

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 and their by-products, pesticides, heavy metals, pharmaceutical products, phytoestrogens and mycotoxins. This can result in such as decline of testosterone levels, sperm deformities and decreased 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

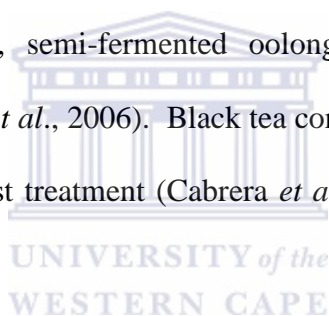
Several compounds are found in plant-based foods, namely phytochemicals or phytoestrogens. Phytochemicals are naturally occurring, biologically active chemical compounds in plants. Phytoestrogens are divided into different groups, isoflavonoids, lignans, and coumestans (Kurzer & Xu, 1997; Miniello *et 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-Iethy & 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. sinensis*), 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 <i>et al.</i> , 2006; Chacko <i>et al.</i> , 2010; Venkateswara <i>et al.</i> , 2011
Total phenolic compounds (µg/100 g)	13	8.9	Thompson <i>et al.</i> , 2006
Total phenols (2.5 g equivalent to:)	165 mg gallic acid	124 mg gallic acid	Lee <i>et al.</i> , 2002
Oxidized phenolic compounds (thearubigins and theaflavins) (% of dry weight)	0	25	Cabrera <i>et al.</i> , 2006; Chacko <i>et al.</i> , 2010; Henning <i>et al.</i> , 2011; Venkateswara <i>et al.</i> , 2011
Lignan content (µg/100g)	12	8.1	Thompson <i>et al.</i> , 2006
Antioxidant capacity (per serving)	436 mg vitamin C equivalents	239 mg vitamin C equivalents	Lee <i>et al.</i> , 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 <i>in vivo</i> , <i>in vitro</i> and in epidemiological studies	Adhami <i>et al.</i> , 2003; Pandey & Gupta, 2009
	Black tea	Protection against androgen-induced prostate cancer <i>in vivo</i> in rat	Siddiqui <i>et al.</i> , 2005
Testicular toxicity	Green tea	Protection against cadmium-induced testicular toxicity <i>in vivo</i> in rat	El-Shahat <i>et al.</i> , 2009
		Protection against reactive oxygen species-induced damage in testicular tissue	Awoniyi <i>et al.</i> , 2011
	Black tea	Protection against sodium fluoride-induced testicular toxicity <i>in vivo</i> in rat	El-Iethy & 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 <i>et al.</i> , 2001
		Protection against nicotine-induced hyperlipidemia and atherogenesis <i>in vivo</i> in rat	Joukar <i>et al.</i> , 2012
Degenerative diseases of nervous system	Green tea	Protection against brain atrophy and cognitive dysfunction in mice	Unno <i>et al.</i> , 2007
	Black tea	Protection against degenerative changes in CNS in mice	Trivedi <i>et al.</i> , 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 <i>et al.</i> , 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 <i>in vitro</i> and <i>in vivo</i> .	Kunishiro <i>et al.</i> , 2001
Colic	Decreases the acetylcholine-induced contractions.	Snyckers & Salemi 1974; Khan & Gilani, 2006
Inflammation	Protects against colitis.	Baba <i>et al.</i> , 2009
Nervous system	Long-term rooibos tea consumption prevents accumulation of lipid peroxides in the brain.	Inanami <i>et al.</i> , 1995
Testicular toxicity	Protects against reactive oxygen species-induced damage in testicular tissue.	Awoniyi <i>et al.</i> , 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).

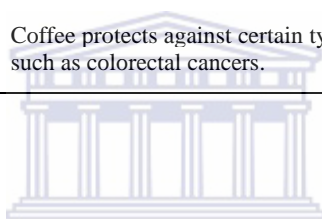
2.4.3 Beneficial Effects of Coffee on Body Systems

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 <i>et al.</i> , 2012
Inflammation and endothelial function	Filtered coffee consumption has an inverse association with inflammation and endothelial dysfunction.	Bonita <i>et al.</i> , 2007
Myocardial infarction	Moderate coffee consumption reduces the risk of myocardial infarction by 31% relative to no consumption.	Panagiotakos <i>et al.</i> , 2003
Anticarcinogenic activity	Coffee protects against certain types of cancers such as colorectal cancers.	Cavin <i>et al.</i> , 2002



