

**Effect of *Eurycoma longifolia* (Tongkat Ali) on the prostate
cancer cell line LNCaP**

By

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A thesis submitted to the Department of Medical Biosciences in
conformity with the requirements for the degree of Master of Science

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DECLARATION

I, HAMZA ABUOHAMRAA, hereby declare that “Effect of *Eurycoma longifolia* (Tongkat Ali) on the prostate cancer cell line LNCaP” is my own work and has not in its entirety, or in part, been submitted for any degree or examination in any other university. All the resources that I have quoted have been indicated and fully acknowledged by complete references.

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DEDICATION

This thesis is dedicated to my father, who taught me that the best kind of knowledge is that which is learned for its own sake. It is also dedicated to my mother, who taught me that even the largest task can be accomplished, if it is done one step at a time. Thank you for giving me the opportunity to prove myself and explore my capabilities. I would also like to dedicate the dissertation to my wife for being with me and supporting me through the whole process and who has made a major contribution to advancing both my personal growth and my academic career.



Key words

Eurycoma longifolia Jack (Tongkat Ali)

Testosterone

DNA fragmentation

Apoptosis

Annexin V-cy3

Viability

Cytotoxicity

LNCaP cells

Prostatic specific antigen (PSA)



Abstract

Introduction

Eurycoma Longifolia Jack, also known as Tongkat Ali (TA) is a tropical plant belonging to the family of Simaroubaceae is widely distributed in South East Asian countries. The extracts of TA have been proven to have cytotoxicity, anti-proliferative and aphrodisiac properties. *In vitro* assays revealed cytotoxicity toward human breast cancer cell lines MCF-7, KB, CaOV-3, RD, DU-145 and HepG2 human liver cancer cells and appear promising as a new chemotherapeutic agent against human cervical carcinoma (HeLa) cells. Although, there are extensive studies reported on its cytotoxicity benefits there are none pertaining to LNCaP human prostate cell line. Therefore, this study aimed at testing the effects of TA on LNCaP cells and prostate specific antigen (PSA) production.

Materials and Methods

This study investigated the effect of different concentrations of TA (0.0025, 0.025, 2.5, 25 and 250 µg/ml) TA on LNCaP human prostate cancer cell line for 24 and 96 hours. The following parameters were investigated: morphology, cell viability (MTT), testosterone modulation, Annexin V-CY3 binding (Apoptosis), DNA fragmentation (TUNEL), caspase 3/7 activity (apoptosis), and PSA production.

Results

When observing the morphological changes of LNCaP cells exposed to TA, a clear increase in detachment and cell death via apoptosis as the concentrations of TA increased. The viability decreased significantly in both 24 and 96 hour treatment of TA at higher dosages (25 and 250 µg/ml). The significant inhibitory effects on testosterone-stimulated cell proliferation were seen at TA concentrations as low as 0.0025 µg/ml TA. At higher concentrations of TA (25 and 250 µg/ml), for all testosterone dosages a decreasing trend in proliferation was found.

Testosterone concentrations of 10 nM showed maximum stimulation of cell proliferation for TA dosages up to 2.5 µg/ml. All concentrations of TA showed significant increase in apoptosis of the cells as dosages increased.

A higher amount of DNA damage found at the highest dosage (250 µg/ml) of TA. The relative caspase 3/7 activation showed significant ($P=0.0043$) activation at the highest concentration (250 µg/ml) of TA. Relative PSA production resulted only a 5% increase with no significant difference at all doses indicating that TA does not change the cell PSA production and the decline in PSA concentration is due to LNCaP cells dying as a result of this exposure to TA.

Conclusion

In summary, the major finding of this study is that Tongkat Ali inhibits the viability of prostate cancer cell lines (LNCaP) through caspase-mediated pathway, as well as increased the level of apoptotic such as DNA fragmentation. In addition, Tongkat Ali also inhibited PSA production. In LNCaP cells, testosterone with the addition of TA does not increase the growth of the cells. However, more *in-vitro* and *in-vivo* studies are needed to establish the exact constituents of the extracts and their mechanism of action. Thus, this study opens perspectives on the use of Tongkat Ali preparations in the treatment of aging male symptoms, prostate cancer prevention or as additional treatment to standard prostate cancer therapy.

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CHAPTER 1

1. INTRODUCTION

1.1 The Human Prostate Gland

The prostate gland is located in the abdominal cavity inferior to the bladder and a vital part of the male reproductive system. Its size and shape are similar to a walnut, with a length of the anterior aspect between 3 and 4 cm and a width between 3.5 and 5 cm weighing around 20 grams. The urethra from the bladder passes through the prostate before entering the penis. The portion of the urethra in the prostate is called the prostatic urethra. Connected to the prostate are two seminal vesicles, which connect via two deferential ducts that originate from the epididymis.

The prostate is surrounded by striated musculature as well as fatty tissue and neurovascular bundles. Post-embryonic growth occurs principally during puberty and the organ develops to encapsulate the urethra (Figure 1) (McNeal, 1981). Embryologically, the glandular part is of epithelial origin (ectodermal) and comprises approximately two thirds of the prostate; while the other third is fibromuscular and mesenchymal (McNeal et al., 1988).

The prostate consists of glandular tissue and muscle tissue. The glandular tissue produce about 20% of seminal fluid and contributes to the viscosity of the semen by secreting proteolytic enzymes, including prostate specific antigen (PSA), prostatic acid phosphatase (PAP), and alkaline phosphatase (AP). A major constituent of prostatic secretion is PSA, along with citrate (18.7 mg/ml) and a high concentration of zinc that is more concentrated (500–1000 times or 488 µg/ml) than in blood (Aumuller et al., 1990). The prostatic fluid also contains compounds such as citrate, spermine (243 mg/ml) and cholesterol (78 mg/ml) (Blandy et al., 1986).

Prostatic fluid which is clear and slightly acidic (pH 6.4) (Meinhardt, 2006), is expelled in the first ejaculate fraction together with spermatozoa and the fluid of the seminal vesicle (Tortora, 1995). The muscular glands of the prostate help to propel the prostate fluid, which improves the motility of spermatozoa. Activity of the prostate tissue is regulated through androgens such as testosterone, which also control its rate of proliferation (McNeal, 1981).

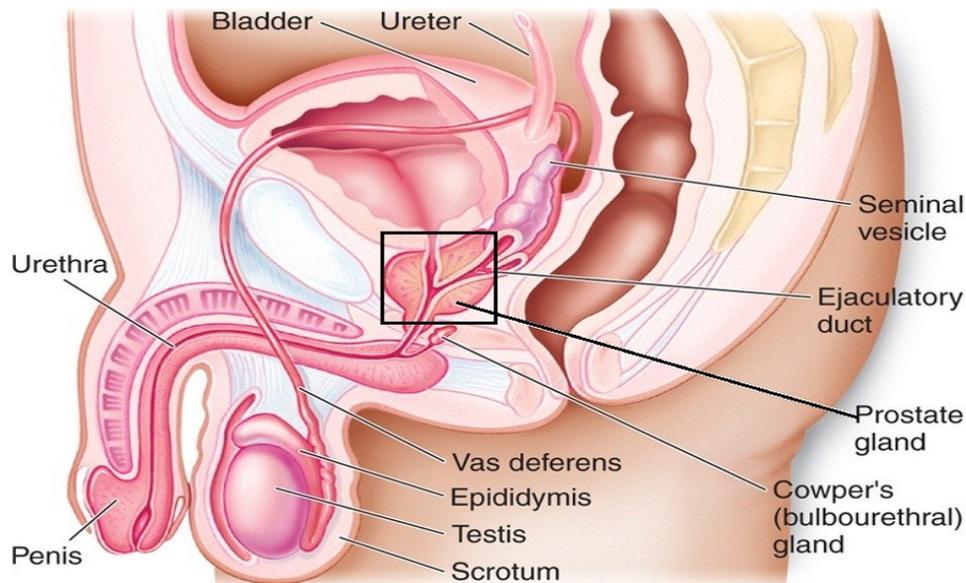


Figure 1: Anatomy of the male pelvis. Anatomical location of the prostate. The prostate is a small muscular gland located beneath the bladder surrounding the urethra (Adapted from McNeal, 1981).

1.2 Anatomy of the Prostate

Anatomically, the prostate is composed of tubuloalveolar glands arranged in lobules surrounded by a stroma. The zone classification is used more often in pathology to identify prostate cancer and was first proposed by McNeal in 1968. According to this classification, the prostate can be divided into 4 zones; the peripheral zone (PZ) and the central zone (CZ) which together comprise <95% of the prostate mass in the prostate of a normal man the periurethral transition zone (TZ) and the anterior fibromuscular zone (AFZ) or stroma and the periurethral glandular zone (Figure 2). Each glandular zone has a specific architecture with varying composition of stromal and epithelial (both basal and differentiated secretory luminal epithelial) cells (reviewed by Cunha et al., 1987; Taplin and Ho, 2001).

1.2.1 The Peripheral Zone

The peripheral zone (PZ) constitutes about 70% of the prostatic volume and contains the majority of the prostate glands. This zone surrounds the distal urethra and approximately 70-80% of prostate cancers originate from this portion of the gland (McNeal et al., 1981). It is also the predominant site for the occurrence of the prostate cancer precursor lesion the prostatic intraepithelial neoplasia (PIN), including high grade PIN (HGPIN) (Bostwick, 1989).

1.2.2 The central zone

The central zone (CZ) contains about 25% of the glandular tissue and is resistant to both carcinoma and inflammation. In comparison with the other zones, cells in the central zone have distinctive morphologic features.

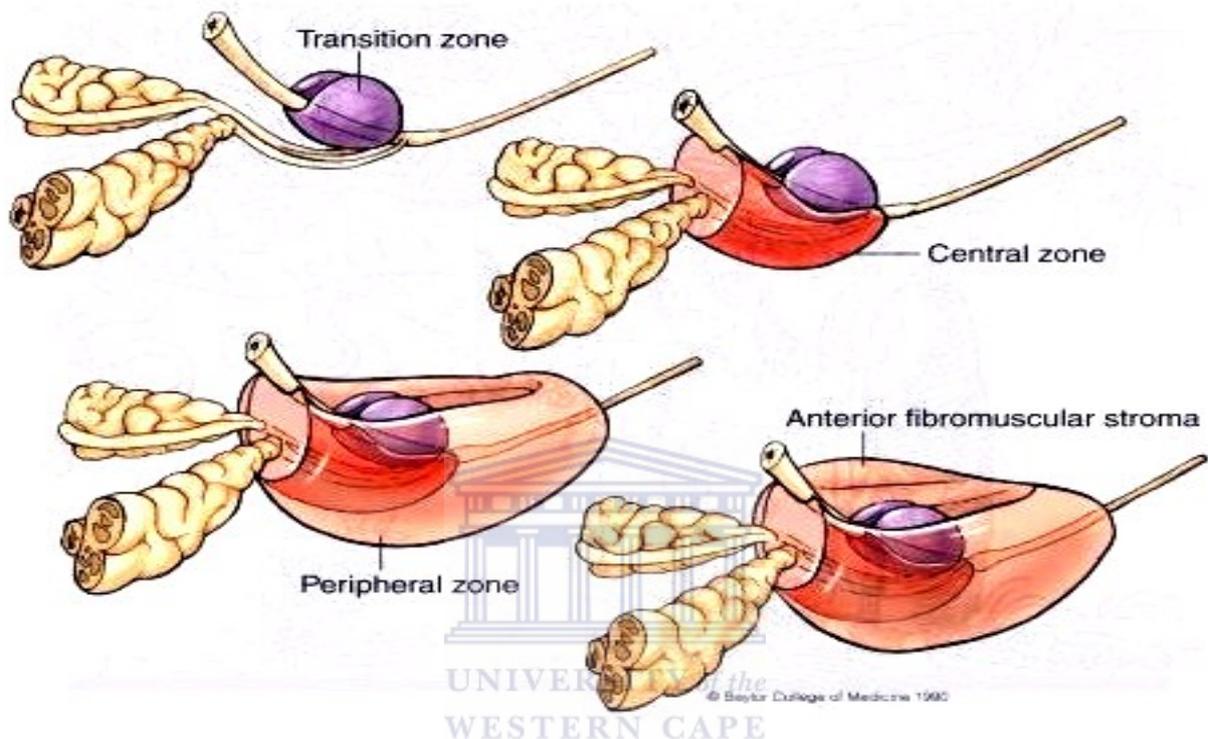


Figure 2: Schematic representation of the prostate. Zonal anatomy of the normal prostate as described by McNeal (1978) (From Campbell 2003).

1.2.3 Transition Zone

The transition Zone (TZ) is the innermost part of the prostate gland that surrounds the urethra and comprises of 5% of the total prostate volume. Approximately 10-20% of prostate cancer originates from this zone (McNeal et al., 1988). It surrounds the proximal urethra and is responsible for benign prostatic hyperplasia (BPH) in older individuals. The parenchymal cells of this zone frequently undergo extensive division and form nodular masses of epithelial cells. Since this zone is in close proximity to the proximity to the prostatic urethra, causing difficulty in urination (McNeal, 1978).

1.2.4 The Anterior Fibromuscular Zone

The Anterior Fibromuscular Zone (AFZ) (or stroma) accounts for approximately 5% of the volume. Additionally, it consists mainly of connective tissues and smooth muscles. Enveloped by a fibro muscular layer that is referred to as the capsule (McNeal, 1981; Villers et al., 1991; McLaughlin et al., 2005). The fibromuscular stroma occupies the anterior surface of the prostate and is principally comprised of smooth muscle (McNeal, 1978).

1.3 Histology of the Prostate Gland

Histologically, the human prostate has a high level of organisation. The glandular component of the prostate is composed of large peripheral ducts. It has bilayered epithelial acini and fibro-muscular stroma, separated from each other by a basement membrane. Three phenotypically distinct cell types can be identified within the epithelial bilayer; the basal cells, the neuroendocrine cells and the luminal cells. Together, these cells are terminally differentiated and sparsely scattered among secretory epithelial cells in the normal prostate (Figure 3) (Sherwood et al., 1991; Bostwick and Dundore, 1997; Lalani et al., 1997; Roy- Burman et al., 2004).

The basal cells comprises of flattened to cuboidal cells at the periphery of the glands separating the secretory epithelial cells from the basement membrane and stroma. It is contractile but also generates many growth factors regulating the glands. These cells are thought to act as stem cells which repopulate the secretory cell layer (Bonkhoff et al., 1994). The majority of investigations show that the basal cells are the non-secretory precursors of the luminal cells in adult prostate (Isaacs and Coffey, 1989; Robinson et al., 1998; Hudson et al., 2000; Richardson et al., 2004; Frame et al., 2010; Ousset et al., 2012), however, contradictory evidence also exists (Wang et al., 2009; Choi et al., 2012).

Neuroendocrine cells lie above the basal cell layer (McNeal, 1988) and reside among the more numerous secretory epithelial cells. The neuroendocrine cells are the least common cell type of the prostatic epithelium. Their function is unknown. However, it has been postulated that they exist to serve an endocrine-paracrine regulatory role in growth and development, similar to neuroendocrine cells in other organs (Bonkhoff et al., 1991; Aprikian et al., 1993), (Abrahamsson and diSant'Agnesse, 1993; Nakada et al., 1993; Rumpold et al., 2002).

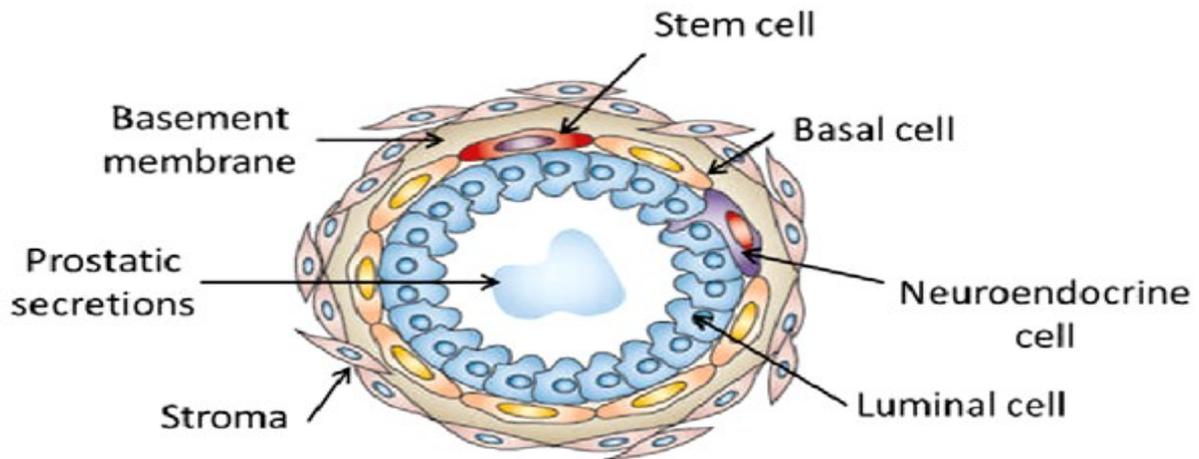


Figure 3: Schematic representation of the prostrate acinus. Prostate gland epithelial is separated from the stroma by well defined basement membrane. Each gland is lined with basal and neuroendocrine cells. Basal cells differentiate into luminal cells that secrete fluid and proteins into the lumen of the gland epithelia (Rane et al., 2012, adapted).

Current evidence suggests that they represent a post-mitotic cell type that is derived from luminal secretory cells (Bonkhoff et al., 1991; Bonkhoff et al., 1994; Bonkhoff et al., 1995). Luminal cells, these are the terminally differentiated columnar cells that are responsible for producing prostatic secretions. They have no proliferative potential and are characterised by the near universal expression of androgen receptor (AR) (Nakada et al., 1993; Wang et al., 2006).

1.4 Hormones in the Prostate

Androgen is a term given to any steroid hormone that primarily influences the growth and development of the male reproductive system. Androgens are involved in normal prostate growth as well as prostate cancer cell growth and proliferation. Testosterone is essential for the normal development of the prostate both during foetal life and during puberty (Macleod et al., 2009; Wilson, 2001). The hormone is also necessary for normal function of the prostate during adulthood and is dominantly (>95%) synthesized in the Leydig cells of the testis and the remaining 5% originates from the adrenal glands. The endocrine pathway that ultimately results in synthesis of androgen begins in the hypothalamus. The hypothalamus synthesizes and releases Gonadotropin Releasing Hormone (GnRH), which stimulates the pituitary to secrete Luteinizing Hormone (LH), Follicle Stimulating Hormone (FSH) and Adrenocorticotrophic Hormone (ACTH) (Figure 4) (Damber, 2005).

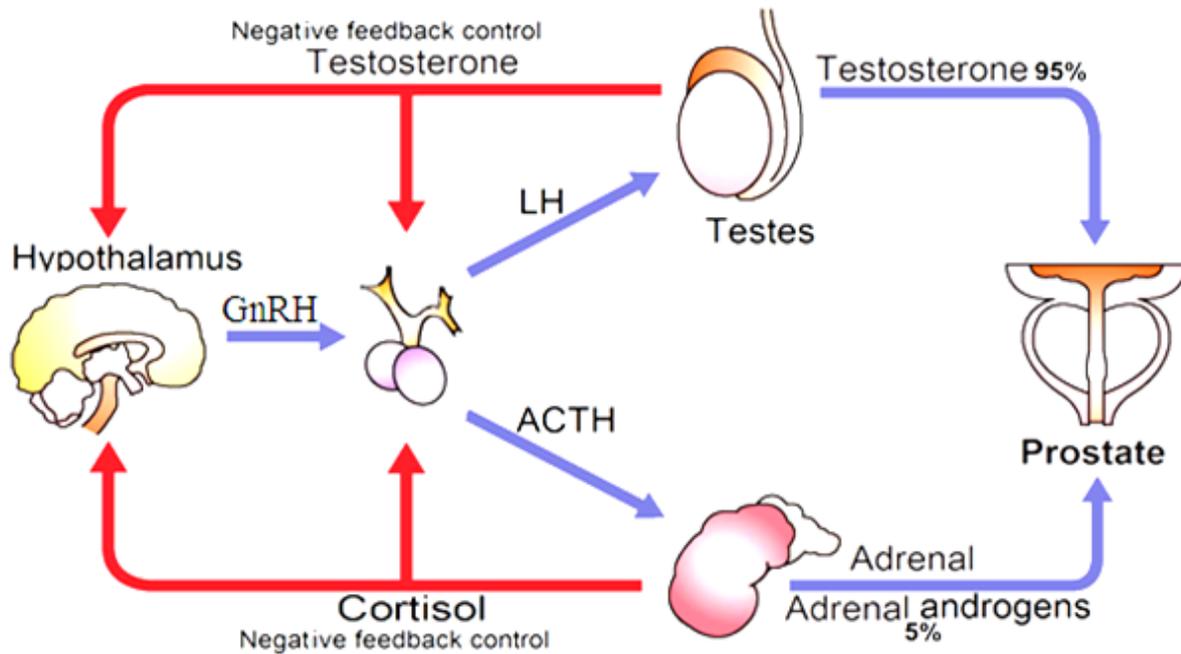


Figure 4: The production of testosterone is under the superior control of the hypothalamus and the pituitary gland. The hypothalamus secretes GnRH and CRH that influences the pituitary to produce LH and ACTH, respectively. LH influences the testes to produce testosterone and ACTH regulates the production of testosterone and other weak androgens from the adrenal glands. The majority of the testosterone originates from the testis. GnRH = gonadotropin-releasing hormone; CRH = corticotropin-releasing hormone; LH = luteinizing hormone; ACTH = adrenocorticotropic hormone, according to (Damber, 2005).

