EFFECTS OF GREEN, BLACK AND ROOIBOS TEA, COFFEE AND BUCHU ON TESTOSTERONE PRODUCTION BY MOUSE TESTICULAR CULTURES

by

Zaroug A.M. Abuaniza

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Department of Medical Biosciences
University of the Western Cape
South Africa

Supervisor: Professor E.J. Pool

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DECLARATION

I, **Zaroug A. M. Abuaniza**, hereby declare that the dissertation "**Effects of Green, Black and Rooibos Tea, Coffee and Buchu on Testosterone Production by Mouse testicular Cultures**" for the Masters degree in Science (MSc) at the University of the Western Cape hereby submitted by me has not been submitted previously at this or any other university, and that it is my own work in design and in execution, and that all materials contained herein have been duly acknowledged by complete references.

Zaroug A. M. Abuaniza **:** .....

Date Signed **:** .....

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ABSTRACT

Modulation of the male reproductive system occurs as a result of exposure to endocrine disrupting compounds (EDCs) in different life stages. The effects of EDCs on the male reproductive system include infertility, decreased sperm count, function and morphology, abnormal development of secondary sex characteristics, reproductive function and sexual behavior, as well as decreased libido. Phytochemicals are naturally occurring, biologically active chemical compounds in plants. They are divided into different groups. Isoflavonoids and lignans, are the two major groups of phytoestrogens. Phytoestrogens of teas, coffee and buchu have many beneficial effects on body systems such as antimutagenic, antidiabetic, anti-inflammatory, antibacterial and antiviral properties. They also elicit many adverse events, for example, heavy consumption of green and black tea may cause liver damage and added unwanted effects when combined with other herbal beverages. Chronic heavy consumption of coffee is positively correlated to acute myocardial infarction and can elevate serum cholesterol levels. Rooibos tea decreases steroidogenesis by steroid secreting cell lines.

This study investigated the effects of these beverages on the male reproductive system, using a minced testes method for determination of cell viability and hormone (testosterone) production. The first objective of this study was to optimize protein supplement for *in vitro* testosterone production using human serum albumin (HSA) and foetal bovine serum (FBS). Testicular cultures were prepared and exposed overnight to different concentrations of both sera and then incubated for 4 hours with or without luteinizing hormone (LH). The results showed that addition of protein

supplements (HSA or FBS) did not have a significant effect on testosterone production. The second objective of this study was to investigate the effects of green tea, black tea, rooibos tea, coffee and buchu on cell viability of testicular cultures. Cells were treated overnight with varying concentrations of the plant extracts followed by incubation with/without LH for 4 hours. The effects of the plant beverages on cellular protein production were determined by the Bradford assay. The results showed that treatment of cells with varying concentrations of the plant extracts (with/without LH-treatment) had no significant effect on total cellular protein. The third objective of this study was to investigate the effects of black, green and rooibos teas, coffee and buchu on testosterone production by testicular cultures. The results obtained from these experiments showed that rooibos tea and buchu did not affect testosterone production in the presence or absence of LH. The results also indicated that green tea, black tea and coffee inhibited testosterone production by mouse testis cultures in the presence of LH, but not in the absence of LH. Black tea was the most potent inhibitor of testosterone synthesis by mouse testis cultures (IC_{50}= 48 µg/ml), followed by coffee (IC_{50}= 64 µg/ml) and green tea (IC_{50}= 173 µg/ml). Green tea, black tea and coffee inhibited LH-stimulated testosterone synthesis, suggesting that these beverages may impair testicular steroidogenesis in mice. Thus, in spite of their acclaimed beneficial effects, consumption of these beverages in high doses raises concerns for their inhibitory effects on male reproductive function. Further *in vitro* and *in vivo* studies are warranted to determine their exact mechanisms of action on the male reproductive system in general and testicular function in particular.

Keywords: male reproductive system, testosterone production, testicular cultures, endocrine disrupting compounds, phytochemicals, green and black tea, rooibos tea, coffee, buchu, consumption, beneficial effects, adverse events.

LIST OF ABBREVIATIONS

ADI	Acceptable Daily Intake
ANOVA	Analysis of Variance
As	Arsenic
ATSDR	Agency for Toxic Substances and Disease Registry
BPA	Bisphenol A
BSA	Bovine Serum Albumin
Bw	Body Weight
cAMP	3'-5'-Cyclic Adenosine Monophosphate
˚C	Degrees Celsius
CANSA	Cancer Association of South Africa
Cd	Cadmium
cells/ml	Cells per Milliliter
CO_2	Carbon Dioxide
Cr	Chromium
Cu	Copper
DBP	Di-butyl-phthalate
DDT	Dichlorodiphenyltrichloroethane or 1,1,1-Trichloro-2,2-Di-(4-Chlorophenyl)-Ethane (IUPAC ID)
DES	Diethylstilbestrol
DHHS	Department of Health and Human Services
DON	Deoxynivalenol
EDCs	Endocrine Disrupting Compounds
EE2	17α-Ethinylestradiol
ELISA	Enzyme-Linked Immunosorbant Assay
EPA	Environmental Protection Agency
$Fe_2Cr_2O_4$	Chromite
FSH	Follicle-Stimulating Hormone

G	Grams
GnRH	Gonadotropin-Releasing Hormone
HCH	Hexachlorocyclohexane
HCPMRA	Health Canada Pest Management Regulatory Agency
3ß-HSD	3ß-Hydroxysteroid Dehydrogenase
17ß-HSD	17ß-Hydroxysteroid Dehydrogenase
HPTA	Hypothalamic-Pituitary-Testicular Axis
LH	Luteinizing Hormone
LOEC	Lowest Observed Effect Concentration
LOAEL	Lowest Observed Adverse Effect Level
µg/dl	Micrograms per Decilitre
µg/g	Micrograms per Gram
µg/kg/day	Micrograms per Kilogram per Day
µg/ml	Micrograms per Millilitre
µg/l	Micrograms per Litre
µl	Microlitre
mg/ml	Milligrams per Millilitre
Ml	Millilitre
mu/ml	Milliunits per Millilitre
MBP	Mono-butyl-phthalate
ng/l	Nanograms per Litre
NOAEL	No Observed Adverse Effect Level
NOEC	No Observed Effect Concentration
OEHHA	The California Office of Environmental Health Hazard Assessment
P450c17	Cytochrome P450 17α-Hydroxylase/C17-20 Lyase
P450SCC	Cytochrome P450 Side-Chain Cleavage Enzyme
Pb	Lead
PBBs	Polybrominated Biphenyls
PBDEs	Polybrominated Diphenyl Ethers

PC	Polycarbonate
PCBs	Polychlorinated Biphenyls
pg/ml	Picograms per Millilitre
PVC	Polyvinyl Chloride
s.c.	Subcutaneous
SGP	Scientific Guidance Panel
StAR	Steroid Acute Regulatory Protein
TCC	Triclocarban
WHO	World Health Organization
Zn	Zinc

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CHAPTER 1

THE MALE REPRODUCTIVE SYSTEM

1.1 Structure and Physiology of the Male Reproductive System

The male reproductive system consists of the testes, epididymis, ductus deferens, seminal vesicles, prostate gland, ejaculatory ducts, bulbourethral glands, penis, urethra and scrotum. The development and maturation of the male reproductive system is regulated by male reproductive hormones. The production and secretion of sex hormones in male are regulated by the hypothalamic-pituitary-testicular axis (HPTA) (Figure 1.1) (Seeley & Stephens, 2003). The hypothalamus secretes gonadotropin releasing hormone (GnRH) which stimulates the release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the pituitary gland (Heindel & Treinen, 1989). LH regulates the synthesis and release of testosterone from Leydig cells. FSH and testosterone bind to specific receptors on Sertoli cells to stimulate spermatogenesis. Sertoli cells secrete inhibin which negatively regulates the synthesis and release of FSH from the anterior pituitary gland (De Kretser *et al*., 2004). Testosterone acts by a negative-feedback mechanism to stop the release of GnRH from the hypothalamus. This inhibits the secretion of LH and FSH from the pituitary gland (Seeley & Stephens, 2003).

1.2 Testosterone

Steroid hormones (estrogens, progestins and androgens) regulate the development of both male and female sexual and reproductive processes in human and animals (Tabb

& Blumberg, 2006). Testosterone is the most important steroid hormone in male reproduction (Kim & Moon Du, 2011). Testosterone is produced by Leydig cells of testes (interstitial cells) in response to stimulation by LH, which is produced by the pituitary gland.

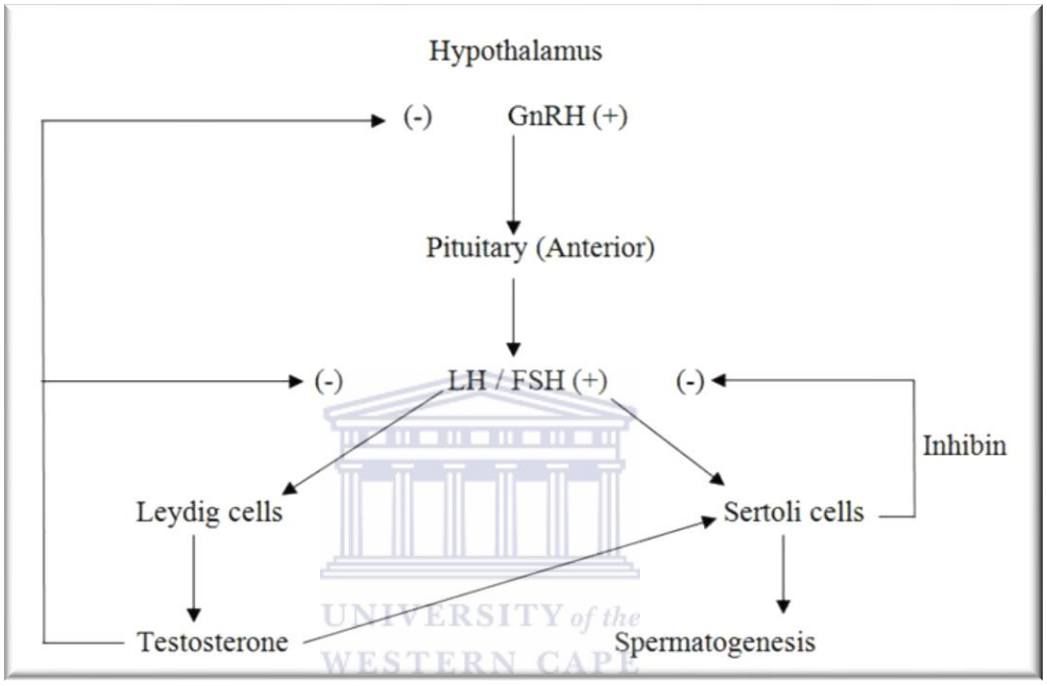

Figure 1.1: Summary of the hypothalamic-pituitary-testicular axis (HPTA) (Seeley & Stephens, 2003).

The production of testosterone can be rapid and is characterized by increased production of a specific protein called steroid acute regulatory protein (StAR). Slow production of testosterone also occurs. It is influenced by the effect of cyclic adenosine monophosphate (cAMP) (Caron *et al*., 1997; Manna & Stocco, 2005). Production of testosterone starts by P450 side chain cleavage of cholesterol, which is the precursor of all steroid hormones. P450 side chain cleavage ($P450_{scc}$) enzyme is located on the inner mitochondrial membrane. This enzyme acts on the side chain of

the cholesterol molecule to produce pregnenolone. This step is called the enzymatic rate-limiting step of steroidogenesis (Walsh *et al*., 2000).

Pregnenolone is then converted to progesterone in the smooth endoplasmic reticulum by the action of 3β-hydroxysteroid dehydrogenase (3β-HSD), after which progesterone is converted to 17α-hydroxyprogesterone, by the action of 17α-hydroxylase-17-20 lyase. The 17α-hydroxyprogesterone is converted to androstenedione by the action of the same enzyme and, finally, the androstenedione is converted to testosterone by the action of 17β-hydroxylase (Figure 1.2). Serum testosterone levels decline gradually with aging in men (Kim & Moon Du, 2011). The decrease in testosterone levels is accompanied by adverse effects such as decreased libido and erectile problems, osteoporosis, and loss of muscle mass (Isidori *et al.*, 2005; Cattabiani *et al.*, 2012).

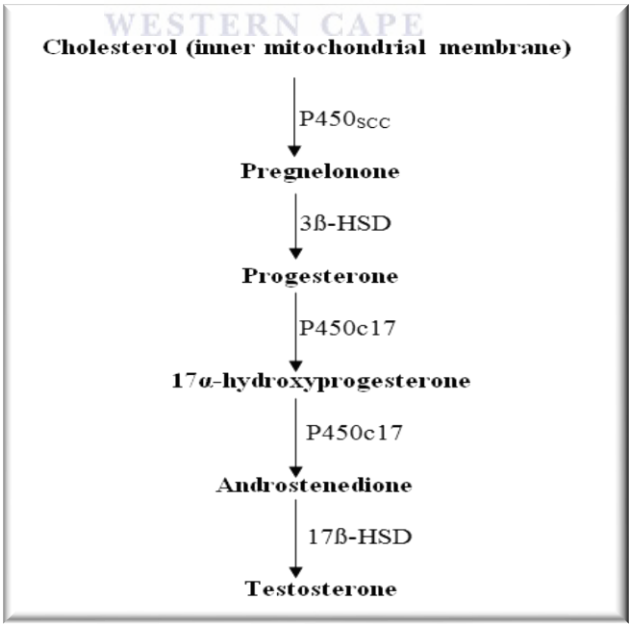

Figure 1.2: Testosterone synthesis from cholesterol within the Leydig cells (Seeley & Stephens, 2003).