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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	<i>Agathosma betulina</i> has hypotensive or antihypertensive potential effects.	Tabassum & Ahmad, 2011
Colic	Buchu oil has spasmolytic action.	Lis-Balchin <i>et al.</i> , 2001
Antimicrobial	Buchu oil has very low antimicrobial activity.	Lis-Balchin <i>et al.</i> , 2001
Anti-inflammatory	Buchu oil has an <i>in vitro</i> anti-inflammatory action	Lis-Balchin <i>et al.</i> , 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. sinensis*), 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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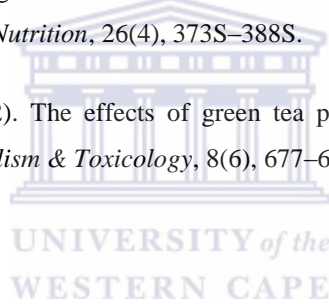
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CHAPTER 3

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).

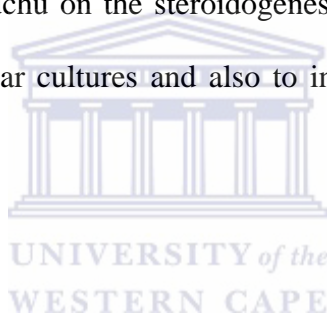
Phytoestrogens are naturally occurring plant-derived phytochemicals (McVey *et al.*, 2004) which can bind estrogen receptors and behave as weak estrogen agonists/antagonists in both human and animals (Hollander, 1996; Bacciottini *et al.*, 2007; Rice & Whitehead, 2008). Studies have demonstrated that 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-Iethey & 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 (CCL₄)-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°.

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° with 5% CO₂ 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° with 5% CO₂ 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.

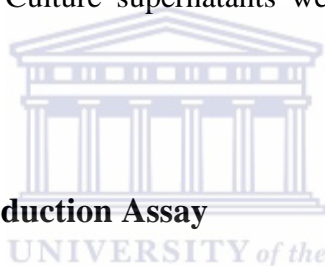
4.3.5 Optimization of Protein Supplements for Testicular Cell Culture

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 (3x10⁵ 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° and 5% CO₂. 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° and 5%

CO₂. 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 (log₂) 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 (4x10⁵ cell/well). The plate was incubated overnight at 37C^o with 5% CO₂. Following the overnight incubation, cultures were incubated at 37C^o with 5% CO₂ 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 % CO₂. 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 (IC₅₀)

In this study, the IC₅₀ 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 IC₅₀ 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.

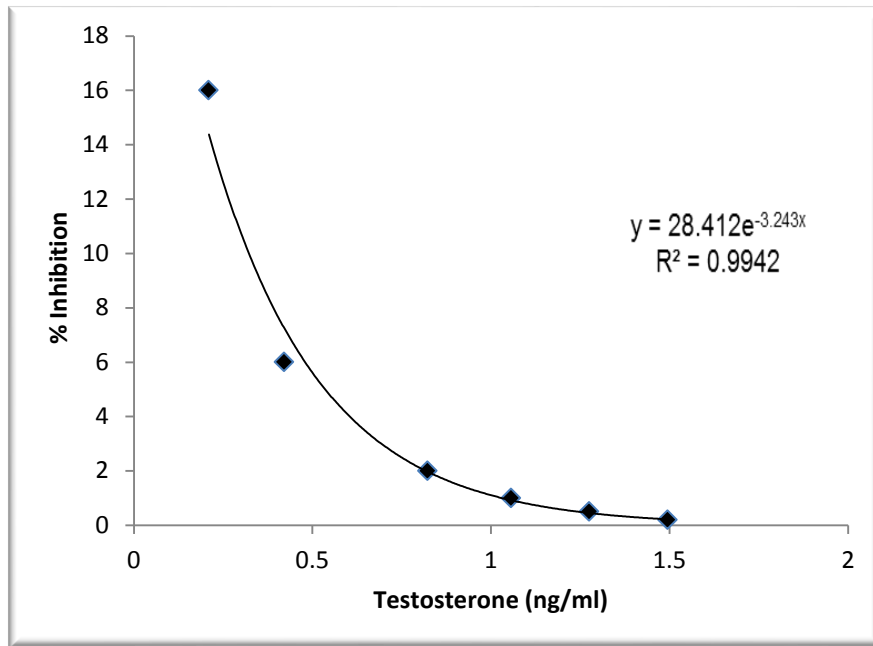
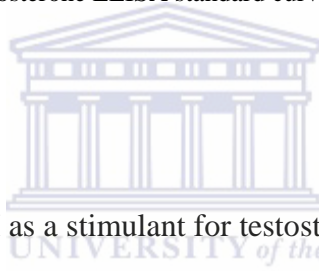


Figure 4.1: The testosterone ELISA standard curve



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.

