

The Effect of *Cissampelos capensis* Extract on Prostate Cancer, Sertoli and Leydig Cell Function

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Declaration

I declare that *The effect of Cissampelos capensis extract on prostate cancer and Sertoli and Leydig cell function* is my own work, that has not been submitted before for any degree or examination in any other university, and that all sources I have used or quoted have been indicated and acknowledged as complete references.

Keenau Mark Pearce

December 2014

Signed:.....



Dedications

This thesis is dedicated to my parents. My father and mother have been examples of what hard work, dedication and passion can achieve, and have taught me to always reach for my goals. They have taught me to be hardworking, understanding, patient, and to never quit on my dreams and aspirations. I would also like to dedicate this thesis to my brother, who has been a unmatched source of inspiration and motivation in perusing my academic goals.



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Key words

Cissampelos capensis (*C. capensis*)

Cytotoxicity

Viability

DNA fragmentation

Testosterone

Doxorubicin

Reactive oxygen species (ROS)

Reactive nitrogen species (RNS)

LNCaP (Prostate cancer cells)

TM3 (Leydig cells)

TM4 (Sertoli cells)



Abstract

Introduction

Cissampelos capensis (*C. capensis*) is a widely used medicinal plant in South Africa, used to treat ailments such as backache, gallstones and stomach aches. *C. capensis* is a rich source of aporphine, morphinane and bisbenzyltetrahydroisoquinoline, which have multiple effects, including inhibition of reactive oxygen species (ROS) and reactive nitrogen species (RNS) production, cytotoxicity towards human lung cancer (A549) and human leukemia (HL-60), and inhibition of tumour metastasis. Additionally, *C. capensis* may be implicated in male reproductive problems, such as poor fertility or prostate problems.

Material and methods

This study investigated the effect of the *C. capensis* extract (0.001, 0.01, 0.1, 1, 10, 100 1000 µg/ml) on LNCaP prostate cancer, TM3 Leydig and TM4 Sertoli cells for 24 and 96 hours. The following parameters were investigated: morphology, cell viability (XTT), testosterone modulation, DNA fragmentation (TUNEL), lactate dehydrogenase activity (LDH), testosterone production, anti-cancer drug combination. In a separate set of experiments, parameters affecting the initiation, progression and metastasis of cancer were investigated. These included the ability of the aqueous *C. capensis* rhizome extract to inhibit of reactive oxygen species (ROS) and reactive nitrogen species (RNS) production, and to inhibit collagenase and elastase activity.

Results

Over 24 hours, LNCaP cells showed dose-dependent increases in cell viability at low concentrations, while decreasing significantly ($P=0.0046$) at the highest concentration (1000 µg/ml). Over 96 hours, LNCaP viability was significantly ($P=0.002$ and $P<0.0001$) decreased at

higher concentrations. However, a biologically significant decrease was only observed at the highest extract concentrations. Moreover, the extract produces a more desirable cytotoxicity toward LNCaP cells when combined with doxorubicin, and when combined with testosterone. Also, the extract produced significant ($P<0.0001$) dose-dependent increases in LNCaP DNA fragmentation over 24 hours, and significant ($P<0.0001$) fragmentation at higher concentrations over 96 hours. The extract was not found to cause any biologically significant increase, or decreases, in TM3 testosterone production. TM4 cells showed a significant ($P<0.0001$) decrease in viability over 24 and 96 hours. Furthermore, the extract produced a significant ($P=0.0273$) increase in LDH release at 1000 $\mu\text{g/ml}$, over 24 hours. Over 96 hours, a significant ($P=0.0002$) increase in LDH release was observed at 100 $\mu\text{g/ml}$, along with the absence of detectable LDH at 1000 $\mu\text{g/ml}$. Additionally, the extract significantly ($P<0.0001$) inhibited RNS production in a dose dependent manner, while only showing exceptional ROS inhibition at higher extract concentration. Also, the extract significantly ($P=0.0001$) inhibited collagenase, while causing no biologically important change in elastase activity.

Conclusion

In summary, the collected data show the extract produced a non-specific cytotoxic effect at high concentrations, had no biologically important effect on elastase activity, and only inhibited collagenase effectively at high extract concentrations. Thus, it can be concluded that the extract is not well suited to treating, and preventing the metastasis of prostate cancer. However, the extract effectively inhibited ROS and RNS production, and thus has potential as a natural antioxidant. However, due to the observed toxicity at high concentrations, isolation of the phytochemicals attributed with antioxidant capabilities is of the utmost importance.

Chapter 1

Introduction

1.1 Factors affecting cancer

1.1.1 Reactive oxygen species

Redox reactions in a biological system refer to those reactions that produce oxidants and antioxidants. The delicate balance between oxidation and reduction, termed redox homeostasis, must be maintained in order to maintain normal bodily function and optimal health (Valko et al. 2007; Pham-Huy et al. 2008). At low concentrations, oxidants, such as reactive oxygen species (ROS) and reactive nitrogen species (RNS), play important roles in maintaining optimal health (Valko et al. 2007; Pham-Huy et al. 2008).

Functionally, low levels of ROS and RNS are necessary for maturation processes of cellular structures and act as defence mechanism against pathogens, such as macrophages, neutrophils and monocytes which release free radicals to destroy invading microbes (Valko et al. 2007; Pham-Huy et al. 2008). Additionally, ROS and RNS play important roles in a number of cellular signalling systems (Valko et al. 2007; Pham-Huy et al. 2008), such as in non-phagocytic cells including fibroblasts, endothelial cells, vascular smooth muscle cells, cardiac myocytes, and thyroid tissue (Valko et al. 2007; Pham-Huy et al. 2008). Nitric oxide, for example, acts as an intercellular messenger for modulating blood flow, thrombosis and neural activity (Pham-Huy et al. 2008). Additionally, Nitric oxide is also important in non-specific host defence, and killing intracellular pathogens and tumours. Another beneficial effect, of free radical molecules exhibited one or more free electrons and functioning as oxidants, is the induction of a mutagenic response (Pham-Huy et al. 2008). It is when levels of oxidants increase beyond antioxidant capacity, that unfavourable consequences, such as oxidative stress, will ensue

(Weiskopf 2011). Thus, antioxidants are vital in maintaining redox homeostasis (Balcerczyk et al. 2014).

Free radicals, characteristically possessing one or more unpaired electron, have a high degree of oxidant capacity (Weiskopf 2011). They can be derived from oxygen, called ROS, or derived from nitric oxide, called RNS. The most biologically important ROS are the superoxide anion, hydroxyl radicals and hydrogen peroxide (Silva et al. 2010). Among RNS are nitric oxide and peroxynitrite (Weiskopf 2011; Kavazis and Powers 2013).

Superoxide is generated as an intermediate in many reactions, such as incomplete reduction of water during oxidative phosphorylation (Silva et al. 2010; Weiskopf 2011; Kavazis and Powers 2013). Once formed, superoxide cannot readily cross cell membranes, is short-lived and is local in its effects. However, superoxide can dismutate, spontaneously or catalysed by superoxide dismutase, to form hydrogen peroxide (Pham-Huy et al. 2008; Kavazis and Powers 2013).

Hydrogen peroxide is a considered more stable form of reactive oxygen species (ROS) in that it is a considered weak oxidizing agent (Kavazis and Powers 2013). In contrast to superoxide, hydrogen peroxide is longer-lasting and is membrane-diffusible (Pham-Huy et al. 2008). At high concentration, due to its ability to generate hydroxyl radicals, hydrogen peroxide can be cytotoxic (Pham-Huy et al. 2008; Kavazis and Powers 2013). Hydroxyl radicals possess a strong oxidizing potential and are highly reactive, thus making them the most dangerous form of ROS (Silva et al. 2010; Weiskopf 2011).

Nitric oxide is the main reactive nitrogen species (RNS) (Editors et al. 2002) and is synthesized readily from the amino acid L-arginine, that occurs through nitric oxide synthase, converting L-arginine into nitric oxide and L-citrulline (Weiskopf 2011; Kavazis and Powers 2013). By itself, nitric oxide is a weak reducing agent but can react with oxygen to form nitric dioxide or hydrogen peroxide to form the highly reactive peroxynitrite (Kavazis and Powers 2013),

which in turn is a strong reducing agent capable of inducing DNA damage and depletion of thiol proteins and nitration proteins (Weiskopf 2011; Kavazis and Powers 2013).

Tumour initiation, promotion and progression form the basis of the current theory of carcinogenesis (Figure 1). Tumour initiation begins in cells when DNA becomes altered, leading to cells replicating at a rate significantly faster than normal (Kavazis and Powers 2013; He et al. 2007; Weiskopf 2011). The tumour promotion stage is associated with extreme proliferation, tissue remodelling and inflammation. Subsequently the tumour progression stage is characterized by the emergence of invasive tumours, as well as further DNA damage, along with gene expression alterations (Pham-Huy et al. 2008; Weiskopf 2011; Kavazis and Powers 2013).

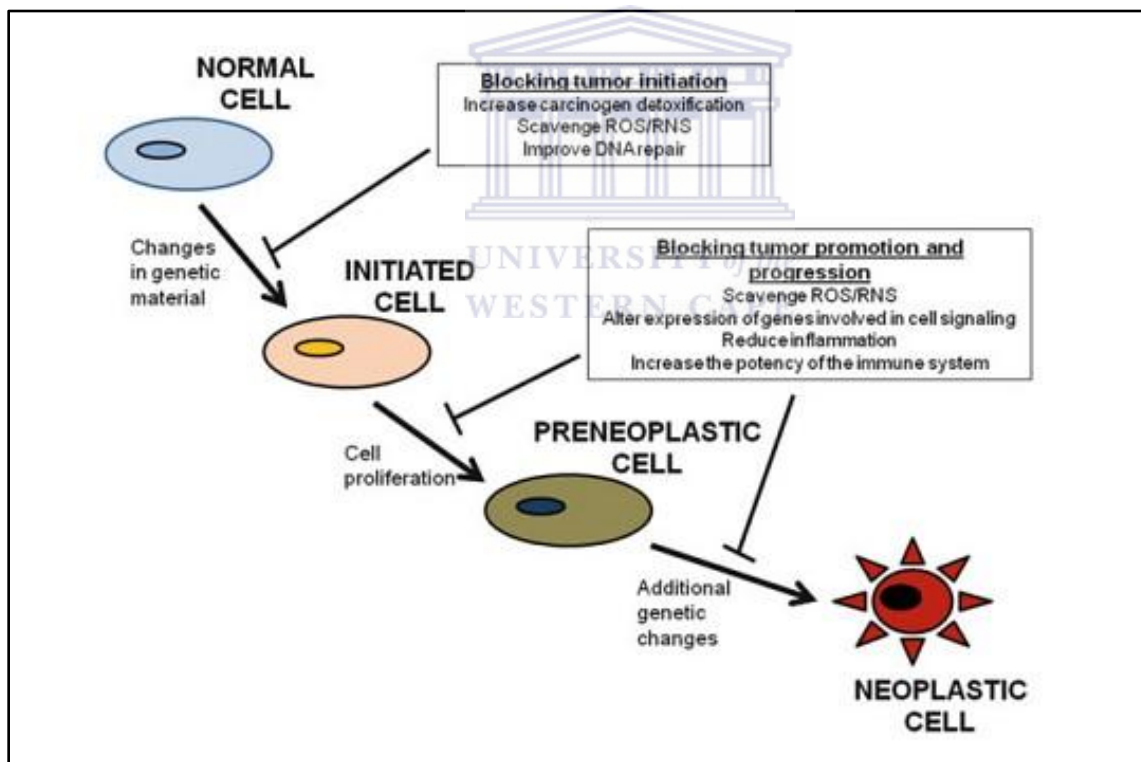


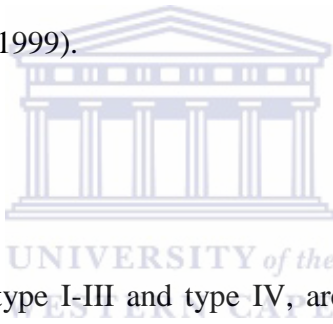
Figure 1: Schematic depiction of the accepted theory of cancer cell formation and possible mechanisms of its prevention (Kavazis and Powers 2013).

Oxidative damage to DNA can occur due to the interaction of DNA and ROS/RNS, in which free radicals, such as hydroxyl radical, react with DNA by additions to bases or abstractions of hydrogen atoms (Devasagayam et al. 2004). In particular, free radicals cause DNA strand breaks, damage to deoxyribose sugar and DNA protein cross links (Devasagayam et al. 2004). Additionally, lipid peroxidation is another example of ROS/RNS induced DNA damage, in which membrane lipids are reacted with free radicals (Devasagayam et al. 2004), which results in decreases in membrane fluidity, changes in phase properties of the membranes, and also causing cross-linking of membrane components, thereby restricting membrane protein mobility (Devasagayam et al. 2004). During the process of lipid peroxidation, free radical attacks result in hydrogen atom abstraction from a methylene group, which leaves an unpaired electron on the carbon atom (Devasagayam et al. 2004). Hereafter, this carbon atom is stabilized by a molecular rearrangement to produce conjugated diene, which in turn can then react with oxygen to produce a lipid peroxy radical (Devasagayam et al. 2004; Barrera 2012). These radicals then further abstract hydrogen atoms from other lipids to form lipid hydroperoxides, while simultaneously further propagating lipid peroxidation (Devasagayam et al. 2004).

Additionally, by-products produced during lipid peroxidation, such as 4-hydroxynonenal (Barrera 2012), have been shown to react with low-molecular weight compounds, such as proteins and DNA (Barrera 2012), eventually causing DNA damage. Nonetheless, since ROS/RNS can directly, and indirectly, damage DNA, which in turn can lead to mutations ultimately resulting in cancer formation (Weiskopf 2011; Barrera 2012; Kavazis and Powers 2013), maintaining the balance of ROS/RNS and antioxidants is essential to create optimal health conditions.

1.1.2 Metalloproteinases

Metalloproteinases (MMPs) are a group of zinc-dependent endopeptidases that are able to degrade almost all components of the extracellular matrix (Polette et al. 2004). During different stages of development, they are expressed in physiological or pathological processes that require cell migration and tissue remodelling (Saarialho-Kere et al. 1992). Physiologically, these processes include tissue morphogenesis, uterine cycle and tissue repair (Saarialho-Kere et al. 1992; Polette et al. 2004). Pathologically, they are involved with rheumatoid arthritis, disorders of the skin, osteoporosis, tumour invasion and metastasis (Saarialho-Kere et al. 1992; Polette et al. 2004). MMPs can be subdivided into different groups, namely collagenases, stromalysins, and gelatinases (Nagase and Woessner 1999).



1.1.3 Collagenases and elastase

Many fibrillar collagens, such as type I-III and type IV, are readily degraded by collagenases (MMP-1, MMP-8, MMP-13) (Polette et al. 2004). Physiologically, MMP-1 is expressed in situations such as embryonic development and tissue repair (Polette et al. 2004). Pathologically, it is expressed in conditions such as chronic cutaneous ulcers and tumors (Saarialho-Kere 1998; Polette et al. 2004).

Polymorphonuclear leukocytes maturing in bone marrow synthesize MMP-8 (Polette et al. 2004; Quintero et al. 2010), which is stored in intracellular granules and released in response to extracellular stimuli (Polette et al. 2004). MMP-8 is also found in articular cartilage chondrocytes and mononuclear fibroblast-like cells in rheumatoid synovia (Quintero et al. 2010). During fetal bone development, postnatal bone remodelling and gingival wound repair, MMP-13 is expressed (Polette et al. 2004). It is also involved in inflammatory conditions such as

osteoarthritis, rheumatoid arthritis, and invasive malignant tumors such as breast and squamous cell carcinoma (Polette et al. 2004).

Elastase (MMP-12) is constantly expressed by macrophages, and is able to degrade extracellular matrix components such as elastin. Additionally, elastase has been shown by to be commonly expressed by macrophages in response to atherosclerotic lesions, abdominal aortic aneurysms and interstitial ulcerations (Polette et al. 2004). Additionally, MMP-12 activity has been linked to squamous cell carcinomas, where macrophages found in tissues that were devoid of normal elastic fibers or with disrupted basement membrane express MMP-12 (Polette et al. 2004).

1.1.5 MMPs and metastasis

When malignant cells detach from the primary tumour, move through stromal tissue, enter the circulation and invade the target organ, metastasis results (Polette et al. 2004). MMP expression and metastatic potential of malignant tumours have been positively correlated in a number of studies. MMP-1 expression was found to be linked to colorectal and oesophageal cancer (Shima et al. 1992), while MMP-2 and MMP-3 was associated with lymph node metastasis and vascular invasion of squamous cell carcinomas of the oesophagus (Peng et al. 2012). Similarly, MMP-13 was associated with metastasis capacity of squamous cell carcinomas of the head, neck and vulva (Culhaci et al. 2004). Local invasion of squamous cell carcinoma of the head and neck was associated with MMP-11 expression (Li et al. 2012). Thus, preventing over expression of MMPs is crucial in the prevention of cancer metastasis.

1.2 Overview of the Male reproductive system

Characteristically, the male reproductive system is comprised of the testes, epididymis, vas deferens, ejaculatory ducts, and penis, that have been termed the major organs of the male reproductive system (Basu 2011). Alongside the major organs are accessory glands, namely the prostate, seminal vesicles, and bulbourethral glands (Basu 2011). These organs and accessory glands make up the basic structures of the male reproductive system (Figure 2) (Basu 2011). Together, the major organs and accessory glands work to produce semen, deposit semen in the female reproductive tract, and to achieve the ultimate goal of fertilization.

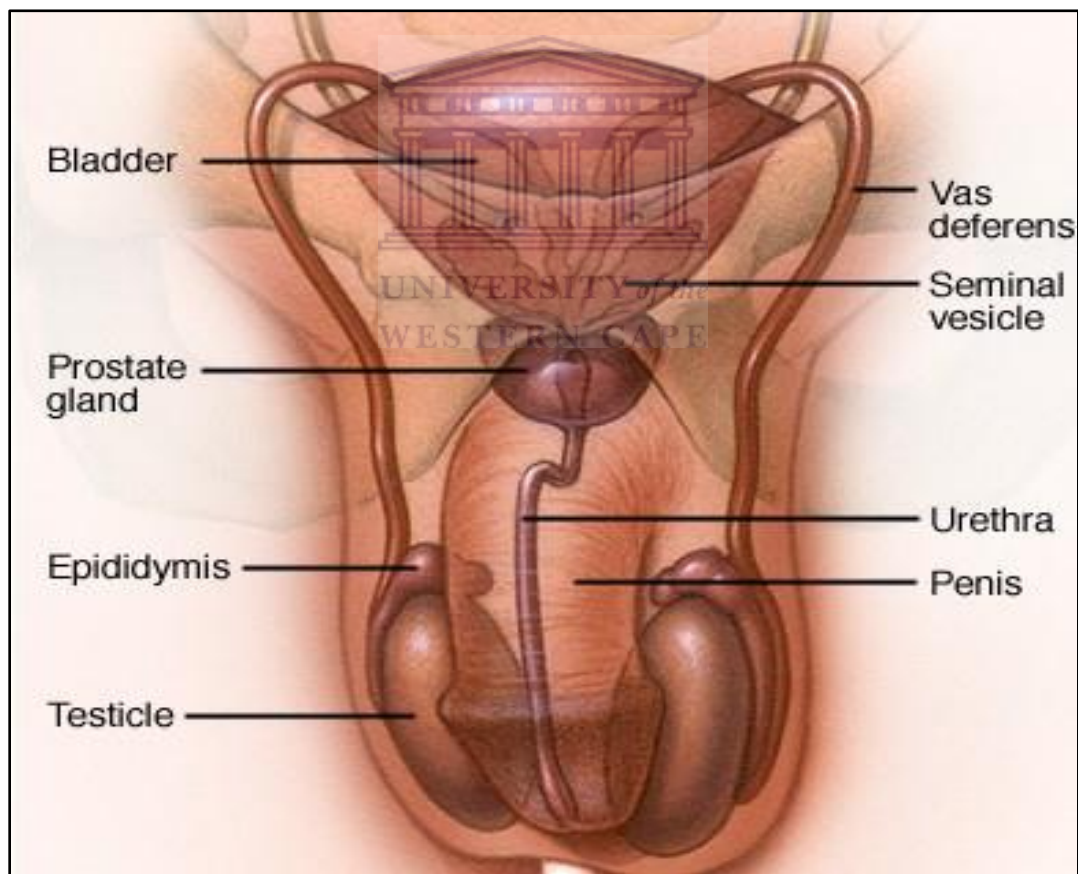


Figure 2: Overview of the male reproductive system (<http://www.mayoclinic.org/male-reproductive-system/img-20008048>)

1.2.2 The Testes

Located in the scrotum, the oval shaped testes, which are the considered primary sex glands, are suspended by the spermatic cord (Basu 2011). Testes are positioned obliquely in the scrotum with the upper end directed forwardly, while the lower end is directed posteriorly and medially. Structurally, the testes consist of the convex anterior border and the flat posterior borders. The anterior border lateral surfaces, or the upper and lower ends, are invested by the visceral layer of the tunica vaginalis; the posterior border receives only partial investment.

Different covering are found in the testes, namely the tunica vaginalis, tunica albuginea and the tunica vasculosa (Basu 2011). Described as a serous covering, the tunica vaginalis is derived from the sac of the peritoneum that precedes the descent of the testes. Next, the tunica albuginea provides the dense fibrous covering of the testes, consisting of bundles of white fibrous tissue (Basu 2011). Finally, the tunica vasculosa consists of a plexus of blood vessels held together by areolar tissue; it covers the inner surface of the tunica albuginea and the different septa in the interior of the gland (Basu 2011).

1.2.2.1 Cell types of the testis:

Histologically, the seminiferous tubules and interstitial tissues are the major structures. Germ cells and Sertoli cells constitute the seminiferous epithelium, while Leydig cells are located in the interstitial tissue, which occupies approximately one-fourth of the total testicular volume (Basu 2011).

1.2.2.2 Sertoli cells

Considered to act in a nurse-like manner, Sertoli cells serve a unique function in the development of germ cells (Johnson et al. 2008). Sertoli cells are a fixed population of cells that play an integral role in the regulation of male fertility (Basu 2011); they are providing structural support and nutrition to developing germ cells (Johnson et al. 2008); phagocytosis of degenerating germ cells and residual bodies (Johnson et al. 2008); release of spermatids at spermiation and production of proteins that regulate, or respond to pituitary hormone release, that influence mitotic activities of spermatogonia (Walker and Cheng 2005; Basu, 2011; Galardo et al., 2014).

Under the influence of follicle stimulating hormone, transport proteins that are secreted by Sertoli cells include transferrin, lactate, ceruloplasmin and Androgen Binding Protein (APB) (Johnson et al. 2008); they are charged with the task of transporting Fe^{3+} , Cu^{2+} and androgens, such as testosterone and dihydrotestosterone (DHT), to germinal cells (Griswold 1988; Walker and Cheng 2005). This is a crucial step in fuelling spermatogenesis (Galardo et al. 2014).

Lactate secretion (Figure 3) is essential to maintaining the acidic environment needed inside the seminiferous tubules and to meet the energy requirements of developing germ cells (Walker and Cheng 2005; Basu 2011). Production of lactate is achieved by Sertoli cell regulation of glucose metabolism, via glycolysis, to yield lactate and then pyruvate under the actions of lactate dehydrogenase (LDH) (Galardo et al. 2014). In part, the need for high concentrations of exogenous lactate, to serve as an energy source, has been shown to be due to the inability of germ to efficiently metabolise glucose (Bardin et al. 1993), along with low glucose concentrations being available for energy *in vivo* (Bardin et al. 1993). Additionally, it has previously been shown that accumulation of lactate inside Sertoli cells is an indicator of cell

toxicity, as this would indicate decreased LDH activity and hence diminished cell function (Senthil-kumar et al. 2004)

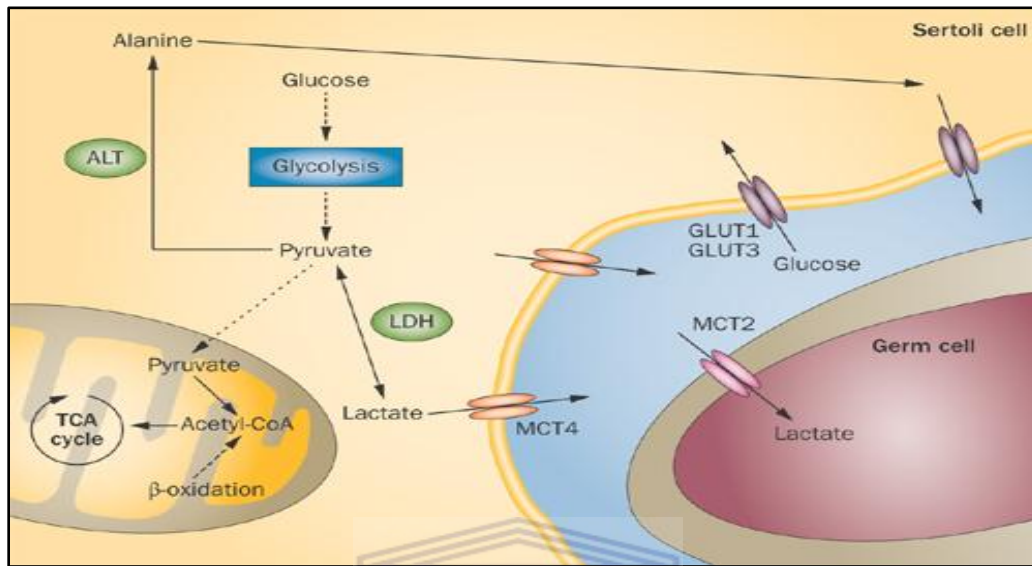


Figure 3: Sertoli cells metabolism. Abbreviations: ALT, alanine aminotransferase; GLUT, glucose transporter; LDH, lactate dehydrogenase; MCT, monocarboxylate transporter; TCA, tricarboxylic acid (Rato et al. 2012).

In addition, Sertoli cells have been shown to secrete inhibin, mullerian-inhibiting substance (MIS), somatomedin C, transforming growth factors alpha and beta, and stem cell growth factor; these proteins possess hormonal or growth-like activities (Griswold 1988; Bardin et al. 1993). Proteins with enzymatic activities, such as plasminogen activator, are also secreted by Sertoli cells. Another class of proteins secreted by Sertoli cells are those contributing to the basement membrane (Griswold 1988; Bardin et al. 1993), such as type IV collagen and laminin. A key feature in structural support, provided by the Sertoli cells, is the blood-testis barrier.

Residing between the tight junctions between adjacent Sertoli cells, this barrier separates spermatogonia and early spermatocytes within the basal compartment, permitting primary spermatocyte movement into the luminal compartment and spermatocyte and spermatid movement into the adluminal compartment (Bardin et al. 1993; Basu 2011). Functionally, this

barrier restricts the para-cellular movement of substances, such as water, electrolytes, ions, nutrients, hormones, paracrine factors, and biological molecules, across the Sertoli cell epithelium into the apical compartment (Chen and Mruk 2012). Additionally, this barrier regulates the movement of potentially harmful substances, such as drugs, environmental toxicants and chemicals, into the apical compartment (Chen and Mruk 2012), and in this manner creating a unique microenvironment for post-meiotic spermatid development (Chen and Mruk 2012; Basu 2011).

Specifically, the key feature of this unique environment is the creation of an immunologic barrier (Bardin et al. 1993), thereby isolating spermatocytes and spermatids from the immune system (Basu 2011). Without this unique separation, their antigens would be recognized as foreign and stimulate the production of anti-sperm antibodies, in turn leading to an autoimmune response and their ultimate destruction (Bardin et al. 1993; Basu 2011).

An autoimmune response of this nature can lead to male infertility (Bardin et al. 1993; Basu 2011). In addition to this barrier, Sertoli cells have been shown to secrete immunosuppressive molecules to block immune responses, to transiently expressed auto-antigens in developing germ cells, thereby working to maintain the immune-privileged nature of the testis (Chen and Mruk 2012).

Breaking down of this barrier can be the result of physical trauma, or harmful substances from the environment, such as heavy metals and chemicals like Bisphenol-A (Chen and Mruk 2012). Nevertheless, disruption of this barrier has been shown to hinder male fertility and disrupt protein expression, reduced semen quality, and has been linked to the occurrence of testicular cancer (Chen and Mruk 2012). Thus, the maintenance of this unique barrier is essential.

1.2.2.3 Leydig cells

Leydig cells, are found adjacent to the seminiferous tubules, and are the primary cells of the interstitium. They are characteristically rich in smooth endoplasmic reticulum and mitochondria (Weinbauer et al. 2010). Important cytoplasmic components of Leydig cells are lipofuscin granules, which are the final product of endocytosis and lysosomal degradation, along with lipid droplets, in which the preliminary stages of testosterone production take place (Weinbauer et al. 2010). Special formations, called Reinke's crystals, are often found in Leydig cells and are thought to be subunits of globular proteins (Weinbauer et al. 2010). Proliferation rates of Leydig cells in the testis are characteristically low and occur under the influence of luteinizing hormone (LH) (Weinbauer et al. 2010; Basu 2011). Under the influence of LH, Leydig cells are considered to be the central site of testosterone production (Figure 4), via the hypothalamic-pituitary-gonadal axis (HPG) axis (Ge et al. 2008; Weinbauer et al. 2010; Basu 2011). In this axis, secretions from the hypothalamus, namely gonadotropin-releasing hormone (GnRH), causes the anterior pituitary to produce LH and follicle stimulating hormone (FSH) (Weinbauer et al. 2010). LH then stimulates Leydig cells, in the testes, to produce testosterone, which in turn is creates a negative feedback loop in which testosterone inhibits the frequency and amplitude of hypothalamic and anterior pituitary secretions (Weinbauer et al. 2010).

Testosterone is crucial to maintaining fertility in men, as it drives spermatogenesis, and to maintain secondary male characteristics (Ge et al. 2008; Basu 2011). However, testosterone deficiencies are commonly associated with men over the age of 40 (Feldman et al. 2002; Wu et al. 2010). Termed late-onset hypogonadism (Wu et al. 2010), the age-associated decrease in testosterone is considered an indicator of natural male ageing, accounting for a decrease of about 1% each year (Feldman et al. 2002). Late-onset hypogonadism can occur as the result of primary

or secondary causes, or as a combination of the two, in men with long-term systemic diseases such as cirrhosis or chronic kidney disease (Wu et al. 2010). Primary hypogonadism is defined as the decline of testosterone production due to abnormalities in the testicles, as these may occur due to infection (Wu et al. 2010).

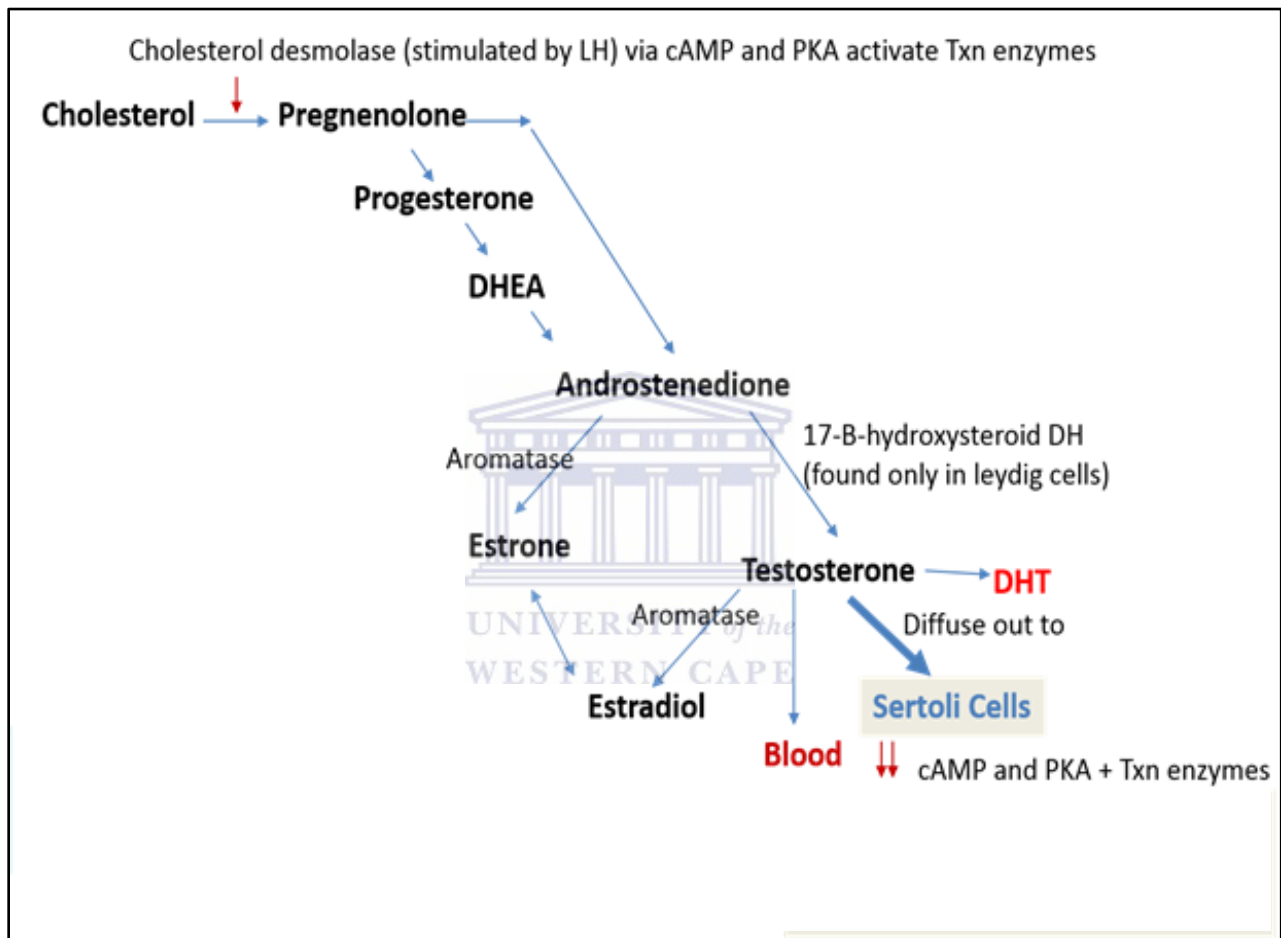


Figure 4: Diagrammatic representation of the process of testosterone synthesis in Leydig cells. Luteinizing hormone (LH) binds to receptors on Leydig cells, leading to activation of adenyly cyclase and thereby products of cAMP. In turn, this activates protein kinase-A, leading to transcriptional activation and thereby activation of enzymes such as cholesterol desmolase. Thereafter, cholesterol is converted to Pregnenolone and the resulting downstream products. (<http://www.pathologyusmle.com/male-and-female-reproductive-pathology-and-pathophysiology-for-usmle-step-1/>).

Often, primary hypogonadism presents with elevated levels of LH, due to the reduced negative feedback effect of testosterone (Wu et al. 2010). However, in the case of secondary hypogonadism, disorders stemming from the hypothalamic-pituitary axis, such as tumours, are quite often the root cause; subnormal levels of LH and testosterone is a characteristic of secondary hypogonadism. Nevertheless, late-onset hypogonadism is associated with deleterious consequences, including a decrease in lean muscle mass, a sharp increase in total body fat mass, and a decrease in muscle strength (Taher 2005; Snyder 2009). Another negative effect experienced by these men is a noticeable decrease in energy levels as well as a decrease in cognitive ability and memory (Taher 2005; Snyder 2009). Insomnia and depression were also reported to be the result of the decline in testosterone (Carnahan and Perry 2004). One of the major impacts of the decrease in testosterone is the collapse of sexual function, characterised by emergence of erectile dysfunction, defined as the inability to achieve or maintain penile erection, for satisfactory sexual performance, along with a decrease in libido in men (Taher 2005). For these reasons, protecting normal Leydig cell function, and combating late-onset hypogonadism, is of the utmost importance to improve the quality of life in these men.

Fortunately, testosterone therapy has been shown to be effective in treating those suffering from late-onset hypogonadism (Hohl et al. 2009; Snyder 2009; Morgentaler et al. 2011). Testosterone replacement therapy is aimed at raising testosterone levels to approximate natural, endogenous levels, with the ultimate goal of improving quality of life (Bassil et al. 2009; Ullah et al. 2014). Conventionally, several types of testosterone replacement therapy exist, namely tablets, injections, transdermal systems, pellets, and buccal preparations of testosterone (Bassil et al. 2009; Ullah et al. 2014). Sadly, side effects of conventional testosterone replacement therapy have been outlined to include an increased risk of prostate cancer, worsening of symptoms of benign prostatic hyperplasia, liver toxicity, worsening symptoms of sleep apnoea, congestive

heart failure, gynecomastia and skin disease (Bassil et al. 2009; Ullah et al. 2014). When these side effects are compared to the ultimate goal of testosterone replacement therapy, that is to improve quality of life (Bassil et al. 2009; Ullah et al. 2014), it is clear that safer alternatives are needed. In addition, many men are unable to accommodate the cost involved in conventional testosterone replacement therapy (Ullah et al. 2014). Thus, the need for cheaper, more accessible, and safer methods of testosterone replacement therapy is needed.

1.2.3 The prostate gland

During the third month of gestation, the prostate gland develops from epithelial invaginations from the posterior urogenital sinus under the influence of the underlying mesenchyme (Hammerich et al. 2008). Normal formation of the prostate requires the presence of dihydrotestosterone, synthesized from foetal testosterone by the action of 5α -reductase (Hammerich et al. 2008). 5α -reductase is an enzyme localised in the urogenital sinus and external genitalia, without which abnormal development of the prostate and external genitalia occur (Hammerich et al. 2008). Hereafter, during the pubertal period, the prostate remains relatively identical but begins to undergo morphological changes into the adult phenotype (Hammerich et al. 2008).

Described as having a walnut-like appearance, the adult prostate gland is triangular in shape, weighs around 20 grams in the average healthy adult male between 25-30 years of age (Hammerich et al. 2008) and is located in the abdominal cavity around the neck of the urinary bladder (Burden et al. 2006; Hammerich et al. 2008). The base of the prostate is located at the neck of the bladder, and the apex of the prostate is located at the urogenital diaphragm (Burden et al. 2006), thus before entering the penis, the urethra passes through the prostate. Along with

the attached seminal vesicles, the prostate gland is separated from the rectum by thin layer of connective tissue known as the Denovilliers fascia (Martínez-Piñeiro 2007).

Functionally, the prostate plays a role in controlling urine output from the bladder and seminal fluid during the process of ejaculation. In addition, the prostate contributes to secretions of the semen (Burden et al. 2006). Secretions from the prostate are a highly complex, heterogeneous mixture of organic and inorganic compounds, accounting for approximately 20-30% of the seminal volume (Grayhack et al. 2002).

Prostatic fluid has been described as clear and slightly acidic (Grayhack et al. 2002), and as the primary source of seminal fluid zinc, magnesium, calcium, and citrate, along with prostate specific antigen and prostatic acid phosphatase (Grayhack et al. 2002). Zinc exists in very high concentrations in the healthy prostate. Secreting concentrations ranging from 150-1000 mg/ml in prostatic fluid (Grayhack et al. 2002). Functionally, prostatic zinc is important for sperm motility, acrosome reaction, capacitation, and stabilizing cell membrane and nuclear chromatin of spermatozoa (Ali et al. 2007). Also, zinc may function to protect the testis against degeneration and act as an antimicrobial agent (Ali et al. 2007; Kelleher et al. 2011). Low zinc levels, which has been correlated with aging (Kelleher et al. 2011), has been associated with decreased fertility in men, as well as prostate disease such as benign prostatic hyperplasia and prostate cancer (Huang et al. 2006; Kelleher et al. 2011) Here, zinc was found to be markedly decreased in malignant prostate tissue, in comparison to normal prostate tissue (Costello and Franklin 2006; Huang et al. 2006; Kelleher et al. 2011). Additionally, zinc has been found to act as an aromatase inhibitor, preventing the production of oestrogens from androgens (Ellem and Risbridger 2006). This inhibition of aromatase by zinc protects the normal functioning of the androgen-dependent prostate, as increases in aromatase have been linked to increased malignancy of the prostate (Ellem and Risbridger 2006).

Citrate is a major component of prostatic fluid present accounting for approximately 376 mg/dl (Grayhack et al. 2002), and is arguably one of the most important anions present in semen (Owen and Katz 2005). Citrate has a high affinity for calcium, magnesium, and zinc (Owen and Katz 2005); and it is thought that it is responsible for the high calcium buffering capacity of semen (Owen and Katz 2005) by being the major regulator of ionized calcium levels in seminal plasma. Similar to zinc, citrate has also been implicated in prostate cancer and benign prostatic hyperplasia (Costello and Franklin 2006), where the citrate concentrations are markedly less than that of normal prostate secretions.

Other compounds found in prostatic fluid include the polyamines, spermine and spermidine (Grayhack et al. 2002), whose enzymatic degradation produces reactive aldehydes, which give semen its characteristic odour (Grayhack et al. 2002). In addition, these polyamines have been found to act as antimicrobials (Grayhack et al. 2002). Phospholipids are also found in prostatic secretions, accounting for 180 mg/dl, along with cholesterol at a concentration of 80 mg/ml (Grayhack et al. 2002). Prostatic acid phosphatase and prostate specific antigen are also found in prostatic fluid (Grayhack et al. 2002; White et al. 2009). They are glycoproteins secreted by prostate epithelial cells that have been used as markers in the characterization of prostate disease (Grayhack et al. 2002; White et al. 2009).

The growth, maintenance, and secretory function of the prostate are regulated by androgens (Burden et al. 2006; Walters et al. 2010; Steers 2011); castration has been shown to reduce prostatic size, prostatic secretion volume, muscarinic receptor expression, and noradrenergic innervation of the prostate (Steers 2011). The major androgen hormone responsible for regulation of the prostate physiology and growth is known as dihydrotestosterone (Burden et al. 2006; Walters et al. 2010; Steers 2011). Within the prostate, dihydrotestosterone concentrations is up to 5 times more than that of testosterone, and is synthesized from

testosterone mainly by the action of type-II 5 α -reductase (Steers 2011). Besides dihydrotestosterone, estrogens and adrenal steroids influence prostate function and growth. Here, testosterone can be converted to estradiol and estrone by aromatase (Steers 2011). Additionally, zinc metabolism, citrate and fructose production, and androgen uptake and metabolism, can be regulated by prolactin (Steers 2011). Oestrogens combined with the effect of prolactin are thought to play a role in the development of benign prostatic hyperplasia (Steers 2011).

1.2.3.1 Histology of the prostate

The prostate gland has a very high level of organization. Large peripheral ducts can be found in the glandular portion of the prostate, which have bi-layered epithelial acini and fibro-muscular stroma, separated from each other by a basement membrane (Burden et al. 2006). This epithelial bi-layer gives rise to three distinct cell types, namely basal cells, luminal cells and neuro-endocrine cells (Burden et al. 2006).

Phenotypically, the basal cells are classified as flattened, cuboidal cells found at the periphery of the gland (Burden et al. 2006; Walters et al. 2010). These cells are contractile, generate many growth factors needed for regulation of the gland and are regarded to act as stem cells that replenish the secretory cell layer (Burden et al. 2006; Walters et al. 2010).

Above the basal cell layer exists the neuro-endocrine cells (Burden et al. 2006; Walters et al. 2010). While not a particularly common cell type, they were found to exist among a vast number of secretory epithelial cells (Walters et al. 2010; Burden et al. 2006). The true function of these cells remains unclear. However, it is thought that they play a role in the regulation of growth and development in an endocrine-paracrine fashion, similar to that of neuro-endocrine cells in other organs (Burden et al. 2006; Walters et al. 2010). Production of prostatic secretions, as well

expression of androgen receptors, is present with the luminal cells. These cells, however, have no proliferative potential (Burden et al. 2006; Walters et al. 2010).

1.2.3.2 Anatomy and histology of the prostate

During the twentieth century, several investigators insisted that the prostate gland was composed of different lobes, despite the inability to observe distinct lobes with the naked eye (Grayhack et al. 2002; Hammerich et al. 2008). Subsequently, McNeal established the most widely accepted concept of various zones, rather than lobes of the prostate (McNeal 1981; Grayhack et al. 2002; Hammerich et al. 2008). In his studies, McNeal observed that the urethra separates the prostate into ventral (fibromuscular) and dorsal (glandular) portions (McNeal 1981). Midway, between the apex and base, the posterior wall of the urethra undergoes a 35-degree ventral angulation that separates the urethra into the proximal and distal segments (Grayhack et al. 2002; Hammerich et al. 2008). Hereafter, the verumontanum and ejaculatory duct exist exclusively in the distal segment (Grayhack et al. 2002; Hammerich et al. 2008). McNeal separated the glandular prostate into four distinct regions; namely the peripheral zone, central zone, and transitional zone (McNeal 1981; Grayhack et al. 2002; Hammerich et al. 2008).

