Investigations on the effects of *Typha capensis* on male reproductive functions

Candidate

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DECLARATION

I declare that the "Investigations on the effects of *Typha capensis* on male reproductive functions" is my own work, that it has not been submitted for any degree or examination at any other university and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

ABDULKAREM ILFERGANE **DATE.....**

SIGN……

DEDICATION

This dissertation is dedicated to my parents for their support and steadfastness towards my education. To my wife for her efforts towards my academic success. To my children: Shaima, Alharith, for inspiration and motivation towards my academic goals. To my city “Benghazi” where I was born, raised and afforded the many wonderful opportunities to better myself and build my future successes.

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PUBLICATIONS

Abstracts and Presentations:

- Ilfergane A, Haines-Arries N, van Zyl L, Henkel R (2015) INVESTIGATIONS ON THE EFFECTS OF TYPHA CAPENSIS ON TM3 LEYDIG CELLS. American Society of Andrology 40th Annual Conference. April 18 - 21, 2015 The Little America Hotel, Salt Lake City, UT.

- Haines-Arries N, Ilfergane A, Henkel R (2015) THE EFFECT OF AN AQUEOUS TYPHA CAPENSIS EXTRACT ON THE AGEING MALE REPRODUCTIVE SYSTEM. American Society of Andrology 40th Annual Conference. April 18 - 21, 2015 The Little America Hotel, Salt Lake City, UT.

- Ilfergane A, Pearce K, Henkel R (2015) EFFECT OF A BIOACTIVE FRACTION OF TYPHA CAPENSIS RHIZOME EXTRACT ON LNCAP AND PWR-1E PROSTATE CELLS. 29th Scientific Meeting of the Malaysian Society of Pharmacology and Physiology. 24-25 August 2015, Shah Alam, Selangor, Malaysia

Investigations on the effects of *Typha capensis* on male reproductive functions

KEY WORDS

Typha capensis (bulrush)

African Traditional medicine

Male reproductive functions

Male infertility

Cytotoxicity

Viability

DNA fragmentation

Testosterone

TM3 (Leydig cells)

LNCaP (Prostate cancer cells)

PWR-1E (Benign prostatic hyperplasia cells)

HPLC

NMR

ABSTRACT

Introduction

Typha capensis, commonly referred to as bulrush also called 'love reed' growing in Southern Africa's wetlands, is one of South Africa indigenous medicinal plants that are traditionally used to treat male fertility problems and various other ailments. Previous studies revealed that *T. capensis* has indeed a beneficial effect on male reproductive functions and aging male symptoms. The *T. capensis* rhizomes are used in traditional medicine during pregnancy to ensure easy delivery, for venereal diseases, dysmenorrhea, diarrhoea, dysentery, and to enhance the male potency and libido. Typha genuses contain flavones and other phenolic compounds, which exhibit anti-oxidative capacity.

Materials and Methods

This study encompasses three parts (part 1: Exposure of different cell lines to crude aqueous extracts of *T. capensis* rhizomes; part 2: HPLC analysis of *Typha capensis* crude rhizome extract and exposure of different cell lines to the F1 fraction of the summer season; part 3: Compound identification by means of NMR spectrometric analysis and exposure of different cell lines to bioactive compounds (Quercetin and Naringenin) isolated from *T. capensis* rhizomes.

Part 1: TM3-Leydig cells and LNCaP cells incubated with different concentrations of crude aqueous extract of *T. capensis* rhizomes (0.01, 0.02, 0.1, 1, 10 and 100 µg/ml) and control (without extract) for 24 and 96 hours, after incubation. The following parameters were evaluated: cell morphology and viability (determined by means of MTT assay).

Part 2: The crude extract HPLC profiles were obtained by preparing the extracts for different seasons (Autumn, Winter, Spring, Summer). TM3-Leydig cells, LNCaP cells and PWR-1E cells incubated with different concentrations *T. capensis* rhizomes extract F1 fraction of the summer season (0.01, 0.02, 0.1, 1, 10 and 100 µg/ml) and control (without extract) for 24 and 96 hours, after incubation. The following parameters were evaluated: cell morphology was observed and recorded, viability

(determined by means of MTT assay), testosterone production (testosterone ELISA test), cell early apoptosis (determined by means of Annexin V-Cy3 binding), DNA fragmentation (determined by means of the TUNEL assay).

Part 3: NMR spectrometric analysis was performed on a ^{13}C spectra were recorded at 400 MHz. TM3-Leydig cells and LNCaP cells incubated with different concentrations of bioactive compounds (Quercetin and Naringenin) isolated from *T. capensis* rhizomes, for acute exposure (24, 96 hours) and chronic exposure (96 hours), after incubation, the following parameters were evaluated: cell morphology and viability (determined by means of MTT assay), testosterone production (testosterone ELISA test), cell early apoptosis (determined by means of Annexin V-Cy3 binding) and DNA fragmentation (determined by means of the TUNEL assay).

Results

Part 1: for TM3-Leydig cells the results reveal no observable morphological changes and no significant influence on cell viability except at highest concentration indicating cellular stress. However, LNCaP cells showed a decline in cell viability at the incubation period 96 hours (-82.4%) more than 24 hours (-64.7%) indicating more cell death.

Part 2: HPLC data showed that the most effective fraction was the F1 fraction from the summer harvest. Results revealed that the *T. capensis* rhizome extract F1 fraction of the summer season significantly enhanced testosterone production in TM3 cells and was more toxic towards cancer cells (LNCaP cells) compared to the normal cell lines (TM3-Leydig, PWR-1E cells).

Part 3: NMR data showed 2 bioactive compounds which were identified as Quercetin and Naringenin. The assays showed that LNCaP cells are more sensitive to the cytotoxic effects and apoptosis induction of both compounds, whereas, the assays resulted in weak effects toward TM3-Leydig cells. However, testosterone production in TM3-Leydig cells was significantly enhanced at low concentrations of Quercetin and Naringenin at all exposure types (acute and chronic) testosterone beak significantly at around 0.100 and 0.125 μg/ml ($P<0.0001$), stimulatory activity in a dose-dependent manner.

Conclusion

Typha capensis enhanced the production of testosterone and might be useful to treat male infertility and aging male problems. Results further reveal that the F1 fraction from the summer harvest had highest biological activity. This study, for the first time, investigated the effects of bioactive compounds (Quercetin and Naringenin) yeilded from aqueous extraction of *Typha capensis* rhizomes in cell lines investigating male reproductive functions. Active compounds present in the rhizomes have caused an increased production of testosterone level in TM3-Leydig cells. Furthermore, the active compounds of *Typha capensis* rhizomes in the high dose had a negative effect on the percentage of DNA fragmentation in LNCaP cells. When compared to the effect of the low dose, the two compounds induced significant apoptosis in cancer cell line (LNCaP) compared with the normal cell line (TM3-Leydig). The isolated compounds are significantly selective towards the cancer cells than the normal cell compared with the exposure of bioactive compounds used in this study.

Chapter 1: Introduction

1.1 Overview of the Male reproductive system

The male reproductive system (Figure 1) it is composed of rete testis, epididymis, ductus deferens, ejaculatory ducts, and urethra, accessory sex glands (seminal vesicles, prostate, and bulbourethral glands), and many other supporting structures, such as the scrotum and the penis, make up the organs of the male reproductive system. The testes are responsible for the production of sperm, along with the secretion of hormones, while the duct system transports and stores sperm. Additionally, the duct system also assists in the maturation of sperm and transports them to the external environment (Benjamin Cummings, 2009).

1.1.1 Testes

The testes, otherwise known as testicles, are a pair of oval shaped glands that are found in the scrotum. Commonly, the testes are roughly 5 cm long and 2.5 cm in diameter, and having an average mass of 10–15 grams per testicle. Developing near the kidneys, specifically in the posterior portion of the abdomen, the testes descend into the scrotum through the inguinal canals when the 7th month of fetal development has been reached. A serous membrane derived from the peritoneum, called the tunica vaginalis, partially covers the testes. Internal to the tunica vaginalis is a white fibrous capsule known as the tunica albuginea, which is composed of dense irregular connective tissue. It extends inward, forming septa that divide the testis into 200-300 internal compartments called lobules. Each lobule contains one to three tightly coiled tubules called seminiferous tubules, in which sperm are produced by a process known as spermatogenesis (Benjamin Cummings, 2009). Two types of cells are found in the seminiferous tubules, namely the spermatogenic cells, which are sperm-forming cells, and Sertoli cells, which have several functions in supporting spermatogenesis. Stem cells, called spermatogonia, develop from primordial germ cells that arise from the yolk sac and enter the testes during the fifth week of development. Toward the lumen of the seminiferous tubule, layers are found which consist of increasingly more mature cells. These include primary spermatocytes, secondary spermatocytes, spermatids, and sperm cells. Once a sperm cell, also known as a spermatozoon, has been successfully formed, it moves into the lumen of the seminiferous tubule.

Found among the various spermatogenic cells are large Sertoli cells. These cells reach from the basement membrane, of the seminiferous tubules, into what is known as the lumen of the tubule. More importantly, tight junctions join neighboring Sertoli cells to one another to form a structure known as the blood–testis barrier. This barrier isolates the developing spermatocytes from blood, thereby preventing a potential auto-immune response. This is important because the surface of spermatogenic cells possess antigens which would be considered foreign by the body's immune system. Also, Sertoli cells provide support and protect the developing spermatocytes by providing much needed nourishment. Furthermore, they phagocytize excess cytoplasm around spermatids during development, and also plays a role in controlling the movement of spermatocytes and the movement of sperm into the seminiferous tubules. They also produce a fluid in which sperm may be transported, along with secreting the hormone inhibin, and regulating the effects of testosterone and follicle-stimulating hormone (FSH). In the spaces between the seminiferous tubules are clusters of cells known as Leydig cells. These cells secrete the hormone testosterone, which is the major androgen of the male reproductive system. An androgen is a hormone that stimulates the development of characteristics associated with masculinity. Testosterone is also responsible for promoting the male sex drive (Benjamin Cummings, 2009).

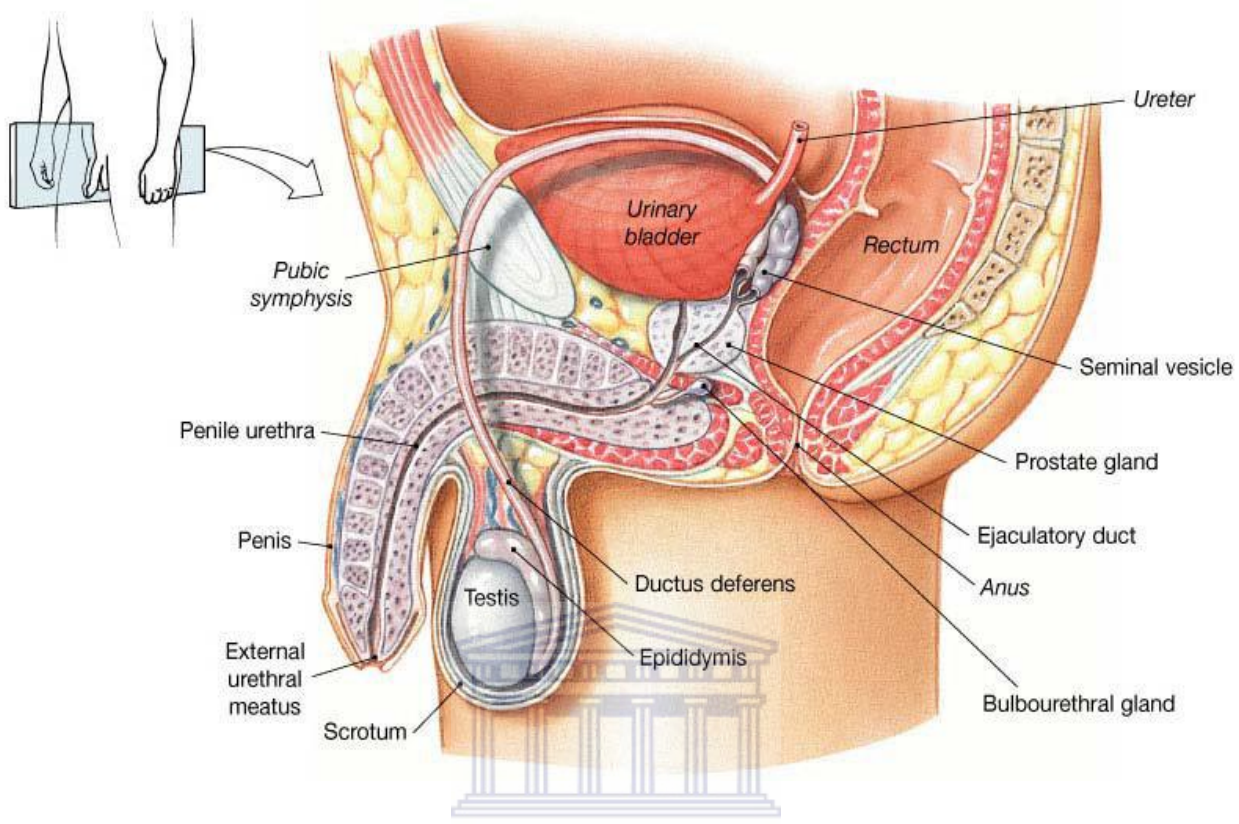


Figure 1: Cross section through the male reproductive system (Pearson Education, 2004).

1.1.2 Supporting cell (Sertoli cells)

Sertoli cells are the supporting cells of the male reproductive system and are found within the seminiferous tubules. These cells are tasked with the creation of a barrier known as the hemato-testicular barrier, along with the nourishment of the developing spermatozoa. These cells have only been found to proliferate during the first year of life in males (Griswold, 1995). Sertoli cells are shaped like Christmas-trees and are found on the basal membrane, in direct contact with one another and with germ cells. Characteristically, these cells have an oval, indented nucleus found perpendicular to the basal membrane. Additionally, the cell nucleolus is clearly visible, providing clear characteristic that may be used to distinguish them from other cell types (Wright et al., 1981).

Junctions between Sertoli cells, known as tight junction, join these cells together to create a divide known as the basal and adluminal compartments. Seroli cell functions are closely regulated by the

hormone FSH. Furthermore, Sertoli cells synthetize many different proteins that play roles in the repductive system of men. Specifically, inhibin, androgen-binding-protein (ABP) and the antimullerian hormone (AMH) are the major proteins produced by the Sertoli cells (Skinner and Griswold, 1980). Androgen-binding globulin has a high affinity for testosterone, thereby making it and is responsible for the maintenance of high intra-tubular testosterone. This is achieved by concentrating testosterone in the lumen of the tubules.

Sertoli cells have also been shown to produce mullerian duct inhibiting factor (MIF), transferrin for transporting iron to tubular cells, ceruloplasmin for the transport of copper, and plasminogen activator which plays a role in mediating the maturation of sperm. Sertoli cells also produce aromatase, an enzyme which is responsible for the conversion of androgens produced by the Leydig cells to estrogens (Papaioannou et al., 2009). Having many functions, Sertoli cells produce many products that are needed for the survival and maturation of sperm cells (Johnson et al., 2008).

1.1.3 Leydig cells

Leydig cells were first described by the German histologist Franz Leydig in 1850 (Glees P. Leydig, 1973). These cells are located in the interstitial compartment of the testis, between and surrounding the seminiferous tubules (Schulze, 1984). Leydig cells are epithelioid and polygonal in shape. The primary function of Leydig cells is to synthesize and secrete androgenic steroids. Testosterone is the main male steroid hormone and is important for spermatogenesis and male sexual characteristics (Hess and De Franca, 2008; Mathur and D'Cruz, 2011). Leydig cells produce steroids, notably the androgens testosterone and dihydrotestosterone (DHT), important hormones involved in male fertility. Low levels of testosterone can result in male infertility (Chen and Zirkin, 2009).

It has been found that testosterone levels in men may decrease as a result of the aging process. Low testosterone can also be seen in adolescent boys experiencing interrupted puberty (hypogonadism) (Haider, 2004). Testosterone is a final product of androgenic hormone biosynthesis, and Leydig cells are known to be the primary source of androgens. In the mammalian testis, two distinct populations of Leydig cells, the fetal and the adult Leydig cells, develop sequentially, and these two cell types

differ both morphologically and functionally. It is well known that the adult Leydig cells maintain male reproductive function by producing testosterone (O'Shaughnessy et al., 2009).

1.1.4 Spermatogenesis

In humans, spermatogenesis takes approximately 65 to 75 days, beginning with cells known as spermatogonia. Most of these spermatogonia will initially undergo the process of mitosis, while some spermatogonia remain in an undifferentiated state so that a reservoir of cells will remain for the production of sperm in the future (Figure 2). Nevertheless, some spermatogonia differentiate into primary spermatocytes. Shortly after forming, each of the newly formed primary spermatocyte will replicate its DNA. Hereafter, meiosis begins. During the first meiotic division, chromosomes arrange themselves in a line at the metaphase plate, and the process of crossing-over ensues. Hereafter, the meiotic spindle pulls one chromosome of each pair to an opposite pole of the dividing cell. Subsequently, the resulting two cells formed by the first meiotic division are known as secondary spermatocytes. Each of these secondary spermatocyte has a total of 23 chromosomes. Each chromosome within a secondary spermatocyte, however, is made up of two chromatids still attached by a centromere. Hereafter, the second meiotic division proceeds, yielding four cells each ciontaining 23 chromosomes. These resulting cells are known as spermatids. In short, one primary spermatocyte results in the formation of four spermatids by two rounds of meiosis. The final stage of spermatogenesis is the development of haploid spermatids into sperm (Ettore, 2011).

During the process of spermiogenesis, each spermatid matures and becomes a single sperm cell. During this process, spermatids become elongated, slender sperm. Next, an acrosome forms on top of the nucleus, a flagellum develops and mitochondria begin to multiply. Finally, spermiation occurs, in which sperm are released from their connections to Sertoli cells. Hereafter, sperm enter the lumen of the seminiferous tubule (Ettore, 2011).

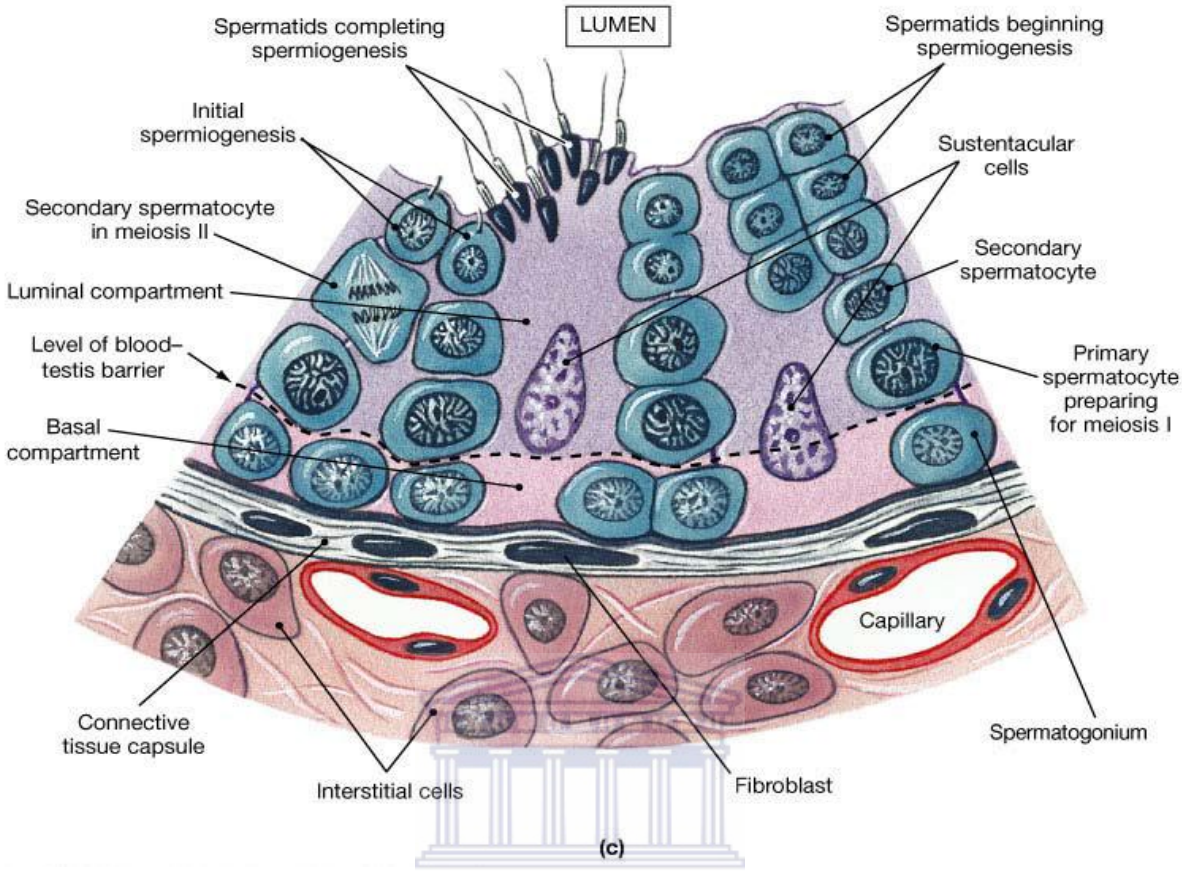


Figure 2: Spermatogenesis, displaying mitotic and meiotic divisions of the cell stages spermatozoa undergoes within the seminiferous tubules (Pearson Education, 2004).

1.1.5 Sperm

In the average male, roughly 300 million sperm are formed each day during spermatogenesis. Measuring on average about 60 micrometer long, sperm contains several structures that make them well suited for both reaching the oocyte and also for the penetration of the oocyte. The major parts making up sperm are known as the head and tail pieces. The head piece normally contains a nucleus with 23 highly condensed chromosomes (Anne, 2011). Covering the nucleus is a vesicle known as the acrosome that is filled with various enzymes. These enzymes are used to help the sperm to penetrate an oocyte so that fertilization may be achieved. Specifically, these enzymes include hyaluronidase and various other proteases. The tail piece of a sperm further subdivided into four different parts (Figure 3). Namely, these parts include the neck, the middle piece, the principal piece, and the end piece. The subdivision known as the neck piece is a region behind the head, which is a

constricted region, and contains structures known as centrioles. Centrioles are responsible for the formation of the microtubules that make up the remainder of the tail piece. The middle piece contains many mitochondria that are typically arranged in a spiral pattern. These mitochondria are responsible for providing the energy for the movement of sperm, along with providing energy for the metabolism of sperm. The principal piece is a tapered portion of the tail and is also the longest portion of the tail piece. After ejaculation has occurred, the survival rates of sperm have been found to be less than 48 hours within the female reproductive tract (Anne, 2011).

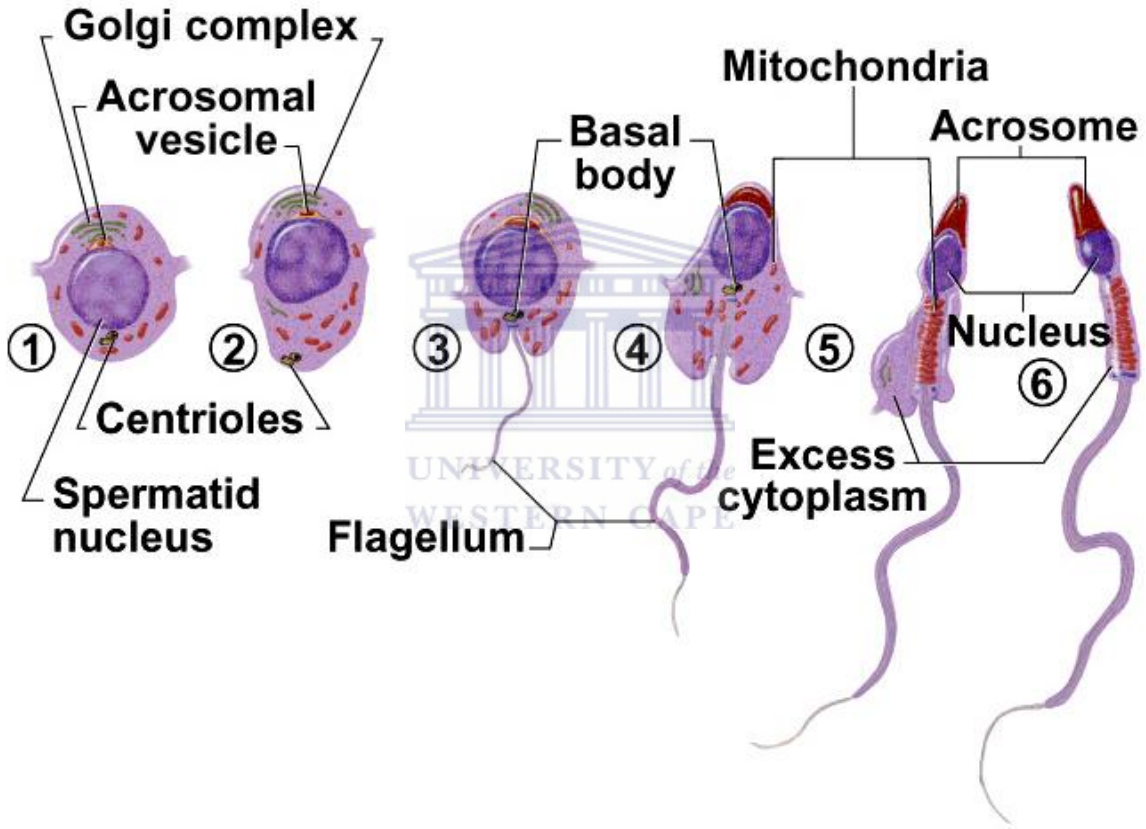


Figure 3: Spermiogenesis and Spermatozoon. Changes that transform spermatids into spermatozoa discarding excess cytoplasm and growing tails (Pearson Education, 2004).

1.1.6 Regulation of the reproductive system

At puberty, certain cells found in the hypthalamus increase their secretion of gonadotropin-releasing hormone (GnRH). This in turn stimulates gonadotrophs found in the anterior pituitary to increase

their secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH). Specifically, LH in turn will stimulate the Leydig cells to produce testosterone, an androgen hormone (Figure 4).

Testosterone is readily produced from cholesterols in the testes and is the major androgen hormone of the male reproductive system. Testosterone has been found to be lipid soluble and thereby easily moves out of Leydig cells into the interstitial fluid and then moves into blood. A negative feedback mechanisms exists in this system, whereby testosterone shuts down the production of LH, which in turn and stops the secretion of GnRH in the hypothalamus. In specific target cells, such as those which are found in the genitals and the prostate, the enzyme known as alpha-reductase will convert the androgen hormone testosterone to a different androgen hormone that is known as dihydrotestosterone (DHT) (Luis, 2005). Nevertheless, FSH acts indirectly to stimulate spermatogenesis. Here, FSH and testosterone synergistic action has been found, whereby the Sertoli cells are stimulated to produce androgen-binding protein (ABP) and release it into the lumen of the seminiferous tubules. By doing this, ABP binds to the circulating testosterone, thereby ensuring a high concentration. Hereafter, testosterone is responsible for the final steps of spermatogenic process that occurs in the seminiferous tubules. Once spermatogenesis has sufficiently occurred, Sertoli cells begin to release inhibin. If spermatogenesis does not proceed at the normal speed, less inhibin is released which in turn results in more FSH secretion and an increased rate of spermatogenesis (Luis, 2005).

Both testosterone and DHT have been found to bind to the same androgen receptors, which are found within the nuclei of target cells. Furthermore, the hormone receptor complex is responsible for the regulation of various gene expressions by resulting in some genes being turned on while other genes are turned off. As a result of these changes the androgen hormones cause many different effects during the development process in males. Before birth, testosterone is responsible for the stimulation of the development of the reproductive system of males, along with the descent of the testes through the inguinal canal. At puberty, testosterone and dihydrotestosterone are responsible for the development and enlargement of the male sex organs, along with the development of male secondary sexual characteristics. The secondary male sex characteristics have been defined as traits that differentiate males or females from one another. Examples of the secondary sex characteristics include the growth of the muscular and skeletal system, whereby men tend to have wider shoulders

and narrower hips, facial hair and chest hair, thickening of the skin, a greatly increased sebaceous gland secretion, and an enlargement of the larynx that result in the characteristic deepened voice in men. Furthermore, androgens contribute to male sexual behavior, spermatogenesis and to sex drive. Androgens are also anabolic hormones, meaning they stimulate protein synthesis. (Luis, 2005).

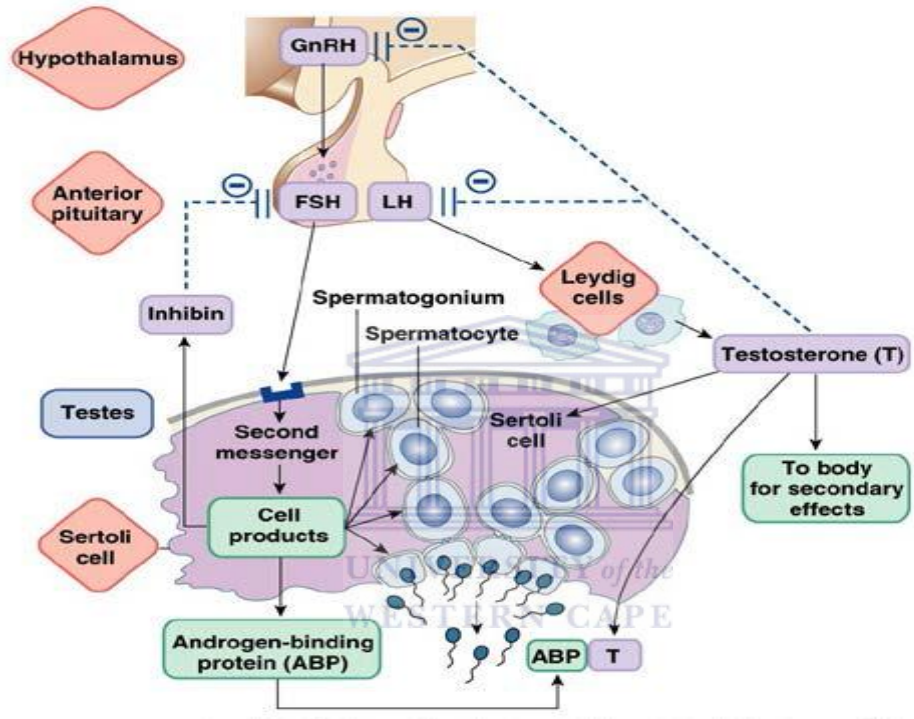


Figure 4: Hypothalamic-Pituitary-Testicular Axis Integrates Testicular Function (Pearson Education, 2007).

1.1.7 Prostate cancer

In comparison to other types of cancer, prostate cancer progresses surprisingly slowly and has been reported to take as long as 30 years before a tumor of the prostate becomes large enough to cause detectable symptoms (Park et al., 2007). Men over the age of 50 are more likely to be diagnosed with prostate cancer, although it has been known to occur in younger men. Typically, cancer is detected in the prostate in most men by the age of 80 (Zhang et al., 2008). However, despite prostate cancer being quite a common occurrence in men over the age of 50, most of these men typically do

not die as a result of the prostate cancer (Ni et al., 2013). Nevertheless, African-American men generally more likely to be diagnosed with prostate cancer at younger ages, along with faster rates of prostate cancer progression, in comparison to men of other racial groups (American Joint Committee on Cancer Prostate, 2010).

Classified as an adenocarcinoma, prostate cancer is a glandular cancer that typically starts when otherwise normal cells of the prostate begin to mutate and change into cancerous cells. The most common region in which cancer of the prostate develops is the peripheral zone. Initially, small clusters of cancerous cells in the prostate stay around otherwise normal glands of the prostate. Hereafter, cancers of the prostate often tend to invade the surrounding lymph nodes, bone and other distant body sites in a process called metastasis (Kyrianou et al., 1994).

Typical diagnosis of prostate cancer is done with a blood test, as prostate cancer has been shown to be linked with elevated prostate-specific antigen (PSA) levels. The use of PSA as a diagnostic tool since the 1980's has drastically improved screening for prostate cancer and significantly reduced the occurrence of metastatic prostate (Bosland et al., 2013). As a result, prostate cancer is now more typically localized when diagnosed, with no sign of metastasis beyonf the region of the pelvis. Many different types of treatment options for localized prostate cancer exists. These treatment options include active surveillance, radical prostatectomy, external beam radiotherapy and brachytherapy. Once prostate cancer reaches a state of metastasis, the deprivation of androgens, known as hormone therapy, is normally the first method of treating the cancer (Kantoff et al., 2010).

1.1.8 Benign prostatic hyperplasia

Also called BPH, benign prostatic hyperplasia is a progressively worsening condition that has been characterized by the enlargement of the prostate that's is coupled with symptoms that manifest in the lower urinary tract, such as difficult passage of urine (Roehrborn et al., 2009). Although BPH has not been found to be a common occurrence in men under the age of 40, up to 50% of men are diagnosed with BPH-related symptoms at the age of 50. It has been found that the incidence of BPH increases by 10% every decade and eventually reaches an incidence of 80% when men reach the age of 80 years old (de la Rosette et al., 2001). Even though BPH has a great impact on the health of the

public, the pathogenesis of BPH remains vastly unresolved. Many theories have been developed, the etiology of BPH still remains largely uncertain in many regards. Many different mechanisms appear to be involved in the development of BPH, yet the ageing process appears to be the major contributing factor involved in the development of BPH. In addition to the ageing process, hormonal changes, metabolic syndrome and inflammation have also been identified as factors contributing to the development of BPH (Untergasser et al., 2005). 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 cellular growth and cell death. It is hypothesised that benign prostatic hyperplasia arises when this homeostasis between cell proliferation and death is disturbed (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). Additionally, 5α-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).

1.2 Fertilization

Fertilization can be defined as the fusion of gametes to produce a new organism. In the human, it involves the fusion of an ovum with the sperm, leading to the formation of an embryo. For fertilization to occur, spermatozoa undergo capacitation in the female reproductive tract, after which they undergo two calcium (Ca^{2+})-dependent physiological processes, namely an acrosome reaction and hyper-activation (Yanagimachi, 1994). Capacitation is a time and species-specific, post-ejaculatory modification of the sperm surface (Van Kooij et al., 1986; Fraser, 1984). The acrosome reaction (AR), also known as acrosome exocytosis (AE), is a terminal morphologic alteration of the spermatozoon, and a synchronized and highly regulated process that is required for fertilization (Knobil and Neill, 1994). This results in the inner acrosomal membrane being exposed at the anterior

sperm head, releasing the content of acrosome such as hydrolytic enzymes, and as a result the sperm head fuse with the oocyte membrane, leading to fertilization (Yanagimachi, 1994; Tollner et al., 2000; Kumi-Diaka and Townsend, 2003). Ca^{2+} is an essential mediator of acrosome reaction (Gonźalez- Martínez et al., 2001), and an efflux of Ca^{2+} from within the acrosome is required in the presence of high cytosolic Ca^{2+} (De Blas et al., 2002). Hyperactivation can be described as the distinct change in sperm motility when the progressively moving spermatozoa become extremely vigorous and less progressive, exhibiting large amplitudes of head displacement (Yanagimachi, 1970). Hyperactivated motility is thought to facilitate the penetration of the zona pellucida or play a role in the transport of spermatozoa in the uterine tube (Yanagimachi, 1994). Excessive production of reactive oxygen species (ROS) have been shown to cause sperm pathology, while a low and controlled concentration of ROS plays important roles in sperm physiology and in the acquisition of sperm fertility (de Lamirande et al., 1997). O^{2-} and H_2O_2 exposed to human spermatozoa increased the development of hyperactivation and capacitation (De Lamirande and Gagnon, 1993). Superoxide dismutase prevented hyperactivation and capacitation in human sperm (De Lamirande and Gagnon, 1993).

A male is generally considered fertile if he has recently sired an offspring and it can thus be assumed that his semen contains a population of fertile spermatozoa. Determine the values for certain semen parameters of fertile males, define the normal ranges of these values and statistically compare the observed values of a semen sample of unknown fertility with the normal ranges reported for fertile males (Mortimer, 1994). Male's fertility potential is based on the evaluation and statistical analysis of certain indirect criteria, it is very important that measurements must be accurate and reproducible. At the same time, the techniques used for assessment must be objective, standardized and sensitive to recognize deviations from the normal ranges (Mortimer, 1994). Normal ranges of selected semen characteristics for humans have been determined and adjusted by the World Health Organization (WHO) during the last two decades (WHO, 2010). Whereas the WHO criteria represent the lower end of normality focus on male infertility, a similar set of normal ranges have been proposed by (Mortimer, 1994).

Fertilization is an extremely complex process involving a series of events. Therefore, one needs to take into account that a considerable amount of factors require thorough investigation. Also, the

weight of each sperm parameter‘s contribution to the multifactorial process that is fertilization (Amann, 2008; Henkel et al., 2005), sperm quality is most certainly an important factor determining successful fertilization (Ombelet et al., 2003). Subsequently, the production of a sufficient amount of mature, motile and functionally competent spermatozoa is of paramount importance for a male to be able to conceive (Kasai et al., 2002). For this reason, the conventional semen analysis is employed in order to determining the quality of spermatozoa within a semen sample. Sperm concentration (Calvo et al., 1994), motility (Ron-el et al., 1991; Robinson et al., 1994) and morphology (Kruger et al. 1988; Menkveld et al. 1996) are some of the parameters that can be evaluated in an attempt to indicate the likelihood of successful fertilization. A number of men showing normal parameters after a standard semen analysis remain unable to conceive (Hull et al., 1985; Baker, 1994). The defective sperm function has been linked to loss of fertilization in males (McLachlan and de Kretser, 2001). The reasons for sperm malfunctioning require extensive examining. In this regard, parameters investigated within this particular study include motility, sperm morphology, sperm mitochondrial membrane potential ($\Delta\psi m$), sperm reactive oxygen species (ROS) production, sperm DNA fragmentation and testosterone concentration (Henkel and Franken, 2011).

1.3 Infertility

Infertility, as defined by the World Health Organization (WHO), is the inability of a sexually active woman, who is not using contraction, to conceive and have a live birth despite trying for 12 or more months (Larsen, 2000; Dyer et al., 2009; Alhassan et al., 2012). Infertility means inability of a couple to conceive. A healthy couple may take about six months to achieve a pregnancy (Thonneau and Spira, 1990). Expected percentage for monthly rate of conceiving among healthy young couples is 25%. Fertility rate decreases in women significantly after age 35. Approximately 15% of the married couples are not able to produce child. Despite this, no clear cause has been found in 15 % of these couples that may be reported by regular clinical and laboratory techniques. Maruyama et al. 2000 explained that 95% of couples trying to conceive should conceive within 13 months. Unidentified cause for childlessness is found in 10–15% of couples (ESHRE Capri Workshop, 1998).

Infertility affects a large number of couples in the U.S. In 2002, it was estimated that 2.1 million married women were infertile (defined as no pregnancy after 12 months or more of not using

contraception) (Chandra et al., 2002). In addition, about 12% of women of reproductive age indicated that they had ever used some kind of medical assistance to help to become pregnant or to maintain a pregnancy. The use of infertility treatment has grown tremendously due to advances in treatment and insurance coverage (Sunderam et al., 2009). Between 1996 and 2005, the number of children born after assisted reproductive technology has increased by 150% and now accounts for over 1% of all children born in the U.S (Brassard et al., 2008). Infertility could be due to a number of different factors, which may originate either in the male or female partner. Maternal factors for infertility include advanced age, tubal factors (post-infection tubal damage, tubal obstruction, pelvic adhesions), ovulatory dysfunctions (including polycystic ovary syndrome), and endometriosis (Brassard et al., 2008).

Male factor infertility includes varicocele, immunological problems (antisperm antibodies), ejaculatory dysfunction, or defective sperm parameters (low concentration, poor motility, or abnormal morphology) (Comhaire et al., 2008). Although estimates vary widely, in about 10-17% of couples no cause is identified (Hull et al., 1985; Templeton et al., 1990). There are also many other possible contributing factors that enhance or help to cause infertility. Some of the more well documented factors include untreated sexually transmitted disease, such as Chlamydia, hormonal imbalances, smoking, radiation exposure, exposure to certain chemicals including pesticides, exposure to lead or other heavy metals, caffeine consumption, and obesity (Hickman et al., 2008; Pasquali et al., 2007). For example, one potential unifying theory of an underlying commonality is oxidative stress (OS), which may be associated with many observed infertility factors (Rowe et al., 2000), and also contributes directly to infertility through various mechanisms.

1.3.1 The psychological impact of infertility

Infertile couples may experience feelings such as aggression, depression, shame, guilt, and anxiety. These feelings can disturb the self-esteem and self-image of couples (Tarlatzis et al., 1993). They feel more depressed and more frustrated. Infertility is naturally stressful it can make difference how they see their sexual relations and their social relations with other people (Rickinson, 1997). Infertility has been described as an often unanticipated crisis situation. Typically, the emotrional strains of testing and treating infertility can be very stressful situation that many people are unable to

cope with (Levin and Theodos, 1997). Prolonged infertility in couples trying to conceive has been reported to bring about the feelings hopelessness and failure (Aghanwa et al., 1999).

The impact that infertility has on men is often very different to the impact it has on women (Greil, 2002). Because many of the medical treatments of childlessness aim at the woman's body, males mostly feel helpless. When the infertility lies with a sperm dysfunction, the male can feel that he is not able to produce baby, impotent or weakly masculine (Kamischke et al., 1998). Due to the strong association of fertility and virility with society, males tend to keep their infertility as secret, worsening their feeling of loneliness. Females can find some support from female friends, while for males it is not common to receive support from friends. Bhatti et al., 1999; Okonufua et al., 1997 describes that males can also suffer from problem of infertility, but male infertility is not discussed usually. Females normally seek treatment for their inability to conceive from religious guides and traditional healers, because they consider it as a supernatural problem. When females remain unsuccessful, they must demand the confidentiality in hospitals. In one society, infertile people are viewed as failed in some basic ways. Women develop fears that their husbands will cast out them for infertility and would not be accepted by the society (Okonufua et al., 1997) suggested a need for society education on the true reasons and treatment of infertility to remove the blame that women carry and to encourage medical treatment.

1.3.2 Types of Infertility

Infertility is divided into following three types (Larsen, 2000). Primary Infertility: The couple is said to be suffering from primary infertility when they have never achieved conception before (Larsen, 2000; Kasia, 1997). Secondary infertility is said for a couple who fails to achieve conception after having conceived once or more (Larsen, 2000). Unexplained infertility if the basic investigations are normal yet there is a persistent failure to conceive, couples are said to have unexplained infertility. This poorly defined entity is usually reported in 10 - 20% of cases (Guzick, 1999). It has been clearly demonstrated that the chance of conception in such couples is most closely related to the duration of their infertility, the age of the female partner and whether the infertility is primary or secondary (Hull et al., 1985; Cheung, 2000). These authors all basically agree that an eventual pregnancy rate of 60-70% will be achieved after 3 years of follow up with no specific treatment. It

has been realized that pregnancy may occur as a natural event unrelated to treatment and no credit for the treatment should be claimed. This concept was clearly formulated by Collins et al. (1995).

1.3.3 The cause of infertility

Failure to produce healthy semen with sufficient number of spermatozoa is commonly responsible for infertility in men (World Health Organization 1995; Devroey et al., 1998). The cause of infertility is attributed to 30% in male, 35% in female, 20% in both male and female, and 15 % is regarded as unknown or idiopathic (Tielemans et al., 2002). Depending on the course of treatment, the cause of male infertility may be defined as either surgical (congenital anatomic anomalies, varicocele, erectile dysfunction and obstruction of the ductal system in the reproductive tract) or medical (may include immunological conditions like antisperm antibodies, infectious disease linked to anatomical obstruction, endocrinopathy, exposure to gonadotoxin and systemic illness, spermatogenic dysfunction and cryptorchidism (Meacham et al., 2007). Decreased semen quality is a primary cause of male infertility and it is characterized by low sperm motility and viability (Banihani et al., 2011). Male infertility may be associated with terms are useful to explain the deficiency in semen with absence of spermatozoa in the semen azoospermia, low sperm production (oligoozoospermia), poor sperm motility (asthenozoospermia) or abnormal sperm morphology (teratozoospermia) or a combination of all three (oligoasthenoteratozoospermia) (Guzick et al., 2001). Idiopathic causes of male infertility have been associated with excessive generation of reactive oxygen species (ROS) by spermatozoa and contaminating leukocytes associated with genito-urinary tract inflammation (Sikka, 2002). The investigation of male infertility starts with a routine semen analyses, in specific, the semen quality examined by means of sperm concentration (Calvo et al., 1994), motility **(**Robinson et al., 1994) and morphology **(**Menkveld et al., 1991).

However, many men who demonstrate normal parameters on standard semen analysis remain unable to induce pregnancy (WHO, 1992; Baker, 1994; Lewis, 2007) reported when determining male infertility these parameters, strictly followed by WHO diagnostics over the past two decades are insufficient. In more 12 recent years, this procedure was complemented by functional parameters (Henkel et al., 2005; Aziz et al., 2007), which aimed at determining the sperm cells' functional capacity. Fertilization is regarded as a multifactorial process (Henkel et al., 2005). Therefore, it is

essential that all facets of the male fertility aspect be thoroughly investigated in this regard. Thus, it is imperative that functional parameters such as capacitation (Sukcharoen et al., 1995) and the ability to undergo stimulated acrosome reaction **(**Henkel et al., 1993) be integrated into the routine fertility analyses (Franken and Oehninger, 2012). Recently, these parameters were complemented by the determination of DNA damage, sperm reactive oxygen species (ROS) and mitochondrial membrane potential (MMP) as abnormalities and have, in the past been shown to be predictive of infertility (Henkel and Franken, 2011). Particularly, sperm DNA damage may contribute to embryonic death (Seli et al., 2004) or the offspring suffering from health complications (Aitken and Krausz, 2001).

In females, conception cannot take place when ovulation is absent. The causes of absent ovulation are amenorrhoea (associated with absence of ovulation) and anovular menstruation that is commonly seen for a few years after menarche and before menopause (Mascarenhas et al., 2012). Due to this factor, infertility is commonly seen in early teens and early forties (Guerra et al., 1998). Congenital absence of vagina and uterus or part of the genital tract creates obstructios and causes amenorrhoea and infertility. The causes of tubal obstruction are infection, spasm of the tubes and endometriosis (Woolcott et al., 1995). In couples who produce healthy spermatozoa and ova, but fail to conceive, the possibility of hostility of female genital tract to the husband's spermatozoa may be the cause. Cervical mucus may become hostile due to production of antibodies against the spermatozoa of the husband. The cervical mucus may interfere with fertility, due to physical or chemical changes in its character. Loss of mucus and faulty direction of cervix are important causative factors in females (McComb et al., 1995).

The exact aetiology of male infertility still remains largely indistinct as almost 50% of cases are classified as idiopathic **(**Sherins, 1995). In an attempt to alleviate or at least shed some light onto this matter, scientists and clinicians employ various assisted reproductive techniques (ART), namely *in vitro* fertilization (IVF) and also intracytoplasmic sperm injection (ICSI). However, these delicate as well as considerably costly procedures have yet to reach an optimum success rate (Gleicher and Barad, 2006).

1.4 Traditional Herbal medicinal plants

1.4.1 Herbal medicine

According to Elvin-Lewis (2001), there are four major types of herbal medicines in existence today according to **(**Elvin-Lewis, 2001), Asian, European, Indigenous African Medicines and Neo-western. The Asian herbal medicinal system has its origins in India, China, and Japan with the Aryuvedic, Unani and Siddha practice from India, Wu-Hsing from China and Kampo from Japan (Feng et al., 2006). Asian and African indigenous herbal medicines are the most intricate systems of herbal medicines and are still routinely practiced by many indigenous cultures. European herbalism has its roots in the Mediterranean civilization and has since evolved to include plants from all over the world. Neo-western herbal medicine is the latest branch in the herbal industry and constitutes a combination of the European medicinal system and indigenous African herbal systems (Feng et al., 2006). Indigenous knowledge and the traditional use of medicinal plants date back centuries. Fossils date the use of traditional plants to approximately 60 000 years ago (Fabricant and Earnsworth, 2001). The use of plants as medicine is evident in the history of many South African cultures and ethnic groups. Various journals have documented the early use of plants for medicinal purposes, particularly by the country's earliest inhabitants, the San (Vayda, 1969).

Of the plants used by the San, approximately 40 species were used for medicinal purposes (Smith et al., 2000). With the process of colonization came the influential penetration of western medicines and its perceived superiority over traditional medicine (Cocks and Dold, 2000), thus eroding existing traditions and cultures of ethnic groups such as the San. Failure to respect indigenous knowledge and culture over the years resulted in the initiation of alien technologies that often undermined local people's confidence (Shelton and Katrinka, 1993), marginalizing and alienating indigenous communities. Currently, more than 1000 species of South African plants are used for medicinal purposes (Mander and Brenton, 2006) and approximately 147 different families of plants are commonly used by various indigenous South African cultures cultures, such as the Zulu, Xhosa and Sotho people, for healing various ailments (Louw et al., 2002). This includes many introduced plants that over generations have been incorporated into South African traditional medicine, such as Dutch, Indian and Chinese medicinal plants (Van Wyk and Gericke, 2000). Some introduced species

include *Glycyrrhiza glabra* (liquorice), *Ruta graveolens* (rue), *Zingiber officinalis* (ginger) and *Acorus calamus* (calamus) (van Wyk and Gericke, 2000). In the absence of sufficient and efficient health care systems, traditional medicines continue to play a vital role among rural communities of most developing countries (Taylor et al., 1995; Grierson and Afolayan 1999; Steenkamp 2003; Verschaeve et al., 2004). The use of traditional medicinal plants is escalating as many individuals, even those from western backgrounds, continue to turn to alternative medicines. This is due to dissatisfaction with conventional medicine, often perceiving it as impersonal or the inability to afford conventional medicine.

For centuries, plants have been identified and used medicinally. Traditional preparation was the medium used to pass all of this acquired knowledge and many different practices from one generation to the next (Taylor et al., 2001). Many people continue to rely heavily on traditional medicinal plants as their main source of healthcare, quite often on a daily basis. Generally, at least 25% of commonly used medications used in conventional medicine have been based on constituents of plants, and many of their synthetically produced analogues. According to the WHO, it is estimated that 80% of the world's population rely on plant derived medicines for their healthcare, although this statistic is primarily derived from developing countries (Gurib-Fakim, 2006).

Traditional medicines, in developing countries, are commonly used due to strong cultural beliefs or as due to an inability to afford conventional medications used in Western medicine (Cunningham, 1988). A continual effort to investigate and categorize the many different medicinal plants used all across Africa has gone largely unrecognized and hasn't been as well documented as Indian and Chinese traditional medicines. It is projected that over 5000 plants, in Africa, have been identified as being used in some form of traditional medicine. Despite this, however, a small percentage of these identified plants have been investigated (Taylor et al., 2001). Extracts made from plants are commonly used in studies that investigate their effects on different biological systems, with regard to the bioactive compound responsible for the observed effect, do provide adequate and accurate analyses. However, it is important that the way these plants are prepared and subsequently the manner in which they are used in traditional medicine is taken into account when active compounds have been any identified from these many extracts (Taylor et al., 2001). To achieve this, the method in which the plants is normally prepared needs to undergo investigation so that the link between the

observed effects and the dosage of plant preparation may be established. It must be mentioned that although the study of plants have the potential to make a significant contribution to modern medicine, often by yielding new drugs with an array of applications, the way these plants are intended to be used in traditional medince mat be entirely different (Taylor et al., 2001).

Conventionally, it is often thought that medicines used to treat many different illnesses , in the form of herbs, pose no negative side effects and a not considered to be toxic. However, it must be mentioned that the constituents of these herbal preparation, such as extracts made from plants, possess many different compounds that hold similarities to synthetically produced chemicals found in conventional medicineat these extracts made from plants may potentially result in undesirable side effects. Despite this not widely recognized, effects such as these, as the result of plant extracts, have previously been recorded (Jowell, 1999). Yet, proponents of medicinal plants often disregard all safety concerns, despite previously recorded incidence of toxicity, and promote the use of these medicines (Bandaranayake, 2006). However, information regarding the safety of herbal medicine is quite limited in comparison to that of synthetically produced medicine, which further exacerbates the disregard for potential harm (Ernst, 1998). Furthermore, information that does exist regarding the safety of herbal medicine rarely accounts for potential dangers when used over long term periods. In addition to this, very limited information regarding stan dard procedure when dosing many of these herbal medicines exist, making it extremely difficult determine the safety. However, despite this, it is thought that these herbal medicines hold many advantages when used to influence the health of patients, in comparison to synthetically produced medicines. Generally, this is thought to be due to synthetically produced medicine only possessing one active compound, whereas herbal medicines possess an array of different compounds which may act in unison (Ernst et al., 1998; Taylor et al., 2001).

1.4.2 The use of traditional medicine

Traditional medicine and its usage in countries that still considered to be developing, often as the main source of healthcare, has been well documented and accepted (UNESCO, 1996). Medicines of a plant origin can often categorized differently. They are sometimes categorized as intricate mixtures which possess an array of different chemical constituents. Generally, infusions, extracts and tinctures

fall within this category. Sometimes, however, isolated active compounds of these medicinal plants are used, forming an entirely new category (Hamburger and Hostettmann, 1991). The use of the isolated active compounds found in medicinal plants is favoured when the desired affects are exponentially more potent in the isolate form, and when accurate dosing is required for these effects. When the observed effects of an active compounds is weak, however, the use of the plant extract is often seen as sufficient (Hamburger and Hostettmann, 1991; Taylor et al., 2001).

Nevertheless, plants that are used in traditional medicine are often made up of many different phytochemicals that have the potential to act alone, or to act together to achieve a desired effect (Gurib-Fakim, 2006). Furthermore, there has been a growing interest in herbal medicine as an alternative to conventional medicine, usually due to the belief that it is much safer (Bent, 2008).

1.4.3 Traditional medicine in Africa

It is thought that traditional systems of medicine that are found in Africa may indeed be the most diverse system in existence. Despite this notion, however, these systems have not been well documented, especially in the scientific sense (Campaign and Richter, 2003). The many different systems that constitute traditional medicine in Africa take on a holistic approach, taking into account both the body as well as the mind of the patient. As such, illnesses are often treated by first considering by evaluating the psychological state of the patient, rather than simply prescribing medicine to treat the symptoms of the illness (Gurib-Fakim, 2006). Furthermore, is has been well recognized that a better ratio of traditional healers to the general population exists, often being responsible for the treatment of 70% of illnesses, in comparison to that of conventional medical doctors (Abdool-Karim et al., 1994). Thus, an intricate network of these traditional healers has been developed and plays an important role in the the wellness of those living in rural areas in particular (Puckree et al., 2002; Zachariah et al., 2002).

The African continent has an enormous wealth of plant resources and traditional medicines play a vital role in the lives of millions of people throughout Africa (Iwu, 1993; Botha et al., 2001). During the latter part of the 20th century and the dawn of post-colonial African societies the use of herbal medicines has again become more important and preferred choice for both many rural and urban dwellers in many African countries (Chavunduka, 1994). Historically, credit for the development of

effective medicine from plant based resources has been attributed to the ancient Egyptians (Nunn, 1996). Specifically, medicines made from these natural resources, often plant extracts, were taken either used orally, inhaled in vapour form or applied directly to an affected area. Many of the natural resources used by the ancient Egyptians, such as plants, were later scientifically backed by (Halberstein, 2005). Subsequently, many plants that have been commonly used in traditional medicine have been previously investigated. These include plants such as *Acacia senegal*, *Agathosma betulina*, *Aloe ferox* and *Aloe vera*, to name a few (Gurib-Fakim, 2006).

Medicinal plants are still an important part of South African cultural heritage as over 60% of the population in urban and rural communities of South Africa is reliant on herbal medicines for their health care needs, as a result of their affordability and accessibility (Manders, 1998). A large number of plant species are used by Zulu traditional healers, with approximately 3000 plant species, from 147 plant families, are known to have medicinal values (Hutchings et al., 1996; van Wyk et al., 1997). Southern Africa has one of the richest plant diversity in the world (Arnold and de Wet, 1993) and a large proportion of these species have been made use of in traditional medicine in the region for centuries (Watt and Breyer-Brandwijk, 1962; Iwu, 1993; Hutchings et al., 1996; Eldeen et al., 2005). In South Africa, the trade in traditional medicinal plants is dominated by material with a long shelf life; bark, roots, bulbs, whole plants, seeds and fruits which are dried and stored in cardboard boxes or sacks (Grace et al., 2002). There are several books and published works of traditional plant medicines in South Africa (Watt and Breyer-Brandwijk 1962, Cunningham 1988, Hutchings 1996, Van Wyk 1997 and Williams et al., 2001).

1.5 Phenolic compounds from medicinal herbs

It is well recognized that many plants that are commonly used in traditional medicines are rich reservoirs of compounds that may also be derived synthetically. These compounds found within these plants, often termed phytochemicals, have been described as bioactive substances that are responsible for medicinal value of these plants (Fukumoto and Mazza, 2000). As defined by the American Cancer Society, phytochemicals are made up of a wide array of different compounds which are of plant origin. Specifically, these include different classes such as flavonoids, alkaloids, sterols and tannins, to name a few (Valpuesta and Botella, 2004). Furthermore, by investigating and

understanding the various mechanisms by which these phytochemicals act may yield greater understanding of how these plants may effect health.

Phytochemicals of the bioactive non-nutrient compounds in fruit, vegetables and other plant based foods have been linked to a reduced risk of major chronic diseases (Kris-Etherson et al. 2002; Liu, 2003). It is estimated that more than 25,000 terpenoids, 12,000 alkaloids and 8,000 phenolics have been identified in plants (Saeed et al., 2005), but a very large number still remain unknown and need to be identified and quantified before their health benefits can be evaluated (Hollman and Arts, 2000). However, recent evidence suggests that the benefits of phytochemicals in fruit and vegetables may be even greater than anticipated because oxidative stress induced by free radicals which is involved in the aetiology of a wide range of chronic diseases can be stabilised by antioxidants (Liu et al., 2003).

1.5.1 Phytochemicals and health effects

Fruits and vegetables that contain significant amounts of bioactive phytochemicals may provide desirable health benefits beyond basic nutrition, and play important roles in the prevention of chronic diseases (Hollman et al., 1996; Galati et al., 2002). The question is whether a purified phytochemical derived from plants has the same health benefit as a whole fruit or vegetable or a mixture of them in which the phytochemical is present. Different parts of plants contain different levels of phytochemicals and bioactivities. For example, it was reported that vitamin C in apple skin accounted for only 0.4% of total antioxidant activity of the fruit even though total antioxidant activity of apples is higher compared to other fruits (Liu, 2003). Researchers have suggested that most of the antioxidant activity of apples may come from phytochemicals such as phenolics and flavonoids rather than vitamin C (Pearson et al., 1999; Chinnici et al., 2004; Crozier et al., 2006). It is also proposed that the additive and synergistic effects of phytochemicals in fruits and vegetables are responsible for their substantial antioxidant and anticancer properties as well as other relevant biological activities in humans (Liu 2003; Kris-Etherton et al., 2004). However, to answer this more research is needed to investigate the relationship of the specific phytochemicals and their contribution to human health.

In recent years, investigation of biological activities of phytochemicals has increased as searches for novel compounds have raised attention to understand the bioactivity and health effects of these chemicals. To this end, a number of methodological approaches have been used (Horan et al. 2003; Kris-Etherton et al. 2004). Early stages of experimental design are of priority as *in vitro* and *in vivo* studies are used to attempt to understand the mechanisms of action before the long-term effect studies. However, this process can take several years before a clinical trial is reached (Gurib-Fakim, 2006). Identification of bioactive compounds from plants and establishing their health effects are a priority as there are exciting prospects that selected beneficial components will reduce the risk of many diseases such as cardiovascular disease and cancer (Ong et al., 2004).

Herbal medicines are widely used in traditional medical practices in the management of a wide variety of illness **(**Jude et al., 2013). Traditional medicines provide a large source of natural antioxidants that might serve as leads for the development of novel drugs. Therefore, much focus has been placed on investigating natural antioxidants and bioactive compounds for preservation of traditional medicines and their use in treating certain human diseases **(**Lin et al., 2010). The use of traditional medicine is widespread and plants still present a large source of natural antioxidants that might serve as leads for the development of novel drugs (Silva et al., 2005).

