



UNIVERSITY *of the*
WESTERN CAPE

**AN IN VITRO INVESTIGATION OF THE EFFECTS OF
CAMELLIA SINENSIS AND *ASPALATHUS LINEARIS* ON
BENIGN (RPWE 1) AND MALIGNANT (LNCaP)
PROSTATE CELL LINES**



by

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

Supervisor: Prof. T. Monsees

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DECLARATION

I declare that an in vitro investigation into the effects of *Camellia sinensis* and *Aspalathus linearis* on benign (RPWE 1) and malignant (LNCaP) prostate cells is my own work and has never ever been submitted before for any degree or examination in any other university and that all sources I have used or quoted have been indicated and acknowledged as complete references.

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.....

Date

.....

DEDICATION.

This thesis is dedicated to my late parents; late Douglas (Snr) and Gladys you built me into a man I am today. Where I went astray you did not tire to guide me. To the best of your abilities you provided for me and supported my every part of life. May the almighty God have mercy on your spirits.



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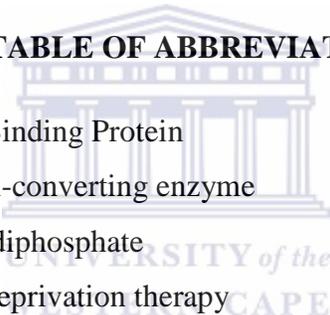
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ABP	Androgen Binding Protein
ACE	Angiotensin-converting enzyme
ADP	Adenosine diphosphate
ADT	Androgen deprivation therapy
AIF	Apoptosis inducing factor
ANT	Adenine nucleotide translocator
AR	Androgen receptor
AREs	Antioxidant response elements
ASP	Aspalathin
ASR	Age Standardised Rate
ATCC	American Type Culture Collection
ATP	Adenosine Triphosphate
B[a]P	Benzo[a]pyrene
BPH	Benign Prostatic Hyperplasia
BTE	Black tea extracts
BT	Black tea
CAT	Catalase

CCl₄	Carbon Tetrachloride
CDK	Cyclin-Dependent Kinase
c-FLIP	Cellular FLICE-like inhibitory protein
CoQ10	Coenzyme Q10
COX-2	Cyclooxygenase
CpG	Cytosine-Phosphate-Guanosine
CRN	Creatinine
CRPC	Castrate Resistant Prostate Cancer
Cu/ZnSOD	Copper Zinc Superoxide Dismutase
Cyp P450	Cytochrome P450 enzyme
CypD	Cyclophilin D
DAPI	4',-6-Diamidino-2-phenylindole dihydrochloride
DCF	2'-7'dichlorofluorescein
DHT	Dihydrotestosterone
DIABLO	Direct IAP Binding protein with low pI
DISC	Death inducing signaling complex
DMSO	Dimethylsulphoxide
DMBA	7, 12-dimethylbenz[a] anthracene
DNA	Deoxyribonucleic acid
DR	Death Receptors
EDTA	Ethyl Diamine Tetra Acetic acid
E2F	Transcription factor
EGCG	Epigallocatechin gallate
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbant assay
ER	Estrogen receptor
ERK2	Extracellular signal-regulated kinases 2
FADD	Fas associated death domain
FBS	Foetal bovine serum
FeO²⁺	Ferryl ion

cFLIP	Cellular FLICE-like inhibitory protein
FR	Fermented rooibos
Glu-P-1	2-Amino-6-methyldipyrido[1,2- a:3',2'-d]imidazole
GPx	Glutathione peroxidase
GSR	Glutathione reductase
GST	Glutathione S-transferase
GT	Green tea
GTA	General transcriptional apparatus
GTCs	Green tea catechins
GTE	Green tea extracts
GTPs	Green tea polyphenols
H₂DCF	2'-7' dichlorofluorescein
H₂O₂	hydrogen peroxide
HOBr	hypobromous acid
HOCl	hypochlorous acid
HPV-18	human papilloma virus-18
HtrA2	Omi/high temperature requirement protein A
IAP	Inhibitor of apoptosis
IGF-I	Insulin-like growth factor-I
JNK1	c-Jun N-terminal kinases
Keap1	Kelch-Like ECH-Associated Protein 1
LNCaP	Malignant prostate cell lines
LPS	Lipopolysaccharide
MAPK	Mitogen activated protein kinase
MMPs	Matrix metalloproteases
MnSOD	Manganese superoxide dismutase
MPT	Mitochondrial permeability transition
MTT	Thiazoyl blue tetrazolium bromide
NF-κB	Nuclear Factor-κap Beta
NMU	Nitrosation of methylurea.

NO	Nitric oxide
NO₂	Nitrogen Dioxide
NQO	Quinone oxidoreductase
Nrf2	Nuclear factor erythroid 2-related factor 2
O₂•-	Superoxide radicals
O₂•-	Superoxide anion radicals
O₃	Ozone
¹O₂	Singlet oxygen
OH•	Hydroxyl ion
PBS	Phosphate Buffered Saline
PERK	Protein kinase RNA-like endoplasmic reticulum kinase
PKC	Protein kinases C
PS	Phosphatidyl serine
PSA	Prostate specific antigen
PT	Permeability transition
PTEN	Phosphatase and tensin homolog
Rb	Retinoblastoma
ROO•	Peroxyl radicals
ROS	Reactive oxygen species
RWPE-1	Human prostate epithelial cell line
SCID	Severe combined immunodeficiency
SHBG	Sex-hormone binding globulin
Smac	Second mitochondria-derived activator of caspase
SOD	Superoxide dismutase
STAT 3	Signal transducer and activator of transcription 3
TNF	Tumor necrosis factor
TPA	12-O-tetradecanoylphorbol-13-acetate
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand
TrxR	Thioredoxin reductase
uPA	Urokinase plasminogen activator

UR	Unfermented rooibos
VDAC	Voltage dependent anion channel
VEGF	Vascular endothelial growth factor
WAFI	cyclin-dependent kinase inhibitor p21



ABSTRACT

The prostate is prone to three pathological processes that include inflammation, benign prostate hyperplasia (BPH) and tumors. According to the center for Disease and Control 1999-2012 report, prostate cancer is the second leading cause of death in the United States. Scientific evidence suggests that up to 30% of men in the general population aged from 50 years and above, irrespective of geographic origin, have foci

of prostate neoplastic growth. Unbalanced ROS production and a dysregulated antioxidant defence system have been implicated in prostate cancer development.

The transformation of a normal cell into cancer takes a very long period. This observation provides the advantage of using nutraceuticals to prevent, arrest or reverse the cellular and molecular processes of carcinogenesis.

Based on scientifically observed positive health roles of green tea (*Cameli sinensis*) and rooibos (*Aspalathus linearis*) on major diseases like atherosclerosis, hepatitis and certain types of cancer, this thesis evaluated the effects of these two teas on benign (RPWE 1) and malignant (LNCaP) prostate cells. This was done through the quantification of reactive oxygen species (ROS) using a fluorescence dye 5,6 CM-H₂DCFDA, total prostate specific antigen (PSA) levels using a PSA ELISA kit, cell viability using the MTT assay, apoptosis using Tali annexin V stain and cell imaging studies using a Zeiss axiovert 200M inverted fluorescence microscope. Statistical analysis was done using graphpad prism.

The findings of this study show that aqueous extracts of green and black tea, fermented and unfermented rooibos and their active compounds epigallocatechin gallate (EGCG) and aspalatin, respectively, are cytotoxic in malignant (LNCaP) prostate cells but exert protective effects in benign (RPWE 1) prostate cells. This thesis implicates the pro-oxidant and anti-oxidant properties of the plant extracts, respectively, for the above mentioned effects. In this regard, tea and rooibos promoted ROS production in malignant (LNCaP) prostate cells, which subsequently promoted cell death of the malignant cells through apoptosis and necrosis. Further to this, tea and rooibos used in this thesis, protected normal prostate cells from the adverse effects of ROS. In this regard, fluorescence microscope photographs showed RPWE 1 cells with low DCF fluorescence compared to the malignant prostate cells. Low magnification light microscope photographs showed RPWE 1 cells with flat polygonal shapes and increased adherence both at low and high concentrations of tea and rooibos. On the contrary, high concentrations of tea and rooibos on malignant

(LNCaP) prostate cells induced stress, which made the cells attain irregular shapes and as the stress levels increased, cells became detached and appeared dead. Flow cytometry confirmed the presence of apoptotic and necrotic cell in malignant (LNCaP) prostate cells. In this thesis, EGCG and aspalathin were responsible for the high rates of apoptosis observed whereas green tea and unfermented rooibos induced the highest rate of necrosis. Further to this, tea and rooibos and the main active compounds EGCG and aspalathin, respectively, significantly promoted the reduction of total serum prostate specific antigen (PSA) in malignant prostate cells. In normal prostate cells, these plant extracts maintained the total serum PSA at its basal physiological level. In this thesis, to the best of our knowledge, we report for the first time the cell-specific effects of fermented rooibos, unfermented rooibos and their main active component aspalathin, on prostate cancer cells. We showed that rooibos and aspalathin exert pro-oxidant effects on malignant LNCaP cells and anti-oxidant effects on benign RPWE 1 cells.

In conclusion, tea (*C. sinensis*) and rooibos (*A. linearis*) and their respective main active compounds, epigallocatechin gallate and aspalathin, are cytotoxic to malignant prostate cells whereas in normal prostate cells, they have protective effects against ROS induced stress. The pro-oxidant and anti-oxidant effects are responsible for the aforementioned effects respectively. The decrease in total serum PSA demonstrate the strong therapeutic effects that tea and rooibos have on malignant (LNCaP) prostate cells.

CHAPTER ONE

LITERATURE REVIEW

1. INTRODUCTION

1.1 Prostate Anatomy and Function

The prostate gland is a walnut-sized gland located between the bladder and pelvic floor in men. The upper portion of the urethra passes through the middle of the prostate gland. The bottom of the prostate gland is closely associated with the urinary sphincter, which is responsible for controlling urine flow.

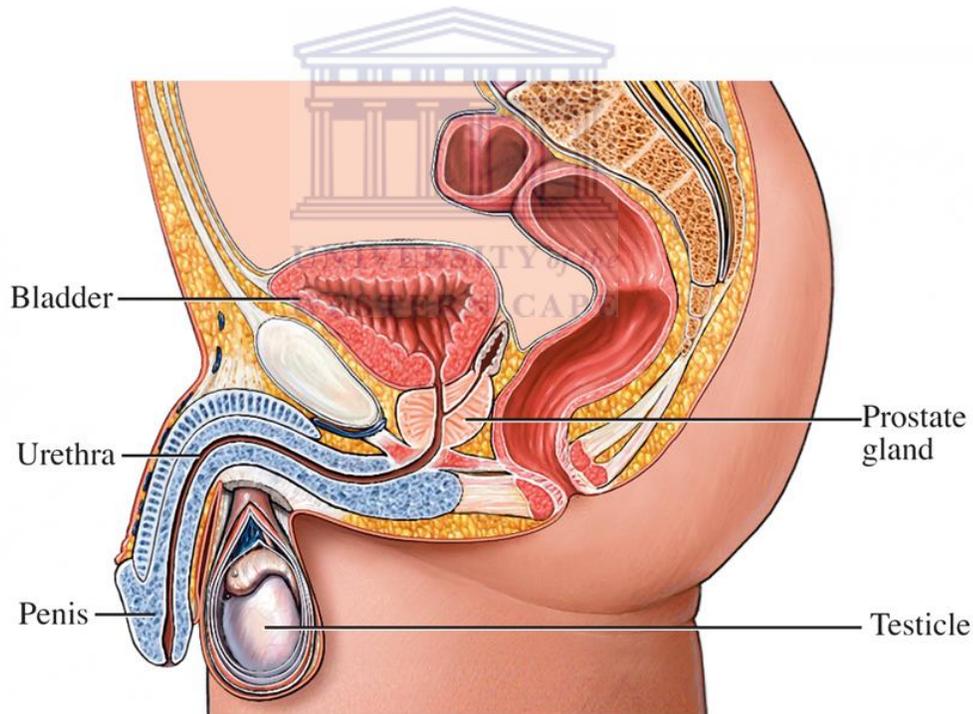


Fig 1-1: Picture of the prostate. Source (Keys, 2015)

The prostate gland is made up of three distinct morphological regions within the human prostate: the peripheral zone, the transition zone, and the central zone (Fine and Reuter, 2012b). The peripheral zone constitutes over 70% of the glandular

prostate. It forms a disc of tissue whose ducts radiate laterally from the urethra, lateral and distal to the verumontanum (anatomical landmark at which ejaculatory ducts empty into the prostatic urethra) all the way to the apex of the prostate (Fine and Reuter, 2012a). Evidence based reports indicate that almost all carcinomas rise from the peripheral zone (McNeal, 1981).

The central zone constitutes 25% of the glandular prostate. Its ducts arise close to the ejaculatory duct orifices and follow these ducts proximally, branching laterally near the prostate base. Its lateral border fuses with the proximal peripheral zone border. Transition zone consists of bilateral regions in the middle to the base of the gland (along the proximal urethra), composed of ducts extending laterally from the urethral wall and curving anteromedially. The anterior fibromuscular stroma forms the entire anterior surface of the prostate as a thick, non-glandular apron, shielding from view the anterior surface of the three glandular regions (Fine and Reuter, 2012b). The significance of this architecture is based upon the relationship of these zones to prostatic disease (Fine and Reuter, 2012a).

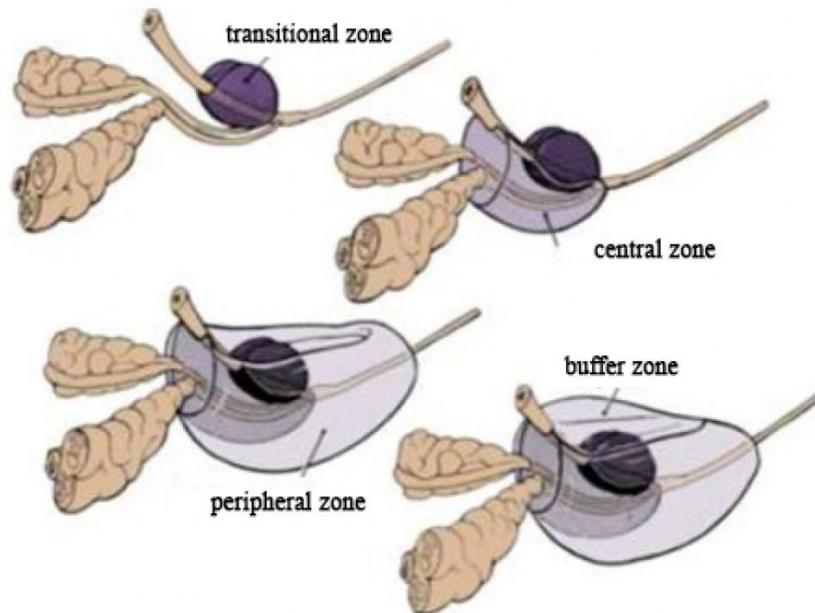


Fig 1-2: The zonal anatomy of the prostate. Source (Fine and Reuter, 2012b).

For example the central zone is relatively resistant to carcinoma and other diseases; the transition zone is the main site of origin of prostate hyperplasia. In all zones, both ducts and acini are lined by secretory epithelium. In each zone, there is a layer of basal cells beneath the secretory lining, as well as interspersed endocrine-paracrine cells. Frequent deviations from normal histology include post-inflammatory atrophy, basal cell hyperplasia, benign nodular hyperplasia, atypical adenomatous hyperplasia, and duct-acinar dysplasia.

1.1.1 Androgen and androgen receptor physiology

Testosterone (T) or its 5 alpha-reduced metabolite, dihydrotestosterone (DHT), is responsible for male reproductive tract development. They mediate the transcriptional activation of the androgen receptor (AR). The physiology of the interactions between androgens and the androgen receptors involves the binding of dihydrotestosterone (DHT) to androgen receptors located in the cytoplasm of the prostate cells. This result in the phosphorylation, dimerization and translocation of the complex to the nucleus where it binds to the androgen response-element of the DNA which in turn activates transcription of specific genes required for prostate cell growth and development (Fig 1.3) (Feldman and Feldman, 2001).

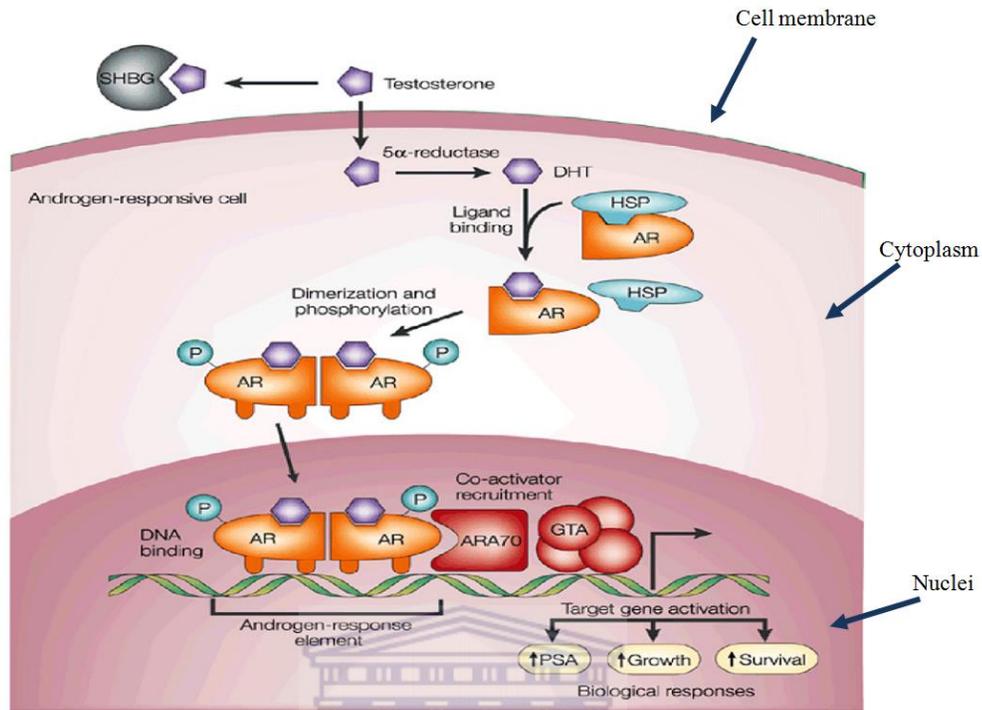


Fig 1-3: A schematic diagram showing the link between androgens and androgen receptors. HSP; heat shock protein. DHT; dehydrotestosterone. AR; androgen receptor. PSA; prostate specific antigen. GTA; general transcriptional apparatus. SHBG; Sex-hormone binding globulin. Source (Feldman and Feldman, 2001)

1.1.2 Prostatic epithelial cell types and their relationship to carcinogenesis

Within the prostatic epithelium, there are at least three distinct cell types that can be distinguished by their morphological characteristics, functional significance, and relevance for carcinogenesis (Fig 1.4). The predominant cell type is the secretory luminal cell, a differentiated androgen-dependent cell that produces prostatic secretory proteins. These luminal cells synthesize and secrete the products of the seminal plasma, including prostatic-specific antigen (PSA) and prostate-specific acid phosphatase, polyamines and prostaglandins (Coffey, 1992). At the molecular level, luminal cells are characterized by their expression of androgen receptor, as well as cytokeratins 8 and 18 and the cell surface marker CD57 (Abate-Shen and Shen, 2000;

Markera et al., 2003). If androgen is removed, these glandular cells undergo apoptosis and the prostate gland becomes regressive. Alternatively, the basal cells only rarely express the AR (Nakada et al., 1993).

The second major epithelial cell type corresponds to the basal cells, which are found between the luminal cells and the underlying basement membrane, and which form a continuous layer in the human prostate. Basal cells express cytokeratins 5 and 14 and CD44 but do not produce prostatic secretory proteins (Abate-Shen and Shen, 2000). These cells are not affected by withdrawal of androgens and do not undergo apoptosis (Isaacs, 1994).

Finally, the third prostatic epithelial cell type is the neuroendocrine cell, a minor population of uncertain embryological origin which is believed to provide paracrine signals that support the growth of luminal cells (di Sant'Agnes, 1992; Abrahamsson 1999).

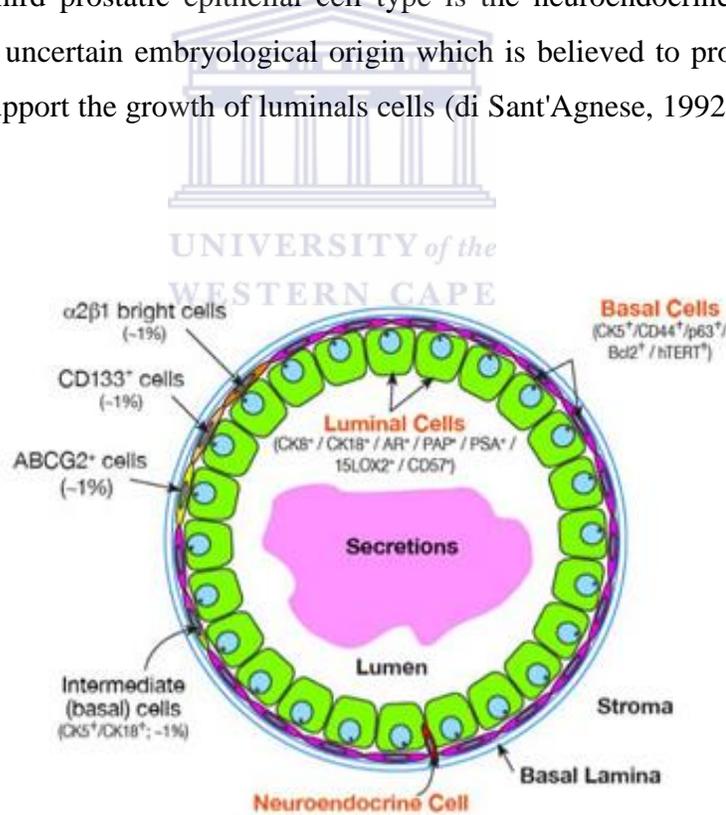


Fig 1-4: Cell types within a human prostatic duct. Source (Abate-Shen and Shen, 2000).

1.2 Prostate pathology

The prostate is prone to three pathological processes which will be discussed below as: prostatitis, benign prostatic hyperplasia (BPH) and tumors (Kumar et al., 2005).

1.2.1 Prostatitis

Despite limited understanding of this disease, prostatitis is surprisingly common, accounting for 8% of visits to urologists and 1% to primary care physicians in the United States (Roberts et al., 1997). A retrospective analysis of clinical data on patients without urinary retention who underwent prostatic biopsy or prostatectomy between January 1999 and March 2009 in the Urology Department of Stellenbosch University and Tygerberg Hospital, South Africa showed histological prostatitis was significantly more prevalent in men with benign prostatic hyperplasia (BPH) than adenocarcinoma (ACP) (Edlin et al., 2012).

While prostatitis affects men of all ages, chronic prostatitis is most prevalent in those who are between 36 to 50 years old, with no apparent racial predisposition. Antimicrobials are prescribed for almost a third of these men, despite the absence of evidence of infection, and this reflects the frustration of physicians in treating chronic prostatitis.

Based on a traditional classification system, prostatitis is defined into four clinical entities. This system was initiated by Meares and Stameyl in 1968 and divides prostatitis into acute, chronic bacterial, chronic nonbacterial, and prostatodynia. The classification is based on the chronology and severity of symptoms, the presence or absence of leukocytes and bacteria in the various segmented lower urinary tract cultures (Roberts et al., 1997).

The most common cause of acute bacterial prostatitis is gram negative organisms. Strains of *Escherichia coli*, *pseudomonas aeruginosa*, *Serratia klebsiella*, *neisseria gonorrhoea tuberculo bacillus* etc are behind the etiology of prostatitis. One theory about this infection is that it occurs as a result of the reflux of infected urine into the ejaculatory and prostatic ducts. Chronic bacterial prostatitis has the same etiological organisms as acute prostatitis. It is more commonly found in older men. It is a relapsing disease with occasional acute exacerbations. It may be diagnosed incidentally from asymptomatic bacteriuria but is frequently associated with a history of recurrent bladder infections, dysuria, testicular and lower back pain (Roberts et al., 1997).

Chronic prostatitis is made up of the non-inflammatory prostatitis, frequently termed as prostatodynia. Patients experience symptoms localized to the prostate but have no obvious inflammation of the prostate. Although it is estimated to be eight times more frequent than bacterial prostatitis. Non-bacterial prostatitis remains a condition of uncertain etiology and is rarely associated with any other infections in the urinary tract. Clinical features are pain, urinary symptoms such as urinary urgency, nocturia, dysuria, weak urinary stream, post void dribbling and sexual dysfunction. It has been reported that prostatitis is carcinogenic and may be important in the etiology of prostate cancer (Dennis et al., 2002).

1.2.2 Benign Prostatic Hyperplasia

BPH has been identified as a common disorder in men over 50 years old (Foster, 2000). The etiology of BPH is multi-factorial and is under the control of the endocrine system. Histologically, BPH can be seen in approximately 20% of men with 40 years old, increasing to 70% by age 60 and to 90% by age 70. Only 50% of those who have microscopic evidence of nodular hyperplasia have clinically detectable enlargement of the prostate, and of these individuals, only 50% develop clinical symptoms world wide (Kumar et al., 2005).

Prostate enlargement has been associated with the action of androgens. In respect of this, testosterone or its more active form, 5 α -dihydrotestosterone (DHT) has been observed to mediate prostate growth. Taking advantage of this, prepubertal castration has been observed to prevent the development of nodular hyperplasia. Research based reports have corroborated the role of 5 α reductase in the conversion of testosterone to DHT in stromal cells. This has qualified stromal cells as the main site for the synthesis of DHT (Wong and Wang, 2000). Once synthesized, DHT has been observed to act either through autocrine signaling of the stromal cells or paracrine signaling by diffusing into nearby epithelial cells. This far, DHT acts as a ligand to nuclear androgen receptor (AR), which controls the transcription of several growth factors that are mitogenic to the epithelial and stromal cells (Fig 1.5).

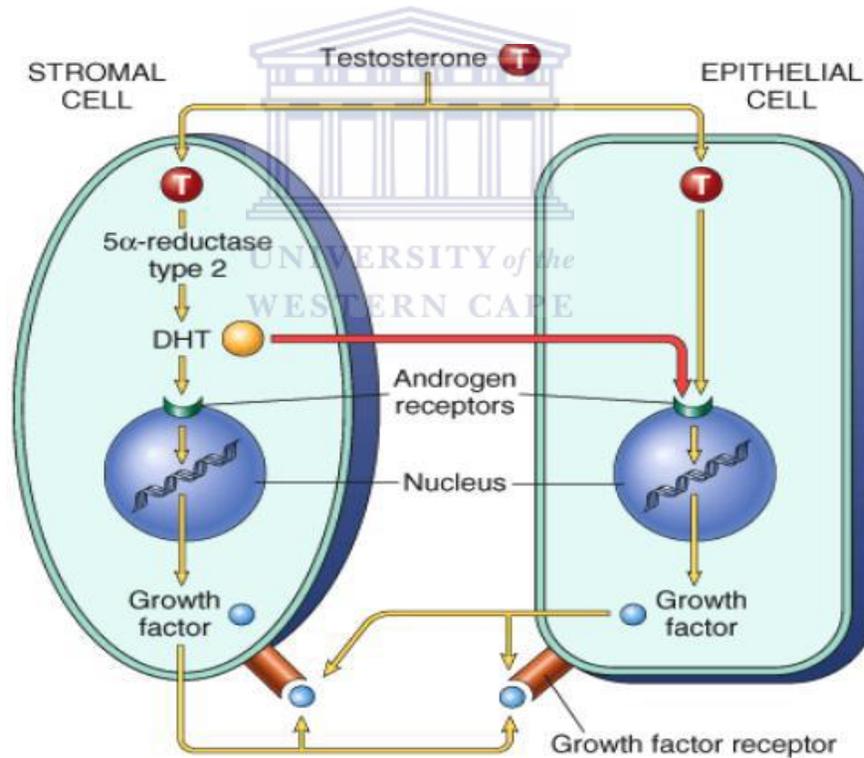


Fig 1-5: Simplified scheme of pathogenesis of nodular prostatic hyperplasia – Role of stromal cells to generate DHT. Source (Kumar et al., 2005).

Besides DHT being the major trophic factor mediating prostatic hyperplasia, estrogens are also involved in rendering prostate cells susceptible to the action of DHT (Wong and Wang, 2000). The role of DHT in the developing of BPH is supported by clinical observations in which therapy with 5 α -reductase inhibitor reduces the DHT content of the prostate, leading to a decrease in prostatic volume and urinary obstruction. Unfortunately, not all patients respond to androgen-depriving therapy, suggesting that prostatic hyperplasia may be etiologically heterogeneous, and probably other factors besides androgens may be involved (McConnell et al., 2003).

Some studies have implicated the transition zone of the prostate gland as the origin of BPH. Development of BPH basically starts with the development of discrete nodules in the periurethral region of the prostate. These nodules, when large enough, compress and narrow the urethral canal leading to partial or complete obstruction of the urethra (Kumar et al., 2005). A cross-sectional view of the gland (especially in areas with primary glandular proliferation) presents tissue with yellow-pink colour accompanied by a flow of milky white prostatic fluid. In fibromuscular regions the nodules are pale gray with no fluid discharge. This region is very distinct from the prostatic capsule (Kumar et al., 2005) (Fig 1.6).

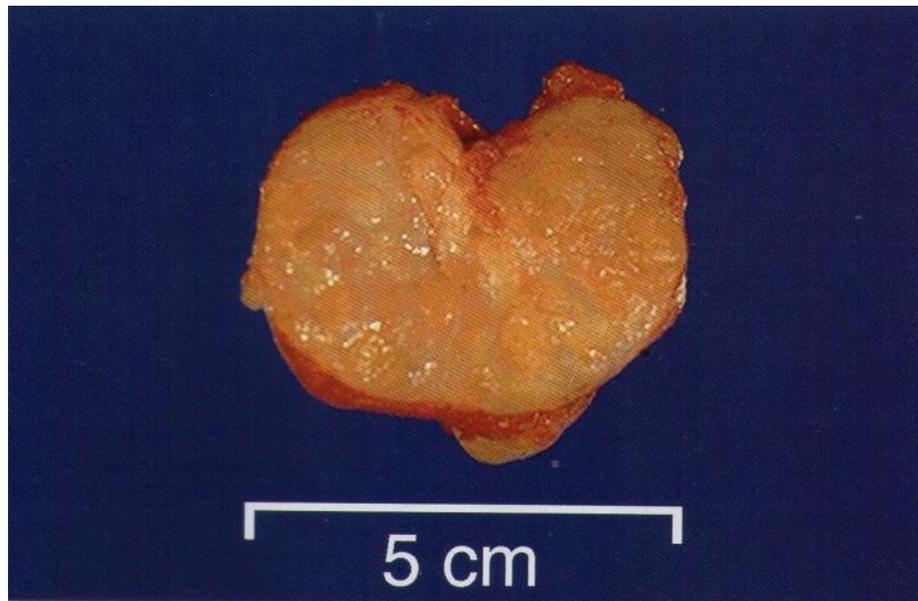


Fig 1-6: Gross appearance of prostatic hyperplasia, which is most pronounced in the lateral lobes. Source (Klatt, 2014).

Microscopically, the main histological feature of BPH is the presence of nodules. These nodules come about due to glandular proliferation and fibrous or muscular proliferation of the stroma. Glandular proliferation consists of aggregations of small to large cystically dilated glands. The glands are lined by two layers (an inner columnar and an outer cuboidal or flattened epithelium) based on an intact basement membrane (Fig 1.7).



Fig 1-7: Microphotograph of prostatic hyperplasia. Source (Klatt, 2014)

Prostatic hyperplasia can involve glands and stroma, although the former are usually more prominent. A large hyperplastic nodule with numerous glands is present here. There is still stroma between the glands. The glands are larger than normal, with more complex infoldings, but still lined by a double layer of uniform columnar cells and basal cuboidal cells that show no atypia. The transitional zone often enables an initial increase in these hyperplastic nodules, although the bulk of prostatic enlargement often comes later from pronounced nodular growth in the peripheral zone (Klatt, 2014).

1.2.2.1 Grading and Staging

The Gleason grading method was devised in the 1960s and 1970s by Dr Donald F Gleason. This grading system is based entirely on the histologic pattern of arrangement of carcinoma cells in H&E-stained sections. This system classifies prostate cancer based on the glandular pattern and differentiation degree as observed under low magnification.

Five basic grade patterns are used to generate a histologic score, which can range from 2 to 10. The lower the number, the more normal the cancer cells look and the lower the grade. The higher the number, the less normal the cancer cells look and the higher the grade. Grades 1 and 2 are not commonly used because the tumor tissue looks and acts like normal tissue. The prostate has neoplastic glands that are uniform with round appearance and are packed into well-circumscribed nodules. On the contrary, grade 5 tumors are not glandular and the tumor cells infiltrate the stroma in the form of cords, sheets and nests (Fig 1.8). The other grades fall in between (McNeal et al., 1990; Epstein, 2000; Gleason and Mellinger, 1974).

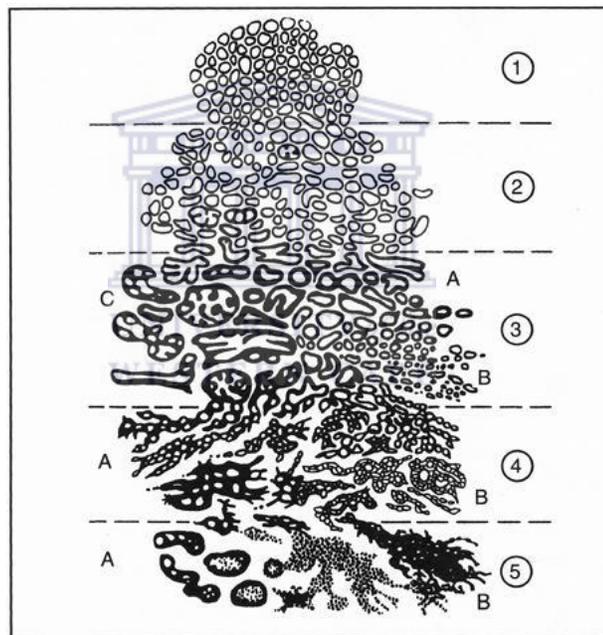


Fig 1-8: Gleason grades 1 to 5. Source (Dehner et al., 2008).

Most prostate tumors are grade 3 or higher. Grade 3 prostate cancer cells are well differentiated, and form well-defined glands. At this grade the tumor has grown into, or invaded, the surrounding prostate tissue. However it is less aggressive and has a favorable prognosis.

At grade 5 the cancer cells are poorly differentiated, to the extent that they don't look and act like normal glands. The cancer is more aggressive and has a less favorable prognosis.

To assign a Gleason score (also called Gleason sum), the pathologist looks at the biopsy sample of the tumour to find the 2 most common types of glandular growth patterns within the tumour. In a case where a tumor contains more than one pattern; a primary grade is assigned to the dominant pattern and a secondary grade to the subdominant pattern. The two numeric grades are then added to obtain a combined Gleason grade or score. For example, a tumor with a dominant grade 3 and a secondary grade 4 would achieve a Gleason score of 7 (McNeal et al., 1990; Epstein, 2000; Epstein and Potter, 2001). The Gleason score is always between 6 and 10. Higher Gleason scores indicate more aggressive tumors.

1.2.2.2 Biomarkers of prostate cancer

Biomarkers have been defined as biological characteristics that can be objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention (Mayeux, 2004). Biomarkers are extensively used as analytical tools to assess biological parameters for a rapid and comprehensive therapeutic analysis. Besides this, biomarkers are also used in the development and evaluation of new therapies (Rolan, 1997). Amongst the many prostate cancer biomarkers available (Fig 1.9) the prostate specific antigen (PSA) is the most common. Once secreted the prostate-specific antigen goes into semen and bloodstream. Diagnostically elevated plasma levels of PSA occur in prostate cancer individuals and are widely used as a screening test for this disease. This 30 kDa serine protease hydrolyzes the sperm motility inhibitor semenogelin in semen and elevated levels have also been observed in benign prostatic hyperplasia and prostatitis.

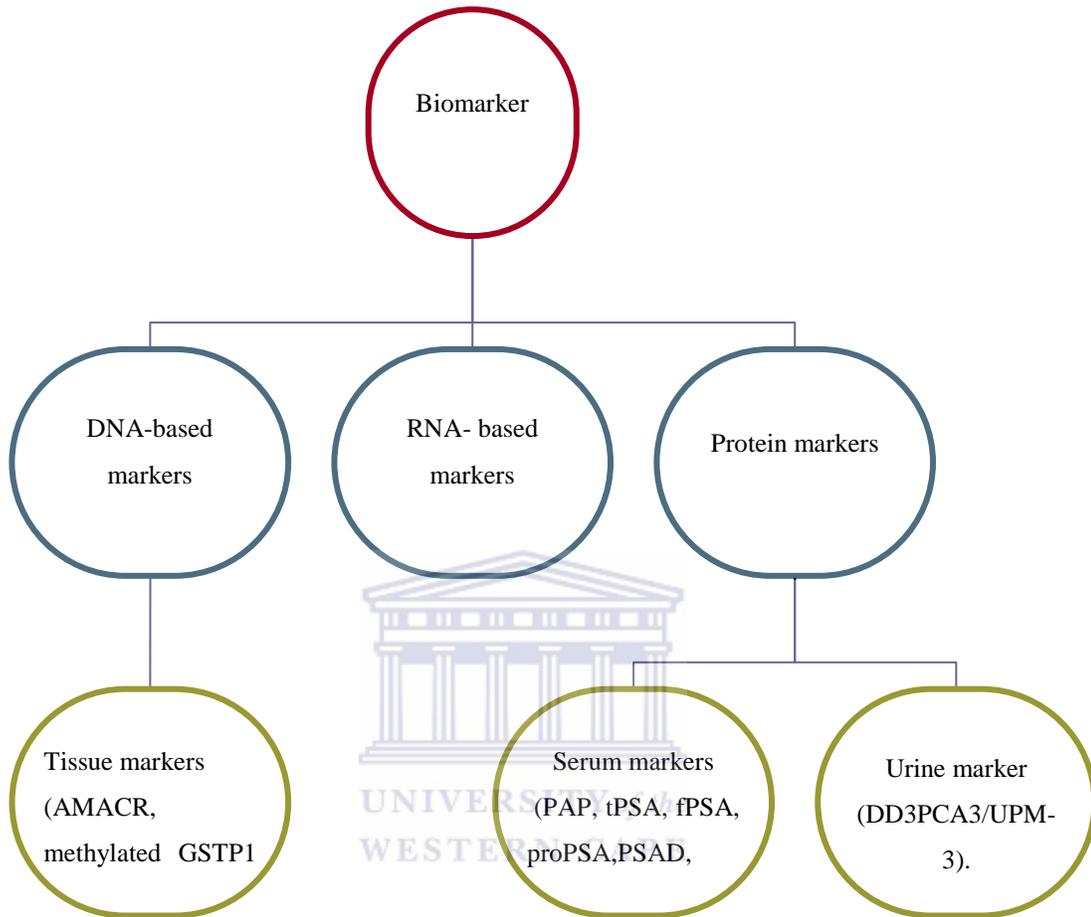


Fig 1-9: General classification of biomarkers based on their description. Source (Ludwig and Weinstein, 2005)

Cancer biomarkers fall into three different categories: prognostic, predictive, and pharmaco-dynamic. Prognostic biomarkers predict the natural course of the cancer, to distinguish the tumor's outcome; help to determine whom to treat, how aggressively to treat, and which candidates will likely respond to a given drug and the most effective dose. Predictive biomarkers evaluate the probable benefit of a particular treatment. Pharmaco-dynamic biomarkers assess the imminent treatment effects of a drug on a tumor and can possibly determine the proper dosage in the early stages of clinical development of a new anticancer drug (Sawyers, 2008).

Among the various proteins used as biomarkers (described in Fig 1.9), PSA has widely been used to assess prostate cancer. The main advantage of PSA testing is its sensitivity. However, the main disadvantage of this test is its low specificity, because some common pathological conditions such as BPH and prostatitis can also increase the levels of PSA (Kufe et al., 2003).

In order to overcome the problems associated with sensitivity and specificity, a variety of diagnostic parameters and tests have been proposed. These include lowering the cut point for defining an abnormal PSA level to below 4.0 ng/mL, age-specific PSA, PSA velocity (PSA-V), volume-adjusted PSA densities, the free-to-total PSA ratio (% of PSA), and bound/complexed PSA (Wilt, 2003). PSA circulates in two forms: free and complexed with molecules as 1- antichymotrypsin (ACT). Men with prostate cancer tend to have a lower percentage of their PSA in the free form compared with men without prostate cancer (Stenman et al., 1994). The free/total PSA measurements might help to differentiate between prostate cancer and normal prostate conditions among men with PSA concentrations between 4.0 ng/mL and 10 ng/mL. PSA levels are normally elevated in older men relative to younger ones, regardless of the absence or presence of cancer. Therefore, a continuous rise in PSA levels over time may be more indicative of cancer than moderately increased PSA (Carter et al., 1992).

1.2.3 Prostate cancer.

Prostate cancer and benign prostatic hyperplasia (BPH) are the two most common neoplastic diseases in men in the US. About 50% of men in their sixth decade and 90% of men by age 85 will develop BPH (Oesterling, 1996).

In 2006 alone, the American Cancer Society reported 234 460 men diagnosed and 27 350 deaths from prostate cancer (Penson and Chan, 2007). This has made prostate cancer to be the most common non-skin cancer and the second leading cause of

cancer death in men in the United States. According to the South African medical journal (2011), data obtained at the Urological Oncology Clinic, Tygerberg Hospital, Western Cape, South Africa, from January 1995 to December 2005; most black men presented with higher grade prostate cancer than white or coloured men. In addition to this, serum PSA levels were also observed to be higher in black men. Using histological grade, grade 1 was the most common grade of prostate cancer in the white (37%) and coloured groups (38%) and grade 2 was the most common in the black group (39%). Further to this, the most common clinical tumour stage at presentation was T1-2 in white patients (53%) and T3-4 in the coloured (61%) and black groups (62%). In addition to this, for patients with known M-stage, metastatic disease was the most common in the black group (53%) and least common in the white group (28%) (Heyns et al., 2011)

Prostate cancer is treated by surgery or radiation when confined to the organ at diagnosis. As it is an androgen-dependent malignancy, androgen deprivation therapy (ADT) is used to control the disease, if disease relapse occurs. Reports show that androgen ablation therapy works positively on metastatic prostate cancer. However, the cancer often recurs leading to the progression from androgen-dependence to androgen independence (Karantanos et al., 2013). Disease progression after initial androgen deprivation therapy (ADT) despite castration levels of testosterone is termed castrate resistant prostate cancer (CRPC) and it can be either metastatic or non-metastatic. This disease progression goes together with an increase in PSA thereby serving as a marker that the androgen axis is still functional despite low circulating levels of serum androgens (Ford III et al., 2003). The following mechanisms have been proposed for the continued activation of AR and the development of CRPC. Intracrine synthesis of androgens, amplification and/or overexpression of AR, overexpression and polymorphism of steroid transporters, mutation of the AR gene, constitutively-active AR splices variants, alteration in AR co-regulators (Suzuki et al., 2003; Egan et al., 2014).

1.2.3.1 Prostate cell lines.

1.2.3.1.1 Benign (RPWE 1) prostate cells.

A human prostate epithelial cell line (RWPE-1) were obtained from the American Type Culture Collection (ATCC) (Rockville, MD, USA). The benign (RWPE 1) prostate cells were chosen because they mimic normal prostatic epithelial cell behavior in their response to growth factors and in their expression of PSA and androgen receptor in response to androgen exposure. In this case, they up-regulate androgen receptors and PSA expression in response to androgen stimulation in a very similar way to the normal prostatic epithelial cells (Bello-DeOcampo et al., 2001).

The RWPE-1 cells have a typical polygonal, epithelial morphology. The RWPE-1 cells were derived by immortalization with human papilloma virus-18 (HPV-18) from epithelial cells isolated from the peripheral zone of a non-neoplastic adult human prostate (Webber et al., 1997). And they respond to growth factors and androgens just as good as normal prostatic epithelial cells. Further to this, RWPE-1 cells do not form tumors when injected into nude mice and even when injected with matrigel instead they grow into glandular acini similar to those seen in the normal prostate (Webber et al., 2001). Based on chromosomal analysis, RWPE-1 cells consists of at least two cell populations, one with a modal chromosome number of 45 (sex chromosome complement (X, - Y) and the other with 51 (XY). Further to this, all RPWE 1 cells express cytokeratins 8 and 18 and this only emphasizes the peripheral zone of the prostate as their origin (Webber et al., 2001).

1.2.3.1.2 Malignant (LNCaP) Cells.

The malignant (LNCaP) prostate cell lines were obtained from the American Type Culture Collection (ATCC) (Rockville, MD, USA). This cell line was used because it

possesses characteristics that are similar to those found in the stratified epithelium of the prostate gland; malignant (LNCaP) prostate cells express AR and PSA. Besides this, its growth is inhibited by androgen withdrawal which is very similar to secretory/luminal cells of the prostate epithelium.

The LNCaP cell line was established from a metastatic lesion of human prostatic adenocarcinoma (Bello et al., 1997). Morphologically they have flat polygonal shapes. They can grow up to 8×10^5 cells/cm² with a doubling time of 3 days. They form clones when grown in semisolid media and are highly resistant to human fibroblast interferon. The LNCaP cells are aneuploid (modal number, 76 to 91) human male karyotype with several marker chromosomes. In short, LNCaP cells are heterogenic and continuously maintain a high degree of polyploidy as well as karyological characteristics in cultures (Horoszewicz et al., 1983).

Besides this, LNCaP cells maintain their malignant properties in culture or animal modal. And when injected in athymic nude mice, tumors develop at the injection site. Both LNCaP cultures and tumors produce acid phosphatase and prostate specific antigen (Papsidero et al., 1981). They possess high-affinity specific androgen receptors in the cytosol and nucleus. They also have estrogen receptors in the cytosol. In addition to this, LNCaP cells require low serum concentrations for cell division and demonstrate anchorage-independent proliferation in semisolid media (Horoszewicz et al., 1983).

1.3 Incidence of prostate cancer

The risk of developing prostate cancer varies across many geographic populations. Studies showed that people from low risk geographic populations such as the Chinese and Japanese, who changed their residence to a place in the Western Nations, had an increased chance of developing prostate cancer (Muir et al., 1991; Klein and Thompson, 2004).

Some authorities have attributed this increase in prostate cancer risk as a result of the latent form of prostate tumour in the migrants before they relocated (Bostwick et al., 2004). However, evidence suggests that up to 30% of men in the general population aged from 50 years and above, irrespective of geographic origin have foci of prostate neoplastic growth (Muir et al., 1991; Nectoux et al., 1991; Klein and Thompson, 2004).

In terms of race, African American men experience disproportionate mortality from prostate cancer (CaP) compared with white American men. The differences seem to come from differences within the androgen axis. However, the serum testosterone levels appear to be similar by race in men with CaP (Gaston et al., 2003). Measurements of androgen receptor (AR) protein expression in malignant and benign prostate tissue from black and white men who underwent radical prostatectomy showed that black Americans are associated with shorter CAG and GGN repeat lengths. The CAG and/or GGN repeats when genomically shortened result in increased expression of AR protein levels. This results in the stimulation of tumors to a greater extent in blacks even if the serum androgen levels are equivalent (Irvine et al., 1995; Stanford et al., 1997).

Reports from another study corroborated the presence of more aggressive prostate cancer PCa among black men than white men. To this extent, histological studies on prostate biopsies for men that had gone for radical prostatectomy showed increased levels of prostate cancer biomarkers (α -methylacyl CoA racemase (AMACR), androgen receptor (AR) and Ki67 in black men than prostate biopsies from white men (Kim et al., 2011).

1.4 Prostate Cancer Risk Factors

All men are at risk of developing prostate cancer. In respect of this, one man in six will be diagnosed with prostate cancer during his lifetime, but only one man in 36

will die of this disease (Dunn and Kazer, 2011). About 80 percent of men who reach age 80 have prostate cancer cells in their prostate. Besides being male, there are other factors, such as age, race, and family history that may contribute to the risk (Hsing and Devesa, 2001).

1.4.1 Age.

The greatest risk factor for prostate cancer is age. This risk increases significantly after the age of 50 in white men who have no family history of the disease and after the age of 40 in black men and men who have a close relative with prostate cancer. About two-thirds of all prostate cancers are diagnosed in men age 65 and older. The older the patient, especially if they are over 70, the less aggressive the disease usually behaves (Haas and Sakr, 1997).

1.4.2 Family history.

Men whose relatives have had prostate cancer are considered to be at high risk. Having a father or brother with the disease more than doubles the risk for prostate cancer. Having a brother with prostate cancer appears to increase the risk more than having an affected father does. That risk is even higher when there are more affected family members (Haas and Sakr, 1997).

1.4.3 Diet.

Dietary fat may be a contributing factor for prostate cancer. The disease is much more common in countries where meat and dairy products are dietary staples compared to countries where the basic diet consists of rice, soybean products, and vegetables (Kolonel, 2001). In this prospective cohort study, the relationship of intake of dietary fat to total prostate cancer incidence was not statistically significant, but consumption of animal fat, especially fat from red meat, was associated with an increased risk of

advanced cancers (Giovannucci et al., 1993). The Fred Hutchinson Cancer Research Center in San Antonio, Texas, reported an increased prostate cancer risk in men with high blood concentrations of long chain omega-3 fatty acid, specifically docosahexaenoic acid (DHA). In the study DHA correlated significantly with high-grade prostate cancer. This far, no explanation has been given as to why high levels of long-chain omega-3 polyunsaturated fatty acids increase prostate cancer risk (Brasky et al., 2013).

1.4.4 Racial differences

Worldwide, the highest age-standardized rate (ASR) for prostate cancer mortality has been reported to occur in Western and Southern Africa and in regions in which most of the people are of African descent, for example, the Caribbean (Rebbeck et al., 2013; Ferlay et al., 2010). In the United States of America, prostate cancer mortality rate ratio has been reported to be higher in African American men than in whites. For example in 2007, black males had an age-adjusted annual death rate from prostate cancer that was 2.4 times greater than whites thus 52.0 vs 21.6 per 100 000 males (Taksler et al., 2012)

Differences in social factors and access to medical services have been implicated for the aforementioned differences. In this regard, lower income, education, lack of health insurance, less frequent PSA screening and comorbidities have been suggested to contribute to more advanced disease at diagnosis and higher mortality amongst black males (Taksler et al., 2012) Some reports have suggested genetics, diet and lifestyle variation and environmental exposure to chronic stress as some of the factors that have promoted the differential mortality rates between African Americans and Whites (Cooperberg, 2013).

In sub-Saharan Africa, cases of prostate cancer were estimated at 26 800 in 2002. This represented 10.6% of all cancers in males. At the same time, Southern Africa

reported 40.5 per 10⁵ males (Ferlay, International Agency for Research on Cancer 2001). Using histological diagnosis, prostate cancer incidence in South Africa was at 40.1 cases per 10⁵ males for white population against 14 per 10⁵ males in the black population. In contrast, the black population was reported to have poorer access to diagnostic and screening facilities (Parkin et al., 2008).

The incidence of prostate cancer in Zimbabwe was at 70 per 10⁵ males for the white population against 25 per 10⁵ males for the black population (Walker et al., 1992). Increasing age or family history of prostate cancer has been implicated as important risk factors for the incidence of PCa in Sub-Saharan African (Baade et al., 2009). This view has been emphasised by Crawford, 2003 to the effect that, 70% of all the cases of prostate cancer have been observed to occur in men older than 65 years and prostate cancer family history being identified as the cause (Crawford, 2003). In addition to this, third world populations have lower prostate cancer rates because most men live a traditional way of life. Because of that PCa rates have been observed to rise in urban populations due to changes in diet and other aspects of life-style (Walker et al., 1992).

1.4.5 Occupation

Industrial workers that are exposed to cadmium are at risk of developing prostate cancer. Cadmium is a trace mineral found in cigarette smoke and alkaline batteries. As such people working in the welding and electroplating occupations are exposed to high levels of cadmium. It has been suggested that cadmium increases the risk for prostate cancer by interacting with zinc. Several enzymes that are involved in the replication and repair of DNA and RNA, such as the polymerases, require zinc to function properly. That's the more reason prostate glands containing cancer have lower levels of zinc than do noncancerous glands (Pienta and Esper, 1993).

1.5 Antioxidant defense mechanisms of the prostate

The antioxidant defense system is there to remove free radicals and reactive oxidants. Factors such as aging, radiation, carcinogens, inflammation and/or activated oncogenes are responsible for the increased production of cellular reactive oxygen species (ROS) which in turn increase the risk of prostate cancer initiation and progression. There are two forms of antioxidants; enzymatic and non-enzymatic antioxidants. Examples of antioxidant enzymes include superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GSR), NADPH: quinone oxidoreductase (NQO) and thioredoxin reductase (TrxR). These antioxidant enzymes maintain redox status by inactivating excessive amounts of reactive oxygen species (ROS). Super oxide (SOD) catalyzes the dismutation of superoxide into H_2O_2 and O_2 , while CAT and GPx remove H_2O_2 .

The two major SOD enzymes in eukaryotic cells are manganese superoxide dismutase (MnSOD) found in mitochondria and copper zinc superoxide dismutase (Cu/ZnSOD) found primarily in the cytoplasm (Thapa and Ghosh, 2012). Non-enzymatic antioxidants such as coenzyme Q10 (CoQ10), glutathione, non-protein thiols, vitamin C and vitamin E play important roles in cellular redox homeostasis. Vitamin E is there to protect cells against lipid peroxidation while ascorbic acid works synergistically with lipoic acid to remove different types of free radicals (Thapa and Ghosh, 2012).

1.6 Chemoprevention.

Chemoprevention was originally defined by Dr. Michael Sporn as the activity of natural forms of vitamin A in preventing the development and progression of epithelial cancer (Sporn and Suh, 2002). It is now being defined as the use of natural or pharmacological agents to suppress arrest or reverse carcinogenesis, at its early stages (Sporn and Suh, 2002). Treatment of early stage prostate cancer can involve

radical prostatectomy, radiation therapy, external beam radiation, brachytherapy, watchful waiting and active surveillance.

Radical prostatectomy is a surgical process that involves removal of the prostate and seminal vesicles and in some cases can involve removal of the pelvic lymphnodes. In addition to this, management of PC can involve use of external beam radiation therapy (Denmeade and Isaacs, 2002). Basically, the therapy involves use of CT scan which then produces a three-dimensional picture of the prostate and seminal vesicles at which a radiologist is able to direct high-energy radiation. Brachytherapy is a PC management technique which involves use of ultrasound to guide the introduction of radioactive pellets in the tumor. However, in other instances, early stage prostate cancer can be managed through extended period of monitoring accompanied by regular digital rectal examinations, PSA tests and prostate biopsies (Dall'Era et al., 2008).

Basically, watchful waiting is mostly used in cases where the likelihood of developing cancer may take long and the man is elderly or the patient is not strong enough for surgery or radiotherapy. Besides this, men who have low-grade early prostate cancer (with a Gleason score ≤ 7 or PSA ≤ 10 ng/ml) are the ones who are placed under active surveillance (Dall'Era et al., 2008). Active surveillance enables the doctors to give treatment to those who will need it rather than to men whose cancer is not going to progress. This prevents potential side effects that include bowel obstruction, erectile dysfunction and incontinence. In order to prevent the patient from undergoing curative intervention (radical prostatectomy, hormonal or radiotherapy) and at the same time experience the side effects that go together with the above mentioned therapies; chemoprevention using pharmacological, dietary biofactors, phytochemicals and even whole plant extracts to prevent arrest or reverse the cellular and molecular processes of carcinogenesis (Neergheen et al., 2010a) can be a better option.

The use of chemoprevention through phytochemicals is being reinforced by the concept of “multistep carcinogenesis” of cancer in which cancer has been observed to develop over a long period of time due to the accumulation of somatic mutations which make a cell to undergo gradual phenotypic changes from a normal to a pre-neoplastic cell that can progress to neoplastic (Russo, 2007). As such a good chemo-preventive agent must be able to provide: (1) primary prevention by protecting the DNA; (2) reverse the promotion stage; (3) prevent tumor growth and metastasis. And above all, it should cure cancer (Russo, 2007).

Scientific reports suggest that an increased consumption of fruit and vegetables is the best approach to prevent the incidence of chronic diseases, such as cancer, cardiovascular diseases and other aging-related pathologies. Another approach is to use dietary pharmaceuticals instead of whole plant foods. However, the challenge is that dietary pharmaceuticals will require chronic administration of low doses of chemo-preventive agents to avoid toxic side effects. Further to this, low doses of the dietary pharmaceuticals may lead to compromised bioavailability (Russo, 2007). It has also been reported that phytochemicals present in a diet rich in fruit and vegetables fall into two main groups: cancer blocking and cancer-suppressing agents (Russo, 2007).

Cancer blocking agents prevent carcinogens from initiating cancer through enhancement of carcinogen detoxification, modification of carcinogen uptake and metabolism, scavenging of reactive oxygen species (ROS) and enhancement of DNA repair (Russo, 2007). Cancer-suppressing agents inhibit cancer promotion and progression after the formation of pre-neoplastic cells by interfering with cell cycle regulation, signal transduction, transcriptional regulation, and apoptosis (Greenwald, 2002; Surh, 2003). It is under this background that this thesis would like to investigate the effects of whole plant products of tea, rooibos and their active polyphenolic compounds on prostate cancer cells.

1.6.1 Dietary phytochemicals inhibit cancer initiation.

The information below, describes the great potential that dietary phytochemicals have in blocking the initiation of carcinogenesis via the induction of phase I and phase II detoxifying/antioxidant enzymes, activation of the mitogen-activated protein kinase (MAPK) signaling pathway and inhibition of a signal transducer and activation of transcription 3 (STAT 3). Dietary phytochemicals block cancer initiation by modulating the levels of biotransformation enzymes such as cytochrome p450, UDP-glucuronosyltransferases, sulfotransferases, N-acetyltransferases, glutathione S-transferases and methyltransferases that promote the elimination of endogenous and environmental carcinogens (Russo, 2007).

In a typical Phase I reaction, a cytochrome P450 enzyme (Cyp P450) uses oxygen and, as a cofactor, NADH, to add a reactive group, such as a hydroxyl radical. This ends in the production of functional groups required for attachment of the phase II polar groups (Russo, 2007). These phase II detoxifying/antioxidant enzymes conjugate oxidants or electrophilic xenobiotics with endogenous ligands, such as glutathione and glucuronide, to reduce their toxicity and reactivity by increasing the solubility of these conjugations and excreting them through urine or bile.

Besides the aforementioned, phytochemicals have also been observed to mediate the induction of the effects of phase II detoxifying agents by activating the nuclear factor erythroid 2-related factor 2 (Nrf2) signaling pathways. The phytochemicals promote the activation of the KEAP 1-Nrf2 complex which is an intracellular sensor against changes in electrophiles or ROS concentrations (Russo, 2007). This promotes the release of Nrf2 in the cytosol and its translocation into the nucleus where it promotes the transcriptional activity of antioxidant response elements (ARE) regulated genes (Yu and Kensler, 2005; Neergheen et al., 2010a). This results in increased production of antioxidant enzymes such as glutathione, superoxide dismutase, catalase thereby enhancing the ability to scavenge reactive oxygen species and inactivate potential

carcinogens (Neergheen et al., 2010b). The above information demonstrates the importance of dietary phytochemicals as chemo-preventive agents in up-regulating the expression of phase I and phase II detoxifying/antioxidant enzymes via the modulation of the Nrf2 signaling pathway (Fig 1.10).

Secondly, the induction effect of phase II detoxifying agents by natural phytochemicals is mediated in part through the activation of Nrf2 signaling pathways. Several intracellular signal transduction pathways, mitogen-activated protein kinases (MAPKs), protein kinase C (PKC), phosphatidylinositol 3-kinase (PI3K) and RNA dependent protein kinase (PKR)-like endoplasmic reticulum kinase (PERK) can activate the KEAP 1–Nrf2 complex. An activated KEAP 1–Nrf2 complex results in the release of Nrf2 in the cytosol thereby allowing it to translocate into the nucleus where it promotes the transcriptional activity of ARE-regulated genes which in turn promotes the encoding of genes necessary for the production of cytoprotective enzymes (Neergheen et al., 2010b) (Fig 1.10). In respect of this, the activation of Nrf2 by EGCG promoted the expression of glutamate–cysteine ligase, manganese superoxide dismutase and heme oxygenase 1 via the Akt and ERK 1/2 signaling pathways in human mammary epithelial cells (Na et al., 2008). In addition to this, anti-oxidative enzymes, anti-inflammatory genes and proteasomal subunits were up-regulated via the Nrf2 pathway (Davies, 2001; Kwak et al., 2003).

Thirdly, phytochemicals have been observed to inhibit tumor growth, angiogenesis and metastasis by removing an inflammatory microenvironment that exists in tumors. Present in tumors are tumor-associated macrophages, tumor-infiltrating lymphocytes which produces proinflammatory cytokines such as tumor necrosis factor (TNF), interleukins 1 and 6 (IL-1, IL-6), growth factors, chemokines like interleukin 8 (IL-8) and signal transducers and activators of transcription (STATs) (Balkwill and Mantovani, 2001; Klampfer, 2008).

These chemokines and cytokines can enhance tumor growth, invasion and metastasis by (i) promoting DNA damage by ROS/RNS, (ii) suppressing DNA repair mechanisms via ROS/RNS, (iii) destabilising the tumor suppressor genes such as p53, (iv) autocrine/paracrine growth and survival factors for malignant cells, (v) promoting vascular permeability and activation of matrix metalloproteinases, (vi) modulation of cell to cell adhesion molecules, (vii) stimulation of angiogenic factors such as VEGF, bFGF, IL-8, MMP (Neergheen et al., 2010b).

Dietary compounds such as guggulsterone, honokiol, curcumin, resveratrol, flavopiridol, and cucurbitacin, have been shown to possess anti-cancer effects in cancer cell lines and xenograft tumors through the inhibition of STAT3 activation (Lee et al., 2013). Plant polyphenols using their antioxidative property work as natural anti-inflammatory agents. As such, plant polyphenols mediate their anticarcinogenic effects by acting as free radical scavengers and metal chelators (Neergheen et al., 2010b).

The anti-inflammatory effects of plant polyphenols can also inhibit the cyclooxygenase activity of COX-2. This results in decreased production of prostaglandins. A decrease in prostaglandin production especially PGE2 receptors using plant polyphenols has been reported to reduce tumor formation in mouse models of colon carcinogenesis (Neergheen et al., 2010b).

