UNIVERSITY of the
WESTERN CAPE

THE POTENTIAL THERAPEUTIC ROLE OF PALM OIL ON PROSTATE CANCER

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.

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May 2019

DECLARATION

I declare that *an in vitro* investigation of **the potential therapeutic role of palm oil on prostate cancer** 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.

Full Name

Hasan Ghanaim

Sign

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Date

May 2019

DEDICATION

This thesis is dedicated to my father, the man who taught me hard work, dedication and passion, and that my goals can always be achieved. It is also dedicated to my mother who taught me to believe in myself and to never quit on my dreams. I would also like to dedicate this thesis to my supervisor, Dr Sahar Abdul-Rasool and thank her for supporting me through the whole process that has made a major contribution to the advancement of my academic career.

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Androgen deprivation therapy **(ADT)**

Androgen receptor **(AR)**

Benign **(PWR-1E)** prostate cell

Benign prostatic hyperplasia **(BPH)**

Crude "red" Palm Oil **(CPO)**

Dihydrotestosterone **(DHT)**

Malignant **(LNCaP)** prostate cell

Prostatic intraepithelial neoplasia **(PIN)**

Prostate-specific antigen **(PSA)**

Polymerase chain reaction **(PCR)**

Reverse transcription **(RT)**

Thiazolyl Blue Tetrazolium Bromide **(MTT)**

Abstract

Prostate cancer is one of the heterogeneous groups of neoplastic diseases originating from the reproductive system of the male naming, the prostate gland. In the west, prostate cancer is the most common cancer affecting African men in older age (over the age of 55) and usually with a family history of the disease. The initiation and progression of this disease is thought to result from the genetic alterations of gene expression in the prostate epithelial cells. Prostate cancer has a very slow progression. This observation provides the advantage of early detection and the notion for using diet to prevent the cellular and molecular processes of carcinogenesis.

Epidemiological research has documented a positive health role for red palm oil on atherosclerosis, arterial thrombosis and several types of cancers. This thesis focuses on investigating the effect of different concentrations of the red palm oil (0.1, 1, 10, 100, 500, 1 000 µg/ml) on malignant (LNCaP) prostate cells and benign (PWR-1E) prostate cells over 24 and 72-hours. The following parameters were investigated: cell morphology and viability (using MTT assay), the expression of androgen receptors and prostate-specific antigen (PSA) via RT-PCR and/or PSA ELISA kit.

The results of this study demonstrate that red palm oil has significant cytotoxic effects on malignant (LNCaP) prostate cells but caused only a slight decrease in cell viability of benign (PWR-1E) prostate cells. Morphologically, we noted a clear increase in detachment and cell death in malignant (LNCaP) cells as the concentrations of red palm oil increased. Moreover, the viability decreased significantly in both 24 and 72-hour treatment of red palm oil. Further to this, red palm oil significantly promoted the reduction of total PSA concentration in malignant (LNCaP) prostate cells whereas in benign (PWR-1E) prostate cells the Red Palm Oil maintained the total serum PSA at its basal physiological level.

In conclusion, red palm oil is significantly cytotoxic to malignant (LNCaP) prostate cells whereas weakly cytotoxic effect toward benign (PWR-1E) prostate cells. The potent inhibition to

mitochondrial dehydrogenase activity is responsible for the aforementioned effects respectively. The decrease in total serum PSA demonstrate the strong therapeutic effects that red palm oil has on malignant (LNCaP) prostate cells

1. Introduction

1.1 Prostate gland

The prostate gland is a part of the male reproductive system. The human gland measures about 3.8 x 2.5 x 3.2 cm (transverse diameter x sagittal diameter x height) and range between 7 grams to 16 grams in weight (Gray, 1974). It's located anterior to the rectum as it wraps around the urethra that works as a tube to allow the urine to flow from the bladder to the penis. The prostate is found in all types of male mammals. Histological studies of the prostate have described three distinct zones; the peripheral zone assimilating 70-75%, the central zone assimilating 20-25% and the transition zone presenting 5-10% of the prostate gland (Cunha et al., 1987). Each zone has coordinated morphogenesis. The first zone is the transition zone that consists of ducts emerging from the urethra and is separated from the surrounding peri-urethral glands by the pre-prostatic sphincter. The second zone is the central zone surrounding the ejaculatory ducts and has typically large acini and more complex ductal branching than the peripheral zone, while later consist of ducts that radiate laterally from the distal prostatic urethra and coincide with the ejaculatory ducts axis (Cunha et al., 1987) (Figure 1). The part of the urethra passing through the prostate is called the prostatic urethra and it merges with the low ejaculatory ducts (McNeal, 1981). The prostate does not have a capsule, but only an integral fibromuscular band that surrounds it. It is sheathed by the muscles of the pelvic floor, which contract during the ejaculatory process.

The prostate gland has a secretory function; it secrets an acidic fluid that made of up to 30% of the semen. The secretory ducts contain basal and luminal epithelial cells, as well as scarce neuroendocrine cells interspersed.

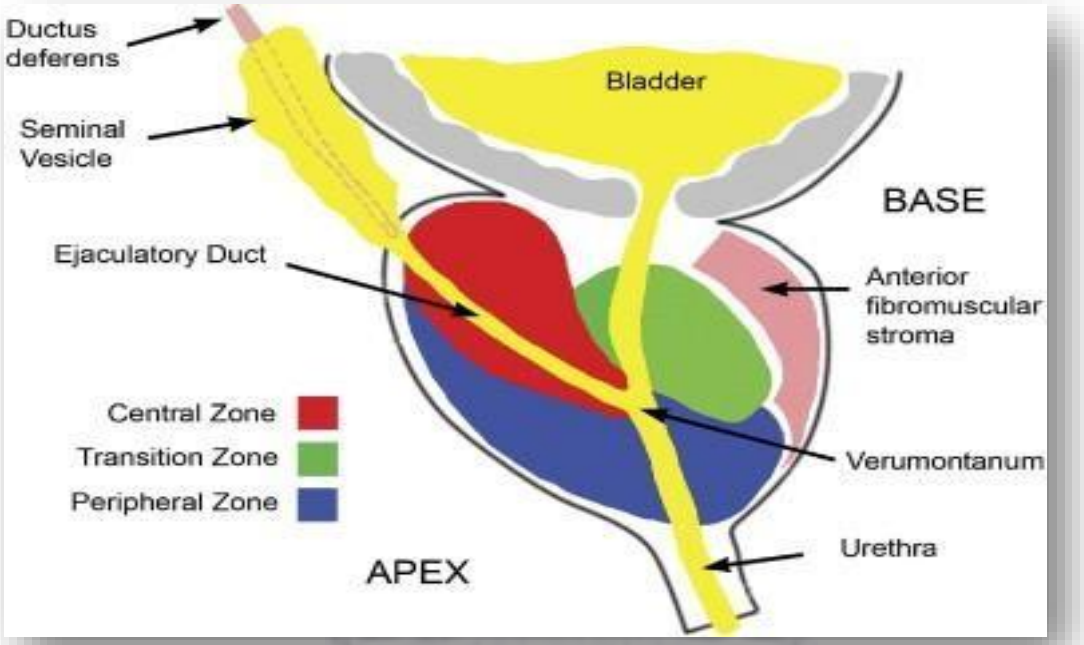

Figure 1: Sagittal view of the prostate gland that specifically illustrates conical central zone and its intimate relationship to ejaculatory ducts and seminal vesicles. Adapted from (McNeal, 1981).The zonal anatomy of the prostate. The Prostate, 2(1), 35–49.

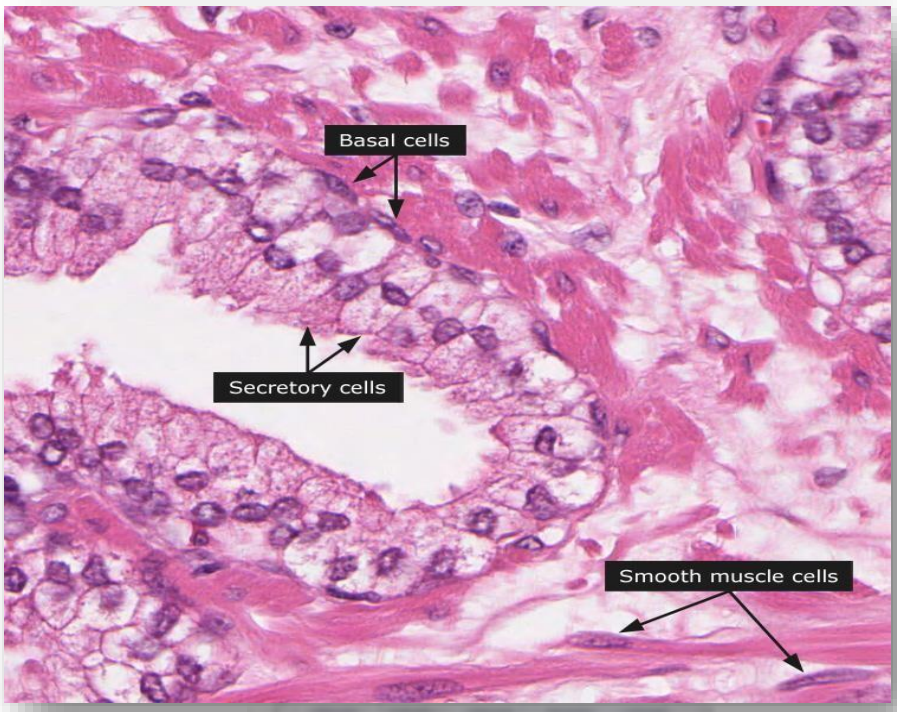

Figure 2 Histology of the human prostate gland. Adapted from (Beltran et al.,2017). Basic Anatomy and Histology of the Prostate. Cambridge University Press.

The human prostate contains stromal and epithelial cells which are separated by the basement membrane and merging in the extracellular matrix. The stromal cells include fibroblasts, smooth muscle cells, minor inflammatory cells, endothelial cells and nerve cells (Wen et al., 2015). The secretory epithelium in the prostate gland is mainly pseudostratified tall columnar cells and basal cells which are supported by the fibroblastic stroma. The stroma is composed of randomly oriented smooth muscle bundles that apply to the bladder. In addition, the epithelium is highly variable and areas of low cuboidal or squamous epithelium are also present, with transitional epithelium in the distal regions of the longer ducts (Fine & Reuter,2012). The postnatal maturation and development of the prostate depend on the mutual interactions between the stromal and the epithelial section (Lamont & Tindall, 2011).

The development of the prostate gland starts during the foetal life and is completed when the individual is sexually mature. This development comes from a sequence of coordinated cellular

events that involves a coordinated ductal branching and canalization in addition to the epithelial and mesenchymal differentiation and proliferation. The developmental process starts when the prostatic buds emerge from the urogenital sinus on the tenth week of development in human foetuses; the space pattern of the emerging prostatic buds provides the foundation of the subsequent development of the human prostate into the histologically organized zones as described earlier.

The prostate developmental events start with the formation of the endodermal urogenital sinus which contains an outer layer of embryonic connective tissue urogenital sinus mesenchyme (UGM) and an inner layer of urogenital sinus epithelium (UGE). The initial stage of the development in UGM involves the differentiation of fibroblasts and smooth muscle cells in response to the UGM androgen receptor signals. Responding to such signal, the urogenital sinus epithelium (UGE) could grow into the surrounding stromal cells and differentiate into epithelial cells as a part of the normal prostate development (Wen et al., 2015).

The androgen-related hormones and androgen receptor signals play a vital role in the prostate developmental events; prostate proliferation, differentiation, morphogenesis and functional maintenance are all influenced by androgen and the androgen receptor signals. The testosterone produced by the foetal testis and the dihydrotestosterone (DHT) which is produced by local conversion of circulating testosterone by the 5α-reductase perform to specify the development of the urogenital sinus into prostate via stimulation of the androgen receptors (AR) (Zhou et al., 2015). Similarly, the reciprocal developmental interactions between the UGM and UGE might be ruled by androgen and the androgen receptor signals, which are fundamental for the development of the normal prostate and might lead to benign prostatic hyperplasia (BPH) and prostate cancer. The epithelial AR and stromal AR function through the epithelial-mesenchymal transition (EMT) to impact prostate development. In the prostate, the AR is expressed in both epithelial and stromal tissues. The trans activated AR in the nucleus could then function through modulation of diverse target genes to impact the development and the maintenance of the prostate. In addition, the expression can impact the cell growth directly, EMT is a process by which epithelial cells lose their cell-cell adhesion and gain migratory properties to become

mesenchymal-like and/or mesenchymal stem cells, where these pluripotent mesenchymal cells could then differentiate into different cell types to impact the progression of benign prostatic hyperplasia (BPH) and prostate cancer (Wen et al., 2015).

Benign prostatic hyperplasia(BPH)

BPH is a common condition featured by the enlargement of the prostate gland. The changes take place in the transition zone of the prostate which leads to pressure on the urethra and ultimately can cause urinary tract problems such as blocking the flow of the urine (Thorpe & Neal, 2003)., BPH has been reported as an extremely common pathology for elderly males that is present in more than 50% of men aged over 60 years (Thiruchelvam, 2014) (Figure 2). Histologically, BPH is presented as increased cellular proliferation of epithelial cells and stromal cells and reduced cell death (Thiruchelvam, 2014).

Although the cause of the benign prostatic hyperplasia remains unclear, it is suggested that androgens hormones play a major role in its development and progression. Furthermore, scientific evidence has shown that androgens must be present for benign prostatic hyperplasia to occur, this has been supported by the fact that men who have had their testicles removed at a young age do not develop benign prostatic hyperplasia (Thorpe & Neal, 2003). As mentioned earlier, the androgen hormones that promote prostate cell proliferation, and are capable of activating the characteristic hyperplastic changes associated with benign prostatic hyperplasia are found to be overexpressed (Thorpe & Neal, 2003). However, the role of the androgens remains unclear and warrant further investigation.

On the molecular level, a study suggested that the stromal cells have to excrete peptide growth factors (androgens) in response to the androgen signalling through the AR in these particular cells, and binding the androgens to the receptors on the basal cells to raise their proliferation and differentiation into luminal cells and creates intracellular signals that would repress the apoptosis pathway to allow the luminal cells to maintain.

While the basal cells can self-renew or differentiate into luminal cells through a multistep process which includes slightly differentiated intermediate or transit-amplifying cells (Wang *et al.,* 2001).

Benign prostatic hyperplasia has also been known to depend on androgen dihydrotestosterone (DHT). A study documented that DHT rather than testosterone is the causative androgen in benign prostatic hyperplasia (Carson & Rittmaster, 2003). DHT is vital to the development and normal growth of the prostate, from the foetal prostate to the development of the male genitalia (Carson & Rittmaster, 2003). However, DHT and the androgens have been hypothesized to commit to the maintenance of a balance between cell proliferation and cell death in the adult prostate (Carson & Rittmaster, 2003). Correspondingly, benign prostatic hyperplasia emerges when the homeostasis between cell proliferation and cell death is disturbed.

The ageing process has its impact on the prostate gland; the concentrations of both total and free testosterone in the plasma decrease with age and correspond with commencement of benign prostatic hyperplasia. At the same time, and when testosterone is decreased with age, DHT remains stable during the time of benign prostatic hyperplasia commencement. This study is permanent with the role of DHT in prostate growth while the testosterone is not seriously involved (Carson & Rittmaster, 2003).

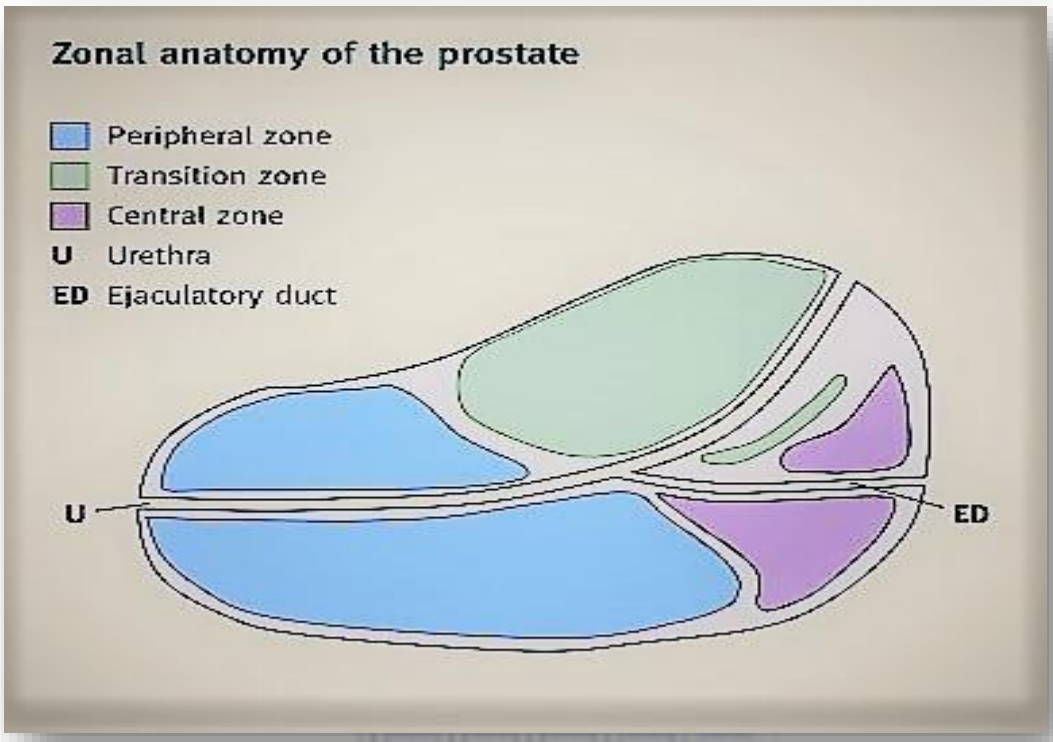

Figure 3: Zonal anatomy of the prostate. Adapted from (McNeal, 1981).The zonal anatomy of the prostate. The Prostate, 2(1), 35–49.

The currently available treatment of BPH using Alpha-blockers is effectively improving the symptoms of being prostatic hyperplasia but do not decrease the prostate size (Carson & Rittmaster, 2003). Moreover, 5α-reductase inhibitors also reduce symptoms and the size of the prostate between 20%-30% (Thiruchelvam, 2014) (Carson & Rittmaster, 2003). However, erectile dysfunction, loss of libido, ejaculation problems and gynecomastia have been reported to be a drug-related side effect (Carson & Rittmaster, 2003). These side effects call for the need to find more suitable alternative therapeutic agents for treating BPH and ultimately prostate cancer.

The correlation between BPH and prostate cancer has been acknowledged since the first autopsy studies of prostate glands (Sommers, 1956). Although, the histological and localization differences, the BPH defined as macroscopic hyperplasia of stromal cells and lesser extent epithelial cells that usually located in the transition zone of the prostate gland (Guess,

2001), while the prostate cancer is an adenocarcinoma that emerges initially from the epithelial cells in the peripheral zone of the prostate gland; only slight portion develops from the epithelial cells in the transition zone (Guess, 2001). Despite the differences, the two diseases share some remarkable features containing hormone-dependent growth and response to antiandrogen therapy. Moreover, several studies have reported that chronic inflammation, metabolic disruption and genetic variation have significant roles in the development of both diseases. Chronic inflammation is a vital factor for a certain amount of common diseases and several types of cancer (Allin et al., 2009), including prostate cancer (De Marzo et al., 2007). It is also thought to act as a driver of disease development or as an indication of inflammatory and immune system response to disease. Chronic inflammation observed either within the tumour or in close proximity to it. Even though the exact role of the inflammation infiltration remains unclear, chronic inflammation could be contributing factor in the development of prostate cancer.

Despite being essential growth factors for normal prostate development, androgens still lead the development of the benign prostatic hyperplasia and cancer. When plasma testosterone is converted into DHT by 5α-reductase, the DHT then binds intracellularly to the androgen receptor in the cell nucleus and triggers cell differentiation and proliferation by activation of various transcription factors (Nacusi & Tindall, 2011). However, the association of the plasma testosterone with the risk of BPH and prostate cancer is still controversial (Ørsted & Bojesen, 2012). This discrepancy might be attributed to the saturation mechanism in the prostate gland that limits the conversion of testosterone to DHT, preventing increased levels of plasma testosterone from leading to increased intraprostatic DHT (Morgentaler & Traish, 2009). Thus, this has led to the development of 5α-reductase inhibitors, which reduce DHT levels for the treatment of BPH and the prevention of prostate cancer (Zhu & Imperato-ginley, 2009).

Metabolic factors have been associated with the development and progression of both BPH and prostate cancer (Nunzio et al., 2012). Insulin-growth factor 1 receptor activation in men with diabetes, central obesity as well as hyperinsulinemia and hypertriglyceridemia, the two components of the metabolic syndrome increase the risk of devolvement of BPH and high-grade

prostate cancer. However, further studies are required to clarify these associations (Ørsted&Bojesen, 2012). Moreover, as a result of developments in genotyping and data analysis over the past few years, genome-wide association studies have detected new genetic variants and associated with prostate cancer incidence, despite the fact that to date little effort has been put into the investigation of the genetic variation associated with BPH (Kote-Jarai et al., 2011).

