $p < 0.05$; $^{\#}$, $p < 0.01$ compared to control.

5.11 ASPARTATE TRANSAMINASE ACTIVITY (AST)

Serum level of aspartate transaminase (AST) activity as shown in figure 5.20 dropped significantly in male rat exposed to 2% and 5 % green tea, 5% black tea and 2% unfermented rooibos ($p < 0.05$). In addition, exposure to 2% black tea, 5% unfermented rooibos and 2 % fermented rooibos revealed a tendency to decreased AST values compared to the control ($p > 0.05$). However, 5% fermented rooibos treated group was shown to have a significant increase in AST activity ($p < 0.05$). ANOVA- trend analysis showed a dose- dependent increase in green tea (P= 0.0050), unfermented rooibos (P= 0.0184) and fermented rooibos (P= 0.0104), while black tea displayed a tendency to a dose- dependent decrease in AST activity (P= 0.5588).

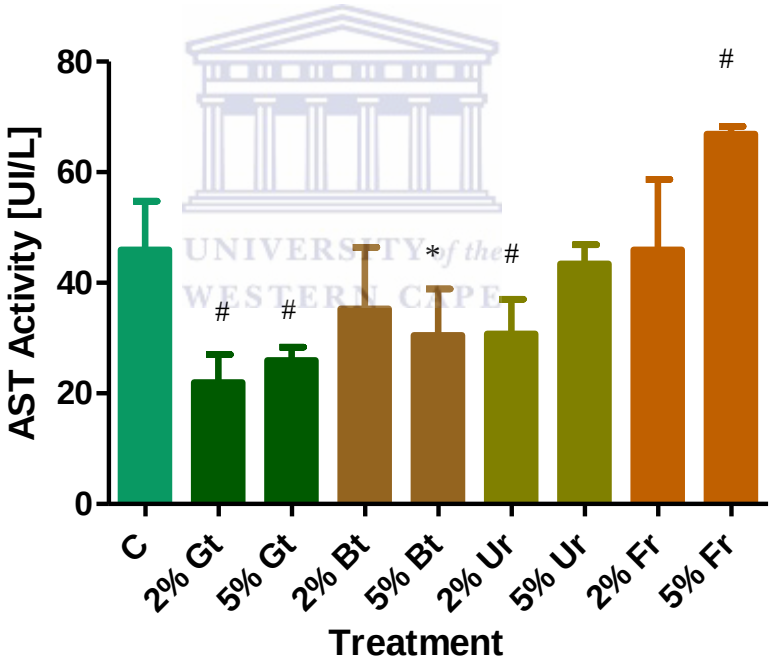


Figure 5.20: Effect of *Camellia sinensis* and *Aspalathus linearis* on Aspartate transaminase (AST) activity in male rat serum.

Values represented are the mean ± SD of 6 animals per group. Abbreviations: C, control; Gt, green tea; Bt, black tea; Ur, unfermented rooibos; Fr, fermented rooibos; *, $p < 0.05$; $^{\#}$, $p < 0.01$ compared to control.

5.12 FERRIC REDUCING ANTIOXIDANT POWER (FRAP) IN MALE RAT SERUM

The sera of male Wistar rats exposed to either *Camellia sinensis* or *Aspalathus linearis* for 52 days displayed no significant difference in FRAP level when compared to the controls ($p > 0.05$; figure 5.21). ANOVA- trend analysis demonstrated a tendency to decreased dose- dependent values in green tea (P=0.5650), unfermented rooibos (P=0.6870) and fermented rooibos (P=0.2602). While a tendency to increased dose- dependent values was observed in the group exposed to black tea (ANOVA- trend analysis: P=0.6137).

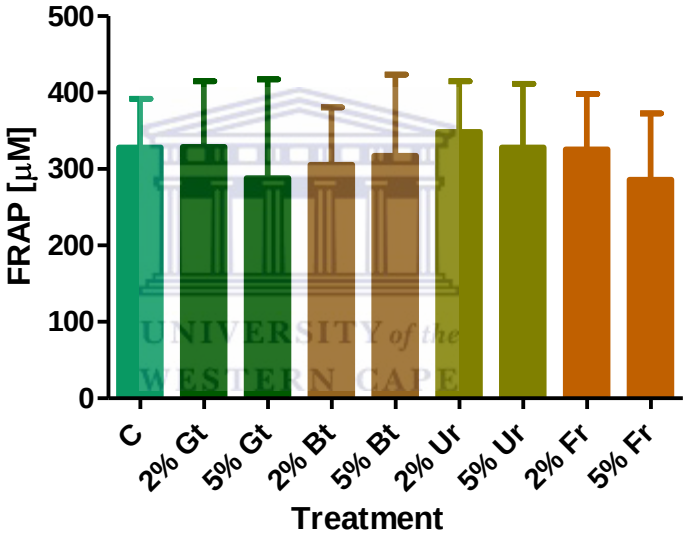


Figure 5.21: Effect of *Camellia sinensis* and *Aspalathus linearis* on ferric reducing antioxidant power (FRAP) in male rat serum.

Values represented are the mean ± SD of 6 animals per group. Abbreviations: C, control; Gt, green tea; Bt, black tea; Ur, unfermented rooibos; Fr, fermented rooibos.

5.13 BIOCHEMICAL ASSAYS ON TESTES, KIDNEY AND LIVER

5.13.1 Lipid Peroxidation (MDA)

Male rats exposed to either *Camellia sinensis* or *Aspalathus linearis* showed no significant difference in the level of lipid peroxidation (LPO) measured as thiobarbituric acid reactive substances (TBARS) in the testes compared to the controls ($p > 0.05$; figure 5.22). 2% green tea tends to decrease the level of TBARS ($p > 0.05$) while 5% green tea tend to increase it by 16.1% ($p > 0.05$), with a dose- dependent increase between teas (ANOVA- trend analysis: P=0.0498. Black tea (2% and 5 %) tends to increase the level of TBARS ($p > 0.05$) by 35.3% and 27.5% respectively. 2% unfermented rooibos showed a tendency to reduced value, and however, at higher concentrations (5%), it tends to have increased the value by 7.7%. Fermented rooibos, both at 2% and 5%, demonstrated a tendency to decreased values when compared to the control (ANOVA- trend analysis: P= 0.2717).

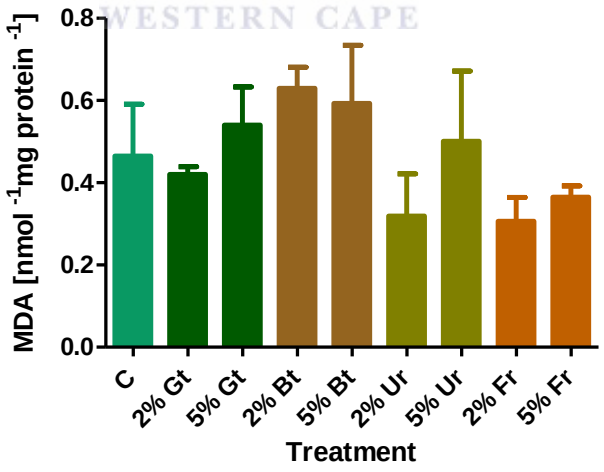


Figure 5.22: Effect of *Camellia sinensis* and *Aspalathus linearis* on lipid peroxidation (MDA) in male rat testis.

Values represented are the mean ± SD of 6 animals per group. Abbreviations: C, control; Gt, green tea; Bt, black tea; Ur, unfermented rooibos; Fr, fermented rooibos.

In the kidneys of male rats exposed to either *Camellia sinensis* or *Aspalathus linearis*, the level of TBARS was not significantly different from the control group ($p > 0.05$; figure 5.23). However, a tendency to a dose- dependent decreased values was observed in the groups exposed to either green (ANOVA- trend analysis: P= 0.7731) or black tea (ANOVA- trend analysis: P= 0.2492). On the other hand, 2% and 5% unfermented rooibos increased its values by 28.7% and 29.4% respectively. In addition, 2% and 5% fermented rooibos increased it by 19% and 44.5% respectively.

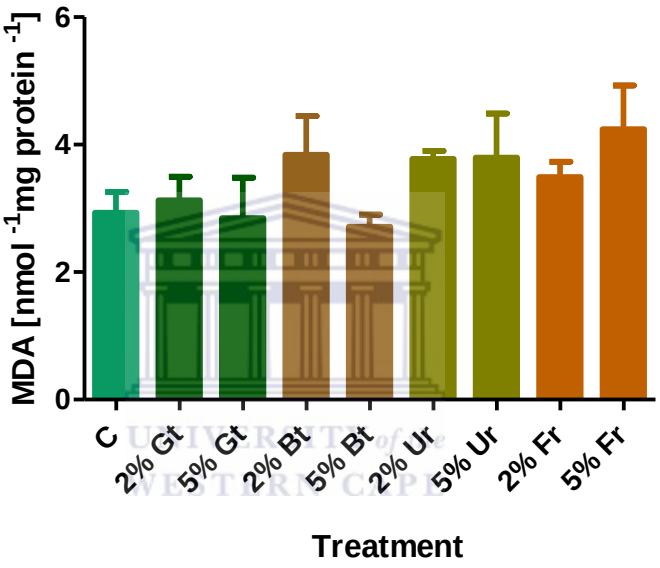


Figure 5.23: Effect of *Camellia sinensis* and *Aspalathus linearis* on lipid peroxidation (MDA) in male rat kidney.

Values represented are the mean ± SD of 6 animals per group. Abbreviations: C, control; Gt, green tea; Bt, black tea; Ur, unfermented rooibos; Fr, fermented rooibos.

TBARS level in the liver of rats exposed to either *Camellia sinensis* or *Aspalathus linearis* showed no significant difference compared to the control ($p > 0.05$; figure 5.24). A tendency to dose- dependent decreased values was observed in the groups exposed to green tea (ANOVA- trend analysis: P= 0.2607) or unfermented rooibos (ANOVA- trend analysis: P= 0.2813) in TBARS level. On the other

hand, a dose- dependent increase in TBARS level was observed in the group exposed to black tea (ANOVA- trend analysis: P= 0.0121) or fermented rooibos (ANOVA- trend analysis: P= 0.0335). 2% black tea and 2% fermented rooibos decreased the level of TBARS by -30.1% and -27% respectively. On the other hand, 5% black tea or 5% fermented rooibos increased the TBARS level in the male rat liver by 3% and 24.2% respectively.

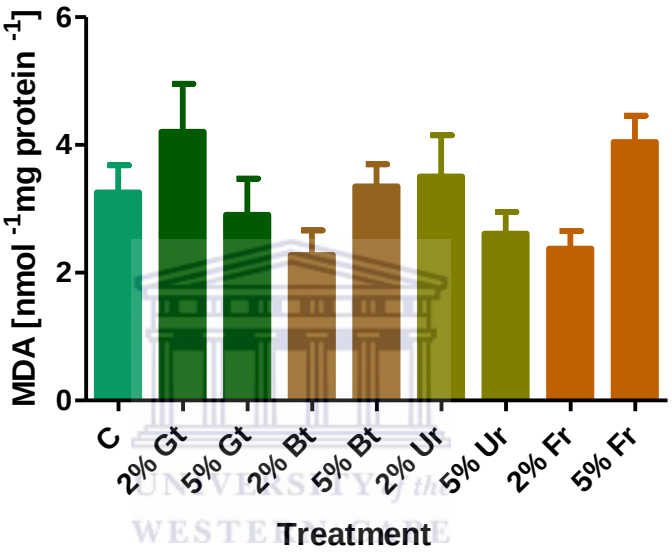


Figure 5.24: Effect of *Camellia sinensis* and *Aspalathus linearis* on lipid peroxidation (MDA) in male rat liver.

Values represented are the mean ± SD of 6 animals per group. Abbreviations: C, control; Gt, green tea; Bt, black tea; Ur, unfermented rooibos; Fr, fermented rooibos.

5.13.2 Glutathione (GSH)

Glutathione (GSH) levels in testes of animals exposed to either *Camellia sinensis* or *Aspalathus linearis* showed no significant differences compared to the controls ($p > 0.05$; figure 5.25).

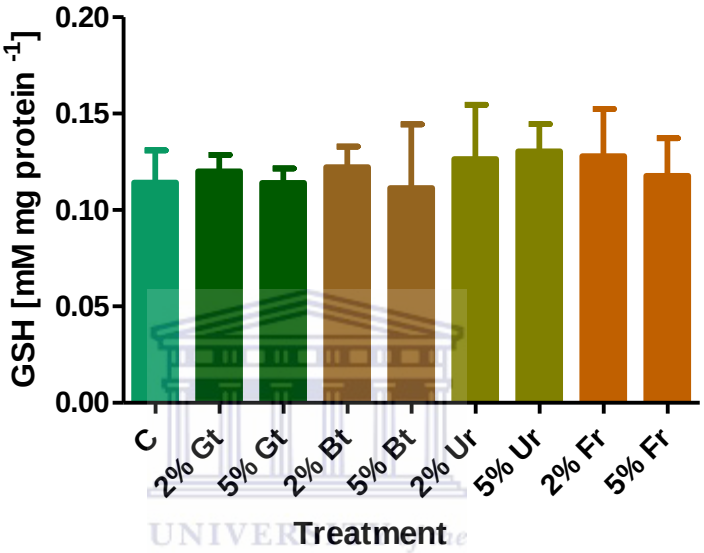


Figure 5.25: Effect of *Camellia sinensis* and *Aspalathus linearis* on glutathione (GSH) in male rat testes.

Values represented are the mean ± SD of 6 animals per group. Abbreviations: C, control; Gt, green tea; Bt, black tea; Ur, unfermented rooibos; Fr, fermented rooibos.

In the male rat kidneys as well, exposure to either *Camellia sinensis* or *Aspalathus linearis* demonstrated no significant effect on the GSH levels compared to the control ($p > 0.05$; figure 5.26).

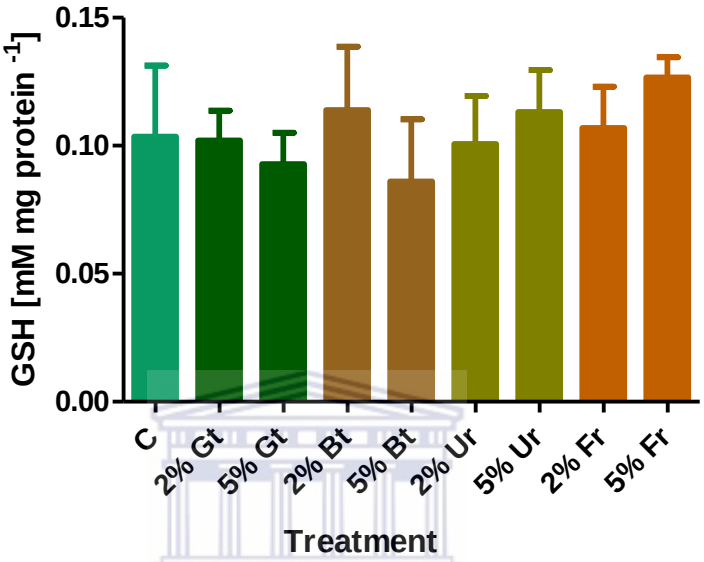


Figure 5.26: Effect of *Camellia sinensis* and *Aspalathus linearis* on glutathione (GSH) in male rat kidney.

Values represented are the mean ± SD of 6 animals per group. Abbreviations: C, control; Gt, green tea; Bt, black tea; Ur, unfermented rooibos; Fr, fermented rooibos.

GSH level in male rat liver exposed to green tea increased significantly at 2% ($p < 0.05$; figure 5.27) with a dose- dependent decrease (ANOVA- trend analysis: P= 0.2602). Black tea showed no significant difference in the level of GSH ($p > 0.05$). Unfermented rooibos, on the other hand, significantly enhanced GSH levels in the liver ($p < 0.05$) with a significant dose- dependent rise (ANOVA- trend analysis: P= 0.0345). In addition, fermented rooibos significantly increased liver GSH values at 5% ($p < 0.05$).

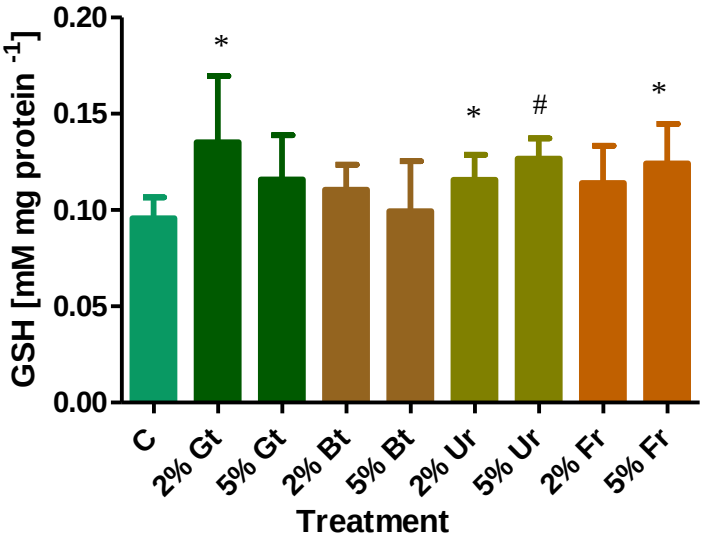


Figure 5.27: Effect of *Camellia sinensis* and *Aspalathus linearis* on glutathione (GSH) in male rat liver.

Values represented are the mean ± SD of 6 animals per group. Abbreviations: C, control; Gt, green tea; Bt, black tea; Ur, unfermented rooibos; Fr, fermented rooibos; *, $p < 0.05$; $^{\#}$, $p < 0.01$ compared to control.

5.13.3 Catalase activity (CAT)

Catalase activity in testes of male rats exposed to *Camellia sinensis* or *Aspalathus linearis* were similar to the control ($p > 0.05$). A tendency to dose- dependent decreased values were observed in the groups exposed to either green tea (ANOVA- trend analysis: P= 0.0044) or unfermented rooibos (ANOVA- trend analysis: P= 0.0919).

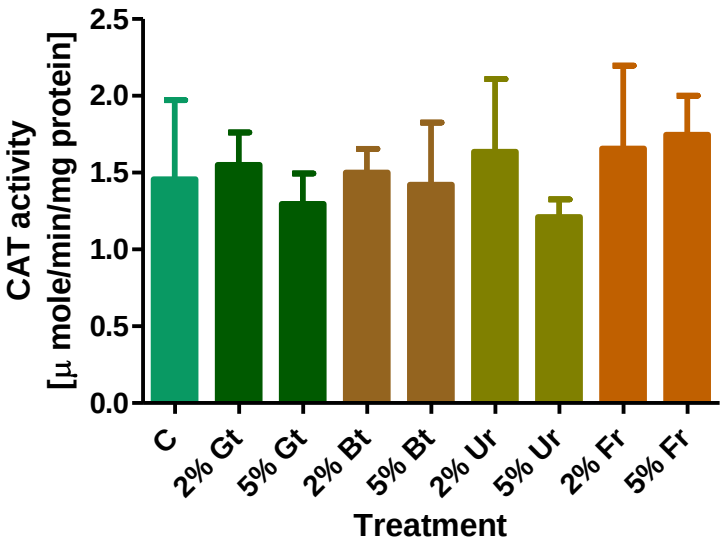


Figure 5.28: Effect of *Camellia sinensis* and *Aspalathus linearis* on catalase activity in the male rat testis.

Values represented are the mean ± SD of 6 animals per group. Abbreviations: C, control; Gt, green tea; Bt, black tea; Ur, unfermented rooibos; Fr, fermented rooibos.

Likewise in the kidney of male rats, *Camellia sinensis* or *Aspalathus linearis* did not have any significant effect on the catalase activity when compared to the control ($p > 0.05$). In addition, a tendency to dose-dependent reduced values was observed in the groups exposed to either green tea, black tea of fermented rooibos while unfermented rooibos on the other hand revealed a tendency to enhanced dose- dependent values.

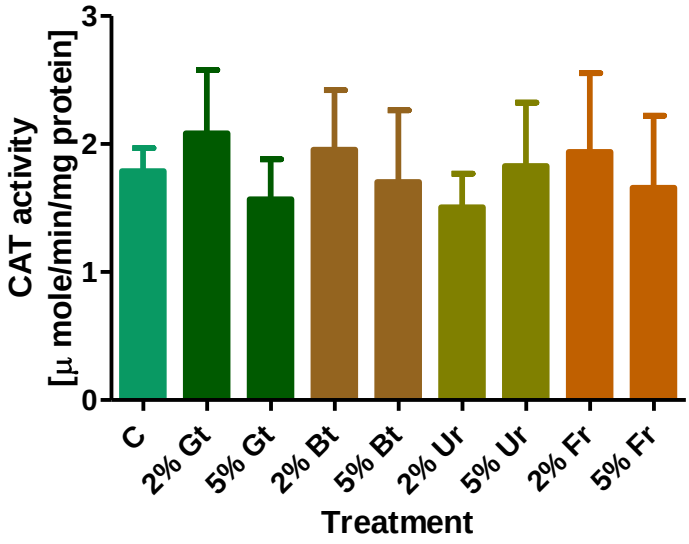


Figure 5.29: Effect of *Camellia sinensis* and *Aspalathus linearis* on catalase activity in the male rat kidney.

Values represented are the mean ± SD of 6 animals per group. Abbreviations: C, control; Gt, green tea; Bt, black tea; Ur, unfermented rooibos; Fr, fermented rooibos.

In the liver of male rat exposed to green tea, black tea or unfermented rooibos, there was no significant difference on the activity of catalase compared to the control ($p > 0.05$). Fermented rooibos on the other hand significantly enhanced catalase activity in the liver ($p < 0.05$).

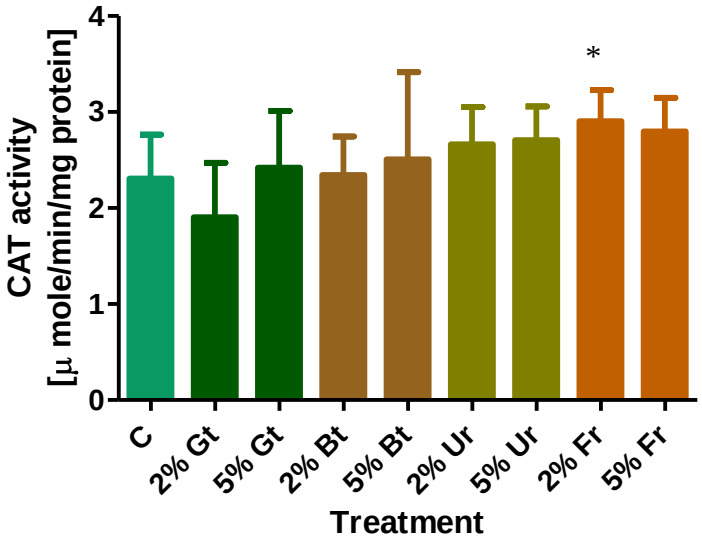


Figure 5.30: Effect of *Camellia sinensis* and *Aspalathus linearis* on catalase activity in the male rat liver.

Values represented are the mean ± SD of 6 animals per group. Abbreviations: C, control; Gt, green tea; Bt, black tea; Ur, unfermented rooibos; Fr, fermented rooibos; *, $p < 0.05$ compared to control.

5.13.4 Superoxide dismutase activity (SOD)

Superoxide dismutase activity (SOD) in the testes of male rats exposed to green tea, black tea or unfermented rooibos showed no significant difference compared to the control ($p > 0.05$; figure 5.31) but increased significantly by 2% fermented rooibos ($p < 0.05$; figure 5.31). However, a tendency to increased activity was observed at the concentrations (2%) of either *Camellia sinensis* or *Aspalathus linearis*. A dose- dependent decrease in SOD activity was observed in black tea (ANOVA- trend analysis: P= 0.0431), and fermented rooibos (ANOVA trend analysis: P= 0.0009).

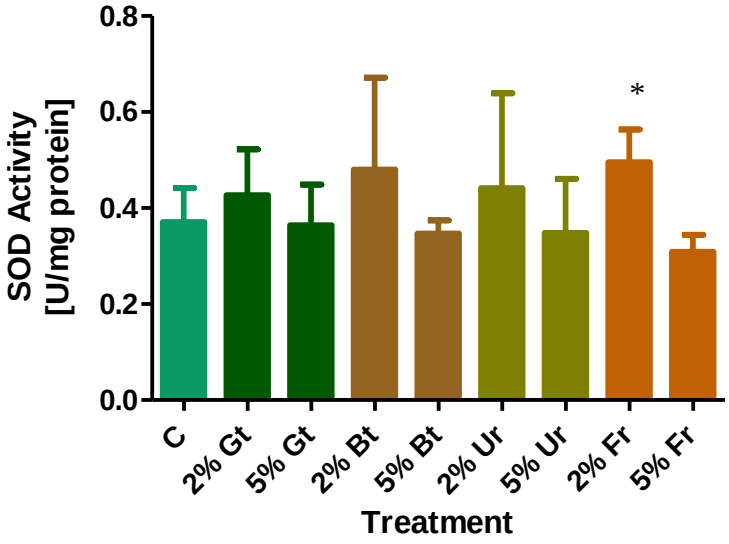


Figure 5.31: Effect of *Camellia sinensis* and *Aspalathus linearis* on superoxide dismutase (SOD) activity in the male rat testis.

Values represented are the mean ± SD of 6 animals per group. Abbreviations: C, control; Gt, green tea; Bt, black tea; Ur, unfermented rooibos; Fr, fermented rooibos; *, $p < 0.05$ compared to control.

In the male kidneys of rats exposed to green tea, unfermented rooibos and fermented rooibos and 5% black tea, SOD activity was similar to the control ($p > 0.05$; figure 5.32). 2% black tea, on the other hand, significantly decreased the activity of SOD ($p < 0.05$).

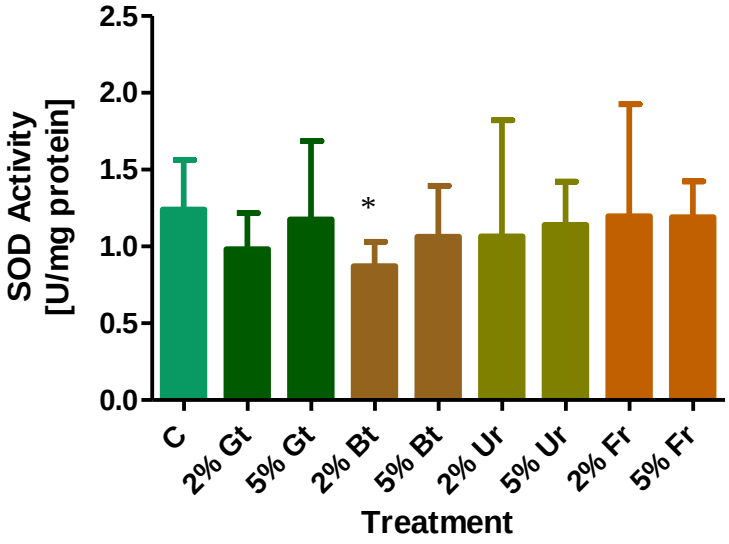


Figure 5.32: Effect of *Camellia sinensis* and *Aspalathus linearis* on superoxide dismutase (SOD) activity in the male rat kidney.

Values represented are the mean ± SD of 6 animals per group. Abbreviations: C, control; Gt, green tea; Bt, black tea; Ur, unfermented rooibos; Fr, fermented rooibos; *, $p < 0.05$ compared to control.

Superoxide dismutase activity in the liver of male rats exposed to either *Camellia sinensis* or *Aspalathus linearis* revealed similar result compared to the control ($p > 0.05$; figure 5.33). However, the lower concentrations (2%) of green tea, black tea and unfermented rooibos tend to produced higher SOD activity compared to the higher concentrations (5%). A dose- dependent decreased SOD activity was not observed in the liver of male rats exposed to green tea (ANOVA- trend analysis: P= 0.4407) and unfermented rooibos (ANOVA- trend analysis: P= 0.2330) but was observed for black tea (ANOVA- trend analysis: P= 0.0465). While a non-statistical increasing trend in SOD activity was observed in the liver of male rats exposed to fermented rooibos (ANOVA- trend analysis: P= 0.2291).

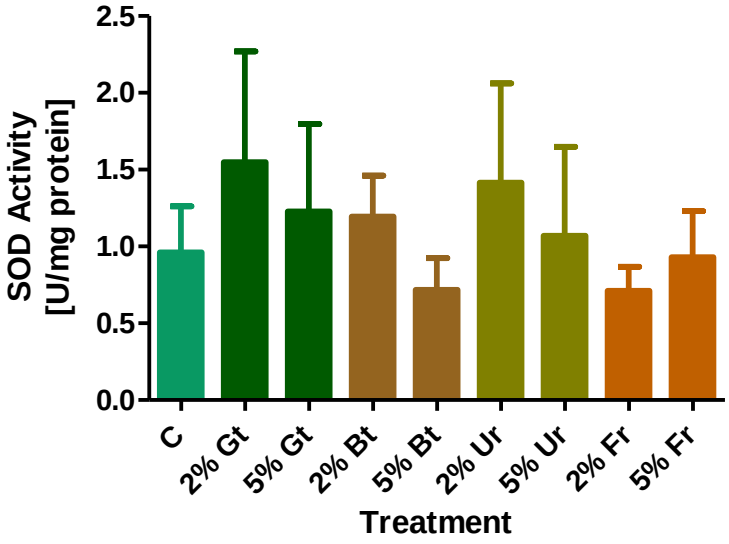


Figure 5.33: Effect of *Camellia sinensis* and *Aspalathus linearis* on superoxide dismutase (SOD) activity in the male rat liver.

Values represented are the mean ± SD of 6 animals per group. Abbreviations: C, control; Gt, green tea; Bt, black tea; Ur, unfermented rooibos; Fr, fermented rooibos.

5.14 HISTOLOGY OF REPRODUCTIVE ORGANS, KIDNEY AND LIVER IN MALE RATS

The histological observation of the testes in rats exposed to either *Camellia sinensis* or *Aspalathus linearis* showed no obvious distortion in the architecture of the cells compared to the control (figures 5.34 and 5.35). All stages of spermatogenesis were present in all treated group as compared to the control. In addition, there are abundant spermatozoa present in the lumen of the seminiferous tubule in all treated groups, like the control.

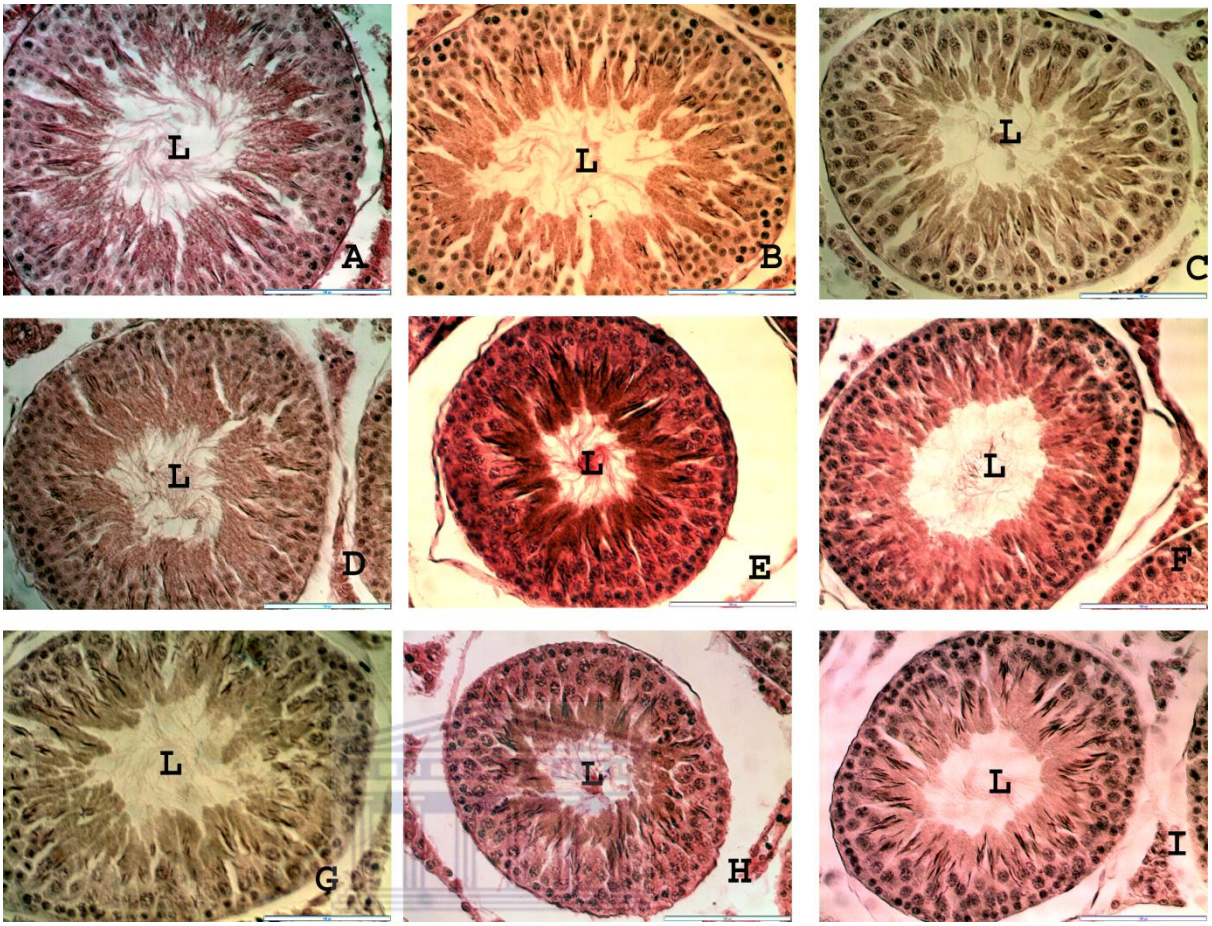


Figure 5.34: Morphology of the male rat testes exposed to *Camellia sinensis* or *Aspalathus linearis* for 52 days using H&E staining. (A) control (B) 2% green tea (C) 5% green tea (D) 2% black tea (E) 2% unfermented rooibos (F) 5% unfermented rooibos (G) 5% black tea (H) 2% fermented rooibos (I) 5% fermented rooibos (L) lumen of seminiferous tubule; Bar = 100 µm.

