# *In vitro* Neuroprotective Effects of *Boophone disticha, Brunsvigia bosmaniae* and *Strumaria truncata* Extracts In SH-SY5Y Cells.

by

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**DECLARATION**

I am aware of and understand the University's policy on plagiarism, and I declare that the thesis titled, "*In vitro* neuroprotective effects of *Boophone disticha, Brunsvigia Bosmaniae* and *Strumaria truncata* extracts in SH-SY5Y cells" is my work that has not been submitted before for any degree and that all the sources I have used or quoted have been referenced accordingly.

Full name: Tusekile Sarah Kangwa Date: September 2021

Sign: ……..

## DEDICATION

*This thesis is dedicated firstly to my ever-loving, promise-keeping and ever-faithful God who has seen me through the duration of my studies and secondly to my amazing Mother, who is my personified strength. I have been able to get to this stage because you have been standing by me through it all, reminding me that I can do anything I set out to do as long as I never forget to involve God in everything.*

"*Commit to the Lord whatever you do, and He will establish your plans*."

**-Proverbs 16: 3-**

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| **6-OHDA** | 6-hydroxy-dopamine |
| **Ach** | Acetylcholine |
| **AChE** | Acetylcholinesterase |
| **AD** | Alzheimer's disease |
| **AIF** | Apoptosis-inducing factor |
| **ATP** | Adenosine triphosphate |
| **BB** | *Brunsvigia bosmaniae* |
| **BBB** | Blood-brain barrier |
| **BD** | *Boophone disticha* |
| **BuChE** | Butyrylcholinesterase |
| **$Ca^{2+}$** | Calcium |
| **CCCP** | Carbonyl cyanide 3-chlorophenylhydrazone |
| **ChAT** | Choline acetyltransferase |
| **$Cl^-$** | Chloride |
| **CNS** | Central nervous system |
| **COMT** | Catechol-o-methyl transferase |
| **DA** | Dopamine agonists |
| **DA** | Dopamine-agonists |
| **DISC** | Death-inducing signalling complex |
| **DMEM** | Dulbecco's Modified Eagle's Medium |
| **DMSO** | Dimethyl sulfoxide |
| **DR** | Death receptor |
| **EAA** | Excitatory amino acid |

| | |
|---|---|
| **eNOS** | Endothelial Nitric oxide synthase |
| **ETC** | Electron transport chain |
| **FBS** | Fetal Bovine Serum |
| **GABA** | Gamma-aminobutyric acid |
| **GAD** | Glutamic acid decarboxylase |
| **Glu** | Glutamate |
| **H2DCFDA** | 2, 7-dichlorofluorescein diacetate |
| **$H_2O_2$** | Hydrogen peroxide |
| **$iCa2^+$** | Intracellular calcium |
| **iNOS** | Inducible Nitric oxide synthase |
| **$K^+$** | Potassium |
| **LBs** | Lewy bodies |
| **LC** | Locus coeruleus |
| **L-DOPA** | L-dihydroxyphenylalanine |
| **LNs** | Lewy neurites |
| ***LRRK2*** | Leucine-rich repeat kinase 2 |
| **MAO** | Monoamine oxidase |
| **MAO-B** | Monoamine oxidase B |
| **MMP** | Mitochondrial membrane potential |
| **$MPP^+$** | 1-methyl-4-phenylpyridinium |
| **MPPP** | 1-methyl-4-phenyl-4-propionoxypiperidine |
| **mtDNA** | Mitochondrial DNA |
| **MTT** | 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide |
| **$Na^+$** | Sodium |

| | |
|---|---|
| **NDDs** | Neurodegenerative diseases |
| **NED** | N-1-naphthylethylenediamine dihydrochloride |
| **NMDA** | N-methyl-D-aspartate |
| **nNOS** | Neuronal Nitric oxide synthase |
| **NO** | Nitric oxide |
| **NOS** | Nitric oxide synthase |
| **NPY** | Neuropeptide Y |
| **·OH** | Hydroxyl radical |
| **$O_2^-$** | Superoxide |
| **OXPHOS** | Oxidative phosphorylation |
| **PARP** | Poly ADP-ribose polymerase |
| **PBS** | Phosphate Buffered Saline |
| **PCD** | Programmed cell death |
| **PD** | Parkinson's disease |
| **PDD** | Parkinson's disease-related dementia |
| ***PINK1*** | PTEN-induced kinase protein 1 |
| **RNS** | Reactive nitrogen species |
| **ROS** | Reactive oxygen species |
| **SEM** | Standard error mean |
| **SN** | Substantia nigra |
| **SNCA** | Alpha-synuclein |
| **SNpc** | Substantia nigra pars compacta |
| **SOD** | Superoxide dismutase |
| **ST** | *Strumaria truncata* |

| | |
|---|---|
| **TCA** | Tricarboxylic acid cycle |
| **TH** | Tyrosine hydroxylase |
| **TMRE** | Tetramethylrhodamine ethyl ester |
| **TRAIL** | Tumour necrosis factor-related apoptosis-inducing ligand |
| **VC** | Vehicle control |
| **$Zn^{2+}$** | Zinc |

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## ABSTRACT

Parkinson's disease (PD), the second most common neurodegenerative disorder after Alzheimer's disease, is one of the leading disability disorders with about 10 million people affected worldwide. The pathological hallmarks of PD are defined by the loss of dopaminergic neurons in the substantia nigra pars compacta of the midbrain with its characteristic clinical motor and non-motor symptoms. However, the loss in dopaminergic neurons causes characteristic clinical manifestations, which include non-motor and motor symptoms. Damage to cholinergic neurotransmitter systems causes non-motor symptoms like sleeping disorders, depression, and a variety of other psychiatric issues, while a malfunctioning nigrostriatal dopaminergic pathway causes such motor symptoms as tremors, stiffness, and postural instability. PD symptoms usually mirror the degree of alteration to neuronal integrity in the affected parts of the brain, but the severity of progression varies with each patient.

Current treatments for PD are largely palliative, and its overall prevalence rates seem to remain high; hence there is a need for novel neuroprotective and therapeutic approaches. This study investigated the potential neuroprotective effects of three Amaryllidaceae plant extracts, namely the *Boophone disticha*, *Brunsvigia bosmaniae*, and *Strumaria truncata*, in an *in vitro* model of PD. The $MPP^+$ neurotoxin was used to induce toxic insult in the SH-SY5Y neuronal cell line pre-treated with the plant extracts.

To achieve the study's aim, SH-SY5Y cells were treated with different concentrations (2.5, 5 and 10 µg/mL) of the three plant extracts and their cytotoxic effects were assessed using the MTT cell viability assay. In addition, the cytotoxic effects of $MPP^+$ were also evaluated to determine the maximum non-toxic concentration, which was then used to induce toxicity to the cells in order to assess the extracts' neuroprotective capabilities. The mechanism of the neuroprotection offered by the extracts against the neurotoxin, was investigated by measuring

intracellular ROS levels, nitric oxide (NO) levels, mitochondrial membrane permeability (MMP) potential, intracellular calcium levels, adenosine triphosphate (ATP) levels, caspase 3 activity, and the autophagy levels in SH-SY5Y cells.

The results obtained showed that the *Boophone disticha, Brunsvigia bosmaniae* and *Strumaria truncata* extracts were able to protect the cells from the imbalance caused by inducing $MPP^+$ toxicity in SH-SY5Y cells. This was demonstrated by their ability to significantly increase cell viability in $MPP^+$-treated cells, attenuate mitochondrial dysfunction by decreasing ROS, calcium, and NO levels, while increasing ATP activity levels. In addition, the extracts attenuated $MPP^+$-induced caspase activity, thereby preventing cell death. Taken together, the plant extracts under study showed neuroprotective potential against the cytotoxic effects of $MPP^+$. These findings suggest the three extracts as a hope for more neuroprotective agents to be used in PD studies owing to their antiapoptotic and antioxidant activity. However, further studies on the active compounds present in the extracts as well as their efficacy in animal models would help validate their use as neuroprotective agents.



*Keywords*: Parkinson's disease; Amaryllidaceae; neuroprotection; *Brunsvigia bosmaniae*; *Boophone disticha*; *Strumaria truncata*

# CHAPTER ONE

# INTRODUCTION

## 1.1. Overview

Neurodegeneration is a complex process that triggers neuronal death in the nervous system and in turn, results in damage and dysfunction of the brain and spinal cord. These neurodegenerative diseases (NDDs) are age-dependent and are not only a major cause of global morbidity and mortality but are also becoming increasingly prevalent (Rahimi and Kovacs, 2014, Gitler et al., 2017). Ageing is a major risk factor for NDDs and also affects neuronal self-repair. Various age-related NDDs are reported to be characterized by the accumulation of misfolded proteins that are disease-specific as well as the progressive loss of vulnerable neurons resulting in behavioural, motor, and cognitive deficits (Hung et al., 2010, Schaffert and Carter, 2020).

The central nervous system (CNS) comprises neurons and glial cells originating from neural precursor cells of a germinal layer known as the ventricular zone (Jessen, 2004). The human brain is a major component of the CNS and contains about 100 billion neurons and over 100 000 km of interconnections (Hofman, 2014). Glial cell dysfunction is known to be the major cause of alterations in neuronal function, thereby contributing to the pathogenesis of several brain disorders (Kaminsky et al., 2016). Microglia which form about 20% of the glial cell population are known to recognize and neutralize foreign objects inside the CNS and are the first cells to respond to a minor pathological change by initiating and sustaining a well-coordinated neuroinflammatory response (Palmer, 2010). Microglial density in the substantia nigra (SN) is higher than in other brain regions like the hippocampus and midbrain (Kim et al., 2000). Studies have elucidated that the increased density of microglia in the SN enhances the release of neurotoxins from activated microglia, which makes the region more susceptible to

inflammation and also decrease the permeability of the blood-brain barrier (BBB), thus playing a vital role in the neurodegeneration of cells (Qin et al., 2007, Wu et al., 2011, Yang et al., 2013, Haruwaka et al., 2019, Liu et al., 2020). Furthermore, in PD-affected brains, the increased inflammatory status is also due to the presence of neurotoxic reactive species that are products of microglia, including nitric oxide and superoxide (Mosley et al., 2006, Yuste et al., 2015, Azam et al., 2021).

Parkinson's disease (PD) is the second most common NDD after Alzheimer's disease (AD), and affects approximately 10 million people worldwide, which is characterized by loss of dopaminergic neurons in the SN pars compacta in the midbrain (Ferreira and Romero-Ramos, 2018). It is reported that about 1% of the world population over the age of 55 is affected by PD, and the risk of developing this movement disorder increases with age, with about 4-5% of patients being 85 years old or above.

PD has also been reported to be more prevalent in males than females, at a ratio of approximately 1.5:1.0 (Rizek et al., 2016, Lewin, 2019) and its symptoms vary from motor to non-motor, with the pathophysiological changes of non-motor features being reported to manifest before the motor features (Goldman and Postuma, 2014). PD's motor symptoms include bradykinesia, tremors, rigidity, postural instability, while non-motor symptoms include bradyphrenia, dysautonomia, sleep disorders, depression, and other psychiatric problems (Williams and Litvan, 2013a, Magrinelli et al., 2016). The motor symptoms are caused mainly by damage to the nigrostriatal dopaminergic system. In contrast, the non-motor symptoms result from damage to such neurotransmitter systems as the cholinergic, noradrenergic, and serotonergic systems (Delaville et al., 2011, Pantcheva et al., 2015).

Since the first description of PD in 1817, many factors have been linked to its etiology, with the interplay among ageing, environmental and genetic susceptibility factors often being

implicated (Pang et al., 2019). For example, females have low PD incidence rates than males, low exposure to the social and behavioral risk factors of PD, low frequency of tremors, and low environmental toxin exposure (Priyadarshi et al., 2001, Pankratz et al., 2002, Bower et al., 2003, Gillies et al., 2004). Studies have also reported a delay in the onset of PD symptoms in women than in men, possibly due to the neuroprotective effects of the female hormone, estrogen (Datla et al., 2003, Gillies et al., 2004, Gillies and McArthur, 2010).

The small deep brainstem nucleus, the locus coeruleus (LC), or 'blue spot', is the main source of noradrenergic neurons, and studies have shown that the loss of these has a vital role in the pathogenesis and progression of movement disorders, including PD (Delaville et al., 2011, Betts et al., 2019, Paredes-Rodriguez et al., 2020). Furthermore, the LC has been reported to be involved in behavioural flexibility, memory, and learning thus, the significant loss of neurons in this nucleus is regarded as a crucial component to consider in NDDs such as PD and AD (Lockrow et al., 2012, Borodovitsyna et al., 2017, Weinshenker, 2018).

Dementia is an umbrella term for a variety of symptoms arising from brain disorders that affect the cognitive and mental functions of the brain (Sun, 2018). Regardless of the debate that still surrounds the neuropathophysiology of PD-related dementia (PDD), there is an estimated sixfold increase in the risk of PD patients developing dementia, with its resultant contribution to the morbidity and mortality rates of PD (Aarsland et al., 2003, Poewe et al., 2008, Leverenz et al., 2009). Although most of the current treatments for PD help prolong life in affected persons by alleviating its symptoms, they do not mitigate the risk of PDD, as PD symptoms are known to worsen with advancing age (Goldman et al., 2018, Fang et al., 2020).

The identification of inherited PD cases has played a significant role in understanding mechanisms involved in disease pathogenesis. This has also contributed to the understanding that PD can either be familial (inherited), a subset of the sporadic form, or idiopathic (non-

inherited) (Pankratz and Foroud, 2007, Chai and Lim, 2013). The familial cases are associated with mutations in various genes such as *SNCA* (PARK1), *PARKIN* (PARK2), PARK3, PARK4, *UCHL1* (PARK5), *PINK* (PARK6), *DJ-1* (PARK7), *LRRK2* (PARK8), *ATP134A2* (PARK9), *GIGYF2* (PARK11), *PLA2G6* (PARK14), *FBX07* (PARK15), *VPS35* (PARK17) and *SYNJ1* (PARK20)(Karimi-Moghadam et al., 2018, Pang et al., 2019, Thao, 2019). Furthermore, the heterogeneity groups observed in familial PD cases suggest that the patients may either undergo slow or rapid disease progression and either autosomal dominant or recessive modes of inheritance (Karimi-Moghadam et al., 2018, Pang et al., 2019).

On the other hand, idiopathic PD, which is considered a spectrum of conditions with no specific cause but multiple causes, may depend on the extended periods between disease initiation and the manifestation of the clinical symptoms (Williams and Litvan, 2013b, Jankovic and Tan, 2020). The pathophysiology of PD involves such mechanisms as protein aggregation, excitotoxicity, neuroinflammation, mitochondrial dysfunction, genetic mutations, and oxidative stress (Tansey et al., 2007, Michel et al., 2016). Mitochondrial dysfunction caused by genetic mutations and environmental toxins leads to compromised mitochondrial membrane potential, decreased ATP levels, disruption of calcium homeostasis, among other effects all resulting in elevated caspase activity and apoptosis, neurodegeneration, and eventually PD (Rego and Oliveira, 2003, Golpich et al., 2017, Yang et al., 2020).

To date, there are no disease-modifying treatments available for PD, as current treatments only offer symptomatic relief of the symptoms associated with the disease. There is increasing evidence to show that the progression of PD is not entirely due to the loss of dopaminergic neurons and depletion of circulating endogenous dopamine, but also by the depletion of gamma-aminobutyric acid (GABA) and excessive levels of other neurotransmitters like acetylcholine and glutamate, as well as neuropeptides like neuropeptide Y and substance P (Adinoff, 2004, Fontoura et al., 2017, Choudhury et al., 2018). For over 40 years, levodopa has

remained the most widely used oral treatment for relieving motor symptoms of PD (Hauser, 2009, Poewe et al., 2010, Salat and Tolosa, 2013) as it is easily decarboxylated into dopamine by the enzyme DOPA decarboxylase and can cross the blood-brain barrier (NCBI, 2021). Other available treatments for PD can be classified according to their modes of action viz, monoamine oxidase B (MAO B)-inhibitors, dopamine-agonists, catechol-o-methyl transferase (COMT) inhibitors, amantadines, and anticholinergics (Zahoor et al., 2018a). Unfortunately, most of these available treatments offer very minimal clinical benefits in terms of the non-motor manifestations of PD (Zahoor et al., 2018b).

For this reason, they enhance the need for more studies to discover more PD drugs that may allow for improved clinical efficacy and improve the adverse side-effects that come with the prolonged use of the drugs, thereby being detrimental to one's health (Seppi et al., 2019). In most parts of the world, there have been developing interests in the use of phytotherapy for neurological and NDDs, mainly because most plant extracts or plant-derived bioactive compounds and nutraceuticals have been reported to either have potent neuroprotective effects (Kumar and Khanum, 2012, da Costa et al., 2020). Compared to chemical drugs, these plant-based medicinal products appear to have a more extensive appeal, especially among many developing countries, considering their affordability, availability, safety, and efficacy profiles (Semenya et al., 2012, Howes et al., 2020). For example, South Africa has a wide variety of medicinal plants, and most rural communities have relied on herbs for the treatment of different ailments for hundreds of years. The availability of vast amounts of medicinal plants in South Africa and their folkloric use have elicited renewed interest in their putative use as sources of potent plant-derived medicines for treating neurological, neurodegenerative, and psychiatric disorders (Enogieru et al., 2018b).

In the present study, the extracts of the Amaryllidaceae family of plants known for their many biological properties, including antioxidant, antiviral, anti-inflammatory, antimalarial,

analgesic, neuroprotective and anti-psychotic effects, were evaluated for their neuroprotective effects in *in vitro* models of neurodegeneration (Bastida et al., 2006, Nair and van Staden, 2013a, Elgorashi, 2019, Omoruyi et al., 2020a). Although widely distributed around the world and consisting of about 800 species and 80 genera, the Amaryllidaceae family is said to be endemic to the southern part of Africa, with the Western Cape province of South Africa having higher numbers of these plant species (Goldblati, 1978, Conrad, 2008, Ibrakaw et al., 2020).

### 1.2. Justification for research

Most neurological and ND diseases have no clearly defined cure, and for PD and other dementia-related problems, symptomatic relief of symptoms may improve memory. However, despite the best available treatment options, there are various challenges with current treatments for NDDs, including drug resistance, non-specific toxicity, and inability to cross the blood-brain barrier. Thus, the use of herbal medicines and plant-derived natural products as alternative treatment sources of very effective and safe neuroprotective compounds that can be easily delivered to the brain is plausible (Kennedy and Wightman, 2011, Ratheesh et al., 2017). Therefore, the present study investigated the potential neuroprotective effects of three South African plants from the Amaryllidaceae family, namely, *Boophone disticha*, *Brunsvigia bosmaniae,* and *Strumaria truncata* in a toxin-induced neuronal toxicity model. Success in this *in vitro* study would contribute to the current multi-disciplinary efforts to develop treatments capable of addressing the limitations associated with current treatment options and provide preliminary evidence that can be validated by *in vivo* techniques and clinical trials.

### 1.3. Aim

This study aimed to investigate the neuroprotective effects of *Boophone disticha, Brunsvigia bosmaniae, and Strumaria truncata* in $MPP^+$-induced toxicity in SH-SY5Y neuroblastoma cells.

## 1.4. Objectives

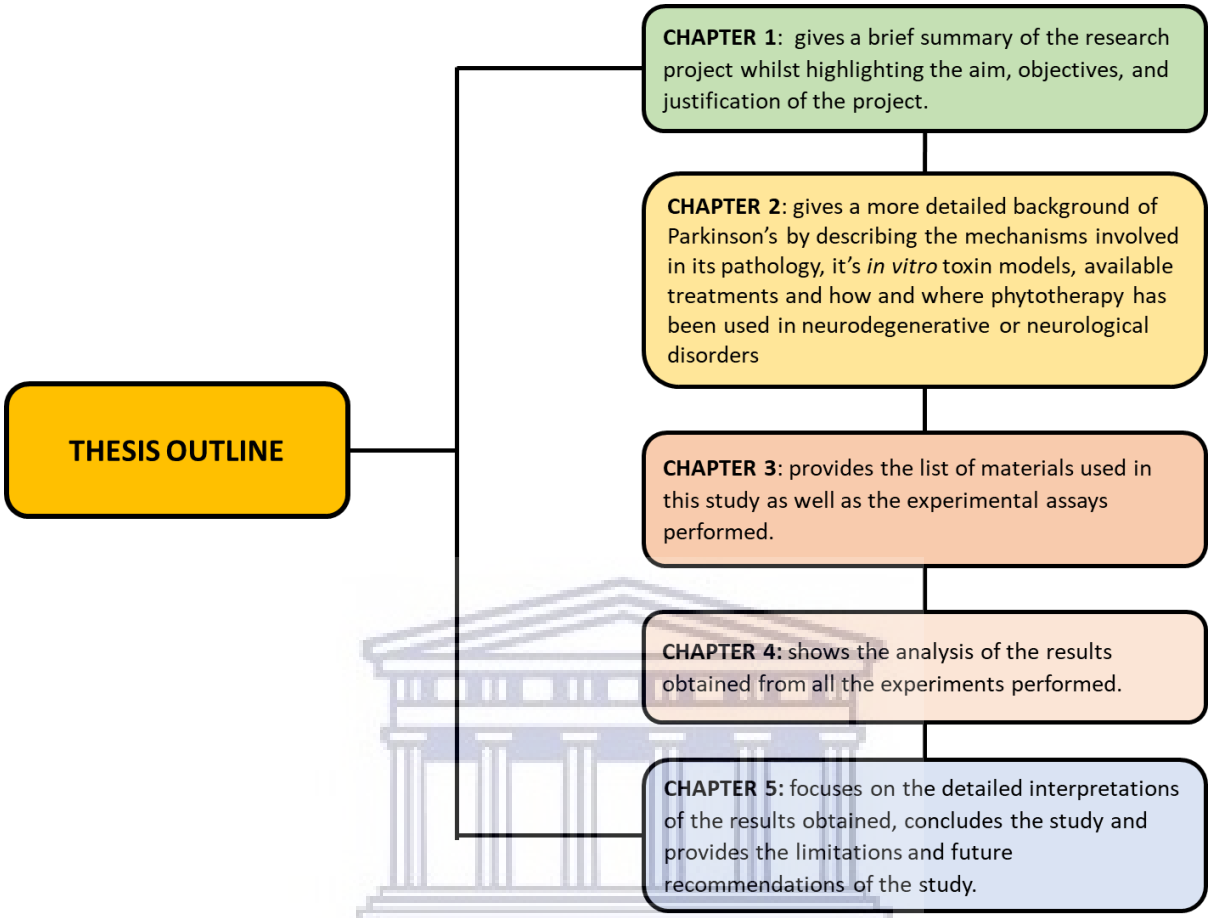
The objectives of the study include:

- To determine the cytotoxicity of plant extracts so as to obtain the optimum concentrations for neuroprotection.
- To determine the optimum concentration of $MPP^+$ that will induce significant neuronal toxicity.
- To establish the mechanisms of neuroprotection of the plant extracts following $MPP^+$ toxicity.

## 1.5. Hypothesis

Based on reports from previous studies and preliminary results of this study, we hypothesized that the *Boophone disticha, Brunsvigia bosmaniae, and Strumaria truncata* would protect SH-SY5Y cells against $MPP^+$-induced neurotoxicity.

## 1.6. Thesis Outline



# CHAPTER TWO

# LITERATURE REVIEW

## 2.1. A Brief History of Parkinson's disease

Parkinson's disease (PD) is a neurodegenerative disease that is associated with a substantially decreased quality of life and significant disability. It was first described by British neurologist James Parkinson whose publication in 1817, titled "*An Essay on the Shaking Palsy"* first described the main clinical features of the disease, which was named after him 50 years later (Parkinson, 2002). In the late 1800s, epidemiological studies by William Gowers showed that PD was more prevalent in males than females and during the 19th century, pathological studies revealed that PD was mainly caused by damage to the substantia nigra (Brissaud, 1899, Obeso et al., 2017a).

About two hundred years after the first essay on the disease, the factors that play significant roles in the pathogenesis of PD are still not fully understood (Duvoisin, 1987, Del Rey et al., 2018). However, studies have shown that the neurotransmitter dopamine present in the substantia nigra, ventral tegmental area, and hypothalamus of the brain plays a key role in aiding the body's coordinated movements. Thus a decrease in dopamine levels results in compromised voluntary and involuntary body movements (Gepshtein et al., 2014) and *parkinsonism* is the motor standpoint for the clinical diagnosis of PD via four PD symptoms: rest tremor, rigidity, bradykinesia, and postural instability (Massano and Bhatia, 2012). In addition to the dopaminergic loss seen in PD there is also the presence of intraneuronal cytoplasmic inclusions, notably Lewy neurites (LNs) and Lewy bodies (LBs), which are the hallmarks of degenerating neurons in PD (Kaidery and Thomas, 2018, Ma et al., 2019).

## 2.2. Epidemiology of Parkinson's Disease

Neurological disorders are reported to be part of the leading disability disorders worldwide, as revealed by a global burden of disease study (Feigin et al., 2017). PD is the second most common neurodegenerative disease after Alzheimer's disease (AD), and is also the fastest-growing disability disorder globally (Chin and Vora, 2014, Feigin et al., 2017). Approximately 10 million people live with PD worldwide, and the global incidence of affected persons increased by 118% making the figure 6.2 million between 1990 and 2015 (Dorsey et al., 2018). The incidence of PD in men is 1.5 times more than in women (Kasten et al., 2007, Muller et al., 2012, Abbas et al., 2017), possibly due to the possible neuroprotective effects of oestrogens on dopaminergic neurons (De Lau and Breteler, 2006, Elbaz et al., 2016). Lower PD incidence rates have been reported in Africans compared to other races, possibly due to fewer disease studies being done on the African continent. In addition, stigmatization of patients with neurological disorders and the preference for traditional treatment methods over allopathic hospital visits in most African cultures, have contributed to late hospital presentations and many undiagnosed cases (Dekker et al., 2020).