In addition, androgens also function as a negative feedback to the pituitary and the hypothalamus to control hormone production (Krongrad and Droller, 1993). In addition, the hypothalamus release corticotropin-releasing hormone (CRH) that induces the secretion of adrenocorticotropic hormone (ACTH) from the pituitary gland.

LH stimulates the testes to release testosterone and ACTH stimulates the adrenal glands to produce the adrenal androgens such as testosterone, dihydrotestosterone (DHE). Both the testicular and adrenal androgens stimulate the prostate, but testosterone is responsible for the primary effects testosterone circulates in the blood either bound to albumin or sex-hormone binding globulin or freely by itself (Krongrad and Droller, 1993). Free testosterone can cross the plasma membrane and reach the prostate by diffusion. Testosterone is converted in the

prostate to the more potent androgen dihydrotestosterone (DHT) by the enzyme 5 α -reductase (Schmidt et al., 2004). DHT binds to the androgen receptor and the activated receptor stimulates cellular growth (So et al., 2003).

Even if testosterone is necessary for a normal function of the prostate and for the development of prostate cancer, no direct link between the actual serum value and the risk of prostate cancer has been established (Roddam et al., 2008). Huggins and Hodges in 1941 have shown the importance of testosterone in prostate cancer. Testosterone contributes to the growth of the tumor, the autocrine and paracrine growth factor-receptor interactions and associated mitogenic signaling as well as defects in apoptotic signaling which are the major contributors in unchecked proliferation and immortalization of prostate cancer cells (Gioeli et al., 1999; Agarwal et al., 2000).

1.5 Prostate Disorders and aging male symptoms

Prostate disorders are usually connected to aging; as the age increases, the probability of developing prostate problems also increases, the size of the prostate varies with age and the prostate of a young man is not comparable to the prostate of an older man (McLaughlin et al., 2005). Its functional anatomy is complicated and the location of the prostate is not ideal and may cause problems with the nearby organs with side-effects such as incontinence, impotence and the inability of the prostate gland to store and secrete its fluid. This is due to the result of the anatomical changes of hypertrophy that normally occur with progression of age. There are three types of prostate diseases: prostatitis, benign prostatic hyperplasia, and prostate cancer.

1.5.1 Prostatitis

Prostatitis is an inflammation of the tissue of the prostate gland. It is the most common genitourinary diagnoses in men aged between 18–50 years, though it can affect men of any age (Collins et al., 1998). Prostatitis can cause symptoms such as painful urination and ejaculation, increased urinary frequency and urgency, pain and discomfort in the lower back region and chronic, recurrent symptoms.

1.5.2 Benign Prostatic Hyperplasia

Benign Prostatic Hyperplasia (BPH) is an extremely common disease in men aged above 60 years and is rarely a threat to life (Djavan et al., 2002). It refers to the enlargement of prostate in the transition zone as a result of benign growth of prostatic stromal and epithelial cells due to increased proliferation. Due to enlargement of prostate, the layer of tissue surrounding it stops it from expanding, resulting in the compression of prostatic part of urethra. The transition zone is the main site for benign prostate hyperplasia (BPH) development, while the peripheral zone is where malignant tumours are found (Blennerhassett et al., 1966).

The cause for BPH is not known, but many potential factors are thought to contribute to BPH. Since BPH cannot occur in the absence of androgen and applying extra androgen does not aggravate the symptoms of BPH. In particular, dihydrotestosterone (DHT), a hormone deriving from testosterone in the prostate, is thought to play a main role in BPH (Isaacs and Coffey, 1989). Even as testosterone drops in older men, DHT can continue to be produced and stored substantially in the prostate. BPH is usually treated with medication such as 5 α -reductase inhibitors in order to shrink the prostate and slowing its growth or with transurethral resection that is performed with the aim to remove the obstructing portion of enlarged prostate tissue (Schroder and Blom, 1989).

1.5.3 Prostate cancer

Prostate cancer is classified as an adenocarcinoma, or glandular cancer, that begins when normal semen-secreting prostate gland cells mutate into cancer cells. The region of prostate gland where the adenocarcinoma is most common is the peripheral zone. Initially, small clumps of cancer cells remain confined to otherwise normal prostate glands, prostate cancers often metastasize in lymph nodes, bone, and other distant sites (Kyrianiou et al., 1994).

In addition to age, factors, such as, genetic susceptibility, diet and lifestyle, infectious agents, inflammation, etc. are all possible instigators for the development of prostate cancer (Coffey et al., 2001; Gonzales et al., 2010; American Cancer Society, 2010). These causal factors may act together or in sequence to initiate or promote the development of cancer.

In the normally functioning prostate, cells will grow, divide, and die on a regular basis. During cancer, the cells of the differentiation process that leads to the accumulation of aberrantly differentiated luminal cancer cells (Nagle et al., 1987) divide and grow

uncontrollably, either because cells do not undergo their regularly scheduled cell death, or because they grow and divide too rapidly. The cancer cells can spread to surrounding tissues, such as the seminal vesicles and bladder and may metastasize to other parts of the body, particularly the bones and lymph node (Coffey and Pienta, 1987).

Prostate cancer is the most common cancer among men in America, and it is the second leading cause of death following only lung cancer (Siegel et al., 2012). In addition, one man in six will be diagnosed with prostate cancer during his lifetime, according to the American Cancer Society (2012). In 2012, it was estimated that 241,740 men were diagnosed with prostate cancer and about 28,170 men died from prostate cancer. African-American males have the highest incidence of prostate cancer in the world (American Cancer Society, 2012).

1.6 Diagnosis of prostate cancer

Diagnosis of prostate cancer is based on the suspected asymmetry of the gland detected by digital rectal examination (DRE), biopsy and serum PSA levels. DRE is a long-established test used by urologist to detect palpable changes in the prostate gland but it can only detect cancers that are relatively large. Another limitation is that the majority of cancers occur in regions that are not accessible by DRE (Selley et al., 1997). Clinical prostate examination also includes: Transrectal Ultra Sonography (TRUS), Computed tomography (CT) scan and Magnetic resonance imaging (MRI) are often used to guide biopsies (Cupp et al., 1993). The field of imaging has expanded from the characterization of locally advanced or metastatic disease to include intra and extra prostatic tumor delineation, including morphology and zonal anatomy which provides basic initial information.

A histological grade of biopsy tissue is assigned using the Gleason grading system (Gleason, 1966) by observing the architectural patterns or degree of differentiation of the gland; that is, whether the cells form glands that resemble the normal prostate. A low grade will be the most differentiated, and scored with a Grade of 1, while a poorly differentiated, rapidly growing cancer will be assigned a Grade of 5 (Figure 5).

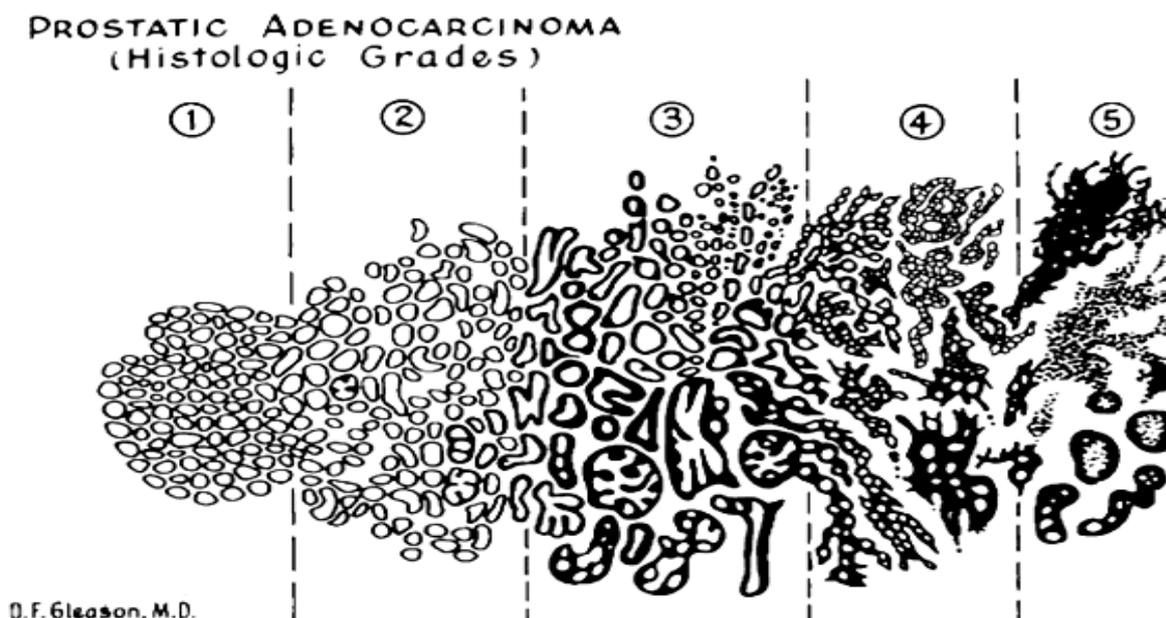


Figure 5: The Gleason grading system for prostatic adenocarcinoma. The original Gleason system describes histological patterns of prostate biopsies which are graded from 1; simple round glands, closely-packed in rounded masses with well-defined edges to 5; anaplastic adenocarcinoma in ragged sheets (Gleason, 1966; Gleason, 1992, adapted).

1.7 Prostate-specific Antigen (PSA)

PSA is a 32-kilodalton (KD) chymotrypsin-like serine protease characterized by (Wang et al., 1979). It was first identified from seminal plasma in the early 1970's (Hara et al., 1971) and was subsequently named prostate-specific antigen by Wang et al, (1979).

Prostate-specific antigen slowly hydrolyzes peptide bonds to cleave semenogelin I and II in the seminal coagulum (Balk et al., 2003), thereby liquefying semen. Some of that enzymes secreted by the prostate can be detected in circulating blood (e.g., PAP, AP and PSA) and used for diagnostic purposes (Stamey et al., 1987; Barichello et al., 1995; Strohmaier et al., 1999). PSA is a serine protease and a member of the tissue kallikrein family of proteases which is secreted into the lumen by prostate ductal and acinar epithelium. The majority of PSA is generated in the transitional zone (TZ) of prostate gland (McNeal et al., 1988). Production of PSA is largely regulated by the androgen-dependent activation of the androgen receptor on prostate cells (Riegman et al., 1991).

Both, normal and malignant prostate epithelial cells produce PSA. In normal prostate, most of the PSA is secreted into the seminal vesicles and only a small amount leaks into circulation. However, in prostate cancer the epithelial cells have an abnormal architecture and more PSA is released into circulation at high concentrations, providing the basis for the PSA serum test (Brawer, 1989; Lalani et al., 1997). Increased leakage may be due to basement membrane disordering, loss of basal cell layer, ductal lumen architecture loss and loss of epithelial cell polarity (Bostwick et al., 1994) (Figure 6).

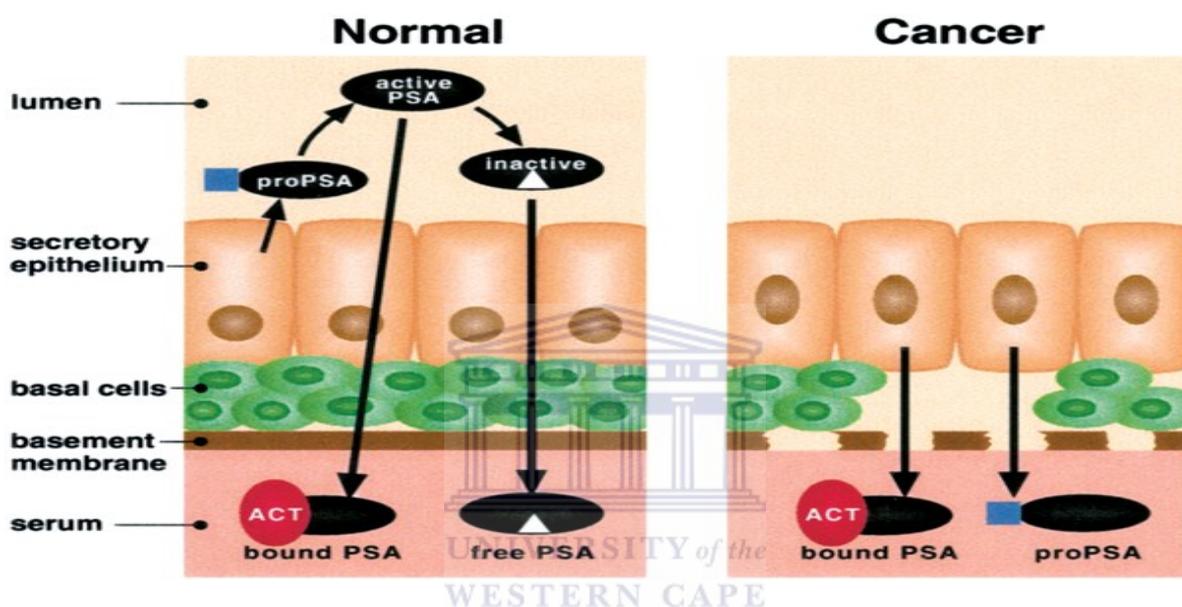


Figure 6: Model of prostate-specific antigen (PSA) biosynthesis in normal prostate epithelium versus cancer. Normal secretory epithelium, surrounded by basal cells and a basement membrane, secretes pro PSA into the lumen where the pro-peptide is removed by hK2 to generate active PSA. A fraction of this active PSA can diffuse into the circulation, where it is rapidly bound by protease inhibitors. The active PSA also undergoes proteolysis in the lumen to generate inactive PSA, which can enter the bloodstream and circulates in an unbound state (free PSA). In prostate cancer loss of basal cells, basement membrane, and normal lumen architecture results in a decrease in the luminal processing of pro PSA to active PSA, and active PSA to inactive PSA, with relative increases in bound PSA and pro PSA in the serum (Balk et al., 2003)

Although PSA is richer a general tissue than a specific prostate marker, the PSA level in serum is a sensitive indicator for prostate cancer. PSA testing not only helps in the early diagnosis but also assists in assessing the response to therapy, determining tumor progression

and in its most controversial role, screening for prostate cancer. In healthy men, the PSA can be found at high concentration, 0.5 to 2 mg/ml in seminal fluid (Wang et al., 1998) and at low concentration in serum, 4 ng/ml (Oesterling and Cooner, 1995). The seminal fluid predominantly contains active, inactive and cleaved forms of PSA proteins (Zhang et al., 1995). In 1980, Papsidero et al. 1980 were the first to detect the serum PSA in men with prostate cancer.

Serum PSA has proven to be a generally reliable indicator in the diagnosis and management of prostate cancer. It has revolutionized the management and follow-up of prostate cancer since its clinical introduction in the late 1980's. Serum PSA remains the best single test for the detection of early prostate cancer, and multiple variations have been studied to improve its sensitivity and specificity first by associating it with the digital rectal examination and then by looking at age-adjusted PSA, PSA density. In 1986, Myrtle and co-workers determined the reference range for PSA assay to be 0.0-4.0 ng/ml (Myrtle et al., 1986). The upper limit of normal PSA (4.0 ng/ml) can also be lowered to 2.5 ng/ml or even lower because 20%-30% of tumours will be missed if the only method of detection is serum PSA with a cut-off of 4.0 ng/ml (Catalona et al., 1997).

PSA levels in blood can be altered by disease conditions like BPH, prostatitis and prostate cancer. Men with advanced prostate cancer may have blood PSA levels of over 100 ng/ml (Lilja et al., 2008). Increased serum PSA levels in prostate cancer are not due to increased production of the protein in the cancerous tissue, but are majorly a consequence of increased leakage of PSA into the circulation. Some studies have also indicated that PSA expression in prostate cancer decreases significantly as compared to PSA produced by benign prostate epithelial cells (John et al., 2001).

1.8 Treatment of Prostate Cancer

Prostate cancer can be generally treated with surgery, radiation therapy, hormonal therapy, occasionally chemotherapy, proton therapy, cryosurgery, or some combination thereof. However, therapy options for men at prostate cancer are often not effective enough to halt the progression of the disease, and frequently induce several side effects.

Although there are several prostate cancer treatments (e.g. prostatectomy, radiation therapy, chemotherapy), androgen deprivation therapy (ADT) has been the cornerstone of therapy ever since its efficacy for treating prostate cancer was first demonstrated by (Huggins and Hodges, 1941). Treatment of prostate cancer relies on multiple strategies depending on the grading and staging of the malignant disease. In the case of locally confined malignant disease, curative treatment in the form of radical resection of the prostate or radiation therapy is possible. Almost half of all prostate cancers are localised at the time of diagnosis.

Chemotherapy has been used for cancer treatment for more than 50 years; sometimes in combination with or parallel to surgery and radiotherapy. After surgical ablation of progressive cancer, metastasized tumor cells continue to progress and this is one of the faultiest associated with surgery. On the other hand, radioactive rays and most anticancer chemotherapeutic agents damage DNA or suppress DNA duplication to kill the rapidly growing tumor cells. At the same time, they also affect normal cells causing serious adverse effects, such as bone marrow function inhibition, bone necrosis, lung fibrosis, ulceration, nausea, and vomiting, renal damage and alopecia (Cassady and Chang, 1981).

One of the main problems with currently available anticancer drugs is their non-selective cytotoxicity which leads to the many adverse effects (Arriagada et al., 2008; Burstein et al., 2008). In recent years, there is an increasing preference for the use of natural products especially from plants in treating and preventing medical complications (Hafidh et al., 2009). Many currently available anticancer drugs such as taxol, vinblastine, vincristine, topotecan, and irinotecan (Allen et al., 2005; Leveque and Jehl, 2007; Magnotta et al., 2007; Choi et al., 2008) derived from plants. The search for novel cost effective anticancer agents with minimal side effects from natural products continues. Thus, it is evident that a wide array of selective and potent components is needed to match the growing problems associated with cancer.

More effective and safer anti-tumor agents for prostate cancer have been developed from herbal plants as potent cancer chemopreventive candidates in complementary and alternative medicine (Campbell et al., 2004; McCann et al., 2005). The development of potential chemopreventative agents for prostate cancer would greatly affect the natural history of this important disease (Khan and Partin, 2003). As chemopreventive compounds from medicinal

plants or herbal extracts might have good antitumor activities with less side effect than conventional antitumor drugs.

1.9 Human Prostate Cancer Cell

The human prostate cancer cells (LNCaP) derived in 1977 from a needle aspirate biopsy of a supraclavicular lymph node from a 50-year-old white male with stage D prostatic cancer (Horoszewicz et al. 1980). LNCaP cells express a mutated form of the androgen receptor, which leads to some alterations in androgenic responses (Veldscholte et al., 1992). However, these cells produce the human prostatic secretory markers prostate specific antigen (PSA) and prostatic acid phosphatase (PAP) both in vitro and when xenografted into nude mice (Chung et al., 1992). LNCaP cells are responsive to androgens in terms of growth yet exhibit aberrant responses to anti-androgens (Wilding et al., 1989).

Proliferation of LNCaP cells can be stimulated by treating them with androgens (Horoszewicz et al., 1983). Androgen treatment of LNCaP cells also increases the production of PSA and hK2 (Montgomery et al., 1992; Young et al., 1992; Grauer et al., 1996). As most of the prostate cancer cell lines express either very little or no AR, LNCaP is perhaps the most relevant cell line for studying the biology of early prostate cancer. Late stage tumors generally develop androgen resistance as a response to hormone therapy (Koivisto et al., 1998).

1.10 Apoptosis

Apoptosis is also called programmed cell death (Kerr et al., 1972). It is a normal process that occurs during development of cell. When stimuli for death are received, cells undergo various morphological changes and die in a regular and controlled manner this is various pathways that are active in order to cause apoptosis (Wyllie et al., 1980). The process of apoptosis can be triggered by a wide variety of physiological and pathological stimuli (Elmore, 2007), associated with morphological changes such as cell shrinkage, membrane blebbing, chromatin condensation, nuclear fragmentation (Hengartner, 2000). In case of cancer, the process of apoptosis is uncontrolled.