The peripheral zone constitutes approximately 75% of the glandular prostate (Grayhack et al. 2002; Burden et al. 2006). Its ductal system enters the urethra along the posterolateral recesses of the urethra, extending from verumontanum distally to the prostate apex (Grayhack et al. 2002; Burden et al. 2006). This zone is a common site for the occurrence of prostate cancer. Wedge-shaped, the central zone, whose base is positioned superiorly at the bladder neck, occupies approximately 20% of the glandular prostate (Grayhack et al. 2002; Burden et al. 2006). Its ductal network closely trails the ejaculatory ducts to the urethra and then empties into the orifices of the ejaculatory ducts on the apex of the verumontanum

(Grayhack et al. 2002; Burden et al. 2006). Inflammation and carcinoma are very rarely observed in this zone (Hammerich et al. 2008).

Accounting for 4-5% of the adult glandular prostate, the transitional zone consists of two modest lobules of para-urethral tissue found anterior to the peripheral zone (Grayhack et al. 2002; Burden et al. 2006). Its ducts empty in the posterior lateral recess of the urethra, just proximal to the peripheral ducts. This zone has been described as another common site for carcinoma and benign prostatic hyperplasia (Grayhack et al. 2002; Burden et al. 2006).

Additionally, the transitional zone is found lateral to McNeal's pre-prostatic sphincter; a smooth muscle cylinder enveloping the proximal urethra from the bladder neck to the base of the verumontanum (McNeal 1981; Grayhack et al. 2002).

Furthermore, the capsule of the prostate is described as a condensation of stromal elements that envelops the underlying parenchyma, much in a uniform manner up until the apex (Grayhack et al. 2002; Hammerich et al. 2008). Intermittently, distinct septa stem from the capsular sheath and breach the interior portion of the gland, which separates it into lobules (Grayhack et al. 2002; Burden et al. 2006). Peripheral aspects of the capsule mainly consist of fibroblasts, collagen, and elastic fibres (Grayhack et al. 2002). Additionally, the prostatic stroma consists mostly of smooth muscle cells, and fibroblasts, which surrounds individual glands and is thought to play an important role in the release of glandular secretions (Grayhack et al. 2002). Furthermore, contractions of smooth muscle surrounding the bladder neck and pre-prostatic sphincter assists the excretion of secretions within the prostatic urethra (Grayhack et al. 2002; Burden et al. 2006). Moreover, the anterior and anterolateral portions of the prostate contain both smooth and skeletal muscle, which joins the fibres of the external sphincter, which contributes to urinary control in that area (Grayhack et al. 2002).

Prostatic excretory ducts are mostly lined by simple or pseudo-stratified columnar epithelium (Grayhack et al. 2002). Additionally, instead of having a main excretory duct, between 16-32 separate ducts with distinct urethral orifices exist (Grayhack et al. 2002). These ducts have been described as having an irregular branching pattern with many cystic outgrowths. Moreover, the ductal networks have been shown to possess both columnar and basal cells, however, without secretory capabilities (Grayhack et al. 2002; Burden et al. 2006). Acinar epithelium is made up of two well-organised cell types; namely the tall, columnar glandular secretory cells that are luminal in orientation, and the non-secretory basal cells that are flattened, cuboidal, and borders a distinct eosinophilic basement membrane (Grayhack et al. 2002). These cells are contractile and are thought to act as stem cells, repopulating the secretory cell layer (Grayhack et al. 2002; Burden et al. 2006). Predominantly, glandular secretory cells possess a number of histological features, such as rarely showing nucleoli and basal cytoplasm harbouring free ribosomes (Grayhack et al. 2002; Burden et al. 2006). Additionally, cells known as neuroendocrine cells are found above the basal layer, residing among the more numerous secretory cells (Rumpold et al. 2002). Being the least common cell type, they are not well understood. It is thought, however, that the neuroendocrine cells serve an endocrine-paracrine regulatory role in growth and development, much like neuroendocrine cells in other organs (Rumpold et al. 2002).

1.2.3.3 Age related changes in the prostate

The prostate gland has been shown to experience two growth spurts (Xia et al. 2002); a rapid growth phase between the ages of 10-30, and a slow growth phase between ages between 30-90 (Xia et al. 2002). Accompanying this growth of the prostate, is an increased rate at which testosterone is converted to dihydrotestosterone in the prostate; leading to a greater build of dihydrotestosterone and thereby an increase in cell growth (Grayhack et al. 2002). As the

prostate enlarges, men begin to experience a few characteristic symptoms, such as difficulty emptying the bladder, increased frequency of night time urination, incontinence and impotence (Grayhack et al. 2002). Additionally, a few diseases are associated with the increased growth of the prostate that occurs as men age; namely prostatitis, benign prostatic hyperplasia and prostate cancer (Grayhack et al. 2002).

1.2.3.4 Prostatitis

Prostatitis, classified as an inflammatory prostate disease, is a common urological occurrence in men under the age of 50. It is a problematic, sometimes serious, challenge that greatly negatively impacts the quality of life; characterised by the occurrence of voiding frequency, reduced urinary flow, perineal pain, and often severe pelvic discomfort and pain (Prezioso et al. 2006; Macaluso 2007). It has been suggested that prostatitis may be caused by an agent capable of inciting inflammation or neurological damage in or around the prostate, leading to pelvic floor and neuromuscular pain (Prezioso et al. 2006; Anothaisintawee et al. 2011). It has also been suggested that prostatitis may be due to a few predisposing factor, such as infection, voiding abnormalities, hormone imbalances, or immunological triggers (Anothaisintawee et al. 2011). Currently, treatment options for prostatitis include antibiotics and anti-inflammatory medications (Anothaisintawee et al. 2011). However, the efficacy of these treatments remains controversial, as clinical evaluation of these treatment options are limited (Anothaisintawee et al. 2011).

1.2.3.5 Benign prostatic hyperplasia

Benign prostatic hyperplasia is a condition characterized by the enlargement of the prostate, along with lower urinary tract symptoms (Schenk et al. 2009). Arising in the peri-urethral and transitional zones, benign prostatic hyperplasia has been described as an inescapable phenomenon for aging males that affects 50% of men by the age of 50, and almost 80% of men by the age of 80 (Schenk et al. 2009). Histologically, benign prostatic hyperplasia is characterised by hyper-proliferation of stromal and epithelial regions of the prostate, causing an array of lower urinary tract symptoms that often prompt men to seek medical attention (Schenk et al. 2009). The pathogenesis of benign prostatic hyperplasia remains poorly understood. However, it is suggested that inflammation plays a role in its development and progression, as evidence of acute and chronic inflammation is found in prostate biopsies of benign prostatic hyperplasia patients (Schenk et al. 2009). Additionally, inflammatory cytokines that function as potent mitogens, capable of inducing the characteristic hyperplastic changes associated with benign prostatic hyperplasia, are found to be overexpressed (Schenk et al. 2009). The underlying cause of inflammation remain unclear, although has been hypothesized to be caused by tissue damage resulting from infection (Carson and Rittmaster 2003; Schenk et al. 2009).

Benign prostatic hyperplasia has also been shown to be androgen-dependent, particularly on dihydrotestosterone. Dihydrotestosterone is essential for normal development of the prostate, from the foetal prostate, to development of the external male genitalia (Carson and Rittmaster 2003). It is hypothesized that dihydrotestosterone and other androgens contribute to maintain homeostasis in the adult prostate, regulating the balance between cell proliferation and cell death. It is hypothesised that benign prostatic hyperplasia arises when this homeostasis between cell

proliferation and death is disturbed (Carson and Rittmaster 2003; Briganti et al. 2009). This arose from the observations that benign prostatic hyperplasia does not develop in men undergoing early castration (Carson and Rittmaster 2003; Briganti et al. 2009). Moreover, the size of the enlarged prostate can be reduced with anti-androgen medications and surgical castration (Carson and Rittmaster 2003; Briganti et al. 2009).

Further observations led to the idea that dihydrotestosterone, rather than testosterone, is the causative androgen in benign prostatic hyperplasia (Carson and Rittmaster 2003; Briganti et al. 2009). Observations showed that concentrations of free testosterone decreased as a function of age that coincides with the onset of benign prostatic hyperplasia. Also, while testosterone decreases with age, dihydrotestosterone concentrations remain constant during the time of benign prostatic hyperplasia onset (Carson and Rittmaster 2003). These observations are consistent with the role of dihydrotestosterone in prostate growth and suggest that testosterone is not critically involved (Carson and Rittmaster 2003; Briganti et al. 2009).

While the precise pathophysiological mechanism of benign prostatic hyperplasia has not been established, evidence supports an important role of dihydrotestosterone (Carson and Rittmaster 2003; Briganti et al. 2009). Additionally, animal and human studies suggest that the development of benign prostatic hyperplasia entails the disruption of dihydrotestosterone-related homeostasis between cell proliferation and cell death (Carson and Rittmaster 2003; Briganti et al. 2009). For some men, benign prostatic hyperplasia is a progressive disease presenting with continual prostate growth and worsening of associated symptoms (Carson and Rittmaster 2003; Briganti et al. 2009).

Treatment of benign prostatic hyperplasia often involves the use of alpha blockers, which improve the symptoms of benign prostatic hyperplasia but do not decrease the prostatic size (Carson and Rittmaster 2003; Briganti et al. 2009). Additionally, 5 α -reductase inhibitors are also

used in the treatment of benign prostatic hyperplasia, which has been shown to stop the progression of the disease, reduce symptoms and decrease the size of the prostate between 20-30% (Carson and Rittmaster 2003; Briganti et al. 2009). However, drug-related side effects were reported, often including erectile dysfunction, decreased libido, ejaculation disorders and gynecomastia. Surgically, benign prostatic hyperplasia is often treated with transurethral resectioning, to remove obstructing portions of the prostate (Carson and Rittmaster 2003; Briganti et al. 2009).

1.2.3.6 Prostate cancer

Prostate cancer, or adenocarcinoma, is the most commonly reported cancer among ageing men and is the second leading cause of cancer-related deaths in men. In 2008, a prevalence of 14% of the total new cases and 6% of the total cancer deaths in men was reported (Jemal et al. 2011). Worldwide incidence rates (Figure 5) vary with the highest rates found in developed countries such as Australia, New Zealand, Western Europe, Western America and Northern Europe (Ferlay et al. 2010). The higher incidence in the developed countries is thought to be due to the higher life expectancy (Taher 2005) and a greater level of prostate specific antigen (PSA) testing (Jemal et al. 2011) which is used as a diagnostic marker (Dimakakos et al. 2014). Nevertheless, it remains clear that prostate cancer is a global concern to aging men over the age of 50 (Ferlay et al. 2010)

Prostate cancer is mainly found in the peripheral zone of the prostate and occurs when the normal prostate gland cells begin to mutate into cancerous cells (Burden et al. 2006). Once cancer of the prostate ensues, metastasis often follows in lymph nodes, other distant sites and in the surrounding bony structures. It is widely accepted that the formation of prostate cancer can

be linked to any one or a combination of different factors including a poor diet, genetic susceptibility, inflammation, age or infectious agents (Nelson et al. 2003; Stangelberger et al. 2008). Prostate cancer was one of the first cancers to be shown to be hormone-dependent and that lead to the concern that a raise in serum testosterone would feed the cancer itself (Snyder 2009). This was based on the observed regression of prostate cancer in androgen deprived males, which was reflected by a decline in PSA levels and a consequent increase in PSA levels with the normalization of testosterone concentration (Huggins et al., 1941; Hohl et al., 2009; Morgentaler, 2009; Morgentaler et al., 2011).



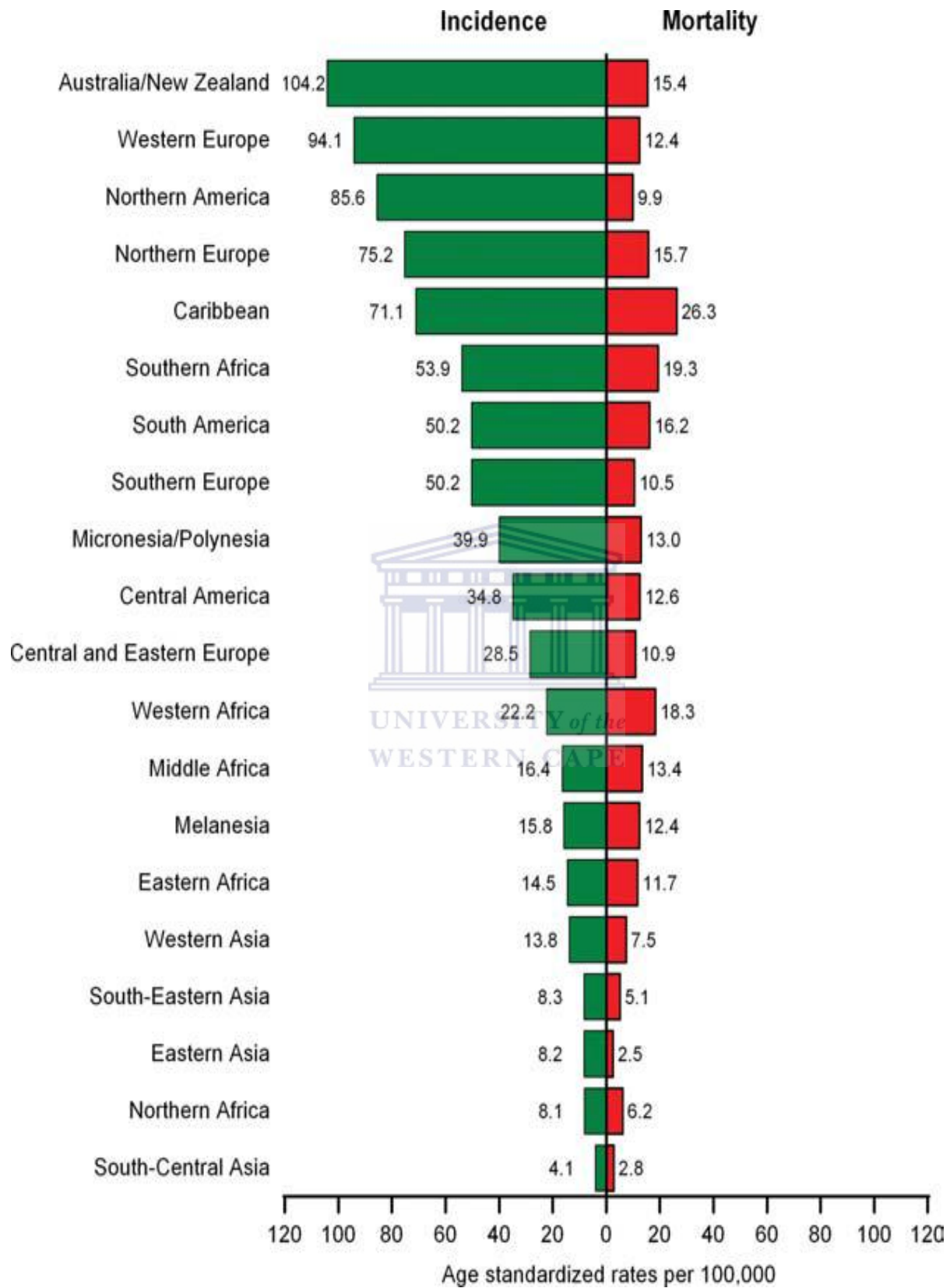


Figure 5: Global incidence and mortality of prostate cancer in 2008 (Ferlay et al. 2010)

Thus, according to the Endocrine Society (Bhasin et al. 2010), testosterone replacement therapy, which would be helpful in alleviating ageing male symptoms, is regarded as an absolute contraindication.

There are many pathways involved in the growth and progression of prostate cancer, such as the interplay between growth factor, receptor tyrosine kinase and androgen receptor pathways. These variables promote abnormal cell signalling that causes excessive cell proliferation, cell-cycle deregulation and increases in cell survival (Rosenberg et al. 2010). Together, these interplaying pathways advance the progression of prostate cancer. The androgen receptor pathway has been implicated in early prostate cancer growth, metastasis, development of hormonal resistance, and increased rate of disease relapse (Rosenberg et al. 2010). In fact, the androgen receptor is expressed throughout all stages of development, in the majority of prostate cancer cases (Rosenberg et al. 2010). Early evidence to support the role of androgens included the observed regression of metastatic prostate cancer in men who underwent surgical castration, population studies showing the rare occurrence of prostate cancer amongst eunuchs, along with the absence of observed prostate cancer in men who present with extremely low levels of dihydrotestosterone (Rosenberg et al. 2010). Hence, treatment of prostate cancer is based on dependence on androgens for development, growth and survival (Rosenberg et al. 2010).

The removal of androgen has been shown to trigger prostate cancer cell death or cell-cycle arrest. However, studies have shown that although medical castration decreased serum testosterone by 94%, intraprostatic testosterone and dihydrotestosterone levels remained high enough (Page et al. 2006), at approximately 20-30% of the control, to prevent any changes to cell proliferation, apoptosis and androgen-related protein expression (Akin et al. 2006; Rosenberg et al. 2010; Jin et al. 2011). Androgens, particularly dihydrotestosterone, and the associated 5 α -reductase, are highly associated with prostate cancer (Rosenberg et al. 2010). Expression of the type 1

5 α -reductase is elevated at different stages of prostate cancer: Prostatic intraepithelial neoplasia (PIN), localized, recurrent and metastatic (Rosenberg et al. 2010).

During the process of malignant transformation, cells gradually evolve from the benign to the malignant phenotype. Prostatic intraepithelial neoplasia (PIN) is a condition defined as the neoplastic growth of epithelial cells in pre-existing benign prostatic accini or ducts (Brawer 2005). PIN satisfies all requirements for premalignant conditions, hence high-grade PIN is accepted as a precursor to prostate cancer (Brawer 2005). Once prostate cancer ensues, spurred on by the disruption of the homeostasis between cell proliferation and death, the peripheral zone becomes the major site of development (Brawer 2005). Previously, it was stated that between 75-85% of cases were occurred in the peripheral zone (Akin et al. 2006).

The transitional zone has also been implicated in prostate cancer, accounting for approximately 25% of cases (Akin et al. 2006). Historically, patients with prostate cancer were categorized based on the stage of the cancer and whether or not they were candidates for surgery (Marciscano et al. 2012). Hence, the term “localized prostate cancer” refers to prostate cancer manageable with local therapy, such as surgery, radiotherapy or active surveillance (Marciscano et al. 2012). Often, following local therapy, patients have been shown to present with rising prostate specific antigen levels in a condition known as biochemically recurrence, which usually precedes metastasis and death (Rosenberg et al. 2010).

The classic model of cancer metastasis, including prostate cancer, is guided by the “seed and soil” hypothesis first proposed by Stephen Paget in 1889. In this model, the “seeds” (tumour cells) metastasize only to “soil” (specific organ) well suited to the tumours growth (Jin et al. 2011). Metastasis of the prostate specifically involved multiple steps, namely angiogenesis, local migration, invasion, extravasation, circulation and extravasation of tumour cells, followed by angiogenesis and colonization in new sites (Jin et al. 2011). Often, the bone is

the common site of metastasis in prostate cancer and is the leading cause of death in advanced prostate cancer (Jin et al. 2011).

1.2.3.7 Diagnosis of Prostate cancer and the Gleason screening

Commonly, the two most used methods for prostate cancer screening are detecting prostate specific antigen (PSA) levels and digital rectal examination (Hoag et al. 2008). PSA is a glycoprotein produced by prostate epithelial cells that may be elevated in men due to disruption of tissue barriers between the prostate gland lumen and capillaries (Hoag et al. 2008). Studies have estimated that elevated PSA levels can precede the onset of prostate cancer by 5-10 years (Hoag et al. 2008). However, elevation in PSA levels has also been shown to be found in prostatitis and benign prostatic hyperplasia (Wolf et al. 2010). However, the accuracy of PSA screening is debatable.

Digital rectal examination (DRE) has long been used in the diagnosis of prostate cancer, identifying nodules, asymmetry or induration (Johnstone et al. 2001). DRE can detect tumours in the posterior and lateral aspects of the prostate. However, only 85% of cancers arise in the periphery, outlying a clear limitation of this technique (Johnstone et al. 2001). In the diagnosis of prostate cancer, ultrasound imaging and prostate biopsies are often used. The classic grey-scale ultrasound imaging is a widely used method in the diagnosis of prostate cancer, but is limited by the fact that approximately half of prostate cancer lesions are invisible on grey scale imaging (Halpern 2006). Additionally, prostatitis and benign prostatic hyperplasia tend to mimic the grey scale image of prostate cancer. Nevertheless, once screened and diagnosed, the severity is graded (Halpern 2006).

All systems aimed at grading the severity of prostate cancer use the acinar pattern. One such system is the Gleason classification scheme (Kozlowski and Grayhack 2002), which is the most widely accepted method of grading prostate cancer, and used low power magnification (40x-100x) to assess the glandular pattern of the tumour in relation to the stromal compartment (Gleason 1966; Kozlowski and Grayhack 2002). In this system, five tumour grades exist (Gleason 1966; Kozlowski and Grayhack 2002). Grade 1 contains a homogenous array of single, oval, tightly packed glands, rarely showing stromal invasions. Grade 2 has an acinar pattern that mimics that of grade 1, without the uniformity in glandular shape and being less tightly packed. Here, a mild amount of stromal invasion can be observed (Gleason 1966; Kozlowski and Grayhack 2002) Grade 3 contains three sub-patterns, such as single, yet irregular, glands separated from each other by more than one gland diameter, along with moderate amounts of stromal invasion. Next, there is a notable presence of a micro-glandular nest of cells that form small groups or cords. Lastly, sharply circumscribed, rounded masses of papillary or cribriform epithelium possessing smooth, sharp edges are observed. Grade 4 contains two sub-patterns (Gleason 1966; Kozlowski and Grayhack 2002). Furthermore, the first is a fashion of ragged glandular masses that exhibit branching, along with stromal invasion. Also, the second is the presence of large cells, with pale, clear cytoplasm similar to that observed in clear-cell carcinoma of the kidney, termed hyperphroid cells (Gleason 1966; Kozlowski and Grayhack 2002). Grade 5 is characterised by irregular infiltrating masses of malignant cells, usually without gland formation.

A second rare variant consists of an irregular cribriform arrangement with central necrosis, similar to that seen in comedocarcinoma of the breast (Gleason 1966; Kozlowski and Grayhack 2002). Recognition that approximately 50% of tumours will present with more than one of Gleason's histological observations, led to the use of the sum of the primary and secondary

patterns as the Gleason pattern score (Kozlowski and Grayhack 2002). Here, a possible score ranges from 2 -10, with 2 being indicative of well-differentiated tumours, and 10 being indicative of undifferentiated tumours (Kozlowski and Grayhack 2002).

1.2.3.8 Treatment of prostate cancer

The increased use of prostate specific antigen screening has allowed prostate cancer to be detected much earlier than previously possible, thus allowing for earlier medical intervention and a greater chance of survival (Wallace et al. 2014). Interventions can be grouped into three broad categories, namely intervention with curative intent, intervention with palliative intent, and the monitoring of the disease and providing treatment when progression is evident (Hegarty et al. 2010; Wallace et al. 2014).

Intervention with curative intent involves the use of radical prostatectomy, radiotherapy, cryo-therapy, or high-intensity ultrasound therapy (Hegarty et al. 2010; Wallace et al. 2014). Radical prostatectomy involves the removal of the entire prostate gland and some surrounding tissue (Hegarty et al. 2010). Although many benefits are associated with this procedure, such as reduced risk of mortality, progression and metastasis, the possible side effects, namely altered urinary function and deteriorated sexual function, left patients with a diminished quality of life (Hegarty et al. 2010).

External-beam radiation therapy makes use of high-speed electrons to split water molecules, thereby producing hydroxyl radicals that cause damage to the DNA of the tumour cells (Torres-Roca 2006). This technique carries two substantial advantages, such as the absence of pain during the procedure and a low clinical failure rate (15% when measured after 2.5 years) (Torres-Roca 2006). However, the list of negative consequences vastly outweighs the positive aspects,

including such as haemorrhoids diarrhoea due to inflammation of the rectum, frequency in urination due to inflammation of the bladder, fatigue and impotency (Mongra et al. 1999; Torres-Roca 2006).

The two main modalities of administering radio therapy are external X-ray beams, known as external-beam radiotherapy, or the implantation of a radioactive sources directly into the tumour, known as interstitial brachytherapy (Koukourakis et al. 2009). Although shown to be an excellent treatment option for localized prostate cancer, there are issues that remain to be further addressed, such as the ideal radiation dose and side effects such as disturbances in urinary function (Koukourakis et al. 2009).

Cryo-therapy involves the use of gasses and probes to rapidly freeze and thaw tumour tissue (Wilt et al. 2008). Currently, cryo-therapy is indicated in low-risk patients as an alternative to radical prostatectomy or radiotherapy. In higher-risk groups, cryo-therapy is used as primary therapy, and in patients who have not responded well to radiation therapy (Simoneau 2006). There are major complication associated with cryo-therapy, such as urethral sloughing, rectal fistula, incontinence, and erectile dysfunction (Simoneau 2006). High intensity ultrasound therapy is a non-invasive technique capable of inducing instantaneous, but irreversible, coagulative necrosis in tissue by thermal effects (Mearini and Porena 2010). It is an attractive approach for the treatment of localized prostate cancer in patients who have a life expectancy of less than 10 years, but do not accept the dangers of radical prostatectomy (Mearini and Porena 2010). Unfortunately, side effects such as prostate swelling and thereby urinary retention, dysuria due to sloughing of necrotic tissue, bladder neck or prostatic urethra constriction, impotence and rectal fistula (Mearini and Porena 2010). Additionally, absolute contraindications exists, such as any condition obstructing trans-rectal probe introduction and intra-prostatic calcification (Mearini and Porena 2010).

Based on the serious side effects associated with the conventional treatments, many patients began turning to complementary and alternative medicine with the belief that this provides a viable treatment option with virtually no side effects (Klempner and Bublely 2012). Thus, many herbs, and their herbal extract derivatives, have been scientifically investigated (Klempner and Bublely 2012). These include the polyphenols found in green tea, epicatechin, epigallocatechin, epicatechin-3-gallate, and epigallocatechin-3-gallate (EGCG), the soy isoflavones, *Scutellaria baicalensis*, β -carotene and lycopene (Klempner and Bublely 2012; Yin et al. 2013). EGCG was able to cause cell cycle arrest in LNCaP and DU145 cells, at the G0 and G1 phase, inhibit metalloproteinase *in vitro*, and lower PSA levels to >50% in androgen-resistant prostate cancer (Yin et al. 2013). However, these positive effects were only seen at serum concentrations of EGCG that were much higher than that seen in humans who consume moderate amounts of green tea.

Additionally, patients presenting with androgen-resistant prostate cancer suffered side effects such as nausea, diarrhoea and fatigue (Yin et al. 2013). Soy isoflavones were shown to reduce 5 α -reductase activity, the enzyme responsible for the conversion of testosterone to dihydrotestosterone (Yin et al. 2013). Additionally, soy isoflavones have been investigated for its use with chemotherapeutics. *Scutellaria baicalensis*, which contains high levels of the flavone baicalin, has been shown to impair proliferation of androgen-independent PC-3 and DU145 prostate cancer cells, inducing cell-cycle arrest at the G0 and G1 phase at concentrations achievable in humans (Yin et al. 2013).

Another plant of interest is *Wedelia chinensis*, an oriental herb containing indole-3-carboxyaldehyde, that was shown to suppress androgen activity (Yin et al. 2013). Moreover, oral administration of this herb impeded tumour formation, as well as disturbing the androgen-

receptor signalling pathway (Yin et al. 2013). Thus, herbal medicine has potential in the treatment of prostate cancer.

Many of the described treatments are aimed at reducing androgens and their actions, thereby contributing to the symptoms of late-onset hypogonadism in these men. While the use of testosterone replacement therapy has been described an absolute contraindication in men suffering from prostate cancer (Bhasin et al. 2010), new evidence suggests otherwise (Atan et al. 2013). A preliminary study showed that in 13 patients with symptomatic hypogonadism and untreated prostate cancer, testosterone replacement therapy caused to change in mean PSA levels, over a 2.5 year period, along with no increase in prostate volume, no progression, and no evidence of metastasis (Morgentaler et al. 2011). Similarly, another study by Morales evaluated the effects of testosterone replacement therapy in 7 hypogonadal men with untreated prostate cancer. Here, 3 of the patients received testosterone-replacement therapy over 33, 96 and 51 months respectively (Morales 2011). Surprisingly, none of the patients presented with metastasis, even if PSA levels became elevated (Morales 2011). Similarly, testosterone replacement therapy was given to men after receiving treatment for prostate cancer. Here, Mulhall et al treated 22 men with symptomatic hypogonadism after radical prostatectomy (Mulhall et al. 2008). Of the 22 patients, only one showed recurrence of prostate cancer. Similarly, Khera et al showed that in 57 patients receiving testosterone replacement therapy after radical prostatectomy, PSA levels remained lowered after 13 months of testosterone replacement therapy (Khera et al. 2009). Similarly, no biochemical recurrence was observed in patients who received testosterone replacement therapy over a 6 month period. In addition to this, studies have shown the inhibition of prostate cancer cells with an increased concentration of testosterone (Chuu et al. 2011). Thus, the use of testosterone replacement therapy in men with prostate cancer may not be an absolute contraindication (Khera et al. 2009; Chuu et al. 2011).

1.3 Traditional medicine

According to the World Health Organisation (WHO), traditional medicine has been defined as “the knowledge, skills and practices based on the theories, beliefs and experiences indigenous to different cultures, used in the maintenance of health and in the prevention, the diagnosis, the improvement or also in the treatment of physical and mental illness” (http://www.who.int/topics/traditional_medicine/en/). Many different structures of traditional medicine exists that follow philosophies and practices based on the environment through which they originated (WHO 2005). A common thread through the many different structures is the emphasis on health, rather than disease, in relation to the body, mind and environment. The health of the individual as a whole is the focal point of traditional medicine, and herbs are an integral part of this philosophy (Schmidt et al. 2008).

With the industrial revolution and advances in medical science, chemically synthesized drugs, and the ability to mass produce these drugs, greatly improved the health care system (WHO 2005). Most rural communities, however, have limited access to this type of medicine, and live in environments that surround them with easily accessible herbs. In fact, many people living in developing countries often live in extreme poverty, and cannot afford conventional Western Medicine as their primary health care (Pal and Shukla 2003). This is due to countries being unable to cope with the rapidly growing urban population, and thereby being unable to provide the appropriate healthcare. Thus, traditional medicine is the primary method of healthcare in these counties (Pal and Shukla 2003).

Studies have shown that when patients were asked for their reason for traditional medicine usage, 15% said that herbs were more effective than conventional medicine, while 6% associated it with fewer side effects (Pal and Shukla 2003). In Peru, 35% of patients using traditional

medicine stated that they used it more than pharmaceuticals (Pal and Shukla 2003; Andel and Carvalheiro 2013). In Nigeria, up to 67% of the population is estimated to use traditional medicine (Pal and Shukla 2003). This high prevalence of traditional medicine was hypothesized to be the result of large rural-to-urban migration, cultural influence, social surroundings, and the belief that natural products pose no risks (Pal and Shukla 2003; Andel and Carvalheiro 2013).

Although very prevalent in developing countries, the use of traditional medicine is also increasing in popularity in developed countries. Barnes et al (2008) suggested that up to 38% of adults in the United States of America used some form of traditional medicine (Barnes et al. 2008). Similarly, it was reported that up to 40% of the population in Hong Kong used a form of traditional medicine, rather than mass produced pharmaceutical drugs (Chan et al. 2003). Approximately half of Southern Australians were estimated to use traditional medicine, reports from Western Europe suggested up to 20% in the Netherlands, and about 49% in France (Andel and Carvalheiro 2013).

Despite conventional medicine being readily available in developed, a notable shift towards traditional medicine is occurring (Andel and Carvalheiro 2013). The proposed reasons for this were to prevent illness, curiosity, the idea that these medicines are safer, and the idea that combining it with conventional medicine would be more beneficial than using either in isolation (Klempner and Bublely 2012; Siegfried and Hughes 2012; Andel and Carvalheiro 2013). Another explanation for the increased usage of traditional medicine in developed countries is the continued use of traditional practices by immigrants (Klempner and Bublely 2012).

Nevertheless, the increased usage of traditional medicine is a global trend in both developing and developed countries, and the underlying reasons are the greater accessibility of these treatments, better affordability of traditional medicine in comparison to conventional medicine, and the perception of traditional medicine being safer or less toxic than conventional medicine (Loya et

al. 2009; Klempner and Bublely 2012; Siegfried and Hughes 2012; Andel and Carvalheiro 2013). Considering the limited insight into the safety, and overall efficacy of traditional medicine, the belief that it is safer may not be entirely true, and thus warrants extensive investigation (Pal and Shukla 2003; Siegfried and Hughes 2012).

1.3.1 Traditional medicine in Africa

The use traditional medicine has become an increasingly popular global trend, due to the belief that it presents less health risks when compared to conventional Western Medicine (Pal and Shukla 2003). In addition to the perceived lower health concerns, it is also significantly cheaper than the pharmaceuticals currently used in Western Medicine (Pal and Shukla 2003). In Africa specifically, conventional health care and pharmaceuticals have become increasingly expensive and thereby unavailable to the vast majority of the population. This has led an increasingly popular usage of traditional medicine, accounting for 80% of the African populations (Siegfried and Hughes 2012).

In South Africa particularly, 70-80% of the population was found to use traditional medicine as a primary source of health care or in conjunction with conventional medicine (Chitindingu et al. 2014). This was linked to the close proximity of traditional healers to their communities and thus their widespread availability, particularly in rural communities (Chitindingu et al. 2014). Additionally, traditional medicine in South Africa is governed by the Traditional Healers Council, which has made great strides into integrating traditional medicine into the health legislative framework (Chitindingu et al. 2014). In fact, the Traditional Practitioners Act of 2007 was passed to regulate traditional medicine, with the main aim being to ensure the efficacy, safety and control of traditional healthcare services (Chitindingu et al. 2014).

South Africa has an array of indigenous plants that have recently been investigated scientifically for their medicinal benefits. These include *Typha capensis*, *Sutherlandia frutescens*, and *Bulbine natalensis*, to name a few, that have shown abilities ranging from affecting sperm function (Henkel et al. 2012), acting as an antioxidant (Katerere and Eloff 2005), boosting testosterone production (Yakubu and Afolayan 2008), on fighting cancer (Chinkwo 2005).

Also, growing interest in the identification of novel pharmaceuticals from plants used in traditional medicine has led to the identification of an estimated 122 drugs from 94 plant species (Chitindingu et al. 2014).

Despite the recent investigations, many plants used in South African traditional medicine remain unevaluated for their reported medicinal benefit. Generally, no information regarding the potential geno-toxic effects after long-term use exists (Chitindingu et al. 2014). Additionally, concerns regarding the potential geno-toxicity, and interference of traditional medicine with conventional medicine, decreasing the effectiveness of conventional medicine, have been raised (Fennell et al. 2004; Chitindingu et al. 2014).

Potential dangers were clearly outlined by Fenell et al (2004) who showed that of the 51 plants used in traditional medicine that are considered to be safe and thus evaluated, many of them showed high levels of toxicity, causing both DNA damage and chromosomal aberration (Fennell et al. 2004). Plants exhibiting toxicity included *Chaetacme aristata*, *Croton sylvaticus*, and *Diospyros whyteana*, to name a few. Additionally, several plants exhibited antigenotoxic effects, such as *Rhamnus prinoides*, *Prunus africana*, and *Syzygium cordatum* (Fennell et al. 2004). Nevertheless, these authors concluded that several plants commonly used in South African traditional medicine can cause damage to genetic material, and could potentially cause long-term damage in patients (Fennell et al. 2004). For that reason, these authors stated that the plant

species tested should be used under caution, along with rigorous toxicological and clinical studies before their wide spread prescription (Fennell et al. 2004).

A similar conclusion was drawn by Taylor et al (2003) who conducted a similar study, evaluating the safety of South African traditional medicine (Taylor et al. 2003). Considering the results produced by Fenell et al (2004), and that a vast amount of plants used in South African traditional medicine remains unevaluated, a clear need for scientific investigated is warranted to ensure the safety of patients.

1.4 *Cissameplos capensis*

The Menispermaceae family consists of 75 genera, to which 520 species belong. Members of this family are found scattered throughout the world and have been used in traditional remedies that are found scattered throughout the world and are well known for their medicinal uses (De Wet and Van Wyk 2008). In South Africa, four species exists under the genus *Cissampelos L*, these include *Cissampelos capensis* (Figure 6), *Cissampelos hirta*, *Cissampelos mucronata* and *Cissameplos torulosa* (De Wet et al. 2011).



Figure 6: Cross section through the rhizomes of *C. capenses*.

Cissampelos capensis (*C. capensis*), commonly known by the Afrikaans name “dawidjie wortel”, is a shrub with thick, divergent branches and twinning stems that is endemic to South Africa. This plant has a long and significant history in Khoisan ethnomedicine, being used to treat many ailments (De Wet et al. 2011). Traditionally, the rhizomes, stems and leaves were used to treat ailments including pain, fever, menstrual cramps, tuberculosis, glandular swelling, gall stones, dysentery, difficult labour, preventing miscarriage, expelling placenta, blood purification, colic, and cancer of the skin or stomach (De Wet and Van Wyk 2008; De Wet et al. 2011). The leaves were used to treat syphilitic sores, ulcers and snake bite wounds (De Wet and Van Wyk 2008; De Wet et al. 2011).

Generally, it is assumed that the medicinal benefits of *C. capensis* are thought to be due to the alkaloids found within the plant (De Wet et al. 2011). These include 12-O-methylcurine, dicentrine, glaziovine, lauroschooltzine, pronuceferine, bulbocapnine, salutaridine, cycleanine, cissacapnine, crotsparine, insularine, reticuline and insulanoline (Figure 7) (De Wet et al. 2011). These alkaloids fall under the classes aporphine alkaloids, morphinane alkaloids and bisbenzyltetrahydroisoquinoline alkaloids (De Wet et al. 2011).

Aporphine alkaloids are endowed with a range of biological activities and have been extensively investigated (Makarasen et al. 2011; Omar et al. 2013). For example, naturally occurring aporphine alkaloids have been investigated as acetylcholine esterase inhibitors, central nervous system receptor ligands, antimicrobial agents (Makarasen et al. 2011; Omar et al. 2013), antimalarial agents, and antiviral agents (Makarasen et al. 2011). Additionally, aporphine alkaloids have been investigated as cytotoxic agents in the treatment of cancer (Makarasen et al. 2011; Omar et al. 2013) and combating the occurrence of multidrug resistance (Min et al. 2006).

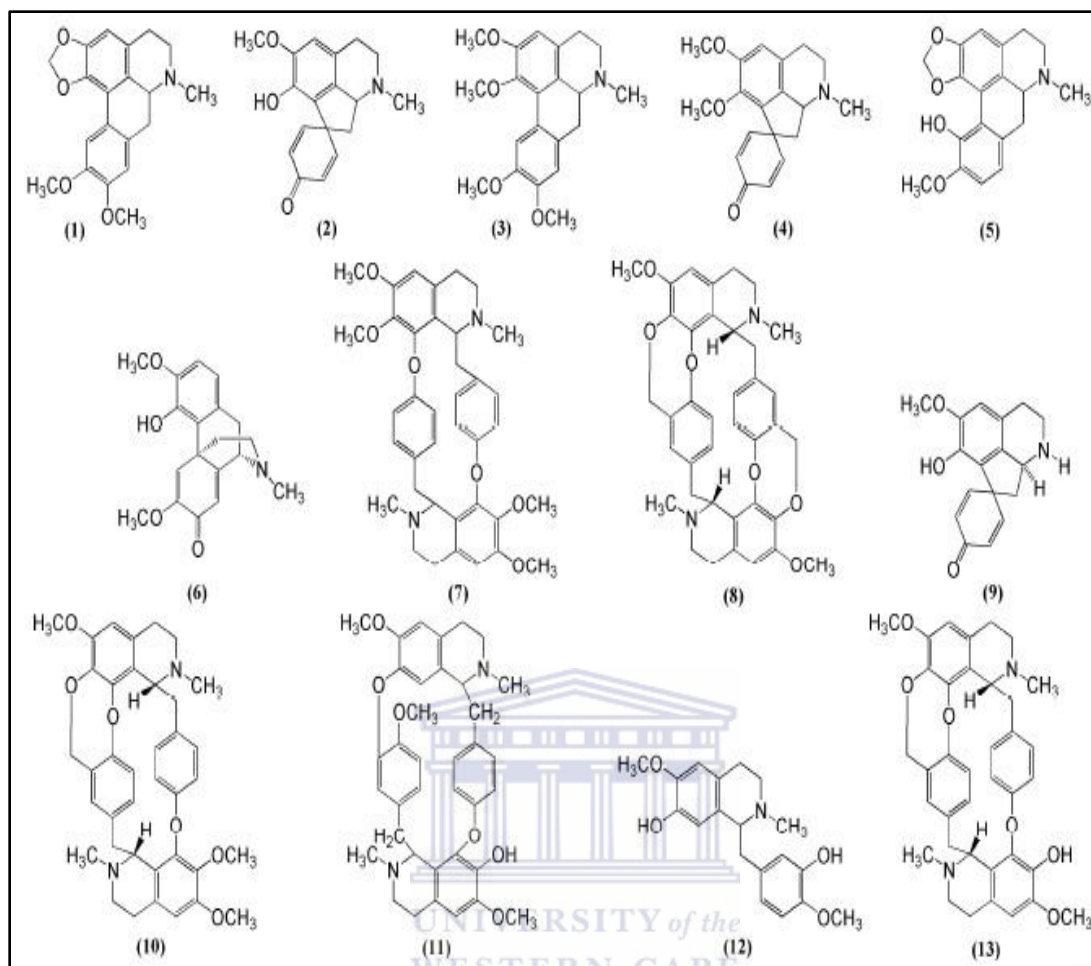


Figure 7: Chemical structures of the major alkaloids isolated from *C. capensis*. Dicentrine (1), glaziovine (2), lauroscholtzine (3), pronuciferine (4), bulbocapnine (5), salutaridine (6), cycleanine (7), cissacapnine (8), crotsparine (9), insularine (10), 12-*O* methylcurine (11), reticuline (12), insulanoline (13) (De Wet et al. 2011).

Also, aporphine alkaloids have been shown to possess antioxidant activity, such as bulbocapnine, apomorphine, gluacine, and isoboldine, to name a few, by removing active oxygen and free radicals, features which are thought to be related to the chemical structure of these alkaloids (Jianxin et al. 2013). Bisbenzyltetrahydroisoquinoline alkaloids, such as cycleanine, have been shown to possess antimicrobial effects, anti-inflammatory, analgesic effects, and have shown

cytotoxicity towards cancer cells (Angerhofer et al. 1999). Also, bisbenzyltetrahydroisoquinoline alkaloids have been shown to possess antioxidant activities, by scavenging free radicals (Cordell 2005). Additionally, a study conducted by Babajidel et al (2010) found tannins, phenols, flavonoids and saponins being present in *C. capensis* extracts and fractions (Babajidel and Mabusela 2010). These phytochemicals have been shown to scavenge ROS/RNS and induce cellular defence enzymes (Lee et al. 2013; Nanda 2014). However, members of the family Menispermaceae, namely *Tinospora cordifolia*, *Tinospora tenera*, and *Cissampelos mucronata* have a traditional use as an aphrodisiac (De Wet and Van Wyk 2008; Ramandeep et al. 2013). In another study, another member of the Menispermaceae family, *Sphenocentrum jollyanum* Pierre, was shown to increase testosterone production (Woode et al. 2009).

Considering the presence of phytochemicals, the many different types of alkaloids, and the traditional use in the treatment of skin and stomach cancer, it can be hypothesized that *C. capensis* may indeed exert an antioxidant effect, protecting against ROS/RNS induced damage, and may prove beneficial in the treatment of prostate cancer. Additionally, considering that other species in the family Menispermaceae are able to affect male sexual health, it is possible that *C. capensis* may act in a similar way. However, no scientifically documented information regarding the effect of *C. capensis* towards these parameters exists. Thus, the present study aimed to investigate these possible effects.

1.5 Aims of the study

C. capensis is a widely used plant in South African traditional medicine, yet very little scientifically documented information regarding its effects exists.

Therefore this study aims investigate:

- The effects of *C. capensis* towards the prostate cancer cell line LNCaP.
- The safety of *C. capensis* with regard to Sertoli and Leydig cell function
- The potential of *C. capensis* to boost testosterone
- The effects of *C. capensis* toward lactate production
- The effects of *C. capensis* toward ROS and RNS
- The ability of *C. capensis* to inhibit elastase and collagenase activity.