1.3 Modulation of the Male Reproductive System

A wide range of substances, both natural and man-made, are thought to cause adverse effects on the male reproductive system, including environmental chemicals such as industrial chemicals and their by-products, pesticides, and heavy metals (Diamanti-Kandarakis *et al.*, 2009), pharmaceuticals and personal care products such as diethylstilbestrol DES (Folmar *et al.*, 2002), and phytoestrogens such as isoflavones, coumestans and lignans (Rice & Whitehead, 2008).

1.3.1 Industrial Chemicals and Their By-Products

These are synthetic chemicals or petroleum products and/or their by-products, such as dioxins, polychlorinated biphenyls (PCBs), and polybrominated biphenyls (PBBs) and polybrominated diphenylethers (PBDEs) (Diamanti-Kandarakis *et al.*, 2009). The structures and effects of industrial chemicals associated with reproductive toxicity are presented in Table 1.1. Dioxins are by-products of many industrial processes, such as manufacturing of pesticides. They can also be the result of natural processes such as volcanic activities and forest fires (WHO, 2010). Dioxins are defined by the World Health Organization (WHO) as a group of related organic chemicals that are highly resistant environmental pollutants with an estimated half-life of 7-11 years inside the body (WHO, 2010).

PCBs are a class of persistent organic pollutants that were widely used in the mid-20th century. Though their production and use was banned by most countries several decades ago, the general population continues to be exposed due to the persistence and bioaccumulation of PCBs. Both PBBs and PBDEs are lipophilic, halogenated very stable organic chemicals.

Table 1.1: The effects of industrial chemicals on the male reproductive system

Name	Effects on male reproduction	References
Dioxins	1 µg/kg/day on gestation day 15 reduces daily sperm production and decreases weight of prostate lobes in male offspring rat.	Simanainen *et al*., 2004
	In utero exposure to dioxins is associated with hypospadias in human and animal new-borns	WHO, 2011
Polychlorinated biphenyls (PCBs)	200 µg/kg/day for 30 days lowers serum testosterone and estradiol levels in rat *in vivo.*	Murugesan *et al*., 2005
	Occupational exposure to PCBs leads to cryptorchidism in newborns and decreases semen quality in adults.	WHO, 2011
	PCBs inhibits basal and LH-stimulated testosterone production from Leydig cell *in vitro.*	Murugesan *et al*., 2008
Polybrominated diphenylethers (PBDEs)	60 or 300 µg/kg on gestation day 6 reduces sperm and spermatid counts in male offspring rat.	Kuriyama *et al.*, 2005
	Occupational exposure to PBDEs leads to cryptorchidism in newborns and decreases semen quality in adults.	WHO, 2011
Bisphenol A (BPA)	100 µg/rat/day for 14 days decreases the plasma free testosterone and increases LH levels in rat.	Tohei *et al.*, 2001
	Exposure to BPA has been linked to low testosterone production and prostate cancer .	CANSA, 2008
	2 µg/kg /day to pregnant mice increases prostate weight in male offspring when they get to adulthood.	Welshons *et al*, 1999
	10 µg/kg/day during the first postnatal period causes prostate cancer in rat.	Diamanti-Kandarakis *et al*., 2009
Phthalates	65000 µg/kg/day reduces sperm counts and induces reproductive tract malformations in rat.	Foster *et al.*, 2000
	750 µg/kg/day from gestational day 14 to postnatal day 3 in rat reduces testis weights and anogenital distance	Gray *et al*., 2000; Parks *et al*., 2000
	50000 µg/kg/day on gestation days 12 to 21 in rat reduces anogenital distance, weight of the testis, epididymis and seminal vesicles.	Mylchreest *et al*., 2000
	Maternal exposure to high levels of phthalates leads to reduced anogenital distance and undescended testes.	WHO, 2011
	A single dose of 50000 µg/kg/day di-n-butyl-phthalate (DBP) or mono-butyl-phthalate (MBP) on gestation day 19.5 decreases intratesticular testosterone levels and cytochrome P450 expression.	Hallmark *et al*., 2007
	Phthalates impair foetal mouse germ cell number *in vitro.*	Lehraiki *et al*., 2009

They are used as flame retardants in many products such as electrical appliances, laptop cabinets and some plastic products to make them more resistant to burning (ATSDR, 2004). The production of PBBs in the US was stopped in 1976 following the accidental contamination in Michigan when PBBs were accidentally given to farm animals as feed additives instead of magnesium oxide.

During the last two decades PBDEs have been detected all over the world in water, air, sediments and soil samples. Lowest observed effect concentration (LOEC) of PBBs is 0.3 mg/kg/day induced blood levels in humans (Vandenberg *et al*., 2012). Bisphenol A (BPA) is an organic industrial chemical that has been used to manufacture polycarbonated polymers, and epoxy resins. BPA plastics and resins are used in the manufacture of milk and food containers, baby formula bottles, water carboys, the interior lining of food cans, and dental resins and composites (Trasande *et al*., 2012).

Exposure to BPA may occur through the consumption of beverages or foods that have been in contact with polycarbonate (PC) plastic containers or epoxy resins in food packaging (Le *et al*., 2008; Geens *et al*., 2011). No observed effects concentrations (NOEC) is 1 μg/l in aquatic organisms (Giudice & Young, 2010), LOEC is 50 mg/kg/day (EPA, 2001; Vandenberg *et al.*, 2009). Phthalates (also known as vinyl) are organic chemical compounds, used as softeners in some plastic products (polyvinyl chloride, PVC) to make the plastic soft and flexible. In pregnant rats, no observed effects level (NOAEL) is 50 mg/kg/day and lowest observed adverse effect level (LOAEL) is 100 mg/kg/day (Mylchreest *et al*., 2000).

1.3.2 Pesticides

Many epidemiological studies have shown that several pesticides adversely affect the male reproductive system (Hollander, 1997). The structures and effects of pesticides associated with reproductive toxicity are presented in Table 1.2. Lindane (hexachlorocyclohexane, HCH*)* is a synthetic organochlorine insecticide used to control external parasites in animals, lice infestations in humans, and as an insecticide in agriculture. Lindane is a major pollutant globally (Walsh *et al*., 2000). Regulatory values of lindane recommended by WHO in 2004 must not exceed 2 µg/l in drinking water. Acceptable daily intake (ADI) of lindane is 5 mg/kg of body weight (WHO, 2004b).

Atrazine is a widely used herbicide. Most people are exposed to atrazine when they manufacture, distribute, mix or use the herbicide. People who live in rural areas may be exposed to low levels through their drinking water. Atrazine was banned in Europe in 2001, but is still used in the US and many other countries (Chevrier *et al*., 2011). LOEC of atrazine is 200 µg/l for amphibians (Vandenberg *et al*., 2012). In drinking water, levels of atrazine must not exceed 2 µg/l (WHO, 2004a).

Methoxychlor is an organochlorine pesticide used against pests and insects that attack crops, vegetables, and livestock. The most probable route of exposure to methoxychlor would be from inhalation or dermal contact by workers involved in the manufacture, handling, or spraying of the pesticide. The lowest observed effect dose (LOED) of methoxychlor is 500 µg/kg for *in utero* and perinatal exposure in rats (Melnick *et al*., 2002; Vandenberg *et al*., 2012). The maximum allowed concentration of methoxychlor in drinking water recommended by WHO in 2004 is 20 µg/l.

Table 1.2: The effects of pesticides on the male reproductive system

Name	Effects on male reproduction	References
Lindane	600 µg/kg/day on day 9 or 14 of lactation or 100 µg/kg/day on days 9 to 14 of lactation to rat dams reduces testicular weight, sperm count and blood testosterone level	Dalsenter *et al.*, 1997
Hexachlorocyclohexane (HCH)	A single dose 600 µg/kg or 5 consecutive doses of 100 µg/kg /day decreases sperm counts, degenerates Sertoli cells and decreases testes	HCPMRA, 2009
	weight in male rat once they reached adulthood	
Atrazine	20000 µg/kg/day for 3 days in rat increases testosterone production, due to a transient stimulation of cAMP in Leydig cells	Pogrmic-Majkic *et al.*, 2010
	5000 µg/kg/day for 3 days in rat reduces serum and intratesticular levels of testosterone	ATSDR, 2002
	12000 µg/kg increases sperm abnormalities, and decreases sperm production, motility and numbers in rat	Farombi *et al*., 2012
Methoxychlor	5000, 10000, and 20000 µg/kg/day for 7 days causes oxidative stress on epididymal sperm leading to reduced fertility in male rats	Latchoumycandae *et al.*, 2002
	20 µg/kg /day to pregnant mice increases prostate weight in male offspring in adulthood	Welshons *et al*, 1999
	2500 µg/kg/day in rat decreases serum testosterone levels	Lafuente *et al*., 2000
Chlorpyrifos	750, 1250 and 1750 µg/kg/day for 30 days in rat causes severe testicular damage and reduces sperm counts	Joshi *et al.*, 2007
	1590 µg/kg and 2120 µg/kg/day on gestation days 6 to 15 in mice decreases the anogenital distance in male and female offspring	Ambali *et al*., 2009
Dichlorodiphenyltrichloroethane (DDT)	2500 µg/liter of mother's blood of DDT causes premature births and low birth weight in humans	Longnecker *et al.*, 2001
	5000 and 10000 µg/kg/day for 10 days in rat decreases testosterone production and number of motile spermatozoa leading to infertility	Ben Rhouma *et al*., 2001)
Vinclozolin	180, 1800, and 18000 µg/kg/day exposure during the sexual organs developmental stages in fish causes low sperm count and prostatitis in offspring	Bayley *et al.*, 2003
	10000 µg/kg/day to pregnant rat causes hypospadias in male offspring of rats fed vinclozolin during gestation and lactation	Christiansen *et al*., 2008
	20000 µg/kg/day of vinclozolin from day 16 to 18 of gestation in rat leads to undescended testes and prepubertal	Shono *et al.*, 2004

Chlorpyrifos is an organophosphate pesticide used on many crops, on golf-courses, non-structural wood treatment and as insecticide for adult mosquitos. Indoor spraying of chlorpyrifos leads to accumulation of the insecticide on children's toys and increases the risk of their exposure to chlorpyrifos (Davis & Ahmed, 1998). chlorpyrifos levels in drinking water must be less than or equal to 30 µg/l (WHO, 2004a).

Dichlorodiphenyltrichloroethane (DDT) is an organochlorine insecticide that has been widely used since the 1940s to control insect-borne diseases such as malaria (Fox *et al*., 1998). DDT was banned in most countries, but still used in South Africa to control malaria (Wells & Leonard, 2006). The LOED of DDT is 5 µg/kg in rodents (Vandenberg *et al*., 2012). The levels of DDT and its metabolites must be less than or equal to 1µg/l in drinking water (WHO, 2004a).

Vinclozolin is a dicarboximide fungicide used to control rots and molds in vineyards and on many fruits and vegetables, and is also sprayed on golf courses. Vinclozolin is an antiandrogenic environmental pollutant, with high affinity to bind the androgen receptors (Bayley *et al*., 2003). Adverse effects resulting from *in utero* exposure to vinclozolin in rats are reversible by administration of a high-dose of testosterone to male offspring at pubertal age (Prue *et al*., 2010).

1.3.3 Heavy Metals

There is growing concern regarding the effects of heavy metals exposure on the male reproductive system. Lead, chromium, arsenic, cadmium, copper and zinc cause irreversible functional and structural toxic effects on the male reproductive system of experimental animals (Mathur *et al*., 2010). The levels and effects of heavy metals

associated with reproductive toxicity are presented in Table 1.3. Lead (Pb) is used in many industrial products such as battery acid, and industries such as plant refineries, smelter and fuel combustion works. Occupational lead exposure occurs during lead mining, smelting, paint removal, demolition, car repair, and recycling. General population exposure to lead can be due to contaminated food and drink and also exhaust fumes. The levels of lead in drinking water must be less than or equal to 10 µg/l (WHO, 2004a). Exposure to lead is associated with many fertility problems in men (Hollander, 1997).

Chromium (Cr) is a naturally occurring element that is mined as iron chromium oxide (chromite) ($Fe_2Cr_2O_4$) ore in many countries, including South Africa, Zimbabwe, Finland, India, Kazakhstan and the Philippines. There are three forms of chromium, namely Cr_2 metallic (0), Cr_2 trivalent (III) and Cr_2 hexavalent (VI). Cr_2 (III) occurs naturally and it is essential for the human body in trace amounts for regulation of blood sugar and fat levels (Anderson, 1997). Cr_2 (0) and Cr_2 (VI) are produced during industrial processing of Cr_2 (III). Cr_2 (VI) is the toxic form of Cr_2 (Cefalu & Hu, 2004). Cr_2 (VI) and its compounds are carcinogenic (Anderson, 1997). The California Office of Environmental Health Hazard Assessment (OEHHA) recommended levels in drinking water of less than 250 µg/l for total Cr and 2 µg/l for Cr (VI) (OEHHA, 2002). Provisional guideline value of Cr in drinking water proposed by WHO and OEHHA is less than 50 µg/l (OEHHA, 2002; WHO, 2004a).

