THE POTENTIAL NEUROPROTECTIVE EFFECTS OF TWO SOUTH AFRICAN PLANT EXTRACTS IN HYDROGEN PEROXIDE-INDUCED NEURONAL TOXICITY

By

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

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November, 2018

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DECLARATION

I declare that the thesis titled "***The Potential Neuroprotective Effects of Two South African Plant Extracts in Hydrogen Peroxide-Induced Neuronal Toxicity***" is my own work, has not been submitted for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged by completed references.

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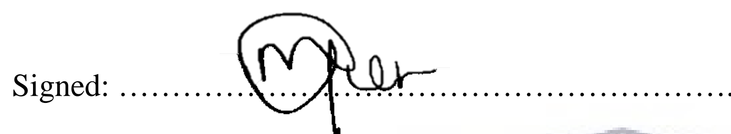

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DEDICATION

I am dedicating this thesis to my fiancé, Brandon who has been a constant source of support and encouragement during the challenges of academics and life. I am truly thankful for having you in my life. This work is also dedicated to my parents, Elxyen and Lorimar who have always loved me unconditionally and whose good examples have taught me to work hard for the things I aspire to achieve.

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ACKNOWLEDGEMENTS

First and foremost, I would like to thank God for letting me live to see this thesis through. Thank you for the wisdom and perseverance bestowed upon me.

I would like to thank my supervisor, Dr. Okobi Ekpo for giving me the opportunity to do this study with him. I am grateful for his scientific guidance and the opportunities he afforded me.

I would also like to thank my co-Supervisor, Prof. Gail Hughes for her contributions to this work. Over the years, she has given me amazing guidance, many insightful suggestions and demonstrated a sincere interest in me.

I would also like to thank Prof. Maryna De Kock who so selflessly offered me her lab space and equipment to conduct some of my experiments.

I would like to acknowledge the members of the Experimental Neurosciences Laboratory who have contributed to the progress I have made. Over the years, Sylvester Ifeanyi Omoruyi and Adaze Peter-Enogieru have taught me several research techniques and offered assistance with my experiments.

To my mentor, Dr. Kareemah Gamieldien, thank you for your continuous support and advice that are helping to shape my professional career. You are the world's #1 boss (in my book at least).

Finally, I would like to thank my friends and family for their continued support, love and encouragement. To the individuals I have met at university who I now consider friends, especially Charnay Cunningham, Lloyd Leach and Monique Engelbrecht, you stood by me when times were hard and made me laugh when I did not even want to smile.

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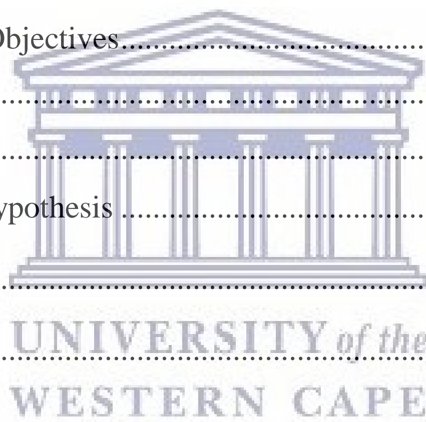
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ABSTRACT

Background: Oxidative stress induced by reactive oxygen species has been strongly associated with many neurodegenerative diseases (NDDs) and many medicinal plant-derived products have been reported to exert potent antioxidant properties. *Sutherlandia frutescens* (SF) and *Carpobrotus edulis* (CE) are two indigenous South African plants with known anti-inflammatory, anti-bacterial, antioxidant and anti-cancer properties. However, the neuroprotective effects of SF and CE have not been extensively studied.

Aims: This study was done to investigate the neuroprotective potentials of *S. frutescens* and *C. edulis* aqueous extracts on hydrogen peroxide (H_2O_2)-induced toxicity in an SH-SY5Y neuroblastoma cellular model of oxidative stress injury.

Methods: The maximum non-toxic dose (MNTD) of SF and CE against SH-SY5Y cells was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Thereafter, the cells were exposed to 250 μM H_2O_2 for 3 hours before treatment with the determined MNTDs of SF and CE extracts respectively and the effects of the treatments on caspase-9 protease activity, intracellular ROS levels, mitochondrial membrane permeability (MMP), nitric oxide (NO) activity, intracellular calcium activity and endogenous antioxidant activity in SH-SY5Y cells was evalaluated using caspase activity kits, DCFH-DA assay, rhodamine 123 fluorescent dye, Griess reagent, Fluo-4 direct calcium reagent, Hoechst staining dye, Superoxide dismutase (SOD) colorimetric and Catalase (CAT) assays, respectively.

Results: Pre-treatment with both extracts of SF and CE showed significant protection against H_2O_2-induced decrease in SH-SY5Y cell viability (apoptotic cell death) and resulted in an increase in caspase-9 activity. In addition, oxidative stress (intracellular ROS), mitochondrial dysfunction, nitric oxide (NO) and intracellular Ca^{2+} levels (excitotoxicity) were respectively attenuated by pre-treatment with the SF and CE extracts.

Conclusion: Thus, the neuroprotective potential of SF and CE extracts could be derived from the summation of anti-apoptotic, antioxidative, anti-excitotoxic and other beneficial effects of these extracts as observed in this study.

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KEYWORDS

Neurodegenerative diseases

Oxidative stress

Hydrogen peroxide

Sutherlandia frutescens

Carpobrotus edulis

Neuroprotection

Antioxidative activity

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ABBREVIATIONS

A-beta (A-β)	Amyloid-beta
AD	Alzheimer's disease
AIDS	Acquired immunodeficiency syndrome
ALS	Amyotrophic lateral sclerosis
ATP	Adenosine Triphosphate
BDNF	Brain-derived neurotrophic factor
CAT	Catalase
CE	*Carpobrotus edulis*
CO_2	Carbon dioxide
CVA	Cerebrovascular accident
CVD	Cardiovascular diseases
DA	Dopamine
DCFH-DA	Dichloro-dihydro-fluorescein diacetate
ddH_2O	Double distilled water
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
FBS	Fetal Bovine Serum
GABA	Gamma amino butyric acid
GPx	glutathione peroxidase
GSH	glutathione
H_2O_2	Hydrogen peroxide
HD	Huntington's disease
HIV	Human immunodeficiency virus
MMP	Mitochondrial membrane potential
MNTD	maximum non-toxic doses
MTT	3-(4,5-dimethylthiazol-2-yl)- 2,5diphenyltretrazolium bromide solution
NADPH	Nicotinamide adenine dinucleotide phosphate
NDDs	Neurodegenerative diseases
NED	N-1-napthylenediamine dihydrochloride
NO	Nitric oxide
$O_2^{\cdot-}$	Superoxide

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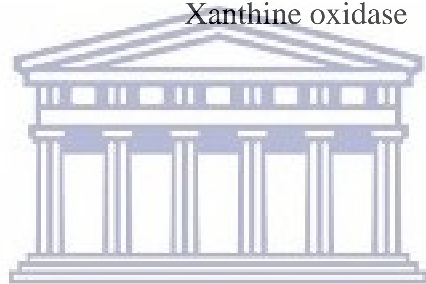
OH˙˙	Hydroxyl radical
OS	Oxidative stress
PBS	Phosphate buffered solution
PD	Parkinson's disease
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SF	*Sutherlandia frutescens*
SNc	Substantia nigra
SOD	Superoxide dismutase
SSA	Sub-Saharan Africa
TNFα	Tissue necrosis factor-alpha
UWC	University of the Western Cape
WHO	World Health Organization
XO	Xanthine oxidase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CHAPTER ONE

INTRODUCTION

1.1 Background

Neurodegenerative diseases are implicated in mortality and morbidity in every country worldwide, with a high prevalence estimated in Africa (Poreau, 2016). Neurodegenerative diseases include various clinical disorders, of which the four major disorders are Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD) and amyotrophic lateral sclerosis (ALS) (Checkoway, Lundin and Kelada, 2016). Neurodegenerative diseases are characterized by memory deficits and impaired cognitive function (Akram and Nawaz, 2017). Such diseases pose a major public health problem, especially in developing countries due increasing prevalence, leading to constraints on caregivers and financial resources. AD is ranked the 6th most common cause of death in the United States of America, while PD is ranked the 2nd most prevalent neurodegenerative disease, affecting approximately 2% of the population (Liu *et al.*, 2017).

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Over the years there has been very little success in discovering cures for neurodegenerative disorders, and the aging population also makes this a very pressing global health matter (Law *et al.*, 2014). While the aetiology of neurodegenerative disorders has yet to be fully understood, some studies have implicated oxidative stress (OS) as a common underlying factor (Han *et al.*, 2014; Kim *et al.*, 2015; Park *et al.*, 2015a). OS is characterized by the imbalance between antioxidants and the accumulation of reactive oxygen species (ROS) (González-Sarrías *et al.*, 2017). While the human body produces ROS as by-products of physiological and biochemical processes, its overproduction can induce OS, resulting in the mitochondrial dysfunction of proteins and lipid membranes, altering their function (Han *et al.*, 2014). Hydrogen peroxide (H_2O_2) is a non-radical form of ROS which can easily be converted into the hydroxyl radical (OH^-), causing damage to the components of most cells, including neurons (Chetsawang and Govitrapong 2010). Mitochondrial dysfunction results in reduced ATP production, calcium mishandling and elevated amounts of ROS, all of which contribute to neuronal cell death in AD and stroke (Uttara *et al.*, 2009; Gray *et al.*, 2015).

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Neuroprotection is a preferred option for managing nervous system disorders, including neurodegenerative diseases and stroke since most neuronal damage is caused by complex biochemical reactions, resulting in apoptotic or necrotic cell death (Hong *et al*., 2014). The use of antioxidant to scavenge free radicals is a plausible approach to modulate apoptosis and many studies have reported that a number of antioxidant-rich plants, foods and dietary supplements have neuroprotective properties when administered prior to suffering an oxidative stressor (Lobo *et al*., 2010; Lalkovičová and Danielisová, 2016). Despite pharmaceutical significant advancements in the development of synthetic drugs to treat neurodegenerative disorders, traditional herbal medicine still remains a valued field of research in most parts of the world due to the biological characteristics of plants and their ability to scavenge free radicals (Ekor, 2014).

Medicinal plants provide a good source of treatment of several illnesses such as neurodegenerative diseases, diabetes mellitus and cancer. Indian and Chinese traditional medicine has identified and recommended numerous plants to treat the symptoms of neurodegenerative diseases (Manoharan *et al.*, 2016). Africa, especially Southern Africa has a rich diversity of plants, with approximately 25% of the total number of plants in the world, hailing from sub-Saharan Africa (SSA) (Wyk, 2008). South Africa has a rich flora and many people still rely on traditional medicines as an alternative therapy due to these being cheaper, accessible and safe for consumption with little or no side-effects (Km and Van Tonder, 2013).

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Carpobrotus edulis (*C. edulis*) and *Sutherlandia frutescens* (*S. frutescens*) are two South African plants, well-known for their use as traditional remedies (Wyk, 2008). *C. edulis*, also known as sour fig is a perennial, succulent plant of the *Aizoaccae* family found in almost all soil types considered to be highly effective against mouth, throat fungal infections, earaches, toothache and oral and vaginal thrush due to its rich antioxidant and antibacterial content (Springfield *et al.*, 2003; Maja, 2009; Ibtissem *et al.*, 2012). C. edulis contains tannins, flavonoids such as rutin, hyperoside, phytosterols and aromatic acids that contributes to its antioxidant and antibacterial properties with many benefits to humans as it has been shown to inhibit platelet aggregation, decrease capillary permeability and, improve blood circulation, making it very useful for the prevention of blood clots, heart attacks and strokes (Hamadi, 2017). Previous studies on *C. edulis* have reported on its antioxidative and antimicrobial properties (Omoruyi, 2014) but studies involving its use in *in vitro* and *in vivo* work are

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scanty. A previous study investigating the antioxidant and phytochemical properties of *C. edulis* exacts against 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), diammonium salt, hydrogen peroxide (H_2O_2), nitric oxide and ferric reducing power yielded satisfactory results as the leaf extracts demonstrated strong H_2O_2 scavenging activity (Omoruyi, Bradley and Afolayan, 2012). The inhibitory effect of the extracts on free radicals may justify the traditional use of this plant to treat common illnesses.

S. frutescens, commonly known as cancer bush is one of the best known medicinal plants endemic to South Africa (Fernandes *et al.*, 2004; Leisching *et al.*, 2015). It is used for a variety of illnesses including influenza, tuberculosis, chronic fatigue syndrome, rheumatoid arthritis, stomach aches, fever, cancer, diabetes, wounds, anxiety, clinical depression and human immunodeficiency virus/ acquired immune deficiency syndrome (HIV/AIDS) (Ifeanyi, 2009). Chemical studies have shown that *S. frutescens* is rich in amino acids and pinitol and contains small amounts of triterpenoid saponins, flavonoids and many other free amino acids, namely L-canavanine and GABA. Canavanine has been documented for its anticancer and antiviral properties and is also known to inhibit the production of nitric oxide (NO) synthase thus proving to be a reliable treatment for septic shock (Leisching *et al.*, 2015). Pinitol is well-known for its use for the treatment of diabetes, cancer and AIDS, while GABA, an inhibitory neurotransmitter, is considered the reason for the use of *S. frutescens* for the management of anxiety and stress (Wyk, 2008). While *S. frutescens* is well-known for its anti-cancer properties, no documented scientific report on the potential benefits and mechanisms of action of *S. frutescens* for the treatment of nervous system disorders exists. (Fernandes *et al.*, 2004).

The current study is aimed at investigating the potential neuroprotective potentials of *S. frutescens* and *C. edulis* in a cellular model of H_2O_2-induced toxicity. The human neuroblastoma cell line SH-SY5Y was used in this model system due to its ability to differentiate into normal neurons (Kovalevich and Langford, 2013; Morelli *et al.*, 2014). Neuroprotection was assumed to occur by treating cells with the extracts before induction of neuronal toxicity cell-damage by the addition of H_2O_2.

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1.2. Rationale for the study

This research deals with the administration of *S. frutescens* and *C. edulis* extracts prior to exposing neurons to cell toxicity. The use of these plants as traditional herbal medicine prompted the initiation of thesis work as there was very little known about the use of these plants as neuroprotective agents. This study will be able to validate or refute epidemiological studies that list *S. frutescens* as a likely neuroprotective agent. It will also provide scientific findings on *C. edulis*- as scientific findings regarding it are very scarce. If the hypothesis holds true, then further validative studies using *in vivo* techniques and clinical trials could be employed to determine the efficacy and mechanisms involved in the administration of each extract.

1.3. Research Aims and Objectives

Aim of the study

To analyse the neuroprotective potential of *S. frutescens* and *C. edulis* separately on H_2O_2-induced human-derived SH-SY5Y cell line. The main mechanism of action addressed include, the effects of *S. frutescens* and *C. edulis* extracts on oxidative stress, excitotoxicity, apoptosis and endogenous antioxidant activities.

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Objectives of the study

The objectives of this study include to:

i. determine the safe and non-toxic doses of *Sutherlandia frutescens* and *Carpobrotus edulis* which maintain the viability of SH-SY5Y cells at different time intervals.

ii. determine the various concentrations of H_2O_2 that could induce the death of at least 50% of the SH-SY5Y cells for different time intervals (the optimal concentration of hydrogen peroxide, hereafter denoted as H_2O_2-OC)

iii. determine the % survival of SH-SY5Y cells following pre-treatment with selected concentrations of *S. frutescens* and *C. edulis* before exposure to H_2O_2-OC for different time intervals.

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iv. evaluate ROS levels and Nitric oxide (NO) activity in SH-SY5Y cells pre-treated with selected concentrations of *S. frutescens* and *C. edulis* before exposure to H_2O_2-OC for different time intervals.

v. evaluate Caspase 9 activity in SH-SY5Y cells following pre-treatment with selected concentrations of *S. frutescens* and *C. edulis* and exposure to H_2O_2-OC for different time intervals

vi. determine the effects of pre-treatment with selected concentrations of *S. frutescens* and *C. edulis* on antioxidant status of SH-SY5Y cells after H_2O_2 toxicity

vii. determine morphological changes in SH-SY5Y cells after pre-treatment with selected concentrations of *S. frutescens* and *C. edulis* and H_2O_2-OC toxicity, using the Hoechst staining technique.

1.4. Research question/ hypothesis

This study hypotheses that the selected concentrations of *S. frutescens* and *C. edulis* separately, will modulate the adverse effects of H_2O_2-induced toxicity *in vitro* on SH-SY5Y human neuroblastoma cells.

1.5. Thesis outline

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Chapter 1 introduces the project (what it's all about, the aims and objectives, techniques used as well as the plants chosen and the reasons for choosing them). Chapter 2 provides a detailed review of literature on neurological disorders, oxidative stress progression and role of traditional herbal medicines and their antioxidative properties that helps alleviate the deleterious effects of ROS on brain cells. Chapter 3 focuses on the methodology used in this study and provides a list of materials, experimental procedures and tests. The statistics used to analyse all data is also described. Chapter 4 presents the key findings of this study while Chapter 5 gives a detailed discussion of results, the conclusion and summary of the entire study as well as limitation of study and future recommendations.

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CHAPTER TWO

LITERATURE REVIEW

2.1 Introduction

Neurodegenerative diseases (NDDs) are a heterogeneous group of illnesses, characterized by the slow progressive decrease in the viability and number of neurons (Penton-Rol and Cervantes-Llanos, 2018). NDDs are becoming more common with a negative impact on countries with longer life expectancies and are ranked the fourth highest source of disease burden in first-world nations (Pérez-Hernández *et al.*, 2016). While the aetiology of most NDDs are not well known, oxidative stress has been implicated (Kim *et al.*, 2015). Many plants used as traditional herbal medicine (THM) contain such compounds as polyphenols, alkaloids and terpenes. It is therefore important that such plants be investigated to scientifically support their traditional use for the treatment of NDDs, especially in most African and Asian traditions.

