THE EFFECT OF ROOIBOS AND GREEN TEA FLAVONOIDS ON THE PHYSIOLOGY OF TM3 LEYDIG CELLS

by

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A dissertation submitted in fulfilment of the requirements for the degree of

Magister Scientiae

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DECLARATION

I, the undersigned, hereby declare that "*The effect of rooibos and green tea flavonoids on the physiology of TM3 Leydig cells*" is my own work and has not previously in its entity, or in part, been submitted for any degree or examination in any other university. All the resources I have quoted have been indicated and acknowledged by complete references.

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ACKNOWLEDGEMENTS

Firstly, sincere thankfulness to my Heavenly Father for providing me the strength and perseverance to complete this study. Lord you made this possible through Your grace. “Trust in the Lord with all your heart and lean not on your own understanding. In all your way acknowledge Him and He shall direct your path” Proverbs 3:5-6.

My supervisor, Professor Thomas Monsees, thank you for your support and guidance throughout my research project. Your valuable input and assistance is sincerely appreciated.

My parents, Shawn and Leotha Webber, thank you for being my pillar of strength and for always encouraging me to accomplish my goals. You sacrificed so much for me and I sincerely appreciate it.

Thank you to my friends and family for always providing moral support. A special thank you to Elric Dieter Raynardt for supporting me and always being there throughout this journey.

To the staff and students of the Medical Biosciences department; Robin Booysen, Eurinah Harris, Nicole Haines, Dr.Tiza Ng’uni, Sameera Slahudeen and so many more, thank you for your assistance and enriching me on an academic and personal level. You are all truly good people.

Special thanks to Professor Mervin Meyer and to Dr Nicole Sibuye for allowing me to use the flow cytometry equipment in your laboratory.

I would sincerely like to thank the National Research Foundation (NRF) for providing funds for this research.

DEDICATION

This thesis is dedicated to my parents Shawn and Leotha Webber. I'm blessed to have you as my parents. I'll be eternally grateful for all that you have done for me.

I LOVE YOU

Table of Contents

ABSTRACT

Camellia sinensis (C.sinesis) and *Aspalathus linearis (A.linearis)* are popular beverages consumed in many countries. Consumption of these plants may protect against cardiovascular disease, neurodegenerative disease, various cancers and osteoporosis. Previous *in vivo* studies demonstrated the beneficial effect of *C.sinesis* and *A.linearis* on various sperm parameters. However, *in vitro* studies demonstrated that these plants may possess anti-androgenic properties that finally result in a reduction of testosterone production in TM3 Leydig cells. *C.sinesis* and *A.linearis* contain an array of phenolic compounds of which the major antioxidant activity is attributed to epigallocathechin-3-gallate (EGCG) and aspalathin, respectively.

Leydig cells are situated in the male reproductive testes, adjacent to the seminiferous tubules. The principal function of these cells is to produce testosterone which is vital for male sexual differentiation, gamete production and the development of secondary sexual characteristics. The aim of this study was to establish the effects of EGCG and aspalathin on TM3 Leydig cell physiology.

TM3 Leydig cells were either exposed to 0.1, 1, 10, 50 or 100µM of EGCG or aspalathin for 24 hours. Cells were subjected to a cytotoxicity analysis by MTT assay and testosterone synthesis was assessed by testosterone ELISA kit. Furthermore, quantification of apoptotic and necrotic cells was determined by Tali® Apoptosis kit (Annexin/PI) for flow cytometry. Mitochondrial membrane potential was evaluated using tetramethylrhodamine ethyl ester (TMRE) in conjunction with flow cytometry and live cell imaging, the latter using the Zeiss axiovert 200M inverted fluorescence microscope.

The results of this study indicate that EGCG had no significant effect on cell viability whereas aspalathin increased mitochondrial dehydrogenase activity at the higher concentrations. Therefore, both EGCG and aspalathin were proven to have no cytotoxic effect, within the tested concentration range. However, they possess the ability to induce cellular stress at the highest concentration. The initial aim for determining the effect of EGCG and aspalathin on testosterone synthesis could not be established as the TM3 Leydig cell steroidogenesis model was defective. Although EGCG and apalathin possess

pro-oxidant properties, they fail to induce significant apoptotic and necrotic cell deaths at the tested concentrations. The results were further confirmed when evaluating the effect of these active components on mitochondrial membrane potential. The results indicate that both EGCG and aspalathin result in a loss of membrane potential and favours depolarization. However, the depolarization was proven to be independent of apoptosis and necrosis. Given the time interval and concentrations used in this study, it may be deduced that EGCG and aspalathin lead to cellular stress without essentially leading to cellular demise.

Keywords: *Camellia sinensis*, *Aspalathus linearis*, TM3 Leydig cells, Epigallocathechin-3-gallate (EGCG), Aspalathin, Tetramethylrhodamine ethyl ester (TMRE), Human chorionic Gonadotropin (hCG).

LIST OF FIGURES

LIST OF ABBREVIATIONS

17β-HSD	17β-Hydroxysteroid dehydrogenase
3β-HSD	3β-Hydroxysteroid dehydrogenase
AA	Arachidonic acid
ADP	Adenosine diphosphate
AIF	Apoptosis inducing factor
ALC	Adult Leydig cell
AR	Androgen receptor
ARTISt	AA related thioesterase involved in steroidogenesis
ASP	Aspalathin
ATP	Adenosine triphosphate
BTB	Blood testis barrier
cAMP	Cyclic adenosine monophosphate
CCCP	Carbonylcyanide-3-chlorophenylhydrazone
CoA	Acyl-coenzyme A
Co-Cells	Compartmentalizing cells
COX	Cyclooxygenase
CREB	cAMP response- element binding protein
DAG	Diacylglycerol
DHEA	Dehydroepiandrosterone
DHT	Dihydrotestosterone

DISC	Death inducing signalling complex
EGCG	Epigallocathechin-3-gallate
EPOX	Epoxygenase
ERK 1/2	Extracellular signal-regulated kinase 1/2
ETC	Electron transport chain
FLC	Fetal Leydig cell
FSH	Follicle-stimulating hormone
GnRH	Gonadotropin-releasing hormone
hCG	Human chorionic gonadotropin
hTERT	Human telomerase reverse transcriptase
ILC	Immature Leydig cell
INSL3	Insulin-like growth factor 3
JNK/SAPK	c-Jun N-terminal kinase/stress-activated protein kinase
LDL	Low-density lipoproteins
LH	Luteinizing hormone
LPOX	Lipoxygenase
MAPK	Mitogen activated protein kinases
MOMP	Mitochondrial outer membrane permeabilization
NNLC	Neonatal Leydig cell
OMM	Outer mitochondrial membrane
P450 scc	P450 Cholesterol side-chain cleavage enzyme

PBR	Peripheral-type benzodiazepine receptor
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
PMA	Phorbol 12 -myristate-13-acetate
PTP	Permeability transition pore
ROS	Reactive oxygen species
SER	Smooth endoplasmic reticulum
StAR	Steroidogenic acute regulatory protein
TMRE	Tetramethylrhodamine ethyl ester
VDAC	Voltage- dependent anion channel

Chapter One: Literature Review

1.1 Male Reproductive System Overview

The anatomy of the human male reproductive system (Figure 1.1) composes of internal and external genitalia. The internal genitalia comprise of the testes, accessory ducts and accessory sex organs, whereas, the external genitalia comprise of the penis and scrotum (McLafferty *et al*., 2014). The physiological purpose of the male reproductive system is to produce sex hormones and transfer semen into the female reproductive tract, to allow successful fertilization (Mawhinney and Mariotti, 2013).

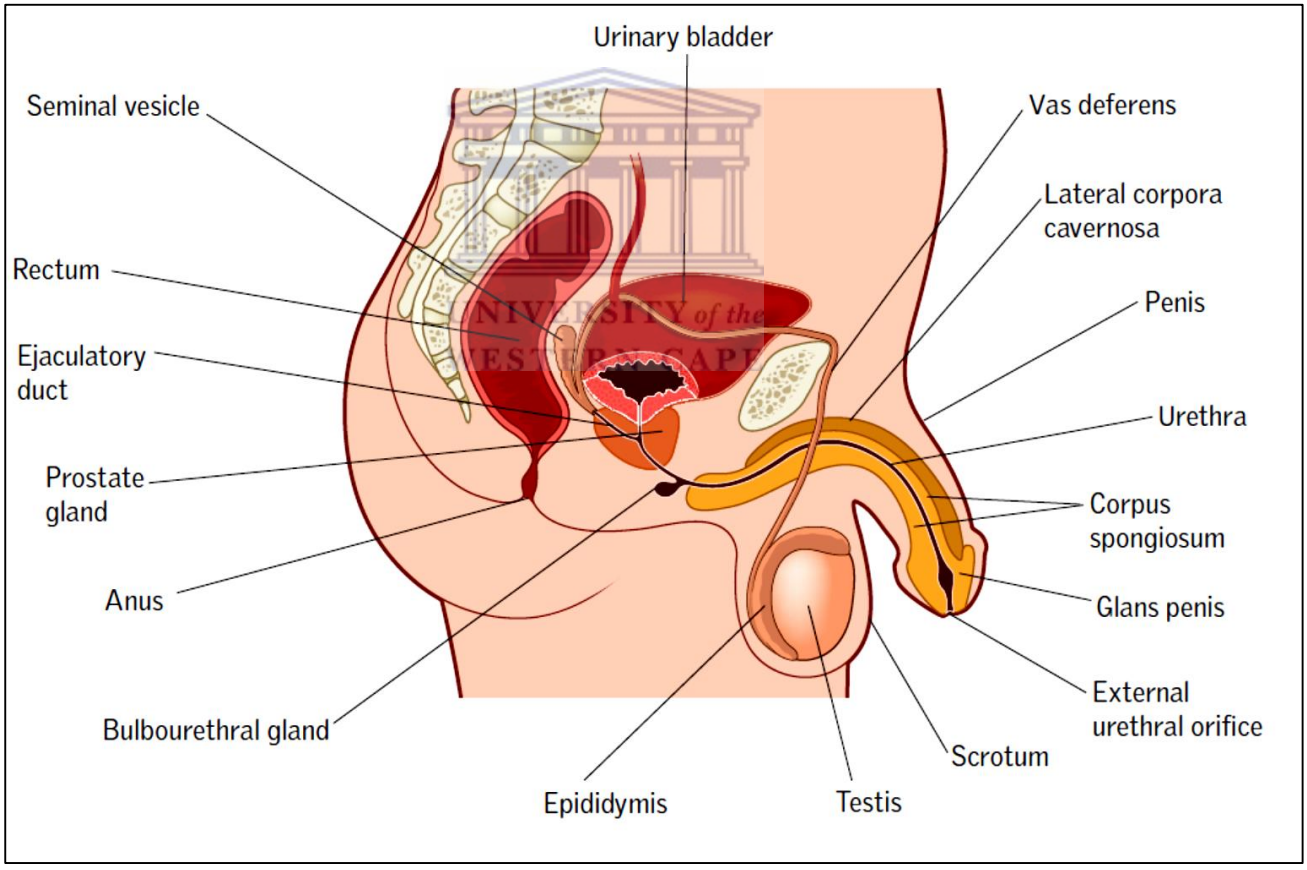


Figure 1.1: Diagram Illustrating the anatomy of the Male Reproductive System (Mawhinney and Mariotti, 2013).

1.1.1 Testes

The testes are two oval, glandular organs that are situated within the scrotum and capsuled within the tunica albuginea, a dense white connective tissue. During fetal development, the testes are situated retroperitoneally within the abdominal cavity. However, the testes and spermatic cord begin to descend through the inguinal canal into the scrotum approximately two months before the birth of the fetus (McLafferty *et al*., 2014).

Characteristically, the testes are organized into a tubular compartment and an interstitial compartment. The tubular compartment consists of seminiferous tubules (tubuli seminiferi) whereas the interstitial compartment (interstitium) is situated between the seminiferous tubules (Weinbauer *et al*., 2010). Spermatogenesis occurs in the seminiferous tubules by the regulation of Sertoli cells and steroidogenesis occurs within the Leydig cells, found in the interstitium (Schlatt *et al*., 1997).

The testes are responsible for producing male gametes and sex steroid hormones. The function of the testes are regulated by the central nervous system and the pituitary gland via a negative feedback loop. Hereby, the secretion of pituitary gonadotropins is stimulated by the gonadotropin-releasing hormone (GnRH) from the hypothalamus and regulated by testicular hormones via the negative feedback mechanism (Amory and Bremner, 2003). The hypothalamus is situated at the base of the brain and is most commonly referred to as the master control unit of the male reproductive system (Emanuele and Emanuele, 1998). Gonadotropin cells of the anterior pituitary are responsible for the secretion of gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (Weinbauer *et al*., 2010). The synthesis of testosterone is stimulated by LH produced via Leydig cells, whereas FSH is responsible for promoting spermatogenesis via specific receptors on Sertoli cells (Amory and Bremner, 2003).

1.1.2 Excretory Ducts

1.1.2.1 Epididymis

The epididymis is a long, thin highly convoluted tubule surrounded by connective tissue and plays a vital role in sperm maturation, sperm concentration, and transportation of sperm to the vas deferens (Turner, 2008).

The medial aspect of the epididymis is attached to the testis by means of the epididymo-testicular connective tissue, while the distal portion is connected to the caudal connective tissue and epididymal fat pad. Characteristically, the epididymis is subdivided into three regions: head (caput), body (corpus) and tail (cauda) (Turner, 2008).

1.1.2.2 Vas Deferens (Ductus deferens)

The vas deferens is a single, tubular structure that consists of thick muscular walls that are situated at the end of the epididymis. The main function of the vas deferens is to transfer spermatozoa from the epididymis, through the ejaculatory duct and into the urethra (Koslov and Andersson, 2013).

The vas deferens originates in the spermatic cord, continues through the inguinal canal and connects with the seminal vesicle in the pelvic cavity. The end portion of the vas deferens dilates into the ampulla, which continues into the ejaculatory duct (Koslov and Andersson, 2013).

1.1.2.3 Ejaculatory Ducts

The ejaculatory duct is a short, straight tubular structure that originates from the merging of the vas deferens and seminal vesicle. It enters the prostate via the posterior aspect, colliculus seminalis, and empties into the prostatic urethra (Goluboff *et al*., 1995).

1.1.2.4 Urethra

The male urethra is a small tube lined with a mucous membrane that forms a passage for both the urinary system and the reproductive system. The function of the urethra is to release fluids from the body, either in the form of urine or semen (Jung *et al*., 2012).

1.1.3 Accessory Glands

1.1.3.1 Seminal Vesicles

The seminal vesicles are paired structures that consist of tubular alveoli and highly folded mucosa. They connect with the ampulla of the vas deferens and are located posterior to the bladder and superior to the prostate gland.

The seminal vesicles provide an energy source to spermatozoa by secreting fluids rich in fructose, including citrate, prostaglandins and proteins. The fluids secreted by the seminal vesicles make up 50% of the total volume of human ejaculate. The secretions play a vital role in coagulation, motility and stability of spermatozoa including suppressing the immune response of the female reproductive system (Gonzales, 2001).

1.1.3.2 Prostate Gland

The prostate gland, walnut or triangular shaped, is the largest accessory gland found within the male reproductive system. It is surrounded by thin fibro-elastic tissue and is situated in the subperitoneal compartment, between the pelvic diaphragm and peritoneal cavity (Bhavsar and Verma, 2014). The gland surrounds the urethra, situated inferior to the bladder and anterior to the rectum.

The prostate gland produces an alkaline fluid that contains proteolytic enzymes, lipids, acid phosphatase and fibrinolysin, which play a vital role in the normal functioning of spermatozoa (Kumar and Majumder, 1995).

1.1.3.3 Bulbourethral Glands (Cowper's Gland)

The paired bulbourethral glands are spherical in shape (3-5mm diameter) and are situated at the base of the penis. During sexual arousal, the bulbourethral glands produce a viscous fluid that is high in glycoproteins, which aids in lubricating the urethral lining (Chughtai *et al*., 2005).

1.1.4 Penis

The penis consists of erectile tissue, corpus cavernosa and corpus spongiosum, that are enclosed by the tunica albuginea. Paired corpora cavernosa tissue is located on the dorsal aspect

of the penis; while the corpus spongiosum is located on the ventral aspect. The penis functions as a passage to release urine and as a copulatory organ that transports semen into the female reproductive tract (Auffenberg *et al*., 2016).

1.1.5 Scrotum

The scrotum is a small, muscular sac that encloses the testes and is located posterior to the penis. The function of the scrotum is to protect the testes and regulation of temperature by means of the cremaster muscles (Patel, 2017).

1.1.6 Sertoli cells

Sertoli cells are somatic, secretory cells that are located in the germinal epithelium. They are situated on the basal membrane and spread into the lumen of the seminiferous tubules, where the Sertoli cells are an integral part of the germinal epithelium. Morphologically, the cytoplasm consists of both smooth and rough endoplasmic reticulum, a distinct Golgi apparatus (transport secretory products), lysosomal granules as well as microtubuli and intermediate filaments (Weinbauer *et al*., 2010).

Sertoli cells provide maintenance of spermatogenesis by providing structural support: they create a distinct nourishing environment for germ cell development and secrete integral factors, which are required for germ cell development and differentiation (O'Shaughnessy *et al.*, 2008). Sertoli cells synthesize and secrete growth factors, anti-apoptotic factors, and seminiferous tubule fluid which contain proteins, steroids, opioids and prostaglandins (Hunter *et al*., 2012; Weinbauer *et al*., 2010). In the absence of Sertoli cells, germ cell differentiation and development would be impossible (Sato *et al*., 2013).

During the early stages of germ cell maturation, germ cells are situated in the basal region and migrate into the adluminal region (Weinbauer *et al*., 2010). When the germ cells progress into the adluminal region, tight junctions are formed between Sertoli cells, which results in the formation of the blood-testis barrier (BTB) (Meng *et al.*, 2005). Components such as junctional adhesion molecules, claudin family proteins, and occludin form part of integral membrane proteins found in the barrier. The BTB is a structure that allows spermatocytes to pass from the basal region into the adluminal region of the testes, therefore the BTB is considered a regulator of spermatogenesis (Puri and Walker, 2013). The BTB also forms an immunological barrier

that blocks the entry of post-meiotic germ cell antigens, which are produced by the immune system.

1.1.7 Leydig cells

Leydig cells were first discovered by Franz Leydig, a German scientist, in 1850. He was considered as one of the pioneers in the discovery and development of histology during the time era. The end of the 19th century brought many advances in the field of light microscopy and led to new discoveries of Leydig cell structure. In 1896, Friedrich Reinke discovered crystalloids in the human Leydig cell by studying the testis of an executed convict. The function of the crystalloids was unknown; he therefore hypothesized that the crystalloids could be involved in the storage of materials, spermatogenesis, and libido (Christensen, 2007).

During 1897 and 1904, Pol Bouin and Paul Ancel made major contributions to the field of reproductive endocrinology. They hypothesized that Leydig cells have an endocrine function that regulates male secondary sexual characteristics within the human body. However, their research received criticism from the scientific community due to a lack of circumstantial evidence. During the 1930's chemical techniques developed considerably and led to an improved understanding of Leydig cell endocrinology (Christensen, 2007).

1.1.7.1 Development of Leydig cells

Human Leydig cells develop in a triphasic pattern by which testosterone is produced; fetal, neonatal and adult maturational phases. During the fetal period, the development of fetal Leydig cells (FLC's) occurs at 14-18 weeks of gestation and results in a testosterone level peak. The human Chorionic Gonadotropin (hCG) hormone has a vital role by regulating the function and activity of the FLC's. Leydig cell numbers become dormant and reduce considerably after 19-23 weeks of gestation, ultimately reducing the quantity of testosterone secretion (Prince, 2001).

The second peak in testosterone levels occurs between 2-3 months postnatally, due to the development of the neonatal Leydig cell (NNLC) population. NNLC's develop from degenerated FLC's and precursor cells that are situated in the interstitium. The activity and function of the NNLC's require stimulus from the hypothalamic-pituitary axis and gonadotrophic hormones. At approximately 4 months, the NNLC population becomes degenerate. Immature Leydig cells (ILC) population develop from the degenerated NNLC

population and remain constant between 3-8 years of age. In comparison to adult Leydig cells, ILC's are smaller in diameter and have an irregularly shaped nucleus (Prince, 2001; Chen *et al*., 2009).

The adult Leydig cell (ALC) population stem from the ILC population and from recruited precursor cells. The ALC population develop at puberty (12-13 years) and results in a testosterone level peak. On average, 6ng/ml of serum testosterone is produced during adulthood. The peak of testosterone is followed by a steady decline in testosterone levels throughout adulthood due to gradual atrophy, reduction in ALC's and decline in the steroidogenic potential of cells (Prince, 2001).

1.1.7.2 Morphology of Leydig cells

Leydig cells form part of a heterogeneous cell population that are situated in the interstitial compartment, between and encompassing the seminiferous tubules (Dong and Hardy, 2004). They are surrounded by blood vessels and compartmentalizing cells (co-cells) that secrete substances similar to glial cells in the central and peripheral nervous system. Leydig cells are separated from these co-cells by a sheath of the extracellular matrix (Dong and Hardy, 2004).

Leydig cells are approximately 15µm in diameter and may differ in appearance that ranges from polygonal, oval or spindle-shaped cells. They consist of one/two distinct nuclei with prominent nucleoli and heterochromatin that are situated on the periphery. The mitochondria and smooth endoplasmic reticulum are the most abundant organelles found in the Leydig cell, a characteristic of an androgen-producing cell (Dong and Hardy, 2004).

1.1.7.3 Physiology of Leydig cells

The review by Christensen, (1975) documented the fundamental research on the functional morphology of the Leydig cell. The Leydig cell has an endocrine function within the testes, under the regulation of LH from the hypothalamic-pituitary-gonadal axis.

In male mammals, Leydig cells are the major source of testosterone synthesis and secretion. Weinbauer *et al*., (2010) stated that human testes consist of approximately 200 X 10^6 Leydig cells. In addition, Leydig cells are also responsible for producing insulin-like factor 3(INSL3). INSL3 is a peptide that has been linked to testicular descent and functions as an indicator for Leydig cell functionality (Ferlin *et al*., 2006).

1.1.7.4 Signal Transduction in Leydig cells

Androgenic hormones are synthesized by acute and chronic regulation of Leydig cells by LH. The acute and chronic response of LH leads to an increase in secondary messenger, cyclic adenosine monophosphate (cAMP) (Payne and Youngblood, 1995).

The acute response of Leydig cells to LH occurs rapidly (in minutes) and involves transferring of cholesterol from the outer to the inner mitochondrial membrane. The translocation of cholesterol into the intermembranous space is referred to as the rate-limiting step, in steroid hormone formation (Allen *et al*., 2006). Subsequently, the cholesterol is converted into pregnenolone by cytochrome P450 cholesterol side-chain cleavage enzyme (P450 scc) and ultimately converted to testosterone by the action of steroidogenic enzymes, within the smooth endoplasmic reticulum (SER) (Allen *et al*., 2006).

There are two carrier proteins that assist the transport of the hydrophobic cholesterol molecule into the aqueous space found between the outer and inner mitochondrial membrane. Steroidogenic acute regulatory protein (StAR), a 30-kDa molecule, plays a vital role in transporting cholesterol into the inner mitochondrial membrane. Jana *et al*., (2008) state that LH stimulation of Leydig cells results in the expression of the StAR gene and the activity of StAR protein affects the testosterone production in Leydig cells. Peripheral-type benzodiazepine receptor (PBR), an 18-kDa outer mitochondrial membrane phosphoprotein, assists the entry, distribution, and availability of cholesterol within the mitochondria (Midzak *et al*., 2007). Krueger and Papadopoulos, (1990) reported that PBR is primarily involved in maintaining mitochondrial cholesterol.

The chronic response of Leydig cells to LH requires increased transcription and translation of genes that encode for steroidogenic enzymes. Chronic stimulation is responsible for sustaining steroidogenic enzyme activity including being an aid to steroidogenic organelles, mitochondrial membrane potential and SER volume (Dong and Hardy, 2004).

1.1.7.4.1 Protein Kinase A (PKA) Pathway

According to Manna *et al*., (2007) the PKA pathway is notably the dominant pathway responsible for steroidogenesis and StAR protein regulation.

The initiation of the PKA pathway occurs when LH binds to a 7-transmembrane G-protein coupled receptor and results in the activation of adenylate cyclase. Subsequently, the increase

in adenylate cyclase activity leads to an elevation of intracellular cAMP. The increase in cAMP activity results in the activation of PKA. PKA phosphorylates cholesteryl ester hydrolase (protein) including transcription factors such as; cAMP response-element binding protein (CREB), steroidogenic factor 1 and GATA-4. In addition, PKA may also result in the *de novo* synthesis of proteins. Binding of the transcription factors to DNA results in the activation of genes associated with steroidogenesis and the StAR protein produced in the nucleus, transported into the mitochondria and then processed to the active form. Once the StAR gene has been activated, the rate limiting occurs and cholesterol is catalyzed to pregnenolone. Furthermore, testosterone is produced via a cascade of reactions involving steroidogenic enzymes (Stocco *et al*., 2005).