Many of the phytochemicals, including terpenoids, alkaloids and phenolics, have been found to have an impact on human health (Pandey and Rizvi, 2009). In contrast, primary metabolites, such as sugars, fats, and amino acids are the nutrients that are essential for plants growth and development. In recent times plant secondary metabolites have become of increasing scientific interest due to their beneficial effects on human health. This can be seen from the large number of publications focusing on phytochemicals, their *in vitro* and *in vivo* activities, and their potential impact on human health (Scalbert et al., 2005). Phenolics are a class of chemical compounds having at least one aromatic ring with one or more hydroxyl group attached to it (Miller and Ruiz-Larrea, 2002). Some plant secondary metabolites are believed to be produced as a result of the plant's interaction with the environment, such as their response to stress and defense against bacterial and viral pathogens. In humans they appear to have desirable medicinal effects, with antioxidant and anticancer properties. In nature, phenolics are usually found conjugated to sugars and organic acids and can be classified into two groups: flavonoid and non-flavonoid compounds.

1.5.2 Phenolic compounds

Phenolic compounds are widely distributed in plants and abundant in fruit and vegetables (Hollman and Arts 2000; Manach et al., 2005; Crozier et al., 2006). One of the major groups of polyphenolic compounds, the flavonoids, is important in contributing to the flavour and colour of many fruits and vegetables (Hertog et al., 1993; Block and Langseth, 1994). Flavonoids are widely distributed in leaves, fruits and parts of the plants (Heim et al., 2002).

Over 5000 types of flavonoids have been identified, from approximately 8000 types of phenolics (Kris-Etherton et al. 2002), mainly as flavones, flavanones, flavan-3-ols, flavonols, anthocyanins, flavonones and isoflavones (Rice-Evans et al., 1996). Flavonoids are classified according to substitutions. They differ in the arrangements of hydroxyl, methoxy, and glycosidic side groups, and in the conjugation between the B- and C-rings. During metabolism, hydroxyl groups are added, methylated, and glycosylated typically forming as 3-*O* glycosides in foods (Heim et al., 2002; Crozier et al., 2006). Flavonoids have been reported to exhibit a wide range of biological effects including antibacterial, antiviral, anti-inflammatory, anti-allergic and vasodilatory actions (Cook and Samman 1996; Di Carlo et al., 1999; Cushnie and Lamb, 2006). In addition, they also inhibit lipid peroxidation, platelet aggregation as well as the activity of enzyme systems including cyclo-oxygenase and lipoxygenase (Korkina and Alfanas'ev 1997; Awad et al., 2001). Flavonoids exert these effects as antioxidants, free radical scavengers, and divalent cations (Afanas'ev et al. 1989; Hollman and Katan, 1997) and are reported to have unique cardio-protective effects (Rajadurai and Prince 2007). For example, rats fed a flavonoid-rich diet are reported to exhibit reduced myocardial post-ischemic damage (Heim et al., 2002). The main classes of phenolic compounds include Flavonols (Kaempferol, quercetin, myricetin), Flavones (Apigenin, luteolin), Flavanones (Naringenin, hesperetin), Flavan-3-ols (Catechins, gallocatechin, Anthocyanidins Pelargonidin, cyanidin, malvidin), Isoflavones (Daidzein, genistein, glycitein) (Miller and Ruiz-Larrea, 2002).

Flavonoids are a group of more than 4,000 phenolic compounds and are ubiquitous in nature (Iwashina, 2000). They are present in fruits, vegetables, and beverages including tea, fruit juices, wines and herbs. They occur in all parts of plants but are not synthesized by humans or fungi. These compounds play an important role in plant development, defense and pollination and they are

responsible for the attractive colours of flowers, fruit, and leaves (de Groot and Rauen, 1998). It has been estimated tonnes of flavonoids and related compounds are produced annually by plants, equivalent to about 2% of all photosynthesized carbon (Andrew and John, 2011). Flavonoids occur primarily conjugated with either one or more sugar residues that are linked to hydroxyl groups, but association with other compounds including carboxylic and organic acids, amines and lipids also takes place. Flavonoids with sugar moieties are referred as the flavonoids glycosides whereas aglycones are flavonoids without a sugar moiety. Flavonoids occur predominantly in plants as glycosides. Glycosylation increases their solubility in water, facilitating their storage in vacuoles of flowers, leaves, stems and roots (Cuyckens and Claeys, 2004). Flavonoids occur as *O*- or *C*-linked glycosidic conjugates. Although any hydroxyl group can be glycosylated, certain positions are favoured; e.g. the 7-hydroxyl group in flavones, flavonones and isoflavones, the 3- and 7-hydroxyls in flavonols and flavan-3-ols, and the 3- and 5-hydroxyls in anthocyanidins (Cavaliere et al., 2005). The structures of flavonoids are based on the flavonoid nucleus, which consists of three phenolics rings. These polyphenolic compounds possess 15 carbon atoms represented by a C6-C3-C6 structure. The basic structure allows for a magnitude of substitution patterns in the A, B and C rings. Flavonoids are classified into several sub-classes depending on the degree of unsaturation and oxidation of basic 15-carbon skeleton. The main groups of dietary importance are flavones, flavonols, flavan-3-ols, flavanones, anthocyanidins and isoflavones.

The different flavonoids that may be found in plants used in traditional medicine have often been reported to possess the ability to act as an antioxidant, making them potential treatments in diseases associated with free radicals. Usually, this antioxidant potential in these flavonoids is due to the presence of hydroxyl groups (Vaya et al., 2003; Kris-Etherton et al., 2002).

On the other hand, alkaloids have previously been described as a structurally diverse class of compounds, with an estimated 12000 different structural configurations that have been identified in plants (Verpoorte and Memelink, 2002; Wink, 2003, 1999). The most famous of these alkaloids are purine alkaloids, tropane alkaloids, isoquinolone, as well as monoterpenoid alkaloids. Generally, these classes of alkaloids are typically differentiated on the bases of their primary metabolite (Marasco and Schmidt-Dannert, 2007). Medicinally, alkalida have proven to be effective in the

treatment of many different ailments. Many of these include the treatment of cancers, malaria or hypertension, to name a few (Rathbone and Bruce, 2002).

Many different alkaloids, which are all nitrogen containing compounds, created via diverse biosynthetic pathways; continue to provide an extensive range of therapeutic compounds (Rathbone et al., 1999). There is much interest in the identification of new alkaloids either as drugs or as pivotal intermediates in the synthesis of new drugs. The use of alkaloids as drugs started in the early 1950s, when camptothecin isolated from the stem of the Chinese ornamental tree *Camptotheca acuminate* was shown to be an effective anticancer agent (Srivastava et al., 2005). Many alkaloids derived from plants have anticancer properties. The anticancer drug Taxol has been reported to exhibit side effects including numbness, nausea and migraine (Singla et al., 2002). This is not uncommon, and as a consequence, there is a continuing search for new anticancer compounds that are active and selective with minimal side effects.

1.5.3 Antioxidants effect

Oxidative stress is caused by the imbalance between oxidants and antioxidants, potentially leading to damage in plants and in humans (Grabmann, 2005). Approximately 13% of the oxygen consumed in human's body is converted to superoxide and other reactive oxygen species (ROS) called free radicals under physiological conditions (Halliwell, 1996). Even though these free radicals perform many important physiological processes such as cell signalling, microbial killing and gene transcription (Dröge 2002; Grabmann, 2005), they may also damage DNA, protein or lipids in the human body (Halliwell, 1996). These deleterious effects are reported to be responsible for chronic diseases mentioned before (Halliwell 1996; Heim et al., 2002). To counteract the threat of free radical induced damage, the human body has developed an antioxidant defence system, which consists mainly of antioxidant enzymes such as superoxide dismutase or catalase and radical scavengers like ascorbic acid or tocopherols (Grabmann, 2005). However, during physical stress, such as exercise or certain disorders, i.e. fever, this antioxidant system is affected and enhanced, which request the body to keep the balance between antioxidants and prooxidants (Clarkson 1991; Clarkson and Thompson, 2000).

Therefore, one easy way improve the antioxidant defense is to increase the dietary intake of antioxidants from food mainly fruits and vegetables, which contain bioactive compounds such as vitamin C, carotenoids and importantly polyphenolics (Harborne and Williams 1998; Kim 2004). Recent studies have emphasized the importance of antioxidants and the mode of action of specific flavonoids as bioactive components of the diet *in vivo* and *in vitro*. It is, therefore, important to have a distinct awareness of the major phenolic antioxidant compounds and their levels in fruit and vegetables, Hollman et al. (1997) and Proteggente et al. (2002) reported the antioxidant capacities of extracts from selected fruit and vegetables assessed using the Trolox Equivalent Antioxidant Capacity, the Ferric Reducing Antioxidant Potential and the Oxygen Radical Absorbance Capacity assays. The results showed that fruits such as strawberries, raspberries, and red plums, anthocyanin-rich, had the highest antioxidant activities, followed by flavanone-rich fruits, such as orange and grapefruit, and flavonols (e.g. green cabbage, spinach, onion, and leek), while the hydroxycinnamate containing fruits (e.g. pear, peach, apple, and tomato) consistently stimulated lesser antioxidant activities (Proteggente et al., 2002). *In vitro* studies have shown the relationships between flavonoid structure and antioxidant activity (Rice-Evans et al., 1996; Awad et al. 2001).

Quercetin, the most potent antioxidant among the flavonoids, has three structural properties which contribute to its activity. First, the number and configuration of hydroxyl groups on A- and B- rings, specifically the dihydroxycathecol structure of the B ring, secondly, the planarity of the molecule and third, the double bond in relation to the 4-oxo group of the C- ring (Rice- Evans et al., 1996). Other antioxidant properties of flavonoids were reported to stabilize unpaired electrons (Duthie et al., 2003), scavenge free radicals from lipid peroxidation (Nijveldt et al., 2001), reduce the incidence of DNA damage (Minelli et al., 2009) and the ability to chelate with transition metal ions, which results in the inhibition of the reactive oxygen species production (Duthie and Crozier, 2000). There is limited information on the antioxidant activities of Malaysian traditional vegetable although recently, the screening of biological activities such as anticancer, antioxidant and anti-inflammatory *in vitro* has been reported (Chang et al., 2000). Extracts from *Centella asiatica* have also been shown to exhibit high antioxidant activities using linoleic acid and TBARS assays (Hussain et al., 2007). Therefore, investigation of the biological activities of these plants should be a priority to understand the potential health effects of Malaysian traditional vegetables. The process of ageing and the pathogenicity of various diseases are thought to be underlined by Reactive oxygen species.

Neurodegenerative disorders, cancer, asthma, liver disease, periodontal disease, muscular degeneration, gastrointestinal inflammatory diseases, cardiovascular disease, cataracts, diabetes, and other inflammatory diseases are thought to be impacted upon by Free Radicals, including hydroxyl radical (OH), lipid peroxide radicals, and the superoxide radical (O^{2-}).

Normal bodily biochemical processes yield reactive oxygen species (ROS). Additionally, increased exposure to xenobiotics, environmental and dietary, produce further ROS (Pourmorad et al. 2006; Kumpulainen and Salonen, 1999). Free radicals induce abnormal protein synthesis, cause depletion of immune system antioxidants, and changes in gene expression as a result of toxins, deep fried foods, environmental pollutants, chemicals, spicy foods radiation, and physical stress. A number of human neurological, and other, disorders are fundamentally underlined by oxidative injury from free radicals. For example, greater oxidative stress coexisting with reduced antioxidant status has been suggested in diabetes. Oxygen free-radicals can initiate peroxidation of lipids. This, in turn, stimulates glycation of protein, inactivation of enzymes and variation in the structure and function of collagen basement and other membranes, therefore, playing an important role in the long term diabetic impairment (Atawodi, 2005; Sabu and Kuttan, 2002). Similarly, in carcinogenesis, ROS are responsible for initiating the multistage carcinogenesis process starting with DNA damage and accumulation of genetic events in one or few cell lines (Atawodi, 2005). It is thought that many overt disease processes are preceded by this free radical-induced damage (Allan and Miller, 1996). Therefore, the potential for many of these disorders to be prevented, delayed or ameliorated exists through therapy using antioxidants, or free-radical scavengers (Delanty and Dichter, 2000). Moreover, as a consequence of the depletion of immune system natural antioxidants in diverse conditions, it may be necessary to consume antioxidants as free radical scavengers (Halliwell, 1996; Kumpulainen and Salonen, 1999).

Interest in the healing capabilities of medicinal plants as antioxidants has recently increased. Apart from renowned and traditionally used natural antioxidants from fruits, wine, tea, spices, and vegetables, some natural antioxidants are already exploited commercially either as antioxidant additives or a nutritional supplements (Schuler, 1990). Moreover, novel antioxidants have been investigated from many other plant species (Oke and Hamburger, 2002). However, there is generally still a demand to gather further information regarding the antioxidant potential of plant species.

Many African medicinal plants have been reported to possess antioxidant properties. The antioxidant potential of a Nigerian beverage additive, *Sacoglottis gabonensis* stem bark, has been reported. The *in vivo* results showed 2, 4 dinitrophenylhydrazine-induced membrane peroxidation. The study concluded that a multifactorial mechanism of antioxidant action existed. It involved the inhibition of catalase. This, in turn, enhanced the superoxide dismutase capability of the liver and red blood cells, and spared tissue utilization of ascorbic acid and tocopherol (Maduka and Okoye, 2002). Their study design made use of a streptozotocin induced non-insulin dependent diabetes mellitus rat model (Ugochukwu and Babadu, 2002). It is known to be one of the best multipurpose medicinal plants used in the treatment of cancer, viral diseases, and inflammatory conditions in South Africa. Their results indicated that hot water extract of *Sutherlandia frutescens* possess superoxide as well as hydrogen peroxide scavenging activities at low concentrations (10 µg/ml). Correspondingly, analysis of methanol extracts of *Rhoicissus tridentate* and *Rhoicissus rhomboidea*, South African medicinal plants used by the Zulu traditional healers, exhibited inhibition of the activities of DPPH (1,1-diphenyl-2-picrylhydrazyl) free radicals, free radical mediated sugar damage and xanthione oxidase and, additionally, prohibited production of thiobarbituric acid reactive substances. However, related plants, such as *Rhoicissus tomentosa and Rhoicissus digitata*, lack these inhibitory properties, unless at excessive concentrations (Ugochukwu and Babadu, 2002).

1.5.4 Anticancer effect of medicinal plants

Medicinal plants have played an significant role in cancer treatment in the last 50 years. A multitude of new clinical applications, geared toward combating cancer, have been developed from plant secondary metabolites and their derivatives. Approximately 35,000 plant samples, from 20 countries, have been collected by The National Cancer Institute. Furthermore, around 114,000 extracts have been screened for anticancer activity (Shoeb, 2006).

Phytochemicals derived from plants have been an important source of several clinically useful anticancer agents such as etoposide, camptothecin derivatives, vincristine, topotecan and irinotecan, vinblastine, and paclitaxel (Cragg and Newman, 2005; Gurib-Fakim, 2006). The pursuit for anticancer agents from plants began in 1950s with the discovery of the vinca alkaloids, vinblastine and vincristine. Consequently, the United States National Cancer Institute instigated an all-

encompassing plant collection programme in 1960 that concentrated mainly on temperate regions, to expand the search for plants with anticancer properties (Cragg and Newman, 2005). This resulted in the uncovering of many novel compounds displaying a variety of cytotoxic activities, including the taxanes and camptothecins, which spanned a period of 30 years from the early 1960s to the 1990s, to be developed clinically (Mukherjee et al. 2001).

The development of new screening technologies led to the revival of plant collections where tropical and sub-tropical regions of the world were focused upon (Gurib-Fakim, 2006). To date, new plant derived clinical anticancer agents have not yet reached the stage of general use but a number of anticancer agents are in a preclinical development and may take several years before they can be fully applied in medical treatment (Mukherjee et al. 2001; Cragg and Newman, 2005). Phenolic compounds have also been reported to possess anticancer properties (Galati et al., 2000; Ren et al., 2001).

The role of phenolic compounds, particularly flavonoids, in the prevention of cancer is associated with their ability to influence cancer inducing processes *in vivo*. quercetin and myricetin have been reported to suppress hydrogen peroxide induced DNA damage in isolated human lymphocytes (Kim et al., 2003), inhibit protein kinase which is responsible for regulating tumour promotion and regulation of cell growth (Srivastava, 2005), exhibit antiproliferative effects and induce apoptosis, a programmed cell death in cancer cells (Iwashita et al., 2000). Between 1940 and 2002, 40% of all available anticancer drugs were natural products or natural product derived. Additionally, a further 8% were considered natural product mimics (Newman et al., 2003). Currently used clinical anticancer agents from medicinal plants may be categorized into four major compound classes: vinca alkaloids, epipodophyllotoxins, taxanes, and camptothecins.

Vincristine and vinblastine, used clinically for over 40 years, were isolated from *Catharanthus roseus* (van Der Heijden et al., 2004). In combination with supplementary chemotherapeutic drugs, they are used in the treatment of leukemias, lymphomas, advanced testicular cancer, lung, and breast cancers (Cragg and Newman, 2005). Their mechanism of action is the inhibition of mitosis with metaphase arrest. This occurs by specific tubulin binding which results in depolymerization. This

mechanism is also accurate for several semi-synthetic derivatives of vinca alkaloids (Okouneva et al., 2003).

Countless derivatives of these four compound classes have been synthesized, with some of them currently used clinically. Natural products have elicited noteworthy biological discoveries, related to their distinctive mechanisms of action (Balunas and Kinghorn, 2005). It is estimated that an excess of 270,000 higher plants currently exist on this planet and it is, therefore, hoped that the discovery of more bioactive compounds useful for activity inhibition of most cancer cells will be achieved through active research into phytochemistry (Houghton, 2005).

1.6 Typha capensis

Typha capensis, commonly referred to as the bulrush, belongs to the family Typhaceae. It is also known as ibuma (Zulu), bulrush (English) and papkuil (Afrikaans). The plant is very common in South Africa, found in all parts, but sparse in the North Western Cape region. It is usually found in wet or seasonally wet places (Van Wyk, 1997). The plant is described as a robust, reed like plant of up to three metres in height. The rhizomes are thick, fleshy and spongy and creep horizontally (Figure 5). Erect stems protrude from the rhizomes to end in thick, strappy, hairless leaves. The characteristic flower stalk has minute male flowers towards the tip with the female flowers packed below the male part in a thick brown mass (Vlotman, 2003). The bulrush 'flower' is formed when the male flowers fall off (Goldblatt and Manning, 2000). Furthermore, *Typha capensis*, being a perennial aquatic plant grows in South Africa‘s wetlands and the country‘s seasonally wet places, can tolerate both acidic and alkaline environments as well as some degree of salinity (Van Wyk et al., 1997). It also holds value as a source of starch. Moreover, the spongy rhizomes are used as a source of nutrition and the pollen may be used as a high protein food. Leaves are also used to make hand brooms and are sometimes used in weaving and thatching by people in rural areas (Chapman and Hall, 2000).

Medicinal uses of the rhizomes of *typha capensis* include prescription during pregnancy to ensure easy delivery, dysmennorhea, venereal diseases, dysentery, diarrhoea, and to enhance the male potency and libido (Shode et al., 2002). Other uses include treatment of genital problems, promotion

of fertility in women and to improve blood circulation. Its use in childbirth stems from the claim that it strengthens uterine contractions and promotes expulsion of the placenta (Watt, 1962; Dunbabin et al., 1992; Pip et al.,1992; Hutchings, 1996). The patient is prescribed one or two cups of a decoction of the roots in boiling water, which is taken daily for a week (Pujol, 1990; Della Greca et al., 1990).

The phytochemistry of several species of *Typha capensis* have noted several flavones and other phenolic compounds (Chapman and Hall, 2000), long chain hydrocarbons as well as various triterpenoids with a steroidal skeleton typhasterol have been identified (Chapman and Hall, 1996; Sick et al. 1983). However, the hexane extract has revealed two new phenolic compounds, namely, typharin and typhaphthalide. The acetone extract has also produced several known flavan-3-ols, which were isolated as mixtures in their free phenolic form. These include afzelechin, epiafzelechin, catechin and epicatechin. All the above compounds have previously been isolated from other plant species.

The crude methanolic extract of *T. capensis* showed inhibition of growth of some of the bacteria, which are believed to be pathogenically responsible for the conditions, which this plant is used to treat (Shode et al., 2002).

Typha capensis has anti-inflammatory,diuretic, immunosuppressive activity and is a well-known traditional medicine. Chemical properties are due to presence of sterol (Della Greca et al., 1990) typhaphthalide, typharin (Shode et al., 2002), flavonolglycoside (Sick et al., 1983), essential fatty acids and phenolic acids (Gallardo et al., 2002). Even though *Typha capensis* has good nutritive properties, its antioxidative property remains untouched. Synthetic antioxidants have untoward side effects, hence, it is essential that a superior drug is isolated from plant sources. Also, an *in vitro* study, investigating the effect of *Typha capensis* on various sperm parameters showed that an aqueous extract of the rhizomes had significant detrimental effects on sperm motility, viability, sperm ROS production as well as on sperm $\Delta\psi m$ (Fransman, 2007).

Figure 5: Images of *Typha capensis* A: Spongy flowers and twisted leaves B: Rhizomes of *Typha capensis*.

1.7 Aim of the study

The aim of the study was to investigate the effect of *Typha capensis* as one of South Africa's indigenous medicinal plants and it's phytochemicals on male reproductive function. This study continued the work that has been carried out before and to further identify specific compounds responsible for the fertility promoting effects. Furthermore, it shed light on a possible seasonality of the production of the active compounds in the plant. The purified compounds underwent cytotoxicity evaluations using *in vitro* assays on various cell lines.

Therefore, the objectives of this study were:

- Fractionation and fingerprinting techniques performed for the characterization of *Typha capensis* active compounds using HPLC.
- The safety of *Typha capensis* extract towards Leydig cell function and the potential of *Typha capensis* to boost testosterone production using the TM3 cell line.
- The effects of *Typha capensis* towards the prostate cancer cell line LNCaP and the Benign prostatic hyperplasia cell line PWR-1E.

Chapter 2: Material and methods

2.1 Chemicals and Equipment

The chemicals used in the study were purchased from the following companies:

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

- Prostate cancer cell line LNCaP
- Leydig cell line TM3
- Benign prostatic hyperplasia cell line PWR-1E

Corning incorporated, New York, USA:

- Tissue culture flasks (25 cm^2, 75 cm^2)
- Eppendorf vials
- Pipette Tips 1000 µl, 200 µl, 10 µl
- Serological pipettes (10ml)

Gibco Invitrogen, Karlsruhe, Germany:

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

Eppingdust, Cape Town, South Africa:

- Ethanol absolute (100%)
- Formaldehyde (40%)

Bio-Rad, Hercules CA, USA:

● DC protein assay reagent A and reagent B

DRG instruments, Marburg, Germany:

● Testosterone 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)

● Super frost slides

Lasec, Cape Town, South Africa:

● Syringes (5, 10 and 25 ml)

Merck, Wadeville Gauteng, South Africa:

● Sodium hydroxide (NaOH)

● Dimethylsulphoxide (DMSO)

● Hydrochloric acid (HCl)

Oxoid, Basingstoke, Hampshire, RG24 SPW, England:

● Phosphate Buffered Saline (PBS) with $Ca^{2+/}Mg^{2+}$

Promega, Madison, USA:

● The DeadEnd™ Colorimetric TUNEL System kit

Sigma-Aldrich, Steinheim, Germany:

● Annexin V-Cy3 MT Apoptosis Detection Kit (Catalogue Number: APOAC)

● Dimethylsulphoxide (DMSO) for freezing medium

● Penicillin

● Streptomycin

● Thiazolyl Blue Tetrazolium Bromide MTT (Catalogue Number: 2128)

● Trypan Blue (TB)

● Triton X-100

● Millex syringe filter units (0.22 μm)

2.2 Equipment and supply

Laminar Flow

● LN Series (Nuve, Ankara, Turkey)

Incubator

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

ELISA-reader

● GloMax Multi Detection System plate reader (Promega Corporation, Madison, USA)

● Labtech System LT 4000 microplate reader (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)

HPLC

● HPLC Ultimate 3000 Pump (THERMO FISCHER- DIONEX, International)

NMR

● The NMR is a Bruker 400 MHz Avance III HD Nanobay NMR spectrometer equipped with a 5 mm BBO probe.

2.3 Study design

This study had two parts, namely the effect of the plant extract harvested in four seasons towards parameters *in vitro* using different cell lines (TM3, LNCaP and PWR-1E) and the identification of the bioactive compounds by means of HPLC and NMR spectrometry. The first part of study was carried out with *T. capensis* crude rhizome aqueous extraction (Figure 6). Thereafter the second part of study was carried out with extract fractioned by HPLC (Figure 7). While the third part of study was carried out with bioactive compounds fractioned by HPLC were used (Figure 8).

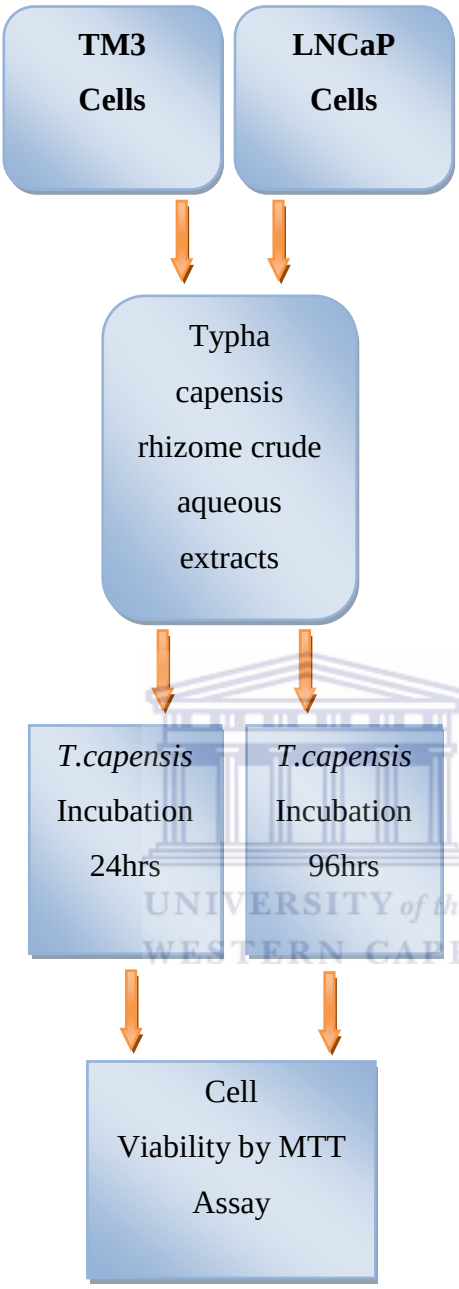


Figure 6: Study design of Part 1 of the study.

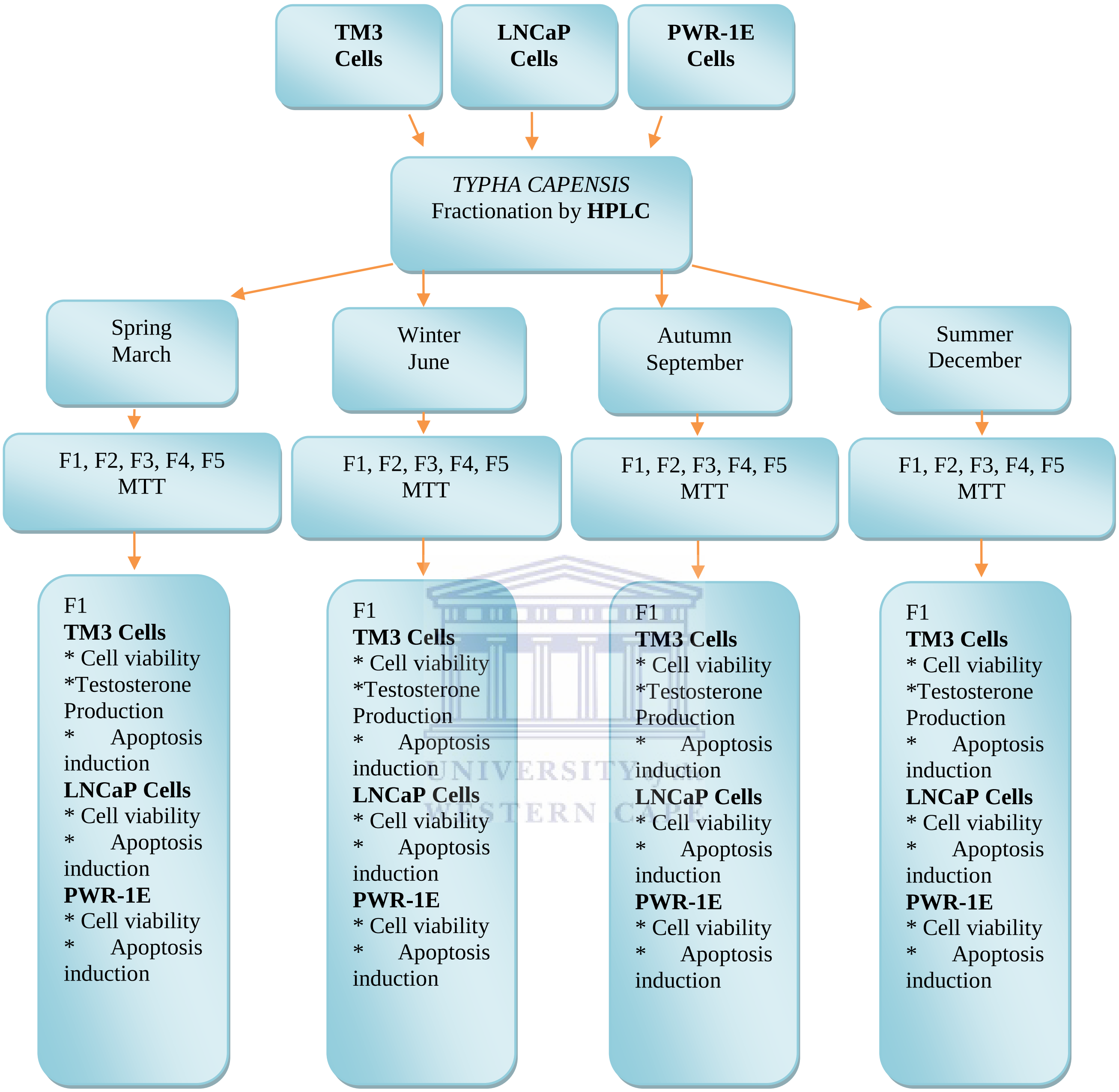


Figure 7: Study design of Part 2 of the study.

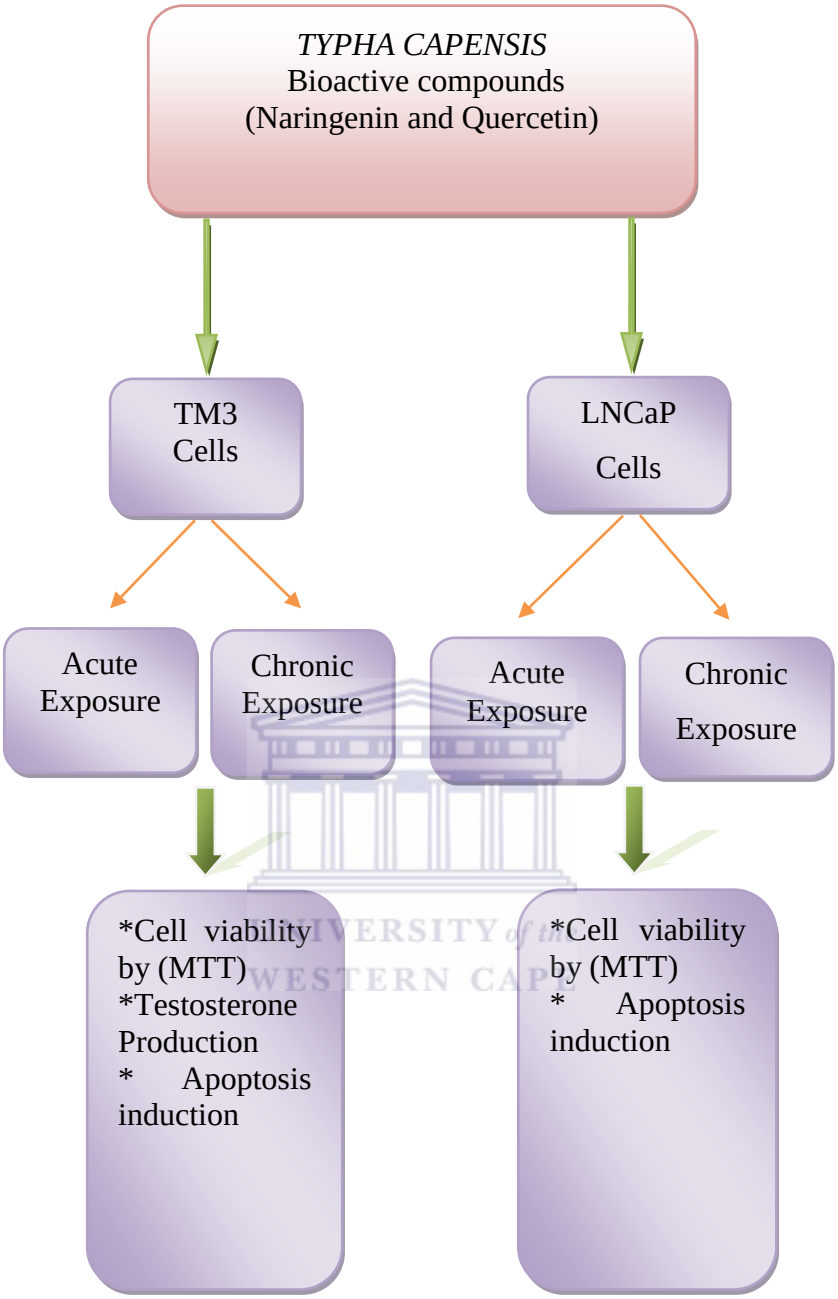


Figure 8: Study design of Part 3 of the study.

2.4 Collection and preparation of plant material

2.4.1 Collection and processing of *Typha capensis* rhizomes

The rhizomes were collected and identified under supervision of Mr. Frans Weitz from the Department of Biodiversity and Conservation Biology at UWC. Rhizomes of *Typha capensis* were collected during the four different seasons in the Cape Nature Reserve, located in the suburb of Belhar in the Western Cape province of South Africa. After collections the rhizomes were washed, chopped into 2-3 cm pieces and allowed to dry at 25ºC in a dried oven. Thereafter, the dried rhizomes were milled into a fine powder for the extraction process.

2.4.2 Aqueous extraction

For preparation of the aqueous rhizome extract the powdered rhizomes were infused in hot (75-80 °C) distilled water (50g dried rhizome extract /1L distilled water). The resulting mixture was allowed to cool down at room temperature for 2 hour, after which it was filtered (Qualitative filter paper 32.0 cm). The filtrate was then frozen at -20ºC and finally freeze-dried using a Vertis-freeze drier to yield the water-soluble extract.

According to traditional healers' suggestion, a handful of dried rhizome is used for the treatment of patients per patient is take in per day. Three handfuls of the rhizomes were weighed out and an average of 70 g was obtained. The aqueous extraction yielded an average of 7.65 g of the extract per 50 g dried rhizome on presumption that an average male weighs 75kg; the extract concentration was calculated according to equation 1:

Equation 1: Extract concentration (g/ml) $= \frac{7.65\ (g)}{75\ 000\ (ml)}$

A stock solution of 10000 µg/ml of *T.capensis* was prepared in Dulbecco's Modified Eagle Medium / Nutrient Mixture F12 Ham (DMEM/F-12, 1:1 mixture) or Roswell Park Memorial Institute (RPMI) 1640 Medium or Keratinocyte-SFM (1X) serum Free Medium for cell culture-related assays (depending on the experimental design and the cells used). These stock solutions underwent serial dilutions to yield different concentrations. The Medium used depended on the cells used the cell culture.

2.4.3 HPLC fractionation

The extract was analysed using a HPLC Ultimate 3000 Pump analysis system, Version 6.80 SR11 Build 3161, comprising an HPLC pump, photodiode array absorbance, detector scanning from 250 to 700 nm, and an autosampler cooled to 10°C (Thermo Finnegan, USA). Separations were carried out using a reverse-phase column with a Phenomenex (Luna 5u c^{18} 100A, USA) C^{18} dimension (250 x 4.6 mm), maintained at 48°C. The chromatographic conditions were as follows: Mobile phase, solvent A: Methanol (MeOH); solvent B: 5% acetic acid (CH_3COOH); Mode: gradient, increasing the organic phase (MeOH) from 20% to 90% over 60 minutes. The flow rate was set at 1.5 mL/min. The conditions for separate phenolic compounds were as follows: 20 min gradient of a 5-40% gradient of acetonitrile in water containing 1.0% formic acid. Phenolics were detected at 365, 350 and 280 nm.

2.4.4 Nuclear magnetic resonance (NMR) spectroscopy

NMR spectra were recorded at 298K using DMSO-d_6 (Dimethyl sulfoxide) as the solvent on a 400 MHz. Bruker Avance III HD Nanobay instrument equipped with a BBO probe (BBO: Bradband Observe, ^{1}H Decoupling Probe). ^{1}H-NMR Proton nuclear magnetic resonance Standard pulse sequences were used to acquire ^{1}D and ^{2}D NMR spectra (1D-NMR One-dimensional nuclear magnetic resonance; 2D-NMR Two-dimensional nuclear magnetic resonance). ^{13}C spectra (^{13}C-NMR Carbon-13 nuclear magnetic resonance) were thus recorded at 100 MHz. The NMR analysis was performed and assisted with by Dr. E. Beukes, Department of Chemistry, UWC.

2.5 Cell culture

TM3 Leydig, LNCaP prostate cancer and PWR-1E benign prostatic hyperplasia cell lines were used to investigate the biological activity of the extract in this study. These cells were cultivated at 37°C in 95% air and 5% CO_2, TM3 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). 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 cm^2 culture flasks. PWR-1E cells were cultured in complete Keratinocyte-SFM (1X) serum Free Medium supplemented with (1 X 25 μg EGF Human Recombinant) and (1X 25mg Bovine Pituitary Extract).

2.5.1 TM3 Leydig cell line

TM3-Leydig cells were obtained from BALB/c mice in 1974 it is an epithelial cell line of Leydig cells which are the primary sites of steroidogenesis in the testis (Foley, 2001). TM3-Leydig cells were used as they produced testosterone, TM3 cells respond to Luteinizing hormone (LH) with an increase in cholesterol metabolism (Matfier, 1980), (Figure 9)

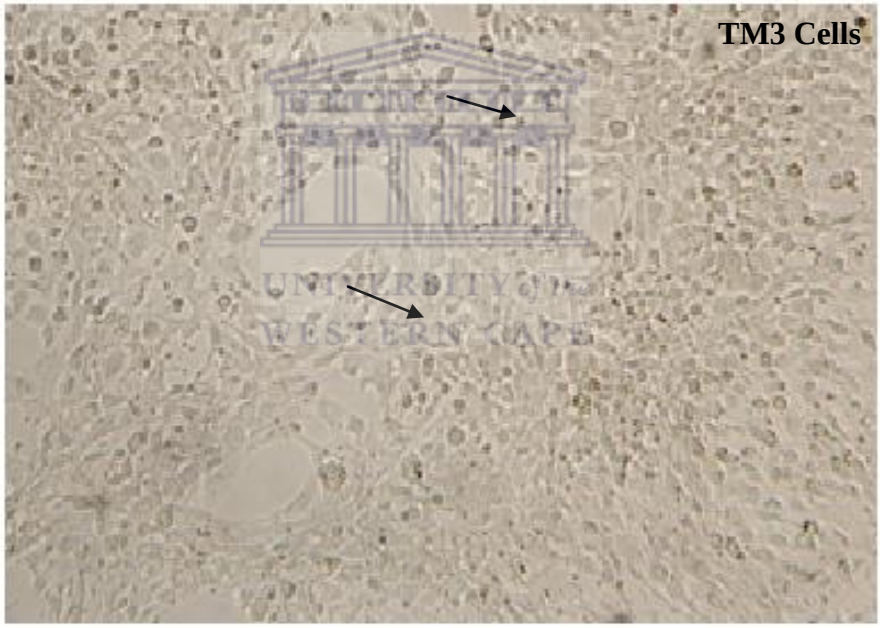


Figure 9: Normal morphology of TM3 Leydig cells. Leydig cells are polyhedral, epithelioid cells with a single eccentrically located ovoid nucleus. The nucleus contains one to three prominent nucleoli and large amounts of dark-staining peripheral heterochromatin. Arrows indicate the cell nucleus structure (10 x magnifications).

2.5.2 LNCaP prostate cancer cell line

The LNCaP prostate cancer cell line derived from a needle aspirate biopsy in 1977 from a 50-year old male, diagnosed with stage D prostate cancer. This cell line was purchased from ATCC. LNCaP cells are an ideal model for investigating early prostate cancer (Horoszewicz et al., 1980) (Figure 10).

Figure 10: Normal morphology of LNCaP prostate cancer cells. Cell are stacking with a slightly less flat and polygonal morphology. Arrows indicate to polygonal cells (10 x magnifications).

2.5.3 PWR-1E Benign prostatic hyperplasia cell line

PWR-1E cells are a benign human prostate epithelial cells line that was purchased from American Type Culture Collection (ATCC, Manassas, VA) and maintained in Kerationocyte Serum Free media prostate epithelial cells from a 68-year-old man with benign prostate hyperplasia. Cells are immortalized with SV-40 large T-antigen and described to express cytokeratins 8, 18, and 19 (but not 14). These cells are reported to metabolize prostatic androgens (Godoy et al., 2011) (Figure 11).

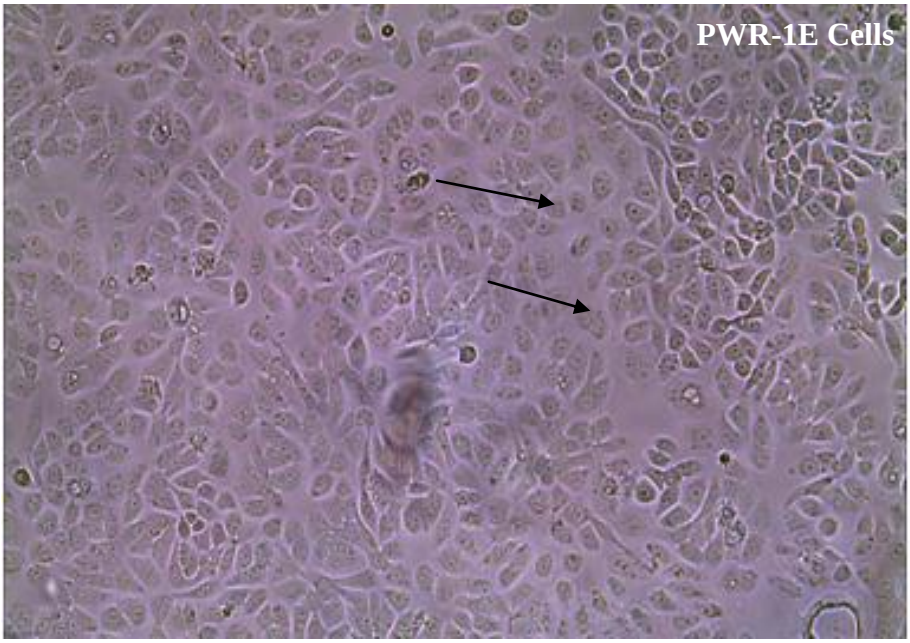


Figure 11: Normal morphology of PWR-1E benign prostatic hyperplasia cell. Arrows indicate to benign prostatic hyperplasia epithelial cells (10 x magnifications).

2.5.4 Culture of TM3 cells

Cells were cultured in 25 and 75 cm^2 flasks, allowed to reach 80-90% 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 2 ml sterile PBS. Following this, 0.5 - 1 ml of 0.25% trypsin/EDTA were added, allowed to cover the surface and gently shaken and incubated at 37ºC for 5 minutes under occasional visual control until cells detached. To neutralize the trypsin, 2 ml of complete growth medium were 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 and the cell pellet re-suspended in 5 ml complete growth medium. Subsequently, 0.5 ml of the resulting suspension were transferred into a new 25 cm^2 flask or 1 ml of the resulting suspension was transferred into a new 75 cm^2 flask, the passage was recorded. The passage gives an indication of the physiological state of the cell line and used to track the age of the cells. Cells were then either passaged into a new 25 cm^2 and 75 cm^2 flasks, respectively, with fresh

medium or seeded into 6-, 24- or 96-well plates. A dilution of cells was made to produce the final cell number required for experiments. Cell morphology was observed and compared with cell viability.

2.5.5 Culture of LNCaP cell line

Cells were cultured in 25 and 75 cm^2 sterile tissue culture flasks and were passaged at regular intervals when cells reached approximately 80-90% confluency. The culture medium was aspirated from the flasks and cells were washed with 2-4 ml sterile PBS. Thereafter, 0.5 - 1 ml 1X 0.25% trypsin/EDTA were added to the flask, gently shaken and under occasional visual control incubated at 37°C until cells began to detach. This process took approximately 4-7 minutes. In order to inactivate the trypsin, 2 ml of RPMI 1640 were added and cells were re-suspended by careful aspiration using a pipette. Finally, the cell suspension was transferred from the flask to a 15 ml test tube and centrifuged at 125 xg for 5 minutes. The supernatant was discarded and the cell pellet re-suspended in 5 ml of RPMI 1640. Following this, 1 ml of the cell suspension was transferred into a new tissue culture flask with fresh RPMI 1640 growth medium and passage recorded. Afterwards, LNCaP cells, which appeared to be growing at a slow rate, were left for up to 48 hours to re-attach. Fresh growth medium was then added to the first flask and incubated for further growth. The cells were then either passaged into a new 25 cm^2 and 75 cm^2 flasks, respectively, with fresh medium or seeded into 6-, 24- or 96-well plates. A dilution of cells was made to produce the final cell number required for experiments. Cell morphology was observed and compared with cell viability.

2.5.6 Culture of PWR-1E cell line

Cells were cultured in 25 and 75 cm^2 sterile tissue culture flasks and were passaged at regular intervals when cells reached approximately 80-90% confluency. The culture medium was aspirated from the flasks and cells were washed with 2-4 ml sterile PBS. Thereafter, 0.5 - 1 ml 1X 0.25% trypsin/EDTA were added to the flask, gently shaken and then incubated at 37°C until cells began to detach. This process took approximately 4-7 minutes under occasional visual control. In order to inactivate the trypsin, 2 ml of Kerationocyte Serum Free media were added and cells were re-suspended by careful aspiration using a pipette. Finally, the cell suspension was transferred from the

flask to a 15 ml test tube and centrifuged at 125 xg for 5 minutes. The supernatant was discarded and the cell pellet re-suspended in 5 ml of Kerationocyte Serum Free media. Following this, 1 ml of the cell suspension was transferred into a new tissue culture flask with fresh Kerationocyte Serum Free media and passage recorded. The cells were then either passaged into a new 25 cm^2 and 75 cm^2 flasks, respectively, with fresh medium or seeded into 6-, 24- or 96-well plates. A dilution of cells was made to produce the final cell number required for experiments; cell morphology was observed and compared with cell viability.

2.5.7 Cell counting and seeding

Cell counts were performed using a hemocytometer so that a specific cell concentration could be reached in 6-well plates or 96-well plates. 50 µl of the cell suspension were added to an equal volume of 2% trypan blue, the solution mixed together and left to stand for 1 minute. To determine the amount of cells needed to perform the experiment, a total cell count was conducted: 10 µl of the suspension were transferred onto a haemocytometer counting chamber and viewed under the microscope. The total cell count result was then calculated according to Equation 2:

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

2.5.8 Cells freezing

2.5.8.1 TM3 cells

In order to detach cells, 1-2 ml 0.25% trypsin/EDTA were added into a 75 cm^2 flask and placed in an incubator at 37ºC for 3-7 minutes. The flask was then examined under the microscope for cell detachment. Thereafter, 2 ml of DMEM/F-12 complete growth medium were added the flask and the cell suspension transferred from the flask to a 15 ml centrifuge tube. After centrifugation at 125 xg for 5 minutes, cells were counted and re-suspended in 1 ml of freezing medium (87.5% DMEM/F-12, 5% Horse serum, 2.5% FBS and 5% DMSO). About 1 million ($1x10^6$) cells suspended in 1.5 ml freezing medium were aliquoted in cryogenic vials, the cells were immediately transferred to -80ºC for 24 hours before being transferred to liquid nitrogen (-196ºC) for long-term storage.

2.5.8.2 LNCaP cells

In order to detach cells, 1-2 ml 0.25% trypsin/EDTA were added into a 75 cm^2 flask and placed in an incubator at 37ºC for 3-7 minutes. The flask was examined under the microscope for cell detachment. Thereafter, 2 ml of RPMI 1640 growth medium were added the flask and the cell suspension transferred from the flask to a 15 ml centrifuge tube. After centrifugation at 125 xg for 5 minutes, cells were counted and re-suspended in 1 ml of freezing medium (50% RPMI 1640, 40% FBS and 5% DMSO). About 1 million ($1x10^6$) cells suspended in 1.5 ml freezing medium were aliquoted in cryogenic vials and stored for future use. The cells were immediately transferred to -80ºC for 24 hours before being transferred to liquid nitrogen (-196ºC) for long-term storage.

2.5.8.3 PWR-1E cells

In order to detach cells, 1-2 ml 0.25% trypsin/EDTA were added into a 75 cm^2 flask and placed in an incubator at 37ºC for 3-7 minutes. The flask was examined under the microscope for cell detachment. Thereafter, 2 ml of RPMI 1640 growth medium were added the flask and the cell suspension transferred from the flask to a 15 ml centrifuge tube. After centrifugation at 125 xg for 5 minutes, cells were counted and re-suspended in 1 ml of freezing medium (50% Kerationocyte Serum Free media, 40% FBS and 5% DMSO). About 1 million ($1x10^6$) cells suspended in 1.5 ml freezing medium were aliquoted in cryogenic vials and stored for future use. The cells were immediately transferred to -80ºC for 24 hours before being transferred into liquid nitrogen (-196ºC) for long-term storage.

2.5.8.4 Cell thawing

Stored vials containing cell lines were removed from liquid nitrogen and cells were rapidly thawed in a 37ºC water bath. The thawed cells were added to 10 ml warmed fresh growth complete medium in a 15 ml tube and then centrifuged for 10 min at 125 xg. The supernatant was discarded and the cell pellet re-suspended were then transferred to a 75 cm^2 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 by means of the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay. This assay has been used to quantify viable cells by observing the reduction of the tetrazolium salt to formazan crystals by the cell mitochondrial dehydrogenenase. Based on the absorbance of the cell samples after the test is carried out, cell viability can be measured in cells (Hansen et al. 1989). A stock solution of 5 mg/ml MTT was prepared by dissolving MTT powder in PBS and wrapped with aluminium foil to prevent exposure to direct light because it is photosensitive. This solution was then stored at 4ºC.

2.6.1.1 TM3 cell viability

TM3 cells were grown to 80% confluency and then trypsinated with 1-2 ml 0.25% trypsin/EDTA. 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, $5x10^3$ cells were used for 24 hours of exposure and $1x10^3$ was used for the 96 hours 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 *T. capensis* rhizome extract for 24 hours and 96 hours, respectively. Thereafter, 20 µl of MTT (5 mg/ml in PBS) were added to each well and plates were incubated 37ºC for an additional 4 hours. Then, the medium was removed from the wells and the formazan crystals formed by the reduction of MTT in living cells were solubilized in 100 µl of dimethylsulfoxide (DMSO). Subsequently, the optical density (OD) of the samples was measured with an ELISA reader (GloMax Multi Detection System). The absorbance was read at 560 nm. The results were expressed as percentage, based on the ratio of the absorbance of treated cells to that of the controls (100%). The inhibitory rate of cell proliferation was calculated according to Equation 3.

Equation 3: Percentage viability $= \dfrac{\text{ABSORBANCE sample X 100}}{\text{ABSORBANCE control}}$

2.6.1.2 LNCaP cell viability

LNCaP cells were grown to 80% confluence and then trypsinated with 2 ml 0.25% trypsin/EDTA. To inactivate, 2 ml of RPMI 1640 growth medium were added. Subsequently, the cells were seeded at 8×10^3 cells/well in 200 µl of complete culture medium for a 24-hours exposure and 3×10^3 cells/well in 200 µl of complete culture medium for a 96-hours exposure in a sterile 96-well plate. After exposing cells to various concentrations of the *T. capensis* rhizome extract for 24 and 96 hours, respectively. Thereafter, 20 µl of MTT (5 mg/ml in PBS) were added to each well. The plates were incubated 37ºC for an additional 4 hours. Then, the medium was removed from the wells and the formazan crystals formed by the reduction of MTT in living cells were solubilized in 100 µl of dimethylsulfoxide (DMSO). Subsequently, the optical density (OD) of the samples was measured with an ELISA reader (GloMax Multi Detection System). The absorbance was read at 560 nm.

2.6.1.3 PWR-1E cell viability

PWR-1E cells were grown to 80% confluence and were the trypsinated with 2 ml 0.25% trypsin/EDTA. To inactivate, 2 ml of RPMI 1640 growth medium were added. Subsequently, the cells were seeded at 8×10^3 cells/well in 200 µl of complete culture medium for a 24-hours exposure and 3×10^3 cells/well in 200 µl of complete culture medium for a 96-hours exposure in a sterile 96-well plate. After exposing cells to various concentrations of the *T. capensis* rhizome extract for 24 and 96 hours, respectively, 20 µl of MTT (5 mg/ml in PBS) were added to each well. The plates were incubated 37ºC for an additional 4 hours. Then, the medium was removed from the wells and the formazan crystals formed by the reduction of MTT in living cells were solubilized in 100 µl of dimethylsulfoxide (DMSO). Subsequently, the optical density (OD) of the samples was measured with an ELISA reader (GloMax Multi Detection System). The absorbance was read at 560 nm.

2.6.2 Determination of apoptosis

2.6.2.1 Determination of early apoptotic events by means of Annexin V-Cy3 binding

Annexin V binds to phosphatidyl-serine, which translocates from the inner to the outer leaflet of the plasma membrane as an early sign of apoptosis. Annexin is a family of calcium binding proteins that bind phospholipids in the presence of calcium (Trotter et al., 1995).

Cell surface phosphatidyl-serine was detected by Annexin V conjugated with the fluorophore Cy3 using the commercially available Annexin V-Cy3.18 conjugate (AnnCy3) apoptosis detection kit. This kit also comprises 6-carboxyfluorescein diacetate (6-CFDA) to detect live cells.

The binding of Annexin V-Cy3 to phosphatidyl-serine is observed as red fluorescence. On the other hand, 6-carboxyfluorescein is used for the detection of living cells. When 6-CFDA enters living cells, cellular esterases hydrolyze it producing a fluorescent compound, 6-carboxyfluorescein (6-CFDA), which is observed as green fluorescence. Therefore, when cells are incubated with both AnnCy3 and 6-CFDA three staining patterns are detected with this double-staining procedure: (1) live cells stain only with 6-CFDA (green), (2) necrotic cells stain only with AnnCy3 (red), and (3) cells in the early stages of apoptosis stain with 6-CFDA (green) and AnnCy3 (red).

Cells were seeded at (8×10^3 cells/well) in 1ml of complete medium for a 24-hour exposure and at (3×10^3 cells/well) for a 96-hour exposure in a sterile 6-well plate, respectively. After this, cells were exposed to the various concentrations of the *T. capensis* rhizome extract, for 24 and 96 hours. After discarding the culture medium, cells were washed with 1ml PBS and trypsinated with 500 µl of 0.25% Trypsin/EDTA. After trypsination, cell suspensions were transferred from 6-well plates to Eppendorf tubes and cell pellets were collected by centrifugation at 125 xg for 5 minutes. Subsequently, cells were re-suspended in 1ml PBS and 50 µl drops were placed on poly-L-lysine-coated slides and allowed to adhere to the slide by incubating for 10 minutes at room temperature, slides were then washed three times in binding buffer (10 mm HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.5) and stained with AnnCy3 (1µg/ml) and 6-CFDA (100 µM) in binding buffer for 10 minutes at room temperature. After this incubation, cells were again washed three times with binding

buffer and observed immediately with a fluorescent microscope. To determine the percentages of cells indicating apoptosis or necrosis, a total of 200 cells were counted and classified according the following scheme: live cells and red stained was counted as apoptotic or necrotic cells (Figure 12, 13 and 14).

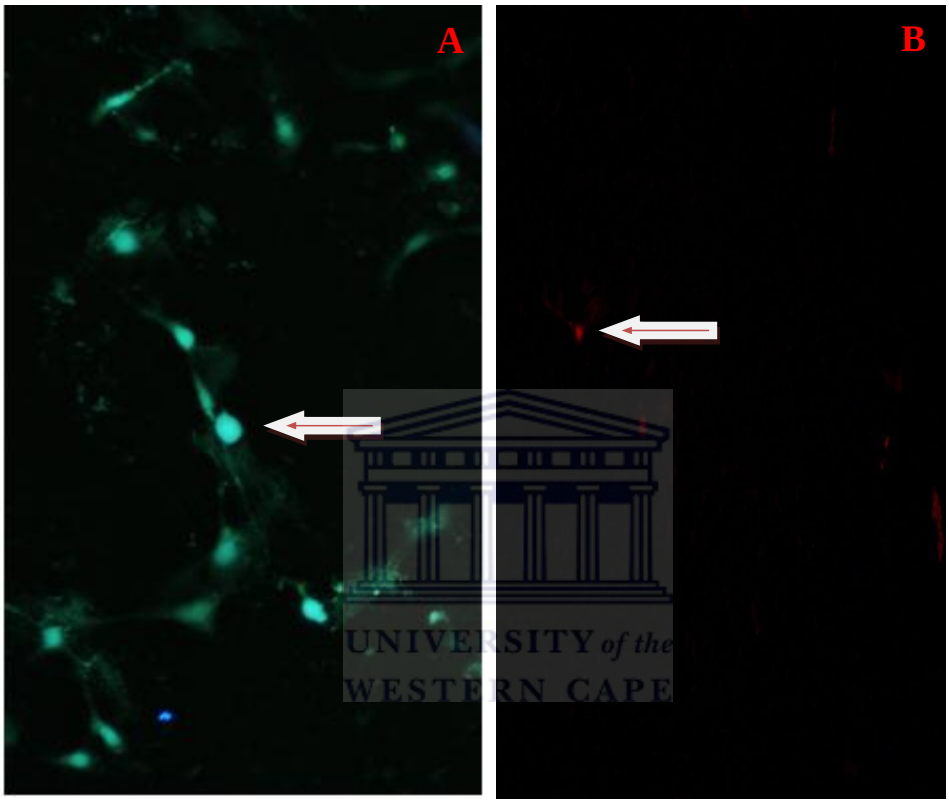


Figure 12: TM3 Cells: Determination of apoptosis by means of Annexin V-Cy3. A: live cells stain only with 6-CFDA (green) (white arrows head), B: necrotic cells stain only with Annexin V-Cy3 (red) (white arrows) after Annexin V Cy3 (20X magnification).

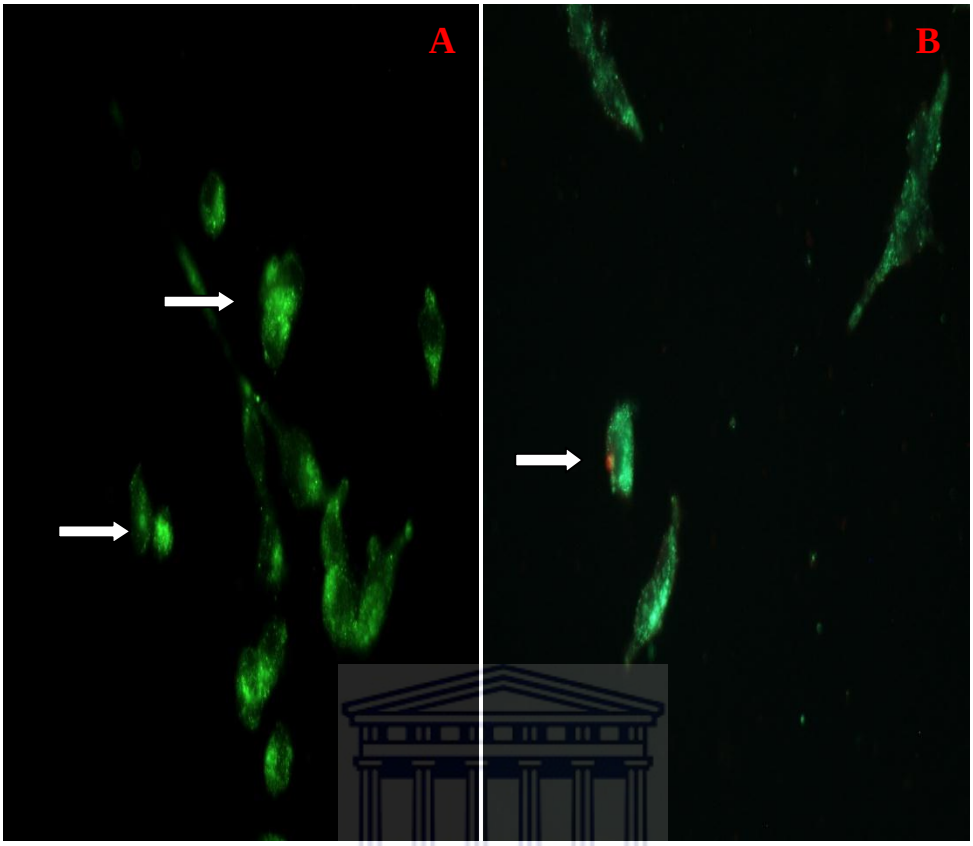


Figure 13: LNCaP Cells: Determination of apoptosis by means of Annexin V-Cy3. A: live cells stain only with 6-CFDA (green) (white arrows head), B: necrotic cells stain only with Annexin V-Cy3 (red) (white arrows) after Annexin V Cy3 (20X magnification).