2.2 Oxidative Stress (OS)

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OS is a condition involving an imbalance between reactive oxygen species (ROS) and antioxidant systems of the body, resulting in excessive build-up of ROS (Birben *et al.*, 2012). OS may be associated with damage of cell membranes due to lipid peroxidation, changes in protein structure and function as a result of oxidation and DNA damage, resulting in chronic diseases such as atherosclerosis, cancer, diabetes, rheumatoid arthritis, cardiovascular diseases (CVD), chronic inflammation, septic shock, aging and NDDs (Figure 2.1). The brain is most susceptible to the harsh effects of ROS due to its high demand for oxygen and glucose, constituting 20% of the body's oxygen supply. Redox-active metals, namely iron and copper are involved in the generation of ROS and oxidative stress-related disorders because of their abundance in the brain and are involved in ROS formation. High levels of polyunsaturated fatty acids in the brain cell membranes also lead to excessive accumulation of ROS as these fatty acids react as substrates for lipid peroxidation (Rivas-arancibia *et al.*, 2011).

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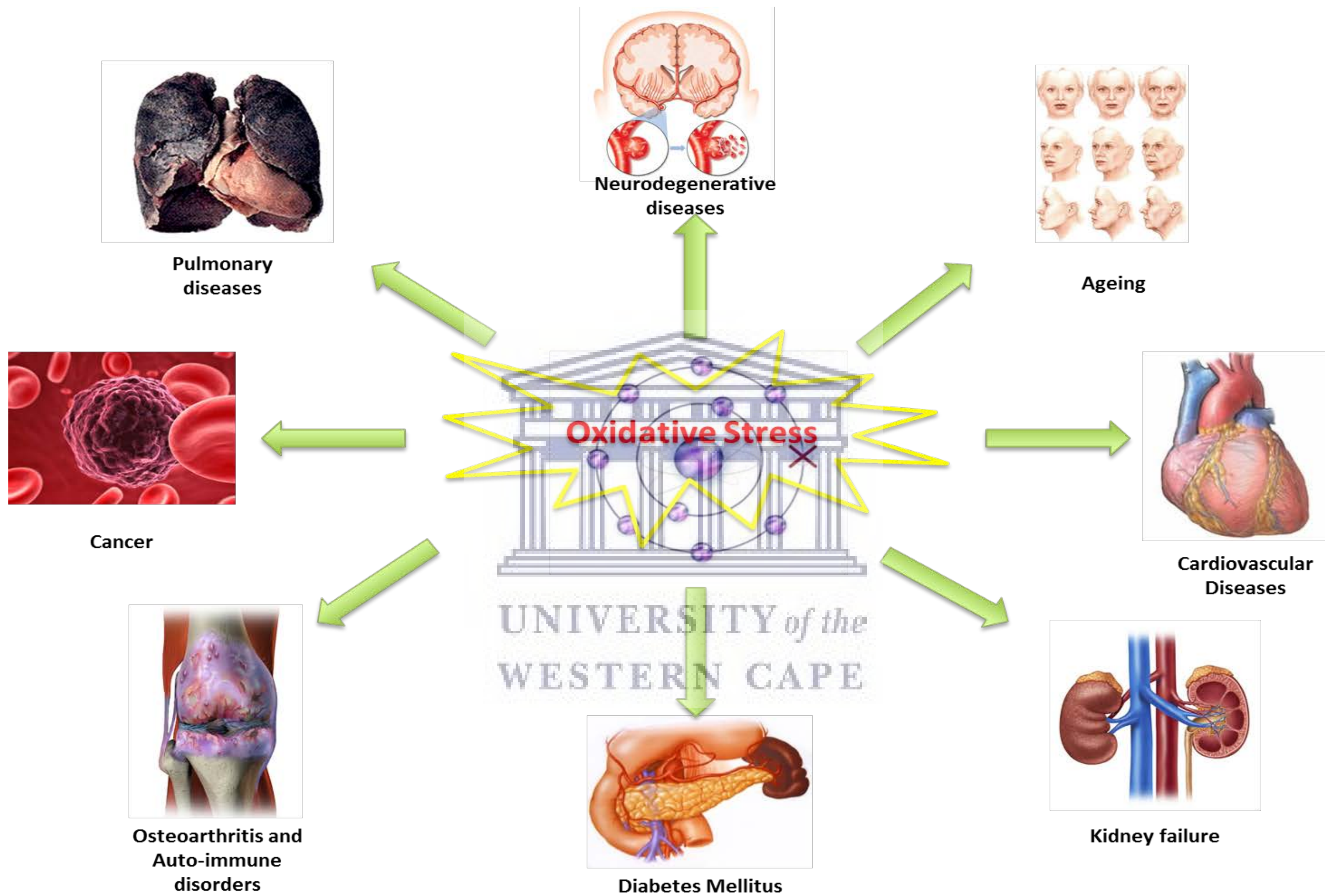

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Figure 2.1.: Diseases in which oxidative stress plays a pathophysiological role

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2.2.1. Types of Reactive Oxygen Species (ROS)

ROS are chemical reactive species derived from oxygen (Gandhi and Abramov, 2012), often short-lived and highly reactive due to their unpaired valence electrons. Oxygen is easily susceptible to free radical formation due to two unpaired electrons present in its outer electron shell. ROS include molecules such as superoxide (O_2^-), hydroxyl radical (•OH) and hydrogen peroxide (H_2O_2) (Figure 2.2) (Uttara *et al.*, 2009). While O_2 is believed to be the instigator in ROS production, it can be transformed into H_2O_2 by the enzyme superoxide dismutase (SOD) and when the production of H_2O_2 exceeds antioxidant defences, it poses a major threat to cells (Castino *et al.*, 2011; Wang *et al.*, 2015). H_2O_2 can then generate highly reactive hydroxyl radicals, responsible for major cytotoxic effects (Kim *et al.,* 2015). Various studies have reported that H_2O_2 play a crucial role of inducing apoptosis in different cell types (Wang *et al*., 2015). Hydroxyl radicals are mainly responsible for the cytotoxic effects of ROS and are therefore of immense interest. Once generated from H_2O_2 and O_2, these radicals are catalysed by iron ions through the Fenton reaction (decomposition of H_2O_2) (Gil-Lozano *et al*., 2017). Such reactive species cause oxidation of lipids, proteins and DNA in the cell.

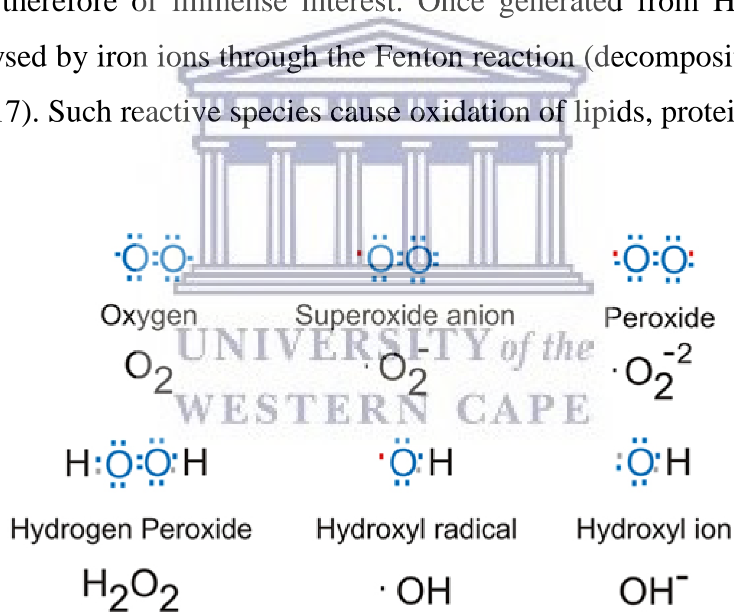

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Figure 2.2: Common reactive oxygen species (Kim *et al.*, 2015)

2.2.2.1 Superoxide anion and Hydroxyl radical

$O_2^{\cdot -}$ is considered a relatively unreactive type of ROS but could be toxic as it initiates reactions that give rise to other ROS. O_2^- can be formed as a product of many reactions such as in flavoprotein reactions, oxidases and hydroxylases (Kim *et al.*, 2015; Wang *et al*., 2018). O2$^{\cdot -}$ radicals can alsoreact with nitric oxide (NO) to generate cytotoxic peroxynitrite anions ($ONOO^-$). Peroxynitrate can react with carbon dioxide (CO_2), resulting in protein damage

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(Klein and Ackerman, 2003). ·OH is considered the most reactive species, formed as a result of exposure to high-energy radiation such as x-rays or gamma rays. H_2O_2 and O_2^- can form the $^-$OH radical *(Wang et al., 2018).*

2.2.2.2 Hydrogen peroxide

H_2O_2 is a ROS that can easily diffuse through cell membranes and is formed by two pathways, firstly is after the direct reduction of oxygen by two unpaired electrons and secondly, from the catalyzation of the superoxide anion by SOD (Singh *et al.,* 2004). Enzymes that produce H_2O_2 from oxygen include xanthine oxide reductase, superoxide dismutase, glucose oxidase, D-amino acid oxidase and uricase. However, the production of H_2O_2 may also occur from the catalyzation of copper (Kim *et al.*, 2015). H_2O_2 can diffuse through membranes and aqueous compartments with ease and can inactivate enzymes at low concentrations. While H_2O_2 is considered a stable ROS molecule, the main threat lies in its ability to generate highly reactive hydroxyl radicals (Haffner, 2000).

2.2.3 Generation of ROS

Free radicals are defined as a molecule containing one ore more unpaired electrons in its outermost atomic orbital and are able to exist alone. ROS and RNS describes free radical species and other non-radical reavtive derivatives (Sen *et al.*, 2010). ROS can be generated by endogenous and exogenous sources. Endogenous ROS are generated within the cells of the body, often mediated by such mitochondrial and non-mitochondrial ROS-generating enzymes as nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Nox), xanthine oxidase (XO), cytochrome P450 and flavin oxidase (from peroxisomes). Exogenous sources of ROS are generated from outside the body and include ultra violet rays, ionizing radiation, drugs and environmental toxins (Kim *et al.*, 2015).

At high concentrations, ROS can be mediators of cellular damage. $O_2^{\cdot}$ radical is responsible for lipid peroxidation and may also have the potential to decrease antioxidant activity of Catalase (CAT) and lutathione peroxide (GPx). $HO_2^{\cdot}$, the pronated form of $O2^{\cdot-}$ is more reactive and can penetrate the membrane,causing tissue damage. H_2O_2, produces toxicity to cell by causing DNA damage, membrane disruption and release calcium iond within cells resulting in the activiation of calcium dependent proteolytic enzymes (Sen *et al.*, 2010).

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2.2.4 Epidemiology of Neurodegenerative Diseases (NDDs)

Universally, life expectancy is becoming longer, including in developing nations. In sub-Saharan Africa, life expectancy at birth has increased by more than 20 years between the years 1950 and 2010 (Lekobou *et al.,* 2015). While an aging population is regarded as a public health success, it also brings with it new challenges in the form of chronic diseases such as cardiovascular diseases, cancers and neurodegenerative diseases. Aging, physical and emotional stress, as well as environmental factors have been implicated as major factors for NDDs.

2.2.4.1. Aging

Aging has been verified in many studies as a major risk factor for neurodegenerative disorders such as stroke, PD and AD (Ataie *et al.*, 2010; Chen *et al*., 2018). To date, there is a sparsity in preventative strategies for neurological disorders and most age-related available treatments only address the symptoms (Shen *et al*., 2013). Hence, research into age-related disorders remains crucial.

2.2.4.2. Stress (physical and emotional)

Modern lifestyles place people under greater amounts of psychological and physical stress (Sotiropoulos, 2014). Stress, is normally regulated through homeostasis, but chronic stress could become problematic since the released glucocorticoids are strongly associated with neuronal atrophy/dysfunction, cognitive impairment as well as mood disorders and depression (Machado *et al*., 2014; Sotiropoulos, 2015). Loss of synapses in the brain due to chronic stress has also been shown to remarkably alter cell-to-cell communication in the nervous system (Mossop, 2011).

2.2.4.3. Environmental factors

Neurotoxic metals such as mercury, aluminium, cadmium and arsenic, as well as pesticides and some metal-based nanoparticles have been implicated in the progression of AD (Chin-chan, Navarro-yepes and Quintanilla-vega, 2015). Heavy metals are well-recognized agents that affect brain development, leading to life-long impairment. Various studies have examined the possible link between aluminium (Al) from antiperspirants and occupational exposures and AD. Epidemiological studies found associations between PD and exposure to

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pesticides and insecticides, however none of these have been validated scientifically (Nandipati and Litvan, 2016).

2.2.5 Oxidative Stress and NDDs

Neurodegenerative disorders result from the progressive loss of neurons within the brain and or spinal cord (Haque and Nazir, 2017). The term encompasses a variety of clinical illnesses, the most common of which are AD and PD. Although much of the pathophysiology of these diseases is not fully understood, altered mitochondrial function, increase in oxidative damage, the presence of abnormal proteins, proteasomes as well as changes in inflammation and excitotoxicity are some of the common physiological alterations (Singh *et al.,* 2004). Oxidized proteins are often removed by proteasomes, while the inhibition of proteasomes also results in the accumulation of abnormal proteins and ROS production. The produced ROS then initiates a cascade of neurodegenerative events including mitochondrial damage, resulting in an increase in Ca^{2+} and an inhibition in the function of proteasomes. Oxygen takes part in the breakdown of glucose in the mitochondria, thus generating energy in the form of ATP. Oxidative stress may have adverse effects on cell proliferation, cell differentiation and cell survival, by activating cell signalling pathways and prolonged exposure often lead to neurotoxicity and neuronal death as seen in such in neurodegenerative diseases as cerebral ischemia, seizures, schizophrenia, PD and AD (Uttara *et al.*, 2009).

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Table 2.1: Characteristic features of well-known neurodegenerative disorders. Adapted from (Singh, Sharad and Kapur, 2004)

Neurodegenerative disorders	Clinical features	Neuropathophysiology
Alzheimer's disease (AD)	Memory loss, progressive deterioration of thought, judgement, language impairment, visual-spatial perception loss	Cortical atrophy Dysfunction and death of neocortex, hippocampus, amygdala, basal forebrain and brainstem
Parkinson's disease (PD)	Bradykinesia Tremors, dementia	Degradation of neurons due to loss of pigmented neurons in the substantia nigra
Huntington's disease (HD)	Cognitive impairments, personality changes	Marked atrophy of the striatum and generalised cortical atrophy with decreased brain weight

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Amyotrophic Lateral Sclerosis (ALS)	Weakness and atrophy of skeletal muscles, weakness of chest muscles and diaphragm and muscle dysfunction in the larynx and pharynx which lead to respiratory problems	Loss of primary motor neurons in the neocortex

2.2.5.1 Alzheimer's disease (AD)

AD is one of the most common neurological disorders, caused by the progressive loss and accumulation of proteins and extracellular senile plaques formed by beta-amyloid (Aβ) oligomers (Gray *et al.*, 2015; Kim *et al.*, 2015). AD is characterized by symptoms such as memory loss, language impairment, cognitive dysfunction and behavioural disturbances including depression, agitation and psychosis (Mahdy *et al.*, 2012) and mitochondrial dysfunction and oxidative stress are considered contributers of cognitive decline and neuronal cell death in AD (Leuner and Reichert, 2012). There are numerous studies that suggest that oxidative imbalance and its resultant neuronal damage may be implicated in AD. Other studies suggest that ROS build-up in neurons may cause oxidation of proteins, DNA and RNA, lipid peroxidation and modification of sugars, resulting in neuronal death in parts of the hippocampus of the brain. The loss of redox homeostasis in an AD-brain leads to chronic oxidative stress that increases the production of ROS and RNS (Huang *et al*., 2016). Other studies have suggested that Aβ-induced oxidative imbalance could increase the byproduct levels related to lipid peroxide, protein oxidation and DNA/RNA oxidation (Wang *et al*., 2014) and causes a reduced level of expression or activity of antioxidants such as uric acid, vitamin C and E as well as antioxidant enzymes such as superoxide dismutase and catalase and have been found in patients with AD. Oxidative damage to DNA and RNA repair may be quite extensive in the hippocampus of the brain as it considered the most severely affected region of the brain.

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2.2.5.2 Parkinson's disease (PD)

Parkinson's disease is the second most common age-related neurological disorder, with approximately 6 million sufferers world-wide (Seoposengwe *et al.*, 2013). Disease prevalence is approximately 2% amongst individuals over 65 years of age, and approximately 4% amongst those older than 80 years of age (De Lazzari *et al*., 2018; Gandhi and Abramov,

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2012). PD, which affects movement is characterized by the selective loss of dopaminergic neurons in the substantia nigra of pars compacta (SNpc) as well as the depletion of dopamine (DA) (neurotransmitter responsible for movement) (Ramkumar *et al.*, 2017). Many studies have shown that an inhibition of redox regulation contributes to PD, but there is still not enough evidence to suggest that ROS is implicated as a primary event or consequence of PD. The overproduction of ROS however is an important factor in cell death, as seen in PD. Changes in antioxidant molecules have been reported in the early stages of PD; glutathione (GSH) has been reported to be reduced in the SNc of PD (Sian-Hülsmann *et al.*, 2011). Higher levels of iron were also observed in the SNc of PD and increased iron levels in DA neurons were also implicated in PD which may allow easier interaction of ferrous irons with H_2O_2 and enhance the production of ^{-}OH (Kim *et al.*, 2015).