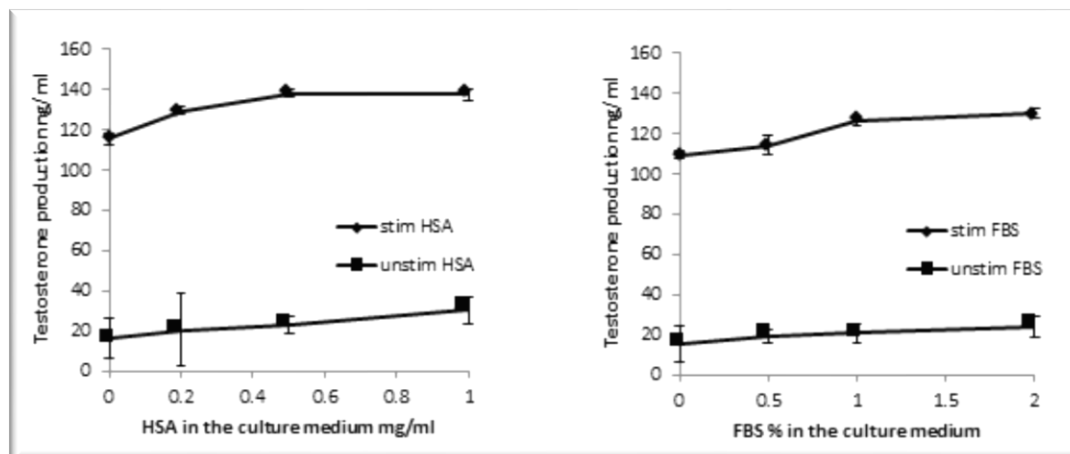


Figure 4.2: The effects of human serum albumin (HSA) and foetal bovine serum (FBS) supplement in medium on testosterone production in mouse testicular cell cultures in the presence and absence of LH. Each point is the mean and standard deviation of 8 replicates.

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

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

Results are means \pm 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 ($\mu\text{g/ml}$)	Protein ($\mu\text{g/ml} \pm \text{SEM}$)				
	Green tea	Black tea	Rooibos	Coffee	Buchu
0	415 \pm 53	411 \pm 50	415 \pm 53	415 \pm 53	411 \pm 50
7.8	417 \pm 52	412 \pm 51	415 \pm 50	417 \pm 54	412 \pm 51
15.6	415 \pm 51	414 \pm 52	415 \pm 51	415 \pm 55	411 \pm 52
31.3	414 \pm 52	419 \pm 54	407 \pm 50	411 \pm 54	415 \pm 54
62.5	409 \pm 50	416 \pm 54	411 \pm 53	417 \pm 52	416 \pm 50
125	408 \pm 51	412 \pm 52	410 \pm 51	408 \pm 51	412 \pm 52
250	411 \pm 52	410 \pm 52	412 \pm 52	417 \pm 54	416 \pm 52
500	408 \pm 52	402 \pm 50	402 \pm 51	409 \pm 52	409 \pm 50

Results are means \pm 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”.