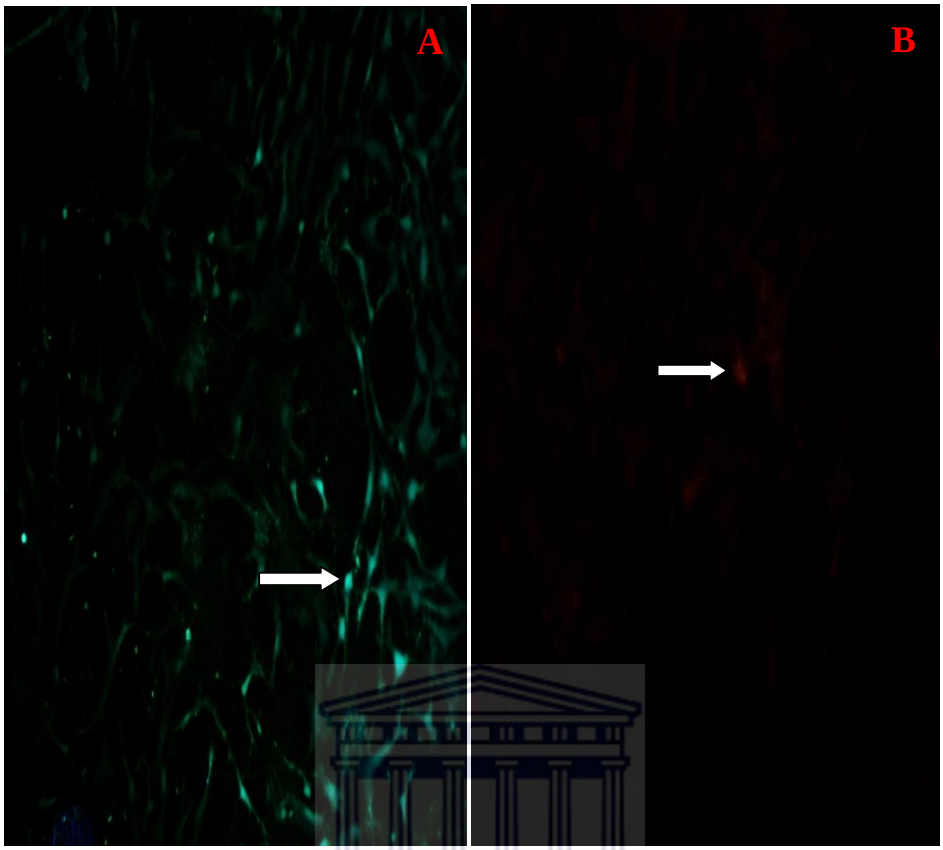


Figure 14: PWR-1E cells: Determination of apoptosis by means of Annexin V-Cy3. A: live cells stain only with 6-CFDA (green) (white arrows head), B: necrotic cells stain only with Annexin V-Cy3 (red) (white arrows) after Annexin V Cy3 (20X magnifications).

2.6.2.2 Determination of DNA fragmentation by means of the TUNEL assay

To determine DNA fragmentation, which indicates an endpoint of apoptosis, the Dead End Colorimetric TUNEL System kit was used. This is a non-radioactive assay which allows for accurate and rapid detection of DNA fragmentation.

For cells better sticking to the slide, poly-L-lysine was diluted 1:10 in distilled water and slides were coated by spreading 200 µl 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.

Cells were seeded at $6x10^5$cells/well in 1 ml of complete growth medium for a 24-hour exposure and at $2x10^5$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 *T. capensis* rhizome extract, the medium was discarded and cells were washed with 1 ml PBS and trypsinated with 500 µl of 0.25% trypsin/EDTA. 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 5 minutes. Subsequently, the supernatant was discarded, the cells were re-suspended in 1 ml PBS and 50 µl 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, 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. Followed by the removal of excess liquid by gently tapping the slides. Cells were covered with 100 µl 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 follows: Tissue paper was used to blot around the equilibrated areas, 100 µl 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 µl 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 µl of the diaminobenzidine (DAB) solution, prepared by combining 50 µl DAB Substrate 20X buffer, 950 µl distilled water, 50 µl DAB 20X chromogen and 50 µl 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 was counted in total on each slide, where brown stained cells were considered TUNEL-positive and unstained cells considered TUNEL-negative Finally, the percentage of TUNEL-positive cells

was calculated (Figure 15, 16 and 17). Recombinant (rTdT) reaction mix was prepared as shown in Table 1.

Table 1: Preperation of rTdT reaction mix

Buffer component	Component volume per standard 100 µl reaction
Equilibration buffer	98 µl
Biotinylated Nucleo tidemix	1 µl
rTdT enzyme	1 µl

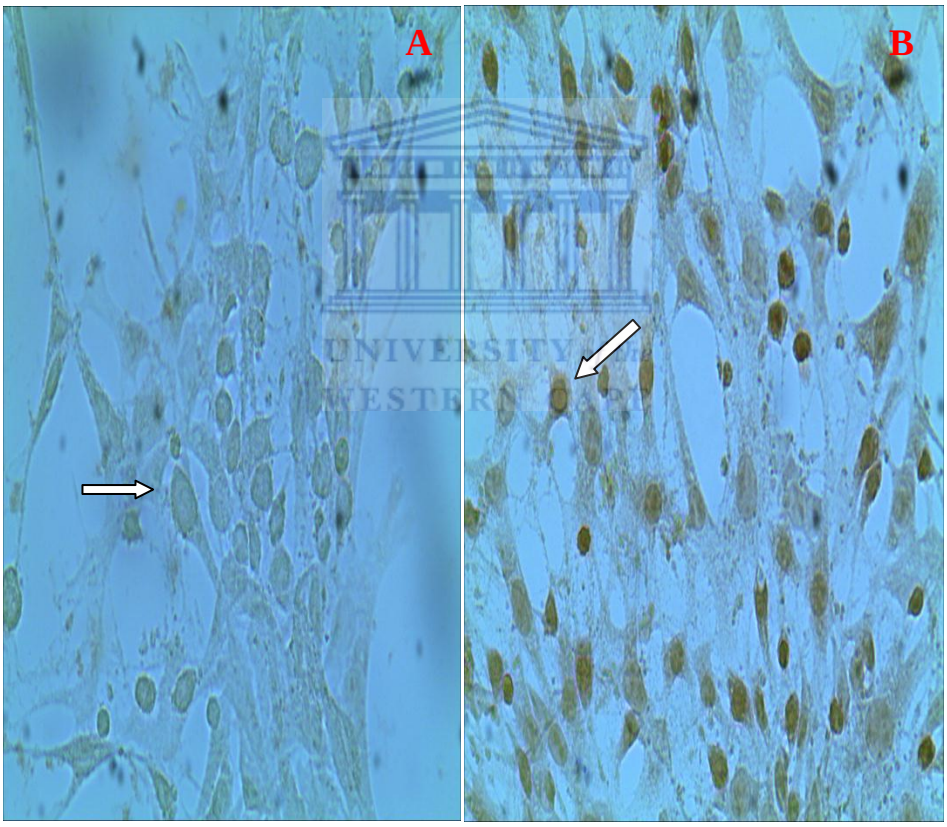


Figure 15: Determination of DNA fragmentation in TM3 cells. Cells considered TUNEL-negative are shown in A (White arrows), whereas cells considered TUNEL-positive are shown in B (White arrows).

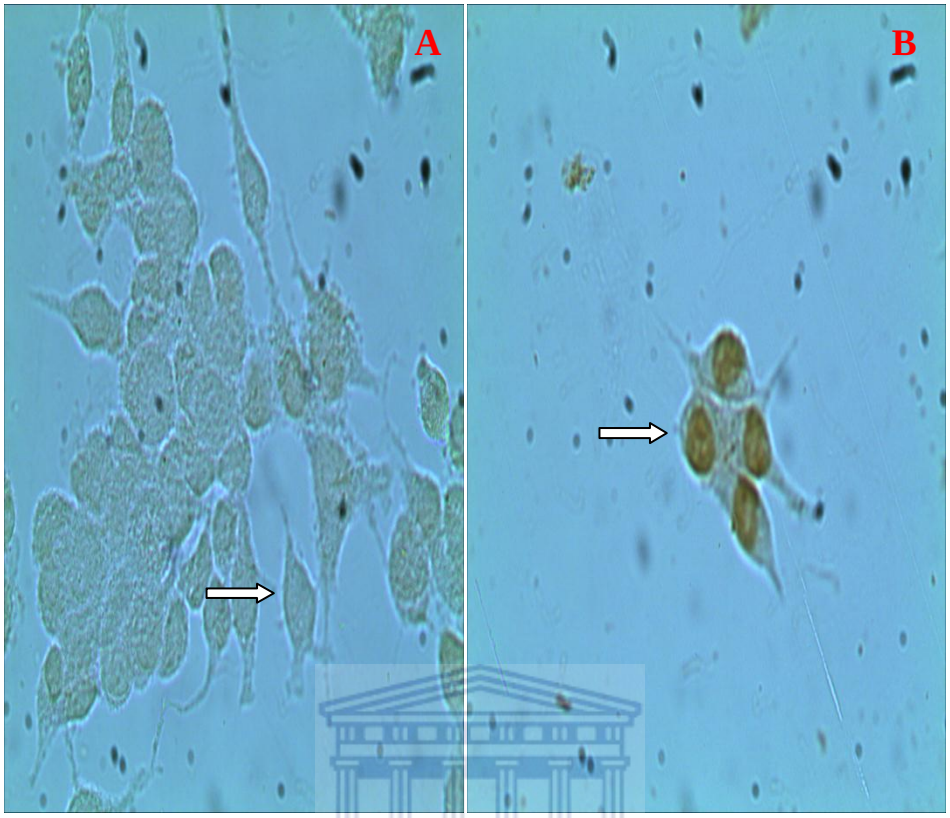


Figure 16: Determination of DNA fragmentation in LNCaP cells. Cells considered TUNEL-negative are shown in A (White arrows), whereas cells considered TUNEL-positive are shown in B (White arrows).

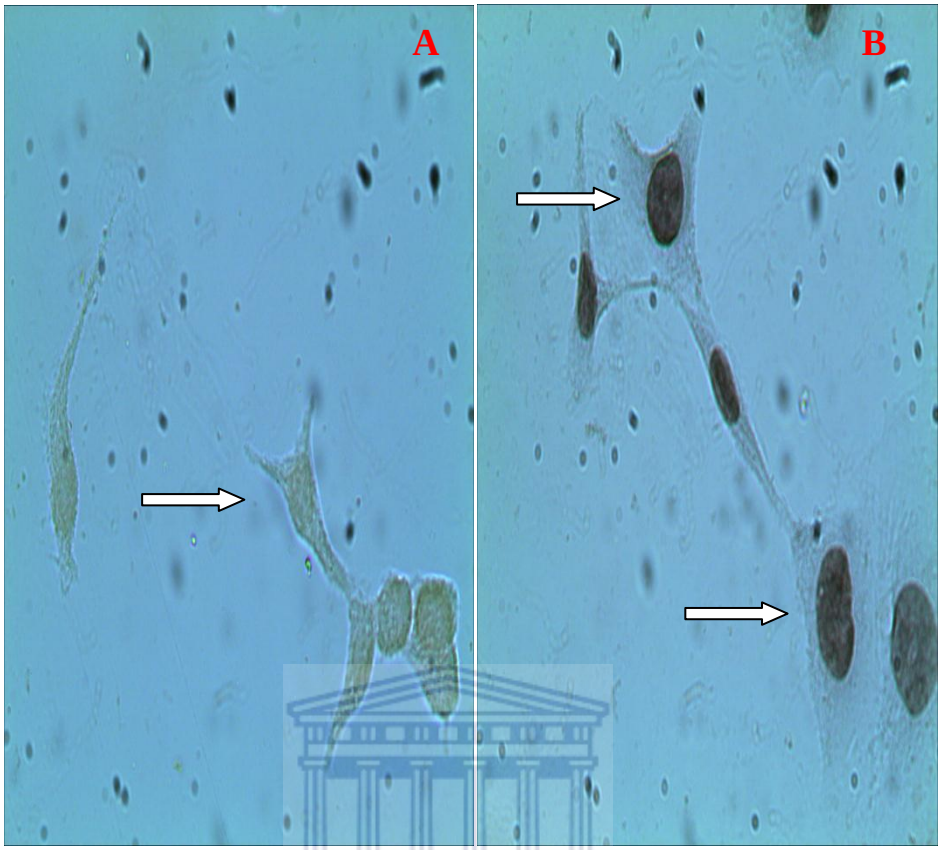


Figure 17: Determination of DNA fragmentation in PWR-1E cells. Cells considered TUNEL-negative are shown in A (White arrows), whereas cells considered TUNEL-positive are shown in B (White arrows).

2.6.3 Determination of protein concentration in TM3-Leydig cells

Protein determination was performed by using the BIO-RAD Protein assay, which is based on the modified method of Lowry et al. (1951). In this process, the dye binds unspecifically to amino groups of any protein within the sample. After the 48 and 96 hours of incubation with the different concentrations of the *T. capensis* rhizome extract and control without the extract, the protein contents were determined for cell proliferation. Medium of each well was removed and 200 µl lysis reagent consisting of 0.5 M NaOH + 0.1% SDS were added. Following the addition of the reagent, the plates were incubated at room temperature for 30 min on a plate shaker on a gentle 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.

2.6.3.1 BSA standard curve

A stock solution of 1400 µg/ml of BSA was prepared by dissolving it in 1 ml of lysis-reagent and further diluted into concentrations of 200, 600 and 1000 µg/ml (Table 2). The blank was lysis-reagent only. Thereafter, the absorbance was read at 690 nm, a standard curve was calculated and the results used to more accurately determine the effects of the *T. capensis* rhizome extract on TM3 Leydig cells (Figure 18). The BSA standard curve was then calculated according to the Equation 4.

Table 2:BSA concentration dilutions for Standard curve

BSA Stock solution (µl)	**Lysis reagent (µl)**	**BSA Concentration (µg/ml)**
100	40	1000
100	133	600
100	600	200
0	600	0 (**blank**)

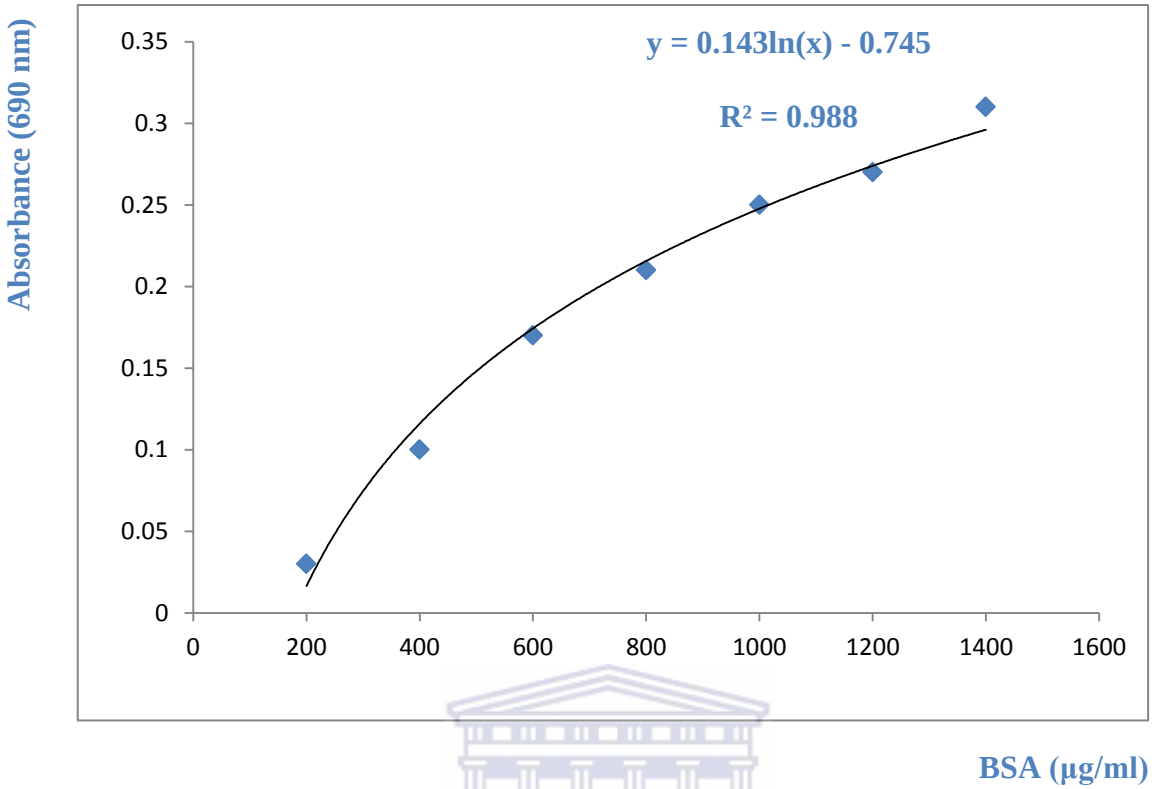


Figure 18: Standard curve for BSA determination

Equation 4: y = (0.143) + (- 0.745) LOG(x)

2.6.4 Determination of testosterone production in TM3-Leydig cells

TM3-Leydig cells were stimulated with human chorionic gonadotropin (hCG) to trigger the production of testosterone. A stock solution was made of hCG with concentration of (50 mIU per 10 µl) and 5 µl/ml were added to each concentration of the *T. capensis* rhizome extract, then TM3-Leydig cells were exposed to the *Typha capesis* extract, for 48 and 96 hours, respectively. Testosterone production was measured by performing the DRG Testosterone ELISA kit assay. All necessary solutions and antibody-coated micro-plate were provided in the kit.

The cells were exposed to different concentrations of the *T. capensis* rhizome extract. Briefly 25 µl of the thawed supernatants and standards were dispensed into selected wells in duplicate. Thereafter, 200 µl enzyme conjugate was added to each well concurrently so as to ensure consistent conditions.

Following that, the microtitre plate was briefly shaken for 10 seconds before an incubation period for 60 minutes at room temperature. After incubation, the solution was decanted and each well was washed 3 times with 400 µL of diluted wash solution. The plate was then gently struck on paper to remove any excess solution. Thereafter, 200 µl of substrate solution was dispensed into the wells and incubated for 15 minutes at room temperature. After that final incubation 100 µl of stop solution was added to terminate the enzymatic activity and resulted in the development of a yellow colour. The plate was then read at 450±10 nm with a Multiskan Ex ELISA reader (Thermo Scientific) within 10 minutes of the addition of the stop solution. As this assay was based on competitive binding, the intensity of the colour was inversely proportional to concentration of testosterone. The concentration values of the samples were determined from the standard curve (which had a range between 0-16 ng/ml) and as expressed in ng/ml (Figure 19). The standard curve for testosterone production in TM3 Leydig cells was then calculated according to the Equation 5.

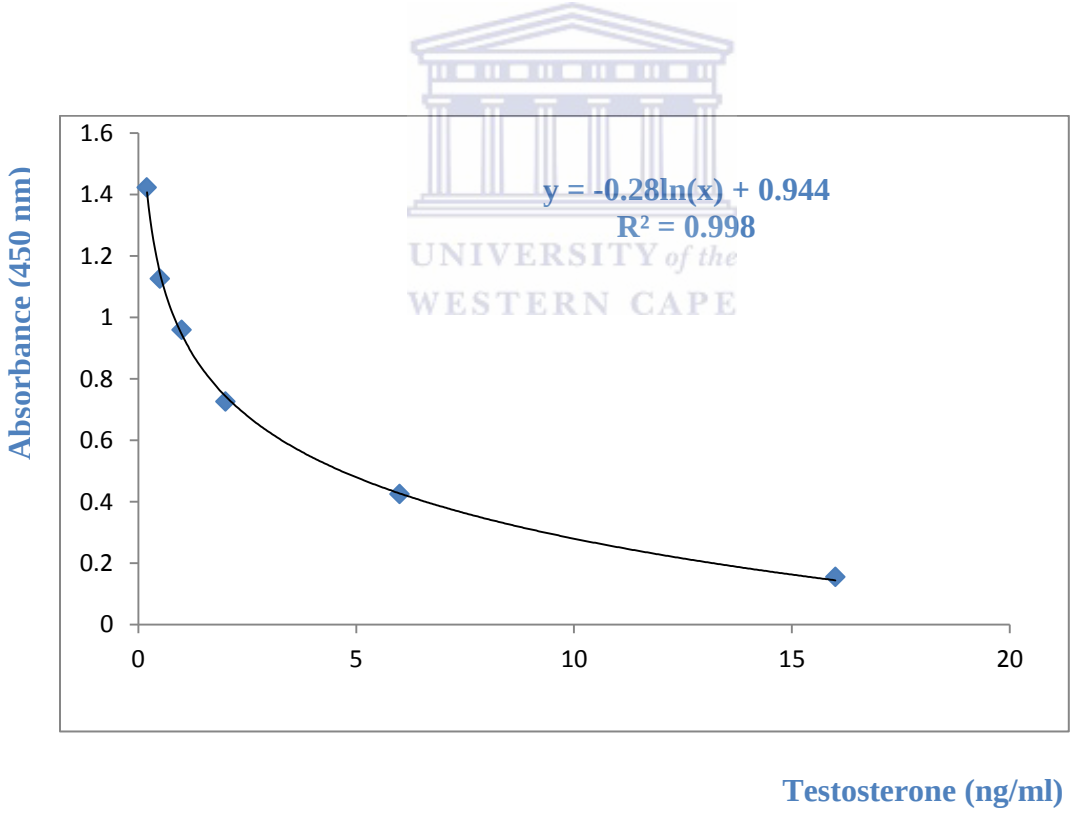


Figure 19: Standard curve for testosterone production in TM3 Leydig cells.

Equation 5: y = (0.944) + (-0.28) LOG(x)

2.7 Statistical Analysis

All statistical calculations were performed using the MedCalc statistical software (Version 12.3; Mariakerke, Belgium). After testing for normal distribution by means of the Kolmogorov-Smirnov test, appropriate statistical tests, either parametric (Pearson correlation, repeated measures one-way ANOVA, paired samples (t-test) or non-parametric (Spearman Rank correlation, Wilcoxon test) were performed. Data were expressed as mean±SEM. A P-value of $P<0.05$ was considered significant.

Chapter3: Results

3.1 Effect of the *Typha capensis* crude rhizome extract on cell viability

3.1.1 Effect of *T. capensis* crude rhizome extract on TM3-Leydig cell viability

TM3-Leydig cell viability was determined after being exposed to different concentrations of *T. capensis* crude rhizome extract (0.01, 0.02, 0.1, 1, 10 and 100 µg/ml). In addition, the cell morphology was observed and recorded. It was found that no observable morphological changes in the flat and polygonal cells had occurred between the control groups and concentrations less than 10 µg/ml, at the higher concentrations 10, 100 µg/ml the cells began showing visible signs of stress (Figure 20).

After TM3-Leydig cells were incubated with different concentrations of *T. capensis* crude rhizome extract (0.01, 0.02, 0.1, 1, 10 and 100 µg/ml) for 24 hours exposure, at concentrations less than 100 µg/ml, the crude rhizome extract showed no significant effect on cell viability compared to the control. At the higher concentrations (>100 µg/ml) it showed an increase in viability of +7.2% compared to the control, indicating cellular stress. One-way ANOVA exhibited no significant trend between the control and 100 µg/ml (ANOVA trend analysis: P=0.127) (Figure 21).

After 96 hours of exposure at concentrations less than 100 µg/ml, the crude rhizome extract showed no significant influence on cell viability compared to the control. However, higher concentration (>100 µg/ml) caused an increase in viability of +10.3% compared to the control, indicating cellular stress. One-way ANOVA exhibited no significant trend between the control and 100 µg/ml (ANOVA trend analysis: P=0.073) (Figure 22).

The results reveal there is no significant difference between 24 and 96 hour incubation period on TM3 cell viability.

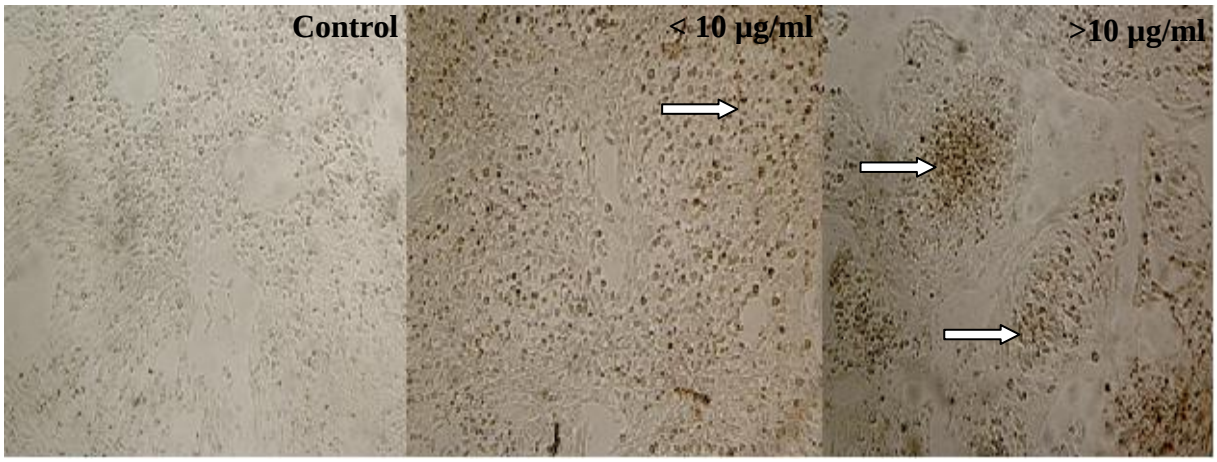


Figure 20: TM3-Leydig cell morphology after exposed to different concentrations of *T. capensis* crude rhizome extract (0.01, 0.02, 0.1, 1, 10 and 100 µg/ml). It was found that no observable morphological changes in the flat and polygonal cells had occurred between the control groups and concentrations less than10 µg/ml, at higher concentrations 10, 100 µg/ml the cells began showing visible signs of stress. The white arrows indicating cellular stress (10 x magnifications).

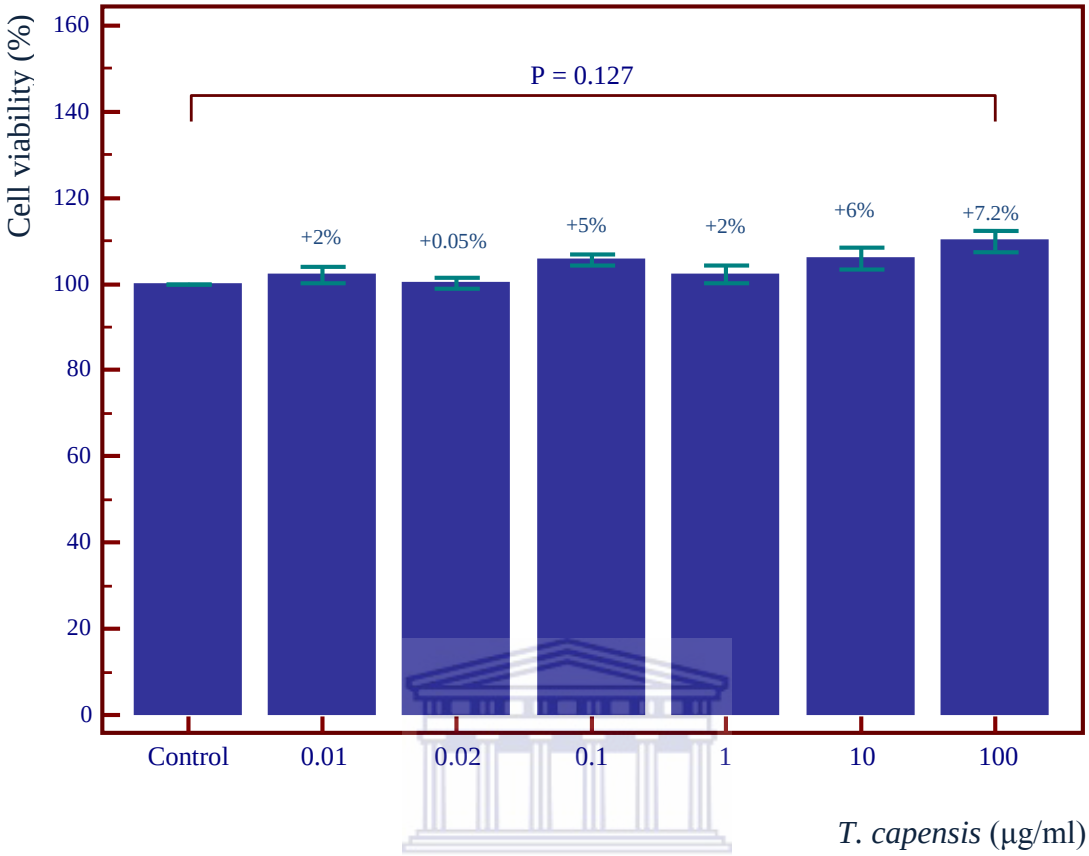


Figure 21: TM3-Leydig cell viability (determined by means of MTT assay) incubated with different concentrations of *T. capensis* crude rhizome extract (0.01, 0.02, 0.1, 1, 10 and 100 µg/ml) for 24 hours. At concentrations less than 100 µg/ml, the crude rhizome extract showed no significant difference in cell viability compared to the control. At higher concentration the crude rhizome extract showed an increase in viability of +7.2% compared to the control, indicating cellular stress. One-way ANOVA exhibited no significant trend between the control and 100 µg/ml (ANOVA trend analysis: P=0.127).

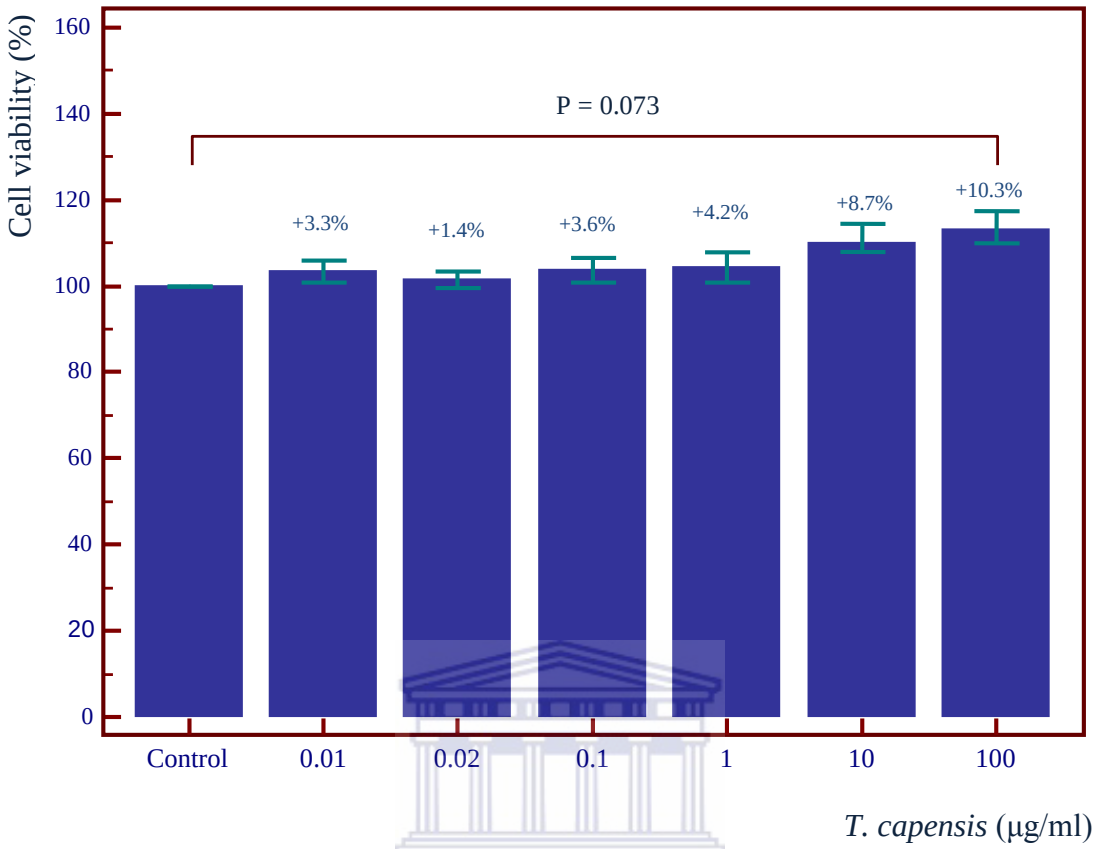


Figure 22: TM3-Leydig cell viability (determined by means of MTT assay) incubated with different concentrations of *T. capensis* crude rhizome extract (0.01, 0.02, 0.1, 1, 10 and 100 µg/ml) for 96 hours. At concentrations less than 100µg/ml, the crude rhizome extract resulted in no significant difference in cell viability compared to the control. At higher concentrations showed an increase in viability of +10.3% compared to the control, indicating cellular stress. One-way ANOVA exhibited no significant trend between the control and 100 µg/ml (ANOVA trend analysis: P=0.073).

3.1.2 Effect of *T. capensis* crude rhizome extract on LNCaP cell viability

The viability of LNCaP cells as well as cell morphology after being exposed to different concentrations of *T. capensis* crude rhizome extract (0.01, 0.02, 0.1, 1, 10 and 100 µg/ml) was observed and recorded. The morphology of LNCaP cells was found to have changed after exposure, there was a clear increase in detachment and clumping of cells and apoptotic bodies could also be seen indicate to cell death (Figure 23).

The viability of LNCaP cells was tested after exposure to different concentrations of *T. capensis* crude rhizome extract (0.01, 0.02, 0.1, 1, 10 and 100 µg/ml) by means of the MTT test. After 24 hours of exposure values at all concentrations decreased. As from 0.01 µg/ml values declined (-5%) and reached a lowest value (-64%) at 100 µg/ml when compared to the control. One-way ANOVA exhibited a significant trend (ANOVA trend analysis: $P<0.0001$) (Figure 24).

The viability of LNCaP cells was tested after exposure to different concentrations of *T. capensis* crude rhizome extract (0.01, 0.02, 0.1, 1, 10 and 100 µg/ml) by means of the MTT test. After 96 hours of exposure viability for all concentrations decreased. As from 0.01 µg/ml, values declined (-7%) and reached a lowest value (-82%) at 100 µg/ml ($P < 0.0001$) when compared to the control. One-way ANOVA exhibited a significant trend (ANOVA trend analysis: $P<0.0001$) (Figure 25).

Results reveal that the viability of LNCaP cells at the highest concentrations (10, 100 µg/ml) showed declines in cell viability at the incubation period 96 hours (-72 and -82.4%) more than 24 hours (-57.8 and -64.7%) indicate to more cell death.

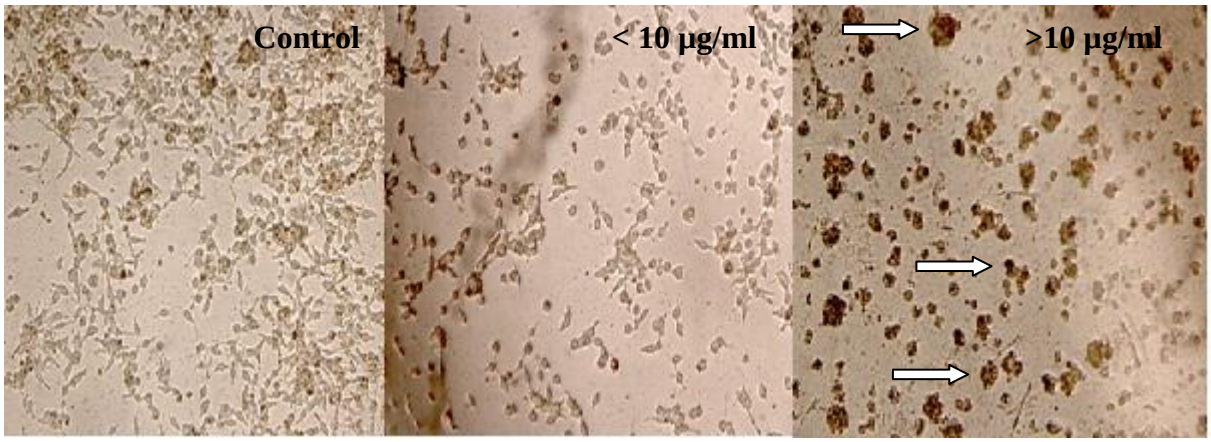


Figure 23: LNCaP cell morphology after exposed to different concentrations of *T. capensis* crude rhizome extract (0.01, 0.02, 0.1, 1, 10 and 100 µg/ml). The morphology of LNCaP cells was found to have changed after exposure. There was a clear increase in detachment and cell death clumping of cells and apoptotic bodies could also be seen indicating to cell death. The white arrows indicate to apoptotic bodies (10 x magnifications).

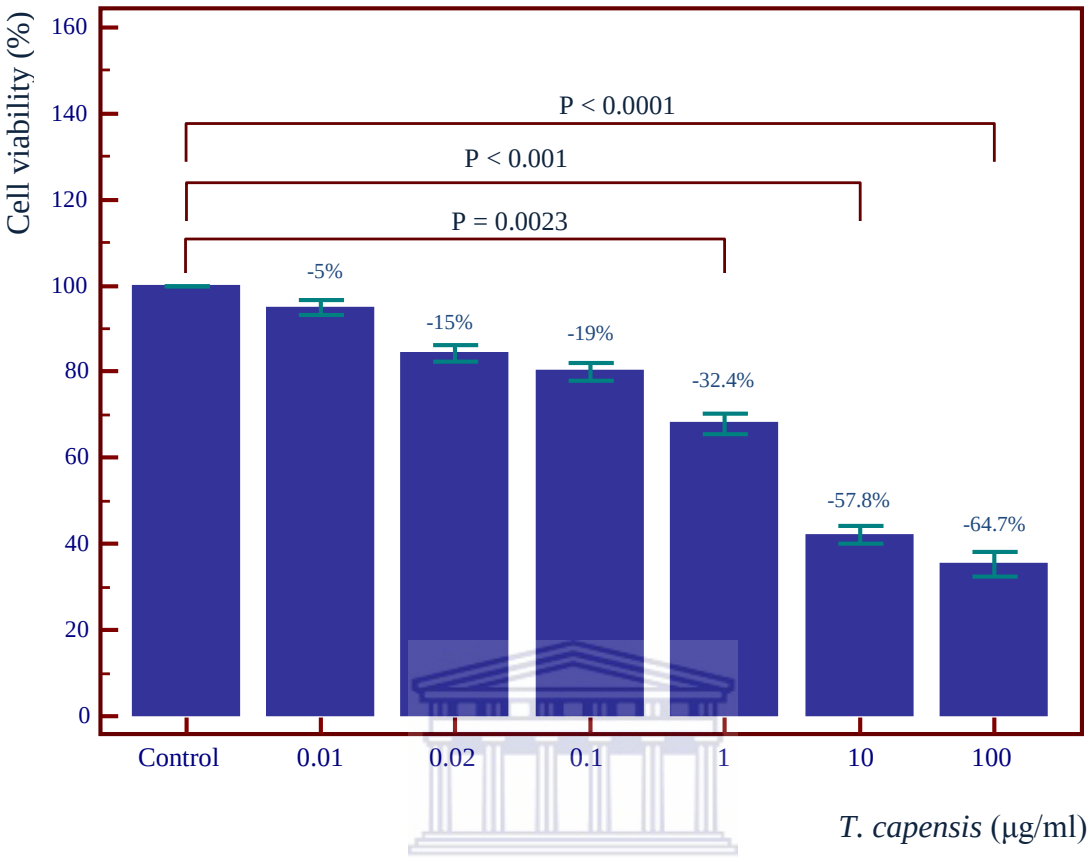


Figure 24: LNCaP cells viability (determined by means of MTT assay). Incubation with different concentrations of *T. capensis* crude rhizome extract (0.01, 0.02, 0.1, 1, 10 and 100 µg/ml) for 24 hours, viability for all concentrations decreased as from 0.01 µg/ml values declined (-5%) reached a lower value (-57%) at 10 µg/ml (P<0.001) when compared to the control. However, at the higher concentrations (100 µg/ml) a significant decrease of -64% (P<0.0001) was observed. One-way ANOVA exhibited a significant trend (ANOVA trend analysis: P<0.0001).

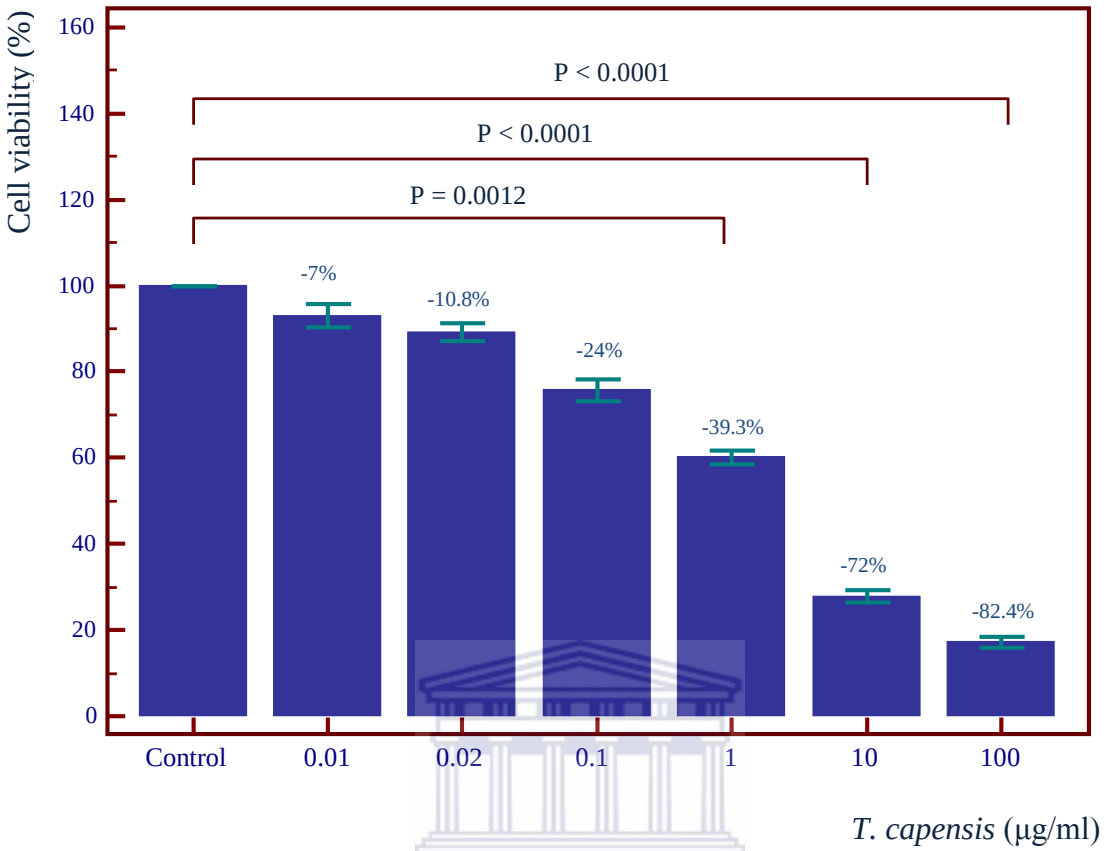


Figure 25:: LNCaP cells viability (determined by means of MTT assay). Incubation with different concentrations of *T. capensis* crude rhizome extract (0.01, 0.02, 0.1, 1, 10 and 100 µg/ml), for 96 hours, viability for all concentrations decreased as from 0.01 µg/ml values declined (-7%) reached a lower value (-72%) at 10 µg/ml ($P < 0.0001$) when compared to the control. However, at higher concentration (100 µg/ml) a significant decrease of -82.4% ($P<0.0001$) was observed. One-way ANOVA exhibited a significant trend (ANOVA trend analysis: $P<0.0001$).

3.2 HPLC analysis of *Typha capensis* crude rhizome extract

3.2.1 HPLC profiling of crude extracts of *Typha capensis*

HPLC data showed that the most effective fraction was the F1 fraction from the summer harvest. The crude extract profiles were obtained by preparing the extracts for different seasons (Autumn, Winter, Spring, Summer) at 100 mg/ml in dH_2O, centrifuging at 8000 xg for 5 minutes, and injecting 30 µl of the supernatant onto a C^{18} analytical column Phenomenex (Luna 5u c18 100A) C18 dimension (250 x 4.6 mm). A gradient of 20 to 90% MeOH with water was run over 40 minutes, at a flow rate of 1 ml/min. The results of the HPLC analysis are shown in the chromatograms as reproduced. The chromatographic profiles show no differences, except for qualitative differences noted with fraction 1 of the summer collection. Fractionation yielded five well separated fractions, which were subsequently collected for four seasons (Figure 26).

3.2.2 HPLC profiling of sub-Fractionation (fraction 1 from Summer Season)

Fraction one (F1) of the summer harvest was subsequently further fractioned several times (Figure 27, 28). The sample was prepared at 20 mg/ml in dH_2O, centrifuged at 8000 xg for 5 minutes, and 30 µl of the supernatant were injected at a time. Phenolic compounds were separated as follows: 20 min gradient of a 5-40% gradient of acetonitrile in water containing 1.0% formic acid, the flow rate was set at 1.5 ml/min. Fractionation yielded two well separated peaks, which were subsequently collected (Figure 29).

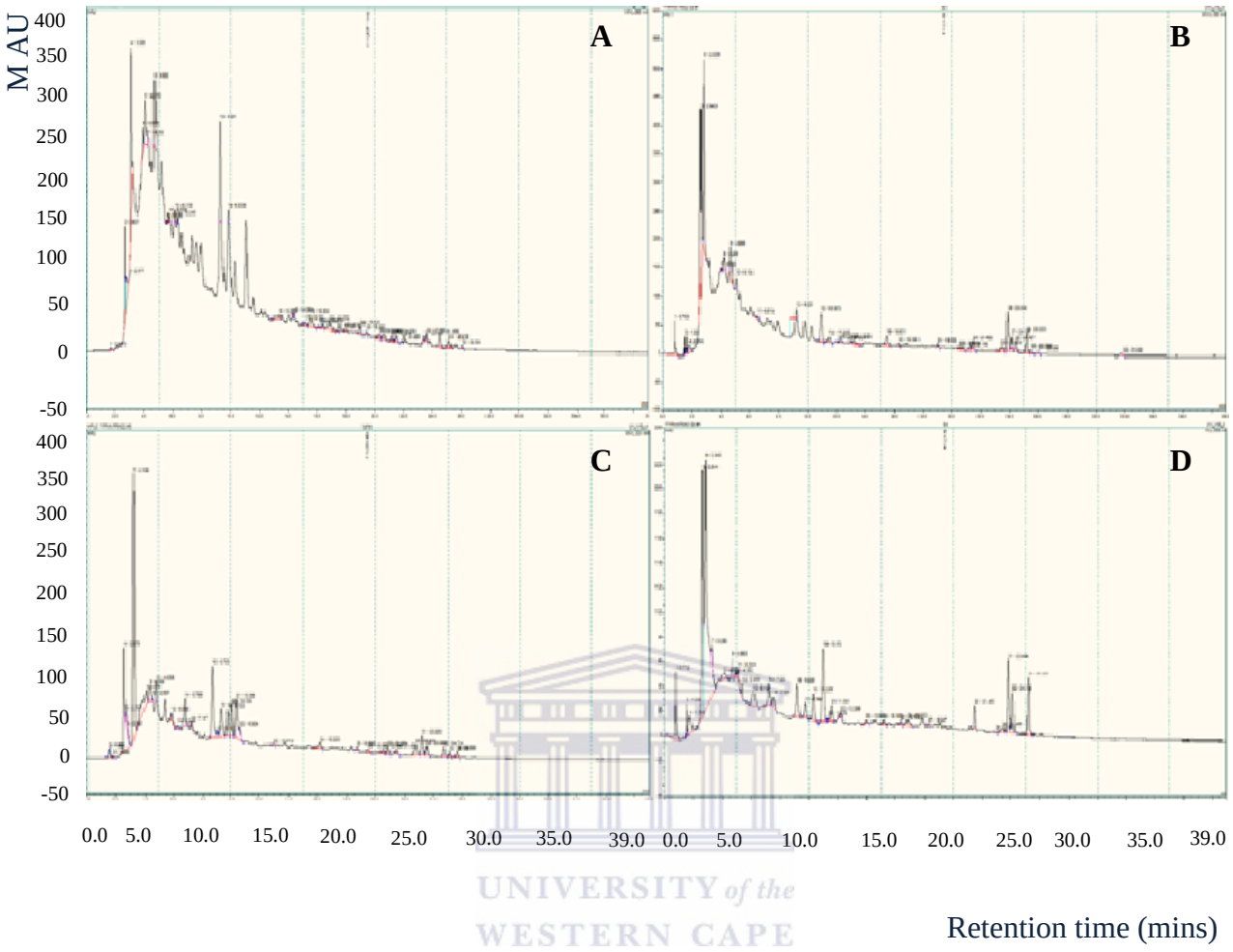


Figure 26: HPLC profiles of *T. capensis* rhizome extract harvested during the different seasons. The chromatograms were recorded at UV 365 nm. Operator: PC Timebase: PC-HP_1 Sequence: HPLC Typha fractionation, Control Program: C18 Luna for Typha, Injection Volume: 30.0µl, Flow rate 1.5 ml/min, Run Time (min): 39.00, Version 6.80 SR11 Build 3161.

A: Summer Season. B: Autumn Season. C: Winter Season. D: Spring Season.

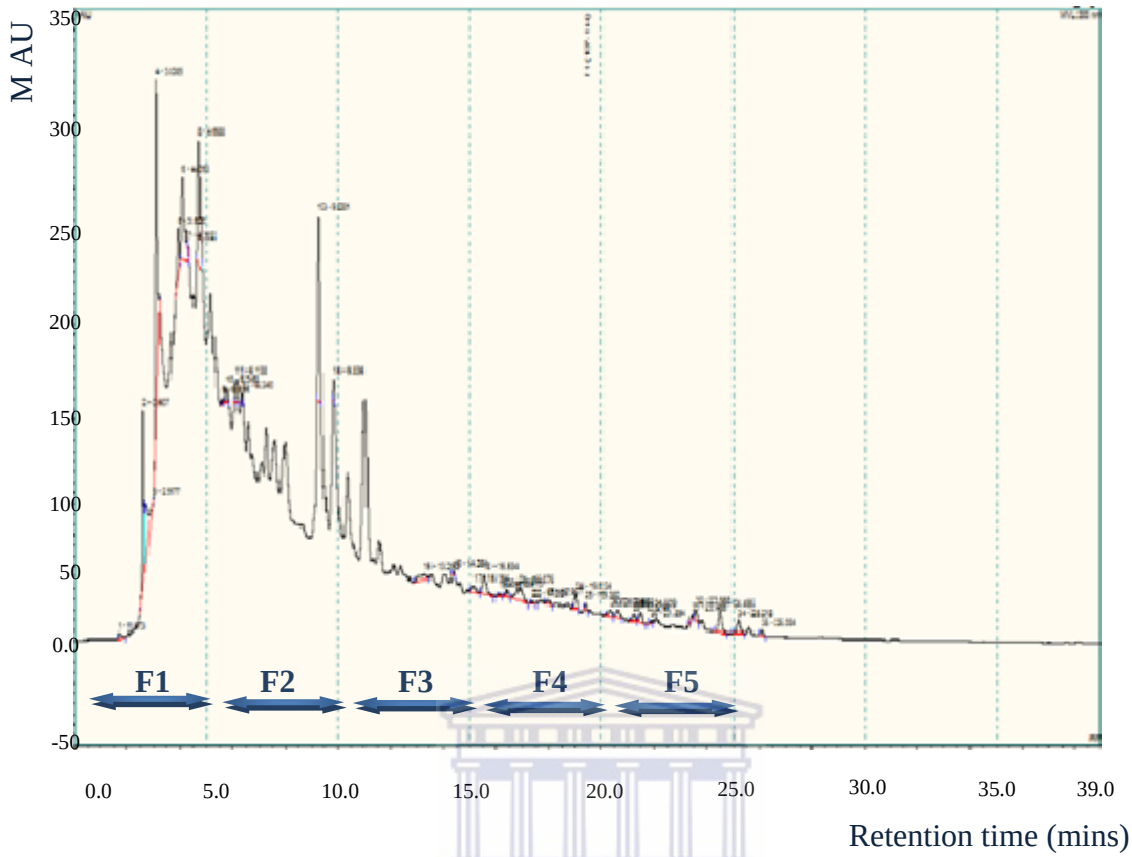


Figure 27: HPLC profile of *T. capensis* rhizome extract from the summer harvest. The chromatograms were recorded at UV 365 nm. Operator: PC Timebase: PC-HP_1 Sequence: HPLC Typha frac, Control Program: C^{18} Luna for Typha, Injection Volume: 30.0 µl, Flow rate 1.5 ml/min, Run Time (min): 39.00, Version 6.80 SR11 Build 3161. The arrows indicating to the area of F1, F2, F3, F4, F5 fraction.

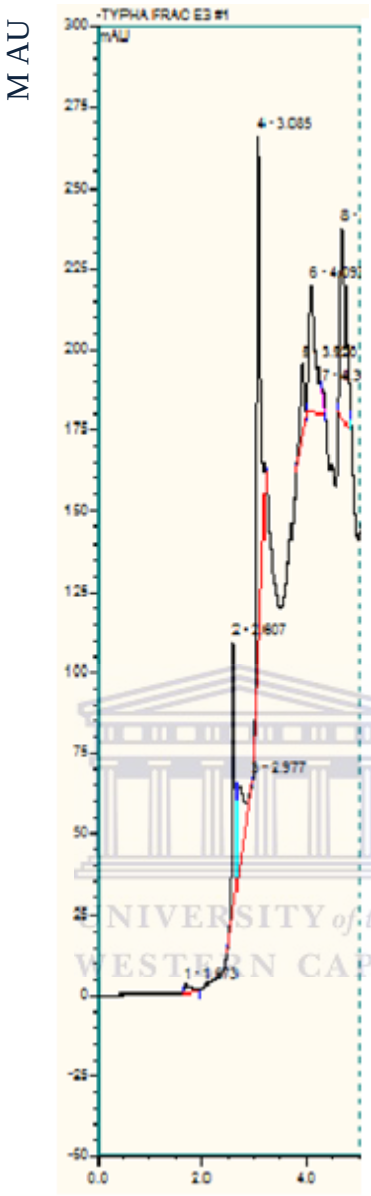


Figure 28: HPLC profile of F1 fraction from the summer harvest of *T. capensis* rhizome extract. The fraction yielded several well separated peaks, which were subsequently collected.

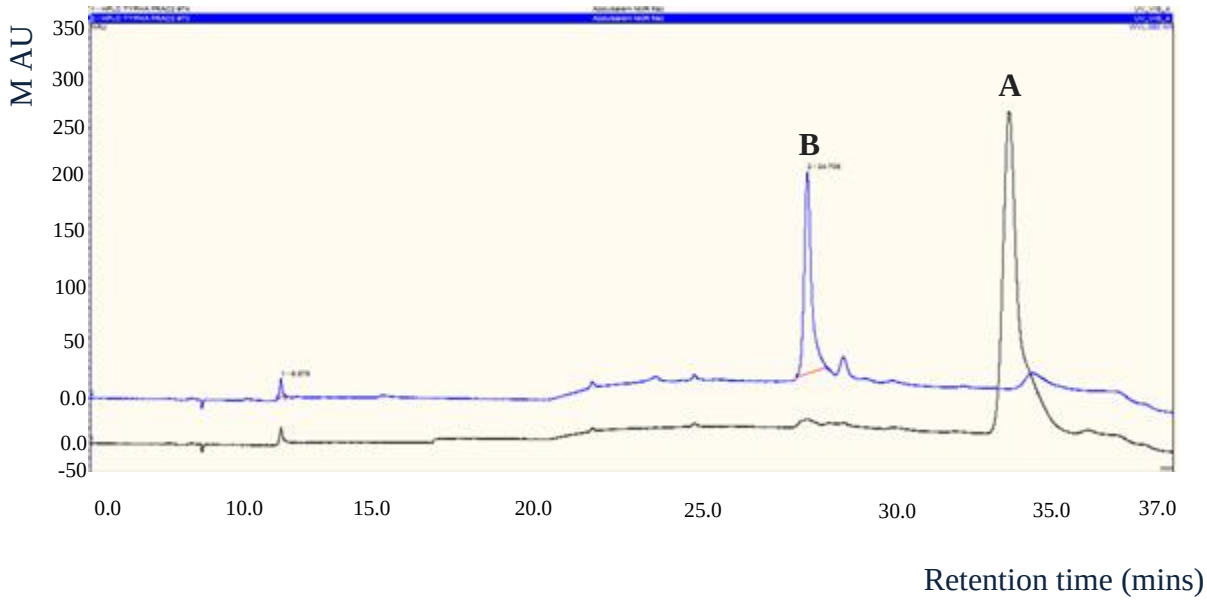


Figure 29: HPLC profile of sub-fraction of F1 fraction from the summer harvest of *T. capensis* rhizome extract. Two active compounds (A & B) were separated. The chromatograms were recorded at UV 350 nm. Operator: PC Timebase: PC-HP_1 Sequence: HPLC Typha frac, Control Program: C18 Luna for Typha, Injection Volume: 30.0µl, Flow rate 1.5 ml/min, Run Time (min): 37.00, Version 6.80 SR11 Build 3161.

3.2.3 Compound identification by means of NMR spectrometric analysis

NMR spectrometric analysis was performed at the Chemistry Department, UWC, on a 400 MHz Bruker Avance III NMR spectrometer with an HD Nanobay of 400 MHz. ^{13}C spectra were thus recorded at 100 MHz. Correlation spectroscopy (COSY), the first and most popular two-dimension NMR experiment is the homonuclear correlation spectroscopy (COSY) sequence, which is used to identify spins which are coupled to each other. Heteronuclear multiple-bond correlation spectroscopy (HMBC), HMBC detects heteronuclear correlations over longer ranges of about 2–4 bonds. Only two of the compounds, structures could be resolved based on *in vitro* experiments.

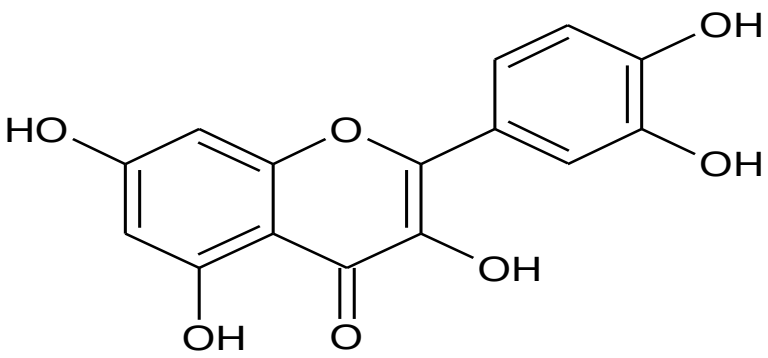
3.2.3.1 Compound A (compound 1): Quercetin (yellow powder)

Table 3 and figures 30 and 31 show the NMR data of compound A, which was identified as Quercetin.