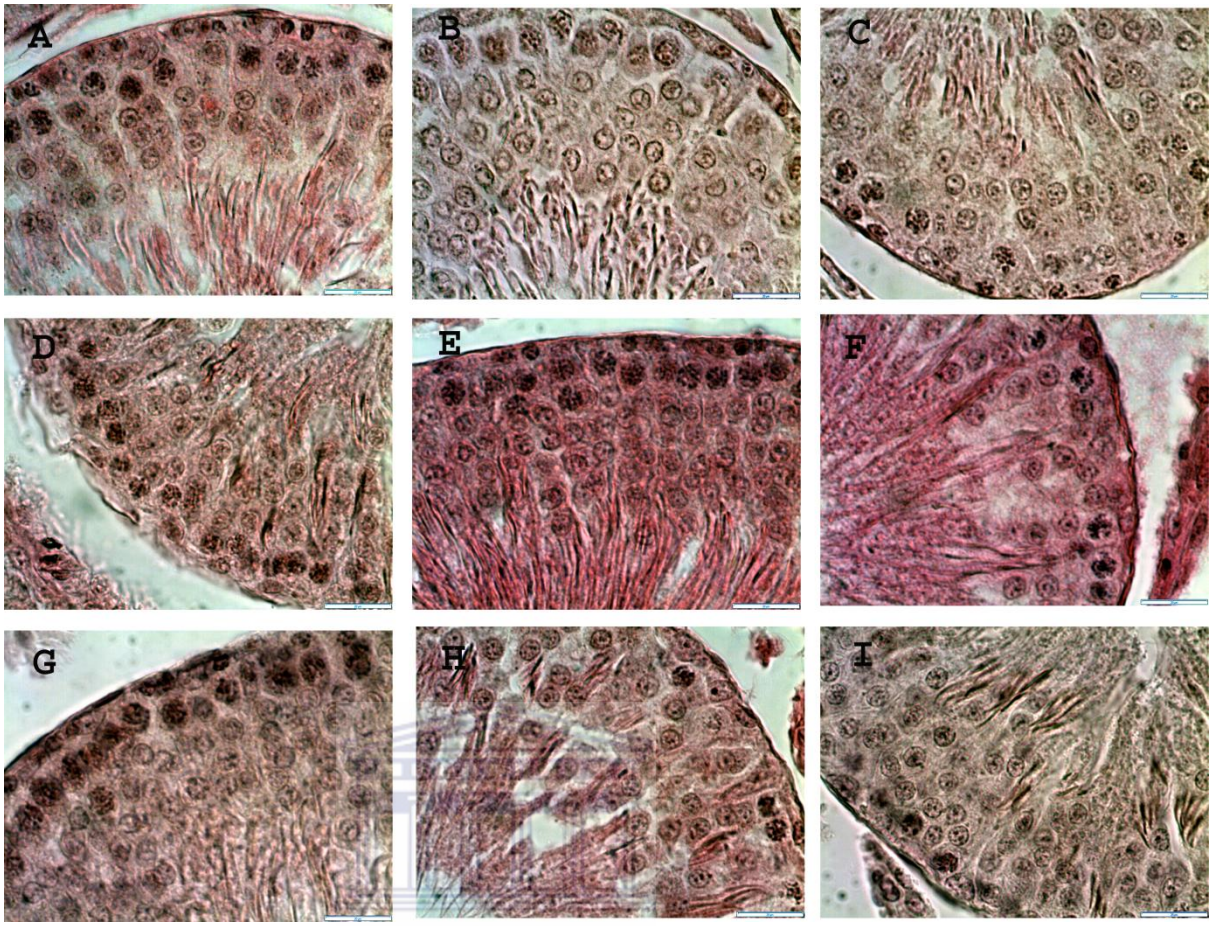


Figure 5.35: Morphology of the male rat testes exposed to *Camellia sinensis* or *Aspalathus linearis* for 52 days using H&E staining. (A) control (B) 2% green tea (C) 5% green tea (D) 2% black tea (E) 2% unfermented rooibos (F) 5% unfermented rooibos (G) 5% black tea (H) 2% fermented rooibos (I) 5% fermented rooibos; Bar = 20 µm.

Sections of the epididymides (cauda and caput) in male rats exposed also to either *Camellia sinensis* or *Aspalathus linearis* showed no difference in structure compared to the control groups (figures 5.36 and 5.37). Both the cauda and caput epithelia of the epididymides appeared normal and contained abundant spermatozoa in their respective lumen in all treatment groups as compared to the control.

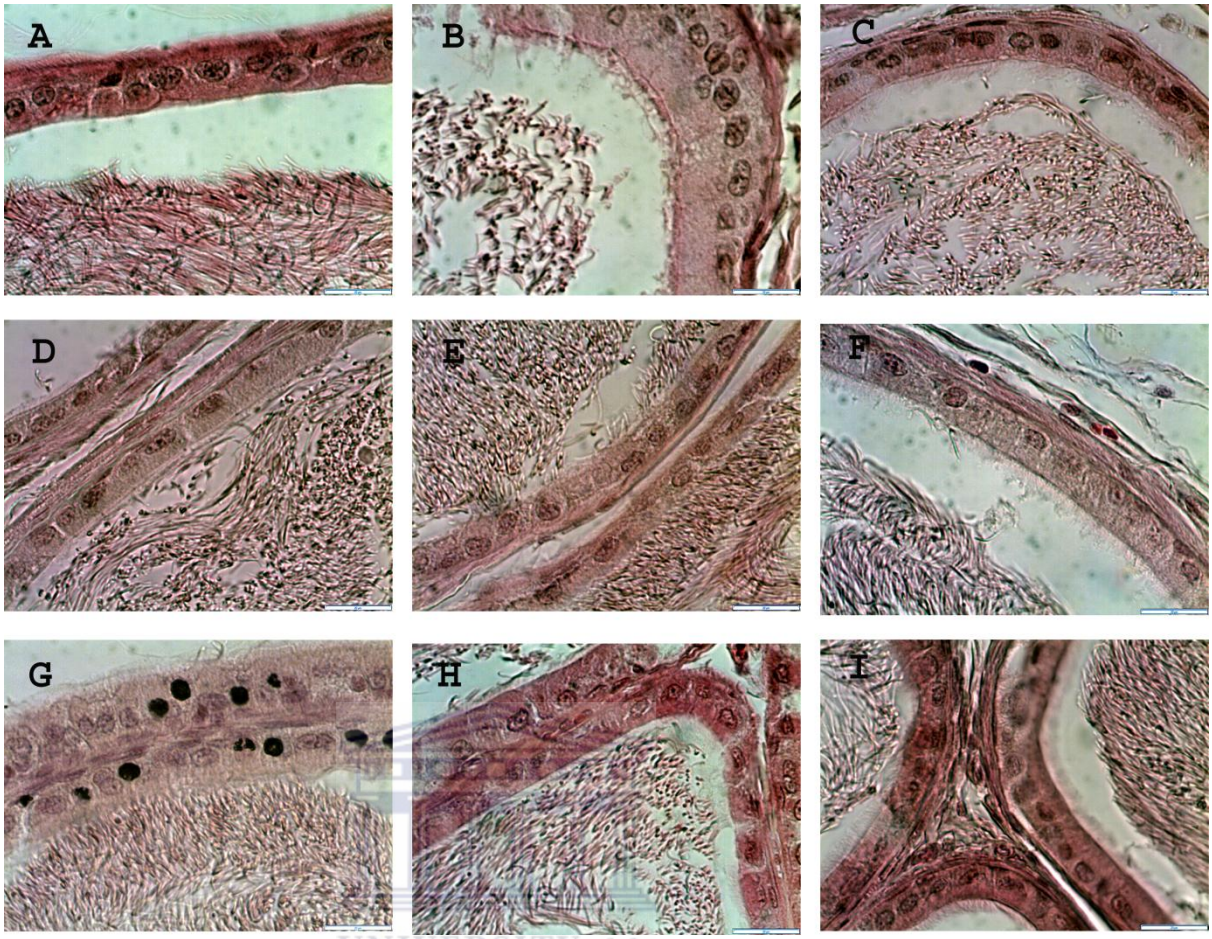


Figure 5.36: Morphology of the male rat cauda epididymides exposed to *Camellia sinensis* or *Aspalathus linearis* for 52 days using H&E staining. (A) control (B) 2% green tea (C) 5% green tea (D) 2% black tea (E) 2% unfermented rooibos (F) 5% unfermented rooibos (G) 5% black tea (H) 2% fermented rooibos (I) 5% fermented rooibos; Bar = 20 µm.

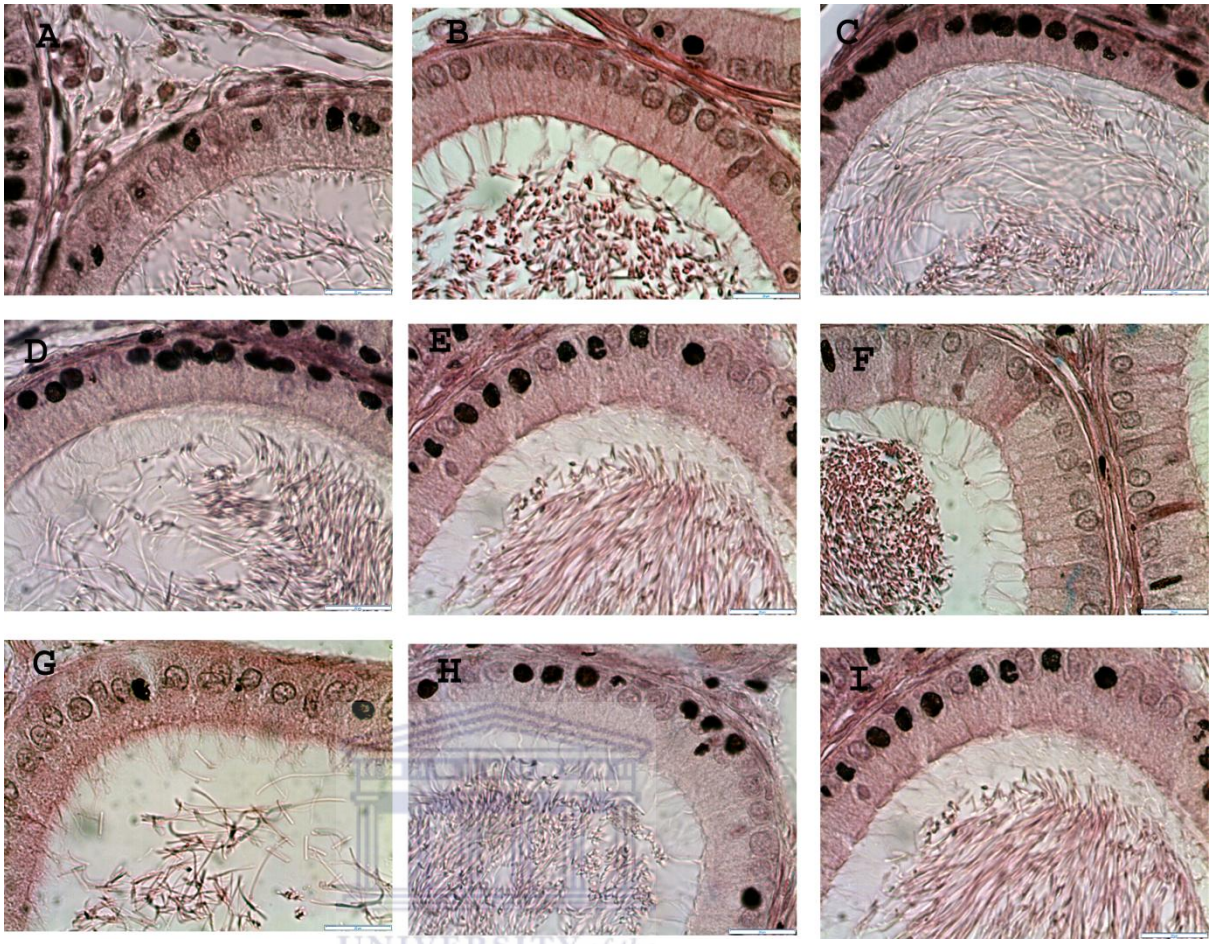


Figure 5.37: Morphology of the male rat caput epididymides exposed to *Camellia sinensis* or *Aspalathus linearis* for 52 days using H&E staining. (A) control (B) 2% green tea (C) 5% green tea (D) 2% black tea (E) 2% unfermented rooibos (F) 5% unfermented rooibos (G) 5% black tea (H) 2% fermented rooibos (I) 5% fermented rooibos; Bar = 20 µm.

The histological observations of the structure of the kidneys in male rats exposed to *Camellia sinensis* or *Aspalathus linearis* showed no alterations compared to the control group (figure 5.38).

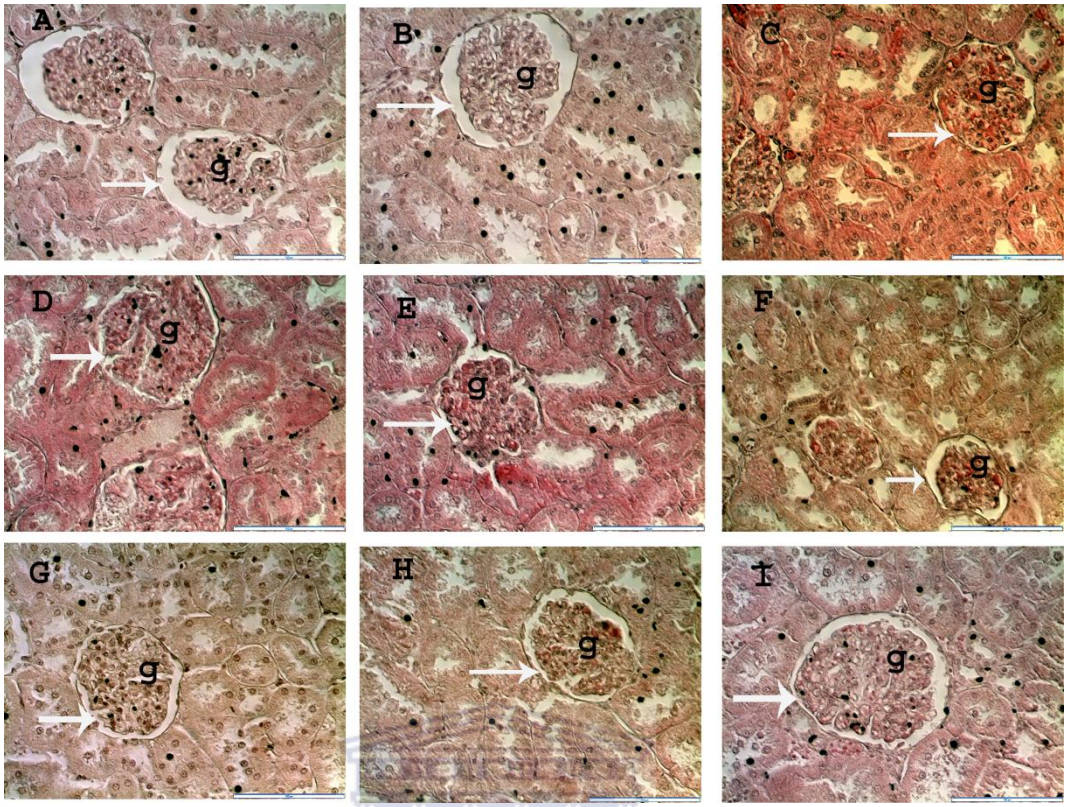


Figure 5.38: Morphology of the male rat kidney exposed to *Camellia sinensis* or *Aspalathus linearis* for 52 days using H&E staining. (A) control (B) 2% green tea (C) 5% green tea (D) 2% black tea (E) 2% unfermented rooibos (F) 5% unfermented rooibos (G) 5% black tea (H) 2% fermented rooibos (I) 5% fermented rooibos; g, glomerulus; white arrow, bowman's capsule; Bar = 100 µm.

Likewise, in the liver, there was no alteration in the architecture of liver in male rats exposed to either *Camellia sinensis* or *Aspalathus linearis* (figure 5.39).

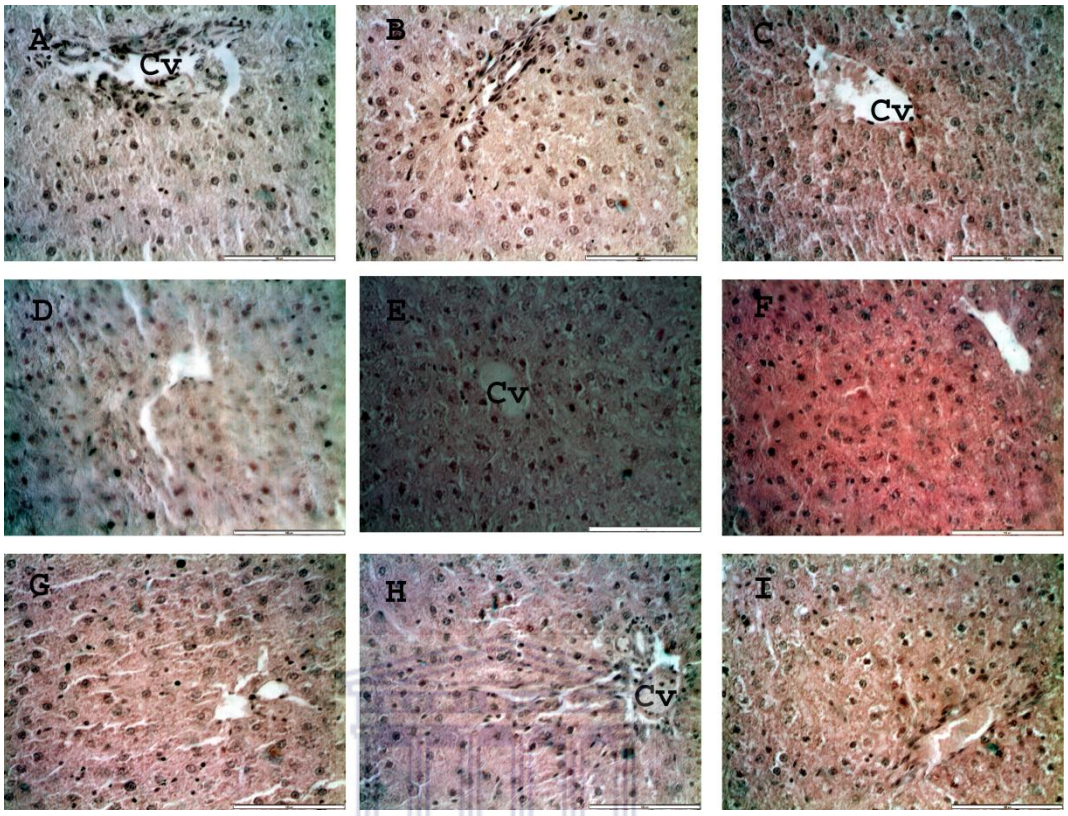


Figure 5.39: Morphology of the male rat liver exposed to *Camellia sinensis* or *Aspalathus linearis* for 52 days using H&E staining. (A) control (B) 2% green tea (C) 5% green tea (D) 2% black tea (E) 2% unfermented rooibos (F) 5% unfermented rooibos (G) 5% black tea (H) 2% fermented rooibos (I) 5% fermented rooibos; Cv, central vein; Bar = 20 µm.

5.15 MORPHOMETRIC MEASUREMENT IN TESTIS AND EPIDIDYMIS

Diameter of seminiferous tubules in male rats exposed to either *Camellia sinensis* or *Aspalathus linearis* was significantly smaller than the control groups ($p < 0.0001$; figure 5.40). Likewise, the epithelial heights of seminiferous tubules in the testes as shown in figure 5.41 were significantly decreased by 2% green tea, 2% and 5% black tea, 5% unfermented rooibos, and 2% and 5% fermented rooibos ($p < 0.05$). 5% green tea and 2% unfermented rooibos showed no significant difference in the epithelial heights compared to the control ($p > 0.05$).

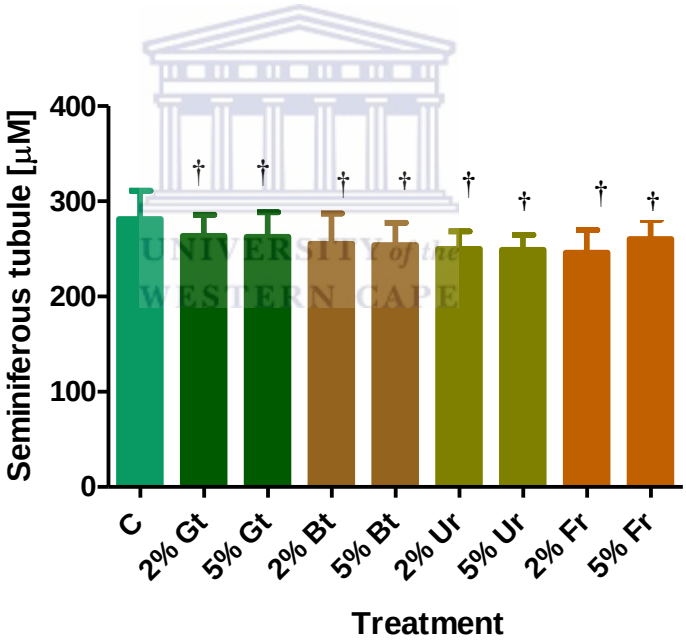


Figure 5.40: Diameter of the seminiferous tubules in rat testis exposed to *Camellia sinensis* or *Aspalathus linearis* after 52 days.

Values represented are the means ± SD of 6 animals per group with 30 tubular profiles measured per animal. Abbreviations: C, control; Gt, green tea; Bt, black tea; Ur, unfermented rooibos; Fr, fermented rooibos; †, p <0.0001 compared to control.

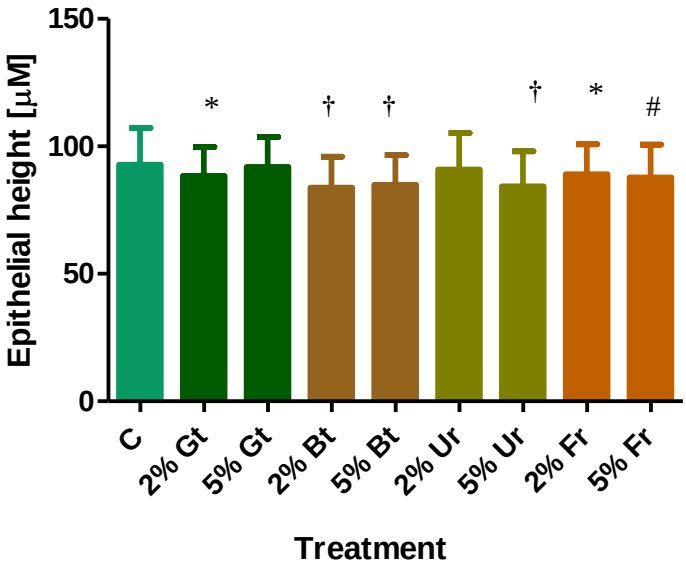


Figure 5.41: Effect of *Camellia sinensis* and *Aspalathus linearis* on the epithelial heights of the seminiferous tubules in rat testis.

Values represented are the means ± SD of 6 animals per group with 30 tubular profiles measured per animal. Abbreviations: C, control, Gt, green tea; Bt, black tea; Ur, unfermented rooibos; Fr, fermented rooibos; *, p <0.05; $^{\#}$, p <0.01; †, p <0.0001 compared to control.

In the epididymis of male rats exposed to *Camellia sinensis* or *Aspalathus linearis*, the caput epithelial height increased significantly in the group exposed to 5% green tea, 2% black tea, 2% and 5% unfermented rooibos of 2% and 5% fermented rooibos (p < 0.01; figure 5.42). The caput epithelial height in male rats exposed to 2% green tea or 5 % black tea showed no significant difference compared to the control (p > 0.05). A significant dose- dependent decrease in the epithelial height (caput) was observed in black tea treated group (ANOVA- trend analysis: P= 0.0081). While a tendency to increased dose-dependent values was observed in the groups exposed to green tea (ANOVA- trend analysis: P= 0.7925), unfermented rooibos (ANOVA- trend analysis: P= 0.1901) or fermented rooibos (ANOVA- trend analysis: P=0.4967).

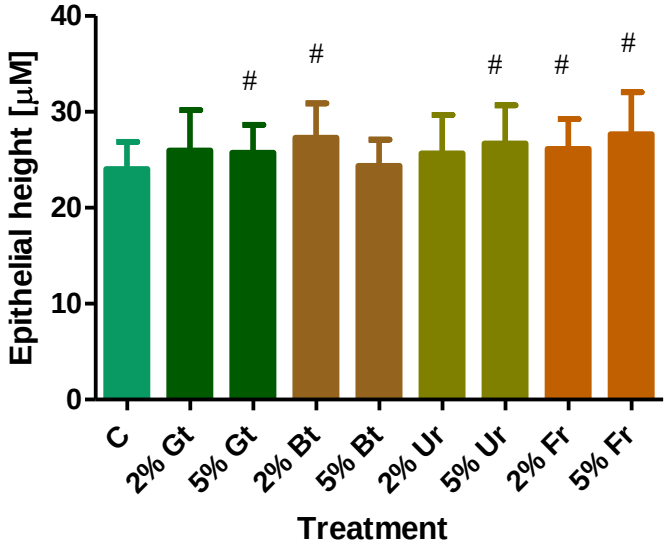


Figure 5.42: Effect of *Camellia sinensis* and *Aspalathus linearis* on the epithelial heights of the epididymis (caput).

Values represented are the means ± SD of 6 animals per group with 10 epithelial heights measured per region per animal. Abbreviations: C, control; Gt, green tea; Bt, black tea; Ur, unfermented rooibos; Fr, fermented rooibos; #, p <0.01 compared to control.

Male rats exposed to *Camellia sinensis* or *Aspalathus linearis*, showed that the cauda epithelial height increased significantly only in the group exposed to 2% green tea (p < 0.01) and 5% black tea (p < 0.0001). In contrast, a significant decrease in the epithelial height (cauda) of male rats exposed 2% unfermented rooibos was observed (p < 0.05) as seen in figure 5.43. 5% green tea, 2% black tea, 2% and 5% fermented or 5% unfermented rooibos showed no significant difference in the epithelial height (cauda) compared to the control group (p > 0.05). A significant dose- dependent increase in epithelial height (cauda) was observed in black tea (ANOVA- trend analysis: P= 0.0013) and unfermented rooibos (ANOVA- trend analysis: P=0.0008). On the other hand, a dose-dependent decrease in epithelial height (cauda) was observed in the groups exposed to green tea (ANOVA- trend analysis: P=0.0338).

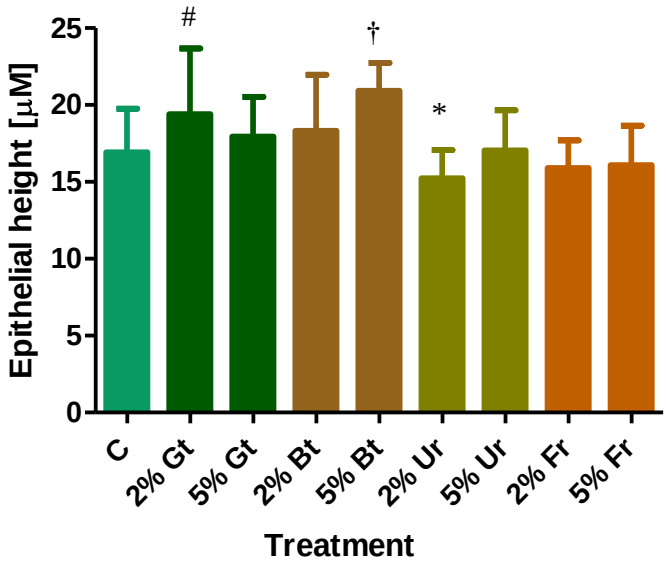


Figure 5.43: Effect of *Camellia sinensis* and *Aspalathus linearis* on the epithelial heights of the epididymis (cauda).

Values represented are the means ± SD of 6 animals per group with 10 epithelial heights measured per region per animal. Abbreviations: C, control; Gt, green tea; Bt, black tea; Ur, unfermented rooibos; Fr, fermented rooibos; *, p <0.05; $^{\#}$, p <0.01; †, p <0.0001 compared to control.

CHAPTER SIX

RESULT OF THE EFFECT OF CAMELLIA SINENSIS AND ASPALATHUS LINEARIS ON THE FEMALE REPRODUCTIVE SYSTEM

6.1 AVERAGE DAILY INTAKE OF FLUID AND EXTRACT PARAMETERS

Fluid intake on weekly basis showed some variations among treatment groups (table 6.1). In the first week, 5% green tea, 2% black tea, and fermented rooibos (2% and 5%) were similar to the control ($p > 0.05$), while the intake of 5% black tea and unfermented rooibos (2% and 5%) were significantly lesser than the control ($p < 0.05$), and 2% green tea intake was significantly more than the control ($p < 0.05$). During the second week, 5% green tea and 5% black tea intake was similar to the control ($p > 0.05$). The other treatment groups (2% green tea, 2% black tea, 2% and 5% unfermented rooibos, and 2% and 5% fermented rooibos) intake was significantly lesser than the control ($p < 0.05$). In the last week (week 3) of treatment, black tea (2% and 5%), unfermented rooibos (2% and 5%), and fermented rooibos (2% and 5%) intake were similar to the control ($p > 0.05$). On the other hand, green tea (2% and 5%) intake was significantly more than the control ($p < 0.05$). On the overall, female Wistar rats' average fluid intake in all treatment groups excluding the 5% unfermented rooibos did not differ significantly from the control groups ($p > 0.05$) after 21 days exposure. 5% unfermented rooibos intake was significantly less than the control ($p < 0.05$).

Average daily intake of fluid (ADI; mg/100 mg BW) increased significantly in the 2% green tea group ($p < 0.05$) while a significant decrease was observed in the groups exposed to black tea (2% and 5%), unfermented rooibos (2% and 5%), and fermented rooibos (2% and 5%) ($p < 0.05$). ADI of 5% green tea was not significantly different from the control ($p > 0.05$) (table 6.2).

ADI (mg/100mg BW) of soluble solids (table 6.2) showed green tea to be the highest on the order: green tea ≥ black tea > unfermented rooibos > fermented rooibos ($p < 0.05$) in both concentrations. In addition, a significant dose-dependent increase was also observed in all teas ($p < 0.05$).

The ADI of total polyphenols was also shown to be more in the green tea (table 6.2). TPP intake in female rats was shown in the order: green tea > black tea ≥ unfermented rooibos > fermented rooibos ($p < 0.05$). Likewise, a significant dose-dependent increase was observed in all tea type ($p < 0.05$).

Flavonol ADI was shown to be highest in the fermented rooibos group in the following order: fermented rooibos > unfermented rooibos > black tea > green tea ($p < 0.05$; table 6.2). In addition, ADI of flavanol was highest in the green tea group in the order: green tea > black tea > unfermented rooibos > fermented rooibos ($p < 0.05$) with a significant dose – dependent increase in all tea type ($p < 0.05$).