A review of the prevalence of PD in thirteen (13) African countries over a 60-year period (1944 to 2004), showed lower prevalence rates than reported for the Americas and Europe (Okubadejo et al., 2006). It has also been predicted that after 2015, increased life expectancy in many African countries will result in increased PD prevalence, as the population of persons aged 65 years and above will increase with the concomitant risk of developing such movement disorders as PD (United Nations, 2015). The Sub-Saharan region is one of the fastest-growing regions in Africa, with prevalence rates ranging from 10 to 235/100 000 and projected to be about 67 million cases among people aged 60 years and above, by 2030 (Velkoff and Kowal, 2006, Oluwole et al., 2019).

In South Africa, one study reported study revealed that out of the fourteen identified cases, there were fewer black patients (3/14) than Indians (5/14) and Whites (6/14) (Cosnett and Bill, 1988). Another study in 2019 reported more PD referral centre prevalence cases than the previous 1988 study by Cosnett and Bill. Despite the absence of significant differences in clinical PD phenotypes in the different ethnic groups, the authors observed more neuropsychiatric symptoms and dyskinesias in White and Indian patients (Amod and Bhigjee, 2019). Due to the increasing numbers of older people in Africa, cases of undiagnosed dementia and other age-related disorders are likely to remain on the increase (Smith, 2018, Dekker et al., 2020).

### 2.2.1. Prevalence of Parkinson's Disease Dementia

An estimated 50% to 80% of individuals with PD will eventually experience PD dementia, which is a decline in cognitive function usually noticed at least one-year post-diagnosis (Meireles and Massano, 2012). The total number of new cases of dementia each year worldwide, is approximately 7.7 million, implying one new case every four seconds (Kumar, 2013). A study by Prince and colleagues (2013) revealed that an estimated 35.6 million people lived with dementia in 2010 globally, with a near doubling of this figure being projected by 2030 (approximately 65.7 million) as well as 115.4 million by 2050. These authors also reported that about 58% of dementia patients are based in middle or low income countries(Prince et al., 2013).

The incidence rate of dementia in PD increases about 4-6 times than usual as the population ages, and at least 75% of PD patients who survive for more than ten years develop dementia (Aarsland and Kurz, 2010). Gender differences do not entirely limit PD or PDD incidence, however available evidence suggests that men tend to have higher incidence rates than women (De Lau and Breteler, 2006, Elbaz et al., 2016). Regardless of the incidence ratio, women tend

to have a faster disease progression and a higher mortality rate (Miller and Cronin-Golomb, 2010, Gillies et al., 2014, Cerri et al., 2019).

### 2.3. Pathophysiology of PD

#### 2.3.1. Mitochondrial dysfunction

Mitochondria play a pivotal role in many functional processes in neurons ranging from energy homeostasis, biogenesis to interrelated or co-dependent cell death, all of which help to maintain neuronal integrity, function and survival (Moreira et al., 2010). Mitochondrial functions are possibly facilitated by the presence of a small amount of genetic material within them, known as the mitochondrial DNA or mtDNA which encode a few vital genes (Logan, 2006). The human mtDNA is a circular molecule of 16569 base pairs and 37 genes, containing 22 tRNAs and 13 polypeptides (Taanman, 1999). It is known that mtDNA is strictly maternally inherited, with a mutation rate that is 5-10 times higher than reported in the nuclear genome (Pyle et al., 2015, Chinnery and Gomez-Duran, 2018). There is however, some research evidence that supports the hypothesis of bi-parental inheritance of mtDNA (Wolff et al., 2013, Luo et al., 2018, McWilliams and Suomalainen, 2019, Vissing, 2019). The vast metabolic demand of the central nervous system (CNS) is based on the highly differentiated nature of neurons hence the need for significant amounts of ATP to maintain ionic gradients across neuronal membranes and for neurotransmission (Fiskum et al., 1999, Ames, 2000).

In the brain, neurons rely on the capacity of mitochondria to build-up membrane excitability and implement complex neurotransmission processes and versatility, thus making neuronal function and survival commonly susceptible to mitochondrial dysfunction (Nicholls and Budd, 2000). Owing to the fact that mitochondria form a compartment that is highly active in function and structure in neurons, any disruption to the functioning causes a disruption in the mitochondrial transport in neuronal segments and an imbalance at sites where $Ca^{2+}$ handling

and ATP supply are necessary (Kann and Kovács, 2007, Lee et al., 2018). Dysfunctional mitochondria produce less ATP and become more proficient at producing reactive oxygen species (ROS), which are molecules like free radicals (superoxide, $O_2^-$), hydroxyl radical (·OH), or non-radicals (hydrogen peroxide, $H_2O_2$) that are short-lived but highly reactive due to unpaired valence electrons (Kim et al., 2015).

The brain is one of the organs considered to be highly vulnerable to ROS challenge due to its high oxygen demand and abundance of peroxidation-susceptible lipid cells (Uttara et al., 2009). During the process of electron transfer, there is an inevitable leakage of electrons which eventually causes the production of anions (superoxides) that play a significant role as part of the endogenous ROS, regardless of an effective cellular antioxidant/mitochondrial system being in place, thus making mitochondria the primary sources of oxidative stress in cells (Kurutas, 2015). Low or moderate amounts of ROS are essential for neuronal function and development, making them products of turnover in the mitochondrial respiratory chain, but excessive ROS levels are very harmful (Beckhauser et al., 2016).



Numerous mitochondrial proteins are frequently under the threat of oxidative damage because they have iron-sulfur clusters for oxidation-reduction reactions. Abnormalities of mitochondrial function and oxidative phosphorylation (OXPHOS) have been identified in neurodegenerative diseases as reported in the literature (Schapira, 2002, Kawamata and Manfredi, 2017, Area-Gomez et al., 2019). Neurons have an exceptionally high number of mitochondrial functions for energy production and are therefore very susceptible to ROS-induced toxicity (Reddy, 2007, Eckert et al., 2011).

In neurodegeneration, the activities of such mitochondrial enzymes as the α-ketoglutarate dehydrogenase and pyruvate dehydrogenase, which are involved in the tricarboxylic acid cycle (TCA), as well as cytochrome oxidase, which is involved in oxidative phosphorylation in the

electron transport chain (ETC) are known to be disrupted (Berg et al., 2002, Tretter and Adam-Vizi, 2005), representing some of the early characteristics of apoptotic cell death (Reddy, 2007). Mitochondrial integrity is known to be firmly preserved by a delicate balance of mitochondrial fission and fusion. Fission allows the restoration and elimination of irreversibly damaged mitochondria, whereas fusion provides faulty mitochondria with replenished supplies of mitochondrial DNA and mitochondrial proteins (Youle and Van Der Bliek, 2012).

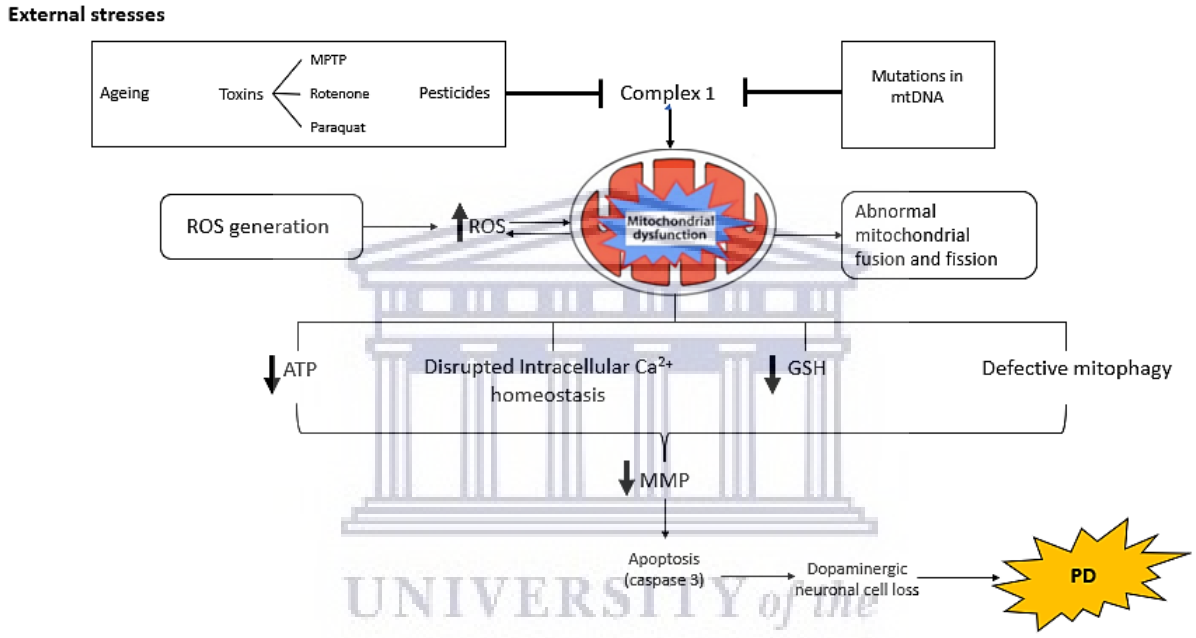



**Figure 2.1**.Summary of the processes involved in the pathophysiology of PD

⊣ inhibit → activates ↑ increases ↓ decreases

### 2.3.2. Oxidative stress in PD

Oxidative stress occurs when there is a discrepancy between ROS production and the body's antioxidant capability (Pizzino et al., 2017, Wei et al., 2018). In the mitochondria, the products of oxidative phosphorylation are such superoxide radicals and oxidants as nitric oxide (NO), hydrogen peroxide ($H_2O_2$), hydroxyl and superoxide radicals. On the other hand, some important antioxidants in humans include catalase, glutathione, and superoxide dismutase (SOD) (Sharma et al., 2012). The ROS generated by microglia and astrocytes in the brain tissue, modulates synaptic and non-synaptic communication between neurons and glia (Popa-

Wagner et al., 2013). In one study, enhanced ROS production or an impaired antioxidant system was shown to tip the cellular redox balance to oxidative imbalance, resulting in the overproduction of ROS. However, when ROS levels exceed the capacity of the antioxidant response system, extensive protein oxidation and lipid peroxidation also occur, resulting in oxidative damage, cellular degeneration and functional decline (Salim, 2017). All these factors have been consistently reported in PD and other neurodegenerative diseases such as AD (Nita and Grzybowski, 2016).

Several factors contribute to the high metabolic activity of the CNS and its susceptible to bioenergetic loss (Flynn and Melov, 2013). It is known that there are fewer antioxidant defense systems in the brain than in other tissues, hence it cannot protect itself against excessive reactive oxygen species (ROS) production. Thus, an imbalance of oxidants and antioxidant agents often leads to oxidative stress and the damage or death of both glial cells and neurons (Schapira, 1998, Gilgun-Sherki et al., 2001). One of the primary production sites for ROS and superoxide radicals is the mitochondrial Complex I (Quinlan et al., 2013, Detaille et al., 2019). In PD, some significant contributors to dopaminergic neuronal loss include excess ROS, an imbalance in neuronal ROS/RNS potential (Dias et al., 2013) as well as defects in the ETC (e.g. damage to Complex I), due to electron leakage (Zhao et al., 2019). Individuals living with PD have been reported to have compromised Complex I activity, which has made ETC dysfunction a vital player in the etiology of PD and other neurodegenerative diseases (Golpich et al., 2017). The damage caused by ROS includes the unsystematic oxidation of proteins, lipids, and mitochondrial DNA (Guo et al., 2013, Nita and Grzybowski, 2016). Superoxides generate hydrogen peroxide ($H_2O_2$) which, when accumulated, interferes with the neuronal redox potential (Kerksick and Zuhl, 2015, Kumar and Pandey, 2015). Tyrosine hydroxylase and monoamine oxidase (MAO) generate ROS, making the dopaminergic neurons vulnerable to oxidative stress (Dias et al., 2013, Meiser et al., 2013b).

The neurotoxins used to model PD such as $MPP^+$ and rotenone are known to inhibit Complex I (**Figure 2.1)** and interfere with the electron flow, resulting in enhanced ROS and decreased ATP production due to the blockage of oxidative phosphorylation (Subramaniam and Chesselet, 2013, Heinz et al., 2017).

### 2.3.3. Nitric Oxide in PD

Nitric oxide (NO) is a well-known, unique, and vital biological signalling molecule that aids in regulating and facilitating various pathways and physiologic roles in the human body. NO also helps to maintain the respiratory, immune, vascular, and nervous systems (Tuteja et al., 2004, Pacher et al., 2007) and also plays a vital role as a mediator for toxic processes in neurodegenerative and neurological disorders (Knott and Bossy-Wetzel, 2009). Nitric oxide synthases (NOSs) are a family of enzymes that catalyze the production of nitric oxide (NO) from l-arginine (Fu, 2014). Three isoforms of the enzyme NO synthase (NOS) are involved in the synthesis of NO, viz, Inducible NOS (iNOS), neuronal NOS (nNOS) and endothelial NOS (eNOS), with the neuronal isoform being the most abundant and has been reported to contribute to NO production that is implicated in $MPP^+$ neurotoxicity (Tieu et al., 2003).



In neurodegenerative diseases such as PD, the modification of proteins through nitrosylation occurs in response to increased levels of NO. The process of S-nitrosylation contributes to the pathology of PD and other neurodegenerative diseases by enhancing insults such as neuronal synaptic damage, endoplasmic reticulum stress, apoptosis, mitochondrial dysfunction, and protein misfolding (Nakamura and Lipton, 2007, Nakamura and Lipton, 2011, Akhtar et al., 2012, Yan and Xu, 2020). Increased NO production can result from the generation of such free radicals as reactive nitrogen species (RNS) in dysfunctional mitochondria, leading to the inhibition of the mitochondrial respiratory complex, induction of lipid peroxidation, and enhancement of the release of $Zn^{2+}$ from intracellular stores (**Figure 2.1**), all of which are

implicated in the progression of PD (Chung, 2006, Lee, 2018). Increased NO levels are reported to enhance the nitration of α-synuclein, resulting in increased oxidative damage and interference with $Ca^{2+}$ levels, which plays a vital role in the activation of such enzymes as nitric oxide synthase that is necessary for the attenuation of the elevated NO (Mosley et al., 2006, Dias et al., 2013, Chang and Chen, 2020).

### 2.3.4. Intracellular Calcium in PD

Over the years, calcium ($Ca^{2+}$) has been shown to be one of the most vital ions for neuronal signalling hence the constant need for its strict control in cells. The excitable nature of neurons is associated with $Ca^{2+}$ homeostasis and is also characterized by their known membrane potential, estimated at -70 mV. However, this potential may change based on some ionic ($Na^+$, $K^+$ and $Cl^-$) changes resulting in the generation action potentials (Calì et al., 2014, Shattock et al., 2015). Increased levels of α-synuclein lead to increased membrane permeability to $Ca^{2+}$, and the disruption of its homeostasis. Thus, the regulation of $Ca^{2+}$ levels, whose cytosolic set point is approximately 100 nM (10 000 times lower than the $Ca^{2+}$ concentration of the extracellular space) is an essential mechanism in neurodegeneration, as the balance aids in the modulation and control of such processes as memory and learning, plasticity, synaptic transmission, excitability, aerobic ATP production and apoptosis (Brini et al., 2014).

When there is low demand for ATP, the mitochondrial sodium-calcium exchanger releases $Ca^{2+}$ into the cytosol from the mitochondrial matrix (**Figure 2.1**) (Shattock et al., 2015) and the mitochondria can provide ATP when the supply is low, for processes that need energy to regulate $Ca^{2+}$. As the concentration of $Ca^{2+}$ increases, the pathologic form of a calcium-sensitive protease known as calpain is activated, leading to the inhibition of optimal neuronal functioning through the promotion of neuronal death (Vosler et al., 2008). Calpain is known to compromise neuronal structure and function, eventually aiding the release of cytochrome c, activating caspases, and initiating apoptosis (Vanderklish and Bahr, 2000). Some *in vitro*

models of PD have demonstrated that increased levels of $Ca^{2+}$ caused by $MPP^{+}$, could inhibit the mitochondrial complex 1, stimulate the mitochondrial nitric oxide synthase and pathologically activate calpain, which then plays a role in enhancing neuronal death (Harbison et al., 2011, Cheng et al., 2018).

### 2.3.5. Apoptosis

The term "programmed cell death" was first used by Lockshin and Williams in 1964, to describe a fixed loss of specific cells observed by an intrinsic cellular suicide programme during insect metamorphosis (Lockshin and Williams, 1964). The single word, "apoptosis," was later used to describe these findings and is considered a natural process that can occur in normal and pathological conditions alike (Kerr et al., 1972). Apoptosis describes the process of cell death triggered by physiological and pathological conditions and regulated by multi-complicated extrinsic and intrinsic ligands. In the absence of such survival signals as peptide growth factors secreted by other cells, all cells experience apoptosis. According to (Zeiss, 2003), apoptosis is a tightly-regulated process that requires a signalling cascade in which caspase activation plays a central role. Alteration of cellular homeostasis and apoptosis regulation at levels of inactive mutations, or the alteration of expressions that include the Bcl-2 protein family, plays a role in neurodegeneration (Akhtar et al., 2004, Roufayel, 2016). The processes involved in neurodegenerative diseases are characterized by the loss of neuronal cell integrity and a compromised protein change in the cytoskeleton (Jellinger, 2010), may cause neuronal cell death by necrosis (cellular accidents) and apoptosis (programmed cell death).

Apoptosis is a highly specialized programmed cell death mechanism that can occur via two main pathways: the intrinsic (internal/ mitochondrial) and extrinsic (external/ death receptor). Another pathway is the granzyme pathway, which induces apoptosis either by granzyme A or B and is associated with T-cell mediated cytotoxicity (Groscurth and Filgueira, 1998, Lieberman, 2010). Apart from the involvement of caspases, some morphological changes are

also known to occur during apoptosis, such as nuclear condensation, DNA degradation, and cellular shrinkage (Elmore, 2007) which make apoptosis the principal means of neuronal death in various neurodegenerative disorders.

All caspases are synthesized in an inactive form and have to undergo an activation process (Amarante-Mendes and Green, 1999, Elmore, 2007, McIlwain et al., 2015). Caspases are grouped functionally into three (3) categories based on their mechanism of action, namely the initiator, executioner, and inflammatory caspases. The initiator caspases are 2, 8, 9 and 10; executioner caspases are 3, 6 and 7 while the inflammatory caspases are 1, 4, 5, 11, 12, 13, and 14 (Amarante-Mendes and Green, 1999). Evidence from both *in vitro* and post-mortem studies supports the role that caspases play to initiate apoptosis in PD, by showing elevated caspase-3 expression and activity in the brain, especially in the substantia nigra pars compacta (SNpc) (Anglade et al., 1997, Hartmann et al., 2000, Mogi et al., 2000). Caspases play a role in the intrinsic and extrinsic apoptosis pathways; while caspase 9 is involved in the intrinsic apoptotic pathway, caspase 8 is implicated in the extrinsic pathway.

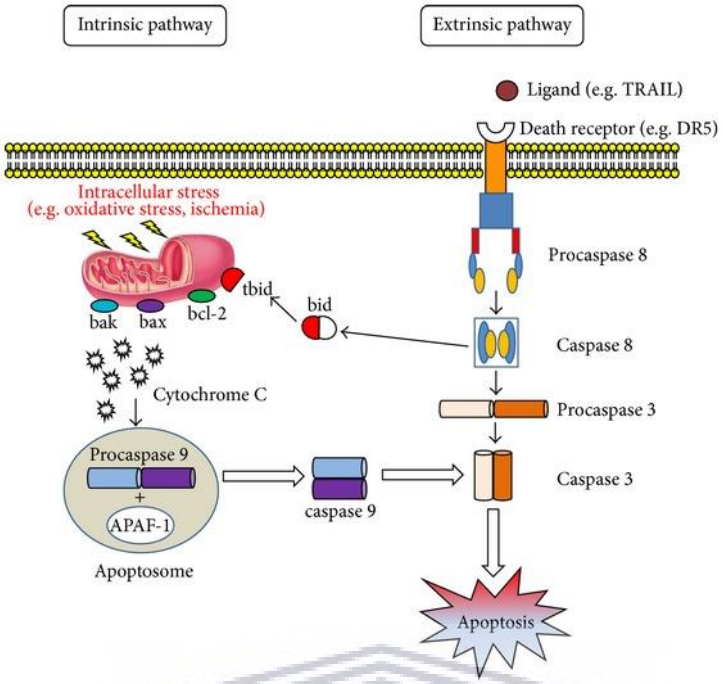



**Figure 2.2.** Illustration of two apoptotic pathways, which are the intrinsic (mitochondrial) and extrinsic (death receptor) pathways, as well as their individual activation mechanisms such as the binding of the ligand (TRAIL) to the death receptor (DR5) in the extrinsic pathway, the binding of the ligand induces the biding of adapter proteins and subsequently enlist caspase 8 into a complex known as the death-inducing signalling complex (DISC) thereby activating it and the release of cytochrome c as a result of various cellular stresses which activates caspase 9 thereby activating caspase 3 which eventually leads to the programmed cell death (PCD) that cells experience. Adapted from (Loreto et al., 2014).



### *2.3.5.1. The Intrinsic pathway*

The intrinsic pathway (also known as the mitochondrial pathway) is controlled by a family of proteins known as the Bcl family and involves the binding of cytoplasmic proteins (BAX and BID) caused by various stress signals to the outer membrane of mitochondria. Bax is the acronym for "Bcl-2 associated X protein" while Bid stands for "BH3 interacting domain death agonist" (Billen et al., 2008, Leibowitz and Yu, 2010). The expressed oncogenes in apoptosis can also be grouped into enhancers (*bcl-x and bax*) and inhibitors (*bcl-xL and bcl-2*) of apoptosis respectively (Gross et al., 1999, Chen et al., 2000, Lev et al., 2003, Kollek et al., 2016). Mediation of the intrinsic pathway is by diverse apoptotic stimuli, which connect at the mitochondria; however, the release of cytochrome c from the mitochondria to the cytoplasm

initiates a caspase cascade (**Figure 2.2**) (Jin and El-Deiry, 2005). This pathway is activated by a range of exogenous and endogenous stimuli, such as DNA damage, ischemia, oxygen deprivation and oxidative stress (Bhattacharyya et al., 2014). The release of cytochrome c from the mitochondria signifies the mitochondrial dysfunction, which leads to the activation of caspase 9 (initiator) and eventually caspase 3 (executor) (**Figure 2.2**) (McIlwain et al., 2015).

#### *2.3.5.2. The Extrinsic pathway*

The extrinsic pathway involves the binding of a death ligand to transmembrane death receptors present on the cell meant to undergo apoptosis. This pathway is also referred to as the death receptor pathway, and is mediated by cell surface death receptors such as the Tumour necrosis factor (TNF) receptor (Jin and El-Deiry, 2005). In this ligand-binding (TRAIL - tumour necrosis factor-related apoptosis-inducing ligand), the death receptor recruits adapter proteins and inactive caspase-8 to form a death-inducing signalling complex (DISC). The formation of this complex activates caspase-8, which also aids in the transition of the extrinsic pathway to the intrinsic pathway in order to intensify the death signal and implement apoptosis; this is achieved by the ability of caspase-8 to cleave Bid, which is responsible for aiding the release of cytochrome c from the mitochondrial intermembrane space (**Figure 2.2**) (Park, 2012, Parrish et al., 2013).

In a cellular model of PD, apoptosis induced by $MPP^+$ toxicity is reported to be associated with characteristic changes such as DNA fragmentation as well as the expression of p53, cleavages of caspase-3 and PARP (poly ADP-ribose polymerase) which is a protein that initiates a response of DNA repair after detecting DNA damage (Erekat, 2018).

### 2.4. The roles of neurotransmitters and neuropeptides involved in PD.

Neurotransmitters are endogenous chemicals responsible for conveying messages between neurons to enable adequate function (Lovinger, 2008). The progressive disruption of

neurotransmitter systems with the presence of intraneuronal inclusions (Lewy bodies), is responsible for the cognitive impairment seen in PD (Barone, 2010). A neurodegenerative disease such as PD is characterized by the significant loss of the neurotransmitter dopamine, in SNpc and the basal ganglia, mainly caused by the degeneration of cholinergic neurons in the extrapyramidal system, which are brain regions associated with cognitive function and motor movement (Alexander, 2004, Dickson, 2012). The cholinergic system is involved in critical physiological processes, such as attention, learning, memory, stress response, wakefulness and sleep, and sensory information (Hampel et al., 2018). A study by Francis and colleagues (1999) reported that in addition to cholinergic dysfunction, other strong correlates of dementia are the chemical and histopathological markers of the excitatory amino acid (EAA)-releasing cortical pyramidal neurons (Francis et al., 1999). The pathogenesis of PD involves several neurotransmitters (dopamine synthesized from L-phenylalanine/L-tyrosine; acetylcholine, from serine; GABA, from glutamate, a by-product of decarboxylation; serotonin, from L-tryptophan and glutamate from glutamine) as well as neuropeptides (cholecystokinin, dynorphin, neurotensin, substance P) (Werner and Coveñas, 2014). An interaction between the neurons of the basal ganglia and those of the substantia nigra via the release of dopamine is vital for the fine-tuning of an organism's movements (Triarhou., 2013) and in PD, decreased dopamine levels result in the disruption of the delicate balance between neurotransmitters and neuropeptides in the brain, specifically the ones within the basal ganglia circuit (Juárez Olguín et al., 2016).

Compromised basal ganglia functions have been associated with PD because the degeneration and depletion of dopamine neurons in the SNpc result in motor and cognitive dysfunctions (Alexander, 2004). Other neurotransmitters may not actively be involved in PD pathogenesis but could be involved in its causation, possibly via their role in PD-associated oxidative stress

(Dias et al., 2013). Dopamine and acetylcholine receptors have a precise subcellular location, ensuring good fine-tuning of synaptic transmission (Vizi et al., 2010).

