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RED PALM OIL AS A THERAPEUTIC AGENT IN TRIPLE-NEGATIVE BREAST CANCER PATIENTS

by

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DECLARATION

I, **Sameera Slahudeen**, declare that thesis on ***"Red palm oil as a therapeutic agent in triple-negative breast cancer patients"*** is my own work, that it has not been submitted for any degree or examination at any other university, that it is free of plagiarism and that all the sources used have been indicated and acknowledged by complete references.

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Date Signed **:** 21 November 2020

DEDICATION

This thesis is dedicated to my wonderful parents. To my dear mother, your support, encouragement and motivation have played a tremendous role in helping me to achieve all my dreams and goals, not only academically, but personally as well. Thank you for always pushing me to uncover my true potential and bestowing a great teaching of never quitting on the things you want most in life. To my late father, your zest for life has been a true inspiration to me to be the best version of myself and you've taught me that even the darkest times can be overcome through perseverance.

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I would also like to dedicate this thesis to anyone who has fallen victim to this grave disease and to survivors who were fortunate to overcome this battle. Your bravery and will to fight is truly admirable. You are forever in our thoughts and prayers.

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"Verily, with every hardship comes ease."

– Quran 94: 5-6.

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ABSTRACT

Purpose: Breast cancer is one of the most frequent and fatal diseases women all around the globe are challenged with today. In women, breast cancer has the highest mortality rate of all cancers and the incidence rate is on the increase. It is estimated that by the year 2025, 19.3 million women will become a victim of this grave health problem. This disease is a result of the formation of malignant tumours caused by genetic alterations that are involved in the proliferation of cells, cellular differentiation and the disturbance in homeostasis which subsequently leads to the abnormal multiplication and growth of cells. Breast cancer is considered a multifactorial disease with various risk factors such as age, radiation exposure, hormone therapy, oral contraceptives, dietary factors, environmental exposure and genetic predispositions. Breast cancers can be subdivided and classified based on their cellular surface receptors such as Estrogen Receptors, Progesterone Receptors and Human Epidermal Growth Factor Receptor 2. Of the various subtypes, the triple-negative breast cancer subtype which is negative for all 3 surface receptors and presents as the most aggressive form of breast cancer with a poor prognosis. Between 10-20% of all breast cancer cases are classified as triple-negative breast cancer. Due to the hormonal status of triple-negative breast cancer, treatment options are limited and thus of great concern. Chemotherapy remains the most common treatment modality, but prognosis is poor with relapse within years ultimately leading to poor survival outcome. Due to this lack of effective treatment plans, an alternative treatment with minimal side effects and better survival remains an imperative area to explore. A wide scope of literature highlights red palm oil and its health benefits, with its growth inhibitory potential drawing great attention. Red palm oil, extracted from the *Elaeis guineensis* palm tree is red in colour due to the abundance of carotenoids, tocotrienols and tocopherols found in the oil. Various compounds make up the oil such as lycopene, carotenes, vitamin E and coenzyme Q_{10}. Most studies have researched the effects of vitamin E extracted from the oil as a contributor to its growth inhibitory activity. This study focuses on the effects of the commercial red palm oil as a whole with all its compounds on the proliferation of breast cancer cells as well as the effect it has on various genes associated with breast cancer.

Method: This study investigated the effect of red palm oil concentrations (1, 10, 100, 500 and 1000 µg/ml) on breast cancer cells—MCF-7 and MDA-MB-231 with comparison to a non-cancerous cell line—MCF-12A for 24-, 48- and 72-hour treatment periods. The parameter investigated was cell proliferation through the CCK-8 cell proliferation assay and the morphology following red palm oil treatment was observed and captured. Additionally, this study also investigated the effect of red palm oil on the expression of Human Mammaglobin (hMAM) and Maspin genes through the PCR assay and results visualised through agarose gel electrophoresis. Data was statistically analysed using GraphPad version 6.0 software.

Results: Following treatment of red palm oil, no apparent changes in the cell morphology was observed despite using variable treatment concentrations over variable times for MCF-7, MDA-MB-231 and MCF-12A cells relative to their respective controls. Immortalised MCF-12A cells showed a significant increase in proliferation with the varying treatment concentrations, but more prominently with the highest concentration at 24, 48 and 72 hours. MCF-7 cells showed significant decreases at 24 and 72 hours. Decreased proliferation was observed at all dosages used, particularly at 10, 100, and 500 µg/ml. Furthermore, MDA-MB-231 cells demonstrated a gradual increase in cell proliferation for the 3 selected time periods in the varying concentrations. Additionally, red palm oil did not alter the gene expression of Maspin at any of the varying treatments for MDA-MB-231 nor MCF-7 cells. However, changes in hMAM gene expression were observed at treatment concentration of 100 µg/ml in MDA-MB-231 cells that were incubated for 24 and 48 hours. However, the hMAM expression was not affected in treated MCF-7 cells.

Conclusion: Red palm oil, as an alternative dietary oil, seems to have potential growth inhibitory properties as demonstrated by the change in the cell proliferation of the MCF-7 cells. Literature show that various individual compounds extracted from red palm oil have anti-proliferative and inhibitory effects on breast cancer cells making them good candidates for therapy. However, this study concludes that red palm oil as a whole component would not be a suitable therapeutic agent for highly aggressive triple-negative breast cancer.

Keywords: breast cancer, triple-negative breast cancer, red palm oil, cell proliferation, human MCF-7 cells, MDA-MB-231 (TNBC) cells, human epithelial MCF-12A cells, PCR, gene expression, GAPDH housekeeping gene, hMAM gene, Maspin gene

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ABBREVIATIONS

BRCA1	Breast cancer 1 gene
BRCA2	Breast cancer 2 gene
CCK-8	Cell counting kit 8
DMEM/F12	Dulbecco's modified eagles medium/F-12
DMSO	Dimethylsulfoxide
EGFR	Epidermal growth factor receptor
EMT	Epithelial-mesenchymal transition
ER	Estrogen receptor
Erk	Extracellular signal-related kinase
FBS	Foetal bovine serum
Fra-1	Fos-related antigen-1
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GIST	Gastrointestinal stromal tumours
HER2	Human epidermal growth factor receptor 2
hBSCs	Human breast milk stem cells
IGFR	Insulin-like growth factor receptor
IHC	Immunohistochemistry
IL-12	Interleukin-12
INF-γ	Interferon-gamma
MAPK	Mitogen-activated protein kinase
NC	Negative control
NCBI	National Centre for Biotechnology Information
NK	Natural killer cells
NLSs	Nuclear localization signals
OPP	Oil palm phenolics
PBS	Phosphate buffer saline
PC	Positive control

PCR	Polymerase chain reaction
PKC	Protein kinase C
PLC	Pregnancy-lactation cycle
PPM	Parts per million
PR	Progesterone receptor
PRKCQ/PKCϴ	Protein tyrosine kinase C theta isoform
qRT-PCR	Quantitative Real-Time Polymerase Chain Reaction
RO	Reverse osmosis
RPO	Red palm oil
SCGB2A1	Secretoglobin family 2A member 1
SERPINB5	Serpin family B member 5
TF	Transcription factor
Th1	T-helper-1
TNF-α	Tumour necrosis factor alpha
TNBC	Triple-negative breast cancer
TRF	Tocotrienol-rich fraction
TSG	Tumour suppressor gene
VC	Vehicle control

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

The purpose of this chapter is to briefly elaborate and illustrate the present understanding of the functional anatomy and physiology of the female breast. It also covers breast cancer with respect to epidemiology, incidences and mortality statistics and the ongoing research attempts aimed towards the prevention and the expansion of targeted molecular and personalized therapies. Furthermore, it incorporates a review of relevant literature to establish and contextualize the specific areas pertaining to breast cancer research, the basis of the therapy of choice and the aims to be investigated.

1.2 Functional Anatomy of the Female Breast

The breasts, also known as the mammary glands or mammae, are dome-shaped structures located between the 2nd and the 6th or 7th ribs anteriorly on the chest (Macéa and Fregnani, 2006; Jones and Lopez, 2013; Peate and Nair, 2016). The base is circular and measures between 10 to 12 cm, but differs in volume or size between individuals. The mass of the non-lactating breast varies from 150 to 255 g whereas the lactating breast may surpass 500 g (Macéa and Fregnani, 2006). They are provided with a significant supply of blood vessels, nerves and lymphatics and are supported by the pectoral muscles (Peate and Nair, 2016). With aging, the volume of the breast decreases and the breast loses its firmness and becomes flatter and pendulant (Macéa and Fregnani, 2006). Figure 1.1 illustrates the various components of the female breast. The breasts are comprised of fibrous connective tissue, adipose tissue and glandular tissue. Each breast holds glandular tissue that is divided into 15 to 20 lobes. These lobes are separated by the suspensory ligaments of Cooper which provide support to the breast, and fat. Each lobe encompasses several lobules, which are the grape-like structures of mammary alveoli (Jones and Lopez, 2013; Mills *et al,* 2016; Peate and Nair, 2016). Figure 1.2 illustrates a single alveolus which is

responsible for milk production and ejection.

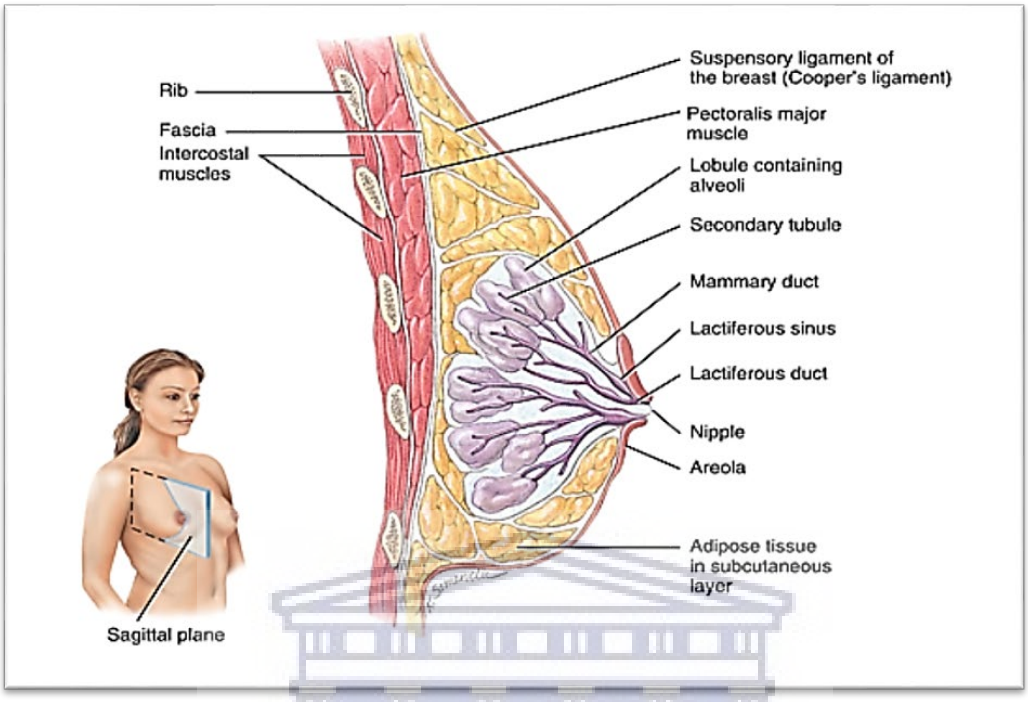

Figure 1.1: Micro-anatomical structure of a female breast shown through the sagittal section (Peate and Nair, 2016).

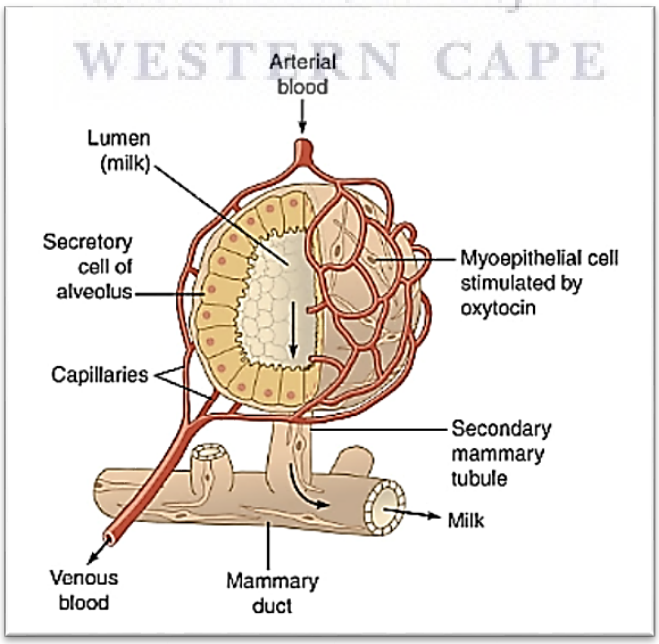

Figure 1.2: Schematic view of a single alveolus located in the lobes of the mammary gland (Jones and Lopez, 2013).

The alveolar glands are the functional unit of mammae and contain milk-secreting cells that are surrounded by myoepithelial cells. The hormone oxytocin causes contractions of the myoepithelial cells which ultimately results in the secretion of milk into the lumen and towards the nipple. Every alveolus receives a substantial blood supply providing raw material for the synthesis of milk. Milk that is made and secreted then enters the secondary mammary tubules. The mammary duct is formed through the joining of secondary mammary tubules from each lobe which then drains into a broader mammary ampulla which allows for the storage of milk. The ampulla then drains into a lactiferous duct and then opens into the nipple (Jones and Lopez, 2013; Mills *et al,* 2016; Peate and Nair, 2016).

The development of breasts during life involves a time course of distinct developmental stages. The main developmental stages involve foetal growth phase, infant (prepubertal) growth phase, pubertal expansion, pregnancy and lactation-affiliated remodelling and the post-lactation and post-menopausal involution (shrinking of the organ) due to changes in the tissue (Russo and Russo, 2004; Geddes, 2007; Hassiotou and Geddes, 2013). From birth to puberty, the breasts contain lactiferous ducts without any alveoli. At puberty, ducts begin proliferating and solid masses of cells are formed. The secreting alveoli appear during pregnancy. In the early weeks of pregnancy, sprouting of ducts and lobular proliferation occurs with an increase in nipple and areolar pigmentation. In the later days of pregnancy, breasts secrete colostrum, a sticky and yellow serous fluid that is then replaced with the proper secretion of milk. The glandular tissue goes back to a resting state once lactation ceases. After menopause, the glandular tissue degenerates, connective tissue becomes less cellular and the volume of collagen declines. This leads to the shrinkage of the breasts (Ellis and Mahadevan, 2013; Mills *et al,* 2016).

While most organs of the human body progress to a relatively mature state through embryonic life, the breast only attains functional maturity during a pregnancy-lactation cycle (PLC) when the breast encounters complete remodelling, resulting in the maturation of a functional milk-secreting organ (Hartmann, 2007). The full development of breasts during lactation is vital to provide the suitable quantity and composition of milk for growth, protection and development of an infant. Furthermore, it has been demonstrated that in the long term, the PLC offers protection against breast cancer

development (Key *et al,* 2001; Hassiotou and Geddes, 2013). However, crosstalk occurs between signalling pathways that fuel the development of a normal mammary during PLC and the oncogenic signals that are associated with the initiation of breast cancer, progression of it and metastasis. Specifically, self-renewal transcription factors (TFs) are shared among human breast milk stem cells (hBSCs) and different types of aggressive tumours. This implies that the generalization about PLC being a protective factor against cancer development should be made with caution (Hassiotou *et al,* 2012; Hassiotou and Geddes, 2013). The onset of the post-menopausal involution phase is paralleled by a decrease in ovarian function and estrogen which leads to the regression and atrophy of the breasts glandular tissue, a build-up of adipose tissue and correlated sequelae following the development of breast cancer (Hassiotou and Geddes, 2013).

1.3 Breast Cancer

Breast cancer continues to exist as the most common and fatal malignancy in females across the globe (Armer *et al,* 2009; Abdulrahman and Rahman, 2012; Lalloo and Evans, 2012; Balekouzou *et al,* 2016; Azubuike *et al,* 2018). Breast cancer is the result of mutations in genes that play a role in processes such as cell proliferation, differentiation and homeostasis which thereby creates an abnormality in breast cells and the uncontrolled cell growth or multiplication thereof, ultimately forming malignant tumours (Apostolou and Fostira, 2013; Guo *et al,* 2014; Shen *et al,* 2018; Waks and Winer, 2019). These tumours generally begin from ductal hyperproliferation, following the development of benign tumours or metastatic carcinomas through constant stimulation by multiple carcinogenic factors (Sun *et al,* 2017).

Typically, breast cancer presents no symptoms when a tumour is minute and is easily treated. This factor makes screening important for early detection. A painless lump is the most common physical sign of breast cancer (American Cancer Society, 2017; Koo *et al,* 2017; Waks and Winer, 2019). Occasionally, the cancer spreads to underarm lymph nodes which causes swelling or a lump. This can occur even before the original tumour is big enough to be felt. Less common symptoms and signs involve heaviness or pain in the breast, persistent changes such as redness of the skin, swelling or thickening, and nipple irregularities such as erosion, retraction or spontaneous discharge especially

where blood is being discharged (Leis, 1980; American Cancer Society, 2017; Koo *et al,* 2017). During a screening examination, breast cancer can be detected before a development of symptoms or after the female notices the lump (American Cancer Society, 2017; Baron *et al,* 2018). Most cell masses are observed on a mammogram and, if lumps turn out to be non-cancerous (benign), they do not go through uncontrollable growth nor spread, and do not pose a threat to life. However, when cancer is suspected, it is necessary to do microscopic analysis of the breast tissue in order to diagnose and determine the stage (the extent of the spread) and to characterize the disease type (McDonald *et al,* 2016; American Cancer Society, 2017). The tissue needed for a microscopic analysis can be procured from a surgical incision or a needle biopsy. Multiple factors which include location and size of the mass, and a patient's preference and resources form the basis of the type of biopsy that is selected and performed (American Cancer Society, 2017; Wang, 2017).

In South African women, breast cancer is said to be the most common cancer, with diverse incidence rates (Langa *et al,* 2015; Moodley *et al,* 2018). According to Moodley *et al,* the South African National Cancer Registry reported an incidence rate of 35 to every 100 000 women in South Africa, with breast cancer being the most prevalent of all cancers (Moodley *et al,* 2018). The incidence rates vary between racial groups with Caucasian women having the highest and African women having the lowest rates of diagnosis, with African women being at a greater risk of breast cancer development in comparison. (Langa *et al,* 2015).

These findings were similarly seen in African-American women with Caucasian women having a better survival rate (Laing *et al,* 1993; Cote *et al,* 2012). Worldwide, breast cancer is the second major cause of mortality following lung cancer with majority of new cases reported in low- and middle-income countries (Ferlay *et al,* 2015; Torre *et al,* 2015; Moodley *et al,* 2018; Black and Richmond, 2019). According to Han and colleagues, breast cancer affects 232, 670 women in the United States while affecting 2360 men (Han and Magliocco, 2016). Bray *et al* reported 2,08 million (11.6%) new breast cancer cases with 626,679 (6.6%) number of deaths in 2018 (Bray *et al,* 2018). An estimated 19.3 million women will be affected by breast cancer by the year 2025 with the majority of women being

from Sub-Saharan Africa (Cumber *et al,* 2017). Figure 1.3 shows a visual representation of incidence of cancer globally while Figure 1.4 shows the mortality of the various cancers with breast cancer being abundantly common.

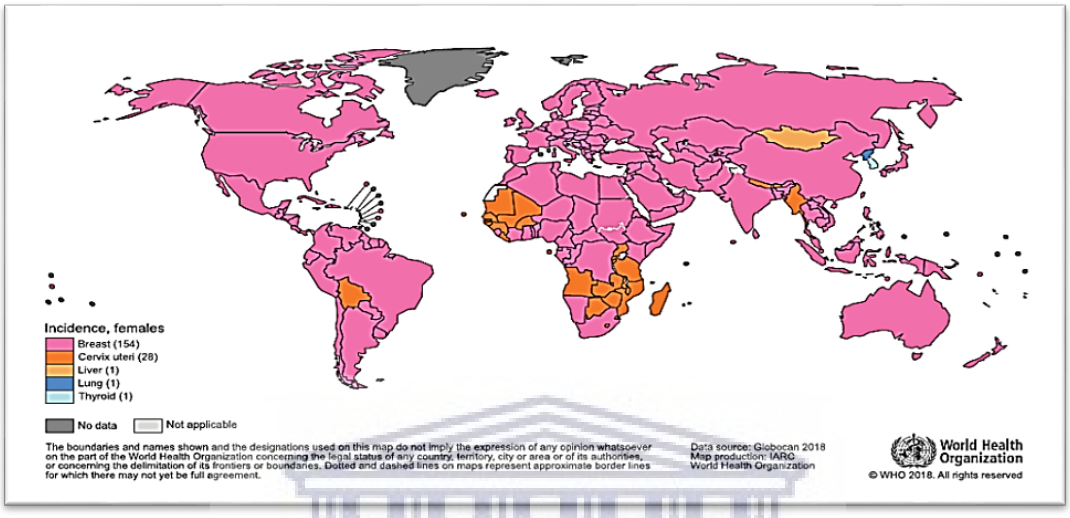

Figure 1.3: Global map showing incidence of the most common cancer types with breast cancer being the most common amongst females in 2018. Data sourced from Globocan 2018 (Bray *et al*, 2018).

1.3.1 Breast Cancer Risk Factors

The development of breast cancer is said to be multifactorial and cannot be linked to one single factor in particular (Casey *et al,* 2008; Ataollahi *et al*, 2015; Ozsoy *et al,* 2017; Momenimovahed and Salehiniya, 2019). Various factors are involved in the pathogenesis of breast cancer across the population and these factors include genetics, acquired factors, exposure to radiation, aging, hormone therapy and environmental factors to name a few (Ataollahi *et al,* 2015; Ozsoy *et al,* 2017; Sun *et al,* 2017; Momenimovahed and Salehiniya, 2019). Figure 1.5 below shows a few risk factors as well as preventative measures. It is imperative to find effective and efficient treatments with minimal damages to target breast cancer. Recent investigations give rise to red palm oil as a new and alternate treatment to breast cancer with anticancerous properties.

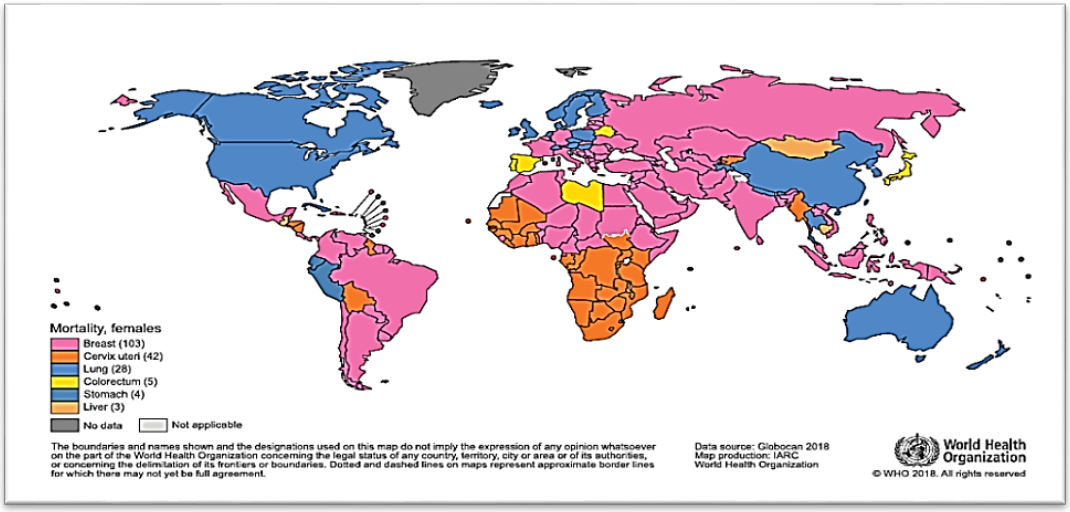

Figure 1.4: Global map showing mortality of the most common cancer types in females in 2018. Data sourced from Globocan 2018 (Bray *et al*, 2018).

While genetics play a key role in the development of breast cancer, there are various factors that come up in the literature. It has been shown that there is an increase in breast cancer incidences with aging and doubles roughly every 10 years (McPherson *et al,* 2000; Kamińska *et al,* 2015; Ozsoy *et al,* 2017). Late age of first birth and nulliparity both give rise to lifetime incidence of breast cancer. The risk of breast cancer between women who give birth to their first baby after 30 years of age and women who give birth before 20 years of age are seen to be doubled. Women who have their first child after 35 years of age are of the highest risk group and appear to be more at risk than nulliparous women (McPherson *et al,* 2000; Kamińska *et al,* 2015).

Women who take oral contraceptives are also at risk for breast cancer (McPherson *et al,* 2000; Marchbanks *et al,* 2002; Casey *et al,* 2008; Ozsoy *et al,* 2017; Wahidin *et al,* 2018). During the consumption of oral contraceptives and for 10 years after termination thereof, there is a minor increase in risk of development (McPherson *et al,* 2000; Marchbanks *et al,* 2002; Casey *et al,* 2008; White, 2018). Women who commence the use of contraceptives before 20 years of age appear to have a greater relative risk than those who commence the use of oral contraceptives at an older age (McPherson *et al,* 2000; Casey *et al,* 2008; White, 2018). Women who begin menstruating earlier in life and have a late

onset of menopause have an increase in risk of breast cancer development (McPherson *et al,* 2000; Beral *et al,* 2010; Ozsoy *et al,* 2017). Women who begin a natural menopause after 55 years of age have double the risk of breast cancer development in comparison to women who go through menopause before 45 years of age.

The incidence of breast cancer is also increased in women who use menopausal hormone therapy (McPherson *et al,* 2000; Beral *et al,* 2010; Surakasula *et al,* 2014; Jones *et al,* 2016; Mørch *et al*, 2017). Estrogen is a known promoter of breast cancer development. Short-term treatment with estrogen only has little to no risk of breast cancer development in comparison to a long-term treatment that has an increased risk (Mørch *et al*, 2017; Brusselaers *et al,* 2018). However, the risk of developing breast cancer seems higher with the combination of estrogen-progestin than only the use of estrogen (McPherson *et al,* 2000; Beral *et al,* 2010; Jones *et al,* 2016; Mørch *et al*, 2017).

Benign breast disease is also considered an important risk factor for breast cancer and can develop in either breast. It comprises a wide range of histological entities generally divided into proliferative lesions without atypia, nonproliferative lesions and atypical hyperplasias proliferative lesions (McPherson *et al,* 2000; Hartmann *et al,* 2005; Cote *et al,* 2012; Zendehdel *et al,* 2018). Literature reveals that women with atypia and familial history had 11 times the risk compared to those with no family history and nonproliferative lesions (McPherson *et al,* 2000; Hartmann *et al,* 2005).

This indicates that the degree of risk differs for the various types of benign breast disease with greater risk with proliferative lesions than that in nonproliferative lesions (Zendehdel *et al,* 2018). Furthermore, women with additional pathologic features such as palpable cysts, fibrosis, duct papillomas, sclerosis adenosis, mastitis, lobular involution, columnar alterations, radical scars and fibroadenomas have a slightly elevated risk of breast cancer in comparison to women without these changes (McPherson *et al,* 2000; Guray and Sahin, 2006; Dorjgochoo *et al,* 2008; Cote *et al,* 2012).

Radiographical appearances of breasts vary between women due to differences in tissue composition and differences in radiographic attenuation characteristics of epithelium, stroma and fat (Boyd *et al,*

2007). Fat is radiographically luminous and appears dark on mammograms. Stroma and epithelium are radiographically dense and light and this appearance is known as mammographic density (Wolfe, 1976; Boyd *et al,* 2007). Women containing dense tissue in 75% or more of her breast have a risk of breast cancer development 4 to 6 times greater than women who have little to no dense tissue (Wolfe, 1976; Byrne *et al,* 1995; Ursin *et al,* 2003; Boyd *et al,* 2007; Tice *et al,* 2015).

Breast exposure to ionising radiation also causes an increase in risk later in life, specifically when exposure occurs during rapid breast formation (McPherson *et al,* 2000; Ronckers *et al,* 2005; Surakasula *et al,* 2014). Teenage girls exposed to radiation showed a doubling in risk of breast cancer during World War 2 (McPherson *et al,* 2000). Females exposed to radiation before 20 years of age are at a greater risk than females after a menopausal age (Thomas *et al,* 1994; Ronckers *et al,* 2005; Washbrook, 2006; Surakasula *et al,* 2014).

Risk of breast cancer also varies between countries. McPherson *et al* noted that migrants assume breast cancer rates of host countries which are indicative of environmental factors (McPherson *et al,* 2000). In recent research, environmental and lifestyle exposures such as poor diet, lack of exercise and physical activity, alcohol consumption, physiological factors and obesity amongst others have also been said to play a role in breast cancer development (Surakasula *et al,* 2014; Cauchi *et al,* 2016; Sun *et al*, 2017).

A plethora of studies outline specific dietary factors that contribute to risk and prevention of breast cancer (Figure 1.5). In a study conducted by Cauchi *et al,* it was noted that consuming canned meat and tomatoes decreases the likelihood of breast cancer development, whereas consuming beans, cabbage and low sodium salt increases the risk of breast cancer even though these items are considered healthy (Cauchi *et al,* 2016). Tomatoes have been considered as a protective component against breast cancer and are known to be rich in lycopene (Rossi *et al,* 2014; Cauchi *et al,* 2016). Lycopene compounds possess antioxidant effects which protect cells against free-radical damage that is associated with the risk of developing breast cancer (Cauchi *et al,* 2016). Other studies revealed that a high fat diet and high intake of red meat, processed or fresh, and independent of preparation method, increased the risk of

breast cancer due to various carcinogenic compounds found in meat (Rossi *et al,* 2014; Shapira, 2017; Taha and Eltom, 2018). Diets containing soy foods and soy beans, skim milk, foods rich in fiber, raw and cruciferous vegetables, fruits, and legumes are said to decrease risk of breast cancer. Furthermore, high consumption of fish, eggs, poultry and nuts may also contribute to a risk reduction of cancer development (Boggs *et al,* 2010; Bao *et al,* 2012; Rossi *et al,* 2014; Shapira, 2017; Taha and Eltom, 2018). The onset of diseases is generally followed by dietary changes (Cauchi *et al,* 2016).

Cauchi *et al* also found that sunlight exposure was linked to a decrease in risk associated with breast cancer. While prolonged exposure to sunlight has damaging effects on the skin, it was seen to reduce risk of developing breast cancer. This could be due to the production of vitamin D and the role it plays in breast cell growth (Garland *et al,* 2006; Shao *et al,* 2012; Rossi *et al,* 2014; Cauchi *et al,* 2016; Kotepui, 2016).

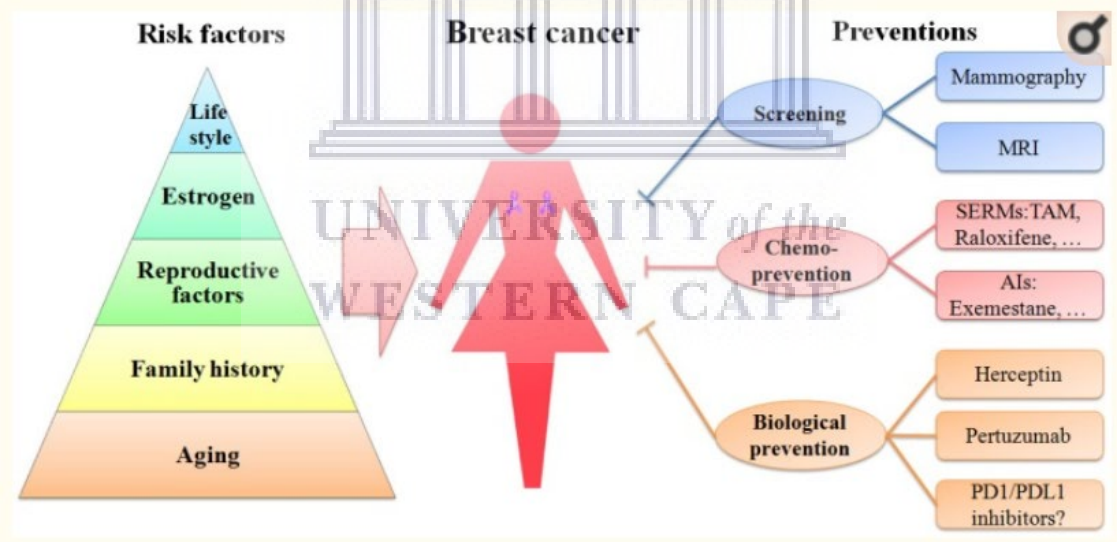

Figure 1.5: Schematic diagram showing various risk factors involved in the development of breast cancer as well as preventative measures (Sun *et al*, 2017).

1.3.2 Genes Associated with Breast Cancer

Breast cancer 1 gene (BRCA1) and Breast cancer 2 gene (BRCA2) mutations are a common cause of hereditary breast cancer which makes up 5-10% of breast cancer occurrences (Smith *et al,* 2007;

Petrucelli *et al,* 2010; Apostolou and Fostira, 2013; Sun *et al*, 2017). BRCA1 and BRCA2 gene mutations not only predisposes to breast cancer, but also other cancers with ovarian cancer being one of them (Easton *et al,* 1995; Claus *et al,* 1998; Antoniou *et al,* 2003; Petrucelli *et al,* 2010; Girardi *et al,* 2018). The risk involved in the development of cancer associated with mutations of BRCA1 or BRCA2 genes vary within families and were obtained through numerous sources which include population-based studies, few affected individuals and multiple-affected individuals in a family. Families with multiple-affected individuals may have enrichment for mutations and is known to have consequential risks for breast cancer as well as ovarian cancer (Claus *et al,* 1998; Choi *et al,* 2004; Smith *et al,* 2007; Petrucelli *et al,* 2010; Girardi *et al,* 2018). Mutations or changes to the two genes, increases the risk of breast cancer, ovarian cancer and prostate cancer development as damaged DNA cannot be repaired, giving rise to further changes and subsequently leading to the various cancers (Mehrgou and Akouchekian, 2016; Sun *et al*, 2017).

The BRCA1 gene, which is located on chromosome 17 at 17q21 as seen in Figure 1.6, contains 24 coding exons which is spread over 80 kb (Easton *et al,* 1995; Dufloth *et al,* 2005; Petrucelli *et al,* 2010; Mehrgou and Akouchekian, 2016; Sun *et al*, 2017). The normal allele generates a 7.8-kb mRNA which encodes susceptibility protein for breast cancer type 1, an 1863 amino acid containing various recognizable protein motifs, a pair of nuclear localization signals (NLSs) found on exon 11, RING-finger domain close to the N-terminus, BRCT domain at C-terminus, and between amino acids 1280-1524 an "SQ" cluster. (Venkitaraman, 2002; Dufloth *et al,* 2005; Lee and Muller, 2010; Petrucelli *et al,* 2010; Mehrgou and Akouchekian, 2016). These features can be seen in Figure 1.7 below. BRCA1 communicates with proteins involved with cellular pathways, which include gene transcription regulation, ubiquitination, cell cycle progression and DNA damage response. BRCA1 helps to maintain genomic integrity serving as a "caretaker" (Venkitaraman, 2002; Petrucelli *et al,* 2010). When BRCA1 is unable to conduct this function, it might allow other genetic defects to accumulate that are directly involved in the development of cancer. In BRCA1, over 1600 mutations have been distinguished leading to framing errors which results in non-functional or missing proteins. The wild-type allele in most cancers are deleted, which strongly suggests BRCA1 is in the tumour suppressor genes class

(Petrucelli *et al,* 2010; Godet and Gilkes, 2017). Functional deficiencies of BRCA1 leads to defects in transcription and DNA repair, abnormal duplication of the centrosome, impaired spindle checkpoint, malfunctioning of G2/M checkpoint regulation in the cell cycle and chromosomal damage (Petrucelli *et al,* 2010).

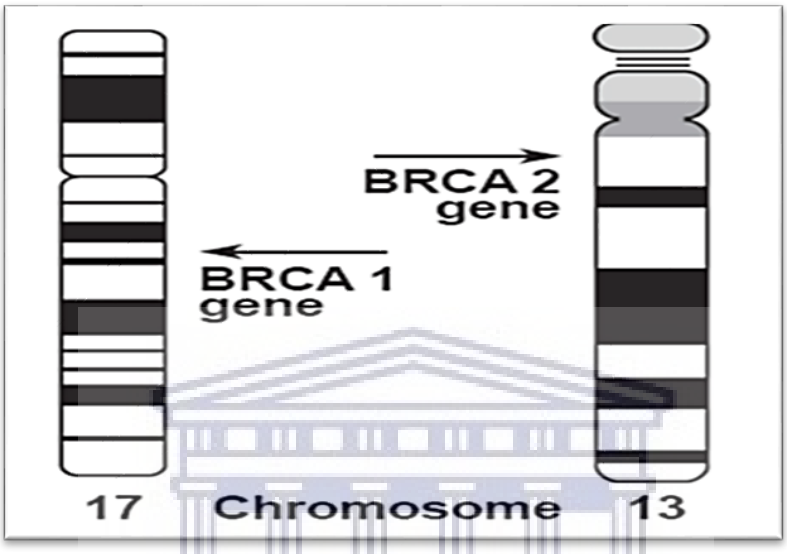

Figure 1.6: Location of breast cancer predisposing genes, BRCA1 and BRCA2, on human chromosomes 17 and 13 respectively (Mehrgou and Akouchekian, 2016).