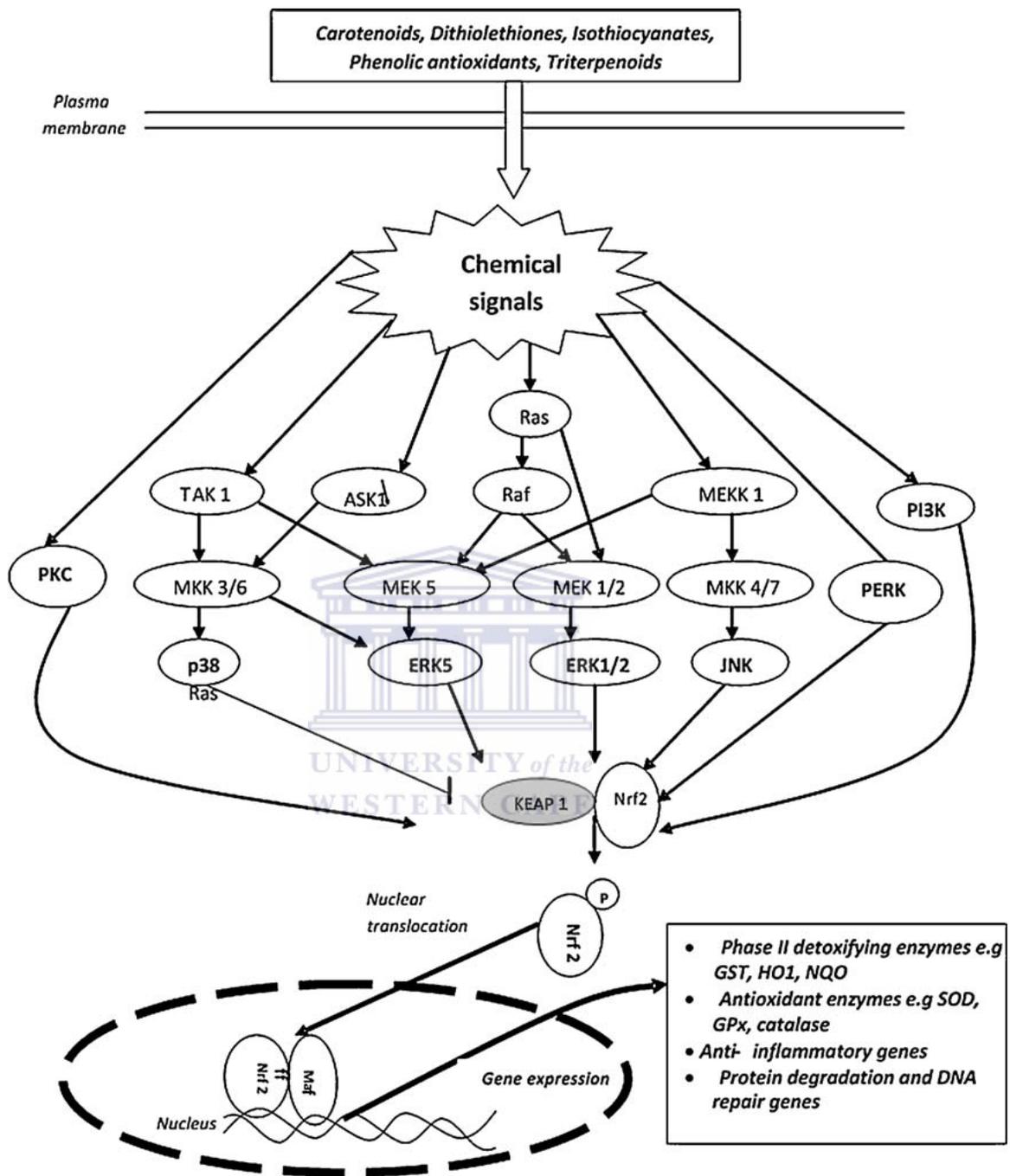


Fig 1-10: Regulation of the Nrf2-mediated pathways by natural phytochemicals. (Neergheen et al., 2010b)

1.6.2 Dietary phytochemicals that suppress cancer progression in initiated tumor cells

Apoptosis or programmed cell death has long been described as a key strategy for the elimination of neoplastic cells. Apoptosis is characterized by typical morphological and biochemical hallmarks including cell shrinkage, chromatin condensation, nuclear DNA fragmentation, membrane blebbing and the formation of apoptotic bodies (Hengartner, 2000). Apoptosis can be triggered by two major pathways: (i) at the plasma membrane upon ligation of the death receptor (extrinsic pathway) and (ii) at the mitochondria (intrinsic pathway) (Hengartner, 2000).

1.6.2.1. Intrinsic/Extrinsic pathway inducing apoptosis

Dietary phytochemicals can induce apoptosis through the extrinsic pathway (Fig 1.10). The extrinsic apoptotic cascade, also called the cell-death pathway, is activated through the binding of extracellular ligands of the tumor necrosis factor (TNF) family of proteins to pro-apoptotic death receptors by forming a death-inducing signaling complex that activates caspases 8 and 10, followed by the activation of caspases 3, 6 and 7 that is the same caspase machinery utilized by the intrinsic pathway (Lee et al., 2013).

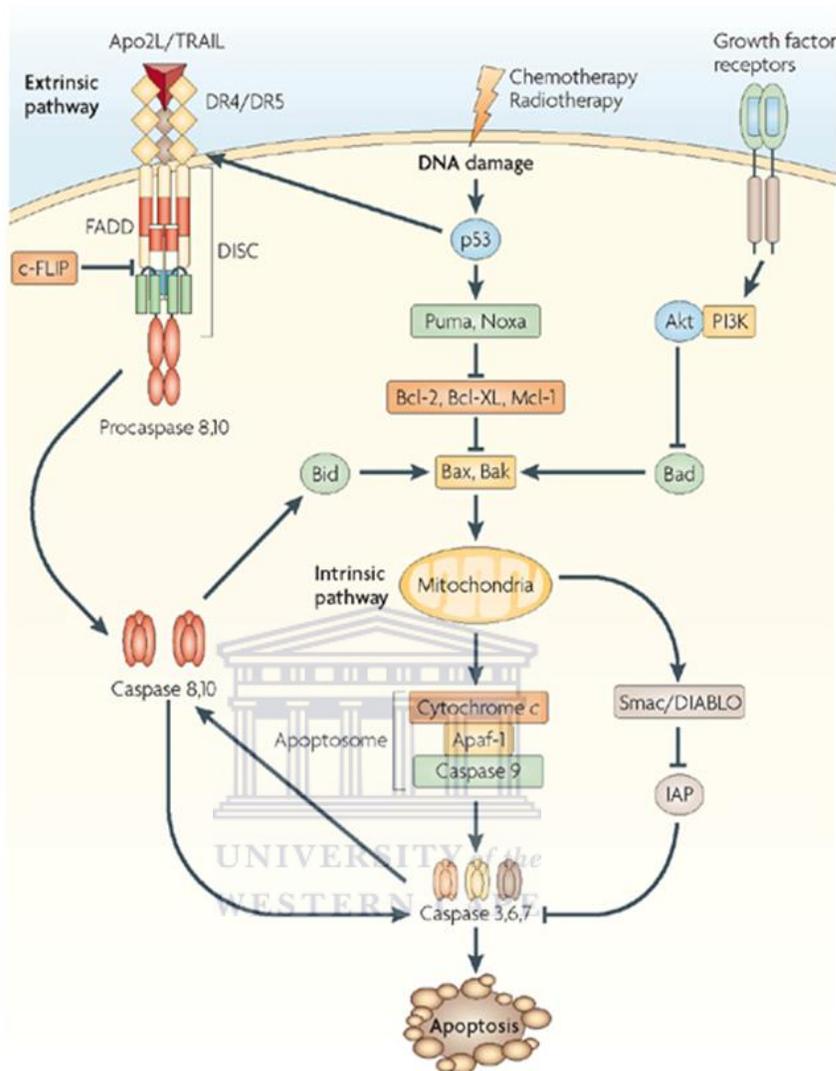


Fig 1-11: Extrinsic and Intrinsic apoptotic pathways. Source (Ashkenazi, 2008)

While some dietary plants are promoting apoptosis through extrinsic pathway a large spectrum of dietary phytochemicals induce apoptosis by regulating the expression of Bcl-2 family proteins via activation of the intrinsic (mitochondrial) apoptotic pathway (Fig 1.10). The mitochondrial pathway is initiated by phosphorylation of the JNK pathway which leads to the release of apoptogenic factors such as cytochrome c, apoptosis inducing factor (AIF), Smac/DIABLO, Omi/HtrA2, endonuclease G, caspase-2 or caspase-9 from the mitochondrial inter membrane space (van Loo et al.,

2002). This is accompanied by the inactivation of anti apoptotic proteins Bcl-2 and Bcl-XL. The release of cytochrome c into the cytosol triggers caspase-3 activation through formation of the cytochrome c/Apaf-1/caspase-9-containing apoptosome complex, while Smac/DIABLO and Omi/HtrA2 promote caspase activation through neutralizing the inhibitory effects to inhibitor of apoptotic proteins (IAPs) (Lee et al., 2013).

Chemoprevention by targeting key components of the apoptosis regulatory pathways which include the antiapoptotic Bcl-2 family of proteins, the inhibitors of apoptosis (IAPs) in particular XIAP, cIAP1, cIAP2, survivin, nuclear factor- κ B (NF- κ B), caspases, tyrosine kinases and key signaling routes (the PI-3K/PKB pathway, the Stat 3/5 pathway and the MAPK pathway) can be the best way to reduce the incidence of cancer (Lee et al., 2013).

1.6.3 Phytochemical Inflammation

Under normal physiological conditions, inflammation is associated with the production of diverse free radicals and oxidants that primarily combat and neutralize invading pathogens and foreign bodies and also destroy the infected host tissue. However chronic inflammation in cancer leads to the continuous production of ROS: mainly superoxide radicals ($O_2^{\bullet-}$), hypochlorous acid (HOCl), hydrogen peroxide (H_2O_2), hypobromous acid (HOBr), hydroxyl radicals (OH^{\bullet}) and RNS like nitric oxide (NO) and nitrogen dioxide ($\bullet NO_2$) (Ohshima et al., 2005). ROS and RNS not only can damage DNA and induce mutations, but also can participate in most carcinogenic processes by activating oncogene products and/or inactivating tumor-suppressor proteins. Plant extracts with high antioxidant index can mediate at least in part their anticarcinogenic effect by acting as free radical scavengers and metal chelators (Neergheen et al., 2010a). Further to this, some of the phytochemicals can work as anti-inflammatory compounds that can inhibit the cyclooxygenase activity of COX-2 thereby decreasing the production of prostaglandins. Prostaglandins, in

particular PGE₂, seems to have a key role in carcinogenesis as activation of several types of PGE₂ receptors trigger other signaling pathways, such as the epidermal growth factor receptor pathway (Han and Wu, 2005; Pai et al., 2002). Furthermore, the genetic or pharmacological disruption of PGE₂ receptors reduces tumor formation in mouse models of colon carcinogenesis (Neergheen et al., 2010a).

1.6.4 Cell cycle arrest in chemoprevention

Cell cycle arrest occurs in response to cellular stress through activation of signal transduction pathways commonly referred to as checkpoints (Hartwell and Weinert, 1989). The checkpoints are activated in the G₁/S phase to prevent replication of damaged DNA or in the G₂/M phase to prevent segregation of damaged chromosomes during mitosis. A wide range of such phytochemicals have been reported to induce growth inhibition of cancer cells by interfering with their deregulated cell cycle progression through modulation of the expression and/or activities of key proteins including cyclin D1, cyclin B1, CDK1, CDK2, p53 and p27^{kip1} (Neergheen et al., 2010a). For example, quercetin exerts its anti-tumor effect through blocking cell cycle progression at the G₀/G₁ interface (Cragg and Newman, 2005).

1.6.5 Angiogenesis: a potential target of chemoprevention

Angiogenesis is the process of new blood vessel formation in tumor growth and metastasis. Plant polyphenols in the presence of abnormal angiogenesis may provide strategies for halting the process of carcinogenesis (Ferrara and Kerbel, 2005). Tumor cells are characterized by the production of angiogenic factors such as vascular endothelial growth factor/vascular permeability factor (VEGF/VPF) and fibroblast growth factor-2 (FGF-2), which switch on an angiogenic phenotype of the tumor implant (Cross and Claesson-Welsh, 2001). In response to this, plant polyphenols block cancer progression by inhibiting the action of matrix metalloproteinases which

in turn block the degradation of the endothelial basement membrane, thereby preventing capillary sprout formation (Mathur et al., 2006). For example, epigallocatechin gallate (EGCG) which is a major component of tea, targets tissue plasminogen activator (t-PA), a proteases that enable tumors to metastasize (Jankun et al., 1997a). The targets of EGCG seem not only to be limited to endothelial cells and their inhibition of angiogenesis may result from the inhibition of matrix metalloproteinases (MMP-2 and MMP-9), as well as urokinase plasminogen activator (u-PA) at higher concentrations while a downregulation of VEGF production in tumor cells and subsequent repression of AP-1, NF- κ B and STAT 1 transcription factor pathways (Jung et al., 2001; Lin et al., 1999).

The major antiangiogenic mechanisms that natural phytochemicals (resveratrol, genistein, diadzein, apigenin) use, involve the down regulation of the angiogenic factors (MMP-9 and VEGF) as well as the up-regulation of tissue inhibitor metalloproteinases (TIMP)-1 which lead to reduction of tumor cell invasion and blood vessel growth (Neergheen et al., 2010a). Reduction of the oxidative stress by polyphenols leads to blockage of ROS formation and alterations in the cellular redox state, resulting in a reduced activation of transcription factors such as AP-1, p53 and NF- κ B, which regulate the expression levels of the key angiogenic factors VEGF. This suggests the importance of antioxidant rich plant extracts or compounds in the management of angiogenesis as a key regulator in chemoprevention (Neergheen et al., 2010a).

1.6.6 Upregulation of gap junctional intercellular communication (GJIC)

Another important target of chemoprevention involves the upregulation of gap junctional intercellular communication (GJIC). GJIC has been hypothesized to regulate growth, differentiation, apoptosis and wound healing and adaptive responses of differentiated cells. Data have shown that transfection of the connexin 32 or connexin 43 gene into GJIC-defective and neoplastic cells resulted in GJIC

restoration and reversion of the transformed phenotype while dominant-negative mutant Cx43 transfected cell has been shown to inhibit GJIC and enhance tumorigenicity (Neergheen et al., 2010a). Tumor cells are therefore characterized by dysfunctional GJIC resulting in a lack of growth control and resistance to apoptosis (Hix et al., 2004). Various tumor promoters and oncogenes downregulate GJIC (Matesic et al., 1994) while several dietary factors and medicinal extracts have been suggested to modulate GJIC by induction of various signal transducing systems which can prevent inhibition of GJIC and therefore suppress tumorigenesis (Neergheen et al., 2010a).

From the information above, dietary phytochemicals has been observed to possess properties against the initiation, promotion and progression of cancer. In line with this, the information below outlines research based information of tea (*Camellia sinensis*) in cancer chemo-prevention and therapy.

1.7 Tea (*Camellia sinensis*)

1.7.1 Background and processing.

Tea essentially signifies two or three leaves and the terminal apical buds of the tropical shrub *Camellia sinensis*, *Camellia assamica* and varieties. The plant was originally discovered and grown in south-east Asia 1000 years ago and according to the Chinese mythology, it was emperor Shen Nung who discovered tea in 2737 b.c. (Harbowy et al., 1997). It is now the most popular beverage, next to water, consumed by over two-thirds of the world's population. It is grown in over 30 countries, exclusively in the subtropical and tropical zones. The per capita worldwide consumption is 120 ml brewed tea per day (Gupta et al., 2002). It is rich in substances with antioxidant properties and contains traces of proteins, carbohydrates, amino acids and lipids, as well as more significant quantities of some vitamins and minerals. Tea is consumed as a hot water extract rather than being eaten as a plant. The three

most popular major tea types are green, black or oolong tea. Black tea constitutes about 80% of the tea manufactured in the world, and is mainly consumed in Western and some Asian countries. Green tea constitutes about 20%, and is mainly consumed in Asia, and a few countries in North Africa and Middle East and the rest 2% is oolong tea consumed in some parts of China and Taiwan (Gupta et al., 2002).

1.7.2 Processing

Green tea is prepared by pan-frying or steaming the tea leaves in order to inactive polyphenol oxidase activity in the leaves. At this stage, steps are taken to prevent the oxidation of green leaf polyphenols. Oolong tea is a partially oxidized product of green tea (Graham, 1992a). Oolong tea is only partly oxidized and because of that it contains reduced amounts of epigallocatechin-3-gallate (EGCG), epicatechin-3-gallate (ECG), epigallocatechin (EGC), epicatechin (EC), galocatechin (GC) (Gupta et al., 2002). By contrast, in the processing of black tea, fresh leaves are crushed and allowed to undergo a polyphenol oxidase mediated oxidation known as “fermentation”. In brief, oxidation of tea leaves is promoted during black tea production. And this results in the oxidation of the catechins to form catechin dimers, known as theaflavins, as well as polymeric thearubigins. These compounds are responsible for the characteristic color and taste of black tea.

1.7.3 Chemical composition

The major active constituents of tea are caffeine and polyphenols. In brewed green tea, caffeine content is 3–6% (Yang et al., 2006). The main classes of polyphenols present in green tea are flavanols and flavonols. These compounds constitute 16–30% of the dry weight of fresh leaf (Graham, 1992b). Catechins (flavan-3-ols) are the predominant form found. Fresh green tea leaves contain five major catechins: catechin (C), (–)-epicatechin (EC), (–)-epicatechin 3-gallate (ECG), (–)-epigallocatechin (EGC) and (–)-epigallocatechin gallate (EGCG). These compounds

are water-soluble and contribute to bitterness and astringency of green tea. Flavonols such as quercetin, kaempferol, myricetin and their glycosides can also be found in green tea (Balentine et al., 1997; Del Rio et al., 2004).

A typical cup of brewed green tea is made using 2 g of tea leaves in 200 mL of hot water and contains approximately 600–900 mg water extractable solids. Of these solids, approximately 30–40% by weight is the tea catechins. (–)-Epigallocatechin-3-gallate (EGCG) is the most abundant catechin and may represent up to 50% of the catechins by weight (Lambert and Elias, 2010). Green tea also contains condensed and hydrolyzable tannins (Engelhardt et al., 2004).

The tea leaves are distinguished by their content of methylxanthines, and polyphenols especially flavonols of the catechin type. Although its chemical composition varies with growing conditions, season, age of the leaves and variety cultivated, the major green tea polyphenols (GTP) are: (–)-epigallocatechin-3-gallate (EGCG), (–)-epicatechin-3-gallate (ECG), (–)-epigallocatechin (EGC), (–)-epicatechin (EC), (+)-gallocatechin (GC), (+)-catechin which together may constitute 30% of the dry leaf weight. Among these, EGCG is believed to be the most protective agent. In addition, caffeine, theobromine, theophylline and phenolic acids, such as gallic acid, are also present as minor constituents of green tea (Sang et al., 2011).

Black tea also contains several polyphenols such as bisflavonols, theaflavins (TF) and thearubigins (TR) (Ahmad and Mukhtar, 1999). Theaflavins contain benzotropolene rings with dihydroxy or trihydroxy substitution systems and exists as catechin dimers while the other polymeric polyphenols often called thearubigins are even more extensively oxidised and polymerised. The catechins from the green fresh leaves are preserved in the final dry green product, while about 80% of the fresh leaf catechins are biochemically oxidized in the manufacture of black tea (Balentine et al., 1997). So differences in the manufacturing process appear to be the only distinction between the three teas. Oolong tea is only partly oxidized and because of that it contains

reduced amounts of epigallocatechin-3-gallate(EGCG), epicatechin-3-gallate (ECG), epigallocatechin (EGC), epicatechin (EC), galocatechin (GC) (Gupta et al., 2002). And due to the partial oxidation oolong tea contains an intermediate content of flavan-3-ols (Kilmartin, 2003). Green tea contains the highest content of almost all identified flavan-3-ols when prepared at 100⁰C and a lower content of the flavan-3-ol monomers is in fully fermented black tea. Some of the minerals found in green tea include potassium, sodium, calcium, fluoride, aluminium, manganese and iron (Reto et al., 2007).

1.7.4 Chemopreventive properties of tea (*Camellia sinensis*)

Tea extracts have been observed to prevent carcinogenesis by inhibiting free radical formation. 12-*O*-tetradecanoylphorbol-13-acetate (TPA) can produce oxygen radicals and peroxides in lymphocytes. Treatment of the lymphocytes with different tea fractions showed that black tea extracts (BTE) and green tea extracts (GTE) inhibited free radical formation (Steele et al., 2000).

Black tea and green tea are being considered as chemoprotective agents because of their ability to protect the cell against initiation and promotion phases of carcinogenesis. All tea fractions for both teas were observed to enhance the induction of phase II enzymes, glutathione-*S*-transferase and quinone reductase (Prochaska and Santamaria, 1988). Tea extracts increase glutathione (GSH) conjugation with electrophiles and in the process decrease the level of reactive electrophiles available to bind to DNA. The inhibition of electrophiles from binding to DNA reduces the likelihood of the DNA damage and possible induction of the carcinogenesis process (Steele et al., 2000).

The possible mechanisms by which green tea polyphenols (GTPs) and epigallocatechin gallate (EGCG) prevent cancer include inhibition of free radical formation and lipid peroxidation (Steele et al., 2000). In addition there have been

reports of the inhibition of cyclooxygenase and lipoxygenase activity by tea compounds (Katiyar et al., 1992; Ho et al., 1992). Green tea catechins have been reported to inhibit 5 α steroid reductase isoenzymes. Besides this, EGCG and ECG were observed to be more potent inhibitors of type I than type II reductase (Liao et al., 1995). Katiyar also showed an induction of phase II glutathione *S*-transferase (GST) and quinone reductase (NADPH: QR) enzyme activity by green tea (Katiyar et al., 1992).

1.7.5 Proposed mechanisms of actions of Catechins

Catechins, especially EGCG, have been found to affect different signal transduction pathways, such as the inhibition of many protein kinases; suppression of the activation of transcription factors, AP-1 and NF- κ B; blocking growth receptor mediated pathways and induction of cell cycle arrest or apoptosis.

1.7.5.1 Modulating the activities of protein kinases

The actions of EGCG in chemoprevention appear to inhibit the phosphorylation of MEK1/2, ERK1/2, and ELK-1 as well as c-Jun (Yang et al., 2000). The ability of EGCG to competitively inhibit the phosphorylation of ELK-1 by ERK1/2 and possibly by competing for the binding site on ERK1/2 have been suggested as a reason for this capability (Siddiqui et al., 2006; Chen et al., 2003). In normal human epidermal keratinocytes, low concentrations of EGCG (less than 1 μ M) have been reported to promote cell proliferation and inhibit UV induced apoptosis through activation of ERK and AKT pathways (Chung et al., 2003; Kwon et al., 2007). The activation of NF- κ B involves the phosphorylation of I- κ B by IKKs; the phosphorylated I- κ B is degraded, setting NF- κ B free and allowing it to translocate into the nucleus to activate NF- κ B responsive genes. EGCG has been shown to inhibit the activation of NF- κ B in several cancer cell lines. This activity has been observed in H891 head and neck carcinoma cells, MDA-MB-231 breast carcinoma

cells, in A431 epidermal carcinoma cells (Yang et al., 2000) and in UV-irradiated normal human epidermal keratinocytes (Afaq et al., 2003). EGCG has also been reported to induce G₀/G₁ cell cycle arrest and this was associated with the inhibition of the activity of CDK2 and CDK4 and related retinoblastoma (Rb) hypophosphorylation. This has been reported to occur in MCF-7 breast cancer cells after treatment with 30 μM EGCG. In head and neck squamous carcinoma cells, EGCG has been observed to reduce the protein levels of cyclin D1, cyclin E, CDK2, CDK4, and CDK6 besides induction of G₀/G₁ phase cell cycle arrest (Tang et al., 2008).

1.7.5.2 Inhibition of DNA methyltransferase and telomerase

DNA methyltransferase is needed for the epigenetic regulation of gene expression by methylating cytosine in the Cytosine-phosphate-Guanosine (CpG) islands of the promoters of many genes. In the development of cancer, many functionally important genes are silenced by this hypermethylation mechanism. It is reported that EGCG inhibited DNA methyltransferase (K_i = 7 μM) and this inhibition resulted in the demethylation of the hypermethylated promoter and the reactivation of tumor suppressor gene p16INK4a, retinoic acid receptor beta, DNA repair genes hMLH1, and methylguanine methyltransferase in human esophageal squamous cell carcinoma KYSE 510 cells (Fang et al., 2003).

Telomerase is one of the enzymes that is over expressed in many human cancers (Artandi et al., 2002). It is responsible for the maintenance of nuclear protein endcaps of the chromosome (Sharpless and DePinho, 2004). Reports show that chronic administration of EGCG (5–10 μM) inhibited telomerase and induced cell senescence (Naasani et al., 1998). Tea polyphenols have also been reported to prevent the antiapoptotic activities of BH3 in the Bcl family of proteins (Yang et al., 2000; Yang et al., 2006). EGCG was reported to inhibit the chymotryptic activity of the 20S proteasome (Nam et al., 2001). And in LNCaP cells EGCG has been reported to

cause cell cycle arrest in the G₀/G₁ stage besides accumulation of p27 and I- κ B inhibitory proteins (Yang et al., 2006).

EGCG and other catechins have been reported to inhibit metastasis and invasion (Jung and Ellis, 2001) through increased expression of the tissue inhibitor of MMPs (TIMP1 and 2). Further to this, green tea polyphenols have been observed to inhibit insulin-like growth factor-1 (IGF-1)-induced signaling in autochthonous mouse model. These changes were found to be associated with decreased protein expression of PI3 kinase and lower levels of phosphorylation of Akt and Erk1/2. Furthermore, the treatment also inhibited markers of angiogenesis and metastasis: vascular endothelial growth factor, urokinase plasminogen activator, and MMPs 2 and 9 (Siddiqui et al., 2004).

1.7.5.4 Anti-mutagenicity

Black tea, green tea extracts and their polyphenols have been observed to inhibit nitrosation of methylurea (NMU). This compound is produced as a result of nitrosation of creatinine (CRN) and has been observed to induce cancer in a number of animal species. To this effect, using a model nitrosation system, Gupta et al., (2002) reports of strong inhibitory properties of tea against the nitrosation of methylurea. The antimutagenic activity of aqueous extracts of green, black, and decaffeinated black tea have been observed against indirect dietary carcinogens such as 2-Amino-6-methyldipyrido[1,2- a:3',2'-d]imidazole (Glu-P-1), Benzo[a]pyrene (B[a]P) and nitrosopyrrolidine. All the three types of tea were observed to prevent the actions of the three promutagens in the presence of an activation system (Bu-Abbas et al., 1996). Further to this, green tea catechins and its major component EGCG have been observed to suppress promutagenic 2-amino-3, 8-dimethylimidazo [4, 5-f] quinoxaline (MeIQx) and 2-amino-1-methyl-6-phenylimidazo [4, 5-b] pyridine (PhIP) found in cooked food (Oguri et al., 1998).

1.7.5.5 Anti-genotoxicity

Below is an outline of the protective effects of tea on DNA damage. 2-Amino-1-methyl-6-phenylimidazo (4, 5-b) pyridine (PhIP) is a pyrolysis product formed during the cooking of proteinaceous animal foods (meat, chicken, and fish). This product has been identified to be a carcinogen in F344 rats. Pre-treatment with green and black tea before gavage of PhiP (50mg/kg) provided chemoprotection against PhIP-DNA adduct levels in the colons of F344 rats (Huber et al., 1997). Besides this, tea and its polyphenols prevented the covalent binding of [3H] *N*-acetoxy PhiP to DNA (Lin et al., 1998). Further to this, green tea has demonstrated the ability to block oxidative DNA damage to the liver as well as hepatotoxicity in rats treated with 2-nitropropane (2NP) (Hasegawa et al., 1995).

1.7.5.6 Anti-oxidants/pro-oxidants

It is also reported that polyphenolic fractions of green tea exhibit antioxidant activity towards hydrogen peroxide and superoxide anion radicals in mouse hepatocytes and human keratinocytes (Zhao et al., 1997; Ruch et al., 1989). MatÉs et al., (1999) reports that the antioxidant defense mechanisms are there to protect cells from oxidative injury and include the enzymatic and non-enzymatic systems. Karihtala and Soini, (2007) mention examples of antioxidant enzymes to include catalase, SOD, glutathione peroxidase (GPx) and peroxiredoxin III (PrxIII) whereas the non-enzymatic antioxidants includes vitamins C, E, and B2, coenzyme Q10, glutathione and b-carotene. These ROS-scavenging systems are required to maintain cellular redox balance: when the amount of ROS exceeds the capacity of the antioxidant machinery oxidative stress occurs (Li et al., 2006). Green, oolong and black teas have been championed to possess greater antioxidant potential (Yen and Chen, 1995; Chan et al., 2011a). Studies have shown that the strong antioxidant properties of green tea are attributed to catechins of EGCG and EGC (Nanjo et al., 1999; Horžić et al., 2009). It has further been mentioned that the three adjacent hydroxyl groups on the

B-ring of EGCG, GCG, EGC, and GC are more effective in scavenging free radicals than the two adjacent OH groups of ECG, CG, and EC (Chan et al., 2011b; Inamdar et al., 2014).

The above chemopreventive and therapeutic effects demonstrated by tea and its catechins on different cancer cell lines have stimulated interest to investigate the effects of rooibos tisane on prostate cancer cell lines.

1.8 Rooibos tea

1.8.1 Background

1.8.1.1 Historical and modern uses

Historically, rooibos was used by the Khoi people of Clanwilliam region of the Western Cape as a beverage and as a source of medicine. Rooibos processing involved harvesting of the shoots during the summer months, followed by chopping using an axe and then crushing them with a mallet. The shoots in the bruised state were then fermented in hollows of stone reefs, followed by sun-drying. In the modern day society rooibos is consumed as herbal tea. It is consumed as a strong, hot brew with milk and sugar added. Away with the business of boiling the leaves and stems in water the modern day consumers use tea bags instead of loose-leaf tea and prepares the infusion in the same manner as Oriental tea, i.e. infusing one tea bag (ca. 2 g) per cup with freshly boiled water for 2–5 min to release flavour and colour, which is then served hot, with or without milk and sugar added according to taste. In South Africa during summer it is also enjoyed cold, usually with lemon juice and sugar added. Vanilla flavoured rooibos in tea bags is more readily available in Europe than unflavoured rooibos (Joubert et al., 2008a).

1.8.1.2 Geographical distribution

Different parts of the world have different climatic conditions. These climatic conditions together with the types of soils, acidity etc determines the type of vegetation that grows in those locations. Similarly the growth of particular herbal teas in a particular geographical area is influenced by the most optimum conditions that can promote their survival and growth.

Aspalathus linearis grows naturally in the Cederberg area encompassing the Citrusdal, Clanwilliam and Nieuwoudtville regions, situated in the western parts of the Western Cape Province of South Africa (Kotina et al., 2012)

1.8.2 Botany

The genus *Aspalathus* includes more than 270 species endemic to South Africa. *Aspalathus linearis* (Kotina et al., 2012) with its needle-like leaves is polymorphic with characteristic morphology and geographical distribution. Some forms are prostrate and remain less than 30cm tall, while other forms grow erect and may reach up to 2m in height. The size, density of branching, development of short shoots, leaf size and flowering time of the biotypes vary considerably (Kotina et al., 2012). The seeds are hard-shelled and need scarification to germinate (Kelly and Van Staden, 1985).

The biotypes can be divided into either reseeder or resprouter (Joubert et al., 2008a). There are also genetic and polyphenolic differences between various populations (Van Der Bank et al., 1995; Van Wyk et al., 1997). The red type, *Aspalathus linearis* currently used for commercial processing is divided into the selected and improved Nortier type (cultivated), and the wild-growing Cederberg type, with its broader and coarser leaves (Morton, 1983). *Aspalathus pendula*, closely related to *Aspalathus linearis*, also has needle-like leaves, but it is rarely used for tea manufacture (Van Heerden et al., 2003).

1.8.3 Phytochemical constituents of rooibos.

Rooibos tea is made from the leaves and fine stems of *Aspalathus linearis*, which is indigenous to South Africa (Morton, 1983). This herbal tea has dietary polyphenols with antioxidant properties (Marnewick et al., 2003). Aspalathin is the principal monomeric flavonoid in unprocessed rooibos tea (Van der Merwe and Debora, 2005). Apart from aspalathin, β -hydroxy-dihydrochalcones, pterosupin and nothofagin have been reported to be present in plant material (Joubert, 1996). Nothofagin is structurally similar to aspalathin except for the hydroxylation pattern of the B-ring. Other phenolic compounds present in rooibos tea are flavones which include iso-orientin, orientin, vitexin, iso-vitexin, chrysoeriol (Rabe et al., 1994) and luteolin (Joubert, 1996). Its flavonols include rutin, iso-quercitrin (Koeppen et al., 1962) and quercetin while the phenolic acids comprise p-hydroxybenzoic, protocatechuic, vanillic, caffeic, p-coumaric and ferulic acids (Rabe et al., 1994).

1.8.4 Biological Properties.

1.8.4.1 Anecdotal Health Effects.

Anecdotal evidence suggests that rooibos tea has healthy benefits based on the absence of alkaloids and low tannin content (Cheney and Scholtz, 1963). It was used in treating colicky babies, cured chronic restlessness, stopped vomiting and stomach cramps. Rooibos was also described to have anti-allergic effect, improved the appetite, reduced nervous tension and promoted sound sleep (Morton, 1983). It also alleviated indigestion, heartburn and nausea (Van Wyk et al., 1997). Topical applications of rooibos extract are believed to alleviate dermatological problems, i.e. eczema, acne and nappy rash, leading to the development of toiletries and cosmetic products, which are sold through supermarkets, farms stalls and beauty shops. Annetjie Theron was the first to develop a range of skin-care products containing

rooibos extract that eventually became well-established in South Africa (Joubert et al., 2008a).

1.8.4.2 Research Based Health Effects.

Evidence based research has proved that Rooibos tea has low tannin levels and does not contain caffeine or any alkaloids. It is because of this that rooibos tea was declared a healthy drink (Cheney and Scholtz, 1963). Further to this, Marnewick et al., (2000) and Hendricks et al., (2010) collaborate that rooibos extracts possess anti-mutagenic, anti-cancer and immune modulating properties. The health benefits have also been observed in individuals at risk of developing cardiovascular disease (Marnewick et al., 2003; Hendricks and Pool, 2010).

Despite the many benefits that one can get from rooibos, numerous studies have shown that flavonoid compounds found in this nutraceutical exhibit phytoestrogenic activity which may promote clinical conditions such as osteoporosis, breast cancer and cardiovascular disease (Arjmandi et al., 1996; Anthony et al., 1996). In this regard, Schlomsa et al. (2012) observed an inhibited steroidogenic activity of H295R cells, a human adrenal carcinoma cell line after being treated with the flavonoid compounds, aspalathin and nothofagin (Schloms et al., 2012). Further to this, some of the cytotoxic effects of rooibos flavonoids have been observed to act as mutagens, pro-oxidants and inhibitors of key enzymes (Skibola, 2000).

1.8.4.3 Anti-oxidant/ pro-oxidant properties

1.8.4.3.1 Antioxidant properties

Studies have reported the antioxidant activity of rooibos tea in cellular systems. Yoshikawa et al. (1990) observed that aqueous *A. linearis* extract inhibits the generation of O_2^- induced by phorbol myristate acetate in human polymorphonuclear

leukocytes (PMA-PMN) (McKay and Blumberg, 2007). In a study done by Ito et al. (1992) a time and concentration-dependent increase in survival rate of mouse leukemia cells was observed following exposure to 40 μM H_2O_2 and pre-incubation with rooibos extract (2.25 g leaves/200 mL water, boiled 20 min) (Inanami et al., 1995). Using a linoleic acid autoxidation system, Hitomi et al. (1999) observed that freshly brewed rooibos tea exhibited strong antioxidant activity in rat liver homogenate while the freeze-dried extract had a strong dose-dependent effect in erythrocyte and microsome systems (McKay and Blumberg, 2007). Further to this, Akaike et al. (1995) demonstrated the ability of rooibos to scavenge alkyl peroxy radicals formed during lipid peroxidation.

1.8.4.3.2 Pro-oxidant Activities.

Rooibos extracts have been observed to possess pro-oxidant activity. Under disease conditions $\bullet\text{OH}$ can be produced in the presence of iron. Low polyphenol concentration will result in low antioxidant activity and increase pro-oxidant activity. To this extent, unfermented rooibos has been observed to contain a high concentration of polyphenols. And using the so-called Fenton reaction, Joubert et al., (2005) found that the dihydrochalcone and flavonoid contents in unfermented rooibos, but not total polyphenol content, resulted in pro-oxidant activity that counteracted the antioxidant effect of the unfermented rooibos extracts (Fig 4.1).

1.8.4.4 Lipid Peroxidation.

Lipid peroxidation refers to a process that causes polyunsaturated fatty acid to turn rancid and is related to many pathological processes which lead to cancer, degenerative disease, and other diseases (Salonen et al., 1992). Lipid peroxidation initiators are reactive oxygen species (ROS) such as hydroxyl ($\text{OH}\bullet$) and peroxy radicals ($\text{ROO}\bullet$) and the superoxide anion radicals ($\text{O}_2\bullet^-$), which are formed by exogenous chemical factors and endogenous metabolic processes in the human body

or in food systems (Gülçin et al., 2004). After chronic administration in rats, rooibos tea was observed to suppress age-related accumulation of lipid peroxides in several regions of their brain (Inanami et al., 1995). As observed by Marnewick, (2003) in rat liver, rooibos tea reduced the levels of oxidized glutathione and increased the levels of reduced glutathione.

1.8.4.5 Anti-mutagenic Effects

The extracts of rooibos were observed to have anti-mutagenic properties. Using *Salmonella* mutagenicity assay against strains TA 98, TA 100 and TA 102 Marnewick et al. (2003) observed that hot water extracts of rooibos teas interfered with carcinogen activation in liver homogenate fractions via interaction with the phase I drug metabolising enzyme, cytochrome P450. Besides interference with carcinogen activation, rooibos provided protection against cancer. After treating rats with fermented and unfermented rooibos Marnewick et al. (2003) observed an increase in the activity of microsomal UDP-glucuronosyl transferase. The oxidative status of the liver was significantly altered by increasing the ratio of reduced glutathione to oxidised glutathione (GSH: GSSG). The modulation of these parameters increased the removal of reactive mutagenic metabolites in vivo via phase II reactions, thereby effectively protecting against carcinogen tissue interactions (Marnewick et al., 2003).

1.8.4.6 Hepatoprotective effects

In order to determine the ability of rooibos to provide hepato-protection Ulicna et al., (2003) administered carbon tetrachloride (CCl₄) to male Wistar rats twice weekly for 10 weeks leading to the development of steatosis and cirrhosis. Follow up experiments done by Kucharska et al., (2004) showed that rooibos increased the antioxidant status of the CCl₄ damaged livers by increasing the tocopherol and reduced coenzyme Q9 levels to values comparable to healthy animals.

1.8.4.7 Phyto-oestrogenic properties

Phyto-oestrogens are non-steroidal, polyphenolic secondary metabolites from plants with a structural and functional similarity to the endogenous human hormone, oestrogen (Kuhnle et al., 2009). Phyto-oestrogens are considered as alternatives for the treatment of menopausal symptoms and steroid hormone dependent cancers such as breast, prostate, endometrial and colon cancer (Joubert et al., 2008a). Following a methanol extract of rooibos Shimamura et al. (2006) reported to have isolated 25 compounds from which nothofagin was observed to have the highest oestrogenic activity, while three other compounds, isovitexin, luteolin-7-glucoside, and hemiphlorin, displayed moderate oestrogenicity. Luteolin, quercetin, eriodictyol and some of their glycosides, were also present, but did not display appreciable oestrogenic activity despite the fact that binding of these molecules to the estrogen receptor (ER) had been reported ((Joubert et al., 2008b).

1.8.4.8 Immune responses.

Kunishiro et al. (2001)) observed that 1-100ug/mL rooibos tea extracts was able to stimulate the antigen-specific antibody production in murine splenocytes. However, it could not stimulate the non-specific antibody response elicited with lipopolysaccharide (LPS). In studies done by Nakano et al. (1997a; 1997b), acid polysaccharides extracted from the leaves of *A. linearis* suppressed the cytopathicity of HIV (HTLV-III) infected MT-4 cells, while polysaccharides from Japanese green tea leaves and a hot water extract of *A. linearis* did not (McKay and Blumberg, 2007).

1.8.4.9 Modulation of carcinogen metabolising enzymes

Rooibos has been observed to protect liver of rats from carcinogens by modulating drug metabolizing enzymes. For example, a 10-week exposure of rats to extracts of fermented and unfermented rooibos as sole source of drinking fluid significantly

increased the activity of the cytosolic glutathione *S*-transferase. The unfermented teas significantly increased the activity of microsomal UDP-glucuronosyl transferase. The oxidative status of the liver was significantly altered by increasing the GSH: GSSG ratio (ratio of reduced glutathione to oxidized glutathione) due to increased GSH and decreased GSSG levels. The modulation of these parameters helped to increase the removal of reactive mutagenic metabolites in vivo via phase II reactions, a development that effectively protected against carcinogen tissue interactions (Marnewick et al., 2003).

1.8.4.10 Antispasmodic effects

Rooibos was observed to initiate K⁺ channel activation and smooth muscle relaxation in asthmatic cases (Gilani et al., 2006). To this effect a concentrated rooibos extract, containing approximately 120 and 199mg quercetin equivalents/g extract of total polyphenols and flavonoids, respectively, exhibited bronchodilatory, antispasmodic and blood pressure lowering effects in experimental animals including rabbits, guinea-pigs and rats. Rooibos was able to relax the K⁺-induced contractions in guinea-pig trachea and rabbit aorta, while the mean arterial blood pressure in rats was significantly reduced in a dose dependent manner (Khan and Gilani, 2006).

1.8.4.11 Vasodilatory effect and improved glucose homeostasis

Persson et al. (2006) investigated the effect of rooibos on the angiotensin-converting enzyme (ACE) which among others, catalyses conversion of angiotensin I to angiotensin II, a potent vasoconstrictor, and production of the vasodilator, nitric oxide (NO), using cultured endothelial cells from human umbilical veins as model system. Incubation of the cells with rooibos for 10 min showed no significant inhibition of ACE, but 24 h incubation resulted in a significant dose dependent increase in NO production. The improvements in the cardiovascular system were also observed by Persson et al. (2010) in that a single oral intake of rooibos significantly inhibited

angiotensin-converting enzyme (ACE) activity. Besides inhibiting ACE, in vivo studies have shown that rooibos stimulates an increased glucose uptake in muscle tissues and insulin secretion from pancreatic cells leading to improved glucose homeostasis and prevention of type II diabetes (Kawano et al., 2009).

1.8.5 Aim of this study

Previous work by different investigators have shown the chemotherapeutic effects of tea (*Camella sinensis*) and rooibos (*Aspalathus linearis*) on different cancer cell lines except those of the prostate. To this extent and to the best of our knowledge no studies investigating the effects of rooibos (*Aspalathus linearis*) have been done on prostate cancer cells. Therefore, the present study investigated the in vitro effects of black and green tea, fermented and unfermented rooibos as well as their main active compounds epigallocatechin gallate (EGCG) and aspalathin, respectively, on prostate cell lines based on the following parameters.

- Cell viability of benign (RPWE 1) and malignant (LNCaP) prostate cell lines.
- Quantification of reactive oxygen species (ROS) following treatment with the different types of teas, rooibos, EGCG and aspalathin.
- Apoptotic effect of tea, rooibos, aspalathin and EGCG on both benign (RPWE 1) and malignant (LNCaP) prostate cells.
- Necrotic effect of tea, rooibos, aspalathin and EGCG on both benign (RPWE 1) and malignant (LNCaP) prostate cells.
- Quantification of prostate specific antigen (PSA) levels; a biomarker for prostate cancer following exposure to tea, rooibos, aspalathin and EGCG.

CHAPTER TWO

2. MATERIALS AND METHODS

2.1 Chemicals

2.2.1 Gibco, Germany supplied:

- Foetal bovine serum (10500)
- Sodium bicarbonate (25080)
- Sodium pyruvate (11360)
- 0.25 % Trypsin / Ethyl Diamine Tetra Acetic acid (EDTA; 25200)
- RPMI 1640 (B-4800)
- Annexin V/PI apoptosis kit (A10788)
- CM H₂DCFDA (C6827)
- Keratinocyte serum free medium (17005-034)

2.1.2 Sigma-Aldrich, Germany supplied:

- Dimethylsulphoxide (DMSO; D2438)
- 4',-6-Diamidino-2-phenylindole dihydrochloride (DAPI)
- Poly-L-Lysin solution
- Thiazoyl blue tetrazolium bromide (MTT)
- Trypan Blue

2.1.5 Roche, Germany supplied:

- MTT.

2.1.6 Oxoid, England supplied:

- Phosphate Buffered Saline (PBS)

2.2 Tissue Culture Ware and Instruments.

2.2.1 Corning, South Africa supplied:

- 96 well plates
- Pipettes

2.2.2 Lasec, Germany supplied:

- Cover slip

2.2.3 Greiner Bio-one, Germany supplied:

- Tissue Culture dishes

2.2.4 Saarchem, South Africa supplied:

- Dimethylsulphoxide (DMSO)

2.2.5 DRG instruments GmbH, Germany.

- PSA total ELISA (EIA- 3719)

2.2.6 Becton, Dickinson and Company (BD).

- BD accuri c6 flowcytometer.

2.3 Plant extracts:

- **Rooibos**

The rooibos tea was a free gift from Rooibos Ltd (Clanwilliam, South Africa). Aspalathin was obtained from HWI Analytik GMBH (product no. 0352-05-85;

Rueizheim, Germany). 2.1 mg of aspalathin was dissolved in 1.6 ml sterile Dimethylsulphoxide (DMSO) (Sigma- Aldrich; St Louis, MO, USA). Since aspalathin does not dissolve in water; DMSO was used to facilitate the dissolution of aspalathin in culture medium. The concentration of the stock solution was 2.9 mM. This stock solution was aliquoted into sterile eppendorf cups at a volume of 50 μ l and stored at -20°C .

- **Black and green tea.**

Black and green teas were commercially obtained in South Africa. Both teas were five roses Ceylon brands (Entyce Beverages, Durban. South Africa). EGCG was purchased from Sigma-Aldrich (product no. E4143; St Louis, MO, USA). EGCG stock solution was prepared by weighing 2.6 mg of the solute and dissolving it in 1.6 ml sterile Dimethylsulphoxide (DMSO) (Sigma- Aldrich; St Louis, MO, USA). EGCG is immiscible in water and in order to facilitate its dissolution in culture medium; EGCG was dissolved in DMSO. The concentration of the stock solution was 3.54 mM. This stock solution was aliquoted into sterile eppendorf at a volume of 50 μ l and stored at -20°C .

2.4 Cell line

A normal human prostate epithelial cell line (RPWE-1 CRL 11609) and a malignant prostate cell line (LNCaP CRL 1740) were obtained from the American Type Culture Collection (ATCC) (Rockville, MD, USA). The malignant (LNCaP) prostate cell line was used because it possesses characteristics that are similar to those found in the stratified epithelium of the prostate gland. Malignant (LNCaP) prostate cells express AR and PSA. Besides this, its growth is inhibited by androgen withdrawal which is very similar to secretory/luminal cells of the prostate epithelium. The benign (RWPE 1) prostate cells were chosen because they mimic normal prostatic epithelial cell

behavior in their response to growth factors and in their expression of PSA and androgen receptor in response to androgen exposure (Bello et al., 1997).

2.5 Preparation of tea extracts

Tea extracts for unfermented and fermented rooibos, green tea and black tea were prepared by weighing 25 g of each tea (fermented and unfermented rooibos, black tea and green tea). Following this, the 25 g of each tea was put in 500 ml of tap boiled water and left to stand for five minutes. This produced tea with a concentration of 5%. The tea was then filtered using cheese cloth. With the help of a vacuum pump the tea extracts were further filtered using whatman filter papers number 4 and 1. The extracts were placed in a freezer at -20°C for two days before mounting on a freeze drying machine for three days whereupon their weight was determined by subtracting the weight of the flasks before freeze drying. After freeze drying, the powdered tea extracts were put in sealed tubes and stored in at -20°C .

2.6 The prostate cell line.

2.6.1 Thawing and Culturing

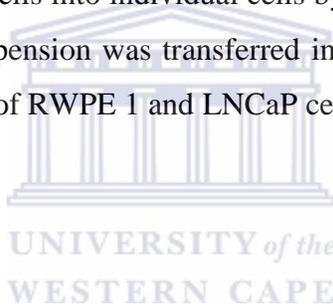
The frozen cells obtained from ATCC were thawed by gentle agitation in a 37°C water bath and sterilized by spraying with 70% ethanol. This was followed by the transfer of 1 ml of LNCaP and RWPE-1 cells to two separate T25 tissue culture flasks each containing 5 ml RPMI-1640 and keratinocyte serum free medium, respectively. All the flasks were supplemented with 10% foetal bovine serum (FBS) and 5 ml of Penicillin (10 000 U) and Streptomycin (10 mg). All the cell lines were maintained at 37°C in a humidified incubator supplied with 5% CO_2 . After 24 hours of resuscitation medium was changed in both cell lines. Thereafter, medium was changed twice every week since it gets acidified rapidly (Horoszewicz et al., 1983).

2.6.2 Sub cultivation of cells

Upon attaining 70-80% confluency, the cell lines were sub cultured to prevent cell death due to over population, lack of space and nutrients. This was done by removing the spent medium with a pipette. The cells were then washed twice with about 3 ml PBS without $\text{Ca}^{2+}/\text{Mg}^{2+}$ to remove toxins and acids that might have accumulated and all traces of serum that contains trypsin inhibitor. After which, 1 ml of 0.25% trypsin or trypsin/EDTA was added to the flask to dislodge the cells and break the cell clusters. To that effect, cells were incubated for about 5 minutes at 37°C. The microscope was used to check if cells were detached from the surface of the flask. The remaining trypsin was inactivated by adding 5 ml of fresh complete medium before re-suspending the cells into individual cells by gentle pipetting using a pipette. Then 2 ml of the cell suspension was transferred into another flask containing 5 ml fresh medium. Each flask of RWPE 1 and LNCaP cells was split into 3 flasks.

2.6.3 Freezing of cells

At 70-80 % confluency of cells, the cells were frozen away following the same procedure as the sub culturing except that after the cells are detached; together with 5 ml of fresh complete culture medium were transferred into a centrifuge tube and centrifuged at 1500 rpm for 10 minutes. After which, the supernatant was removed leaving a pellet behind. This was followed by the addition of 1ml complete medium with 10% DMSO (freezing medium) and aspirated before transferring into a cryovial. The cryovials were then put onto ice (-20°C) in a styrofoam box for 30 minutes. Slow cooling was done in order to prevent the development of water crystals which can kill the cells. The cells were then transferred and stored at -80°C overnight. The next day, cells were removed from the Styrofoam box and stored at -80°C.



2.6.4 Plating of cells

At 70-80% confluency, spent medium was removed with a pipette and the cells were washed twice with 3 ml PBS without $\text{Ca}^{2+}/\text{Mg}^{2+}$. To each flask, 1 ml trypsin/EDTA was added, followed by incubation of the cells for 5 minutes at 37°C . The cells were then examined under the microscope to make sure that all the cells were detached from the surface of the flask.

To inactivate trypsin, 3 ml of the culture medium was added to each flask and the suspended cells were transferred into a centrifuge tube and centrifuged at 1500 rpm for 10 minutes. Thereafter, the medium was removed from the tube and to the pellet 1 ml fresh medium was then added and aspirated. To count cell numbers 10 μl of trypan blue and 10 μl cell suspensions were put into an eppendorf cup thereby give a dilution factor of 2. Following that, 10 μl of this cell suspension was transferred to a haemocytometer counting chamber in order to count the total number of cells. The formula below was used to calculate the total number of cells in 1 ml.

$$\text{Mean} * 10000 * \text{dilution factor (2)} = \text{cells/ml}$$

The number of cells to be plated on a 96 well plate was found using the formulae below:

$$\frac{\text{Number of cells needed} * \text{Total volume} * 1000}{\text{Total number of cells counted}} = \text{volume of cell required } (\mu\text{l})$$

Total number of cells counted

The preliminary experiments used 4000 LNCaP cells per 300 μl in 96 well plates

2.7 Determination of Cell Viability.

2.7.1 MTT Assay

Viability of the malignant (LNCaP) and benign (RPWE 1) prostate cells was measured using the MTT assay. MTT assay is dependent on the cellular reduction of yellow MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] by mitochondrial dehydrogenases of viable cells to a blue, insoluble formazan product which can be measured spectrophotometrically.

In order to determine the viability of the cells, 5 000 cells/ml (i.e. 1000 cells/200 μ l culture medium) were cultured in a 96 well plate for 24 hours to allow cellular attachment. The next day, the medium was removed and the adherent cells were exposed to different concentrations (125, 250, 500, 1 000, 5 000, 10 000 μ g/ml) of EGCG and aspalathin. Besides exposing both cell lines to the above mentioned concentrations of black tea, green tea, fermented, unfermented rooibos, EGCG and aspalathin the cell lines were also exposed to 50 000 μ g/ml of tea and rooibos for 24 and 72 hours, respectively. The negative control consisted of untreated cells in culture medium and the positive control consisted of cells treated with 6% DMSO. The cells were incubated for 24 and 72 hours in order to investigate the effects of acute and chronic exposure of the prostate cells to the teas. 50 000 μ g/ml of EGCG or aspalathin was not used as our preliminary experiments showed a drastic reduction in cell numbers and a huge increase in damaged cells.

MTT stock solution was prepared by dissolving 1 mg MTT/ ml PBS. The dissolving process was facilitated by rotating for approximately 1 hour at room temperature. MTT solution was filtered using a syringe filter of 0.1 μ m. First of all tea extracts were removed from all the wells and there after the cells were washed with PBS. Subsequently 200 μ l new medium was added. To this medium, a volume of 20 μ l MTT was added (i.e. making one-tenth of the culture volume) and incubated for 3

hours at 37⁰C. Thereafter, the supernatant was removed by aspiration and the plate was tapped upside down on paper towels to remove any remaining fluid. 100 µl of DMSO was then added to each well to dissolve the precipitated dye. Absorbance of the dye was measured with an ELISA reader (Thermo electron corporation, South Africa) at a wavelength of 560 nm with a reference wavelength of 750 nm.

2.7.2 Light microscopy

In order to determine the viability of the cells using light microscopy, cells were seeded at a density of 5 000 cells per ml of culture medium for the 24 and 72 hour exposure periods, respectively. The cells were cultured in sterile 24-well plates. The cells were then allowed to attachment for 24 hours. Subsequently, the medium was removed and adherent cells were exposed to different concentrations (125, 250, 500, 1 000, 5 000, 10 000 µg/ml) of EGCG and aspalathin. On top of the above mention concentrations; both cell lines were exposed to a further 50 000 µg/ml of black tea, green tea, fermented and unfermented rooibos for 24 and 72 hours, respectively. After discarding the culture medium, cells were washed with 1 ml PBS. Fresh medium (1ml) was then added followed by the capture of 10 representative phase contrast photographs at 100x magnification. The negative control consisted of untreated cells in culture medium and the positive control consisted of cells treated with 6% DMSO. The cells were incubated for 24 and 72 hour in order to test the effects of acute and chronic exposure to the teas. 50 000 µg/ml of EGCG or aspalathin was not used as our preliminary experiments showed a drastic reduction in cell numbers and a huge increase in damaged cells.

2.8 Determination of Reactive Oxygen Species (ROS) using 5-(and-6)-Chloromethyl-2', 7'-Dichlorodihydrofluorescein diacetate, acetyl ester (CM-H₂DCFDA).

Determination of reactive oxygen species levels was done using CM-H₂DCFDA dye. CM-H₂DCFDA is a chloromethyl derivative of H₂DCFDA that is used as an indicator for reactive oxygen species (ROS) in cells. CM-H₂DCFDA has the property of diffusing passively into cells. In this form this dye is non fluorescent and only becomes fluorescent after cellular oxidation and removal of acetate groups by cellular esterases. The presence of an additional thiol reactive chloromethyl group enhances the ability of the compound to bind to intracellular components and in the process prolongs the dye's cellular retention.

In brief, the protocol used in this ROS assay involved dissolving 50 µg CM-H₂DCFDA in 150 µl DMSO. This stock solution was used to prepare 12.36 ml of 7 µM CM-H₂DCFDA. Prostate cell lines were seeded in 24 well plates at a density of 5 000 cells per ml. After 24 hours (following attachment) the cells were then treated with black tea, green tea, fermented rooibos, unfermented rooibos, EGCG and aspalathin. LNCaP positive control cells were treated with 50 µM Hydrogen peroxide (H₂O₂) for 30 minutes. RPWE 1 positive control cells were treated with 100 µM H₂O₂ for 10 hours. Initially, 4 different concentrations of H₂O₂ (25 µM, 50 µM, 100 µM and 200 µM) were used. In the final analysis, after repeating this experiment for 3 times, 100 µM H₂O₂ was adopted as a positive control inducer. The 50 µM H₂O₂ solution was prepared by dissolving 100µl 30% H₂O₂ into 5.9 ml media (diluent). This produced a 0.5% H₂O₂ solution = 50 µM H₂O₂. The negative control, positive control and 72 hour treated cells were harvested from the 24 well plates using 0.25% trypsin into 15 ml centrifuge tubes, spun down for 6 minutes at 1500 rpm.

The pellet formed was then re-suspended in 400 ul PBS containing 7 µM CM-H₂DCFDA and incubated at 37°C in the dark for 30 minutes to load the dye. This was

followed by the addition of 4 ml PBS to the tube in order to remove excess dye. The tube was then centrifuged at 1500 rpm for 6 minutes. The supernatant was discarded and the pellet re-suspended in 400 µl PBS before transferring to eppendorf tubes for analysis on the FL 1 channel of the BD accuri 6 flow cytometer. The excitation frequency was between 492 and 495 nm whereas the emission frequency was between 517 and 527 nm. (N.B. Untreated cells not loaded with dye were used as a negative control to examine cellular auto-fluorescence).

ROS was also analysed using an inverted Zeiss Axiovert 200M fluorescence microscope (Zeiss, Göttingen, Germany) was used to determine ROS. For this to be achieved benign (RPWE 1) and malignant (LNCaP) prostate cells cultured in an 8-well Ibidi slide were exposed to increasing concentrations of GT, BT, FR, UR, EGCG and ASP for 24 and 72 hours. The cells were then incubated with 7 µM CM 5,6 CM H₂DCFDA for 45 minutes at 37⁰C. levels. In this thesis, 10 representative photographs were taken for each sample at 100x magnification. The principle of fluorescence microscope is based on the specimen being illuminated with light of a specific wavelength which is then absorbed by the fluorophores, In this case; 490 nm excitation wavelength and 530 nm emission wavelength were used. The illumination light is separated from the much weaker emitted fluorescence through the use of a spectral emission filter. The green filter set was used together with 490 nm excitation wavelength and 530 nm emission wavelengths.

2.9 Determination of early apoptotic events by means of Alexa fluor 488 annexin V and Propidium iodide Tali Apoptosis Kit using flow cytometry.

The principle behind the annexin V apoptotic assay is that in normal live cells, phosphatidyl serine (PS) is located on the cytoplasmic surface of the cell membrane. However, in apoptotic cells, PS is translocated from the inner to the outer leaflet of the plasma membrane. This exposes the PS to the external cellular environment, (van Engeland et al., 1998) thereby marking the cell for recognition and phagocytosis by

macrophages (Fadok et al., 1992; Fadok et al., 1993). In respect of this, the human anticoagulant, annexin V, which is biotinylated with a fluorophore and has a high affinity for PS, is able to identify apoptotic cells by binding to PS exposed on the outer leaflet (Koopman et al., 1994).

For this apoptosis assay we used a commercially available Alexa Fluor® 488 annexin V/Dead Cell Apoptosis Kit with Alexa® Fluor 488 annexin V and PI for flow cytometry. The kit contains recombinant annexin V conjugated to the green fluorescent Alexa Fluor® 488 dye. In addition, the kit contains a red-fluorescent Propidium iodide (PI) nucleic acid binding dye. This dye is impermeant to live cells and apoptotic cells, but stains dead cells with red fluorescence, binding tightly to the nucleic acids in the cell. After staining a cell population with Alexa Fluor® 488 annexin V and PI in the provided binding buffer, apoptotic cells show green fluorescence, dead cells show red and green fluorescence, and live cells show little or no fluorescence. The above mentioned populations of cells were monitored using the BD accuri flow cytometer using an excitation wavelength of 488 nm.

The set up of the experiment involved preparation of samples for treatments and compensation tests. The compensation samples consisted of two negative controls (cells with no dye and cells stained with both annexin V and PI), three samples for necrosis (cells stained with annexin V only, cells stained with PI only and cells stained with both annexin V and PI) and three positive control samples (apoptotic cells stained with annexin only, apoptotic cells stained with PI only and apoptotic cells stained with both PI and annexin V).

To induce apoptosis, 6% DMSO was used on both RPWE 1 and LNCaP cells. In a previous study, 5% DMSO in RPMI was used in LNCaP cells in order to inhibit cryopreservation-induced apoptosis (Baust, 2005). In another previous study to evaluate the possible apoptotic effects of monoclonal antibodies, C5-I, and C5-II, on DU-145 and LNCaP cells; 10% DMSO in complete medium and 3 hour incubation (10% in CM10 for 3 hours) was used as a positive control (Nejatollahi et al., 2013).

Further to this, DMSO induces apoptosis by promoting the collapse of the mitochondrial membrane potential, release of cytochrome c from the mitochondria besides activation of caspase 9 and 3 and downregulation of Bcl- 2 (Liu et al., 2001).

In RPWE 1 cells necrosis was induced using 100 μM H_2O_2 (positive control) with 15 hour incubation whereas in LNCaP cells, 50 μM H_2O_2 (positive control) with 60 minute incubation produced the expected results. The above results were achieved after performing four independent experiments using 20 μM – 1000 μM H_2O_2 and each experiment was done in triplicate. Freitas et al. (2012) reported that 500 μM H_2O_2 inhibited cell proliferation in RPWE 1 and its effects were more pronounced following 72 hours of treatment. This concentration produced necrotic cell death in HPV10. In another previous study, using endothelial cells (Lelli et al., 1998) observed apoptosis after exposure to 100 μM H_2O_2 without an alteration in ATP levels. However, when 5 mM H_2O_2 was used there was a significant decrease in ATP levels and the cells went into necrotic cell death. In the present study, treated samples consisted of cells treated with different concentrations of black tea, green tea, fermented rooibos, unfermented rooibos, EGCG and aspalathin. For the treated samples, three independent experiments were performed and each treatment was prepared in triplicate.

The cells were seeded at a density of 5 000 cells per ml of complete RPMI 1640 medium for 24 or 72 hour exposure period in sterile 24-well plate, respectively. Cells were then exposed to 1 000 $\mu\text{g/ml}$ of black tea, green tea, fermented rooibos, unfermented rooibos, EGCG and aspalathin. Our preliminary studies using (125 – 50 000 $\mu\text{g/ml}$) BT, GT, FR, UR, ASP and EGCG showed that 1 000 $\mu\text{g/ml}$ of the aforementioned samples was enough to induce at least 50% of apoptotic deaths in malignant LNCaP cells. Based on this, we considered using this concentration as a frame of reference to determine the percentage of apoptotic and necrotic prostate cells. In brief, after discarding the culture medium, cells were washed with 1ml PBS and trypsinated with 250 μl of 0.25% trypsin/EDTA. After trypsination, cell

suspensions were transferred from 24-well plates to 15 ml centrifuge tubes and cell pellets were collected by centrifugation at 1500 rpm for 6 minutes.