### 2.4.1. Dopamine

Dopamine, also known as 3,4-Dihydroxytyramine, belongs to a family of catecholamines, owing to its possession of an amine and catechol group (Nagatsu, 2007), and is the most abundant neurotransmitter that exerts its action by binding to the five dopamine receptors found in the brain (Tarazi, 2001, Maramai et al., 2016). Dopamine synthesis by dopaminergic neurons can be broken down into parts (i) from the amino acid L-tyrosine using the tyrosine hydroxylase enzyme that catalyzes the transformation of L-tyrosine to L-DOPA (L-dihydroxyphenylalanine), after which (ii) a carboxylic acid group is removed from the ethyl side chain linked to the amine group thus resulting in dopamine (Nagatsu, 2007, Daubner et al., 2011, Taveira-da-Silva et al., 2019). Its four significant pathways make its distribution from one brain region possible (Molinoff and Axelrod, 1971, Tarazi, 2001). The nigrostriatal pathway is one of the essential pathways that accounts for 70% of the total dopamine in the brain, hence any dysfunction to this pathway or degeneration of its neurons will contribute to movement disorders as seen in PD because most of the neurons in this pathway are projected from the substantia nigra pars compacta (Alexander, 2004, Drui et al., 2014).

Levodopa (approved by the USFDA in 1975) is the most widely used pharmacological treatment for PD and was first discovered after being isolated from a bean seedling of Vicia faba in 1913. About thirty years later, Peter Holtz found that levodopa could be converted to the active form of catecholamine dopamine by the L-dopa decarboxylase enzyme mechanism of action (Blaschko, 1939, Hornykiewicz, 2010). A 1961 study reported that 20 PD patients with marked dopamine depletion in their caudate nucleus, had improved motor symptoms when treated intravenously with the levodopa precursor L-dihydroxyphenylalanine (Hornykiewicz, 2010, Hornykiewicz, 2017, Ovallath and Sulthana, 2017). This study triggered interest in the

clinical use of dopamine and a 1967 study finally paved the way for the successful use of levodopa as a therapeutic agent, administered in gradual, incremental doses (Cotzias and Papavasiliou, 1967, Taylor et al., 2015). The prolonged use of levodopa has, however been shown to have side effects that cause more harm than good (Wang et al., 2017a).

Levodopa is usually prescribed in combination with other drugs such as dopa decarboxylase inhibitors (e.g., carbidopa), to potentiate its efficacy. Apart from being a dopamine metabolic precursor, levodopa can cross the BBB and get converted to dopamine in the brain. Co-administration with carbidopa enhances the effect of levodopa by inhibiting its early metabolism by peripheral decarboxylase enzymes before it reaches the brain to generate dopamine (Seeberger and Hauser, 2009). Dopamine agonists (DA) are a class of drugs used in PD for their ability to mimic the activity of the neurotransmitter dopamine when deficient (Borovac, 2016). This class of drugs attains its role of mimicking dopamine by targeting and binding to the dopamine D2- type receptors with side effects including nausea, fibrosis, hallucinations, somnolence, and peripheral edema (Etminan et al., 2003). Examples of these drugs are pramipexole (mirapex) and rotigone (neupro). The treatment approaches for PD as commonly used in clinical setups and practice are mostly levodopa, dopamine agonists and monoamine oxidase type B (MAO-B) inhibitors as well as catechol-O-methyltransferase (COMT) inhibitors. These drugs can be used as monotherapy or adjunctive therapy, depending on the stage of the disease. MAO-B inhibitors and COMT inhibitors, generally prevent the peripheral oxidation of levodopa (Münchau and Bhatia, 2000).

### 2.4.2. Acetylcholine

Acetylcholine is one of the main neuromodulators responsible for the fine-tuning activity of the basal nuclei and exerts profound effects on behavioral manifestation (Picciotto et al., 2012). Acetylcholine (Ach) influences cellular and synaptic physiology, thus switching network dynamics resulting in behavioral transitions ranging from sleep to wakefulness, distraction to

attention, and learning and recall (Colangelo et al., 2019). The cholinergic neurotransmitter, Ach is synthesized by choline acetyltransferase (ChAT), and its action is terminated by acetylcholinesterase and butyrylcholinesterase (Vijayaraghavan et al., 2013). The human brain contains two significant cholinesterases: Acetylcholinesterase (AChE) encoded by a gene on chromosome 7) and Butyrylcholinesterase (BuChE) encoded by a gene on chromosome 3, with AChE being the more abundant of the two (Mesulam et al., 2002). AChE is an enzyme that is a specific esterase belonging to a family of enzymes known as carboxylesterase, as it mainly hydrolyses ACh. In PD, just like AChE, BuChE is also expressed in the frontal cortex but at lower levels. In contrast, higher AChE and BuChE levels would be observed in patients with Parkinson's disease dementia (PDD) (Nordberg et al., 2013). AChE activity is higher in motor neurons than in sensory neurons.

Historically, the first available class of drugs available for PD were anticholinergics and are still being used as secondary treatments for PD in conjunction with other antiparkinsonian drugs (Brocks, 1999). Anticholinergics are reported to act by counterbalancing the reduced dopamine in predominant tremor cases. In 1867, a student (Léopold Ordenstein) of Jean-Martin Charcot's (the first man to suggest the use of the term "*Parkinson's disease*" instead of "*shaking palsy*"), discovered the antiparkinsonian properties of anticholinergics after administering to some to patients to dry their mouths and stop the excessive drooling and salivation (Lang and Blair, 1989). Trihexyphenidyl, also known as benzhexol and trihex (brand names - Artane, Parkin, Pacitane, Hexymer; approved by FDA in 2003), is an anticholinergic drug used in PD to inhibit the parasympathetic nervous system and directly relieve muscle spasms (Houghton et al., 2008). It has also been reported that Trihexyphenidyl should be used with caution as its side effects include hallucinations, blurred vision, drowsiness, dry mouth, irritability, decreased urination; its long term use has also been reported to contribute to altering neuroimmune responses and inflammation (Funakawa and Jinnai, 2005, Huang et al., 2016b,

Mahal et al., 2018, Zahoor et al., 2018b). Benztropine (brand name – Cogentin; approved by the FDA in 2007) is another anticholinergic drug used in PD and aids in blocking the action of acetylcholine and the uptake of dopamine to prolong dopamine effects, thus alleviating PD symptoms (Dong et al., 2016). Just like Trihexyphenidyl, its clinical use is limited as the side effects seem to outweigh the therapeutic benefits; thus, these drugs are best used as adjunct therapeutics to alleviate the adverse side effects caused by other antiparkinsonian drugs.

### 2.4.3. Glutamate

The most abundant excitatory neurotransmitter in the mammalian CNS is glutamate which is extensively distributed and exclusively located intracellularly (Zhou and Danbolt, 2014). It improves cognitive behaviour and has been beneficial in several neurological disorders, including epilepsy and mental retardation (Fonnum, 1984). This excitotoxin has the ability to destroy CNS neurons by excessive activation of excitatory receptors on dendritic and somal surfaces (Newcomer et al., 2000). With this, glutamate and its receptors facilitate most of the excitatory neurotransmission while playing central roles in neurogenesis, neurodegeneration, synaptic plasticity underlying learning and memory (Danbolt, 2001). This degeneration of the dopaminergic nigrostriatal pathway causes a desensitization of the receptors with ultimate synaptic depression by activating synaptic N-methyl-D-aspartate (NMDA) receptors (ion-channel receptors found at most excitatory synapses, where they respond to the neurotransmitter glutamate) and NR2B receptors which are considered to be responsible for synaptic plasticity impairment (Yao and Zhou, 2017). NMDA receptors present in the striatum are crucial for dopamine-glutamate interactions. However, excessive activation of NMDA receptors causes excitotoxicity and neurodegeneration, which is as a result of the enhanced release of glutamate in the synaptic cleft or a decrease in the removal of glutamate from the synaptic cleft, both of which result in an influx of calcium ($Ca^{2+}$) thus causing cell death by apoptosis or necrosis (Pankratov and Lalo, 2014, Bukke et al., 2020).

Amantadine (brand name- Gocovri; approved by FDA in 2017 or 2003) is an uncompetitive NMDA-receptor antagonist that has been developed and approved to be an adjunctive therapy to levodopa in PD with the aim of treating the dyskinesia seen in affected individuals (Paquette et al., 2012). This drug was initially developed in the early 1960s and approved as an anti-influenza drug in 1966 (Field, 1999). The mechanisms of action of amantadine in PD include enhancement of the partial blockage of NMDA receptors in the presence of excess glutamate. This is achieved by controlling the irregular calcium influx using Magnesium as a means of blocking the excessive calcium release, thus obtaining an optimum environment required for the storage of chemical information (Blanpied et al., 2005, Deutschenbaur et al., 2016).

### 2.4.4. GABA

The gamma-aminobutyric acid (GABA) neurotransmitter system is the central and peripheral nervous system's primary inhibitory system and is mainly generated by the decarboxylation of glutamate (Glu) by glutamic acid decarboxylase (GAD) (Obata, 2013). GABA was first discovered in 1950 by paper chromatography, in human and animal brains, as a derivative of glutamic acid (Awapara et al., 1950, Roberts and Frankel, 1950). The prolonged use of levodopa has, however been shown to have side effects that cause more harm than good (Wang et al., 2017a). The involvement of GABA in regulating calcium-dependent mechanisms emphasizes the role it plays in maintaining the various cellular metabolic functions such as mitochondrial function and oxidative stress, all of which play a vital role in neurodegeneration (Schaffer and Kim, 2018). Abnormal intracellular calcium levels cause a dysfunction in the $Ca^{2+}$/GABA control, thus enhancing various cascading processes that result in elevated calcium levels and the accumulation of Lewy bodies, a pathological mark of PD (Hermes et al., 2010, Błaszczyk, 2016).

Various studies have been carried out to validate the role of GABA in the pathogenesis of PD; a study by De Jong in 1984 observed that in a group of healthy individuals, GABA levels were

significantly low, and the same levels were almost similar to the levels seen in PD-affected individuals who were either receiving levodopa or an anticholinergic drug (De Jong et al., 1984). Another study by Chassain and colleagues (2010) used an $MPP^+$-induced toxicity model in mice and reported increased GABA levels in the striatal cells. The administration of levodopa helped attenuate the increased GABA content (Chassain et al., 2010). A study by Yao also reported that GABA levels in the cortex of rats induced with 6-hydroxyDA (6-OHDA) toxin, was higher compared to the control rats (Huang et al., 2019). The disruption in the functioning of the GABA neurotransmitter is a major factor in all neurodegenerative diseases and the non-motor symptoms observed in the early stages of PD have been linked to GABA deficiency (Murueta-Goyena et al., 2019).

### 2.4.5. Neuropeptide Y (NPY)

Neuropeptides are generally protein-like molecules produced and released by neurons of the central and peripheral nervous systems and act on neural substrates (Belzung et al., 2006, Peng et al., 2017). In 1982, Tatemoto and colleagues isolated and sequenced the neuropeptide Y from a porcine brain (Tatemoto et al., 1982). Neuropeptide Y (NPY) belongs to the pancreatic polypeptide family and is a 36 amino acid peptide, that derives its name from the single-letter code (Y) for the amino acid tyrosine owing to the numerous tyrosine residues present (Larhammar et al., 1993, Beck, 2006). NPY is one of the most conserved peptides expressed by various neuronal systems in regions spanning from the medullary brainstem to the cerebral cortex, and is also one of the most abundant neuropeptides in the brain, known to contribute to the regulation of essential physiological functions (Holzer et al., 2012). One study with animal models showed that the compromised nigrostriatal dopamine pathway caused a significant increase of the NPY-expressing cells in the striatum (Kerkerian et al., 1986) and the study by Cannizzaro and colleagues (2003), also reported high levels of NPY in PD patients compared to healthy individuals (Cannizzaro et al., 2003). In another study, increased levels of NPY

caused an inhibition in the release of glutamate, thus offering protection to the dopamine neurons by preventing the excitotoxicity caused by excessive levels of glutamate (Goto et al., 2013). The fundamental role of this neuropeptide in the pathology of PD is yet to be fully understood; however, studies still support the idea that increasing levels of NPY can offer neuroprotection to dopaminergic neurons (Li et al., 2019, Zheng et al., 2021). Increased NPY levels have been reported to (i) act as a paracrine and autocrine immune mediator (Farzi et al., 2015) and (ii) regulate calcium levels, modulate neurogenesis, and attenuate neuroinflammation (Duarte-Neves et al., 2016, Li et al., 2019).

### 2.5 Genes involved in PD

Multiple risk factors have been linked to the pathology of PD, one of which is genetic predisposition, as various monogenic forms and genetic risk factors have been identified (Singleton et al., 2013). Identifying the genes that are mostly compromised in PD has played a significant role in understanding the underlying pathogenesis of the disease. Such genes as *PARK2* (parkin), *DJ-1*, *SNCA (encoding α-synuclein protein), LRRK2 (Leucine-rich repeat kinase 2),* and *PINK1 (PTEN-induced kinase protein 1)* have been considered therapeutic targets in most studies (Nuytemans et al., 2010).



Understanding of the role that mutations on these genes play in the compromised dopaminergic system that leads to PD is important (Nuytemans et al., 2010, Yonova-Doing et al., 2012).

#### 2.5.1. α-Synuclein (SNCA)

The genetic link of the pre-synaptic neuronal protein SNCA to the pathogenesis of PD, dates back to 1997 when antibodies were developed against the protein, which recurred in PD investigations as the core component of the disease (Spillantini et al., 1997, Stefanis, 2012a). SNCA is a small protein with 140 amino acid residues and a 117 kb span, located on chromosome 4q21. It has six exons initially reported in an Italian American family linked with

familial PD, and the pattern observed in the inheritance was confirmed to be autosomal-dominant (Vaughan, 2001). Mutations of the α-synuclein protein are predominantly associated with the formation of aggregates that are critical to the formation of Lewy bodies and Lewy neurites, known to play a pivotal role in the neuropathological diagnosis of sporadic PD (Xu and Pu, 2016, Shimozawa et al., 2017, Mahul-Mellier et al., 2020).

### 2.5.2. Leucine-rich repeat kinase 2 (LRRK2)

Another autosomal dominant gene involved in PD is the large gene *LRRK2* associated with *PARK8*. Its mutations happen to be the principal and common cause of familial and sporadic PD despite the central role of α-synuclein in PD (Kumar and Cookson, 2011). This gene encodes a protein of 2527 amino acids with a 144 kb span located on chromosome 12p12 with 51 exons (Selvaraj and Piramanayagam, 2019). *LRRK2* involves two enzymes: GTPases and kinases, that aid in influencing its role in signalling. Although initially discovered in a Japanese family of the Sagamihara kindred, the variants of this gene have been identified in European families and other Asian populations. This plethora enhances its importance as a monogenic cause of PD in various people (Corti et al., 2011). The clinical features associated with PARK8 parkinsonism have been described as a broad spectrum of pathological phenotypes. The patients who identify with this gene are those in the late-onset stage of PD (Li et al., 2014). In the pathogenesis of PD, α-synuclein and *LRRK2* are reported to act synergistically, and the disruption in the normal functioning of *LRRK2* enhances the accumulation and pathology of the α-synuclein thus altering cellular functions and causing neuronal death (Liu et al., 2012, O'Hara et al., 2020, Rivero-Ríos et al., 2020).

### 2.5.3. Parkin (PARK2)

Causative genes involved in the autosomal recessive form of PD include parkin, a ubiquitin E3 ligase, which was initially discovered in Japanese samples with juvenile parkinsonism, thus accounting for most of the cases reported for this rare form of PD that presents before 21 (Klein

and Westenberger, 2012). The parkin gene encodes a protein of 465 amino acids with a 144 kb span and is located on chromosome 6q25-q27 with 12 exons (Selvaraj and Piramanayagam, 2019). Mutations in this gene contribute to sporadic cases (early-onset before 45). The normal function of *parkin* involves the clearance of dysfunctional mitochondria through autophagy or mitophagy; hence, when inactivated and mutated, its normal function is inhibited, leading to disruption in protein degradation and mitochondria maintenance (Chai and Lim, 2013). Many pathogenic factors trigger the inactivation of *parkin*, including oxidative, nitrosative and dopaminergic stress, which are major compromising factors in the sporadic form of PD which eventually lead to the toxic accumulation of abnormal proteins and mitochondrial dysfunction (Tsang and Chung, 2009, Chai and Lim, 2013, Ge et al., 2020). Toxicity of α-synuclein has been reported to be reduced by the overexpression of *parkin*, in the cellular, drosophila and rat models of PD (Petrucelli et al., 2002, Bonifati et al., 2005, Khandelwal et al., 2010).

### 2.5.4. (PTEN)-induced kinase 1 (PINK1)

The *PINK1* gene was identified in 2001 within a large Italian pedigree after a homozygous missense, and nonsense mutation and was adjudged to be responsible for PARK6-associated autosomal recessive PD (Lohmann et al., 2003, Bonifati et al., 2005). This gene was the second gene discovered for autosomal recessive PD after *parkin*, and it encodes 581 amino acid proteins, located on chromosome 1p35-p36 with eight exons (Pickrell and Youle, 2015). Encoding a serine/threonine-protein kinase localized to the mitochondria is the *PINK1* which in its normal function, aids in protecting mitochondrial homeostasis, thereby offering neuroprotection (Wang et al., 2011). Like the parkin gene, mutations in *PINK1* result in increased calcium and ROS levels due to altered mitochondrial function, ultimately inducing neuronal cell death (Matsuda et al., 2013). A decrease in the membrane potential of the mitochondria causes an accumulation of *PINK1* in the outer mitochondrial membrane, which then recruits *parkin*, whose ubiquitin-proteasome system enhances the activity of mitophagy

and eventually causes mitochondrial degradation (Ashrafi and Schwarz, 2013, Quinn et al., 2020).

### 2.5.5. DJ-1

The first link of the DJ-1 gene to PD was in 2003 in a Dutch and Italian family by deletion and homozygous point mutations (Bonifati et al., 2003, Repici and Giorgini, 2019). This small gene codes for 189 amino acids with seven exons, and its mutation accounts for 1-2% of the autosomal recessive early-onset PD (Hernandez et al., 2016). Being a multifunctional protein, the most vital role is protecting the brain from oxidative stress (Klein and Westenberger, 2012). In PD-affected persons, the levels of DJ-1 in the cerebrospinal fluid are high; thus, it has been a helpful biomarker in neurodegenerative disease. It is present mainly in the early stages of the diseases (Pankratz et al., 2006). Studies have reported and confirmed that similar to *parkin*, the overexpression of the *DJ-1* gene can inhibit the aggregation of α-synuclein but the not the oligomerization of fibril formation in animal and cellular models PD (Batelli et al., 2008, Madison, 2017, Dolgacheva et al., 2019, Chia et al., 2020).

**Table 1.** Summary of PD main risk genes, their chromosomal location, and deterministic factors

| Locus | Gene | Chromosome Location | Mode of Inheritance | Disease onset | Distinctive clinical features | Possible role in PD | Type of Mutation |
|---|---|---|---|---|---|---|---|
| *PARK1* | *SNCA* | 4q21 | Autosomal dominant; rarely sporadic | Early-onset | -Rapid progression<br>-Less tremor | Aggregates into clumps called LB | -Missense<br>-Point |
| *PARK8* | *LRRK2* | 12q12 | Autosomal dominant; sporadic | Late-onset | -Typical middle to late onset parkinsonism | It is implicated in signalling pathways, including apoptosis and regulation of the cytoskeleton. | -Missense |
| *PARK2* | *parkin* | 6q25-q27 | Autosomal recessive; sporadic | Early onset | -Slow progression<br>-Dystonia<br>-Dyskinesia | Mutated form inhibits the clearance of degraded protein, causing the accumulation. | -Missense<br>-Nonsense<br>-Frameshift<br>-Point |
| *PARK6* | *PINK1* | 1p35-p36 | Autosomal recessive | Early-onset | -Typical early onset Parkinsonism<br>-L-dopa associated dyskinesis | Loss of PINK1 function may result in toxicity and cell death | -Missense<br>-Nonsense<br>-Frameshift<br>-Point |
| *PARK7* | *DJ-1* | 1p36 | Autosomal recessive | Early-onset | -Psychiatric symptoms with early-onset | Mutated form disrupts protection from | -Missense<br>-Nonsense<br>-Point |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | | | | -Slow progression<br>-Focal dystonia | oxidative stress and leads to death | -Exon deletion |

*Adapted and modified from: (Tan and Jankovic, 2006, Saiki et al., 2012, Kim, 2013)*

## 2.6. Epigenetics of PD

In 1942, Conrad Waddington first used the term 'epigenetics' to describe the processes in which genotype gives rise to phenotype (Jobe and Zhao, 2017). The word "epigenetics" was first used to describe interactions involved in differentiation and development in higher organisms (Handy et al., 2011). Epigenetic changes involve heritable gene expression without coding sequence modifications (Gibney and Nolan, 2010) and information can be transferred from one generation to the next at the cellular or whole organism levels (Halušková, 2010). Epigenetic changes involve DNA methylation, modification of histones, and non-coding RNA (Nowacka-Zawisza and Wiśnik, 2017). In a study by Moosavi and Ardekani (2016), epigenetic mechanisms were said to be involved in the regulation of the biological process from conception to death, with a significant role in brain development and disease owing to factors such as disordered chromatin organization in both early childhood and adult neurodegenerative disorders (Moosavi and Ardekani, 2016). Therefore, the rapid increment in of chromatin-modifying drugs with incredible therapeutic potential for the nervous system degenerative diseases led to increased interest in the field of epigenetics in the nervous system (Moosavi and Ardekani, 2016).

Fundamental cellular processes in the nervous system, such as synaptic plasticity and complex behaviors like learning and memory, make the epigenetic code very critical in the nervous system (Gräff and Mansuy, 2008). Initially, epigenetics and its role in diseases was only associated and discussed in oncology but this has been extended to neurodevelopment and neurodegenerative diseases (Zoghbi and Beaudet, 2016). As the biological mechanisms of PD remain inconclusive, epigenetics has been an increasing area of interest in biomedical research,

which leading to the discoveries of new histone variants and modifications. Some environmental factors such as hazardous exposures, diet, and life events can impact gene expression, which implies that epigenetics can act as an intersection for the pathophysiology and risk factors of PD (Qiao et al., 2015). So far, the SNCA gene happens to be the most studied gene of potential epigenetic alterations in PD (Miranda-Morales et al., 2017)

Apart from co-locating with neuronal loss sites, misfolded α-synuclein comprises Lewy bodies (LB) which are considered the pathologic hallmark of PD (Stefanis, 2012b). Regardless of the primary toxicity exhibited by the α-synuclein in neuronal nuclei, the α-synuclein has also been reported to contribute to the organizational distress and distribution of DNA histone epigenetic modifications (**Figure 2.3**) (Labbé et al., 2016). In a study by Jowaed *et al.*, the SNCA promoter was found to be methylated leading to regulated gene expression in cultured cells, and hypomethylated in the PD brains when compared to normal tissue. The observed reduced methylation caused increased expression of the SNCA gene (**Figure 2.3**) (Jowaed et al., 2010). This study was in line with other studies that have reported that increased expression of the SNCA contributes significantly to the risk of sporadic PD (Maraganore et al., 2006, Mizuta et al., 2006, Tagliafierro and Chiba-Falek, 2016, Campêlo and Silva, 2017).

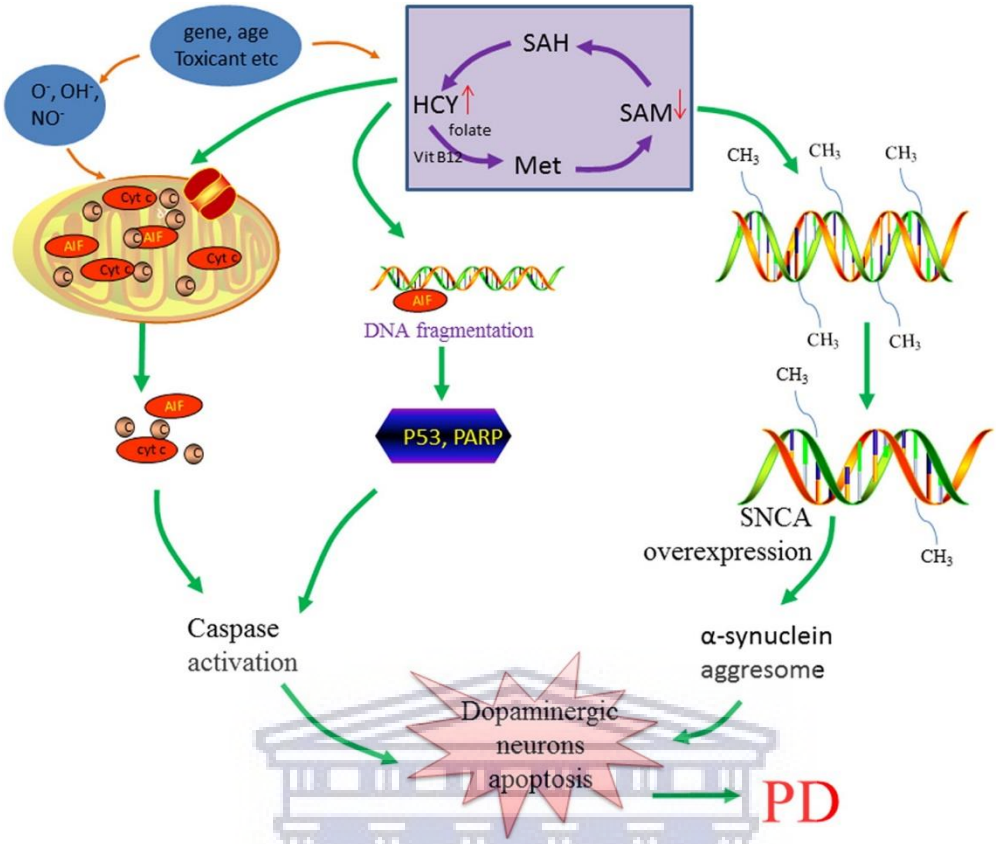



**Figure 2.3.** The mitochondrial function and integrity of the cells are compromised by the increased levels of homocysteine which triggers the release of cytochrome c and apoptosis-inducing factor (AIF), resulting in the activation of caspases, thus causing dopaminergic neuronal cell death. Oxidative stress plays a significant role in elevated levels of homocysteine which causes DNA fragmentation, enhancing p53 induction and PARP activation, which eventually activate caspases to implement dopaminergic neuronal cell death. Impairment in the regulatory function of DNA methylation, which a deficiency in folates can trigger, contributes to the alpha-synuclein (SNCA) overexpression, thus forming aggresomes (similar to Lewy Bodies) and eventually causing PD. Adapted from (Feng et al., 2015).



