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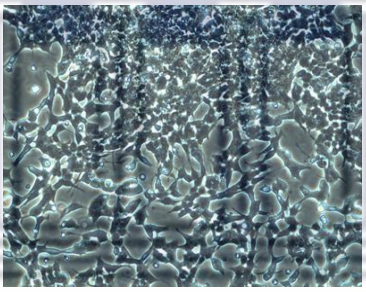
In vitro Neuroprotective Potential of Phyllanthin in MPP^+-Induced Toxicity in SH-SY5Y Neuroblastoma Cells



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A thesis submitted in fulfilment of the requirements for the degree of Master of Science (MSc) in Medical Bioscience, in the Department of Medical Biosciences Faculty of Natural Sciences, University of the Western Cape.

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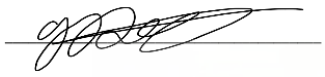
June 2023

DECLARATION

I, Joshua Delport, declare that the thesis titled *"In vitro* Neuroprotective Potential of Phyllanthin in MPP^+-Induced Toxicity in SH-SY5Y Neuroblastoma Cells*"* is my own work, that it has not been submitted for any degree or examination at any other university, that it is free of plagiarism and that all the sources used have been indicated and acknowledged by complete references.



15 June 2023

Joshua Delport — Date

DEDICATION

This thesis is dedicated to my belated grandmother and loving mother, Shirley Rosina Maria Delport, who has been a constant source of support and encouragement during the challenges of academics and life. I am truly grateful and in awe of your patience, strength and of the sacrifices which you have made. I know it was not easy to raise me as a single parent.

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List of Abbreviations

6-OHDA	6-hydroxy-dopamine
α-synuclein	Alpha-synuclein
Ach	Acetylcholine
AChE	Acetylcholinesterase
AD	Alzheimer's disease
AIF	Apoptosis-inducing factor
ALS	Amyotrophic Lateral Sclerosis
ATP	Adenosine triphosphate
BBB	Blood-brain barrier
BuChE	Butyrylcholinesterase
Ca^{2+}	Calcium
CCCP	Carbonyl cyanide 3-chlorophenylhydrazone
Cl^-	Chloride
CNS	Central nervous system
CT	Computed Tomography
DAT	Dopamine Transporter
DBS	Deep Brain Stimulation

DIP	Drug Induced Parkinsonism
DI	Discrimination index
DLB	Dementia with Lewy Bodies
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DRT	Dopamine replacement therapy
Fe^{2+}	Iron
FUS	Fused in sarcoma
eNOS	Endothelial Nitric oxide synthase
ETC	Electron transport chain
FBS	Fetal Bovine Serum
GABA	Gamma-aminobutyric acid
GAD	Glutamic acid decarboxylase
GBA1	Glucocerebrosidase 1
GCase1	β-glucocerebrosidase 1
Glu	Glutamate
GPx	Glutathione peroxide
H_2O_2	Hydrogen peroxide
HD	Huntington's Disease
HIV	Human Immunodeficiency Virus

HY	Hoehn and Yahr
iNOS	Inducible Nitric oxide synthase
K^+	Potassium
LBs	Lewy bodies
L-DOPA	L-dihydroxyphenylalanine
LCIG	levodopa carbidopa intestinal gel
LRRK2	Leucine-rich repeat kinase 2
MAO	Monoamine oxidase
MAO-B	Monoamine oxidase B
MDS	Movement Disorder Society
MMP	Mitochondrial membrane potential
MPP^+	1-methyl-4-phenylpyridinium
MPPP	1-methyl-4-phenyl-4-propionoxypiperidine
MRI	Magnetic Resonance Imaging
MS	Multiple Sclerosis
MSA	Multiple System Atrophy
mtDNA	Mitochondrial DNA
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
Na^+	Sodium
NADH	Nicotinamide adenine dinucleotide

NCEs	New chemical entities
NDDs	Neurodegenerative disorders
NFTs	Neurofibrillary tangles
nNOS	Neuronal Nitric oxide synthase
NO	Nitric oxide
NOS	Nitric oxide synthase
·OH	Hydroxyl radical
O_2^-	Superoxide
PBS	Phosphate Buffered Saline
PCD	Programmed cell death
PC12	Pheochromocytoma cells 12
PD	Parkinson's disease
PINK1	PTEN-induced kinase protein 1
REM	Rapid Eye Movement
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
SA	South Africa
SSA	Sub-Saharan Africa
SWEDDs	Scans Without Evidence of Dopaminergic Deficits
TB	Tuberculosis
TDP-43	TAR DNA-binding protein 43
TCA	Tricarboxylic acid cycle
TH	Tyrosine hydroxylase
TMRE	Tetramethylrhodamine ethyl ester
TRAIL	Tumour necrosis factor-related apoptosis-inducing ligand
UK	United Kingdom
UN	United Nations
UPDRS	Unified Parkinson's Disease Rating Scale
USA	United States of America
VPS35	Vacuolar Protein Sorting 35
WHO	World Health Organization

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Research Outputs

Omoruyi, S. I.; **Delport, J**.; Kangwa, T. S.; Ibrakaw, A. S.; Cupido, C. N.; Ekpo, O. E. & Hussein, A. A. 2020. In vitro neuroprotective potential of *Clivia miniata* and *Nerine humilis* (Amaryllidaceae) in MPP^+-induced neuronal toxicity in SH-SY5Y neuroblastoma cells. *South African Journal of Botany*, *136***,** 110-117. DOI: https://doi.org/10.1016/j.sajb.2020.06.028.

Abstract

Parkinson's disease (PD) is the second most common age-related neurodegenerative disease after Alzheimer's disease, with an estimated 9 million people projected to be affected worldwide by the year 2030. PD is associated with numerous motor symptoms such as tremor, bradykinesia (slowness of movements), hypokinesia (reduction in movement amplitude) and akinesia (absence of normal unconscious movements). However, in addition to these motor symptoms, several non-motor manifestations are common, such as sensory symptoms (pain, tingling), hyposmia, sleep disturbance, depression, and cognitive impairment.

Although the etiology of PD is not fully understood, its pathological hallmarks are characterized by the presence of Lewy bodies (abnormal protein aggregates containing α-synuclein), microgliosis (the accumulation of activated microglial cells) and the excessive death of dopaminergic neurons in the substantia nigra pars compacta region of the brain. Currently, there is no known cure for PD with dopamine replacement therapy (mainly levodopa) being the gold standard for treating PD-associated motor symptoms. Long-term levodopa treatment often leads to disabling side effects, hence there is a need for alternative treatment methods.

This study investigated the potential neuroprotective effects of Phyllanthin, the bioactive compound in *Phyllanthus amarus*, using an *in vitro* model of PD, the 1-methyl-4-phenylpyridinium (MPP^+)-induced SH-SY5Y neuroblastoma cellular model. The cytotoxic effects of phyllanthin and MPP^+ were assessed using the MTT cell viability assay, which was also used to determine the optimum concentrations of phyllanthin and MPP^+ to be used in further experiments. The effects of phyllanthin against MPP^+-induced nitric oxide (NO) levels, mitochondrial membrane potential (MMP), intracellular calcium levels, adenosine triphosphate (ATP) levels and caspase 3/7 activities in the SH-SY5Y cells were also investigated, using appropriate assay kits. Furthermore, molecular docking was performed to determine the interaction of phyllanthin with the enzymes acetylcholinesterase (AChE), butyrylcholinesterase (BuChE) and monoamine oxidase-B (MAO-B).

The results obtained showed that phyllanthin could protect the cells from MPP^+-induced toxicity, significantly increased the viability of MPP^+-treated cells, attenuated mitochondrial dysfunction by increasing ATP production in the cells and preventing cell death through the inhibition of

MPP^+-induced increase in caspase 3/7 activities. In addition, the results from the *in silico* studies and enzymatic assays, showed that phyllanthin poorly binds to AChE, BuChE and MAO-B and displayed poor inhibitory activities on these enzymes. Taken together, the results tend to suggest that phyllanthin has neuroprotective potential against the cytotoxic effects of MPP^+, thus supporting its use as a neuroprotective agent in other experimental models of neurotoxicity. More experiments are needed to elucidate its mechanisms of action.

Keywords: Phyllanthin, SH-SY5Y, MPP^+, Parkinson's Disease, Neuroprotection.

CHAPTER ONE

Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disease, first described by James Parkinson in his 1817 monograph "Essay on the Shaking Palsy" (Dauer and Przedborski, 2003). It is the second most common age-related neurodegenerative disease after Alzheimer's disease, with an estimated 9 million people to be affected by 2030, worldwide (de Lau and Breteler, 2006). PD presents with numerous motor symptoms such as tremor, which occurs at rest but decreases with voluntary movement, increased rigidity of PD patient's limbs, postural and gait impairments, bradykinesia (slowness of movements), hypokinesia (reduction in movement amplitude) and akinesia (absence of normal unconscious movements) which in itself manifests as a variety of symptoms, including paucity of normal facial expression, decreased voice volume, drooling and failure to swallow without thinking about it (Lopes et al., 2010). In addition to motor symptoms, several non-motor manifestations are common, such as sensory symptoms (pain, tingling), hyposmia, sleep disturbance, depression, and cognitive impairment (Nystrom, 2016). The pathological hallmarks of PD are characterized by the immense death of dopaminergic neurons in the substantia nigra, the presence of Lewy bodies (abnormal protein aggregates containing α-synuclein) and microgliosis (the accumulation of activated microglial cells) (Xicoy, Wieringa and Martens, 2017).

The burden of PD worsens as age progresses with the initial onset of the disease occurring at around age 60, affecting only 2% of the population and rises to about 12% at age 70, with men being at a 50% higher risk of contracting PD than females (Lopes et al., 2010; Dauer and Przedborski, 2003). Despite the incidence of PD being strongly related to age, a cross sectional study by Wickremaratchi et al., 2009 found that approximately 30% of the patients are younger than 65 years of age at the time of diagnosis (Wickremaratchi et al., 2009). To date the treatment for PD is quite challenging, hence the need to explore other therapeutic options including medicinal plants, bioactive compounds and other natural products.

Plants have been used as medicines for thousands of years. these medicines initially took the form of crude drugs such as poultices, teas, powders, and other herbal formulations (Greenwell and Rahman, 2015). Studies show that plant extracts from the Amaryllidaceae family (*Clivia miniate*

and Nerine humilis), and *Boophone haemanthoides* have neuroprotective capabilities as they were able to inhibit apoptosis and decreased toxin-induced oxidative stress, in an MPP^+-induced SH-SY5Y model of PD (Omoruyi et al., 2020, Ibrakaw et al., 2020). In addition to *in vitro* studies, many medicinal plants and their compounds have been successful in alleviating the symptoms associated with neurodegenerative disorders (NDD) (Luthra and Roy, 2021, Rathees et al., 2017). Since ancient times, *Mucuna pruriens* (*M. pruriens*) has been used in ayurvedic medicine to treat the symptoms of PD (Ovallath and Deepa, 2013). Studies in both animal and human subjects have demonstrated that treatment with *M. pruriens* seed improves locomotor behaviour (Jansen et al., 2014, Singhal, Lalkaka and Sankhla, 2003). Further studies reported that *M. pruriens* also improves synaptic and mitochondrial functions, essential for neuronal survival (Poddighe et al., 2014). In traditional Chinese medicine, the bark of *Magnolia Officinalis* (talauma) is used to treat neurosis, anxiety, stroke, and dementia. (Singhal, Bangar and Naithani, 2012). *Ginkgo biloba (G.biloba),* another plant used in traditional Chinese medicine has been reported to have neuroprotective capabilities (Maitra et al., 1995; Marcocci et al., 1994). Also, EGb 761 or Ginkor, a standardized extract of *G. biloba*, has been shown 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 (Singh et al., 2019, Abd El-Aziz, 2012).

In addition to *G. biloba*, *Curcuma longa* (*C. longa*), commonly known as turmeric, has also been reported to have neuroprotective potential. *C. longa* protects against damage to the blood-brain barrier, neural cell death and brain aging (Jiang et al., 2007, Bala, Tripathy and Sharma, 2006). Further studies have shown that curcumin, an important component of *C. longa*, maintains mitochondrial complex I activity and protects against nitrosative stress *in vitro* (Mythri et al., 2011). Another study, using a 6-OHDA-induced rat model of PD, reported that curcumin protects against neuronal anomalies in the SN region of the brain (Song et al., 2016). *Withania somnifera* (*W. somnifera*), another plant used to treat PD, attenuates the increased oxidative stress in the MPTP-induced mouse model of PD (RajaSankar et al., 2009). Similarly, *Bacopa monnieri* (*B. monnieri*) exhibits antioxidative and neuroprotective properties. *B. monnieri* has also been reported to enhance memory and cognitive functions (Kumar et al 2016, Mathur et al., 2010). Additionally, *Phyllanthus amarus* (*P. amarus*) has also been reported to produce a dose-dependent improvement in the memory scores of mice. Furthermore, *P. amarus* also successfully reduced brain

cholinesterase activity and reversed scopolamine and diazepam induced amnesia (Joshi and Parle, 2007).

In addition to the multitude of cognitive benefits, numerous studies have also demonstrated the motor benefits of medicinal plants. Jahromy et al., 2014 showed that the oral administration of *Nigella sativa* hydroalcoholic seeds extract (100 and 200 mg/kg) improved perphenazine-induced muscle rigidity score in mice (Jahromy et al., 2014). In 2017, Aruna, Rajeswari and Sankar tested the neuroprotective potential of *Oxalis corniculate* (*O. corniculate*), in MPTP-induced mice. Their study demonstrated that treatment with *O. corniculate* overturned the changes in muscle coordination induced by MPTP (Aruna, Rajeswari and Sankar, 2017). Additionally, a 2020 study to assess the effect of *Phyllanthus muellarianus* (*P. muellarianus*) on motor function and coordination showed that aqueous leaf extract of *P. muellarianus* significantly attenuated ciprofloxacin-mediated increases in narrow beam, gait scores and landing foot splay distance in male rats (Ibitoye, Aliyu and Ajiboye, 2020). Furthermore, in 2022, Wu et al demonstrated that *Artemisia argyi* reversed MPP^+-induced impaired motor function in an MPTP PD mouse model (Wu et al., 2022). Finally, a 2023 study by Enemali et al showed that Aqueous leaf extract of *Phyllanthus amarus* protected the motor activity of wistar rats against paraquat exposure.

1.1 Rationale for the Study

Parkinson's disease (PD) is the most common neurodegenerative movement disorder, with an estimated 9 million people to be affected by the year 2030, worldwide (de Lau and Breteler, 2006). Normal motor function is heavily dependent on the coordinated synthesis and release of dopamine by the neurons projecting from the substantia nigra to the corpus striatum (Chase et al., 1998). Degradation of these dopaminergic neurons are mainly responsible for the movement disturbances associated with Parkinson's disease (Nagatsu et al., 2000, Chase et al., 1998). The use of levodopa as dopamine-replacement therapy is highly effective in treating the symptoms of PD and remains the standard drug to which other therapies are compared. However, prolonged use of levodopa and dopamine can generate reactive oxygen species and induce the degeneration of cultured dopaminergic neurons (Ogawa, 1994). Considering this, concerns have been raised that levodopa could enhance oxidative stress and speed up the degeneration of residual dopaminergic neurons in patients with PD. Hence there is the need for new treatment options and herbal remedies have

received considerable interest due to the broad spectrum of their pharmacological properties which can be explored for the clinical management of neurodegenerative diseases (Omoruyi et al., 2020). Therefore, the current study investigated the neuroprotective potential of phyllanthin, the primary active ligan of *Phyllanthus amarus* (*P. amarus*), a plant that has recently received increasing attention because of its remarkable antioxidant, anti-inflammatory properties and other properties (Wannannond et al., 2011). Success in this *in vitro* study would contribute to the ongoing efforts to develop alternative treatments capable of addressing the limitations associated with current treatment options. Additionally, the study would provide preliminary evidence that can be validated by *in vivo* techniques.