The pellet was then re-suspended in 200 ml of binding buffer (provided by the kit). Annexin V staining was accomplished following the product instruction (Clontech, Palo Alto, CA). In brief, 5 μ L Alexa Fluor® 488 annexin V (Component A) and 1 μ L 100 μ g/mL PI working solution (prepared in step 4) were added to each 100 μ L of cell suspension cells were incubated at room temperature for 15 minutes. After the incubation period, 400 μ L 1X annexin-binding buffer was added and mixed gently. All the samples were kept on ice and then immediately taken for flow cytometry analysis using a fluorescence emission at 530 nm (green) for the FL1 channel and 585 nm (red) for the FL3 channel. The population of cells was separated into three groups: live cells, apoptotic cells and dead cells.

2.10 Determination of Prostate Specific Antigen (PSA) using ELISA kit

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 (Lilja et al., 1991).

In order to determine PSA levels, both RPWE 1 and LNCaP cells were plated at a density of 3×10^4 cells / well in 1 ml of keratinocyte serum free media and complete RPMI 1640 medium, respectively. The cells were cultured in sterile 24-well plates and grown to 80% confluency. Culture media were discarded after the incubation period and cells were washed with PBS. Cells were then treated with black tea, green tea, fermented rooibos, unfermented rooibos, EGCG and aspalathin for 24 and 72 hours, respectively. Thereafter, the cell culture supernatants were collected, put in 96 well culture plates and frozen at -20°C until PSA determination. In this study, we

were expected to determine PSA levels both at 24 and 72 hours. However, because of financial constraints we did not assess the PSA levels at 24 hours. Instead we determined the PSA levels at 72 hours only.

On the day of PSA determination; all reagents, zero standards, low and high controls from the PSA kit and samples were brought to room temperature. Meanwhile a design of how the samples were to be loaded on the microplate was prepared with each sample to be tested in triplicate. Positions of wells together with their respective samples, zero standards and low and high control were documented to ensure proper identification later. Unused micro well modules were zipped into a lock bag with a desiccant and stored at 5 °C. After this, 25 µL of standards, controls or samples were pipetted into each well. Samples and the standards were then incubated for 5 minutes at room temperature.

This was followed by the addition of 100 µL of PSA conjugate into each well. The plate was moved on the table for 10 seconds in order to mix thoroughly. The plate was then incubated for 1 hour at room temperature (18 °C - 25 °C) whereupon solution from the wells was removed by aspirating the liquid using a 12 channel multi-well pipette. Thereafter, the plate was tapped on an adsorbent paper to remove residual liquid. The wells were then washed 6 times using 250 µL distilled water per well. With every wash the water was allowed to settle in the wells for 15 seconds before removing it. 100 µL Tetramethylbenzidine (TMB) substrate solution was then pipetted into each well followed by 20 minute incubation at room temperature (18 °C - 25 °C). Thereafter, 100 µL/well stop solution was added to the wells in the same order as the substrate solution. The absorbance were read at 450 nm with a spectrophotometer plate reader (Offenburg, Germany) and the concentration of PSA in LNCaP and RPWE 1 were determined according to the standard curves (Fig 2.1 and 2.2) respectively.

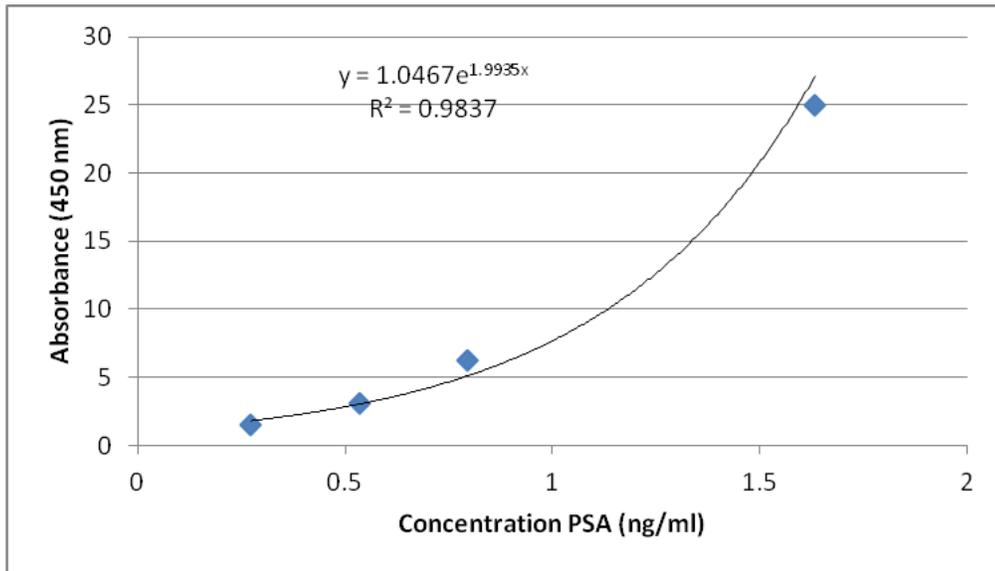


Fig 2-1: Standard curve for total serum prostate specific antigen ELISA in RPWE 1 cells. The standard curve shows that there is a good correlation ($R^2 = 0.9837$) between absorbance and PSA concentration.

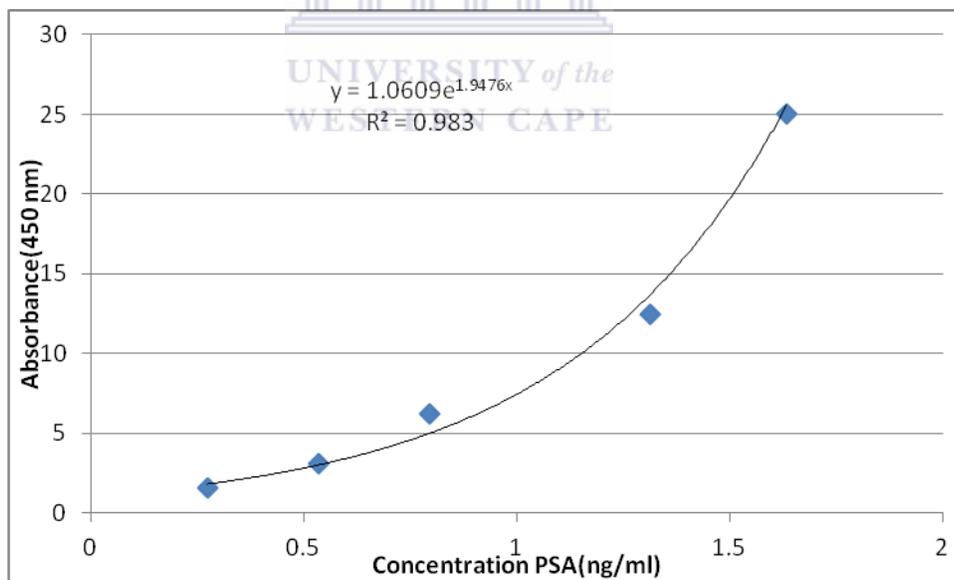
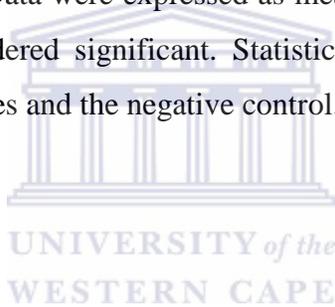


Fig 2-2: Standard curve for total serum prostate specific antigen ELISA in LNCaP cells. The standard curve shows that there is a good correlation ($R^2 = 0.983$) between absorbance and PSA concentration.

2.11 Statistical analysis

All statistical calculations were done using GraphPad Prism (Version 5.03, San Diego, USA). After testing for normal distribution using Kolmogorov-Smirnov test, appropriate statistical tests were done using either parametric, one-way ANOVA, repeated measures ANOVA or paired samples t-test. One-way ANOVA's (two-tailed) was used to test for significant group differences followed by the Tukey–multiple comparison tests in order to establish which groups differed significantly. This went together with the creation of statistical tables for each. The paired t-Test, a non-parametric analogue to the one-way ANOVA was used to test for group differences when the data was not normally distributed. Statistical significance was at the 5% level ($P < 0.05$). Data were expressed as mean \pm SD. A p-value of $p = 0.001$ and $P < 0.05$ was considered significant. Statistical comparisons were conducted between the treated samples and the negative control.



CHAPTER THREE

3. RESULTS

3.1 Cell Viability

LNCaP as well as RPWE 1 cells were exposed to increasing concentrations of aqueous extracts of *Camellia sinensis*, *Aspalathus linearis* and their active compounds epigallocatechin gallate (EGCG) and aspalathin, respectively, for 24 and 72 hours. During this period, morphological studies were done with the aim of investigating possible cytotoxic effects of black tea (BT), green tea (GT), fermented rooibos (FR), unfermented rooibos (UR), epigallocatechin gallate (EGCG) and aspalathin (ASP) on malignant (LNCaP) or benign (RPWE 1) prostate cells using light microscopy and the MTT assay. In this study, both prostate cell lines were exposed to 125 – 10 000 µg/ml of aspalathin and EGCG and to 125 – 50 000 µg/ml of BT, GT, FR and UR.

The highest concentration of 50 000 µg/ml aspalathin and EGCG was not used in this study because our preliminary experiments had proved this concentration to be very cytotoxic. This concentration completely damaged and killed both the normal RPWE 1 and malignant LNCaP prostate cells. All experiments were repeated as at least three independent experiments.

3.1.1 Malignant (LNCaP) prostate cell viability

3.1.1.1 Effect of black tea (BT) on malignant (LNCaP) prostate cells following 24 hour incubation.

The results obtained after exposing malignant (LNCaP) prostate cells to BT for 24 hours show no obvious changes in cell morphology between treated groups 125-1 000 $\mu\text{g/ml}$ BT (C-F) compared to the control (A). The cells maintained their flat and polygonal shapes. However, at higher concentrations 5 000 and 10 000 $\mu\text{g/ml}$ BT (G-H) cells started to show signs of stress. Cells attained irregular shapes appeared in clumps and clusters when compared to the control (A). Clear characteristics of dead cells were observed at 50 000 $\mu\text{g/ml}$ BT (I) as most cells were detached and looked similar to those of the positive control (6% DMSO) (B) (Fig 3.1).

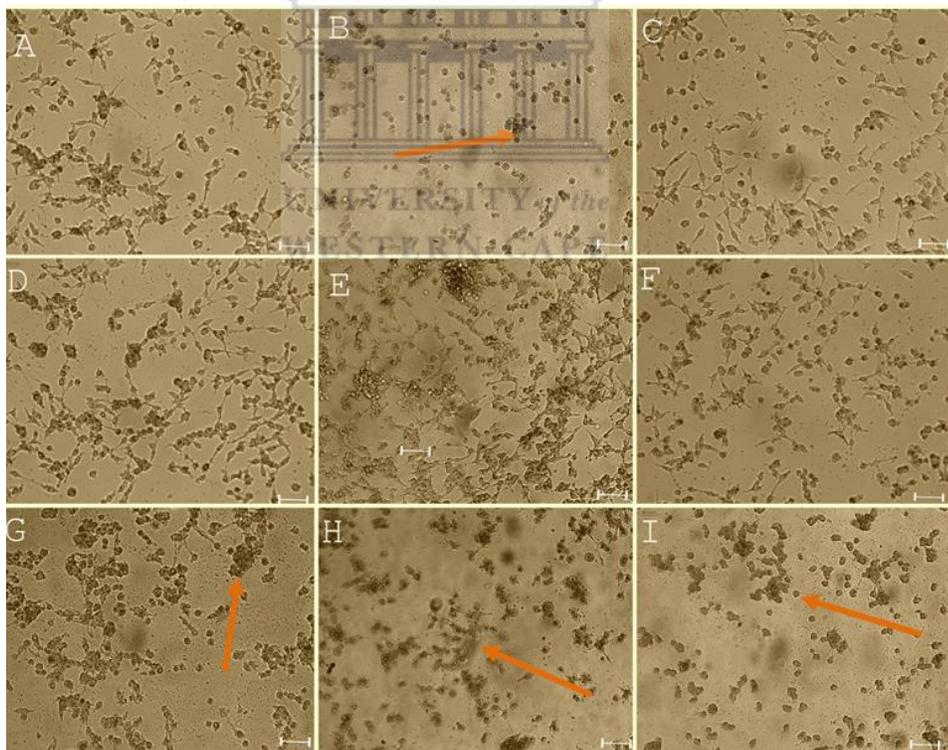


Fig 3-1: Light microscope micrographs (A-I) showing cell morphology of malignant (LNCaP) prostate cells following 24 hour incubation with black tea.

(A) 0 $\mu\text{g/ml}$, (B) 6% DMSO (C) 125 $\mu\text{g/ml}$, (D) 250 $\mu\text{g/ml}$, (E) 500 $\mu\text{g/ml}$, (F) 1 000 $\mu\text{g/ml}$, (G) 5 000 $\mu\text{g/ml}$, (H) 10 000 $\mu\text{g/ml}$ and (I) 50 000 $\mu\text{g/ml}$ A= Negative Control; B= Positive Control; Arrows illustrate rounded up and clustered cells. 100x Magnification

3.1.1.2 Effect of green tea on malignant (LNCaP) prostate cells following 24 hour incubation.

Malignant (LNCaP) prostate cells were exposed to green tea for 24 hours and micrographs showed no obvious changes in cell morphology between treated groups 125-500 $\mu\text{g/ml}$ GT (C-D) compared to the control (A). The cells maintained their flat and polygonal shapes. However, at 1 000 and 5 000 $\mu\text{g/ml}$ GT (F and G) cells started to show signs of stress. Cells appeared irregular in shape and at 10 000 – 50 000 $\mu\text{g/ml}$ GT (H and I) most of the cells were detached and appeared similar to the positive control (B) (Fig 3.2)

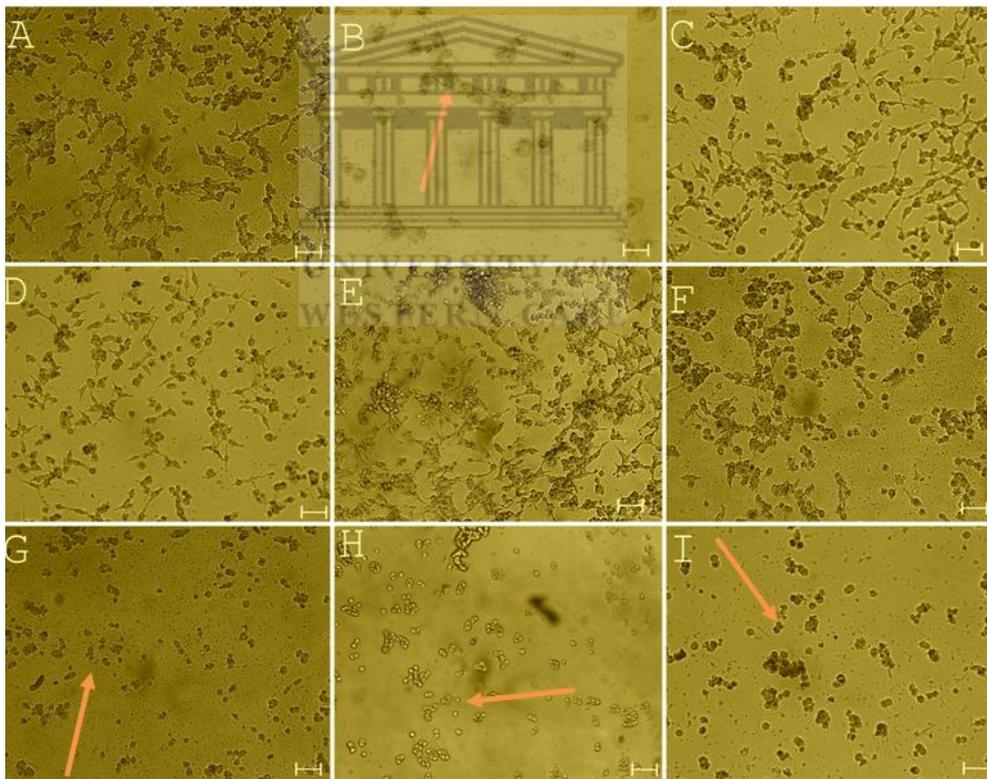


Fig 3-2: Light microscope micrograph showing the effect of green tea on cell viability of malignant (LNCaP) prostate cells after 24 hours.

(A) negative control, (B) positive control (6%DMSO), (C) 125 $\mu\text{g/ml}$ (D) 250 $\mu\text{g/ml}$ (E) 500 $\mu\text{g/ml}$ (F) 1 000 $\mu\text{g/ml}$ (G) 5 000 $\mu\text{g/ml}$ (H) 10 000 $\mu\text{g/ml}$, (I) 50 000 $\mu\text{g/ml}$. 100x magnification. DMSO; Arrows indicate morphological changes that include loss of flat polygonal shape, cluster formation, shrinkage and irregular shape. Bar = 50 μm

3.1.1.3 Effect of fermented rooibos on malignant (LNCaP) prostate cells following 24 hour incubation.

Malignant (LNCaP) prostate cells were exposed to fermented rooibos for 24 hours and micrographs showed no obvious changes in cell morphology between treated groups 125-5 000 $\mu\text{g/ml}$ FR (C-F) compared to the control (A). The cells maintained their flat and polygonal shapes comparable to the negative control (A). However at 10 000 and 50 000 $\mu\text{g/ml}$ FR (H and I) cells became stressed, attained irregular shape besides forming clumps and clusters similar to the positive control (6% DMSO) (B) (Fig 3.3).

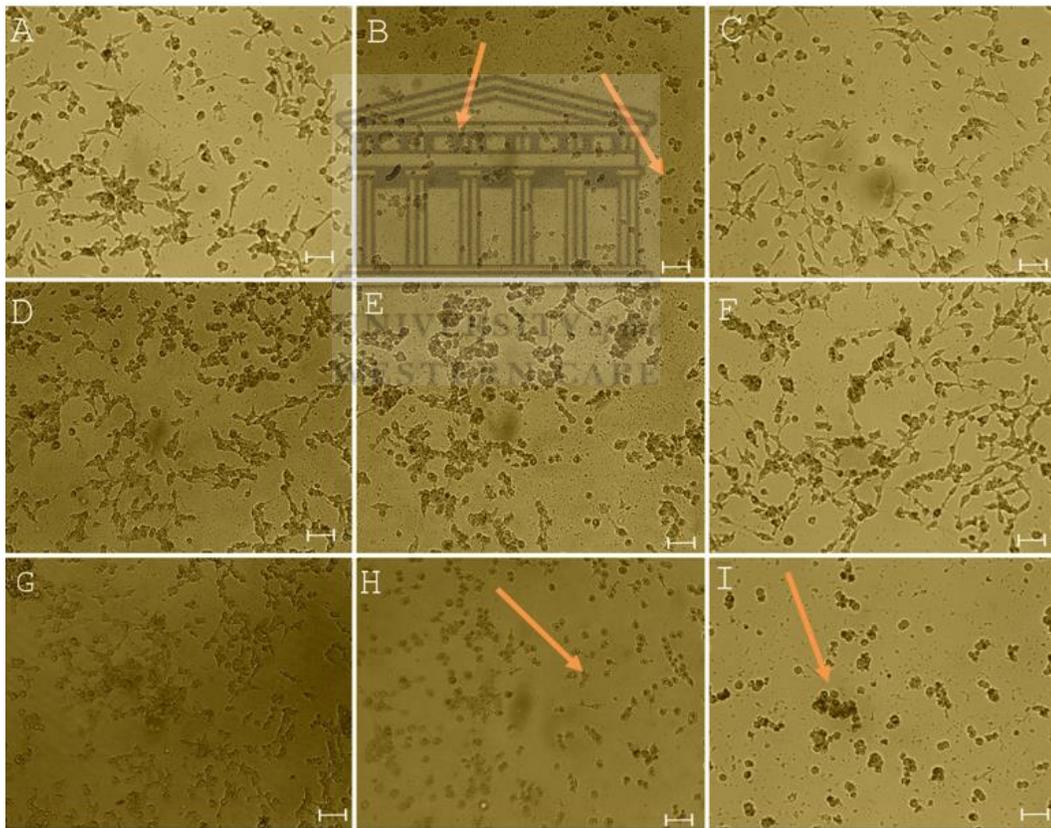


Fig 3-3: Light microscope micrographs (A-I) showing cell morphology of malignant (LNCaP) prostate cells following 24 hour incubation with fermented rooibos.
(A) 0 $\mu\text{g/ml}$, (B) 6% DMSO (C) 125 $\mu\text{g/ml}$, (D) 250 $\mu\text{g/ml}$, (E) 500 $\mu\text{g/ml}$, (F) 1 000 $\mu\text{g/ml}$, (G) 5 000 $\mu\text{g/ml}$, (H) 10 000 $\mu\text{g/ml}$ and (I) 50 000 $\mu\text{g/ml}$ A= Negative Control, B= Positive Control, DMSO; Arrows illustrate rounded up, clustered and detached cells. 100x magnification. Bar = 50 μm

3.1.1.4 Effect of unfermented rooibos on malignant (LNCaP) prostate cells following 24 hour incubation.

Malignant (LNCaP) prostate cells were exposed to unfermented rooibos for 24 hours and micrographs showed no obvious changes in cell morphology between treated groups 125-5000 $\mu\text{g/ml}$ UR (C-F) compared to the negative control (A). The cells maintained their flat and polygonal shapes. However, at 5 000 $\mu\text{g/ml}$ UR (G) cell started to show signs of stress. Cells started to lose shape and most of them shrunk. At 10 000 and 50 000 $\mu\text{g/ml}$ (H and I) most of the cells were detached and were comparable to the positive control (6% DMSO) (B) (Fig 3.4)

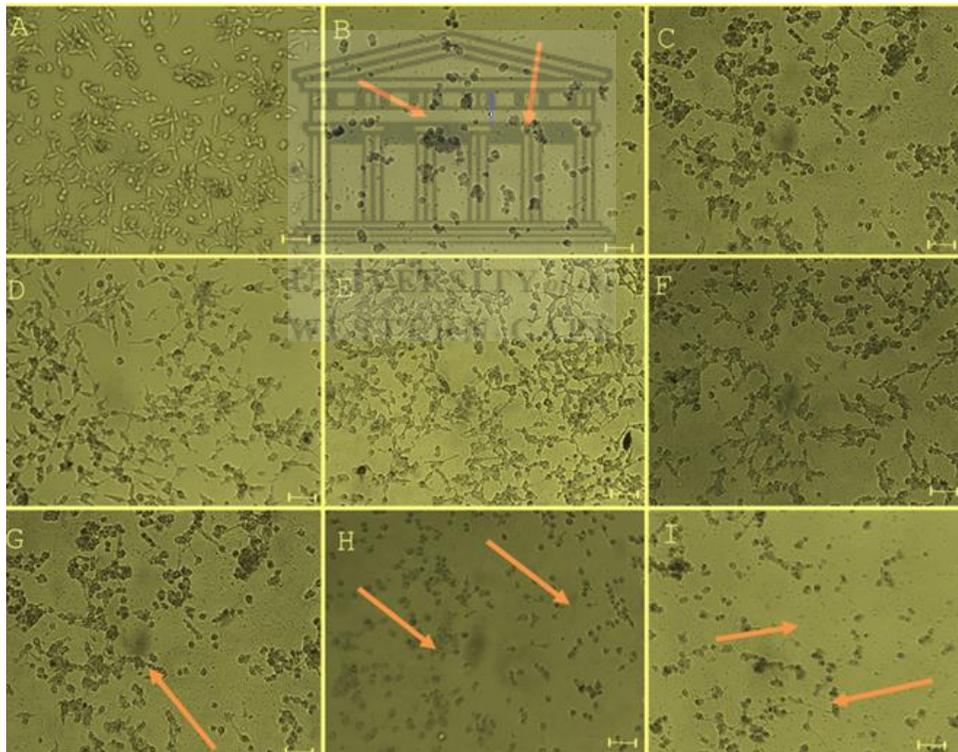


Fig 3- 4: Light microscope micrographs (A-I) showing cell morphology of malignant (LNCaP) prostate cells following 24 hour incubation with unfermented rooibos. (A) 0 $\mu\text{g/ml}$, (B) 6% DMSO (C) 125 $\mu\text{g/ml}$, (D) 250 $\mu\text{g/ml}$, (E) 500 $\mu\text{g/ml}$, (F) 1 000 $\mu\text{g/ml}$, (G) 5 000 $\mu\text{g/ml}$, (H) 10 000 $\mu\text{g/ml}$ and (I) 50 000 $\mu\text{g/ml}$. A= Negative Control; B= Positive Control; DMSO; Arrows show rounded up and clustered cells. Magnification: 100x. Bar = 50 μM .

3.1.1.5 Effect of aspalathin on malignant (LNCaP) prostate cells following 24 hour incubation.

Malignant (LNCaP) prostate cells were exposed to aspalathin for 24 hours and micrographs showed no obvious changes in cell morphology at 125-1 000 $\mu\text{g/ml}$ ASP (C-F) compared to the negative control (A). The cells maintained their flat and polygonal shapes. However, at 5 000 $\mu\text{g/ml}$ ASP (G), cells started to show signs of stress, such that they appeared round in shape. Most of the cells were detached and appeared dead at 10 000 $\mu\text{g/ml}$ ASP (H) and were comparable to the cells of the positive control (6% DMSO) (B). The highest concentration of 50 000 $\mu\text{g/ml}$ aspalathin was not used because our preliminary experiments showed most of the cells dead and detached (Fig 3.5).

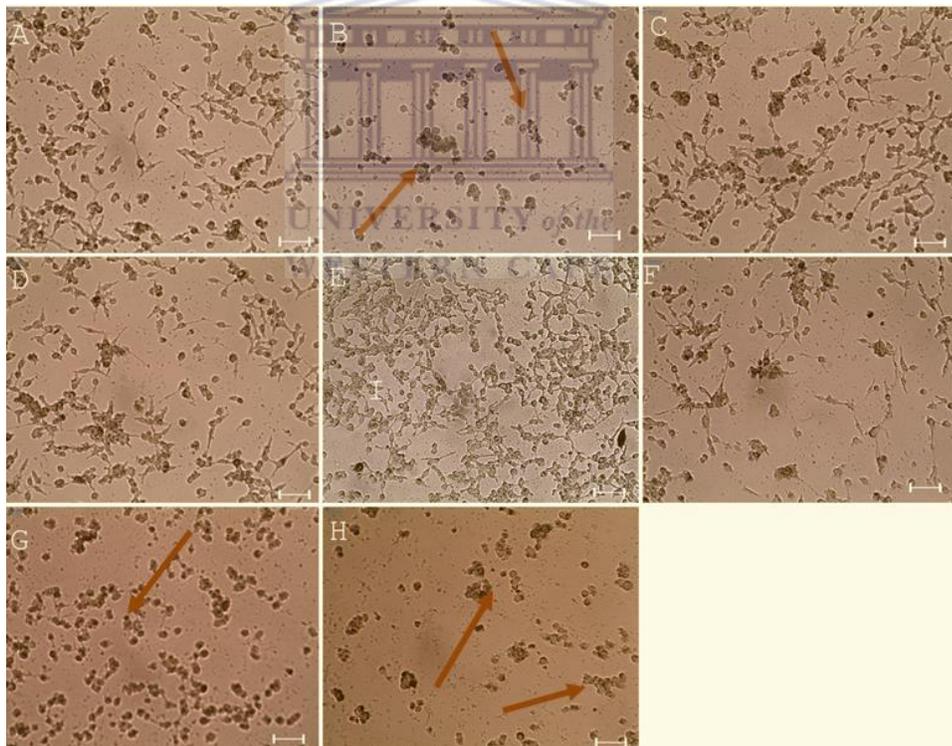


Fig 3-5: Light microscope micrographs (A-H) showing cell morphology of malignant (LNCaP) prostate cells following 24 hour incubation with aspalathin. (A) 0 $\mu\text{g/ml}$, (B) 6% DMSO (C) 125 $\mu\text{g/ml}$, (D) 250 $\mu\text{g/ml}$, (E) 500 $\mu\text{g/ml}$, (F) 1 000 $\mu\text{g/ml}$, (G) 5 000 $\mu\text{g/ml}$. (H) 10 000 $\mu\text{g/ml}$ A= Negative Control; B= Positive Control; DMSO; Arrows illustrate rounded up and clustered cells. 100x Magnification Bar = 50 μm .

3.1.1.6 Effect of EGCG on malignant (LNCaP) prostate cells following 24 hour incubation.

Malignant (LNCaP) prostate cells were exposed to EGCG for 24 hours and micrographs showed no obvious changes in cell morphology at 125-5 000 $\mu\text{g/ml}$ EGCG (C-F) with flat polygonal shapes compared to the negative control (A). However, at 5 000 $\mu\text{g/ml}$ EGCG (G) the cells started to show signs of stress. Cells started to lose shape and most of the cells attained round shapes, formed clumps and clusters when compared to the negative control (A). Most of the cells at 10 000 $\mu\text{g/ml}$ EGCG (H) were detached and looked similar to those of the positive control (B). The highest concentration of 50 000 $\mu\text{g/ml}$ EGCG was not used because our preliminary experiments showed most of the cells dead and detached (Fig 3.6).

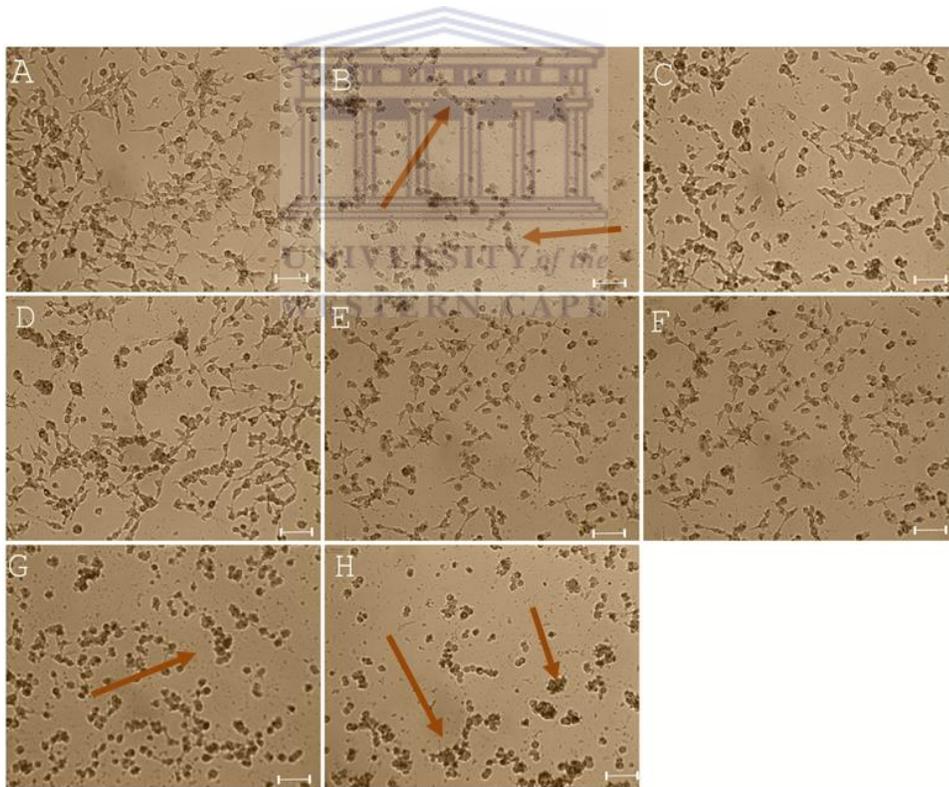


Fig 3-6: Light microscope micrographs (A-H) showing cell morphology of malignant (LNCaP) prostate cells following 24 hour incubation with EGCG. (A) 0 $\mu\text{g/ml}$, (B) 6% DMSO (C) 125 $\mu\text{g/ml}$, (D) 250 $\mu\text{g/ml}$, (E) 500 $\mu\text{g/ml}$, (F) 1 000 $\mu\text{g/ml}$, (G) 5 000 $\mu\text{g/ml}$, (H) 10 000 $\mu\text{g/ml}$. A= Negative Control; B= Positive Control, DMSO; Arrows illustrate rounded up and clustered cells, detached dead cells, 100x Magnification. Bar = 50 μm

3.1.1.7 Effect of black tea on malignant (LNCaP) prostate cells following 72 hour incubation.

The results obtained after exposing the malignant (LNCaP) prostate cells to black tea for 72 hours produced no obvious observable changes in cell morphology between treated groups 125 and 500 $\mu\text{g/ml}$ BT (C-D) compared to the negative control (A). The cells maintained their flat and polygonal shapes. However, at higher concentrations (E-I) the cells started to show signs of stress. At 5 000, 10 000 and 50 000 $\mu\text{g/ml}$ BT (G, H and I) the cells attained irregular shape; formed clumps and clusters and some cells appeared dead comparable to the positive control (B) (Fig 3.7).

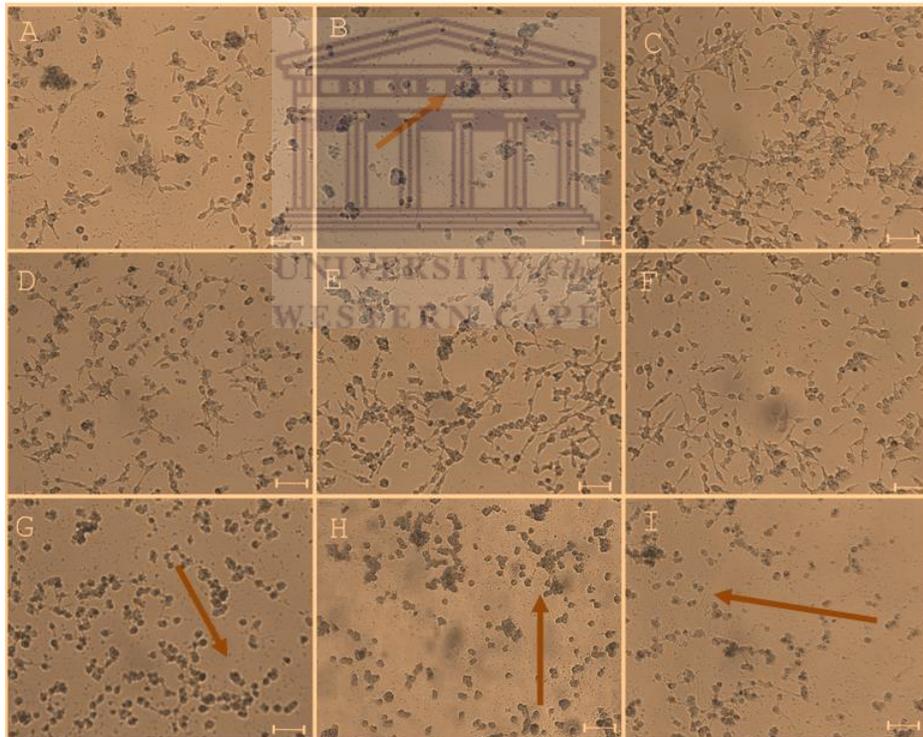


Fig 3-7: Light microscope micrographs (A-I) showing cell morphology of malignant (LNCaP) prostate cells following 72 hour incubation with black tea for 72 hours.

(A) Negative control, (B) Positive Control (6%DMSO), (C) 125 $\mu\text{g/ml}$ (D) 250 $\mu\text{g/ml}$ (E) 500 $\mu\text{g/ml}$ (F) 1 000 $\mu\text{g/ml}$ (G) 5 000 $\mu\text{g/ml}$ (H) 10 000 $\mu\text{g/ml}$, (I) 50 000 $\mu\text{g/ml}$. Magnification: 100x. Changes showing characteristics of apoptosis are indicated by arrows and include loss of flat polygonal shape, cluster formation, shrinkage, clumping and irregular shape. Bar = 50 μm .

3.1.1.8 Effect of green tea on malignant (LNCaP) prostate cells following 72 hour incubation.

The results obtained after exposing the malignant (LNCaP) prostate cells to green tea for 72 hours produced no obvious changes in cell morphology between treated groups 125 and 500 $\mu\text{g/ml}$ GT (C-D) compared to the negative control (A). The cells maintained their flat and polygonal shapes. However, at higher concentrations (E-I) cells started to show signs of stress. Stress levels were more pronounced at 5 000, 10 000 and 50 000 $\mu\text{g/ml}$ GT (G, H and I) with cells attaining an irregular shape, clumped together and appeared in clusters. Clear signs of cell death were also observed as most cells were detached and appeared similar to the positive control (6% DMSO) (B) (Fig 3.8)

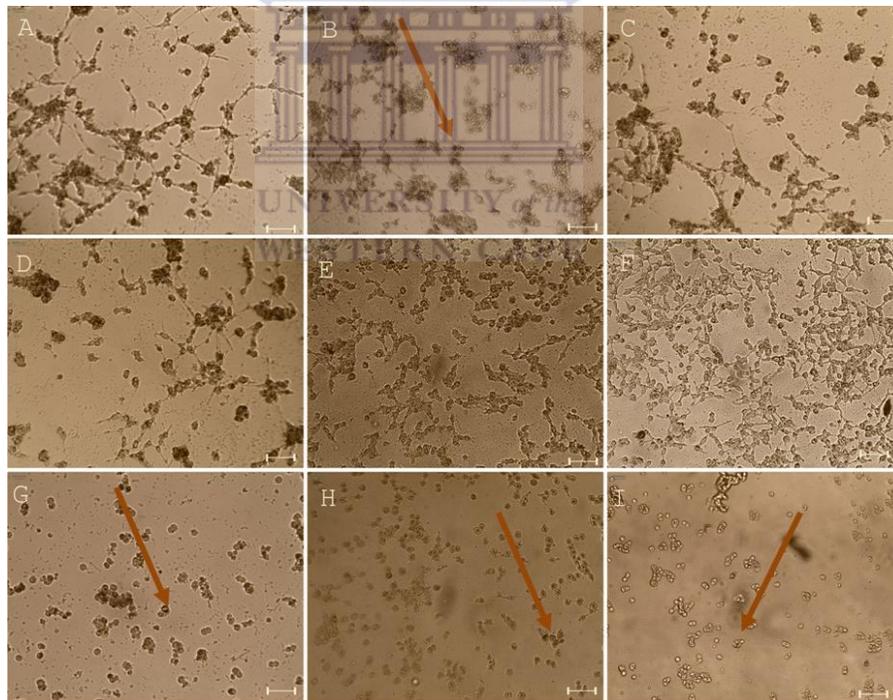


Fig 3-8: Light microscope micrographs (A-I) showing cell morphology of malignant (LNCaP) prostate cells following 72 hour incubation with green tea.

(A) 0 $\mu\text{g/ml}$, (B) 6% DMSO (C) 125 $\mu\text{g/ml}$, (D) 250 $\mu\text{g/ml}$, (E) 500 $\mu\text{g/ml}$, (F) 1 000 $\mu\text{g/ml}$, (G) 5 000 $\mu\text{g/ml}$, (H) 10 000 $\mu\text{g/ml}$ and (I) 50 000 $\mu\text{g/ml}$. A= Negative Control; B= Positive Control; DMSO; Arrows illustrate rounded up and clustered cells. Magnification: 100x. Bar = 50 μM

3.1.1.9 Effect of fermented rooibos on malignant (LNCaP) prostate cells following 72 hour incubation.

The results obtained after exposing the malignant (LNCaP) prostate cells to fermented rooibos for 72 hours produced no observable changes in cell morphology between treated groups 125, 500 and 1 000 $\mu\text{g/ml}$ FR (C-E) compared to the negative control (A). The cells maintained their flat and polygonal shapes. However, at higher concentrations (F-I) cell started to show signs of stress. The cells appeared more stressed at 5 000, 10 000 and 50 000 $\mu\text{g/ml}$ FR (G, H and I) with most of them attaining an irregular shape, formed clumps and appeared in clusters similar to the positive control (B) (Fig 3.9).

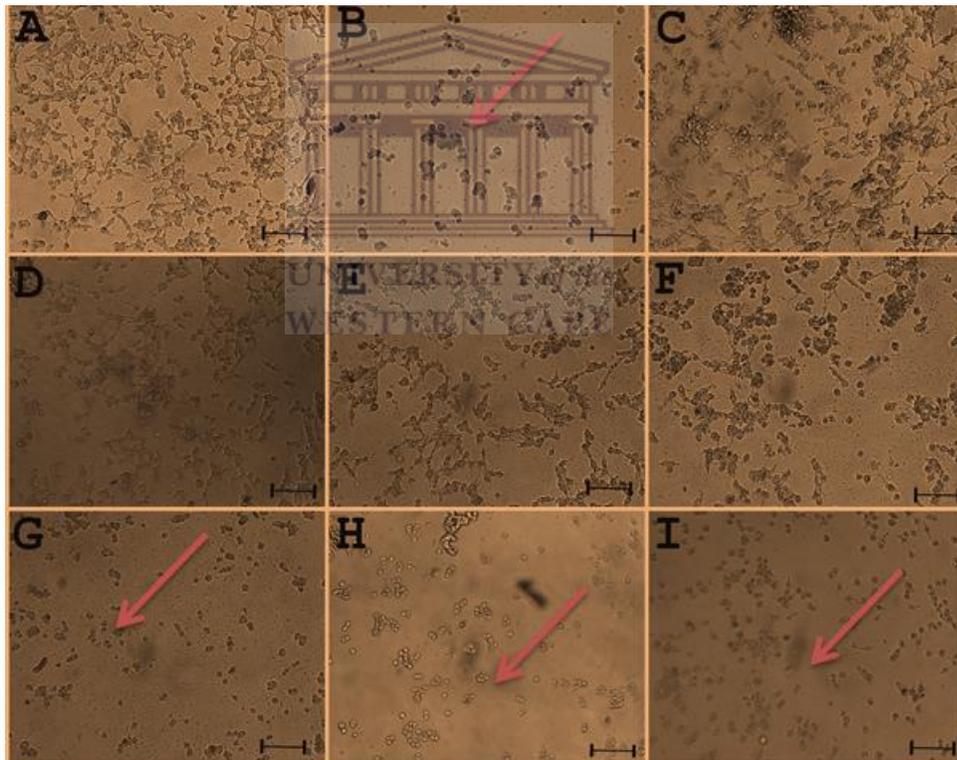


Fig 3- 9: Light microscope micrographs (A-I) show cell morphology of malignant (LNCaP) prostate cells following 72 hour incubation with fermented rooibos for 72 hours. (A) Negative control, (B) Positive Control (6% DMSO), (C) 125 $\mu\text{g/ml}$ (D) 250 $\mu\text{g/ml}$ (E) 500 $\mu\text{g/ml}$ (F) 1 000 $\mu\text{g/ml}$ (G) 5 000 $\mu\text{g/ml}$ (H) 10 000 $\mu\text{g/ml}$, (I) 50 000 $\mu\text{g/ml}$. Magnification: 100x. Changes showing characteristics of apoptosis are indicated by arrows and include loss of flat polygonal shape, cluster formation and irregular shape. Bar = 50 μm .

3.1.1.10 Effect of unfermented rooibos on malignant (LNCaP) prostate cells following 72 hour incubation.

The results obtained after exposing the malignant (LNCaP) prostate cells to unfermented rooibos for 72 hours produced no observable changes in cell morphology between treated groups 125 and 500 $\mu\text{g/ml}$ UR (C-D) compared to the negative control (A). The cells maintained their flat and polygonal shapes. However, at higher concentrations 5 000, 10 000 and 50 000 $\mu\text{g/ml}$ UR (G, H and I) cells attained an irregular shape, formed clumps and clusters similar to the positive control (B) (Fig 3.10).

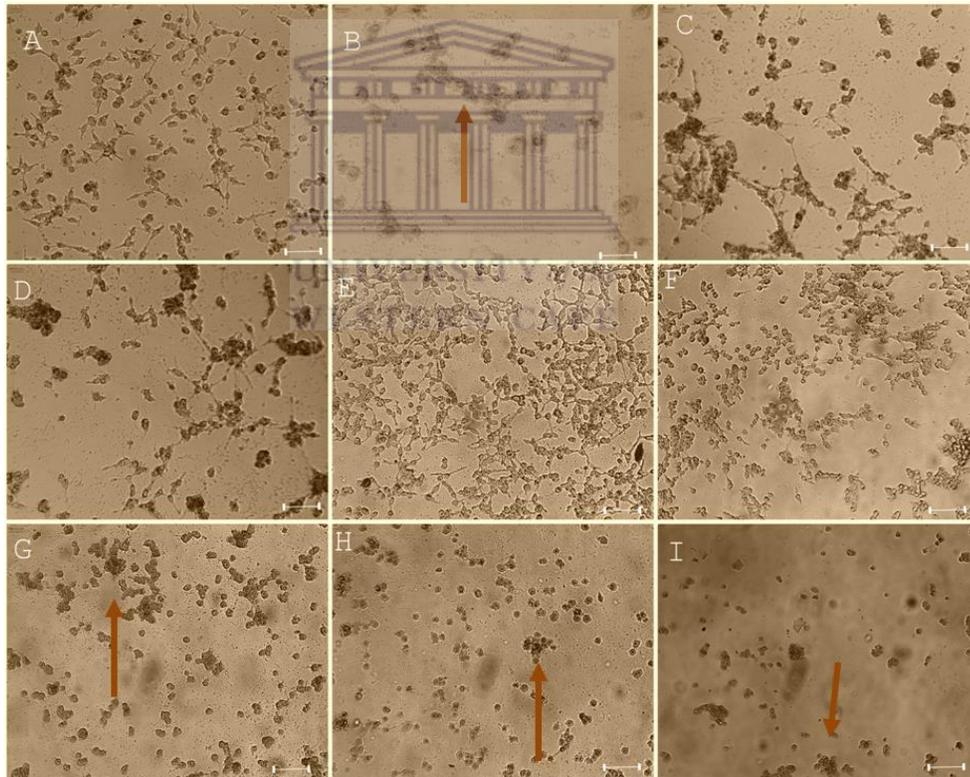


Fig 3- 10: Light microscope micrographs (A-I) showing cell morphology of malignant (LNCaP) prostate cells following 72 hour incubation with unfermented rooibos for 72 hours. (A) Negative Control, (B) Positive Control (6% DMSO), (C) 125 $\mu\text{g/ml}$ (D) 250 $\mu\text{g/ml}$ (E) 500 $\mu\text{g/ml}$ (F) 1 000 $\mu\text{g/ml}$ (G) 5 000 $\mu\text{g/ml}$ (H) 10 000 $\mu\text{g/ml}$, (I) 50 000 $\mu\text{g/ml}$. Changes showing characteristics of apoptosis are indicated by arrows and include loss of flat polygonal shape, cluster formation, shrinkage and irregular shape. Magnification: 100x. Bar = 50 μm .

3.1.1.11 Effect of aspalathin on malignant (LNCaP) prostate cells following 72 hour incubation.

The results obtained after exposing malignant (LNCaP) prostate cells to aspalathin for 72 hours produced no observable changes in cell morphology between treated groups 125 – 1 000 $\mu\text{g/ml}$ ASP (C-G) compared to the negative control (A). The cells maintained their flat and polygonal shapes. However, at 5 000 $\mu\text{g/ml}$ ASP cells started to show signs typical of stressed cells when compared to the negative control (A). Cells attained irregular shape and the stress was more pronounced at 10 000 $\mu\text{g/ml}$ ASP (H) with cells forming clumps and clusters that were comparable to the positive control (6% DMSO) (B) (Fig 3.11).

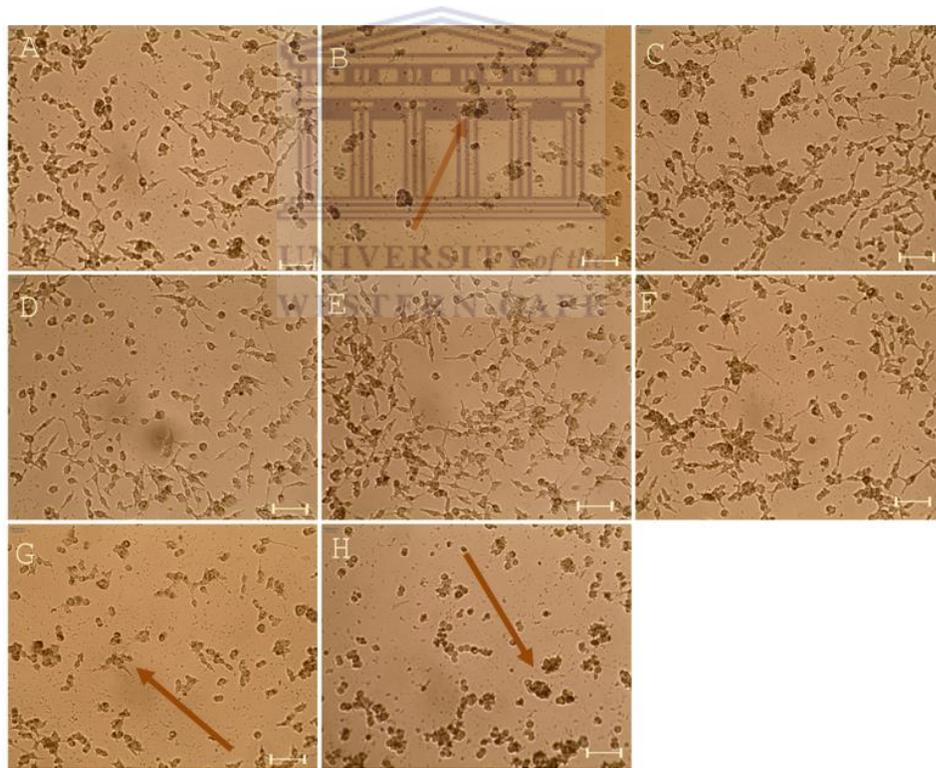


Fig 3- 11: Light microscope micrographs (A-H) showing cell morphology of malignant (LNCaP) prostate cells following 72 hour incubation with aspalathin for 72 hours.

(A) Negative control, (B) Positive Control (6%DMSO), (C) 125 $\mu\text{g/ml}$ (D) 250 $\mu\text{g/ml}$ (E) 500 $\mu\text{g/ml}$ (F) 1 000 $\mu\text{g/ml}$ (G) 5 000 $\mu\text{g/ml}$ (H) 10 000 $\mu\text{g/ml}$. ASP = Aspalathin; DMSO; Changes showing characteristics of apoptosis are indicated by arrows and include loss of flat polygonal shape, cluster formation, shrinkage and irregular shape. Magnification: 100x. Bar = 50 μm .

3.1.1.12 Effect of EGCG on malignant (LNCaP) prostate cells following 72 hour incubation.

Below are light microscope micrographs of malignant (LNCaP) prostate cells following exposure to EGCG for 72 hours. There were no observed changes in cell morphology between treated groups 125-500 $\mu\text{g/ml}$ EGCG (C-G) compared to the negative control (A). The cells maintained their flat and polygonal shapes. However, at 1 000 $\mu\text{g/ml}$ EGCG (F) cells started to lose the flat polygonal shape due to stress and at 5 000 and 10 000 $\mu\text{g/ml}$ EGCG (G and H) cells attained an irregular shape, formed clumps and clusters. Some cells appeared detached and dead with characteristics similar to the positive control (6% DMSO) (B) (Fig 3.12).

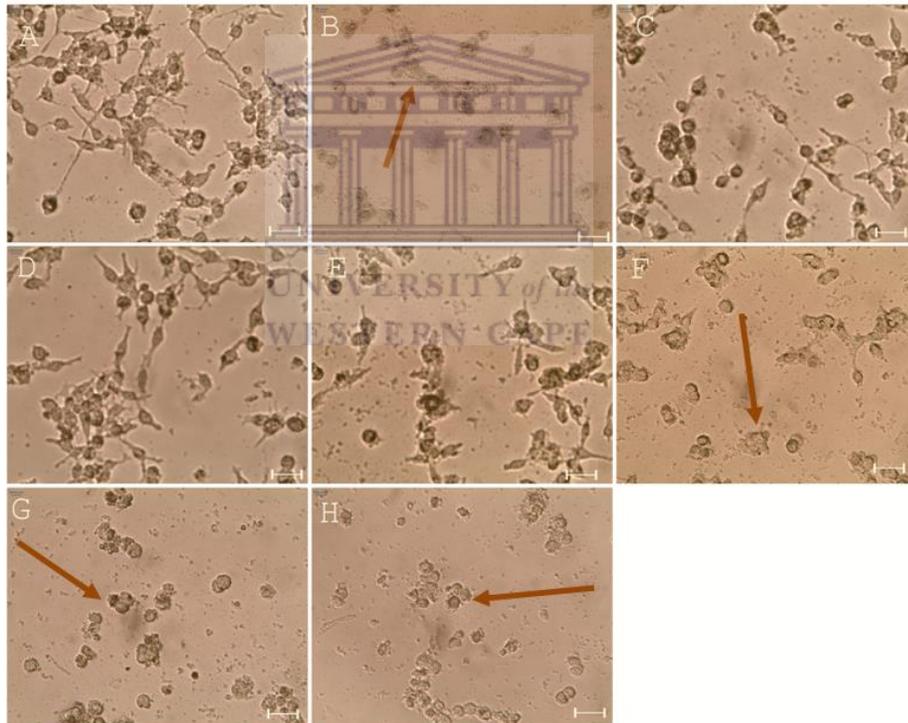
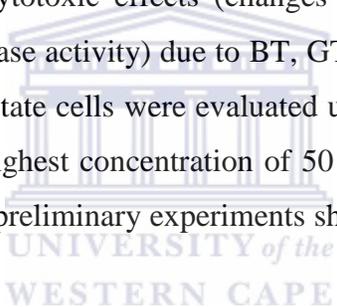


Fig 3- 12: Light microscope micrographs (A-I) showing cell morphology of malignant (LNCaP) prostate cells following 72 hour exposure to EGCG.

(A) Negative Control, (B) Positive control (6%DMSO), (C) 125 $\mu\text{g/ml}$ (D) 250 $\mu\text{g/ml}$ (E) 500 $\mu\text{g/ml}$ (F) 1 000 $\mu\text{g/ml}$ (G) 5 000 $\mu\text{g/ml}$ (H) 10 000 $\mu\text{g/ml}$, (I) 50 000 $\mu\text{g/ml}$. 200x magnification. EGCG; DMSO; Arrows indicate morphological changes which include shrinkage and irregular shape. Bar = 50 μm

3.1.2 Benign (RPWE 1) cell viability.

Benign (RPWE 1) prostate cells were exposed to increasing concentrations of aqueous extracts of *Camellia sinensis*, *Aspalathus linearis* and their active compounds epigallocatechin gallate (EGCG) and aspalathin respectively for 72 hours. Our preliminary studies involving the acute exposure (24 hours) of RPWE 1 cells to black tea, green tea, fermented rooibos, unfermented rooibos, EGCG and aspalathin showed cells with no clear observable signs of stress. As such chronic exposure of RPWE 1 cells to the above mentioned treatment samples did not have any cytotoxic effects. In this regard, the cells were exposed for 72 hours in order to determine the effects of chronic exposure of the the benign (RPWE 1) cells to the nutraceuticals. During this period, the cytotoxic effects (changes in morphology and changes in mitochondrial dehydrogenase activity) due to BT, GT, FR UR, EGCG and aspalathin on Benign (RPWE 1) prostate cells were evaluated using light microscopy and MTT assay. In this study, the highest concentration of 50 000 µg/ml aspalathin or EGCG was not used because our preliminary experiments showed most of the cells dead and detached.



3.1.2.1 Benign (RPWE 1) prostate cells exposed to black tea for 72 hours.

The results obtained after exposing benign (RPWE 1) prostate cells to black tea for 72 hours produced no observable changes in cell morphology between all the treated groups (C- I) compared to the negative control (A). The cells maintained their flat and polygonal shapes. However, at the positive control (B) cells looked stressed with most of them losing their flat polygonal shape hence attaining an irregular shape, formed clumps and clusters (Fig 3.13).

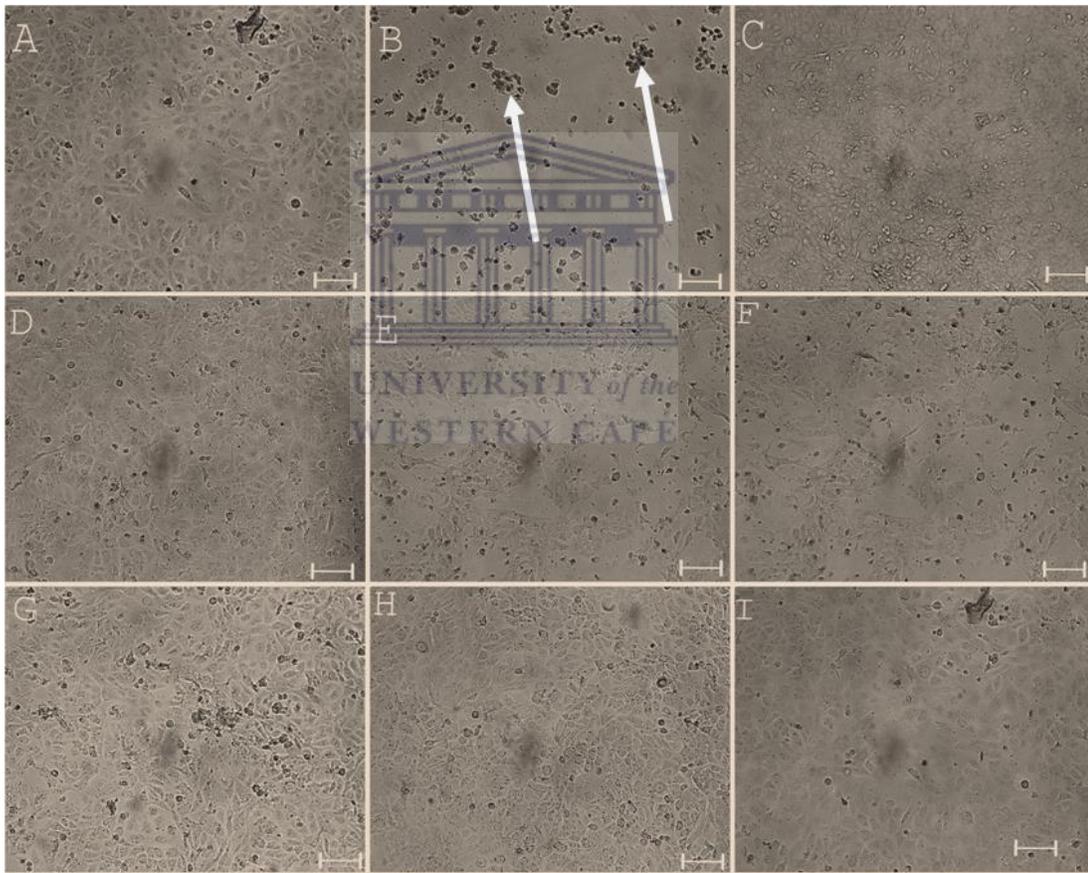


Fig 3-13: Light microscope micrographs showing cell viability of benign (RPWE 1) prostate cells treated with black tea for 72 hours.

(A) Negative control, (B) Positive control (6% DMSO), (C) 125 $\mu\text{g/ml}$ (D) 250 $\mu\text{g/ml}$ (E) 500 $\mu\text{g/ml}$ (F) 1 000 $\mu\text{g/ml}$ (G) 5 000 $\mu\text{g/ml}$ (H) 10 000 $\mu\text{g/ml}$, (I) 50 000 $\mu\text{g/ml}$. 100x magnification. Changes showing characteristics of apoptosis are indicated by arrows and include loss of flat polygonal shape, cluster formation, shrinkage, clamping and irregular shape plus cell detachment. Bar = 50 μm .

3.1.2.2 Benign (RPWE 1) prostate cells exposed to green tea for 72 hours.

The results obtained after exposing benign (RPWE 1) prostate cells to green tea for 72 hours produced no observable changes in cell morphology between treated groups 125, 250, 500, 1 000 and 5 000 $\mu\text{g/ml}$ GT (C- G) compared to the negative control (A). The cells maintained their flat and polygonal shapes. However, at 10 000 and 50 000 $\mu\text{g/ml}$ GT (H and I) the cells looked stressed with most of them attaining an irregular shape besides forming clusters that were comparable to the positive control (B) (Fig 3.14).

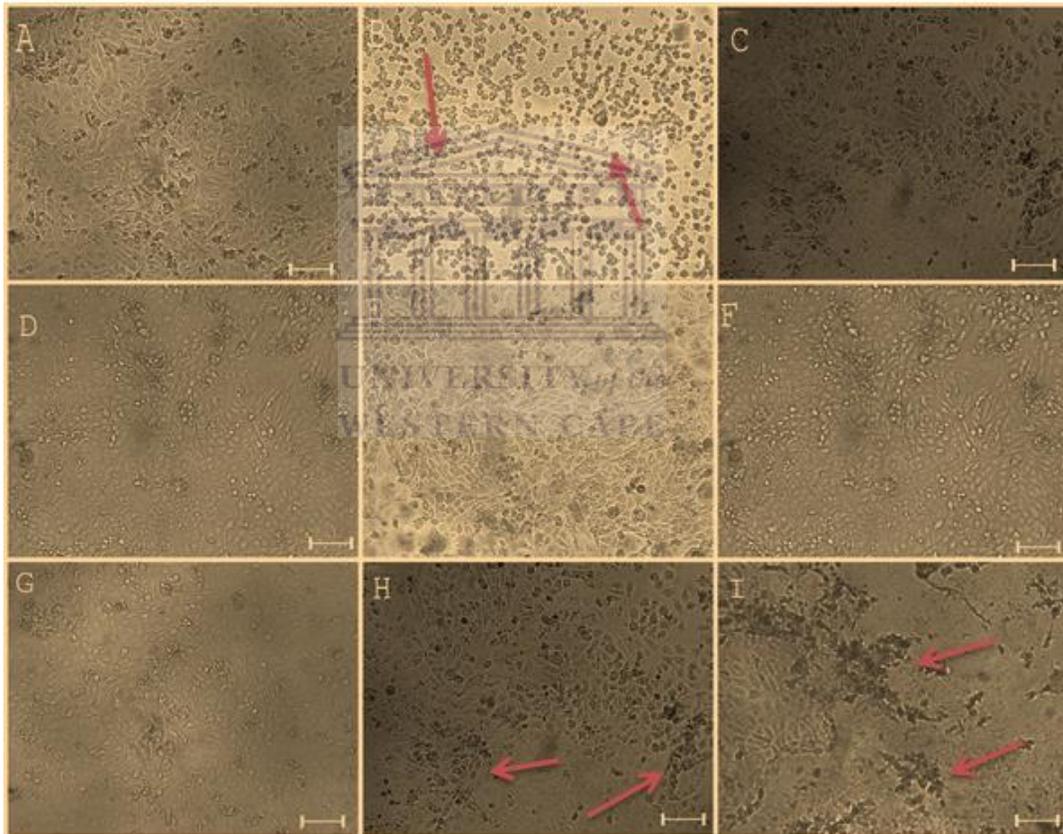


Fig 3-14: Light microscope micrographs showing cell viability of benign (RPWE 1) prostate cells exposed to green tea for 72 hours.

(A) Negative control, (B) Positive control (6% DMSO), (C) 125 $\mu\text{g/ml}$ (D) 250 $\mu\text{g/ml}$ (E) 500 $\mu\text{g/ml}$ (F) 1 000 $\mu\text{g/ml}$ (G) 5 000 $\mu\text{g/ml}$ (H and I) 10 000 $\mu\text{g/ml}$. 100x magnification. Changes showing characteristics of apoptosis are indicated by arrows and include loss of flat polygonal shape, cluster formation, shrinkage, clumping and irregular shape and cell detachment. Bar = 50 μm .

