

# Potential for modulation of cancer-associated oxidative stress: an *in vitro* investigation

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## ABSTRACT

While cancer is a chronic, complex disease associated with a multitude of steps in its initiation and progression, all cancers generally have similar underlying maladaptive physiological mechanisms which may be addressed in order to prevent or treat this disease. Interest in the context of this thesis was in two of these maladaptive mechanisms, namely inflammation and oxidative stress. Both unresolved or severe inflammation and oxidative stress have been associated with cancer aetiology, and therefore administration of an anti-inflammatory or antioxidant could potentially prevent the initiation and progression of cancer. Current conventional cancer therapies are associated with a wide range of adverse side effects, leading to much interest in plant-based alternatives. For the purpose of this thesis,  $\Delta$ -7 mesembrenone, a potent antioxidant isolated from *Sceletium tortuosum* and Cannabidiol, an antioxidant and anti-inflammatory extracted from *Cannabis sativa*, were studied in the context of breast cancer treatment.

The potential anti-cancer activity of  $\Delta$ -7 mesembrenone and Cannabidiol were investigated in three breast cell models *in vitro*. MCF12A, MCF7 and MDA-MB-231 cells were treated with varying doses of  $\Delta$ -7 mesembrenone and Cannabidiol in isolation or in combination for a period of 24 hours, and the effects on cell viability and mitochondrial reductive capacity were assessed. Following this, the ROS production and the GSH/GSSG ratio were determined.

$\Delta$ -7 mesembrenone in isolation resulted in cytotoxicity and increased ROS production across all cell models. Cannabidiol exposure in estrogen receptor positive breast cancer resulted in reductions in cell viability and mitochondrial reductive capacity, corresponding to an increased ROS production in these cells. No toxic effects of CBD were evident in the estrogen receptor negative breast cancer or normal breast cells at lower doses. Finally, combination treatments resulted in adverse effects across all cell models and at combined high doses, the negative effects were cumulative.

We conclude that  $\Delta$ -7 mesembrenone has no benefit in the context of breast cancer, as it exhibited significant levels of cytotoxicity in normal healthy breast cells at all concentrations reducing cancer cell survival, which could not be countered by Cannabidiol co-treatment. Cannabidiol showed more promise as an anti-cancer drug due to its high levels of cytotoxicity

and the increased ROS production it induced in the estrogen receptor positive breast cancer cell line. Both the normal breast cells and the estrogen receptor negative cells exhibited little side effects than can be ascribed to Cannabidiol, illustrating the importance of this treatment in certain types of breast cancer only. Further research is warranted to better elucidate the cellular mechanisms involved and potential for treatment with this extract.

## UITREKSEL

Hoewel kanker 'n chroniese en komplekse toestand is wat gepaardgaan met verskeie stappe in terme van oorsprong en progressive, het all kankers ooreenstemmende onderliggende wanaanpassings van fisiologiese sisteme wat geadresseer kan word om die siekte te behandel of te voorkom. In die konteks van hierdie tesis is twee van hierdie wanaangepaste meganismes, inflammasie en oksidatiewe stres, van spesifieke belang. Beide hierdie meganismes word geassosieer met kanker etiologie. Dus kan toediening van anti-inflammatoriese or anti-oksidadant middels potensieel teen kanker beskerm. Huidige konvensionele kankerterapie word gekenmerk deur 'n verskeidenheid newe-effekte, wat gelei het na belangstelling in plantverwante alternatiewe. Vir die doel van hierdie tesis, is  $\Delta$ -7 mesembrenoon, 'n sterk anti-oksidadant geïsoleer uit *Sceletium tortuosum*, en kannabidiol, 'n anti-oksidadant en anti-inflammatoriese middel uit *Cannabis sativa*, in die konteks van borskanker bestudeer.

Die moontlike teenkanker aktiwiteit van  $\Delta$ -7 mesembrenoon en kannabidiol is *in vitro* in drie borskanker selmodelle ondersoek. MCF12A, MCF7 en MDA-MB-231 selle is met 'n verskeidenheid dosisse van  $\Delta$ -7 mesembrenoon en kannabidiol, in isolasie of in kombinasie, behandel vir 24 uur, gevolg deur 'n assessering van die effekte op sel lewensvatbaarheid en mitokondriale reduktiewe kapasiteit. Hierna is reaktiewe suurstof spesie (RSS) produksie en die GSH/GSSG verhouding bepaal.

$\Delta$ -7 mesembrenoon op sy eie was sitotoksies en het verhoogde RSS produksie in alle seltepes tot gevolg gehad. Kannabidiol blootstelling in estrogeen reseptor positiewe borskankerselle het verlaagde seloorlewing en mitokondriale reduktiewe kapasiteit veroorsaak, wat ooreenstem met die verhoogde RSS produksie in hierdie sellyn. Geen toksiese effekte van kannabidiol was sigbaar in estrogeen negatiewe selle by laer dosisse nie. Laastens het die kombinasie behandeling newe-effekte gehad in alle modelle – hierdie effekte was kumulatief by hoër dosisse.

Ons gevolgtrekking is dat  $\Delta$ -7 mesembrenoon geen voordeel in die konteks van borskanker inhou nie, aangesien dit toksies was vir normale selle by alle konsentrasies wat kankersel oorlewing beperk het. Hierdie negatiewe effek kon nie voorkom word deur byvoeging van

kannabidiol nie. Kannabidiol het meer belofte ingehou as teenkankermiddel deur verhoogde sitotoksiteit en RSS produksie wat dit in estrogeen reseptor positiewe selle tot gevolg gehad het. Beide normale en estrogeen negatiewe selle het min nuwe-effekte op kannabidiol gewys, wat die potensiaal van kannabidiol behandeling in sekere kankertipes uitwys. Verdere navorsing sal help om die meganismes betrokke op sellulêre vlak, verder te belig.

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## LIST OF ACRONYMS AND ABBREVIATIONS

5-HT	Serotonin
AIDS	Acquired Immunodeficiency Syndrome
ARV	Antiretroviral
ATP	Adenosine Triphosphate
BRCA1	Breast Cancer susceptibility gene 1
BRCA2	Breast Cancer susceptibility gene 2
CAT	Catalase
CBC	Cannbichromene
CBD	Cannabidiol
CBG	Cannabigerol
CBN	Cannabinol
CNS	Central Nervous System
CO <sub>2</sub>	Carbon Dioxide
COX-2	Cyclooxygenase 2
DMEM	Dulbeccos Modified Eagle Medium
DNA	Deoxyribonucleic Acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
E2	Estradiol
ER-	Estrogen Receptor negative
ER+	Estrogen Receptor positive
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
GSH	Reduced glutathione
GSHPx	Glutathione peroxidase
GSSG	Oxidised glutathione
H <sup>+</sup>	Proton
H <sub>2</sub> O	Water

H <sub>2</sub> O <sub>2</sub>	Hydrogen peroxide
HIF-1	Hypoxia Inducible Factor 1
HIF-2 $\alpha$	Hypoxia Inducible Factor 2 $\alpha$
HIV	Human Immunodeficiency Virus
HPLC	High Performance Liquid Chromatography
IL-1	Interleukin 1
IL-1 $\alpha$	Interleukin 1 $\alpha$
IL-1 $\beta$	Interleukin 1 $\beta$
IL-6	Interleukin 6
IL-8	Interleukin 8
MCF12A	Normal human mammary gland cell
MCF7	Human breast adenocarcinoma (ER+)
MDA-MB-231	Human breast adenocarcinoma (ER-)
MRA	Monoamine Releasing Agent
NADPH	Nicotinamide Adenine Dinucleotide Phosphate Hydrogen
NF- $\kappa\beta$	Nuclear Factor $\kappa\beta$
NO	Nitric oxide
NOX	Nitrogen Oxides
O <sub>2</sub>	Oxygen
O <sub>2</sub> <sup>-</sup>	Superoxide anion
OH $\bullet$	Hydroxyl radical
ONO <sub>2</sub> <sup>-</sup>	Peroxynitrite
PDE4	Phosphodiesterase 4
PenStrep	Penicillin Streptomycin
PMS	N-methyl dibenzopyrazine methyl sulfate
Prx	Peroxiredoxin
RLU	Relative Light Units
ROS	Reactive Oxygen Species
SOD	Superoxide dismutase

SSRI	Selective Serotonin Reuptake Inhibitor
TNF	Tumour Necrosis Factor
USA	United States of America
VEGF	Vascular Endothelial Growth Factor
WHO	World Health Organisation
XTT	Sodium 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5- [(phenylamino)-carbonyl]-2H-tetrazolium) salt
$\Delta^9$ -THC	$\Delta^9$ -Tetrahydrocannabinol

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## CHAPTER ONE: INTRODUCTION

Cancer is a chronic disease responsible for more than 20% of the deaths occurring each year worldwide. This already massive disease burden is only anticipated to become more severe, with millions of new cases predicted annually. Breast cancer specifically is the most frequently diagnosed cancer in women, and upwards of 500 000 deaths each year are as a result of breast cancer.

Cancer in general is a highly complex disease, characterised by a multistep process resulting in tumorigenesis, progression and invasion. While varying types of cancer all have their own disease specific triggers, all cancers have the same maladaptive physiological mechanisms and processes, and once these may be addressed, tumour initiation and progression may be limited or inhibited entirely. Of particular interest in the context of this study was inflammation and oxidative stress, both implicated directly in the development and progression of carcinogenesis.

Both inflammation and oxidative stress are important biological processes necessary for survival and are beneficial to the host at a certain threshold. Acute inflammation which is resolved quickly is a key component of innate immunity, tissue repair and detoxification, while oxidative stress is an important mediator of signalling and host defence. However, when inflammation and oxidative stress remain unresolved, potential cellular targets may become oxidised and degraded resulting in detrimental effects, one of which is carcinogenesis. Therefore, in order to prevent or treat carcinogenesis, antioxidant and anti-inflammatory agents may prove very beneficial.

Current traditional cancer treatments such as chemotherapy and radiation have been associated with many adverse side effects, high expenses and secondary toxicity in response to anti-cancer drugs. For this reason, a large body of current research has shifted its focus to plant-based therapies for as potential anti-cancer agents. Plant-based medication is associated with far fewer adverse side effects and has shown potential in treatment of other inflammatory and oxidative stress-based diseases, such as neurodegeneration, arthritis and inflammatory bowel disease.

For the purpose of this thesis, two plant-based compounds were studied, namely  $\Delta$ -7 mesembrenone isolated from *Scelletium tortuosum* and Cannabidiol isolated from *Cannabis sativa*.  $\Delta$ -7 mesembrenone has recently been determined to be a powerful antioxidant, and Cannabidiol has both anti-inflammatory and antioxidant properties. These compounds were studied in the context of treatment of breast cancer, and the resolution of the oxidative stress associated with this disease.

Aims of this study included determination of the effect of exposure with two plant-based extracts,  $\Delta$ -7 mesembrenone isolated from *Scelletium tortuosum* and Cannabidiol isolated from *Cannabis sativa*, in isolation and in combination, on normal breast (MCF12A) and breast cancer (MCF7 and MDA-MB-231) cell viability. In addition to this, the study aimed to elucidate the relevant cellular mechanisms involved related to redox status by which the extracts exert their effects on cell survival. Finally, we aimed to assess potential interaction effects of the two plant extracts in this context.

This thesis begins with a review of all the relevant literature studied in order to better understand the mechanisms involved in oxidative stress, inflammation and their role in carcinogenesis, as well as the potential of phytomedicines in this context. Following this, the methods and the materials used for this study are described, after which the results obtained are displayed. This thesis then ends off with a discussion of the results attained, and the relevant conclusions drawn from this study.

## CHAPTER TWO: LITERATURE REVIEW

### 2.1 Introduction

Chronic disease, and more specifically the chronic non-communicable diseases, typically have a relatively long maladaptive progression before clinical onset, and are the result of physiological, environmental, genetic and behavioural factors (World Health Organisation, 2018). The 4 main non-communicable diseases are cardiovascular disease, cancer, chronic respiratory diseases and diabetes, and risk factors for these diseases include lack of physical activity, unhealthy diets, tobacco use and harmful alcohol consumption. People globally from all countries and regions, both developing and developed, are affected by non-communicable diseases, however a higher prevalence is observed in developing countries. According to the World Health Organisation (WHO), these diseases are responsible for 71% of the deaths worldwide annually, with 42% of these deaths being the result of cardiovascular disease and 22% due to cancer (World Health Organisation, 2018).

Within South Africa specifically, a significant health transition is occurring, with an approximate 400% increase in the incidence of chronic disease in both rural and urban areas within the last 20 years. This growing burden has placed an ever-growing pressure on both short and long-term healthcare services nationwide (Mayosi *et al.*, 2009). While a massive increase in non-communicable disease burden has already been experienced, this burden is only expected to become more severe, partially due to the emergence of anti-retroviral medication (ARVs). This treatment has resulted in higher mortality and prolonged death in HIV/AIDS infected patients, with more than 3.4 million people in South Africa alone using ARV treatments, making it the largest ARV treatment programme in the world (Moyo *et al.*, 2018). Expansion in these health programs have resulted in a decrease in the amount of deaths in South Africa that are due to communicable disease, such as HIV and tuberculosis. This in part has resulted in an increase in the age of the population, which lends itself to an increase in the incidence of chronic disease, such as cancer and cardiovascular disease. Focus now has shifted to combat these non-communicable diseases (Statistics South Africa, 2017). For the purpose of this thesis, I will focus on cancer, and in particular, breast cancer.

## 2.2 Cancer

### 2.2.1 Epidemiology

Cancer is the second leading cause of death worldwide, and thus poses a large burden economically on developing and developed countries alike (Torre *et al.*, 2015). This burden of cancer is increasing every year, and the WHO predicts 20 million new cancer cases annually from as early as 2025, with continuation of this escalation over the following decades (World Health Organisation, 2011). Developing countries are typically characterised by late diagnosis and limited treatment access, resulting in more deaths from chronic disease (Torre *et al.*, 2015). Factors such as increased population age, as well as cancer-promoting lifestyle choices also contribute to the poor prognosis of cancer (World Health Organisation, 2008).

Breast cancer is the most frequently diagnosed cancer in women worldwide, as well as the leading cause of cancer deaths in females. In 2012 alone, there were 1.7 million cases of breast cancer, and 521900 deaths caused by this disease around the world (Torre *et al.*, 2012). Year after year, more and more health data are collected globally which demonstrate the ever-increasing mortality, incidence and economic burden of breast cancer, and this is only predicted to increase annually (Coughlin *et al.*, 2009).

### 2.2.2 Breast cancer risk factors

There are multiple risk factors associated with developing breast cancer. Such risk factors include firstly, age, whereby the risk is doubled in women every 10 years up until menopause is reached, after which the risk dissipates dramatically (Willett *et al.*, 2000). Secondly, the age at which women begin mensuration and the age at which menopause starts also plays a role. Studies have shown that women who menstruate early in life, or women who develop menopause after the age of 55 are at a higher risk of developing the disease (McPherson, *et al.*, 2000). Thirdly, family history also plays a vital role, and up to 10% of breast cancer cases are related to a genetic predisposition and are generally inherited through genes such as BRCA1 or BRCA2 (DeMichele and Weber, 2000). However, in the context of this these, the role of estrogen in the development of breast cancer and the resulting oxidative stress is of particular interest.

Sex hormones are implicated in the carcinogenesis of many different types of human cancers, and there is a plethora of experimental data across multiple studies obtained over decades which indicates that estrogen plays a critical role in the development and progression of breast cancer (van Leeuwen *et al.*, 2000). Increasing evidence suggests that an increase in estrogen exposure is associated with a greater risk of developing breast cancer, and estrogens may act as carcinogens through mechanisms involving oxidative stress in the kidney, liver and breast tissues of various rodent models (Harvel *et al.*, 2000). In comparison to the oxidative stress levels seen in normal breast tissue, cancerous breast tissue has a much higher amount of oxidative DNA damage, and this has a strong correlation with the estrogen receptor status of the breast tissue studied (Mussarat *et al.*, 1996). Estrogen receptors are responsible for retaining estrogen intracellularly and have a very high affinity for estrogen binding (Kuiper *et al.*, 1997). There is evidence to suggest that breast cancer cells which are estrogen receptor positive (ER+) have a greater sensitivity to oxidative stress levels, as they do not metabolise the reactive oxygen species as efficiently as estrogen receptor negative (ER-) breast cancers (Mobley and Brueggemeier, 2004). Furthermore, the presence of estradiol (E2), one of the primary estrogens in women, has been linked to increased sensitivity of breast cancer cells to oxidative damage (Liehr, 1999).

### 2.2.3 Cancer aetiology

Extensive cancer research has generated a large body of knowledge about the disease in general, and how tumorigenesis is characterised by multistep processes resulting in genetic mutations, which are the driving force behind the change from normal, healthy cells to highly malignant derivatives of the host (Hanahan and Weinberg, 2000). Most notably, there are mutations in the genome resulting in an acquisition of function of oncogenes, while a loss of function is seen in tumour suppressor genes, resulting in the promotion of cancer progression (Bishop and Weinberg, 1996).

Multiple theories exist in which the causation and origin of cancer are described, most notably and commonly described of which is Hanahan and Weinbergs “Hallmarks of Cancer”. This theory describes certain biological capabilities that are acquired during the process of tumour development, allowing initiation of the tumour development as well as metastasis of

the cancer. These hallmarks are self-sufficiency of growth signals, insensitivity of anti-growth signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis, invasion and metastasis, deregulated metabolism, genome instability, immune system evasion and inflammation (Hanahan and Weinberg, 2011). This theory demonstrates that while all types of cancer have their own unique aetiology, all cancers share similar maladaptive physiological mechanisms and processes. If these maladaptation's may be addressed, tumour initiation and cancer progression could potentially be limited or removed entirely.

For this study, one of the most interesting hallmarks is that of inflammation, due in part to the fact that it is crucial, as inflammation is implicated directly in the perpetuation of many of the other hallmarks of cancer (Hanahan and Weinberg, 2011). Interlinked with the hallmark of inflammation is that of oxidative stress, as oxidative stress results from the inflammation occurring during cancer, and may also contribute to tumour initiation and progression. Therefore, in the following section I will be reviewing these two important processes that are interlinked in the context of cancer. While these are two general cancer-related processes I will be focussing on, it is important to note that all types of cancer have their own unique range of disease-specific triggers and factors that culminate to result in their own carcinogenesis and tumour progression pathways. However, this falls outside of the scope of this thesis, and for the purpose of my study I will be focussing specifically on oxidative stress and how it is related to inflammation.

## **2.3 Common exacerbating role players in cancer**

### *2.3.1 Oxidative stress*

Increased oxidative stress is associated with the pathogenic mechanisms of many diseases, including non-communicable diseases such as cancer and diabetes mellitus; inflammatory diseases; neurodegenerative diseases such as Parkinson's and Alzheimer's; as well as the process of ageing (Durackova, 2010). Interestingly, cumulative oxidative damage and repair cycles – which is the basis for physiological ageing – seems to be upregulated in all non-communicable diseases and seems to result from “accelerated ageing” (Petersen and Smith, 2016). This common aetiology suggests that an understanding of the process of oxidative

stress, as well as knowledge on how to limit or counter it, may be applicable more widely than to cancer alone.

Oxidative stress may be defined as the imbalance between the production of free radicals, namely reactive oxygen species (ROS), and their removal via antioxidant systems, functioning to protect important biomolecules and organs from potential damage resulting from this imbalance (Reuter *et al.*, 2010). Therefore, stress begins to occur when the net influx of ROS is greater than the particular cells ability to detoxify the detrimental reactive species (Wellen and Thompson, 2010). The term reactive oxygen species (ROS) includes among others, the superoxide anion ( $O_2^-$ ), hydroxyl radical ( $OH^\bullet$ ), hydrogen peroxide ( $H_2O_2$ ), peroxyxynitrite ( $ONO_2^-$ ) and nitric oxide (NO) (Ingram and Diotallevi, 2017). Formation of free radicals generally starts with  $O_2$  and results in the formation of reactive species such as  $H_2O_2$  and  $OH^\bullet$  as seen in the reactions illustrated in Figure 2.1.

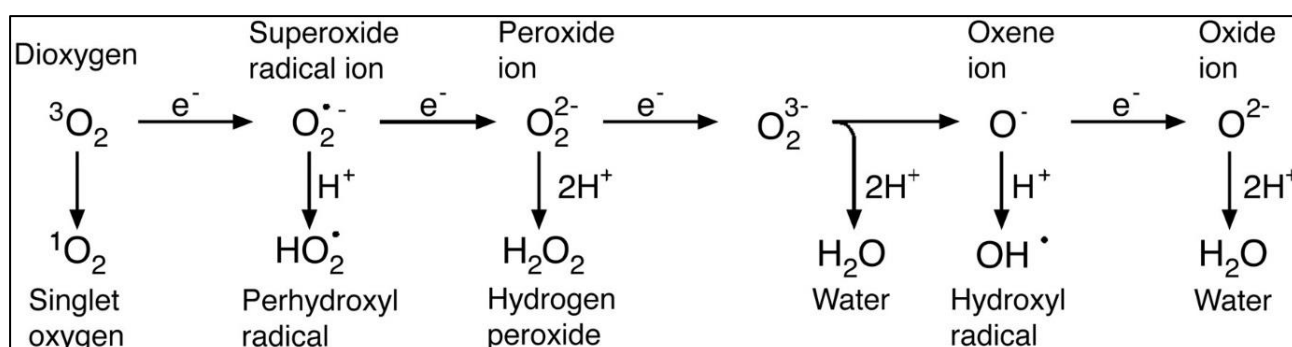


Figure 2.1 – Types of Reactive Oxygen Species (ROS) (Rodriguez and Redman, 2005)

### 2.3.1.1 Sources of free radicals

Free radicals may either come from endogenous sources, such as the mitochondria, peroxisomes and the endoplasmic reticulum, or they can originate from exogenous sources such as tobacco smoke, pesticides and alcohol. While these free radicals have many important biological functions, an alteration in the balance of these reactive species may result in increased oxidative stress.

One of the primary sources of endogenous free radical production are the mitochondria. Oxidative phosphorylation is the main source of energy production in eukaryotic cells, and it

occurs via the reduction of  $O_2$  at the mitochondria to  $H_2O$  (Sorbara and Girardin, 2011). The reduction of  $O_2$  generates an  $H^+$  gradient which drives the synthesis of ATP, and this process results in the formation of many intermediate products, such as  $H_2O_2$ . This occurs during the electron transport chain, whereby the membrane-impermeable superoxide anion ( $O_2^-$ ) is generated within the inner membrane of the mitochondria. This anion is then converted to  $H_2O_2$  via mitochondrial dismutase, and then moves into the cytoplasm out of the mitochondria.