Arsenic (As) occurs naturally in the environment in toxic inorganic forms combined with oxygen, chlorine and sulfur. In animals and plants, arsenic occurs in harmless organic forms combined with hydrogen and carbon. It is used for many industrial purposes such as wood preservation, pigments, pesticides and poison gases. Arsenic is

one of the most common water pollutants and its levels in drinking water must be equal to or less than 1 µg/l (WHO, 2004a).

Table 1.3: The effects of heavy metals on the male reproductive system

Name	Effects on male reproduction		References
Lead (Pb)	Blood levels > 30-40 µg/dl in lab animals and > 40 µg/dl in adult humans	Results in low sperm count, volume, motility and morphological sperm defects.	Alexander *et al*., 1996; Apostoli *et al.*, 1998; Telisman *et al*., 2000; Pizent *et al*., 2012
Chromium Cr_2 hexavalent (VI)	40 µg/kg/day for 26 days in rats	Causes low testosterone levels and reduces sperm counts and reduces sex organ weight in rats.	Chandra *et al.*, 2007
	100 or 200 µg/kg/day for 15 days in rats	Increasess FSH and decreases LH and testosterone blood levels.	Marouani *et al*., 2012
Arsenic (As)	500 µg/kg/day for 6 days/week for 4 weeks in rats	Arsenic causes effects on the male reproductive system similar to that caused by estradiol treatment, including testicular toxicity and impairment of androgen production.	Moiseeva, 1984
	Blood level higher than 0.58 µg/dl in humans	Associated with low sperm motility, semen volume and LH.	Meeker *et al*., 2008; Pizent *et al*., 2012
		Induces malignant tumor of prostate epithelial cells *in vitro*.	Benbrahim-Tallaa *et al*., 2007
Cadmium (Cd)	5000 µg/kg/day for 45 days in mice	Decreases sperm counts, motility, and maturity, and levels of testosterone.	Monsefi *et al.*, 2010
	Urinary cadmium levels >10 µg/L	High LH and testosterone levels.	Zeng *et al.*, 2002
	Oral exposure to cadmium	Causes prostate cancer in experimental animals.	Telisman *et al.*, 2000; Diamanti-Kandarakis *et al*., 2009

Continued/…

Table 1.3: The effects of heavy metals on the male reproductive system (Continued)

Name	Effects on male reproduction		References
	2000-3000 µg/kg/day for 26 days in rats	Reduces serum testosterone, FSH and LH, and decreases sex organ weight in a dose-dependent manner.	Chattopadhyay *et al*., 2005
Copper (Cu)	2000-3000 µg/kg/day for 120 days in rats		Chattopadhyay *et al*., 1999
	Seminal plasma levels from 7890 to 27460 µg/l	Zinc deficiency in human and animals leads to low serum testosterone level and oligospermia.	Prasad, 2008; Gilabert *et al*., 1996
Zinc (Zn)		Negatively affects sperm motility and viability	Dissanayake *et al*., 2010
		Zinc deficiency in mice impairs spermatogenesis	Croxford *et al*., 2011

Cadmium (Cd) is a highly toxic element that can bio-accumulate and has a long biological half-life in mammals. General population exposure to Cd is associated with contaminated drinking water, food and cigarette smoke (Zhou *et al*., 2004). Industrial Cd exposure occurs as a result of mining, smelting, and combustion of fossil fuels (Henson & Chedrese, 2004; Zhou *et al*., 2004). Levels of Cd in drinking water recommended by WHO in 2004 is less than 0.3 µg/l.

Copper (Cu) is an essential element needed by the adult human body at a dose of 1.5-3.0 mg/day to prevent anaemia and for healthy skeletal, reproductive, nervous system and metabolic processes (Chattopadhyay *et al*., 2005). Copper levels in drinking water must not exceed 2000 µg/l (WHO, 2004a). In Germany, chronic copper intoxication from tap water has been linked to many severe diseases such as liver cirrhosis and chronic gastrointestinal diseases (Eife *et al*., 1999).

Zinc (Zn) is an essential trace element. It is important for normal metabolism, reproduction and immune system (Prasad, 2007; Prasad, 2008). The adult human body contains 1–3 g of zinc, approximately 0.1% of which is replenished daily (Maret & Sandstead, 2006; Dissanayake *et al*., 2010). Zinc in drinking water is not of health concerns at levels normally observed (WHO, 2004a).

1.3.4 Pharmaceuticals and Personal Care Products

Estrogenic pharmaceutical products are used in estrogen-replacement therapy, contraceptive formulations and as growth enhancement products in animals (Arcand-Hoy *et al*., 1998). Many pharmaceuticals, such as birth control pills and corticosteroids have endocrine disrupting properties. Pharmaceuticals associated with reproductive toxicity are presented in Table 1.4.

17α-Ethinylestradiol (EE2) is an estrogenic pharmaceutical that is found in female contraceptive pills (Mazellier *et al*., 2008). EE2 has been detected in the environment at different concentrations in many countries. In Germany, EE2 concentrations in rivers were up to 5 ng/l (Kuch & Ballschmiter, 2001). In 2000 in the United States, EE2 concentrations in 139 streams across 30 states ranged from 5 to 273 ng/l (Kolpin *et al*., 2002). In the Netherlands, in surface water EE2 detected concentrations was 0.3 ng/l (Vethaak *et al*., 2005).

Diethylstilbestrol (DES) is a synthetic estrogen that was prescribed to women between the 1940s-1970s to prevent miscarriages and premature birth (Hollander, 1997; Marty *et al*., 2011). DES was detected in water and sediments of three rivers in Tianjin area, northern China, in concentrations below 10 ng/l (Lei *et al.*, 2009).

Table 1.4: The effects of pharmaceuticals and personal care products on the male reproductive system

Name	Effects on male reproduction	References
17α-Ethinylestradiol (EE2)	0.002 µg/kg/day in pregnant women impairs development of the testes and prostate in sons.	Thayer, 2002
	0.296 ng/l to 296.4 µg/l in water disrupts male sexual behaviour in amphibians	Kristensen *et al*., 2005); Hoffmann & Kloas, 2012
	200 ng/l in water reduces sperm count, testis weight and causes feminization in fish.	Kristensen *et al.* , 2005
Diethylstilbestrol (DES)	0.02 and 0.2 µg/kg/day on day 11 to 17 of pregnancy in mice increases prostate weight in male offspring at adulthood.	Welshons *et al*, 1999
	200 µg/kg/day on day 11 to 17 of pregnancy in mice decreases anogenital distance in male and female offspring and decreases prostate weight in male offspring in adulthood.	Vom Saal *et al*., 1997; Palanza *et al.*, 2001;
	Maternal exposure in rats causes hypospadias, cryptorchidism, micropenis, and increases transmitted susceptibility to malignancies.	Vom Saal *et al*., 1997
	Occupational exposure to DES leads to cryptorchidism in in newborns and decreases semen quality in adults.	Li *et al*., 2003; WHO, 2011
Triclocarban (TCC)	75 mg/kg/day orally for 24 in rat leads to testicular degeneration	SGP, 2010
	Exposure to TCC enhances testosterone-induced effects and enlarges accessory sex organs in castrated male rats.	Chen *et al.*, 2008
	0.25% in diet for 10 days in rats causes hyperplasia of accessory sex organs.	Duleba *et al*., 2011

Concentrations of diethylstilbestrol in ambient air samples from plants that manufacture diethylstilbestrol ranged from 0.02 to 24 µg/l (DHHS, 2011). DES was used as a growth promoter for sheep and cattle. People could have been exposed to DES at concentrations of up to 10 ppb in meat produced from DES-treated animals (DHHS, 2011). The LOED (dose typically administered to pregnant women) is 30–130 µg/kg/day (Vandenberg *et al*., 2012). Oral LOAEL is 0.02 µg/kg/day, and NOEL is 0.002 µg/kg/day in mice (EPA, 2001). DES increases the incidence of breast cancer in exposed women, and increases the risk of reproductive tract deformities in their progeny (Marty *et al*., 2011).

Triclocarban (3, 4, 4'- trichlorocarbanilides or TCC) is an antibacterial agent used in numerous personal care products such as bar soaps, body washes, detergents and in many other household products. TCC is a very persistent environmental pollutant with a half-life of 450 days in sediment (Halden & Paull, 2005). In California, TCC has been reported at 0.22 µg/l in recycled water (Anderson *et al.*, 2010). Concentrations of TCC detected in the US surface water is 0.25 µg/l (Chalew & Halden, 2009). In streams with known inputs of raw wastewater levels of up to 6.75 µg/l were detected (Halden & Paull, 2005). NOEC of TCC is 0.101 µg/l for aquatic organisms (Giudice & Young, 2010). Human reproductive toxicity, oral LOAEL is 15000 µg/kg/day (EPA, 2008), and oral NOEL of TCC is 2500 µg/kg/day (SGP, 2010).

1.3.5 Mycotoxins

Zearalenone is an estrogenic mycotoxin produced by many fungal species of the genus *Fusarium*. *Fusarium* is found in the soil in temperate and warm climates and often contaminate cereal crops (Zinedine *et al*., 2007). The NOEL of zearalenone for reproductive and teratogenic effects in pregnant rat is less than 100 µg/kg (Collins *et al*., 2006).

Deoxynivalenol (DON) is a mycotoxin which commonly contaminates cereal and cereal-based foods worldwide. DON occurs in grains such as wheat, barley, oats, rye, and maize. DON is produced by numerous strains of *Fusarium* and some other fungi. It induces vomiting in swine and dogs at very low doses 0.05 to 0.1 mg/kg body weight (bw) (Pestka & Smolinski, 2005). Effects of mycotoxins on the male reproductive system are summarized in Table 1.5.

Table 1.5: The effects of mycotoxins on the male reproductive system

Name	Effects on male reproduction	References
Zearalenone	In pregnant rat 8 mg/kg/day on gestation day 6 to19 increases anogenital distance in male and female offspring.	Collins *et al*., 2006
Deoxynivalenol	Potential effects on steroidogenesis and alteration in gene expression *in vitro*.	Ndossi *et al*., 2012
	5 mg/kg/day for 28 days in rats reduces testosterone concentration, weight of accessory sex organs and semen quality.	Sprando *et al*., 2005

1.3.6 Phytoestrogens

Phytoestrogens are naturally occurring plant-derived phytochemicals (McVey *et al*., 2004). Their common biological roles are to act as part of a plant's defense mechanism. Although composed of a wide group of non-steroidal compounds of diverse structure, phytoestrogens have been shown to bind estrogen receptors and to behave as weak agonists/antagonists in both animals and humans (Hollander, 1997; Bacciottini *et al*., 2007; Rice & Whitehead, 2008). Their name was derived from their ability to bind the estrogen receptors and induce estrogenic/antiestrogenic effects. Phytoestrogens are divided into different groups, isoflavonoids, lignans, and coumestans (Kurzer & Xu, 1997; Miniello *et al*., 2003; Branca & Lorenzetti, 2005).

Human consumption of isoflavones has the largest impact because of its availability and variety in soy-derived food products. They have a lower estrogenic activity than endogenous estrogens, are quickly excreted and do not bio-accumulate (Hollander, 1997). Studies on various animal species have demonstrated that high level intake of phytoestrogens can induce adverse effects on reproductive health. These studies have also shown that exposure to high levels of phytoestrogens during developmental stages can adversely affect the nervous and reproductive system in rodents (Humfrey, 1998).

Isoflavones are present in legumes, lentils, chickpeas as well as soy products which include soy milk, soybeans, soy flour and soy-based infant formulas (McVey *et al*., 2004). Genistein, daidzein, and glycitein are the principal isoflavones in soy (Messina & Wood, 2008). Phytoestrogens associated with male reproductive toxicity are presented in Table 1.6. Genistein is an isoflavone found in many plant-based food items, mainly soy and soy-derived food. Genistein is the major phytoestrogen in soy used as an alternative therapy for several diseases such as cancer, cardiovascular diseases, menopausal symptoms and osteoporosis. Typical Asian diets contain 150 µg/kg/day of isoflavone mixture while Western diets contain less than 20 µg/kg/day isoflavones (Coward *et al*., 1993; Cooke *et al*, 2006).