3.1.2.3 Benign (RPWE 1) prostate cells exposed to fermented rooibos for 72 hours.

The results obtained after exposing benign (RPWE 1) prostate cells to fermented rooibos for 72 hours produced no observable changes in cell morphology between treated groups 125, 500 and 5 000 $\mu\text{g/ml}$ FR (C- G) compared to the negative control (A). The cells maintained their flat and polygonal shapes. However, at concentrations of 10 000 and 50 000 $\mu\text{g/ml}$ FR (H and I), respectively, the cells looked stressed with most of them attaining an irregular shape, formed clumps comparable to the positive control (B) (Fig 3.15).

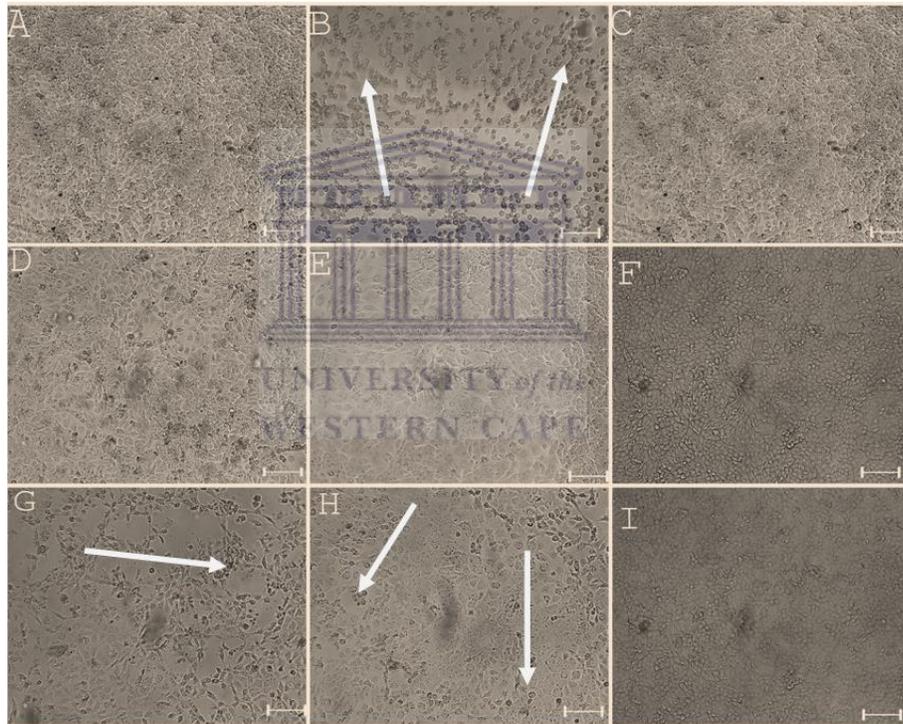


Fig 3-15: Light microscope micrographs show cell viability of benign (RPWE 1) prostate cells following exposure to fermented rooibos for 72 hours.

(A) Negative control, (B) Positive control (6%DMSO), (C) 125 $\mu\text{g/ml}$ (D) 250 $\mu\text{g/ml}$ (E) 500 $\mu\text{g/ml}$ (F) 1 000 $\mu\text{g/ml}$ (I) 5 000 $\mu\text{g/ml}$ (G)10 000 $\mu\text{g/ml}$, (H) 50 000 $\mu\text{g/ml}$. magnification : 100x. FR: fermented rooibos. Changes showing characteristics of apoptosis are indicated by arrows and include loss of flat polygonal shape, cluster formation, shrinkage, loss of shape and cell detachment. Bar = 50 μm .

3.1.2.4 Benign (RPWE 1) prostate cells exposed to unfermented rooibos for 72 hours.

The results obtained after exposing benign (RPWE 1) prostate cells to unfermented rooibos for 72 hours produced no observable changes in cell morphology between treated all treated groups (C- I) compared to the negative control (A). The cells looked normal and were dividing whilst maintaining their flat and polygonal shapes (Fig 3.16).

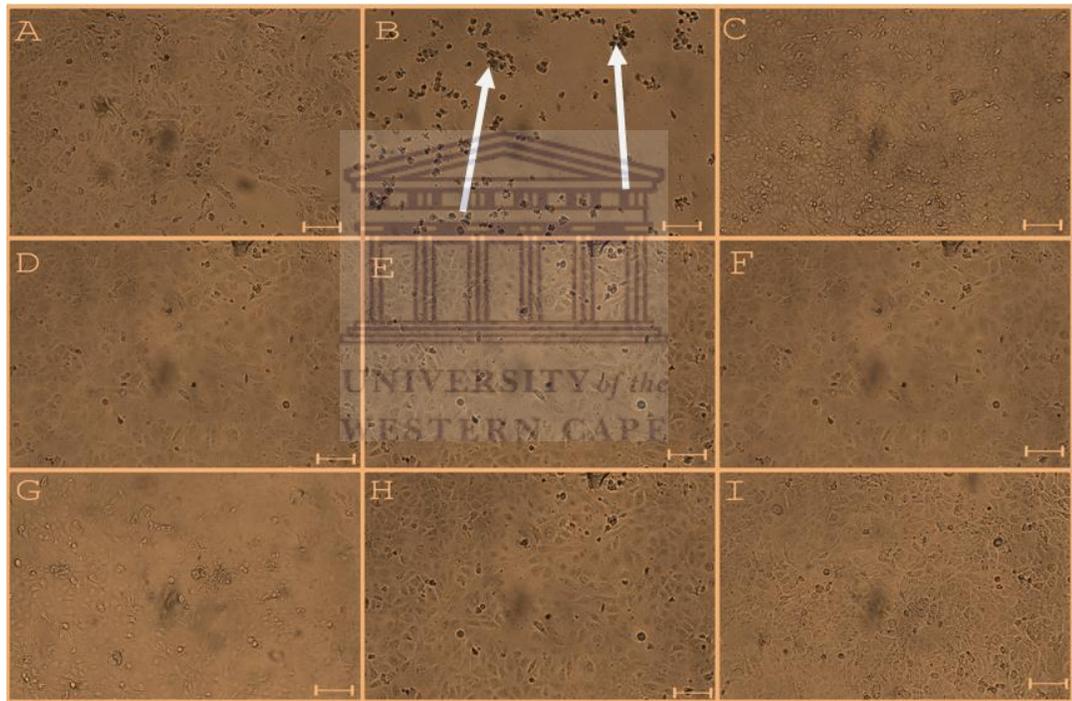


Fig 3-16: Light microscope micrographs showing cell viability of benign (RPWE 1) prostate cells with unfermented rooibos for 72 hours.

(A) Negative control, (B) positive control (6%DMSO), (C) 125 µg/ml (D) 250 µg/ml (E) 500 µg/ml (F) 1 000 µg/ml (G) 5 000 µg/ml (H)10 000 µg/ml, (I) 50 000 µg/ml. 100x magnification. Changes showing characteristics of apoptosis are indicated by arrows and include loss of flat polygonal shape, shrinkage, clumping and irregular shape. Bar = 50 µm.

3.1.2.5 Benign (RPWE 1) prostate cells exposed to aspalathin rooibos for 72 hours.

The results obtained after exposing benign (RPWE 1) prostate cells to aspalathin for 72 hours produced no observable changes in cell morphology between treated groups 125, 500 and 5 000 $\mu\text{g/ml}$ ASP (C- G) compared to the negative control (A). The cells maintained their flat and polygonal shapes. However, at 10 000 $\mu\text{g/ml}$ ASP (H) the cells looked stressed with most of them attaining an irregular shape, formed clumps and clusters similar to the positive control (B) (Fig 3.17).

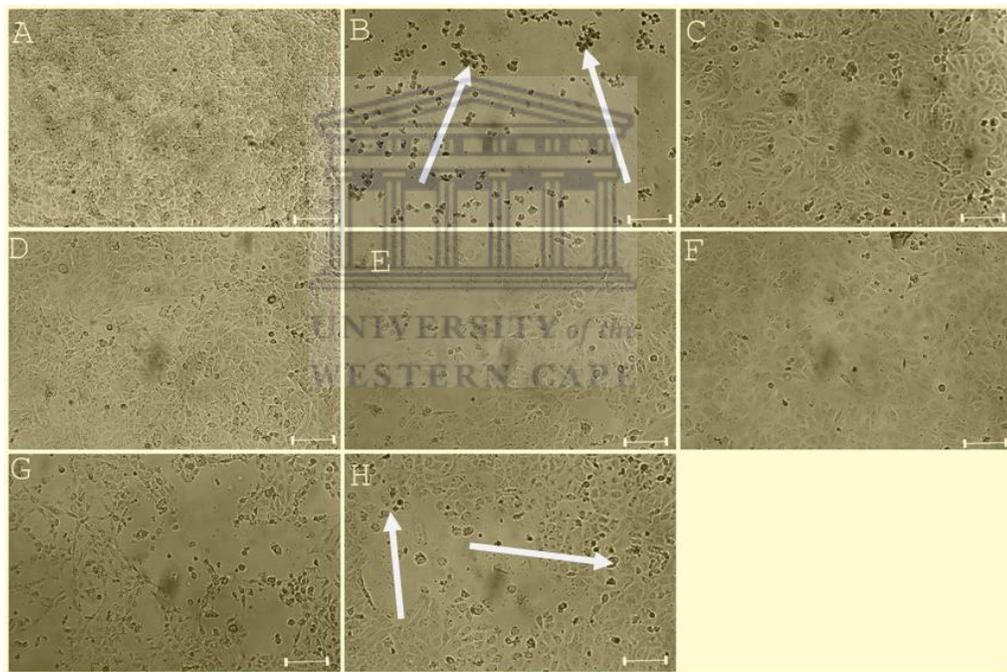


Fig 3-17: Light microscope micrographs showing cell viability of benign (RPWE 1) prostate cells treated with aspalathin for 72 hours.

(A) Negative control, (B) positive control (6% DMSO), (C) 125 $\mu\text{g/ml}$ (D) 250 $\mu\text{g/ml}$ (E) 500 $\mu\text{g/ml}$ (F) 1 000 $\mu\text{g/ml}$ (G) 5 000 $\mu\text{g/ml}$, (H) 10 000 $\mu\text{g/ml}$. magnification: 100x. Changes showing characteristics of apoptosis are indicated by arrows and include loss of flat polygonal shape, cluster formation, shrinkage and irregular shape. Bar = 50 μm .

3.1.2.6 Benign (RPWE 1) prostate cells exposed to EGCG for 72 hours.

The results obtained after exposing benign (RPWE 1) prostate cells to EGCG for 72 hours produced no observable changes in cell morphology between treated groups 125, 500 and 5000 $\mu\text{g/ml}$ EGCG (C- G) compared to the negative control (A). The cells maintained their flat and polygonal shapes. However, at 10 000 $\mu\text{g/ml}$ EGCG (H) the cells looked stressed with most of them attaining an irregular shape, formed clumps and formed clusters comparable to the positive control (B) (Fig 3.18)

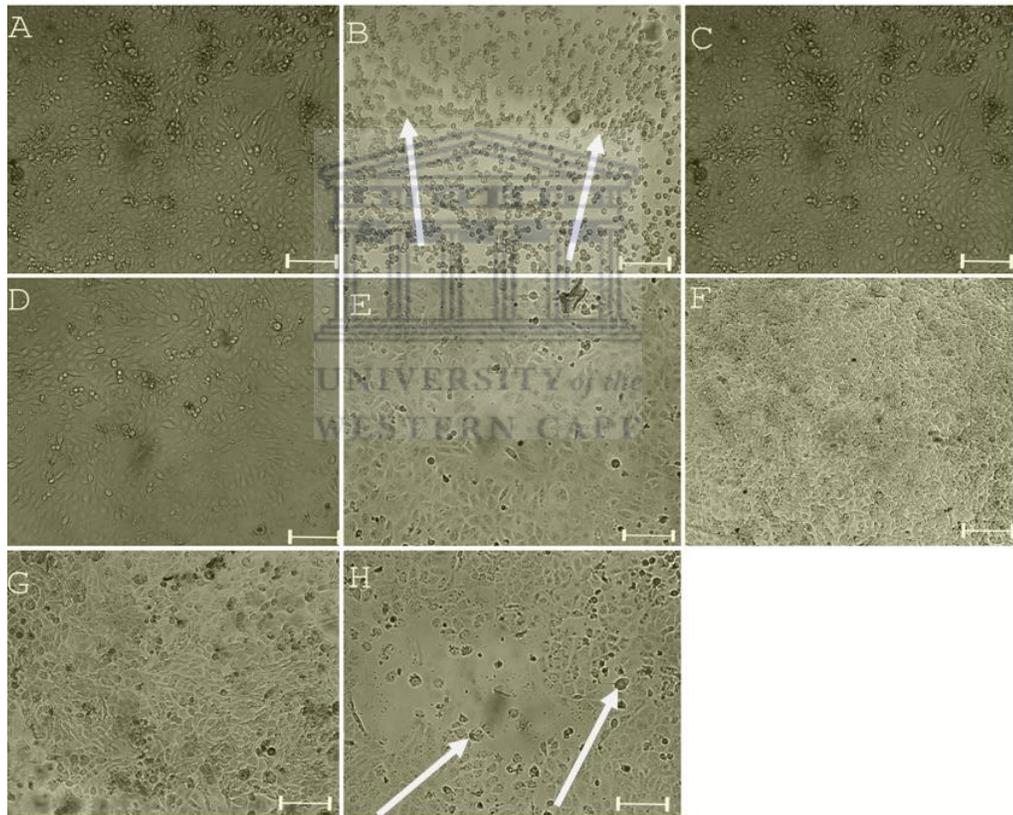


Fig 3-18: Light microscope micrographs show cell viability of benign (RPWE 1) prostate cells with EGCG for 72 hours.

(A) Negative control, (B) positive control (6%DMSO), (C) 125 $\mu\text{g/ml}$ (D) 250 $\mu\text{g/ml}$ (E) 500 $\mu\text{g/ml}$ (F) 1 000 $\mu\text{g/ml}$ (G) 5 000 $\mu\text{g/ml}$ (H) 10 000 $\mu\text{g/ml}$ magnification: 100x. Arrows indicate morphological changes that include loss of flat polygonal shape, cluster formation, shrinkage and irregular shape. Bar = 50 μm .

3.2 MTT ASSAY

Shown below are percentages of viable benign (RPWE 1) and malignant (LNCaP) prostate cells after incubation with increasing concentrations of GT, BT, FR, UR, EGCG and ASP 125-50 000 µg/ml for 24 and 72 hours, respectively.

3.2.1 Viability of malignant LNCaP and benign (RPWE 1) prostate cells measured by MTT ASSAY

3.2.1.1 Incubation of malignant (LNCaP) prostate cells with black tea for 24 and 72 hours.

After subjecting the data to a normality test, a two-tailed paired t-test showed that incubation of malignant (LNCaP) prostate cells for 24 hours with 125-1 000 µg/ml BT promoted a non-significant ($p > 0.05$) increase in cell viability whereas higher concentration of BT 5 000 and 10 000 µg/ml caused a non - significant ($p > 0.05$) decrease in cell viability respectively compared to the negative control. The positive control produced caused a significant ($p < 0.001$) decrease in cell viability compared to the negative control.

At 72 hours incubation period one-way ANOVA statistical analysis showed a concentration- dependent increase in mitochondrial dehydrogenase activity which was initially non-significant ($p > 0.05$) at 125 µg/ml BT and then became significant ($p < 0.01$, $p < 0.01$) after incubating the cells with 250, 500 and 1 000 µg/ml BT, respectively, compared to the negative control. This was followed by a concentration - dependent significant ($p < 0.001$) decrease in mitochondrial dehydrogenase activity at 5 000, 10 000 and 50 000 µg/ml BT, respectively. The positive control (6% DMSO) produced a significant ($p < 0.001$) reduction in mitochondrial dehydrogenase activity compared to the control.

Two-way ANOVA statistical analysis followed by Sidak's multiple comparison test showed that malignant (LNCaP) prostate cells exposed to 125, 250, 500 and 1 000 µg/ml BT for 72 hours induced a significant ($p < 0.01$, $p < 0.001$, $p < 0.001$, $p < 0.001$) increase in mitochondrial dehydrogenase activity compared to 24 hour of incubation. However, during the same incubation period (72 hours) 5 000 µg/ml BT produced no clear change in mitochondrial dehydrogenase activity compared to 24 hour incubation. In addition to this, incubation for 72 hours with 10 000 and 50 000 µg/ml BT resulted in significant ($p > 0.01$) decrease in mitochondrial dehydrogenase activity compared to 24 hour incubation. Incubation with (6% DMSO) positive control for 72 hours showed an enhanced significant ($p < 0.001$) decreased mitochondrial dehydrogenase activity compared to 24 hours (Fig 3.19).

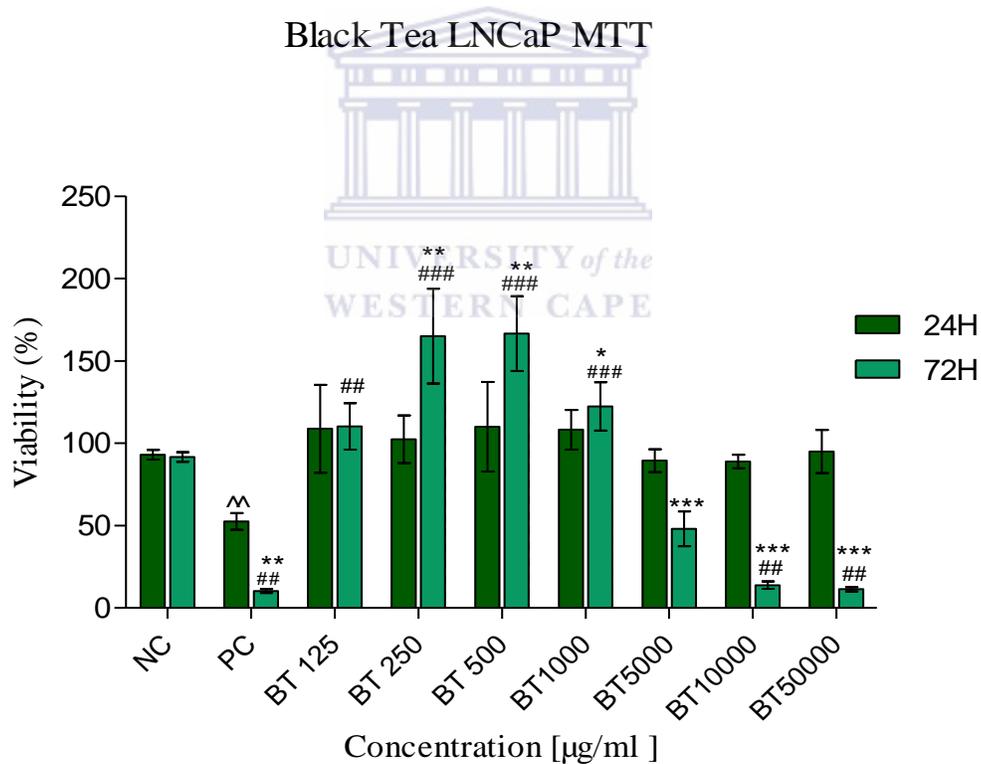


Fig 3- 19: Viability of LNCaP cells exposed to black tea for 72 hours.

Values represent mean \pm SD, n=9. (BT) Black tea; NC= negative control (medium); PC= positive control (6% DMSO) ### $p < 0.001$; ## $p < 0.01$, # $p < 0.05$; *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$; ^^ $p < 0.001$; ^^ $p < 0.01$; ^ $p < 0.05$; ^: represent p- value at 24 hours compared to its negative control. *: represent p – value at 72 hours compared to its negative control. #: represent p- value at 72 hours compared to 24 hours.

3.2.1.2 Incubation of malignant (LNCaP) prostate cells with green tea for 24 and 72 hours.

Incubation for 24 hours resulted in a significant increase in mitochondrial dehydrogenase activity in the range from 125, 250, 500 and 1 000 $\mu\text{g/ml}$ GT, which peaked at 250 $\mu\text{g/ml}$ GT compared to the negative control. This was followed by a significant ($p < 0.001$) concentration-dependent decrease in cell viability at 5 000, 10 000 and 50 000 $\mu\text{g/ml}$ GT. At the same time, the positive control produced a significant ($p < 0.001$) drop in mitochondrial dehydrogenase activity compared to the negative control.

One - way ANOVA statistical analysis showed that exposure of malignant (LNCaP) prostate cells to green tea for 72 hours resulted in a non-significant ($p > 0.05$) increase in mitochondrial dehydrogenase activity at 125-500 $\mu\text{g/ml}$ GT compared to the negative control. This was followed by a significant ($p < 0.01$) increase in mitochondrial dehydrogenase activity at 1 000 $\mu\text{g/ml}$ GT followed by a sharp and significant ($p < 0.001$) drop between 5 000 and 50 000 $\mu\text{g/ml}$ GT. The positive control produced stronger and significant ($p < 0.001$) decrease in cell viability compared to the negative control.

Two way ANOVA statistical analysis followed by Sidak's multiple comparison test showed that malignant (LNCaP) prostate cells exposed to 125-1,000 $\mu\text{g/ml}$ GT for 72 hours induced a significant decrease ($p < 0.001$) in cell viability compared to 24 hour incubation. However, during the same incubation period (72 hours) 5 000-50 000 $\mu\text{g/ml}$ GT resulted in a non-significant ($p > 0.05$) decrease in mitochondrial dehydrogenase activity compared to 24 hour incubation period. Incubation with positive control for 72 hours showed a significant sharp drop ($p < 0.001$) in cell viability compared to 24 hours (Fig 3.20).

Green Tea LNCaP MTT

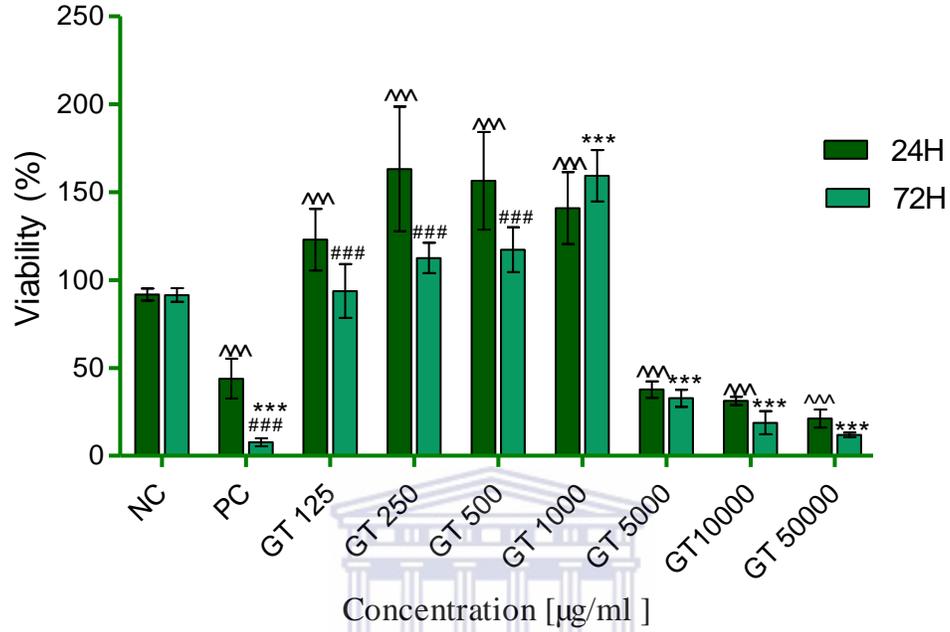


Fig 3- 20: Viability of malignant (LNCaP) prostate cells exposed to green tea for 24 and 72 hours. Values represent mean \pm SD n=9. (GT) Green tea; NC= negative control (medium); PC= positive control (6% DMSO) ### p < 0.001; ## p < 0.01, # < 0.05; ***p < 0.001; ** p < 0.01; * p < 0.05; ^^p < 0.001; ^^ p < 0.01; ^ p < 0.05; ^ : represent p- value at 24 hours compared to its negative control. *: represent p – value at 72 hours compared to its negative control. #: represent p- value at 72 hours compared to 24 hours.

3.2.1.3 Incubation of malignant (LNCaP) prostate cells with fermented rooibos for 24 and 72 hours.

After subjecting the data to normality test, two - tailed paired t-test showed that incubation of malignant (LNCaP) prostate cells for 24 hours with 125, 250, 5 000, 10 000 and 50 000 $\mu\text{g/ml}$ FR stimulated a non-significant ($p > 0.05$) decrease in mitochondrial dehydrogenase activity compared to the control. Whilst treatment with 500 and 1 000 $\mu\text{g/ml}$ FR produced non-significant ($p > 0.05$) increase in mitochondrial dehydrogenase activity respectively compared to the control. The positive control showed a significant ($p < 0.001$) drop in cell viability compared to the negative control.

One way ANOVA showed that incubation of malignant (LNCaP) prostate cells for 72 hours with 125, 250, 500 and 1 000 $\mu\text{g/ml}$ FR induced a non-significant ($p > 0.05$) generalised increase in mitochondrial dehydrogenase activity compared to the negative control. However, exposure of the cells to 5 000 and 10 000 $\mu\text{g/ml}$ FR produced a significant ($p < 0.01$) increase in mitochondrial dehydrogenase activity. Further to this, a significant sharp drop in cell viability was observed at 50 000 $\mu\text{g/ml}$ FR. The positive control showed a significant ($p < 0.001$) decrease in mitochondrial dehydrogenase activity compared to the negative control.

Two way ANOVA statistical analysis followed by Sidak's multiple comparison test showed that malignant (LNCaP) prostate cells exposed to 125, 250, 500 and 1 000 $\mu\text{g/ml}$ FR for 72 hours produced no significant ($p > 0.05$) changes in mitochondrial dehydrogenase activity when compared to 24 hour incubation. However, incubation for 72 hours with 5 000 and 10 000 $\mu\text{g/ml}$ FR produced significant ($p < 0.001$) increase in mitochondrial dehydrogenase activity respectively followed by a sharp significant decrease with 50 000 $\mu\text{g/ml}$ FR when compared to 24 hour incubation. Incubation with positive control (6% DMSO) for 72 hours showed a significant ($p <$

0.001) decrease in mitochondrial dehydrogenase activity compared to 24 hours (Fig 3.21).

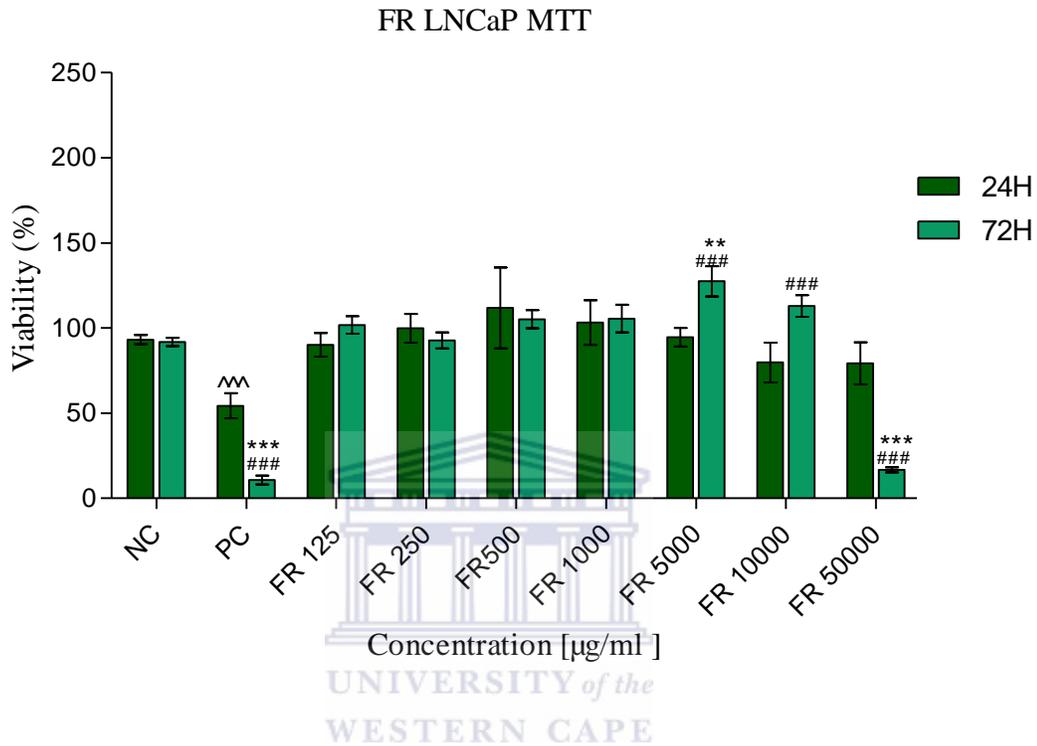


Fig 3- 21: Viability of malignant (LNCaP) prostate cells exposed to a fermented rooibos for 72 hours. Values represent mean \pm SD, n=9. (FR) fermented rooibos, NC= negative control (medium); PC= positive control (6% DMSO) *** p < 0.001; ** p < 0.01, * p < 0.05; *** p < 0.001; ** p < 0.01; * p < 0.05; $^{^^}$ p < 0.001; $^{^^}$ p < 0.01; $^{\wedge}$ p < 0.05; $^{\wedge}$: represent p- value at 24 hours compared to its negative control. * : represent p – value at 72 hours compared to its negative control. $^{\#}$: represent p- value at 72 hours compared to 24 hours.

3.2.1.4 Incubation of malignant (LNCaP) prostate cells with unfermented rooibos for 24 and 72 hours.

After subjecting the data to a normality test, two tailed paired t-test showed that incubation of malignant (LNCaP) prostate cells for 24 hours with 125-500 µg/ml UR produced a non-significant ($p > 0.05$) decrease in mitochondrial dehydrogenase activity compared to the negative control. However, a significant ($p < 0.01$) increase in mitochondrial dehydrogenase activity was shown at 1 000-10 000 µg/ml UR which peaked at 5 000 µg/ml UR and significantly ($p < 0.001$) decreased at 50 000 µg/ml UR compared to the negative control. The positive control showed a significant ($p < 0.001$) decrease in cell viability compared to the negative control.

One-way ANOVA statistical analysis showed that incubation of malignant (LNCaP) prostate cells for 72 hours showed non-significantly ($p > 0.05$) lower values of cell viability in lower concentrations of UR 125-1 000 µg/ml. Thereafter, there was a concentration-dependent significant ($p < 0.001$) decrease in cell viability starting from 1 000 µg/ml UR to 50 000 µg/ml UR. The positive control showed a significant ($p < 0.001$) decrease in cell viability compared to the negative control.

Two way ANOVA statistical analysis followed by Sidak's multiple comparison test showed that malignant (LNCaP) prostate cells exposed to 125, 250 and 500 µg/ml UR for 72 hours produced a non-significant ($p > 0.05$) change in mitochondrial dehydrogenase activity when compared to 24 hour incubation. However, during the same incubation period (72 hours) 1000, 5 000 and 10 000 µg/ml UR showed a significant ($p < 0.001$) decrease in mitochondrial dehydrogenase activity respectively when compared to 24 hour incubation. Further to this, incubation with (50 000 µg/ml UR) for 72 hours resulted in a significant ($p > 0.01$) decrease in mitochondrial dehydrogenase activity compared to 24 hour incubation. Incubation with (6% DMSO) positive control for 72 hours showed an enhanced significant ($p < 0.001$) decreased mitochondrial dehydrogenase activity compared to 24 hours (Fig 3.22).

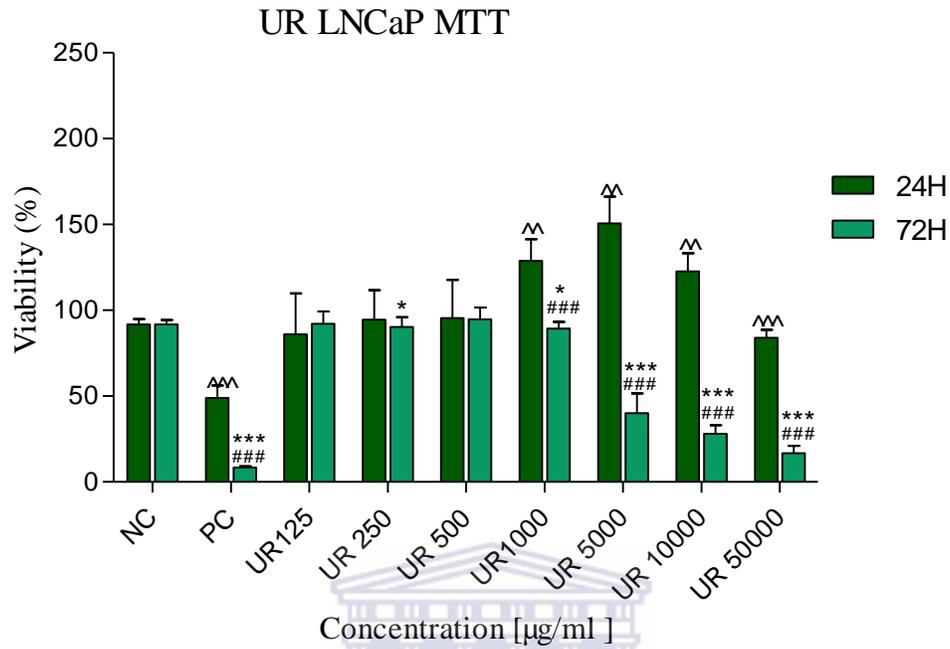


Fig 3- 22: Effect of unfermented rooibos on the viability of malignant (LNCaP) prostate cells following exposure for 24 and 72 hours.

Values represent mean \pm SD n=9. (UR) unfermented rooibos; NC= negative control (medium); PC= positive control (6% DMSO) ### p < 0.001; ## p < 0.01, # < 0.05; ***p < 0.001; ** p < 0.01; * p < 0.05; ^^^p < 0.001; ^^ p < 0.01; ^ p < 0.05; ^: represent p- value at 24 hours compared to its negative control. *: represent p – value at 72 hours compared to its negative control. #: represent p- value at 72 hours compared to 24 hours.

3.2.1.5 Incubation of malignant (LNCaP) prostate cells with aspalathin for 24 and 72 hours.

The normality test followed by a two-tailed paired test was used to analyse data obtained after exposing malignant (LNCaP) prostate cells to 125, 250, 500, 1 000, 5 000 and 10 000 µg/ml ASP for 24 hours. The results obtained showed a non-significant decrease in mitochondrial dehydrogenase activity when compared to the control. Incubation for 24 hours with 10 000 µg/ml ASP produced significant ($p < 0.01$) decrease in mitochondrial dehydrogenase activity. Besides this, the positive control showed a significant ($p < 0.001$) decrease in cell viability when compared to the negative control.

One-way ANOVA statistical analysis of data obtained after exposing the cells to increasing concentrations of aspalathin for 72 hours showed a significant ($p < 0.05$, $p < 0.01$) increase in mitochondrial dehydrogenase activity at 250 and 500 µg/ml ASP, respectively. This was followed by a marked significant ($p < 0.001$) increase in mitochondrial dehydrogenase activity at 1 000 and 5 000 µg/ml ASP, respectively, compared to the negative control. The mitochondrial dehydrogenase activity then significantly ($p < 0.01$ and $p < 0.001$) decreased both at 10 000 and 50 000 µg/ml ASP, respectively compared to the negative control. The positive control showed a significant ($p < 0.001$) drop in cell viability compared to the negative control.

Two way ANOVA statistical analysis followed by Sidak's multiple comparison test showed that malignant (LNCaP) prostate cells exposed to 250, 500, 1000, 5 000 and 10 000 µg/ml ASP for 72 hours induced an enhanced significant ($p < 0.001$) increase in mitochondrial dehydrogenase activity respectively when compared to 24 hour incubation. However, incubation with 125 and 50 000 µg/ml ASP, respectively, produced no significant ($p > 0.05$) changes in cell viability compared to 24 hour incubation period. The positive controls 72 hours did not show any significant ($p >$

0.05) change in mitochondrial dehydrogenase activity when compared to 24 hours (Fig 3.23).

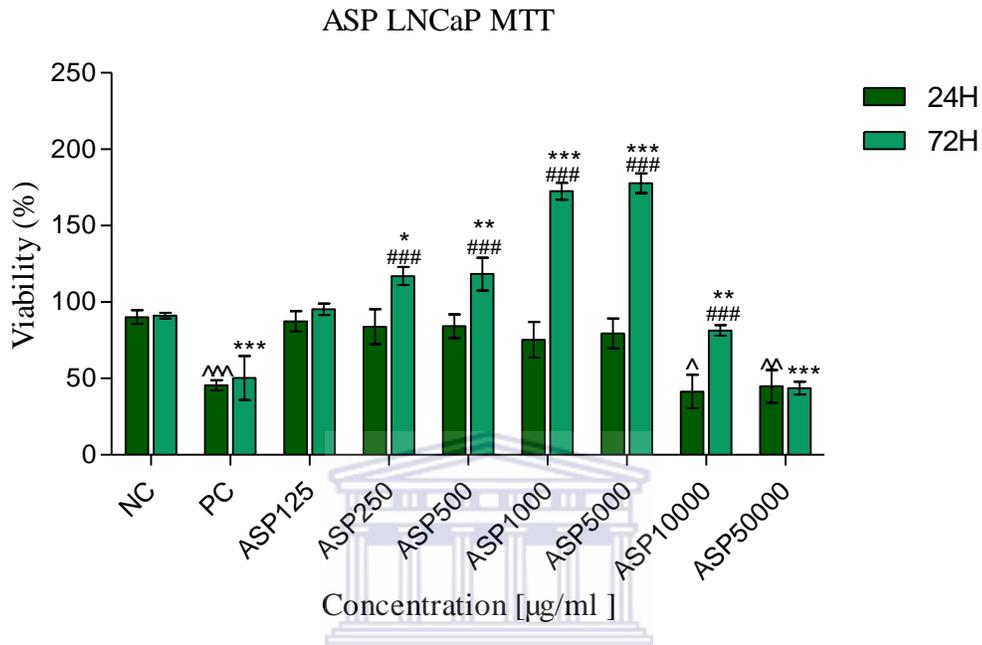


Fig 3- 23: Viability of malignant (LNCaP) prostate cells exposed to aspalathin for 24 and 72 hours. Values represent mean \pm SD, n=9. (ASP) aspalathin; NC= negative control (medium); PC= positive control (6% DMSO) ### p < 0.001; ## p < 0.01, # < 0.05; ***p < 0.001; ** p < 0.01; * p < 0.05; ^^p < 0.001; ^^ p < 0.01; ^ p < 0.05; ^: represent p- value at 24 hours compared to its negative control. *: represent p – value at 72 hours compared to its negative control. #: represent p- value at 72 hours compared to 24 hours.

3.2.1.6 Incubation of malignant (LNCaP) prostate cells with EGCG for 24 and 72 hours.

A normality test followed by a two-tailed paired t-test showed that incubation of malignant (LNCaP) prostate cells for 24 hours with 125-10 000 $\mu\text{g/ml}$ EGCG showed no significant ($p > 0.05$) changes in mitochondrial dehydrogenase when compared to the control. Further increase in the concentration of EGCG, showed a significant ($p < 0.001$) drop in mitochondrial dehydrogenase activity at 50 000 $\mu\text{g/ml}$ EGCG. Besides this, the positive control showed a significant ($p < 0.001$) decrease in mitochondrial dehydrogenase activity compared to the negative control.

One way ANOVA showed that incubation of the malignant (LNCaP) prostate cells with EGCG for 72 hours results in a significant ($p < 0.05$, $p < 0.001$, $p < 0.001$) increase in mitochondrial dehydrogenase activity at 250, 500 and 1 000 $\mu\text{g/ml}$ EGCG, respectively, compared to the negative control. This was followed by a rapid concentration-dependent significant ($p < 0.001$) decrease in mitochondrial dehydrogenase activity at 5 000, 10 000 and 50 000 $\mu\text{g/ml}$ EGCG, respectively. Besides this, the positive control showed a significant ($p < 0.001$) decrease in mitochondrial dehydrogenase activity compared to the negative control.

Two-way ANOVA statistical analysis followed by Sidak's multiple comparison test showed that malignant (LNCaP) prostate cells exposed to 125 $\mu\text{g/ml}$ EGCG for 72 hours induced a significant ($p < 0.001$) increase in mitochondrial dehydrogenase activity compared to 24 hour incubation. However, incubation with 250 $\mu\text{g/ml}$ EGCG showed no change in mitochondrial dehydrogenase activity compared to 24 hour incubation period. In contrast, 500, 1000, 5 000, 10 000 and 50 000 $\mu\text{g/ml}$ EGCG resulted in significant ($p > 0.001$) decreased mitochondrial dehydrogenase activity compared to 24 hour incubation period. The positive controls (6% DMSO) at 24 and 72 hours did not show any significant ($p < 0.001$) change in cell viability compared to 24 hours (Fig 3.24)

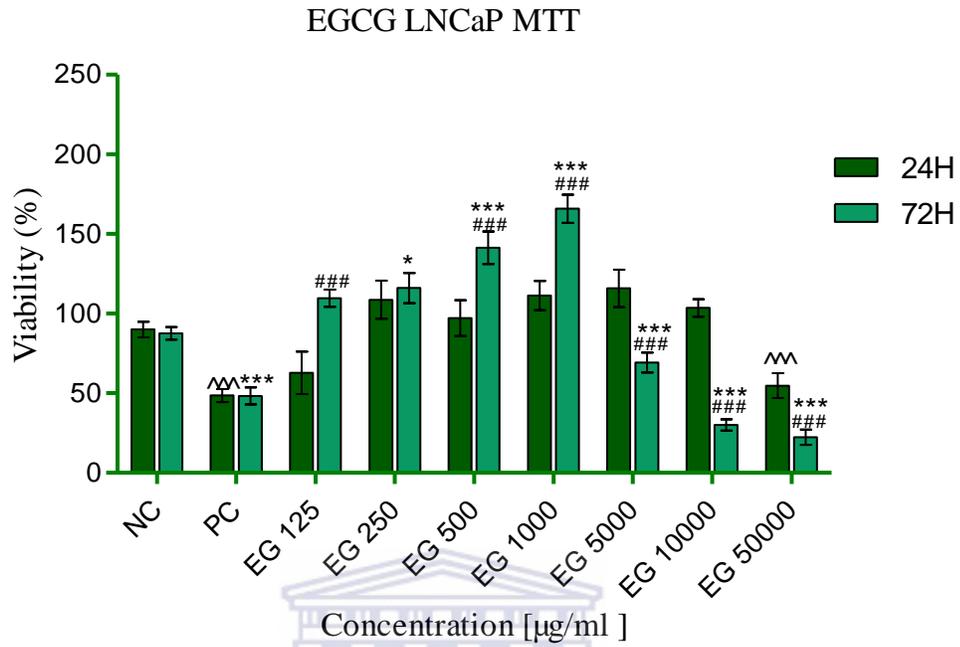


Fig 3- 24: Viability of malignant (LNCaP) prostate cells exposed to EGCG for 72 hours. Values represent mean \pm SD, n=9. (EGCG) epigallocatechin gallate; NC= negative control (medium); PC= positive control (6% DMSO) ### p < 0.001; ## p < 0.01, # < 0.05; ***p < 0.001; ** p < 0.01; * p < 0.05; ^^p < 0.001; ^^ p < 0.01; ^ p < 0.05; ^: represent p- value at 24 hours compared to its negative control. *: represent p – value at 72 hours compared to its negative control. #: represent p- value at 72 hours compared to 24 hours.

3.2.1.7 Incubation of Benign (RPWE 1) prostate cells with black tea for 24 and 72 hours.

One- way ANOVA showed that incubation of benign (RPWE 1) prostate cells for 24 hours with 125-1 000 $\mu\text{g/ml}$ BT promoted a non-significant ($p > 0.05$) increase in mitochondrial dehydrogenase activity compared to the control. In addition to this, 5000, 10 000 and 50 000 $\mu\text{g/ml}$ BT produced a significant ($p < 0.01$, $p < 0.001$ and $p < 0.001$) increase in mitochondrial dehydrogenase activity compared to the control. At the same time, the positive control produced a strong and significant ($p < 0.001$) decrease in mitochondrial dehydrogenase activity compared to the control.

At 72 hour incubation period, one way ANOVA showed a non-significant ($p > 0.05$) rise in mitochondrial dehydrogenase activity at 1 000 $\mu\text{g/ml}$ BT compared to the negative control. This was followed by a concentration - dependent significant ($p < 0.001$) increase in mitochondrial dehydrogenase activity after exposure to 5000, 10 000 and 50 000 $\mu\text{g/ml}$ BT, respectively, compared to the control. At the same time the positive control produced a sharp and significant ($p < 0.001$) decrease in mitochondrial dehydrogenase activity compared to the control.

Two- way ANOVA statistical analysis followed by Sidak's multiple comparison test showed that benign (RPWE 1) prostate cells exposed to 125-10 000 $\mu\text{g/ml}$ BT for 72 hours produced a non-significant ($p > 0.05$) change in mitochondrial dehydrogenase activity compared to 24 hour incubation. However, incubation with 50 000 $\mu\text{g/ml}$ BT for 72 hours resulted in significant ($p < 0.01$) increased mitochondrial dehydrogenase activity compared to 24 hour incubation period. The positive controls (6% DMSO) at 72 hours did not show any significant ($p > 0.05$) change in mitochondrial dehydrogenase activity compared to 24 hours (Fig 3.25)

3.2.1.8 Incubation of benign (RPWE 1) prostate cells with green tea for 24 and 72 hours.

One way ANOVA showed that incubation of benign (RPWE 1) prostate cells with 125-5000 µg/ml GT for 24 hours produced no effect in mitochondrial dehydrogenase activity whereas 10 000 and 50 000 µg/ml GT produced a significant ($p < 0.001$) concentration-dependent increase in mitochondrial dehydrogenase activity, respectively, compared to the negative control. The positive control produced a significant ($p < 0.001$) decrease in mitochondrial dehydrogenase activity compared to the control.

After incubating the cells for 72 hours, one-way ANOVA statistical analysis showed a significant ($p < 0.01$) increase mitochondrial dehydrogenase activity at 10 000 and 50 000 µg/ml GT, respectively, compared to the control. At the same time the positive control produced an enhanced significant ($p < 0.001$) decrease in mitochondrial dehydrogenase activity compared to the control.

Two-way ANOVA statistical analysis followed by Sidak's multiple comparison test showed that benign (RPWE 1) prostate cells exposed to 125-5 000 µg/ml GT for 72 hours showed no significant ($p > 0.05$) changes in mitochondrial dehydrogenase activity compared to 24 hour incubation. However, incubation with 10 000 or 50 000 µg/ml GT for 72 hours produced a significant ($p < 0.05$) increase in mitochondrial dehydrogenase activity compared to 24 hour incubation period. Incubation with GT for 72 hours did not produce any significant differences compared to 24 hour exposure. The positive controls (6% DMSO) at 72 hours did not show any significant ($p > 0.05$) change in mitochondrial dehydrogenase activity compared to 24 hours (Fig 3.26)

3.2.1.9 Incubation of Benign (RPWE 1) prostate cells with fermented rooibos for 24 and 72 hours.

One-way ANOVA showed that incubation of benign (RPWE 1) prostate cells with 5000 µg/ml FR for 24 hours promoted a significant ($p < 0.05$) increase in mitochondrial dehydrogenase activity whereas 10 000 and 50 000 µg/ml FR produced a significant ($p < 0.01$, $p < 0.001$) concentration-dependent increase in mitochondrial dehydrogenase activity, respectively, compared to the negative control. The positive control caused a significant ($p < 0.05$) drop in mitochondrial dehydrogenase compared to the negative control.

At 72 hours incubation period, one-way ANOVA showed a significant ($p < 0.001$) mitochondrial dehydrogenase activity at 1000 to 50 000 µg/ml FR, respectively, compared to the control. The positive control produced a significant ($p < 0.001$) decrease in mitochondrial dehydrogenase activity compared to the negative control.

Two-way ANOVA statistical analysis followed by Sidak's multiple comparison test showed that benign (RPWE 1) prostate cells exposed to 125-500 µg/ml FR for 72 hours showed a non-significant ($p > 0.05$) increase in mitochondrial dehydrogenase activity compared to 24 hour incubation. However, incubation for 72 hour with 1000, 5 000 or 10 000 µg/ml FR produced a significant ($p < 0.001$) increase in mitochondrial dehydrogenase activity respectively compared to 24 hour incubation. Besides this, incubation with 50 000 µg/ml FR produced no significant ($p > 0.05$) change in mitochondrial dehydrogenase activity compared to 24 hours. The positive control at 72 hours showed a significant ($p < 0.001$) decrease in mitochondrial dehydrogenase activity compared to 24 hours (Fig 3.27).

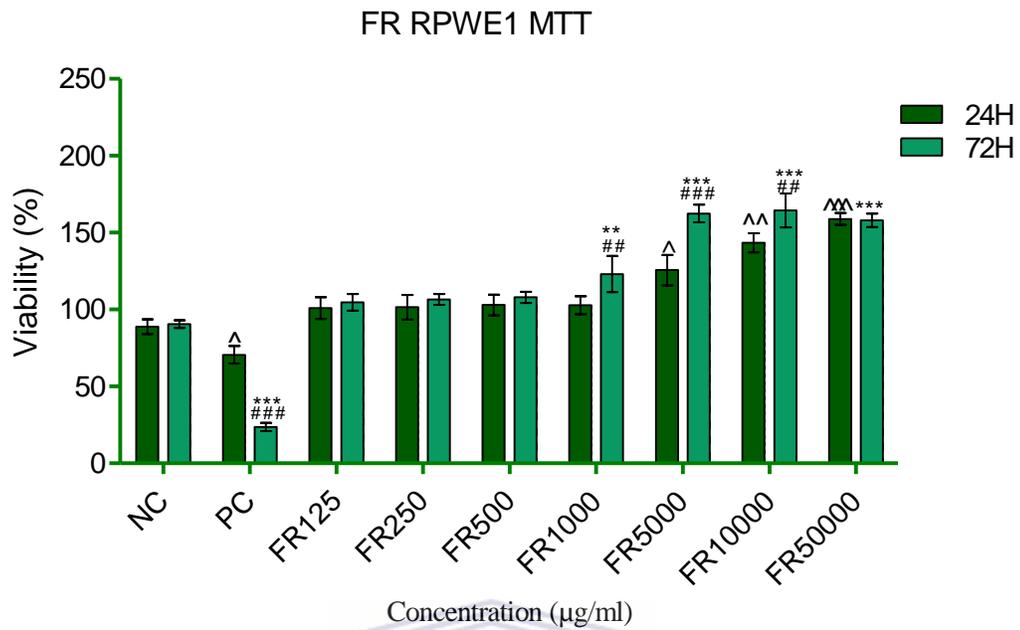


Fig 3- 27: Viability of benign (RPWE 1) prostate cells following exposure to fermented rooibos for 24 and 72 hours.

Values represent mean \pm SD n=9. (FR) fermented rooibos; NC= negative control (medium); PC= positive control (6% DMSO) ### p < 0.001; ## p < 0.01, # < 0.05; ***p < 0.001; ** p < 0.01; * p < 0.05; ^^p < 0.001; ^^ p < 0.01; ^ p < 0.05; ^: represent p- value at 24 hours compared to its negative control. *: represent p – value at 72 hours compared to its negative control. #: represent p- value at 72 hours compared to 24 hours.

3.2.1.10 Incubation of Benign (RPWE 1) prostate cells with unfermented rooibos for 24 and 72 hours.

One way ANOVA statistical analysis showed that incubation of benign (RPWE 1) prostate cells for 24 hours with 125-1 000 µg/ml UR did not change mitochondrial dehydrogenase activity compared to the control. In contrast, 5 000, 10 000 and 50 000 µg/ml UR produced a significant ($p < 0.01$, $p < 0.001$) concentration-dependent increase in mitochondrial dehydrogenase activity. The positive control produced a significant ($p < 0.001$) decrease in mitochondrial dehydrogenase activity compared to the control.

After 72 hours of incubation, one- way ANOVA statistical analysis showed again no change in mitochondrial dehydrogenase activity when benign (RPWE 1) prostate cells were exposed to 125, 250, 500 and 1 000 µg/ml UR compared to the control. However, this was followed by a significant ($p < 0.01$, $p < 0.001$) a concentration-dependent increase in mitochondrial dehydrogenase activity at 5 000, 10 000 and 50 000 µg/ml UR, respectively. The positive control produced significant ($p < 0.001$) drop in mitochondrial dehydrogenase activity.

Two- way ANOVA statistical analysis followed by Sidak's multiple comparison test showed that benign (RPWE 1) prostate cells exposed to 125, 250, 500, 1 000, 5 000, 10 000 µg/ml UR for 72 hours did not show significant ($p > 0.05$) changes in mitochondrial dehydrogenase activity respectively compared to 24 hour incubation. However, 72 hour incubation with 50 000 µg/ml UR induced a significant ($p < 0.001$) increase in mitochondrial dehydrogenase activity compared to 24 hours. The positive controls at 72 hours did not show any significant ($p > 0.05$) change in mitochondrial dehydrogenase activity compared to 24 hours (Fig 3.28).

UR RPWE 1 MTT

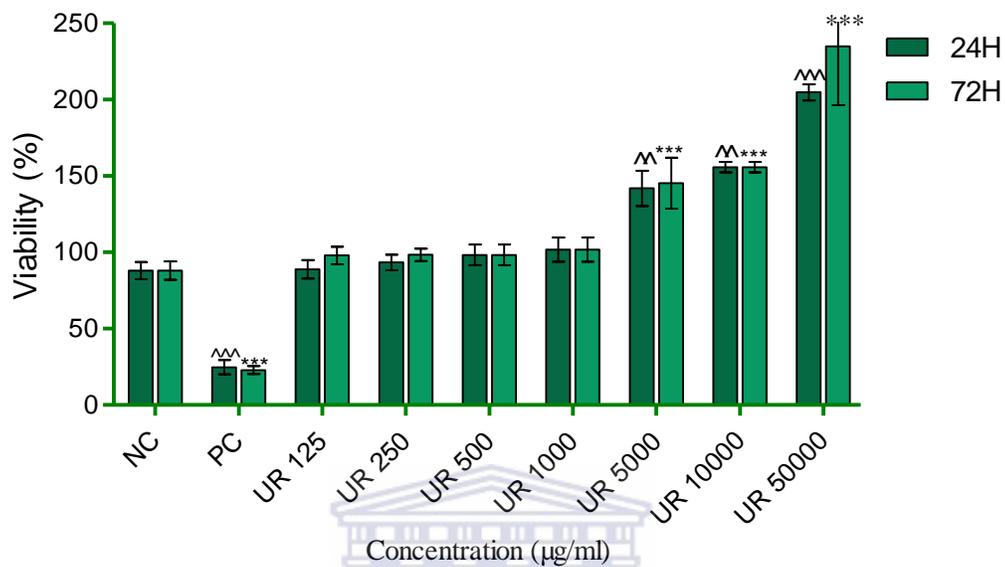


Fig 3- 28: Viability of benign (RPWE 1) prostate cells following exposure to unfermented rooibos for 24 and 72 hours.

Values represent mean \pm SD n=9. (UR) unfermented rooibos; NC= negative control (medium); PC= positive control (6% DMSO) ### p < 0.001; ## p < 0.01, # < 0.05; ***p < 0.001; ** p < 0.01; * p < 0.05; ^^p < 0.001; ^ p < 0.01; ^ p < 0.05; ^ : represent p- value at 24 hours compared to its negative control. *: represent p – value at 72 hours compared to its negative control. #: represent p- value at 72 hours compared to 24 hours.

3.2.1.11 Incubation of Benign (RPWE 1) prostate cells with aspalathin for 24 and 72 hours.

One-way ANOVA statistical analysis showed that 24 hour incubation of benign (RPWE 1) prostate cells with 125-50 000 µg/ml ASP promoted significantly lower mitochondrial dehydrogenase activity from 1 000 µg/ml ASP onwards compared to the negative control. The positive control produced a significant ($p < 0.001$) decrease in mitochondrial dehydrogenase activity compared to the negative control.

At 72 hours incubation period one-way ANOVA statistical analysis showed lower cell viability at 125 µg/ml ASP, thereafter, 250 – 10 000 µg/ml ASP caused a concentration-dependent increase in mitochondrial dehydrogenase activity, that was significant ($p < 0.001$) from 500 µg/ml ASP onwards. The positive control produced a drastic significant ($p < 0.001$) drop in mitochondrial dehydrogenase activity compared to the negative control.

Two-way ANOVA statistical analysis followed by Sidak's multiple comparison test showed that benign (RPWE 1) prostate cells exposed to 125- 250 µg/ml ASP for 72 hours produced did not differ in mitochondrial dehydrogenase activity compared to 24 hour incubation. However, incubation for 72 hour with 500, 1 000, 5 000 and 10 000 µg/ml ASP produced a significant ($p < 0.001$) increase in mitochondrial dehydrogenase activity respectively when compared to 24 hour incubation. The positive controls at 72 hours did not show any significant ($p > 0.05$) change in mitochondrial dehydrogenase activity compared to 24 hours (Fig 3.29)

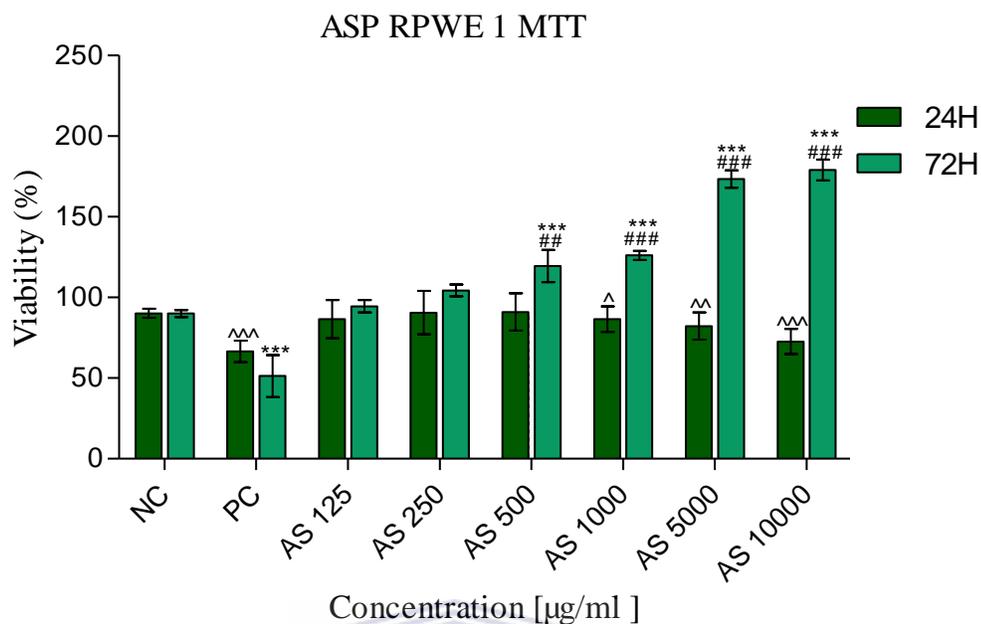


Fig 3- 29: Effect of aspalathin on the viability of benign (RPWE 1) prostate cells after 24 and 72 hour incubation period.

Values represent mean \pm SD n=9. (ASP) aspalathin; NC= negative control (medium); PC= positive control (6% DMSO) ### p < 0.001; ## p < 0.01, # < 0.05; ***p < 0.001; ** p < 0.01; * p < 0.05; ^^p < 0.001; ^^ p < 0.01; ^ p < 0.05; ^ : represent p- value at 24 hours compared to its negative control. *: represent p – value at 72 hours compared to its negative control. #: represent p- value at 72 hours compared to 24 hours.

3.2.1.12 Incubation of Benign (RPWE 1) prostate cells with EGCG for 24 and 72 hours.

One-way ANOVA statistical analysis showed that incubation of benign (RPWE 1) prostate cells for 24 hours with 125-5000 µg/ml EGCG had no effect on cell viability, thereafter, there was a concentration-dependent significant ($p < 0.01$) decrease in cell viability at 1 000 µg/ml EGCG, onwards compared to the negative control. The positive control produced a drastic significant ($p < 0.001$) decrease in mitochondrial dehydrogenase activity compared to the negative control.

In contrast, at 72 hours incubation period one-way ANOVA statistical analysis showed a concentration-dependent significant ($p < 0.001$) increase in mitochondrial dehydrogenase activity at 10 000 µg/ml EGCG, respectively, compared to the negative control. The positive control produced a drastic significant ($p < 0.001$) decrease in mitochondrial dehydrogenase activity compared to the negative control.

Two-way ANOVA statistical analysis followed by Sidak's multiple comparison test showed that benign (RPWE 1) prostate cells exposed to 125- 500 µg/ml EGCG for 72 hours caused no significant ($p > 0.05$) changes in mitochondrial dehydrogenase activity compared to 24 hour incubation. However, incubation for 72 hour with 1 000, 5 000 and 10 000 µg/ml EGCG caused a significant ($p < 0.001$) concentration-dependent increase in mitochondrial dehydrogenase activity respectively when compared to 24 hour incubation. The positive controls at 72 hours caused significant ($p < 0.001$) decrease in mitochondrial dehydrogenase activity compared to 24 hours (Fig 3.30)

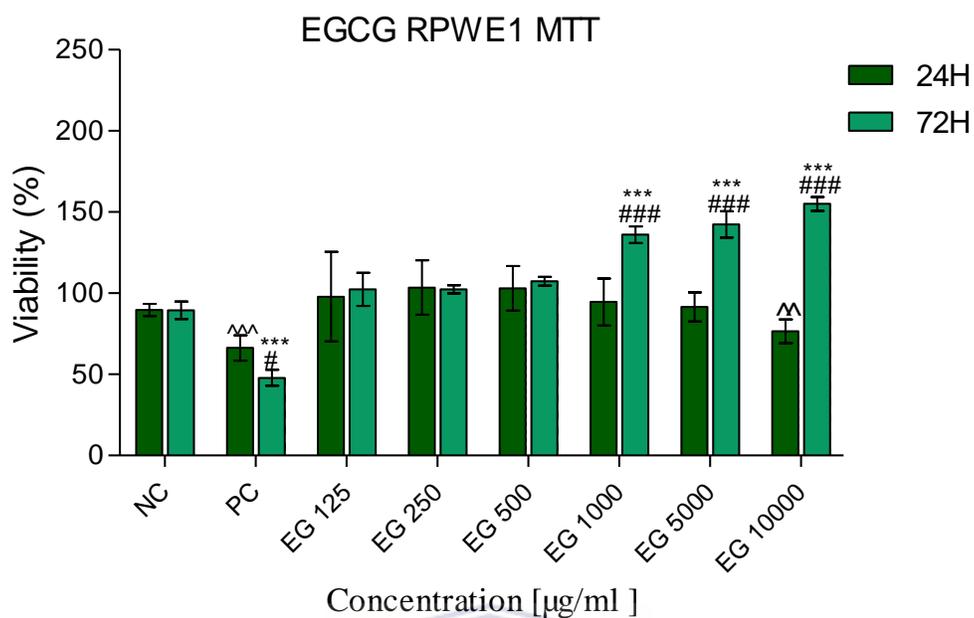


Fig 3-30: Viability of benign (RPWE 1) prostate cells following exposure to EGCG for 24 and 72 hours.

Values represent mean \pm SD n=9. (EGCG) epigallocatechin gallate; NC= negative control (medium); PC= positive control (6% DMSO) ### p < 0.001; ## p < 0.01, # < 0.05; ***p < 0.001; ** p < 0.01; * p < 0.05; ^^p < 0.001; ^ p < 0.01; ^ p < 0.05; ^: represent p- value at 24 hours compared to its negative control. *: represent p – value at 72 hours compared to its negative control. #: represent p-value at 72 hours compared to 24 hours.