2.2.6. Pathophysiology

Though the aetiology of most NDDs is still unknown, several in vivo studies have shown that redox imbalance, excessive release of glutamate causing excitotoxicity, an increase in Ca^{2+} activating mitochondrial dysfunction and more oxidative and inflammatory responses lead to neuoral death (Zhong *et al.*, 2016). Redox imbalance and chronic inflammation are major implicators of neuronal damage and death and the overproduction of ROS by mitochondria and NADPH oxidase in oxidative stress is thought to be the cause for tissue damage in NDDs (Zhang *et al.,* 2007). Reduced ATP levels results in the cessation of the electron transport chain activity in mitochondria, resulting in the disruption of ionic pump systems, causing increase in intracellular Na^{+}, Ca^{2+} and Cl concentration and the efflux of K^{+}. These actions cause the depolarization of neurons, leading to excess neurotransmitter release, causing neuronal excitotoxicity. Mitochondrial dysfunction may also lead to neuronal oxidative damage in the progression of neurodegenerative diseases. Since these cellular organelles generate ROS, produce ATP, undergo calcium homeostasis, membrane lipid concentration as well as membrane permeability transition. Mitochondrial diseases involving neurodegeneration will likely implicate some or all of the above-mentioned functions. Previous studies have shown that alterations in mitochondrial integrity affect oxidative phosphorylation and ATP production, and increase susceptibility to apoptosis and accumulation of damaged mitochondria with unstable DNA (Lepetsos and Papavassiliou, 2016).

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2.2.7. Neuroprotection

Neuroprotection is a complex term, used in reference to mechanisms that are able to protect the nervous system against acute and chronic injuries (Iriti *et al.*, 2010). Limitations placed on current treatments for NDDs have led to investigations of novel strategies to finding compounds and non-pharmacological treatments that would ameliorate these conditions, whilst decreasing side effects. Such strategies involve preventing cell death and restoring function to damaged neurons, and restoring or increasing neuronal numbers (Monteiro *et al.*, 2017). Despite differences in NDD associated symptoms and injuries, many of the mechanism causing neurodegeneration are the same (i.e.: oxidative stress, mitochondrial dysfunction, excitotoxicity, inflammation, protein aggregation). Of these mechanism, neuroprotective mechanism often target inflammation, excitotoxicity and oxidative stress- all of which are highly associated with NDDs.

Neuroinflammation is one of the major features in acute and chronic NDDs, in which microglia and astrocytes are contributors to inflammation, therefore therapeutic mechanisms would aim to modulate function of the innate immune system (Glass *et al.*, 2010). Anti-excitotoxicity is one of the most important mechanisms involved in the prevention or treatment of excitotoxcity in NDDs. The role of these is to inhibit the binding of glutamate to NMDA receptors, preventing the increase of Ca^{2+}. Caspase inhibitors are used primarily for their anti-apoptotic effects, while antioxidants are known as the primary treatment used to control OS levels and they work to eliminate ROS.

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The development of new therapeutics for NDDs has proven very difficult over the past decade. Since the pathogenesis of most NDDs involves oxidative stress, therapies that involve the management of OS have been widely considered to be useful in ameliorating some of the symptoms associated with NDDs. The human body however, has a rich endogenous antioxidant system (Figure 2.3.) which includes enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT), as well as non-enzymatic molecules such as uric acid, glutathione (GSH) and ascorbic acid (Aguilar *et al.*, 2008). Cellular ROS levels may be reduced through the defense mechanisms of antioxidant enzymes and antioxidants, while O_2^- can be inactivated by SOD to produce H_2O_2 which can then be further removed by the action of glutathione peroxidases, catalase and peroxiredoxins (Sofo *et al.*, 2015).

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Catalase, SOD and GPx are the three primary enzymes involved directly in the elimination of ROS such as O_2^-, •OH, NO and H_2O_2, while GSH, glucose-6-phosphate dehydrogenase and cytosolic GST are secondary enzymes which help in the detoxification of ROS by depleting the peroxide levels or maintaining a steady supply of glutathione and NADPH which is necessary for primary antioxidants to function properly (Singh, Sharad and Kapur, 2004). The mode of action of antioxidants depends largely on their ability to scavenge free radical species as well as their ability to reduce iron (Omoruyi, Bradley and Afolayan, 2012).

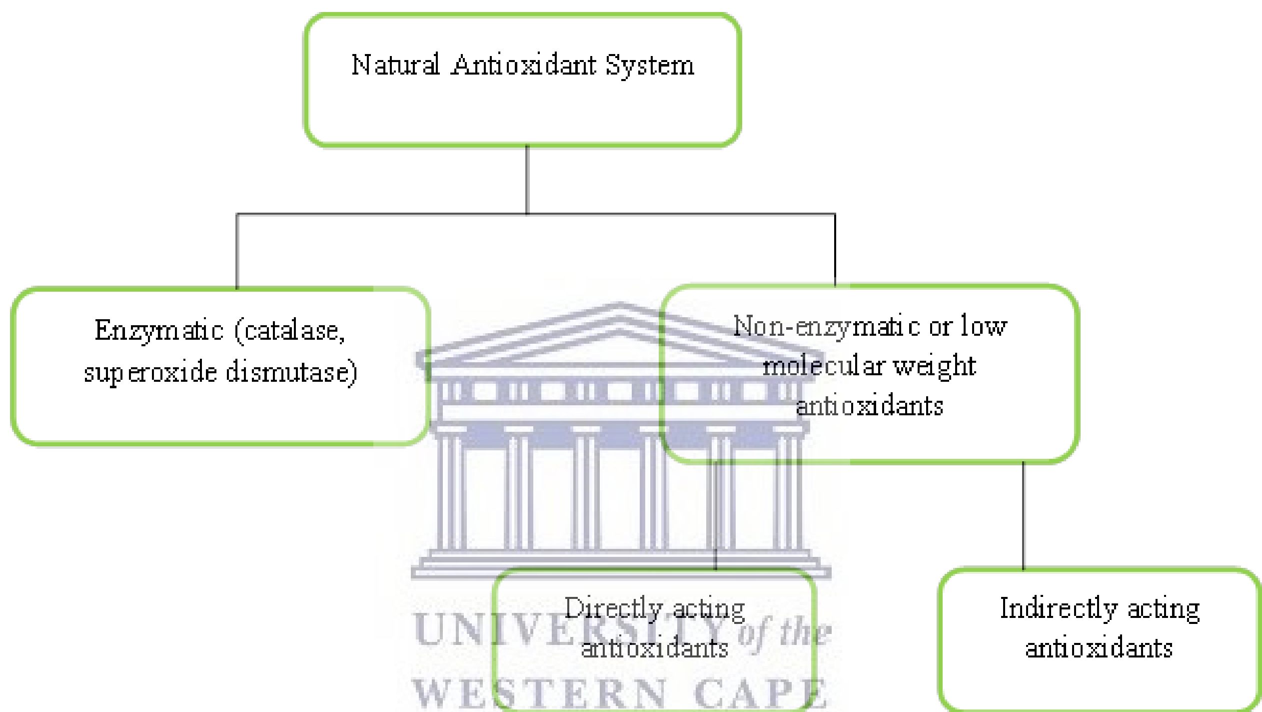

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Figure 2.3: Categorization of natural antioxidants, adapted from (Uttara *et al.*, 2009)

2.2.7.1. Superoxide dismutase (SOD)

SOD is a key player in the breakdown of O_2^- radical to the less reactive H_2O_2 and oxygen. There are three distinct types of SOD, the first is cytosolic copper/zinc-SOD, the second, mitochondrial manganese SOD and the third is extracellular SOD. These have been identified as SOD1, SOD2 and SOD3, respectively and SOD1 and SOD2 are the main derivatives involved in the elimination of O_2^- radical in the cytosol and mitochondria, respectively(Cavallaro *et al.*, 2017).

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2.2.7.2. Catalase (CAT) and Glutathione Peroxidase

CAT is responsible for the conversion of H_2O_2 to water (H_2O) and oxygen (O_2) using iron or manganese as a cofactor (Haffner, 2000). CAT is located in peroxisomes and also found in the cytoplasm and mitochondria (Kim *et al.*, 2015). Catalase is considered important in severe OS by reducing intracellular H_2O_2. Glutathione peroxidase play a significant role in oxidant balance in the body. It reduces H_2O_2 and lipid peroxidases to H_2O and lipid alcohols. Inadequate expression or dysfunction of glutathione peroxidase can contribute to OH· formation by not detoxifying H_2O_2. At lower levels of expression, glutathione peroxidase also may allow for lipid peroxidase reaction with free metals to form lipid peroxyl radicals (Wolin *et al*., 2002; Wassmann *et al,*. 2004).

2.2.7.3. Peroxiredoxins (PRx)

PRx are thiol-specific peroxidases that catalyse the reduction of H_2O_2. PRx make up approximately 1% of cellular proteins and are highly reactive. They are responsible for the reduction of approximately 90% of mitochondrial H_2O_2 and 100% of cytoplasmic H_2O_2 (Kim *et al.*, 2015).

2.2.7.4. Glutathione (GSH)

GSH is a peptide molecule synthesized from glutamate, cysteine and lycine. It executes protective functions of cells against oxidative stress GSH can react with O_2- and OH for the removal of ROS, therefore it is considered an important neuroprotective agent against NDDs such as AD and PD (Aoyama *et al.*, 2012). It can also donate an electron for the reduction of peroxide in the GPx reaction. Numerous studies have reported that GSH is involved in inhibiting apoptotic cell death and DNA damage in brain cells following oxidative stress (Kim *et al.*, 2015).

2.2.7.5. Vitamin C and Vitamin E

Vitamin C is water-soluble antioxidant involved in the removal of free radicals by electron transfer and acts as a cofactor for antioxidant enzymes (Paulsen, 2010). Vitamin E is a lipid-soluble antioxidant that can attenuate the effects of peroxide and protect against lipid peroxidation in cell membrane (Paulsen, 2010; Rizvi *et al.*, 2014).

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2.3. SHSY5Y Neuroblastoma cell line

An *in vitro* model widely used in NDDs is the neuroblastoma SHSY5Y cell line which is a derivative of Sk-N-SH cell line (Ferguson and Subramanian, 2016), established in the 1970's from a bone marrow biopsy of a 4-year old neuroblastoma patient and is presumed to arise from neural crest cells. SH-SY5Y cells can be differentiated from a neuroblast-like state into mature human neurons through a variety of mechanism including the use of Retinoic acid, phorbol esters and neurotrophins such as brain-derived neurotrophic factor (BDNF) (Teppola *et al*., 2016). There are very important differences between SH-SY5Y cells in their undifferentiated and differentiated states. When undifferentiated, they rapidly proliferate and appear to be non-polarized with few, short processes. They also grow in clumps, expressing markers of immature neurons. When differentiated, these cells extend long, branched processes, decrease in proliferation (Xicoy, Wieringa and Martens, 2017).

2.4. Traditional Herbal Medicine (THM)

2.4.1. Introduction

Many plant species from across the world have pharmacological properties and have been used since ancient times (Shrestha and Dhillion, 2003; Chun *et al.,* 2008; Balkrishna, 2017). Today, more than 60% of the world's population depends on plants for their primary medical needs, with more than 80% of those being in developing nations (Shrestha and Dhillion, 2003). Herbal medicine remains a crucial part of various cultures and traditions such as in the Chinese, Indian and African. It is the knowledge of these plants that has led to the discovery of new cures (Soni *et al.*, 2012).

In South Africa, medicinal plants are used as either a complement or alternative to Western medication (Maja, 2009), mainly due to the cultural diversity as well as the country's massive floral biodiversity. South Africa has more than 30,000 plant species with about 3 000 of these species being used as traditional medicine (Thring, Springfield and Weitz, 2007). More than 80% of the South African population depend on at least one traditional remedy and this can be attributed to the fact that such remedies are often cheaper, locally available and easy to administer as simple preparations (Ghuman and Coopoosamy, 2011; Seoposengwe *et al.*, 2013). THMs form a crucial part of complementary and/or alternative medicine as most show promising responses due to their active chemical ingredients (Surveswaran, *et al*., 2006; Soni

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et al., 2012). Over the past two decades, numerous chemical analyses have revealed potential beneficial properties of many plant species. These are attributed to polyphenols, flavonoids, tannins and essential oils present in these plants, which present a wide spectrum of biological activity such as anti-inflammatory, anti-cancer, anti-allergic and antioxidant effects (Othman, *et al.*, 2017).

2.4.2. Polyphenols

Polyphenols are naturally-occurring compounds found in majority of plants, fruits, vegetables, cereals and beverages (Pandey and Rizvi, 2009). Polyphenols are classified as secondary metabolites of plants and are generally involved in the defense against ultra violet (UV) radiation or pathogens (Pérez-Hernández *et al.*, 2016).

More than 8000 polyphenolic compounds have been identified in plant species. Polyphenols are classified based on the number of phenol rings they contain and on the basis of structural elements that bind the rings together. The main classes include phenolic acids, flavonoids, stilbenes and ligans (Figure 2.4) (Pérez-Hernández *et al.*, 2016).

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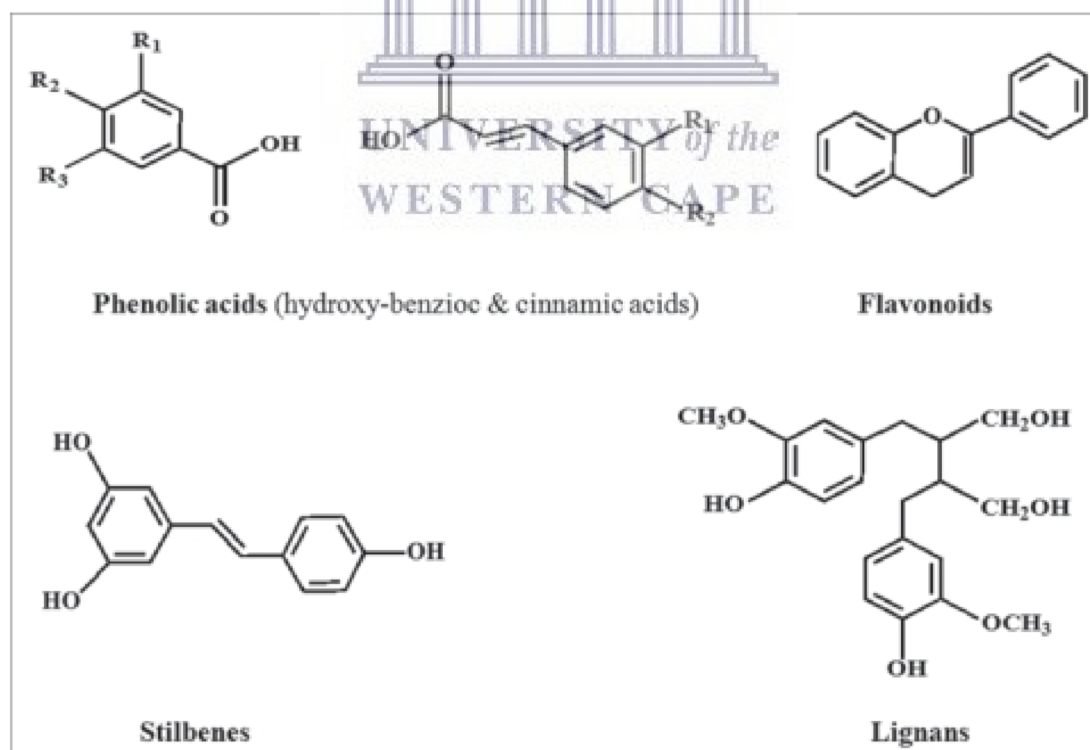

Figure 2.4: Chemical structure of the classes of polyphenols (Pandey and Rizvi, 2009)

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2.4.2.1 Phenolic acids

Phenolic acids are found in food and are divided into two classes: derivatives of benzoic acid and derivatives of cinnamic acid (Pandey and Rizvi, 2009).

2.4.2.2 Flavonoids

Flavonoids are characterized by two or more aromatic rings bearing at least one aromatic hydroxyl group connected to a carbon bridge. These are sub-divided into five categories, namely: flavanols, flavones, flavanones and anthocyanins (Figure 2.5) (Canda, 2012) and the divisions are based on the saturation of the flavan ring as well as their hydroxylation (Surveswaran *et al.,* 2006). Flavonoids are mostly present in flower-producing plants, fruits and vegetables (Rakoma, 2016).