During early phase of apoptosis, there are families of proteins called caspases that are activated (Chinnaiyan and Dixit, 1996). Caspases are a family of cysteine proteases that play a key role in the apoptotic process and are highly conserved during development and aging,

Caspase-3, the effector caspase in apoptosis, represents a convergence point for two different caspase-dependent apoptotic pathways: the mitochondrial (intrinsic) pathway and the death receptor (extrinsic) pathway (Cohen, 1997; Zhang et al., 2004; Hsu et al., 2008). Direct activation of such executioner caspases is thought to be an anticancer strategy, which may prove beneficial in treating many cancers in which procaspase-3 concentrations are elevated (Putt et al., 2006).

1.10.1 The Intrinsic Pathway

The intrinsic or mitochondrial pathway is initiated within the cell, and is triggered by stimuli such as irreparable genetic damage, hypoxia, and severe oxidative stress are some triggers of the initiation of the intrinsic mitochondrial pathway (Karp, 2008).

Regardless of the stimuli, this pathway is the result of increased mitochondrial permeability and the release of pro-apoptotic molecules such as cytochrome-c into the cytoplasm (Danial and Korsmeyer, 2004). This pathway is closely regulated by a group of proteins belonging to the Bcl-2 family, There are two main groups of the Bcl-2 proteins, namely the pro-apoptotic proteins (e.g. Bax, Bak, Bad, Bcl-Xs, Bid, Bik, Bim and Hrk) and the anti-apoptotic proteins (e.g. Bcl-2, Bcl-XL, Bcl-W, Bfl-1 and Mcl-1) (Reed, 1997). While the anti-apoptotic proteins regulate apoptosis by blocking the mitochondrial release of cytochrome-c, the pro-apoptotic proteins act by promoting such release. It is not the absolute quantity but rather the balance between the pro- and anti-apoptotic proteins that determines whether apoptosis would be initiated (Green and Reed, 1998).

1.10.2 The Extrinsic Pathway

The extrinsic or death receptor pathway triggers apoptosis in response to the binding of a death ligand with specific death receptors on the cell surface (Ashkenazi & Dixit, 1998). Although several death receptors have been described, the best known death receptors is the type 1 TNF receptor (TNFR1) and a related protein called Fas (CD95) and their ligands are called TNF and Fas ligand (FasL) respectively (Hengartner, 2000). These death receptors have an intracellular death domain that recruits adapter proteins such as TNF receptor-associated death domain (TRADD) and Fas-associated death domain (FADD), as well as cysteine proteases like caspase 8 (Schneider and Tschopp, 2000).

Binding of the death ligand to the death receptor results in the formation of a binding site for an adaptor protein and the whole ligand-receptor-adaptor protein complex is known as the death-inducing signalling complex (DISC) (O'Brien and Kirby, 2008). The activated form of the enzyme, caspase 8 is an initiator caspase, which initiates apoptosis by cleaving other downstream or executioner caspases (Karp, 2008). Caspases are divided into two groups, the upstream initiator caspases (caspase-9, caspase-2, caspase 8, and caspase 10) and the downstream executor caspases (caspase-3, caspase 6 and caspase-7) (Enari et al., 1998).

1.10.3 Apoptosis and Anti-Cancer Therapy

Apoptosis provides a number of useful clues when generating effective therapies and many chemotherapeutic agents exert their anticancer effects by inducing apoptosis in cancer cells (Schuchmann and Galle, 2004). Therefore, induction of apoptosis has become a principal mechanism by which anticancer therapy is effective (Kundu et al., 2005). The deregulation of prostate growth in prostate cancer cells is notable by apoptotic evasion, loss of differentiation, and uncontrolled proliferation. For the treatment of advanced metastatic prostate cancer and the appearance of therapeutic resistance of prostate tumors, the challenges in the implementation of effective therapeutic strategies involve functional significance of anti-apoptotic pathways (Reynolds and Kyprianou, 2006).

Apoptosis is an important process in the cell development and maintenance of tissue homeostasis, as well as an effective mechanism by which harmful cells can be eliminated (Green and Reed, 1998; Kaufmann and Hengartner, 2001). Recently, the relationship between apoptosis and cancer has been emphasized, with increasing evidence suggesting that the related processes of neoplastic transformation, progression and metastasis involve the alteration of normal apoptotic pathways (Bold et al., 1997). This mechanism provides a number of clues with respect to effective anticancer therapy, and many chemotherapeutic agents exert their anticancer effects by inducing apoptosis in cancer cells (Kamesaki, 1998). Therefore, the induction of apoptosis has become a principal mechanism by which anticancer therapy is effective (Kundu et al., 2005). Homeostasis between cell death and cell proliferation is required to maintain normal state. Disruption of this cellular balance or dysregulation of controlling mechanisms can lead to human disease including cancer. Hence clinically many diseases are the ultimate result of either deficient apoptosis or excessive apoptosis (Thompson, 1995).

Cancer cells acquire resistance to apoptosis by over expression of anti-apoptotic proteins (Bcl-2, IAPs and FLIP) and/or by the down-regulation or mutation of pro-apoptotic proteins (Bax, Apaf-1, caspase 8 and death receptors) (Gosslau and Chen, 2004). In cancer therapy, one approach that suppresses the tumor growth is by activating the apoptotic machinery in the cell (Lowe and Lin, 2000). Evidence obtained during recent years is beginning to establish that a large majority of cancer chemotherapy agents affect tumor cell killing *in vivo* and *in vitro* through launching the apoptosis cascade (Makin and Dive, 2001).

1.11 Herbal Medicine

Herbal medicine is the traditional or folk medicine practice based on the use of plants or plant extracts. People in all nations have used many kinds of herbs for the treatment of ailments since prehistory until now. In the 19th century, scientists started with extracting the active ingredients from plants to form medicines, but over the time and due to the development of chemistry, chemists began making their own version of plant compounds. Therefore, a transition from raw herbal compounds to modern pharmaceutical drug usage occurred with subsequent decline in the herbal medicine usage. However, recently, people are coming back to the herbal medicines. They believe that herbal medicines are safe because they are natural. Furthermore, medicinal plants comprise a significant part of the biodiversity and form an inextricable link between biological and cultural diversity and forms the basis for the sustainable exploitation and conservation of biodiversity according to the World Health Organization (WHO) (MacLennan et al., 1996; Eisenberg et al., 1998; Woods, 1999; Ernst and White, 2000; Ernst, 2000; Khan et al., 2001; Taha, 2002; WHO, 2002; Zaidi, 2002; Saras, 2003). It is believed that herbal treatment assist in boosting their immune system, prolong life, relieve symptoms and ameliorate the desirable side effects of modern therapies (Wirth et al., 2005).

1.11.1 Herbal Medicine and Prostate Cancer

Medicinal plants provide an inexhaustible source of anticancer drugs in terms of both variety and mechanism of action (Yano et al., 1994). There is increasing evidence for an association between a high consumption of fruits and vegetables and reduced risk of cancer (La Vecchia et al., 1997; Morse et al., 2000). Plants have a long history of use in the treatment of cancer (Hartwell, 1982), though the efficacy such treatments have should be viewed with some scepticism because cancer, as a specific disease entity, is likely to be poorly defined in terms of folklore and traditional medicine (Cragg et al., 1994).

Previous studies revealed that more than 3350 plant species are used in traditional medicine against cancer (Graham et al., 2000). Compounds derived from natural products offer a means of evaluation of new chemical classes of anticancer agents as well as novel and potentially relevant mechanisms of action (da Rocha et al., 2001).

There has long been a keen interest in herbal or alternative therapies for prostate cancer. Traditional medicine is thought to maintain the health and vitality of individuals, and also cure diseases, including cancer without causing toxicity. More than 50% of all modern drugs in clinical use are of natural origin, many of which have the ability to control cancer cells and have contributed considerably to approximately 60 available cancer chemotherapeutic drugs (Kelloff, 1994; Kinghorn, 1999). Several studies have established that extracts from several herbal medicines or mixtures had an anticancer potential *in vitro* or *in vivo* (Bonham et al., 2002; Hu et al., 2002; Kao et al., 2001; Lee et al., 2002; Yano et al., 1994).

The need to develop more effective anti-tumor drugs has prompted investigators to explore new sources of pharmacologically active compounds, especially from natural products. The success of these drugs and the increasing need for improved anti-cancer therapies against difficult to treat or highly malignant forms of cancers have spurred an intensive search for other bioactive plant-derived anti-cancer compounds. Increasing popularity of the use of phytotherapy-based medicines as preventive medicines or for health management has also driven research in these areas (Raskin et al., 2002).

Plant alkaloids are considered highly active chemotherapeutic agents in various cancers including those of the breast and prostate (Obasaju et al., 2001; Saloustros et al., 2008). The use of herbal intervention is widespread in all regions of the developing world and is rapidly growing in developed countries (Cassileth, 1995; Yan et al., 2006). In spite of the extensive use of herbal therapies, there is insufficient scientific evidence validating their efficacy and safety. Thus, basic research aimed at elucidating the underlying mechanisms of any potential herbal effects are very important for the use of herbal medicine.

Recently, scientists have focused on the potential role of extracts of traditional Chinese medicinal herbs as alternative and complementary medications for cancer treatment. The extracts of Chinese medicinal herbs are often used together with traditional cancer therapy to improve the survival rate and quality of life, as the use of herbal extracts are much less expensive than the standard antineoplastic therapies currently available (Cha et al., 1997; Liu et al., 2001; Zou and Liu, 2003; Han et al., 2003; Hao et al., 2007).

1.12 *Eurycoma Longifolia* Jack

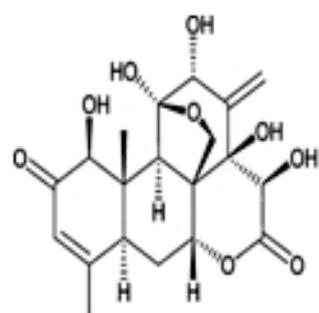
Tongkat Ali (TA, *Eurycoma Longifolia*, Figure 7) is a tropical plant belonging to the order of Geraniales, family of Simaroubaceae (Kamarudin and Latiff, 2002; Aziz et al., 2003). It is an evergreen tree growing up to 10-15m in height. It is commonly found in understory of the lowland forests up to 500 m above sea level (Goh et al., 1995), and is widely distributed in South East Asian countries such as Malaysia, India, China, Indonesia, Myanmar, Vietnam, Cambodia, Philippines and Thailand. Depending on the places and countries that it is found in the local names given vary, these include: Long Jack, Malaysian Ginseng, Local Ginseng, Natural Viagra, Pasak Bumi, Payung Ali, Setunjang Bumi, Tongkat Baginda, Tongkat Ali Hitam, Cay ba binh, Ian-don, and Jelaih (Bhat and Karim, 2010; Medicinal Plants, 2011). Each of the leaves is about 20-40 cm long and the fruit is ovoid in shape, when ripe turns dark brown in colour (Nurhanan, 2005).

Eurycoma longifolia is an important medicinal plant and has always been regarded as one of the most important traditional remedies in these South-East Asian countries. Every part of the plant is utilized for medicinal purposes (Jiwajinda et al., 2002; Osman et al., 2003). However, the most valuable component is the roots of this plant, which is used to treat various ailments, including, aches, fever, malaria, sexual insufficiency, glandular swelling as well as a general tonic (Ang et al., 2003; Kuo et al., 2003, 2004; Tambi and Imran, 2010; Tambi et al., 2012).

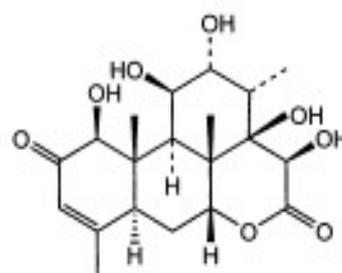


Figure 7: The tree of Tongkat Ali (*Eurycoma longifolia* Jack) and the root.
From: <http://www.tongkatali.org>

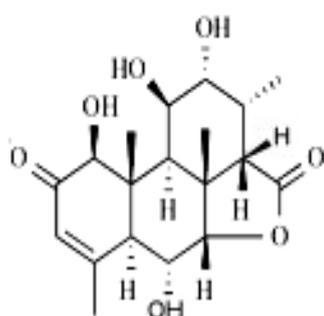
The extract of TA roots is a brownish powder and containing 22.0% bioactive eurypeptide, 41.1% glycosaponin and 1.6% eurycomanone, quassinoids and some alkaloids that have been proven to possess anti-malarial, anti-ulcer, antipyretic, cytotoxic, antiproliferative, aphrodisiac properties and plant growth inhibition activities (Morita et al., 1990; Zhari et al., 1999 Kuo et al., 2003; Nurhanan et al., 2005; Chan et al., 2005; Tee and Azimzhtol, 2005; Hout et al., 2006). Many studies have been performed to scientifically prove the healing properties which have lead to the isolation of the bioactive components which include; eurycomanone (A), 14,15 β -dihydroxyklaineaneone (B), longilactone (C), 11- dehydroklaineaneone (D), 6-dehydrolongilactone (E), 15 β - hydroxyklaineaneone (F), 15 β -O-acetyl-14-hydroxyklaineaneone (G) , eurycomanol (H), and phenolic components, tannins, and triterpenes, as depicted in Figure 8.



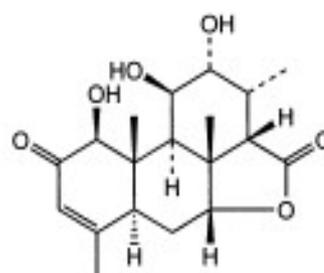
(A) eurycomanone



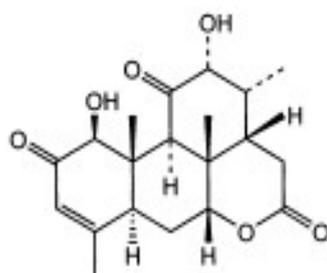
(B) 14,15β-dihydroxyklaineaneone



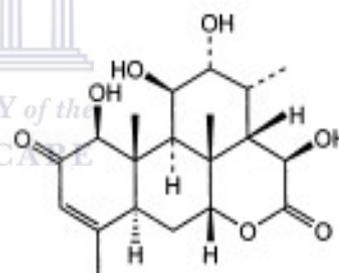
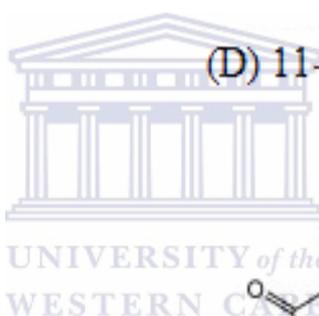
(C) longilactone



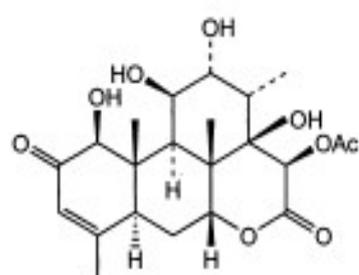
(D) 11-dehydroklaineaneone



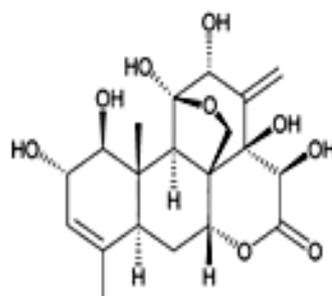
(E) 6-dehydrolongilactone



(F) 15β-hydroxyklaineaneone



(G) 15β-O-acetyl-14-hydroxyklaineaneone



(H) eurycomanol

Figure 8: Some of the chemical constituents isolated from the root of *Eurycoma longifolia* Jack (Source: Ismail et al., 1999). (A) Eurycomanone (B) 14,15β-dihydroxyklaineaneone (C) Longilactone (D) 11-dehydroklaineaneone (E) 6-dehydrolongilactone (F) 15β-hydroxyklaineaneone (G) 15β-O-acetyl-14-hydroxyklaineaneone (H) eurycomanol

In *vitro* assays revealed cytotoxicity, anti-HIV and anti-malarial activities of approximately 65 compounds from the roots of TA (Kardono et al., 1999). Among the compounds evaluated, approximately eight displayed cytotoxicity toward the human lung cancer cell lines (A-549), while seven exhibited strong cytotoxicity towards the human breast cancer cell lines (MCF-7) (Kuo et al., 2003). Two compounds namely eurycomanone and 7-methoxy-p-carboline induce apoptosis in breast cancer cells (Tee et al., 2007).

In addition, the chloroform, n-butanol, methanol and water extracts obtained from the root indicated cytotoxic effect against KB, CaOV-3, RD, MCF-7, DU-145, and MDBK cell lines (Nurhanan et al., 2005). These results indicate that, except for the water-soluble extract, all the other compounds produced significant cytotoxic effect on these cell lines with no significant cytotoxic effect on the MDBK (kidney) normal cell line (Nurhanan et al., 2005). Eurycomanone is one of the novel compounds of TA, which appear promising as a new chemotherapeutic agent against human cervical carcinoma (HeLa) cells as anti-proliferative properties and induces apoptosis through the up-regulation of p53 as the mode of death observed in these cells (Nurkhasanah et al., 2008).

1.13 Aim of Study

Studies have also reported *E. longifolia* extracts improved the sexual performance in rats (Ang and Sim, 1998; Zanolini et al., 2009), increased the sperm quality (Noor et al., 2004), and reversed-estrogen induced infertility (Wahab et al., 2010). In addition, clinical studies have shown that the sperm quality of idiopathic infertile males and the testosterone level of late-onset hypogonadism were improved when treated with the plant aqueous extract (Tambi and Imran, 2010; Tambi et al., 2012). Thus, triggering the discussion of its usage as potential testosterone replacement therapy for aging men (George and Henkel, 2014). In turn, this raises concerns about the prostate health as testosterone deprivation has been shown to cause prostate cancer (Huggins and Hodges, 1941). Therefore, despite the fact that there is no scientific evidence that testosterone replacement therapy feeds an existing or triggers a latent prostate cancer (Morgentaler et al., 2008), the Endocrine Society (Bassil et al., 2009) regards prostate cancer as an absolute contraindication for testosterone replacement therapy (TRT). In the light of the above, 80% of men experiencing aging males' symptoms, including fatigue, loss of libido or osteoporosis (Tambi, 2007), a therapy that elevates testosterone levels and has anti-cancer properties would be of great advantage.

Therefore, this study aimed at:

- To investigate the cytotoxicity against human prostate cancer cell *in vitro* and the mode of action.
- Providing clarity on the mechanism of action of TA on prostate cancer with regard to early, late and pathway specific apoptosis to reduce or eliminate prostate cancer.
- Determine the effect of TA on the viability of LNCaP cells.
- Determine the effect of TA on induction of apoptosis in LNCaP cells.
- Determine the caspase 3/7 activity of TA *in vitro* using LNCaP cells.
- Determine the effect of TA on the prostate-specific antigen (PSA) of LNCaP cells.



Chapter 2

MATERIALS AND METHODS

2.1 Chemicals and supply

In this study, all chemicals used, were of analytical or cell culture grade.

Abcam, London, UK:

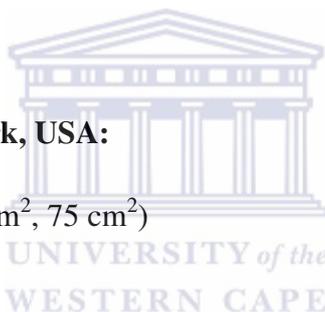
- Prostate Specific Antigen Human ELISA Kit (PSA).

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

- Prostate cancer cell line LNCaP

Corning incorporated, New York, USA:

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



Eppingdust, Cape Town, South Africa:

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

Gibco Invitrogen, Karlsruhe, Germany:

- Roswell Park Memorial Institute (RPMI 1640) Medium
- Fetal Bovine Serum (FBS)
- 0.25% Trypsin/Ethyl Diamine Tetra Acetic acid (EDTA)

Greiner Bio-One, Frickenhausen, Germany:

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

Knittel Gläser, Braunschweig, Germany:

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

Lasec, Cape Town, South Africa:

- Syringes (5, 10 and 25 ml)

**Merck, Wadeville Gauteng, South Africa:**

- Dimethylsulphoxide (DMSO)
- Hydrochloric acid (HCl)

Promega, Madison, USA:

- Caspase-Glo™ 3/7 assay kit (Catalogue Number: G8091)
- The DeadEnd™ Fluorometric TUNEL System kit (terminal deoxynucleotide transferase mediated dUTP nick-end labelling)

Oxoid, Basingstoke, Hampshire, RG24 SPW, England:

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

Sigma-Aldrich, Steinheim, Germany:

- Annexin V-Cy3 MT Apoptosis Detection Kit (Catalogue Number: APOAC)
- Dimethylsulphoxide (DMSO) for freezing medium
- Penicillin
- Streptomycin
- Testosterone purum $\geq 99.0\%$ (HPLC) (Catalogue Number: 86500)
- Thiazolyl Blue Tetrazolium Bromide MTT (Catalogue Number: 2128)
- Trypan Blue (TB)
- Triton X-100
- Millex syringe filter units (0.22 μm)

2.2 Equipments and supply*ELISA-reader*

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

Laminar Flow

- LN Series (Nuve, Ankara, Turkey)

Incubators

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

Scale

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

Microscope

- Inverted System Microscope (Lasec, Cape Town, South Africa)
- Nikon Eclipse TS60, Nikon, Tokyo, Japan

2.3 Study design

LNCaP cells were exposed to different concentrations of TA for 24 and 96 hours according to the study design depicted is (Figure 9).