## 2.7. Neurotoxins used in PD models

### 2.7.1. 6-Hydroxy-Dopamine (6-OHDA)

The first dopaminergic neurotoxin to be discovered was the 6-OHDA (chemical structure shown in **Figure 2.4**). It was developed for use as a PD model by Ungerstedt, who administered it into the substantia nigra of rats in 1968 to investigate and exploit its neurotoxicity (Ungerstedt, 1968, Hernandez-Baltazar et al., 2017). The cytotoxic effects of 6-OHDA have been reported to induce degeneration in noradrenergic and dopaminergic neurons (Wąsik et al., 2018). This catecholamine neurotoxin not only accumulates in the cytosol, but also uses the

same transport system as dopamine (González-Hernández et al., 2004, Meiser et al., 2013a).Despite the inability of 6-OHDA to cross the BBB, once it is delivered in the body, it possesses high selectivity for dopaminergic cells, thereby disrupting the nigrostriatal system due to excess neurotoxicity (Carvey et al., 2005). The toxicity of 6-OHDA is similar to that of $H_2O_2$, and the mechanism of action in causing neurodegeneration after it inhibits complex I and IV activity involves glutathione depletion, lipid peroxidation, oxidative stress, protein oxidation, and mitochondrial dysfunction, all of which generate a reduction in the membrane potential of dopaminergic neurons (Kulich et al., 2007, Simola et al., 2007, Massari et al., 2016, Puspita et al., 2017).

### 2.7.2. Rotenone

Rotenone (chemical structure shown in **Figure 2.4**) is one of the most potent members of the rotenoid family and is a naturally-occurring complex ketone pesticide (Caboni et al., 2004). It is highly lipophilic, readily crosses biological membranes and the BBB without the use of transporters and is a commonly used toxin in cell culture and animal models of PD that inhibit complex I activity (Duty and Jenner, 2011, Keane et al., 2011). Other than its action on the mitochondrial complex I, this toxin has been reported to impair the synthesis of microtubules from tubulin, resulting in excess tubulin monomers that are vital in the pathology of neurodegeneration (Cabeza-Arvelaiz and Schiestl, 2012). The action of rotenone on mitochondria is achieved by its ability to inhibit the activity of the NADH-ubiquinone reductase, thus impairing oxidative phosphorylation (Heinz et al., 2017). Other studies have shown that rotenone caused proteasome dysfunction, aggregation of α-synuclein, and oxidatively modified the DJ-1 gene (Betarbet et al., 2006).

### 2.7.3. 1-Methyl-4-Phenyl-1,2,3,6-Tetrahydropyridine (MPTP)

The simple compound 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) was first identified as a potential PD-causing toxin in the early 1980s when heroin addicts in California

accidentally administered it to themselves, and developed an acute form of parkinsonism, with symptoms that were indistinguishable from sporadic PD (Langston et al., 1983, Hisahara and Shimohama, 2011). MPTP is a non-toxic by-product in the synthesis of the narcotic, meperidine analog 1-methyl-4-phenyl-4-propionoxypiperidine (MPPP) (Ziering and Lee, 1947), and has lipophilic properties that allow it to cross the blood-brain barrier (BBB), to become toxic after undergoing biotransformation inside brain astrocytes, through catalysis by the enzyme monoamine oxidase B (MAO B) (Kopin, 1987, Sian et al., 1999, Reith, 2016). The mediated biotransformation by MAO B involves the conversion of MPTP to $MPDP^{+,}$ which is then oxidized into its toxic form 1-methyl-4-phenylpyridinium ($MPP^+$) (chemical structure shown in **Figure 2.4**) (Fowler et al., 2002). When dopaminergic neurons absorb the toxic $MPP^+$ via the dopamine transporter, inhibition of the complex I enzyme of the mitochondrial electron transport chain (ETC) occurs, subsequently leading to the disruption of the flow of electrons and a decrease in ATP levels and oxidative stress (Martinez and Greenamyre, 2012, Subramaniam and Chesselet, 2013).

Various studies have been reported to support the role that plants have played in ameliorating $MPP^+$ induced toxicity. A study by Liu et al. reported that osthole, a compound extracted from the plant-derived medicine *Cnidium monnieri* was used on PC12 cells. It showed neuroprotective potential against cytotoxicity induced by $MPP^+$ by inhibiting ROS production and death signalling proteins (Liu et al., 2010). Ojha et al. showed that curcuminoids, active polyphenols of the rhizome *Curcuma longa*, exerted neuroprotective potential through its anti-inflammatory action against $MPP^+$ induced toxicity in an *in vivo* PD model (Ojha et al., 2012). Another study by Wang et al. studied the impact of a popular primary plant flavonoid known as *Pinocembrin*, isolated from various plants. Their study reported that pinocembrin ameliorated the $MPP^+$ induced neurotoxicity in SH-SY5Y cells by inhibiting the expression of death signalling proteins (Wang et al., 2014). Paeoniflorinin isolated from the Chinese

herb *Radix Paeoniae alba* was used in an *in vivo* PD model with $MPP^+$ induced neurotoxicity and was able to attenuate the effects of the toxin on the dopaminergic cells by enhancing the expression of tyrosine hydroxylase, inhibiting apoptotic pathways and enhancing the dopamine transporter protein expression thus being reported as a promising neuroprotective agent (Zheng et al., 2017). *Carpobrotus edulis* was used in a study conducted by Enogieru and colleagues in which their findings reported that the extract was able to offer neuroprotection to SH-SY5Y cells following exposure to toxin $MPP^+$ by decreasing toxin-induced oxidative stress and inhibiting the death signalling proteins (Enogieru et al., 2018a).

In a study carried out by Omoruyi and colleagues, an extract from the Amaryllidaceae family, *Crossyne guttata,* showed neuroprotective capabilities against toxicity induced by $MPP^+$ in a neuroblastoma cell line (Omoruyi et al., 2019). *Holothuria scabra* was also reported to have neuroprotective and neurorestorative potential against $MPP^+$ induced damage in a mouse model by inhibiting death signalling proteins and enhancing the expression of tyrosine hydroxylase, thus reported to exert neuroprotective capabilities in a cellular model of PD using $MPP^+$ (Noonong et al., 2020). In another study, Enogieru et al. investigated the aqueous leaf extract of *Sutherlandia frutescens* against $MPP^+$ neurotoxicity in SH-SY5Y cells. They reported that the extract was able to attenuate the toxic effects of the neurotoxin via inhibiting the apoptotic pathway and inhibiting the depolarization of mitochondrial membrane potential, thus being categorized as a potential neuroprotective agent (Enogieru et al., 2020). Two plant extracts from the Amaryllidaceae family, *Nerine humilis* and *Clivia miniata,* were investigated as neuroprotective agents by Omoruyi et al., and the study reported that the extracts exerted neuroprotective potential as they were able to ameliorate the toxic effects of the toxin by enhancing the decreased ATP levels and inhibiting apoptosis (Omoruyi et al., 2020a). Furthermore, another study reported the neuroprotective potential of *Boophone haemanthoides* extract and its bioactive compounds against $MPP^+$ induced toxicity in an *in vitro* PD model

using SH-SY5Y cells as the extract and its bioactive compounds decreased toxin-induced oxidative stress and inhibited death signalling proteins (Ibrakaw et al., 2020).

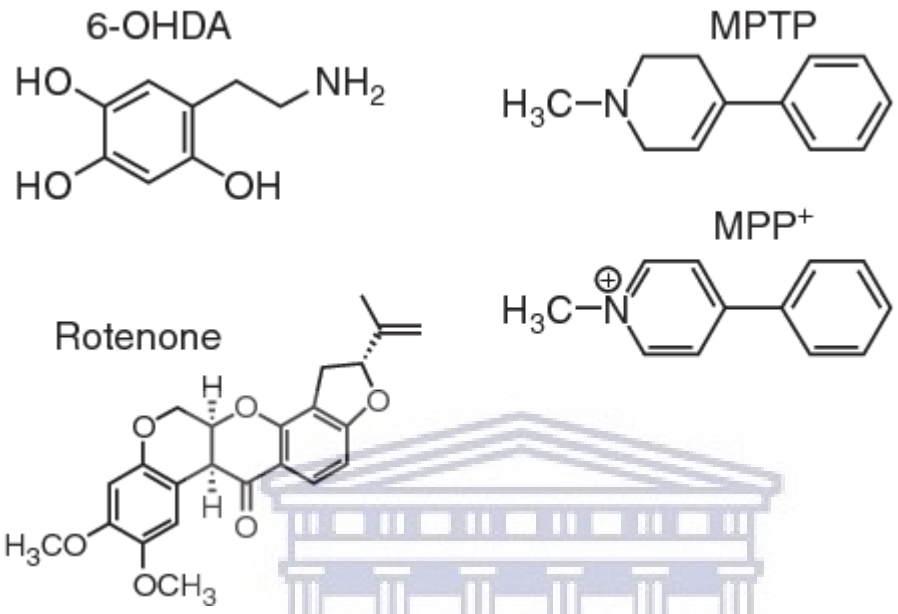



**Figure 2.4**. Chemical structures of the PD neurotoxins 6-OHDA, Rotenone and $MPP^+$ (Tieu, 2011).



## 2.8. Medicinal plants and bioactive compounds used for PD

As there is no cure for PD, the drugs currently used for symptomatic relief of the symptoms of PD are known to have adverse side effects, highlighting the need to explore safe alternatives. Ethnobotanical evidence suggests that traditional and complementary medical practices involving the use of different herbal medicines may be useful therapeutic strategies for PD (Poewe et al., 2010). Investigations of the pharmaceutical properties of medicinal plants have increased over the years as plants are considered to be significant sources of therapeutic bioactive compounds (Pham-Huy et al., 2008b). The African traditional healthcare system is one of the oldest therapeutic methods that is fundamentally preventive, protective, nutritive, and curative in nature and usually considered to be safe and harmless because patients experience fewer or no side effects (Mahomoodally, 2013).

Over the past few years, more scientific literature has emerged focusing on herbal extracts and their phytochemicals, also referred to as secondary metabolites (Kennedy and Wightman, 2011). Since minimal quantities in terms of concentration dosage of plant material are required when screening for bioactivity during various drug development strategies, using plants as a source of medicine is enthusiastic for the pharmaceutical industry (Bodeker et al., 1997). Different medicinal plants and natural compounds have been considered helpful in alleviating neurodegenerative diseases in addition to relieving neurological symptoms, as reported *in vivo* studies or clinical trials, including *Baccopa monnieri*, *Ginkgo biloba*, *Withania somnifera (ashwagandha)*, curcumin, and resveratrol (Ratheesh et al., 2017, da Costa et al., 2020, Luthra and Roy, 2021).

The *Ginkgo biloba* extract has been reported to reduce neuronal loss by preventing apoptosis, reducing excitotoxicity possibly as a result of an excessive influx of calcium (Schwarzkopf et al., 2013, Tanaka et al., 2013, Nash and Shah, 2015, Huang et al., 2016a, Percário et al., 2020). The extract has also been reported to mediate neuroprotection in PD models by regulating iron homeostasis, inhibiting the activity of the monoamine oxidase enzyme, and alleviating oxidative stress, which is a vital cause of PD (Ahlemeyer and Krieglstein, 2003, Ao et al., 2006, Abd El-Aziz, 2012, Omar, 2013, Wang et al., 2017b, Chen et al., 2019, Singh et al., 2019b, Percário et al., 2020).

Furthermore, the extract of *Withania somnifera* has also been reported to offer neuroprotection for PD and other neurodegenerative diseases in both *in vitro* and *in vivo* models by attenuating the accumulation of toxic free radicals, which results from the dysfunction of the free-radical scavenging enzymes namely, superoxide dismutase, catalase, glutathione peroxidase (Bhattacharya et al., 1997, Ahmad et al., 2005, Kumar and Kumar, 2009, Surendran and Raja Sankar, 2010, Prakash et al., 2013, Manjunath, 2015). Also, extracts of *Bacopa monnieri* have also been used in *in vitro* and *in vivo* models of various neurodegenerative diseases, and have

been shown to improvement cognitive functions in PD and AD by reversing the effect of toxin-induced injuries and ameliorating the motor symptoms (Simpson et al., 2015, Mathur et al., 2016). Its mechanism of action involves inhibiting apoptotic pathways of dopaminergic neurons, enhance complex I activity, decreasing the levels of tyrosine hydroxylase (TH) immunoreactivity, increase dopamine levels, enhancing antioxidative enzyme (SOD) activity, enhances learning and memory, increase dopamine levels, and slow progression of PD (Bhattacharya et al., 2000, Jyoti and Sharma, 2006, Viji et al., 2010, Charles et al., 2011, Meena et al., 2012, Simpson et al., 2015, Singh et al., 2017a, Singh et al., 2021).

Plant-derived bioactive compounds used as nutraceuticals or herbal supplements have also been studied for their potential neuroprotective and therapeutic effects against PD. Curcumin is a polyphenol extracted from the rhizomes of turmeric, an ancient Indian herb used in curry powder. It has been studied widely for its many beneficial effects in different diseases, including its anti-inflammatory, antioxidant, pro-neurogenesis, microgliogenesis-inhibiting, iron-chelating, anti-plaque formation, anticancer and neuroprotection properties (Darvesh et al., 2012, Du et al., 2012, Lee et al., 2013, Cui et al., 2016, Jha et al., 2016). Curcumin has significantly contributed to the novel studies of PD and other neurodegenerative disorders owing to its ability to cross the BBB. It is lipophilic in nature, crosses the blood brain barrier and binds to plaques (Shabbir et al., 2021) and has been reported to attenuate α-synuclein, induced oxidative stress, inhibit fibril formation and formation of α-synuclein oligomers in PD models (Du et al., 2012, Singh et al., 2013, Cui et al., 2016, Jha et al., 2016, Singh et al., 2017b, Wang et al., 2017b). However, its efficacy as a therapeutic agent is hindered by its hydrophobic nature, low bioavailability, poor stability, and chronic consumption, often leading to hepatoxicity (Wang et al., 2017b, Maiti and Dunbar, 2018, Yavarpour-Bali et al., 2019).

Resveratrol, a polyphenolic compound found in several plants, has been reported to have various medicinal properties, including anticancer, anti-inflammatory, anti-oxidation, energy

homeostasis regulation, downregulation of iNOS expression, and promotion of eNOS (Xia et al., 2019). Resveratrol has also been reported to increase ATP production, decrease lactate content, enhance complex I activity, promote mitochondrial oxidative function to decrease oxidative stress (Hussein, 2011, Liu et al., 2017, Arbo et al., 2020, Annaji et al., 2021). Resveratrol also acts synergistically with levodopa at low doses to attenuate toxin-induced injury in PD models, thereby offering neuroprotection (Tsai et al., 2007, Jin et al., 2008, Huang et al., 2011, Magalingam et al., 2015, Liu et al., 2019, Xia et al., 2019). Similar to curcumin, the limitations of resveratrol as a therapeutic agent are attributed to its poor solubility, short half-life, instability, low bioavailability, and unfavorable degradation (Walle et al., 2004, Tellone et al., 2015, Shindikar et al., 2016, Shaito et al., 2020).

### 2.9. South African medicinal plants with anti-Parkinson's activity

South Africa is home to over one thousand plant species owing to its diverse plant flora, and it is estimated that about 80% of the South African population uses plant-based medicines as the initial treatment option for most disease conditions (Street and Prinsloo, 2013, Semenya and Potgieter, 2014, Mothibe and Sibanda, 2019). With increasing focus on natural products in scientific research, South Africa's large-scale indigenous floral species provides more opportunities to discover novel bioactive chemicals (Louw et al., 2002, Al-Bader et al., 2009). Plant extracts and their metabolites play a significant role in the production of cosmetics, nutraceuticals, dietary supplements and pharmaceutical products which could be used to manage neurodegenerative diseases as evidenced by *in vitro* and *in vivo*, studies as well as clinical trials (Pohl and Kong Thoo Lin, 2018). Such proteins as α-synuclein, PINK1 and parkin are known to play an essential role in the pathogenesis of PD, hence phytotherapeutic agents are currently being developed to attenuate the dysfunction caused by these proteins (George et al., 2009, Shahpiri et al., 2016, Shan et al., 2018, Rabiei et al., 2019).

### 2.9.1. Amaryllidaceae Family

Amaryllidaceae is a plant family that is prevalent in the southern African region, harbouring at least 250 of the approximately 850 species known worldwide (Meerow and Snijman, 1998, Nair and van Staden, 2013b). The majority of the family members are distributed through the Capensis floral kingdom of the Western Cape and the species in this family are divided among the 75 genera and have been reported to be a good source of alkaloids that possess anticancer, neuroprotection, antibacterial, antiviral, antimalarial, and antioxidant properties (Nair et al., 2013, Nair and van Staden, 2013b, Nair and Van Staden, 2014, Cimmino et al., 2017). In this Amaryllidaceae family are the *Strumaria, Boophone,* and *Brunsvigia* genera which are the plants used in the current research project.

Various Amaryllidaceae plants and their alkaloids have been investigated in *in vitro* cytotoxicity studies and the most commonly used alkaloids from this family of plants are lycorine, galantamine, narciclassine, and tazettine (Koutová et al., 2020). The reported neuroprotective properties of this plant family have enhanced their exploration for specific pharmacological benefits in PD and some studies have reported good inhibiting potential of some of these plant extracts on cell viability at varying concentrations using different cell lines (Adewusi et al., 2012, Adewusi et al., 2013, Lepule, 2017, Lepule et al., 2019, Ibrakaw et al., 2020, Omoruyi et al., 2020a, Omoruyi et al., 2020b). Other studies have reported the that the Amaryllidaceae alkaloids showed promising potential in inhibiting the viability of some cancer cells (Griffin et al., 2007, Evidente and Kornienko, 2009, Doskočil et al., 2015, Reis et al., 2019, Breiterová et al., 2020, Nair and Van Staden, 2021).

#### ***2.9.1.1.*** *Strumaria truncata*

*Strumaria* (commonly known as Cape snowflake) is second only to *Cyrtanthus* in terms of biological species' rarity amongst the Southern African Amaryllidaceae. It is reasonably diverse group of small, bulbous plants that is endemic to southern Africa, extending from

south-western Namibia throughout the winter rainfall zones of South Africa's Northern and Western Cape, to the central summer rainfall zones in eastern Free State and western Lesotho (Duncan, 2010). However, high species concentrations are found in the uplands of Namaqualand near Springbok and on the Bokkeveld Escarpment near Nieuwoudtville (Snijman, 2013). This genus has a species called *Strumaria truncata* (commonly known as the Namaqualand snowflake), a charming bulbous plant with a curious aerial sheath and occurs in the western parts of the Northern Cape, usually on south-facing slopes of rocky outcrops, sandy or clay soil (Snijman, 2008). Its funnel-shaped flowers are typically bright white and vary in shades of pink, depending on the wild locality with conspicuously protruding, long stamens (**Figure 2.5A**) (Duncan, 2000). The *Strumaria truncata* plant has little or no reported studies except the preliminary screenings of the family; neither has there been any traditional uses to report specific beneficial properties further.

**2.9.1.2.** *Brunsvigia bosmaniae*

This geophyte with rounded candelabra-like pink flower heads (**Figure 2.5B***)* belongs to the genus *Brunsvigia* which has about 20 species, has huge bulbs with tunics that are tan-coloured and brittle (Dee Snijman, 2005). Despite their ability to grow in various habitats, *Brunsvigia* plants thrive best during the summer rainfall period (Du Plessis and Duncan, 1989). *Brunsvigia* plants are extensively distributed in the Drankensburg, KwaZulu-Natal province of South Africa (Dee Snijman, 2005) and *Brunsvigia bosmaniae* occurs on rocky outcrops, coastal sand, loamy, and clay soils and grows mainly in winter rainfall regions. Grown in locations such as the Western Karoo, Tygerberg, and Namaqualand, the plant flowering heads are reportedly strongly scented at night (Goldblatt and Manning, 2000). The traditional uses are mainly centered around local diviners to enhance the accuracy of their readings. The San people initially used the bulbs to influence and affect the behavior and mind of the person to which they are administered (Rust, 2009). Similar to the *Strumaria truncata*, there have been little or

no studies on this plant except the preliminary screenings of the family that have been reported to show various beneficial properties.

***2.9.1.3.*** *Boophone disticha*

The Boophone genus has two species, namely the *Boophone disticha* and *Boophone haemanthoides*. *Boophone* originates from the Greek *bous* meaning ox, and *phone* meaning death, thus referring to the poisonous properties of the bulb of this plant (Lithudzha and Reynolds, 2005). The specific name *disticha* means leaves erect in a fan shape. *Boophone disticha* is extensively distributed in all provinces of South Africa as well as tropical Africa. It has large, round heads, which are sometimes on such short stems, making them appear to grow directly from the bulb, almost at ground level (**Figure 2.5C**). It occurs in dry grassland and on rocky slopes, mainly in summer rainfall regions, and flowers between July and October. The Khoi/San people initially used this plant as a narcotic as well as an arrow poison but the everyday use of this plant amongst the Sotho, Xhosa, and Zulu people is for narcotic purposes such as inducing stupor in newly circumcised initiates, for sedation of psychotic patients, and to produce hallucinatory effects during rituals (divination) (De Smet, 1996, Cheesman et al., 2012). Traditionally the bulbs and roots of this plant have been extensively as an outer dressing in circumcision, for rashes, burns, and skin conditions such as eczema; for pain alleviation and pus drawer in septic wounds and boils, and as a reliever in arthritic swelling, sprains and strains (Neergaard et al., 2009, Cheesman et al., 2012, Tonisi et al., 2020) (Du Plooy *et al.,* 2001; Botha *et al.,* 2005). Other traditional uses include calming and sedating psychotic patients and being ingested by conventional healers for divinatory purposes to induce hallucinations (Sobiecki, 2002).

The biological activities of *Boophone disticha* have been validated by previous studies in which the methanol extract was shown to possess antioxidant properties that may be advantageous when treating neurological disorders (Adewusi et al., 2012). A recent study reported the

neuroprotective activity of *Boophone disticha* in an *in vitro* PD model induced by the neurotoxin 6-hydroxydopamine (6-OHDA), with an increase in the cell density of SH-SY5Y reported (Lepule et al., 2019). Isolated alkaloids from *Boophone disticha* such as buphanamine, distichamine, and buphanidrine have also been reported to possess neuroprotective properties and can be utilized as a remedy for anxiety and depression due to their serotonin transporter mimicking effects (Sandager et al., 2005, Cheesman et al., 2012, Adewusi et al., 2013, Masi et al., 2018, Pote et al., 2018, Ibrakaw et al., 2020).


**Figure 2.5.** A representation of (A) Strumaria truncata (B) Brunsvigia bosmaniae (C) Boophone disticha (PlantZAfrica, SANBI)



With the background that has been highlighted in this chapter and more, therapeutic interventions for PD remain a severe need to aid in the management of disease as the actual mechanisms of the disease pathophysiology are still being studied and understood. Consequently, this present study investigates the neuroprotection activity of extracts from the Amaryllidaceae family, whose alkaloids have been reported to possess various therapeutic properties and have motivated the need to explore extracts belonging to this family. The next chapter will highlight the assays carried out to test and validate the neuroprotective efficacy of the extracts in an *in vitro* PD model.