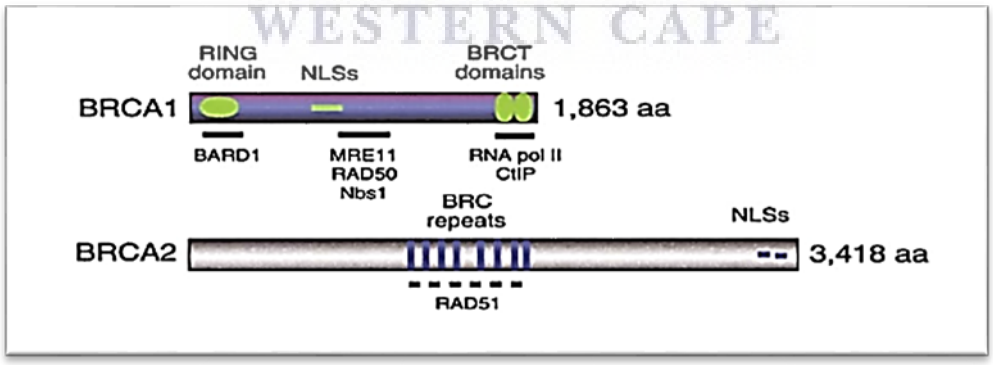

Figure 1.7: Features of BRCA1 and BRCA2 genes. BRCA1 showing the N-terminal RING domain, C-terminal BRCT domain, nuclear localisation signals (NLSs) and 1863 amino acids. BRCA2 showing NLSs and 6 of 8 protein motifs that can bind to RAD51 and 3418 amino acids (Venkitaraman, 2002).

The BRCA2 gene, which is located on chromosome 13 at 13q12 as seen in Figure 1.6, contains 27 coding exons (Dufloth *et al,* 2005; Petrucelli *et al,* 2010; Mehrgou and Akouchekian, 2016; Sun *et al*, 2017). The normal allele generates a 10.4-kb mRNA which encodes susceptibility for breast cancer type 2, a 3418 amino acid containing eight 30-40 residue motifs located in exon 11, mediating BRCA2 binding to RAD51 (Venkitaraman, 2002; Dufloth *et al,* 2005; Petrucelli *et al,* 2010; Mehrgou and Akouchekian, 2016). These features can be seen in Figure 1.7. BRCA2 is generally located in the nucleus and possesses phosphorylated residues. The susceptibility protein of breast cancer type 2 has no evident association to the susceptibility protein of breast cancer type 1 and contains no recognizable protein motifs. Nevertheless, BRCA1- and BRCA2-encoded proteins seem to share numerous functional similarities which may suggest why these gene mutations lead to a distinct hereditary predisposition to breast cancer (Petrucelli *et al,* 2010). BRCA2 seems to partake in the DNA repair process. Similar to BRCA1, BRCA2 acts as a "caretaker" serving to maintain genomic integrity (Venkitaraman, 2002; Petrucelli *et al,* 2010). Over 1800 mutations have been distinguished which consist of frameshift insertions, deletions or nonsense mutations which lead to early shortening of protein transcription (Petrucelli *et al,* 2010; Godet and Gilkes, 2017). Cells which lack BRCA2 are deficient in repairing double stranded DNA breaks, as shown by hypersensitivity to ionizing radiation (Petrucelli *et al,* 2010).

Tumour Suppressor Gene proteins are produced by BRCA1 and BRCA2, and are referred to as TSGs (Mehrgou and Akouchekian, 2016). Variation in genes such as somatic mutations and deletions, interference with TSG expression and functional loss is considered the main driving force for tumorigenesis and malignancy promotion (Osborne *et al,* 2004; Lee and Muller, 2010; Guo *et al,* 2014; Zhu *et al,* 2015; Wang *et al,* 2018). According to Knudson's two-hit model hypothesis, both TSG copies or alleles must become mutated or functionally inactivated to result in the loss of functionality of the TSG for the development of tumours and unmasking the malignant phenotype (Evans and Prosser, 1992; Osborne *et al,* 2004; Lee and Muller, 2010; Zhu *et al,* 2015; Wang *et al,* 2018). Several pathways are often deregulated in breast cancer as a result of these genetic mutations. These include the mechanisms or pathways involving vital lipid and protein kinases which play a key role in the proper

functioning of cell cycle machinery, cellular growth and their survival, pathways involved in DNA damage response and apoptosis (Osborne *et al,* 2004; Lee and Muller, 2010; Wang *et al,* 2018).

1.3.3 Molecular Markers of Malignant Transformation

The development of breast cancer is largely due to the inactivation of TSGs and activation of proto-oncogenes which result in malignant transformation of cells (Xia *et al,* 2019). The progression of breast cancer involves a series of intermediate processes which begins with ductal hyperproliferation and eventually leading to metastatic disease. With the variability in breast cancer development and progression, identifying markers that could anticipate behaviour of cells and tumours play a vital role. Molecular markers are defined as characteristics that are unbiasedly measured as a standard of normal or pathogenic processes. While most molecular markers are protein markers, in recent research gene markers have also become prominent tumour makers based on their expression patterns (Banin Hirata *et al,* 2014). Thus, it is important to look at the molecular markers involved in malignant transformation and progression such as SCGB2A1 gene also known as human mammaglobin (hMAM) and SERPINB5 gene also known as Maspin.

SCGB2A1 or hMAM, located on chromosome 11q12.2, is a glycoprotein containing 93 amino acids and is a member belonging to the secretoglobin-uteroglobin superfamily which includes nine human secretoglobins (Lacroix, 2006; Munakata *et al,* 2014; Yadav *et al,* 2015 Hassan *et al,* 2017; Zhang *et al,* 2020). The complete functions of hMAM remains unclear, but have been closely associated with immune functions, cell secretion, regulating host steroid metabolism and transport, tissue repair and tumorigenesis associated functions which include proliferation, migration and invasion (Koh *et al,* 2014; Hassan *et al,* 2017; Zhang *et al,* 2020). While this gene is greatly identified and associated with breast cells, it is also found in other organs which include lung, uterus, ovary, sweat gland, salivary gland, prostate and the thyroid (Zuo *et al,* 2009; Monsalve-Lancheros *et al,* 2019; Zhang *et al,* 2020). HMAM expresses in normal breast cells, but the overexpression of this marker is closely associated with the formation of breast tumours as seen in breast cancer cells as well as metastatic lymph nodes (Nunez-Villar *et al,* 2003; Ouellette *et al,* 2004; Lacka *et al,* 2012).

Furthermore, gene expression of hMAM was established to be a widely used and specific tool in detecting metastatic cells circulating in the peripheral blood of patients with breast cancer (Grünewald *et al,* 2000; Nunez-Villar *et al,* 2003). Based on the high specificity and expression pattern of hMAM, this tumour marker is believed to be a good candidate for detecting and managing breast cancer (Ouellette *et al,* 2004; Yadav *et al,* 2015).

Maspin, located on chromosome 18q21.3-q23, is a member belonging to the serine protease inhibitor family and consists of 2584 nucleotides that codes for a 42-kDa peptide (Maass *et al,* 2000; Berardi *et al,* 2013; Helal and El-Guindy, 2017; Sun *et al,* 2017). This protease inhibitor is affiliated with other inhibitors that include plasminogen activator inhibitor and α-1-antitrypsin, in addition to non-inhibitor serine proteins for instance ovalbumin. Protease inhibitors and proteases are known to portray vital roles in metastatic and tumour invasion processes. A protease degrades the extracellular matrix while prevention of this process is done by the protease inhibitor (Maass *et al,* 2000). In normal mammary glands, Maspin may be found at the cell surface, in the nucleus, cytoplasm and in the extracellular matrix of cells. This gene is responsible for mammary gland development and the cell adhesion and mobility during the embryogenesis process (Machowska *et al,* 2014).

Maspin is characterized as a tumour suppresser gene in the second class due to its ability to exert inhibitory effects on invasion, antiangiogenic properties, antimetastatic activity, reducing tumour vascularization and pro-apoptotic promoter. Research implicates Maspins capability to inhibit angiogenesis through the ability of blocking neovascularization and causing a reduction in tumour-related microvessels (Maass *et al,* 2000; Bailey *et al,* 2005; Helal and El-Guindy, 2017; Banias *et al,* 2019). In breast, gastric, prostate and melanoma cancer, Maspin is downregulated whereas in gallbladder, thyroid, colorectal and pancreatic cancer Maspin is overexpressed (Maass *et al,* 2000; Machowska *et al,* 2014; Dabiri *et al,* 2016). Therefore, the evaluation and implementation of molecular markers are capable of playing a pivotal role in generating a more efficient and effective therapeutic strategy for breast cancer patients (Banin Hirata *et al,* 2014).

1.3.4 Subgroups of Breast Cancer

Molecularly, breast cancer divides into subgroups on the basis of cellular surface receptors, which forms components of Human Epidermal Growth Factors and includes Progesterone Receptors (PR), Estrogen Receptors (ER) and Human Epidermal Growth Factor Receptor 2 (HER2) that is visualized through immunohistochemistry (IHC) (Mehrgou and Akouchekian, 2016; Dai *et al,* 2017; Ellingjord-Dale *et al,* 2017). The detection of these various surface receptors plays a key role in prognosis of breast cancer and assessment of the type of tumour (Parker *et al,* 2009; Kondov *et al,* 2018). Breast cancer is divided into five-six dominant subgroups by most studies. These subgroups include: Claudin-low, Normal-like, Luminal A, Luminal B, HER2 type and triple-negative (basal-like) as seen in Figure 1.8 and are collectively known as intrinsic subtypes of breast cancer (Parker *et al,* 2009; Daemen *et al,* 2013; Mehrgou and Akouchekian, 2016; Dai *et al,* 2017; Abubakar *et al,* 2018; Gulzar *et al,* 2018). Breast cancer molecular subtypes may be constructive in determining treatment strategies and new alternate therapies.

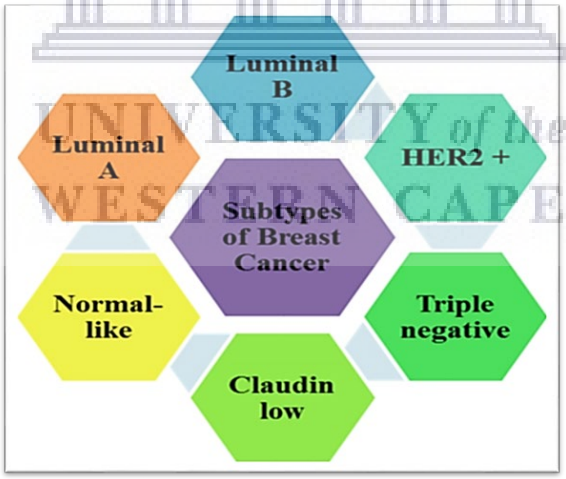

Figure 1.8: Six main subgroups of breast cancer (Mehrgou and Akouchekian, 2016).

Normal-like tumours are generally small and are more likely to have a high-quality prognosis. Roughly 6–10% of breast cancer cases are categorized as normal-like tumours (Mehrgou and Akouchekian, 2016). These tumours are positive for ER, PR and HER2 (Reis-Filho and Pusztai, 2011). Luminal tumour cells are seemingly breast cancer cells dawning in the inner cells that line the breast ducts.

Luminal A tumour cells tend to be PR- and ER-positive whilst being negative for HER2. These types of tumours have great prognosis, low recurrence rates and high survival rates (Kittaneh and Glück, 2011; Han and Magliocco, 2016; Mehrgou and Akouchekian, 2016; Gulzar *et al,* 2018). Roughly 40–60% of breast cancer cases are placed into the Luminal A category (Kittaneh *et al,* 2013). Luminal B tumours tend to be ER positive and/or PR positive. The majority of these kinds of tumours are positive for HER2, as opposed to Luminal A cells (Kittaneh and Glück, 2011; Han and Magliocco, 2016; Mehrgou and Akouchekian, 2016; Gulzar *et al,* 2018). Luminal B tumours in women are detected at younger ages in comparison to Luminal A tumours. However, Luminal B tumours have a poor prognosis due to lymph node involvement, lower grade tumours and larger tumour size (Mehrgou and Akouchekian, 2016). Roughly 15–20% of breast cancer is placed into the Luminal B category (Kittaneh *et al,* 2013).

HER2 tumours tend to be PR- and ER-negative with an overexpression of HER2 (Kittaneh and Glück, 2011; Han and Magliocco, 2016; Mehrgou and Akouchekian, 2016; Gulzar *et al,* 2018). This tumour accompanies the involvement of lymph nodes and lower tumour grade. These tumours are detected at a younger age in women in comparison to Luminal A or Luminal B tumours. HER2 tumours have a low prognosis and are susceptible to early and repetitive recurrences and metastasis. Roughly 10–15% of breast cancer cases are placed in this category (Han and Magliocco, 2016; Mehrgou and Akouchekian, 2016).

Claudin-low tumours are often triple-negative. These types of tumours are negative for PR, ER and HER2 (Kittaneh and Glück, 2011; Prat and Perou, 2011; Mehrgou and Akouchekian, 2016). However, lymphocyte infiltration is frequently seen and the expressions of E-cadherin, cell-cell adhesion proteins are reduced making claudin-low tumours different to others. This kind of tumour also has attributes of stem cells and mesenchymal cells (Mehrgou and Akouchekian, 2016; Marotti *et al,* 2017). These types of tumours generally have a poor prognosis and due to its hormone receptor negative status, therapy continues to be a major concern much like triple-negative breast cancer tumours (Prat and Perou, 2011).

1.3.5 Triple-Negative Breast Cancer

Multiple pathways are implicated in developing triple-negative breast cancer (TNBC) from breast cancer cells that are basal-like. One of the main pathways includes losing expression of various receptors through BRCA1-related pathways or through mutations that occur at random (Al-Mahmood *et al,* 2018). Triple-negative breast cancer is ER-, PR- and HER2-negative, thus making it a triple-negative type of tumour (Lehmann *et al,* 2011; Kim *et al,* 2016; Mehrgou and Akouchekian, 2016; Hubalek *et al,* 2017). These biological markers, involvement of lymph nodes, the histological characteristics and size of tumours, and absence or presence of metastases, play a role in determining prognosis and strategies involved in therapeutic management (Marotti *et al,* 2017). TNBCs carry on average 1.68 somatic mutations per Mb of the coding regions which are approximately 60 somatic mutations in every tumour. Some tumours may have a higher mutation burden (greater than 4.68 somatic mutations per Mb) with the mutation burden not being uniform across TNBC (Bianchini *et al,* 2016).

There are numerous categories for TNBC tumours. The subtypes of TNBC were identified with regard to the molecular complexity. These subtypes include basal-like 1, basal-like 2, mesenchymal, mesenchymal stem-like, immunomodulatory and luminal androgen receptor (Andre and Zielinski, 2012; Mehrgou and Akouchekian, 2016; Bareche *et al,* 2018; Santonja *et al,* 2018). The category known as basal-like is due to this type of tumour having attributes comparable to the outer cells (basal) that lines the breast ducts (Andre and Zielinski, 2012; Mehrgou and Akouchekian, 2016). TNBC and basal-like breast cancer share phenotypical features which are sequentially highly aggressive and with a poor result (Fayaz *et al,* 2014).

While the terms TNBC and basal-like breast cancer are interchangeably used, they are not the same (Perou *et al,* 2000; Bertucci *et al,* 2008; Santonja *et al,* 2018). TNBC refers to immunophenotypes of breast cancer which is immunologically negative to PR, ER and HER2, while basal-like breast cancer refers to molecular phenotypes of tumours that are characterized by cDNA microarrays (Ismail-Khan and Bui, 2010; Cinkaya *et al,* 2016). Based on cDNA microarrays, Perou and colleagues described four

subtypes of breast cancer, which included the basal-like subtype with an estimation of 75% of TNBC grouped in this subtype (Perou *et al,* 2000; Mehanna *et al,* 2019). When examined morphologically, cancers that are basal-like have poor differentiation and are normally high grade, invasive, ductal carcinomas and are classified histologically accordingly. Generally, basal-like carcinomas of breast cancer have a morphological consistency with a high mitotic count, high nuclear grade and necrosis as seen in Figure 1.9 (Ismail-Khan and Bui, 2010; Timmerman *et al,* 2013; Marotti *et al,* 2017). Research has also shown that nearly 82% of breast cancers that are basal-like express p53 compared to luminal A subgroup where 13% express p53 (Ismail-Khan and Bui, 2010).

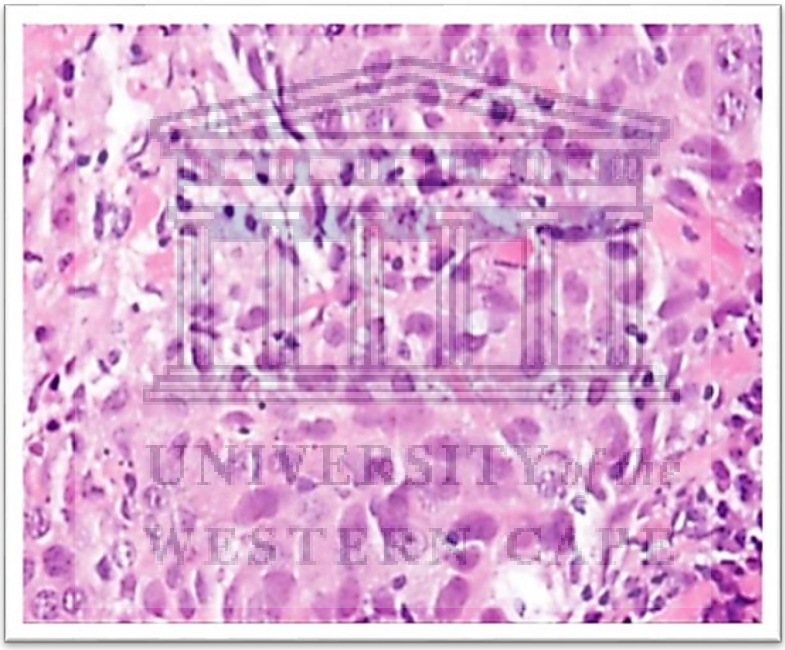

Figure 1.9: An example of basal-like carcinoma of TNBC, high nuclear grade (grade 3 invasive ductal carcinoma). HE, x400 (Ismail-Khan and Bui, 2010).

A subdivision of basal-like breast cancer and TNBC of a lower histological grade includes acinic cell, apocrine, secretory and adenoid cystic breast carcinoma. Basal-like breast cancer tend to express cytokeratins (cytokeratin 5, 6, 8, 14, 17 and 18) affiliated with basal cancer types due to them arising from an outer basal layer whereas luminal subtypes express genetic markers of normal breast cells and luminal epithelial cells as well as expressing high volumes of luminal cytokeratins. Tumour depicting

an immunohistochemical marker CK5 can be seen in Figure 1.10. Vimentin, aB-crystallin, epidermal growth factor receptor (EGFR), fascin, p-cadherin, caveolins 1 and 2, p63, c-kit, and other growth factors which include insulin-like growth factor receptor (IGFR) is generally expressed in this type of breast cancer (Ismail-Khan and Bui, 2010; Marotti *et al,* 2017). Research indicates that 23% of basal-like carcinomas are HER2+ as defined by studies of gene expression, thus making not every basal-like carcinoma HER2-. Hence, HER2 immunoreactivity cannot be utilized to eliminate a basal-like carcinoma (Ismail-Khan and Bui, 2010).

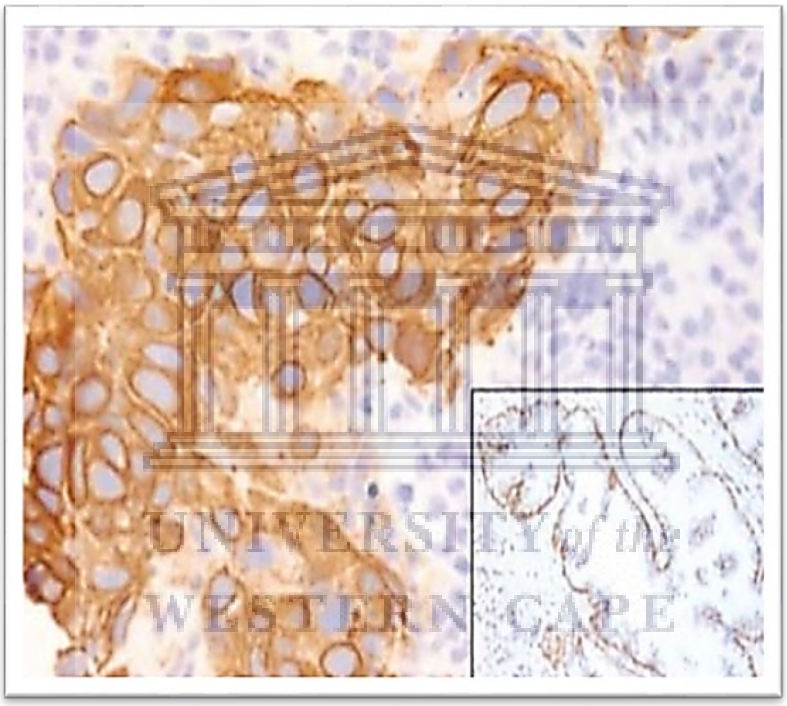

Figure 1.10: Tumour showing CK5 positivity, typical for basal-like breast cancer. Immunohistochemical CK5 stain, x400. Insert: CK5 stain highlighting basal cells of a normal control slide. HE, x200 (Ismail-Khan and Bui, 2010).

Of the vast diagnosis of breast cancer, over 170,000 are classified as triple-negative. Triple-negative tumours have a poor prognosis and are often highly aggressive tumours (Timmerman *et al,* 2013; Hubalek *et al,* 2017). TNBC constitutes for roughly between 10–20% of breast cancer cases. Approximately 75% of TNBC cases are “basal-like” (Chavez *et al,* 2010; Lehmann *et al,* 2011; Ahn *et al,* 2016).

TNBC is highly prevalent in African-American women and premenopausal women, and is noted that the incidence of this disease in African American women is more than double than in white women. In African American women, 47% of the tumours are triple-negative while triple-negative tumours are comprised of 22% in white women (Ismail-Khan and Bui, 2010; Andre and Zielinski, 2012; Mehrgou and Akouchekian, 2016; Vanderpuye *et al,* 2017). Most cases of breast cancer associated with the predisposing gene BRCA1, is basal-like and triple-negative; and is prone to be of a high grade (Ismail-Khan and Bui, 2010; Apostolou and Fostira, 2013; Mehrgou and Akouchekian, 2016; Godet and Gilkes, 2017).

1.3.5.1 Oncogenic Growth Promoters of TNBC

Oncogenes are genes that are able to promote cell proliferation in which normal cells grow uncontrollably due to an abnormal increase in the rate of cell division (Evans and Prosser, 1992; Osborne *et al,* 2004; Shen *et al,* 2018). These genes can become activated through the alteration of the genetic material or mutations (Wang *et al,* 2018). Oncogenes perform opposite functions to tumour suppresser genes, but have been found to work in conjunction with one another to promote cancerous cell development. The gain of function or overexpression of oncogenes and the loss of function or disruption of tumour suppresser genes, have been noted to be the major mechanism or driving force for tumour development (Osborne *et al,* 2004; Lee and Muller, 2010; Guo *et al,* 2014; Shen *et al,* 2018).

Byerly and colleagues conducted a study on promoters of oncogenic growth and cell death resistance of TNBC cells and found that the protein kinase C (PKC) family had various roles in cancerous cells (Byerly *et al,* 2016). PKC demonstrates vital regulatory roles in numerous fundamental cellular processes. These processes include gene expression regulation, cell cycle control, signal transduction, angiogenesis, apoptosis and cellular proliferation to name a few (Kang, 2014; Yin *et al,* 2014). A dramatic alteration to these processes may be achieved through an abnormal overexpression of PKC or the activation and/or localisation thereof, thereby altering the cellular growth processes (Yin *et al,* 2014).

Protein tyrosine kinase C theta isoform (PRKCQ/PKCϴ) was identified as a potential regulator for the anchorage-independent durability of breast cancer cells. This member of the PKC family is distinguished by an idiosyncratic protein domain structure which consists of diacylglycerol binding sites and typically lacking calcium binding sites (Belguise *et al,* 2012; Kang, 2014; Byerly *et al,* 2016). Throughout the haematopoietic system, PRKCQ/PKCϴ is extensively expressed chiefly in mast cells, T cells, platelets and natural killer (NK) cells, as well as in the liver, thymus, skeletal muscle and nervous system (Meller *et al,* 1998; Byerly *et al,* 2016). PRKCQ is reported to have an expressive role in solid tumours such as gastrointestinal stromal tumours (GIST) and, recently, including breast cancer with ER-negative tumours in particular. ERα expression in breast cancer cells is directly suppressed by PRKCQ expression which is needed for tumorigenesis (Belguise and Sonenshein, 2007; Byerly *et al,* 2016).

Upon further research, it had been revealed that Fos-related antigen-1 (Fra-1) plays an important role in oncogenesis as well as the progression and maintenance of various tumour types. High concentrations of Fra-1 has been reported in multiple cancer cell lines as well as tissues which includes breast, brain, bladder, endometrial, lung, prostate and colon carcinomas. Furthermore, Fra-1 has an effective role in cellular growth, motility and cell morphology of breast cancer and cell migration is stimulated by PRKCQ through stabilizing expression of Fra-1 in TNBC cells (Kustikova *et al,* 1998; Belguise *et al,* 2012; Byerly *et al,* 2016). PRKCQ may also contribute to forming or maintaining breast cancer stem cell population through boosting expression of genes affiliated with epithelial-mesenchymal transition (EMT). This process is mediated through direct chromatin interactions (Zafar *et al,* 2014; Byerly *et al,* 2016).

Byerly *et al* also showed that PRKCQ is adequate for driving growth-factor-independent proliferation, relocation and cell death resistance of MCF-10A breast epithelial cells (Byerly *et al,* 2016). Proliferation is promoted through PKC stimulating the activation of extracellular signal-related kinase (Erk) or activity of mitogen-activated protein kinase (MAPK) in a manner dependent on kinase activity (Kang, 2014; Byerly *et al,* 2016). While PRKCQ is adequate to promote such phenotypes in MCF-10A cells,

it is also necessary *in vivo* and *in vitro* growth of a subtype of TNBC cells. In comparison to other subtypes, expression of PRKCQ is relatively larger in human triple-negative carcinomas. Invasiveness and growth of TNBC cells are impaired through inhibiting PRKCQ kinase activity. Inhibiting PRKCQ, either by inhibiting kinase activity or downregulating the expression, causes a suppression in growth of TNBC which promotes the potential for PRKCQ inhibitors as a therapeutic strategy such as the use of PRKCQ small molecule kinase inhibitors thus supporting PRKCQ as a potential therapeutic target (Byerly *et al,* 2016).

1.3.5.2 Constraints and Challenges with TNBC Therapy

With TNBC being such an aggressive subtype, treatment options remain limited and prognosis remains poor (Andre and Zielinski, 2012). Hormonal therapy or trastuzumab-based therapy is not beneficial to women with TNBC due to the loss of ER, PR and HER2 target receptors. This makes chemotherapy, radiotherapy and surgery one of the few and common treatment modalities for TNBC (Wahba and El-Hadaad, 2015; Uscanga-Perales *et al,* 2016). Studies suggest TNBC responds better to chemotherapy compared to other breast cancer types, but the prognosis remains poor (Ismail-Khan and Bui, 2010; Wahba and El-Hadaad, 2015, Cinkaya *et al,* 2016). This is attributable to a shortened disease-free interval in adjuvant and neoadjuvant settings as well as an aggressive course in a metastatic setting (Wahba and El-Hadaad, 2015).

Adjuvant chemotherapy is generally recommended for patients who have a tumour size that is less than 0.5 cm or are node-positive regardless of the size of this tumour. Neoadjuvant chemotherapy is particularly used in patients that have locally advanced breast cancer at the point of diagnosis (Cinkaya *et al,* 2016). A preferred chemotherapeutic regimen in the adjuvant or neoadjuvant has not been established yet. However, high-dose and dose-dense regimens are considered to be more effective in TNBC patients (Bianchini *et al,* 2016). Current therapeutic strategies for managing TNBC include targeting of DNA repair complex like (taxanes and platinum compounds), cell proliferation like (regimens containing anthracycline), p53 like (taxanes) and targeted therapy (Berrada *et al,* 2010; Wahba and El-Hadaad, 2015).

Anthracyclines and taxanes are known to be effective treatments for TNBC (Bianchini *et al,* 2016). The efficacy of taxanes and anthracyclines in metastatic BC is greater in tumours that are ER negative; therefore, both of these are implemented as first-line treatment in patients with TNBC, even if it is accompanied by a short-lasting benefit (Mustacchi and De Laurentiis, 2015). Various literature showed that taxanes and anthracyclines in combination were more effective in comparison to a non-anthracycline regimen. However, the overall survival and progression-free survival is said to be worse in TNBC (Mustacchi and De Laurentiis, 2015; Pandy *et al,* 2019).

Other drugs such as the platinum derivatives carboplatin and cisplatin are said to have activity in TNBC treatment. In a neoadjuvant setting, adding the platinum derivatives have been noted to improve pathologic complete response as well as disease-free survival. While the inclusion of platinum compounds has the ability to increase pathologic complete response rates, the usage remains controversial in the neoadjuvant therapy regimen and is not routinely recommended.

Some patients have a great response to neoadjuvant chemotherapy with a high pathologic complete response post-surgery. However, other patients portray no response to the neoadjuvant therapy regimen and overall suffers with early relapses (Caramelo *et al,* 2019). This indicates that no treatment regimen can be singled out as a standard treatment for patients with TNBC as the responses varies between patient to patient.

With the lack of efficient treatment modalities for TNBC, finding alternative and effective treatments with minimal side effects remain a great concern. Chemotherapeutic drugs that are generally used have unfavourable effects that damage normal cells. Natural plant extracts that are said to possess anticancerous properties with fewer side effects would be a good alternate to the harsh drugs currently being used to deal with this harsh disease. Various studies suggest red palm oil would be a suitable therapeutic reagent with anticancerous properties due to the good composition of the palm oil (Loganathan *et al,* 2013).

1.4 Red Palm Oil (RPO)

Palm oil and palm kernel oil are derived from the *Elaeis guineensis* palm tree and is abundantly found in west and central Africa. The tree belonging to the Palmae family has an unbranched stem and can grow up to 20 or 30 meters in height with a 25 to 30-year economic lifespan. A female bunch produced possess a weight of approximately 30 to 40 kg and can obtain roughly 2000 fruitlets that have a black colour when young and turns orange-red upon ripening. Oil palm produces two distinct oils, namely palm oil (the major oil) and palm kernel oil (the minor oil). The palm oil is extracted either through wet or dry processes from the orange-red mesocarp of the palm fruit while palm kernel oil is extracted from the seeds of the fruit and is a light yellow oil (Ebong *et al,* 1999; Edem, 2002; Mukherjee and Mitra, 2009; Mancini *et al,* 2015).

In the original form, palm oil has orange-redness in colour due to carotenes, tocopherols and tocotrienols present in the oil, hence being known as red palm oil (RPO) (Kritchevsky, 2000; Imoisi *et al,* 2015). Red palm oil contains various compounds and is a source for natural antioxidants, contains fatty acids that are both saturated (50%) and unsaturated (40% monosaturated and 10% polysaturated) in proportions that are almost equivalent to one another with the fatty acids being glyceryl laurate, linoleate, linolenate, myristate, oleate, palmitate and stearate as summarised in Table 1.1, anti-oxidative carotenoids such as alpha-, beta- and gamma-carotenes, lycopenes, vitamin E such as tocopherols and tocotrienols, and ubiquinones such as coenzyme Q_{10} with levels of 10–80 ppm (Ebong *et al,* 1999; Nagendran *et al,* 2000; Edem, 2002; Wergeland *et al,* 2011; Imoisi *et al,* 2015).

Palm kernel oil contains higher proportions of lauric acid and mystiric acid which predominantly make palm kernel oil highly saturated (Wergeland *et al,* 2011; Imoisi *et al,* 2015). When comparing palm oil to fats in their natural state of being unhydrogenated, it is noted that palm oil contains greater proportions of palmitic acid with respect to the constituent fatty acids. Palm stearin (solid fraction 30-35%) and palm olein (liquid fraction 65-70%) share the same main fatty acids, specifically oleic acid, linoleic acid and palmitic acid. However, palm olein has less palmitic acids and relatively more linoleic and oleic acids than palm stearin (Imoisi *et al,* 2015).

Due to palm oil being semi solid at room temperature, it is not necessary for hydrogenation, while other oils may need to be hydrogenated during manufacturing processes of certain products. Hydrogenated fats frequently contain significant portions of trans fatty acids but excessive intakes and the effects thereof on health may be uncertain (Edem, 2002; Imoisi *et al,* 2015).

Table 1.1: Fatty acids found in red palm oil

Fatty Acid	Type	Percentage (%)
Glyceryl laurate	Saturated	0.1
Linoleate	Polyunsaturated	10
Linolenate	Polyunsaturated	0.3
Myristate	Saturated	1
Oleate	Monounsaturated	39
Palmitate	Saturated	44
Stearate	Saturated	5

(Data sourced and adapted from Imoisi *et al,* 2015).

1.4.1 Carotenoids in Red Palm Oil

The various components of red palm oil have various effects on the body. Palm oil possess 500-700 ppm of carotenoids making it the richest natural source of this compound and gives the red colour to the oil (Gee, 2007; Mancini *et al,* 2015). The same nutrient is responsible for giving carrots, tomatoes, and various fruits and vegetables their characteristic rich colours (Imoisi *et al,* 2015). Alpha- and beta-carotenes are the major carotenes found in red palm oil along with other minor carotenoid components. The most abundant carotenoid found is beta-carotene (Edem, 2002).

Table 1.2 displays the carotenoid composition. Rich sources of carotenoids are said to enhance the functions of the immune system as well as improving cardiovascular health, ageing, and possible inhibitory effects on cancer development and prevention (Nagendran *et al,* 2000; Mukherjee and Mitra, 2009; Imoisi *et al,* 2015; Mancini *et al,* 2015). Carotenoids are members that are part of a biological network of antioxidants that convert free fatty peroxy radicals and highly reactive radicals to lesser active species (Edem, 2002).

Table 1.2: Composition of carotenoids found in red palm oil

Carotene	Composition (%)
β-Carotene	56.02
α-Carotene	35.16
cis-α-Carotene	2.49
Lycopene	1.30
Phytoene	1.27
δ-Carotene	0.83
ζ-Carotene	0.69
cis-β-Carotene	0.68
γ-Carotene	0.33
Neurosporene	0.29
β-Zeacarotene	0.23
Phytofluene	0.06

(Data sourced and adapted from Gee, 2007).

These compounds play a key potential role in which they act as biological antioxidants where they protect cells and tissues from free radicals and their damaging effects (Ebong *et al,* 1999; Mukherjee and Mitra, 2009; Imoisi *et al,* 2015). Exposure to free radicals may come from different sources which include unbalanced diets, stress, residue of insecticides and pesticides in food and water, industrial pollution, pollutants found in cigarette smoke and various other environmental influences. An accumulation of free radicals is associated with debilitating diseases such as cancer and heart disease, as well as affecting the ageing process. The antioxidants act as a buffer against the free radicals and are said to play a role in protecting cellular ageing, cancer, Alzheimer's disease, atherosclerosis and arthritis—thus, making the antioxidants found in palm oil a good preventative measure against free radicals (Mukherjee and Mitra, 2009; Imoisi *et al,* 2015). Carotenoids, such as alpha- and beta-carotene in particular, are vitamin A precursors and red palm oil is able to retain about 80% of its original carotenoids. This attribute also makes the oil an extraordinary source of vitamin A allowing it to combat various other diseases associated with vitamin A deficiencies, aids in tissue maintenance, prevents night blindness and promotes growth (Ebong *et al,* 1999; Mukherjee and Mitra, 2009; Imoisi *et al,* 2015).

Alpha- and gamma-carotenes yield one vitamin A molecule each while beta-carotene is able to yield two vitamin A molecules. In the intestine, beta-carotene is oxidatively cleaved yielding the isopropanoid compound containing a 6-membered carboxylic ring as well as an 11-carbon side chain (Edem, 2002). During the process of refining, bleaching and deodorization, the carotenes are mostly destroyed which consequently produces the light-coloured oil that is desired by the consumers (Edem, 2002; Gee, 2007). However, there are processes that are available that allow for the refining of the red palm oil without the destruction of carotenoids. In Malaysia, there are at least three corporations that produce refined red palm oil (Gee, 2007).

1.4.2 Vitamin E in Red Palm Oil

Palm oil contains an enriching source of vitamin E and it is noted that no other common vegetable or animal oil contains the quantity of vitamin E in comparison. Roughly 559-1000 ppm of the essential vitamin E compounds is found in palm oil and is summarised in Table 1.3.

Table 1.3: Vitamin E levels found in red palm oil

Component	Parts per million (PPM)
Vitamin E	716
α-tocopherol	158
α-tocotrienol	143
δ-tocotrienol	86
γ-tocotrienol	329

(Data sourced and adapted from Ebong *et al*, 1999).