1.2 Aim of the Study

The aim of this study was to investigate the neuroprotective potential of Phyllanthin, the major bioactive compound in *Phyllanthus amarus* plant extract, against MPP^+-induced SH-SY5Y neuroblastoma toxicity.

1.3 Objectives of the Study

The objectives of this study were to:

i. determine the cytotoxicity of Phyllanthin in SH-SY5Y cells after 24 hours exposure.
ii. determine the concentration of MPP^+ that induced approximately 50% cell death in the SH-SY5Y cells after 24 hours.
iii. ascertain the survival of SH-SY5Y cells following pre-treatment with selected concentrations of Phyllanthin prior to MPP^+ exposure.
iv. observe morphological changes in SH-SY5Y cells after pre-treatment with selected concentrations of Phyllanthin prior to MPP^+ exposure.
v. ascertain changes in Nitric Oxide (NO) levels in SH-SY5Y cells pre-treated with selected concentrations of Phyllanthin prior to MPP^+ exposure.
vi. determine changes in the mitochondrial membrane potential of SH-SY5Y cells after pre-treatment with selected concentrations of Phyllanthin prior to MPP^+ exposure.
vii. measure levels of ATP production in SH-SY5Y cells after pre-treatment with selected concentrations of Phyllanthin prior to MPP^+ exposure.

viii. measure the intracellular calcium concentration in SH-SY5Y cells after pre-treatment with selected concentrations of Phyllanthin prior MPP^+ exposure after 24 hours.

ix. ascertain levels of Caspase 3/7 activities in the SH-SY5Y cells following pre-treatment with selected concentrations of Phyllanthin prior to MPP^+.

1.4 Research Question/ Hypothesis

This study hypothesized that the selected concentrations of Phyllanthin will prevent the adverse effects of MPP^+-induced toxicity *in vitro* on SH-SY5Y human neuroblastoma cells.

1.5 Thesis Outline

- Chapter 1 introduces the project by stating the rationale behind the study, the aims and objectives, techniques used, and the compound chosen.
- Chapter 2 provides a detailed review of the literature on neurodegenerative disorders and the role of traditional herbal medicines and their antioxidative properties that help to alleviate the deleterious effects of MPP^+-induced toxicity on neural cells.
- Chapter 3 focuses on the methodology used in this study and provides a list of materials, experimental procedures and tests done.
- Chapter 4 presents the key findings of this study.
- Chapter 5 gives a detailed discussion of results, the conclusion and summary of the entire study as well as limitations of study and future recommendations.

CHAPTER TWO

Literature review

2.1 Neurodegenerative Disorders (NDDs)

Neurodegenerative disorders (NDDs) result from a deterioration of neurons which in turn leads to a progressive loss of selectively vulnerable population of neurons. NDDs can be grouped based on primary clinical features (e.g., parkinsonism, motor neuron disease or dementia) and anatomic distribution of neurodegeneration (e.g., spinocerebellar degenerations, extrapyramidal disorders, or frontotemporal degenerations), with pyramidal and extrapyramidal movement disorders as well as behavioral and cognitive disorders being the commonest. Most patients display mixed clinical features, with very few having pure syndromes. Additionally, NDDs are also defined by specific protein accumulations, and protein abnormalities that define NDDs may be present prior to the onset of clinical features (Dugger et al., 2017).

Motor neuron disease (MND), Alzheimer's disease (AD) and Parkinson's disease (PD) are the common degenerative brain disorders with substantial economic implications to the society. More than 20 million people worldwide are affected by the severe consequences of these three diseases (Ben-Shlomo, Whitehead and Smith, 1995) ref. AD is defined by progressive dementia and is known to also display some of the pathological changes and motor features found in Parkinson's disease. Similarly, both PD, primarily a disorder of motor function and movement involving the neostriatum and basal ganglia, and MND, a disease characterized by muscle weakness and wasting involving the lower and upper motor neurons of the brainstem and spinal cord, can also be accompanied by progressive dementia (Mayeux, 2013). Regardless of the differences in their distinct clinical manifestations, these 3 diseases share numerous features, including occurrence as either a sporadic or inherited disorder, onset at middle age or during the later years of life, extra- or intracellular aggregation as well as the deposition of altered proteins (Taylor, Hardy and Fischbeck, 2002).

2.1.1 Alzheimer's Disease (AD)

AD is an incurable, chronic and progressive NDD caused by the accumulation of proteins and extracellular senile plaques (Gray et al., 2015, Kim et al., 2015). Progressive damage to the brain cells results in cognitive dysfunctions that leads to memory impairments. Other symptoms include

language and cognitive impairment, psychosis, depression and agitation (Mahdy et al., 2012). Oxidative stress and mitochondrial dysfunction are considered to be the major causes of neuronal cell death in AD (Leuner and Reichert, 2012). The loss of redox homeostasis in an AD-brain leads to chronic oxidative stress that increases the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Huang et al., 2016). ROS build-up in neurons leads to the modification of sugars, oxidation of proteins and lipid peroxidation, ultimately resulting in neuronal cell death in parts of the hippocampus.

2.1.2 Multiple Sclerosis (MS)

MS is a chronic disorder characterized by the demyelination of neurons in both grey and white matter of the brain and spinal cord, although the observed demyelination is a lot more prominent in white matter (Kutzelnigg and Lassmann, 2014). MS is also categorized as a disorder of the immune system, in which T cells target the central nervous system CNS self-antigen in genetically susceptible individuals. Initial lesions are primarily formed in focal regions of demyelinated white matter; these focal regions are called plaques. The pathological symptoms of MS may vary with the locations of the plaques but are basically associated with the infiltration of T cells across the blood-brain barrier ADD SYMPTOMS OF MS (Polman et al., 2011). This demyelination is thought to impair neural communication.

2.1.3 Amyotrophic Lateral Sclerosis (ALS)

ALS is a chronic heterogeneous neurological disorder characterized by the degeneration of both the lower and upper motor neurons (Logroscino et al., 2010). ALS has two major forms; familial and sporadic. The sporadic form is most common despite having no hereditary history, whereas the familial type has a genetically inherited component (Zarei et al., 2015, Abhinav et al., 2007). Apart from cellular stress, it is hypothesized that the accumulation of intraneuronal proteins superoxide dismutase (SOD1), TAR DNA-binding protein 43 (TDP-43), and fused in sarcoma (FUS) disrupts normal protein homeostasis, inducing ALS. These proteins are frequently identified in animal models and pathological studies of patients suffering from ALS (Morgan and Orrell, 2016). Initial symptoms of ALS include twitching, cramping, muscle tenderness, and muscle impairment (Goetz, 2000). Later in the advanced stages of the disease, patients experience

difficulty swallowing, speaking and breathing (dysphagia, dysarthria and dyspnea) (Kori et al., 2016).

2.1.4 Huntington's Disease (HD)

HD is an autosomal dominant neurological disease. Due to its progressiveness, autosomal dominant inheritance pattern, and the combination of cognitive, motor and behavioral impairments, HD is traumatizing for both patients and their relatives (Bates et al., 2015). Pathologically, HD is the result of an expanded trinucleotide repeat of CAG sequence in the gene HTT5 on chromosome 4, encoding the abnormal pathogenic multifunctional protein named Huntingtin. The mutant protein has an unusual polyglutamine sequence, correlating with the expanded CAG repeat, which has been shown to be toxic in nature, and leads to neuronal dysfunction and death. Neurons in the striatum are particularly susceptible to this mutant protein. However, HD has been documented as a disorder affecting the whole brain and body. Abnormality of the huntingtin protein leads to neuronal death via several mechanisms key of which are: the tendency of the mutant protein to form aggregates with direct effect on protein homeostasis, axonal transport and mitochondrial function (Bates et al., 2014, Ross et al., 2014). Glutamate excitotoxicity and a decrease in brain-derived neurotrophic factors are the other hypotheses involved in neural damage of HD (Bates et al., 2015).

2.2 Parkinson's Disease (PD)

PD is the second most common neurodegenerative disease after Alzheimer's disease, with a predicted prevalence of 9 million people worldwide by 2030 ADD CURRENT STATS (de Lau and Breteler, 2006). The initial manifestations of the disease occur at approximately 60 years, affecting a mere 2% of the population rising to 12% at 70 years of age (Lopes et al., 2010). It presents with motor and non-motor symptoms that worsen with advancing age, leading to a need for assistance with all daily activities. Disease manifestation is characterized by the presence of Lewy bodies (abnormal protein aggregates containing α-synuclein), the death of dopaminergic neurons in the substantia-nigra and micro gliosis (an accumulation of activated microglial cells) (Xicoy, Wieringa and Martens, 2017).

2.2.1 Proteins Involved in Progression of PD

The most frequently occurring neurodegenerative disorders are tauopathies, amyloidosis' alpha-synucleinopathies and transactivation-response DNA-binding protein 43 (TDP-43) proteinopathies. The abnormal protein structure in these disorders, paired with their neuroanatomical and cellular distribution, are the major histopathologic features required for making a specific neuropathological diagnosis. Examples of these protein accumulations in neurons include alpha-synuclein in lewy bodies, tau in neurofibrillary tangles (NFTs) or Pick bodies, and TDP-43 in neuronal cytoplasmic and intranuclear inclusions. Protein accumulations in oligodendroglia include alpha-synuclein in glial cytoplasmic inclusions and tau in coiled bodies. Protein accumulations in astrocytes include astrocytic plaques, tau in tufted astrocytes and thorn-shaped astrocytes. These abnormal protein aggregates are made of intrinsic proteins and other neuro-cellular components, unlike the neuronal inclusions found within viral infections where the protein is foreign.

2.2.2 Synucleinopathies and PD

This class of NDD is characterized by the accumulation of the presynaptic protein, alpha-synuclein (α-synuclein), within neuronal cells, mainly oligodendroglia. α-Synuclein is a 140- amino-acid protein originally discovered as a non-amyloid component of senile plaques in AD. It is hypothesized to have a role in synaptic vesicle trafficking (Sudhof and Rizo, 2011). In 1997, mutations in the gene coding for α-synuclein, were discovered in familial PD (Polymeropoulos et al. 1997). Subsequently, α-synuclein has been found to be the major component of Lewy bodies (Spillantini et al., 1997). Lewy bodies are neuronal inclusions found in numerous NDD, including PD. In addition to Lewy body disorders, the pathological accumulation of α-synuclein is found in multiple system atrophy (MSA) (Lantos, 1998). In MSA, the aggregates are primarily found within oligodendrocytes, whereas in Lewy body diseases, they are predominantly found within neurons. α-Synuclein is significantly associated with PD, as the protein is a fundamental component of lewy bodies, and its mutated forms cause familiar PD (Cookson, 2005). Furthermore, both humans and mice with the familial A53T mutation of α-synuclein display tau filamentous inclusions. This is consistent with previously described pathological findings in idiopathic PD, that tau and α-synuclein co-localize (Ishizawa et al., 2003). Mice with the familial A30P mutation of α-synuclein display an increase in tau phosphorylation at Ser396/404 (Frasier et al., 2005). It has been

hypothesized that this is regulated by α-synuclein and GSK3complex. Interestingly, MPP^+ also causes alpha synuclein regulated phosphorylation of tau at Ser396/404 (Duka et al., 2009). This may have implications for idiopathic PD proper, in which α-synuclein may recruit tau protein by phosphorylating Ser396, to participate in neurodegeneration (Muntane et al., 2008).

The abnormal accumulation of aggregated host proteins, prions, β-amyloid and α-synuclein activates inflammasomes that function as intracellular sensors for both host-derived danger signals and microbial pathogens. Once activated, inflammasomes induce an innate immune response by releasing inflammatory cytokines. In addition to this, inflammasomes also induce pyroptosis, an inflammatory mediated lytic form of cell death, that releases additional inflammatory mediators leading to neuroinflammation and ultimately neurodegeneration.

2.2.3 History of PD

The first clear medical description of PD was written in 1817 by James Parkinson in his essay titled, Essay of the Shaking Palsy (Goetz, 2011). . However, in the mid-1800s, Jean-Martin Charcot another pioneer of PD, was the foremost figure responsible for expanding and refining the early description of Parkinson's disease internationally (Massano and Bhatia, 2012). Charcot along with his students described the clinical spectrum of PD, noting two prototypes, the rigid/akinetic and the tremorous form. They fully described the arthritic changes, pain and dysautonomia that can accompany PD. In addition to this, Charcot recognized that not all PD patients necessarily have tremor or are markedly weak and was the first to recommend the use of the term "Parkinson's disease", rejecting the original designation of shaking palsy or paralysis agitans (Lees, 2007). Prior to Charcot, the classifications of neurological diseases were primitive, disorders were mainly grouped by primary symptoms, such as tremors or weakness. Charcot's first significant contribution to the study of PD was his distinction of PD from other tremorous disorders. Examining patients at the Salpeˆtrie`re Hospital in Paris, Charcot developed a protocol for observing tremor at rest and then during movement. He noticed that the patients with action tremor had accompanying features of spasticity, weakness, and visual disturbance. However, patients with rest tremor differed in having slowed movements, rigidity, very soft speech, and a typical hunched posture. Charcot's tremor studies were highly publicized and helped to distinguish PD as a distinct neurological disorder that could be confidently diagnosed. Charcot distinguished PD from multiple

sclerosis (MS) and other disorders characterized by tremor. Additionally, he recognized cases that would later be classified among Parkinsonism-plus syndromes (Goetz, 2011).

2.2.4 Parkinson's Disease in Africa

The African continent has been experiencing rapid changes in recently, with increases in life expectancy. In Sub-Saharan Africa (SSA), life expectancy has increased by more than 20 years between 1950 and 2010 with the overall average life expectancy increasing from 46 years in 1990 to 55 in 2017 (Lekobou et al., 2015). While an aging population can be regarded as a public health success, it also brings with it new challenges in the form of chronic diseases such as cancers, cardiovascular and neurodegenerative diseases. Aging has been identified in numerous studies as a major risk factor for neurodegenerative disorders such as Parkinson's Disease (Chen et al., 2018, Ataie et al., 2010). In Africa, Parkinson's disease (PD) is thought to be less common than elsewhere in the world. This may be attributed to the diagnostic criteria used, as African countries face unique challenges pertaining to the diagnosis and management of PD. Firstly, there are significantly fewer neurologists in African countries than in developed countries, 0.03/100 000 in Africa compared to 4.84/100,000 in Europe.

The world health organization (WHO) recommends at least one neurologist per 100,000 people (WHO., 2004). However, it is estimated that approximately 270 million Africans live in countries where there are less than five neurologists per country. Additionally, more than half of SSA countries do not have a neurological society. This limited access to neurologists' results in the underdiagnosis and treatment of patients suffering with neurological disorders like PD (Bower et al., 2014). Secondly, screening equipment designed and validated in developed countries are primarily in English and is therefore not suitable for much of Africa, which has hundreds of local languages and dialects making the screening for PD using questionnaires validated in countries like the UK and the USA challenging in Africa (Dotchin, Msuya and Walker, 2007). Finally, the perception of PD and other neurological disorders in Africa tends to be different from other areas. Patients with disorders such as epilepsy or leprosy are often seen as 'cursed' and as a result are cast out of society (Huth et al., 2019). The cultural norm is to consult traditional healers prior to seeking professional medical help, as a result, patients in Africa tend to present much later than those in developed countries.