Chapter 2

Materials and Methods

2.1 Chemical Supply

The chemicals used in the current study were of the highest possible quality, and were purchased from the following companies:

American Type Cell Culture (ATCC), Manassas, USA:

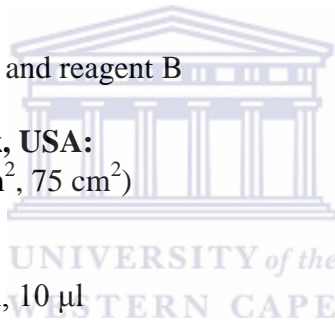
- Prostate cancer cell line LNCaP
- Leydig cell line TM3
- Sertoli cell line TM4

Bio-Rad, Hercules CA, USA:

- DC protein assay reagent A and reagent B

Corning incorporated, New York, USA:

- Tissue culture flasks (25 cm², 75 cm²)
- Eppendorf vials
- Pipette Tips 1000 µl, 200 µl, 10 µl
- Serological pipettes (10ml)



DRG instruments, Marburg, Germany:

- Testosterone assay kit

Eppingdust, Cape Town, South Africa:

- Ethanol absolute (100%)

Gibco Invitrogen, Karlsruhe, Germany:

- Roswell Park Memorial Institute (RPMI 1640) Medium
- Dulbecco's Modified Eagle Medium / Nutrient Mixture F12 Ham (DMEM/F12, 1:1 mixture)
- Fetal Bovine Serum (FBS)
- Horse Serum
- Trypsin/Ethyl Diamine Tetra Acetic acid (EDTA) (0.25%)
- Formaldehyde (37%)

- Enzchek elastase assay kit
- Enzchek collagenase assay kit

Greiner Bio-One, Frickenhausen, Germany:

- Tissue culture plates (6-, 24- and 96-well plates)
- Test tubes (15 ml and 50 ml)

Knittel Gläser, Braunschweig, Germany:

- Cover slips (22 x 22 mm)
- Microscope slides (76 x 26 mm)
- Superfrost slides

Knight Scientific Limited, Plymouth, UK:

- ABEL[®] Antioxidant Test Kit for superoxide
- ABEL[®] Antioxidant Test Kit for peroxynitrite

Lasec, Cape Town, South Africa:

- Syringes (5, 10 and 25 ml)

Merck, Wadeville Gauteng, South Africa:

- Sodium hydroxide (NaOH)

Promega, Madison, USA:

- The DeadEnd[™] Colorimetric TUNEL System kit

Oxoid, Basingstoke, Hampshire, RG24 SPW, England:

- Phosphate Buffered Saline (PBS) with Ca²⁺/Mg²⁺

Sigma-Aldrich, Steinheim, Germany:

- Dimethylsulphoxide (DMSO) for freezing medium
- Penicillin
- Streptomycin
- Testosterone purum ≥99.0% (HPLC)
- 2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT)
- Trypan Blue (TB)
- Triton X-100

- Millex syringe filter units (0.22 μm)
- Doxorubicin (DOX)
- Lactate dehydrogenase assay kit
- Glycerol
- Bovine serum albumin (BSA)
- Sodium dodecyl sulphate (SDS)

2.2 Equipment and supply

ELISA-reader

- GloMax Multi Detection System plate reader (Promega Corporation, Madison, USA)
- Labtech System LT 4000 microplate reader (Lasec, Cape Town, South Africa)

Laminar Flow

- LN Series (Nuve, Ankara, Turkey)

Incubator

- Series 2000 (Lasec, Cape Town, South Africa)

Scale

- WAS 160/X (Lasec, Cape Town, South Africa)

Plate shaker

- 96-well flat bottomed plate (Greiner Bio-One, Frickenhausen, Germany).

Centrifuge

- Hermle Z200a (Labortechnik, Wehingen, Germany)

Microscope

- Inverted System Microscope (Lasec, Cape Town, South Africa)



2.3 Study design.

In order to investigate the effect of the plant extract towards parameters affecting prostate cancer, LNCaP cell viability, DNA fragmentation, anti-cancer drug combination, along with the combined effect of the extract and testosterone, was determined (Figure 9). With regard to the effect of the extract on non-cancerous cells TM3 and TM4 cells, the testosterone production and lactate secretion were investigated as functional parameters, along with viability of TM3 Leydig cells and TM4 Sertoli (Figure 9).

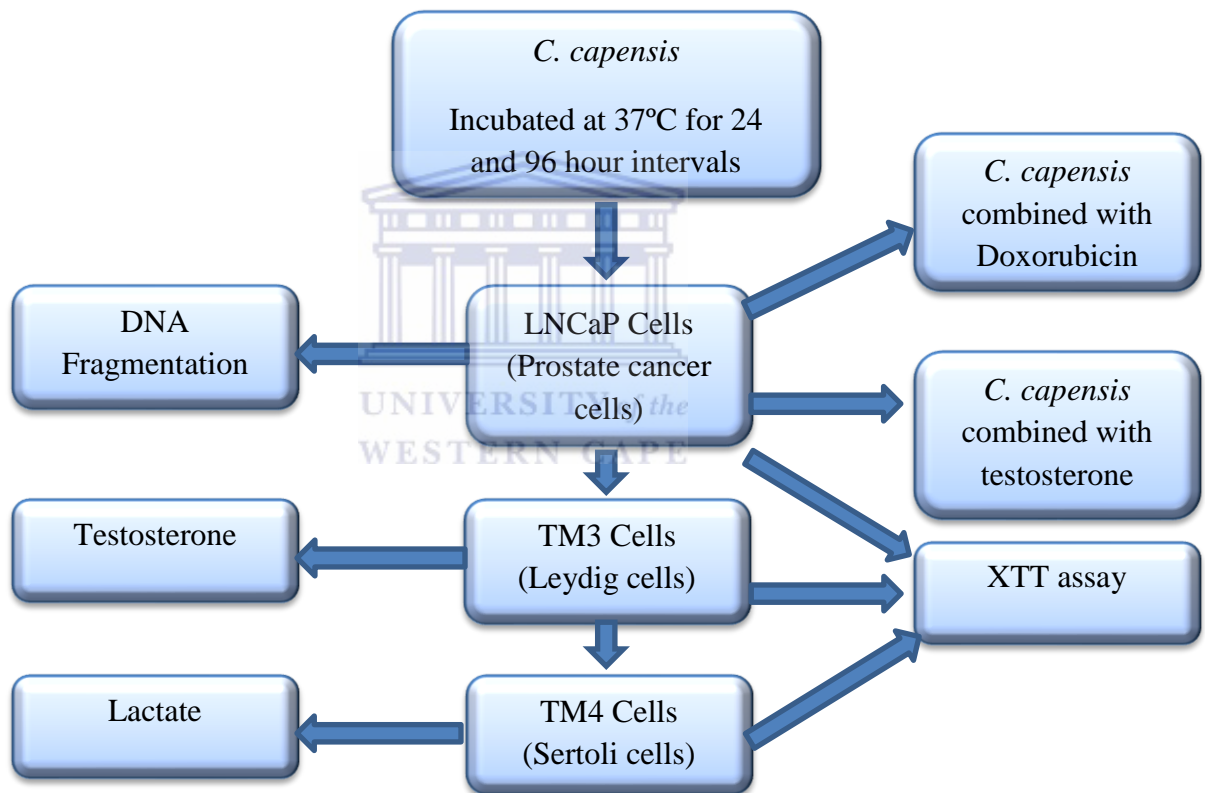


Figure 9: Study design for the investigation of the effect of the extract on cancerous and non-cancerous cells to evaluate the physiology of an average ageing male. Cells were exposed to *C. capensis* at concentrations of 0.001/ 0.01/ 0.1/ 1/ 10/ 100/ 1000 µg/ml and the cell viability (XTT (2,3-bis[2-methoxy-4-nitro-5-ulfophenyl]-2H-tetrazolium-5-carboxanilide), DNA fragmentation, testosterone production and lactate production were tested.

In a separate set of experiments, parameters affecting the initiation, progression and metastasis of cancer were investigated. These included the ability of the aqueous *C. capensis* rhizome extract to inhibit of reactive oxygen species (ROS) and reactive nitrogen species (RNS) production, and to inhibit collagenase and elastase activity (Figure 10).

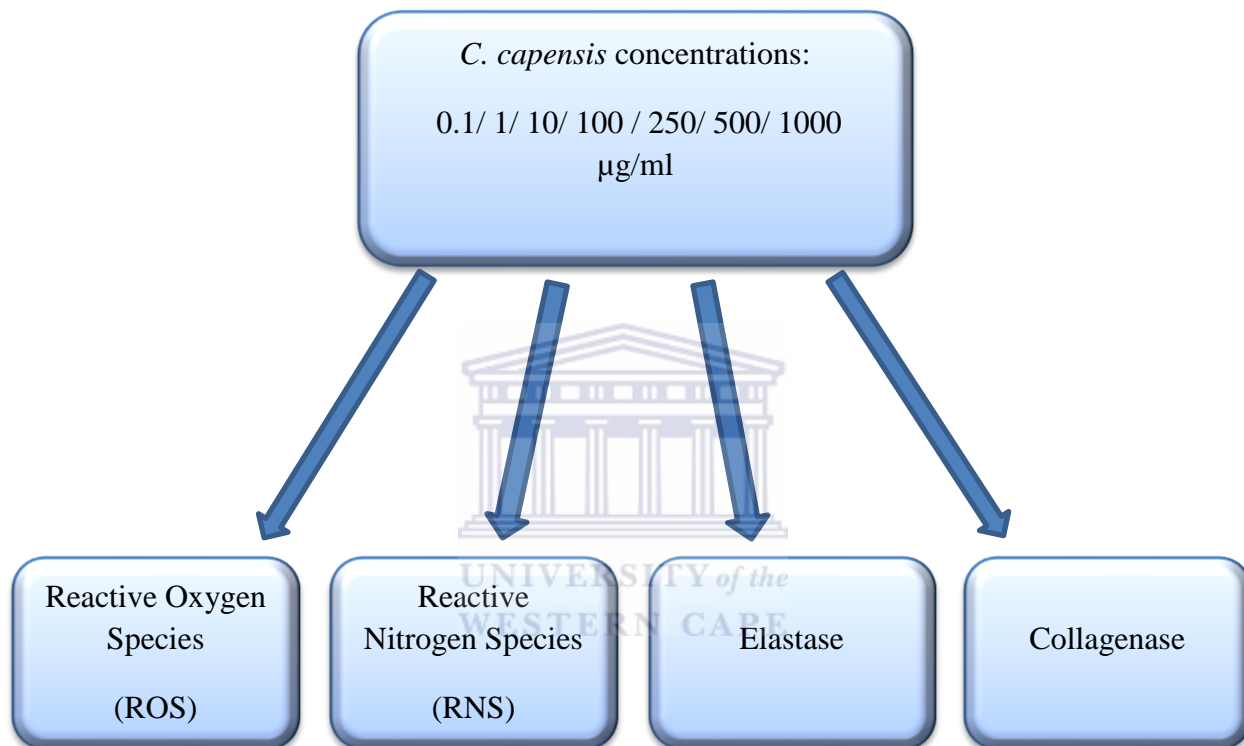


Figure 10: Study design to investigate the effects of the aqueous *C. capensis* rhizome extract toward the initiation, progression and metastasis of cancer, chemiluminescent tests were used. Concentrations of 0.1/ 1/ 10/ 100 / 250/ 500/ 1000 µg/ml were used to investigate inhibition of elastase activity, collagenase activity, reactive oxygen species, and reactive nitrogen species.

2.4 Plant extract

2.4.1 Rhizome collection

Under the supervision of Mr. Frans Weitz, from the Department of Biodiversity and Conservation Biology at UWC, the rhizomes of *C. capensis* were collected and identified during the summer months in the Cape Nature Reserve, located in the suburb of Belhar in the Western Cape province of South Africa. Once collected, the rhizomes were washed, chopped into 1-2 cm pieces and allowed to dry at 25°C in an oven. Thereafter, the dried rhizomes were milled into a fine powder for the extraction process.

2.4.2 Extract preparation

To prepare an aqueous rhizome extract of *C. capensis*, the powdered rhizomes were infused in distilled water that was heated to approximately 70-75°C. The resulting mixture was allowed to stand at room temperature for 1 hour, after which it was filtered. The filtrate was then frozen at -20°C and finally freeze-dried under the supervision of Mr. Lilburn Cyster, Department of Biodiversity and Conservation Biology, using a Vertis-freeze drier to yield the water-soluble extract.

Commonly, traditional healers will prescribe a handful of the dried rhizome for the treatment of patients. In order to establish the amount a patient is ingesting per day, three handfuls of the rhizomes were weighed out and an average of 88.3 g was obtained. Following this, aqueous extraction yielded an average of 17.72 g of the extract per 100 g. On the assumption that the average male weighs 80kg, an extract concentration of 221.5 µg/ml, was calculated according to equation 1.

Equation 1: Extract concentration (g/ml) = $\frac{17.71 \text{ (g)}}{80\,000 \text{ (ml)}}$

To account for the effects below and beyond 221.5 µg/ml, a scale ranging from 0.001 µg/ml – 1000 µg/ml was used. Based on this, a 10 000 µg/ml stock solution was made in Roswell Park Memorial Institute (RPMI) 1640 Medium or Dulbecco's Modified Eagle Medium / Nutrient Mixture F12 Ham (DMEM/F-12, 1:1 mixture) for cell culture-related assays. These stock solutions underwent serial dilutions to yield concentrations of 0.001/ 0.01/ 0.1/ 1/ 10/ 100/ 1000 µg/ml for all cell culture based assays. For all chemi-luminescent tests, however, the extract was diluted to concentrations of 0.1/ 1/ 10/ 100/ 250/ 500/ 1000 µg/ml. All controls contained no extract and were treated with medium only.

2.5 Cell culture

LNCaP prostate cancer, TM3 Leydig and TM4 Sertoli cell lines were used for the purpose of the study. They were cultivated at 37°C in 95% air and 5% CO₂, following standard aseptic work procedures. LNCaP cells were cultured in complete RPMI 1640 growth medium, supplemented with 10% fetal bovine serum (FBS), 1% penicillin (100 IU/ml) and streptomycin (100 µg/ml), in 75 cm² culture flasks. TM3 and TM4 cells were cultured in complete DMEM/F-12 growth medium supplemented with 2.5% Fetal Bovine Serum (FBS), 5% Horse serum and 1% penicillin (100 IU/ml) and streptomycin (100 µg/ml).

2.5.1 LNCaP prostate cancer cell line

LNCaP is a human prostate cancer cell line derived from a needle aspirate biopsy in 1977 from a 50-year old male, diagnosed with stage D prostate cancer (Horoszewicz et al. 1980). These cells express a mutated form of the androgen receptor, resulting in some differences in androgenic response (Horoszewicz et al. 1983). Most prostate cancer cell lines express little to no androgen

receptor, making LNCaP the ideal model for investigating early prostate cancer (Horoszewicz et al. 1983).

2.5.2 TM3 Leydig and TM4 Sertoli cell line

These two distinct cell lines were isolated from the testis of immature BALB/c mice in 1974. Both are epithelial cell lines that exhibit unique characteristics (Matfier 1980). TM3 cells respond to Luteinizing hormone (LH) with an increase in cyclic-AMP production and cholesterol metabolism (Matfier 1980). These cells do not, however, respond to follicle stimulating hormone (FSH). TM4 cells respond to FSH with an increase in cyclic-AMP production. These cells do not, however, respond to LH stimulation (Matfier 1980).

2.5.3 Culture of LNCaP cells

Cells were cultured in 75 cm² flasks, allowed to grow to 80% confluency and finally passaged once this was reached. To remove compounds that may interfere with the actions of trypsin, the growth medium was discarded and the cells rinsed with 5 ml sterile PBS. Subsequently, 1-2 ml of 0.25% trypsin were added, allowed to cover the surface of the flask, and incubated at 37°C until cells began to detach. This took approximately 5 minutes and was performed under intermittent visual control.

Once cells detached, 2 ml of complete growth medium was added to neutralize the action of the trypsin. Cells were then carefully re-suspended by repeated aspiration and then finally transferred to a 15 ml conical tube to be centrifuged at 125 x g for 5-10 minutes. Following this, the supernatant was removed and the cell pellet re-suspended in 5 ml complete growth medium. Thereafter, 1 ml of the resulting suspension was transferred into a new 75 cm² flask, containing

complete growth medium, and the passage was recorded to track the age and physiology of the cells. Additionally, cell morphology was observed and compared with cell viability.

2.5.4 Culture of TM3 and TM4 cells

Cells were cultured in 75 cm² flasks, allowed to reach 80% confluency and finally passaged once this was reached. To remove compounds that may interfere with the actions of trypsin, the growth medium was discarded and cells washed with 5 ml sterile PBS. Following this, 1-2 ml of 0.25% trypsin was added, allowed to cover the surface of the flask and incubated at 37°C until cells detached. This took approximately 10 minutes and was performed with intermittent visual control. To neutralize the trypsin, 2 ml of complete growth medium was added, the cells re-suspended and finally transferred to a 15 ml conical tube to be centrifuged at 125 x g for 5 minutes. Thereafter, the supernatant was removed, the cell pellet re-suspended in 5 ml complete growth medium and 1 ml of the resulting suspension was transferred into a new 75 cm² flask, containing complete growth medium. Passage numbers were recorded to track the age and physiology of the cells. Additionally, cell morphology was observed and compared with cell viability

2.5.5 Cell counting and seeding

Following the detachment of the cells with trypsin and re-suspension in fresh growth medium, cell counts were performed using a hemocytometer so that a specific cell concentration could be reached in 6-well plates or 96-well plates. This was achieved by combining 50 µl of cell suspension with an equal volume of 2% trypan blue and transferring 10 µl of this mixture to a hemocytometer counting chamber. The chamber was viewed under a microscope and the total

cell count for each experiment was calculated according to equation 2. Following this, a dilution of cells was made according to the final cell number needed for each experiment as needed.

Equation 2: Volume of cells required (μl) = $\frac{\text{Number of cells needed} \times 100}{\text{Total number of cells counted}}$

2.5.6 Cell freezing

2.5.6.1 LNCaP cells

Cells were grown to 80% confluency prior to cell freezing. To remove compounds that may interfere with the actions of trypsin, the growth medium was discarded and cells rinsed with 5 ml sterile PBS. Subsequently, 1-2 ml of 0.25% trypsin were added, allowed to cover the surface of the flask, and incubated at 37°C until cells began to detach. This took approximately 5 minutes and was performed under intermittent visual control.

Once cells detached, 2 ml of complete growth medium was added to neutralize the action of the trypsin. Cells were then carefully re-suspended by repeated aspiration, counted and then finally transferred to a 15 ml conical tube to be centrifuged at 125 x g for 5-10 minutes. Following this, the supernatant was removed, the cell pellet re-suspended in 5 ml freezing medium (50% RPMI 1640, 40% FBS and 5% DMSO), dispensed into cryogenic vials and immediately frozen at -80°C for 24 hours. Thereafter, the vials were transferred to liquid nitrogen for long-term storage.

2.5.6.2 TM3 and TM4 cells

Cells were grown to 80% confluency prior to cell freezing. To remove compounds that may interfere with the actions of trypsin, the growth medium was discarded and the cells rinsed with 5 ml sterile PBS. Subsequently, 1-2 ml of 0.25% trypsin were added, allowed to cover the

surface of the flask, and incubated at 37°C until cells began to detach. This took approximately 5 minutes and was performed under intermittent visual control.

Once cells detached, 2 ml of complete growth medium was added to neutralize the action of the trypsin. Cells were then carefully re-suspended by repeated aspiration, counted and then finally transferred to a 15 ml conical tube to be centrifuged at 125 x g for 5-10 minutes.

Hereafter, the supernatant was removed and the cell pellet was re-suspended in 10 ml freezing medium (87.5% DMEM/F-12, 5% Horse serum, 2.5% FBS and 5% DMSO), aliquoted into cryogenic vials and immediately frozen at -80°C for 24. Thereafter, the vials were transferred to liquid nitrogen for long-term storage.

2.5.7 Cell thawing

2.5.7.1 LNCaP cells

To propagate the cells, the vial containing the frozen LNCaP cells was placed into a water bath heated to 37°C to thaw the cells rapidly. Once thawed, the cells were transferred to a 15 ml conical tube containing 10 ml RPMI 1640 medium supplemented with penicillin, streptomycin, and FBS. The suspension was then centrifuged at 125 x g for 5 minutes, after which the supernatant was aseptically removed, the pellet re-suspended in 10 ml fresh RPMI 1640 and finally transferred to a 75 cm² culture flask and placed into the incubator.

2.5.7.2 TM3/TM4 cells

To propagate the cells, the vials containing the frozen TM3 and TM4 cells were placed into a water bath heated to 37°C to thaw the cells rapidly. Once thawed, the cells were transferred to a 15 ml conical tube containing 10 ml DMEM/F-12 medium supplemented with penicillin,



streptomycin, and FBS. The suspension was then centrifuged at 125 x g for 5 minutes, after which the supernatant was aseptically removed and the pellet was re-suspended in 10 ml fresh DMEM/F-12 medium. The cells were then transferred to a 75 cm² flask and left undisturbed in the incubator for 48 hours.

2.6 Test parameters

2.6.1 Determination of cell viability

Cell viability was determined using the 2,3-bis [2-methoxy-4-nitro-5-sulphophenyl]-2H-tetrazolium-5-carboxanilide (XTT) assay. This is a colorimetric assay which allows for the detection of viable and dead cells. The detection of viable and dead cells is achieved by measuring cellular metabolic activities, such as the reduction of the yellow XTT salt to a highly colored formazan dye (Wang et al. 2011). The reduction of the tetrazolium salt to the formazan dye occurs due to the action of mitochondrial reductases. These enzymes are only present in viable cells as they become deactivated shortly after cell death (Roehm et al. 1991). Therefore, the degree in the color change, and consequently the amount of formazan dye being produced, is proportional to the number of viable cells in the sample.

In short, cultures were removed from the incubator at the appropriate times and the XTT working solution was prepared by thawing and combining 100 µl of the XTT electron coupling solution with 5 ml of the XTT labeling solution. If sediment appeared, the solution was heated to 37°C and swirled until no longer opaque. Then, 100 µl of this solution were added to each well and placed into the incubator for 2-4 hours. Finally, the absorbance was read at 450 nm Glomax Multi Detection System plate-reader (Promega Corporation, Madison, U.S.A) and results were

expressed as percentage viability. This was calculated according to the absorbance of treated cells versus the absorbance of the controls, according to equation 3.

Equation 3: Percentage viability =
$$\frac{\text{ABSORBANCE sample} \times 100}{\text{ABSORBANCE control}}$$

2.6.1.1 LNCaP cell viability

LNCaP cells were grown to 80% confluency and were then trypsinated with 1-2 ml 0.25% trypsin. Thereafter, the trypsin was inactivated by adding 2 ml of complete growth medium and a cell count was performed. Following this, cells were seeded into sterile 96-well plates at 8×10^3 cells/well in 200 μ l of complete growth medium, for a 24 hour exposure, and 3×10^3 cells/well in 200 μ l of complete growth medium for a 96-hours exposure. After exposing cells to various concentrations of the aqueous *C. capensis* rhizome extract for 24 hours and 96 hours, respectively, 100 μ l of XTT were added to each well. The plates were incubated at 37°C for an additional 2 hours. Subsequently, the absorbance of the samples was measured at 450 nm with an ELISA reader (GloMax Multi Detection System).

2.6.1.2 TM3 and TM4 cell viability

The TM3 and TM4 cells were grown to 80% confluency and were the trypsinated with 1-2 ml 0.25% trypsin. Thereafter, the trypsin was inactivated by adding 2 ml of complete growth medium and a cell count was performed and cells seeded into sterile 96-well plates. For the TM3 cell line, 5×10^3 cells were used for 24 hours of exposure and 1×10^3 was used for the 96 hour exposure. When seeding the TM4 cell line, 6×10^3 cells were used for the 24 hours of exposure and 1×10^3 cells were used for the 96-hour exposure.

Once cells were seeded into 96-well plates, they were left to attach for 24 hours and then exposed to various concentrations of the extract for 24 hours and 96 hours, respectively. Thereafter, 100 μ l of the working XTT solution were added to each well and the plates were incubated at 37°C for an additional 2 hours. Subsequently, the absorbance of the samples was measured at 450 nm with an ELISA reader (GloMax Multi Detection System).

2.6.2 The effect of the aqueous *C. capensis* rhizome extract on testosterone-stimulated LNCaP cells

LNCaP cells were grown to 80% confluency and were trypsinated with 1-2 ml 0.25% trypsin. Thereafter, the trypsin was inactivated by adding 2 ml of complete growth medium and a cell count was performed. Following this, cells were seeded into sterile 96-well plates at 8×10^3 cells/well in 200 μ l of complete growth medium, for 24-hour exposure, and 3×10^3 cells/well in 200 μ l of complete growth medium for a 96-hour exposure.

Prior to seeding of cells, testosterone was dissolved in 100% ethanol to produce a stock solution. Following this, the stock solution was diluted to obtain concentrations of 1 nM, 10 nM, 100 nM and 1000 nM. The final ethanol concentration was 1% and controls were subsequently treated with 1% ethanol without testosterone. Each concentration of the extract was tested in the presence of testosterone for both 24-hour and 96-hour exposures.

Thereafter, the 2,3-bis [2-methoxy-4-nitro-5-ulfophenyl]-2H-tetrazolium-5-carboxanilide (XTT) assay was performed to measure the reduction of the yellow XTT salt to a highly colored formazan dye, which is an indication of the cell viability. For both, the 24-hour and the 96-hour exposure, 100 μ l of the XTT working solution were added to each well and allowed to incubate

for 2 hours at 37°C. Following this, the absorbance was determined at 450 nm using an ELISA plate reader. Finally, the percentage of cell viability was calculated for each set of experiments.

2.6.3 Effect of the aqueous *C. capensis* rhizome extract on LNCaP cells in combination with doxorubicin

The chemotherapeutic agent doxorubicin (DOX) was dissolved in DMSO to arrive at a 100 µM solution. The stock solution was diluted to yield concentrations ranging from 0.001 nM- 100 000 nM.

Following this, LNCaP cells that were grown to 80% confluency and then trypsinated with 1-2 ml 0.25% trypsin. Thereafter, the trypsin was inactivated by adding 2 ml of complete growth medium and a cell count was performed. Subsequently, cells were seeded into sterile 96-well plates at 8×10^3 cells/well in 200 µl of complete growth medium and treated with the varying concentrations of DOX. After the treatment with DOX, the cells were washed with PBS and supplied with fresh growth medium. Next, the 2,3-bis [2-methoxy-4-nitro-5-ulfophenyl]-2H-tetrazolium-5-carboxanilide (XTT) assay was performed to measure the reduction of the yellow XTT salt to a highly coloured formazan dye. The degree in the colour change, and consequently the amount of formazan dye being produced, is proportional to the number of viable cells in the sample. To accomplish this 100 µl of the XTT working solution was added to each well and allowed to incubate for 2 hours. Following this, the absorbance was determined at 450 nm and the IC₅₀ value for DOX was calculated. Hereafter, the IC₅₀ value was combined with each concentration of the extract, in an equi-Molar fashion, for 24 and 96 hour incubation periods and the combined effect on cell viability was determined by performing the aforementioned XTT assay.

2.6.4 Determination of DNA fragmentation

To determine DNA fragmentation, which indicates an endpoint of apoptosis, the Dead end Colorimetric Tunnel System kit was used. This is a non-radioactive assay which allows for accurate and rapid detection of DNA fragmentation. For the assay, poly-L-lysine was diluted 1:10 in distilled water and slides were coated by spreading 200 μ l of the solution in water onto the surface of a clean glass slide. Once dry, slides were rinsed in distilled water and allowed to air dry for approximately 30 minutes and then stored at 4°C.

LNCaP Cells were seeded at 6×10^5 cells/well in 1 ml of complete growth medium for a 24-hour exposure and at 2×10^5 cells/well in 1 ml of complete growth medium for a 96-hours exposure in sterile 6-well plates. After exposure of cells to various concentrations of the extract, the medium was discarded and cells were washed with 1 ml PBS and trypsinated with 500 μ l of 0.25% trypsin. After trypsination, cell suspensions were transferred from 6-well plates to Eppendorf tubes and cell pellets were collected by centrifugation at 125 x g for 8 minutes. Subsequently, the supernatant was discarded, the cells were re-suspended in 1 ml PBS and 50 μ l were placed on poly-L-lysine-coated slides and allowed to air dry prior to fixation. Fixation was carried out for 25 minutes in 4% formaldehyde solution in PBS. Afterwards, slides were washed for 5 minutes in fresh PBS at room temperature.

To permeabilize cells, the slides were immersed in 0.2% triton X-100 solution in PBS for 5 minutes at room temperature and then rinsed in fresh PBS for 5 minutes at room temperature. After removing excess liquid was removed by gently tapping the slides, cells were covered with 100 μ l equilibration buffer to equilibrate at room temperature for 10 minutes. During this time, the biotinylated nucleotide mix was thawed and sufficient amounts of Terminal Deoxynucleotidyl Transferase, Recombinant (rTdT) reaction mix was prepared as shown in Table 1.

Table 1: Preparation of rTdT reaction mix

Buffer component	Component volume per standard 100 μ l reaction
Equilibration buffer	98 μ l
Biotinylated Nucleotide mix	1 μ l
rTdT enzyme	1 μ l

Tissue paper was used to blot around the equilibrated areas, 100 μ l of rTdT reaction mix was added and slides were covered with plastic cover slips to ensure an even spread of the reagent. Then, slides were incubated at 37°C for 60 minutes inside a humidified chamber. Subsequently, slide were incubated in 2x SSC in a Coplin jar for 5 minutes to stop the reaction. Thereafter, slides were washed in fresh PBS for 5 minutes at room temperature and then immersed in 0.3% hydrogen peroxide in PBS for 5 another minutes at room temperature to block all endogenous peroxidases.

Following this, slides were washed for 5 minutes in PBS at room temperature, after which 100 μ l horseradish peroxidase-labelled streptavidin (Streptavidin HRP) solution (provided with the kit), diluted 1:500 in PBS were added to the slides and slides incubated for 30 minutes at room temperature. Then, slides were washed in PBS for 5 minutes. Following this, 100 μ l of the diaminobenzidine (DAB) solution, prepared by combining 50 μ l DAB Substrate 20X buffer, 950 μ l distilled water, 50 μ l DAB 20X chromogen and 50 μ l hydrogen peroxide 20X, were added to each slide until a light brown background develops. Finally, slides were rinsed repeatedly in distilled water and mounted in glycerol. Staining was then observed using a light microscope and up to 200 cells were counted in total on each slide, where brown stained cells

were considered TUNEL-positive and unstained cells considered TUNEL-negative (Figure 11). Finally, the percentage of TUNEL-positive cells was calculated.

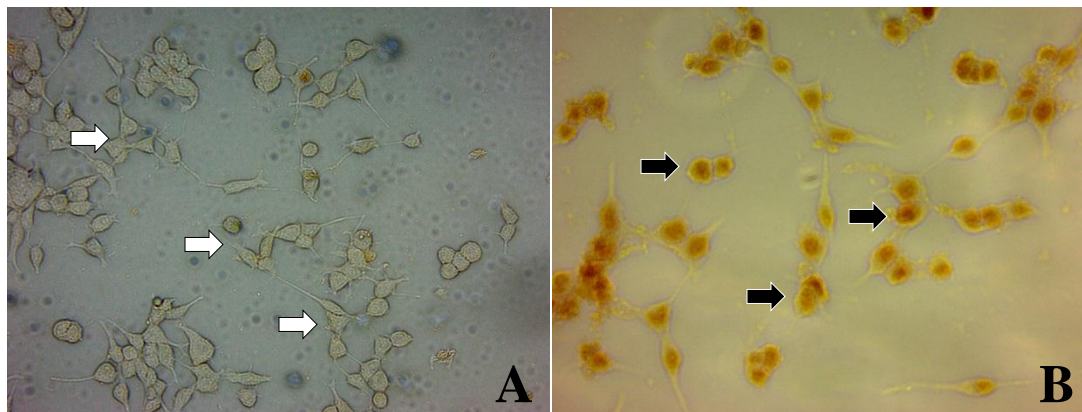


Figure 11: Determination of DNA fragmentation in LNCaP cells after exposure to the extract at different concentrations. Cells considered TUNEL-negative are shown in A (White arrows), whereas cells considered TUNEL-positive are shown in B (Black arrows).

2.6.5 Determination of protein concentration

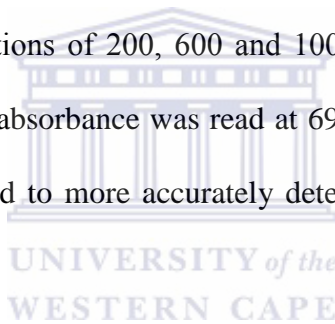
To accurately measure the complete biochemical parameters, the protein concentration of the cells was determined used as a reference. This was done by performing the BIO-RAD Protein assay in which the dye binds un-specifically to amino groups of any protein within the sample. After 24 hours and 96 hours of incubation with the various concentrations of the extract, the protein contents were determined for cell proliferation.

The medium of each well was removed and 200 μ l lysis-reagent, consisting of 0.5 M NaOH + 0.1% SDS, were added. Following this, the plates were incubated at room temperature for 30 minutes while being gently rocked on a plate shaker.

Thereafter, 20 μl of the lysate was transferred in duplicate into non-sterile 96-well plate, along with 25 μl of reagent A and 200 μl of reagent B. The resulting mixture was incubated for 30 minutes at room temperature. Thereafter, the absorbance was measured at 690 nm using an ELISA plate reader (GloMax Multi Detection System). This was then compared to a standard curve which was established by using BSA under the same conditions of the BIO-RAD Protein assay.

2.6.6 BSA standard curve

A stock solution of 1400 $\mu\text{g/ml}$ of BSA was prepared by dissolving it in 1 ml of lysis-reagent and further diluted into concentrations of 200, 600 and 1000 $\mu\text{g/ml}$ (Table 2). The blank was lysis-reagent only. Thereafter, the absorbance was read at 690 nm, a standard curve (Figure 12) was calculated and the results used to more accurately determine the effects of the extract on TM3 Leydig cells.



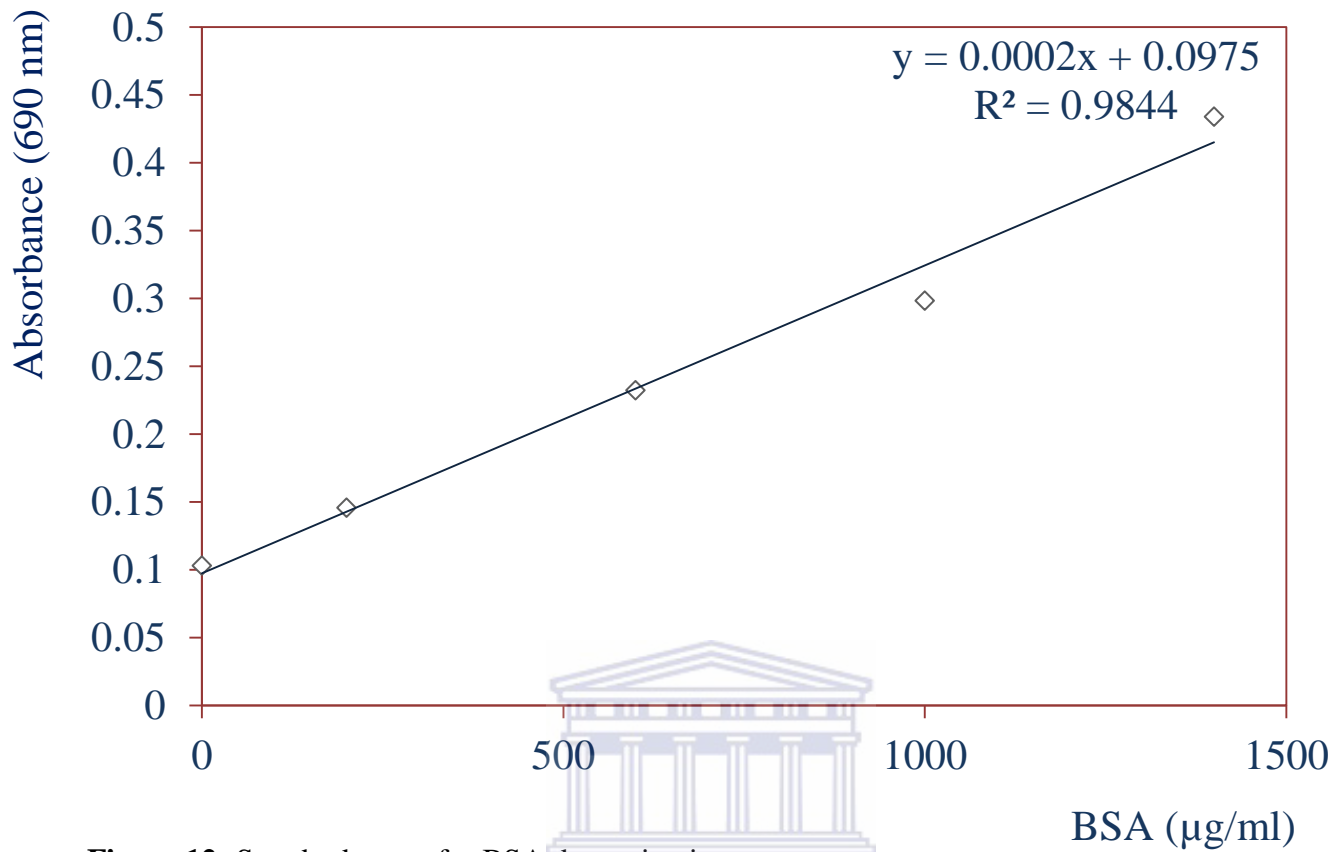


Figure 12: Standard curve for BSA determination

Table 2: BSA preparation for standard curve

BSA stock + Lysis reagent	BSA concentration
100 + 40	1000
100 + 133	600
100 + 600	200
0 + 600	0

2.6.6 Determination of testosterone production in TM3-Leydig cells

For the purpose of this study, TM3-Leydig cells were stimulated with human chorionic gonadotropin (hCG) to trigger the production of testosterone. A stock solution was made of hCG and 5 µl/ml was added to each concentration of the extract. After TM3-Leydig cells were exposed to the extract for of 24 hours and 96 hours, respectively, testosterone production was measured by performing the Testosterone ELISA kit assay. All necessary solutions and antibody-coated micro-plate were provided by the manufactures and a standard curved (Figure 13) was calculated with each set of experiments.

To prepare the wash solution 30 ml of the 40x concentrated solution were added to 1170 ml of deionized water to a final volume of 1200 ml. The testing procedure required 25 µl samples to be transferred into the 96-well plate, provided by the manufacturer, with the addition of 200 µl Enzyme Conjugate mixed thoroughly for 10 seconds and incubated for 1 hour at room temperature. During this time, the wash solution was prepared by adding 30 ml of the 40x concentrated solution to 1170 ml of deionized water to a final volume of 1200 ml. After the incubation period, the contents of the micro-plate were shaken out briskly, rinsed 3 times with 400 µl of diluted wash solution and then struck sharply on paper towel to remove excess solution. Following this, 200 µl of the Substrate solution were added to each well and incubated for 5 minutes at room temperature, after which 100 µl of Stop solution was added to stop the enzymatic process. Finally, absorbance was determined at 450 nm with an ELISA plate reader and the concentration of testosterone production was determined.

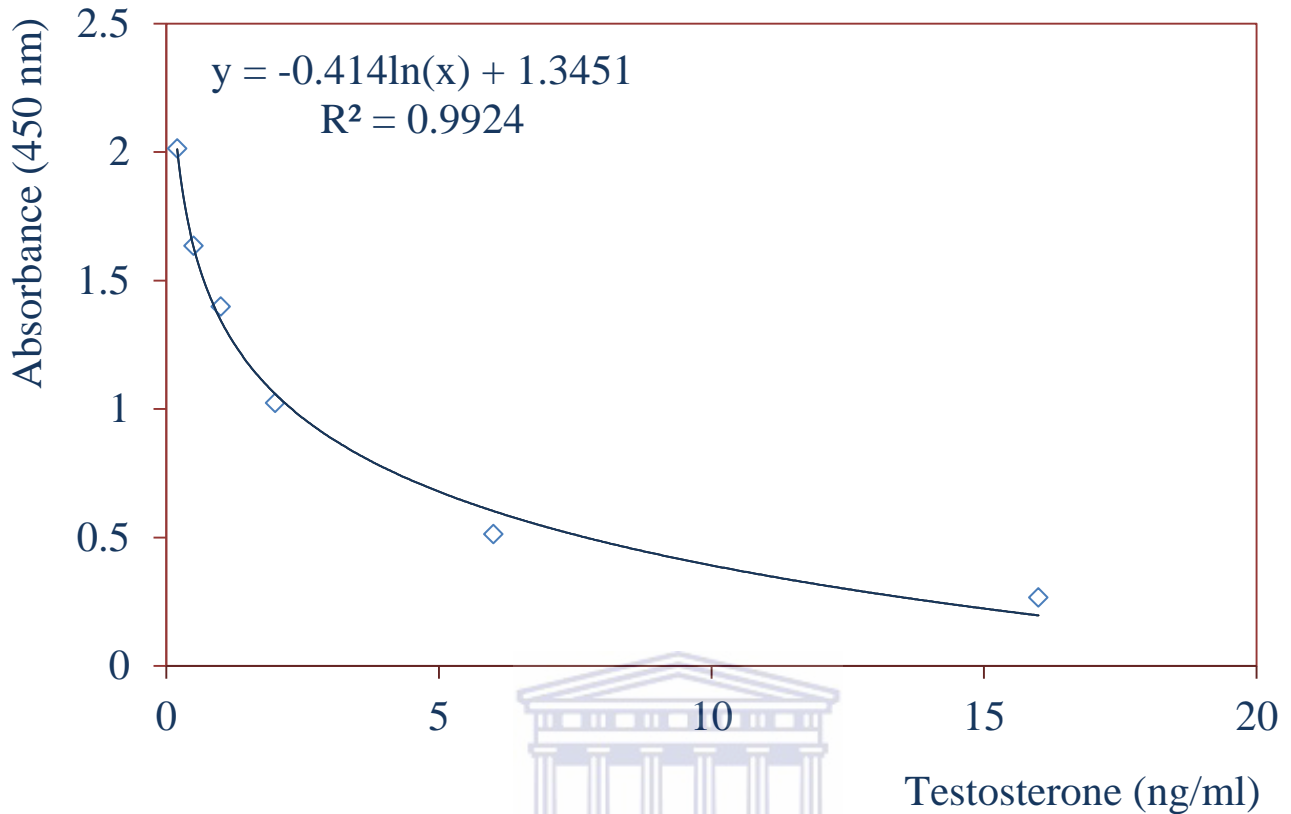


Figure 13: Standard curve for testosterone production in TM3 Leydig cells.

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2.6.7 Determination of lactate dehydrogenase (LDH) secretion of TM4-Sertoli cells

Following the exposure of TM4 cells to the extract for 24- and 96-hour periods, the lactate secretion was determined by means of measuring the lactate dehydrogenase activity. This assay measures the inter-conversion of pyruvate and lactate by the oxido-reductase enzyme lactate dehydrogenase (LDH). All reagent required were provided by the manufacture and a standard curve (Figure 14) was calculated with each set of experiments. Prior to performing the experiment, the LDH assay buffer was allowed to reach room temperature and the LDH substrate mix was reconstituted by adding 1 ml of distilled water to the vial provided with the kit. The standard was reconstituted by adding 400 µl of distilled water to generate the 1.25 mM solution. The testing procedure required 50 µl samples to be added in duplicate to a 96-well plate.

Thereafter, 50 μl of the master reaction mix (Table 3) was added to each well. Following this, the plates were incubated for 2-3 minutes at 37°C before the initial measurement (T_{initial}) was taken at 450 nm using a plate reader. Next, the plates were incubated at 37°C and read every 5 minutes at 450 nm until the most active sample exceeded the highest standard (12.4 nmole/well). This reading was considered as T_{final} and the LDH activity was calculated.