# CHAPTER THREE

# MATERIALS AND METHODS

## 3.1. Materials

| **Chemical reagents and consumables** | **Suppliers** |
|---|---|
| 2, 7-dichlorofluorescein diacetate (H2DCFDA) | Sigma-Aldrich, St Louis MO, USA |
| 96-well tissue culture-treated plates (transparent, white, black) | SPL Life Sciences, Korea |
| Autophagy kit | Sigma-Aldrich, St Louis MO, USA |
| Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) | Thermo Fisher Scientific, Waltham, USA |
| Caspase 3/7 kit | Promega, Madison, WI, USA |
| Cell culture dish (35, 60 and 100 mm) | SPL Life Sciences, Korea |
| Cell viability reagent MTT | Sigma-Aldrich, St Louis MO, USA |
| Cell culture centrifuge tubes (15 mL and 50 mL) | SPL Life Sciences, Korea |
| Cryovials | SPL Life Sciences, Korea |
| Dimethyl sulfoxide (DMSO) | Sigma-Aldrich, St Louis MO, USA |
| Dulbecco's modified eagles' medium (DMEM) | Gibco, Life Technologies Corporation, Paisley, UK |
| Ethanol | Kimix, Cape Town, South Africa |
| Fetal bovine serum (FBS) | Gibco, Life Technologies Corporation, Paisley, UK |
| FITC - Annexin V Apoptosis Kit | Invitrogen, Paisley, UK |
| Fluo-4 Direct $Ca^{2+}$ kit | Invitrogen, Paisley, UK |
| Griess reagent kit | Promega, Madison, WI, USA |
| Mitochondrial ToxGlo ATP kit | Promega, Madison, WI, USA |
| Neurotoxin $MPP^{+}$ | Sigma-Aldrich, St Louis MO, USA |
| Penicillin-streptomycin solution | Lonza Group Ltd., Verviers, Belgium |
| Phosphate buffered saline (1X, without Ca or Mg and pH 7.4) | Sigma-Aldrich, St Louis MO, USA |
| Pipette tips (white tips: 2-10 µl, yellow tips: 10-200 µl and blue tips: 200-1000 µl) | SPL Life Sciences Gyeonggi-do, Korea |
| SH-SY5Y cell line | Donation from the Blackburn Laboratory, University of Cape Town, South Africa |
| Sterile serological pipettes | SPL Life Sciences, Gyeonggi-do, Korea |
| Tetramethylrhodamine ethyl ester perchlorate (TMRE) | Thermo Fisher Scientific, Waltham, USA |
| 0.4% Trypan blue | Sigma-Aldrich, St Louis MO, USA |
| Trypsin-Versene (EDTA) Mixture | Lonza Group Ltd., Verviers, Belgium |
| | |
| **Equipment** | **Suppliers** |
| Multi-mode plate reader (PolarStar Omega) | BMG Labtech Omega® POLARStar |
| Water-jacketed incubator at 37°C with 5% $CO_2$ | Labtech, Germany |

## 3.2. Experimental design

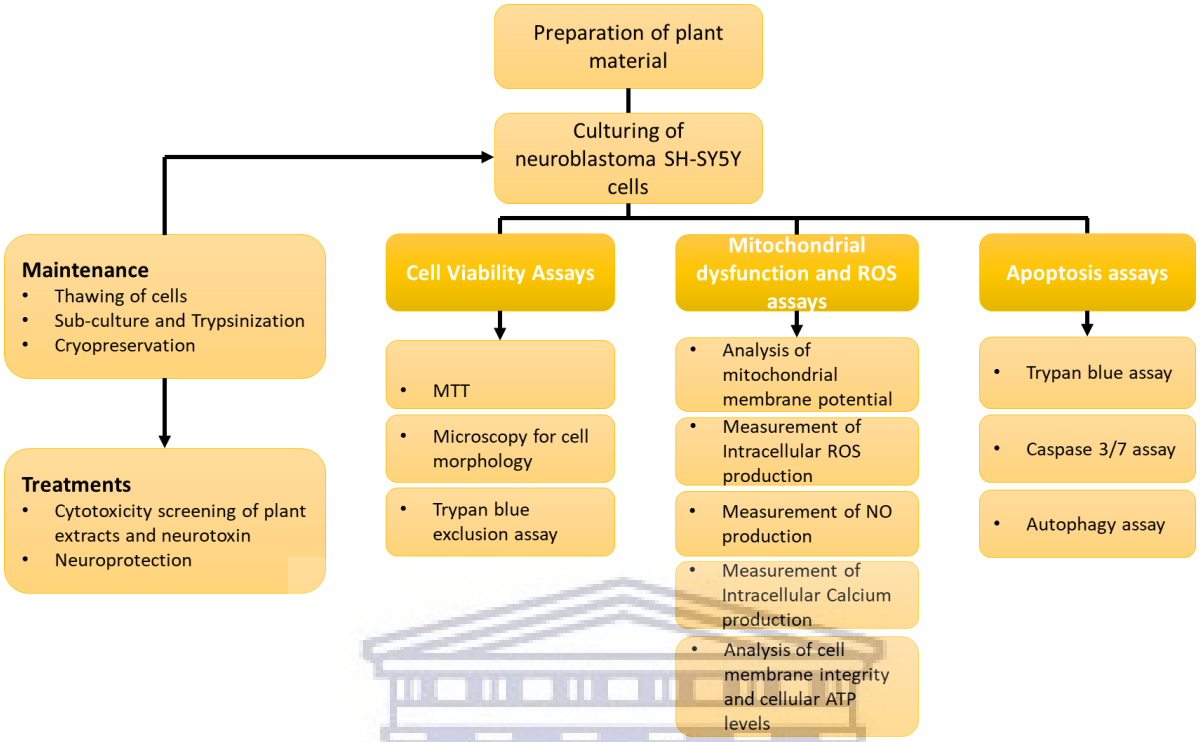



**Figure 3.1***:* Illustration of the experimental design

## 3.3. Methodology

### *3.3.1.* Preparation of plant material

The selection and preparation of the plant extracts were carried out by the collaborators of this project from the Department of Chemistry at Cape Peninsula University of Technology (CPUT). The selected plant material was weighed, and 200g of the plant bulb was dissolved in methanol, extracted at room temperature for 48 hours and filtered. After the filtration process; the extracts were evaporated with reduced pressure at 45°C, and allowed to dry. Once the extracts were solvent-free, they were stored in Eppendorf's at 4°C for later use.

### *3.3.2* Cell culture and maintenance

The human neuroblastoma SH-SY5Y cells that were used in this study were generously donated by the Blackburn Laboratory, University of Cape Town.

#### *i) Thawing cells*

This neuronal cell line was stored at -80°C until time for use, and thawed in a 37°C water bath. The thawed cells in cryovials were re-suspended in 9 mL of the growth medium, Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Life Technologies Corporation, Paisley, UK), supplemented with 10% Fetal Bovine Serum (FBS, Gibco, Life Technologies Corporation, Paisley, UK) and 1% Penicillin/Streptomycin (Lonza Group Ltd., Verviers, Belgium). This was done in order to dilute the Dimethyl sulfoxide (DMSO, Sigma-Aldrich, St Louis MO, USA) in a 15 mL centrifuge tube. The cells were centrifuged at 2500 revolutions per minute (rpm) for 5 minutes; the supernatant was discarded, and the pellet was re-suspended in 10 mL of DMEM in a 100mm petri dish (SPL Life Sciences, Korea). Cells were maintained in a water-jacketed $CO_2$ incubator at 37°C with 5% $CO_2$ (Labtec, Germany) until 70-80% confluence was attained. An inverted phase-contrast microscope was used to view the cells routinely, and this

was done to ensure that the cells had the right morphology and did not exceed the desired confluency level.

### *ii) Sub-culture and Trypsinization*

Upon attaining the desired confluency, the growth medium was discarded, and cells were washed with 1ml of 1X Phosphate Buffered Saline solution (PBS, pH 7.4; Sigma-Aldrich, St Louis MO, USA). The PBS was then discarded, and 1 mL of trypsin EDTA (Lonza Group Ltd., Verviers, Belgium) was added to the dish to allow for detachment of the cell monolayer, followed by incubation, and gently tapping the lower surface of the flask while viewing under the microscope to confirm complete detachment. The cells were thereafter lifted by adding 3 mL of the growth medium, to deactivate any traces of trypsin and the cells were re-grown in a 100mm dish, after gentle re-suspension to obtain single cells. Cells were maintained and grown to confluence, and the medium was changed every 48-72 hours until ready to be used for experiments.

**Figure 3.2:** An illustration of the cell culture performed in Petri dishes. *[Adapted from (Heiden, 2015)]*

### *iii) Cryopreservation*

Confluent cells were trypsinized as described above, and the excess cell suspension was transferred to well-labelled cryovials ensuring each vial contained 900 µL of the cell

suspension. A 100 µL of DMSO was added to each cryovial to obtain 1 mL aliquots, and this was immediately kept on ice and stored at -80°C.

### 3.3.3. Treatments

#### *i) Cytotoxicity screening*

Cells were seeded in 96-well plates and incubated overnight for attachment to occur. The cells were then exposed to increasing concentrations of the extracts by replacing the growth medium with fresh medium containing the appropriate extract concentrations, after which the plates were incubated again for 24 hours at 37°C in a $CO_2$ incubator (Labtec, Germany). The plant extracts were first dissolved in DMSO (Sigma-Aldrich, St Louis MO, USA) to prepare the 40 mg/mL stock solutions from which further dilutions were made in growth medium to prepare the 2.5 to 10 µg/mL, 10 to 40 µg/mL concentrations as well as the 25, 50, 75 and 100 µg/mL concentrations (results not shown). The rest of the stock solution was stored at -20°C. Sixteen (16) plant extracts in total from the Amaryllidaceae family were screened for cytotoxicity to determine which of the plants would be a good candidate for neuroprotection studies. This was to be determined by how well the extracts were able to increase cell viability relative to the control. Some cells were treated with only the DMSO concentration used to prepare the highest concentration of the extracts and served as the vehicle control.

To prepare for the neurotoxicity study, the 50 mM stock solution of $MPP^+$ (Sigma-Aldrich, St Louis MO, USA) was prepared in unsupplemented medium, and further diluted with supplemented medium to make up the desired concentrations ranging from 500 µM to 2500 µM. For the toxicity testing, the cells were seeded and kept overnight, and their growth medium replaced with fresh $MPP^+$-containing medium while the cells that were not exposed to any $MPP^+$ were used as control. All treatments were incubated for 24 hours.

The 2.5, 5 and 10 µg/mL extracts concentrations of *Boophone disticha* (BD), Brunsvigia *bosmaniae* (BB) and *Strumaria truncata* (ST) were selected, because extract toxicity was

minimal, but cell viability was optimal at these concentrations. Also, the screening studies showed that 2000 µM of $MPP^+$ was the optimal concentration, and this was selected for use in subsequent experiments.

***ii) Neuroprotection***

In most neurological diseases, the neuronal structure or function is compromised, thus causing irreversible damage. This progressive degeneration of neuronal elements has enhanced the study of more novel therapies with the hope of finding therapeutic agents that will aid in the modification and relatively preserve neuronal elements (Joy and Johnston Jr, 2001). This therapeutic strategy is known as neuroprotection. Once screening of the extracts was completed and the $MPP^+$ neurotoxin concentration optimized, the SH-SY5Y cells were seeded in a 96-well plate and pre-treated with 2.5, 5 and 10 µg/mL of the extracts (BD, BB and ST) for 2 hours after which 2000 µM of the $MPP^+$ neurotoxin was added to the extract-containing wells. Some cells were treated with growth medium only and served as controls. All the plates were incubated for 24 hours and analysed for cell viability using the MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] assay, as described below.



### 3.3.4. Determination of cell viability (MTT assay)

Tetrazolium compounds have been extensively used in cell viability detection, cytotoxicity testing, as well as for the estimation of cell proliferation and activation. The ability of tetrazolium compounds to cleave the formazan dye ensures a high degree of accuracy with this assay. The commonly used tetrazolium compound assay, also known as MTT assay, was first described by Mosmann in 1983 and has since been modified by various investigators (Mosmann, 1983).

In the present study, 10 µL of the MTT working solution (5 mg/mL in PBS; Sigma-Aldrich, St Louis MO, USA) was added to each treated well. For the cytotoxicity screening, the volume

per well was 100 µL; therefore, only 10 µL of MTT was added to each well whereas, for the neuroprotection plates, 20 µL of the MTT working solution was added to each well as the volume per well was 200 µL. All plates were incubated for 4 hours, after which the growth medium was aspirated, and the formed formazan crystals were solubilized by adding 100µL of DMSO to each well. Furthermore, the intensity of the dissolved formazan crystals (purple colour) was quantified using the PolarStar Omega (BMG Labtech Omega® POLARStar) microplate reader at an absorbance of 570 nm and the percentage cell viability was calculated relative to the control and the maximum non-toxic doses were obtained.

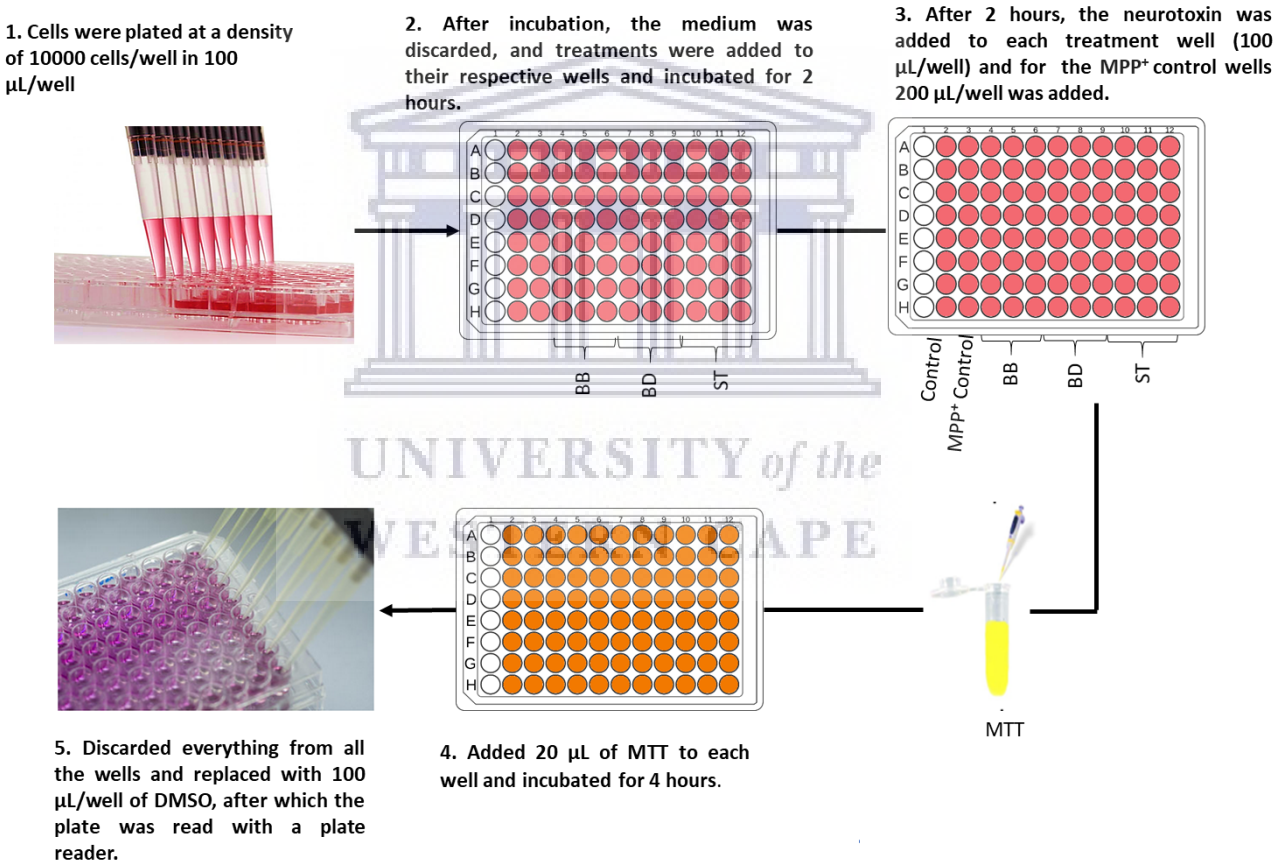



**Figure 3.3:** Schematic illustration of the neuroprotection assay using MTT reagent and showing the formed purple formazan crystals being solubilized with DMSO before reading the plate.

### 3.3.5. Changes in cell morphology

For the morphology studies, the SH-SY5Y cells were seeded in 96-well plates and treated with the selected concentrations of all three extracts (BD, BB and ST) as well as with the selected

concentration of $MPP^+$. Post-treatment, the cells were visualized using the 10 X objective lens of a Zeiss inverted light microscope and the Zeiss software Version 2.3 was used to obtain the images of the control cells, as well as the $MPP^+$-treated and extract-treated cells, respectively.

### 3.3.6. Trypan blue dye exclusion assay

This assay was used to assess the impact of treatments on cell viability, in addition to the MTT assay. The trypan blue dye selectively enters only the dead cells due to compromised cell membrane integrity and binds to intracellular proteins. However, the live cells do not pick up the trypan blue dye while the dead cells stain blue and can be distinguished (Aslantürk, 2018). For this assay, cells were seeded at a density of $2.5 \times 10^5$ /dish in 35mm dishes, treated with all three extracts as described above, and incubated for 24 hours. Post incubation, cells were detached by trypsinization, centrifuged, and the pellet resuspended in fresh medium. From the cell suspension, 20 µL was transferred to an Eppendorf tube, to which an equivalent volume of 0.4% trypan blue dye (Sigma-Aldrich, St Louis MO, USA) was added and loaded onto the counting chambers of a haemocytometer after which the percentage of live cells was determined.



### 3.3.7. Measurement of the mitochondrial membrane potential

A commonly used mitochondrial dye, tetramethylrhodamine ethyl ester perchlorate (TMRE; Thermo Fisher Scientific, Waltham, USA) was used to measure the changes in the mitochondrial membrane potential ($\Delta\Psi m$) (Nisar et al., 2015). This positively charged, red-orange dye accumulates in active mitochondria due to attraction by the negatively charged inner mitochondrial membrane. However, when there is membrane depolarization, the net negative charge across a healthy mitochondrion (180 mV) is disrupted and a decrease in its membrane potential occurs, as evidenced by a reduction in fluorescence signal when analysed. To perform this assay, the protocol previously used by Pariyar and colleagues was applied, with some modifications (Pariyar et al., 2017).

Cells were seeded at 10 000cells/well in a black 96-well microplate and treated as described in the neuroprotection section. For the positive control, carbonyl cyanide 3-chlorophenylhydrazone (CCCP; Thermo Fisher Scientific, Waltham, USA) was used, as it has been reported to be a proton gradient uncoupling agent, which can reduce the electrochemical potential as a way of disrupting the mitochondrial membrane potential ($\Delta\Psi m$) and altering the mitochondrial morphology (Zhang et al., 2016, Miyazono et al., 2018). After treatment with the plant extracts and $MPP^+$, CCCP (1000 μM; made up in growth medium) was added to the positive control wells at 100 μL per well and incubated for 10 minutes. The culture medium was then discarded from all the wells and the cells were then stained with TMRE (400 nM; prepared in growth medium), excluding the column containing untreated cells as they received no stain. The negative control received no treatment but was stained with TMRE. After staining, cells were further incubated for 25 minutes, the dye-containing medium discarded and cells washed three times with PBS before reading with a microplate reader (BMG Labtech Omega® POLARStar) at an excitation wavelength of 544 nm and an emission wavelength of 590 nm. The fluorescence of treated cells was expressed as percentages of control.



### 3.3.8. Measurement of Intracellular ROS production

To measure the levels of production of intracellular reactive oxygen species (ROS), the cell-permeable molecular probe 2, 7-dichlorofluorescein diacetate ($H_2$DCFDA; Sigma-Aldrich, St Louis MO, USA) was used. The non-polar and non-ionic DCFH-DA is converted to the polar derivative DCFH which is eventually converted to DCF when oxidized by intracellular reactive oxygen species or peroxides (Rastogi et al., 2010, Yoon et al., 2018). To perform this assay, a protocol by Ibrakaw and colleagues was used, with slight modifications (Ibrakaw et al., 2020).

Cells were seeded at 10 000cells/well in a black 96-well microplate and treated for 24 hours as described in the neuroprotection section. Cells to be used as positive control were then incubated with 10% hydrogen peroxide ($H_2O_2$) for the last 10 minutes of the experiment. The

DCFH-DA stock was prepared in DMSO, from which a dilution was carried out in serum-free medium (un-supplemented) to prepare 20 μM of the dye. All medium was discarded after the 10-minute incubation, and cells were washed with PBS. Thereafter, 100 μL of the made-up working solution of the dye was added to each well and incubated for an additional 1 hour at 37°C. Post incubation, the solution was discarded, and cells were washed gently with PBS followed by measurement of DCF fluorescence intensity using a microplate reader (BMG Labtech Omega® POLARStar) at excitation wavelength 485 nm and emission wavelength 538 nm. Fluorescence intensity was expressed as a percentage of control.

### 3.3.9. Measurement of Nitric Oxide production

The enzyme nitric oxide synthase (NOS) catalyzes the production of nitric oxide (NO) from L-arginine (Förstermann and Sessa, 2012), and the NO becomes lethal after prolonged exposure to it (Wink et al., 2011). Increased levels of nitric oxide levels promote inflammatory responses, thus causing apoptosis or secondary tissue necrosis (Tripathi et al., 2007). One stable end-product of nitric oxide metabolism is nitrite, which is usually measured to verify the NO production in cells, using the Griess reagent system kit (Promega, Madison, WI, USA).



The Griess assay was carried out according to the manufacturer's instructions; cells were seeded at 10 000cells/well and treated as described in the neuroprotection section. Before removing the cells from the incubator, a 100 μM nitrite solution prepared by diluting the provided 0.1M nitrite standard solution in the medium, was added to a 96 well plate in varying amounts: 100 μL to 3 wells in the first row, and 50 μL of DMEM only was added to the rest of the wells aligned with the top 3. Thus, a 6 serial twofold dilution in triplicate was carried out all the way down the three designated columns, and this was done to obtain the reference curve for the nitrite standard. The last row did not receive any nitrite solution. Once this was done, the final volume was 50 μL/well with concentrations ranging from 100, 50, 25, 12.5, 6.25, 3.13 and 1.56 μM. Cell-free supernatants from experimental samples were then added to the plate

in triplicates at a volume of 50 µL/well, and to this, 50µL of Sulfanilamide (1% Sulfanilamide in 5% phosphoric acid) solution was added to all the experimental and dilution series wells. The plate was incubated at room temperature for 10 minutes away from light after which, 50 µL of the NED (0.1% N-1-naphthylethylenediamine dihydrochloride in water) solution was added to all wells and incubated for 10 minutes at room temperature away from light. A magenta/purple colour change was observed immediately, and absorbance was measured using a microplate reader (BMG Labtech Omega® POLARStar) at 520nm and 550 nm wavelengths, respectively.

### 3.3.10. Measurement of Intracellular Calcium production

The increase in intracellular calcium ($iCa2^{+}$) can be attributed to ROS-mediated stress (Tan et al., 2018). In this study, $iCa^{2+}$ levels were measured using a fluo-4 direct $Ca^{2+}$ detection assay kit (Invitrogen, Paisley, UK), according to manufacturer's instructions. Cells were seeded at 10 000 cells/well in a white 96-well microplate and treated for 24 hours as described in the neuroprotection section. A 250 mM stock solution of probenecid was prepared by adding 1 mL of fluo-4-direct calcium assay buffer to a vial containing 77 mg of water-soluble probenecid and vortexed to ensure complete dissolution. A 2X calcium reagent loading solution was then prepared by adding 5mL of the assay buffer and 1 mL of the 250 mM stock solution to one bottle of calcium reagent. With each well having a total volume of 200 µL, 150 uL was discarded to ensure that only 50 µL was remaining, to which an equal volume of the reagent loading solution was added. Incubation was carried out for 60 minutes at 37°C, and fluorescence was measured using a microplate reader (BMG Labtech Omega® POLARStar) at excitation wavelength of 494 nm and emission wavelength of 516 nm.

### 3.3.11. Determining cell membrane integrity and cellular adenosine triphosphate levels

The neurotoxin $MPP^{+}$ is reported to cause inhibition of mitochondrial electron transport chain (ETC) complex I, thus disrupting the mitochondrial energy metabolism resulting in a loss of

adenosine triphosphate (ATP) production (Martinez and Greenamyre, 2012). To investigate the changes in cell membrane integrity and cellular ATP levels, a Mitochondrial ToxGlo ATP assay kit (Promega, Madison, WI, USA) was used according to the manufacturer's instructions. Briefly, cells were seeded at 10 000 cells/well in a white 96-well microplate and treated for 24 hours as described in the neuroprotection section. With the wells having a total volume of 200 µL, 100 µL was discarded. A volume of 20 µL of the 5X cytotoxicity reagent was added to each well and mixed briefly on a plate shaker at 500-700 rpm for 1 minute. This was incubated for 30 minutes at 37°C, after which fluorescence intensity was measured using a microplate reader (BMG Labtech Omega® POLARStar) at excitation wavelength 485 nm and emission wavelength 520 nm. Furthermore, the plate was equilibrated to room temperature for 5 minutes, after which 100 µL of ATP detection reagent was added to each well and the plate then placed on a plate shaker for 5 minutes at 500-700rpm to ensure that the reagent and sample were properly mixed. Luminescence intensity was measured using a microplate reader (BMG Labtech Omega® POLARStar) at a wavelength of 520 nm.

### 3.3.12. Caspase 3/7 apoptosis assay

To investigate apoptosis in the cells, the caspase 3/7 assay kit (Promega, Madison, WI, USA) was used, according to the manufacturer's instructions. Cells were seeded at 10 000 cells/well in a white 96-well microplate and treated as described in the neuroprotection section for 24 hours. With the wells having a total volume of 200 µL, 100 µL was discarded to ensure that only 100 µL was left, and to this, an equal volume of the Caspase-Glo 3/7 reagent solution was added per well. The microplate was placed on a plate shaker for 30 seconds at 300-500 rpm, to gently mix the contents. The plate was then incubated at room temperature for 1 hour, after which luminescence was measured using a microplate reader (BMG Labtech Omega® POLARStar) at a wavelength of 520 nm.

### 3.3.13. Autophagy assay

To investigate autophagy in a cell, the Autophagy Assay kit (Sigma-Aldrich, St Louis MO, USA) was used according to the manufacturer's instructions. Cells were seeded at 10 000 cells/well in a black 96-well microplate and treated for 24 hours, as described in the neuroprotection section. Prior to the commencement of the experiment, the working solution of the autophagosome detection reagent was prepared by diluting 10 µL of the 500X detection reagent into 5 mL of the stain buffer. All the medium from the cells was discarded and replaced with 100 µL/well of the working solution of the autophagosome detection reagent. The plate was incubated for 1 hour at 37°C. After incubation, the detection reagent was discarded, and cells were gently washed thrice, with the wash buffer provided, by adding 100 µL per well. Fluorescence intensity was measured using a microplate reader (BMG Labtech Omega® POLARStar) at excitation wavelength 360 nm and emission wavelength 520 nm.

## 3.4 Statistical Analysis

All experiments in this study were performed in triplicates, and the analysis of parameters in this study was achieved by utilizing the three-parameter logistic equation of GraphPad Prism 7 statistical software. The data obtained were then presented as means ± standard error of the mean (SEM).

# CHAPTER FOUR

# RESULTS

## 4.1. Cytotoxicity screening of plant extracts to determine the optimum concentration

The cytotoxic effects of the extracts of *Strumaria truncata, Boophone disticha,* and *Brunsvigia bosmaniae* on the SH-SY5Y neuronal cell line were assessed and evaluated using the MTT assay after 24 hours of treatment. To determine the optimum concentration for each extract, only concentrations that enhanced cell viability and showed minimal toxicity to the SH-SY5Y cells were selected. The screening began with the 25-100 µg/mL concentration range for each extract, followed by the 10 - 40 µg/mL concentration range and finally 2.5 to 10 µg/mL concentration range. The results for the 25-100 µg/mL and 10 - 40 µg/mL concentration ranges were discarded because of high toxicity. However, the results of the screening with the 2.5 to 10 µg/mL concentration range showed significant increase in cell viability for all extracts especially at the 2.5 and 5 µg/mL concentrations, when compared to the vehicle control (**Figure 4.1)**. Based on these results, only the 2.5, 5 and 10 µg/mL concentrations of *Boophone disticha, Brunsvigia bosmaniae* and *Strumaria truncata* respectively, were selected for further studies.