Table 3: ^{1}H (400 MHz) and ^{13}C (125 MHz) NMR spectrometric data for Compound A (Quercetin)

	^{13}C, (DEPT135)	**^{1}H (multiplicity, integration, *J*=Hz)**	**COSY**	**HMBC**
1	175.8 (s)			
2	163.8 (s)			
3	160.7 (s)			
4	156.1 (s)			
5	147.6 (s)			
6	146.7 (s)			
7	145.0 (s)			
8	135.7 (s)			
9	121.9 (s)			
10	120.0 (d)	7.537 (dd, 1H, *J*=8.44 Hz, *J*=2.18 Hz)	H-11	C-5, C-7 (lr), C-12
11	115.6 (d)	6.883 (d, 1H, *J*=8.51 Hz)	H-10	C-5, C-7, C-9, C-12 (lr)
12	115.0 (d)	7.674 (d, 1H, *J*=2.18 Hz)	-	C-5, C-7, C-9(lr), C-10
13	102.9 (s)			
14	98.1 (d)	6.185 (d, 1H, *J*=1.95 Hz)	H-15 (lr)	C-2, C-3, C-13, C-15
15	93.3 (d)	6.405 (d, 1H, *J*=1.95 Hz)	H-14 (lr)	C-1, C-2, C-4, C-13, C-15
	OH-1	12.486 (s, 1H)	-	C-3, C-13, C-14
	OH-2	10.777 (br s, 1H)	-	
	OH-3	9.583 (br s, 1H)	-	
	OH-4	9.354 (br s, 2H)*	-	
	OH-5	9.309 (br s, 2H)*	-	

*overlapped protons. lr = long range



Molecular Formula = $C_{15}H_{10}O_7$

Formula Weight = 302.2357

Figure 30: Chemical structures, molecular formula and formula weight of the Quercetin.

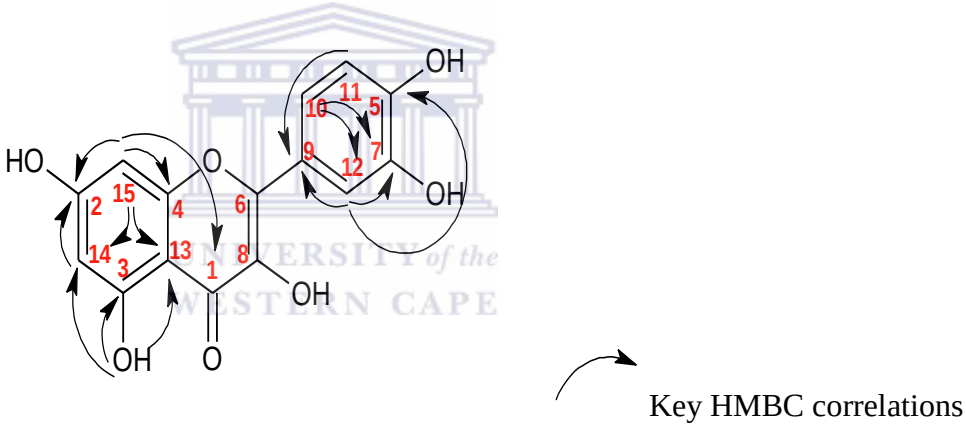


Figure 31: Key HMBC (2D) correlation which led to confirmation of the structure for Quercetin.

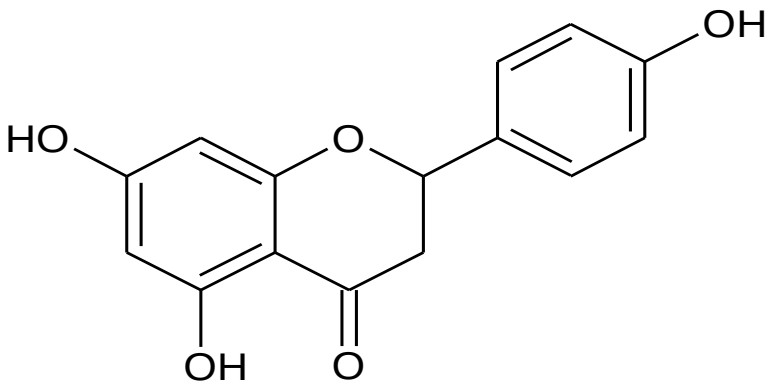
3.2.3.2 Compound B (compound 2): Naringenin (white powder)

Table 4 as well as figures 32 and 33 depict the NMR data of compound B, which was identified as Naringenin.

Table 4: ^{1}H (400 MHz) and ^{13}C (125 MHz) NMR spectrometry data for Compound B (Naringenin)

	^{13}C, (DEPT135)	^{1}H (multiplicity, integration, *J*=Hz)	COSY	HMBC
1	196.4 (s)			
2	166.6 (s)			
3	163.5 (s)			
4	162.9 (s)			
5	157.7 (s)			
6	128.8 (s)			
7	128.3 (d)	7.314 (d, 2H, *J*=8.62 Hz)	H-8	C-5, C-7, C-8, C-12
8	115.1 (d)	6.792 (d, 2H, *J*=8.62 Hz)	H-7	C-5, C-7, C-8, C-12
9	101.8 (s)			
10	95.8 (d)[#]	5.882 (s, 2H)*	-	C-1, C-2, C-3, C-9, C-11
11	94.9 (d)[#]	5.882 (s, 2H)*	-	C-1, C-2, C-3, C-9, C-11
12	78.4 (d)	5.435 (dd, 1H, *J*=12.98 Hz; *J*=2.88 Hz)	H-13a, H-13b (lr)	C-1, C-4 (lr), C-7, C-13
13	42.0 (t)	**a**) 3.263 (dd, 1H, *J*=17.27 Hz; *J*=12.74 Hz)	H-12, H-13b	C-1, C-7, C-12
		b) 2.678 (dd, 1H, *J*=17.27 Hz; *J*=2.83 Hz)	H-12 (lr), H-13a	C-1, C-7(lr), C-9
	OH-1	12.148 (s, 1H)	-	C-1, C-2, C-3, C-9, C-10
	OH-2	10.779 (br s, 1H)	-	-
	OH-3	9.584 (br s, 1H)	-	-

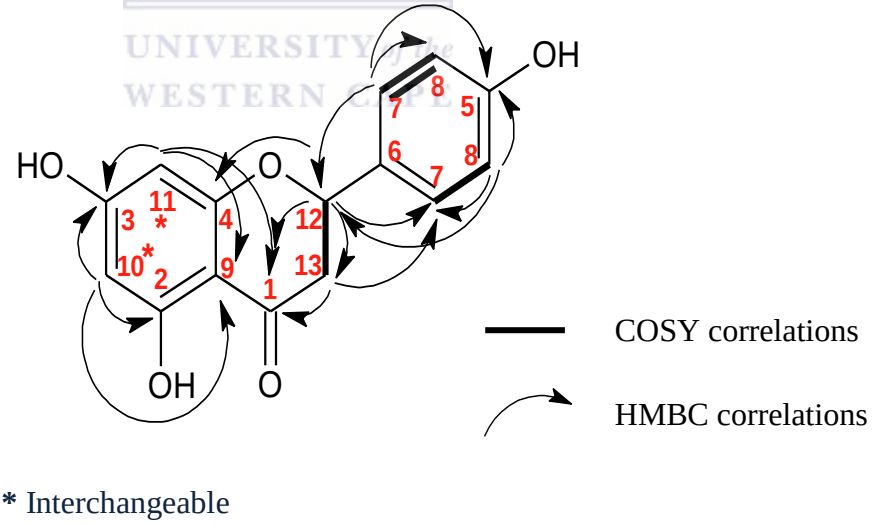
*overlapped protons.
[#]assignments are interchangeable.
lr = long range



Molecular Formula = $C_{15}H_{12}O_5$

Formula Weight = 272.25278

Figure 32: Chemical structures, molecular formula and formula weight of the Naringenin.



* Interchangeable

Figure 33: Key HMBC (2D) correlation which led to confirmation of the structure for Naringenin.

3.3 Effect of *Typha capensis* rhizome extract fractions after HPLC fractionation

3.3.1 Effect of *T. capensis* rhizome extract fractions on cell viability

After exposure of different cell lines (TM3-Leydig cells, LNCaP cells and RWP-1E cells) to different fractions of the *Typha capensis* rhizome extract derived from different seasons. At different concentrations (0.01, 0.02, 0.1, 1, 10 and 100 µg/ml) for 24 and 96 hours, data show that the most effective fraction was the F1 fraction.

3.3.1.1 Effect of *T. capensis* rhizome extract F1 fraction from four seasons on TM3-Leydig cell viability

TM3 cell viability was determined by means of the MTT assay. After 24 hours of exposure to different concentrations of F1 fraction of the *T. capensis* rhizome extract (0.01, 0.02, 0.1, 1, 10 and 100 µg/ml) harvested in different seasons, no significant difference in cell viability compared to the control was observed. At higher concentrations, viability increased compared to the control, indicating cellular stress. Results showed no significant between the concentration of 100 µg/ml in F1 fraction of the summer season to different seasons (P=0.096, P=0.084 and P=0.076, respectively) (Figure 34).

After 96 hours of exposure, the F1 fraction showed no significant difference in cell viability compared to the control. At higher concentrations, viability increased compared to the control, indicating cellular stress. Results revealed a significant between the concentration of 100 µg/ml in F1 fraction of the summer season harvest to different seasons (P=0.049, P=0.047 and P=0.042, respectively) (Figure 35). The results reveal that the most effective fraction was the F1 fraction from the summer harvest.

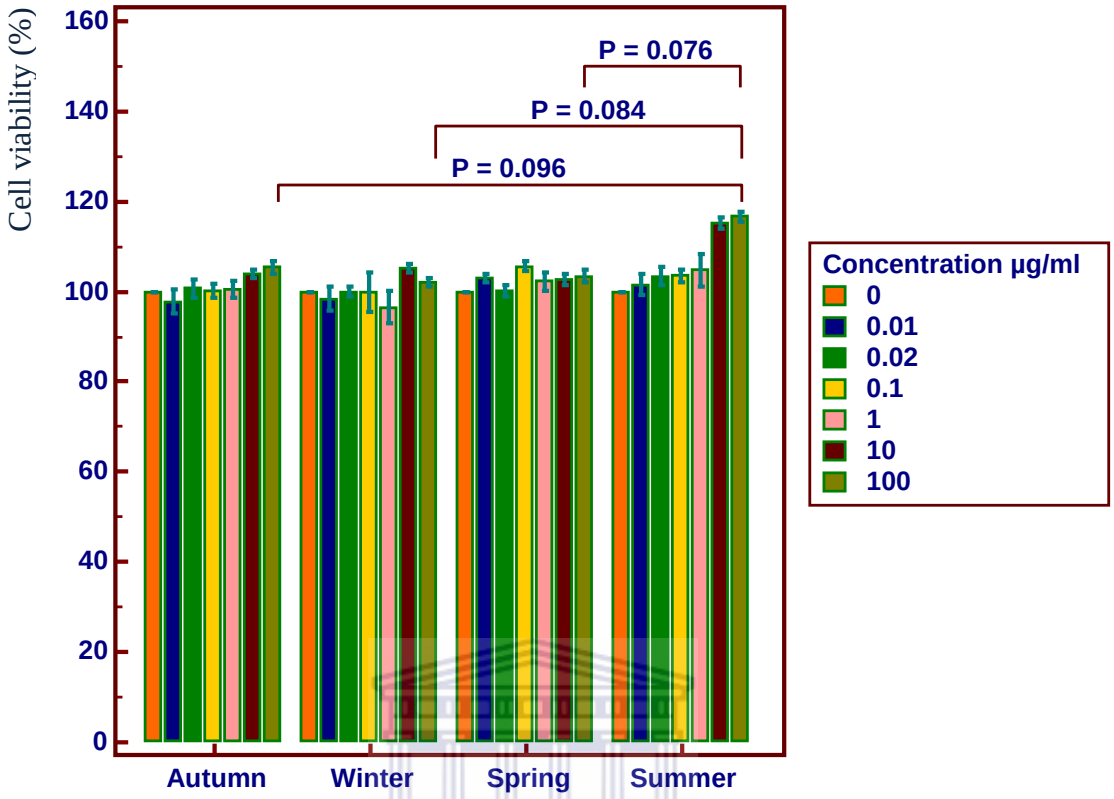


Figure 34: TM3-Leydig cell viability (determined by means of the MTT assay). After 24 hours of exposure to different concentrations of F1 fraction of the *T. capensis* rhizome extract (0.01, 0.02, 0.1, 1, 10 and 100 µg/ml) harvested in different seasons. The F1 fraction showed no significant effect on cell viability compared to the control. At higher concentrations, viability increased compared to the control, indicating cellular stress. Results showed no difference between the concentrations of 100 µg/ml in F1 fraction of the summer season to different seasons (P=0.096, P=0.084 and P=0.076, respectively).

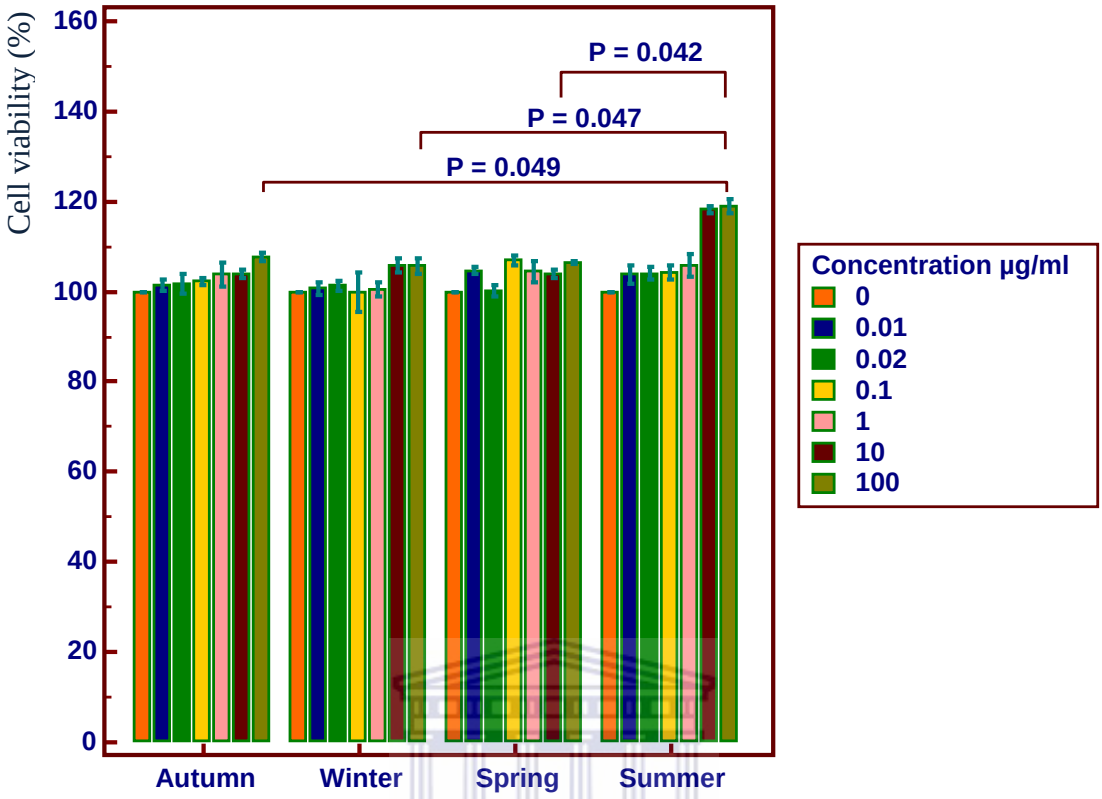


Figure 35: TM3-Leydig cell viability (determined by means of the MTT assay). After 96 hours of exposure to different concentrations of F1 fraction of the *T. capensis* rhizome extract (0.01, 0.02, 0.1, 1, 10 and 100 µg/ml) harvested in different seasons, the F1 fraction showed no significant difference on cell viability compared to the control. At higher concentrations, viability increased compared to the control, indicating cellular stress. Results revealed a significant difference between the concentration of 100 µg/ml in F1 fraction of the summer season to other seasons (P=0.049, P=0.047 and P=0.042, respectively).

3.3.1.2 Effect of *T. capensis* rhizome extract F1 fraction from four seasons on LNCaP cell viability

LNCaP cell viability was determined by means of the MTT assay. After 24 hours of exposure to different concentrations of F1 fraction of the *T. capensis* rhizome extract (0.01, 0.02, 0.1, 1, 10 and 100 µg/ml) harvested in different seasons. Viability for all concentrations decreased when compared to the control. However, at higher concentrations (10, 100 µg/ml) a significant decrease was observed compared to the control, indicating cellular death. A significant difference between the concentration of 100 µg/ml in F1 fraction of the summer season to different seasons can be seen (P=0.0023, P=0.0032 and P=0.0019, respectively) (Figure 36).

After 96 hours of exposure to the F1 fraction, cells showed a decrease in cell viability at all concentrations when compared to the control. However, at higher concentrations (10, 100 µg/ml) a significant decrease was observed compared to the control, indicating cellular death. Results showed a significant difference between the concentration of 100 µg/ml in F1 fraction of the summer harvest to other seasons (P=0.0021, P=0.0026 and P=0.0031, respectively) (Figure 37). The results reveal that the most effective fraction was the F1 fraction from the summer harvest.

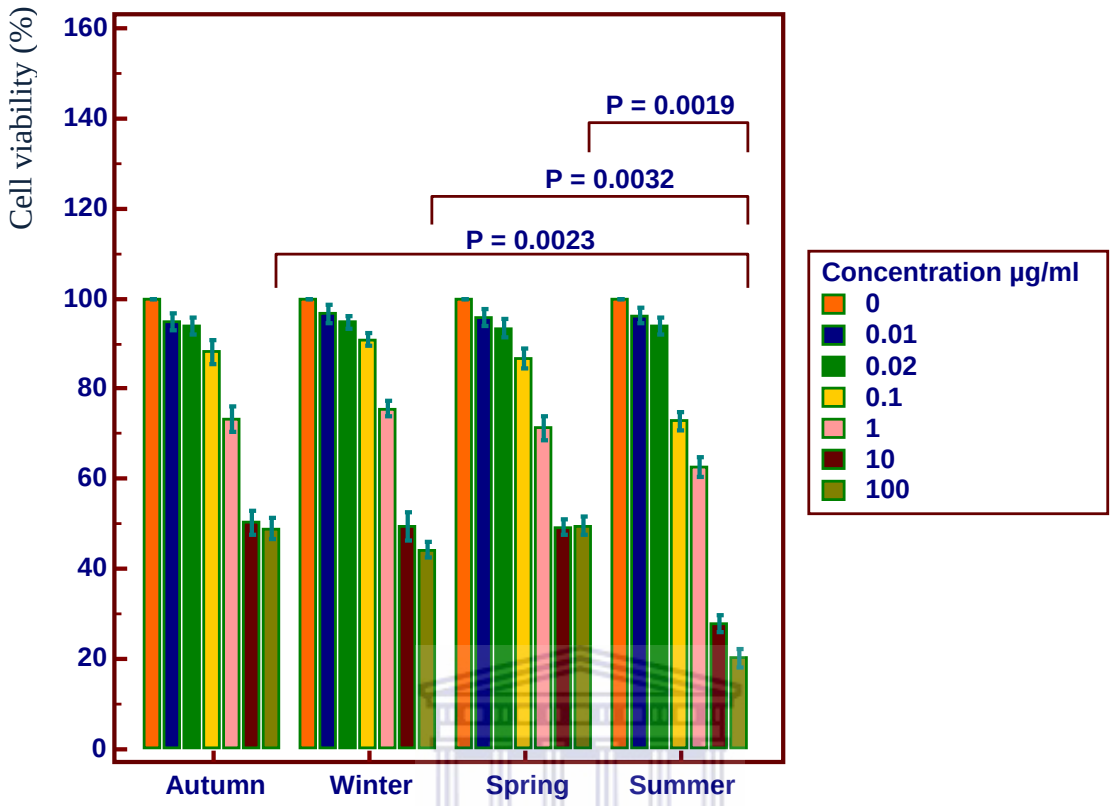


Figure 36: LNCaP cell viability (determined by means of the MTT assay). After 24 hours of exposure to different concentrations of F1 fraction of the *T. capensis* rhizome extract (0.01, 0.02, 0.1, 1, 10 and100 µg/ml) harvested in different seasons, viability for all concentrations decreased when compared to the control. However, at higher concentrations (10, 100 µg/ml) a significant decrease was observed compared to the control, indicating to cellular death. A significant difference between the concentration of 100 µg/ml in F1 fraction of the summer season to other seasons can be seen (P=0.0023, P=0.0032 and P=0.0019, respectively).

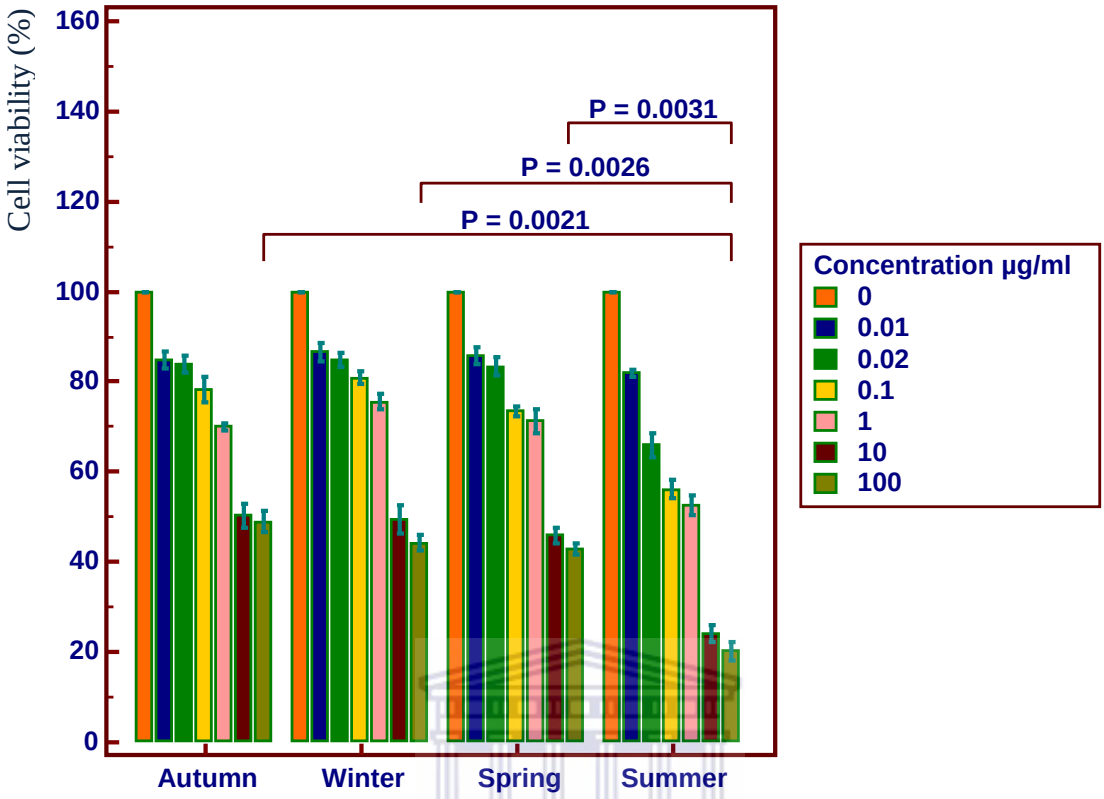


Figure 37: LNCaP cell viability (determined by means of the MTT assay). After 96 hours of exposure to different concentrations of F1 fraction of the *T. capensis* rhizome extract (0.01, 0.02, 0.1, 1, 10 and 100 µg/ml) harvested in different seasons, viability for all concentrations decreased when compared to the control. However, at higher concentrations (10, 100 µg/ml) a significant decrease was observed compared to the control, indicating cellular death. Results showed a significant difference between the concentration of 100 µg/ml in F1 fraction of the summer season to other seasons (P=0.0021, P=0.0026 and P=0.0031, respectively).

3.3.1.3 Effect of *T. capensis* rhizome extracts F1 fraction from four seasons on PWR-1E cell viability

PWR-1E cell viability was determined by means of the MTT assay. After 24 hours of exposure to different concentrations of F1 fraction of the *T. capensis* rhizome extract (0.01, 0.02, 0.1, 1, 10 and 100 µg/ml) harvested in different seasons, no significant difference in cell viability compared to the control was observed. At higher concentration 100 µg/ml, viability decreased compared to the control, indicating cellular death. Results revealed no significant difference between the concentration of 100 µg/ml in F1 fraction of the summer season to different seasons (P=0.096, P=0.102 and P=0.136, respectively) (Figure 38).

After 96 hours of exposure, the F1 fraction showed no significant effect on cell viability compared to the control. At the highest concentration, 100 µg/ml, viability decrease compared to the control, indicating to cellular stress. No significant between the concentration of 100 µg/ml in F1 fraction of the summer season to other seasons was observed (P=0.128, P=0.131 and P=0.213, respectively) (Figure 39).

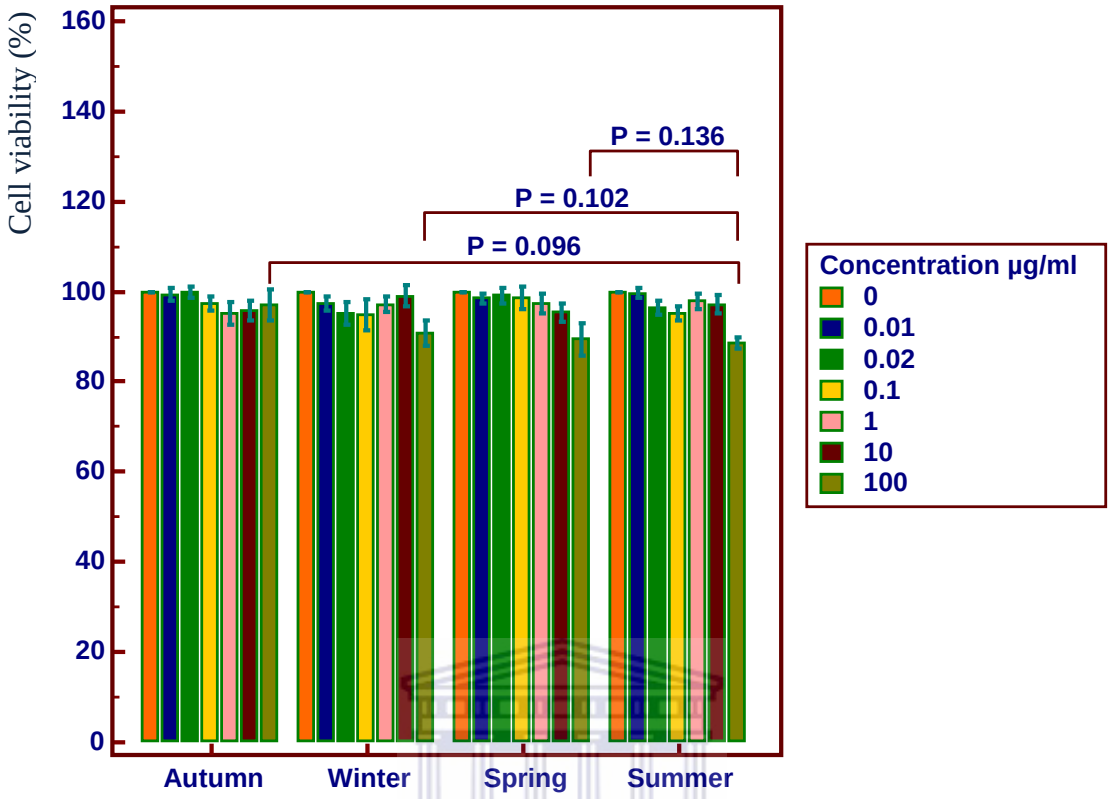


Figure 38: PWR-1E cell viability (determined by means of the MTT assay). After 24 hours of exposure to different concentrations of F1 fraction of the *T. capensis* rhizome extract (0.01, 0.02, 0.1, 1, 10 and 100 µg/ml) harvested in different seasons, no significant difference in cell viability compared to the control can be seen. At highest concentration 100 µg/ml, viability decreases compared to the control, indicating cellular death. Results revealed no significant difference between the concentration of 100 µg/ml in the F1 fraction of the summer season to other seasons (P=0.096, P=0.102 and P=0.136, respectively).

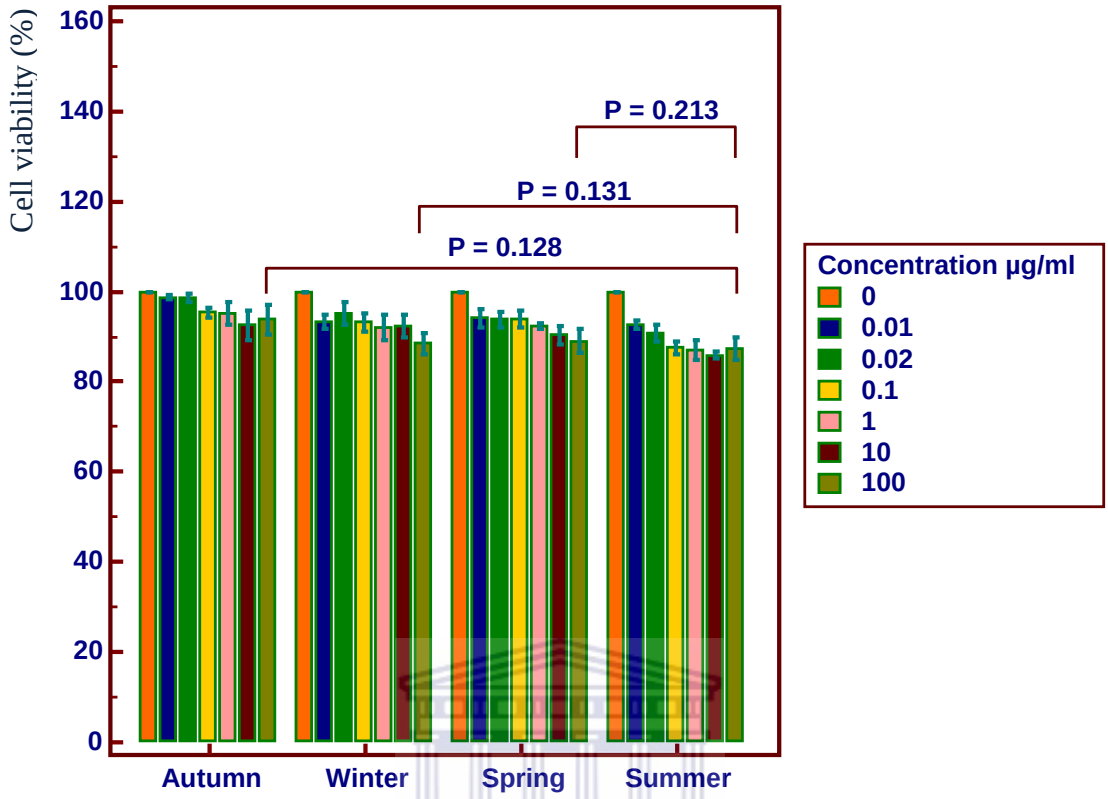


Figure 39: PWR-1E cell viability (determined by means of the MTT assay). After 96 hours of exposure to different concentrations of F1 fraction of the *T. capensis* rhizome extract (0.01, 0.02, 0.1, 1, 10 and 100 µg/ml) harvested in different seasons, no significant difference in cell viability can be seen. At 100 µg/ml, viability decreased compared to the control, indicating cellular death. No difference between the concentration of 100 µg/ml in F1 fraction of the summer season to other seasons was observed (P=0.128, P=0.131 and P=0.213, respectively).

3.3.2 Effect of *T. capensis* rhizome extract F1 fraction of the summer season on cell viability

3.3.2.1 Effect of *T. capensis* rhizome extract F1 fraction of the summer season on TM3-Leydig cell viability

TM3-Leydig cells were cultured in complete DMEM-F12 medium under 5 % CO_2 and 95 % air at a temperature of 37 ºC until 70-80 % confluency in culture flasks. TM3-Leydig cells were seeded at concentrations of ($6x10^4$cells/ml) for 24 hours exposure to the F1 fraction of the summer season, and ($2x10^4$cells/ml) for 96 hours exposure to the F1 fraction of the summer harvest in 96-well plates. After 24 and 96 hours incubation, respectively, the TM3-Leydig cells were exposed to different concentrations of the *T. capensis* rhizome extracts F1 fraction of the summer harvest (0.01, 0.02, 0.1, 1, 10 and 100 µg/ml). Cell viability was determined by means of the MTT assay.

After being exposed to different concentrations of *T. capensis* rhizome extracts F1 fraction of the summer season, the cell morphology was observed and recorded. It was found that no observable morphological changes in the flat and polygonal cells had occurred between the control groups and concentration less than10 µg/ml. At highest concentrations (10, 100 µg/ml), the cells began showing visible signs of stress (Figure 40).

After TM3-Leydig cells were incubated with the different concentrations of the F1 fraction of the summer season (0.01, 0.02, 0.1, 1, 10 and 100 µg/ml) for 24 hours at concentrations less than 10 µg/ml, the fraction showed no significant difference in cell viability compared to the control. At highest concentrations (10 and 100 µg/ml), however, viability showed increases of +11.5% and +13.3%, respectively, indicating cellular stress. One-way ANOVA exhibited no trend (ANOVA trend analysis: P=0.098) (Figure 41).

After TM3-Leydig cells were incubated with the different concentrations of the F1 fraction of the summer season (0.01, 0.02, 0.1, 1, 10 and 100 µg/ml) for 96 hours, at concentrations less than 10µg/ml, the F1fraction showed no significant difference in cell viability compared to the control. At higher concentrations (10, 100 µg/ml), viability showed an increase in viability of +15.7% and

+19.4%, respectively, indicating cellular stress. One-way ANOVA exhibited no trend (ANOVA trend analysis: P=0.062) (Figure 42).

The results reveal there is no significant difference between 24 and 96 hour incubation period on TM3 cell viability.

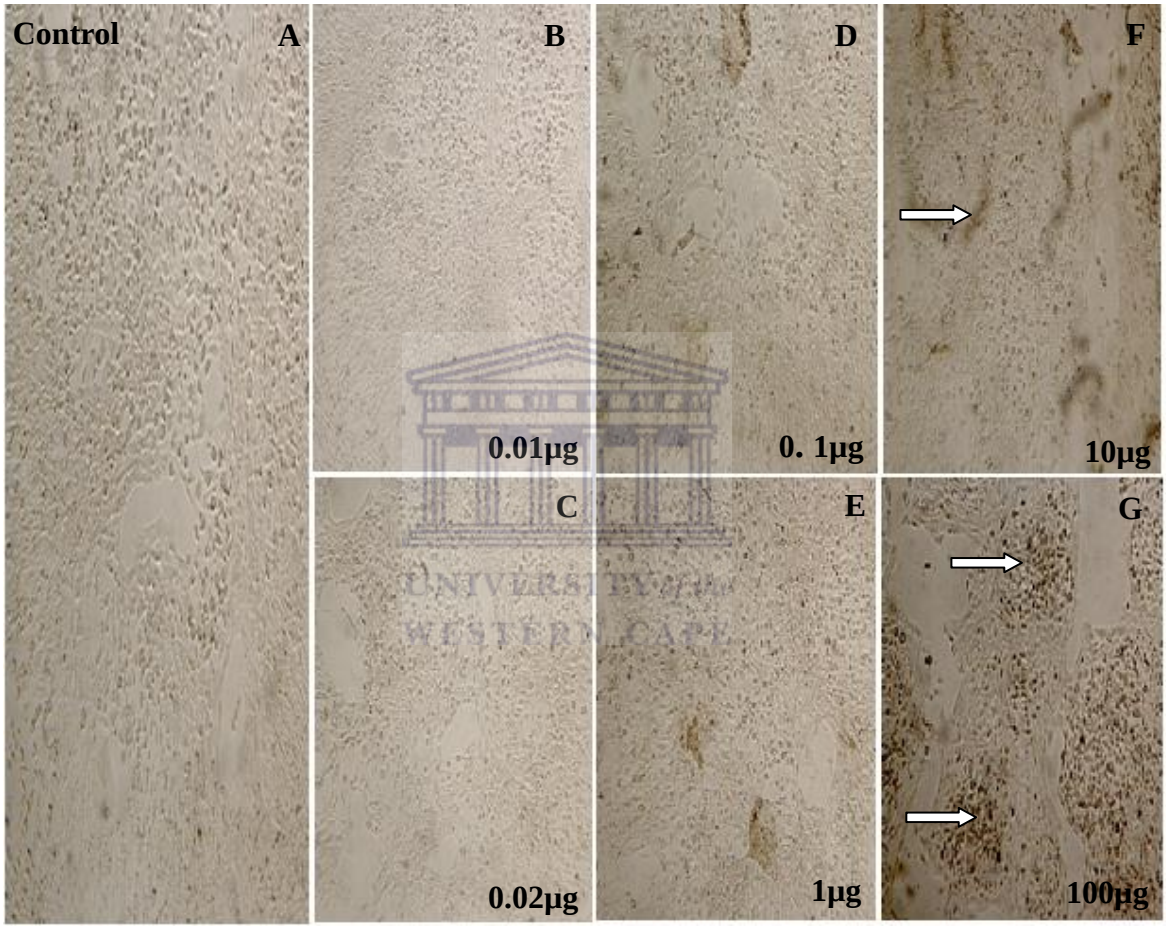


Figure 40: TM3 cells after exposed to different concentrations of *T. capensis* rhizome extracts F1 fraction of the summer harvest (0.01, 0.02, 0.1, 1, 10 and 100 µg/ml), the cell morphology was observed and recorded. It was found that no observable morphological changes in the flat and polygonal cells had occurred between the control groups and concentration up until 1 µg/ml (A-E), at higher concentrations 10, 100 µg/ml (F-G) the cells became rounder than usual and cells began showing visible signs of stress. The white arrows indicating cellular stress (10 x magnifications).

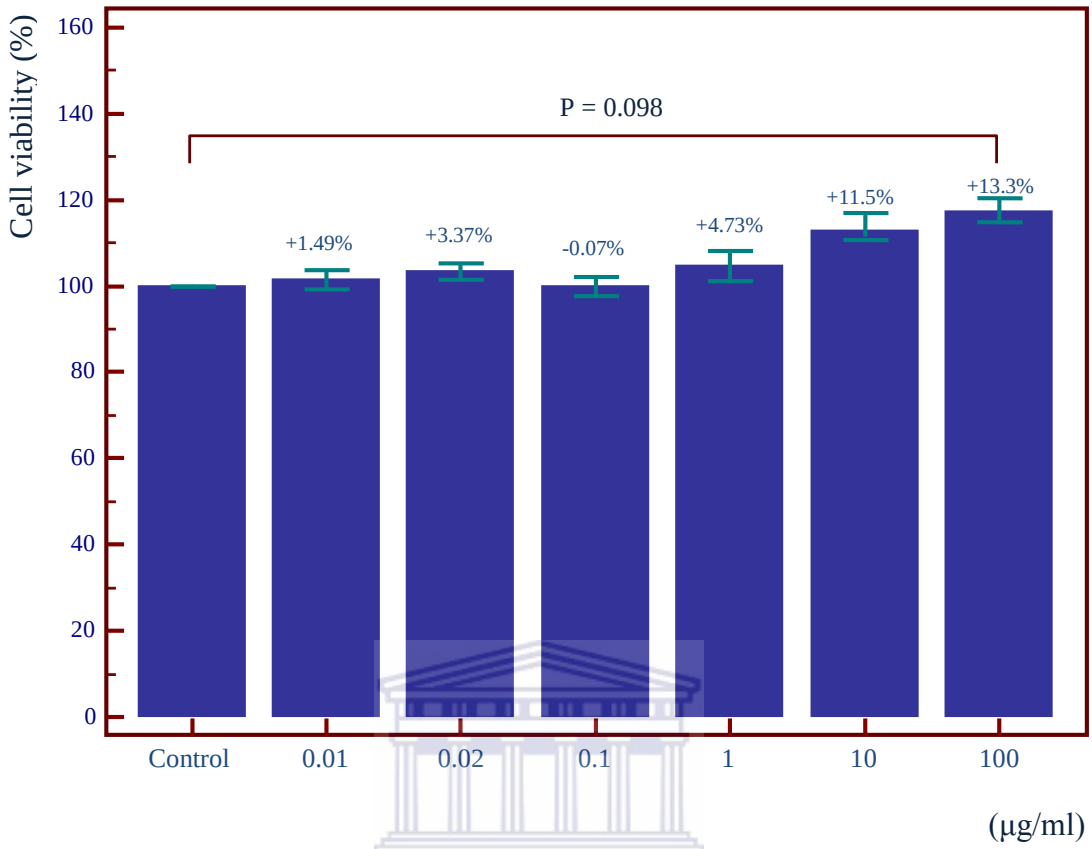


Figure 41: TM3-Leydig cell viability (determined by means of the MTT assay) after incubation of cells with different concentration of *T. capensis* rhizome extracts F1 fraction of the summer harvest (0.01, 0.02, 0.1, 1, 10 and 100 µg/ml) for 24 hours. At concentrations less than 10µg/ml, the F1 fraction of the summer season showed no significant difference in cell viability compared to the control. At higher concentrations, viability showed an increase of +11.5% and +13.3%, respectively, indicating cellular stress. One-way ANOVA exhibited no trend (ANOVA trend analysis: P=0.098).

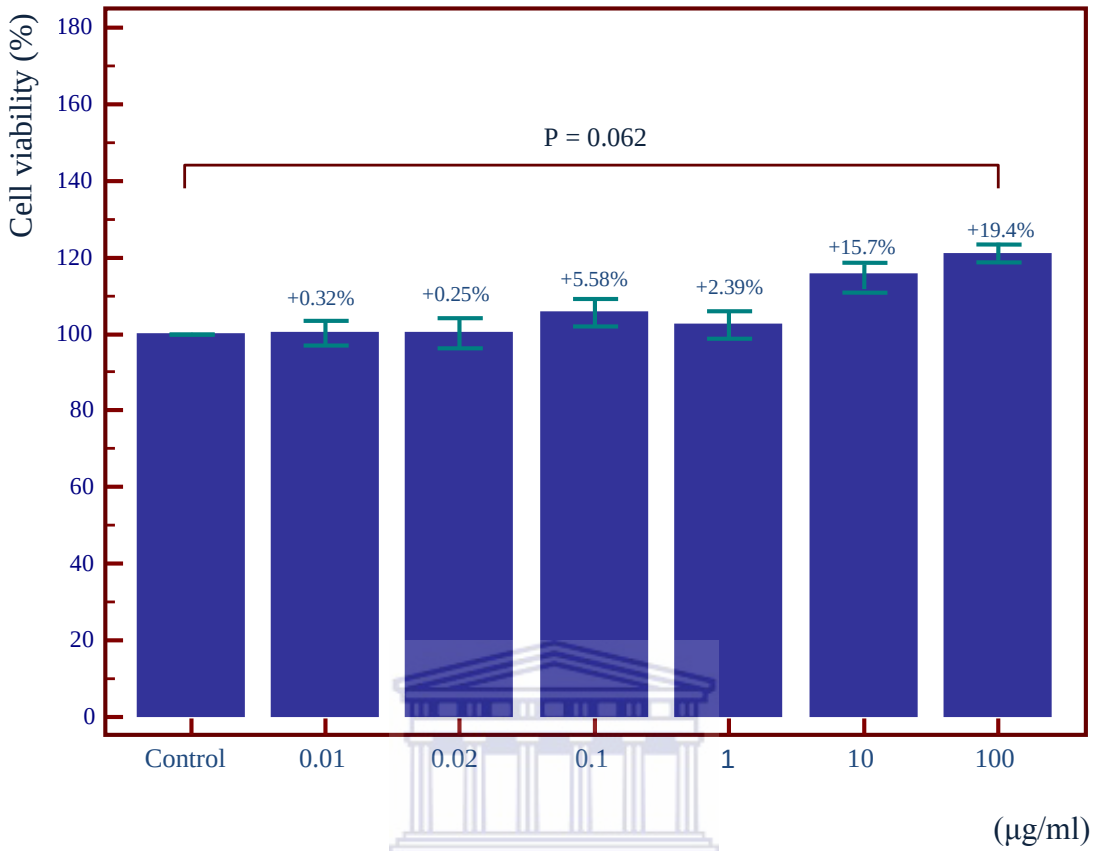


Figure 42: TM3-Leydig cell viability (determined by means of the MTT assay) after incubation of cells with different concentration of *T. capensis* rhizome extracts F1 fraction of the summer harvest (0.01, 0.02, 0.1, 1, 10 and 100 µg/ml) for 96 hours. At concentrations less than 10µg/ml, the fraction showed no significant difference in cell viability compared to the control. At higher concentrations, viability showed an increase of +15.7% and +19.4%, respectively, indicating cellular stress. One-way ANOVA showed no trend (ANOVA trend analysis: P=0.062).

3.3.2.2 Effect of *T. capensis* rhizome extracts F1 fraction of the summer season on LNCaP cell viability

The prostate cancer cell line LNCaP was cultured in RPMI 1640 Medium in 75 cm^2 culture flask at 37˚C under a 5% CO_2 and 95% air. LNCaP cells were seeded at concentrations of ($6x10^4$cells/ml) for 24 hours exposure to the F1 fraction of the summer season, and ($2x10^4$cells/ml) for 96 hours exposure to the F1 fraction of the summer season into 96-well plates. After 24 hours incubation, the LNCaP cells were exposed to different concentrations of *T. capensis* rhizome extracts F1 fraction of the summer season (0.01, 0.02, 0.1, 1, 10 and 100 µg/ml). Cell viability was determined by means of the MTT assay.

After exposure to different concentrations of *T. capensis* rhizome extracts F1 fraction of the summer season, the morphology of LNCaP cells changes markedly. There was a clear increase in detachment and cell death clumping. Apoptotic bodies could also be seen indicating cell death (Figure 43).

The viability of LNCaP cells was tested after exposure to different concentrations of F1 fraction of the summer harvest (0.01, 0.02, 0.1, 1, 10 and 100 µg/ml) by means of the MTT test. After 24 hours of exposure, cell viability for all concentrations decreased when compared to the control. However, at higher concentrations (10, 100 µg/ml) a significant decrease of -70.7% ($P<0.0001$) and -77.1% ($P<0.0001$) was observed compared to the control. One-way ANOVA exhibited a significant trend (ANOVA trend analysis: $P<0.0001$) (Figure 44).

After 96 hours of exposure cell viability for all concentrations decreased when compared to the control. However, at higher concentrations (10, 100 µg/ml) a significant decrease of -72.2% ($P<0.0001$) and -82.8% ($P<0.0001$) was observed compared to the control. One-way ANOVA exhibited a significant trend (ANOVA trend analysis: $P<0.0001$) (Figure 45).

The results reveal that the viability of LNCaP cells at higher concentrations 100 µg/ml showed declines in cell viability at incubation period 96 hours (-82.8%) more than 24 hours (-77.1%) indicating more cell death.

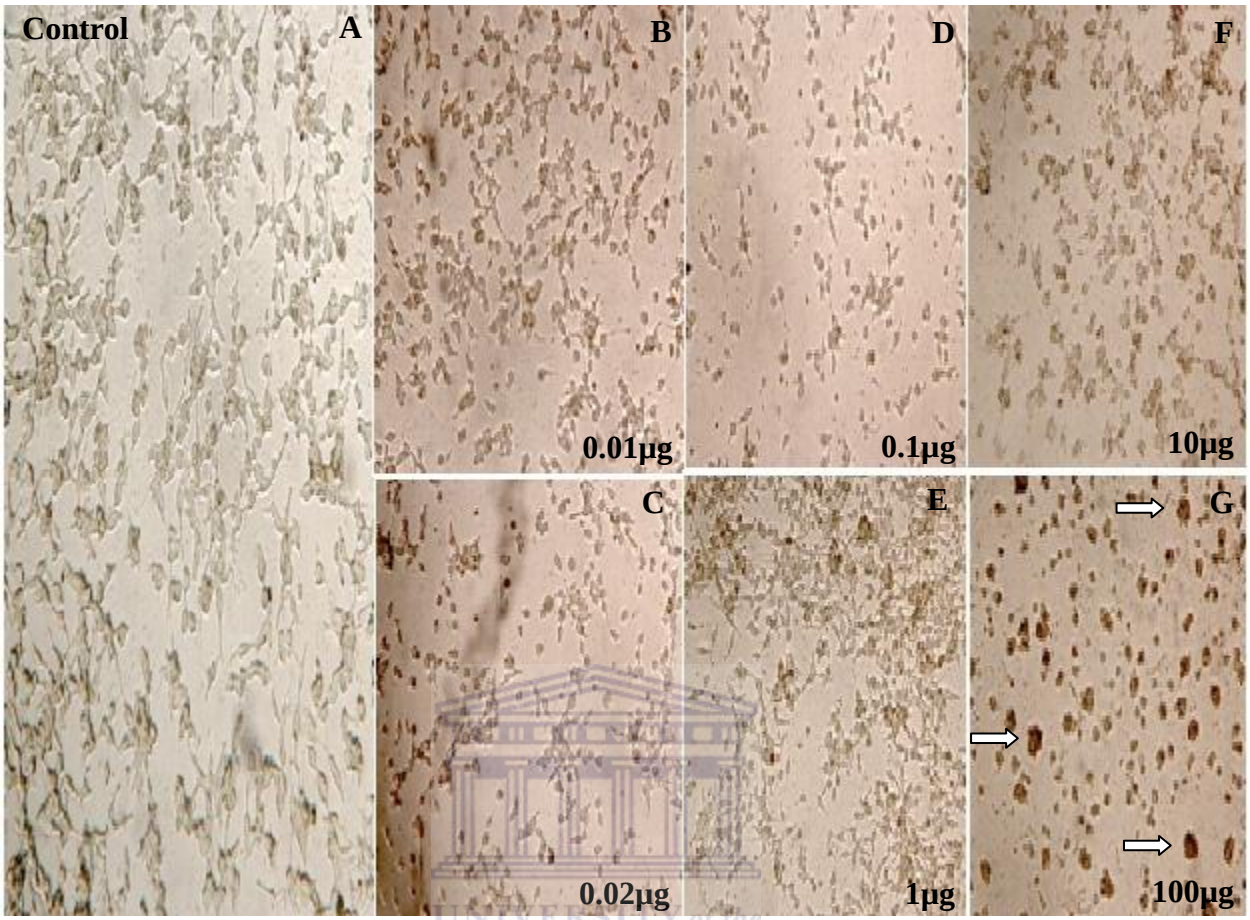


Figure 43: Cell morphology of LNCaP cell after exposure to increasing concentrations of the F1 fraction of the summer season (0.01, 0.02, 0.1, 1, 10 and 100 µg/ml). Cellular morphology changed there was a clear increase in detachment between the control groups and concentration up until 1 µg/ml (A-E). At higher concentrations 10, 100 µg/ml (F-G) there was a clear increase in clumping of cells and apoptotic bodies could also be seen indicating cell death. The white arrows indicating to apoptotic bodies (10 x magnifications).

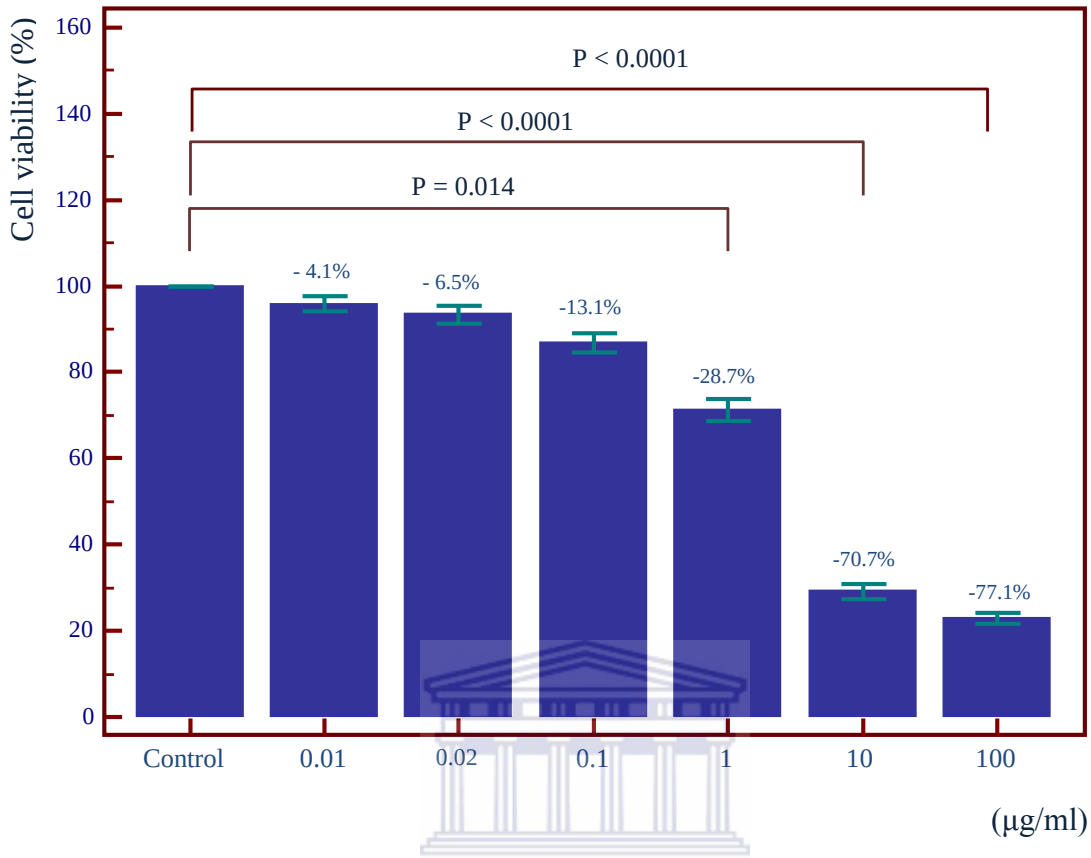


Figure 44: LNCaP cell viability (determined by means of the MTT assay). After 24 hours of exposure to different concentrations of the F1 fraction of the summer season (0.01, 0.02, 0.1, 1, 10 and 100 µg/ml), viability for all concentrations decreased. However, at higher concentrations (1, 10 and 100 µg/ml) a significant decrease of -70.7% (P<0.0001) and -77.1% (P<0.0001) was observed compared to the control. One-way ANOVA exhibited a significant trend (ANOVA trend analysis: P<0.0001).

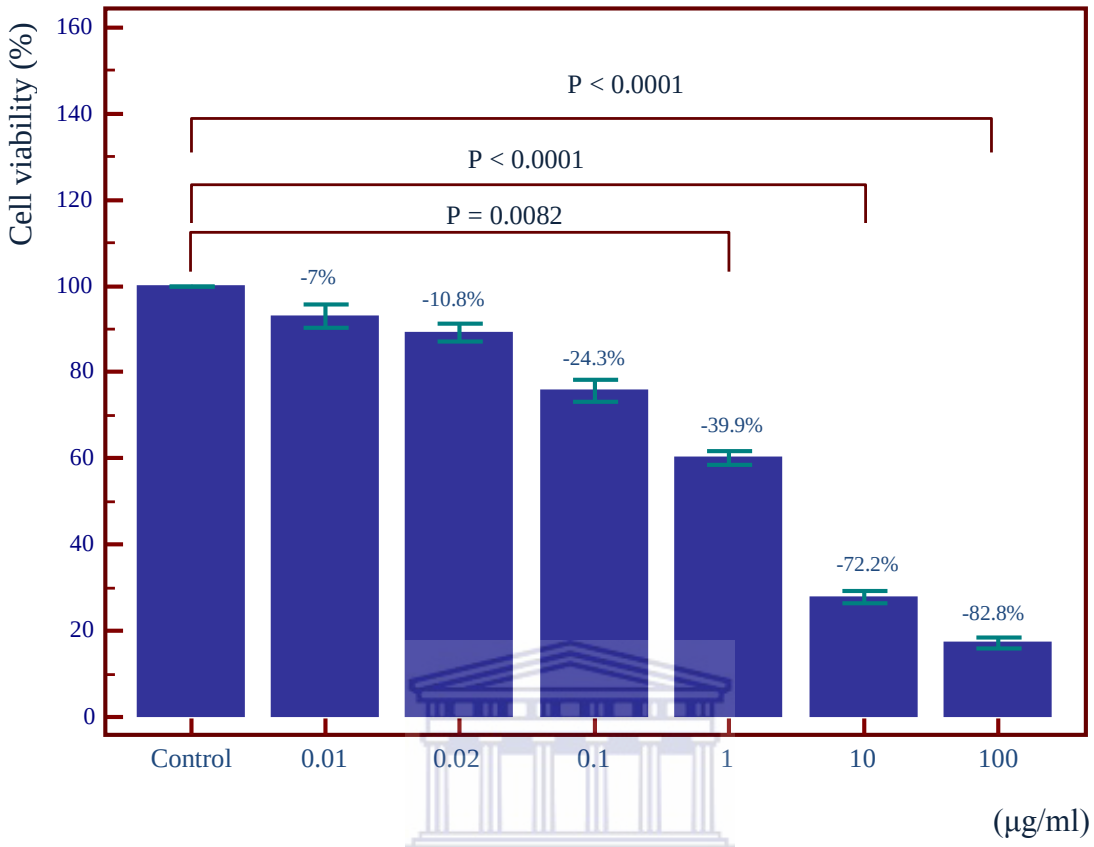


Figure 45: LNCaP cell viability (determined by means of the MTT assay). After 96 hours of exposure to different concentrations of of the F1 fraction of the summer harvest (0.01, 0.02, 0.1, 1, 10, 100 µg/ml), viability for all concentrations decreased when compared to the control. However, at higher concentrations (10, 100 µg/ml) a significant decrease of -72.2% (P<0.0001) and -82.8% (P<0.0001) was observed. One-way ANOVA exhibited a significant negative trend (ANOVA trend analysis: P<0.0001).

3.3.2.3 Effect of *T. capensis* rhizome extracts F1 fraction of the summer season on PWR-1E cell viability

The benign prostatic hyperplasia cell line PWR-1E was cultured in complete Keratinocyte-SFM (1X) serum Free Medium in 75 cm^2 culture flask at 37˚C under a 5% CO_2 and 95% air. PWR-1E cells were seeded at concentrations of ($6x10^4$cells/ml) for 24 hours exposure and ($2x10^4$cells/ml) for 96 hours exposure into 96-well plates. After 24 and 96 hours incubation, the PWR-1E cells were exposed to different concentrations of *T. capensis* rhizome extract F1 fraction of the summer harvest (0.01, 0.02, 0.1, 1, 10 and 100 µg/ml). Cell viability was determined by means of MTT assay

After being exposed to different concentrations of *T. capensis* rhizome extract F1 fraction of the summer harvest, the cell morphology was observed and recorded. It was found that no observable morphological changes in the flat and polygonal cells had occurred between the control groups and concentration less than10 µg/ml. At higher concentrations >100 µg/ml the cells began showing an increase in detachment and cell death (Figure 46).

The viability of PWR-1E cells was tested after exposure to different concentrations of F1 fraction of the summer season (0.01, 0.02, 0.1, 1, 10 and 100 µg/ml) by means of the MTT test. After 24 hours of exposure, cell viability for all concentrations showed no significant changes when compared to the control. At higher concentrations >100 µg/ml, however, a significant decrease of -28.6% (P=0.0089) was observed. One-way ANOVA exhibited no trend (ANOVA trend analysis: P=0.139) (Figure 47).

After 96 hours of exposure cell viability for all concentrations showed no significant effect on cell viability when compared to the control. At higher concentrations >100 µg/ml, a significant decrease of -36.2% (P=0.0019) was observed when compared to the control. One-way ANOVA exhibited no trend (ANOVA trend analysis: P=0.128) (Figure 48).

The results reveal that the viability of PWR-1E cells at higher concentrations 100 µg/ml showed declines in cell viability at an incubation period of 96 hours (-36.2%) more than 24 hours (-28.6%) indicate to more cell death.