Vitamin E forms part of a biological antioxidant network and while palm oil contains a low amount of phospholipids (5–130 ppm), in the presence of vitamin E they are capable of functioning as antioxidant synergists (Ebong *et al,* 1999; Edem, 2002). This important antioxidant confers stability to the oil against oxidative deterioration (Nagendran *et al,* 2000). This antioxidant serves to seize the oxidative degradation of cellular membranes from lipid peroxidation that were catalysed by free radicals (Ebong *et al,* 1999; Edem, 2002; Kannappan *et al,* 2012). Free radicals that are uncontrollably produced are

correlated to damaging effects of the function and structure of a cell (Nesaretnam *et al,* 2004). Biological systems are protected against carcinogenic and oxidative stress due to vitamin E containing free radical scavenger properties. Through the neutralization of free radical damage, vitamin E is capable of inhibiting the development of cardiovascular disease and cancer. Vitamin E is not only imperative for its lipid lowering and cellular antioxidant properties, but also plays a key role as an anti-proliferating agent (Edem, 2002; Nesaretnam *et al,* 2004; Kannappan *et al,* 2012).

1.4.3 Tocopherols in Red Palm Oil

Natural vitamin E is mainly composed of tocopherols and tocotrienols (Ebong *et al,* 1999; Mukherjee and Mitra, 2009: Kannappan *et al,* 2012). Tocotrienols and tocopherols are collectively known as tocochromanols and occur in eight different isomers divided into four tocotrienols and four tocopherols. These isomers that exist are alpha-, beta-, gamma- and delta-tocotrienols and alpha-, beta-, gamma- and delta-tocopherols (Nagendran *et al,* 2000; Mukherjee and Mitra, 2009; Imoisi *et al,* 2015). Tocochromanols obtain a similar structure with a common chromanol ring and at the C-2 position they contain a side chain. Tocopherols and tocotrienols only differ in their side chains and contain substituted methyl groups on the chroman ring at an identical position. Tocopherols contain saturated phytyl carbon chains while tocotrienols contain unsaturated isoprenoid side chains accompanied by double bonds in positions 3', 7' and 11' (Ramdas *et al,* 2011; Kannappan *et al,* 2012; Ling *et al,* 2012). These compounds act as strong antioxidants which make them reasonably stable to oxidation and through their chromanol ring are able to scavenge peroxyl radicals (Ebong *et al,* 1999; Edem, 2002; Ling *et al,* 2012).

Tocopherols constitute approximately 18–22% of the vitamin E present in palm oil and only exist as free chromanols. They are abundantly found in seeds, leaves, nuts and oils of most plants and other common vegetables. Tocopherols are more widespread in plant tissues (Edem, 2002; Ramdas *et al,* 2011; Ling *et al,* 2012; Urvaka *et al,* 2019). Tocopherols are fat soluble biomolecules that possess a lipophilic nature (Kannappan *et al,* 2012; Azzi, 2019; Urvaka *et al,* 2019). They are comprised of a 15-carbon saturated tail and a chromanol ring derived from phytyl diphosphate and homogentisate, respectively, at positions 5′, 7′ and 8′ (Sen *et al,* 2010; Ahsan *et al,* 2015). In plants, biosynthesis of

tocopherols is catalysed through homogentisate phytyltransferase which enables a condensation reaction of phytyl diphosphate and homogentisate (Sen *et al,* 2010). Tocopherols contain four isoforms, namely alpha-, beta-, delta- and gamma-tocopherol with the chemical structures exhibited in Figure 1.11 (Kannappan *et al,* 2012; Ling *et al,* 2012; Azzi, 2019; Urvaka *et al,* 2019). These isomers are of significant importance based on its unique antioxidant and physiochemical properties. The tocopherol content is able to stabilize the palm oil against oxidation (Nagendran *et al,* 2000; Imoisi *et al,* 2015; Urvaka *et al,* 2019).

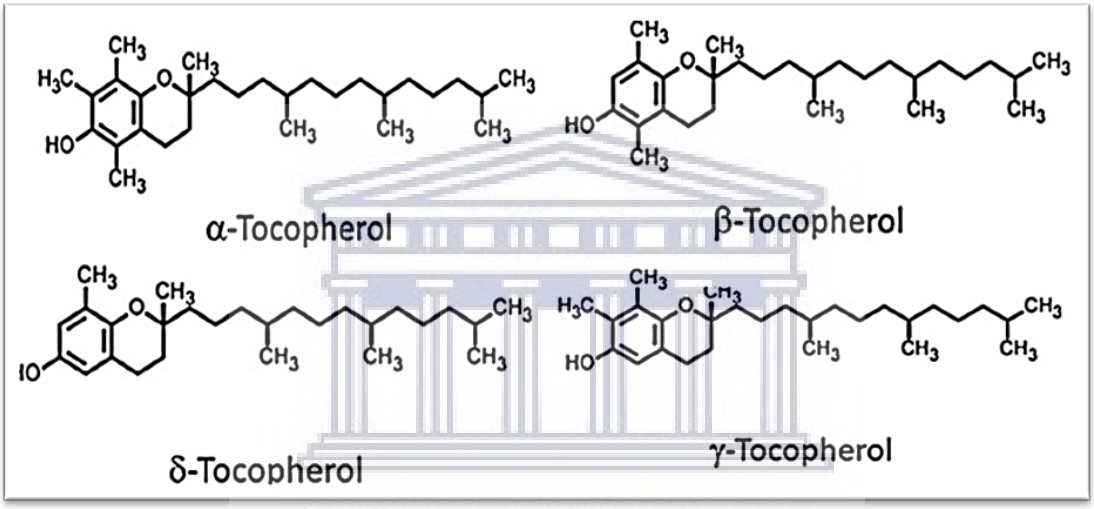

Figure 1.11: Chemical structures of the different forms of Tocopherols with the differences being the position and number of methyl groups located on their chromanol ring (Azzi, 2019).

Tocopherols are persistent in high temperatures such as in cases of deep frying due to tocopherols being less volatile (Imoisi *et al,* 2015). Tocopherols are able to disturb lipid oxidation by inhibiting the formation of hydroperoxide in the chain propagation process or disturbing the decomposition process whereby it inhibits aldehyde formation. Apart from its activity towards free radicals, alpha-tocopherol has high reactivity towards singlet oxygen molecules and is able to protect the oil with regard to photosensitised oxidation (Sundram *et al,* 2003). On average, 50–60% of tocopherol is retained after the oil has been refined (Imoisi *et al,* 2015).

1.4.4 Tocotrienols in Red Palm Oil

Tocotrienols constitute approximately 78–82% of the vitamin E present in palm oil and are unsaturated analogues of tocopherol that occur in esterified forms (Edem, 2002; Szulczewska-Remi *et al,* 2005; Ramdas *et al,* 2011). They are found in natural sources such as wheat germ, rice bran, walnut, rye, maize, poppy, hazelnut, flax seed, pumpkin seed, grape seed and are most abundantly found in palm oil (Ramdas *et al,* 2011; Kannappan *et al,* 2012; Ling *et al,* 2012). Palm oil is a rich source for tocotrienols and is noted to be the only vegetable oil with such appreciable quantities available on the market (Edem, 2002; Comitato *et al,* 2009; Ahsan *et al,* 2015). Tocotrienols are less ubiquitous in the plant kingdom (Sen *et al,* 2010). Much like tocopherols, tocotrienols are fat soluble molecules and possess a similar structure (Kannappan *et al,* 2012; Azzi, 2019). As stated earlier, tocotrienols have 3 trans double bonds at positions 3′, 7′, and 11′ and the 15-carbon phytyl tail originates from geranylgeranyl diphosphate (Sen *et al,* 2010; Ahsan *et al,* 2015). Condensation of geranylgeranyl diphosphate and homogentisate is catalysed by homogentisate geranylgeranyl transferase (Sen *et al,* 2010). Tocotrienls contain four isoforms, namely alpha-, beta-, delta- and gamma-tocopherol with the chemical structures exhibited in Figure 1.12 (Kannappan *et al,* 2012; Ling *et al,* 2012; Azzi, 2019).

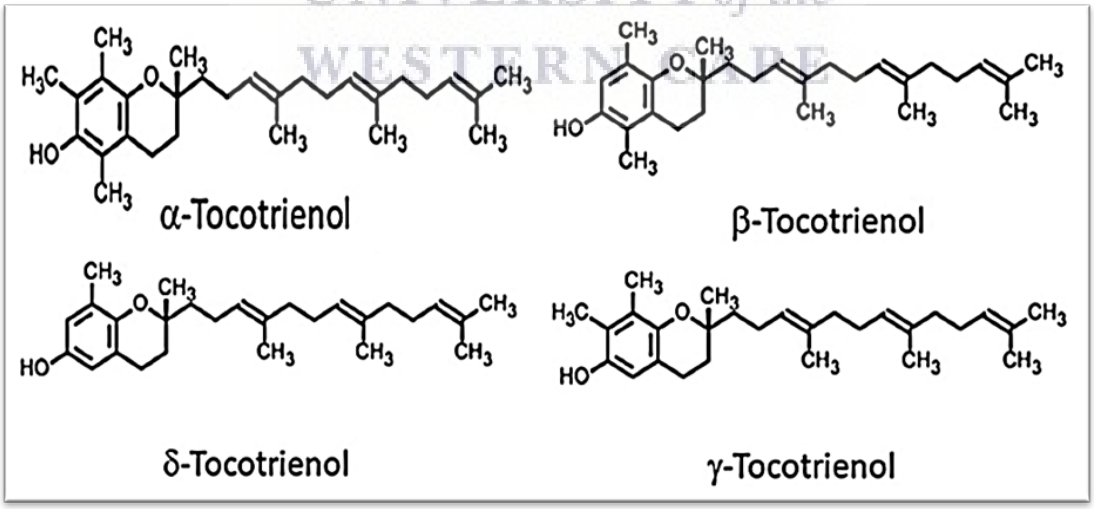

Figure 1.12: Chemical structures of the different forms of Tocotrienols with the differences being the position and number of methyl groups located on their chromanol ring (Azzi, 2019).

These vitamin E isomers possess potent antioxidant properties that grant the oxidative stability to the oil (Nagendran *et al,* 2000). Great health benefits are associated with tocotrienols, including immunomodulatory, antihypertensive, neuroprotective properties, as well anticholesterolaemic and anticancer properties (Lim *et al,* 2014). These molecules are natural inhibitors of platelet aggregation and the biosynthesis of cholesterol. In humans and animals, they are capable of reducing the concentrations of apolipoprotein B, platelet factor 4, thromboxane B_2 and are able to lower plasma cholesterol through the inhibition of HMG-CoA reductase activity (Ebong *et al,* 1999; Nagendran *et al,* 2000; Edem, 2002; Mancini *et al,* 2015).

The literature revealed that tocotrienols could potentially be better for chemoprevention and chemotherapeutic agents for diseases that are degenerative (Gee, 2007). While all the isomers contain the same chemical structure, it was observed that the location and substitution of the methyl groups of the isomer at the head region play a key role in the determination of anti-apoptotic and anti-proliferative activities of an individual isoform. Palm oil contains a rich source of gamma-tocotrienol and has shown to possess anti-proliferative properties in various types of cancerous cells *in vitro* which include colon, lung, prostate, melanoma and breast cancer (Edem, 2002; Lim *et al,* 2014; Ji *et al,* 2015). Suppressing the progression of cancer types, particularly the inhibition of breast cancer cells makes the components of red palm oil an encouraging therapeutic agent (Nagendran *et al,* 2000).

1.4.5 The Tocotrienol-Rich Fraction (TRF)

Tocotrienol-rich fraction (TRF) contains roughly 22–30% of alpha-tocopherols and roughly 70% of tocotrienols which are comprised of 18% alpha-tocotrienol, less than 12% beta-tocotrienol, 12% delta-tocotrienol and 36% gamma-tocotrienol (Hafid *et al,* 2013; Lim *et al,* 2014; Shahidi and De Camargo, 2016). The TRF in vitamin E are believed to demonstrate anticancer and antioxidant activities with immune enhancing effects. The anticancer effects include antimetastic, anti-proliferative and pro-apoptotic effects. It is able to significantly reduce tumours induced in mice (*in vivo*) as well as being capable of inducing apoptosis and inhibiting cell proliferation in a variety of cancerous cells in *in vitro* (Guthrie *et al,* 1997; Comitato *et al,* 2009; Comitato *et al,* 2010; Ramdas *et al,* 2011). *In vivo* studies

have shown that the administration of TRF to mouse models caused the reductions of tumour numbers in mice with liver carcinogenesis. TRF was also shown to supress the formation of lung tumours. Furthermore, a daily supplementation for a 16-week period with TRF revealed a lower incidence of the formation of prostate tumours in rats. Additionally, antitumour properties were enhanced and tumour growth was inhibited in mouse models with human pancreatic cancer through administration of TRF. It has been demonstrated that the consumption thereof has the ability to decrease oxidative DNA damage and improves the cognitive function of rats (Kannappan *et al,* 2012; Ling *et al,* 2012).

While TRF contains up to 800 mg/kg weight of the gamma- and alpha-tocotrienol isotypes, gamma-tocotrienol as well as delta-tocotrienol exhibit greater activity against the production of tumours (Nesaretnam *et al,* 2004; Sen *et al,* 2010; Imoisi *et al,* 2015). Various *in vitro* studies have revealed that tocotrienols have a greater antioxidant effect in comparison to tocopherols. Tocotrienols have demonstrated inhibiting effects on the growth of several cancers which include colon, prostate, liver and importantly breast cancer (Szulczewska-Remi *et al,* 2005; Ling *et al,* 2012; Lim *et al,* 2014).

To determine the efficacy of individual tocotrienols with varying concentrations, ER positive and ER negative breast cancer cells of humans are commonly used. Individual tocotrienols display greater inhibitory effects on cancerous cells at considerably smaller concentrations than TRF as gamma-tocotrienol was shown to be more effective in inhibiting the proliferation of MDA-MB-435, estrogen negative cells (Guthrie *et al,* 1997; Sundram *et al,* 2003). Based on a sequence of studies, it was found that palm TRF and toctrienols inhibited proliferation of human MCF-7 cells more effectively. These tocotrienols proved to be effective in inhibiting p53-mutant MDA-MB-231 (estrogen receptor negative) and p53 wild type MCF-7 (estrogen receptor positive). Tocotrienols are able to prevent harmful cell growth and is capable of doing so in the presence of estradiol as well as the absence of it, therefore allowing protection against various types of breast cancer and hormone-related breast cancer. This indicates that tocotrienols demonstrate inhibitory effects irrespective of p53 and estrogen receptor status which further suggests different mechanisms of action for these cancerous cells (Guthrie *et al,* 1997; Imoisi *et al,* 2015; Tran *et al,* 2015).

While some authors have demonstrated apoptotic activity in numerous cancerous cell lines without inhibiting the growth of non-tumour cells such as human MCF-10A cells, research also provides evidence for tocotrienols being capable of inhibiting growth or killing normal cells, but solely in extremely high dosages (Imoisi *et al,* 2015; Tran *et al,* 2015). Upon further investigation, Ramdas *et al* conducted a study on tocotrienol-treated MCF-7 human breast cancer cells and found that vitamin E exerted ubiquitous effects on MCF-7 cells and up regulated and down regulated genes that were affiliated with breast cancer biology. Tumour progression has been said to be facilitated by dysfunctions in the apoptotic pathways by expressing cancer cells resistance to death mechanisms that are relevant to metastasis (Ramdas *et al,* 2011; Kannappan *et al,* 2012). Cell accumulation may also be promoted by a dysregulation of apoptotic pathways which may contribute to neoplastic diseases through the prevention or the delay of normal cell turnover (Ramdas *et al,* 2011).

Ramdas *et al* determined that treating MCF-7 cells with TRF, tocotrienol isomers and alpha-tocopherol produced wide effects on a great number of genes which covers various molecular functions which could potentially lead to apoptosis (Nesaretnam *et al,* 2004; Ramdas *et al,* 2011). It was also noted that TRF possess immunostimulatory effects with potential clinical benefits that could enhance immune responses to vaccine. Evidence suggests a daily supplementation of TRF could induce a potent cell-mediated response such as T-helper-1 (Th1) responses. This action of TRF would be of great benefit in fighting viral infections as well as cancer.

Therefore, there is a growing indication that tocotrienols are capable of modulating a comprehensive variety of transcriptional responses of genes affiliated with different cancer types. The up-regulation of MIG6 and down-regulation of API5 provides promising roles for tocotrienols being able to target signalling pathways of genes associated with breast cancer for chemopreventative programmes. In turn, it is imperative to identify the most effective concentrations of compounds of vitamin E that is able to affect protein or gene expression for the development of cancer-specific gene target therapy that possess little to no toxicity towards normal cells (Ramdas *et al,* 2011).

It is known that dendritic cells (DCs) are highly developed antigen-presenting cells which play a pivotal role in the mediation of antitumour immune responses. Various studies have indicated that tumour antigen-pulsed DCs which is also referred to as cancer vaccines, are able to induce the activation and the proliferation of cytotoxic T-lymphocytes and T-helper cells which mediates the antitumour immune responses. However, many studies noted that the efficacy of these cancer vaccines appeared to be low in mice and human tumours. Therefore, there is a necessity for improving the efficacy of DC-based vaccines potentially through using adjuvants (Hafid *et al,* 2013).

Hafid *et al* found that supplementation with TRF as a combined approach inhibited the growth of tumours and generated tumour specific responses. Their study suggested the use of TRF as an adjuvant therapy in order to boost anticancer and immune enhancing effects of DC-based vaccines. TRF was found to increase CD40 and CD80 expression which are responsible for maturation and activation of dendritic cells and necessary for T-cell activation and survival. This gives rise to TRF treatment being a facilitator for dendritic cell maturation and activation which sequentially improves antigen presenting ability (Hafid *et al,* 2013). Splenocytes from animals with TRF adjuvanted therapy showed a greater production of interleukin-12 (IL-12) and interferon-gamma (INF-γ), cytokines that stimulates cell mediated responses for example tumour specific cytotoxic T-lymphocyte activities. IL-12 and INF-γ promotes the development of Th1 immune responses, which plays an important role in the production of antitumour immune responses (Ling *et al,* 2012; Hafid *et al,* 2013). IL-12 is recognised as a T-cell stimulating factor and is produced by macrophages and dendritic cells in response to stimulation of antigens. IL-12 is also able to stimulate INF-γ and tumour necrosis factor alpha (TNF-α) production by natural killer cells and T-lymphocytes which improves cytotoxic activity (Hafid *et al,* 2013).

1.4.6 Preparation of Tocochromanol Concentrates

Szulczewska-Remi *et al* conducted a study on the preparation of tocochromanol concentrates found in red palm oil using a three-step crystallization process and compared it to supercritical fluid extractions with carbon dioxide. The crystallization process of acylglycerols was used to obtain concentrates of tocochromanols. Nucleation and crystal growth were seen during the cooling process. The oil phases

and the crystals were split into stearin and olein through vacuum filtration. Results obtained through these procedures were then analysed (Szulczewska-Remi *et al,* 2005). It was found that total content of the tocols was remarkably higher in the concentrate with almost 28 mg/g in comparison to crude palm oil with approximately 1 mg/g. With that said, there was a greater contribution of tocotrienols with 26 mg/g in the overall number of tocochromanol homologs. Delta-tocopherol was observed to have the highest increase by almost 57 times and delta-tocotrienol with 51 times. Gamma-tocotrienol was observed to have a notable increase by 29 times. The differences in solubility during crystallization of the tocochromanols homologs are largely dependent on chemical structures in addition to chemical and physical properties. Delta homologs contain the smallest amount of methyl groups that facilitates in its crystallization. Furthermore, tocotrienols that contain double bonds in the tails are extracted more easily. In comparison, using the supercritical fluid extractions process demonstrated that significant changes were not observed in the tocochromanols compositions. (Szulczewska-Remi *et al,* 2005).

The three-step crystallization process is able to obtain concentrates with tremendously high quantities of tocotrienols and tocopherols as well as beta-carotenes which make this procedure highly efficient. After the first crystallization, highest loss of crude sample was noted followed by significantly smaller losses after second and third crystallization steps. Fractioning of crude red palm oil allows for examining alterations in fatty acid profiles. In particular, the transformation between the plastic form of crude palm oil which has 50% unsaturated and 50% saturated fatty acids to the liquid form of concentrate with 12% saturated fatty acids. The completed product after crystallization was characterized by obtaining 5 times greater amounts of beta-carotene, with tocotrienols being almost 30 times higher and tocopherols being 10 times more (Szulczewska-Remi *et al,* 2005).

1.4.7 Palm Oil Characteristics and Utilization of the Oil

Palm oil contains various characteristics which are important in determining the incorporation into food and other products. Palm oil has a high solid-glyceride quantity which gives the appropriate consistency without undergoing hydrogenation (Ebong *et al,* 1999; Imoisi *et al,* 2015). The level of triglycerides with high melting points in combination with a comparatively low solid content at 10°C assists in the

formulation of products with a broad plastic range making it suitable for certain industrial applications and hot climates. Due to the broad plastic range, the oil has proportionally slow melting properties. Palm oil has a tendency to crystallize in the smaller beta prime crystals. This characteristic is preferable for certain applications such as cakes and for margarines. The slow crystallization properties may lead to structural hardening in the completed product and tendencies for recrystallization (Edem, 2002; Imoisi *et al,* 2015).

This oil is used for the manufacturing of margarine, ice-creams that are vegetable oil based, shortenings, cocoa butter equivalents in certain products like chocolate, mayonnaise preparation, instant noodles and oatmeals, microwave popcorn, peanut butter, salad dressings, preparing soups and stews, and deep fat frying (Ebong *et al,* 1999; Edem, 2002; Mancini *et al,* 2015). This oil is also a raw material that can be used in the production of soaps and oleochemical products that are used in the manufacturing of various detergents and surfactant products as well as waxes, cosmetics, toothpastes and lubricants. The oil is exceptionally resistant to oxidation and stable to temperature thus giving it a long shelf life (Ebong *et al,* 1999; Edem, 2002).

For over 5000 years, palm oil was used for preparation of foods and other dietary requirements. Currently, palm oil has a worldwide appeal for cooking due to the oil being free of trans-fats that are responsible for clogging arteries (Mukherjee and Mitra, 2009). According to Wergeland colleagues, diets supplemented with red palm oil during treatment with other drugs, played a role in stabilizing biochemical changes and functioning of the heart in comparison to only using drug treatments.

Various studies indicated that red palm oil was affiliated with a better recovery and better protection of a heart that was exposed to reperfusion induced stress/ischaemia which was most likely due to the antioxidants present in red palm oil (Wergeland *et al,* 2011). While red palm oil is said to play a role in a number of diseases, recent research makes mention to the anticancerous properties in red palm oil making it important to understand the composition of this oil and the role it would play in targeting cancerous cells.

1.4.8 Essential Oils and Anticancer Activity

One of the rapidly growing areas of research is the exploration of natural plant-based products as a method to finding newer chemical components and compounds as anticancer agents. A variation of plant products grouped as alkaloids, glycosides, polyphenols, saponins and triterpenes, to name a few, has shown encouraging anticancer properties in both *in vivo* and *in vitro*. Over 1000 plants have been recorded to obtain significant anticancer properties. Camptothecin, colchicine, ellipticine, etoposide, irinotecan, lepachol, paclitaxel, podophyllotoxin, taxol, vinblastine and vincristine are a few examples of compounds derived from plants that are found to have a widespread application in cancer therapeutics. Products derived from plants are expected to induce fewer side effects in comparison to synthetic drugs. It has also been reported that essential oils from aromatic plants possess anticancer properties and these oils are said to improve a cancer patient's quality of life (Gautam *et al,* 2014).

Essential oils are concentrated liquids that are hydrophobic and have specific aromas that are produced by aromatic plants. They are also referred to as ethereal oils or volatile oils. The biological properties and composition of essential oils are dependent on their constituents. These constituents include aromatic compounds, terpenes and other compounds of diverse origins and are classified according to their chemical structures. Essential oils from plants that grow in various environments vary in composition thus have different uses. Some oils are specifically used for aromas in perfumes or as a flavouring agent for food products.

These oils are also used in aromatherapy, baths and massages for the overall improvement on health. The lipophilic nature of the oils allows them to cross membranes of cells easily and extend into the cell. Essential oils are depicted as strong antioxidants and antimicrobials with a use for managing severe diseases such as diabetes, Alzheimer's, cardiovascular disease, various other diseases and cancer. The anticancer prevention strategies identified include apoptosis, DNA repair mechanism and cell cycle arrest. The oils reduce metastasis, multidrug resistance and proliferation of cancer cells (Figure 1.13) (Gautam *et al,* 2014).

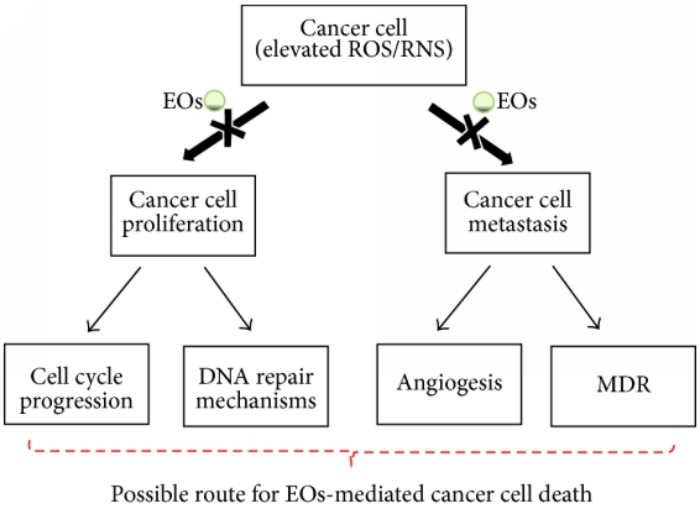

Figure 1.13: Essential oils possible route and multitargeted role in cancer prevention (Gautam *et al*, 2014).

Chen *et al* investigated possible anticancer activities of essential oils acquired from two species, namely myrrh and frankincense. Research suggests myrrh exhibits analgesic, anti-inflammatory, anti-parasitic, hypolipidemic, cytotoxic and anticancer activities. Research also suggests that frankincense exhibits numerous health supporting properties which includes anti-bacterial, anti-inflammatory, anti-fungal, treatment of rheumatoid arthritis and anticancer activities. The constituents identified in the essential oils of frankincense and myrrh included alcohols, esters, monoterpenes and sesquiterpenes. Chen *et al* noted a significant inhibitory effect in cell lines following myrrh essential oil treatment in comparison to frankincense treatment and the combination of the essential oils. This suggests that apoptosis could be a vital contributor to the biological efficacy of MCF-7 breast cancer cells. The rate of apoptosis was found to be higher in myrrh essential oil groups in comparison to frankincense and the combination of essential oils at three concentrations. Their findings suggested that breast cancer cells exhibited an increased sensitivity to myrrh essential oil. This makes essential oils possible candidates for anticancerous activity and therapies (Chen *et al,* 2013). Certain oils pose as good candidates in the prevention and treatment of cancerous cells. Based on the literature, red palm oil looks to be a strong and promising candidate for treating a grave and harsh disease such as breast cancer.

1.5 Aims of the Study

Red palm oil is one of the most abundantly used cooking oils in Africa and is said to possess properties that may be beneficial in targeting cancerous cells.

Therefore, this study aims to investigate:

- The growth-related *in vitro* effects of red palm oil on MCF-7 breast cancer cells, MDA-MB-231 triple-negative breast cancer cells, and a non-cancerous breast cell line, MCF-12A.
- The effect of red palm oil on gene expression of hMAM, Maspin and GAPDH Housekeeping genes in these cell lines.

CHAPTER 2

MATERIALS AND METHODS

2.1 Introduction

This chapter summarizes the materials, equipment and methods used in order to maintain the various cell lines used for the selected assays. This chapter also focuses on the preparation of the red palm oil used to conduct the proliferation assay and the PCR of the selected genes.

2.2 Chemicals

All chemicals used in the current study were of the highest possible grade and were obtained from the following companies:

Bio-Smart Scientific

- All tissue culture grade plastic consumables needed for cellular and molecular biology.

Gibco, Life Technologies; Grand Island, NY, USA & Gibco, Life Technologies; Paisely, UK

- Penicillin and streptomycin
- Dulbeco's Modified Eagle Medium F/12 Nutrient Mix (DMEM/F12)
- Heat-Inactivated Fetal Bovine Serum (FBS)
- Phosphate Buffered Saline (PBS)
- Trypsin-EDTA 0.25%

Inqaba Biotechnical Industries (Pty) Ltd, Pretoria, SA

- Forward and Reverse Primers for: hMAM, Maspin and GAPDH housekeeping gene

Lonza; Basal, Switzerland

- SimplyLoad® 100 bp DNA Ladder

Lonza, WhiteSci; Rockland, USA

- Seakem® LE Agarose

Lonza, WhiteSci; Walkersville, USA

- 0.4% Trypan Blue

Malaysian Palm Oil Board Ltd; Selangor, Malaysia

- Nutro Palm Oil

Merck; Darmstadt, Germany

- Chloroform
- Ethanol (Molecular Grade)
- Tris (hydroxymethyl) aminomethane

Merck; Gauteng, SA

- Boric Acid powder
- EDTA Disodium Salt Dihydrate

Promega; Madison, WI, USA

- 5X Colourless Go Taq® Flexi buffer
- 25 mM $MgCl_2$
- Blue/Orange 6X Loading Dye
- Ethidium Bromide Solution (Molecular Biology Grade)
- Go Taq® G2 Flexi DNA Polymerase
- PCR Nucleotide Mix (dNTPs)

Roche; Indianopolis, IN, USA

- TriPure Isolation Reagent

Separations; Cape Town, SA

- Nuclease-free water (PCR Grade)

Sigma-Aldrich; St. Louis, USA

- 2-propanol (isopropanol)
- Cell Counting Kit-8 (CCK-8)
- Dimethyl Sulfoxide (DMSO)

Takara Bio Inc.; Tokyo, Japan

- PrimeScript™ Reverse Transcription Reagent Kit

2.3 Equipment and Supplies

Bioer Technology Co., LTD; Hangzhou, China

- Little Genius Thermal Cycler

Lasec; Cape Town, SA

- Hermle Z 206 A Centrifuge
- NuAire NU-5510E autoflow incubator
- NuAire NU-201-430E laminar flow
- WAS 160/X scale

Promega; Madison, USA

- GloMax Multi-Detection System plate reader

Takara Bio Inc.; Tokyo, Japan

- Advance Mupid – One system

ThermoFisher Scientific

- Nanodrop ND-2000 spectrophotometer

UVP, Inc.; San Gabriel, CA, USA

- UV Transilluminator

Zeiss; GmbH, Germany

- Carl Zeiss PrimoVert phase-contrast Microscope

2.4 Cell Culture

All tissue culture operations were conducted in an AireGard NU-201-430E horizontal laminar airflow table top workstation which provided a HEPA-filtered clean work area (NuAire) under the appropriate aseptic techniques that are essential for tissue culture. The cells were cultivated in a model NU-5510E NuAire DHD autoflow automatic CO_2 air-jacketed incubator maintained at 37°C in 95% air and 5% CO_2. All items used in the laminar and incubator were kept sterile through the use of 70% ethanol.

2.4.1 Cell Lines

2.4.1.1 MCF-12A

The MCF-12A cells used in the present study was generously donated by Prof M. Meyer's lab (University of the Western Cape). The MCF-12A cell line was established at the Michigan Cancer Foundation which gave rise to the MCF series. These cells were derived from a 63-year-old Caucasian post-menopausal nulliparous woman and the tissue was taken at a reduction mammoplasty (Sweeney *et al*, 2018). MCF-12A cells are immortalised breast epithelial cells and are better referred to as non-tumorigenic or non-transformed cells (Neve *et al,* 2006; Stander *et al,* 2009; Sweeney *et al,* 2018). Previous studies indicate that MCF-12A cells are negative for estrogen and progesterone receptors (Neve *et al,* 2006; Kenny *et al,* 2007). However, in more recent literature, studies showing that MCF-12A cells are estrogen receptor positive (ER+) and progesterone receptor positive (PR+) are on the increase (Gelfand *et al,* 2016; Sweeney *et al,* 2018). This immortalised cell line belongs to the Basal B subtype and exhibit a typical luminal epithelial phenotype with the ability to produce dome formation in confluent cultures (Kenny *et al,* 2007; Weber-Ouellette *et al,* 2018). Figure 2.1 demonstrates the regular cell morphology of MCF-12A cells captured using a Carl Zeiss PrimoVert phase-contrast Microscope.

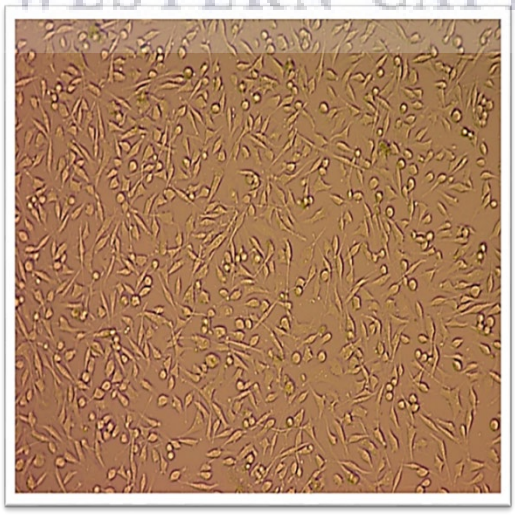

Figure 2.1: Normal cellular morphology of non-transformed MCF-12A cells with a flat and polygonal appearance characteristic to a typical luminal epithelial morphology. (Magnification: 40X, Carl Zeiss PrimoVert phase-contrast Microscope).

2.4.1.2 MCF-7

The MCF-7 cells used in the present study were generously donated by Prof M. de Kocks lab (university of the Western Cape). Established in 1973, a primary culture was isolated from a pleural effusion from a woman 69 years of age with metastatic mammary carcinoma. MCF-7 cells were established by Dr Soule and colleagues from the Michigan Cancer Foundation thus giving their name as MCF cells (Brooks *et al,* 1973; Soule *et al,* 1973; Holliday and Speirs, 2011; Comsa *et al,* 2015). MCF-7 cells belong to the molecular subtype group namely Luminal A and is positive for estrogen (ER+) and progesterone (PR+) receptors (Neve *et al,* 2006; Kenny *et al,* 2007; Comsa *et al*, 2015). MCF-7 cells were able to retain various characteristics of differentiated mammary epithelium which included cytoplasmic estrogen receptors and their ability of dome formation (Soule *et al*, 1973). Figure 2.2 shows the regular morphology of MCF-7 cells captured using a Carl Zeiss PrimoVert phase-contrast Microscope.

Figure 2.2: Normal cell morphology of MCF-7 cells depicting a flat and polygonal phenotype. (Magnification: 40X, Carl Zeiss PrimoVert phase-contrast Microscope).

2.4.1.3 MDA-MB-231

The MDA-MB-231 cells used in the present study were generously donated by Dr S. Prince's lab (University of Cape Town). In the 1970s, the MDA series was established at M.D. Anderson Hospital

and Tumor Institute (Cailleau *et al,* 1978; Chavez *et al,* 2010; Dai *et al,* 2017). MDA-MB-231 was derived from a Caucasian female, 51 years of age, through a pleural effusion (Cailleau *et al,* 1978). MDA-MB-231 cells are negative for estrogen receptors (ER-), progesterone receptors (PR-) as well as human epidermal growth factor receptor 2 (HER2-) which is the rationale behind these cells being referred to as a triple-negative breast cancer (TNBC). MDA-MB-231 is an invasive and highly aggressive cell line (Liu *et al,* 2003; Neve *et al,* 2006; Kenny *et al,* 2007; Chavez *et al,* 2010). Initially the cell line was classified as a Basal B cell type due to the lack of receptor expression, it but has now been recognized as a Claudin-low molecular subtype (Holliday and Speirs, 2011; Harrell *et al,* 2014; Dai *et al,* 2017). The cell line depicts an endothelial-like morphology with growth typically in a stellate pattern (Kenny *et al,* 2007, Chavez *et al,* 2010; Harrell *et al,* 2014). Figure 2.3 shows the regular morphology of MDA-MB-231 cells captured using a Carl Zeiss PrimoVert phase-contrast Microscope.

Figure 2.3: Normal morphology of MDA-MB-231 cells displaying a stellate pattern and are phenotypically spindle shaped cells. (Magnification: 40X, Carl Zeiss PrimoVert phase-contrast Microscope).

2.4.2 Maintenance of MCF-12A, MCF-7 and MDA-MB-231 Cell Cultures

All cell cultures were maintained following standard cell culture procedures under sterile conditions. MCF-12A cells were cultured using GIBCO® Dulbecco's Modified Eagles Medium/F-12 (DMEM/F12) supplemented with 10% heat-inactivated and gamma-irradiated foetal bovine serum

(FBS), 1% penicillin-streptomycin, 20 ng/ml human epidermal growth factor, 100 ng/ml cholera toxin, 0.01 mg/ml bovine insulin and 500 ng/ml hydrocortisone, and 5% horse serum while MCF-7 and MDA-MB-231 cells were cultured in GIBCO® DMEM/F12 supplemented only with 10% FBS and 1% penicillin-streptomycin. Culture medium was periodically changed and cells were seeded into 96 well plates for experimentation upon reaching 80-90% confluency.

2.5 Preparation of Red Palm Oil for Experimental Procedures

The Nutro Palm Oil was a generous gift from the Malaysian Palm Oil Board Ltd (Selangor, Malaysia). Table 2.1 shows the contents of the Nutro Palm oil.