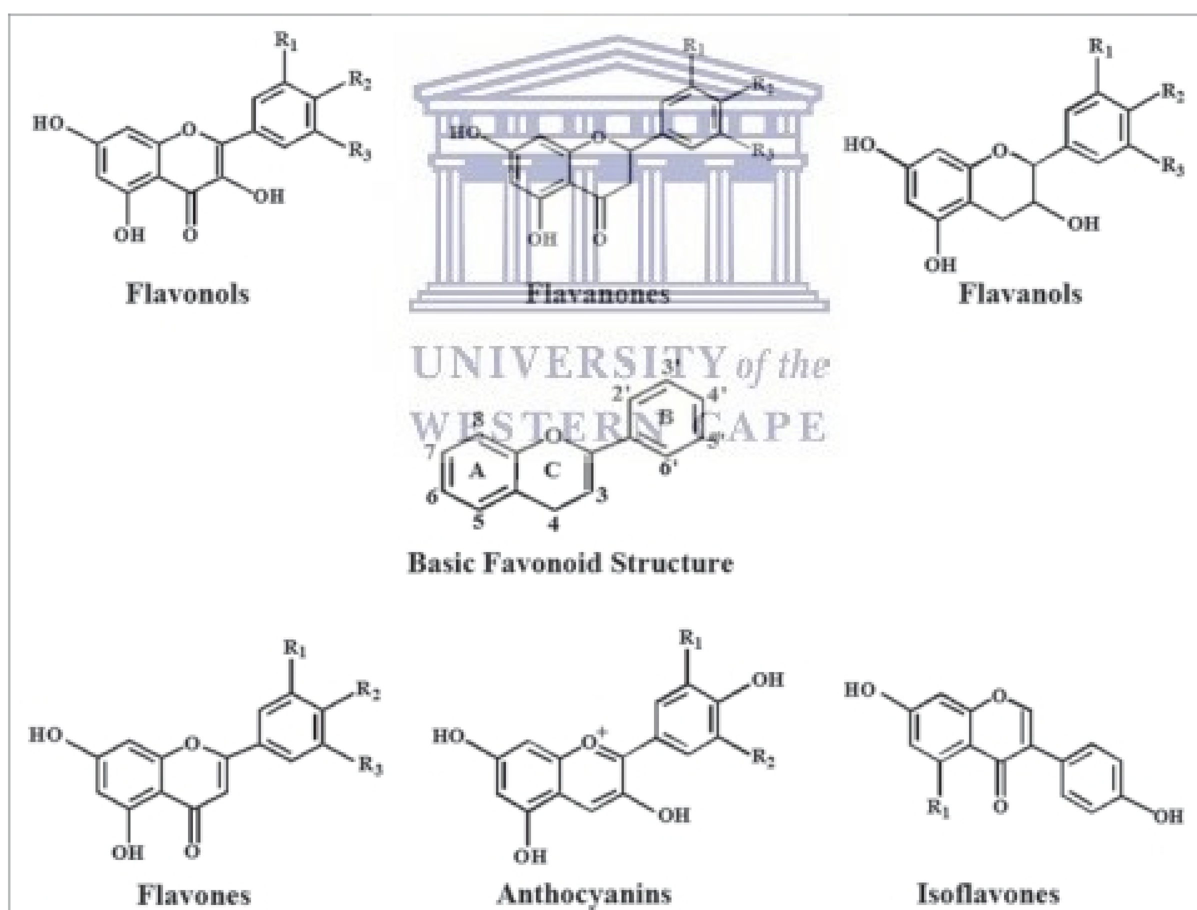

Figure 2.5: Chemical structures of sub-classes of flavonoids (Pandey and Rizvi, 2009)

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2.4.2.3 Stilbenes and Ligans

Stilbenes contain two phenyl moieties connected by a two-carbon methylene bridge. Most stilbenes in plants act as antifungal phytoalexins. A well-known stilbene is resveratrol which is found in grapes. Ligans are diphenolic compounds that contain a 2,3-dibenzylbutane structure that is formed by the dimerization of two cinnamic acid residues (Pandey and Rizvi, 2009).

2.4.3 Neuroprotective effects of polyphenols

Oxidative stress is one of the mechanisms identified in the progression of NDDs and due to the antioxidative activity of polyphenols, their consumption is said to provide protection in NDDs. A previous study revealed that people drinking three to four glasses of wine per day had 80% decreased incidence of dementia and AD compared to those who drank less or did not drink at all (Vassallo, 2008). This was attributed to the polyphenolic stilbene, resveratrol which is abundant in wine and is known to scavenge O_2^- and •OH in brain cells (Pandey and Rizvi, 2009). Over the past few years, plant extracts have become very popular candidates for studying the antioxidant and anti-inflammatory properties due to their active compounds. Plant extracts rich in polyphenols and alkaloids can function as scavengers due to their many phenolic hydroxyl and nitrogen groups. These groups act as electron donors to the aromatic ring by catching free radicals and reacting with ROS such as superoxide, peroxyl, hydroxyl radicals, NO, peroxynitrite and singlet oxygen (Pérez-Hernández *et al.*, 2016).

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2.4.4 Plants used traditionally to treat age-related/neurological disorders

Approximately 3500 species of plants are used as traditional medicine in Southern Africa (Adewusia, Foucheb and Steenkamp, 2013) and most of these plants contain chemical substances with interesting pharmacological effects used to treat age-related and NDDs (Gericke, 2002). *Zingiber officinale* (Ginger) is a well-known important plant used in Chinese, Ayurvedic and Tibia-Unani traditional medicine; its root is known for anti-inflammatory and antioxidant properties. A previous study revealed that ginger extract significantly reduced NO production, prostaglandin E2, IL-1β, IL-6 and TNFα in BV2 microglial cells (Pérez-Hernández *et al.*, 2016). A herbal mixture called Toki To prepared from 10 different types of plants (*Angelicae radix*, *Pinelliae tuber*, *Cinnamomi cortex*, *Ginseng radix*, *Zanthoxyli fructus*, *Zingiberis siccatum*, *Rhizoma* and *Glycyrrhizae radix*) is

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known for its beneficial effects against PD. Toki To administered orally was found to reduce motor symptoms such as bradykinesia and prevented dopaminergic neuron loss in the substantia nigra (Pérez-Hernández *et al.*, 2016).

2.4.5. Description of medicinal plants used in this study

2.4.5.1. *Carpobrotus edulis*

Carpobrotus edulis, more commonly known as sour fig, Cape fig, (English), ghaukum (khoi), suurvy (Afrikaans), igcukuma (Xhosa) and umgongozi (Zulu) is a water-wise succulent plant with large daisy-like flowers. The name is of Greek origin and is derived from karpos "fruit" and brotos "edible", thus referring to fruits that are edible. The genus consists of approximately 20 accepted species, of which most are endemic to the Eastern, Western and Northern Cape provinces of South Africa. *C. edulis* mainly inhabits the sandy coastal regions in mild Mediterranean climates but can also be seen inland in sandy areas. The plant is used by traditional healers to treat tuberculosis, diabetes mellitus, sores, high blood pressure, intestinal worms and constipation (Omoruyi, Bradley and Afolayan, 2012).

There is a strong likelihood that *C. edulis* may contain bioactive secondary metabolites that work against opportunistic infections such as HIV/AIDS (Smith-Palmer, Stewart and Fyfie, 1998). Limited information exists on the antioxidant activity of *C edulis* (Omoruyi, Bradley and Afolayan, 2012) and even a recent study on the phytochemical properties of the methanolic extracts of different parts of this plant but did not report on the antioxidant activity of the extracts (Hanen *et al*., 2011). *C edulis* belongs to the family Aizoaceae and consist of approximately 20 species, of which the majority are confined to South Africa. It is a group-creeping plant with succulent leaves and typical yellow flowers, fading to pink plant stems are either spreading or prostrate and can grow up to 3 meters long. The leaves grow between 4 and 14 cm in length, and 8 to 17 mm wide, and are bright green, waxy and often appear red along the edges.

The leaves of C.edulis contain flavonoids (rutin, neohesperidin and hyperoside), catechin, ferulic acid and catechol tannins (SANBI).

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Figure 2.6: A representation of succulent *C. edulis* plants (Malan and Notten, 2006)

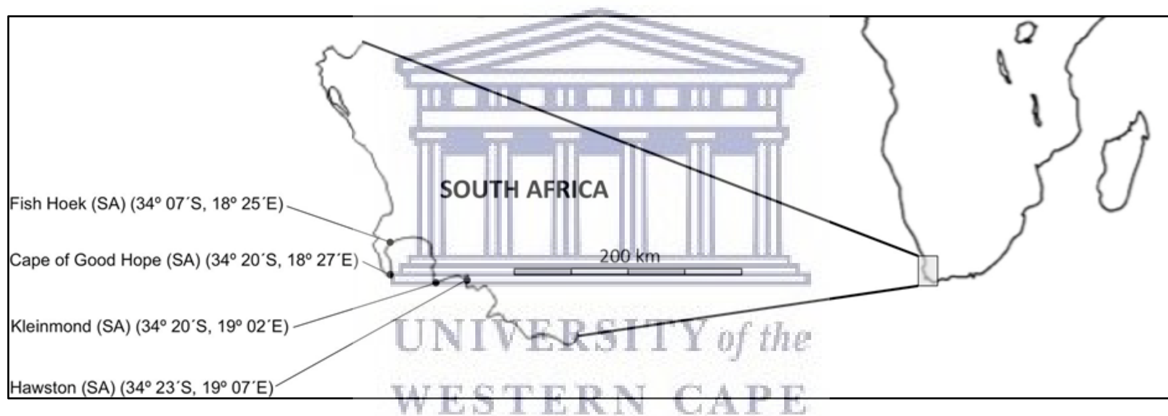

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Figure 2.7: Geographical sites in the Western Cape region of South Africa where *C. edulis* grows (www.frontiersin.org)

2.4.5.2 *Sutherlandia frutescens*

Sutherlandia frutescens is regarded as one of South Africa's most versatile medicinal plants (Fernandes *et al.*, 2004) and it belongs to the Fabaceae family which is endemic to the South Western Cape and Northern Cape provinces of South Africa (Figure 2.8). Leaves, stems, flowers and roots are used to make infusions or decoctions of this plant for washing wounds and eyes as well as to reduce fevers. The infusions made from leaves and stems are used to treat cancers, fever, diabetes, kidney and liver problems, and rheumatism and stomach illnesses (Tobwala *et al.*, 2014). Traditional healers prepare the muti according to the type of disease indicated by the patient. *S. frutescens* is also used to treat depression and stress. Plants

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are usually collected fresh, then dried and stamped or mashed into powder between two stones and the preparations then infused with cold or boiling water.

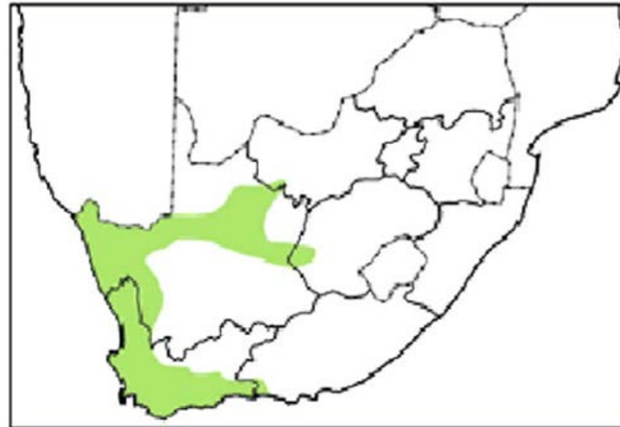

Figure 2.8: Geographical sites in the Western Cape and Northern Cape provinces of South Africa (shown in green) where *S. frutescens* grows (Rakoma, 2016).

The Facaceae family consists of more than 600 genera and approximately 1200 species. *S. frutenscens* consist of five species namely, *S. microphylla*, *S. montana*, *S. tomentosa*, *S. humillis* and *S. frutescens* (Faleschini, 2015). The term *Sutherlandia* refers to a shrubby plant and is known by many names such as balloon pea, cancer bush (English); kankerbos, blaasbossie (Afrikaans); umnwele (Xhosa), Insiswa (IsiZulu); Musa-Pelo, Motlepelo and Phethola (Sotho). It has a bitter taste and its leaves have an aromatic scent. The height of the plant varies between 0.2-2.5 m (Rakoma, 2016).

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Figure 2.9: representation of a *S. frutescens* plant, (Rakoma, 2016)

S. frutescens has become increasingly popular over the years, resulting in its commercialization with products ranging from anti-viral and adaptogenic tonics, capsules, tablets, powders, sprays, gels and teas currently being sold on the South African and international markets (Figure 2.10).

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Figure 2.10: Various products manufactured from Sutherlandia and sold commercially. A: anti-viral tonic, B: immune booster tablets for energy and vitality, C: Sutherlandia capsules, D: nasal spray, E: Sutherlandia tea, F: Sutherlandia gel used as an application for wounds

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Phytochemical studies on *S. frutescens* showed that it contains a significant amount of gamma amino butyric acid (GABA) and L-canavanine, pinitol, flavonol glycosides and asparagine (Phulukdaree *et al*., 2010). L-canavanine is a very potent non-protein amino acid and and is an L-arginine antagonist with antiviral, anti-bacterial and anti-fungal and anti-cancer properties (Rakoma, 2016). It is also a selective inhibitor of inducible nitric oxide synthase, making it a possible form of treatment for septic shock and chronic inflammation (Anfossi *et al*., 1999). This complex compound can be toxic in excessive amounts but in lesser amounts it is considered powerful against many illnesses.

Pinitol is an anti-diabetic agent and has been isolated from the leaves of *S. frutescens*. Pinitol may hold strong clinical applications for the treatment of muscle wasting in cancer and HIV/AIDS patients. GABA, isolated from dry *S. frutescens* leaves is an inhibitory neurotransmitter that could be applied in the treatment of anxiety and stress as well as the improvement of mood disorders (Phulukdaree *et al*., 2010).

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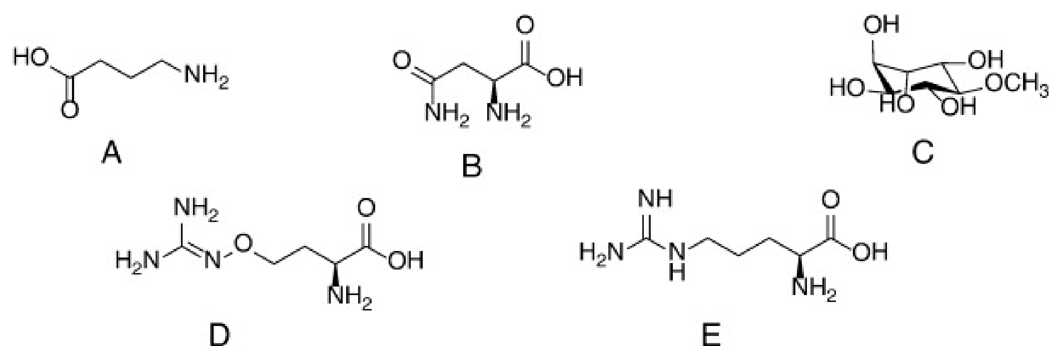

Figure 2.11: Chemical structures of active compounds founds in *S. frutescens.* A: γ-aminobutyric acid; B: L-asparagine; C: d-pinitol; D: L-canavanine; E: L-arginine (Mncwangi and Viljoen, 2012).

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CHAPTER 3

MATERIALS AND METHODOLOGY

3.1. Introduction

In this study, we used an in vitro technique to explore the potential neuroprotective effects of S.frutescens and C. edulis water extracts on SH-SH5Y cells. The materials used and methodologies performed are outlined below.

3.2. Materials

Chemicals: H_2O_2, Dimethyl sulfoxide (DMSO) and Rhodamine 123 mitochondrial specific fluorescent dye were purchased from Sigma-Aldrich (St. Louis, USA). Caspase 9 protease activity kit, Catalase colorimetric activity kit, Griess reagent, Dulbecco's Modified Eagles Medium (DMEM), fetal bovine serum (FBS), fluo-4 direct Calcium activity kit, Penicillin-streptomycin, Superoxide dismutase (SOD) colorimetric activity kit were purchased from Thermo Fisher, (Johannesburg, ZA). Phosphate buffered solution (PBS) and Trypsin (1X) EDTA were purchased from WhiteSci (Cape Town, ZA).

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Consumables: 96-well plates, 10 ml serelogical pipettes, 23 cm cell scrapers, 2ml microcentrifuge tubes, 15 ml centrifuge tubes and 50 ml conical tubes were purchased from BioSmart (Cape Town, ZA). Reagent resevoirs (50 ml) and nitrile powder-free examination gloves were purchased from Lasec (Cape Town, ZA). NEST tissue culture dishes (60 mm and 100 mm, respectively) were purchased from WhiteSci (Cape Town, ZAR).

Plants: Dry *S. frutescens* leave milled powder and dry *C. edulis* leave milled powder was generously gifted from Mr. Sylvester Omoruyi and Mr. Dipo David at the department of Medical Biosciences at the University of the Western Cape (UWC) (Bellville, ZA). S. frutescens dry milled powder was purchased from Big Treee Health Products (Fish Hoek, ZA).

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3.3. Methods

3.3.1 Preparation of extracts

Stock solution was prepared by weighing out 2 mg of each extract and mixing it with 1 ml ddH_2O, respectively. Stock solution was aliquoted into sterile 2 ml microcentrifuge tubes at a volume of 100 µl and stored at – 20°C.

3.3.2 Cell culture

SH-SY5Y cells are a human neuroblastoma derived cell line with neuron-like characteristics. These cells were used for this study because they share numerous functional and biochemical characteristics with innate neurons (Seoposengwe, van Tonder and Steenkamp, 2013). SH-SY5Y cells were obtained from the Department of Medical Imaging and Clinical Oncology, Stellenbosch University as a kind donation to the Experimental Neuroscience Laboratory at the UWC. Cells were cultured with DMEM, supplemented with 10% FBS and 1% penicillin-streptomycin in a humidified incubator with 5% CO_2 at 37°C. Cells were left to attach and acclimatize for 24 hours prior to each experiment.

3.3.3 Screening for maximum non-toxic doses (MNTD) of plant extracts

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SH-SY5Y cells ($6x10^3$ cells/well) were seeded into flat bottom 96-well plates. Cells were incubated for 24 hours to adhere and acclimatize. Once 60-70% confluence was attained, the cells were treated with various concentrations ranging from 5, 10, 15, 20, 25, 50, 100, 200 and 400 µg/ml of SF and CE, respectively for 24 hours. Control cells were grown in supplemented DMEM, without any treatment. Subsequently, 10 µl of 3-(4, 5-dimethlthiazol-2-yl)-2,5-diphenyltretrazolium bromide (MTT) solution was added to each well and cells were then incubated at 37°C for 4 hours. Media was aspirated carefully and 100 µL DMSO added to each well. Absorbance was read at 570 nm using a Polarstar Omega BMG Labtech. To determine the MNTD, graphs of the percentage cytotoxicity against the concentrations of the extracts were plotted.

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3.3.4 Determination of optimal concentration of hydrogen peroxide (H_2O_2)

SH-SY5Y cells were plated into 96-well plates and treated with various concentrations (50, 250, 500, 750 and 1000 µM) of H_2O_2 for 1 hour and 3 hours, respectively to induce oxidative stress. The working H_2O_2 solution was prepared from 30% stock solution prior to each experiment. Control cells were grown in supplemented DMEM, without H_2O_2 (Zhong *et al.*, 2016). MTT solution (10 µl) was added to each well and cells were incubated at 37°C for 4 hours. Media was aspirated carefully and 100 µL DMSO was added to each well. Absorbance was read at 570 nm using a (Polarstar Omega BMG Labtech) microplate reader.