Many African countries are also overburdened by HIV, TB, malaria, and other infectious outbreaks, which neurological disorders, despite being on the increase, tend to play second fiddle to. Hence very few facilities and resources are dedicated to non-communicable illnesses. Furthermore, medication is only accessible by 12.5% of Africans with PD compared to 79% in Europe (Bower et al., 2014). According to United Nations (UN) data, SAs population was approximately 59,308,690 people at mid-year 2020. It is estimated that between 16-17% of the population have private medical aid (Govender et al., 2021), while the rest rely on state facilities for health care. However, only three PD medications are currently available in state hospitals, namely: Amantadine, Levodopa, and dopamine agonists. In numerous PD patients, invasive treatment methods are eventually needed to manage motor complications and other challenges brought upon by the disease. Three different invasive options are currently available: continuous duodenal administration of levodopa carbidopa intestinal gel (LCIG), continuous subcutaneous administration of apomorphine, and deep brain stimulation (DBS). Of these, DBS has the largest level of evidence, however there is a lack of large studies directly comparing these options (Giugni and Okun, 2014). It is estimated that approximately 160,000 patients with Parkinson's disease have had DBS surgery worldwide. This is rapidly increasing at a rate of approximately 12,000 procedures annually (Lee et al., 2019). In a recent survey, 74 percent of South African neurologists reported that they have referred patients for DBS surgery for the management of a movement disorder. Unfortunately, in SA only three tertiary state neurological centers are equipped to perform DBS surgery, but still lack adequate funding for devices. Four private sector practices offer DBS surgery, but these are general neurology practices, not specializing in one specific disorder. Given the ratio of patients to neurologist and the need to cover the whole spectrum of neurology, no singular movement disorders center currently exists in SA. As such, most South African neurologists rely on the few available DBS centers to treat Parkinson's Disease. However, one of these centers reported performing only five procedures over the last five years (Huth et al., 2019).

Compared to other populations, Africans display greater levels of genetic diversity which has evolved in response to exposure to infectious diseases and diverse climates (Campbell and Tishkoff, 2010). The G2019S mutation of the LRRK2 gene is the most common mutation associated with PD. However, a 2019 study found that none of the 91 Black South African

participants in their study had this mutation (it should be noted that this study had a relatively small sample size) (du Toit et al., 2019). Additionally, a 2009 study by Bardien et al concluded that mutations in the Parkin gene, a common cause of genetic PD globally, were not a significant cause of PD in SA (Bardien et al., 2009). This shows that some of the most common PD-associated genetic mutations globally are not necessarily relevant in SSA populations. Furthermore, a 2016 South African study found that the akinetic-rigid variant of PD is more common in blacks than in whites, and this result was reproduced by Modi and Smith in a larger cohort. The study further demonstrated that black patients seem to develop PD symptoms at a younger age along with a decrease in cognitive ability (Modi and Smith, 2016).

2.2.5 Diagnosis of PD

The diagnosis of PD requires many neurological examinations, key of which is gene testing and brain structural imaging, either by magnetic resonance imaging (MRI) or computed tomography (CT). In addition to the neurological tests, specialists also investigate a number of physical and psychological symptoms (Gupta et al., 2018). The psychological inspection examines the emotional state of the patient and also tests for dementia and depression. The physical inspection focuses on movement difficulties, namely bradykinesia, resting tremor and rigidity (Mostafa et al., 2019). Diagnosing idiopathic PD can be straightforward in patients with no atypical features and a classic history of PD. However, misdiagnosis is common, with error rates varying from 15% to 24%. An analysis of 11 clinico-pathological studies revealed that the clinical diagnosis accuracy of PD was only 80.6% (Tolosa et al., 2021). Furthermore, even with strict diagnostic criteria, 10% of patients diagnosed with PD had other underlying conditions. The biggest challenge that experts face is being able to differentiate PD from atypical parkinsonian disorders in the early stages. Atypical Parkinson's is an umbrella term used to describe various NDDs with prominent parkinsonian symptoms, but they differ from PD in terms of their clinical presentation, underlying pathology, progression, and prognosis (Rajput & Rajput, 2014). PD may be confused with a few other motor disorders, most commonly the conditions described below:

A). Vascular parkinsonism: In this condition rest tremor is uncommon. However, gait tends to be impaired as parkinsonian symptoms are predominant in the lower limbs, hence the designation "lower body parkinsonism". Other indications of brain vascular lesions may be present, such as

pseudobulbar palsy, spasticity and hemiparesis. Patients are usually irresponsive to levodopa and structural brain imaging is crucial to support or rule out this diagnosis (Lees et al., 2009).

B). Drug-induced parkinsonism (DIP): In DIP, a coarse, postural tremor is commonly seen and parkinsonian symptoms tend to present symmetrically. Other drug-induced disorders may be present, such as tardive dystonia, orolingual dyskinesias, or akathisia, especially when the culprit drug is an antipsychotic. For the diagnosis of DIP, it is crucial that symptoms appear after the drug has been introduced (Alvarez and Evidente, 2008).

C). Dementia with Lewy bodies (DLB): In contrast to PD, dementia is the hallmark of this disorder, whereas parkinsonism is observed either early on or as the disease progresses. Patients, often elderly, suffer from daily fluctuations in cognition and alertness, as well as extremely detailed visual hallucinations, involving human figures and animals. Other features commonly seen are extreme sensitivity to neuroleptic drugs, rapid eye movement (REM) sleep behavior disorder, and dysautonomia (Geser et al., 2005).

D). Tremor disorders: Tremulous PD is often confused with essential tremor (Jain et al., 2006), but with careful observation the correct diagnosis can be made. Tremulous PD is characterized by the prevalence of rest tremor over other motor symptoms. Furthermore, tremulous PD is associated with slower progression and consequently decreased cognitive decline (Stebbins et al., 2013).

E). Another group of tremor patients often misdiagnosed as PD was characterized by Schneider et al. (2007), Schwingenschuh et al. (2010). These patients have SWEDDs (scans without evidence of dopaminergic deficits), so named because the dopamine transporter (DAT) imaging is normal, ruling out striatal presynaptic degeneration. These patients frequently display tremor at rest, as well as head tremor. However, no true akinesia is seen. This group shows electrophysiological and clinical characteristics resembling dystonia, which should be actively searched for clinically (Schneider et al., 2007, Schwingenschuh et al., 2010).

2.2.6 Genetic Mutations Involved in PD

SNCA/PARK1

A missense mutation in the protein α-synuclein (A53T), encoded by the SNCA/PARK1 gene, was initially identified to cause a familial form of PD. Soon afterward, series of missense point mutations on the N-terminal of α-synuclein, including A30P, A53E, H50Q, E46K and G51D were

also identified. SNCA-linked PD is commonly of early onset and usually progresses rapidly (Zeng et al., 2018). α-synuclein is the major component in Lewy bodies and most is phosphorylated at Ser129 of α-synuclein, which facilitates α-synuclein fibril uptake in neurons and exacerbates the progression of PD (Sato et al., 2011). Mutations of α-synuclein are toxic to dopaminergic neurons as they alter a series of intracellular signaling programs.

LRRK2

Leucine-rich repeat kinase 2 (LRRK2) is a multi-domain protein, linked with "classical" late-onset PD and accounts for 4% of hereditary PD (Steger et al., 2017). The LRRK2/PARK8 gene represent the highest risk of familial PD, causing autosomal dominant PD (Ferreira and Massano, 2017). Morbidity of PD is significantly elevated among LRRK2 G2019S mutation carriers as aging progresses (Healy et al., 2008). LRRK2 G2019S mutation elevates mobility of α-synuclein and enhances α-synuclein accumulation in cultured primary neurons and in dopaminergic neurons in the substantia nigra of a PD brain (Volpicelli-Daley et al., 2016) In addition, LRRK2 G2019S mutation enhances the kinase activity and results in the impairment of synaptic vesicle trafficking selectively in ventral midbrain neurons, including dopaminergic neurons (Pan et al., 2017).

PINK1 and Parkin

PTEN-induced putative kinase (PINK) 1 is a mitochondrial protein kinase that is a critical protein associated with the pathogenesis of PD. PINK1 deficiency results in the autosomal recessive PARK6 variant of PD. PINK1 mutations have been shown to account for approximately 1–3% of early onset PD in populations of European ancestry, 2.5% of early onset PD in a sample of ethnic Chinese, Malays, and Indians and 8.9% of autosomal recessive PD in a sample of Japanese families (Bekris et al., 2010).

Protein Deglycase DJ-1

Mutations of Protein Deglycase DJ-1, encoded by the PARK7 gene, have been found to be linked with the early onset of recessive PD, which was first identified in 2 European families with an age of onset between 20–40 years (Bonifati et al., 2003). DJ-1 has also been found to interact with α-synuclein, regulating its aggregation by weak hydrophobic interaction and reestablishing α-synuclein-induced cellular toxicity (Zondler et al., 2014). Currently, the most recognized role of

DJ-1 in the pathophysiology of PD is its neuroprotective function against oxidative stress (Biosa et al., 2017). DJ-1 mutations mostly affects a protein linked with intracellular oxidation-reduction (Ishikawa et al., 2009).

Vacuolar Protein Sorting 35 (VPS35)

Mutations of the Vacuolar Protein Sorting 35 (VPS35) gene were originally discovered in an Austrian family with late-onset PD and accounts for 1% of familial PD (Bonifacino and Hurley, 2008). VPS35 D620N mutation has been reported to be pathogenic in patients with PD in Asian, American and European families (Chen et al., 2017). VPS35 D620N mutation results in a decrease of enzymatic activity in mitochondrial complex I and II of PD patients and causes mitochondrial dysfunction by recycling dynamin-like protein 1 complexes finally resulting in the loss of dopaminergic neurons (Tang et al., 2015).

GBA1

The homozygous and heterozygous mutations of the glucocerebrosidase 1 (GBA1) gene are linked to PD, with a surplus of 10% of individuals carrying GBA1 mutations developing PD before the age of 80 (Migdalska-Richards et al., 2016). GBA1 encodes β-glucocerebrosidase 1 (GCase 1). Activation of GCase 1 by using a small-molecule modulator restored the function of lysosome and then cleared the accumulation of pathological α-synuclein in midbrain neurons of PD patients (Mazzulli et al., 2016), indicating the potential importance of GCase 1 in the development of idiopathic PD. It is estimated that approximately 10–25% of PD cases carry a GBA1 mutation, in which L444P and N370S are the most common mutations (Sidransky et al., 2009).

2.2.7 Molecular Imbalances Involved in PD

2.2.7.1 Calcium imbalance

Intracellular calcium (Ca^{2+}) regulates many cellular processes and is essential for signal transduction. In neurons, Ca^{2+} acts as the main secondary messenger to transmit the depolarization status and synaptic activity to the biochemical machinery of neurons (Gleichmann and Mattson, 2011). The concentration of cytosolic free Ca^{2+} in resting neurons is 10,000 times lower than the concentration of Ca^{2+} in the extracellular space. This concentration gradient results in a significant increase in cytosolic Ca^{2+} after depolarization, making Ca^{2+} regulation a critical process in neurons

(Brini et al., 2014). To maintain Ca^{2+} homeostasis, the Ca^{2+} entering neurons is either sequestered in intracellular organelles, such as the endoplasmic reticulum and mitochondria, or pumped back across the plasma membrane, both requiring large amounts of energy in the form of ATP. The ability to sequester Ca^{2+} is a fundamental property of mitochondria. The accumulation of Ca^{2+} in the mitochondria catalyzes oxidative phosphorylation causing an increase in ATP production, thus assisting with the high metabolic demands of neuronal electrical activity (Gleichmann and Mattson, 2011). During normal synaptic activity, intracellular Ca^{2+} levels increase only for a short period and have no negative effects on neurons. However, in contrast to most of the neurons in the brain, SNpc dopaminergic neurons are independently active, generating action potentials in a clock-like manner without synaptic input. The pace-making activity of these neurons is powered by voltage-dependent L-type Ca^{2+} channels, resulting in prolonged elevations in cytosolic Ca^{2+} concentrations in these neurons (Chan et al., 2007). The large Ca^{2+}-buffering burden created by the autonomous pace-making activity of SNpc dopaminergic neurons compromises mitochondrial function, leading to mitochondrial oxidative stress and oscillations in mitochondrial potential which is associated with impaired ATP production (Guzman et al., 2010). Supporting the pathogenic claim of an increased Ca^{2+} concentration linked to the autonomous pace-making activity of SNpc dopaminergic neurons, the L-type Ca^{2+} channel antagonist, Isradipine, is able to reduce rotenone-induced dendritic loss in adult ventral midbrain slices and to attenuate SNpc dopaminergic neurodegeneration in MPTP-intoxicated mice (Chan et al., 2007). These observations suggest that prolonged mitochondrial Ca^{2+} overload in SNpc dopaminergic neurons might make these cells vulnerable to PD. To further support this notion, neighboring dopaminergic neurons in the ventral tegmental area, which does not rely on L-type Ca^{2+} channels for pace-making, are relatively protected in PD (Chan et al., 2010).

Furthermore, several studies also supported the role of alterations in mitochondrial Ca^{2+} homeostasis in PD. A 1997 study by Sheehan et al found that cybrid cells containing mtDNA from PD patients displayed lower mitochondrial Ca^{2+} sequestration than control cells following carbachol-stimulated Ca^{2+} entry (Sheehan et al., 1997). Similarly, the PD-inducing neurotoxins rotenone and MPP^+ decreases mitochondrial Ca^{2+} uptake and increases cytosolic free Ca^{2+} in cultured cells (Wang and Xu 2005, Sousa et al. 2003). In addition, a study by Gandhi et al demonstrated that the PD-related protein PINK1 regulates the physiological release of Ca^{2+} from the mitochondria through the mitochondrial Na^+/Ca^{2+} exchanger (Gandhi et al., 2009). Removal

of PINK1 in dopaminergic neurons impairs Ca^{2+} efflux from mitochondria, leading to the accumulation of mitochondrial Ca^{2+}, increased production of ROS in the mitochondria, decreased mitochondrial respiration and a decreased threshold for Ca^{2+} dependent opening of the mitochondrial permeability transition pore complex, ultimately resulting in increased apoptosis (Gandhi et al., 2009). Finally, Crocker et al demonstrated that genetic or pharmacological inhibition of Ca^{2+}-sensitive proteases decreased dopaminergic neurodegeneration in MPTP-intoxicated mice, further supporting a role of increased Ca^{2+} load in PD-related cell death in vivo (Crocker et al., 2003).

2.2.7.2 Oxidative stress

Oxidative stress can be defined as a disequilibrium between the amount of reactive oxygen species (ROS) produced and the inability of a biological system to detoxify the reactive intermediates, thus creating a perilous state resulting in cellular damage. Due to its increased demand for oxygen and glucose, the brain is highly susceptible to the destruction brought upon by ROS. Increased levels of polyunsaturated fatty acids in the brain's cell membranes also lead to the accumulation of ROS since these fatty acids react as substrates for lipid peroxidation (Rivas-arancibia et al., 2011). The link between dopaminergic neuronal degeneration and oxidative stress is further supported by modeling the motor aspects of PD in animals using toxins that cause oxidative stress such as rotenone, MPTP, paraquat and 6-OHDA (Richardson et al., 2005). In addition to PD, many other neurodegenerative disorders including Amyotrophic lateral sclerosis, Alzheimer's disease and Huntington's disease are associated with oxidative stress as well, despite having distinct clinical and pathological features suggesting that oxidative stress is a common mechanism contributing to neuronal degeneration (Lin and Beal., 2006; Anderson, 2004).