Prostate cancer (adenocarcinoma)

Prostate cancer is more common in men over 55. In America; 1 in 8 men will be diagnosed with prostate cancer at some point of their lives. The incidence increases with age; it was reported that only 1 in 10.000 under the age 45 will be diagnosed with adenocarcinoma and that this incidence increase to 1 in 14 for men of the age 65 to 69. In fact, mostly 60% of all prostate cancer is diagnosed in men over the age of 65. Therefore, age is the biggest risk factor for prostate cancer. Prostate cancer is a very slow progressing disease; many men die of old age without even knowing they had prostate cancer. Genetics is an important risk indicator as the family history might indicate the possibility of being at risk; higher incidence has been reported in men whose family members were diagnosed before age 65. Men often also are at high risk of prostate cancer when they have a strong family history of other cancers such as colon cancer, breast cancer or pancreatic cancer. Other risk factors like race must be also considered with the African race being more prone. According to the United States Preventive Services Task Force, the incidence of prostate cancer in African American men is almost 60 % higher and the mortality rate is two to three times greater than it is in Caucasians (Shenoy et al., 2016).

Moreover, lifestyle and nutritional habits might play a role in the development of the disease (Nordqvist, 2016). Many of these could lead to aggressive and fatal cases. For example, certain physical characteristics such as overweight are more likely to be the main reason for having prostate cancer. Furthermore, high calcium intake and adverse habits like smoking can lead to prostate cancer. Requesting a family medical history in terms of prostate cancer tends to be a crucial approach.

The histological changes are the earliest indication of the malignant transformation that might take place in a healthy prostate. Several experts propose that prostate cancer begins with changes in the shape and the size of the prostate gland called prostatic intraepithelial neoplasia "PIN" (Heidenreich et al., 2011). Physicians say that about 50% of all 50 years old men have PIN (Nordqvist, 2016). Those cells do not seem to have moved elsewhere, but these changes can be detected under a microscope. On the other hand, the cancer cells would have moved into other parts of the prostate. Clinicians have ranked the prostate gland cell changes on a scale from low-grade to a high-grade, where the high-grade represent the presence of more cancer cells in a histological section while in a low-grade section more normal cells are present (Nordqvist, 2016).

The diagnosis of prostate cancer has developed over the years. Doctors depend on many tests to diagnose prostate cancer. They also use tests to find cancer has spread to another part of the body from the prostate, a process known as metastasis (Barry, 2001). For prostate cancer, a biopsy is one way to find whether the patient has cancer or not. A biopsy is performed by taking a small sample of the prostate tissue for testing in the laboratory. However, if a biopsy is not possible, the doctor may suggest other tests that will help to make the diagnosis such as PSA blood test or Trans-rectal ultrasound (TRUS) (Barry, 2001).

The TNM classification system is used by the doctors to describe prostate cancer growth or spread, as well as its location. The TMN system provides proper new categories for the modulation of inconspicuous cancers detected by PSA and ultrasound (Edge & Compton, 2010). The "T" plus a number from 0 to 4 is used to describe the size and the location of the tumour. The "N" used to describe whether if cancer has spread to the regional lymph nodes and the "M" indicates whether the prostate cancer has spread to other parts of the body, such as the lungs or the pancreas. Doctors assign the stage of cancer by combining the T, N, and M classifications, and also includes the PSA level and Grade Group (Edge & Compton, 2010). The classification achieves the purposes of the staging system, it reflects the pathologic characteristics of cancer in each sub-stage and even appears to reflect prognosis of this tumour (Ohori et al., 1992). The TNM system is trustworthy and includes precious new categories essential to the logical classification of prostate cancer.

Prostate cancer is treated by surgery, radiation or hormone when limited to the organ at the time of diagnosis. If disease relapse occurs, androgen deprivation therapy (ADT) is used to control the disease. Studies show that androgen deprivation therapy acts effectively on metastatic prostate cancer. However, two to three year after treatment, many of the prostate cancer treated cases show resistance to treatment and cancer often recurs which lead to the progression from androgen-dependence to androgen independence (Karantanos et al., 2013). Cancer progression after initial ADT is termed castrate-resistant prostate cancer (CRPC) and it can be either metastatic or non-metastatic. The cancer progression and the increase in PSA levels serve as a guide that the androgen axis is still functional despite low circulating levels of serum androgens (Tran et al., 2009).

Prostate-Specific Antigen (PSA)

Human prostate-specific antigen (PSA) has become the most sensitive tool for the early detection of prostate cancer and monitoring therapy (William et al., 1991). Since 1979 numerous articles have been published, both on the biochemical features of the PSA molecule and the relationship between PSA and prostate cancer (Kuriyama et al., 1981).

PSA is a product of the human glandular Kallikrein gene family, localized on the long arm of chromosome 19, that is almost selectively expressed by prostate tissue (Riegman et al., 1992). Figure.4. The molecular characteristics of PSA have been proven by amino acid and cDNA sequencing techniques. It was stated that PSA contains 237 amino acids with a molecular mass of one single polypeptide chain of 26,079 Da containing approximately 7% (wt/wt) carbohydrate (Wang et al., 1979). PSA is synthesized in the epithelial cells of the prostate tissues (normal, benign hypertrophic, and cancerous) and is secreted into the seminal fluid. It has been detected in the endoplasmic reticulum only in the epithelial cells of the prostatic ductal element by a specific antibody to PSA (William et al., 1991).

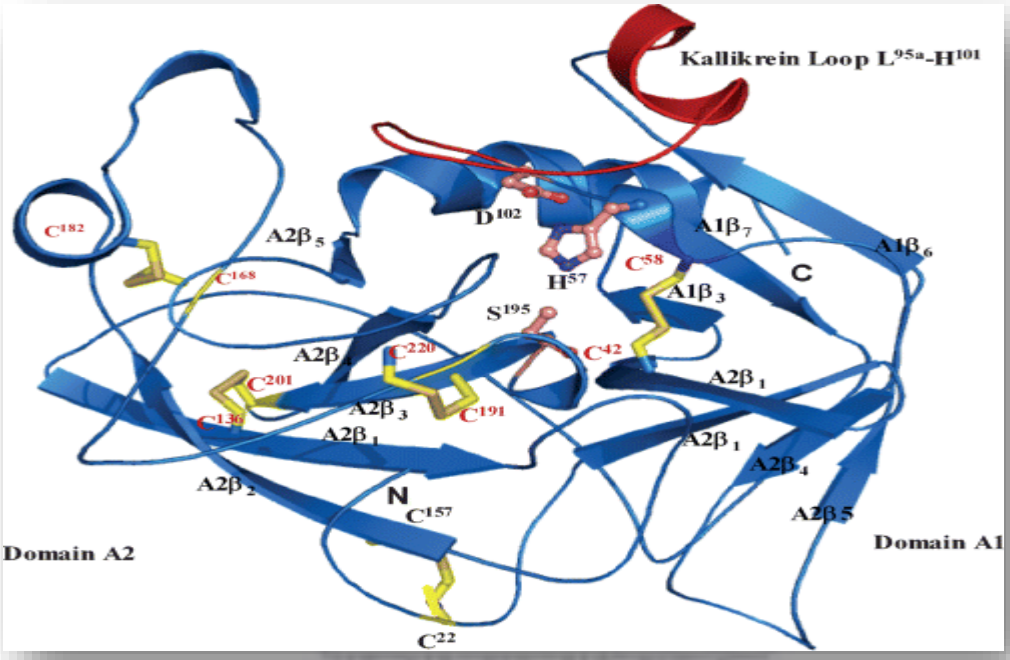

Figure 4: Overall structure of human prostate-specific antigen, the picture was adapted from (Hassan & Kumar, 2007).

The PSA test used to measure the level of the PSA in blood. Serum levels can be raised when there are abnormal activities in the prostate gland, this including prostate cancer as well as benign prostatic hyperplasia (BPH), enlarged prostate and prostatitis resulting from inflammation or infection of the prostate. Furthermore, physical activities might raise the PSA levels, therefore these activities must be avoided before people go for PSA testing. Doctors can look at various measures of the PSA, such as absolute level and measure the change over time while comparing it to the prostate size, to decide if a biopsy is needed. Also, the version of the PSA test allows measuring a specific component called the "free PSA", which can sometimes help to find if the tumour is cancerous or noncancerous (Schröder et al., 2012).

Until recently, many doctors and professional organizations recommended yearly PSA testing for men beginning at age 50. Some organizations encouraged those men who are at higher risk of prostate cancer such as the African men or men whose father or brother had prostate cancer to begin testing at age 40 or 45 (Lucia et al., 2004). However, there is no specific normal or abnormal

level of PSA in the blood. Most doctors considered PSA levels of 4.0 ng/ml and lower as normal, so if a man had a PSA level above 4.0 ng/ml doctors would often recommend a prostate biopsy to determine whether prostate cancer was present (Lucia et al., 2004). If the PSA of the man continues to rise, the doctor may recommend additional tests to determine the nature of the problem. A urine test may be recommended to check for a urinary tract infection. They may also recommend imaging test like for example ultrasound, X-ray or cystoscopy.

Androgen receptor (AR)

The androgen receptor is also known as NR3C4 (nuclear receptor 3, group C, member 4). It is a nuclear receptor that has the ability to bind to DNA and regulate the expression of relative genes. In response, the androgen receptor is activated by binding either testosterone or dihydrotestosterone DHT, which are the male sex hormones. Testosterone is synthesized primarily by the Leydig cells in the testes under the regulation of the luteinizing hormone (LH) produced by the anterior pituitary gland (Tan et al., 2015).

The androgens are mediated via the AR, which has a variety of biological actions including important roles in development and maintenance of the reproductive, cardiovascular, immune and neural systems. AR signalling may also be involved in the development of tumours in the prostate, bladder, liver, lung and kidney (Davey & Grossmann, 2016). However, the ideal development and maintenance of the prostate gland are totally dependent on the action of the androgens through the AR. Furthermore, the action of the androgens can be exerted via the AR in DNA binding-dependent manner to regulate the target gene transcription, or in a non DNA binding-dependent manner to initiate rapid transcription via cellular events such as the phosphorylation of the 2nd messenger signalling cascades (Heinlein & Chang, 2002).

Androgen receptor structure

The AR gene is located on the X chromosome and expressed in a variety range of tissues such as in the bone, muscle, prostate and adipose tissue. Understanding of the AR structure and function

may lead to design plans that inhibit the AR function at specific stage of the disease. The AR is a part of the nuclear receptors superfamily that function as ligand-inducible transcription factors which mediate the expression of target genes in response to ligand-specific to each receptor including steroids, vitamin D, thyroid hormone and xenobiotic agents (Heinlein & Chang, 2002).

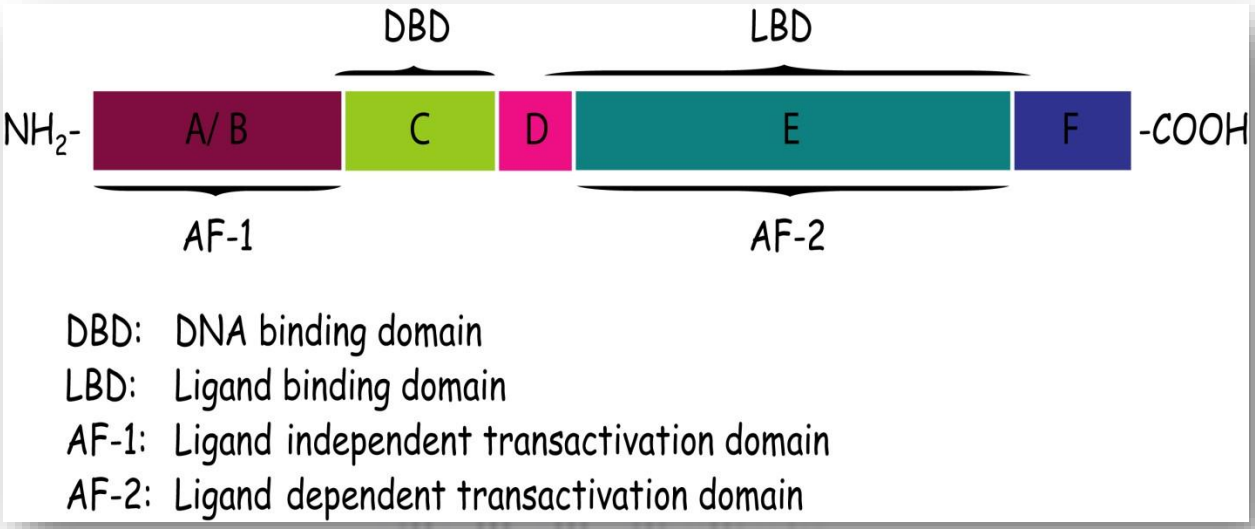

Figure 5: Functional domains of the androgen receptor (AR): N-terminal domain, DNA binding domain (DBD), Ligand binding domain (LBD) and hinge region (D).

The nuclear receptors had been subdivided into three general types (McKenna *et al.,* 1999, Mangelsdorf *et al.,* 1995). The classical steroid receptors such as the AR and progesterone receptor (PR) are nuclear receptors typically from ligand-induced homodimers which binds to inverted to repeat DNA response elements (Heinlein & Chang, 2002). The second type of the nuclear receptors dimerize with the 9-cis retinoic acid receptors for vitamin D_3 (VDR), thyroid hormone (TR) and the peroxisome proliferation-activated receptors (PPAR), whilst the DNA response elements of this type of nuclear receptors are characteristically direct repeats (McKenna *et al.,* 1999). The last type of nuclear receptors are the orphan receptors such as testicular receptor4 (TR4), testicular receptor2 (TR2) and chicken ovalbumin upstream promoter transcription factor (COUP-TF). The phosphorylation has been shown to modify the ligand-induced activity of steroid receptors. However, it has become clear recently that at least some

nuclear receptors may also turn to active transcription independently of their relative ligand through phosphorylation (Kato *et al.,* 1995), even though the physiological influence of ligand-independent activation has yet to be established (Heinlein & Chang, 2002).

The AR embraces four functional domains: The N-terminal transcriptional regulation domain, the DNA binding domain (DBD), a short hinge region and COOH-terminal domain (CTD) which includes both the AR ligand-binding domain (LBD) and AF-2 co-activator binding surface (Dehm & Tindall, 2007) (Figure 5). The N-terminal domain (NTD) is the most variable between all nuclear receptors in terms of length and sequence, whilst the DNA binding domain (DBD) is the most conserved region between all the steroid hormone nuclear receptors family. The DBD of all the steroid hormone nuclear receptors consists of two zinc fingers that recognize particular DNA consensus sequences. Where these zinc fingers work to facilitate direct DNA binding of the AR to the promoter and enhancer regions of AR-regulated genes, that way allows the activation functions of the N-terminal and ligand-binding domains to stimulate or inhibit the transcription of these genes (Heinlein & Chang, 2002).

The DNA binding domain (DBD) is connected to the ligand-binding domain (LBD) by a hinge region. The ligand-binding domain (LBD) also has an identical structure between the nuclear receptors and mediates the interaction between the AR heat shock and chaperone proteins, additionally interacting with the N-terminus of the AR to stabilize bound androgens (Heinlein & Chang, 2002). Inside the AR, two transcriptional activation functions have been identified: the ligand-independent AF-1, located in the N-terminal domain which is desired for maximum activity of the AR (Trapman et al., 1988). The ligand-dependent AF-2, located in the ligand-binding domain (LBD) which is required for forming the co-regulators binding site as well as mediating direct interactions between the N-terminal and the ligand-binding domains (N/C interactions) (Gottlieb et al., 2001).

The role of the AR in prostate cancer

The AR has been widely studied to clarify its biological role in the development and progression of prostate cancer. The differentiation, growth and maintenance of the prostate tissue are dependent on ARs and the androgens in general, over-activation of the AR signalling pathway will lead to the progression of prostate cancer. Although the androgen deprivation results in dramatic depression of a number of prostate secretory cells through apoptosis, there is still some evidence to indicate that prostate cancer cells acquire a relative resistance to androgen ablation-induced apoptosis early in transformation and androgen primarily regulates the proliferation of the prostate cancer cells (Heinlein & Chang, 2004). Several studies for human prostate cancer cells obtained recently after the inception of the androgen deprivation has demonstrated that prostate cancer appears to be morphologically changed by the androgen deprivation but is not subjected to necrosis or apoptosis (Reuter, 1997).

The amplification of the AR gene has been proposed as a technique which enables prostate cancer cells to be sensitive to the reduced level of androgens present after the androgen ablation therapy, therefore with the association between AR amplification and hormone-refractory prostate cancer some investigators suggest that a selection for increased AR gene copy number may occur under conditions of androgen deprivation because an elevated level of AR gene expression could contribute to the ability of cancer cells to proliferate in a reduced androgen environment (Koivisto et al., 1997) (Wallén et al., 1999). Even though the AR amplification has resulted in increased AR protein expression, but it does not seem to affect survival after androgen deprivation for advanced prostate cancer.

Moreover, the transcriptional activity of the AR can be affected by coregulators, which have an influence on a number of functional properties of the AR including the ligand specificity and the DNA-binding capacity. These coregulators could significantly modify the events of prostate cancer development and progression, but do not bind to the DNA. Recently, the AR ligand-dependent transcriptional factor has been found to be modifying by large number of coregulators

(Rahman *et al.,* 2004). Table 1 summarizes those AR coregulators that can affect the AR transactivation in prostate cancer cells.

Table 1: Summarizes the androgen receptor AR coregulators and the target region (Rahman et al., 2004).

Coregulator	AR region
ARA24	**N-term**
ARA54	**LBD**
ARA55	**LBD**
ARA70	**DBD-LBD**
ARA160	**N-term**
ARA267	**N- and C-term**
β-Catenin	**ND**
Gelsolin	**LBD**
Supervillin	**N- and C-term**
Smad3/Smad4	**ND**
ARA67	**N-term**

***NOTE: ND means Not Determined.**

Data partially obtained and adapted from (Rahman *et al.,* 2004).

Ultimately, the AR coregulators could be required for optimal AR function in the progression of any AR-related diseases as well as hormonal resistance to prostate cancers. As the AR coregulators physically interact with the androgen receptor and modulate its transcriptional activity, the interruption of the AR coregulator interactions reveals a need for developing new therapeutic agents to regulate both androgen-dependent and androgen-independent prostate tumours (Rahman et al., 2004).

Apoptosis

Apoptosis characterized by distinct morphological features such as shrinking of the cell, nuclear fragmentation, protrusion (blebbing) of the cell membrane and phagocytosis by neighbouring cells (Hiramine, 1997). The apoptosis is recognized by alteration in the size and the shape of the cells as the cells are smaller, the cytoplasm is dense and the organelles are tightly packed, as well as thickening of the nuclear chromatin which becomes marginated against the nuclear membranes. Afterwards, the nucleus becomes condensed and breaks up (Karyorrhexis), and subsequently the cells detach from the surrounding tissue to form extensions (apoptotic bodies). The apoptotic bodies have well-preserved membrane and mitochondria which give those bodies the ability to phagocytosed into neighbouring cells. If the crumble cell is not phagocytosed, it would undergo degradation which features a process called secondary necrosis (Kerr et al., 1994). Most of these changes are recognizable by light microscopy.

Apoptosis is linked to a number of proteolytic enzymes called caspases, which have important roles in protein digestion. Caspases trigger cell death by splitting specific proteins in the cytoplasm and nucleus. Caspases have been found in all cells as procaspases (inactive precursors), which can usually be activated through cleavage by other caspases (Alberts *et al;* 2002). The activation process is initiated either by intracellular death signals which are regulated by members of the Bcl-2 and IAP (inhibitor of apoptosis) protein families, or extracellular death signals.

The palm oil

The scientific relation between dietary fats and cancers has pointed out a growing research interest in palm oil, which is the second-largest consumed vegetable oil in the world. Palm oil contains 50% saturated fatty acids that do not promote adverse effects such as atherosclerosis, arterial thrombosis and oxidation, and it also has high amount of antioxidants and vitamin E (Ebong et al., 1999).

The palm oil has been extracted from the palm tree "Elaeis guineensis" from the orange-red mesocarp of the fruit, the tree has an unbranched stem and belongs to the Palmae family (Edem, 2002). These trees produce large quantities of red palm oil. Two distinct oils are produced by palm tree (Palm Kernel Oil and Crude Palm Oil), both of which are important in world trade (Edem, 2002). The “Palm Kernel Oil” (PKO) is extracted from the seeds of the palm fruit which contains about 50% oil, it is a light yellow oil (Mancini et al., 2015). The second type is the “Crude red Palm Oil” (CPO) is the fresh oil obtained from the mesocarp which has healthy beneficial compounds, such as triacylglycerols (TAGs), vitamin E, carotenoids and tocotrienols which are potent fat-soluble, as well as impurities such as free fatty acids (FFAs) phospholipids and lipid oxidation products (Mancini et al., 2015). Physical refining of CPO is preferred over the chemical refining. The latter takes place at the palm oil refinery to remove the undesirable impurities and involves steps like bleaching and deodorizing which are an integral process of refining palm oil and can only exceptionally be erased if the oil to be refined is very low in components requiring removal. (Gee, 2007).