1.1.7.4.2 Protein Kinase C (PKC) Pathway

The role of the PKC pathway in steroidogenesis has been under controversy due to contradictory literature reports stating that PKC pathway may be an inhibitor, stimulator or ineffective in the steroidogenic process.

The PKC pathway initiates when LH binds to the G-coupled protein receptor, leading to the stimulation of phospholipase C (PLC). In addition, PLC activates the downstream PKC pathway. The PKC pathway can be activated by diacylglycerol (DAG) and phorbol 12-myristate-13-acetate (PMA) (Liu *et al*., 2006; Stocco *et al*., 2005). Stocco *et al*., (2005) reported that PMA initiates moderate steroid hormone synthesis in certain steroidogenic cells. In an MA-10 Leydig cell line, PMA activates mitogen-activated protein kinase (MAPK)/ ERK 1/2 and leads to phosphorylation of the transcription factor, CREB protein. Additionally, PMA results in decreased expression of the repressor, DAX-1. In the MA-10 and mLTC cell lines, PMA activation of the PKC pathway results in an increase in CREB phosphorylation and StAR expression (Jo *et al*., 2005).

Subsequently, the PKC pathway results in the transcription and translation of genes that encode for StAR protein expression. However, the PKC pathway fails to phosphorylate the StAR protein and renders the protein inactive. Failure to activate StAR prohibits the cholesterol transfer and ultimately steroidogenesis. Furthermore, the PKA pathway results in phosphorylation of the StAR protein and leads to gene activation. Once activated, the steroidogenic process can proceed and the steroid hormones can be synthesized (Stocco *et al*., 2005).

1.1.7.4.3 Mitogen-Activated Protein Kinase (MAPK) Pathway

Mitogen-activated protein kinases (MAPKs) are associated with serine/threonine kinases that are considered conserved enzymes which play a vital role in the foundation stages of intracellular signalling pathways. There are four divisions of MAPKs that exist in mammals, namely; extracellular signal-regulated kinase 1/2 (ERK 1/2), p38, c-Jun N-terminal kinase/ stress–activated protein kinases (JNK/SAPKs) and ERK5/ Big MAPK (BMK1) (Manna and Stocco, 2007).

Functionally, MAPKs are responsible for transmitting signals from growth factors, hormones, cytokines, and neurotransmitters. In addition, they regulate a vast number of intracellular functions which include development, proliferation, differentiation, gene expression and steroidogenesis. However, the role of MAPKs in steroidogenesis has not been established as there are many contradicting literature reports (Manna and Stocco, 2007).

1.1.7.4.4 Arachidonic Acid (AA) Pathway

Arachidonic acid (AA) has a vital role in LH-stimulated steroid biosynthesis. In addition to cAMP, AA and calcium ions act as secondary messengers involved in regulating steroidogenesis (Ronco *et al*., 2002). In the MA-10 mouse Leydig tumour cell line it was reported that AA was crucial for StAR gene expression (Wang *et al*., 2000).

Once LH binds to cell receptors, initiation of phospholipase 2 (PLA2) occurs which leads to the release of intracellular AA from phospholipids (Stocco *et al*., 2005). Cooke *et al*., (1991) demonstrated that in rat testicular Leydig cells, the release of AA occurs within 1 minute. Subsequently, AA gets metabolized through either the cyclooxygenase (COX), lipoxygenase (LPOX) or the epoxygenase (EPOX) enzymatic pathways. The metabolites of LPOX and EPOX have been shown to have a stimulatory effect on 3β-HSD and 17β- HSD enzymes. In addition, an inhibitory effect on LPOX and EPOX results in an inhibition of StAR expression and steroid biosynthesis (Stocco *et al*., 2005).

AA can be synthesized via an alternate pathway that involves acyl-coenzyme A (CoA). CoA is classified as an AA-related thioesterase involved in steroidogenesis (ARTISt) and results in the release of AA by the aid of arachidonyl - CoA (Stocco *et al*., 2005). Once AA is inhibited, the inhibition results in LH and StAR expression. However, the addition of AA results in an increase in StAR protein expression. AA plays a regulatory role and a positive signal in the

expression of the StAR gene at the level of transcription and may increase sensitivity of Leydig cells to LH/hCG stimulation (Wang *et al*., 2000).

1.1.7.5 Steroidogenesis

In Leydig cells, the process of steroid biosynthesis is essential for the production of androgenic hormones (Allen *et al*., 2006). The synthesis of these hormones occurs through a Δ4 pathway or Δ5 pathways, under the regulation of LH. The Δ4 pathway occurs in rodents such as rats and mice, whereas the Δ5 pathway occurs in humans, dogs, primates, pigs and rabbits (Stocco and McPhaul, 2006).

In the Δ4 pathway, cholesterol is cleaved into pregnenolone by P450 scc (side-chain cleavage). Pregnenolone is further converted into progesterone by 3β- hydroxysteroid dehydrogenase enzyme (3β-HSD). Progesterone is catalyzed by P450 17α- hydroxylase enzyme (17α – hydroxylase) into androstenedione. 17β – hydroxysteroid dehydrogenase enzyme (17β- HSD) catalyzes androstenedione into testosterone (Stocco and McPhaul, 2006).

In the Δ5 pathway, P450 scc (side-chain cleavage) catalyzes the conversion of cholesterol into pregnenolone. Firstly, 17α – hydroxylase catalyzes pregnenolone into 17α - hydroxypregnenolone and cleaves 17α – hydroxypregnenolone into dehydroepiandrosterone (DHEA). 17β- HSD catalyzes DHEA into androstenedione and thereafter, 3β-HSD converts androstenedione into testosterone (Stocco and McPhaul, 2006; Suh *et al*., 2008).

Testosterone is the primary hormone produced by steroid biosynthesis but can be further catalyzed into dihydrotestosterone and 17β- estradiol by 5α- reductase and aromatase, respectively (Dong and Hardy, 2004).

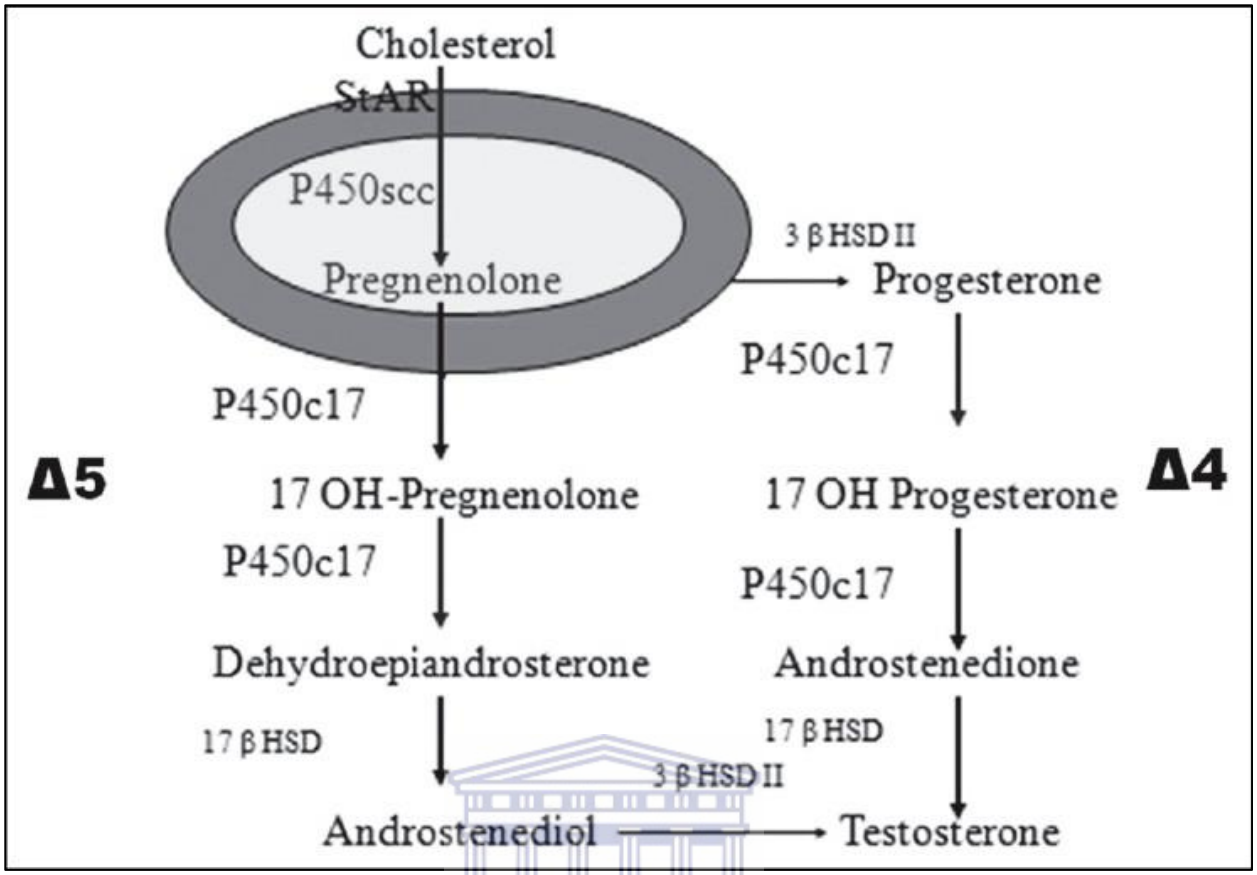


Figure 1.2: Steroid biosynthesis in Leydig cells (Kumar *et al.*, 2014).

1.1.8 Structure and Function of Testosterone

Testosterone consists of 19 Carbon atoms (C-19 molecule) and the skeleton structure of the molecule consists of 17 C atoms. The androstane skeleton consists of three six-carbon rings and a five-carbon ring that form a complex structure.

Testosterone is the fundamental androgen synthesized by Leydig cells, within the testes. Approximately 95% of testosterone is synthesized within the testes, while 5% of the hormone is synthesized within the adrenal gland. Androgens, particularly testosterone, are vital for male sexual differentiation, gamete production, and development of secondary sexual characteristics. The secondary sexual characteristics can be classified as an androgenic and anabolic response. The androgenic effect is associated with larynx enlargement, deepening of the voice and an increase in exterior hair growth and *libido*. Anabolic effects refer to the development of skeletal muscle that encourages linear growth which further leads to epiphysis closure (Kicman, 2008).

1.1.8.1 Mechanism of Testosterone Transport in Blood

In the bloodstream, approximately 54% of testosterone binds to albumin, 44% to human sex hormone binding-globulin (hSHBG) while 2% remains unbound to protein (free testosterone) (Weinbauer *et al*., 2010). Testosterone has a high affinity for hSHBG binding; however, hSHBG- bound testosterone is incapable of binding to androgen receptors in target cells. It was presumed that the unbound testosterone could be the only active form of the hormone that elicits a response in target cells. However, Heinrich-Balard *et al*., (2015) state that both the free and albumin-bound testosterone could be an indication of the bioavailable testosterone.

The free testosterone and albumin-bound testosterone, found in the intravascular space, diffuse into the interstitial space and bind to target cells. There are two mechanisms by which testosterone can elicit a response in target cells, either by genomic changes or non-genomic changes. Genomic changes are associated with direct binding of testosterone to the androgen receptor (AR), whereas non-genomic changes are associated with either AR binding or secondary messenger pathways. Once testosterone binds to an AR, the hormone diffuses into the cell membrane and binds to cytoplasmic receptor proteins. The complex migrates to the nucleus where testosterone binds to the nuclear receptor, thereby forming a hormone-receptor complex. The hormone-receptor complex binds to certain regions of DNA, resulting in the transcription of specific proteins that are necessary for eliciting the desired response (Heinrich-Balard *et al.,* 2015; Weinbauer *et al*., 2010).

In addition, testosterone can mediate a response in target cells by conversion to 5α-dihydrotestosterone (DHT) via 5α-reductase or to estradiol via aromatase. DHT has a more potent effect when compared to testosterone and is vital for the development of the external genitalia in males (Weinbauer *et al*., 2010).

Inactivation of testosterone occurs by oxidation to androstenedione via 17β- hydroxysteroid dehydrogenase (17β-HSD) or by reduction to DHT. The excess testosterone, including DHT, undergoes glucuronidation and sulfation to aid the release of testosterone through the urinary tract (Weinbauer *et al*., 2010).

1.2 Male Infertility- Prevalence and Incidence

According to the World Health Organization, infertility is termed as an inability of a couple to conceive or successfully achieve parturition after regular unprotected sexual intercourse for

one or more years. Under normal circumstances, 80-85% of couples can successfully achieve conception within the one-year period (Moslemi and Tavanbakhsh, 2011).

However, infertility is becoming a growing concern in many cultures and societies across the world as 10-15% of couples are affected (Deka and Sarma, 2010). According to guidelines on male infertility, approximately 15% of couples that cannot conceive in a given year tend to seek infertility treatment and 25% to 50% of infertility in couples is associated with the male as the contributing factor (Walczak–Jedrzejowska *et al*., 2013).

The pathophysiology of male infertility can be associated with idiopathic oligozoospermia (reduced sperm count), varicocele, male accessory gland infection and immunological infertility. Environmental factors, such as exposure to harmful chemicals and genetic factors also contribute immensely to male fertility status (Irvine, 1998).

1.3 Medicinal Plants

Medicinal plants have been used globally by many traditional cultures for centuries, due to their therapeutic properties. Therapeutically, medicinal plants are used to maintain a healthy status as well as to treat various health ailments and diseases (Mahomoodally, 2013).

According to the World Health Organization statistics (WHO), 80% of the population in developing countries depend on the beneficial properties of medicinal plants as their sole health care source (Ekor, 2014). The popularity of medicinal plants has grown over the past few decades because of reduced side-effects and their affordability, compared to pharmaceutical medicine (Mahmoud and Gairola, 2013).

1.4 Herbal Teas

Tea is the most commonly consumed beverage in the world (Chen *et al*., 2013). The growing popularity of tea may be attributed to the fact that it is rich in polyphenol antioxidants, which benefit the human body by preventing diseases and combatting the production of reactive oxygen species (ROS). Low quantities of ROS are required for sperm to function normally but abnormally high amounts of ROS have an adverse effect on the quality and fertilizing ability of spermatozoa (Agarwal *et al*., 2014).

Green tea consumption has been shown to prevent the formation of certain cancers, cardiovascular diseases, prevents damage to cell structures and acts as an anti-inflammatory,

antibacterial and antiviral agent (Chacko *et al*., 2010). The most prevalent polyphenol in *Aspalathus linearis* is aspalathin while *Camellia sinensis* contains EGCG (Canda *et al*., 2014; Chaturvedula and Prakash, 2011).

In an *in vitro* study conducted by Opuwari and Monsees, (2015), it was established that *A.linearis* and *C.sinensis* have an anti- androgenic effect on TM3 Leydig cells. It was deduced that the herbal teas result in a significant reduction in the synthesis of steroid hormones, particularly testosterone. They hypothesized that *A.linearis* and *C.sinensis* may have a direct effect on steroidogenesis by reducing the synthesis of pregnenolone from cholesterol via P450 scc (side-chain cleavage) enzymes, cAMP level reduction or by interfering with 3β-HSD and 17β-HSD synthesis.

In an *in vivo* study conducted by Opuwari and Monsees, (2014) it was deduced that *A.linearis* improves sperm concentration, motility, and viability possibly due to the tea's antioxidant properties. However, excessive consumption of rooibos may lead to minor structural changes within the male reproductive system and early acrosome reaction in spermatozoa, which may negatively affect male infertility.

1.5 Polyphenols

Polyphenols are naturally occurring polyhydroxylated compounds that are secondary metabolites of plants (Pandey and Rizvi, 2009). The polyphenolic structure is described by Quideau *et al*., (2011) as originating from shikimate phenylpropanoid and/or polyketide pathway, containing several phenol rings and lacking nitrogenous functional groups in their basic chemical structure. Presently, there are over 8000 phenolic compounds discovered in plant extracts (Lewandowska *et al*., 2013).

Dietary polyphenols are ubiquitously found in fruits, tea, vegetables, wine, dry legumes, chocolate and cereals (Lewandowska *et al*., 2013; Scalbert *et al*., 2005). These phenolic compounds contribute to the unique taste, colour and nutritional value of fruits (El Gharras, 2009). They are classified into various groups based on the number of phenolic rings and structural elements that allow these rings to bind together (Kumar *et al*., 2014). Furthermore, polyphenols are classified into phenolic acids, flavonoids, curcuminoids and stilbenes (Figure 1.3).

1.5.1 Antioxidant and Pro-oxidant properties of dietary polyphenols

ROS and free radicals are normal by-products of cellular metabolism, are vital in assisting cell functions and are actively involved in apoptosis (Joubert *et al*., 2005). However, excessive ROS leads to oxidative stress and may lead to adverse modifications to lipid, protein and nucleic acid compounds (Han *et al*., 2007).

Polyphenols possess antioxidant properties which are considered to be the most abundantly found in daily nutrition. These polyphenols play a vital role in decreasing the activity of ROS such as peroxides, superoxides and hydroxyl radicals (Bramati *et al*., 2002). The protective effects of polyphenols against oxidative stress, cardiovascular disease, cancer, diabetes, aging and degenerative diseases have been well documented in literature (Pandey and Rizvi, 2009; Han *et al*., 2007; Hu, 2007).

The polyphenolic antioxidants are potent free radical scavengers because they directly block certain enzymes such as xanthine oxidase and PKC, which are responsible for catalyzing redox reactions. They are also responsible for chelating iron (II)/ copper(I) and iron (III)/copper (II) ions and prevents the formation of hydroxyl radicals that are synthesized via the Haber-Weiss/ Fenton reactions (Quideau *et al*., 2011). In addition, polyphenols function as antioxidants by prohibiting the oxidation of low-density lipoproteins (LDL's), platelet aggregation and red blood cell damage (El Gharras, 2009).

Additionally, the beneficial effects of antioxidants are evident but they also possess pro-oxidant properties (Joubert *et al*., 2005). These pro-oxidant properties may also result in oxidative stress within the cell (Galati and O'Brien, 2004) and might further lead to the destruction of cellular components including DNA (Heim *et al*., 2002).

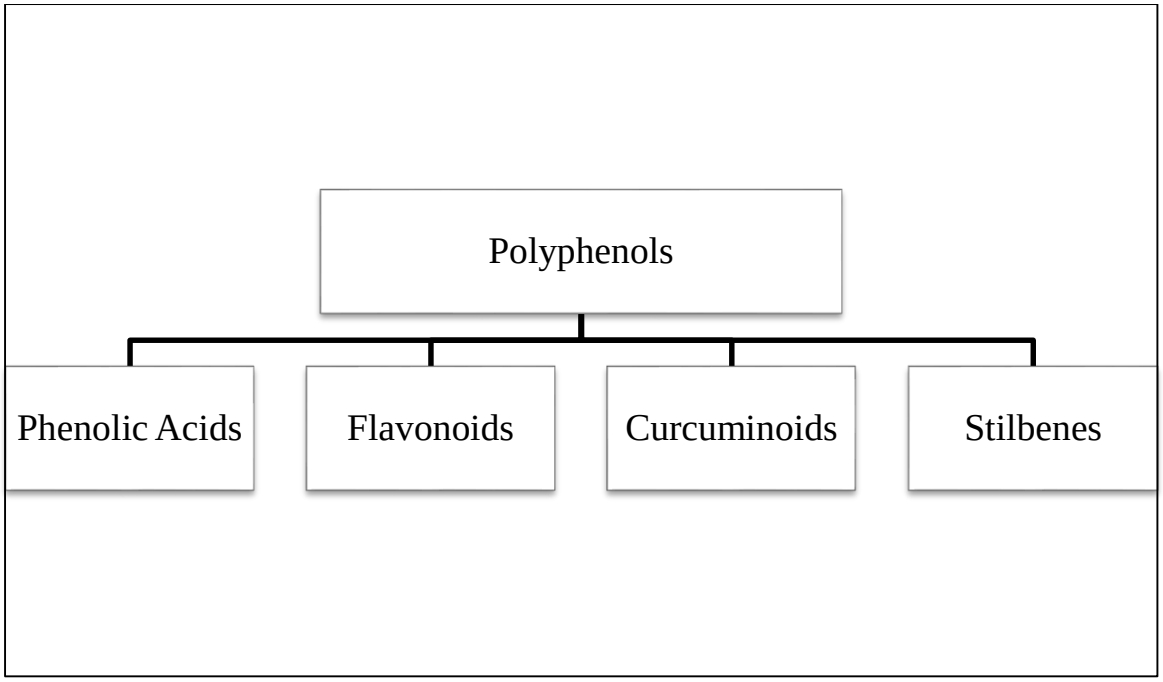


Figure 1.3 Schematic of the various subclasses of polyphenols (Lall *et al*., 2015).

1.5.2 Phenolic Acids

Phenolic acids are categorized into hydroxybenzoic and hydroxycinnamic acids, derivatives of benzoic acid and cinnamic acids, respectively. The phenolic acids are commonly found in plants, particularly present in fruits with an acidic taste (Lall *et al*., 2015).

Hydroxybenzoic acid forms part of hydrolyzable tannins that are present in mangoes as gallotannins and as ellagitannins in strawberries, raspberries, and blackberries. Gallic acid, a component of hydroxybenzoic acid, has a gallate moiety that forms an integral part of (-) – epi-gallocatechin-3-gallate (EGCG) and most commonly found in beverages such as green tea (Eslami *et al*., 2010).

Hydroxycinnamic acids are the most frequently found phenolic acids when compared to the hydroxybenzoic acids (Pandey and Rizvi, 2009). The hydroxycinnamic acid category contains caffeic, coumaric, ferulic and sinapic acids. They are found in free form in processed foods that have been frozen, sterilized and fermented. The caffeic acids (free and esterified) are regarded as the most common and constitute 75-100% of overall hydroxycinnamic acids found in fruit. In cereal grain, ferulic acid is the most common hydroxycinnamic acid and the acid content ranges from 0.8-2g kg^{-1} dry weight of wheat (El Gharras, 2009).

1.5.3 Flavonoids

Flavonoids are a group of phenolic compounds found in various plants and form an integral part of the human diet. The earliest consumption of flavonoids, by humans, dates back to approximately 4 million years ago (Kumar and Pandey, 2013). Presently, there are over 4000 different forms of flavonoid compounds and they are considered the most abundant dietary polyphenols (Lall *et al*., 2015). They are situated in the leaves, flowers, stems and barks, which are important as they provide the colour to flowers, fruits and leaves of plants (Pandey and Rizvi, 2009). Flavonoids have a pivotal role in plants, by acting as a defense mechanism when microbial infection or injury occurs (Kumar and Pandey, 2013).

Structurally, flavonoids are produced via the phenylpropanoid pathway and have a benzo-γ-pyrone form, consisting of a fifteen- carbon skeleton comprising two attached benzene rings joined by a heterocyclic pyrane ring (Kumar and Pandey, 2013). Flavonoids naturally exist as aglycones, their primary form, as glycosides or as methylated compounds. Their structures differ depending on the degree of alkylation and/or glycosylation as well as the amount of hydroxyl groups attached (Pandey and Rizvi, 2009). According to Lall *et al*., (2015), flavonoids have seven classes: Flavanols, flavones, flavanones, flavonols, isoflavones, anthocyanidins and chalcones.

Flavonoids are beneficial as they improve the quality of health and may prevent certain chronic diseases (Birt and Jeffery, 2013). Physiologically, flavonoids have the ability to act as an antioxidant, antimicrobial and an anti – inflammatory agents. They have a vital role in liver protection and in the treatment of diabetes. In Parkinson's disease, the flavonoids found in berries may potentially aid in preventing memory loss in the elderly (Kumar and Pandey, 2013).

1.5.3.1 Flavanols

Flavanols are categorized into four subclasses: Catechins, proanthocyanidins, theaflavins and thearubigins (Lall *et al*., 2015). The catechins are monomers and consist of catechin, epicatechin, gallocatechin, epigallocatechin, catechin gallate, epicatechin gallate, gallocatechin gallate and epigallocatechin gallate (Sies *et al*., 2012). Catechins are found in various fruits and red wine, however, green tea and chocolate contain the largest quantity of these phenolic compounds (El Gharras, 2009). Tea, legume plants, and grapes all contain gallocatechin, epigallocatechin gallate and epigallocatechin.

Theaflavins are black tea pigments that are synthesized once tea leaves undergo fermentation (oxidation); thereby resulting in the formation of these complex condensed polyphenolic compounds (Menet *et al*., 2004).