Table 1.6: The effects of phytoestrogens on the male reproductive system

Name	Effects on male reproduction	References
Genistein	In adult male mouse 250 µg s.c./kg/day for 9 days reduces serum LH, testosterone levels and prostate weight	Strauss *et al*., 1998
	600 µg/g isoflavones for 5 weeks in rat decreases testosterone and androstenedione blood levels	Weber *et al*., 2001
Lignans	14 and 28 mg/kg/day for 6 weeks increases weight of the testes and epididymis, improves storage capacity of the epididymis for the spermatozoa, increases testosterone levels, decreases FSH and improves sperm parameters.	Shittu & Bankole, 2007; Shittu *et al*., 2008
Quercetin	50, 100 and 200 µg/L quercetin till 1 month postmetamorphosis in amphibians leads to male feminization and impaired testicular development.	Cong *et al*., 2006

The increased isoflavone intake by Asians has been linked to the lower incidence of cancer amongst the population group. Genistein has adverse effects on the endocrine system such as impairment of the thyroid function (Sosić-Jurjević *et al*., 2010) and thymic atrophy (Cooke *et al*, 2006). Lignans are phytoestrogens found in many plants such as flax and sesame. Lignans play a role in plant growth and act as antioxidants in human and animal metabolism. Lactational exposure to 10% flax seed or equivalent

levels of its lignans to rats, and 50000 µg/day in humans, reduces breast cancer risk at adulthood and causes no reproductive toxicity or severe adverse effects (Ward *et al*, 2001; Chen *et al*, 2003). Quercetin is a flavonoid (catechin) that occurs mainly in tea leaves. Quercetin has been shown to afford protective effects against diesel exhaust particle-induced reproductive toxicity (Izawa *et al*., 2008). Quercetin improves sperm motility, viability and concentration (Taepongsorat *et al*, 2008).

1.4 Adverse Outcomes on the Male Reproductive System Due to EDCs

Many adverse effects of the male reproductive system have been linked to exposure to endocrine disruptors such as increased incidence of testicular and prostate cancer, deterioration of sperm quality, high incidence of cryptorchidism and hypospadias (Giwercman *et al*., 2007). Several epidemiological studies have linked the occupational and environmental exposure to EDCs to adverse reproductive effects.

1.4.1 Testicular and Prostate Cancer

In the UK, the incidence of prostate cancer associated with endocrine disruptors has increased between 1975 and 2007 from 33 per 100,000 to 97 per 100,000 (De Coster & Van Larebeke, 2012). Low-dose exposure to bisphenol A during developmental stages was linked to adverse effects on the male reproductive system such as prostate cancer (Ho *et al*, 2006). DES an endocrine disrupting medicine has been linked to prostate and testicular cancer in human and animals (Imaida & Shirai, 2000).

1.4.2 Hypospadias and Cryptorchidism

Hypospadias is a displacement of the urethral meatus onto the underside of the shaft of the penis. Cryptorchidism is a disorder whereby the testis fails to descend into its normal position in the scrotum. The occupational exposure of pregnant women to endocrine disrupting chemicals such as dioxins and furans, polychlorinated biphenyls, organochlorine pesticides, phthalate esters, brominated flame-retardants and some heavy metals, increases the risk of hypospadias (Morales-Suárez-Varela *et al*., 2011). An epidemiological study in 2007 has demonstrated that the risk for hypospadias and cryptorchidism is increased relative to the combined effect of *in utero* occupational exposure to environmental estrogens such as pesticides and organohalogenated compounds (Fernandez *et al*., 2007). Li *et al*. (2003) and Palmer *et al*. (2009), have demonstrated that *in utero* exposure to DES is associated with hypospadias, cryptorchidism, micropenis, and epididymal cysts. Prenatal exposure to phthalate at environmental levels causes developmental adverse effects on the male reproductive system, including reduced anogenital distance, impairment of Leydig cell function, and hypospadias (Swan *et al*., 2005), cryptorchidism and reduced fertility (Bay *et al*., 2006; Sharpe, 2006).

1.4.3 Low Semen Quality

Meta-analysis studies have shown a decrease in semen quality of 40% worldwide during the last decades because of the exposure of males to the endocrine disruptors (Eertmans *et al*., 2003). Approximately 6% of men are thought to be infertile, of which 40-90% are characterized by low sperm count, due to unknown causes or endocrine disruptors (Sinclair, 2000).

1.5 Summary

The male reproductive system consists of the testes, epididymis, ductus deferens, seminal vesicles, prostate gland, ejaculatory ducts, bulbourethral glands, penis, urethra and scrotum. The function of male reproductive system (steroidogenesis and spermatogenesis) is regulated by the hypothalamic-pituitary-testicular axis (HPTA). Testosterone, the most important sex hormone in male reproduction, is primarily produced by Leydig cells of the testes, and is responsible for normal reproductive function, secondary sexual characteristics, development of reproductive organs, spermatogenesis maintenance and sexual behaviour. Modulation of the male reproductive system happens as a result of exposure to endocrine disrupting compounds (EDCs) such as industrial chemicals and their by-products, pesticides, heavy metals, pharmaceuticals and personal care products, phytoestrogens and mycotoxins. This can result in many problems such as decline of testosterone levels, sperm deformities and decreased sperm count, testicular and prostate cancer, hypospadias, decreased libido, infertility and loss of muscle mass.

1.6 References

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CHAPTER 2

THE EFFECTS OF COMMONLY CONSUMED PHYTOBEVERAGES ON HEALTH

2.1 Introduction

Several compounds are found in plant-based foods, namely phytochemicals or phytoestrogens. Phytochemicals are naturally occurring, biologically active chemical compounds in plants. Phytoestrogens are divided into different groups, isoflavonoids, lignans, and coumestans (Kurzer & Xu, 1997; Miniello *et al*., 2003; Branca & Lorenzetti, 2005). Phytoestrogens give these plants definitive physiological actions on the human and animal body. The increased phytoestrogens intake has been linked to the lower incidence of cancer (Coward *et al*., 1993; Ward *et al*, 2001; Chen *et al*, 2003; Cooke *et al*, 2006).

Polyphenols have been demonstrated to protect and prevent diseases of the male reproductive system that resulted by high stress of reactive oxygen species (ROS) (Shi, Dalal, & Jain, 1991; Adhami *et al*., 2003; Siddiqui *et al*., 2005; Pandey & Gupta, 2009; El-Shahat *et al*., 2009; Awoniyi *et al*., 2011; El-lethey & Shaheed, 2011; Corrêa *et al*., 2012). In this chapter, details of phytochemicals of green and black tea, rooibos tea, coffee and buchu as well as their biological activities on the male reproductive system are reviewed.

2.2 Green and Black Tea

2.2.1 Introduction

After water, tea is the second most consumed drink in the world. Green tea and black tea are consumed by between 20-22% and 73-78% of the world's population, respectively (Graham, 1992; Krishnan & Maru, 2006; Cabrera *et al*., 2006; Henning *et al.*, 2011). Green tea and black tea are produced from *Camellia sinensis (C. senensis)*, a plant of the family *Theaceae*. The plant is native to southern and east Asia. *C. sinensis* leaves contain polyphenols, including an enzyme polyphenol oxidase which is activated when the leaves are cut, and this results in the polyphenols being oxidized. Different fermentation processes of the leaves produce different kinds of tea, namely non-fermented green tea, semi-fermented oolong tea, and fermented black tea (Graham, 1992; Cabrera *et al*., 2006). Black tea contains less catechins than green tea because of the post-harvest treatment (Cabrera *et al*., 2006; Unachukwu *et al*., 2010; Henning *et al.*, 2011).

2.2.2 Phytochemicals of Green and Black Tea

Tea leaves contain high levels of antioxidant compounds known as catechins (monomeric flavonoids). The prominent catechins in the green tea are catechin (C), epicatechin (EC), epigallocatechin (EGC), epicatechin gallate (ECG), epigallocatechin gallate (EGCG) and gallocatechin gallate (GCG). These catechins are oxidized to polymeric theaflavins (TFs) such as theaflavin (TF1), theaflavin-3-gallate (TF2A), theaflavin-3'-gallate (TF2B) and theaflavin-3,3'-digallate (TF3) and thearubigins during the fermentation process of the black tea production (Leung *et al*., 2001; Henning *et al.*, 2011). Comparison between green and black tea composition is presented in Table 2.1. Tea polyphenols have been demonstrated to have

antimutagenic, antidiabetic, anti-inflammatory, antibacterial and antiviral properties (Cabrera *et al*., 2006; Nune *et al*., 2009).

Table 2.1: Comparison between green and black tea chemical composition

Compound	Green tea	Black tea	References
Total phenolic compounds (% of dry weight)	30	5	Graham, 1992; Cabrera *et al*., 2006; Chacko *et al*., 2010; Venkateswara *et al*., 2011
Total phenolic compounds (µg/100 g)	13	8.9	Thompson *et al*., 2006
Total phenols (2.5 g equivalent to:)	165 mg gallic acid	124 mg gallic acid	Lee *et al.*, 2002
Oxidized phenolic compounds (thearubigins and theaflavins) (% of dry weight)	0	25	Cabrera *et al*., 2006; Chacko *et al*., 2010; Henning *et al.*, 2011; Venkateswara *et al*., 2011
Lignan content (µg/100g)	12	8.1	Thompson *et al.*, 2006
Antioxidant capacity (per serving)	436 mg vitamin C equivalents	239 mg vitamin C equivalents	Lee *et al.*, 2002
Caffeine (g/kg dry weight)	3.4	3.5	Hilal & Engelhardt, 2007

2.2.3 Beneficial Effects of Green and Black Tea on Body Systems

Compared to green tea, the antioxidant activity of black tea has not been extensively studied. Black tea contains less polyphenols than green tea, and is regarded as having a weaker antioxidant activity (Lee *et al.*, 2002; Gawlik & Czajka, 2007). A summary of the beneficial biological activities of green and black tea are given in Table 2.2. There is a growing body of concern about the adverse effects of heavy consumption of tea, and about food additives related to *C. sinensis* that cause serious injuries such as acute hepatic toxicity (Sarma *et al*., 2008).

Table 2.2: The biological activities of green and black tea

Description	Tea type	Effects	References
Prostate cancer	Green tea	Prevention and treatment of prostate cancer *in vivo*, *in vitro* and in epidemiological studies	Adhami *et al*., 2003; Pandey & Gupta, 2009
	Black tea	Protection against androgen-induced prostate cancer *in vivo* in rat	Siddiqui *et al*., 2005
Testicular toxicity	Green tea	Protection against cadmium-induced testicular toxicity *in vivo* in rat	El-Shahat *et al*., 2009
		Protection against reactive oxygen species-induced damage in testicular tissue	Awoniyi *et al*., 2011
	Black tea	Protection against sodium fluoride-induced testicular toxicity *in vivo* in rat	El-lethey & Shaheed, 2011
Cardiovascular system	Green tea	There is a positive correlation between cardiovascular health and green tea consumption.	Wolfram, 2007; Babu & Liu, 2008
	Black tea	Short and long intake of black tea decreases cardiovascular disease events.	Duffy *et al*., 2001
		Protection against nicotine-induced hyperlipidemia and atherogenesis *in vivo* in rat	Joukar *et al*., 2012
Degenerative diseases of nervous system	Green tea	Protection against brain atrophy and cognitive dysfunction in mice	Unno *et al*., 2007
	Black tea	Protection against degenerative changes in CNS in mice	Trivedi *et al*, 2012
Inflammations and cancers of digestive system	Green tea	Protection against oesophageal, stomach and colonic cancer, and prevention of gastrointestinal disorders	Koo & Cho, 2004
	Black tea	Prevention of colitis in mice	Song *et al*., 2011
		Protection against pesticides-induced hepatic injury	Khan, 2006

2.2.4 Adverse Effects of Green and Black Tea on Body Systems

Heavy consumption of green and black tea may cause some adverse effects such as liver damage (Sarma *et al*., 2008), potential interaction with prescribed drugs and disruption of metabolic processes (Yang & Pan, 2012), alteration of therapeutic efficacy and the possibility of causing problems when combined with other herbal beverages (Schönthal, 2011).

2.3 Rooibos Tea

2.3.1 Introduction

Aspalathus linearis, commonly known as rooibos tea or red bush tea, is an indigenous South African plant, naturally decaffeinated, with low levels of tannin (Shimamura *et al*, 2006; Baba *et al*., 2009). It grows in the Cederberg, Clanwilliam, and neighbouring mountains of the Western Cape, South Africa. Traditionally, rooibos tea is used as colic relief for infants, in cosmetic and slimming products, as colouring and flavouring agents for baking products, for relief of allergies and as a bronchodilator in asthma (Joubert *et al*., 2008; Van Wyk, 2011). *Aspalathus linearis* is rich in antioxidant substances such as phenolic acids, polyphenols and flavonoids which scavenge free radicals, and thereby prevent oxidative damage to cells (Joubert *et al*., 2008).

2.3.2 Phytochemicals of Rooibos Tea

Many phytoestrogens have been isolated from rooibos tea, including spalathin, which is the main flavonoid of *Aspalathus linearis.* The spalathin concentration is 1 mg/g in fermented rooibos and up to 50 mg/g in unfermented rooibos (McKay & Blumberg, 2007; Van Wyk, 2011). Other flavonoids that have been isolated from rooibos tea include aspalalinin, nothofagin, orientin, iso-orientin, isovitexin, dihydro-orientin, dihydro-iso-orientin, hemiphlorin, quercetin, quercetin-3-robinobioside, hyperoside, isoquercetrin and rutin (Bramati *et al*., 2002; Shimamura *et al*., 2006; McKay & Blumberg, 2007; Joubert *et al*., 2008). The total antioxidant activity of the green rooibos is 2.8-fold higher than that of the fermented rooibos tea due to the degradation of green rooibos phenolic compounds during fermentation (Standley *et al*., 2001; Schulz *et al*., 2003). Due to its antioxidant properties, rooibos tea has received a lot of attention, particularly with respect to beneficial effects on health.