The main component of crude palm oil is triacylglycerol, where all unsaturated fatty acids in palm oil triacylglycerol are Cis configuration. 1-palmitoyl-2,3-dioleoyl-sn-glycerol and 1,2dipalmitoyl-2-oleyl-sn-glycerol are the main triacylglycerols in palm oil. The TAGs composition reveals that sn-2 position of the palm oil triacylglycerols is mainly esterified with unsaturated fatty acid (oleic acid and linoleic acid). Therefore the fatty acids at the sn-1 and sn-3 position are hydrolysed into fatty acids and 2-monoacyl-sn-glycerols during digestion, while the sn-2 position of the palm oil triacylglycerols will remain intact when converted into body fat (Gee, 2007).

In particular, CPO is the most valuable natural source of carotenoids and tocotrienols. The characteristic colour of the red palm oil is due to the plenty of these carotenoids (500-700mg/L). The main carotenoids present in CPO are β-carotene (56%) and α-carotene (35%). Both β-carotene and α-carotene are provitamin A, which can be converted to vitamin A that plays an important role in genetic regulation. Table 2 summarizes the carotenoid composition of CPO.

Table 2: Carotenoid composition of CPO

Carotene	composition %
β-carotene	56.02
α-carotene	35.16
ϒ-carotene	0.33
δ-carotene	0.83

Data partially obtained and adapted from (Gee, 2007).

The carotenoids are destroyed during the regular refining processes. Processes are available to refine red palm oil without destroying the carotenoids (Ji et al., 2015). Furthermore, the carotenoids jointly with vitamin E, ascorbic acid, enzymes and proteins are members of the biological antioxidant network, because of their ability to act as effective quenchers of singlet oxygen. Thereby protecting against oxidative damage to the cells.

Carotenoids have been suggested to have possible inhibitory effects on the development of certain types of cancers. Their roles in inhibiting the proliferation of several types of cancers such as oral, lung, brain, stomach and pancreatic cancers have been investigated (Nagendran et al., 2000) (Kamat & Devasagayam, 1995). Besides it was found that α-carotene has more potent inhibitor for skin and liver cancer than β-carotene. Interestingly, the whole bouquet of palm carotenoids had greater inhibitory effect than the isolated carotenoids.

The palm oil is the only vegetable oil available in the world market that is rich in tocotrienols. The tocotrienols have been highlighted in the prevention and therapy of certain forms of cancers (Kamat & Devasagayam, 1995) (Srivastava & Gupta, 2006). More than 80% of the vitamin E (Tocopherols and Tocotrienols) present in crude palm oil, the four major types of vitamin E present as α-tocopherols, α-tocotrienol, ϒ-tocotrienol, and δ-tocotrienol. In particular, their inhibitory effect on human prostate cancer cells is encouraging, as well as an important role in suppressing the progression of many types of cancers (Srivastava & Gupta, 2006). Tables 3 summarize the vitamin E composition of CPO.

Table 3: Vitamin E composition of CPO.

Component	(ppm)
Vitamin E	716
α-tocopherol	158
α-tocotrienol	143
ϒ-tocotrienol	329
δ-tocotrienol	86

Data partially obtained and adapted from (Gee, 2007).

An insight into the association between palm oil and solid tumours.

Tocopherols and tocotrienols have been demonstrated to possess anticancer properties in several studies (Nesaretnam, 2008). Tocomin is a commercially available mixture of naturally containing 78% of tocotrienols and 22% of tocopherols which is extracted from the palm oil. As the palm oil is mostly rich in tocotrienols, it has been shown to have more powerful anticancer and antioxidant properties than the tocopherols. The effects of the Tocomin on human breast cancer cell lines (MCF-7 and MDA-MB231) and human non-transformed epithelial cells (MCF-10A) viability and proliferation were determined (Tran et al., 2014). This study demonstrated that Tocomin inhibits the cell proliferation and induces apoptosis in breast cancer cells (MCF-7 and MDA-MB231), but did not observe any cell death with Tocomin in the noncancerous (MCF-10A) cells.

A study has reported that the tocotrienols are potent cancer chemotherapeutic and preventive agents in both *in vitro* and *in vivo* conditions, yet their accurate mechanisms of action on cell death and other inhibitory pathways are unknown (Ling et al., 2012). Different mechanisms have been suggested including modulation of the immune response, blocking oxidative stress or suppression of the ceramide synthesis (Kannappan et al., 2012).

Although studies for tocotrienols and its bioactivity are scarce, a group of researchers have shown evidence that β- tocotrienol had also inhibited the growth of both human lung and brain cancer cell lines. The study investigated the human lung adenocarcinoma A549 and the glioblastoma U87MG cells (Lim el at., 2014). These cancer cells were incubated with β-tocotrienol and have

demonstrated the hallmarks of apoptosis including membrane blebbing, chromatin condensation and formation of apoptotic bodies. The study confirmed the anti-proliferation effect and DNA damages on both lung and brain cancer cell lines without significantly affecting the normal cell lines. Hence, the apoptotic properties of the tocotrienols were significantly approved of its capability to induce the double-strand DNA breaks (DSBs) without involving the single-strand DNA breaks (SSBs) (Lim et al., 2014).

For a decade, epidemiological researchers specified a significant association between the palm oil components and the anti-proliferative effects on the cancer cells. Moreover, it has been reported that a palm oil-derived product called Bio-12 has anti-proliferation properties against human malignant melanoma cell lines (MeWo), the Bio-12 identifies as lipid esters derived from a range of fatty acid of palm oil (Komarasamy & Sekaran, 2012). The Bio-12 has successfully suppressed the growth of the melanoma cells (MeWo) in a concentration and time-dependent manner but without any effects for the human normal skin fibroblasts. Therefore, the Bio-12 was also tested whether could cause cell cycle redistribution, interestingly the results showed that at different concentrations and time frames the BIO-12 brings about significant cell cycle arrest at the S-phase and then induction of apoptosis in melanoma cells (MeWo) (Komarasamy & Sekaran, 2012).

The deregulation of the cell cycle is one of the critical events that drive the cancer cells into uncontrolled proliferation which is a hallmark of cancer (Evan & Vousden, 2001). The important roles of deregulation in tumorigenesis, the cell cycle regulatory molecules serve as ultimate targets for therapeutic intervention in cancers. In this regard, the palm oil contains functional components which have been reported to possess certain biological activities such as high antioxidant and anticancer properties.

Aim of the study

Recent research data by different investigators have pointed out the chemotherapeutic effects of red palm oil on various cancer cell lines, however, there is a paucity of relevant data on the

investigation of the effects of red palm oil on prostate cancer cells. Therefore, the current study seeks to evaluate the *in vitro* effects of red palm oil on testosterone-sensitive prostate LNCaP cancer cells and benign hyperplasia prostate PWR-1E (epithelial) cells based on the following parameters

- To evaluate the effect of red palm oil on cell proliferation using MTT assay.

- To test the effect of red palm oil on the expression of prostate-specific antigen (PSA) in transformed and non-transformed prostate cell lines using ELISA techniques.

- To gain an insight into the effect of red palm oil on the expression profile of 3 genes (POU3F2, PEG10 and SRRM4) related to cancer progression and tumour resistance using RT-PCR.

The ultimate aim is to gain an insight into the potential value of red palm oil as a natural anti-cancerous agent for the treatment of prostate cancer.

2. Material and Methods

Testing the effect of red palm oil on cell proliferation

Collection and preparation of the Palm Oil

The Nutro Palm Oil (high in vitamin A & E) was a free gift from Malaysian Palm Oil Board Ltd (Selangor, Malaysia). 200,000 µg of the palm oil was dissolved in 1 ml sterile Dimethyl sulfoxide (DMSO) (Merck- Wadeville; Gauteng, South Africa). The DMSO was used to facilitate the dissolution of the red palm oil in a culture medium. The concentration of the stock solution was 200,000 µg/ml. This stock was further diluted with culture medium so that the final highest concentration of DMSO in all samples was less than 0.5%. The control samples contained the same concentration of DMSO diluted in culture medium. All preparations were performed under sterile working conditions inside a laminar flow, aliquoted into sterile Eppendorf tubes and stored at 40C until further use.

Cell culture

LNCaP prostate cancer and PWR-1E benign prostatic hyperplasia cell lines were used to investigate the biological effect of the extract in this study. These cells were cultured at 37^0C under a 5% CO_2 humidified atmosphere. LNCaP cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium, supplemented with 10% fetal bovine serum (FBS), 1% penicillin (100 IU/ml) and streptomycin (100 µg/ml), in 25 or 75 cm^2 culture flasks. PWR-1E cells were cultured in Keratinocyte Serum-Free Medium (K-SFM), supplemented with 0.05 mg/ml bovine pituitary extract (BPE) and 5 ng/ml human recombinant epidermal growth factor (EGF).

LNCaP prostate cancer cell line

The LNCaP cell line is an androgen-sensitive human prostate adenocarcinoma cell derived from the left supraclavicular lymph node metastasis from a 50-year-old male in 1977. This cell line was

established from a metastatic lesion of human prostatic adenocarcinoma, and it is an ideal model for investigating early prostate cancer (Horoszewicz *et al.,* 1980) Figure 6.

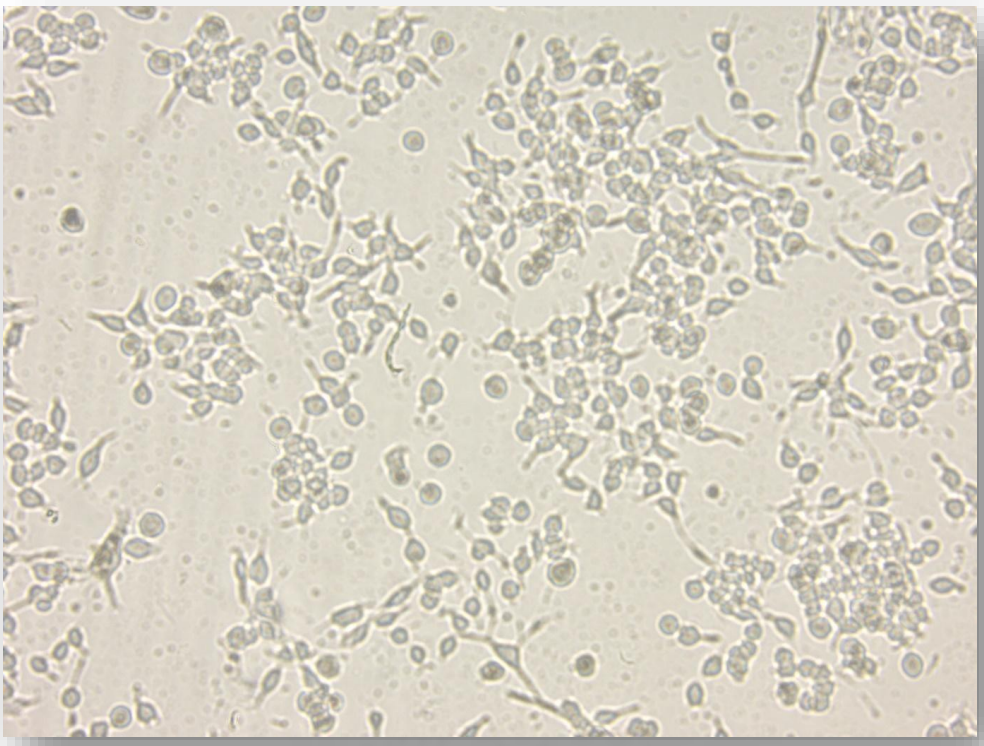

Figure 6: Normal morphology of LNCaP prostate cancer cells

Benign (PWR-1E) prostate cell

PWR-1E has established to express many characteristics of normal prostatic epithelial cells. The most remarkable characteristics of PWR-1E cells are growth stimulation, increased expression of androgen receptor and induction of PSA expression in response to androgens, which indisputably establish their prostatic epithelial origin. (Webber *et al.,* 1996) Figure 7.

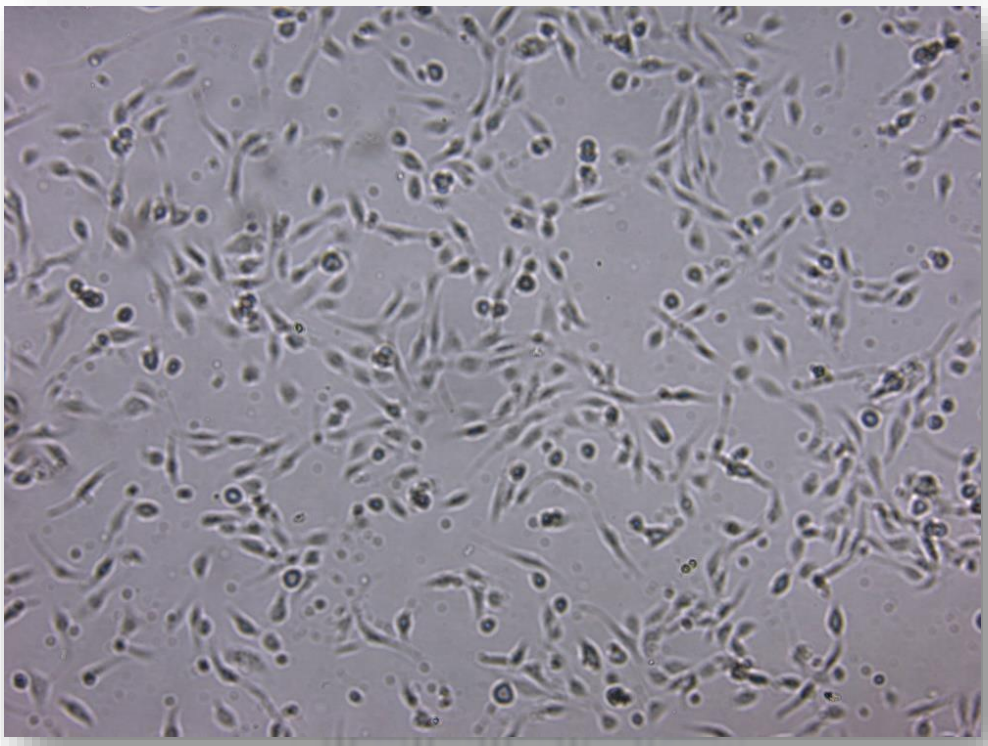

Figure 7: Normal morphology of PWR-1E Benign prostatic hyperplasia cells stacking with a slightly less flat and polygonal morphology.

Culture of LNCaP cell line

Cells were cultured in 25 or 75 cm^2 sterile tissue culture flask and were passaged at regular intervals when cells reach approximately 80-90% confluency. The culture medium was aspirated from the flasks and cells were washed with 2-4 ml sterile PBS (phosphate-buffered saline). Thereafter, 2-3 ml 1×0.25% trypsin/EDTA were added to the flask, gently shaken and then incubated at 37^0C under a 5% CO_2 humidified atmosphere until cells began to detach. This process took approximately 4-7 minutes. In order to inactivate the trypsin, 3-4 ml of RPMI 1640 containing 10% fetal bovine serum were added and cells were re-suspended by careful aspiration using a pipette. Eventually, the cell suspension was transferred from the flask to 15 ml test tube and centrifuged at 100× g for 10 minutes.

The supernatant was discarded and the cell pellet suspended in 4 ml of RPMI 1640 containing 10% FBS. Following this, 2 ml of the cell suspension was transferred into a new tissue culture flask with fresh RPMI 1640 growth medium with supplements as mentioned previously and passage recorded. Afterwards, LNCaP cells, which appeared to be growing at a slow rate, were left for up to 48 hours to re-attach. Fresh medium was then added to the first flask and incubated for further growth. The cells were then either passaged into new 25 and 75 cm^2 flasks, respectively, with fresh medium or seeded into 6-, 24- or 96-well plates. A dilution of cells was made to produce the final cell number required for experiments.

Culture of PWR-1E cell line

Cells were cultured in 25 or 75 cm^2 sterile tissue culture flasks and were passaged at regular intervals when cells reached approximately 80-90% confluency. The culture medium was aspirated from the flasks and cells were washed with 2-4 ml sterile PBS. Thereafter, 2-3 1×0.25% trypsin/EDTA was added to the flask, gently shaken and then incubated at 37^0C under a 5% CO_2 humidified atmosphere until cells began to detach. This process took approximately 4-7 minutes under occasional visual control. In order to inactivate the trypsin, 4 ml of keratinocyte serum-free media were added and cells were re-suspended by careful aspiration using a pipette. Finally, the cell suspension was transferred from the flask to 15 ml test tube and centrifuged at 125 ×g for 5 minutes. The supernatant was discarded and the cell pellet re-suspended in 5 ml keratinocyte serum-free media. Following this, 2 ml of the cell suspension was transferred into a new tissue culture flask with fresh keratinocyte serum-free media and passage recorded. The cells were then either passaged into a new 25 and 75 cm2 flasks, respectively, with fresh medium or seeded into 6-, 24- or 96-well plates. A dilution of cells was made to produce the final cell number required for experiments; cell morphology was observed and compared with cell viability.

Cell counting and seeding

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

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

Cell freezing

LNCaP cells

Cells were grown approximately 80-90% confluency. The growth medium was removed and cells washed with 4 ml sterile PBS. Thereafter, 1-2 ml of 0.25% trypsin were added into cell culture flask and incubated at 37°C under a 5% CO_2 humidified atmosphere until cells began to detach. This process took 4-7 minutes and was performed under intermittent visual control.

Once cells detached, 2 ml of complete growth medium was added then carefully re-suspended by aspiration, counted and then transferred to a 15 ml test tube to be centrifuged at 100 × g for 10 minutes. Subsequently, the supernatant was removed, and the cell pellet suspended in 5 ml freezing medium (85% RPMI 1640, 10% FBS and 5% DMSO), cells suspended into cryogenic vials. The cryogenic vials were placed into a Styrofoam box at 4^0C for 30 minutes and then immediately transferred to -80^0C for 24 hours before transferred to liquid nitrogen for long-term storage.

PWR-1E Cells

Cells were grown approximately 80-90% confluency. The growth medium was removed and cells washed with 4 ml sterile PBS. Thereafter, 1-2 ml of 0.25% trypsin were added into cell culture flask and incubated at 37^0C under a 5% CO_2 humidified atmosphere until cell began to detach. This process took 4-7 minutes and was performed under intermittent visual control.

Once cells detached, 2 ml of completed growth medium was added then carefully re-suspended by aspiration, counted and then transferred to a 15 ml test tube to be centrifuged at 125 × g for 5 minutes. Subsequently, the supernatant was removed and cell pellet re-suspended in 5 ml freezing medium (supplemented Keratinocyte Serum Free K-SFM and 7.5% DMSO), cells suspended into cryogenic vials. The cryogenic vials were placed into a Styrofoam box at 4^0C for 30 minutes and then immediately transferred to -80^0C for 24 hours before transferred to liquid nitrogen for long-term storage.

Cell thawing

LNCaP Cells

To plate the cells, the stored vial containing the frozen LNCaP cells was rapidly placed into a water bath heated to 37^0C. Once thawed, the cells were transferred to a 15 ml test tube containing 10 ml (RPMI 1640 medium supplemented with 10% FBS and 1% penicillin, streptomycin). The suspension was then centrifuged at 100 × g for 10 minutes. The supernatant was discarded and the cell pellet re-suspended in 1 ml complete fresh medium and finally transferred to a 75 cm^2 culture flask and placed into the incubator for 48 hours.

PWR-1E

To plate the cells, the stored vial containing the frozen PWR-1E cells was rapidly placed into a water bath heated to 37^0C. Once thawed, the cells were transferred to a 15 ml test tube

containing 10 ml (supplemented keratinocyte serum-free media). The suspension was then centrifuged at 125 × g for 5 minutes. The supernatant was discarded and the pellet suspended in 1 ml complete fresh medium and finally transferred to 75 cm^2 culture flask and placed into the incubator for 48-hours.

Determination of cell viability

The viability of the malignant LNCaP and benign PWR-1E prostate cells was measured using MTT (Thiazoyl blue tetrazolium) assay. This colourimetric assay uses reduction of a yellow tetrazolium salt to measure cellular metabolic activity as a proxy for cell viability. MTT assay dependents on the cellular reduction of yellow MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) by mitochondrial dehydrogenases of viable cell to a blue formazan product which can be measured spectrophotometrically.

MTT stock solution was prepared by dissolving 1mg MTT/ml PBS. The MTT solution was gently mixed and filtered. Then, was wrapped with aluminium foil to prevent exposure to direct light because the solution is photosensitive, and this solution was stored at -20^{0}C.

The volume of 20µl was added to each well plate containing 200µl of the extracts and incubated for 3 hours at 37^{0}C. Thereafter, the supernatant was removed and 150µl sterile DMSO was added to each well to dissolve the precipitated dye.