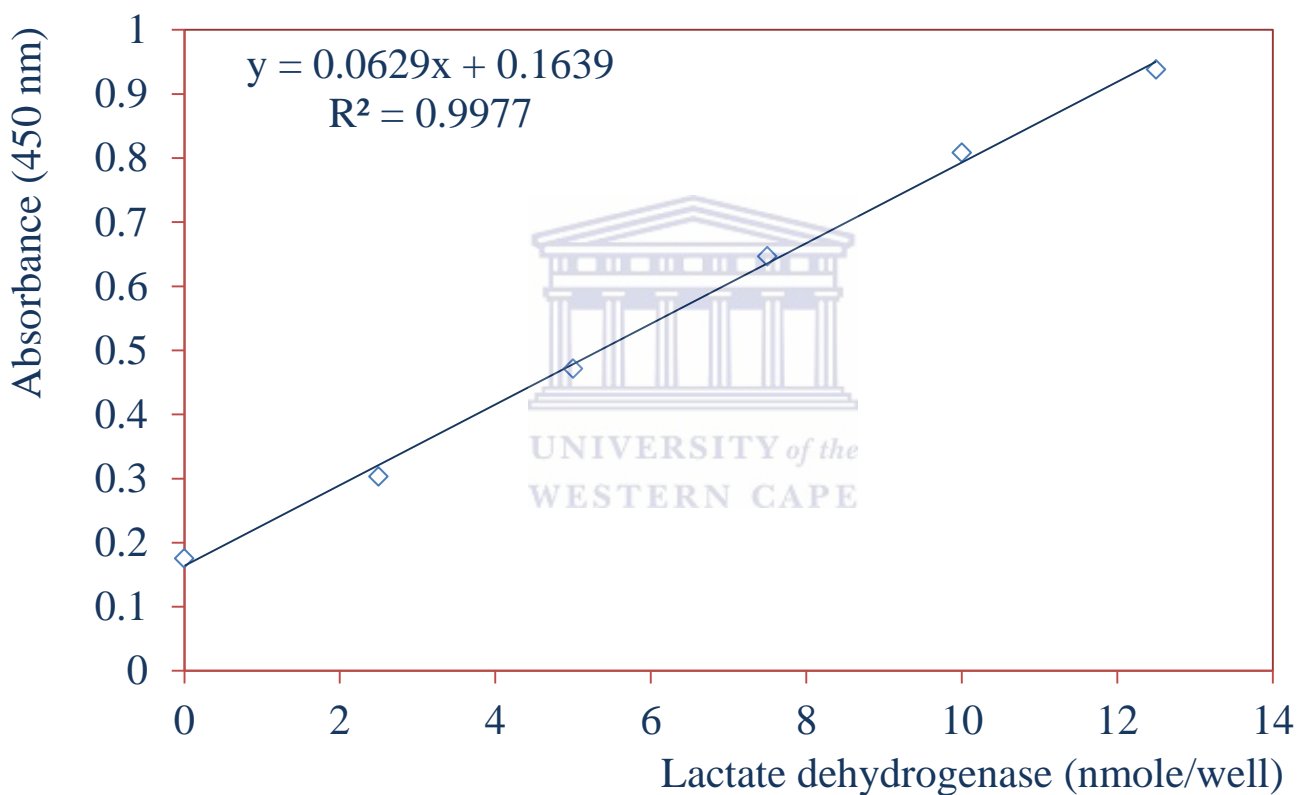


Figure 14: Standard curve for the determination of Lactate dehydrogenase activity in TM4 Sertoli cells.

Table 3: Preparation of Master Reaction Mix

Reagent	Master reaction mix per 50 μ l reaction
LDH assay buffer	48
LDH substrate mix	2

2.6.8 Determination of ROS and RNS inhibiting activity

The capability of the extract to scavenge ROS and RNS was assessed using chemi-luminescent ABEL[®] Antioxidant Test Kits containing Pholasin[®] specific for oxygen radicals such as superoxide anion and nitrogen species like peroxy nitrite anion. Pholasin[®] is a photo protein isolated from the mollusc *Pholas dactylus*, which emits light in the presence of certain oxidants. The antioxidant capacity of the sample under test is expressed as the percentage reduction of peak luminescence of Pholasin[®] observed during the course of the test in the presence and absence of the sample.

2.6.8.1 Superoxide

All reagents needed to perform the chemi-luminescent test for superoxide were provided with the kit, which was carried out following the manufacturer's instructions. In short, the assay buffer, samples, antioxidant Pholasin[®] and solution A were combined in a micro-plate, while solution B was only injected into each well when the plate was in the light measuring position. To perform the assay, 15 μ l of assay buffer and 10 μ l of the various concentrations of the extract were added to each well. For control groups, 25 μ l of assay buffer without the extract were used. Next, 50 μ l of antioxidant Pholasin[®] was added to each well along with 100 μ l of solution A.

Thereafter, 25 µl of solution B were injected into each well immediately before measurement while the micro-plate was in the light measuring position. The measurement of luminescence was carried out using the LUMI star Galaxy plate reader (BMG Labtech), Ortenburg, Germany). The antioxidant capacity of a sample was expressed as percentage reduction of peak luminescence according to equation 4:

$$\text{Equation 4: Inhibition (\%)} = \frac{\text{PEAK control} - \text{PEAK sample}}{\text{PEAK control}}$$

2.6.8.2 Peroxynitrite

All reagents needed to perform the chemi-luminescent test for peroxy-nitrite were provided with the kit, which was carried out following the manufacturer's instructions. Initially, the assay buffer, samples and antioxidant Pholasin[®] were combined in a micro-plate, while the SIN-1 solution was only injected into each well when the plate was in the light measuring position.

For the assay, 100 µl of the peroxy-nitrite reconstitution and assay buffer without extract were added to each well for control groups or 95 µl of assay buffer and 5 µl of the different concentrations of the extract. Next, 50 µl of the SIN-1 solution was injected into each well using an automatic dispenser, after the assay had commenced. The measurement of luminescence was carried out using the LUMIstar Galaxy plate reader (BMG Labtech) and the antioxidant capacity of a sample was according to equation 5.

$$\text{Equation 5: Inhibition (\%)} = \frac{\text{PEAK control} - \text{PEAK sample}}{\text{PEAK control}}$$

2.6.9 Determination of collagenase inhibiting activity

Collagenase activity was determined using the Collagenase/Gelatinase Assay Kit and all reagents and samples were prepared according to the manufacturer's instructions.

In short, the lyophilized protein standards were reconstituted by combining one vial containing the gelatin substrate with 1 ml of deionized water to yield a 1.0 mg/ml stock solution. A 1x reaction buffer was prepared by combining 2 ml of the 20x reaction buffer with 18 ml deionized water. A 1000 U/ml stock solution of collagenase was prepared by adding to 0.5 ml deionized water to one vial. For experiments, the collagenase stock solution was diluted to 0.1 U/ml in the reaction buffer, 0.1 M Tris-HCl, pH 8.0, containing 0.2 mM sodium azide and 0.5% bovine serum albumin, while the extract was diluted in 1x reaction buffer at various concentrations.

The assay was run by adding 80 µl of the diluted *C. capensis* to each well, followed by adding 20 µl of the gelatin substrate. Next, 100 µl collagenase solution (0.1 U/ml) were added to each well and the fluorescence was measured continuously for up to 2 hours at room temperature. This was accomplished using a fluorescence plate reader (FLUOstar Galaxy, BMG Labtech). The increase in fluorescence measured is proportional to the proteolytic activity. Fluorescence in the sample wells is decreased compared to the control if the sample is able to inhibit the collagenase activity. The measurement is stopped when the collagenase control reaches its maximum. Collagenase activity in the samples was calculated using the fluorescence intensity of the sample wells in percentage of the control fluorescence intensity at this time point according to equation 6:

Equation 6: Collagenase activity (%) = $100 \times \frac{\text{SAMPLE}_{\text{time max}}}{\text{CONTROL}_{\text{time max}}}$

2.6.10 Determination of Elastase inhibiting activity

Elastase was taken from the EnzChek Elastase Assay Kit. The lyophilized protein standards were reconstituted as recommended in the manufacturers' instructions. In short, one vial containing the elastin substrate was added to 1 ml of deionized water to produce a 1 mg/ml stock solution. To prepare the 1x reaction buffer needed, 6 ml of the 10x reaction buffer was added to 45 ml of deionized water. The vial containing porcine pancreatic elastase was prepared by dissolving the contents in 0.5 ml distilled water to yield a 100 U/ml stock solution. For experiments, this stock solution was diluted to 0.1 U/ml in the reaction buffer, 0.1 M Tris-HCl, pH 8.0, containing 0.2 mM sodium-azide and 0.5% bovine serum albumin, and *C. capensis* was diluted in the 1x reaction buffer. The elastin stock solution was diluted tenfold with the 1x reaction buffer, to yield a 100 µg/ml working solution

When performing the experiment, 50 µl of the diluted the extract was added to each well, as well as 50 µL of the DQ elastin substrate. This was followed by the addition of 100 µl elastase solution (0.1 U/ml) to each well. The fluorescence was measured continuously for up to 2 h, at room temperature (excitation wavelength: 495 nm, emission wavelength: 515nm), using a fluorescence plate reader (FLUOstar Galaxy, BMG Labtech). The increase in fluorescence measured is proportional to the proteolytic activity. Fluorescence in the sample wells is decreased compared to the control if the sample is able to inhibit the elastase activity. Elastase activity in the samples was calculated using the fluorescence intensity of the sample wells in percentage of the control fluorescence intensity at this time point according to equation 7:

Equation 7: Elastase activity (%) = $100 \times \frac{\text{SAMPLE time max}}{\text{CONTROL time max}}$

2.7 Statistical Analysis

Data generated in the present study were recorded and analysed statistically using MedCalc for Windows, version 12.7.5.0 (MedCalc Software, Mariakerke, Belgium). All experiments were run in triplicate and sample sized were kept to 10.

After calculating the summary stats, including the Kolmogorov-Smirnov test for normal distribution, data were analyzed by means of the independent t-test if normally distributed. If the samples were not normally distributed, the Mann-Whitney test was used. To test for a trend between parameters, the repeated measures and one-way ANOVA was performed. A P-value of less than 0.05 was considered significant.



Chapter 3

Results

3.1 Cell Viability

3.1.1 LNCaP Cell Viability

After being exposed to increasing concentrations of the aqueous *C. capensis* rhizome extract, the cell morphology was observed and recorded. It was found that no observable morphological changes in the flat and polygonal cells had occurred between control groups and 10 µg/ml (A-F). It was only at higher concentrations (G-H) that cells began showing visible signs of stress (Figure 15).

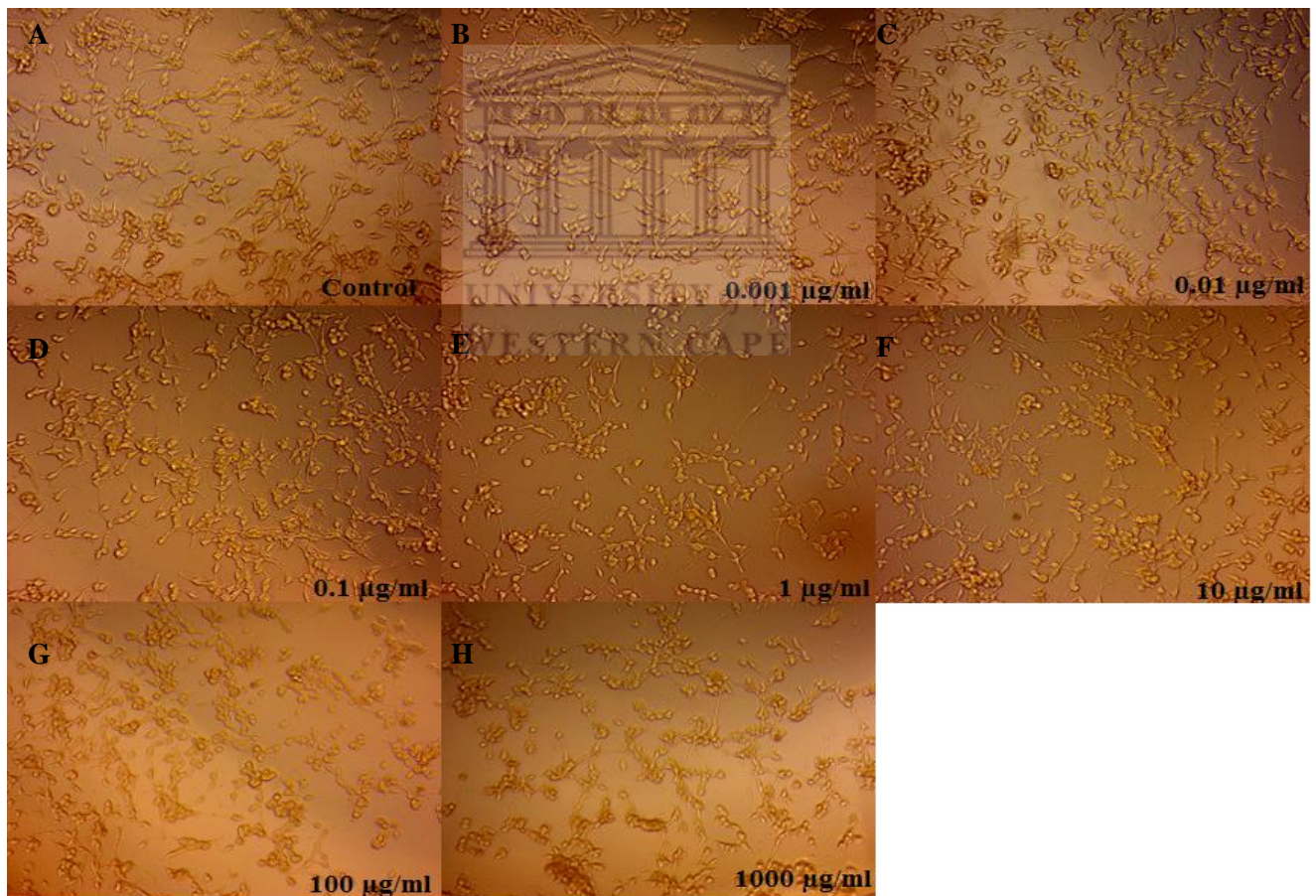


Figure 25: LNCaP cell morphology after being exposed to increasing concentrations of the aqueous *C. capensis* rhizome extract over 24 hours. Morphology remained unchanged up until 100µg/ml (A-F). At higher concentrations (G-H) stress became evident.

The XTT assay (Figure 16) revealed statistically significant ($P=0.0025$, $P=0.0002$, $P=0.0003$) increases in the viability between the control and 0.1 $\mu\text{g/ml}$, along with a statistically significant ($P=0.0046$) decrease in the viability between the control and 1000 $\mu\text{g/ml}$. Both, the observed increase between the control and 0.1 $\mu\text{g/ml}$, along with the decrease between 0.1 $\mu\text{g/ml}$ and 1000 $\mu\text{g/ml}$, occurred in a dose-dependent manner. When analyzed further, the repeated measures ANOVA displayed an initial significant ($P=0.003$) positive trend bellow between control and 0.1 $\mu\text{g/ml}$, followed by a significant ($P=0.0037$) negative trend between 0.1 $\mu\text{g/ml}$ and 1000 $\mu\text{g/ml}$. One-way ANOVA exhibited a significance ($P<0.001$) trend between the control and 1000 $\mu\text{g/ml}$.

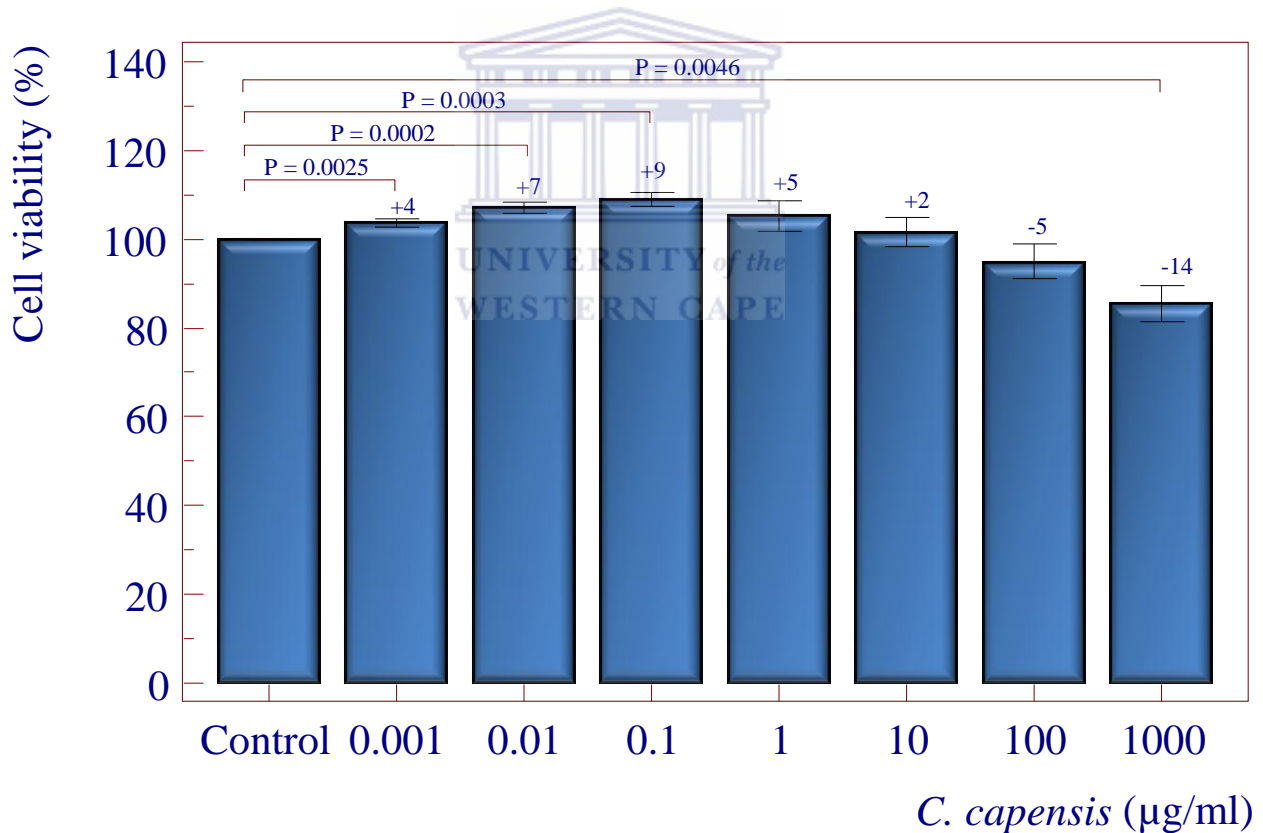


Figure 16: Cell viability of LNCaP cells as determined by XTT assay for an exposure time of 24 hours. Cells showed a significant ($P=0.0025$, $P=0.0002$ and $P=0.0003$) increase in percentage viability (≤ 0.1 $\mu\text{g/ml}$), followed by a significant ($P=0.0046$) decrease beyond 0.1 $\mu\text{g/ml}$.

After being exposed to the aqueous *C. capensis* rhizome extract for 96 hours, the cell morphology was observed and recorded. It was noted that low levels of stress, observed as cell stacking with a slightly less flat and polygonal morphology, yet survival, was apparent between the control groups and 10 $\mu\text{g/ml}$ (A-F). It was only at concentrations higher than 10 $\mu\text{g/ml}$ (G-H) that cell rounding and clumping, and therefore stress (G), along with cell death (H) were evident (Figure 17).

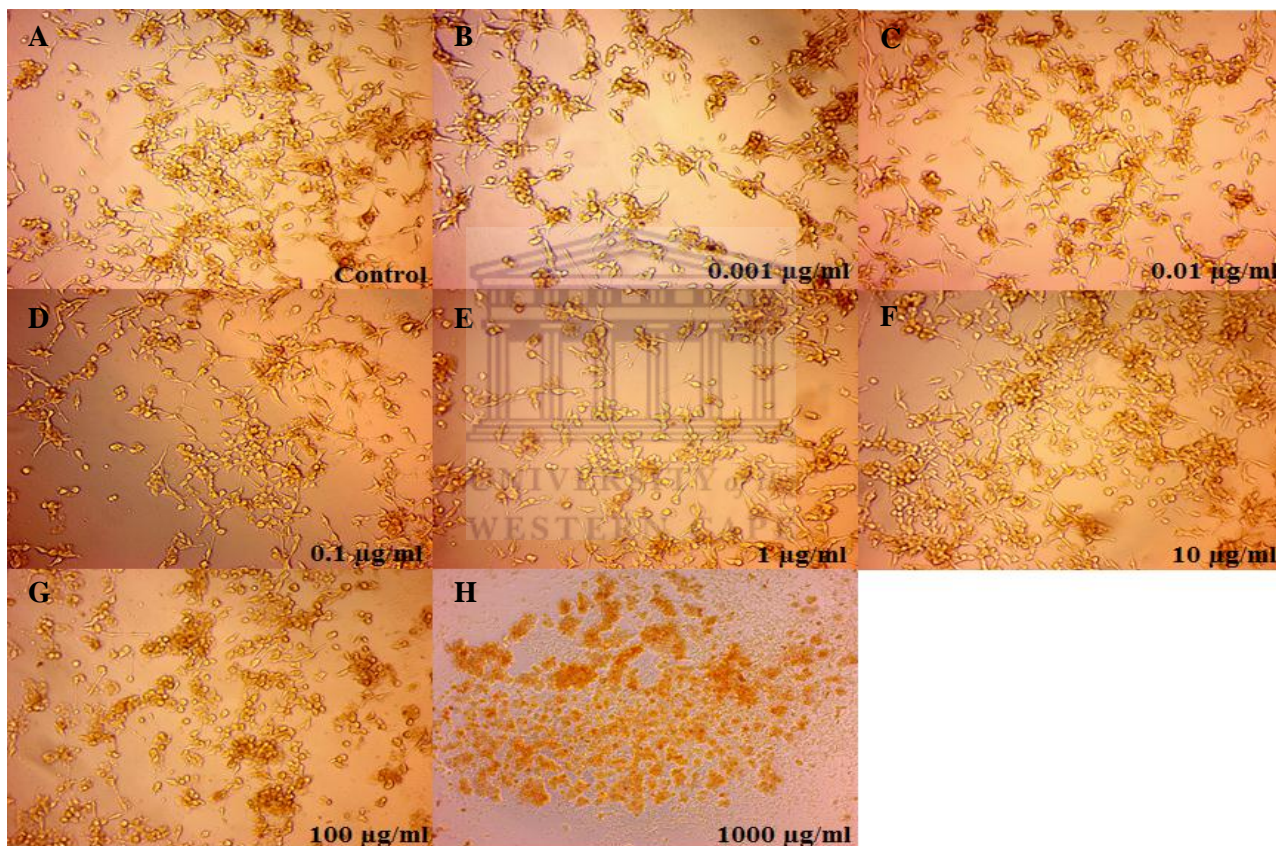


Figure 17: Observed LNCaP cell morphology after being exposed to increasing concentrations of the aqueous *C. capensis* rhizome extract over 96 hours. Slight changes in morphology, from flat and polygonal to slightly less flat and stacked, were observed up until 10 $\mu\text{g/ml}$ (A-F). All higher concentrations (G-H) showed a changed in cell morphology from flat and polygonal to rounded and clumped (G), along with total cell death (H).

Similarly, the XTT assay revealed only minor decreases in the viability between the control groups and 10µg/ml (Figure 18). At higher concentrations, a steady, dose-dependent decline in the viability was observed. A statistically significant ($P=0.0002$) decline was observed at 100 µg/ml, along with a further marked, statistically significant ($P<0.0001$) decline at the highest concentration used in this study (1000 µg/ml). The repeated measures ANOVA showed a significant ($P<0.0001$) negative trend between the control and 1000 µg/ml, along with One-way ANOVA, revealing significance ($P<0.001$) between the control and 1000 µg/ml.

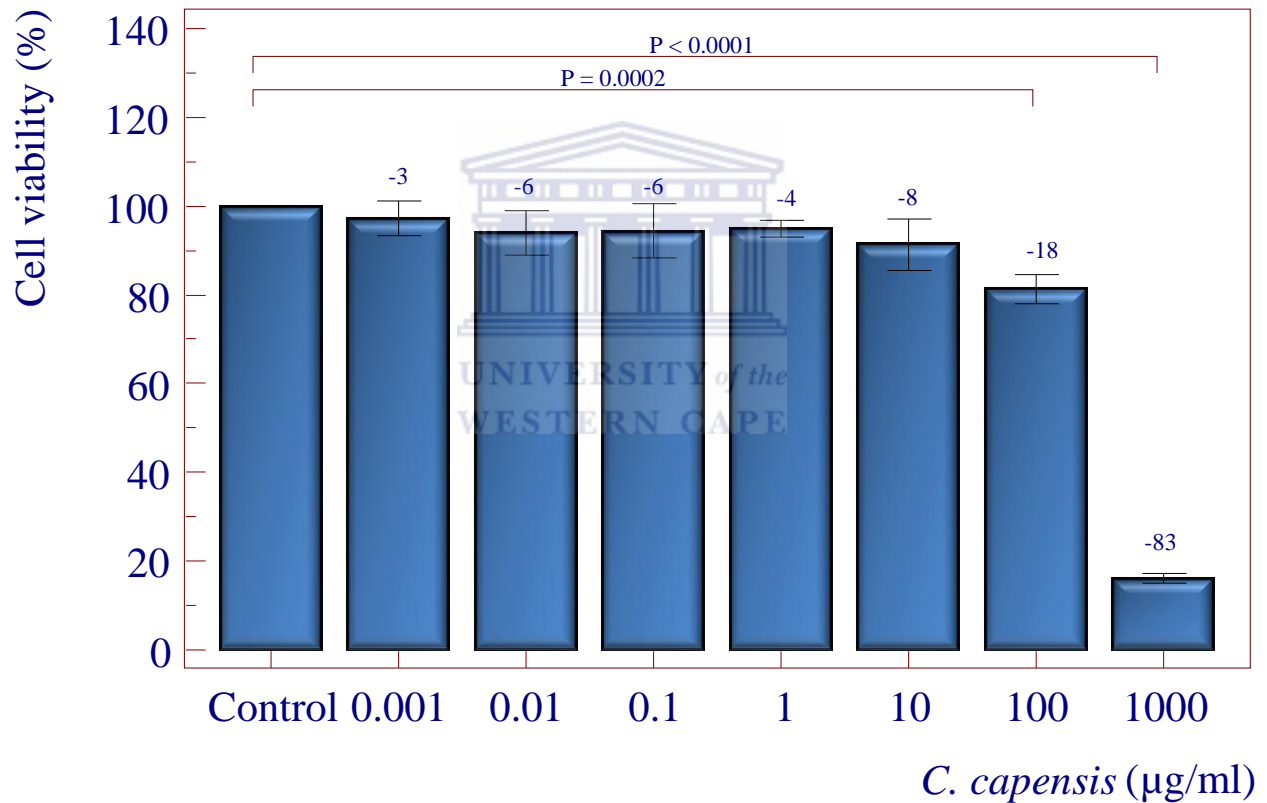


Figure 18: Cell viability of LNCaP cells as determined by the XTT assay for an exposure time of 96 hours. Cells showed no significant change in percentage viability below 10 µg/ml, while showing a significant ($P=0.0002$ and $P<0.0001$) decrease in percentage viability beyond this concentrations (10 µg/ml-1000 µg/ml).

3.1.2 TM3 Cell viability

After being exposed to increasing concentrations of the aqueous *C. capensis* rhizome extract over 24 hours, the cell morphology was observed and recorded (Figure 19). It was found that no observable changes in the typical rounded, and clustered morphology had occurred between the control groups and 10 $\mu\text{g/ml}$ (A-F). At higher concentrations (G-H), cells became notably less clustered, more sparsely spaced, and rounder than usual (G), along with showing signs of cell death (H).

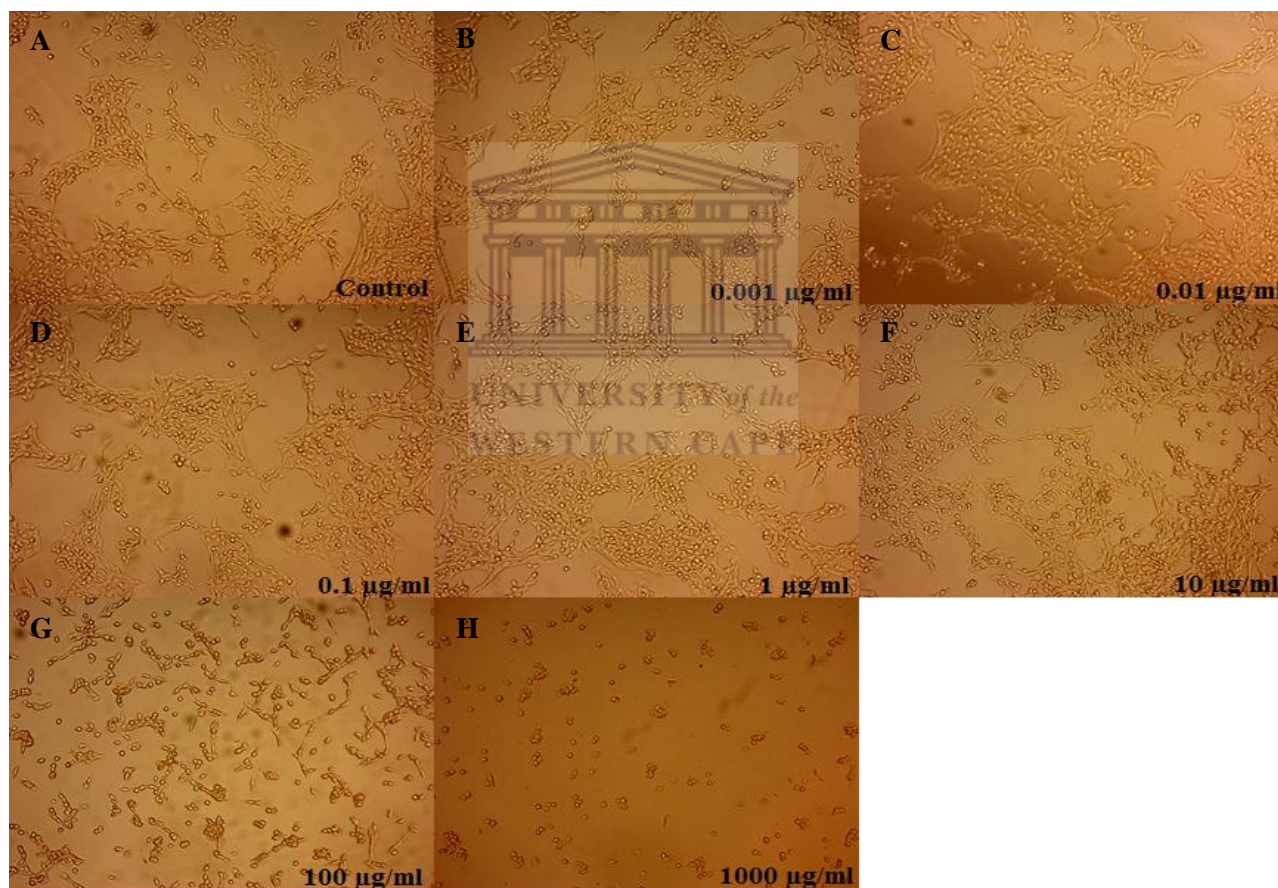


Figure 19: TM3 cell morphology after being exposed to increasing concentrations of the aqueous *C. capensis* rhizome extract over 24 hours. Morphology remained unchanged up until 10 $\mu\text{g/ml}$ (A-F). At higher concentrations (G-H) cells became rounder than usual and more sparsely spaced (G), and showed evidence of cell death (H).

The XTT assay revealed no change in percentage viability between the control groups and 100 µg/ml (Figure 20). It was only at concentrations as of 1000 µg/ml that the viability increased, with a statistically significant ($P=0.0147$) increase in viability observed at 1000 µg/ml. When analyzed further, the repeated measures ANOVA revealed an insignificant (P) positive trend between the control and 1000 µg/ml, along with one-way ANOVA showing an insignificant ($P=0.054$) trend between the control and 1000 µg/ml.

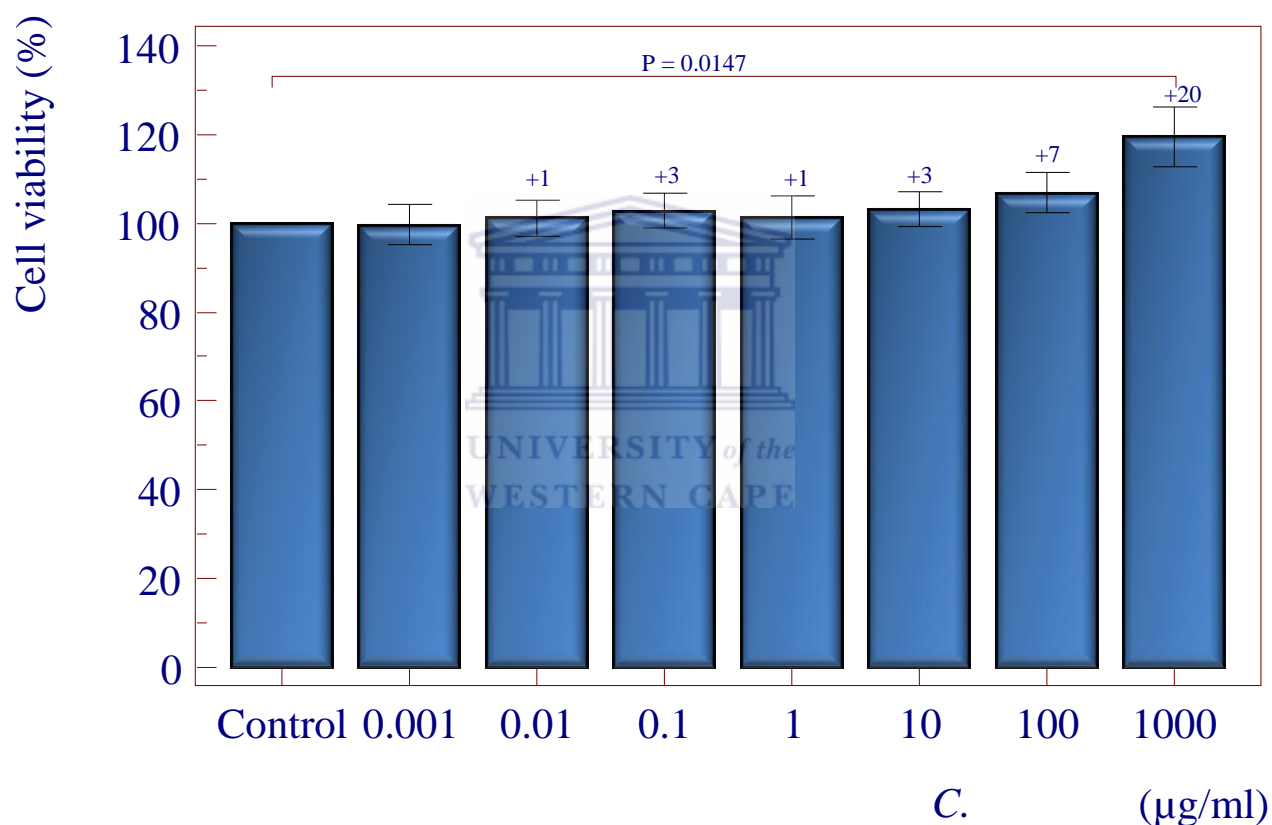


Figure 20: TM3 cell viability as determined by the XTT assay over a 24 hour exposure. Cells showed no significant change in percentage viability, up until 1000 µg/ml was reached. It was only at this concentrations that a significant ($P=0.0147$) increase in viability was found.

After being exposed to the extract for 96 hours, the cell morphology was observed and recorded (Figure 21). It was observed that the cell morphology remained typically rounded and clustered, and therefore unchanged between the control groups and 10 $\mu\text{g/ml}$ (A-F). At higher concentrations (G-H), cell rounding typical of cell death was observed (G) along with total cell death (H).

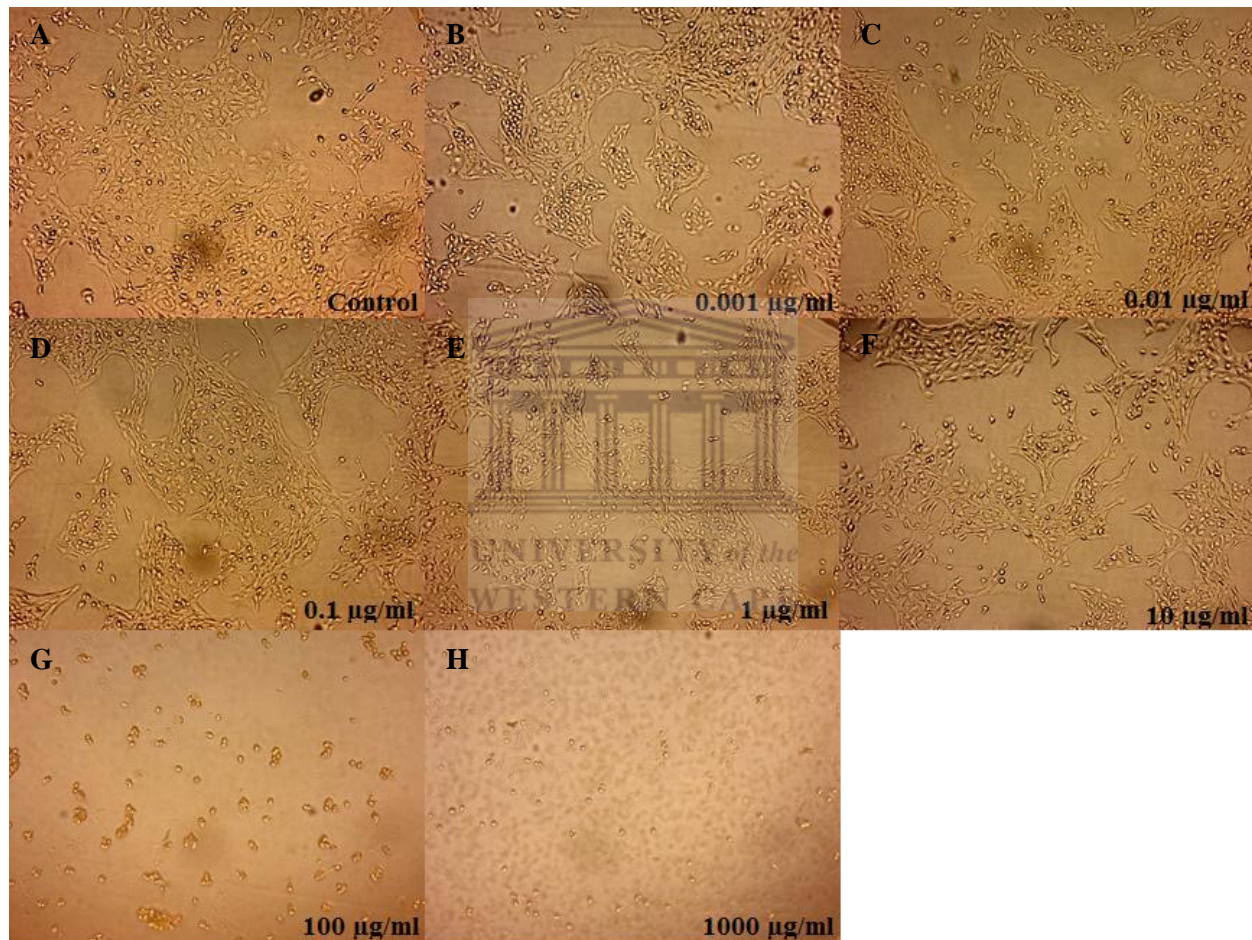


Figure 21: TM3 cell morphology after being exposed to increasing concentrations of the aqueous *C. capensis* rhizome extract over 96 hours. Cell morphology remained typically rounded and clustered, and therefore unchanged up until 10 $\mu\text{g/ml}$ (A-F). At higher concentration (G-H) cells became atypically rounded and showed clear signs of cell death.

Similarly, the XTT assay displayed only a minor elevation in viability at 0.001 $\mu\text{g/ml}$ and 0.01 $\mu\text{g/ml}$, with no change in viability between the control group and 0.1 $\mu\text{g/ml}$ and 1 $\mu\text{g/ml}$ (Figure 22). As of 10 $\mu\text{g/ml}$, viability began to decline in a dose-dependent manner that was statistically significant ($P < 0.0001$) at 100 $\mu\text{g/ml}$ and 1000 $\mu\text{g/ml}$, respectively. The repeated measures ANOVA that revealed a significant ($P < 0.0001$) trend between the control and 1000 $\mu\text{g/ml}$, as well as one-way ANOVA, revealing a significant ($P < 0.001$) trend between the control and highest concentration used in this study (1000 $\mu\text{g/ml}$).

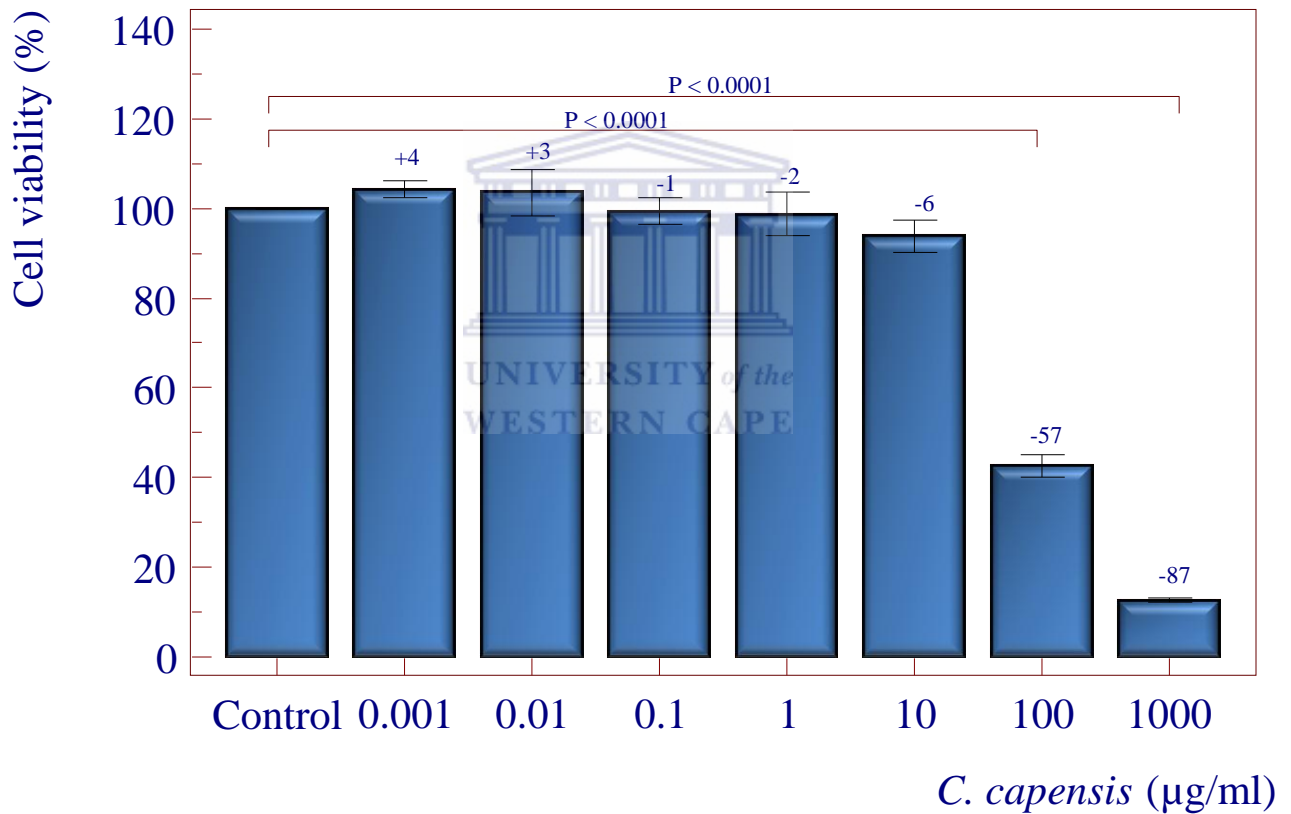


Figure 22: TM3 cell viability as determined by the XTT assay over a 96 hour exposure. Cells showed no significant change in percentage viability, up until 100 $\mu\text{g/ml}$ and 1000 $\mu\text{g/ml}$ were reached. It was only at these concentrations that a significant ($P < 0.0001$) decrease in percentage viability was found.

3.1.3 TM4 cell viability

After being exposed to increasing concentrations of the aqueous *C. capensis* rhizome extract over 24 hours, the cell morphology was observed and recorded (Figure 23). No observable changes in the typical flat, polygonal and clustered morphology had occurred between the control groups and 10 $\mu\text{g/ml}$ (A-F). At higher concentrations (G-H), cells became less clustered and slightly less flat and polygonal (G), as well as becoming atypically rounded and even less clustered (H).