While the mitochondria are a rich source of ROS formation, peroxisomes are also main producers of free radicals. Peroxisomes are small organelles which have a vital role in lipid metabolic pathways, such as fatty acid  $\alpha$ - and  $\beta$ -oxidation and ether-phospholipid biosynthesis. Peroxisomes contain certain enzymes, such as urate oxidase and polyamine oxidase, that are responsible for the production of  $H_2O_2$ , which forms a part of their normal catalytic cycle (Antonenkov *et al.*, 2010). These organelles also contain xanthine oxidase and nitric oxide synthase, which result in the production of  $O_2^-$  and  $H_2O_2$ , and NO respectively. Finally, another rich source of free radical generation is the NOX family of NADPH oxidases. These are enzymes involved in the transport of electrons across plasma membranes, as well as the production of the superoxide anion and other ROS. Therefore, NADPH oxidases serve almost exclusively as ROS-generating enzymes. NADPH oxidases are found in many areas throughout the body, such as in phagocytes, fibroblasts, tumour cells and vascular smooth muscle (Szatrowski and Nathan, 1991).

#### *2.3.1.2 Oxidative damage in cells*

Production of free radicals is necessary and beneficial for the survival and functioning of the organism for several reasons. Firstly, ROS are key players in host defence, and they are involved in the digestion of invading pathogens and debris (Oliveira *et al.*, 2010). Secondly, they function as signalling molecules through reversibly oxidising protein thiol groups, and are involved in processes such as signal transduction, disulphide bond formation, gene expression and control of the caspase activity which is activated during apoptotic mechanisms (Sosa *et al.*, 2013). These growth and repair mechanisms are achieved through use of the mitochondrial electron transport chain, as well as through use of several enzymes within the



host, namely cytochrome P450, xanthine oxidase, the NADPH-oxidase enzyme complex, amongst several others. Studies have also revealed that endoplasmic reticulum-generated ROS have important functions in protein folding (Santos, *et al.*, 2009).

While ROS are very important for certain fundamental cellular events, their reactivity may be harmful if the wrong targets are oxidised. Potential targets leading to undesired outcome include macromolecules, such as DNA, lipids and proteins. Free radicals can oxidise polyunsaturated fatty acids in lipids, and initiate lipid peroxidation, which can ultimately result in loss of fluidity in the membrane and thus disrupt membrane proteins (Cabiscol *et al.*, 2000). DNA is also a major target for free radicals in which both the base and the sugar molecules may be oxidised, as seen in figure 2.2, resulting in double and single stranded breaks in the backbone and within the base and sugar groups, and crosslinks may be formed with other molecules, thereby blocking replication of certain genes (Sies, 1992). This type of damage to DNA is associated with mutagenesis and carcinogenesis (Breen and Murphy, 1995).

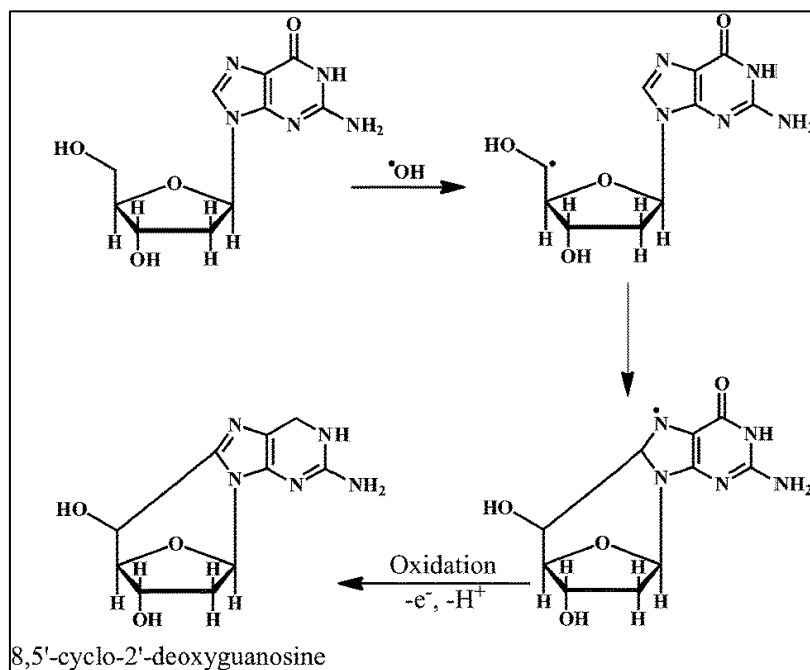


Figure 2.2. – Harmful reaction of the hydroxyl free radical with the sugar moiety of DNA (Nimse and Pal, 2015)

### 2.3.1.3 ROS generation in cancer cells vs normal cells

Increased oxidative stress and ROS have been associated with cancer for a long period of time, in both the initiation and progression of cancer, the phenotypic behaviour of cancer cells, as well as the response to treatment interventions (Sabharwal and Schumacker, 2014). This association can be highly complex, and sometimes even paradoxical.

Firstly, it has been observed that there is a relationship between ROS and the genesis of cancer (Schumacker, 2015). Within what is known as the “mitochondrial paradigm” in cancer, it has been demonstrated that mutations within either nuclear or mitochondrial DNA associated with the electron transport chain, potentially caused by hypoxia, can result in an increase in the production of reactive species (Wallace, 2005). This then results in a build-up of electrons within the chain, which can then be used to form superoxide, which can be converted into H<sub>2</sub>O<sub>2</sub> by superoxide dismutase (Schumacker, 2006). This H<sub>2</sub>O<sub>2</sub> is then free to leave the mitochondria, whereby it can oxidise potentially harmful targets, such as certain macromolecules, like DNA, introducing genetic instability. Over time, as the mitochondrial ROS production increases, there is an increase in Hypoxia Inducible Factor 2 $\alpha$  (HIF-2 $\alpha$ ), a transcription factor which suppresses DNA mismatch/repair processes, further perpetuating and accumulating the malignant DNA mutations, driving the transformation of the cell to becoming malignant (Ralph *et al.*, 2010). Another Hypoxia inducible factor, HIF-1, has also been linked to cell survival in the presence of increased ROS, as its activation by reactive species enhanced the survival and development of tumours through gene upregulation involving glycolysis, angiogenesis and other cell metabolic pathways (Gao *et al.*, 2007).

Secondly, there is evidence to suggest that certain cancer types are characterised by an increase in ROS production, in comparison to that of a normal, non-cancerous cell type. This is predicted to be as a result of the constitutive activation of cell proliferation pathways (Trachootham *et al.*, 2006). Cell proliferation in normal cells is as a result of growth factor stimulation, and this requires ROS for cell signalling. Therefore, it stands to reason that in cancer cells, characterised by an increased proliferative rate, there would be increased ROS present for cell signalling to occur. However, there has been some speculation that this increased ROS production could actually be due to a higher metabolic rate, rather than an oncogenic transformation (Ferreira, L.M., 2010). More research is required to fully elucidate this.

Although the pathways described above drive the malignancy towards proliferation and survival, they do eventually generate a level of oxidative stress that becomes too severe for the cancer cells to cope with. This introduces the third difference between cancerous and non-cancerous cells, whereby there is a greater expression of the endogenous antioxidant thioredoxin reductase within cancerous cell types (Schumacker, 2006). The thioredoxin system, made up of NADPH, thioredoxin reductase and thioredoxin, is one of the systems involved in the proliferation, viability and apoptosis of cancer cells (Lu *et al.*, 2007). This system is upregulated through a shift in the tumorigenic cells glycolytic pathway, where it is reprogrammed to increase flux through the pentose phosphate pathway to ensure an adequate supply of NADPH, one of the main driving forces of the cells antioxidant capacity (Sabwharal and Schumacker, 2014). Following this, thioredoxin reductase is expressed in response to elevated concentrations of free radicals, and it is translocated to the nucleus to ensure maintenance of a reducing environment. This allows for binding of transcription factors with DNA, allowing gene expression to continue even when oxidative stress levels are increased (Holmgren, 2008). This endogenous antioxidant is typically over expressed in malignant cells, further driving them towards tumour progression via cell proliferation, despite increased levels of oxidative stress.

Although these mechanisms manipulate the endogenous ROS levels to ensure tumour cell survival and progression, cancerous cells can be killed by agents which increase ROS levels even further, beyond the point of survival of the malignancy, which is the usual strategy for current traditional therapy. This can either be achieved through addition of a chemical agent which would increase ROS production, such as histone deacetylase inhibitors, or by decreasing the capability of the cell to scavenge free radicals, such as  $\beta$ -phenylethyl isothiocyanate (Adachi *et al.*, 2004). This appears to be selectively toxic to tumour cells, as they have an already increased level of ROS production, essential for their rate of growth and signalling, whereas normal cells have a much lower oxidant stress baseline, and therefore addition of these compounds resulted in a less severe change in oxidant signalling levels (Schumacker, 2015). This illustrates the delicate nature of the redox status of the cell in question, and how changes in oxidative stress or ROS levels may potentially make the difference between a cellular microenvironment that is tumour promoting or one which is tumour suppressing, as illustrated in figure 2.3.

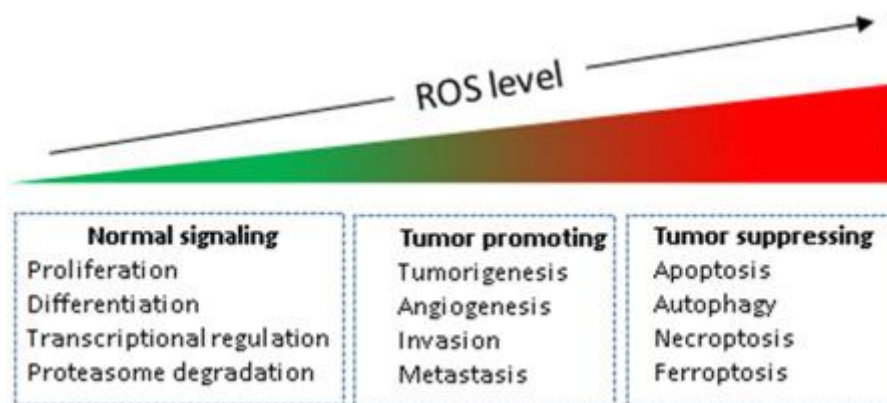


Figure 2.3 – The relationship between cellular oxidative stress and tumorigenesis/tumour suppression. (Galadari *et al.*, 2017).

#### 2.3.1.4 Endogenous antioxidant systems

Most cells or host organisms possess intercellular mechanisms to evade or diminish the threat and damage caused by these free radicals, namely antioxidants. Antioxidants are substances, enzymatic and non-enzymatic, which when present at low concentrations in comparison to the oxidative species substrate, may significantly delay or prevent oxidation of that substrate (Sies, 1997). Enzymatic antioxidants found within the hosts natural defence mechanism includes glutathione peroxidase, superoxide dismutase (SOD), glutathione and several others. Antioxidants can also be obtained through dietary intake, either through daily supplements or via eating healthy foods such as fruits and vegetables. The fine balance between antioxidants and free radicals illustrates the importance of these antioxidants, and if the balance is shifted towards a molecular environment with higher concentrations of ROS, targets necessary for maintaining homeostasis start to become oxidised, creating potential downstream adverse effects.

Antioxidants may be enzymatic or non-enzymatic and both modulate the free radical reactions. Typically, enzymatic antioxidants react with free radicals and either break them down or leave them non-reactive, and most often are functioning to reduce the levels of lipid hydroperoxide and  $H_2O_2$ . Thus, they are vital for maintaining the integrity of cellular membranes and prevention of lipid peroxidation. The main enzymatic antioxidants are catalase (CAT), Glutathione peroxidase (GSHPx), Superoxide Dismutase (SOD) and

Peroxiredoxin (Prx) (Nimse and Pal, 2015). SODs are typically located in the cytosol and mitochondria and are responsible for the conversion of the superoxide anion into oxygen and  $H_2O_2$  using a metal cofactor (Copper, Zinc etc.). CAT is found in peroxisomes and is responsible for the formation of water and oxygen from  $H_2O_2$  (Zhan *et al.*, 2004). GSHPx and Prx are located primarily in the cytosol and are used to convert  $H_2O_2$  into water (Cabsicol *et al.*, 2000). Below is a table summarising these reactions mentioned.

Enzymatic antioxidant	Cellular location	Substrate	Reaction
Mn/Cu/Zn SOD	Mitochondrial matrix (Mn SOD) cytosol (Cu/Zn SOD)	$O_2^{\cdot -}$	$O_2^{\cdot -} \rightarrow H_2O_2$
CAT	Peroxisomes cytosol	$H_2O_2$	$2H_2O_2 \rightarrow O_2 + H_2O$
GSHPx	Cytosol	$H_2O_2$	$H_2O_2 + GSH \rightarrow GSSG + H_2O$
Prx-I	Cytosol	$H_2O_2$	$H_2O_2 + TrxS_2 \rightarrow Trx(SH)_2 + H_2O$

Figure 2.4 – The main enzymatic antioxidants, their cellular locations and the reactions they catalyse (Nimse and Pal, 2015).

Non-enzymatic antioxidants are most often what are known as chain-breaker antioxidants, as they are involved in halting the cascade for the formation of free radicals (Bolann and Ulvik, 1997). Examples of non-enzymatic antioxidants are vitamin E, vitamin C and vitamin A (Tafazoli *et al.*, 2005), bioflavonoids such as flavone and flavanol (Pietta, 2000) and carotenoids such as  $\beta$ -carotene (Olsen and Krinsky, 1995). These chain-breaking antioxidants are typically involved with preventing the propagation and elongation steps in the formation of free radicals from lipids, and thus prevent the generation of these free radicals.

It is important to note that beneficial antioxidant capacity of antioxidants may be lost if the antioxidant in question is administered at a dose which is too high. This may result in the loss of their radical-scavenging function, and may shift the antioxidant behaviour towards a pro-oxidant, which exacerbates the problem of oxidative stress and may result in further cellular damage occurring (Burkitt, 2001). Therefore, antioxidants should be administered with caution, and at doses which are safe in order to achieve the desired free radical scavenging effects.

There is evidence to suggest that treatment with antioxidants can serve a protective role as preventative agents against cancer development and tumour initiation, as well as serve as effective treatment in combating tumour growth and progression (Block *et al.*, 2008).

#### 2.3.1.5 Assessing redox status

There are many *in vitro* and *in vivo* methods for assessing the redox status of a cell/organism, but for the purpose of this study I will be focussing on assessing the H<sub>2</sub>O<sub>2</sub> reactive species production *in vitro* as well as the antioxidant capacity of an endogenous antioxidant, namely glutathione, *in vitro*.

In order to elucidate the level of oxidative stress within cells, one can determine the amount of H<sub>2</sub>O<sub>2</sub> present within the cell. This is a convenient way, as H<sub>2</sub>O<sub>2</sub> has the longest half-life of all ROS species produced within a cell, and various other reactive species are converted into H<sub>2</sub>O<sub>2</sub> (Newsholme *et al.*, 2012). An example of this would be the conversion of the superoxide anion to H<sub>2</sub>O<sub>2</sub> and water via SOD. Therefore, a change in the H<sub>2</sub>O<sub>2</sub> levels within the cell could be indicative of changes in the oxidative stress levels being experienced within the cells. A potential assay that could be used to detect the levels of H<sub>2</sub>O<sub>2</sub> within cells is the ROS-Glo™ H<sub>2</sub>O<sub>2</sub> Assay from Promega (Promega, USA).

Another effective way of determining the redox status of a cell is to determine the endogenous antioxidant capacity of that cell. This could then give insight as to the susceptibility of that certain cell type to increases in oxidative stress levels, and the capacity of the cell type to deal with different levels of oxidative stress. An example of this would be using the GSH/GSSG-Glo™ Assay from Promega (Promega, USA). This assay allows one to determine the ratio between reduced Glutathione (GSH) and oxidised Glutathione (GSSG) within a cell following exposure to increased levels of oxidative stress. Glutathione is an abundant antioxidant present in eukaryotic cells, most of which exists as GSH. Certain chemicals react with GSH to form GSSG, decreasing the ratio of GSH/GSSG present in the cell. Changes in the ratio of GSH/GSSG is associated with a diseased state of the cell, and indicates higher levels of oxidative stress.

### 2.3.2 Inflammation

Just as oxidative stress plays a key role in normal healthy functioning and host survival, inflammation is a vital process involved in normal body functioning. Sources of inflammation are widespread, and include bacterial or viral infections, exposure to allergens, radiation or toxic chemicals, autoimmune and chronic diseases, obesity, consumption of alcohol, tobacco use, and a high calorie diet (Aggarwal *et al.*, 2009; Scetter *et al.*, 2010). Inflammation is largely non-specific, but it interacts with many other innate and adaptive immune processes to strengthen the body's resistance to pathogens and mutated cells.

The initial stage of inflammation, known as acute inflammation, is mediated through activation of the immune system. This inflammation is resolved after a short period of time and has shown many benefits within the host organism (Reuter, *et al.*, 2010). Some of these benefits include detoxification, facilitation of the healing process, tissue repair and removal of infection. If inflammation persists for a longer period, it is classified as chronic inflammation, and this can be detrimental to the host and may result in development of a wide array of chronic diseases, including cancer. The longer the inflammation persists, the higher the risk of cancer (Reuter *et al.*, 2010). The process of inflammation is a complex, multifaceted process. However, a comprehensive review on inflammation is beyond the scope of this thesis. Therefore, rather, the next section will focus specifically on how inflammation may exacerbate cancer progression, and more specifically how it links to oxidative stress in this context.

#### 2.3.2.1 Inflammation in cancer

Despite the fact that inflammation serves as a localised protection for tissue irritation or infection and thereby is vital for optimum function and protection of the host organism, it also has a negative impact in many chronic diseases such as cancer, diabetes, etc. Chronic inflammation is one of the hallmarks of many different types of cancers, and it has been observed that chronic inflammation over long periods of time results in a 15-20% higher risk of developing cancer in our lifetime (Del Prete *et al.*, 2011). Unresolved inflammation due to any failure in the immune response may disrupt the cellular microenvironment, which could result in genetic mutations in cancer-related genes, DNA repair mechanisms, and

posttranslational modifications in proteins involved in the cell cycle. Furthermore, inflammatory cells themselves may also contribute to tumour progression within the tumour microenvironment, as well as to angiogenesis and metastasis (Eiro and Vizoso, 2012).

In terms of cancer initiation and inflammation, one of the first links is the presence of macrophages at the site of tumour initiation and development and may be found in areas demonstrating hyperplasia, a very early stage in cancer initiation (Mantovani *et al.*, 2006). Macrophages are an essential part of the hosts immunity, and in the context of regular inflammation and day to day normal functioning, they play a very crucial role in immune regulation, wound healing, removal of invading microbes, etc. However, in the context of cancer, macrophages can contribute to the process of carcinogenesis. Macrophages are associated with promoting tumour growth through creation of an inflammatory environment which is mutagenic via the generation of reactive oxygen species, an example of the link which exists between oxidative stress and inflammation (Qian and Pollard, 2010). This mutagenic environment may result in activation of certain oncogenes or cancer suppressing genes, as well as certain transcription factors involved in tumour initiation and progression.

In addition to macrophages, neutrophils and mast cells can also drive the process of tumorigenesis. Neutrophils are an essential player in the innate immune response and are often the first responders to sites of infection and function to remove invading microbes or pathogens, and thus are extremely important in the inflammatory response. Mast cells also play an important role in host defence and are activated during the inflammatory response to secrete mediators, cytokines and histamine, all necessary to remove invading pathogens and infection. However, in the context of cancer, both neutrophils and mast cells may upregulate the production of non-specific pro-inflammatory cytokines such as tumour necrosis factor (TNF), interleukin-1 $\alpha$  (IL-1 $\alpha$ ) or IL-1 $\beta$ , or IL-6, present during an immune or inflammatory response, and this contributes to carcinogenesis (Aggarwal *et al.*, 2006). Although TNF contributes to carcinogenesis (Wang and Lin, 2008), this factor also has an important role in inflammation, and is involved in cell survival, cell proliferation and cell death. However, in terms of carcinogenesis, TNF exerts its cancer effects through signalling the transcription factor nuclear factor- $\kappa$ B (NF- $\kappa$ B) (Feller *et al.*, 2013). This has been observed to be one of the primary links between inflammation and tumour initiation and has been observed to allow preneoplastic as well as malignant cells to evade apoptosis (Karin and



Naugler, 2008). NF- $\kappa$ B has also been implicated in the expression of several pro-inflammatory gene products, such as IL-1 $\beta$ , IL-1 $\alpha$ , IL-8, chemokines, Cyclooxygenase-2 (COX-2), etc., all of which play a critical role in suppressing apoptosis, promoting angiogenesis, metastasis and invasion (Avalle, *et al.*, 2017). It is therefore no surprise that NF- $\kappa$ B has been found to be constitutively active in most tumours, and again illustrates the link between inflammation and cancer.

Following tumour development, a different mechanism of inflammation can ensue which may allow for the survival of the tumour in the host. Most malignancies trigger an inflammatory response, creating a tumour-promoting microenvironment (Mantovani, *et al.*, 2008). Oncogenes, such as the RAS gene, when expressed can result in the recruitment of leukocytes and tumour-promoting chemokines, further perpetuating the malignancy. In addition to this, an “angiogenic” switch may be activated, allowing angiogenesis to occur (Soucek *et al.*, 2007). Following growth of the tumour, the malignancy eventually becomes nutrient deprived and experience hypoxia due to a limited blood supply. This causes necrosis to occur at the core of the tumour, which results in the expression of pro-inflammatory cytokines, such as IL-1 and vascular endothelial growth factor (VEGF) (Vakkila and Lotze, 2004). This inflammatory response then triggers angiogenesis, allowing the remaining cancer cells to be provided with the nutrients and growth signals necessary for their survival (Grivennikov *et al.*, 2010).

While many epidemiological studies have shown that chronic inflammation predisposes individuals to certain cancers, they have also demonstrated that non-steroidal anti-inflammatory agents may protect against several tumours as well as carcinogenesis (Mantovani, 2005). An example of this would be anti-inflammatory agents which suppress NF- $\kappa$ B. Because NF- $\kappa$ B is so active in tumour cells, suppressing its activity could potentially have anti-cancer effects because this transcription factor is involved in the expression of many pro-inflammatory cytokines and factors which help to drive tumorigenesis in the context of cancer. If the expression of NF- $\kappa$ B is down regulated by an anti-inflammatory drug, it may therefore inhibit tumour growth or carcinogenesis.

### 2.3.2.2 *The link between Inflammation and oxidative stress in cancer*

Inflammation and oxidative stress work in synergy to perpetuate carcinogenesis and tumour progression in the context of cancer. While this topic is complex and has many contributing factors towards carcinogenesis, three main examples will be explained.