The absorbance of the dye was measured with an ELISA reader (GLoMax Multi Detection System) at a wavelength of 560 nm with a reference wavelength of 750 nm. The optical density (OD) was calculated by subtracting the absorbance of the reference wavelength from the absorbance of the test wavelength. Absorbance readings from test samples then divided by the average of the control and multiplied by 100 to get percentage cell viability.

$$\text{Percentage viable cells} = \frac{\text{Absorbance sample}}{\text{Absorbance control}} \times 100$$

Average of the control sample

Testing the effect of the Red Palm Oil on LNCaP viability

LNCaP cells were grown to 80% confluency and then trypsinated with 2 ml 0.25% trypsin/EDTA and incubated for 4-7 minutes. Afterwards, the trypsin was inactivated by adding 2 ml of RPMI 1640 growth medium and cell counting was performed. Subsequently, cells were seeded into sterile 96-well plates at 5 × 103 cells/well and 2×103 cells/well in 200 µl of complete growth medium for a 24-hours and 72-hours exposure, respectively. The cells were then allowed to attach for 24 hours, following this, the medium was removed and the cells were exposed to different concentrations (0.1, 1, 10, 100, 500, 1000 µg/ml) of the Red Palm Oil for 24-hours and 72-hours, the final Dimethyl sulfoxide (DMSO) concentration was 0.5%. Control was treated with 0.5% dimethyl sulfoxide (DMSO) without the red palm oil. After 24-hours post-treatment, the medium was removed and 200 µl fresh growth medium was added, in addition to 20 µl of MTT (1mg MTT/ml PBS) to each well. The plates were incubated at 37^0C for an additional 3 hours. Subsequently, the medium was removed from the wells and the formazan crystals formed by the reduction of MTT in living cells were solubilized in 100 µl of dimethylsulfoxide (DMSO). Thereafter, the absorbance of the samples was measured at a wavelength of 560 nm with a reference wavelength of 750 nm.

Testing the effect of the Red Palm Oil on PWR-1E viability

PWR-1E cells were grown to 80% confluency and then trypsinated with 2 ml 0.25% trypsin/EDTA and incubated for 4-7 minutes. Afterwards, the trypsin was inactivated by adding 2 ml of keratinocyte serum-free media and cell counting was performed. Subsequently, cells were seeded into sterile 96-well plates at 5×10^3 cells/well and 2×10^3 cells/well in 200 µl of complete growth medium for a 24-hours and 72-hours exposure, respectively. The cells were then allowed to attachment for 24 hours, following this, the medium was removed and the cells were exposed to different concentrations (0.1, 1, 10, 100, 500, 1000 µg/ml) of the Red Palm Oil for 24-hours and 72-hours, the final dimethylsulfoxide (DMSO) concentration was 0.5%. Control was treated

with 0.5% dimethylsulfoxide (DMSO) without the red palm oil. After 24-hours post-treatment, the medium was removed and 200 µl fresh growth medium was added, in addition to 20 µl of MTT (1mg MTT/ml PBS) to each well. The plates were incubated at 37^0C for an additional 3 hours. Subsequently, the media was removed from the wells and the formazan crystals formed by the reduction of MTT in living cells were solubilized in 100 µl of dimethylsulfoxide (DMSO). Thereafter, the absorbance of the samples was measured at a wavelength of 560 nm with a reference wavelength of 750 nm.

The expression of prostate-specific antigen (PSA) via ELISA

The production of PSA is largely regulated by the androgen-dependent activation of the androgen receptor in prostate cells both, normal and malignant prostate epithelial cells (Yousef & 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). PSA Levels of 4.0 ng/ml or higher are strong indicators of the possibility of prostatic cancer (Lucia et al., 2004).

In order to determine PSA levels, both LNCaP and PWR-1E cells were plated at a density of 5x104 cells / well in 1 ml of complete RPMI 1640 medium (supplemented with 10% foetal bovine serum (FBS) and 1% penicillin, streptomycin) and keratinocyte serum-free media, 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 red palm oil at different concentration for 24 and 72 hours, respectively. Thereafter, the cell culture supernatants were collected, stored in Eppendorf vials at -20^0C until PSA determination.

On the day of PSA determination, all reagents, standards and samples were brought to room temperature (18^0C - 25^0C). In the meantime, the standards and the samples were loaded on the microplate to be tested. Samples were tested in duplicates. Positions of wells together with their respective samples, standards and controls were documented to ensure later identification. Unused microwell modules were zipped into a locked bag with desiccant and stored at 5^0C.

Following this, 25 µl of standards controls or samples were pipetted into each well. Samples and standards were then incubated at the room temperature for 5 minutes.

Thereafter, 100 µl of PSA conjugate was added into each well. The plate was agitated manually on the table for 10 seconds in order to mix thoroughly. The plate was then incubated for 1 hour at room temperature (18^0C - 25^0C) whereupon solution from the wells was removed by decanting the plate and tapped on absorbent paper in order to remove residual liquid. Thereafter, the wells were washed 5-6 times using 250 µl distilled water per well. In every wash, the water was allowed to settle in the wells for 15 seconds before removing it. Following this, 100 µl Tetramethylbenzidine (TMB) substrate solution was then pipetted into each well followed by 20-minute incubation at room temperature (18 °C - 25 °C). Subsequently, 100 µL/well stop solutions were added to the wells in the same order as the substrate solution. Eventually, the absorbance was read at 450 nm with an ELISA reader (GLoMax Multi Detection System) and the concentration of PSA in LNCaP and PWR-E 1 were determined according to the standard curve below Figure 8.

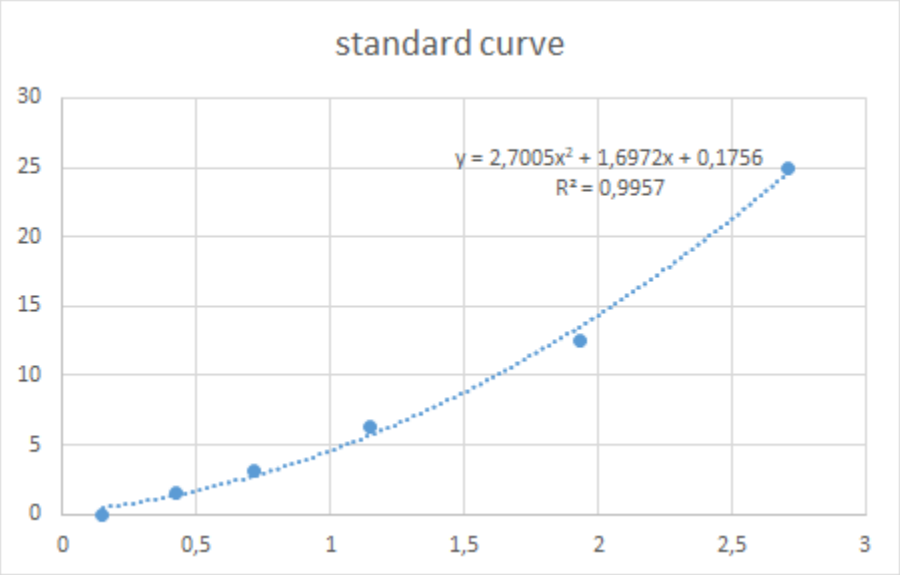

Figure 8: Standard curve for total serum prostate-specific antigen ELISA in LNCaP and RPW-E1 cells. The standard curve shows that there is a good correlation (R^2 = 0.9957) between absorbance and PSA concentration.

The expression of androgen receptors and prostate-specific antigen (PSA) via PCR:

Primer Design

Species-specific primer was designed for each of the 3 target genes of interest; the member of the POU homeobox 2 of neural transcription factors class 3 (POU3F2 also known as BRN2), paternally expressed 10 (PEG 10), serine/arginine repetitive matrix 4 (SRRM4) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a housekeeping gene.

The NCBI (National Centre for Biotechnology Information) gene database was used to design the primers. The NCBI gene database can be accessed at https://www.ncbi.nlm.nih.gov./gene. Using the primer BLAST, primers designed for mRNA of the target genes, verifying that the primers span the exon-exon junctions. Table 4 summarizes the primers information and Appendix II for more information regarding the primers. Subsequently, the primers were purchased from (Inqaba Biotech Industries “Pty” Ltd), and were diluted to obtain 100 µM stock solution then stored at -20^0C until required.

Table 4: Table presenting the primer sequences, predicted product length and melting temperatures used for the target genes of interest.

Gene	Product Size	NCBI Reference Number	Primer Sequence	Tm
GADPH	290bp	NM_001289745	Forward 5'-AGAAGGCTGGGGCTCATTTG-3' Reverse 5'-AGGGGCCATCCACAGTCTTC-3'	53.83^{0}C 55.88^{0}C
POU3F2	260bp	NM_005604.3	Forward 5'-TTTGCAAGATAAATGGTGAC-3' Reverse 5'-CTGTAGACACATTCTCTGAA-3'	54.25^{0}C 56.30^{0}C
PEG 10	253bp	NM_001040152.1	Forward 5'-CAACCTATATAAGGCTCACA-3' Reverse 5'-CTTATTTCACGCGAGGAC-3'	56.30^{0}C 57.62^{0}C
SRRM 4	310bp	NM_194286.3	Forward 5'-ACAAGAAACAATCTCGAAGC-3' Reverse 5'-GCTGTTTTGGTAAAGAGGT-3'	56.30^{0}C 55.85^{0}C

Cell Culture

Both LNCaP and PWR-1E cells were cultured in T75 cell culture flasks containing complete RPMI 1640 medium (supplemented with 10% fetal bovine serum and 1% penicillin, streptomycin) and Keratinocyte serum-free medium, respectively. Moreover, they were both preserved at 37^{0}C under a 5% CO2 humidified atmosphere. Media was replaced when cell growth reached 80-90% confluency. Hence, once the cells were ready for splitting, the old media was aspirated from the flasks and the cells were washed with 2-4 ml sterile PBS (phosphate-buffered saline). Thereafter, 2-3 ml 1×0.25 trypsin EDTA was added to the flasks, and then incubated at 37^{0}C until the cells began to detachment (not longer than 5 minutes). Once the detachment of cells microscopically confirmed, the trypsin was inactivated by adding 3-4 ml of complete RPMI 1640 media and Keratinocyte serum-free media to the malignant LNCaP and normal PWR-1E prostate cells, respectively. Following that, the cells suspension was transferred from the flask to 15 ml test tube and centrifuged at 125×g for 5 to 7 minutes. Thereafter, the supernatants were discarded and the pellets were suspended in 1 ml of the appropriate media. A 20µl of the concentrated cells suspension was then mixed with 20µl of the trypan blue, then transferred to a

haemocytometer to calculate the number of cells. The concentrated cells suspension was then diluted to produce a concentration of 1,000,000 cells/ml. Thus these new cells suspension were cultured into T75 flasks (1 ml per flask), 5 ml of complete RPMI 1640 medium and Keratinocyte serum-free media were added to the LNCaP and PWR-1E flasks, respectively. These flasks were then incubated at 37^0C under a 5% CO_2 humidified conditions for 24 hours to allow the cells to re-attach and recover from the trypsinization process.

After the 24-hours recovery period, old media were removed and replaced with 5 ml of fresh medium with the Red Palm Oil at both IC_{25} and IC_{75} values based on MTT data analysis using the latest GraphPad Prism software. Considering the Red Palm Oil samples were solubilized in dimethylsulfoxide (DMSO), the final dimethylsulfoxide (DMSO) concentration was 0.5% for all treatments. Thereafter, the treatments used were as follows; negative control (medium with 0.5% DMSO), IC_{25} as 400 µg/ml Red Palm Oil in media with 0.5% DMSO and IC_{75} as 1043 µg/ml Red Palm Oil in media with 0.5% DMSO. These flasks were then incubated at 37^0C under a 5% CO2 humidified conditions for another 24 hours.

RNA Extraction

After 24-hour of treatment with red palm oil, the following steps were carried out under non-sterile conditions. The treatment medium was removed and each flask was washed with 3 ml of PBS (phosphate-buffered saline). The PBS was also removed and replaced with 2 ml of Tripure Isolation Reagent (Munich, Germany) to each flask, smoothly flashed for a few times with the tri-RNA reagent until all the cells were detached. Subsequently, each 1 ml of the tri-RNA-cell supernatant was transferred to an appropriately labelled 2 ml centrifuge tube. Those tubes were then vortexed for a minute before being incubated for five minutes at room temperature. Following this, 200 µl of chloroform was added to each centrifuged tube and vortexed for 15 seconds before being incubated for 5-10 minutes at room temperature. The solutions were then separated into three phases by centrifugation at 12,000×g for 15 minutes at 4^0C. Consequently, 400-500 µl of the upper clear solution was transferred to appropriately labelled 1.5 ml centrifuge tubes. After this, 500 µl of isopropanol was added to each 1.5 centrifuge tube and completely

mixed, then allowed to precipitate the RNA overnight at -20^{0}C. In the following day, each tube was centrifuged at 12,000×g for 10 minutes at 4^{0}C and the supernatant was completely discarded.

Thereafter, 1 ml of 80% ethanol solution was added to each RNA pellet, vortexed for a minute and then centrifuged at 7,500×g for 5 minutes. Once again, the supernatant was carefully removed and discarded; without allowing the pellets to dry completely. After this, the RNA pellets were suspended in 100 µl of nuclease-free water. A sample from each RNA solution was then measured using NanoDrop 1000 (Thermo Scientific, USA) before determining how to dilute the RNA solutions for the reverse-transcription reaction. Eventually, the RNA samples were stored at -20^{0}C until required.

Reverse transcription reaction

Reverse transcription (RT) is a technique used to synthesize a complementary DNA (cDNA) from an RNA template with the use of the reverse transcriptase enzyme. The reverse transcription (RT) was carried out through the Takara Prime Script™ RT reagent kit (Takara, China) according to the manufacturer's instructions. The reverse transcription mix was prepared according to the table below.

Table 5: Table presenting the makeup of the master mix used to perform the reverse transcription reaction

Reagent	Initial concentration	Final concentration
5×PrimeScript Buffer	5×	1×
Prime Script RT enzyme Mix1	20×	1×
Oligo dT Primer	50 µM	25 pmol
Random hexamers	100 µM	50 pmol

Once the reverse transcription mix was prepared, it was diluted into aliquots of 3,5 µl into appropriately labelled PCR tubes. Next, the thawed RNA samples were added to a proper tube,

diluted with nuclease-free water to obtain a final concentration of 0.1 µg RNA/µl so that each tube contain 10 µl of the reverse transcription mix. Hence, the RNA together with the nuclease-free water reached to 6.5 µl in total per tube.

Thereafter, the reverse transcription mixes were replaced in a thermocycler and under the following temperature fluctuations: 37^0C for 15 minutes (reverse transcription), 85^0C for 5 seconds (heat inactivation of reverse transcriptase) and 4^0C for 5 minutes. Finally, once the RT reaction was completed, all the cDNA samples were stored at -20^0C until required.

Polymerase chain reaction PCR

The polymerase chain reaction was carried out by using the GoTaq**R** G2 Flexi DNA polymerase PCR kit (Promega, USA) according to the manufacturer's instructions, which set-up in a volume of 25 µl per sample. (2 µl) Thawed cDNA was transferred to PCR tubes containing the following PCR mix reagents in Table 6. A blank control was included in the sample set, it contained nuclease-free water instead of a cDNA sample.

Table 6: Table presenting the makeup of the master mix used to perform the polymerase chain reaction

Reagent	Final concentration	1× (µl)	12× (µl)
Water	N/A	17,275	201,3
Buffer	5×	5	60
MgCl2	1mM	1	12
dNTPs (10mM)	0.5mM	0,5	6
Taq polymerase	1.25u	0.125	1,5
Forward Primer (100mM)	0.2 µM	0.05	6
Reverse Primer (100mM)	0.2 µM	0.05	6
Total		25	292,8

Subsequently, the PCR tubes were placed in a thermocycler for 40 PCR cycles. Yet, this was progressed by 3 minutes' exposure at 95^0C for denaturation. The PCR cycles initiated with a 45

seconds exposure to 95^{0}C for denaturation, followed by 45 seconds at 52^{0}C for annealing and another 45 seconds at 72^{0}C for an extension.

Agarose gel electrophoresis

During the PCR cycling, a 1% agarose gel was prepared for agarose gel electrophoresis (AGE). Initially, a 10 × TBE (Tris, boric acid and EDTA) buffer which was prepared as follows: 54g of Tris base was added to 27,5g of boric acid and 4,65g of EDTA and made up to 500 ml reverse-osmosis water. Afterwards, the 10× TBE was diluted to 1× using Reverse Osmosis (RO) water.

1,5g of agarose powder was prepared and mixed with 150 ml of 1× TBE buffer. However, this was heated at the high power in a microwave for 2 minutes. Subsequently, the 1% agarose-TBE mixture was allowed to cool down before placing into a gel template. The gel was set at room temperature for approximately 3 minutes before transferring the gel to the electrophoresis chamber and covering the gel in 1 × TBE.

Finally, a 20 µl of each cDNA sample was mixed with 7 µl of Novel juice (GeneDirex, Taiwan), before transferring to the proper wells on the gel. In addition to 100 bp ladder (Promega, USA) was included in the gel; by mixing 5 µl of the ladder with 7 µl Novel juice before transferring to it's allocated well in the gel.

The gel was run at 100 V for 150 minutes before viewing. The gel was cooled in ice before transferring to BLook LED transilluminator (GeneDirex, Taiwan) and viewed in a dark room. The gels were captured to validate the PCR data.

Statistical analysis

All the statistical calculation was recorded and analysed statistically using the MedCalc statistical software version 18.2.1 (MedCalc Software, Mariakerke, Belgium). After testing for normal distribution by means of the Kolmogorov-Smirnov test, appropriate statistical tests, either

parametric (Pearson correlation, one-way ANOVA, repeated-measures ANOVA, t-test or nonparametric (Spearman Rank correlation, Wilcoxon test) were performed. Tukey tests were done to detect outliers. Data were expressed as mean±SD or mean±SEM. A P-value of P=0.0001 and P<0.05 was considered significant.

3. Results

Cell culture results

Effect of Red Palm Oil on Cell Viability

LNCaP, as well as RPW-E 1 cells, were exposed to increasing concentrations of aqueous dilution of Red Palm Oil for 24 and 72 hours respectively. During this period, morphological studies and colourimetric assay were done with the aim of investigating possible cytotoxic effects of red palm oil on malignant (LNCaP) or benign (RPW-E 1) prostate cells using light microscopy and the MTT assay. In this study, both prostate cell lines were exposed to different concentrations of red palm oil (0.1, 1, 10, 100, 500, 1 000 µg/ml) for 24 hours ($5x10^4$ cells/ml) and 72 hours ($2x10^4$ cells/ml).

The highest concentration of 1 000 µg/ml red palm oil was not used in later experiments because our data had proven this concentration to be very cytotoxic. This concentration completely damaged and killed both the normal RPW-E 1 and malignant LNCaP prostate cells. All experiments were repeated independently at least three times.

Malignant (LNCaP) prostate cell viability

After malignant LNCaP cells were exposed to increasing concentration of the red palm oil for 24 hours, the cell morphology was detected and recorded. No obvious changes in cell morphology were observed and cells maintained their flat and polygonal shapes in the negative control group and the cells treated with 10 µg/ml red palm oil extract. However, at higher concentrations (100, 500 and 1 000 µg/ml) and in the positive control (10 % DMSO), the cells began to show increasing visible signs of stress, such as loss of flat polygonal shape, shrinking and clumping indicating cellular death (Figure 3.9).