2.2.7.2.1 Reactive Oxygen Species (ROS)

Reactive Oxygen Species (ROS) are short-lived, highly reactive chemical species derived from oxygen. ROS reactivity stems from their unpaired valence electrons (Gandhi and Abramov, 2012). ROS includes the following molecules: the hydroxyl radical (·OH), superoxide anion radical (O_2^2) and hydrogen peroxide (H_2O_2) (Uttara et al., 2009). The superoxide anion, which is primarily produced by mitochondrial complexes I and III of the electron transport chain (ETC), is very reactive and can easily cross the inner mitochondrial membrane (Muller, Liu and Van Remmen,

2004). Superoxide is thought to be the catalyst in ROS production, O_2^{2-} can be converted to H_2O_2 via the enzyme superoxide dismutase (SOD) and when H_2O_2 production exceeds antioxidant defenses, it poses a major threat to cells (Wang et al., 2015, Castino et al., 2011). Besides being produced by mitochondria, H_2O_2 can also be produced by peroxisomes. Under normal conditions, peroxisomes contain catalase, which converts H_2O_2 to water, preventing its accumulation. However, when peroxisomes are damaged and their enzymes down-regulated, H_2O_2 escapes to the cytosol and contributes to oxidative stress (Muller, Liu and Van Remmen, 2004). Various studies have reported that H_2O_2 plays a crucial role in inducing apoptosis in numerous cell types (Wang et al., 2015). In the presence of reduced metals such as Fe^{2+}, H_2O_2 Is transformed, by the Fenton reaction, into the highly reactive hydroxyl radical. The hydroxyl radical is the most harmful of all ROS and is primarily responsible for the cytotoxicity of ROS (Valko et al., 2007). Nevertheless, at high concentrations, all ROS can be catalysts of cellular damage. The superoxide radical causes lipid peroxidation and also decreases the antioxidant capacity of glutathione peroxide (GPx) and catalase (CAT). Hydrogen peroxide induces cytotoxicity through membrane disruption, DNA damage and catalyzing the release of calcium ions within cells, leading to the activation of calcium dependent proteolytic enzymes (Sen et al., 2010).

2.2.7.2.2 Nitric Oxide

In addition to ROS, there is also evidence supporting the involvement of reactive nitrogen species (RNS) in regulating nitrosative stress (Malkus, Tsika and Ischiropoulos, 2009). RNS is generated by a reaction of superoxide with nitric oxide (NO), which produces large volumes of peroxynitrite (Carr, McCall and Frei, 2000). Peroxynitrite induces a dose-dependent decrease in dopamine synthesis (Szabo, Ischiropoulos and Radi, 2007). NO is produced by the enzyme, NO synthase (NOS), which has three isoforms, inducible NOS (iNOS) identified in glial cells, endothelial NOS (eNOS) and neuronal NOS (nNOS) identified in neurons (Vincent, Tilders and Van Dam, 1998, Murphy et al, 1993). NO inhibits numerous enzymes as well as complexes I and IV of the mitochondrial ETC, leading to ROS generation (Carr, McCall and Frei, 2000). The involvement of NO in PD is supported by postmortem brain tissue analyses displaying increased expression of nNOS and iNOS in basal ganglia structures using immunohistochemical studies and in situ hybridization (Eve et al, 1998, Hunot et al, 1996). Additionally, in the MPTP model of PD, the gliosis in the substantia nigra is associated with significant over-expression of iNOS, while

inhibition of nNOS safeguards against MPTP toxicity. Taken together, these observations suggest that NO may play a role in PD (Liberatore et al., 1999, Schulz et al., 1995).

2.2.8 Mitochondrial Disfunction

Although many studies show the presence of oxidative stress in PD, it remains unclear whether the accumulation of ROS is a primary event or a result of other cellular dysfunctions. Since mitochondria has dual functionality as both the source and target of ROS, numerous studies suggest that mitochondrial dysregulation is a significant factor in the pathogenesis of PDREF. One of the expected outcomes of impaired mitochondrial function is bioenergetic failure due to a reduction in ATP production. Supporting this view, MPP^+ has been found to cause a rapid and significant reduction of cellular ATP levels, in both brain synaptosomal preparations and whole mouse brain tissues (Chan et al., 1991, Scotcher et al., 1990). Besides their role in energy production, mitochondria are also involved in cell death pathways, calcium homeostasis and stress response. Therefore, impairing mitochondrial function causes cellular damage and ultimately neurodegeneration (Zhu and Chu, 2010).

2.2.9 Environmental Causes of PD

Numerous environmental factors have been hypothesized to increase the risk of PD, but few have been found to be significant and consistent (Adami et al., 2011). However, living in a rural environment appears to increase the risk of developing PD REF. Additionally, some epidemiological studies have reported a correlation between exposure to wood preservatives and pesticide use (Davie, 2008). Moreover, modern lifestyles tend to place people under tremendous psychological and physical stress (Sotiropoulos, 2014). Stress is generally regulated through homeostasis, but chronic stress becomes problematic since the released glucocorticoids are strongly associated with neuronal atrophy/dysfunction, cognitive impairment as well as mood disorders and depression (Sotiropoulos, 2015, Machado et al., 2014).

2.2.10 Progression of Parkinson's Disease

Due to the insidious nature of neurodegeneration in PD, the actual 'onset' of disease has not been clearly defined (Marek and Jennings, 2009). However, 5 stages of PD progression have been identified. During the initial stage, the person presents with mild symptoms that generally do not interfere with daily activities. Tremor and other movement symptoms only occur on one side of

the body. Changes in walking, posture and facial expressions also occur during this stage. During stage two symptoms start to worsen. Rigidity, tremor, and other movement symptoms now affect both sides of the body. Poor posture and walking problems may be apparent. Slowness of movements and loss of balance are the hallmarks of stage 3. Falls are more common during this "mid-stage". The person is still fully independent, but symptoms drastically impair activities such as eating. At stage four, symptoms are severe and limiting. Although, it's possible to stand without assistance, movement almost always requires assistance. The person requires help with most activities of daily living and is unable to live alone. Stage five is the most advanced and debilitating stage and rigidity of the legs may render it impossible to stand or walk. The person is bedridden or requires a wheelchair and may experience hallucinations, constant nursing is also required for all activities (Parkinson's Foundation, 2019). To assess and describe the severity of PD, multiple clinical scales have been developed (Jankovic, 2008). The 2 commonly used rating scales mainly focus on motor symptoms. They are the Hoehn and Yahr (HY) and the Unified Parkinson's Disease Rating Scale (UPDRS). The Hoehn and Yahr scale, originally introduced in 1967 is used to describe how motor symptoms progress in PD. the scale rates symptoms on a scale of 1 to 5, where 1 and 2 represent early-stage, 2 and 3 represents mid-stage, and 4 and 5 represents advanced-stage PD (Goetz et al., 2004). The Unified Parkinson's Disease Rating Scale (UPDRS) was originally designed in the 1980's and later revised by the Movements Disorders Society (MDS). The new version is now termed MDS-UPDRS and is currently the most widely used clinical rating scale for PD. The UPDRS is a multidimensional tool that includes both questionnaire parts as well as a clinical examination. (Goetz et al., 2008).

2.2.11 Consequences of PD

Given that the current therapies available for PD are unable to cure or adequately suppress all the symptoms associated with PD, particularly not in the long term, the disease will have negative consequences for the patient with regards to daily life (Shulman et al., 2008). Several studies have investigated the impact of PD on quality of life, particularly from a health-related perspective, consistently reporting disability and overall disease severity (as measured by UPDRS or the HY scale) to be associated with a poorer quality of life. Among specific symptoms of PD, gait impairments, depression and complications of therapy was found to have a particular impact on the health-related quality of life (Soh, Morris and McGinley, 2011). A stronger impact of PD on

the quality of life has been reported in younger patients than compared to older patients. Among patients with an early onset of PD, disability related unemployment and early retirement is common, as such socioeconomic consequences may contribute to a decreased quality of life (Schrag et al., 2003).

2.2.12 Models of PD

Provided that the current therapies available for PD cannot cure nor adequately suppress most of the symptoms associated with PD, particularly not in the long term, it is necessary to have sufficient models for both *in vitro* and *in vivo* studies to help further understand the pathophysiological mechanisms underlying PD and develop truly effective disease-modifying therapies (Xicoy, Wieringa and Martens, 2017). These models can generally be categorized into animal, genetic and cellular models.

Zebrafish are promising animals for modeling PD because their dopaminergic nervous system has been well characterized and they have more similar PD gene homology to humans than most animals (Flinn et al., 2008). Zebrafish also have the potential to address some of the more traditional animal model limitations as they have a shorter generation time and produce an enormous number of offspring. Furthermore, zebrafish provides the possibility to produce mutant strains, providing further insight into the molecular pathways involved in PD (Johnson and Bobrovskaya, 2015).

Studies assessing the genetic defects linked with inherited cases of PD are of great value because they offer great insight into PD pathogenesis and aid to identify novel treatment targets (Gasser, 2001). Many studies have linked familial PD with five main genes: PARK2, SNCA, PTEN-induced kinase 1 (PINK1), leucine-rich repeat kinase 2 (LRRK2) and DJ-1. Furthermore, genome-wide association studies have also recently found that polymorphic variations in these genes result in an increased risk of late-onset sporadic PD (Lesage and Brice, 2012). The way that the gene–environment interactions lead to sporadic cases of PD can be explored using genetic PD models in combination with exposure to environmental factors such as the toxins like MPP^+. Such models might generate evidence of how different risk factors interact and provide a better understanding of PD pathogenesis.

2.2.13 SH-SY5Y Cell Line

Primary mesencephalic neurons serve as a good source of dopaminergic neurons, the cells primarily affected in PD. However, human primary neurons are extremely difficult to obtain, handle and culture. Furthermore, the ethical difficulties in acquiring appropriate and sufficient amounts of human primary neurons further limit the application of this cell source. Nevertheless, the cellular models of PD mainly include: primary mesencephalic neurons, non-neuronal tumor cell lines such as pheochromocytoma cells (PC12) and neuronal tumor cell lines, primarily human neuroblastoma (SH-SY5Y) cells (Hong-rong, Lin-sen and Guo-yi, 2010). An ideal cellular model of PD is one that is established in cells susceptible to the neurotoxins produced during PD and is capable of addressing the questions pertaining to the selective loss of the dopaminergic neurons in the substantia nigra. SH-SY5Y cells can be differentiated from a neuroblast-like state into mature human neurons through a variety of mechanisms including the use of Retinoic acid, phorbol esters and neurotrophins such as brain-derived neurotrophic factor (Teppola et al., 2016).

The human neuroblastoma (SH-SY5Y) cell line is a thrice-replicated subline of SK-N-SH cells, originally established in the early 1970's from a bone marrow biopsy of a neuroblastoma patient. This cell line is regularly chosen because of its ease of maintenance, human origin and catecholaminergic (though not strictly dopaminergic) neuronal properties (Xicoy, Wieringa and Martens, 2017). Furthermore, numerous properties of the SH-SY5Y cell line validate its use as a model for dopaminergic neurons: firstly, SH-SY5Y cells can synthesize norepinephrine and dopamine. Secondly, the cells express dopamine transporter (DAT), a protein which regulates dopamine homeostasis by way of specific uptake and sequestration of dopamine. Additionally, DAT is essential for MPP^+ incorporation into neurons, as such the SH-SY5Y cell line is widely used to analyze the mechanisms of MPP^+-induced neurotoxicity as well as the pathogenesis underlying MPP^+-induced Parkinson's disease (Hong-rong, Lin-sen and Guo-yi, 2010). To create SH-SY5Y-derived cellular models that replicate PD, drug treatment and/or genetic approaches aimed at the manipulation of candidate genes expressed in genetic studies of PD-families are used (Kovalevich and Langford, 2013). The most frequently used compounds in drug-based approaches are rotenone, 6-hydroxydopamine (6-OHDA) and 1-methyl-4-phenylpyridinium (MPP+), which dysregulate numerous cellular pathways, focusing on oxidative stress and mitochondrial dysfunction (Xicoy, Wieringa and Martens, 2017).

2.2.14 1-methyl-4-phenylpyridinium (MPP^+)

MPP^+ is the toxic metabolite of 1-methyl-4phenyl-1,2,3,6-tetrahydropyridine (MPTP), a by-product in the synthesis of 1-methyl-4-phenyl-4-propionoxy-piperidine (MPPP), a synthetic analog of heroin, which induces severe parkinsonism in humans when injected intravenously. (Xicoy, Wieringa and Martens, 2017). MPTP readily infiltrates the blood-brain barrier and enters the brain cells. However, MPTP itself does not appear to be toxic, but its oxidized byproduct, MPP^+ has been shown to be toxic, and to induce a syndrome clinically and pathologically similar to PD. MPTP is converted to MPP^+ by monoamine oxidase B in non-neuronal cells, such as glial cells and astrocytes, after which MPP^+ causes selective impairment of dopaminergic neurons (Przedborski et al., 2004). MPP^+ is actively transported into dopaminergic neurons via the plasma membrane in a dopamine transporter (DAT) dependent manner. A 1997 study by Gainetdinov et al has shown that DAT is essential for MPP^+ neurotoxicity *in vivo*. In their experiments, Gainetdinov et al found that exposure to a DAT antagonist greatly impaired the toxicity of MPP+ as it does *in-vivo* and in primary mesencephalic neurons (Gainetdinov et al., 1997).

Although the precise mechanisms underlying the mode of MPP^+ toxicity remains unknown, it is hypothesized that the toxicity of MPP^+ relies on a mitochondrial concentrating mechanism. The mitochondrial uptake of MPP^+ creates sufficiently high concentrations of the toxin to obstruct mitochondrial respiration by interfering with the first step of the ETC (Richardson et al., 2006). Obstruction of mitochondrial respiration has two major cytotoxic consequences. Firstly, it reduces ATP production, inhibiting energy-dependent processes, key of which is ion transport (Przedborski et al., 2004). The disruption of calcium ion (Ca^{2+}) homeostasis plays a critical role in MPP^+ toxicity. This causes an increase in intracellular Ca^{2+}, resulting in the activation of Ca^{2+}-dependent enzymes, such as calpains I and II and protein kinase, which disrupts normal cell function, causing cell damage (Sian et al., 1999). Secondly, MPP^+ appears to assist the frequency of oxidative stress as it also generates free iron and reactive oxygen radicals (Hare et al., 2013).

Furthermore, MPP^+ and mitochondrial nicotinamide adenine dinucleotide (NADH) dehydrogenase have been hypothesized to yield toxic hydroxyl radicals derived from hydrogen peroxide. In monkeys, MPP^+ has been shown to release toxic iron (II) (Fe^{2+}), which may react with hydrogen peroxide to produce hydroxyl radicals via the Fenton reaction (He et al., 2003). The iron-mediated Fenton chemistry of the cell enables the continued production of hydroxyl radicals via

the oxidation of Fe^{2+} in the presence of hydrogen peroxide, while constantly regenerating via reduction with the same hydrogen peroxide species. Assuming an adequate source of iron is available, the reaction continues, overpowering cellular antioxidant mechanisms resulting in cellular death (Hare et al., 2013). Hydroxyl, Fe^{2+} and other free radical species have been greatly implicated in the pathogenesis of PD. More importantly, as observed in rodents, MPTP catalyzes a reduction of glutathione content, another fundamental nigral biochemical change distinctive of Parkinson's disease (Sian et al., 1999).