Firstly, ROS may be produced following stimulus from pro-inflammatory cytokines in phagocytic and nonphagocytic cells via activation of certain kinases used for signalling. In phagocytes, NADPH oxidase is an enzyme used to remove bacterial or microbial invaders as part of the immune response. Once the phagosome becomes activated following interaction with an invading pathogen, superoxide free radicals accumulate within the phagosome, which are then converted to H<sub>2</sub>O<sub>2</sub>. This creates an acidic environment within the phagosome, allowing the invading pathogen to begin to break down and become destroyed. While this immune response is necessary for the survival of the host, the generation of the free radicals necessary to degrade pathogens may also have carcinogenic effects. In addition, TNF- $\alpha$  is associated with production of ROS via neutrophils, and along with IL-1 $\beta$  and interferon, also pro-inflammatory cytokines, may result in expression of nitric oxide synthase (Federico *et al.*, 2007). The elevated ROS production may also contribute to activation of transcription factors such as NF- $\kappa$ B, which may activate many pro-inflammatory factors as well as promote cell proliferation during cancer initiation and progression (Laurent *et al.*, 2005).

Secondly, free radicals generated may react with the phospholipids found in cellular membranes, resulting in the formation of hydroperoxide and lipoperoxides (Marnett *et al.*, 2003). This alters the properties of the membrane, particularly the membrane permeability, and causes lipid peroxidation to occur (Vernier, *et al.*, 2009). Mutations to neighbouring epithelial cells can also occur due to the increase in free radicals produced through the peroxidation. This could result in the recruitment of inflammatory cells such as neutrophils to the site of lipid peroxidation, which in turn may result in an increase in free radicals, further perpetuating carcinogenesis.

Thirdly, sustained inflammation in cancer is associated with angiogenesis, as explained previously. Macrophages and monocytes are a major source of angiogenic-promoting factors such as proinflammatory cytokines, NO, prostaglandins and VEGF (Zouki *et al.*, 2001). These factors can induce the formation of ROS and contribute to the perpetuation of tumorigenesis

via angiogenesis. When arachidonic acid, found in the membranes of cells, undergoes oxidative metabolism, as described above, prostaglandins are generated, which in turn may induce the expression of pro-inflammatory cytokines, enhancing the production of ROS species (Baron and Sandler, 2000). COX-2 is one of the primary enzymes responding to the production of prostaglandins and are found to be upregulated in many different types of cancer, including breast carcinomas (Gupta and Dubois, 2001).

From the literature reviewed here, the role of the self-propagating oxidative stress-inflammation loop in cancer progression is undeniable. Despite many decades of cancer research, no generally applicable effective therapy has been identified, perhaps due to the lack of these therapies to address these processes specifically. Given the shift in consumer bias towards natural medicines, the next section provides a brief overview of progress made in terms of plant-derived natural medicines in the context of cancer treatment or prevention.

#### **2.4 Phytomedicine in cancer**

Biomedical or “Western” approaches to medicine are typically associated with the physical body of an individual, with treatment based on technology, science, clinical analysis and knowledge. This is quite different to that of traditional medicine, which may be defined as “the sum total of all knowledge and practices, whether explicable or not, used in diagnosis, prevention and elimination of physical, mental, or societal imbalance, and relying exclusively on practical experience and observation handed down from generation to generation, whether verbally or in writing” (Richter, 2003). Although contrasting in approach, both play an imperative role in the field of medicine.

In several countries worldwide, particularly those of low- and middle- income status, it has been found that the number of biomedical practitioners may not be enough to meet the needs of the population, showing the importance of traditional medicine as a health resource (Oyebode *et al.*, 2016). It has been estimated that within South Africa alone, there are at least 200 000 traditional healers, and only 25 000 biomedical doctors (Matomela, 2004). This almost 10:1 ratio demonstrates the fact that the South African population has a higher accessibility to these traditional healers, and therefore the important role these healers have

in addressing the physical and mental health needs of a large portion of the population (Morris, 2001).

The main source of medication used and prescribed by these traditional healers are plant-based and herbal remedies, with more than 770 plant species being traded and used in South Africa alone (Mander *et al.*, 2007). Trade within this industry is said to contribute at least R29 million to the economy each year, signifying just how essential this industry is (Mander *et al.*, 2007). However, not only are plant-based medications popular amongst traditional healers and their patients, they are also commercially more popular due to the increased consumer demand for phyto-pharmaceutical therapies (Patnala and Kanfer, 2012). Humans have been using products or materials from nature, such as plants, microorganisms and animals, as medication for different diseases for thousands of years. Increased demand for plant-based medicine has also been contributed to the fact that development of novel pharmaceutical medicines appears to be slowing down significantly, and thus over the last decade more focus has been placed on natural products and combining them with the high-throughput technology used in medicine today (Yuan *et al.*, 2016). Plant-based therapies have several advantages over therapies based on biomedical treatments or pharmaceutical drugs, and one main example of this is the unique chemical range of plant-based products. This lends itself towards diversity in their mode of action, and potentially could allow for treatment in a wide array of diseases due to their multiple molecular targets.

Within the context of cancer specifically, plant-based products have become an attractive alternative to traditional treatment, most commonly chemotherapy and radiation. Traditional therapies have been associated with many pitfalls such as high costs of treatment, secondary toxicity of anti-cancer drugs, and adverse drug reactions such as vomiting, pain, nausea, constipation, diarrhoea etc., which may require additional medical attention (Nurgali, *et al.*, 2018). Thus, the risk often outweighs the benefit when it comes to the use of traditional chemotherapeutic agents, which has resulted in many cancer sufferers to consider alternative therapies, one of which is the use of plant-based medication.

Plant-based medications have been used for prevention and treatment of cancer for centuries, first seen in Africa, Asia and Europe centuries ago. Many plant extracts have been believed to prevent carcinogenesis, decrease tumour size and help prevent cancer-related symptoms (Greenwell and Rahman, 2015). Certain pure phytochemical extracts have already

been approved by the Food and Drug Administration (FDA) or have at least advanced to clinical trials for use as anti-cancer treatments, such as resveratrol obtained from grape skins, curcumin from turmeric, pomegranate extract, lycopene extracted from tomatoes, etc. (Paller *et al.*, 2016). This illustrates the potential for use of plant-based therapies and phytochemicals for the prevention and treatment of cancer and is indicative of their ability to withstand scientific testing.

In terms of common anti-cancer mechanisms of medicinal plants for which scientific data is available, a comprehensive review of medical plants used in cancer therapy has recently been published (Oyenihi and Smith, 2018). Some of the plants reviewed included *Vitis vinifera* (grapes), *Curcuma longa* (turmeric), *Azadirachata indica* (Indian lilac), *Glycine max* (soybeans), *Olea europaea* (olives), etc. It was demonstrated that these plants, among others reviewed, all had a high polyphenol content, and these plant-based polyphenol compounds are of the most prominence in the context of anti-cancer medication. A major advantage seen with the use of polyphenolic compounds is their ability to interact with multiple molecular targets (Kruger *et al.*, 2014), and therefore make use of many different mechanisms of prevention or treatment of carcinogenesis. For the plants commonly used in cancer treatment, scientific data illustrates anti-inflammatory and/or antioxidant functioning for the majority.

For the purpose of this thesis, I would like to focus on two specific plant-extracts which are commonly consumed in combination and which anecdotally have anti-cancer effects, namely  $\Delta$ -7-mesembrenone (isolated from *Sceletium tortuosum*) and cannabidiol (CBD) (isolated from *Cannabis sativa*).

#### 2.4.1 *Sceletium tortuosum*

This plant has many anecdotally claimed uses, including natural medicine, dietary supplements, raw materials, use in veterinary treatments, and pharmacology (Gericke and Viljoen, 2008). *Sceletium* is a small groundcover plant which is indigenous to South Africa, particularly the Western Karoo area (figure 2.5), belonging to the family of plants known as *Mesembryanthemaceae*. Plants belonging to this family are distinguished by their dry leaves which appear to be “skeletonised” due to its pronounced lignified leaf vein structure,

accounting for the name *Sceletium*, deriving from the Latin word *Sceletus* (figure 2.6a) (Patnala and Kanfer, 2017). In addition to these dried-out leaves, *Sceletium* plants also have succulent leaves surrounding their flowers (figure 2.6b), whose petals may vary between white, light pink and yellow in colour. Currently, 8 species of the *Sceletium* plant are recognised, and can be divided into two “types” – the *Tortuosum* type and the *Emarcidum* type. The *Tortuosum* type includes: *Sceletium tortuosum*, *Sceletium expansum*, *Sceletium crassicaule* and *Sceletium varians*, while the *Emarcidum* type includes: *Sceletium exalatum*, *Sceletium emarcidum* and *Sceletium rigidum* (Patnala and Kanfer, 2017) Of these, *Sceletium tortuosum* has been used in natural products and has been studied the most extensively of the 8 species.



Figure 2.5 – Geographical map indicating the distribution of *Sceletium* in South Africa (Gerike and Viljoen, 2008)



Figure 2.6a – *Sceletium* plant and its “skeletonised appearance” of the dried leaves (Gericke and Viljoen, 2008)



Figure 2.6b – *Sceletium tortuosum* plant surrounded by its white flowers (Patnala and Kanfer, 2007)

#### 2.4.1.1 *Sceletium* and its alkaloids

Traditionally, *Sceletium tortuosum*, also known as “Kanna” or “kougoed”, has been used for centuries by the San hunter-gatherers and the Nama population of Southern Africa, many years before the first uses of these plants by European settlers (Van Wyk, 2011). Historically, this plant has been used as a masticator for a variety of reasons, such as the anecdotal relief from hunger or thirst, relief from abdominal pain, relief from toothache, to treat colic in infants, and has even been said to have a sedative effect (Gericke and Viljoen, 2008).

There are four different classes of alkaloids found within the *Sceletium* plants, namely, (1) the 3a-aryl-*cis*-octahydroindole class, (2) the *C*-secomesembrine alkaloids, (3) the alkaloids containing a 2,3-disubstituted pyridine moiety and (4) a ring *C*-seco *Sceletium* alkaloid A<sub>4</sub> group (Gericke and Viljoen, 2008).

*Sceletium* is currently one of the only known genus of plants with their species containing high levels of the alkaloid Mesembrine, one of the most pharmacologically-active alkaloids in this plant (Krstenansky, 2016). *Sceletium tortuosum* is known to contain mesembrine, mesembrenone, mesembranol, mesembrenol, alkaloid A<sub>4</sub>, chennanine and tortuosamine



(Gericke and Viljoen, 2008). It has been reported that only the *tortuosum* type of *Sceletium* plants contain these alkaloids, where as those of the *emarcidum* type are said to be totally void of mesembrine, or any other types of alkaloids associated with the species. The four major alkaloids that define the alkaloidal composition of *Sceletium tortuosum* are mesembrine ((3aS, 7aS)-3a-(3,4-dimethoxyphenyl)-1-methylhexahydro-1H-indol-6(2H)-one), mesembranol, ((3aS,6R,7aS)-3a-(3,4-dimethoxyphenyl)-1-methyloctahydro-1H-indol-6-ol) mesembrenone ((3aR,7aS)-3a-(3,4-dimethoxyphenyl)-1-methyl-3,3a,7,7a-tetrahydro-1H-indol-6(2H)-one) and mesembrenol ((3aR,6S,7aS)-3a-(3,4-dimethoxyphenyl)-1-methyl-2,3,3a,6,7,7a-hexahydro-1H-indol-6-ol) (Shikanga *et al.*, 2012; Krstenansky, 2017) (Figure 2.8). *Sceletium tortuosum* extracts differ in their potential for various medical treatments or preventative agents in accordance with their alkaloidal composition, and extracts higher in certain alkaloidal content have been found to have different medicinal properties.

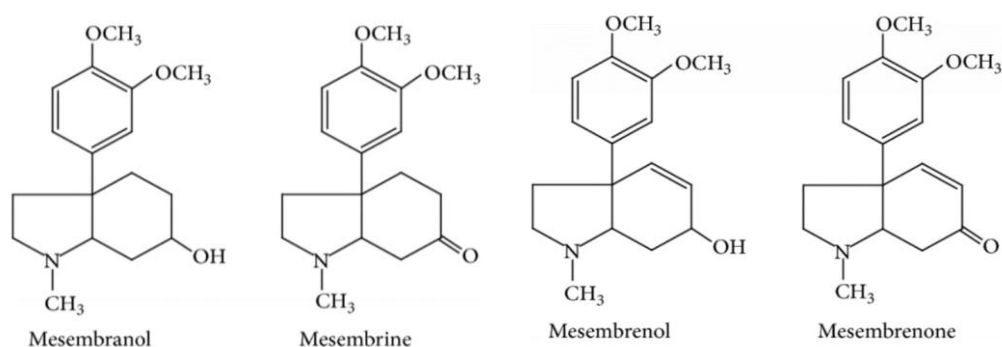


Figure 2.7 – The most abundant alkaloids found within *Sceletium tortuosum* (Chiu, *et al.*, 2014)

#### 2.4.1.2 The alkaloidal content of *Sceletium* determines its properties

Today, *Sceletium tortuosum* has a variety of uses, one of which is often to treat neurological disorders and neurodegeneration, due to its ability to suppress the central nervous system (Shikanga *et al.*, 2012). Recently, it has been discovered that *Sceletium tortuosum* high in mesembrine alkaloid content acts primarily as a monoamine releasing agent (MRA) and has a secondary function as a selective serotonin reuptake inhibitor (SSRI), two of the most common classes of drugs used when treating anxiety and depression (Coetzee *et al.*, 2015).



Zembrin is a standardised extract of *Sceletium tortuosum* high in Mesembrine which has recently been identified as to interact with the Central Nervous System (CNS) through prevention of blockage of the Serotonin (5-HT) transporter and inhibition of phosphodiesterase-4 (PDE4) activity (Terburg *et al.*, 2013). In this same study Zembrin was also shown to reduce anxiety-related amygdala reactivity and attenuate amygdala-hippocampus coupling, both of which helping to reduce the hyper-responsivity to mild threats, as seen in anxiety sufferers. In addition to its potential anti-depressant effects, Zembrin has also shown potential benefit in the treatment of schizophrenia via dual targeting of both PDE4 activity and serotonin activity, resulting in improvement of the negative symptoms and cognitive deficits of schizophrenia (Chiu *et al.*, 2017).

In addition to its anxiolytic and anti-depressant effects, *Sceletium tortuosum* has been shown to have both mild anti-inflammatory, as well as potent antioxidant effects, depending on the alkaloidal content of the extract used. Firstly, *Sceletium tortuosum* containing a high mesembrine alkaloid content, known as Trimesemine™, was shown to possess a mild anti-inflammatory and cytoprotective effect in the context of acute inflammation in the peripheral compartment, ultimately resulting in increased monocytic cell viability and limited basal (unstimulated) production of pro-inflammatory cytokines (Bennett and Smith, 2018). A second *Sceletium tortuosum* extract, high in  $\Delta$ -7 mesembrenone alkaloid content, was found to be a potent antioxidant, as it acted as a powerful inhibitor of tyrosinase activity, and had a similar effect on this enzyme as kojic acid at high doses (Bennett and Smith, 2018). The antioxidant activity of the  $\Delta$ -7 mesembrenone extract was further corroborated by performing a 2,2-diphenyl-1-picrylhydrazyl (DPPH) inhibition assay in which the antioxidant capacity of  $\Delta$ -7 mesembrenone was very similar to ascorbic acid, which is a well-known antioxidant (Allen and Bayraktutan, 2009). Finally, the polyphenol content of  $\Delta$ -7 mesembrenone was determined to be relatively high, 20-fold higher than the polyphenol content of Trimesemine™ (Bennett and Smith, 2018). These studies highlight the potential different mechanisms and functions of *Sceletium tortuosum* extracts containing different alkaloidal compositions. These studies do however also illustrate the potential of *Sceletium tortuosum* as a supplement in the setting of inflammation related chronic disease, and the prospective prevention of these diseases through the mild anti-inflammatory and antioxidant

mechanisms. However, the potential anti-cancer efficacy of especially the potent antioxidant  $\Delta$ -7 mesembrenone is still unknown.

#### 2.4.2 *Cannabis sativa*

*Cannabis sativa* is an annual dioecious plant belonging to the family Cannabaceae (Fischedick *et al.*, 2010). *Cannabis sativa* is possibly one of the oldest plants cultivated by man, but has remained controversial throughout its history. *Cannabis sativa* is a plant originating from central Asia and has gained popularity worldwide, cultivated in many different habitats and geographical regions (Merlin, 2003). For more than 5000 years, *Cannabis* has been used as a textile fibre, in traditional medicine, and as a source of food (Mikuriya, 1969). Evidence suggests that many different cultural groups cultivated *Cannabis sativa* thousands of years ago, such as the Chinese, the Egyptians and the Romans (Mercuri *et al.*, 2002). The plant arrived in India approximately 1000 years ago, and due to its narcotic properties, it was used in many religious rituals, as well as an anaesthetic (Fankhauser, 2002).

Within Western medicine specifically, *Cannabis* had gained popularity as a medicinal plant during the 19<sup>th</sup> Century, and by early 20<sup>th</sup> Century, pharmaceutical companies had started producing several *Cannabis*-based medicinal products, such as Merck (Germany), Parke-Davis (USA), Burroughs-Wellcome (England) and Eli Lilly (USA) (Aldrich, 1997). These *Cannabis* products were prescribed by doctors for medical issues such as asthma, whooping cough, pain and as a sedative (Fankhauser, 2002). However, this eventually was stopped a few years later due to varying potencies of the *Cannabis* extracts, unpredictable responses across individuals, as well as side effects of anxiety and cognitive impairment (Zuardi *et al.*, 2006). In addition, it was during this time that other significant medical advancements were made, such as the development of certain vaccines, the emergence of aspirin and the development of hypodermic syringes which allowed for injection of drugs such as morphine (Li and Lin, 1974). However, for various reasons, *Cannabis* is still widely consumed for both recreational and medicinal applications.

### 2.4.2.1. Cannabinoids

Phytocannabinoids are a group of terpenphenolic compounds predominantly produced in *Cannabis*. The most common and abundantly present cannabinoids within *Cannabis sativa* are  $\Delta^9$ -Tetrahydrocannabinol ( $\Delta^9$ -THC), Cannabidiol (CBD), Cannabinol (CBN), Cannabigerol (CBG), and Cannbichromene (CBC) (Andre *et al.*, 2016).

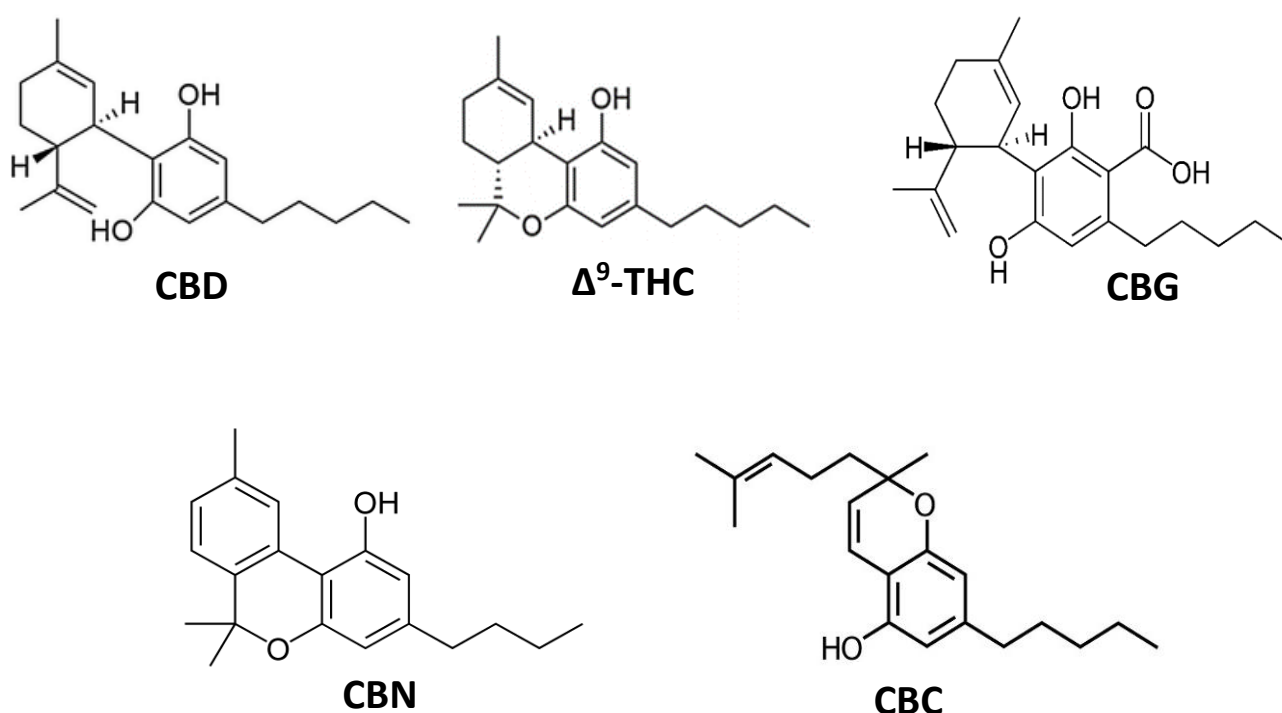


Figure 2.8 – The most abundant cannabinoids found within *Cannabis sativa* (Adapted, Palazzoli, 2017).

Of these cannabinoids mentioned,  $\Delta^9$ -THC and CBD are the most commonly studied in the context of phytomedicine.  $\Delta^9$ -THC has been involved in many different medical experiments and has shown promising results in the context of a variety of medical diseases. An example of this is the therapeutic use of  $\Delta^9$ -THC in AIDS patients as an appetite stimulant, as well as to prevent disease-associated nausea and vomiting (Izzo *et al.*, 2009). A *Cannabis* extract containing  $\Delta^9$ -THC and CBD in a ratio of 1:1, known as Sativex, has also been marketed and used for pain relief in patients with multiple sclerosis, and as an additional treatment in

advanced cancer therapy (Russo, *et al.*, 2007). While  $\Delta^9$ -THC has a variety of benefits associated with its use, it is also the cannabinoid with the greatest psychotropic properties, and with this has been associated with a range of adverse side effects, such as memory loss, impaired brain function, psychosis, dizziness and dry mouth to name a few (McGee *et al.*, 2002; Ferguson *et al.*, 2008). It is for this reason that the other non-psychotropic cannabinoids have gained interest and popularity within the context of plant-based medication and therapy. To date, CBD in particular has gained much popularity in the field of medicine and has shown to have potential in a variety of diseases, with no psycho-stimulating properties and very few known side effects (Pertwee, 2008; Zuardi, 2008).