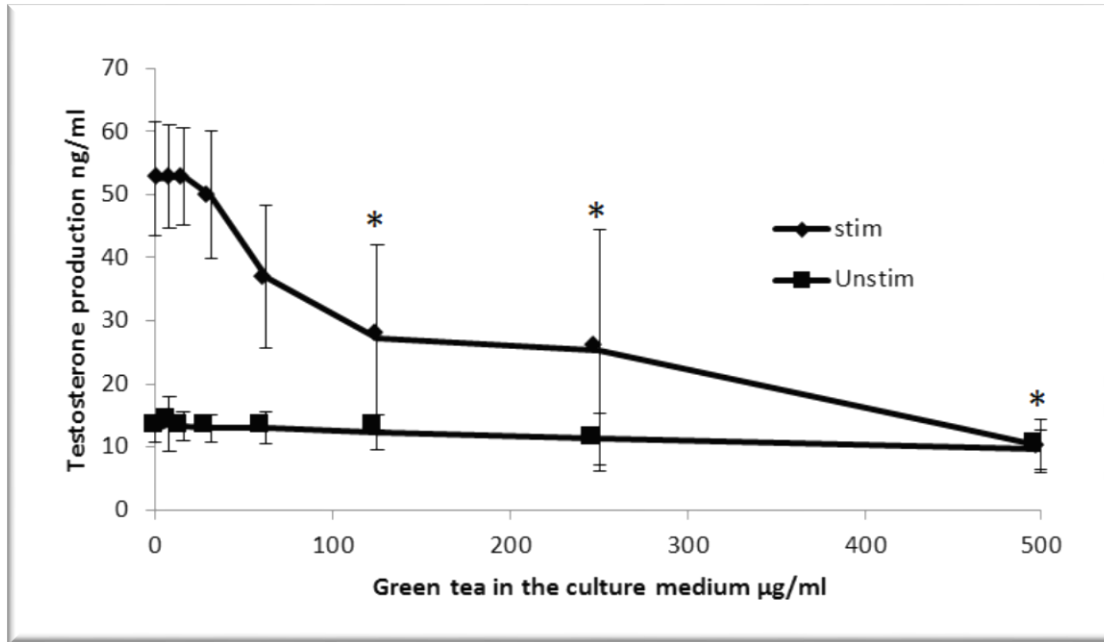


Figure 4.3: The effects of green tea extract on testosterone production by mouse testicular cell cultures. Results are the mean and standard deviation of 6 replicates. (* $P < 0.010$ relative to the control).

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.

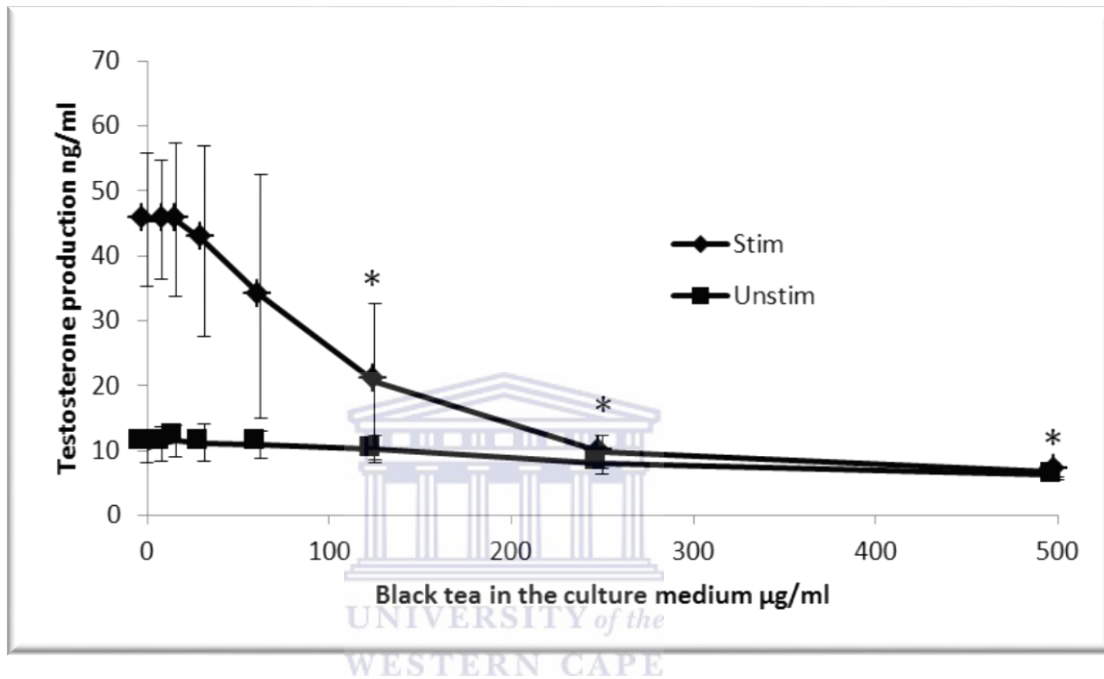


Figure 4.4: The effects of black tea extract on testosterone production by mouse testicular cell cultures. Results are the mean and standard deviation of 6 replicates. (* $P < 0.010$ relative to the control).