3.3 Detection of reactive oxygen species (ROS) in benign (RWPE 1) and malignant (LNCaP) benign (RPWE 1) prostate cells using 5,6 CM H₂DCFDA and fluorescence microscope.

To investigate if tea or rooibos have a potential influence on the ROS level in prostate cells, benign (RPWE 1) and malignant (LNCaP) prostate cells were exposed to increasing concentrations of GT, BT, FR, UR, EGCG or ASP for 24 and 72 hours respectively. The fluorophore CM 5,6 CM H₂DCFDA 7 μM and inverted Zeiss Axiovert 200M fluorescence microscope (Zeiss, Göttingen, Germany) were used to determine ROS levels. In this thesis, 10 representative photographs were taken for each sample at 100x magnification. In brief, after loading cells with the ROS dye in an 8-well Ibidi slide, we aimed at identifying cells that were emitting the DCF fluorescence. Cells with this fluorescence, represented cells that were able to oxidise 2'-7' dichlorofluorescein (H₂DCF) to 2'-7' dichlorofluorescein (DCF). The diacetate form, H₂DCFDA when taken up by cells is acted upon by non-specific cellular esterases and in the process cleaves off lipophilic groups (Kalyanaramana et al., 2012). This results in the formation of a charged compound that gets trapped inside the cell. High DCF fluorescence intensity represents high ROS levels and vice versa for low DCF fluorescence intensity.

The results obtained in this thesis, did not meet the above principle, to the extent that there were a lot of disparities in the amount of fluorescence shown by photographs taken. There were a lot of variations in fluorescence intensity between different fields (micrographs) for each concentration. Firstly, different light intensities required different exposure times for one single treatment which may have contributed to this problem. Secondly, micrographs of RPWE 1 prostate cells showed fluorescence intensity that was comparable to that of LNCaP prostate cells. In this regard, LNCaP cells were expected to demonstrate the presence of high ROS through increased DCF fluorescence intensity compared to RPWE 1 cells. The disparities in fluorescence intensity were also observed within each of the two cell lines; micrographs of

untreated cells showed normal dividing cells plus high DCF fluorescence intensity whereas micrographs containing irregular shaped and highly stressed cells (those treated with higher concentrations of tea or rooibos) showed low fluorescence intensity. On the other hand, the cell morphology and spreading pattern as seen in those micrographs clearly showed that in general the benign RPWE 1 prostate cells coped well with high levels of ROS whereas the malignant LNCaP cells did not. In brief, after trying various interventions; quantification of ROS using fluorescence microscopy was not achieved. Micrographs of LNCaP and RPWE 1 cells are shown in Figs 3.31 - 3.55.



3.3.1 Detection of reactive oxygen species (ROS) in benign (RPWE 1) prostate cells after incubation with black tea for 24 hours using a fluorescence microscope.

Below are low magnification fluorescence microscope micrographs taken after 24 hour incubation of RPWE 1 cells with BT. Fig 3.31 (I) show cells incubated with 50 000 $\mu\text{g/ml}$ BT. The cells seem to be stressed as demonstrated by the increased cell volume (swollen) compared to the negative control (A). Furthermore, using DCF fluorescence, (I) showed increased fluorescence intensity compared to the negative control. In addition to this, Fig 3.31 (G) and (H) displayed cells which emitted fluorescence of similar intensity to the positive control (B), however the cells on these micrographs did not show any change in cell morphology. This means that these cells were not stressed and could cope with the ROS that was detected. In this study, we expected to see a concentration-dependent increase in fluorescence intensity. On the contrary, cells on micrographs C – H did not produce a concentration dependent fluorescence instead these cells produced fluorescence which similar to that of the negative control. In brief, all concentrations of BT, except 50 000 $\mu\text{g/ml}$, did not induced stress in RPWE 1 cells at 24 hours (Fig 3.31).

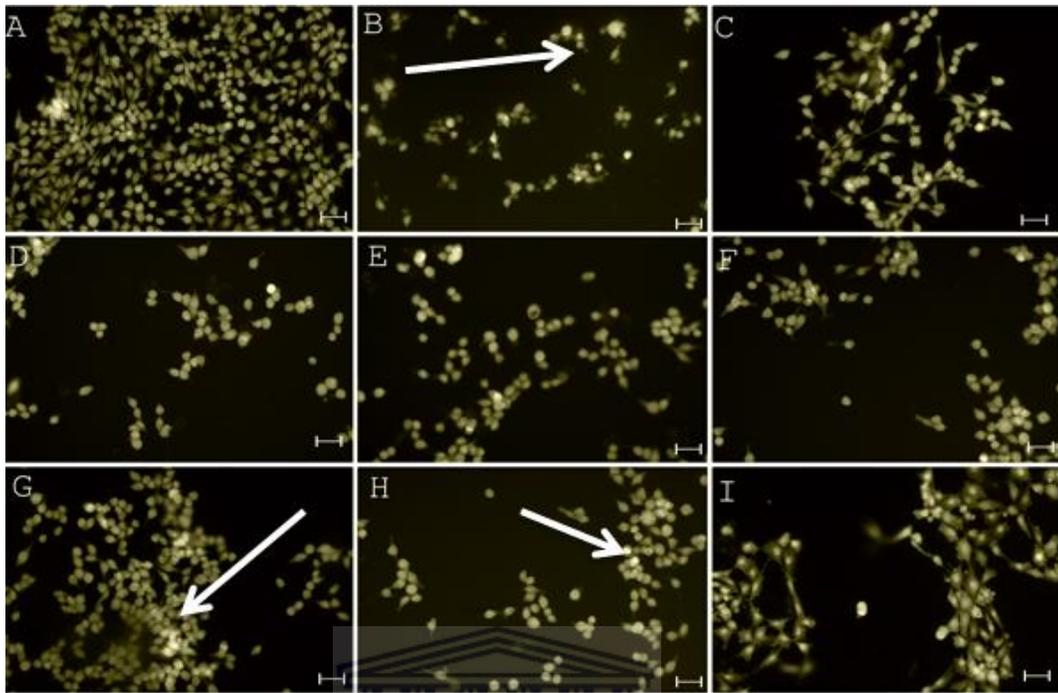
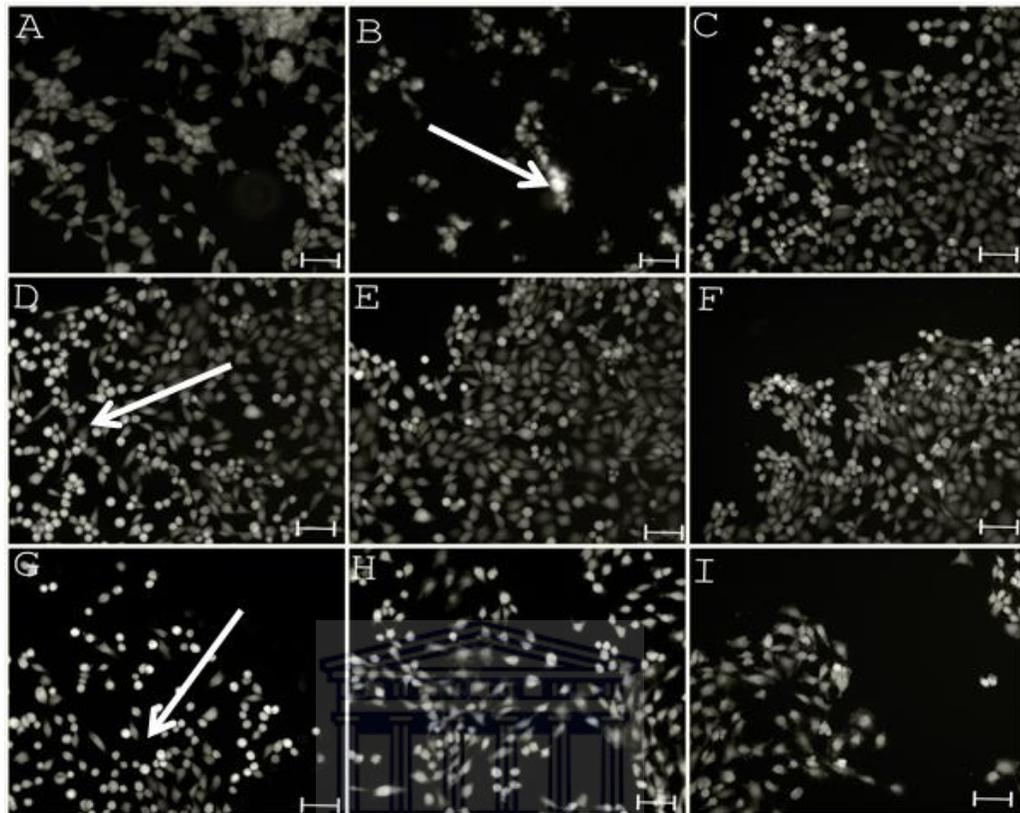


Fig 3-31: ROS quantity expression in benign (RPWE 1) prostate cells after 24 hour exposure to black tea using a fluorescence microscope.

(A) Negative control (B) PC (6% DMSO) (C) 125 µg/ml (D) 250 µg/ml (E) 500 µg/ml (F) 1 000 µg/ml (G) 5 000 µg/ml (H) 10 000 µg/ml (I) 50 000 µg/ml. Magnification: 100x. Bar = 50µm. Arrows illustrate stressed cells. Cells were stained with 7 µM CM 5, 6 CM H₂DCFDA.

3.3.2 Detection of reactive oxygen species (ROS) levels in benign (RPWE 1) prostate cells after incubation with green tea for 24 hours using a fluorescence microscope.

Below are low magnification fluorescence microscope micrographs taken after incubating RPWE 1 cells for 24 hour with green tea. Morphologically, the micrographs showed normal dividing and adherent cells. The cells did not show any obvious signs of stress, changes in cell volume when compared to the negative control (A). However, the degree of fluorescence intensity was not proportional to the concentration of the treatment. In this thesis, we expected highly stressed cells (cells treated with high concentration of green tea) to produce more ROS and fluorescence compared to cells which are less stressed (treated with lower concentrations of green tea). On the contrary, all the cells in these micrographs had low and highly variable fluorescence pattern which was not directly proportional to the concentration of the treatment. Further to this, cells on micrographs D and G appear to emit the same fluorescence intensity and yet they have been treated with different concentrations of GT (500 or 10 000 µg/ml). In addition to this, cells on micrographs A, B, C, E and F appeared to emit fluorescence of comparable intensity making it difficult to rate the ROS levels with reference to the concentration of GT. Furthermore, the fluorescence intensity of these micrographs was similar to that of the negative control. Cells on all micrographs were able to be seen because they were all producing some degree of ROS. However, the fluorescence was not intense probably because of low levels of ROS and the presence of antioxidants such as catalase, superoxide dismutase and glutathione. Otherwise, we expected a concentration-dependent increase in fluorescence intensity (Fig 3.32).



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Fig 3-32: ROS quantity expression in benign (RPWE 1) prostate cells after 24 hour exposure to green tea using a fluorescence microscope.

(A) Negative control (B) PC (6% DMSO) (C) 125 µg/ml (D) 250 µg/ml (E) 500 µg/ml (F) 1 000 µg/ml (G) 5 000 µg/ml (H) 10 000 µg/m (I) 50 000 µg/ml. Cells were stained with 7 µM CM 5, 6 CM H₂DCFDA. Arrows illustrate cells emitting DCF fluorescence. Magnification: 100x. Bar = 50µm.

3.3.3 Detection of reactive oxygen species (ROS) in benign (RPWE 1) prostate cells after incubation with fermented rooibos for 24 hours using a fluorescence microscope.

Low magnification fluorescence microscope micrographs were taken after 24 hour incubation with fermented rooibos and all micrographs, except the positive control (B), showed normal dividing and adherent benign (RPWE 1) prostate cells when compared to the untreated cells (A). After loading the cells with H₂DCFDA, the micrographs showed cells which were emitting variable degrees of fluorescence. Notably, the intensity of the fluorescence was not directly proportional to the concentration of the treatment. In this thesis, we expected highly stressed cells to produce more ROS and in turn fluorescence more. On the contrary, all the cells in these micrographs had low fluorescence pattern which is an indication of low production of ROS. For example micrographs C, F, D and G displayed increased fluorescence than the negative control. Morphologically, the cells looked normal without any sign of stress when compared to the positive control. The presence of antioxidants, such as catalase, superoxide dismutase and glutathione has been implicated for this state of ROS levels. However, micrographs H and I treated with higher concentrations of FR showed cells with low DCF fluorescence than the rest of the micrographs. Otherwise, we expected highly stressed cells to fluorescence more than the untreated normal prostate cells (A) (Fig 3.33).

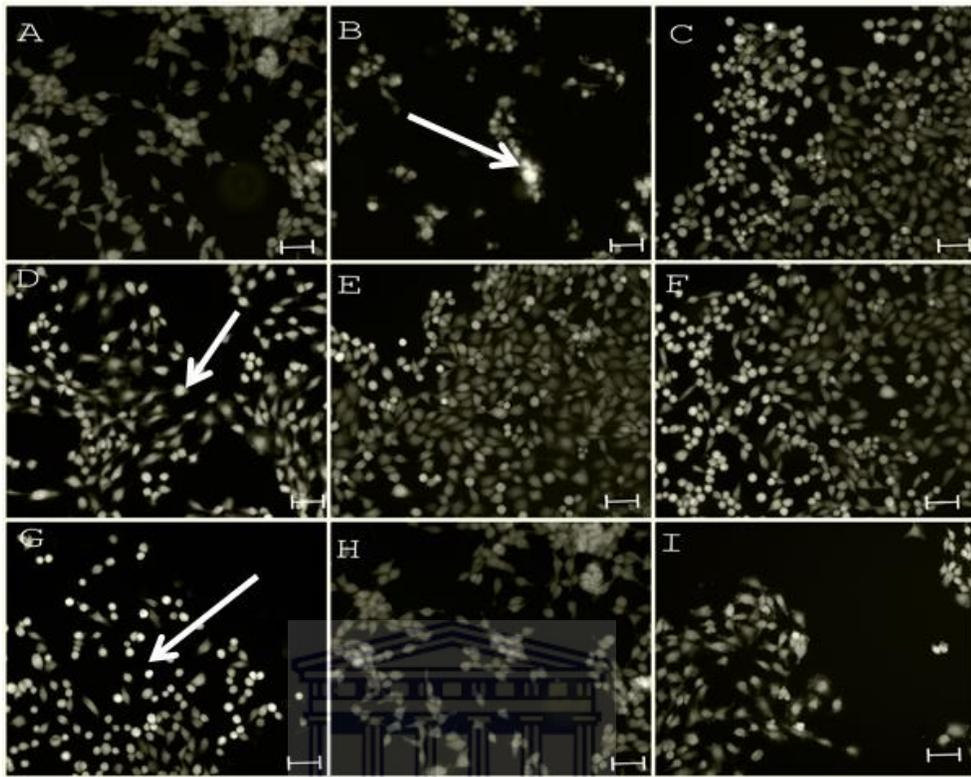
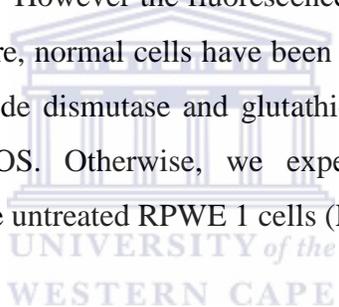
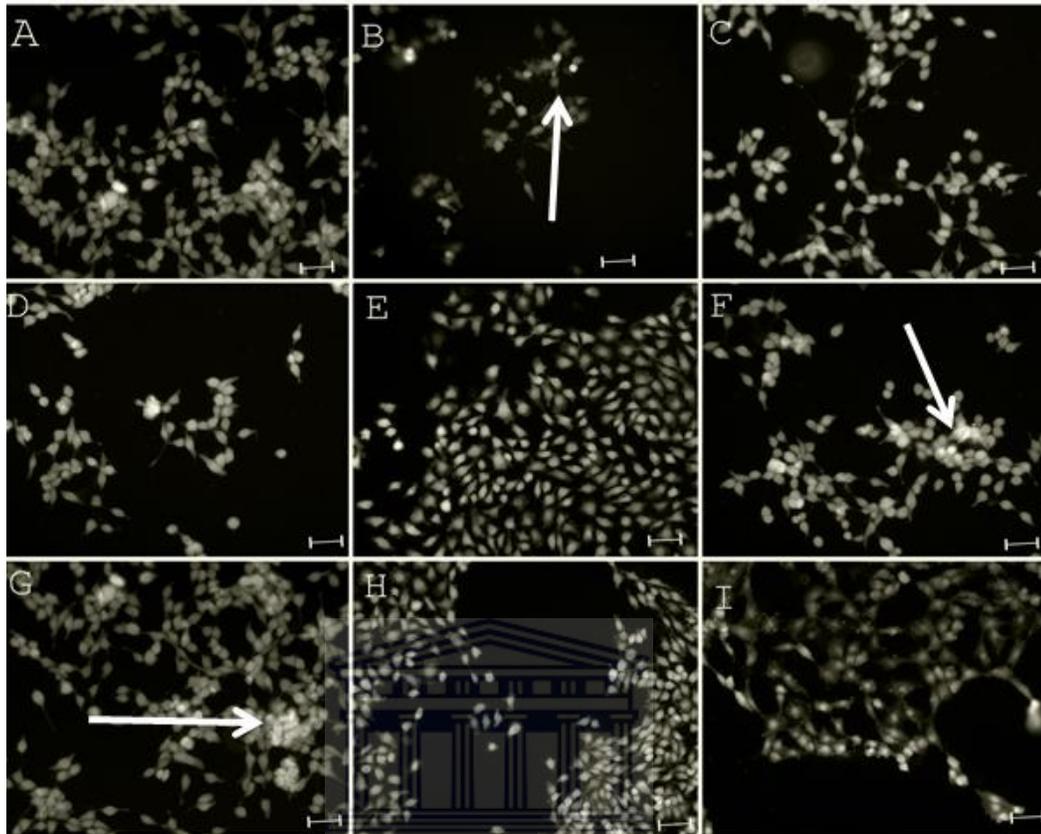


Fig 3-33: ROS quantity expression in benign (RPWE 1) prostate cells after 24 hour exposure to fermented rooibos using a fluorescence microscope. (A) Negative control (B) PC (6% DMSO) (C) 125 µg/ml (D) 250 µg/ml (E) 500 µg/ml (F) 1 000 µg/ml (G) 5 000 µg/ml (H) 10 000 µg/m (I) 50 000 µg/ml. Magnification: 100x. Bar = 50µm. Arrows illustrate stressed cells. Cells were stained with 7µM CM 5, 6 CM H₂DCFDA.

3.3.4 Detection of reactive oxygen species (ROS) in benign (RPWE 1) prostate cells after incubation with unfermented rooibos for 24 hours using a fluorescence microscope.

Below are low magnification fluorescence microscope micrographs taken after incubating RPWE 1 cells for 24 hour with unfermented rooibos. Subsequent staining of the cells with H₂DCFDA showed cells which emitted low fluorescence intensity. The DCF fluorescence produced by these cells was not directly proportional to the concentration of the unfermented rooibos. Morphologically, the cells did not show any obvious signs of stress, changes in cell volume when compared to the positive control (B). In this study, we expected highly stressed cells to produce more ROS and in turn fluorescence more. However the fluorescence was not intense because of low levels of ROS. Furthermore, normal cells have been reported to contain antioxidants, such as catalase, superoxide dismutase and glutathione. These antioxidants remove much of the excess ROS. Otherwise, we expected highly stressed cells to fluorescence more than the untreated RPWE 1 cells (Fig 3.34).





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Fig 3-34: ROS quantity expression in benign (RPWE 1) prostate cells after 24 hour exposure to unfermented rooibos using a fluorescence microscope. (A) Negative control (B) PC (6% DMSO) (C) 125 µg/ml (D) 250 µg/ml (E) 500 µg/ml (F) 1 000 µg/ml (G) 5 000 µg/ml (H) 10 000 µg/m (I) 50 000 µg/ml. Magnification: 100x. Bar = 50µm. Cells were stained with 7µM CM 5, 6 CM H₂DCFDA.

3.3.5 Detection of reactive oxygen species (ROS) in benign (RPWE 1) prostate cells after incubation with aspalathin for 24 hours using a fluorescence microscope.

Below are low magnification fluorescence microscope micrographs taken after incubating RPWE 1 cells for 24 hour with aspalathin. Morphologically, the Fig 3.35 showed normal dividing and adherent cells with no obvious signs of stress or any changes in cell volume when compared to the negative control (A). However, H₂DCFDA stain showed cells with low but variable fluorescence intensity. In this thesis, we expected highly stressed cells (cells treated with high concentration of aspalathin) to produce more ROS and fluorescence compared to cells which are less stressed (treated with lower concentrations of aspalathin). On the contrary, cells on micrograph C, D, G and H had increased DCF fluorescence compared to the untreated cells (A). Morphologically the cells on these micrographs looked unstressed when compared to the positive control (B). At the same time micrographs E and F showed no change in fluorescence when compared to the negative control. The low fluorescence pattern demonstrated by the RPWE 1 cells was not intense probably because of low levels of ROS and the presence of antioxidants such as catalase, superoxide dismutase and glutathione. In summary, incubation of RPWE 1 cells with aspalathin rooibos for 24 hours did not produce a concentration-dependent fluorescence pattern. In this study, the highest concentration of 50 000 µg/ml aspalathin was not used because our preliminary experiments had proved this concentration to be very cytotoxic to the extent that most of the cells died and became detached (Fig 3.35).

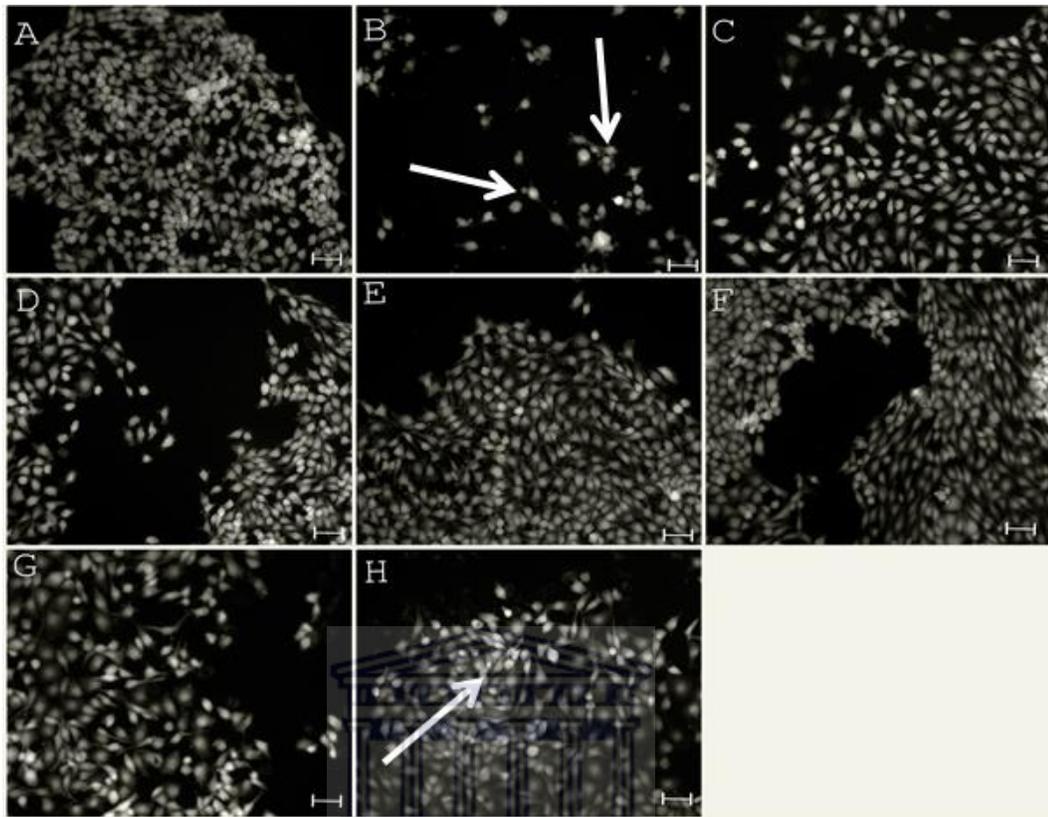


Fig 3-35: ROS quantity expression in benign (RPWE 1) prostate cells after 24 hour exposure to aspalathin using a fluorescence microscope. (A) Negative control. (B) PC (6% DMSO) (C) 125 µg/ml (D) 250 µg/ml (E) 500 µg/ml (F) 1 000 µg/ml (G) 5 000 µg/ml (H) 10 000 µg/ml. Magnification: 100x. Bar = 50µm. Arrows illustrate stressed cells. Cells were stained with 7 µM CM 5, 6 CM H₂DCFDA.

3.3.6 Detection of reactive oxygen species (ROS) levels in benign (RPWE 1) prostate cells after incubation with EGCG for 24 hours using a fluorescence microscope.

Below are low magnification fluorescence microscope micrographs taken after incubating RPWE 1 cells for 24 hour with EGCG. Morphologically, all micrographs showed normal dividing and adherent cells when compared to the negative control (A). The CM - H₂DCFDA stain showed cells with some degree of fluorescence; however there was not concentration-dependent fluorescence. We expected highly stressed cells (cells treated with high concentration of EGCG) to produce more ROS and fluorescence compared to cells which are less stressed (treated with lower concentrations of EGCG). On the contrary, all the cells in these micrographs had low and highly variable fluorescence patterns which was not directly proportional to the concentration of the treatment. For example, cells on micrograph C - F emitted fluorescence of comparable intensity to each other and yet these micrographs showed cells which were treated with increasing concentrations of EGCG (125 – 1 000 µg/ml).

All micrographs showed cells that were all producing low levels of ROS. This was a result of normal cellular physiological processes. However, treatment of the cells with EGCG did not result in increased fluorescence intensity probably because normal cells have been reported to contain low levels of ROS due to the presence of high levels of antioxidant enzymes such as catalase, superoxide dismutase and glutathione. These remove much of the excess ROS. However, increased fluorescence was observed on micrograph G and I treated with 5 000 and 10 000 µg/ml EGCG, respectively, compared to the negative control (A). Morphologically, the cells on these two photographs looked normal and were not stressed despite showing increased fluorescence intensity. Otherwise, we expected a concentration-dependent increase in fluorescence intensity in this assay. In this study, the highest concentration

of 50 000 $\mu\text{g/ml}$ EGCG was not used because our preliminary experiments showed most of the cells dead and detached (Fig 3.36).

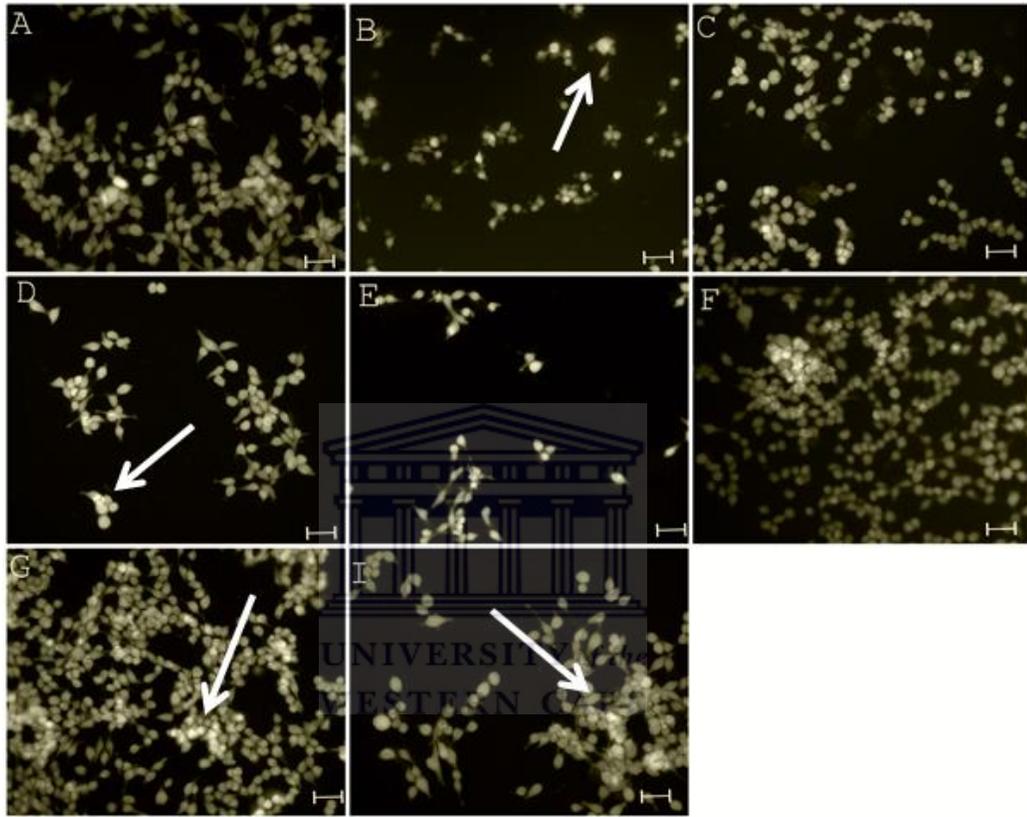


Fig 3-36: ROS quantity expression in benign (RPWE 1) prostate cells following 24 hour exposure to epigallocatechin gallate (EGCG) using a fluorescence microscope.
(A) Negative control (B) PC (6% DMSO) (C) 125 $\mu\text{g/ml}$ (D) 250 $\mu\text{g/ml}$ (E) 500 $\mu\text{g/ml}$ (F) 1 000 $\mu\text{g/ml}$
(G) 5 000 $\mu\text{g/ml}$ (H) 10 000 $\mu\text{g/ml}$. Magnification: 100x. Bar = 50 μm . Arrows illustrate stressed cells. Cells were stained with 7 μM CM 5, 6 CM H₂DCFDA.

3.3.7 Detection of reactive oxygen species (ROS) levels in benign (RPWE 1) prostate cells after incubation with black tea for 72 hours using a fluorescence microscope.

Below are micrographs of RPWE 1 cells taken following incubation with black tea for 72 hours. After loading these cells with CM-H₂DCFDA Fig 3.37 A to I showed cells with variable fluorescence pattern. The fluorescence was not directly proportional to the concentration of black tea used. For example cells treated with 50 000 µg/ml BT showed decreased fluorescence compared to the negative control. Morphologically the cells were detached with broken membrane surfaces compared to the negative control (B). Micrograph treated with 10 0000 µg/ml BT showed increased fluorescence compared to the negative control. Morphologically despite being attached the cells stressed as demonstrated by the increased cell volume (swollen). In this thesis, we expected highly stressed cells including the positive control (B) to produce more ROS and in turn fluorescence more. However, the fluorescence of these cells was not intense because of low levels of ROS. In addition to this, the presence of the antioxidants, such as catalase, superoxide dismutase and glutathione scavenged most of the ROS. This protected the cells from ROS induced stress (Fig 3.37).

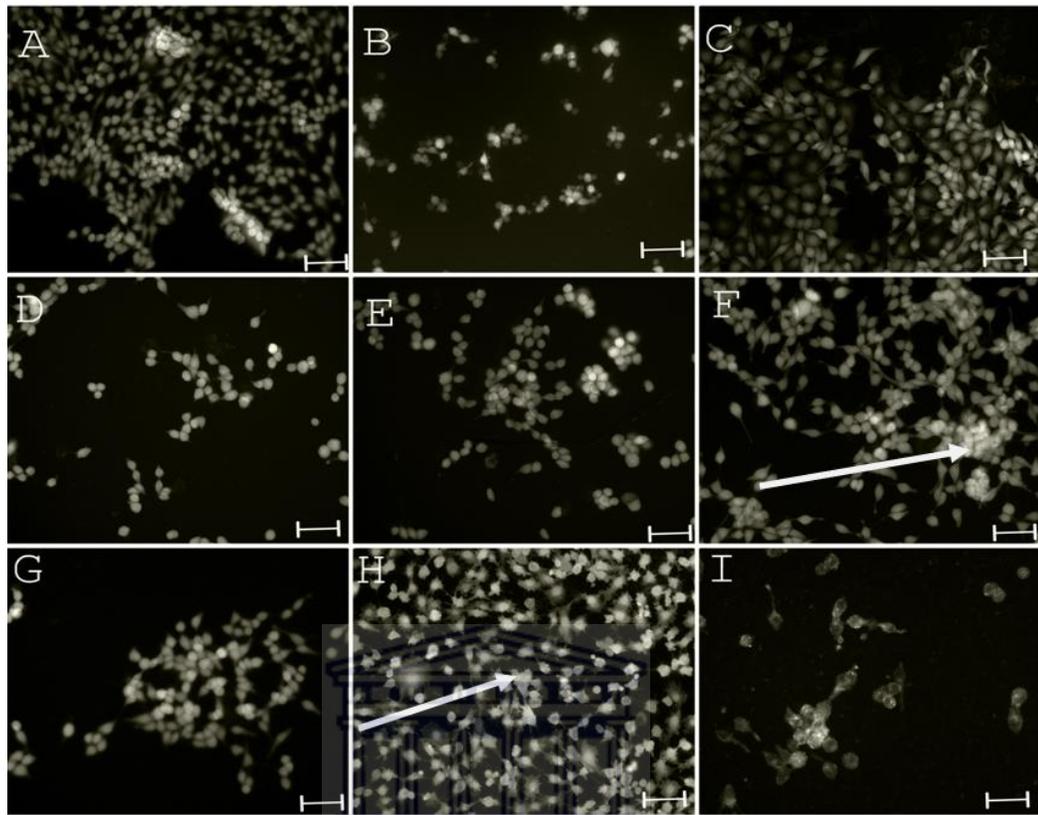


Fig 3-37: ROS quantity expression in benign (RPWE 1) prostate cells after 72 hour exposure to black tea using a fluorescence microscope.

(A) = Negative Control (B) PC (6% DMSO) (C)125 µg/ml (D) 250µg/ml (E) 500µg/ml (F) 1 000 µg/ml (G) 5 000 µg/ml (H) 10 000 µg/ml (I) 50 000 µg/ml. Cells were stained with 7µM CM 5, 6 CM H₂DCFDA. Magnification: 100x. Bar = 50µm.

3.3.8 Detection of reactive oxygen species (ROS) levels in benign (RPWE 1) prostate cells after incubation with green tea for 72 hours using a fluorescence microscope.

Below are low magnification fluorescence microscope micrographs taken after incubating RPWE 1 cells for 72 hours with GT. Morphologically, Fig 3.38 C to H showed normal dividing and adherent cells. The cells did not show any obvious signs of stress, changes in cell volume when compared to the control (A). Subsequent staining of the cells with CM-H₂DCFDA, showed cells with green fluorescence.

However, the fluorescence intensity was not proportional to the concentration of the treatment. In this thesis, we expected highly stressed cells to produce more ROS and in turn more fluorescence. On the contrary, the fluorescence pattern was highly variable and not directly proportional to the concentration of the green tea used. Further to this, the fluorescence was not intense because of low levels of ROS produced. In this regard, the presence of the antioxidants, such as catalase, superoxide dismutase and glutathione might have scavenged much of the ROS generated. Otherwise, we expected highly stressed cells to fluorescence more than the untreated cells. In respect of this, micrographs C, D, F and G treated with 125 - 5 000 µg/ml GT showed fluorescence intensity which was comparable to the untreated cells. Micrograph H, treated with 10 000 µg/ml GT showed cells which were swollen as determined by the increase in cell volume and were stressed compared to the negative control.

These cells produced more fluorescence intensity compared to the positive control. Subsequent increase in the concentration of the concentration of GT at 50 000 µg/ml (I) showed cells which were heavily stressed. Some of the cells had broken surfaces and were detached from the surface. The cells on this micrograph despite being heavily stressed produced decreased fluorescence intensity compared to the negative

control (A) The low cell population on this photograph might have contributed to the overall total fluorescence on this micrograph (Fig 3.38).

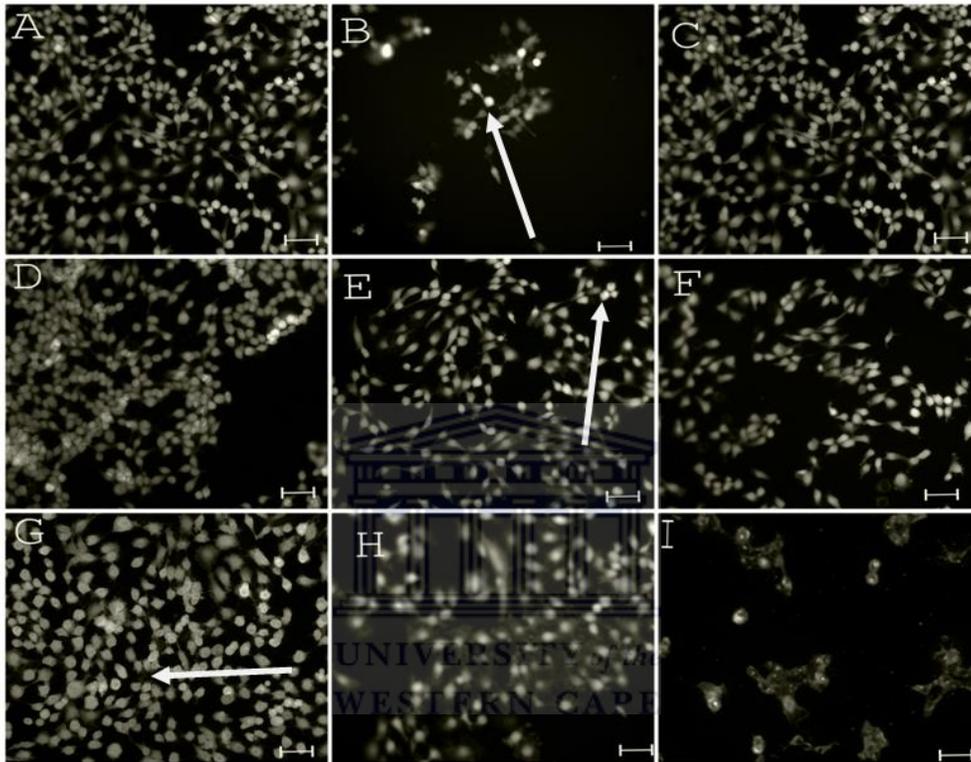


Fig 3-38: ROS quantity expression in benign (RPWE 1) prostate cells after 72 hour exposure to green tea using fluorescence microscope.

(A) Negative control (B) PC (6% DMSO) (C) 125 µg/ml (D) 250 µg/ml (E) 500 µg/ml (F) 1 000 µg/ml (G) 5 000 µg/ml (H) 10 000 µg/ml (I) 50 000 µg/ml. Cells were stained with 7 µM CM 5, 6 CM H₂DCFDA. Arrows illustrate stressed cells and emitting DCF fluorescence. Magnification: 100x. Bar = 50µm.

3.3.9 Detection of reactive oxygen species (ROS) levels in benign (RPWE 1) prostate cells after incubation with fermented rooibos for 72 hours using a fluorescence microscope.

Below are low magnification fluorescence microscope micrographs taken after incubating RPWE 1 cells for 72 hours with fermented rooibos. After loading these cells with H₂DCFDA Fig 3.39 C to F showed cells with low but variable fluorescence pattern. The fluorescence was not directly proportional to the concentration of fermented rooibos used. Morphologically the cells looked normal with flat polygonal shape; meaning that they were not stressed. In this thesis, we expected highly stressed cells to produce more ROS and in turn fluorescence more. However, the fluorescence of these cells was not intense probably because of low levels of ROS.

In addition to this, the presence of the antioxidants, such as catalase, superoxide dismutase and glutathione might have scavenged most of the ROS. This protected the cells from ROS induced stress. However, at 1 000 or 5 000 µg/ml FR (F and G) cells showed increased fluorescence intensity compared to the negative control (A). Subsequent incubation with 50 000 µg/ml FR (I) showed cells with decreased fluorescence intensity similar to the positive control (B). Morphologically cells on this micrograph appeared highly stressed, swollen and showed broken cell membrane surfaces. The number of cells on this micrograph were much lower compared to the negative control (A) (Fig 3.39).

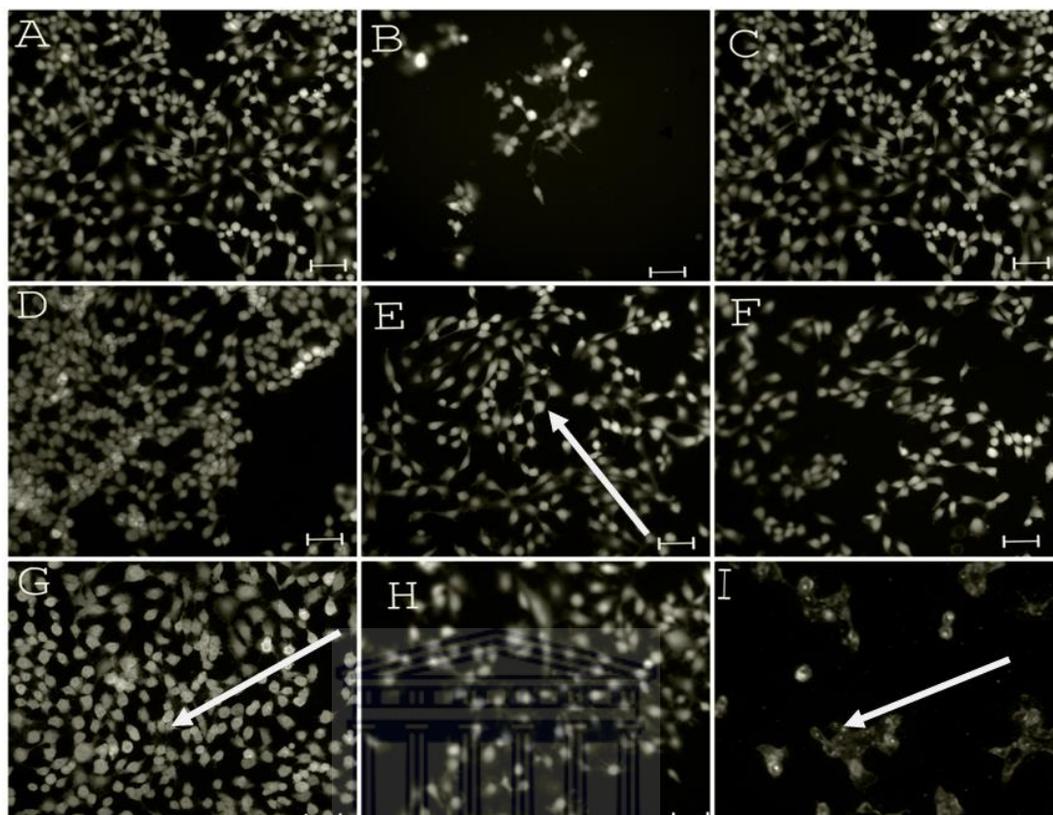


Fig 3-39: ROS quantity expression in benign (RPWE 1) prostate cells following 72 hour exposure to fermented rooibos using a fluorescence microscope.

(A) Negative control (B) PC (6% DMSO) (C) 125 µg/ml (D) 250 µg/ml (E) 500 µg/ml (F) 1 000 µg/ml (G) 5 000 µg/ml (H) 10 000 µg/ml (I) 50 000 µg/ml. Arrows illustrate stressed cells. Cells stained with CM H₂DCFDA. Magnification: 100x. Bar = 50µm.

3.3.10 Detection of reactive oxygen species (ROS) levels in benign (RPWE 1) prostate cells following incubation with unfermented rooibos for 72 hours using a fluorescence microscope.

Below are low magnification fluorescence microscope micrographs taken after incubating RPWE 1 cells for 72 hours with unfermented rooibos. Morphologically, the cells looked flat and polygonal in shape. They were adherent cells (micrograph C to F). In this thesis, we expected highly stressed cells to produce more ROS and in turn fluorescence more. On the contrary, all the cells in these micrographs had low and highly variable fluorescence pattern which was not directly proportional to the concentration of unfermented rooibos used. The fluorescence was not intense probably because of low levels of ROS.

Normal cells have been reported to contain low ROS levels as a result of the presence of the antioxidants, such as catalase, superoxide dismutase and glutathione. These anti-oxidants might have scavenged most of the ROS thereby protecting the cells from ROS induced stress. Otherwise, we expected highly stressed cells (treated with high UR concentration) to fluorescence more than the less stressed cells (treated with low UR concentration). However, at 5 000 or 10 000 $\mu\text{g/ml}$ UR (F and G) showed increased fluorescence intensity compared to the negative control (A). Subsequent incubation with 10 000 $\mu\text{g/ml}$ UR (H) showed highly stressed and swollen cells. Incubation with 50 000 $\mu\text{g/ml}$ UR, micrograph I showed highly stressed cells with most of cells detached and the fluorescence produced by these cells was below that of the positive control(B) (Fig 3.40).

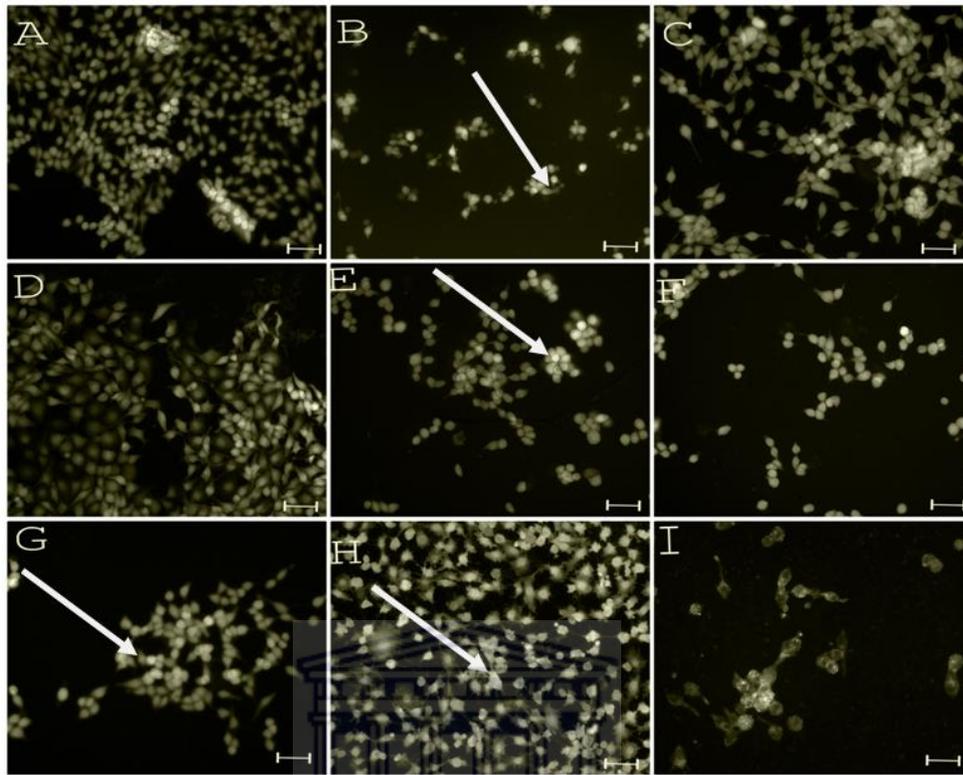


Fig 3-40: ROS quantity expression in benign (RPWE 1) prostate cells following 72 hour exposure to unfermented rooibos.

(A) Negative control (B) PC (6% DMSO) (C) 125 µg/ml (D) 250 µg/ml (E) 500 µg/ml (F) 1 000 µg/ml (G) 5 000 µg/ml (H) 10 000 µg/m (I) 50 000 µg/ml. Cells were stained with 7 µM CM 5, 6 CM H₂DCFDA. Arrows illustrate stressed cells and emitting DCF fluorescence. Magnification: 100x. Bar = 50µm.

3.3.11 Detection of reactive oxygen species (ROS) in benign (RPWE 1) prostate cells following exposure to aspalathin for 72 hours using a fluorescence microscope.

Below are low magnification fluorescence microscope micrographs taken after incubating RPWE 1 cells for 72 hour with aspalathin. Morphologically, Fig 3.41 C – H showed normal dividing and adherent cells in all the micrographs. The cells did not show any obvious signs of stress, changes in cell volume when compared to the negative control (A). However, subsequent staining of the cells with H₂DCFDA cells showed cells with low but highly variable fluorescence pattern. Besides this, the fluorescence was not directly proportional to the concentration of aspalathin used. For example, cells on micrographs D - F treated with 250 - 1 000 µg/ml ASP emitted fluorescence of the same intensity.

However, these cells were expected to produce a concentration- dependent fluorescence compared to negative control. Furthermore, micrographs B and C treated with 125 and 250 µg/ml aspalathin appear to emit fluorescence of the same intensity as the negative control cells. Further to this, micrograph H showed stressed cells as indicated by the increased cell volume compared to the positive control (B). Further to this, the fluorescence on micrograph H, treated with 50 000 µg/ml ASP was comparable to the positive control (B). The above mentioned observations are trying to justify the inconsistencies that this assay encountered in trying to determine the ROS levels using the Zeiss fluorescence microscope.

However, cells on all micrographs emitted low levels of fluorescence which is a sign that they were producing ROS. Notably, the fluorescence was not intense probably because of low levels of ROS. Besides this, the presence of antioxidants such as catalase, superoxide dismutase and glutathione might have scavenged most of the ROS. Above all, we expected a concentration-dependent increase in fluorescence

intensity. The highest concentration of 50 000 $\mu\text{g/ml}$ aspalathin was not used based on the fact that our preliminary experiments resulted in death and detachment of the cells (Fig 3.41).

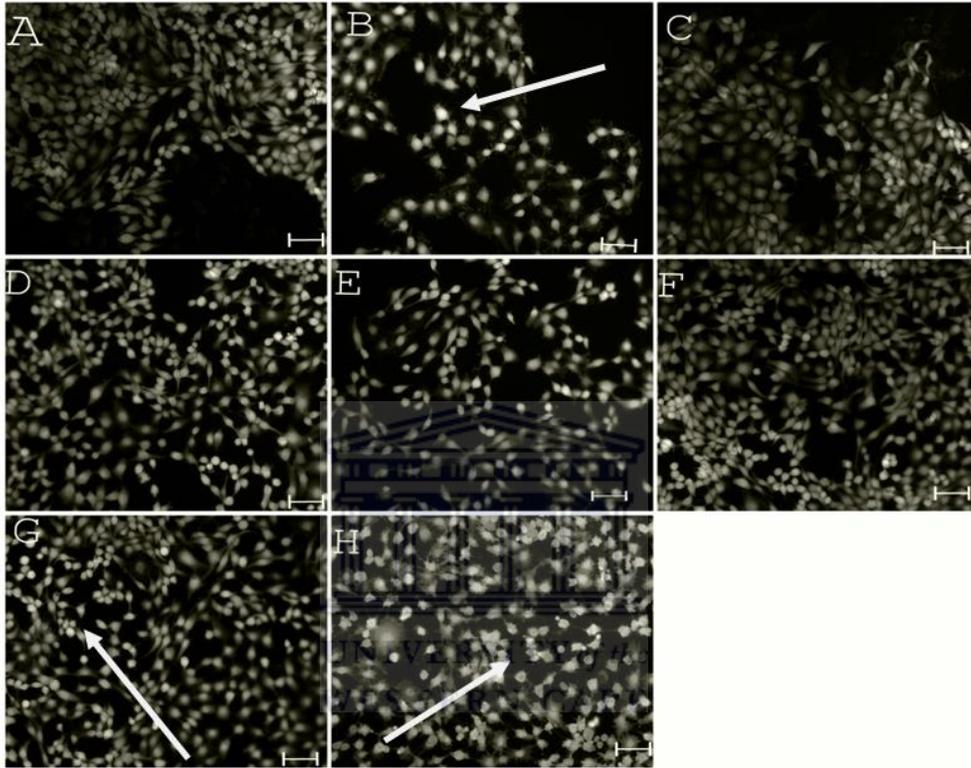


Fig 3-41: ROS quantity expression in benign (RPWE 1) prostate cells after 72 hour exposure to aspalathin using a fluorescence microscope. (A) = Negative Control (B) PC (6% DMSO) (C) 125 $\mu\text{g/ml}$ (D) 250 $\mu\text{g/ml}$ (E) 500 $\mu\text{g/ml}$ (F) 1 000 $\mu\text{g/ml}$ (G) 5 000 $\mu\text{g/ml}$ (H) 10 000 $\mu\text{g/ml}$. Arrows illustrate stressed cells. Cells were stained with 7 μM CM 5, 6 CM H₂DCFDA. Magnification: 100x. Bar = 50 μm .

3.3.12 Detection of reactive oxygen species (ROS) levels in benign (RPWE 1) prostate cells after incubation with EGCG for 72 hours using a fluorescence microscope.

Below are low magnification fluorescence microscope photographs taken after incubating RPWE 1 cells for 72 hour with EGCG. Morphologically, the cells looked normal in shape and adherent to the surface without any obvious sign of stress compared to the negative control (A). Upon staining the cells with CM H₂DCFDA, green fluorescence was emitted by cells on all the micrographs. However, the degree of fluorescence was not directly proportional to the concentration of EGCG. In this thesis, we expected highly stressed cells (cells treated with high concentration of EGCG) to produce more ROS and fluorescence compared to less stressed cells (treated with lower concentrations of EGCG. For example, cells on micrograph E and G emitted more fluorescence when compared to the negative control (A).

Micrographs C, D and H showed fluorescence pattern which was comparable to that of the negative control and yet these cells were subjected to stress through EGCG treatment. Basically, RPWE 1 cells are coping with the EGCG induced stress and the low levels of fluorescence are due to the presence low levels of ROS; which are reported to be present in normal respiring cells. Besides this, the presence of antioxidants such as catalase, superoxide dismutase and glutathione might have been responsible for the regulation of the ROS levels. Although they are normal prostate cells, we expected a concentration-dependent increase in fluorescence intensity. The highest concentration of 50 000 µg/ml aspalathin was not used based on the fact that our preliminary experiments resulted in death and detachment of the cells (Fig 3.42).

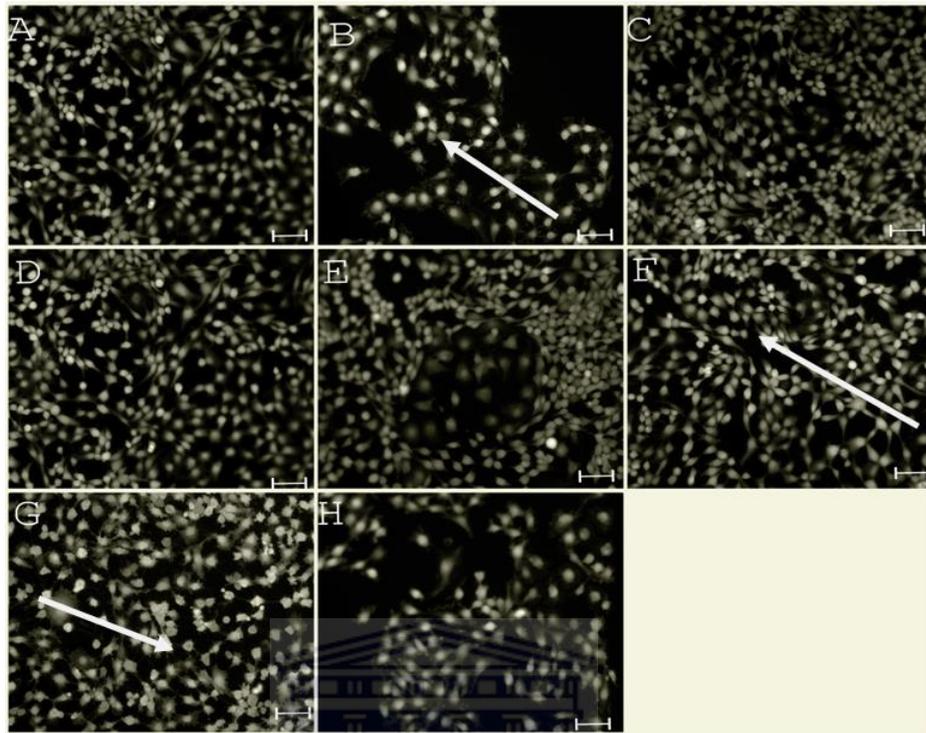


Fig 3- 42: ROS quantity expression in benign (RPWE 1) prostate cancer cells following 72 hour exposure to EGCG using a fluorescence microscope.

(A) = Negative Control (B) PC (6% DMSO) (C) 125 $\mu\text{g/ml}$ (D) 250 $\mu\text{g/ml}$ (E) 500 $\mu\text{g/ml}$ (F) 1 000 $\mu\text{g/ml}$ (G) 5 000 $\mu\text{g/ml}$ (H) 10 000 $\mu\text{g/ml}$. Arrows illustrate stressed cells. Cells were stained with 7 μM CM 5, 6 CM H₂DCFDA. Magnification: 100x. Bar = 50 μm .

3.3.2 Detection of reactive oxygen species (ROS) production in malignant (LNCaP) prostate cells using a fluorescence microscope.

To assess the concentration of ROS in malignant (LNCaP) prostate cells were exposed to different concentrations of GT, BT, FR, UR, EGCG and ASP for 24 and 72 hours. The highest concentration of 50 000 µg/ml aspalathin or epigallocatechin gallate was not used based on the fact that our preliminary experiments resulted in death and detachment of the cells. The fluorophore CM 5,6 CM H₂DCFDA and inverted Zeiss Axiovert 200M fluorescence microscope (Zeiss, Göttingen, Germany) were used to determine ROS levels. In brief, after loading cells with the ROS dye in an 8-well Ibidi slide, we aimed at identifying cells that were emitting the DCF fluorescence.

Cells that emitted fluorescence, represented cells that were able to oxidise 2'-7' dichlorofluorescein (H₂DCF) to 2'-7' dichlorofluorescein (DCF). The diacetate form, H₂DCFDA when taken up by cells is acted upon by non-specific cellular esterases and in the process cleaves off lipophilic groups. This results in the formation of a charged compound that gets trapped inside the cell. High DCF fluorescence intensity represent high ROS levels and vice versa for low DCF fluorescence intensity. In this thesis, 10 representative micrographs were taken for each sample at 100x magnification.

3.3.2.1 Detection of reactive oxygen species (ROS) in malignant (LNCaP) prostate cells after incubating with black tea for 24 hours using a fluorescence microscope.

Below are low magnification fluorescence microscopy micrographs taken after incubating malignant (LNCaP) prostate cells for 24 hour with black tea. Morphologically all the micrographs for the treated samples showed cells that were stressed. Micrographs D, E and G showed cells that have lost the flat and polygonal

shape and had become rounded. Micrographs C, F, H and I showed cells that were swollen and looked stressed when compared to the untreated cells. After staining the cells with H₂DCFDA, the ROS dye showed increased fluorescence production on micrographs F and H; cells incubated with 1 000 or 10 000 µg/ml BT. Micrograph I showed heavily stressed cells, as indicated by the decreased cell numbers following detachment. Interestingly these cells did not fluoresce much when compared to the negative control (A) (Fig 3.43).

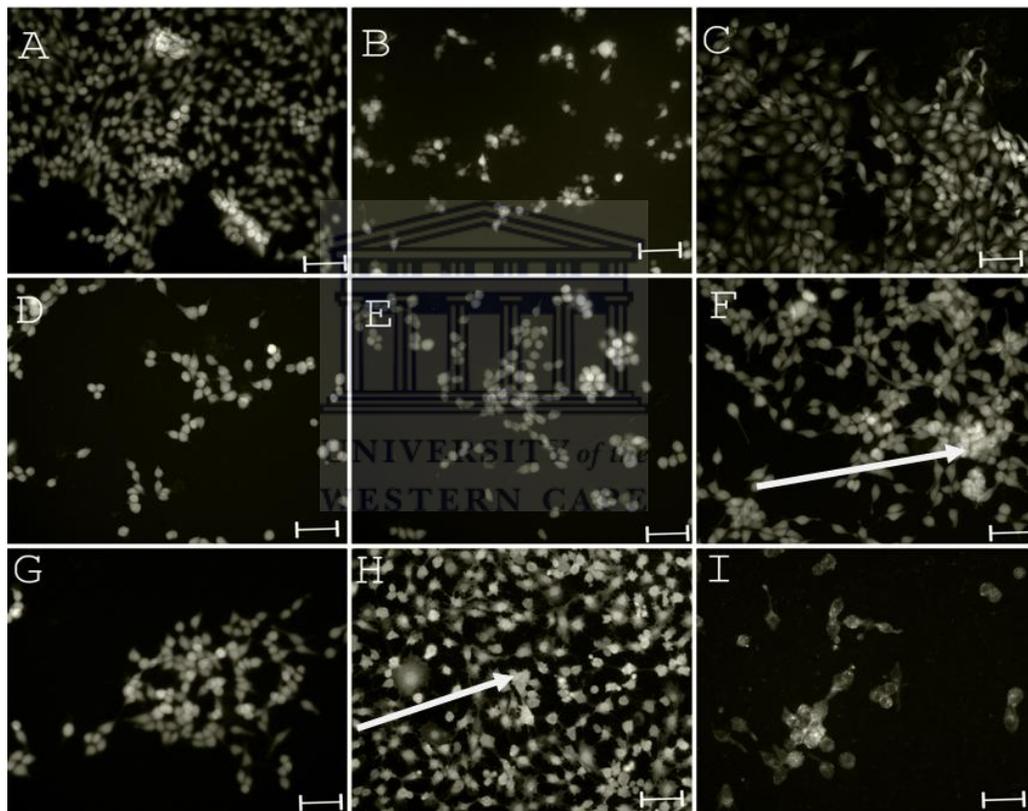


Fig 3-43: ROS level expression in malignant (LNCaP) prostate cells after 24 hour exposure to black tea using a fluorescence microscope. (A) Negative control (B) PC (6% DMSO) (C) 125 µg/ml, (D) 250 µg/ml (E) 500 µg/ml, (F) 1 000 µg/ml, (G) 5 000 µg/ml (H) 10 000 µg/ml (I) 50 000 µg/ml. Magnification: 100x. Bar = 50µm. Cells were stained with 7 µM CM 5, 6 CM H₂DCFDA. Arrows also illustrate highly stressed cells emitting DCF fluorescence.

3.3.2.2 Detection of reactive oxygen species (ROS) levels in malignant (LNCaP) prostate cells after incubating with green tea for 24 hours using a fluorescence microscope.

Below are low magnification fluorescence microscopy photographs taken after incubating malignant (LNCaP) prostate cells for 24 hour with green tea. Fig 3.44 C-G contain micrographs of cells treated with 125, 250, 500, 1 000 and 5 000 $\mu\text{g/ml}$ GT, respectively. Morphologically the cells appeared to be normally dividing and adherent when compared to the negative control (A). After staining the cells with H_2DCFDA , the cells emitted higher fluorescence compared to the negative control. Incubation of cells with 10 000 - 50 000 $\mu\text{g/ml}$ GT (H-I) showed cells that were stressed as observed by the increased higher fluorescence intensity. Morphologically, these stressed cells looked similar to the positive control cells (B). In this assay we expected a concentration- dependent increase in fluorescence. Instead there was no clear pattern of fluorescence (Fig 3.44).