#### 2.4.2.2. Cannabidiol in cancer

Within cancer specifically, there have been a variety of uses for *Cannabis sativa*. Preparations containing  $\Delta^9$ -THC and CBD have been used in cancer patients to help with an increase in appetite, to help treat nausea following chemotherapy, and to alleviate cancer-associated pain (Hall *et al.*, 2005). However, given the adverse effects of whole *Cannabis* or  $\Delta^9$ -THC on for example memory, CBD has gained popularity as preferred medicinal *Cannabis* isolate.

Cannabidiol has been shown to exert anti-proliferative as well as pro-apoptotic effects in a variety of human cancer cell lines, such as breast carcinoma, prostate carcinoma, gastric adenocarcinoma and colorectal carcinoma (Ligresti, *et al.*, 2006). Furthermore, CBD has the potential to increase the production of ROS via the regulation of intracellular  $[Ca^{2+}]$  in tumour cells, subsequently inducing apoptosis (Ryan, 2009). Treatment with CBD also resulted in the down-regulation in expression of Id-1, a gene involved in the regulation of the metastatic potential of cancer, thereby causing a reduction in the aggressiveness of the cancer (McAllister, 2007). Finally, and of most relevance to this study, Cannabidiol has been shown to act both as an anti-inflammatory and an antioxidant, thereby assisting in the prevention of initiation or progression of cancer (Turner, 1981).

### 2.4.2.3. CBD as an anti-inflammatory

As mentioned previously, cancer is a chronic disease associated with chronic inflammation over a long period of time, and the resulting oxidative stress that comes with it. It would thus make sense that use of an anti-inflammatory agent could potentially alleviate the inflammatory load in the host and prevent cancer initiation or further progression of the disease. Studies have recently been conducted which suggest that *Cannabis sativa*, and more specifically CBD, can act as an anti-inflammatory agent and have immunosuppressive properties (Klein, 2005). In this study by Klein, it was shown that the endocannabinoid system is involved in host inflammatory response and plays a direct role in cytokine production and leukocyte formation. Further studies have indicated that CBD has the potential to decrease the production and expression of TNF- $\alpha$ , IL-6, IL-1 $\beta$  and IL-8, all pro-inflammatory cytokines in human and animal models (Mecholulam, 2007). This potential of CBD as a pro-inflammatory cytokine mediator could be of great benefit within the context of cancer and alleviating the symptoms of inflammation. In addition to this, Cannabidiol may decrease inflammation via enhanced adenosine signalling mediated through inhibition of adenosine uptake (Burstein, 2015). It has been suggested in previous studies that adenosine A2A receptors may down-regulate immune cells that may be over active, resulting in a decline in the inflammatory response, and may be another mechanism through which CBD decreases inflammation. Finally, exposure to CBD in human and rat models has found a reduction in the immune response, whereby T-cell response and proliferation was suppressed and neutrophil migration was inhibited (Barichello *et al.*, 2012).

While inflammation related to cancer remains the focus of this study, the anti-inflammatory potential of CBD is also useful in the treatment and prevention of many other different diseases and disease-related symptoms. Recently, it was determined that CBD has potential anti-inflammatory benefits in the treatment of inflammation-related diseases such as Psoriasis, arthritis, Parkinson's Disease, Alzheimer's disease, Huntington's disease and analgesia (Wilkinson and Williamson, 2007; Capasso *et al.*, 2008; Costa, 2007).

#### 2.4.2.4 CBD as an antioxidant

In addition to its anti-inflammatory effects, Cannabidiol is also a potent antioxidant. CBD has been shown to have protective antioxidant activity similar to that of ascorbate or  $\alpha$ -tocopherol, both of which are well-characterised potent antioxidants (Hampson, 1998). Previous studies have demonstrated Cannabidiol's ability to limit or inhibit glutamate toxicity mediated via its ability to reduce oxidative stress (Marsicano *et al.*, 2002). In addition to this, when administered concurrently with binge ethanol consumption, CBD protected against oxidative stress-induced neurodegeneration with the same efficacy as  $\alpha$ -tocopherol (Hamelink *et al.*, 2005). Finally, further studies have shown that CBD has the capability to regulate the production of  $\text{Ca}^{2+}$  by the mitochondria, which may also mediate the production of ROS production and regulate intracellular oxidative stress levels (Ryan *et al.*, 2009).

Study of the literature has therefore clearly indicated that Cannabidiol and other cannabinoids such as  $\Delta^9$ -THC may act as potent antioxidants, however little is known about the antioxidant effects of Cannabidiol within the context of cancer. This has set up the focus for this study, in order to better understand the antioxidant potential of Cannabidiol in the context of breast cancer treatment and prevention and to potentially elucidate the cellular mechanisms involved.

### 2.5 Drug-Drug Interactions

Medical drugs are chemical compounds which perform a biological function once ingested and incorporated into the body. These compounds mimic the behaviours and functions of certain biomolecules in order to carry out a physiochemical function, after which they are generally excreted. When two or more drugs are administered at the same time, they naturally will influence the function, duration and strength of their respective pharmacological actions via interaction with one another (Yoshikawa *et al.*, 2004).

Drug interactions may have desired, reduced or unwanted effects, and the incidence of drug-drug interactions increases with increasing number of drugs administered at the same time. Possible drug-drug interactions include an additive effect, whereby administration of both medicinal drugs may increase the absorption and effect of each separate drug. This results in

a greater effectiveness of one or both drugs and is associated with a favourable treatment outcome. In contrast to this, drugs may also interact antagonistically to one another, resulting in a decrease in the function or uptake of one or both drugs. This may result in one or more of the drugs have a diminished function or a loss of function all together, an unfavourable outcome. Finally, adverse drug reactions occur when two or more drugs are taken in combination, resulting in negative side effects when taken in combination to one another. These side effects have the potential to be dangerous and may result in hospitalisation or even death. Therefore, when administering two drugs simultaneously, it is important to study their interactions with one another and to determine the side effects of their concurrent use.

## 2.6 Summary

Cancer is a chronic, non-communicable disease responsible for 22% of deaths worldwide annually. While all cancers have their own set of unique triggers, carcinogenesis and tumour progression, most cancers are also associated with chronic inflammation and high levels of oxidative stress. Within breast cancer specifically, estrogen receptor status and oxidative stress levels have been linked to both carcinogenesis and progression of the disease. This has been thought to be due to the unresolved oxidative stress within ER+ cells, while ER- cells may resolve oxidative stress more efficiently. Within the context of cancer therapy, plant-based medicines could be promising therapeutic avenues, due to their high antioxidant and anti-inflammatory modes of action, reducing the load of inflammation or oxidative stress and therefore either preventing or slowing cancer progression. Exposure of breast cancer cells to an antioxidant such as  $\Delta$ -7-mesembrenone or Cannabidiol may result in cell death, and this could occur for a number of reasons. Firstly, an increase in antioxidant capacity within the cell may result in an increased ability to quench free radicals and subsequently a reduction in the oxidative stress levels, therefore preventing them from initiating carcinogenesis or preventing tumour progression. Alternatively, administration of an antioxidant at a dose too high may result in a shift towards a pro-oxidant status. This would cause the antioxidant to lose its radical scavenging ability and further drive oxidative stress. This may push the cell beyond its ability to survive the increased oxidative stress, resulting in cell death. Two plant-based extracts were investigated in this study, namely  $\Delta$ -7 mesembrenone isolated from *Sceletium tortuosum* and Cannabidiol extracted from *Cannabis sativa*. These extracts were chosen due

to their increase in popularity and consumption in the last decade, and because they are often used in conjunction with one another. We could find no literature published in the context of cancer-specific investigations for  $\Delta$ -7 mesembrenone, and little experimental data exists in which the redox ability of Cannabidiol is described within the context of cancer specifically. This has set up the focus for this thesis, in order to better elucidate the redox capabilities of these two plant-based extracts within the context of breast cancer prevention or treatment.

## 2.7 Hypothesis and aims

### 2.7.1 Hypothesis

We hypothesised that both  $\Delta$ -7-mesembrenone from *Scelletium tortuosum* and Cannabidiol (CBD) from *Cannabis sativa* would have an anti-cancer effect when exposed to two breast cancer cell lines, MCF7 (ER+) and MDA-MB-231 (ER-).

### 2.7.2. Aims

In order to test our hypothesis, we formulated the following aims:

- a) To determine the effect of exposure with two plant-based extracts, namely  $\Delta$ -7-mesembrenone from *Scelletium tortuosum* and Cannabidiol from *Cannabis sativa*, in isolation and in combination, on normal breast cell (MCF12A) and breast cancer (MCF7 and MDA-MB-231) cell viability.
- b) To elucidate cellular mechanisms related to redox status, by which these plant products may achieve their effects on cell survival.
- c) To assess potential interaction effects of the two plant extracts in this context.



## CHAPTER 3: METHODS AND MATERIALS

### 3.1 Cell Culture

The following cell lines were used for the purpose of this study:

1. MCF12A – A non-tumorigenic epithelial cell line of normal mammary tissue
2. MCF7 – Human breast cell adenocarcinoma (ER+, PR+, HER2-)
3. MDA-MB-231 – Human breast cell adenocarcinoma (ER-, PR-, HER2-)

Cultures of MCF12A, MCF7 and MDA-MB-231 cells were maintained at 37°C and 5% CO<sub>2</sub> in a humidified incubator. MCF12A cells were maintained in DMEM-F12 medium (Lonza, Germany) supplemented with 10% FBS (Fetal Bovine Serum) (Gibco, Life Technologies Corp., USA), 100ng/mL cholera toxin (Sigma-Aldrich, USA), 500ng/mL hydrocortisone (Sigma-Aldrich, USA) and 1mg/mL human insulin (Sigma-Aldrich, USA). MCF7 and MDA-MB-231 cells were maintained in high glucose DMEM (Gibco, USA) supplemented with 10% FBS (Gibco, Life Technologies Corp., USA).

For each experiment, each cell line was exposed to a variation of doses of either  $\Delta$ -7 mesembrenone, CBD or a combination of the two extracts, for a period of 24 hours. After this exposure, the relevant experiments were carried out. Doses used for  $\Delta$ -7 mesembrenone were 0.25mg/mL, 0.5mg/mL or 0.75mg/mL; and CBD doses were 5ng/mL, 10ng/mL, 20ng/mL or 100ng/mL. Doses used for the  $\Delta$ -7 mesembrenone were selected based on recommendations by the supplier of the extract (Verve Dynamics, South Africa), and a preliminary study was conducted by our research group in order to determine the most effective doses of the extract within the context of this study. Cannabidiol doses were selected based on what was consistent with recent literature. Three combinations of the two extracts were also used, namely 0.5mg/mL  $\Delta$ -7-mesembrenone and 20ng/mL CBD (Combination 1); 0.75mg/mL  $\Delta$ -7-mesembrenone and 20ng/mL CBD (Combination 2); and 0.75mg/mL  $\Delta$ -7-mesembrenone and 100ng/mL CBD (Combination 3).

Cell numbers were determined before execution of each experiment by use of a haemocytometer following trypsinisation of each culture. Cells were seeded in 48 well plates

at a density of 50 000 cells per well for the trypan blue and XTT (Sodium 2,3,-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)-carbonyl]-2H-tetrazolium) salt) assays, while 96 well plates were seeded at a density of 25000 cells per well for the ROS-Glo and GSH/GSSG assays.

The trypan blue and XTT assays were performed in triplicate and a minimum of three times. ROS-Glo and GSH/GSSG assays were also performed in triplicate.

### **3.2 Characterisation of *Sceletium tortuosum* extract rich in $\Delta$ -7 mesembrenone and Cannabidiol extracted from *Cannabis sativa***

The  $\Delta$ -7 mesembrenone rich extract was prepared from the *Sceletium tortuosum* plant, and this was selectively extracted to obtain approximately 60%  $\Delta$ -7 mesembrenone alkaloidal content using proprietary methods (Verve Dynamics Pty, Somerset West, South Africa). The Cannabidiol, extracted from *Cannabis sativa*, was procured from Echo Pharmaceuticals B.V. (Weesp, Netherlands) after obtaining a permit from the Department of Health, Republic of South Africa (Permit No: POS 231/2017/2018). Both extracts were kindly donated by Mr Richard Davies (Verve Dynamics Pty, Somerset West, South Africa) for the purpose of this study.

High-performance Liquid Chromatography (HPLC) was employed to further characterize both the  $\Delta$ -7 mesembrenone and Cannabidiol extracts. The Shimadzu HPLC system equipped with LC-10AT single pump and SPD-10A UV-VIS detector as well as a Luna 5  $\mu$ m, C18 (2) 100Å, 150 x 4.6 mm column (Phenomenex, California, USA) was used. Products were dissolved in HPLC-grade absolute methanol and filtered with 0.45  $\mu$ m syringe filter before separations were carried out under isocratic conditions. A mobile phase containing absolute methanol: glacial acetic acid: deionized water (70%:15%:15%) was used for the Cannabidiol extract; and that containing deionized water: acetonitrile (70%:30%) + 1 mL/L aqueous ammonia was used for the  $\Delta$ -7-mesembrenone extract. The flow rate was set at 1 mL/L; detector wavelength at 250 nm; and temperature maintained at 25 °C for the 15 minutes run time of the Cannabidiol extract. For the  $\Delta$ -7-mesembrenone extract analysis, the flow rate was also set at 1 mL/L; detector wavelength at 280 nm; temperature at 25 °C for the 14 minutes run time. 20  $\mu$ L of each sample was injected for both extracts.

### 3.3 XTT mitochondrial reductive capacity assay

The XTT assay was used to determine the effects of the  $\Delta$ -7-mesembrenone, CBD or the various combination doses on the mitochondrial reductive capacity of each cell line, which could then be used to obtain a better understanding of the viability of each cell type following exposure. In brief, MCF7, MCF12A and MDA-MB-231 cells were exposed to either  $\Delta$ -7-mesembrenone, CBD or combination dosages for a period of 24 hours. At the end of the incubation period, media and extracts were removed, and the tetrazolium dye, sodium 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)-carbonyl]-2H-tetrazolium salt (XTT) was added to each well at a concentration of 10mg/mL. An intermediate electron carrier, N-methyl dibenzopyrazine methyl sulfate (PMS), was then added to each well at a concentration of (20 $\mu$ L/mL), to assist with reduction of XTT to a highly pigmented formazan product, as first described (Scudiero *et al.*, 1988). Cells were then incubated for 4 hours at 37°C in a 5% CO<sub>2</sub> humidified incubator. The absorbance of each well was then determined at 490nm, and results were expressed as a percentage of the control for each respective cell line.

### 3.4 Trypan blue cell viability assay

Absolute cell viability was determined using the trypan blue assay as more direct measure of cell viability, to further corroborate results seen in the XTT assay. Each cell type was seeded in 48 well plates at 50000 cells per well and was allowed to grow and adjust for a period of 24 hours. Following this, cells were exposed to the respective doses of either  $\Delta$ -7-mesembrenone, CBD or a combination of the two extracts for a period of 24 hours. Cells were then trypsinised from the wells, the trypan blue azo dye (Thermo Fisher, USA) was added to each sample and 10 $\mu$ l dyed cell suspension loaded into Countess counting chamber slides. Cell viability was subsequently determined using the Countess automated cell counter (Thermo Fisher, USA). The final cell viability was expressed as a percentage of the control for each cell type.

### 3.5 ROS-Glo H<sub>2</sub>O<sub>2</sub> assay

Reactive Oxygen Species (ROS) are generated in all cells and may act as molecules for signalling, however in excess they may lead to cell damage or even cell death. Therefore, measuring ROS production would give a good indication as to what events were occurring within the different cells following exposure to the various extracts and combinations. The ROS-Glo H<sub>2</sub>O<sub>2</sub> assay (Promega, USA) was used to determine the amount of H<sub>2</sub>O<sub>2</sub> generated within the cells, as many types of ROS are converted into H<sub>2</sub>O<sub>2</sub> (Alfadda and Sallam, 2012). Thus, a change in H<sub>2</sub>O<sub>2</sub> may indicate a shift in the ROS levels being generated within the different cell types. This assay was performed after the MCF7, MCF12A and MDA-MB-231 cells were exposed to either  $\Delta$ -7-mesembrenone, CBD or combination dosages for a period of 18 hours. A H<sub>2</sub>O<sub>2</sub> substrate was then added to each well and incubated for a further 6 hours at 37°C in a 5% CO<sub>2</sub> humidified incubator. This H<sub>2</sub>O<sub>2</sub> substrate reacts with H<sub>2</sub>O<sub>2</sub> to generate a luciferin precursor. In the final step, a ROS-Glo detection solution buffer was added, which contained Ultra-Glo™ Recombinant luciferase as well as D-Cysteine, and was incubated for 20 minutes. The D-Cysteine serves to convert the luciferin precursor to luciferin, following which the Ultra-Glo™ recombinant luciferase reacts with the luciferin to generate a luminescent signal in proportion with the H<sub>2</sub>O<sub>2</sub> generated. This luminescent signal was read using a Glo-Max luminometer (Promega, USA). Results were expressed as Relative Light Units (RLU).

### 3.6 GSH/GSSG-Glo assay

Glutathione is present in all eukaryotic cells and serves as an abundant antioxidant source (Sies, 1999). Most Glutathione is found as its reduced form, GSH, whereby the sulfhydryl group of the cysteine is not linked to a second Glutathione. Small amounts of Glutathione exist in its oxidised form, GSSG, and is present as a dimer of two molecules linked through a disulphide bond between the two cysteine groups on both molecules (Pompella, 2003). Glutathione that has been oxidised is indicative of oxidative stress, and a decrease in the ratio between GSH and GSSG is associated with aging, chronic disease and certain signalling events between cells (Ghezzi, 2005). Thus, determining the ratio of GSH:GSSG in all cell types following exposure to either  $\Delta$ -7-mesembrenone, CBD or combination exposures would give

a good indication of the level of oxidative stress occurring within the various cell types, as well as the antioxidant capacities of the different cell lines. The GSH/GSSG-Glo assay (Promega, USA) was used to quantify the total amount of Glutathione within the cells (GSH and GSSG), as well as the amount of oxidised Glutathione, and a ratio was then calculated from these readings. Briefly, MCF7, MCF12A and MDA-MB-231 cells were exposed to either  $\Delta$ -7-mesembrenone, CBD or combination dosages for a period of 24 hours. Following the incubation period, media and extracts were removed, and either Total Glutathione Lysis Reagent (GSH+GSSG) or Oxidised Glutathione Lysis Reagent (GSSG) was added to the cells. The Total Glutathione Lysis reagent lysed the cells and converts all Glutathione present in those cells to its reduced form, GSH. In contrast, the Oxidised Glutathione Lysis reagent lysed the cells and released the GSH and GSSG present in the cells, but it also contains N-Ethylmaleimide, which reacts with GSH specifically and prevents it from generating a luminescent signal. A Luciferin generation agent was subsequently added to all cells and incubated on a shaker at room temperature for 30 minutes. This generation agent creates one mole of luciferin from the GSH probe, and two moles of luciferin per GSSG probe. Finally, Luciferin Detection agent was added to all of the cells and incubated for a further 15 minutes on a shaker at room temperature. The detection agent stops the generation of luciferin and creates a luminescent signal which is proportional to the amount of GSH from the total Glutathione (GSH+GSSG), or to GSSG only. The luminescence of each well was read using a Glo-Max luminometer, and GSH:GSSG ratios calculated from the Relative Light Units (RLUs).

### **3.7 Statistical Analysis**

All statistical analyses were conducted using Graphpad Prism Version 6 software (Graphpad software, La Jolla, CA, USA), and results were expressed as the mean  $\pm$  SD. Two-way ANOVAs were used, as well as Bonferroni *post-hoc* tests, in order to elucidate any differences seen between the cell types and the different dosages of the relevant extract used. Differences between groups and dosages were considered statistically significant at  $P < 0.05$ .

## CHAPTER 4: RESULTS

### 4.1 Characterisation of $\Delta$ -7-mesembrenone extract from *Sceletium tortuosum* and Cannabidiol extract from *Cannabis sativa*

The  $\Delta$ -7-mesembrenone product extracted from *Sceletium tortuosum* was analysed using HPLC methods to determine the polyphenol content of the extract. As depicted in Figure 4.1, there are 3 main peaks seen on the chromatogram, which represent  $\Delta$ -7-mesembrenone, mesembrenone and mesembrine respectively. Quantitatively, 62.2% of this extract was comprised of the  $\Delta$ -7-mesembrenone alkaloid, while 27.9% was made up of mesembrine and 9.8% mesembrenone. This demonstrates the  $\Delta$ -7-mesembrenone rich composition of the extract used in this study.