Proanthocyanidins are polymers of catechins and are considered condensed tannins. They are largely present in grapes and seeds, particularly in the skin. These tannins contribute greatly to the taste of certain fruits (grapes and peaches), drinks (beer, cider, and teas) and to the bitter taste of chocolate. The quality of the flavour depends on the maturation and ripening processes (El Gharras, 2009).

1.5.3.2 Flavones

Flavones are a group of phenolic compounds that are considered to be one of the major flavonoids such as flavanols, isoflavones, and flavonols. They are crystalline substances that range from colourless to yellow when they are dissolved (Singh *et al*., 2014).

Flavones have a 2-phenylchromen-4-one backbone structure and mainly contain luteolin and apigenin glycosides. The glycosides of flavones are largely found in fruits, leafy vegetables, particularly in celery and herbs (Lagiou *et al*., 2006). The *C*- glycosides of flavones are commonly found in millet and wheat (cereals) (El Gharras, 2009).

1.5.3.3 Flavanones

Flavanones are one of the main flavonoids and they initiate the formation of the other flavonoid classes. Presently, there are 350 flavanone aglycones and 1000 flavanone glycosides that have been discovered (El Gharras, 2009).

The flavanones are present in various species and are most commonly found in the stems, branches, flowers, roots and peels. The most abundant flavanones are found in citrus fruits, however, these compounds are also found in tomatoes and mint plants (El Gharras, 2009). In the genus *Citrus*, the main flavanones are hesperetin, naringenin, and eriodictyol (El Gharras, 2009; Khan and Dangles, 2014).

1.5.3.4 Flavonols

Flavonols are the most predominant compounds present in plant food when compared to the various flavonoid classes. They are mainly classified into quercetin, kaempferol and myricetin

glycosides (Aherne and O'brien, 2002). Literature reports that quercetin glycosides are extensively studied and are found in higher quantities, in comparison to other flavonol compounds (El Gharras, 2009; Lea, 2015).

They are more commonly present in onions, Spanish cherry tomatoes, grapes, broccoli, blue berries and curly kale (Aherne and O'brien, 2002; El Gharras, 2009). The skin of fruits and vegetables have been shown to possess the highest concentrations of flavonols, particularly quercetin glycosides (Aherne and O'brien, 2002).

1.5.3.5 Isoflavones

Isoflavones are phytoestrogenic flavonoid compounds most commonly found in leguminous plants. *In vivo* and *in vitro* studies reported that elevated levels of estrogen may be due to the isoflavones activating the aromatase enzyme (Zhang *et al*., 2016).

Isoflavones are commonly found in soy food products and mainly contain genistin, daidzin, glycitin, equol and biochanin A (Setchell, 1998; Zhang *et al*., 2016). Soy bean has gained much popularity within the research community over the past 22 years due to the presence of isoflavone compounds (Wang *et al*., 2013).

1.5.3.6 Anthocyanidins

Anthocyanidins are water-soluble pigments that are commonly found in vascular plants. Vascular plants possess specialized tissues (xylem and phloem) that enable the transport of water, minerals and photosynthetic by-products through the plant. They provide colour to certain fruits and vegetables, ranging from orange, pink, violet, blue, and red. When compared to carotenoids, the anthocyanidins are unstable compounds and the stability of the compound relies on pH, temperature and chemical composition (Castañeda -Ovando *et al*., 2009).

The anthocyanidins are found in berries, grapes, cabbages and various plants as delphinidin and cyanidin glycosides (Lazze *et al*., 2003). According to Lila, (2004) anthocyanidins are considered to be bioflavonoids, due to their beneficial properties in treating human ailments/diseases.

1.5.3.7 Chalcones

Chalcones are flavonoid compounds that have been used in ancient times for herbal medicinal practices. They can be converted to flavanones via the chalcone isomerase enzyme, hence the close relationship between these two compounds within plant species (Yazdan *et al*., 2015).

Fruits, vegetables, spices and teas are rich sources of chalcones and are present as glycosides or free aglycons (Gutierrez *et al*., 2015). In addition, chalcones are more abundantly found as naringenin chalcone in citrus fruits. These naringenin chalcones have a vital role in adding to total polyphenol content and in the biosynthetic pathway of flavonoids (Orlikova *et al*., 2011).

1.5.4 Curcuminoids

Curcuminoids are a class of natural polyphenols that consist of two aromatic rings with hydroxyls linked to carboxyl groups via an aliphatic chain (Han *et al*., 2007). Recently, they were extensively studied as nutraceuticals and most commonly utilized as spices to add flavour and colour to various food dishes.

Characteristically, curcuminoids are a yellow powder derived from the rhizomes of *Curcuma longa*, commonly known as tumeric (Gupta *et al*., 2013). Curcumin, demethoxycurcumin (DMC), bisdemethoxycurcumin (BMDC), 5'- methoxycurcumin and dihydrocurcumin are biological components of curcuminoids (Lall *et al*., 2015; Henrotin *et al*., 2013). *Curcuma longa* (tumeric) possesses 5% of curcumin, which is considered the most abundant compound present in tumeric (Henrotin *et al*., 2013).

The beneficial properties of curcumin have been used by Asian cultures for many centuries to treat various ailments. Studies have proven that curcumin and its analogues have antioxidant, antimicrobial, anti-inflammatory, hypoglycaemic and wound healing properties (Gupta *et al*., 2013). In an *in vivo* and *in vitro* study, curcumin was shown to possess chemopreventive and chemotherapeutic properties. The mechanism of action results in the down-regulating of epidermal growth factor receptor (EGFR) families, insulin-like growth factor type-1 receptor (IGF-1R), sonic hedgehog and further signalling cascades. They lead to suppression of the apoptotic regulators Bcl-2 and Bcl- xL while activating Bax and Bad to initiate apoptosis (Mimeault and Batra, 2011).

1.5.5 Stilbenes

The chemical structure of stilbenes consists of two phenyl moieties bonded to a two carbon methylene bridge, 1,2 – diphenylethylene being their core structure and exist as monomers or oligomers (Pandey and Rizvi, 2009; Han *et al*., 2007). Stilbenes naturally exist in plants as phytoalexins; substances produced upon injury or infection and are also considered as stress metabolites (Lall *et al*., 2015).

The most widely studied stilbene compound is resveratrol (3,4',5-trihydroxystilbene), commonly found in grapes and red wine (Pandey and Rizvi, 2009). Resveratrol has growing popularity within the research community due to its potential medicinal properties. Stilbenes possess cardio-protective, anti-cancer, anti-oxidant and anti-inflammatory properties (Flamini *et al*., 2013).

Pterostilbene (trans-3,5-dimethoxy-4-hydroxystilbene) is an additional component of the stilbenes and occurs as a dimethylated derivative of resveratrol. The compound has anti-oxidant properties and is most commonly found in blueberries and grapes. They perform a chemoprotective and therapeutic role in cancerous cells by changing the cell cycle, initiating apoptosis and by inhibiting metastasis (McCormack and McFadden, 2012).

Piceatannol (trans-3, 4, 3', 5' tetrahydroxystilbene) is a hydroxylated form of resveratrol that occurs in rhubarb, grape skin, sugar cane, white tea, passion fruit, Japanese knotwood and peanuts (Lall *et al*., 2015; Piotrowska *et al*., 2012). In relation to resveratrol, piceatannol possesses antioxidant, anti-inflammatory, cardioprotective and anti-cancer properties. It has the ability to inhibit the formation of latent membrane protein 2A (LMP2A), a viral protein tyrosine kinase associated with Leukemia, Non-Hodgkins's lymphoma and Epstein - Barr virus (EBV) (Flamini *et al*., 2013).

1.5.6 Bioavailability of dietary polyphenols

Bioavailability refers to the quantity of nutrients that may be digested, absorbed and metabolised through physiological processes (Pandey and Rizvi, 2009).

Dietary polyphenols are complex compounds and occur in the form of esters, polymers or as glycosides. Therefore, these compounds undergo hydrolysis by intestinal enzymes or colonic microflora to enable absorption (Pandareesh *et al*., 2015). The absorption of polyphenols

occurs at various sites within the gastro-intestinal tract (Figure 1.4) and rely on the modifications during metabolism (Pandey and Rizvi, 2009).

Upon entry into the oral cavity, polyphenols associate with salivary proteins enriched with proline and form soluble polyphenol-protein aggregates. The interaction between the polyphenol-protein aggregates result in changes in protein tertiary structure and may ultimately impact on polyphenol-protein activity. In the stomach, polyphenols are subjected to an acidic environment, which may affect their stability. Studies show that resveratrol, quercetin and catechins compounds remain stable despite the low pH environment. In addition, the stomach wall possess transporters that allow the absorption of phenolic acids, anthocyanins and flavonoid aglycones (Lewandowska *et al*., 2013).

In the small intestine, glycosylated polyphenolic compounds are converted to aglycones by action of endogenic β- glucosidase, lactase- phlorizin hydrolase (LPH) and cytosolic β-glucosidase (CBG). The lipophilicity of aglycones enable them to enter epithelial cells via passive diffusion. The small intestine is the prime site for conjugation reactions; glucuronidation, methylation and sulfation. The purpose of conjugation reactions is to restrict the action of chemical compounds and to increase their solubility, which facilitates their excretion in bile and urine (D'Archivio *et al*., 2007).

Polyphenolic compounds travel to the liver via the portal vein, where they undergo further conjugation reactions. Moreover, bile metabolites may be transported via enterohepatic circulation to the small intestine. They are converted to less polar aglycones by intestinal enzymes, thereby allowing for reabsorption or elimination in faeces (D'Archivio *et al*., 2007).

The large intestine plays a vital role in absorption and catabolism of polyphenolic compounds by action of colonic microflora. Metabolites are absorbed and travel via the portal vein to the liver where they undergo further conjugation reactions. The conjugated metabolites are eliminated in urine, while the unabsorbed metabolites are eliminated in faeces (Lewandowska *et al*., 2013).

Evidently, polyphenol compounds undergo extensive modifications during the absorption process. Therefore, the chemical form entering plasma and tissue differ considerably when compared to the food/diet before ingestion (Pandareesh *et al*., 2015).

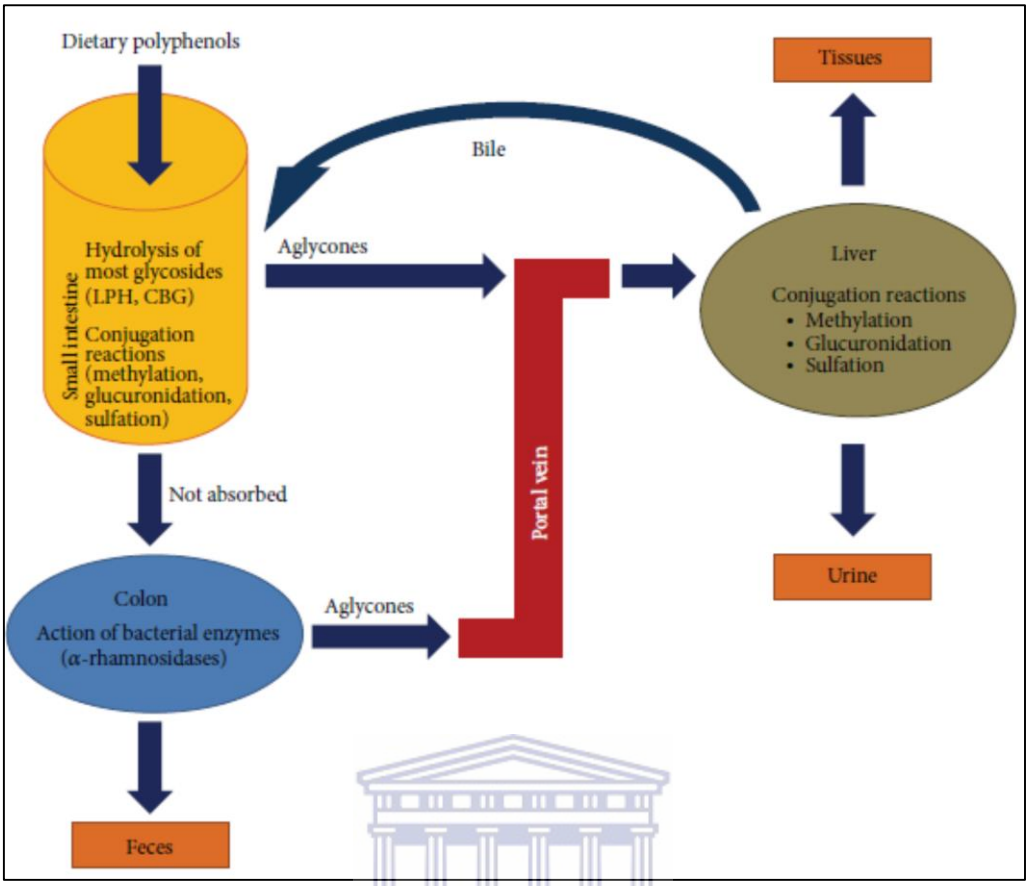


Figure 1.4: The metabolic process of dietary polyphenols within humans (Marin *et al.*, 2015).

1.6 Aspalathus linearis (Rooibos)

Aspalathus linearis belongs to the Fabaceae (Leguminosae) family and consist of 278 various species (Dahlgren, 1988). *A.linearis* was first discovered by the Cape Khoi tribes and has been utilized for over 230 years. Commercially, the popularity of *A.linearis* has grown from 524 tons in 1955 to 10 600 tons in 2003 (Joubert and Schultz, 2006).

A.linearis is characterized as a leguminous shrub, indigenous to the Cedarberg region in the Western Cape of South Africa (Mckay and Blumberg, 2007). The shrub grows to a height of approximately 2m and produces green needle-like leaves that have a length of 15-60mm and a width of 0.4 to >1.0mm. The branches bare a yellow pea-shaped flower that blossoms at the commencement of spring until early summer. The flower contains a hard, dicotyledonous seed that develops a red colour once the seed ripens. *A.linearis* has a tap-root system which prevents

desiccation and consists of nodules with nitrogen-fixing bacteria that play a vital role in converting atmospheric nitrogen to ammonia (Joubert and Schultz, 2006).

A. linearis can be classified into red type, black, grey, and red-brown. The red type can be further classified into Nortier and wild growing Cederberg types that are cultivated and prepared for processing. However, the black and grey types are not processed due to the unsavoury flavour and the poor quality tea they produce (Joubert and Schultz, 2006).

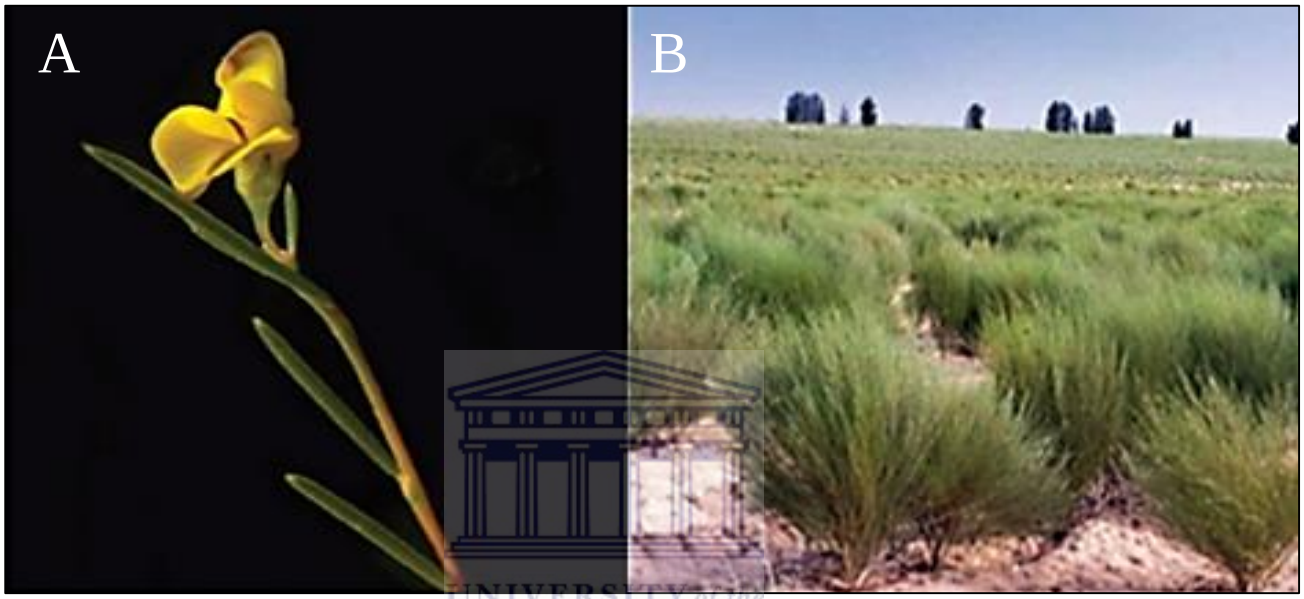


Figure 1.5: (A) Yellow pea-shaped flower on the branch of *Aspalathus linearis*. (B) Rooibos shrubs found in the Cederberg region (Van Niekerk and Viljoen, 2008; Erickson, 2003).

1.6.1 The processing of *A. linearis*

The leaves and fine stems of *A.linearis* are used to produce the polyphenol-rich herbal tea, Rooibos (Jagangi and Wheeler, 2003). Harvesting of the leaves and stems occurs during the months of December and March (Morton, 1983). Once leaves and steams are harvested, they undergo a fermentation process that involves shredding and bruising which initiates the oxidation process. Furthermore, the addition of water to the reaction results in rapid oxidation of the plant material. The duration of fermentation varies between 12-14 hours and results in the characteristic red-brown colour and honey aroma. Once fermentation is completed, the tea undergoes steam-pasteurization to ensure that the microbial population adheres to the set guidelines (Joubert and Schultz, 2006).

The unfermented form of rooibos, known as green rooibos, has a distinct green colour when compared to the red-brown colour of fermented rooibos. The processing of green rooibos involves minimal fermentation, which produces the characteristic colour and results in higher antioxidant levels in comparison to fermented rooibos (Erickson, 2003).

1.6.2 Polyphenols present in Rooibos

The polyphenolic properties present in rooibos can be characterized into the category of flavonoids and phenolic acids. The flavonoids are further classified into dihydrochalcones, flavones, and flavonols (Marnewick *et al*., 2003). The dihydrochalcones found in rooibos are aspalathin and nothofagin (Bramati *et al*., 2002), the flavones orientin, iso-orientin, vitexin, iso-vitexin, luteolin and chrysoeriol as well as the flavonols rutin, isoquercetin and quercetin (Joubert and Schulz, 2006).

The phenolic acids found in rooibos have antioxidant abilities and comprise of benzoic acid, caffeic acid, ferulic acid, protocatechuic acid, *p*-hydroxybenzoic acid, *p*-coumaric acid, syringic acid, vanillic acid, and cinnamic acid (Joubert and Schulz, 2006).

1.6.3 Beneficial properties of Rooibos

Rooibos tea is a caffeine-free beverage that contains low levels of tannins and can benefit pregnant women, children, and individuals that are caffeine sensitive (Hong *et al*., 2014). The growing popularity of rooibos can be attributed to its distinct taste, versatility and beneficial qualities as a health drink (Jaganyi and Wheeler, 2003).

Rooibos tea contains vital dietary antioxidants and its growing popularity within the research community is likely due to its antioxidant properties (Jaganyi and Wheeler, 2003). Rooibos aids in the relief of insomnia, anxiety, stress and physiological ailments that are associated with elevated amounts of the stress hormone, cortisol (Jaganyi and Wheeler, 2003). In cardiovascular research, Cha *et al*., (2009) reported that Chrysoeriol, an antioxidant present in rooibos, has the ability to prevent atherosclerosis by inhibiting smooth muscle migration within the aorta.

In a study performed on diabetic rats, researchers concluded that rooibos might have a protective effect against vascular complications associated with diabetes (Uličná *et al*., 2006).

Rooibos has also been shown to have an anti-mutagenic effect by suppressing mutations that may lead to cancer (Van der Merwe *et al*., 2006).

Gilani *et al*., (2006) showed that rooibos has an antispasmodic effect and is commonly used for the treatment of gastrointestinal disorders.

The effect of rooibos on the male reproductive system varies between *in vivo* and *in vitro* studies. The antioxidant properties of rooibos proved to be beneficial in the process of spermatogenesis, leading to improved viability and motility of sperm. However, excessive consumption of rooibos may lead to early acrosome reaction of spermatozoa, which may negatively affect male infertility (Opuwari and Monsees, 2014).

In an *in vivo* study, Awoniyi *et al*., (2012) showed that rooibos extracts (green rooibos and fermented rooibos) improved sperm quality, protected sperm against induced oxidative stress and increased antioxidant enzymes.

In the process of steroidogenesis, an *in vitro* study showed that rooibos resulted in a significant reduction in testosterone levels (Opuwari and Monsees, 2015). A study on H295R Adrenal cells showed that rooibos and certain flavonoids inhibited cytochrome P450 enzyme (involved in steroidogenesis) and cortisol production (Scholms and Swart, 2014).

1.7 Aspalathin

Aspalathin is a C– glycosidic dihydrochalcone present in *A.linearis*. The chemical structure of aspalathin is unique due to the lack of O – glycosidic linkage, which attaches a single or dimeric sugar moiety to a vast number of flavonoids (Courts and Williamson, 2009).

The unfermented rooibos (green rooibos) extracts contain higher quantities of aspalathin, when compared to fermented rooibos (Kreuz *et al*., 2008; Han *et al*., 2013). Literature states that 4-12% of aspalathin glycosides may be present in green rooibos (Han *et al*., 2013). Approximately 43% of the total antioxidant capacity in unfermented rooibos (aqueous extracts) is contributed by aspalathin (Joubert *et al*., 2005).

Once *A.linearis* undergoes fermentation, the quantity of aspalathin content is reduced to below 7%. Hence, the popularity of green rooibos is evident and in high demand by nutraceutical and cosmetic companies, due to the high antioxidant properties (Joubert *et al*., 2005). However, little is known about the absorption and metabolism of aspalathin in humans, due to the stability

of the bond between the sugar and flavonoid structure, which is resistant to enzymes and acids (Kreuz *et al*., 2008).

Aspalathin has potent radical scavenging properties to combat ROS and possess similar scavenging abilities to other flavonoids such as quercetin and epigallocathechin gallate (Joubert *et al*., 2005). Recent studies also suggest that aspalathin induces glucose uptake in muscle tissue and stimulates insulin secretion from pancreatic β – cells (Han *et al*., 2013). Muller *et al*., (2012) reported that aspalathin resulted in reduced hyperglycemia and glucose intolerance, as well as increased glucose uptake and insulin secretion in db/db mice (a model for obesity, diabetes and dyslipidemia).

1.8 *Camellia sinensis* (Tea)

Camellia sinensis (*C.sinensis*) is an evergreen plant or tree belonging to the Theaceae family. *C.sinensis* plants are indigenous to China and later extended to India, Japan, and Europe (Sharangi, 2009). There are two distinct botantical types: *C sinensis* var. sinensis, a bush-like plant that is commonly found in China and Southeast Asia, and *C.sinensis* var. assamica, a large-leaved tree found in a state of India known as the Assam region (Dias *et al*., 2013).

The leaves of *C.sinensis* are cultivated for the preparation of tea, a popular beverage consumed since 3000BC (Sharangi, 2009). Tea can be found in more than 30 countries and an estimated 18-20 billion cups are consumed on a daily basis, across the world (Fernando and Soysa, 2015). Black tea is mainly consumed in Western counties, while green tea is preferred in Japan and Northern China. The consumption of oolong tea mainly occurs in Taiwan and Southern China (Gramza *et al*., 2005). The popularity of tea may be attributed to their low cost, stimulatory effects, sensorial properties and health benefits (Dias *et al*., 2013).

1.8.1 The processing of *C.sinensis*

The leaves of *C.sinensis* undergo various levels of oxidation (fermentation) and polymerisation to produce white, green, oolong or black teas (Figure 1.6). These teas differ based on their organoleptic taste, chemical content, flavour and appearance (Sharangi, 2009).

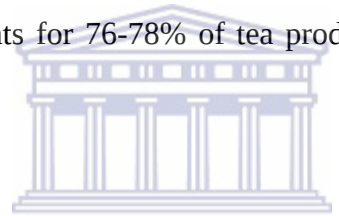
In the production of white tea, young leaves or buds are annually harvested in spring. The buds are prevented from growing in sunlight, thereby reducing chlorophyll formation, resulting in the characteristic white appearance of the leaves. Immediately after harvesting, the leaves are

steamed and dried to prevent oxidation. White teas possess the highest levels of antioxidants and least caffeine when compared to green, oolong and black teas (Dias *et al*., 2013; Sharangi, 2009).