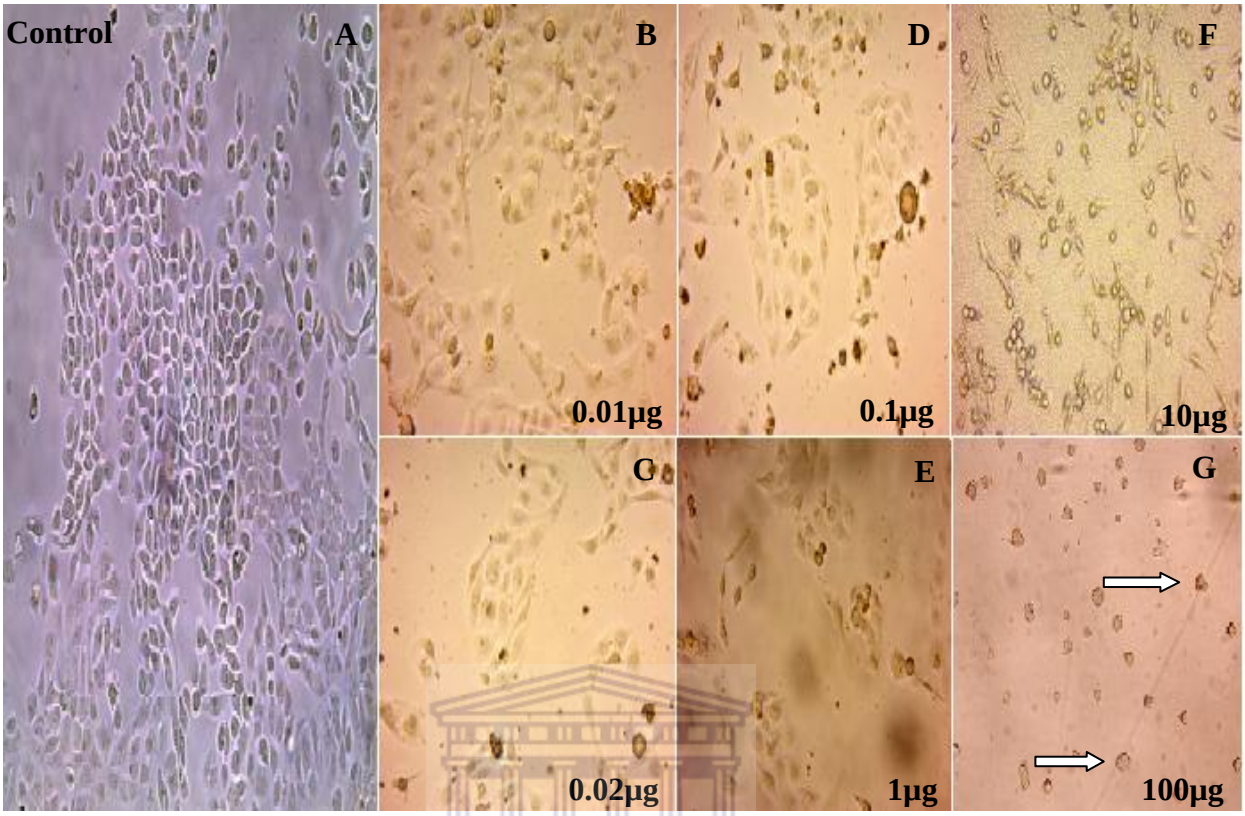


Figure 46: PWR-1E cells after being exposed to different concentrations of of *T. capensis* rhizome extract F1 fraction of the summer season (0.01, 0.02, 0.1, 1, 10, 100 µg/ml). It was found that no observable morphological changes in the flat and polygonal cells had occurred between the control groups and concentration up until 1 µg/ml (A-E). At higher concentrations 10, 100 µg/ml (F-G), the cells began showing an increase in detachment and cell death. The white arrows indicate cell death (10 x magnifications).

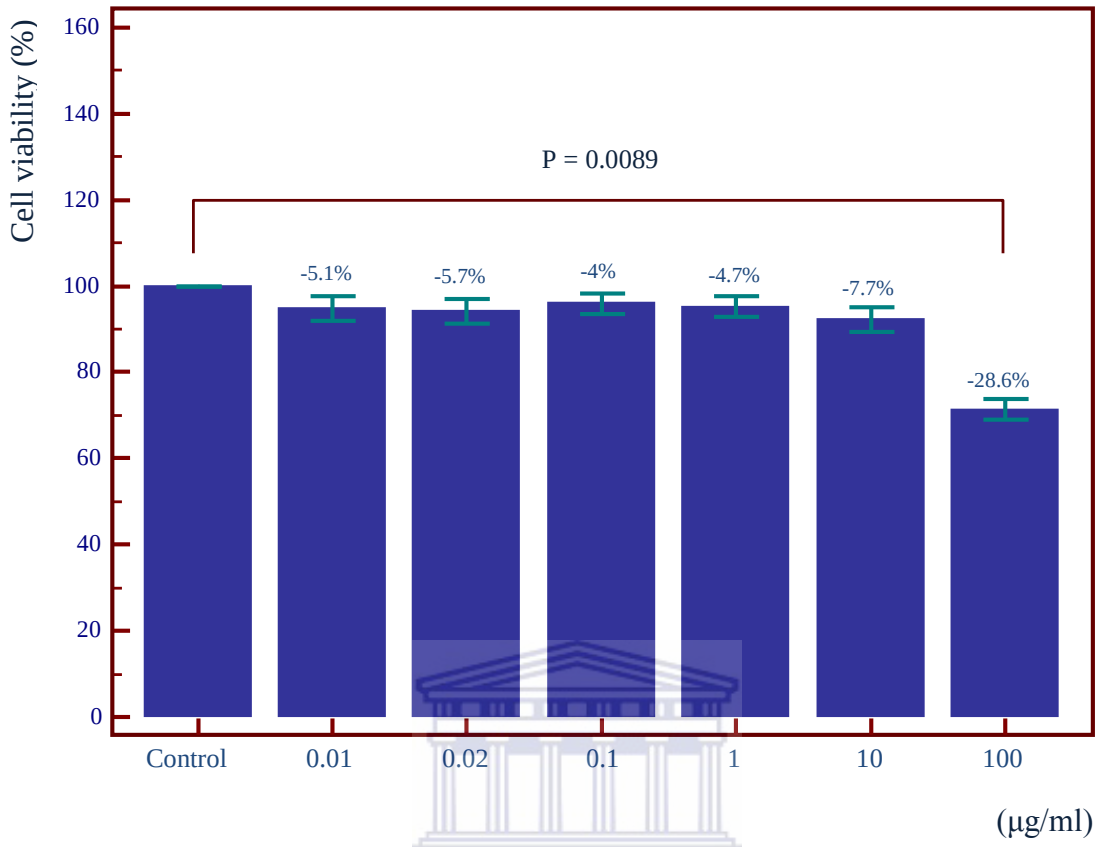


Figure 47: PWR-1E cell viability (determined by means of the MTT assay). After 24 hours of exposure to different concentrations of *T. capensis* rhizome extracts F1 fraction of the Summer season (0.01, 0.02, 0.1, 1, 10 and 100 µg/ml), viability for all concentrations showed no significant effect on cell viability when compared to the control, at higher concentrations (100 µg/ml) a significant decrease of -28.6% (P=0.0089) was observed when compared to the control. One-way ANOVA exhibited no trend (ANOVA trend analysis: P=0.139).

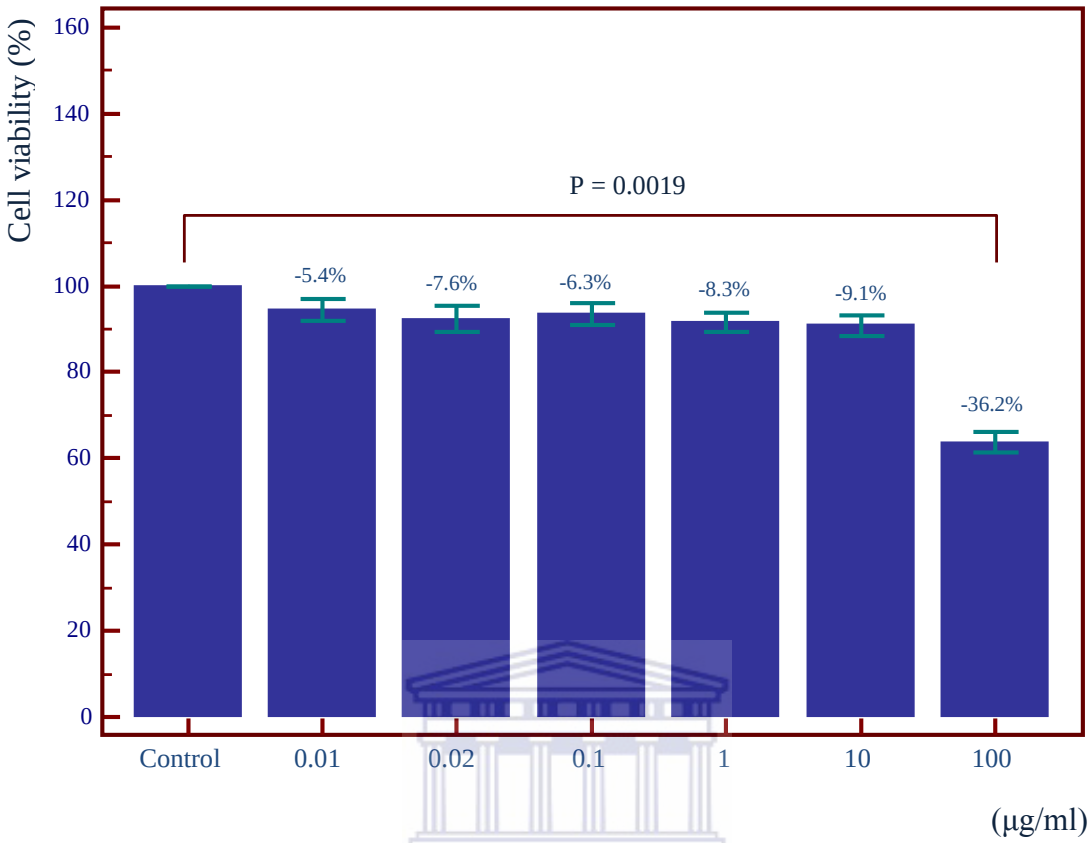


Figure 48: PWR-1E cell viability (determined by means of the MTT assay). After 96 hours of exposure to different concentrations of *T. capensis* rhizome extracts F1 fraction of the summer season (0.01, 0.02, 0.1, 1, 10 and 100 µg/ml), viability for all concentrations showed no significant effect on cell viability when compared to the control, at higher concentrations (100 µg/ml) a significant decrease of -36.2% (P=0.0019) was observed when compared to the control. One-way ANOVA exhibited no trend (ANOVA trend analysis: P=0.128).

3.3.3 Effects of *T. capensis* rhizome extract F1 fraction from four seasons on testosterone production in TM3-Leydig cells

TM3 cell testosterone production was determined after exposure to extract harvested in the four seasons (Autumn, Winter, Spring and Summer). After 48 hours of exposure to different concentrations of F1 fraction of the *T. capensis* rhizome extract (0.01, 0.02, 0.1, 1, 10 and 100 µg/ml) from different seasons, the concentration of testosterone increased when compared to different concentrations of F1 fraction. A significant peak in the testosterone production was found at 0.1µg/ml for all seasons. At higher concentrations, testosterone production declined again. A significant increase between the concentration of 0.1 µg/ml in F1 fraction of the summer season to different seasons was found (P=0.0013, P=0.0021 and P=0.001, respectively) (Figure 49).

Testosterone production after 96 hours of exposure to different concentrations of F1 fraction of the *T. capensis* rhizome extract (0.01, 0.02, 0.1, 1, 10 and 100 µg/ml) from different seasons increased when compared the different concentrations of F1 fraction to the control peaking at an extract concentration of 0.1 µg/ml. At higher concentrations, testosterone production declined again. A significant increase between the concentration of 0.1 µg/ml in F1 fraction of the summer season to different seasons was observed ($P < 0.001$, $P < 0.001$ and $P < 0.001$, respectively) (Figure 50). The results reveal that the most effective fraction was the F1 fraction from the summer harvest.

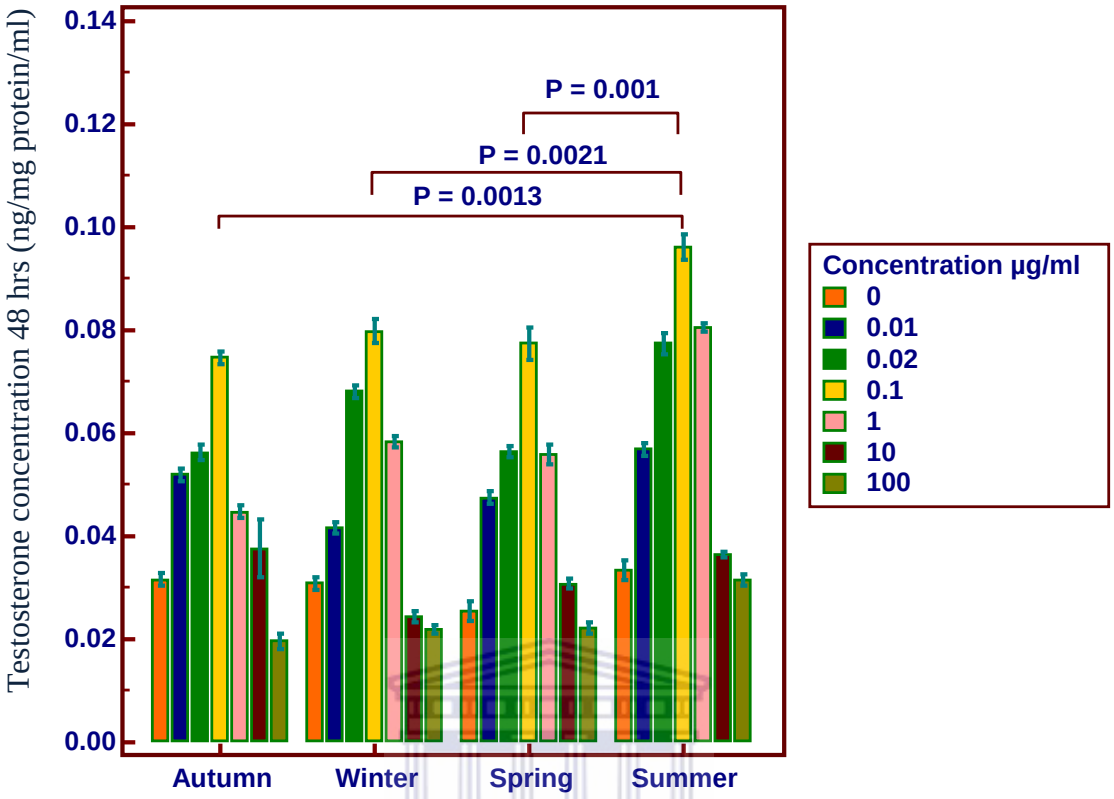


Figure 49: TM3 cell testosterone production after exposure to increasing concentrations of *T. capensis* rhizome extract from different seasons (Autumn, Winter, Spring and Summer). After 48 hours of exposure to different concentrations of F1 fraction of the *T. capensis* rhizome extract (0.01, 0.02, 0.1, 1, 10 and 100 µg/ml) from different seasons, the concentration of testosterone increased when compared the different concentrations of F1 fraction to the control. A significant peak in the testosterone production was found at 0.1µg/ml ($P<0.0001$) for all seasons. At higher concentrations, testosterone production declined again. A significant increase between the concentration of 0.1 µg/ml in F1 fraction of the summer season to different seasons was found ($P=0.0013$, $P=0.0021$ and $P=0.001$, respectively).

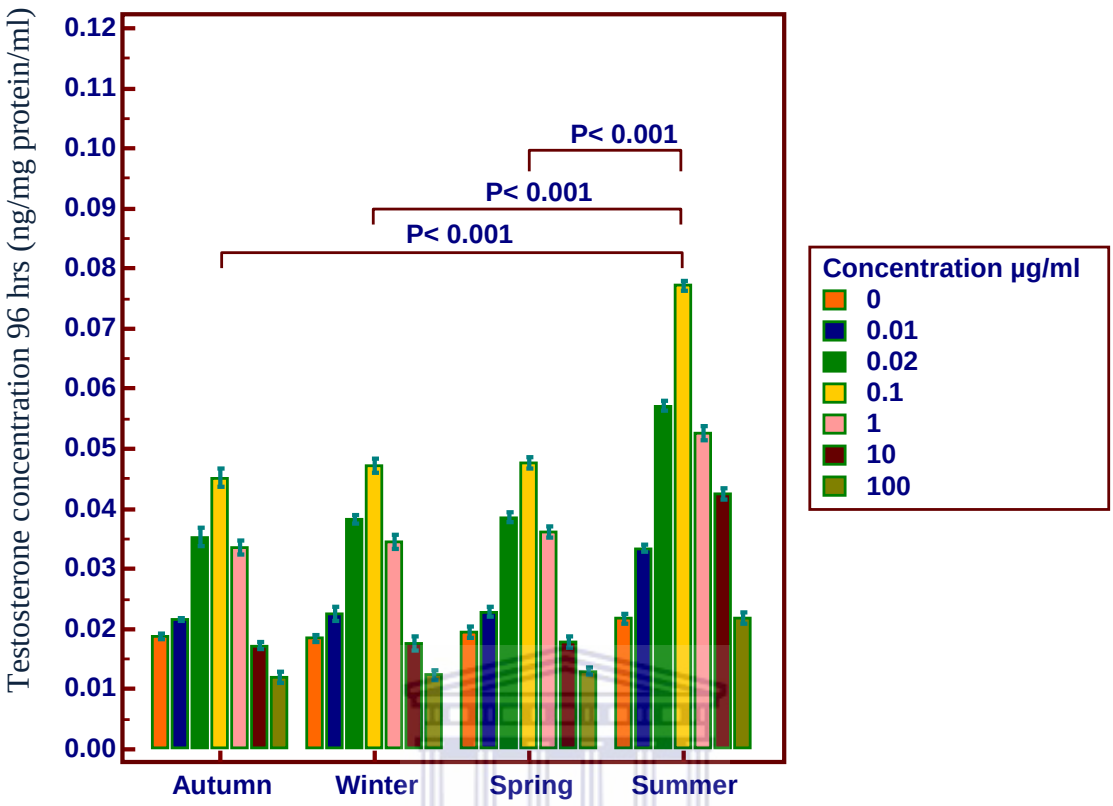


Figure 50: TM3 cell testosterone production after exposure to increasing concentrations of *T. capensis* rhizome extract from different seasons (Autumn, Winter, Spring and Summer). After 96 hours of exposure to different concentrations of F1 fraction of the *T. capensis* rhizome extract (0.01, 0.02, 0.1, 1, 10 and 100 µg/ml) the concentration of testosterone increased when compared the different concentrations of F1 fraction to the control peaking at an extract concentration of 0.1 µg/ml this increase is significant (P<0.0001). At higher concentrations, testosterone production is declining again. A significant increase between the concentration of 0.1 µg/ml in the F1 fraction of the summer season to different seasons can be seen (P<0.001, P<0.001and P<0.001, respectively).

3.3.3.1 Effects of *T. capensis* rhizome extract F1 fraction of the summer season on testosterone production in TM3-Leydig cells

TM3-Leydig cells were analysed for testosterone production and protein content. Testosterone concentrations obtained were subsequently compared and normalized to protein content in each well to obtain the amount of testosterone (ng/mg protein/ml).

After TM3 cells were exposed for 48 hours to different concentrations of *T. capensis* rhizome extract F1 fraction of the summer season (0.01, 0.02, 0.1, 1, 10 and 100 µg/ml), the concentration of testosterone increase when comparing the different concentrations of F1 fraction to the control. significant increases of +67.3 and +125.6%, respectively, peaking at concentrations of 0.02 and 0.1 µg/ml were found (P=0.009, P<0.0001, respectively). At higher concentrations (10 and 100 µg/ml), a decrease in testosterone production to -12.6% and -33.9% was observed when compared to the control (Figure 51).

After TM3 cells were exposed for 96 hours to different concentrations of F1 fraction of the summer season (0.01, 0.02, 0.1, 1, 10 and 100 µg/ml), testosterone shows significant increases of +34.7% (P=0.0019), +97% (P=<0.0001) and +157.8% (P=<0.0001) at extract concentrations of 0.01, 0.02 and 0.1 µg/ml. At higher concentrations (10 and 100 µg/ml) a decrease in testosterone production of -17 and -45% was observed when compared to the control (Figure 52).

Results reveal that the testosterone production at extract concentrations of 0.01, 0.02 and 0.1 µg/ml after 96 hours of incubation increased (+34.7%, +97% and +157.8%). These increases were higher than after 48 hours of incubation (+15.8%, +67.3% and +125.6%).

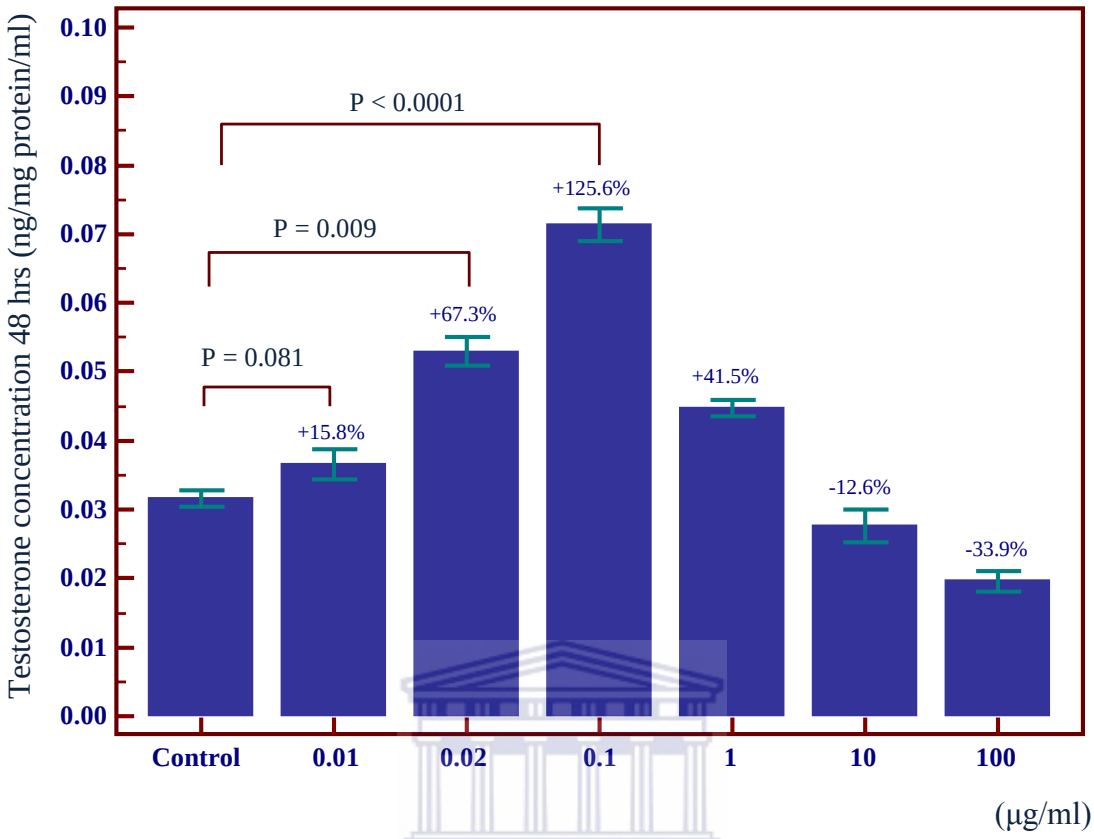


Figure 51: Testosterone production of TM3 cells over a 48 hours incubation period. After TM3 cells were exposed for 48 hours to different concentrations of *T. capensis* rhizome extract F1 fraction of the summer season (0.01, 0.02, 0.1, 1, 10 and 100 µg/ml), the concentration of testosterone increased when comparing the different concentrations of F1 fraction to the control. A significant increases of +67.3% and +125.6% peaking at concentrations of 0.02 and 0.1 µg/ml was found (P=0.009, P<0.0001, respectively). At higher concentrations (10 and 100 µg/ml), a decrease of testosterone production -12.6% and -33.9% was observed when compared to the control.

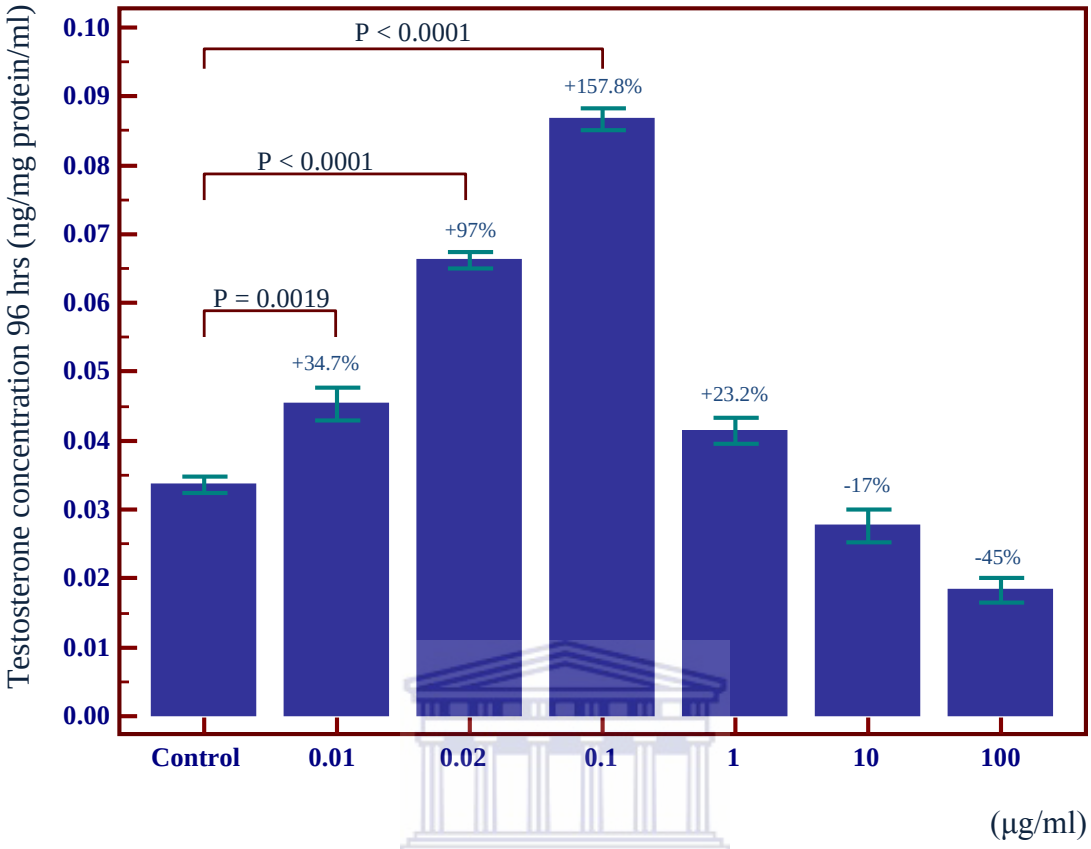


Figure 52: Testosterone production of TM3 cells over a 96 hours incubation period. After TM3 cells were exposed for 96 hours to different concentrations of F1 fraction of the summer season (0.01, 0.02, 0.1, 1, 10 and 100 µg/ml), the concentration of testosterone shows significant increases of +34.7% (P=0.0019), +97% (P=<0.0001) and +157.8% (P=<0.0001). At higher concentrations (10 and 100 µg/ml) a decrease of testosterone production of -17 and -45%,respectively, was observed.

3.3.4 Effects of *T. capensis* rhizome extract F1 fraction of the summer season on cell early apoptosis

3.3.4.1 Effect of *T. capensis* rhizome extract F1 fraction of the summer season on Annexin V-Cy3 binding in TM3-Leydig cells

The effect of *T. capensis* rhizome extract F1 fraction of the summer season on Annexin V-Cy3 binding to TM3 cells as an indicator of apoptosis was analysed after exposure to different concentrations of F1 fraction (0.01, 0.02, 0.1, 1, 10 and 100 µg/ml). After exposure for 24 hours, exposure at low concentrations (0.01, 0.02, 0.1 and 1 µg/ml) for 24-96 hours showed no increase in early apoptosis when compared to the control. However, at higher concentrations (10, 100 µg/ml), increases in the percentage of cells with signs of early apoptosis of 2.67% and 3.5%, respectively, were found. One-way ANOVA exhibited no trend (ANOVA trend analysis: P=0.502) (Figure 53).

After exposure for 96 hours, exposure at low concentrations (0.01, 0.02, 0.1 and 1 µg/ml) caused no increase in early apoptosis when compared to the control. At higher concentrations (10, 100 µg/ml) however, a 3.5% and 4.3% increase in the percentage of cells with signs of early apoptosis was observed. One-way ANOVA exhibited no trend (ANOVA trend analysis: P=0.408) (Figure 54).

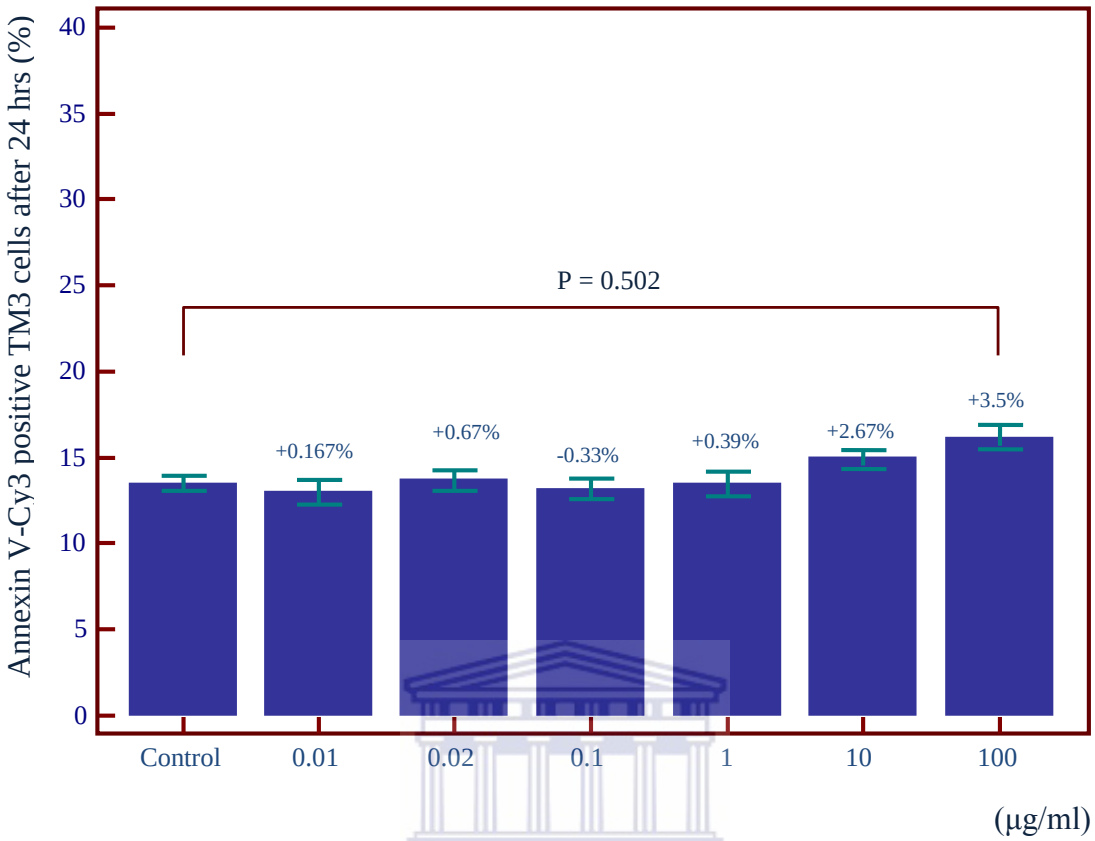


Figure 53: Effect of *T. capensis* rhizome extract F1 fraction of the summer season (0.01, 0.02, 0.1, 1, 10 and 100 µg/ml) on Annexin V-Cy3 indicating cell apoptosis in TM3 cells. After 24 hours, exposure at low concentrations (0.01, 0.02, 0.1 and 1 µg/ml) showed no increase in early apoptosis when compared to the control. However, at higher concentrations (10, 100 µg/ml), an increase of 2.67% and 3.5%, respectively, in the percentage of cells with signs of early apoptosis was found. One-way ANOVA exhibited no trend (ANOVA trend analysis: P=0.502).

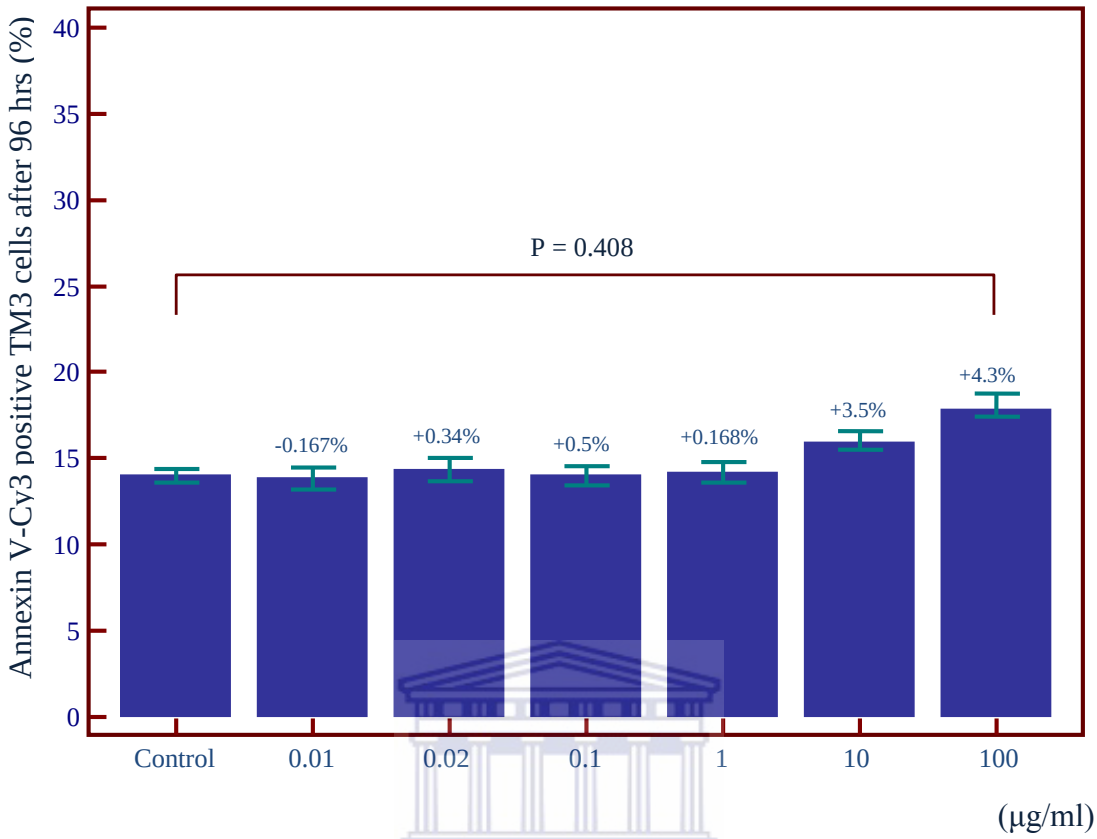


Figure 54: Effect of *T. capensis* rhizome extract F1 fraction of the summer season (0.01, 0.02, 0.1, 1, 10 and 100 µg/ml) on Annexin V-Cy3 indicating cell apoptosis in TM3 cells. After 96 hours of exposure at low concentrations (0.01, 0.02, 0.1 and 1 µg/ml), no increase in early apoptosis when can be seen. At higher concentrations (10, 100 µg/ml), however, revealed a 3.5% and 4.3%, respectively, increase in the percentage of cells with signs of early apoptosis can be seen. One-way ANOVA exhibited no trend (ANOVA trend analysis: P=0.408).

3.3.4.2 Effect of *T. capensis* rhizome extract F1 fraction of the summer season on Annexin V-Cy3 binding in LNCaP cells

The effect of *T. capensis* rhizome extract F1 fraction of the summer season on Annexin V-Cy3 binding to LNCaP cells as an indicator of apoptosis was analysed after exposure to different concentrations (0.01, 0.02, 0.1, 1, 10 and 100µg/ml). After being exposed for 24 hours at low concentrations (0.01, 0.02, 0.1 and 1 µg/ml), an increase of Annexin V-Cy3 binding indicated early signs of apoptosis when compared to the control. At higher concentrations (10 and 100µg/ml), increases of +28% (P=0.00199) and +33.8% (P=0.00163), respectively in the percentage of cells with signs of early apoptosis were significant. One-way ANOVA exhibited a significant positive trend (ANOVA trend analysis: P<0.0001) (Figure 55).

After 96 hours of exposure, all concentrations caused a dose-dependent increase in early apoptosis in the cells. At higher concentrations (10 and 100 µg/ml), a significant increase of 38.8% (P=0.009) and 52.3%(P<0.001), respectively, in the percentage of cells with signs of early apoptosis was significant. One-way ANOVA exhibited a significant position trend after exposure for 96 hours (ANOVA trend analysis: P<0.0001) (Figure 56).

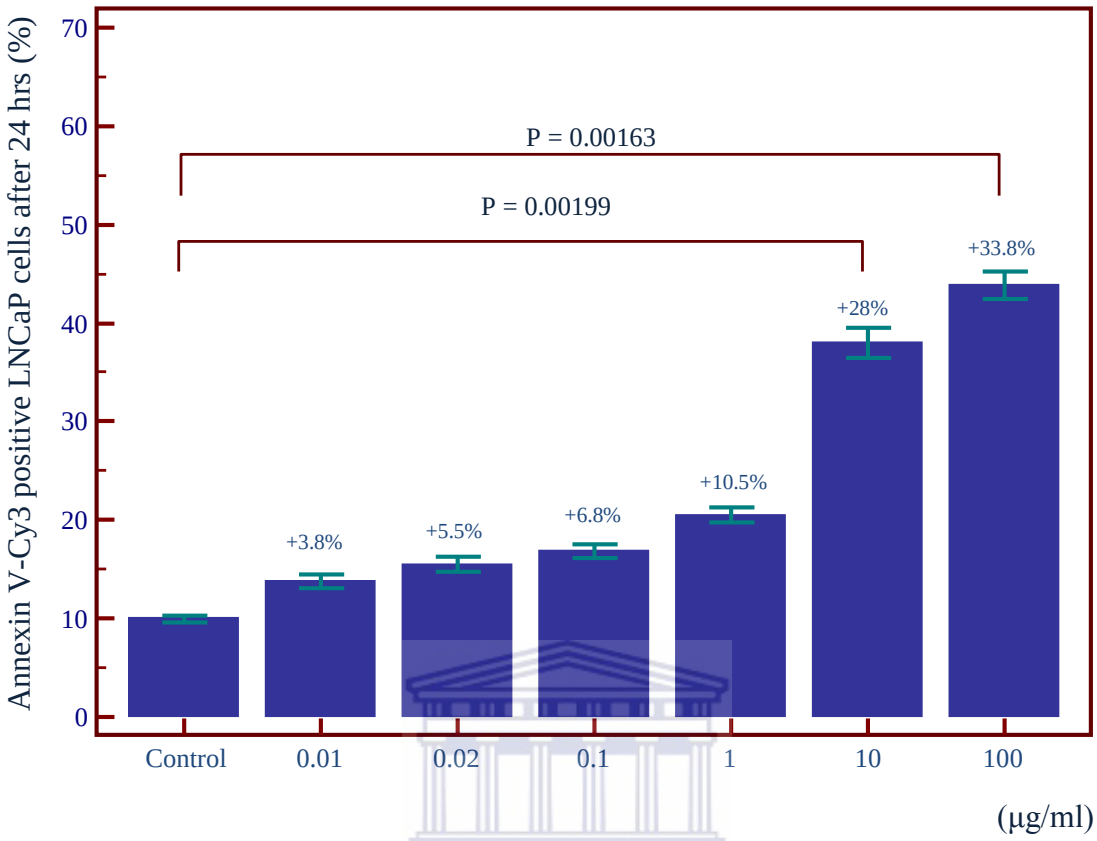


Figure 55: Effect of *T. capensis* rhizome extract F1 fraction of the summer season (0.01, 0.02, 0.1, 1, 10 and 100 µg/ml) on Annexin V-Cy3 binding indicating cell apoptosis in LNCaP cells. After exposure for 24 hours at low concentrations (0.01, 0.02, 0.1 and 1 µg/ml), LNCaP cells showed signs of early apoptosis. At higher concentrations (10 and 100 µg/ml), the increase of +28% (P=0.00199) and +33.8% (P=0.00163), respectively, in the percentage of cells with signs of early apoptosis is sinificant. One-way ANOVA exhibited a significant positive trend (ANOVA trend analysis: P<0.0001).

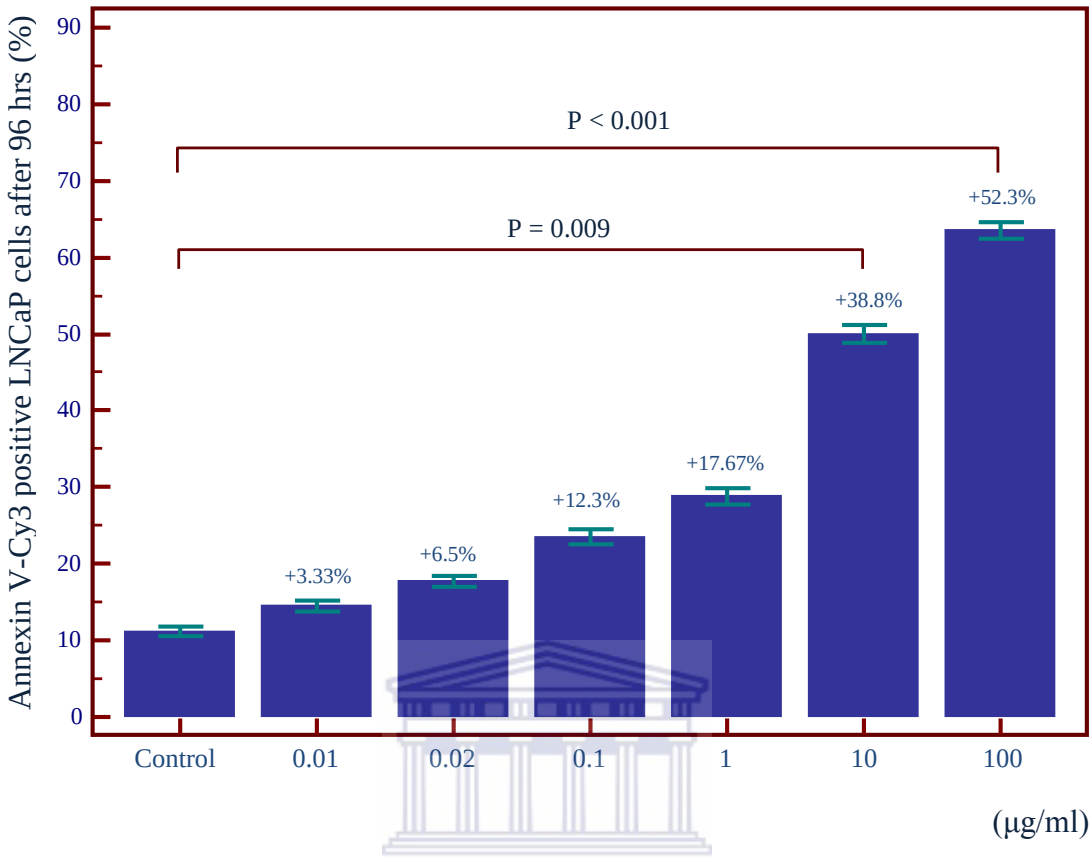


Figure 56: Effect of *T. capensis* rhizome extract F1 fraction of the summer season (0.01, 0.02, 0.1, 1, 10 and 100 µg/ml) on Annexin V-Cy3 binding indicating cell apoptosis in LNCaP cells. After 96 hours of exposure, cells showed a dose-dependent increase in early apoptosis when compared to the control. At higher concentrations (10 and 100 µg/ml) a significant increase of +38.8% (P=0.009) and +52.3% (P<0.0001), respectively in the percentage of cells with signs of early apoptosis is evident. One-way ANOVA exhibited a significant positive trend after exposure for 96 hours (ANOVA trend analysis: P<0.0001).

3.3.4.3 Effect of *T. capensis* rhizome extract F1 fraction of the summer season on Annexin V-Cy3 binding in PWR-1E cells

The effect of *T. capensis* rhizome extract F1 fraction of the summer season on Annexin V-Cy3 binding on Annexin V-Cy3 binding to PWR-1E cells as an indicator of apoptosis was analysed after exposure to increasing concentrations of the F1 fraction of the summer season (0.01, 0.02, 0.1, 1, 10 and 100 µg/ml). After exposure for 24 hours at low concentrations (0.01, 0.02, 0.1 and 1 µg/ml) no change in Annexin V-Cy3 binding compared to the control was observed. At higher concentrations (100 µg/ml), a slight increase of 3.67% (P=0.077) in the percentage of cells with signs of early apoptosis was found. One-way ANOVA exhibited no trend after exposure for 24 hours (ANOVA trend analysis: P=0.507) (Figure 57).

After exposure for 96 hours, exposure at low concentrations (0.01, 0.02, 0.1 and 1 µg/ml) revealed no change in Annexin V-Cy3 binding when compared to the control. At higher concentrations (100 µg/ml), an increase of 5.36% (P=0.046) in the percentage of cells with signs of early apoptosis was found. One-way ANOVA exhibited no trend after exposure for 96 hours (ANOVA trend analysis: P=0.316) (Figure 58).

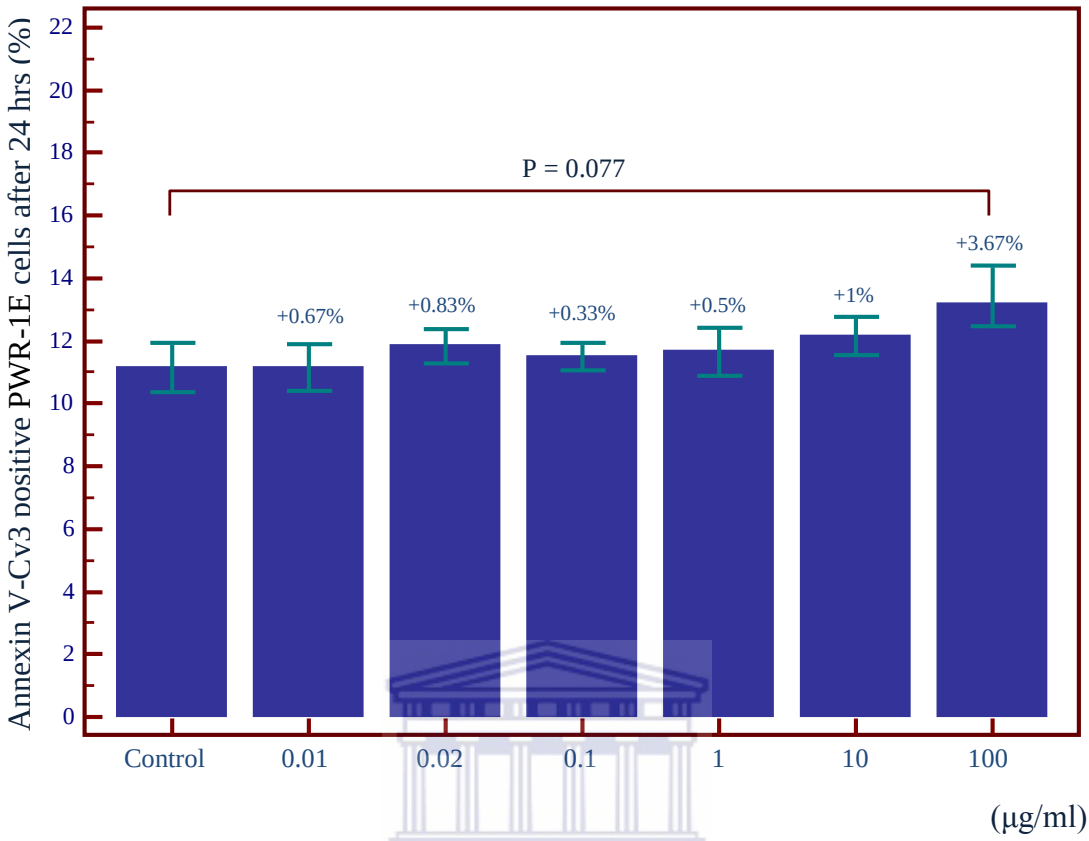


Figure 57: Effect of *T. capensis* rhizome extract F1 fraction of the summer season (0.01, 0.02, 0.1, 1, 10 and 100 µg/ml) on Annexin V-Cy3 binding as indicator of apoptosis in PWR-1E cells. After exposure for 24 hours at low concentrations (0.01, 0.02, 0.1 and 1 µg/ml), no change in Annexin V-Cy3 binding when was observed. At higher concentrations (100 µg/ml), an increase of 3.67% (P=0.077) in the percentage of cells early apoptosis were found. Yet, this increase was not significant. One-way ANOVA exhibited no trend after exposure for 24 hours (ANOVA trend analysis: P=0.507).

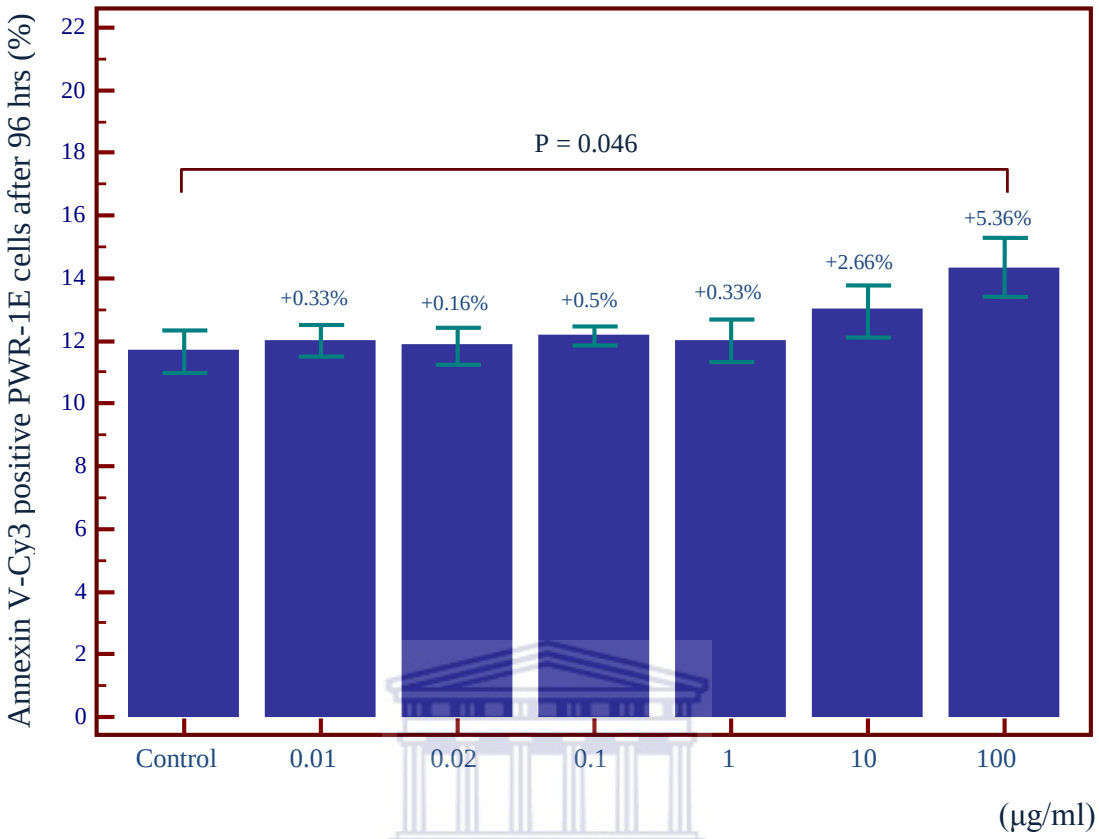


Figure 58: Effect of *T. capensis* rhizome extract F1 fraction of the summer season (0.01, 0.02, 0.1, 1, 10 and 100 µg/ml) on Annexin V-Cy3 binding in PWR-1E cells. After exposure for 96 hours at low concentrations (0.01, 0.02, 0.1 and 1 µg/ml), no change in Annexin V binding is evident. At higher concentrations (100 µg/ml), a significant increase of 5.36% (P=0.046) in the percentage of cells with signs of early apoptosis is found. One-way ANOVA exhibited no trend after exposure for 96 hours (ANOVA trend analysis: P=0.316).

3.3.5 Effects of *T. capensis* rhizome extract F1 fraction of the summer season on DNA fragmentation determined by means of the TUNEL assay

3.3.5.1 DNA fragmentation in TM3-Leydig cells

TM3-Leydig cells were incubated with increasing concentrations of *T. capensis* rhizome extract F1 fraction of the summer season (0.01, 0.02, 0.1, 1, 10 and 100 μg/ml) and analysed for DNA fragmentation.

After exposure for 24 hours at higher concentrations (10, 100 μg/ml), TM3 cells showed an increase of +2.16% (P=0.095) and +3.4% (P=0.079), respectively, TUNEL-positivity indicating DNA damage. Yet these increases were not significant. One-way ANOVA exhibited no trend (ANOVA trend analysis: P=0.612) (Figure 59).

Exposure for 96 hours to higher concentrations (10, 100 μg/ml), caused increase of +2.33% and +6.5%, respectively, TUNEL-positivity indicating DNA damage, still these increasing were not significant at of 10 μg/ml (P=0.072); it become significant as of 100 μg/ml (P=0.031). One-way ANOVA exhibited no significant trend yet after 96 hours exposure (ANOVA trend analysis: P=0.411) (Figure 60).

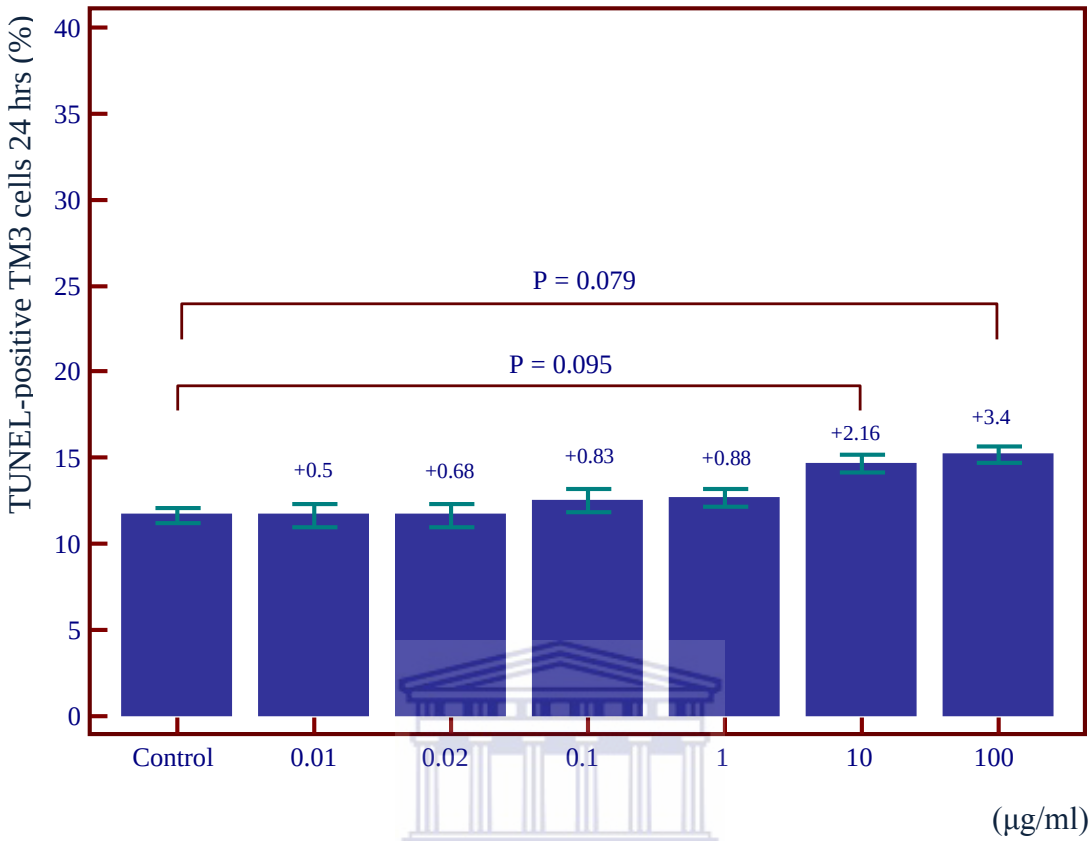


Figure 59: Effect of *T. capensis* rhizome extract F1 fraction of the summer season (0.01, 0.02, 0.1, 1, 10 and 100 µg/ml) on DNA fragmentation in TM3-Leydig cells. After exposure for 24 hours at higher concentrations (10, 100 µg/ml) TM3 cells showed an increase of +2.16% (P=0.095) and +3.4% (P=0.079), respectively, TUNEL-positivity indicating DNA damage. This increase is not significant. One-way ANOVA exhibited no trend (ANOVA trend analysis: P=0.612).

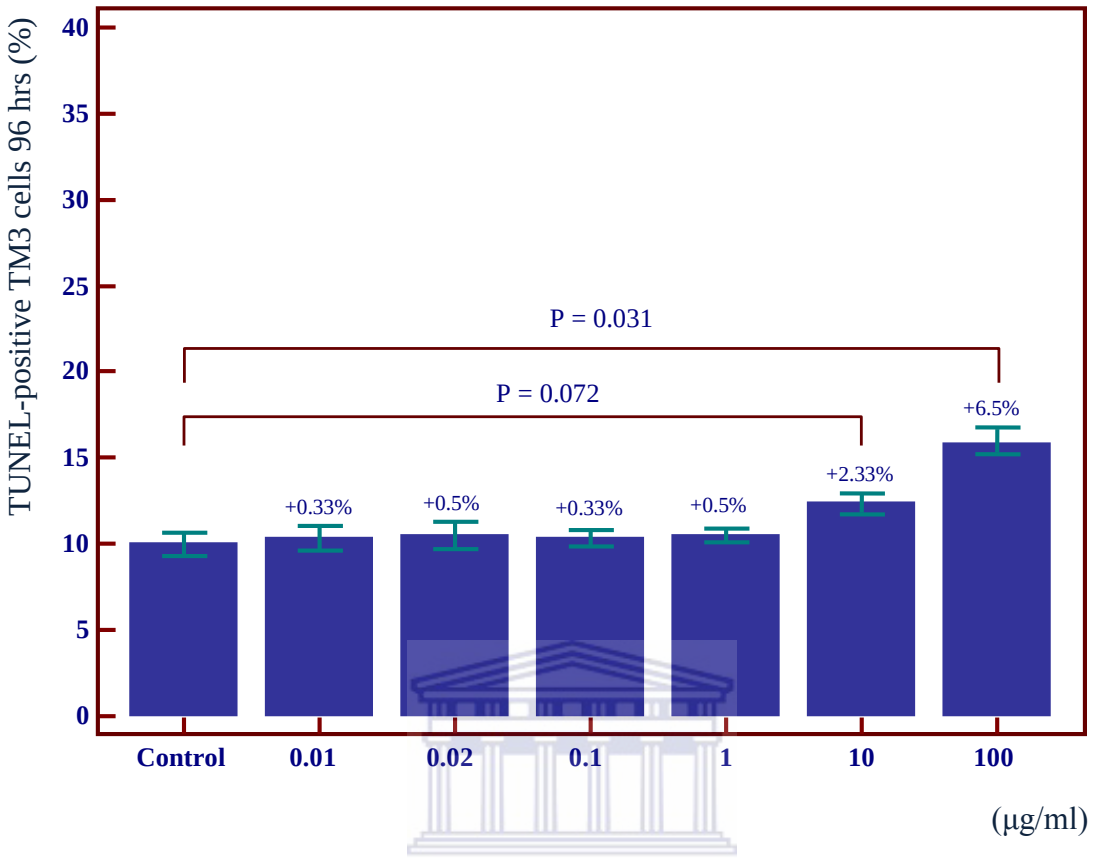


Figure 60: Effect of *T. capensis* rhizome extract F1 fraction of the summer season (0.01, 0.02, 0.1, 1, 10 and 100µg/ml) on DNA fragmentation in TM3-Leydig cells. Exposure for 96 hours to higher concentrations (10, 100 µg/ml) caused increases of +2.33% and +6.5%, respectively, TUNEL-positivity indicating DNA damage. Yet, these increases are not significant as at concentration of 10 µg/ml (P=0.072). Only from 100 µg/ml, the change was significant (P=0.031). One-way ANOVA exhibited no significant trend after 96 hours exposure (ANOVA trend analysis: P=0.411).

3.3.5.2 DNA fragmentation in LNCaP cells

LNCaP cells were incubated with increasing concentrations of *T. capensis* rhizome extract F1 fraction of the summer season (0.01, 0.02, 0.1, 1, 10 and 100 μg/ml) and analysed for DNA fragmentation.