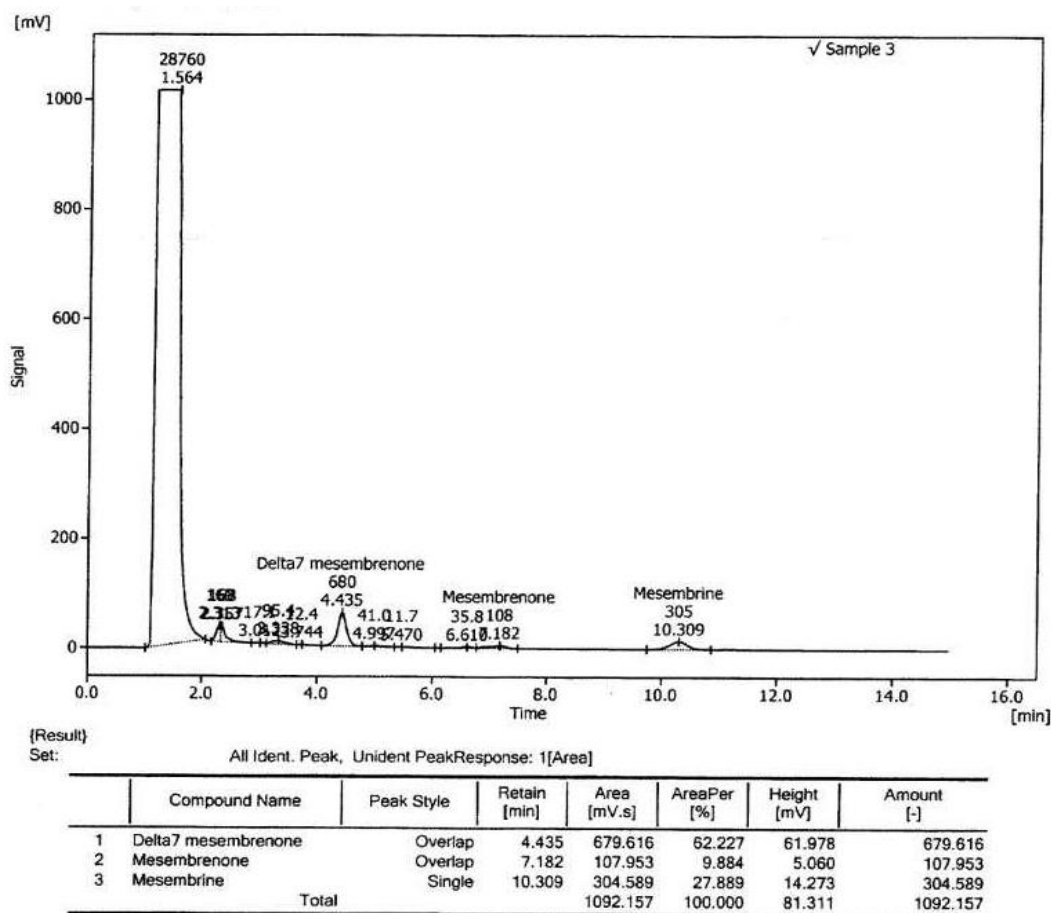
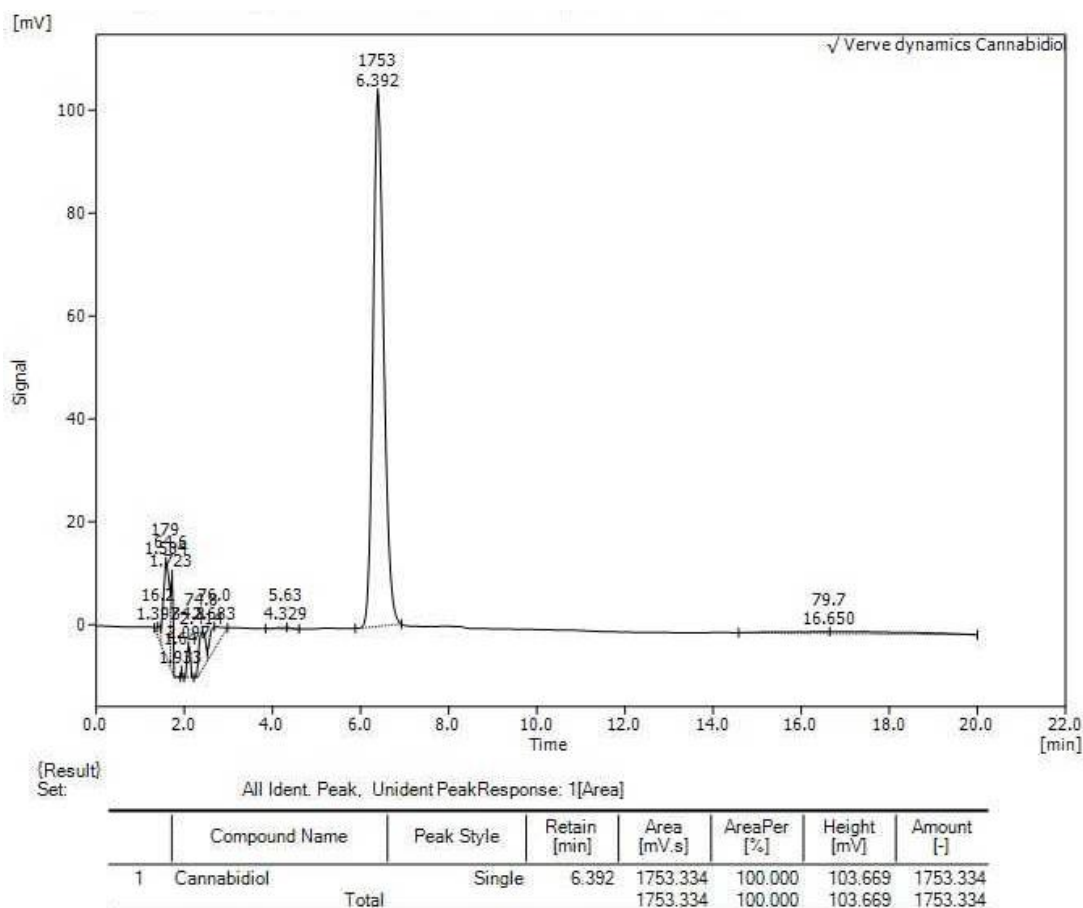


Figure 4.1 – Chromatogram indicating the polyphenol content of the  $\Delta$ -7-mesembrenone extract obtained from *Sceletium tortuosum*

The Cannabidiol extract from *Cannabis sativa* was also analysed using HPLC methods used for the  $\Delta$ -7-mesembrenone product. As illustrated in Figure 4.2, there is 1 peak on the chromatogram, representing 100% Cannabidiol content of this extract.

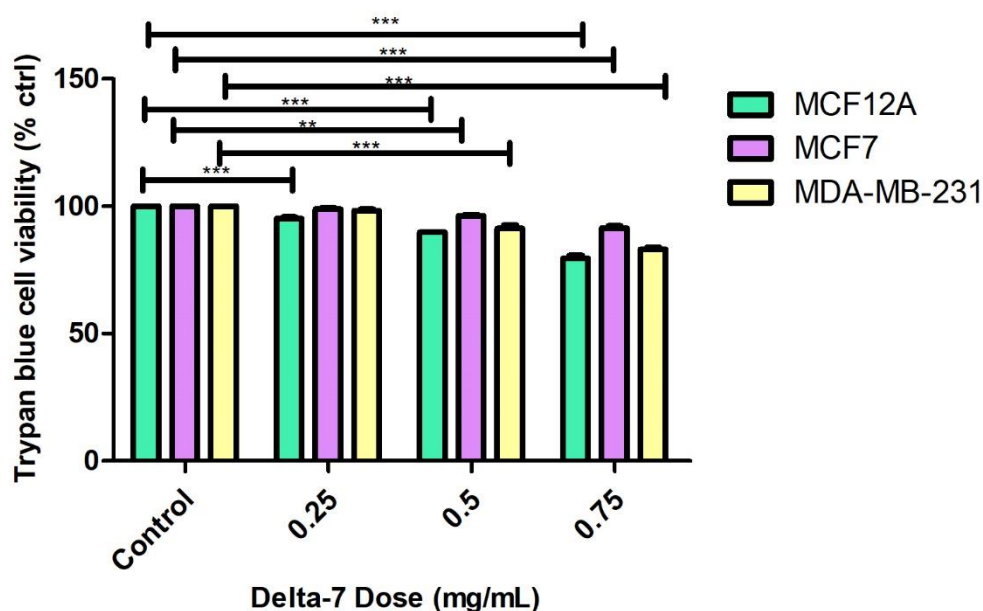


**Figure 4.2 – Chromatogram indicating that the Cannabidiol product extracted from *Cannabis sativa* is 100% CBD**

#### **4.2 Effects of $\Delta$ -7-mesembrenone, CBD or combination treatments on cytotoxicity of MCF12A, MCF7 and MDA-MB-231 cell models**

The Trypan Blue cell viability assay was used to determine the cytotoxicity encountered by the MCF12A, MCF7 and MDA-MB-231 cell models following exposure to  $\Delta$ -7-mesembrenone, CBD or combinations of both extracts. Following exposure to  $\Delta$ -7-mesembrenone, the MCF12A cell line experienced a statistically significant dose-dependent decrease in absolute cell viability across all doses of  $\Delta$ -7-mesembrenone (Figure 4.3). Both MCF7 and MDA-MB-231 cell types also experienced a statistically significant decrease in cell viability for the two

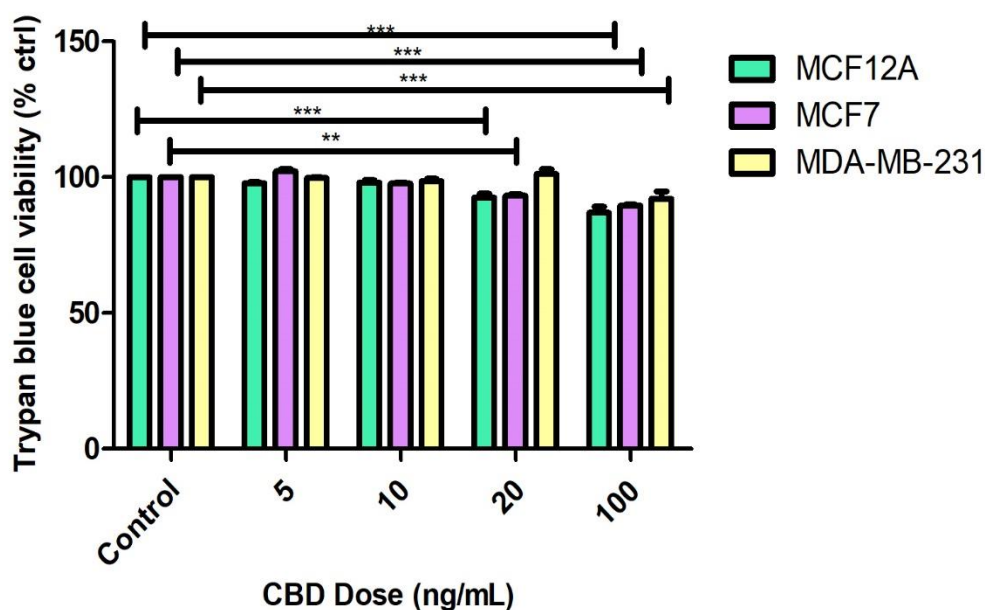
highest doses of  $\Delta$ -7-mesembrenone (0.5mg/mL and 0.75mg/mL). The MDA-MB-231 cell line experienced a larger decrease in cell viability in comparison to control than that of the MCF7 cell line. Following performance of a two-way ANOVA, the main effect of dose had a significance value of  $P < 0.001$ .



**Figure 4.3 – Cell viability of MCF12A, MCF7 and MDA-MB-231 following treatment with increasing doses of  $\Delta$ -7-mesembrenone.** The data are presented as a percentage of the untreated controls for each cell type. Doses used were 0.25mg/mL, 0.5mg/mL and 0.75mg/mL. Statistics were obtained in the form of a two-way ANOVA and a Bonferroni post-hoc test (\*\* =  $P < 0.01$ ; \*\*\* =  $P < 0.001$ ).

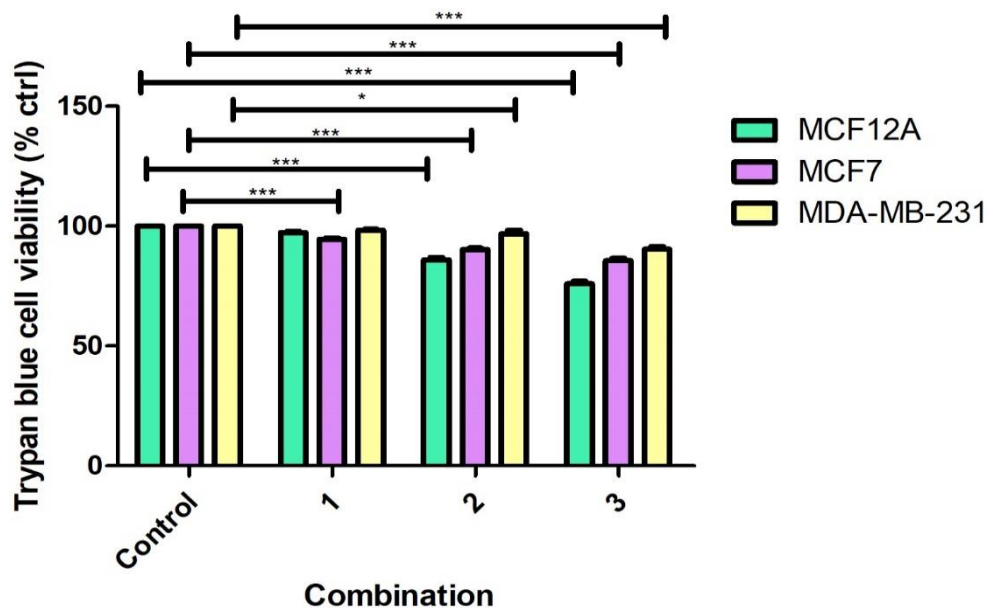
Cell viability of the three cell types was then determined following exposure to CBD at four different doses (Figure 4.4). Statistically significant cytotoxicity was only observed at the two highest doses of CBD (20ng/mL and 100ng/mL) for the MCF12A and MCF7 cell lines, and only at the highest dose (100ng/mL) in the MDA-MB-231 cell line. Results of the two-way ANOVA indicated a main effect of dose significance value of  $P < 0.001$ .





**Figure 4.4 – Cell viability of MCF12A, MCF7 and MDA-MB-231 following treatment with increasing doses of CBD.** The data are presented as a percentage of the untreated controls for each cell type. Doses used were 5ng/mL, 10ng/mL, 20ng/mL and 100ng/mL. Statistics were obtained in the form of a two-way ANOVA and a Bonferroni post-hoc test (\*\* =  $P < 0.01$ ; \*\*\* =  $P < 0.001$ ).

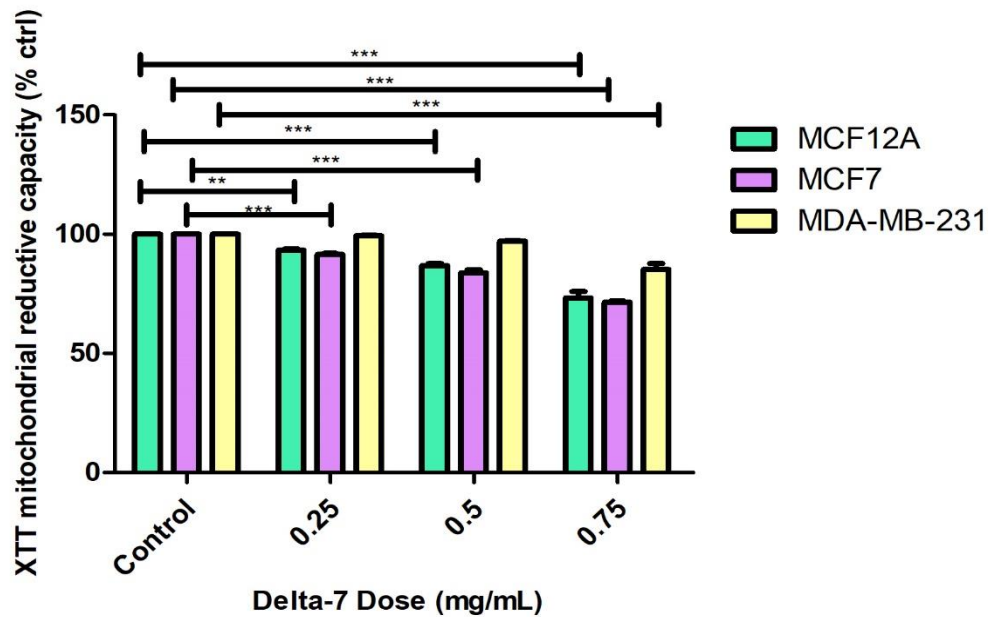
Finally, cell viability was determined following exposure to three different combinations of  $\Delta$ -7-mesembrenone and CBD (Figure 4.5). For the MCF12A and MDA-MB-231 cell lines, significant decreases in cell viability was observed in combination 2 (0.75mg/mL  $\Delta$ -7-mesembrenone, 20ng/mL CBD) and combination 3 (0.75mg/mL  $\Delta$ -7-mesembrenone, 100ng/mL CBD). The MCF7 cell line appeared to be more susceptible to combination treatments, with significant decreases in viability observed across all combination treatments. Following assessment using a two-way ANOVA, main effect of combination doses was  $P < 0.001$ .



**Figure 4.5 – Cell viability of MCF12A, MCF7 and MDA-MB-231 following treatment with 3 different combinations of  $\Delta$ -7-mesembrenone and CBD.** The data are presented as a percentage of the untreated controls for each cell type. Combinations used are 0.5mg/mL  $\Delta$ -7-mesembrenone and 20ng/mL CBD (1); 0.75mg/mL  $\Delta$ -7-mesembrenone and 20ng/mL CBD (2); and 0.75mg/mL  $\Delta$ -7-mesembrenone and 100ng/mL CBD (3). Statistics were obtained in the form of a two-way ANOVA and a Bonferroni post-hoc test (\* =  $P < 0.05$ ; \*\* =  $P < 0.01$ ; \*\*\* =  $P < 0.001$ ).

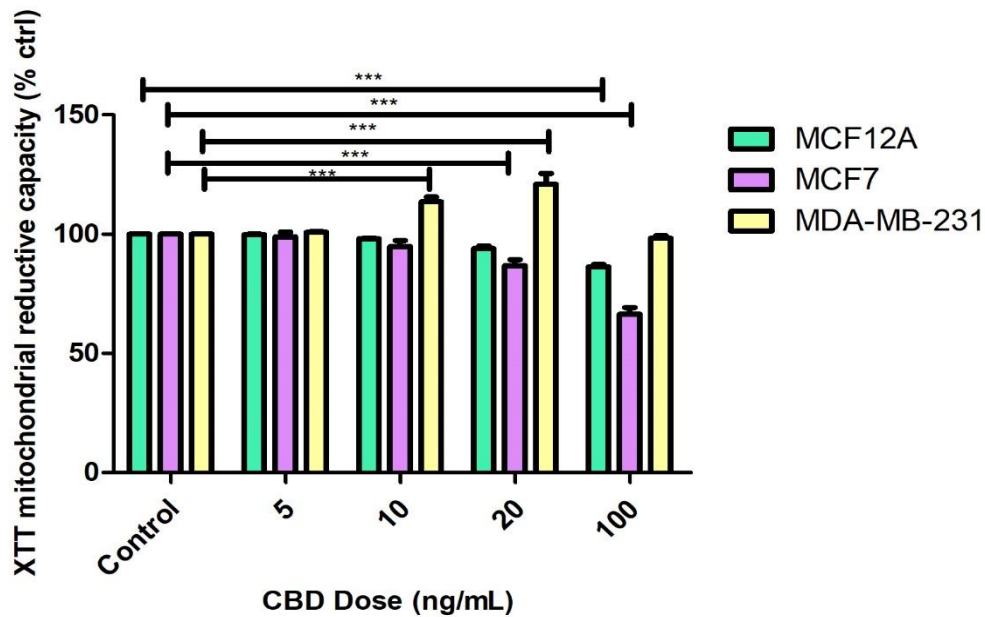
#### **4.3 Effects of $\Delta$ -7-mesembrenone, CBD or combination treatments on the mitochondrial reductive capacity of MCF12A, MCF7 and MDA-MB-231 cell models**

The XTT assay was used to determine the mitochondrial viability of the MCF12A, MCF7 and MDA-MB-231 cell lines following treatment with either  $\Delta$ -7-mesembrenone, CBD or a combination of the two. Once again, following exposure to  $\Delta$ -7-mesembrenone, the MCF12A cell line displayed a statistically significant dose-dependent decrease in mitochondrial reductive capacity for all doses of  $\Delta$ -7-mesembrenone tested (Figure 4.6). The MCF7 cell type also exhibited the same dose-dependent, statistically significant decrease in reductive capacity across all  $\Delta$ -7-mesembrenone doses, and experienced a slightly greater decrease in reductive capacity than the MCF12A cells. The MDA-MB-231 cells only experienced a statistically significant decrease in mitochondrial reductive capacity at the highest dose of  $\Delta$ -7-mesembrenone (0.75mg/mL). The main effect of dose assessed using a two-way ANOVA once again had a statistical significance of  $P < 0.001$ .



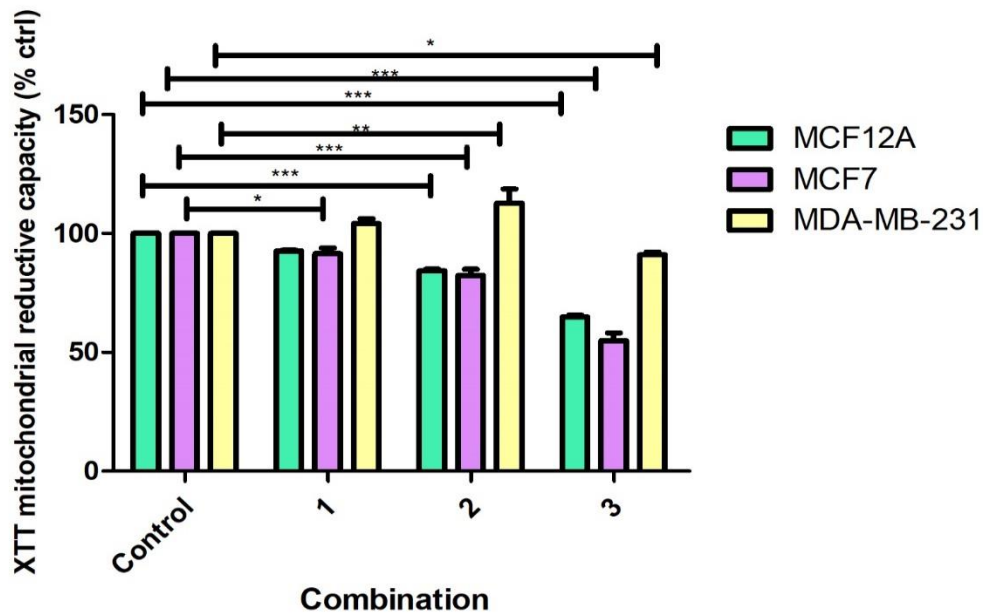
**Figure 4.6 – Mitochondrial reductive capacity of MCF12A, MCF7 and MDA-MB-231 following treatment with increasing doses of  $\Delta$ -7-mesembrenone.** The data are presented as a percentage of the untreated controls for each cell type. Doses used were 0.25mg/mL, 0.5mg/mL and 0.75mg/mL. Statistics were obtained in the form of a two-way ANOVA and a Bonferroni post-hoc test (\*\* =  $P < 0.01$ ; \*\*\* =  $P < 0.001$ ).

A statistically significant reduction in mitochondrial viability was seen in MCF12A cells only at the highest dose of CBD (100ng/mL) (Figure 4.7). However, in the MCF7 cell line, a decrease in reductive capacity was observed at the two highest doses, and experienced a greater reduction in this reductive capacity compared to that seen in the MCF12A cell line. In contrast to this, the MDA-MB-231 cells experienced a significant dose-dependent increase in mitochondrial reductive capacity at 10ng/mL and 20ng/mL CBD, with this reductive capacity appearing to return to approximately the same as the control at the highest dose of CBD. Main effect of dose was statistically significant ( $P < 0.001$ ) according to the results of the two-way ANOVA.