2.2.15 Treatment of PD

Normal motor function is heavily dependent on the coordinated synthesis and release of dopamine by the neurons projecting from the substantia nigra to the corpus striatum (Chase et al., 1998). Degradation of these dopaminergic neurons are mainly responsible for the movement disturbances associated with Parkinson's disease (Nagatsu et al., 2000). The symptoms linked to PD only become apparent when approximately 70% of the striatal dopamine and about 50% of the dopaminergic neurons are lost (Dunnett and Bjorklund, 1999). Therefore, symptomatic management of PD with levodopa and other dopaminergic drugs dominates current therapy and is very effective in managing the early stages of PD. Most motor symptoms of PD initially respond well to dopaminergic replacement therapy (DRT), but most patients are affected by complications, such as dyskinesia or motor fluctuations within 5 years of treatment (LeWitt, 2008). For 40 years, a combination of levodopa and a peripheral decarboxylase inhibitor has been the gold standard for the treatment of PD. However, the benefits achieved usually come at a price as long-term levodopa treatment often leads to disabling side effects (Davie, 2008).

In numerous PD patients, invasive treatment methods are eventually needed to manage motor complications and other challenges brought upon by the disease. Three different invasive options are currently available: continuous duodenal administration of levodopa carbidopa intestinal gel (LCIG), continuous subcutaneous administration of apomorphine, and deep brain stimulation (DBS). Of these, DBS has the largest level of evidence, however there is a lack of large studies directly comparing these options (Giugni and Okun, 2014). DBS is a functional neurosurgical technique, which differs from the other 2 invasive options in that it does not directly involve DRT but focuses on diminishing the need of drugs. The most common drawback of DBS is infection, occurring in approximately 15% of surgeries. Other complications reported after DBS surgery

include impaired gait and speech, cognitive decline, and psychiatric disturbance (e.g., depression, mania, behavioral disorders) (Bronstein et al., 2011). As such, ongoing severe psychiatric symptoms and cognitive impairments are contraindications for DBS surgery, though it is difficult to distinguish whether the worsening of such features are adverse effects of DBS, or rather part of the PD progression (deSouza et al., 2013).

To date, the pathophysiological processes underlying levodopa-induced complications are still not fully understood. In animal studies, the development of dyskinesias and motor fluctuations has been linked to the degree of neuronal loss in the substantia nigra and the dose and duration of levodopa therapy, as independent factors (Jenner, 2008). Furthermore, a study by Williams and lees, 2009 had reported that motor complications occur very early following the initiation of levodopa therapy in monkeys and in humans with MPTP-induced parkinsonism, who display severe depletion of dopaminergic neurons (Ballard et al., 1985). It is therefore vital to search for novel therapies for Parkinson's disease with potentially minimal complications which medicinal plants represent, considering their vast chemical composition and other bioactive compounds (Jain and Jain, 2018, Freyssin et al., 2018).

2.2.16 Plants Used in Traditional Medicine

Considering concerns that have been raised about levodopa and its potential to enhance oxidative stress and speed up the degeneration of residual dopaminergic neurons in patients with PD, there is a need for new treatment options and overtime herbal remedies have received considerable interest due to the broad spectrum of pharmacological properties that can be explored for the clinical management of neurodegenerative diseases (Omoruyi et al., 2020).

Natural products have played a fundamental role as new chemical entities (NCEs). Approximately 28% of NCEs between 1981 and 2002 were either natural products or natural-product derived. Another 20% of NCEs during this period were natural product mimics, meaning that the synthetic compound was derived from the study of natural products (Newman et al., 2003). Combining these categories, research on natural products accounted for approximately 48% of the NCEs reported from 1981–2002. In both 2001 and 2002, approximately one quarter of the bestselling drugs worldwide were natural products or derived from natural products (Butler, 2004).

Today, more than 60% of the world's population depends on plants for their primary medical needs (Shrestha and Dhillion, 2003). More than 80% of South Africans rely on at least one traditional remedy. This is because traditional remedies are often much cheaper, locally available, and easier to administer as simple preparations (Seoposengwe et al., 2013, Ghuman and Coopoosamy, 2011). South Africa has more than 30,000 plant species with approximately 3 000 of these species being used either as an alternative or to complement western medication (Thring, Springfield and Weitz, 2007). Most of these plants have chemical substances with pharmacological effects used in the treatment of age-related and NDDs (Gericke, 2002). Herbs have antioxidant and anti-inflammatory activities that may be used in the treatment of NDDs. Toki To IS THIS USED IN AFRICA, a herbal mixture prepared with Angelicae radix, Cinnamomi cortex, Pinelliae tuber, Zingiberis siccatum, Zanthoxyli fructus, Ginseng radix, Rhizoma and Glycyrrhizae radix is known for its protective effects against PD. When administered orally, Toki To prevents neuronal loss in the substantia nigra, reducing motor symptoms (Pérez-Hernández et al., 2016).

The clinical benefits of *Ginkgo biloba* another plant used in traditional Chinese medicine have been reported and knowledge of their medicinal properties date back to 5000 years ago. Numerous studies have demonstrated the antioxidant and free radical scavenging potential of EGb 761, a standardized extract of *Ginkgo biloba* (Maitra et al., 1995, Marcocci et al., 1994). An earlier study by Ramassamy et al demonstrated that EGb 761 inhibits the alteration of the neuronal dopamine uptake system and modifications in membrane fluidity induced by a pro-oxidant system ascorbic acid/Fe^{2+} in the striatum of mice (Ramassamy et al., 1993, 1992). This protection was also observed *in-vivo*, as dopaminergic neurons were protected against MPTP when mice received EGb 761 two weeks before MPTP was infused peripherally via an osmotic mini-pump (Ramassamy et al., 1990)

The bark of *Magnolia Officinalis* (talauma) is used as a traditional memory enhancing agent in Chinese medicine for the treatment of neurosis, anxiety, stroke, and dementia. Magnolia Officinalis inhibits the memory impairment induced by scopolamine through the inhibition of acetylcholinesterase AChE. The ethanolic extracts of *M. officinalis*, magnolol and honokiol, are reported to have antioxidant activity *in vitro* and *in vivo* (Singhal, Bangar and Naithani, 2012). Furthermore, Galantamine, used in the treatment of Alzheimer's disease has been isolated from the Amaryllidaceae family of plants. Alzheimer's patients have an acetylcholine deficiency, a

neurotransmitter that plays a key role in cognitive function and reasoning. The brains of those with mild-to-moderate Alzheimer's disease have abnormally low acetylcholine concentrations. This implies that any compound that enhances the cholinergic system in the brain may be useful in treating Alzheimer's disease and similar brain malfunctions (Harald et al., 2018).

2.2.17 Phyllanthin

Phyllanthus amarus (*P. amarus*) is extensively studied for its hepatoprotective properties but has recently received increasing attention because of the antioxidant and anti-inflammatory properties of the plant. However, the antioxidant and anti-inflammatory effects of *P. amarus* on the central nervous system, leading to protection against structural and functional changes, remains relatively unexplored. The results from a study by Wannannond and colleagues suggest that *P. amarus* extract may strengthen the recovery of peripheral nerves after injury, partly via the decreased oxidation stress. Phyllanthin and hypophyllanthin have been widely reported to be the primary active ligans in *P. amarus* (Wannannond et al., 2011).

Figure 1 A representation of Phyllanthus amajrus (Charles, 2019)

Joshi and Parle, 2007 reported that *P. amarus* produced a dose- dependent improvement in the memory scores of both younger and older mice. Additionally, *P. amarus* also successfully reversed

the amnesia induced by scopolamine (0.4 mg/kg, i.p.) and diazepam (1 mg/kg, i.p.). Brain cholinesterase activity was also reduced.

Alagan et al., 2019 sought to investigate the neuro-protective effects of *P. amarus* extract against immune responses and memory impairment in LPS-induced neuroinflammation in rodents. The hippocampus and cerebral cortex were sectioned and stained. The *P. amarus* extract treated group and control group did not show any inflammatory changes in the hippocampus and cerebral cortex, when treated at three different doses. In addition, no morphological changes were seen in the neurons and pyramidal cells. When investigating the effects of the *P. amarus* extract on cognitive behavioral activities, the discrimination index (DI) was calculated using the formula DI = (B − A3)/ (B + A3). The results revealed that the group that only received lipopolysaccharide (LPS) showed negative DI values, suggesting non-spatial memory impairment. Pre-treatment with *P. amarus* extract (100, 200, and 400 mg/kg) for 14 days showed positive DI values, suggesting that the rats were able to significantly discriminate the novel from familiar objects. However, following 28 days of treatment, only the groups treated with 200 and 400 mg/kg of *P. amarus* extract displayed a positive DI. In the same way, the positive control group also demonstrated a positive DI value. Taken together, it is evident that treatment with *P. amarus* extract at 200 and 400 mg/kg protects against LPS-induced memory impairment. To study the effects of the *P. amarus* extract on lipopolysaccharide-Induced pro-inflammatory cytokines, the concentrations of IL-1β and TNF-α proteins were measured in rat brains. An increase of pro-inflammatory cytokines in the LPS-induced group was observed and taken as evidence of neuroinflammation. However, *P. amarus* extract treated groups displayed a significant decrease in IL-1β and TNF-α levels than the vehicle control in 14- and 28-day treatments. Additionally, the LPS-treated group displayed small and numerous slender cell morphological features, as compared with other treated groups, which is indicative of activated microglia. However, pre-treatment with *P. amarus* extract of 200 and 400 mg/kg in 14 and 28 days showed a significant reduction in CD11b/c integrin expression, like that of the positive control. The CD11b/c integrin is expressed in activated microglia as a surface marker. It is an effective marker to recognize microglial activation at the onset of neurodegeneration.

In another study conducted by Tao and colleagues to investigate the potential of the standardized extract of *P. amarus* in PTZ-induced convulsion and kindling associated post-ictal depression in

mice found that chronic administration of PTZ significantly induced kindling in the mice reflected by elevated seizure severity score as compared to normal mice. Administration of diazepam (5 mg/kg) significantly decreased PTZ induced kindling in the mice as compared to PTZ control mice. *P. amarus* (100 and 200 mg/kg) treatment also significantly reduced seizure severity score over a duration of 15 days as compared to PTZ control mice. However, when compared with *P. amarus* treated mice, administration of diazepam showed more significant attenuation in PTZ-induced kindling in mice. Furthermore, the study found PTZ-induced seizures to be associated with an increase in nitric oxide and malondialdehyde levels and a decrease in superoxide dismutase and glutathione levels. Pretreatment with *P. amarus* inhibited reactive nitrogen species and reactive oxygen species induced lipid peroxidation, indicated by a decrease in malondialdehyde levels. Taken together, the results from this study is evidence of the neuroprotective capabilities of *P.amarus* against PTZ-induced seizures. Phyllanthin was identified in the phytochemical analysis of the *P. amarus* methanol extract by HPLC and is thought to be responsible for the anticonvulsant potential of *P. amarus* (Tao et al., 2020). The antiepileptic potential of phyllanthin is thought to be mediated through the balancing inhibitory (GABA) and excitatory (glutamate) brain monoamines, voltage gated ion channels (NaþKþ/Caþ2-ATPase) and the inhibition of the NF-kB/ TLR-4 pathway to ameliorate neuroinflammation (TNF-a, IL-1b and COX-2). In addition, the results of this study support the findings of Reddy and colleagues who found that the administration of phyllanthin from the standardized extract of *P. amarus* attenuates the elevated brain oxido-nitrosative stress (Reddy et al., 2018).

CHAPTER THREE

Methodology and Materials

3.1 Cell culture

The human neuroblastoma SH-SY5Y cells were a generous donation from the Blackburn Laboratory, University of Cape Town. Cells were grown in Dulbecco's Modified Eagle's medium (DMEM Gibco, Life Technologies, Milan, Italy), supplemented with 10% fetal bovine serum, (FBS, Sigma-Aldrich, St Louis MO, USA), 100U/mL penicillin and 10mg/mL streptomycin (Lonza). Cultures were incubated at 37°C in humidified air with 5% CO_2 with a medium change every 3 days. Cells were sub-cultured when they attained 70 to 80 percent confluency using a solution of 1×trypsin-EDTA (Lonza).

3.2 Treatments

3.2.1 Phyllanthin

Phyllanthin was purchased from Sigma-Aldrich, St Louis MO, USA. Stock solutions of Phyllanthin were prepared by dissolving weighed amounts of Phyllanthin in Dimethyl sulfoxide (Sigma-Aldrich, St Louis MO, USA) from which final concentrations of 5µM; 10µM; 15µM; 20µM and 25µM; 12.5µM; 6.25µM; 3.125µM were respectively made in cell growth medium.

3.2.2 MPP^+ Induction of Neurotoxicity

Stock solutions of MPP^+ (Sigma-Aldrich, St Louis MO, USA.) were prepared by dissolving pre-weighed amounts of the MPP^+ in un-supplemented DMEM, to a concentration of 50 mg/ml from which final concentrations of 1000 to 2500 µM were made in complete growth medium. Thereafter, the SH-SY5Y cells were seeded at a density of 10×10^4 and treated with the final concentration range of MPP+ for 24 hours to obtain the concentration of MPP+ that reduced cell viability to about 50%. Following MTT assays, the 2500µM concentration was chosen for further studies.

3.2.3 Neuroprotection by Phyllanthin

The SH-SY5Y cells were seeded at a density of 10 000 cells per well in a 96 well plate and incubated for 24 hours to allow for the attachment of cells to the plate surface. After attachment, the expended medium was carefully discarded and cells were pre-treated with 100µl of phyllanthin at 5µM; 10µM; 15µM; 20µM and 3.125µM; 6.25µM; 12.5µM; 25µM before the introduction of 100µl of MPP^+ (2500µM) to each well. The vehicle treated cells and the cells treated with MPP+ alone served as control and the plates were left to incubate at 37°C for 24 hours before cell viability assays were done and cell morphology analyzed.

3.2.4 MTT Cell Viability Assay

Following treatment with both Phyllanthin and MPP^+, the MTT (Sigma-Aldrich, St Louis MO, USA) assay was used to measure the viability of cells. This assay measures the mitochondrial activity of living cells based on their ability to reduce the yellow MTT tetrazolium salt into a purple formazan crystal mainly by mitochondrial dehydrogenases. Briefly, cells were seeded into 96-well plates and treated as mentioned above after which the MTT assay was performed by adding 10 or 20 µl (depending on well volume) of 5 mg/mL MTT solution to each well and left to incubate for 4 hours at 37°C in the dark. After the incubation period, the medium containing the MTT dye was removed, and the resulting purple formazan formed was solubilized with 100 µl of DMSO and absorbance was measured using a microplate reader (BMG Labtech Omega POLARStar) at a wavelength of 570 nm and percentage cell viability was calculated relative to the control.

3.2.5 Cell Morphology

To visualize changes in the morphology of the SH-SY5Y cells after the respective treatments, cells were seeded and treated as mentioned above and left to incubate for 24 hours. Following incubation, changes in morphology for the various treatment conditions were observed using the Zeiss inverted light microscope with 10X objective lens. Images were captured using the Zeiss software version 2.3.

3.2.6 Adenosine Triphosphate (ATP) Assay

The Mitochondrial ToxGlo ATP assay kit (Promega, USA) was used to determine ATP levels in the cells. Briefly, cells were seeded in a white 96-well plate at a density of 10 000 cells per well and left to attach, after which the cells were treated as per neuroprotection assay above. Following treatment, the cells were processed according to the manufacturer's protocol and luminescence intensity was read using the microplate reader (BMG Labtech Omega POLARStar) and readings were expressed as percentages of control.