Green tea is produced from the freshly dried leaves of *C.sinensis* (Nagle *et al*., 2006). The leaves are rolled and steamed to prevent fermentation before the drying process. Green tea contains the richest concentrations of polyphenols (i.e. EGCG) in comparison to white, oolong and black teas (Forester and Lambert, 2011).

In the production of oolong tea, the leaves of *C.sinensis* are partially fermented. The flavour and health benefits of oolong tea are similar to green and black teas. Oolong tea plays a role in protecting healthy skin cells and reducing the aging process (Sharangi, 2009).

The production of black tea involves the withering of *C.sinensis* leaves to reduce the moisture content. The leaves are rolled and crushed to commence fermentation. Black tea is the most abundantly produced and accounts for 76-78% of tea production worldwide (Fernando and Soysa, 2015).



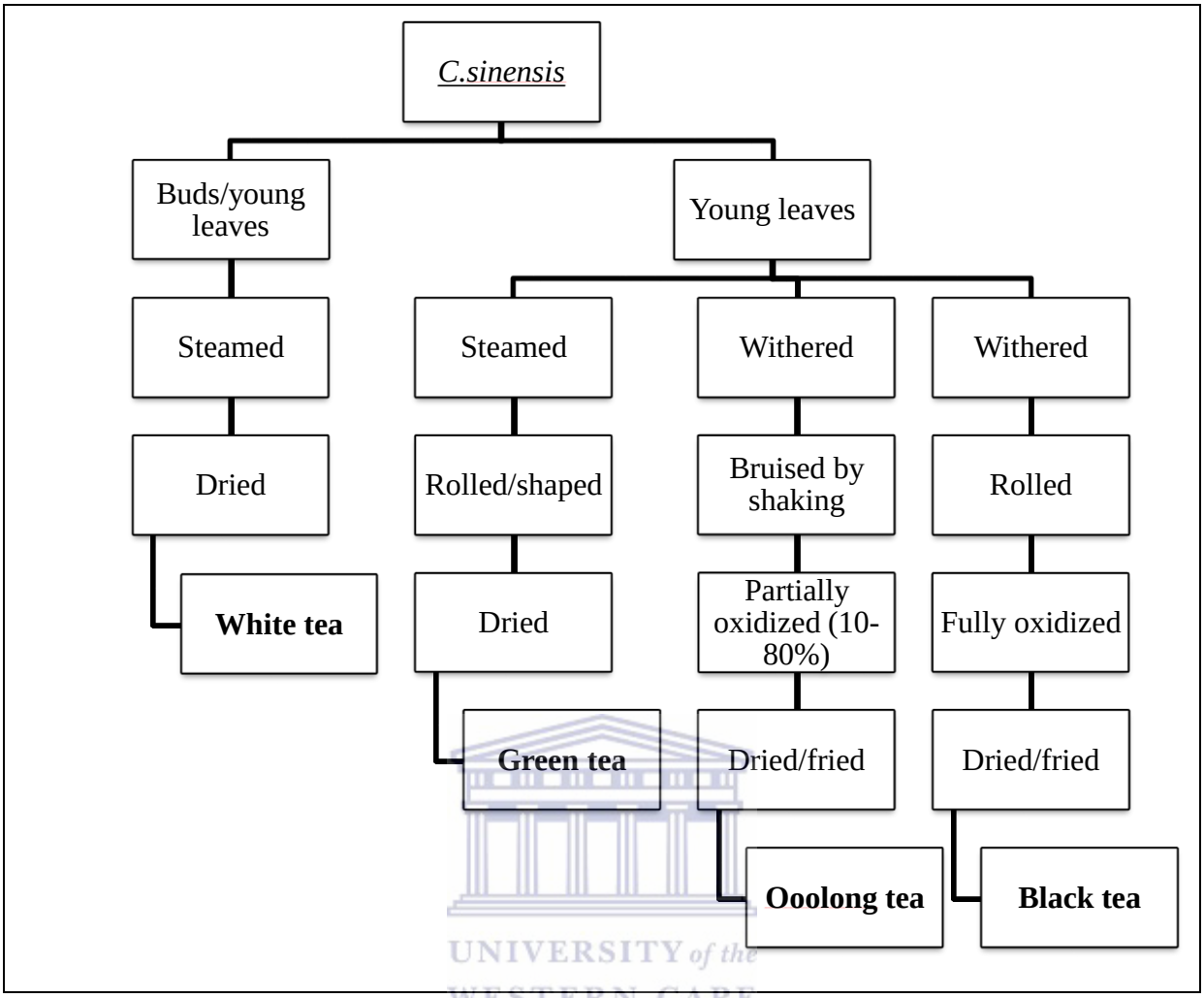


Figure 1.6: The processing of *C.sinensis* (Dias *et al*., 2013).

1.8.2 Polyphenols present in *C.sinensis*

The major component of *C.sinensis* leaves are the polyphenolic compounds, which constitute 25-35% of the dry weight (Balantine, 1997). The polyphenols present in tea leaves are flavanols (catechins), anthocyanidins, flavones, flavonols and phenolic acids (Chaturvedula and Prakash, 2011).

The catechins are predominantly present in tea leaves, specifically (-)-epicatechin (EC), (-)-epicatechin gallate (ECG), (-)-epigallocatechin (EGC), (-)-epigallocatechin gallate (EGCG), (+)-catechin (C), and (+)-gallocatechin (GC) (Chaturvedula and Prakash, 2011). It has been reported that EGCG is the most bioactive component and abundantly present in tea leaves (Sharangi, 2009; Gramza *et al*., 2005). The catechins contribute to the bitterness, astringency and sweet taste of tea (Chaturvedula and Prakash, 2011).

In addition to polyphenols, alkaloids (caffeine, theobromine, and theophylline), volatile oils, polysaccharides, amino acids, vitamins and inorganic elements are present in tea leaves (Sharangi, 2009).

1.8.3 Beneficial properties of *C.sinensis*

The beneficial properties of tea polyphenols have been well documented in the literature, over many decades. Tea polyphenols exert many physiological effects by acting as stimuli as well as an antioxidant, anti-atherosclerotic, anti-mutagenic, anti-hypertensive, antimicrobial, anti-diabetic and neuro-protective compounds (Dias *et al*., 2013).

Although many *in vivo* and *in vitro* studies have been conducted on tea polyphenols, it remains evident that tea polyphenols combat free radicals within the human body (Chaturvedula and Prakash, 2011). Green and black teas have greater antioxidant effects on peroxyl radicals than 22 common vegetables (Gramza *et al*., 2005). Sharangi, (2009) reported that tea catechins possessed greater antioxidant properties than vitamin C and E, tocopherol and carotene.

Buzzini *et al*., (2009) concluded that green tea polyphenols, particularly catechins, possessed an array of molecules that had antioxidant activity. In addition to combatting free radicals, tea antioxidants increase the activity of detoxifying enzymes glutathione peroxidase, glutathione reductase, glutathione –S-transferase, catalase and quinine reductase in the small intestine, liver and lungs.

Yang and Wang, (2010) investigated the effects of green tea and green tea polyphenols on cancer formation using animal models. They concluded that these compounds had an inhibitory effect on the formation and development of tumours in the oral cavity, liver, colon, pancreas, bladder, prostate and mammary glands. In addition, Lambert and Elias, (2010) reported that green tea and EGCG may have inhibitory effects on tumorigenesis stages.

Black and green teas possess antimicrobial properties and have been shown to reduce *Helicobacter pylori*, a bacterium associated with gastric, peptic and duodenal diseases (Chaturvedula and Prakash, 2011).

In studies performed on animal models, consumption of tea was also shown to prevent atherosclerosis, reduce blood triglycerides, total cholesterol content and high blood pressure (Khan and Mukhtar, 2007; Chaturvedula and Prakash, 2011).

1.9 Epigallocathechin-3-gallate

The chemical structure of epigallocatechin- 3-gallate (EGCG) has two tri-hydroxyl groups at carbon 3', 4' and 5' on the B-ring structure and a gallate moiety esterified at carbon 3 on the C-ring (Dias *et al*., 2013).

EGCG has a pyrogallol-type structure on the B- ring, which is mainly responsible for initiating apoptosis and has antioxidative properties, which undergo autooxidation, leading to the formation of reactive oxygen species (Mereles and Hunstein, 2011). The gallolyl moiety (D-ring gallate group) is an essential structure that results in the inhibition of a fatty acid synthase, which ultimately leads to a cytotoxic effect on cancerous human cells (Mereles and Hunstein, 2011).

EGCG is a major catechin that represents 50%-80% of the overall catechins found in green tea (Singh *et al*., 2011). The beneficial properties of EGCG have been extensively studied and established by associating its chemical structure with its function. EGCG is an antioxidant that possess potent radical scavenging properties that act against a variety of ROS (Mitrica *et al*., 2012).

Murakami *et al*., (2002) stated that in the HepG2 cell line, EGCG reduced cytotoxicity initiated by H_2O_2 and elevated enzymes involved in oxidative stress, leading to elevation of cellular GSH. Moreover, Taylor *et al*., (2005) concluded that EGCG possess antimicrobial properties that suppressed the growth of various gram negative and gram positive bacterial species.

Through various *in vitro* and *in vivo* cancer treatment models, the potential of EGCG was established. These studies showed the mechanisms by which EGCG inhibited initiation, promotion, and progression of cancer. In A/J mice with lung tumours, EGCG was shown to decrease proliferation, induce apoptosis and decrease angiogenesis (Singh *et al*., 2011).

In a study conducted in male rats, Figueiroa *et al*., (2009) concluded that EGCG had a reducing effect on the weight of the testes and accessory organs as well as reduced the level of LH and

testosterone in circulation. In addition, EGCG was shown to inhibit PKA and PKC signalling pathways as well as inhibit P450 scc (side-chain cleavage) and 17β- HSD activity, which are both essential in steroidogenesis.

Male Sprague Dawley rats treated with EGCG (approximately 85mg/kg body weight) for 7 days showed a 70% reduction in circulating testosterone and a reduction in the growth of accessory sexual organs, particularly the prostate gland (Kao *et al*., 2000).

Roychoudhury *et al*., (2017) reported that EGCG (2µM and 20µM) increased sperm motility, viability and promoted sperm capacitation in males. However, higher concentrations of EGCG (60µM) induced the opposite effect on sperm cells.

Human Sertoli cells treated with 50µM EGCG showed a reduction in oxidative damage within their protein and lipid structures. However, 50µM EGCG resulted in a decrease in lactate production. Lactate has a vital role in the survival and development of germ cells by providing energy and protecting germ cells. Furthermore, the same concentration of EGCG decreased the mitochondrial membrane potential of Sertoli cells, which may ultimately lead to a disruption in ATP (adenosine triphosphate) synthesis (Dias *et al*., 2017).

1.10 Apoptosis

Apoptosis is an energy dependant co-ordinated pathway that leads to cellular demise, during normal development and cellular damage. The process is characterised by cell shrinkage, pyknosis, plasma membrane blebbing and the shedding of apoptotic bodies (Nikoletopoulou *et al*., 2013). Cysteine aspartyl proteases (Caspases) are responsible for the initiation and execution of apoptosis either by an extrinsic or intrinsic apoptotic pathway (Wong, 2011).

1.10.1 Extrinsic Pathway

The initiation of the extrinsic pathway begins when a pro-apoptotic ligand binds to a death receptor on the extracellular surface of the cell. The most widely known receptors belong to the tumour necrosis factor (TNF) family, namely TNFR1 and Fas with the corresponding ligands TNF and Fas (Elmore, 2007). These receptors have an intracellular death domain such as TNF receptor associated death domain (TRADD) or Fas-associated death domain (FADD). Once the ligand binds to the death receptor, an adaptor protein with associated death effector domain (DED) and pro-caspase 8 forms the death inducing signalling complex (DISC). This

complex results in the activation of pro-caspase 8 into the active caspase 8, which is responsible for initiating the execution of apoptosis (Wong, 2011).

1.10.2 Intrinsic Pathway

The onset of the intrinsic pathway can be initiated by intracellular triggers (Figure 1.7) such as DNA damage, toxins, hypoxia, oxidative stress and radiation. These triggers lead to mitochondrial outer membrane permeabilization (MOMP) and result in the release of cytochrome C from the mitochondrial intermembrane space into the cytosol. The released cytochrome C binds and activates the adaptor protein Apaf-1 and procaspase 9, forming the apoptosome complex. The active caspase 9 is responsible for the cleaving and activation of caspases 3,6 and 7 (Galluzzi *et al*., 2012). Ultimately, these caspases execute the process of apoptosis (Loreto *et al*., 2014). Apoptosis inducing factor (AIF), second mitochondria-derived activator of caspase (Smac), direct IAP binding protein with low pI (DIABLO) and Omi/ high temperature requirement protein A (HtrA2) are additional proteins released from the mitochondrial intermembrane space. Smac/DIABLO and Omi/HtrA2 are responsible for blocking the inhibitors of apoptosis proteins (IAP), thereby promoting apoptosis (Elmore, 2007; Wong, 2011).

The Bcl-2 family of proteins regulate the intrinsic pathway by controlling the release of cytochrome C from the mitochondria. These Bcl-2 proteins are either anti-apoptotic (Bcl-2, Bcl-x, Bcl-XL) or pro-apoptotic (Bax, Bak, Bid) and are situated on the mitochondrial membrane. These families of proteins are strictly regulated by the tumour suppressor protein P53 (Loreto *et al*., 2014).

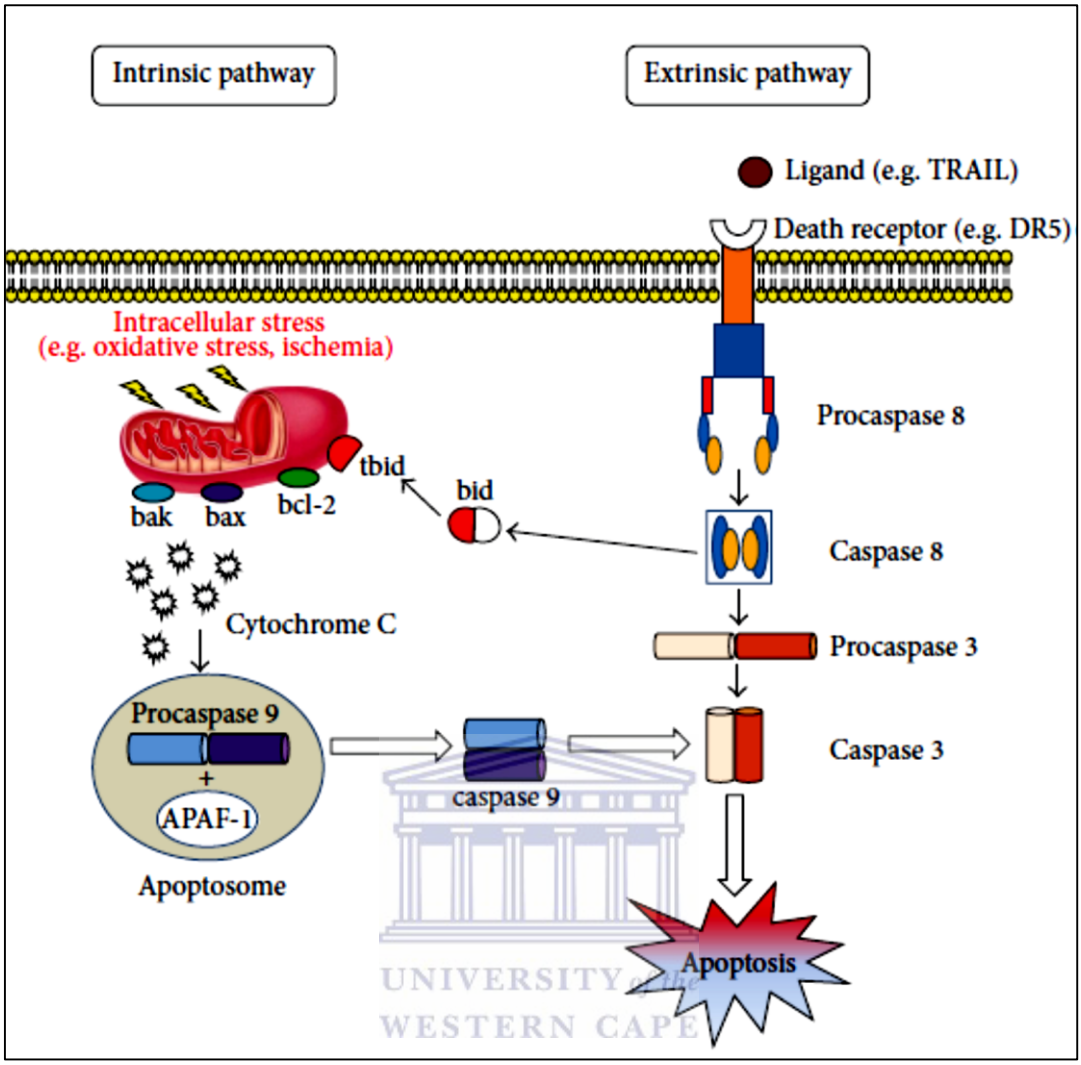


Figure 1.7: The apoptotic pathways (Loreto *et al*., 2014).

1.11 Aims of this study

Camellia sinensis (tea) and *Aspalathus linearis* (rooibos) may possess the ability to reduce testosterone production *in vitro* (Opuwari and Monsees, 2015). Therefore, further investigation was prompted to determine the role of the major active flavonoids of these teas on TM3 Leydig cell physiology.

The present study is aimed at investigating the *in vitro* effects of EGCG (tea component) and aspalathin (rooibos component) on TM3 Leydig cell functions based on the following parameters:

- Cytotoxicity of EGCG or aspalathin on basal and hCG stimulated TM3 Leydig cells.
- Quantification of testosterone levels after treatment with EGCG or aspalathin.
- Evaluation of the mitochondrial membrane potential.
- Apoptotic effects of EGCG or aspalathin on TM3 Leydig cells.
- Necrotic effects of EGCG or aspalathin on TM3 Leydig cells

Chapter Two: Materials and Methods

2.1 Chemicals

2.1.1 Sigma-Aldrich; St Louis, Missouri, USA:

- Epigallocathechin gallate (EGCG; E4143)
- Thiazoyl blue tetrazolium bromide (MTT)
- Trypan Blue
- Dimethylsulphoxide (DMSO) (D2438)
- Dextran (31390)
- Charcoal (C9157)

2.1.2 Lonza; Basel, Switzerland:

- Dulbecco's Modified Eagle's Media F-12 (1:1 mix) (3398)
- Trypsin / Ethyl Diamine Tetra Acetic acid (EDTA)

2.1.3 Ibidi GmbH; Martinsried, Munich, Germany:

- 60 µ-Dish (80136)

2.1.4 HWI Analytik GmBH; Rulzheim, Germany:

- Aspalathin (Cas No. 6027436)

2.1.5 Saarchem; Honeydew, South Africa:

- Dimethylsulphoxide (DMSO)

2.1.6 Becton, Dickinson and Company (BD); Franklin Lakes, New Jersey, USA:

- BD Accuri C6 Flow Cytometer

2.1.7 DRG Instruments Gmbh; Marburg, Germany:

- Testosterone ELISA (EIA- 1559)

2.1.8 Life Technologies Corporation; California, USA:

- Phosphate buffer saline (PBS)

2.1.9 Hyclone Laboratories; Logan, Utah, USA:

- Fetal bovine serum
- Horse serum

2.1.10 Molecular Probes; Eugene, Oregon, USA:

- CM-H_2DCFDA (C6827)
- Tali Apoptosis Kit – Annexin V Alexa Flour 488 (A10788)
- Tetramethylrhodamine (TMRE; T669)

2.1.11 Nest Biotechnology; Wuxi, Jiangsu, China:

- 12 and 96 well plates

2.1.12 AppliChem GmbH; Darmstadt, Germany:

- Tris-Hydrochloride (Tris-HCl)

2.2 Preparation of Aspalathin

Aspalathin was purchased from HWI Analytik GmBH (Cas No. 6027436; Rulzheim, Germany). The stock solution was prepared in sterile DMSO to a final concentration of 20mM. The stock solution was stored in aliquots at -20^{0}C.

2.3 Preparation of Epigallocathechin-3- gallate (EGCG)

EGCG was purchased from Sigma- Aldrich (E4143; St Louis, MO, USA). The stock solution was prepared in sterile DMSO to a final concentration of 20mM. The stock solution was stored in aliquots at -20^{0}C.

2.4 Cell line

The TM3 cell line derived from *Mus musculus* was purchased from American Type Cell Culture (CLR- 1714; USA). The TM3 cell line is reported to respond to luitenizing hormone (LH) and may result in an increased cAMP production, but does not respond to FSH. In the presence of LH, the cell may effectively metabolize cholesterol (Mather *et al.*, 1980). Many research articles reported that TM3 Leydig cells produce testosterone upon stimulation with hCG (Edjenguele *et al*., 2014; Opuwari an Monsees, 2015; Chen *et al*., 2015).

2.5 TM3 Leydig cell line

2.5.1 Cell culture and Thawing

The cells were thawed by gently rotating the cryovial in a 37^0C water bath and sterilized by spraying the cryovial with 70% isopropanol. Subsequently, 1ml of TM3 cells was transferred into a 75cm^2 cell culture flask containing 25ml Dulbecco's Modified Eagles Media F-12 (DMEM) with 15mM Hepes and L-Glutamine. The DMEM media was supplemented with 2.5% fetal bovine serum (FBS) and 5% horse serum (HS). Cells were grown at 37^0C in a 5% CO_2 incubator and examined after 24 hours. Upon reaching 70-80% confluency, the cells were prepared for sub-culturing.

2.5.2 Sub-cultivation of cells

Once reaching 70-80% confluency, cells were sub-cultured to prevent cell death. Firstly, the media was removed and the cells washed with 2ml phosphate buffer saline (PBS). Thereafter, 2ml 0.25% trypsin/EDTA was added to the flask and incubated for 2 minutes at 37^0C.

The detachment of cells from the surface of the flask wall was examined using an inverted light microscope. The trypsin was inactivated by adding 4ml complete media to the flask. Subsequently, 1ml of the cell suspension was transferred into a separate flask containing 20ml media.

2.5.3 Freezing of cells

Upon reaching 70-80% confluency, the cells were frozen according to sub-culturing procedure. After trypsin inactivation, the cell suspension was transferred to a centrifuge tube and centrifuged at 260 x g for 5 minutes. The supernatant was removed and the remaining pellet was re-suspended in freezing media (complete media containing 5% DMSO). The cell suspension was gently aspirated and transferred into cryovials. The cryovials were placed into a styrofoam box at 4^0C for 30 minutes and then transferred to a -80^0C for overnight storage. The following day, the cryovials were stored in liquid nitrogen. The rationale for slow freezing is to reduce the risk of ice crystal formation and prevent cell lyses.

2.5.4 Plating of cells

Once cells reached 70-80% confluency, the depleted media was removed and the cells were washed with 2ml PBS without Ca^{2+}/Mg^{2+}. After the removal of PBS, 2ml of trypsin/EDTA was added to the cells. The flask was incubated for 2 minutes and examined for detachment under an inverted microscope. Subsequently, 4ml complete media was added to the cells for trypsin/EDTA inactivation.

The cells were transferred into a 15ml tube and centrifuged at 260 x g for 5 minutes. After centrifugation, the media was removed without disturbing the pellet and replaced with 1ml complete media.

Firstly, the supernatant was gently aspirated and 10µl of the cell suspension was transferred to an eppendorf cup. Thereafter, 10µl of trypan blue was added to the eppendorf cup, creating a dilution factor of 2. In order to count the number of cells, 10µl of cell suspension was transferred to a haemocytometer counting chamber. The following formula was used to determine the number of cells in 1ml complete media:

Mean *2 (dilution factor) *10000 = cells/ml

The cell number for the various plates was determined using the following equation:

$$\text{Volume of cells required (µl)} = \frac{\text{concentration of cells needed (cells/ml)} * \text{total volume(µl)}}{\text{concentration of cells counted (cells/ml)}}$$

2.6 Determination of Cytotoxicity

2.6.1 MTT Principle

MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) is a colorimetric yellow tetrazolium dye that measures the activity of mitochondrial dehydrogenase. This is a reflection of the cell viability and proliferation. The yellow tetrazolium form is reduced to purple formazan crystals by action of mitochondrial dehydrogenase enzymes in metabolically active cells (Van Tonder *et al*., 2015).

2.6.2 Experimental Procedure

The MTT working solution was prepared by dissolving 1mg/ml in PBS. The solution was allowed to rotate at room temperature for approximately 1 hour. Subsequently, the solution was sterile filtered and wrapped in aluminium foil to protect from light.