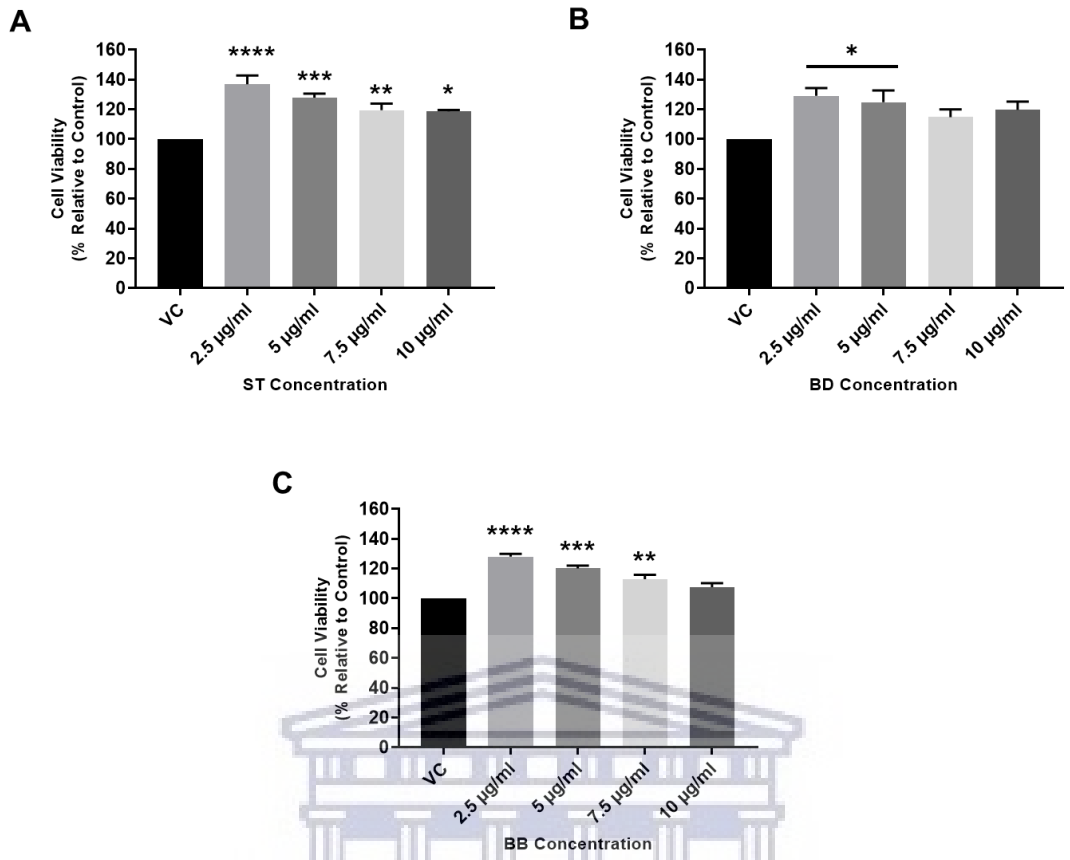



**Figure 4.1. Evaluation of the impact of extracts on cell viability**. The graphs show the cytotoxic effects of the plant extracts (A), Strumaria truncata (ST), Boophone disticha (BD), and Brunsvigia bosmaniae (BB) extracts on the viability of the SH-SY5Y cells following a 24-hour exposure. Control cells were treated with DMSO, and the viability was evaluated using the MTT cell viability assay. Each bar represent mean ± SEM of three experiments (n=3) and statistical significance was denoted by asterisks (* $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$ and ****$p < 0.0001$). VC = vehicle control.



## 4.2. Cytotoxic effect of $MPP^+$ on SH-SY5Y cell viability

The **Figure 4.2** shows the effect of the neurotoxin $MPP^+$ on SH-SY5Y cell viability evaluated using the MTT assay following a 24-hour exposure when compared to the control. The cells were treated with increasing concentrations of 500, 1000, 1500, 2000, and 2500 µM $MPP^+$ and the results indicate significant toxic effects of $MPP^+$ on the SH-SY5Y cells at all the treatment concentrations, with the lowest concentration (500 µM) having the least toxicity (77.8%) to the cells, while the highest concentration (2500 µM) reduced the cell viability to about 33%. However, at the 2000 µM $MPP^+$ concentration, there was significant reduction in cell viability by approximately 51% which was considered an optimum concentration to induce minimum

toxicity to the cells and test the neuroprotective effects of the plant extracts. Thus the 2000 μM $MPP^+$ concentration was selected for all the neuroprotection experiments.

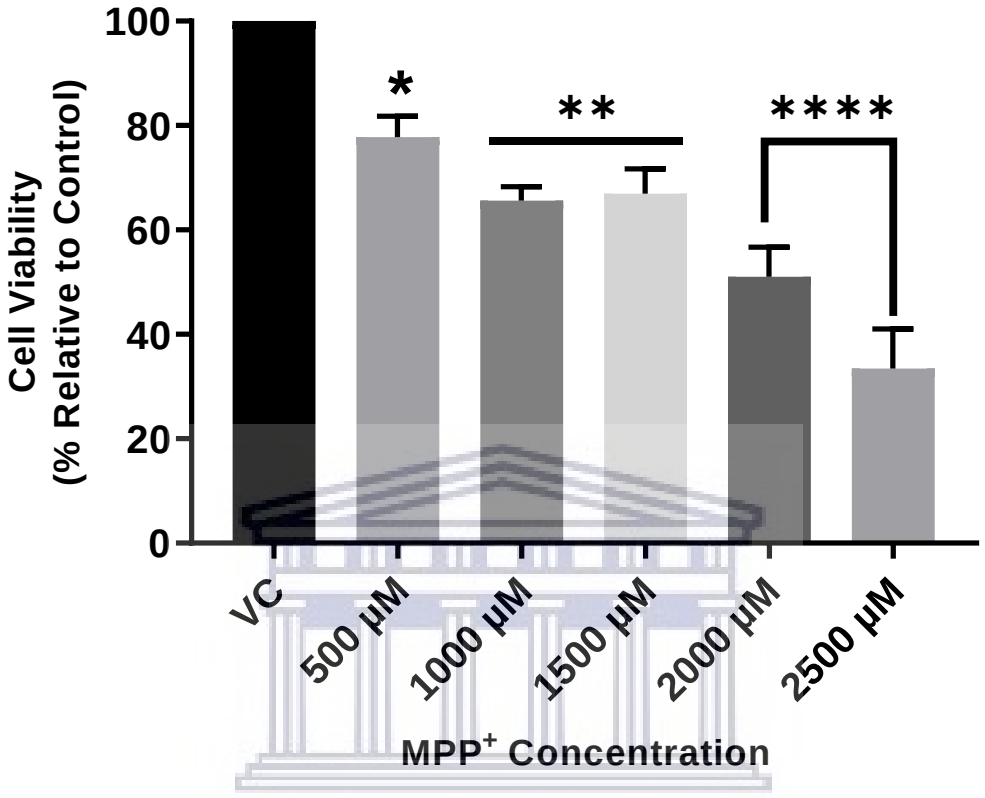



**Figure 4.2. Dose-response of $MPP^+$.** The graph shows the toxicity of $MPP^+$ on the viability of SH-SY5Y cells following a 24-hour exposure. The control cells were left untreated, and the viability was evaluated using the MTT cell viability assay. The graphs were prepared as mean ± SEM of three experiments (n=3) using GraphPad Prism 7 statistical software. The statistically significant changes are denoted by asterisks (* $p < 0.05$; ** $p < 0.01$, and ****$p < 0.0001$). VC = vehicle control.



### 4.3. Neuroprotective effects of the plant extracts against $MPP^+$ induced SH-SY5Y cytotoxicity

Following the selection of the optimum concentrations for the plant extracts (**Figure 4.1**) and the neurotoxin $MPP^+$ (**Figure 4.2**), the SH-SY5Y cells were pre-treated with the 2.5, 5, and 10 μg/mL extracts concentrations of BD, BB and ST, for two hours before the introduction of $MPP^+$ (2000 μM) followed by 24-hour incubation. **Figure 4.3** shows that whereas cells treated with $MPP^+$ alone significantly reduced cell viability to approximately 50%, pre-treatment with the plant extracts significantly attenuated the toxicity induced by $MPP^+$ when compared with

the control. In **Figure 4.3A**, cells pre-treated with ST had significant increases in viability (approximately 98%, 91% and 91.5% for the 2.5 µg/mL, 5 µg/mL, and 10 µg/mL concentrations respectively), when compared to cells treated with only $MPP^+$. Furthermore, the cells pre-treated with BD had increased cell viability (89%, 88% and 94% for the 2.5 µg/mL, 5 µg/mL, and 10 µg/mL concentrations, respectively (**Figure 4.3B**). Similarly, treatment with the BB extract also resulted in increased cell viability in a concentration-dependent manner (93%, 78% and 72% for the 2.5 µg/mL, 5 µg/mL, and 10 µg/mL concentrations, respectively when compared to the $MPP^+$- only treated cells (**Figure 4.3C**). Together, these results have shown the neuroprotective potential of the plant extracts.

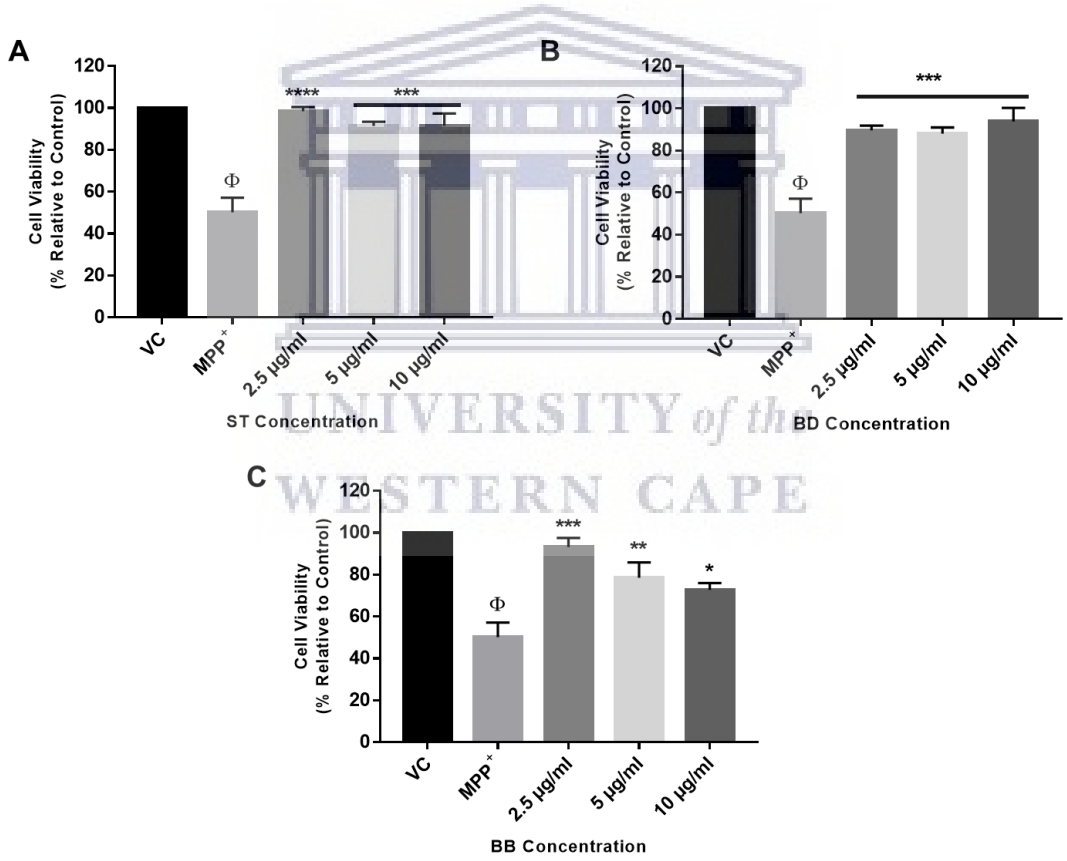
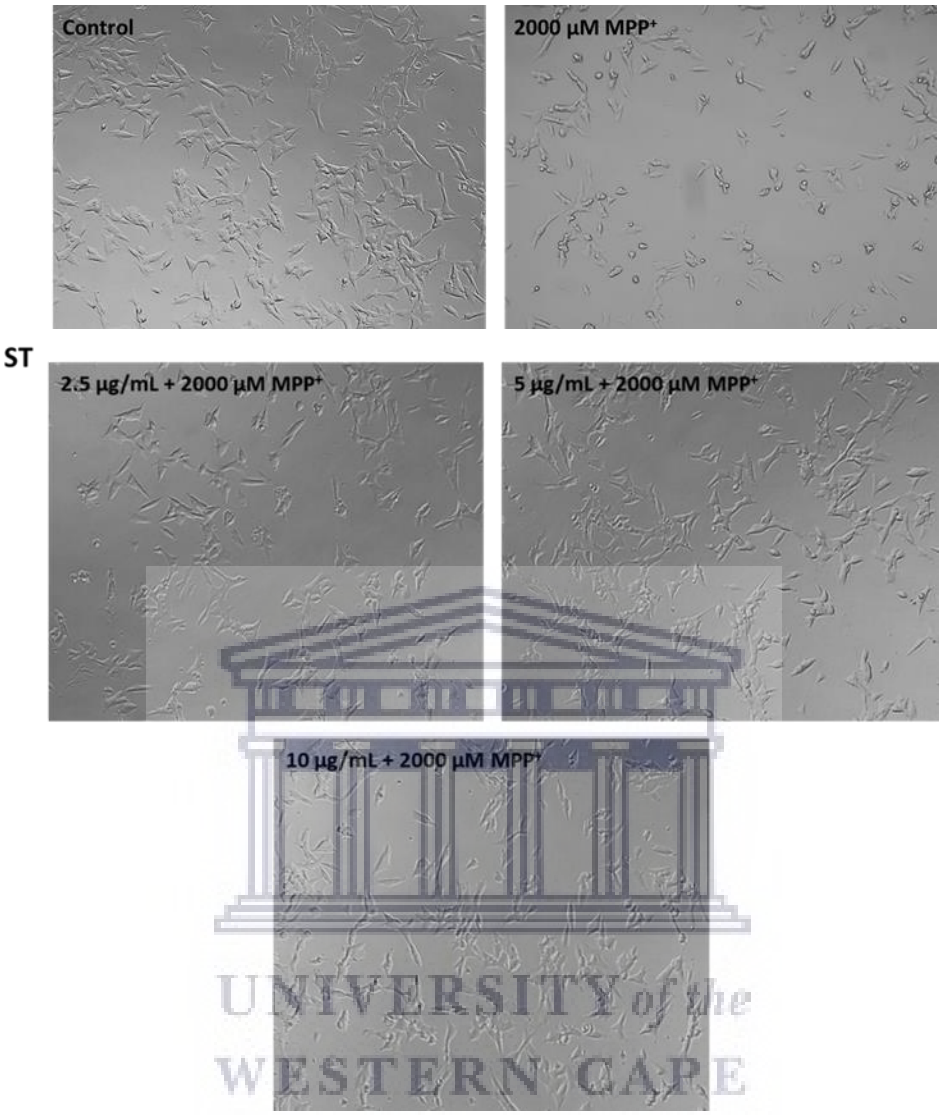
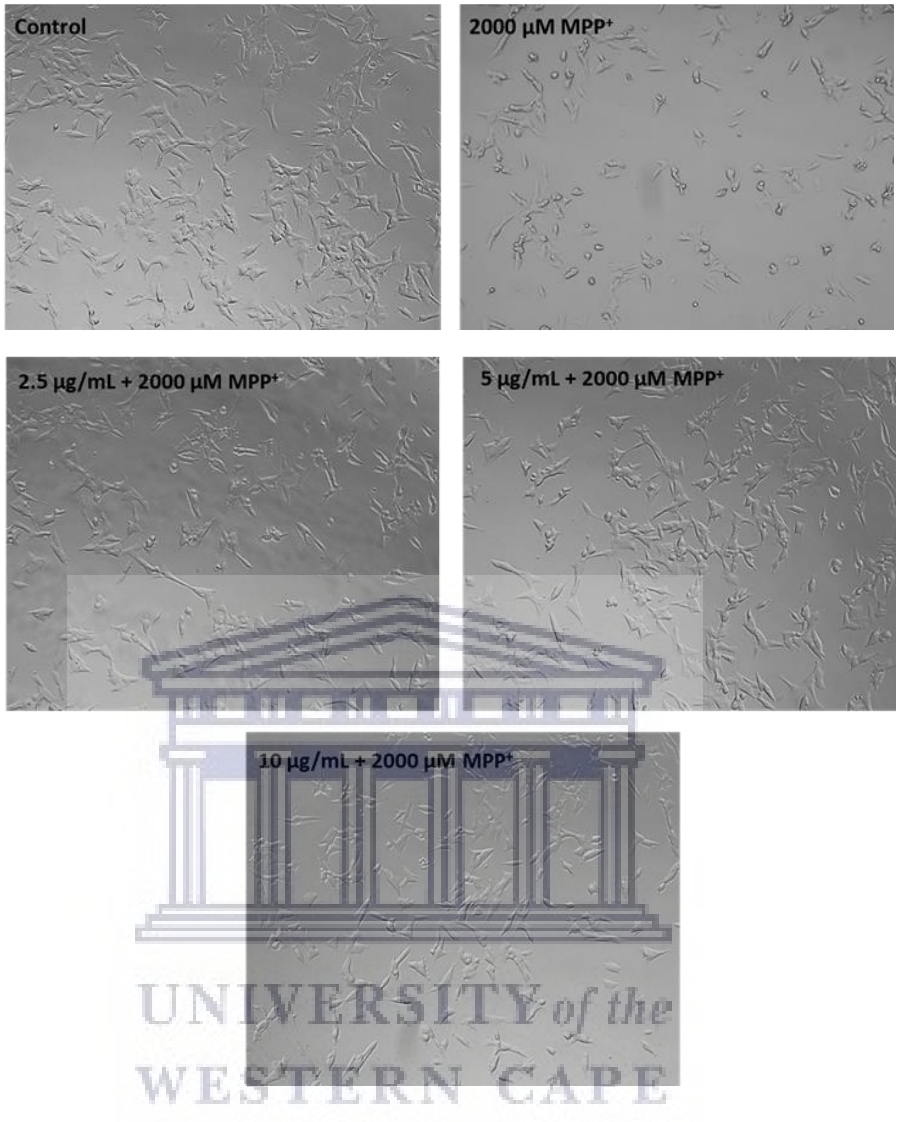



**Figure 4.3. Neuroprotective effects of the extracts on cell viability after exposure to $MPP^+$.** The graphs illustrate the neuroprotective capabilities of the plant extracts (A) Strumaria truncata (ST), (B) Boophone disticha (BD), and (C) Brunsvigia bosmaniae (BB) when pre-treated SH-SY5Y cells are exposed to MPP+ for 24 hours. The control cells were left untreated, and the viability was evaluated using the MTT cell viability assay. The graphs were prepared as mean ± SEM of three experiments (n=3) using GraphPad Prism 7 statistical software. The statistically significant changes are denoted by asterisks (* $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$ and ****$p < 0.0001$). Control cells vs MPP+ only treated cells is represented by Φ. VC = vehicle control.

### 4.4. Effects of extracts on $MMP^+$-induced morphological changes in SH-SY5Y cells

The changes in the cell morphology were investigated as SH-SY5Y cells were plated in 96 well plates and exposed to treatments as was done for the neuroprotection experiments, after which the changes in cell morphology were observed using an inverted light microscope at a magnification of X100 (**Figure 4.4A, B and C**). The results showed that the $MPP^+$-treated cells showed altered morphology compared to control cells, including the loss of neuronal projections, elongated and rounded shapes as well as a reduction in cell density, some of which are reminiscent of cells undergoing programmed cell death. However, following pre-treatment with the extracts of ST, BD and BB, there was remarkable retention of cell morphology and density characteristics, and the cells looked similar to the untreated control cells.





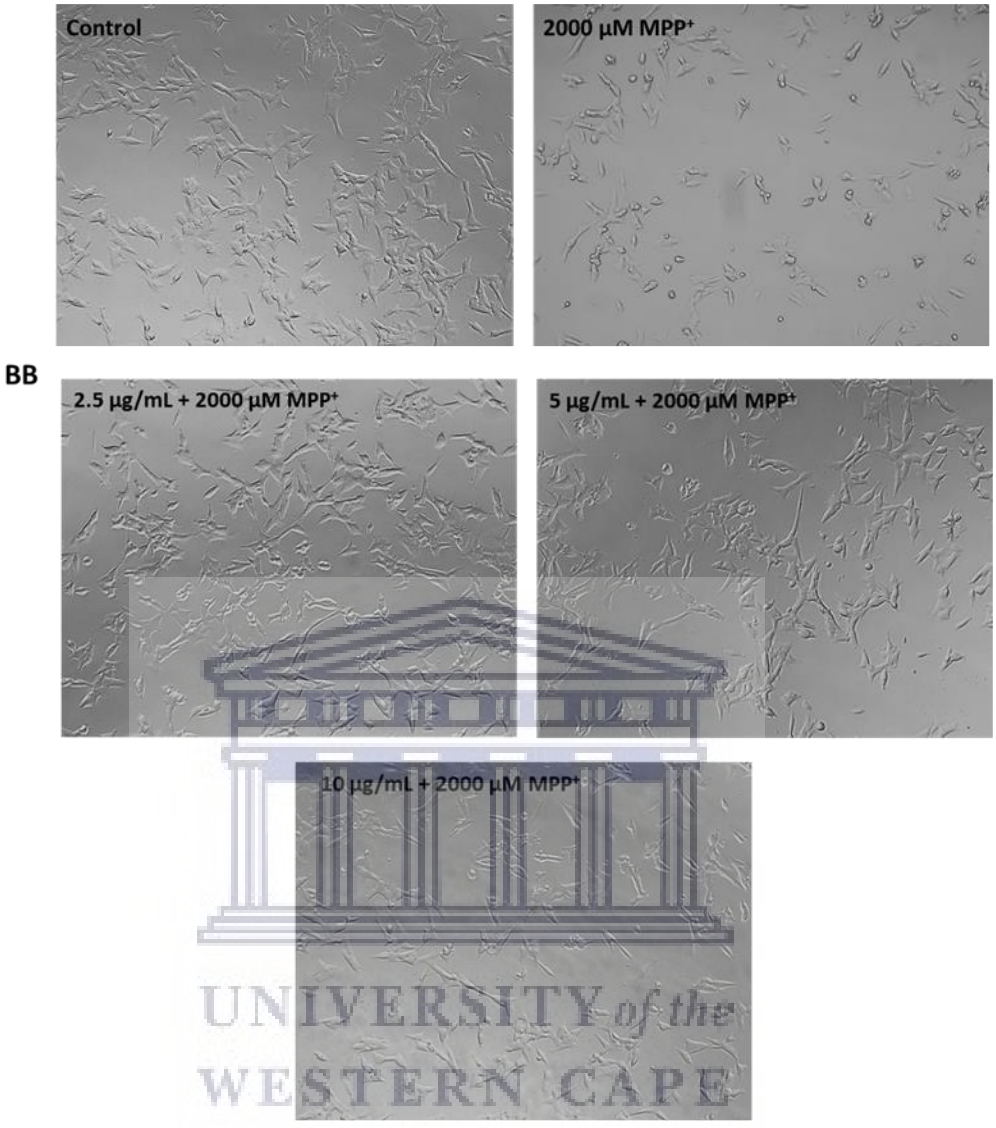





**Figure 4.4. Microscopic morphological evaluation of SH-SY5Y cells.** The morphological analysis of plant extracts Strumaria truncata (ST), Boophone disticha (BD), and Brunsvigia bosmaniae (BB) when pre-treated SH-SY5Y cells are exposed to 2000 µM $MPP^+$ for 24 hours.

## 4.5. Neuroprotective Effects of ST, BD, and BB Extracts Against $MPP^+$-Induced Decrease in Mitochondrial Membrane Potential (MMP) in SH-SY5Y Cells

Altered mitochondrial membrane potential is observed as part of the mechanisms of action of $MPP^+$, and this can be linked to an increase in oxidative stress and could also act as a marker for apoptotic stimuli (Kim and Kim, 2018). In the present study, the effects of treatment with the plant extracts on $MPP^+$-induced alterations in the MMP of SH-SY5Y cells was investigated

using the TMRE dye and the mitochondrial uncoupler CCCP (carbonyl cyanide m-chlorophenylhydrazone) was used as a positive control because it depolarizes the membrane potential. The results showed that treatment with $MPP^+$ only, resulted in a decrease in MMP similar to the effects of CCCP. However, pre-treatment with the plant extracts attenuated the effects of $MPP^+$ albeit not significant (**Figure 4.5**). Together, these results indicate that the restoration of MMP in the SH-SY5Y cells may be a neuroprotection mechanism following $MPP^+$ toxicity.