**Figure 4.7 – Mitochondrial reductive capacity of MCF12A, MCF7 and MDA-MB-231 following treatment with increasing doses of CBD.** The data are presented as a percentage of the untreated controls for each cell type. Doses used were 5ng/mL, 10ng/mL, 20ng/mL and 100ng/mL. Statistics were obtained in the form of a two-way ANOVA and a Bonferroni post-hoc test (\*\*\*) =  $P < 0.001$ ).

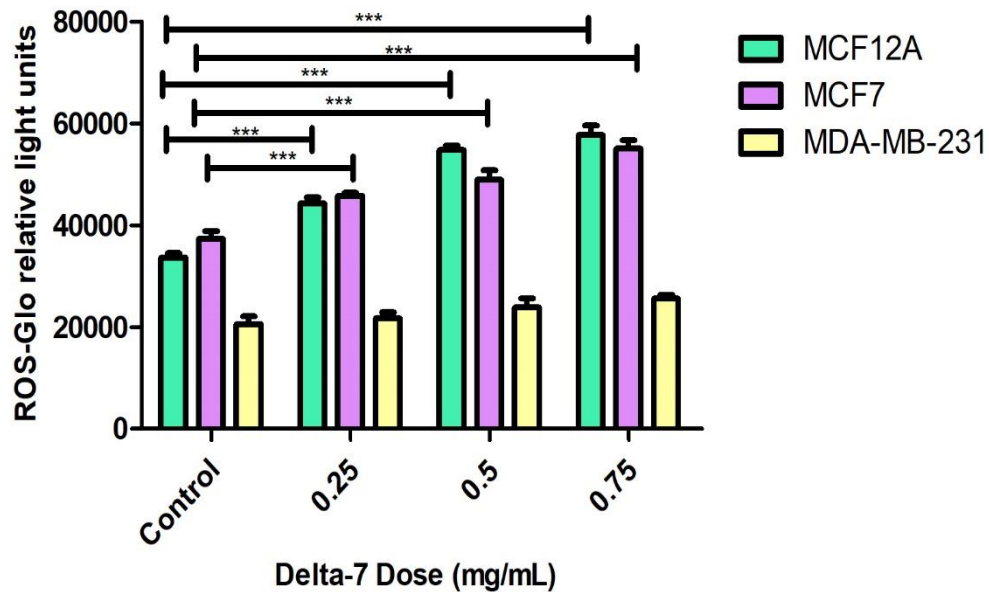
The combination doses yielded results similar to those seen in the trypan blue viability data. The MCF12A cells experienced a significant reduction in mitochondrial viability for both combinations 2 and 3, while the MCF7 cell line experienced a decrease in mitochondrial reductive capacity across all 3 combinations (Figure 4.8). The MDA-MB-231 cells experienced a statistically significant increase in mitochondrial reductive capacity when exposed to combination 2, and a statistically significant decrease in reductive capacity when exposed to combination 3. A statistically significant effect of combination used was determined using a two-way ANOVA, and a P value less than 0.001 was obtained.



**Figure 4.8 – Mitochondrial reductive capacity of MCF12A, MCF7 and MDA-MB-231 following treatment with 3 different combinations of  $\Delta$ -7-mesembrenone and CBD.** The data are presented as a percentage of the untreated controls for each cell type. Combinations used are 0.5mg/mL  $\Delta$ -7-mesembrenone and 20ng/mL CBD (1); 0.75mg/mL  $\Delta$ -7-mesembrenone and 20ng/mL CBD (2); and 0.75mg/mL  $\Delta$ -7-mesembrenone and 100ng/mL CBD (3). Statistics were obtained in the form of a two-way ANOVA and a Bonferroni post-hoc test (\* =  $P < 0.05$ ; \*\* =  $P < 0.01$ ; \*\*\* =  $P < 0.001$ ).

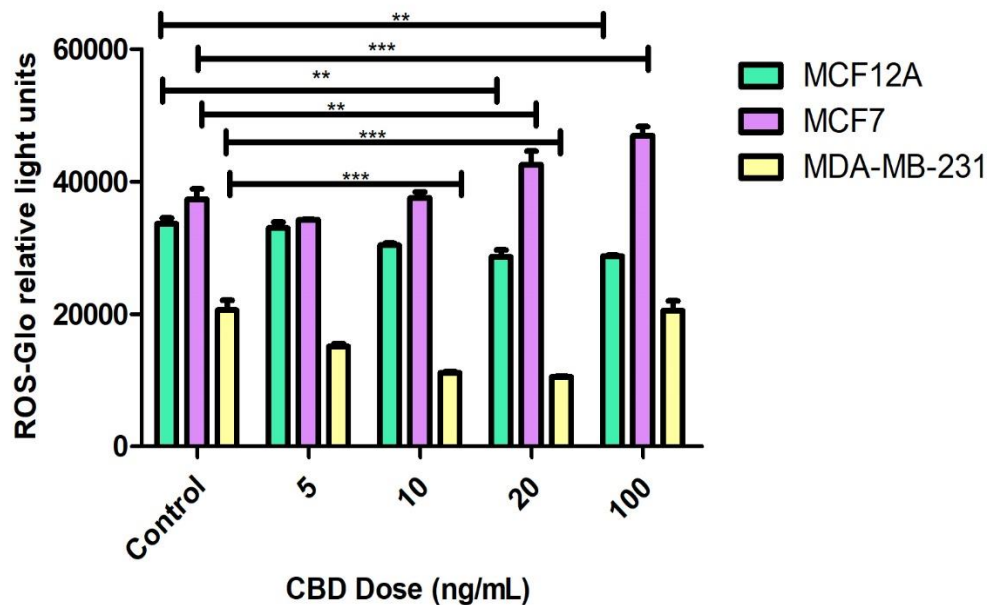
#### 4.4 Determination of ROS production in MCF12A, MCF7 and MDA-MB-231 cell models following exposure to $\Delta$ -7-mesembrenone, CBD or combination treatments

ROS production was determined using the ROS-Glo  $H_2O_2$  assay from Promega in each cell type following treatment with either  $\Delta$ -7-mesembrenone, CBD or a combination of both extracts. ROS production increased significantly in a dose-dependent manner for both MCF12A and MCF7 cell lines for all  $\Delta$ -7-mesembrenone doses assessed (Figure 4.9). There was no statistically significant increase in ROS production seen in the MDA-MB-231 cell line for any of the  $\Delta$ -7-mesembrenone doses assessed. Main effect of dose assessed using a two-way ANOVA was significant, with  $P < 0.001$ .



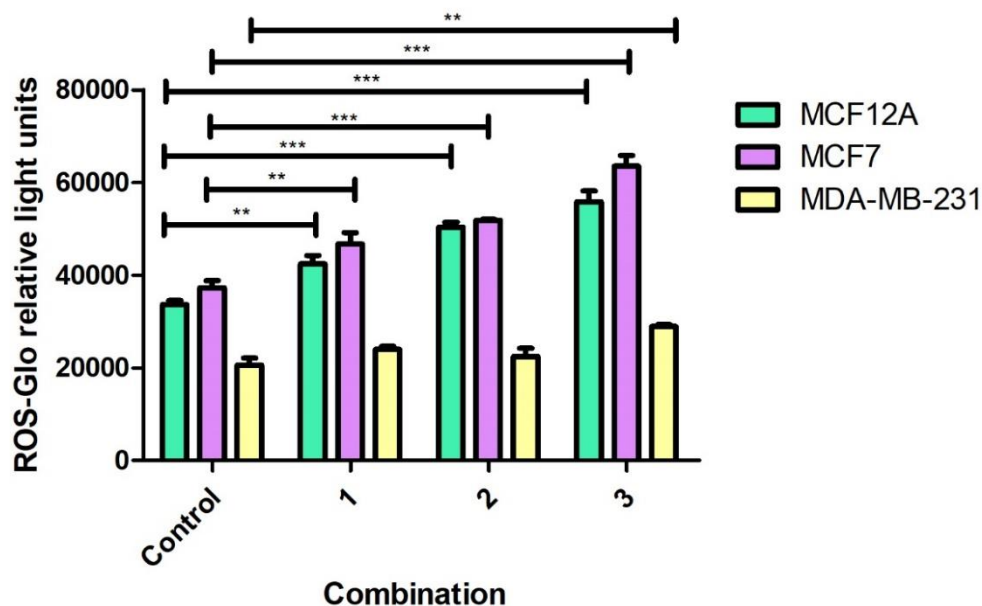
**Figure 4.9 – ROS production of MCF12A, MCF7 and MDA-MB-231 following treatment with increasing doses of  $\Delta$ -7-mesembrenone.** The data are presented as relative light units determined using a Glo-Max luminometer. Doses used were 0.25mg/mL, 0.5mg/mL and 0.75mg/mL. Statistics were obtained in the form of a two-way ANOVA and a Bonferroni post-hoc test (\*\*\*) =  $P < 0.001$ ).

When exposed to CBD, the MCF12A cells showed a dose-dependent decrease in ROS production when compared to the control, which reached statistical significance for the two highest doses of CBD (20ng/mL and 100ng/mL) (Figure 4.10). A similar effect was seen in the MDA-MB-231 cell line, with ROS production significantly reduced at the 10ng/mL and 20ng/mL doses, although in this cell line, ROS production appeared to increase to control levels after exposure to the 100mg/mL dose. In contrast, the MCF7 cells exhibited a dose-dependent increase in production of ROS, which reached statistical significance at 20ng/mL and 100ng/mL doses of CBD. Results of a two-way ANOVA indicated that the main effect of dose had a significance of  $P < 0.001$ .



**Figure 4.10 – ROS production of MCF12A, MCF7 and MDA-MB-231 following treatment with increasing doses of CBD.** The data are presented as relative light units determined using a Glo-Max luminometer. Doses used were 5ng/mL, 10ng/mL, 20ng/mL and 100ng/mL. Statistics were obtained in the form of a two-way ANOVA and a Bonferroni post-hoc test (\*\* =  $P < 0.01$ ; \*\*\* =  $P < 0.001$ ).

Exposure to combination doses of  $\Delta$ -7-mesembrenone and CBD resulted in similar ROS production trends in all 3 cell types as that of the  $\Delta$ -7-mesembrenone alone (Figure 4.11). The MCF12A and MCF7 cell lines both experienced significant increases in ROS production for all 3 combinations tested. In line with the  $\Delta$ -7-mesembrenone results, the MDA-MB-231 cells seemed relatively less sensitive to the combination treatments, with ROS production only significantly increased after exposure to combination 3. Main effect of combination, determined using a two-way ANOVA, was statistically significant ( $P < 0.001$ ).

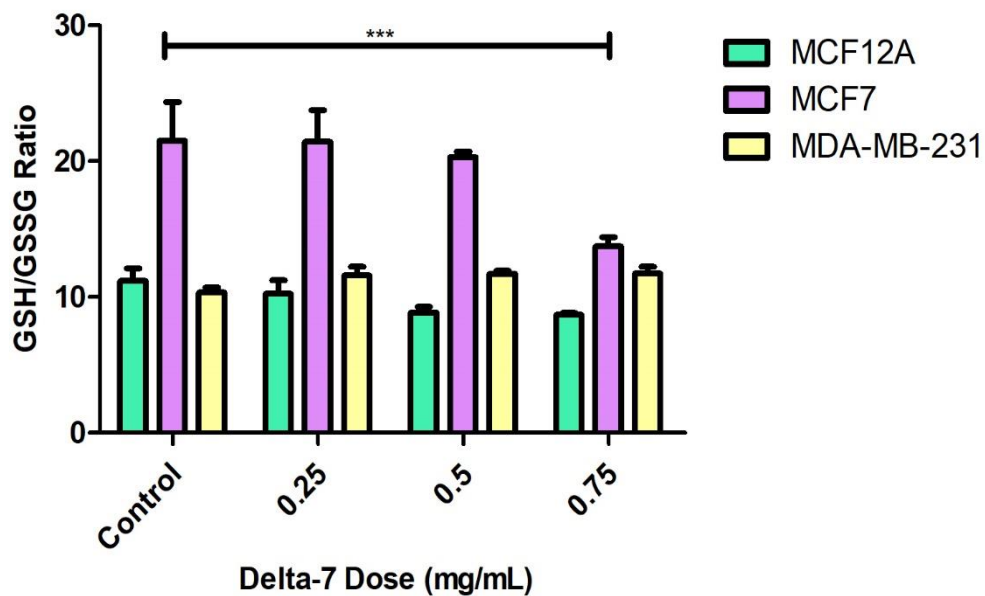


**Figure 4.11 – ROS production of MCF12A, MCF7 and MDA-MB-231 following treatment with 3 different combinations of  $\Delta$ -7-mesembrenone and CBD.** The data are presented as relative light units determined using a Glo-Max luminometer. Combinations used are 0.5mg/mL  $\Delta$ -7-mesembrenone and 20ng/mL CBD (1); 0.75mg/mL  $\Delta$ -7-mesembrenone and 20ng/mL CBD (2); and 0.75mg/mL  $\Delta$ -7-mesembrenone and 100ng/mL CBD (3). Statistics were obtained in the form of a two-way ANOVA and a Bonferroni post-hoc test (\*\* =  $P < 0.01$ ; \*\*\* =  $P < 0.001$ ).

#### **4.5 Determination of the ratio of reduced to oxidised glutathione in MCF12A, MCF7 and MDA-MB-231 cell models following exposure to $\Delta$ -7 mesembrenone, CBD or combination treatments**

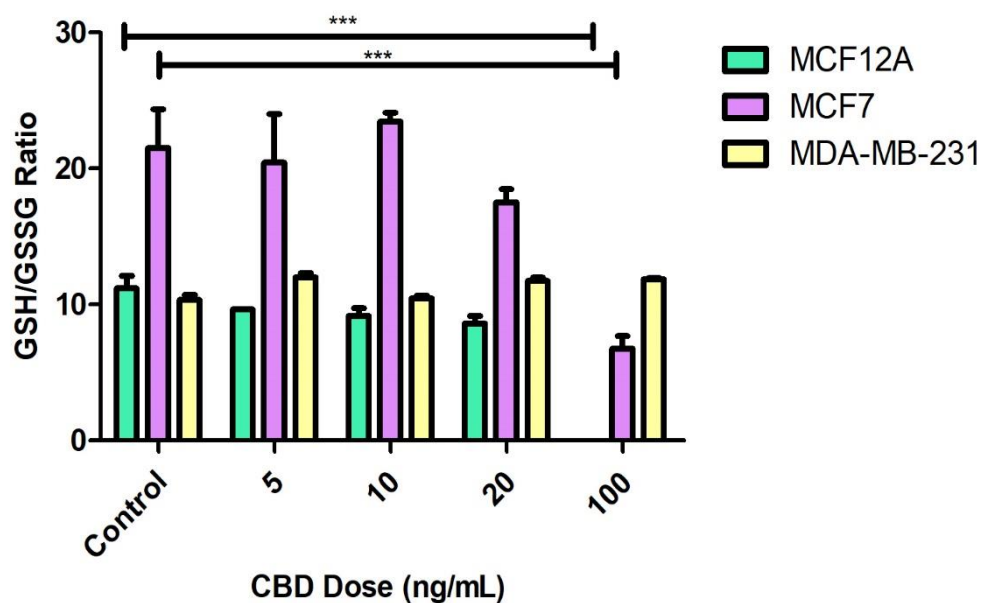
The ratio of reduced Glutathione (GSH) to oxidised Glutathione (GSSG) was determined using the GSH/GSSG-Glo assay from Promega in order to assess antioxidant capacity of the cell types. A statistically significant decrease in the GSH/GSSG ratio was only observed for the MCF7 cell type at the highest dose of  $\Delta$ -7-mesembrenone (Figure 4.12). There were no significant differences in the GSH/GSSG ratio for MCF12A and MDA-MB-231 cell types across all  $\Delta$ -7-mesembrenone doses assessed. A two-way ANOVA was performed, and main effect of dose was  $P < 0.05$ .





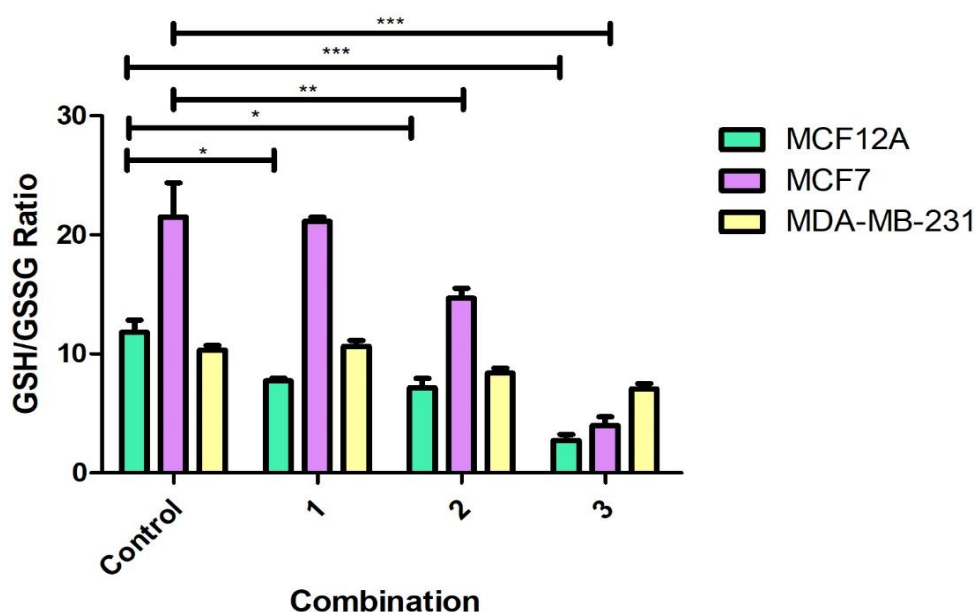
**Figure 4.12 – GSH/GSSG ratio of MCF12A, MCF7 and MDA-MB-231 following treatment with increasing doses of  $\Delta$ -7-mesembrenone.** The data are presented as a ratio determined using a standard curve after raw data was determined using a Glo-Max luminometer. Doses used were 0.25mg/mL, 0.5mg/mL and 0.75mg/mL. Statistics were obtained in the form of a two-way ANOVA and a Bonferroni post-hoc test (\*\*\*) =  $P < 0.001$ ).

CBD exposure resulted in a significant decrease in the GSH/GSSG ratio in the MCF12A and MCF7 cells at the highest dose of CBD (Figure 4.13). No significant changes were observed in this ratio in the MDA-MB-231 cells following exposure to CBD. Main effect of dose was assessed using a two-way ANOVA and had a P value less than 0.001.



**Figure 4.13 – GSH/GSSG ratio of MCF12A, MCF7 and MDA-MB-231 following treatment with increasing doses of CBD.** The data are presented as a ratio determined using a standard curve after raw data was determined using a Glo-Max luminometer. Statistics were obtained in the form of a two-way ANOVA and a Bonferroni post-hoc test (\*\*\*) =  $P < 0.001$ ).

Finally, MCF12A cells exhibited significant, decreases in the GSH/GSSG ratios for all 3 combination doses (Figure 4.14). The MCF7 cells showed a decrease in GSH/GSSG ratios that was statistically significant for combinations 2 and 3. A two-way ANOVA indicated that the effect of different combinations used was statistically significant ( $P < 0.001$ ). Again, the MDA-MB-231 cells showed no significant difference in the GSH/GSSG ratios in response to any of the combinations assessed.



**Figure 4.14 – GSH/GSSG ratio of MCF12A, MCF7 and MDA-MB-231 following treatment with 3 different combinations of  $\Delta$ -7-mesembrenone and CBD.** The data are presented as a ratio determined using a standard curve after raw data was determined using a Glo-Max luminometer. Combinations used are 0.5mg/mL  $\Delta$ -7-mesembrenone and 20ng/mL CBD (1); 0.75mg/mL  $\Delta$ -7-mesembrenone and 20ng/mL CBD (2); and 0.75mg/mL  $\Delta$ -7-mesembrenone and 100ng/mL CBD (3). Statistics were obtained in the form of a two-way ANOVA and a Bonferroni post-hoc test (\* =  $P < 0.05$ ; \*\* =  $P < 0.01$ ; \*\*\* =  $P < 0.001$ ).

## CHAPTER 5: DISCUSSION

$\Delta$ -7 mesembrenone, isolated from *Sceletium tortuosum*, and Cannabidiol, extracted from *Cannabis sativa*, used in isolation or in combination, was studied in the context of their effect on cellular oxidative stress and survival in an *in vitro* cancer model.

We report differential effects for the two extracts assessed. While  $\Delta$ -7 mesembrenone seemed to exhibit more adverse than beneficial effects, cannabidiol data were more positive.

### 5.1 $\Delta$ -7 mesembrenone in isolation exhibited high risk for toxicity

In terms of the anti-cancer capacity of  $\Delta$ -7 mesembrenone in MCF12A, MCF7 and MDA-MB-231 cells at three different doses (0.25-, 0.5-, and 0.75mg/mL), cytotoxicity was evident in the MCF12A and MCF7 cell lines across all doses of  $\Delta$ -7 mesembrenone, while the MDA-MB-231 cells had a reduction in cell viability only at the highest doses of  $\Delta$ -7 mesembrenone. A similar trend was also observed when examining the mitochondrial reductive capacity of the cells following exposure to  $\Delta$ -7 mesembrenone.

While  $\Delta$ -7 mesembrenone is an antioxidant, exposure to an antioxidant at a dose that is too high may also be detrimental to the cells. This may result in a reduction in their ability to scavenge free radicals, shifting the antioxidant to a pro-oxidant, further exacerbating the problem of oxidative stress (Burkitt, 2001). However, it is also known that cancer cells have been associated with a naturally higher level of ROS and oxidative stress than non-cancerous cell types and increased ROS has a direct link to both the initiation and propagation of the tumour as explained previously. Therefore, cancer cells may potentially survive increased levels of oxidative stress with a greater efficiency than that of other types of normal human cells (Trachootham *et al.*, 2006). With this in mind, the relevant cellular mechanisms at play following exposure of the three cell lines to  $\Delta$ -7 mesembrenone may be elucidated.

Firstly, in comparison to that of the other two cell lines, the MCF12A cells experienced the greatest decrease in cell and mitochondrial viability. This cell death may be explained by the high levels of ROS production experienced following treatment with this extract, indicating

that  $\Delta$ -7 mesembrenone acted as a pro-oxidant in these cells. There appeared to be little glutathione antioxidant activity, and endogenous antioxidant capacity was unable to reduce the effects of the high levels of oxidative stress.