3.3.5 Treatments

In order to investigate the neuroprotective effects of SF and CE on H_2O_2-induced apoptosis, the SH-SY5Y cells were exposed to six different treatment combinations as shown in Table 3.1. Dry extracts of SF and CE were prepared by dissolving them into 1ml ddH_2O, respectively.Cells were pre-treated with 5, 10, 15 and 20 µg/ml SF and CE for 24 hours before exposure to the optimal H_2O_2 concentration of 250 µM for 1 hour and 3 hours, respectively, as predetermined in section 3.2.4 above.

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Table 3.1: Treatment groups used in the investigation of neuroprotective effects of two South African plant extracts on H_2O_2 - induced apoptosis in SH-SY5Y cells.

Group	*Sutherlandia frutescens (SF)*	*Carpobrotus edulis (CE)*
1	Control (untreated cells)	Control (untreated cells)
2	H_2O_2 only (250 µM)	H_2O_2 only (250 µM)
3	SF 5 µg/ml + 250 µM H_2O_2	CE 5µg/ml + 250 µM H_2O_2
4	SF 10 µg/ml + 250 µM H_2O_2	CE 10 µg/ml + 250 µM H_2O_2
5	SF 15 µg/ml + 250 µM H_2O_2	CE 15 µg/ml + 250 µM H_2O_2
6	SF 20 µg/ml + 250 µM H_2O_2	CE 20 µg/ml + 250 µM H_2O_2

H_2O_2- Hydrogen peroxide; SF- *Sutherlandia frutescens*; CE- *Carpobrotus edulis*

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3.3.6 Measurement of cell viability

MTT solution (20 µl) was added to all treatment groups (Table 3.1) and incubated for 4 hours. MTT solution was removed and 100 µl DMSO was added and absorbance read at 570 nm using a (Polarstar Omega BMG Labtech) microplate reader. Based on cell viability studies, the extract dosage showing the best level of protection against H_2O_2-induced toxicity was selected for subsequent experiments (in sections 3.2.7- 3.2.13 below).

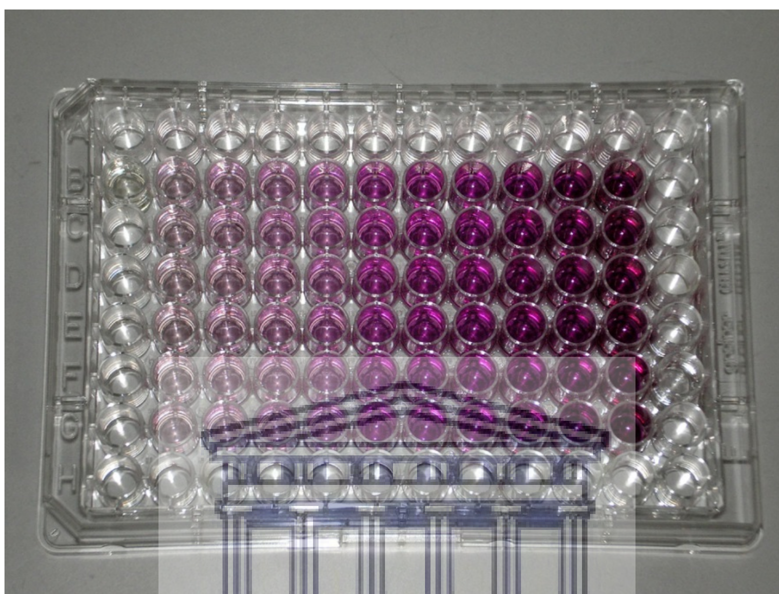

Figure 3.1: Example of MTT assay. The image shows a 96-well plate in which the MTT solution was removed and the formazan solvent DMSO was added, producing a purple colour.

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3.3.7 Measurement of Caspase 9 Protease activity

The induction of apoptosis via exposure of cells to H_2O_2 has been shown to increase the activity of the caspase class of enzymes. Caspase 9 is involved in the intrinsic pathway of apoptosis (González-Sarrías *et al.*, 2017). Cells were seeded into 4 tissue culture dishes, plated 1.2 x10^6 cells/dish and incubated for 24 hours at 37°C. One dish labelled SF and another labelled CE were pre-treated with 10 µg/ml extract for 24 hours prior to treating with 250 µM H_2O_2. Cells were then suspended in 30 µl of chilled lysis buffer and incubated for 10 minutes followed by centrifugation at 10 000 x g for 1 minute in a (Dlab) centrifuge machine DM0412. Supernatant was transferred into microcentrifuge tubes and kept on ice. Supernatant was then diluted to a concentration of 50-200 µg protein per 50 µl cell lysis buffer and the Reaction buffer (2X) was aliquoted into a tube. Dithiothreitol (DTT) was added to the 2X reaction buffer before use and 50 µl of the 2X reaction buffer was then added

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to each sample. Afterwards, 5 µl 4mM LEHD- pNA substrate was added to the samples and incubated at 37°C for 2 hours before reading at 400nm or 405nm in a (Polarstar Omega BMG Labtech) microplate reader.

3.3.8 Assessment of Intracellular ROS

Levels of intracellular ROS were determined using the fluorescent probe DCFH-DA with some modifications (Wang and Xu, 2005). Briefly, SH-SY5Y cells ($6x10^3$ cells/well) were seeded in 96-well plates and pre-treated with 10µg/ml SF and CE, respectively for 24 hours. Cells were then incubated with 250µM H_2O_2 for 3 hours. DCFH-DA (10µM) was prepared with 20ml unsupplemented DMEM and 100µl added to each well. Cells were incubated for 60 minutes then washed once with sterile PBS and the fluorescence intensity of DCF was measured using a (Polarstar Omega BMG Labtech) microplate-reader at excitation wavelength 485 nm and emission wavelength 538 nm.

3.3.9 Measurement of mitochondrial membrane potential (MMP)

Mitochondrial membrane potential (MMP) was determined by flow cytometry using the fluorescent dye rhodamine 123. Cells were seeded at 1.2×10^6 cells/dish into cell culture dishes, and incubated overnight and later pre-treated with extracts for 24 hours. The optimal H_2O_2 concentration of 250 µM was then added and incubated at 37°C for 3 hours. Cells were thereafter centrifuged at 3000 revolutions per minute (rpm) for 3 minutes, the supernatant removed and 2 ml media added to the pellets. Cell pellets were then re-suspended, after which 2 µl Rhodamine 123 dye was added and incubated for 30 minutes. The cells were centrifuged at 3000 rpm for 3 minutes and supernatant decanted afterward. Cell pellets were resuspended in 500 µl PBS and transferred into 1 ml Eppendorf tubes. Fluorescent intensity was measured using a (BD Accuri CSampler) Flow Cytometer.

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3.3.10 Nitric oxide activity

Nitric oxide activity was measured using Griess reagent as it measures the levels of nitrite (NO_2^-) in solutions. Equal volumes of N-1-napthylethylenediamine dihydrochloride (NED) and sulfanilic acid were mixed together to form Griess reagent. 50 µL of Griess reagent was added to each well of a 96-well plate and 100 µL of each sample added in afterward and incubated for 30- 45 minutes in the dark at room temperature. Absorbance was read at 458 nm using a Polarstar Omega BMG Labtech microplate reader.

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3.3.11 Fluo-4 Direct Intracellular Calcium detection assay

A total of 6 x 10^5 cells were seeded in 96-well plates overnight and then pre-treated with 10 µg/ml of SF and CE respectively for 24 hours. The H_2O_2 only group and respective extract groups were then treated with 250 µM H_2O_2 for 3 hours. Thereafter, 100 µl of the 2X Fluo-4 Direct calcium reagent loading solution was added to each well of the 96-well plates and incubated at 37°C for 1 hour. Fluorescence was read at 494 nm for excitation and 516 nm for emission in a microplate reader.

3.3.12 Superoxide dismutase (SOD) colorimetric assay

Cells were seeded into NEST 100 mm tissue culture dishes (1.2x10^6 cells) and incubated for 24 hours and after acclimatization, the cells were pre-treated with 10 µg /ml of SF and CE for 24 hours. The H_2O_2 - only group and the respective extract groups were then treated with 250 µM H_2O_2 for 3 hours. Cells were centrifuged at 250 x g for 10 minutes at 4°C and supernatants were discarded. Adherent cells were rinsed in the culture dish with cold PBS, then scrapped and collected using a cell scrapper. Cells were transferred into 15 mL tubes and centrifuged using a (DLab centrifuge machine DM0412) at 300 x g for 5 minutes and the supernatants discarded. Cells were resuspended in 20 µL ice-cold PBS and transferred to microcentrifuge tubes on ice and then centrifuged using an Eppendorf centrifuge machine 5417R at 3000 rpm for 20 minutes at 4°C. Supernatants were collected and assayed immediately. Standards were prepared by adding 250 µL Assay buffer while 75 µL Assay Buffer was added to each of 7 tubes labelled 2, 1, 0.5, 0.25, 0.125, 0.0625 and 0 mU/ mL SOD respectively. Serial dilutions of the standards were made and used immediately. The assay was performed by adding 10 µL of standards or diluted samples and 50 µL 1X Substrate into the appropriate wells. Subsequently, 25 µL 1X Xanthine Oxidase was added to each well and cells were incubated for 20 minutes at room temperature. Absorbance was read using a (Polarstar Omega BMG Labtech) at 450 nm.

3.3.13 Catalase (CAT) Assay

Cells (1.2x10^6) were seeded into NEST 100 mm tissue culture dishes and incubated for 24 hours and after acclimatization, cells were pre-treated with 10 µg /ml of SF and CE for 24 hours. The H_2O_2 - only group and extract groups were then treated with 250 µM H_2O_2 for 3 hours followed by centrifugation at 250 x g for 10 minutes at 4°C. Supernatant was discarded and 1 ml cold 1X Assay buffer was added to cells followed by immediate sonication and

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centrifugation at 3000 rpm for 20 minutes. Standards were prepared by adding 10 µL Catalase standard to one tube containing 190 µL 1X Assay buffer while 100µL 1X Assay buffer was added to 6 tubes labelled: 2.5, 1.25, 0.625, 0.313, 0.156, and 0 U/ml catalase respectively and serial dilutions of the standard were made. Thereafter, 25 µL of standards or diluted samples were added to the appropriate wells and 25 µL of Hydrogen Peroxide reagent was added into each well and incubated for 30 minutes at room temperature. Then, 25 µL of substrate was added into each well, followed by 25 µL of 1X Horserasdish Peroxidase Concentrate and incubation for 15 minutes at room temperature. Absorbance was read at 560 nm using a (Polarstar Omega BMG Labtech) microplate reader.

3.3.14 Hoechst Staining

Hoechst is a fluorescent dye that penetrates into the nucleus of cells and binds to DNA. This was done to observe nuclear and apoptotic changes in the cells as previously described by (Wang *et al.*, 2015), with some modifications made. Cells, $3x10^5$ cells /dish were seeded into 60 mm NEST tissue culture dishes and incubated for 24 hours at 37°C. Cells were then pre-treated with extracts for 24 hours and 250 µM H_2O_2 was added and incubated at 37°C for 3 hours. In order to determine fluorescence, cells were recovered from a culture flask and centrifuged at 3000 rpm using a DLab centrifuge machine DM0412. Supernatant was removed and 400 µl PBS added. Eppendorf tubes were used to collect 200 µl of cell suspension. In order to stain, 100 µl Hoechst 33258 staining solution was added to the same tube and incubated at 37°C for 30 minutes with protection from light after which10 µl of cell and staining solution was placed on a glass slide and covered with a cover slip and viewed for fluorescent imaging using a fluorescence microscope.

3.3.15 Statistical analysis

All experiments in this study were performed in triplicates and repeated accordingly. The statistical analysis of the differences between the control, H_2O_2 and SF and CE- treated groups was determined using one-way analysis of variance (ANOVA) with Tukey's multiple comparison tests. The analyses were done with GraphPad PRISM software version 5.02, CA, USA. The data were presented at means ± standard error of mean (SEM) and $p < 0.05$ were considered statistically significant.

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CHAPTER FOUR

RESULTS

4.1. Introduction

In this study, OS was induced in SH-SY5Y cells with 250 µM H_2O_2 after being pre-treated with SF and CE extracts at various concentrations. Outcomes from the H_2O_2 only group was compared with the control and SF and CE treated groups. The study was done to evaluate the potential of the two South African plants in preventing/reducing the severity of H_2O_2-induced neurotoxicity. Findings from this study are presented below:

4.2. Cytotoxicity screening of Sutherlandia Frutescens (SF) plant extract

To determine the effects of SF treatment on cell viability, SH-SY5Y cells were seeded into 96-well plates and cultured for 24 hours. Cells were thereafter treated with SF at a concentration range of 5-400 µg/ml for 24 hours. SF treatment was found to have no significant effects at lower concentrations up to 200 µg/ml but the 400 µg/ml concentration significantly decreased cell viability (see Figure 4.1). Therefore, the SF concentration range of 5-20 µg/ml that did not induce cytotoxicity in SH-SY5Y cells was chosen for subsequent studies that examine the neuroprotective effects of SF against H_2O_2-induced cytotoxicity.

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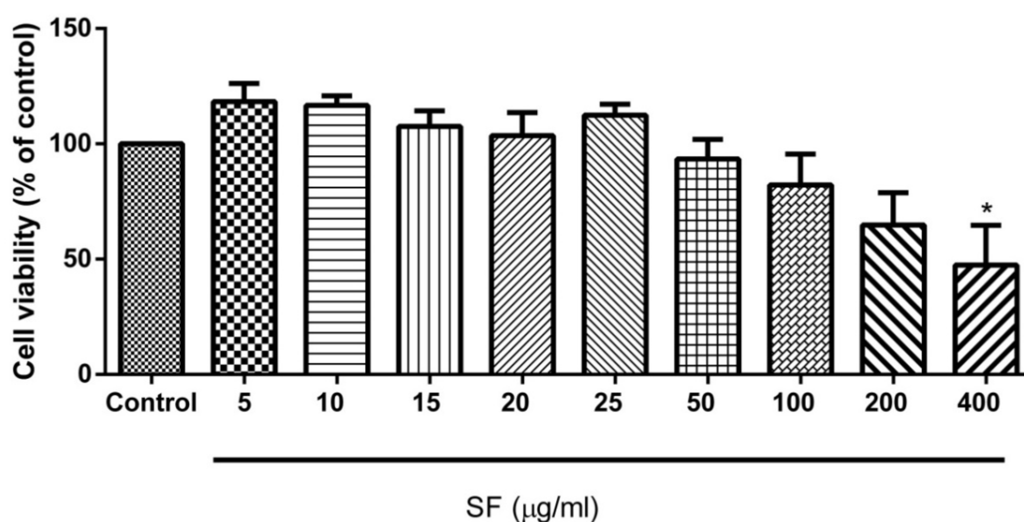

Figure 4.1: Effects of *S. frutescens* (SF) on viability of SH-SY5Y neuroblastoma cells using the MTT assay.Values are shown as means ± SE; *p<0.05.

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4.3. Cytotoxicity screening of CE plant extract

To investigate the effects of *C. edulis* (CE) extract on cell viability, SH-SY5Y cells were seeded into 96-well plates and cultured for 24 hours, before exposure to CE at a concentration range of 5-400 µg/ml for another 24 hours. As shown in Figure 4.2, CE treatment had no significant effects on viability of SH-SY5Y cells hence the non-toxic concentration range of 5-20 µg/ml was chosen to evaluate the neuroprotective effects of CE against H_2O_2-induced cytotoxicity.

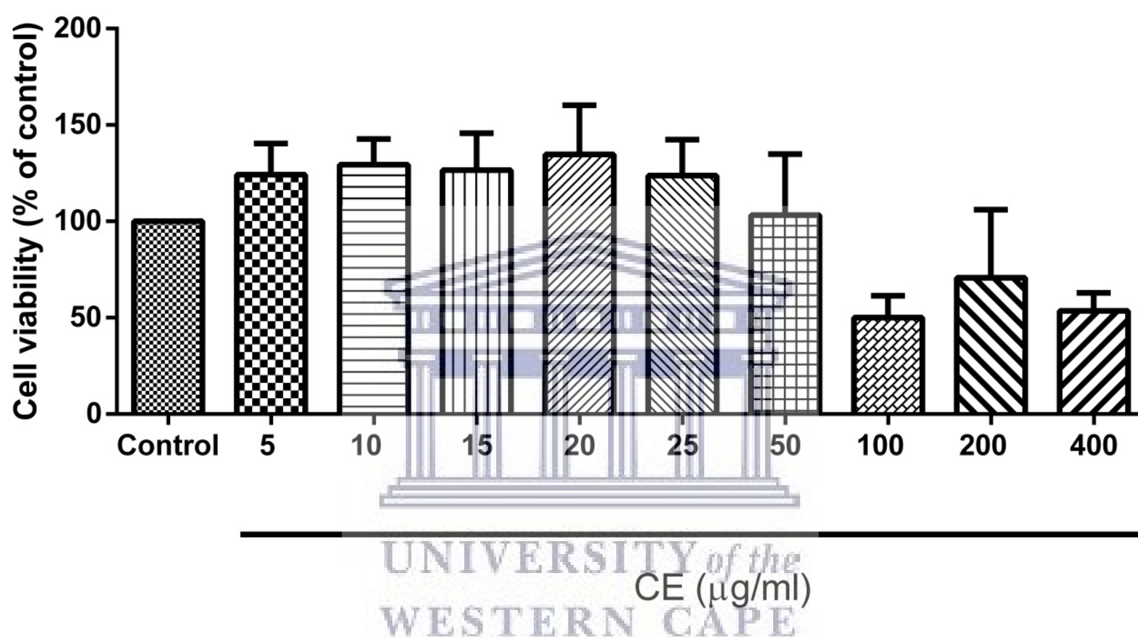

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Figure 4.2: Effects of *C. edulis* on viability of SH-SY5Y neuroblastoma cells using the MTT assay values are shown as means ± SE; $^*p<0.05$.