2.3.3 Beneficial Effects of Rooibos Tea on Body Systems

Rooibos tea is a popular beverage in South Africa and has a growing worldwide market. Compared to green and black tea, few studies are available related to the biological activities of rooibos tea. The biological activities of rooibos tea are summarized in Table 2.3. Rooibos tea, as a rich source of antioxidants, is recommended as a safe, commercially available and effective hepatoprotector to patients with hepatic lesions.

Table 2.3: The biological activities of rooibos tea

Description	Effects	References
Cellular immunity	Rooibos tea stimulates splenocytes *in vitro* and *in vivo*.	Kunishiro *et al*., 2001
Colic	Decreases the acetylcholine-induced contractions.	Snyckers & Salemi 1974; Khan & Gilani, 2006
Inflammation	Protects against colitis.	Baba *et al*., 2009
Nervous system	Long-term rooibos tea consumption prevents accumulation of lipid peroxides in the brain.	Inanami *et al*., 1995
Testicular toxicity	Protects against reactive oxygen species-induced damage in testicular tissue.	Awoniyi *et al*., 2011
Asthma	Bronchodilator effects in congestive respiratory	Khan & Gilani, 2006
Blood pressure	Blood pressure lowering effects	Khan & Gilani, 2006

2.3.4 Adverse Effects of Rooibos Tea on Body Systems

Marnewick *et al*. (2003) have evaluated a wide spectrum of safety indices in Fischer rats exposed to rooibos tea (2 g/100 ml water for 10 weeks), including liver and kidney functions, serum enzymes total and unconjugated bilirubin, total protein, total cholesterol and iron status, but could not demonstrate any adverse effects at this level. Studies on phytoestrogens of rooibos tea showed that rooibos tea can cross-react with

natural estrogens in ELISA (Shimamura *et al.*, 2006), and can also decrease steroidogenesis by steroid secreting cell line (Schloms *et al*., 2012)

2.4 Coffee

2.4.1 Introduction

Coffee is one of the most popular beverages consumed daily throughout the world. Coffee is prepared from the roasted seeds of the coffee plant of the family *Rubiaceae*. Coffee is a truly tropical shrub native to Ethiopia (Gómez-Ruiz *et al*., 2007). The two main species grown are arabica coffee and robusta coffee (Bonita *et al*, 2007; Vignoli *et al.*, 2011; Gunalan *et al*., 2012). Coffee also stands out as a dietary source of potential antioxidant compounds such as caffeine.

2.4.2 Phytochemicals of Coffee

The amount of polyphenols in coffee vary with species and with different degrees of roasting (Daglia *et al*., 2000). The most important phenolic compounds of coffee that may affect human health are caffeine, cafestol and kahweol, and chlorogenic acid (Higdon & Frei, 2006; Bonita *et al*, 2007), caffeic acid, hydroxyhydroquinone (Butt & Sultan, 2011). Many studies have reported that the coffee consumption may prevent some chronic conditions such as type II diabetes mellitus, liver cirrhosis, hepatocellular carcinoma and Parkinson's disease (Kang *et al*., 2009; Butt & Sultan, 2011; Kang *et al*., 2011).

2.4.3 Beneficial Effects of Coffee on Body Systems

Seven cups of coffee/day may lower the chance to develop type II diabetes by about 50% (Van Dam & Feskens, 2002). Epidemiological studies showed that, moderate

daily consumption of filtered coffee does not induce any adverse effects on the cardiovascular system (Ranheim & Halvorsen, 2005). Beneficial biological activities of coffee are presented in Table 2.4.

Table 2.4: The biological activities of coffee

Description	Effects	References
Antioxidant properties	Coffee antioxidant activity is important to protect biological systems and reducing the risk of diseases related to reactive oxygen species.	Shi, Dalal, & Jain, 1991; Corrêa *et al*., 2012
Inflammation and endothelial function	Filtered coffee consumption has an inverse association with inflammation and endothelial dysfunction.	Bonita *et al*, 2007
Myocardial infarction	Moderate coffee consumption reduces the risk of myocardial infarction by 31% relative to no consumption.	Panagiotakos *et al*., 2003
Anticarcinogenic activity	Coffee protects against certain types of cancers such as colorectal cancers.	Cavin *et al*., 2002

2.4.4 Adverse Effects of Coffee on Body Systems

A positive relationship between acute myocardial infarction and coffee consumption has been confirmed (Panagiotakos *et al*., 2003; Greenland, 2013). Chronic heavy consumption of coffee may increase blood pressure (Noordzij *et al*., 2005). Previous studies have also shown an inverse association between coffee consumption and other major causes of death such as diabetes, inflammatory diseases, stroke, and injuries and accidents (Freedman *et al*., 2012). The two compounds, namely cafestol and kahweol, are the cause of serum cholesterol elevation due to coffee consumption (Rustan *et al*., 1997; Bonita *et al*., 2007).

2.5 Buchu

2.5.1 Introduction

Agathosma betulina (round-leaf buchu or short buchu), and *Agathosma crenulata* (oval-leaf buchu or long-leaf buchu), are referred to as buchu. *Agathosma betulina* and *Agathosma crenulata* are indigenous South African shrubs. They grow in the Cederberg mountains of The Western Cape, South Africa (Moolla & Viljoen, 2008). Buchu has been used traditionally to treat several ailments (Moolla & Viljoen, 2008; Van Wyk, 2011). Nowadays *buchu* preparations are used to treat many conditions such as colic, urinary tract infections, cough, fever and rheumatism (Simpson, 1998). Both buchu species are rich in flavonoids which are responsible for the benefits of buchu oil.

2.5.2 Phytochemicals of Buchu

Very few studies have been done on the non-volatile fractions of the *Agathosma* species. Buchu oil extracted from *Agathosma betulina* contains approximately 31% (iso)menthone, 41% (psi)-diosphenol and, and 3% cis- and trans-8-mercapto-p-menthane-3-ones, while the *Agathosma crenulata* oil contains 54% pulegone and 7% trans-8-acetylthio-p-menthan-3-one (Posthumus *et al.*, 1996).

2.5.3 Beneficial Effects of Buchu on Body Systems

Despite its long historic use as a phytomedicine, the biological activities of buchu have not been extensively studied. Table 2.5 summarizes the biological activities of buchu.

2.5.4 Adverse Effects of Buchu on Body Systems

Very few studies have been performed on buchu and no adverse effects of buchu consumption have been reported in human or animal models.

Table 2.5: The biological activities of buchu

Description	Effects	References
Blood pressure	*Agathosma betulina* has hypotensive or antihypertensive potential effects.	Tabassum & Ahmad, 2011
Colic	Buchu oil has spasmolytic action.	Lis-Balchin *et al*., 2001
Antimicrobial	Buchu oil has very low antimicrobial activity.	Lis-Balchin *et al*., 2001
Anti-inflammatory	Buchu oil has an *in vitro* anti-inflammatory action	Lis-Balchin *et al*., 2001

2.6 Summary

Several compounds are found in our plant-based foods, namely phytochemicals or phytoestrogens which are naturally occurring, biologically active chemical compounds divided into different groups, isoflavonoids, lignans, and coumestans. Green tea and black tea are produced from *Camellia sinensis* (*C. senensis*), a plant of the family *Theaceae*. The plant is native to Southern and East Asia. *Aspalathus linearis*, commonly known as rooibos tea or red bush tea, is an indigenous South African plant, naturally decaffeinated, with low levels of tannin. Coffee is prepared from the roasted seeds of the coffee plant of the family *Rubiaceae*. Coffee is a truly tropical shrub native to Ethiopia. The two main species grown are Arabica coffee and Robusta coffee. *Agathosma betulina* (round-leaf buchu or short buchu), and *Agathosma crenulata* (oval-leaf buchu or long-leaf buchu), are referred to as buchu. They are indigenous South African shrubs.

Tea polyphenols have antimutagenic, antidiabetic, anti-inflammatory, antibacterial and antiviral properties. Coffee has antimutagenic, antioxidant and anti-inflammatory effects. Rooibos tea has antioxidant activities, bronchodilator effects in asthma and blood pressure lowering effects. Buchu has antihypertensive, antimicrobial and anti-inflammatory effects. They also have many adverse effects - heavy consumption of

green and black tea may cause liver damage and may cause serious health problems when combined with other herbal beverages. Chronic heavy consumption of coffee is positively related to acute myocardial infarction and can elevate serum cholesterol levels. Rooibos tea decreases steroidogenesis by steroid secreting cell lines. No adverse effects of buchu consumption have been reported in human or animal models.

2.7 References

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CHAPTER 3

AIM OF THE STUDY

3.1 Introduction

Steroid hormones (e.g. oestrogens, progestines and androgens) regulate the developmental, sexual and reproductive processes in human and animals (Evans, 2009). Testosterone is the most important steroid hormone in male reproduction as it is responsible for normal reproductive function, secondary sexual characteristics, development of reproductive organs, spermatogenesis maintenance and sexual behaviour (Seeley & Stephens, 2003; Kim & Moon Du, 2011). Serum testosterone levels decline due to many causes such as aging, and endocrine disruption (Kim & Moon Du, 2011). Disruption of the male reproductive system is associated with many problems such as sperm deformities and decreased sperm count, testicular and prostate cancer, decreased libido, infertility and loss of muscle mass (Isidori *et al*., 2005; Siddiqui *et al.*, 2005; Cattabiani *et al*., 2012).

Because of its vital role in the normal sexual and reproductive processes of males, testosterone should always be maintained at the normal levels. Meta-analysis studies have shown a decrease in semen quality of 40% worldwide during the last few decades (Eertmans *et al*., 2003). Approximately 6% of men are thought to be infertile of which 40-90% are characterized by low sperm count due to unknown causes (Sinclair, 2000). There is increasing evidence that endocrine disruptors can disturb the optimal functioning of the reproductive system in human and animals. A wide range of substances, both natural and man-made, are thought to cause adverse effects on the

male reproductive system, including environmental chemicals, pesticides, and heavy metals (Diamanti-Kandarakis *et al*., 2009), pharmaceuticals and personal care products (Folmar *et al*., 2002), phytoestrogens (Rice & Whitehead, 2008) and mycotoxins (Zinedine *et al*., 2007).

Phytoestrogens are naturally occurring plant-derived phytochemicals (McVey *et al*., 2004) which can bind estrogen receptors and behave as weak estrogen agonists/antagonists in both human and animals (Hollander, 1996; Bacciottini *et al*., 2007; Rice & Whitehead, 2008). Studies have demonstrated that high intake levels of phytoestrogens can adversely affect the nervous and reproductive system in rodents (Humfrey, 1998).

The most important route by which endocrine disrupting chemicals enter the body is through food and drink. Tea and coffee are the most popular consumed beverages in the world after water. Rooibos tea and buchu are popular beverages in South Africa and have a growing worldwide market. Because of their consumption worldwide, and their broad benefits/and or adverse effects that have been approved in previous studies, it is interesting, from both a public and a scientific perspective, to investigate their potential benefits or adverse effects on the male reproductive system.

Tea leaves and coffee beans are rich in phytoestrogens. Many phytoestrogens have been isolated from both rooibos and buchu as explained in Chapter 2. Studies conducted on the effects of teas on metal toxicity in testes showed that green and black tea protect against metal-induced testicular toxicity (El-Shahat *et al*., 2009; El-lethey & Shaheed, 2011). Green tea can alter the morphology and histology of the male

reproductive system in rats and causes decrease in serum testosterone levels (Chandra *et al*., 2011).

Studies on phytoestrogens of rooibos tea showed that rooibos tea can cross-react with natural estrogens in ELISA (Shimamura *et al.*, 2006), and can also decrease steroidogenesis by steroid secreting cell line (Schloms *et al*., 2012). *In utero* studies showed that exposure to high doses of caffeine impairs gonadal development in male offspring rats and decreases serum testosterone levels (Dorostghoal *et al*., 2012). No data are available on the effects of teas, coffee and buchu on steroidogenesis in testis culture. This study aimed to investigate the direct effects of green tea, black tea, rooibos tea, coffee and buchu on the steroidogenesis of male reproductive system *in vitro* using mouse testicular cultures and also to investigate their cytotoxicity in the same system.

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CHAPTER 4

EFFECTS OF POPULAR HOT BEVERAGES (GREEN TEA, BLACK TEA, ROOIBOS TEA, COFFEE AND BUCHU) ON TESTOSTERONE PRODUCTION BY MOUSE TESTICULAR CULTURES

4.1 Abstract

This study aimed to investigate the effects of green tea, black tea, rooibos tea, coffee and buchu on testosterone synthesis by basal and LH-stimulated mouse testicular cell cultures. Balb/C testicular cell cultures were prepared and incubated overnight with different concentrations of beverage extracts. Cell cultures were then incubated for a further 4 hours under basal or LH-stimulated conditions. Supernatants were collected and testosterone production was assessed using a competitive ELISA. The results showed that rooibos tea and buchu did not have significant effects on both basal and LH-stimulated testosterone production. Green tea, black tea and coffee inhibited LH-stimulated testosterone production in a dose-dependent manner. At a concentration of 500 µg/ml, green tea, black tea and coffee testosterone inhibition was 81%, 85% and 81%, respectively. At 250 µg/ml green tea, black tea and coffee inhibited testosterone production by 52%, 78% and 65%, respectively. At 125 µg/ml green tea, black tea and coffee inhibited testosterone production by 48%, 55% and 37%, respectively. No significant effects on the basal testosterone production were observed with green tea, black tea and coffee. Results of this study showed that green tea, black tea and coffee may impair the normal steroidogenesis in mouse testis and thus their consumption at

relatively high doses raises concern of their effects on male reproductive function in spite of their other beneficial effects.