Table 2.1: Contents of commercial Nutro Palm Oil

Component	14 g (1 Tablespoon) serving	Per 100 g serving
Calories	120 kcal	900 kcal
Protein	0 g	0 g
Carbohydrates	0 g	0 g
Fat	14 g	100 g
Saturated	5.6 g	39.7 g
Monounsaturated	6.4 g	46 g
Polyunsaturated	2 g	14.3 g
Vitamin E (tocopherols and tocotrienols)	15.4 mg	110 mg
Carotenoids (Pro-Vitamin A)	7.7 mg	55 mg
Trans fat	0 mg	0 mg
Cholesterol	0 mg	0 mg

(Data sourced from Nutro Palm Oil bottle)

Due to the inability of red palm oil to dissolve in growth medium alone, the oil had to be dissolved in a suitable solvent. Various solvents were used to determine the solubility of red palm oil. Different percentages of red palm oil ranging from 20-80% were used in order to determine the highest percentage of red palm oil that was successfully dissolved in a particular solvent. These solvents included butanol, chloroform, DMSO, ethanol, isopropanol, methanol, propanol, Tween-20 and Tween-80. Some agents

were able to fully dissolve the oil while others only partially dissolved the oil or did not dissolve the oil at all. The ability of the oil to mix in with the solvent and not show separated phases in the solution was considered a positive result while the inability of the oil to mix was regarded as a negative result. The same was considered when the red palm oil and solvents were added to the media (summarized in Table 2.2 and can be seen in Figures 2.4 to 2.9).

Table 2.2: Dissolution of red palm oil in various solvents and growth media

Solvent	Composition	Dissolution in Solvent	Dissolution in Medium Following Solvent
Butanol	20% oil + 80% butanol	Positive	Negative
	30% oil + 70% butanol	Positive	Negative
	40% oil + 60% butanol	Positive	Negative
	50% oil + 50% butanol	Positive	Negative
	60% oil + 40% butanol	Positive	Negative
	70% oil + 30% butanol	Positive	Negative
	80% oil + 20% butanol	Positive	Negative
	90% oil + 10% butanol	Positive	Negative
Chloroform	20% oil + 80% chloroform	Positive	Negative
	30% oil + 70% chloroform	Positive	Negative
	40% oil + 60% chloroform	Positive	Negative
	50% oil + 50% chloroform	Positive	Negative
	60% oil + 40% chloroform	Positive	Negative
	70% oil + 30% chloroform	Positive	Negative
	80% oil + 20% chloroform	Positive	Negative
	90% oil + 10% chloroform	Positive	Negative
DMSO	20% oil + 80% DMSO	Positive	Positive
	25% oil + 75% DMSO	Partial Dissolution	Negative
	30% oil + 70% DMSO	Partial Dissolution	Negative
	35% oil + 65% DMSO	Partial Dissolution	Negative
	40% oil + 60% DMSO	Negative	Negative

/continued

Table 2.2: Dissolution of red palm oil in various solvents and growth media (continued)

DMSO	45% oil + 55% DMSO	Negative	Negative
	50% oil + 50% DMSO	Negative	Negative
Ethanol	20% oil + 80% ethanol	Negative	Negative
Isopropanol	20% oil + 80% isopropanol	Positive	Negative
	30% oil + 70% isopropanol	Positive	Negative
	40% oil + 60% isopropanol	Positive	Negative
Isopropanol	50% oil + 50% isopropanol	Positive	Negative
	60% oil + 40% isopropanol	Positive	Negative
	70% oil + 30% isopropanol	Positive	Negative
	80% oil + 20% isopropanol	Positive	Negative
	90% oil + 10% isopropanol	Positive	Negative
Methanol	20% oil + 80% methanol	Negative	Negative
Propanol	20% oil + 80% propanol	Positive	Negative
	30% oil + 70% propanol	Positive	Negative
	40% oil + 60% propanol	Positive	Negative
	50% oil + 50% propanol	Positive	Negative
	60% oil + 40% propanol	Positive	Negative
	70% oil + 30% propanol	Positive	Negative
	80% oil + 20% propanol	Positive	Negative
	90% oil + 10% propanol	Positive	Negative
Tween-20	20% oil + 80% Tween-20	Negative	Negative
Tween-80	20% oil + 80% Tween-80	Negative	Negative

After dissolving the oil, appropriate concentrations were diluted in medium in order to obtain suitable working concentrations. Upon dilution, the oil separated in the medium except for one concentration containing 20% oil with 80% DMSO. The concentration of 20% oil and 80% DMSO was used as the basis in order to prepare a stock concentration of 200 000 µg/ml. 200 000 µg of red palm oil was weighed out and added to DMSO to make up a 1ml stock solution of 200 000µg/ml of red palm oil. A serial dilution was performed from the stock concentration of 200 000µg/ml to obtain the various

working concentrations (ranging from 1–1000 µg/ml) needed in order to carry out the required experiments and the DMSO concentration was kept at less than 0.5% in all treatment concentrations as this percentage exhibits no cytotoxic effects and is an accepted percentage for breast cancer cell lines (Shebaby *et al,* 2014; Adefolaju *et al,* 2015; Jamalzadeh *et al,* 2016; Nguyen *et al,* 2020).

Red palm oil was dissolved in varying concentrations of butanol (Figure 2.4); ranging from 20% oil and 80% butanol (A), 30% oil and 70% butanol (B), 40% oil and 60% butanol (C), 50% oil and 50% butanol (D), 60% oil and 40% butanol (E), 70% oil and 30% butanol (F), 80% oil and 20% butanol (G), and 90% oil and 10% butanol (H).

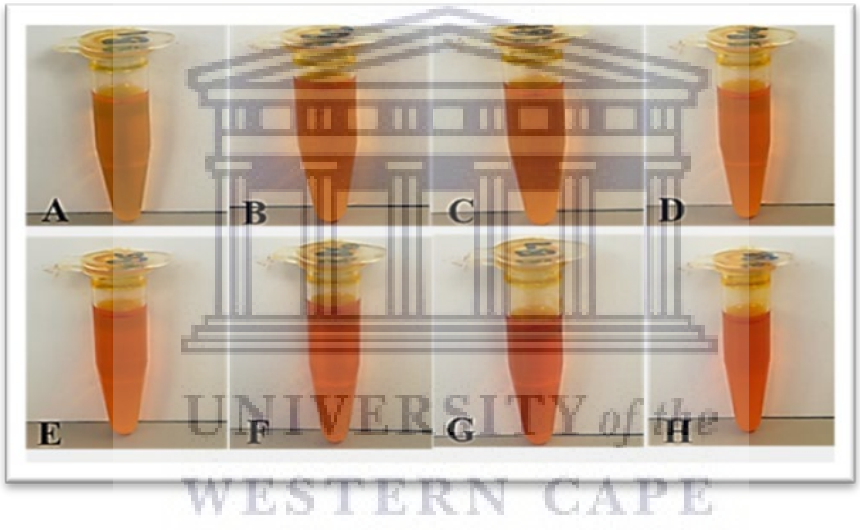

Figure 2.4: Varying concentrations of Red palm oil dissolved in butanol.

Red palm oil was dissolved in varying concentrations of chloroform (Figure 2.5); ranging from 20% oil and 80% chloroform (A), 30% oil and 70% chloroform (B), 40% oil and 60% chloroform (C), 50% oil and 50% chloroform (D), 60% oil and 40% chloroform (E), 70% oil and 30% chloroform (F), 80% oil and 20% chloroform (G), and 90% oil and 10% chloroform (H).

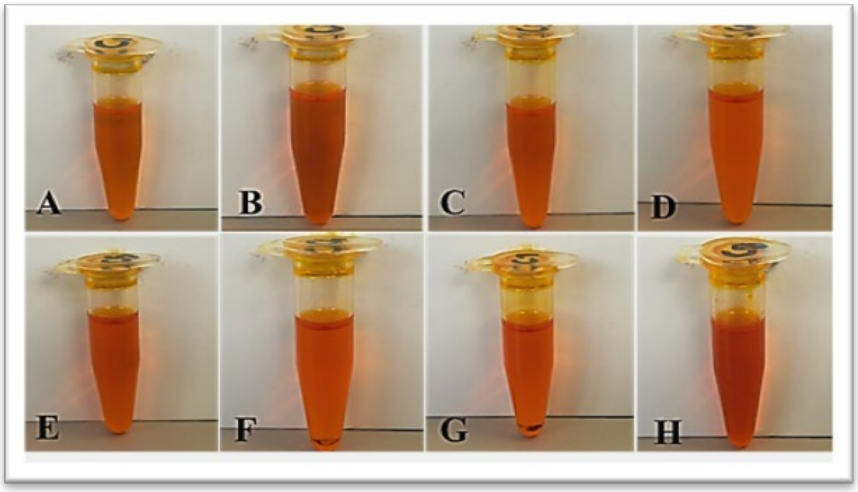

Figure 2.5: Varying concentrations of Red palm oil dissolved in chloroform.

Red palm oil was dissolved in varying concentrations of DMSO (Figure 2.6); ranging from 20% oil and 80% DMSO (A), 25% oil and 75% DMSO (B), 30% oil and 70% DMSO (C), 35% oil and 65% DMSO (D), 40% oil and 60% DMSO (E), 45% oil and 55% DMSO (F), and 50% oil and 50% DMSO (G).

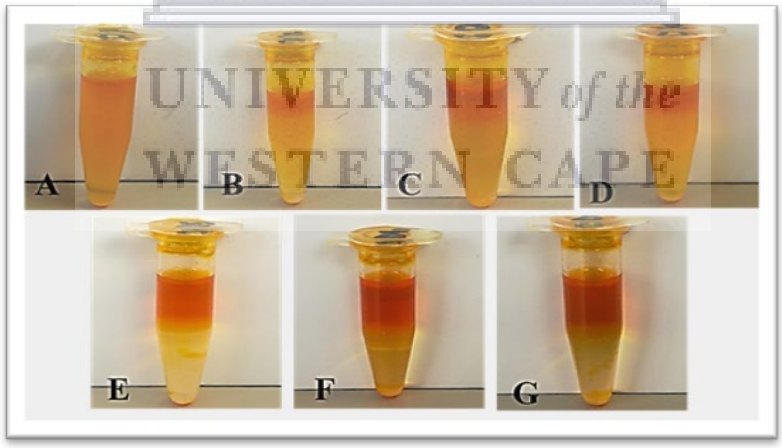

Figure 2.6: Varying concentrations of Red palm oil dissolved in DMSO.

Red palm oil was dissolved in varying concentrations of isopropanol (Figure 2.7); ranging from 20% oil and 80% isopropanol (A), 30% oil and 70% isopropanol (B), 40% oil and 60% isopropanol (C), 50% oil and 50% isopropanol (D), 60% oil and 40% isopropanol (E), 70% oil and 30% isopropanol (F), 80%

oil and 20% isopropanol (G), and 90% oil and 10% isopropanol (H).

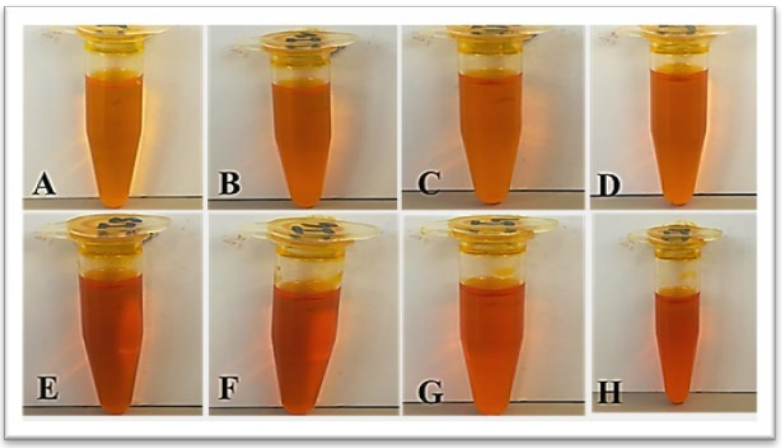

Figure 2.7: Varying concentrations of Red palm oil dissolved in isopropanol.

Red palm oil was dissolved in varying concentrations of propanol (Figure 2.8); ranging from 20% oil and 80% propanol (A), 30% oil and 70% propanol (B), 40% oil and 60% propanol (C), 50% oil and 50% propanol (D), 60% oil and 40% propanol (E), 70% oil and 30% propanol (F), 80% oil and 20% propanol (G), and 90% oil and 10% propanol (H).

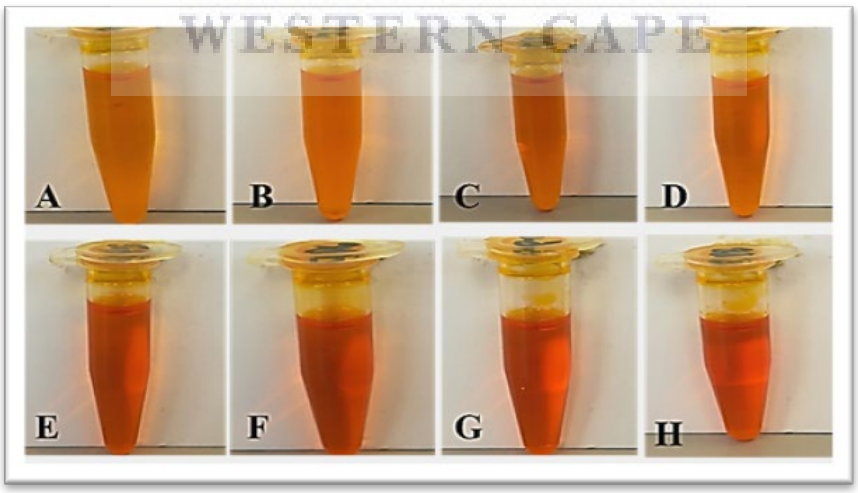

Figure 2.8: Varying concentrations of Red palm oil dissolved in propanol.

Red palm oil was dissolved in other solvents which showed no dissolution (Figure 2.9). These included

20% oil and 80% ethanol (A), 20% oil and 80% methanol (B), 20% oil and 80% Tween-20 (C), and 20% oil and 80% Tween-80 (D).

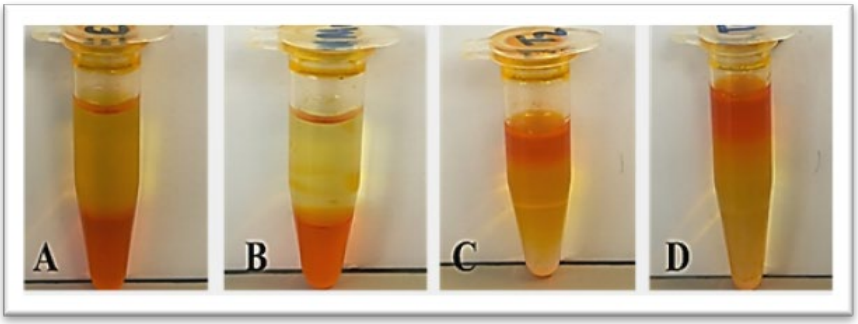

Figure 2.9: Dissolution of Red palm oil in other solvents.

2.6 Cell Proliferation Assay

Cell proliferation of MCF-12A, MCF-7 and MDA-MB-231 cells was determined using a 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H tetrazolium, monosodium salt (WST-8) which is commonly known as the cell counting kit 8 (CCK-8) assay. This assay is a colorimetric assay in which WST-8 is reduced in cells by dehydrogenases to produce an orange water soluble formazan dye (coloured product) demonstrated in Figure 2.10. The number of living cells is directly proportional to the production of formazan dye generated through the dehydrogenase activity in the cells. This assay provides high stability and high sensitivity which permits a precise assay (Ishiyama *et al,* 1997; Tominaga *et al,* 1999; Ginouves *et al,* 2014).

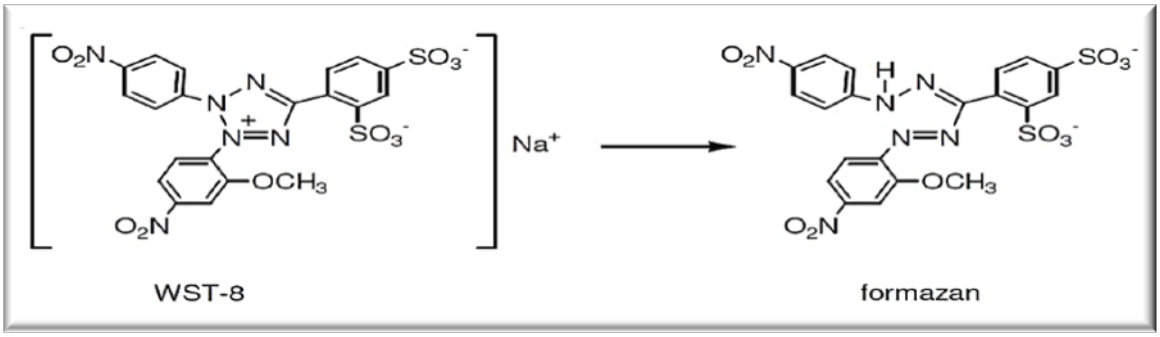

Figure 2.10: Chemical structure of WST-8 and reduction to formazan (Tominaga *et al*, 1999).

In short, cells were seeded and left to attach for 24 hours. Cells were then treated with appropriate treatment concentrations and exposed over 24, 48 and 72 hours. Then, cultures were removed from the incubator and the cell morphology of treated cells was viewed in relation to the non-treated control group using a Carl Zeiss PrimoVert phase-contrast Microscope and the image was captured. Thereafter, 10 µl of CCK-8 solution were added to each well. Cultures were placed back in the incubator for an additional 1-4 hours, based on the cell line. Finally, the absorbance was read at 450 nm using an ELISA plate reader (GloMax Multi-Detection System). The results were calculated using Equation 1 and expressed as percentage live cells.

Equation 1: Percentage Live Cells = $\frac{\text{Absorbance sample - absorbance blank}}{\text{Average negative control}}$ x 100

2.6.1 MCF-12A, MCF-7 and MDA-MB-231 Cell Proliferation Assay

Cells were grown to 80% confluency and were trypsinized with 1 ml 0.25% trypsin-EDTA. The trypsin was then inactivated by adding 2 ml complete growth medium. Cells were collected, centrifuged and resuspended in 1ml fresh growth medium. A cell count was performed per Equation 2 and diluted per Equation 3 for seeding. For MCF-12A, cells were seeded into 96-well plates at densities of 4.5 x 10^3 cells/well in 100 µl of complete growth medium for 24-hour exposure, 3 x 10^3 cells/well in 100 µl of complete growth medium for 48-hour exposure and 2 x 10^3 cells/well in 100 µl for 72-hour exposure. For MCF-7, cells were seeded into 96-well plates at densities of 3 x 10^3 cells/well in 100 µl of complete growth medium for 24-hour exposure, 2 x 10^3 cells/well in 100 µl of complete growth medium for 48-hour exposure and 1 x 10^3 cells/well in 100 µl for 72-hour exposure. For MDA-MB-231, cells were seeded into 96-well plates at densities of 5 x 10^3 cells/well in 100 µl of complete growth medium for 24-hour exposure, 3 x 10^3 cells/well in 100 µl of complete growth medium for 48-hour exposure and 2 x 10^3 cells/well in 100 µl for 72-hour exposure.

Cells were incubated for 24 hours to allow attachment of cells. Following 24-hour attachment, the medium was removed and cells were then exposed to varying concentrations of red palm oil treatment consisting of 1 µg/ml, 10 µg/ml, 100 µg/ml, 500 µg/ml and 1000 µg/ml in 100 µl growth medium

solution. The DMSO concentration was less than 0.5% in each treatment concentration. The negative control (NC) contained solely complete growth medium, whereas the vehicle control (VC) contained 0.5% DMSO to demonstrate no cytotoxic effects on cells at this percentage and the positive control (PC) contained 10% DMSO. Blank wells containing appropriate treatment solutions without cells were used to blank correct the absorbance reading.

Following the appropriate exposure times, cells in each well was viewed using Carl Zeiss PrimoVert phase-contrast Microscope to compare the cell morphology following treatment to the untreated control group and the image was captured. Thereafter, 10 µl CCK-8 was added to each well and incubated at 37ºC for 1-2 hours. Subsequently, the absorbance of the samples in the CCK-8 solution was measured at 450 nm with a plate reader (GloMax Multi-Detection System) and the percentage live cells were calculated per Equation 1.

Equation 2: Cells/ml = Mean x 2 (dilution factor) x 10^4

Equation 3: Volume of cells required = $\frac{\text{Concentration of cells needed (cells/ml) x Total volume}}{\text{Concentration of cells counted (cells/ml)}}$

2.7 Expression of hMAM, Maspin and GAPDH Housekeeping Genes

2.7.1 RNA Extraction from Cultured MCF-12A, MCF-7 and MDA-MB-231 Cells

The data collected from the cell proliferation assay following treatment was used to obtain the optimal concentrations needed to expose each cell line in order to test the effect of red palm oil on the gene expression of hMAM, Maspin and GAPDH housekeeping genes Following the cell proliferation assay, two concentrations (10 µg/ml and 100 µg/ml) were selected for treatment as these were the optimal concentrations per the cell proliferation results. Cells were grown and treated for 24, 48 and 72 hours. The DMEM/F12 growth medium was aspirated and 1–2 ml PBS was used to wash the cells. RNA was extracted from untreated and treated cell cultures by adding 1 ml of cold TriPure isolation reagent to

the flask and homogenized. The TriPure reagent allows for the simultaneous extraction of proteins, RNA and DNA from cells and tissues due to phenol and guanidine thiocyanate found in the reagent (Chomczynski, 1993). Each ml of the homogenate was transferred to a sterile 2-ml Eppendorf tube and incubated for 5 minutes at room temperature. Then, 0.2 ml of chloroform was added per ml of Tripure and vortexed for 15 seconds. Tubes were incubated for 5–10 minutes at room temperature.

Tubes were centrifuged at 12 000 x g for 15 minutes at 4°C to separate the solution into 3 phases. 350-500 µl of the colourless upper aqueous phase (RNA) was transferred to a sterile 1.5-ml Eppendorf tube and 0.5 ml isopropanol added to each tube containing the RNA solution and mixed thoroughly. The RNA was precipitated overnight at -20°C. The following day, the RNA solution was centrifuged at 12 000 x g for 10 minutes at 4°C and the supernatant was removed. Then, 1 ml of 80% ethanol was added to the RNA pellet, vortexed and centrifuged at 7500 x g for 5 minutes at 4°C. The supernatant was aspirated and resuspended in 0.1 ml nuclease-free water. The RNA was quantified with a Nanodrop ND-2000 spectrophotometer (ThermoFisher Scientific) which determined the ratio of absorbance at 260 nm and 280 nm to calculate the concentration and purity of RNA.

2.7.2 Reverse Transcription of RNA—cDNA Synthesis of Total RNA

The RNA was prepared for reverse transcription by heat-shock-cooling. The RNA was heated to 90°C for 5 minutes followed by shock-cooling at 0°C for 5 minutes. Thereafter, the RNA was ready to be added to the PrimeScript™ Reverse Transcription kit for the synthesis of cDNA. Total RNA was reversed transcribed into cDNA in a 10-µl reaction. A 3.5 µl master mix was prepared using 2 µl of 5X PrimeScript buffer, 0.5 µl PrimeScript RT enzyme mix, 0.5 µl Oligo dT Primer (50 µM) and 0.5 µl of Random 6 mers (100 µM). Total RNA of 1 µg/µl was added to the master mix and nuclease-free water was added to make a final volume of 10 µl.

The final reaction was run for 1 cycle at 37°C for 15 minutes followed by 85°C for 5 seconds. Thereafter, 4°C for 5 minutes using a thermal cycler (Bioer Technology). The cDNA was quantified with a Nanodrop ND-2000 spectrophotometer (ThermoFisher Scientific) which determined the ratio of

absorbance at 260 nm and 280 nm to calculate the concentration and purity of cDNA.

2.7.3 Primer Design

Specific primers were designed for a housekeeping gene and 2 target genes of interest, viz., Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping gene, Secretoglobin family 2A member 1 (SCGB2A1 also known as hMAM) and Serpin family B member 5 (SERPINB5 also known as Maspin). The primers used in this study were designed using the Ensembl genome browser (https://www.ensembl.org/index.html) and the National Centre for Biotechnology Information (NCBI) gene database (https://www.ncbi.nlm.nih.gov./gene).

The criteria for selected primers included: Product length of 200-250 bp, GC% content, Tm and primers spanning the exon-exon junctions were specified. The selected primers are summarised in Table 2.3 and Annexures 1–4 depict the full gene summary. Primers were purchased from Inqaba Biotechnical Industries (Pty) Ltd, South Africa.

Table 2.3: Summary of the selected primers

Gene	Product size (bp)	Gene bank Accession number	Primer sequence	Tm	GC %
GAPDH	227	NM_001289745.3	F 5'- AGAAGGCTGGGGCTCATTTG- 3'	53.83	55
			R 5'- GATGGCATGGACTGTGGTCA - 3'	53.83	55
hMAM	200	NM_002407.2	F 5' - CAGATTCTGGCTGCAAACTC -3'	51.78	50
			R 5'- CACTGTATGCATCATCAGTCCA - 3'	52.97	45
Maspin	227	NM_002639.4	F 5' - TGTTCCTTTTCCACGCATTT - 3'	47.68	40
			R 5'- AATGAAGAACCTGTCCAATTT - 3'	46.55	33

2.7.4 Polymerase Chain Reaction (PCR)

In the 1980s, Kary Mullis developed the polymerase chain reaction (PCR) method that allowed for the amplification of specific fragments of DNA over 1 billion-fold (Deepak *et al,* 2007; Seifi *et al,* 2012). This enzymatic assay is highly sensitive as only minute amounts of DNA fragments are needed for a PCR to produce enough copies (Garibyan and Avashia, 2013). Every PCR assay is dependent on and

requires a DNA template, nucleotide mix, two oligonucleotide primers and a DNA polymerase. DNA polymerase is the vital enzyme that links together the individual nucleotides thereby creating the PCR product. The polymerase enzymes are located in all living organisms and their function is to duplicate genetic material as well as proofreading and correcting the copies.

Taq polymerase is one of the most common and efficient enzymes used in PCR and is isolated from *Thermus aquaticus* (a thermophilic bacterium). This bacterium thrives and undergoes reproduction in environments that are lethal to many other organisms. Their ability to survive in very high temperatures makes them the most suitable choice for this assay.

PCR consists of 3 important steps:

1. Denaturation - heating the reaction temperature to 90–96°C whereby the strands of the genetic material are separated,

2. Annealing or hybridisation - lowering the reaction temperature to allow the binding of the specific primers to the target sequence, and

3. Extension – raising the temperature again which allows the DNA polymerase to extend the primers and generate the PCR product.

After every cycle, the number of copies of the target DNA doubles thereby creating billions of copies in a rapid and simple reaction (Erlich, 1989; Powledge, 2004; Garibyan and Avashia, 2013). The PCR reaction was set up to a final volume of 25 µl which consisted of 5 µl of Go Taq® Flexi buffer (5X), 1 µl $MgCl_2$ (25 mM), 0.125 µl Go Taq® G2 Flexi DNA Polymerase (5 U/µl), 0.5 µl PCR Nucleotide Mix (dNTPs) (10 mM), 0.375 µl forward primer (10 µM) and 0.375 µl reverse primer (10 µM) (primers summarised in Table 2.3), 1 µl cDNA template and 16.625 µl nuclease-free water to make up the final volume of 25 µl (reaction mix summarised in Table 2.4).

Table 2.4: Preparation of PCR reaction mix

Reaction components	Starting Concentration	Volume in µl	Final Concentration
Go Taq® Flexi buffer	5X	5	1X
$MgCl_2$	25 mM	1	1 mM
Go Taq® G2 Flexi DNA Polymerase	5 U/µl	0.125	0.025 U/µl
PCR Nucleotide Mix (dNTPs)	10 mM	0.5	0.2 mM
Forward Primer	10 µM	0.375	0.15 µM
Reverse Primer	10 µM	0.375	0.15 µM
cDNA template	Varying per sample	1	<0.05 µg/µl
Nuclease-free water	N/A	8.2	N/A
Final volume		**25 µl**	

The non-template control contained nuclease-free water in place of cDNA. The controls and samples were amplified using a thermal cycler (Bioer Technology) under the following conditions: 1 cycle of pre-denaturation at 95°C for 3 minutes, followed by 40 cycles consisting of denaturation at 95°C for 45 seconds, annealing at 50°C (based on the melting temperature and optimisation of each molecular marker) for 45 seconds and extension at 72°C for 45 seconds (cycling conditions summarised in Table 2.5). The PCR products were then visualized through gel electrophoresis.

Table 2.5: Cycling conditions for PCR

Steps	Temp	Time	Cycles
Pre-Denaturation	95ºC	3 min	1
Denaturation	95ºC	45 sec	40
Annealing	50ºC	45 sec	
Extension	72ºC	45 sec	

2.7.5 Agarose Gel Electrophoresis

PCR products were separated using a 1.5% agarose gel. 10X TBE buffer was prepared using 108 g Tris-base, 55 g Boric acid and 9.3 g EDTA and adjusted to 1L using Reverse Osmosis (RO) water. 1X TBE

buffer was prepared using 50 ml 10X TBE buffer and 450 ml RO water. The 1.5% agarose gel was prepared using 2.25 g agarose powder and 150 ml 1X TBE buffer. Agarose powder was dissolved in the 1X TBE buffer by microwaving for 2–3 minutes. The solution was allowed to cool for a few minutes and 3 µl Ethidium Bromide was added. The casting tray was prepared by adding well combs. The semi-cooled gel was carefully poured into the casting tray to avoid air bubbles and allowed to solidify for approximately 30 minutes. Once the gel had set in the casting tray the well comb was removed. The gel was carefully removed from the casting tray and placed in the electrophoresis chamber containing the remaining 1X TBE buffer.

7.5 µl DNA ladder was added to the first lane of the gel. 10 µl of each cDNA sample was mixed with 2 µl loading dye (6X) and the 12 µl samples were transferred to the appropriately allocated lanes. The same was done for the non-template water control. The gel was allowed to run for 80–90 minutes at 100V in the Advance Mupid – One gel electrophoresis system (Takara Bio Inc.). Upon completion, the gel was viewed using a UV Transilluminator (UVP, Inc.) and the image was captured.

2.8 Statistical Analysis

Data accumulated in this study was recorded and statistically analysed using GraphPad Prism version 6 for Windows (GraphPad Software, San Diego, California USA, www.graphpad.com). All experiments were conducted in triplicate. Outliers, if any was first detected in GraphPad, after which, the Kolmogorov-Smirnov testing method was performed in order to check for normal distribution of the data. If data was normally distributed, parametric test was conducted by independent t-test. If data was not normally distributed, non-parametric test was conducted by Mann-Whitney test. One-way ANOVA trend analysis was performed to test for a trend between parameters. $p<0.05$ was considered statistically significant.

CHAPTER 3

DATA ANALYSIS AND RESULTS

3.1 Introduction

This chapter focuses on all results obtained from the cell proliferation assay and the PCR assay. All findings are recorded, displayed and analysed.

3.2 Effect of Red Palm Oil on MCF-12A, MCF-7 and MDA-MB-231 Cell Proliferation and Cell Morphology

3.2.1 Analysis of MCF-12A Cell Morphology and Proliferation

Following 24-hour exposure to 1-1000 µg/ml of red palm oil, cell morphology was observed and recorded (Figure 3.1, D=1, E=10, F=100, G=500 and H=1000 µg/ml red palm oil). No apparent signs consistent with cell stress or death such as cell shrinkage and cell rounding were seen in the 0.5% DMSO vehicle control (VC) (B) when compared to the negative control (NC) (A). In comparison to the control group (A), the group treated with 10% DMSO positive control (PC) (C) showed cells that appeared rounded and fewer cells were observed. The experimental groups (D-H) showed no obvious signs consistent with cell stress or death such as cell shrinkage or cell rounding between the lowest (D) and highest (H) concentrations. Cells treated with red palm oil (D-H) and both the negative control (A) and 0.5% DMSO vehicle control (VC) (B) displayed flat and polygonal cells characteristic to their luminal epithelial morphology. The CCK-8 assay (Figure 3.2) showed statistically significant ($p=0.0013$, $p=0.0104$, $p=0.0282$, and $p=0.0085$) increases in cell proliferation between negative control (NC) and red palm oil treatments of 1, 10, 500 and 1000 µg/ml concentrations, respectively, with 100 µg/ml yielding no statistically significant ($p<0.05$) increase in cell proliferation. When analysed further, one-way ANOVA showed no statistically significant ($p<0.05$) upward trend from the negative control to 1000 µg/ml.

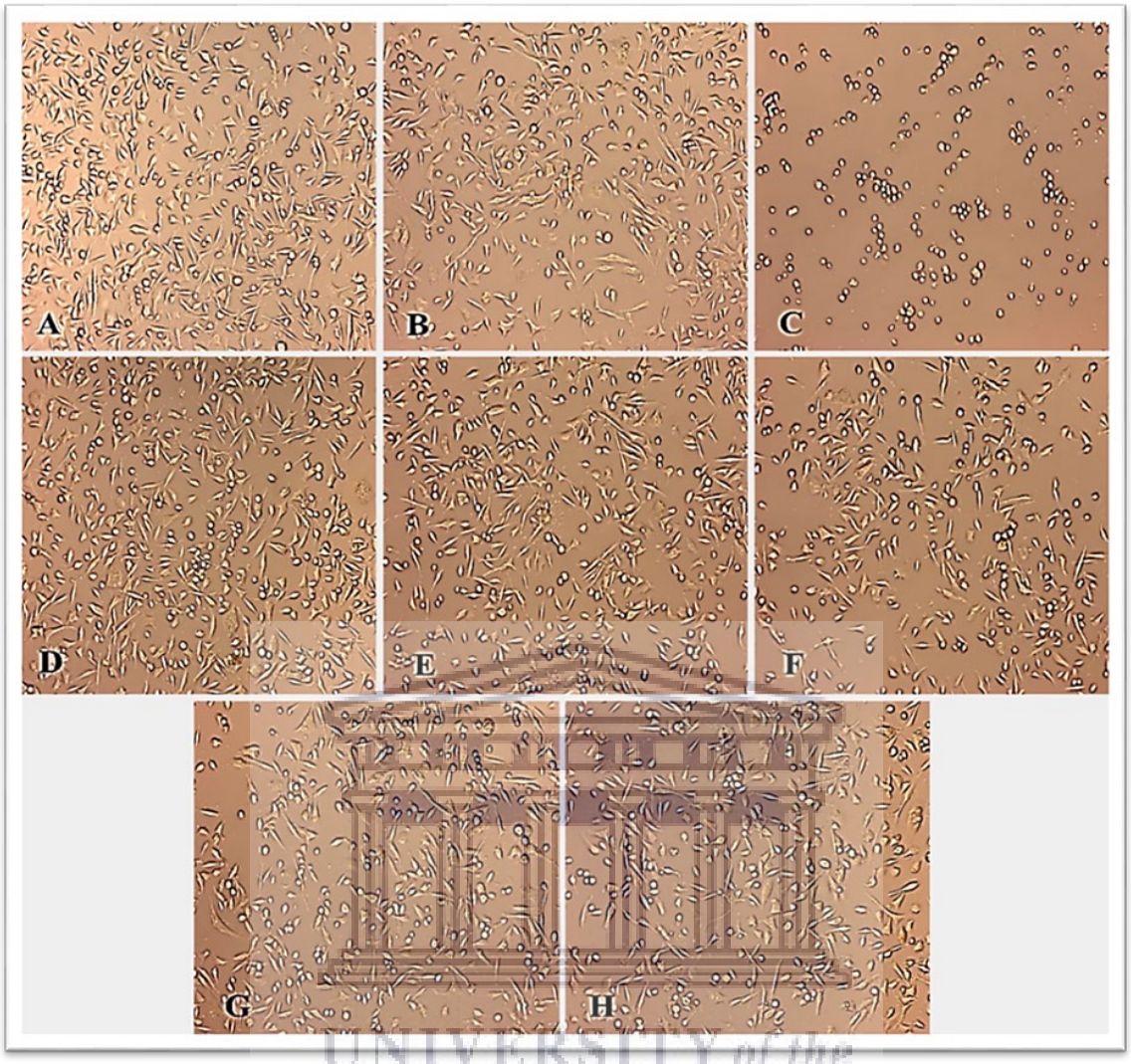

Figure 3.1: MCF-12A cell morphology following treatment with 1–1000 µg/ml of red palm oil over 24 hours (D-H), in comparison to the negative (A), vehicle (B), and positive control (C) samples. Cell morphology apparently showed no changes at each concentration of RPO used (D-H), maintaining their flat and polygonal morphology characteristic to epithelial cells. Positive control (C) shows a change in morphology with a rounding of cells. A=Negative Control (NC), B=0.5% DMSO Vehicle Control (VC), C=10% DMSO Positive Control (PC), D=1, E=10, F=100, G=500 and H=1000 µg/ml red palm oil. (Magnification: 40X, Carl Zeiss PrimoVert phase-contrast Microscope).