4.4. Determination of optimal concentration of H_2O_2

In order to determine the optimal concentration of H_2O_2 that could induce 50% cell death, SH-SY5Y cells were exposed to 50, 250, 500, 750 and 1000 µM H_2O_2 for 1 and 3 hours respectively. Results obtained show that at lower concentrations (50-250 µM), H_2O_2 had no significant effect on SH-SY5Y cell viability for the 1-hour exposure but higher concentrations (500-1000 µM) significantly ($^{****}p<0.0001$) decreased cell viability (Figure 4.3). For the 3-hour H_2O_2 exposure, there was a significant decrease in cell viability across all concentrations (50-1000 µM), however, an optimal concentration of 250 µM H_2O_2 was selected was considered for the study.

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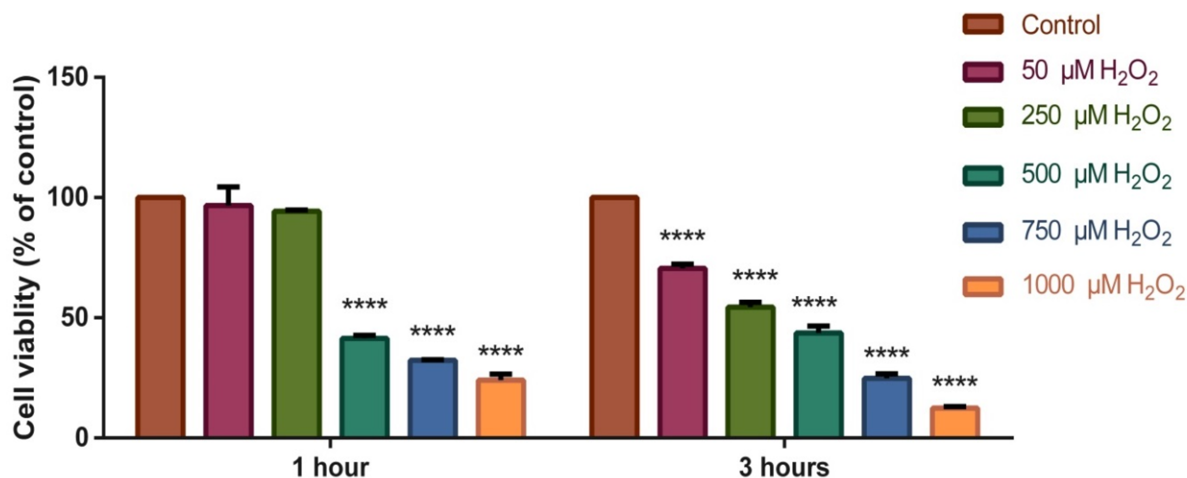

Figure 4.3: Effects of H_2O_2 on the viability of SH-SY5Y cells after 1 hour and 3 hours incubation respectively. Values are shown as means ± SE; ****p<0.0001.

4.5. Protective effects of SF against H_2O_2-induced cell death

SH-SY5Y cells were pre-treated with 5-20 µM of SF for 24 hours followed by exposure to 250 µM H_2O_2 for 1 hour and 3 hours respectively. Figure 4.4A shows that cells were still very viable following pre-treatment with all the concentrations of SF and subsequent exposure to H_2O_2. Even cells treated with only H_2O_2 showed no significant death when compared to the control group. Figure 4.4B shows that cells treated with only H_2O_2 had significantly decreased cell viability compared to the control while only the 10 µg/ml SF concentration significantly attenuated H_2O_2-induced cytotoxicity. Thus, the maximum non-toxic SF treatment concentration of 10 µg/ml was used for all subsequent experiments.

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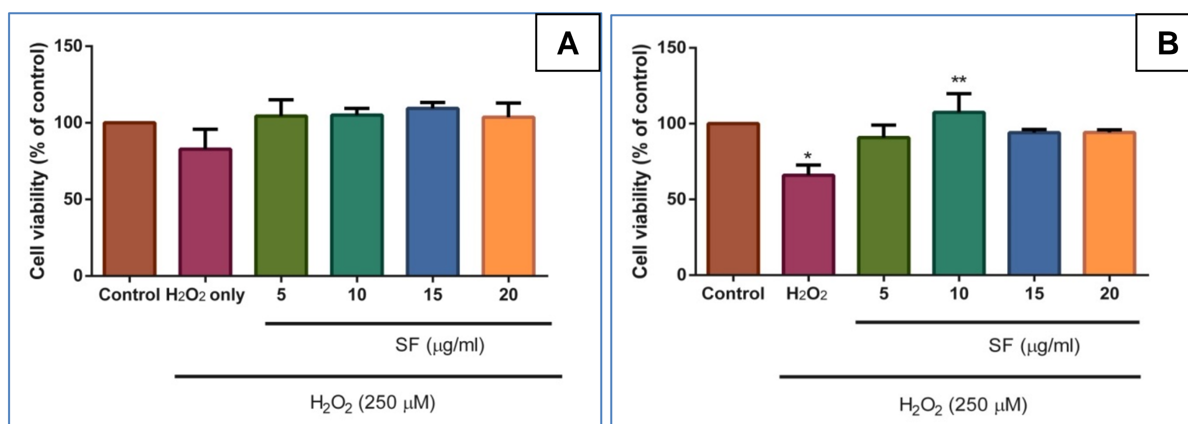

Figure 4.4: Effects of 24-hour pre-treatment with *Sutherlandia frutescens* on induced death of SH-SY5Y neuroblastoma cells after a 1-hour (Figure A) and a 3-hour (Figure B) incubation in H_2O_2. Values are shown as means ± SE, *p<0.05 and **p<0.01.

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4.6. Protective effects of CE against H_2O_2-induced cell death

The protective effects of *C. edulis* against H_2O_2-induced SH-SY5Y death was also examined. The cells were pre-treated with various concentrations of CE for 24 hours, followed by incubation with 250 µM H_2O_2 for 1 hour and 3 hours respectively. Figure 4.5A shows that no significant changes were observed in all treatments including H_2O_2 treatment. In Figure 4.5B, after 3 hours of exposure to H_2O_2, cell viability decreased significantly in cells treated with H_2O_2 only while the 10, 15 and 20 µg/ml concentrations of CE significantly attenuated the H_2O_2-induced cytotoxicity when compared to the control. Thus, the maximum non-toxic CE treatment concentration of 10 µg/ml was used for all subsequent experiments.

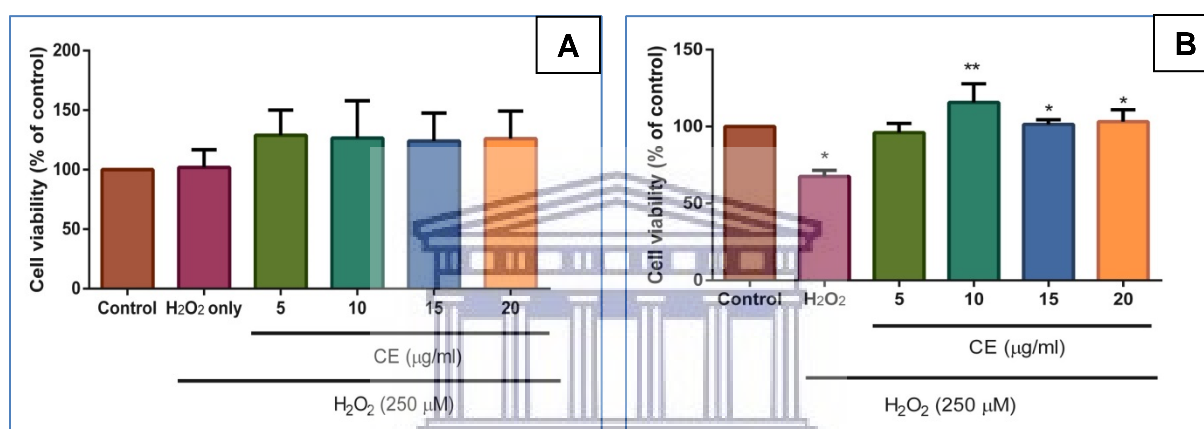

Figure 4.5: Effects of 24-hour pre-treatment with *Carpobrotus edulis* on induced death of SH-SY5Y neuroblastoma cells after a 1-hour (Figure A) and 3-hour (Figure B) incubation in H_2O_2. Values are shown as means ± SE, $*p<0.05$ and $**p<0.01$

4.7. Effects of pre-treatment of extracts SF and CE on intracellular ROS levels in SH-SY5Y cells after H_2O_2 incubation

H_2O_2 is known to interact with various cell complexes causing a blockage of ATP production and the promotion of oxygen free radicals (Wang *et al.*, 2015). In this study, the degree of ROS accumulation was evaluated in SH-SY5Y cells pre-treated with 10 µg/ml of SF and CE for 24 hours followed by 3 hours incubation in 250 µM H_2O_2 . As shown in Figure 4.6, H_2O_2–only treatment resulted in anincrease in DCF intensity by 3.848-fold relative to the control. Treatment with the SF extract was found to reduce ROS generation relative to the H_2O_2-only treatment, but the observed reduction was not statistically significant. On the other

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hand, treatment with the CE extract significantly reduced ROS generation by 1.761-fold relative to the H_2O_2-only treatment.

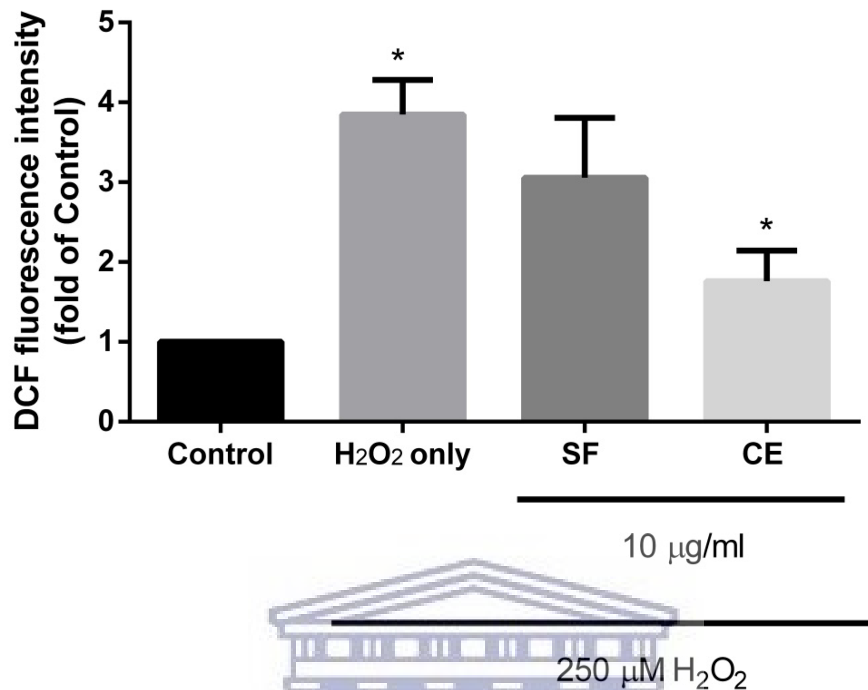

Figure 4.6: Effects of *S. frutescens* and *C. edulis* plant extracts at the maximum non-toxic dose on intracellular reactive oxygen species (ROS) levels in SH-SY5Y neuroblastoma cells after 3 hours incubation in H_2O_2. Values are shown as means ± SE, *$p<0.05$.

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4.8. Effects of SF and CE extracts pre-treatment on Nitric Oxide (NO) production by SH-SY5Y cells exposed to H_2O_2 for 3 hours

The production of NO is an important inflammatory response in neurons (Sylvie et al., 2014). in the present study, the protective effects of SF and CE plant extracts on NO production evaluated in SH-SY5Y cells pre-treated with 10 µg/ml of SF and 10 µg/ml CE respectively for 24 hours followed by 3 hours incubation in 250 µM H_2O_2. In the H_2O_2-only treated cells, NO production significantly increased by 1.062-fold relative to the control while only pre-treatment with the 10 µg/ml CE extract significantly attenuated the H_2O_2-induced increase in NO production by 1.011-fold relative to the control.

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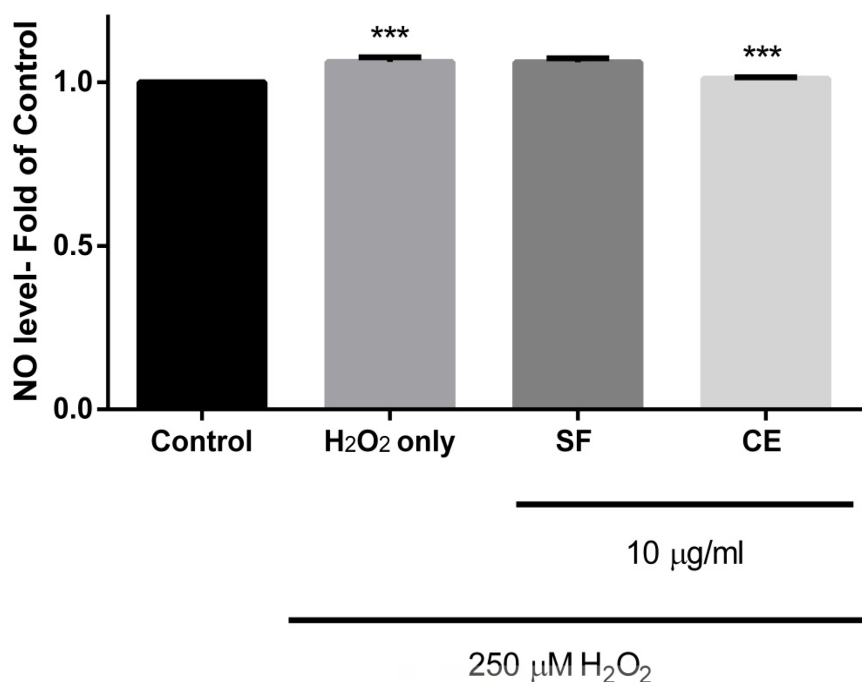

Figure 4.7: Effects of *S. frutescens* and *C. edulis* plant extracts at the maximum non-toxic dose on Nitric Oxide production by SH-SY5Y cells after 3 hours incubation in H_2O_2. Values are shown as means ± SE, ***$p<0.001$.

4.9. Effects of SF and CE extracts pre-treatment on intracellular Calcium (Ca^{2+}) handling by SH-SY5Y cells exposed to H_2O_2 for 3 hours

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Mitochondrial dysfunction and increased levels of ROS could disrupt calcium homeostasis in the brain and changes in these pathways are often observed in patients with AD or cerebral ischemia (Gray *et al.*, 2015). To study intracellular calcium levels, cells were pre-treated with 10 µg/ml concentration of SF and CE plant extract respectively for 24 hours followed by 250 µM H_2O_2 for 3 hours.

Results obtained show that the H_2O_2-only treated cells had significantly higher intracellular calcium than the control group while treatment with both plant extracts significantly lowered intracellular calcium relative to the control, better with CE than SF (Figure 4.8).

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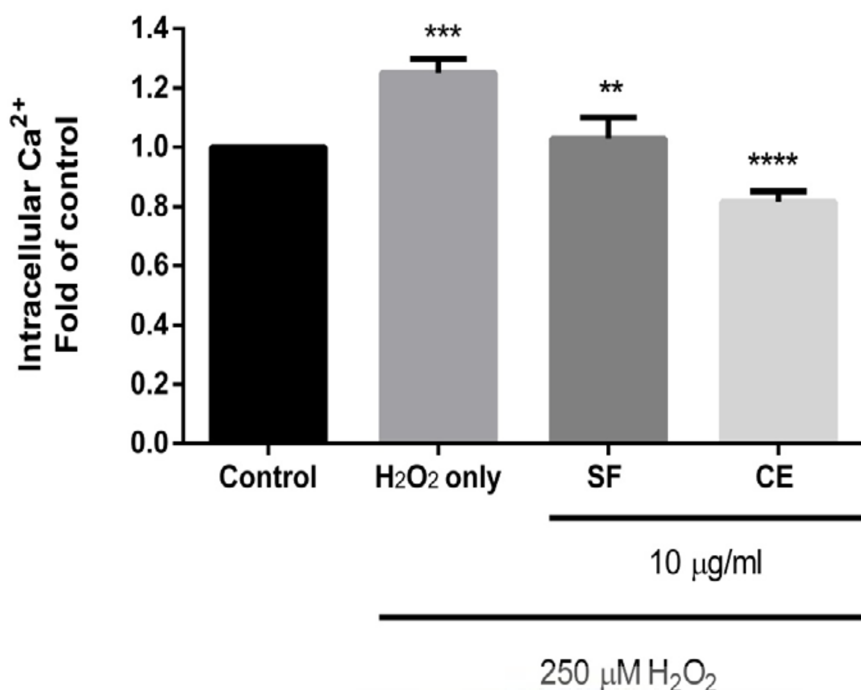

Figure 4.8: Effects of *Sutherlandia frutescens* and *Carpobrotus edulis* plant extracts at the maximum non-toxic dose on intracellular calcium levels in SH-SY5Y cells after 3 hours incubation in H_2O_2. Values are shown as means ± SE, **$p<0.01$, ***$p<0.001$ and **** $p<0.0001$.

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4.10. Effects of SF and CE extracts pre-treatment on H_2O_2-induced caspase-9 activation in SH-SY5Y cells exposed to H_2O_2 for 3 hours

Caspases are known as the molecular machinery that drives apoptosis (Wang and Xu, 2005). As caspase-9 is an important biomarker of the apoptosis process, its activity was examined in this study. an increase in caspase-9 activity was observed to 308.3% of the control. The addition of SF and CE attenuated H_2O_2-induced caspase-9 activation, respectively (132.5% and 95.98%).