Secondly, cytotoxicity was experienced at the highest doses of  $\Delta$ -7 mesembrenone assessed in the MCF7 cell model, with reductions in the mitochondrial reductive capacity seen across all doses. This translated into an increased ROS production across all doses assessed, once again demonstrating the pro-oxidant activity of  $\Delta$ -7 mesembrenone in these cells. Endogenous glutathione antioxidant capacity was also decreased in the MCF7 cells, potentially illustrating a greater endogenous antioxidant capacity than that of the MCF12A cells, and the innate ability of cancer cells to tolerate oxidative stress at a higher level than non-cancerous cells, consistent with the findings of many studies (Pelicano, 2004; Cairns *et al.*, 2011; Gupte *et al.*, 2008; Hileman *et al.*, 2003).

Finally, within the MDA-MB-231 cells, cytotoxicity and mitochondrial toxicity was only experienced at the highest doses of  $\Delta$ -7 mesembrenone assessed, with no changes in ROS production or endogenous antioxidant activity. This can be explained by the increased tolerance of cancer cells to ROS, as mentioned previously. In addition to this, the MDA-MB-231 cell line is the only cell line assessed in this study which does not express the estrogen receptor (ER-). Previous studies (Mobley and Brueggemeier, 2004; Liehr, 1999; Wani *et al.*, 1998) have indicated that cells that are ER- have a greater ability to metabolise free radicals and reduce oxidative stress, while the presence of estrogen receptors increase the sensitivity to oxidative stress.

From these findings it is clear that in terms of breast cancer therapy, any potential anti-cancer benefit of  $\Delta$ -7 mesembrenone is outweighed by the risk to normal cells associated with its exposure. No literature currently exists about  $\Delta$ -7 mesembrenone, except that it is a very potent antioxidant (Bennett and Smith, 2017). Much lower doses of  $\Delta$ -7 mesembrenone may be assessed in the context of an antioxidant supplement, however, toxicity associated with its exposure in normal cells rules it out as a potential cancer treatment. It is also important to note that this study was conducted *in vitro* and may not translate directly to *in vivo* models. However, doses selected for this study were relevant to work previously conducted using  $\Delta$ -7 mesembrenone (Bennet and Smith, 2017), and due to the cytotoxicity experienced in the

normal cells even at the lowest dose, use of different doses would not be relevant in this context.

## **5.2 Cannabidiol may provide anti-cancer benefits in certain types of breast cancer**

In contrast to the poor prognostic value of  $\Delta$ -7 mesembrenone, data for Cannabidiol seems more positive. Firstly, in terms of the normal breast cell line, reductions in cell viability and mitochondrial reductive capacity only occurred significantly at the highest dose assessed. However, exposure to higher doses of CBD (20- or 100ng/mL) resulted in a decrease in the ROS production in this cell type, which could be explained by the antioxidant potential of Cannabidiol (Hampson, 1998; Mariscano *et al.*, 2002). While high concentrations of free radicals may damage cells and result in cell death, a certain concentration of free radicals are important for cell metabolism (Droge, 2002). If too many free radicals are quenched, effects may be detrimental to the cells. This may be the case here, in which Cannabidiol is very potent, resulting in a decrease in reactive species that actually becomes detrimental to the cells at the highest dose of 100ng/mL CBD. Finally, endogenous antioxidant capacity of the MCF12A cell line was exhausted at 100ng/mL of CBD, which may suggest that the Cannabidiol loses its beneficial effects and pattern of drug response at a certain threshold, and this may require further investigation.

When considering results in MCF7 cells exposed to CBD cell viability and mitochondrial reductive capacity were both decreased significantly at the highest doses assessed. This reduction in mitochondrial reductive capacity was more severe than the one seen in the MCF12A cell line and was associated with large increases in ROS production. There are several reasons as to why this may occur in the MCF7 cell line. Firstly, this cell line is estrogen receptor positive, as previously mentioned, and has an increased sensitivity to reactive species. Secondly, literature has indicated that MCF7 cells specifically experienced a decrease in cell proliferation upon exposure to Cannabidiol (Caffarel *et al.*, 2012). This study indicated that cell cycle progression in MCF7 cells is arrested, and transition from the G1 to the S phase is inhibited, in conjunction with inhibition of adenyl cyclase expression, resulting in the activation of Raf-1/ERK/MAPK cascade, ultimately downregulating prolactin expression (Nisson *et al.*, 2011). This effectively prevents cell proliferation in the MCF7 cell line specifically. Thirdly, additional studies have also implicated CBD exposure in increased ROS production in certain tumour cell lines specifically, mediated via increased intracellular  $[Ca^{2+}]$

controlled by inhibition of mitochondrial  $\text{Ca}^{2+}$  reuptake or increased release of  $\text{Ca}^{2+}$  (Izzo *et al.*, 2009). Interestingly, additional studies have indicated that metabolism of CBD in certain tumour cells results in formation of an intermediate known as CBD hydroxyquinone, which also generates ROS and contributes to cytotoxicity (Wu and Yan, 2010). This is of interest when considering the reduction in the GSH/GSSG ratio of the MCF7 cells following CBD exposure (figure 4.12), as CBD hydroxyquinone has been recently observed to form a complex with endogenous glutathione, resulting in glutathione depletion (Wu and Yan, 2010). This provides an explanation for the significantly greater decrease in endogenous glutathione in the MCF7 cell line following CBD exposure when compared to the normal control cells. Additionally, endogenous glutathione may also have been depleted via scavenging of increased reactive species generated in this cell line.

A very different result was found upon exposure of the MDA-MB-231 cells to the CBD extract. Cytotoxicity was only experienced at the highest dose, and mitochondrial reductive capacity increased at doses of 10- and 20ng/mL CBD, with reductions in reductive capacity only experienced at the highest dose. This could be indicative of the CBD acting as an antioxidant, removing free radicals and allowing for better signalling, proliferation and survival of the MDA-MB-231 cells. Similar to the MCF12A cells, the MDA-MB-231 cell model experienced a significant decrease in ROS production at 10- and 20ng/mL CBD doses, with ROS levels appearing to return to normal at the 100ng/mL dose. This could be explained by the CBD offering a protective effect mediated via free radical removal and decreases in oxidative stress to a level more beneficial to the cell. The lack of estrogen receptors also allows MDA-MB-231 cells to better metabolise reactive species, as previously discussed. Finally, no changes were observed in the ratio between GSH and GSSG, which is in line with decreased production of ROS. Endogenous antioxidant capacity was not depleted, and the MDA-MB-231 cells were protected from any adverse effects.

Literature has shown conflicting results regarding this topic, and some studies have observed anti-proliferative effects of CBD on MDA-MB-231 (Ligriesti *et al.*, 2006; McAllister *et al.*, 2011), where other studies have reported no effect of CBD exposure (Velasco *et al.*, 2012), as experienced in this study, and some have reported pro-tumorigenic effects of CBD exposure (Zhu *et al.*, 2000).

Despite the large body of literature that currently exists regarding *Cannabis sativa* and its Phytocannabinoids such as cannabidiol and  $\Delta^9$ -THC in the context of medicine and disease treatment, very little exists in the context of oxidative stress mediated cancer therapy and the antioxidant capacity of CBD in this context. This alone illustrates the importance of research in this area, and the contribution of this study to this topic. Overall, exposure to cannabidiol may be a favourable treatment for breast cancers that are estrogen receptor positive, as the MCF7 cell line showed the greatest cytotoxicity and adverse effects when exposed to this extract across all doses assessed. The MCF12A cell line experienced fewer adverse effects than the MCF7 model and were able to better tolerate the cannabidiol extract. However, MDA-MB-231 cells also experienced an increased survival upon exposure to CBD, with no toxic effects. Thus, cannabidiol should not be used as a treatment for estrogen receptor negative cancers, indicating the importance in considering the stage and estrogen status of the breast cancer prior to treatment. This warrants further research in this context, however a maximum dose needs to be determined in the context of breast cancer, because predictable dose response was lost at a certain threshold in the MCF12A and MDA-MB-231 cell lines. Finally, it is also important to note that studies *in vitro* may not always translate directly *in vivo*, but results obtained in this study regarding Cannabidiol are promising.

### 5.3 Insights on drug interactions

Very little literature exists in the context of combination therapies using CBD in the treatment of cancer or specifically, breast cancer. In order to gain a better understanding of this, literature from other niches had to be considered and studied. One study assessed the cardioprotective effects of Cannabidiol when taken in combination with doxorubicin in cancer (Fouad *et al.*, 2013). Cannabidiol, when taken in combination with doxorubicin reduced the expression of TNF levels associated with doxorubicin exposure, in addition to reduced NO levels, cardiac malondialdehyde expression and troponin-T levels. This resulted in a cardioprotective effect, reducing the cardiotoxicity associated with doxorubicin exposure, in part of which was mediated by decrease inflammation and oxidative stress, which is in line with the scope of this study. In addition to this, there is a large body of work that examines the neuroprotective effect of CBD. One such study examined the protective effect of Cannabidiol in the context of neurotoxicity, in which retinal ganglion cells were exposed to *N*-



methyl-D-aspartate (NMDA), resulting in increased peroxynitrite accumulation and cell death (El-Remessy *et al.*, 2003). Cells which were exposed to NMDA and CBD in combination had reduced neurotoxicity as a result of decreased peroxynitrite production, and suggests Cannabidiol may have a potential use in Glaucoma treatment. This further demonstrates the potential protective effect of Cannabidiol.

Using information gained from previous studies, the drug interactions between  $\Delta$ -7 mesembrenone and Cannabidiol were considered. Combinations with high dose  $\Delta$ -7 mesembrenone (2 and 3) resulted in significant decreases in cell viability and mitochondrial reductive capacity in the MCF12A that were not countered by exposure to high doses of Cannabidiol. In addition to this, all combinations resulted in increased ROS production similar to that generated by  $\Delta$ -7 mesembrenone in isolation, further indicating that CBD was not potent enough to counter these negative effects, even at high doses. In this case, combination exposure was even more detrimental to MCF12A cells and the highest  $\Delta$ -7 mesembrenone and CBD combination resulted in total depletion of endogenous glutathione activity. Combinations of both antioxidants may have resulted in an antioxidant overload, resulting in an increased production of free radicals that could not be scavenged entirely.

MCF7 cells had similar results upon exposure to the combination doses as the MCF12A cell model in terms of cell viability and mitochondrial reductive capacity and increased ROS production. As previously mentioned, this may be as a result of a variety of factors. Firstly, the estrogen receptor status of this cell line and its increased sensitivity to ROS may be a contributing factor. Secondly, the fact that certain tumour cells metabolise CBD specifically via a pathway promoting ROS generation, as well as a potential antioxidant overload in these cells, shifting towards a pro-oxidative state. In addition to this, as previously mentioned, MCF7 cells have a particular sensitivity to CBD exposure, resulting in cell cycle arrest and inhibition of proliferation (Caffarel *et al.*, 2012). Finally, endogenous glutathione was depleted almost entirely when the extracts were used in their highest combinations, which correlates with the increased ROS production observed.

Results for MDA-MB-231 cells were not so severe, but again indicate that CBD was unable to remove the negative effects associated with  $\Delta$ -7 mesembrenone exposure. Significant changes in ROS production was only observed in combination 3, which may be due to an antioxidant over load considering the high doses of  $\Delta$ -7 mesembrenone and CBD used in this

combination. This increase in ROS production correlates with the decrease in cell viability. No changes were observed in the ratio between GSH and GSSG across any of the combinations assessed, which may indicate that the cells are able to tolerate higher levels of ROS as previously discussed.

While Cannabidiol has been able to counter toxicity induced by other chemicals or drugs in previous studies, it was unable to counter the negative effects associated with  $\Delta$ -7 mesembrenone exposure. This may be due to the fact that CBD was not potent enough to protect the cells from  $\Delta$ -7 mesembrenone exposure, and the beneficial effects of CBD in isolation were totally lost when used in these combinations. In some cases, at the highest doses of CBD and  $\Delta$ -7 mesembrenone, a cumulative effect was observed, further perpetuating oxidative stress and antioxidant depletion. This indicates that not enough information is known about each extract to validate their compound use, and this is in line with a previous paper by Casedas and co-workers, in which it states that certain extracts do not have enough information about them to validate their responsible compounding (Casedas, *et al.*, 2018). Not enough is understood about the conditions under which seemingly beneficial supplements may become detrimental.

#### **5.4 Concluding remarks**

In the context of cancer therapy or prevention,  $\Delta$ -7 mesembrenone isolated from *Sceletium tortuosum* was not effective. Exposure to  $\Delta$ -7 mesembrenone resulted in detrimental effects to the normal breast cell line which outweighed any potential anti-cancer benefits observed in both the breast cancer cell models. However, further research may be conducted in the context of  $\Delta$ -7 mesembrenone at lower doses as a potential antioxidant supplement. Cannabidiol showed more promise in this context, where healthy breast cells showed less adverse effects upon exposure to CBD, with the estrogen receptor positive breast cancer exhibiting more detrimental effects. This is a beneficial outcome, whereby normal breast tissue remains healthy upon exposure to CBD, where the MCF7 breast cancer did not. Further research could be done in order to better understand the effect of Cannabidiol treatment in estrogen receptor positive breast cancer therapy. However, this treatment had no anti-cancer activity when the estrogen receptor negative breast cell line was exposed to increasing doses

of Cannabidiol. Finally, in terms of a combination therapy, the Cannabidiol was unable to counter the harsh side effects of  $\Delta$ -7 mesembrenone exposure. Therefore, the combination treatments would have little benefit in the context of breast cancer therapy.

### **5.5 Future research**

Future research should be conducted to further investigate the anti-cancer potential of Cannabidiol in the treatment and prevention of estrogen receptor positive breast cancers. In addition to this, the dose at which the Cannabidiol shows detrimental effects within the normal breast tissue should also be elucidated and better understood. Investigations may also be conducted *in vivo* in order to determine whether the results obtained in the *in vitro* experiments translate to animal models, which is not always the case.

Furthermore, investigations should be conducted into the redox status of each breast cancer cell type, as well as any other cell type exposed to these two extracts, in order to better understand the cellular mechanisms at play. Each cell type has its own inherent oxidative stress basal level, as well as their own endogenous antioxidant mechanisms and capacities. This would need to be studied prior to further treatment in order to elucidate whether the relevant treatment would be effective or not.

## CHAPTER 6: REFERENCES

Aggarwal, B.B., Shishodia, S., Sandur, S.K., Pandey, M.K., Sethi, G., 2006. Inflammation and cancer: How hot is the link? *Journal of Biochemical Pharmacology* 72, pp. 1605-1621.

Aggarwal, B.B., Vijayalekshmi R.V., Sung, B., 2009. Targeting inflammatory pathways for prevention and therapy of cancer: short-term friend, long-term foe. *Clinical Cancer Research* 15, pp. 425-430.

Aldrich, M., 1997. History of therapeutics in Cannabis. *Cannabis in Medical Practice* pp. 35-55.

Alfadda, A., Sallam, R., 2012. Reactive oxygen species in health and disease. *Journal of Biomedicine and Biotechnology*.

Allen, C.L., Bayraktutan, U., 2009. Antioxidants attenuate hyperglycaemia-mediated high brain endothelial cell dysfunction and blood-brain barrier hyperpermeability. *Journal of Diabetes Obesity and Metabolism* 11, pp. 480-490.

Andre, C.M., Hausman, J., Guerriero, G., 2016. *Cannabis sativa*: The plant of a thousand and one molecules. *Journal of Frontiers in Plant Science* 7(19).

Antonenkov, V.D., Grunau S., Ohlmeier, S., Hiltunen, J.K., 2010. Peroxisomes are oxidative organelles. *Journal of antioxidants and redox signalling* 13, pp. 525-537.

Avalle, L., Camporeale, A., Camperi, A., Poli, V., 2017. STAT3 in cancer: A double-edged sword. *Cytokine* 98, pp. 42-50.

Baron, J.A., Sandler, R.S., 2000. Nonsteroidal anti-inflammatory drugs and cancer prevention. *Annual Review of Medicine* 51, pp. 511-523.

Bedard, K., Krause, K., 2007. The NOX family of ROS-Generating NADPH oxidases: physiology and pathophysiology. *Journal of Physiological Reviews* 87(1), pp. 245-313.

- Bennett, A.C., Van Camo, A., Lopez, L., Smith, C., 2018. *Sceletium tortuosum* may delay chronic disease progression via alkaloid-dependent antioxidant or anti-inflammatory action. *Journal of Physiology and Biochemistry* 74(4), pp1-9.
- Bishop, J.M., Weinberg, R., 1994. Scientific American molecular oncology. *New York Scientific American*.
- Blake, D.R., Robson, P., Ho, M., Jubbs, R.W., McCabe, C.S., 2006. Preliminary assessment of the efficacy, tolerability and safety of cannabis-based medicine (Sativex) in the treatment of pain in Rheumatoid arthritis. *Oxford Academic Journal of Rheumatology* 45(1), pp. 50-52.
- Block, K.I., Koch, A.C., Mead, M.N., Tothy, P.K., Newman, R.A., Gyllenhaal, C., 2008. Impact of antioxidant supplementation on chemotherapeutic toxicity: a systematic review of the evidence from randomised controlled trials. *International Journal of Cancer* 123, pp. 1227-1239.
- Bolann, B.J., Ulvik, R.J., 1997. How do antioxidants work? *Journal of the Norwegian Medical Association* 117(13), pp. 1928-1932.
- Breen, A.P., Murphy, J.A., 1995. Reactions of oxyl radicals with DNA. *Free radical biology medicine* 18, pp. 1033-1077.
- Burkitt, M., 2001. Too much of a good thing? *Journal of Natural Biotechnology* 19, pp. 811-812.
- Cabiscol, E., Tamarit, J., Ros, J., 2000. Oxidative stress in bacteria and protein damage by reactive oxidative species. *Journal of International Microbiology* 3(3), pp. 3-8.
- Cairns, R.A., Harris, I.S., Mak, T.W., 2011. Regulation of cancer cell metabolism. *Nature Reviews Cancer* 11, pp. 85-95.
- Capasso, R., 2008. Cannabidiol, extracted from *Cannabis sativa*, selectively inhibits inflammatory hypermobility in mice. *Brazilian Journal of Pharmacology* 154, pp. 1001-1008.
- Chiu, S., Gericke, N., Woodbury, M., Badmaev, V., Raheb, H., Terpstra, K., Antongiorgi, J., Bureau, Y., Cernovsky, Z., Hou, J., Sanchez, V., Williams, M., Copen, J., Husni, M., Goble, L., 2014. Proof of concept randomized controlled study of cognition effects of the proprietary extract *Sceletium tortuosum* (Zembrin) targeting Phosphodiesterase-4 in cognitively healthy

subjects: Implications for Alzheimer's Dementia. *Journal of Evidence-based Complementary and Alternative Medicine* 2014.

Chiu, S., Raheb, H., Terpstra, K., Vaughan, J., Carrie, A., Farina-Woodbury, M., Bureau, Y., Cernovsky, Z., Hou, J., Copen, J., Husni, M., Badmaev, V., Shad, M., Suntras, Z., Gericke, N., 2017. Exploring standardised Zembrin extracts from the South African plant *Sceletium tortuosum* in dual targeting of Phosphodiesterase-4 (PDE-4) and serotonin reuptake inhibition as potential treatment of schizophrenia. *International Journal of Complementary and Alternative Medicine* 6(5), pp. 2-9.

Coetzee, D.D., Lopez, V., Smith, C., 2015. High-Mesembrine *Sceletium* extract (Trimesimine™) is a monoamine releasing agent, rather than only a selective serotonin reuptake inhibitor. *Journal of Ethnopharmacology* 177, pp. 111-116.

Costa, B., 2007. The non-psychoactive cannabis constituent cannabidiol is an orally effective therapeutic agent in rat chronic inflammatory and neuropathic pain. *European Journal of Pharmacology* 556, pp. 75-83.

Coughlin, S.S., Ekwueme D.U., 2009. Breast cancer as a global health concern. *Cancer Epidemiology* 33(5), pp.315-318.

DeMichele, A., Weber, B.L., 2000. Inherited genetics factors. *Journal of diseases of the breast*, pp. 221-236

Del Prete, A., Allavena, P., Santoro, G., Fumarulo, R., Massimiliano, C.M., Mantovani, A., 2011. Molecular pathways in cancer-related inflammation. *Biochimica Medica* 21(3), pp.264-275.

Droge, W., 2002. Free radicals in the Physiological Control of Cell Function. *Physiological Reviews* 82(1), pp. 47-95.

Durackova, D., 2010. Some current insights into oxidative stress. *Physiological Research* 59, 459-469.

Eiro, N., Vizoso, F.J., 2012. Inflammation and cancer. *World Journal of Gastrointestinal Surgery* 4(3), pp. 62-72.

Fankhauser, M., 2002. History of Cannabis in Western medicine. *Cannabis and Cannabinoids*, pp. 37-51

- Federico, A., Morgillo, F., Tuccillo, C., Ciardello, F., Loguerico, C., 2004. Chronic inflammation and oxidative stress in human carcinogenesis. *International Journal of Cancer* 121, pp. 2381-2386.
- Fishedick, J.T., Hezekamp, A., Erkelens, T., Choi, Y.H., Verpoote, R., 2010. Metabolic fingerprinting of *Cannabis sativa* L., cannabinoids and terpenoids for chemotaxonomic and drug standardization purposes. *Journal of Phytochemistry* 71, pp. 2058-2073.
- Fransen, M., Nordgren, M., Wang, B., Apanasets, O., 2012. Role of Peroxisomes in ROS/RNS-metabolism: Implications for human disease. *Molecular Basis of Disease* 1822(9), pp.1363-1372.
- Feller, L., Alitini, M., Lemmer, J., 2013. Inflammation in the context of oral cancer. *Oral Oncology* 49(9), pp.887-892.
- Ferguson D.M., Boden, J.M., 2008. Cannabis use and later life outcomes. *Society for the Study of Addiction* 103(6), pp. 969-976.
- Ferreira, L.M., 2010. Cancer metabolism: The Warburg effect today. *Experimental and Molecular Pathology* 89(3), pp. 372-380.
- Gaffney, C.D., 2006. A study of Mesembryanthemaceae alkaloids. *MSc Thesis, University of Johannesburg South Africa*.
- Galadari, S., Rahman, A., Pallichankandy, S., Thayyullathil, F., 2017. Reactive oxygen species and cancer paradox: To promote or to suppress? *Free Radical Biology and Medicine* 104, pp. 144-164.
- Gao, P., Zhang, R., Dinavahi, R., Li, F., Xiang, Y., Raman, V., Bhujwalla, Z.M., Felsher, D.W., Cheng, L., Pevsner, J., 2007. HIF-Dependent anti-tumorigenic effect of antioxidants in vivo. *Cancer Cell* 12(11), pp.230-238.
- Gericke, N., Viljoen, A.M., 2008. Sceletium – a review update. *Journal of Ethnopharmacology* 119, pp. 653-663.
- Ghezzi, P., 2005. Regulation of protein function by glutathionylation. *Journal of Free radical research communications* 39, pp. 573-580.