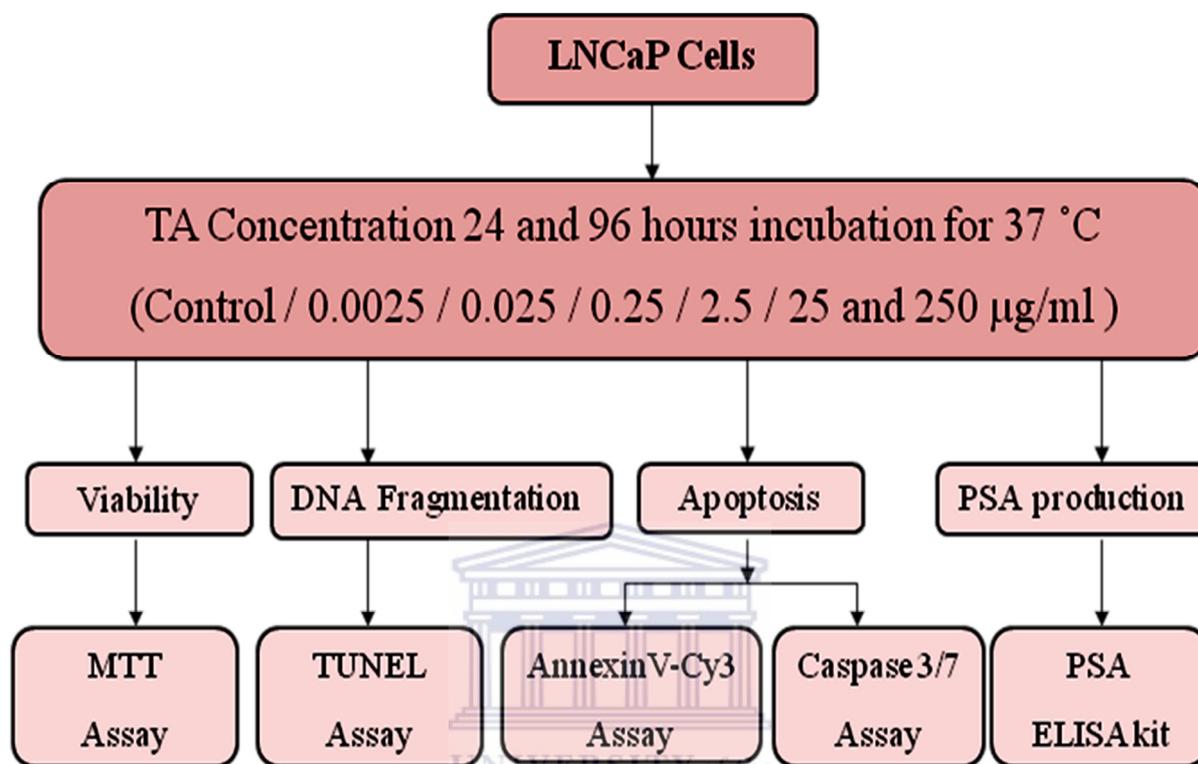


Figure 9: Study design for LNCaP cells and MTT: (3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide).

TUNEL: Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labelling. Annexin V-Cy3: Cell surface phosphatidyl-serine was detected by Annexin V conjugated with Cy3. Caspase 3/7: Luminescent assay that measures caspase-3 and -7 activities in purified enzyme preparations or cultures of adherent cells. PSA: Prostate Specific Antigen.

2.4 Plant extract used (Tongkat Ali, *Eurycoma longifolia* Jack)

A powdered, patented (patent number: WO0217946), standardized, water-soluble extract of Tongkat Ali (TA) provided by Biotropics Malaysia Berhad (Kuala Lumpur, Malaysia) was used in this study. A stock solution of 12.1 mg/ml TA was prepared and mixed with fresh RPMI 1640 media to achieve the desired final concentrations, namely: 0.0025, 0.025, 2.5, 25, 250 µg/ml and control (without extract). All preparations were performed under sterile working conditions in a laminar flow, aliquoted in 15 ml sterile test tubes and frozen at 4°C till further use.

2.5 Phosphate buffered saline (PBS)

Dulbecco's A PBS contained 0.15 M NaCl, 2.5 mM KCl, 10 mM Na₂HPO₄ and 18 mM Na₂HPO₄, pH 7.4. Prepared in ready to used tablets one tablet was dissolved per 100 ml of distilled water according to manufacturer's instructions. This buffer will be referred to throughout as PBS.

2.6 Cell Culture

The prostate cancer cell line LNCaP was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA), and was cultured in Roswell Park Memorial Institute (RPMI) 1640 Medium in 75 cm² culture flask at 37°C under a 5% CO₂ humidified atmosphere. Cells from Passage 2 were grown in RPMI 1640, supplemented with 10% fetal bovine serum (FBS), 1% penicillin (100 IU/ml) and streptomycin (100 µg/ml).

2.6.1 Culture of LNCaP cell line

Cells were cultured in 75 cm² sterile tissue culture flasks and were passaged at regular intervals when cells reached approximately 80% confluency. The culture medium was aspirated from the flasks and cells were washed with 5 ml sterile PBS. Thereafter, 2 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 2-5 minutes and was performed under occasional visual control. In order to inactivate the trypsin, 2 ml of RPMI 1640 containing 10% fetal bovine serum 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 8 minutes.

The supernatant was discarded and the cell pellet re-suspended in 5 ml of RPMI 1640 containing 10% FBS. Following this, 1 ml of the cell suspension was transferred into a new tissue culture flask with fresh RPMI 1640 growth medium with supplements as mentioned previously and passage recorded. Recording the passage gives an indication of the physiological state of the cell line and used to track the age of the cells.

Following this, 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 1:

Equation 1:
$$\frac{\text{Number of cells needed} \times 100}{\text{Total number of cells counted}} = \text{volume of cells required } (\mu\text{l})$$

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 cm² and 75 cm² 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.

2.6.2 Freezing cells

In order to detach cells, 2 ml 0.25% trypsin/EDTA were added into a 75 cm² flask and placed in an incubator at 37°C for 2-5 minutes. The flask was tapped gently and 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 8 minutes, cells were counted and re-suspended in 1 ml of freezing medium (50% RPMI 1640, 40% FBS and 5% DMSO).

About 2 million cells suspended in 1.5 ml freezing medium were aliquoted in cryogenic vials (Corning incorporated, New York, USA) and stored for future use. The cells were immediately transferred to -80°C for 24 hours before being transferred to liquid nitrogen (-196°C) for long-term storage.

2.6.3 Thawing Cells

Stored vials containing cell lines were removed from liquid nitrogen and cells were rapidly thawed in a 37°C water bath. The thawed cells were added to 9 ml pre-warmed RPMI 1640 growth medium in a 15 ml tube and then centrifuged for 8 min at 125 xg. The supernatant was discarded and the cell pellet re-suspended in fresh medium and seeded into a 75 cm² culture flask.

2.7 Test parameters

2.7.1 Morphological changes induced by Tongkat Ali

LNCaP cells (6×10^5 cells/well) grown on 6-well plates were treated with Tongkat Ali (TA) extract at different concentrations of 0.0025, 0.025, 2.5, 25 and 250 $\mu\text{g/ml}$ for 24 hours and 2×10^5 cells/well for a 96 hours, respectively. Cells without treatment were considered as control. After exposure, the morphological changes were observed under an inverted microscope.

2.7.2 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 tetrazolium salt to formazan crystals by the cells mitochondrial dehydrogenase. Based on the absorbance of the cell samples after the test is carried out, cell viability can be measured in cells (Mosmann, 1983).

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 4°C .

LNCaP 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 Tongkat Ali for 24 and 96 hours, respectively, 20 μl of MTT (5 mg/ml in PBS) were added to each well. The plates were incubated 37°C 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 2:

$$\text{Equation 2: Growth inhibition} = \frac{\text{OD control} - \text{OD treated sample}}{\text{OD control}} \times 100$$

2.6.3 The effect of TA on LNCaP viability in the presence or absence of testosterone

The LNCaP (lymph node carcinoma of the prostate) human prostate cancer cell line is a well-established, androgen-dependent cell line (Culig et al., 1999). Therefore provides a good model for changes that occur in prostate tumor cells as the cells are subjected to androgen deprivation both *in vitro* and *in vivo* (Horoszewicz et al., 1983; Chuu et al., 2007). As they are able to retain most of the characteristics of human prostatic carcinoma, like the dependence on androgens (Negri and Motta et al., 1994), this cell line is an attractive model to study hormonal and cytotoxicity therapies.

LNCaP cells were grown to 80% confluence and then trypsinated with 2 ml 0.25% trypsin/EDTA for 3-5 mins under occasional visual control. To inactivate the trypsin, 2ml of RPMI 1640 growth medium were added. Subsequently, the cells were seeded at 8×10^3 cells/well in 200 μ l of complete cutler medium for the 24 hours exposure and 3×10^3 cells/well for 96-hours exposure.

Prior to seeding of cells, testosterone (T) (Sigma-Aldrich) was dissolved in 100% ethanol to arrive at a stock solution of T. Following this, the stock solution was diluted to obtain final concentrations of 1 nM, 10 nM and 100 nM and 1000 nM. The final ethanol concentration was 1%. Controls were treated with 1% ethanol without T and samples were cultivated in triplicate.

At 24 hours post-treatment, the medium was removed and cells washed twice with PBS. To study the modulatory effects of TA on testosterone-induced alterations, the cells were treated again in triplicate either with 100% ethanol alone (vehicle control) or with various testosterone concentrations pre-dissolved in ethanol (1 nM, 10 nM, 100 nM and 1000 nM). Thus, each concentration of TA (0.0025–250 μ g/ml) was tested in the presence of 1 nM, 10 nM, 100 nM and 1000 nM for either 24 or 96 hours.

The MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide) assay was used to quantify viable cells by observing the reduction of tetrazolium salt to formazan crystals by the cells mitochondrial dehydrogenase. Based on the absorbance of the cell samples after the test is carried out, cell viability can be measured in cells (Mosmann, 1983).

For 24- and 96-hour experiments, respectively, 20 μ l of MTT (5 mg/ml in PBS) were added to each well. The plates were incubated 37°C 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 μ l of dimethylsulfoxide (DMSO). Subsequently, the optical density (OD) of the samples was measured with an ELISA reader (GloMax Multi Detection System) at 560 nm. Finally, the percentage of viability refund to the control as 100% was calculated for each set of experiments, namely those experiments employing T which was dissolved in ethanol and those without T and did not contain ethanol (see result) considering that 1% ethanol affected cell growth of LNCaP cells, all comparison between the control without T (and without ethanol) and the T- treatment were refund to their respective controls (with/without ethanol). The respective percent values were then compared.

2.7.4 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., 1994, 1995). Cell surface phosphatidyl-serine was detected by Annexin V conjugated with Cy3 using the commercially available Annexin V-Cy3 apoptosis detection kit. This kit also comprises hand, 6-carboxyfluorescein to detect live cells.

The binding of Annexin V-Cy3 to phosphatidyl-serine is observed as red fluorescence. On the other hand, 6-carboxyfluorescein (6-CFDA) 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 the 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 Annexin V-CY3 (red), and (3) cells in the early stages of apoptosis stain with 6-CFDA (green) and Annexin V-CY3 (red) (Figure 10).

The cells were seeded at (6×10^4 cells/well) in 1ml of complete RPMI 1640 medium for a 24-hour exposure and at 2×10^4 cells/well for a 96-hour exposure in a sterile 6-well plate, respectively. After this, cells were exposed to the various concentrations of Tongkat Ali (TA) for 24 and 96 hours. After discarding the culture medium, cells were washed with 1ml PBS and trypsinated with 500 μ l 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 8 minutes. Subsequently, cells were re-suspended in 1ml PBS and 50 μ l drops were placed on poly-L-lysine-coated slides and allowed to adhere to the slide by incubating for 10 minutes at room temperature breaks in an enzymatic reaction by labelling the free 3'-OH and the modified nucleotides with terminal deoxynucleotidyl transferase. Slides were then washed three times in binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl_2 , pH 7.5) and double stained with AnnCy3 (1 μ g/ml) and 6-CFDA (100 μ M) 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 10) in each experiment.

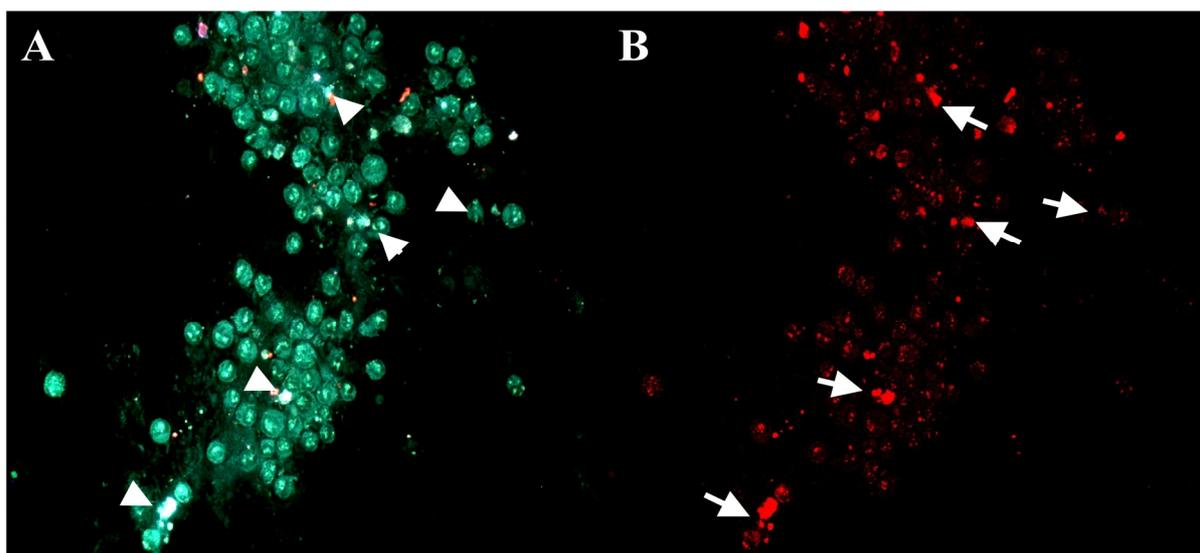


Figure 10: Determination of apoptosis by means of Annexin V-Cy3. **A:** live cells stain only with 6-CFDA (green) (white arrows head), **B:** necrotic cells stain only with Annexin V-Cy3 (red) (white arrows) after Annexin V Cy3 (20X magnifications).

2.7.5 Determination of DNA fragmentation by means of the TUNEL assay

The DNA laddering technique is used to visualize the endonuclease cleavage products of apoptosis (Wyllie, 1980). Since DNA fragmentation occurs in the later phase of apoptosis, the absence of a DNA ladder does not eliminate the potential that cells are undergoing early apoptosis (Kressel and Groscurth, 1994; Ito and Otsuki, 1998).

The presence of apoptosis was determined by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labelling (TUNEL) using the DeadEnd™ Fluorometric TUNEL System kit from Promega (Madison, WI, USA). This kit detects apoptotic cells *in situ* by specific end labelling and detection of DNA fragments produced by the apoptotic process. This assay is very sensitive, allowing detection of single- and double-strand DNA breaks in an enzymatic reaction by labelling the free 3'-OH and the modified nucleotides with terminal deoxynucleotidyl transferase.

Cells were seeded at (6×10^5 cells/well) in 1ml of complete RPMI 1640 medium for a 24-hours exposure and at (2×10^5 cells/well) in 1ml of complete RPMI 1640 medium for a 96-hours exposure in sterile 6-well plate. After exposure of cells to various concentrations of TA, the medium was discarded and cells were washed with 1 ml PBS and trypsinated with 500 μ l of 0.25% Trypsin/EDTA. After trypsination, cell suspensions were transferred from 6-well plates to Eppendorf vials and cell pellets were collected by centrifugation at 125 xg for 8 minutes and then discarded supernatant. Subsequently, cells were re-suspended in 1 ml PBS and 50 μ l drops were placed on poly-L-lysine-coated slides.

Air dried slides were fixed in 4% formaldehyde in PBS (pH 7.4) for 25 minutes at 4°C. After fixation, slides were washed in PBS for 5 minutes at room temperature and permeabilized in 0.2% Triton™ X-100 in PBS for 5 minutes at room temperature. The slides were then rinsed twice in PBS for 5 minutes at room temperature. Afterwards, 100 μ l of equilibration buffer were added to each slide and allowed to equilibrate for 10 minutes. Subsequently, 20 μ l of TdT incubation buffer were added to each slide, covered with a plastic cover slip and slides were incubated for 60 minutes at 37°C in a humidified chamber away from light for 1 hour at 37°C. Subsequently, the cover slips were carefully removed, and the reaction terminated by immersion in 2X SSC for 15 minutes.

Slides were then washed in PBS three times and immediately analyzed using a fluorescence microscope. To determine the percentages, of cells indicating apoptosis, a total of 200 cells were counted and the cells that fluoresce brightly green were counted as TUNEL-positive cells (Figure 11) in each experiment.

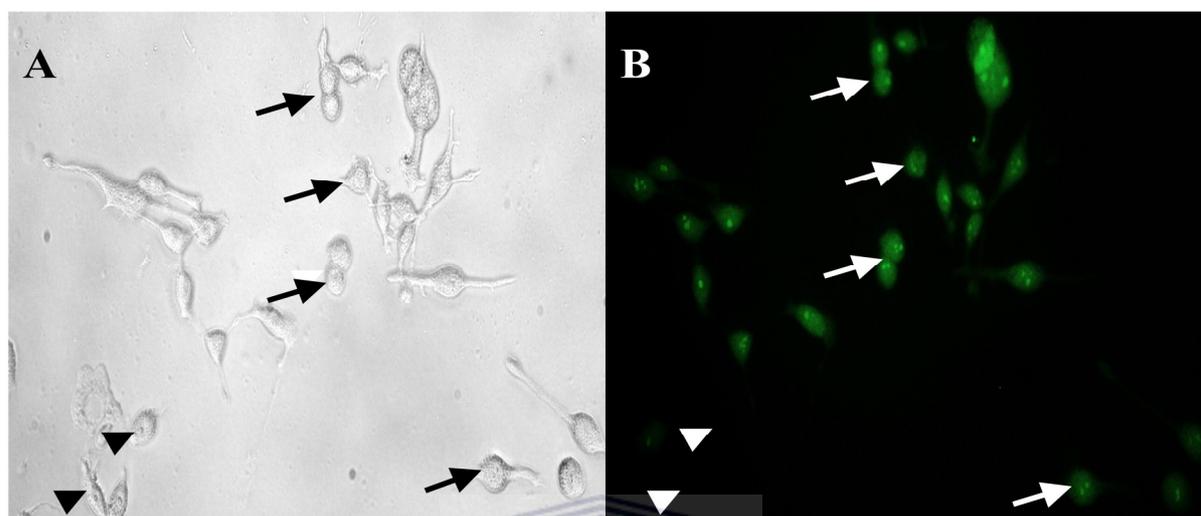


Figure 11: Determination of DNA fragmentation in LNCaP cells after exposure to different concentration of TA. A) Bright field microscopic image. B) Fluorescence image.

TUNEL-negative cells: (arrows head); TUNEL positive-cells (white arrows)
(20 X magnifications).

2.7.6 Determination of caspase activation

The activity caspase 3/7 was determined by the luminescent caspase-Glo 3/7 assay kit. After cells were treated with TA, LNCaP cells were seeded in white-walled 96-well tissue culture plates at a density of 5×10^3 cells/well in 100 μ l of RPMI 1640 growth medium and allowed to adhere for 24 hours after seeding. Subsequently, cells were treated with Tongkat Ali at the different concentrations for 24 hours, experiments were carried out in duplicate.

Assay procedures were conducted according to the manufacturer's instructions. After treatment of the cells with TA, the caspase-Glo™ reagent was prepared by mixing the caspase-Glo™ substrate and caspase-Glo™ buffer at room temperature and was protected from light. Thereafter, 100 μ l of this mixture were added to each well in the dark (1:1 ratio of medium to mixture), mixed and then incubated in the dark for 1-2 hours at room temperature. The plate was then read using a GloMax Multi Detection System plate reader

for luminescence. The level of luminescence observed corresponds to the level of caspase 3/7 activity.