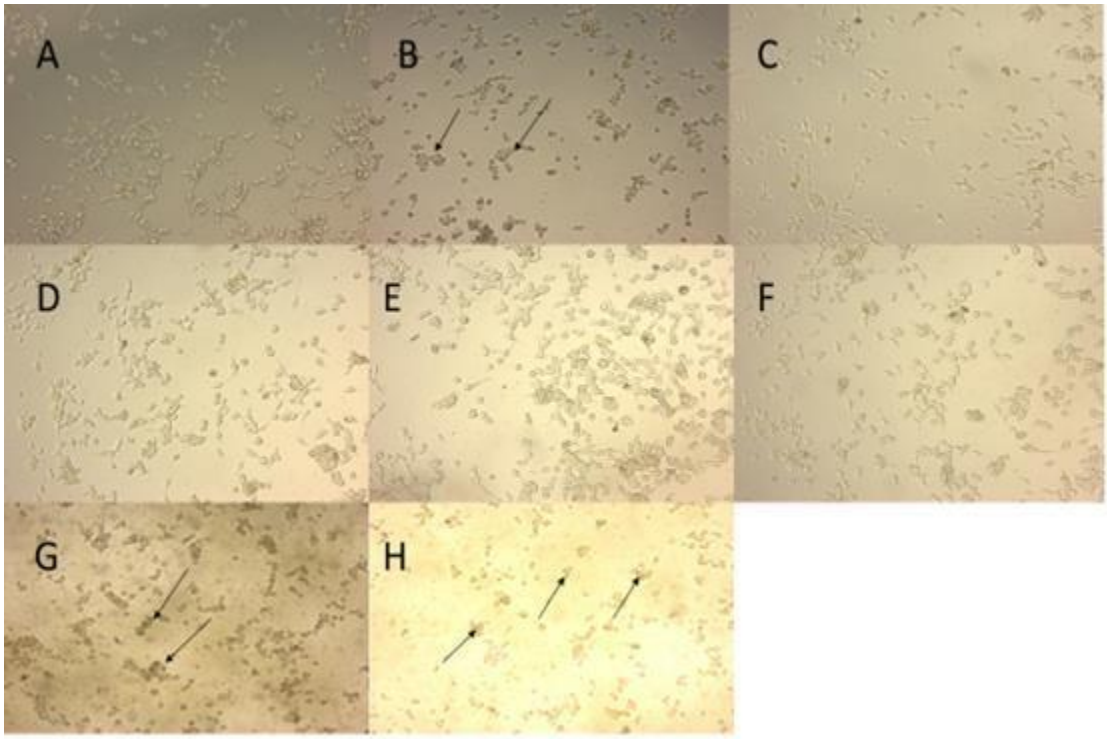

Figure 3.9: Micrographs (inverted light-microscope) illustrating the morphological changes (400 x magnification) in the LNCaP cells after 24- hour exposure to different concentrations of red palm oil extract. A = Negative control, B = Positive control 10 % DMSO, C = 0.1 µg/ml, D = 1 µg/ml, E = 10 µg/ml, F = 100 µg/ml, G = 500 µg/ml, H = 1000 µg/ml. Arrows (B, G and H) indicate apoptotic characteristics; loss of flat polygonal shape, shrinking and clumping of cells

In figure 3.10, the MTT assay performed on the LNCaP cells revealed significant ($P<0.0001$, $P=0.01$, $P<0.0001$) decreases in their viability between the negative control and 500 µg/ml, along with a statistically significant ($P=0.01$) decrease in the viability between the negative control and 1 000 µg/ml. In addition, ANOVA-Analysis showed an initial significant ($P<0.01$) negative trend between 500 µg/ml and 1 000 µg/ml. One-way ANOVA exhibited a significant ($P<0.01$) negative trend between the negative control and 1 000 µg/ml.

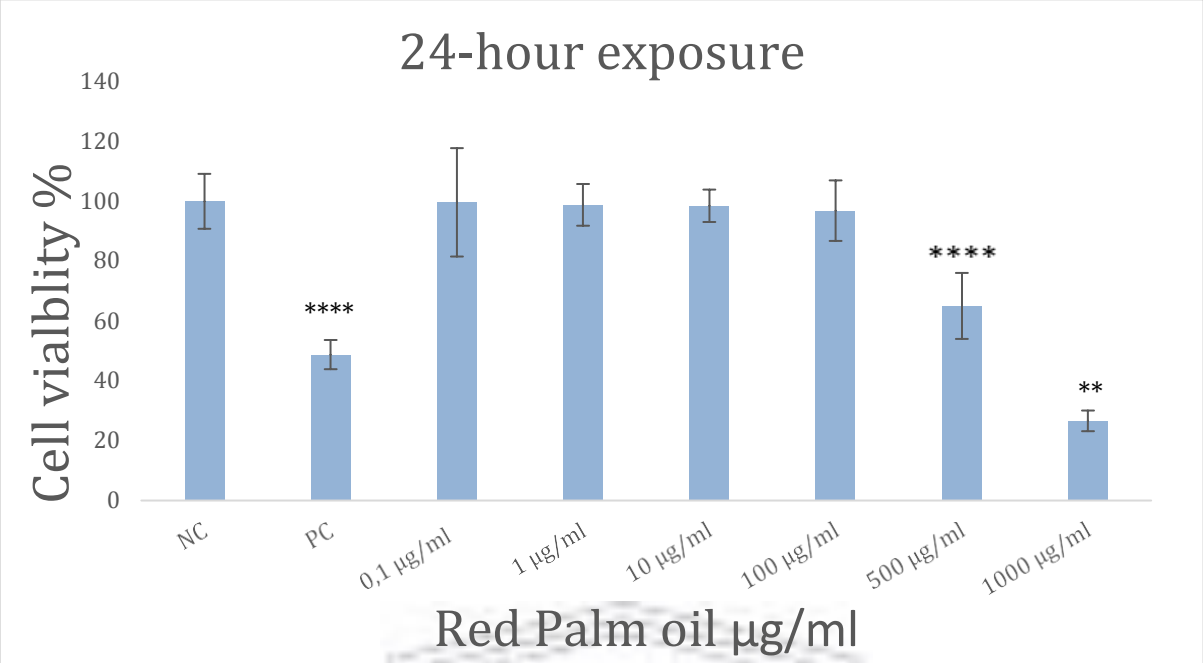

*Figure 3.10: Graph depicting the effect of red palm oil (0.1 µg/ml – 1 000 µg/ml) on cell viability of the LNCaP cells using the MTT assay after 24 – hour exposure. Data represented as mean ± SD (n = 8). NC = Negative control (growth media with 0.05 % DMSO), PC = 10 % DMSO. * indicates P-value < 0.05, ** P < 0.01, *** P < 0.001 and **** P < 0.0001. Cells showed a negative trend between 500 µg/ml – 1 000 µg/ml*

After the cells were exposed to increasing concentrations of the red palm oil extract for 72 hours, the cell morphology was detected and recorded. It was shown that no obvious changes in cell morphology with low levels of stress and polygonal morphology, yet survival, was visible between the control groups and 10 µg/ml. However, it was detected that at higher concentrations (100 µg/ml - 1 000 µg/ml), cells start clumping due to high levels of stress as well as cell death (Figure 3.11).

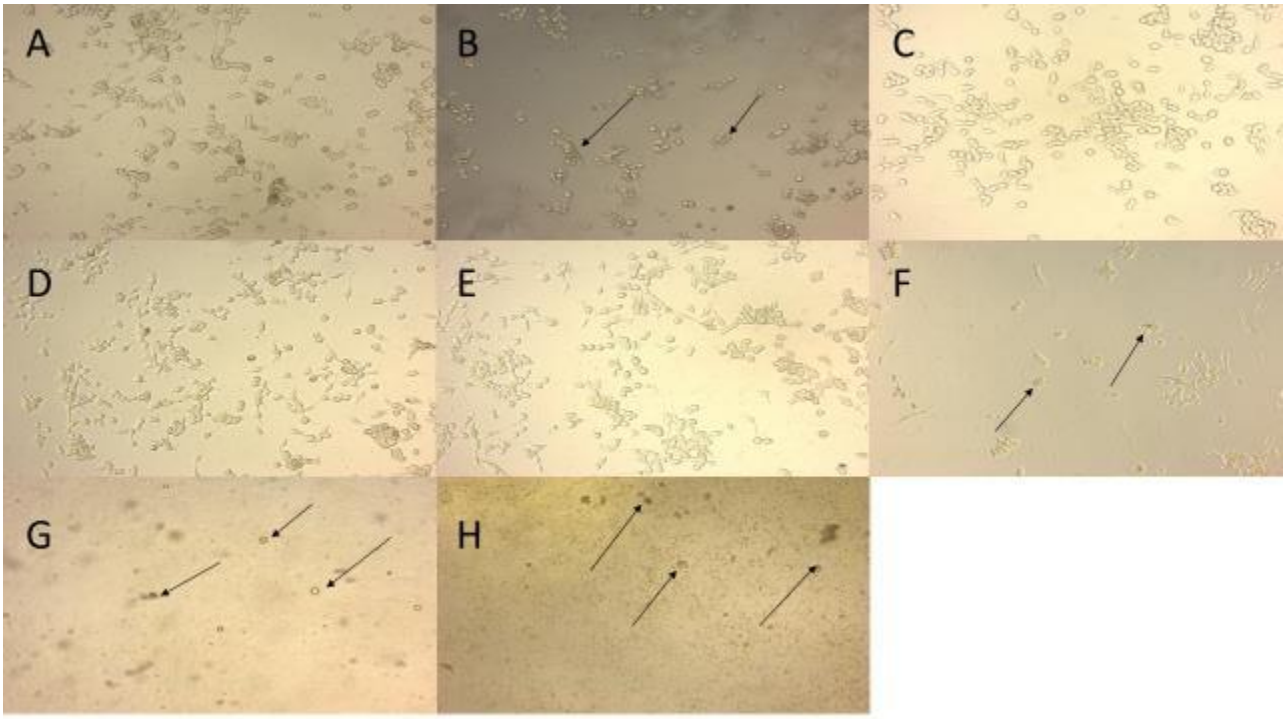

Figure 3.11: Micrographs (inverted light-microscope) illustrating the morphological changes (400 x magnification) in the LNCaP cells after 72-hour exposure to different concentrations of red palm oil extract. A = Negative control, B = Positive control 10 % DMSO, C = 0.1 µg/ml, D = 1 µg/ml, E = 10 µg/ml, F = 100 µg/ml, G = 500 µg/ml, H = 1 000 µg/ml. Arrows (B, F, G and H) indicate apoptotic characteristics; loss of flat polygonal shape, shrinking and clumping of cells.

Correspondingly, the MTT assay (Figure 3.12) revealed only a slight decrease in the viability between the negative control group and 10 µg/ml. The decline at higher concentrations was much higher revealing a dose-dependent relationship in the cell viability. A statistically significant (P= 0.0056) decline was detected at 100 µg/ml in comparison to the negative control, with a further marked statistically significant (P< 0.0001, P< 0.0001) decline at the highest concentrations used in this study (500 µg/ml, 1 000 µg/ml). ANOVA-Analysis showed a significant (P< 0.0001) negative trend between the negative control and 500 µg/ml, along with one-way ANOVA, showing significant (P< 0.0001) negative trend between the negative control group and 1 000 µg/ml.

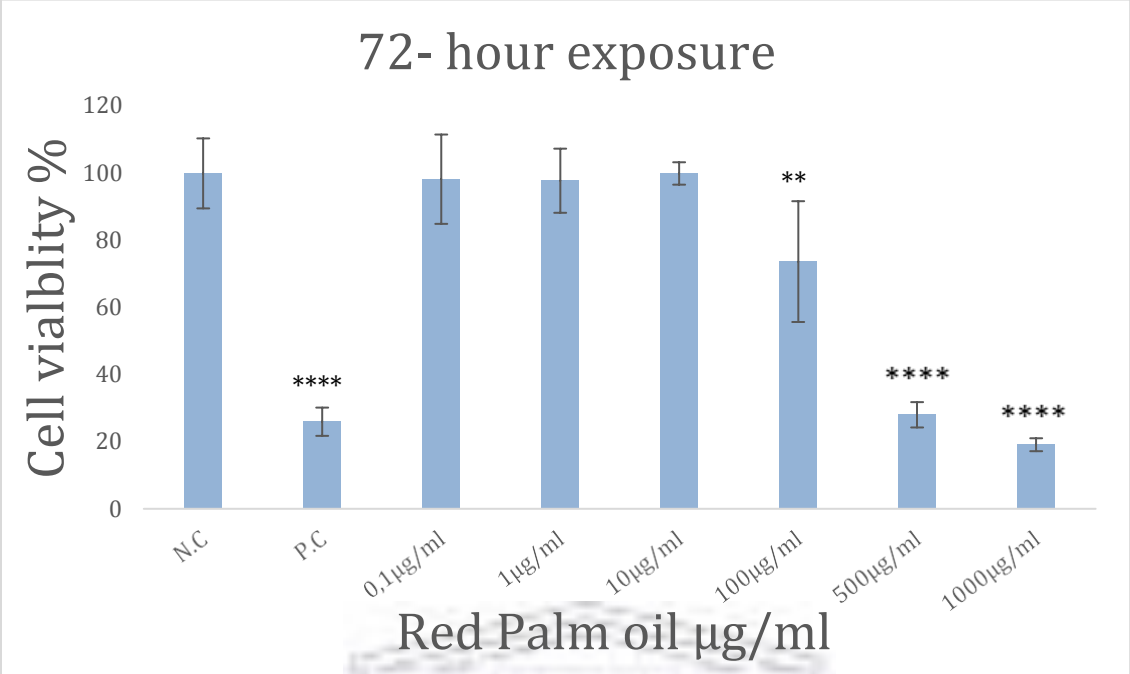

*Figure 3.12: Graph depicting the effect of red palm oil (0.1 µg/ml – 1 000 µg/ml) on cell viability of the LNCaP cells using the MTT assay after 72 – hour exposure. Data represented as mean ± SD (n = 8). NC = Negative control (growth media with 0.05 % DMSO), PC = 10 % DMSO. * indicates P-value < 0.05, ** P < 0.01, *** P < 0.001 and **** P < 0.0001. Cells showed a negative trend between 100 µg/ml – 1 000 µg/ml*

Benign (PWR-1E) prostate cell viability

After benign (PWR-1E) prostate cells were exposed to increasing concentration of the red palm oil for 24 hours, the cell morphology was detected and recorded. No obvious changes in cell morphology were noted and cells maintained their flat and polygonal shapes between the control groups and 100 µg/ml. Only at concentrations higher than 500 µg/ml, the cells began to show visible signs of stress, indicating cellular death (Figure 3.13).

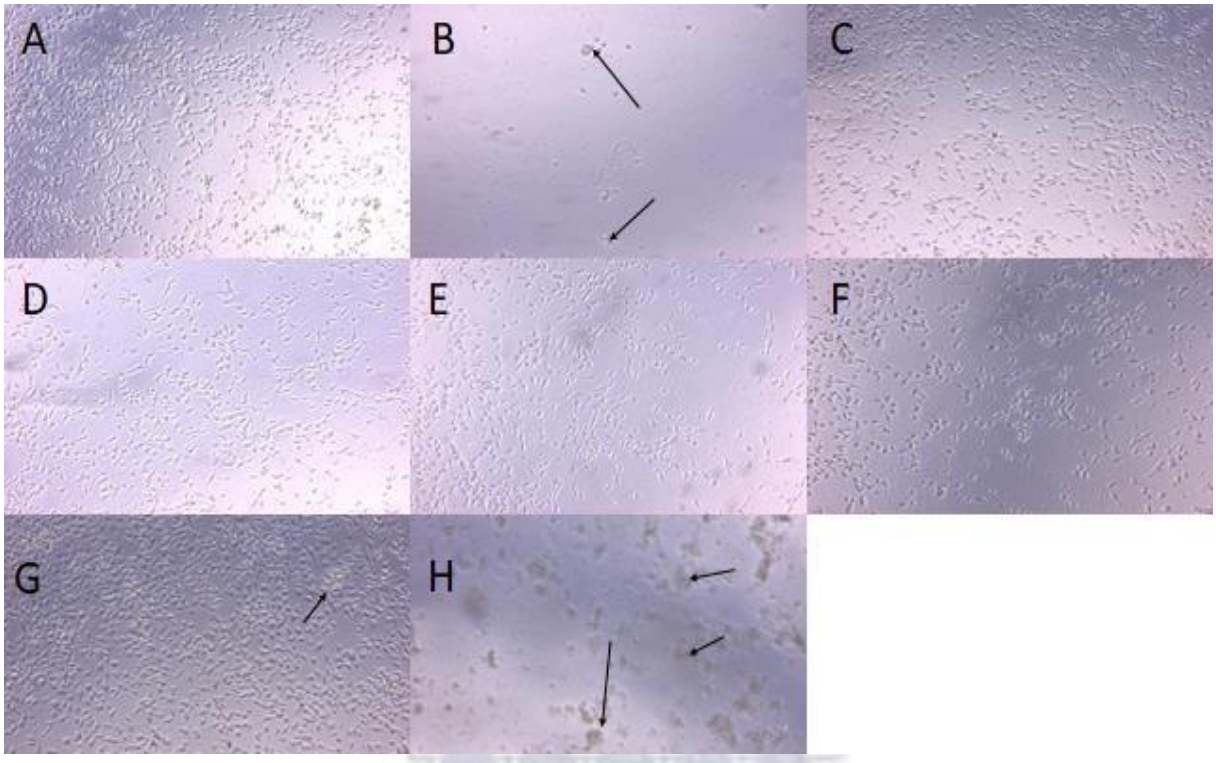

Figure 3.13: Micrographs (inverted light-microscope) illustrating the morphological changes (400 x magnification) in the PWR-1E cells after 24-hour exposure to different concentrations of red palm oil extract. A = Negative control, B = Positive control 10 % DMSO, C = 0.1 µg/ml, D = 1 µg/ml, E = 10 µg/ml, F = 100 µg/ml, G = 500 µg/ml, H = 1 000 µg/ml. Arrows (B, G and H) indicate apoptotic characteristics; loss of flat polygonal shape, shrinking and clumping of cells.

Moreover, the MTT Assay (figure 3.14) revealed no significant decreases in the cell viability between the negative control and 500 µg/ml (P = 0,139). At the highest concentration (1 000 µg/ml), the extract produced a significant decrease (P = 0,0035) in the cell viability compared to the negative control. In addition, the ANOVA-Analysis showed a non - significant (P = 0,7225, P = 0,5048, P = 0,3566, P = 0,1327 and P = 0,139 respectively) negative trend between the negative control and 0.1 µg/ml - 500 µg/ml.

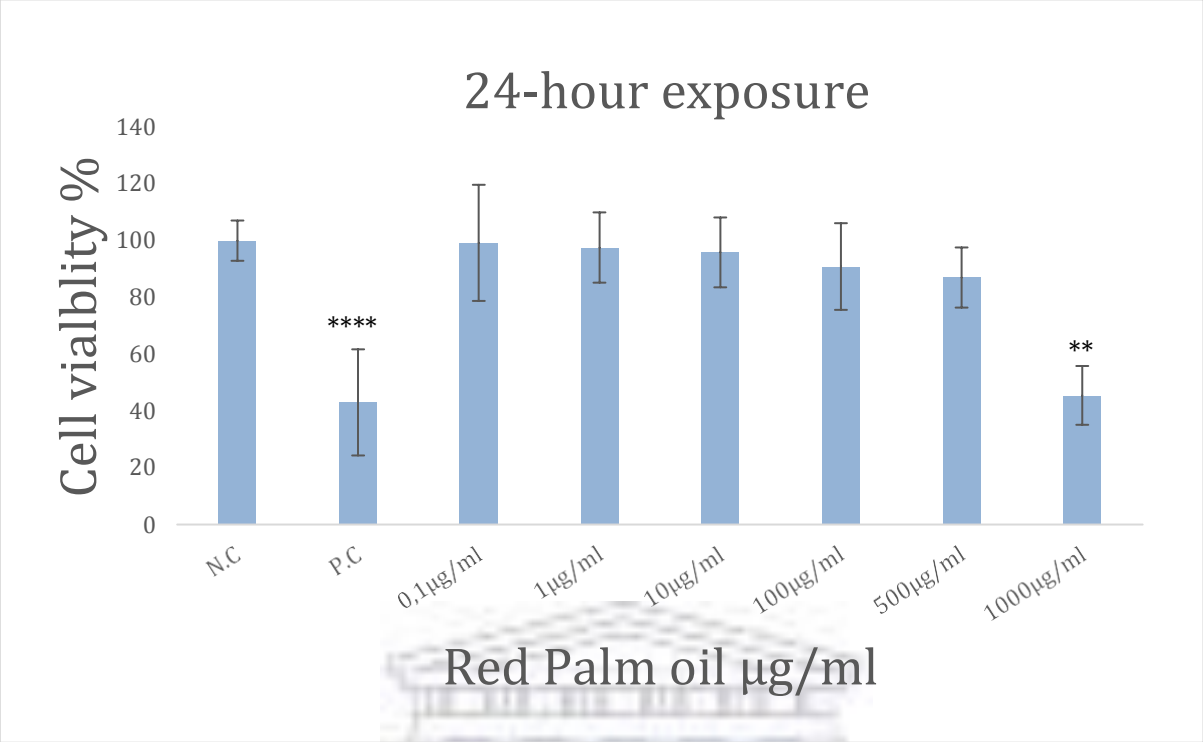

*Figure 3.14: Graph representing the effect of red palm oil (0.1 µg/ml – 1 000 µg/ml) on cell viability of the PWR-1E cells using the MTT assay after 24 – hour exposure. Data represented as mean ± SD (n = 8). NC = Negative control (growth media with 0.05 % DMSO), PC = 10 %. * indicates P – value < 0.05, ** P < 0.01, *** P< 0.001 and **** P < 0.0001.*

After the cells were exposed to increasing concentrations of the red palm oil extract for 72 hours, the cell morphology was observed and recorded. No significant changes in cell morphology were observed with low levels of treatment as cells showed polygonal morphology, hence, survival was visible between the control groups and 100 µg/ml. However, it was detected that at higher concentrations (500 µg/ml and 1 000 µg/ml), cells start clumping due to high levels of stress as well as cell death (Figure 3.15).