3.2.7 Caspase 3/7 Apoptosis Assay

Apoptosis is a tightly regulated process that requires a signaling cascade in which caspase activation plays a central role (Zeiss, 2003). To measure apoptosis within the cells, the Caspase 3/7 assay kit (Promega, USA) was used to estimate the level of caspase 3/7 activity within the cells according to manufacturer's instructions. Briefly, cells were seeded in a white 96-well plate at a density of 10 000 cells per well and left to attach, after which the cells were treated as per neuroprotection assay above. Following treatment, equal volumes of Caspase 3/7 assay mix were added to each well and luminescence intensity was read with a microplate reader (BMG Labtech Omega POLARStar). Luminisence intensities of treated cells were expressed as percentages of control.

3.2.8 Measurement of Mitochondrial Membrane Potential (MMP)

TMRE (tetramethylrhodamine, ethyl ester) is a positively charged cationic dye capable of penetrating the cell membrane. Under normal conditions, TMRE accumulates in cellular mitochondria in response to their high membrane potential and relative negative charge. However, inactive and depolarized mitochondria have decreased membrane potential and fail to sequester TMRE (Kwon et al., 2017). Cells were seeded at 10 000cells/well in a black 96-well microplate and treated as described in the neuroprotection section. Carbonyl cyanide 3-chlorophenylhydrazone (CCCP; Thermo Fisher Scientific, Waltham, USA) was used for the positive control, as it has been reported to be an effective mitochondrial membrane disruptor that depolarizes the mitochondrial membranes of cells, making them unable to retain the fluorescent signal of TMRE (Zhang et al., 2016). Following treatment with phyllanthin and MPP^+, 100 µL of

CCCP (1000 μM; made up in growth medium) was added to the positive control wells and left to incubate for 10 minutes. The culture medium was then discarded from all the wells, after which all the treated and negative control cells were stained with TMRE (400 nM; prepared in growth medium). The cells were then incubated for 25 minutes. Following incubation, the stained medium was discarded, and the 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.

3.2.9 Nitric Oxide Activity

Nitric oxide activity was analyzed using Griess reagent based on its ability to measure the levels of nitrite in solutions. The Griess assay was carried out according to the manufacturer's instructions. Briefly, the cells were seeded at 10 000 cells/well and treated as described in the neuroprotection section. To obtain a reference curve for the nitrate standard, a 100 μM nitrite solution was prepared by diluting the provided 0.1M nitrite standard solution in DMEM. After incubation the solution was added to a 96 well plate as follows: 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. A twofold serial dilution was then performed in the three designated columns, with the last row not receiving 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 (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 for 10 minutes at room temperature, away from any light. Hereafter, 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 any light. Absorbance was measured using a microplate reader (BMG Labtech Omega® POLARStar) at 520nm and 550 nm wavelengths, respectively.

3.2.10 Fluo-4 Direct Intracellular Calcium Detection Assay

In neurons, Ca^{2+} acts as the main secondary messenger to transmit the depolarization status and synaptic activity to the biochemical machinery of neurons (Gleichmann and Mattson 2011). The

PD-inducing neurotoxins rotenone and MPP^+ decreases mitochondrial Ca^{2+} uptake (Wang and Xu 2005). In the present study intracellular Ca^{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. Following treatment, 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 Ca^{2+} 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 Ca^{2+} reagent. Medium was discarded from each well until 50µl/well remained, to which 50µl of the reagent loading solution was added. The plate was then incubated at 37°C for 60 minutes, and fluorescence measured using a microplate reader (BMG Labtech Omega® POLARStar) at excitation wavelength of 494 nm and emission wavelength of 516 nm.

3.2.11 Cholinesterase (ChE) assay

A modified Ellman's method was employed to determine the cholinesterase (ChE) inhibitory activity of phylanthin (Ellman and Courtney, 1961). The reagents used include acetylcholinesterase from the *electric eel* (*eletrophorus electricus*; *ee*AChE, product number C3389), *equine serum* butyrylcholinesterase (C1057), 5,5'-dithiobis-(2-nitrobenzoic acid) commonly known as Ellman's reagent (DTNB- D8130), *S*-butrylthiocholine iodide (BTCI-B3253), acetylthiocholine iodide (ATCI- A5751), trizma hydrochloride (tris- T3253), cholinesterase inhibitors (donepezil hydrochloride- D6821 and tacrine hydrochloride- A79922) were all purchased from Sigma Aldrich®, Merck. The *ee*AChE, *eq*BuChE, DTNB, BTCI and ATCI were diluted with tris buffer solution (50 mM, pH 8) to their specified assay concentrations, and to each well of the clear, flat-bottom 96 well plate, the following was added: 148µL of 1.5 mM DTNB, 50 µL of either 0.22 U/mL *ee*AChE or 0.12 U/ml *eq*BuChE and 2 µL of either phyllanthin, positive control (donepezil for *ee*AChE or tacrine for *eq*BuChE) or vehicle control (DMSO). Both phyllanthin and positive controls were dissolved in DMSO to obtain concentrations of 100 µM, 70 µM, 50 µM, 30 µM and 10 µM. The 96 well plates were then shaken and incubated at 25 °C for 20 min in the BioTek Synergy H1 multimode microplate reader. Thereafter, 30 µL of either the 15 mM ATCI or 15 mM BTCI substrate was added to each well using the BioTek Synergy H1 multimode microplate reader dispenser, and absorbance was read at 405 nm every 20 seconds for

5 minutes for the *ee*AChE assay, and every 1 minute for one hour, for the *eq*BuChE assay. The absorbance values used to determine the percentage inhibition of the compounds and positive control were read at 5 minutes for AChE and one hour for BuChE, respectively. Thereafter, the percentage inhibition for the compounds and positive controls was calculated using the following equation: [absorbance of negative control - absorbance of test compound or positive control) / absorbance of negative control (DMSO only)] x 100.

3.2.12 Monoamine Oxidase-B (MAO-B assay)

The *in vitro* monoamine oxidase B (MAO-B) inhibitory activities of phyllanthin was determined using the *human* MAO-B (*h*MAO-B, M7442, Sigma Aldrich, St Louis, USA) and the kynuramine dihydrobromide substrate (K3250, Sigma Aldrich, St Louis, USA). Briefly, the *h*MAO-B (0.075 mg/ml) and kynuramine substrate (30 µM) were dissolved in a potassium phosphate buffer solution (KH_2PO_3 100 mM, pH 7.4, 0.9% w/v NaCl), and the test compounds and positive control were dissolved in DMSO to obtain various concentrations (1000 µM, 700 µM, 500 µM, 300 µM and 100 µM). Each concentration of phyllanthin and the positive controls were conducted in triplicate to ensure consistent results. The experiments were performed in black, flat-bottom 96 well plates with each well containing the following: 207.5 µL of potassium phosphate buffer, 2.5 µL of negative control (DMSO) or phyllanthin and 25 µL of *h*MAO-B. The plate was shaken and incubated at 37 °C for 10 minutes in the BioTek Synergy H1 multimode microplate reader. Thereafter, 15 µL of the kynuramine substrate was added using the BioTek Synergy H1 multimode microplate reader dispenser, further shaken and incubated at 37 °C for 20 minutes in the BioTek Synergy H1 multimode microplate reader. Subsequently, 50 µL of sodium hydroxide (NaOH) was added to each well to stop the reaction and fluorescence was read at an excitation/emission wavelength of 310 nm/400 nm. The compound's percentage inhibition was calculated relative to the negative control (DMSO only).

3.2.13 Statistical Analysis

Data generated by the study was analyzed with the GraphPad Prism 6 statistical package. The results were expressed as standard error of means (SEM) of three independent experiments performed in quadruplicate wells. To determine levels of significance, one-way analysis of

variance (ANOVA) was used, data sets with a p value less than 0.05 was considered significant when MPP+ -treated cells were compared to either the extract-treated or control cells.

3.3 Chemicals

Hydrogen peroxide (H_2O_2), Dimethyl sulfoxide (DMSO) and Rhodamine 123 mitochondrial specific fluorescent dye were purchased from Sigma-Aldrich (St. Louis, USA). Caspase 3/7 protease activity kit, Griess reagent, Phosphate buffered solution (PBS), Dulbecco's Modified Eagles Medium (DMEM), fluo-4 direct Calcium activity kit, fetal bovine serum (FBS), Penicillin and streptomycin. and Trypsin (1X) EDTA were purchased from WhiteSci (Cape Town, ZA).

3.4 Consumables

Different sizes of 96-well plates, 2ml & 1.5ml Eppendorf's, 10 ml serological pipettes, 15ml & 50 ml conical tubes were purchased from BioSmart (Cape Town, ZA). Nitrile powder-free examination gloves and reagent reservoirs (50 ml) were purchased from Lasec (Cape Town, ZA). NEST tissue culture dishes (60 mm and 100 mm, respectively) were purchased from WhiteSci (Cape Town, ZAR).

3.5 Molecular Docking Studies

3.5.1 Preparation of Protein and Ligands

The AChE (PDB: *4EY7*) (Cheung et al., 2012), BuChE (PDB: *4BDS*) (Nachon et al., 2013) and MAO-B (PDB: *2V5Z*) (Binda et al., 2007) holo x-ray structures obtained from the protein data bank (PDB, https://www.rcsb.org) (accessed on 17 October 2021), were utilized for molecular docking analysis (Schöning-Stierand et al., 2020). The *4EY7* x-ray structure has a resolution < 2.35 Å and R-values < 2.11, The *4BDS* x-ray structure has a resolution < 2.10 Å and R-values < 2.09 and the *2V5Z* x-ray structure has a resolution < 1.60 Å and R-values < 2.27. The most appropriate protein chain of the x-ray homodimer structure was selected using PDB residue-property plots, native ligand binding interactions. Chain A was chosen for molecular docking as it contained the least number of outliers on the residue property plots, accurate binding interactions and appropriate ligand model. The most appropriate ligand model was determined using the following parameters: goodness of fit percentage = 88 % (AChE), 49 % (BuChE) and 70 % (MAO-B), real space correlation coefficient = 0.978 (AChE), 0.884 (BuChE) and 0.913 (MAO-B) as well as ProteinPlus

EDIA (https://proteins.plus) (accessed on 20 December 2021) (Fährrolfes et al., 2017, Meyder et al., 2017, Schöning-Stierand et al., 2020) median score 0.955 (AChE), 0.970 (BuChE) and 0.960 (MAO-B). The x-ray crystal protein structures were also selected as their co-crystallized ligands are known potent inhibitors of the respective proteins ((*4EY7* (donepezil, IC_{50} = 80 nM), *4BDS* (tacrine, IC_{50} = 50 nM) and *2V5Z* (safinamide, IC_{50} = 450 nM)) (Binda et al., 2007). The protein structure was prepared and The Molecular Operating Environment (MOE) 2020 software suite, Version 2020.09. http://www. chemcomp.com) was used for docking studies with the following protocol: The unselected protein chains and the respective co-crystallized ligand, solvent and co-factors were removed. Thereafter, the water molecules further than 4.5 Å from the ligand were removed. Atoms further than 8 Å from the ligand were fixed and the receptor residues were tethered with a constraint value of 0.25 Å. The tethering of the protein residue heavy atoms within 8 Å of the ligand ensures that no artificial movements from the original coordinates will occur during energy minimization (Bhattacharya and Cheng, 2013). The proteins were structurally prepared and protonated through the utilization of the built-in MOE structure preparation and Protonate3D software tools using default parameters and the following pH's: 7.0, 6.5 and 7.4 for AChE, BuChE and MAO-B respectively. Finally, partial charges were corrected, and energy minimization was conducted utilizing the following parameters: forcefield: Amber12: EHT, solvation: R-field and gradient: 0.01. Once the structures were optimized, the fixed and tethered constraints were removed for molecular docking. The docking algorithm chosen for these experiments was based on induced fit docking to allow for flexible interactions of the test ligand with the protein active site sidechains. Hence, the constraints were removed to ensure the active site side chains were able to flex during induced fit docking. The prepared protein structures were saved in .moe file format. The ligands used for molecular docking were drawn using the ACD/ChemSketch (ACD/ChemSketch, version 2020.2.1, Advanced Chemistry Development, Inc., Toronto, ON, Canada, www.acdlabs.com, 2021) package. Protonation and energy minimization of the ligands was conducted utilizing the following parameters: forcefield: MMFF94x, solvation: R-field and gradient: 0.0001. The protonation state of each ligand was conducted at the specified pH values of each enzyme.

3.5.2 Molecular Docking of Phyllathin

Self-docking was used to validate the docking protocol as well as to determine the protein structure's suitability to successfully dock the native ligand. The native ligands donepezil, tacrine and safinamide were docked into their respective protein active sites using the docking parameters; placement: triangle matcher, placement score algorithm: London dG, returned poses: 100, refinement: induced fit, iterations: 1000, refinement score algorithm: GBVI/WSA dG, scored poses: 5. The success of the docked ligands was determined using a root median square deviation (RMSD)-based criterion between the docked native ligands and the crystallographic ligands. A RMSD value of > 2 Å for both the top pose (lowest binding affinity score pose) and average RMSD across the top five docked poses was used to validate the reliability of the docking protocol to predict realistic binding conformations and interactions. The self-docked donepezil, tacrine and safinamide ligands top pose in their respective protein active sites obtained a RMSD of 0.09 Å, 0.52 Å and 0.29 Å respectively. The average RMSD over the top five docked poses of donepezil, tacrine and safinamide was 1.11 Å, 1.30 Å and 0.97 Å respectively. Therefore, the validated molecular docking protocol was employed in this study. Phyllanthin was imported into a combined database and docked into each prepared protein individually using the validated docking protocol. The best docked ligand conformation of each phyllanthin was selected using the following criteria: lowest binding affinity score within the top 5 binding conformations and best interactions with important active site residues. The best binding pose of phyllanthin was visually inspected and the interactions with the binding pocket residues were analyzed using the MOE 2020 software suite and MOE ligand interaction tool (Molecular Operating Environment (MOE), Version 2020.09. http://www. chemcomp.com.). The build-in scoring function of MOE, S-score, was used to predict the binding affinity score (kcal/mol) of each ligand within the protein active site after docking.

CHAPTER FOUR

Results

4.1 Cytotoxicity Screening of Phyllanthin

To determine the optimum concentration of phyllanthin to be used for neuroprotection studies, the MTT cell viability assay was performed on the SH-SY5Y cells exposed to increasing concentrations of phyllanthin (5µM; 10µM; 15µM; 20µM) for 24 hours. The results show that treatment with phyllanthin increased the cell viability across all concentrations, with the 5 µM concentration having the highest viability. Cell viability obtained was 114, 109.31, 107.27 and 105.31 for the 5 µM, 10 µM, 15 µM and 20 µM respectively. Together, these concentrations did not induce any form of toxicity to the cells and the 5 µM, 10 µM, and 20 µM were selected for neuroprotection studies.