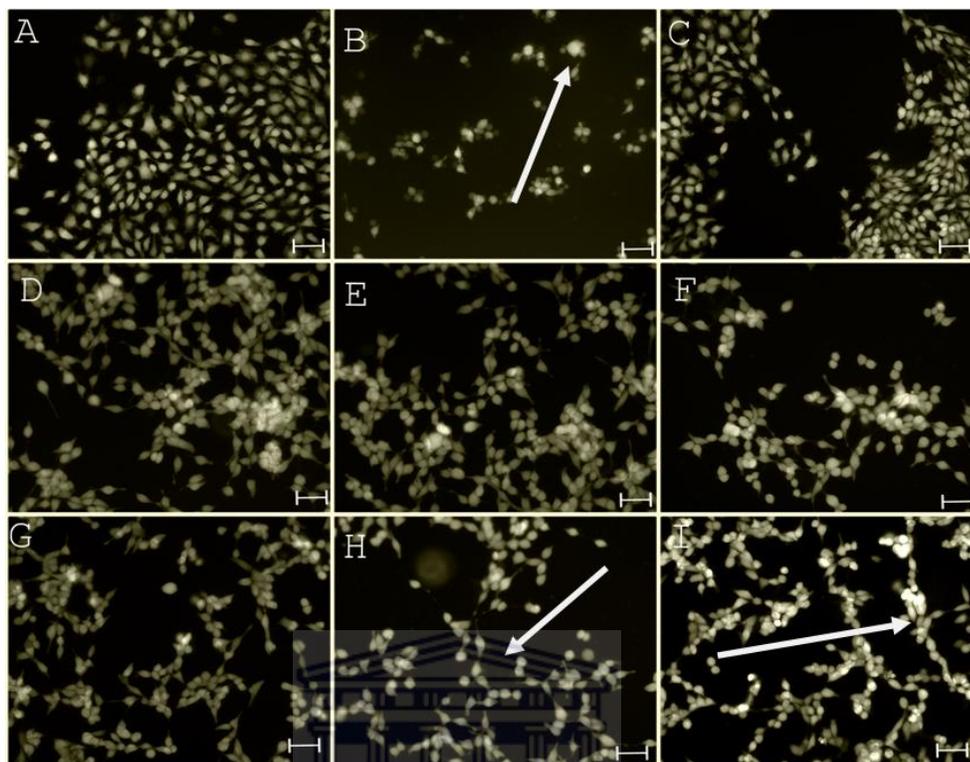


Fig 3-44: ROS quantity expression in malignant (LNCaP) prostate cancer cells after 24 hour exposure to green tea using fluorescence microscope.

(A) Negative control (B) PC (6% DMSO) (C) 125 µg/ml (D) 250 µg/ml (E) 500 µg/ml (F) 1 000 µg/ml (G) 5 000 µg/ml (H) 10 000 µg/ml (I) 50 000 µg/ml. Magnification: 100x. Bar = 50µm. Arrows illustrate cells that are swollen and have lost adhesion to other surrounding cells. Cells were stained with 7 µM CM 5, 6 CM H₂DCFDA. Arrows also illustrate highly stressed cells emitting DCF fluorescence.

3.3.2.3 Detection of reactive oxygen species (ROS) levels in malignant (LNCaP) prostate cells after incubating with fermented rooibos for 24 hours using a fluorescence microscope.

Below are low magnification fluorescence microscopy micrographs taken after incubating malignant (LNCaP) prostate cells for 24 hour with fermented rooibos. Micrographs C- F showed cells treated with 125 – 1 000 $\mu\text{g/ml}$ FR. The cells on these micrographs did not show any obvious signs of stress. The cells appeared normal and adherent. The H_2DCFDA dye detected ROS positive cells. In this regard, exposure to 5 000, 10 000 or 50 000 $\mu\text{g/ml}$ FR (G- I) showed significant increase in fluorescence compared to the negative control. However, this fluorescence appeared to be lower than that of the positive control (B). Morphologically, the cells appeared slightly stressed as shown by the increase in cell volume (swelling) compared to the negative control (A) (Fig 3.45)

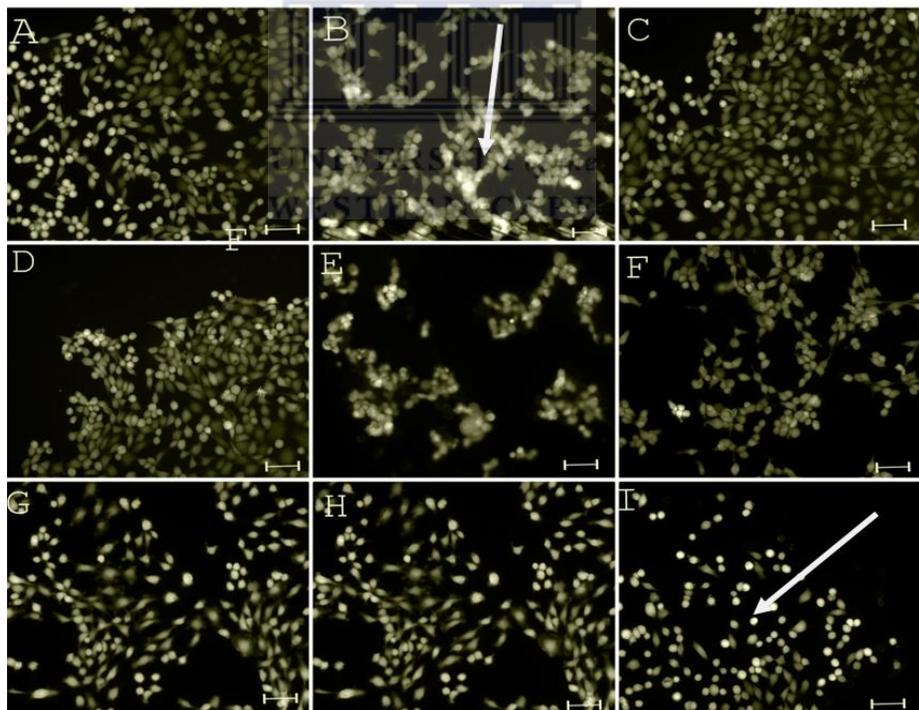


Fig 3-45: ROS quantity expression in LNCaP cells after 24 hour exposure to fermented rooibos using a fluorescence microscope.

(A) Negative control (B) PC (6% DMSO) (C) 125 $\mu\text{g/ml}$ (D) 250 $\mu\text{g/ml}$ (E) 500 $\mu\text{g/ml}$ (F) 1 000 $\mu\text{g/ml}$ (G) 5 000 $\mu\text{g/ml}$ (H) 10 000 $\mu\text{g/ml}$ (I) 50 000 $\mu\text{g/ml}$. Magnification: 100x. Bar = 50 μm . Cells were stained with 7 μM CM 5, 6 CM H_2DCFDA . Arrows illustrate highly stressed cells emitting DCF fluorescence.

3.3.2.4 Detection of reactive oxygen species (ROS) production in malignant (LNCaP) prostate cells after incubating with unfermented rooibos for 24 hours using a fluorescence microscope.

Below are low magnification fluorescence microscopy micrographs taken after incubating malignant (LNCaP) prostate cells for 24 hour with unfermented rooibos. All the treated samples except micrographs C and E (treated with 125 or 500 $\mu\text{g/ml}$ UR) showed the presence of stressed cells. The ROS dye, H_2DCFDA showed some levels of fluorescence as a sign that they were respiring. Morphologically micrographs D, F, G, H, and I showed cells with increased cell volume (swollen) compared to the negative control. This demonstrated the presence of UR induced stress. Basically, we expected a concentration-dependent fluorescence production unfortunately this was not the case (Fig 3.46).

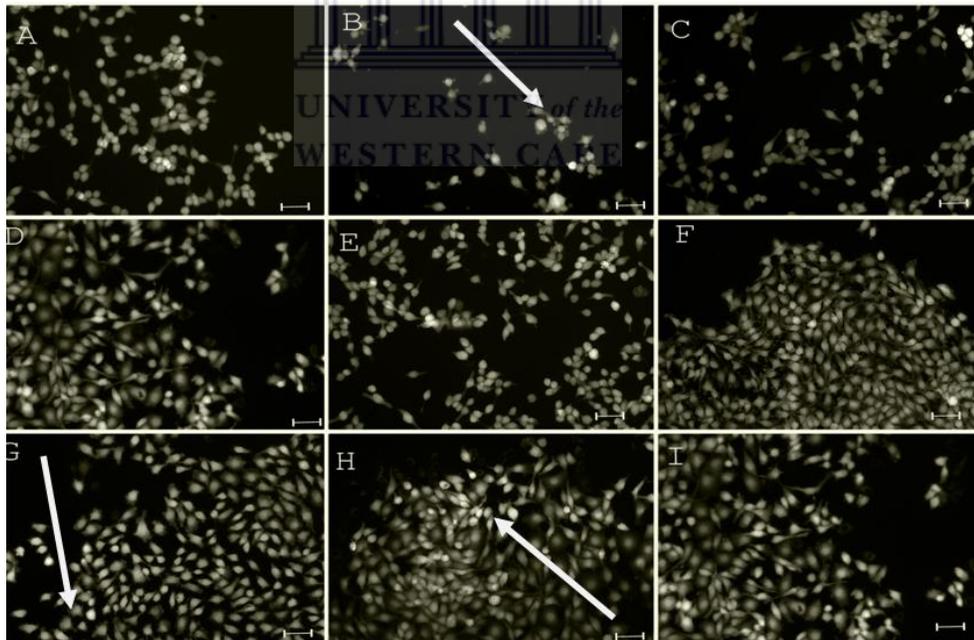


Fig 3-46: ROS levels in malignant (LNCaP) prostate cells after 24 hour exposure to unfermented rooibos using a fluorescence microscope. (A) Negative control (B) PC (6% DMSO) (C) 125 $\mu\text{g/ml}$ (D) 250 $\mu\text{g/ml}$ (E) 500 $\mu\text{g/ml}$ (F) 1 000 $\mu\text{g/ml}$ (G) 5 000 $\mu\text{g/ml}$ (H) 10 000 $\mu\text{g/ml}$ (I) 50 000 $\mu\text{g/ml}$. Magnification: 100x. Bar = 50 μm . Cells were stained with 7 μM CM 5, 6 CM H_2DCFDA . Arrows also illustrate highly stressed cells emitting DCF fluorescence.

3.3.2.5 Detection of reactive oxygen species (ROS) in malignant (LNCaP) prostate cells after incubating with aspalathin for 24 hours using a fluorescence microscope.

Below are low magnification fluorescence microscopy micrographs taken after incubating malignant (LNCaP) prostate cells for 24 hour with aspalathin. Micrographs C- E showed cells treated with 125, 250 or 500 $\mu\text{g/ml}$ ASP, respectively. All the cells on these micrographs did not show any obvious signs of stress. The cells appeared to be normally dividing and adherent. After staining them with the ROS dye, the cells demonstrated basal levels of fluorescence compared to the negative control. However, 1000 and 10 000 $\mu\text{g/ml}$ ASP (F and H) showed significant increased fluorescence than the negative control (Fig 3.47).



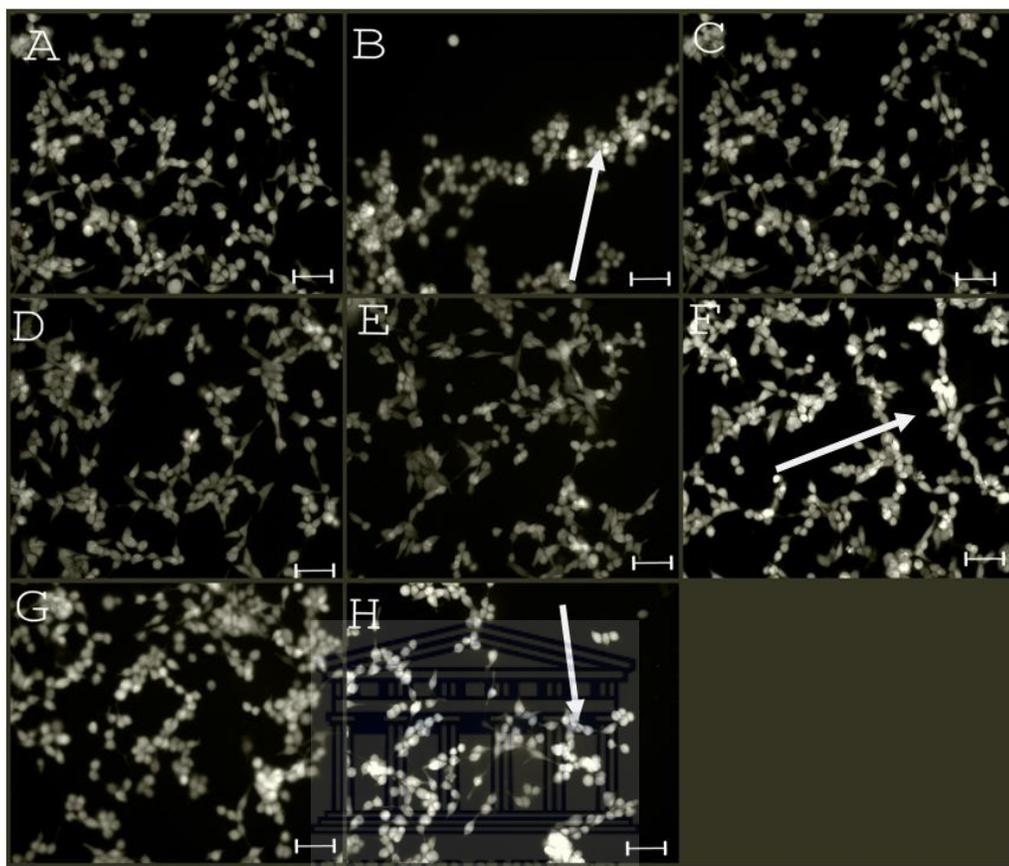


Fig 3-47: ROS quantity expression in LNCaP cells after 24 hour exposure to aspalathin using fluorescence microscope.

(A) Negative control (B) PC (6% DMSO) (C) 125 $\mu\text{g/ml}$ (D) 250 $\mu\text{g/ml}$ (E) 500 $\mu\text{g/ml}$ (F) 1 000 $\mu\text{g/ml}$ (G) 5 000 $\mu\text{g/ml}$ (H) 10 000 $\mu\text{g/ml}$. Magnification: 10x Bar = 50 μm . Arrows illustrate stressed cells. Cells were stained with 7 μM CM 5, 6 CM H₂DCFDA. Magnification: 100x Bar = 50 μm .

3.3.2.6 Detection of reactive oxygen species (ROS) production in malignant (LNCaP) prostate cells after incubating with EGCG for 24 hours using a fluorescence microscope.

Below are low magnification fluorescence micrographs taken after incubating malignant (LNCaP) prostate cells for 24 hours with EGCG. Micrographs C - H showed cells that looked normal and not stressed. The morphology of these cells was comparable to that of the negative control (A). The ROS dye showed lower DCF fluorescence in all the micrographs. Of great note was the high fluorescence shown by micrographs E and H following treatment with 5 000 or 10 000 $\mu\text{g/ml}$ EGCG. Cells on these two micrographs showed increased fluorescence production despite not demonstrating any signs of stress (Fig 3.48).

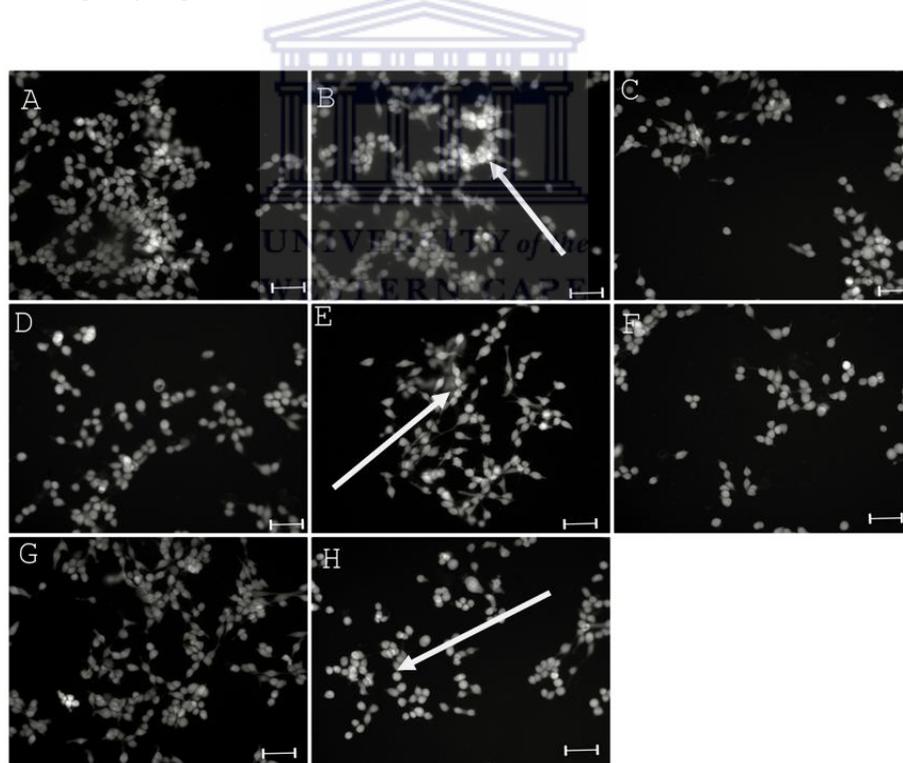


Fig 3-48: ROS quantity expression in malignant (LNCaP) prostate cells following 24 hour exposure to epigallocatechin gallate (EGCG) using a fluorescence microscope. (A) Negative control (B) PC (6% DMSO) (C) 125 $\mu\text{g/ml}$ (D) 250 $\mu\text{g/ml}$ (E) 500 $\mu\text{g/ml}$ (F) 1 000 $\mu\text{g/ml}$ (G) 5 000 $\mu\text{g/ml}$ (H) 10 000 $\mu\text{g/ml}$. Arrows illustrate cells that are emitting fluorescence. Cells were stained with 7 μM CM 5, 6 CM H₂DCFDA. Magnification: 100x Bar = 50 μm

3.3.2.7 Detection of reactive oxygen species (ROS) in malignant (LNCaP) prostate cells after incubating with black tea for 72 hours using a fluorescence microscope.

Below are low magnification fluorescence microscopy micrographs (C and D) taken after incubating malignant (LNCaP) prostate cells with 125 or 250 $\mu\text{g/ml}$ BT, respectively, for 72 hours. The micrographs show normal dividing and adherent malignant (LNCaP) prostate cells with fluorescence comparable to that of the untreated cells (A). However, after treating the LNCaP cells with 500 – 50 000 $\mu\text{g/ml}$ BT (E - H) showed a concentration – dependent increase in stress levels. However, instead of the cells emitting high fluorescence levels there was a decrease in fluorescence when compared to the positive control (micrograph B). Morphologically, stress levels appeared to increase with each increase in concentration. Further to this, the number of cells continued to decrease as the concentration of BT increased. Because of this, the intensity of DCF fluorescence continued to decrease as BT concentration increased.

Briefly, we expected a concentration-dependent increase in ROS levels. And because of this, highly stressed LNCaP cells (those treated with higher BT concentration) were supposed to produce more ROS than the less stressed cells (those treated with low concentrations of BT). In the final analysis, a concentration-dependent increase in fluorescence intensity could not be achieved due to several factors. The probable reasons for the increased fluorescence intensity could come about because of the pro-oxidant effect of UR on LNCaP cells which may have induced increased stress and generation of more ROS. Besides this, high physiological levels of ROS have been reported in tumor cells. This accompanied by a compromised anti-oxidant defense system made up of very low levels of manganese dioxide, catalase, glutathione and superoxide dismutase etc may have promoted elevated ROS levels in cancer cells.

The Zeiss fluorescence microscope used in this thesis, produced different exposure times and light intensities for cells treated with the same concentration. After several interventions, this thesis, could not determine the ROS levels using fluorescence microscopy. It is the more reason that flow cytometry was used to determine ROS levels in both LNCaP and RPWE 1 cells (Fig 3.49).

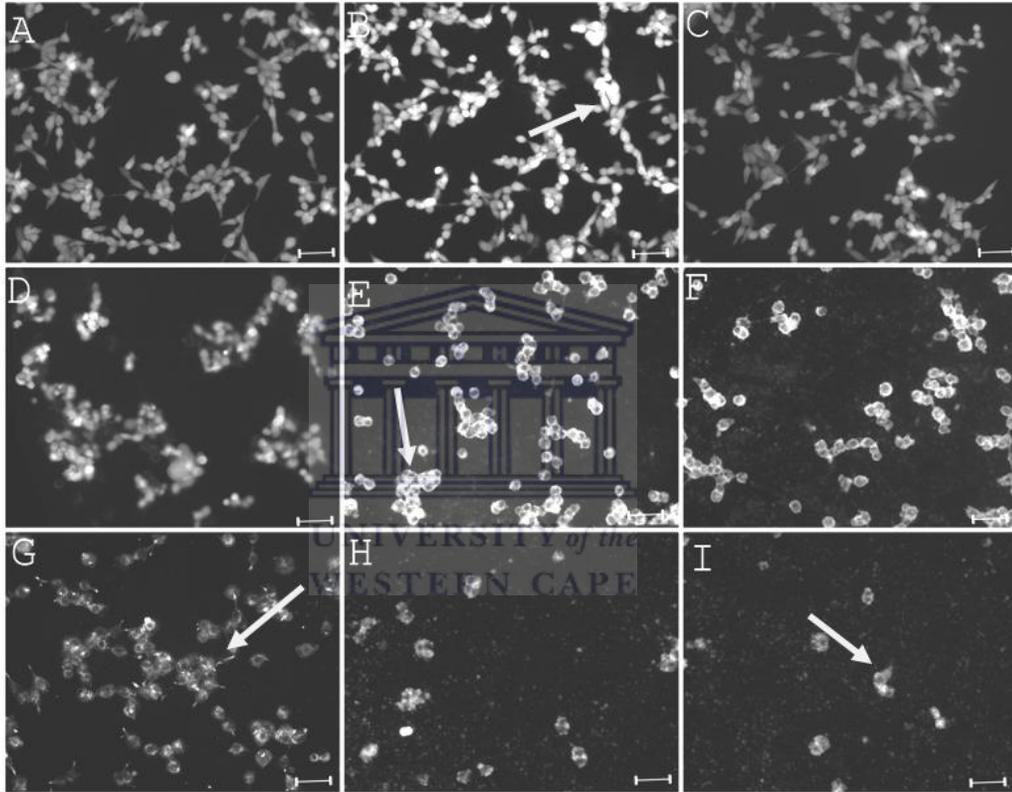


Fig 3-49: ROS quantity expression in malignant (LNCaP) prostate cells after 72 hour exposure to black tea using a fluorescence microscope.

(A) Negative control (B) PC (6% DMSO) (C) 125 $\mu\text{g/ml}$ (D) 250 $\mu\text{g/ml}$ (E) 500 $\mu\text{g/ml}$ (F) 1 000 $\mu\text{g/ml}$ (G) 5 000 $\mu\text{g/ml}$ (H) 10 000 $\mu\text{g/ml}$ (I) 50 000 $\mu\text{g/ml}$. Magnification: 100x Bar = 50 μm . Arrows illustrate cells that are swollen and have lost adhesion to other surrounding cells. Cells were stained with 7 μM CM 5, 6 CM H₂DCFDA. Magnification: 100x Bar = 50 μm

3.3.2.8 Detection of reactive oxygen species (ROS) in malignant (LNCaP) prostate cells after incubating with green tea for 72 hours using a fluorescence microscope.

Below are low magnification fluorescence microscopy micrographs taken after incubating malignant (LNCaP) prostate cells for 72 hour with green tea. Micrographs A, C and D showed cells treated with 0 µg/ml GT, 125 µg/ml GT or 250 µg/ml GT, respectively, with normal dividing and adherent malignant (LNCaP) prostate cells. After, staining the cells with H₂DCFDA the cells emitted low fluorescence intensity compared to the positive control (micrograph B). However, exposure to 500 -50 000 µg/ml GT; micrographs E - I showed few cells that were emitting high fluorescence. Of notable interest is that whilst there was a decrease in the number of cells on the micrographs; individual cells seemed to be highly stressed and produced more DCF fluorescence. A morphological analysis, confirmed the presence of a concentration-dependent increase in stress levels. And because of this, most of the cells appeared round, besides having broken cell membranes. Most of them had lost attachment to the surface and other surrounding cells. The reduced cell numbers negatively affected the total fluorescence emitted per micrograph.

Briefly, we expected a concentration-dependent increase in ROS levels. And in line with this, highly stressed LNCaP cells (those treated with higher GT concentration) were expected to produce more ROS than the less stressed cells (those treated with low concentrations of GT). This would have resulted in a concentration-dependent increase in fluorescence intensity. The increased fluorescence intensity has been suggested to occur as a result of the pro-oxidant effect of GT on LNCaP cells which promoted the generation of more ROS. Besides this, high physiological levels of ROS have been reported in tumor cells. This accompanied by a compromised anti-oxidant defense system promoted elevated ROS levels in LNCaP cells. After several interventions, this thesis, could not determine the ROS levels using fluorescence microscope (Fig 3.50).

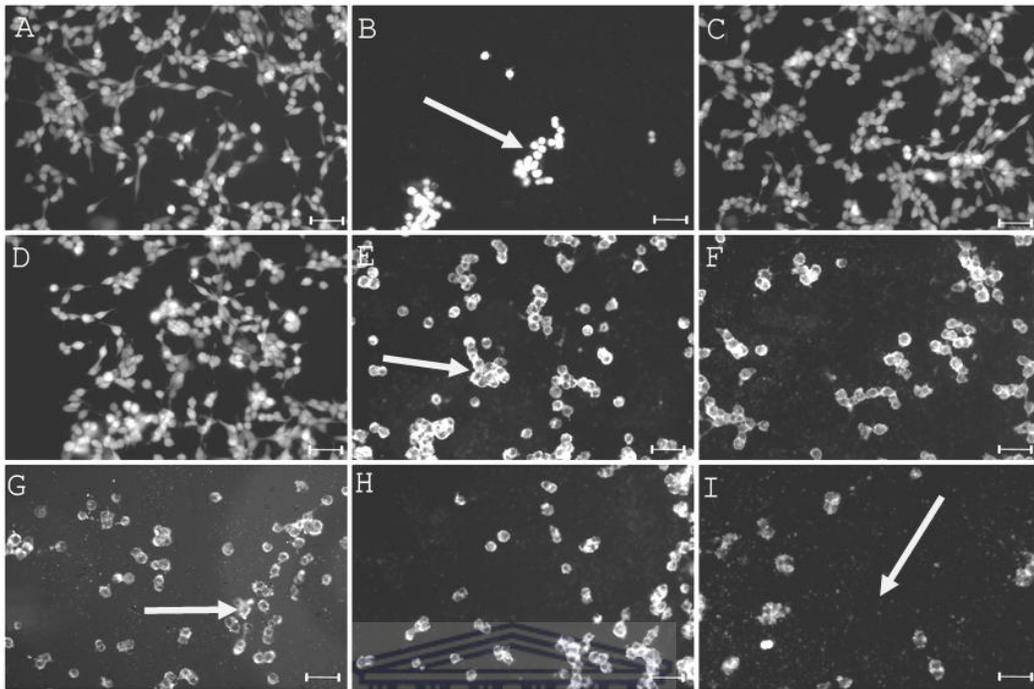


Fig 3-50: ROS quantity expression in malignant (LNCaP) prostate cancer cells after 72 hour exposure to green tea using a fluorescence microscope.

(A) Negative control (B) PC (6% DMSO) (C) 125 µg/ml (D) 250 µg/ml (E) 500 µg/ml (F) 1 000 µg/ml (G) 5 000 µg/ml (H) 10 000 µg/ml (I) 50 000 µg/ml. Magnification: 100x Bar = 50µm. Cells were stained with 7 µM CM 5, 6 CM H₂DCFDA. Arrows illustrate stressed cells emitting DCF fluorescence or empty space left by detached cells.

3.3.2.9 Detection of reactive oxygen species (ROS) in malignant (LNCaP) prostate cells after incubating with fermented rooibos 72 hours using a fluorescence microscope.

Below are low magnification fluorescence microscopy micrographs taken after incubating malignant (LNCaP) prostate cells for 72 hour with fermented tea. Micrographs C, D and F showed cells treated with 125, 500 or 500 $\mu\text{g/ml}$ FR, respectively, with normal dividing and adherent malignant (LNCaP) prostate cells. After, staining the cells with H_2DCFDA the cells emitted high fluorescence intensity compared to the negative control. However, exposure to 500 $\mu\text{g/ml}$ FR (E) showed a decrease in fluorescence compared to the negative control (A).

Morphologically, the cells appeared slightly stressed with round shape. Of notable interest is that there was a decrease in the number of cells on the micrographs as the concentration of FR increased. This was accompanied by a decrease in DCF fluorescence. Subsequent increase in concentration at 5 000, 10 000 or 50 000 $\mu\text{g/ml}$ FR; micrographs G, H and I, respectively, showed heavily stressed cells. In brief, a morphological analysis, confirmed the presence of a concentration-dependent increase in stress levels. And because of this, most of the cells appeared round, with broken cell membranes and detached. Above all, there was a very big drop in fluorescence compared to the negative control.

Briefly, we expected a concentration-dependent increase in ROS levels. And because of that highly stressed LNCaP cells (those treated with higher FR concentration) were supposed to produce more ROS than the less stressed cells (those treated with low concentrations of FR). This would have resulted in a concentration-dependent increase in fluorescence intensity. The reason for the increased fluorescence intensity could have been due to the pro-oxidant effect of FR on LNCaP cells which would have resulted in increased stress and generation of more ROS. Besides this, high physiological levels of ROS have been reported in tumor cells. This accompanied by

a compromised anti-oxidant defense system made up of very low levels of manganese dioxide, catalase, glutathione and superoxide dismutase etc may have promoted elevated ROS levels in cancer cells. After several interventions, this thesis, has failed to determine the ROS levels using fluorescence microscopy (Fig 3.51).

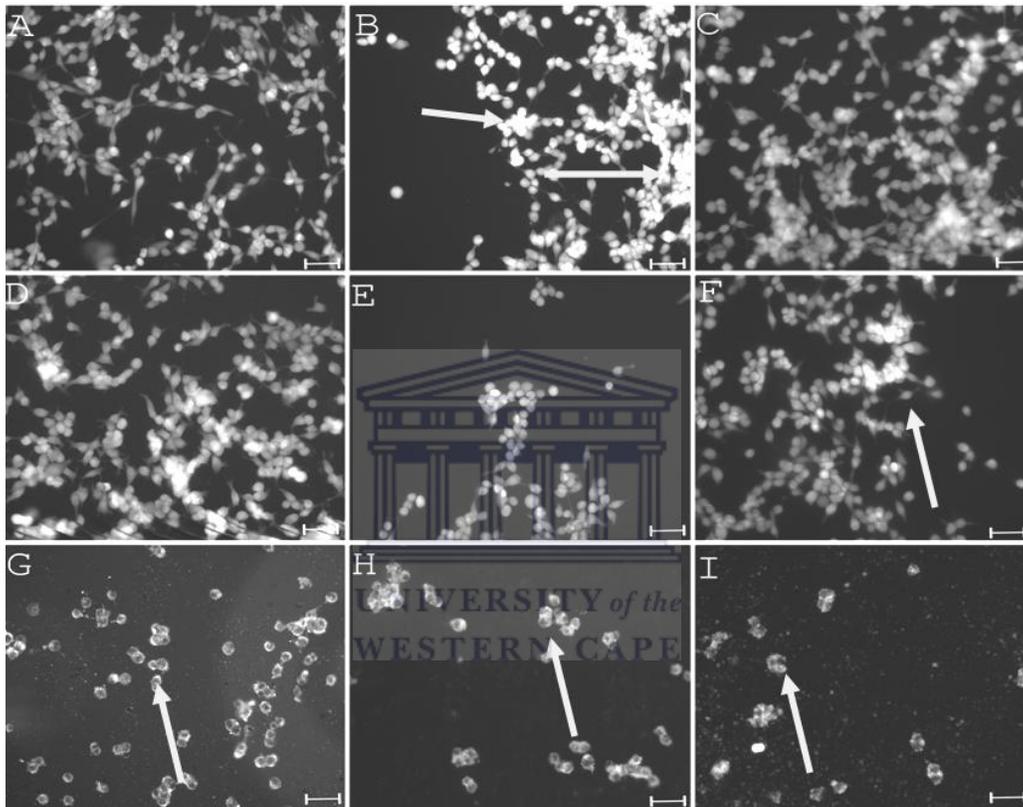


Fig 3-51: ROS quantity expression in LNCaP cells after 72 hour exposure to fermented rooibos using fluorescence microscope.

(A) Negative control (B) PC (6% DMSO) (C) 125 µg/ml (D) 250 µg/ml (E) 500 µg/ml (F) 1 000 µg/ml (G) 5 000 µg/ml (H) 10 000 µg/ml (I) 50 000 µg/ml. Magnification: 100x Bar = 50 µm. Cells were stained with 7 µM CM 5, 6 CM H₂DCFDA.

3.3.2.10 Detection of reactive oxygen species (ROS) in malignant (LNCaP) prostate cells after incubating with unfermented rooibos for 72 hours using a fluorescence microscope.

Below are low magnification fluorescence microscopy micrographs (A and C) taken after incubating malignant (LNCaP) prostate cells with 0 or 125 $\mu\text{g/ml}$ UR, respectively, for 72 hours. The micrographs showed low fluorescence emission coming from normal dividing and adherent malignant (LNCaP) prostate cells. The fluorescence increased rapidly after treating the cells with 250 or 500 $\mu\text{g/ml}$ UR compared to the untreated cells (A). Subsequent exposure of cells to 1 000 - 50 000 $\mu\text{g/ml}$ UR; micrographs F and I showed heavily stressed cells which produced decreased fluorescence intensity compared to the negative control.

Morphologically, stress levels appeared to increase with each increase in concentration. Further to this, the number of cells continued to decrease as the concentration of UR increased. Because of this, the intensity of DCF fluorescence continued to decrease as UR concentration increased. Briefly, we expected a concentration-dependent increase in ROS levels. And because of this, highly stressed LNCaP cells (those treated with higher UR concentration) were supposed to produce more ROS than the less stressed cells (those treated with low concentrations of UR). In the final analysis, a concentration-dependent increase in fluorescence intensity could not be achieved due to several factors. The probable reasons for the increased fluorescence intensity could come about because of the pro-oxidant effect of UR on LNCaP cells which may have induced increased stress and generation of more ROS.

Besides this, high physiological levels of ROS have been reported in tumor cells. This accompanied by a compromised anti-oxidant defense system made up of very low levels of manganese dioxide, catalase, glutathione and superoxide dismutase etc may have promoted elevated ROS levels in cancer cells. The Zeiss fluorescence

microscope used in this thesis, produced different exposure times and light intensity to the cells treated with the same concentration. After several interventions, this thesis, has not achieved the set objective of determining ROS levels using fluorescence microscopy (Fig 3.52).

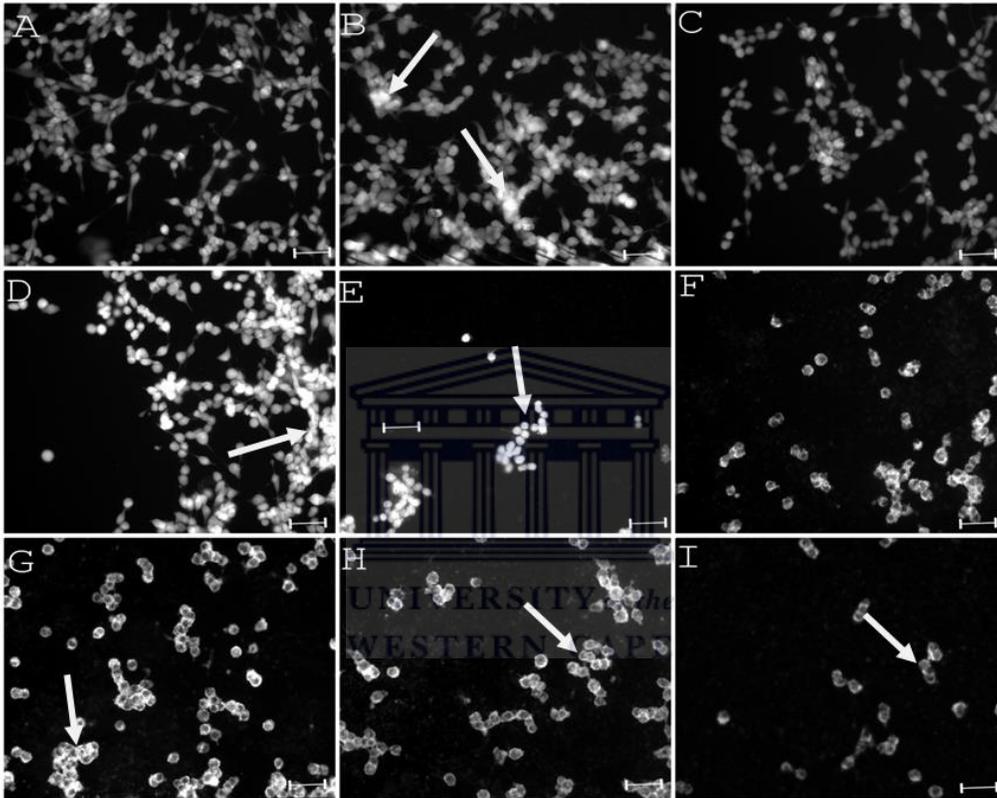


Fig 3-52: ROS quantity expression in malignant (LNCaP) prostate cells after 72 hour exposure to unfermented rooibos using fluorescence microscope.

(A) Negative control (B) PC (6% DMSO) (C) 125 µg/ml (D) 250 µg/ml (E) 500 µg/ml (F) 1 000 µg/ml (G) 5 000 µg/ml (H) 10 000 µg/ml (I) 50 000 µg/ml. Magnification: 100x Bar = 50µm. Arrows illustrate cells that are swollen and have lost adhesion to other surrounding cells. Cells were stained with 7µM CM 5, 6 CM H₂DCFDA. Magnification: 100x Bar = 50µm

3.3.2.11 Detection of reactive oxygen species (ROS) in malignant (LNCaP) prostate cells after incubating with aspalathin for 72 hours using a fluorescence microscope.

Below are low magnification fluorescence microscopy micrographs taken after incubating malignant (LNCaP) prostate cells for 72 hour with aspalathin. Micrographs C and D showed cells treated with 125µg/ml ASP or 250 µg/ml ASP, respectively, with normal dividing and adherent malignant (LNCaP) prostate cells. After being loaded with the ROS dye, H₂DCFDA LNCaP cells treated with 500 – 10 000 µg/ml ASP; micrographs E and F showed a concentration - dependent decrease in fluorescence compared to the positive control (B).

Morphologically, stress levels appeared to increase with each increase in concentration. Further to this, the number of cells continued to decrease as the concentration of ASP increased. Because of this, the intensity of DCF fluorescence continued to decrease as ASP concentration increased. Briefly, we expected a concentration-dependent increase in ROS levels. And because of this, highly stressed LNCaP cells (those treated with higher ASP concentration) were supposed to produce more ROS than the less stressed cells (those treated with low concentrations of ASP). In the final analysis, a concentration-dependent increase in fluorescence intensity could have been achieved. The probable reasons for the increased fluorescence intensity would have been due to the pro-oxidant effect of ASP on LNCaP cells which may have induced increased stress and generation of more ROS.

Besides this, high physiological levels of ROS have been reported in tumor cells. This accompanied by a compromised anti-oxidant defense system made up of very low levels of manganese dioxide, catalase, glutathione and superoxide dismutase etc may have promoted elevated ROS levels in cancer cells. After several interventions, this thesis, could not determine the ROS levels using fluorescence microscopy. The

highest concentration of 50 000 $\mu\text{g/ml}$ aspalathin was not used because our preliminary experiments showed death and detachment of the cells (Fig 3.53).

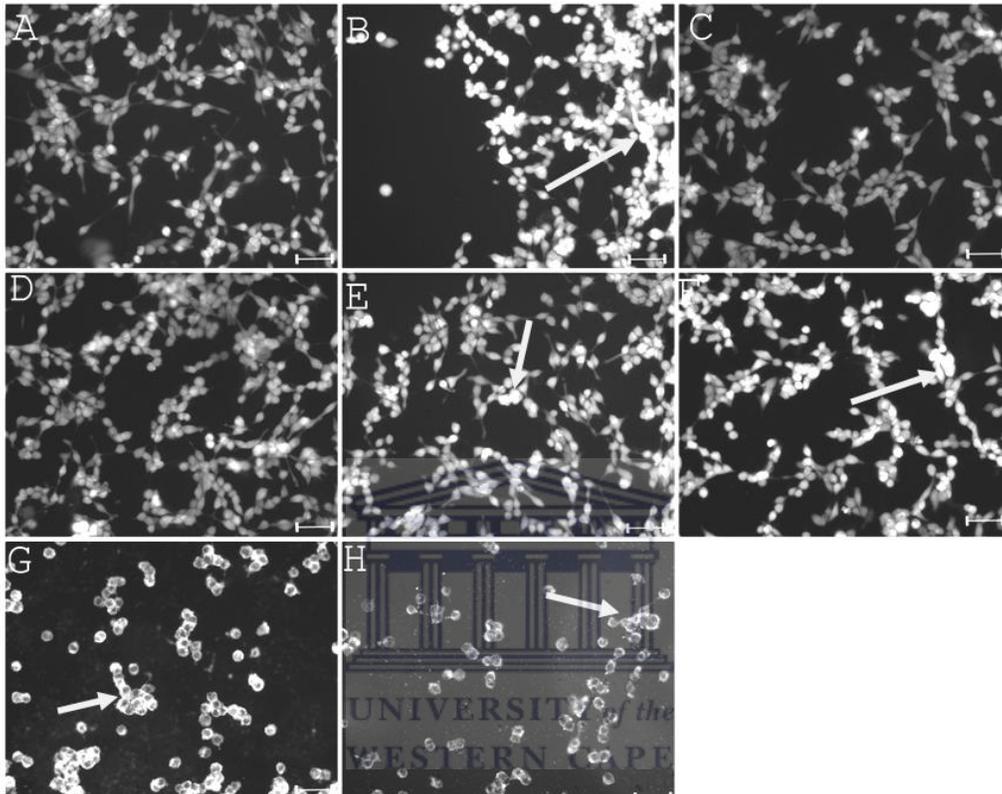


Fig 3-53: ROS quantity expression in LNCaP cells after 72 hour exposure to aspalathin using a fluorescence microscope.

(A) Negative control (B) PC (6% DMSO) (C) 125 $\mu\text{g/ml}$ (D) 250 $\mu\text{g/ml}$ (E) 500 $\mu\text{g/ml}$ (F) 1 000 $\mu\text{g/ml}$ (G) 5 000 $\mu\text{g/ml}$ (H) 10 000 $\mu\text{g/ml}$ (I) 50 000 $\mu\text{g/ml}$. Magnification: 10x Bar = 50 μm . Cells were stained with 7 μM CM 5, 6 CM H_2DCFDA . Arrows illustrate stressed and swollen cells with surfaces that are broken and have lost cellular components.

3.3.2.12 Detection of reactive oxygen species (ROS) in malignant (LNCaP) prostate cells after incubating with EGCG for 72 hours using a fluorescence microscope.

Below are low magnification fluorescence microscopy micrographs (C, D and E) taken after incubating malignant (LNCaP) prostate cells with (125, 250 and 500 $\mu\text{g/ml}$ EGCG), respectively, for 72 hours. After being loaded with the ROS dye, H_2DCFDA LNCaP cells, demonstrated high levels of fluorescence intensity compared to the negative control. However, exposure to 1 000 -10 000 $\mu\text{g/ml}$ EGCG; micrographs F - H showed LNCaP cells that emitted low fluorescence intensity compared to the positive control (micrograph B). Morphologically, the micrographs showed LNCaP cells which were more stressed as the concentration of EGCG increased. However, the stress levels were inversely proportional to the DCF fluorescence.

Briefly, we expected a concentration-dependent increase in ROS levels. And because of this, highly stressed LNCaP cells (those treated with higher EGCG concentration) were supposed to produce more ROS than the less stressed cells (those treated with low concentrations of EGCG). In the final analysis, a concentration-dependent increase in fluorescence intensity could have been achieved. The probable reasons for the increased fluorescence intensity would have been due to the pro-oxidant effect of EGCG on LNCaP cells which may have induced increased stress and generation of more ROS. Besides this, high physiological levels of ROS have been reported in tumor cells. This accompanied by a compromised anti-oxidant defense system made up of very low levels of manganese dioxide, catalase, glutathione and superoxide dismutase etc may have promoted elevated ROS levels in cancer cells. After several interventions, this thesis, has failed to determine the ROS levels using fluorescence microscopy. The highest concentration of 50 000 $\mu\text{g/ml}$ EGCG was not used because our preliminary experiments showed death and detachment of the cells (Fig 3.54).

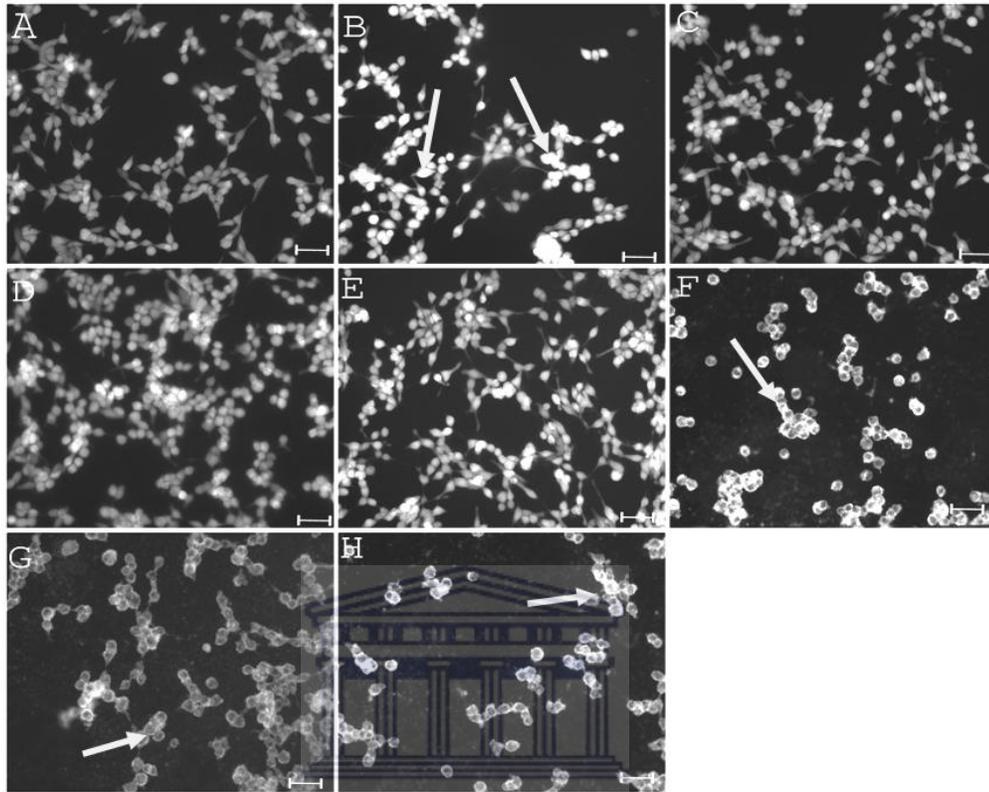


Fig 3-54: ROS level expression in malignant (LNCaP) prostate cells after 72 hour exposure to epigallocatechin gallate (EGCG) using a fluorescence microscope. (A) Negative control (B) PC (6% DMSO) (C) 125 µg/ml (D) 250 µg/ml (E) 500 µg/ml (F) 1 000 µg/ml (G) 5 000 µg/ml (H) 10 000 µg/ml. Arrows illustrate cells that are swollen and have lost adhesion to other surrounding cells. Cells were stained with 7µM CM 5, 6 CM H₂DCFDA. Magnification: 100x Bar = 50µm

3.3.2.13 Comparison of fluorescence levels between LNCaP and RPWE 1 cells using H₂DCFDA stained micrographs

Below are representative micrographs of RPWE 1 cells (A1 – 7) and LNCaP cells (B1-7) showing the extent to which each of these cell lines emitted DCF fluorescence after being treated with *C. sinensis* or *A. linearis* for 72 hour. The micrographs below show RPWE 1 and LNCaP cells treated with green tea. Morphologically RPWE 1 cells appear normal and seem to be coping well with the exposure to the tea induce stress in all the treatments(125-50 000 µg/ml) GT. H₂DCFDA stain show ROS production in all the samples through partial fluorescence in all the micrographs. A concentration-dependent fluorescence pattern was expected to be seen. Probably, very little ROS is being produced and at the same time it is being removed by the up to date antioxidant system which is characteristic of normal cells.

On the contrary, LNCaP cells appear stressed at 125 µg/ml GT and the fluorescence is comparable to that of the negative control. Higher concentrations demonstrate a concentration dependent increase in stress. Of great notice is that the fluorescence is not adding up with the concentration that has been used. There is a lot of variability in fluorescence to the extent that it has no specific pattern. Overall, LNCaP cells appear very stressed morphologically. On top of this the fluorescence is decreasing as the concentration is increasing partly because of the decrease in the cell numbers (Fig 3.55).

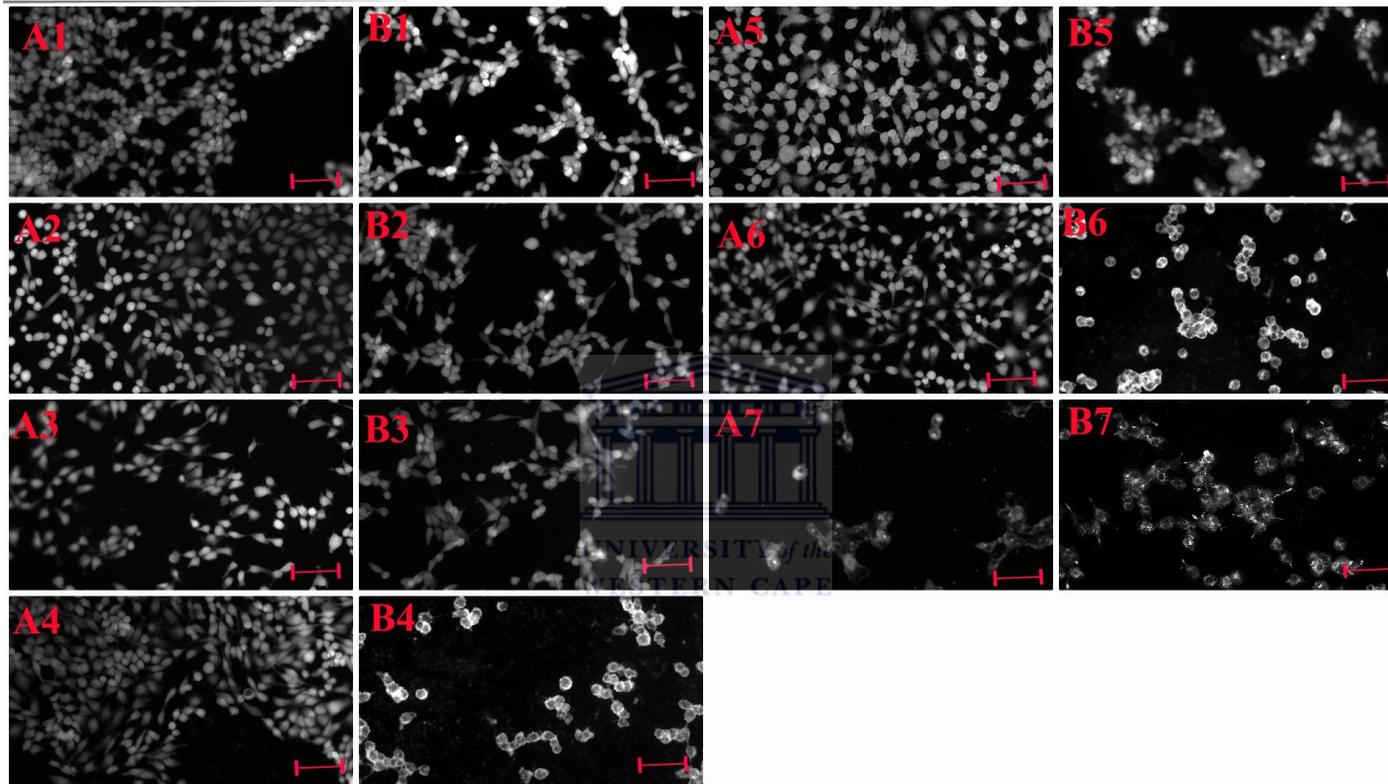


Fig 3-55: A comparison of ROS levels in RPWE 1 and LNCaP cells following exposure to green tea for 72 hours. (A1 & B1) Negative control, (A2 & B2) 125 µg/ml, (A3 & B3) 250 µg/ml, (A4 & B4) 500 µg/ml, (A5 & B5) 1 000 µg/ml, (A6 & B6) 5 000 µg/ml, (A7 & B7) 10 000 µg/ml. Magnification: 100x Bar = 50 µm. Arrows illustrate cells that are swollen and have lost adhesion to other surrounding cells. Cells were stained with 7 µM CM 5, 6 CM H₂DCFDA. Magnification: 100x Bar = 50 µm. A1-7 represents RPWE 1 cells. B1-7 represents LNCaP cells.

3.4 Reactive oxygen species (ROS) quantity expression in malignant (LNCaP) and benign (RPWE 1) prostate cells following exposure to *Camellia sinensis*, *Aspalathus linearis*, EGCG and aspalathin for 72 hours using flow cytometry.

After exposing malignant (LNCaP) and benign (RPWE 1) prostate cells to tea, rooibos tisane and their respective active compounds EGCG and aspalathin, ROS levels were determined. Cells loaded with 7 μM of the fluorescence dye CM H₂DCFDA were counted using the BD 6 accuri FACS using the FL1 channel. Lack of cytotoxic effects of tea and rooibos after acute (24 hours) exposure of RPWE 1 cells and insignificant morphological changes (cytotoxic effects) observed in LNCaP cells; we considered determining ROS levels in both cell lines after chronic exposure (72 hours) to the two nutraceuticals. The graphs below show the quantity of ROS in benign RPWE 1 and malignant LNCaP cells after chronic (72 hour) exposure of the cells to BT, GT, FR, UR, EGCG and aspalathin.

3.4.1 ROS quantity expression in benign (RPWE 1) prostate cells following exposure to black tea for 72 hours using flow cytometry.

A two way ANOVA statistical analysis showed that incubation of benign (RPWE 1) prostate cells for 72 hours with 125, 250, 500 and 1 000 $\mu\text{g/ml}$ BT did not result in any significant ($p > 0.05$) changes (5.3%, 5.9%, 5.6% and 6.5%, respectively) in ROS production when compared to the negative control (4.46%). Higher concentrations of BT (5 000, 10 000 and 50 000 $\mu\text{g/ml}$) produced significantly ($p < 0.001$) elevated ROS levels (19.5%, 27.6% and 27.7%, respectively) in a concentration dependent manner. The positive control (17.5%) produced significantly ($p < 0.001$) increased ROS levels compared to the negative control (4.46%).

Statistical analysis also showed that incubation of malignant (LNCaP) prostate cells for 72 hours with low concentrations of BT (125, 250, 500 and 1000 $\mu\text{g/ml}$) did not result (19%, 11.3%, 13.8% and 14%, respectively) in any significant ($p > 0.05$)

changes in ROS production when compared to the negative control (6.01%). However, incubation with higher BT concentration (5 000 and 10 000 and 50 000 µg/ml BT) produced significantly ($p < 0.001$) higher ROS levels (45.83%, 47.4% and 53.66%, respectively). The positive control produced significantly ($p < 0.001$) increased ROS levels (35.9%) compared to the negative control (6.01%).

Further to this, when ROS levels in malignant (LNCaP) and benign (RPWE 1) prostate cells were compared, two way ANOVA statistical analysis showed that exposure of these cell lines to all concentration treatments (125, 250, 500, 1 000, 5000, 10 000 and 50 000 µg/ml BT) for 72 hours resulted in a generalised higher ROS production by malignant (LNCaP) prostate cells compared to benign (RPWE 1) prostate cells. This increase was statistically significant ($p < 0.001$) when malignant (LNCaP) prostate cells were treated with 5 000- 50 000 ng/ml BT when compared to benign prostate cells. Besides this, there was significant ($p < 0.001$) increased ROS production in malignant negative and positive control cells, respectively, compared to benign prostate cells (Fig 3.56).

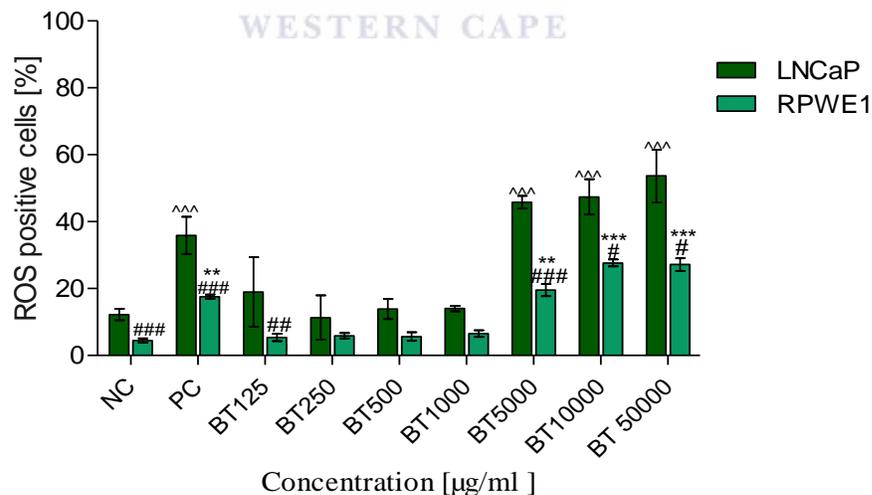


Fig 3- 56: ROS production in benign (RPWE 1) and malignant (LNCaP) prostate cells following exposure to black tea for 72 hours.

Values represent mean \pm SD n=9. (BT) black tea; PC, Positive control = 50 µM H₂O₂ for LNCaP and 200 µM H₂O₂ for RPWE 1. NC, negative control; ###.p < 0.001;## p < 0.01, # < 0.05 ***p < 0.001; ** p < 0.01; * p < 0.05; ^^^p < 0.001; ^^ p < 0.01; ^ p < 0.05; ^ : represent LNCaP vs Negative control. *: represent RPWE 1 vs Negative control. #: represent 72 hours compared to 24 hours.

3.4.2 ROS quantity expression in benign (RPWE 1) prostate cells following exposure to green tea for 72 hours using flow cytometry.

A two way ANOVA statistical analysis showed that incubation of benign (RPWE 1) prostate cells for 72 hours with low concentration of GT (125, 250, 500 and 1000 µg/ml) did not result in any significant ($p > 0.05$) changes in ROS production (2.03%, 2.56%, 3% and 4.4%, respectively) when compared to the negative control (1.56%). Subsequent exposure of RPWE 1 cells to 5 000, 10 000 and 50 000µg/ml GT produced significantly ($p < 0.001$) higher ROS levels (7.7%, 11.63% and 11.06%) respectively compared to the negative control (1.56%). In addition to this, the positive control (17.57%) produced significantly ($p < 0.001$) increased ROS levels.

Secondly, a two way ANOVA statistical analysis showed that incubation of malignant (LNCaP) prostate cells for 72 hours with 125, 250 and 500 µg/ml GT did not result in any significant ($p > 0.05$) changes in ROS production (3.93%, 5.73%, and 5.93%) respectively. Thereafter, 1 000, 5 000, 10 000 and 50 000µg/ml GT exposure caused a significant increase (9.1%, 23.43%, 30.43% and 36.82%, respectively) in ROS positive cells.

Further to this, when ROS levels in malignant (LNCaP) and benign (RPWE 1) prostate cells were compared, two way ANOVA statistical analysis showed within the tested concentration range (125, 250, 500, 1000, 5000, 10000 and 50000 µg/ml GT) a generalised statistically higher ROS production in malignant (LNCaP) prostate cells compared to the benign (RPWE 1) prostate cells. This increase was statistically significant ($p < 0.001$) when malignant (LNCaP) prostate cells were treated with 1 000- 50 000 µg/ml GT when compared to benign prostate cells. Besides this, there was significant ($p < 0.001$) increased ROS production in malignant negative and positive control cells, respectively, compared to benign prostate cells (Fig 3.57).

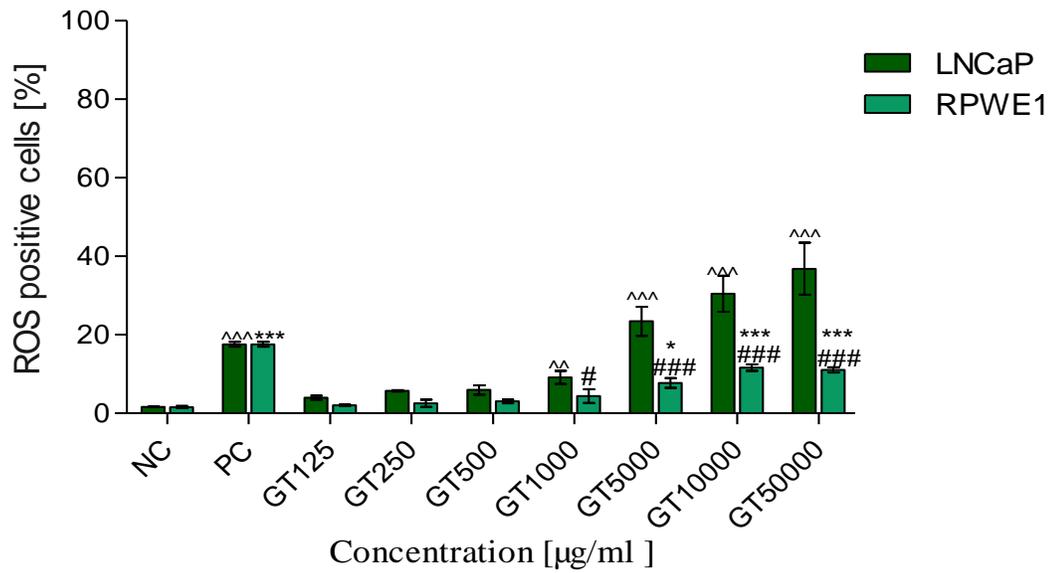
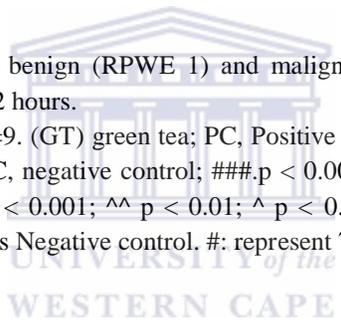


Fig 3- 57: ROS production in benign (RPWE 1) and malignant (LNCaP) prostate cells following exposure to green tea rooibos 72 hours.

Values represent mean \pm SD n=9. (GT) green tea; PC, Positive control = 50 μ M H₂O₂ for LNCaP and 200 μ M H₂O₂ for RPWE 1. NC, negative control; ###, p < 0.001; ## p < 0.01, # < 0.05 ***p < 0.001; ** p < 0.01; * p < 0.05; ^^^p < 0.001; ^^ p < 0.01; ^ p < 0.05; ^ : represent LNCaP vs Negative control. *: represent RPWE 1 vs Negative control. #: represent 72 hours compared to 24 hours.



3.4.3 ROS quantity expression in benign (RPWE 1) prostate cells following exposure to fermented rooibos for 72 hours using flow cytometry.