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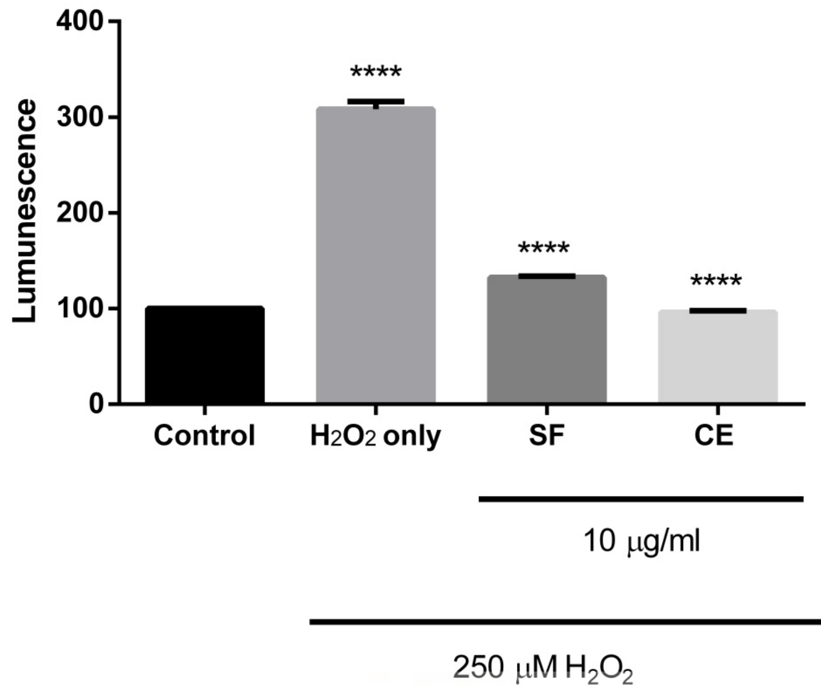

Figure 4.9: Effects of *S. frutescens* and *C. edulis* plant extracts at the maximum non-toxic dose on caspase 9 activity in SH-SY5Y cells after 3 hours incubation in H_2O_2. Values are shown as means ± SE, ****$p<0.0001$.

4.11. Effects of SF and CE extracts pre-treatment on H_2O_2-induced changes in nuclear morphology of SH-SY5Y cells exposed to H_2O_2 for 3 hours

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Changes in nuclear morphologywere assessed using the Hoechst 33324 stain. As shown in Figure 4.10, the normal SH-SY5Y cells nuclei had a regular oval shape. Apoptotic nuclei appeared condensed and fragmented after exposure to 250 µM H_2O_2 for 3 hours. Pre-treating cells with SF and CE for 24 hours, respectively, decreased the H_2O_2 –induced nuclear damage.

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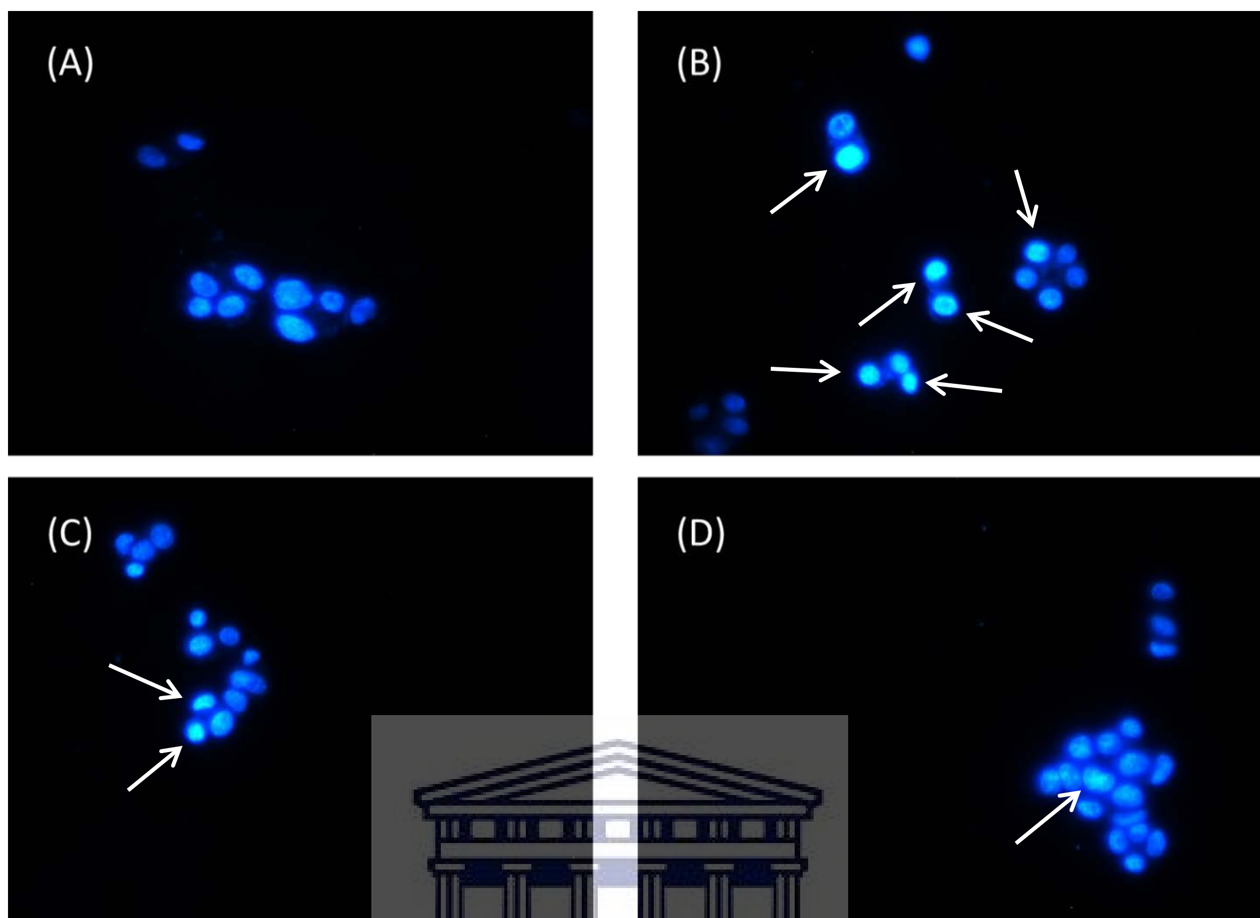

Figure 4.10: Nuclear morphological analyses of SH-SY5Y cells by fluorescence microscopy. The figure shows the fluorescence micrographs of Hoechst 3324 stained nuclear morphology. SH-SY5Y cells were untreated (A), treated with 250 µM H_2O_2 for 3 hours (B), treated with 10 µg/ml SF for 24 hours + H_2O_2 3hours (C), or treated with 10 µg/ml CE for 24 hours + H_2O_2 for 3 hours (D). Apoptotic cells exhibited morphological changes in the nuclei typical of apoptosis. Arrows represent apoptotic cells.

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4.12. Effects of SF and CE extracts pre-treatment on the Mitochondrial Membrane Potential (MMP) of SH-SY5Y cells exposed to H_2O_2 for 3 hours

Rhodamine 123 was used as a molecular probe to determine the MMP of SH-SY5Y. A decrease in the intensity of rhodamine 123 fluorescence indicated loss of MMP. Figure 4.11A shows that H_2O_2 caused breakdown of MMP due to a shift to the left in fluorescence intensity while Figure 4.11B shows that H_2O_2 significantly caused a collapse of mitochondrial membrane potential as seen by a shift to the left in fluorescence intensity. In Figure 4.11B, the histogram shows that SF attenuated the H_2O_2-induced increase as there was no shift to the left in fluorescence intensity. Figure 4.11C, revealed that CE prevented the H_2O_2-induced increase of MMP by decreasing the percentage of cells.

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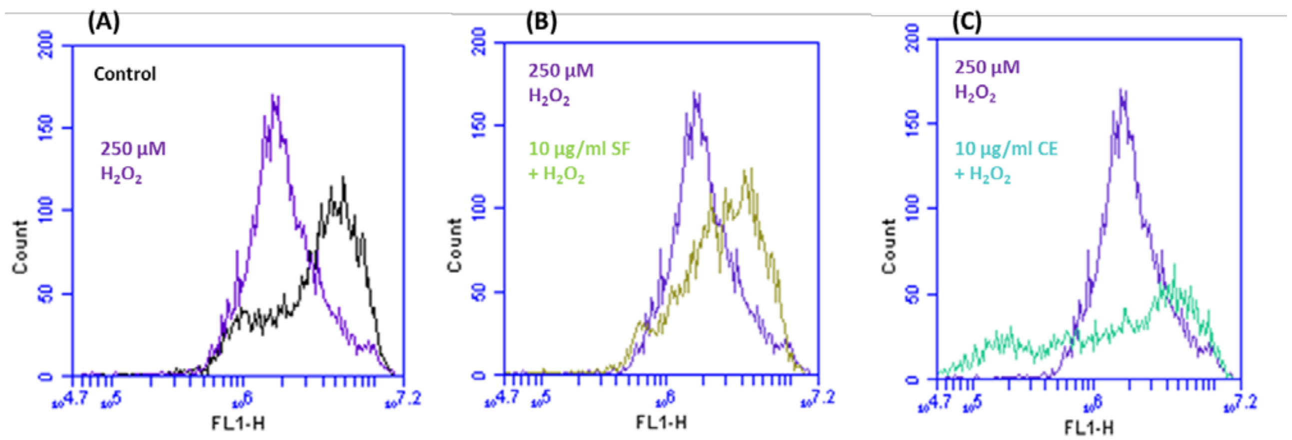

Figure 4.11A: Effects of *Sutherlandia frutescens* and *Carpobrotus edulis* plant extracts at the maximum non-toxic dose on MMP of SH-SY5Y cells after 3 hours incubation in H_2O_2. MMP was measured by flow cytometry using rhodamine 123 staining (A-C). Values are shown as means ± SE; *p<0.05.

Figure 4.11B shows that pre-treating cells for 24 hours with 10 µg/ml SF and CE plant extracts respectively significantly prevented the H_2O_2-induced increase of MMP from 56.70% to 75.87% (SF) and 64.89% (CE) (relative to control %).

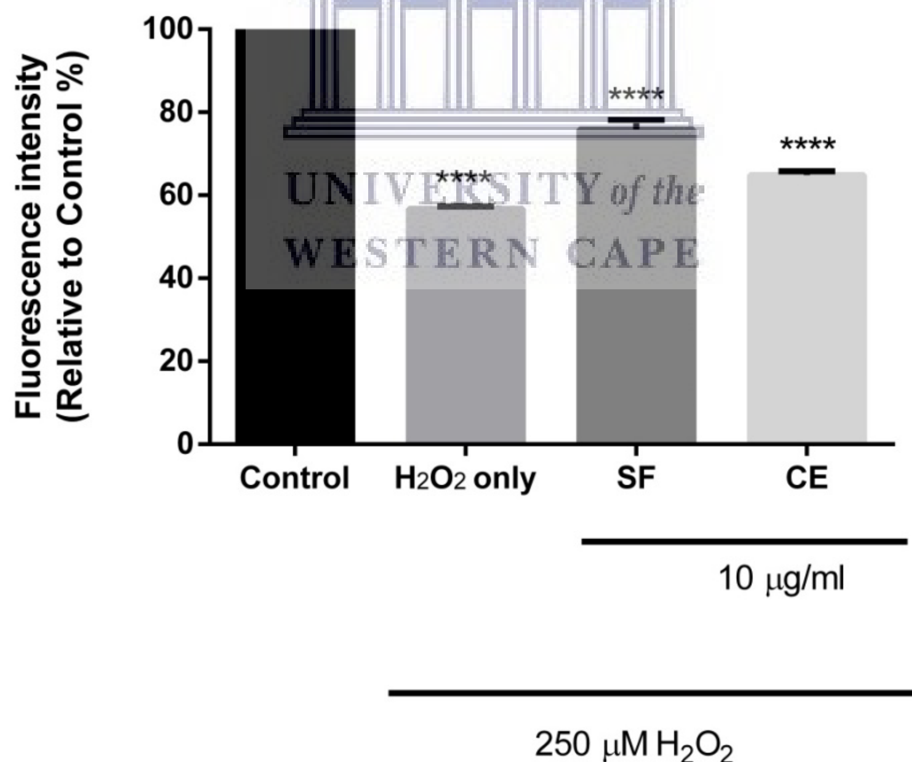

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Figure 4.11B: Effects of SF and CE plant extracts at the maximum non-toxic dose on mitochondrial membrane permeability. Treatment with H_2O_2-only cells was significantly different from control cells. Treatment of cells with SF and CE was significantly different from H_2O_2-only cells. Data shown are the means ± SE, ***p<0.001.

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4.13. Effects of SF and CE extracts pre-treatment on Superoxide dismutase (SOD) activity in SH-SY5Y cells exposed to H_2O_2 for 3 hours

Figure 4.12 shows that cells treated with H_2O_2 only had significantly lower SOD activity (1.048) when compared to the control, the SF-treated cells (1.655) and the CE-treated cells (1.777).

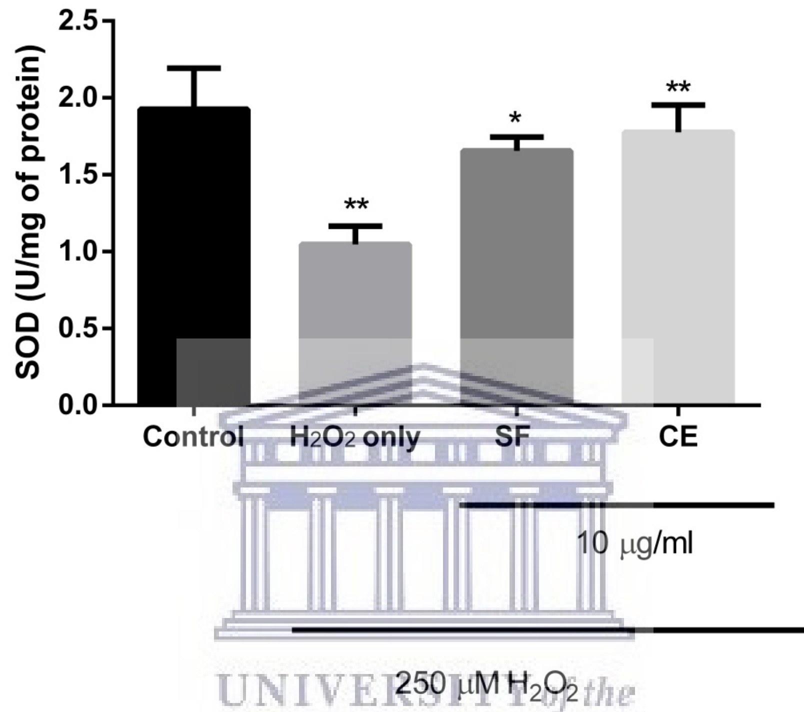

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Figure 4.12: Effects of *Sutherlandia frutescens* and *Carpobrotus edulis* plant extracts at the maximum non-toxic dose on on SOD activity in SH-SY5Y cells after 3 hours incubation in H_2O_2. Values are shown as means ± SE; *p<0.05 and p<0.01.

4.14. Effects of SF and CE extract pre-treatment on Catalase (CAT) activity in SH-SY5Y cells exposed to H_2O_2 for 3 hours

As shown in Figure 4.13, catalase activity in H_2O_2-only cells was significantly lower (3.482) than the control whileSF-treated cells had a higher catalase activity (4.963) compared to cells with H_2O_2-only and the CE-treated cells had a significantly higher catalase activity (5.192) compared toH_2O_2-only treated cells.

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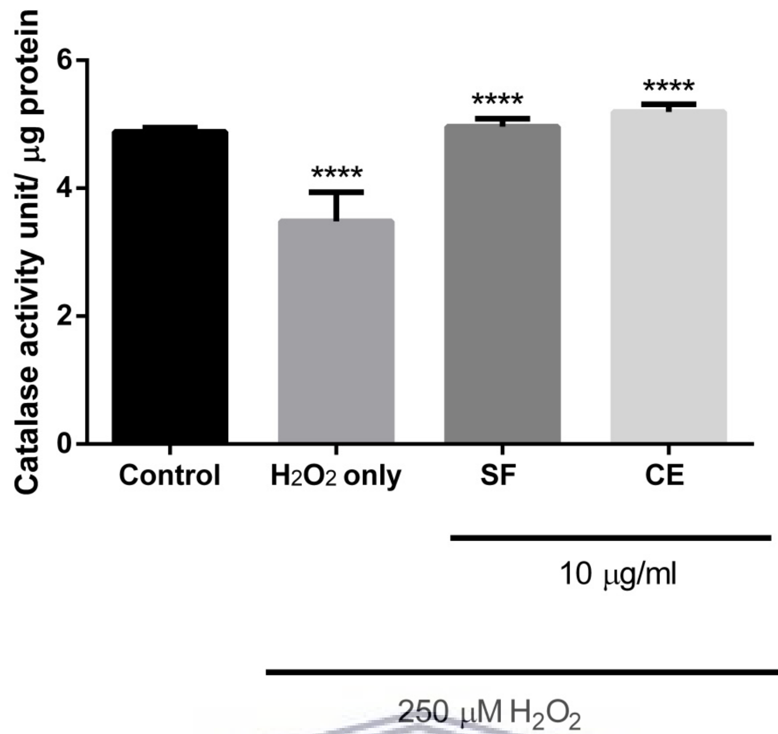

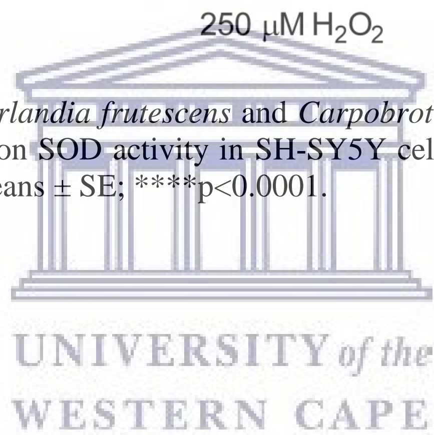
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Figure 4.13: Effects of *Sutherlandia frutescens* and *Carpobrotus edulis* plant extracts at the maximum non-toxic dose on on SOD activity in SH-SY5Y cells after 3 hours incubation in H_2O_2. Values are shown as means ± SE; ****p<0.0001.