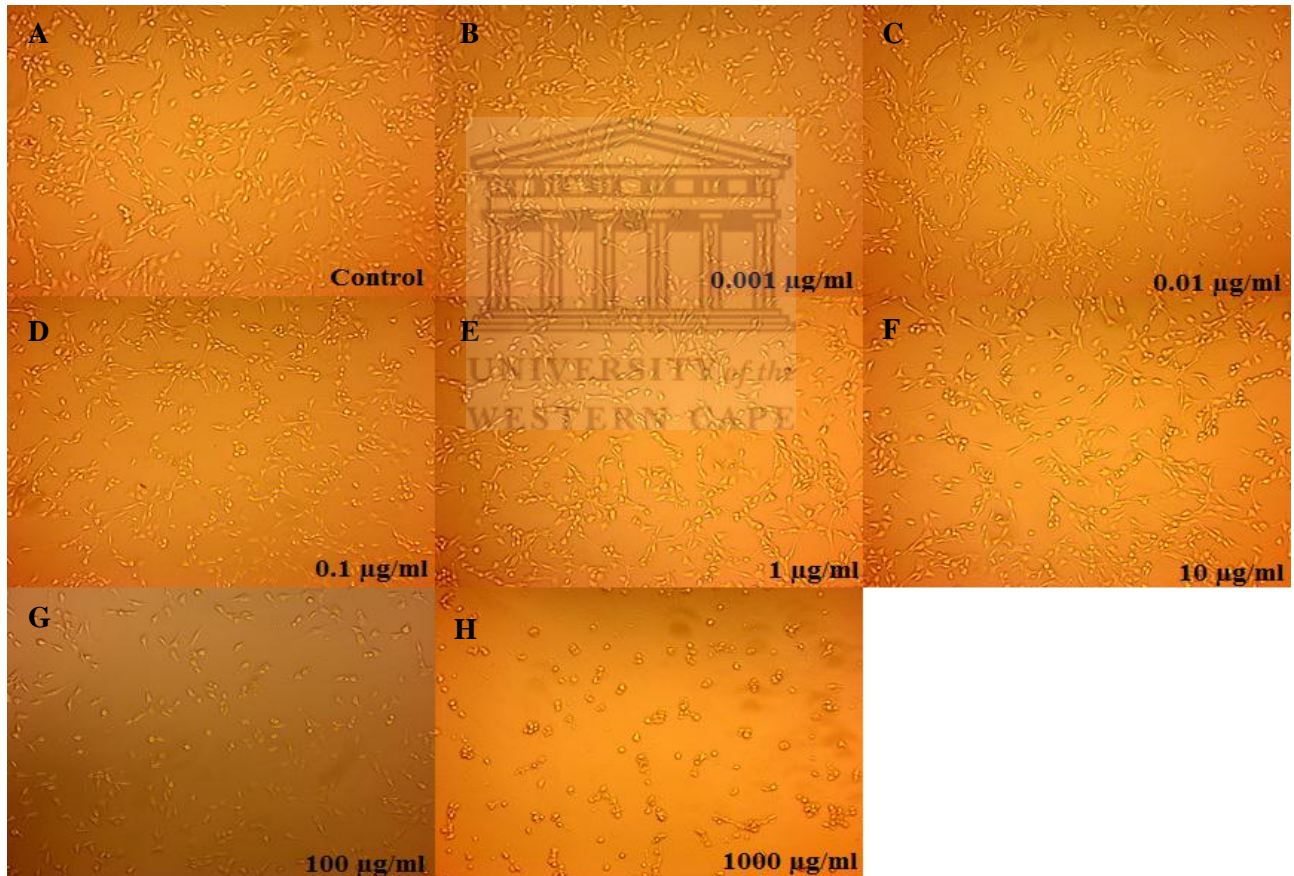


Figure 23: TM4 cell morphology after being exposed to increasing concentrations of the extract over 24 hours. Morphology remained unchanged, flat, polygonal and clustered, up until 10 $\mu\text{g/ml}$ (A-F). Higher concentrations (G-H) showed signs of less flat, polygonal and clustered cells (G) along with cell rounding (H).

The XTT assay revealed a slight, but steady, dose-dependent increase in the cell viability between control groups and 100 µg/ml (figure 24). At 1000 µg/ml, a sharp, significant ($P < 0.0001$) drop in the viability was observed. Trend analysis using the repeated measures ANOVA revealed a significant ($P = 0.005$) positive trend between control and 100 µg/ml, and a significant ($P < 0.0001$) negative trend between 100 µg/ml and 1000 µg/ml. When analyzed further, one-way ANOVA also yielded significance ($P < 0.001$) between the control and 1000 µg/ml.

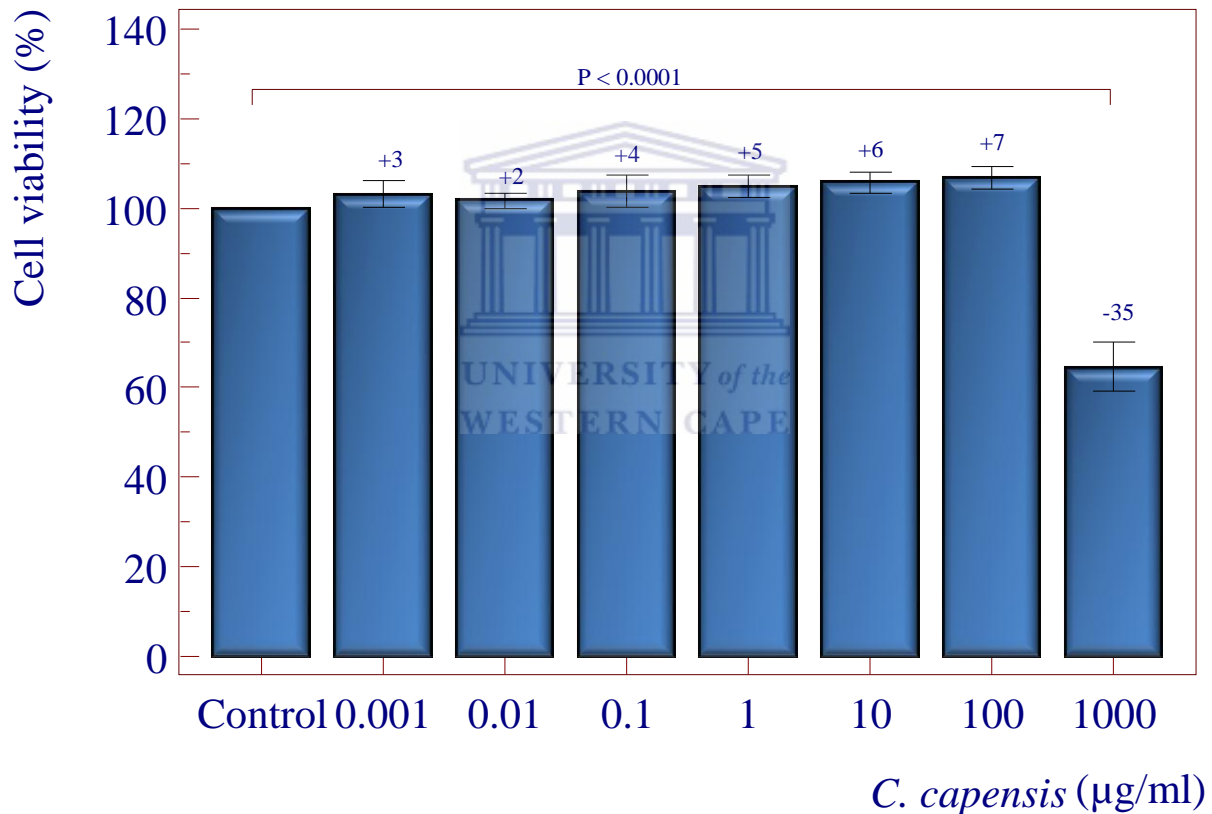


Figure 24: TM4 cell viability as determined by the XTT assay over a 24-exposure period. Cells showed minor, dose-dependent increases in the viability up until 100 µg/ml was reached. At 1000 µg/ml, a sharp, significant ($P < 0.0001$) decrease in cell viability was observed.

After being exposed to the aqueous *C. capensis* rhizome extract for 96 hours, the cell morphology was observed and recorded (Figure 25). Cell morphology remained flat, polygonal, clustered, and therefore unchanged between the control groups and 10 $\mu\text{g/ml}$ (A-F). At higher concentrations (G-H), cell morphology was notably different, showing scattered, slightly rounded cells (G), as well as evidence of cell death (H).

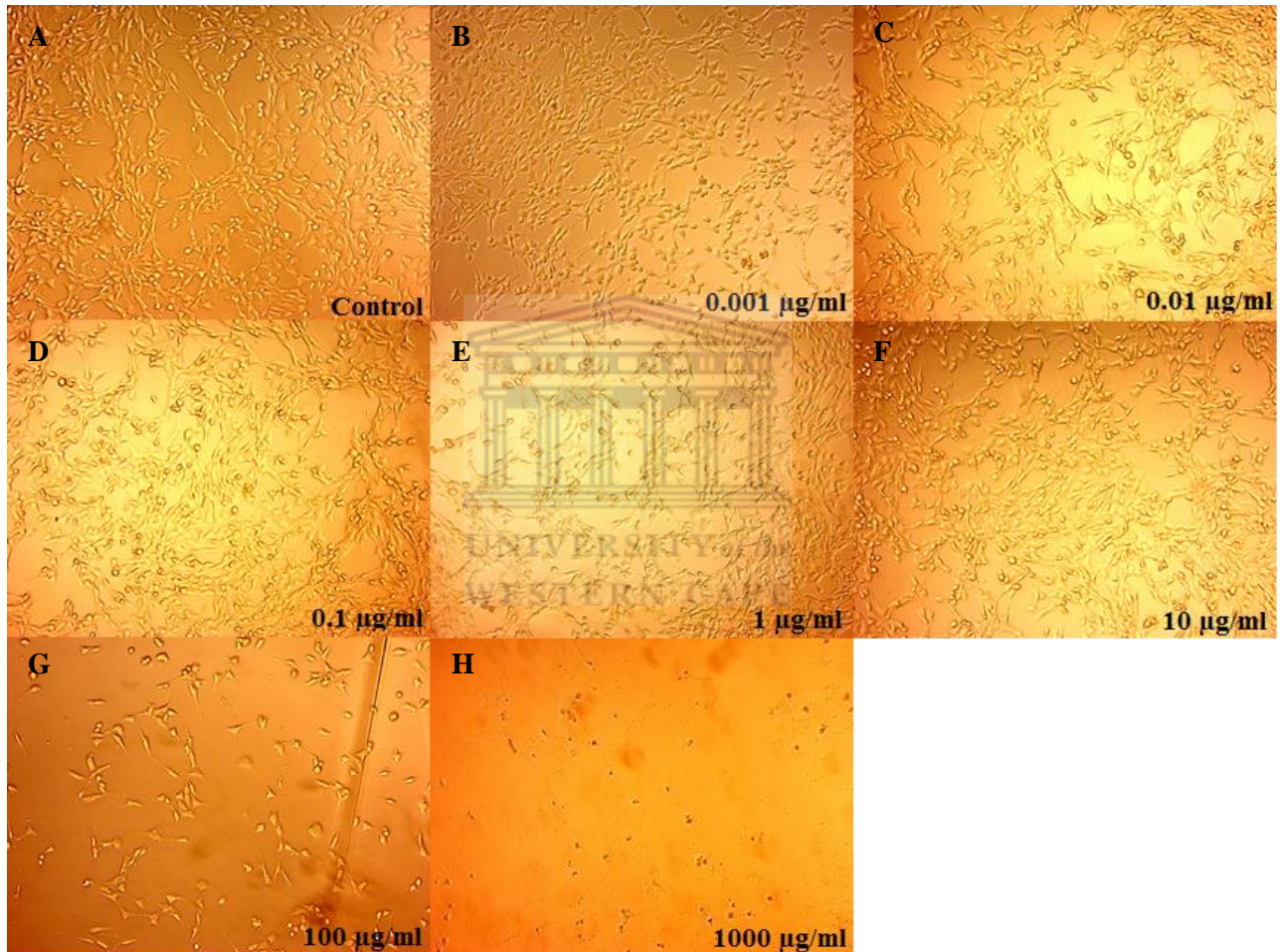


Figure 25: TM4 cell morphology after being exposed to increasing concentrations of the extract over 96 hours. Cell morphology remained flat, polygonal, clustered, and therefore unchanged up until 10 $\mu\text{g/ml}$ (A-F). At higher concentration (G-H) cells began to show evidence of stress, characterized by cells being more scattered and slightly rounded (G), along with evidence of cell death (H).

Similarly, the XTT assay revealed relatively no change in cell viability between the control and 10 µg/ml (Figure 26). Hereafter, at the higher concentrations, cell viability began declining in a dose-dependent manner, yielding significance ($P < 0.0001$) at 100 µg/ml and 1000 µg/ml of the extract, respectively. Trend analysis using the repeated measures ANOVA produced a significant ($P < 0.001$) positive trend between the control and 1000 µg/ml. Further analyses using the one-way ANOVA also yielded significance ($P < 0.001$) between the control and 1000 µg/ml of the extract.

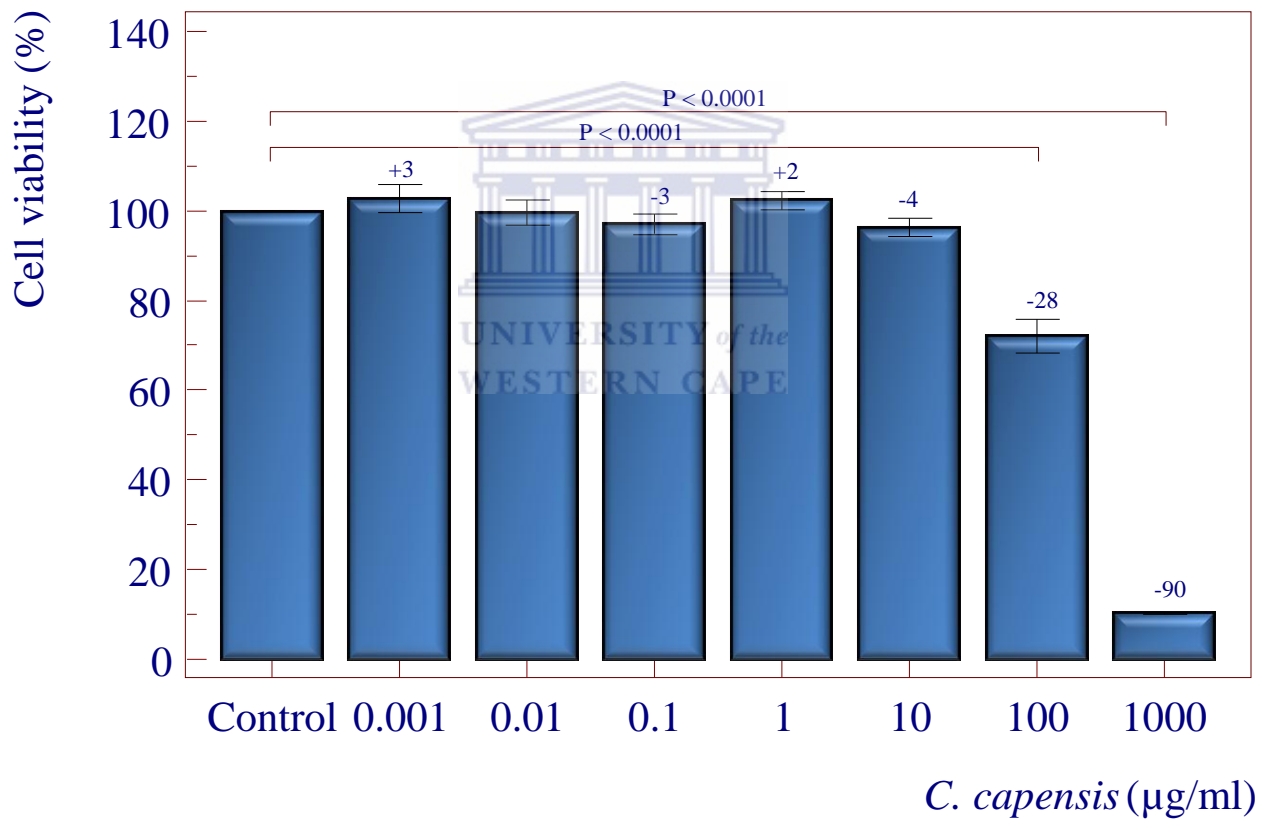


Figure 26: TM4 cell viability as determined by the XTT assay over a 96-hour exposure period. Cells showed no significant change in cell viability, up until 10 µg/ml was reached. Concentrations of 100 µg/ml and 1000 µg/ml produced a significant ($P < 0.0001$) dose-dependent decline in cell viability.

3.1.4 The combined effect of the extract and doxorubicin

LNCaP cell morphology was observed and recorded after being exposed to increasing concentrations of the aqueous *C. capensis* rhizome extract (1-1000 µg/ml) combined with 21.3µM of Doxorubicin (DOX), over a 24-hour exposure period (Figure 27). It was observed, that in the presence of 21.3 µM of DOX, each concentrations of the extract presented with an altered LNCaP cell morphology, changing from flat and polygonal to rounded and clumped (A-H). It must be noted that although cells have become rounded and clumped, total cell death was not observed.

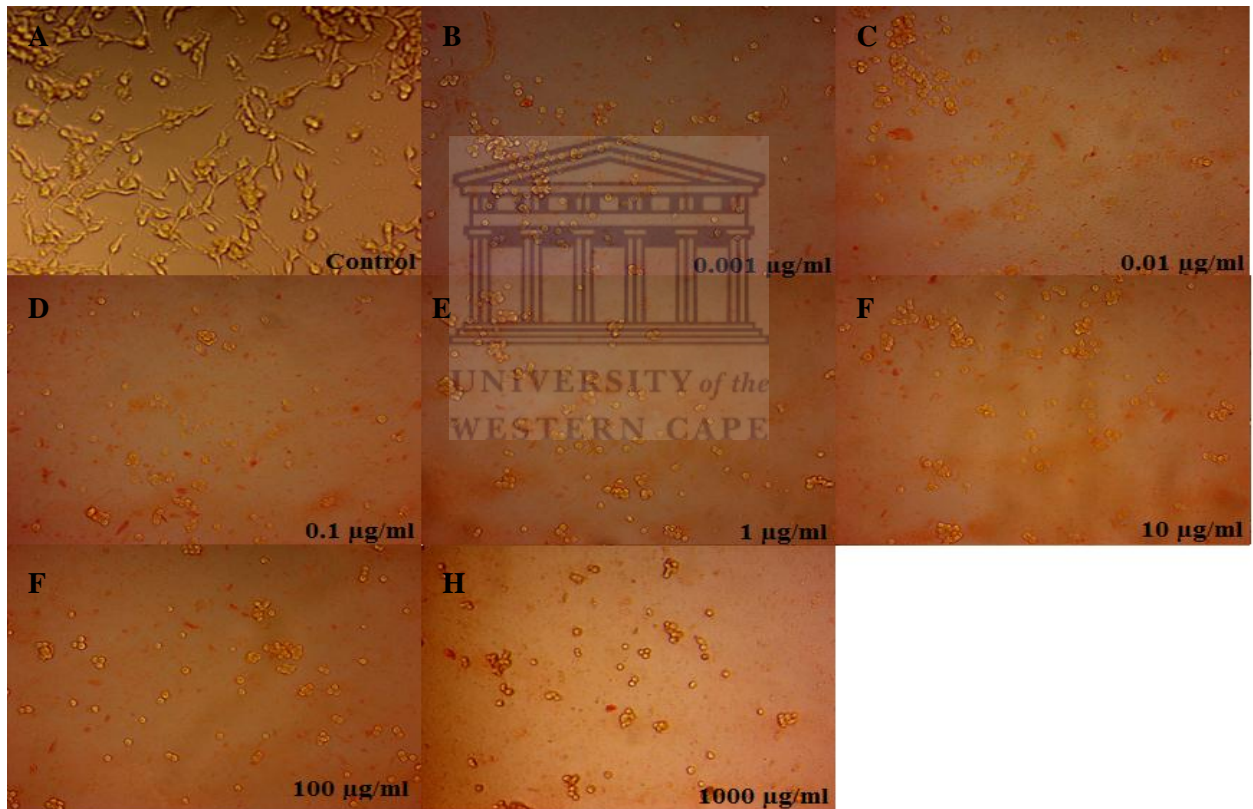


Figure 27: Effects of the aqueous *C. capensis* rhizome extract combined with the 21.3 µM of Doxorubicin (DOX) over a 24-hour exposure period. Each concentration of the extract showed altered LNCaP cell morphology, changing from flat and polygonal to round and more clumped. Total cell death, however, was not observed.

When LNCaP cells were exposed to the extract alone, a slow but steady increase in cell viability was observed between the control and 10 $\mu\text{g/ml}$ (Figure 28). Hereafter, a steady, dose-dependent decrease was observed between 10-1000 $\mu\text{g/ml}$. Subsequently, when the extract was combined with 21.3 μM of Doxorubicin (DOX), a steady, dose-dependent decrease in cell viability was observed between concentrations of 0.001-1000 $\mu\text{g/ml}$ of the extract. However, when the effect of the extract alone was compared with the combined effect of the extract with DOX, it was clear that a greater decrease in cell viability was achieved at each concentration of the extract. Significance ($P < 0.0001$, $P = 0.0018$, $P = 0.0206$, $P = 0.0176$, $P = 0.0018$, $P = 0.0036$, $P = 0.0071$, $P = 0.0033$) between the extract alone and the extract in combination with DOX was observed at each concentration of the extract.

Trend analysis using the repeated measures ANOVA revealed a negative trend, albeit insignificant ($P = 0.0678$), between the control group and the highest concentration of the extract only, 1000 $\mu\text{g/ml}$. One-way ANOVA revealed significance ($P < 0.001$) between the control and highest concentration of the extract alone. Trend analysis, using the repeated measures ANOVA, of the combined effect of the extract and DOX revealed an insignificant, negative trend between the control group and highest concentration of the combination, 1000 $\mu\text{g/ml}$ of the extract. One-way ANOVA revealed an insignificant ($P = 0.502$) trend between the control groups and highest concentration of extract combined with DOX.

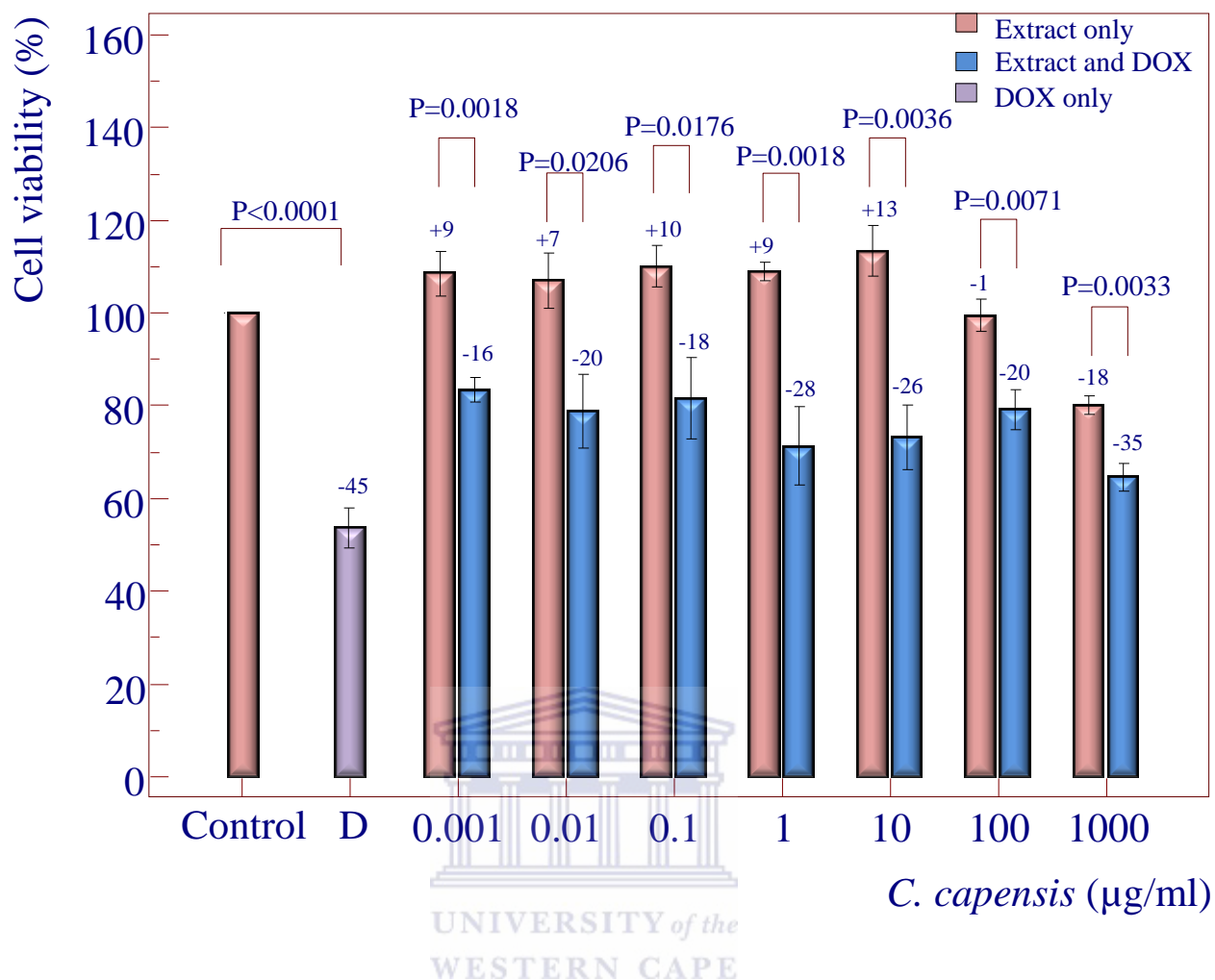


Figure 28: Effects of 21.3 µM of Doxorubicin (DOX) combined with the extract on LNCaP cells over a 24-hour exposure period. DOX alone is shown by (D). When evaluating the extract alone, a steady increase in cell viability was observed between the control and 10 µg/ml, followed by a steady, decrease in cell viability between 10-1000 µg/ml. Subsequently, when the extract was combined with 21.3 µM of Doxorubicin (DOX), a dose-dependent decrease in cell viability was observed between concentrations of 0.001-1000 µg/ml of the extract. However, when the effect of the extract alone was compared with the combined effect of the extract with DOX, it was clear that a greater decrease in cell viability was achieved at each concentration of the extract. Significance (P<0.0001, P=0.0018, P=0.0206, P=0.0176, P=0.0018, P=0.0036, P=0.0071, P=0.0033) between the extract alone and the extract in combination with DOX was observed.

Over a 96-hour exposure period (Figure 29)., the combined effect of the aqueous *C. capensis* rhizome extract and 21.3 μM of Doxorubicin (DOX) produced altered cell morphology in LNCaP cells, changing from flat and polygonal, to more rounded and stressed (A-F). Additionally, observed cell numbers were much lower between the control and 10 $\mu\text{g/ml}$ *C. capensis* (A-F). At the higher concentrations of the extract, 100-1000 $\mu\text{g/ml}$, total cell death was observed (H).

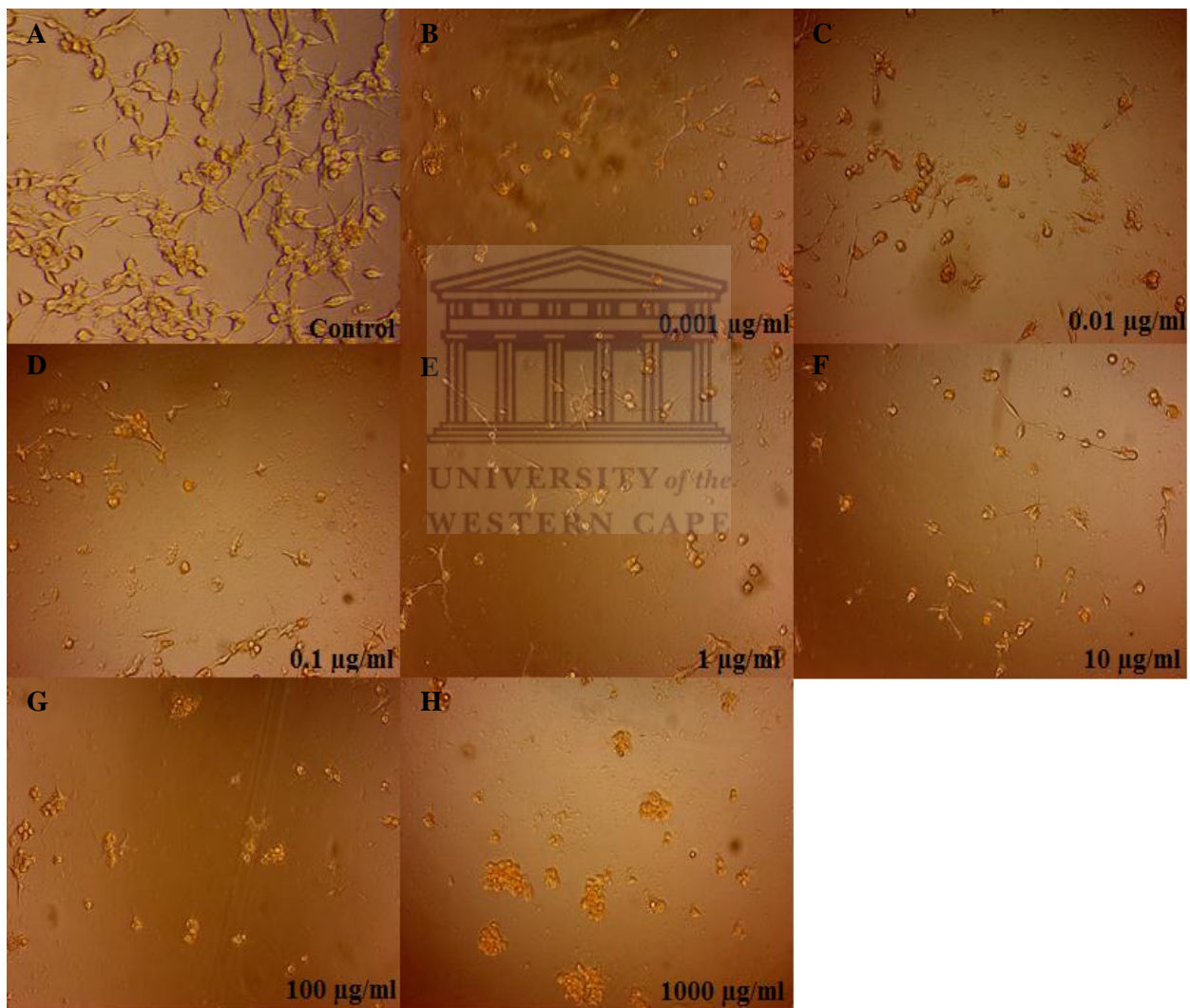


Figure 29: Effects of increasing concentrations of the aqueous *C. capensis* rhizome extract combined with the 21.3 μM of Doxorubicin (DOX) on LNCaP cells over a 96-hour exposure period, largely showing evidence of cell death at each concentration.

Over a 96-hour exposure period, the extract alone showed a dose-dependent increase in cell viability between the control and a concentration of 0.01 µg/ml the extract. Hereafter, a further dose-dependent decrease in cell viability was observed between concentrations of 0.1-1000 µg/ml the extract. In comparison, the combined effect of the extract and DOX revealed a steady, dose-dependent decrease in cell viability between concentrations of 0.001-1000 µg/ml of the extract. Additionally, a clear difference in the percentage decrease in cell viability was observed between the extract alone and the extract combined with DOX, Where DOX yielded a greater observed percentage decrease in cell viability. A Significant ($P < 0.0001$ and $P = 0.002$) difference between the extract alone and the extract under the influence of DOX was found at each concentration of the extract.

Trend analysis using the repeated measures ANOVA, with regard to the extract alone, revealed a significant ($P = 0.00001$), negative, trend between the control group and 1000 µg/ml of the extract. One-way ANOVA also revealed significance ($P = 0.002$). When analyzing the extract combined with DOX, trend analysis using the repeated measures ANOVA revealed a significant ($P = 0.0486$), negative, trend between the control group and 1000 µg/ml of the extract. Similarly, one-way ANOVA revealed significance ($P < 0.001$) between the control and 1000 µg/ml of the extract.

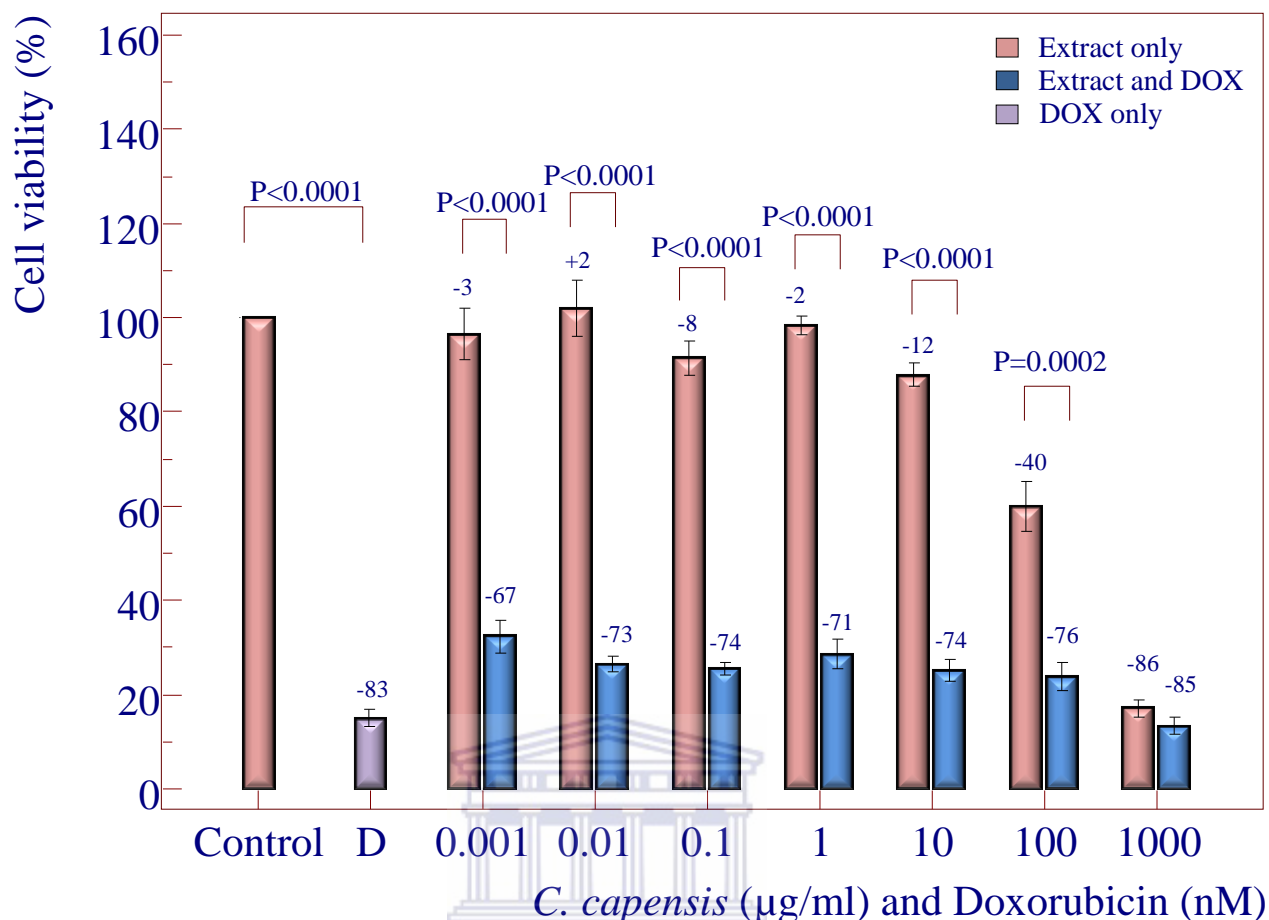


Figure 30: Effects of DOX and the aqueous *C. capensis* rhizome extract on LNCaP cells as determined by the XTT assay over 96 hours. DOX alone is shown by (D). The extract alone showed a dose-dependent increase in cell viability between the control and a concentration of 0.01 µg/ml of the extract, followed by a dose-dependent decrease in cell viability was between concentrations of 0.1-1000 µg/ml of the extract. In comparison, the combined effect of the extract and DOX revealed a steady, dose-dependent decrease in cell viability between concentrations of 0.001-1000 µg/ml of the extract. Additionally, a clear difference in the percentage decrease in cell viability was observed between the extract alone and the extract combined with DOX, where DOX yielded a greater observed percentage decrease in cell viability. A Significant ($P < 0.0001$ and $P = 0.002$) difference between the extract alone and the extract under the influence of DOX was found at each concentration of the extract.

3.1.5 The effect of the extract on testosterone stimulated LNCaP cells

LNCaP cells are androgen sensitive, thereby making it the perfect model for investigating the effects of testosterone. Thus, increasing concentrations of the aqueous *C. capensis* rhizome extract were tested in the presence of different concentrations of testosterone, ranging from 0.001-1000 nM, for 24 and 96 hour periods and cell morphology was recorded (Figure 32 and Figure 33).

Legend to Figure 32 and Figure 33

LNCaP cell morphological changes under the influence of *C. capensis* and testosterone over 24 and 96 hours. LNCaP morphology at each concentration of testosterone only is shown by A-F. The effect of each testosterone concentration in combination with increasing concentrations of *C. capensis* is shown by G-V1.

Over 24 hours (Figure 32), it was observed that low concentrations of testosterone (A-D) showed a proliferative effect, whereas as high concentrations of testosterone (E-F) showed an inhibitory effect, characterized by cell stress and eventual early signs of death. When the increasing concentrations of testosterone were combined with increasing concentrations of the extract, cell stress and eventual cell death became progressively more evident. The greatest inhibitory effects were observed at 1000 nM of testosterone at each concentration of the extract.

Over 96 hours (Figure 33), testosterone continued to show proliferative effects at low concentrations (A-D) and inhibitory effects at higher concentrations (E-F). Again, when the increasing concentrations of testosterone were combined with increasing concentrations of the extract, cells became progressively more stacked, less flat and polygonal, rounded and eventually showed signs of cell death. The greatest inhibitory effect was seen at 1000 nM testosterone.

- A: Testosterone without *C. capensis* (0.01 nM)
 B: Testosterone without *C. capensis* (0.1 nM)
 C: Testosterone without *C. capensis* (1 nM)
 D: Testosterone without *C. capensis* (10 nM)
 E: Testosterone without *C. capensis* (100 nM)
 F: Testosterone with *C. capensis* (1000 nM)
- G: Testosterone with *C. capensis* (0.01 nM + 0.001 µg/ml *C. capensis*)
 H: Testosterone with *C. capensis* (0.1 nM + 0.001 µg/ml *C. capensis*)
 I: Testosterone with *C. capensis* (1 nM + 0.001 µg/ml *C. capensis*)
 J: Testosterone with *C. capensis* (10 nM + 0.001 µg/ml *C. capensis*)
 K: Testosterone with *C. capensis* (100 nM + 0.001 µg/ml *C. capensis*)
 L: Testosterone with *C. capensis* (1000 nM + 0.001 µg/ml *C. capensis*)
- M: Testosterone with *C. capensis* (0.01 nM + 0.01 µg/ml *C. capensis*)
 N: Testosterone with *C. capensis* (0.1 nM + 0.01 µg/ml *C. capensis*)
 O: Testosterone with *C. capensis* (1 nM + 0.01 µg/ml *C. capensis*)
 P: Testosterone with *C. capensis* (10 nM + 0.01 µg/ml *C. capensis*)
 Q: Testosterone with *C. capensis* (100 nM + 0.01 µg/ml *C. capensis*)
 R: Testosterone with *C. capensis* (1000 nM + 0.01 µg/ml *C. capensis*)
- S: Testosterone with *C. capensis* (0.01 nM + 0.1 µg/ml *C. capensis*)
 T: Testosterone with *C. capensis* (0.1 nM + 0.1 µg/ml *C. capensis*)
 U: Testosterone with *C. capensis* (1 nM + 0.1 µg/ml *C. capensis*)
 V: Testosterone with *C. capensis* (10 nM + 0.1 µg/ml *C. capensis*)
 W: Testosterone with *C. capensis* (100 nM + 0.1 µg/ml *C. capensis*)
 X: Testosterone with *C. capensis* (1000 nM + 0.1 µg/ml *C. capensis*)
- Y: Testosterone with *C. capensis* (0.01 nM + 1 µg/ml *C. capensis*)
 Z: Testosterone with *C. capensis* (0.1 nM + 1 µg/ml *C. capensis*)
 A1: Testosterone with *C. capensis* (1 nM + 1 µg/ml *C. capensis*)
 B1: Testosterone with *C. capensis* (10 nM + 1 µg/ml *C. capensis*)
 C1: Testosterone with *C. capensis* (100 nM + 1 µg/ml *C. capensis*)
 D1: Testosterone with *C. capensis* (1000 nM + 1 µg/ml *C. capensis*)
- E1: Testosterone with *C. capensis* (0.01 nM + 10 µg/ml *C. capensis*)

F1: Testosterone with *C. capensis* (0.1 nM + 10 µg/ml *C. capensis*)
G1: Testosterone with *C. capensis* (1 nM + 10 µg/ml *C. capensis*)
H1: Testosterone with *C. capensis* (10 nM + 10 µg/ml *C. capensis*)
I1: Testosterone with *C. capensis* (100 nM + 10 µg/ml *C. capensis*)
J1: Testosterone with *C. capensis* (1000 nM + 10 µg/ml *C. capensis*)

K1: Testosterone with *C. capensis* (0.01 nM + 100 µg/ml *C. capensis*)
L1: Testosterone with *C. capensis* (0.1 nM + 100 µg/ml *C. capensis*)
M1: Testosterone with *C. capensis* (1 nM + 100 µg/ml *C. capensis*)
N1: Testosterone with *C. capensis* (10 nM + 100 µg/ml *C. capensis*)
O1: Testosterone with *C. capensis* (100 nM + 100 µg/ml *C. capensis*)
P1: Testosterone with *C. capensis* (1000 nM + 100 µg/ml *C. capensis*)

Q1: Testosterone with *C. capensis* (0.01 nM + 1000 µg/ml *C. capensis*)
R1: Testosterone with *C. capensis* (0.1 nM + 1000 µg/ml *C. capensis*)
S1: Testosterone with *C. capensis* (1 nM + 1000 µg/ml *C. capensis*)
T1: Testosterone with *C. capensis* (10 nM + 1000 µg/ml *C. capensis*)
U1: Testosterone with *C. capensis* (100 nM + 1000 µg/ml *C. capensis*)
V1: Testosterone with *C. capensis* (1000 nM + 1000 µg/ml *C. capensis*)

WESTERN CAPE

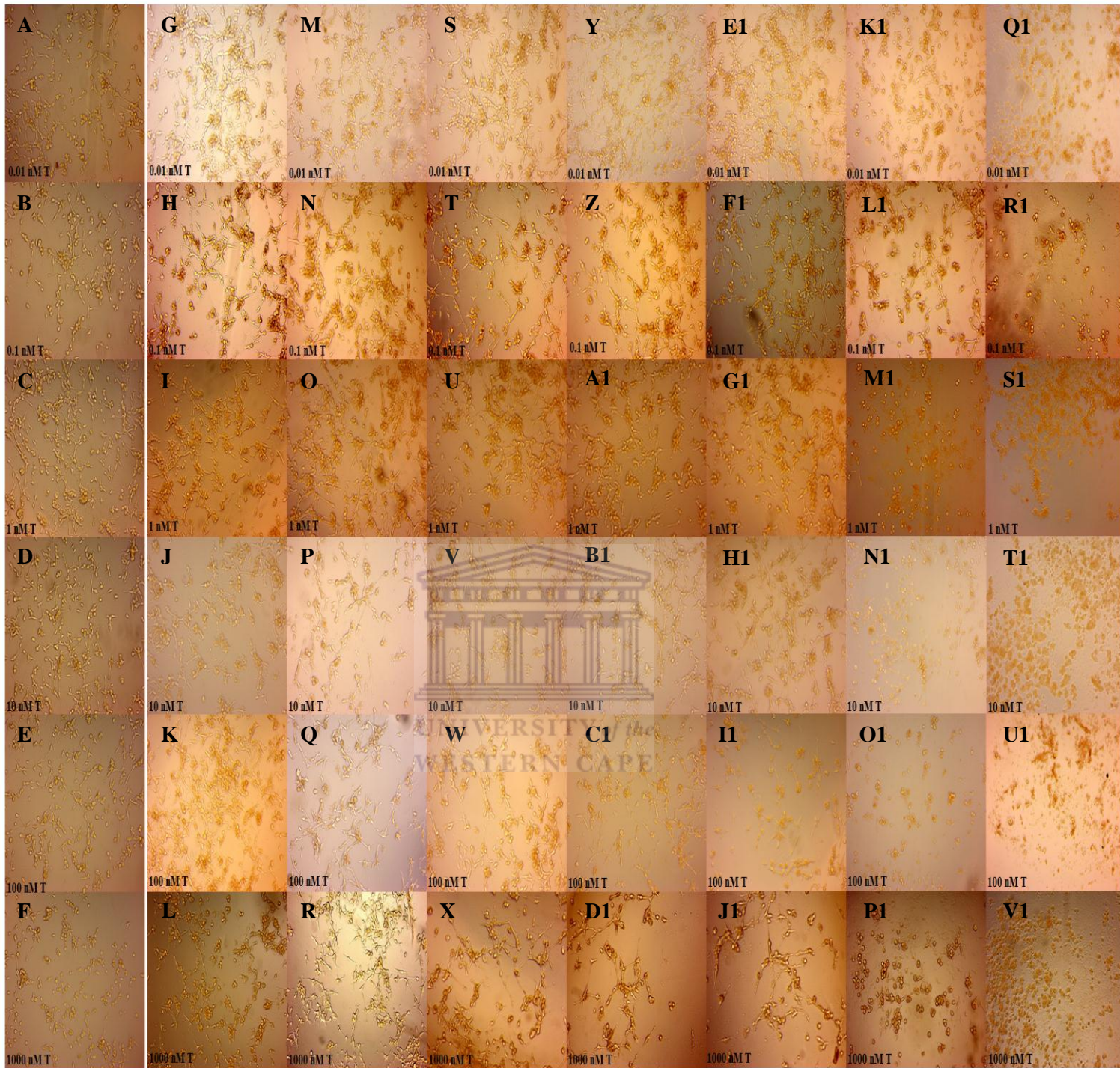


Figure 32: LNCaP cell morphology after being exposed to increasing concentrations of the aqueous *C. capensis* rhizome extract (0.001 $\mu\text{g/ml}$ -1000 $\mu\text{g/ml}$) and testosterone (0.01 nM-1000 nM) over 24 hours. At each concentration of the extract, it was observed that 1000 nM testosterone showed an inhibitory effect, whereby signs of cell stress became evident.