2.7.6 Determination of Prostate Specific Antigen (PSA)

The production of prostate-specific antigen (PSA) is largely regulated by the androgen-dependent activation of the androgen receptor on prostate cells both, normal and malignant prostate epithelial cells (Yousef and Diamandis, 2001).

PSA is a 33 kD serine proteinase which, in human serum, is predominantly bound to α -1 antichymotrypsin and α -2 macroglobulin. Current methods of screening men for prostate cancer utilize the detection of the major α -1 antichymotrypsin (Lilja et al, 1991). PSA Levels of 4.0 ng/ml or higher are strong indicators of the possibility of prostatic cancer (Catalona et al., 1993).

In order to determine PSA levels, LNCaP cells were plated at (6×10^4 cells/well) in 1 ml of complete RPMI 1640 medium for a 24-hours exposure in a sterile 24-well plate and grown to 80% confluency. Culture media were discarded after the incubation period and cells washed with PBS. Cells were then treated with Tongkat Ali at different concentrations for 24 hours, in duplicate. Thereafter, the cell culture supernatants were collected, stored in Eppendorf vials and frozen until PSA determination. On the day of PSA determination, all reagents and samples were brought to room temperature (25°C).

2.7.6.1 ELISA for PSA in culture supernatants

Prior to PSA determination, various solutions had to be prepared. The wash buffer was diluted 5-fold with distilled water. Following this, the PSA standard was prepared by briefly spinning the vial containing recombinant human PSA before the addition of 400 μ l 1X concentrated buffer into the vial to prepare a 50 ng/ml standard concentration. This solution was then mixed gently. Subsequently, a series of dilutions was performed (Figure 12), which involved the initial transfer of 50 μ l PSA-total standard (50 ng/ml) from the vial containing the recombinant human PSA solution into a tube with 950 μ l 1X concentrated buffer to prepare a 2.500 pg/ml standard solution. Then, 300 μ l 1X concentrated buffer were transferred into 7 tubes. Next, 200 μ l of the 2.500 pg/ml standard solutions were transferred to a test tube to produce the 1000 pg/ml concentration. After thoroughly mixing, this process was repeated, resulting in a concentration range of 0-2.500 pg/ml. The 1X concentrated buffer

served as the zero standards. Solutions in Eppendorf vials were mixed gently between transfers.

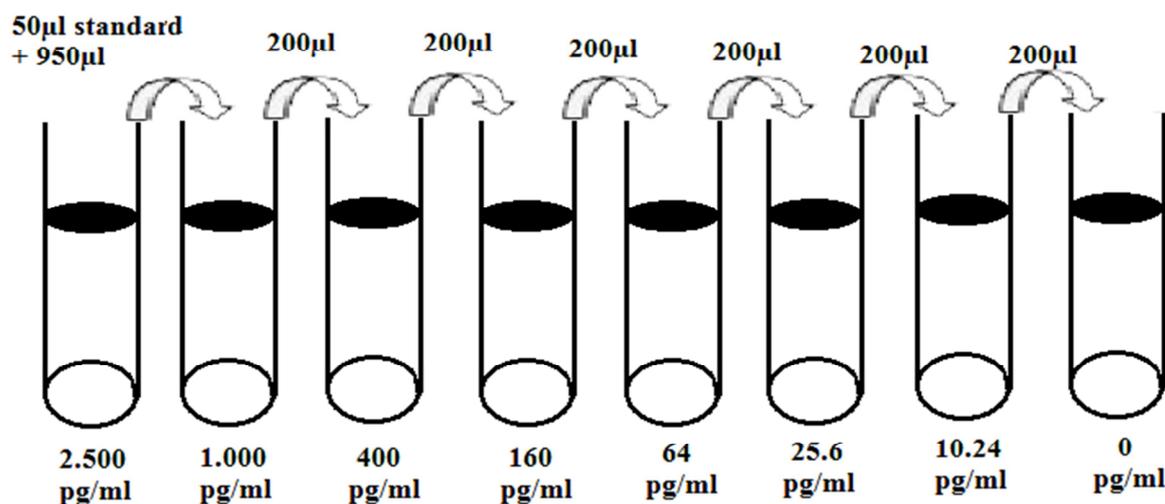


Figure 12: Serial dilution for PSA Standard

Since the wash buffer concentrate (20X) contained visible crystals it was warmed at room temperature with gentle mixing until all visible crystals were dissolved. Thereafter, the solution was diluted in distilled water at a ratio of 1:99 prior to use. The detection antibody concentrate was prepared by the addition of 100 µl of 1X concentrated buffer with gentle mixing. Following this, the detection antibody concentrate was further diluted 80-fold with 1X concentrated buffer. The vial containing HRP-Streptavidin concentrate was mixed gently. Thereafter, 25 µl of HRP-Streptavidin concentrate was transferred into a tube with 12.5 ml 1X concentrated buffer to prepare a 500-fold diluted HRP Streptavidin solution for use in the assay.

The testing procedure required that 100 µl of each standard and sample be transferred into the 96-well microtiter plate (which is coated with an antibody specific for human PSA). Thereafter, the plate was covered with a plastic cover provided in the kit and incubated for 2.5 hours at room temperature. After incubation, the solution in the wells was discarded and the wells were washed 4 times with 300 µl/ml 1X wash solution. After the last wash, all remaining solutions were removed and the plate was blotted against clean paper towels. Finally, 100 µl of 1X prepared biotinylated antibody were added to each well and then incubated for 1 hour at room temperature with gentle shaking.

Following the 1 hour incubation period, the solution was discarded and wells were washed 4 times as described above with gentle blotting of the plate to remove excess solution. Then, 100 μ l of the prepared streptavidin solution were added to each well before the plate was further incubated for 45 minutes at room temperature with gentle shaking. The washing step was once again repeated and the plate blotted on paper towel. Thereafter, 100 μ l of 3, 3', 5, 5'-tetramethylbenzidine (TMB) in buffer solution was added to each well and incubated for 30 minutes at room temperature in the dark with gentle shaking resulting in the development of a blue colour. Finally, 50 μ l of the stop solution were added to each well resulting in the colour changing from blue to yellow (Figure 13A and B). The absorbance was read at 450 nm with an ELISA plate reader and the concentration of PSA in the media determined was according to the standard curve (Figure 14) using Equation 3.

Equation 3: $Y = \text{Exp}(5.2194) + (3.5241) \text{LOG}(x)$.

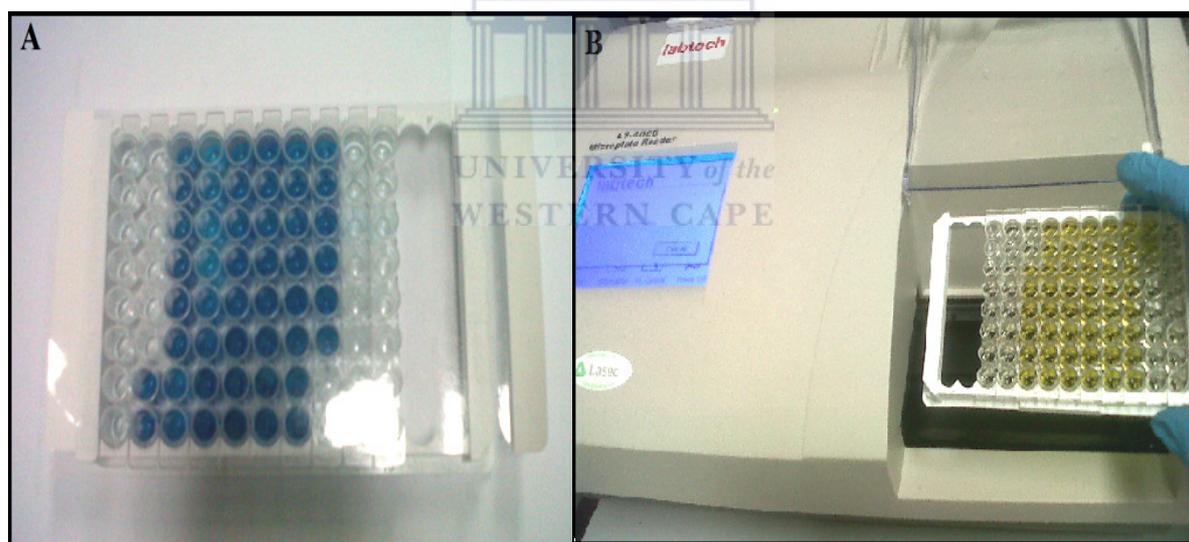


Figure 13: Prostate Specific Antigen (PSA). **A:** typical 96-well ELISA plate before stop solution added (blue); **B:** Typical 96-well plates after adding stop solution (yellow). The absorbance was determined at 450 nm with an ELISA plate reader (Labtech).

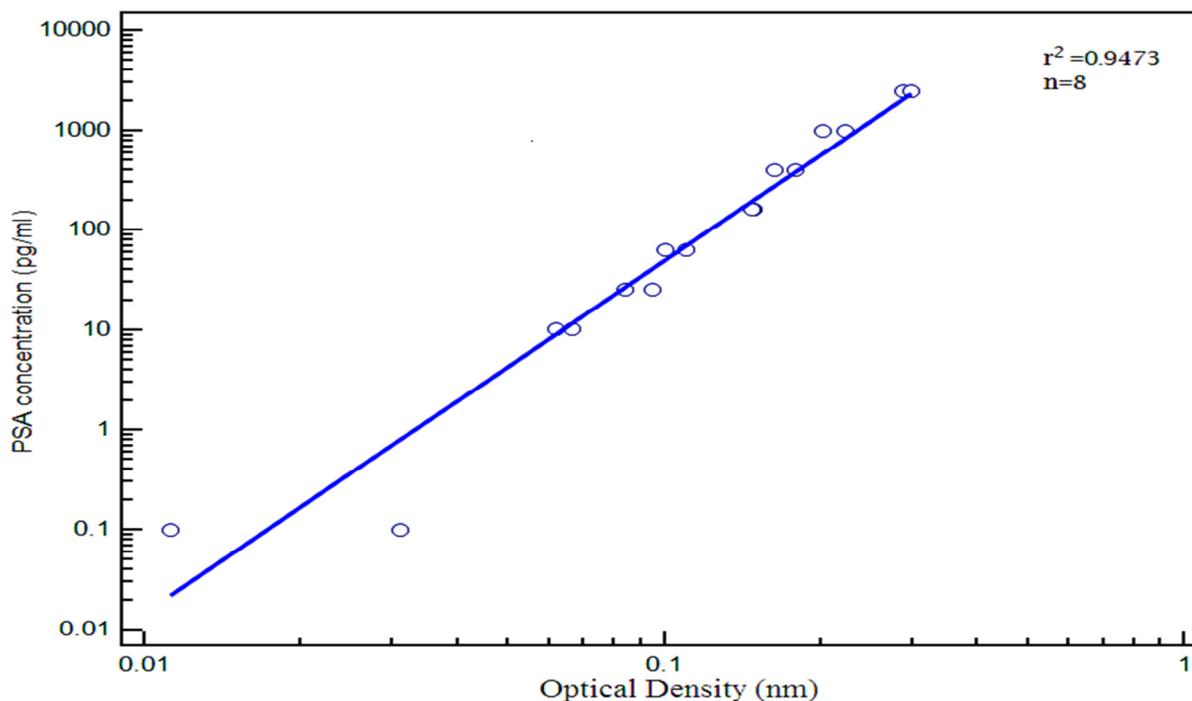


Figure 14: Standard curve for the determination of PSA. The standard curve shows a double-logarithmic relationship between the PSA concentration and the optical density. The correlation coefficient r^2 value=0.9473.

2.8 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, one-way ANOVA, repeated measures ANOVA, paired samples t-test) or non-parametric (Spearman Rank correlation, Wilcoxon test) were performed. Tukey tests were carried out to detect outliers. Data were expressed as mean \pm SD or mean \pm SEM. A P-value of P=0.0001 and P<0.05 was considered significant. Were calculated from the effects of TA concentrations of the compound by Statistical analysis was done by one-way ANOVA. In addition GraphPad Prism Version 6.03 was also used for comparative analysis (GraphPad Prism 6.03, GraphPad Software Incorporation, San Diego, USA).

Chapter 3

RESULTS

The prostate cancer cell line LNCaP was cultured in RPMI 1640 Medium in 75 cm² culture flask at 37°C under a 5% CO² humidified atmosphere and supplemented with 10% Fetal Bovine Serum (FBS). LNCaP cells were exposed to different concentrations of TA (0.0025, 0.025, 2.5, 25 and 250 µg/ml) for 24 hours (6x10⁴cells/ml) and 96 hours (2x10⁴cells/ml). The following parameters were tested: morphology, viability (%), annexin V-CY3 binding (%), DNA fragmentation (%), caspase 3/7 activation (RUL) and PSA production (pg/ml), relative PSA and viability (pg/ml/%).

3.1 Effect of TA on cell morphology

When observing the morphological changes of LNCaP cells after 24 hours of exposure to increasing concentration of TA, there was a clear increase in detachment and cell death (Figure 15). Clumping of cells and apoptotic bodies could also be seen.

3.2 Effect of TA on cell viability

The viability of LNCaP cells was tested after exposure to different concentrations of TA by mean of the MTT test. After 24 hours of exposure, viability appeared to increase at lower concentrations (Figure 16A). Yet, this increase was not significant. For higher concentrations, viability decreased again reaching significant (P=0.0226) at a TA concentrations of 250 µg/ml.

After 96 hours of exposure, viability for all concentrations decreased when compared to the control (Figure 16B). However, a significant decrease (P=0.0010) could only be observed for the highest concentration 250 µg/ml.

When comparing both exposure periods, cells exposed to TA for 24 hours showed an initial increase in viability of 9-22%, indicating cellular stress. As from 2.5 µg/ml TA, values declined (21%) reached lower value (-35%) at 250µg/ml TA. In contrast, after 96 hours of exposure, all concentrations of TA led to a decrease in viability ranging from 1-47% indicating cell death toward higher concentrations over

a longer period of time. ANOVA trend analysis revealed significant trends for both exposure periods (ANOVA trend analysis: $P=0.0004$ and $P=0.0012$, respectively).

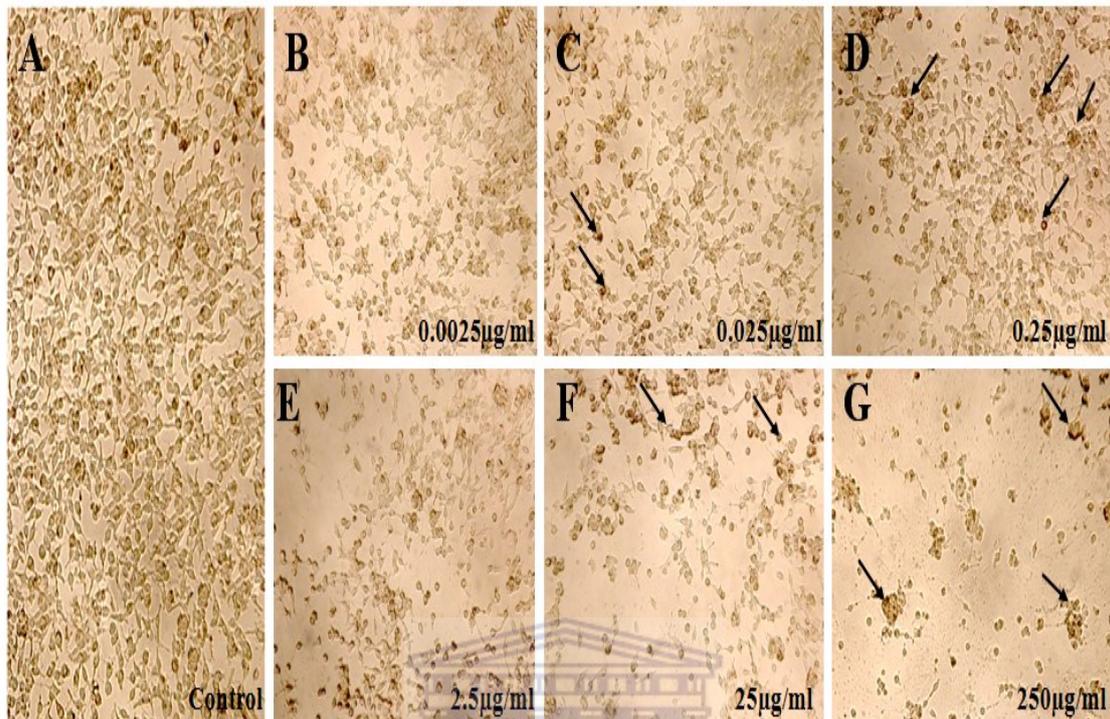


Figure 15: Morphological changes in the LNCaP cells after 24 hours of treatment with various concentrations of TA: Apparently, cells are undergoing apoptosis. (10 x magnifications). Arrows indicate apoptotic bodies and clumping.

A: Control without Tongkat Ali (TA)

B: Tongkat Ali (TA 0.0025 µg/ml)

C: Tongkat Ali (TA 0.025 µg/ml)

D: Tongkat Ali (TA 0.25 µg/ml)

E: Tongkat Ali (TA 2.5 µg/ml)

F: Tongkat Ali (TA 25 µg/ml)

G: Tongkat Ali (TA 250 µg/ml)

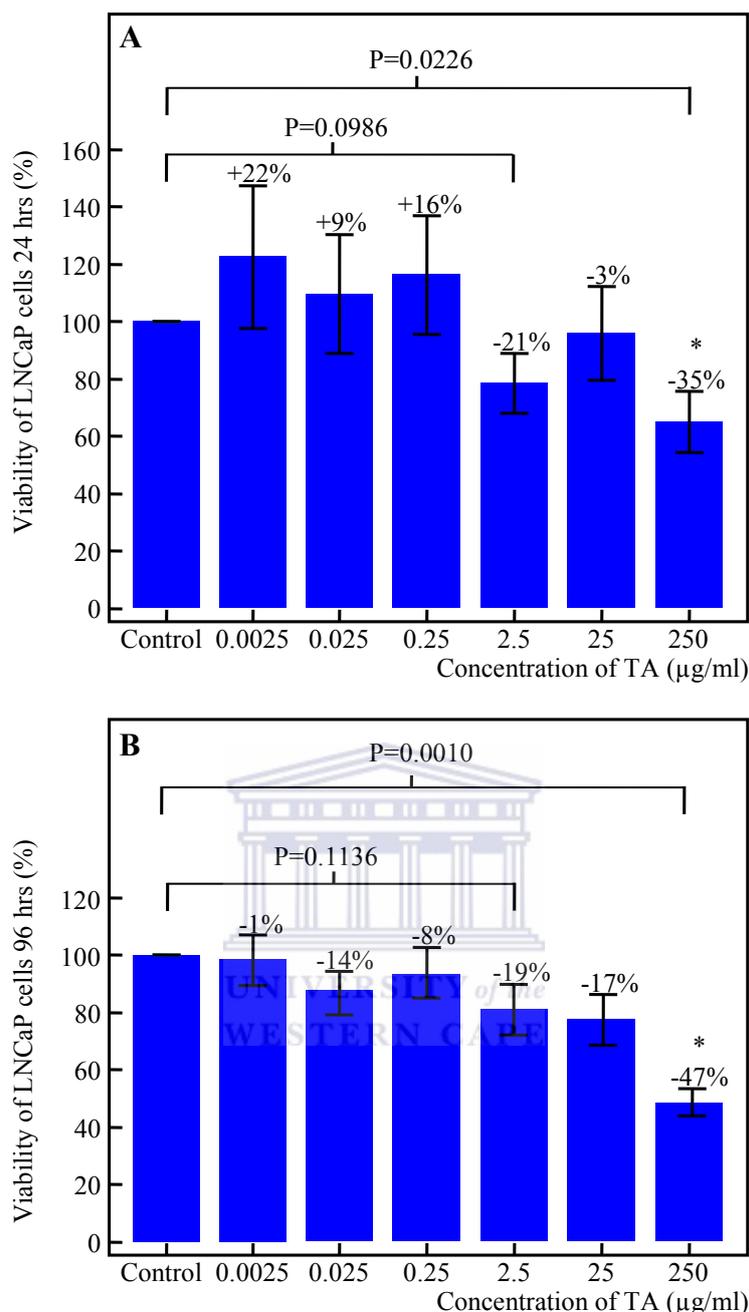


Figure 16: The effect of TA (0.0025, 0.025, 0.25, 2.5, 25 and 250 $\mu\text{g/ml}$) on LNCaP cells viability *in vitro*. Values are shown as mean \pm SEM (n=8), increase [+] or decrease [-] with statistical significance established at $P < 0.05$ [*].