Greenwell, M., Rahman, P.K.S.M., 2015. Medicinal plants: Their use in anticancer treatment. *International Journal of Pharmaceutical Sciences and Research* 6(10), pp. 4103-4112.

Grivennikov, S. I., Greten, F. R., Karin, M., 2010. Immunity, Inflammation, and Cancer. *Cell* 140, pp. 883-899.

Gupta, R.A., Dubois, R.N., 2001. Colorectal cancer prevention and treatment by inhibition of cyclooxygenase-2. *Nature Reviews Cancer* 1, pp.11-12.

Gupte, A., Mumper, R.J., 2008. Elevated copper and oxidative stress in cancer cells as a target as a target for cancer treatment. *Cancer Treatment Reviews* 35(1), pp. 32-46.

Hall, W., Christie, M., Currow, D., 2005. Cannabinoids and cancer: causation, remediation and palliation. *The Lancet Journal of Oncology* 6(2), pp. 35-42.

Hamelink, C., Hampson, A., Wink, D.A., Eiden, L.E., Eskay, R.L., 2005. Comparison of Cannabidiol, antioxidants and diuretics in reversing binge ethanol-induced neurotoxicity. *The journal of Pharmacology and Experimental Therapeutics* 368(1), pp. 780-788.

Hampson, A.J., Grimaldi, M., Axelrod, J., Wink, D., 1998. Cannabidiol and delta9-tetrahydrocannabinol are neuroprotective antioxidants. *Proceedings of the National Academy of Sciences of the United States of America* 95(14), pp. 8268-8273.

Hanahan, D., Weinberg, R.A., 2000. The Hallmarks of Cancer. *Cell* 100, pp. 57-70.

Hanahan, D., Weinberg, R.A., 2011. Hallmarks of cancer: the next generation. *Cell*, Volume 144, Issue 5, pp. 646-674.

Harvel, D.M., Streckter, T.E., Tochacek, M., Xie, B., Pennington, K.L., McComb, R.D., Roy, S.K., Shull, J.D., 2000. Rat strain-specific actions of 17beta-estradiol in the mammary gland: correlation between estrogen-induced lobuloalveolar hyperplasia and susceptibility to estrogen-induced mammary cancers. *Proceedings of the National Academy of Sciences of the United States of America* 97, pp. 2779-2784.

Harvey, A.L., Young, L.C., Viljoen, A.M., Gericke, N.P., 2011. Pharmacological actions of the South African medicinal and functional food plant *Sceletium tortuosum* and its principal alkaloids. *Journal of Ethnopharmacology* 137, pp. 1124-1129.



- Hileman, E.O., Liu, J., Albitar, M., Keating, M.J., Huang, P., 2003. Intrinsic oxidative stress in cancer cells: a biochemical basis for therapeutic selectivity. *Cancer Chemotherapy and Pharmacology* 53(3), pp. 209-219.
- Holmgren, A., 2008. Antioxidant function of thioredoxin and glutaredoxin systems. *Antioxidants and redox signalling* 2(4).
- Ingram, S., Diottallevi, M., 2017. Reactive Oxygen Species: Rapid fire in inflammation. *Biochemical Society* 39(4), pp. 30-34.
- Izzo, A.A., Borrelli, F., Capasso, R., Di Marzo, V., Mechoulam, R., 2009. Non-psychotropic plant cannabinoids: new therapeutic opportunities from an ancient herb. *Cell* 30(10), pp. 515-527
- Karin, M., Naugler, W.E., 2008. NF-KappaB and cancer-identifying targets and mechanisms. *Current Opinions in Genetic Development* 18, pp. 19-26.
- Klein, T., 2005. Cannabinoid-based drugs as anti-inflammatory therapeutics. *Nature Reviews Immunology* 5, pp. 400-411.
- Krstenansky, J., 2016. Mesembrine alkaloids: Review of their occurrence, chemistry and pharmacology. *Journal of Ethnopharmacology* 195, pp.10-19.
- Kuiper, G., Carlson, B., Grandien, K., Enmark, E., Nilsson, S., 1997. Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors  $\alpha$  and  $\beta$ . *Endocrinology* 138, pp. 863-870.
- Laurent, A., Nicco, C., Chereau, C., 2005. Controlling tumour growth by modulating endogenous production of reactive oxygen species. *Cancer Research* 65, pp. 948-956.
- Liehr, J.G., 1999. 4-Hydroxylation of estrogens as a marker for mammary tumours. *The Biochemical Society Transactions* 27, pp. 318-323.
- Ligresti, A., 2006. Antitumour activity of plant cannabinoids with emphasis on the effect of cannabidiol on human breast carcinoma. *Journal of Pharmacology and Experimental Therapeutics* 318, pp. 1375-1387.
- Liu, R.H., 2013. Health-promoting components of fruits and vegetables in the diet. *Journal of Advances in Nutrition* 4, pp. 384-392.

Lu, J., Chew, E., Holmgren, A., 2007. Targeting thioredoxin reductase is a basis for cancer therapy by arsenic trioxide. *Proceedings of the National Academy of Sciences of the United States of America* 104(30), pp. 12288-12293.

Mander, M., Ntuli, L., Dierderichs, N., Mavundla, K., 2007. Economics of the Traditional Medicine Trade in South Africa. *South African Health Review* 2007(1), pp 189-196.

Mantovani, A., 2005. Cancer: Inflammation by remote control. *Nature* 435, pp. 752-753.

Mantovani, A., Allavena, P., Sica, A., Balkwill, F., 2008. Cancer-related inflammation. *Nature* 454, pp. 436-444.

Mantovani, V., Schioppa, T., Porta, C., Allavena, P., Sica, C., 2006. Role of tumour-associated macrophages in tumour progression and invasion. *Cancer Metastasis Reviews* 25, pp. 315-322.

Marnett, L.J., Riggins, J.N., West, J.D., 2003. Endogenous generation of reactive oxidants and electrophiles and their reactions with DNA and protein. *Journal of Clinical Investigations* 111, pp. 583-593.

Marsicano, G., Moosmann B., Hermann, H., Lutz, B., Behl, C., 2002. Neuroprotective properties of cannabinoids against oxidative stress: the role of the endocannabinoid receptor CB1. *Journal of Neurochemistry* 80(3), pp. 448-456.

Matomela, N., 2004. Traditional medicine, culture and health. *The AIDS Foundation of South Africa*.

Mayosi, B.M., Flisher, A.J., Lalloo, U.G., Sitas, F., Tollman, S.M., Bradshaw, D., 2009. The burden of non-communicable diseases in South Africa. *The Lancet* 374(9693), pp. 934-947.

McAllister, S.D., 2007. Cannabidiol as a novel inhibitor of Id-1 gene expression in aggressive breast cancer cells. *Journal of Molecular Cancer Therapeutics* 6, pp. 2921-2927.

McAllister, S.D., Murase, C., Christian, R.T., 2011. Pathways mediating the effects of cannabidiol on the reduction of breast cell proliferation, invasion and metastasis. *Breast cancer Research Treatment*

- McGee R., Williams, S., Poulton, R., Moffitt, T., 2002. A longitudinal study of cannabis use and mental health from adolescence to early childhood. *Society for the Study of Addiction* 95(4), pp. 491-503.
- McPherson, K., Steel, C.M., Dixon, J.M., 2000. Breast cancer- epidemiology, risk factors and genetics: A Clinical review. *British Medical Journal* 321, pp. 624-628.
- Mechoulam, R., 2007. Cannabidiol recent advances. *Journal of Chemical Biodiversity* 4, pp. 1678-1692.
- Mecuri, A.M., Accorsi, C.A., Mazzanti, M.B., 2002. The long history of *Cannabis* and its cultivation by the Romans in central Italy, shown by pollen records from Lago Albano and Lago di Nemi. *Vegetation History and Archaeobotany* 11(4), pp.236-276.
- Merlin, M., 2003. Archaeological evidence for the tradition of psychoactive plant use in the old world. *Journal of Economic Botany* 57(3), pp. 295-323.
- Mikuriya, T.H., 1969. Marijuana medicine: past, present and future. *Journal of California Medicine* 110, pp. 34-40.
- Mobley, J.A., Brueggemeier, R.W., 2004. Estrogen receptor-mediated regulation of oxidative stress and DNA damage in breast cancer. *Oxford Academic Journal of Carcinogenesis* 25(1), pp. 3-9.
- Morris, K., 2001. Treating HIV in South Africa – a tale of two systems. *Lancet* 357, pp. 1190.
- Moyo S, Young PW, Gouws E, Naidoo I, Wamicwe J, Mukui I., 2018. Equity of antiretroviral treatment use in high HIV burden countries: Analyses of data from nationally-representative surveys in Kenya and South Africa. *PLoS one* 13(8): e0201899.
- Mussarat, J., Arezina-Wilson, J., Wani, A.A., 1996. Prognostic and aetiological relevance of 8-hydroxyguanosine in human breast carcinogenesis. *European Journal of Cancer* 32, pp. 1209-1214.
- Newsholme, P., Abdulkader, F., Krause, M., 2012. Reactive oxygen and nitrogen species generation, antioxidant defences and  $\beta$ -cell function: A critical role for amino acids. *Journal of Endocrinology* 214, pp. 11-20.

Nurgali, K., Jagoe, R.T., Abalo, R., 2018. Adverse effects of cancer chemotherapy: Anything new to improve tolerance and reduce sequale? *Journal of Frontiers in Pharmacology* 9.

Oliveira, B.F., Nogueira-Machado, J.A., Chaves, M.M., 2010. The role of oxidative stress in the aging process. *The Scientific World Journal* 10, pp. 1121-1128.

Olson, J.A., Krinsky, N.I., 1995. Introduction: The colourful fascinating world of the carotenoids: important physiologic modulators. *Federation of American Societies of Experimental Biology* 9, pp. 1547-1550.

Oyebode, O., Kandala, N., Chilton, P., Lilford, R., 2016. Use of traditional medicine in middle-income countries: a WHO-SAGE study. *Health Policy and Planning* 31(8).

Palazzoli, F., Citti, C., Viella, A., Luciata, M., 2017. Development of a simple and sensitive liquid chromatography triple quadrupole mass spectrometry (LC-MS/MS) method for the determination of Cannabidiol (CBD),  $\Delta^9$ -tetrahydrocannabinol (THC) and its metabolites in rat whole blood after oral administration of a single high dose of CBD. *Journal of Pharmaceutical and Biomedical analysis* 150.

Paller, C.J., van Die, M.D., Bone, K.M., Emery, J., Williams, S.G., Pirotta, M.V., 2016. Phytotherapeutic interventions in the management of biochemically recurrent prostate cancer: a systematic review of randomised trials. *British Journal of Urology* 117(4), pp.17-34.

Patnala, S., Kanfer, I., 2012. Chemotaxonomic studies of mesembrine-type alkaloids in *Sceletium* plant species. *South African Journal of Science* 109(3).

Patnala, S., Kanfer, I., 2017. *Sceletium* plant species – Alkaloidal components, chemistry and ethnopharmacology. *Journal of Ethnopharmacology* 121(1), pp. 86-91.

Pelicano, H., Carney, D., Huang, P., 2004. ROS stress in cancer and therapeutic implications. *Drug Resistance Updates* 7(2), pp. 97-110.

Petersen, K., Smith, C., (2016). Ageing-associated oxidative stress and inflammation are alleviated by products from grapes. *Oxidative medicine and cellular longevity*, 216.

Pertwee, R.G., 2008. The diverse CB1 and CB2 receptor pharmacology of three plant cannabinoids: delta9-tetrahydrocannabinol, cannabidiol and delta9-tetrahydrocannabivarin. *British Journal of Pharmacology* 153, pp. 199-215.

- Pietta, P.G., 2000. Flavonoids and antioxidants. *Journal of Natural Products* 63, pp. 1035-1042.
- Pompella, A., 2003. The changing faces of Glutathione, a cellular protagonist. *Journal of Biochemical Pharmacology* 66(8), pp. 1499-1503.
- Ralph, S.J., Rodriguez-Enriquez, S., Neuzil, J., Saveedra, E., Moreno-Sanchez, R., 2010. The causes of cancer revisited: "Mitochondrial malignancy" and ROS-induced oncogenic transformation – Why mitochondria are targets for cancer therapy. *Molecular Aspects of Medicine* 31, pp. 145-170.
- Reuter, S., Gupta, S.C., Chaturvedi, M.M., Aggarwal, B. B., 2010. Oxidative stress, inflammation and cancer: how are they linked? *Free Radical Biology and Medicine* 49, pp. 1063-1616.
- Richter, M., 2003. Traditional medicines and traditional healers in South Africa. *Treatment actions campaign and AIDS law project* 17, pp. 4-19.
- Robson, P.J., 2018. Therapeutic aspects of Cannabis and cannabinoids. *The British Journal of Psychiatry* 178, pp. 107-115.
- Rocha, F.C.M., Stefano, S.C., De-Cassia Haeik, R., Oliviera, L.M.Q., Da Silveira, D.X., 2008. Therapeutic use of *Cannabis sativa* on chemotherapy-induced nausea and vomiting among cancer patients: A systematic review and meta-analysis. *European Journal of Cancer Care* 17(5), pp. 431-443.
- Rodriguez, R., Redman, R., 2005. Balancing the generation and elimination of reactive oxygen species. *Proceedings of the National Academy of Sciences of the United States of America* 102(9), pp. 3175-3176.
- Russo, E.B., Guy, G.W., Robson, P.J., 2007. Cannabis, Pain and Sleep: Lessons from therapeutic clinical trials of *Sativex*, a Cannabis-based medicine. *Journal of Chemistry and Biodiversity* 4(8), pp. 1729-1743.
- Ryan, D., 2009. Cannabidiol targets mitochondria to regulate intracellular  $Ca^{2+}$  levels. *Journal of Neuroscience* 29, pp. 2053-2063.
- Sabharwal, S.S., Schumacker, P.T., 2014. Mitochondrial ROS in Cancer: Initiators, amplifiers, or an Achilles heel? *Nature Reviews Cancer* 14(11), pp. 709-721.

- Santos, C.X., Tanaka, L.Y., Wosniak, J., Laurindo, F.R., 2009. Mechanisms and implications of oxidative species generation during the unfolded protein response: roles of endoplasmic reticulum oxidoreductases, mitochondrial electron transport, and NADPH oxidase. *Journal of antioxidants and redox signalling* 11, pp.2409-2427.
- Schetter, A.J., Heegaard, N.H., Harris, C.C., 2010. Inflammation and cancer: interweaving microRNA, free radical, cytokine and p53 pathways. *Carcinogenesis* 31 pp. 37-49.
- Shikanga, E.A., Kamatou, G.P.P., Chen, W., Combrink, S., Viljoen, A.M., 2012. Validated RP-UHPLC PDA and GC-MS methods for the analysis of psychoactive alkaloids in *Scelletium tortuosum*. *South African Journal of Botany* 82, pp. 99-107.
- Schumacker, P.T., 2006. Reactive oxygen species in cancer cells: Live by the sword, die by the sword. *Cancer Cell* 10(3), pp. 175-176.
- Schumacker, P.T., 2015. Reactive oxygen species in cancer: A dance with the devil. *Cancer Cell* 27(2), pp. 156-157.
- Scudiero D.A., Shoemaker R.H., Paull K.D., Monks A., Tierney S., Nofziger T.H., Currens M.J., Seniff D., Boyd M.R., 1988. Evaluation of a soluble tetrazolium/formazan assay for cell growth and drug sensitivity in culture using human and other tumour cell lines. *American Association for Cancer Research* 48(17), pp. 4827-4833.
- Sies, H., 1992. Singlet oxygen induced DNA damage. *Journal of Mutation Research* 275, pp. 367-375.
- Sies, H., 1997. Oxidative stress: Oxidants and Antioxidants. *Experimental Physiology* 82, pp. 291-295.
- Sies, H., 1999. Glutathione and its role in cellular functions. *Free radical biology and medicine* 27, pp. 922-935.
- Sorbara, M., Girardin, S.E., 2011. Mitochondrial ROS fuel the inflammasome. *Cell Research* 21, pp. 558-560.
- Sosa, V., Moline, T., Somoza, R., Paciucci, R., Kondoh, H., Lleonart, M. E., 2013. Oxidative stress and cancer: An overview. *Aging Research Reviews* 12, pp. 376-390.

Soucek, L., Lawlor, E.R., Soto, D., Shchors, K., Swigart, L.B., Evan, G.I., 2007. Mast cells are required for angiogenesis and macroscopic expansion of Myc-induced pancreatic islet tumours. *Journal of Natural Medicine* 13, pp. 1211-1218.

Statistics South Africa, 2017. New mid-year estimates reveal an aging population. *Statistics South Africa*.

Swart, A.C., Smith, C., 2016. Modulation of glucocorticoid, mineralocorticoid and androgen production in H295 cells by Trimesemine, a mesembrine-rich *Sceletium* extract. *Journal of Ethnopharmacology* 177, pp. 35-45.

Szatrowski, T.P., Nathan, C.F., 1991. Production of large amounts of Hydrogen peroxide by human tumour cells. *Journal of Cancer Research* 51(3), pp. 794-798.

Trachootham, D., Zhou, Y., Zhang, H., Demizu, Y., Chen, Z., Pelicano, H., Chiao, P.J., Achanta, G., Arlinghaus, R.B., Liu, J., Huang, P., 2006. Selective killing of oncogenically transformed cells through a ROS-mediated mechanism by  $\beta$ -phenylethyl isothiocyanate. *Cancer Cell* 10(3), pp.241-252.

Tafazoli, S., Wright, J.S., O'Brien, P.J., 2005. Prooxidant and antioxidant activity of Vitamin E analogues and Troglitazone. *Journal of Chemical Research in Toxicology* 18, pp. 1567-1574.

Torre, L.A., Bray, F., Siegel, R.L., Ferlay, J., Lortet-Tieulent J., Jemal, A., 2015. Global cancer statistics, 2012. *CA: A Cancer Journal for Clinicians*, 65(2).

Turner, C.E., Elsohly, M.A., 1981. Biological activity of cannibichromene, its homologs and isomers. *Journal of Clinical Pharmacology* 21, pp. 283-291.

Vakkila, J., Lotze, M.T., 2004. Inflammation and necrosis promote cell growth. *Nature Reviews Immunology* 4, pp. 641-648.

Van Leeuwen, F.E., Rookus, M.A., Verloop, J., 2000. Prevalence of Gynecologic Cancer in Women Exposed to Diethylstilbestrol in Utero. *The New England Journal of Medicine* 342, pp. 1838-1839.

Van Wyk, B.E., 2011. The potential of South African plants in the development of new medicinal products. *South African Journal of Botany* 77, pp. 812-819.

Van Wyk, B.E., Wink, M., 2004. Medicinal Plants of the world. *Pretoria: Briza Publications*

Velasco, G., Sanchez, C., Guzman, M., 2012. Towards the use of cannabinoids as antitumour agents. *Nature Reviews Cancer*

Wade, D.T., Makela, P.M., House, H., Bateman, C., Robson, P., 2006. Long-term use of a cannabis-based medicine in the treatment of spasticity and other symptoms in multiple sclerosis. *Multiple Sclerosis Journal* 12(5), pp. 639-645.

Wallace, D.C., 2005. A mitochondrial paradigm of metabolic and degenerative diseases, aging and cancer: A new dawn for evolutionary medicine. *Annual Review of Genetics* 39, pp. 359-407.

Wang, X., Lin, Y., 2008. Tumour necrosis factor, friend or foe? *Acta Pharmalogica Sinca* 29, pp. 1275-1288.

Wellen, K.E., Thompson, C.B., 2010. Cellular metabolic stress: Considering how cells respond to nutrient excess. *Journal of Molecular and Cellular Biology* 40, pp. 323-332.

Wilkinson, J.D., Williamson, E.M., 2007. Cannabinoids inhibit human keratinocyte proliferation through non-CB1/CB2 mechanisms and have a potential therapeutic value in the treatment of psoriasis. *Journal of Dermatological Sciences* 45, pp. 87-92.

Willet, W.C., Rockhill, B., Hankinson, S.E., 2000. Epidemiology and non-genetic causes of breast cancer. *Journal of Diseases of the Breast* 21, pp. 175-220

World Health Organisation, 2008. The Global Burden of Disease: 2004 Update. *Geneva: World Health Organisation*.

World Health Organisation, 2011. Number of deaths (World) by cause. *World Health Organisation: Global health observatory data repository*.

Wu, H., Yan, T., 2010. Cannibidol hydroxyquinone-induced apoptosis of splenocytes is mediated predominantly by thiol depletion. *Toxicology letters* 195, pp. 68-74.

Yoshikawa, S., Satou, K., Konagaya, A., 2004. Drug Interaction Ontology (OTO) for Inferences of Possible Drug-Drug Interactions. *Medinfo* pp. 454-459.

Yuan, H., Qianqian, M., Ye, L., Piao, G., 2016. The traditional medicine and modern medicine from natural products. *Molecules* 21(5).



Zhan, C.D., Sindhu, R.K., Pang, J., Ehdaie, A., Vaziri, N.D., 2004. Superoxide dismutase, catalase and glutathione peroxidase in the spontaneously hypertensive rat kidney: effect of antioxidant-rich diet. *Journal of Hypertension* 22(10), pp. 2025-2033.

Zhang, H., Wang, S., Zhang, Y.C., Ye, Y.J., Cui, Z.R., Fang, W.G., 2005. Correlation between Stat3 signal transduction pathway and expression of cyclooxygenase-2 in colorectal cancer cells. *The Chinese Medical Journal* 85, pp. 2899-2904.

Zhu L.X., Sharma, S., Stolina, M., 2000. Delta-9-tetrahydrocannabinol inhibits anti-tumour immunity by a CB2 receptor mediated, cytokine dependent pathway. *Journal of Immunology* 165, pp. 373-380.

Zouki, C., Jozsef, L., Ouellet S., Paquette, Y., Filep, J.G., 2001. Peroxynitrite mediates cytokine-induced IL-8 gene expression and production by human leukocytes. *Journal of Leukocyte Biology* 69, pp. 815-824.

Zuardi, A.W., 2006. History of *Cannabis* as a medicine: a review. *Brazilian Journal of Psychiatry* 28(2), pp. 1509-1516.

Zuardi, A.W., Crippa J.A.S., Hallak, J.E.C., Moreira F.A., Guimaraes, F.S., 2006. Cannabidiol, a *Cannabis sativa* constituent, as an antipsychotic drug. *Journal of Medical Biology Research* 39(4), pp. 421-429

Zuardi, A.W., 2008. Cannabidiol: From an inactive cannabinoid to a drug with a wide spectrum of action. *Brazilian Journal of Psychiatry* 30, pp. 271-280.