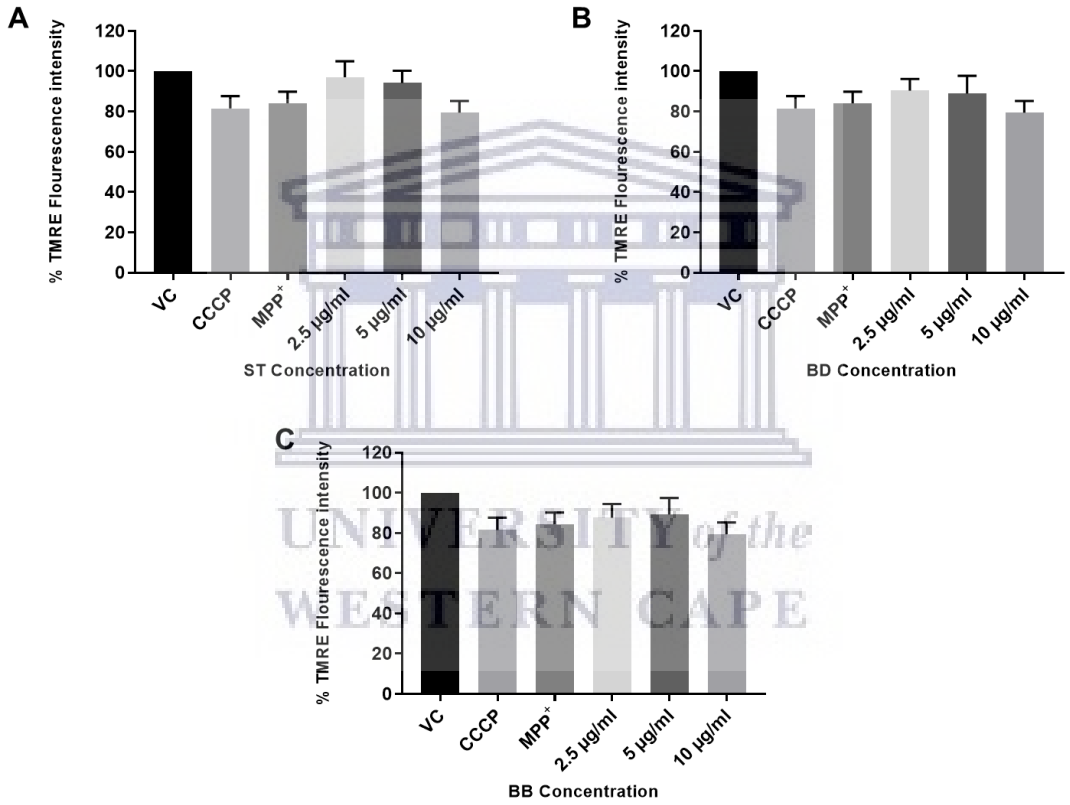



**Figure 4.5. Mitochondrial membrane potential evaluation in untreated and pre-treated SH-SY5Y cells after exposure to $MPP^+$.** The graphs illustrate the effect of the plant extracts (A) *Strumaria truncata (ST)*, (B) *Boophone disticha (BD)*, and (C) *Brunsvigia bosmaniae (BB)* on $MPP^+$ (2000 µM) induced damage to the mitochondrial membrane potential in SH-SY5Y cells after 24 hours shown by the fluorescence intensity of TMRE. Cells treated with CCCP were used as positive control while the negative control cells were left untreated. The graphs were prepared as mean ± SEM of three experiments (n=3) using GraphPad Prism 7 statistical software. VC = vehicle control.

### 4.6. Neuroprotective Effects of ST, BD, and BB Extracts Against $MPP^+$-Induced Increase in ROS levels in SH-SY5Y Cells

Neurotoxins are reported to generate ROS by inhibiting complex I (Drechsel and Patel, 2008). Therefore, to investigate increased ROS generation in $MPP^+$ toxicity, a fluorescence dye, DCFDA was used to evaluate ROS accumulation in pre-treated SH-SY5Y cells exposed to $MPP^+$. Hydrogen peroxide was used as a positive control and the untreated cells served as the normal control and was set at 100%. The results show that both $MPP^+$ and hydrogen peroxide significantly increased ROS levels in the cells to about 178% and 200% respectively, compared to the control. However, the cells pre-treated with the extracts showed a reduction in ROS levels. The ST extract (**Figure 4.6A**) showed a reduction in ROS activities, which was statistically significant at the 2.5 μg/mL concentration with a percentage ROS level (99 %) being approximate to the control. Similarly, Figure **4.6B** shows that the BD extract attenuated ROS levels in the cells with the most significant reduction observed at the 5 μg/mL concentration. In addition, the BB extract attenuated the ROS levels in the cells in a concentration-dependent manner as an increase in concentration resulted in reduced ROS activities, which were most significant at 10 μg/mL concentration (**4.6C**). Taken together, these results suggest that inhibition of $MPP^+$-induced increase in ROS activity is involved in the neuroprotection of the Amaryllidaceae extracts under study.

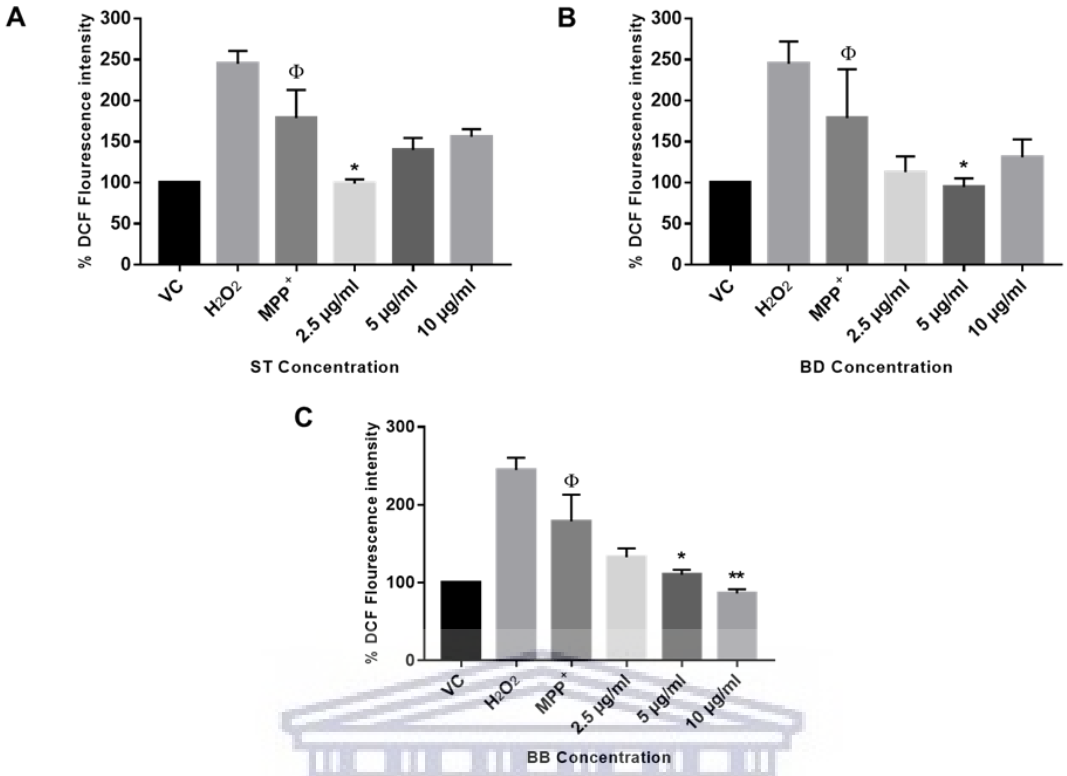



**Figure 4.6. Evaluation of ROS generation in toxin-induced untreated and pre-treated SH-SY5Y cells.** The graphs illustrate the effect of the plant extracts (A) *Strumaria truncata (ST),* (B) *Boophone disticha (BD),* and (C) *Brunsvigia bosmaniae (BB)* on $MPP^+$ (2000 µM) induced dysfunction of intracellular reactive oxygen species in SH-SY5Y cells after 24 hours shown by the fluorescence intensity of DCF. Cells treated with $H_2O_2$ were used as positive control while the negative control cells were left untreated. The graphs were prepared as mean ± SEM of three experiments (n=3) using GraphPad Prism 7 statistical software. The statistically significant changes are denoted by asterisks (* $p < 0.05$; ** $p < 0.01$). Control cells vs $MPP^+$-only treated cells are represented by Φ. VC = vehicle control.



## 4.7. Neuroprotective Effects of ST, BD, and BB Extracts Against $MPP^+$-Induced Increase in Nitric Oxide (NO) in SH-SY5Y Cells

Despite being a vital unique intracellular messenger, NO tends to be toxic to the cells when there is excessive production, and its levels are altered (Tripathy et al., 2015). The $MPP^+$ mechanism has been reported to involve increased levels of NO (Cleeter et al., 2001, Keane et al., 2011). To ascertain the impact of the respective treatments on NO levels in the SH-SY5Y cells, the cells were plated in 96-well plates and exposed to treatments as was done for the neuroprotection experiments, and the Griess kit was used to measure NO levels. The results show that whereas treatment with $MPP^+$ increased NO levels in the cells when compared to the control, only pre-treatment with the BD extract (**Figure 4.7B**) significantly reduced NO levels

at all three concentrations. In contrast, treatment with the ST extract (**Figure 4.7A**) showed only slight reductions that were not statistically significant and treatment with the BB extracts showed very minimal to no reduction compared to the control (**Figure 4.7C**). Together these results indicate that reduction of NO levels induced by $MPP^+$ is part of the mechanisms of neuroprotection by the Amaryllidaceae extracts.

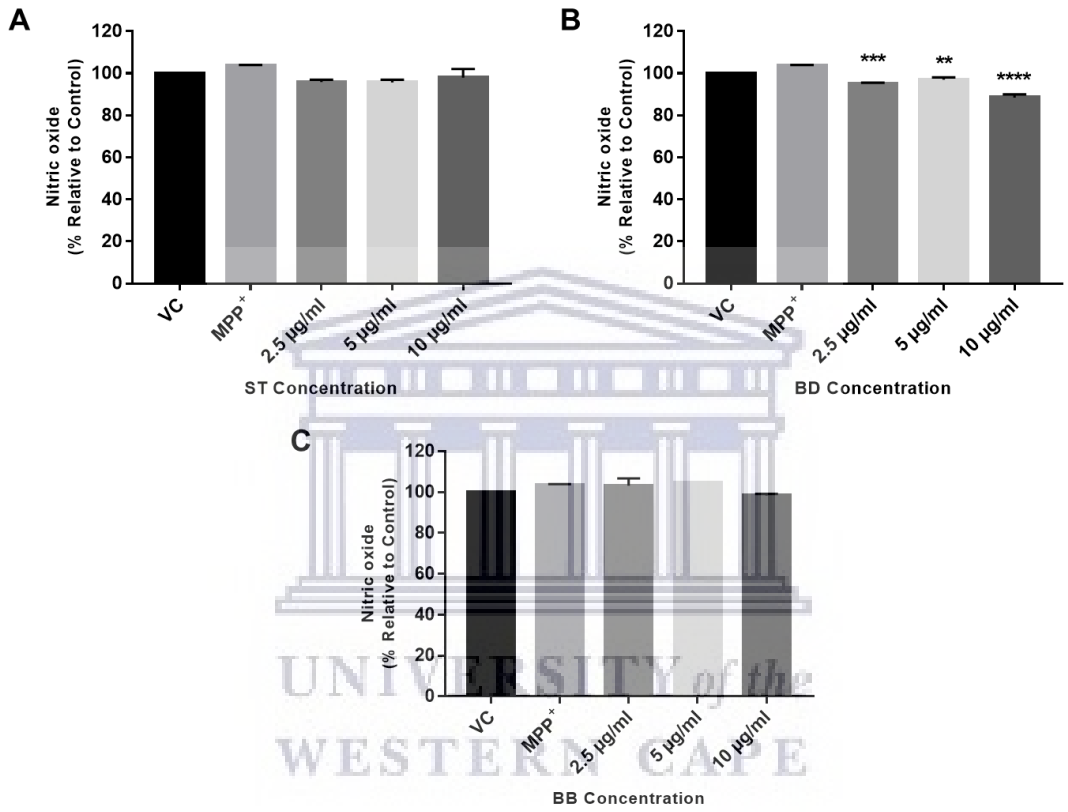



**Figure 4.7. Investigating NO production following exposure of untreated and pre-treated SH-SY5Y cells to $MPP^+$.** The graphs illustrate the effect of the plant extracts (A) *Strumaria truncata (ST),* (B) *Boophone disticha (BD),* and (C) *Brunsvigia bosmaniae (BB)* on $MPP^+$ (2000 µM) induced Nitric oxide production in SH-SY5Y cells after 24 hours. The graphs were prepared as mean ± SEM of three experiments (n=3) using GraphPad Prism 7 statistical software. The statistically significant changes are denoted by asterisks (** $p < 0.01$, *** $p < 0.001$ and ****$p < 0.0001$). VC = vehicle control.

## 4.8. Neuroprotective Effects of ST, BD, and BB Extracts Against $MPP^+$-Induced Depletion of Cellular ATP

One of the contributing factors to the cell death caused by $MPP^+$ is the impairment of ATP production (Subramaniam and Chesselet, 2013). To ascertain if plant extracts prevented ATP depletion in the cells following $MPP^+$ toxicity, the mitotox ATP assay kit was used. The results

obtained in **Figure 4.8** show that when compared to the control, the cells treated with $MPP^+$ showed a significant reduction in ATP levels to about 65%. Interestingly, all extract concentrations tested significantly increased ATP levels in the cells (**Figure 4.8**). These results show that inhibition of ATP depletion is involved in the neuroprotective activities of the plant extract tested, with the *Boophone disticha* extract having the most significant ameliorative effects.

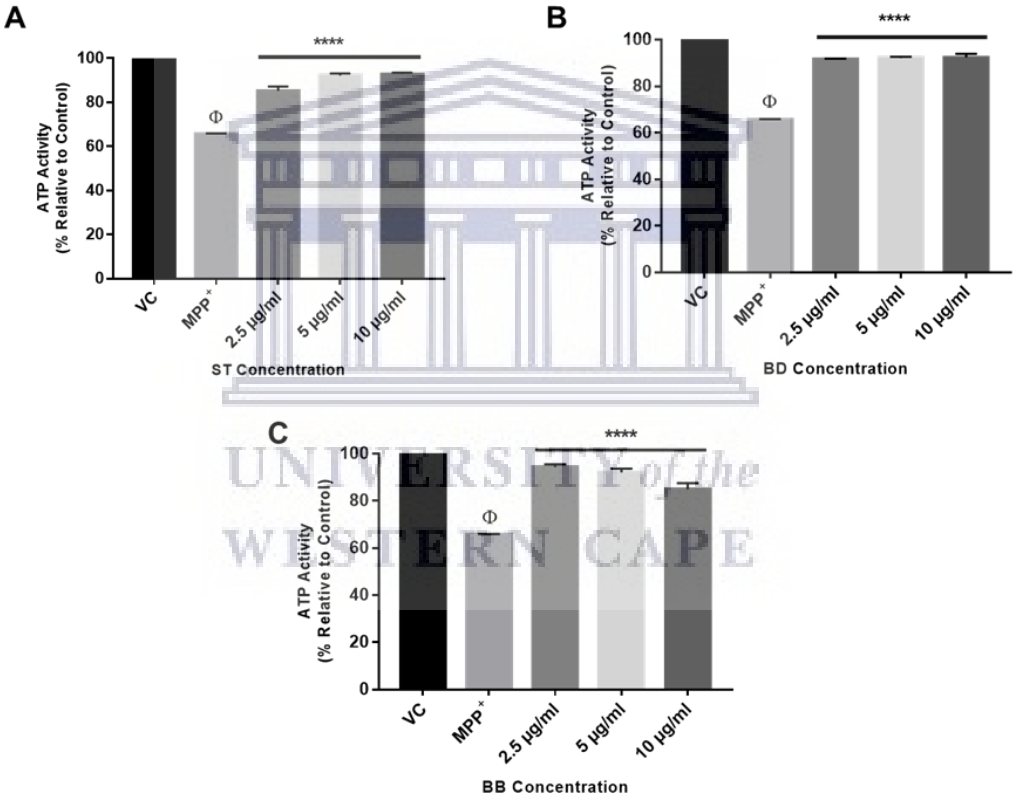



**Figure 4.8**. **Evaluating the effect of $MPP^+$ on ATP production.** The graphs illustrate the effect of the plant extracts (A) *Strumaria truncata (ST),* (B) *Boophone disticha (BD),* and (C) *Brunsvigia bosmaniae (BB)* on $MPP^+$ (2000 µM) induced decrease of ATP levels in SH-SY5Y cells after 24 hours. The graphs were prepared as mean ± SEM of three experiments (n=3) using GraphPad Prism 7 statistical software. The statistically significant changes are denoted by asterisks ****$p < 0.0001$). Control cells vs $MPP^+$ only treated cells is represented by Φ. VC = vehicle control.

### 4.9. Neuroprotective Effects of ST, BD, and BB Extracts Against $MPP^+$-Induced Intracellular Calcium Level Dysfunction

Signalling molecules play vital roles in the body and one major signalling molecule is calcium, which is also known to be a common denominator in various cellular dysfunctions as it can become toxic and cause cell death at extreme levels (Bagur and Hajnóczky, 2017). As an essential element, alteration of neuronal calcium levels has been implicated in various neuropathological conditions such as PD (Wojda et al., 2008, Morales et al., 2016). Exposure to $MPP^+$ causes significant disturbances in dopaminergic cells, and it has been reported that the dysregulation of the neuronal calcium balance by $MPP^+$ is one mechanism by which it exerts its toxicity to cause loss of dopaminergic neurons via apoptosis (Mapa et al., 2018, Jung et al., 2019).

In this study, the intracellular calcium levels were measured using the Flou-4-direct kit. Cells were pre-treated with plant extracts for two hours before adding $MPP^+$ (2000 μM) and all treatments lasted for 24 hours**. Figure 4.9** shows that although the $MPP^+$-induced calcium levels increased slightly above the control, which was not significant. **Figure 4.9A** shows that treatment with the ST extract, decreased calcium levels slightly, albeit not significant. The BD (**Figure 4.9B**) and BB (**Figure 4.9C**) extracts showed an increasing concentration-dependent attenuation, albeit not significant. Altogether, these results tend to suggest that increased intracellular calcium levels are not strongly implicated in $MPP^+$ toxicity in the present study.

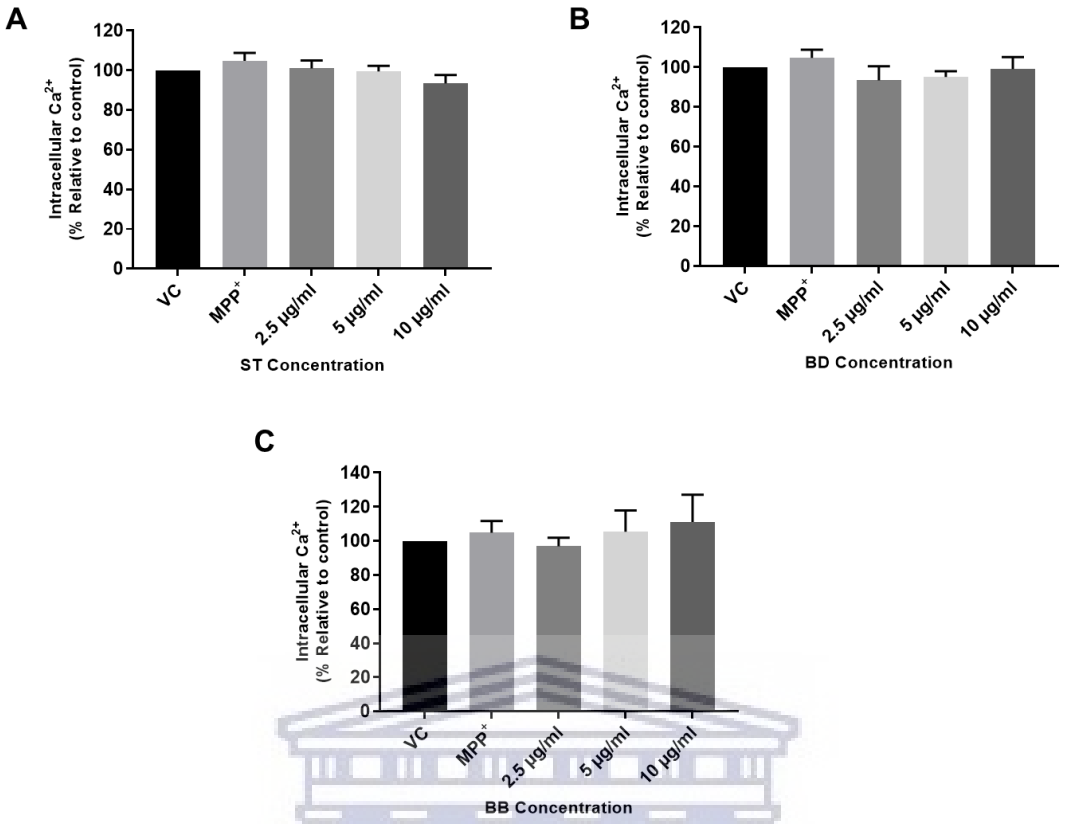



**Figure 4.9. Evaluation of calcium homeostasis following exposure of untreated and pre-treated SH-SY5Y cells to MPP$^+$.** The graphs illustrate the effect of the plant extracts (A) *Strumaria truncata (ST),* (B) *Boophone disticha (BD),* and (C) *Brunsvigia bosmaniae (BB)* on MPP$^+$ induced dysregulation of intracellular $Ca^{2+}$ homeostasis in SH-SY5Y cells after 24 hours. The graphs were prepared as mean ± SEM of three experiments (n=3) using GraphPad Prism 7 statistical software. VC = vehicle control.

## 4.10. Neuroprotective Effects of ST, BD, and BB Extracts Against MPP$^+$ -Induced Cell Death in SH-SY5Y Cells.

Trypan blue was used to determine the number of viable or live cells in the pre-treated cells following their exposure to MPP$^+$ for 24 hours. The cells treated with MPP$^+$ only showed the least viability, while the cells pre-treated with ST and BB showed increased cell viability in a concentration-dependent trend with the 2.5 µg/mL concentration exerting the most protective potential. Finally, the extract BD exerted its most protection at the highest concentration, 10 µg/mL. Altogether, the results show that all three extracts significantly increased cell viability by ameliorating the toxic effects of MPP$^+$.

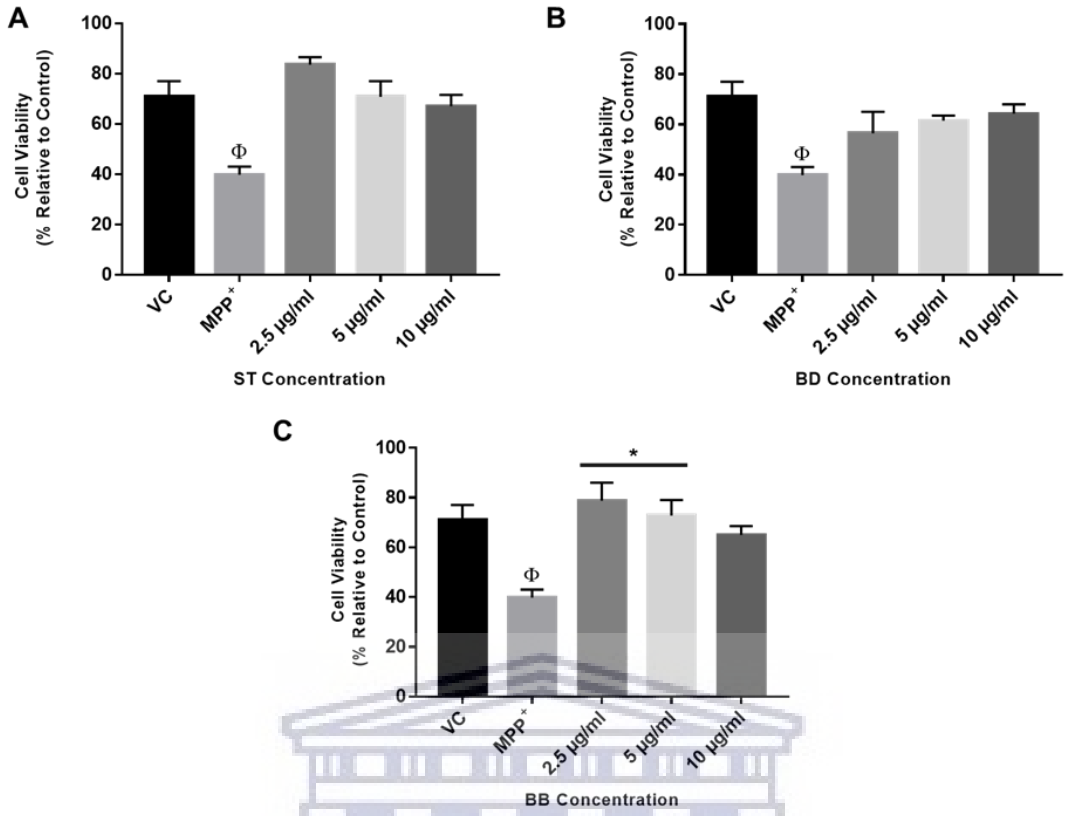



**Figure 4.10. Cell viability determination following exposure of untreated and pre-treated SH-SY5Y cells to MPP$^+$.** The graphs illustrate the effect of the plant extracts (A) *Strumaria truncata (ST)*, (B) *Boophone disticha (BD)*, and (C) *Brunsvigia bosmaniae (BB)* on MPP$^+$ (2000 µM) induced cell death in SH-SY5Y cells after 24 hours and trypan blue was used for this investigation. Cells left untreated were used as a control. The graphs were prepared as mean ± SEM of three experiments (n=3) using GraphPad Prism 7 statistical software. The statistically significant changes are denoted by asterisks (*$p < 0.05$). Control cells vs MPP$^+$ only treated cells is represented by Φ. VC = vehicle control.



## 4.11. Neuroprotective Effects of ST, BD, and BB Extracts Against MPP$^+$ -Induced Autophagic activity in SH-SY5Y Cells.

Recycling of damaged organelles is usually achieved via a process called autophagy (Mizushima, 2007). MPP$^+$ leads to decreased neuronal survival by stimulating dysfunctional autophagic machinery or an enhanced expression of autophagic flux in the absence of biogenesis (Cherra and Chu, 2008, Dagda et al., 2013). PD toxins cause a dysregulation in the autophagy pathway and are considered autophagy inducers, causing an autophagic flux that may be detrimental to neuronal survival (Mizushima, 2007, Chen et al., 2012a, Dagda et al., 2013). Therefore, the levels of autophagy were investigated in the control cells, MPP$^+$-only

treated cells, and pre-treated cells that were exposed to $MPP^+$ for 24 hours. **Figure 4.11** shows that, in the cells treated with $MPP^+$, there was a decreased autophagy activity but pre-treatment with the extracts attenuated the effects of $MPP^+$ at all treatment concentrations, by increasing autophagic activity. Altogether, these results suggest that the ability of the extracts to significantly increase the autophagic levels that the $MPP^+$ decreased may aid their neuroprotective mechanism.