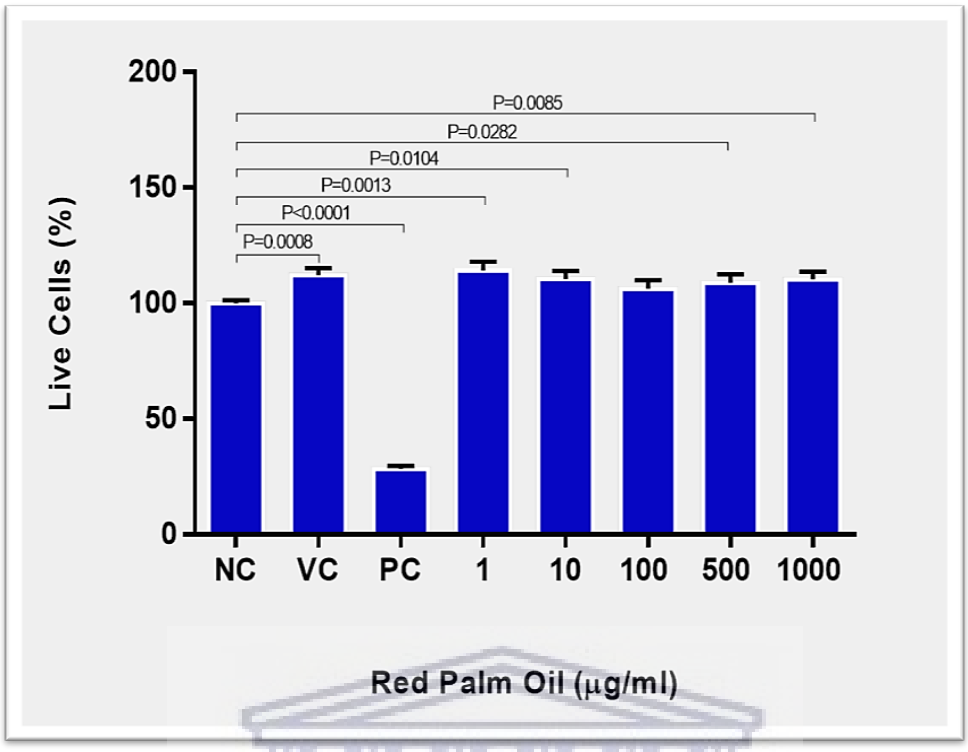

Figure 3.2: Dose-response effect on cell proliferation of MCF-12A cells following 24 hours of exposure to red palm oil. Cells showed statistically significant (p=0.0013, p=0.0104, p=0.0282, and p=0.0085) increases in cell proliferation between the negative control (NC) and 1, 10, 500 and 1000 µg/ml red palm oil treatments, respectively. NC=Negative Control, VC=0.5% DMSO Vehicle Control and PC=10% DMSO Positive Control.

Following a 48-hour exposure to 1-1000 µg/ml of red palm oil (D-H), cell morphology was observed and recorded (Figure 3.3). When compared to the negative control (A), the group treated with the 0.5% DMSO vehicle control (VC) (B) maintained their luminal epithelial morphology with a polygonal and flat appearance, and indicated no apparent signs consistent with cell stress or death such as cell rounding or shrinkage of cells, while cell rounding and fewer cells were observed for the 10% DMSO positive control (PC) (C). The experimental group (D-H) remained comparable to the control (A), whereby exhibiting no obvious cell shrinkage or cell rounding, signs that are consistent with cell stress or death between the lowest and highest concentrations (D-H) and their flat and polygonal luminal epithelial morphology remained unchanged.

The CCK-8 assay (Figure 3.4) showed statistically significant ($p=0.0037$, $p=0.0220$ and $p<0.0001$) increases in cell proliferation between the negative control (NC) and 1, 500 and 1000 µg/ml red palm oil treatments, respectively. Statistically significant ($p=0.0030$) increase in cell proliferation was observed in the 0.5% DMSO vehicle control (VC) whereas a statistically significant ($p<0.0001$) decrease in cell proliferation was observed in the 10% DMSO positive control (PC). Furthermore, one-way ANOVA showed a statistically significant ($p=0.0048$) upward trend between negative control (NC) and the highest red palm oil concentration.

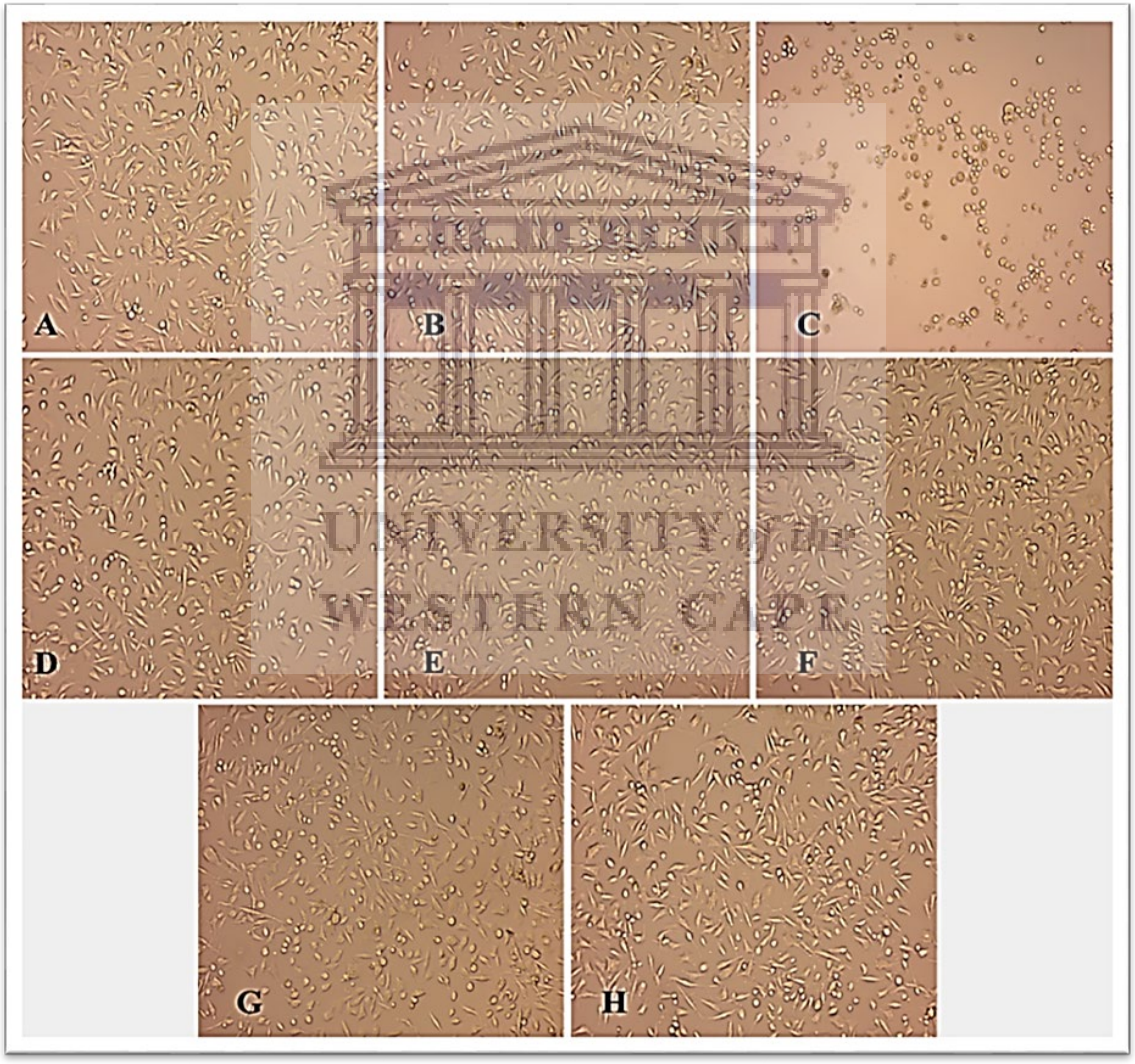

Figure 3.3: MCF-12A cell morphology following treatment with 1-1000 µg/ml of red palm oil over 48 hours (D-H), in comparison to the negative (A), vehicle (B), and positive control (C) samples. Cell morphology apparently showed no changes and cells maintained their characteristic epithelial morphology with a flat and polygonal appearance with each red palm oil concentration (D-H). Positive control (C) shows a change in morphology to rounded cells.

A=Negative Control, B=0.5% DMSO Vehicle Control (VC), C=10% DMSO Positive Control (PC), D=1, E=10, F=100, G=500 and H=1000 µg/ml red palm oil. (Magnification: 40X, Carl Zeiss PrimoVert phase-contrast Microscope).

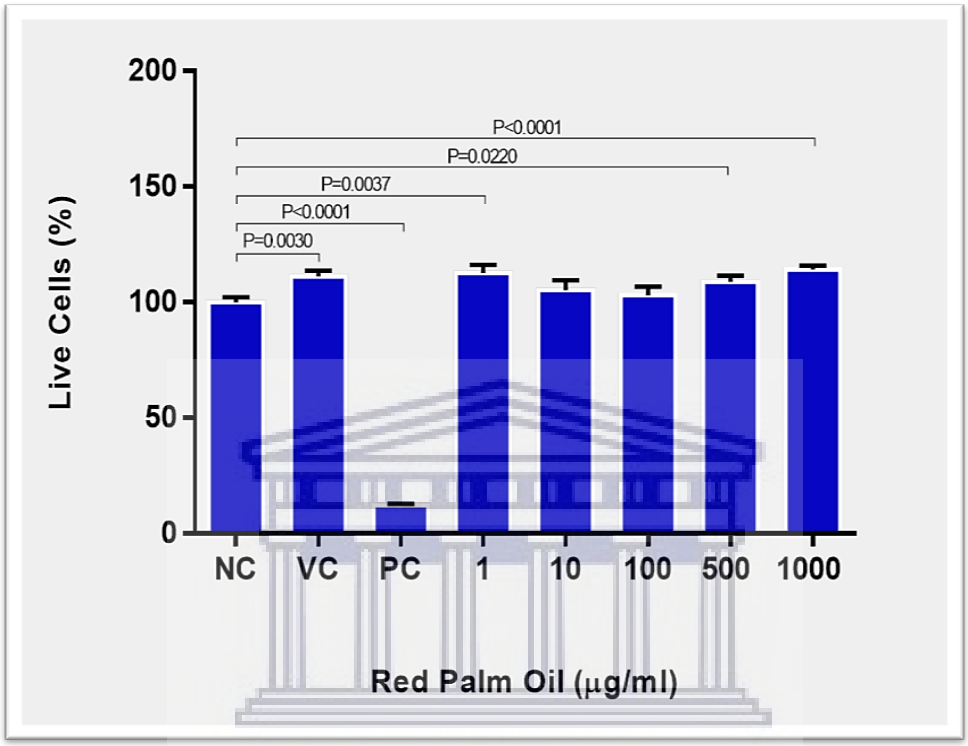

Figure 3.4: Dose-response effect on cell proliferation of MCF-12A cells following 48 hours of exposure to red palm oil. Cells showed statistically significant ($p=0.0037$, $p=0.0220$ and $p<0.0001$) increases in cell proliferation between the negative control (NC) and 1, 500 and 1000 µg/ml treatments, respectively. NC=Negative Control, VC=0.5% DMSO Vehicle Control and PC=10% DMSO Positive Control.

Following a 72-hour exposure to 1-1000 µg/ml of red palm oil (D-H), cell morphology was observed and recorded (Figure 3.5). When compared to the negative control (A), the group treated with the vehicle control (0.5% DMSO) (B) showed no apparent signs consistent with cell stress or cell death such as shrinkage of cells or rounding of cells, thus maintaining their flattened and polygonal appearance. However, the group treated with a positive control (10% DMSO) (C) showed signs consistent with cell stress and cell death where cells appeared rounded and fewer cells were observed.

The experimental groups (D-H) exhibited no obvious cell shrinkage or cell rounding, signs consistent with cell stress or death, between the lowest (D) and highest (H) concentrations. Cells treated with red palm oil at varying concentrations (D-H) maintained their characteristic epithelial morphology with a polygonal and flat appearance. The CCK-8 assay (Figure 3.6) showed statistically significant ($p=0.0251$, $p=0.0228$ and $p<0.0001$) increases in cell proliferation between the negative control (NC) and 1, 500 and 1000 µg/ml of red palm oil, respectively. Statistically significant ($p=0.0070$) increase in cell proliferation was observed in the 0.5% DMSO vehicle control (VC) and a statistically significant ($p<0.0001$) decrease in cell proliferation was observed in the 10% DMSO positive control (PC). Upon further analysis, one-way ANOVA showed a statistically significant ($p<0.0001$) upward trend between negative control (NC) and the highest red palm oil concentration.

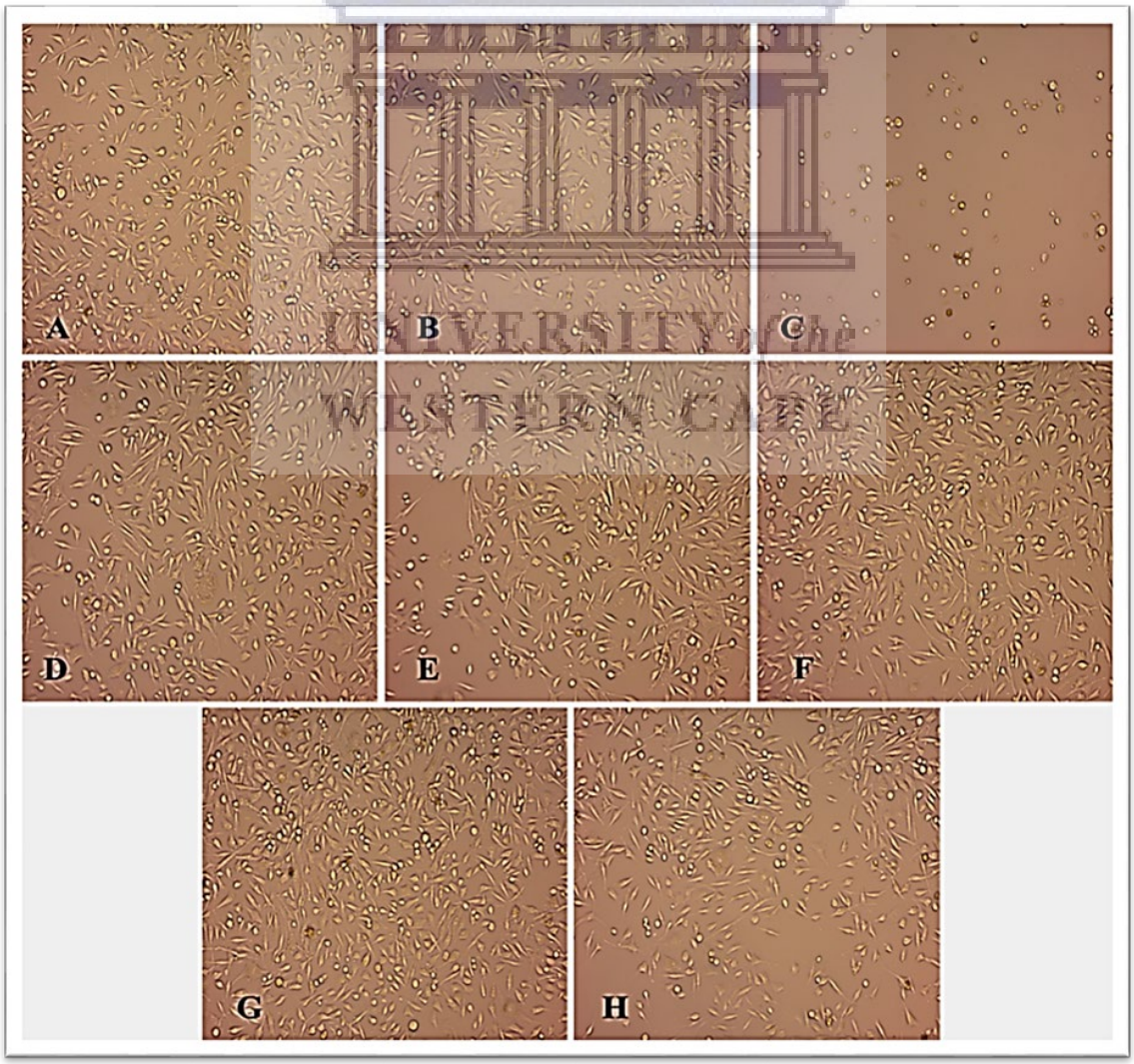

Figure 3.5: MCF-12A cell morphology following treatment with 1-1000 µg/ml of red palm oil over 72 hours (D-H), in comparison to the negative (A), vehicle (B), and positive control (C) samples. Cell morphology apparently showed no change and cells maintained their flat and polygonal morphology characteristic to luminal epithelial cell morphology with each concentration of red palm oil (D-H). Positive control (C) shows a change in morphology with cell rounding. A=Negative Control (NC), B=0.5% DMSO Vehicle Control (VC), C=10% DMSO Positive Control (PC), D=1, E=10, F=100, G=500 and H=1000 µg/ml red palm oil. (Magnification: 40X, Carl Zeiss PrimoVert phase-contrast Microscope).

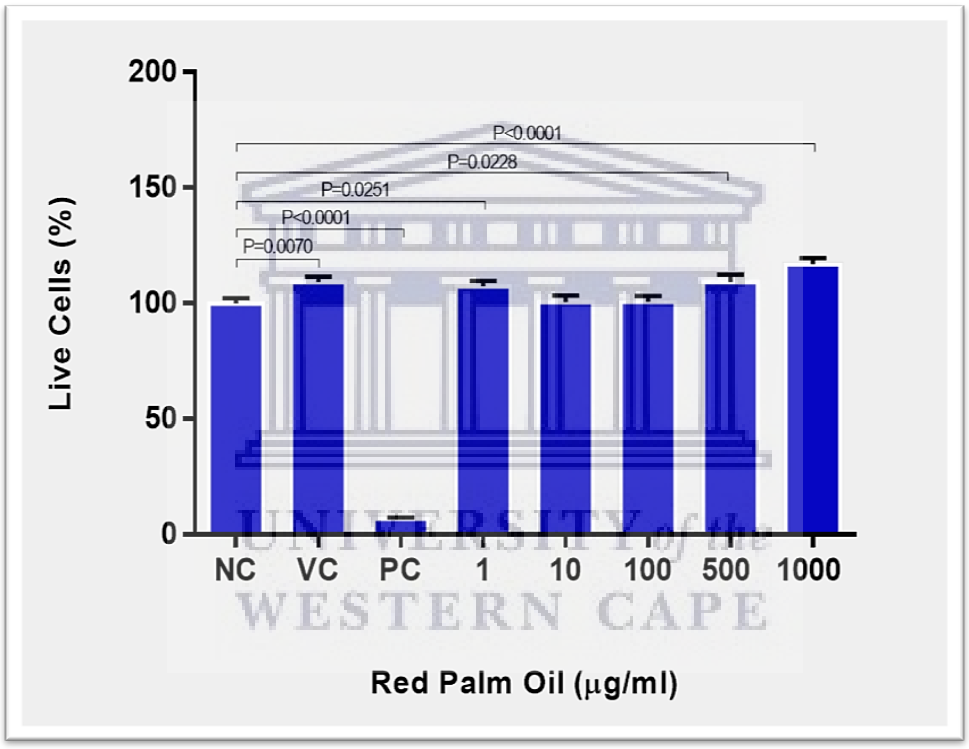

Figure 3.6: Dose-response effect on cell proliferation of MCF-12A cells following 72 hours of exposure to red palm oil. Cells showed statistically significant (p=0.0251, p=0.0228 and p<0.0001) increases in cell proliferation between the negative control (NC) and 1, 500 and 1000 µg/ml treatments, respectively. NC=Negative Control, VC=0.5% DMSO Vehicle Control and PC=10% DMSO Positive Control.

Compared cell proliferation of MCF-12A cells following 24, 48 and 72 hour incubations with 1–1000 µg/ml of red palm oil (Figure 3.7). Statistically significant (p<0.05) increases in cell proliferation are

indicated by asterisks (*) showing level of significance with specific p values shown in (Figures 3.2, 3.4 and 3.6). * shows p<0.05, ** shows p<0.01, *** shows p<0.001 and **** shows p<0.0001. Following a 24-hour exposure, an increase in cell proliferation was observed between the negative control (NC) and 1000 µg/ml. Furthermore, a 48-hour exposure showed an increase in cell proliferation between the negative control (NC) and highest concentration with the 72 hour exposure to red palm oil following suit. Overall, an increase in cell proliferation was noticed between the negative control (NC) and red palm oil concentrations at each treatment period.

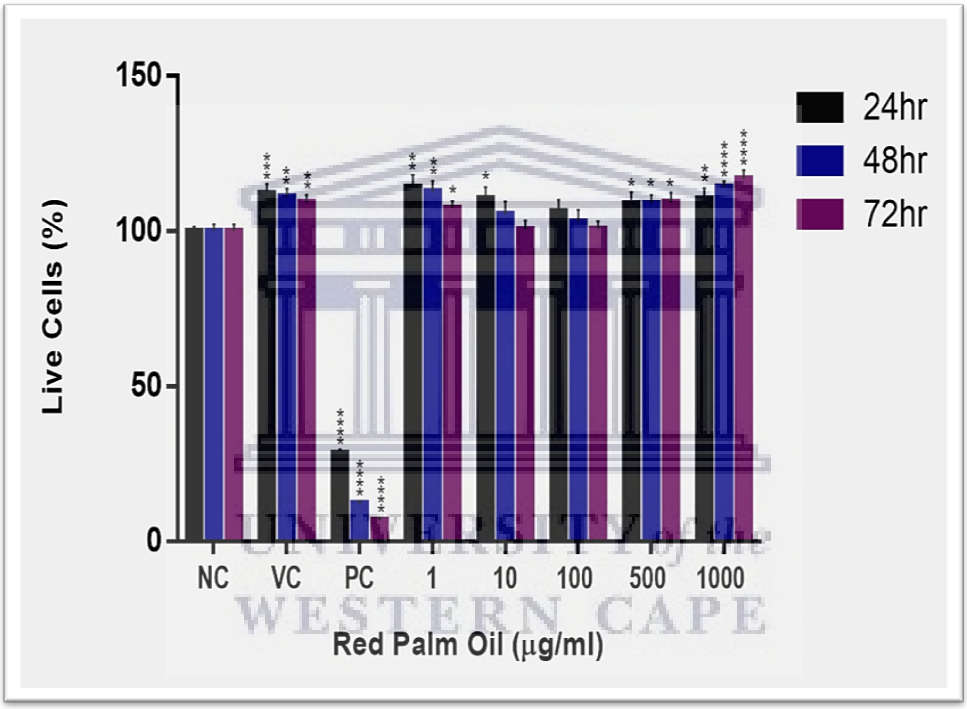

Figure 3.7: Compared cell proliferation of MCF-12A cells following a 24-, 48- and 72-hour incubations with 1-1000 µg/ml of red palm oil. Statistically significant (p<0.05) increases in cell proliferation observed and shown by asterisks (*) where * = p<0.05, ** = p<0.01, *** = p<0.001 and **** = p<0.0001. NC=Negative Control, VC=0.5% DMSO Vehicle Control and PC=10% DMSO Positive Control.

3.2.2 Analysis of MCF-7 Cell Morphology and Proliferation

Following 24 hours of exposure to 1-1000 µg/ml of red palm oil (D-H), cell morphology was observed and recorded (Figure 3.8). When compared to negative control (NC) (A), the group treated with the

0.5% DMSO vehicle control (VC) (B) remained characteristically flat and polygonal, exhibiting no obvious signs consistent with cell stress or death which include cell rounding and cell shrinkage, while signs consistent with cell death was observed for the 10% DMSO positive control (PC) (C) which showed cell rounding and fewer cells were observed. The experimental group remained comparable to the control (A), displaying no apparent changes in cell morphology between the lowest and highest concentration (D-H) which maintained their polygonal and flat appearance.

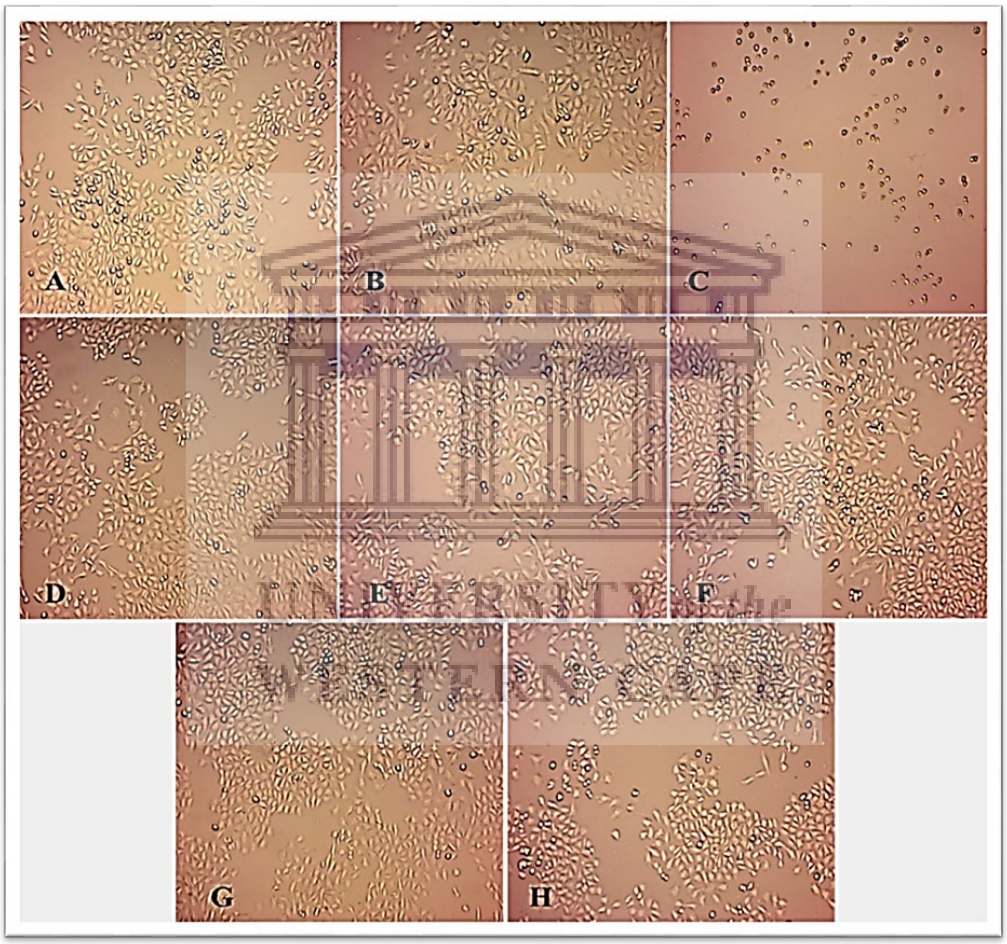

Figure 3.8: MCF-7 cell morphology following treatment with 1-1000 µg/ml of red palm oil over 24 hours (D-H), in comparison to the negative (A), vehicle (B), and positive control (C) samples. Cell morphology apparently remained unchanged at each concentration of RPO (D-H) used, remaining flat and polygonal. Change in cell morphology can be seen in the positive control (C) with cells more rounded in appearance. A=Negative Control (NC), B=0.5% DMSO Vehicle Control (VC), C=10% DMSO Positive Control (PC), D=1, E=10, F=100, G=500 and H=1000 µg/ml red palm oil. (Magnification: 40X, Carl Zeiss PrimoVert phase-contrast Microscope).

The CCK-8 assay (Figure 3.9) showed statistically significant (p=0.04, p=0.0064, p=0.0223, p<0.0001 and p=0.0046) decreases in cell proliferation between negative control (NC) and all concentrations of red palm oil following a 24-hour treatment. When analysed further, one-way ANOVA showed a statistically significant (p=0,005) downward trend from the negative control (NC) to 1000 µg/ml. No statistically significant (p<0.05) increase in cell proliferation was observed between the negative control (NC) and the 0.5% DMSO vehicle control (VC), while the 10% DMSO positive control (PC) yielded a statistically significant (p<0.0001) reduction in cell proliferation.

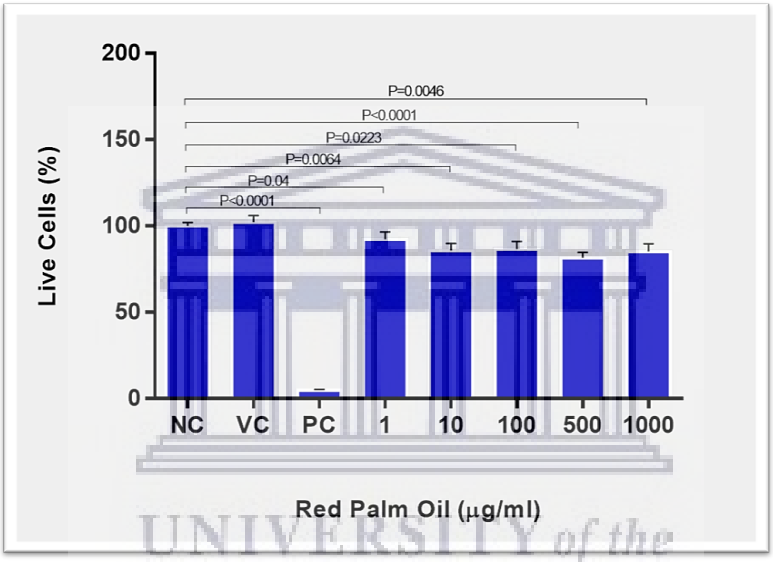

Figure 3.9: Dose-response effect on cell proliferation of MCF-7 cells following 24 hours of exposure to red palm oil. Cells showed statistically significant (p=0.04, p=0.0064, p=0.0223, p<0.0001 and p=0.0046) decreases in cell proliferation between the negative control (NC) and all the concentrations of red palm oil tested. NC=Negative Control, VC=0.5% DMSO Vehicle Control and PC=10% DMSO Positive Control.

Following a 48-hour of exposure to 1-1000 µg/ml of red palm oil (D-H), cell morphology was observed and recorded (Figure 3.10). When compared to negative control (A), the group treated with the 0.5% vehicle control (VC) (B) remained characteristically flat and polygonal, and indicated no apparent signs consistent with cell stress or death such as cell rounding, while signs consistent with cell death was observed for the positive control (10% DMSO) (C) whereby cells appeared more rounded and fewer

cells were observed. The experimental group remained comparable to control, which showed no obvious signs consistent with cell stress or death between the lowest and highest concentration (D-H) maintaining their characteristic flat and polygonal cell morphology. The CCK-8 assay (Figure 3.11) showed no statistically significant ($p<0.05$) increases in cell proliferation between the negative control (NC) and concentrations containing red palm oil. Statistically significant ($p=0.0001$) increase in cell proliferation was seen in the 0.5% DMSO vehicle control (VC) while statistically significant ($p<0.0001$) decrease in cell proliferation was observed in the 10% DMSO positive control. Upon further analysis, one-way ANOVA yielded no statistically significant ($p<0.05$) upward trend from the negative control to 1000 µg/ml.

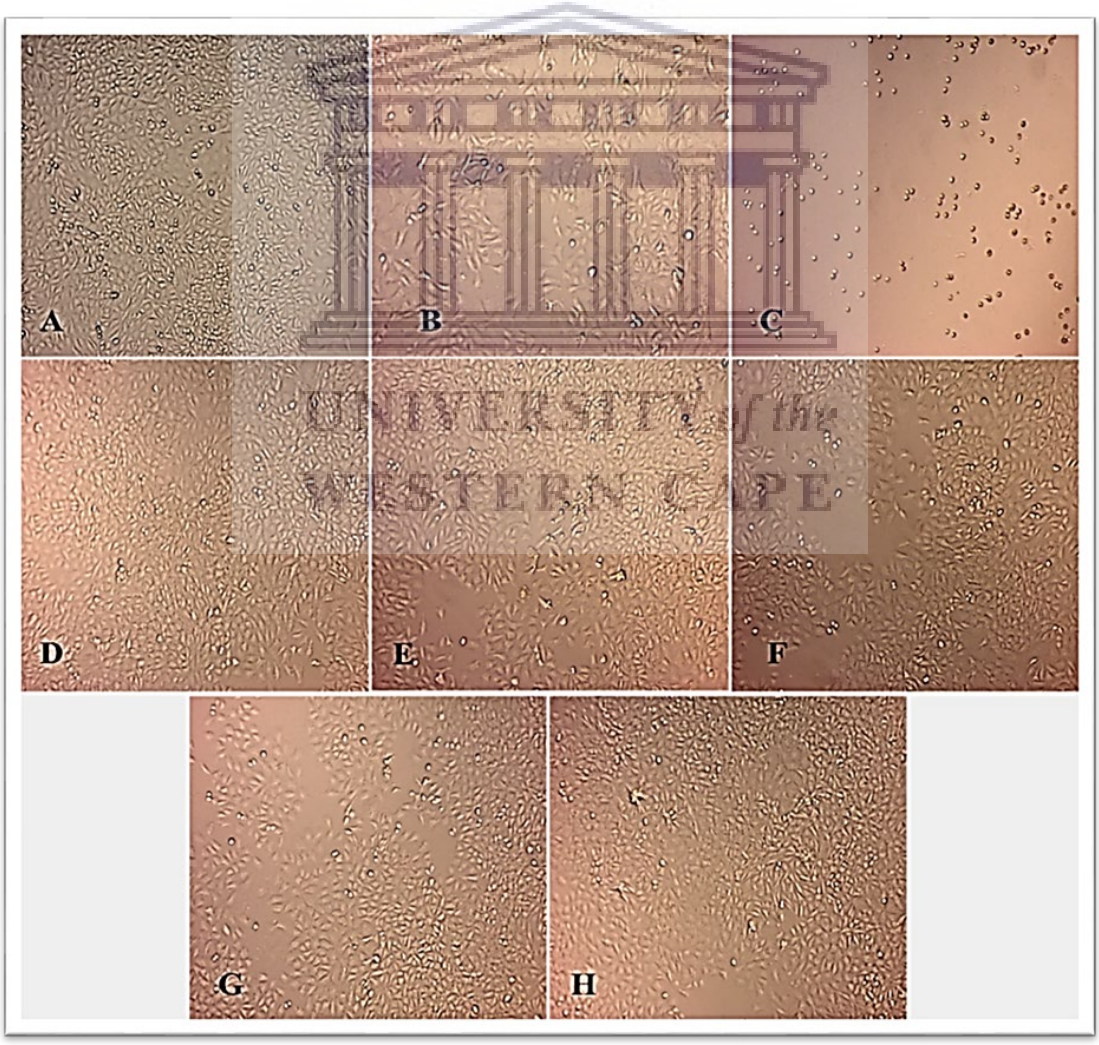

Figure 3.10: MCF-7 cell morphology following treatment with 1-1000 µg/ml of red palm oil over 48 hours (D-H), compared to the negative (A), vehicle (B), and positive control (C)

samples. Cell morphology apparently showed no change at each concentration of RPO (D-H) used, remaining flat and polygonal. Change in cell morphology observed in the positive control (C) with rounded cells. A=Negative Control (NC), B=0.5% DMSO Vehicle Control (VC), C=10% DMSO Positive Control (PC), D=1, E=10, F=100, G=500 and H=1000 µg/ml red palm oil. (Magnification: 40X, Carl Zeiss PrimoVert phase-contrast Microscope).

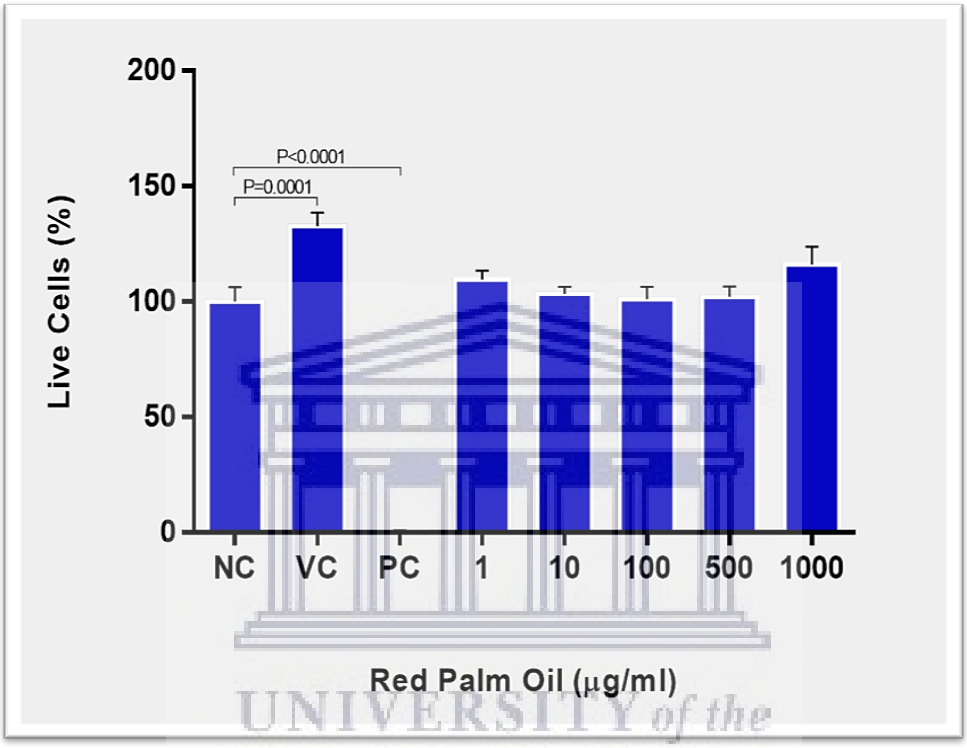

Figure 3.11: Dose-response effect on cell proliferation of MCF-7 cells following 48 hours of exposure to red palm oil. Cells showed statistically significant (p=0.0001) increase in cell proliferation in the vehicle control (VC) whereas statistically significant (p<0.0001) decrease in cell proliferation in positive control (PC). No statistically significant increases seen between concentrations containing red palm oil. NC=Negative Control, VC=0.5% DMSO Vehicle Control and PC=10% DMSO Positive Control.