Table 6.1: Average daily intake (mL/100g BW) and total body weight gain over 21days in female rats

Treatment	Mean fluid intake over 3 weeks period (mL/100g BW)			Mean fluid intake (mL/100g BW)
	Wk(1)	Wk(2)	Wk(3)	
Control	14.23±1.05[a]	15.64±0.97[a]	13.71±0.81[a]	14.53±0.94[a]
2% Gt	15.91±0.70[b]	13.81±0.57[b]	15.94±0.84[b]	15.22±0.70[a]
5% Gt	14.14±1.41[a]	15.48±0.92[a]	15.17±1.05[b]	14.93±1.13[a]
2% Bt	13.66±0.86[a]	13.29±0.68[b]	12.66±0.91[a]	13.20±0.82[a]
5% Bt	11.00±1.60[b]	15.27±1.38[a]	12.65±0.97[a]	12.98±1.32[a]
2% Ur	12.29±0.67[b]	12.91±0.28[b]	13.95±1.41[a]	13.05±0.79[a]
5% Ur	12.67±0.76[b]	11.58±1.89[b]	12.50±1.14[a]	12.45±1.26[b]
2% Fr	13.47±1.94[a]	13.71±0.99[b]	12.53±1.27[a]	13.24±1.40[a]
5% Fr	12.84±1.26[a]	11.85±1.11 [b]	13.26±1.30[a]	12.65±1.22[a]

Values represented are the mean ± SD of 6 animals per group. Abbreviations: C, control; Gt, green tea; Bt, black tea; Ur, unfermented rooibos; Fr, fermented rooibos; Wk, week. Means in the column followed by the same letter do not differ significantly. If the letter differs, then $p < 0.05$ compared to control.

Table 6.2: Average intake parameters of the female rats receiving the respective tea extract during 21 days treatment

Samples	Fluid Intake		Soluble Solids [Ss]		Total Polyphenols		Flavonol		Flavanol	
	ADI	ADI		ADI		ADI		ADI		ADI
	[ml]	[mg/100g BW]	[mg/ml]	[mg/100g BW]	[%SS]	[mg/100g BW]	[%SS]	[mg/100g BW]	[%SS]	[mg/100g BW]
Control	32.36±0.02^{a}	14.53±1.23^{a}	-	-	-	-	-	-	-	-
2% Gt	33.00±1.30^{a}	15.22±1.22^{b}	4.86±1.21^{a}	73.96±5.95^{a}	33.31±2.88^{a}	24.64±2.13^{a}	0.28±0.07^{a}	0.21±0.05^{a}	29.48±5.68^{a}	21.80±4.20^{a}
5% Gt	29.62±1.18^{b}	14.93±1.23^{a}	11.81±2.28^{bh}	176.36±14.48^{b}	31.84±3.96^{a}	56.16±6.99^{b}	0.09±0.01^{b}	0.16±0.02^{ac}	35.29±8.62^{a}	62.23±15.21^{b}
2% Bt	30.33±0.10^{b}	13.20±0.88^{b}	5.68±1.44^{ai}	74.99±5.01^{a}	25.93±1.66^{be}	19.45±1.25^{c}	0.27±0.08^{a}	0.20±0.06^{a}	6.07±2.11^{bh}	4.56±1.57^{c}
5% Bt	23.60±0.35^{b}	12.98±2.21^{b}	10.21±1.69^{ch}	132.48±22.51^{c}	27.22±6.25^{ae}	36.06±8.28^{dg}	0.16±0.01^{c}	0.21±0.02^{adh}	14.62±1.21^{c}	19.37±1.61^{a}
2% Ur	32.41±0.10^{a}	13.05±1.11^{b}	2.55±0.60^{d}	33.28±2.84^{d}	40.06±4.69^{cfg}	13.33±1.56^{e}	0.80±0.16^{d}	0.27±0.05^{aeh}	8.82±2.89^{dhi}	2.94±0.96^{dh}
5% Ur	24.14±1.56^{b}	12.25±1.36^{b}	7.10±0.47^{e}	86.97±9.64^{e}	32.16±5.25^{af}	27.97±4.57^{ag}	0.30±0.09^{a}	0.26±0.08^{ai}	8.73±0.85^{ehi}	7.60±0.74^{e}
2% Fr	28.83±0.11^{b}	13.24±1.46^{b}	2.05±0.55^{f}	27.1±3.00^{f}	40.10±2.41^{dg}	10.88±0.65^{f}	2.02±0.35^{e}	0.55±0.09^{bf}	3.78±1.01^{fhj}	1.03±0.27^{f}
5% Fr	27.65±0.75^{b}	12.65±1.30^{b}	5.69±0.54^{gi}	71.97±7.41^{a}	33.47±2.07^{ac}	24.09±1.49^{a}	0.49±0.05^{f}	0.35±0.04^{bgi}	3.21±0.44^{gj}	2.31±0.32^{gh}

Values represented are the mean ± SD of 6 animals per group. Abbreviations: C, control; Gt, green tea; Bt, black tea; Ur, unfermented rooibos; Fr, fermented rooibos. Means in the column followed by the same letter do not differ significantly. If the letter differs, then $p < 0.05$

6.2 BODY WEIGHT GAIN

In the female rats, the weekly weight gain did not differ significantly in the treated groups compared to the control ($p > 0.05$; figure 6.1) and also showed a progressive increase in weight gain with increasing time (week). In addition, the total weight gain of female rats exposed to the various types of tea and herbal infusion and concentrations for 21 days showed no significant difference compared to the control ($p > 0.05$; figure 6.2).

A tendency to dose- dependent decreased values in weight gain was observed in female rats exposed to black tea (ANOVA- trend analysis: P= 0.6764) and unfermented rooibos (ANOVA trend analysis: P= 0.6161). On the other hand, a tendency to an increased dose-dependent values in weight gain was observed in the group exposed to green tea (ANOVA trend analysis: P= 0.1673) and fermented rooibos (ANOVA trend analysis: P= 0.1059).

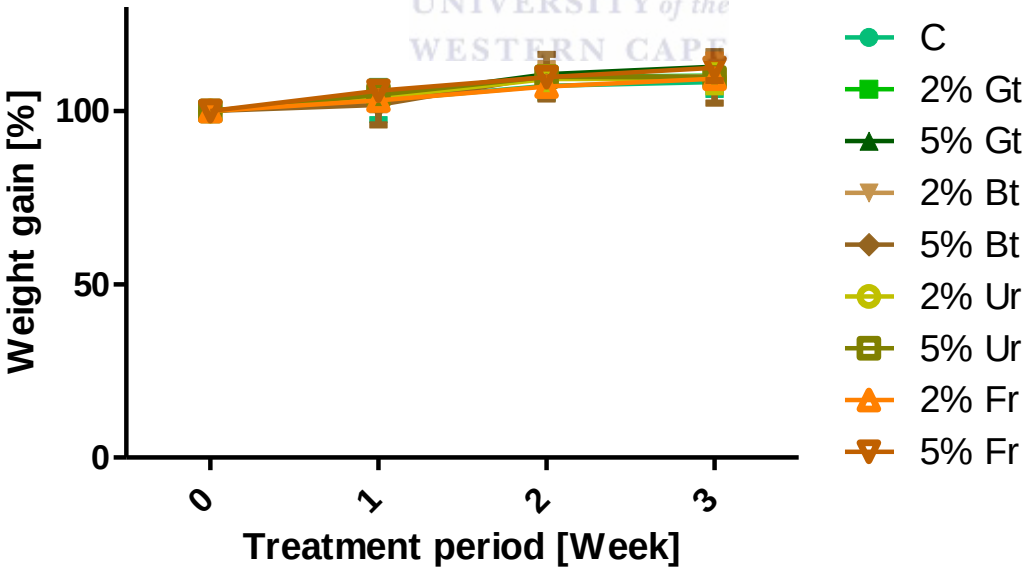


Figure 6.1: Weekly weight gain for the period of treatment in female rats exposed to *Camellia sinensis* and *Aspalathus linearis.*

Values represented are the mean ± SD of 6 animals per group. Abbreviations: C, control; Gt, green tea; Bt, black tea; Ur, unfermented rooibos; Fr, fermented rooibos.

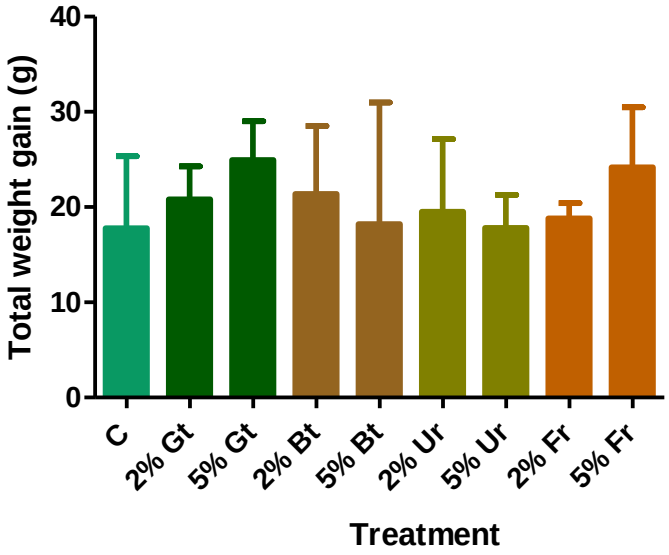


Figure 6.2: Effect of *Camellia sinensis* and *Aspalathus linearis* on total weight gain in female rats after 21 days exposure.

Values represented are the mean ± SD of 6 animals per group. Abbreviations: C, control; Gt, green tea; Bt, black tea; Ur, unfermented rooibos; Fr, fermented rooibos; Wk, week.

6.3 REPRODUCTIVE ORGAN, LIVER AND KIDNEY WEIGHTS

Absolute weight of ovaries in most treatment groups were similar to control ($p > 0.05$), however, groups exposed to either 5 % unfermented or 5% fermented rooibos significantly decreased it ($p < 0.05$; table 6.3). The relative weight of ovaries significantly reduced in the group exposed to 5 % fermented rooibos ($p < 0.05$), the rest of the treatment groups showed no significant difference compared to the control ($p > 0.05$) as shown in table 6.3.

The absolute weight of the uteri was significantly increased by unfermented rooibos (2% and 5%), and 2% fermented rooibos ($p < 0.05$). However, green tea (2% and 5 %), black tea (2% and 5%), and 5 % fermented rooibos did not differ significantly with the control ($p > 0.05$). In addition, the relative weight of the uterus was significantly increased by 5% unfermented

rooibos ($p < 0.01$), while the rest of the treatment groups showed no significant difference ($p > 0.05$) (table 6.3).

Absolute weight of liver showed no significant difference in the groups exposed to green tea (2% and 5%), 5% black tea, 5 % unfermented rooibos and fermented rooibos (2% and 5%) ($p > 0.05$), however, a significant increase was observed in the groups exposed to 2% unfermented rooibos or 2% black tea ($p < 0.05$). Relative weight of liver was significantly increased in groups exposed to 2% or 5% of either black tea or unfermented rooibos ($p < 0.05$), but showed no significant difference in the groups exposed to green tea or fermented rooibos ($p > 0.05$) (table 6.3).

Furthermore, no significant difference in the absolute weight of kidney was observed in animals exposed to either *Camellia sinensis* or *Aspalathus linearis* ($p > 0.05$). On the other hand, 5% black tea significantly increased the relative weight of female rat kidney ($p < 0.05$), while the rest of the treatment groups showed no significant difference ($p > 0.05$) (table 6.3).

Table 6.3: Weight of reproductive organs, liver and kidneys after 21 days treatment in female rats

Organ/ Treatment	Control	2% Gt	5% Gt	2% Bt	5% Bt	2% Ur	5% Ur	2% Fr	5% Fr
Ovaries									
Absolute	0.19±0.03	0.20±0.01	0.18±0.02	0.15±0.07	0.18±0.03	0.18±0.02	0.15±0.02*	0.16±0.01	0.13±0.02#
Relative	0.08±0.01	0.09±0.01	0.08±0.01	0.07±0.00	0.08±0.01	0.08±0.01	0.07±0.01	0.07±0.01	0.06±0.01*
Uteri									
Absolute	0.60±0.14	0.48±0.06	0.75±0.26	0.75±0.23	0.82±0.39	0.95±0.35*	0.81±0.10*	0.80±0.04*	0.51±0.06
Relative	0.26±0.07	0.24±0.05	0.34±0.12	0.32±0.09	0.38±0.17	0.40±0.14	0.40±0.05#	0.34±0.06	0.23±0.05
Liver									
Absolute	10.60±0.68	10.80±0.92	10.88±0.80	11.62±0.66*	10.87±0.89	11.73±0.60*	10.04±0.75	10.65±0.72	10.25±1.32
Relative	4.63±0.25	4.84±0.32	4.97±0.30	4.93±0.19*	5.12±0.38*	5.07±0.28*	4.98±0.24*	4.81±0.27	4.67±0.22
Kidneys									
Absolute	2.16±0.22	2.17±0.05	2.20±0.16	2.28±0.09	2.20±0.18	2.17±0.13	1.92±0.11	2.02±0.19	2.07±0.16
Relative	0.95±0.08	0.97±0.04	1.01±0.08	0.97±0.04	1.03±0.03*	0.94±0.04	0.95±0.05	0.91±0.05	0.95±0.07

Values represented are the mean ± SD of 6 animals per group. Abbreviations: C, control; Gt, green tea; Bt, black tea; Ur, unfermented rooibos; Fr, fermented rooibos; Wk, week; *, $p < 0.05$; #, $p < 0.01$ compared to control. Relative organ weight, organ weight/final body weight x 100

6.4 FOLLICLE STIMULATING AND LUTEINIZING HORMONE

Follicle stimulating hormone level in female rats exposed to either *Camellia sinensis* or *Aspalathus linearis* for 21 d showed no significant difference compared to the control ($p > 0.05$; figure 6.3). A tendency to increased dose-dependent values was observed in groups exposed to green tea (ANOVA-trend analysis: P=0.1689) or black tea (ANOVA-trend analysis: P=0.2736).

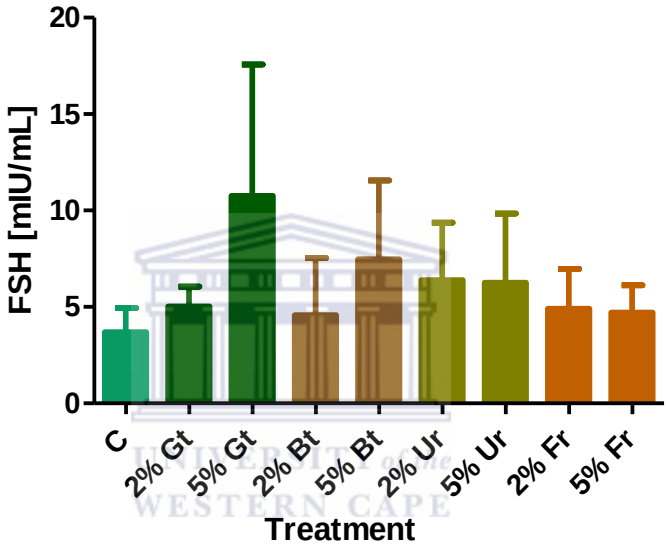


Figure 6.3: Effect of *Camellia sinensis* and *Aspalathus linearis* on follicle stimulating hormone in female rats after 21 days exposure.

Values represented are the mean ± SD of 6 animals per group. Abbreviations: C, control; Gt, green tea; Bt, black tea; Ur, unfermented rooibos; Fr, fermented rooibos.

The level of luteinizing hormone in female rats exposed to either *Camellia sinensis* or *Aspalathus linearis* for 21 d (figure 6.4), showed that 2% and 5% green tea significantly decreased its level ($p < 0.05$). In addition, 5% black tea was also shown to significantly decrease the level of LH in female rats ($p < 0.01$). Unfermented rooibos (2% and 5%), fermented rooibos (2% and 5%), and 2%

black tea showed no significant difference in the level of LH when compared to the control ($p > 0.05$). A tendency to decreased dose- dependent values was observed in the groups exposed to green tea (ANOVA- trend analysis: P=0.5649), black tea (ANOVA- trend analysis: P=0.1546) and unfermented rooibos (ANOVA- trend analysis: P=0.9804). Fermented rooibos showed a tendency to increased dose- dependent values (ANOVA- trend analysis: P=0.7826).

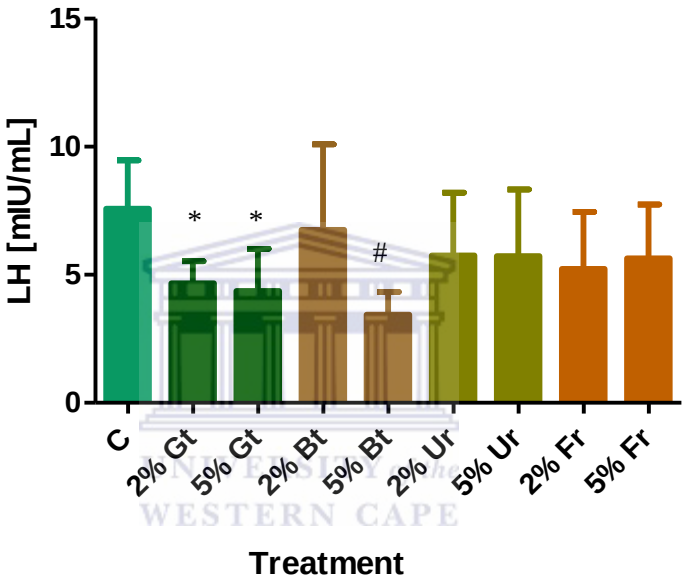


Figure 6.4: Effect of *Camellia sinensis* and *Aspalathus linearis* on Luteinizing hormone in female rats after 21 days exposure.

Values represented are the mean ± SD of 6 animals per group. Abbreviations: C, control; Gt, green tea; Bt, black tea; Ur, unfermented rooibos; Fr, fermented rooibos; *, $p < 0.05$; $^{\#}$, $p < 0.01$ compared to control.

6.5 CREATININE ACTIVITY

Creatinine activity in female rat serum showed no significant difference compared to the control ($p > 0.05$), except for 5 % fermented rooibos which significantly increased it ($p < 0.05$) as shown in figure 6.5. A tendency to increased dose-dependent values was observed in green tea (ANOVA-trend analysis: P=0.4040), black tea (ANOVA- trend analysis: P=0.0646), unfermented rooibos (ANOVA-trend analysis: P=0.4621), and fermented rooibos (ANOVA- trend analysis: P= 0.8743).

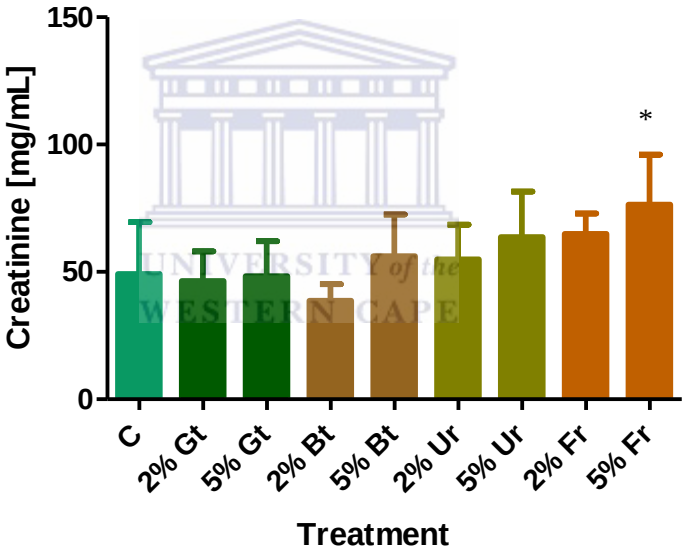


Figure 6.5: Effect of *Camellia sinensis* and *Aspalathus linearis* on creatinine activity in female rats after 21 days exposure.

Values represented are the mean ± SD of 6 animals per group. Abbreviations: C, control; Gt, green tea; Bt, black tea; Ur, unfermented rooibos; Fr, fermented rooibos; Wk, week; *, $p < 0.05$ compared to control.

6.6 ALANINE TRANSAMINASE ACTIVITY (ALT)

Alanine transaminase (ALT) activity in serum of female rat exposed to either *Camellia sinensis* or *Aspalathus linearis* was shown to decrease significantly in the groups exposed to either 5% green tea, 2% and 5% black tea or 2% unfermented rooibos ($p < 0.05$). While in 2% green tea, 5% unfermented rooibos, 2% and 5 % fermented rooibos there was no significant difference compared to the control ($p > 0.05$) (figure 6.6). ANOVA trend analysis showed a tendency to a dose- dependent decrease in ALT activity in serum of female rats exposed to black tea (P=0.0433). On the other hand, a tendency to a non-statistical dose-dependent increase in ALT activity was observed in the groups exposed to either unfermented rooibos (ANOVA-trend analysis: P=0.921) or fermented rooibos (ANOVA- trend analysis: P=0.2721).

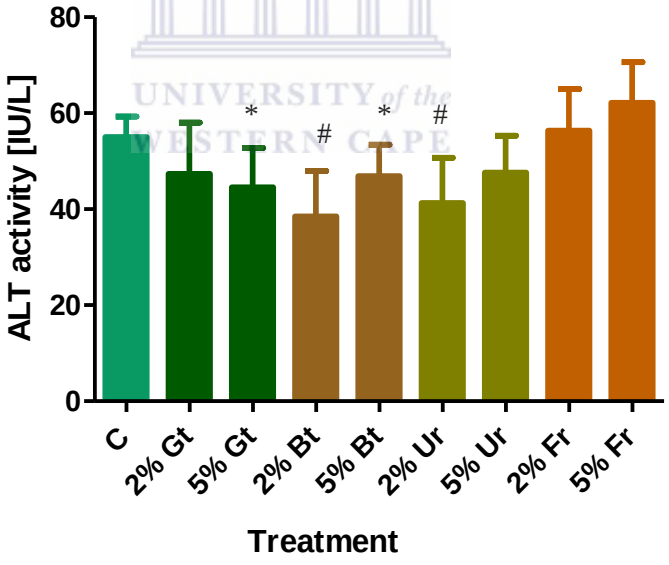


Figure 6.6: Effect of *Camellia sinensis* and *Aspalathus linearis* on alanine transaminase (ALT) activity in female rats after 21 days exposure.

Values represented are the mean ± SD of 6 animals per group. Abbreviations: C, control; Gt, green tea; Bt, black tea; Ur, unfermented rooibos; Fr, fermented rooibos; Wk, week; *, $p < 0.05$; $^{\#}$, $p < 0.01$ compared to control.

6.7 ASPARTATE TRANSAMINASE ACTIVITY (AST)

Aspartate transaminase (AST) activity in serum of female rats exposed to either *Camellia sinensis* or *Aspalathus linearis* for 21 days showed no significant difference compared to the control ($p > 0.05$) except 5 % fermented rooibos which significantly increased its activity ($p < 0.01$) (figure 6.7). A tendency to decreased dose- dependent values was observed in the groups exposed to green tea (ANOVA- trend analysis: P=0.0008). On the other hand, ANOVA- trend analysis, showed a non-statistical increased dose dependent values in the groups exposed to unfermented rooibos (P=0.1650) or fermented rooibos (P=0.1288).

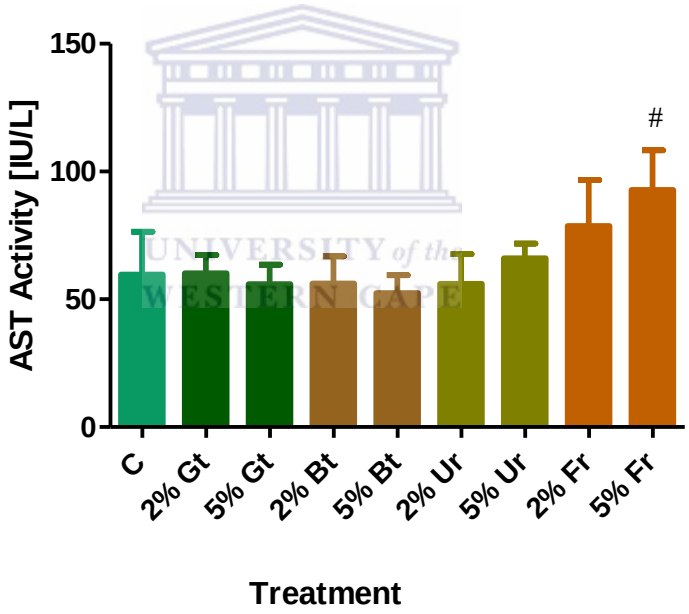


Figure 6.7: Effect of *Camellia sinensis* and *Aspalathus linearis* on aspartate transaminase (AST) activity in female rats after 21 days exposure.

Values represented are the mean ± SD of 6 animals per group. Abbreviations: C, control; Gt, green tea; Bt, black tea; Ur, unfermented rooibos; Fr, fermented rooibos; Wk, week; $^{\#}$, $p < 0.01$ compared to control.

6.8 FERRIC REDUCING ANTIOXIDANT POWER (FRAP) IN FEMALE RAT SERUM

Sera of female Wistar rats exposed to either *Camellia sinensis* or *Aspalathus linearis* for 21 days showed no significant difference in the FRAP when compared to the control ($p > 0.05$; figure 6.8).

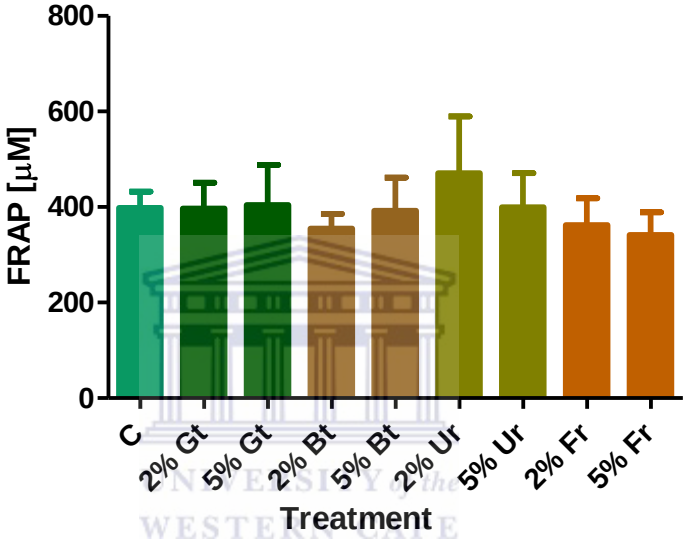


Figure 6.8: Effect of *Camellia sinensis* and *Aspalathus linearis* on ferric reducing antioxidant power (FRAP) in female rats after 21 days exposure.

Values represented are the mean ± SD of 6 animals per group. Abbreviations: C, control; Gt, green tea; Bt, black tea; Ur, unfermented rooibos; Fr, fermented rooibos; Wk, week.

6.9 BIOCHEMICAL ASSAYS ON KIDNEY AND LIVER

6.9.1 Lipid peroxidation (MDA)

Female rats exposed to either *Camellia sinensis* or *Aspalathus linearis* for 21 d showed no significant difference in the level of lipid peroxidation (LPO) measured as thiobarbituric acid reactive substances (TBARS) in the kidney compared to the controls ($p > 0.05$) (figure 6.9). Green tea was shown to have a tendency to decreased dose- dependent values (ANOVA- trend analysis: P=0.5057). On the other hand, a tendency to a dose- dependent increased values was observed in the groups exposed to unfermented rooibos (ANOVA-trend analysis: P=0.1137), and fermented rooibos (ANOVA-trend analysis: P=0.1790).

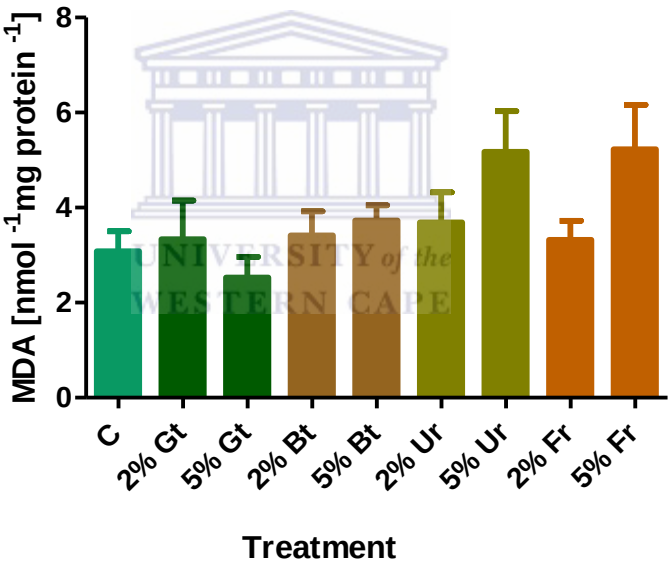


Figure 6.9: Effect of *Camellia sinensis* and *Aspalathus linearis* on lipid peroxidation (MDA) in female rat kidney.

Values represented are the mean ± SD of 6 animals per group. Abbreviations: C, control; Gt, green tea; Bt, black tea; Ur, unfermented rooibos; Fr, fermented rooibos.

MDA levels in female liver exposed to *Camellia sinensis* or *Aspalathus linearis* for 21 d did not differ significantly from the control ($p > 0.05$; figure 6.10). However, a tendency to a dose- dependent decrease values was observed in groups exposed to green tea (ANOVA-trend analysis: P=0.4393), black tea (ANOVA-trend analysis: P=0.0814), unfermented rooibos (ANOVA-trend analysis: P=0.2325) and fermented rooibos (ANOVA-trend analysis: P=0.0109).

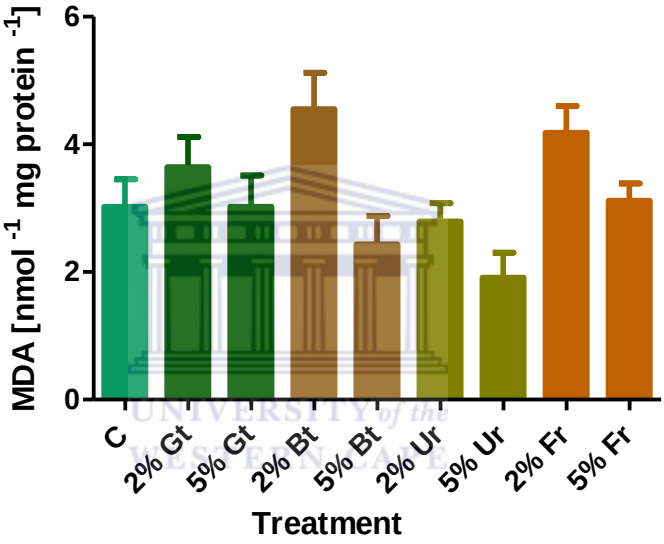


Figure 6.10: Effect of *Camellia sinensis* and *Aspalathus linearis* on lipid peroxidation (MDA) in female rat liver.

Values represented are the mean ± SD of 6 animals per group. Abbreviations: C, control; Gt, green tea; Bt, black tea; Ur, unfermented rooibos; Fr, fermented rooibos.

6.9.2 Glutathione (GSH)

GSH level in female rat kidneys exposed to *Camellia sinensis* or *Aspalathus linearis* showed no significant difference compared to the control group ($p > 0.05$; figure 6.11). However, unfermented and fermented rooibos showed a tendency to decreased GSH level dose- dependently (ANOVA- trend analysis: P=0.1213; P=0.0450 respectively).

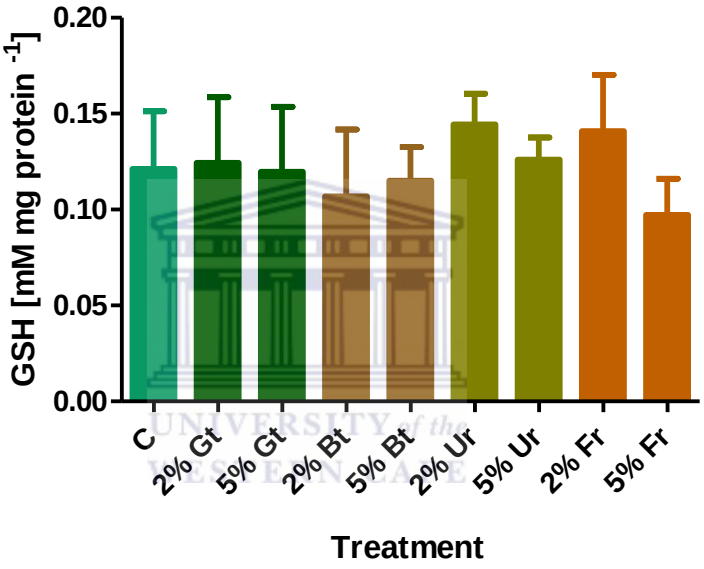


Figure 6.11: Effect of *Camellia sinensis* and *Aspalathus linearis* on glutathione (GSH) in female rat kidney.

Values represented are the mean ± SD of 6 animals per group. Abbreviations: C, control; Gt, green tea; Bt, black tea; Ur, unfermented rooibos; Fr, fermented rooibos.

GSH level in the liver of female rats exposed to *Camellia sinensis* or *Aspalathus linearis* for 21 d showed no significant difference compared to the control ($p > 0.05$; figure 6.12). However, a dose- dependent decreased GSH values was

observed in the groups exposed to green tea (ANOVA- trend analysis: 0.1014) and unfermented rooibos (ANOVA- trend analysis: P=0.2879).