A: 24 hours exposure: Lower concentrations show increases of 9-22% in viability. However, at concentration of 250 $\mu\text{g/ml}$, a significant decrease of 35% can be seen. A significant trend (ANOVA trend analysis: $P=0.0004$) towards higher concentrations in decreased viability could be found.

B: 96 hours exposure: All concentrations show decreases in viability with a significant decrease ($P=0.0010$) of 47% at 250 $\mu\text{g/ml}$. A significant trend (ANOVA trend analysis: $P=0.0012$) in a dose-dependent manner could be established.

3.3 Effect of TA and testosterone on LNCaP Cells

Determining the effect of testosterone on TA activity on LNCaP cells is shown by data in Figure 16. The treatment of cells with testosterone resulted in a dose-dependent induction of cell proliferation. The significant inhibitory effects on testosterone-stimulated cell proliferation were seen at TA concentrations as low as 0.0025 $\mu\text{g/ml}$ TA. At higher concentrations of TA (25 and 250 $\mu\text{g/ml}$), cell proliferation was inhibited for all testosterone dosages used. Cells appear sparser and apoptosis is clearly evident by clumping (Figure 17 Y-F1).

Testosterone concentrations of 10 nM showed maximum stimulation of cell proliferation for TA dosages up to 2.5 $\mu\text{g/ml}$. Apoptosis is visible for testosterone and ethanol only treated cells (Figure 16 E-H). An initial drop in the cell number in the control, which included the vehicle for testosterone solubilisation, was due to the solvent mediator 1% ethanol.



Legend to Figure 17: Morphological changes in the LNCaP cells exposed to different concentrations of TA (0.0025-250 $\mu\text{g/ml}$) and T (1-1000 nM) after 24 hours of treatment undergoing various stages of apoptosis (10 x magnifications). Testosterone was dissolved by aid of 1% ethanol.

A: Control Medium without testosterone only (T0)

B: Control Medium without testosterone only (T0)

C: Control Medium with Ethanol.

D: Control Medium with Ethanol.

E: Testosterone without Tongkat Ali (1 nM+ TA 0 $\mu\text{g/ml}$)

F: Testosterone without Tongkat Ali (10 nM+ TA 0 $\mu\text{g/ml}$)

G: Testosterone without Tongkat Ali (100 nM+ TA 0 $\mu\text{g/ml}$)

H: Testosterone without Tongkat Ali (1000 nM+ TA 0 $\mu\text{g/ml}$)

I: Testosterone with Tongkat Ali (TA 0.0025 $\mu\text{g/ml}$ + T 1 nM)

J: Testosterone with Tongkat Ali (TA 0.0025 $\mu\text{g/ml}$ + T 10 nM)

K: Testosterone with Tongkat Ali (TA 0.0025 $\mu\text{g/ml}$ + T 100 nM)

L: Testosterone with Tongkat Ali (TA 0.0025 $\mu\text{g/ml}$ + T 1000 nM)

M: Testosterone with Tongkat Ali (TA 0.025 $\mu\text{g/ml}$ + T 1 nM)

N: Testosterone with Tongkat Ali (TA 0.025 $\mu\text{g/ml}$ + T 10 nM)

O: Testosterone with Tongkat Ali (TA 0.025 $\mu\text{g/ml}$ + T 100 nM)

P: Testosterone with Tongkat Ali (TA 0.025 $\mu\text{g/ml}$ + T 1000 nM)

Q: Testosterone with Tongkat Ali (TA 0.25 $\mu\text{g/ml}$ + T 1 nM)

R: Testosterone with Tongkat Ali (TA 0.25 $\mu\text{g/ml}$ + T 10 nM)

S: Testosterone with Tongkat Ali (TA 0.25 $\mu\text{g/ml}$ + T 100 nM)

T: Testosterone with Tongkat Ali (TA 0.25 $\mu\text{g/ml}$ + T 1000 nM)

U: Testosterone with Tongkat Ali (TA 2.5 $\mu\text{g/ml}$ + T 1 nM)

V: Testosterone with Tongkat Ali (TA 2.5 $\mu\text{g/ml}$ + T 10 nM)

W: Testosterone with Tongkat Ali (TA 2.5 $\mu\text{g/ml}$ + T 100 nM)

X: Testosterone with Tongkat Ali (TA 2.5 $\mu\text{g/ml}$ + T 1000 nM)

Y: Testosterone with Tongkat Ali (TA 25 $\mu\text{g/ml}$ + T 1 nM)

Z: Testosterone with Tongkat Ali (TA 25 $\mu\text{g/ml}$ + T 10 nM)

A1: Testosterone with Tongkat Ali (TA 25 $\mu\text{g/ml}$ + T 100 nM)

B1: Testosterone with Tongkat Ali (TA 25 $\mu\text{g/ml}$ + T 1000 nM)

C1: Testosterone with Tongkat Ali (TA 250 $\mu\text{g/ml}$ + T 1 nM)

D1: Testosterone with Tongkat Ali (TA 250 $\mu\text{g/ml}$ + T 10 nM)

E1: Testosterone with Tongkat Ali (TA 250 $\mu\text{g/ml}$ + T 100 nM)

F1: Testosterone with Tongkat Ali (TA 250 $\mu\text{g/ml}$ + T 1000 nM)

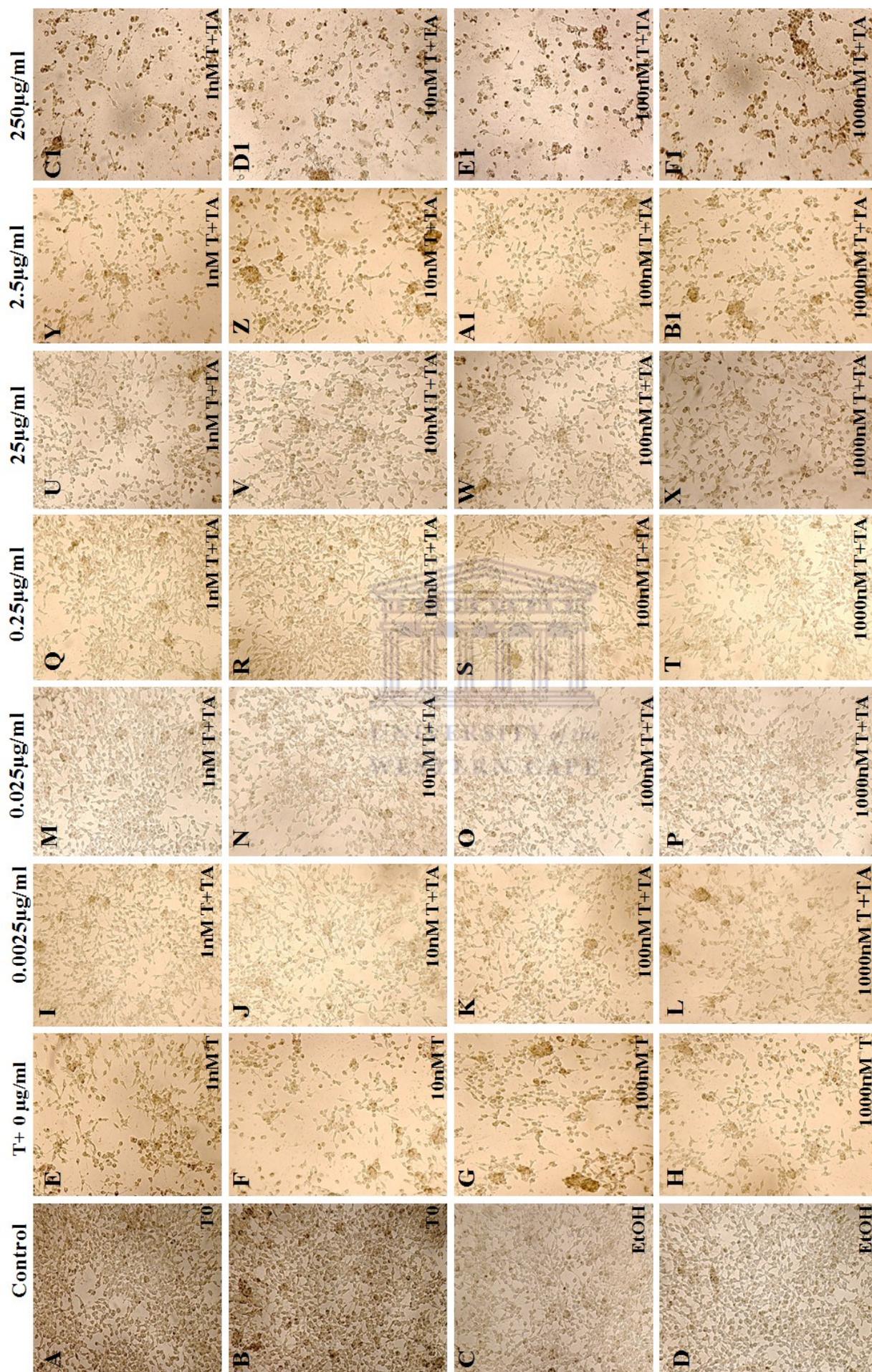


Figure 17:

To study the modulatory effects of TA on testosterone-induced alterations, the cells were treated with testosterone concentrations pre-dissolved in ethanol (1 nM, 10 nM, 100 nM and 1000 nM) and concentration of TA (0.0025–250 µg/ml) for 24 and 96 hours.

The MTT assay was used to quantify viable cells (previously explained) for 24- and 96-hour experiments, respectively. The percentage of viability referred to the control as 100% was calculated for each set of experiments with T- treatment and referenced to their respective controls (with/without ethanol). The respective percentage values were then compared.

After 24 hours of treatment (Figure 18A), cells treated with TA concentrations of 0.0025-2.5 µg/ml showed an increase in viability, continued by a significant decline at 250 µg/ml TA ($P < 0.0001$) in the presence of T. At a concentration of 10 nM T, cell stimulation reached a maximum (Figure 17 J, N, R, V, Z, and D1)

When comparing (T + TA) and (TA without T), it is clear that T plays a significant modulatory role in proliferation. A significant dose-dependent trend (ANOVA trend analysis: $P = 0.0024$) toward a declining viability for TA was evident.

For the 96-hour exposure (Figure 18B), similar results could be observed as that of 24 hours. When comparing the control with 2.5 and 25 µg/ml TA+ 1000 nM T, significant decreases in viability were seen ($P = 0.0014$ and $P = 0.0002$, respectively). For the treatment group 250 µg/ml TA+ 1000 nM T result become being highly significant ($P < 0.0001$). A similar trend for the dose-dependent manner of the effect could be found (ANOVA trend analysis: $P = 0.0003$).

Legend to Figure 18: To study the modulator effects of TA on testosterone-induced alterations, the cells were treated with testosterone concentrations pre-dissolved in ethanol (1 nM, 10 nM, 100 nM and 1000 nM) and concentration of TA (0.0025, 0.025, 0.25, 2.5, 25 and 250 µg/ml) on LNCaP cells *in vitro*. Values are shown as Mean ± SEM (n=8), increase [+] or decrease [-] and statistical significance at P<0.05 [*] and highly significant P<0.0001 [**].

A: 24 hours exposure: A significant increase in viability seen at 0.25 µg/ml TA of 23% and highly significant increases as from 2.5 to 250 µg/ml (P<0.0001). A highly significant trend (ANOVA trend analysis: P<0.0001) in dose dependency could be found. At the highest TA concentration (250 µg/ml) a 75% significant decrease (P<0.0001) is depicted. At concentration 25 µg/ml only a borderline significance (P<0.0001) is reached at a decrease of 40%. A highly significant trend (ANOVA trend analysis: P=0.0003) in the absence of T dependant manner could be established.

B: 96 hours exposure: All concentrations show significant increases. A highly significant trend (ANOVA trend analysis: P<0.0001) was established as well. At the highest TA concentration (250 µg/ml) a 72% significant decrease (P<0.0001) is depicted. At concentration 25 µg/ml only a borderline significance (P=0.0089) is reached at a decrease of 34%. A highly significant trend (ANOVA trend analysis: P=0.0002) in a concentration dependant manner could be established.

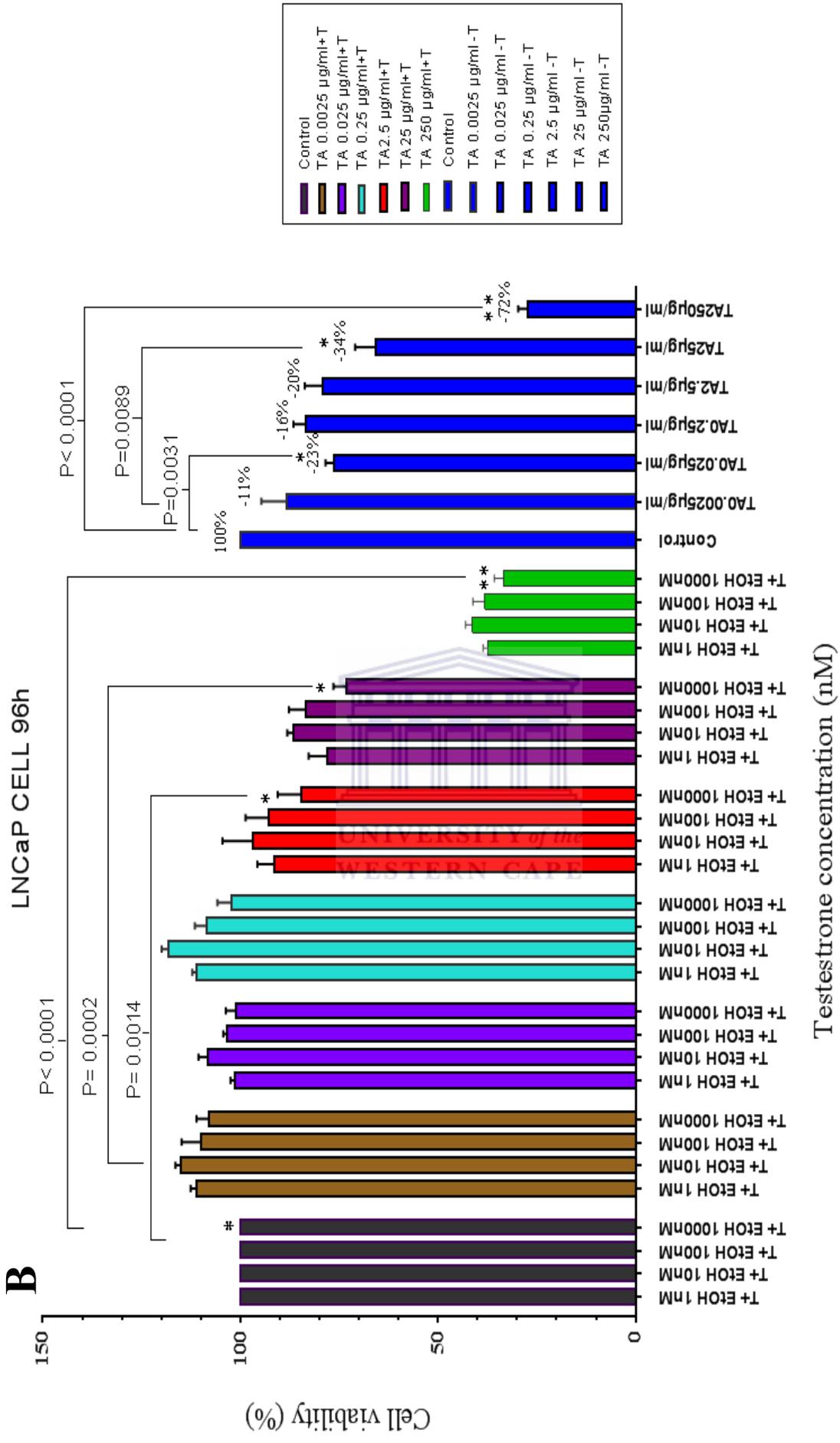


Figure 18 B:

3.4 Effect of TA on Annexin V-Cy3 binding

Annexin V-Cy3 binding to LNCaP cells as indicator of apoptosis was analyzed after exposure to different concentrations of TA. After exposure for 24 hours, lower dosages of TA revealed no change in annexin V binding. However, as the concentrations of TA increased a significantly higher annexin V binding accrued, indicating early apoptosis (Figure 19A). A dose-dependent trend could be shown by ANOVA trend analysis (ANOVA trend analysis: $P < 0.0001$). At 24 hours, the significance in percentage of apoptotic cells was much lower, ranging from 10-32% of annexin V-positive cells than those in cells incubated for 96 hours (8-41%).

After 96 hours of exposure, all concentrations showed a significant increase in early apoptosis in the cells as dosages increased (Figure 19B).

3.5 Effect of TA on DNA fragmentation

LNCaP cells were incubated with different concentrations of TA and analyzed for DNA fragmentation. After 24 hours of exposure, DNA damage (TUNEL-positivity) has shown to significantly increase with each concentration when compared to the control (Figure 20A). All concentrations revealed highly significant values ($P < 0.0001$) except at concentration 0.025 $\mu\text{g/ml}$, which reached a lower level of significance ($P = 0.0002$). An ANOVA trend analysis also revealed a highly significant dose-dependency ($P < 0.0001$).

After LNCaP cells were exposed to TA for 96 hours, similar results were observed (Figure 20B). All concentrations showed highly significant increases in DNA damage when compared to the control.

Although, both exposure periods revealed an increase in the percentage of DNA-damaged cells as concentrations increased, it did, however, not reach a level of more than 50%. At 24 hours, a maximum of 46% DNA fragmentation was recorded, while after 96 hours 49% of the cells showed DNA fragmentation at the highest concentration (250 $\mu\text{g/ml}$).

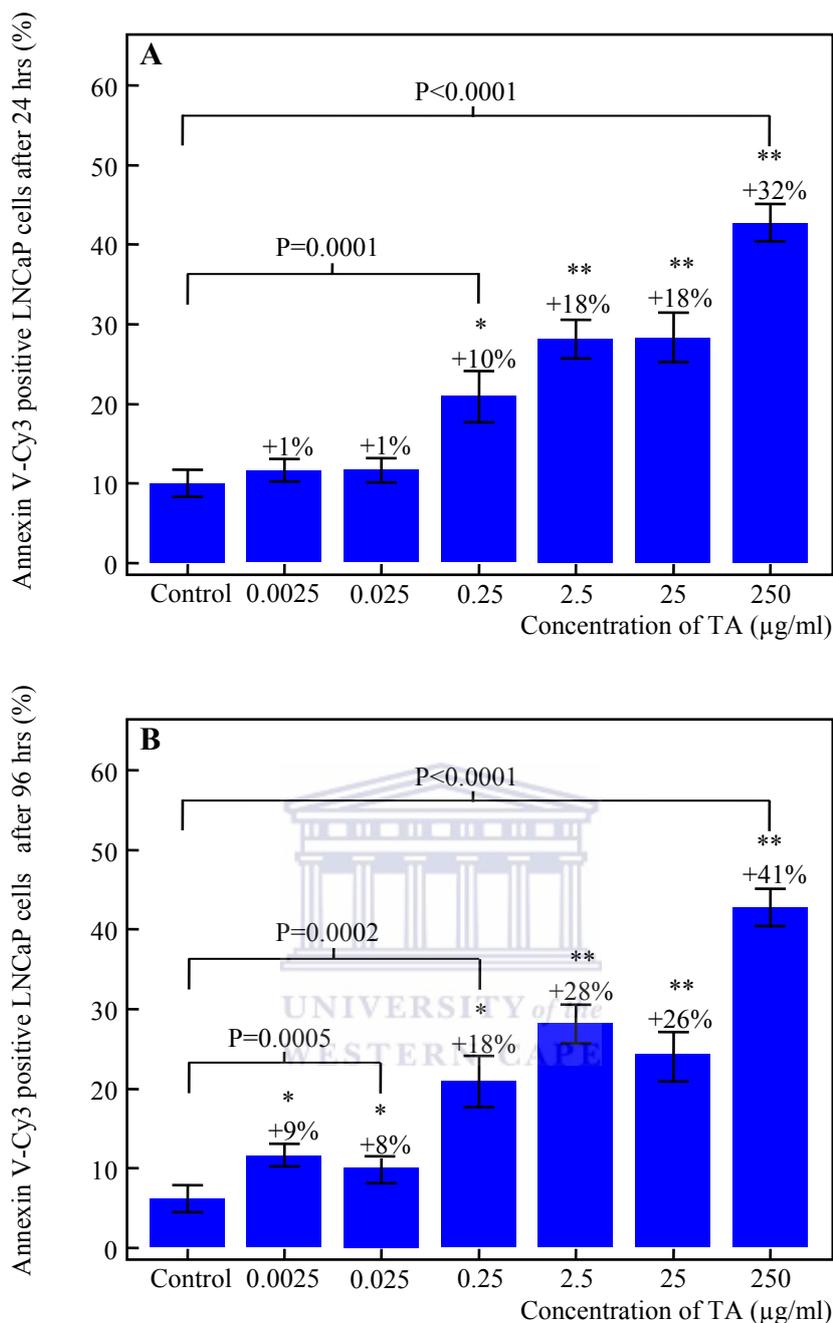


Figure 19: The effect of TA (0.0025, 0.025, 0.25, 2.5, 25 and 250µg/ml) on Annexin V-Cy3 indicating cells apoptosis. Values are shown as mean \pm SEM (n=8), increase [+] or decrease [-] with statistical significance established at $P<0.05$ [*] and highly significant $P<0.0001$ [**].