TM3 cells were assessed by using the MTT assay. Cells were plated into 96 well plates at a density of 20 000 cells/ml and incubated for 24 hours. The cells were either stimulated with or without 25mIU hCG for 24 hours at concentrations of 0.1µM, 5µM, 10µM and 100µM of EGCG and aspalathin, respectively. The negative control consisted of media containing 0.5% DMSO and the positive control consisted of 10% DMSO. The following day, supernatants were removed and cells were washed with 200µl PBS and replaced with 200µl fresh media. Subsequently, 20µl of MTT was added to each well and incubated at 37^0C for 4 hours. The supernatant was removed and replaced with 100µl DMSO to dissolve the dye. The plate was transferred to a shaker for approximately 10 minutes to homogenize the solution. The absorbance was read using a microplate reader at 560nm with a background subtraction of 750nm.

2.6.3 Morphological changes using light microscopy

The effect of EGCG and aspalathin on morphological properties and survival rate of basal and stimulated TM3 Leydig cells was assessed. The cells seeded for MTT analysis were observed under inverted microscopy after 24 hour treatment with either EGCG or aspalathin.

2.7 Testosterone determination using Enzyme linked immunosorbent Assay (ELISA)

2.7.1 ELISA Principle

Testosterone ELISA is a solid phase assay and based on the principle of competitive binding. The microtiters wells are coated with a monoclonal (mouse) antibody with high specificity/affinity to the testosterone molecule. The competition occurs between the endogenous testosterone sample and testosterone horseradish peroxidase conjugate for binding to the coated antibody. The quantity of bound peroxidase conjugate is reversely proportional to the concentration of testosterone in the sample.

2.7.1 The preparation of charcoal stripped sera

The protocol was adapted from Dang and Lowik, (2005) with minor modifications. The removal of steroid hormones was necessary for testosterone ELISA analysis.

The initial steps involved the preparation of a one litre 0.1M Tris hydrochloride (Tris-HCl) buffer solution by adding 15.76g Tris-HCl to 900ml reverse osmosis (RO) water. The solution was titrated with 1M hydrochloric acid (HCL) and/or 1M sodium chloride (NaOH) to pH 8, the one litre was completed using RO water. Subsequently, 250g dextran and 2.5g charcoal was added to the 0.1M Tris-HCl buffer solution. The solution was allowed to mix overnight at 4-5^{0}C. The HS and FBS were heat inactivated at 56^{0}C for 45 minutes. The Tris-HCl-dextran-charcoal solution was centrifuged at 1000 x g for 10 minutes and the supernatant was discarded. The heat inactivated sera was added to the pellet, gently mixed and further incubated at 45^{0}C for 45 minutes. The sera solutions were centrifuged at maximum speed of 4180 x g for 10 minutes. The supernatant was removed and sterile filtered. Thereafter, the supernatant was aliquoted and stored at -20^{0}C.

2.7.3 Experimental Procedure

Testosterone levels were determined by performing an ELISA assay. Preliminary experiments were conducted to determine whether the cells respond optimally to the hCG stimulus. The TM3 Leydig were either grown in complete media, un-supplemented media or media containing charcoal stripped sera. The TM3 Leydig cells grown in complete media were seeded at 4000 cells/well and allowed to attach for 24 hours. Thereafter, cells were exposed to four concentrations of hCG (12,24,36 and 48mIU) for 24 hours in a 37^{0}C, 5% CO_2 incubator. The supernatants were frozen at -20^{0}C until the ELISA assay was performed.

In the un-supplemented group, TM3 Leydig cells were seeded with complete media at 2500 cells/well for 24 hours. Subsequently, the media was removed and replaced with media containing half the quantity of sera (1.25% FBS and 2.5% HS). The cells were slowly weaned off to prevent cellular stress. After 24 hours, the cells were treated with the un-supplemented media (no sera). The following day, TM3 cells were exposed to 12.5mIU and 25mIU hCG for 48 hours. The supernatants were frozen at -20^{0}C until the ELISA assay was performed.

In the charcoal stripped media group, TM3 Leydig cells were seeded with complete media at 2500 cells/well for 24 hours. The media was removed and replaced with complete media for a further 24 hours. Thereafter, TM3 Leydig cells were treated with media containing charcoal

stripped sera for 24 hours. The following day, TM3 cells were exposed to 12.5mIU and 25mIU hCG for 48 hours. The supernatants were frozen at -20^{0}C until the ELISA assay was performed.

Briefly, 25µl of samples and testosterone standards (0, 0.2, 0.5, 1, 2, 6, 16ng/ml) were added to the corresponding wells. 200µl enzyme conjugate was added to each well, contents were mixed well for 10 seconds and incubated at room temperature for 60 minutes. After incubation, the contents were briskly shaken and then discarded. Each well was then washed with 400µl wash solution and the process repeated 3 times. The residual droplets were removed by striking the wells sharply on absorbent paper. Then, 200µl substrate solution was added to each well and incubated for 15 minutes at room temperature. The reaction was terminated by adding 100µl of stop solution to each well and the absorbance was read at 450nm within 10 minutes.

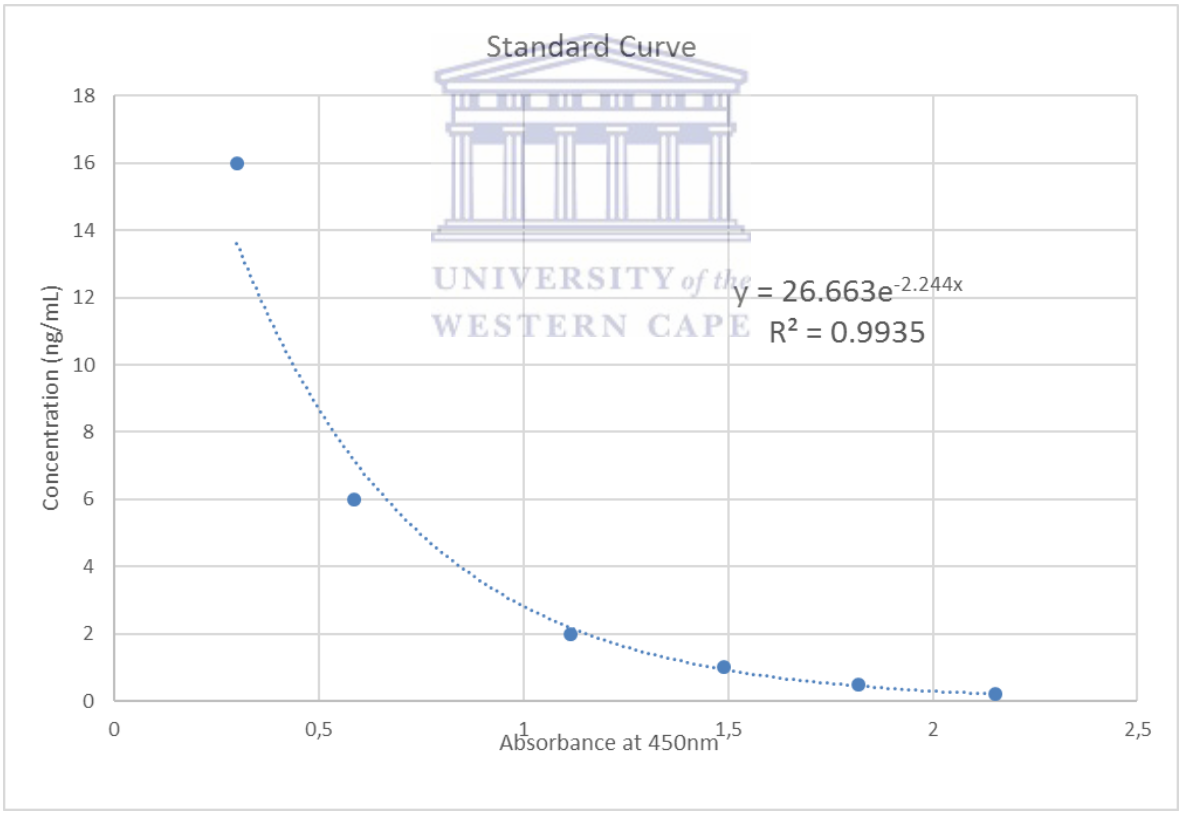


Figure 2.1: Typical standard curve for testosterone production in TM3 Leydig cells.

2.8 The determination of apoptotic cells using Annexin V Alexa Fluor 488 with Propidium iodide Tali Apoptosis Kit

2.8.1 Annexin/PI principle

A normal, healthy cell plasma membrane has an asymmetrical arrangement of a phospholipid bilayer. The phosphatidylcholine phospholipids are situated on the external membrane/surface of the cell and the phosphatidylserine phospholipids on the inner membrane/surface. The onset of apoptosis results in the translocation of these phosphatidylserine phospholipids to the external membrane/surface of the cell. Annexin V is a biotinylated fluorochrome that binds to phosphatidylserine in the presence of calcium, resulting in green fluorescence. Hence, Annexin V may be considered a marker for apoptotic cells and detection can be established by fluorescent microscopy and flow cytometry (Wlodkowic *et al*., 2011).

Propridium iodide (PI) passes through the membrane of damaged cells and binds to the nucleic acids. PI cannot penetrate live or apoptotic cells and only stains dead cells with red fluorescence, due to the increased permeability of the damaged cell membrane (Rieger *et al*., 2011).

2.8.2 Experimental procedure

Cells were plated into 12 well plates at a density of 100 000 cells/ml for 24 hours. The experimental set up involved two 0.5% DMSO negative control groups (unstained and stained with Annexin/PI), two positive control groups for apoptosis (Annexin/PI and Annexin only) and two positive control groups for necrosis (Annexin/PI and PI only). Cells were also treated with 5 and 100µM EGCG and aspalathin for 24 hours, respectively. Apoptosis was induced by treating cells with 6% DMSO for 24 hours (Qi *et al*., 2008). Whereas, necrosis was induced with 2mM H_2O_2 for 1 hour (Troyano *et al*., 2003).

In brief, cells were harvested using 0.25% trypsin for 1 minute and inactivated with complete media. The cells were transferred to eppendorf tubes and centrifuged at 260 x g for 4 minutes. The pellet of each sample was re-suspended in 20µl 1X Annexin binding buffer. Thereafter, 1µl Annexin V Alexa Fluor 488 was added to the appropriate tubes and incubated in the dark for 20 minutes at room temperature. Each sample tube was centrifuged at 260 x g for 4 minutes and the supernatant removed. The pellet was re-suspended in 100µl 1X Annexin binding buffer and 1µl Tali Propridium Iodide was added to the appropriate tubes. The samples were gently

mixed and incubated for 5 minutes in the dark. The tubes were centrifuged at 260 x g for 4 minutes and re-suspended in 300µl Annexin binding buffer.

The samples were placed on ice and analysed using the FL 1 and FL 3 channel of the BD accuri flow cytometer. Live cells, apoptotic and necrotic cells were the particular groups analysed. A minimum of 10 000 cells per sample were analysed.

2.9 The evaluation of mitochondrial membrane depolarization using Tetramethylrhodamine ethyl ester (TMRE) fluorescence dye.

2.9.1 TMRE Principle

TMRE is a positively charged, red-orange dye that penetrates cells with active mitochondria and results in red-orange fluorescence. A loss or disruption of the mitochondrial membrane potential (Δψm) leads to a diminished red-orange fluorescence of cells. Depolarization of the Δψm could be an indicator for apoptosis and necrosis (Lemasters *et al*., 1999).

2.9.2 Experimental Procedure for Flow Cytometry

The cells were plated into 12 well plates at a density of 100 000 cells/ml and were allowed to attach for 24 hours in a 37^0C, 5% CO_2 incubator. Cells were then treated with, 5 and 100µM EGCG and aspalathin for 24 hours, respectively. In addition, cells were treated with a negative control of 0.5% DMSO for 24 hours, while the positive control of 1% DMSO was exposed for 24 hours. According to Yuan *et al*., (2014), 1% DMSO was used as a positive control to impair mitochondrial integrity. Furthermore, the cells were harvested from the 12 well plates using 0.25% trypsin for 1 minute and inactivated with complete media. The cells were then transferred to 15ml tubes and centrifuged at 260 x g for 4 minutes.

The cells were stained with 300µl of 1µM TMRE (stock solution of 3034.21µM) dye and incubated in the dark for 30 minutes at 37^0C. Subsequently, 4ml PBS was added to the tube to remove the dye and centrifuged at 260 x g for 4 minutes. The supernatant was discarded and the pellet was re-suspended in 400µl PBS. The cell suspension was transferred to an eppendorf tube and analysed using the FL 3 channel of the BD accuri flow cytometer. A minimum of 10 000 cells were analysed per sample.

2.9.3 Experimental Procedure for Fluorescence Imaging

Fluorescence microscopy is a technique that typically requires the attachment of fluorophores to specific cell structures and then observing the fluorophores under the microscope with the aid of specific filters. The technique involves exciting a fluorophore with light of a specific wavelength; subsequently the fluorophore emits light at a lower wavelength, the latter being what is viewed. For the purpose of this thesis, the principle was applied to detect depolarization within the mitochondria of TM3 Leydig cells.

Firstly, the cells were seeded into Ibidi 60µ-Dish at a density of 50 000 cells/400µl and were allowed to attach for 1 hour, thereafter, an additional 400µl media was added to the dish. The cells were grown over a 24 hour period in a 37^0C incubator. Cells were then treated with 5 and 100µM EGCG and aspalathin, respectively. The cells were treated with a negative control of 0.5% DMSO for 24 hours and a positive control of 1% DMSO for 24 hours (Yuan *et al*., 2014).

The supernatant was removed from each well and gently washed with 800µl PBS. The cells were stained with 800µl/well of 1µM TMRE dye for 30 minutes at 37^0C. Subsequently, cells were washed thrice with 800µl PBS to remove excess TMRE dye. 800µl PBS was added to each well and viewed immediately on the Zeiss Axiovert 200M Inverted microscope (Zeiss, Germany) using the AxioVision40 software (version 4.8.2.0). The red filter set was used with an excitation wavelength of 584nm and an emission wavelength of 607nm.

2.10 Statistical Analysis

Statistical analysis was performed using GraphPad prism (version 5.03) statistical software (GraphPad Software, San Diego, CA, USA). One-way ANOVA trend analysis was performed followed by Turkey multiple comparison test. The data expressed as mean±SD and $P<0.05$ was considered statistically significant. All experiments were repeated with similar findings. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Chapter Three: Results

3.1 Cytotoxicity

In order to investigate the effect of EGCG and aspalathin on basal and stimulated (hCG) TM3 cells, the morphological changes and mitochondrial dehydrogenase activity was determined by light microscopy and MTT viability assay, respectively.

3.1.1 The morphology of basal TM3 Leydig cells after 24 hour exposure to EGCG

The results obtained after treating TM3 Leydig cells with EGCG for 24 hours showed no visible changes in cell morphology (C-G) when compared to the negative control (A). Cells maintained their flat polygonal morphology with no obvious cell rounding observed. Distinct cell clusters with rounded and shrunken morphology were observed in cells exposed to 10% DMSO, indicating dead cells (B) (Figure 3.1).

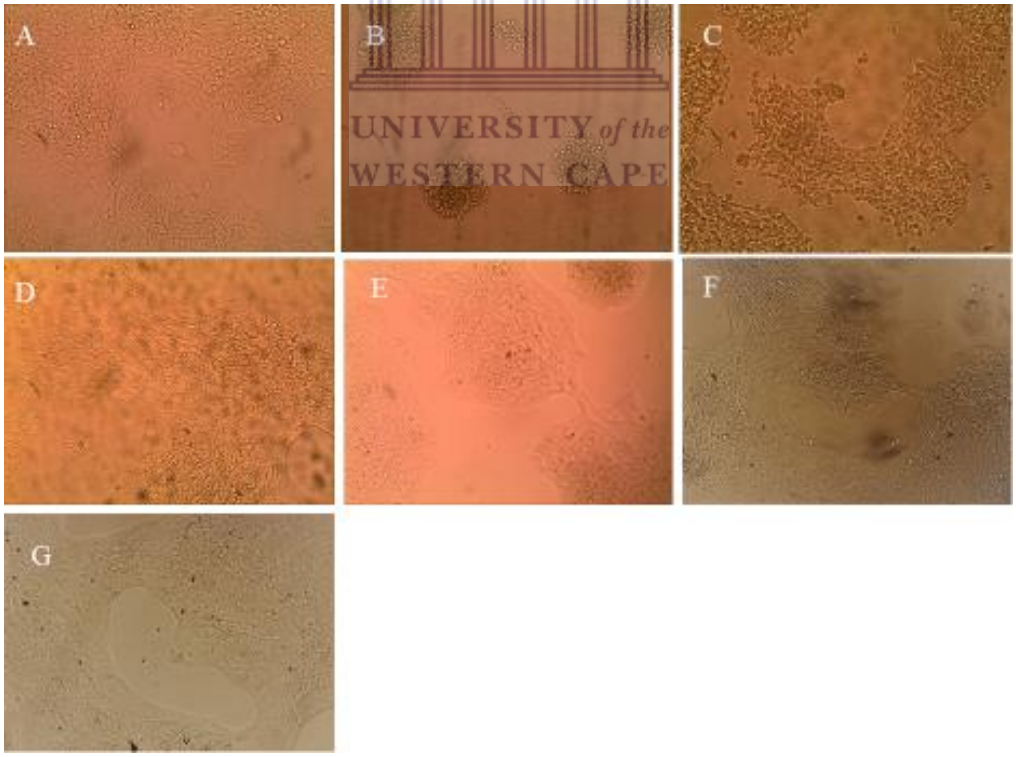


Figure 3.1: Micrographs showing the effect of EGCG on basal TM3 Leydig cells after 24 hour exposure. Abbreviations: (A) negative control (0.5% DMSO), (B) positive control (10% DMSO) (C) 0.1µM (D) 5µM (E) 10µM (F) 50µM (G) 100µM. Magnification = 100X.

3.1.2 The morphology of hCG-stimulated TM3 Leydig cells after 24 hour exposure to EGCG

Stimulated TM3 Leydig cells treated with EGCG for 24 hours showed no observable differences in cell morphology (C-G) when compared to the negative control (A). Cells maintained their flat polygonal morphology with no obvious cell rounding observed. Distinct cell clusters with rounded and shrunken morphology were observed in cells exposed to 10% DMSO, indicating dead cells (B) (Figure 3.2).

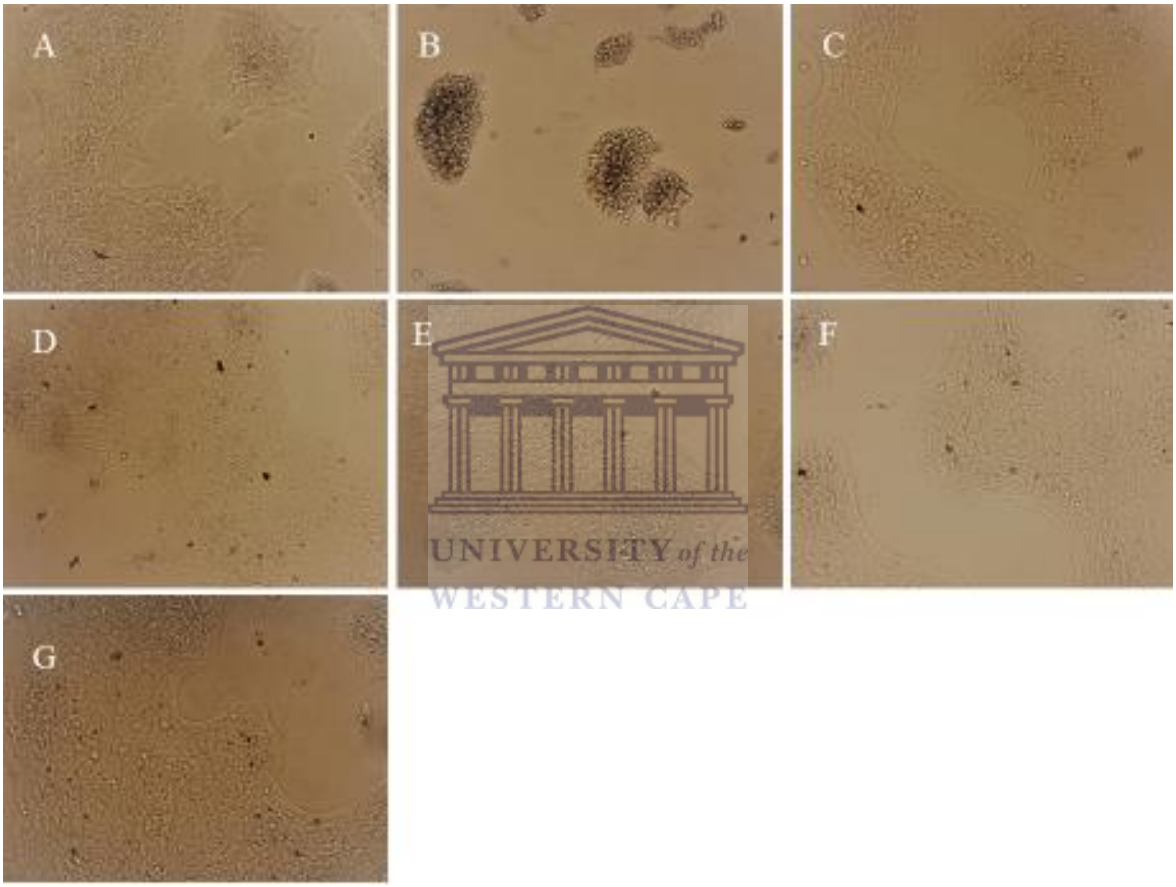


Figure 3.2: Micrographs showing the effect of EGCG on hCG-stimulated (25mIU) TM3 Leydig cells after 24 hour exposure. Abbreviations: (A) negative control (0.5% DMSO) (B) positive control (10% DMSO) (C) 0.1µM (D) 5µM (E) 10µM (F) 50µM (G) 100µM. Magnification = 100X.

3.1.3 The morphology of basal TM3 Leydig cells after 24 hour exposure to aspalathin

The results obtained after treating TM3 Leydig cells with aspalathin for 24 hours showed no visible changes in cell morphology (C-G) when compared to the negative control (A). Cells maintained their flat polygonal morphology with no visible cell rounding observed. Distinct cell clusters with visible rounded and shrunken morphology were observed in cells exposed to 10% DMSO, indicating dead cells (B) (Figure 3.3).

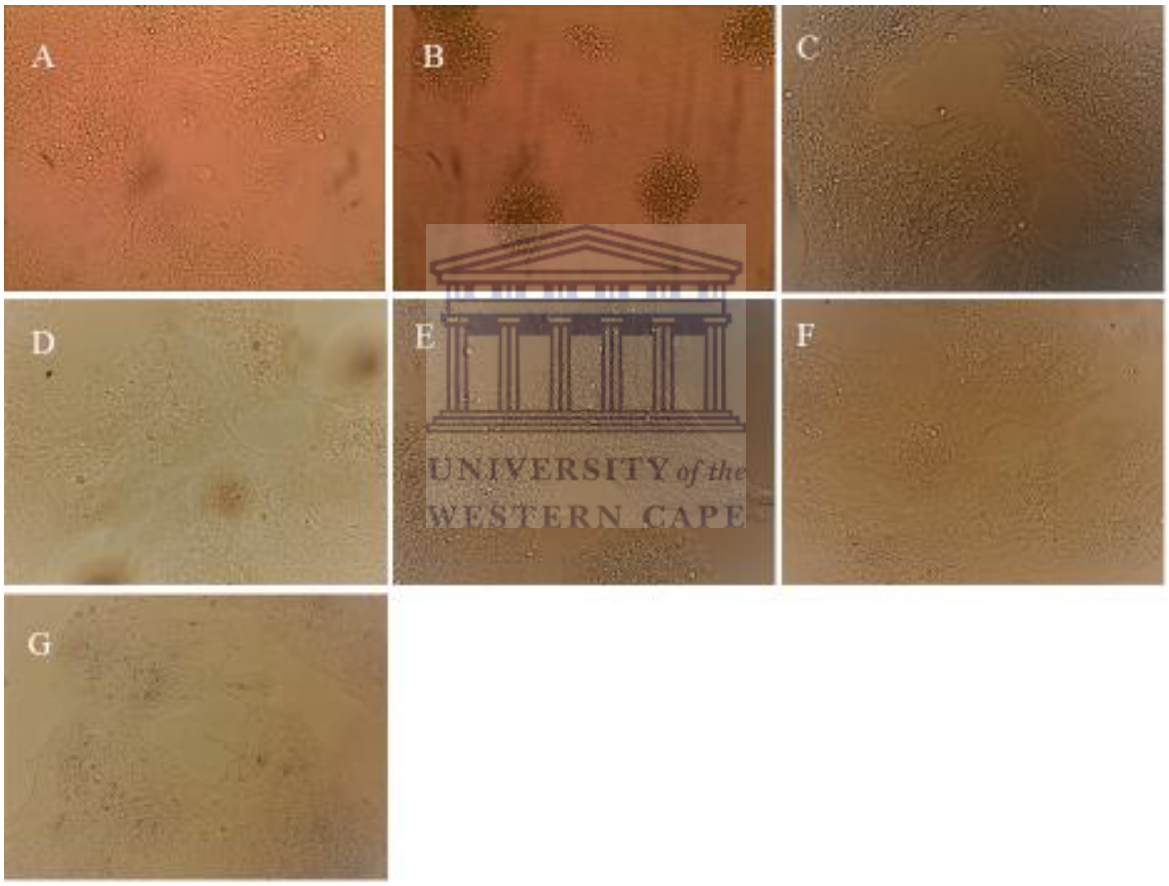


Figure 3.3: Micrographs showing the effect of aspalathin on basal TM3 Leydig cells after 24 hour exposure. Abbreviations: (A) negative control (0.5% DMSO) (B) positive control (10% DMSO) (C) 0.1µM (D) 5µM (E) 10µM (F) 50µM (G) 100µM. Magnification = 100X.