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).

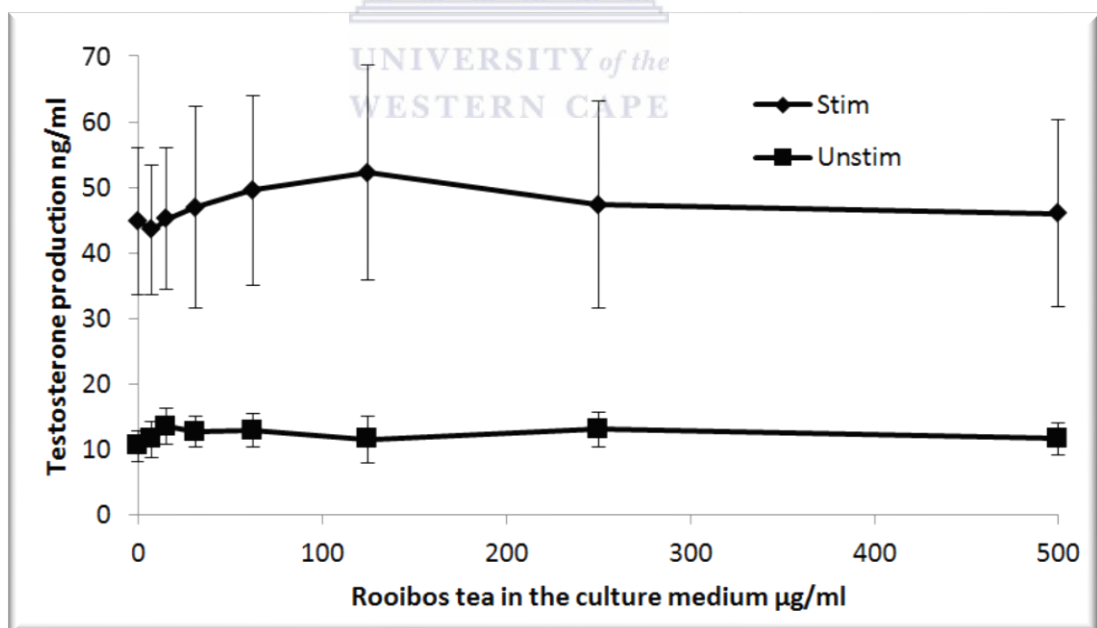


Figure 4.5: The effects of rooibos tea extract on testosterone production by mouse testicular cell cultures. Results are the mean and standard deviation of 6 replicates. ($P > 0.001$).

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 IC₅₀ 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.