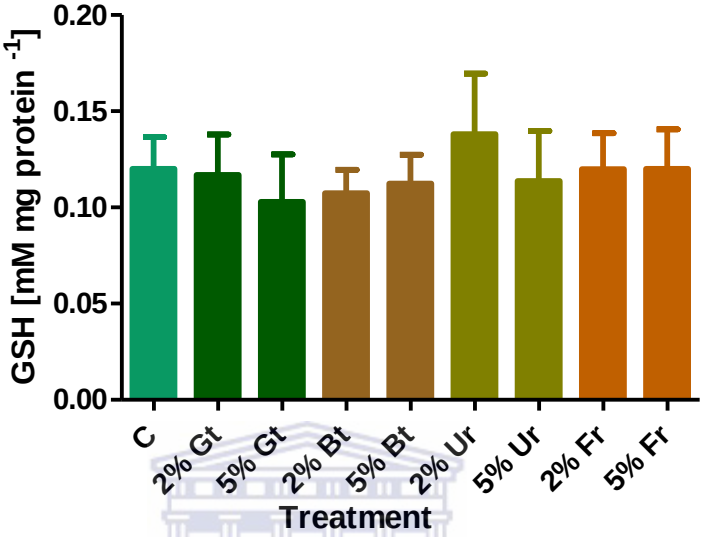


Figure 6.12: Effect of *Camellia sinensis* and *Aspalathus linearis* on glutathione (GSH) in female rat liver.

Values represented are the mean ± SD of 6 animals per group. Abbreviations: C, control; Gt, green tea; Bt, black tea; Ur, unfermented rooibos; Fr, fermented rooibos.

6.9.3 Catalase Activity (CAT)

Catalase activity in kidney of female rats exposed to green tea, black tea or fermented rooibos was not significantly different from the control ($p > 0.05$; figure 6.13). However, a significant increase in its activity was observed in the kidneys of female rats exposed to 2% unfermented rooibos. In addition, a dose-dependent reduced values was also observed in the groups treated with unfermented rooibos (ANOVA- trend analysis: P= 0.0009).

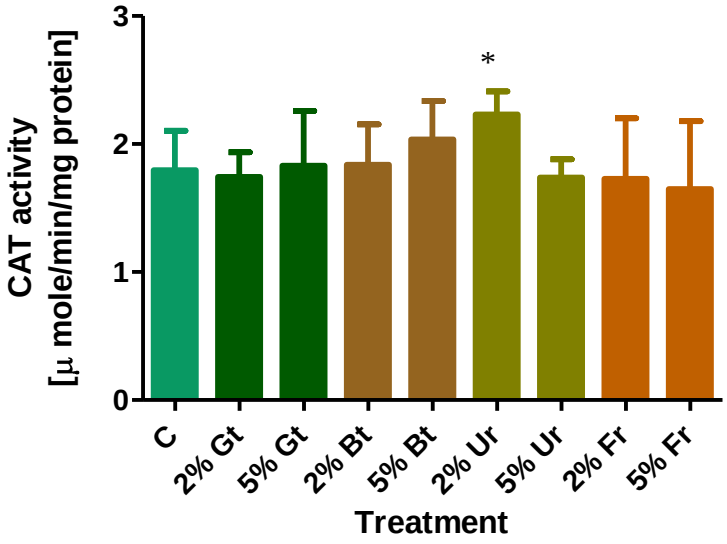


Figure 6.13: Effect of *Camellia sinensis* and *Aspalathus linearis* on catalase activity in female rat kidney.

Values represented are the mean ± SD of 6 animals per group. Abbreviations: C, control; Gt, green tea; Bt, black tea; Ur, unfermented rooibos; Fr, fermented rooibos; *, $p < 0.05$ compared to control.

Liver of female rats exposed to green tea, fermented rooibos, 5% black tea or 2% unfermented rooibos showed no significant difference in the level of catalase activity compared to the control ($p > 0.05$; figure 6.14). However, 2% black tea was shown to significantly enhance its activity and a dose - dependent decrease in activity was observed in the black tea treated group (ANOVA- trend analysis: P= 0.0142). Unfermented rooibos (5%), on the other hand significant increased the activity of catalase in the female rat liver ($p < 0.05$).

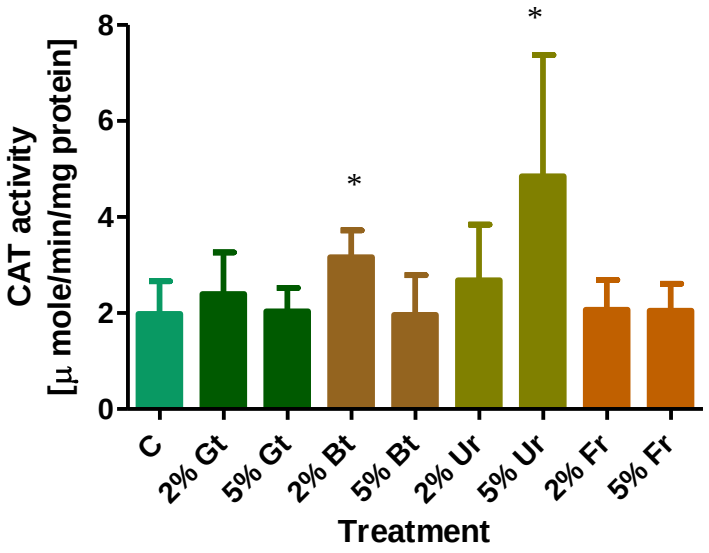


Figure 6.14: Effect of *Camellia sinensis* and *Aspalathus linearis* on catalase activity in female rat liver.

Values represented are the mean ± SD of 6 animals per group. Abbreviations: C, control; Gt, green tea; Bt, black tea; Ur, unfermented rooibos; Fr, fermented rooibos; *, $p < 0.05$ compared to the control.

6.9.4 Superoxide dismutase activity (SOD)

The kidneys of female rats exposed to either *Camellia sinensis* or *Aspalathus linearis* revealed no significant difference in the activity of superoxide dismutase ($p > 0.05$).

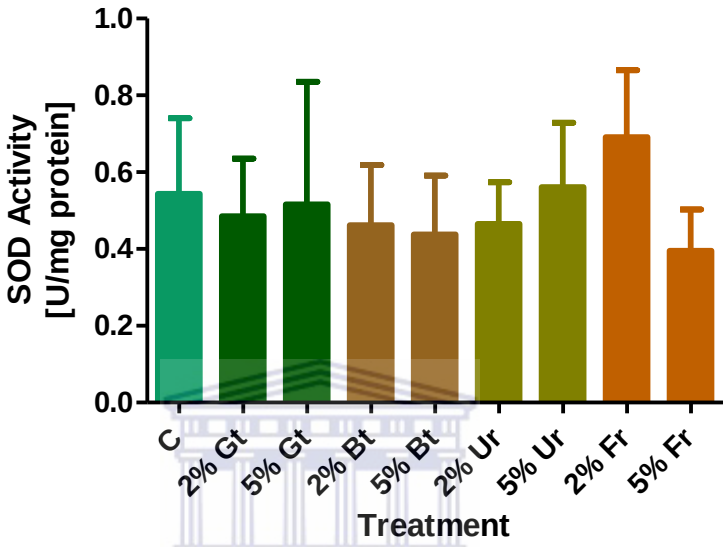


Figure 6.15: Effect of *Camellia sinensis* and *Aspalathus linearis* on superoxide dismutase activity in female rat kidney.

Values represented are the mean ± SD of 6 animals per group. Abbreviations: C, control; Gt, green tea; Bt, black tea; Ur, unfermented rooibos; Fr, fermented rooibos.

Superoxide dismutase activity in the liver of female rats exposed to either *Camellia sinensis* or *Aspalathus linearis* also revealed no significant difference compared to the control ($p > 0.05$).

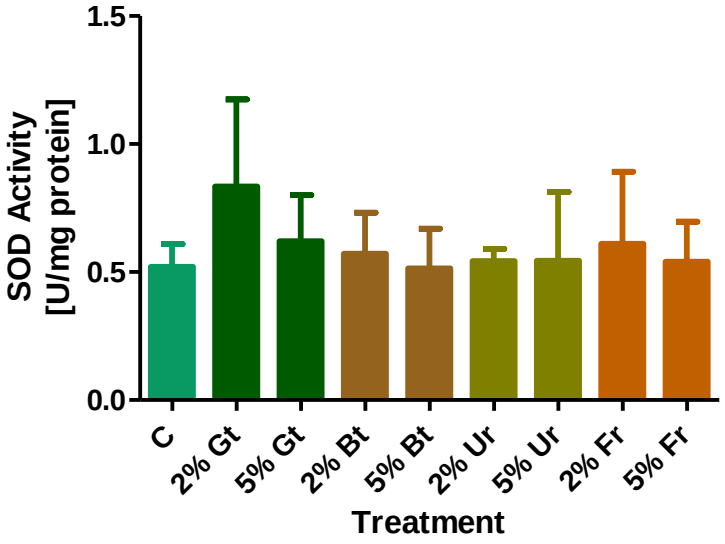


Figure 6.15: Effect of *Camellia sinensis* and *Aspalathus linearis* on superoxide dismutase activity in female rat liver.

Values represented are the mean ± SD of 6 animals per group. Abbreviations: C, control; Gt, green tea; Bt, black tea; Ur, unfermented rooibos; Fr, fermented rooibos.

6.10 HISTOLOGY OF THE REPRODUCTIVE ORGANS, KIDNEY AND LIVER IN THE FEMALE RATS

At the end of 21d exposure to either *Camellia sinensis* or *Aspalathus linearis*, the ovaries of the female rats contained follicles at all stages of development and had normal appearance of corpus luteum (CL) in all treated group with marke central fibrous tissue formation, which was comparable to the control group. In addition, there was no significant sign of edema, cystic follicles, or retained oocytes (figure 6.16).

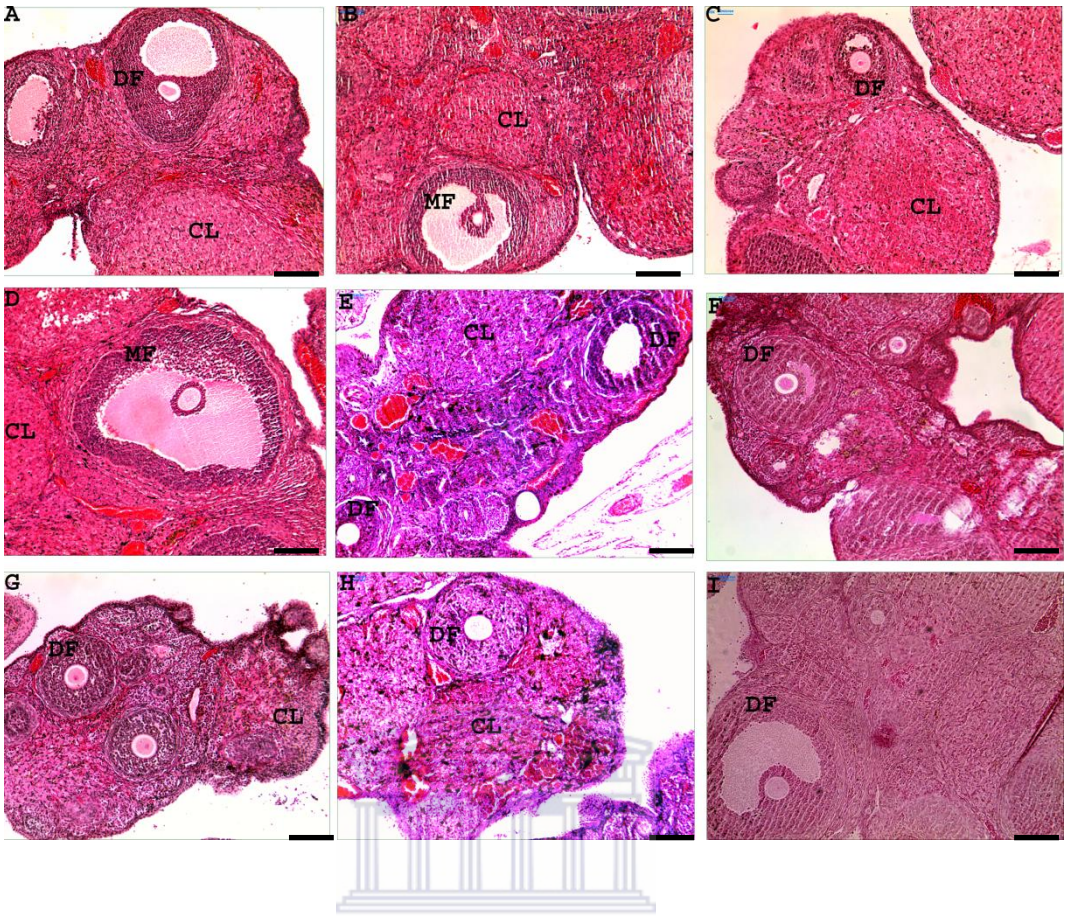


Figure 6.16: Morphology of the female rat ovaries after exposure to either *Camellia sinensis* or *Aspalathus linearis* after 21 days. (A) control (B) 2% green tea (C) 5% green tea (D) 2% black tea (E) 2% unfermented rooibos (F) 5% unfermented rooibos (G) 5% black tea (H) 2% fermented rooibos (I) 5% fermented rooibos; CL, corpus luteum; DF, developing follicle; MF, mature follicle; Bar = 50 µm.

The uterus of the female rats on the day of proestrus exposed to either *Camellia sinensis* or *Aspalathus linearis* after 21 days showed normal morphology in the endometrium with the presence of endometrial glands, myometrium and perimetrium (figure 6.17).

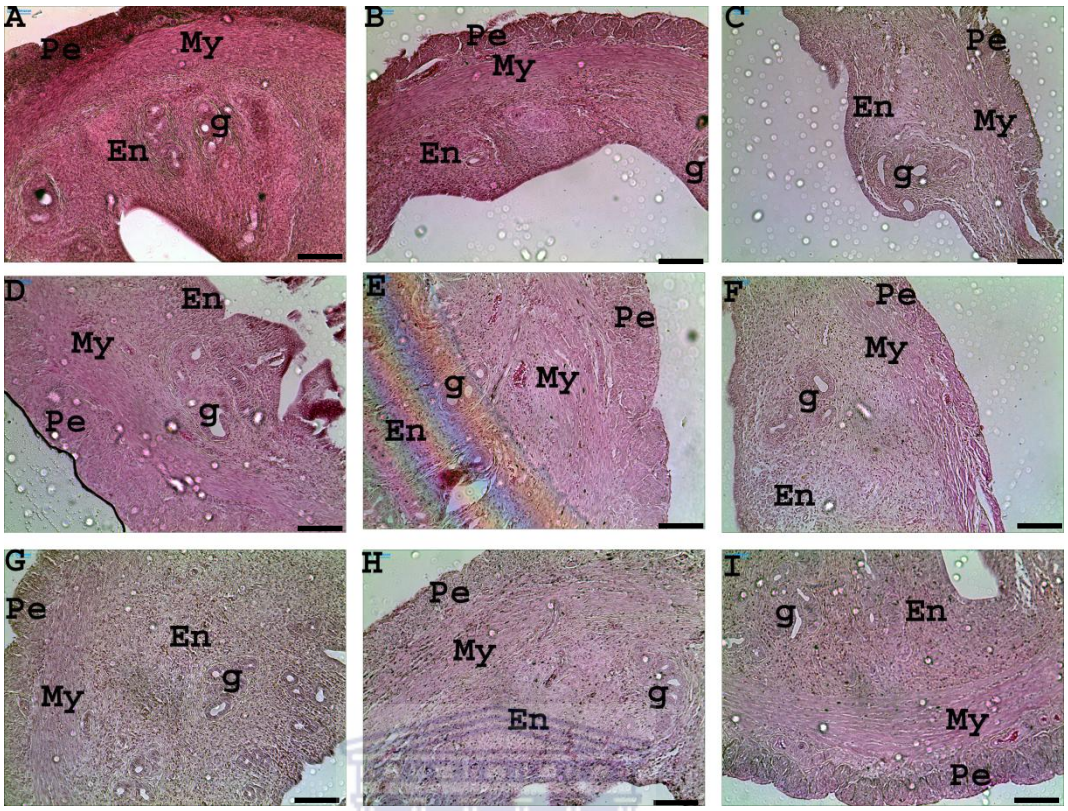


Figure 6.17: Morphology of the female rat uterus after exposure to either *Camellia sinensis* or *Aspalathus linearis* after 21 days. (A) control (B) 2% green tea (C) 5% green tea (D) 2% black tea (E) 2% unfermented rooibos (F) 5% unfermented rooibos (G) 5% black tea (H) 2% fermented rooibos (I) 5% fermented rooibos; Pe, perimetrium; My, myometrium; En, endometrium; g, endometrial gland; Bar = 50 µm.

In the kidney also of female rats exposed to either *Camellia sinensis* or *Aspalathus linearis*, a normal architecture in all treated rats compared to the control was observed (figure 6.18).

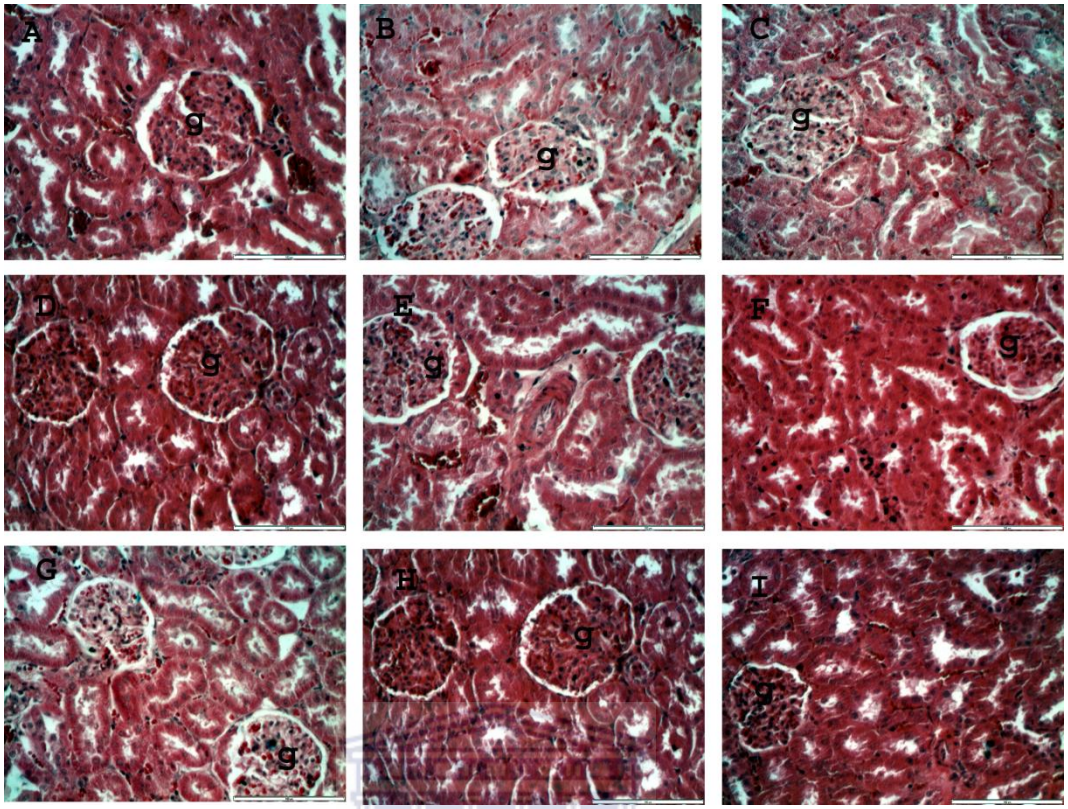


Figure 6.18: Morphology of the female rat kidney after exposure to either *Camellia sinensis* or *Aspalathus linearis* after 21 days. (A) control (B) 2% green tea (C) 5% green tea (D) 2% black tea (E) 2% unfermented rooibos (F) 5% unfermented rooibos (G) 5% black tea (H) 2% fermented rooibos (I) 5% fermented rooibos; g, glomerulus; Bar = 100 µm.

In addition, after the 21d exposure of female rats to either *Camellia sinensis* or *Aspalathus linearis*, the architecture of the liver showed no obvious alteration compared to the control (figure 6.19).

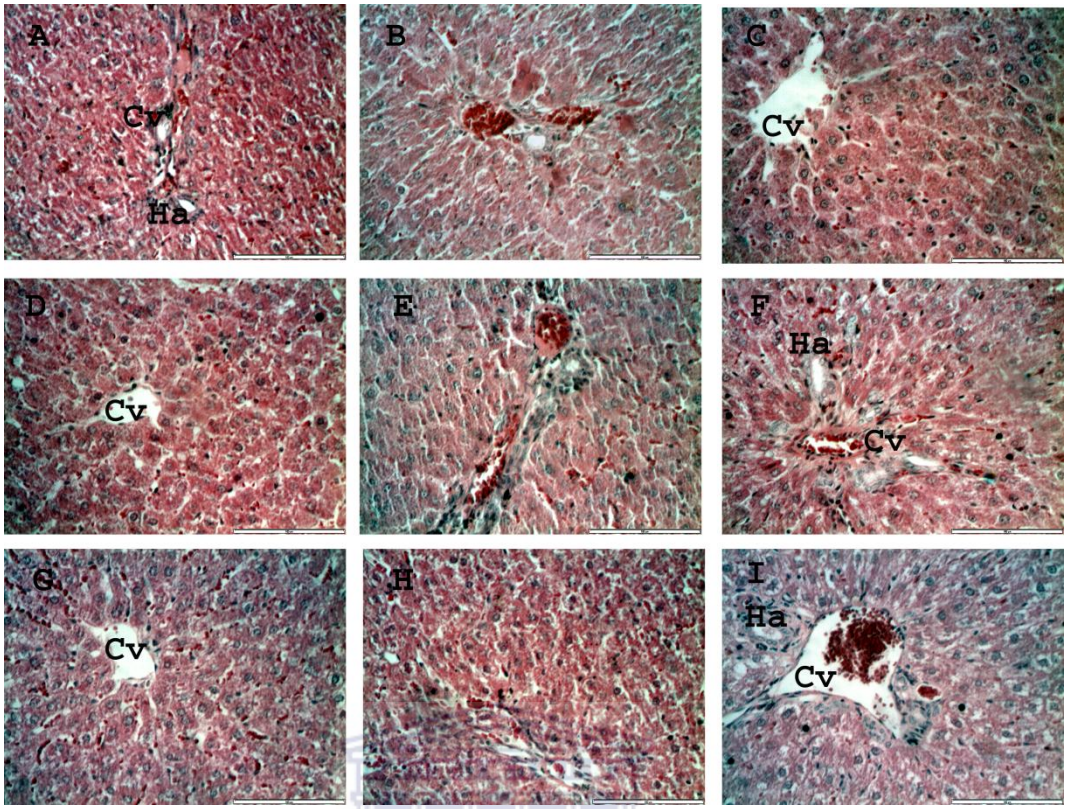


Figure 6.19: Morphology of the female rat liver after exposure to either *Camellia sinensis* or *Aspalathus linearis* after 21 days. (A) control (B) 2% green tea (C) 5% green tea (D) 2% black tea (E) 2% unfermented rooibos (F) 5% unfermented rooibos (G) 5% black tea (H) 2% fermented rooibos (I) 5% fermented rooibos; Cv, central vein; Ha, hepatic artery; Bar = 100 µm.

CHAPTER SEVEN

EFFECT OF CAMELLIA SINENSIS AND ASPALATHUS LINEARIS ON MALE SEXUAL BEHAVIOUR AND FERTILITY

7.1 MALE SEXUAL BEHAVIOUR

The time it took each male to reach the female in all treatment groups was similar to the control ($p > 0.05$); however, 2% green tea and unfermented rooibos tended to require less time (figure 7.1). The number of snifs by male rats exposed to unfermented rooibos and 2% fermented rooibos increased significantly ($p < 0.05$) while male rats exposed to green tea, black tea and 5% fermented rooibos was similar to the control ($p > 0.05$) (figure 7.2). Mount frequency was increased significantly by 5% green tea, 2% black tea, 2% unfermented rooibos and fermented rooibos ($p < 0.05$), and showed no significant difference in the groups exposed to 2% green tea, 5% black tea, and 5% unfermented rooibos ($p > 0.05$) compared to the control (figure 7.3). Mount latency in all treatment groups was similar to the control; however, groups exposed to 2% black tea and 2% unfermented rooibos tend to have a decreased value compared to the control ($p > 0.05$) (figure 7.4).

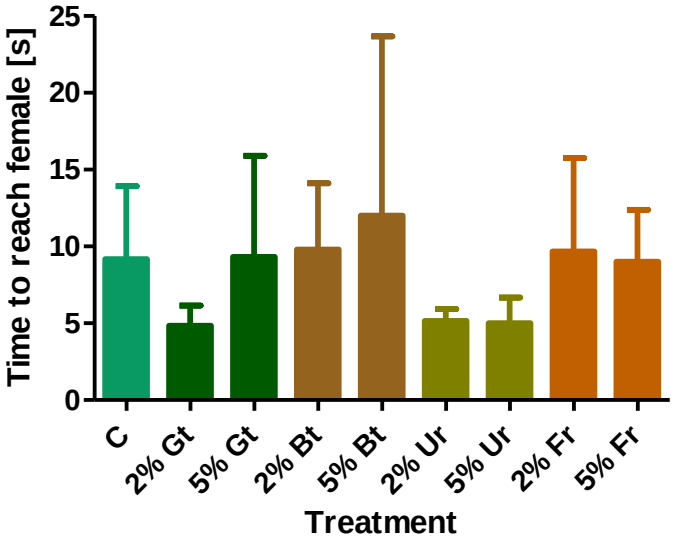


Figure 7.1: Time to reach female after exposure to *Camellia sinensis* or *Aspalathus linearis.*

Values represented are the mean ± SD of 6 animals per group. Abbreviations: C, control; Gt, green tea; Bt, black tea; Ur, unfermented rooibos; Fr, fermented rooibos.

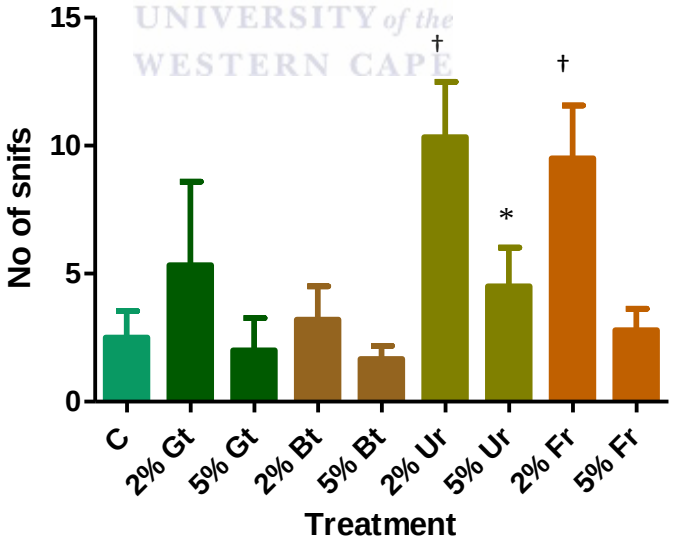


Figure 7.2: No of snifs in male rats exposed to *Camellia sinensis* or *Aspalathus linearis.*

Values represented are the mean ± SD of 6 animals per group. Abbreviations: No of snifs, number of snifs after 3 min of pairing with female rats; C, control; Gt, green tea; Bt, black tea; Ur, unfermented rooibos; Fr, fermented rooibos; *, $p < 0.05$;$^{\dagger}p < 0.001$ compared to control.

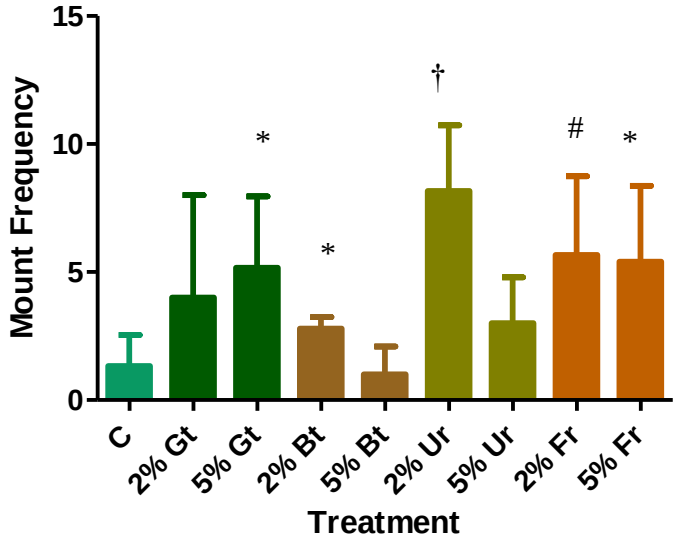


Figure 7.3: Mount frequency of male rats exposed to *Camellia sinensis* or *Aspalathus linearis.*

Values represented are the mean ± SD of 6 animals per group. Abbreviations: C, control; Gt, green tea; Bt, black tea; Ur, unfermented rooibos; Fr, fermented rooibos; *, $p < 0.05$; $^{\#}$, $p < 0.01$; †, $p < 0.001$ compared to control.

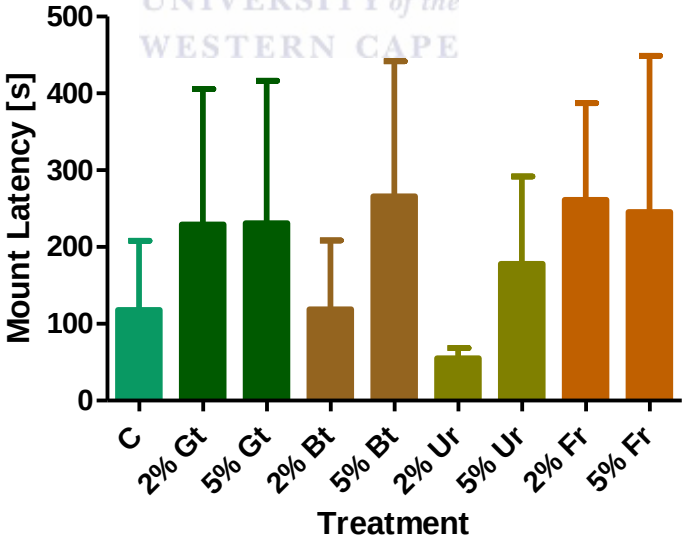


Figure 7.4: Mount latency of male rats exposed to *Camellia sinensis* or *Aspalathus linearis.*

Values represented are the mean ± SD of 6 animals per group. Abbreviations: C, control; Gt, green tea; Bt, black tea; Ur, unfermented rooibos; Fr, fermented rooibos.

7.2 MALE FERTILITY

From table 7.1, the index of libido in male rats exposed to either *Camellia sinensis* or *Aspalathus linearis* was comparable to the control group. Quantal pregnancy (percentage of pregnant female rats per number mated), implantation index (percentage of total implantation obtained per number mated) and fertility index (percentage of pregnant female rats per number paired) was decreased by green tea and 2% unfermented rooibos, 5% unfermented rooibos and fermented rooibos produced a similar result, while black tea increased these parameters. Green tea and 2% unfermented rooibos had a 100% pre- implantation loss (% of pregnancy loss before implantation) and 0% post implantation loss (% of pregnancy loss after implantation), this as a result of the no pregnancy outcome. The pre-implantation loss in the groups exposed to 5% unfermented rooibos and fermented rooibos was similar to the control, while black tea decreased it. In addition, post-implantation loss in black tea was similar to the control, while 5% unfermented rooibos, and fermented rooibos decreased it.

Table 7.1: Fertility and reproductive performance of male rats exposed to *Camellia sinensis* or *Aspalathus linearis*

Parameters (%)	C	2% Gt	5% Gt	2% Bt	5% Bt	2% Ur	5% Ur	2% Fr	5% Fr
Index of libido	100	100	100	100	100	100	100	100	100
Quantal pregnancy	11.1 (1/9)	0 (0/8)	0 (0/9)	50 (4/8)	55.6 (5/9)	0 (0/8)	11.1 (1/9)	12.5 (1/8)	12.5 (1/8)
Implantation index	44.4	0	0	662.5	666.7	0	166.7	150	137.5
Pre-implantation loss	96.0	100	100	50.8	53.8	100	88.9	91.2	94.5
Post-implantation loss	25	0	0	24.2	22.0	0	0	8.3	18.2
Fertility index	8.3	0	0	50	58.3	0	8.3	8.3	10

Abbreviations: C, control; Gt, green tea, Bt, black tea; Ur, unfermented rooibos; Fr, fermented rooibos.

CHAPTER EIGHT

RESULT OF THE IN VITRO EFFECT OF CAMELLIA SINENSIS AND ASPALATHUS LINEARIS ON TM3 LEYDIG CELL VIABILITY AND TESTOSTERONE PRODUCTION

8.1 EFFECT OF CAMELLIA SINENSIS AND ASPALATHUS LINEARIS ON TM3 LEYDIG CELL VIABILITY

In order to determine the effect of tea and the herbal infusion on cell viability, MTT assay was used to investigate the activity of mitochondrial dehydrogenase on TM3 cells. From figure 8.1, a significant decrease in mitochondrial dehydrogenase activity was observed in cells exposed to 4% DMSO ($p < 0.05$), which served as the positive control.