3.1.4 The morphology of hCG-stimulated TM3 Leydig cells after 24 hour exposure to aspalathin

The results obtained after treating stimulated TM3 Leydig cells with aspalathin for 24 hours showed no visible changes in cell morphology (C-G) when compared to the negative control (A). Cells maintained their flat polygonal morphology with no visible cell rounding observed. Distinct cell clusters with visible rounded and shrunken morphology were observed in cells exposed to 10% DMSO, indicating dead cells (B) (Figure 3.4).

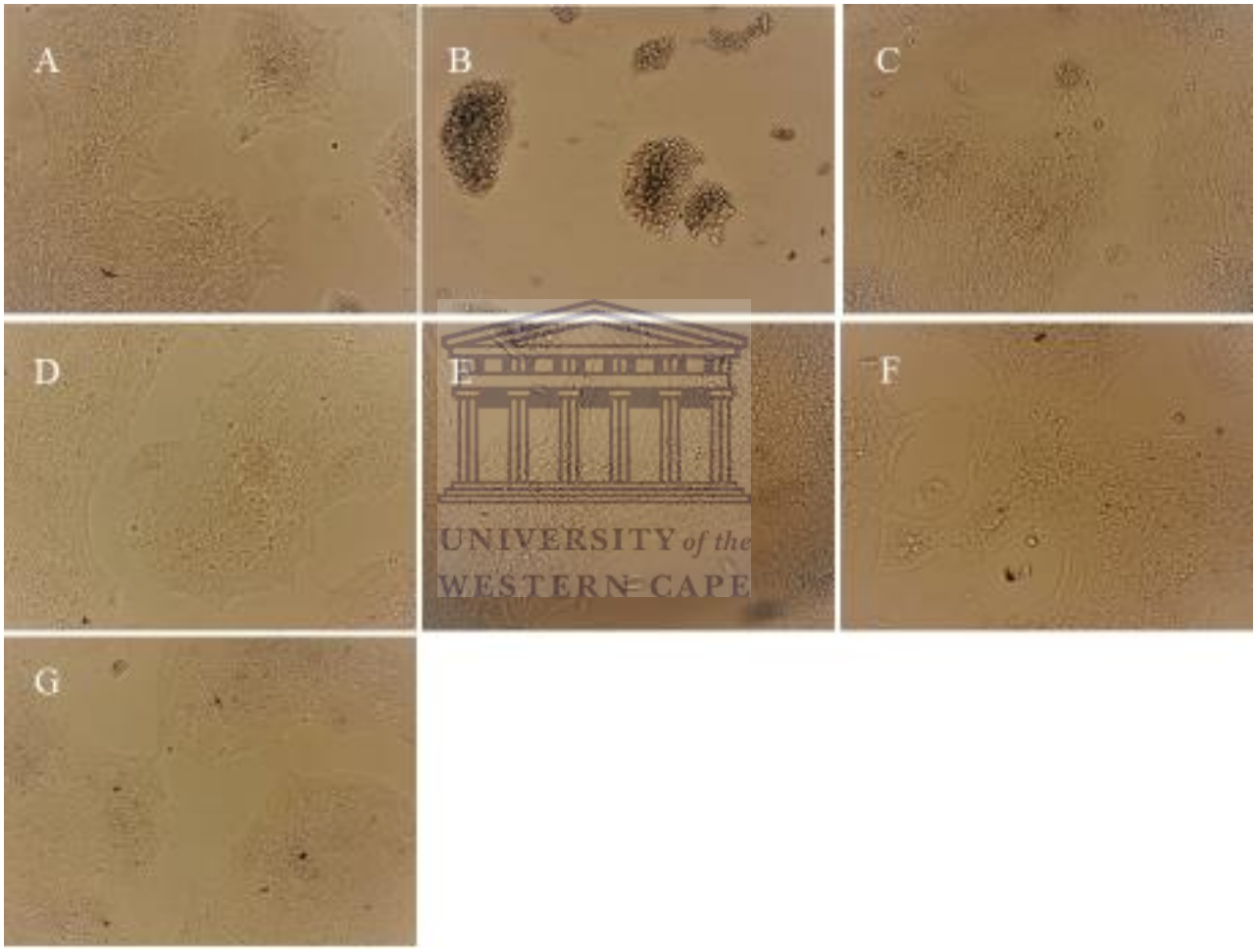


Figure 3.4: Micrographs showing the effect of aspalathin on hCG-stimulated (25mIU) TM3 Leydig cells after 24 hour exposure. Abbreviations: (A) negative control (0.5% DMSO), (B) positive control (10% DMSO) (C) 0.1µM (D) 5µM (E) 10µM (F) 50µM (G) 100µM. Magnification = 100X.

3.1.5 The effect of EGCG on basal and hCG-stimulated TM3 cells after 24 hour exposure

Overall, mitochondrial dehydrogenase activity slightly decreased in basal and hCG-stimulated TM3 cells between the range of 0.1-100µM, when compared to the negative control (Figure 3.5 and Figure 3.6). Basal and hCG-stimulated groups showed a significant decrease at 0.1µM ($p<0.01$) and ($p<0.05$), respectively. In the basal group, further significant reduction in enzyme activity was observed at 50µM ($p< 0.001$) and at 100µM ($p< 0.001$). The hCG-stimulated group did not display any significant changes between 50µM and 100µM when compared to the negative control. In both groups the positive control showed a significant ($p< 0.001$) reduction in mitochondrial dehydrogenase activity in relation to the negative control.

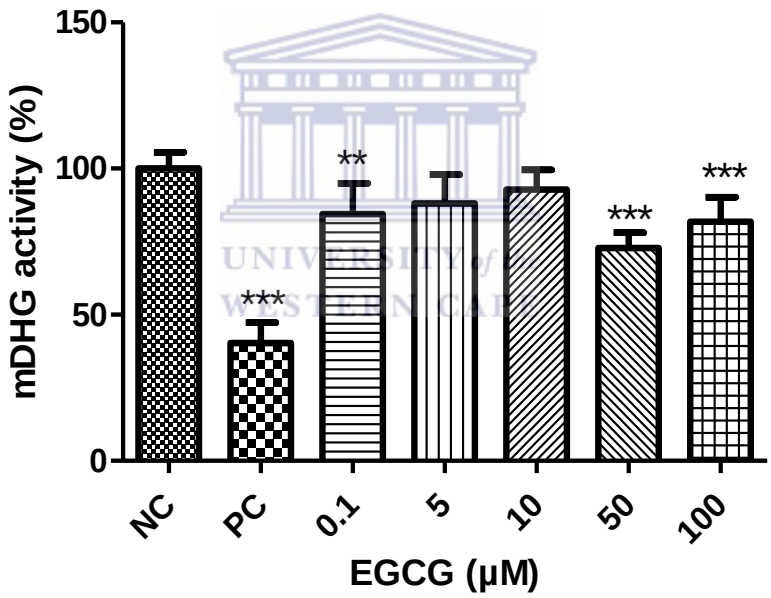


Figure 3.5: Relative mitochondrial dehydrogenase (mDHG) activity of basal TM3 cells exposed to EGCG for 24 hours. Values represent mean ± SD, n=8. Abbreviations: epigallocatechin- 3-gallate (EGCG), NC-negative control (0.5% DMSO), PC-positive control (10% DMSO); ** p<0.01, *** p<0.001, compared to the negative control.

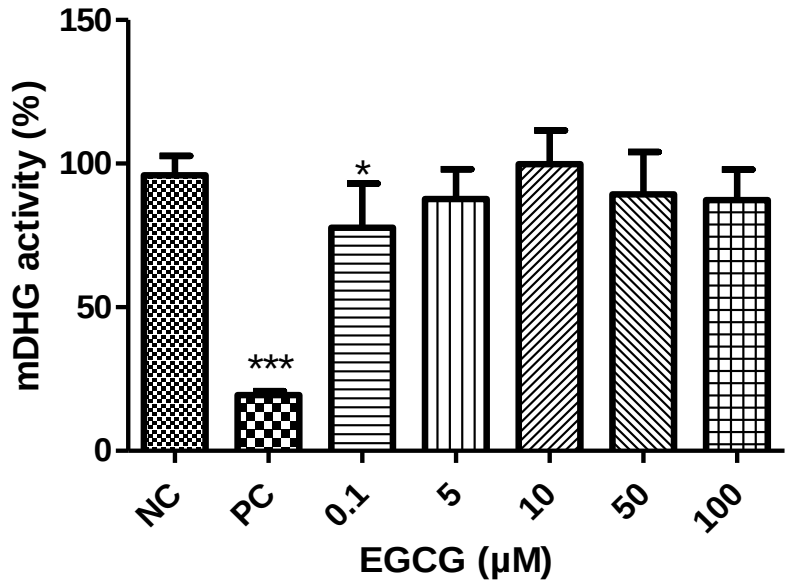


Figure 3.6: Relative mitochondrial dehydrogenase (mDHG) activity of hCG-stimulated TM3 cells exposed to EGCG for 24 hours. Values represent mean ± SD, n=8. Abbreviations: epigallocatechin- 3-gallate (EGCG), NC-negative control (0.5% DMSO), PC-positive control (10% DMSO); * p<0.05, *** p<0.001, compared to the negative control.

3.1.6 The effect of aspalathin on basal and hCG-stimulated TM3 cells after 24 hour exposure

The basal group showed a significant decrease in mitochondrial dehydrogenase activity after exposure to 0.1-10µM aspalathin. At the highest aspalathin concentration, the mitochondrial dehydrogenase activity displayed a non-significant increase. In contrast, hCG-stimulated Leydig cells did not show any differences in mitochondrial dehydrogenase activity compared to the negative control. However, enzyme activity increased at the exposure to 50µM aspalathin and was statistically significant ($p<0.001$) at 100µM. A significant reduction in enzymatic activity was observed at the positive control ($p<0.001$) in both groups, compared to the negative control (Figure 3.7 and Figure 3.8).

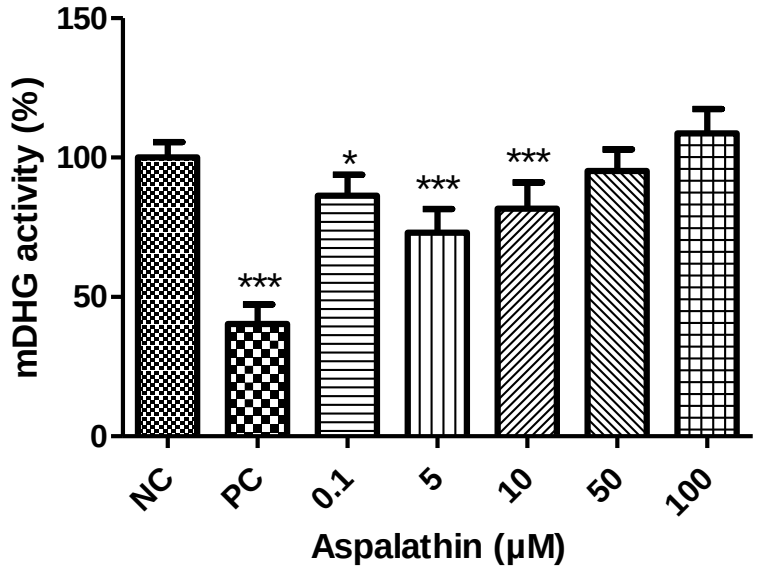


Figure 3.7: Relative mitochondrial dehydrogenase (mDHG) activity of basal TM3 cells exposed to aspalathin for 24 hours. Values represent mean ± SD, n=8. Abbreviations: NC-negative control (0.5% DMSO), PC-positive control (10% DMSO); * p<0.05, *** p<0.001, compared to the negative control.

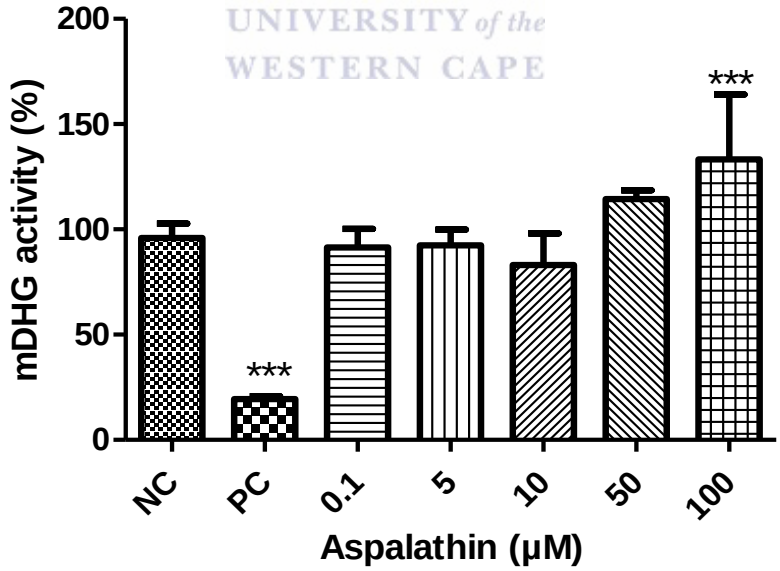


Figure 3.8: Relative mitochondrial dehydrogenase (mDHG) activity of hCG-stimulated TM3 cells exposed to aspalathin for 24 hours. Values represent mean ± SD, n=8. Abbreviations: NC-negative control (0.5% DMSO), PC-positive control (10% DMSO); *** p<0.001, compared to the negative control.

3.2 Testosterone production

3.2.1 Testosterone production in TM3 Leydig cells using supplemented media

Preliminary experiments were conducted to determine whether the cells respond optimally to the hCG stimulus. The TM3 Leydig cells were exposed to four concentrations of hCG for 24 hours. The supernatants were removed and analysed for testosterone synthesis.

There was no significant difference in testosterone synthesis observed at each hCG concentration when compared to the negative control (Figure 3.9). As TM3 Leydig cells previously responded well to hCG treatment, with a significant increase in testosterone production (Edjenguele *et al*., 2014; Opuwari an Monsees, 2015; Chen *et al*., 2015) it may be possible that the specific batch of TM3 Leydig cells ceased to produce sufficient testosterone.

Therefore, further methods were performed to validate the results in figure 3.9 and to test if other batches of TM3 Leydig cells were also unresponsive for the purpose of this study. Different batches of TM3 Leydig cells were used for the results depicted in figure 3.10 and figure 3.11. In addition, the experimental conditions were slightly altered as this may have an effect on steroid production (Engeli *et al*., 2018).

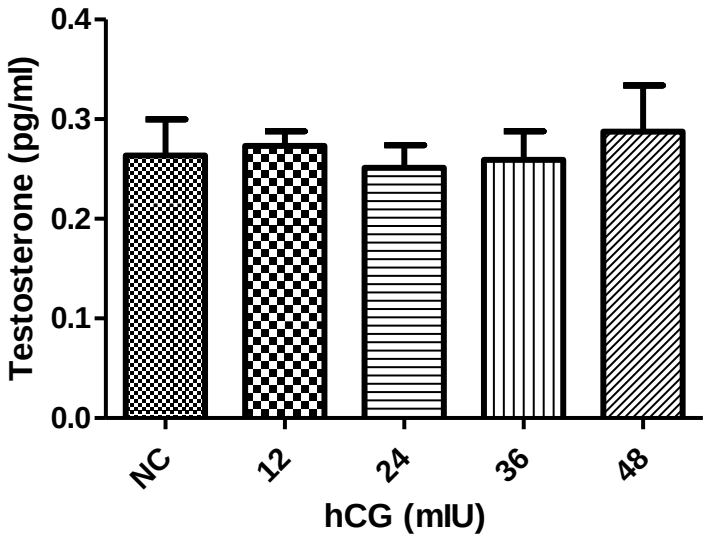


Figure 3.9: Testosterone production in TM3 cells after exposure to various concentrations of hCG for 24 hours using supplemented media. Values represent mean ± SD, n=5. Abbreviations: NC-negative control (media and cells only).

3.2.2 Testosterone production in TM3 Leydig cells using supplemented media containing half of the sera concentration

TM3 Leydig cells were exposed to un-supplemented media and 12.5mIU or 25mIU hCG for 48 hours. In figure 3.10, no significant difference was observed at the various hCG concentrations when compared to the negative control.

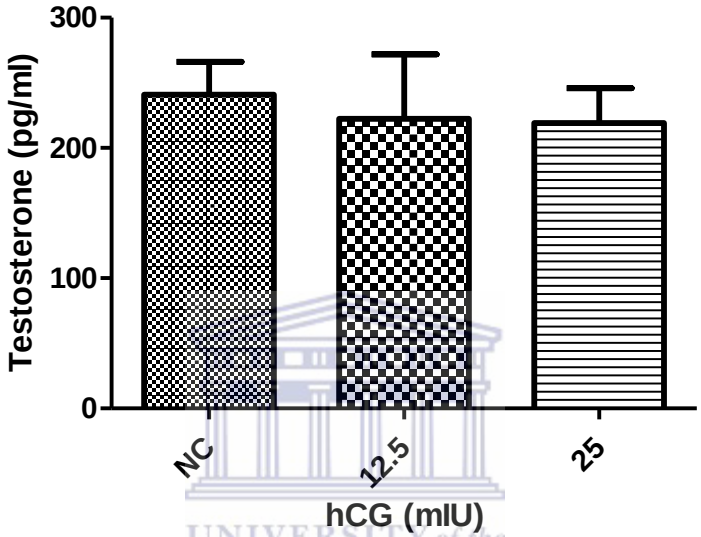


Figure 3.10: Testosterone production in TM3 cells after exposure to various concentrations of hCG for 24 hours using un-supplemented media. Values represent mean ± SD, n=4. Abbreviations: NC-negative control (media and cells only).

3.2.3 Testosterone production in TM3 Leydig cells using media containing charcoal stripped sera

TM3 Leydig cells were exposed to media containing charcoal stripped sera and 12.5mIU or 25mIU hCG for 48 hours. Figure 3.11 depicts a slight concentration dependent increase in testosterone production. However, this increase was not significant compared to the negative control (unstimulated Leydig cells). It can be deduced that all these TM3 cell batches used in this study were unresponsive to the hCG stimulus. Therefore, further investigation on the effect of EGCG and aspalathin on testosterone synthesis was avoided, as the results obtained would be inconclusive.

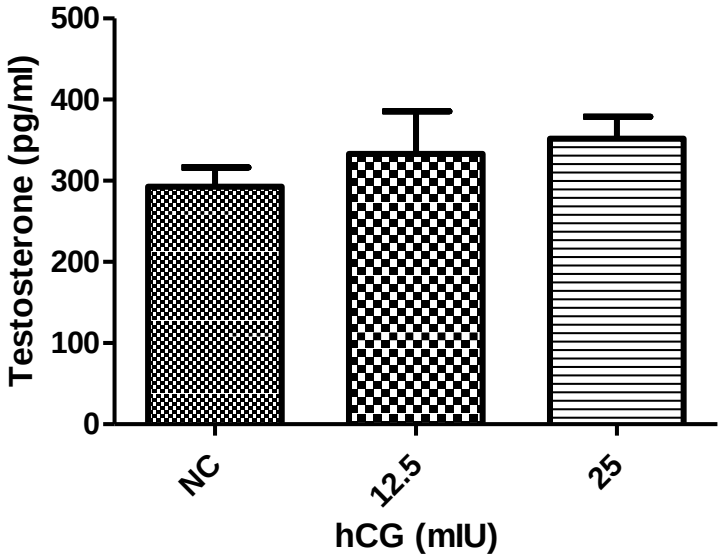


Figure 3.11: Testosterone production in TM3 cells after exposure to various concentrations of hCG for 24 hours using media containing charcoal stripped sera. Values represent mean ± SD, n=4. Abbreviations: NC-negative control (media and cells only).

3.3 Apoptosis and Necrosis

Annexin V is a flurochrome that detects apoptotic cells by binding to the external phosphatidylserine on the cell surface whereas PI binds to the nucleic acids of cells with damaged membranes. Hence, the Annexin/PI assay is suitable for quantifying apoptotic and necrotic cell death. The results below indicate the percentage of apoptotic and necrotic TM3 Leydig cells after incubation with EGCG or aspalathin for 24 hours as determined by an Annexin/PI assay using flow cytometry.

When compared to the negative control (1.4%), EGCG at 5µM (1.3%) and aspalathin at 5µM (1.7%) showed minimal differences in the quantity of apoptotic cells. While EGCG at 100µM (2.7%) and aspalathin at 100µM (3.3%) showed a non-significant increase in comparison to the negative control (Figure 3.12 and Figure 3.13).

The 6% DMSO positive control (61.6%) resulted in a highly significant increase of apoptotic cells when compared to the negative control. A previous study conducted on rat organotypic cultures showed that 6% DMSO induces apoptosis after 24 hours (Qi *et al*., 2008).

The results of the present study indicate that 5µM and 100µM EGCG have no effect on inducing necrosis when compared to the negative control (Figure 3.14 and Figure 3.15). In contrast, there is a non-significant reduction in necrotic cell number at 5µM (6.9%) and 100µM (12.8%) EGCG in respect to the negative control (15.9%). TM3 Leydig cells treated with 5µM (15.6%) and 100µM (13.7%) aspalathin showed minimal variability in the quantity of necrotic cells with respect to the negative control (15.9%).

The positive control of 2mM H_2O_2 lead to a significant increase in necrotic cells (47.2%) in relation to the negative control. A study conducted on U-937 human promonocytic cells proved that 2mM H_2O_2 successfully induced necrosis (Troyano *et al*., 2003). In preliminary studies, the TM3 Leydig cells were treated with 2mM H_2O_2 for 30 minutes. However, these cells maintained their morphology and minimal necrosis was detected. Therefore, the 2mM for 1 hour was adopted and resulted in the significant increase of necrotic cells.

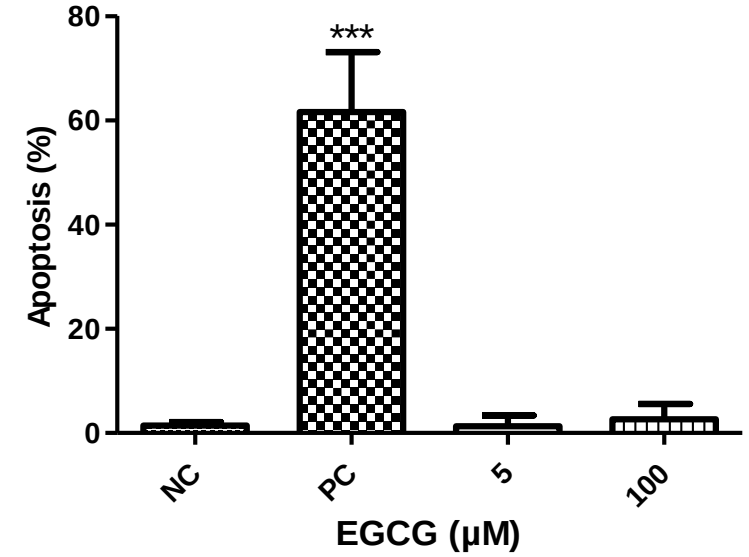


Figure 3.12: Quantification of apoptotic cells after 24 hour exposure to EGCG. Values represent mean ± SD, n=6. Abbreviations: NC-negative control (0.5% DMSO), PC-positive control (6% DMSO); *** p<0.001, compared to the negative control.