4.2 Introduction

Reproduction is a very important process that is required for species survival. Steroid hormones (e.g. oestrogens, progestines and androgens) regulate the development of sexual and reproductive processes in human and animals (Evans, 2009). Testosterone is the most important steroid hormone in male reproduction. It is responsible for growth and development of sex and reproductive organs of male (penis, testes, epididymis, vas deferens, scrotum, prostate and seminal vesicle), and the development of secondary sex characteristics such as muscle mass and hair patterns (Rahiman & Pool, 2010). Serum testosterone levels decline due to many causes such as aging, and endocrine disruption (ED) (Kim & Moon Du, 2011). Disruption of the male reproductive system is associated with many problems such as sperm deformities and decreased sperm count, testicular and prostate cancer, decreased libido, infertility and loss of muscle mass (Isidori *et al*., 2005; Siddiqui *et al.*, 2005; Cattabiani *et al*., 2012). The adverse effects of endocrine disrupting chemicals (EDCs) are due to their ability to antagonize the effect of endogenous hormones, mimic the effect of endogenous hormones, disrupt the synthesis and metabolism of endogenous hormones, or disrupt the synthesis of hormone receptors (Chou, 2005). Phytoestrogens are naturally occurring plant-derived phytochemicals (McVey *et al*., 2004). They can bind estrogen receptors and act as weak agonists/antagonists in both animals and humans (Hollander, 1996; Bacciottini *et al*., 2007; Rice & Whitehead, 2008). Studies on various animal species have demonstrated that high intake levels of phytoestrogens can induce adverse effects on reproductive health, and have also shown that exposure to high

levels of phytoestrogens during developmental stages can adversely affect nervous and reproductive system in rodents (Humfrey, 1998).

Green tea and black tea are produced from *Camellia sinensis.* Leaves of *C. sinensis* contain certain polyphenols and polyphenol oxidase. Polyphenol oxidase is activated when the leaves are cut and results in the polyphenols being oxidized. Different fermentation processes of the leaves produce different kinds of tea, namely non-fermented green tea, semi-fermented oolong tea, and fermented black tea (Graham, 1992; Cabrera *et al*., 2006). Green tea contains more catechins than black tea because of the post-harvest treatment (Cabrera *et al*., 2006; Henning *et al*., 2011). Green tea extract has been used in Chinese traditional medicine for treatment and prevention of many disease conditions (Liao, 2001). Polyphenols of green tea have antioxidant effects, which give green tea its effects in many diseases that are linked to the presence of reactive oxygen species (Zaveri, 2006). Green tea polyphenols have been proved to treat and prevent prostate cancer (Pandey & Gupta, 2009), and to stimulate human hair growth *in vitro* (Kwon *et al*., 2007). Black tea extract protects against pesticide-induced liver damage (Khan, 2006), and against androgen-induced prostate damage (Siddiqui *et al*., 2005). Black tea has anti-cancer properties and is also used for heart disease prevention (Ruxton, 2009).

Aspalathus linearis, commonly known as rooibos tea or red bush tea, is naturally decaffeinated, and rich in antioxidants such as phenolic acids, polyphenols and flavonoids which scavenge free radicals, and thereby prevent oxidative damage to cells (Joubert *et al*., 2008). Traditionally, rooibos tea has been used as colic relief for infants, in cosmetic and slimming products, as colouring and flavouring agents of baking products, as an anti-allergic agent and as a bronchodilator in asthma (Joubert *et*

al., 2008; Van Wyk, 2011). Rooibos tea is a rich source of antioxidants and is recommended as a safe, commercially available and effective hepatoprotector to patients with hepatic lesions (Ulicná *et al*., 2003; Kucharská *et al*., 2004). Administration of rooibos tea to carbon tetrachloride (CCL_4)-damaged rats showed antifibrotic effects (Ulicná *et al*., 2003; Kucharská *et al*., 2004). Rooibos tea can be used as a supportive therapy in diseases where free radicals are involved in the pathological processes, such as damage of ocular vessels in diabetic patients (Ulicná *et al*., 2006; Baba *et al*., 2009). Rooibos tea aqueous extract has bronchodilatory and blood pressure lowering effects *in vivo* and *in vitro* (Khan & Gilani, 2006).

Coffee is one of the most widely consumed beverages worldwide. Coffee is a tropical shrub native to Ethiopia from where it has been distributed all over the world (Gómez-Ruiz *et al*., 2007). Studies have shown inverse associations between coffee consumption and major causes of death such as diabetes, inflammatory diseases, stroke, and injuries and accidents (Freedman *et al*., 2012). Epidemiological studies showed that moderate daily consumption of filtered coffee does not induce any adverse effects on the cardiovascular system (Ranheim & Halvorsen, 2005). Coffee consumption may prevent some chronic conditions such as type II diabetes mellitus, liver cirrhosis, hepatocellular carcinoma and Parkinson's disease (Kang *et al*., 2009; Butt & Sultan, 2011; Kang *et al*., 2011).

Agathosma betulina and *Agathosma crenulata,* commonly referred to as buchu are indigenous South African shrubs native to the Cederberg mountains of the Western Cape, South Africa (Moolla & Viljoen, 2008). Buchu has been used traditionally to treat a wide range of ailments (Moolla & Viljoen, 2008; Van Wyk, 2011). Nowadays buchu preparations are used to treat many conditions such as colic, urinary tract

infections, cough, fever and rheumatism (Simpson, 1998). Both buchu species are rich in flavonoids which are responsible for the benefits of buchu oil.

There is a growing concern about adverse effects of EDCs on human and animal health. This prompted extensive research into the development of screening tests of EDCs. These screening tests involve assessing the effects of known and potential EDCs on reproductive function, sexual development and hormone production (Timm, 2005). Different methods are employed to assess the effects of EDCs on the reproductive system including *in vitro*, *in vivo* and *ex vivo* methods.

In vitro methods have been suggested as a screening tool for EDC monitoring because of low costs, reduced animal needs, and the ability to screen a large number of samples at the same time with multiple endpoints (Timm, 2005). *In vitro* methods to screen the effects of EDCs on the male reproductive system include the whole testis method, the sectioned or minced testes method, and the isolated and cultured testicular cell method (Timm, 2005). The sectioned or minced testes method is recommended by the EPA as a potential screening tool for EDCs that affect the male reproductive system (Timm, 2005). The minced or sliced testis method has been designed to screen compounds that can disrupt any of the intracellular pathways involved in the testicular steroidogenesis. The assay is based on the steroidogenic activity of testicular tissue, which primarily occurs in the Leydig cells.

The aim of this study was to employ a minced testes method to screen phytochemicals in green, black and rooibos tea, coffee and buchu samples for male reproductive toxicity and steroid production.

4.3 Materials and Methods

4.3.1 Chemicals and Reagents

All chemicals, reagents and solvents were purchased from Sigma (Germany), unless otherwise stated in the text. All reagents were of analytical grade. Rooibos tea bags (Fresh Pack™ rooibos tea MD 09.12.11 11:32 BB 08.03.13), black tea bags (Five Roses™ black tea MD 21.11.11 13:46 BB 20.11.12 (10)), green tea bags (Vital™ Chinese green tea MNF 18JAN12 G B/BEFORE 01:2015), buchu tea bags (Cape Moondance™ BB 08/08/2014) and coffee were purchased from a supermarket.

4.3.2 Animals

Pathogen-free, two months old Balb/C male mice were purchased from The University of Cape Town (South Africa). The mice were kept in a well-ventilated animal house with a 12:12 hour light/dark cycle. The mice were fed standard mouse feed (Medical Research Council, Cape Town, South Africa) with free access to normal drinking water.

4.3.3 Preparation of Beverage Extracts

Each product extract was prepared by adding 50 ml of boiling tap water to a 2.5 g prepacked bag of the product. After 3 minutes the bag was removed and the extract volume re-adjusted to 50 ml. The samples were prepared as 50 mg dry weight/ml water extracts (50 mg/ml). Extracts were then left to cool down to room temperature. Extracts were sterilized using cellulose acetate membrane syringe filters. Aliquots of the extracts were stored at -4 C^{o}.

4.3.4 Mouse Testes Cell Preparation and Culture

Medium was prepared (0.5 ml of glutamax and 0.5 ml of antibiotic and antimycotic and 49 ml of RPMI-1640 medium) under aseptic conditions in a laminar flow cabinet. After obtaining approval from the institutional animal ethics committee, mice were sacrificed by cervical dislocation and testes were dissected out under aseptic conditions, minced and mixed with 10 ml of medium in a 15 ml tube (Greiner Bio-one). Large clumps and debris were allowed to settle to the bottom of the tube (1 minute). The medium layer was transferred to another fresh 15 ml tube and the volume was made up to 10 ml using fresh medium. The cells were then incubated at 37C^{o} with 5% CO_2 for 1 hour. After that the cell suspension was centrifuged at 4000 x g for 10 minutes. The supernatant was discarded and cells were resuspended again in 10 ml of medium. After that cells were incubated at 37C^{o} with 5% CO_2 for 30 minutes. Following the incubation period, the cell suspension was centrifuged at 4000 x g for 10 minutes. The supernatant was discarded and the cell pellet was suspended in 8 ml of medium.

4.3.5 Optimization of Protein Supplements for Testicular Cell Culture

Human serum albumin (HSA) and foetal bovine serum (FBS) were evaluated as protein supplements for *in vitro* testosterone production by mouse testicular cell cultures. Cells were cultured in 96-well plates at 50 µl/well ($3x10^5$ cells/well), with different concentrations of FBS (0, 0.5, 1 and 2% in medium volume) or HAS (0, 0.2, 0.5 and 1 mg/ml of medium) and were incubated overnight at 37C^{o} and 5% CO_2. After the overnight incubation, LH (10 mU/ml) in the same culture medium was added to some cultures (100 µl/well), while duplicate cultures used as control only received medium (100 µl/well). Cultures were again incubated for 4 hours at 37C^{o} and 5%

CO_2. Supernatants were collected after the incubation period and were used for ELISA.

4.3.6 Effects of Extracts of Green, Black and Rooibos Tea, Coffee and Buchu

Medium containing 2% FBS was used to prepare a double (log_2) dilution series of the samples. The prepared samples were transferred to a 96 well plate at 50 µl/well. Cells were then added at 50 µl/well ($4x10^5$ cell/well). The plate was incubated overnight at $37C^o$ with 5% CO_2. Following the overnight incubation, cultures were incubated at $37C^o$ with 5% CO_2 with 100 µl of the LH stimulus (10 mU/ml in medium) or 100 µl of medium for four hours. Culture supernatants were collected after the stimulation period.

4.3.7 Testosterone Production Assay

After the 4 hour incubation period, supernatant from LH-treated and non-treated cells were assayed for testosterone concentrations using commercially available ELISA kit (DRG Instruments, GmbH, Germany) to assess the effect of plant extracts on hormone production. The assays were performed as per the manufacturer's instructions. The range of the testosterone assay were between 0 – 16 ng/ml.

4.3.8 Cell Viability Assay

Cytotoxicity assays are widely used in *in vitro* studies. The effects of the plant beverages on cell viability were determined by the Bradford assay. Testicular cells were cultured in Eppendorf tubes (50 µl/well) with varying concentrations of beverages, incubated overnight at 37 ˚C with 5 % CO_2. After the incubation period, cultures were incubated again for 4 hours with or without LH. Tubes were centrifuged

at 4000 x g for 10 minutes. Cell pellets were collected, washed by PBS and used for the Bradford assay.

4.3.9 The Half Maximal Inhibitory Concentration (IC_{50})

In this study, the IC_{50} is described as the half maximal inhibitory concentration of beverage extract required to achieve 50% *in vitro* response inhibition. The Masterplex ReaderFit 2010 software (version 2.0, Miraibio, http://www.miraibio.com) was used to determine a four or five-parameter nonlinear regression model equation to calculate IC_{50} values of green and black tea, roobos tea, and coffee and buchu on testosterone production in mouse testicular cell culture.

4.3.10 Statistical Analysis

One-way analysis of variance (ANOVA) was used to compare results with the controls. All data are presented as mean ± standard deviation (SD). $P<0.001$ was considered as significant.

4.4 Results and Discussion

4.4.1 Validation of Methods to Monitor Testosterone Production by Testicular Cells

Testosterone was used as a biomarker to determine the effects of plant beverages on steroidogenesis in testicular cells. Testosterone was analyzed using a competitive ELISA. The standard curve for the testosterone ELISA is shown in Figure 4.1. The standard curve was used to calculate the concentrations of testosterone in the medium. The standard curve shows that there is a good correlation (R^2= 0.9942) between the absorbance and testosterone concentration.