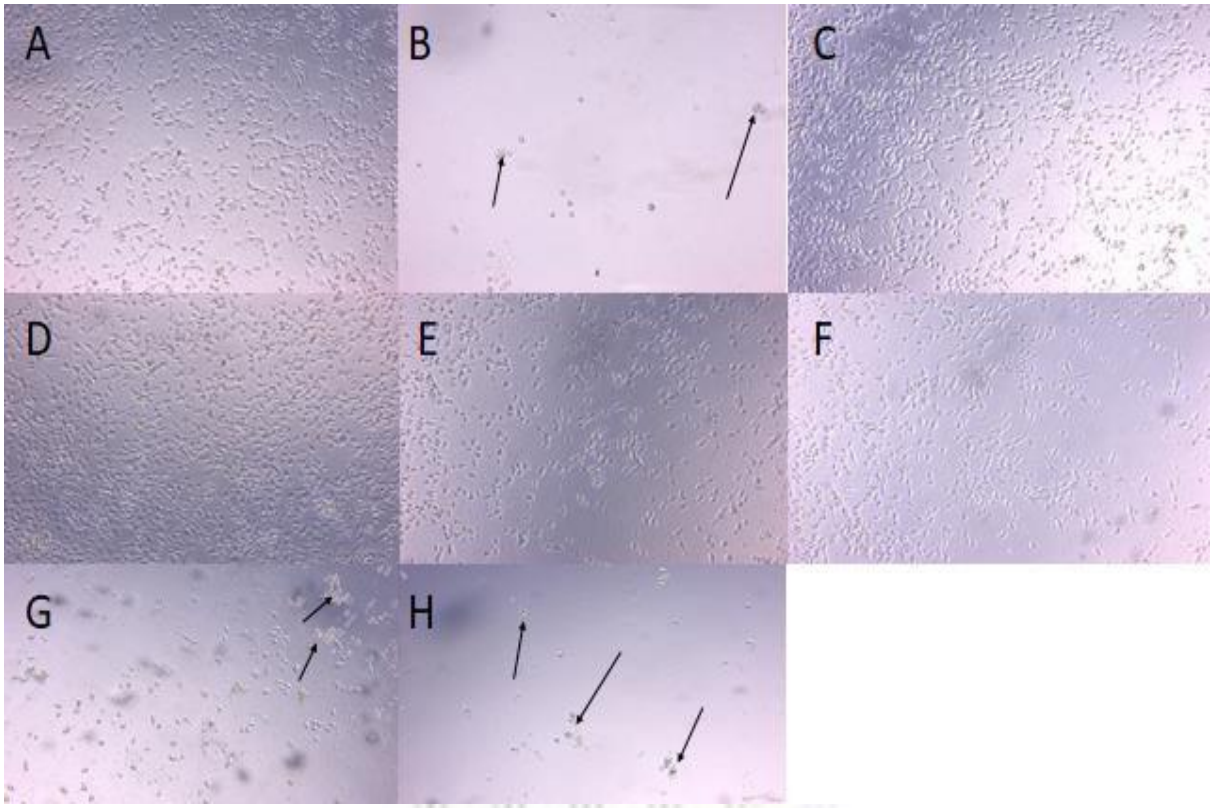

Figure 3.15: Micrographs (inverted light-microscope) illustrating the morphological changes (400 x magnification) in the PWR-1E cells after 72 -hour exposure to different concentrations of red palm oil extract. A = Negative control, B = Positive control 10 % DMSO, C = 0.1 µg/ml, D = 1 µg/ml, E = 10 µg/ml, F = 100 µg/ml, G = 500 µg/ml, H = 1 000 µg/ml. Arrows (B, G and H) indicate apoptotic characteristics; loss of flat polygonal shape, shrinking and clumping of cells.

Correspondingly, the MTT assay (Figure 3.16) revealed non-significant decrease in the viability between the negative control group, 10 µg/ml and 100 µg/ml (P = 0,2737 and P = 0,4060 respectively). However, the decline at higher concentrations was much higher revealing a dose-dependent relationship in the cell viability. A statistically significant (P = 0,0001) decline was detected at 500 µg/ml in comparison to the negative control, with a further marked statistically significant (P< 0.0001) decline at the highest concentrations used in this study 1 000 µg/ml.

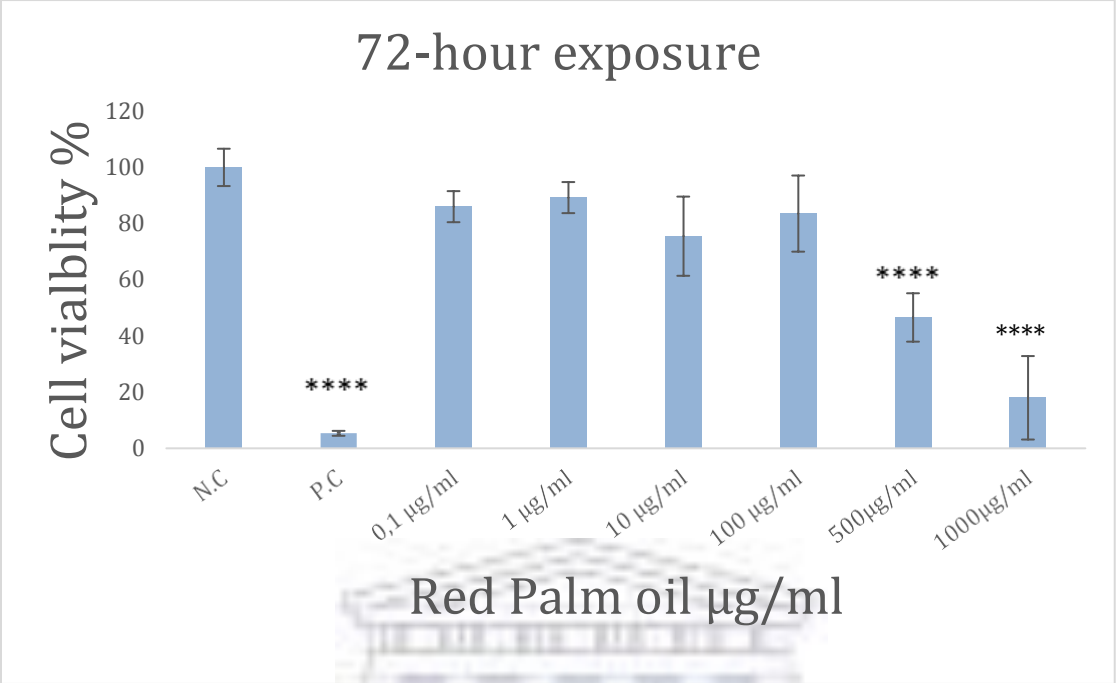

*Figure 3.16: Graph representing the effect of red palm oil (0.1 µg/ml – 1 000 µg/ml) on cell viability of the PWR-1E cells using the MTT assay after 24 – hour exposure. Data represented as mean ± SD (n = 8). NC = Negative control (growth media with 0.05 % DMSO), PC = 10 %. * indicates P-value < 0.05, ** P < 0.01, *** P < 0.001, and **** P < 0.0001. Cells showed a negative trend between 500 µg/ml – 1 000 µg/m.*

Effect of Red Palm Oil on Total Serum PSA in prostate cells

Prostate-specific antigen (PSA) is widely used to screen for prostate cancer. Malignant (LNCaP) and benign (PWR-1E) prostate cells were exposed to increasing concentrations of aqueous dilution of Red Palm Oil for 24 and 72 hours respectively. Total serum PSA ELISA kit was used to determine the concentration of the prostate-specific antigen.

Total Serum PSA in Malignant (LNCaP) prostate cell

After malignant LNCaP cells were exposed to increasing concentration of the red palm oil for 24 hours, and the production of PSA was analysed. Here, one-way ANOVA statistical analysis showed a steady decline in PSA secretion was reached at the concentration of Red Palm Oil (0.1, 1 and 10

µg/ml) (Figure 3.17). However, a major and significant ($p < 0.001$) drop in total PSA of malignant (LNCaP) prostate cells was observed after exposing them to higher concentrations of the Red Palm Oil (100 and 500 µg/ml).

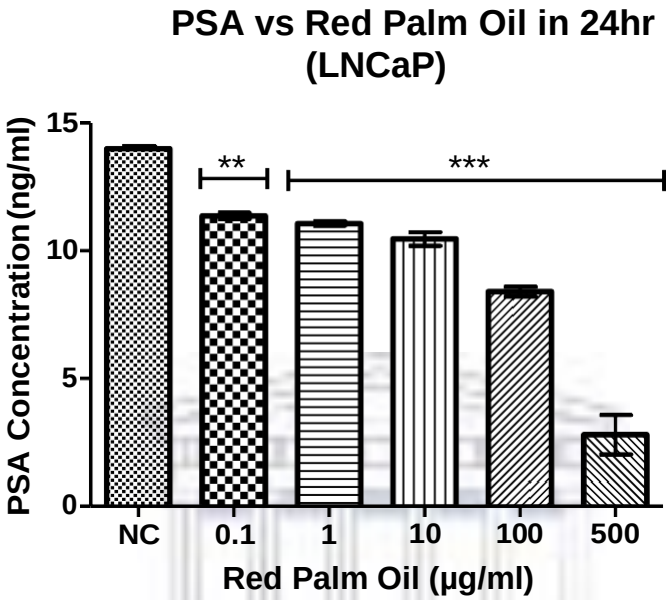

*Figure 3.17: The effect of Red Palm Oil on total serum PSA in malignant (LNCaP) prostate cells after the 24-hour incubation period. Data represented as mean ± SD. NC = Negative control (growth media with 0.05 % DMSO). * indicates p-value < 0.05, ** p < 0.01, *** p < 0.001 and **** p <0.00001. Cells showed a negative trend between 0.1 µg/ml – 500 µg/ml*

Correspondingly, after the cells were exposed to increasing concentrations of the red palm oil extract for 72 hours, and the production of PSA was analysed. The decrease in total serum PSA level was compared to the negative control became significant ($p < 0.001$) from 0.1 µg/ml onwards. Palm Oil concentration of 10 µg/ml onwards caused an additional sharp drop in PSA concentration. (Figure 3.18).

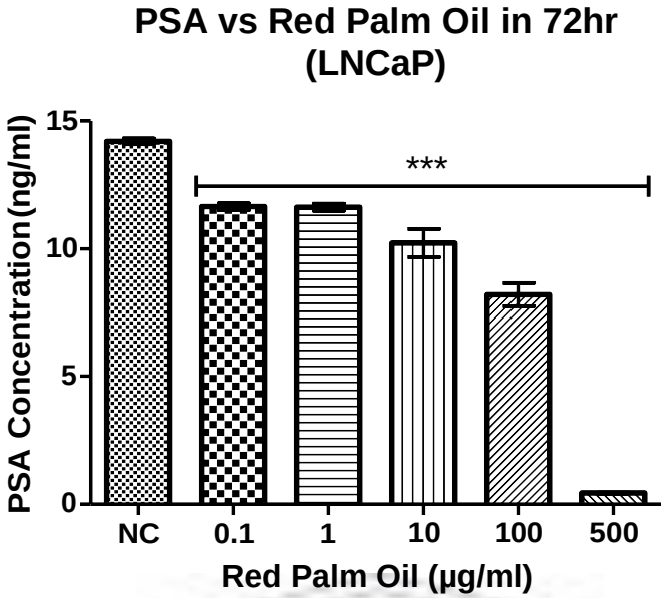

*Figure 3.18: The effect of Red Palm Oil on total serum PSA in malignant (LNCaP) prostate cells after 72-hour incubation period. Data represented as mean ± SD. NC = Negative control (growth media with 0.05 % DMSO). * indicates p-value < 0.05, ** p < 0.01, *** p < 0.001 and **** p < 0.0001. Cells showed a negative trend between 0.1 µg/ml – 500 µg/ml.*

Total Serum PSA in Benign (PWR-1E) prostate cell

After benign (PWR-1E) cells were exposed to increasing concentration of the red palm oil for 24 hours, the production of PSA was analysed and recorded. However, one-way ANOVA statistical analysis showed no significant ($p < 0.359$) increase in the level of total serum PSA at (1 µg/ml) when compared to the negative control (Figure 3.19).

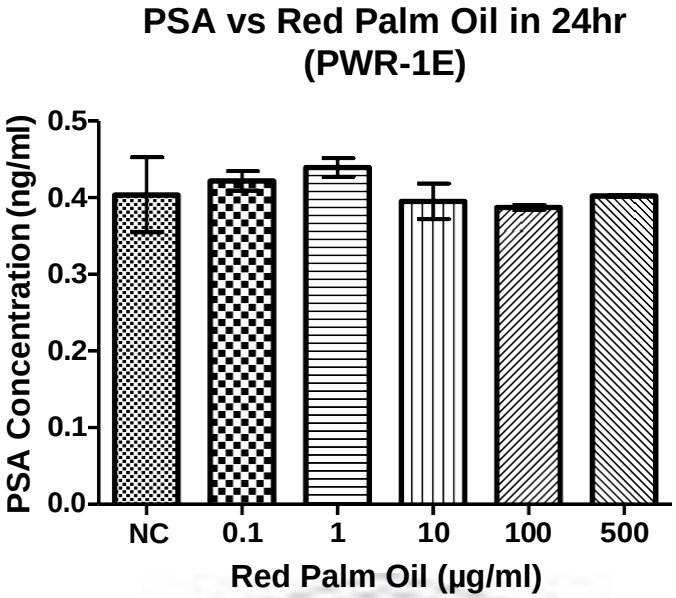

*Figure 3.19: The effect of Red Palm Oil on total serum PSA in benign (PWR-1E) prostate cells after 24-hour incubation period. Data represented as mean ± SD. NC = Negative control (growth media with 0.05 % DMSO). * indicates p - value < 0.05, ** p < 0.01, *** p < 0.001 and **** p < 0.0001.*

In addition, the benign (PWR-1E) prostate cells were exposed to increasing concentrations of Red Palm Oil for 72-hour, and the production of PSA was also analysed and recorded. Likewise, the benign (RPWE 1) prostate cells showed only non-significant increases in the level of total PSA following exposure to 500 µg/ml of the Red Palm Oil when compared to the negative control. There was no change in the level of total PSA in samples treated with 0.1, 1 10 100 µg/ml of the oil respectively (Figure 3.20).

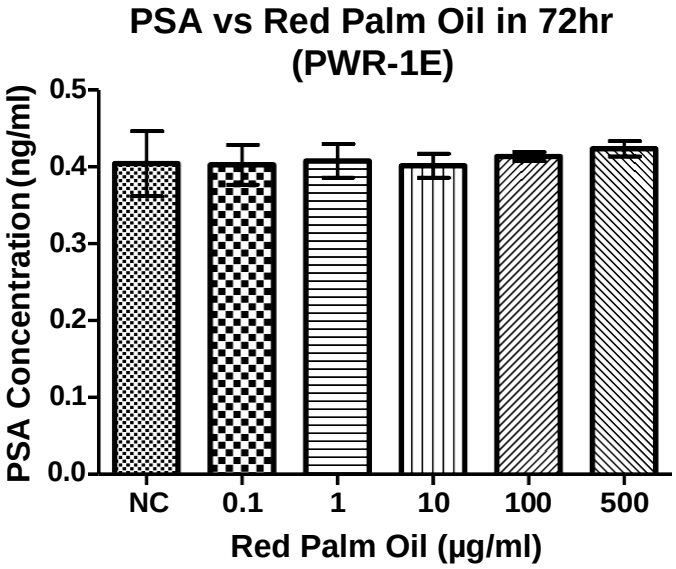

*Figure 3.20: The effect of Red Palm Oil on total serum PSA in benign (PWR-1E) prostate cells after 72-hour incubation period. Data represented as mean ± SD. NC = Negative control (growth media with 0.05 % DMSO). * indicates p-value < 0.05, ** p < 0.01, *** p < 0.001 and **** p < 0.0001.*

3.2 Molecular Biology

GAPDH

The first PCR which identified the presence and general expression of PSA, a visualisation of the PSA gene was considered necessary. Visualisation of target gene expression requires a gene with unchanged expression in tissues to normalize the expression of the target gene. The reference gene used in this study glyceraldehyde-3-phosphate dehydrogenase (GAPDH), this gene is referred to as a "housekeeping".

The first PCR was carried out on the housekeeping gene Gapdh, to confirm that the PCR was in fact working. The PCR runs were succeeded, and appear to show amplification of the intended product (290 bp). Thereafter, the Novel Juice was used and only the last successful PCR is shown below Figure 3.21.

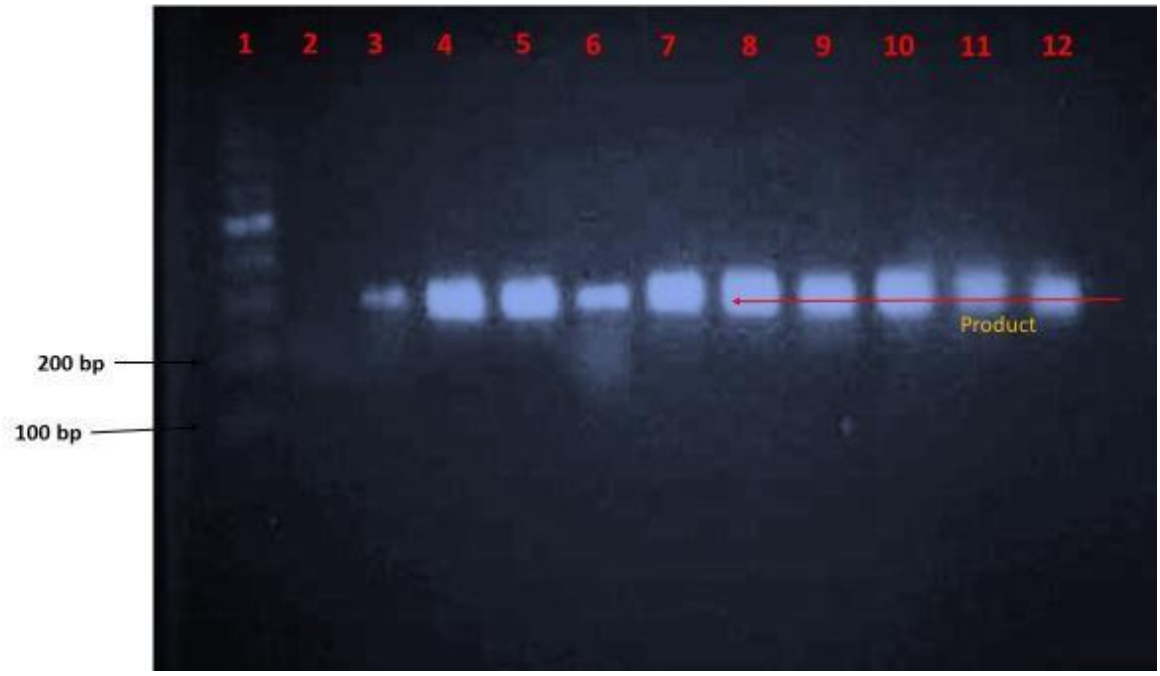

Figure 3.21: Agarose gel electrophoresis of the Housekeeping gene (GAPDH), showing bands of the intended product. Red arrow indicates the product size (290 bp). The gel represented as line 1: 100pb

Marker, line 2: N.C (Nuclease-free water), line 3: untreated LNCaP, line 4: treated at IC_{25} for 24h LNCaP, line 5: treated at IC_{75} for 24h LNCaP, line 6: treated at IC_{25} for 72h LNCaP, line 7: treated at IC_{75} for 72h LNCaP, line 8: untreated PWR-1E, line 9: treated at IC_{25} for 24h PWR-1E, line 10: treated at IC_{75} for 24h PWR-1E, line 11: treated at IC_{25} for 72h PWR-1E, line 12: treated at IC_{75} for 72h PWR-1E.

POU3F2

The AGE for POU3F2 PCR appears to show amplification of the intended product (260 bp). However, Figure 3.22 shows bans in all of the lanes; albeit in slightly different locations.

Figure 3.22: Agarose gel electrophoresis of the POU3F2 gene, showing bands of the intended product. Red arrow indicates the product size (260 bp). The gel represented as line 1: 100pb Marker, line 2: N.C (Nuclease-free water), line 3: untreated LNCaP, line 4: treated at IC_{25} for 24h LNCaP, line 5: treated at IC_{75} for 24h LNCaP, line 6: treated at IC_{25} for 72h LNCaP, line 7: treated at IC_{75} for 72h LNCaP, line 8: untreated PWR-1E, line 9: treated at IC_{25} for 24h PWR-1E, line 10: treated at IC_{75} for 24h PWR-1E, line 11: treated at IC_{25} for 72h PWR-1E, line 12: treated at IC_{75} for 72h PWR-1E.

PEG 10

The amplification of The PEG 10 gene showed no visible bands (results not shown). Therefore, optimization of PCR reactions was carried out in an attempt to improve the binding. A differential

annealing temperatures were achieved by the use of gradient cycle, and the amplification demonstrated a non-specific binding (results not shown). Eventually, further optimization involved regulation of PEG 10 primer concentration. A primer concentration of 0.3 µM was integrated with the PCR mixture, which yielded to appears bands at the intended product (253 bp) result shown in figure 3.23.