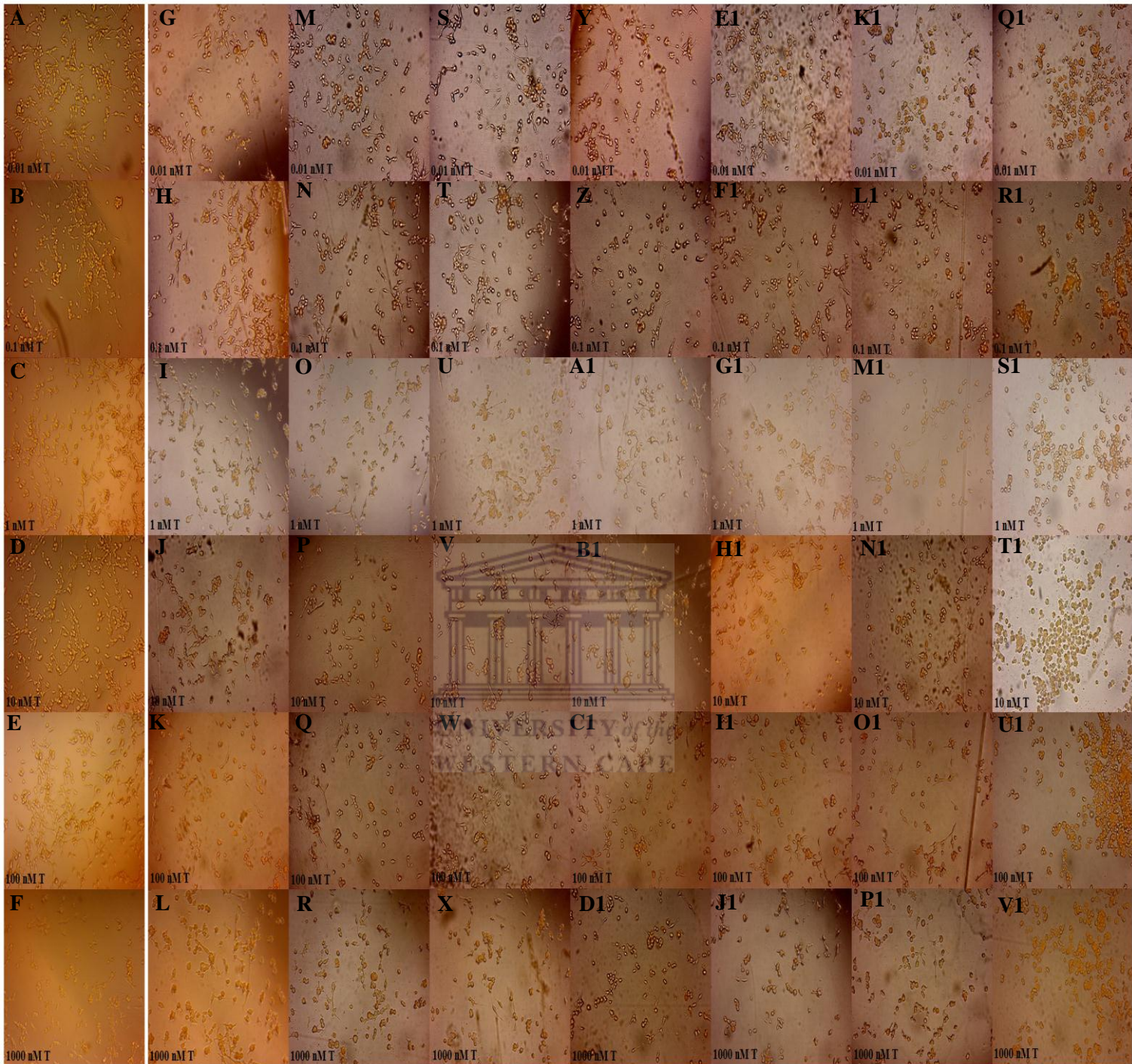


Figure 33: LNCaP cell morphology after being exposed to increasing concentrations of the aqueous *C. capensis* rhizome extract (0.001 µg/ml-1000 µg/ml) and testosterone (0.01 nM-1000 nM) over 96 hours. At each concentration of the extract it was observed that 100-1000 nM testosterone showed an inhibitory effect, whereby signs of cell stress and death became evident.

Over 24 hours (Figure 34), the XTT assay revealed an apparent proliferation of LNCaP cells at extract concentrations between 0.001 µg/ml-0.1 µg/ml, along with an apparent inhibition of LNCaP cells at extract concentrations between 1 µg/ml-1000 µg/ml. Concentrations of 0.01-100 nM testosterone produced minor elevations of cell viability at 0.001 µg/ml of the extract, and no changes in cell viability at 0.01 µg/ml of the extract. Hereafter, 0.01-1000 nM testosterone produced a dose-dependent increase in cell viability when combined with 0.1 and 1 µg/ml of the extract, while showing an apparent dose-dependent decrease in cell viability when combined with 10-1000 µg/ml of the extract. While 1000 nM testosterone produced an increase in cell viability when combined with 0.001 µg/ml of the extract, and a progressively, dose-dependent decrease in cell viability when combined with 0.01-1000 µg/ml of the extract.

Significance (P=0.0341 and P=0.0352) was exhibited at extract concentrations of 0.01 µg/ml and 1000 nM testosterone, and at 0.1 µg/ml extract and 100 nM testosterone. Similarly, significance (P=0.0214, P=0.024, P=0.022, P=0.0121, P=0.0187, P=0.0113, P=0.0271, P=0.0107, P=0.041, P=0.0065, P=0.0084, P=0.009, P=0.0225, P=0.0009, P=0.0007) was observed at 1 µg/ml of the extract and 1000 nM testosterone, at 10 µg/ml of the extract and 10-1000 nM testosterone, at 100 µg/ml extract and 0.01-1000 nM testosterone and at 1000 µg/ml extract and 0.1-1000 nM testosterone, respectively. It must be noted that the greatest decreases in cell viability were observed when extract concentrations between 1-1000 µg/ml were combined with 1000 nM of testosterone.

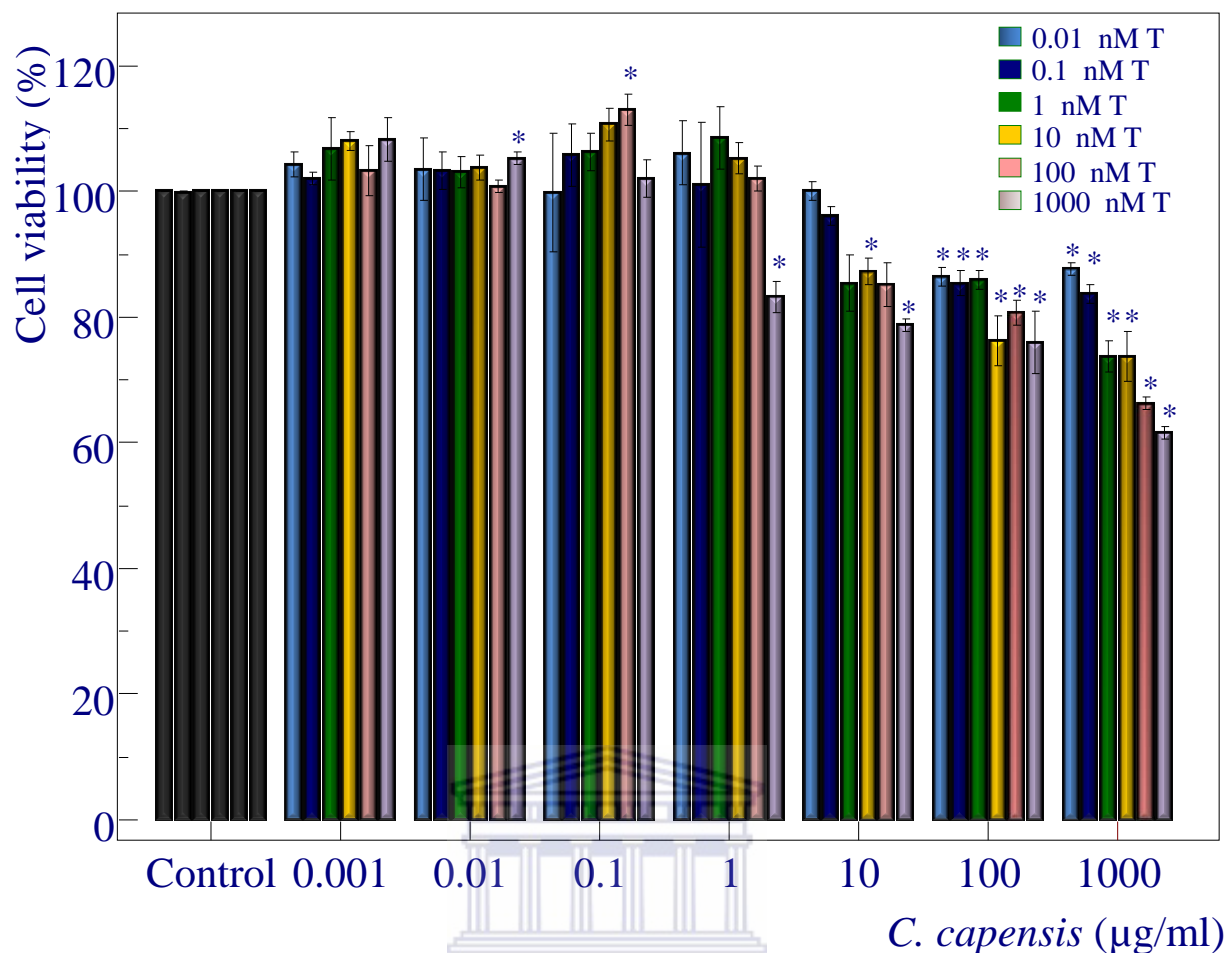


Figure 34: Combined effect of the aqueous *C. capensis* rhizome extract and testosterone on LNCaP cells as determined by the XTT assay over 24 hours. An apparent proliferation of LNCaP cells at extract concentrations between 0.001 µg/ml-0.1 µg/ml, along with an apparent inhibition of LNCaP cells at extract concentrations between 1 µg/ml-1000 µg/ml was observed. Concentrations of 0.01-100 nM testosterone produced minor elevations of cell viability at 0.001 µg/ml of the extract, and no changes in cell viability at 0.01 µg/ml of the extract. Hereafter, 0.01-1000 nM testosterone produced a dose-dependent increase in cell viability when combined with 0.1 and 1 µg/ml extract, while showing an apparent dose-dependent decrease in cell viability when combined with 10-1000 µg/ml extract. While 1000 nM testosterone produced an increase in cell viability when combined with 0.001 µg/ml extract, and a progressively, dose-dependent decrease in cell viability when combined with 0.01-1000 µg/ml extract.

After 96 hours, (Figure 35), 0.001-0.1 $\mu\text{g/ml}$ of the extract combined with 0.01 nM-10 nM testosterone produced minor declines in cell viability, while 100-1000 nM testosterone produced increases in cell viability when combined with 0.001-0.01 $\mu\text{g/ml}$ of the extract. Hereafter, 0.01-10 nM testosterone continued to show minor declines in cell viability when combined with 1-10 $\mu\text{g/ml}$ of the extract, while showing further, dose-dependent declines in cell viability between 100-1000 $\mu\text{g/ml}$ of the extract. 100-1000 nM testosterone exhibited a steady, dose-dependent decrease in cell viability when combined with 1-1000 $\mu\text{g/ml}$ of the extract. Significance ($P=0.047$, $P=0.0185$ and $P=0.0013$) was observed at 0.001 $\mu\text{g/ml}$ extract combined with 0.1-1000 nM testosterone, and 0.01 $\mu\text{g/ml}$ extract combined with 1000 nM testosterone, respectively. Significance ($P=0.008$, $P=0.0071$, $P=0.0292$, $P=0.0380$, $P=0.0255$, $P=0.0111$, $P=0.0402$, $P=0.0049$, $P=0.0029$, $P<0.0001$, $P=0.0018$, $P<0.0001$, $P=0.0001$ and $P=0.0008$) was also observed at 0.001 $\mu\text{g/ml}$ of the extract combined with 0.01 nM and 10 nM testosterone, at 0.01 $\mu\text{g/ml}$ of the extract combined with 0.01 nM and 1 nM testosterone, 10 $\mu\text{g/ml}$ of the extract combined with 0.1 nM testosterone, 100 $\mu\text{g/ml}$ of the extract combined with 0.01 nM, 0.1 nM and 1 nM testosterone, and at 1000 $\mu\text{g/ml}$ of the extract combined with 0.01-1000 nM testosterone, respectively. The greatest decreases in cell viability were observed when concentrations of extract between 10-1000 $\mu\text{g/ml}$ were combined with 1000 nM testosterone.

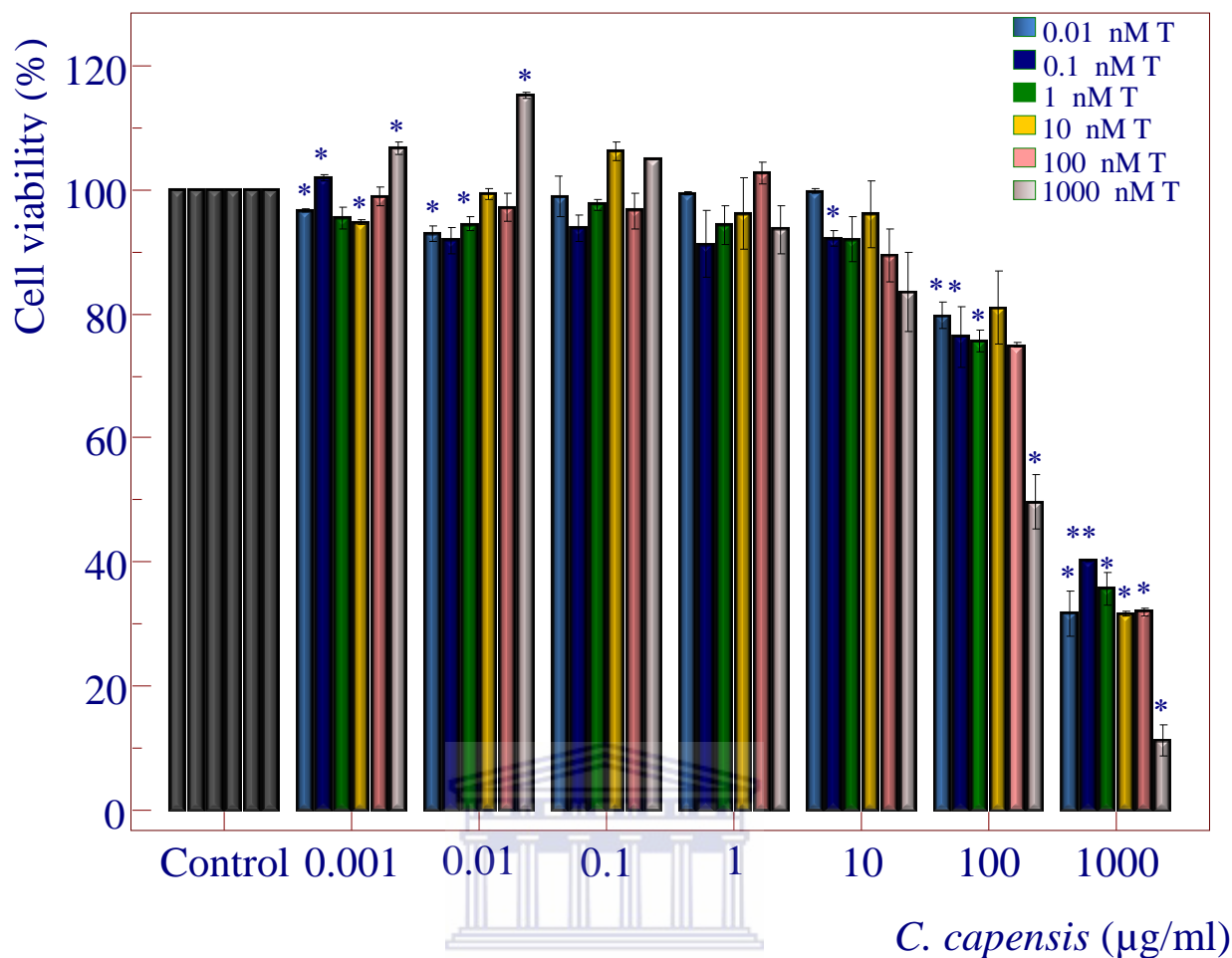


Figure 35: Combined effect of the aqueous *C. capensis* rhizome extract and testosterone on LNCaP cells as determined by the XTT assay over 96 hours. Low concentrations, 0.001-0.1 µg/m of the extract, combined with 0.01 nM-10 nM testosterone produced minor declines in cell viability, while 100-1000 nM testosterone produced increases in cell viability when combined with 0.001-0.01 µg/m of the extract. Hereafter, 0.01-10 nM testosterone continued to show minor declines in cell viability when combined with 1-10 µg/m of extract, while showing further, dose-dependent declines in cell viability between 100-1000 µg/m of the extract. 100-1000 nM testosterone exhibited a steady, dose-dependent decrease in cell viability when combined with 1-1000 µg/m of the extract.

The effect of 1000 nM testosterone in combination with the extract was subsequently tested over 24 and 96 incubation periods.

When exposing the LNCaP cells to increasing concentrations of the extract over 24 hours (Figure 36), a general trend emerged, whereby cell viability steadily increased in a dose-dependent manner to a concentration of 0.1 µg/ml extract, after which the cell viability steadily declines in a dose-dependent manner to the highest concentrations used in this study, 1000 µg/ml extract. Additionally, the highest increase in cell viability produced by the extract alone accounted for 111%, while the greatest decrease in cell viability accounted for 81%.

When the extract was combined with 1000 nM of testosterone over a 24-hour period (Figure 36), the same general trend was observed with an increase in cell viability between the control groups and 0.1 µg/ml extract was observed; followed by a progressive, dose-dependent decrease in cell viability between 1-1000 µg/ml of the extract.

Additionally, when percentages were considered, it was clear that at each concentration of the extract, 1000 nM testosterone produced a higher observed percentage increase between the control and 0.1 µg/ml of the extract, along with a greater percentage decrease between 1-1000 µg/ml of the extract. Statistically, significance ($P=0.0018$) was observed between the difference in viability between the extract alone and the extract in combination with 1000 nM testosterone.

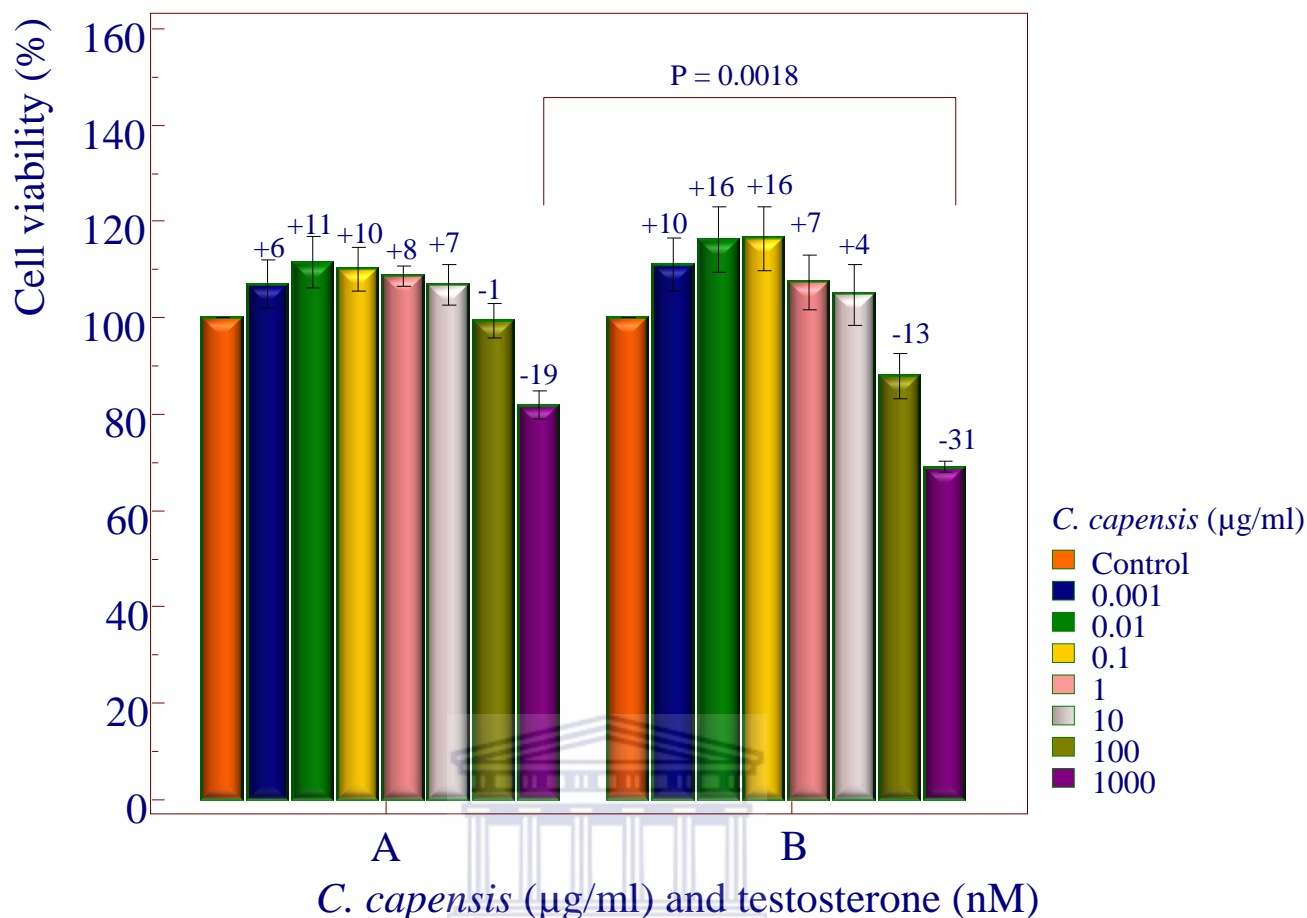


Figure 36: The combined effect of the extract and 1000 nM testosterone, as determined by the XTT assay over 24 hours. (A) shows the effect of the extract on LNCaP cells in absence of testosterone, while (B) shows the effect of the extract on LNCaP cells in the presence of 1000 nM testosterone. Observably, the same general trend emerged, whereby there is an increase in cell viability between the control groups and 0.1 µg/ml extract, followed by a steady decrease in cell viability between 1-1000 µg/ml of the extract. However, when comparing the actual percentages, it was noted that a greater percentage increase was observed between control groups and 0.1 µg/ml, as well as a greater percentage decrease between 1-1000 µg/ml, when the extract was combined with 1000 nM testosterone.

Similarly, when the LNCaP cells were exposed to the extract alone (Figure 37), an increase in cell viability was observed between the control groups and 0.01 µg/ml of the extract, while a steady, dose-dependent decrease in cell viability was observed between 0.1-1000 µg/ml of the extract. When the extract was combined with 1000 nM of testosterone over a 96-hour period (Figure 37), it was found that an identical trend was produced, whereby an increase in observed cell viability was found between the control and 0.01 µg/ml of the extract, while a steady, dose-dependent decrease in cell viability was observed between 0.1-1000 µg/ml of the extract.

Moreover, when comparing the actual percentages produced by the combination of the extract and 1000 nm testosterone to the effect of the extract alone, it was clear that the combined effect of the extract and testosterone produced a higher cell viability at each concentration between the control and 0.01 µg/ml of the extract along with a much sharper, dose-dependent decline in percentage viability at each concentration between 0.1-1000 µg/ml of the extract. Significance ($P=0.0179$ and $P=0.0441$) was also revealed between the differences in viabilities produce between the cells exposed to the extract only and those exposed to the combined effect of the extract and 1000 nM testosterone, observed at 0.01 µg/ml and at 1000 µg/ml extract, respectively.

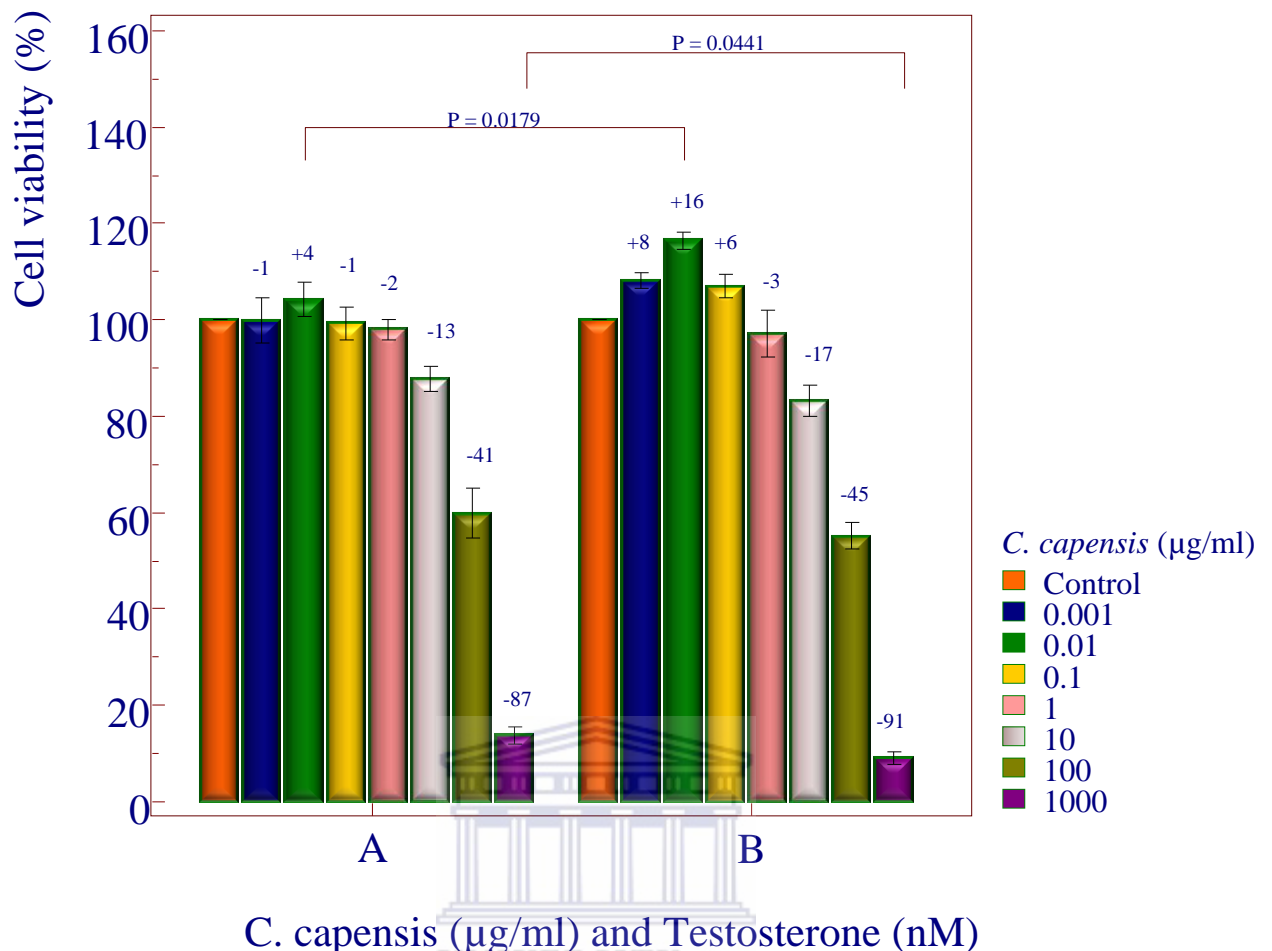


Figure 37: Combined effect of the aqueous *C. capensis* rhizome extract and 1000 nM testosterone on LNCaP cells, as determined by the XTT assay over 96 hours. The effect of the extract alone is shown by (A), while the effect of the extract in combination with 1000 nM testosterone is shown by (B). When the effect of the extract alone is compared to the combined effect of the extract and testosterone, it is clear that the same trend emerges, whereby there is an increase in cell viability between the control and 0.01 µg/ml, while there is a decrease in cell viability between 0.1-1000 µg/ml. However, when the actual percentages are considered, it is clear that the combined effect of the extract and testosterone produces a higher increase in percentage viability between control and 0.01, along with a sharper decrease in percentage viability between 0.1-1000 µg/ml.

3.2 DNA fragmentation in LNCaP cells

After exposing LNCaP cells to different concentrations of the aqueous *C. capensis* rhizome extract for 24 hours, DNA fragmentation was determined (Figure 38). A dose-dependent increase in TUNEL-positive cells was observed between the control groups and 1000 $\mu\text{g/ml}$ of the extract. Although increasing in a dose-dependent manner, it appeared that TUNEL-positive cells were still able to maintain their normal flat and polygonal cell morphology up until 10 $\mu\text{g/ml}$ was reached (A-F). It was observed that at 100 $\mu\text{g/ml}$, cell morphology became less flat and polygonal, and thereby visibly stressed (G), while at 1000 $\mu\text{g/ml}$ cells presented with an abnormal, rounded morphology, typical of cell death (F).

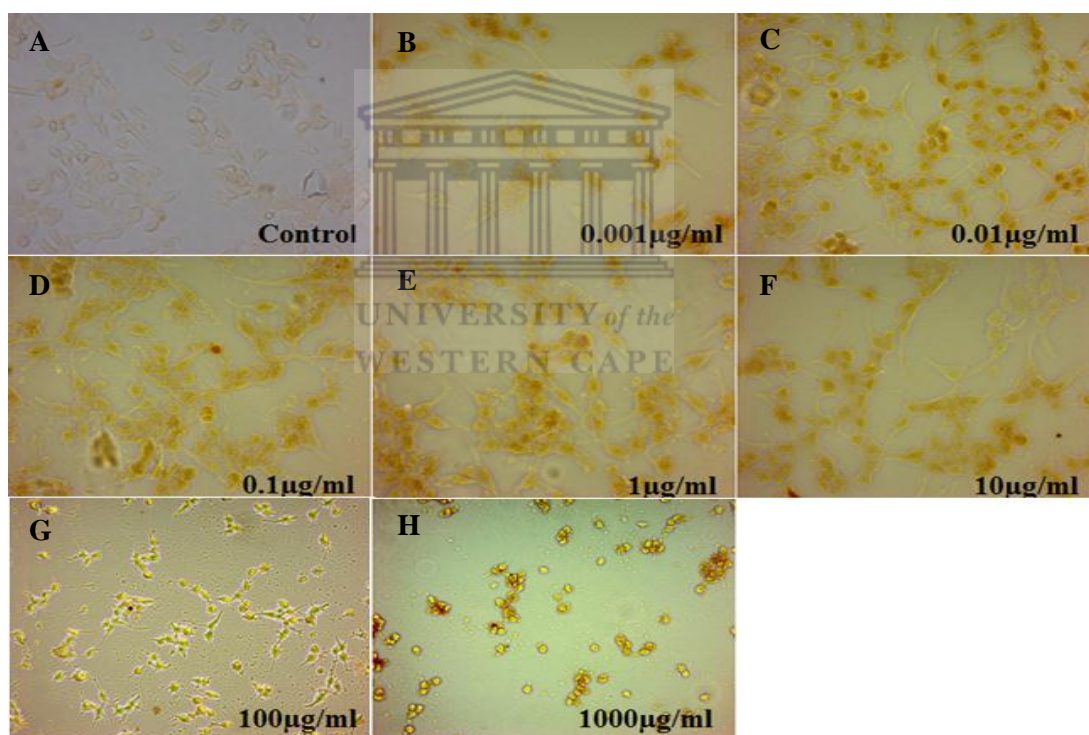


Figure 38: Effect of the aqueous *C. capensis* rhizome extract toward LNCaP cell DNA fragmentation over 24 hours. Cells showed a dose-dependent increase in observed DNA fragmentation between the control and 10 $\mu\text{g/ml}$, with apparent normal morphology (A-F). Hereafter, cells showed visible signs of stress at 1000 $\mu\text{g/ml}$, presenting with abnormal, less flat and polygonal morphology (G). At 1000 $\mu\text{g/ml}$, cells presented with an altered, rounded morphology that is indicative of the presence of cell death (F).

The percentage of observed TUNEL-positive cells was confirmed by the repeated measures ANOVA that revealed a significant ($P < 0.001$) positive trend between the control and highest concentration, 1000 $\mu\text{g/ml}$ of the extract (Figure 39). Similarly, one-way ANOVA revealed a significant ($P < 0.0001$) trend between the control and 1000 $\mu\text{g/ml}$. It is important to note that even the lowest concentration used in this study (0.001 $\mu\text{g/ml}$) resulted in a significant ($P = 0.0013$) increase in observed TUNEL-positive cells.

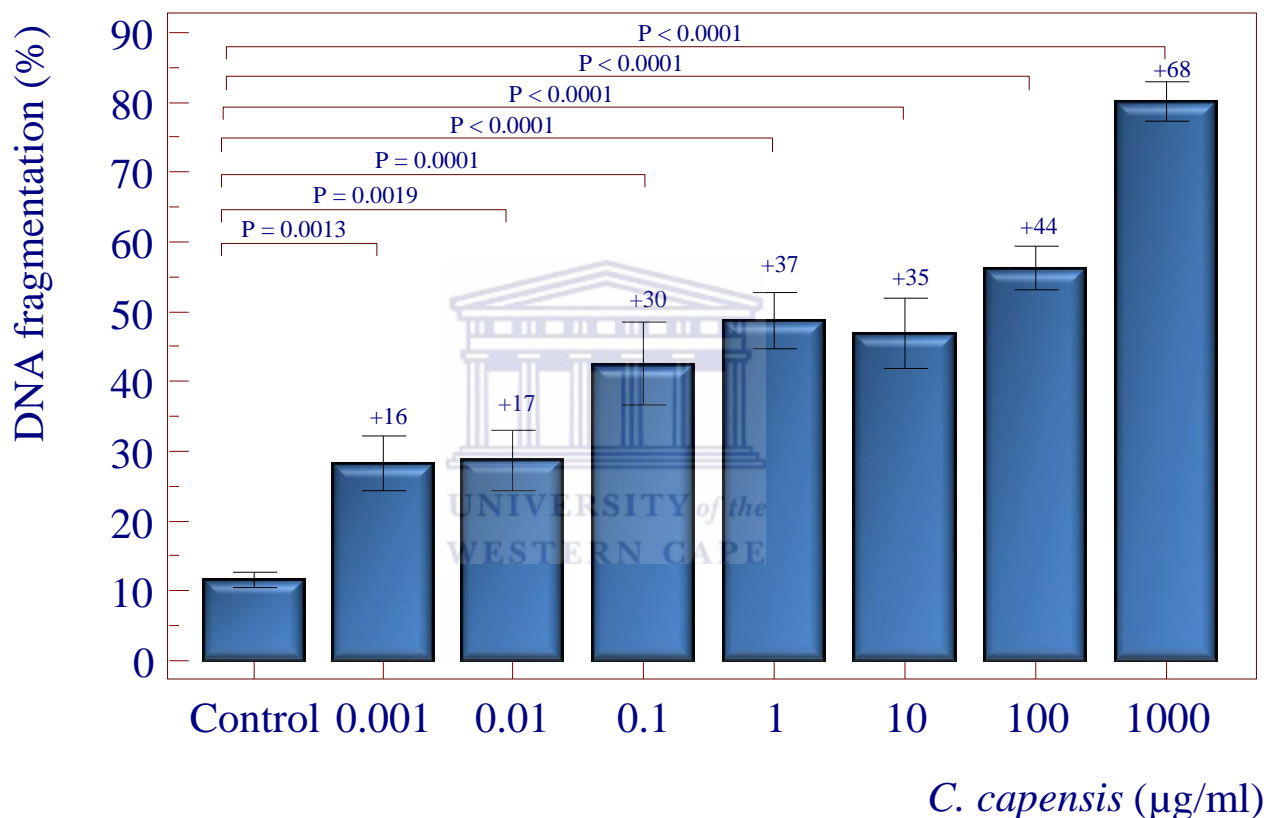


Figure 39: Effect of aqueous *C. capensis* rhizome extract on DNA fragmentation in LNCaP cells after 24 hours of exposure. A significant ($P = 0.0013$, $P = 0.0019$, $P = 0.0001$ and $P < 0.0001$) progressive, dose-dependent increase in observed TUNEL-positive cells was found between the control and 1000 $\mu\text{g/ml}$ when compared to the control. One-way ANOVA also yielded significance ($P < 0.001$) between control and 1000 $\mu\text{g/ml}$.

After 96-hours of exposure (Figure 40), the dose-dependent increase in observed TUNEL-positive cells was no longer observed between the control and 1000 $\mu\text{g/ml}$ (A-H). Instead, an apparent adaptation and proliferation in the presence of the extract was observed, along with normal flat and polygonal morphology (A-F). It was only at a concentration of 100 $\mu\text{g/ml}$ the extract that TUNEL-positive cells were observed (G). These cells were no longer flat and polygonal, but rather showed a rounded morphology indicative of cell death (G). At 1000 $\mu\text{g/ml}$, total cell death had occurred and only fragments of cells could be found stained TUNEL-positive (H).

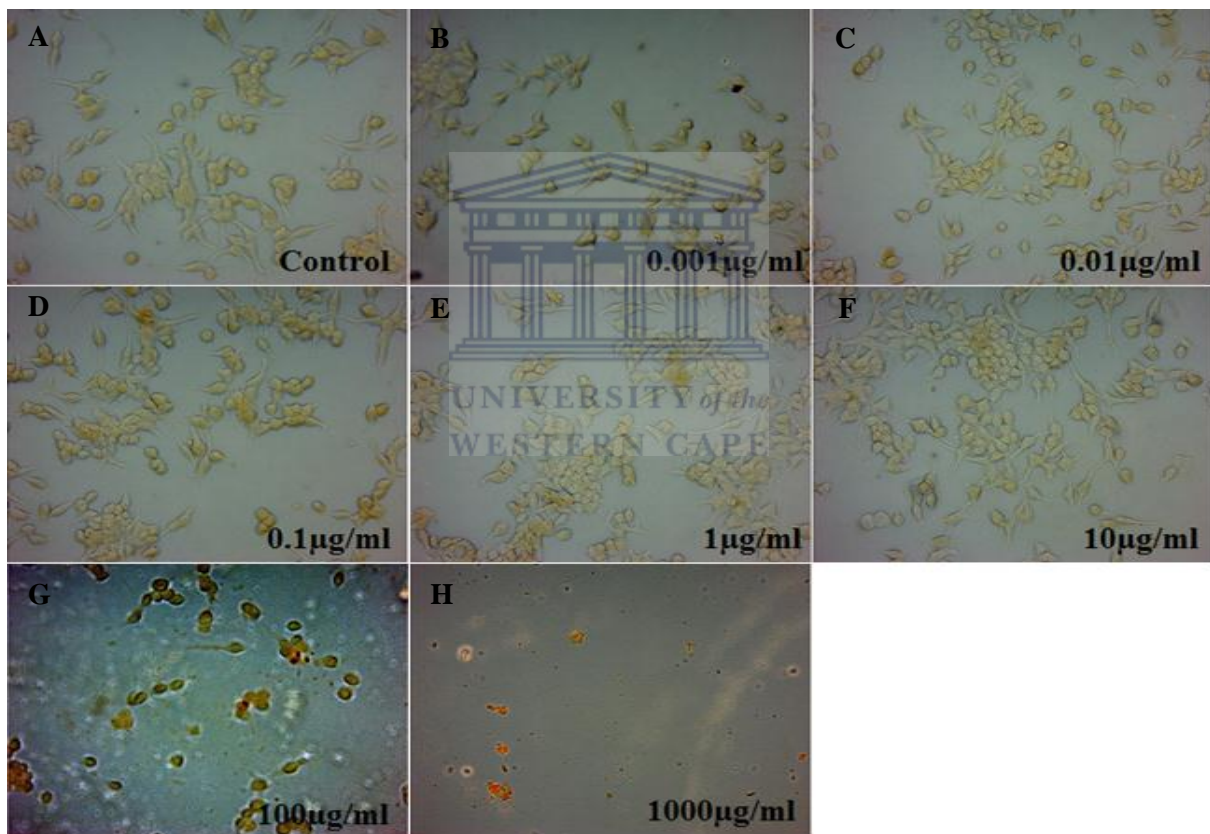


Figure 40: Effect of aqueous *C. capensis* rhizome extract toward LNCaP cell DNA fragmentation over 96-hours of exposure. The dose-dependent increase in observed TUNEL-positive cells was no longer exhibited between control and 10 $\mu\text{g/ml}$., but rather adaptation and proliferation in the presence of the extract (A-F). It was only at 100 $\mu\text{g/ml}$ that TUNEL-positive cells were found, with an altered morphology. At 1000 $\mu\text{g/ml}$, only fragments of cells could be found, all of which were stained positive.

The repeated measures ANOVA yielded an insignificant ($P=0.16$) positive linear trend between control and 10 $\mu\text{g/ml}$, and a significant ($P<0.0001$) positive trend thereafter (Figure 41). One-way ANOVA revealed significance ($P<0.0001$) between the control and 1000 $\mu\text{g/ml}$ of the extract. Between the control and 10 $\mu\text{g/ml}$, low levels of TUNEL-positive cells are observed, that slightly increase in a dose-dependent manner. It is not until 100–1000 $\mu\text{g/ml}$ that a greater dose-dependent increase in TUNEL-positive cells can be observed. At 100 $\mu\text{g/ml}$, a significant ($P<0.0001$) sharp and sudden increase in TUNEL-positive cells can be observed, accounting for 80%. Similarly, at 1000 $\mu\text{g/ml}$, a further, significant ($P<0.0001$) increase in TUNEL-positive cells can be observed accounts for 100%.

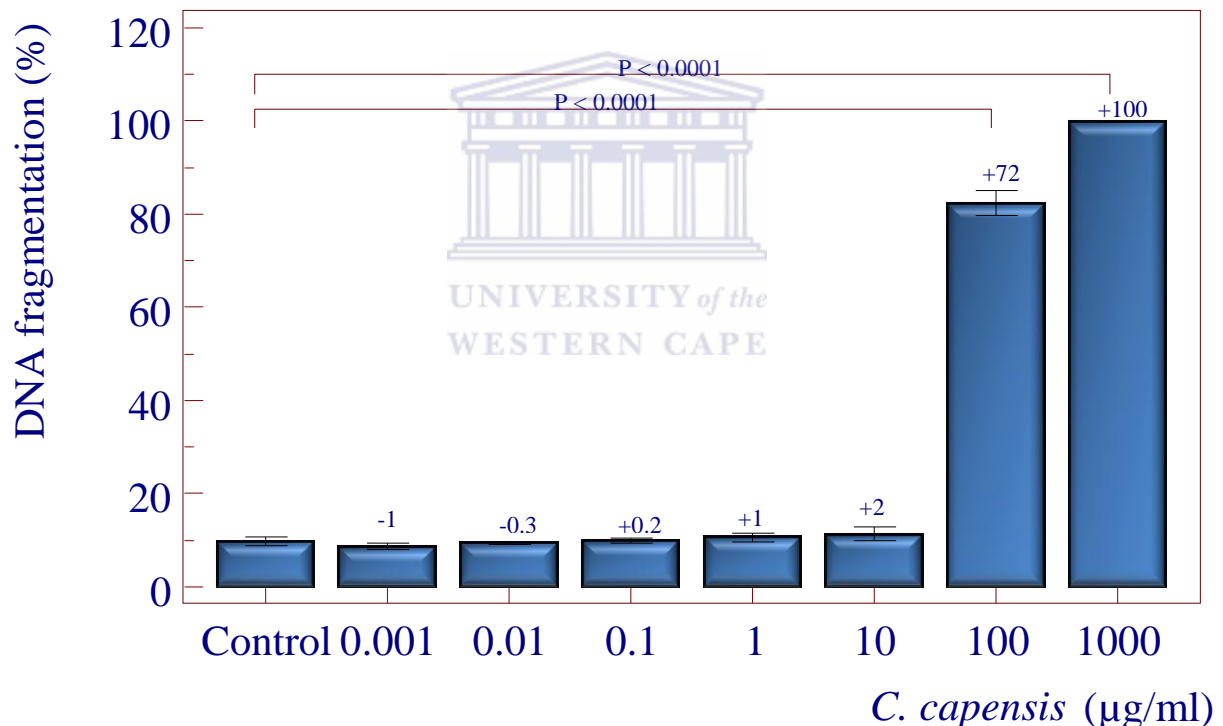


Figure 41: Effect of the extract on DNA fragmentation in LNCaP cells after 96-hours of exposure. Low levels of TUNEL-positive cells, increasing very slightly in a dose-dependent manner, were observed between the control group and 10 $\mu\text{g/ml}$ of the extract. It was not until 100-1000 $\mu\text{g/ml}$ of the extract that a greater dose-dependent increase in TUBEL-positive cells were observed, where 100 $\mu\text{g/ml}$ showed a sharp, significant ($P<0.0001$) increase. At 1000 $\mu\text{g/ml}$, TUNEL-positive cells significantly ($P<0.0001$).