Following 72 hours of exposure 1-1000 µg/ml of red palm oil (D-H), cell morphology was observed and recorded (Figure 3.12). In comparison to the negative control (NC) (A), the group treated with the 0.5% vehicle control (VC) (B) remained characteristically flat and polygonal, showing no apparent signs consistent with cell stress or death such as rounding of cells, while signs indicative of cell death were observed for the 10% DMSO positive control (PC) (C) whereby cells appeared more rounded and

fewer cells were observed. The experimental group remained comparable to control, showing no obvious signs consistent with cell stress or death between the lowest and highest concentration (D-H) maintaining their polygonal and flat appearance.

The CCK-8 assay (Figure 3.13) showed statistically significant ($p=0.0123$, $p<0.0001$, $p<0.0001$, $p<0.0001$ and $p=0.0093$) decreases in cell proliferation between the negative control (NC) and all the concentrations of red palm oil tested. No statistically significant ($p<0.05$) increase in cell proliferation was observed between negative (NC) and 0.5% DMSO vehicle control (VC) whereas, statistically significant ($p<0.0001$) decrease in cell proliferation was seen in the 10% DMSO positive control (PC). Furthermore, one-way ANOVA showed a statistically significant ($p<0.0001$) downward trend between negative control (NC) and the highest red palm oil concentration.

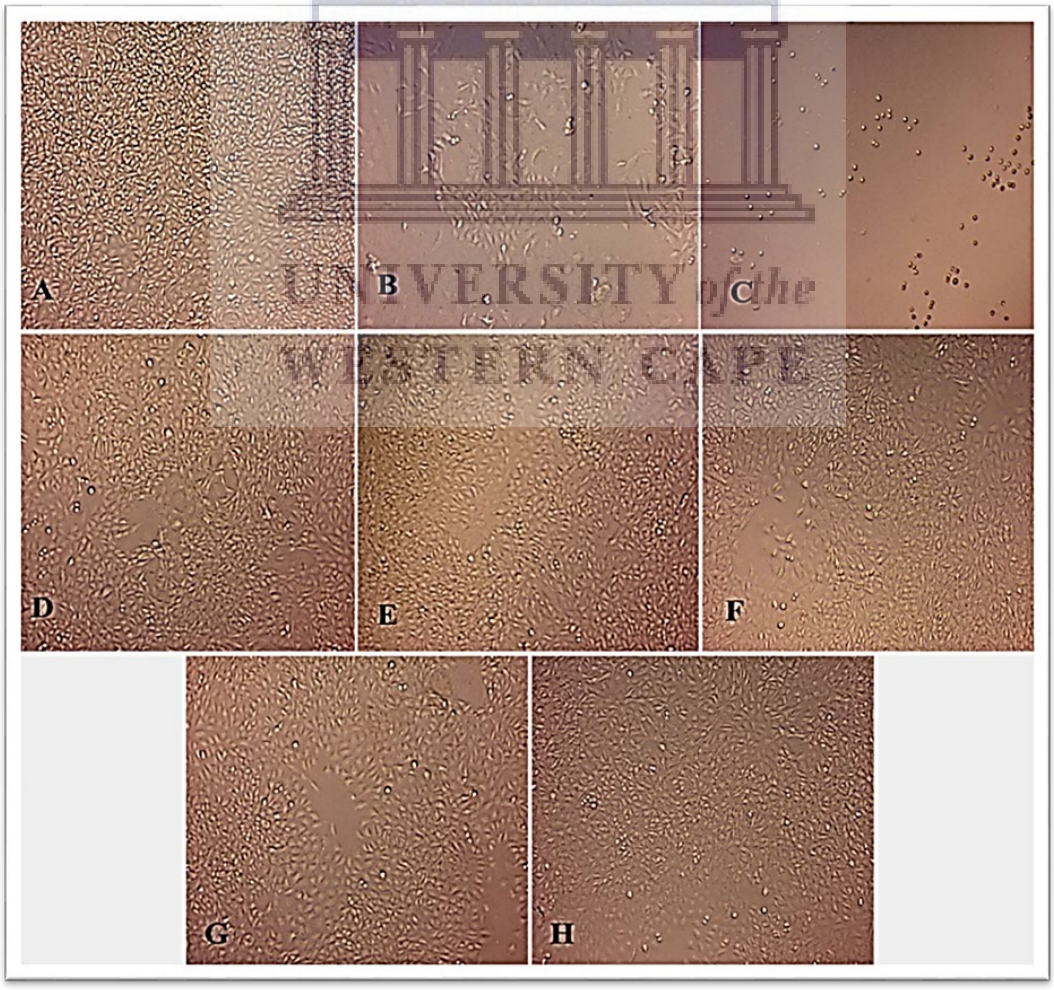

Figure 3.12: MCF-7 cell morphology following treatment with 1-1000 µg/ml of red palm oil

over 72 hours (D-H), in comparison to the negative (A), vehicle (B), and positive control (C) samples. Cell morphology apparently showed no changes at each concentration of RPO (D-H) used thus remaining flat and polygonal. Change in cell morphology was observed in the positive control (C) with a rounding of cells. A=Negative Control (NC), B=0.5% DMSO Vehicle Control (VC), C=10% DMSO Positive Control (PC), D=1, E=10, F=100, G=500 and H=1000 µg/ml red palm oil. (Magnification: 40X, Carl Zeiss PrimoVert phase-contrast Microscope).

Comparison of cell proliferation of MCF-7 cells following 24, 48 and 72 hour incubations with 1-1000 µg/ml of red palm oil is shown in Figure 3.14. Statistically significant ($p<0.05$) decreases in cell proliferation are indicated by asterisks (*) showing level of significance with specific p values shown in (Figures 3.9, 3.11 and 3.13). * shows $p<0.05$, ** shows $p<0.01$, *** shows $p<0.001$ and **** shows $p<0.0001$. Following 24 hour exposure, a decrease in cell proliferation was observed between the negative control and 1000 µg/ml, whereas 48 hour exposure revealed observable increases in cell proliferation at 1-1000 µg/ml. Subsequently, 72 hour exposure exhibited a noticeable decrease in cell proliferation at each concentration.

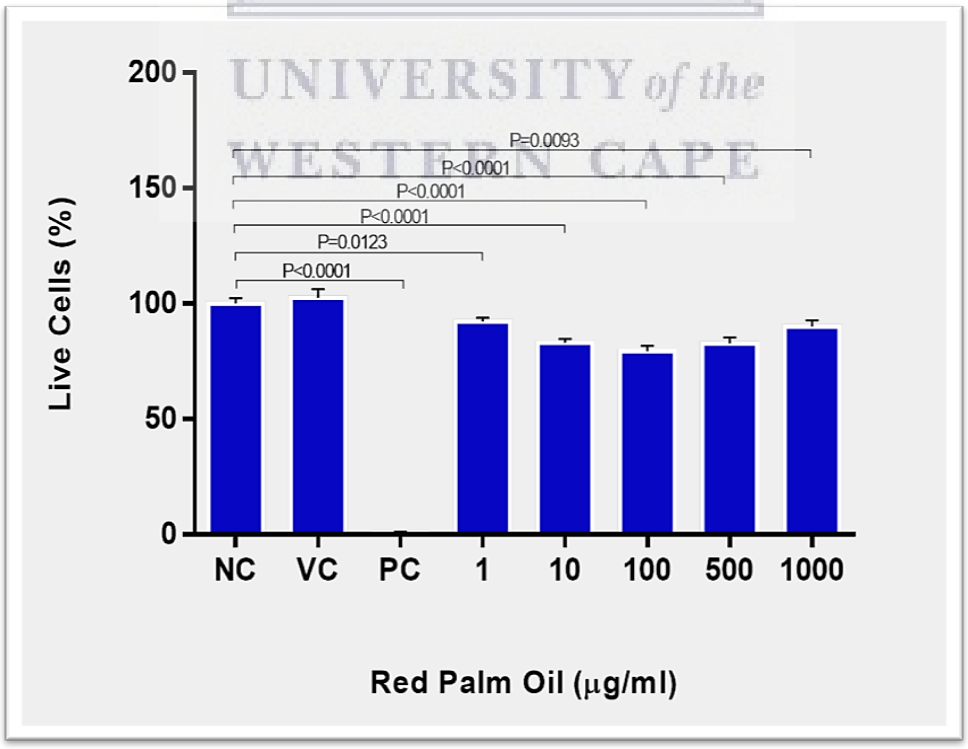

Figure 3.13: Dose-response effect on cell proliferation of MCF-7 cells following 72 hours of exposure to red palm oil. Cells showed statistically significant ($p=0.0123$, $p<0.0001$, $p<0.0001$,

p<0.0001 and p=0.0093) decreases in cell proliferation between the negative control (NC) and all the concentrations of red palm oil tested. NC=Negative Control, VC=0.5% DMSO Vehicle Control and PC=10% DMSO Positive Control.

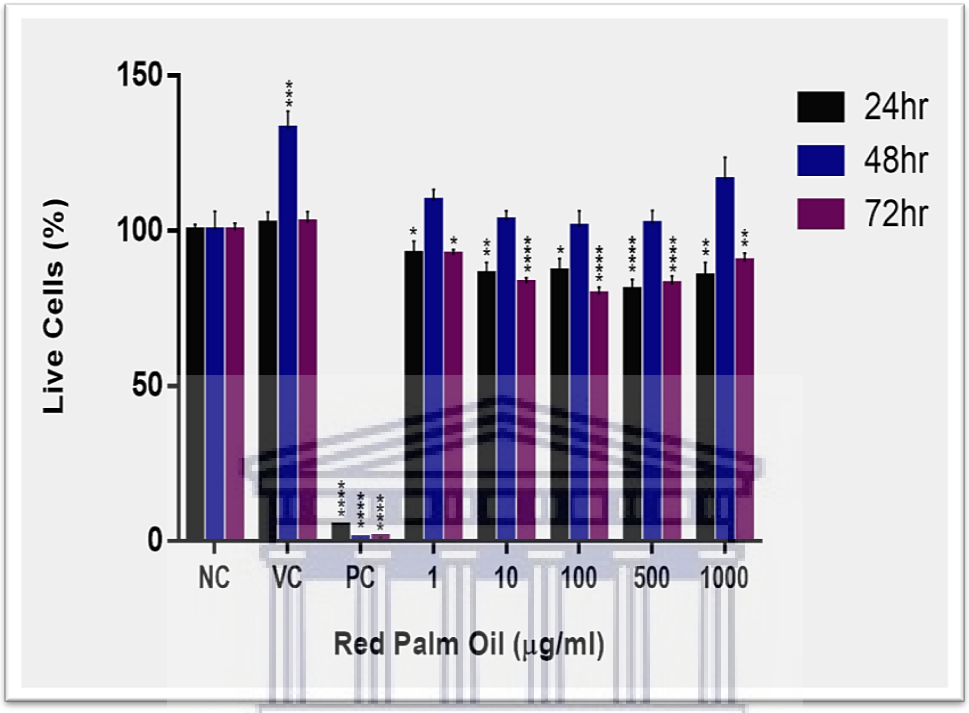

Figure 3.14: Compared cell proliferation of MCF-7 cells following 24, 48 and 72 hour incubations with 1-1000 µg/ml of red palm oil. Statistically significant (p<0.05) decreases in cell proliferation observed and shown by asterisks (*) where * = p<0.05, ** = p<0.01, *** = p<0.001 and **** = p<0.0001. NC=Negative Control, VC=0.5% DMSO Vehicle Control and PC=10% DMSO Positive Control.

3.2.3 Analysis of MDA-MB-231 Cell Morphology and Proliferation

Following 24 hour exposure to 1-1000 µg/ml of red palm oil (D-H), cell morphology was observed and recorded (Figure 3.15) In comparison to the negative control (NC) (A), the group treated with the 0.5% DMSO vehicle control (VC) (B) showed no apparent signs consistent with cell stress or cell death such as cell rounding and shrinking whereas the group treated with a 10% DMSO positive control (PC) (C) showed signs consistent with cell stress and cell death with a change in normal morphology from spindle shape and stellate pattern to rounded cells. The experimental groups (D-H) showed no obvious

signs consistent with cell stress or death between the lowest (D) and highest (H) concentrations maintaining their spindle shape appearance.

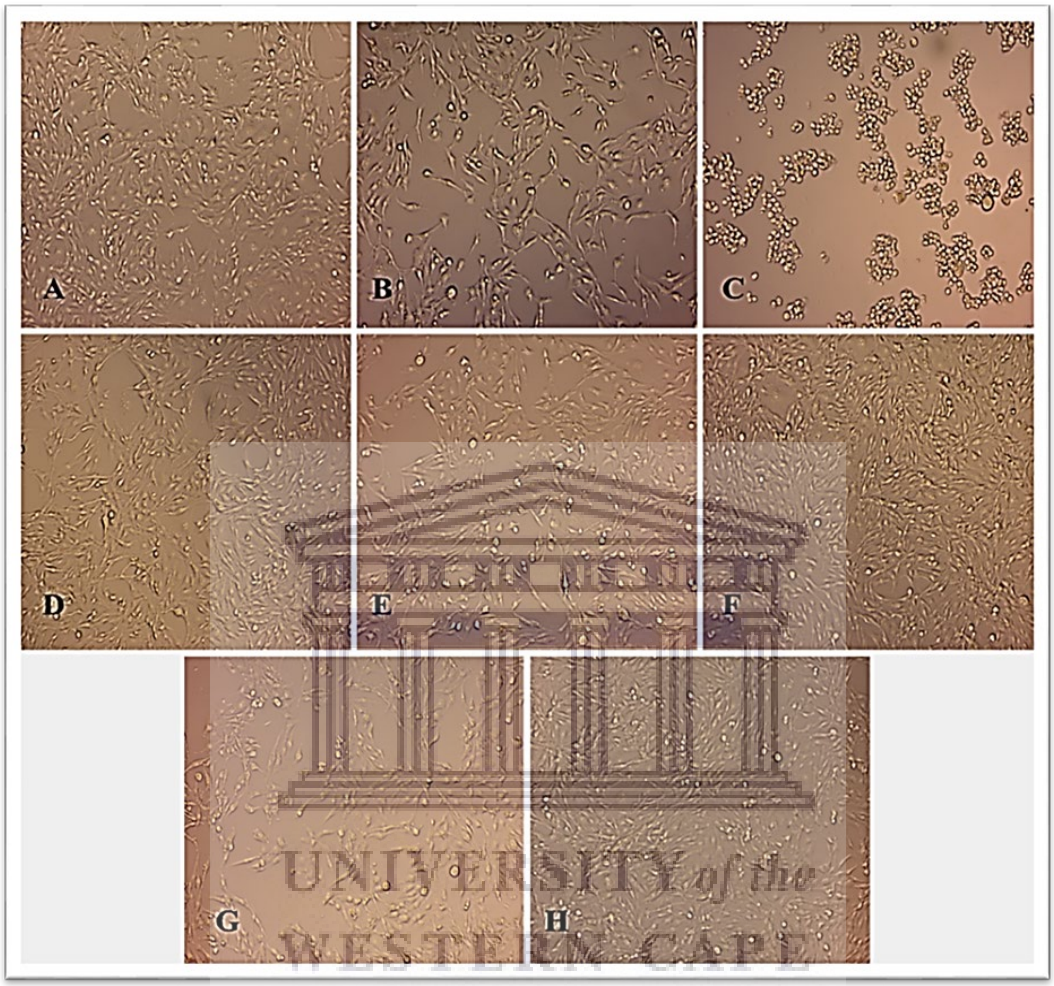

Figure 3.15: MDA-MB-231 cell morphology following treatment of 1-1000 µg/ml of red palm oil over 24 hours (D-H) in comparison to the controls; Negative (A), vehicle (B) and positive control (C) samples. Cell morphology apparently remained unchanged in each experimental group (D-H) maintaining their spindle shape and stellate pattern. Positive control (C) shows change in cell morphology from spindle shape to rounded cells. A=Negative Control (NC), B=0.5% DMSO Vehicle Control (VC), C=10% DMSO Positive Control (PC), D=1, E=10, F=100, G=500 and H=1000 µg/ml red palm oil. (Magnification: 40X, Carl Zeiss PrimoVert phase-contrast Microscope).

The CCK-8 assay (Figure 3.16) showed statistically significant (p=0.0468) increase in cell proliferation between the negative control (NC) and red palm oil concentration containing 1 µg/ml. No statistically

significant ($p<0.05$) increases in cell proliferation were observed in the higher concentrations of red palm oil. The 0.5% vehicle control (VC) did not exhibit statistically significant ($p<0.05$) increase in cell proliferation while the 10% DMSO positive control (PC) yielded a statistically significant ($p<0.0001$) reduction in cell proliferation. When further analysed, one-way ANOVA showed no statistically significant ($p<0.05$) upward trend between the negative control and 1000 µg/ml.

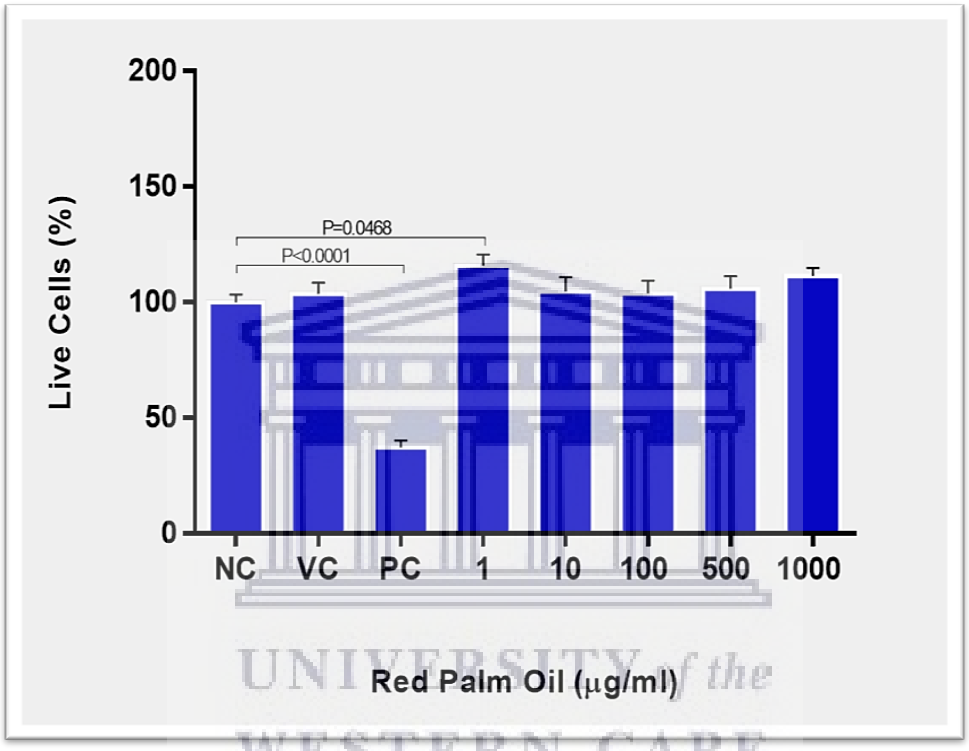

Figure 3.16: Dose-response effect on cell proliferation of MDA-MB-231 cells following 24 hours of exposure to red palm oil. Cells showed statistically significant ($p=0.0468$) increase in cell proliferation between the negative control (NC) and 1 µg/ml. No statistically significant increases were seen in higher concentrations of red palm oil. NC=Negative Control, VC=0.5% DMSO Vehicle Control and PC=10% DMSO Positive Control.

Following a 48-hour exposure to 1-1000 µg/ml of red palm oil (D-H), cell morphology was observed and captured (Figure 3.17). The group treated with the 0.5% vehicle control (VC) (B) indicated no apparent signs consistent with cell stress and death such as cell rounding and shrinking whereas the 10% DMSO positive control (PC) (C) showed signs indicative of cell stress and death whereby fewer

cells were observed and cells appeared rounded in comparison to the negative control (A). Cell morphology in the experimental groups (D-H) remained unchanged and showed no obvious signs associated with cell stress or death between the lowest (D) and highest concentrations (H) maintaining their characteristic spindle shape and stellate appearance.

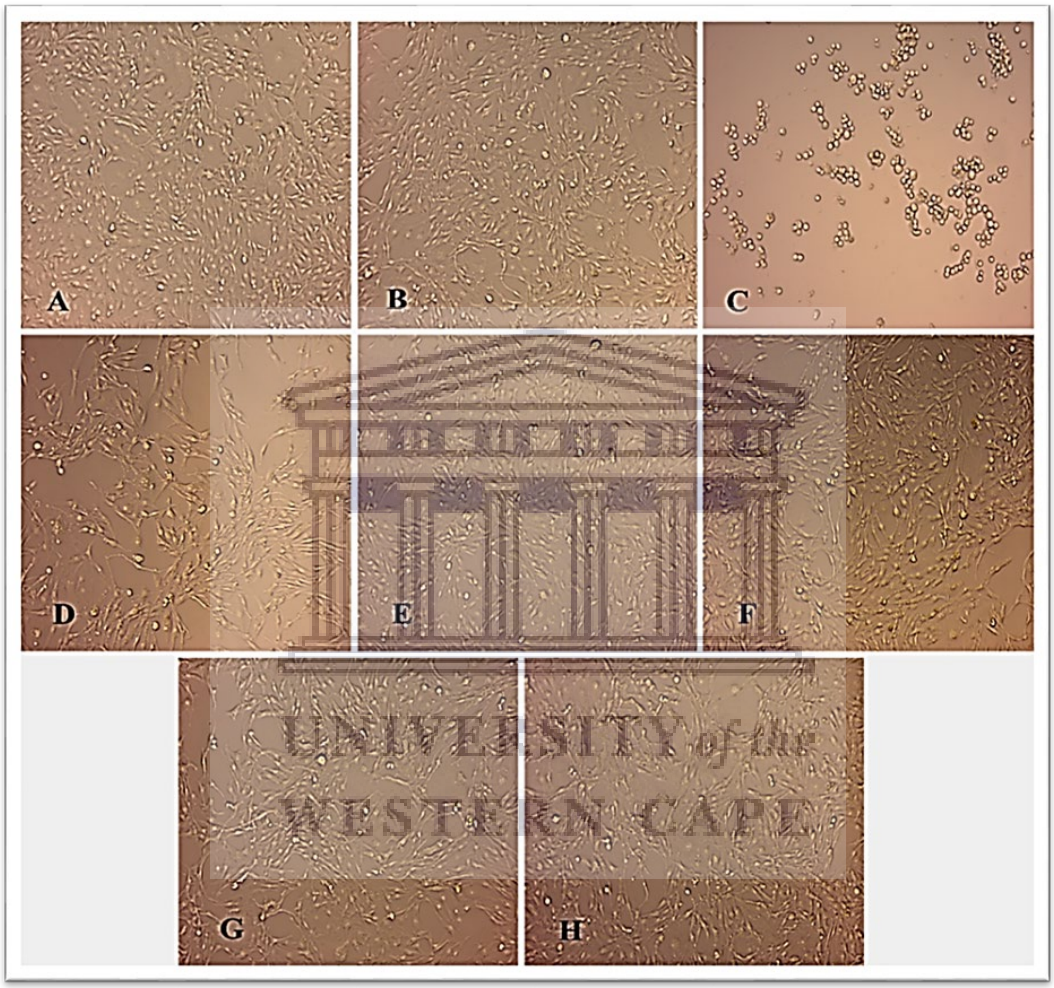

Figure 3.17: MDA-MB-231 cell morphology following treatment of 1-1000 µg/ml of red palm oil over 48 hours (D-H) in comparison to the controls; Negative (A), vehicle (B) and positive control (C) samples. Cell morphology apparently showed no changes in each experimental group (D-H) and maintained their normal spindle shaped morphology and stellate pattern. A change in cell morphology was observed from spindle shape to rounded cells in the positive control (C). A=Negative Control (NC), B=0.5% DMSO Vehicle Control (VC), C=10% DMSO Positive Control (PC), D=1, E=10, F=100, G=500 and H=1000 µg/ml red palm oil. (Magnification: 40X, Carl Zeiss PrimoVert phase-contrast Microscope).

The CCK-8 assay (Figure 3.18) showed statistically significant ($p<0.0001$) increases in cell proliferation between negative control (NC) and all red palm oil concentrations. A statistically significant ($p=0.0033$) increase in cell proliferation was noted between negative (NC) and 0.5% DMSO vehicle control (VC). Furthermore, statistically significant ($p<0.0001$) decrease in cell proliferation was observed between negative (NC) and 10% DMSO positive control (PC). When analysed further, one-way ANOVA showed a statistically significant ($p<0.0001$) upward trend between negative control (NC) and highest concentration of red palm oil.

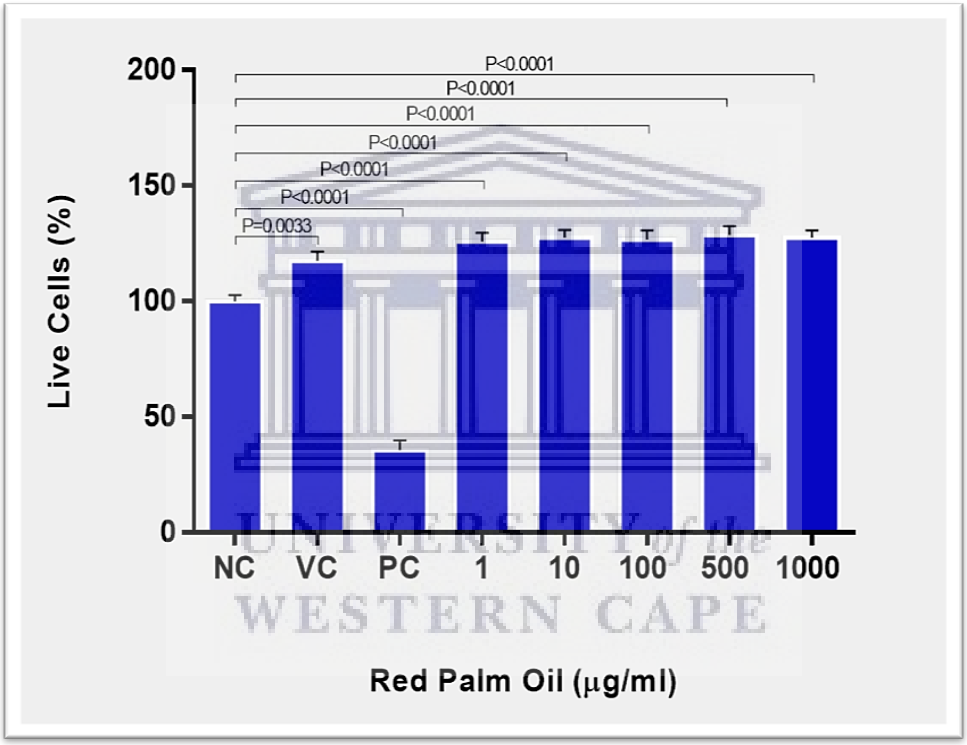

Figure 3.18: Dose-response effect on cell proliferation of MDA-MB-231 cells following 48 hours of exposure to red palm oil. Cells showed statistically significant ($p<0.0001$) increases in cell proliferation between the negative control (NC) and all concentrations of red palm oil tested. NC=Negative Control, VC=0.5% DMSO Vehicle Control and PC=10% DMSO Positive Control.

Following a 72-hour exposure to 1-1000 µg/ml of red palm oil (D-H), cell morphology between control groups (A-C) and experimental groups (D-H) were observed and recorded (Figure 3.19). The

experimental groups showed no obvious signs associated with cell stress or death such as cell shrinking and rounding between the lowest (D) and highest (H) concentrations with an unchanged morphology of their stellate pattern and spindle shape appearance consistent with the negative (A) and 0.5% DMSO vehicle control (VC) (B). The 10% positive control group (PC) (C) showed signs consistent with cell stress and cell death whereby cells appeared rounded and fewer cells were observed. The CCK-8 assay (Figure 3.20) showed statistically significant ($p=0.0015$, $p=0.0273$, $p=0.0323$, $p=0.007$ and $p=0.0438$) increases in cell proliferation between the negative control (NC) and all the concentrations of red palm oil tested. No statistically significant ($p<0.05$) increase in cell proliferation was observed between negative (NC) and 0.5% DMSO vehicle control (VC) while a statistically significant ($p<0.0001$) decrease in cell proliferation was seen between negative (NC) and 10% DMSO positive control (PC). Upon further analysis, one-way ANOVA showed no statistically significant ($p<0.05$) upward trend from the negative control and the highest concentration of red palm oil.

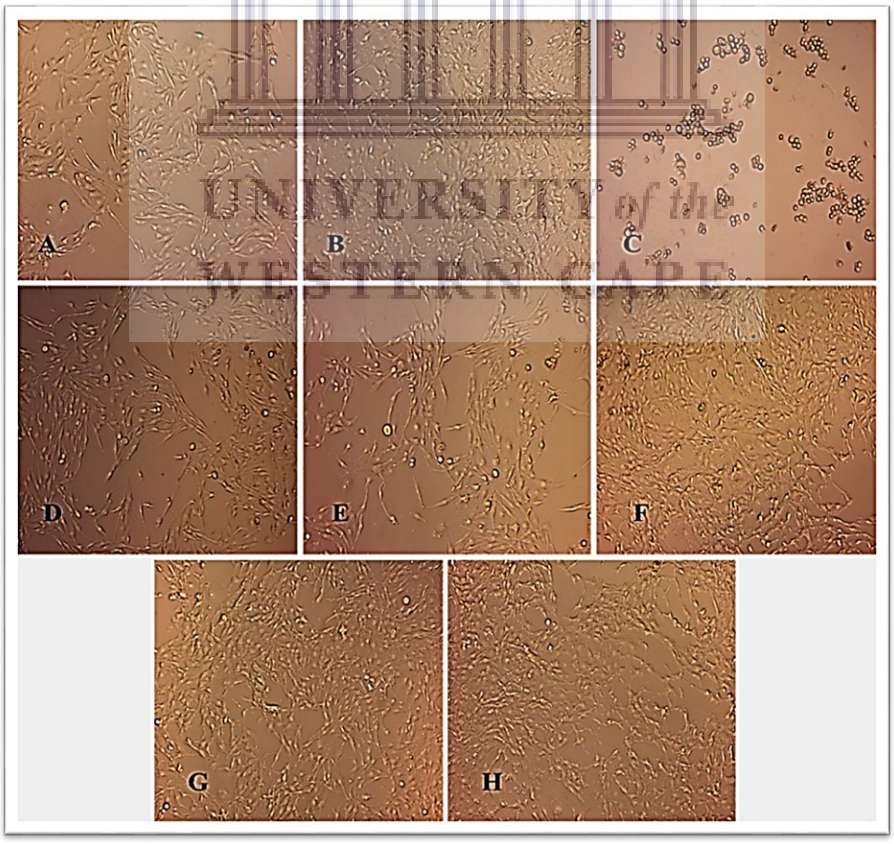

Figure 3.19: MDA-MB-231 cell morphology following treatment of 1-1000 µg/ml of red palm oil over 72 hours (D-H) in comparison to the control groups; Negative (A), vehicle (B) and

positive control (C) samples. Cell morphology apparently remained unchanged in each experimental group, thus marinating their stellate pattern and spindle shape. A change in morphology from spindle shape to rounding of cells were observed in the positive control (C) A=Negative Control (NC), B=0.5% DMSO Vehicle Control (VC), C=10% DMSO Positive Control (PC), D=1, E=10, F=100, G=500 and H=1000 µg/ml red palm oil. (Magnification: 40X, Carl Zeiss PrimoVert phase-contrast Microscope).

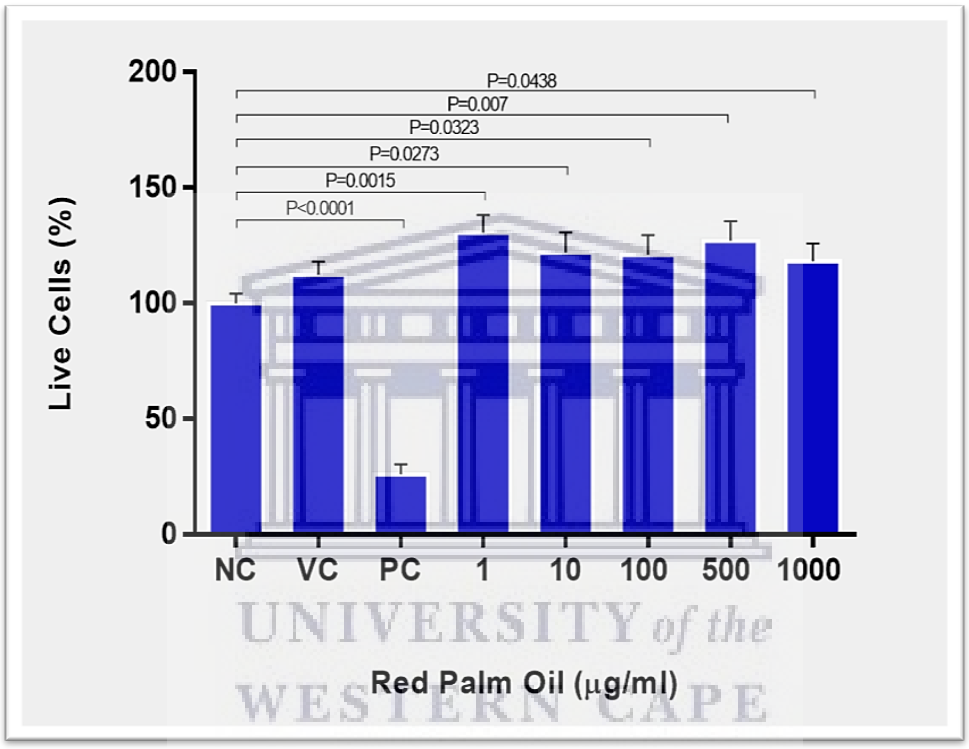

Figure 3.20: Dose-response effect on cell proliferation of MDA-MB-231 cells following 72 hours of exposure to red palm oil. Cells showed statistically significant (p=0.0015, p=0.0273, p=0.0323, p=0.007 and p=0.0438) increases in cell proliferation between the negative control (NC) and all the concentrations of red palm oil tested. NC=Negative Control, VC=0.5% DMSO Vehicle Control and PC=10% DMSO Positive Control.

Comparison of cell proliferation of MDA-MB-231 cells following 24, 48 and 72 hour incubations with 1-1000 µg/ml of red palm oil is shown in Figure 3.21. Statistically significant ($p<0.05$) increases in cell proliferation are indicated by asterisks (*) showing level of significance with specific p values shown in Figures 3.16, 3.18 and 3.20. * shows $p<0.05$, ** shows $p<0.01$, *** shows $p<0.001$ and **** shows

p<0.0001. Following a 24-hour exposure, an increase in cell proliferation was observed between the negative control (NC) and 1-1000 µg/ml treatment concentrations. Furthermore, a 48-hour exposure showed an increase in cell proliferation between the negative control (NC) and highest concentration with the 72-hour exposure to red palm oil following suit. An overall increase in cell proliferation was noticed between the negative control and red palm oil concentrations at each treatment.

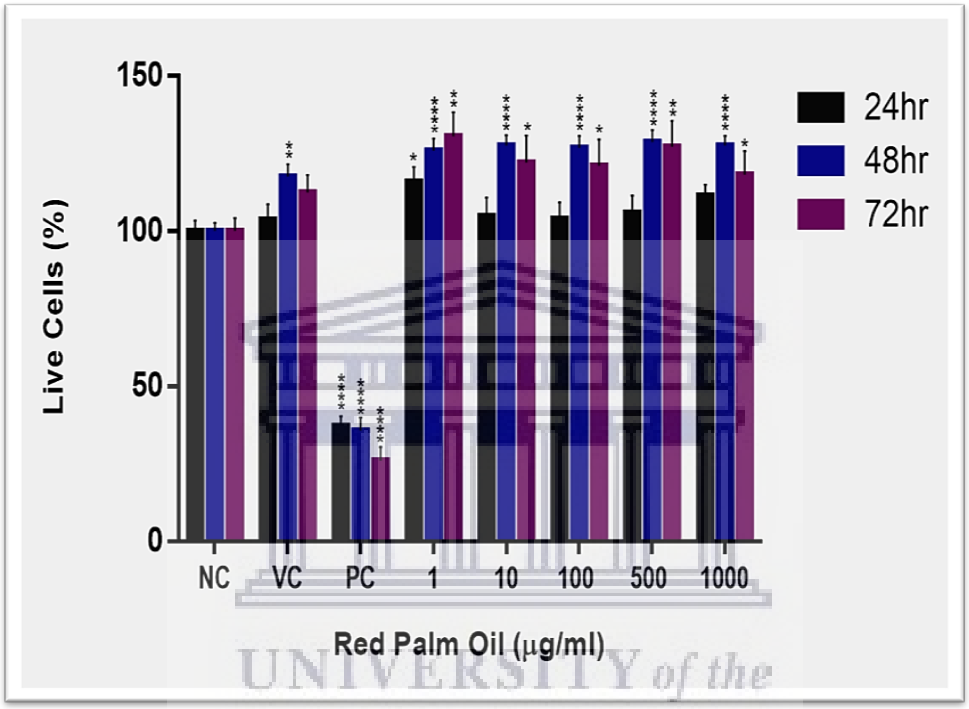

Figure 3.21: Compared cell proliferation of MDA-MB-231 cells following 24, 48 and 72 hour incubations with 1-1000 µg/ml of red palm oil. Statistically significant (p<0.05) increases in cell proliferation observed and shown by asterisks (*) where * = p<0.05, ** = p<0.01, *** = p<0.001 and **** = p<0.0001. NC=Negative Control, VC=0.5% DMSO Vehicle Control and PC=10% DMSO Positive Control.