The activity of mitochondrial dehydrogenase in non-stimulated TM3 cells exposed to green tea was shown to increase in a dose- dependent manner, however, at 0.5% there was a significant decrease ($p < 0.05$) compared to the negative control. In addition, the 0.5% and 0.1% stimulated group were similar to the negative control ($p > 0.05$), while a significant decrease was observed in the cells exposed to either 0.025% or 0.5% ($p < 0.05$). On the overall, the level of mitochondrial dehydrogenase activity was shown to significantly increase in the non- stimulated cells compared to the stimulated ones ($p < 0.05$) (figure 8.1).

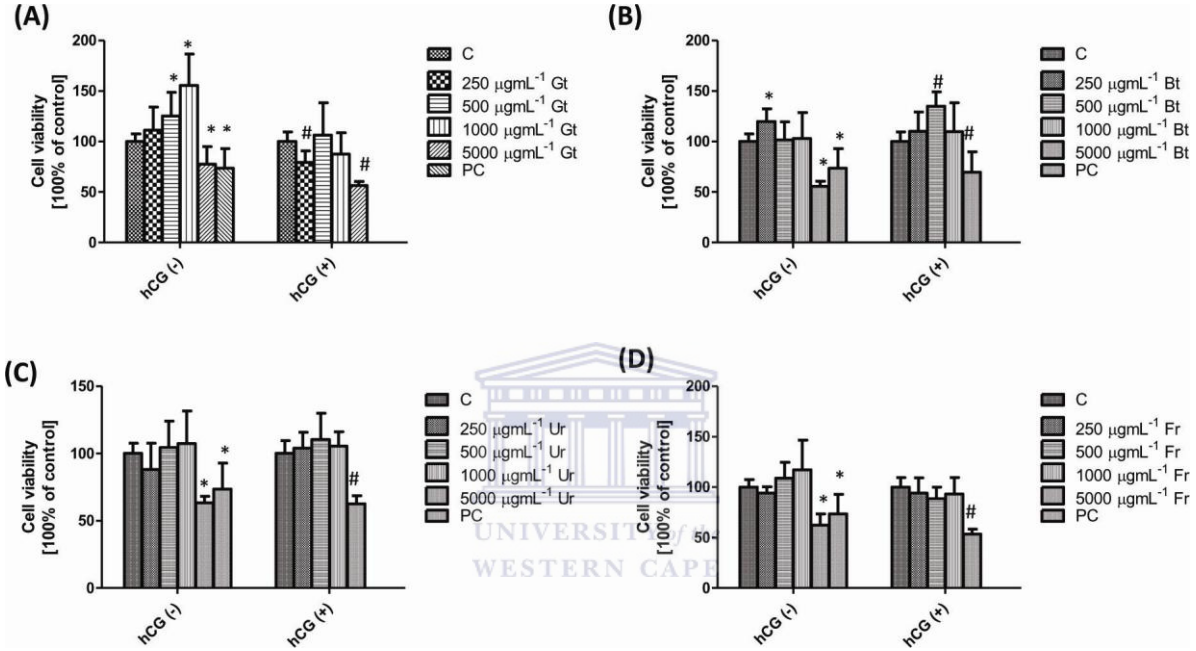


Figure 8.1: Viability of TM3 Leydig cells exposed to *Camellia sinensis* or *Aspalathus linearis* in the presence or absence of HCG. Values represented are the mean ± SD of six determinations repeated in two independent experiments. Abbreviations: (A) green tea; (B) black tea; (C) unfermented rooibos; (D) fermented rooibos; C, control; Gt, green tea; Bt, black tea;Ur, unfermented rooibos; Fr, fermented rooibos; *, $p < 0.05$, when non - stimulated treated groups are compared to the negative control; $^{\#}$, $p < 0.05$, when stimulated treated groups are compared with the positive control.

Black tea increased and decreased the activity of mitochondrial dehydrogenase in the 0.025 and 0.5 % non - stimulated cells respectively ($p < 0.05$), while at 0.05 and 0.1% , there was no significant difference compared to the control. In general, a dose- dependent decrease was observed ($p > 0.05$) in the non - stimulated treated cells. In the stimulated groups, a tendency to increased values ($p > 0.05$) or significant increase ($p < 0.05$) was observed in 0.025 to 0.1 % groups. However, a significant decrease in the enzyme activity was observed in 0.5 % of black tea. On the overall, a tendency to enhanced values or significant increase was observed in the stimulated treated groups compared to the non – stimulated treated groups excluding 0.025%, where the reverse was observed (figure 8.1).

Unfermented rooibos, showed a tendency to a dose- dependent increase in the activity of mitochondrial dehydrogenase in both the non – stimulated and stimulated groups (figure 8.1). However, a significant decrease in the enzyme activity was observed at 0.5% in non – stimulated and stimulated group ($p < 0.05$). A more or less similar result was observed when comparing the stimulated with the non – stimulated treated groups ($p > 0.05$).

Fermented rooibos showed no significant effect on the activity of mitochondrial dehydrogenase in most of the non – stimulated treated group, however, a tendency to a dose – dependent increased values was observed with a significant decrease at 0.5 % ($p < 0.05$; figure 8.1). The stimulated group at 0.025 and 0.1% was similar to the control ($p > 0.05$), while at 0.05 and 0.5 %; there was a significant decrease ($p < 0.05$). On the overall, the non - stimulated group tend to have higher enzyme activity compared to the stimulated group.

Taken together, the highest concentration of *Aspalathus linearis* and *Camellia sinensis* (0.5%) is cytotoxic for TM3 Leydig cells.

8.2 EFFECT OF CAMELLIA SINENSIS AND ASPALATHUS LINEARIS ON THE MORPHOLOGY OF TM3 LEYDIG CELLS

Under the phase contrast microscope, the non- stimulated or stimulated control group revealed the presence of normal dividing cells, while cells exposed to 4% DMSO (positive control) showed characteristic changes in the morphology of the cells which included cell shrinkage and irregular shape (figures 8.3- 8.10). From figure 8.3 and 8.4, the morphology of TM3 cells exposed to green tea in the absence or presence of HCG, showed normal appearance of dividing cells at concentrations between 0.025 and 0.1%. However, at 0.5% there was less number of cells with their morphology similar to the positive control, 4% DMSO, due to its toxic effect. Hence, this concentration of green tea (0.5%) is cytotoxic to TM3 Leydig cells.

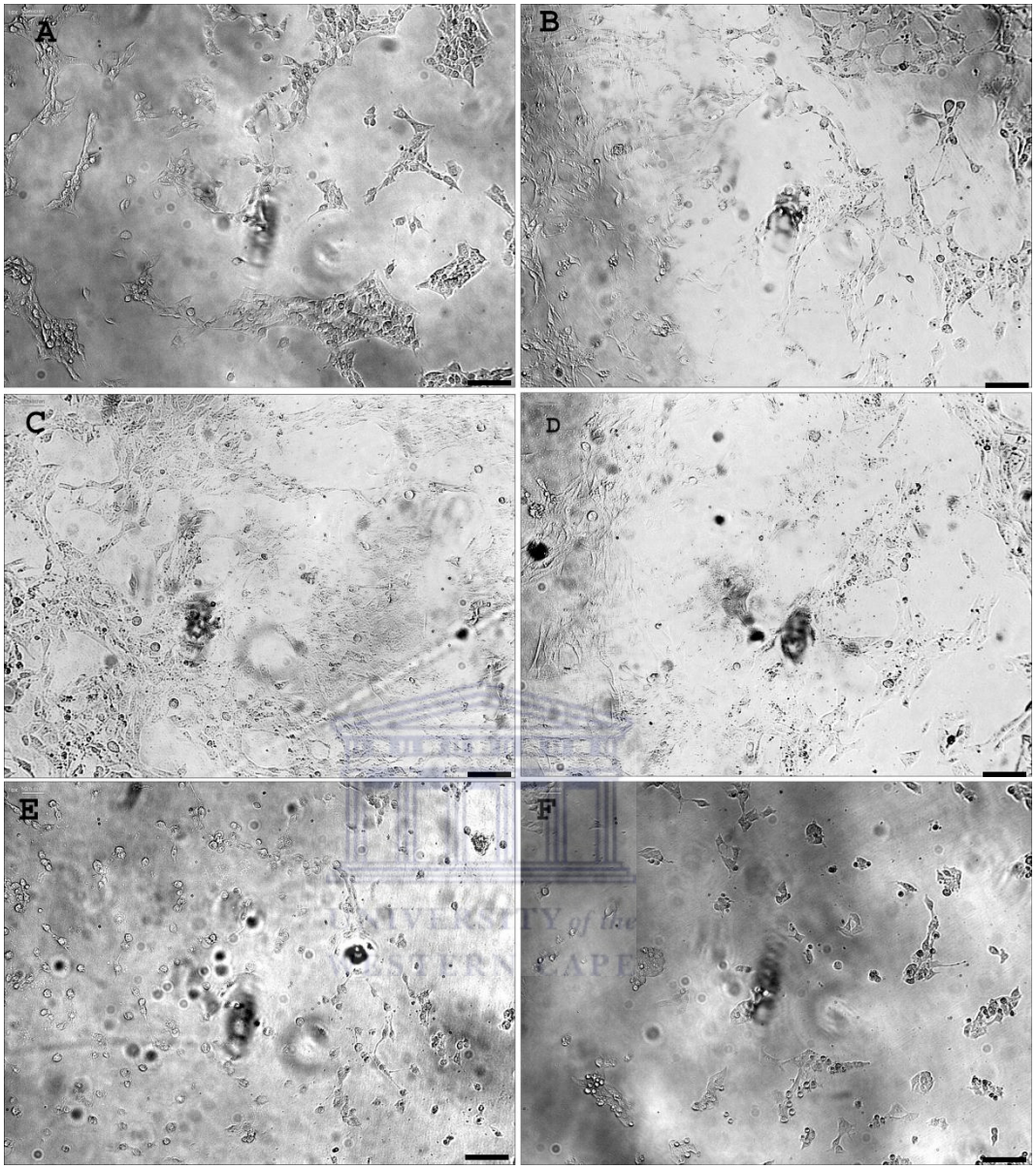


Figure 8.3: Morphology of TM3 Leydig cells exposed to green tea in the absence of HCG. Abbreviations: (A) control without HCG (B) 0.025% (C) 0.05% (D) 0.1% (E) 0.5% (F) 4% DMSO; Bar = 50 µm.

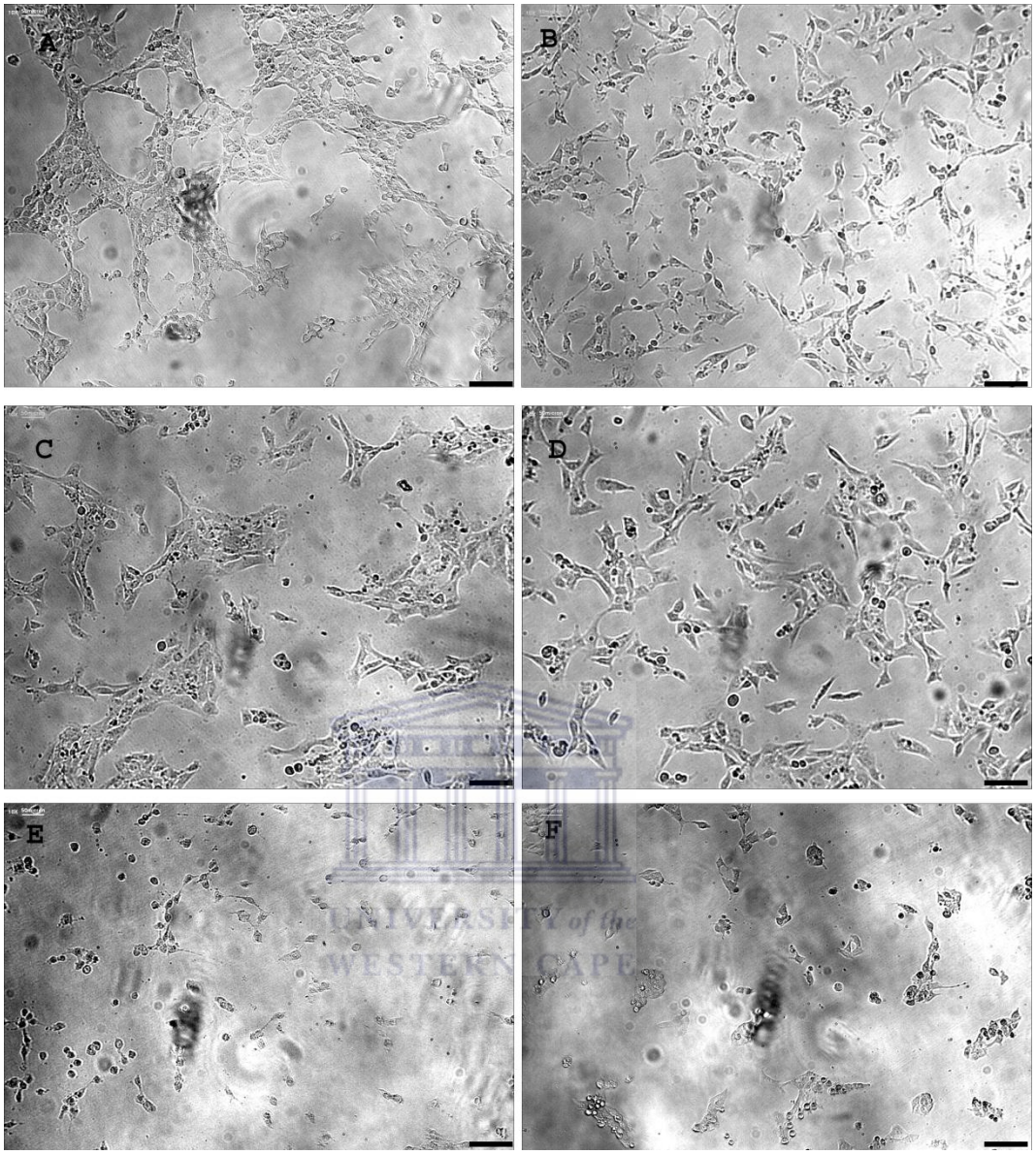


Figure 8.4: Morphology of TM3 Leydig cells exposed to green tea in the presence of HCG. Abbreviations: (A) control without HCG (B) 0.025% (C) 0.05% (D) 0.1% (E) 0.5% (F) 4% DMSO; Bar = 50 μm.

Similar to green tea, the cells exposed to black tea, revealed normal morphology of cells compared to the control either in the absence or presence of HCG between 0.025 and 0.1%, while at 0.5%, there appeared to be cell death as the cells were characterized as shrunken and irregularly shaped like the positive control (figures 8.5 and 8.6).

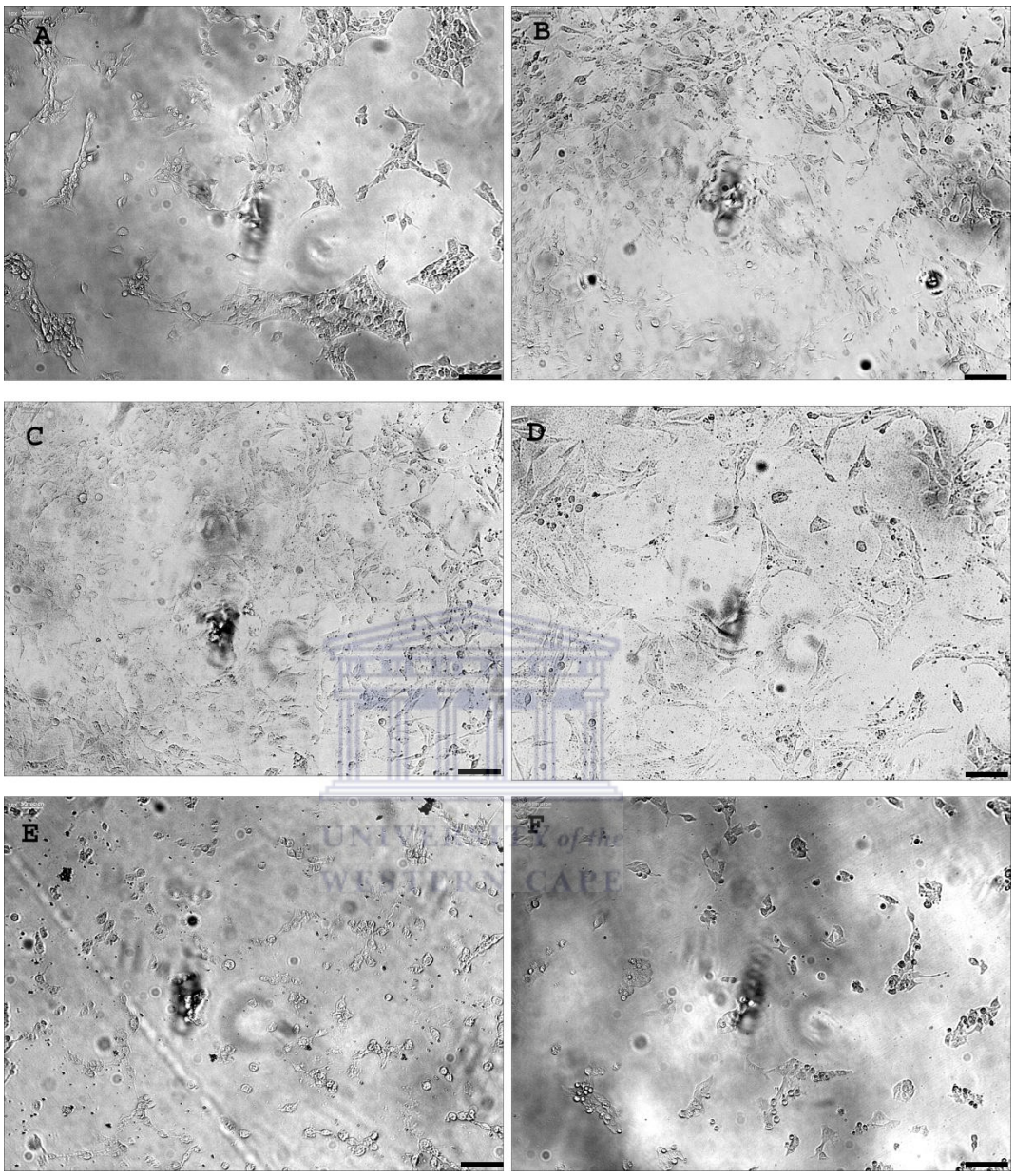


Figure 8.5: Morphology of TM3 Leydig cells exposed to black tea in the absence of HCG. Abbreviations: (A) control without HCG (B) 0.025% (C) 0.05% (D) 0.1% (E) 0.5% (F) 4% DMSO; Bar = 50 μm.

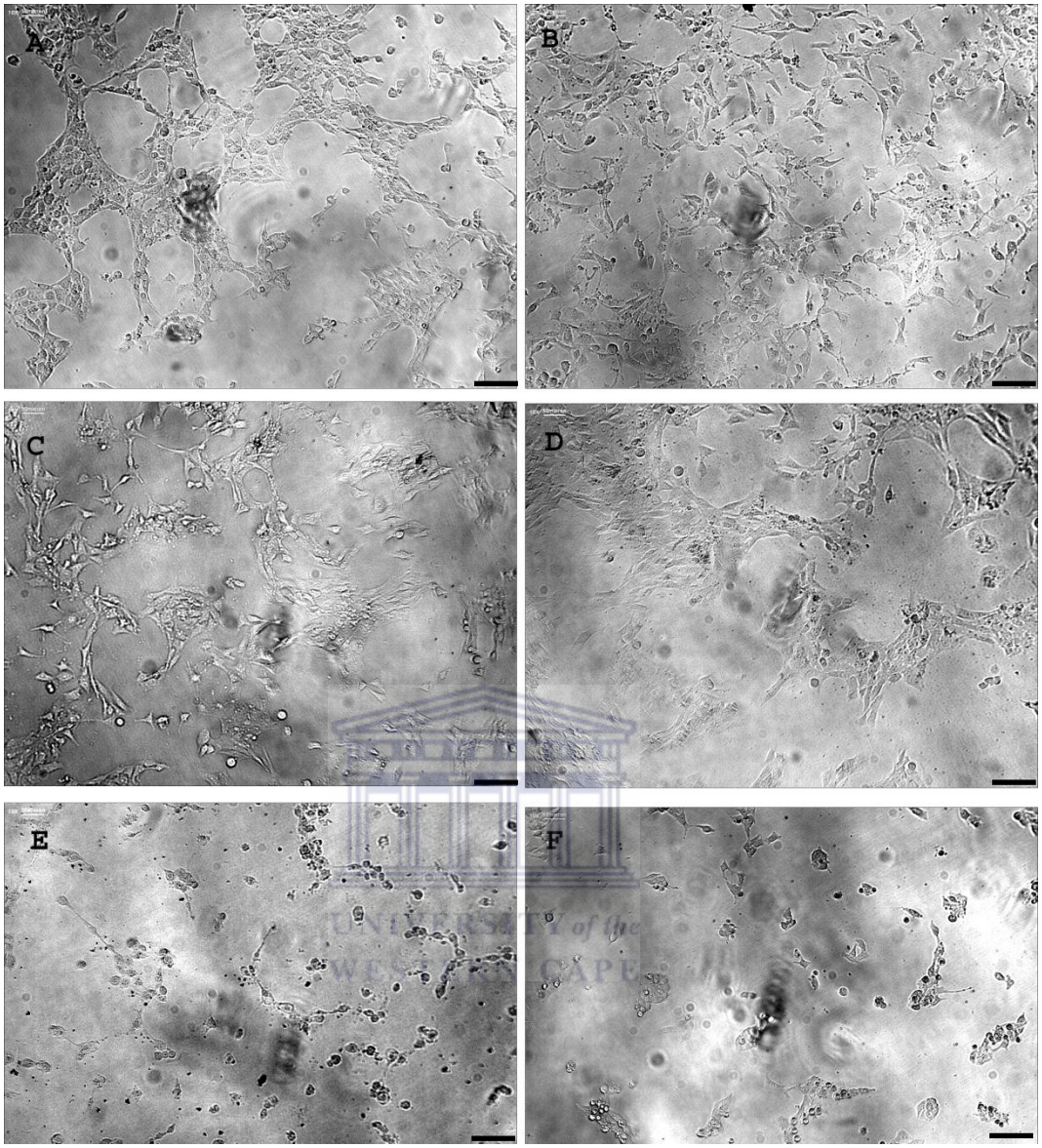


Figure 8.6: Morphology of TM3 Leydig cells exposed to black tea in the presence of HCG. Abbreviations: (A) control without HCG (B) 0.025% (C) 0.05% (D) 0.1% (E) 0.5% (F) 4% DMSO; Bar = 50 µm.

TM3 Leydig cells exposed to unfermented rooibos between 0.025 and 0.1% in the absence or presence of HCG showed normal morphology of cells compared to the control. However, at 0.5%, there were changes in the cell morphology which were similar in appearance to the positive control (figures 8.7 and 8.8). The changes in the morphology reveal that unfermented rooibos are cytotoxic to the cells at 0.5%.

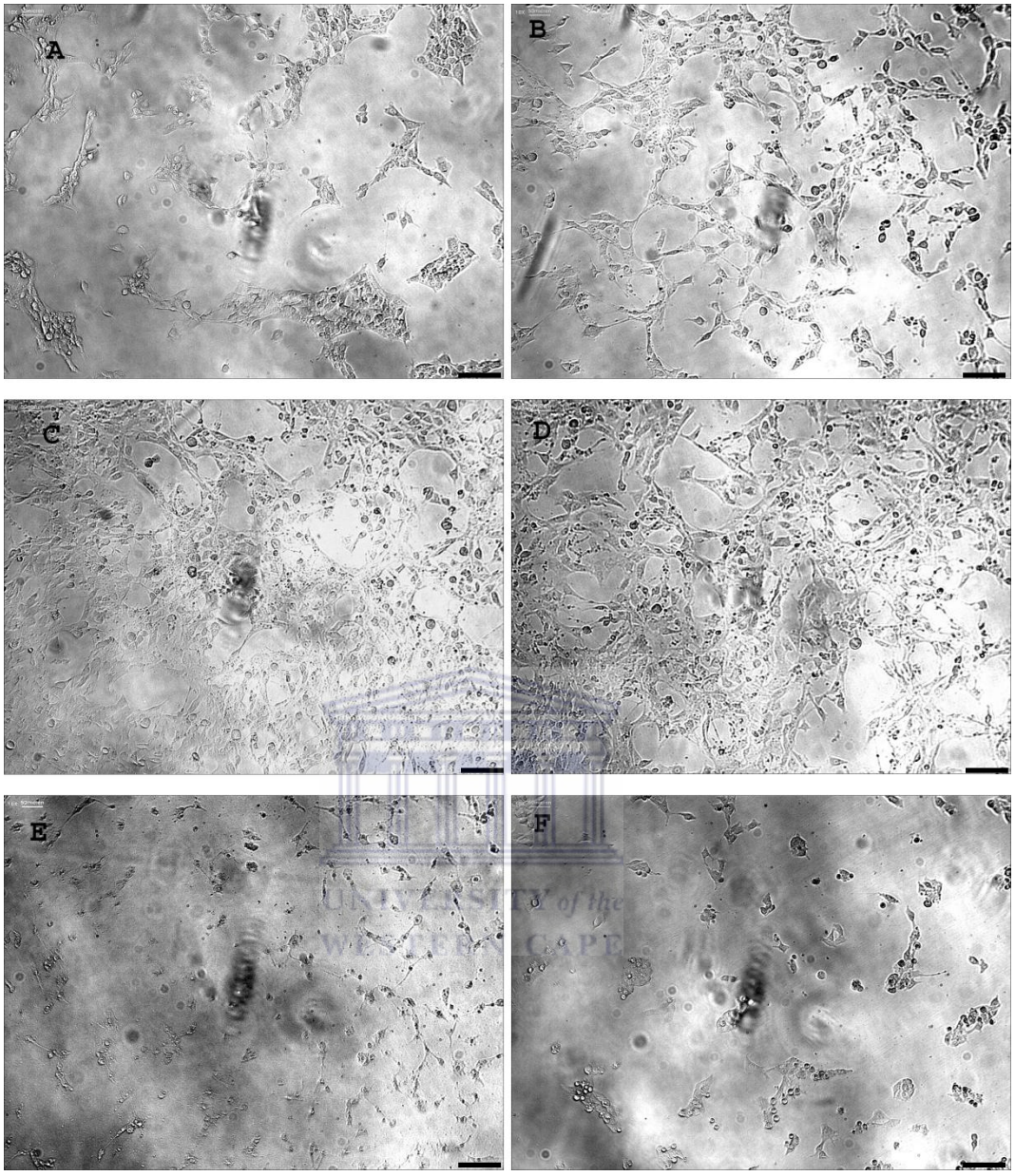


Figure 8.7: Morphology of TM3 Leydig cells exposed to unfermented rooibos in the absence of HCG. Abbreviations: (A) control without HCG (B) 0.025% (C) 0.05% (D) 0.1% (E) 0.5% (F) 4% DMSO; Bar = 50 µm.

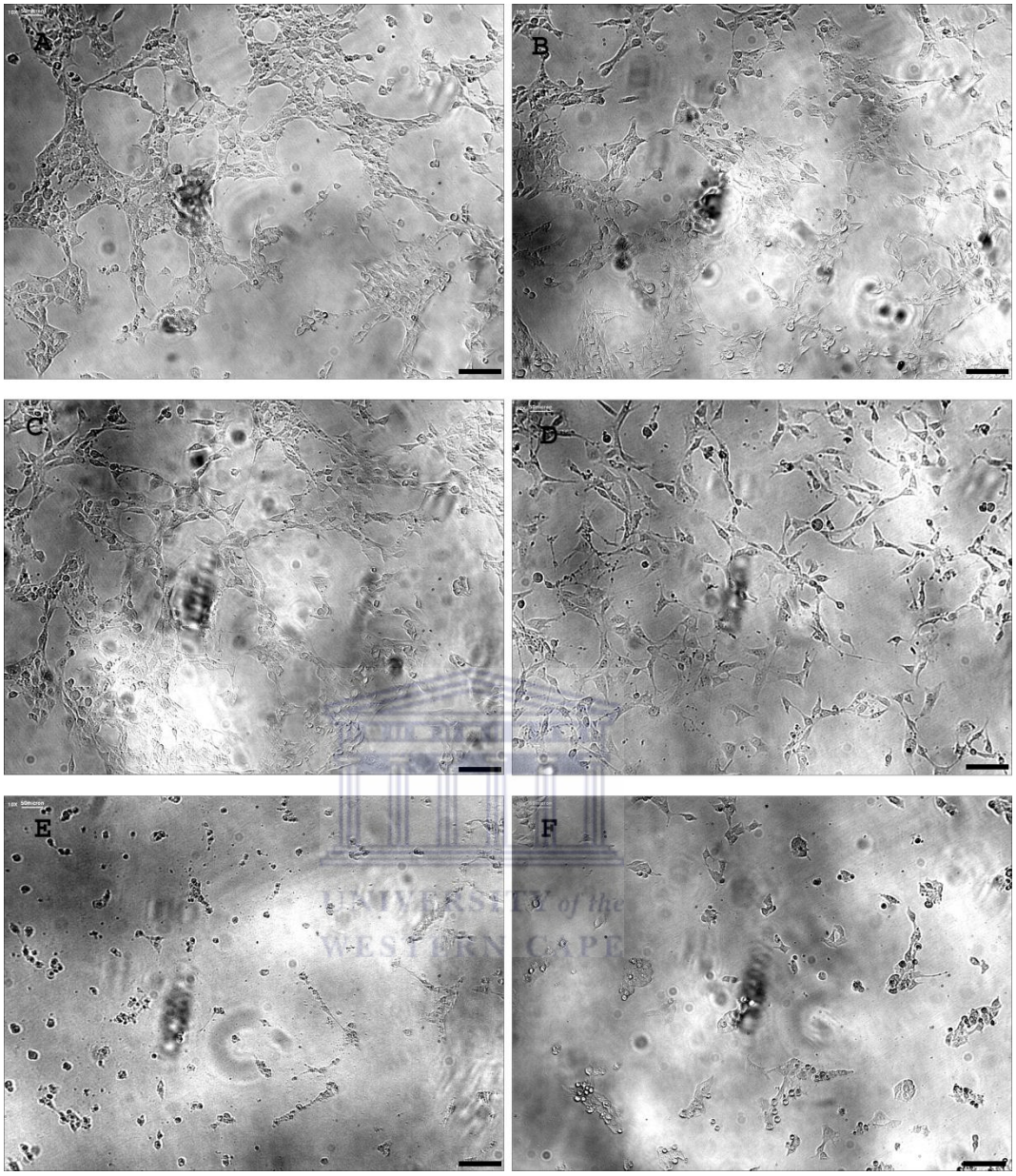


Figure 8.8: Morphology of TM3 Leydig cells exposed to unfermented rooibos in the presence of HCG. Abbreviations: (A) control without HCG (B) 0.025% (C) 0.05% (D) 0.1% (E) 0.5% (F) 4% DMSO; Bar = 50 µm.

Finally, exposure of fermented rooibos to TM3 cells in the absence or presence of HCG resulted in no changes in morphology compared to the control between 0.025 and 0.1%. At the highest concentration of 0.5%, a change in the morphology of the cells was also observed and was similar to the cells exposed to

4% DMSO (figures 8.9 and 8.10). Again, fermented rooibos at 0.5% is cytotoxic to the TM3 Leydig cells.