3.3 Testosterone production

A slight, but not significant, elevation in testosterone production between the control group and the experimental group treated with 10 $\mu\text{g/ml}$ of the extract was observed over a 24-hour exposure period. This was followed by a steady, dose-dependent decrease thereafter, as from 100-1000 $\mu\text{g/ml}$ (Figure 42). Statistically, significance ($P=0.0009$ and $P=0.0006$) was revealed at 100 and 1000 $\mu\text{g/ml}$ of the extract. Trend analysis using the repeated measures ANOVA showed no relationship up to a concentration of 10 $\mu\text{g/ml}$ of the extract. From there, a significant ($P=0.0005$) negative trend was observed. One-way ANOVA also revealed significance ($P<0.0001$) between the control and 1000 $\mu\text{g/ml}$ extract.

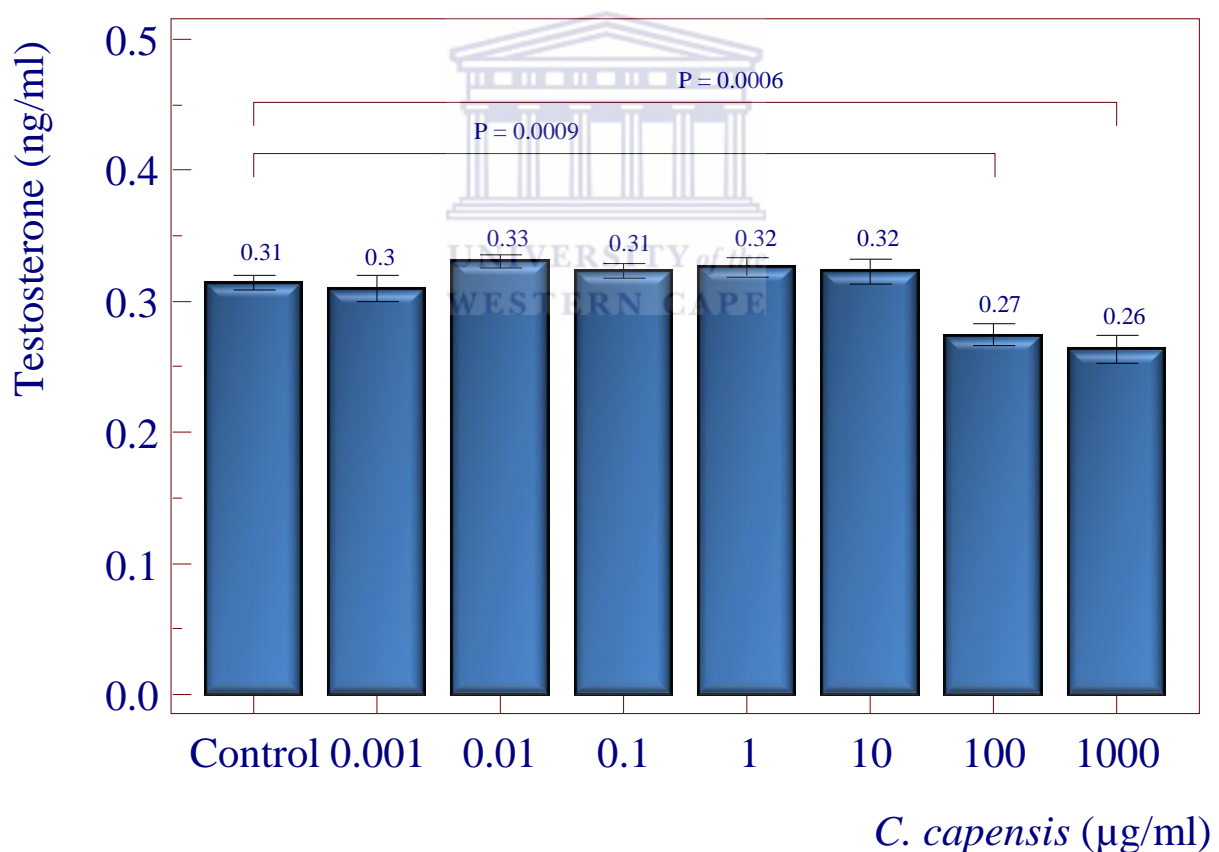


Figure 42: TM3 testosterone production over a 24-hour incubation period. Cells showed only slight increases in testosterone production between the control and 10 $\mu\text{g/ml}$ that was not significant, while showing significant ($P=0.0009$ and $P=0.0006$) decrease in testosterone production at 100 $\mu\text{g/ml}$ and 1000 $\mu\text{g/ml}$, respectively.

After 96-hours (Figure 43), testosterone production was found to be slightly, but not significantly, elevated between the control and a concentration of 0.001 $\mu\text{g/ml}$ of the extract. Subsequently, a steady, dose-dependent decline was observed between concentration of 1-1000 $\mu\text{g/ml}$ extract, with significance ($P=0.0306$ and $P=0.0023$) observed at 100 and 1000 $\mu\text{g/ml}$ extract. Trend analysis using the repeated measures ANOVA revealed a significant ($P<0.0001$) negative trend between the control and 1000 $\mu\text{g/ml}$, while one-way ANOVA yielded no significance ($P=0.232$) between the control and 1000 $\mu\text{g/ml}$ extract.

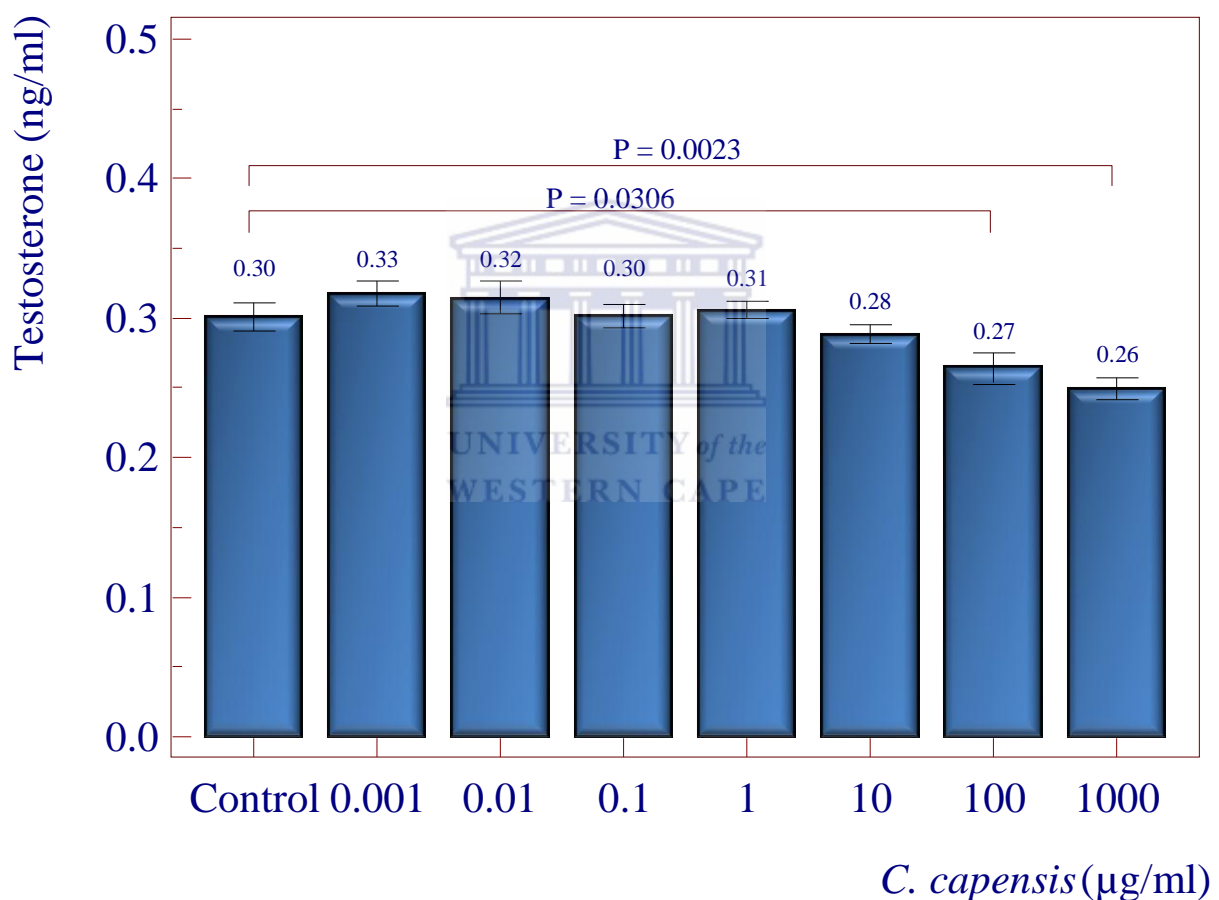


Figure 43: TM3 testosterone production over a 96-hour incubation period. Testosterone production remained only slightly elevated between control and 0.001 $\mu\text{g/ml}$, while showing a dose-dependent, decrease in testosterone production between 0.01-1000 $\mu\text{g/ml}$. Significance ($P=0.0306$ and $P=0.0023$) was observed at 100 $\mu\text{g/ml}$ and 1000 $\mu\text{g/ml}$, respectively.

3.4 Lactate Dehydrogenase (LDH) activity

Slight, but not significant, decreases in lactate dehydrogenase (LDH) activity was observed in TM4 Sertoli cells over a 24-hour exposure period, between the control and 10 µg/ml of the aqueous *C. capensis* rhizome extract (Figure 44). Hereafter, a sudden, dose-dependent increase in LDH activity was observed between 100-1000 µg/ml of the extract, with significance ($P=0.0273$) being revealed at 1000 µg/ml of the extract. Trend analysis using the repeated measures ANOVA revealed a significant ($P=0.012$) positive trend produced by the repeated measures ANOVA between the control and the highest concentration (1000 µg/ml). One-way ANOVA also revealed significance ($P<0.0001$) between control and 1000 µg/ml extract.

After exposure to the extract for 96-hours, TM4 cells (Figure 45), LDH activity was slightly, but not significantly, lowered at concentrations of 0.001 and 1 µg/ml of the extract, while being slightly, but not significantly, elevated between concentrations of 0.01 and 0.1 µg/ml extract. LDH release became increasingly elevated between concentrations of 10 and 100 µg/ml of the extract, with significance ($P=0.0002$) revealed between the control and 100 µg/ml. Hereafter, a sudden, sharp drop in LDH release was observed at a concentration of 1000 µg/ml extract, that was found to be statistically significant ($P<0.0001$). Trend analysis using the repeated measures ANOVA showed a significant ($P=0.0121$) positive trend between the control and 100 µg/ml extract, while showing a sharp significant ($P<0.0001$) negative trend between 100 µg/ml and 1000 µg/ml of the extract. One-way ANOVA also revealed significance between control and 1000 µg/ml of the extract.

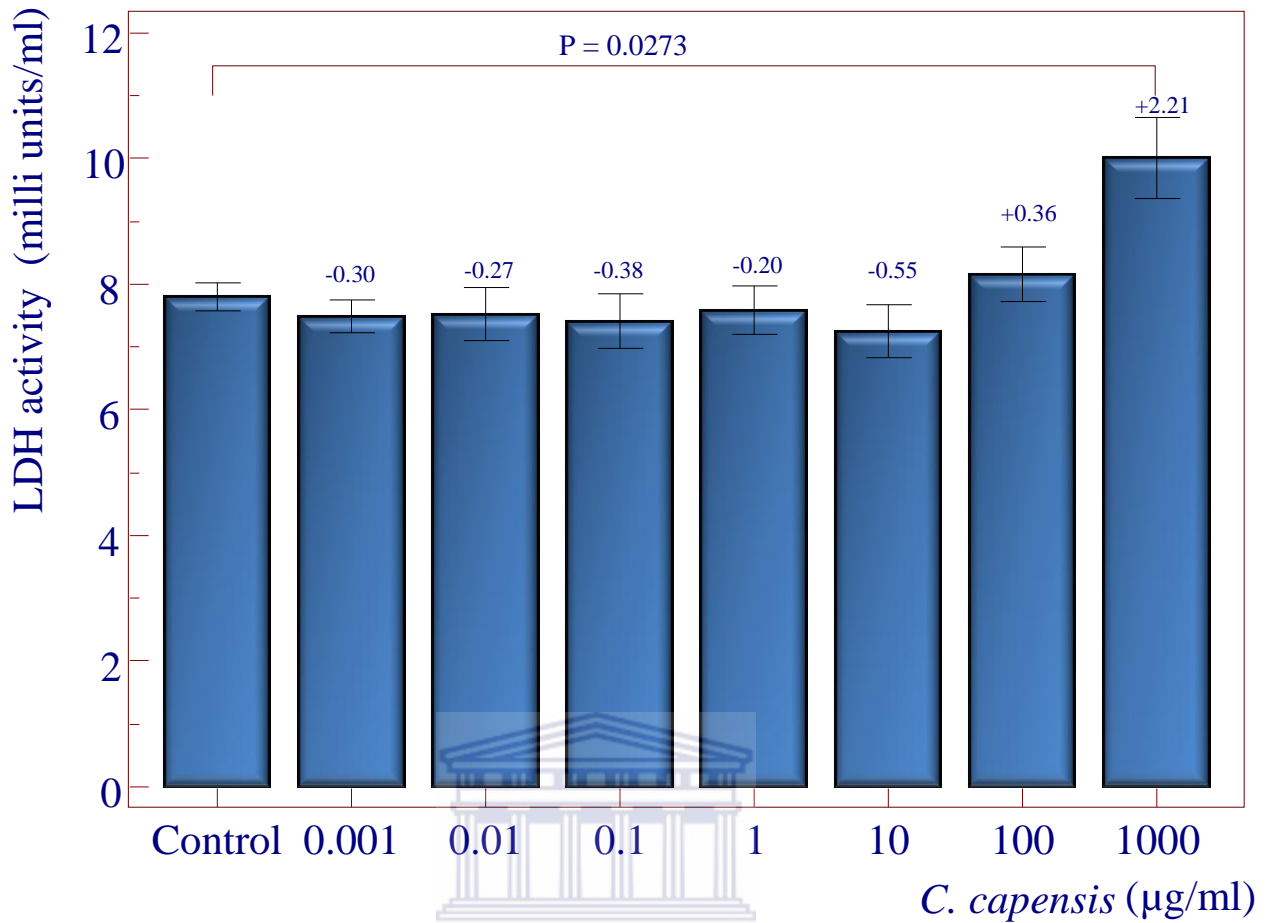


Figure 44: Lactate dehydrogenase (LDH) activity in TM4 cells over a 24 hour period. Cells slight, but not significant, decreases in LDH release between the control and 10 µg/ml of the extract. Hereafter, LDH release began to increase in a dose-dependent manner, between concentrations of 100-1000 µg/ml of the extract, with significance ($P=0.0273$) being observed at 1000 µg/ml of the extract. One-way ANOVA yielded significance ($P<0.0001$) between the control and 1000 µg/ml of the extract.

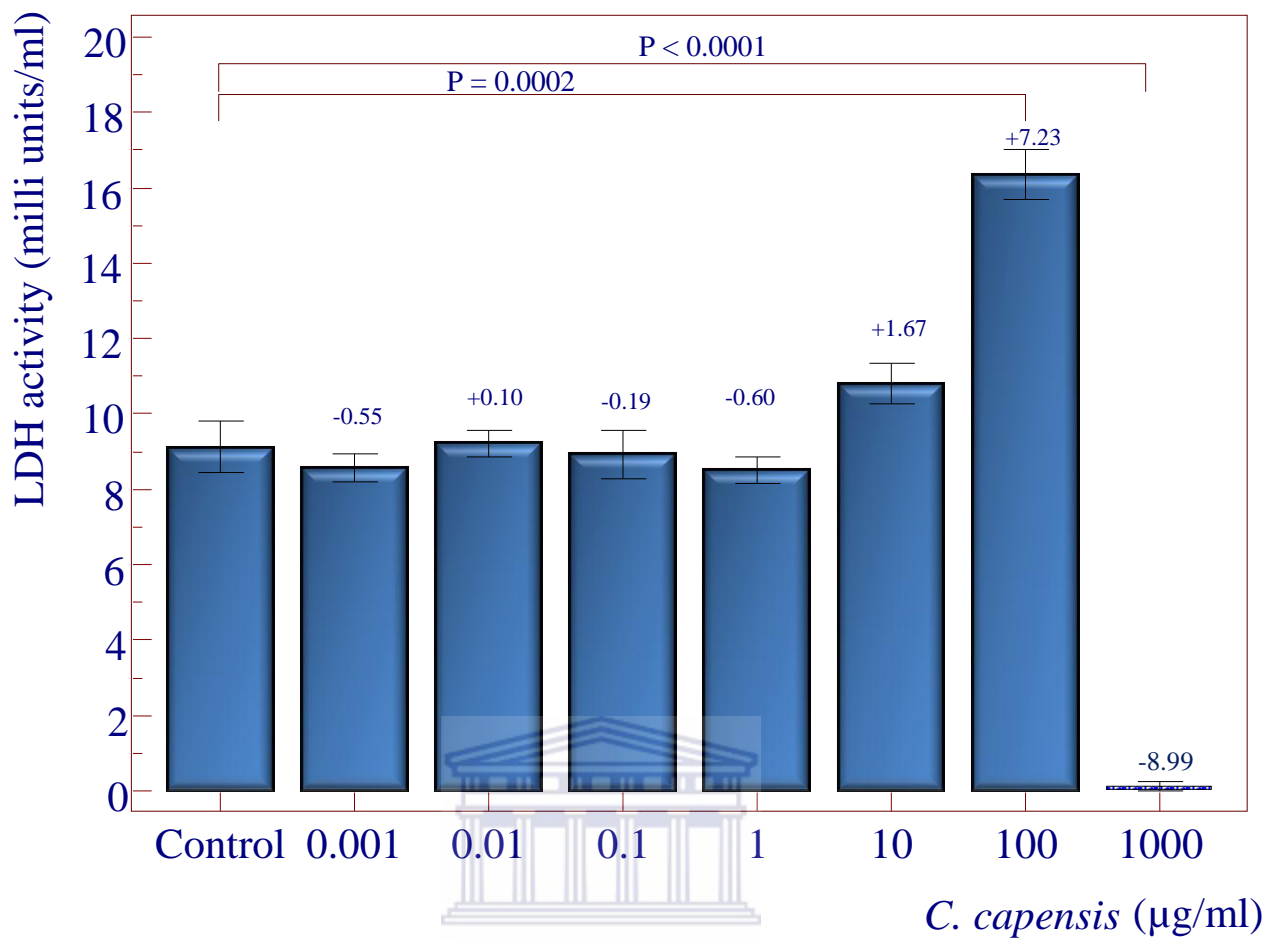


Figure 45: LDH activity in TM4 cells over a 96-hour exposure period. LDH release was observed to be slightly, but not significantly, lowered at concentrations of 0.001 and 1 µg/ml of the extract, while being slightly, but not significantly, elevated between concentrations of 0.01 and 0.1 µg/ml of the extract. Following this, LDH release became increasingly elevated between concentrations of 10 and 100 µg/ml of the extract, with significance (P=0.0002) revealed between the control and 100 µg/ml of the extract, while exhibiting a significant (P<0.0001) sudden, sharp drop in LDH at an extract concentration of 1000 µg/ml. This sharp drop in LDH activity coincides with the observed TM4 cell death, which occurred at 1000 µg/ml of the extract. One-way ANOVA yielded significance (P<0.0001) between the control and 1000 µg/ml extract.

3.5 RNS and ROS inhibition

Increased inhibition of reactive nitrogen species (RNS) production was observed with increasing concentrations of the aqueous *C. capensis* rhizome extract, in a dose-dependent manner (Figure 46). Although increasing in a dose-dependent manner, it must be noted that between the control and 1 µg/ml of the extract, only slight inhibition of RNS, that was not found to be significant, was observed. It was only at concentrations between 10 and 1000 µg/ml of the extract that RNS was significantly ($P=0.0002$ and $P<0.0001$) and substantially inhibited in a dose-dependent manner. Trend analysis using the repeated measures ANOVA revealed a significant ($P=0.0001$), positive trend between the control and 1000 µg/ml of the extract. Similarly, one-way ANOVA revealed significance ($P<0.0001$) between the control and 1000 µg/ml of the extract.

Similarly, the extract was able to inhibited reactive oxygen species (ROS) production (Figure 47). However, it must be noted that an absence of ROS inhibition was observed 0.1 and 10 µg/ml of the extract, along with an apparent decrease in ROS inhibition between 1-250 µg/ml extract. It was only between 250-1000 µg/ml of the extract that ROS inhibition steadily increased in a dose-dependent manner. Nevertheless, significance ($P=0.0198$, $P=0.0425$, $P=0.0185$, $P<0.0001$) was observed between the control and 1, 100, 250, 500 and at 1000 µg/ml of the extract. Subsequently, trend analyses using the repeated measures ANOVA revealed a significant ($P=0.0136$), positive, trend between the control and 1000 µg/ml of the extract. Similarly, one-way ANOVA yielded significance ($P=0.001$) between the control and 1000 µg/ml extract.

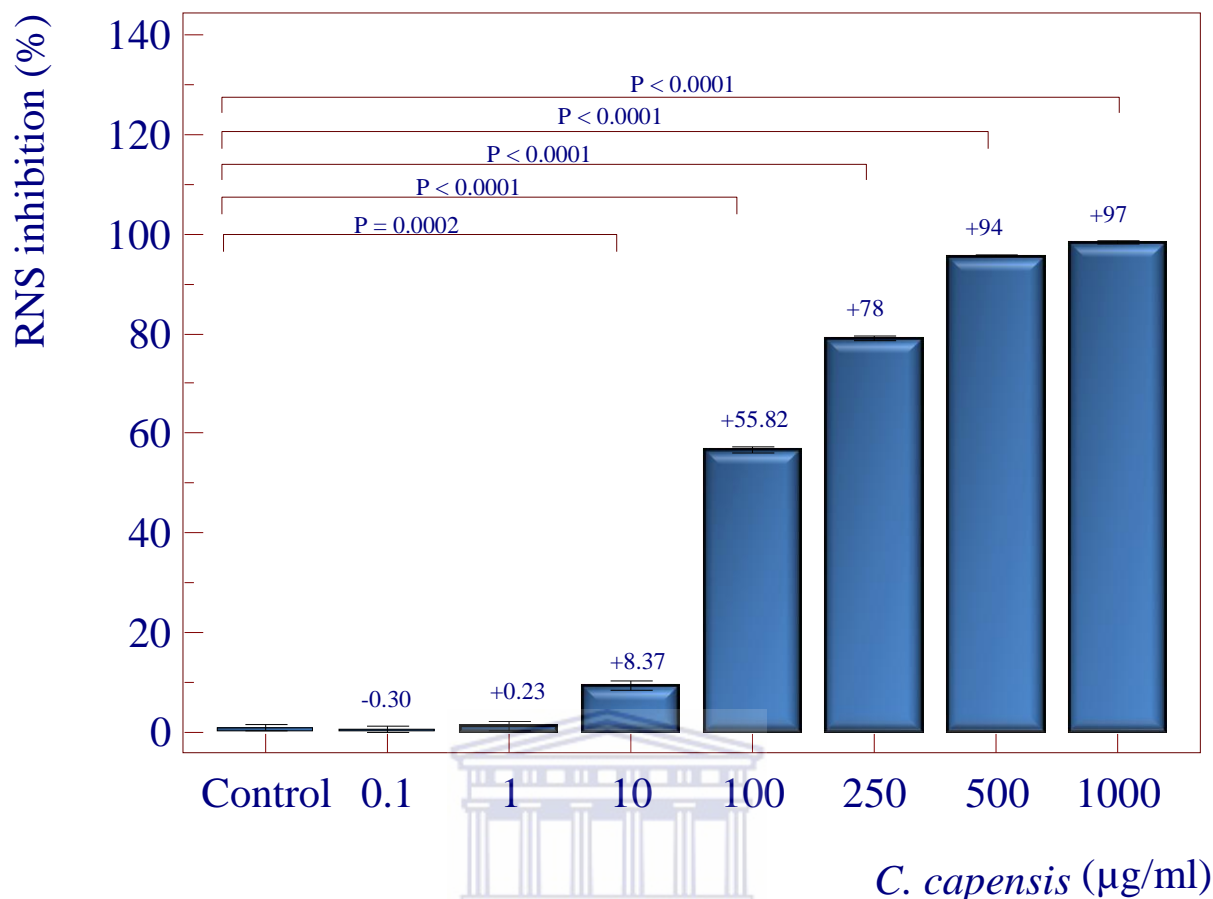


Figure 46: Reactive Nitrogen Species (RNS) inhibition as determined by the ABEL antioxidant test kit using peroxyntirite. Although increasing in a dose dependent manner, it must be noted that between the control and 1 µg/ml of the extract, only slight inhibition of RNS, that was not found to be significant, was observed. It was only at concentrations between 10 and 1000 µg/ml of the extract that RNS was significantly ($P=0.0002$ and $P<0.0001$) and substantially inhibited in a dose-dependent manner. One-way ANOVA yielded significance ($P<0.0001$) between the control and 1000 µg/ml extract.

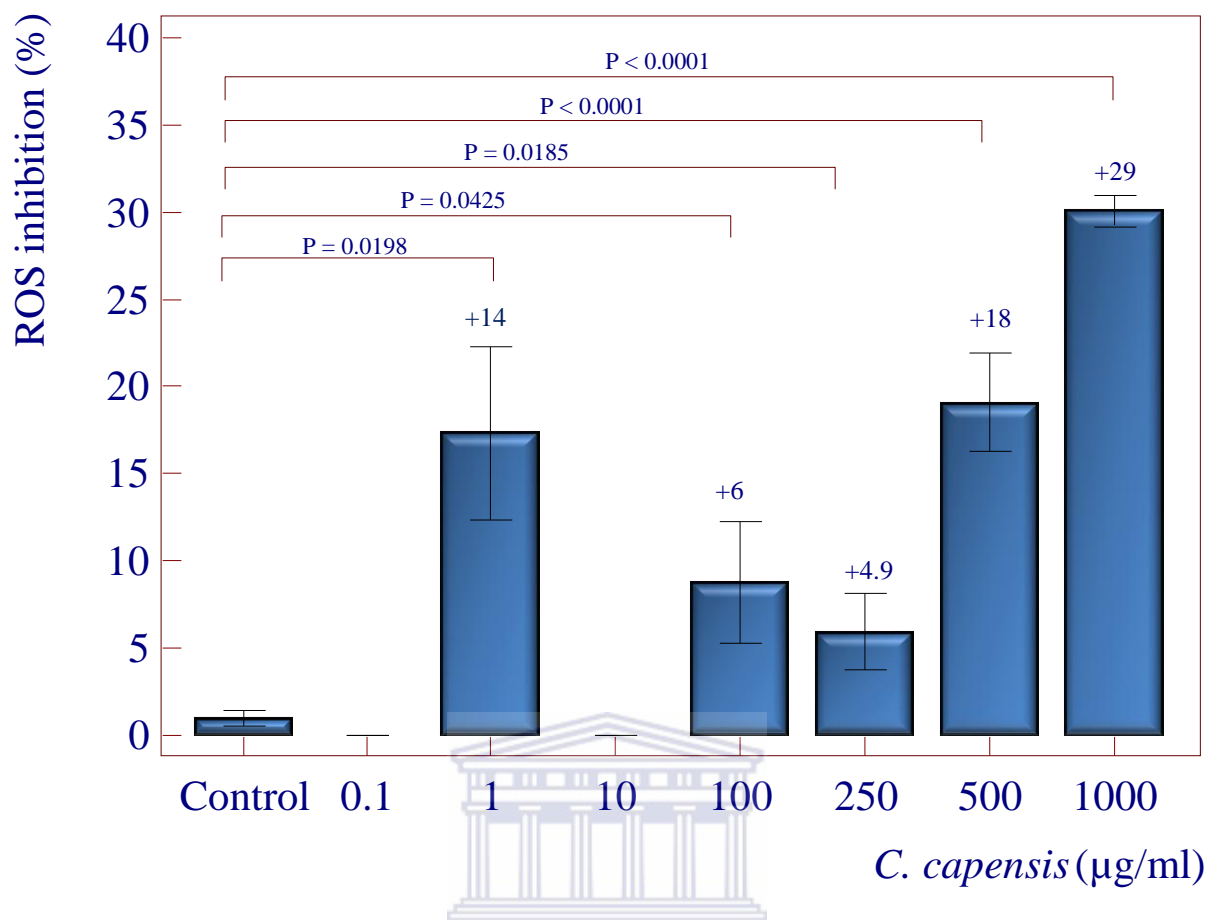


Figure 47: ROS inhibition as determined by the ABEL antioxidant test kit using superoxide. An absence of ROS inhibition was observed 0.1 and 10 µg/ml of the extract, along with an apparent decrease in ROS inhibition between 1-250 µg/ml of the extract. It was only between 250-1000 µg/ml of the extract that ROS inhibition steadily increased in a dose dependent manner. Nevertheless, significance ($P=0.0198$, $P=0.0425$, $P=0.0185$, $P<0.0001$) was observed between the control and 1, 100, 250, 500 and at 1000 µg/ml of the extract. One-way ANOVA yielded significance ($P=0.001$) between control and 1000 µg/ml.

3.6 Inhibition of collagenase activity

A significant ($P=0.0001$) dose-dependent inhibition of collagenase activity was observed at each concentration between the control and 1000 $\mu\text{g/ml}$ of the extract (Figure 48). Very little change in collagenase inhibition occurred between 0.1 and 250 $\mu\text{g/ml}$ of the extract. It was only between 250 and 1000 $\mu\text{g/ml}$ of the extract that collagenase activity was more substantially. Repeated measures ANOVA revealed a significant ($P=0.0002$), positive trend between the control and 1000 $\mu\text{g/ml}$, while one-way ANOVA also yielded significance ($P<0.0001$) between the control and 1000 $\mu\text{g/ml}$ extract.

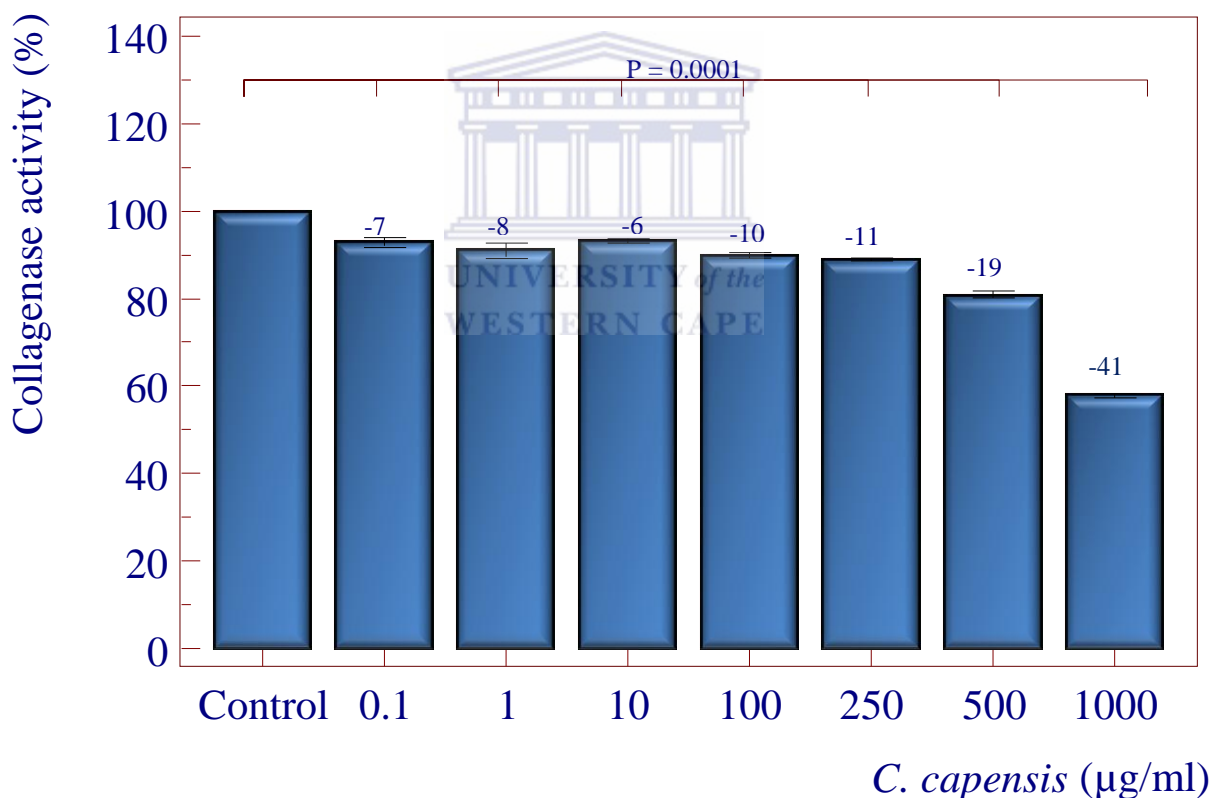


Figure 48: Effect of the extract towards collagenase activity, showing a significant ($P=0.0001$) dose-dependent inhibition of collagenase activity at all concentrations. The greatest inhibition of collagenase activity was observed at 500 $\mu\text{g/ml}$ and 1000 $\mu\text{g/ml}$, respectively.

3.7 Inhibition of Elastase activity

The extract was found to produce very slight decreases in elastase activity at concentrations between 0.1-10 µg/ml of the extract, while resulting in no change in elastase activity at concentrations between 100 and 1000 µg/ml of the extract (Figure 49). Statistically, the decreases in elastase activity observed between 0.1-10 µg/ml of the extract were found to be statistically significant ($P=0.0003$ and $P<0.0001$). Trend analysis using the repeated measures ANOVA revealed an insignificant ($P=0.0829$), positive trend, while ANOVA revealed significance ($P<0.001$) between the control and 1000 µg/ml of the extract.

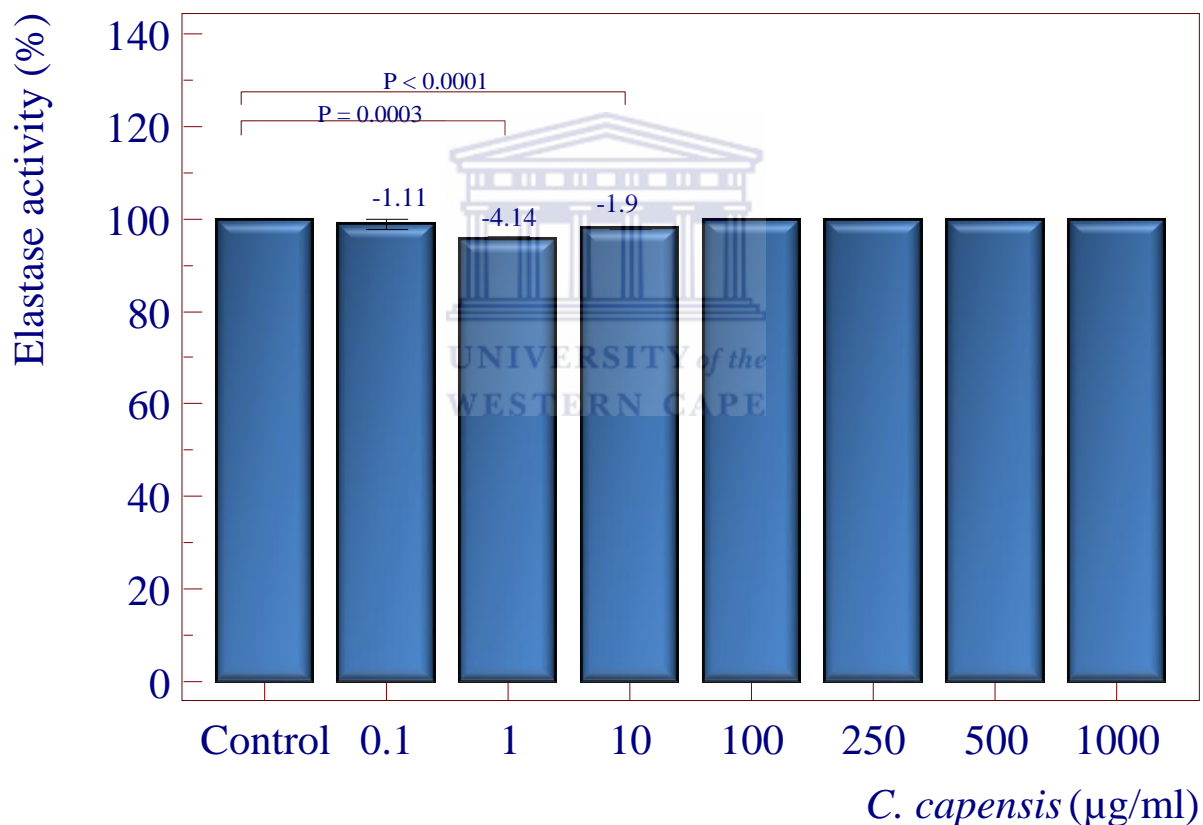


Figure 49: Effect of the extract towards elastase activity showing a significant ($P=0.0003$ and $P<0.0001$) decrease in elastase activity at 1 µg/ml and 10 µg/ml extract, respectively. No further decrease in elastase was observed between concentrations of 0.1-10 µg/ml extract.

Chapter 4

Discussion

4.1 Introduction

The use of traditional medicine has become an increasingly popular global trend (Siegfried and Hughes 2012). This is mostly due to traditional medicine being more readily available, easily accessible, forming part of cultural practices, and the belief that it presents less health risks when compared to conventional Western Medicine (Pal and Shukla 2003; Siegfried and Hughes 2012). In addition to the lower perceived health concerns, it is also significantly cheaper than the pharmaceuticals currently used in Western Medicine (Pal and Shukla 2003), making it an attractive alternative.

In Africa, the vast majority of the population is unable to cope with the cost involved in conventional health care and pharmaceutical, making this system unavailable (Chitindingu et al. 2014). Without the availability of conventional medicine, people began turning to the cheaper and more easily accessible traditional medicine, often in desperation (Chitindingu et al. 2014). Subsequently, this has led to an increasingly popular usage of traditional medicine among the African population, accounting for approximately 80% (Siegfried and Hughes 2012).

In addition to the cost involved, other reasons for the increased usage of traditional medicine are strong cultural beliefs, increased curiosity and interest in a more holistic approach to medicine, and the belief that traditional medicine is natural, and therefore safer than pharmaceutical drugs (Siegfried and Hughes 2012). While this belief is becoming more widespread, it is not necessarily true, as shown by Fennel et al (2004) who demonstrated toxicity of plant extracts that are commonly used in traditional medicine

(Fennell et al. 2004). Thus, extensive investigation into the safety, and efficacy, of plants used in traditional medicine is vital.

Despite recent investigations, many plants that are commonly used in South African traditional medicine remain unevaluated for their reported medicinal benefit, or possible dangers. *Cissampelos capensis* falls within this category.

4.2 Cell Viability

South African Menispermaceae species have been used in traditional medicine to treat a range of different ailments, as these plant species contain a wide variety of alkaloids (De Wet et al. 2011). *C. capensis* specifically is used in traditional medicine to treat cancer. However, scientifically documented information regarding *C. capensis* and its medicinal benefits are extremely limited. Thus, De Wet et al (2009) set out to gain insight into medicinal benefits of *C. capensis*, among other South African Menispermaceae species, in terms of cancer treatment by testing crude rhizome and leaf alkaloidal extracts against breast cancer (MCF-7), melanoma (UACC62) and renal cancer cells (TK10) (De Wet et al. 2009). De Wet et al (2009) showed that despite the traditional use of *C. capensis* in the treatment of cancer, both the leaf and rhizome extracts of produced surprisingly weak cytotoxic effects toward all three cancer cell lines used in the study. The weak cytotoxicity observed by De Wet is in line with the results of the current study, using the prostate cancer cell line LNCaP.

When the LNCaP cells were exposed to the aqueous *C. capensis* rhizome extract over 24 hours, it was noted that at low concentrations of the extract, a slight increase in cell viability was evident, followed by a decrease in viability at higher concentrations. Although slight, the increase in viability observed at low concentrations was significant, and apparently due to cellular stress, characterized by an increased mitochondrial dehydrogenase activity (Berridge et al. 1996). At higher concentrations of the extract, a significant decrease in viability,

accounting for 14%, was evident, indicating a reduced mitochondrial dehydrogenase activity (Berridge et al. 1996). In addition to this, there were observably less cells at the highest concentration, indicating the death of these cells.

When the cells were exposed to the extract over 96 hours, however, there was no longer observable stress at lower concentrations of the extract. Instead, slightly lowered cell viability was evident, indicating a subtle reduction in mitochondrial dehydrogenase activity (Berridge et al. 1996). Additionally, cells were not observably fewer and no characteristic signs of cell death were evident at these concentrations, indicating adaptation to the extract and survival of these cells. However, at higher concentrations of the extract, a more substantial decrease in viability, and thus mitochondrial dehydrogenase activity (Berridge et al. 1996), was evident; accounting for a 83% reduction in viability at the highest concentration. Furthermore, there were substantially fewer cells observed at the highest concentration, indicating the death of these cells. When comparing the percentage reduction in viability, it is clear that biologically significant cytotoxicity only occurred at the highest concentration over an extended period of time. Based on this necessity of a high concentration of the extract over an extended period of time to cause biologically significant cytotoxicity, the aqueous *C. capensis* rhizome extract can be said to have exhibited weak cytotoxicity toward prostate cancer LNCaP cells. This is similar to the previously mentioned weak cytotoxicity observed by De Wet et al (2009), using crude alkaloidal rhizome extract, and may be the result of the specific alkaloids present within the rhizomes of *C. capensis*.

Previously, De Wet et al (2011) characterized the alkaloids present in the leaves, stems and rhizomes of *C. capensis*, and showed that the rhizomes were a source of bisbenzyltetrahydroisoquinoline, proaporphine and aporphine alkaloids. Specifically, the identified alkaloids included cycleanine, 12-O-methylcurine, cissacapine, glaziovine, pronuciferine, bulbocapnine and insularine (De Wet et al. 2011). The bisbenzyltetrahydroisoquinoline alkaloids; cycleanine, 12-O-methylcurine and cissacapnine,

were identified as the major alkaloids found in the rhizomes of *C. capensis* (De Wet et al. 2011). Of the identified *C. capensis* rhizome alkaloids, cycleanine is the only alkaloid that has previously been shown in studies to produce cytotoxicity (Schiff 1991; Zi et al. 2007; De Wet et al. 2011)

Specifically, Schiff (1987) investigated the general effects of cycleanine and reported that isolated cycleanine produced analgesic, anti-inflammatory and muscle relaxant effects. In addition, the same author also reported that cycleanine possessed anti-cancer abilities (Schiff 1991). Subsequently, Zi et al (2007) set out to investigate the proposed anticancer abilities of cycleanine and reported potent cytotoxicity against human lymphoma cells (Ramos RA-1), along with modest cytotoxicity against human lung cancer (A549), murine leukemia (P388) and human leukemia (HL-60) cells.