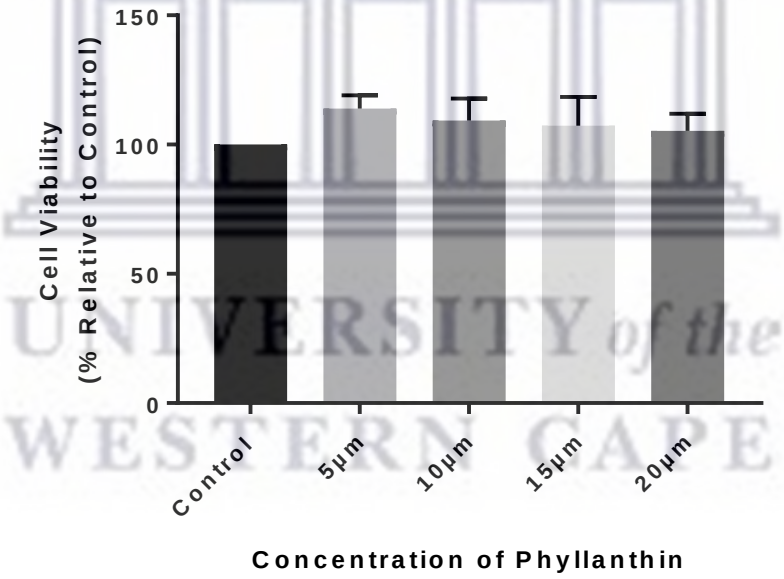

Figure 2: Evaluation of the cytotoxicity of phyllanthin on SH-SY5Y cell viability*. The graphs illustrate the cytotoxic effects of phyllanthin on the viability of the SH-SY5Y cells following a 24-hour exposure. Cell viability was evaluated using the MTT cell viability assay. The graph was prepared as mean ± SEM of three experiments (n=3) using GraphPad Prism 7 statistical software.*

4.2 Cytotoxicity Screening of MPP^+ on SH-SY5Y

To measure the cytotoxicity of MPP^+ exposure on the SH-SY5Y cell line, the MTT cell viability assay was used, 24 hours after treatment of cells with increasing concentrations of 500, 1000, 1500,

2000, and 2500 μM MPP^+ (Figure 3). The results indicate that MPP^+ induced significant toxicity on the SH-SY5Y cells at all the treatment concentrations, with the lowest concentration (500 μM) having the least toxicity (84.14% cell survival) to the cells, while the highest concentration (2500 μM) reduced the cell viability to about 45%. Therefore, the 2500 μM MPP^+ concentration was selected for all the neuroprotection experiments in order to induce toxicity in the cells (Figure 3).

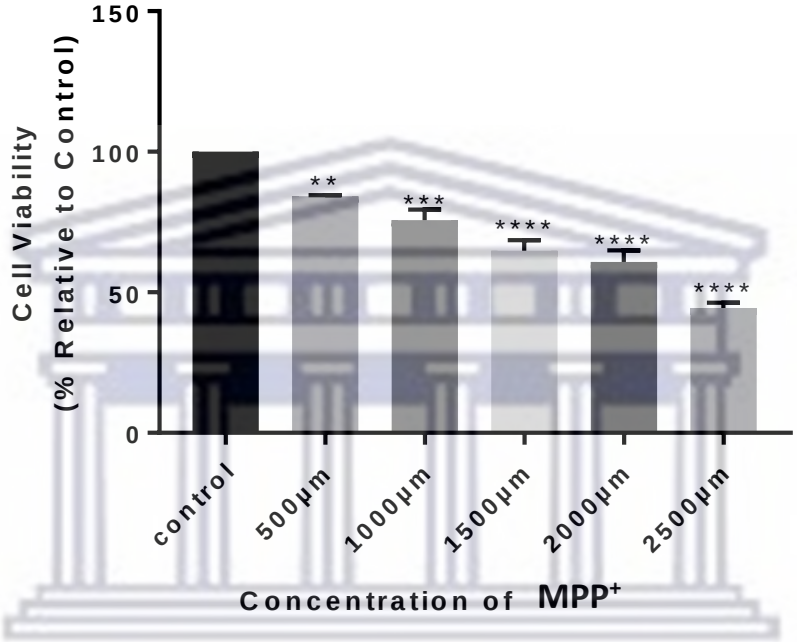

Figure 3: Evaluation of the cytotoxicity of MPP⁺ on the SH-SY5Y cell line. *The graph illustrates the toxicity of MPP⁺ on the viability of SH-SY5Y cells following a 24-hour exposure. The control cells were left untreated, and the viability was evaluated using the MTT cell viability assay. The graph was 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 < 0.0001).*

4.3 Neuroprotective Effects of Phyllanthin Against MPP^+-induced Toxicity in SH-SY5Y Cells

To determine the neuroprotective potential of phyllanthin against MPP^+-induced toxicity in SH-SY5Y cells, the cells were pre-treated with the selected concentrations of phyllanthin (5; 10; 15 and 20μM), for two hours prior to the introduction of MPP^+ (2500 μM). The MTT assay was used to determine cell viability after 24-hour incubation. Figure 4 shows that the exposure to MPP^+ alone significantly reduced cell viability by approximately 50%. However, pre-treatment with phyllanthin attenuated the toxicity induced by MPP^+, with all the pretreated samples having a cell

viability above 80% and a viability of 99.3% and 94.7% recorded at 10 and 20µM phyllanthin respectively. Taken together, these results show that phyllanthin at the tested concentrations protect the SH-SY5Y cells from MPP^+ toxicity.

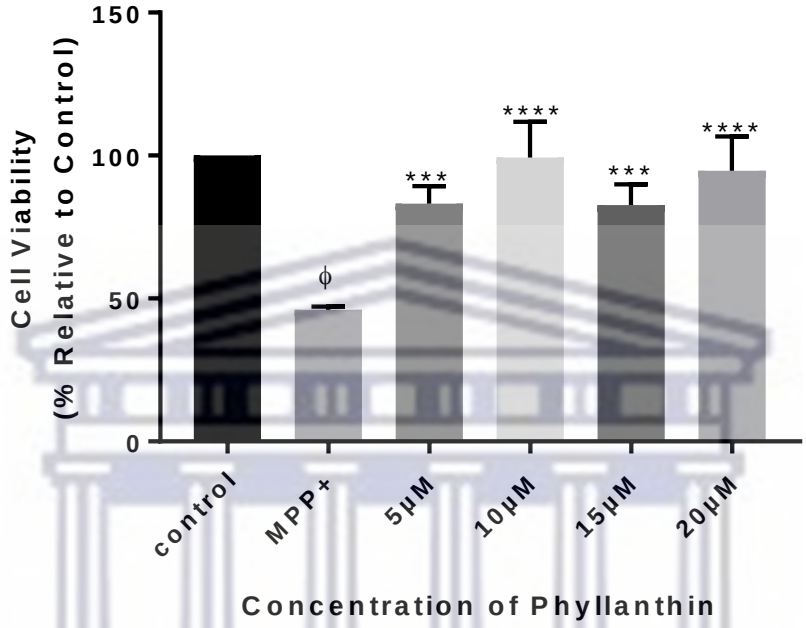

Figure 4: Evaluation of the neuroprotective potential of phyllanthin in SH-SY5Y cells following a 24-hour exposure to MPP+. *The graph illustrates the neuroprotective capabilities of SH-SY5Y cells pretreated with phyllanthin, following exposure to MPP+ for 24 hours. The control cells were left untreated and cell 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 < 0.0001).*

4.4 Effects of Phyllanthin on MMP^+-induced Morphological Changes in SH-SY5Y Cells

To investigate the neuroprotective potential of phyllanthin on MPP^+-induced toxicity, morphological changes in the SH-SY5Y cells were observed using an inverted light microscope at a magnification of X100 after treatment of cells as was done for the neuroprotection experiments (Figure 5). The results showed that the MPP^+-treated cells displayed an altered morphology compared to control cells. The cells displayed a decrease in neuronal projections and a more rounded morphology, similar to cells undergoing programmed cell death (Langley, 2007). However, pre-treatment with phyllanthin appears to prevent the morphological changes induced by MPP^+, restoring the characteristic neuronal projections and density of the cells.

Figure 5: Evaluation of the neuroprotective potential of phyllanthin in SH-SY5Y cells against MPP+-induced morphological damage in SH-SY5Y cells. *The images illustrate the neuroprotective capabilities of SH-SY5Y cells pretreated with phyllanthin, following exposure to MPP+ for 24 hours. Changes in morphology were observed using the Zeiss inverted light microscope (100x magnification). Images were captured using the Zeiss software version 2.3.*

4.5 The Effect of Phyllanthin on Mitochondrial Membrane Potential in SH-SY5Y Cells Following Exposure to MPP^+

The neuroprotective potential of phyllanthin on MPP^+-induced alterations in the MMP of SH-SY5Y cells was investigated using the fluorescent probe, TMRE. The charge and solubility of TMRE enables it to accumulate in the matrix of the mitochondria. Additionally, Carbonyl cyanide m-chlorophenylhydrazone (CCCP) was used as a positive control. CCCP has been reported to be an effective mitochondrial membrane disruptor that depolarizes the mitochondrial membranes of cells, making them unable to retain the fluorescent signal of TMRE (Kwon et al., 2017). Figure 6 shows that treatment with MPP^+ only resulted in a slight decrease in MMP. However, pre-treatment with phyllanthin at the 20 µM concentration increased MMP to a value almost similar to the control cells.

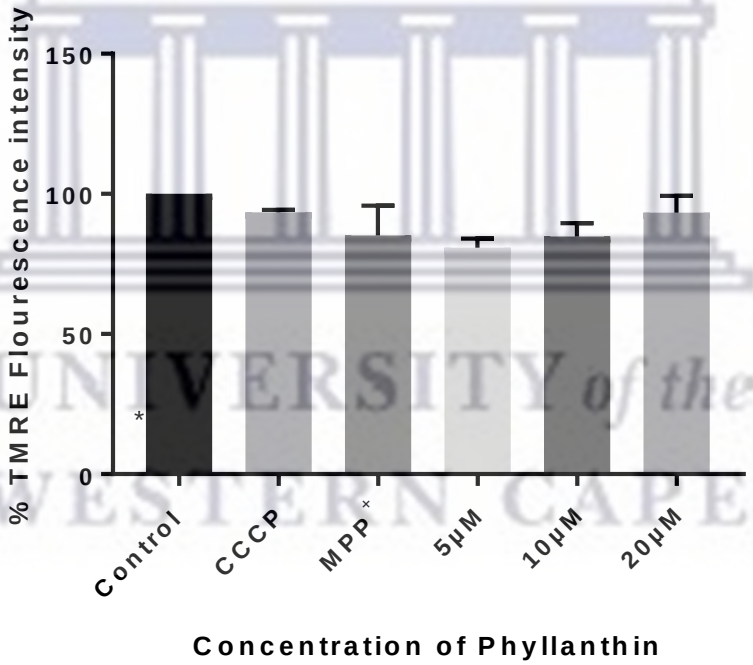

Figure 6: The effect of Phyllanthin on mitochondrial membrane potential in SH-SY5Y cells following a 24-hour exposure to MPP^+. *The graph illustrates the effect of phyllanthin on MPP^+ (2500 µM) induced damage to the mitochondrial membrane potential in SH-SY5Y cells after 24 hours shown by the fluorescence 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.*

4.6 The Effect of Phyllanthin on Nitric Oxide Production in SH-SY5Y Cells Following Exposure to MPP^+

There is evidence supporting the involvement of reactive nitrogen species RNS in regulating nitrosative stress (Malkus, Tsika and Ischiropoulos, 2009). NO inhibits numerous enzymes as well as complexes I and IV of the mitochondrial ETC (Carr, McCall and Frei, 2000). NO activity was analyzed using Griess reagent according to the manufacturer's protocol after treating the cells in a 96 well plate and absorbance was read at 458 nm. The results show that there was no significant change in NO across all treatment concentrations (Figure 7).

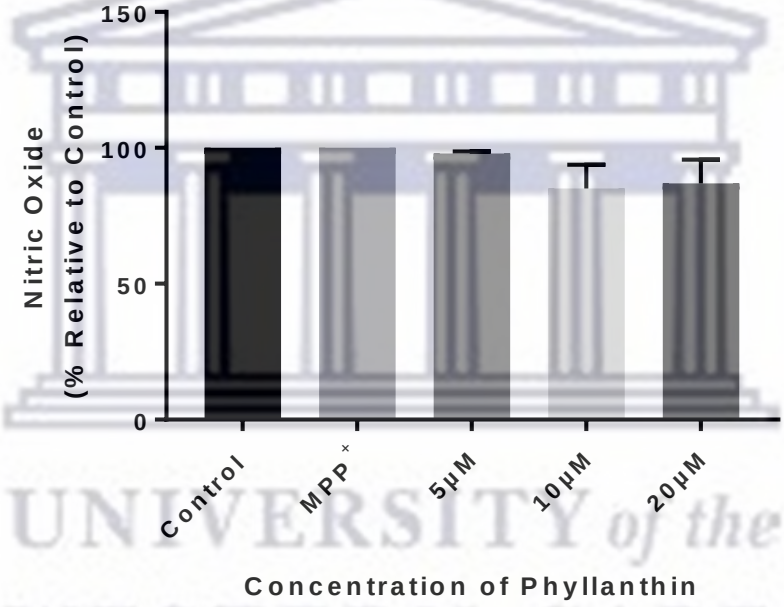

Figure 7: The effect of phyllanthin on nitric oxide production in SH-SY5Y cells following a 24-hour exposure to MPP^+. *The graph illustrates the effect of phyllanthin on MPP^+ (2500 µM) induced nitric oxide production in SH-SY5Y cells after 24 hours. The control cells were left untreated and NO production was evaluated using the Griess reagent. The graphs were prepared as mean ± SEM of three experiments (n=3) using GraphPad Prism 7 statistical software.*

4.7 The Effect of Phyllanthin on ATP Production in SH-SY5Y Cells Following Exposure to MPP^+.

The toxicity of MPP^+ relies on a mitochondrial concentrating mechanism, interfering with the first step of the electron transport chain. One of the expected outcomes of impaired mitochondrial function is bioenergetic failure due to a reduction in ATP production (Richardson et al., 2006). Considering this, we investigated the changes in ATP levels to ascertain if phyllanthin will inhibit

the depletion of ATP in the SH-SY5Y cells. Results obtained showed that MPP^+ treatment significantly reduced ATP levels (45.69%) in the cells when compared to control (100 %), but pre-treatment with phyllanthin significantly increased ATP levels and this was highest for the 5 µM concentration (71.19%). Altogether, inhibition of ATP degeneration is involved in the neuroprotection conferred by phyllanthin.

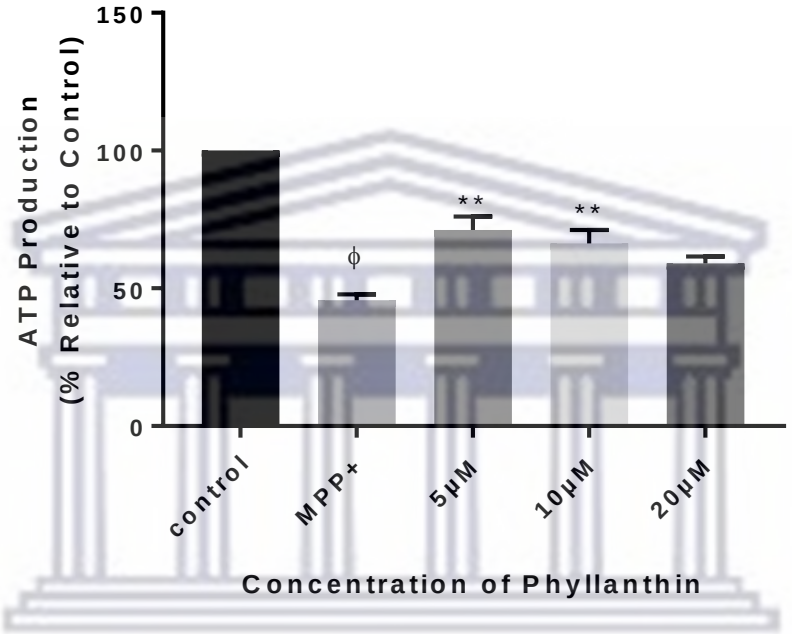

Figure 8: The effect** of phyllanthin on ATP production in SH-SY5Y cells following a 24-hour exposure to MPP^+. The graph illustrates the effects of pretreatment with phyllanthin on ATP production in SH-SY5Y cells, following exposure to MPP^+ for 24 hours. The control cells were left untreated and ATP production was evaluated using the mitochondrial ToxGlo ATP assay kit. 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 < 0.001 and ****p < 0.0001).*

4.8 The Effect of Phyllanthin on Calcium Production in SH-SY5Y Cells Following Exposure to MPP^+.

Intracellular calcium regulates many cellular processes and is essential for signal transduction (Gleichmann and Mattson 2011). The PD-inducing neurotoxins rotenone and MPP^+ decreases mitochondrial Ca^{2+} uptake (Wang and Xu 2005). The Fluo-4 Direct Calcium detection assay was used to measure intracellular calcium. The cells were seeded and treated with Phyllanthin as mentioned before, 2 hours before exposure to MPP^+ (2500 µM). Fluorescence was read at 494 nm

for excitation and 516 nm for emission in a microplate reader. Figure 9 shows that there was no change in intracellular calcium across all treatment concentrations.