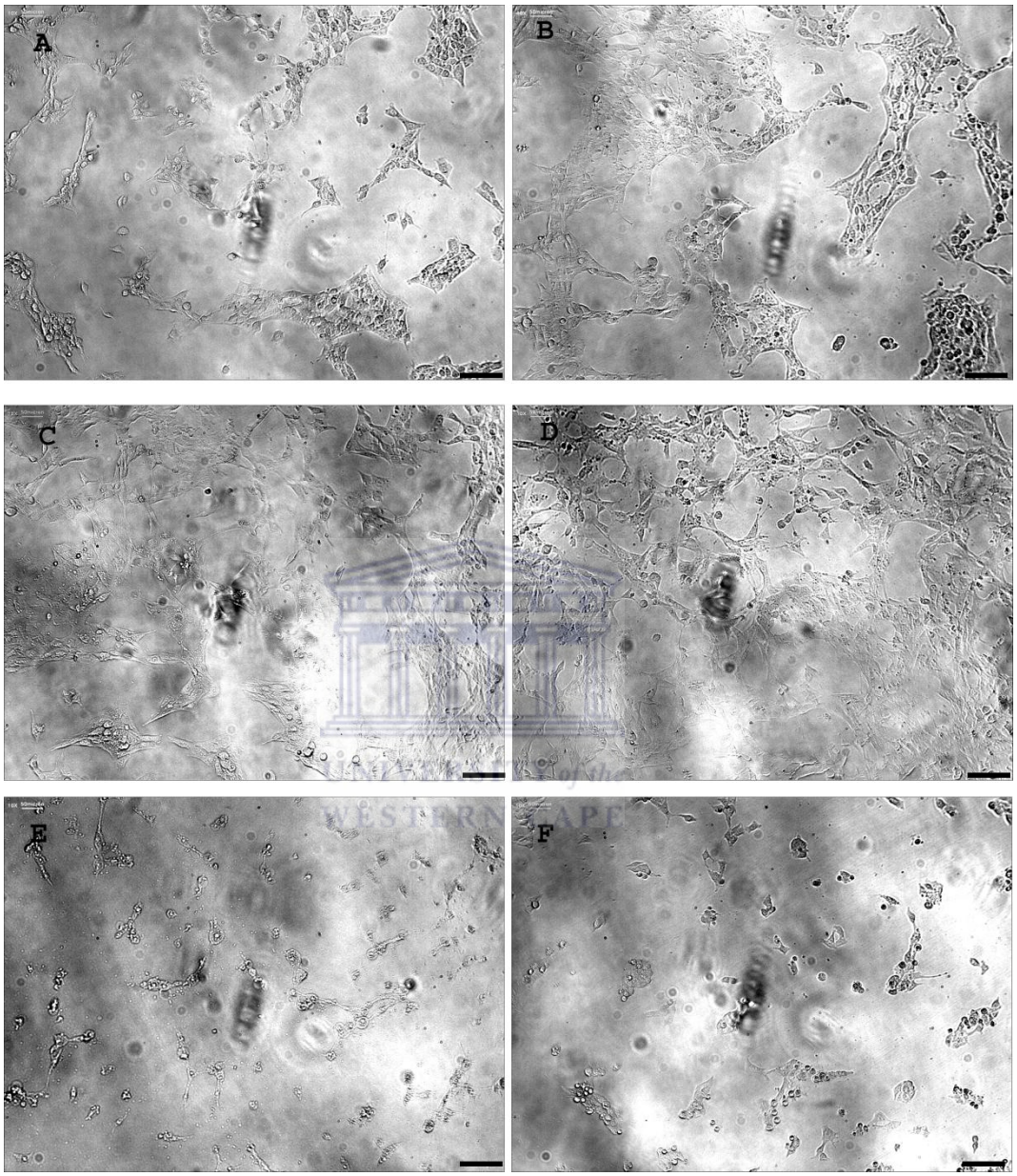


Figure 8.9: Morphology of TM3 Leydig cells exposed to fermented rooibos in the absence of HCG. Abbreviations: (A) control without HCG (B) 0.025% (C) 0.05% (D) 0.1% (E) 0.5% (F) 4% DMSO; Bar = 50 μm.

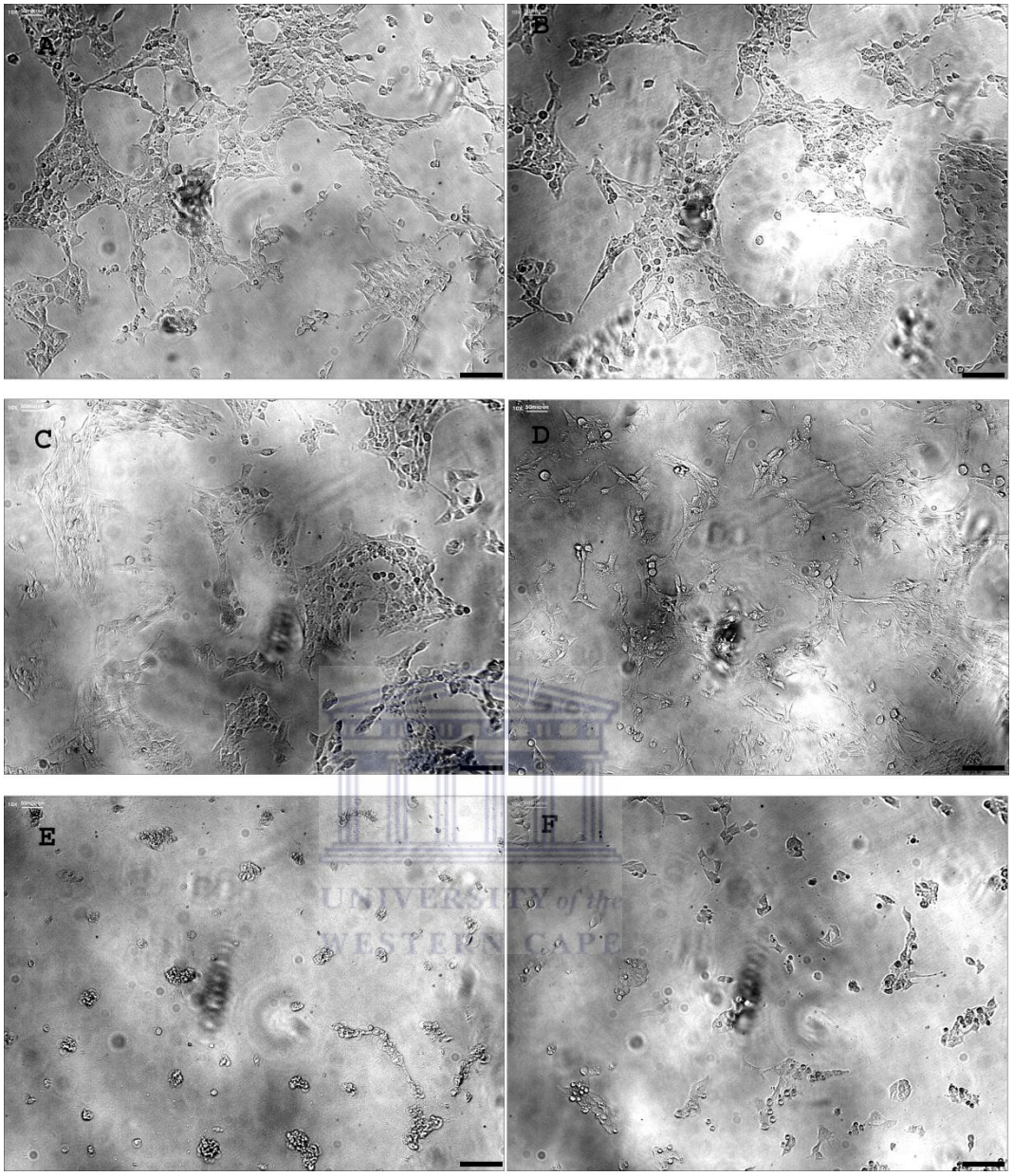


Figure 8.10: Morphology of TM3 Leydig cells exposed to fermented rooibos in the presence of HCG. Abbreviations: (A) control without HCG (B) 0.025% (C) 0.05% (D) 0.1% (E) 0.5% (F) 4% DMSO; Bar = 50 µm.

In summary, lower concentrations of green and black tea, and unfermented and fermented rooibos (0.025 – 0.1%) resulted in no obvious changes in the morphology of non – stimulated or stimulated TM3 Leydig cells, while at high

concentration (0.5%), changes in its morphology and much lower cell numbers was observed.

8.3 EFFECT OF CAMELLIA SINENSIS AND ASPALATHUS LINEARIS ON TESTOSTERONE PRODUCTION IN TM3 LEYDIG CELLS

In order to determine the androgenic property and inhibition of HCG steroidogenesis by *Aspalathus linearis* and *Camellia sinensis*, TM3 Leydig cells were exposed to different concentrations of green tea, black tea, unfermented rooibos or fermented rooibos for 24 h in the absence or presence of HCG respectively. The supernatants were collected and analyzed for the production of testosterone.

Stimulated control (positive control) was shown to produce significant high amounts of testosterone compared to the non – stimulated (negative control; $p < 0.05$) (figures 8.11). This clearly demonstrates the capacity of this batch of TM3 cells to synthesize and release testosterone. On the overall, *Aspalathus linearis* and *Camellia sinensis* revealed a tendency to decreased values or significantly decreased the level of testosterone produced by the TM3 cells in the absence or presence of HCG.

From figure 8.11, TM3 cells exposed to green tea showed that production of testosterone was similar to the negative control ($p > 0.05$) except at 0.05% and 0.5% where a significant decrease was observed ($p < 0.05$). When comparing the stimulated cells to the positive control, a significant decrease was observed ($p < 0.05$). In addition, the stimulated treated cells were also similar to the negative control group ($p > 0.05$). In general, the stimulated treated cells had higher testosterone levels compared to the non – stimulated TM3 cells (figure 8.11).

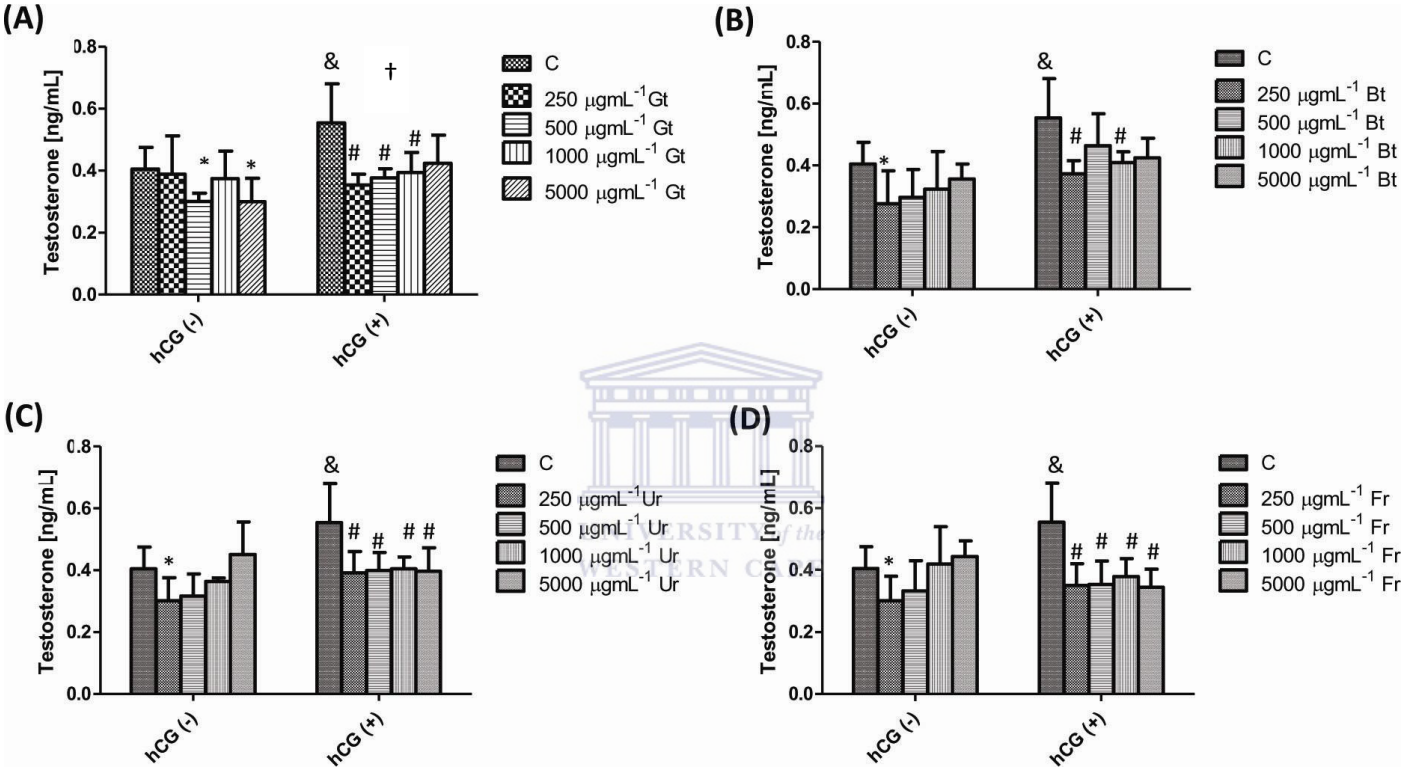


Figure 8.11: Testosterone production in TM3 Leydig cells exposed to *Camellia sinensis* or *Aspalathus linearis* in the presence or absence of HCG.

Values represented are the mean ± SD of six determinations repeated in two independent experiments. Abbreviations: (A) green tea; (B) black tea; (C) unfermented rooibos; (D) fermented rooibos; C, control; Gt, green tea; Bt, black tea;Ur, unfermented rooibos; Fr, fermented rooibos; *, $p < 0.05$, when non - stimulated treated groups are compared to the negative control; $^{\#}$, $p < 0.05$, when stimulated treated groups are compared with the positive control; $^{\&}$, $p < 0.05$, when the negative control is compared with the positive control.

Black tea, at lower concentrations (0.025 and 0.05%; figure 8.11) caused a significant decrease in testosterone production in the non – stimulated treated group ($p < 0.05$). Compared to the positive control, the stimulated treated group showed a tendency to decrease (0.05 and 0.5%; $p > 0.05$) or significantly decrease (0.025 and 0.1%; $p < 0.05$) the production of testosterone. The treated stimulated cells were more or less similar to the negative control ($p > 0.05$).

Unfermented rooibos was shown to significantly decrease the level of testosterone in non – stimulated TM3 cells at 0.025% compared to the negative control ($p < 0.05$). However, a dose- dependent increased value was observed ($p > 0.05$). Compared to the positive control, a significant decrease was observed in all the stimulated treated groups ($p < 0.05$). Stimulated cells were shown to also be similar to the non - stimulated control ($p > 0.05$). While comparing the level of testosterone between the non - stimulated and stimulated treated groups, a tendency to increased values was observed in the stimulated group from 0.025 to 0.1%. However, at the highest concentration (0.5%), the reverse was observed (figure 8.11).

Fermented rooibos significantly decreased the level of testosterone in the non – stimulated treated cells at 0.025% compared to the negative control (figure 8.4; $p < 0.05$). However, a dose- dependent increase was observed ($p > 0.05$). Compared to the positive control, the stimulated treated cells significantly decreased the level of testosterone ($p < 0.05$). A similar result was observed in the stimulated treated groups compared to the negative control ($p > 0.05$). While comparing the level of testosterone between the stimulated and non – stimulated treated cells, it was observed that at lower concentrations (0.025 – 0.1%), the stimulated cells produced more ($p > 0.05$), while at higher concentration (0.5%), the non - stimulated treated groups significantly enhanced testosterone production ($p < 0.05$) (figure 8.11).

CHAPTER NINE

DISCUSSION

9.1 ANTIOXIDANT CAPACITY OF CAMELLIA SINENSIS AND ASPALATHUS LINEARIS

Imbalance in the generation and scavenging abilities and time of production of reactive oxygen species are shown to impair functions of the male and female reproductive systems (Iwasaki & Gagnon, 1992; Sikka 1996; de Lamirande et al., 1997; Agarwal & Allamaneni, 2004; Agarwal et al., 2006). Antioxidants are also known to scavenge and suppress the formation of ROS and lipid peroxidation (Sikka, 1996). Furthermore, *Camellia sinensis* and *Aspalathus linearis* have gained popularity for its antioxidative properties (von Gadow et al., 1997). In this present study, analysis of antioxidant level in tea and herbal infusion showed *Camellia sinensis* to be higher than *Aspalathus linearis*, similar to the findings from previous studies (von Gadow et al., 1997; Marnewick et al., 2000). In this study, soluble solids, total polyphenols, flavanol content (catechin) and FRAP revealed that green tea had the highest amount, followed by black tea, unfermented rooibos, and fermented rooibos. In line with previous studies, green tea was shown to have higher antioxidant capacity than black tea, as was also shown by the total phenolic contents in green and black tea (Yokozawa et al., 1998; Lee et al., 2002; Atoui et al., 2005). In general, a dose- dependent increase in antioxidant level was observed in the respective teas and herbal infusions. However, the high amount of antioxidants capacity in these plants was not associated with significant changes in the serum antioxidant capacity when analyzed using FRAP, after the 21 or 52 day period of treatment in the female or male rats respectively. This was also reported in previous studies (Widlansky et al., 2005; Coimbra et al., 2006; Marnewick et al., 2011). The fluid intake in all treatment groups of either the male or female rats were more or less similar to the control, excluding the 5% Ur group that was significantly lesser in both the female and male rats.

9.2 *IN VIVO* EFFECTS OF CAMELLIA SINENSIS AND ASPALATHUS LINEARIS ON MALE RAT REPRODUCTIVE SYSTEM, KIDNEY AND LIVER FUNCTIONS

A 52d treatment in male rats was chosen for this study, as the duration of spermatogenesis in rat takes about 52 days (França et al., 1998). In this study, the weekly body weight gain in all treatment groups was similar to the control and increased progressively throughout the study period. Likewise, the total weight gain of the treated male rats was similar to the control. A previous *in vivo* study of daily intake of 2% unfermented and fermented rooibos as sole source of drinking for 3 weeks, also demonstrated that rooibos caused no significant difference in the body weight gain (Marnewick et al., 2009), similar to our findings. However, Jurani et al. (2008), revealed a significant decrease in body weight in male Japanese quail birds exposed to rooibos. On the other hand, oral administration of green tea extract at 2.5% and 5% concentrations to male albino rats at a dose of 1 mL/100g body weight for 26 days was shown to decrease the net gain in body weight (Chandra et al., 2011). In addition, Zheng et al. (2004) showed that green tea had a suppressive effect on body weight gain increase and fat accumulation owing to its caffeine and theanine components. However, similar to our findings, Sato et al. (2010) showed that green tea intake resulted in no significant difference in body weight gain. Intake of black tea in this study revealed no significant effect on body weight gain, similar to previous findings (Greger & Lyle, 1988; de Vos et al., 2003). However, other studies revealed that intake of black tea resulted in a significant decreased body weight gain (Hamdaoui et al., 1997; Wang et al., 2011). In addition, this present study also revealed that increased concentrations of either black tea or fermented rooibos showed a trend to decreased weight gain.

In this study we found that the level of testosterone produced in the sera of treated male rats was similar to the control excluding the 5% Gt group which increased

significantly. However, it was shown from a previous study that male rats exposed to green tea had significantly decreased testosterone level (Chandra et al., 2011), which is in contrast to our finding but similar to Satoh et al. (2002). Inhibitory effect of the green tea components (EGC, EGCG, ECG, GCG and CG), in human placental aromatase activity was also observed in a dose-dependent manner.This enzyme is responsible for the aromatization of androgens into estrogens (Satoh et al., 2002). Hence, an inhibitory aromatase activity might result in an accumulation of testosterone as seen for green tea in same studies. Black tea, on the other hand, was shown to increase testosterone level significantly (Ratnasooriya & Fernando, 2008), however, a tendency to a dose-dependent decrease was observed in this PhD study. Rooibos has flavonoids, as its major compound, (Joubert, 1996) of which many are classified as endocrine disruptors (Patisaul & Jefferson, 2010). This present study showed that unfermented and fermented rooibos tend to decrease the production of testosterone in a dose-dependent manner. Scholms et al. (2012), revealed in an *in vitro* study that H295R cells (a human adrenal carcinoma cell line, capable of producing many steroids) exposed to 1mg/mL unfermented rooibos, and dihydrochalcones (10µM aspalathin or 10µM notofagin) for 48h significantly decreased A4 (which is a primary precursor of testosterone) and testosterone level. Flavonoids such as daidzein, genistein and apigenin exposed individually or as a mixture to H295R cells for 24h also showed a significant dose- dependent inhibition of testosterone secretion (Ohlsson et al., 2010). Ohno et al. (2003) again showed in an *in vivo* study that subcutaneous administrations of genistein to male Wistar rat tend to decrease serum testosterone level.

Testosterone sensitive organs (testis, epididymis, seminal vesicles and prostate) in the treated male rats were more or less similar to the control, similar to findings from a previous study (Satoh et al., 2002). However, other studies had shown a decrease in the weight of testes, accessory reproductive organs, circulating level of LH and testosterone in intact rats exposed to green tea (Kao et al., 2000; Chandra et al., 2011). Increased testis weight but a tendency to a decreased

epididymal weight in rats exposed to black tea was observed in a study by Wang et al. (2011). In this present study, the weight of the testis did not differ significantly, however, a tendency to a decreased epididymal weight was observed. To the best of my knowledge this is this first finding on the effect of rooibos on testosterone sensitive organs mentioned earlier.

Histological examination of the testes and epididymis showed no obvious alterations in the treated groups compared to the control. That is similar to the findings from a previous study (Sato et al., 2010). However, morphometric measurements of the epithelial heights of the seminiferous tubules in the testes showed a significant decrease in the groups exposed to 2% green tea, black tea, 5% unfermented rooibos or fermented rooibos. In contrast, no significant difference was observed in the groups exposed to 5% green tea and 2% unfermented rooibos. In addition, the diameter of the seminiferous tubules in male rats exposed to *Camellia sinensis* or *Aspalathus linearis* was also significantly decreased compared to the control. In the epididymis, however, a significant increase in the epithelial height of the caput was observed in the groups exposed to 5% green tea, 2% black tea, unfermented and fermented rooibos. 2% green tea and 5% black tea did not affect it significantly. On the other hand, the epithelial height of the cauda epididymides of male rats treated with 2% green tea or 5% black tea did increase significant and decreased significantly by 2% unfermented rooibos. No significant difference was observed in the rest of the group. In the epididymal epithelium, 5-α-reductase converts testosterone to dihydrotestosterone (DHT), which is the dominant androgen present in the epididymis. The caput region has a higher amount androgens concentration (dihydrotestesterone and testosterone) than the cauda epididymis, with the later having higher levels of testosterone/DHT ratio (Jean-Faucher et al., 1986). Hence the epithelium of the cauda epididymis might be more sensitive to the reduced level of testosterone found in this study after the treatment of rats with unfermented and fermented rooibos. Certain reproductive toxicants (cypermethrin, permethrin, and 3-phenoxybenzoic acid) were shown to significantly reduce the perimeter and

number of cell layers of seminiferous tubules as well as sperm motility in male rats *in vivo* and *in vitro* (Elbetieha et al., 2001; Yuan et al., 2010). In this study, only in the morphometric measurements were reductions observed, whereas sperm counts, motility and vitality were clearly enhanced. This rather speaks against a reproductive toxic effect.

Male infertility have been associated with poor semen quality, which may be characterized by low sperm motility and viability (Banihani et al., 2011) or low sperm production (oligoozoospermia), poor sperm motility (asthenozoospermia) or abnormal sperm morphology (teratozoospermia) or a combination of all three (oligoasthenoteratozoospermia) (Guzick et al., 2001). In this study, sperm concentration was shown to improve by the unprocessed tea and herbal infusion (tisane) as green tea and unfermented rooibos significantly increased it while black tea and fermented rooibos showed no significant difference compared to the control. From previous studies, unfermented and fermented rooibos was also shown to significantly increase sperm concentration (Awoniyi et al., 2012) while green tea was shown to decrease it in a dose- dependent manner (Chandra et al., 2011). Sato et al. (2010) showed that intake of green tea in male rats had no significant effect on sperm concentration but increased sperm motility significantly. This thesis showed that sperm viability improved significantly in all treatment groups. Sperm motility was increased significantly by 2% green tea, black tea and unfermented rooibos, however, a dose- dependent decrease in sperm motility was observed with green tea. Of the total motile sperm, only 2% black tea and unfermented rooibos significantly improved the number of progressive motile sperm, while a dose- dependent decrease was observed with green tea. Accordingly, 2% green tea, black tea and unfermented rooibos significant reduced the number of static sperm present, while fermented rooibos and 5% green tea did not differ significantly. These improved semen parameters after the treatment of rats with *Camellia sinensis* or *Aspalathus linearis* suggest that these tea or tisane may be regarded to enhance fertility.

Sperm velocity parameters (VCL, VSL and VAP) directly measure the motion of sperm (Horimoto et al., 2000). In this study, sperm velocity parameters which included VCL, VSL, VAP, ALH, LIN, STR, WOB and BCF showed no significant alteration compared to the control, however, a tendency to increased values was observed. A correlation was shown to exist between the fertilizing rate and these sperm velocity parameters (Moore & Akhondi, 1996), with VCL and BCF being indicators of sperm viability; VAP, VSL, STR and LIN markers of sperm progression, while, STR and LIN in addition describes swimming patterns (Schettgen et al., 2002; Duty et al., 2004). These also point to the fact that both *Camellia sinensis* and *Aspalathus linearis* could enhance the fertilizing capacity of the sperm.

Spontaneous acrosome reaction was shown to increase significantly in male rats exposed to green tea, 5% black tea and fermented rooibos with no significant effect in unfermented rooibos group. In order to trigger further sperm maturation steps such as motility or acrosome reaction, a physiological amount of ROS is needed (Henkel, 2011). Due to the high antioxidant activity of *Aspalathus linearis* and *Camellia sinensis*, a scavenging ability of ROS levels would be expected, thus reducing damages to the sperm plasma membrane. As a result, sperm viability and motility should be enhanced while acrosome reaction should be inhibited. As discussed earlier, both tea and herbal infusion significantly improved sperm motility, viability and concentration. However, the percentage of spontaneous reacted acrosome was significantly increased by green tea, black tea and fermented rooibos. This enhanced acrosome reaction might thus be triggered by a different mechanism induced by the tea. A premature acrosome reaction can reduce the capability of sperm to penetrate the zona-pellucida of the oocyte, thus inhibiting fertilization (Tesarik et al., 1992; Hsu et al., 1999). Thus, despite the beneficial effects of *Aspalathus linearis* and *Camellia sinensis*, the increased spontaneous acrosome reaction seen would negatively affect the fertilization of an oocyte.

The concentration of serum creatinine is used as a measure of the glomerular filtration rate (GFR), an index of renal function (Perrone et al., 1992). From our study, the weight of kidneys of male rats exposed to either *Camellia sinensis* or *Aspalathus linearis* was increased significantly only at high concentrations (5%). This was evident in the increased level of creatinine in sera of the treated animals. As green tea showed a tendency to increased values, black tea increased from 2%, and unfermented and fermented rooibos significantly increased serum creatinine. However at either concentration, there were no obvious changes in the structures of the kidneys in all treated groups. Never the less, consumption of either *Camellia sinensis* or *Aspalathus linearis* in large amount could be detrimental to the normal functioning of the kidney.

ALT and AST are specific markers for liver function and the activities of both enzymes are high in tissues such as the liver, heart and muscles (Rej, 1997). Any damage to the hepatocytes results in increased activity of ALT and AST in the serum (Goldberg & Watts, 1965) and the level of these enzymes are monitored in clinical diagnosis of disease and damage to structural integrity of the liver (Amin & Hamza, 2005). In this study, liver weight of the male rats in all treated groups was similar to the control. This finding is in agreement with the level of ALT and AST found in the green and black tea treated groups. However, the level of ALT or AST in serum of male rats exposed to fermented rooibos showed a tendency to increased values or increased significantly respectively. In a previous study, where volunteers were exposed to 6 cups of fermented rooibos per day over 6 weeks, a significant increase in the serum level of creatinine, ALT and AST (Marnewick et al., 2011) was noticed. Higher activities of AST and ALT in serum were found to be in response to oxidative stress induced by hyperthyroidism (Chattopadhyay et al., 2007; Subudhi et al., 2008). Messarah et al. (2010) also associated hyperthyroidism accompanied by oxidative stress with increased hepatic lipid peroxidation and cell damage markers (AST, ALT, LDH and ALP) activities.

Aspalathus linearis and *Camellia sinensis* are known for their high level of antioxidant activity. Antioxidants are known to scavenge or suppress the formation of ROS (Sikka, 1996; von Gadow et al., 1997). This present study revealed that despite the high antioxidant level in the teas and herbal infusions *in vitro*, there was no significant difference in the ferric reducing antioxidant power (FRAP) *in vivo*. This is in accordance with published data (Widlansky et al., 2005; Coimbra et al., 2006; Marnewick et al., 2011). Furthermore, the level of GSH in the testis or kidney was not altered by either plant extract. However, a significant increase in GSH level was observed in the liver of the male rats exposed to 2% green tea, unfermented rooibos (2% and 5%) and 5% fermented rooibos. GSH plays a role in scavenging free radicals and peroxide produced during normal cellular respiration, which would otherwise cause oxidative damage to lipids, proteins, and nucleic acids (Schuck et al., 2008). A depletion in intracellular GSH is a hallmark indicator of oxidative stress, which is mostly the case in cancer cells (Pelicano et al., 2004), which is not the case in this study and could be associated with the antioxidant property of *Camellia sinensis* or *Aspalathus linearis.* Catalase activity in the testis, kidney and liver of the male rats were also comparable to the controls. Only 2% fermented rooibos increased the activity of catalase significantly in the liver. Superoxide dismutase activity increased significantly only in the testis of male rats exposed to 2% fermented rooibos, others where similar to the control but tend to increase in the lower concentrations. In the kidney and the liver, there were no significant changes in SOD activity compared to the control. In addition, the level of lipid peroxidation was not altered in the testes, liver or kidney of male rats exposed to either *Camellia sinensis* or *Aspalathus linearis*. These effects may reflect the antioxidant effects of the plants in these tissues. Furthermore, Awoniyi et al. (2011) revealed that 2% unfermented and fermented rooibos *ad libitum* in epididymal sperm of male rats resulted in similar MDA level but caused significantly increased CAT, SOD and GSH levels, while 2% green tea led to similar MDA, CAT, and GSH levels but resulted in significantly increased SOD levels compared to the control groups (Awoniniyi et al., 2012). However, from a previous study an increased

lipid peroxidation in the liver of mice following treatment with high dose of EGCG was observed and this increase was attributed to the prooxidative effect of green tea (Lambert et al., 2010), as previous studies mentioned that some of the biological activities of EGCG may be due to induction of oxidative stress (Hou et al., 2005; Galati et al., 2006).

9.3 *IN VIVO* EFFECTS OF CAMELLIA SINENSIS AND ASPALATHUS LINEARIS ON THE FEMALE REPRODUCTIVE, KIDNEY AND LIVER FUNCTION

Female rats were exposed to either *Camellia sinensis* or *Aspalathus linearis* for 21 d. This treatment period was chosen based on the duration of the oestrous cycle (4-5 days) in the female rat and was also used in previous studies (Telefo et al., 1998; Oyedeji & Bolarinwa, 2010). Exposure of female rats to either *Camellia sinensis* or *Aspalathus linearis* did not alter the weight gain during the period of treatment. Contrary to this present study, consumption of green tea by female rats was shown to suppress weight gain (Chan et al., 2010; Lu et al., 2012). Huang et al. (2009) revealed that female CF-1 mice fed with high fat diet and exposed to 0.2% black tea extract for 10 weeks resulted in an anti-obesity effect by suppressing body weight gain and adipose tissue formation. In addition, a trend towards increased body weight in female Japanese quail bird exposed to 0.175% rooibos was observed (Jurani et al., 2008). These varying results may be a result of different experimental conditions.

The ovary is made up *inter alia* of stroma, follicles and corpus luteum, hence the net weight of these tissues constitute the net weight of the ovary (Oyedeji & Bolarinwa, 2010). Antiestrogenic substances were shown to increase ovarian weight and decrease uterine weight (Matsuda et al., 1997; Kang et al., 2005);

however, this could be affected by oedema or by the number of *corpora lutea* present per ovary (Rockette et al., 2006). In this study however, the weight of ovaries of female rats exposed to *Camellia sinensis* or *Aspalathus linearis* did not differ significantly compared to the control. Only by 5% fermented rooibos, was there a decrease in the ovarian weight. This non-significant change in the ovary weight could imply that the constituent of the ovary remained intact after the treatment. El-Samannoudy et al. (1980) demonstrated that subcutaneous administration of β-sitesterol, a phytoestrogen, in immature sheep also reduced the ovarian weight. However, histological sections of the ovaries in this present study revealed no structural changes in the treated groups compared to the control.

Furthermore, the weight of the uteri in the treated groups did not significantly differ from the control group with exception to the 5% unfermented rooibos, where a significant increase was observed. In addition, histological sections of the uteri in all treated groups revealed no structural changes compared to the controls. Estrogenic compounds were shown to increase the weight of the uterus (Turner, 1971); in addition, the stromal cells in the endometrium are known to proliferate and increase in size under the influence of estrogens (Yuan, 1991). This might explain the observed increased weight of the uterus after treatment with 5% unfermented rooibos. Furthermore, Shimamura et al (2006) revealed that rooibos possessed 25 compounds with estrogenic properties, with notofaghin possessing estrogenic activity similar to genistein, while aspalathin possessed half the activity of notofaghin, even though both dihydrocalchones are the major constituent of *Aspalathus linearis* (Marais et al., 2000; Bramati et al., 2002). In this study, *Aspalathus linearis* seems to possess weak estrogenic properties based on the results obtained on the weights of the ovaries and uterii.

In this study, FSH levels in female rats exposed to either *Camellia sinensis* or *Aspalathus linearis* did not differ significantly from the control. On the other hand, LH levels decreased significantly in the groups exposed to *Camellia sinensis*, while *Aspalathus linearis* had no significant effect. In response to the rising levels of estrogen a pronounced peak in LH occurs during the proestrus phase of the estrous cycle of the female rats, which eventually results in ovulation. Any alterations in LH level could potentially reduce or delay ovulation, resulting in reduced litter size (Tyler & Gorski, 1980). From a previous study, EGCG in green tea was shown to negatively affect reproductive function in swine by inhibiting the proliferation of granulose cells, steroidogenesis and follicular angiogenesis by reducing vesicular endothelial growth factor (VEGF) production (Basini et al., 2005). Hence, from findings of this study, *Camellia sinensis* may delay the onset of ovulation.