Being one of the major alkaloids present in the rhizomes of *C. capensis*, and the only one shown to induce cytotoxicity in previous studies, it can be hypothesized that cycleanine was responsible for the cytotoxicity observed at high extract concentrations in the current study. However, due to the necessity of a high extract concentration, in the current study, to produce biologically significant cytotoxicity, it can also be hypothesized that low levels of cycleanine existed within the aqueous *C. capensis* rhizome extract. This notion may be supported by the study conducted by De Wet et al (2011) that not only characterized the alkaloids present in *C. capensis* rhizomes, stems, and leave, but also described their specific percentage yields. In their study, De Wet et al (2011) showed that there was a substantial amount of plant to plant alkaloid quantity variation, which in the case of cycleanine ranged anywhere from a low of 14% to a high of 77%. For this reason, it is possible that the aqueous *C. capensis* rhizome extract used in the current study may have had a cycleanine content that fell within the lower end of the scale. Should this be true, it would indicate that a high extract concentration was needed in order to accumulate enough cycleanine to incite a cytotoxic effect.

Based on the previously mentioned studies describing the potent cytotoxicity of isolated cycleanine (Schiff 1991; Zi et al. 2007), it could then be hypothesized that the isolation of cycleanine from *C. capensis* would be more beneficial in the treatment of prostate cancer, as opposed to the aqueous *C. capensis* rhizome extract used in the current study. However, the safety of isolated cycleanine is questionable when the effects of the extract toward the non-cancerous TM3 Leydig and TM4 Sertoli cells observed in the current study are considered.

When the TM3 cells were exposed to the extract over 24 hours, it was noted that slight elevations in cell viability were evident at low extract concentrations, followed by a significant, more substantial increase in viability at the higher concentrations, accounting for a 20 % increase. This elevation in cell viability indicates the presence of cellular stress, characterized by an elevation in mitochondrial dehydrogenase activity (Berridge et al. 1996). When these cells were exposed to the extract over 96 hours, low extract concentrations exhibited a slight elevation in cell viability was evident, accounting for a 4% increase at best, followed by a dose-dependent decrease in cell viability that was only significantly lower at the highest concentrations, accounting for a 57% and 87% decrease, respectively. Cells were thus minimally stressed at lower concentrations, characterized by a slight increase in mitochondrial dehydrogenase activity (Berridge et al. 1996), while the highest concentrations exhibited more substantial decreases in viability, thereby a reduced mitochondrial dehydrogenase activity (Berridge et al. 1996), along with observably fewer cells and hence cell death.

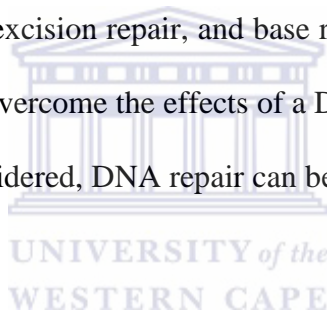
Similarly, when the TM4 cells were exposed to the extract over 24 hours, low extract concentrations exhibited a slight dose-dependent increase in cell viability, accounting for a 7% increase at best. Cell mitochondrial dehydrogenase activity was thus minimally, but progressively increased, showing a progressive occurrence of cell stress. Following this, there was a decline in cell viability at the highest concentrations, accounting for 35%. Additionally,

fewer cells were observed at this concentration, indicating cell death. Over 96 hours, cell viability fluctuated between a 3% increase and a 4% decrease in viability at low extract concentrations. Despite the fluctuation, no difference in observable cell numbers or morphology could be observed, indicating cell adaptation and survival at these concentrations. Cell viability decreased significantly at higher extract concentrations, accounting for 28% and 90% reduction, respectively. At these concentrations, cells were observably fewer and morphologically atypical, indicating cell stress and cell death. Again, it is clear that the extract was only able to produce biologically significant cytotoxicity at the highest concentration, over an extended period of time in TM3 Leydig and TM4 Sertoli cells. Unfortunately, this occurred at the same extract concentration that is necessary to produce cytotoxicity in prostate cancer LNCaP cells. Therefore, the extract produced cytotoxicity in a non-specific manner at high concentrations. However, if cycleanine is the alkaloid responsible for the cytotoxic effect, it stands to reason that isolated cycleanine might also produce non-specific cytotoxicity. Nevertheless, the need for a high extract concentration to produce biologically significant cytotoxicity in prostate cancer cells, that is also capable of producing the same effect in non-cancerous cells, indicates that the aqueous *C. capensis* rhizome extract may not be suited to the treatment of prostate cancer.

4.3 DNA fragmentation

Previously, a study conducted by Geske and Nelson (2000) investigated the occurrence of DNA repair in early stages of apoptosis using cultured murine mammary epithelial cells. These authors reported that cell repair mechanisms became activated when cells were confronted with an DNA-damaging apoptotic stimulus (Geske and Nelson 2000). Additionally, they suggested that if the stimulus did not induce enough DNA damage, to overwhelm the repair mechanism of the cells, then transcription and translation could

re-initiate and the cell would not be driven toward apoptosis, and hence survive (Geske and Nelson 2000). It was only when the DNA damage exceeded the capabilities of repair mechanisms, or when these mechanisms were deactivated by caspase proteolysis, that cells were observed to be driven toward apoptosis (Geske and Nelson 2000). Subsequently, the ability of cells to repair DNA until repair mechanisms are overwhelmed, which then leads to apoptosis, was also reported in a study conducted by Gordon et al (2002), investigating the interaction of DNA damage and repair mechanisms in photoreceptor degeneration. More recently, Martin et al (2008) outlined the effects of DNA repair mechanism in the resistance of cancer cells to chemotherapeutic agents. They stated that five repair mechanisms existed to protect cellular DNA from injury; namely nucleotide excision repair, mismatch repair, double-strand break repair, base-excision repair, and base repair. Nevertheless, it is clear that the possibility exists for cells to overcome the effects of a DNA damaging agent, and survive. When the current results are considered, DNA repair can be hypothesized to have occurred in prostate cancer LNCaP cells.



When LNCaP cells were exposed to the aqueous *C. capensis* rhizome extract for 24 hours, low extract concentrations yielded and increase in observed DNA fragmentation, characterized by stained cell nuclei, in a dose-dependent fashion. It is essential to note, however, that despite cell nuclei taking up the stain at these concentrations, cell morphology remained identical to that of control groups, and therefore unchanged. The lack of morphological change at low concentrations, despite taking up the stain, may indicate the ability of the extract to incite low levels of DNA damage in LNCaP cells. At higher concentrations, observed DNA fragmentation climbed dramatically in a dose-dependent manner, along with obvious changes in LNCaP cell morphology and observably less cells. If the extract indeed caused DNA damage, then, in accordance with the observations of Geske and Nelson (2000), it can be hypothesized that high extract concentrations produced

sufficient DNA damage to drive the cells towards cell death. The observed effect of the extract toward LNCaP cells over 96 hours may further support this notion.

When LNCaP cells were exposed to the extract for 96 hours, DNA fragmentation, characterized by stained cell nuclei, were scarcely observed at low extract concentrations. In fact, the observed fragmentation at low extract concentrations was comparable to that observed in control groups. Additionally, cell morphology remained flat, polygonal, and otherwise normal. Should the hypothesized extract-related DNA damage be true, then the absence of DNA fragmentation, along with the normal cell morphology, may further support the proposed low levels of DNA damage at low extract concentrations. Assuming this to be true, it would be in line with the previously mentioned reports of cell survival, rather than cell death, when the amount of DNA damage was within the capacity cell repair mechanisms. At the higher extract concentrations, observed DNA fragmentation abruptly increased, accounting for a 72% and 100% increase, respectively. Furthermore, cells were observably fewer at these concentrations, with characteristic signs of cell death. It is essential to note that these are the same extract concentrations that produced substantial fragmentation and alterations in cell morphology over 24 hours.

Thus, this may indicate that the extract caused enough DNA damage at high concentration to overcome the capabilities of the cell repair mechanisms and drive the cell toward cell death, while failing to do so at lower concentrations. With this in mind, it can only be hypothesized which compound within the extract was responsible for these observed effects. It stands to reason that the compound most likely responsible for the hypothesized extract-induced DNA damage would be cycleanine, as this alkaloid has previously been shown to be cytotoxic towards cancer cells. However, studies reporting the cytotoxic effects of cycleanine did not investigate the possible mode of action, and thus it cannot be said with certainty to directly cause DNA damage. However, it would serve to explain the necessity of a high concentration to drive the LNCaP cells toward cell death, and be in line with the previously hypothesized

low levels of cycleanine within the extract. In addition, most chemotherapeutic agents cause DNA damage, resulting in single-stranded or double-stranded breaks. If cycleanine was the causative alkaloid, then it can be hypothesized to cause single-stranded or double-stranded breaks in DNA, especially since cells can recover from these specific types of DNA damage. Alternatively, the findings of Babajidel and Mabusela (2010) may provide a different explanation for the hypothesized extract-related DNA damage in LNCaP cells. In addition to alkaloids, Babajide and Mabusela (2010) revealed *C. capensis* to be a source of other phytochemicals, such as phenols, tannins, saponins and flavonoids. These types of phytochemicals are known to act as antioxidants, scavenging free radicals and thus helping to maintain the previously mention redox homeostasis. However, in a recent study conducted Lu et al (2013), it was demonstrated, using the flavonoid epigallocatechin (ECGC), that excessive antioxidant supplementation could cause adverse effects in humans. Specifically, these authors showed that excessive amounts of ECGC resulted in reductive DNA damage, precisely double stranded breaks, in human lung cancer cells (A549) and non-cancerous human skin cells (GM05757).

Furthermore, these observations were in line with those observed in a study conducted by Wang et al and a study conducted by Nyugen and co-writers, who demonstrated that the weakly bound electrons found in antioxidants could cause direct DNA damage in cells (Wang et al. 2009; Nguyen et al. 2011). Moreover, these authors showed that this was due to the dissociation of the weakly bound electron to the guanine bases, resulting chemical bond breakages and thus single and double stranded DNA breaks (Wang et al. 2009; Nguyen et al. 2011). For these reasons, it is possible that the LNCaP cells used in the current study were exposed to excessive amount of antioxidants present within the extract, and hereby exhibited DNA damage.

Based on the observation by Lu et al (2013), it is possible that antioxidant present in the aqueous *C. capensis* rhizome extract also resulted in single or double stranded DNA breaks in

LNCaP cells. It must be mentioned that the study conducted by Babajidel and Mabusela (2010) merely screened for antioxidants in *C. capensis* extracts, but did not quantify them. Thus, the actual amounts of antioxidants present within the extract used in the current study may be low, thus dictating a need for a high extract concentration to induce cell death. Additionally, another possibility is that low levels of antioxidants and cycleanine acted synergistically to produce the observed DNA damage. However, without further investigation it is not possible to definitively identify the mechanism by which the damage occurred.

4.4 Combined effect of testosterone and extract

An early study conducted by Huggins and Hodges in 1941 investigated the link between carcinoma of the prostate and androgen hormones by observing the serum marker acid phosphatase (Huggins and Hodges 1941). These authors demonstrated that castration in men with prostate cancer caused a decrease in acid phosphatase and regression of the disease, whereas administration of testosterone caused progression of the disease. Subsequently, studies also suggested that administration of testosterone to men with prostate cancer increased the severity of the disease, and observed mortality (Prout and Brewer 1967; Fowler and Whitmore 1981). Thus, testosterone administration was believed to be contraindicated in men suffering from prostate cancer.

Since then, many studies investigating this relationship have reported conflicting results (Morgentaler 2006; Morgentaler 2009; Chuu et al. 2011). Morgentaler (2006) clearly outlined contradiction in the available literature, stating that administration of testosterone to men with, or suspected of having prostate cancer may not be contraindicated, as previously believed. An example used by Morgentaler was the rare occurrence of prostate cancer during the peak testosterone years of the early 20's, despite the common presence of microfoci in these young men (Morgentaler 2006). Subsequently, a study was conducted by

Khera et al (2009) investigated the effects of testosterone replacement therapy in men who underwent radical prostatectomy. Khera et al (2009) demonstrated that testosterone replacement therapy in these men did not increase prostate-specific antigen (PSA) levels, and thus did not aggravate prostate cancer. Additionally, a review of available literature by Rhoden and Averbeck (2009) also clearly outlined that no compelling scientific evidence supported the belief that higher testosterone levels worsened the occurrence of prostate cancer. More recently, a study conducted by Song and Khera (2014) investigated the effects of normal physiological levels testosterone toward androgen-sensitive prostate cancer cells (LNCaP and MDA PCa 2b), along with androgen insensitive prostate cancer cells (PC-3 and DU145). They demonstrated that prostate cancer cells would grow optimally at low levels of testosterone, while their growth was inhibited at higher levels of testosterone (Song and Khera 2014). These observations are in line with the current study, when LNCaP cells were exposed to both increasing concentrations of the aqueous *C. capensis* rhizome extract and increasing concentration of testosterone. Observably, low concentrations of testosterone caused overall improved growth, characterized by an observed greater cell number. Also, cell morphology remained flat and polygonal, and thus normal. At higher concentrations of testosterone, the LNCaP cells began to show morphological changes from flat and polygonal to rounded, and thus stressed. These observations continued over a 96-hour period, whereby low levels of testosterone caused an observed LNCaP cells proliferation and high concentrations resulted in inhibition.

When the increasing concentrations of the extract were added to the increasing concentrations of testosterone over 24 hours, it was observed that low concentrations of the extract produced slight dose-dependent elevations in cell viability when testosterone concentrations increased. The observed increase in cell viability is indicative of increase mitochondrial dehydrogenase activity, and hence cell stress (Berridge et al. 1996). It is important to note that at these lower extract concentrations, low testosterone concentrations observably resulted in overgrowth of

the cells, characterized by cell stacking. For this reason, the observed elevation in viability, and thereby stress, was most likely due to cell overgrowth rather than extract toxicity. As the testosterone concentration increased, cells became progressively less stacked, presented with slightly rounded morphology, and marginally fewer observed cells, indicating slight cell death. When extract concentrations of 0.1 and 1 $\mu\text{g/ml}$ were reached, however, it was noted that lower testosterone concentrations continued to cause slight elevations in cell viability, along with the observed overgrowth and thereby cell stress, while higher testosterone concentrations caused a reduction in cell viability. Also, cell morphology became progressively rounder, and showed further signs of cell death, as testosterone concentrations increased.

Over 96 hours, low extract concentrations combined with low testosterone concentrations exhibited slight reductions in LNCaP cell viability, while higher testosterone concentrations produced substantially elevated cell viability. Cells were observably slightly fewer, indicating a small amount of cell death, and were rounded. As the extract concentration increased, it was noted that low testosterone concentrations slowly started showing further slight reductions in cell viability, while higher testosterone concentrations showed greater reductions in cell viability. It must be noted that the most biologically significant increases and decreases in cell viability were produced by the highest concentration of testosterone, indicating its ability to induce the greatest increase in mitochondrial dehydrogenase activity and thereby cell stress (Berridge et al. 1996).

It is also important to note that even at the highest concentration of testosterone, substantially lowered cell viability was only produced when with the highest concentration of the extract. In fact, when a comparison was drawn between the effect of the extract alone, and in combination with the highest concentration of testosterone, it was clear that a much greater inhibitory effect could be achieved when the two were combined. However, even with this combined effect, biologically significant reductions in viability were only observed at the

highest extract concentration, over an extended period of time. When previous characterization of the major *C. capensis* rhizome alkaloid is considered, it can be hypothesized to be responsible for the observed combined effect of testosterone and the extract in LNCaP cells.

In a study conducted by Tian and Pan (1997), combating multidrug resistance in cancer cells using cycleanine, insularine and insulanoline was investigated. These authors reported that cycleanine was able to down-regulate P-glycoprotein expression in these resistance-acquired cancer cells, and thus reduce multi-drug resistance (Tian and Pan 1997). Considering that cycleanine is one of the major alkaloids present in the rhizomes of *C. capensis*, it may be possible that it affected the LNCaP cells used in the current study in a similar way, resulting in P-glycoprotein inhibition. This notion may be further supported by the observations of Fedoruk et al (2004). Fedoruk et al (2004) investigated the role of P-glycoprotein in androgen modulation in prostate cancer cells. In their study, they demonstrated in PC-3 and LNCaP cells that androgen accumulation was both dependent on function androgen receptors, and was modulated by the P-glycoprotein (Fedoruk et al. 2004). Furthermore, these authors also concluded that the consequences of P-glycoprotein overexpression would result in decreased androgen accumulation, leading to a decreased cell proliferation. If cycleanine indeed caused inhibition of LNCaP P-glycoprotein in the current study, it can be hypothesized, based on the findings of Fedoruk et al (2004), that testosterone would have accumulated within these cells at a greater concentration than normal.

Should this be true, it would result in an even greater proliferative effect at low levels of testosterone, which would serve to explain the combined effects of the extract and testosterone in the current study. Furthermore, it may also indicate that as the concentration of the extract, and thus cycleanine, increased, the inhibition of P-glycoprotein also increased. Should this be correct, it can be hypothesized that a much greater intracellular androgen accumulation would occur at low levels of testosterone, eventually resulting in proliferative

testosterone concentrations becoming inhibitory. If cycleanine could indeed result in proliferative testosterone concentrations becoming inhibitory, then the stigma attached to testosterone in prostate cancer would no longer pose a problem. However, without further investigation this cannot be said with certainty.

4.5 Combined effect of doxorubicin and the extract

Previously, De Wet et al (2011) characterized the alkaloids present in *C. capensis*, describing the major alkaloids found within the rhizomes to be 12-O-methylcurine and cycleanine. Cycleanine is a bisbenzyltetrahydroisoquinolone alkaloid that has previously been shown to produce cytotoxicity toward cancer cells. Additionally, the study conducted by Tian and Pan (1997), investigating multidrug resistance, demonstrated that cycleanine was able to down-regulate P-glycoprotein expression in drug-resistant cancer cells, resulting in a greater accumulation of intracellular doxorubicin (DOX) and thus cell cytotoxicity. Furthermore, the study conducted by Fedoruk et al (2004) demonstrated that P-glycoprotein was active in LNCaP cells, playing an integral role in intracellular androgen accumulation. Considering that cycleanine is a major *C. capensis* rhizome alkaloids, and thereby most likely present in the extract used in the current study, it would stand to reason that combining the extract with DOX would allow for a greater intracellular DOX accumulation and thus more substantial cell death.

In the current study, the prostate cancer LNCaP cells were exposed to increasing concentrations of the extract in the presence of 21.3 nM of DOX (the calculated IC₅₀ value), and compared to the effect of the extract alone. Over 24 hours, the extract in isolation produced slight increases in cell viability at low concentration, indicating an increased mitochondrial dehydrogenase activity and hence cell stress (Berridge et al. 1996). At higher

concentrations, the extract in isolation caused slight decreases in viability in a dose-dependent manner, accounting for an 18% decrease at best.

Although causing a decrease at the higher extract concentration, the actual percentage decrease does not indicate biological significance. However, this is in direct contrast to the effect of the extract in combination with DOX. When the extract was combined with DOX over 24 hours, it was noted that cell viability was decreased at each concentration, ranging from a 16-35% decrease. Additionally, the combined effect of the extract and DOX produced a more substantial decrease in viability at each concentration than what the extract alone achieved. This may support the notion that potentially low levels of cycleanine present within the extract down-regulated the P-glycoprotein, caused intracellular DOX accumulation and thus caused greater cell death. However, when the percentage decrease in viability of the combination is compared to the effect of DOX alone, it is clear that DOX in isolation produced more substantial cytotoxicity. Thus, it is possible that other compounds within the extract may have interfered with the actions of DOX, overshadowing the potential helpful effects of cycleanine.

Previously, Babajidel and Mabusela (2010) showed that *C. capensis* was a rich source of phytochemicals, namely phenols, tannins, flavonoids and saponins. Lee et al (2013) previously showed that these types of phytochemicals have the ability to scavenge free radicals, and act as antioxidants. This may serve to explained the aforementioned observations in LNCaP cells in the current study, as Lamson and Brignall (1999) suggested that compounds acting as antioxidants were able to interfere with the actions of DOX. These authors suggested that antioxidants prevented oxidative stress to cells, the mechanisms by which DOX induces damage to cancerous cells (Lamson and Brignall 1999). Subsequently, Conklin (2004) clearly outlined the ability of antioxidants to prevent DOX-induced oxidative stress to cells, whilst preserving the drugs antineoplastic activity. This was also reported in

subsequent studies that showed antioxidants were able to prevent DOX-induced cardiotoxicity.

Considering that the combined effect of the extract and DOX produced substantial cell death, albeit less than DOX alone, it can be hypothesized that the antioxidant capacity of the extract may act in a similar manner described by Conklin (2004). This may be further supported by the combined effect of the extract and DOX over 96 hours. When the LNCaP cells were exposed to DOX combined with the extract over 96 hours, it was noted that cell viability was further decreased at each concentration, with the highest decrease in viability observed at the highest concentration, accounting for an 85% decrease.

Although, DOX alone produced a greater reduction in viability in comparison to the extract-DOX combination, each concentration of DOX combined with the extract resulted in a substantial, biologically significant decrease in viability, in comparison to the extract alone which only showed substantial, biologically significant decreases in viability at higher concentrations. Thus, it is indeed possible that the antioxidant capacity of the extract may also prevent DOX-induced oxidative stress while preserving the antineoplastic effects of the drug. Should this be true, the extract, or perhaps isolated antioxidants from the extract, may have place in preventing DOX-induced cardiac side effects. However, further investigations are necessary to substantiate this assumption.

4.6 Testosterone production

Surampudi et al (2012) clearly outlined the clinical manifestation termed late-onset hypogonadism. These authors described late-onset hypogonadism as a syndrome characterized by low circulating serum testosterone levels that resulted in symptoms such as low libido, muscle weakness, impaired erectile function, depression, and decreased vitality (Surampudi et al. 2012). Furthermore, they demonstrated through available literature that late-onset hypogonadism is a common manifestation among ageing males, who very often

present with testosterone levels far lower than what is considered normal in healthy, young males (Surampudi et al. 2012). Moreover, these authors described the underlying causes of late-onset hypogonadism, stating that the observed decline in testosterone levels were subject to many factors. Specifically, these factors included a declined Leydig cell function, decline in pituitary-hypothalamic axis function with loss of circadian variation, changes in testosterone receptor sensitive, and changes in cardiometabolic and inflammatory markers (Surampudi et al. 2012). More recently, Huhteneimi (2014) also clearly outlined these factors affecting the occurrence of late-onset hypogonadism, as well as the negative effects late-onset hypogonadism has toward quality of life.

However, the clinical manifestation known late-onset hypogonadism can be managed by means of testosterone replacement therapy. According to Surampudi et al (2012), the goal of testosterone replacement therapy is to raise serum testosterone levels into the mid-normal serum range, roughly between 400-477 ng/dl, to effectively treat and reduce the associated symptoms. Thus, the ultimate goal of testosterone replacement therapy is to improve quality of life in hypogonadal men (Khera and Lipshultz 2007; Surampudi et al. 2012). Khera and Lipshultz (2007) described testosterone replacement therapy being beneficial in treating signs and symptoms of late-onset hypogonadism, improving sexual function, muscle mass, bone density, cognition and mood, and thus substantially improving quality of life. Similarly, a recent study conducted by Okada et al (2014) investigated testosterone replacement therapy in late-onset hypogonadism in 50 ageing men also outlined the benefits associated with it. These authors accomplished this by measuring parameters such as international index of erectile function, international prostate symptom score, self-rating depression scale and ageing male symptoms, before and after the administration of testosterone replacement therapy over a six month period (Okada et al. 2014). Furthermore, they demonstrated that the administration of testosterone had increased the scores the of

international index of erectile function, international prostate symptom score, self-rating depression scale and ageing male symptoms, and resulted in an overall improvement in the health and quality of life in these men (Okada et al. 2014). Thus, these authors concluded that testosterone replacement therapy is a safe, effective means of improving the health and quality of life in late-onset hypogonadism.

A study conducted by Arver et al (2014) stated that long term testosterone replacement therapy was both affordable and sustainable for men in Sweden. However, this may not hold true for developing countries. In developing countries, access to conventional health care is very limited, due to poverty and increased population movement from rural to urban areas (Pal and Shukla 2003). Thus, it stands to reason that men experiencing late-onset hypogonadism in developing countries may not have access to testosterone replacement therapy, let alone be able to cope with the costs involved.

However, many plants, such as *Lepidium meyenii* (Maca) (Gonzales et al. 2002), *Ptychopetalum olacoides* (muira pauma) (Waynberg 1994) and *Eurycoma longifolia* (Tongkat Ali) (Tambi et al. 2011; Solomon et al. 2014), have shown the ability to improve sexual function and libido, as well as boosting testosterone levels. The ability of plants to improve sexual function and boost testosterone is significant, considering that people living in developing countries, such as South Africa, rely on traditional medicine as a primary source of healthcare (Kofi 2004; Chitindingu et al. 2014). However, the results obtained in the current study suggest that *C. capensis* may not be suited to this purpose.

In the current study, TM3 Leydig cells were exposed to the aqueous *C. capensis* rhizome extract. When the Leydig cells were exposed to the extract for 24 hours, low extract concentrations produced a very slight increase in testosterone production, accounting for a 0.02 ng/ml increase, at best. At higher extract concentrations, a significant decrease in testosterone production was observed, accounting for a 0.06 ng/ml decrease, at best. While a decrease in testosterone production of this nature is not biologically

significant, any decrease in testosterone production, with regard to late-onset hypogonadism, is not desirable. When Leydig cells were exposed to the extract for 96 hours, testosterone production remained marginally increased when compared to the control groups, accounting for a 0.03 ng/ml increase, at best. Additionally, higher extract concentrations did not produce a further decrease in testosterone production when compared to that observed over 24 hours. Thus, it is clear that the aqueous *C. capensis* rhizome extract does not induce testosterone production in a biologically significant manner. As such, it would thereby not be able to assist men suffering from late-onset hypogonadism as the result of impaired Leydig cell function. Additionally, a closer inspection of the alkaloids present in the rhizomes of *C. capensis* may further demonstrate that this plant is not well suited in treating low serum testosterone.

According to De Wet et al (2011), *C. capensis* was shown to be a source of the aporphine alkaloids. Previously, a study conducted by Shin et al (1998) demonstrated that bulbocapnine had the ability to reduce dopamine levels in PC-12 neuronal cells. In fact, bulbocapnine resulted in a 42.2% dopamine inhibition in these cells (Shin et al. 1998). Moreover, this was demonstrated at a bulbocapnine concentration of 20 μ M (Shin et al. 1998). The ability of bulbocapnine to inhibit dopamine presents a problem, as dopamine and testosterone have a direct relationship.

Under normal circumstances, dopamine counteracts the secretion of prolactin releasing hormone (PRH) by promoting the secretion of prolactin inhibiting factor (PIF) (Zeitlin and Rajfer 2000). In turn, PIF inhibits the release of prolactin, which under normal circumstances inhibits the release of Gonadotropin Releasing Hormone GNRH (Zeitlin and Rajfer 2000). Thereby, inhibition of prolactin by dopamine results in more GNRH secretion, and hence more testosterone production (Zeitlin and Rajfer 2000). Thus, without dopamine, testosterone levels would decline.

De Wet et al (2011) showed that bulbocapnine only accounted for 1-6% of the total alkaloid yield in their sample, which is not a substantial amount. However, Shin et al (1998) was able to produce a 42.2% dopamine inhibition at a low bulbocapnine concentration of 20 μM (Shin et al. 1998). In addition, the amount of bulbocapnine present in the extract used in the current study is virtually unknown. Thus, it is possible that a *C. capensis* rhizome extract would contain enough bulbocapnine to mimic the dopamine inhibition observed by Shin et al (1998). Should this be true, it would suggest that the *C. capensis* rhizome extract is not suited to treating late-onset hypogonadism, and would in fact threaten normal testosterone production, even in healthy males. However, without further investigation, and quantification of bulbocapnine within rhizome extracts, this cannot be said with certainty.

4.7 Lactate dehydrogenase (LDH) activity

Yi et al (2011) described lactate dehydrogenase (LDH) as a soluble, catalysing enzyme found in the cell cytoplasm that participates in cell glycolysis. Moreover, LDH has also been identified as being responsible for the production of lactate in Sertoli cells by regulating glucose transport and catalysing the conversion of pyruvate to lactate (Marion et al. 2002; Walker and Cheng 2005). More recently, Galardo et al (2014) clearly outlined the role of LDH in regulating glucose metabolism, via glycolysis, to yield lactate and then pyruvate. Furthermore, lactate is essential to maintain the acidic environment of the seminiferous tubules and also provides the essential energy required for development of spermatogonia (Marion et al. 2002; Walker and Cheng 2005). Thus, it is clear that LDH is an important enzyme needed in male fertility. Additionally, Yi et al (2011) demonstrated that during events causing damage to cell membranes, such as in necrosis, LDH has been found to leak out of cell cytoplasm. Chan et al (2013) described necrosis as a type of cell death that is characterised by cell swelling, rupturing and breakage of the plasma membrane. Moreover,

rupturing of cells was described to cause the release of intracellular contents into the surrounding environment, such as LDH (Chan et al. 2013). Thus, measuring LDH leakage in TM4 Sertoli cells provides an indication of their normal function, and hence would provide insight into the effect of the *C. capensis* rhizome extract toward their normal cell function (Yi et al. 2011).

In the current study, TM4 Sertoli cells were exposed to the aqueous *C. capensis* rhizome extract for 24 and 96 hours. It was noted, over 24 hours, that low concentrations of the extract resulted in very slight decreases in LDH release, accounting for a 0.55 milli units/ml, at best. It is essential to note that a decrease of this nature holds no biological significance. At higher extract concentrations, however, LDH release suddenly increased. It is essential to note that the observed LDH release coincides with the extract concentrations that yielded TM4 cell stress and death. Furthermore, when the TM4 cells were exposed to the extract for 96 hours, LDH remained slightly decreased at low extract concentrations, accounting for a 0.6 milli units/ml decrease, at best. Conversely, higher extract concentrations yielded a further increase in LDH release. Once again, these higher extract concentration coincided with those that produced decreased TM4 cell viability.

It is essential to note that LDH was undetectable at the highest extract concentration, the same concentration at which considerable TM4 cell death was observed. Based on these observations, it is clear that the effect of the aqueous *C. capensis* extract toward TM4 LDH activity coincides with the effect of the extract toward TM4 cell viability. For this reason, it can be hypothesized that low concentrations of the extract results in little-no cell damage, and therefore no LDH leakage at low concentrations, while causing cell damage, death and thereby LDH leakage and absence at high concentrations. Thereby, it can also be reasoned that *C. capensis* would not interfere with normal actions of LDH in lactate production.

However, the present study was limited to a 96 hour incubation period, and therefore it remains unevaluated what may happen beyond this time frame.

4.8 ROS and RNS inhibitory effect

Wiseman and Halliwell (1996) described reactive oxygen species (ROS) as a collective term for oxygen radicals, such as superoxide and other non-radicals that may act as oxidizing agents or that may be readily converted into radicals like peroxyxynitrite. Similarly, these authors describes reactive nitrogen species (RNS) as a collective term that included Nitric oxide radicals, nitrogen dioxide radical, and products arising when nitric oxide reacts with superoxide. These authors outlined that ROS/RNS have many carcinogenic characteristics, including included the ability to cause structural alterations to DNA, affecting cytoplasmic and nuclear signal transduction, and modulating activity of proteins and genes that respond to stress. Furthermore, these authors went on to demonstrate that ROS/RNS induced damage contributed to the formation of many human diseased states, such as cancer, poor fertility, inflammatory disease and the general ageing process (Wiseman and Halliwell 1996). More recently, Dalle-Donne et al (2006) also outlined the negative effects of ROS/RNS toward normal health, demonstrating that ROS/RNS contributes to the normal ageing process, as well as diseases such as atherosclerosis and neurodegenerative disease. Additionally, these authors further demonstrated that ROS/RNS induced damage, such as DNA structural alterations, was a contributing factor to cancer formation (Dalle-Donne et al. 2006). Since then, more recent studies have also demonstrated the ability of ROS/RNS to cause DNA damage, either directly or indirectly, which in turn can lead to mutations ultimately resulting in cancer formation, as well as contribute to diseased states in humans (Weiskopf 2011; Barrera 2012; Kavazis and Powers 2013). However, it is essential to note that many of the negative effects associated with ROS/RNS only arise when they become unbalanced with the

body's defence mechanism. Nevertheless, the negative effects associated with ROS/RNS have been found to be counteracted by antioxidants (Weiskopf 2011; Barrera 2012; Kavazis and Powers 2013).

An antioxidant is a compound that disposes, scavenges, and suppresses the formation and actions of ROS/RNS. Maestri et al (2006) stated that a large proportion of antioxidants typically feature one or more phenolic hydroxyl groups attached to a carbon ring. Moreover, it was outlined that the presence of these hydroxyl groups allows these compounds to scavenge away free radicals (Maestri et al. 2006; Gülçin et al. 2010), by acting as chain breakers and receptors (Wiseman and Halliwell 1996; Gülçin et al. 2010). Thus, the use of antioxidants in preventing the actions of ROS/RNS has become common practice. However, serious doubts exist regarding the safety of prolonged use of traditional antioxidants, namely butylated hydroxyanisole, butylated hydroxytoluene, propylgallate and tert-butyl hydroquinone (Gülçin et al. 2010). Not surprisingly, this has led to an increasingly growing interest in safer, plant derived sources of antioxidants (Gülçin et al. 2010).

A study conducted by Babajidel and Mabusela (2010) screened indigenous South African medicinal plants for phytochemicals. In their study, they demonstrated that their samples of *C. capensis* tested positive for different phytochemicals, namely flavonoids, tannins, phenols and saponins. Lee et al (2013) very clearly outlined the ability of these types of phytochemicals to directly scavenge ROS/RNS, and in that manner prevent the formation associated diseases, such as cancer. Similarly, Nanda (2014) also outlined the potential of these phytochemicals, often found in medicinal plants and vegetables, to directly scavenge radical species, such as ROS/RNS. The reported antioxidant abilities of these phytochemicals (Lee et al. 2013; Nanda 2014) are in line with the observed antioxidant abilities produced by the aqueous *C. capensis* rhizome extract used in the current study.

When the extract was investigated for its ability to inhibit RNS formation, it was observed that at low extract concentrations exhibited a very weak ability to inhibit RNS formation, accounting for an 8.37% inhibition, at best. However, as the extract concentration increased, so did its ability to effectively inhibit RNS formation, in a dose-dependent manner. Additionally, the degree to which the extract inhibited RNS formation at higher concentrations can be said to be of biological significance. Based on these observations, it can be hypothesized that the phytochemicals reported by Babajidel and Mabusela (2010) may not exist in very high quantities within the extract used in the current study, and thus a higher extract concentration was needed. Nevertheless, it is clear that the extract was effectively able to inhibit RNS formation.

When the extract was investigated for its ability to inhibit ROS formation, it was observed that extract concentrations ranging from 0.1-100 µg/ml produced varying levels of ROS inhibition, with a great deal of outliers, and sometimes a total absence of ROS inhibition. However, at the highest concentrations, the extract exhibited the ability to inhibit ROS in a dose-dependent manner. It is essential to note that the highest observed ROS inhibition only accounted for a 29% at best, whereas the highest inhibition of RNS accounted for 97% at best. Additionally, the assays used tested the extracts ability to inhibit either superoxide (ROS) or peroxynitrite (RNS). Based on this, it can be hypothesized that the phytochemicals within the extract had a higher affinity toward peroxynitrite inhibition, rather than superoxide inhibition. However, the study conducted by Babajidel and Mabusela (2010) only screened for the presence of phytochemicals, and did not quantify them or provide insight into their chemical structure. Thus, without further investigation into the specific structures and of each phytochemical within *C. capensis*, it is not possible to definitively explain why a greater affinity toward RNS inhibition was observed. In addition to phenols tannins, saponins and flavonoids, De Wet et al (2011) demonstrated that *C. capensis* rhizomes contained aporphine and bisbenzyltetrahydroisoquinolone alkaloids, bulbocapnine, glaziovine and 12-O-

methylcurine. When the chemical structures of each of these alkaloids were reviewed, it was clear that each of these alkaloids featured a hydroxyl group. Based on the statement that many antioxidants possess a hydroxyl group (Maestri et al. 2006), it is possible that these alkaloids contributed to the observed antioxidant effect of the extract, possibly working synergistically with the reported phenols, tannins, saponins and flavonoids.

On the other hand, a study conducted by Shalaweh et al (2014) clearly demonstrated that an aqueous *C. capensis* rhizome extract was able to induce ROS production in spermatozoa in a dose-dependent manner. Moreover, they concluded that the alkaloids within the extract triggered intrinsic superoxide production (Shalaweh et al. 2014).

Previously, a study conducted by Li et al (2003) showed that inhibition of mitochondrial complexes I and II resulted in elevated ROS production in human leukaemia cells (HL-60). Similarly, a study conducted by Chen et al (2007) also demonstrated that inhibition of mitochondrial complexes I and II yielded elevated ROS production in embryonic kidney cells (HEK-293), cervical cancer cells (Hela), glioblastoma cells (U-87), human leukaemia cells (HL-60). Additionally, studies have also shown that bisbenzyltetrahydroisoquinoline alkaloids have the ability to inhibit mitochondrial complexes I and II (Storch et al. 2000; Granell et al. 2004). De Wet et al (2011) showed that *C. capensis* rhizomes were a source of the bisbenzyltetrahydroisoquinoline alkaloids cycleanine, cissacapine and insularine. Thus, it is possible that cycleanine, cissacapine and insularine is present within the extract and may work to induce ROS production, while other alkaloids such as bulbocapnine, glaziovine and 12-O-methylcurine may work to prevent ROS induced damage.

Nevertheless, it is clear that the extract used in the current study possessed the ability to act as an antioxidant. However, Poljsak et al (2013) clearly outlined clinical observation in which antioxidant supplementation in excess presented no benefits, but instead cause negative effects such as DNA damage, increased mortality and contributed to cancer formation. Considering that the study conducted by Babajidel and Mabusela (2010) did not quantify the

phytochemical present within their *C. capensis* samples, and that the phytochemical quantity of the extract that was currently used is unknown, it is unclear if the extract would provide excessive amounts of antioxidants. Additionally, the highest ROS and RNS inhibition was observed at the same high concentrations that produced decreases in cell viability in TM3, TM4 and LNCaP cells, making the safety of the extract as an antioxidant questionable. Thus, the quantification of phytochemicals within the extract, along with the identification of their chemical structure is essential. Additionally, when the observations of Shalaweh et al (2014) are considered, the isolation of these phytochemicals may prove to be more beneficial, and safer, in comparison to using the extract as a natural antioxidant.

4.9 Collagenase and elastase inhibitory effect

Collagenase and elastase have previously been identified as enzymes that are part of the family metalloproteinases, and that are responsible for degrading the major the components of connective tissue; namely collagen and elastin (Chubinskaia 1990; Xiang and Sang 1998; Ala-aho and Kähäri 2005). Ala-aho and Kähäri (2005) outlined metalloproteinases as a family of zinc-dependent neutral endopeptidases capable of degrading all components of the extracellular matrix, thus playing an essential role in physiological situation requiring extracellular matrix remodelling, such as in embryonic development, wound repair and tissue regeneration.

The metalloproteinase family has also been shown to be involved in pathological conditions ranging from rheumatoid arthritis to aortic aneurysms and chronic ulcerations (Ala-aho and Kähäri 2005; Hong et al. 2014). Furthermore, metalloproteinases are also involved in tumour growth, invasion and metastasis (Polette et al. 2004; Ala-aho and Kähäri 2005; Iizuka et al. 2014). Degradation of the extracellular matrix and basement membrane by metalloproteinases have been shown to be the first steps in invasion and metastasis

(Polette et al. 2004; Ala-aho and Kähäri 2005; Iizuka et al. 2014). An example of this would be the observations made by Polette et al (2004) that elastase plays a role in squamous cell carcinoma, whereby tissues expressing elastase were found devoid of normal elastic fibres and has disrupted basement membranes. Recently, Ho et al (2014) revealed elastase as a diagnostic marker and demonstrated that this enzyme contributes to the progression, invasion and metastasis of cancer. Similarly, type-IV collagenase have been shown to be promoters of cancer invasion and metastasis in breast, colon and brain tumours (Deryugina and Quigley 2006; Kähäri and Ala-aho 2009).

Reportedly, type-IV collagenases have been expressed in both benign and malignant prostate tissue (Brehmer et al. 2003; Ozden et al. 2013). A study conducted by Zhang et al (2004) measured the enzymatic activities of type-IV collagenases in men with and without prostate cancer, and demonstrated that type-IV collagenases were associated with progression and metastasis of prostate cancer. Subsequently, Zhang et al (2008) demonstrated the role of type-IV collagenases in prostate cancer, showing that they were elevated in men with prostate cancer and was involved with perineural invasion (Zhang et al. 2008).

More recently, Lin et al (2014) not only demonstrated that type-IV collagenases were involved in prostate cancer invasion and metastasis, but also demonstrated that type-IV collagenases could affectively be inhibited by a plant-base flavonoid known as naringenin. Similarly, Lee et al (2007) also demonstrated that plant-based antioxidants, or phytochemicals, isolated from *Pinus densiflora* were able to inhibit the activity of type-IV collagenases, and attributed this to the presence of the flavonoids epigallocatechin gallate (EGCG) and epigallocatechin (EGC).

A study conducted by Thring et al (2009) demonstrated that phytochemicals within green tea extract, a source of flavonoids and tannins, to inhibit both collagenase and elastase activity.

When the extract used in the current study was investigated for its ability to inhibit collagenase activity, specifically type-IV collagenase, it was observed that the extract was

able to produce a dose-dependent decrease in collagenase activity. However, it is essential to note that at lower concentrations, the extract was only able to slightly decrease collagenase activity, accounting from a 6-11% decrease, at best. A decrease in collagenase activity of this nature cannot be said to be biologically significant. At higher extract concentrations, further decreases in collagenase activity were observed, with the highest decrease accounting for 41 %. While this may be seen as biologically significant, it is essential to note that this decrease was observed at 1000 µg/ml, the same extract concentration that produced cell death over time in TM3, TM4 and LNCaP cells. For this reason, the concentration needed to produce biologically significant collagenase inhibition is overshadowed by the toxic effects towards cells observed at that concentration. Furthermore, the extract only produced slight decreases in collagenase activity within the non-toxic dose range of the extract, which may indicate low levels of flavonoids and tannins within the extract. Thus, the extract used in the current study can be said to be a weak collagenase inhibitor.

When the extract was investigated for its ability to inhibit elastase activity, it was observed that a marginal decrease in elastase activity was produced, accounting for a 4.14% decrease, at best. A decrease in elastase activity of this manner cannot be said to be of biological significance, and thus the extract cannot be said to be an inhibitor of elastase activity. However, when the actual percentages are considered, it is clear that the extract was more suited toward collagenase inhibition rather than elastase. Similar observations were made by Thring et al (2009) that demonstrated that the green tea extract was able to inhibit collagenase activity by 47.17%, while only inhibiting elastase activity by 9.99%, which they attributed to the presence of EGCG.

Flavonoids, such as the EGCG, have previously been found to strongly inhibit collagenase activity by acting as a metal chelator, while marginally inhibiting elastase activity by acting as protease inhibitors. For this reason, it is possible that the flavonoid EGCG exists within *C. capensis*, and thus within the extract used in the present study. Should this be true, it

would serve to explain the observed affinity toward collagenase inhibition, rather than elastase. However due to the low level of collagenase inhibition, it would indicate a low level of EGCG within the extract. Alternatively, other flavonoids capable of acting as a metal chelators and protease inhibitors may be responsible for the observation, rather than EGCG specifically.

Tannins have been shown to strongly inhibit collagenase activity (Hong et al. 2014). Babajidel and Mabusela (2010) demonstrated the presence of tannins within *C. capensis*, it is possible that the extract used in the current study also contained tannins. However, considering the low level to which collagenase was inhibited, it can be hypothesized that low levels of tannins existed within the extract.

4.10 Conclusion and further outlooks

Based on the results obtained in this study, the extract was only able to produce biologically significant decreases in LNCaP cell viability at high concentrations, and over an extended period of time. For this reason, the extract can be said to be weakly cytotoxic toward LNCaP cells, and may not be suited for the treatment of prostate cancer. Accordingly, patients should be careful when relying on this traditional anti-cancer remedy. Furthermore, the extract was not found to be a viable inhibitor of elastase and collagenase and is thus not suited to the prevention of cancer metastasis. Additionally, the concentrations needed to produce LNCaP cell death also produced cell death in TM3 Leydig and TM4 Sertoli cells, consequently producing non-specific cytotoxicity. However, low extract concentrations did not negatively impact testosterone production or LDH activity, exhibiting no negative effect toward normal TM3 and TM4 cell function. The extract was able to inhibit both reactive oxygen species (ROS) and reactive nitrogen species (RNS) production and thus has potential as a natural antioxidant.

It is also crucial to note that a substantial amount of plant to plant variation was reported between plants found in different geographical location, as well as within the same population. It is for this reason that standardisation of the extract, along with all reported phytochemicals, may be a crucial next step. Subsequently, further investigations to clarify the discrepancies to the traditional claims have to be conducted.



Chapter 5

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