3.3 Gene Expression Analysis of GAPDH, hMAM and Maspin Genes

3.3.1 GAPDH Gene Analysis

Following the PCR assay for GAPDH housekeeping gene with MCF-12A, MDA-MB-231 and MCF-7 samples, gel electrophoresis imaging was captured (Figure 3.22). The non-template controls (lanes 2,

10 and 18) showed no amplification indicating no contamination of any other genetic material. The untreated and treated samples of MCF-12A (lanes 3-9), MDA-MB-231 (lanes 11-17) and MCF-7 (lanes 19-25) showed gene expression of the intended target with a size of ~227 bp.

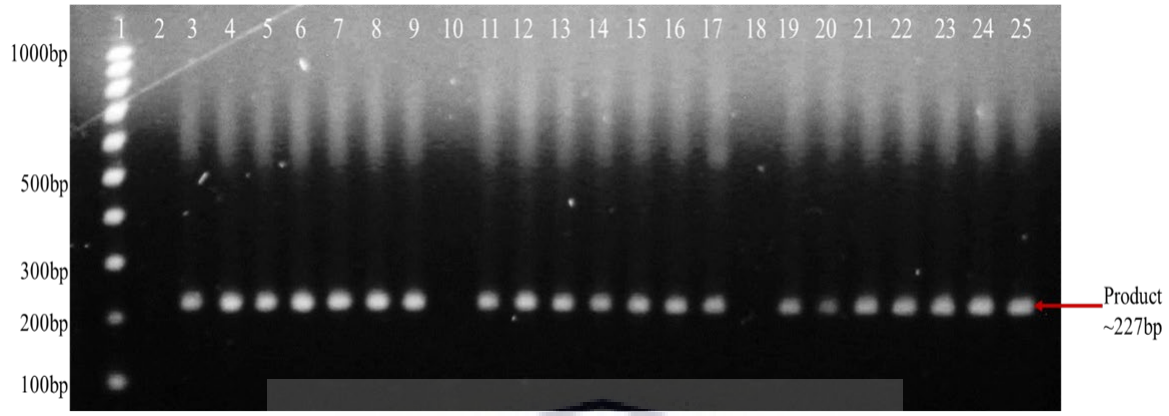

Figure 3.22: Gel electrophoresis imaging of GAPDH gene with MCF-12A, MDA-MB-231 and MCF-7 untreated and treated samples showing bands of intended target (~227 bp). Gene expression of GAPDH was observed in both treated and untreated samples of all 3 cell lines. Lanes 1-25 are as follows; Lane 1: molecular ladder, Lane 2: Non-template control, Lane 3; MCF-12A untreated, Lane 4-9: MCF-12A treated (T24 10, T24 100, T48 10, T48 100, T72 10 and T72 100 respectively), Lane 10: Non-template control, Lane 11: MDA-MB-231 untreated, Lane 12-17: MDA-MB-231 treated (T24 10, T24 100, T48 10, T48 100, T72 10 and T72 100 respectively), Lane 18: non template control, Lane 19: MCF-7 untreated, Lane 20-25: MCF-7 treated (T24 10, T24 100, T48 10, T48 100, T72 10 and T72 100 respectively). T24, T48 and T72 represent the number of hours the samples were treated, with 10 and 100 representing the concentrations (in µg/ml) at which the samples were treated.

3.3.2 hMAM gene Analysis

Gel electrophoresis imaging of the hMAM gene with MCF-12A, MDA-MB-231 and MCF-7 was captured (Figure 3.23). The non-template controls (lanes 2, 10 and 18) showed no gene expression indicative of a non-contaminated assay of other genetic material. The untreated and treated samples of MCF-12A (lanes 3-9) and 3 treated samples of MDA-MB-231 (lanes 13-15) showed gene expression of the intended target with a size of ~200 bp. However, lanes 22-25 of MCF-7 samples showed bands at different regions.

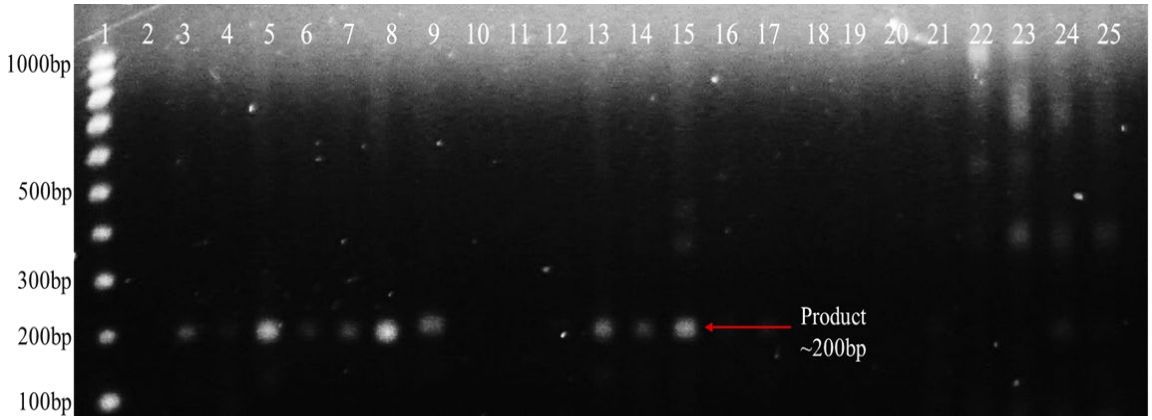

Figure 3.23: Gel electrophoresis imaging of hMAM gene with MCF-12A, MDA-MB-231 and MCF-7 untreated and treated samples. Gene expression of hMAM were observed in MCF-12A samples (lanes 3-9) and treated MDA-MB-231 samples (lanes 13-15) with target of interest at ~200 bp. Non-specific amplification was observed in the MCF-7 treated cells (lanes 22-25). Lanes 1-25 are as follows; Lane 1: 100 bp molecular ladder, Lane 2: Non-template control, Lane 3; MCF-12A untreated, Lane 4-9: MCF-12A treated (T24 10, T24 100, T48 10, T48 100, T72 10 and T72 100 respectively), Lane 10: Non-template control, Lane 11: MDA-MB-231 untreated, Lane 12-17: MDA-MB-231 treated (T24 10, T24 100, T48 10, T48 100, T72 10 and T72 100 respectively), Lane 18: non template control, Lane 19: MCF-7 untreated, Lane 20-25: MCF-7 treated (T24 10, T24 100, T48 10, T48 100, T72 10 and T72 100 respectively). T24, T48 and T72 represent the number of hours the samples were treated, with 10 and 100 representing the concentrations (in µg/ml) at which the samples were treated.

3.3.3 Maspin Gene Analysis

Gel electrophoresis imaging of the Maspin gene with MCF-12A, MDA-MB-231 and MCF-7 was captured (Figure 3.24). The non-template controls (lanes 2, 10 and 18) showed no gene expression which demonstrates that the assay was free from contamination of other genetic material. Gene expression was observed in the untreated and treated samples of MCF-12A (lanes 3-9) at the intended target of ~227 bp. No gene expression was observed in the MDA-MB-231 samples (lanes 11-17) as well as the MCF-7 samples (lanes 19-25).

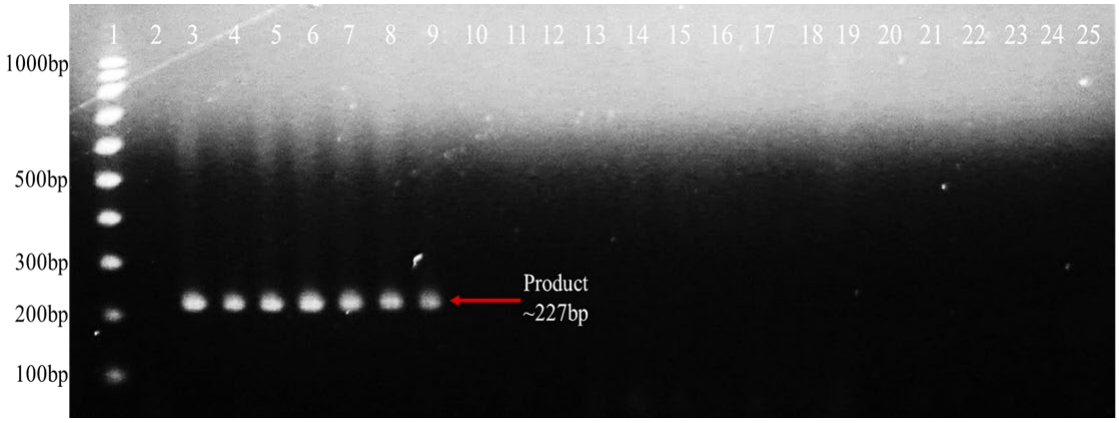

Figure 3.24: Gel electrophoresis imaging of Maspin gene with MCF-12A, MDA-MB-231 and MCF-7 untreated and treated samples. Gene expression of Maspin was observed in both treated and untreated MCF-12A samples with a product size of ~227 bp. No gene expression was observed in MDA-MB-231 and MCF-7 samples. Lanes 1-25 are as follows; Lane 1: 100 bp molecular ladder, Lane 2: Non-template control, Lane 3; MCF-12A untreated, Lane 4-9: MCF-12A treated (T24 10, T24 100, T48 10, T48 100, T72 10 and T72 100 respectively), Lane 10: Non-template control, Lane 11: MDA-MB-231 untreated, Lane 12-17: MDA-MB-231 treated (T24 10, T24 100, T48 10, T48 100, T72 10 and T72 100 respectively), Lane 18: non template control, Lane 19: MCF-7 untreated, Lane 20-25: MCF-7 treated (T24 10, T24 100, T48 10, T48 100, T72 10 and T72 100 respectively). T24, T48 and T72 represent the number of hours the samples were treated, with 10 and 100 representing the concentrations (in µg/ml) at which the samples were treated.

CHAPTER 4

DISCUSSION, CONCLUSIONS AND FUTURE PERSPECTIVES

4.1 Introduction

This chapter provides a brief background to the importance of the treatment of choice used in this study. The chapter focuses on the results obtained in Chapter 3 whereby (1): 3 cell lines were exposed to varying concentrations of red palm oil over 3 time periods where the concentrations were selected based on the percentage of oil as well as the DMSO present at non-cytotoxic concentrations and the time periods selected were based on the literature that indicated suitable time periods for the oil to exert effects, and (2): the genetic expression analyses of a breast epithelial marker (hMAM) and a marker of metastasis (Maspin) following treatment of red palm oil were observed. The ultimate aim was to determine whether red palm oil exerts anti-proliferative effects on the breast cancer cell lines studied.

4.2 Background

Globally, breast cancer continues to be a grave problem with an approximation of 1 in 8 females developing this disease during their lifetime (Ferrini *et al,* 2015). To date, it is known as the most commonly occurring cancer and the second leading cause of death of the various cancer forms (Ferrini *et al,* 2015; Momenimovahed and Salehiniya, 2019; Domeyer and Sergentanis, 2020). The rate of incidence as well as mortality is on the increase and the burden created by this harsh disease is expected to worsen in the years to come (Black and Richmond, 2019; Momenimovahed and Salehiniya, 2019). Additionally, studies have suggested that while breast cancer was prevalent in high income and developed countries, the mortality and incidence rates are rapidly increasing in lower- to middle-income countries which could be a result of various risk factors associated with breast cancer as well as the overall population growth and ageing (Torre *et al,* 2015; Rocha-Brischiliari *et al,* 2017; Black and Richmond, 2019).

While breast cancer comprises many subtypes, the triple-negative breast cancer subtype, or TNBC, is known to be the most aggressive of all and is linked to a greater mortality rate in comparison to any of the other subtypes. TNBC encompasses approximately 10–20% of all breast cancer cases and present more commonly in younger patients. The lack of ER, PR and HER2 creates a significant challenge for safe and effective treatment regimens (Uscanga-Perales *et al,* 2016; Pandy *et al,* 2019). Due to the lack of the aforementioned receptors, the treatment options are limited to radiotherapy, surgery and chemotherapy (Uscanga-Perales *et al,* 2016; Neophytou *et al,* 2018; Mehanna *et al,* 2019). Over the last few decades, chemotherapy has been regarded as one of the main therapeutic measures.

However, TNBC is associated with a greater risk of distant recurrence, an increase in probability of relapse, high rates of metastases and a poor overall survival. This dire outcome is generally due to metastases development in secondary organs which includes the brain, lungs and bones (Mustacchi and De Laurentiis, 2015; Neophytou *et al,* 2018; Mehanna *et al,* 2019). Furthermore, it is said that not more than 30% of patients survive more than 5 years with metastatic TNBC despite the initial favourable response to the chemotherapy and ultimately nearly all patients will succumb to this disease (Lehmann *et al,* 2011; Bianchini *et al,* 2016). Therefore, there is a growing urge in finding suitable and effective strategies that allow for the prevention of cancer or alternative therapeutic methods that can eradicate this disease and provide a better quality of life.

In recent research, naturally occurring compounds that possess potential health benefits have become desirable therapeutic targets for various ailments and diseases (Nesaretnam, 2008). There has been a growing interest in red palm oil and its potential anticancerous properties. In recent years, interest in palm oil has grown rapidly growing among and it is now one of 17 major oils being manufactured (Koushki *et al,* 2015). Palm oil is the most abundantly used vegetable oil today with the majority of usage being in various food products and cooking preparations (May and Nesaretnam, 2014; Gesteiro *et al,* 2019). In comparison to other oils, palm oil has the highest yield per unit of crop with an annual production of more than 50 million tonnes. Indonesia and Malaysia are known to be the leading manufacturers of this oil (Koushki *et al,* 2015; Loganathan *et al,* 2017).

Red palm oil, produced from the fruit of the *Elaeis guineensis* oil palm tree, obtains its red hue due to the presence of a high content in carotenoids such as beta carotene and lycopene (Cassiday, 2017; Gesteiro *et al,* 2019). Additionally, palm oil provides a rich source of various other phytonutrients which include the vitamin E isomers (tocotrienols and tocopherols), phytosterols, coenzyme Q_{10} and squalene (May and Nesaretnam, 2014; Shahidi and De Camargo, 2016). While these phytonutrients are considered the minor components of red palm oil constituting to a percentage less than 1, they exhibit health benefits that exceed their antioxidant functions. It is reported that the various phytonutrients, particularly carotenes and the vitamin E compounds, can provide anticancer activity (May and Nesaretnam, 2014; Loganathan *et al,* 2017).

A vast array of studies suggest red palm is a suitable candidate for targeting breast cancer, specifically triple-negative breast cancer, based on the possible anticancer properties of the various components of the oil (Edem, 2002; Comitato *et al,* 2009; Sekaran *et al,* 2010; May and Nesaretnam, 2014; Tran *et al,* 2015). There is a growing need to find a preventative or a safe therapeutic agent for cancer patients. Therefore, this study looked at the effect of red palm oil on breast cancer cells as well as its effect on normal breast epithelial cells. Furthermore, this study sought to understand how red palm oil influences the expression of genes that are associated with breast cancer and metastases.

4.3 Impact of Red Palm Oil on Cell Proliferation

This study looked at the effects of red palm oil at varying concentrations on breast cancer cells MCF-7 and transformed MDA-MB-231 as well as a normal immortalised cell line, MCF-12A. The cell morphology of all 3 cell lines were observed following treatment of red palm oil over 24, 48 and 72 hours and the cell proliferation was evaluated through the CCK-8 cell proliferation assay with treatment concentrations ranging from 1-1000 µg/ml (D=1, E=10, F=100, G=500 and H=1000 µg/ml). Three control groups were added for each assay; a negative control (NC), a vehicle control containing 0.5% DMSO (VC) and a 10% DMSO positive control (PC). When MCF-12A cells were exposed to red palm oil, apparently no change in cellular morphology was observed in any of the treatment groups (group D-H) at 24, 48 and 72 hours (Figures 3.1, 3.3, and 3.5, respectively).

The positive control (group C) which contained a high, possibly cytotoxic, dose of DMSO showed signs associated with cell death or apoptosis such as cell rounding and fewer cells were observed (Elmore, 2007; Niranjana *et al,* 2015). MCF-12A cells exhibited their typical flat and polygonal appearance characteristic to their typical luminal epithelial phenotype. In comparison, the same observations were made in MCF-7 and MDA-MB-231 breast cancer cells. MCF-7 cells maintained its flat and polygonal shape (Figures 3.8, 3.10 and 3.12) while MDA-MB-231 cells exhibited their spindle shape and stellate pattern (Figures 3.15, 3.17 and 3.19). No signs, consistent with cell death or cell stress such as cell rounding, in any of the cell lines with any of the treatment concentrations over the 24, 48 and 72 hour treatment periods were observed.

Upon further investigation, the CCK-8 cell proliferation assay, revealed significant increases in MCF-12A cell proliferation between the negative control and the highest concentration of 1000 µg/ml at 24, 48 and 72 hours (Figures 3.2, 3.4 and 3.6, respectively). Significant increases in cell proliferation were also observed at lower concentrations with the exception of 100 µg/ml for 24 hours as well as 10 and 100 µg/ml for 48 and 72 hours. One-way ANOVA showed a significant upward trend between the negative control and 1000 µg/ml at 48 and 72 hours, but not at 24 hours. A comparison of each treatment period can be collectively seen in Figure 3.7. These findings suggest that red palm oil does not cause inhibition of cell proliferation in normal non-cancerous cells but rather a potential stimulatory effect on MCF-12A cells.

Most studies look at the effect of individual components of red palm oil on normal breast epithelial cells similar to MCF-12A and found that these cells were unaffected by the individual palm oil treatments (Patacsil *et al,* 2012; Parajuli *et al,* 2015; Tran *et al,* 2015). While normal breast epithelial cells are unaffected by the red palm oil compounds, the current study shows an increase in cell proliferation which could be potentially explained by the whole palm oil being unable to work synergistically and producing an antagonistic effect resulting in the proliferation of cells (Loganathan *et al,* 2013). The effects of red palm oil on MCF-12A cells remain novel and require further investigation.

Similar findings were observed in the transformed MDA-MB-231 cancer cells to that of MCF-12A cells. Although a significant increase in proliferation of 31% was observed in 1 µg/ml in comparison to the negative control, no other significant increases in proliferation for cells exposed to higher concentrations were observed at the 24-hour treatment (Figure 3.16). Furthermore, no significant increase in cell proliferation was found between the 1000 µg/ml concentration and the negative control. In summary, increases in cell proliferation were observed in MDA-MB-231 cells with every treatment concentration (1–1000 µg/ml) after 48 and 72 hours (Figures 3.18 and 3.20, respectively), with a statistically significant upward trend observed in the 48 hour treatment and no statistically significant upward trend observed in the 72-hour treatment between the negative control and the highest concentration.

Overall, the 48-hour treatment shows the highest percentage proliferation among the chosen time periods as seen in Figure 3.21. Therefore, we conclude that the cell growth of MDA-MB-231 was not inhibited by the red palm oil. Other studies show a widespread of evidence in which individual components of red palm oil is capable of producing an inhibitory effect on MDA-MB-231 cell proliferation. These studies used individual compounds of the oil such as tocopherols and tocotrienols and found a decrease in cell proliferation (Hsieh *et al,* 2010; Tran *et al,* 2015; Idriss *et al,* 2020). However, a study conducted by Prakash *et al,* showed an increase in MDA-MB-231 cell proliferation when using beta-carotene from red palm oil (Prakash *et al,* 2001).

The findings by Prakash *et al,* could possibly explain the result seen in the current study where MDA-MB-231 cells increased in cell proliferation possibly due to the beta-carotene present in the whole red palm oil which may suppress the inhibitory effects associated with the vitamin E component present in the oil. However, the mode of action in which the whole red palm oil produces any effects is not yet known and requires further research. Additionally, due to an increase in proliferation of the aggressive MDA-MB-231 cells, the whole red palm oil would not be a beneficial preventative or treatment option for the MDA-MB-231 cancer cells.

In contrast, MCF-7 cancer cells exposed to red palm oil for 24 hours (Figure 3.9) showed *significant decreases in cell proliferation* in every treatment concentration in comparison to the negative control. Furthermore, the treatment concentration of 500 µg/ml showed an 18% reduction of cell proliferation, which was the highest percentage decrease in cell proliferation after 24-hour exposure. One-way ANOVA revealed a significant downward trend between the negative control and highest treatment concentration. Additionally, similar decreases in cell proliferation were observed after the 72 hour incubation period, but 100 µg/ml showed a 21% decrease in cell proliferation, which was the highest percentage decrease in cell proliferation after 72-hour exposure (Figure 3.13).

However, MCF-7 cells exposed for 48 hours (Figure 3.11) showed no significant increases in cell proliferation between the negative control and any treatment concentrations of red palm oil. Furthermore, Figure 3.14 shows the quantification of cell proliferation combined where MCF-7 cells show a decline in cell proliferation after 24 hours followed by an increase in cell proliferation after 48 hours and another decline in cell proliferation after 72 hours with a decrease more prominent at *concentrations of 10, 100 and 500 µg/ml*. Overall, these results obtained in the current study could potentially be explained through the vast array of studies using various isolated compounds found in red palm oil to target the cancerous cells instead of using the oil as a collective compound.

Sekaran *et al* conducted a study on the effects of oil palm phenolics on MCF-7 cells. The oil palm phenolics (OPP) are water-soluble and are mostly made up of phenolic acids which were isolated from palm oil. 200, 500, 1000, 1500 and 2000 µg/ml treatment concentrations were used. They found a dose-dependent decrease in the MCF-7 cell line showing the distinct inhibitory effect on cell proliferation. This decrease began after 24 hour incubation with a prominent decrease in the proliferation at the last incubation period, similarly to the results seen in the current study however not in a dose dependent manner. Furthermore, the normal control was unaffected indicating no cytotoxic effects of OPP on normal cells whereas the red palm oil caused a 10% increase in MCF-12A cell proliferation after 24 hours and a 17% increase in cell proliferation at the last incubation period at the highest treatment concentration observed in the present study (Sekaran *et al,* 2010).

Other red palm oil compounds such as the tocotrienol-rich or TRF containing both tocotrienols and tocopherols have been noted to exhibit great activity through inhibiting cell proliferation. A study conducted by Tran *et al* sought to demonstrate the effect of tocotrienols and tocopherols (denoted as Tocomin® in their study) in combination on MDA-MB-231 and MCF-7 cancer cells. They observed that the cell proliferation of both cell lines was significantly decreased when cells obtained treatment concentrations of 80 and 100 µg/ml after 24 hours. No cytotoxic effects were observed in the control cell line (Tran *et al,* 2015). In the current study, MCF-7 cells also showed a decrease in cell proliferation with a 13% reduction when cells were exposed to 100 µg/ml for 24 hours. While the results of the current study also demonstrates the decrease in proliferation in MCF-7 cells when using the whole red palm oil, the opposite was observed in MDA-MB-231 cells whereby an increase of 4% in cell proliferation was observed at 100 µg/ml after 24 hours. Additionally, an increase of 6% in cell proliferation of the normal MCF-12A cells was also observed.

In another study, Ramdas *et al* demonstrated the effects of TRF, individual tocotrienol isomers (alpha, delta- and gamma-) as well as alpha-tocopherol on breast cancer cells. Ramdas *et al* found that the tocotrienol isomers inhibited the proliferation of MCF-7 cells while alpha-tocopherol exerted no effects below the 20 µg/ml concentration and higher concentrations were needed to exhibit the same decrease in cell proliferation in comparison to the different tocotrienols. The TRF had greater potency than gamma- and delta- tocotrienol but were less potent than alpha-tocotrienol (Ramdas *et al,* 2011). Ramdas *et al* proved that low dosages were capable of inhibiting the cell proliferation of MCF-7; the current study found that the whole red palm oil was also capable of producing anti-proliferative effects on MCF-7 cells at a low dosage. In the current study, decreases in cell proliferation of MCF-7 of 14 and 17% were seen at a low treatment concentration of 10 µg/ml for 24 and 72 hours respectively.

Numerous other studies using just individual vitamin E isomers were found with a vast majority looking at the effects of gamma-tocotrienol. Parajuli *et al* looked at the effects of both gamma-tocotrienol and alpha-tocopherol on MCF-7 cells. The isomers were diluted using absolute ethanol followed by suspension in sterile 10% bovine serum albumin. Treatment concentrations of 1–10 µM were used for

gamma-tocotrienol and 20–100 μM for alpha-tocopherol. A significant decrease in the cell numbers was observed in the 6–8 μM concentration of gamma-tocotrienol on the MCF-7 cells in comparison to the control group. At 7.8 μM gamma-tocotrienol treatment, 50% inhibition was observed. No significant effects were observed on the control MCF-10A cell line showing that this compound was not toxic to normal cells. However, the same analogy was made for the alpha-tocopherol treatment further confirming that lower doses of alpha-tocopherol could not exhibit a great inhibitory effect (Parajuli *et al,* 2015). In the current study, MCF-7 cell proliferation was significantly decreased with the whole red palm oil containing tocopherol and tocotrienol. However, the control cell line showed an increase in cell proliferation which indicates a resistance to the growth inhibitory effects of the compounds present in the red palm oil.

A study by Patacsil *et al* compared the effects of gamma-tocotrienol to alpha-tocotrienol on MDA-MB-231 cells as well as MCF-7 cells. The tocotrienols were diluted in DMSO followed by treatment concentration preparation ranging from 10–40 μM. Both tocotrienols exerted effects on the cancerous cell lines but gamma-tocotrienol exerted a slightly more outspoken inhibitory effect on both MCF-7 and MDA-MB-231 cells. Their findings indicate that inhibition through tocotrienol treatment on breast cancer cells are independent of ER and p53 status which could affect the sensitivity to treatment in various cancer cells (Patacsil *et al,* 2012). In contrast, the MDA-MB-231 cells showed an increase in cell proliferation whereas MCF-7 cell proliferation was decreased in the current study when the whole oil was used as a treatment. This implies that the differences in cellular response could be due to the mode of action through which red palm oil exerts its effects.

In a more recent study, Idriss *et al* compared beta-tocotrienol to gamma-tocotrienol using MDA-MB-231 cells and MCF-7 cells to compare the effect of both compounds on the two different cell lines. Cells were treated with 10–50 μM doses of beta- and gamma-tocotrienol treatment for 24 and 48 hours. The results show a dose-dependent and time-dependent decline in cell proliferation in both breast cancer cell lines. However, beta-tocotrienol exerted a greater effect than gamma-tocotrienol. The 50% inhibitory effect (IC_{50}) and thus potency was also lower for beta-tocotrienol than for gamma-tocotrienol

at 24 and 48 hours, indicating a faster anti-proliferation effect. The same observation was made for MCF-7 cells between the two treatment groups, but the MDA-MB-231 cells were seen to be more susceptible to the beta-tocotrienol group (Idriss *et al,* 2020). In contrast, the current study showed an increase in cell proliferation of MDA-MB-231 cells regardless of dosage after 24 and 48 hours, further indicating no sensitivity to the red palm oil treatment. While a decrease in cell proliferation of MCF-7 cells was seen in the present study, the effect occurred in a manner that was not dose dependent.

Hsieh *et al* undertook a study to compare alpha-, delta- and gamma-tocotrienols to each other and these compounds were either diluted in ethanol or DMSO. MCF-7 and MDA-MB-231 cells were exposed to 1, 5 and 10 μM concentrations for each treatment group for a 72-hour period. They found that of the 3 compounds, delta- and gamma-tocotrienol showed a significant inhibition in cellular growth of both cell lines in the treatment concentration of 10 μM in comparison to alpha-tocotrienol which was also demonstrated in a study mentioned previously. However, all 3 compounds exerted inhibitory effects on the cancerous cell lines further confirming that tocotrienols are capable of exhibiting effects independent of estrogen status (Hsieh *et al,* 2010). In the present study, MCF-7 cells showed a decrease of 9% in cell proliferation at the highest red palm oil treatment after 72-hour exposure whereas MDA-MB-231 cells showed an increase of 18% in cell proliferation which showed the compounds present in the whole red palm oil cannot produce the same inhibitory effects between the two distinct cell lines.

While the vitamin E isomers have vastly demonstrated inhibitory potential, other studies focused on the effects of compounds such as carotenes and lycopene as a potential therapeutic agent in targeting breast cancer cells. Ng *et al* conducted a comparative study of the effect of carotenoids on both estrogen dependent MCF-7 and triple-negative MDA-MB-231 cells. They treated the cells with concentrations of 10^{-7} M and 10^{-6} M for 3 days and found that proliferation of MCF-7 cells was significantly inhibited with the higher carotenoid treatment. In contrast, the carotenoid concentrations showed no inhibitory effects on the MDA-MB-231 cells which could possibly be explained due to its ER status. Furthermore, carotenoids can be converted to vitamin A which could target estrogen dependent MCF-7 cells and not MDA-MB-231 cells (Ng *et al,* 2000).

A study by Prakash *et al* further demonstrated the effects of beta-carotene on these breast cancer cells in comparison to lycopene as well. The compounds were diluted in tetrahydrofuran and treatment concentrations did not exceed 0.1% of this solvent when the treatment concentrations were prepared at 1, 3, 7, 10 and 20 µM. Prakash *et al* found that the lycopene treatment was capable of inhibiting both MCF-7 and MDA-MB-231 cell growth. Additionally, they found a significant reduction in the growth of MCF-7 cells with beta-carotene treatment. However, MDA-MB-231 cells showed a significant increase in cell proliferation with the beta-carotene treatment (Prakash *et al,* 2001). A similar observation was made in the current study whereby the MDA-MB-231 cells showed an increase in cell growth with the red palm oil. The current study showed a decrease in MCF-7 cell proliferation of 14 and 9% for 24 and 72 hours respectively when the highest concentration of palm oil was applied, while an increase in MDA-MB-231 cell proliferation of 11, 27 and 18% for 24, 48 and 72 hours respectively were observed. These findings imply that estrogen is not an essential factor in its therapeutic role in these cells and the compounds found in the whole palm oil when used together does not produce the same inhibitory effects on MDA-MB-231 cells (Prakash *et al,* 2001).

A similar study was later conducted by Gloria *et al*. Beta-carotene and lycopene concentrations of 0.5–10 µM were tested on MCF-7 and MDA-MB-231 cells. They demonstrated that both the MCF-7 and MDA-MB-231 cell number was significantly decreased at the highest concentration of beta-carotene but the effect was much greater after 24 hours for MCF-7 in comparison to its 96-hour period. The decrease in MDA-MB-231 cell number was similar for both time points. Furthermore, they too found a reduction in cell proliferation with lycopene treatment. Here they reported that lycopene treatment was greater after 96 hours for both cell lines indicating that lycopene works in a time-dependent manner (Gloria *et al,* 2014). In the current study, after 48-hour treatment at higher treatment concentrations, MDA-MB-231 showed the highest overall increase in cell proliferation with increases of 18-30% at varying concentrations of red palm oil. The findings of the current study further suggest that the whole palm oil with all its components cannot produce growth inhibitory effects in comparison to individual compounds. While compounds of red palm oil showed outspoken anti-proliferative activity on breast cancer cells, various studies also demonstrated the effects of these compounds on other cancer types,

including colon, liver, lung, melanoma, pancreatic and prostate cancer.

In a study conducted by Xu *et al,* the effects of gamma-tocotrienol on HT-29 human colon adenocarcinoma cells were determined. The gamma-tocotrienol was prepared in ethanol not exceeding 0.15% and treatment concentrations of 15, 30, 45 and 60 µM were used. They observed a decrease in cell proliferation by 26.8%, 52.3%, 64.2% and 68.4% as the treatment concentration increased at the 48-hour time point. For 24, 48 and 72 hours, the 30 µM concentration showed a decrease in cell proliferation with time. They noted that 31.7 µM inhibited 50% of cell proliferation. Furthermore, they demonstrated notable changes in the cells morphology whereby the cell showed shrinkage and rounding, chromatin condensation and apoptotic bodies in accordance with apoptosis at the treatment concentration of 60 µM for 48 hours. They concluded that treatment concentrations of 30-60 µM of gamma-tocotrienol demonstrated significant inhibition of HT-29 cell proliferation in a dose-dependent and time-dependent treatment plan (Xu *et al,* 2009).

Qureshi *et al* conducted a study on liver cancer cells and the effect of delta-tocotrienol on them. The treatment concentrations of delta-tocotrienol used were 2.5, 5, 10, 20, 40 and 80 µM for a 48 hour period. They observed anti-proliferative activity at 5 µM, but noted that the most effective doses were between 20–80 µM with greater than 50% decrease in proliferation. They reported that delta-tocotrienol were highly effective and stimulated apoptosis (Qureshi *et al,* 2018). The cause of apoptosis in liver cells was suggested to be the result of a down regulation in the expression of proteasomal subunits and TNF-α following delta-tocotrienol treatment which could potentially be a good preventative or therapeutic agent for liver cancer (Abdullah and Atia, 2018; Qureshi *et al,* 2018).

Another study was conducted on the effects of OPP. Ji *et al* determined its effects on pancreatic cancer cell lines, PANC-1 and BxPC-3. Cells were treated with varying concentrations of OPP ranging from 20-50 µl/ml for 72 hours. They observed that an increase in treatment dosage resulted in a decrease of cell proliferation in both cancer cell lines. A higher cell inhibition percentage was observed in PANC-1 in comparison to BxPC-3 as the doses increased. The inhibition percentage for BxPC-3 were 12%,

30%, 54% and 75% whereas PANC-1 demonstrated percentages of 35%, 51%, 75% and 91% with the increase in the treatment concentrations (Ji *et al,* 2015). In the study by Sekaran *et al,* OPP was also tested on human lung carcinoma cells (A549). They found a decrease in cell proliferation as the amount of treatment days increased. Cells were less viable and proliferative at greater doses of OPP with a maximum inhibition observed at the highest treatment dosage. The effect of OPP on these cells were both time and dose dependent. OPP had no inhibitory effects on normal non-cancerous cells (Sekaran *et al,* 2010).

A study conducted by Lim *et al* tested the effects of beta-tocotrienol on A549 cancer cells. These cells were treated with concentrations of 1-100 μM for 24, 48 and 72 hours. They reported that beta-tocotrienol caused a significantly inhibitory effect on the cancer cells in a dose and time dependent manner. The increase in dosage directly decreased the cell number in cancer cells but not in a non-cancerous cell line. (Lim *et al,* 2014). Individual compounds of red palm oil has shown no inhibitory or stimulatory effects on other non-cancerous cell lines while the present study shows a possible stimulatory effect on normal breast epithelial cells when the whole red palm oil was used.

In the study by Qureshi *et al* as previously mentioned, the effect of delta-tocotrienol on lung cancer cells were also observed. They found that smaller doses ranging from 2.5–10 μM were capable of cell inhibition greater than 50% when compared to its control which was also similarly noted in other cancerous cell lines. The capability of inhibiting cell proliferation at low dosages further supports these tocotrienols as potent anti-proliferative compounds (Qureshi *et al,* 2018). Teodoro *et al* also looked at the effects of lycopenes on A549 cells with treatment concentrations ranging from 1–5 μM for 48 and 96 hours. They demonstrated that after 48 hours no significant inhibition of cell proliferation had occurred but after 96 hours they witnessed a significant decrease in cell growth which shows lycopene to inhibit growth in a time dependent manner (Teodoro *et al,* 2012).

Marelli *et al* undertook a study to determine the effects of delta-tocotrienol on two human melanoma cell lines, namely A375 and BLM. Normal human melanocytes were used as the control and the anti-

proliferative effects for all 3 cell lines were tested. These cells were incubated for 24 and 48 hours with treatment concentration ranging from 5–20 µg/ml. They found that delta-tocotrienol inhibited proliferation in a dose dependent manner at both time periods. 10-20 µg/ml treatment concentration proved to be the most prominent in this inhibitory effect. Moreover, no inhibitory effects were observed in the normal melanocytes (Marelli *et al,* 2016). The inhibitory effects of delta-tocotrienol were also confirmed in the study conducted by Qureshi *et al.* They too found that delta-tocotrienol was highly potent and effective on these cells and were capable of inhibiting more than 50% of cell growth at low concentrations (Qureshi *et al,* 2018).

Yap *et al* conducted a study to demonstrate the effects of the various vitamin E isomers on prostate cancer cells using the androgen independent PC-3 cell line and the androgen dependent LNCaP cell line in comparison to PZ-HPV-7 immortalised cells. All 3 cell lines were treated with the various isomers at concentrations of 20, 40, 60, 80 and 100 µM. The isomers exerted no effects on the immortalised cell line while a significant suppression of cell growth was detected in both PC-3 and LNCaP cells. Toctotrienols, gamma-tocotrienol in particular, exhibited greater effects on the PC-3 cells in a time and dose dependent manner while delta-tocotrienol was found to be much more potent for LNCaP cells. The order of potency of the various isomers ranging from lower potency to higher potency is beta-tocopherol ≈ alpha-tocopherol ≈ alpha-tocotrienol ≈ delta-tocopherol < gamma-tocopherol < beta-tocotrienol < delta-tocotrienol < gamma-tocotrienol (Yap *et al,* 2008).