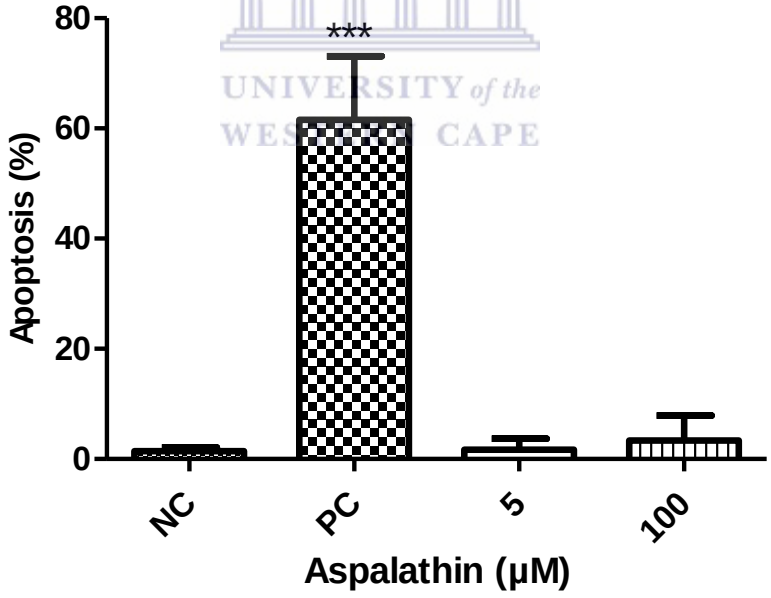


Figure 3.13: Quantification of apoptotic cells after 24 hour exposure to aspalathin. Values represent mean ± SD, n=6. Abbreviations: NC-negative control (0.5% DMSO), PC-positive control (6% DMSO); *** p<0.001, compared to the negative control.

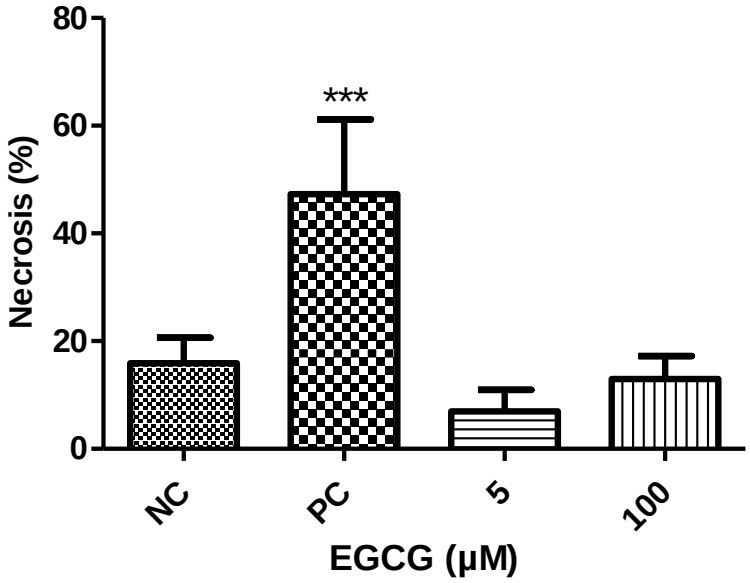


Figure 3.14: Quantification of necrotic cells after 24 hour exposure to EGCG. Values represent mean ± SD, n=6. Abbreviations: NC-negative control (0.5% DMSO), PC-positive control (1 hour exposure to 2mM H_2O_2); *** p<0.001, compared to the negative control.

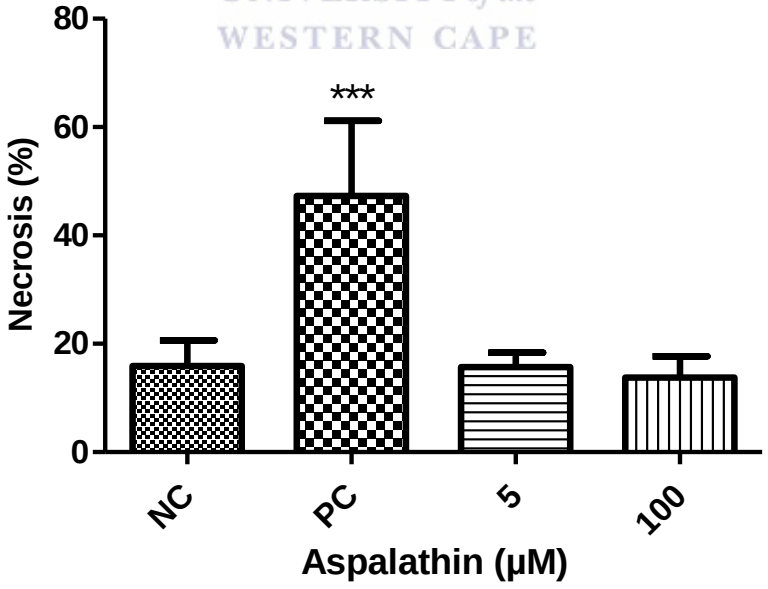


Figure 3.15: Quantification of necrotic cells after 24 hour exposure to aspalathin. Values represent mean ± SD, n=6. Abbreviations: NC-negative control (0.5% DMSO), PC-positive control (1 hour exposure to 2mM H_2O_2); *** p<0.001, compared to the negative control.

3.4 Evaluation of mitochondrial depolarization

3.4.1 Evaluation of mitochondrial depolarization in TM3 Leydig cells by flow cytometry analysis

TMRE is a red-orange fluorophore that penetrates cells with actively respiring mitochondria. A loss in mitochondrial membrane potential leads to a decrease in fluorescence intensity. In order to assess potential changes in the mitochondrial membrane potential, TM3 Leydig cells were treated for 24 hours with 5µM and 100µM EGCG or aspalathin. Thereafter, cells were treated for 30 minutes with TMRE and analysed by flow cytometry and fluorescence microscopy.

The results in figure 3.16 showed a significant increase in mitochondrial depolarization at 5µM ($p<0.05$**)** and 100µM ($p<0.05$**)** when compared to the negative control. However, there is no clear difference in mitochondrial depolarization between 5µM and 100µM EGCG. The figure 3.17 indicates that cells treated with aspalathin showed a non-significant increase in depolarization at 5µM and 100µM when compared to the negative control. The positive control of 1% DMSO showed a significant increase in mitochondrial depolarization in both groups. A study conducted on mouse astrocytes confirmed that 1% DMSO induces mitochondrial depolarization after 24 hours (Yuan *et al*., 2014).

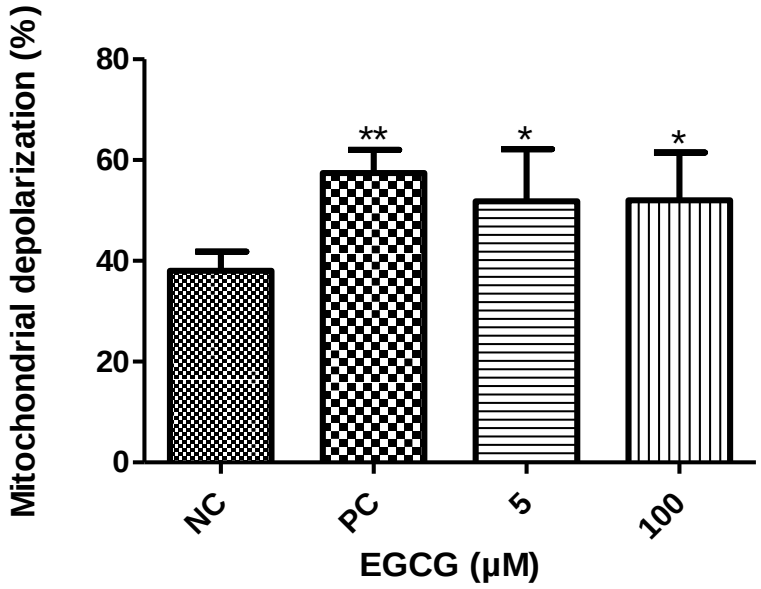


Figure 3.16: Evaluation of mitochondrial depolarization of TM3 Leydig cells exposed to EGCG for 24 hours. Values represent mean ± SD, n=6. Abbreviations: NC-negative control (0.5% DMSO), PC-positive control (1% DMSO); * p<0.05, ** p<0.01, compared to the negative control.

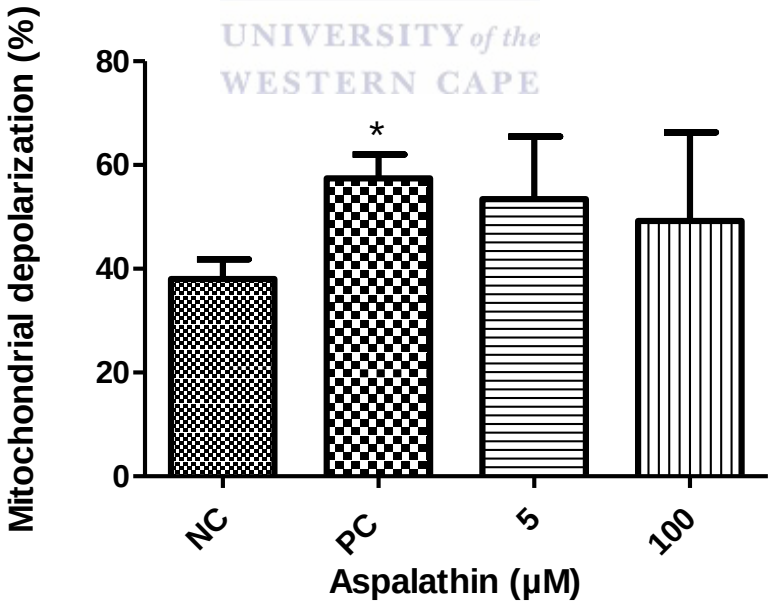


Figure 3.17: Evaluation of mitochondrial depolarization of TM3 Leydig cells exposed to aspalathin for 24 hours. Values represent mean ± SD, n=6. Abbreviations: NC-negative control (0.5% DMSO), PC-positive control (1% DMSO); * p<0.05, compared to the negative control.

3.4.2 Evaluating mitochondrial depolarization in TM3 Leydig cells after incubation with EGCG for 24 hours using fluorescence microscopy

The TMRE fluorophore results in bright red-orange fluorescence in cells with functional mitochondria. Whereas a loss in the intensity of the red-orange fluorescence indicates depolarization of the mitochondria.

The fluorescence micrographs depicting TM3 Leydig cells after exposure to EGCG. The negative control (A) in figure 3.18 and figure 3.19 showed a bright red fluorescence, which indicates healthy and functional mitochondria. The intensity of the red fluorescence diminished drastically and cell clumping occurred within the positive control (B), this indicates a loss in the mitochondrial membrane potential and mitochondrial damage. At both magnifications, 5µM EGCG (C) showed no visible difference to the negative control, whereas at 100µM (D), a clear loss in fluorescence intensity was observed.

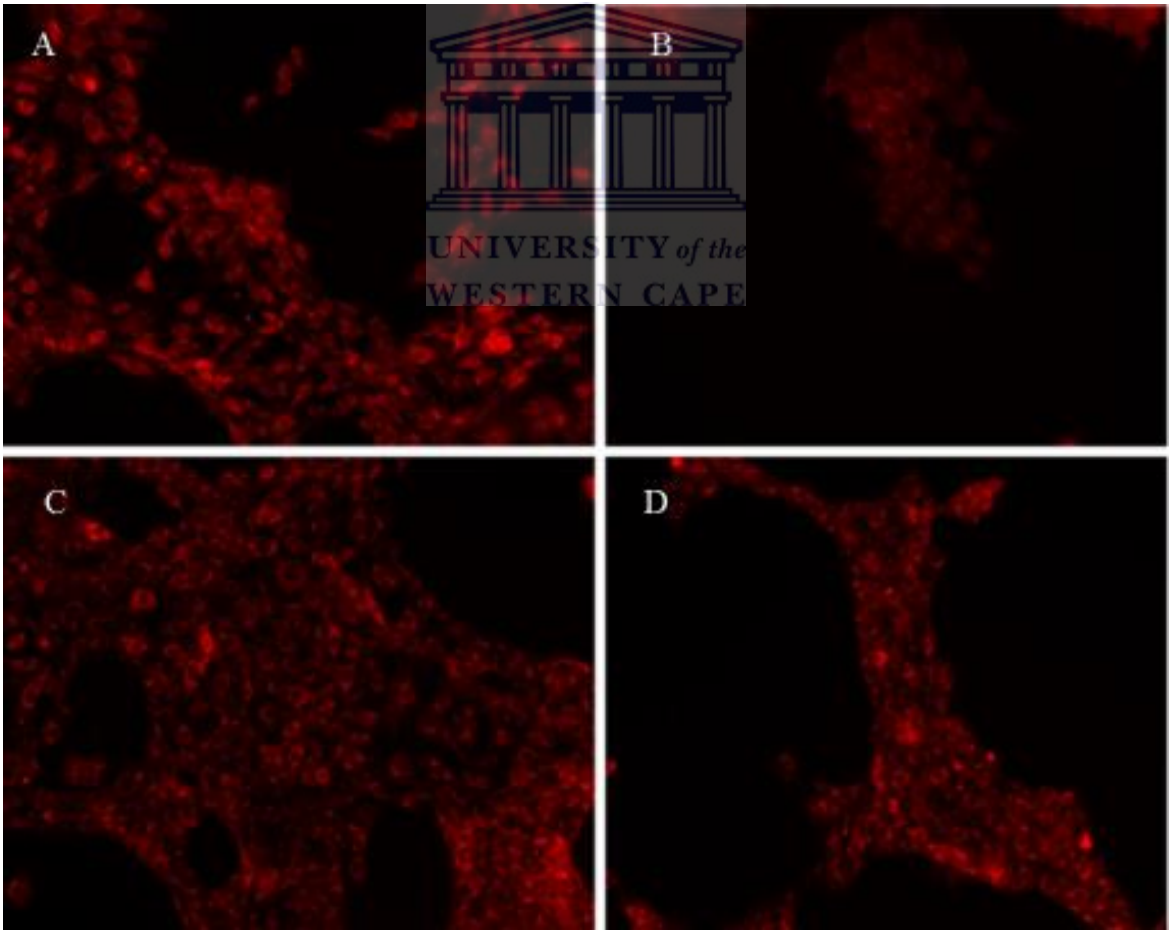


Figure 3.18: Mitochondrial membrane potential (Δψm) of TM3 cells exposed to EGCG for 24 hours using fluorescence microscopy. (A) NC-negative control (0.5% DMSO) (B) PC-positive control (1% DMSO) (C) 5µM (D) 100µM. Magnification: 100X.

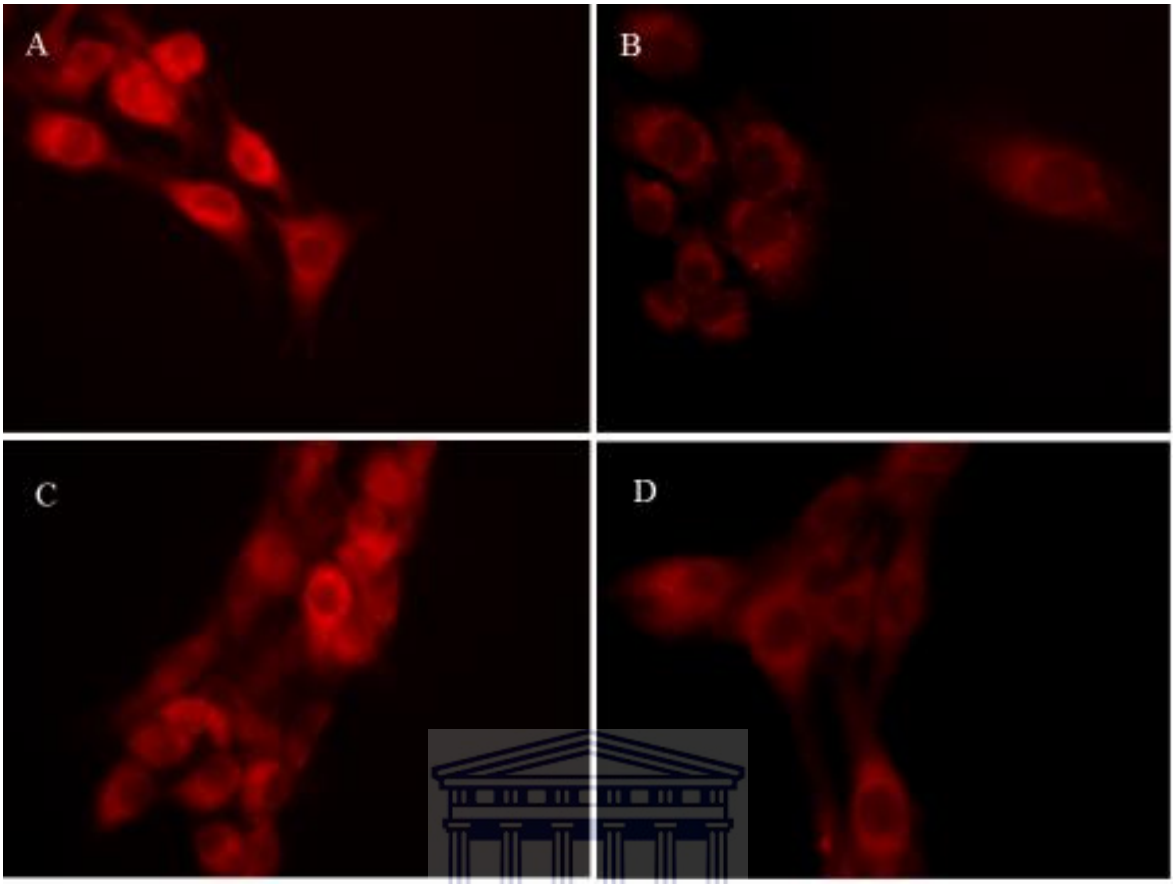


Figure 3.19: Mitochondrial membrane potential (Δψm) of TM3 cells exposed to EGCG for 24 hours using fluorescence microscopy. (A) NC-negative control (0.5% DMSO) (B) PC-positive control (1% DMSO) (C) 5µM (D) 100µM. Magnification: 400X.

3.4.3 Evaluating mitochondrial depolarization in TM3 Leydig cells after incubation with aspalathin for 24 hours using fluorescence microscopy

The fluorescent micrographs depicting TM3 Leydig cells after exposure to aspalathin (Figure 3.20 and Figure 3.21). The negative control (A) shows bright red fluorescence, which indicate healthy and functional mitochondria. The intensity of the red fluorescence diminished drastically and cell clumping occurred within the positive control (B), indicative of loss of membrane potential and mitochondrial damage. At both magnifications, 5µM (C) aspalathin showed no visible difference to the negative control, whereas at 100µM (D), a clear loss in fluorescence intensity was observed.

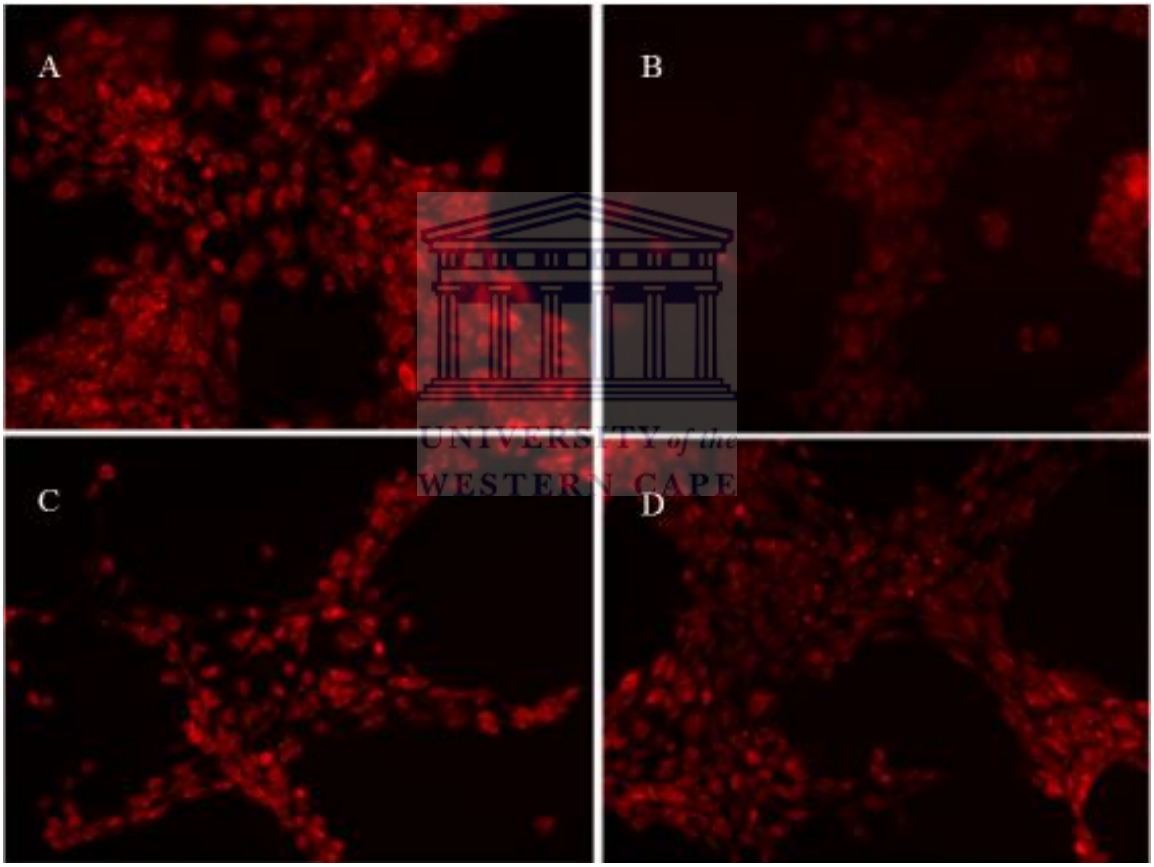


Figure 3.20: Mitochondrial membrane potential (Δψm) of TM3 cells exposed to aspalathin for 24 hours using fluorescence microscopy. (A) NC-negative control (0.5% DMSO) (B) PC-positive control (1% DMSO) (C) 5µM (D) 100µM. Magnification: 100X.

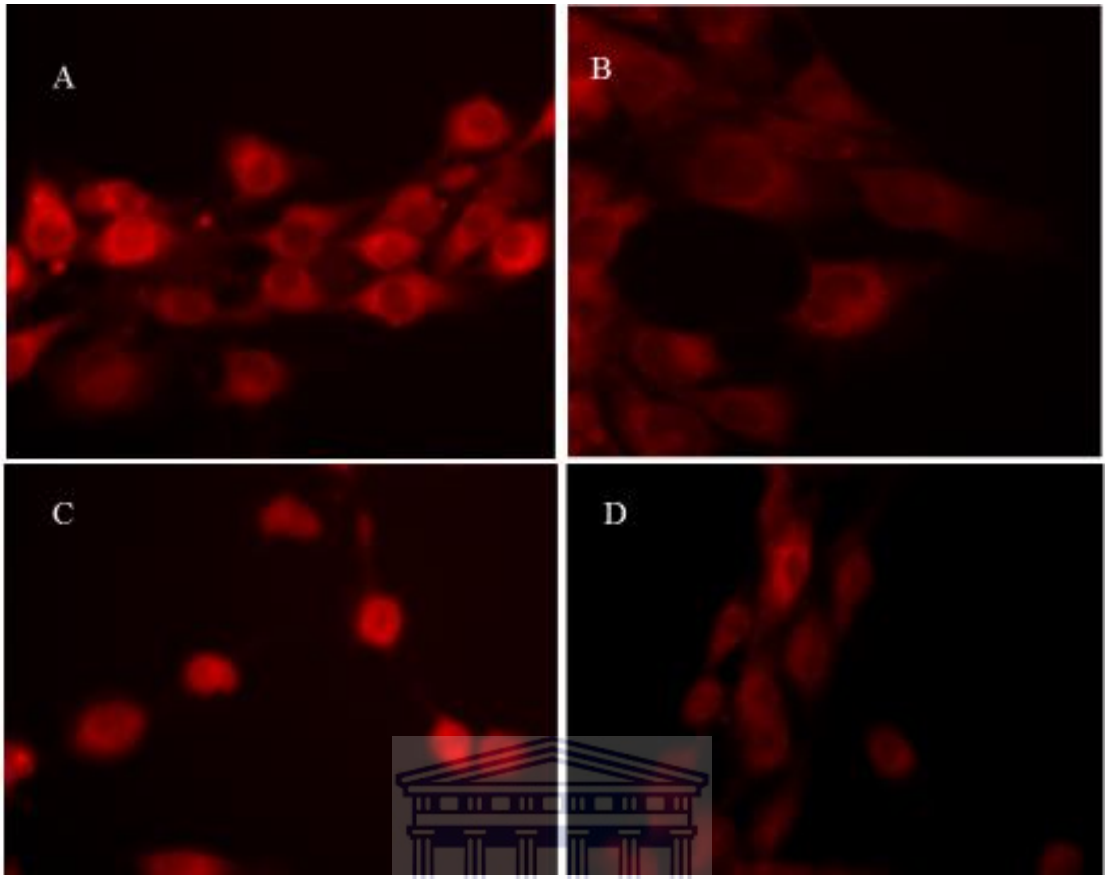


Figure 3.21: Mitochondrial membrane potential (Δψm) of TM3 cells exposed to aspalathin for 24 hours using fluorescence microscopy. (A) NC-negative control (0.5% DMSO) (B) PC-positive control (1% DMSO) (C) 5µM (D) 100µM. Magnification: 400X.