After exposure of LNCaP cells for 24 hours at higher concentrations (1, 10, 100 μg/ml) the percentage of TUNEL-positive cells increased by +14%, +21.6% and +29%, respectively, in a dose-dependent manner. No significant change between the control and 0.1 μg/ml (P=0.066) was found. However, a significant dose-dependent increase in TUNEL-positive cells was found between the control and 1, 10 and 100 μg/ml (P=0.0047, P=0.003 and P=0.001, respectively). One-way ANOVA exhibited a significant trend (ANOVA trend analysis: P<0.0001) (Figure 61).

After exposure for 96 hours, LNCaP cells exhibited an increasing percentage of TUNEL-positive cells at higher concentrations (1, 10, 100 μg/ml) of +16.67, +25.6 and +34%, respectively. No significant effect between the control and 0.1 μg/ml (P=0. 0624). However, a significant increase of TUNEL-positive cells was found between the control and 1, 10, 100 μg/ml (P=0.0028, P=0.001 and P<0.0001, respectively). One-way ANOVA exhibited a significant trend (ANOVA trend analysis: P<0.0001) (Figure 62).

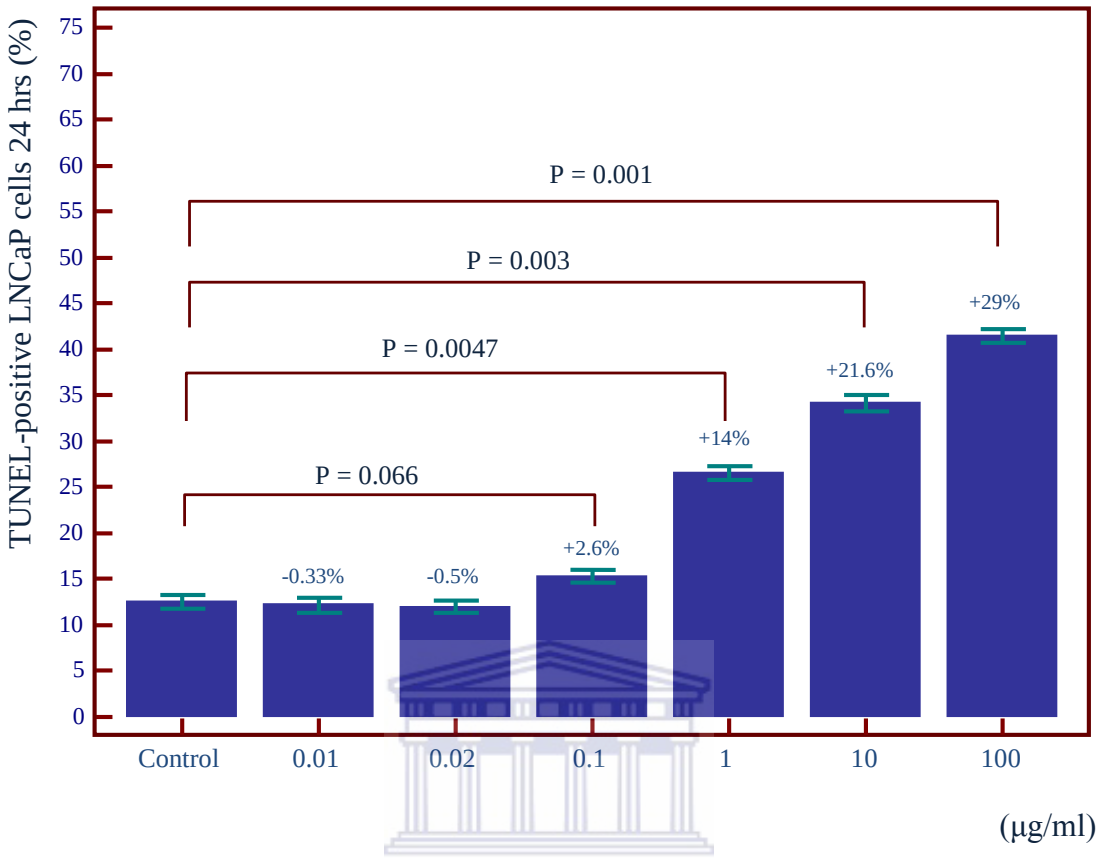


Figure 61: Effect of *T. capensis* rhizome extract F1 fraction of the summer season (0.01, 0.02, 0.1, 1, 10 and 100 µg/ml) on DNA fragmentation in LNCaP cells. After exposure of LNCaP cells for 24 hours at higher concentrations (1, 10, 100 µg/ml), a dose-dependent increase in the percentage of TUNEL-positive cells by 14%, 21.6% and 29%, respectively, is seen. This is no differance between the control and the extract concentration of 0.1 µg/ml (P=0.066). However, a significant dose-dependent increase of TUNEL-positive cells was found between the control and 1, 10 and 100 µg/ml (P=0.0047, P=0.003 and P=0.001, respectively). One-way ANOVA exhibited a significant trend (ANOVA trend analysis: P<0.0001).

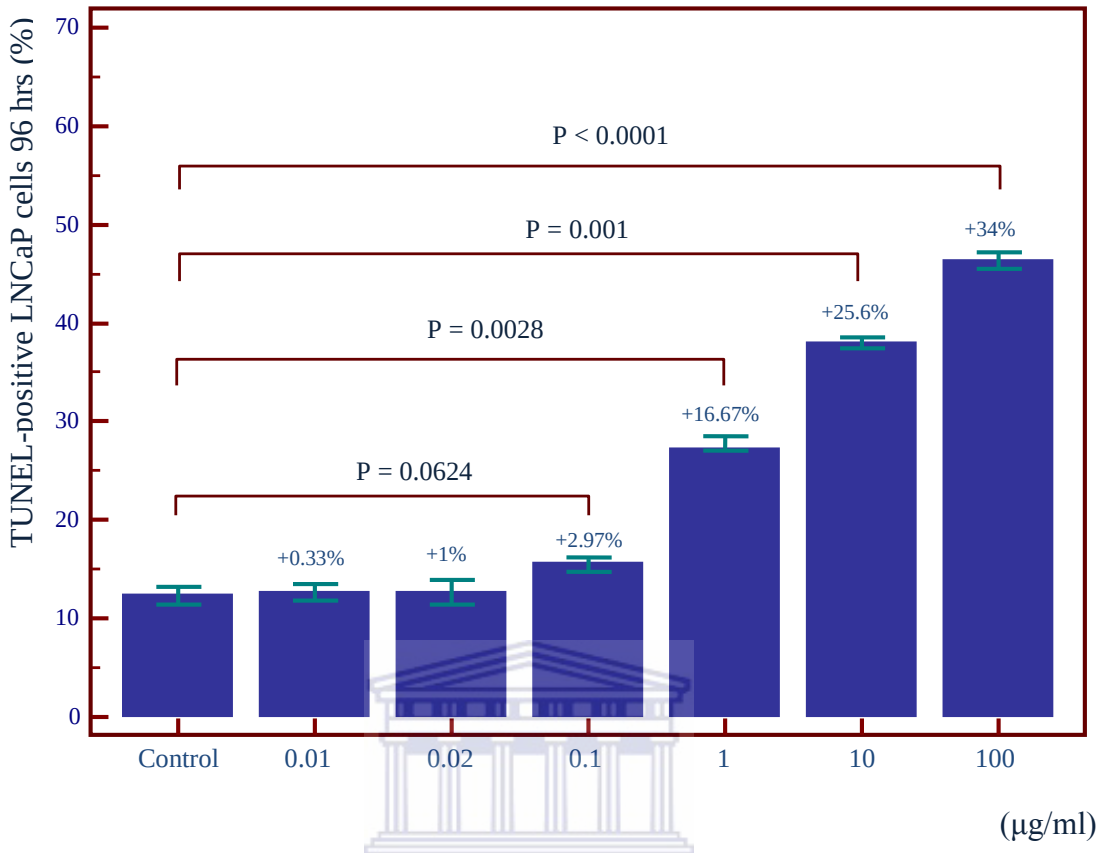


Figure 62: Effect of *T. capensis* rhizome extract F1 fraction of the summer season (0.01, 0.02, 0.1, 1, 10 and 100 µg/ml) on DNA fragmentation in LNCaP cells. After exposure for 96 hours, LNCaP cells exhibited an increasing percentage of TUNEL-positive cells at higher concentrations (1, 10, 100 µg/ml) of 16.67, 25.6 and 34%, respectively. No significant effect between the control and 0.1 µg/ml (P=0.0624) is evident. However, a significant increase of TUNEL-positive cells was found between the control and 1, 10, 100 µg/ml (P=0.0028, P=0.001and P<0.0001, respectively). One-way ANOVA exhibited a significant trend (ANOVA trend analysis: P<0.0001).

3.3.5.3 DNA fragmentation in PWR-1E cells

PWR-1E cells were incubated with increasing concentrations of *T. capensis* rhizome extract F1 fraction of the summer season (0.01, 0.02, 0.1, 1, 10 and 100 µg/ml) and analysed for DNA fragmentation.

After exposure of PWR-1E cells for 24 hours to the extract at low concentrations (0.01, 0.02, 0.1, 1 and 10 µg/ml) no increase in the percentage of TUNEL-positive cells was found. At the highest concentration (100 µg/ml), a slight, not significant, increase of 3.25% (P=0.087) indicated signs of DNA damage. One-way ANOVA exhibited no trend for TUNEL-positive cells. (ANOVA trend analysis: P=0.657) (Figure 63).

After 96 hours of exposure at low concentrations (0.01, 0.02, 0.1, 1 and 10 µg/ml), no increase in the percentage of TUNEL-positive cells was observed. At higher concentrations (100 µg/ml), a significant increase in the percentage of TUNEL-positive cells of 7.1% (P=0.0024) indicates DNA damage. One-way ANOVA exhibited no trend (ANOVA trend analysis: P=0. 422) (Figure 64).

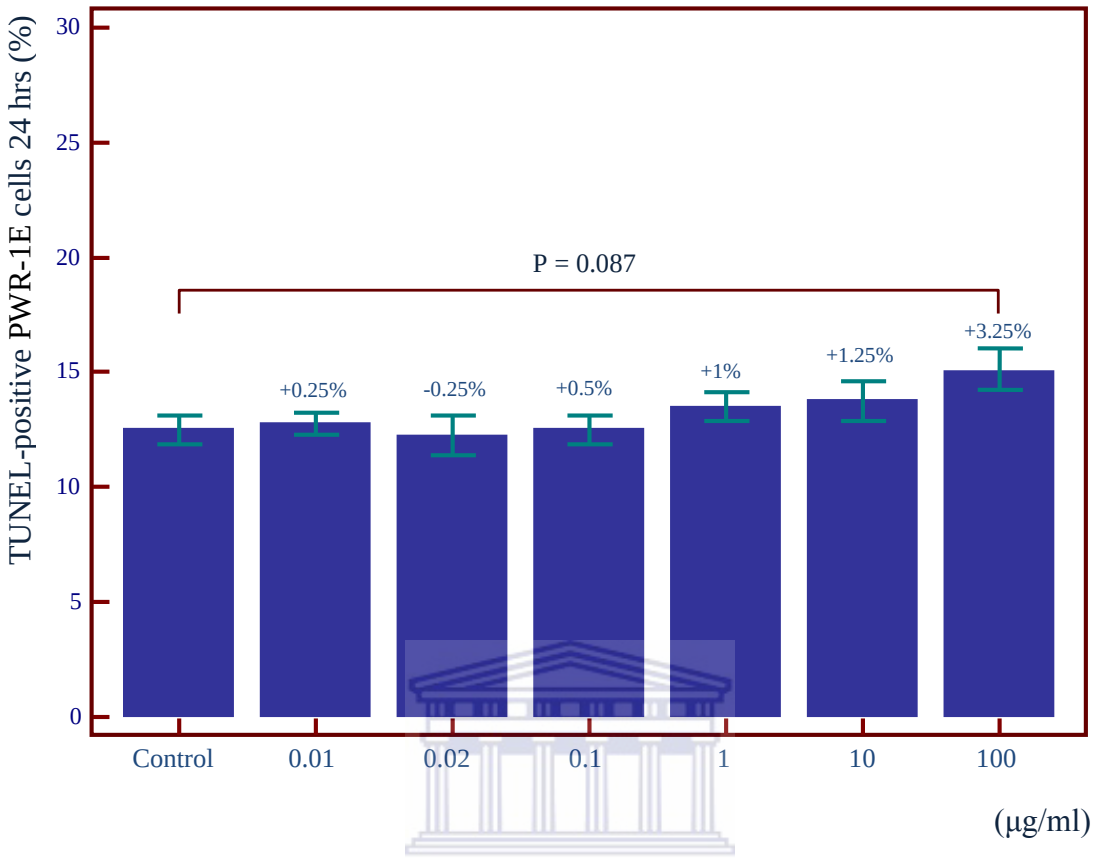


Figure 63: Effect of *T. capensis* rhizome extract F1 fraction of the summer season (0.01, 0.02, 0.1, 1, 10 and 100 µg/ml) on DNA fragmentation at PWR-1E cells. After exposure of PWR-1E cells for 24 hours at low concentrations (0.01, 0.02, 0.1, 1 and 10 µg/ml) no increase in the percentage of TUNEL-positive cells can be observed. At the highest concentration (100 µg/ml), a slight, not significant, increase of 3.25% (P=0.087) indicates signs of DNA damage. One-way ANOVA exhibites no trend (ANOVA trend analysis: P=0.657).

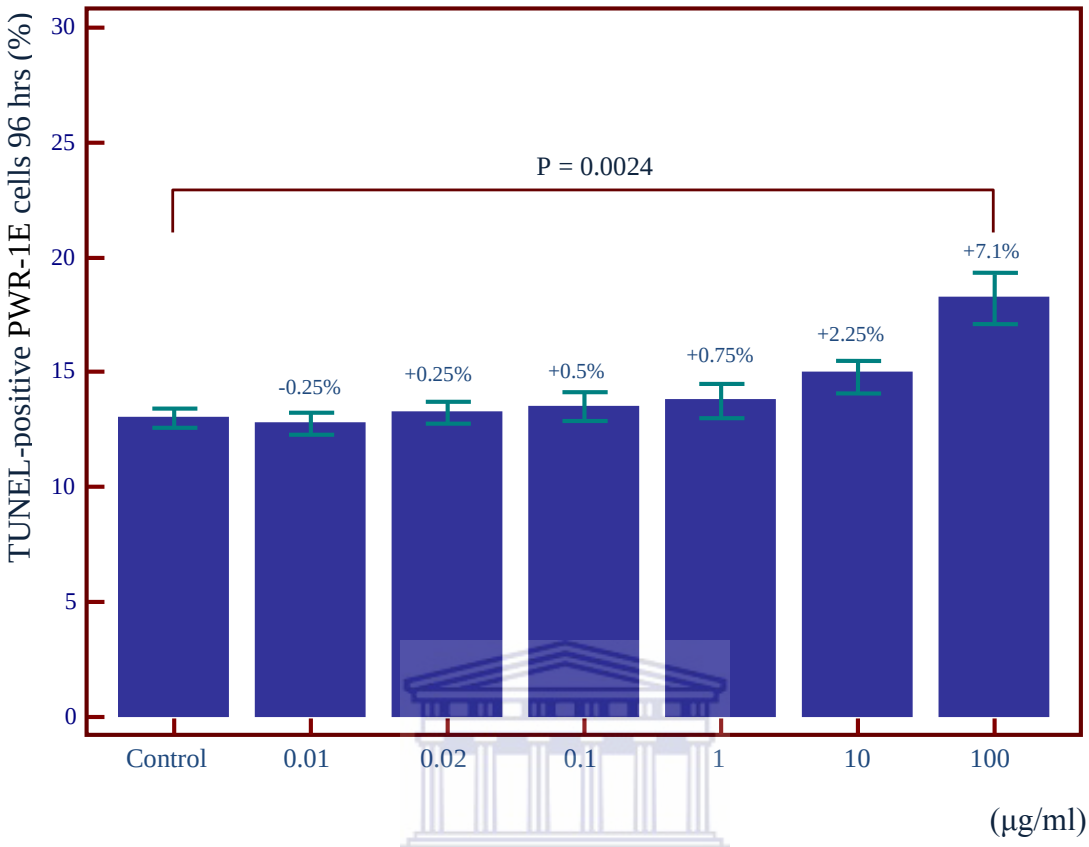


Figure 64: Effect of *T. capensis* rhizome extract F1 fraction of the summer season (0.01, 0.02, 0.1, 1, 10 and 100 µg/ml) on DNA fragmentation of PWR-1E cells. After 96 hours of exposure at low concentrations (0.01, 0.02, 0.1, 1 and 10 µg/ml), no increase in the percentage of TUNEL-positive cells can be seen. At higher concentrations (100 µg/ml), a significant increase of 7.1% (P=0.0024) in the percentage of TUNEL-positive cells indecates DNA damage. One-way ANOVA exhibited no trend (ANOVA trend analysis: P=0.422).

3.4 Effect of active compounds (Quercetin and Naringenin) on TM3-Leydig cells and LNCaP cells viability (acute and chronic exposure)

In this particular study, HPLC analysis of 1g of *T. capensis* rhizome extract yielded about 200 mg of F1 fraction. According to the HPLC profile of sub-fraction of F1 fraction from the summer harvest of *T. capensis* rhizome extract, 63% was Quercetin and 37% was Naringenin. The concentrations of Querceting and Naringenin used in the *in vitro* study were calculated according to the actual amounts of these compounds found in the extact and their respective ratios.

3.4.1 Effect of Quercetin on TM3-Leydig cell viability

After TM3-Leydig cells were incubated (acute exposure) with increasing concentrations, (0.012, 0.037, 0.062, 0.087, 0.112 and 0.137 µg/ml), of quercetin, which is one of the biologically active compounds in *T. capensis* rhizomes for 24 and 96 hours, cell viability was tested by means of the MTT test and cell morphology was observed and recorded. After 24 and 96 hours of acute exposure, quercetin resulted in no observable morphological changes of the flat and polygonal TM3 cells. However, after 96 hours of chronic exposure, morphological changes in the flat and polygonal cells had occurred between the control groups and higher concentrations (> 0.112 µg/ml) indicating cellular stress (Figure 65).

After 24 hours exposure, quercetin showed no significant effect on cell viability at all concentrations. No significant effect between the control and the highest concentration used 0.137 µg/ml was observed (P=0.063) (Figure 66 A).

After 96 hours of exposure (acute exposure), cell viability was observed and recorded. At all concentrations, quercetin showed no significant effect on cell viability compared to the control. In addition, there was no significant difference between the control and the highest concentration, 0.137 µg/ml (P=0.054) (Figure 66 B).

After 96 hours of chronic quercetin exposure, cell viability for all concentrations decreased when compared to the control. The cells began showing visible signs of stress. A significant decrease of

-21% was observed between the control and the highest concentration, (0.137 µg/ml) (P=0.001) (Figure 66 C).

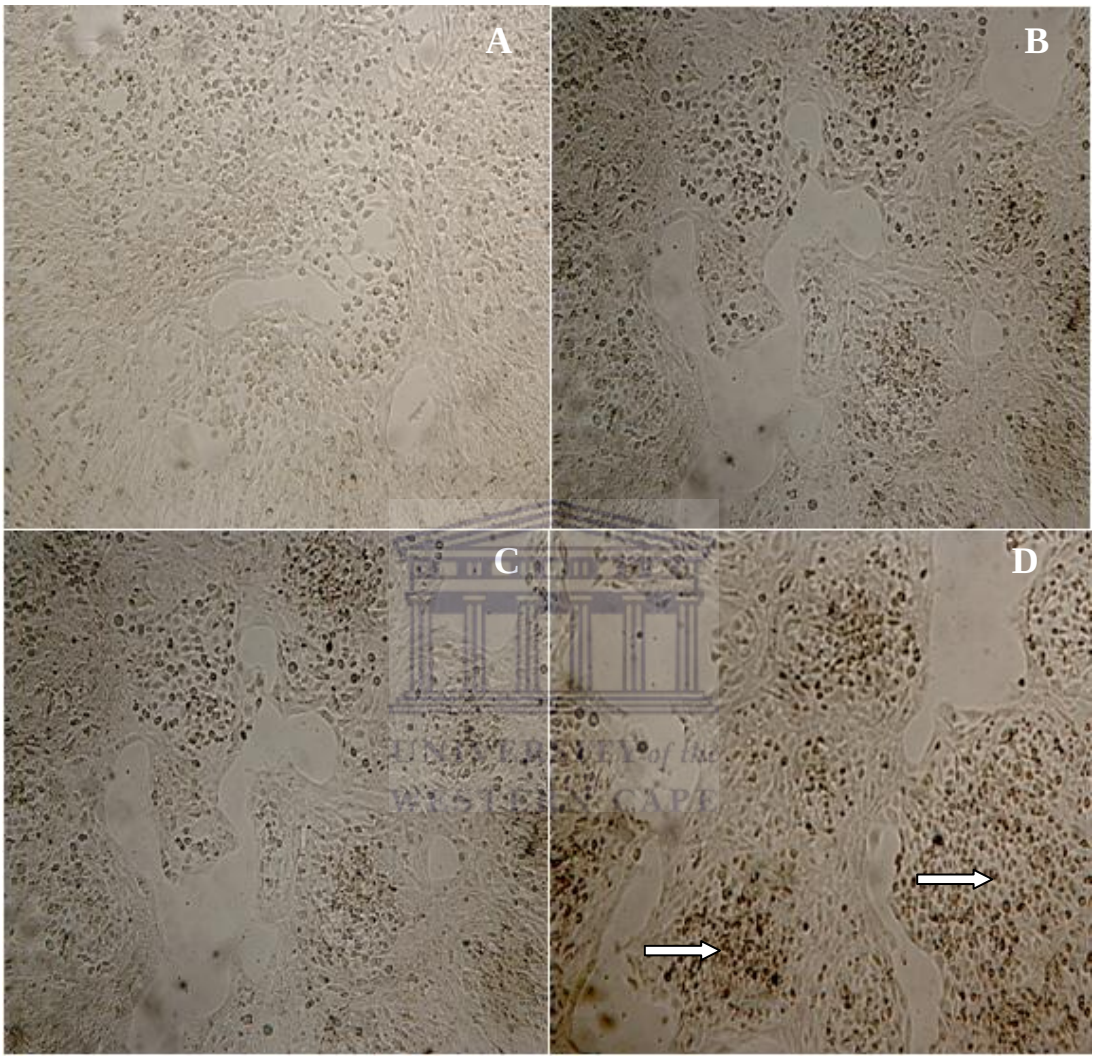


Figure 65: TM3-Leydig cell morphology after being exposed with increasing concentrations of Quercetin (0.012, 0.037, 0.062, 0.087, 0.112 and 0.137 µg/ml). After 24 and 96 hours of acute exposure, Quercetin resulted in no observable morphological changes in the flat and polygonal cells of TM3 cells. However, after 96 hours of chronic exposure, morphological changes in the flat and polygonal cells had occurred at higher concentrations (> 0.112 µg/ml) indicating cellular stress. The white arrows indicating cellular stress (10 x magnifications). A: control. B: 24 hours acute exposure > 0.112 µg/ml. C: 96 hours acute exposure > 0.112 µg/ml. D: 96 hours acute exposure > 0.112 µg/ml.

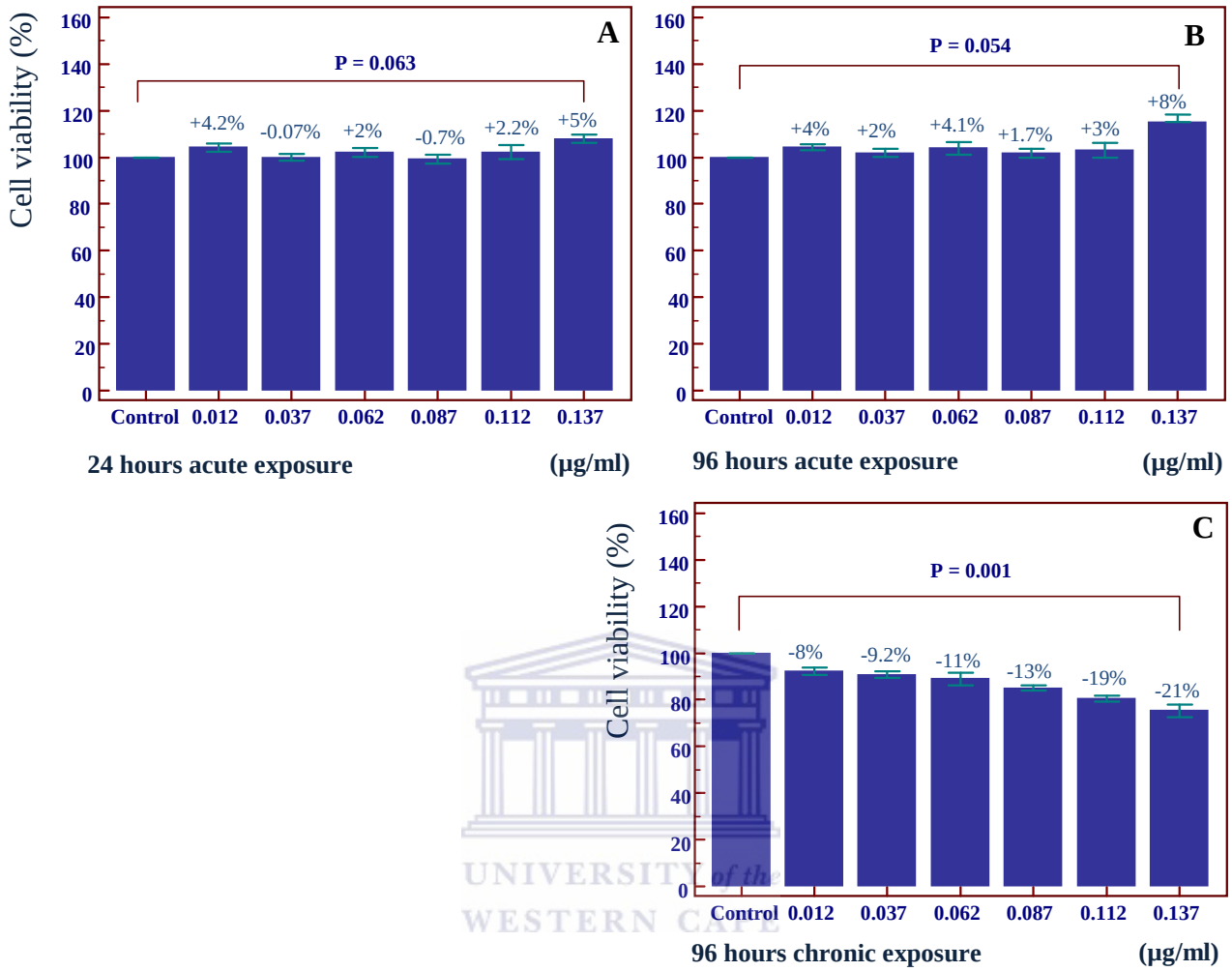


Figure 66: TM3-Leydig cell viability (determined by means of the MTT assay) after Quercetin exposure.

A: Incubation for 24 hours of acute exposure with Quercetin. At all concentrations, no significant difference in cell viability compared to the control is evident. No significant effect between the control and the highest concentration, (0.137 µg/ml) (P=0.063) can be seen.

B: Incubation for 96 hours of acute exposure with Quercetin. At all concentrations, no significant effect in cell viability can be seen.

C: Incubation for 96 hours of chronic exposure with Quercetin. Viability for all concentrations decreased significantly in dose-dependent manner, with a maximum effect at higher concentration used (0.137 µg/ml) (P=0.001).

3.4.2 Effect of Naringenin on TM3-Leydig cell viability

TM3-Leydig cells were incubated with different concentrations (0.013, 0.038, 0.063, 0.088, 0.113 and 0.138 µg/ml) of Naringenin, which is the other bioactive compound that was identified in *T. capensis* rhizomes extract. After 24 hours exposure, cell viability was tested by means of the MTT test. At all concentrations of Naringenin, no significant change in cell viability was observed. No significant effect between the control and the highest concentration (0.138 µg/ml) (P=0.095) (Figure 67 A) could be found.

After 96 hours of acute exposure, the cell viability was observed and recorded. At all concentrations of Naringenin, no significant change in cell viability compared to the control was found (Figure 67 B).

After 96 hours of chronic Naringenin exposure, viability for all concentrations decreased when compared to the control. The cells began showing visible signs of stress. A significant decrease of -20% was found between the control and 0.138 µg/ml (P=0.0028) (Figure 67 C).

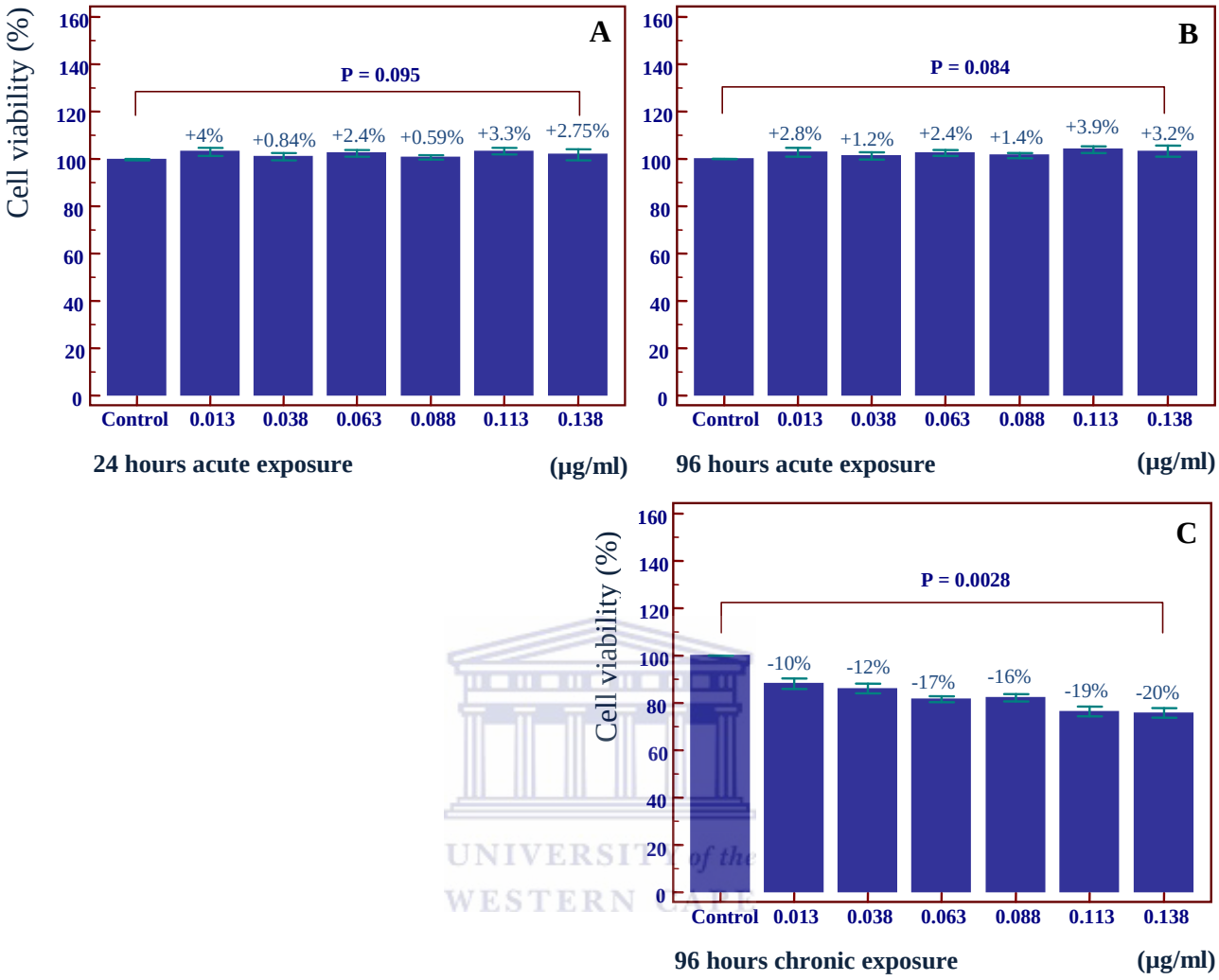


Figure 67: TM3-Leydig cell viability (by means of the MTT assay) after Naringenin exposure.

A: Incubation for 24 hours with Naringenin (acute exposure). At all concentrations of Naringenin, no significant change in cell viability can be seen.

B: Incubation for 96 hours with Naringenin (acute exposure). At all concentrations of Naringenin, no significant change in cell viability observed.

C: Incubation for 96 hours with Naringenin (chronic exposure). Viability at all concentrations decreased in a dose-dependent manner with a maximum effect of -20% between the control and 0.138 µg/ml (P=0.0028).

3.4.3 Effect of mixture (Quercetin and Naringenin) on TM3-Leydig cell viability

After TM3-Leydig cells were incubated with increasing concentrations of a mixture of Quercetin and Naringenin (0.025, 0.050, 0.075, 0.100, 0.125, 0.150, 0.175 and 0.200 µg/ml) for 24 hours, cell viability was determined by means of the MTT test. At all concentrations, no effect on cell viability could be observed (Figure 68 A).

After 96 hours of acute exposure, cell viability was observed and recorded. At all concentrations, the mixture Quercetin and Naringenin exhibited no significant effect on cell viability (Figure 68 B).

After 96 hours of chronic exposure of TM3 cells to the mixture of Quercetin and Naringenin, viability for all concentrations decreased in a dose-dependent manner. The cells began showing visible signs of stress. With a biggest decrease of -24% at the higher concentration (0.200 µg/ml) ($P<0.001$) (Figure 68 C).

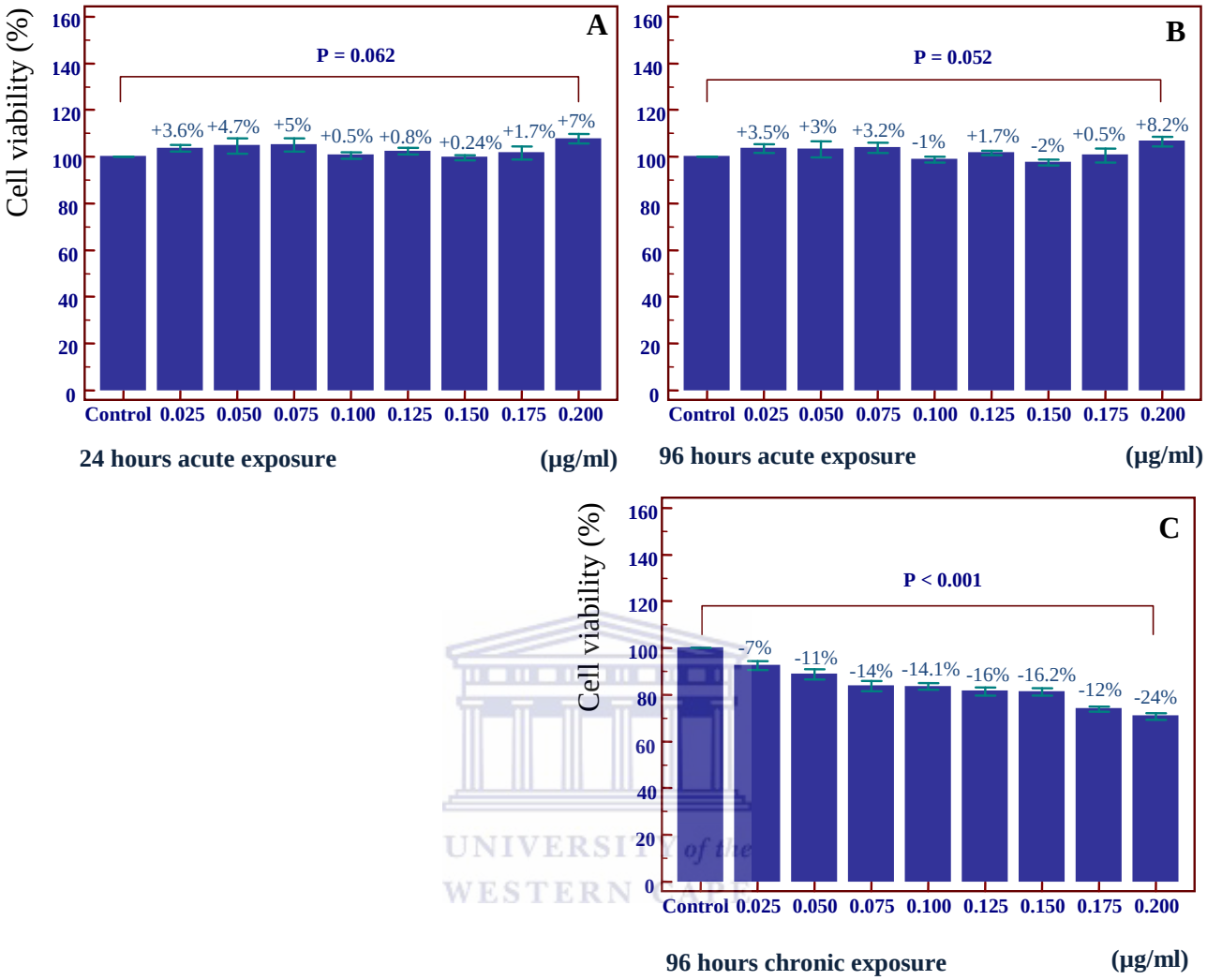


Figure 68: TM3 cell viability (determined by means of the MTT assay) after exposure to the mixture of Quercetin and Naringenin.

A: Incubation for 24 hours with the mixture (acute exposure). At all concentrations, no effect on cell viability can be seen. No significant effect can be found between the control and the highest concentration, (0.200 µg/ml) (P=0.062).

B: Incubation for 96 hours of acute exposure. At all concentrations, no effect on cell viability can be observed.

C: Chronic exposure for 96 hours. Viability for all concentrations decreased significantly and dose-dependently with a maximum effect of -24% between the control and 0.200 µg/ml (P=0.001).

3.4.4 Effect of Quercetin on LNCaP cell viability

After LNCaP cells were incubated with increasing concentrations of Quercetin (0.012, 0.037, 0.062, 0.087, 0.112 and 0.137 μg/ml) for 24 and 96 hours, cell viability was tested by means of the MTT test and the cell morphology was observed and recorded. At 24 and 96 hours of acute exposure, Quercetin caused a significant decrease in cell viability, with changes in cell morphology particularly at the higher concentrations (0.112 and 0.137 μg/ml). There was a clear increase in detachment and cell death. At 96 hours of chronic exposure, cell viability for all concentrations decreased dose-dependently. There was a clear increase in detachment and clumping of cells and apoptotic bodies could also be seen indicating cell death. In particular at higher concentrations (0.112 and 0.137 μg/ml) there was a clear increase in detachment and cell death (Figure 69).

After 24 hours of acute exposure, cell viability was tested by means of the MTT test. All concentrations showed a significant dose-dependent decrease in cell viability up to -31% and -34%, respectively, at the highest concentrations (0.112 and 0.137 μg/ml). A significant decrease was observed between the control and the highest concentration, 0.137 μg/ml (P =0.0014) (Figure 70 A).

After 96 hours of acute Quercetin exposure, cell viability was observed and recorded. At all concentrations, a significant, dose-dependent decrease in cell viability was evident, particularly at highest concentrations, with a decrease of -35% and -40%, respectively. This decrease was significant (P<0.0001) (Figure 70 B).

After 96 hours of chronic exposure to Quercetin, cell viability decreased at all concentrations when compared to the control, with a significant maximum decrease of -58% and -62%, respectively, at the highest concentrations, 0.112 μg/ml (P<0.0001) and 0.137 μg/ml (P<0.0001) (Figure 70 C).

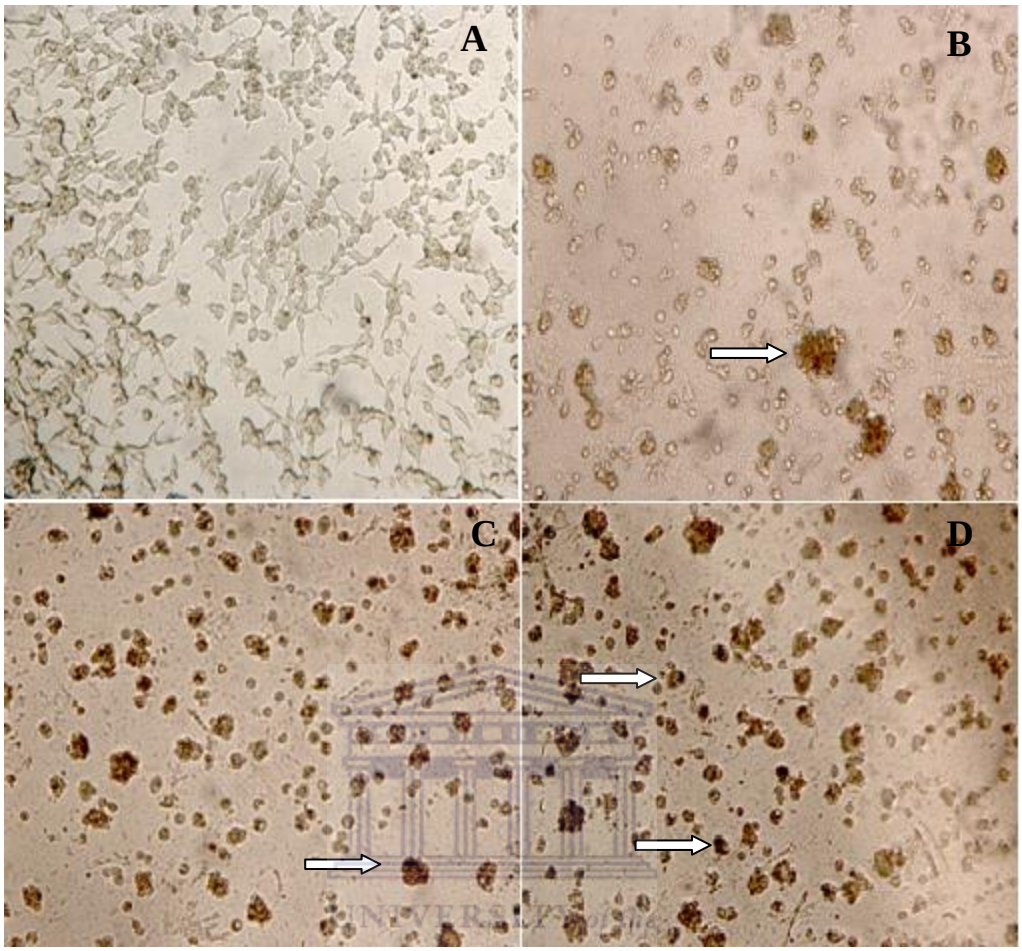


Figure 69: LNCaP cell morphology after cells being exposed to increasing concentrations of Quercetin (0.012, 0.037, 0.062, 0.087, 0.112 and 0.137 µg/ml). The morphology of LNCaP cells was found to have changed dramatically after the exposure. There was a clear increase in detachment and clumping of cells and apoptotic bodies could also be seen indicating cell death particularly at higher concentrations (0.112 and 0.137 µg/ml). There was a clear increase in detachment and cell death. The white arrows are indicating apoptotic bodies (10 x magnifications). A: control. B: 24 hours acute exposure > 0.112 µg/ml. C: 96 hours acute exposure > 0.112 µg/ml. D: 96 hours acute exposure > 0.112 µg/ml.

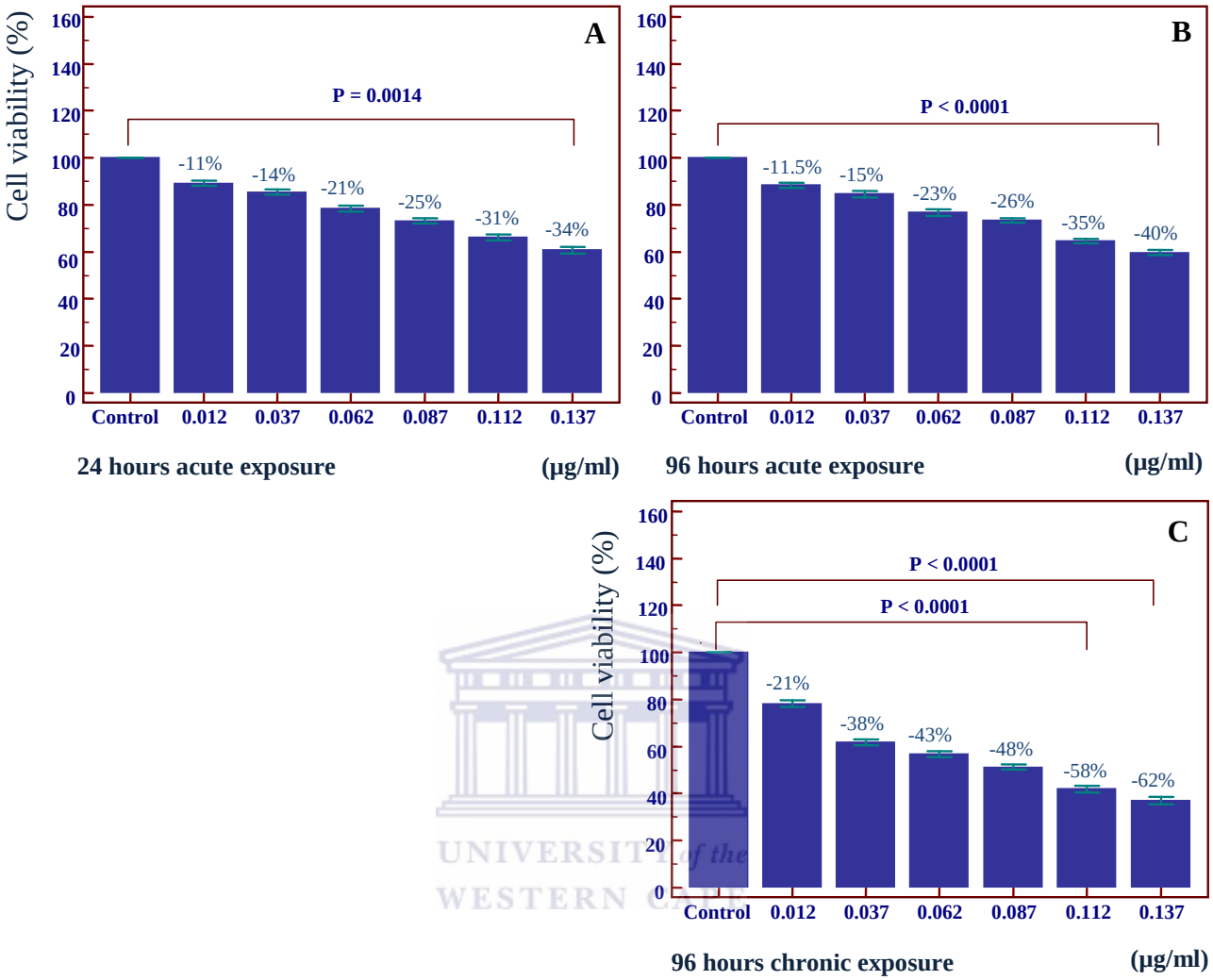


Figure 70: LNCaP cell viability (determined by means of the MTT assay) after Quercetin exposure.

A: Incubation for 24 hours with Quercetin (acute exposure). All concentrations showed a significant dose-dependent decrease in cell viability compared to the control of up to -31% and -34%, respectively, at the highest concentrations (0.112 and 0.137 µg/ml).

B: Incubation for 96 hours with Quercetin (acute exposure). At all concentrations, a significant, dose-dependent decrease in cell viability was evident, particularly at the highest concentrations with decreases of -35% and -40%, respectively.

C: Incubation for 96 hours with Quercetin (chronic exposure). Cell viability decreased at all concentrations when compared to the control, with a significant maximum decrease of -58% and -62%, respectively, at the highest concentrations, 0.112 µg/ml (P<0.0001) and 0.137 µg/ml (P<0.0001).

3.4.5 Effect of Naringenin on LNCaP cell viability

After LNCaP cells were incubated with different concentrations of Naringenin (0.013, 0.038, 0.063, 0.088, 0.113 and 0.138 µg/ml) for 24 hours, cell viability was determined by means of the MTT test. At all concentrations, a significant, dose-dependent decrease in cell viability with maximum values of -34% and -39%, respectively, at higher concentrations (0.113 µg/ml and 0.138 µg/ml) was obvious. (Figure 71 A).

After 96 hours of acute Naringenin exposure, cell viability was observed and recorded. At all concentrations, a significant decrease in cell viability could be seen, with maximum decreases of -36% and -41%, respectively, at the highest concentrations (0.113 and 0.138 µg/ml) (Figure 71 B).

After 96 hours of chronic Naringenin exposure, cell viability for all concentrations decreased when compared to the control, with maximum decreases of -52% and -62%, respectively, at highest concentrations (0.113 and 0.138 µg/ml) (Figure 71 C).

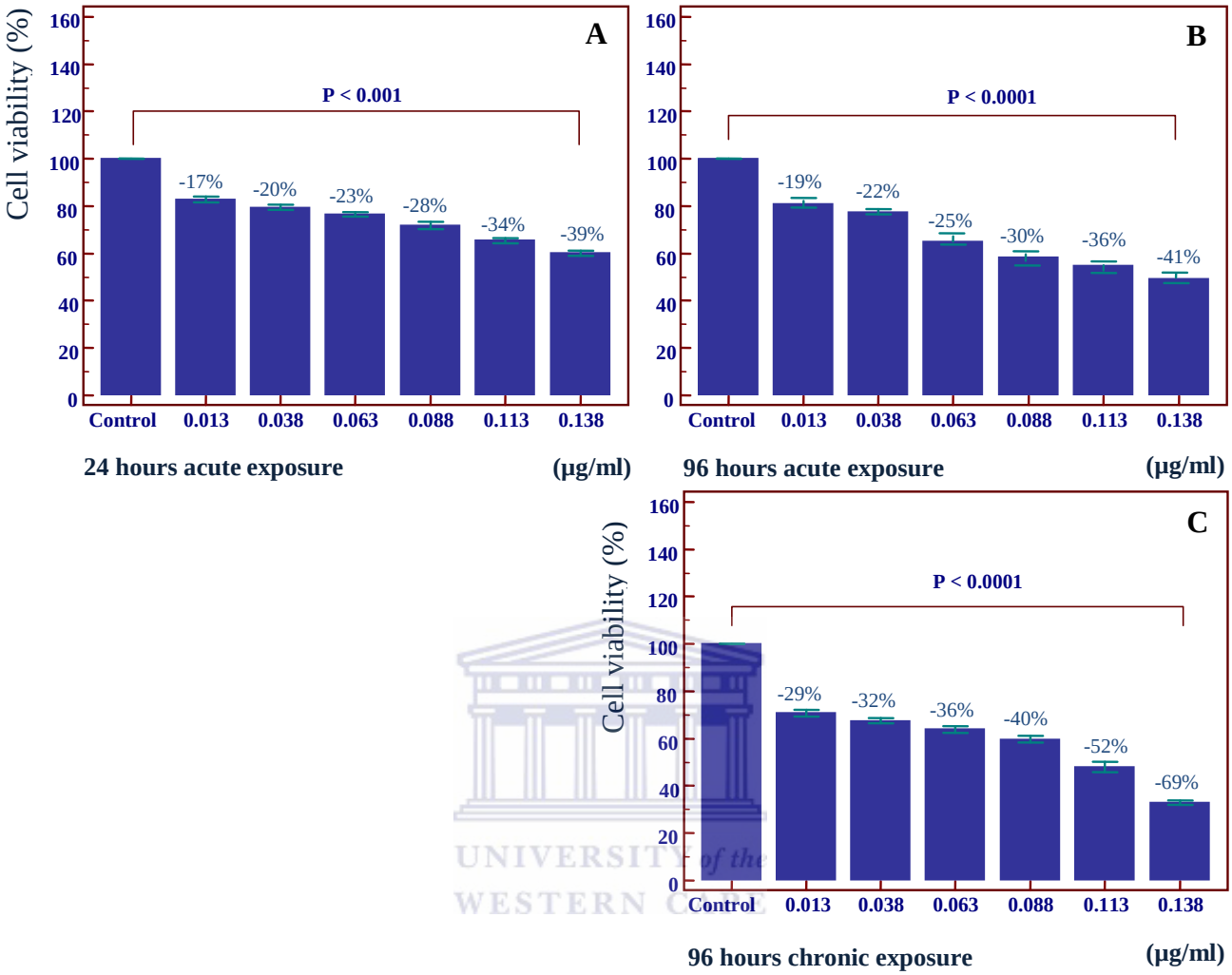


Figure 71: LNCaP cell viability (determined by means of the MTT assay) after Naringenin exposure.

A: Incubation for 24 hours with Naringenin (acute exposure). At all concentrations, a significant dose-dependent decrease in cell viability, with a significant difference between the control and the highest concentration (0.138 µg/ml) (P<0.001) is obvious.

B: Incubation for 96 hours with Naringenin (acute exposure). At all concentrations, a significant decrease in cell viability can be seen. The decrease between the control and the highest concentration (0.138 µg/ml) (P<0.0001) is significant.

C: Incubation for 96 hours with Naringenin (chronic exposure). Cell viability for all concentrations decreased when compared to the control, with maximum decreases of -52% and -62%, respectively, at highest concentrations (0.113 and 0.138 µg/ml). A significant dose-dependent decrease was found between the control and 0.138 µg/ml (P<0.0001).

3.4.6 Effect of mixture (Quercetin and Naringenin) on LNCaP cell viability

After LNCaP cells were incubated with increasing concentrations of a mixture (0.025, 0.050, 0.075, 0.100, 0.125, 0.150, 0.175 and 0.200 µg/ml) of Quercetin and Naringenin for 24 hours, cell viability was determined by means of the MTT test. At all concentration a significant dose-dependent decrease in cell viability compared to the control was seen, with decreases of -41% and -43%, respectively, at the highest concentrations (0.175 and 0.200 µg/ml). A significant decrease was found between the control and the highest concentration, 0.200 µg/ml ($P<0.0001$) (Figure 72 A).

After 96 hours of acute exposure, cell viability also showed a significant dose-dependent decrease in cell viability compared to the control, with maximum values of -51% and -57%, respectively, at the highest concentrations (0.175 and 0.200 µg/ml). A significant decrease observed between the control and the highest concentration, 0.200 µg/ml ($P<0.0001$) (Figure 72 B).

After 96 hours of chronic exposure of the cells to the mixture of Quercetin and Naringenin, cell viability decreased significantly and dose-dependently, with maximum values of -75% and -79%, respectively, at the highest concentrations (0.175 and 0.200 µg/ml). A significant decrease could be seen between the control and the highest concentration, 0.200 µg/ml ($P<0.0001$) (Figure 72 C).

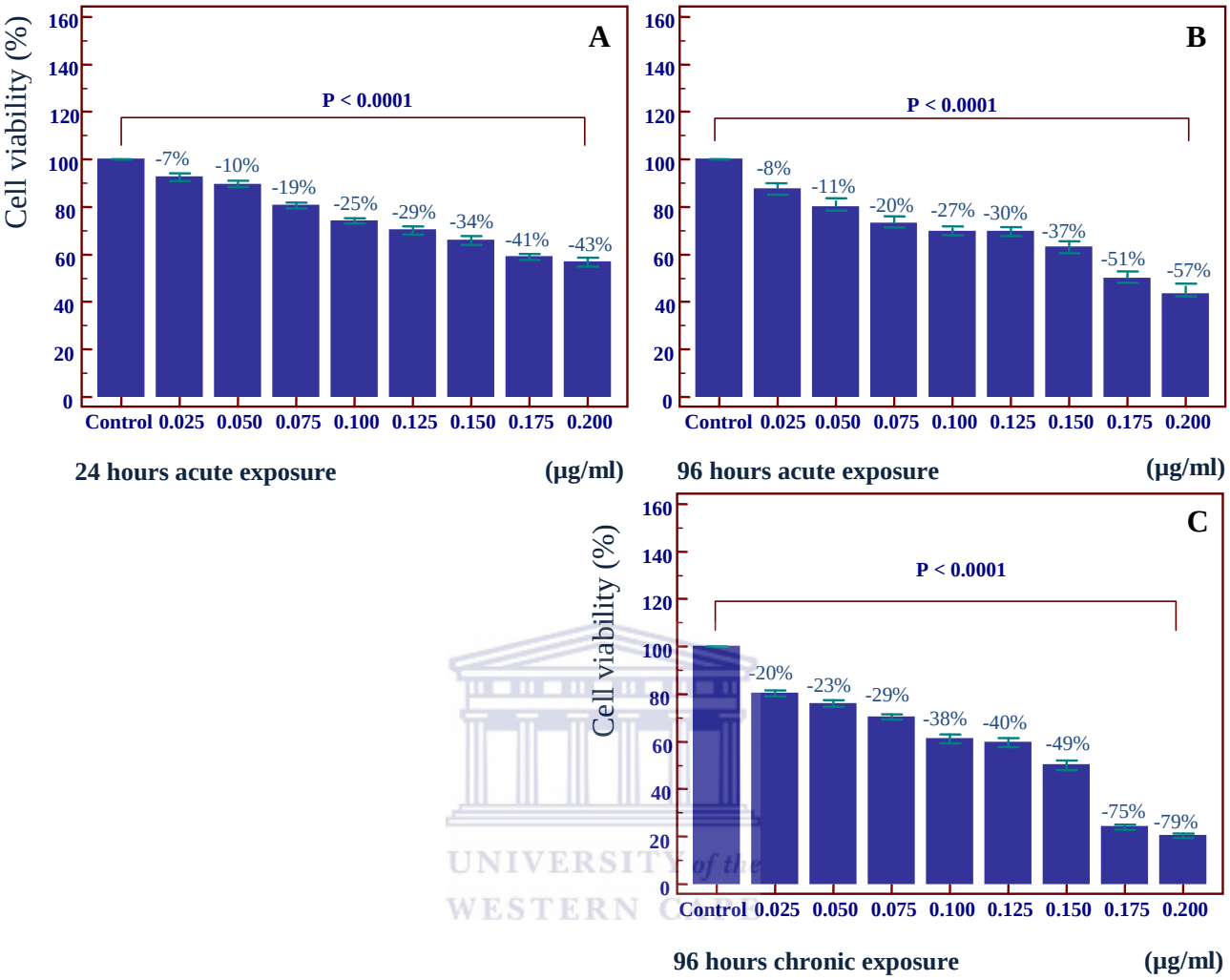


Figure 72: LNCaP cell viability (determined by means of the MTT assay) after exposure to the mixture of Quercetin and Naringenin.

A: Incubation for 24 hours acute exposure with the mixture. At all concentration a significant dose-dependent decrease in cell viability compared to the control is seen. A significant decrease was found between the control and the highest concentration, 0.200 µg/ml ($P<0.0001$).

B: Incubation for 96 hours acute exposure with the mixture. Cell viability also showed a significant, dose-dependent decrease in cell viability compared to the control. A significant decrease observed between the control and the highest concentration, 0.200 µg/ml ($P<0.0001$).

C: Incubation for 96 hours chronic exposure with the mixture. Viability for all concentrations decreased when compared to the control. A significant decrease could be seen between the control and the highest concentration, 0.200 µg/ml ($P<0.0001$).

3.5 Effect of active compounds (Quercetin and Naringenin) on testosterone production in TM3-Leydig cells

3.5.1 Effect of Quercetin on testosterone production in TM3-Leydig cells

After TM3 cells were exposed for 48 hours to different concentrations of Quercetin (0.025, 0.050, 0.075, 0.100, 0.125, 0.150, 0.175 and 0.200 µg/ml), testosterone production increased at concentrations of (0.025, 0.050, 0.075, 0.100 and 0.125 µg/ml) when compared to the control. The increase found at the concentration of (0.125 µg/ml) was significant ($P < 0.0001$). At the Quercetin highest concentrations (0.150, 0.175 and 0.200 µg/ml), testosterone production decreased dose-dependent (Figure 73).

After TM3 cells were exposed for 96 hours with Quercetin (acute exposure) at increasing concentrations (0.025, 0.050, 0.075, 0.100, 0.125, 0.150, 0.175 and 0.200 µg/ml), testosterone production shows a dose-dependent increase peaking at the concentration of (0.100 µg/ml) when compared to the control. This increase was significant ($P < 0.0001$). At higher concentrations (0.150, 0.175, and 0.200 µg/ml), testosterone production decreased again in a dose-dependent manner when compared to the peak at 0.100 µg/ml (Figure 73).

After TM3 cells were exposed with Quercetin for 96 hours (chronic exposure) at increasing concentrations (0.025, 0.050, 0.075, 0.100, 0.125, 0.150, 0.175 and 0.200 µg/ml), testosterone production increased peaking at 0.125 µg/ml when compared to the control. This increase was significant ($P < 0.0001$). At the highest concentration (0.150, 0.175 and 0.200 µg/ml), testosterone production decreased again in a dose-dependent manner when compared to the pesk at 0.125 µg/ml (Figure 73).