A: 24 hours exposure: A significant increase in apoptosis is seen at 0.25 µg/ml TA of 10% and highly significant increases as from 2.5 to 250 µg/ml ($P<0.0001$). A highly significant trend (ANOVA trend analysis: $P<0.0001$) in dose dependency could be found.

B: 96 hours exposure: All concentrations show significant increases. A highly significant trend (ANOVA trend analysis: $P<0.0001$) was established as well.

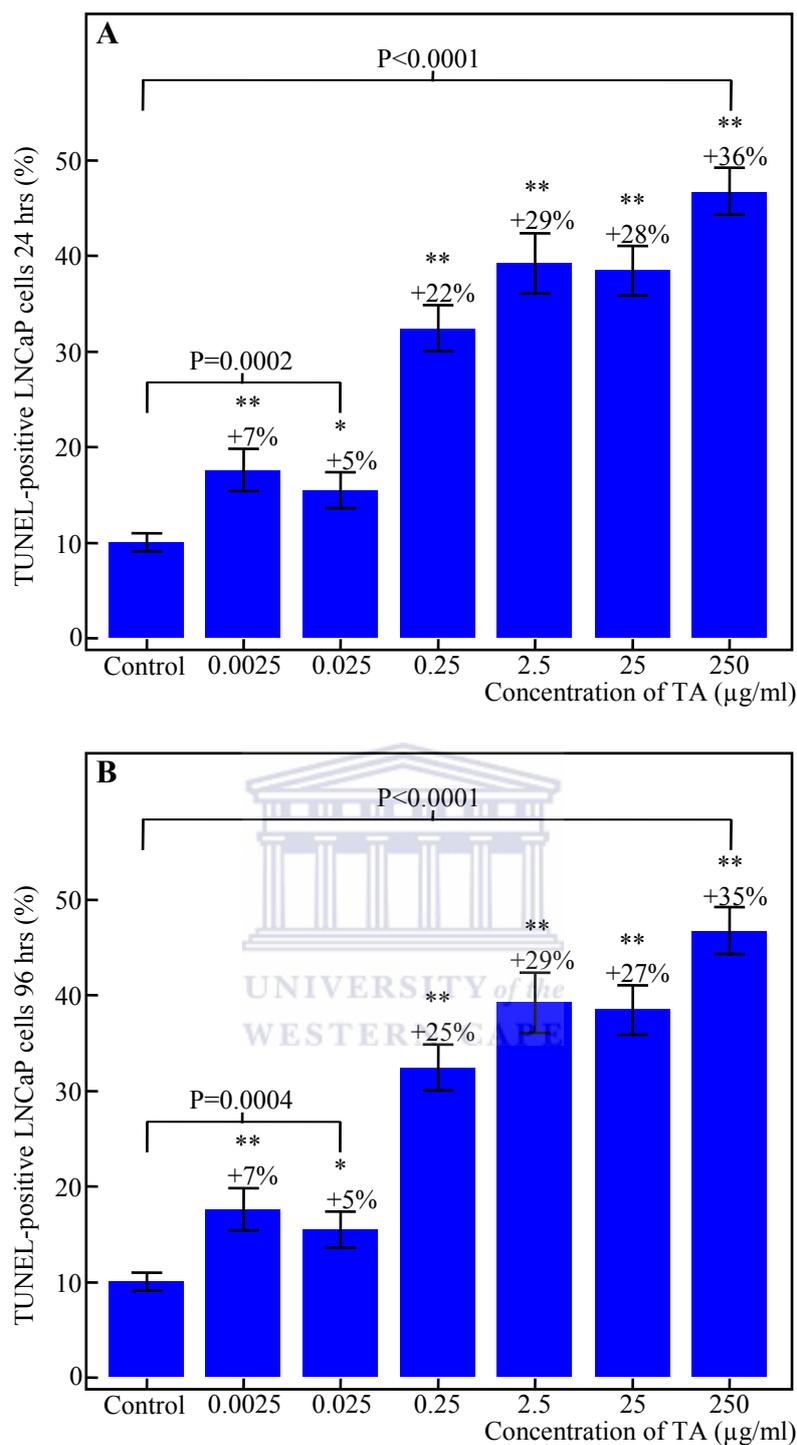


Figure 20: The induction of DNA damage (TUNEL-positivity) of TA (0.0025, 0.025, 0.25, 2.5, 25 and 250 $\mu\text{g/ml}$) in cells. Values shown as mean \pm SD (n=8). Statistical difference shown as $P < 0.05$ [*] and highly significant $P < 0.0001$ [**].

A: 24 hours exposure: All concentrations show significant increases. A highly significant trend (ANOVA trend analysis: $P < 0.0001$) could also be found.

B: 96 hours exposure: All dosages show significant increases with a 35% at the highest dose. A highly significant trend (ANOVA trend analysis: $P < 0.0001$) can be established.

3.6 Effect of TA on Caspase 3/7 activation

After LNCaP cells were exposed to different concentrations of TA, the detection of Caspase 3/7 activation was analyzed. At lower concentrations up to 25 $\mu\text{g/ml}$, no significant change was observed. Only after incubation with TA 250 $\mu\text{g/ml}$ TA a highly significant increase ($P < 0.0001$) was observed when compared to the control (Figure 21A). The decrease in Caspase 3/7 activity at 2.5 $\mu\text{g/ml}$ TA was significant but counterbalanced by an increase of 19% at 25 $\mu\text{g/ml}$. A trend towards higher Caspase activation could also be observed (ANOVA trend: $P = 0.0001$). The relative Caspase activity (caspase activity related to viability; Figure 22B) showed significant ($P = 0.0043$) activation at the highest concentration with a significant positive dose dependence (ANOVA trend analysis: $P = 0.0299$), while at lower concentrations an initial increase was followed by a decrease at 25 $\mu\text{g/ml}$.

3.7 Effect of TA on PSA secretion

LNCaP cells were exposed to TA at different concentrations and the production of prostatic specific antigen (PSA) was analyzed. A steady decline in PSA secretion was which reached significance ($P = 0.0132$) at the highest concentration of TA (250 $\mu\text{g/ml}$) used in the study was seen (Figure 23). ANOVA trend analysis revealed that the decline took place in a dose-dependent manner (ANOVA trend: $P = 0.0055$).

Considering that TA caused cell death in a dose-dependent manner, the relative PSA concentration (PSA per percentage viable cells) was calculated. Here, no significant change was observed (Figure 24). Although the increase at the highest concentration compared to the control was not significant, ANOVA trend analysis resulted in a borderline significance ($P = 0.0768$). Yet, the value is not significant.

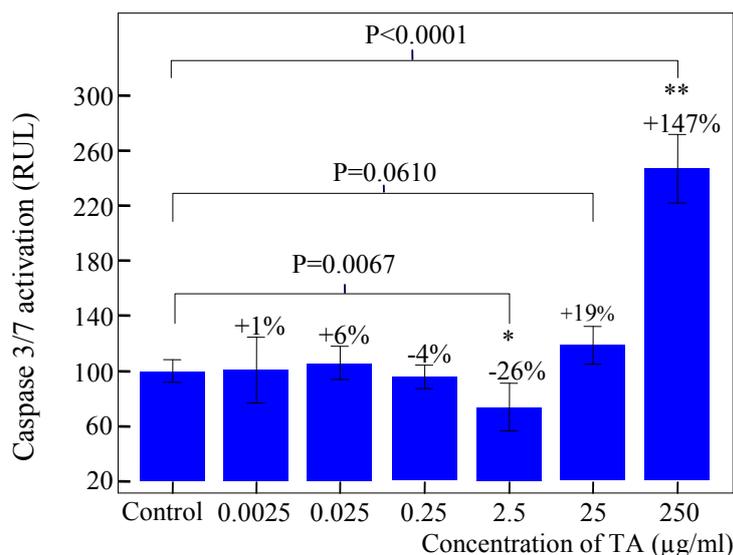


Figure 21: The Effect of different concentrations of TA (0.0025, 0.025, 0.25, 2.5, 25 and 250 µg/ml) in relative Caspase 3/7 activation. Values shown as mean \pm SD (n=8). Statistical difference shown as $P<0.05$ [*] and highly significant $P<0.0001$ [**]. A highly significant trend (ANOVA trend analysis: $P=0.0001$) could be established.

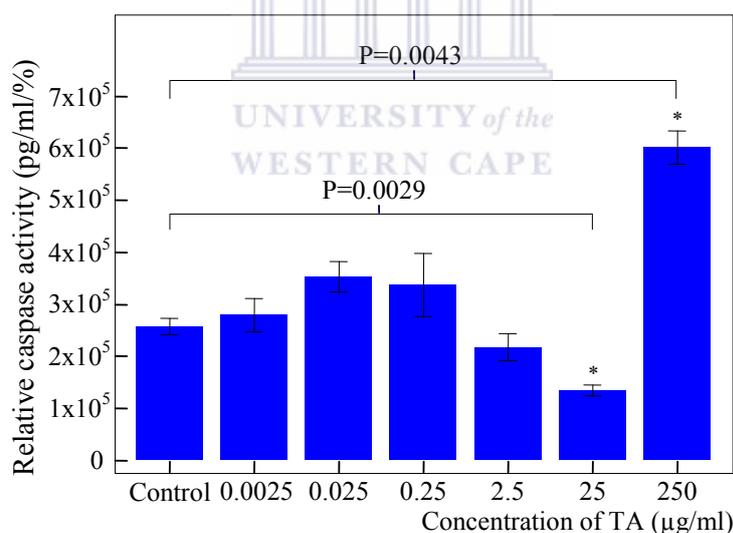


Figure 22: The Effect of different concentrations of TA (0.0025, 0.025, 0.25, 2.5, 25 and 250 µg/ml) in relative Caspase 3/7 activation. Values shown as mean \pm SD (n=8). Statistical difference shown as $P<0.05$ [*] and highly significant $P<0.0001$ [**]. A highly significant trend (ANOVA trend analysis: $P=0.0001$) could be established. Effect of on Relative Caspase 3/7 activation (Caspase 3/7 related to viability [µg/ml/%]) shows significant increase ($P=0.0043$) activation at the highest concentration with significant dose dependence (ANOVA trend analysis: $P=0.0299$).

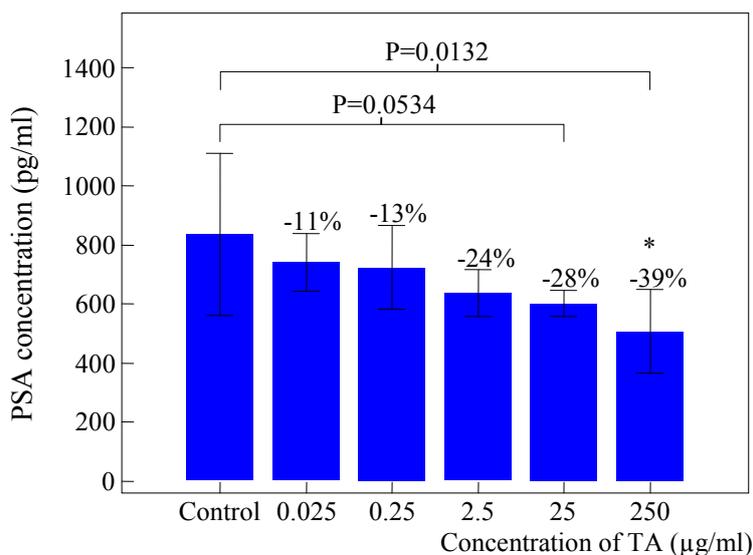


Figure 23: The effect of TA (0.025, 0.025, 0.25, 2.5, 25 and 250 µg/ml) on PSA concentration in LNCaP cells *in vitro*. Values are shown as mean \pm SEM (n=8), increase [+] or decrease [-] and statistical significance at $P < 0.05$ [*]. At the highest TA concentration (250 µg/ml) a 39% significant decrease ($P = 0.0132$) is depicted. At concentration 25 µg/ml only a borderline significance ($P = 0.0534$) is reached at a decrease of 28%. A highly significant trend (ANOVA trend analysis: $P = 0.0055$) in a concentration dependant manner could be established.

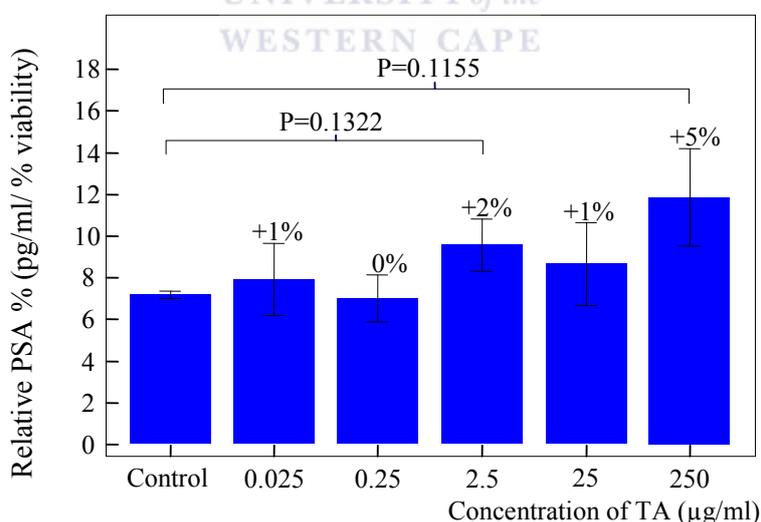


Figure 24: The effect of TA (0.025, 0.025, 0.25, 2.5, 25 and 250 µg/ml) on the relative PSA concentration in LNCaP cells *in vitro*. Values are shown as mean \pm SEM (n=8), increase [+] or decrease [-] and statistical significance at $P < 0.05$ [*]. The related PSA concentration PSA % shows no change. Only a 5% increase is depicted at the highest concentration. ANOVA trend analysis revealed no significance ($P = 0.0768$).

Chapter 4

Discussion

The use of herbal medicines as alternative treatments has become increasingly popular throughout the world as conventional medical approaches are either failing to produce positive treatment results or are too expensive to afford for countless people, particularly in poorer countries. Herbal medicines are also perceived by the public to have lesser and relieve side effects, boost the immune system, or prolong life (Wirth et al., 2005) as compared to conventional medicine. Thus, many people are turning back to natural remedies, especially herbal medicine, as a solution for maintaining health and for the treatment of disease (Castleman, 1995). The World Health Organization (WHO, 2002) estimates that 80% of the people in developing countries of the world rely on traditional medicine for their primary health care, and about 85% of traditional medicine involves the use of plant extracts (Farnsworth, 1988).

Between 1960 and 1982, the National Cancer Institute screened around 114,000 extracts from an estimated 35,000 plant samples for anticancer activity (Cragg, 1993). This screening then initiated a further screening program of 28,800 plant samples collected from over 20 countries to investigate anticancer activity in human cancer cell lines during 1987 and 1991 (Cragg, 1993). The increasing popularity of the use of phytotherapy-based medicines as preventive medicines or for health management has also driven research (Raskin et al. 2002) and plant-derived anti-cancer drugs that are used in standard chemotherapeutic regimens for various forms of cancers could be attributed to bioactive plant-derived anti-cancer compounds such as Vinca alkaloids, epidophyllotoxins, camptothecins and taxanes (Cragg and Newman, 2005). However, there is still inadequate scientific evidence of the health benefit, efficacy, safety and mechanism of cancer cell death resulting from their consumption. Furthermore, highly malignant and resistant forms of cancers are increasing and the majority of the available drugs have limited anti-solid tumor activity (Parasnis, 2004). Therefore, there is a need for more effective anticancer agents, which has prompted investigators to explore new sources of pharmacologically-active compounds, especially from natural products.

In Malaysia, *Eurycoma longifolia* Jack (Tongkat Ali, TA) is commonly used in traditional medicine against a variety of diseases including male reproduction and aging males' problems (Ang et al., 1995; Chan et al., 1995; Ang and Sim, 1997; Hamzah and Yusof, 2003). After pharmacological evaluation it has been shown to possess cytotoxic properties for various cancer cell lines (Morita et al., 1990; Kardono et al., 1991; Itokawa et al., 1992; Morita et al., 1993) largely attributed to eurycomanone, a compound of the quassinoid group (Ang et al., 2000; Jiwajinda et al., 2002).

Previous studies of eurycomanone on Hela cells and MCF-7 breast cancer cells revealed apoptotic effects (Nurkhasanah et al., 2008; Cheah and Azimahtol, 2004). Yet, in another investigation eurycomanone showed minimum effect on non-cancerous, Mardin-Darby Bovine Kidney MDBK cells and kidney epithelial cells, Vero cells (Nurhanan et al., 2005) and non-cancerous breast cells (MCF-10A) (Cheah and Azimahtol, 2004). An extract and some fractions of *Eurycoma longifolia* had cytotoxic effects against two mammalian cell lines Vero and human skin fibroblast cells Hs27. Cytotoxic effects of *Eurycoma longifolia* extracts have been shown in the human cell lines Hep2 and HFL1 (Mohd-Fuat et al., 2007).

More recently, an investigation with an aqueous extract of TA on TM3-Leydig and TM4-Sertoli cells showed its selective cytotoxicity to non-cancerous cells (Erasmus, 2012). However, considering that TA has conflicting cytotoxic mechanisms of action for selective cells, TA boosts testosterone that prostate problems including prostate cancer are aging males' problems and TA can be used to treat these, no study has been performed on androgen-dependant human prostate cancer.

4.1 Determination of suitable *in vitro* concentrations of *Eurycoma longifolia* aqueous extract for LNCaP prostate cancer cells

In the studies performed by Zakaria (2009) on HepG2 cells and Nurhanan (2005) on KB, DU-145, RD, MCF-7, CaOV-3, MDBK cell lines to determine the cytotoxic effect of TA, the findings indicated that the extract exerted cytotoxic effect with an IC_{50} of ≤ 20 $\mu\text{g/ml}$. Therefore, in the current study a final concentration of 250 $\mu\text{g/ml}$ was serially diluted in ten fold to concentrations 0.0025, 0.025, 0.25, 2.5, 25 $\mu\text{g/ml}$

to establish a cut-off value, which includes the range of IC₅₀ and dosages which could be comparative to an effect of resistant cancer tumors. In addition, a clinical trial performed by Shaiful et al. (2012) concluded that Physta[®] freeze-dried water extract of TA is tolerable and safe at an administered dose of 300 mg per individual per day. On this base, the concentrations used for the current study are within the range required for experimentation as *in vivo* concentrations are about 10 times higher in comparison to *in vitro* concentrations.

4.2 Cytotoxic effect of Tongkat Ali on LNCaP cell viability

Early stage prostate cancer distinctively relies on androgens for proliferation, and the blocking of the androgen receptor (AR) pathway induces tumor regression (Peehl, 2005). However, as prostate cancer progresses into later stages, the cells become androgen-independent, and no restorative therapy exists for this now unmanageable disease (Molina and Beldegrun, 2011). Reactivation of the AR coordinates the procession and plays an important role (Heemers et al., 2009). Several mechanisms could describe this procession namely; (i) the hyperactivation of AR pathway could result from hypersensitivity to androgen, (ii) irregular androgen synthesis, (iii) abnormal activation by co-regulator alterations, or (iv) alternative pathways, such as growth factors, receptor tyrosine kinases (Yamaoka et al., 2010; Cai et al., 2011).

In the present study, the viability of androgen-sensitive LNCaP human prostate cancer cells was tested after exposure to different concentrations of TA using the MTT assay by the cell's mitochondrial dehydrogenase (Mosmann, 1983). For practical measures, 24 hours of exposure was related to early stage prostate cancer and a 96-hours exposure as late stage. After 24 hours of exposure, the results showed an initial increase in the percentage of cellular viability at lower dosages of TA. Yet, at higher concentrations of TA (250 µg/ml) during the same 24-hour period, viability significantly decreased (P=0.0226), which clearly indicates cellular death. This increase in viability must be revealed to as cellular stress number of cells did not and proliferation. However, after 96 hours of exposure, all concentrations revealed a decrease, in cell viability and at highest concentration (250 µg/ml) 53% of the cells were dead.