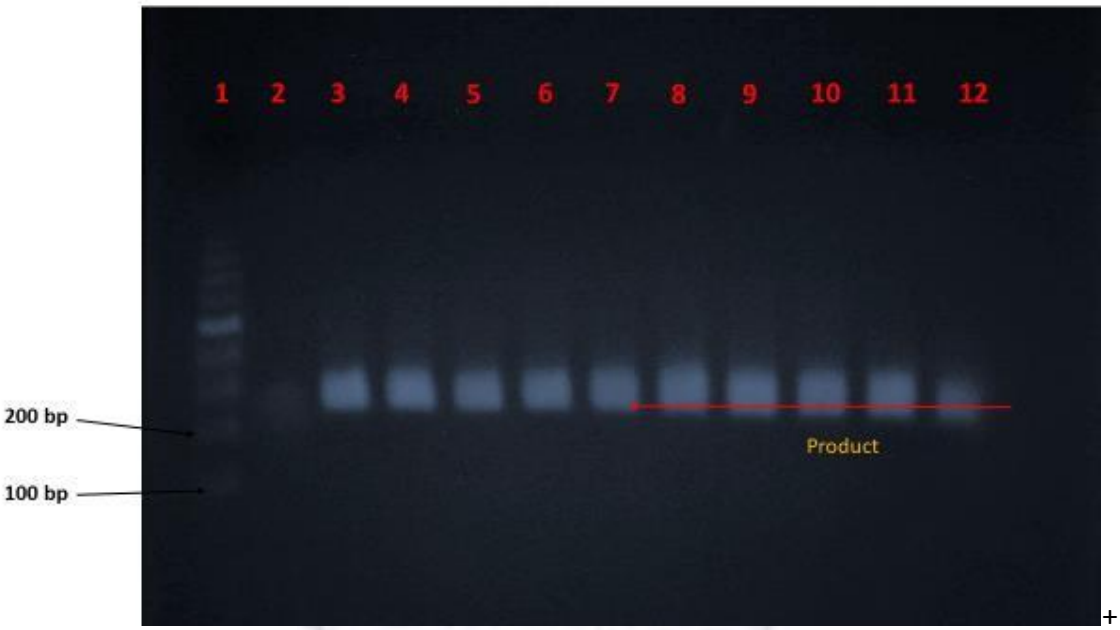

Figure 3.23: Agarose gel electrophoresis of the PEG 10 gene, showing bands of the intended product. Red arrow indicates the product size (253 bp). The gel represented as line 1: 100pb Marker, line 2: N.C (Nuclease-free water), line 3: untreated LNCaP, line 4: treated at IC_{25} for 24h LNCaP, line 5: treated at IC_{75} for 24h LNCaP, line 6: treated at IC_{25} for 72h LNCaP, line 7: treated at IC_{75} for 72h LNCaP, line 8: untreated PWR-1E, line 9: treated at IC_{25} for 24h PWR-1E, line 10: treated at IC_{75} for 24h PWR-1E, line 11: treated at IC_{25} for 72h PWR-1E, line 12: treated at IC_{75} for 72h PWR-1E.

SRRM4

Conventional PCR was carried out on SRRM4 gene, but no bands were visible in any of the lines (results not shown). Further optimization involved the annealing temperature demonstrates non-specific product binding. Additional optimization implicated the regulation of the SRR4 gene to eliminate the formation of non-specific bands. A primer concentration of 0.3 µM was integrated into the PCR mixture, but this neither resulted in a significant difference nor elimination of primer dimers. Eventually, optimization was required, i.e., a decrease to 0.1 µM in primer concentration which yielded to decline to eliminate the formation of non-specific bands (Figure 3.24).

Figure 3.24: Agarose gel electrophoresis of the SRRM4 gene, showing non-specific bands. Red arrow indicates the non-specific bands. The gel represented as line 1: 100pb Marker, line 2: N.C (Nuclease-free water), line 3: untreated LNCaP, line 4: treated at IC_{25} for 24h LNCaP, line 5: treated at IC_{75} for 24h LNCaP, line 6: treated at IC_{25} for 72h LNCaP, line 7: treated at IC_{75} for 72h LNCaP, line 8: untreated PWR-1E, line 9: treated at IC_{25} for 24h PWR-1E, line 10: treated at IC_{75} for 24h PWR-1E, line 11: treated at IC_{25} for 72h PWR-1E, line 12: treated at IC_{75} for 72h PWR-1E.

4. Discussion

The red palm oil is one of the largest consumed vegetable oils throughout the world. The oil extracted from the fruit of the tropical palm tree (Elaeis guineensis) is red because it contains carotenes, tocopherols, and tocotrienols. The palm oil tocopherol-rich fraction (TRF) is what makes red palm oil both unique and valuable clinically due to its biological and nutritional properties such as anti-oxidant, hepato-protective and anti-cancer effects. The palm oil is one of the traditionally used cooking oils by the West and Central Africans, where local populations used it for cooking. Currently, Malaysia and Indonesia are leading the producers and palm oil is the most widely produced oil in the world (Fattore & Fanelli, 2013).There is accumulating evidence that TRF of red palm oil can inhibit tumour growth (Zhang et al., 2015). Many research groups have reported on the relationship between palm oil and various cancers. However, in the last few years doubts have been raised about its usefulness which warrants the need for further studies to prove its anti-cancerous and anti-metastatic qualities.

Despite the fact that dietary fats are linked to cancer, few studies have been done to assess its anti-cancer effects on the prostate. Therefore, this thesis is meant to address its potential usefulness in treating prostate cancer. *In vitro* assays were used to assess the therapeutic effect of red palm oil. To achieve this, the malignant (LNCaP) and benign (PWR-1E) prostate cells were used to investigate the anti-cancer effects of red palm oil and its main active compounds, tocopherols, tocotrienols and carotenoids on the prostate. Another objective was to investigate the cytotoxic effect of the oil. PSA, the biomarker for prostate cancer was determined using total PSA ELISA kit with the aim of determining the chemo-therapeutic effects of the red palm oil. Furthermore, a molecular analysis is conducted for testing the expression of three genes known to be involved in cancer metastasis and these are: the member of the POU homeobox 2 of neural transcription factors class 3 (POU3F2 also known as BRN2), paternally expressed 10 (PEG 10), serine/arginine repetitive matrix 4 (SRRM4). These genes were selected several studies have pointed out that they are expressed during the metastatic process in solid tumours *(Larue et al.,* 2010); (Akamatsu *et al.,* 2015); (Li *et al.,* 2017).

In the following sections, the main findings in this thesis will be discussed and the published data of our research groups will be acknowledged.

Cell Viability via MTT assay

In this study, the cell viability of androgen-sensitive LNCaP human prostate cancer cells and benign (RPWE1) prostate cells was evaluated after exposure to different concentrations of the red palm oil using MTT assay. The results achieved after incubating androgen-sensitive LNCaP human prostate cancer cells for 24-hours with red palm oil demonstrated a significant decrease in the percentage of cellular viability at higher concentrations of red palm oil (Figure 3.10). Moreover, the decrease in cell viability at higher concentrations of red palm oil (500- 1 000 µg/ml) during the same 24-hours period was evident. We found that the viability was significantly decreased ($P=0.01$, $P<0.0001$), which clearly indicates cellular death characterized by the reduction of mitochondrial dehydrogenase activity. (Berridge and Tan., 1993).

The benign (PWR-1E) prostate cells were exposed to red palm oil for 24-hour. There was no statistically decrease in cell viability between the control groups and cells treated at a concentration of 100 µg/ml. However, at higher concentration (500 µg/ml), cells showed signs of stress, as indicated by a reduction in mitochondrial dehydrogenase activity (Figure 3.14) (Berridge and Tan., 1993). In the present study, assessing the cell viability indicated clearly that biologically significant decrease in the viability only occurred at the highest concentration of red palm oil. Moreover, and after 24-hour incubation of prostate cells with red palm oil specifically at 500 µg/ml, we noticed that the androgen-sensitive LNCaP human prostate cancer cells had shown more stress-related changes than the benign (RPW-1E) prostate cells.

Morphological studies of both androgen-sensitive LNCaP human prostate cancer cells and benign (RPW-1E) prostate cells demonstrated a normal attachment and differentiation with the cells showing flat polygonal shapes. Comparing the cell numbers of both LNCaP and RPW-1E cell lines on the micrographs to those of their respective negative control, showed that the red palm oil did not really affect their viability at lower concentrations. However, at 500 µg/ml, the androgen-

sensitive LNCaP human prostate cancer cells appeared clumped, irregular in shape and the cell numbers were decreased due to death and detachment, whilst benign (RPW-1E) prostate cells showed fewer changes in morphology (Figures 3.9- 3.13). Assessing cell viability with the MTT assay had clearly indicated that at a concentration of 500 µg/ml and with 24-hour incubation, the red palm oil is decreasing viability to the androgen-sensitive LNCaP human prostate cancer cells and less viability effects to the benign (RPW-1E) prostate cells.

Furthermore, the incubation of the malignant LNCaP prostate cells for 72-hour has demonstrated a significant decrease in the mitochondrial dehydrogenase activity at the concentrations from (100 µg/ml up to 1000 µg/ml) used when compared to the negative control (Figure 3.12). Micrographs of the malignant LNCaP prostate cells exposed to the highest concentrations (100, 500, and 1000 µg/ml) of red palm oil revealed a decrease in cell numbers as well as changes in morphology like loss of regular shape and death (Figure 3.11). Concisely, both graphs and micrographs results proved that the red palm oil is highly suppressed to malignant LNCaP prostate cells viability and produced significant cellular stress at the mitochondrial level.

The incubation of the benign (PWR-1E) prostate cells for the same period of 72-hour at low concentrations (10 and 100 µg/ml) showed a non-significant decrease in mitochondrial dehydrogenase activity, followed by a rapid decrease in mitochondrial dehydrogenase activity at higher concentrations (500 and 1000 µg/ml) (Figure 3.16). In addition, the benign (PWR-1E) prostate cells maintained their shape and the cell numbers slightly increased at 100 µg/ml, but only at the higher concentrations the cells clumped together and had shown signs of death on the micrographs compared to the control (Figure 3.15). To summarize our findings, cytotoxicity of the red palm oil to the benign (PWR-1E) prostate cells only appeared at the highest concentrations (500 to 1000 µg/ml) indicating considerable cellular stress at the mitochondrial level.

A study (Sen & Baltimore, 1985) mentioned that the transcription factor NF-jB is closely connected to mitochondrial activities and the process of tumorigenesis, as it's linked with the conversion of normal cells, and is fundamentally active in most tumour cells. Another study has clearly indicated that the suppression of the NF-jB activation in both malignant LNCaP and benign (PWR-1E) prostate cells decreased the mitochondrial dehydrogenase activity (Schmidt et al., 2010). In this context, our study has shown that the absorbance readings have featured a decrease in mitochondrial dehydrogenase activity with loss of cell viability. Moreover, the MTT assay and morphological micrographs showed that malignant (LNCaP) prostate cells appear to be more suppressed than benign (RPWE 1) prostate cells. This result leads to establishing that Red Palm Oil does not play a rule in promoting stimulation and proliferation of late-stage prostate cancer cells but rather as a possible anti-cancer factor. However, as the cell's mitochondrial dehydrogenase activity were used to test the viability, other possible explanation could have been the cell's suppression to proliferation.

A study has looked into the antiproliferative effects and primary apoptotic mechanisms of β-tocotrienol on human lung adenocarcinoma (Lim et al., 2014). A549 and glioblastoma U87MG cells were investigated. It was evidenced that β-tocotrienol had inhibited the growth of both A549 (GI50=1.38±0.334μM) and U87MG (GI50=2.53±0.604μM) cells at rather low concentrations. Cancer cells incubated with β-tocotrienol were also found to exhibit hallmarks of apoptotic morphologies including membrane blebbing, chromatin condensation and formation of apoptotic bodies. The study suggested that the apoptotic properties of β-tocotrienol in both A549 and U87MG cells were the results of its capability to induce significant ($P<0.05$) double-strand DNA breaks (DSBs) without involving single-strand DNA breaks (SSBs). β-Tocotrienol is reported to induce activation of caspase-8 in both A549 and U87MG cells guided by no activation when caspase-8 inhibitor, z-IETD-fmk was added. The study reported on the disruption of the mitochondrial membrane permeability of the cells in a concentration- and time-dependent manner. The induction of apoptosis by β-tocotrienol in A549 and U87MG cells was confirmed to involve both the death-receptor mediated and mitochondria-dependent apoptotic pathways.

Another study has investigated the effects of a palm oil-derived nanopolymer called Bio-12, a

lipid esters derived from a range of fatty acids of palm oil against human malignant melanoma (Komarasamy & Sekaran, 2012). The study aimed to identify the anti-proliferative properties of Bio-12 against human malignant melanoma cell line (MeWo) and to elucidate the mode of actions whereby Bio-12 brings about cell death. Bio-12 significantly inhibited the growth of MeWo cells in a concentration- and time-dependent manner with a median inhibitory concentration (IC_{50}) value of 1/25 dilution after 72 h but was ineffective on human normal skin fibroblasts (CCD-1059sk). The mode of actions of Bio-12 on MeWo cells was investigated and cell cycle flow cytometry demonstrated that MeWo cells treated with increasing concentrations of Bio-12 resulted in S-phase arrest, accompanied by the detection of sub-G1 content, indicative of apoptotic cell death. The induction of apoptosis was further confirmed via caspase (substrate) cleavage assay which showed induction of early apoptosis in MeWo cells. DNA strand breaks which were evident through increase of TUNEL positive cells and formation of a characteristic DNA ladder on agarose gel electrophoresis. Moreover, treatment of MeWo cells with Bio-12 induced significant increase in lactate dehydrogenase (LDH) activity. Their results showed that Bio-12 possesses the ability to suppress proliferation of human malignant melanoma cells and this suppression is at least partly attributed to the initiation of the S-phase arrest, apoptosis and necrosis, suggesting that the red palm oil is indeed worth for further investigations.

A more relevant study has attributed the decrease in cell viability to cell cycle arrest (Srivastava & Gupta, 2006). They used normal human prostate epithelial cells (PrEC), virally transformed normal human prostate epithelial cells (PZ-HPV-7), and human prostate cancer cells (LNCaP, DU145, and PC-3). They evaluated the growth-inhibitory and apoptotic effects of the tocotrienol-rich fraction (TRF) extracted from palm oil. Their TRF treatment to PrEC and PZ-HPV-7 resulted in almost identical growth-inhibitory responses of low magnitude. They found that TRF treatment resulted in significant decreases in cell viability and colony formation in all three prostate cancer cell lines. The IC (50) values after 24h TRF treatment in LNCaP, PC-3, and DU145 cells were in order 16.5, 17.5, and 22.0 µg/ml. Moreover, they found that TRF treatment resulted in significant apoptosis in all the cell lines as evident from (i) DNA fragmentation, (ii) fluorescence microscopy, and (iii) cell death detection ELISA, whereas the PrEC and PZ-HPV-7 cells did not undergo apoptosis, but showed modestly decreased cell viability only at a high dose of 80 microg/ml. Their study of cell cycle analysis, TRF (10-40 µg/ml) resulted in a dose-dependent G0/G1 phase arrest

and sub G1 accumulation in all three cancer cell lines but not in PZ-HPV-7 cells. Their results suggest that the palm oil derivative TRF is capable of selectively inhibiting cellular proliferation and accelerating apoptotic events in prostate cancer cells and that TRF offers significant promise as a chemopreventive and/or therapeutic agent against prostate cancer.

Further to this, we report that in this thesis, red palm oil demonstrated significant cytotoxic effects on malignant (LNCaP) prostate cells and lower cytotoxic effects on normal (PWR-1E) prostate cells (Figure 3.9 and Figure 3.13). The mitochondrial dehydrogenases activity was used in this thesis to test the viability of the prostate cells in the presence of the red palm oil, other possible pathways might have been involved in the inhibition of cell proliferation.

Prostate-specific antigen (PSA) via ELISA

PSA has been described as a member of the tissue kallikrein family of proteases that is fundamentally synthesized in the normal epithelial cells of the prostate tissues and secreted into the lumen of the seminal fluid which responsible for the cleavage of the most abundant proteins and semenogelin in the seminal coagulum, thereby causing liquefaction of the seminal plasma coagulum and facilitating spermatozoa progression (Lilja et al., 1987). PSA expression is regulated by androgens, consistently expressed in prostate cancer. PSA levels have been widely used as a biomarker for early detection of prostate cancer (Pezzato et al., 2004).

There is a scarcity in research reporting on red palm oil treatment and measuring PSA levels in prostate cancer. In the current study, PSA levels of malignant LNCaP and begin PWR-1E prostate cells were measured using a PSA total ELISA kit. Both transformed prostate cancer cells, LNCaP and benign prostate cancer cells, PWR-1E were exposed to increasing concentrations of red palm oil for 24 and 72 hours, respectively. The findings obtained from this assay showed reduction in total PSA concentration in LNCaP cells (Figure 3.14 and Figure 3.15) in a dose-dependent manner when exposed to red palm oil. While no observable change in total serum PSA concentration by benign PWR-1E cells were noted (Figure 3.16 and Figure 3.17). These findings suggest its potential anti-cancerous properties.

In normal prostate cells the basal cell layer and basement membrane are not compromised, leading to normal prostate cells have serum PSA levels less than 4 ng/ml (Bello *et al.,* 1997). The normal prostate cells have two tumour suppressor proteins; p53 and retinoblastoma (Rb) proteins (Thangavel *et al.,* 2015). The benign PWR-1E cells are E7 immortalized cells and possess characteristics of normal cells (Monini *et al.,* 1996). Hence, in the presence of androgens and up-regulated AR, these cells secrete PSA like normal prostate epithelial cells (Bello *et al.,* 1997). Ultimately, this result in both the non-transformed PWR1-E and the transformed LNCaP prostate epithelial cells being capable of producing PSA (Balk *et al.,* 2003) In prostate cancer, the disruption of the basal cell layer and basement membrane may allow easier leak of PSA to the circulation and thus result in higher serum levels (Porter, 1993). This leakage of PSA is used as a sensitive tumour biomarker for prostate cancer and therefore regarded as an oncological indicator of disease and response to prostate cancer treatment (Stamey *et al.,* 1989).

Moreover, the AR modulates the development and functioning of male reproductive organs and play a significant role in the progression of prostate cancer. It stimulates dependent protein kinase (PKA) that promote the G1-S phase of the cell cycle, induces inactivation of the retinoblastoma tumour suppressor (RB), and eventually regulate the androgen-dependent proliferation of prostate cells. Androgen stimulation in prostate cells triggers rapid activation of the PKA pathway, which induces a mitogenic response *(Pollack et al.,* 2009). We suggest that PKA regulates the activation of AR which leads to an increased PSA expression in an androgen-independent fashion (Sarwar *et al.,* 2012), thereby effective AR inhibition is indicative of a loss of detectable serum PSA.

The red palm oil used in this study plus its respective active compounds produced a concentration-dependent decrease in total PSA concentration in malignant (LNCaP) prostate cells whereas in benign (PWR-1E) prostate cells the Red Palm Oil concentrations did not significantly change.

Reports from previous studies demonstrate a direct relationship between a reduction in PSA and prostate cancer growth (Brausi et al., 1995). Considering only living cells produce PSA, the reduction in the PSA concentration is due to cell death and might indicate losing shape and

decreasing in cell numbers. (Zhang et al., 2002) reported significant reduction in serum levels of PSA in malignant LNCaP prostate cells by influencing the translation of AR after a brief treatment with vitamin E.

The anti-metastatic properties of Red Palm Oil: The expression of genes implicated in metastasis

The exact molecular mechanisms by which red palm oil exhibit its anti-cancerous properties as well as its role in prostate cancer is not fully understood and remains an active area for research. The impact of prostate cancer is due to the lack of methods for early diagnosis and prognosis, resulting in delayed clinical treatment for this disease. For example, the biomarker currently used for prostate cancer diagnosis is the PSA, which is considered the best tumour biomarker available, yet it is a biomarker with many drawbacks. Thus, new and better molecular biomarkers are urgently needed for early diagnosis and adequate prediction of the clinical course and outcome of the disease, since effective treatments apply only for early stages, but it is practically incurable in advanced stages (Bettin *et al.,* 2016).