A two way ANOVA statistical analysis showed that incubation of benign (RPWE 1) prostate cells for 72 hours with (125 – 500 µg/ml FR) did not result in any significant ($p > 0.05$) changes in ROS production (4.33%, 3.93% and 9.76%, respectively) when compared to the negative control (1.23%). However, 1 000 µg/ml FR was shown to produce significant ($p < 0.01$) higher ROS levels (13.1%) compared to the negative control (1.23%). Incubation of benign (RPWE 1) prostate cells with 5 000, 10 000 and 50 000 µg/ml FR produced a concentration dependent significant ($p < 0.001$) rise in ROS levels (20.2%, 31.5% and 33.79%, respectively). In addition to this, the benign (RPWE 1) positive treated prostate cells produced significantly ($p < 0.001$) increased ROS levels (17.57%) compared to the negative control (1.23%).

Further to this, a two way ANOVA statistical analysis showed that exposure of malignant (LNCaP) prostate cells with 125 and 250 µg/ml FR for 72 hours did not result in any significant ($p > 0.05$) changes in ROS production (13.73% and 15.7%) when compared to the negative control (8.33%). Exposure to 500 and 1 000 µg/ml FR for 72 hours was shown to produce significantly ($p < 0.05$) higher ROS levels (17.23% and 23.63%). Higher concentrations of FR 5 000, 10 000 and 50 000 µg/ml caused a concentration dependent significant ($p < 0.001$) increase in ROS levels (31.43%, 55.76% and 57.5%, respectively) compared to the negative control (8.33%). In addition to this, the positive control produced significantly ($p < 0.001$) increased ROS levels (8.33%).

When ROS levels in malignant (LNCaP) and benign (RPWE 1) prostate cells were compared, two way ANOVA statistical analysis showed that exposure of these cell lines to 125, 250, 500, 1000, 5000, 10 000 and 50 000 µg/ml FR for 72 hours resulted in a significantly higher ROS production by malignant (LNCaP) prostate cells in comparison to benign (RPWE 1) prostate cells. The negative and positive control of

malignant (LNCaP) prostate cells produced statistically significant ($p < 0.001$) higher ROS levels than the benign (RPWE 1) prostate cells (Fig 3.58).

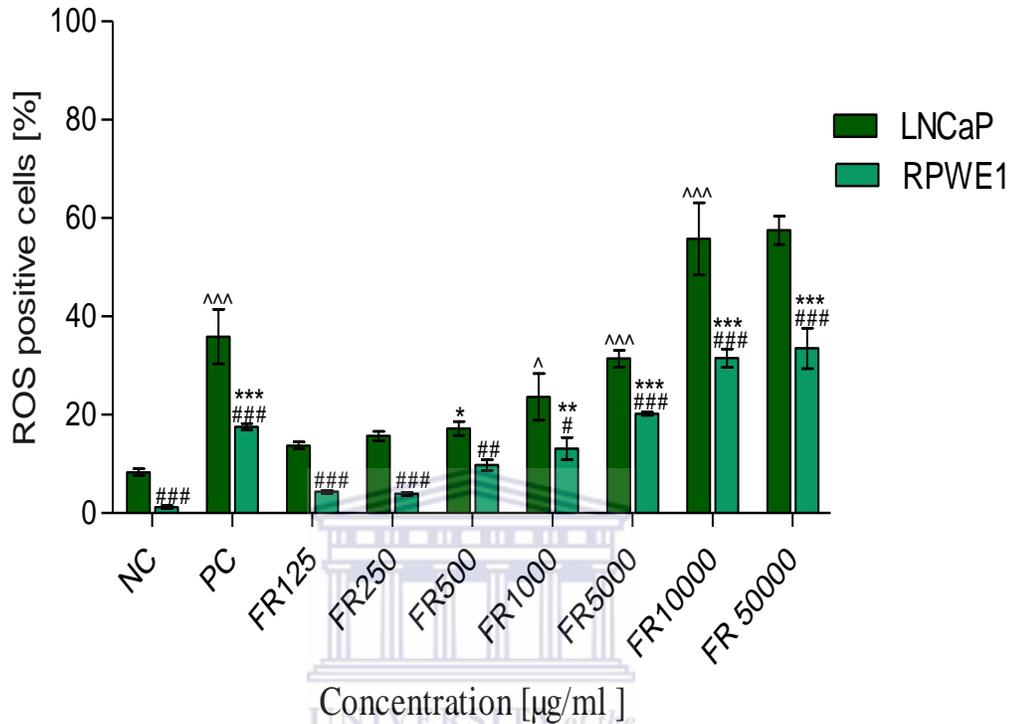


Fig 3- 58: ROS production in benign (RPWE 1) and malignant (LNCaP) prostate cells following exposure to fermented rooibos 72 hours using flow cytometry.

Values represent mean \pm SD n=9. (FR) fermented rooibos; PC, Positive control = 50 μ M H₂O₂ for LNCaP and 200 μ M H₂O₂ for RPWE 1. NC, negative control; ### p < 0.001; ## p < 0.01, # < 0.05; ***p < 0.001; ** p < 0.01; * p < 0.05; ^^^ p < 0.001; ^^ p < 0.01; ^ p < 0.05; ^ : represent LNCaP vs Negative control. *: represent RPWE 1 vs Negative control. #: represent 72 hours compared to 24 hours.

3.4.4 ROS quantity expression in benign (RPWE 1) prostate cells following exposure to unfermented rooibos for 72 hours using flow cytometry.

A two way ANOVA statistical analysis showed that incubation of benign (RPWE 1) prostate cells for 72 hours with 125, 250, 500 µg/ml UR did not result in any significant ($p > 0.05$) changes in ROS production respectively when compared to the negative control. However, incubation with 1000, 5 000, 10 000 and 50 000 µg/ml UR produced significant ($p < 0.001$) higher ROS levels respectively compared to the negative control. In addition to this, the positive control produced significantly ($p < 0.001$) increased ROS levels compared to the negative control.

Secondly, a two way ANOVA statistical analysis showed that incubation of malignant (LNCaP) prostate cells for 72 hours with 125, 250 and 500 µg/ml UR did not result in any significant ($p > 0.05$) changes in ROS production when compared to the negative control. However, 1000, 5 000, 10 000 and 50 000 µg/ml UR produced significant ($p < 0.001$) elevated ROS levels respectively compared to the negative control. In addition to this, the positive control produced significantly ($p < 0.001$) increased ROS levels.

Further to this, when ROS levels in malignant (LNCaP) and benign (RPWE 1) prostate cells were compared, two way ANOVA statistical analysis showed that exposure of these cell lines to 125, 250, 500 µg/ml UR for 72 hours malignant (LNCaP) prostate cells did not produce any significant ($p > 0.05$) changes in ROS levels when compared to the benign (RPWE 1) prostate cells. However, a notable finding was the statistically significant ($p < 0.05$) increase in ROS level production in malignant (LNCaP) prostate cells following treatment with 1 000 µg/ml UR when compared to RPWE 1. Further incubation of malignant (LNCaP) prostate cells with higher concentration (5 000, 10 000 and 50 000 µg/ml) of UR produced significantly ($p < 0.001$) increased ROS concentrations respectively when compared with the benign (RPWE 1) prostate cells. Besides this, there was significant ($p < 0.001$)

increased ROS production by malignant (LNCaP) positive control prostate cells compared to those in positive control treated normal prostate cells. Similarly, malignant (LNCaP) untreated prostate cells showed significant ($p < 0.001$) higher ROS level concentration than benign (RPWE 1) untreated prostate cells (Fig 3.59).

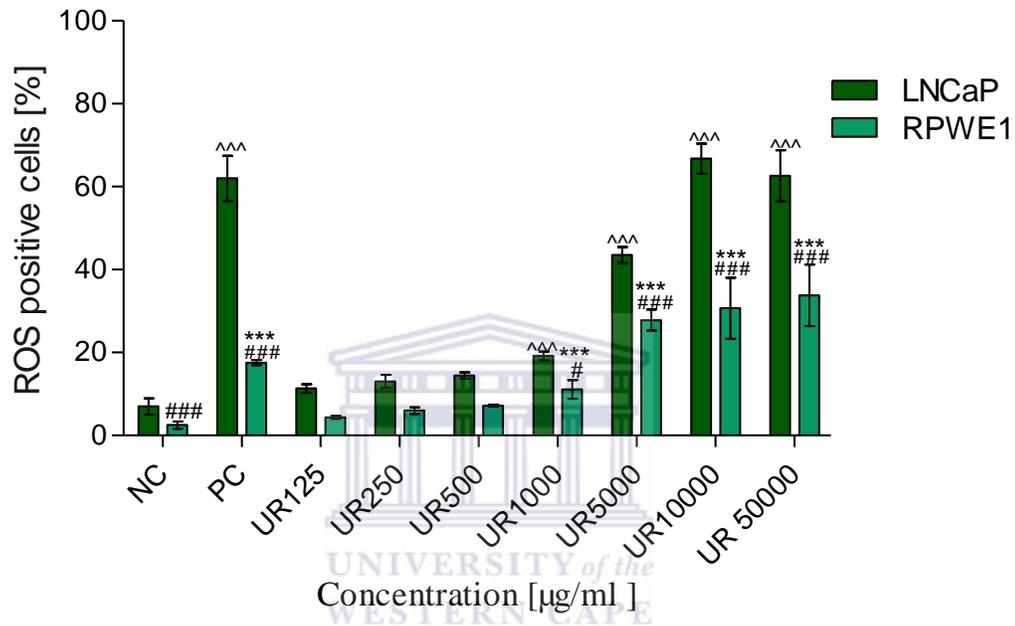


Fig 3- 59: ROS production in benign (RPWE 1) and malignant (LNCaP) prostate cells following exposure to unfermented rooibos 72 hours using flow cytometry. Values represent mean \pm SD; n=9. (UR) unfermented rooibos; PC, Positive control; 50 μ M H₂O₂ for LNCaP and 100 μ M H₂O₂ for RPWE 1. NC, negative control; ###.p < 0.001;## p <0.01, # < 0.05 ***p < 0.001; ** p < 0.01; * p < 0.05; ^^^ p < 0.001; ^^ p < 0.01; ^ p < 0.05; ^ : represent LNCaP vs Negative control. *: represent RPWE 1 vs Negative control. #: represent 72 hours compared to 24 hours.

3.4.5 ROS quantity expression in benign (RPWE 1) prostate cells following exposure to aspalathin for 72 hours using flow cytometry.

A two way ANOVA statistical analysis showed that incubation of benign (RPWE 1) prostate cells for 72 hours with 125, 250 and 500 µg/ml ASP did not result in any significant ($p > 0.05$) changes in ROS production when compared to the negative control. However, 1000, 5 000 and 10 000 µg/ml ASP produced significant ($p < 0.001$) higher ROS levels compared to the negative control. In addition to this, the positive control produced significantly ($p < 0.001$) increased ROS levels.

Regarding malignant (LNCaP) prostate cells, a two way ANOVA statistical analysis showed that incubation over 72 hours with 125 and 250 µg/ml ASP did not result in any significant ($p > 0.05$) changes in ROS production, respectively, compared to the negative control. Higher concentration of ASP (1 000 µg/ml) caused a concentration-dependent significant rise in ROS positive cells.

Further to this, when ROS levels in malignant (LNCaP) and benign (RPWE 1) prostate cells were compared, two way ANOVA statistical analysis showed that exposure of these cell lines to all concentrations tested (125, 250, 500, 1000, 5000, 10000 and 50 000 µg/ml) ASP for 72 hours resulted in a generalised significant ($p < 0.001$) increased ROS production in malignant (LNCaP) prostate cells when compared to benign (RPWE 1) prostate cells. Besides this, there was significant ($p < 0.001$) increased ROS production in malignant negative and positive control cells, respectively, compared to benign prostate cells (Fig 3.60).

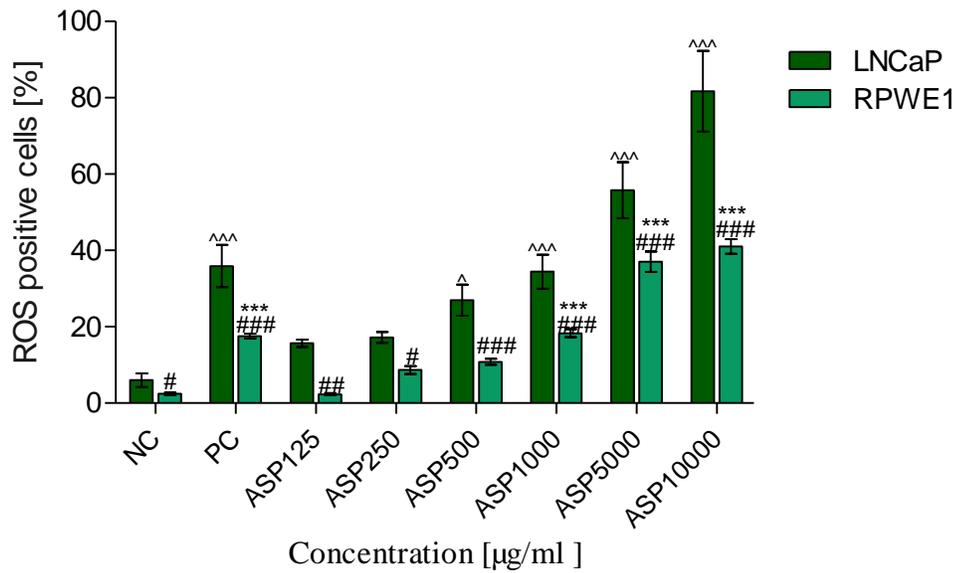


Fig 3- 60: ROS production in benign (RPWE 1) and malignant (LNCaP) prostate cells following exposure to aspalathin for 72 hours using flow cytometry. Values represent mean \pm SD n=9. (ASP) aspalathin; PC, Positive control = 50 μ M H₂O₂ for LNCaP and 200 μ M H₂O₂ for RPWE 1. NC, negative control; ###.p < 0.001;## p <0.01, # < 0.05 ***p < 0.001; ** p < 0.01; * p < 0.05; ^^p < 0.001; ^^ p < 0.01; ^ p < 0.05; ^ : represent LNCaP vs Negative control. *: represent RPWE 1 vs Negative control. #: represent 72 hours compared to 24 hours.

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3.4.6 ROS quantity expression in benign (RPWE 1) prostate cells following exposure to EGCG for 72 hours using flow cytometry.

A two way ANOVA statistical analysis showed that incubation of benign (RPWE 1) prostate cells for 72 hours with 125, 250 and 500 µg/ml EGCG did not result in any significant ($p > 0.05$) changes in ROS production when compared to the negative control. Exposure of the benign (RPWE 1) prostate cells to 5 000 and 10 000 µg/ml EGCG produced significantly ($p < 0.001$) higher ROS levels. In addition to this, the positive control produced significantly ($p < 0.001$) increased ROS levels compared to the negative control.

Secondly, a two way ANOVA statistical analysis showed that incubation of malignant (LNCaP) prostate cells with 125, 250 and 500 µg/ml EGCG for 72 hours did not result in any significant ($p > 0.05$) changes in ROS production respectively when compared to the negative control. However, incubation with 1 000, 5 000 and 10 000 µg/ml EGCG produced significantly ($p < 0.001$) elevated ROS levels respectively compared to the negative control. In addition to this, the positive control produced significantly ($p < 0.001$) increased ROS levels compared to the negative control.

Further to this, when ROS levels in malignant (LNCaP) and benign (RPWE 1) prostate cells were compared, two way ANOVA statistical analysis showed that exposure of these cell lines to all tested concentrations (125, 250, 500, 1000, 5 000, 10 000 and 50 000 µg/ml EGCG) for 72 hours resulted in significantly ($p < 0.001$) higher ROS production in malignant (LNCaP) prostate cells when compared to the benign (RPWE 1) prostate cells. This increase was statistically significant ($p < 0.001$) when malignant (LNCaP) prostate cells were treated 125 - 50 000 µg/ml EGCG when compared to benign prostate cells. Besides this, there was significant ($p < 0.001$) increased ROS production in malignant negative and positive control cells, respectively, compared to benign prostate cells (Fig 3.61).

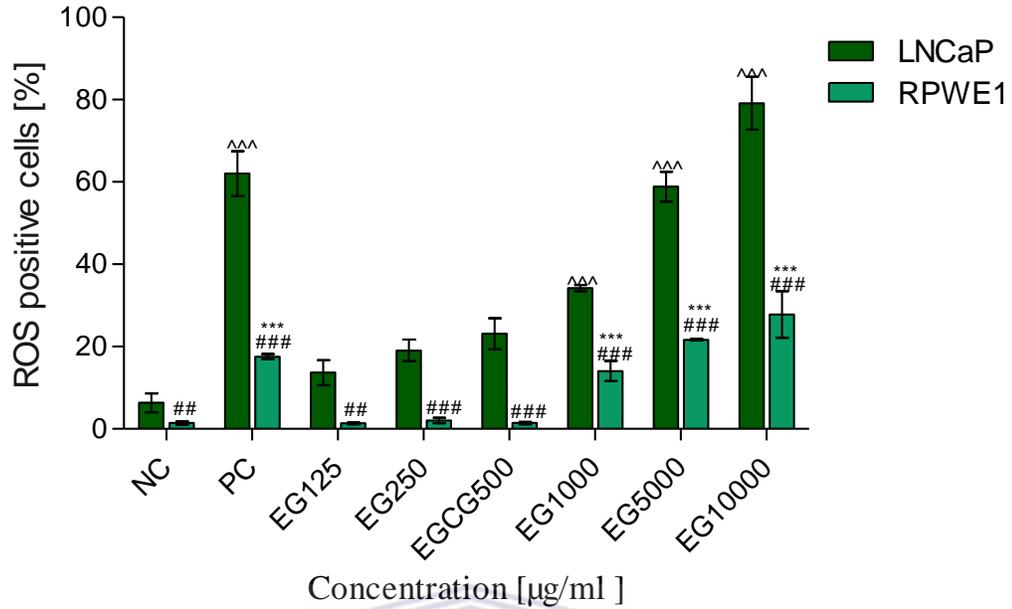


Fig 3- 61: ROS production in benign (RPWE 1) and malignant (LNCaP) prostate cells following exposure to EGCG for 72 hours. Values represent mean \pm SD n=9. (EGCG) epigallocatechin gallate; PC, Positive control = 50 μ M H₂O₂ for LNCaP and 200 μ M H₂O₂ for RPWE 1. NC, negative control; ###.p < 0.001; ## p < 0.01, # < 0.05; ***p < 0.001; ** p < 0.01; * p < 0.05; ^^p < 0.001; ^^ p < 0.01; ^ p < 0.05; ^: represent LNCaP vs Negative control. *: represent RPWE 1 vs Negative control. #: represent 72 hours compared to 24 hours.

3.5 Necrosis and Apoptosis

The results below show the percentage of apoptotic malignant (LNCaP) and benign RPWE 1 prostate cells after incubating them with 1 000 $\mu\text{g/ml}$ tea, rooibos and their main active compounds epigallocatechin gallate and aspalathin for 24 and 72 hours, respectively. In this thesis, our preliminary studies showed that 1 000 $\mu\text{g/ml}$ of BT, GT, FR, UR, ASP and EGCG induced at least 50% of apoptotic deaths in malignant LNCaP cells. Based on this, we considered to use this concentration in the preparation of our treatment samples for the Annexin V assay.

In the present thesis in order to induce apoptosis in both RPWE 1 and LNCaP cells, 6% DMSO was used. And in order to induce necrosis in RPWE 1 cells, 100 μM H_2O_2 with 15 hour incubation was used whilst in LNCaP cells, 50 μM H_2O_2 with 60 minute incubation induced necrosis. The above results were achieved after performing four preliminary independent experiments using 20 μM – 1000 μM H_2O_2 with each experiment being done in triplicate.

In a previous study, 5% DMSO in RPMI was used in LNCaP cells in order to inhibit cryopreservation-induced apoptosis (Baust, 2005). In addition to this, 10% DMSO in complete medium and 3 hour incubation (10% in CM10 for 3 hours) induced apoptosis in DU-145 and LNCaP cells (Nejatollahi et al., 2013).

Freitas et al. (2012) reported that 500 μM H_2O_2 inhibited cell proliferation in RPWE 1 and its effects were more pronounced following 72 hours of treatment. In another previous study, endothelial cells became apoptotic after exposing them to 100 μM H_2O_2 without altering ATP levels (Lelli et al., 1998). However, when 5 mM H_2O_2 was used there was a significant decrease in ATP levels and the cells went into necrotic cell death.

As such, at least 10 000 events were acquired on the flow cytometer in order to identify the percentage of prostate cells that picked annexin V dye following externalization of the interior part of the cell membrane. In a similar manner, we endeavored to identify the cells that had compromised cell membranes and allowed propidium iodide to enter the cell and nucleus and intercalate with the DNA. In this regard, the FL1 (green) and FL3 (red) channels read the percentage of apoptotic cells and necrotic cells, respectively.

3.5.1 Apoptosis in malignant (LNCaP) prostate cells.

In this regard, a comparison of the percentage of LNCaP apoptotic cells at 24 hours compared to the negative control using two-way ANOVA statistical analysis, showed no significant ($p > 0.05$) increase in the percentage of apoptotic cells following incubation with 1 000 $\mu\text{g/ml}$ BT, FR, EGCG or ASP, respectively. A notable point was a significant ($p < 0.05$ and $p < 0.001$) increase in percentage of apoptotic deaths following incubation with 1 000 $\mu\text{g/ml}$ UR and GT for 24 and 72 hours, respectively. Furthermore, the positive control (6% DMSO) showed a sharp significant ($p < 0.001$) increase in the percentage of apoptotic cells at 24 hour incubation compared to the negative control.

In addition to this, a two-way ANOVA statistical analysis of the data obtained after incubating the cells with 1 000 $\mu\text{g/ml}$ FR for 72 hours showed no significant ($p > 0.05$) increase in the percentage of apoptotic cells compared to the negative control. However, 1 000 $\mu\text{g/ml}$ BT produced a significant ($p < 0.05$) increase in the percentage of apoptotic cells as a result of exposure for 72 hours. This type of cell death increased significantly ($p < 0.001$) after incubating these cells with 1 000 $\mu\text{g/ml}$ GT, UR, ASP or EGCG, respectively compared to the negative control. Furthermore, the positive control (6% DMSO) showed a greatly enhanced significant ($p < 0.001$) increase in the percentage of apoptotic cells at 72 hours compared to the negative control.

Further to this, two way ANOVA statistical analysis followed by Sidak's multiple comparison test showed a general increase in the percentage of apoptotic cells after 72 hours in all the treated samples compared to the 24 hour incubation period. In this thesis, 1 000 µg/ml GT produced a significant ($p < 0.05$) increase in the percentage of apoptotic cells at 72 hours (48.03%) compared to 24 hours (37.96%). The percentage of apoptotic cells also increased significantly following exposure of the malignant (LNCaP) prostate cells for 72 hours (26.23%) to 1 000 µg/ml UR compared to 24 hours (12.63%). Besides this, the positive control, 6% DMSO, produced a higher upward significant ($p < 0.001$) increase in the percentage of apoptotic cells between 24 hour (34%) and 72 hour incubation period (63.83%).

A highly significant ($p < 0.001$) increase in the percentage of apoptotic cells was produced by both EGCG (28.23%; 7.7%) and ASP (45.43%; 7.5%) at 72 hours relative to 24 hour. However, the two plant extracts 1 000 µg/ml BT (12.4%; 4.6%) and 1 000 µg/ml FR (12.4%; 5.3%) produced a non-significant increase ($p > 0.05$) in the percentage of apoptotic cells respectively at 72 hours compared to 24 hours. There was no significant increase in the percentage of apoptotic cells for the negative control at 72 hours compared to 24 hours. At the same time, there was a significant ($p < 0.001$) increase in apoptotic death between the positive controls at 72 hours compared to 24 hours. The plant extracts when arranged in order of decreasing ability to induce apoptosis in malignant (LNCaP) prostate cells showed; GT > ASP > EGCG > UR > BT > FR (Fig 3.62)

3.5.2 Apoptosis in benign (RPWE 1) prostate cells.

The results below present the percentage of apoptotic benign (RPWE 1) prostate cells after incubation with 1 000 µg/ml tea or rooibos for 24 and 72 hours, respectively.

After incubating RPWE 1 cells for 24 hours, the percentage of apoptotic dead cells in the treated samples was compared to the negative control. And results of the two-way ANOVA statistical analysis showed a significant drop in the percentage of apoptotic cells in all the treated samples compared to the negative control. This demonstrated the protective effects of tea, rooibos and their main active compounds have on normal prostate cells. The positive control showed a significant ($p < 0.001$) increase in the number of apoptotic dead cells compared to the negative control.

Further to this, after incubating the cells for 72 hours, two-way ANOVA statistical analysis followed by Sidak's multiple comparison showed lower values of apoptotic dead cells compared to the negative control. Similar to 24 hours, the normal prostate cells appear to cope with the teas, rooibos and their main active compounds. The positive control showed a significant ($p < 0.001$) increase in the percentage of apoptotic deaths compared to negative control.

Two-way ANOVA statistical analysis followed by Sidak's multiple comparison test showed a non-significant ($p > 0.05$) increase in the percentage of apoptotic cells in all the treated sample at 72 hours compared to 24 hour exposure time. In this thesis, GT (0.93%; 7.66%); EGCG (1.46%; 7.66%); ASP (0.9%; 2.5%); UR (0.76%; 4.66%) FR (0.96%; 1.2%) BT (0.2%; 2.7%). A notable observation, was the exposure of benign (RPWE 1) prostate cells to 1 000 µg/ml GT for 72 hours; this produced a significant ($p < 0.01$) increase in the percentage of apoptotic cells (7.66%) compared to 24 hours (0.93%). Besides this, the positive control, produced a non-significant ($p > 0.05$) increase in the percentage of apoptotic cells between 24 hour (28.4%) and 72 hour exposure time (31.73%) (Fig 3.63).

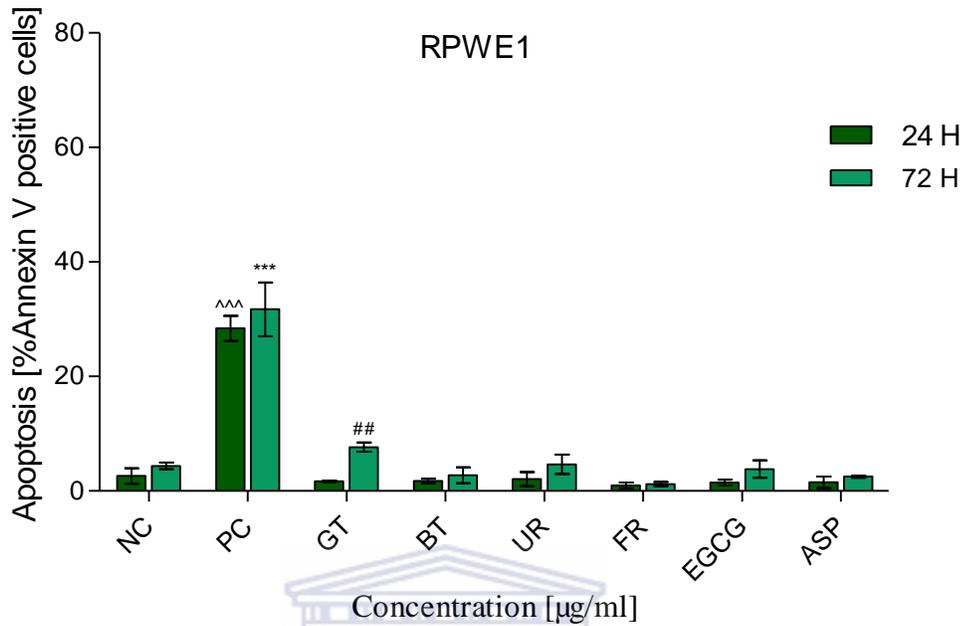


Fig 3- 63: Determination of apoptotic effect of 1 000 µg/ml GT, BT, UR, FR, EGCG and ASP on benign (RPWE 1) prostate cancer cells following 24 and 72 hours incubation period, respectively, using flow cytometry.

Values represent mean \pm SD, n= 8. ASP; aspalathin; EGCG; epigallocatechin gallate, UR; unfermented rooibos, FR; fermented rooibos, BT; Black tea, GT; Green tea. NC= negative control (medium); PC= positive control (6% DMSO). ^^^ p < 0.001; ## p < 0.01, # < 0.05; ***p < 0.001; ** p < 0.01; * p < 0.05; ^^p < 0.001; ^p < 0.01; ^ p < 0.05; ^: represent p- value at 24 hours compared to its negative control. *: represent p – value at 72 hours compared to its negative control. #: represent p- value at 72 hours compared to 24 hours.

3.5.3 Necrosis in malignant (LNCaP) prostate cells.

The results below show the percentage of malignant (LNCaP) prostate cells that became necrotic following exposure to 1 000 µg/ml GT, BT, UR, FR, EGCG and ASP for 24 and 72 hours. After incubating malignant (LNCaP) prostate cells for 24 hours, the percentage of necrotic cells in the treated samples was compared to the negative control. Two-way ANOVA statistical analysis followed by Sidak's multiple comparison test showed no significant ($p > 0.05$) increase in the percentage of necrotic deaths following exposure to 1 000 µg/ml of BT or FR, respectively, compared to the negative control. Incubation with 1 000 µg/ml of GT, UR, FR and EGCG, significantly ($p < 0.001$) increased the percentage of necrosis respectively. 1 000 µg/ml of ASP also induced significant ($p < 0.001$) increased necrotic cell death compared to the negative control. Furthermore, the positive control promoted significantly ($p < 0.001$) more necrosis compared to the negative control.

After incubating the cells for 72 hours, two-way ANOVA statistical analysis followed by Sidak's multiple comparison test showed no significant ($p > 0.05$) increase in the percentage of necrotic deaths following exposure to 1 000 µg/ml of FR compared to the negative control. Incubation with 1 000 µg/ml of BT promoted a significant ($p < 0.05$) increase in necrosis percentage; and subsequent exposure to 1 000 µg/ml GT, UR, EGCG or ASP significantly ($p < 0.001$) increased the percentage of necrotic cells respectively compared to the negative control. The positive control showed a significant ($p < 0.001$) increase in the percentage of necrotic deaths compared to negative control.

Two-way ANOVA followed by Sidak's multiple comparison test was used to analyse the difference between the two time periods following exposure to 1 000 µg/ml of GT, BT, UR, FR, EGCG or ASP. Incubation of these malignant (LNCaP) prostate cells with 1 000 µg/ml EGCG and 1 000 µg/ml GT produced a significant ($p < 0.001$) increase in the percentage of necrotic cells compared to incubation for 24 hours GT (48.03%; 37.96%) and EGCG (28.23%; 7.7%). Further to this, there was

significant ($p < 0.01$) increased percentage of necrotic cells (26.23%) following 72 hour exposure of the malignant (LNCaP) prostate cells to 1 000 $\mu\text{g/ml}$ UR compared to 24 hours (12.63%). Similarly, 72 hour exposure of these cells to 1 000 $\mu\text{g/ml}$ ASP produced significant ($p < 0.01$) increase in the percentage of necrotic cells (26.86%) compared to 24 hour incubation (18.8%). Besides this, the positive control, 100 μM H_2O_2 produced a significant ($p < 0.001$) increase in the percentage of apoptotic cells between 24 hour (34%) and 72 hour incubation period (63.83%). Subsequent incubation with 1 000 $\mu\text{g/ml}$ BT for 72 hours produced significant ($p < 0.01$) increase in the percentage of necrotic cells compared to 24 hours. However 1 000 $\mu\text{g/ml}$ FR did not produce any significant ($p > 0.05$) increase in necrosis when compared to 24 hours (Fig 3.64).

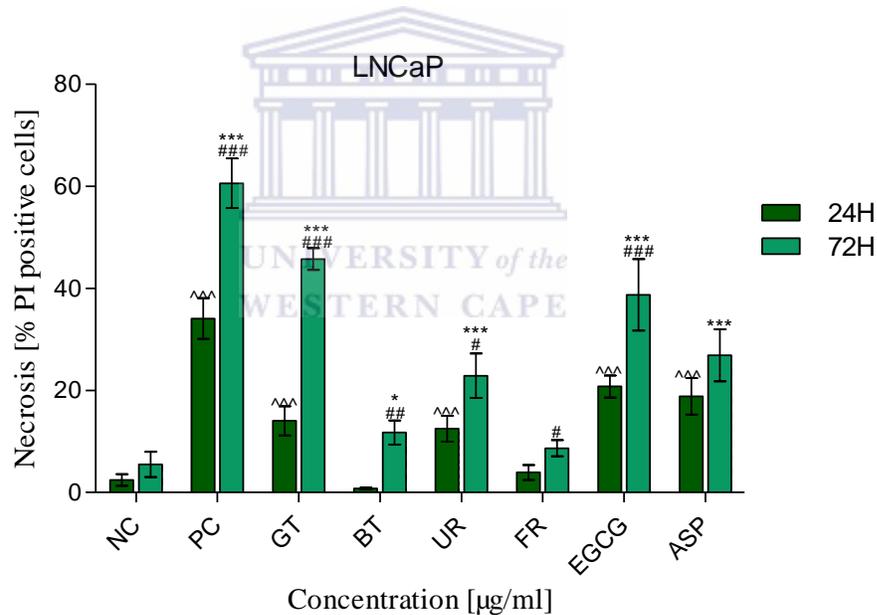


Fig 3- 64: Determination of the necrotic effect of 1 000 $\mu\text{g/ml}$ GT, BT, UR, FR, EGCG and ASP on malignant (LNCaP) prostate cancers following 24 and 72 hours incubation period, respectively, using flow cytometry.

Values represent mean \pm SD, $n = 8$. ASP; aspalathin; EGCG; epigallocatechin gallate, UR; unfermented rooibos, FR; fermented rooibos, BT; Black tea, GT; Green Tea. NC= negative control (medium); PC= positive control (100 μM H_2O_2). ### $p < 0.001$; ## $p < 0.01$, # $p < 0.05$; *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$; ^^ $p < 0.001$; ^^ $p < 0.01$; ^ $p < 0.05$; ^: represent p - value at 24 hours compared to its negative control. *: represent p – value at 72 hours compared to its negative control. #: represent p - value at 72 hours compared to 24 hours.

3.5.4 Necrosis in benign (RPWE 1) prostate cells.

Results below show the percentage of necrotic prostate cells after incubation with 1 000 $\mu\text{g/ml}$ GT, BT, UR, FR, EGCG or ASP for 24 and 72 hours, respectively. After incubating the RPWE 1 cells for 24 hours, the percentage of necrotic dead cells in the treated samples was compared to the negative control. And results of, two-way ANOVA statistical analysis followed by Sidak's multiple comparison test showed a no significant decrease in the percentage of necrotic cells in all the treated samples compared to the negative control. GT, BT, UR, FR, EGCG and ASP seemed to protect normal prostate cells against necrotic death. Furthermore, the positive control promoted significantly ($p < 0.001$) more necrosis compared to the negative control.

Further to this, after incubating the cells for 72 hours, two-way ANOVA statistical analysis followed by Sidak's multiple comparison test showed no significantly ($p > 0.05$) increased percentage of necrotic cells compared to the negative control. Similar to 24 hour incubation, the normal prostate cells appeared to cope well with GT, BT, UR, FR, EGCG and aspalathin. The positive control showed a significant ($p < 0.001$) increase in the percentage of necrotic deaths compared to negative control.

Two way ANOVA statistical analysis followed by Sidak's multiple comparison test showed that incubation of benign (RPWE 1) for 72 hours produced a significant ($p < 0.001$) increase in the percentage of necrotic cells in all the treatments compared to 24 hour incubation. Further to this, the positive control, 100 μM H_2O_2 produced a significant ($p < 0.001$) increase in the percentage of necrotic cells between 24 hour (28.1%) and 72 hour (50.8%) exposure times. Freitas et al. (2012) reported that 500 μM H_2O_2 inhibited cell proliferation in RPWE 1 and its effects were more pronounced following 72 hours of treatment. In another previous study, using endothelial cells (Lelli et al., 1998) observed necrotic cell death when 5 mM H_2O_2 was used (Fig 3.65).

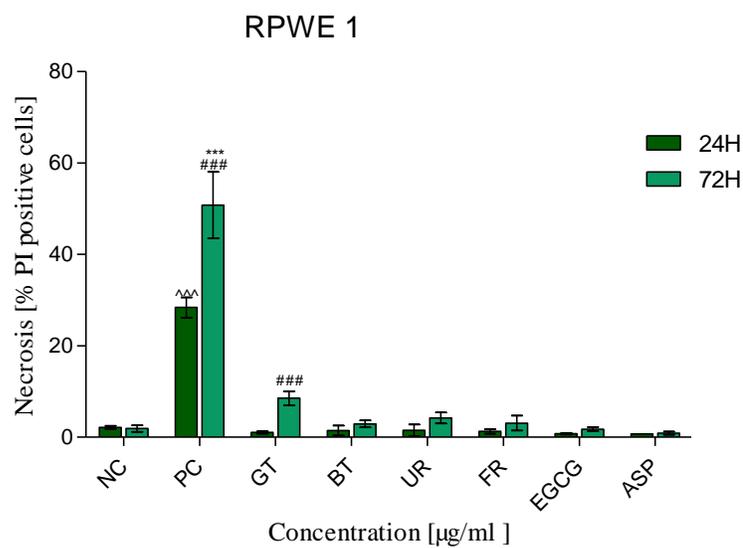


Fig 3- 65: Determination of necrotic effect of 1 000 µg/ml GT, BT, UR, FR, EGCG and ASP on benign (RPWE 1) prostate cancers following 24 and 72 hours incubation period respectively using flow cytometry.

Values represent mean \pm SD, n= 8. ASP; aspalathin; EGCG; epigallocatechin gallate, UR; unfermented rooibos, FR; fermented rooibos, BT; Black tea, GT; Green Tea. NC= negative control (medium); PC= positive control (100 µM H₂O₂). ### p < 0.001; ## p < 0.01, # < 0.05; ***p < 0.001; ** p < 0.01; * p < 0.05; ^^p < 0.001; ^^ p < 0.01; ^ p < 0.05; ^: represent p- value at 24 hours compared to its negative control. *: represent p – value at 72 hours compared to its negative control. #: represent p- value at 72 hours compared to 24 hours.

3.5.5 Comparison of apoptosis and necrosis in malignant LNCaP prostate cells.

The histograms below show the effect of exposing benign LNCaP prostate cells to 1 000 µg/ml BT, GT, FR, UR, EGCG or ASP for 72 hours. The *C. sinensis* and *A. linearis* and their main active ingredients induced apoptotic and necrotic deaths in malignant LNCaP prostate cells. In all the apoptosis graphs, the negative control showed very few (4.64%) apoptotic cells and very few (2.93%) necrotic cells. The positive control (green colour) showed more (80.66%) apoptotic cells and necrotic cells (89.04%). The histograms of all the treatment samples showed a generalised higher percentage of apoptotic cells and necrotic cells.

In this regard, exposure of LNCaP prostate cells to 1 000 µg/ml BT resulted in higher (61.09%) apoptotic and necrotic deaths (56.65%). In this regard, the histogram for black tea (purple colour) was symmetrical and bimodal with more apoptotic cells similar to the positive control (green colour). The graph for necrosis showed a symmetrical distribution of necrotic cells same as the positive control (green colour).

In a similar manner, green tea induced more apoptotic (63.16%) and necrotic deaths (54.23%). The treatment histogram for green tea (purple colour) was symmetrical and bimodal with more cells dead similar to the positive control (green colour). The histogram for necrosis showed a less spread and symmetrically distributed population of necrotic cells with some outliers.

Further to this, exposure of malignant LNCaP cells to fermented rooibos induced 63.63% of apoptotic deaths besides promoting necrotic deaths (31.72%). In respect of this, the histogram for fermented rooibos showed the number of apoptotic cells that were more spread and symmetrically distributed similar to the positive control. At the

same time, the histogram for necrosis showed a less spread and symmetrically distributed population of necrotic cells.

Exposure of the malignant LNCaP prostate cells to unfermented rooibos induced 70.63% of apoptotic deaths besides inducing necrotic deaths (48.21%). In respect of this, the histogram for unfermented rooibos showed the number of apoptotic cells that were asymmetrical and bimodal in their distribution. The positive control was more spread and symmetrically distributed. The histogram for necrosis showed a less spread and symmetrically distributed population of necrotic cells.

Further to this, aspalathin induced more apoptotic (66.58%) and necrotic deaths (27.08%). The aspalathin histogram (purple colour) showed a population of apoptotic cells that was less spread, symmetrical and bimodal with most of the cells dead similar to the positive control (green colour). The graph for necrosis showed a less spread and symmetrically distributed population of necrotic cells.

Finally, EGCG induced more apoptotic (69.62%) and necrotic deaths (17.28%). The EGCG histogram (purple colour) showed a population of apoptotic cells that was less spread, asymmetrical and polymodal in the distribution of apoptotic cells similar to the positive control (green colour). The graph for necrosis showed a less spread and symmetrically distributed population of necrotic cells (Fig 3.66).

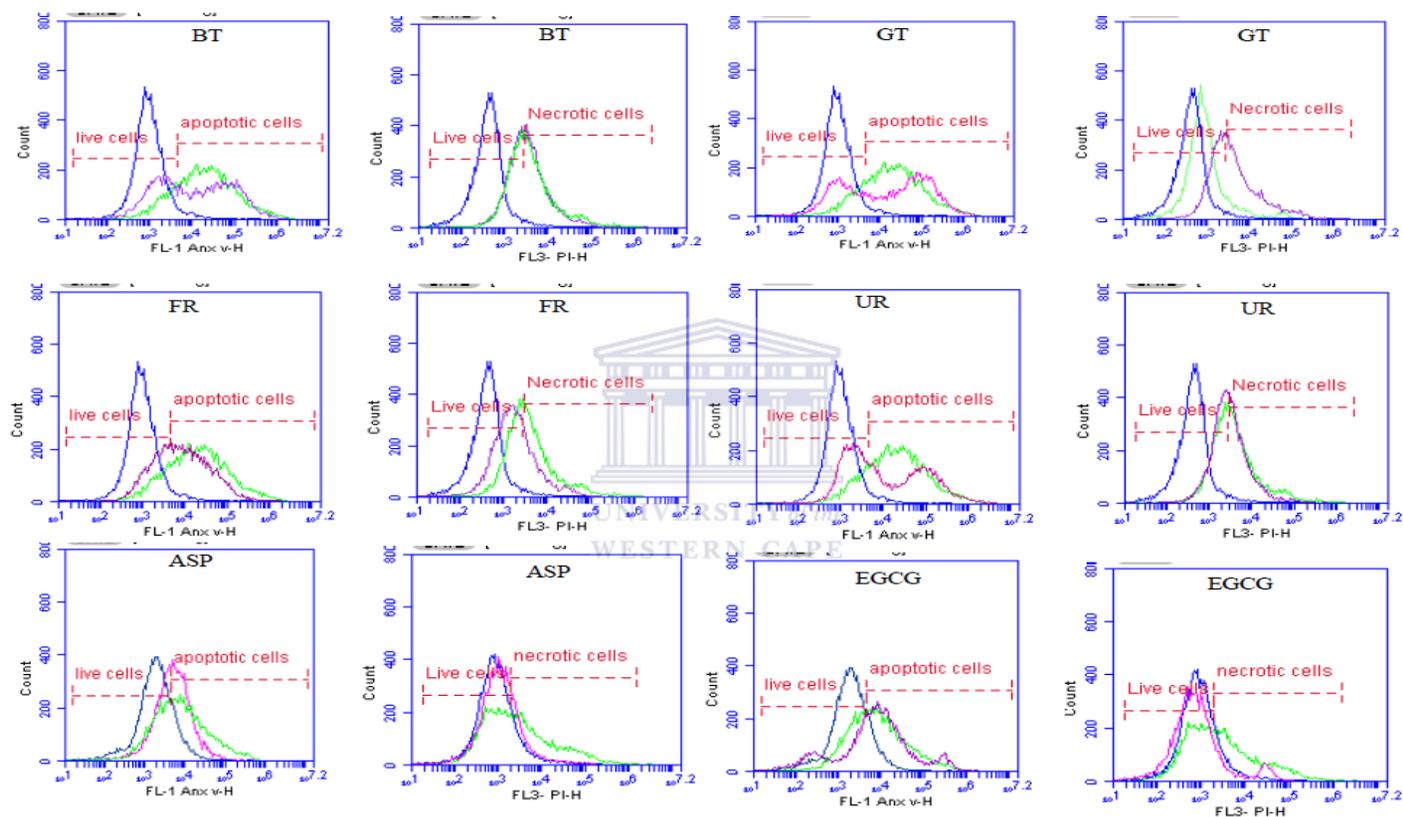


Fig 3.66: Histogram displaying the effect of incubating LNCaP cells with 1 000 $\mu\text{g/ml}$ *Camellia sinensis*, *Aspalathin linearis* or their major active ingredients for 72 hours. Blue graph represents untreated cells; green graph represents positive control (6% DMSO) and purple graph represents treatment with tea, rooibos or their active ingredients (1 000 $\mu\text{g/ml}$). BT, black tea; GT, green tea; FR, fermented rooibos; UR, unfermented rooibos; ASP, aspalathin; EGCG, epigallocatechin gallate.

3.5.6 Comparison of apoptosis and necrosis in benign RPWE 1 prostate cells.

The histograms below show the effect of exposing benign RPWE 1 prostate cells to 1 000 µg/ml BT, GT, FR, UR, EGCG or ASP for 72 hours. The *C. sinensis* and *A. linearis* and their main active ingredients induced non-significant percentages of apoptotic and necrotic deaths in the benign RPWE 1 prostate cells. In all the apoptosis micrographs, the negative control (blue colour) showed very few (3.45%) apoptotic cells and very few (1.79%) necrotic cells. The positive control (green colour) showed more apoptotic cells (90.09%) and necrotic cells (66.88%). As regards the treatment samples all histograms showed a generalised non-significant percentage of apoptotic cells and necrotic cells.

In this regard, exposure of benign RPWE 1 prostate cells to 1 000 µg/ml BT produced apoptotic (3.45%) and necrotic deaths (14.39%). The histogram for black tea (purple colour) was less spread, symmetrical and bimodal with non-significant percentage of apoptotic cells similar to the negative control (blue colour). The histogram for necrosis showed a symmetrical distribution of necrotic cells same as the negative control (blue colour).

Exposure of benign RPWE 1 prostate cells to 1 000 µg/ml GT produced apoptotic (3.38%) and necrotic deaths (27.57%). The histogram for green tea (purple colour) was less spread, symmetrical with non-significant percentage of apoptotic cells similar to the negative control (blue colour). The histogram for necrosis showed a right skewed distribution of necrotic cells same as the negative control (blue colour).

Further to this, exposure of benign RPWE 1 prostate cells to 1 000 µg/ml FR produced apoptotic (8.96%) and necrotic deaths (7.92%). The histogram for fermented rooibos (purple colour) was less spread, symmetrical with non-significant percentage of apoptotic cells similar to the negative control (blue colour). The

histogram for necrosis showed a symmetrical distribution of necrotic cells similar to the negative control (blue colour).

In addition to this, exposure of benign RPWE 1 prostate cells to 1 000 $\mu\text{g/ml}$ unfermented produced apoptotic (4.96%) and necrotic deaths (7.92%). The histogram for unfermented rooibos (purple colour) was less spread, symmetrical with non-significant percentage of apoptotic cells similar to the negative control (blue colour). The histogram for necrosis showed a symmetrical distribution of necrotic cells similar to the negative control (blue colour).

Further to this, exposure of benign RPWE 1 prostate cells to 1 000 $\mu\text{g/ml}$ aspalathin produced apoptotic (3.71%) and necrotic deaths (2.36%). The histogram for aspalathin (purple colour) was less spread, symmetrical with non-significant percentage of apoptotic cells similar to the negative control (blue colour). The graph for necrosis showed a symmetrical distribution of necrotic cells similar to the negative control (blue colour).

The non-significant percentages in apoptotic and necrotic deaths were also observed following exposure of the benign RPWE 1 prostate cells to EGCG. In respect of this, EGCG induced apoptotic (7.3%) and necrotic deaths (4.38%). The histogram for EGCG (purple colour) was less spread, symmetrical with non-significant percentage of apoptotic cells similar to the negative control (blue colour). The graph for necrosis showed a symmetrical distribution of necrotic cells and was similar to the negative control (blue colour) (Fig 3.67).

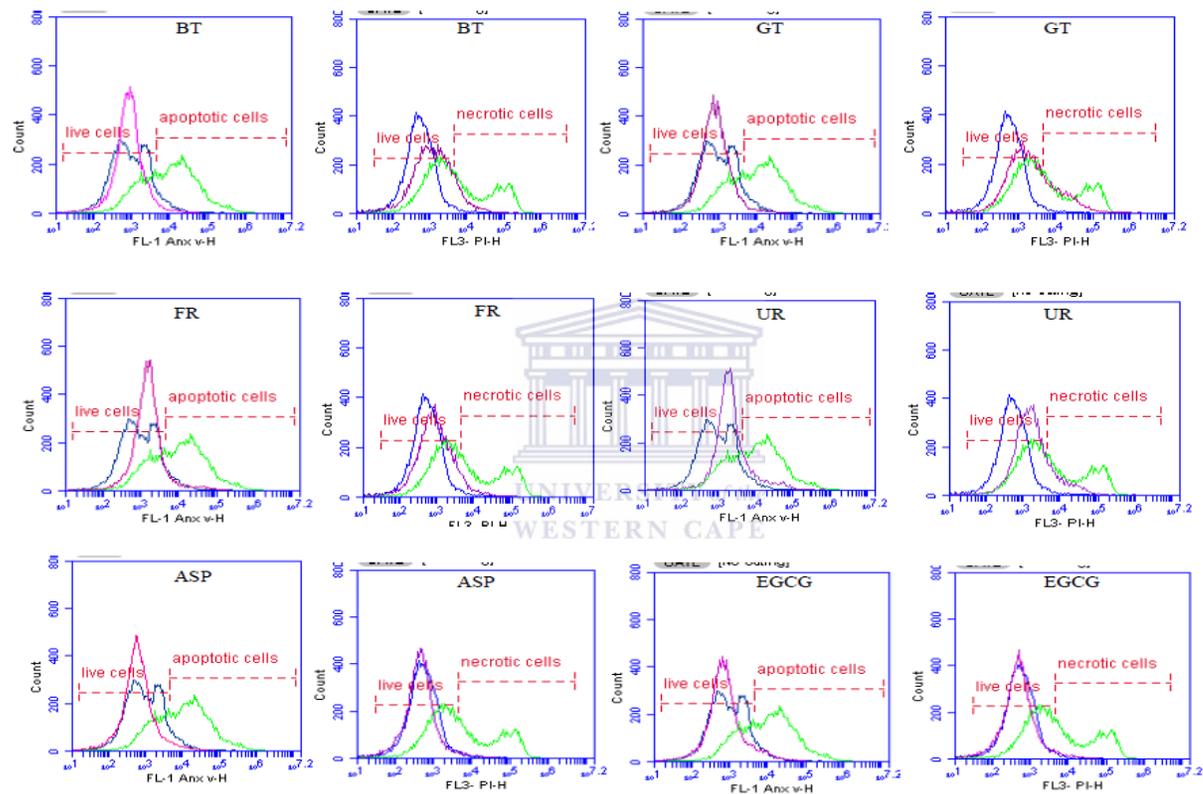


Fig 3.67: Histogram displaying the effect of incubating RPWE 1 cells with 1 000 μg/ml *Camellia sinensis*, *Aspalathin linearis* or their major active ingredients for 72 hours. Blue graph represents untreated cells, green graph represents positive control (100 μM H₂O₂) and purple graph represents treatment with tea, rooibos or their active ingredients (1 000 μg/ml). BT, black tea; GT, green tea; FR, fermented rooibos; UR, unfermented rooibos; ASP, aspalathin; EGCG, epigallocatechin gallate.

3.6 Effect of *C. sinensis*, *Aspalathin linearis*, EGCG and aspalathin on Total Serum PSA in prostate cells.

Prostate specific antigen (PSA) may be used to screen for prostate cancer. Malignant (LNCaP) and benign (RPWE 1) prostate cells were exposed to GT, BT, FR, UR, EGCG and aspalathin for 72 hours. In this study, we were expected to determine PSA levels following acute and chronic exposure of the prostate cell lines to BT, GT, FR, UR, ASP and EGCG. However, because of financial constraints we only succeeded in determining the PSA levels at 72 hours only.

In this regard, Total serum PSA ELISA kit was used to determine the concentration of prostate specific antigen. In brief, the teas and rooibos used in this study plus their respective active compounds produced a concentration-dependent decrease in total serum PSA concentration in malignant (LNCaP) prostate cells whereas in benign (RPWE 1) prostate cells the tea and tisane did not produce any significant observable change in the level of total serum PSA.

3.6.1 Effect of black tea on total PSA in malignant (LNCaP) and benign (RPWE 1) prostate cells.

One way ANOVA statistical analysis showed that exposure of malignant (LNCaP) prostate cells to black tea produced a concentration - dependent decrease in total serum PSA level compared to the negative control which became significant from 250 µg/ml onwards. BT concentration of 5 000 µg/ml onwards caused an additional sharp drop in PSA concentration. On the other hand, the benign (RPWE 1) prostate cells showed only non-significant ($p > 0.05$) changes in the level of total serum PSA in all the treated samples (250-50 000 µg/ml BT) when compared to the negative control. Further to this, the PSA concentration in RPWE 1 was much lower than in LNCaP cells (Fig 3.68).

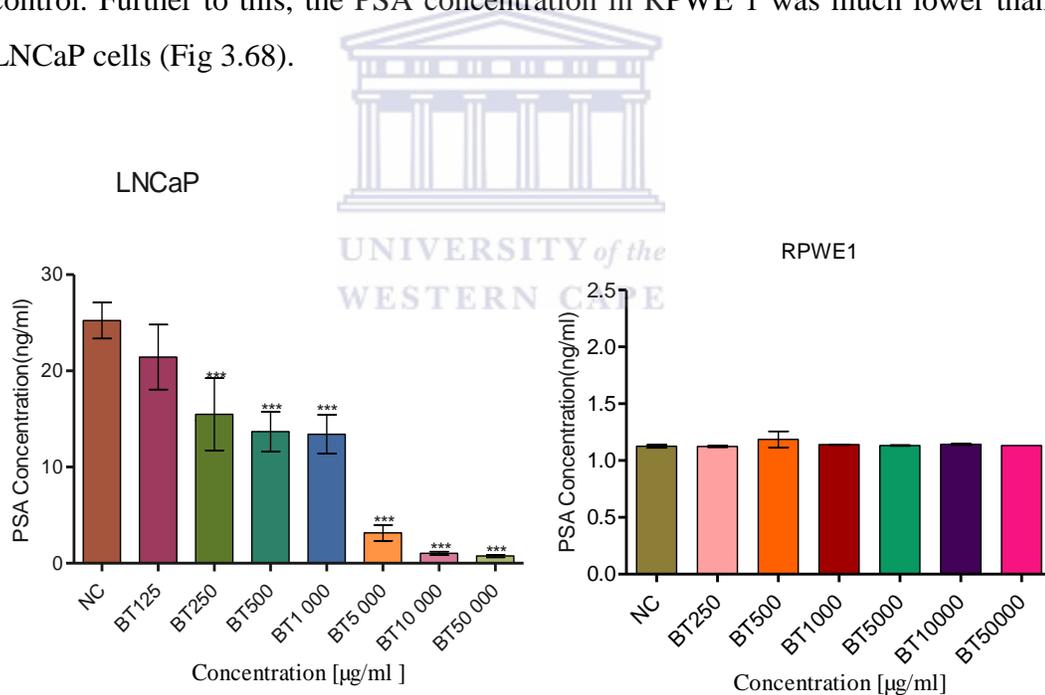


Fig 3- 68: The effect of black tea on total serum PSA in malignant (LNCaP) and benign (RPWE 1) prostate cells after 72 hour incubation period.

Values represented are the mean \pm SD of 7 samples. BT; black tea. NC = Negative Control. *** $p < 0.001$; ** < 0.01 ; * $p < 0.05$.

3.6.2 Effect of green tea on total PSA in malignant (LNCaP) and benign (RPWE 1) prostate cells.

One way ANOVA statistical analysis showed that exposure of malignant (LNCaP) prostate cells to green tea (125, 250 and 500 µg/ml) produced a concentration - dependent decrease in total serum PSA level compared to the negative control. This was followed by a drastic drop in total PSA following exposure of LNCaP cells to a GT concentration range of 1 000 – 50 000 µg/ml. In addition to this, the benign (RPWE 1) prostate cells showed no significant ($p > 0.05$) increase in the level of total serum PSA at (1 000 µg/ml) GT when compared to the negative control (Fig 3.69).

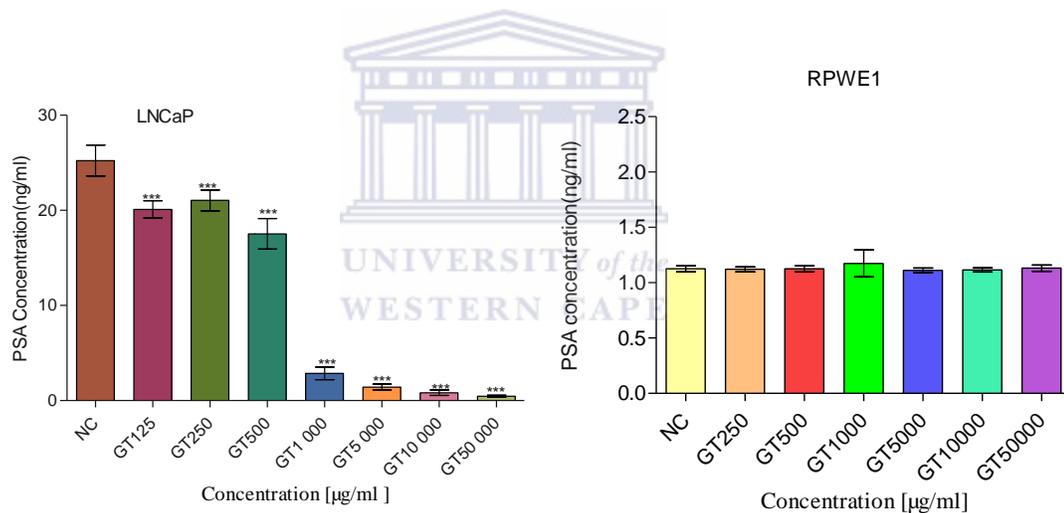


Fig 3- 69: The effect of green tea on total serum PSA in malignant (LNCaP) and benign (RPWE 1) prostate cells after 72 hour incubation period.

Values represented are the mean \pm SD of 7 samples. GT; green tea. NC = Negative Control. *** $p < 0.001$; ** < 0.01 ; * $p < 0.05$.

3.6.3 Effect of fermented rooibos on total PSA in malignant (LNCaP) and benign (RPWE 1) prostate cells.

One way ANOVA statistical analysis showed that incubation of LNCaP cells with 250 µg/ml FR displayed no alteration in the level of total PSA when compared to the negative control. However, a notable finding was the major and significant ($p < 0.001$) drop in total PSA of malignant (LNCaP) prostate cells after exposing them to higher concentrations of FR (500, 1 000, 5 000, 10 000 and 50 000 µg/ml). The benign (RPWE 1) prostate cells showed only non-significant ($p > 0.05$) increase in the level of total PSA following exposure to 5 000 µg/ml FR when compared to the negative control. There was no change in the level of total PSA in samples treated with 250, 500, 1 000, 10 000 and 50 000 µg/ml FR respectively. Although the level of total serum PSA formed a plateau in RPWE 1, its concentration was much lower than in LNCaP cells (Fig 3.70).

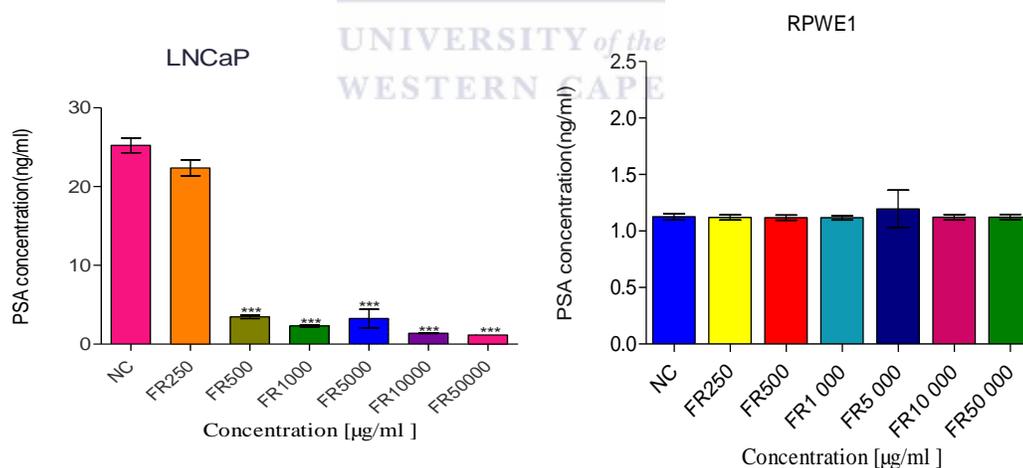


Figure 3- 70: The effect of fermented rooibos on total serum PSA in malignant (LNCaP) and benign (RPWE1) prostate cells after 72 hour incubation period.

Values represented are the mean \pm SD of 7 samples. FR; fermented rooibos. NC = Negative Control. *** $p < 0.001$; ** < 0.01 ; * $p < 0.05$.

3.6.4 Effect of unfermented rooibos on total PSA in malignant (LNCaP) and benign (RPWE 1) prostate cells.

One way ANOVA statistical analysis, showed that incubation of LNCaP cells with unfermented rooibos in concentrations from 125 to 1 000 $\mu\text{g/ml}$ caused a significant decrease in PSA levels of more than 50%. Higher concentration (500 – 50 000 $\mu\text{g/ml}$) UR caused a further significant drop.

The benign (RPWE 1) prostate cells showed only non-significant ($p > 0.05$) increase in the level of total PSA after being treated with (5 000 $\mu\text{g/ml}$) UR when compared to the negative control. Although the level of total serum PSA formed a plateau in RPWE 1; its concentration was much lower than in LNCaP cells (Fig 3.71)

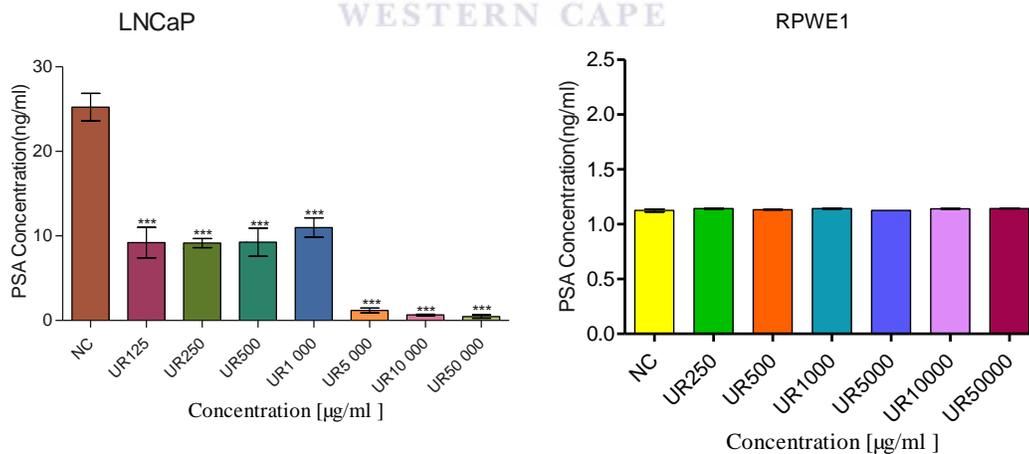


Fig 3- 71: The effect of unfermented rooibos on total serum PSA in malignant (LNCaP) and benign (RPWE 1) prostate cells after 72 hour incubation period. Values represented are the mean \pm SD of 7 samples. UR; unfermented rooibos. NC = Negative Control. *** $p < 0.001$; ** < 0.01 ; * $p < 0.05$.