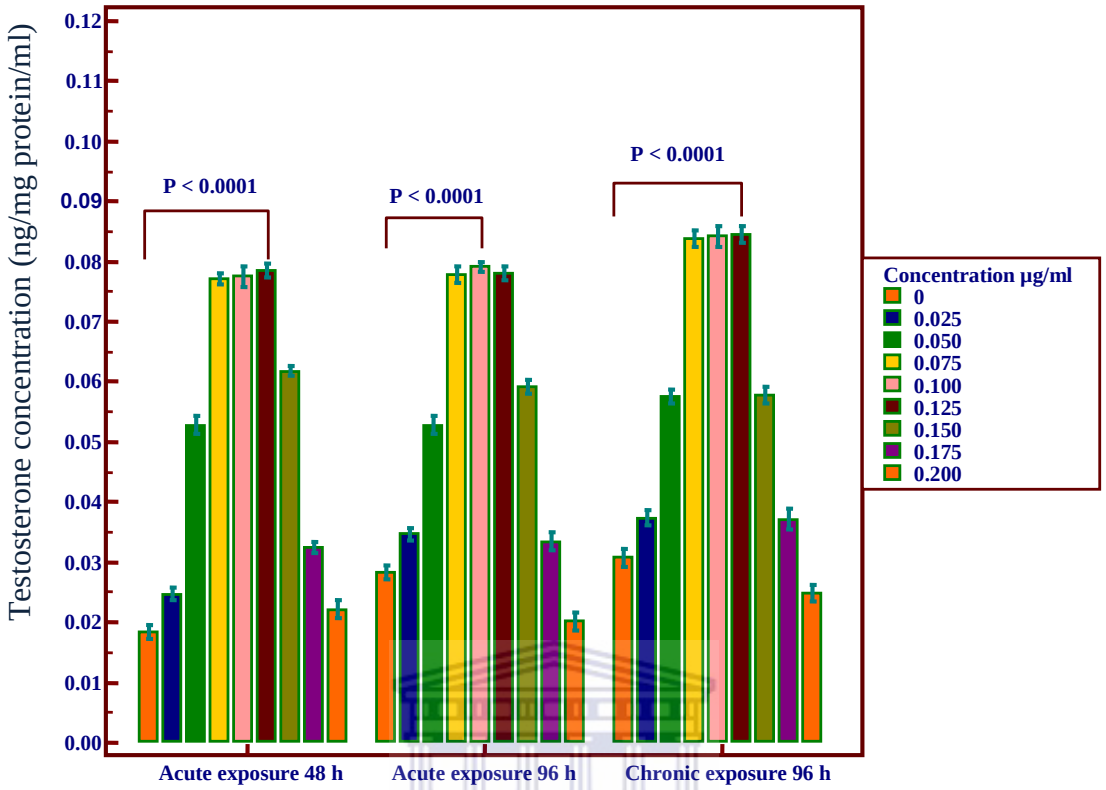


Figure 73: Effect of Quercetin on testosterone production in TM3-Leydig cells after different periods of incubation (acute exposure 48 hours, acute exposure 96 hours, and chronic exposure 96 hours), at different concentrations of Quercetin (0.025, 0.050, 0.075, 0.100, 0.125, 0.150, 0.175 and 0.200 µg/ml). Testosterone production increased dose-dependently to a maximum and declined afterwards (biphasic data). This increase was significant at the concentration of (0.100 and 0.125 µg/ml) ($P < 0.0001$ and $P < 0.0001$, respectively).

3.5.2 Effect of Naringenin on testosterone production in TM3-Leydig cells

After TM3 cells were exposed (acute exposure) for 48 hours to different concentrations of Naringenin (0.025, 0.050, 0.075, 0.100, 0.125, 0.150, 0.175 and 0.200 µg/ml), the concentration of testosterone increased in a dose-dependent manner peaking at a maximum Naringenin concentration of 0.100 µg/ml. This increased was significant ($P < 0.0001$). Thereafter, it decreased again at higher concentrations (0.150, 0.175 and 0.200 µg/ml) (Figure 74).

After TM3 cells were exposed for 96 hours to Naringenin (acute exposure) at different concentrations (0.025, 0.050, 0.075, 0.100, 0.125, 0.150, 0.175 and 0.200 µg/ml), the concentration of testosterone increased and peaked with a significant increase at the concentration of 0.100 µg/ml ($P<0.0001$). At higher concentrations (0.150, 0.175 and 0.200 µg/ml), a decrease in the testosterone production was observed when compared to the peak at 0.100 µg/ml (Figure 74).

After TM3 cells were exposed for 96 hours to Naringenin (chronic exposure) at increasing concentrations (0.025, 0.050, 0.075, 0.100, 0.125, 0.150, 0.175 and 0.200 µg/ml), the concentration of testosterone production increased significantly at 0.125 µg/ml Naringenin ($P<0.0001$). At higher concentrations (0.150, 0.175 and 0.200 µg/ml), a decrease in the testosterone production was observed when compared to the peak at 0.125 µg/ml (Figure 74).

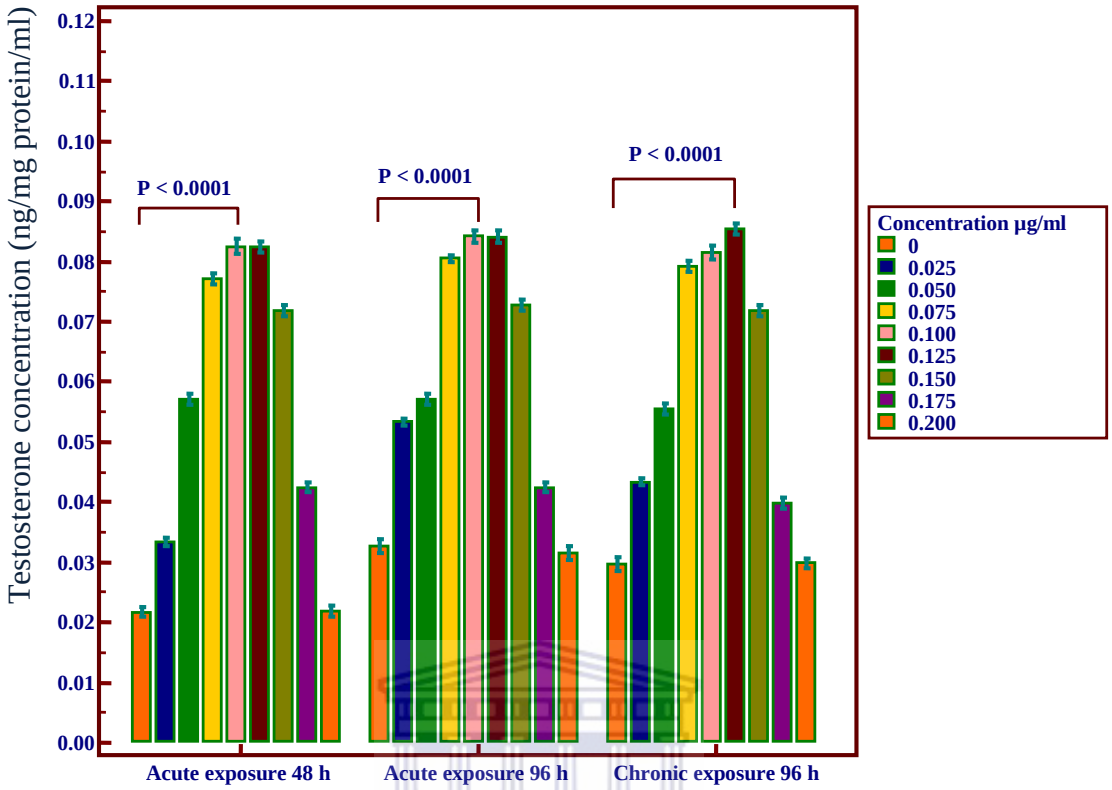


Figure 74: Effect of Naringenin on testosterone production of TM3-Leydig cells after different periods of incubation (acute exposure 48 hours, acute exposure 96 hours, and chronic exposure 96 hours). For all exposure types, testosterone concentrations peak at around 0.100 and 0.125 µg/ml, ($P<0.0001$). At the highest concentrations (0.150, 0.175 and 0.200 µg/ml), a dose-dependent decrease of testosterone production was observed.

3.5.3 Effect of mixture a of Quercetin and Naringenin on testosterone production of TM3-Leydig cells

After TM3 cells were exposed for 48 hours to different concentrations of the mixture of Quercetin and Naringenin (0.025, 0.050, 0.075, 0.100, 0.125, 0.150, 0.175 and 0.200 µg/ml), the concentration of testosterone showed a biphasic response peaking at a maximum concentrations of 0.125 µg/ml ($P<0.0001$). At the highest concentration (0.150, 0.175 and 0.200 µg/ml), a decrease of testosterone production was observed (Figure 75).

After TM3 cells were exposed for 96 hours to the mixture (acute exposure) at different concentrations (0.025, 0.050, 0.075, 0.100, 0.125, 0.150, 0.175 and 0.200 µg/ml), testosterone production showed an increase with a peak at at the concentration of 0.125 µg/ml of the mixture when compared to the control. This increase was found to be significant ($P<0.0001$). At the highest concentrations of the mixture (0.150, 0.175 and 0.200 µg/ml), testosterone production declined again (Figure 75).

After TM3 cells were exposed for 96 hours to the mixture (chronic exposure) at different concentrations (0.025, 0.050, 0.075, 0.100, 0.125, 0.150, 0.175 and 0.200 µg/ml), testosterone production increased to peak at a mixture concentration of 0.125 µg/ml. This increase was significant ($P<0.0001$). At the highest concentrations (0.150, 0.175 and 0.200 µg/ml), testosterone production declined again (Figure 75).

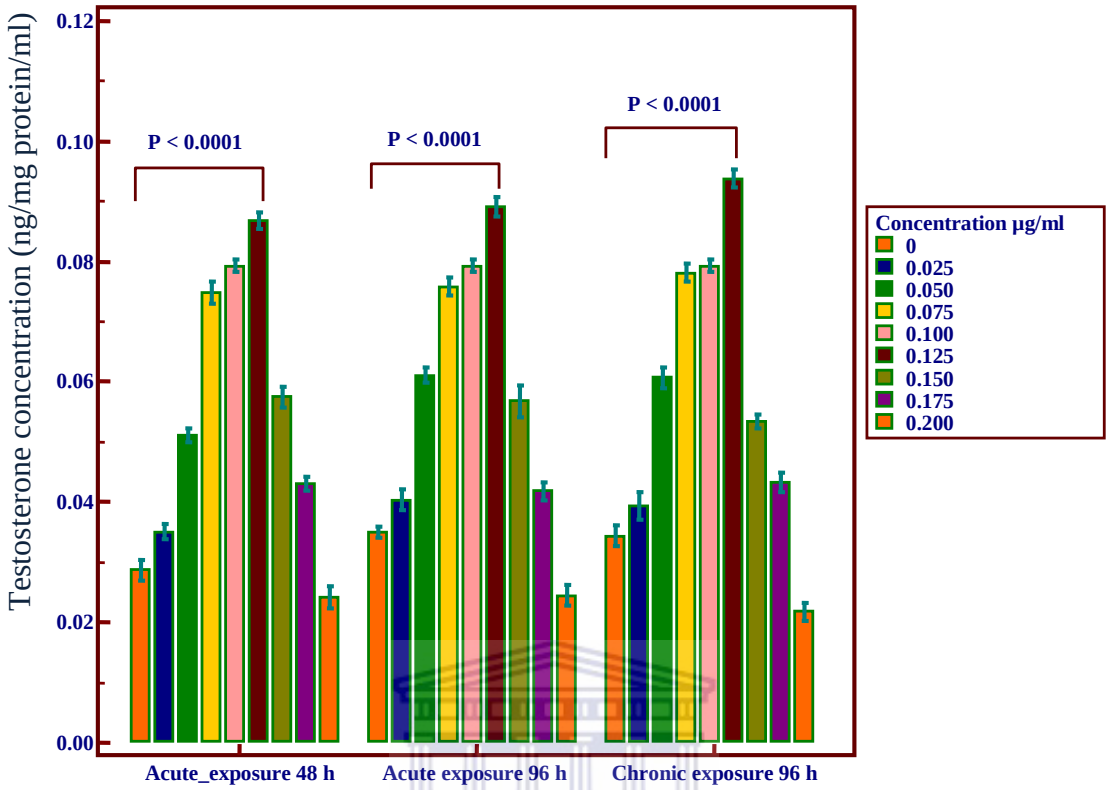


Figure 75: Effect of the mixture (Quercetin and Naringenin) on testosterone production of TM3-Leydig cells after different periods of incubation (acute exposure 48 hours, acute exposure 96 hours, and chronic exposure 96 hours), at different concentrations of the mixture of Quercetin and Naringenin (0.025, 0.050, 0.075, 0.100, 0.125, 0.150, 0.175 and 0.200 µg/ml). Testosterone concentrations increase to peak at 0.125µg/ml. This increase is significant (P<0.0001). At the highest concentrations of the mixture in a dose-dependent manner (0.150, 0.175 and 0.200 µg/ml), testosterone production declined again.

3.6 Effect of the bioactive compounds (Quercetin and Naringenin) on early apoptosis in TM3-Leydig cells and LNCaP cells

3.6.1 Effect of Quercetin Annexin V-Cy3 binding in TM3-Leydig cells

After TM3 cells were exposed for 24 hours to different concentrations of Quercitin (0.025, 0.050, 0.075, 0.100, 0.125, 0.150, 0.175 and 0.200 µg/ml), no increase in signs of early apoptosis was observed. No significant difference between the control and the highest concentration, 0.200 µg/ml (P=0.1099) (Figure 76).

After TM3 cells were exposed for 96 hours to Quercetin (acute exposure) at different concentrations (0.025, 0.050, 0.075, 0.100, 0.125, 0.150, 0.175 and 0.200 µg/ml), only slight elevation in early signs of apoptosis was found at the highest concentration of Quercetin indicating cellular stress. A significant increase between the control and the highest concentration, 0.200 µg/ml (P=0.0112) (Figure 76).

After TM3 cells were exposed for 96 hours to Quercitin (chronic exposure) at different concentrations (0.025, 0.050, 0.075, 0.100, 0.125, 0.150, 0.175 and 0.200 µg/ml), no increase in early apoptosis was observed at low Quercitin concentrations. However, at higher concentrations (0.150, 0.175 and 0.200 µg/ml) a marked, dose-dependent increase was obvious significant (P=0.0220, P=0.0007, P=0.0001, respectively) (Figure 76).

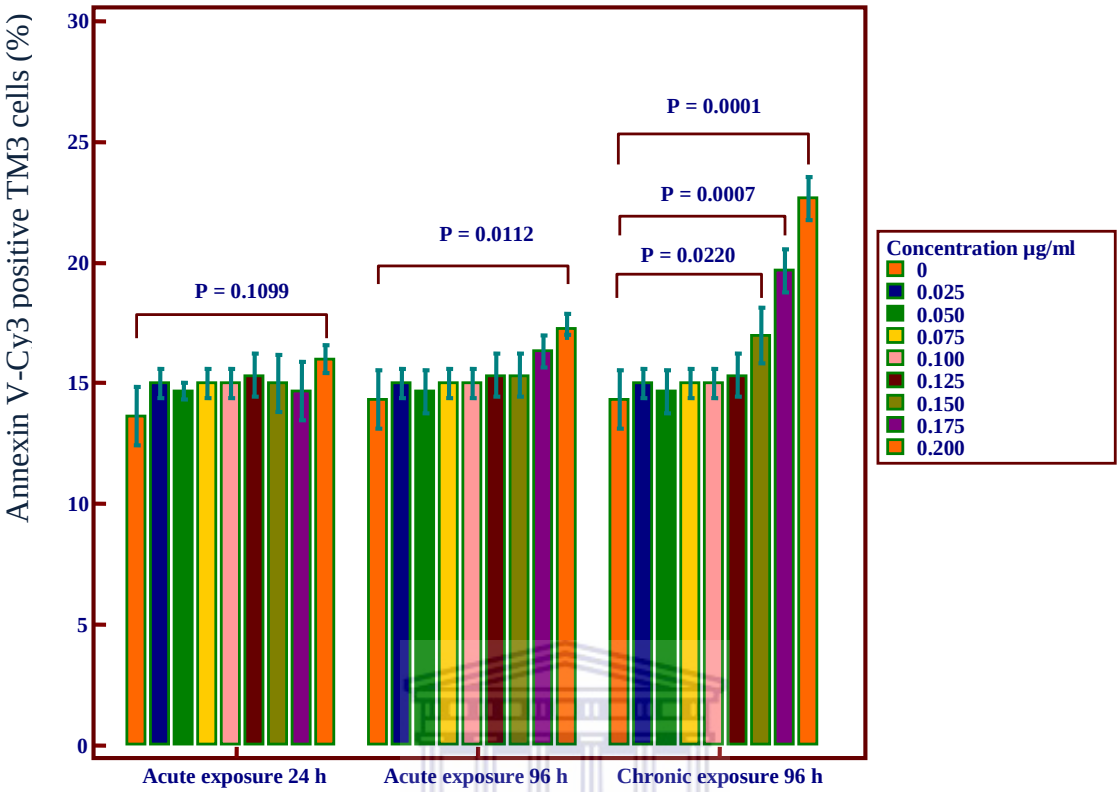


Figure 76: Effect of Quercetin on early apoptosis as determined by means of Annexin V-Cy3 binding in TM3-Leydig cells after different periods of incubation (acute exposure 24 hours, acute exposure 96 hours, and chronic exposure 96 hours) at different concentrations of Quercetin (0.025, 0.050, 0.075, 0.100, 0.125, 0.150, 0.175 and 0.200 µg/ml). After 24 hours of exposure, cells showed no increase in early apoptosis when compared to the control. No significant difference between the control and the highest concentration, 0.200 µg/ml (P=0.1099). After 96 hours of acute exposure, also no increase in early apoptosis can be seen except a slight non- significant increase at the higher concentration. A significant increase between the control and the highest concentration, 0.200 µg/ml (P=0.0112). After 96 hours of chronic exposure no increase in early apoptosis can be seen at low concentrations. However, at higher concentrations (0.150, 0.175 and 0.200 µg/ml) a marked, dose-dependent increases was obvious significant (P=0.0220, P=0.0007, P=0.0001, respectively).

3.6.2 Effect of Naringenin on Annexin V-Cy3 binding in TM3-Leydig cells

After TM3 cells were exposed for 24 hours (acute exposure) to different concentrations of Naringenin (0.025, 0.050, 0.075, 0.100, 0.125, 0.150, 0.175 and 0.200 µg/ml), no increase in early apoptosis was found. There was no significant difference between the control and the highest concentration, 0.200 µg/ml (P=0.6291) (Figure 77).

After TM3 cells were exposed for 96 hours to Naringenin (acute exposure) at different concentrations (0.025, 0.050, 0.075, 0.100, 0.125, 0.150, 0.175 and 0.200 µg/ml), also no increase in early apoptosis was observed, only a slight increase at the highest concentrations. This increase was not significant (P=0.084) (Figure 77).

After TM3 cells were exposed for 96 hours to Naringenin (chronic exposure) at different concentrations (0.025, 0.050, 0.075, 0.100, 0.125, 0.150, 0.175 and 0.200 µg/ml), no change in early apoptosis could be observed at low concentrations, with a significant increase in highest concentrations (0.175 and 0.200 µg/ml) (P=0.0002, P<0.0001, respectively) (Figure 77).

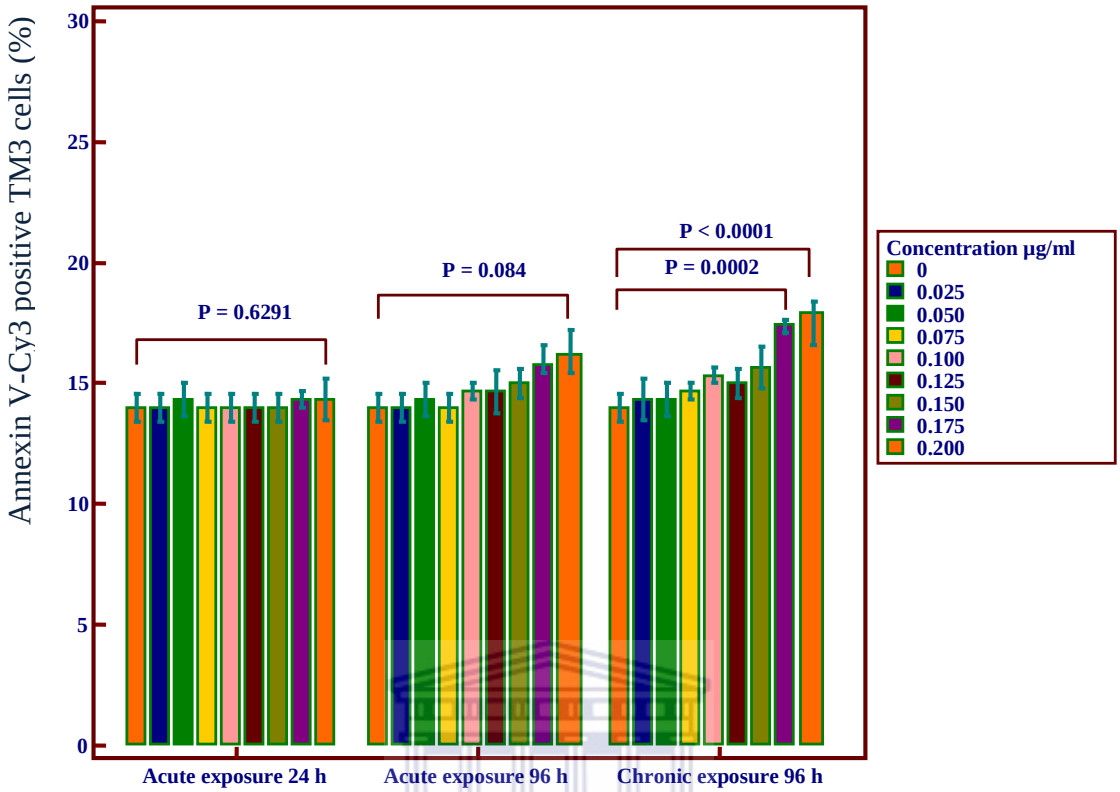


Figure 77: Effect of Naringenin on early apoptosis as determined by means of Annexin V-Cy3 binding in TM3-Leydig cells after different periods of incubation (acute exposure 24 hours, acute exposure 96 hours, and chronic exposure 96 hours) at different concentrations of Naringenin (0.025, 0.050, 0.075, 0.100, 0.125, 0.150, 0.175 and 0.200 µg/ml). After 24 hours of exposure, no increase in early apoptosis can be seen. No significant difference between the control and the highest concentration, 0.200 µg/ml (P=0.6291). After 96 hours of acute exposure also no increase in early apoptosis is evident, with a slight increase at the highest concentrations. This increase was not significant (P=0.084). After 96 hours of chronic exposure at low concentrations no increase in early apoptosis can be seen, with a significant increase in highest concentrations (0.175 and 0.200 µg/ml) (P=0.0002, P<0.0001, respectively).

3.6.3 Effect of the mixture of Quercetin and Naringenin on Annexin V-Cy3 binding in TM3-Leydig cells

After TM3 cells were exposed (acute exposure) for 24 hours to different concentrations of a mixture of Quercetin and Naringenin (0.025, 0.050, 0.075, 0.100, 0.125, 0.150, 0.175 and 0.200 µg/ml), no increase in early apoptosis was observed. No significant difference between the control and the highest concentration, 0.200 µg/ml (P=0.079) was observed (Figure 78).

After TM3 cells were exposed for 96 hours to the mixture of Quercitin and Naringenin (acute exposure) at different concentrations (0.025, 0.050, 0.075, 0.100, 0.125, 0.150, 0.175 and 0.200 µg/ml), also no increase in early apoptosis was found, with a slight increase at higher concentrations. This increase between the control and the highest concentration, 0.200 µg/ml was significant (P=0.039) (Figure 78).

After TM3 cells were exposed for 96 hours to the mixture (chronic exposure) at different concentrations (0.025, 0.050, 0.075, 0.100, 0.125, 0.150, 0.175 and 0.200 µg/ml), no increase in early apoptosis at low concentrations was found. However, at higher concentrations (0.125, 0.150, 0.175 and 0.200 µg/ml), Annexin V binding increased significantly (P=0.0224, P=0.001, P<0.0001, P<0.0001, respectively) (Figure 78).

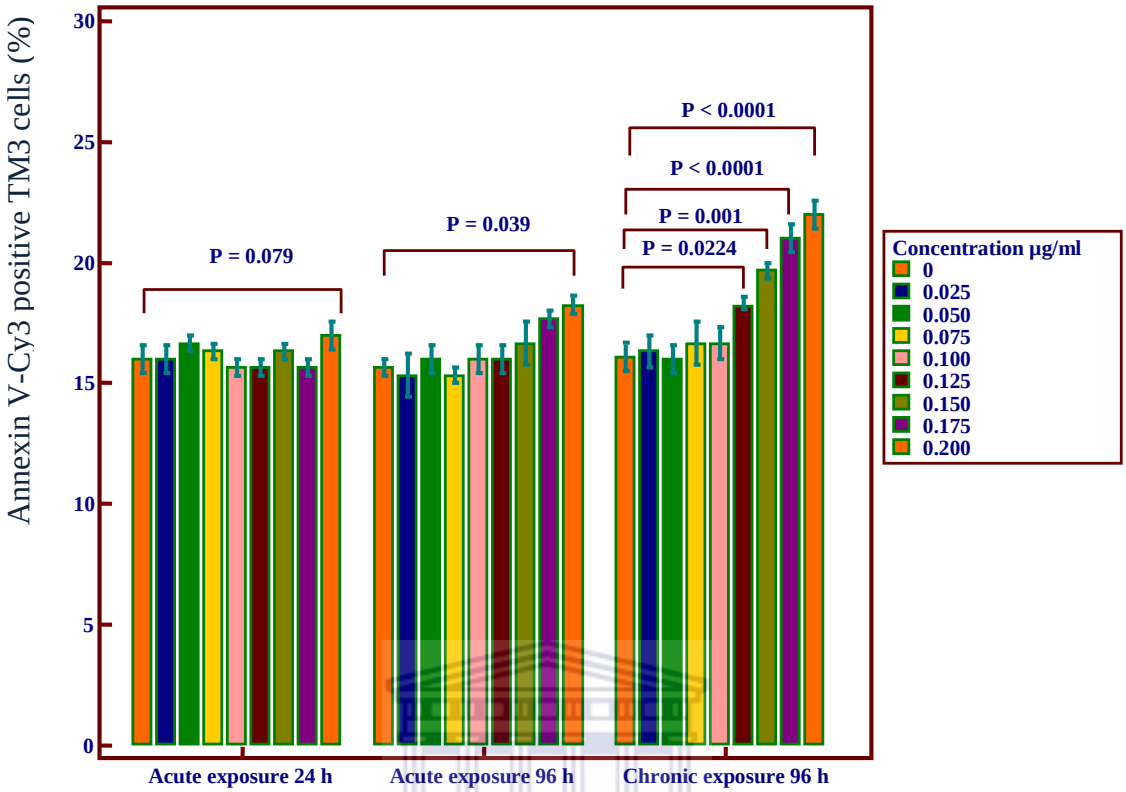


Figure 78: Effect of the mixture of Quercetin and Naringenin on early apoptosis as determined by means of Annexin V-Cy3 binding in TM3-Leydig cells after different periods of incubation (acute exposure 24 hours, acute exposure 96 hours, and chronic exposure 96 hours) at different concentrations of the mixture (0.025, 0.050, 0.075, 0.100, 0.125, 0.150, 0.175 and 0.200 µg/ml). After being exposed for 24 hours, cells showed no increase in early apoptosis when compared to the control. No significant difference between the control and the highest concentration, 0.200 µg/ml (P=0.079). After 96 hours acute exposure no increase in early apoptosis can be seen, except for a slight increase at the highest concentrations. This increase between the control and the highest concentration, 0.200 µg/ml was significant (P=0.039). After 96 hours of chronic exposure no increase in early apoptosis is obvious. However, at higher concentrations (0.125, 0.150, 0.175 and 0.200 µg/ml), Annexin V binding increased significantly (P=0.0224, P=0.001, P<0.0001, P<0.0001, respectively).

3.6.4 Effect of Quercetin on Annexin V-Cy3 binding in LNCaP cells

After LNCaP cells were exposed (acute exposure) for 24 hours to different concentrations of Quercetin (0.025, 0.050, 0.075, 0.100, 0.125, 0.150, 0.175 and 0.200 µg/ml), all concentration resulted in an increase in early apoptosis when compared to the control. This increase between the control and the highest concentration, 0.200 µg/ml was significant ($P<0.0001$) (Figure 79).

After LNCaP cells were exposed for 96 hours to Quercetin (acute exposure) at different concentrations (0.025, 0.050, 0.075, 0.100, 0.125, 0.150, 0.175 and 0.200 µg/ml), all concentration resulted in an increase in early apoptosis when compared to the control, with increase being significant at the highest concentrations 0.175 and 0.200 µg/ml ($P<0.0001$, $P<0.0001$, respectively). One-way ANOVA exhibited a significant trend increase (ANOVA trend analysis: $P<0.0001$) (Figure 79).

After LNCaP cells were exposed for 96 hours to Quercetin (chronic exposure) at different concentrations (0.025, 0.050, 0.075, 0.100, 0.125, 0.150, 0.175 and 0.200 µg/ml), all concentration led to an increase in early apoptosis when compared to the control, the increases at the highest concentrations 0.175 and 0.200 µg/ml is significant ($P<0.0001$, $P<0.0001$, respectively). One-way ANOVA exhibited a significant trend increase (ANOVA trend analysis: $P<0.0001$) (Figure 79).

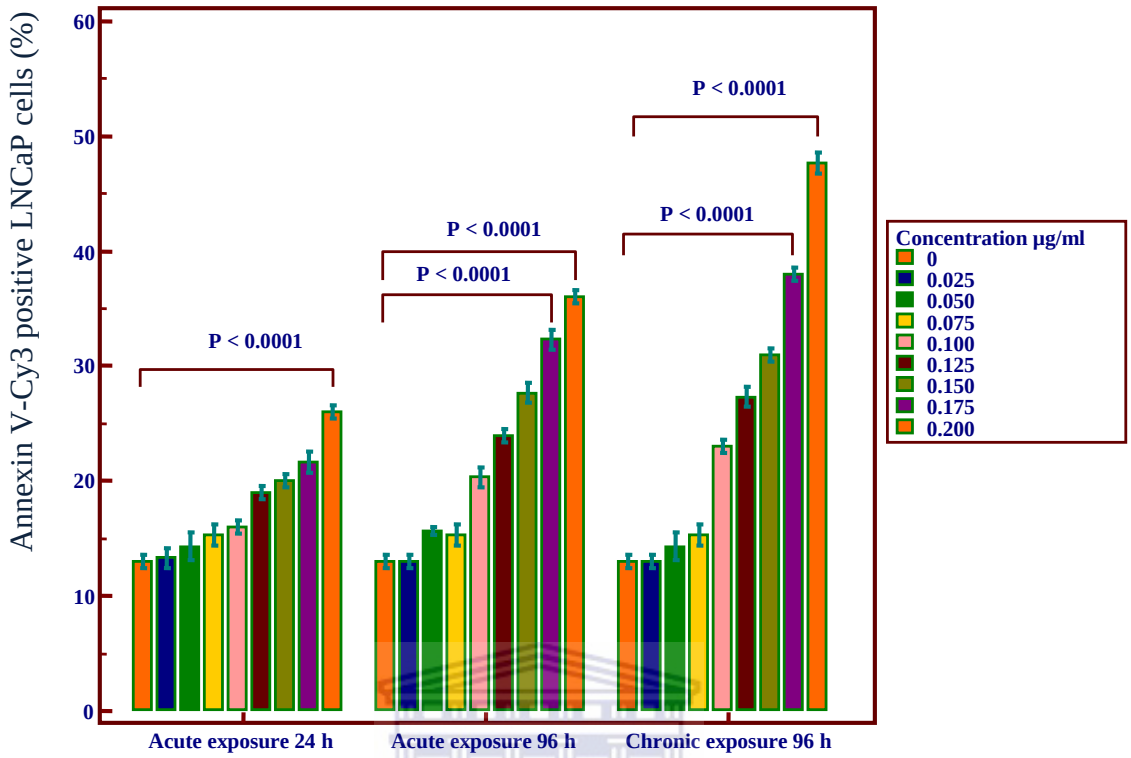


Figure 79: Effect of Quercetin on early apoptosis as determined by means of Annexin V-Cy3 binding in LNCaP cells after different periods of incubation (acute exposure 24 hours, acute exposure 96 hours, and chronic exposure 96 hours) at different concentrations of Quercetin (0.025, 0.050, 0.075, 0.100, 0.125, 0.150, 0.175 and 0.200 µg/ml). After 24 hours of acute exposure, all concentration showed an increase in early apoptosis when compared to the control. This increase between the control and the highest concentration, 0.200 µg/ml was significant (P<0.0001) . After 96 hours of acute exposure all concentrations showed an increase in early apoptosis when compared to the control, with increase being significant at the highest concentrations 0.175 and 0.200 µg/ml (P<0.0001, P<0.0001, respectively). After 96 hours of chronic exposure, all concentration led to an increase in early apoptosis when compared to the control. The increases at the highest concentrations 0.175 and 0.200 µg/ml is significant (P<0.0001, P<0.0001, respectively).

3.6.5 Effect of Naringenin on Annexin V-Cy3 binding in LNCaP cells

After LNCaP cells were exposed (acute exposure) for 24 hours to different concentrations of Naringenin (0.025, 0.050, 0.075, 0.100, 0.125, 0.150, 0.175 and 0.200 µg/ml), all concentration revealed an increase in early apoptosis when compared to the control. A significant increase between the control and the highest concentration, 0.200 µg/ml was significant (P=0.001) (Figure 80).

After LNCaP cells were exposed for 96 hours to Naringenin (acute exposure) at increasing concentrations (0.025, 0.050, 0.075, 0.100, 0.125, 0.150, 0.175 and 0.200 µg/ml), all concentration resulted in an increase in early apoptosis (Figure 80).

After LNCaP cells were exposed for 96 hours to Naringenin (chronic exposure) at different concentrations (0.025, 0.050, 0.075, 0.100, 0.125, 0.150, 0.175 and 0.200 µg/ml), all concentrations caused an increase in early apoptosis with a highly significant increase between the control and the highest concentration, 0.200 µg/ml (P<0.0001) (Figure 80).

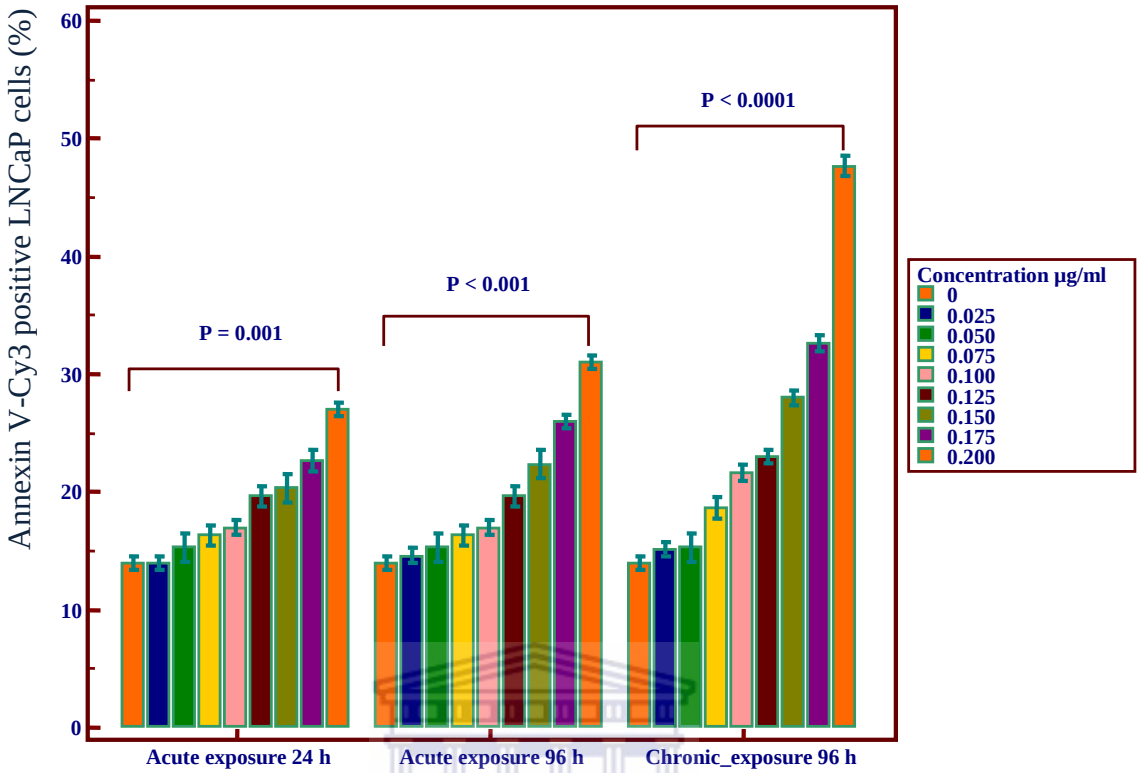


Figure 80: Effect of Naringenin on early apoptosis as determined by means of Annexin V-Cy3 binding in LNCaP cells after different periods of incubation (acute exposure 24 hours, acute exposure 96 hours, and chronic exposure 96 hours) at different concentrations of Naringenin (0.025, 0.050, 0.075, 0.100, 0.125, 0.150, 0.175 and 0.200 µg/ml). After 24 hours of acute exposure, all concentration showed an increase in early apoptosis when compared to the control. A significant increase between the control and the highest concentration, 0.200 µg/ml was significant (P=0.001). After 96 hours of acute exposure all concentrations showed an increase in early apoptosis. After 96 hours of chronic exposure all concentrations resulted in an increase in early apoptosis with a highly significant increase between the control and the highest concentration, 0.200 µg/ml (P<0.0001).

3.6.6 Effect of the mixture of Quercetin and Naringenin on Annexin V-Cy3 binding in LNCaP cells

After LNCaP cells were exposed for 24 hours (acute exposure) to different concentrations of a mixture of Quercitin and Naringenin (0.025, 0.050, 0.075, 0.100, 0.125, 0.150, 0.175 and 0.200 µg/ml), at all concentrations an increase in early apoptosis was evident. A significant increase was found between control and highest concentration, 0.200 µg/ml (P=0.0012) (Figure 81).

After 96 hours of a mixture of Quercetin and Naringenin acute exposure at increasing concentrations (0.025, 0.050, 0.075, 0.100, 0.125, 0.150, 0.175 and 0.200 µg/ml), all concentrations resulted in an increase in early apoptosis (Figure 81).

For 96 hours chronic exposure of a mixture of Quercetin and Naringenin at different concentrations (0.025, 0.050, 0.075, 0.100, 0.125, 0.150, 0.175 and 0.200 µg/ml), an increase in early apoptosis was observed at all concentrations with a highly significant increase between the control and the higher concentrations, 0.175 and 0.200 µg/ml (P<0.0001, P<0.0001, respectively) (Figure 81).

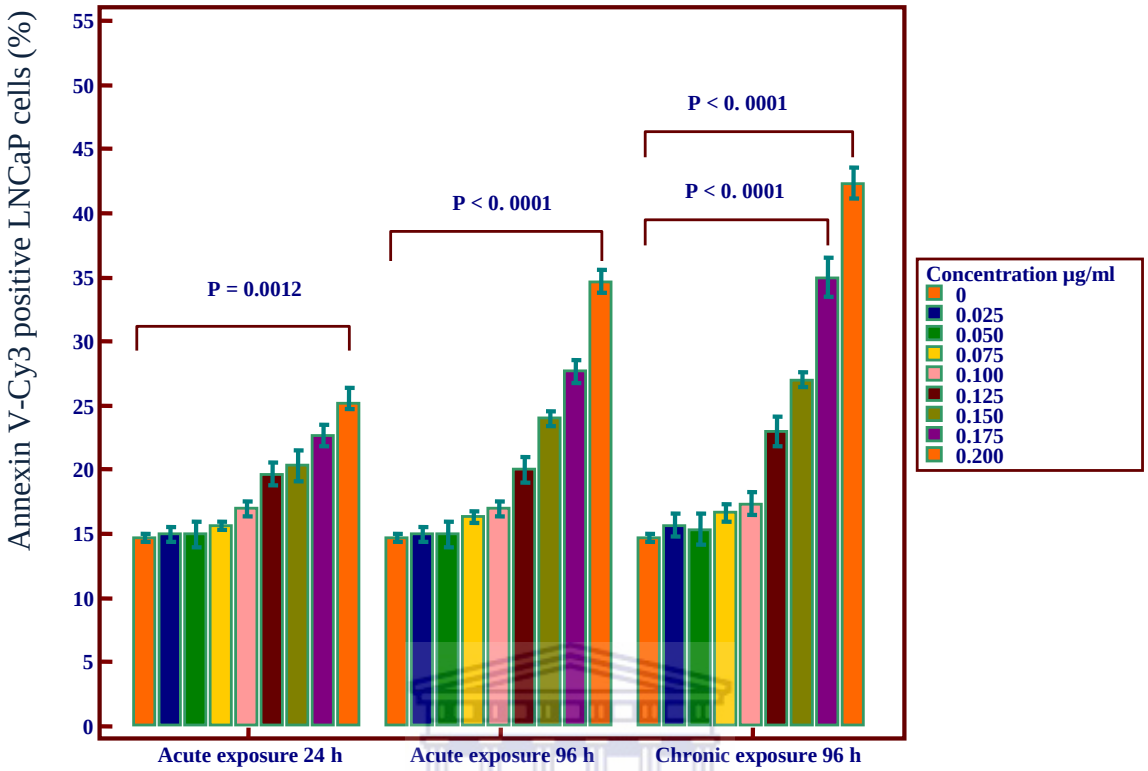


Figure 81: Effect of a mixture of Quercetin and Naringenin on early apoptosis as determined by means of Annexin V-Cy3 binding in LNCaP cells after exposure for different periods (acute exposure 24 hours, acute exposure 96 hours, and chronic exposure 96 hours) for different concentrations of Quercetin (0.025, 0.050, 0.075, 0.100, 0.125, 0.150, 0.175 and 0.200 µg/ml). After 24 hours of acute exposure, all concentration led to an increase in early apoptosis when compared to the control. A significant increase was found between control and highest concentration, 0.200 µg/ml (P=0.0012). After 96 hours of acute exposure all concentrations resulted in an increase in early apoptosis. After 96 hours of chronic exposure an increase in early apoptosis was observed, with a highly significant increase at the highest concentrations 0.175 and 0.200 µg/ml (P<0.0001, P<0.0001, respectively). Indicating early apoptosis.

3.7 Effect of bioactive compounds (Quercetin and Naringenin) on DNA fragmentation in TM3-Leydig cells and LNCaP cells

3.7.1 Effect of Quercetin on DNA fragmentation in TM3-Leydig cells

After TM3 cells were exposed for 24 hours (acute exposure) at different increasing concentrations of Quercitin (0.025, 0.050, 0.075, 0.100, 0.125, 0.150, 0.175 and 0.200 µg/ml), all concentrations showed no increase in the percentage of TUNEL-positive cells. No signifigant difference between control and highest concentration, 0.200 µg/ml (P=0.305) (Figure 82).

For 96 hours of acute exposure with Quercetin at different concentrations (0.025, 0.050, 0.075, 0.100, 0.125, 0.150, 0.175 and 0.200 µg/ml), also no increase in the percentage of TUNEL-positive cells was observed (Figure 82).

After TM3 cells were chronically exposed for 96 hours with Quercetin at increasing concentrations (0.025, 0.050, 0.075, 0.100, 0.125, 0.150, 0.175 and 0.200 µg/ml), results showed a dose-dependent increase in the percentage of TUNEL-positive cells with a significant increase between control and the highest concentration, 0.200 µg/ml (P=0.001). One-way ANOVA exhibited a significant trend increase (ANOVA trend analysis: P=0.0037) (Figure 82).

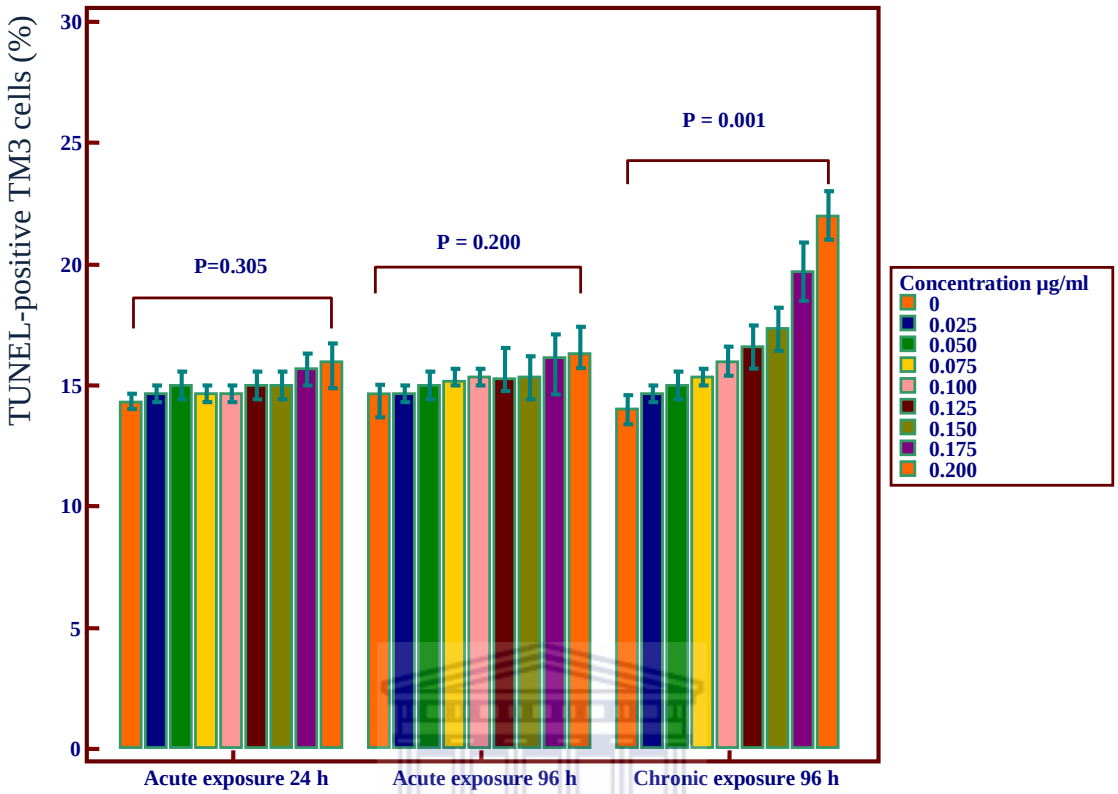


Figure 82: Effect of Quercetin on DNA fragmentation as determined by means of the TUNEL assay in TM3-Leydig cells after different periods of incubation (acute exposure 24 hours, acute exposure 96 hours, and chronic exposure 96 hours) at different concentrations of Quercetin (0.025, 0.050, 0.075, 0.100, 0.125, 0.150, 0.175 and 0.200 µg/ml). After 24 hours, all concentrations showed no increase in the percentage of TUNEL-positive cells between control and highest concentration, 0.200 µg/ml (P=0.305). For 96 hours of incubation with Quercetin (acute exposure) at different concentrations (0.025, 0.050, 0.075, 0.100, 0.125, 0.150, 0.175 and 0.200 µg/ml), also no increase in the percentage of TUNEL-positive cells were observed. After TM3 cells were chronically exposed for 96 hours with Quercetin at increasing concentrations (0.025, 0.050, 0.075, 0.100, 0.125, 0.150, 0.175 and 0.200 µg/ml), data show a significant dose-dependent increase in the percentage of TUNEL-positive cells with a significant increase between the control and highest concentration, 0.200 µg/ml (P=0.001).

3.7.2 Effect of Naringenin on DNA fragmentation in TM3-Leydig cells

After TM3 cells were exposed for 24 hours (acute exposure) at different concentrations of Naringenin (0.025, 0.050, 0.075, 0.100, 0.125, 0.150, 0.175 and 0.200 µg/ml), no change in the percentage of TUNEL-positive cells was evident. No significant difference between control and highest concentration, 0.200 µg/ml (P=0.194) was observed (Figure 83).

Acute exposure of TM3 cells with Naringenin at increasing concentrations (0.025, 0.050, 0.075, 0.100, 0.125, 0.150, 0.175 and 0.200 µg/ml) for 96 hours, also led to no change in the percentage of TUNEL-positive cells (Figure 83).

After TM3 cells were exposed for 96 hours to chronic Naringenin exposure, at different concentrations (0.025, 0.050, 0.075, 0.100, 0.125, 0.150, 0.175 and 0.200 µg/ml), also no significant change in the percentage of TUNEL-positive cells was observed. Yet, a slight increase at the highest concentrations (0.175 and 0.200 µg/ml) was obvious. This increase was significant (P=0.017, P=0.001, respectively) (Figure 83).

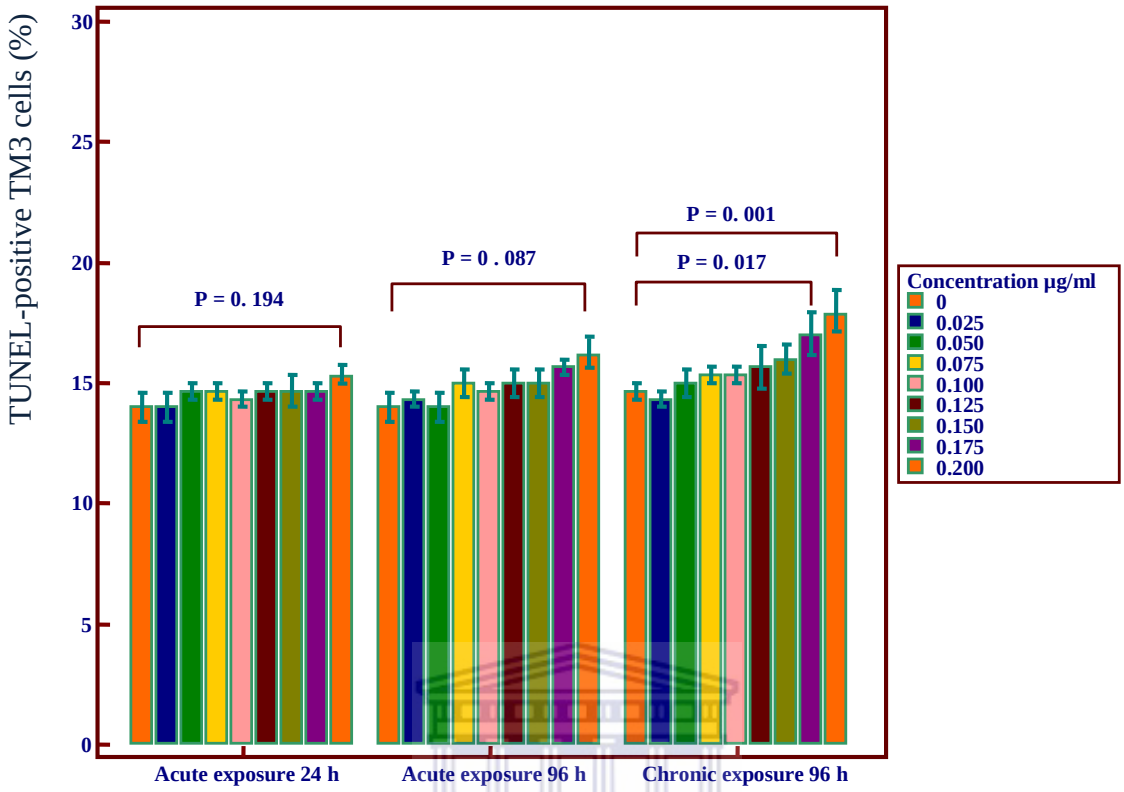


Figure 83: Effect of Naringenin on DNA fragmentation as determined by means of the TUNEL assay in TM3-Leydig cells after different periods of incubation (acute exposure 24 hours, acute exposure 96 hours, and chronic exposure 96 hours) at different concentrations of Naringenin (0.025, 0.050, 0.075, 0.100, 0.125, 0.150, 0.175 and 0.200 µg/ml). For the 24-hours acute exposure, no change in the percentage of TUNEL-positive cells was evident, with no significant difference between control and highest concentration, 0.200 µg/ml (P=0.194). After 96 hours of acute Naringenin exposure, also no change in the percentage of TUNEL-positive cells was observed. After TM3 cells were exposed for 96 hours to chronic Naringenin exposure, at different concentrations (0.025, 0.050, 0.075, 0.100, 0.125, 0.150, 0.175 and 0.200 µg/ml), also no significant change in the percentage of TUNEL-positive cells was observed. Yet, a slight increase at the highest concentrations (0.175 and 0.200 µg/ml) is evident. This increase was significant (P=0.017, P=0.001, respectively).

3.7.3 Effect of the mixture of Quercetin and Naringenin on DNA fragmentation in TM3-Leydig cells

After TM3 cells were exposed for 24 hours to the mixture of Quercetin and Naringenin (acute exposure) at different increasing concentrations (0.025, 0.050, 0.075, 0.100, 0.125, 0.150, 0.175 and 0.200 µg/ml), no change in the percentage of TUNEL-positive cells was observed. With no difference between control and highest concentration, 0.200 µg/ml (P=0.104) (Figure 84).

After TM3 cells were exposed for 96 hours to the mixture of Quercetin and Naringenin (acute exposure) at different concentrations (0.025, 0.050, 0.075, 0.100, 0.125, 0.150, 0.175 and 0.200 µg/ml), also no increase in TUNEL-positive cells could be seen (Figure 84).

However, incubation for 96 hours with the mixture of Quercetin and Naringenin (chronic exposure) at different concentrations (0.025, 0.050, 0.075, 0.100, 0.125, 0.150, 0.175 and 0.200 µg/ml) resulted in a significant increase in the percentage of TUNEL-positive cells at all concentrations when compared to the control in particular at the highest concentrations, 0.175 and 0.200 µg/ml (P=0.0022, P=0.0021, respectively). One-way ANOVA exhibited a significant trend increase (ANOVA trend analysis: P=0.014) (Figure 84).

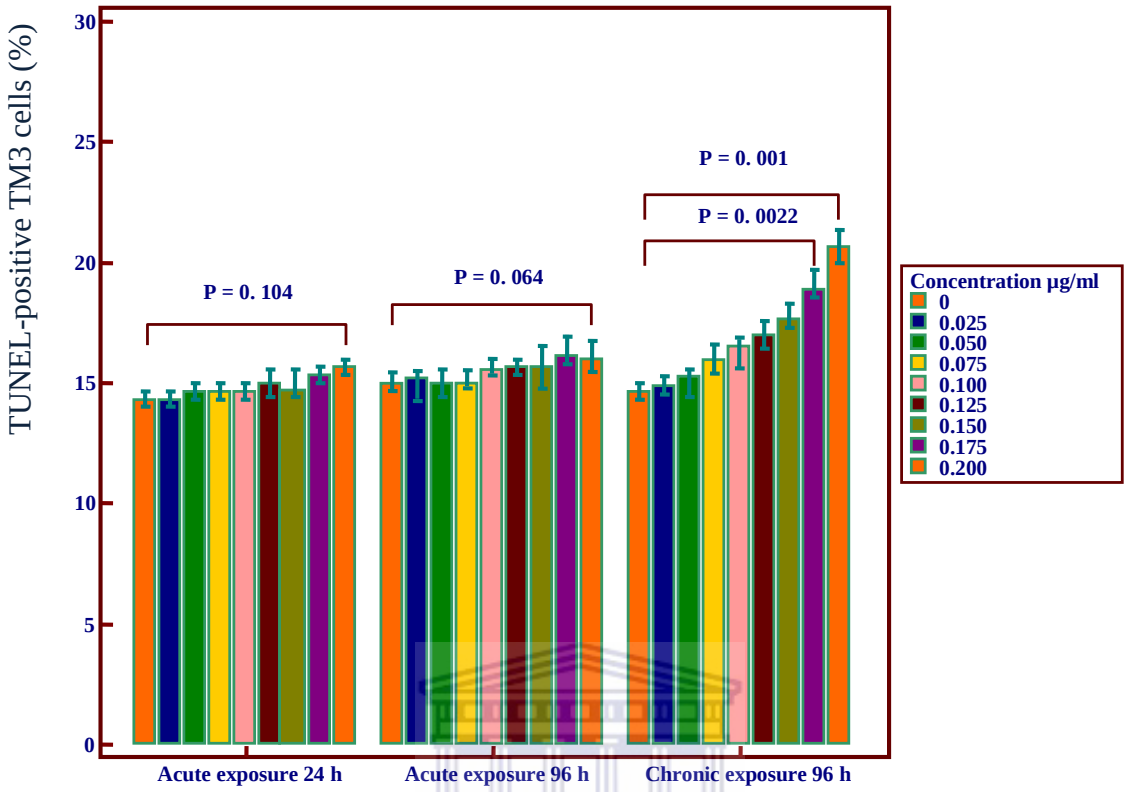


Figure 84: Effect of the mixture of Quercetin and Naringenin on DNA fragmentation as determined by means of the TUNEL assay in TM3-Leydig cells after different periods of incubation (acute exposure 24 hours, acute exposure 96 hours, and chronic exposure 96 hours) for different concentrations of the mixture (0.025, 0.050, 0.075, 0.100, 0.125, 0.150, 0.175 and 0.200 µg/ml). After 24 hours of incubation with the mixture of Quercetin and Naringenin (acute exposure) at different concentrations, no change in the percentage of TUNEL-positive cells were observed. With no significant difference between control and highest concentration, 0.200 µg/ml (P=0.104). After 96 hours acute exposure also no change in the percentage of TUNEL-positive cells could be seen. After 96 hours chronic exposure, a significant increase in the percentage of TUNEL-positive cells at all concentrations when compared to the control in particular at the highest concentrations, 0.175 and 0.200 µg/ml (P=0.0022, P=0.0021, respectively) was evident.

3.7.4 Effect of Quercetin on DNA fragmentation in LNCaP cells

After LNCaP cells were exposed for 24 hours (acute exposure) to Quercetin at different concentrations (0.025, 0.050, 0.075, 0.100, 0.125, 0.150, 0.175 and 0.200 µg/ml), a dose-dependent increase in TUNEL-positivity was caused at all concentrations. A significant increase between control and highest concentration, 0.200 µg/ml ($P<0.001$) was observed (Figure 85).

After LNCaP cells were exposed for 96 hours to Quercetin (acute exposure) at increasing concentrations (0.025, 0.050, 0.075, 0.100, 0.125, 0.150, 0.175 and 0.200 µg/ml), an increase in the percentage of TUNEL-positive cells was observed. A highly significant increase was found between control and highest concentration, 0.200 µg/ml ($P<0.0001$) (Figure 85).

Chronic exposure of the cells to Quercitin for 96 hours resulted in an increase in TUNEL-positivity at all concentrations. This was a highly significant increase at the highest concentrations 0.175 and 0.200 µg/ml ($P<0.0001$, $P<0.0001$ respectively) (Figure 85).