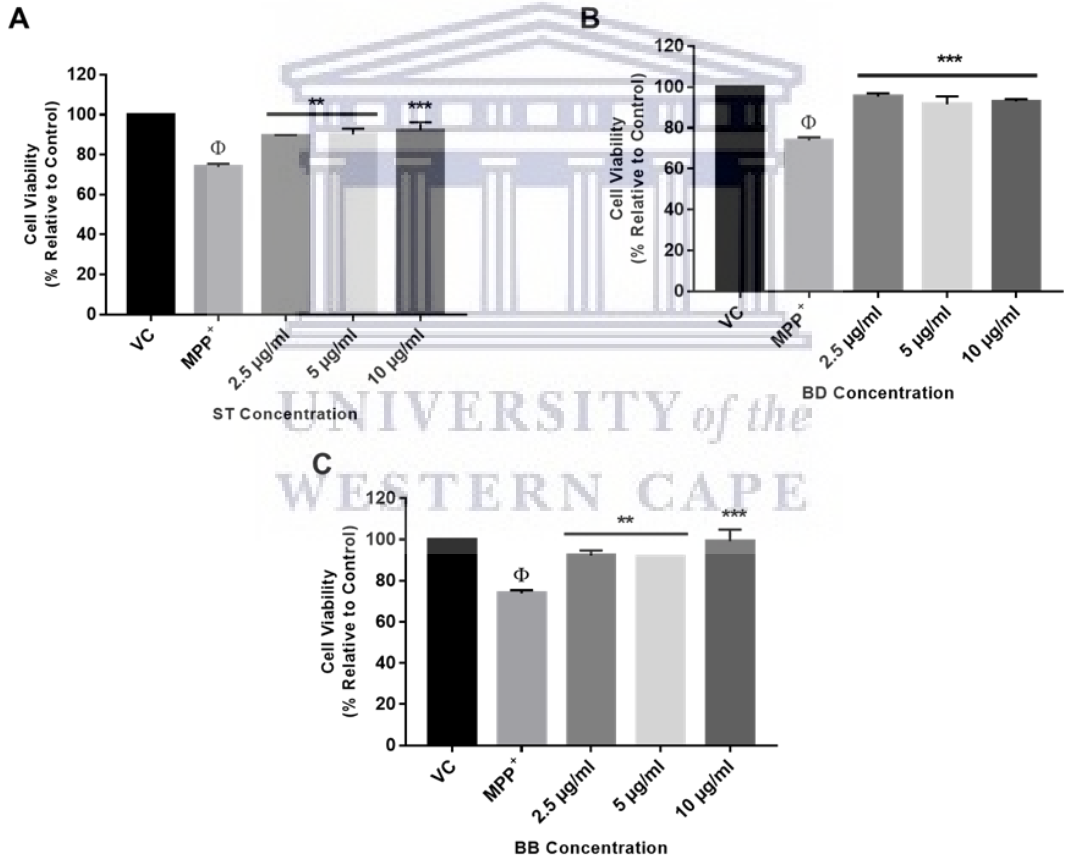



**Figure 4.11**. **Investigating autophagic activity of extracts following exposure of untreated and pre-treated SH-SY5Y cells to $MPP^+$.** The graphs illustrate the effect of the plant extracts (A) *Strumaria truncata (ST)*, (B) *Boophone disticha (BD)*, and (C) *Brunsvigia bosmaniae (BB)* on $MPP^+$ (2000 µM) induced autophagy in SH-SY5Y cells after 24 hours. Cells left untreated were used as a control. The graphs were prepared as mean ± SEM of three experiments (n=3) using GraphPad Prism 7 statistical software. The statistically significant changes are denoted by asterisks (** $p < 0.01$, and *** $p < 0.001$). Control cells vs $MPP^+$ only treated cells is represented by Φ. VC = vehicle control.

### 4.12. Neuroprotective Effects of ST, BD, and BB Extracts Against $MPP^+$ -Induced Apoptosis

Caspases 3/7 being executors of apoptosis have been identified as important events of neuronal apoptosis and serve as vital biomarkers implicated in neuronal death (McIlwain et al., 2015). In addition, various neuronal toxins have been reported to enhance the expression of caspase activity (Glushakova et al., 2017). Therefore, the caspase 3/7 kit was used to investigate apoptosis on the control cells, $MPP^+$ only treated cells and pre-treated cells that were exposed to $MPP^+$ for 24 hours. The results (**Figure 4.12**) show high levels of caspase activity 3/7 (251 %) in the cells treated with $MPP^+$ only when compared to control cells which were set at 100%. However, pre-treatment with the extracts reduced the activities of caspase 3/7. The cells pre-treated with the extract ST attenuated the toxic insult of $MPP^+$ in a concentration-dependent manner (**Figure 4.12A**), while the BD extract also showed a marked reduction of caspase 3/7 activities with the 5 µg/mL showing the most reduction in caspase activity, and the BB extract showed the most significant reduction at the 2.5 µg/mL concentration. Altogether the results indicate that the neuroprotection mechanisms of these extracts include anti-apoptotic capabilities demonstrated by inhibition of $MPP^+$-induced increase in caspase-3 activity.

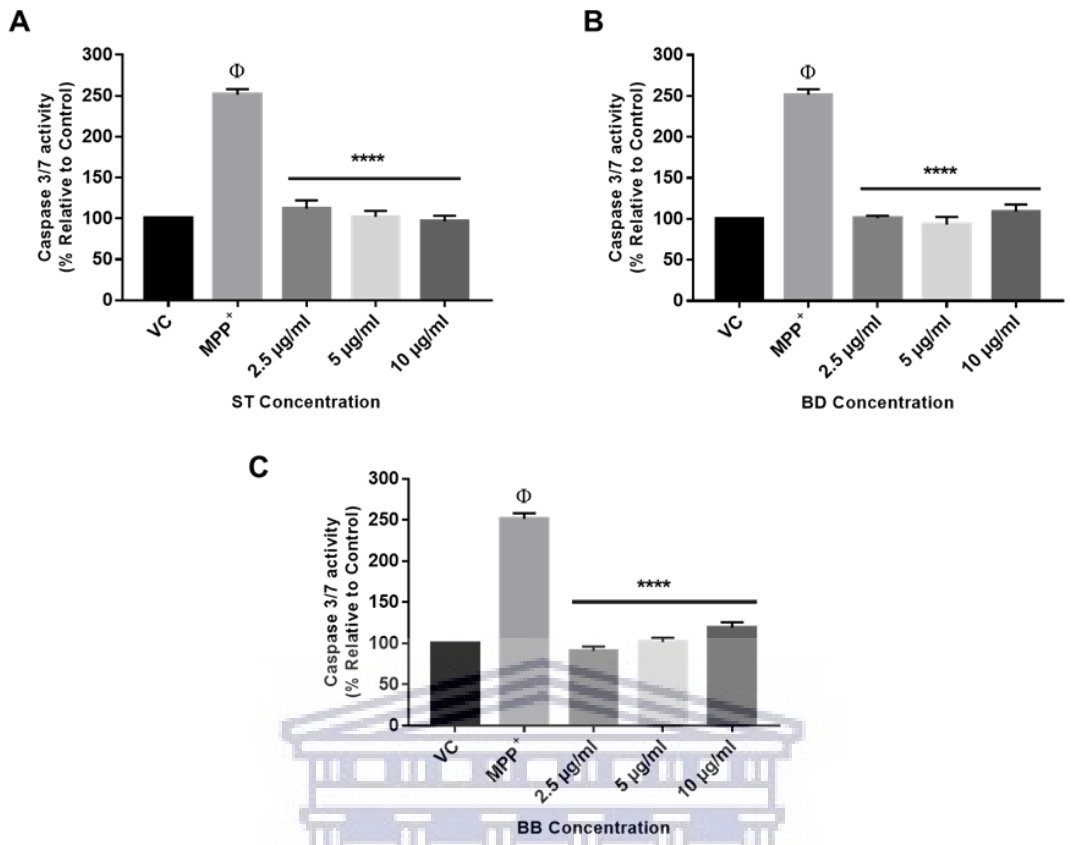



**Figure 4.12**. **Investigating caspase 3/7 activity in toxin-induced untreated and pre-treated SH-SY5Y cells.** The graphs illustrate the effect of the plant extracts (A) *Strumaria truncata (ST)*, (B) *Boophone disticha (BD)*, and (C) *Brunsvigia bosmaniae (BB)* on $MPP^+$ (2000 µM) induced apoptosis. The activity of caspase 3/7 was investigated in SH-SY5Y cells after 24 hours. The graphs were prepared as mean ± SEM of three experiments (n=3) using GraphPad Prism 7 statistical software. The statistically significant changes are denoted by asterisks (****$p < 0.0001$). Control cells vs $MPP^+$ only treated cells is represented by Φ. VC = vehicle control.

# CHAPTER FIVE

# DISCUSSION AND CONCLUSION

## 5.1. Discussion

With the increasing global burden of neurological and neurodegenerative diseases, there is a need for more effective therapeutic approaches. Despite the lack of complete understanding of the mechanisms involved in the pathogenesis of Parkinson's disease (PD), multidisciplinary research in the past decades especially that which involves the development of new treatment techniques, has triggered new hope for novel treatments of neurological conditions (Obeso et al., 2017b, Del Rey et al., 2018, Stoker and Barker, 2020). From several theories, the process of neurodegeneration leading to PD can be attributed to seven main factors which include oxidative stress (ROS, free radicals, NO), proteolysis defects, protein misfolding (α-synuclein protein), immunological reactions, iron metabolism disorders (toxic iron accumulation), mitochondrial dysfunction and apoptosis (Gurunnaselage Don, 2016, Amro et al., 2018, Abramov et al., 2020).



Limitations in the treatment of PD have been attributed to the heterogeneity of syndromes; however, the vast range of factors involved in its etiology provides countless opportunities for more specific treatment options (Kouli et al., 2018). Thus, agents that address different mechanisms of PD can be combined in one, to produce an effective treatment. The application of herbal medicines for treating neurological and non-neurological diseases is the oldest and most widely used medical system, dating back to 2200 years ago (Kim et al., 2012). Despite advancements in medical research, recent studies have shown that herbal medicines are still used widely (Benzie and Wachtel-Galor, 2011, Ekor, 2014). With more plants being investigated for medicinal purposes, there is increasing possibility of developing more effective and less toxic PD drugs from natural sources considering that plants contain bioactive

secondary metabolites that can inhibit different PD mechanisms (Krause and Tobin, 2013, More et al., 2013, Sharifi-Rad et al., 2020).

In the present study, the neuroprotective activities of extracts from the Amaryllidaceae herbaceous family were investigated in an $MPP^+$-induced neurotoxicity model, in SH-SY5Y cells. Cytotoxicity assessment of the plant extracts was carried out to establish the optimum concentrations used in the study independently. Findings from this study show that all extracts were found to increase cell viability compared to the control, in the SH-SY5Y cells. Additionally, the trend observed in the screening experiments showed that the lowest concentration (2.5µg/mL) positively impacted cell viability and this is similar to the findings from a previous study on some Amaryllidaceae species (Ingrassia et al., 2008, Havelek et al., 2014, Lepule, 2017, Lepule et al., 2019, Omoruyi et al., 2020b). Furthermore, when the viability of SH-SY5Y cells was assessed following exposure to the $MPP^+$ toxin and pre-treatment with the plant extracts, the findings also showed that plant extracts protected the cells from the toxicity induced by $MPP^+$. In addition, the images obtained after treatments appeared to show the restoration of cellular morphology and support the neuroprotection potential of the extracts. Other previous studies have also shown that Amaryllidaceae extracts offered protection on SH-SY5Y cells when exposed to harmful toxins (Adewusi et al., 2013, Seoposengwe et al., 2013, Castillo et al., 2016, Lepule, 2017, Lepule et al., 2019, Ibrakaw et al., 2020, Omoruyi et al., 2020a). Therefore, the neuroprotective effects observed in this present study could be attributed to the bioactive compounds in the Amaryllidaceae plant family.

In order to understand the diverse mechanisms of neuroprotection offered by treatment with the Amaryllidaceae extracts, different mechanistic parameters were evaluated. In living cells, the mitochondrial membrane potential (MMP) is considered to be very sensitive to various stress factors (Harshkova et al., 2019) and is critical for cellular homeostasis and survival and also plays an essential role in apoptosis. Thus it is considered a more accurate depiction of

mitochondrial integrity (Osellame et al., 2012). It is known that the neurotoxin $MPP^+$ causes depolarization in membrane integrity due to its inhibitory activities on the mitochondrial complex I function which causes inhibition of ATP production, and leads to compromised membrane potential (Subramaniam and Chesselet, 2013, Kaidery and Thomas, 2018). In healthy mitochondria, the ability of the fluorescent probe TMRE to accumulate, is illustrated by the high TMRE fluorescence intensity observed in the extract pre-treated cells plus $MPP^+$. The charge and solubility of the probe enable it to accumulate in the matrix of the mitochondria. In this study, the CCCP that was used as a positive control has been reported to be an effective mitochondrial membrane disruptor that depolarizes the mitochondrial membranes as cells are not able to retain the fluorescent signal of TMRE (Chalmers and McCarron, 2008, Narendra et al., 2010, Perry et al., 2011, Sivandzade et al., 2019).

Untreated cells exposed to CCCP in this study, showed decreased membrane potential, which is in line with previous reports in literature (Brenner-Lavie et al., 2008, Gómez-Sánchez et al., 2014, Cheng et al., 2016) and a similar decrease was seen in cells exposed to $MPP^+$ only compared with untreated cells, indicating a compromised mitochondrial membrane potential. Cells pre-treated with ST, BD, and BB extracts before $MPP^+$ exposure showed a similar trend in their ability to attenuate the induced toxicity caused by $MPP^+$. All three extracts showed the most effective attenuating potential at lower concentrations than at the highest concentration. This was observed by the minimal attenuating effect the extracts showed on the toxin-induced decrease in membrane potential. Previous studies have also reported that $MPP^+$ exposure compromised the potential of the mitochondrial membrane and decreased the viability of the SH-SY5Y neuronal cells (Chen et al., Kim et al., 2018, Zhong et al., 2018, Enogieru et al., 2020). The data obtained in this present study suggests that the Amaryllidaceae extracts attenuated mitochondrial membrane potential in the SH-SY5Y cells following $MPP^+$ toxicity.

Oxidative stress is a leading event that eventually results in mitochondrial dysfunction and neuroinflammation and the various mechanisms involved in increased ROS production play a vital role in the pathogenesis of PD (Singh et al., 2019a). Free radicals are beneficial when in low amounts, but increased amounts cause membrane lipid peroxidation (Pham-Huy et al., 2008a, Gupta et al., 2014). It has been reported that low levels of ROS can activate cellular survival processes and, while death processes are activated by elevated ROS levels (Poljsak et al., 2013, Lee et al., 2021). The primary source of ROS is mitochondria and excessive ROS production by brain mitochondria could disrupt normal redox signaling (Borza et al., 2013, Kumar and Pandey, 2015). ROS is implicated in various neurodegenerative diseases and its non-free radical form $H_2O_2$, promotes cell death in neuronal cells (Chen et al., 2012b) and intracellular ROS is known to oxidize H2DCF to its fluorescent form DCF (Wang and Roper, 2014, Souza et al., 2019). In the present study, treatment of SH-SY5Y cells with $H_2O_2$ resulted in high levels of ROS production compared to the untreated control cells, which showed high DCF fluorescence intensity. The findings also show that the $MPP^+$-only treatment caused an increase in ROS levels while treatment with the extracts significantly attenuated the $MPP^+$-induced increased ROS activity. This study confirms literature reports that PD neurotoxins are ROS-inducing agents that result in reduced cellular antioxidant status leading to cell death (Drechsel and Patel, 2008). The results obtained are also in line with what has been reported from previous studies that exposure to $MPP^+$ increased ROS levels (Janhom and Dharmasaroja, 2015, Gong et al., 2017, Kim and Park, 2018, Yan et al., 2018, Zhong et al., 2018, Enogieru et al., 2020, Ibrakaw et al., 2020). This study suggests that the attenuation of toxin-induced increase in ROS levels validates the antioxidant properties of Amaryllidaceae extracts.

Nitric oxide (NO) has been reported to play a vital role in enhancing synaptic transmission, regulating ATP channels, potassium channels, and calcium-activated potassium channels in the CNS (Kawano et al., 2009). Regardless of the plethora of physiological roles that NO has, it is

essential to acknowledge that elevated NO levels are harmful and could induce dopaminergic neuronal death (Pacher et al., 2007, Galluzzi et al., 2018), by blocking electron transport chain activity and depleting cellular energy, which damages the mitochondria, causing a release of cytochrome c, activation of caspase activity, and eventually apoptosis (Perier and Vila, 2012, Di Meo et al., 2016). The current study shows that the $MPP^+$ toxin caused only a slight, non-significant increase in NO levels compared to the control, which is in variance with reports from previous studies that showed extremely high intracellular NO levels in $MPP^+$-only treated SH-SY5Y cells (Wang et al., 2016, Singh et al., 2018, Yang et al., 2018). Altogether, these findings tend to suggest that although $MPP^+$ did not entirely elevate NO production as seen in other studies, even the minimal increase observed was attenuated by pre-treatment with the extracts under study.

The $Ca^{2+}$ pathway is involved in the release of neurotransmitters, intersecting with oxidative stress and mitochondrial function (Alexander, 2004, Moghaddam et al., 2017). Ageing and pathological conditions alter intracellular $Ca^{2+}$ signaling and cause significant changes in neuronal excitability, thereby negatively affecting neuronal network activity and metabolism (Gleichmann and Mattson, 2011, Zündorf and Reiser, 2011). Elevated intracellular $Ca^{2+}$ levels may be highly toxic to cells because they lead to the opening of the mitochondrial permeability transition pore (mPTP) which in turn causes a release of cytochrome C, eventually leading to apoptosis (Norenberg and Rao, 2007, Redza-Dutordoir and Averill-Bates, 2016). Neurotoxins have been reported to alter $Ca^{2+}$ homeostasis. A study by Sukumaran and colleagues (2018) elucidated that the disturbance of $Ca^{2+}$ levels by neurotoxins ($MPP^+$ and 6-OHDA) caused a decreased expression of the Transient Receptor Potential Canonical (TRPC-1) by inhibiting its binding to NF-kB. Their study revealed that the decreased expression of TRPC-1 decreases cell survival by promoting apoptosis and inhibiting autophagy (Sukumaran et al., 2018a). In this study, the elevated levels of $Ca^{2+}$ induced by $MPP^+$ treatment were differentially inhibited

by pre-treatment with all the extracts, and this was similar to findings from a previous study in which pre-treatment with the extracts of BD modulated the calcium levels elevated by a parkinsonian toxin 6-OHDA (Lepule et al., 2019).

High levels of cellular energy (ATP) are required to maintain optimum levels of $Ca^{2+}$ therefore, the disruption caused by PD toxins on $Ca^{2+}$ also suggests a likely inhibition in ATP synthesis (Zaichick et al., 2017, Zolkipli-Cunningham and Falk, 2017). Of all the electron transport chain (ETC) products, ATP is one of the most significant products, and it is vital for the maintenance of cellular functions (Bonora et al., 2012). Upon its uptake by dopaminergic neurons, $MPP^+$ induces toxicity by disrupting electron flow, resulting in an inhibition of ATP production (Storch et al., 2000, Subramaniam and Chesselet, 2013, Choi et al., 2015). In the present study, $MPP^+$ lead to ATP degradation in the cells and upon pre-treatment with plant extracts, a reversal of these effects was observed, which confirms their neuroprotective potentials. These results are similar to those in studies that reported that Amaryllidaceae plants showed great potential in restoring ATP production following toxin-induced suppression (Lepule, 2017, Lepule et al., 2019, Ibrakaw et al., 2020, Omoruyi et al., 2020a).



In 1963, the work of Christian de Duve brought the literal meaning of the cellular death mechanism "autophagy" into bear, as he demonstrated that autophagy was indeed a system that involved delivering of intracellular materials to lysosomes for degradation (Mizushima, 2018). Various conditions are implicated in the induction of autophagy, including low energy supply, lack of nutrients, and decreased oxygen levels (Codogno and Meijer, 2005). Autophagy can be neuroprotective (to promote neuronal cell survival in situations of inflammation, degeneration, and other stresses) or destructive in which case there is increased and dysfunctional removal of unwanted cellular material causing an overactivation of autophagy leading to the degradation of essential proteins and organelles (Michel et al., 2016, Anding and Baehrecke, 2017).

In this study, the ability of BB, BD and ST to induce autophagy following exposure to $MPP^+$ was investigated and the extracts were found to confer protection to the cells following $MPP^+$-induced toxicity, by enhancing the effects of autophagy, which may be one of the underlying mechanisms by which the extracts offer neuroprotection to the cells. These results are similar to a previous *in vitro* study by Kim et al. (2014) on SH-SY5Y cells in which chebulagic acid was found to offer protection following $MPP^+$-induced toxicity to the cells. The study reported that chebulagic acid upregulated the expression of autophagic inducers, which in turn enhanced autophagy without inducing toxicity to the cells, thus offering neuroprotection (Kim et al., 2014). Together, these results suggest that $MPP^+$, like other neurotoxins used in PD models, impairs autophagy (Li et al., 2015, Moors et al., 2017, Sukumaran et al., 2018b).

Apoptosis, which is programmed cell death, is a common vital form of dopaminergic neuronal death in PD, employed to regulate cell proliferation (Chi et al., 2018). Additionally, low doses of $MPP^+$ and other neurotoxins have been reported to trigger neuronal cell death via apoptosis, while at higher doses, they activate neuronal cell death via necrosis (Elmore, 2007, Orrenius et al., 2010). In *in vitro* and *in vivo* experimental models, neurotoxins such as $MPP^+$ have been implicated in inducing neurotoxicity by activating the caspase 3 pathway (Zeng et al., 2018), usually via an apoptosome complex that leads to the release of cytochrome c, hyper-activated microglial response, enhanced neurotoxicity and eventually neuronal death (Parrish et al., 2013). In this study, the caspase 3/7 assay was used to determine the apoptotic pathway induced by the neurotoxin $MPP^+$ in the SH-SY5Y cells. Our results showed that cells treated with $MPP^+$ only, had an elevated caspase activity when compared to the control cells, and this is similar to previous report that showed that $MPP^+$ toxicity is mediated by the activation of caspase 3 activity leading to the death and depletion of dopaminergic neurons (Chu et al., 2005, Ramachandiran et al., 2006, Zeng et al., 2006, Sukumaran et al., 2018b). In this study, all three

extracts significantly ameliorated the enhanced caspase activity induced by the toxin, possibly by inhibiting caspase 3 activity.

### 5.2 Conclusion

Based on the results of this study, the BB, BD and ST extracts, all promoted increase in cell viability by ameliorating toxicity induced by $MPP^+$ on the SH-SY5Y neuroblastoma cell line, suggesting that the extracts can offer neuroprotection. Although pre-treatment with the plant extracts produced no major significant difference between the $MPP^+$ and control cells when NO levels were investigated, their effects in ameliorating $MPP^+$-induced suppression of ATP production, decrease in the elevated calcium levels, decrease in the elevated ROS levels, elevated caspase activity, confer scientific validation for their use as potent therapeutic options for PD. In addition, enhancement of autophagy by these plant extracts potentially inhibits the loss of dopaminergic neurons, hence playing a vital role in slowing the progression of the disease. It is plausible to suggest that pre-treatment with the extracts induced autophagy as a survival mechanism while still conferring anti-apoptotic properties.



In conclusion, the neuroprotective effects of the three Amaryllidaceae plant extracts against $MPP^+$ toxicity was confirmed by the present study, attributable to their ability to enhance antioxidant defence mechanisms and demonstrate anti-apoptotic effects, thereby downregulating the oxidative stress highlighted in cellular models of PD. As there is a need for the development of drugs that ameliorate especially oxidative damage in PD (Calderón et al., 2010, Benedec et al., 2018, Cortes et al., 2018, Leporini et al., 2018, Lepule et al., 2019, Omoruyi et al., 2019, Omoruyi et al., 2020a), the promising antioxidant effects of the Amaryllidaceae plant extracts make them promising novel natural product candidates for the treatment of PD. These low-cost and readily available plants require further extensive *in vitro* and *in vivo* scientific studies including phytochemical, pharmacological and toxicological

standardization, and optimization processes, that would lead to the production of nutraceutical and botanical products for PD, that can possibly undergo clinical trials, to prepare them for the market value chain.

### 5.2.1 Study limitations

The inability to perform more assays and the use molecular techniques to ascertain the potential mechanism of actions of the extracts were some limitations experienced in this study and these could be attributed to time restraints and limited funds. In addition, the objective of this study was to study only the effects of the crude extracts and not the active compounds in the plant extracts.

### 5.2.2 Future Recommendations

Future recommendations for this study would be:

1. Screening other neurotoxins like rotenone and paraquat to determine the one that best models PD.
2. Isolation of the bioactive active compounds from all three plant extracts and evaluation of their neuroprotective efficacy in modulating PD-related antioxidant effects, based on superoxide dismutase (SOD), malondialdehyde (MDA), catalase (CAT), glutathione (GSH) and lactate dehydrogenase (LDH) activity assays.
3. To investigate protein aggregation and protein misfolding using such molecular techniques as western blotting, which could also aid in measuring autophagic biomarkers enhanced.
4. After isolating bioactive compounds, molecular docking would be recommended to aid in understanding the ability of the isolated bioactive compounds to interact with the actual biological molecules that are involved in the ligand-binding complexes in body systems, relevant to the drug delivery design strategy (Ma et al., 2011, De Ruyck et al., 2016, Pinzi and Rastelli, 2019). This would also aid in drug optimization and repositioning.

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