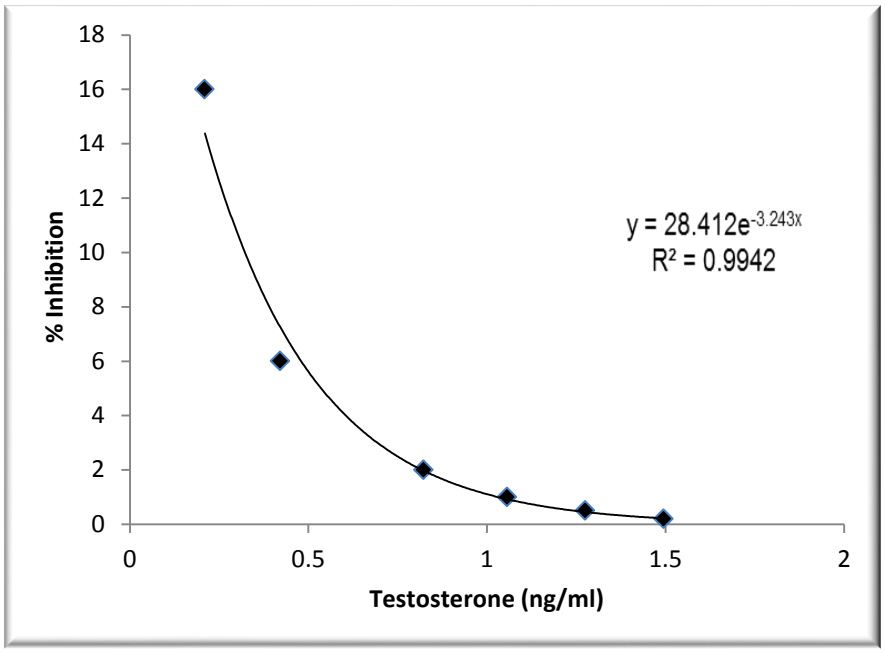

Figure 4.1: The testosterone ELISA standard curve

The rationale for using LH as a stimulant for testosterone production is based upon the biological process by which testosterone production occurs. LH treatment significantly increased testosterone production at all concentrations of HSA and FBS tested. Addition of protein supplements (HSA or FBS) did not have a significant effect on testosterone production. Results are shown in Figure 4.2. These findings showed that neither FBS nor HSA is required for testosterone production.

4.4.2 Effects of Beverages on Total Cellular Protein

Treatment of cells with varying concentrations of the plant extracts (with and without LH-treatment) had no significant effect on total cellular protein. Data are given in Tables 4.1 and 4.2 for unstimulated and stimulated testicular cultures, respectively.

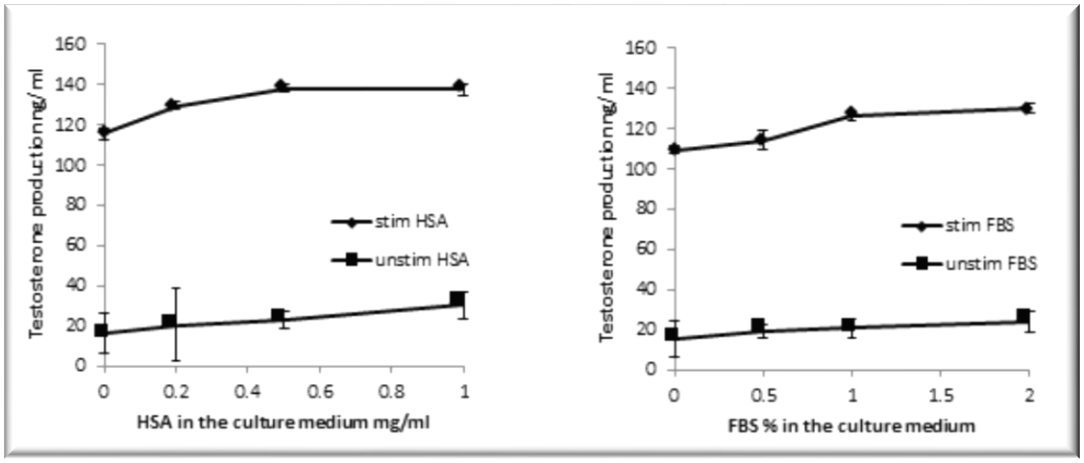

Figure 4.2: The effects of human serum albumin (HSA) and foetal bovine serum (FBS) supplement in medium on testosterone production in mouse testicular cell cultures in the presence and absence of LH. Each point is the mean and standard deviation of 8 replicates.

Table 4.1: The effects of plant extracts on total cellular protein recovery in unstimulated testicular cultures.

Concentration of teas (µg/ml)	Protein (µg/ml ± SEM)				
	Green tea	Black tea	Rooibos	Coffee	Buchu
0	441 ± 70	439 ± 73	435 ± 67	438 ± 69	422 ± 56
7.8	440 ± 72	438 ± 70	435 ± 68	442 ± 71	423 ± 58
15.6	438 ± 69	437 ± 69	433 ± 67	441 ± 65	421 ± 57
31.3	441 ± 67	443 ± 70	430 ± 68	444 ± 69	420 ± 56
62.5	434 ± 72	438 ± 70	435 ± 68	437 ± 70	420 ± 57
125	430 ± 66	437 ± 70	431 ± 67	439 ± 69	418 ± 56
250	431 ± 67	434 ± 69	433 ± 67	439 ± 69	422 ± 58
500	428 ± 69	427 ± 68	431 ± 67	436 ± 69	415 ± 57

Results are means ± SEM of four replicates. No significant differences were found between the different treatments ($P > 0.05$).

Table 4.2: The effects of plant extracts on total cellular protein recovery in stimulated testicular cultures.

Concentration of teas (µg/ml)	Protein (µg/ml ± SEM)				
	Green tea	Black tea	Rooibos	Coffee	Buchu
0	415 ± 53	411 ± 50	415 ± 53	415 ± 53	411 ± 50
7.8	417 ± 52	412± 51	415 ± 50	417 ± 54	412 ± 51
15.6	415 ± 51	414 ± 52	415 ± 51	415 ± 55	411 ± 52
31.3	414 ± 52	419 ± 54	407 ± 50	411 ± 54	415 ± 54
62.5	409 ± 50	416 ± 54	411 ± 53	417 ± 52	416 ± 50
125	408 ± 51	412 ± 52	410 ± 51	408 ± 51	412 ± 52
250	411 ± 52	410 ± 52	412 ± 52	417± 54	416 ± 52
500	408 ± 52	402 ± 50	402 ± 51	409 ± 52	409 ± 50

Results are means ± SEM of four replicates. No significant differences were found between the different treatments ($P > 0.05$).

After cell death, cell membranes disintegrate resulting in leaking of proteins. The loss of intracellular protein and its release into the culture medium can result in the decrease of total cellular protein of treated cultures compared to control. In Tables 4.1 and 4.2 it is evident that the total cellular protein had not been affected after treatment with plant extracts. These findings suggest that plant extracts are not cytotoxic to the testicular cultures.

4.4.3 Effects of Beverages on Testosterone Production

The effects of increasing concentrations of green tea, black tea, rooibos tea, coffee and buchu in culture medium on testosterone production in unstimulated and stimulated testicular cultures are shown in Figures 4.3, 4.4, 4.5, 4.6 and 4.7, respectively. For experimental details, see "Materials and Methods".

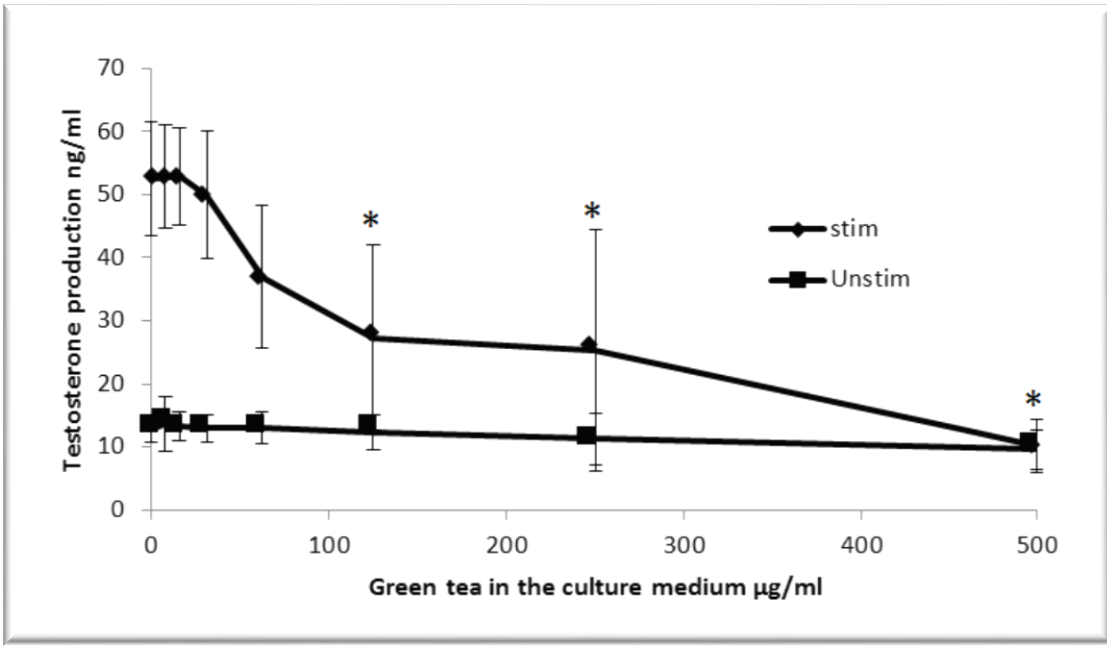

Figure 4.3: The effects of green tea extract on testosterone production by mouse testicular cell cultures. Results are the mean and standard deviation of 6 replicates. (* P<0.010 relative to the control).

In the presence of LH, high concentrations of green tea inhibited testosterone production (Figure 4.3). At 500 µg/ml, green tea inhibited testosterone production by 81%. At 250 µg/ml the inhibition was by 52%. At 125 µg/ml green tea inhibited testosterone production by 48%. The 50% inhibitory concentration (IC_{50}) for green tea was 173 µg/ml. Green tea had no effect on testosterone production in the absence of LH. These results showed that green tea extracts were not cytotoxic to the testicular cultures. This indicates that green tea inhibition of testosterone production in the presence of LH is due to modulation or inhibition of testosterone pathways.

In the stimulated cultures, high concentrations of black tea inhibited testosterone production (Figure 4.4). At 500 µg/ml black tea inhibited testosterone production by 85%. At 250 µg/ml the testosterone inhibition was by 78%. At 125 µg/ml black tea inhibited testosterone production by 55%. The IC_{50} for black tea is 48 µg/ml. Black

tea had no effect on testosterone production in the absence of LH. These results showed that black tea extracts were not cytotoxic to the testicular cultures. This indicates that black tea inhibition of testosterone production in the presence of LH is due to modulation or inhibition of enzymatic reactions required for steroidogenesis.

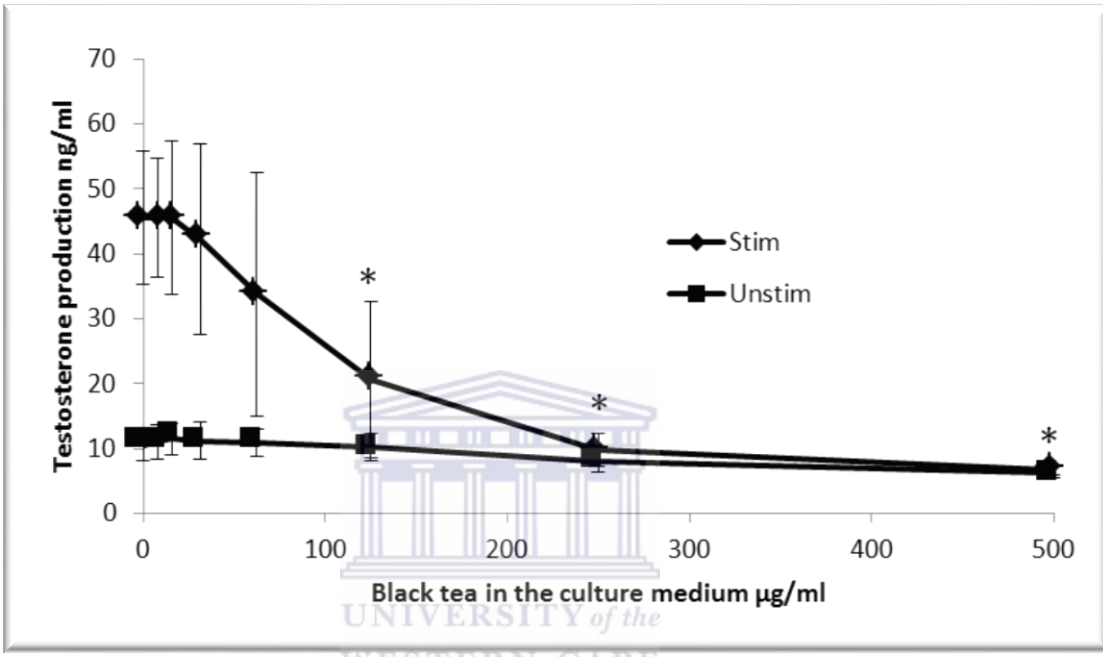

Figure 4.4: The effects of black tea extract on testosterone production by mouse testicular cell cultures. Results are the mean and standard deviation of 6 replicates. (* P<0.010 relative to the control).