5% black tea was shown to significantly increase the weight of the kidney in the female rats, however, creatinine which is a kidney function marker (Perrone et al., 1992) only increased significantly by 5% fermented rooibos, while green tea, black tea and unfermented rooibos presented a non significant dose- dependent increased values in serum. However, histological sections of the kidneys revealed no obvious morphological alteration.

In the female rats, the weights of the liver increased significantly in the groups exposed to black tea and unfermented rooibos, while green tea and fermented rooibos showed a tendency to increased values. ALT level was shown to decrease in sera of female rats exposed to green tea, black tea or unfermented rooibos, while fermented rooibos tend to increase it. In addition, AST level did not differ from the control in most of the treated groups except in 5% fermented rooibos, where it increased significantly. ALT and AST are liver function markers (Rej, 1997; Amin & Hamza, 2005). This increase is similar to the findings by

Marnewick et al. (2011), which may suggest that large intake of fermented rooibos over a long period of time may have a detrimental effect on the functioning of the liver and kidney. However, the histological sections in this study presented no alteration in the architecture of the liver in female rats exposed to either *Camellia sinensis* or *Aspalathus linearis.*

As mentioned earlier, this present study revealed that despite the high level of antioxidants in the teas and herbal infusions *in vitro,* there was no signicant difference in the ferric reducing antioxidant power (FRAP) in sera of female rats exposed to either *Camellia sinensis* or *Aspalathus linearis in vivo*. GSH levels in both kidney and liver of the female rats were not altered in all treated groups compared to the control. Catalase activity in the kidney was similar to the control in all treated groups except in 2% unfermented rooibos, where a significant increase was observed. Furthermore, the activity of catalase in the liver was also similar to the control in most treated groups besides the 2% black tea and 5% unfermented rooibos, where a significant increase was observed. Superoxide dismutase activity in both the kidney and liver of the female rats exposed to either *Camellia sinensis* or *Aspalathus linearis* was similar to the control. In addition, MDA level in the kidney was also similar to the control, however, at higher concentrations of either unfermented or fermented rooibos, although not significant, increased values were observed. In the female rat liver, the level of lipid peroxidation in the treated group was similar to the control, and a tendency to decreased dose- dependent values was observed in all treated groups. These effect seen could be attributed the antioxidative properties in either *Camellia sinensis* or *Aspalathus linearis*.

9.4 *IN VIVO* EFFECTS OF CAMELLIA SINENSIS AND ASPALATHUS LINEARIS ON MALE RAT SEXUAL BEHAVIOUR AND FERTILITY OUTCOME

From this present study, although not significant, male rats treated with 2% green tea and unfermented rooibos (2% and 5%) took a shorter time to reach the receptive female rat, while male rats treated with 5% black tea, required a longer time to reach the female. In addition, it was shown that the lower concentration of tea and the herbal infusion increased the number of sniffs in the male rats compared to the higher concentrations. Similarly, the mount frequency increased in all treatment groups, with increased dose- dependent values in green tea, and dose- dependent decreased values in those exposed to black tea, unfermented and fermented rooibos. Male rats exposed to 2% black tea or 2% unfermented rooibos required shorter time to mount (mount latency) the female rats, while the rest of the treatment group took longer time. In general, the sexual behaviour or competence in each tea and herbal infusion treated rat group was improved at lower concentrations compared to the higher concentrations. El- Lethey & Shaheed (2011) showed that administration of 2% black tea for 14 weeks to male rat increased its sexual drive as seen by the decreased mount latency and increased total mount frequency. In addition, it was also shown that black tea possessed aphrodisiac tendencies, which is marked by shortening of mounts and intromission latencies and increased frequencies of mounts and intromissions (Ratnasooriya & Dharmasiri, 2000; Yakubu et al., 2007; Ratnasooriya and Fernando, 2008). Sexual behaviour has been classified as an androgen dependent behaviour (Schiavi et al., 1997; El- lethey & Shaheed, 2011). The result obtained on the sexual behaviour in this study corresponds to the level of testosterone produced in the male rats, as green tea produced the highest level, followed by unfermented and fermented rooibos, with black tea been the least. To the best of my knowledge, this study provides the first result on the effect of *Aspalathus linearis* and green tea on male sexual behaviour.

All treatment groups showed that there was no impairment in the index of libido. Fertility index, quantal pregnancy and implantation index improved in male rats exposed to black tea, 5% unfermented rooibos and fermented rooibos while green tea and 2% unfermented rooibos presented a decreased value.

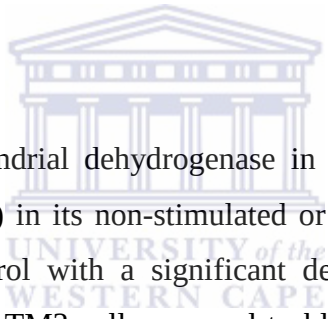
As a result of the poor fertility outcome obtained in the control group (quantal pregnancy of 11.1%), the results obtained here cannot be conclusive as it could be as a result of the length of mating, the inexperienced male rats, or the model of female rats used (siblings of the male rats). Hence, a repeat of the experiment is highly recommended by either increasing the length of time of mating or using another model of female rats. Unfortunately, due to the restriction of funding for this research project, a repeat was not feasible. However, for future studies, this might be looked into.

9.5 *IN VITRO* EFFECTS OF CAMELLIA SINENSIS AND ASPALATHUS LINEARIS ON CELL VIABILITY AND TESTOSTERONE PRODUCTION IN TM3 LEYDIG CELL LINE

TM3 Leydig cell line is derived from mouse BALB/c testis. Leydig cells are located in the interstitial spaces of the seminiferous tubules and account for about 95% of androgen synthesis and secretion (Shen et al., 2012). Chen et al. (2002) suggested that functional changes in the Leydig cell rather their loss results in the reduction of serum testosterone. In this study, the ability of *Camellia sinensis* and *Aspalathus linearis* to inhibit steroidogenesis by TM3 Leydig cells *in vitro* was examined by measuring their effects on testosterone production.

Figueiroa et al. (2009) revealed that green tea extract and isolated EGCG inhibited testosterone level in both basal and stimulated purified Leydig cells in a dose-dependent manner. This decreased level of testosterone was not due to a cytotoxic

effect, as the number of viable cells exposed to GTE (6.92 - 69.2 μg/mL) or EGCG (10 – 100 μg/mL) remained unchanged. However, at 692 μg/mL of GTE, fewer numbers of viable cells was observed and thought to contribute to the decreased level of testosterone produced (Figueiroa et al., 2009). In this present study, however, lower concentrations of green tea did not alter the production of testosterone in the non-stimulated cells but decreased significantly at 0.5%, which corresponded to the number of viable cells observed. In addition, the level of testosterone produced by the stimulated cells exposed to green tea decreased significantly compared to the control. Study of the morphology of the cells revealed that green tea did not alter their structures at lower concentrations, but resulted in cell death and reduced number of cells at 0.5%, which was similar to the effect observed by 4% DMSO.



The activity of mitochondrial dehydrogenase in TM3 Leydig cells exposed to black tea (0.025 – 0.1%) in its non-stimulated or stimulated state were more or less similar to the control with a significant decrease at 0.5%. The level of testosterone produced in TM3 cells exposed to black tea revealed a tendency to decreased values or was significantly reduced in non-stimulated and stimulated conditions. Similar to the number of viable cells, the morphology of TM3 cells in either state was similar to the control except at 0.5%, where cell death was visible and comparable to the effect from 4% DMSO.

Unfermented rooibos produced no significant effect on the activity of mitochondrial dehydrogenase except at the highest concentration (0.5%), where a significant decrease was observed in non-stimulated and stimulated cells. Similarly, the level of testosterone produced by the non-stimulated cells was similar to the negative control but displayed a tendency to decreased values. Furthermore, stimulated TM3 Leydig cells exposed to unfermented rooibos revealed a significant decrease in the level of testosterone produced compared to

the positive control. Similar to the teas, 0.5% unfermented rooibos rulted in cell death and reduced number of cells which was similar to the effect in the TM3 cells exposed to 4% DMSO.

Furthermore, the level of testosterone produced by non-stimulated TM3 cells exposed to fermented rooibos did not result in any significant change, although a tendency to reduced values was observed. In addition, stimulated TM3 Leydig cells exposed to fermented rooibos was shown to produce a significantly reduced level of testosterone at all concentrations. The activity of mitochondrial dehydrogenase was more or less similar to the control in both conditions of the cells. At 0.5% however, fermented rooibos significantly reduced the enzyme activity in the non- stimulated or stimulated conditions of the cells. Similar to this study, Noh et al. (2012) showed that lower concentrations of fermented rooibos (10- 50 μg/mL) resulted in no changes in TM3 cell viability.

In isolated Leydig cells, Figueiroa et al. (2009) demonstrated that the mechanisms underlying the inhibitory effect of green tea extract and EGCG on testosterone production involved the inhibition of the PKA/PKC signaling pathways, as well as the inhibition of P450scc enzyme and 17β-hydroxysteroid dehydrogenase function. In the Leydig cells, the steroidogenic pathway begin when LH binds to the receptors on the plasma membranes of the cells, thereby initiating a cascade of events which results in the conversion of cholesterol to pregnenolone by P450 scc (Stocco & Clark, 1996). Of which the rate-limiting step for testosterone synthesis is considered to be the translocation of cholesterol from the outer to the inner mitochondrial membrane. This is activated in response to hCG stimulation via increased levels of cAMP (Stocco & Clark, 1996; Stocco, 1998).

Taken together, these results illustrated that *Aspalathus linearis* and *Camellia sinensis* displayed a tendency to inhibit the level of testosterone produced in the non-stimulated condition. This indicates their antiandrogenic activity. One of the possible mechasims in which this is carried out is by inhibiting HCG stimulated steroidogenesis, which was demonstrated by the significantly reduced levels of testosterone produced by the stimulated cells. Both *Camellia sinensis* and *Aspalathus linearis* have been shown to possess antiproliferative effects due to their antioxidative properties (Marnewick et al., 2000; Devaliya et al., 2007; Shcuck et al., 2008). The decrease in cell viability at the highest concentrations of either plant extracts observed in this study thus confirms this property.

9.6 CONCLUSION AND RECOMMENDATION

As mentioned earlier, several studies have shown that both *Camellia sinensis* and *Aspalathus linearis* are rich in antioxidants. These antioxidants are also thought to enhance reproductive functions and fertility, as they are able to scavenge and suppress the formation of reactive oxygen species. However, for normal physiological functions, a balance between the level of antioxidants and formation of reactive oxygen species needs to be established.

This present study demonstrated that *Camellia sinensis* and *Aspalathus linearis* could enhance reproductive functions and fertility. Beneficial effect of both plants was displayed by the positive effect on sperm concentration, viability and motility, which may be linked to their antioxidant properties. But due to the increased spontaneous acrosome reaction caused by these plants, fertilization of the oocyte might be affected. Despite the possible beneficial effects in reproduction, this study also revealed that large intake of either *Camellia sinensis* or *Aspalathus linearis* over a long period of time could result in subtle structural

changes in the male reproductive system and impair the liver and kidney function as seen by the increased levels of ALT and AST and creatinine.

Furthermore, this study demonstrated that both *Aspalathus linearis* (rooibos) and *Camellia sinensis* (tea) possess anti-androgenic property on TM3 Leydig cells. The mechanism of action by which both plants decreased testosterone production needs to be investigated and clarified. However, we hypothesize here that this might involve any of the following: by reducing 1) conversion of cholesterol to pregnenolone via the P450scc enzymes, 2) lowering the level of cAMP, 3) inhibition of 3β- and/or 17β-hydroxysteroid dehydrogenase. To the best of our knowledge, this is the first time in which the anti-androgenic property of rooibos and black tea on TM3 Leydig cells is demonstrated.

Camellia sinensis and *Aspalathus linearis* were shown to possess estrogenic properties as illustrated by the changes in the weights of the uteri and ovaries and hormonal levels of FSH and LH. To clarify the estrogenic properties in both plants, a detailed study on the adult and neonatal female rats is needed, including the possible effects on their oestrous cycle. Furthermore, detailed studies needs to be carried out to clarify the fertility outcome in both the male and female.

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APPENDICES

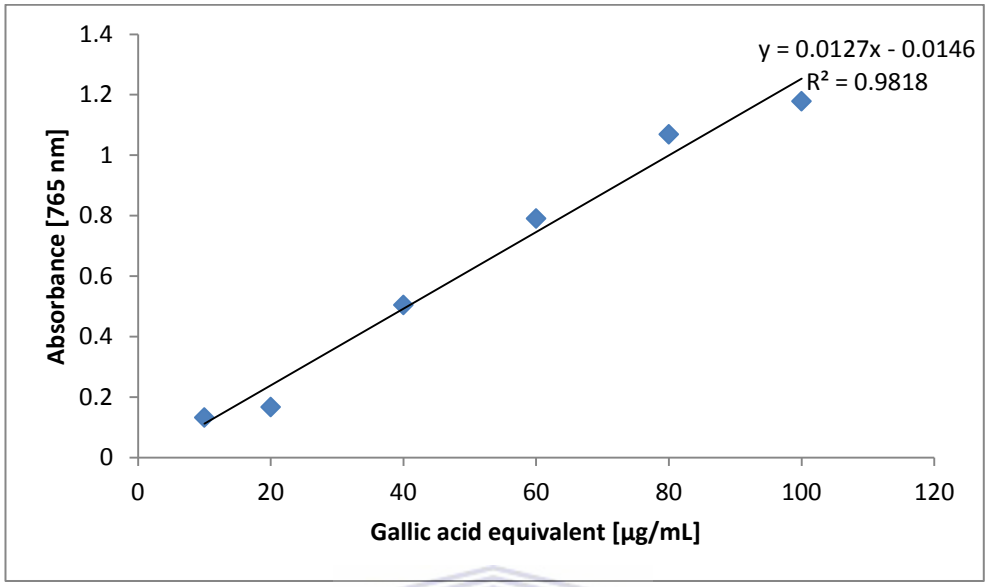
Appendix A: Gallic acid standard preparation for total polyphenol determination

Std Solution	Vol. of 0.1% Gallic acid (µL)	Vol. of dH_2O (mL)	Final vol. (mL)	Final dilution (µg/mL)
S1	16.7	1.65	1.67	10
S2	35	1.63	1.67	20
S3	66.7	1.6	1.67	40
S4	100	1.57	1.67	60
S5	133.3	1.53	1.67	80
S6	166.7	1.5	1.67	100

Appendix B: Preparation of tea and herbal infusions concentrations using the suggested dilutions for total polyphenols determination

Test tubes	Dilution	Tea (µL)	Solvent (µL)	Total Vol. (mL)
Green	25x	40	960	1
	45x	22	978	1
	65x	15.4	984.6	1
Black	25x	40	960	1
	45x	22	978	1
	65x	15.4	984.6	1
Unfermented Rooibos	15x	66	934	1
	25x	40	960	1
	35x	28	972	1
Fermented Rooibos	25x	40	960	1
	35x	28	972	1
	45x	22	978	1

Appendix C: Standard curve for the determination of total polyphenols



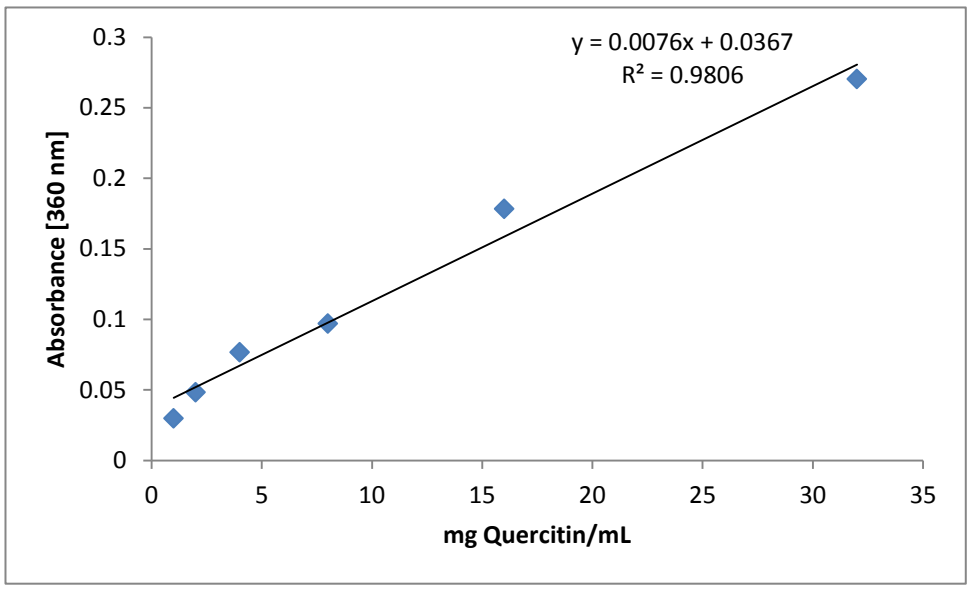
Appendix D: Quercitin standard for determination of flavonol

Test tubes	Quercitin stock (µL)	95% EtOH (µL)	Quercitin EtOH (mg/mL)	Final volume (mL)
Blank	-	1000	-	1
S1	200	800	8	1
S2	100	900	4	1
S3	50	950	2	1
S4	25	975	1	1

Appendix E: Dilution of tea and herbal infusion for the determination of flavonol

Test tubes	Dilution	Plant extract (µL)	Solvent (µL)	Final volume (mL)
Blank	-	-	1000	1
Green tea	5x	200	800	1
	10x	100	900	1
	15x	70	930	1
Black tea	5x	200	800	1
	10x	100	900	1
	15x	70	930	1
Unfermented rooibos	5x	200	800	1
	10x	100	900	1
	25x	40	960	1
Fermented rooibos	10x	100	900	1
	15x	70	930	1
	25x	40	960	1

Appendix F: Standard curve for the determination of flavonol



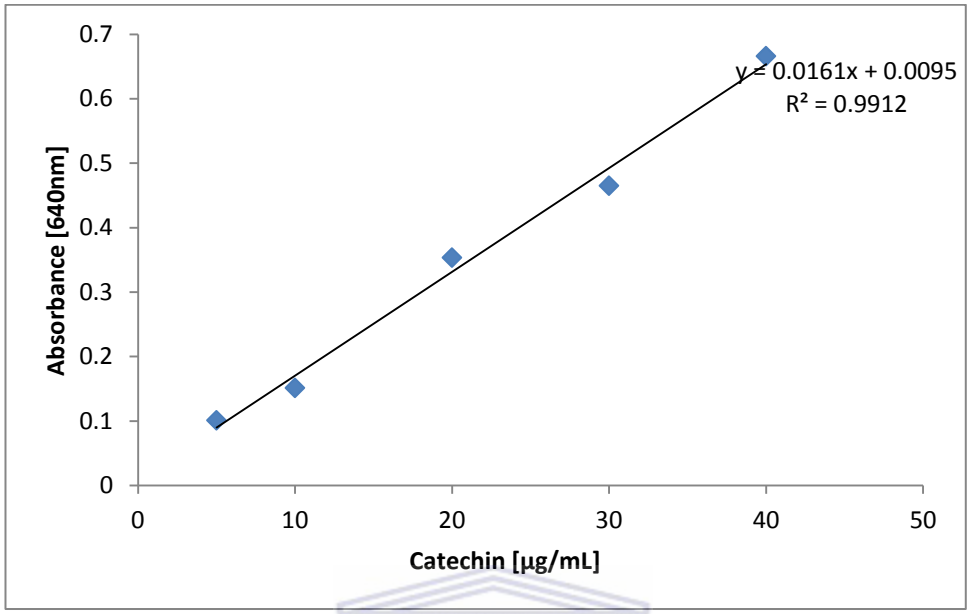
Appendix G: Catechin standard for determination of flavanol

Test tubes	Catechin stock (µL)	MeOH (µL)	Catechin (µg/mL) MeOH	Final dilution	Final volume (mL)
S1	5	995	5	200x	1
S2	10	990	10	100x	1
S3	20	980	20	50x	1
S4	30	970	30	33.33x	1
S5	40	960	40	25x	1

Appendix H: Dilution of tea and herbal infusions for determination of flavanol

Test tubes	Dilution	Plant extract (µL)	Solvent (mL)	Total volume (mL)	Suggested incubation time (min)
Blank	-	-	1.0	1.0	-
Green tea	150x	0.007	0.993	1.0	5
	180x	0.006	0.994	1.0	5
	200x	0.005	0.995	1.0	5
Black tea	80x	0.013	0.987	1.0	5
	100x	0.01	0.99	1.0	5
	120x	0.008	0.992	1.0	5
Unfermented rooibos	100x	0.01	0.99	1.0	15
	110x	0.009	0.991	1.0	15
	120x	0.008	0.992	1.0	15
Fermented rooibos	15x	0.067	0.933	1.0	15
	30x	0.033	0.967	1.0	15
	45x	0.022	0.978	1.0	15

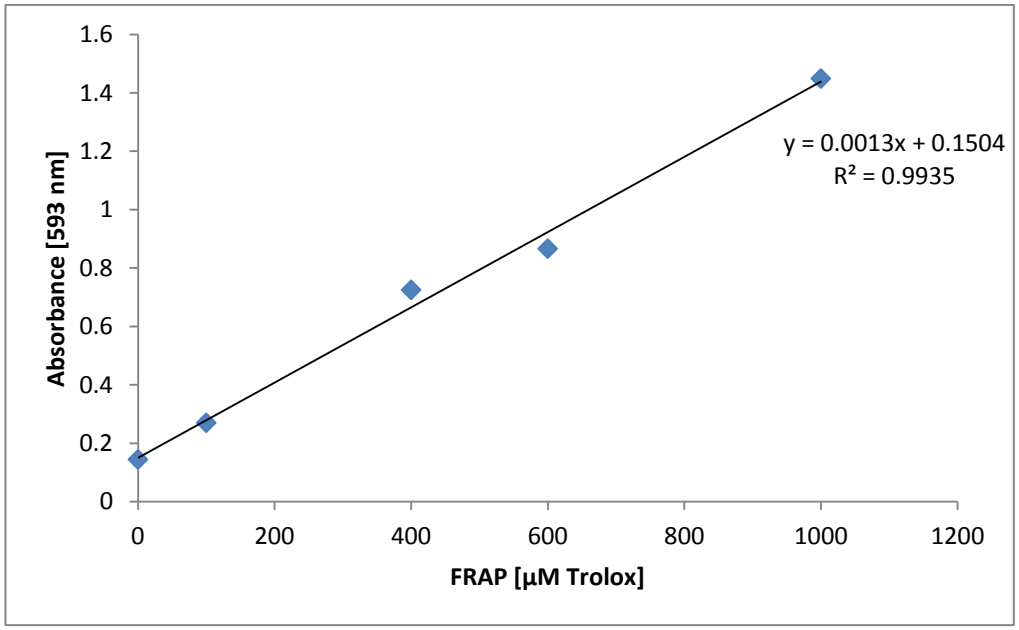
Appendix I: Standard curve for the determination of flavanol.



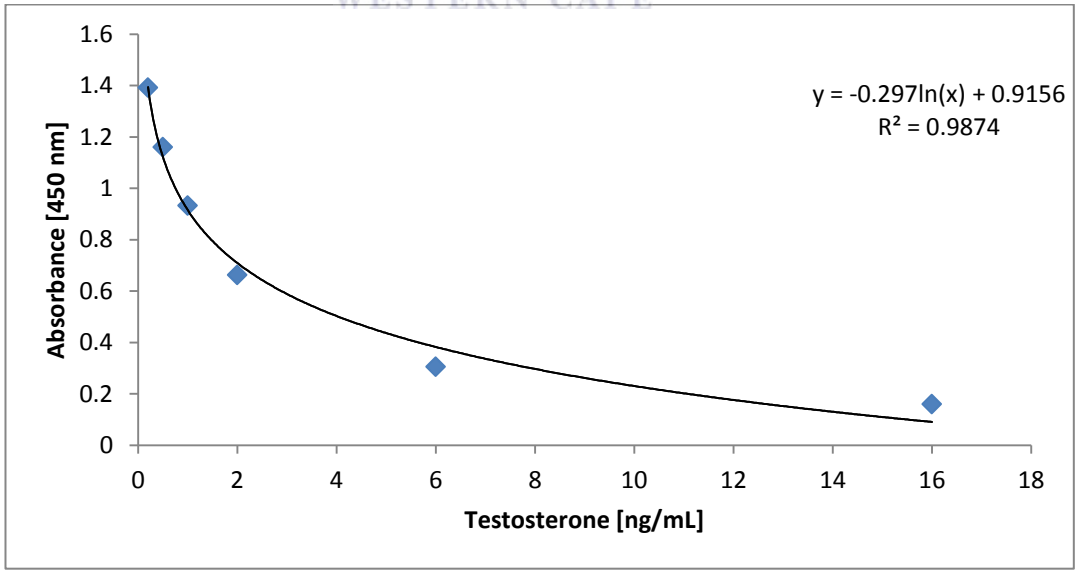
Appendix J: Preparation of standards for ferric reducing antioxidant power (FRAP)

Standards	Trolox (µM)	Vol. of trolox (µL)	Vol. of MeOH (µL)	Final volume (mL)
S1	100	20	980	1
S2	150	30	970	1
S3	200	40	960	1
S4	400	80	920	1
S%	600	120	880	1
S6	1000	200	800	1

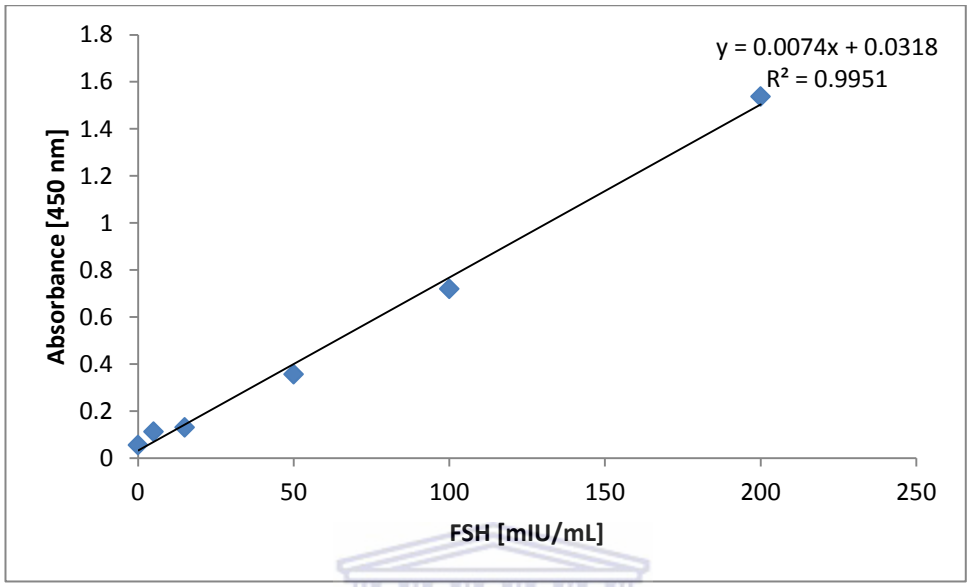
Appendix K: Standard curve for the determination of FRAP



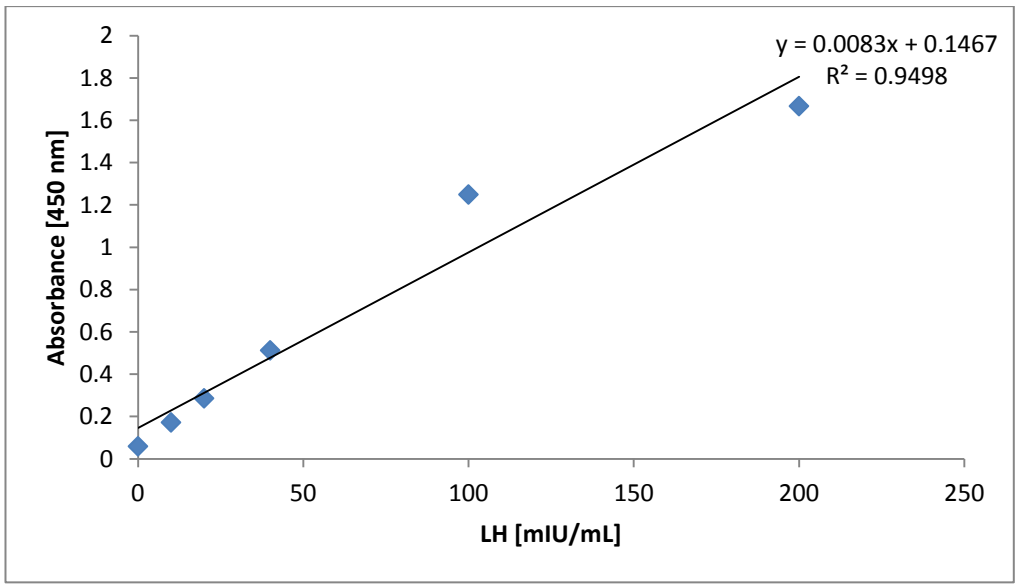
Appendix L: Standard curve for the determination of testosterone production



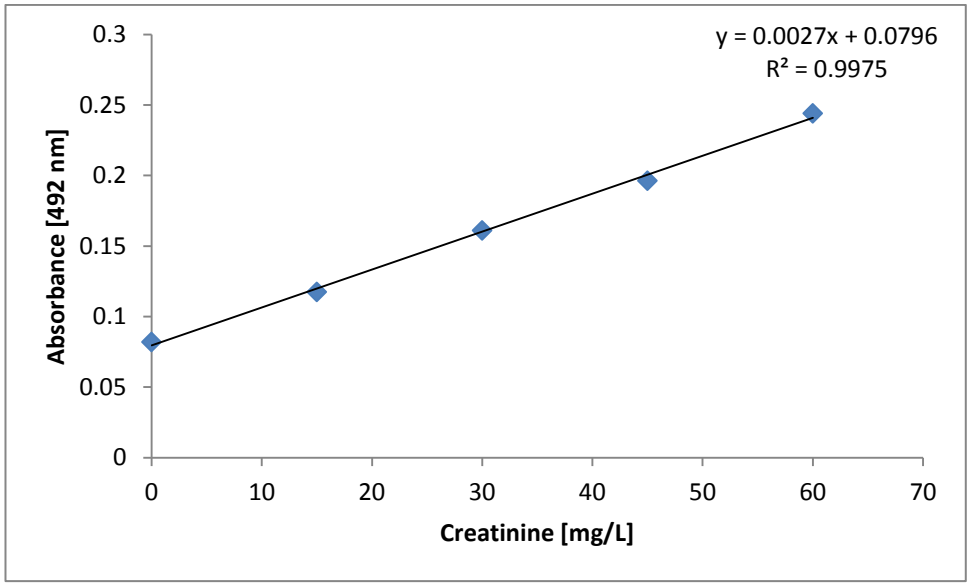
Appendix M: Standard curve for the determination of follicle stimulating hormone production



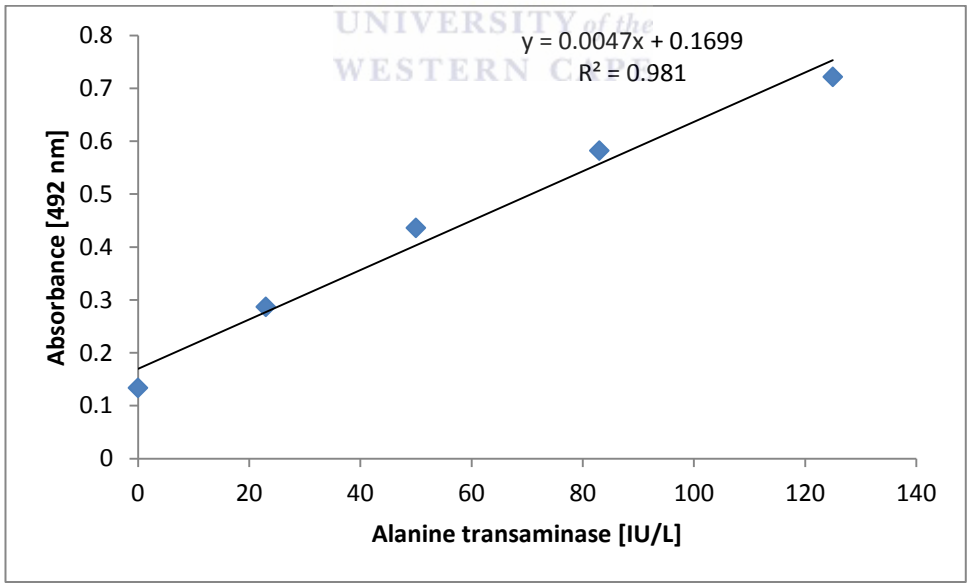
Appendix N: Standard curve for the determination of luteinizing hormone production