3.6.5 Effect of aspalathin on total PSA in malignant (LNCaP) and benign (RPWE 1) prostate cells.

The results below show a concentration-dependent decrease in the level of total prostate specific antigen (PSA) produced by malignant (LNCaP) prostate cells following their exposure to aspalathin for 72 hours. One way ANOVA statistical analysis showed significantly ($p < 0.05$) decreased total serum PSA levels following malignant (LNCaP) prostate cells incubation with 250, 500 and 1 000 $\mu\text{g/ml}$ ASP, respectively, when compared to the negative control. There were further significant ($p < 0.01$ and $p < 0.001$) decreases in total serum PSA after exposing the cells to 5 000 and 10 000 $\mu\text{g/ml}$ ASP, respectively, when compared to the negative control. Incubation of benign (RPWE 1) prostate cells with 250 and 10 000 $\mu\text{g/ml}$ ASP produced no change in the concentration of PSA. However, 500, 1 000 and 5 000 $\mu\text{g/ml}$ ASP showed a non-significant ($p > 0.05$) increase in the level of total serum PSA in the three treated samples, respectively, when compared to the negative control. In summary, PSA levels in RPWE 1 were lower than in LNCaP cells (Fig 3.72).

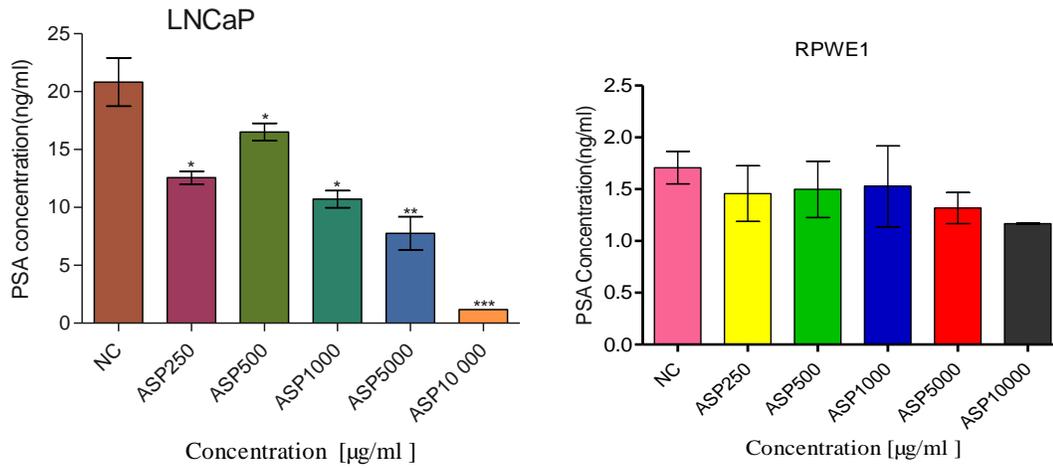
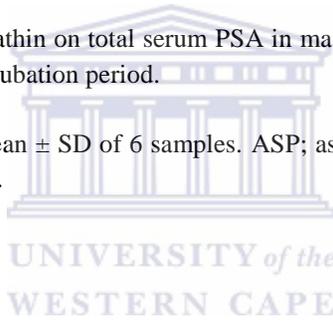


Fig 3- 72: The effect of aspalathin on total serum PSA in malignant (LNCaP) and benign (RPWE 1) prostate cells after 72 hour incubation period.

Values represented are the mean \pm SD of 6 samples. ASP; aspalathin. NC = Negative Control. *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$.



3.6.6 Effect of EGCG on total PSA in malignant (LNCaP) and benign (RPWE 1) prostate cells.

One way ANOVA statistical analysis showed that exposure of malignant (LNCaP) prostate cells to 250, 500, 1 000 µg/ml EGCG produced non-significant ($p > 0.05$) changes in the level of total PSA, respectively, when compared to the negative control. Higher concentration caused a significant and sharp drop in PSA level. Incubation of benign (RPWE 1) prostate cells with the whole concentration range of 250, 500, 1 000 and 10 000 µg/ml EGCG produced a non-significant ($p > 0.05$) decrease in PSA concentration compared to the negative control. However, the total serum PSA concentration in benign RPWE 1 was much lower than in LNCaP prostate cells (Fig 3.73).

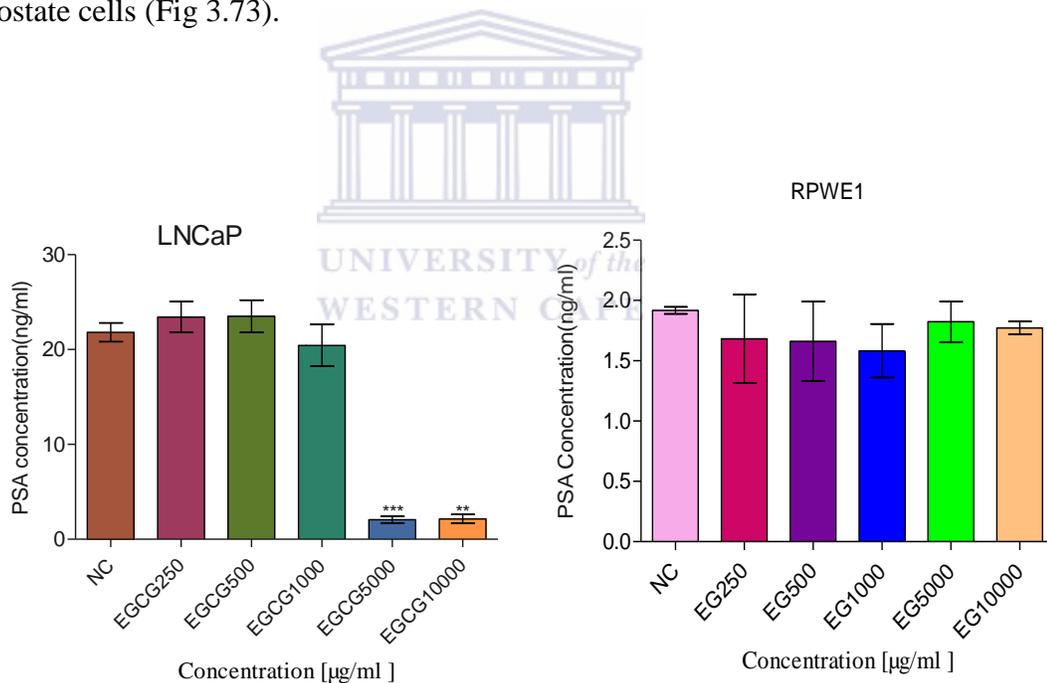


Fig 3- 73: The effect of EGCG on total serum PSA in malignant (LNCaP) and benign (RPWE 1) prostate cells after 72 hour incubation period.

Values represented are the mean \pm SD of 6 samples. EGCG; epigallocatechin gallate. NC = Negative Control. *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$.

CHAPTER FOUR

4. DISCUSSION

4.1 Cell viability and MTT assay.

In recent years, several reports have documented that polyphenolics, which include curcumin, resveratrol, and gallo-tannins such as tannic acid, gallic acid, epigallocatechin, epicatechin-3-gallate, and epigallocatechin-3-gallate, induce apoptosis in various cancer cell lines (Hadi et al., 2000a). Gallo-tannins are the constituents of green tea, the consumption of which is considered to reduce the risk of various cancers such as those of bladder, prostate, oesophagus, and stomach (Ahmad et al., 1997).

In brief, green tea polyphenols (GTPs) found in the tea plant (*Camellia sinensis*), have been observed to induce apoptosis in many types of tumor cells and have been proposed as chemo-preventive or therapeutic agents (Yamamoto et al., 2003). Several lines of evidence in the literature strongly suggest that it is the pro-oxidant action of plant-derived polyphenolics rather than their antioxidant activity that may be an important mechanism for their anticancer and apoptosis-inducing properties. It is under this background that the present study assessed the cytotoxic effects of black and green tea, fermented and unfermented rooibos and their respective compounds aspalathin and EGCG on malignant (LNCaP) and benign (RPWE 1) prostate cells.

In respect of this, MTT assay was done in order to assess the cytotoxic effects of black and green tea, fermented and unfermented rooibos, epigallocatechin gallate (EGCG) and aspalathin on malignant (LNCaP) and benign (RPWE 1) prostate cells. The results obtained after incubating benign (RPWE 1) prostate cells for 24 hours with tea and rooibos showed no significant changes in mitochondrial dehydrogenase activity, for concentrations 125 – 1 000 µg/ml EGCG which is an indicator of

unchanged cell viability (Fig 3.30). No significant change was observed in all samples treated with EGCG, aspalathin and FR for 24 hours. However, significant increases in mitochondrial dehydrogenase activity were observed in benign (RPWE 1) prostate cells that were treated with higher concentrations (5000, 10000 and 50 000 µg/ml) of BT, GT, FR and UR respectively, compared to the control (Fig 3.25, 3.26, 3.27 and 3.28).

Whilst there were no significant observable changes in mitochondrial dehydrogenase activity of benign (RPWE 1) prostate cells treated with lower concentrations (between 125-1000 µg/ml) of tea and rooibos, malignant (LNCaP) prostate cells produced a generalised increase in mitochondrial dehydrogenase activity compared to the negative control. After treatment with higher concentrations (5 000 and 50 000 µg/ml) of teas and rooibos, malignant (LNCaP) prostate cells registered a significant decrease in mitochondrial dehydrogenase activity (Fig 3.19-3.24). In brief, these quantitative results showed that 24 hour incubation of prostate cells with tea and rooibos specifically the higher concentrations induced more stress in malignant (LNCaP) prostate cells than in benign (RPWE 1) prostate cells.

Morphology studies of both benign (RPWE 1) and malignant prostate cells show normal adherent and dividing cells with flat polygonal shapes. The cell numbers on the micrographs of both cell lines were comparable to those of their respective negative control, meaning that the tea and rooibos did not affect their viability. However, at 50 000 µg/ml malignant (LNCaP) prostate cells appeared shrunk, irregular or round in shape and the cell numbers were reduced due to detachment and death whilst benign (RPWE 1) prostate cells did not show any change in morphology. This is in line with the MTT assay; depicting that 50 000 µg/ml tea and rooibos extracts with 24 hour incubation are cytotoxic to the malignant (LNCaP) prostate cells but to a much lesser extent to benign (RPWE 1) prostate cells (Figs 3.1 – 3.6).

Incubation of RPWE 1 cells for 72 hours produced a generalised concentration-dependent increase in mitochondrial dehydrogenase activity for all the treatments relative to the negative control (Figs 3.25 – 3.30). Furthermore, micrographs of RPWE 1 cells exposed to the highest concentrations (5 000 , 10 000 and 50 000 µg/ml) of black and green tea, fermented and unfermented rooibos did not show a significant decrease in cell numbers despite observable changes in morphology like loss of regular shape and stress (Figs 3.13 – 3.18). In other words, the teas and rooibos at 5 000, 10 000 and 50 000 µg/ml and 72 hours incubation were not yet cytotoxic to the normal prostate cells but produced considerable cellular stress at the mitochondrial level.

During the same 72 hour incubation period, malignant (LNCaP) prostate cells at low concentrations (250 – 1000 µg/ml) underwent a rapid increase in mitochondrial dehydrogenase activity (indicative of cellular stress) and this was followed by a rapid decrease in mitochondrial dehydrogenase activity after exposure to higher concentrations (5 000 – 50 000 µg/ml) to give a biphasic graph (Figs 3.19 – 3.24). Further to this, the cells looked highly stressed, round in shape and clumped together and there were very few cell numbers on the micrographs compared to the control (Figs 3.7– 3.12). In brief, both quantitative (graphs) and qualitative (micrographs) results show that the tea and rooibos are more cytotoxic to malignant (LNCaP) prostate cells than the normal (RPWE 1) prostate cells.

Reports from Hegner et al., (1978), mention that oxidative stress is responsible for the general increase in mitochondrial dehydrogenase activity and in itself is a product of an inefficient reduction of oxygen which generates superoxide (Berridge and Tan, 1993). In this case, live malignant (LNCaP) and benign (RPWE 1) prostate cells have active mitochondria which generates NADH; a reducing agent that transfers electrons to MTT (Berridge and Tan, 1993). In support of this, previous studies have shown that cellular reduction of MTT is more related to NADH production through glycolysis than to respiration (Berridge and Tan, 1993). In this case, the absorbance

readings represent increased mitochondrial activity coming from cells that are alive whilst the decrease in mitochondrial dehydrogenase activity points towards loss of cell viability.

From the MTT assay and morphological micrographs, malignant (LNCaP) prostate cells appear to be more stressed than normal (RPWE 1) prostate cells. Tumor cells have been observed to contain diminished amounts of manganese superoxide dismutase and lower copper-zinc superoxide dismutase activity. The lower activity of these two enzymes is accompanied by increased levels of superoxide radicals (Ashkenazi et al., 1999; Oberley and Buettner, 1979). The diminished amounts of manganese superoxide dismutase make it impossible for cancer cells to remove the superoxide radicals from the cytoplasm. On the contrary, normal (RPWE 1) prostate cells have an efficient antioxidant system and are able to scavenge much of the excess free radicals.

The superoxide (O_2^-) radicals formed can follow several pathways, some of which result in the formation of hydroxyl ion (OH^-) and singlet oxygen (1O_2). In addition to this, SOD can dismutate to form H_2O_2 plus ground-state oxygen (Oberley and Buettner, 1979). Thirdly, superoxide radicals can donate electrons to metals and change their oxidation state. For instance, O_2^- can react with Fe_3^+ to produce Fe_2^+ . The Fe_2^+ generated from O_2^- can catalyze a Fenton type production of hydroxyl radicals (OH^-) from H_2O_2 (Fig 4.1). Further to this, the reaction of H_2O_2 with Fe_2^+ in the presence of O_2^- can also produce 1O_2 . Both OH^- and 1O_2 have been shown to cause lipid peroxidation and DNA fragmentation (Oberley and Buettner, 1979). In the present thesis, BT, GT, FR, UR, aspalathin and EGCG seem to be responsible for the induction of oxidative stress, change in morphology and apoptotic death of the malignant (LNCaP) prostate cells.

In this case, our tea and rooibos extracts are oxidatively stressing the malignant (LNCaP) prostate cells more than the normal (RPWE 1) prostate cells through the

production of high levels of superoxide radicals and hydrogen peroxide besides the basal physiological levels of ROS already available in the malignant cancer cells. Since tumor cells apparently have lower levels of catalase, MnSOD and Cu-Zn SOD, the basal physiological levels of ROS are relatively higher than in normal cells. Although both cell lines were treated with the same concentrations of tea or rooibos, the ROS levels in malignant (LNCaP) prostate cells were quickly up-regulated to cytotoxic physiological levels, whereas in the normal prostate cells they remained lower. Hence the increased mitochondrial dehydrogenase activity and more apoptotic cell deaths in malignant (LNCaP) prostate cells than normal (RPWE 1) prostate cells. The different extent of cell viability between the normal and malignant prostate cells following treatment with BT, GT, FR, UR, aspalathin and EGCG has been justified by some previous studies. For example, Elbling et al., (2005) or Nakazato et al., (2005) reported that inhibition of cancer cell viability and induction of apoptosis by green tea polyphenols in vitro appear to be, in part, due to the generation of hydrogen peroxide and superoxide anion.

Further to this, ECG, EGCG, and EGC have been shown to inhibit the viability of prostate cancer cells by inducing apoptosis, an effect attributed to ROS (H₂O₂ and superoxide anion) formation. The above observation has been further corroborated by a study in which EGCG was observed to strongly inhibit various cancer cell lines more than other green tea polyphenols and appeared to have no effect on normal cells. This was possible because of greater oxidative stress in cancer cells than in normal cells and lower catalase levels in cancer cells than in normal cells (Forester and Lambert, 2011).

According to Shankar et al. (2007), the ability of BT and GT to inhibit tumor cell proliferation, was demonstrated as a decrease in malignant (LNCaP) prostate cell proliferation after culturing them in media containing patient serum collected after BT and GT consumption. From the study, it was concluded that tea polyphenols and theaflavins are bio-available in the prostate where they may be involved in the

prevention of prostate cancer. Green tea and its major constituent EGCG inhibit the growth of a variety of human prostate cancer-cell lines.

In line with this, treatment of prostate cancer cells with EGCG has been reported to result in a concentration-dependent inhibition of cell growth through cell cycle arrest and induction of apoptosis (Shankar et al., 2007). In support of this is a preclinical study which demonstrated the accumulation of tea polyphenols (PP) and theaflavins in the small and large intestine, liver, and prostate of C57BL/6 mice which had received a decaffeinated black tea diet (Henning et al., 2006).

In addition to this is a human clinical trial, which found that tea plant polyphenols were greater in prostate samples from men consuming black (BT) and green tea (GT) than in men consuming a caffeine-matched soda control (Henning et al., 2006). From Figs 3.19 – 3.24 it is clear that the lower concentrations of our treatments had either no influence or caused a rise in the mitochondrial dehydrogenase activity (especially after 72 hours of exposure). Further to this, the higher concentrations of teas increased mitochondrial dehydrogenase activity in benign (RPWE 1) cells. We conclude that tea and rooibos are cytotoxic to the malignant (LNCaP) prostate cells, but promote cell “viability” of normal prostate cells. Further to this, to best of our knowledge, this thesis reports for the first time the lack of cytotoxic effects of FR, UR and aspalathin on benign (RPWE 1) prostate cells (Figs 3.27, 3.28 and 3.29). In addition to this, UR and aspalathin have demonstrated enhanced cytotoxic effects on malignant (LNCaP) prostate cells (Fig 3.22 and 3.23).

The cytotoxic effects of the unfermented rooibos and its main active compound, aspalathin seem to match the already reported cytotoxic effects of GT and EGCG, on LNCaP cells, respectively (Fig 3.20 and 3.24). Further to this, we report that in this thesis, FR demonstrated the least cytotoxic effects on malignant (LNCaP) prostate cells and did not show any cytotoxic effects on normal prostate cells (Figs 3.21 and 3.27). Whilst mitochondrial dehydrogenases activity was used in this thesis to test the

prostate cells' viability in the presence of the teas and rooibos, other possible pathways might have been involved in the inhibition or stimulation of cell proliferation.

4.1.1 Other possible mechanisms that may influence cell viability of prostate cells.

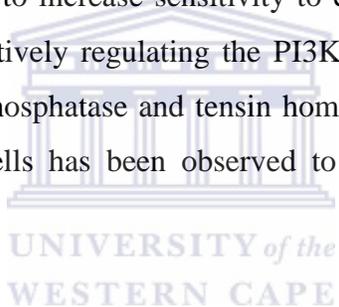
Cell cycle dysregulation leads to cell cycle progression and allows cancer cells to continue to proliferate indefinitely; therefore, the inhibition of cell proliferation is a major mechanism that works in concert with cell cycle arrest to inhibit malignant growth. In addition to this, green tea, rich in epigallocatechin gallate (EGCG), has demonstrated a significant reduction of several factors that promote cancer cell proliferation by inhibiting DNA synthesis, de-differentiation and angiogenesis (Thomas et al., 2015).

Prostate cell growth involves the conversion of testosterone (T) to the more active dihydrotestosterone (DHT) by the enzyme 5α -reductase. The binding of DHT to AR in the presence of 5α -reductase results in prostate cancer development and its progression (Lee et al., 1995). In vitro studies using rats have shown that EGCG and ECG are potent inhibitors of 5α -reductase and their effect were confirmed following reduction in the proliferation of rat prostates (Liao and Hiipakka, 1995). Similarly, EGCG treatment of androgen-sensitive prostate cancer cells switched DHT from a growth promoter to a growth inhibitor and sensitized cancer cells to apoptosis and green tea infusions reduced DHT levels in severe combined immunodeficiency mice (SCID) injected with LNCaP xenografts (Connors et al., 2012).

Previous investigations have reported that green tea EGCG and black tea theaflavins (TF3 and TF2B) inhibit the production of 5α -reductase and at the same time alter the binding capacity of the androgen receptor (AR) to dehydrotestosterone (DHT) with the net effect of modulating the androgen-responsive genes that are responsible for

prostate growth and PSA production (Lee et al., 1995). In addition to this, EGCG has also been reported to down-regulate the expression of the AR (Ren et al., 2000).

Other studies have reported of the ability of black tea theaflavins (TF3 and TF2B) to inhibit testosterone-induced cell growth, PSA secretion and FAS protein expression in LNCaP cells (Lee et al., 1995). In addition to this, GT has been shown to block ornithine decarboxylase, an enzyme which signals cells to proliferate faster and bypass apoptosis (Iqbal et al., 2003). Further to this, scientific reports describe PTEN (phosphatase and tensin homolog) as a tumor suppressor gene identified on human chromosome 10q23 (Shankar et al., 2007). This gene is frequently deleted or mutated in a wide range of human cancers including that of prostate (Cairns et al., 1997). PTEN has been observed to increase sensitivity to cell death in response to several apoptotic stimuli by negatively regulating the PI3K/Akt pathway (Stambolic et al., 1998). Because of this, phosphatase and tensin homolog (PTEN) over-expression in human prostate cancer cells has been observed to induce apoptosis (Chen et al., 2001).



In the present thesis, to the best of our knowledge, we report for the first time that FR, UR and aspalathin managed to inhibit cancer cell viability and induce apoptosis in prostate cancer cells. The already investigated mechanisms used by GT, BT and EGCG are being suggested as the possible mechanism that might have been used by UR, FR and aspalathin and in this case, rooibos might have potentiated the sensitivity of the PTEN gene to apoptotic death besides blocking the ornithine decarboxylase enzyme.

Investigations have reported a marked decrease in the prevalence and extent of high-grade intra-epithelial neoplasia in prostates after androgen-deprivation therapy compared with untreated prostates. This decrease was accompanied by epithelial hyperplasia, cytoplasmic clearing, and prominent acinar atrophy, with a decreased ratio of acini to stroma (Ferguson et al., 1994). One way by which prostate cancer can

be treated is by inhibiting urokinase (uPA). Urokinase plasminogen activator is a proteolytic enzyme that is commonly over expressed in different human cancers. It is responsible for proteolytic degradation of the extracellular matrix. Because of this, uPA mediates tumor cell invasion into adjacent tissues which leads to metastasis (Conese et al., 1995). Reduction of this enzyme decreases tumour size and reverses cancer. In line with this observation, EGCG in green tea extracts has been reported to inhibit the enzyme urokinase (u-plasminogen activator) (Jankun et al., 1997b). Reports indicate that EGCG achieves this by binding to urokinase and in the process blocks phosphorylation of His 57 and Ser 195 of the urokinase catalytic triad thereby extending it toward Arg 35 and in the process creating a positively charged loop of urokinase. This posture and localization of EGCG interferes with the ability of uPA to recognize its substrates and thus inhibits the enzyme activity (Mukhtar et al., 2000).

Insulin-like growth factor-I (IGF-I) is responsible for the promotion of cell proliferation and has been implicated as a risk factor for the development of prostate cancer. However, previous *in vitro* studies have shown inhibited cell viability following treatment of prostate cancer cells with black tea polyphenols. In brief, BT polyphenols achieved this by inhibiting the IGF-I signal transduction pathway, through reduced autophosphorylation of the IGF-I receptor-1. Further to this, epidemiological studies have reported reduced incidence of prostate cancer in populations with high intake of black tea (Klein et al., 2002). In the present thesis, BT has been observed to induce increased viability then death. Only after 72 hours BT significantly stressed the cells and this induced cytotoxic effects that resulted in the inhibition of cell viability of the malignant (LNCaP) prostate cancer cells.

4.2 Reactive Oxygen Species (ROS).

Reactive Oxygen species (ROS) have been defined as products of normal cellular metabolism and are important in stimulation of signaling pathways in response to

changing intra- and extracellular environmental conditions (Klebanoff, 1980; Thannickal and Fanburg, 2000). ROS can be in the form of : a) oxygen radicals such as superoxide ($O_2^{\cdot-}$), hydroxyl ($\cdot OH$), peroxy (RO_2^{\cdot}), and alkoxy ($RO\cdot$) and b) non-radicals that can be reduced or get converted into radicals, such as hypochlorous acid (HOCl), ozone (O_3), singlet oxygen (1O_2), and hydrogen peroxide (H_2O_2). Mitochondria are considered as the most important cellular source of ROS and may be susceptible to oxidative damage. ROS can modify cellular protein, lipid, and DNA, which results in altered functions of the cell (Klaunig et al., 1998). It has been reported that ROS, when present at high levels, may play a key role in the mechanisms of initiation and progression of diseases such as carcinogenesis and diseases associated with aging (Venkataraman et al., 2005). However, when present at normal levels, ROS have important physiological functions, such as regulation of signal transduction pathways, mitosis, cell differentiation and activation of gene transcription factors (Venkataraman et al., 2005).

Figs 3.7 – 3.12 showed malignant (LNCaP) prostate cells that are stressed as characterized by significant losses in cell volume, shape and adhesion to the surface at concentrations as low as 500 $\mu g/ml$ of tea and rooibos. At higher concentrations (5 000-50 000 $\mu g/ml$) of tea or rooibos, the malignant (LNCaP) prostate cells appeared swollen, with membrane surfaces broken, cytosolic components were outside probably a sign of a dead cell (Figs 3.7 – 3.12). Morphologically, RPWE 1 micrographs (Fig 3.13 – 3.18) showed normal dividing cells that were not under stress due to ROS. In the present thesis, high ROS levels were cytotoxic to malignant (LNCaP) prostate cells whereas low ROS levels did not produce any observable change in cell numbers of both LNCaP and RPWE 1 prostate cells. Micrographs of both cell lines treated with low concentrations (125-500 $\mu g/ml$) of tea and rooibos on face value, showed cell numbers that are comparable to those of their respective negative controls.

From a previous study, (Klaunig et al., 2010) observed that high levels of ROS resulted in the induction of apoptosis or necrosis and exposure to low or transient ROS promoted cell proliferation. This observation is also corroborated by (Fiorani et al., (1995) and Frenkel, (1992) who observed that treatment of LNCaP cells with low or transient exposure to ROS results in oxidative stress which promoted an increase in cell proliferation whereas high ROS levels induced apoptosis or necrosis. In brief, they found that low concentration of tea polyphenols produced ROS which activated the stress gene NF- κ B leading to phosphorylation of I κ B in the presence of I κ B kinase. This resulted in the degradation of I κ B and release of NF- κ B. The free NF- κ B then translocated to the nucleus where it trans-activated oncogenes such as c-myc, c fos and c jun (Khan et al., 2006). This promoted increased cell proliferation through altered expression of growth factors and proto-oncogenes (Khan et al., 2006; Frenkel, 1992; Fiorani et al., 1995).

Reports have indicated that cellular levels of ROS influence the selective activation of transcription factors besides promoting differential induction of cell death or survival between malignant (LNCaP) and benign (RPWE 1) prostate cells. This report is in line with the present study to the extent that low ROS levels have been observed to promote cell survival of both malignant (LNCaP) and normal (RPWE 1) prostate cells; however, high levels of ROS induced increased apoptotic death in malignant (LNCaP) prostate cells compared to benign (RPWE 1) prostate cells. Also corroborating this observation is work from a previous investigation which found that the effect of ROS on cell growth depend on the concentration and cell type and could promote normal cells to proliferate but kill tumor cells (Laurent et al., 2005). Basically, low catalase activity and high ROS levels in the malignant (LNCaP) prostate cells induce cell deaths, whereas high catalase activity and low ROS levels in benign (RPWE 1) prostate cells promote the survival of RPWE 1 cells (Yamamoto et al., 2003).

In normal cells, up-regulated ROS levels could activate the JNK pathway resulting in apoptosis, whereas low concentrations or transient high levels of ROS promote cell proliferation of normal cells through the activation of the ERK pathway (Nicco et al., 2005). In cancer cells, increased levels of ROS cause tumor cell growth arrest and apoptotic death because of their high basal level of ROS. In normal cells, the basal level of H_2O_2 is low and an initial increase is first associated with cell growth. In other words, tumor cells have high levels of H_2O_2 which is associated with a rapid cell growth. Any further increase in intracellular H_2O_2 inhibits tumor cell proliferation; whereas any decrease in H_2O_2 concentration stimulates tumor cell growth (Nicco et al., 2005). In tumor cells, ROS potentiates the mitogen activated protein kinase (p38 MAPK) and induces its hypersensitivity to stress signals. Thus, the fate of tumor cells is tightly correlated with the duration of ROS stimulation and depends on the basal redox status of the cell (Nicco et al., 2005).

The above information seem to suggest that BT, GT, FR, UR, EGCG and aspalathin created differential oxidative environments between benign (RPWE 1) and malignant (LNCaP) prostate cells through exploitation of a compromised redox homeostasis in the tumor cells. Previous studies reported that most of the plant polyphenols possess both antioxidant as well as pro-oxidant properties (Azmi et al., 2006). Further to this, pro-oxidant action of these polyphenols are responsible for the mobilization of endogenous copper ions and the consequent pro-oxidant action leads to the generation of super oxide anion and hydrogen peroxide which are further converted into hydroxyl radicals (Azmi et al., 2006). Low levels of superoxide dismutase and catalase in malignant LNCaP cells results in diminished conversion of superoxide anion and H_2O_2 to H_2O and O_2 and hence the increased levels of ROS in cancer cells. These high ROS levels are responsible for the cytotoxic actions of the polyphenols on cancer cells (Hadi et al., 2000a). The above observations are very much in agreement with the qualitative data obtained in this study. Micrographs of malignant (LNCaP) prostate cells when compared to those of normal (RPWE 1) show malignant (LNCaP)

prostate cells heavily stressed due to high levels of ROS than the normal (RPWE 1) prostate cells (Figs 3.7 – 3.12 and Figs 3.13 – 3.18).

Corroborating the above observations are reports from Kumar et al., (2008) as well as Szatrowski and Nathan, (1991) which mention that prostate cancer cells generate more ROS and NADPH oxidase (Nox) enzymes compared to the normal prostate tissue. The reason behind this is the presence of ectopic sources of ROS in prostate cancer cells. The sources include various forms of Nox (Nox1, Nox2, Nox3, Nox4 and Nox5) which are not present in normal prostate cells (Kumar et al., 2008). Further to this, in normal cells there is low production of O_2^- and ROS is mainly generated by the cytosolic NADPH oxidase. In contrast, in tumor cells O_2^- is mainly generated by mitochondrial respiratory chain (by complex I and III) (Laurent et al., 2005). The hyperactivity of mitochondria in tumor cells can be related to their elevated metabolic activity. In addition, the permanent oxidative stress in tumor cells, leads to the decrease in SOD and catalase activities that, in turn, favour the accumulation of ROS (Nicco et al., 2005).

In this study, quantification of ROS using Zeiss fluorescence microscope did not achieve the expected outcome. In brief, the Zeiss 200M inverted fluorescence microscope was used to assess ROS. It is equipped with Axiovision software. Prostate cells grown in an 8 well Ibidi slide were incubated with the CM- H_2DCFDA for 45 minutes and a specific green filter set was chosen. The microscope, with the help of the software, sets the required exposure time and light intensity. Of great note, was the lack of consistency in the exposure times and light intensities that were being produced for each micrograph taken for cells exposed to same concentration of tea or rooibos. In other words, different fields of the same treatment were subjected to different exposure times and light intensities. Further to this, there were instances where micrographs of untreated cells (with normal dividing and unstressed cells) showed high fluorescence intensity compared to micrographs containing stressed cells; cells treated with higher concentration of tea or rooibos. This might have

contributed to the differences in DCF fluorescence intensity hence the disparities in quantification of ROS levels between differently stressed cells within a cell line and indeed between LNCaP and RPWE 1 cells (Figs 3.31 – 3.54).

In this thesis, we expected increased ROS generation in LNCaP cells compared to RPWE 1 cells. Because of this, an increased DCF fluorescence intensity was expected to be observed in LNCaP cells than in RPWE 1 cells. Secondly, we expected to see a concentration- dependent increase in fluorescence intensity in both prostate cell lines. Basically, the oxidation of 2',7'-dichlorofluorescein (H_2DCF) to 2',7'-dichlorofluorescein (DCF) helps to quantify H_2O_2 . The diacetate form, H_2DCFDA when taken up by cells is acted upon by non- specific cellular esterases and in the process cleaves off lipophilic groups. This results in the formation of a charged compound that gets trapped inside the cell. In short, oxidation of H_2DCF by ROS converts the molecule to 2',7'-dichlorodihydrofluorescein (DCF), which is highly fluorescent (Uy et al., 2011). After trying out various interventions the quantification of ROS using this method was not achieved and that is why the BD accuri 6 flow cytometer (Ann Arbor, MI, USA) was used.

In the present thesis, BT, GT, FR, UR, EGCG and aspalathin generated higher ROS levels in malignant (LNCaP) than in normal (RPWE 1) prostate cells. Flow cytometry results confirmed this effect after exposing both prostate cell lines for 24 and 72 hours to tea and rooibos (Figs 3.56 - 3.61). LNCaP cells have been reported to possess a dysfunctional antioxidant system which is characterized by low catalase and dismutase levels than RPWE 1 cells. Apart from the presence of a defective antioxidant system in cancer cells, high ROS production may come about as a result of the activation of oncogenes, aberrant metabolism, mitochondrial dysfunction, and loss of functional p53. In addition to this, growth factors and cytokines have also been reported to be responsible for the production of ROS (Liou and Storz, 2010).

The present study now reports the pro-oxidant effects of rooibos (FR, UR and the isolated active compound aspalathin) which resulted in the production of high ROS levels in malignant (LNCaP) prostate cells. Interestingly, from this thesis, we report the presence of weak pro-oxidant effect by UR, FR, BT, ASP, GT, and EGCG in RPWE 1 prostate cells. In respect of this are flow cytometry results which showed low ROS levels in RPWE 1 cells both at 24 and 72 hours (Figs 3.56 - 3.61). In addition to this, flow cytometry analysis of RPWE 1 cells for apoptotic death using annexin V showed significantly reduced percentage of apoptotic RPWE 1 cells compared to the negative control. These results, further confirms the absence of a strong significantly marked influence of pro-oxidant effects by tea and rooibos in normal prostate cells. By extension it also confirms the absence of ROS induced necrotic deaths.

From the above information, we can safely say that GT, BT, UR, FR, EGCG and aspalathin behaved as anti-oxidants in RPWE 1 cells. In this thesis, plant extracts protected normal prostate cells from ROS induced apoptotic death. Von Gadow et al., (1997) reported that GT has the highest antioxidant capacities amongst all tea polyphenols. This antioxidant capacity has been attributed to the presence of gallic acid such as gallic acid and epigallocatechin gallate. The loss of EGCG in black tea as a result of the oxidation process (fermentation) results in its decreased antioxidant capacity. In this regard, the oxidation of the gallic acids by polyphenol oxidases and subsequent formation of thearubigins and theoflavins account for BT decreased antioxidant capacity (Robertson, 1992).

From the information above, GT should be a better antioxidant than black tea. Similarly, UR has a higher antioxidant capacity compared to FR. This is the case because UR contains more aspalathin than FR (Koeppen and Roux, 1966). Aspalathin is the major flavonoid in unfermented rooibos. This flavonoid gets oxidised during fermentation leaving only 7% of the aspalathin in FR (Joubert and Ferreira, 1996). This observation is not in agreement with our observed results. In this thesis, we

observed that the effects of GT, BT, UR, FR, EGCG and aspalathin are cell specific (they depend on the type of cell). Tea and rooibos are cytotoxic to prostate cancer cells through ROS generation (pro-oxidant effects) and protect normal prostate cells against ROS induced death probably through the removal of excess ROS. Further to this, plant extracts that demonstrated strong pro-oxidants effect in malignant (LNCaP) prostate cells displayed weak anti-oxidant effects in normal (RPWE 1) cells. In this case, GT, UR, EGCG and aspalathin which are very strong pro-oxidants in malignant (LNCaP) prostate cells demonstrated very weak anti-oxidant activity in normal (RPWE 1) prostate cells. GT and UR offered little protection against apoptosis in RPWE 1 cells compared to BT and FR. In similar manner, EGCG and ASP provided RPWE 1 cells less protection against necrosis (Fig 3.65). In the final analysis, BT and FR which are weak pro-oxidants in LNCaP cells gave the highest protection against ROS induced necrosis and apoptosis in RPWE 1 cells. In other words BT and FR are great anti-oxidants in normal prostate cells and serve as weak pro-oxidants in LNCaP cells (Figs 3.62 and 3.64).

From the above information, this thesis reports for the first time the presence of cell specific pro-oxidant and anti-oxidant effects by tea and rooibos; thus tea and rooibos are pro-oxidants in LNCaP cells while acting as anti-oxidants in RPWE 1 cells. Furthermore, when arranged in order of decreasing pro-oxidant capacity, the rank and file is as below: GT > UR > EGCG > ASP > FR > BT. In summary, GT and UR are very strong pro-oxidants in causing apoptotic death in LNCaP cells whereas UR and ASP are very strong pro-oxidants in promoting necrotic cell death in LNCaP cells (Table 4.1). At the same time, FR and BT are very strong anti-oxidants and offered the greatest protection against ROS induced apoptotic death of RPWE 1 cells. In the same manner, ASP, EGCG, FR and BT protect RPWE 1 cells from ROS induced necrosis of RPWE 1 cells. When arranged in order of decrease anti-oxidant capacity; the rank and file is as below: BT > FR > ASP > EGCG > UR > GT.

4.3 Apoptosis and Necrosis.

Apoptosis is defined as a controlled type of cell death that can be induced by a variety of physiologic and pharmacological agents, was first coined by (Kerr et al., 1972) on the basis of the following main morphological criteria: cellular shrinkage, condensation and margination of the nuclear chromatin, DNA fragmentation, cytoplasmic vacuolization and cell lysis. Apoptosis can also be defined as a representation of a discrete manner of cell death that differs from necrotic cell death and is regarded as an efficient way to eliminate damaged cells (Kanduc et al., 2002). Basically apoptosis is the protective mechanism through which unwanted cells are eliminated from the system. This is essential for normal development, turnover and replacement of cells in the living system and serves as the protective mechanism against cancer (Hengartner, 2000).

In the present thesis, malignant (LNCaP) and benign (RPWE 1) prostate cells were exposed to 1 000 µg/ml of BT, GT, FR, UR, aspalathin and EGCG, respectively (Fig 3.62). Arranged in order of decreasing apoptotic death (percentage) induced; EGCG and aspalathin induced more apoptotic deaths in malignant (LNCaP) prostate cells than the rest of the flavonoids (Table 4.1). The tea and rooibos used in this thesis may have induced apoptotic death using two major pathways; the death receptor (extrinsic pathway) and the mitochondria (intrinsic pathway) (Hengartner, 2000). In this regard, reports indicate that EGCG activates caspase-3 and caspase-9, regulates mitochondrial functions (release of cytochrome c and Smac/DIABLO, and depolarization of mitochondrial membranes), and cleaves PARP. In addition to this, EGCG also invokes Bax oligomerization and depolarization of mitochondrial membranes to facilitate cytochrome c release into cytosol. These physiological events are critical for the mitochondrial-dependent apoptosis or cell-intrinsic pathway of apoptosis (Shankar et al., 2007).

It's reported that EGCG induces cell cycle arrest and apoptosis in many cancer cells without affecting the normal cells (Ahmad et al., 1997; Yang et al., 2002). EGCG does this through the induction of the expression of Cdk inhibitors p21/WAF1/CIP1, and p27/KIP1, besides decreasing the expression of cyclin D1; it also inhibits Cdk2 and Cdk4 kinases (Gupta et al., 2003; Liang et al., 1999). In brief, EGCG either exerts its growth-inhibitory effects by modulating the activities of G₁ regulatory proteins such as Cdk2 and Cdk4 or by mediating the induction of p21 and p27.

In addition to this, EGCG could possibly have induced apoptosis in malignant prostate (LNCaP) cells via modulation of two related pathways: (a) stabilization of p53 by phosphorylation on critical serine residues and p14ARF-mediated down regulation of murine double minute 2(MDM2) protein, and (b) negative regulation of NF- κ B activity, thereby increasing the expression of the pro-apoptotic protein Bcl-2 (Khan et al., 2006). Further to this, EGCG may have induced the stabilization of p53 besides up-regulation in its transcriptional activity, thereby resulting in activation of its downstream targets p21/WAF1 and Bax. Thus, EGCG had a concurrent effect on two important transcription factors p53 and NF- κ B, could have changed the ratio of Bax/Bcl-2 to induce apoptosis. This may have altered the expression of Bcl-2 family members triggering the activation of initiator caspases 9 leading to the activation of a cascade of caspases and resulting in apoptosis. Taken together, the data indicate that EGCG may possibly induce apoptosis in human prostate carcinoma cells by shifting the balance between pro- and anti-apoptotic proteins in favor of apoptosis (Adhami et al., 2005).

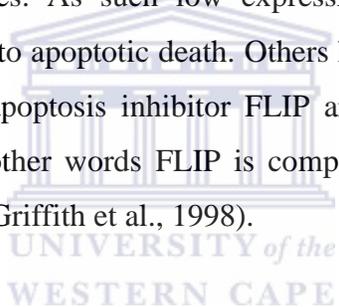
It has also been reported that green tea catechins (GTCs), particularly EGCG, inhibits the chymotrypsin-like activity of the proteasome resulting in the accumulation proteasome targets p21, p27, Bax, and I κ B α . The accumulation of cell cycle regulators, p21 and p27 will cause cell cycle arrest; while the accumulation of the pro-apoptotic protein, Bax, contributes to apoptosis. Additionally, the elevation of I κ B α expression inhibits the translocation of the oncogenic protein, NF κ B, to the

nucleus, resulting in reduced expression of its target genes, Bcl-xL and Bcl-2, cyclin D and cyclin E, vascular endothelial growth factor (VEGF) and matrix metalloproteases (MMPs). Reductions in these proteins further drive the processes of cell cycle arrest, decreased cell proliferation, and apoptosis, as well as the inhibition of tumor cell invasion and metastasis, respectively. The cumulative effect of these mechanisms leads to the inhibition of prostate cancer progression and metastasis (Connors et al., 2012).

Results of the present thesis, showed EGCG and aspalathin as the highest ROS inducers followed by GT, UR, BT and FR in both LNCaP and RPWE 1 (Table 4. 1). Using plant polyphenols as apoptotic agents, a model has been proposed that underlines the potential of DNA- associated copper in cells to activate phenolic compounds by way of a copper-redox reaction, whose net effect is generation of reactive oxygen species and electrophilic phenolic intermediates that result in oxidative DNA base modifications, DNA strand breaks, and DNA adducts of phenolic intermediates (Hadi et al., 2000b; Li and Trush, 1994). The results that we have obtained in this thesis via the annexin V/apoptosis assay (Figs 3.62 and 3.63) together with the principle behind the model proposed by Li and Trush (1994) reinforce the fact that the tea and rooibos used in this thesis produced ROS which led to the induction of apoptosis. Phosphatidyl serine (PS) is located on the cytoplasmic surface of the cell membranes activate an enzyme scramblase that flips PS from the inner leaflet of the cell membrane to the outside thus binding annexin V (Fadok et al., 1992; Fadok et al., 1993). Annexin V has a high affinity for PS and is able to identify apoptotic cells by binding to PS exposed on the outer cell membrane (Koopman et al., 1994). PI enters non-intact cell membranes and all the necrotic cells picked the red colour of propidium iodide.

Chinnaiyan et al., (2000) describes the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) as an endogenous activator of the cell death pathway which works by activating the cell surface death receptors 4 and 5 (DR4 and DR5).

Chemotherapy or ionizing radiation in combination with TRAIL have been observed to have a synergistic effect against breast cancer and this synergistic effect is p53 dependent and may be induced through the up-regulation of the TRAIL-receptor DR5. The apoptotic deaths through TRAIL have been observed to occur differentially between cancer and normal cells. Cancer cells are very sensitive to TRAIL induced apoptosis whereas normal cells are remarkably resistant (Ashkenazi et al., 1999). However some investigators feel that the molecular basis for this difference is controversial and unresolved such that some have proposed that the expression of decoy receptors, molecules that bind TRAIL but fail to signal death, as the ones that confer resistance to normal tissues. From previous studies Ichikawa et al., (2001) reported that cancerous tissues express higher levels of DR5 mRNA and protein than do normal cells or tissues. As such low expression of DR5 contributes to the resistance of normal cells to apoptotic death. Others have suggested that the levels of the intracellular caspase/apoptosis inhibitor FLIP are the one that protects normal cells from apoptosis; in other words FLIP is comparatively highly up-regulated in normal than cancer cells (Griffith et al., 1998).



Transcripts of TRAIL have been identified in the spleen, thymus, prostate and lung tissues. This far, 4 type I trans-membrane receptors have been identified and include DR4 (TRAIL-R1), DR5 (TRAIL-R2), DcR1 (TRAIL-R3) and DcR2 (TRAIL-R4) (Vindrieux et al., 2011). In the present thesis, the potential increased expression of DR4 (TRAIL 1) and DR5 (TRAIL 2) in the malignant (LNCaP) prostate cells relative to the normal (RPWE 1) prostate cells may have potentiated GT, BT, FR, UR, EGCG and aspalathin to induce more apoptotic deaths in the tumor cells.

Another investigation into why TRAIL confers resistance in normal cells and not in tumor cells was reported by (Zhang and Fang, 2005). They observed that the ratio of expression between death receptors DR4 and DR5 and decoy receptors DcR1 and DcR2 did not have any correlation with the sensitivity of hepatocytes to TRAIL. In line with this, is a proposal made to the effect that other molecules in the signaling

pathway of TRAIL- induced apoptosis are responsible for deciding the fate of normal cells in response to TRAIL. In this regard over-expression of Cellular FLICE-like inhibitory protein (c-FLIP) and inhibitor of apoptosis (IAP) were reported to be responsible for the protection of certain human normal cells such as melanocytes, lung and foreskin fibroblasts from TRAIL-induced apoptosis (Zhang and Fang, 2005).

Siddiqui et al. (2008) reported that green tea polyphenol EGCG sensitizes LNCaP cells to undergo apoptosis by TRAIL. In the study, a combination of EGCG and TRAIL significantly enhanced the protein expression levels of DR4. This led to the subsequent inhibition of expression of FADD and FLIP and XIAP and cIAP, a group of protein inhibitors of apoptosis (IAPs). Further to this, EGCG in combination with TRAIL synergistically inhibited SMAC/ DIABLO, a direct IAP binding protein. In addition to this, there was an up-regulation in poly (ADP-ribose) polymerase cleavage and pro-apoptotic Bcl2 family of proteins.

In a study to assess whether green and black tea polyphenols had any effect on the expressions of pro-apoptotic proteins, p53 and Bax as well as anti-apoptotic protein, Bcl-2 and mutant p53, in a 7, 12-dimethylbenz[a] anthracene (DMBA) induced mouse skin tumorigenesis; both green and black tea polyphenols were found to induce apoptosis through an enhanced increased expression of wild type p53 and Bax and decreased expression of Bcl-2 and mutant p53. Both GT and BT polyphenols induced apoptosis via cytochrome *c* release, caspase activation and PARP cleavage (Ramos 2007).

In line with this, expression levels of cytochrome *C* and Apaf-1 were found to be lower in DMBA exposed group in comparison with the control. Survivin directly inhibits apoptosis and its expression is frequently high in cancer cells and correlate with resistance to chemotherapy. Low levels of survivin were observed in tea polyphenols supplemented groups over DMBA exposed group (Roy et al., 2009).

Roy et al. (2011) examined the inhibitory effect of green tea polyphenol (GTP) and black tea polyphenol (BTP) on the development of mammary tumors- induced by 7, 12-dimethylbenz (a) anthracene (DMBA) in female, Wistar rats. In the study, GTP and BTP supplementation as a sole source of drinking solution scavenged reactive oxygen species (ROS). This inhibited cyclooxygenase-2 (Cox-2) and led to the inactivation of phosphorylated forms of nuclear factor kappa B (NF-kB) and Akt. GTP and BTP delayed the onset of mammary tumorigenesis, reduced the total number of tumors, average number of tumors per tumor bearing animal and significant reduction in the ROS generation. In brief, GTP and BTP supplementation suppressed the activation of pro-inflammatory gene Cox-2 by down-regulating NF-kB, Akt, mutant-p53 and up-regulating wild type-p53. In other words, green and black tea scavenged ROS and counteracted the cancer effects of DMBA-induced mammary glands of the mice.

Enhanced activity of tyrosine kinases, increased expression of the EGFR or erbB family of RTKs have been implicated in a wide variety of human malignancies. In human PCA, aberrant expression of the erbB family of RTKs, such as EGFR (also known as erbB1), erbB2, and erbB3, has been reported to be present at higher levels in prostatic intraepithelial neoplasia and invasive PCA. In addition, epidermal growth factor, TGF- α and erbB1 have been shown to be associated with the regulation of prostatic cell mitogenesis. In human prostate carcinoma DU145 cells silymarin induced cell cycle arrest followed by cell growth inhibition. In this regard, silymarin was observed to exert its effects on erbB-1-Shc-ERK1/2 signaling and cell cycle regulators. Basically, exposure of prostate carcinoma DU145 cells to silymarin inhibited erbB, Shc and ERK1/2 activation. Impairment of erbB1-Shc activation resulted in the inhibition of MAPK/ERK1/2 activation. At the same time, silymarin promoted a significant induction of cell cycle regulatory molecules CDKs Cip1/p21 and Kip1/p27 followed by their increased binding to CDKs. This prevented RB hyperphosphorylation and kept it bound to transcription factor E2F (E2F) in a

hypophosphorylated state. Basically, silymarin induced cell cycle arrest of DU 145 cells in G₁ phase (Agarwal, 2000).

In the present thesis, FR, UR and aspalathin induced apoptosis in malignant LNCaP cells. Previous studies using black tea polyphenols (BPT), green tea polyphenols (GTP), silymarin and EGCG as presented above have reported signaling pathways that they respectively modulated in order to induce cell cycle arrest and apoptotic death. The present thesis would like to suggest the aforementioned as possible pathways which may have been used by FR, UR and aspalathin to induce apoptotic deaths in the malignant LNCaP cells.

In the same manner, treatment of both cell lines with 1 000 µg/ml of each of the teas and rooibos resulted in more GT and UR induced necrotic deaths in malignant (LNCaP) prostate cells than was registered with EGCG, aspalathin, BT and FR. With respect to apoptosis, 1 000 µg/ml of the tea or rooibos induced more apoptotic deaths in EGCG- and aspalathin- treated samples than GT and UR, respectively. From a past study, Lelli et al., (1998) reported that necrosis is manifested by swelling and disruption of internal organelles and plasma membrane lysis resulting in the liberation of denatured proteins, DNA fragments, lysosomal contents, and other cellular debris from the cytoplasm into the extracellular space. Kroemer et al. (1995) described apoptosis in terms of cells having intact plasma membranes and cytoplasmic organelles (e.g., mitochondria) whilst in the late phases of apoptosis have the internal contents including fragmented DNA packaged into membrane-bound apoptotic bodies.

Leist and Nicotera, (1997) reported necrosis and apoptosis as representations of biochemical and morphologic patterns of cell death in a sense that the same initiating agent (depending on dose) can induce either apoptosis or necrosis, and the level of cellular ATP may define which pattern of death occurs. In other words, the amount of ATP at the time of cell death will define whether a cell is apoptotic or necrotic. If it

has enough ATP, the cell will be apoptotic and if it has no ATP, the cell will become necrotic. In the absence of ATP, apoptosis is blocked and using the same upstream pro-apoptotic signals necrotic cell death is induced. In brief, ATP supply mediates a switch from necrosis to apoptosis (Kim et al., 2003). From the results of the present thesis, it has become clear that *Camellia sinensis* and *Aspalathus linearis* have induced death of the prostate cells via apoptosis and necrosis.

Basically, the percentage of apoptotic and necrotic cells obtained following treatment with 1 000 µg/ml of either GT, BT, UR, FR, EGCG or aspalathin suggest that the above treatments may have modulated the opening of permeability transition (PT) pore through the production of ROS which induced the opening of the voltage dependent anion channel (VDAC) from the outer membrane and the adenine nucleotide translocator (ANT) from the inner membrane in the presence of cyclophilin D (CypD) to allow the release of cytochrome C from the mitochondria. This in turn promotes the downstream processes that led to the activation of caspase 3 and depending on ATP levels some cells stained red with Propidium iodide (PI) and were read by the FL 3 channel as necrotic (no ATP) and those cells that exposed phosphatidylserine (PS) through membrane flip to allow the binding of annexin V and were read on FL1 of the BD accuri flow cytometer as apoptotic (with ATP).

Results from the present thesis, have shown that high ROS levels generated by GT, BT, FR, UR, EGCG or aspalathin (Fig 3.62) induced apoptosis in malignant prostate cells. In agreement with this observation, reports indicate that high levels of ROS cause apoptosis and it comes about as a result of ROS triggering the opening of the mitochondrial permeability transition pore and release of pro-apoptotic factors. The mitochondrial permeability transition pore complex consists of an inner membrane segment, the adenine nucleotide translocator (ANT), which imports ADP and exports ATP, cyclophilin D, intermembrane creatinine kinase, and the outer membrane voltage dependent ion channel (VDAC) (Fruehauf, 2007).

Chemotherapy agents have been observed to modulate the opening of this pore primarily by triggering DNA damage response pathways at cell cycle checkpoints (Hanahan and Weinberg, 2000). In a similar fashion the tea and rooibos used in this thesis may trigger apoptosis and necrosis in the malignant (LNCaP) cells. Tea and rooibos might have been responsible for the opening of the MTP in LNCaP cells. Just as is the case with drug-induced apoptosis GT, BT, UR, FR, EGCG and aspalathin may have up-regulated the cytosolic concentrations of pore opening proteins, such as Bax and Bak above their critical threshold and in the processes targeted to destabilize of VDAC by chaperones such as Bid and Bim. VDAC destabilization increases ROS generation and promotes ion influx and ultimate mitochondrial membrane rupture, causing the release of the pro-apoptotic protein groups including cytochrome c, apoptosis inducing factor, Smac/Diablo, procaspases, and Endo G (Faustin et al., 2004).

4.4 Prostate specific antigen (PSA)

Prostate-specific antigen (PSA) has been defined as a kallikrein-like serine-protease that is constitutively secreted by normal epithelium of the prostatic gland and is the major protein in semen responsible for the cleavage of the most abundant proteins, semenogelin and fibronectin, thereby triggering liquefaction of the seminal plasma coagulum and facilitating spermatozoa progression. PSA, whose expression is regulated by androgens, has been found to be raised in benign prostatic hyperplasia and neoplastic. In line with this, PSA levels are used as a biomarker for early detection of prostate cancer (Pezzato et al., 2004).

Androgens play a critical role in regulating the growth, differentiation and survival of epithelial cells in many androgen-responsive organs, such as prostate and skin. The enzyme steroid 5 α -reductase catalyzes the conversion of testosterone (T) to a more active androgen, dihydrotestosterone (DHT). DHT then binds to androgen receptors (AR) and functions in the nucleus to regulate specific gene expression. Androgens via

their cognate receptor may be involved in the development and progression of benign prostate hyperplasia, prostate cancer, hirsutism, male pattern alopecia and acne (Lee and Peehl, 2004).

The androgen receptor (AR) is responsible for the development and progression of prostate cancer. The androgen receptor is one of the controllers of G₁-S phase of the cell cycle. It induces signals that promote G₁ cyclin-dependent kinase (CDK) activity, induce phosphorylation/inactivation of the retinoblastoma tumor suppressor (RB), and finally control androgen-dependent proliferation of prostate cells.

Androgen stimulation in AR-positive cells triggers rapid activation of the MAPK pathway, which then induces a mitogenic response. Basically treatment can start with the removal of AR function through ligand depletion. This can be done through bilateral orchiectomy or by using GnRH agonists. This can be followed by the use of direct AR antagonists such as bicalutamide (Chodak, 2005; Klotz, 2000). Firstly, these agents compete for DHT binding. Secondly, selected AR antagonists trigger the recruitment of transcriptional co-repressors (e.g., NCoR) to androgen response elements (AREs), thereby fostering active repression of AR target gene expression (Shang et al., 2002). Effective AR inhibition is indicative of a loss of detectable serum PSA.

In this study, PSA levels in LNCaP and RPWE 1 cells were quantified using a PSA total ELISA kit. Malignant (LNCaP) and benign (RPWE 1) prostate cells were exposed to increasing concentrations of GT, BT, UR, FR, EGCG and aspalathin for 72 hours. The results obtained from this assay showed malignant (LNCaP) prostate cells (Figs 3.66 - 3.71) producing a concentration-dependent decrease in total serum PSA concentration in response to tea and rooibos, whereas in RPWE 1 cells there was no observable change in total serum PSA levels (Fig 3.66 - 3.71).

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 and Yousef et al., 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, 1994). This leakage of PSA is used as a sensitive and specific tumor biomarker for prostate cancer screening and assessment (Stamey et al., 1989a and Stamey et al., 1989b) and is therefore regarded an oncological indicator of disease and response to prostate cancer therapy (Cadeddu et al., 1998).

In normal prostate cells the basal cell layer and basement membrane are not compromised. And because this, individuals with normal prostate cells have serum PSA levels which are less than 4 ng/ml (Bello et al., 1997). In this regard, the benign RPWE 1 cells have two tumor suppressor proteins; p53 and retinoblastoma (Rb) proteins (Rhim et al., 1994). Further to this, benign RPWE 1 cells are E7 immortalized cells and retain characteristics of normal cells (Monini, 1995). These cells have also been reported to possess prostatic epithelial cell-associated PSA and androgen receptor (AR) expression as their functional markers. In the presence of androgens and upregulated AR, these cells secrete PSA like normal prostate epithelial cells (Bello et al., 1997).

In the current thesis, malignant LNCaP cells showed a concentration-dependent decrease in the concentrations of PSA following exposure to black tea, green tea, fermented rooibos, unfermented rooibos, EGCG and aspalathin. Reports from previous studies describe presence of a direct relationship between a decline in PSA and the shrinkage of prostate cancer (Brausi et al., 1995). Since 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, our treatment samples did not affect the PSA levels in the benign (RPWE 1) cells. The benign RPWE 1 cells did not die, hence the absence of an observable change in PSA levels.

Mclarty et al., (2009) reported significant reduction in serum levels of PSA, HGF, IGF-1, IGFBP-3 and VEGF in men with prostate cancer after a brief treatment with EGCG (Polyphenon E) with no elevation of liver enzymes.

PSA is regulated by androgens (Christensson and Lilja, 1994; Wolf et al., 1992). PSA may also be directly involved in the invasive ability of prostate cancer cells. It degrades gelatin, type IV collagen and activates MMP-2. EGCG treatment has been reported to inhibit these activities in prostate cancer cells. Besides this, EGCG treatment of malignant (LNCaP) prostate cells resulted in decreased PSA expression in vitro and decreased tumor PSA expression in R1Ad tumor xenografts in mice (Chuu et al., 2009).

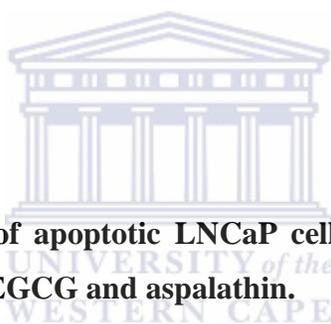


Table 4- 1: Percentage of apoptotic LNCaP cells following exposure to 1 000 µg/ml BT, GT, FR, UR, EGCG and aspalathin.

Number	Polyphenol	Apoptotic (%)
1	Black tea	2.7
2	Green tea	7.66
3	Fermented rooibos	3.2
4	Unfermented rooibos	5.6
5	Aspalathin	22.5
6	Epigallocatechin gallate	21

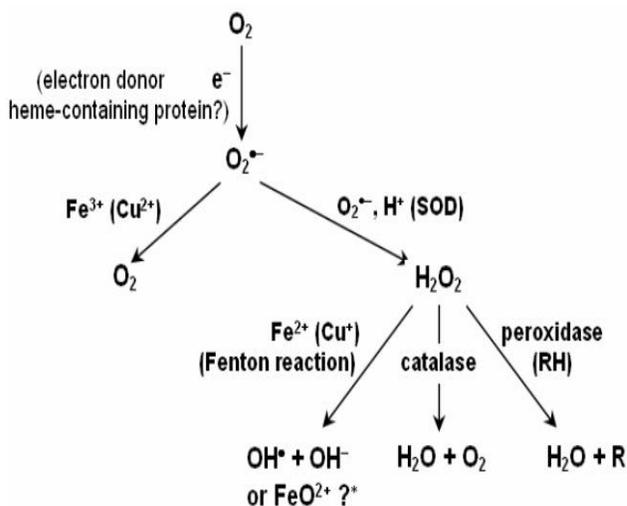


Fig 4- 1: Fenton reaction in biological systems. (Barbusiński, 2009)

Schematic representation of the sequence of events involved in Fenton reaction. Initially, electron donors can convert oxygen to superoxide anion ($\text{O}_2^{\bullet -}$) which is rapidly converted to hydrogen peroxide. Hydrogen peroxide can further form hydroxyl radicals (OH^\bullet) or ferryl ion (FeO^{2+}) in the actual Fenton reaction in the presence of ferrous or cuprous ions (which are simultaneously oxidized to ferric or cupric ions). SOD - superoxide dismutase; RH/R - reducing agent in oxidized and reduced form.

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CONCLUSION

Tea, *Camellia sinensis* (*C. sinensis*), is the most popular beverage consumed throughout the world. Rooibos, *Aspalathus linearis* (*A. linearis*), a native of Northern and Western Cape South Africa has gained popularity as a health drink both from within and without. Both of these teas possess physiological and pharmacological properties such as anti-oxidant, hepato-protective, anti-mutagenic, immunomodulating and anti-cancer effects. So far, there is a lot of literature reporting the effects of tea in prostate cancer but not rooibos. Despite rooibos showing so many positive health benefits; no studies seem to have been done to assess its anti-cancer effects on the prostate.

For this reason, this thesis used in vitro assays to assess the effects of rooibos and tea on prostate cancer. To achieve this, two prostate cell lines, the malignant (LNCaP) and benign (RPWE 1) prostate cells were used to investigate the anti-cancer effects of these two beverages and their main active compounds, EGCG and aspalathin on the prostate. Cytotoxic studies were done first, followed by the determination of ROS levels. In the final analysis, PSA, the biomarker for prostate cancer was determined using total serum PSA ELISA kit with the aim of determining the chemo-therapeutic effects of these beverages.

This thesis, reports that tea and rooibos are cytotoxic to malignant prostate cells and protect normal prostate cells against ROS-induced cell death. Furthermore, green tea and unfermented rooibos are potent pro-oxidants with a very good capacity to induce apoptotic death in malignant (LNCaP) prostate cells whereas the main active components EGCG and aspalathin are good promoters of necrosis. This thesis also reports that black tea and fermented rooibos are very good anti-oxidants with very efficient protective effects against ROS induced death in benign (RPWE 1) prostate cells. The knowledge gained through this study has the potential to facilitate individuals with making decisions on whether or not to use tea and rooibos in chemo-prevention and therapy. However, in light of the necrotic deaths promoted by the main active compounds, EGCG and aspalathin, more research needs to be done on whether chronic use of isolated active compounds should be recommended in chemo-prevention and therapy. The results of this study confirm previous reports that suggested that plant polyphenols can potentially prevent cancer. Tea and rooibos can be used as dietary supplements to prevent the initiation, reverse the promotion and above all stop the progression of cancer.

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