Late clinical detection leads to the fact that metastasis the most likely cause of death in prostate cancer. The metastatic process involves dissemination of tumour cells from the primary tumour to distant organs and subsequent growth in the new tissue microenvironment, invasion of the extracellular matrix, formation of new blood vessels from a pre-existing vasculature (angiogenesis) and colonization of distant organs. In the present study, we determined the mRNA

expression profiles of three genes implicated in the metastatic process; the first gene is PO3F2 which is a transcription factor that is implicated in apoptosis and metastasis in melanoma but not researched in prostate cancer. The second gene is the placental gene 10 (PEG 10) located on human chromosome 7. PEG 10 is an oncogene that has a role in tumour progression and is implicated in the proliferation, apoptosis and metastasis of tumours. Moreover, a study has found that PEG 10 promotes the progression of neuroendocrine prostate cancer which is a very aggressive and drug-resistant AR negative type of prostate cancer (Akamatsu *et al.,* 2015). PEG 10 has been found to be positively expressed prostate cancer and its expression is associated with poor clinical outcome as it promotes tumour proliferation and inhibition of apoptosis. The third gene is the Serine Argenine repetitive Matrix 4 (SRRM4) which is expressed in neuroendocrine prostate cancer (Li *et al.,* 2017). The molecular analysis has shown that PO3F2 is neither expressed in prostate malignant cells nor the benign prostate cells and therefore is not a suitable marker to study cancer progression in the prostate gland. The other two markers namely PEG 10 and SRRM4 were highly expressed in both malignant and benign cells and there was no difference in expression between the treated cells and non- treated which might indicate that red palm oil does not affect/prevent the metastatic process. The molecular results require further optimization through quantitative experimentation with real-time quantitative polymerase chain reactions (qRT-PCR) to determine a cut-off value for the expression in benign and malignant prostate cells before commenting on the role of these markers in prostate cancer progression. A useful guideline for a gene expression analysis in prostate cancer is published recently (Bettin *et al.,* 2016). The aim of this study was to evaluate the gene expression profiles of a set of prostate cancer-associated genes in prostate cancer cell lines, to determine their association with different cancer phenotypes and identify potential novel biomarkers for this disease. A quantitative real-time PCR was used to determine the expression profiles of 21 prostate cancers–associated genes in the human prostate cancer cell lines PC-3 and LNCaP, using the nontumorigenic cell line PWR-1E as control cell line. Genes evaluated were ESM-1, SERPINE2, CLU, BGN, A2M, PENK, FMOD, CD81, DCN, TSPAN8, KBTBD10, F2RL1, TMSB4X, SNCG, CXXC5, FOXQ1, PDPN, SPN, CAV1, CD24 and KLK3. Several of the evaluated genes showed significantly altered expression in the prostate cancer cell lines when compared with nontumorigenic PWR-

1E cells. Further evaluation of FMOD transcript in prostate clinical samples from patients diagnosed with benign or malignant prostatic disease identified a significant difference in the expression levels of this proteoglycan. The study suggested that the FMOD gene is a potential biomarker from this set of genes, and can be further evaluated in clinical samples from patients diagnosed with benign or malignant prostatic disease.

Conclusion

The results of this study have shown that the red palm oil produces a biologically significant decrease in malignant (LNCaP) cell viability over an extended period of time. In addition, the oil has minimal cytotoxic effect on the benign (PWR-1E) prostate cells. Furthermore, red palm oil is a potent inhibitor to mitochondrial dehydrogenase activity inducing death in malignant (LNCaP) prostate cells. This might be attributed to the main active components' tocopherols, tocotrienols and carotenoids are good anticancer agents. In addition, red palm oil significantly decreases the level of total serum PSA in malignant (LNCaP) prostate cells without any observable change in benign (PWR-1E) prostate cells. The knowledge gained through this study has the potential to provide a background for clinical trials using the red palm oil in chemoprevention and therapy.

However, to determine which of the active compounds (i.e. tocopherols, tocotrienols and carotenoids) has resulted in prostate cancer cell death. More research needs to be done on whether the chronic use of isolated active compounds should be recommended in prostate cancer. The findings of this study confirm previous reports that suggested that red palm oil can be used as dietary supplements to prevent and stop the progression of cancer. More studies should be done to support the growing contention that red palm oil can be a useful and healthful addition to the human diet (Kritchevsky, 2010).

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Appendix I

Chemical Supply

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

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

Prostate cancer cell line LNCaP

Normal prostate epithelial cell PWR-1E

Merck, Wadeville Gauteng, South Africa:

Dimethyl Sulphoxide Dimetilsulfoxido (DMSO)

Sigma-Aldrich, Louis, Missouri, United States:

Thiazolyl Blue Tetrazolium Bromide MTT (Catalogue Number:

Trypan Blue (TB)

Lonza, Verviers, Belgium:

Roswell Park Memorial Institute (RPMI 1640) Medium

Fetal Bovine Serum (FBS)

Greiner Bio-One, Frickenhausen, Germany:

Test tubes (15 ml and 50 ml)

Tissue culture flasks (25 cm2, 75 cm2)

Techno Plastic Products (TPP), Trasadingen, Switzerland:

Tissue culture plates (12-, 24- and 96-well plates)

Lasec, Cape Town, South Africa:

Syringes (5, 10 and 25 ml)

Syringe filters unit (0.20 µm)

Kimix, Cape Town, South Africa:

Serological pipettes 25 ml, 10 ml, 5 ml

Pipette Tips 1000 µl, 200 µl, 10 µl

Eppendorf vials

Equipment and supply

ELISA-reader

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

Lasec, Cape Town, South Africa:

Laminar Flow

Incubator

Scale

Centrifuge

Zeiss, Cape Town, South Africa:

Microscope

Appendix II

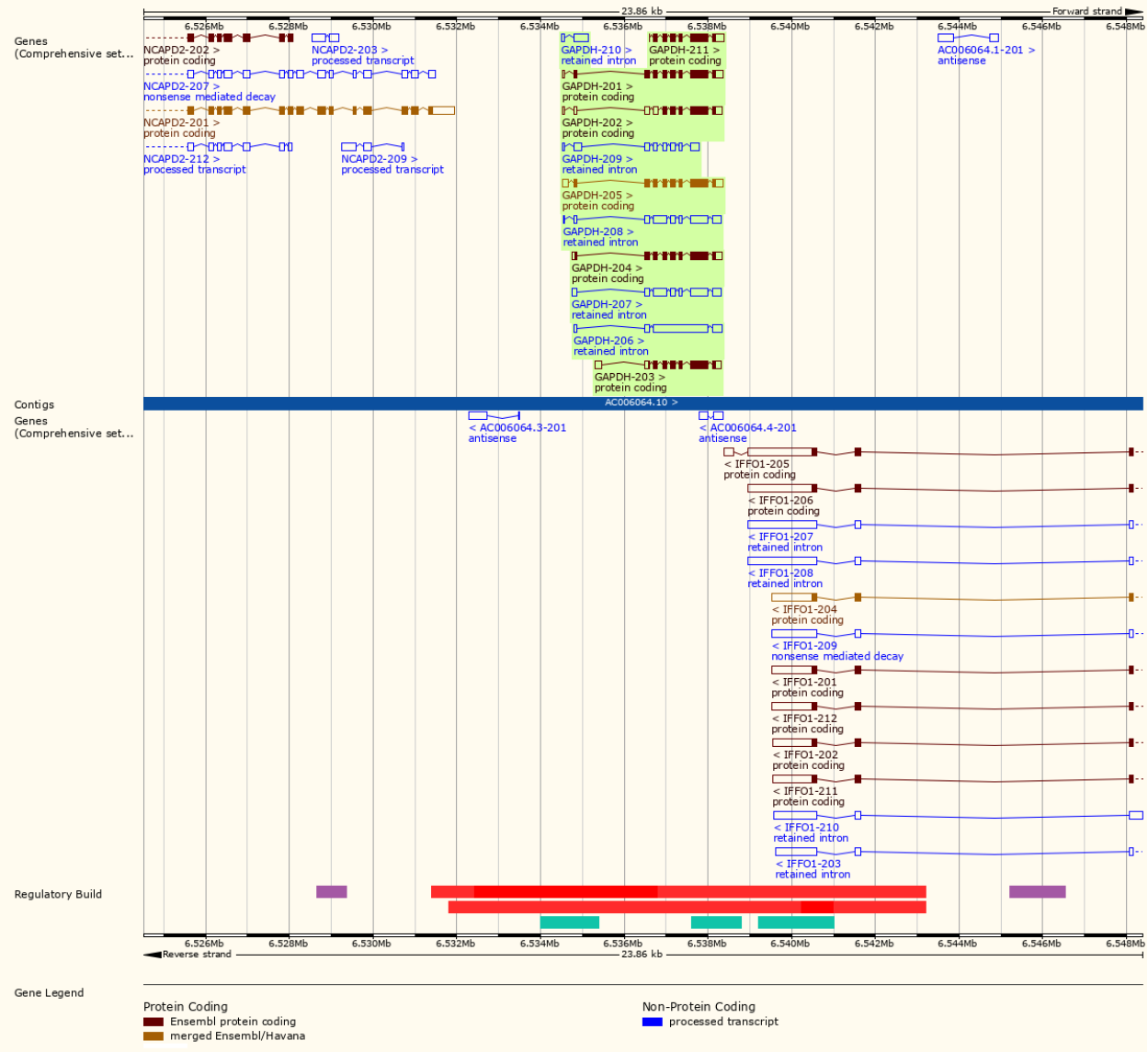
Name gene / ID	Description	Location	Aliases	MIM
GAPDH **ID: 2597**	glyceraldehyde-3-phosphate dehydrogenase [*Homo sapiens* (human)]	Chromosome 12, NC_000012.12 (6534517..6538375)	G3PD, GAPD, HEL-S-162eP	138400
POU3F2 **ID: 5454**	POU class 3 homeobox 2 [*Homo sapiens* (human)]	Chromosome 6, NC_000006.12 (98834704..98838790)	BRN2, N-Oct3, OCT7, OTF-7, OTF7, POUF3, brn-2, oct-7	600494
PEG10 **ID: 23089**	paternally expressed 10 [*Homo sapiens* (human)]	Chromosome 7, NC_000007.14 (94656325..94669695)	EDR, HB-1, MEF3L, Mar2, Mart2, RGAG3, RTL2, SIRH1	609810
SRRM4 **ID: 84530**	serine/arginine repetitive matrix 4 [*Homo sapiens* (human)]	Chromosome 12, NC_000012.12 (118981495..119163051)	KIAA1853, MU-MB-2.76, nSR100	613103

The Position of each primer used in this thesis.

Target gene: GADPH

NCBI Reference Sequence: NM_001289745

Forward primer 5'-AGAAGGCTGGGGCTCATTTG-3'
Reverse primer 5'-AGGGGCCATCCACAGTCTTC-3'



ORIGIN

```
1 gctctctgct cctcctgttc gacagtcagc cgcatcttct tttgcgtcgc caggtgaaga

61 cgggcggaga gaaacccggg aggctaggga cggcctgaag gcggcagggg cgggcgcagg

121 ccggatgtgt tcgcgccgct gcgggccgag ccacatcgct cagacaccat ggggaaggtg

181 aaggtcggag tcaacggatt tggtcgtatt gggcgcctgg tcaccagggc tgcttttaac

241 tctggtaaag tggatattgt tgccatcaat gacccccttca ttgacctcaa ctacatggtt

301 tacatgttcc aatatgattc cacccatggc aaattccatg gcaccgtcaa ggctgagaac

361 gggaagcttg tcatcaatgg aaatcccatc accatcttcc aggagcgaga tccctccaaa

421 atcaagtggg gcgatgctgg cgctgagtac gtcgtggagt ccactggcgt cttcaccacc
```

```
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541 tctgctgatg cccccatgtt cgtcatgggt gtgaaccatg agaagtatga caacagcctc
601 aagatcatca gcaatgcctc ctgcaccacc aactgcttag cacccctggc caaggtcatc
661 catgacaact ttggtatcgt ggaaggactc atgaccacag tccatgccat cactgccacc
721 cagaagactg tggatggccc ctcagggaaa ctgtggcgtg atggccgcgg ggctctccag
781 aacatcatcc ctgcctctac tggcgctgcc aaggctgtgg gcaaggtcat ccctgagctg
841 aacgggaagc tcactggcat ggccttccgt gtccccactg ccaacgtgtc agtggtggac
901 ctgacctgcc gtctagaaaa acctgccaaa tatgatgaca tcaagaaggt ggtgaagcag
961 gcgtcggagg gccccctcaa gggcatcctg ggctacactg agcaccaggt ggtctcctct
1021 gacttcaaca gcgacaccca ctcctccacc tttgacgctg gggctggcat tgccctcaac
1081 gaccactttg tcaagctcat ttcctggtat gacaacgaat ttggctacag caacagggtg
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1201 aagagcacaa gaggaagaga gagaccctca ctgctgggga gtccctgcca cactcagtcc
1261 cccaccacac tgaatctccc ctcctcacag ttgccatgta gacccccttga agaggggagg
1321 ggcctaggga gccgcacctt gtcatgtacc atcaataaag taccctgtgc tcaaccagtt
1381 aaaaaaaaaa aaaaaaaaaa a
```

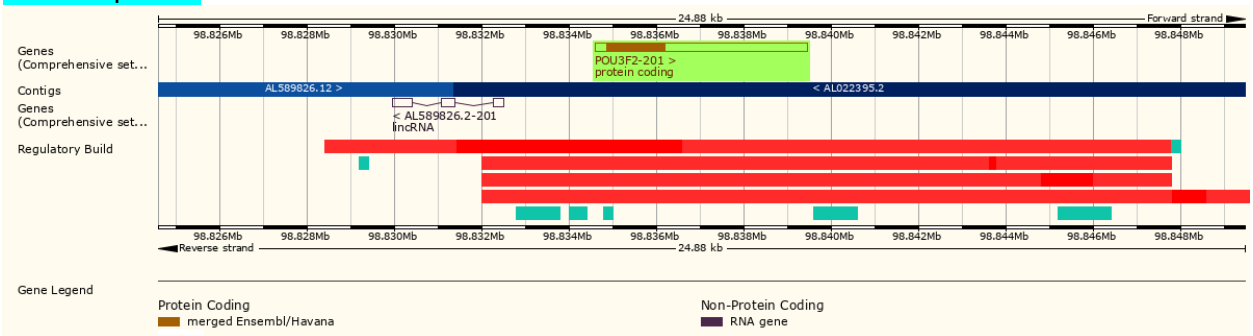
Forward primer

Reverse primer

Target gene: POUF3

NCBI Reference Sequence: NM_005604.3

Forward primer 5’-TTTGCAAGATAAATGGTGAC-3’
Reverse primer 5’-CTGTAGACACATTCTCTGAA-3’



ORIGIN

```
1 agtaatagca ggagcagcaa cagaaggcgt cggagcgggc gtcggagctg cccgctgtgg
61 gagagagagg agacagaaag agcgagcgag gagagggagc ccgaggcgaa aaagtaactg
```

```
121 tcaaatgcgc ggctccttta accggagcgc tcagtccggc tccgagagtc atggcgaccg
181 cagcgtctaa ccactacagc ctgctcacct ccagcgcctc catcgtgcac gccgagccgc
241 ccggcggcat gcagcagggc gcgggggggct accgcgaagc gcagagcctg gtgcagggcg
301 actacggcgc tctgcagagc aacggacacc cgctcagcca cgctcaccag tggatcaccg
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481 agccctcggt ggtggtgcag cagggcggcc gcggagacga gctgcacggg ccaggcgccc
541 tgcagcagca gcatcagcag cagcaacagc aacagcagca gcaacagcag caacagcagc
601 agcagcagca gcaacagcgg ccgccgcatc tggtgcacca cgccgctaac caccacccgg
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721 ccaacggcgg cttgctctac tcgcagccca gcttcacggt gaacggcatg ctgggcgccg
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961 cgccgacctc ggacgacctg gagcagttcg ccaagcagtt caagcagcgg cggatcaaac
1021 tgggatttac ccaagcggac gtggggctgg ctctgggcac cctgtatggc aacgtgttct
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Forward primer

Reverse primer

Target gene: PEG10

NCBI Reference Sequence: NM_001040152.1

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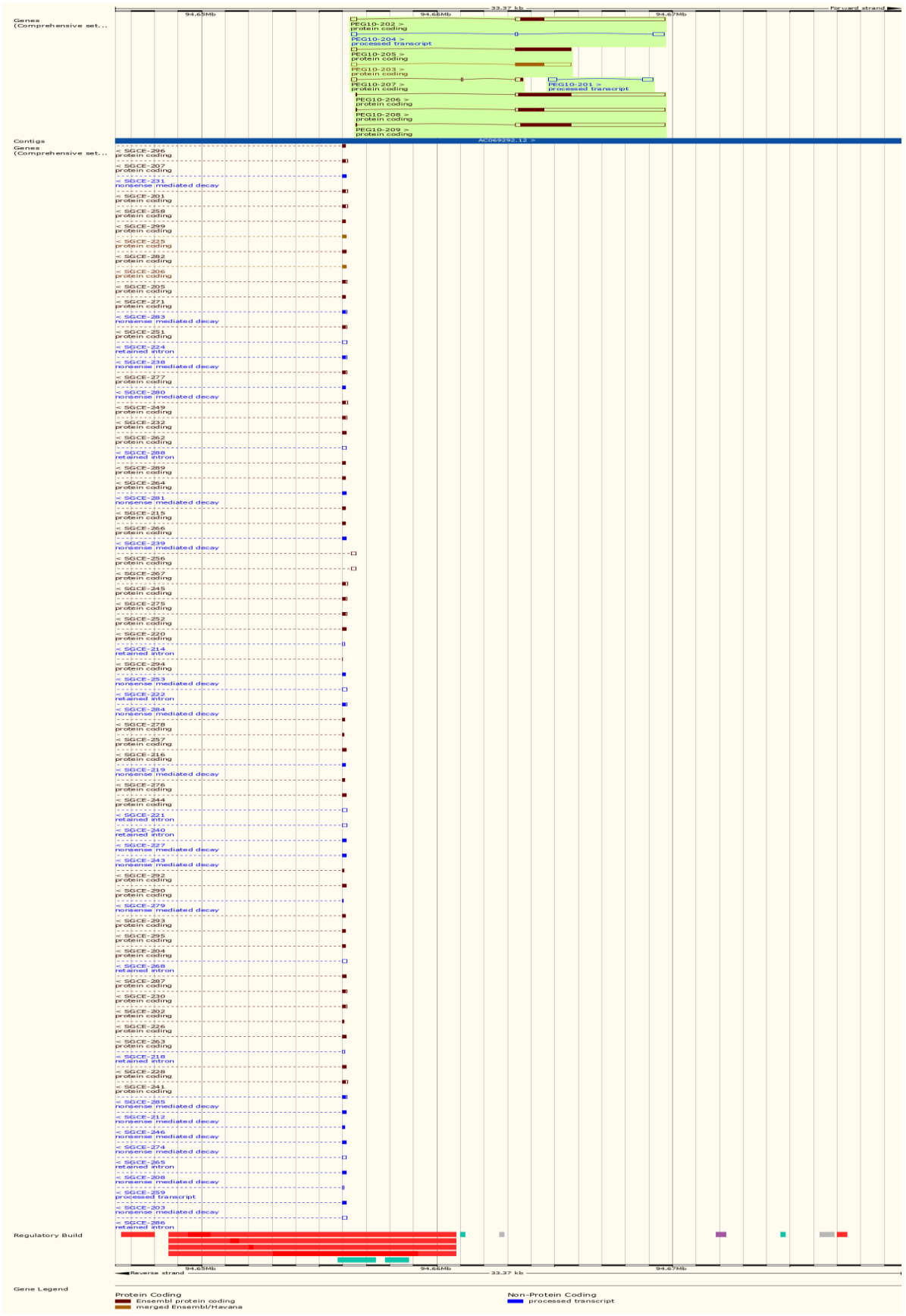

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Forward primer

Reverse primer

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Target gene: SRRM4

NCBI Reference Sequence: NM_194286.3

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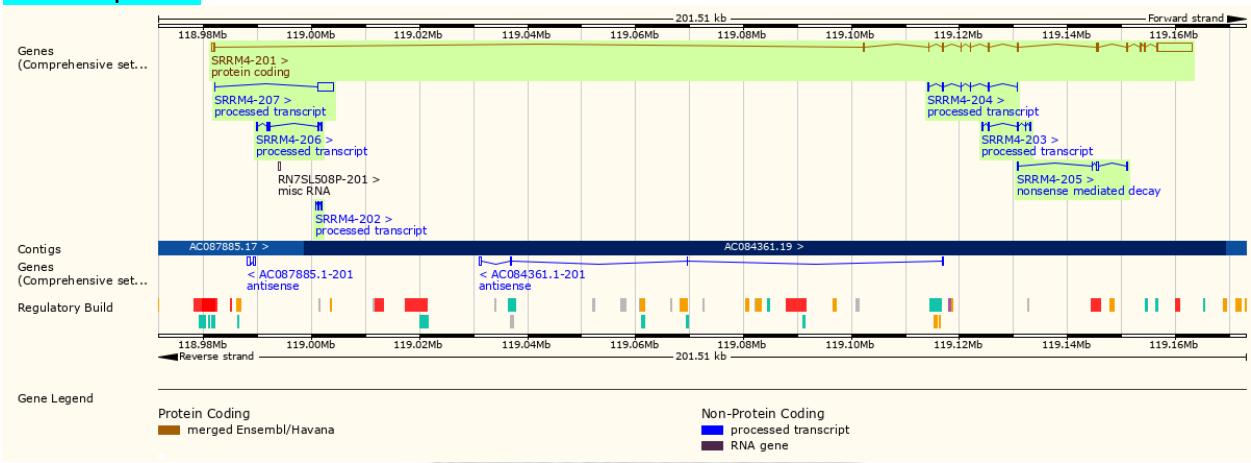

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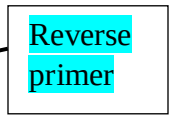


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