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CHAPTER FIVE

DISCUSSION, CONCLUSION AND FUTURE RECOMMENDATIONS

5.1. Introduction

Reactive oxygen species are regarded as important causative factors of OS, (Han *et al.*, 2014) which is implicated in the development of most disease conditions including NDDs and CVDs (Auddy *et al*., 2003). The underlying mechanism in OS is however not fully understood. Phytochemicals have for many decades been recognized for their therapeutic potential, mainly because of their antioxidant, anti-allergic, anti-inflammatory, anti-viral, anti-proliferative and anti-carcinogenic properties (Anand *et al*., 2017). Some plants contain significant amounts of antioxidants to prevent oxidative stress, and thus represent a potential source of new compounds with antioxidative activity modulation of neurodegenerative-related OS (Kasote *et al.*, 2015). Nutritional supplements have also been recently promoted as commercially available sources of antioxidants (Pereira-netto and Pereira-netto, 2018).

The current study was done to investigate the potential of *S. frutescens* (SF) and *C. edulis* (CE) extracts in preventing H_2O_2-induced neurotoxicity in brain cells, SH-SY5Y neuroblastoma which have been used previously in similar studies. *S. frutescens* or *C. edulis* extracts were found to protect against neuronal loss, overproduction of ROS, activation of caspase-9 proteases, production of NO, intracellular calcium mishandling and mitochondrial dysfunction, as well as increased levels of endogenous antioxidants. Cells also had elevated levels of endogenous antioxidants.

5.2. Cytotoxicity studies

In the present study, SH-SY5Y neuroblastoma cells were treated with varying concentrations of SF extract ranging from 5 µg/ml to 400 µg/ml for 24 hours, as shown in Fig. 4.1 and 4.2 to determine MNTD which shows cell-proliferation or very little to no cell death. Cells exposed to low dosages of the plant extract showed cell proliferation beyond 100%. These concentrations were 5-20 µg/ml and were further used in the investigation against their protective effects against H_2O_2-induced cell death. A previous study investigating the effects of hot water SF on luminol and lucigenin enhanced chemiluminescence of neutrophils as well

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as its ability to scavenge superoxide and hydrogen peroxide activities indicated that SF extracts possesses the ability to scavenge both radicals at concentrations as low as 10 µg/ml (Fernandes *et al.*, 2004). Previous literature on SF mainly shows its involvement as an anticancer treatment (Rakoma, 2016). In the present study, SH-SY5Y cells were treated with 5 µg/ml - 400 µg/ml CE extracts for 24 hours. Cells responded better to low dosages of CE (5- 20 µg/ml) and these were used to determine at which volume that will confer significant attenuation against H_2O_2-induced cell death. A previous study investigating the neuroprotective effects of Melandrii Herba, also made use of SH-SY5Y cells as it is the most commonly used in vitro model for investigating neurotoxic cell death, and for assessing the neuroprotective effects of natural products. Their data showed that Melandrii Herba by itself demonstrated no cytotoxicity on SH-SY5Y cell viability at low dosages (Lee *et al.*, 2017). Anaother study assessing the protective effect of total phenolic compounds (TPC) from Inuila helenium on H_2O_2-induced SH-SY5Y cells showed that of the cells treated within a concentration rangw of 0.5-50 µg/ml TPC for 24 hours. The results from their study showed that low dosages (0.5-10 µg/ml) was nontoxic to SH-SY5Y cells according to cell viability, thus supporting the findings in our present study which suggest that low doses of plant extracts are non-toxic to SH-SY5Y cells (Wang *et al.*, 2015).

H_2O_2 could serve as an oxidative inducer in the model of oxidative stress and H_2O_2-induced oxidative stress plays an important role in apoptosis when present or exogenously applied to nucleated eukaryotic cells (Polimeno *et al.*, 2009; Wang *et al.*, 2015). Numerous studies have revealed that H_2O_2-induced apoptotic cell death depends on the concentration and exposure time of H_2O_2 (Han *et al.*, 2014; González-Sarrías *et al.*, 2017; Lee *et al.*, 2017). In our study, optimal concentration of H_2O_2 was determined by treating SH-SY5Y cells with various concentrations of H_2O_2 ranging from 50- 1000 µM for 1 and 3 hours. Only the 3 hours exposure group showed a significant decline in cell viability In this study we confirmed that SH-SY5Y cells treated with 50-250 µM H_2O_2 for 1 hour exhibited a very poor loss of cell viability. SH-SY5Y cells exposed to the same concentrations for 3 hours exhibited a significant loss of cell viability from 50- 1000 µM H_2O_2. Three hours exposure at 500 µM – 1000 µM would be too high to evaluate the neuroprotective effects of SF and CE because only 20-50 % cells survived. Therefore, 250 µM H_2O_2 was utilized (approx. 30 % inhibition) to examine the neuroprotective potential of SF and CE extracts in SH-SY5Y cells, respectively in subsequent studies. Data determined that SF and CE pre-treatment (10 µg/ml) showed the best level of prevention against H_2O_2-induced cell death. Therefore, SF and CE

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had a neuroprotective effect on H_2O_2-treated SH-SY5Y cells. A previous study reported 35% SH-SY5Y cell death when incubated with 200 µM H_2O_2 for 24 hours (Wang *et al.*, 2015).

5.3. Neuroprotection by SF and CE extracts

SH-SY5Y cells pre-treated with 5-20 µg/ml of SF extract for 24 hours, was exposed to 250 µM H_2O_2 for 1 hour and 3 hours, respectively. Our data determined that SF (10 µg/ml) and H_2O_2 exposure at 3 hours showed the best level of prevention against H_2O_2-induced cell death. Therefore, SF had a neuroprotective effect on H_2O_2-treated SH-SY5Y cells.

Cells were also pre-treated with 5-20 µg/ml of CE extract for 24 hours, then exposed to 250 µM H_2O_2 for 1 hour and 3 hours, respectively. CE extract (10 µg/ml) and H_2O_2 exposure at 3 hours showed the best level of prevention against H_2O_2-induced cell death. Therefore CE had a neuroprotective effect on H_2O_2-treated SH-SY5Y cells. (Wang *et al.*, 2015) also demonstrated that low dosages (0.5-10 µg/ml) of their plant extract was nontoxic to SH-SY5Y cells at 24 hour exposure.

5.4. Intracellular ROS

Oxidative stress may affect cell proliferation, differentiation and survival via the activation of cell signalling pathways. However, chronic exposure of high levels of oxidative stress can cause the impairment of cellular functions resulting in neurotoxicity and neuronal cell death in neurodegenerative diseases and aging (Park *et al.*, 2015b). This is attributed to ROS being able to oxidize vital cellular components such as lipids, proteins and DNA and the alteration of signalling pathways that ultimately promote cell damage and death (Zhou *et al.*, 2008). The ROS generated by the cells are determined by using the non-ionic and non-polar fluorescent dye 2,7-dichlorfluoresceindiacetate (DCFH-DA). The ROS that is produced during the oxidative stress converts the DCFH-DA into DCF which emits fluorescence that is directly proportional to the amount of ROS generated (Masilamani *et al.*, 2017). There is a direct correlation between the fluorescence and the OS induced by H_2O_2. The fluorescence intensity in SH-SY5Y cells treated with 250 µM H_2O_2 was 3.8 times compared to the control group. However, in cells treated with SF, followed by H_2O_2, we observed a decrease, though not significant. Cells treated with CE, followed by H_2O_2 showed a significant decrease in ROS, indicating the ROS inhibitory effect of CE. These results are in accordance with a previous study where Bikaversin, an active ingredient from *Fusarium oxysporum*

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demonstrated inhibitory effects on ROS generation (Nirmaladevi *et al*., 2014). Another study demonstrates that treatment of SH-SY5Y cells with 200 µM H_2O_2 lead to a significant increase in intracellular ROS in comparison to the control (Wang *et al.,* 2015).

5.5. Nitric Oxide (NO) production

Nitric oxide, a neurotoxic inflammatory mediator is a short-lived (half-life 3–30 s) lipophilic colourless gas that can very easily diffuse between cells (Chen *et al*., 2015). Although it does not interact directly with biomolecules, NO can react with oxygen to produce stable intermediates such as, NO_2, N_2O_4, N_3O_4, and peroxynitrite when reacting with superoxide. This reaction is considered toxic. A previous study focusing on the antioxidant potentials of SF showed that water extract of SF had a high NO scavenging ability (Tobwala *et al.*, 2014). Another study observing the antioxidant potential of CE determined that water extract of CE also maintained a high NO scavenging ability (Omoruyi, Bradley and Afolayan, 2012). In the present study, our data revealed that SF did not exhibit a considerable NO scavenging ability, however, CE did.

5.6. Intracellular Ca^{2+} handling

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The dysfunction of mitochondria as well as the increase in ROS production can also disrupt Ca^{2+} homeostasis (Gray *et al.*, 2015). Ca^{2+} is a second messenger and plays a vital role in regulating the release if neurotransmitters, synaptic plasticity, neuronal excitation and gene transcription. Changes in intracellular Ca^{2+} concentration may directly affect neuronal excitability (Monteiro *et al*., 2017). Changes in all three of these pathways are observed in previous studies involving AD patients as well as numerous animal and cell culture models of AD. In the present study, we observed that H_2O_2 increased intracellular Ca^{2+} levels significantly, but SF and CE attenuated this H_2O_2-induced alterations in intracellular Ca^{2+} levels. Numerous studies have indicated that an increase in excitotoxic glutamate can increase the influx of Ca^{2+} and is thought to be acommon pathway to cell death.

5.7. Caspase-9 Protease activity

We examined whether H_2O_2-induced oxidative stress resulted in cell death. We confirmed that H_2O_2 treatment induced cell death by caspase-9 protease activity and Hoechst 33324

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staining. Apoptosis is well-known in the pathophysiological cascade of neurodegenerative diseases such as AD and stroke (Zhou *et al.*, 2008). In patients suffering a stroke, apoptosis is triggered following cerebral ischemia by numerous death signals such as production of free radicals, DNA damage, and mitochondrial injury. In apoptotic processes, cytochrome c is released from the mitochondria to the cytosol. The released cytochrome c activates caspase-9 which in turn triggers the activation of caspase-3 which induces cell death (Han *et al.*, 2014). However, our study showed that pre-treatment with SF and CE extracts reduced SH-SY5Y death, suggesting that these extracts acted as anti-apoptotic agents by modulating ROS and mitochondrial function under H_2O_2-induced neurotoxic conditions.

5.8. Mitochondrial Membrane Permeability

It is well-known that H_2O_2-induced oxidative stress disrupts MMP and results in mitochondrial dysfunction. MMP provide a valuable clues of cells health and functional status (Wang *et al.*, 2015). Rhodamine 123 (RH-123), a mitochondrial dye was used to monitor the membrane permeability of mitochondria as it induces the quenching of RH-123 fluorescence, and the rate of fluorescence deterioration is proportional to MMP. In the present study, cells treated with 250 μM H_2O_2 showed a 32% decrease of MMP compared to that of the control, indicating the depolarization of MMP. However we observed MMP being restored by SF and CE pre-treatment, followed by H_2O_2. A previous study showed MMP decreased in SH-SY5Y cells after exposure to 200 μM H_2O_2 compared to the control, while pre-treatment with TPC significantly reduced the decrease of MMP amd decrease in ATP production (Wang *et al.*, 2015). Another study showed that Bikaverin inhibited MMP disruption, providing protection against H_2O_2-induced OS (Nirmaladevi *et al.*, 2014).

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5.9. Antioxidants (SOD and CAT)

Cells are equipped with lots of antioxidants such as glutathione peroxidase (GSH-Px), catalase (CAT) and superoxide dismutase (SOD) which serve to prevent damage caused by ROS (Koo *et al.*, 2004). H_2O_2 formed by OS is removed by catalase. The present result showed that H_2O_2-induced decrease in cell survival was correlated with a reduction in SOD and CAT activities. When SH-SY5Y cells were pre-treated with SF and CE, an increase in activities of SOD and CAT were observed. This suggests that cytoprotective effects of SF

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and CE are involved against intermediate concentrations of H_2O_2-induced neurotoxicity of SH-SY5Y cells.

The antioxidant activity of polyphenol compounds in the plant depends on the free radical scavenging capabilities to reduce iron. While there is limited information regarding the antioxidant activity of CE, a recent study determined that aqueous extracts appeared to be one of the best solvents for antioxidant activity (Omoruyi, Bradley and Afolayan, 2012). In our present study, SH-SY5Y cells were also treated with various concentrations of CE to determine the maximum non-toxic dose showing cell proliferation. Cells were treated for 24 hours and those exposed to low CE dosages were further used in their investigation of their protective effects against H_2O_2-induced cell death. The data in the study revealed that CE aqueous extract attenuated cell death of 3 hours exposure to H_2O_2 at three different concentrations of CE but the dosage that was most significant was 10 µg/ml.

5.10. Cell morphology

In the present study, H_2O_2 caused apoptotic cell death with morphological nuclear changes (Figure. 4.10), consistent with a previous study (Lee *et al*., 2018). The Hoechst 33324 staining method which separates the live and apoptotic cells by showing brightly stained nuclei was conducted to morphologically observe apoptosis. Microscopic imaging showed that H_2O_2 treatment induced neuronal death whereas SF- and CE- treated cells showed decreased chromatin condensation and cell shrinkage. The results indicate that SF and CE inhibited the morphological changes against H_2O_2-induced cell damage. Another study explored whether salidroside may protect against neuronal cell apoptosis. H_2O_2-treated cells stained with fluorescent DNA, binding dye, Hoechst 33342 showed morphological features as seen in apoptosis (Zhang *et al*., 2007).

5.11. CONCLUSION

This study showed that both SF and CE extracts ameliorated H_2O_2-induced neurotoxicity in SH-SY5Y cells, possibly through the bioactivity of polyphenolic compounds present in them. The protective effects were demonstrated through anti-apoptotic effects, the modulation of

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ROS levels, nitric oxide production and intracellular calcium mishandling as well as the restoration of SOD and CAT activity in SH-SY5Y cells. Thus, both SF and CE may be potential sources of neuroprotective agents that can be used to slow-down or ameliorate neurodegenerative and ageing-induced diseases. Further *in vivo* investigations and possibly clinical trials are suggested to validate these findings.

5.12. LIMITATIONS

This study was done to investigate the possible neuroprotective effects of SF and CE plant extracts in H_2O_2 toxicity, using only a few assays and techniques to provide basic information on the extracts of SF and CE. Other assays often used to elucidate the mechanisms of action of most neuroprotective compounds were not done in this study.

Stress combined with protein aggregation could trigger a cascade of events that lead to cell death as seen in most NDDs. Protein aggregation is a common pathological hallmark of most NDDs (Jellinger, 2010), and occurs due to the production of toxic substances, soluble oligomers and protofibrillar derivatives of misfolded proteins (Kahl *et al.*, 2018). While the exact mechanisms are not fully understood, genetic and environmental factors have been implicated. Thus, protein extraction and western blot analyses could be the mechanism of action for both SF and CE but this was not included in the objectives of this study. In addition, caspase-3 as the final executor of apoptosis in the cell signalling and apoptotic pathways was not studied. The present study only investigated caspase-9 protease activity due to challenges with the instability of the caspase-3 kit purchased.

Injuries to nervous tissue will usually affect both cell types (neurons and glial cells) present in this type of tissue. Only a neuronal model (dopaminergic SH-SY5Y neuroblastoma cells) was used in all our assays, representing a possible limitation of this study. Astrocytes are specialized glial cells that perform homeostatic functions to maintain the survival of nervous tissue and are the most commonly studied glial cells for the determination of neuroprotective, angiogenic and antioxidative properties in literature. However, astrocytes could not be used in this study due to challenges with procuring new cells.

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5.13. FUTURE RECOMMENDATIONS

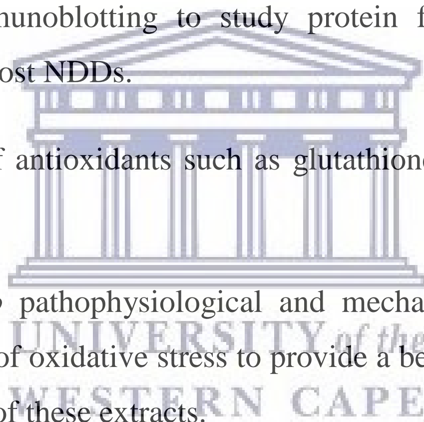
Future studies are recommended to incorporate the following aspects:

1. Determination of the most active compounds in SF and CE extracts through phytochemical analysis and evaluation of the neuroprotective potential of these products against H_2O_2-induced neurotoxicity.

2. Study the effects of SF and CE extracts and their active compounds on astrocytes, and animals/primates, namely rats and monkeys, respectively.

3. Investigation of plant extracts/active compounds on enzymes such as cholinesterase and monoamine oxidase A should be carried out to determine whether these two enzymes play a significant role in the progression of NDDs.

4. Use of the Western immunoblotting to study protein folding and aggregation that accompany cell death in most NDDs.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5. Determination of levels of antioxidants such as glutathione (GSH) in and neurons glial cells.

6. Evaluation of the *in vivo* pathophysiological and mechanistic effects of SF and CE extracts in animal models of oxidative stress to provide a better understanding of possible neuroprotective functions of these extracts.

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