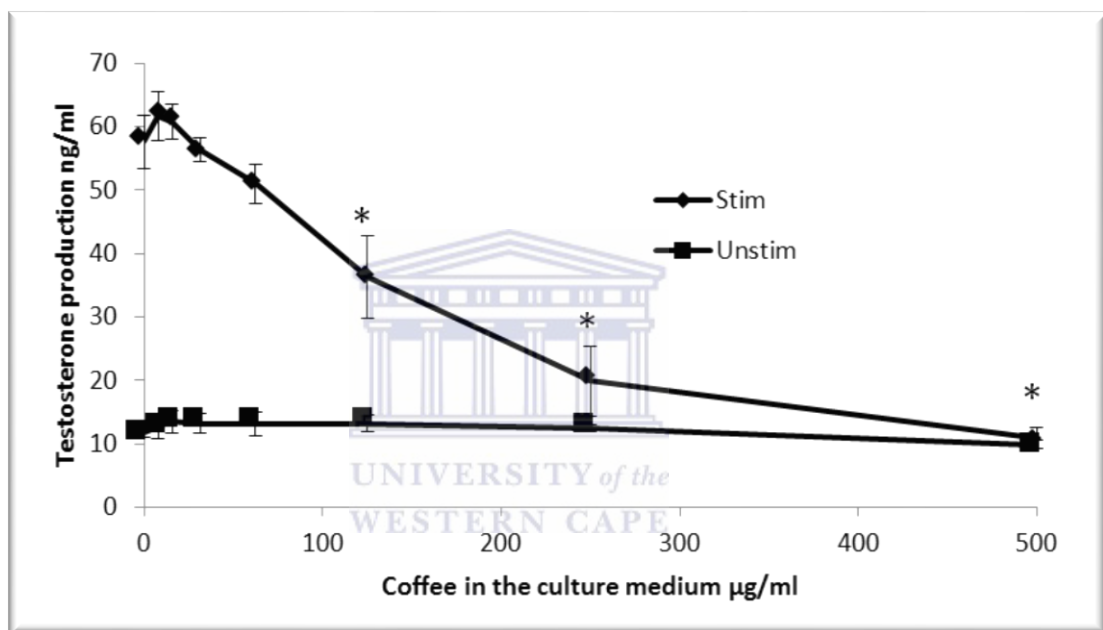


Figure 4.6: The effects of coffee extract on testosterone production by mouse testicular cell cultures. Results are the mean and standard deviation of 6 replicates. (* P<0.010 relative to the control).

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.

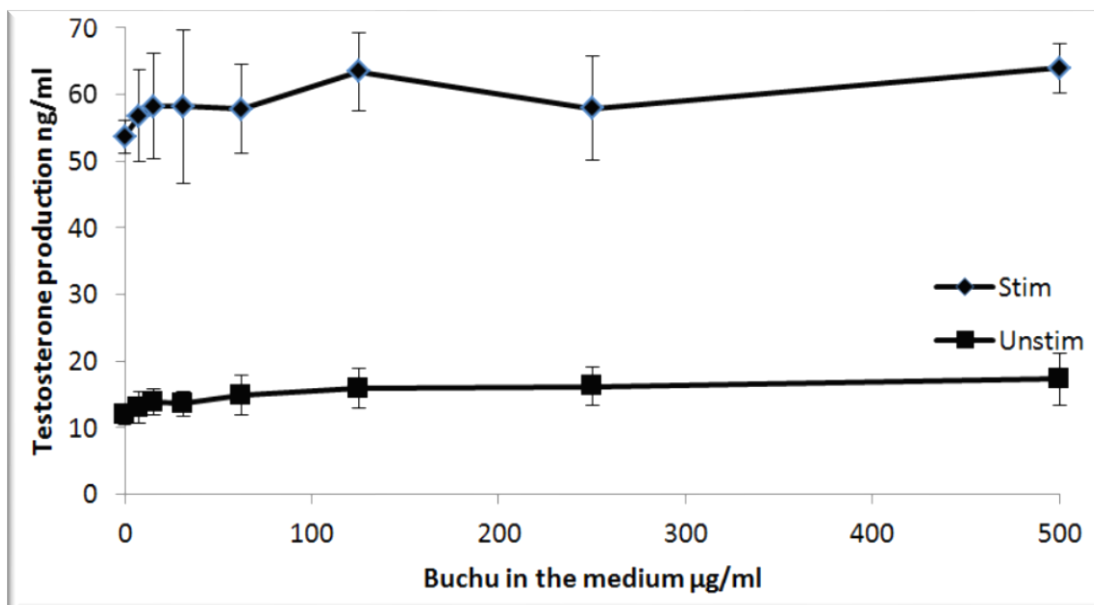


Figure 4.7: The effects of buchu extract on testosterone production by mouse testicular cell cultures. Results are the mean and standard deviation of 6 replicates. ($P > 0.001$).

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 ($IC_{50} = 48 \mu\text{g/ml}$) followed by coffee ($IC_{50} = 64 \mu\text{g/ml}$) and the green tea ($IC_{50} = 173 \mu\text{g/ml}$). 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

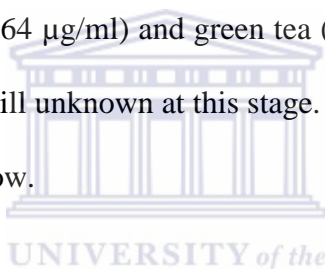
CONCLUSIONS AND FUTURE PERSPECTIVES

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 ($IC_{50} = 48 \mu\text{g/ml}$), followed by coffee ($IC_{50} = 64 \mu\text{g/ml}$) and green tea ($IC_{50} = 173 \mu\text{g/ml}$). 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.