Chapter Four: Discussion

Polyphenols are dietary antioxidants that are commonly found in fruit, vegetables wine, coffee and tea. These natural compounds are of particular interest within the scientific community due to their role in cancers, diabetes, cardiovascular and neurodegenerative diseases.

Flavonoids are a subclass of polyphenols that has been implicated in affecting the physiology of various cell lines. EGCG is classified into the cathechin clan of flavonoids while aspalathin is classified into the chalcone (dihydrochalcone) clan. They possess pro-oxidant and mutagenic abilities that may result in cytotoxicity (Schloms and Swart, 2014). These flavonoids are referred to as phytoestrogens due to their chemical structure that share similar characteristics to 17-β-estradiol (Ly *et al*., 2014). Hence, they have the ability to bind to membrane and estrogen receptors thereby promote estrogenic activity. This may have implications in various hormone related cancers and may alter reproductive functions (Ly *et al*., 2014; Schloms and Swart, 2014).

4.1 Cell viability

In this study, MTT viability assay was undertaken to assess the cytotoxic effect of EGCG and aspalathin on basal and hCG stimulated TM3 Leydig cells.

Mitochondrial dehydrogenase activity is an indicator for measuring viability of cells. An increase in the mitochondrial dehydrogenase activity may indicate cellular stress (Monsees *et al*., 2000).

Morphological studies of basal and hCG-stimulated TM3 Leydig cells showed normal, adherent and flat polygonal shaped cells. The cell numbers in each micrograph at the various concentrations are similar to the cell numbers observed in the negative control. Furthermore, EGCG and aspalathin had no effect on the morphology of TM3 Leydig cells. It may be deduced that EGCG and aspalathin are not cytotoxic to TM3 Leydig cells after 24 hour incubation but may induce cellular stress.

In the basal control group (Figure 3.5), EGCG showed no increase in mitochondrial dehydrogenase activity when compared to the negative control. However, a significant reduction in enzyme activity occurred at 0.1µM, 50µM and 100µM. The hCG-stimulated group showed a significant reduction at 0.1µM (Figure 3.6). The overall lowered cell viability was

indicated by the reduced activity of mitochondrial dehydrogenase. The cells survived and may have adapted to EGCG as no characteristic signs of cell death was observed.

In the basal group, aspalathin led to an elevation in mitochondrial dehydrogenase activity at 100µM in respect to the negative control. In addition, the stimulated group showed the increase in enzyme activity at 100µM (Figure 3.7 and Figure 3.8). The increase in mitochondrial dehydrogenase activity above the negative control may indicate cellular stress.

EGCG and aspalathin were reconstituted in DMSO; however, the final DMSO concentration did not exceed 0.5%. In order to control for the DMSO, a vehicle control was included in the study and the final DMSO concentration had no cytotoxic effect. This was to ensure that the results depicted are a true reflection of the effect of the flavonoids (EGCG or aspalathin) and not that of the DMSO.

The results indicate that neither EGCG or aspalathin, within the tested concentration range, have a cytotoxic effect on basal and hCG-stimulated TM3 Leydig cells. However, these polyphenolic compounds have the potential to induce cellular stress after 24 hours.

4.2 Testosterone Production

Testosterone has a vital function in the growth and development of male sex organs. Leydig cells are responsible for the synthesis and secretion of testosterone under the regulation of LH (Hwang *et al*., 2011).

In previous research conducted by Opuwari and Monsees, (2015) they showed that TM3 Leydig cells possess the ability to produce elevated levels of testosterone when stimulated with hCG after 24 hours. In addition, Edjenguele *et al*., (2014) proved that after 24 hours TM3 cells produce elevated levels of testosterone compared to the basal control (without hCG). In another study conducted on TM3 Leydig cells, Chen *et al*., (2015) showed that gene expression of P450 scc (side-chain cleavage), 3β-HSD, 17β-HSD and P450-17α enzymes increased upon stimulation with hCG for 24 hours. These enzymes have a vital role in the steroidogenic pathway and ultimately testosterone production.

In this thesis, TM3 Leydig cells were exposed to varying concentrations of hCG (12, 24, 36, 48mIU) for 24 hours (Figure 3.9). There was no significant difference between the hCG stimulated cells and the control. This indicates that these particular cells, undertaken for the research study, are unresponsive to hCG stimulation after 24 hours. In figure 3.10 and figure 3.11 no significance was found between the hCG concentrations and the negative control. Different approaches were adapted to confirm the preceding results. Various concentrations of hCG stimuli were tested on the TM3 Leydig cells and the media was adapted in order to establish a suitable protocol. Furthermore, different batches of TM3 Leydig cells were obtained from another research laboratory group at the University of the Western Cape. However, the results obtained were proven to be similar and further confirmed the findings. Therefore, the effect of EGCG and aspalathin on testosterone levels could not be investigated.

The unresponsive nature of these cells are a limitation of this study, however, previous research has been conducted on the role of these flavonoids on reproductive organs, steroidogenic enzymes and testosterone production. Figueiroa *et al*., (2009) reported that EGCG may reduce testes, accessory reproductive organs and result in the reduction of LH and testosterone in the rat male reproductive system. They concluded that EGCG reduced testosterone production in basal and stimulated rat Leydig cells *in vitro*. The rationale for the effect of EGCG on testosterone production may be linked to the gallate group present within the chemical structure, enabling EGCG to elicit an inhibitory effect on testosterone synthesis. Furthermore,

these authors reported that EGCG may influence PKA/PKC signalling pathways and have a direct or indirect inhibitory action on P450 scc (side-chain cleavage) and 17β-HSD.

Schloms and Swart, (2014) investigated the effect of rooibos flavonoids (dihydrochalcones and flavones) on adrenal steroidogenic enzymes. They observed that structural differences of these flavonoids play a vital role in regulating the process on steroid biosynthesis. These factors include the quantity and position of hydroxyl groups within the chemical structure, structural flexibility and number of glucose moieties. Although these flavonoids have structural differences, it was deduced that they alter and inhibit the activity of key enzymes involved in adrenal steroidogenesis.

The rationale for the loss of functionality in TM3 Leydig cells steroidogenesis has not been well documented in literature. The immortalized Leydig cells used for the purpose of this study are derived from epithelial tissue of 11-13 day old mice and are classified as pre-pubertal (immature). Furthermore, a plethora of literature using primary cell culture report that old (21-24 months) Leydig cells in Brown Norway rats lose their steroidogenic ability over time when compared to young (4-6 months) Leydig cells. These functional changes of Leydig cells may be through a defective signal transduction pathway and/or a reduction in the activity of steroidogenic enzymes (Chen *et al*., 2002; Culty *et al*., 2002).

The defect in the signal transduction pathway may be related to the LH and cAMP association. Chen *et al*., (2002) reported that aged Leydig cells may have a defective LH-cAMP signal, which reduces their sensitivity to LH stimulation. The inability of Leydig cells to produce cAMP results in a cascade of events that includes the reduction of the StAR protein and steroidogenic enzymes (Chen *et al*., 2002).

In a study conducted on Brown Norway rats showed that cholesterol transport to the mitochondria and mRNA expression of the PBR protein significantly decreased in old (24 months old) Leydig cells when compared to young (4 months old) Leydig cells. Therefore, any alterations in cholesterol transport and PBR protein may contribute to testosterone decreases observed in old Leydig cells (Culty *et al*., 2002). In addition, Leers-Sucheta *et al*., (1999) proved that old (23-24 months old) Male Sprague Dawley rat Leydig cells showed reduced expression of the StAR protein, therefore, the reduction in testosterone synthesis may be linked to reduced StAR protein expression.

Passage numbers have been reported to influence the cells characteristics, particularly their morphology and response to stimuli. Literature has shown that physiologically, cell lines of a lower passage differ significantly to the higher passage. In a study conducted on Caco-2 cells, a significant difference was observed in the biological characteristics and transport abilities of these cells as passage numbers increased (Yu *et al*., 1997). Opuwari and Monsees, (2015) reported that TM3 Leydig cells may lose their ability to synthesize testosterone. Therefore, passage number may affect the steroidogenic potential of TM3 Leydig cells.

Recently, Engeli *et al*., (2018) reported that under basal conditions TM3, MA-10 and BLTK1 Leydig cells are unable or synthesize only low quantities of testosterone. In the stimulated groups, these cells produced a small quantity of testosterone coupled with large quantities of progesterone. The rationale for the low levels of testosterone was linked to minimal expression of 17β-HSD enzyme. This enzyme has a vital role in catalyzing the final conversion of androstenedione into testosterone. Hence, these three murine Leydig cell lines are inappropriate models for investigating testosterone synthesis disruption. Furthermore, these findings confirm and support the results obtained in this study.

There are commercially available Leydig cell lines that also fail to produce sufficient quantities of testosterone. The only viable alternative would be to use primary (animal testes) Leydig cell culture to ensure a large yield of testosterone. However, primary culture requires ethical clearance, cell isolation is a time consuming process and expensive, as the procedure requires enzymes and hormones to purify cells. In addition, animal testis could be acquired from an abattoir but the testes may not be readily available as these abattoirs only slaughter animals once a week and testes sample may be prone to degradation (transport and storage).

4.3 Apoptosis and Necrosis

Apoptosis is the process of cell death associated with morphological and biochemical changes within the cell. These changes are related to cell shrinkage, nuclear alterations, the translocation of phosphatidylserine to the outer membrane and chromatin condensation. Cells undergoing apoptosis are engulfed by phagocytes or neighbouring cells to prevent cell lysis and this ensures that no inflammation occurs (Leist *et al*., 1997).

Necrosis refers to morphological characteristics associated with premature membrane rupture, cell lysis, dilation of organelles such as endoplasmic reticulum and mitochondria (Nikoletopoulou *et al*., 2013). Cells exposed to extreme environmental conditions or genetic changes may be subjected to undergo necrosis (Syntichaki and Tavernaraki, 2002). The process of necrosis occurs independent of ATP production and results in a loss of mitochondrial function (Karch and Molkentin, 2015). Fink and Cooksen, (2005) reported the necrotic process as cells that have undergone cell death. The necrotic cells are subjected to a macropinocytotic mechanism whereby the phagocytes only engulf particular areas of the cell and not the entire cell as in apoptosis. Furthermore, cellular components are released during necrotic cell lysis, thereby alerting the innate immune system to induce the inflammatory response (Golstein and Kroemer, 2006).

The results of this study show that TM3 Leydig cells exposed to 5µM and 100µM EGCG (Figure 3.12) for 24 hours resulted in few apoptotic deaths when compared to the negative control. In addition, 5µM and 100µM aspalathin elicited the same effect on TM3 Leydig cells with minimal apoptotic cells observed (Figure 3.13). Therefore, it may be deduced that these polyphenolic compounds did not induce significant apoptotic death in TM3 Leydig cells at the respective concentrations.

In a study conducted on lymphoma cells, DMSO concentrations between 1-2% prevented apoptosis while increased concentrations resulted in apoptosis (Lin *et al*., 1995). Research conducted on rat cochlear organotypic cultures, indicated that a 6% DMSO concentration led to apoptotic cell death after 24 hours. DMSO induces an apoptotic response by resulting in a decrease of Bcl-2 protein, decreased mitochondrial membrane potential, allows the release of cytochrome C and initiates caspase 9 and caspase 3 (Qi *et al*., 2008).

The results in figure 3.14 indicate similar percentages of necrotic cells between the negative control and 100µM EGCG, with a slight reduction in necrotic cells at 5µM EGCG, when compared to the negative control. Evidently, similar results are observed in figure 3.15, where TM3 Leydig cells treated with aspalathin show minimal differences in necrotic percentages when compared to the negative control. The results indicate that EGCG and aspalathin are not inducers of necrosis at 5µM and 100µM, after 24 hours.

The effect of these polyphenolic compounds, particularly EGCG, on apoptosis differ in cancerous and non-cancerous cell lines. In non-cancerous cell lines, EGCG was reported to have a protective effect against exposure to toxins, hypoxia and irradiation (Kim *et al*., 2015). However, Lu *et al*., (2013) reported that EGCG results in DNA damage and apoptosis in non-cancerous (normal) human lung and skin cells. They also established that EGCG promotes the mutation frequency within these cells. This indicates that EGCG may possess pleiotrophic characteristics and the effect of EGCG may depend on the particular cell line of interest (Hagen *et al*., 2013).

However, a plethora of literature reports the significance of EGCG in various cancerous cell lines with respect to apoptosis. In a study conducted on five gastric cell lines, Onoda *et al*., (2011) reported that EGCG induced apoptotic cell death by inhibiting survivin. In human colorectal cancer, EGCG was shown to significantly promote cell cycle arrest in the G1 phase and result in apoptosis (Du *et al*., 2012). Furthermore, Hagen *et al*., (2013) reported that EGCG induces apoptosis in the PC3 Prostate cancer cell line and modifies caspase 9, thereby favouring pro-apoptotic isoforms.

The distinct difference in non-cancerous and cancerous cell lines may be linked to the Human telomerase reverse transcriptase (hTERT). The expression of hTERT occurs in more than 90% of cancers and are absent in non-cancerous cell lines. Min *et al*., (2012) reported that EGCG results in down-regulation of hTERT and increased ROS production in cancerous cells while not inducing the same effect in non-cancerous cells.

Furthermore, EGCG and aspalathin led to minimal apoptotic and necrotic cell deaths. It can be deduced that these polyphenolic compounds are unable to induce apoptosis and necrosis in TM3 Leydig cells at the concentrations of 5µM and 100µM after 24 hours.

4.4 Evaluation of mitochondrial depolarization

Mitochondria are complex double membrane organelles that are associated with bioenergetics, cell signalling, apoptosis and necrosis (Lum and Nagley, 2003; Krohn *et al*., 1999). Their main functional role involves synthesizing adenosine triphosphate (ATP), calcium ion homeostasis, fatty acid synthesis and the production of haem and iron-sulphur proteins (Tait and Green, 2012).

Mitochondria are the site for oxidative phosphorylation, which occurs via the electron transport chain (ETC) to produce ATP from adenosine monophosphate (ADP) and inorganic phosphate (Althoff *et al*., 2011). ETC influences redox state, ROS production, mitochondrial membrane potential, cell signalling and apoptosis (Birsoy *et al*., 2015). The ETC is composed of four protein Complexes (I-IV) that allow the transfer of electrons from donor molecules (Birsoy *et al*., 2015). As these electrons move through Complex I-IV, they produce energy that permit the transfer of hydrogen protons from the mitochondrial matrix into the intermembrane space (Birsoy *et al*., 2015; Perry *et al*., 2011). This constant flow of protons results in the formation of the electrochemical gradient. In addition, these protons flow back into the mitochondrial matrix via ATP-generating ATPase (Complex V) and produce ATP. Mitochondrial membrane potential and the mitochondrial pH gradient are considered the force that propels these protons into the mitochondria (Perry *et al*., 2011).

Alterations in the mitochondrial membrane potential may be linked to the mitochondrial permeability transition pore (PTP). The PTP is a channel across the inner mitochondrial membrane that, once opened, leads to depolarization of the mitochondria (Lum and Nagley, 2002). The PTP consist of peripheral benzodiazepine (PBR), voltage-dependent anion channel (VDAC), adenine nucleotide translocator (ANT) and cyclophilin D (CyD). The PTP allows the free passage of molecules and ions under 1.5 kDa. Furthermore, an increase in Ca^{2+} and pro-oxidants promote the opening of PTP (Krohn *et al*., 1999).

Voltage dependent anion channel (VDAC) is a protein located on the outer mitochondrial membrane (OMM). Functionally, the protein is responsible for binding pro and anti-apoptotic proteins from the Bcl-2 family and creates a contact site between the outer and inner mitochondrial membrane. VDAC regulates the transfer of metabolites (ADP, ATP) necessary for mitochondrial function across the outer mitochondrial membrane (OMM) into the intermembrane space. These metabolites are available to the inner membrane space to perform the necessary cellular functions (Robey and Hay, 2006).

VDAC interacts with an important enzyme, hexokinase, which has binding sites on the VDAC system. Further upstream, hexokinase is regulated by serine/threonine kinase Akt/PKB (protein kinase B). Hexokinase plays a vital role in glucose metabolism by phosphorylating glucose to produce glucose-6-phosphate. In addition, the enzyme regulates cell survival and apoptotic signalling by maintaining mitochondrial integrity and cytochrome C release, respectively. Hexokinases compete with Bak or Bax for binding sites on VDAC, thereby, preventing apoptosis by restricting the binding and aggregation of Bak or Bax to VDAC on the OMM. Disruption of hexokinase enables Bak or Bax to bind to the VDAC system and ultimately leads to impaired mitochondrial function, outer membrane permeabilization and cytochrome C release (Robey and Hay, 2006; Pastorino and Hoek, 2008).

Mitochondria have been implicated in the initiation and activation of apoptosis in various mammalian cell lines. The anti-apoptotic and pro-apoptotic Bcl-2 family of proteins are situated on the mitochondria whereby they are responsible for activating caspases. The release of cytochrome C from the mitochondria plays an active role in the formation of initiator caspase 9. Furthermore, mitochondrial proteins have a vital function in normal cell growth and not only involved in cellular demise (Wang and Youle, 2009).

The role of mitochondria in Leydig cells are of particular interest due to their crucial function in steroid biosynthesis. The rate-limiting step of steroidogenesis occurs within the mitochondria, whereby cholesterol is transferred from the outer to the inner mitochondrial membrane by action of StAR. This initiates the cascade of events that involves the conversion of cholesterol into pregnenolone by P450 scc (side-chain cleavage) and ultimately the synthesis of testosterone (Allen *et al*., 2006). In a study conducted on MA-10 Leydig cells, authors reported that any alteration in mitochondrial state, particularly the ETC, may affect the regulation of steroid biosynthesis (Midzak *et al*., 2007). Allen *et al*., (2006) reported that mitochondrial membrane potential, mitochondrial ATP production and mitochondrial pH have a crucial impact on the production of steroids. Furthermore, these authors deduced that mitochondria should be energized, polarized and actively respire to allow successful Leydig cell steroid biosynthesis (Allen *et al*., 2006; Haider, 2007; Midzak *et al*., 2007).

Jones *et al*., (2003) assessed the protective roles of simvastatin on mitochondrial membrane potential using TMRE by subjecting cardiac myocytes to oxidative stress. They reported a loss in mitochondrial membrane potential after exposing cardiac myocytes to H_20_2 and concluded

that simvastatin displays a protective role in these cells by preserving mitochondrial membrane potential after initiating oxidative stress.

Walsh *et al*., (2017) assessed the mitochondria of human breast cancer and human lung cancer by live cell imaging using TMRE. They exposed these cell lines to radiation with either carbon or protons and reported an instantaneous depolarization within the mitochondria.

The results of the present study, using TMRE, confirmed an increase in mitochondrial depolarization in TM3 Leydig cells after 24 hour exposure to 5µM ($p<0.05$**)** and 100µM ($p<0.05$**)** EGCG (Figure 3.16). The Leydig cells exposed to aspalathin for 24 hours showed a non-significant increase in depolarization at 5µM and at 100µM (Figure 3.17). The increase in mitochondrial depolarization may indicate that EGCG and aspalathin are producing excessive levels of intracellular ROS. These polyphenolic compounds are beneficial as an antioxidant, however, they possess the ability to undergo auto-oxidation that result in highly unstable biomolecules under cell culture conditions (Joubert *et al*., 2005; Valenti *et al*., 2013). This process enables these biomolecules to possess pro-oxidant properties, particularly H_2O_2, that produce elevated levels of intracellular ROS. Hence, high EGCG and aspalathin concentrations may adversely affect ETC components of mitochondria and result in mitochondrial depolarization.

In Figures 3.18-3.21, live cell imaging indicates no visible change in fluorescent intensity at 5µM EGCG (C) and 5µM aspalathin (C), respectively. However, at 100µM EGCG (D) and 100µM aspalathin (D) diminished red fluorescence is observed in relation to the negative control. Furthermore, a clear reduction in red/orange intensity of the 1% DMSO positive controls is observed (B) when compared to the negative controls (A).

The positive control of 1% DMSO after 24 hours resulted in significant depolarization of the mitochondria in respect to the negative control. Yuan *et al*., (2014) reported impaired mitochondrial integrity and mitochondrial membrane potential in cultured mouse astrocytes after exposure to 1% DMSO for 24 hours. DMSO has amphiphilic characteristics that increase the fluidity and thinning of the membrane, results in loss of phospholipid membrane integrity and the forms water pores in the membrane bilayer. Authors concluded that DMSO affects mitochondrial membrane potential due to their high membrane fluidity (Yuan *et al*., 2014).

Although EGCG and aspalathin affect the TM3 Leydig cell mitochondria, the results of this study indicate that mitochondrial depolarization may occur independent of apoptosis. A body

of literature reports the link between mitochondrial dysfunction and the initiation of the apoptotic pathway. However, Krohn *et al*., (1999) examined the mitochondrial membrane potential of rat neuronal cells during staurosporine induced apoptosis using TMRE. They concluded that mitochondrial depolarization could occur without initiating the apoptotic process. Furthermore, this may indicate that the VDAC-Hexokinase system on the OMM may be fully functional (absence of Bak or Bax) and the release of cytochrome C into the cytosol has not occurred after 24 hours. Hence, no apoptosis was detected even though mitochondrial depolarization occurred.

In addition, Ly *et al*., (2003) reported that changes in the mitochondrial membrane potential may not automatically lead to apoptosis. Authors concluded that any disturbance in mitochondrial membrane potential could lead to apoptosis; however, this ultimately depends on the particular cell system of interest and the apoptotic stimuli (Ly *et al*., 2003).

The MTT assay assesses the relative activity of mitochondrial dehydrogenase activity (mDHG), particularly succinate dehydrogenase. This enzyme is located on the inner mitochondrial membrane and an indicator for cellular viability (Green and Narayana, 1980). The link between the TMRE and MTT assay both relate to the mitochondria. However, the TMRE assesses the integrity of the inner mitochondrial membrane while the MTT assay specifically related to mitochondrial enzymatic activity within the inner mitochondrial membrane. At the tested concentrations, EGCG was shown to produce an overall lowered mDHG activity while aspalathin resulted in an increase in mDHG. The MTT data indicate that after 24 hours EGCG may have elicited a response within the mitochondria of these cells coupled with cellular stress when treated with aspalathin for 24 hours. For both flavonoids, a loss in the mitochondrial membrane potential was detected, with EGCG resulting in significant depolarization. This indicates that mDHG activity may be an indicator for detecting inner mitochondrial membrane depolarization.

The present study shows that EGCG and aspalathin leads to mitochondrial depolarization without the initiation of apoptosis. Therefore, TM3 cells exposed to 5µM and 100µM EGCG and aspalathin, respectively, may not necessarily lead to cellular demise. However, these flavonoids may disrupt the ETC and negatively impact ATP production.

Chapter Five: Conclusion

In this research study, the *in vitro* effects of EGCG and aspalathin were investigated on TM3 Leydig cell physiology.

In summary, EGCG had no significant effect on cell viability whereas aspalathin increased mitochondrial dehydrogenase activity at the higher concentrations. Therefore, both EGCG and aspalathin were proven to have no cytotoxic effect, within the tested concentration range. However, they possess the ability to induce cellular stress at the highest concentration. The initial aim for determining the effect of EGCG and aspalathin on testosterone synthesis could not be established as the TM3 Leydig cell steroidogenesis model was defective. In contrast to previous reports in literature but also data published within this particular research group, the current populations of TM3 Leydig cells used in this study failed to produce detectable amounts of testosterone. Even stimulation with various concentrations of hCG failed to elevate testosterone levels. This phenomenon was also reported in literature and hints to a defect in steroidogenesis that can develop in Leydig cell culture models.

Although EGCG and apalathin possess pro-oxidant properties, they fail to induce significant apoptotic and necrotic cell deaths at the tested concentrations. The results were further confirmed when evaluating the effect of these active components on mitochondrial membrane potential. The results indicate that both EGCG and aspalathin result in a loss of membrane potential and favours depolarization. However, the depolarization was proven to be independent of apoptosis and necrosis. Given the time interval and concentrations used in this study, it may be deduced that EGCG and aspalathin lead to cellular stress without essentially leading to cellular demise.

Future recommendations:

Determine the appropriate cell line model for conducting studies on steroid hormone interruption.

Increase the amount of time intervals and a higher concentration to accurately determine the cytotoxic effect of these flavonoids on Leydig cell physiology.

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