This finding leads to establishing that TA's androgenic properties does not play a role in further stimulation and proliferation of late stage prostate cancer but rather as a possible antigrowth agent. In addition, as the cells' mitochondrial dehydrogenases were used to test the viability, other possible pathways could have factored into the cell's inhibition to proliferate. This may be supported by previous investigations of the cytotoxic effect of TA to cancer cells (Nurhanan et al., 2005; Nurkhasanah et al., 2009; Zakaria et al., 2009). These studies concluded that cells treated with eurycomanone showed obvious morphological changes, including the loss of adhesion, rounding, and sporadically distribution and there was clear concentration-dependent response, indicating the possibility of apoptosis (Nurkhasanah et al., 2009). A general consensus could be drawn that the antiproliferative and cytotoxic effect may be contributed by apoptotic pathways, an assumption which was investigated in the current study.

4.3 Effect of Tongkat Ali on LNCaP cell release of PSA

LNCaP cells are one of the best *in vitro* models for human prostate cancer studies because they possess an aneuploid male karyotype, produce PSA, and are responsive to androgenic stimulation (Hsieh et al., 1997). Both, normal and malignant prostate epithelial cells produce PSA (Balk et al., 2003). PSA is produced by the secretory epithelial cells in the acini and ducts, and secreted directly into the lumen of the prostate gland (Henttu et al., 1992; Yousef and Diamandis, 2001). In prostate cancer, a distinctive initial trait is the disruption of the basal cell layer and basement membrane allowing the leakage out into the lumen and serum (Brawer et al., 1989; Bostwick et al., 1994). This leakage of PSA is used as a sensitive and specific tumor biomarker for prostate cancer screening and assessment (Stamey et al., 1989a, 1989b and 1989c) and is therefore regarded oncological indicator of disease and response to prostate cancer therapy (Cadeddu et al., 1998).

In the current study, PSA production by LNCaP cells showed decreased concentrations in a dose-dependent manner when exposed to TA. In the past, trials have shown a direct relationship between a decline in PSA and the shrinkage of prostate cancer (Brausi et al., 1995). A reduction in serum PSA levels has also been suggested to be an endpoint biomarker for hormone-refractory human prostate cancer intervention (Lilja et al., 1987; 2008). A positive indication may be brought to light

that TA may be responsible in disrupting the tumor cell membrane as the cell viability of the current investigation also indicates a similar dose-dependent decrease signalling cell death response. However, in effect, as only viable cells can produce PSA, the decline in the PSA concentration is due to cell death, and might indicate shrinkage of a prostate cancer tumor. On the other hand, the relative PSA concentration remained the same, without significant change, indicating that the cellular PSA production and therefore the physiology of the cells did not change.

Zi and Agarwal, (1999) suggested a mechanism by which TA decreased PSA level in LNCaP cells could decrease by milk thistle, *Silybum marianum* extract. The seeds of milk thistle contain silymarin, a polyphenol composed mainly of silibinin, with small amounts of other stereoisomers isosilybin, dihydrosilybin, silydianin, and silychristin (Wagner et al., 1974). Silibinin has been shown to decrease intracellular and secreted levels of PSA in human prostate cancer LNCaP cells under both serum- and androgen-stimulated conditions associated with inhibition of cell growth via a G1 arrest in cell cycle progression, the G1 arrest by silibinin does not lead to apoptosis but causes neuroendocrine differentiation of the cells (Zi and Agarwal, 1999). Based on these findings it was concluded that silibinin has a strong potential to be developed as an antiproliferative differentiating agent for the intervention of hormone-refractory human prostate cancer (Zi and Agarwal, 1999).

Nevertheless, the use of PSA screening remains contentious as the value is not relative for all men as quoted by Lilja et al. (2008) "...standard serum total PSA tests lack the sensitivity and specificity to detect a large fraction of early-stage tumors". However, PSA biology in normal prostate and prostate cancer still promises to improve prostate cancer detection.

4.4 Effect of Tongkat Ali on LNCaP Cells viability in the presence or absence of testosterone

Prostate cancer is an androgen-dependent tumor and testosterone administration is thought to stimulate its growth (Holmang et al., 1993). Thus, the Endocrine Society Clinical Practice Guidelines (Bhasin et al., 2010), recommend testosterone replacement therapy (TRT) as a contraindication for prostate cancer patient. However, this concept is based on the study nearly 62 years ago, performed by Huggins and

Hodges (1941) On the other hand, these reports were severely criticized for being flawed (Morgentaler et al., 2006; Morgentaler et al., 2010). Yet, several studies failed to establish a clear link between an increased risk of prostate cancer development, progression and recurrence in patients treated with testosterone (Isbarn et al., 2009; Traish et al., 2011).

Nevertheless, no scientific evidence for the claim that testosterone replacement therapy (TRT) is triggering or supporting prostate cancer has been reported, thus far. Considering that *Eurycoma longifolia* extract is increasing the serum testosterone concentrations, there might be a potential risk that a TA treatment of elderly men might cause prostatic problems. On the other hand, the randomized, double-blind, placebo-controlled clinical trial by Ismail et al. (2012) revealed no difference between the placebo and the verum group for serum PSA levels and the question remains what the protective status of these patients was. In addition, there are indications that the aqueous extract of TA has cytotoxic activity on several cancer cell lines (Nurhanan et al., 2005; Tee et al., 2007; Zakaria et al., 2009; Wong et al., 2012; George & Henkel, 2014). If this anti-cancer activity of TA would be confirmed, there might be the possibility that both testosterone “normalizing” and anti-proliferative activities could be combined in this natural product, providing an excellent treatment option for aging males’ symptoms in terms of a herbal hormone replacement therapy.

The 2006 Clinical Practice Guidelines by the Endocrine Society states “Testosterone therapy in adult men with androgen deficiency syndromes” suggest that the so-called late-onset hypogonadism should be regarded as a clinical and biochemical state with rising age, characterized by particular symptoms such as loss of libido and erectile dysfunction, together with a low level of serum testosterone (Bhasin et al., 1995). The authors advise not to treat androgen deficiency in general, but do not advise against the treatment of older men with low testosterone and clear symptoms of hypogonadism (Bhasin et al., 1995). The guidelines and the evidential basis for the diagnosis and treatment of low testosterone in elderly men have, however, been debated and a more clear differentiation between age-related hypotestosteronemia and late-onset hypogonadism is warranted (Shames et al., 2007; Handelsman, 2011).

Testosterone deficiency in older men can result in clinical manifestations that have also been described in hypogonadal younger men. They include progressive decrease in muscle mass and strength, osteoporosis, fatigue and depression, loss of libido, and erectile dysfunction (Vikan et al., 2009; Roddam et al., 2008). With regard to the prostate, exogenous testosterone administrations is thought of stimulating growth of prostate cancer (Holmang et al., 1993) and worsen symptoms of benign prostatic hypertrophy (Siiteri & Wilson, 1970) as prostate cancer is androgen-dependent tumor.

This failure to find a distinct link between prostate cancer growth and serum testosterone levels has recently been explained with the concept of a saturation model, according to which serum androgen levels below a not yet clearly defined point of maximum testosterone binding to the androgen receptor in prostate cells will result in considerable changes in prostate cancer growth. Once a maximum binding is achieved, further increased androgen concentrations will have no or only little effect (Morgentaler & Traish, 2009).

In the present study, testosterone at different concentrations (1 nM-1000 nM) was added to different dosages of TA (0.0025–250 µg/ml) to investigate whether testosterone modulates LNCaP cell proliferation. The data collected suggest that testosterone in conjunction with ethanol only decreases the viability of the cells. In addition, the combination of testosterone with TA for both periods, 24 and 96 hours, only a slight increase in cell proliferation at lower concentrations of TA was observed, especially 10 nM testosterone showed modulation of potential optimal growth stimulation.

However, at higher dosages of TA and testosterone, viability decreased drastically and clumping caused by apoptosis was seen. Thus, these findings may support reports by Morgentaler et al. (2006, 2010) that testosterone does not increase the growth of prostate cancer. In this current case, even though testosterone was added as a modulator, no rapid growth of prostate cancer was found and thus with TA it may again be supported as a maintainer or restorer of testosterone (Chaing et al., 1994; Talbott et al., 2013; Erasmus, 2013). Nevertheless, it is recommended that patients showing elevated levels of prostate-specific antigen (PSA), increased PSA velocity or suspicious rectal examination results undergo a prostate biopsy (McGill et al., 2012).

In the present investigation, the relative PSA concentration did not increase with the treatment of TA.

This study is also the first that investigated the effects of TA supplementation on parameters that have to be watched during testosterone replacement therapy as they represent possible contraindications for a therapy that increases serum testosterone levels, which could lead e.g. to muscle damage in physically active individuals (Tambi & Imran, 2010; Tambi et al., 2012; Henkel et al., 2013).

The conventional testosterone replacement therapy was found to improve mood and well-being, and reduce fatigue and irritability in hypogonadal men (Wang et al., 1996; Lunenfeld & Nieschlag, 2007). TA has recently been recognized as a traditional remedy in late-onset hypogonadism, an age related decline in serum testosterone levels affecting quality of life in men (Tambi et al., 2012) as well as an alternative treatment for idiopathic male infertility (Tambi & Imran, 2010). Proper clinical studies on the safety and efficacy of Tongkat Ali treatment were not established until recently (Tambi et al., 2012; Ismail et al., 2012).

With regards, to the psychological effects of TA, Ang and Cheang (1999) have demonstrated the anxiolytic effect of this herbal extract in mice. In the human, a randomized placebo-controlled study including 32 men and 32 women was conducted by Talbott et al. (2013). The authors showed significant improvements in moderately stressed subjects. All mood parameters such as tension, anger and confusion improved significantly. This is thought to be due to changes in the hormonal profile as testosterone levels increased and cortisol levels decreased leading to a significantly improved cortisol testosterone ratio in the TA group.

A recent study on the oral administration of a quassinoid-rich *Eurycoma longifolia* extract in male rats has shown that the hypothalamic-pituitary-gonadal axis was triggered to decrease plasma oestrogen, increased testosterone, luteinizing hormone and follicle stimulating hormone levels (Low et al., 2013). Thus, the effect of TA on the testosterone and oestrogen release may explain the *in vivo* fertility improvement, testosterone elevation and anti-oestrogenic properties of *Eurycoma longifolia* as previously reported (Chan et al., 2009; Zanolli et al., 2009; Abdulghani et al., 2012).

The increase of the testosterone level following the administration of the plant extract. However, has provoked warning on the safety of chronic consumption of the plant (Bosland, 2000).

Tongkat Ali is known to advance quality of life by improving vitality, physical activity and a sense of general wellbeing (Lana et al., 2006), has an anti-aging effect seen in the Aging Male Score (AMS) that are attributed to testosterone supplementation (Morley et al., 1997; Lunenfeld & Nieschlag, 2007). Taken together, based on the current findings, it appears that Tongkat Ali may be a safer and cheaper alternative treatment of aging males' symptoms for the negative effects of testosterone deficiency. Nevertheless, further studies have to confirm safety and clinical indications for such treatment. A significant advantage will be the form of administration as this herbal remedy is available in capsules.

4.5 Apoptotic effect of Tongkat Ali on LNCaP prostate cancer cell

Apoptosis is an active physiological process of programmed cell death, resulting in cellular self-destruction that involves specific morphological and biochemical changes in the nucleus and cytoplasm (Mans et al., 2000) It can be characterized by cell shrinkage, chromatin condensation inter-nucleosomal DNA fragmentation, and the formation of apoptotic bodies (D'Agostini et al., 2005). Apoptosis can further be classified into two fundamental pathways: (1) the mitochondrial or intrinsic pathway; and, (2) the death receptor or extrinsic pathway (Yoon and Gores, 2002). It can be even further being determined as early or late on set apoptosis (Chiu et al., 2003).

Apoptosis is arguably one of the most potent forms of defence against cancer (Ghavami et al., 2009). In cancer therapy, one approach that suppresses the tumor growth is by activating the apoptotic machinery in the cell (Lowe and Lin, 2000) due to the effect of anticancer agents (Kerr et al., 1994). Therefore, the induction of apoptosis has been recognized as a strategy for the identification of anticancer drugs (Powell et al., 2003). There is substantial evidence that alteration in the cellular and molecular pathways that control the cell cycle and apoptosis may change the sensitivity and resistance to anticancer agents (Pezzuto, 1997). Apoptotic agents that suppress the proliferation of malignant cells by inducing apoptosis may represent a

useful mechanistic approach to both, cancer chemoprevention and chemotherapy (Alshatwi et al., 2011).

While many anticancer agents have been developed, unfavorable side effects and resistance are serious problems (Khan and Mlungwana, 1999). Thus, there is growing interest in the use of plant materials for the treatment of various cancers and the development of safer and more effective therapeutic agents (Panchal, 1998). These plant extracts often contain phytoestrogens. Phytoestrogens possess antioxidant activity, and, therefore, possess the potential for exerting an influence on hormone-dependent cancers including prostate cancer, which may also contribute to cell growth (Adlercreutz et al., 1986; Morton et al., 1997). Two groups of phytoestrogens, polyphenol flavonoid antioxidants and lignans, are receiving attention for the prevention and intervention of human cancers including prostate cancer (Adlercreutz et al., 1986; Morton et al., 1997; Sun et al., 1998).

In the present study, androgen-dependant LNCaP human prostate cancer cells were treated with TA. As it is known, TA's cytotoxic properties, attributed to its eurycomanone content or high similarity of quassinoids (Kardono et al., 1991) are more dominantly displayed as an anticancer promoter. The antitumor activity is one of the most impressive medicinal properties of quassinoids and has been well researched (Jiwajinda et al 2002; Miyake et al 2010). Many quassinoids display antitumor activity in different potencies (Guo et al., 2005). The mechanism of action is believed to be that quassinoids can inhibit protein synthesis by inhibition of the ribosomal peptidyl transferase activity (Hall et al., 1982; 1983).

In the current investigation, the detection of early signs of apoptosis using Annexin V Cy3-binding and late signs of apoptosis using DNA fragmentation (TUNEL assay) were determined in order to establish specific indications of the mechanism of action or pathways involved in anti-proliferation and anticancer properties. Annexin V is a Ca^{2+} -dependent phospholipids binding protein that detects phosphatidylserine externalization of the plasma membrane (Vermes et al., 1995). This process is related to apoptosis and signals for engulfment by phagocytes (Fadok and Henson, 2003). When LNCaP cells were exposed to TA, Annexin V-Cy3 stained cells exhibited morphological changes displaying punctuated cells, typical of apoptotic cells. In

addition, after 24 hours of treatment with TA cells showed an increase in the percentage of DNA damaged cells of 32% at highest concentration of TA (250 µg/ml) and 40%, after 96 hours of incubation. This confirms that TA induces early signs of apoptosis after 24 hours, but cell death takes longer. This strongly correlates to the study performed by Nurkhasanah et al. (2008) after exposure of Hela cells with eurycomanone, the percentage of apoptotic cells in the annexin +/PI- quadrant increased from 24 to 48 hours of exposure, thus indicating that eurycomanone induced apoptosis in Hela cells.

Furthermore, it was found that treatment with TA induced DNA fragmentation by (TUNEL) assay in LNCaP cells as well, in a time- and concentration-dependant manner. This assay is widely used for detection of DNA fragmentation in the investigations previously mentioned on HepG2, CaOV3, Hela, A549, and MCF-7 (Kuo et al., 2004; Tee et al., 2007; Zakaria et al., 2009).

Caspase 3/7 activity was also determined after LNCaP cells were treated with TA and shown to increase in a dose-dependent manner with significant increase at the highest concentration (250 µg/ml). This suggests the extrinsic pathway of apoptosis is initiated by TA. Therefore, it can be said that there are ligand interactions that sequentially lead to cleavage of initiator Caspase 8, which cleaves executioner Caspase 3, resulting in apoptosis (Scaffidi et al., 1998), and eventually cell death. Caspase-3 is a downstream adaptor caspase which can be proteolytically activated by caspase 8 via mitochondrial or cell death receptor signaling pathways (Cohen, 1997; Zhang et al., 2004; Hsu et al., 2008).

4.6 Conclusion and further outlook

Data accumulated from this research propose that dietary phytochemicals, like Tongkat Ali have the potential to manage deregulated signaling pathways or restore checkpoint pathways of apoptosis in cancer. Biological activities of Tongkat Ali show a promising future in treatment of prostate cancer. However, this requires further investigation to figure out the mode of action of Tongkat Ali and additional research to determine the amount of regular intake. In addition, the type of treatment plan with Tongkat Ali for the patients, aggressive or prolonged, will have to be considered.

In summary, the major finding of this study is that Tongkat Ali exhibits cytotoxicity on LNCaP prostate cancer cells through a caspase-mediated pathway, leading to DNA fragmentation and eventually cell death. In addition, Tongkat Ali treatment also significantly decreases the PSA concentration in the medium of LNCaP cells. These data suggest that changes in serum PSA levels in individual patients during the Tongkat Ali treatment would not be an effect specific for PSA secretion, as treated LNCaP cells, when incubated with Tongkat Ali, did not decrease the PSA secretion. The results presented here, rather show by analogy that constant or decreasing serum PSA levels would be indicative of decreasing size and /or number of prostate cancer tumor cells. Thus, Tongkat Ali treatment might lead to a shrinkage or, at least, to a non-progression of a tumor.

Thus, a treatment of patients with Tongkat Ali or its extract might not only be beneficial to patients in terms of prostate cancer, but also with regard to a testosterone replacement therapy in elderly or hypogonadal men as this treatment combines two effects, boosting or restoring testosterone secretion and anti-proliferative effects on the prostate. In fact, recent clinical and scientific evidence has shown that not all prostate cancer or benign prostatic hyperplasia originated from elevated testosterone levels (Morgentaler, 2006). As suggested by George and Henkel (2014), this opens new treatment perspectives for aging men suffering from aging males' symptoms and who might be at risk for the development of a prostate cancer. However, more *in vitro* and *in vivo* studies are needed to identify the active constituents of the extract including their mechanism of action within the prostate gland as well. The final goal can then be the development of standardized Tongkat Ali preparations to be used in prostate cancer prevention or as a supplementary treatment to standard prostate cancer therapy. A significant advantage will be the form of administration as this herbal remedy is already available on the market in capsules.

Chapter 5

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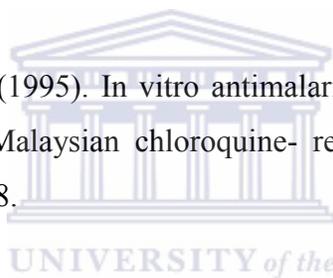
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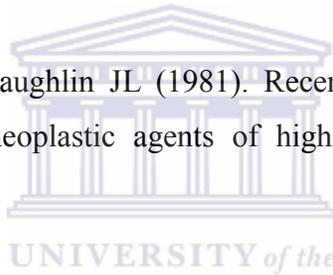
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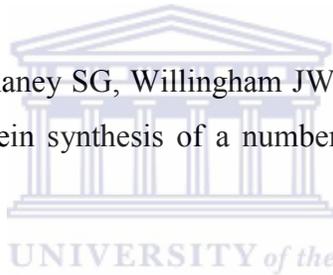
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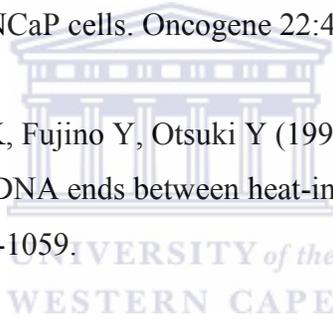
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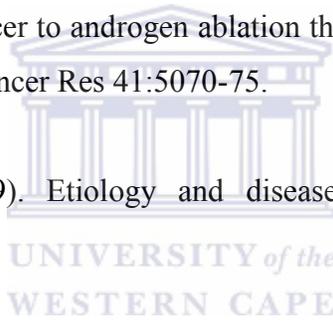
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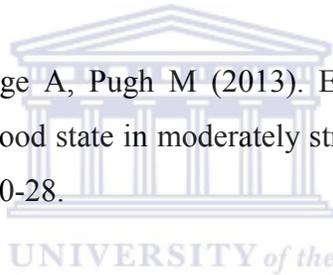
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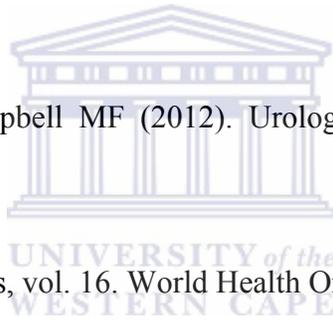
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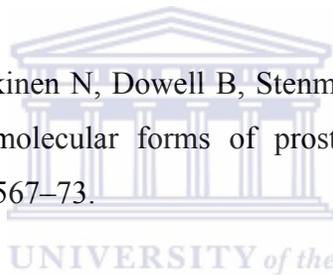
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