The abovementioned studies provide some insight into the effects of individual components of palm oil on various cancer cells as there is a scarcity of research on the effects of the whole red palm oil. Based on the aforementioned studies, there is an accumulation of evidence that supports individual components isolated from red palm oil that causes inhibition of cancer cell growth in various cell lines without causing cell death to normal immortalised cells. However, the present study demonstrated a stimulatory effect on the normal MCF-12A cell line and the lack of inhibitory effects using red palm oil with all its phytonutrients on the triple-negative MDA-MB-231 cell line.

In an extensive study conducted by Loganathan *et al,* a similar observation was made on the MDA-MB-231 cells. Loganathan and colleagues tested out MCF-7 and MDA-MB-231 cancer cell lines using various individual compounds isolated from red palm oil and compared that to a combined phytonutrient mixture. The individual compounds tested were TRF, alpha-tocopherol, tocotrienols (alpha-, delta- and gamma-), coenzyme Q10, squalene and carotenoid complex. The phytonutrient mixture tested had a combination of vitamin E, coenzyme Q10, squalene, carotenes, phospholipids and polyphenols much like the red palm oil that was used in this study. They reported that the individual compounds produced much higher levels of anti-proliferative effects in comparison to the combined phytonutrient mixture with little to no effects on the cancerous cells (Loganathan *et al,* 2013).

They also demonstrated that the TRF and its isomers caused the complete suppression of proliferation in both cell lines in a dose dependent manner. In contrast, the other individual compounds such as coenzyme Q_{10}, squalene and carotenoids were unable to completely suppress cell growth which further demonstrates that tocotrienols are the vital components in anti-proliferative activity. They noted that there have been no reports of synergism between palm oil vitamin E and the other phytonutrients which could explain the lack of inhibitory effects on MDA-MB-231 cell proliferation seen in the present study. Furthermore, Loganathan *et al* combined tocotrienols with individual compounds of coenzyme Q_{10}, squalene and carotenoids, and found a weaker anti-proliferation pattern in comparison to tocotrienols on their own or that of the phytonutrients itself, i.e., each compound exhibited better anti-proliferation activity individually than in combination. The difference in the inhibitory effects implies that each individual compound uses different mechanisms to obtain a decrease in cell proliferation with pathways being competitive and antagonistic (Loganathan *et al,* 2013).

A possible explanation for the difference in inbitory effects lies in mechanisms of the cell cycle. In a combined-agent treatment it is likely that the compounds modulate alternative genes or pathways that may influence gene products that are involved in cell proliferation and cell death. Another possible explanation could involve the direct interaction of receptors. A different or complementary binding site would be required for an efficient binding of combined treatment compounds to produce a great effect.

If common binding sites of the compounds are shared, this could lead to compound competition and the inhibition of treatments resulting in anti-proliferative effects not being achieved (Loganathan *et al,* 2013).

These findings provide a potential explanation as to why red palm oil was unable to produce an inhibitory effect on the triple-negative breast cancer cell line MDA-MB-231 while causing an inhibitory effect on MCF-7 cells. The MCF-7 cells may be responsive to lower percentages of the active compounds (such as tocotrienols, tocopherols and carotenes) present in the whole red palm oil while MDA-MB-231 cells may require much higher percentages of these compounds in order to achieve a decrease in cell proliferation. Additionally, the normal cell line, MCF-12A had a similar response to the red palm oil as the triple-negative cancer cell line. The red palm oil could potentially cause the stimulatory effect on the two cell lines through the same mode of action. The mode of action through which red palm oil was able to produce an inhibitory effect on MCF-7cells while causing an increase in MDA-MB-231 cells and the normal MCF-12A cells, need to be investigated.

4.4 Influence of Red Palm Oil on Gene Expression

The precise molecular mechanism through which red palm oil exhibits its renowned inhibitory activity as well as the role it plays in breast cancer and its subtypes is not yet known. The consequences associated with breast cancer, particularly triple-negative breast cancer, stems from a multitude of factors which include insufficient methods for early diagnosis and prognosis, management of the tumours due to its aggressive nature, high rate of metastases to visceral organs, as well as the limitation in target therapies available for TNBC patients which subsequently leads to death. It is imperative to obtain biomarkers that are able to identify TNBC reliably and efficiently as these biomarkers may be a beneficial tool in prognosis or as a predictive indicator of TNBC. Furthermore, these biomarkers may also provide possible targets for therapy (Yadav *et al,* 2015; Bao *et al,* 2019). In recent research, a plethora of biomarkers have been affiliated with triple-negative cancer. However, with regard to gene expression very few have been compared. Some of these biomarkers have been validated with promising results in independent series, but others remain controversial (Ilie *et al,* 2018; da Silva *et al,*

2020). Therefore, there is a substantial need to research how possible therapeutic agents such as red palm oil may influence the genetic expression of various genes that are associated with breast cancer development.

In this study, following the administration of red palm oil, the mRNA expression levels of the genes of interest, hMAM and Maspin, as well as the GAPDH housekeeping gene were determined. A reference or housekeeping gene ideally is expressed at medium to high levels in order to make detection easy, shows consistent expression levels in different cell types as well as showing expression within the same cell type even while undergoing various treatments or experimental parameters and is generally used to test the cDNA quality (Zainuddin *et al,* 2010; Caracausi *et al,* 2017; Nguyen and Dao, 2019). The molecular analysis of GAPDH in this study showed that this housekeeping gene adhered to its standard criteria whereby expression was observed between normal non-cancerous cells as well as the 2 cancerous cell types. Furthermore, expression was also observed between the varying treatment concentrations of red palm oil. Following the analysis of the reference gene, the 2 targets of interest, hMAM and Maspin were explored and examined.

Studies suggest that a higher level of hMAM gene expression is observed in cancerous breast cells in comparison to normal and healthy breast cells (Al Joudi, 2014; Monsalve-Lancheros *et al,* 2019). However, in this study there was a notable difference in the expression between the normal and cancerous cell types. Gene expression of the mRNA between MCF-12A and MDA-MB-231 cells were visually similar. In MCF-12A cells, expression was observed in the untreated control cell as well as the cells treated with varying concentrations of red palm oil. In contrast, in MDA-MB-231 cells no expression was observed in the untreated control cell but observable expression in cells treated with 3 different treatment concentrations of the oil. Additionally, gene expression of MCF-7 cells showed some non-specificity and no expression at the intended target. The lack of hMAM expression in the cancerous cell types could potentially suggest that red palm oil may have exerted an effect possibly down-regulating the hMAM gene. However, these molecular results require further investigation through quantitative experimentation using quantitative real-time PCR (qRT-PCR) in order to

determine an appropriate cut-off value for expression of cancerous and non-cancerous cells before making a final deduction of the role of hMAM with a red palm oil treatment.

Several studies have investigated hMAM as a molecular marker for breast cancer and the development of targeted therapeutic tools. Koh *et al* investigated the role of hMAM in relation to migration and invasion of cancer cells. It was reported that MCF-7 and MDA-MB-231 breast cancer cells exhibited higher expression levels in comparison to normal MCF-10A cells which subsequently lead to a decrease in migration and invasion in breast cancer cells while the inverse was observed in normal cells. These findings propose that metastasis can be regulated through the expression of hMAM (Koh *et al,* 2014).

In another study, Nguyen and colleague examined the expression of hMAM on 4 cancer cell lines such as MDA-MB-231, MCF-7, BT-474 and KPL-4. It was found that MDA-MB-231 did not express hMAM whereas the other 3 cell types showed expression. Through RT-PCR and Northern Blot assays, the detection of hMAM was observed in 90% malignant tissue, whereas little to no detection was observed in benign or healthy tissue. Their study also showed no significant statistical differences in the expression of hMAM with regards to distant metastasis, tumour size or the stage of the disease (Nguyen and Dao, 2019). Similarly, research conducted by Al Joudi, noted that in breast cancer the rate of detection of hMAM mRNA expression was unrelated to familial history of breast cancer, tumour size and the presence of ER, PR and HER2 receptors. Furthermore, while secretoglobins are generally known to be modulated by steroid hormones, it was deduced that expression levels of hMAM is not induced by breast cancer cell lines that are ER positive such as MCF-7 which implies that hMAM is nearly solely expressed independently of steroid hormones in the mammary gland (Al Joudi, 2014). This gives rise to a promising biomarker especially for TNBC due to its hormonal status. However, further research into understanding the mechanism and role of this gene is warranted.

Studies indicate that a decrease in Maspin production level, specifically in breast cancer, leads to the progression and its transition from non-invasive cancer to invasive cancer. In a study conducted by Machowska *et al,* it was demonstrated that Maspin showed no expression in cancerous cells such as

MDA-MB-231 and MCF-7 whereas expression was seen in normal MCF-10A cells (Machowska *et al,* 2014). In the present study, the mRNA expression of Maspin in normal MCF-12A, MDA-MB-231 and MCF-7 cancer cells corroborated the findings of Machowska *et al*. Furthermore, expression was observed in both the untreated control and the red palm oil treated samples of the normal MCF-12A cell line. No expression was observed in untreated or treated samples of the cancerous cells. This indicates that red palm oil exerted no regulatory effects on the Maspin gene with the inability to prevent cancerous activity or the metastatic process.

Various researchers have investigated Maspin expression *in vitro* in different tissue and between normal gland metastatic diseases. A reduction in the expression of Maspin was observed in the advanced stages of breast cancer. Berardi *et al* and Banias *et al,* both demonstrated that Maspin was directly correlated with high histologic grade, younger age, a larger tumour size, ER and PR expression and a poor prognosis. Therefore, there is a substantial need for further investigation in order to clarify the mechanism of Maspin and its potential role for being a prognostic marker (Berardi *et al,* 2013; Banias *et al,* 2019). Furthermore, the study conducted by Machowska *et al,* explained that the lack of expression in cancer cells such as MDA-MB-231 and MCF-7 was a result of gene silencing through epigenetic processes. They also noted anti-proliferative activity of Maspin on MCF-7 cells were the strongest in cells containing nuclear Maspin in comparison to cytoplasm Maspin. This indicates a higher nuclear Maspin level is affiliated with better survival in breast cancer patients which gives rise to nuclear Maspin being a potential new marker for breast cancer prognosis (Machowska *et al,* 2014).

In the present study, the level of expression of hMAM and Maspin genes were not detected in the MCF-7 cancer cell line. The conventional PCR method is of low sensitivity which therefore warrants further quantitative analysis of these molecular markers through qRT-PCR. Real-time PCR is dependent on a cut-off value making it a more sensitive and specific quantification of nucleic acids in real time during the amplification process. This sensitivity allows for the detection of a single molecule making diagnostics feasible with reduced amounts of intricate biological materials in comparison to conventional PCR (Deepak *et al,* 2007).

Additionally, Arya *et al* and Valones *et al* indicated RT-PCR to be more beneficial than conventional PCR based on the reliability in detecting and measuring of products as they accumulate which allows quantitation of the products in the reactions exponential phase, a greater reproducibility of product and provides a rapid analysis with high precision of less than 2% standard deviation and a high technical sensitivity of less than 5 copies reported by Arya *et al* demonstrating the specificity of RT-PCR. Moreover, no post PCR steps are necessary which lowers the risk of possible cross-sample contamination (Arya *et al,* 2005; Valones *et al,* 2009). Real-time PCR is a more superior detection method and would be advantageous in determining the role of these markers in the progression of breast cancer.

4.5 Conclusion and Future Perspectives

The percentage of live cells was still relatively high even after the longest incubation period used in this study. However, red palm oil inhibited the proliferation of MCF-7 cancer cells which showed that MCF-7 cells were more sensitive in response to the oil as growth was inhibited, in comparison to the triple-negative MDA-MB-231 cancer cells. Based on the overall findings in this study, it can be deduced that red palm oil has a potential inhibitory effect on MCF-7 breast cancer cells while producing a stimulatory effect on MDA-MB-231 and MCF-12A cells. While apparent through extensive literature, individual components extracted from red palm oil can induce cell death and decrease cancer cell proliferation and progression in MDA-MB-231 cells, the same was not observed when the whole red palm oil containing all its compounds was used. Therefore, these findings suggest that red palm oil would not be an appropriate therapeutic agent in the prevention or treatment regimen for the highly aggressive triple-negative cancer subtype.

In future, longer treatment periods could be used to determine if red palm oil exerts better anti-proliferative effects over a longer treatment period particularly in MCF-7 cells. A more effective and efficient method in the dissolution of red palm oil should be looked at in order to obtain a higher oil to solvent ratio which in turn will allow for higher treatment concentrations of red palm oil to be investigated. More assays such as apoptosis should be investigated. Moreover, further optimization of

the hMAM molecular marker should be conducted with possibly lower primer concentrations and different annealing temperature to obtain better expression of the target. Additionally, it would be essential to perform quantitative real-time PCR on the molecular markers to obtain quantitative data for this study.

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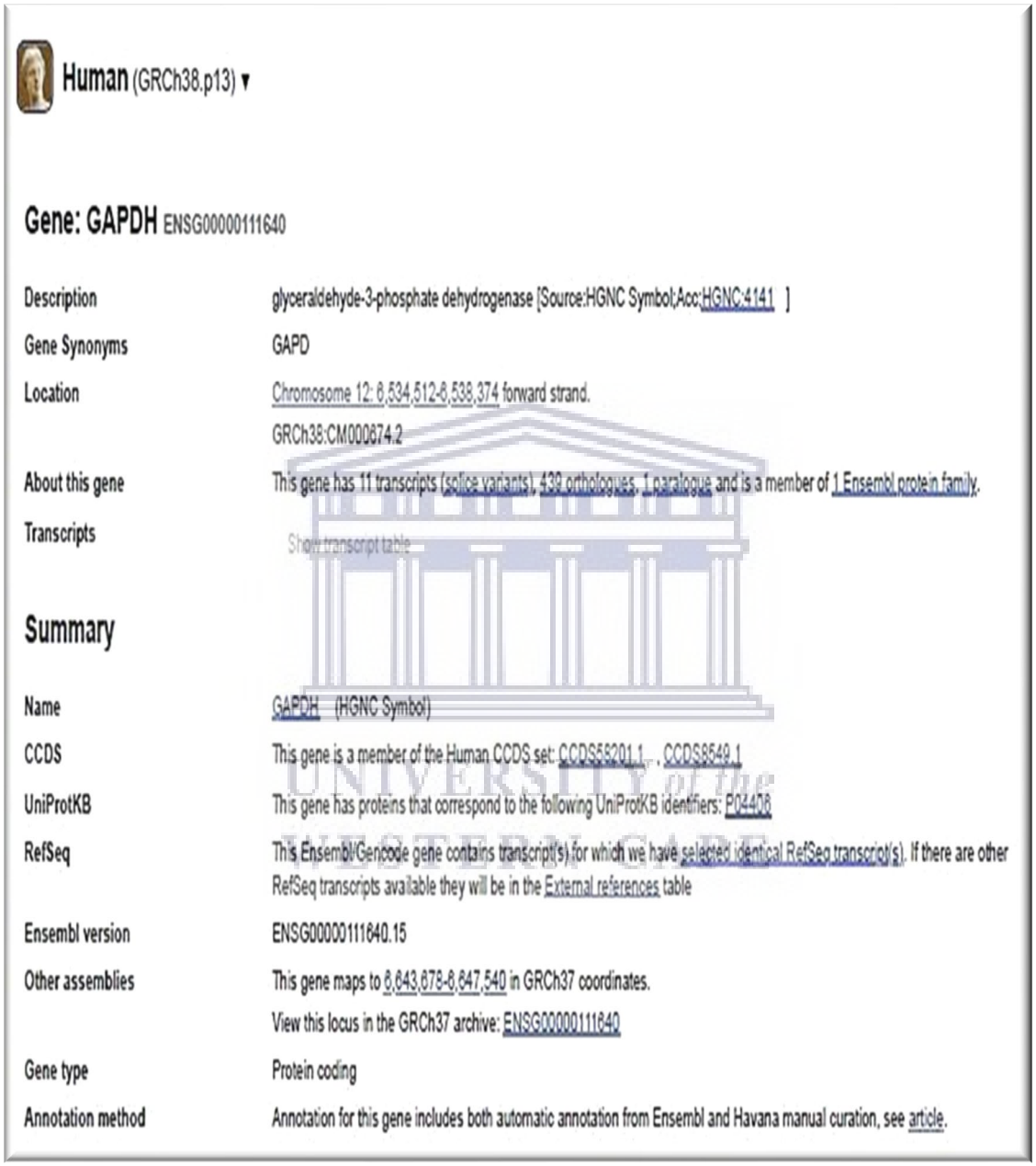
ANNEXURES

ANNEXURE 1: DETAILS OF THE SELECTED GENES

Name/Gene ID	Term used in study	Description	Location	Aliases	MIM
GAPDH ID: 2597	GAPDH	Glyceraldehyde-3-phosphate dehydrogenase [*Homo sapiens* (human)]	Chromosome 12, NC_000012.12 (6534517..6538371)	G3PD, GAPD, HEL-S-162eP	138400
SCGB2A1 ID: 4246	hMAM	Secretoglobin family 2A member 1 [*Homo sapiens* (human)]	Chromosome 11, NC_000011.10 (62208673..62213943)	LPHC, LPNC, MGB2, UGB3	604398
SERPINB5 ID: 5268	Maspin	Serpin family B member 5 [*Homo sapiens* (human)]	Chromosome 18, NC_000018.10 (63476958..63505085)	PI5, maspin	154790

ANNEXURE 2: GAPDH GENE AND TRANSCRIPT SUMMARY



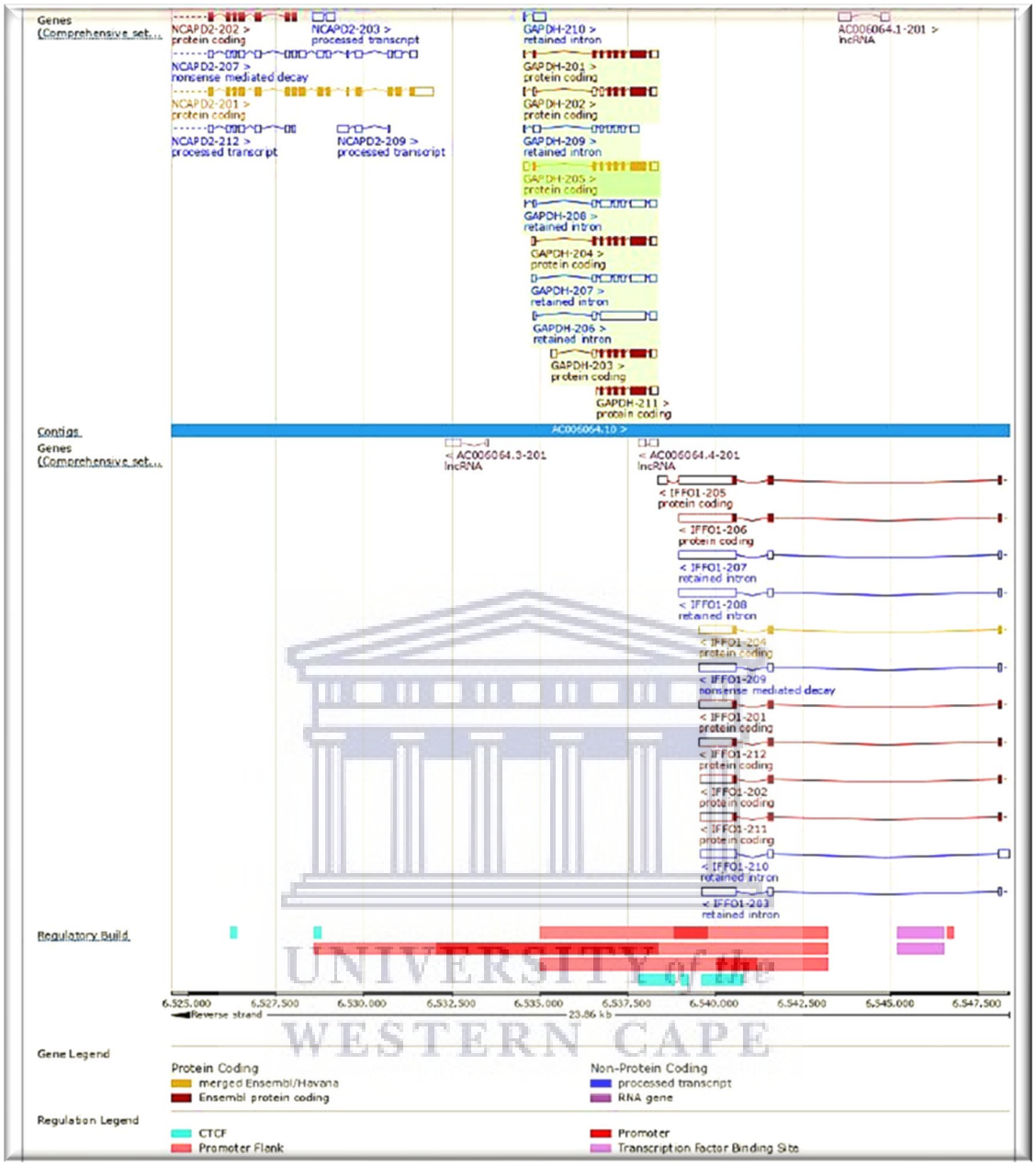
Human (GRCh38.p13) ▾

Gene: GAPDH ENSG00000111640

Description	glyceraldehyde-3-phosphate dehydrogenase [Source:HGNC Symbol;Acc:HGNC:4141]
Gene Synonyms	GAPD
Location	Chromosome 12: 6,534,512-6,538,374 forward strand. GRCh38:CM000674.2
About this gene	This gene has 11 transcripts (splice variants), 439 orthologues, 1 paralogue and is a member of 1 Ensembl protein family.
Transcripts	Show transcript table

Summary

Name	GAPDH (HGNC Symbol)
CCDS	This gene is a member of the Human CCDS set: CCDS58201.1 , CCDS8549.1
UniProtKB	This gene has proteins that correspond to the following UniProtKB identifiers: P04406
RefSeq	This Ensembl/Gencode gene contains transcript(s) for which we have selected identical RefSeq transcript(s). If there are other RefSeq transcripts available they will be in the External references table
Ensembl version	ENSG00000111640.15
Other assemblies	This gene maps to 6,643,678-6,647,540 in GRCh37 coordinates. View this locus in the GRCh37 archive: ENSG00000111640
Gene type	Protein coding
Annotation method	Annotation for this gene includes both automatic annotation from Ensembl and Havana manual curation, see article.

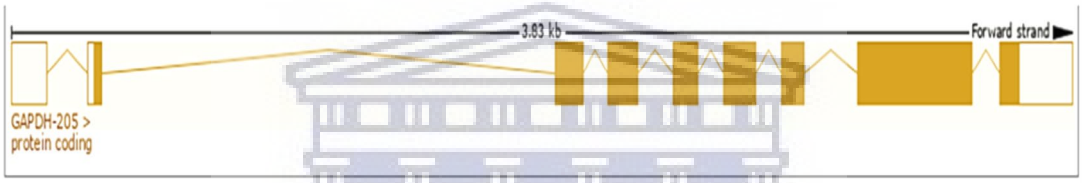


Human (GRCh38.p13) ▾

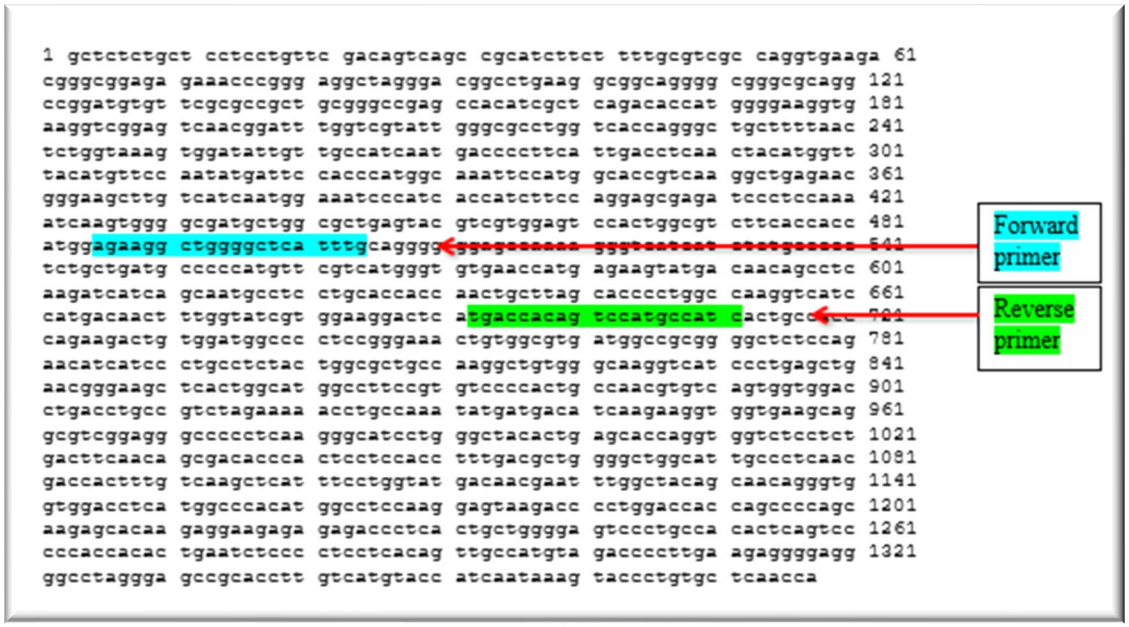
Transcript: GAPDH-205 ENST00000396861.5

Description	glyceraldehyde-3-phosphate dehydrogenase [Source:HGNC Symbol;Acc:HGNC:4141]
Gene Synonyms	GAPD
Location	Chromosome 12: 6,534,534-6,538,359 forward strand.
About this transcript	This transcript has 9 exons, is annotated with 50 domains and features, is associated with 1852 variant alleles and maps to 1364 oligo probes.
Gene	This transcript is a product of gene ENSG00000111640.15 Show transcript table

Summary



Statistics	**Exons:** 9, **Coding exons:** 8, **Transcript length:** 1,348 bps, **Translation length:** 335 residues
CCDS	This transcript is a member of the Human CCDS set: CCDS8549
Uniprot	This transcript corresponds to the following Uniprot identifiers: P04406
Transcript Support Level (TSL)	TSL:5
Version	ENST00000396861.5
Type	Protein coding
Annotation Method	Transcript where the Ensembl genebuild transcript and the Havana manual annotation have the same sequence, for every base pair. See article.
GENCODE basic gene	This transcript is a member of the Gencode basic gene set.



ANNEXURE 3: HMAM GENE AND TRANSCRIPT SUMMARY

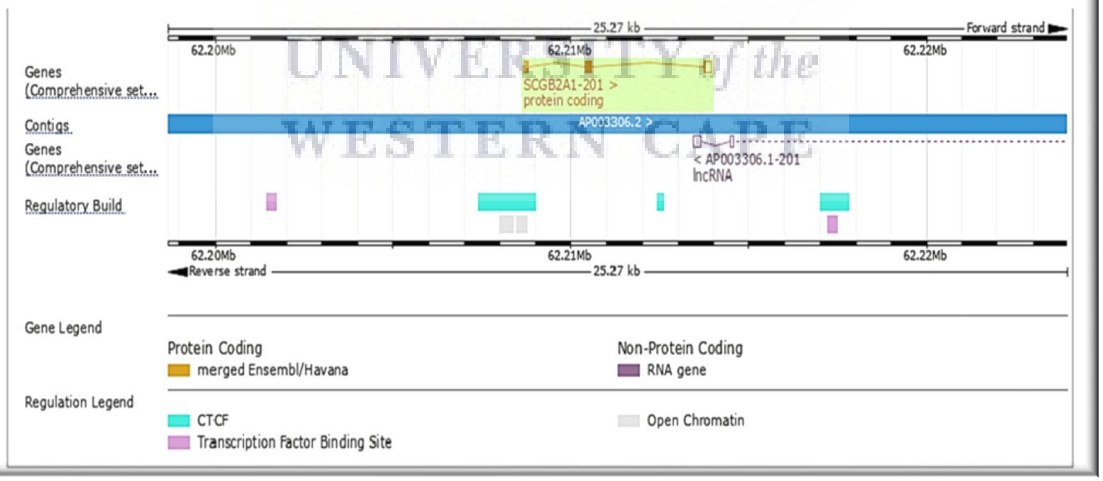
Human (GRCh38.p13) ▾

Gene: SCGB2A1 ENSG00000124939

Description	secretoglobin family 2A member 1 [Source:HGNC Symbol;Acc:HGNC:7051]
Gene Synonyms	LPHC, MGB2, MGC71973, UGB3
Location	Chromosome 11: 62,208,673-62,213,943 forward strand. GRCh38:CM000673.2
About this gene	This gene has 1 transcript (splice variant), 134 orthologues, 1 paralogue and is a member of 1 Ensembl protein family.
Transcripts	Show transcript table

Summary

Name	SCGB2A1 (HGNC Symbol)
CCDS	This gene is a member of the Human CCDS set: CCDS8016.1
UniProtKB	This gene has proteins that correspond to the following UniProtKB identifiers: O75556
RefSeq	This Ensembl/Gencode gene contains transcript(s) for which we have selected identical RefSeq transcript(s). If there are other RefSeq transcripts available they will be in the External references table
Ensembl version	ENSG00000124939.6
Other assemblies	This gene maps to 61,976,145-61,981,415 in GRCh37 coordinates. View this locus in the GRCh37 archive: ENSG00000124939
Gene type	Protein coding
Annotation method	Annotation for this gene includes both automatic annotation from Ensembl and Havana manual curation, see article.



Human (GRCh38.p13) ▾

Transcript: SCGB2A1-201 ENST00000244930.6

Description	secretoglobin family 2A member 1 [Source:HGNC Symbol;Acc:HGNC:7051]
Gene Synonyms	LPHC, MGB2, MGC71973, UGB3
Location	Chromosome 11: 62,208,673-62,213,943 forward strand.
About this transcript	This transcript has 3 exons, is annotated with 8 domains and features, is associated with 1948 variant alleles and maps to 220 oligo probes.
Gene	This transcript is a product of gene ENSG00000124939.6 Show transcript table

Summary

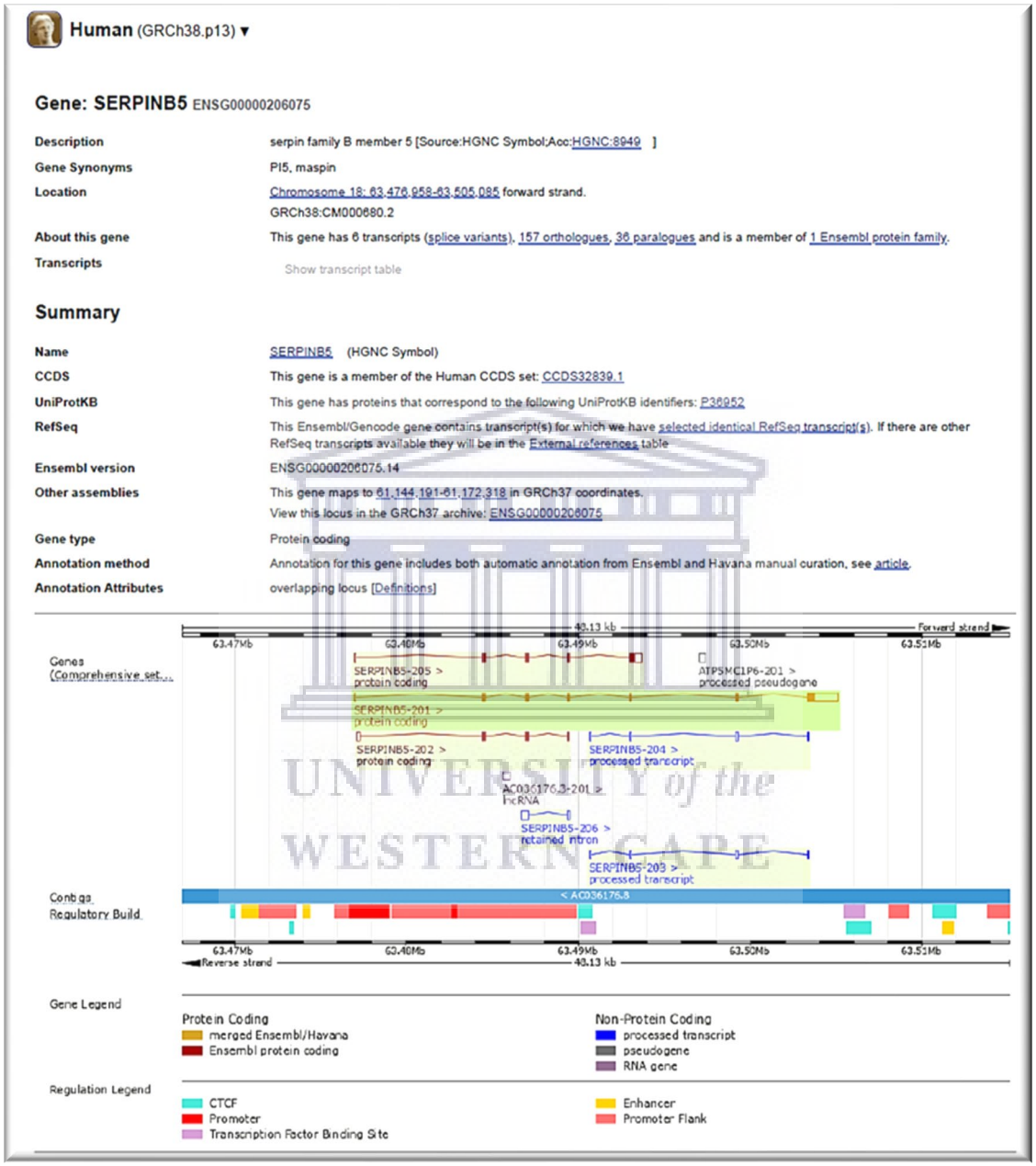
Statistics	**Exons:** 3, **Coding exons:** 3, **Transcript length:** 520 bps, **Translation length:** 95 residues
CCDS	This transcript is a member of the Human CCDS set: CCDS8016
Uniprot	This transcript corresponds to the following Uniprot identifiers: Q75556
Transcript Support Level (TSL)	TSL:1
Version	ENST00000244930.6
Type	Protein coding
Annotation Method	Transcript where the Ensembl genebuild transcript and the Havana manual annotation have the same sequence, for every base pair. See article.
GENCODE basic gene	This transcript is a member of the Gencode basic gene set.


```
1 cctccacagc aacttccttg atccctgcca cgcacgactg aacacagaca gcagccgcct
61 cgccatgaag ctgctgatgg tcctcatgct ggcggccctc ctcctgcact gctatgcaga
121 ttctggctgc aaactcctgg aggacatggt tgaaaagacc atcaattccg acatatctat
181 acctgaatac aaagagcttc ttcaagagtt catagacagt gatgccgctg cagaggctat
241 ggggaaattc aagcagtgtt tcctcaacca gtcacataga actctgaaaa acttcggact
301 gatgatgcat acagtgtacg acagcatttg gtgtaatatg aagagtaatt aactttaccc
361 aaggcgtttg gctcagaggg ctacagacta tggccagaac tcatctgttg attgctagaa
421 accacttttc tttcttgtgt tgtcttttta tgtggaaact gctagacaac tgttgaaacc
481 tcaaattcat ttccatttca ataaactaac tgcaaatcac t
```

Forward primer

Reverse primer

ANNEXURE 4: MASPIN GENE AND TRANSCRIPT SUMMARY

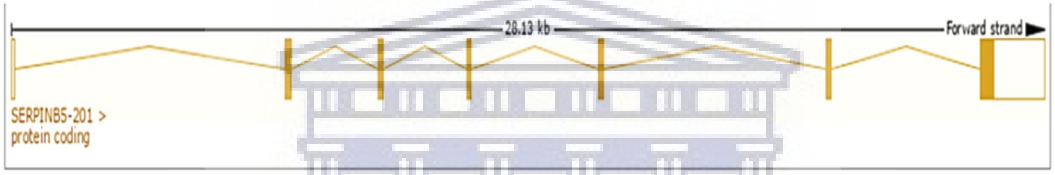


Human (GRCh38.p13) ▾

Transcript: SERPINB5-201 ENST00000382771.9

Description	serpin family B member 5 [Source:HGNC Symbol;Acc:HGNC:8949]
Gene Synonyms	PI5, maspin
Location	Chromosome 18: 63,476,958-63,505,085 forward strand.
About this transcript	This transcript has 7 exons, is annotated with 25 domains and features, is associated with 7214 variant alleles and maps to 367 oligo probes.
Gene	This transcript is a product of gene ENSG00000206075.14 Show transcript table

Summary



Statistics	**Exons:** 7, **Coding exons:** 6, **Transcript length:** 2,586 bps, **Translation length:** 375 residues
CCDS	This transcript is a member of the Human CCDS set: CCDS32839
Uniprot	This transcript corresponds to the following Uniprot identifiers: P36952
Transcript Support Level (TSL)	TSL:1
Version	ENST00000382771.9
Type	Protein coding
Annotation Method	Transcript where the Ensembl genebuild transcript and the Havana manual annotation have the same sequence, for every base pair. See article.
GENCODE basic gene	This transcript is a member of the Gencode basic gene set.