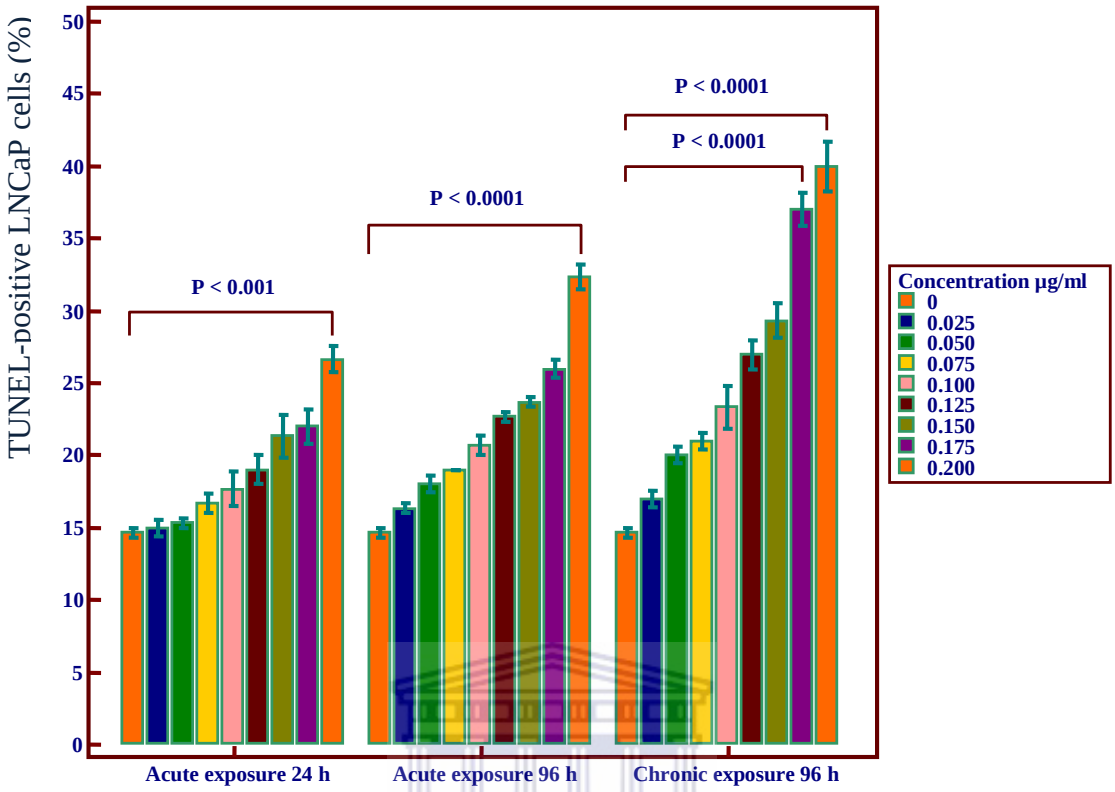


Figure 85: Effect of Quercetin on DNA fragmentation as determined by means of the TUNEL assay in LNCaP cells, after different periods of incubation (acute exposure 24 hours, acute exposure 96 hours, and chronic exposure 96 hours) at different concentrations of Quercetin (0.025, 0.050, 0.075, 0.100, 0.125, 0.150, 0.175 and 0.200 µg/ml). The 24-hour acute exposure led to a dose-dependent increase in the percentage of TUNEL-positive cells when compared to the control. A significant increase between control and highest concentration, 0.200 µg/ml ($P<0.001$) was observed. After 96 hours acute exposure, an increase in TUNEL-positivity cells is evident. Highly significant increase was found between control and highest concentration, 0.200 µg/ml ($P<0.0001$). Chronic exposure of the cells to quercetin for 96 hours resulted in an increase in TUNEL-positivity at all concentrations. This was a highly significant increase at the highest concentrations 0.175 and 0.200 µg/ml ($P<0.0001$, $P<0.0001$ respectively).

3.7.5 Effect of Naringenin on DNA fragmentation in LNCaP cells

After LNCaP cells were exposed for 24 hours (acute exposure) to different concentrations of Naringenin (0.025, 0.050, 0.075, 0.100, 0.125, 0.150, 0.175 and 0.200 µg/ml), a dose-dependent increase in TUNEL-positivity could be seen. A significant increase between control and highest concentration, 0.200 µg/ml (P=0.001) was found (Figure 86).

After LNCaP cells were exposed to 96 hours to Naringenin (acute exposure) at increasing concentrations (0.025, 0.050, 0.075, 0.100, 0.125, 0.150, 0.175 and 0.200 µg/ml), all concentration resulted in an increase in the percentage of TUNEL-positive cells. A significant positive and dose-dependent increase between control and highest concentration, 0.200 µg/ml (P<0.0001) was obvious (Figure 86).

For the 96-hour chronic exposure with Naringenin at different concentrations (0.025, 0.050, 0.075, 0.100, 0.125, 0.150, 0.175 and 0.200 µg/ml), an increase in the percentage of TUNEL-positive cells was observed, with a highly significant increase at the highest concentrations 0.175 and 0.200 µg/ml (P<0.0001, P<0.0001, respectively). One-way ANOVA exhibited a significant positive trend (ANOVA trend analysis: P<0.0001) (Figure 86).

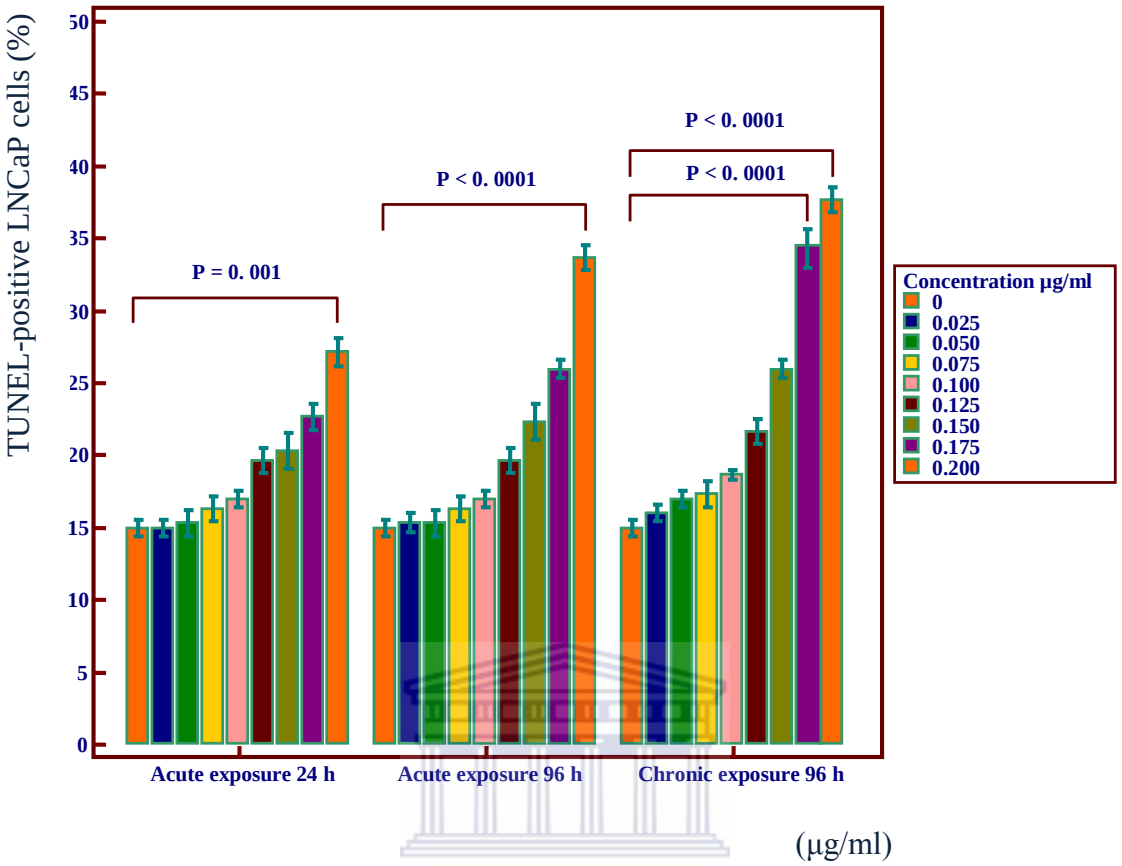


Figure 86: Effect of Naringenin on DNA fragmentation as determined by means of the TUNEL assay in LNCaP cells, after different periods of incubation (acute exposure 24 hours, acute exposure 96 hours, and chronic exposure 96 hours) at increasing concentrations of Naringenin (0.025, 0.050, 0.075, 0.100, 0.125, 0.150, 0.175 and 0.200 µg/ml). After 24 hours exposure, all concentrations resulted in an increase of TUNEL-positive cells. A significant increase between control and highest concentration, 0.200 µg/ml (P=0.001) was found. After 96 hours of acute exposure, a dose-dependent increase in TUNEL-positive cells is obvious. A significant positive increase between control and highest concentration, 0.200 µg/ml (P<0.0001) was obvious. After 96 hours chronic exposure an increase in the percentage of TUNEL-positive cells were observed, with a highly significant increase at the highest concentrations 0.175 and 0.200 µg/ml (P<0.0001, P<0.0001, respectively).

3.7.6 Effect of the mixture of Quercetin and Naringenin on DNA fragmentation in LNCaP cells

After LNCaP cells were exposed for 24 hours of (acute exposure) to increasing concentrations of a mixture of Quercetin and Naringenin (0.025, 0.050, 0.075, 0.100, 0.125, 0.150, 0.175 and 0.200 µg/ml), a significant, dose-dependent increase in TUNEL-positive cells was observed. A significant increase between control and highest concentration, 0.200 µg/ml ($P<0.001$) was observed (Figure 87).

After LNCaP cells were exposed for 96 hours to the mixture (acute exposure) at different concentrations (0.025, 0.050, 0.075, 0.100, 0.125, 0.150, 0.175 and 0.200 µg/ml), also an increase in TUNEL-positivity at all concentrations could be seen with a highly significant increase between control and highest concentration, 0.200 µg/ml ($P<0.0001$) was found (Figure 87).

After LNCaP cells were exposed for 96 hours to the mixture of Quercetin and Naringenin (chronic exposure) at increasing concentrations (0.025, 0.050, 0.075, 0.100, 0.125, 0.150, 0.175 and 0.200 µg/ml), all concentration resulted in an increase in TUNEL-positivity, with a highly significant increase at the highest concentrations 0.175 and 0.200 µg/ml ($P<0.0001$, $P<0.0001$, respectively). This increase was even steeper than that after an acute exposure. One-way ANOVA exhibited a significant positive trend (ANOVA trend analysis: $P<0.0001$) (Figure 87).

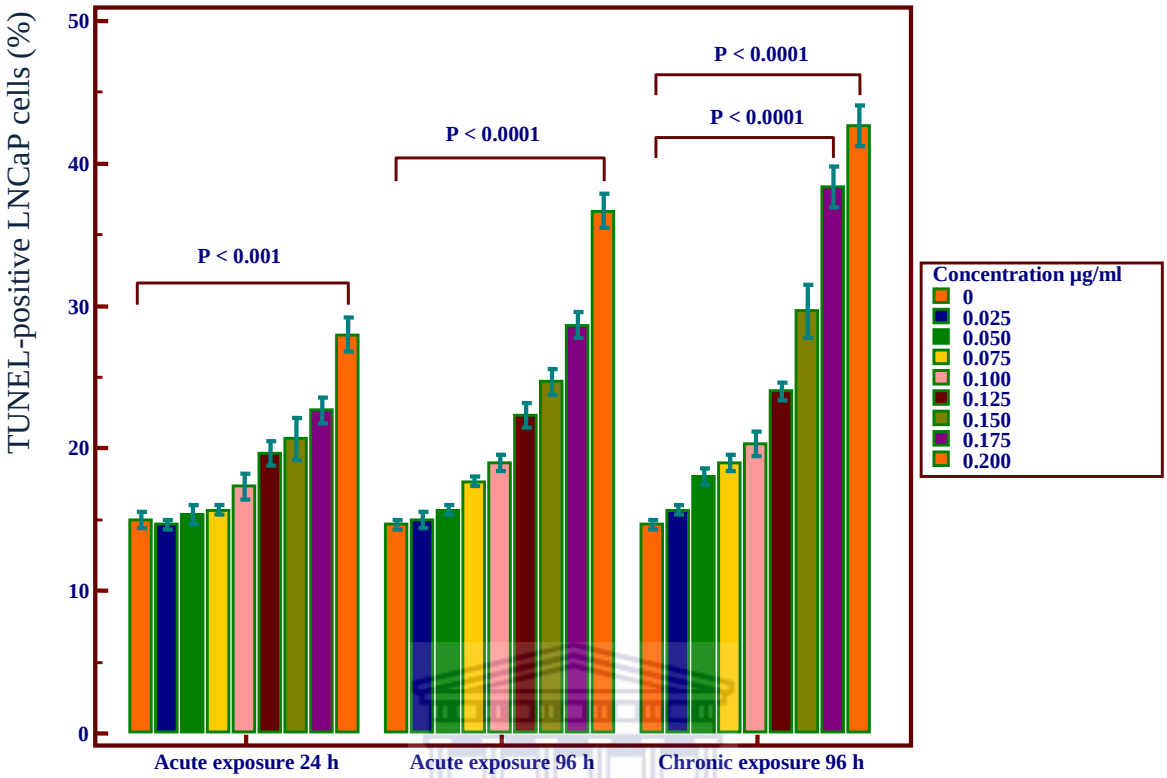


Figure 87: Effect of the mixture of Quercetin and Naringenin on DNA fragmentation as determined by means of the TUNEL assay in LNCaP cells after different periods of incubation (acute exposure 24 hours, acute exposure 96 hours, and chronic exposure 96 hours) at different concentrations of the mixture (0.025, 0.050, 0.075, 0.100, 0.125, 0.150, 0.175 and 0.200 µg/ml). Acute exposure for 24 hours showed an increase in the percentage of TUNEL-positive cells A significant increase between control and highest concentration, 0.200 µg/ml ($P<0.001$) was observed. After 96 hours of acute exposure a dose-dependent increase in TUNEL-positivity cells at all concentrations could be seen with a highly significant increase between control and highest concentration, 0.200 µg/ml ($P<0.0001$). After 96 hours of chronic exposure, an even steeper increase in TUNEL-positivity, with a highly significant increase at the highest concentrations 0.175 and 0.200 µg/ml ($P<0.0001$, $P<0.0001$, respectively) is obvious. One-way ANOVA exhibited a significant positive trend increase (ANOVA trend analysis: $P<0.0001$).

Chapter 4: Discussion

4.1 Introduction

The use of plants as medicine is evident in the history of many cultures and ethnic groups. Various reports have documented the early use of plants for medicinal purposes, particularly by the country's earliest inhabitants (Watt and Breyer-Brandwijk, 1962; Vayda, 1969; Hutchings, 1989). The use of herbs in traditional medicine, in spite of being used for thousands of years, has been given more and more attention in Western societies in recent years. Additionally, there has been a growing interest in medicinal plants, specifically when focusing on the phytochemical material. Medicinal plants have been introduced into a number of conventional treatments in various fields of medicine as a growing number of people are looking for alternative treatments for various diseases and ailments. In excess of 25% of the professional prescriptions contain extracts from plants and active ingredients derives from plants, sometimes even without the knowledge of the doctors. Yet, little is known in Western medicine about these traditional methods of disease control (Castleman, 1995). Notwithstanding, science has introduced only a very small number of conventional treatments in conventional medicine.

Typha capensis, commonly also called 'love reed', has been described as the roots of male virility by Sangomas (herbal medical practitioner) or Izinyanga (herbs) (Van Wyk et al., 1997). It is believed that this plant can enhance male fertility (Hutchings et al., 1996). The rhizomes are used in traditional medicine during pregnancy to ensure easy delivery. They are also used for venereal diseases, dysmenorrhea, diarrhoea, dysentery, and in addition to enhance the male potency and libido, amongst others (Watt and Breyer-Brandwijk, 1962; Hutchings et al., 1996). Other uses include treatment of genital problems, promotion of fertility in women and to improve blood circulation.

Its use in childbirth stems from the claim that it strengthens uterine contractions and promotes expulsion of the placenta (Watt, 1962; Hutchings, 1996; Pip et al. 1992; Dunbabin et al., 1992). The patient is prescribed one or two cups of a decoction of the rhizome in boiling water, which is taken daily for a week (Pujol, 1990; Della Greca et al., 1990). The phytochemistry of several species of the

Typha genus has several flavones and other phenolic compounds (Chapman and Hall, 2000), long chain hydrocarbons as well as various triterpenoids with a steroidal skeleton typhasterol have been identified (Chapman and Hall, 1996; Sick et al., 1983). More specifically, the *Typha* genus contains flavones and other phenolic compounds, which exhibit an anti-oxidative capacity (Chapman and Hall, 2000).

For *T. capensis* many important effects of phenolic compounds, which have a direct impact on the ability of antioxidants have been reported (Chapman and Hall, 2000), for reactive oxygen species (ROS). However, it is largely dependent on the amount of phenolic compounds contained in the extract. ROS have been considerably described as detrimental and as a major cause of cell death, decreased sperm motility and male infertility (Aitken and Clarkson, 1988; Sharma et al., 2001; Henkel et al., 2005). However, *T. capensis* appears to exert some inhibitory effect toward collagenase activity (Henkel et al., 2012). This is significant as it has been reported that the inhibition of collagen activity can reduce the invasive ability of cancer cells, and additionally, it may affect the formation of the basement membrane *in vivo* (Boghaert et al., 1994; Liu and Rose, 1995). In addition to this, possible anti-cancer effects from *T. capensis* extracts have previously been eluded to, increasing the necessity for investigation (Henkel et al., 2012). However, scientifically documented information regarding these parameters are very limited.

Thus, this is the first study, aimed at scientifically investigating the male reproductive function and isolation, purification and structural identification of bioactive compounds extracted from *T. capensis* rhizome, as well as its possible usage as traditional remedy.

4.2 Effects of *T. capensis* aqueous rhizome extract on functional parameters of TM3-Leydig and LNCaP cells

4.2.1 Cytotoxic effects of *T. capensis* aqueous rhizome extract on TM3-Leydig cells and LNCaP cells

Previously, a study conducted by De Wet et al. (2009) investigated the possibility of cytotoxicity of crude alkaloid extracts from the leaves and rhizomes of all the South African members of the family

Menispermaceae species. Each of these plants was tested against MCF7 (breast), UACC62 (melanoma) and TK10 (renal) cancer cell lines. Extracts of ten of the total thirteen species used in their study showed positive cytotoxic activity against all three cancer cell lines, with significant inhibition of cellular growth along with total growth inhibition. These authors also suggested that despite the traditional use of *Cissampelos capensis,* for example, 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, demonstrating the possibility of a falsely perceived efficacy toward cancer by traditional healers.

Nevertheless, many plants investigated for suggested anti-cancer abilities often prove true. A study conducted by Nurkhasanah et al. (2009), for example, investigated anticancer claims of eurycomanone which is a cytotoxic compound found in *Eurycoma longifolia* Jack. These authors demonstrated the cytotoxicity of eurycomanone, killing 50% of HeLa cells, reducing viability and proliferation significantly, thus making it a potent anti-proliferative agent. Moreover, Zakaria et al. (2009) demonstrated the cytotoxicity of eurycomanone against human hepato carcinoma cells, suggesting that eurycomanone is cytotoxic on cancerous liver cells, HepG2 and less toxic on normal cells Chang's liver and WLR-68. Similarly, Mahfudh et al. (2008) reported that eurycomanone was cytotoxic on cancerous cells (CaOv-3, HeLa, HepG2, HM3KO, MCF-7) and less toxic on normal cells (MDBK, Vero), demonstrating the potential of plants to act as effective anticancer agents.

This possibility of anticancer effects is true for many plants as Slambrouck et al. (2007) determined, showing the effects of crude aqueous extracts of a panel of medicinal plants on the growth and invasion of cancer cells, showing that extracts of *Larrea tridentata* (Creosote Bush) and *Juniperus communis* (Juniper Berry) significantly decreased the growth of MCF-7/AZ breast cancer cells.

Furthermore, Arora et al. (2013) demonstrated the effects of aqueous extracts of 3 edible medicinal mushroom species, namely, *Auricularia polytricha*, *Macrolepiota procera*, and *Pleurotus ostreatus*. These extracts were carried forward to study cytotoxic, antiproliferative, and antiapoptotic effects on breast (MCF-7), colon (COLO-205), and kidney (ACHN) cancer cell lines. Among all the extracts, the aqueous extract of *Pleurotus ostreatus* and the ethanolic extract of *Macrolepiota procera* showed the highest cytotoxic effect on all 3 cancer cell lines, especially COLO-205. Data obtained

demonstrate that the aqueous extracts of all 3 species of mushrooms have significant antiproliferative effects on the cancerous cells (COLO-205) compared with other cancer cells.

More interestingly, Sigstedt et al. (2008) showed that the crude extract of dandelion leaf (DLE) decreased the growth of MCF-7/AZ breast cancer cells in an ERK-dependent manner, whereas the aqueous extracts of dandelion flower (DFE) and root (DRE) had no effect on the growth of either cell line. Furthermore, DRE was found to block invasion of MCF-7/ AZ breast cancer cells while DLE blocked the invasion of LNCaP prostate cancer cells. Similarly, Levy et al. (2014) demonstrated that the viability of LNCaP cells was significantly decreased in a dose-dependent manner following a 24-hour treatment with the *Urtica dioica* extract. This clearly shows that plants have the ability to act as anticancer agents, but more specifically toward prostate cancer cells. This is in line with the current study.

In the current study, the MTT assay was used to determine the cytotoxicity of *T. capensis*. The biochemical process is dependent on the activity of mitochondrial dehydrogenase which is only active in viable cells (Berridge et al., 1996). When TM3 cells were exposed to the aqueous *T. capensis* rhizome extract over 24 and 96 hours it was noted that no effect in cell viability was evident at low extract concentrations, whereas at the higher concentrations (>10 μg/ml) an increase in cell viability could be seen. This elevation in cell viability indicates the presence of cellular stress. The results present that *T. capensis* it has no, or only weak, cytotoxic effects on cell viability of TM3-Leydig cells at all concentrations.

On the other hand, when the LNCaP cells were exposed to the aqueous *T. capensis* rhizome extract over 24 and 96 hours respectively, a significant decrease in cell viability was noted at all concentrations of the extract, indicating a reduced mitochondrial dehydrogenase activity. Moreover, at both the 24-hour and 96-hour exposure, LNCaP cells revealed a remarkable dose-dependent decrease in viability, cellular death is clearly evident at 10 and 100 μg/ml. The results present that the cancer cells, in contrast to the non-cancerous TM3 cells, are stressed and dying under the cytotoxic effects of *T. capensis* at all concentrations. This confirms the idea that this extract has anticancer abilities and warrants its use in this manner.

4.3 Effect of *T. capensis* rhizome extract fractions after HPLC fractionation

Phytochemical studies of the cytotoxic fractions of *Typha capensis* extracts from the different seasons resulted in four fractions, after performing HPLC. Results reveal that the most effective fraction was F1 fraction from the summer harvest.

4.3.1 Effect of *T. capensis* rhizome extract F1 fraction of the summer season on functional parameters of TM3-Leydig, LNCaP and PWR-1E cells

4.3.1.1 Cytotoxic effects of *T. capensis* rhizome extract F1 fraction of the summer season on TM3-Leydig, LNCaP cells and PWR-1E cells

The cytotoxic activities of the isolated fractions were evaluated using the MTT assay. After TM3 cells were exposed to *T. capensis* rhizome extract F1 fraction of the summer season for 24 and 96 hours, at low concentrations the fraction showed no significant difference in cell viability. However, at the highest concentrations (10 and 100 µg/ml) viability showed increases between of +11.5% and +19.4%, respectively, indicating cellular stress. The *T. capensis* rhizome extract F1 fraction of the summer season resulted in no significant difference between 24 and 96 hour incubation periods on TM3 cell viability.

However, when the cancer cell line LNCaP was exposed to *T. capensis* rhizome extract F1 fraction of the summer season for 24 and 96 hours respectively, cell viability significantly, dose-dependently, decreased for all concentrations with declines of up to 82% after 96 hours of exposure at the highest concentration (100 µg/ml) used.

While for the non-cancerous TM3-cells, cytotoxic effects of *T. capensis* rhizome extract F1 fraction of the summer season on PWR-1E cells after 24 and 96 hour, also resulted in no significant changes when compared to the control, and only at the highest concentration (100 µg/ml), revealed decreases of -28.6% and -36.2%, respectively, were evident for both incubations, the 24- and 96-hour period. This is similar to studies conducted on many other planst which show similar results.

Teriflunomide (TFN) is an inhibitor of de novo pyrimidine synthesis and the active metabolite of leflunomide (Chemoprevention, 2010). Leflunomide is prescribed to patients worldwide as an immunomodulatory and anti-inflammatory disease-modifying prodrug. Leflunomide inhibited the growth of human prostate cancer xenographs in mice, and leflunomide or TFN promoted cytostasis and apoptosis in cultured cells. These findings suggest that TFN could be useful in prostate cancer chemoprevention (Chemoprevention, 2010). However, investigation of the possible mechanistic aspects of this tenet by characterizing the effects of TFN using premalignant PWR-1E and malignant DU-145 human prostate epithelial cells showed that TFN promoted a dose- and time-dependent cytostasis or apoptosis induction in these cells (Chemoprevention, 2010).

Rice et al. (2008) characterized the effects of a soy-derived isoflavone concentrate (ISF) on growth and gene expression profiles in the LNCaP, an androgensensitive human prostate cancer cell line. ISF caused a dose-dependent decrease in viability ($P<0.05$) and DNA synthesis ($P<0.01$) as well as an accumulation of cells in G2/M, and G0/G1 phases of the cell cycle compared with controls. However, ISF inhibits the growth of LNCaP cells through the modulation of cell cycle progression and the differential expression of androgen-regulated genes. Thus, ISF treatment serves to identify new therapeutic targets designed to prevent proliferation of malignant prostate cells.

In a study by Jayaprakasha et al. (2012), fresh Nagami kumquats (*Fortunella margarita*) were subjected to hydrodistillation using a Clevenger-type apparatus to obtain volatile oil. Kumquat volatile oil inhibits proliferation of androgen-dependent human prostate cancer (LNCaP) cells through induction of apoptosis and inhibition of inflammation. Results of the current study suggest that the volatile principles of kumquats have great potential for the prevention of cancer.

Glycyrrhetinic acid (GA) is the active metabolite of glycyrrhizic acid, one of the components of liquorice extract (Hawthorne et al., 2008). It has been shown to possess anti-inflammatory activity and to inhibit hepatic tumour growth. In this preliminary study, shown that GA could significantly reduce the rate of proliferation of LNCaP androgen dependent prostate cancer cells, whereas it had no effect on proliferation of PC3 and DU145 androgen-independent prostate cancer cells. Additionally, GA could significantly reduce the production of prostate-specific antigen by LNCaP cells maintained in-vitro (Hawthorne et al., 2008).

4.3.1.2 Effects of *T. capensis* rhizome extract F1 fraction of the summer season on testosterone production in TM3-Leydig cells

Testosterone is the male hormone secreted by the Leydig cells located within the interstitium of the testis. Among the other functions of this hormone, it is also responsible for the maintenance of sperm cell production and is known to influence sexual behaviour (Seeley et al., 2003).

Testosterone production by TM3-Leydig cells was investigated after the exposure of *T. capensis* rhizome extract F1 fraction of the summer season at different concentrations for 48 and 96 hours. The testosterone production after the 48-hour exposure exhibited significant increases of +67.3% and +125.6%, ($P=0.009$, $P<0.0001$, respectively) peaking at concentrations of 0.02 and 0.1 µg/ml when comparing to the control. At higher concentrations, a decrease in testosterone production was observed, whereas after 96 hours of exposure the testosterone production significantly increased at the concentrations 0.01, 0.02 and 0.1 µg/ml by +34% ($P=0.0019$), +97% ($P<0.0001$), and +157% ($P<0.0001$). However, testosterone production declined again at higher concentrations <10 µg/ml.

Results revealed that testosterone production of TM3 cells after exposure to *T. capensis* rhizome extract F1 fraction of the summer season at both exposure periods, 48- and 96-hours, appeared to a biphasic effect with a peak at 0.1 µg/ml of the extract. This confirms that *T. capensis* rhizome extract can be used to induce testosterone production in TM3 cells in a biologically significant manner.

This is the first study showing the capacity of *Typha capensis* to enhance testosterone production in TM3 cells. Although there are many plant extracts reportedly having aphrodisiac properties and the ability to improve male sexual functions, only a miniscule proportion has been scientifically proven to have a positive effect on increasing testosterone production (Patel et al., 2011).

Hence, in the present study the ability of *Typha capensis* extract F1 to stimulate testosterone production was shown to increase by 157%. The F1 extract was acting directly on ageing Leydig cells and promoting the production of testosterone, which is supported by the observed increase in serum testosterone concentrations. Additionally, the increase in testosterone was not an affect by LH secretion by the pituitary gland. Therefore, the F1 extract was acting directly on the Leydig cells and

not through the hypothalamic-pituitary-gonadal (HPG) axis. This increase is in line with previous studies on this plant (Fransman, 2007; Haines, 2012).

Specifically, Fransman (2007) proved that aqueous *Typha capensis* crude extract is able to increase the levels of testosterone and decrease body weight in comparison to the control, *in vivo.* Similarly, Chen et al. (2002) concluded that the inability of aged rat Leydig cells (from 21-24 month old rats) to produce testosterone in the presence of LH *in vitro* was possibly due to inefficient signal transduction during steroidogenesis. This may be explained by considering this process of raising testosterone by other plants.

Other plants such as *Eurycoma longifolia* (Tongkat Ali), *Hibiscus macranthus* and *Basella alba,* are also reported to boost testosterone levels, thus exhibiting aphrodisiac properties and increasing sexual motivation and performance (Moundipa et al., 2005; Nantia et al., 2011; Tambi et al., 2011; Manfo et al., 2014; Solomon et al., 2014). Longjack root (Tongkat Ali) is used traditionally in Malaysia, Indonesia, and Vietnam, where the root of the plant is boiled and consumed as a tonic for increased sexual potency (Ang et al., 1989).

Studies have provided evidence in support of the claim that a particular compound called eurycomanone, extracted from Longjack root, can increase testosterone levels in rats (Zanoli et al., 2009). Multiple mechanisms of action have been proposed concerning the increase in testosterone levels with Longjack root supplementation. The earliest possible mode of action was described by Small (2000), whereby Longjack root was found to increase cAMP levels (Small et al. 2000). This increase in cAMP enhances glucose utilization and may have a significant contribution to the energy increasing effects.

In a more recent study, Longjack root extract induced testosterone synthesis and elevated LH and FSH but reduced oestrogen levels in the plasma (Low et al., 2013). This finding provides evidence that treatment with Longjack root extract may potentially down-regulate the oestrogen-mediated feedback effect on LH and LSH secretion in the hypothalamic-pituitary-gonadal axis (Prakash et al. 2007). The same study suggested that the enhanced production of testosterone by Leydig cell

explants is the mechanism of action, owing to the inhibition of phosphodiesterase and aromatase by eurycomanone (Low et al., 2013).

Tribulus terrestris, commonly known as a tropical plant found growing readily in America, Europe, Asia, Australia, and Africa. Traditionally, *Tribulus terrestris* has a history in increasing testosterone levels in men (Gauthaman and Ganesan, 2008). However, as this herb has been used for centuries to promote urinary health, increased sexual desire, and as a general tonic. This botanical agent has gained renewed interest due to claims that it can enhance testosterone. A limited number of animal studies have found that TT significantly increases testosterone levels. For example, two of three recent studies utilizing Wistar rats found serum testosterone levels to be significantly increased following varying dosages of TT taken once daily (Shukla et al., 2009; Martino-Andrade at el., 2010; Ghosian Moghaddam at el., 2013).

Basella alba is a plant commonly used by traditional healers in the West Cameroon region to treat infertility and boost potency in men. Furthermore, methanol extracts made from this plant (MEBa) have been shown to greatly stimulate testosterone production in both testicular sections and Leydig cell cultures. Additionally, these methanolic extracts were found to boost testosterone production in normal adult albinos male rats (Nantia et al., 2007; Moundipa et al., 2006). Conversely, Moundipa et al. (2005) showed that a methanol extract of *Basella alba* stimulates testosterone production by Leydig cells after 12 hours of incubation, with a maximum effect at 10 μg/mL. Recent results published by Nantia et al. (2011) showed that this extract did not affect Leydig cell viability.

A study by Tambi et al. (2011) showed that serum testosterone concentration in 76 late-onset hypogonadism patients before and after treatment with 200mg standardised water-soluble extract of *Eurycoma longifolia* for 1 month resulted in highly significant increase in the testosterone concentration when statistically calculated with a with $P<0.0001$.

Rauvolfia vomitoria is a plant used by traditional healers in Cameroon for the treatment of various ailments, namely diarrhoea, malaria, hypertension and male infertility. Interestingly, Lembe et al. (2014) investigated the effects of *Rauwolfia vomitoria* bark extract on reproductive functions of male rats, and the results showed that testosterone level was significantly observed at the dose of 200

mg/kg (P<0.01). However, in this study it was explained that the significant increase in testosterone levels in blood indicated an increase in androgen level in all treated animals.

4.3.1.3 Effects of *T. capensis* rhizome extract F1 fraction of the summer season on cell early apoptosis in TM3-Leydig, LNCaP and PWR-1E cells

Annexin V is a Ca^{2+}-dependent phospholipids binding protein that detects phosphatidylserine externalization of the plasma membrane indicating early signs of apoptosis (Vermes et al. 1995). TM3-Leydig cells after 24 and 96 hours of treatment with *T. capensis* rhizome extract F1 fraction of the summer season, respectively, at low concentrations cells showed no significant difference in the percentage of early signs of apoptosis. At higher concentrations, however, slight increases, but not siginificant, of the percentage of apoptotic cells were found, indicating cellular stress. This confirms that *T. capensis* rhizome extract F1 fraction of the summer season does not induce early signs of apoptosis in TM3-Leydig cells.

When LNCaP cells were exposed to *T. capensis* rhizome extract F1 fraction of the summer season for 24 and 96 hours, LNCaP cells shown to increase in a dose-dependent manner with significant increase at the higher concentration. All concentrations yielded an increase in percentages of early signs of apoptosis. At higher concentrations (10 and 100 μg/ml), a significant increase between of +28% to +52% in the percentage of early signs of apoptosis was significant (P<0.001). This confirms that *T. capensis* rhizome extract F1 fraction of the summer season induces early signs of apoptosis in LNCaP cells.

After PWR-1E cells were exposed to *T. capensis* rhizome extract F1 fraction of the summer season over 24 and 96 hours, it was noted that *T. capensis* rhizome extract F1 fraction does not have the ability to induce early signs of apoptosis in PWR-1E cells. However, at the highest concentration (100 μg/ml), it was noted that slight elevations in the percentage of early signs of apoptosis between of +3.67% to +5.36% became evident. This elevation in cell early apoptosis indicates the presence of cellular stress.

4.3.1.4 Effects of *T. capensis* rhizome extract F1 fraction of the summer season on DNA fragmentation in TM3-Leydig, LNCaP and PWR-1E cells

Apoptosis occurs normally during development, aging, and as a homeostatic mechanism to maintain cell populations in tissues. Apoptosis also occurs as a defence mechanism such as in immune reactions or when cells are damaged by disease or noxious agents (Norbury and Hickson, 2001). Apoptosis is a programmed cell death characterized by morphological features and extensive DNA fragmentation (Collins at el., 1997).

In the present study determining DNA fragmentation, the TUNEL assay was used to investigate the late signs of apoptosis. However, in this study, no effect of the *T. capensis* rhizome extract F1 fraction on cell DNA fragmentation could be observed in both cell lines TM3-Leydig and PWR-1E cells after exposed over 24 and 96 hours, and recorded low DNA fragmentation levels as an effect of apoptosis process. Therefore, the *T. capensis* rhizome extract F1 fraction can be said to have no destructive effects on DNA fragmentation of normal cell lines (TM3-Leydig and PWR-1E cells). However, after exposing LNCaP cells were exposed for 24 and 96 hours the *T. capensis* rhizome extract F1 fraction caused enough DNA damage characterized by stained cell nuclei at high concentrations (1, 10 and 100 µg/ml) to drive the cell toward decreasing capacity of cell repair mechanisms, fragmentation and cell death. This confirms that treatment with *T. capensis* rhizome extract F1 fraction induced DNA fragmentation in the cancer cell line LNCaP in a concentration-dependant manner.

Stolarczyk et al. (2013) concluded that LNCaP cells treated with extracts from *Epilobium parviflorum* and *Epilobium hirsutum*. Incubation of LNCaP cells with aqueous *Epilobium* extracts (20, 50, 70 mg/ml). Cell apoptosis was determined by staining with propidium iodide (PI) and Annexin V–fluorescein isothiocyanate. This resulted in a reduction of proliferation in a concentration-dependent manner. The percentage of late apoptotic cells was significantly increased, in a dose-dependent manner, and apoptotic cells in the early phase of apoptosis (Annexin V+/PI–). Cells treated with extracts, especially in lower concentration of 20 mg/ml, were necrotic the percentage of necrotic cells was statistically significant.

In a study conducted by Cristina et al. (2014), the cytotoxic and apoptotic activities of *Ficus pseudopalma* (FP) Blanco leaf extracts were investigated against the human prostate PRST2 cancer cell line. A significant decrease in the viability of PRST2-treated cells ($P < 0.001$) in a concentration-dependent manner was observed. Using APO-BrdU TUNEL assay, it was observed that the apoptotic activities of the extracts increased in a concentration-dependent manner.

A study conducted by Chiu et al. (2015) determined the effects of *Hibiscus sabdariffa* leaf extract (HLE) on LNCaP cells. It was shown that HLE, in a dose-dependently manner, inhibited the migration and invasion of human prostate cancer LNCaP. The results showed that HLE exerted an inhibitory effect on the activity and expressions of matrix metalloproteinase-9 (MMP-9). The HLE-inhibited MMP-9 expression appeared to be a consequence of nuclear factor-kappaB (NF-κB) inactivation because its DNA-binding activity was suppressed by HLE. These findings suggested that the inhibition of MMP-9 expression by HLE may act through the suppression of the Akt/NF- κB signalling pathway, which in turn led to the reduced invasiveness of the cancer cells.

Lin et al. (2012) examined the anticancer properties of *Hibiscus sabdariffa* leaf extract (HLE). It was revealed that the effect of HLE in LNCaP cells might be mediated via both intrinsic (Bax/cytochrome c-mediated caspase 9) and extrinsic (Fas-mediated caspase 8/t-Bid) apoptotic pathways. These also implied that HLE combined the two death pathways, triggering redundant signalling to induce cell apoptosis. The data also showed that HLE induced apoptotic signal in LNCaP cells *in vitro* and *in vivo.*

4.4 Isolation, Characterization, Cytotoxicity, Testosterone production, Apoptosis induction of phenolic active compounds (Quercetin and Naringenin) from *T. capensis* rhizome extract F1 fraction of the summer season

Previous studies into the specific phytochemical composition of *Typha capensis* and its relation to the reported cytotoxic fractions resulted in two major phenolic compounds, specifically flavonoids, which have been identified as quercetin and naringenin. Reportedly, these two specific bioactive phenolics, quercetin flavanols and naringenin flavonoids, were identified based on their NMR spectra, specifically ^{1}H and ^{13}C. It is important to note that this study represents the first time that the

two phenolics responsible for the cytotoxic effects of *Typha capensis*, specifically Quercetin and Naringenin, have been successfully isolated from *Typha capensis* rhizomes.

Traditional healers would give a male seeking treatment for infertility a handful of *Typha* rhizomes to boil in water and the infusion is taken daily for about a week. Several handfuls of *Typha* rhizomes were weighed and an average was calculated which represents the weight of *Typha* extract per kg of body weight that a potential male would take as prescribed treatment. The hypothesized concentrations were tested for cytotoxicity to determine whether they would be toxic to cells in an in vitro system. In this particular study HPLC analysis of 1g of *T. capensis* rhizome extract yielded about 200 mg of F1 fraction. According to the HPLC profile of sub-fraction of F1 fraction from the summer harvest of *T. capensis* rhizome extract, 63% was Quercetin and 37% was Naringenin. However, according to this, percentage concentrations were estimated to be prescribed treatment in an *in vitro* system.

According to results obtained in the current study, the most effective concentrations of Quercetin and naringenin, for boosting testosterone, were ranged between 0.075 to 0.125 µg/ml *in vitro*, which is considerably lower in comparison to typical concentrations prescribed by Sangomas, ranging from 2.537 to 1.487 µg/ml *in vivo*. This is likely due to the oral bioavailability of quercetin being low in humans and highly variable (0-50%) (Pedersen and Miller, 1980). Bioavailability has previously been defined as a subcategory of absorption and is the fraction of an administered dose of an unchanged drug that reaches the systemic circulation (Russo et al., 2014). In addition, the bioflavonoid Naringenin is difficult to absorb on orally. At best, approximately 15% of the total ingested naringenin will be absorbed in the human gastrointestinal tract (Choudhury et al., 1999). Following oral administration, it is thought that naringenin is converted to the aglycone naringenin, most likely in the gut (Choudhury et al., 1999). Furthermore, it has been demonstrated that naringenin inhibits CYP3A4 activity in human liver microsomes (Fuhr et al., 1993). In addition, the action of the liver, which is obviously not available in the *in vitro* system, has to be taken into consideration when comparing the effective concentrations of these compounds.

Previously, two new phenolics, namely typhaphthalide and typharin were isolated and identified from hexane extract produced from the rhizomes of *Typha capensis* (Shode et al., 2002). In addition

to this, certain relevant species, such as *Typha domingensis*, have been shown to isolate phytotoxic compounds from aqueous extracts, and structural elucidation by NMR spectroscopy. These phytotoxins were identified as essential fatty acids, namely inoleic acid and alinolenic acid, along with phenolic compounds of known phytotoxic activity, such as caffeic acid, caffeic, p-coumaric, and gallic acid (Gallardo-Williams at el., 2002). However, in *Typha angustifolia*, 11 major flavonoids were isolated and by HPLC-PDA-MS and (^{1}H NMR, ^{13}C NMR and MS) methods. These 11 compounds that were previously identified are quercetin-3-O-(2G-α-L-rhamnosyl) rutinoside, quercetin-3O-neohesperidoside, kaempferol-3-O-(2G-α-L-rhamnosyl)rutinoside, isorhamnetin-3-O-(2G-α-L-rhamnosyl)-rutinoside, typhaneoside, kaempferol-3-O-neohesperidoside, isorhamnetin-3-O-neohesperidoside, isorhamnetin-3-O-rutinoside, quercetin, naringenin, kaempferol, and isorhamnetin (Tao et al., 2011).

By definition, phenolics are a group of compounds which are described as possessing one or more aromatic rings to which is attached at least one hydroxyl group. Furthermore, phenolic compounds can be subcategorized into different groups, such as flavonoids and non-flavonoid phenolic compounds (Jaganath and Crozier, 2009). These types of compounds have been reported to be a large and complex group of chemical constituents of most plants and are well known for their diverse pharmacological efficacies, which not only affect their quality, but also contribute to their beneficial health effects (de Beer et al., 2002). In recent years, due to a growing interest in medicinal plants, these natural phenolic compounds have received increasing interest, since a great amount of them can be found in plants, and consumption of vegetables and beverages with a high level of such compounds may reduce the risk of development of several diseases due to their antioxidant power, among other factors (Reis Giada, 2013). Moreover, it has been reported that the consumption of flavonoids, especially from natural sources, has anti-tumor capabilities. In addition, these flavonoids have been reported to have many other beneficial functions, such as the inactivation of many different types of carcinogens, induction of cell cycle arrest and promotion of apoptosis, the inhibition of angiogenesis (Kanadaswami et al., 2005), the effective reversal of multi-drug resistance, or a combination of these previously mentioned activities (Kanadaswami et al., 2005).

The compound known as quercetin has previously been reported to be a very important, and beneficial bioflavonoid, which is most beneficial in the human diet as far as as polyphenolic

flavonoids go (Lamson and Brignall, 2000). An example of why this is true are previously conducted epidemiological studies reviewing the effects of quercetin, which have found an inverse relationship between the dietary intake of quercetin and occurrence of cardiovascular disease (Larson et al., 2010). Furthermore, These findings have led to *in vitro, in vivo*, and clinical research to attempt to determine the exact mechanism by which quercetin may exert these reported cardioprotective effects (Larson et al., 2010).

In recent years, however, research into quercetin has expanded beyond the reported cardioprotective effects and is now being investigated for promise as a potential anti-cancer agent (Lamson and Brignall, 2000). Nevertheless, the estimated average daily dietary intake of quercetin by an individual in the United States is 25 mg (Lamson and Brignall, 2000). Furthermore, the reputation of quercetin as an antioxidant compound stems from the reactivity of phenolic compounds with free radical species to form phenoxy radicals which are considerably less reactive. It can therefore be said that quercetin has importance in terms of ethnopharmacology such as its use as antioxidant, anticancer and neuroprotective, it has been reported as an efficient free radical scavenger, known as an antioxidant molecule (Maalik et al., 2014). It has been reported that quercetin has radical scavenging potential, and this may give it the ability to prevent the formation of cancer induced by excessive oxidative stress (Baghel et al., 2012).

Among flavonoids, quercetin is considered to be an excellent free-radical scavenging antioxidant, even if such an activity strongly depends on the intracellular availability of reduced glutathione. Apart from the reportedly strong antioxidant activity, quercetin has also been reported to exert a direct, pro-apoptotic effect toward different tumor cells, and has been shown to block the growth of several human cancer cell lines at different phases of the cell cycle. Both these effects have been documented in a wide variety of cellular models as well as in animal models (Gibellini et al., 2011). Additionally, it has been reported that while quercitine exerts cytotoxic effects towards cancerous cells, it remains relatively non-toxic towards non-cancerous cells, making it an ideal, selectively toxic agent in the treatment of cancers (Gibellini et al., 2011).

Indeed, the ability of quercetin to exert anti-proliferative and pro-apoptotic effects on normal cells only at very high concentrations sharply contrasts with the low concentrations needed to exert the

same effects on cancer cells that are, for example, 3.5 μM for the B16-BL6 murine melanoma cell line (Caltagirone et al., 2000), 25 μM for PC-3 (p53-null cells) and DU-145 (p53- mutated cells) human prostate cancer cell lines (Nair et al. 2004) and 10 μM for SK-Br3, MDA-MB-453 and MDA-MB-231 human breast carcinoma cells (Jeong et al., 2009). Interestingly, the proliferation of MCF-10A cells, which are normal breast epithelial cells, is not affected by 10 μM of quercitin (Jeong et al. 2009) and, similarly, in the normal fibroblast cell line BG-9, quercetin does not affect cell growth (Nair et al. 2004). Quercetin that can be obtained with a diet rich in these flavonoids are capable of exerting significant effects on tumor cells, while not affecting cell cycle or cell activation of normal, non-transformed cells (Gibellini et al., 2011).

Previous studies of quercetin and its reported effects toward cellular models offer an extensive and nearly exhaustive explanation of the possible mechanisms that may possibly link quercetin to the oxidative cell balance and to the control of cell-cycle phases. Furthermore, very promising results have been obtained in the evaluation of the biological effects of quercetin on both cancer and normal cells (Gibellini et al., 2011). Specifically, the high toxicity of quercetin for cancer cells, along with the characteristic to exert anti-proliferative and pro-apoptotic effects on normal cells only at high concentrations are crucial aspects in the field of anticancer research, whose important goal is the identification of drugs that selectively kill tumor cells without damaging normal cells (Gibellini et al., 2011).

Naringenin belongs to the flavanones and is mainly found in fruits such as grapefruit and oranges, and many different vegetables. Pharmacologically, it has been shown to have anti-cancer, anti-mutagenic, anti-inflammatory, anti-oxidant, anti-proliferative and anti-atherogenic activities (Ameer et al., 1996; Cavia-Saiz et al., 2010; Dou et al., 2013).

Many previous studies have suggested that Naringenin supplementation is very beneficial for the treatment of obesity, diabetes, hypertension, and metabolic syndrome (Sumathi et al., 2015). Furthermore, Naringenin and its derivatives have previously been shown to exhibit strong anti-oxidant potential along with many different protective effects for the improvement of many different aspects of human health. Several *in vitro* and *in vivo* experimental results support their beneficial effects, Naringenin is used for the treatments of osteoporosis, cancer and cardiovascular diseases,

and showed lipid-lowering and insulin-like properties and anti-inflammatory capacity (Yilma et al., 2013; Sumathi et al., 2015). In addition to this, Naringenin has also been reported to have anti-carcinogenic properties (Frydoonfar et al., 2003). Specifically, the effects of Naringenin on cell proliferation of an HT-29 colon cancer cell line showed that there was a significant inhibition of cell proliferation when these cells were exposed to Naringenin (Sumathi et al., 2015).

4.4.1 Cytotoxic effects of bioactive compounds isolated from *T. capensis* on TM3-Leydig and LNCaP cells

The cytotoxic activities of the isolated compounds were evaluated using the MTT assay. The cytotoxic effects of these compounds against TM3-Leydig and LNCaP cells, along with normal somatic cells and cancer cells, after cells were incubated with increasing concentratios of quercetin and naringenin, which are two of the biologically active compounds in *T. capensis* rhizomes. Here, the assay showed that LNCaP cells are more sensitive to the cytotoxic effects of both of these compounds, whereas, the assay resulted in markedly weaker effects toward TM3-Leydig cells which are normal somatic, non-cancerous cells. However, it is evident that the isolated compounds are significantly selective towards the cancer cells than non-cancerous cells, as compared with the exposure of bioactive compounds used in this study.

It is important to note that this is the first time that cytotoxicity has been reported when the isolated compounds from the *T. capensis* rhizomes were used against these cell lines, specifically the non-cancerous TM3-Leydig cells and LNCaP prostate cancer cells. Furthermore, the cytotoxic effects of quercetin have been tested previously in the JCA-1 and LNCaP cells (Nakanoma et al., 2001). Here, quercetin was reported by these authors to enhance heat-induced inhibitory effects on cell growth in both JCA-1 and LNCaP cells. Moreover, these results suggested that quercetin may possibly enhance the heat-induced cytotoxicity in prostate cancer cells, perhaps through the inhibition of hsp70 production. Moreover, when exposed to ovarian cancer C13* cells, quercetin used at low concentrations was shown to promote the survival of the ovarian cancer C13* cells treated with cisplatin and varying degrees of attenuation of cytotoxicity of cisplatin treatment, when combined with cisplatin (Li et al., 2014). However, a study conducted by Gibellini et al. (2011) showed that quercetin exerts a direct, pro-apoptotic effect toward tumor cells, and can indeed quite effectively

block the growth of several human cancer cell lines at different phases of the cell cycle. Both these effects have been documented in a wide variety of cellular models as well as in animal models. The high toxicity exerted by quercetin on cancer cells perfectly matches with the almost total absence of any damages for normal, non-transformed cells. This was effectively shown in a study conducted by Knowles et al. (2000) demonstrating that an exposure to increasing concentrations of quercetin and Kaempferol led to an apparent dose-dependent decrease in PC-3 prostate cancer cell proliferation.

Nevertheless, the cytotoxic effect of naringenin have been tested previously in the human cervical HeLa cancer cells (Krishnakumar et al. 2011) by means of the MTT-based colorimetric assay, which revealed higher cytotoxic efficacy of naringenin in HeLa cells. However, a study conducted by Ayob et al. (2014) demonstrated that the highest concentrations of kaempferol and naringenin were detected in leaves extracted from Mersing. The effects of kaempferol and naringenin were examined on breast cancer cell lines, MDA-MB-231 and MDA-MB-468, using MTT assay showed high cytotoxicity against breast cancer cells. Whereas, naringenin was also shown to inhibit both human colorectal, SW1116 and SW837, and breast cancer, HTB26 and HTB132 cell growth in a dose- and time-dependent manner (Abaza et al., 2015). However, in a study showing the effects of naringenin on the HT29 human colon cancer cell line, naringenin used at concentrations ranging from 0.02 to 0.09 mmol caused an increased proliferation of HT29 colon cancer cell lines (Frydoonfar et al., 2003). Significant inhibition of cell proliferation was observed in HT29 colon cancer cells exposed to Naringenin at doses greater than 0.71 mmol. Furthermore, naringenin exposure has also been shown to significantly reduce cell viability of A431 human epidermoid carcinoma cells with a concomitant increase in nuclear condensation and DNA fragmentation in a dose-dependent manner (Ahamad et al., 2014).

A study conducted by Song et al. (2015) confirmed that the naringenin effectively inhibited the cell proliferation in HCT116 and SW480 cells, as well as decreased the level of cyclin D1 protein in human colorectal cancer cell lines, HCT116 and SW480. However, the findings of this study demonstrated that naringenin-induced proteasomal degradation of cyclin D1 might inhibit the cell proliferation in human colorectal cancer cells. Furthermore, this study provides valuable information on molecular events for the anti-cancer activity of Naringenin. (Kusharyanti et al. 2011) Addtionally, a study investigating the cytotoxic effect and potential apoptosis induction of naringenin in

combination with doxorubicin on HeLa cells revealed that naringenin and doxorubicin showed cytotoxic effect on HeLa cells with their IC50 values of 195 μM and 1 μM, respectively. Whereas combination of naringenin-doxorubicin showed greater cytotoxicity compared the single treatment of doxorubicin. Doxorubicin and naringenin inhibited the growth of HeLa cells in dose-dependent manner.

4.4.2 Effects of bioactive compounds isolated from *T. capensis* on testosterone production in TM3-Leydig cells

In the current study, testosterone production by TM3-Leydig cells was investigated after the exposure of bioactive compounds quercetin and naringenin, at different concentrations for 48 and 96 hours, respectively. Testosterone production was significantly enhanced at low concentrations by quercetin and naringenin, at both acute and chronic exposures, and testosterone production was shown to peak significantly at around 0.100 and 0.125 μg/ml ($P<0.0001$), demonstrating stimulatory activity in a dose-dependent manner. Whereas, at the highest concentrations a dose-dependent decrease of testosterone productions was observed. It must be noted that this is the first study showing that both quercetin and naringenin boost testosterone levels in TM3-Leydig cells.

Previously, in a study investigating the testosterone production, it was found that in order to isolate the active fractions of the methanol extract of *Basella alba* (MEBa) and to investigate the action mechanism underlying their effects on testosterone production, TM3 Leydig cells were treated with nine fractions of MEBa, resulted in. MEBa and all fractions significantly increased testosterone production in a dose-dependent manner (Edjenguele et al., 2014).

4.4.3 Effects of bioactive compounds isolated from *T. capensis* on cell early apoptosis in TM3-Leydig and LNCaP cells

In this study, inductions of early apoptosis by the isolated compounds (quercetin and naringenin) were evaluated using Annexin V Cy3-binding. The results shown after different types and period of exposures for 24-96 hours reveal that the two compounds induced significant apoptosis in cancer cell line (LNCaP) compared with the normal cell line (TM3-Leydig). Significant early phase apoptosis

was observed compared to non-significant effects on normal cell line with the use of Annexin-binding for the two compounds. However, TM3-Leydig cells after chronic exposure only at highest concentrations resulted in an increase in early phase apoptosis this increase was significant by ($P<0.001$). This confirms that quercetin and naringenin induce early signs of apoptosis after 96-hours of chronic exposure in normal cell line, but cell induced apoptosis takes longer than cancer cell line.

A study conducted by Gibellini et al. (2011) showed that quercetin has also received greater attention as pro-apoptotic flavonoid with a specific and almost exclusive activity on tumor cell lines rather than normal, non-transformed cells. Furthermore, direct pro-apoptotic effects of quercetin collectively, the pro-apoptotic effects of quercetin may result from multiple pathways. First, in MDA-MB-231 cells, quercetin treatment increases cytosolic Ca^{2+} levels and reduces the mitochondrial membrane potential (MMP), thus promoting activation of caspase-3, -8 and -9. The capability of quercetin to induce apoptosis via mitochondrial pathway has been confirmed in U937 cell line. Moreover, quercetin has also been shown to act as an anti-tumour drug *in vitro* by exerting a strong pro-apoptotic activity on leukemic cells but not on immune cells, as several chemotherapeutic drugs do (Lugli et al., 2009). Moreover, Quercetin treatment did not sensitize peripheral blood mononuclear cells to CD95-induced apoptosis. However, inhibition of lymphocyte proliferation and activation damp hopes on its possible use in cancer therapy without inducing interference on normal cellular functions.

Furthermore, it was also indicated that pre-incubation of cells with quercetin followed by cisplatin treatment was very effective in induction of apoptosis in HeLa cells (Jakubowicz-Gil et al., 2005). The pro-apoptotic activity of quercetin was mediated by several mechanisms: activation of caspase-3, inhibition of expression and nuclear translocation of Hsp72 and inhibition of MRP level.

It has also previously been shown that quercetin induced the apoptosis of MCF-7 cells and inhibited the proliferation of the MCF-7 breast cancer cells in a time- and concentration-dependent manner (Zheng, 2013). Here, quercetin inhibited the growth of MCF-7 cells and promoted apoptosis by inducing G0/ G1 phase arrest. It also regulated the expression of survivin mRNA in MCF-7 cells, which may be the mechanism underlying its antitumor effect. Similarly, it was confirmed that

naringenin dose-dependently induced apoptosis in both Caco-2, colon cancer, and HL-60, leukemia, cell lines, suggesting that the cytotoxic activity of naringenin in Caco-2 and HL-60 cells occurs via apoptosis (Kanno et al., 2005).

Moreover, Caco-2 cells required a higher concentration of naringenin than HL-60 cells to undergo apoptosis. Previously, naringenin was tested to ascertain the DNA damage following exposure of various concentrations of compound to A431 cells (Human Squamous Carcinoma) the results obtained from the DNA fragmentation assay showed the undamaged DNA in control cells whereas naringenin-treated cells represent DNA fragmentation which was increased in a dose dependent manner (Ahamad et al., 2014). Also, evidence has been exhibited that naringenin is highly effective in inhibiting cell proliferation and inducing apoptosis cell death in human hepatocellular carcinoma Hep G2 cells and naringenin may be a promising candidate for hepatocarcinogenesis treatment (Arul and Subramanian, 2013).

Furthermore, the cytotoxic effect and apoptosis induction of naringenin in combination with doxorubicin on HeLa cells (cervical cancer) the result showed both 0,5 μM doxorubicin and 100 μM Naringenin induced apoptosis in HeLa cells and there was not apoptotic occurrence in cell control (Kusharyanti et al., 2011). Combination of 0.5 μM doxorubicin-100 μM Naringenin resulted the highest occurrence of apoptotic cells and the lowest density of cells.

4.4.4 Effects of bioactive compounds isolated from *T. capensis* on DNA fragmentation in TM3-Leydig and LNCaP cells

In this study, the effects of the isolated compounds Quercetin and Naringenin on the function of the cell apoptosis were evaluated using late signs of apoptosis DNA fragmentation, investigated by means of the TUNEL assay.

When TM3 cells were exposed to the Quercetin and Naringenin for different types and period of exposures for 24-96 hours, it was noted that for 24-96 hours acute exposure, no change in the percentage of TUNEL-positive cells was observed. However, after chronic exposure 96 hours produced slight elevations in the percentage of TUNEL-positive cells were evident at the highest

compounds concentrations. This elevation in cell apoptosis indicates the presence of cellular stress, is clear that the phenolic compounds of Quercetin and Naringenin were only able to produce biologically significant apoptotic effect on DNA fragmentation at the highest concentration, over an extended period of time and chronic exposure in TM3 Leydig cells. Moreover, the quercetin and naringenin can be said to have exhibited weak induction of DNA fragmentation toward normal somatic TM3 Leydig cells.

When LNCaP cells were exposed to the quercetin and naringenin for different types and period of exposures for 24-96 hours, the results reveal that the two compounds of Quercetin and Naringenin induced significant late phase apoptosis in cancer cell line LNCaP, this may indicate that the quercetin and Naringenin caused enough DNA damage at all concentrations to overcome the capabilities of the cell repair mechanisms and drive the cell toward cell death, and increase in observed DNA fragmentation, characterized by stained cell nuclei, in a time- and concentration-dependant manner. A study conducted by Knowles et al. (2000) showed that DNA fragmentation was not induced after exposure to 100 μM quercetin or kaempferol. However, prolonging the exposure to quercetin or kaempferol did not influence the degree of DNA fragmentation.

4.5 Conclusion:

The effect of seasonality has been successfully studied, along with many reproductive parameters, according to several time periods. Specifically, the objective of this study was to investigate the effect seasonal changes had toward cell viability and testosterone production. Results obtained clearly demonstrated that TM3-Leydig and LNCaP cell viability were significantly higher after exposure to fractions of the summer harvest, as compared to the other seasons. However, PWR-1E cells showed no significant difference in viability between summer, spring, autumn and winter months. Furthermore, testosterone concentrations were found to be significantly higher after exposure to fractions of the summer harvest, when compared to the other seasons. Thus, the results obtained in the present study demonstrated that clear seasonal differences exist in the concentration of the bioactive compounds in the rhizomes. Therefore, further investigations into the seasonality should be conducted as this might have economic implications for farmers when harvesting the

plant, should *T. capensis* possibly be approved by the Medicines Control Council for the treatment of aging male symptoms.

Moreover, this study, for the first time, isolated, identified and investigated the effects of bioactive compounds that were isolated from an aqueous extract of *Typha capensis* rhizomes toward male reproductive functions, health and towards the treatment of prostate cancer. The isolated active compounds present in the rhizomes have clearly resulted in an increased production of testosterone in TM3-Leydig cells, indicating the potential usage in testosterone replacement therapy. Furthermore, high dosages of the active compounds were shown to have negative effect on the percentage of DNA fragmentation in LNCaP cells when compared to the effect of the low dose. This may indicate that although seemingly effective toward the treatment of prostate cancer, further investigation into the ideal dosage, along with the safety of the dose may be required.

Chapter 5: References

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