Green and black tea polyphenols have antimutagenic, antidiabetic, anti-inflammatory, antibacterial and antiviral properties (Cabrera *et al*., 2006; Nune *et al*., 2009; Cui *et al*., 2012). However, not enough data are available on their effects on steroidogenesis of the male reproductive system. Green and black tea extracts adversely affect testosterone production in rats both *in vivo* (Chandra *et al*., 2011), and inhibited testosterone production *in vitro* by rat Leydig cells (Figueiroa *et al*., 2009). Epigallocatechin gallate (EGCG) of green tea inhibited testosterone production *in vivo* when injected intraperitoneal into rats (Kao *et al*., 2000). The results of the present

investigation are consistent with results obtained for these previous studies. On the contrary, reports suggest that black tea extracts increases the serum testosterone levels *in vivo* (Ratnasooriya & Fernando, 2008; Yu *et al*., 2010). This study has shown that black tea extracts inhibited testosterone production by mouse Leydig cells.

Rooibos tea did not affect testosterone production in the presence or in the absence of LH (Figure 4.5). Previous studies suggest that rooibos tea could improve reproduction and health as it is a rich source of scavenging agents (McKay & Blumberg, 2007; Awoniyi *et al*., 2012). This study showed that rooibos tea had no adverse effects on testosterone production by testicular cells. On the contrary, recent studies on a testosterone-secreting cell line demonstrated that rooibos tea decreased steroidogenesis (Schloms *et al*., 2012).

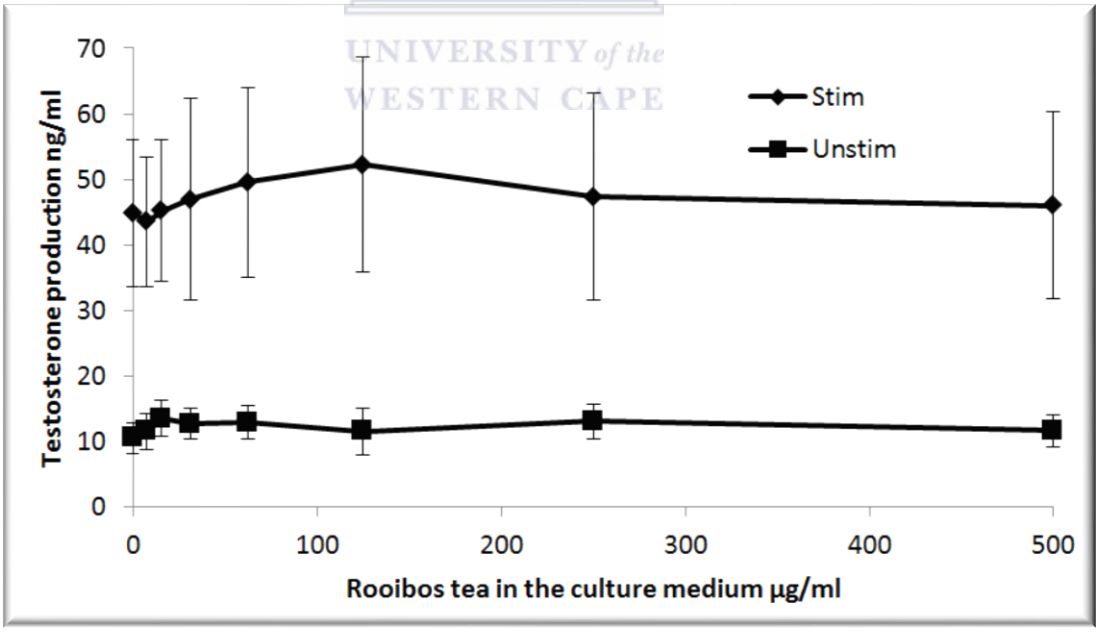

Figure 4.5: The effects of rooibos tea extract on testosterone production by mouse testicular cell cultures. Results are the mean and standard deviation of 6 replicates. (P>0.001).

Coffee inhibited LH-stimulated testosterone production by mouse testicular cells at 500 µg/ml, 250 µg/ml and at 125 µg/ml by 81%, 65% and 37%, respectively (Figure 4.6). The IC_{50} for coffee is 64 µg/ml. Coffee did not affect basal testosterone production. These results showed that coffee extracts were not cytotoxic to the testicular cultures. This indicates that coffee inhibition of testosterone production in the presence of LH is due to a modulation of the pathways involved in steroidogenesis.

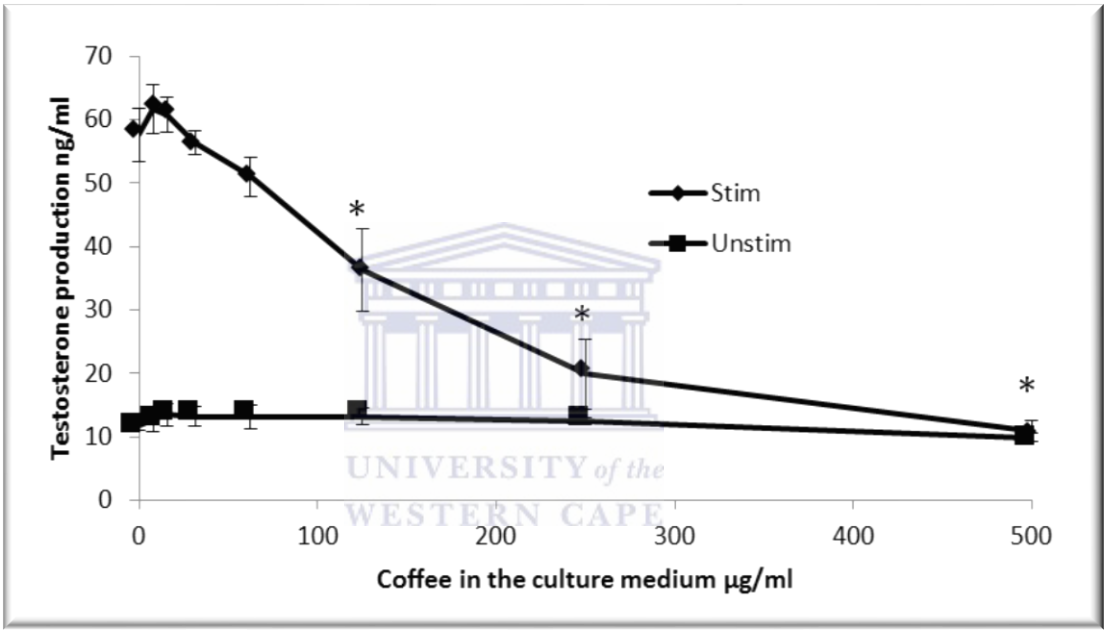

Figure 4.6: The effects of coffee extract on testosterone production by mouse testicular cell cultures. Results are the mean and standard deviation of 6 replicates. (* P<0.010 relative to the control).

Buchu did not affect testosterone production neither in the presence of LH nor in the absence of LH (Figure 4.6). To our knowledge, no studies have yet been published on the effects of buchu on male reproduction.

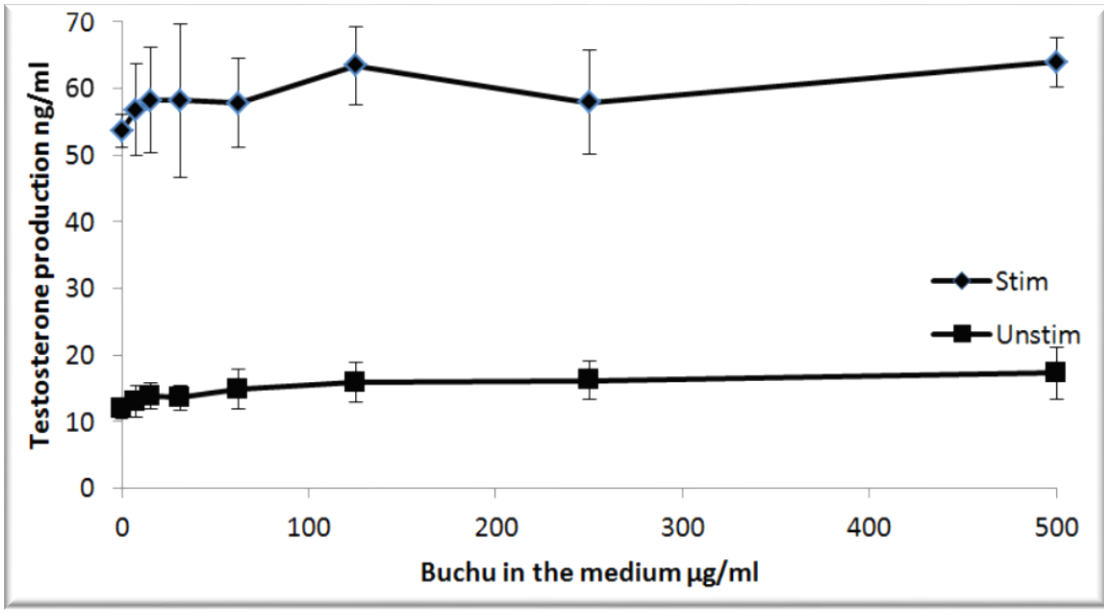

Figure 4.7: The effects of buchu extract on testosterone production by mouse testicular cell cultures. Results are the mean and standard deviation of 6 replicates. (P>0.001).

4.5 Conclusions

The present study showed that the extracts of green tea, black tea and coffee are not cytotoxic to the testicular cultures. However extracts of these beverages inhibited LH stimulated testosterone production. This study also shows that black tea is the most potent inhibitor of testosterone synthesis (IC_{50}= 48 µg/ml) followed by coffee (IC_{50}= 64 µg/ml) and the green tea (IC_{50}= 173 µg/ml). The cause of testosterone inhibition is still unknown at this stage. It could possibly be due to an inhibition of the StAR protein expression which carries cholesterol to the P450 enzyme system (Houk *et al*., 2004) or due to a modulation of the enzymatic reactions of steroidogenesis (cytochrome P450 enzyme system) (Akingbemi *et al*., 2004). Furthermore, the extracts could be impairing the action of LH (Chandra *et al*., 2011), or possibly due to other unknown reason/s. These findings suggest that green tea, black tea and coffee may potentially inhibit testosterone production. Further studies are warranted to

determine and clarify the exact mechanisms involved and the fractions of teas that cause the inhibition. *In vivo* studies are also needed to confirm these results. If *in vivo* studies confirm these effects, maximum recommended daily intake levels should be made available to consumers to warn them of potential risks associated with these beverages.

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CHAPTER 5

CONCLUSIONS AND FUTURE PERSPECTIVES

This study investigated the effects of green, black and rooibos tea, coffee and buchu on testosterone production using mouse testicular cultures. The thesis consists of 5 chapters. A summary of the chapters follows.

Chapter 1 outlined the anatomy and physiology of the male reproductive system with special emphasis on testosterone biosynthesis and regulation, modulation of male reproductive function due to exposure to EDCs and the adverse effects of EDCs on the male reproductive system. Chapter 2 provided an overview of the most commonly consumed plant beverages (teas, coffee and buchu). It looked at the phytochemicals in these beverages and the potential effects of these beverages on different physiological systems. The medicinal and adverse effects of the plant beverages were also summarized in this chapter. Chapter 3 covered the aim of this study and offered a general motivation why research needs to be done on the effects of plant beverages on testosterone production.

Chapter 4 presented new research on the effects of plant beverages on testosterone synthesis by testicular cultures. This chapter will be submitted for publication to a peer-reviewed scientific journal. The first objective of this study was to optimize culture conditions for *in vitro* testosterone production by testicular cultures. The results of this study showed that the addition of protein supplements to the medium did not affect testosterone production. The study also confirmed that LH stimulation of testicular cultures resulted in upregulated testosterone synthesis. The second objective

of this study was to investigate the effects of black, green and rooibos teas, coffee and buchu on cell viability of mouse testicular cultures. These experiments showed that none of the beverages were cytotoxic at the concentrations investigated. The third objective of this study was to investigate the effects of black, green and rooibos teas, coffee and buchu on testosterone production by testicular cultures. The results obtained from these experiments showed that rooibos tea and buchu did not affect testosterone production in the presence or absence of LH. The results also indicated that green tea, black tea and coffee inhibited testosterone production by mouse testis cultures in the presence of LH, but not in the absence of LH. Black tea was the most potent inhibitor of testosterone synthesis by mouse testis cultures (IC_{50}= 48 µg/ml), followed by coffee (IC_{50}= 64 µg/ml) and green tea (IC_{50}= 173 µg/ml). The cause/s of testosterone inhibition is still unknown at this stage. Some of the conceivable opinions of inhibition are given below.

Suppression of testosterone synthesis could possibly be due to inhibition of the StAR protein expression which carries cholesterol to the P450 enzyme system or due to modulation of the enzymatic reactions of steroidogenesis (cytochrome P450 enzyme system). Alternatively, the extracts could be impairing the action of LH due to receptor binding modulation or intracellular messaging mechanisms or possibly due to other unknown reason/s. These findings suggest that green tea, black tea and coffee may potentially inhibit testosterone production. Future studies must be done to determine if these effects also manifest *in vivo*. If *in vivo* experiments confirm *in vitro* data pertinent research will be required to minimize risks to consumers and also to elucidate the exact mechanism/s whereby these beverages inhibit testosterone production.