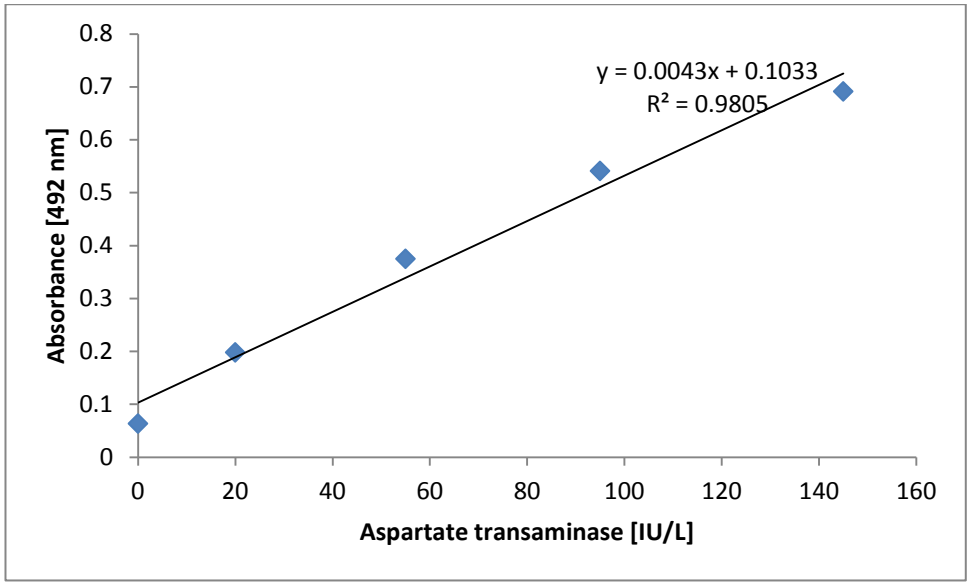
Appendix O: Standard curve for the determination of creatinine in serum



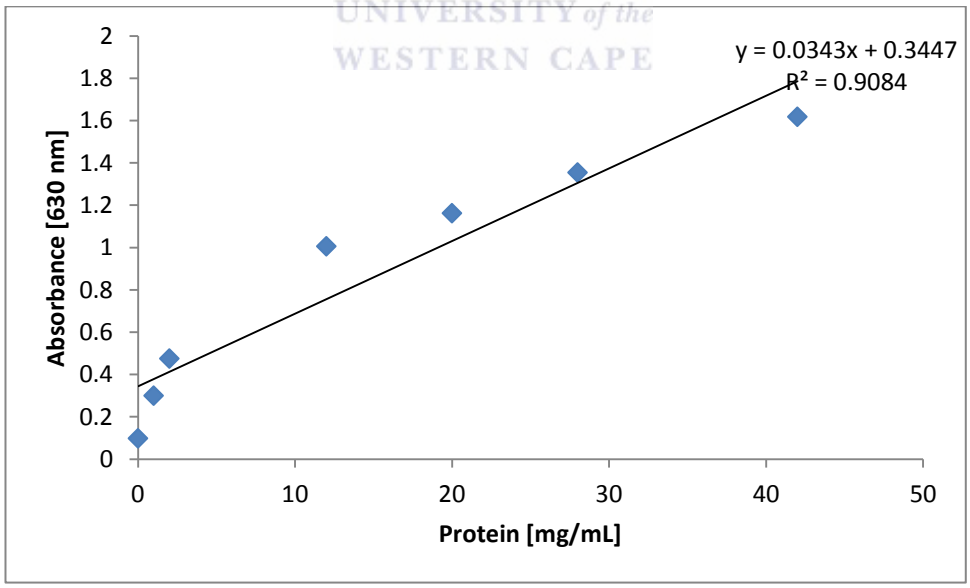
Appendix P: Standard curve for the determination of alanine transaminase in serum



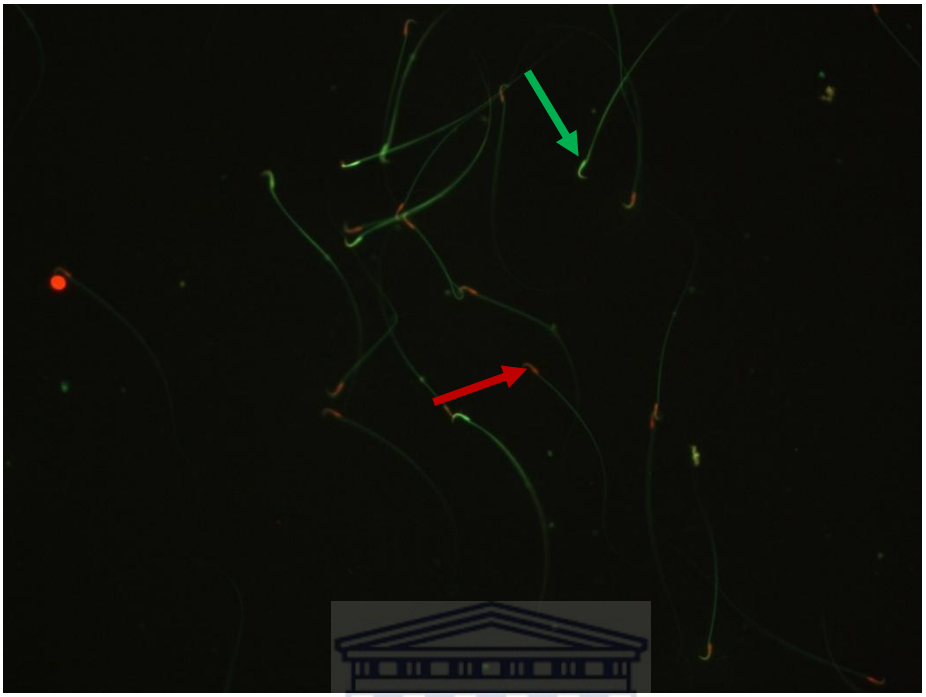
Appendix Q: Standard curve for the determination of aspartate transaminase in serum



Appendix R: Standard curve for protein determination



Appendix S: Vitality test on rat sperm



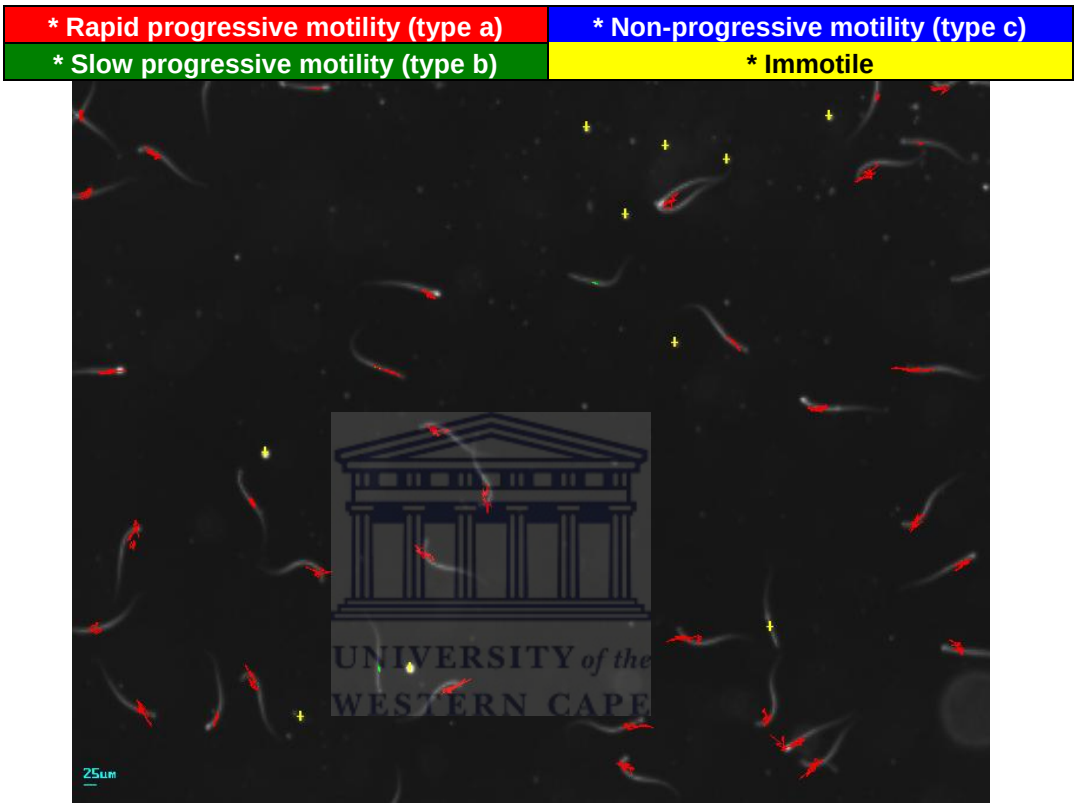
Green arrow (live sperm) and red arrow (dead sperm)

Appendix T: Definition of sperm parameters*

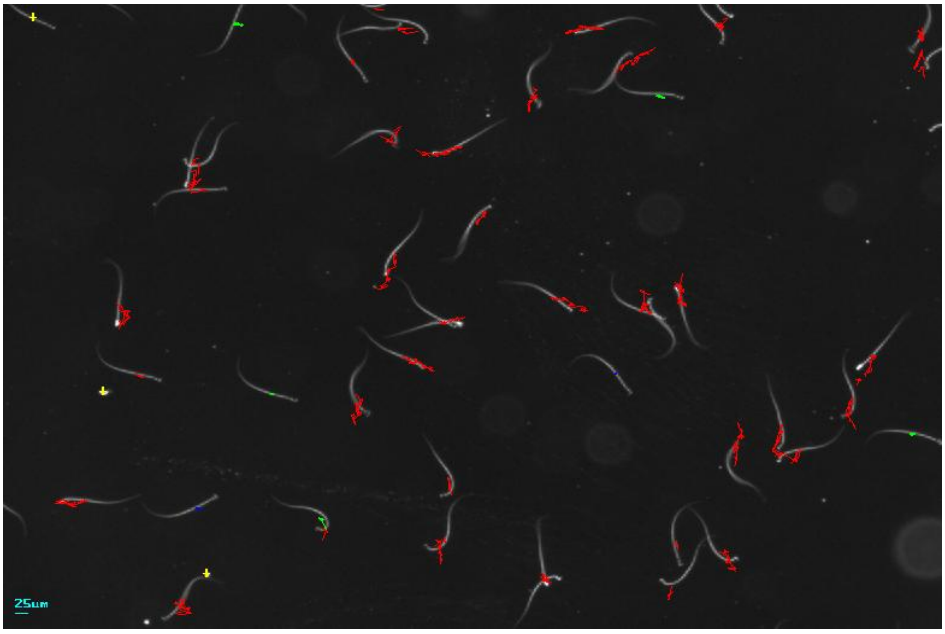
Parameter	Abbreviation	Definition	Unit
Total motility	-	Percentage motility of spermatozoa swimming with a VCL > 20 µm/s and VSL > 9 µm/s and the rest were regarded as immotile	%
Progressive motility	-	Percentage motility on only spermatozoa swimming in a progressively forward direction with a LIN> 30%, VAP > 20 µm/s and VSL > 30 µm/s	%
Curvilinear velocity	VCL	Time- average velocity of sperm head along its actual path	µm/s
Straight line velocity	VSL	Time- average velocity of sperm head projected along straight line between its first and last detected position	µm/s
Average path velocity	VAP	Time- average velocity of sperm head projected along its average spatial trajectory	µm/s
Linearity	LIN	Ratio of projected length to total length of curvilinear trajectory; LIN= VSL/VCL	%
Wobble	WOB	Expression of the degree of oscillation of the curvilinear path about its spatial average path; WOB = VAP/VCL	%
Amplitude of lateral head displacement	ALH	Maximum amplitude of lateral distance of the sperm head trajectory about its spatial average path	µm
Straightness	STR	Expression of the straightness of average path; STR = VSL/VAP	%
Beat cross frequency	BCF	Frequency of sperm head crossing the sperm average path in either direction	Hz

*From van der Horst et al., 1999 with slight modification.

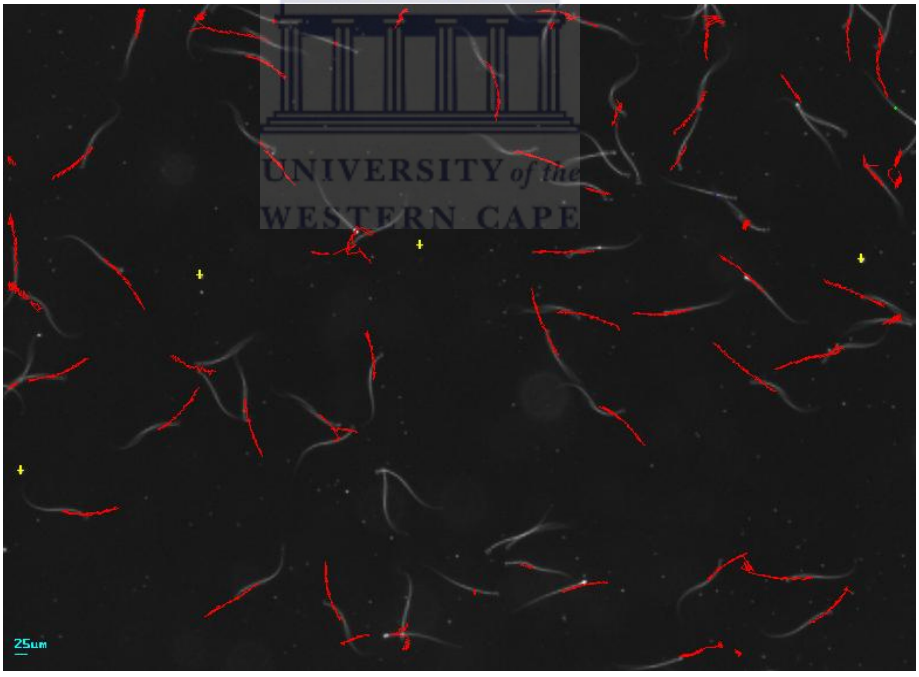
Appendix U: Random representation of motility of rat sperm in the various treatment groups.



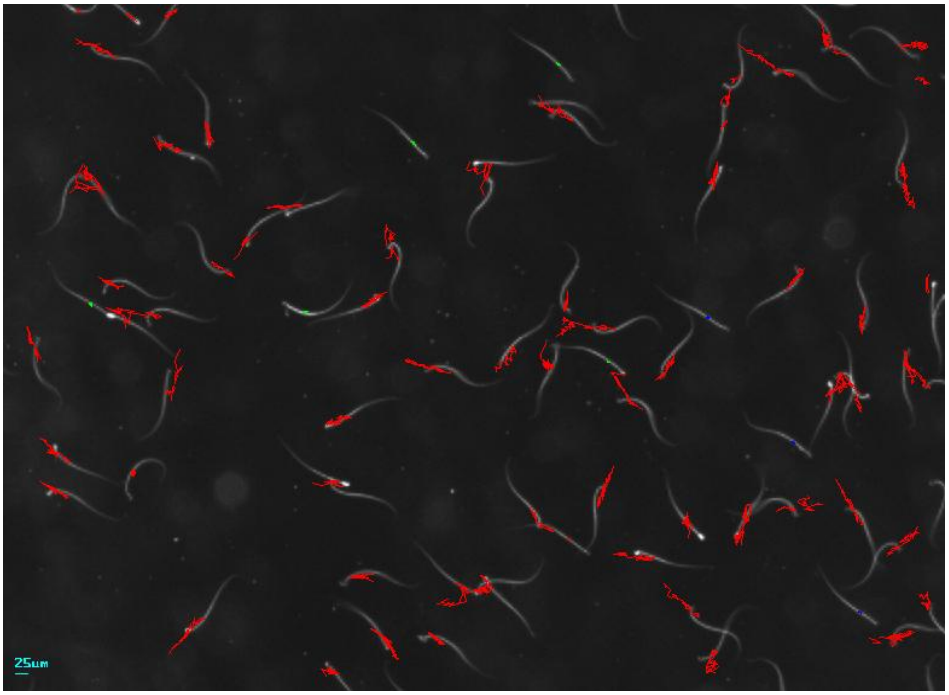
Control



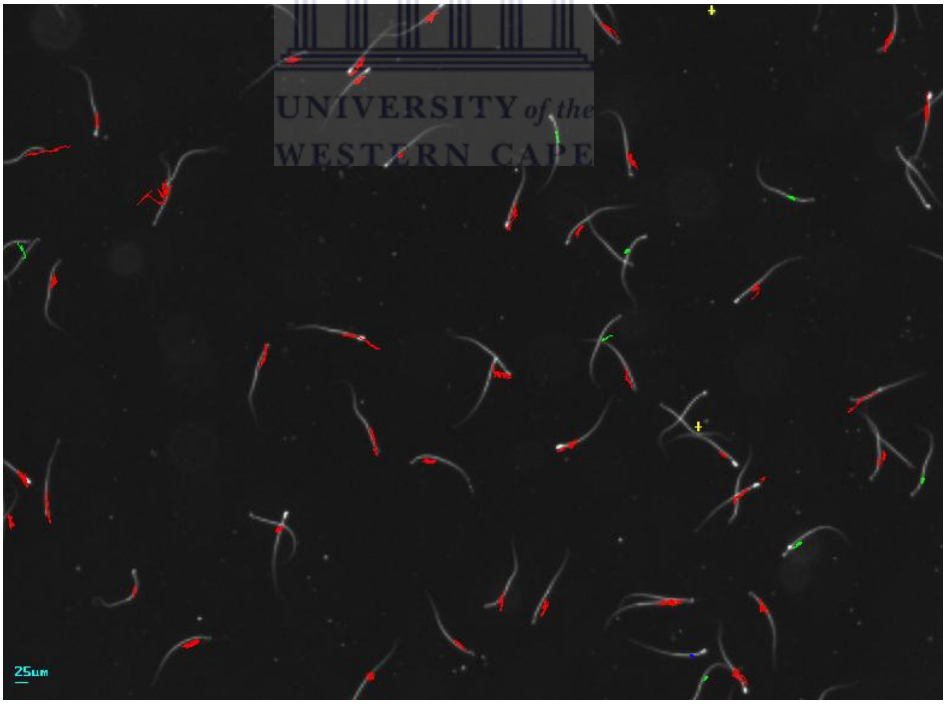
2% Green tea



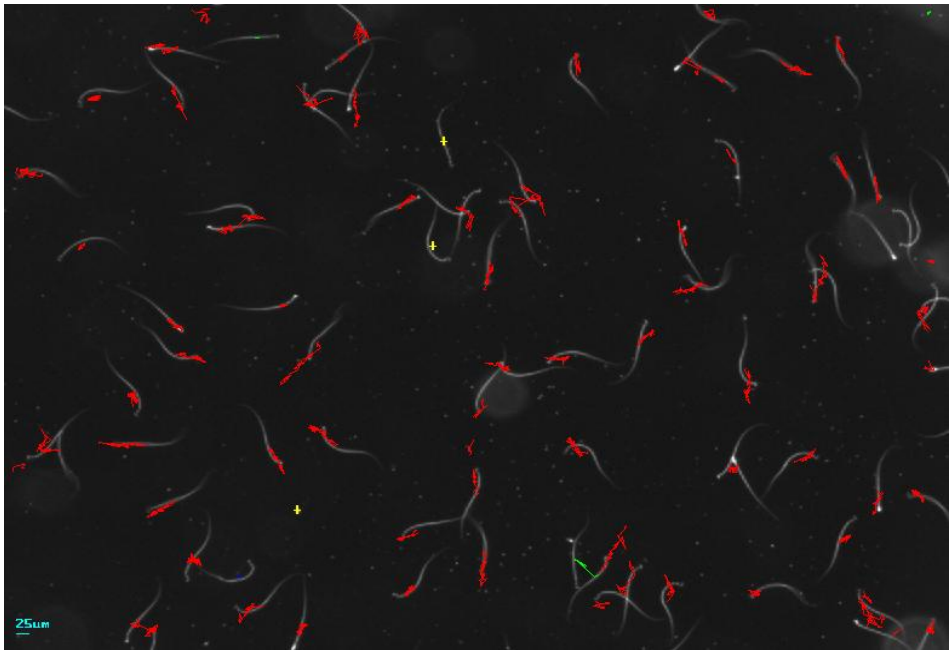
5% Green tea



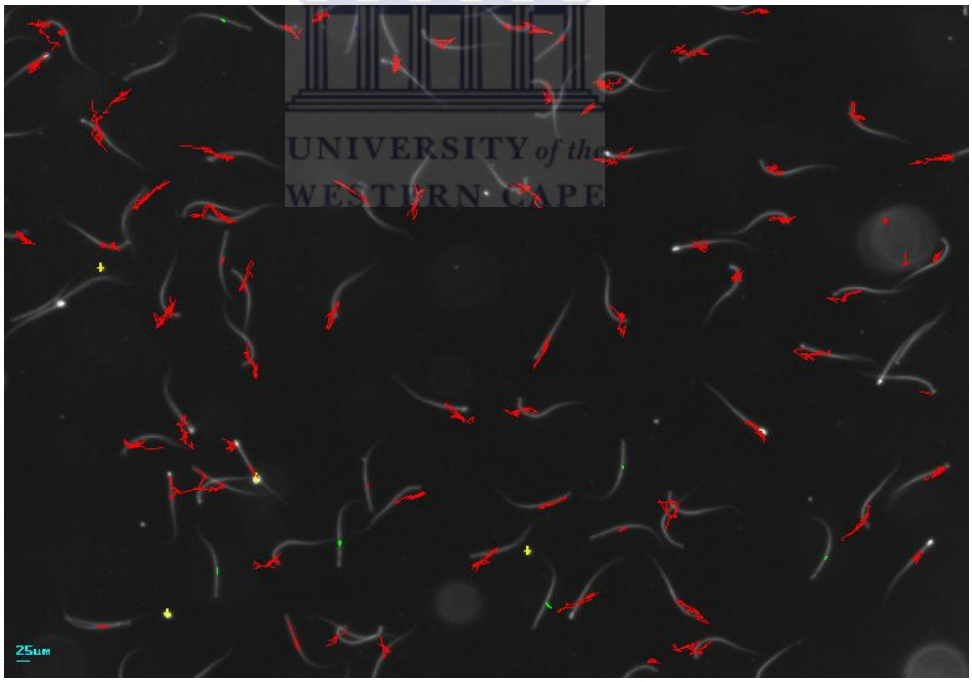
2% Black tea



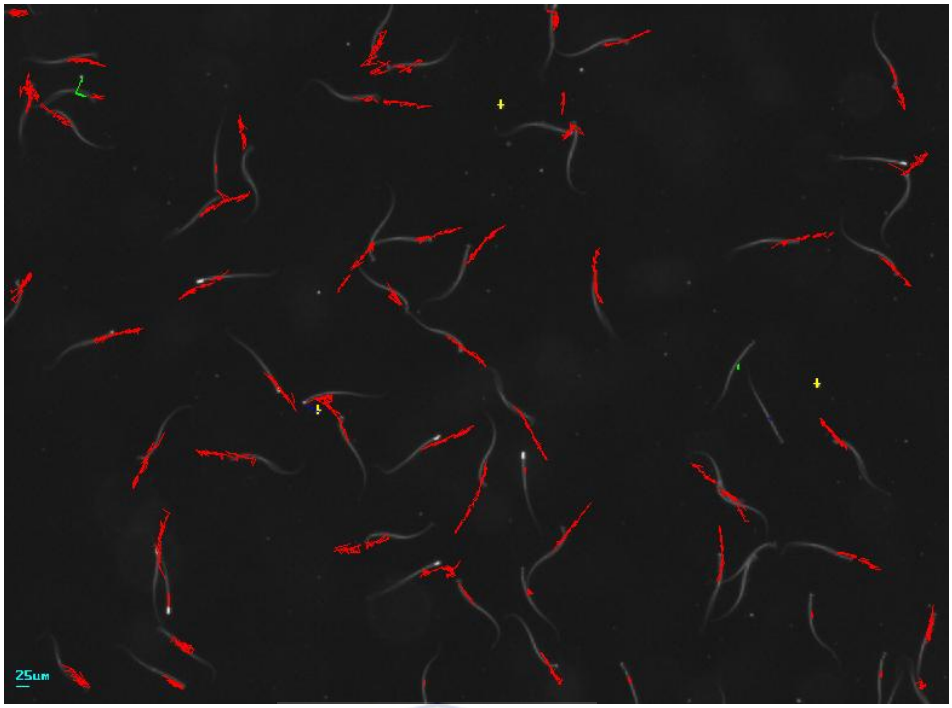
5% Black tea



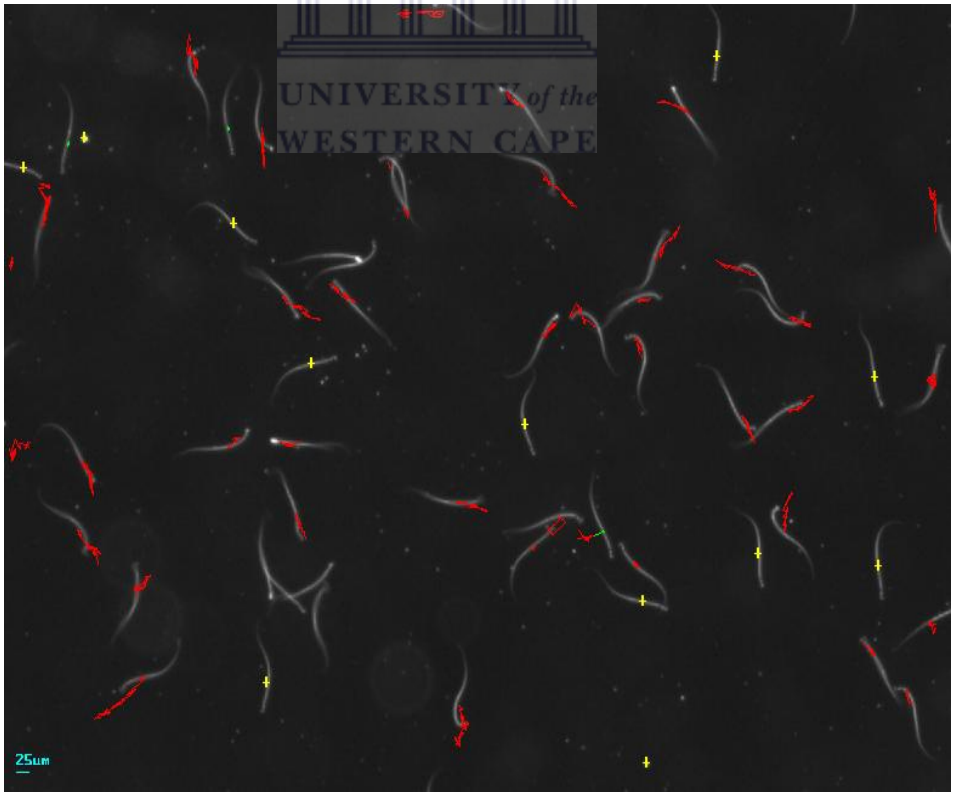
2% Unfermented rooibos



5% Unfermented rooibos

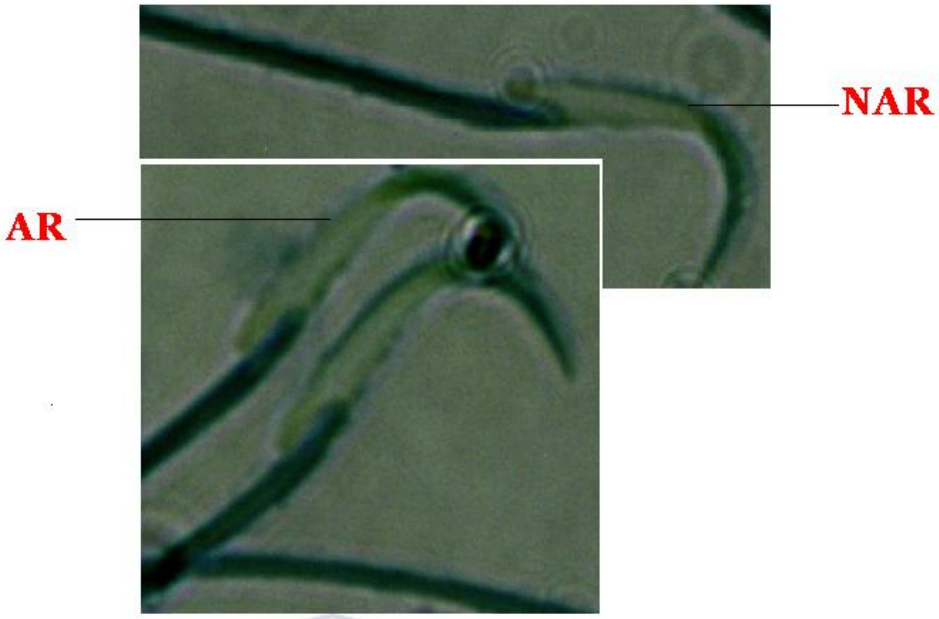


2% Fermented rooibos



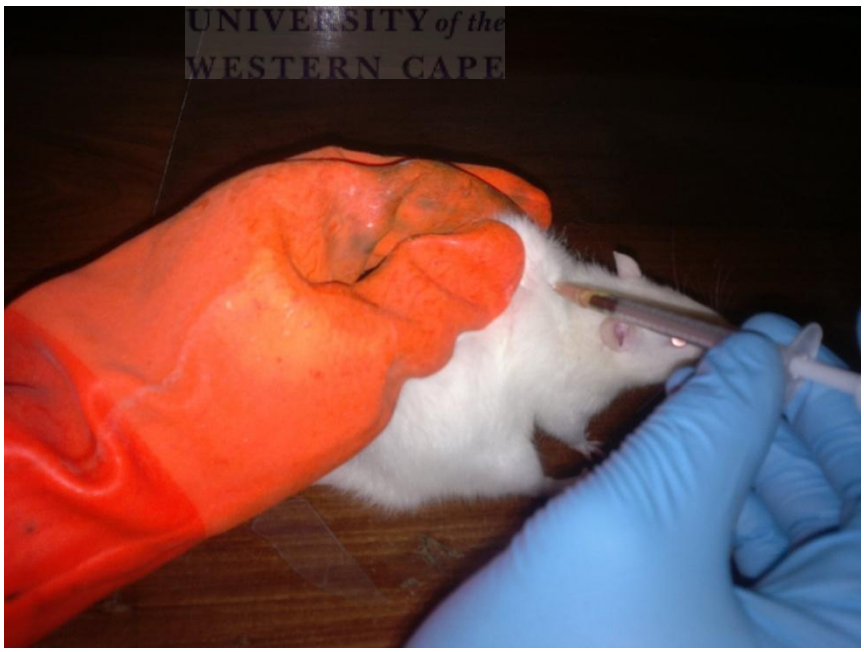
5% Fermented rooibos

Appendix V: Acrosome reaction in rat sperm

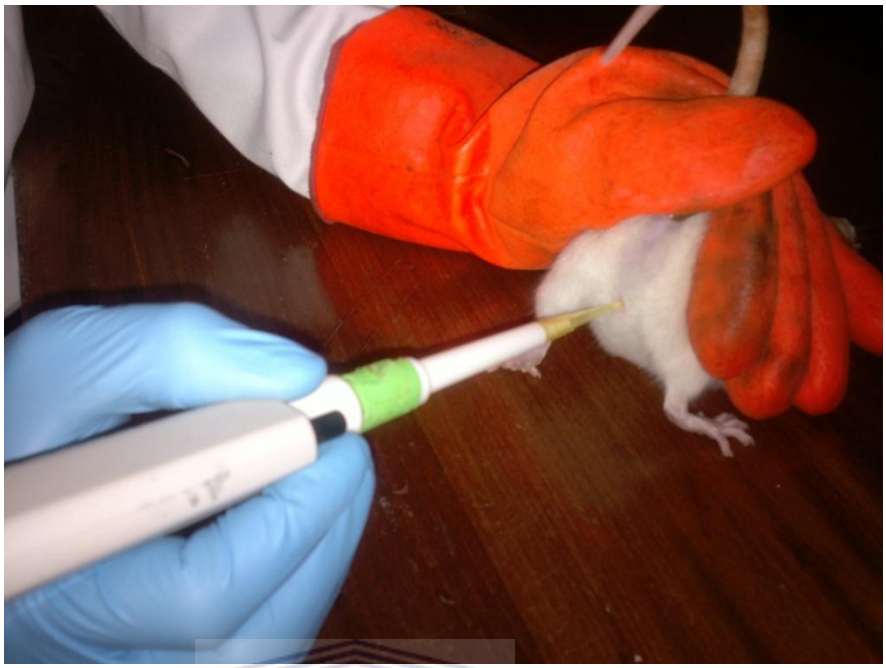


AR, Acrosome reacted spermstozoa; NAR, non acrosome reacted spermatozoa

Appendix W: Subcutaneous injection in female rat



Appendix X: Procedure for obtaining vaginal smear in the female rat



Appendix Y: Dissected pregnant female rat