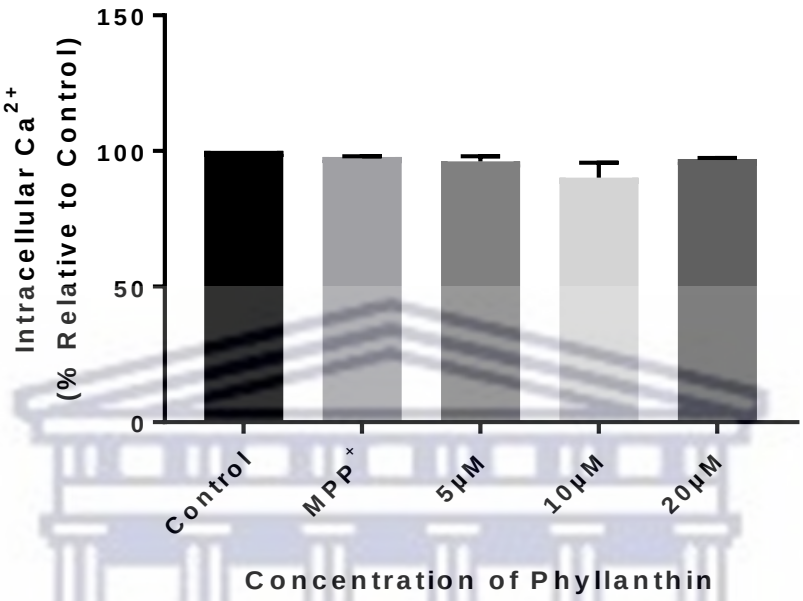

Figure 9: The effect of phyllanthin on calcium production in SH-SY5Y cells following a 24-hour exposure to MPP+. *The graph illustrates the effect of phyllanthin on MPP+ (2500 µM) induced dysregulation of intracellular Ca^{2+} homeostasis in SH-SY5Y cells after 24 hours. Ca^{2+} production was evaluated using the Fluo-4 Direct Calcium detection assay. The graphs were prepared as mean ± SEM of three experiments (n=3) using GraphPad Prism 7 statistical software.*

4.9 The Effect of Phyllanthin on Caspase3/7 Production in SH-SY5Y Cells Following Exposure to MPP+

Mitochondrial damage leads to the release of numerous mitochondrial proteins that mediate programmed cell death (PCD). Thus, to further investigate the mechanism of neuroprotection by phyllanthin, cells were treated as previously described for the neuroprotection experiments, and the activities of caspase 3/7 was determined as an indicator for apoptosis. Figure 10 shows that following MPP^+ treatment alone, elevated level of caspase 3/7 activities was evident. However, all of the phyllanthin samples have substantially less caspase 3/7 activity than the MPP^+ well (5µM: 140.57%; 10µM: 176.26% and 20µM phyllanthin: 231.38%) compared to MPP^+ (401.51%). These results were in a concentration dependent manner with the caspase 3/7 activity in the cells treated with 5µM phyllanthin being nearly 3 times less than that of the MPP^+ sample. Taken together, these results suggest that phyllanthin has the potential to protect against MPP^+ induced apoptosis.

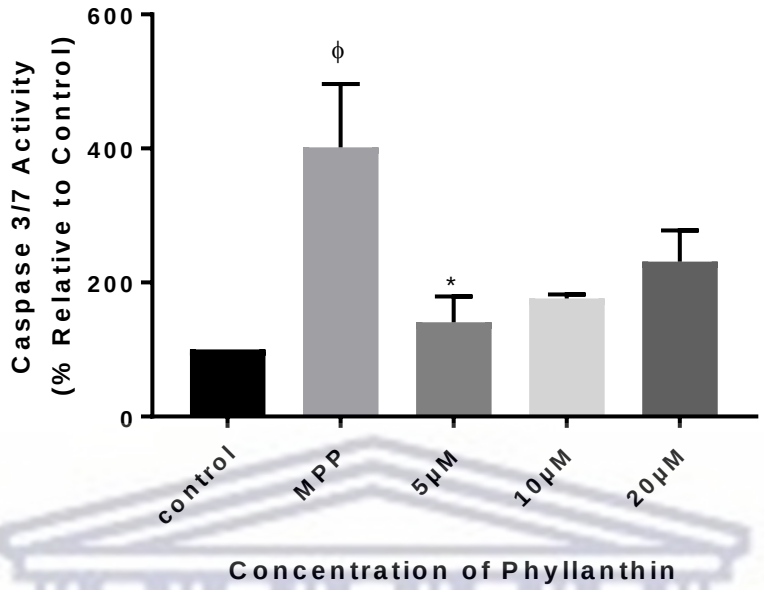

Figure 10: The effect of Phyllanthin of caspase3/7 production in SH-SY5Y cells following a 24-hour exposure to MPP+ induced SH-sy5y cells. *The graph illustrates the effect of phyllanthin on MPP⁺ (2500 µM) induced apoptosis. The activity of caspase 3/7 was investigated in SH-SY5Y cells after 24 hours. The control cells were left untreated and the caspase activity was evaluated using the Caspase 3/7 assay kit. 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).*

4.10 Effects of Phyllanthin on Acetylcholinesterase, Butyrylcholinesterase, and Monoamine oxidase-B (MOA-B)

Patients suffering from NDDs often have acetylcholine deficiency. Acetylcholine is a neurotransmitter that plays a key role in fine-tuning the activity of basal nuclei and exerts profound effects on behavioral manifestation (Picciotto et al., 2012). The human brain has two significant cholinesterases: Acetylcholinesterase (AChE) and Butyrylcholinesterase (BuChE), with AChE being the more abundant of the two and is essential for acetylcholine degeneration (Mesulam et al., 2002). To support the neuroprotection results, the inhibitory effect of phyllanthin on AChE, BuChE and MAO-B was investigated using enzymatic assays. The results show that 100 µM phyllanthin inhibited AChE (0%), BuChE (14.9%) and MAO-B (25.8%). Although the concentration used is above the ones selected for the neuroprotection studies, we can however not speculate what impact the exact concentrations used in this study will have on these enzymes.

Table 1: The inhibiting potential of phyllanthin on AChE, BuChE and MAO-B.

Compounds	AChE (IC_{50}, µM)	BuChE (IC_{50}, µM)	MAO-B (IC_{50}, µM)
Phyllanthin	0 % inhibition at 100µM	14.9 % inhibition at 100µM	25.8 % inhibition at 100µM
Donepezil	0.08 *	-	-
Tacrine	-	0.05 *	-
Safinamide	-	-	0.45 [a]

(* = p-value: < 0.05)

Molecular docking results of phyllanthin

Molecular docking is a computational technique that predicts the binding pose with the lowest binding affinity of a ligand within a protein active site. Molecular docking provides further insights into the structure activity relationships between a ligand and protein (Meng et al., 2011). Acetylcholinesterase (AChE) metabolizes the neurotransmitter acetylcholine (ACh) and has been shown to play a crucial role in the development of various neurodegenerative diseases e.g., Alzheimer's Disease (Talesa, 2001; Marucci et al., 2021). AChE has a narrow active site gorge of 20 Å in length and contains two anionic sites ((peripheral anionic site (PAS) and catalytic anionic site (CAS)) shown to be essential for the catalytic activity of the protein (Colletier et al., 2006; Sussman et al.,1991). Phyllanthin was docked across both the PAS and CAS of AChE (figure 11B and 11 D) and obtained a binding affinity score of -9.24 kcal/mol (table 2) compared to donepezil's (-10.70 kcal/mol).

Table 2: Binding interaction types between phyllanthin and binding site residues as well as binding affinity score and IC50's for AChE, BuChE and MAO-B.

Enzymes	Types of binding interactions and residues interaction with phyllanthin			Binding affinity score (kcal/mol)	IC_{50} (µM)
	Hydrogen	Hydrogen-π	π-π stacking		
AChE [a]	-	Tyr341 [d]	-	-9.24	0 % inhibition at 100 µM
BuChE [b]	Asp70	-	Trp82, Tyr332	-10.60	14.9 % inhibition at 100 µM
MAO-B [c]	-	Ile199	-	-11.59	25.8 % inhibition at 100 µM

a = acetylcholinesterase, b = butyrylcholinesterase, c = monoamine oxidase B, d = two of the same protein-ligand interactions with the same residue

BuChE is cholinesterase (ChE) enzymes and is responsible for metabolizing the ACh neurotransmitter. BuChE shares a similar structural composition to AChE with both enzymes containing PAS and CAS active sites and the active sites are 20 Å in length (Colletier et al., 2006; Sussman et al.,1991; Marakovic et al., 2016 and Lane, Potkin and Enz, 2006). BuChE binding site contains more aliphatic rich amino acids compared to AChE resulting in a binding pocket volume of 200 $Å^3$ larger than the AChE active site (Lane, Potkin and Enz, 2006). Phyllanthin was docked within the BuChE active site and obtained a similar binding affinity score of -10.60 kcal/mol (table 2) when compared to tacrine (-11.56 kcal/mol).

The enzyme Monoamine oxidase (MAO) has two isoforms MAO-A and MAO-B, with MAO-B the most dominant enzyme in the brain. The MAO enzymes are responsible for the metabolism of various substrates namely, dopamine, serotonin, norepinephrine, and epinephrine (Lane, Potkin and Enz, 2006 and Mzezewa et al., 2021). MAO-B has also shown to cause the formation of

neurotoxic free radicals and radical oxidative species that have shown to play a significant role in the development of neurological diseases and disorders (Yiannopoulou and Papageorgiou 2013; Riederer, 2004 and Blennow et al., 2015). The docked ligand obtained a superior molecular docking binding affinity score of -11.59 kcal/mol (table 2) compared to safinamide (-10.74 kcal/mol). Details of phyllanthin binding to AChE, BuChE and MOA-B are shown in figures 11-13 and more explanations on the figures will be done in the discussion section..

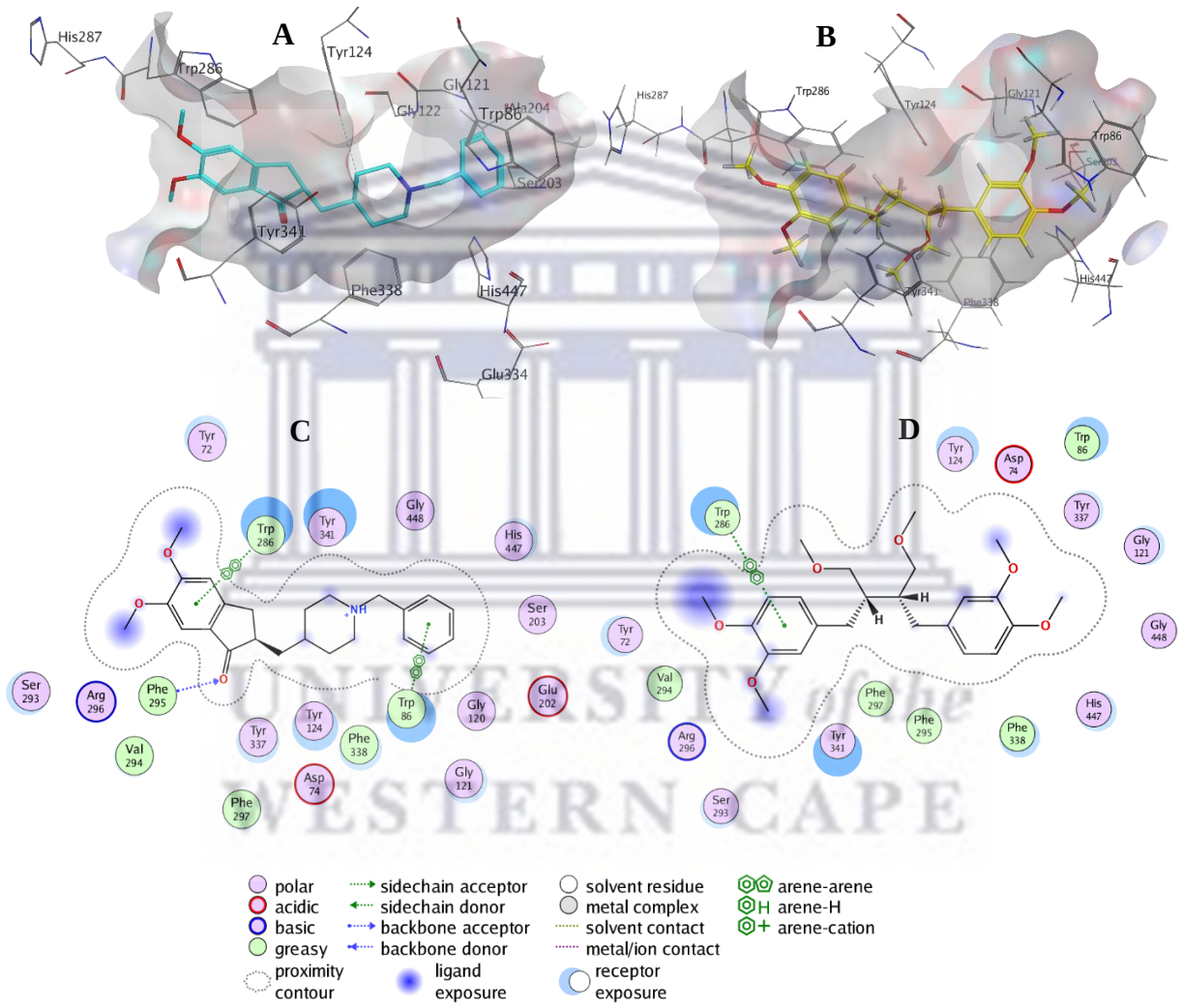

Figure 11: The three-dimensional representations of the binding orientations of donepezil (A) and phyllanthin (B) and the two-dimensional binding interaction map of donepezil (C) and phyllanthin (D) within the AChE active site within the AChE active site using the MOE 2020 software suite *(Molecular Operating Environment, 2020). Phyllanthin was docked across both the PAS and CAS of the AChE active site The two-dimensional binding interaction map of donepezil (C) and phyllanthin (D) within the AChE active site. The dimethoxy benzene moiety Phyllanthin was orientated within the PAS and aliphatic linker orientated in the mid-section of the AChE active site formed a hydrogen-π interaction with mid-section residue Tyr341.*

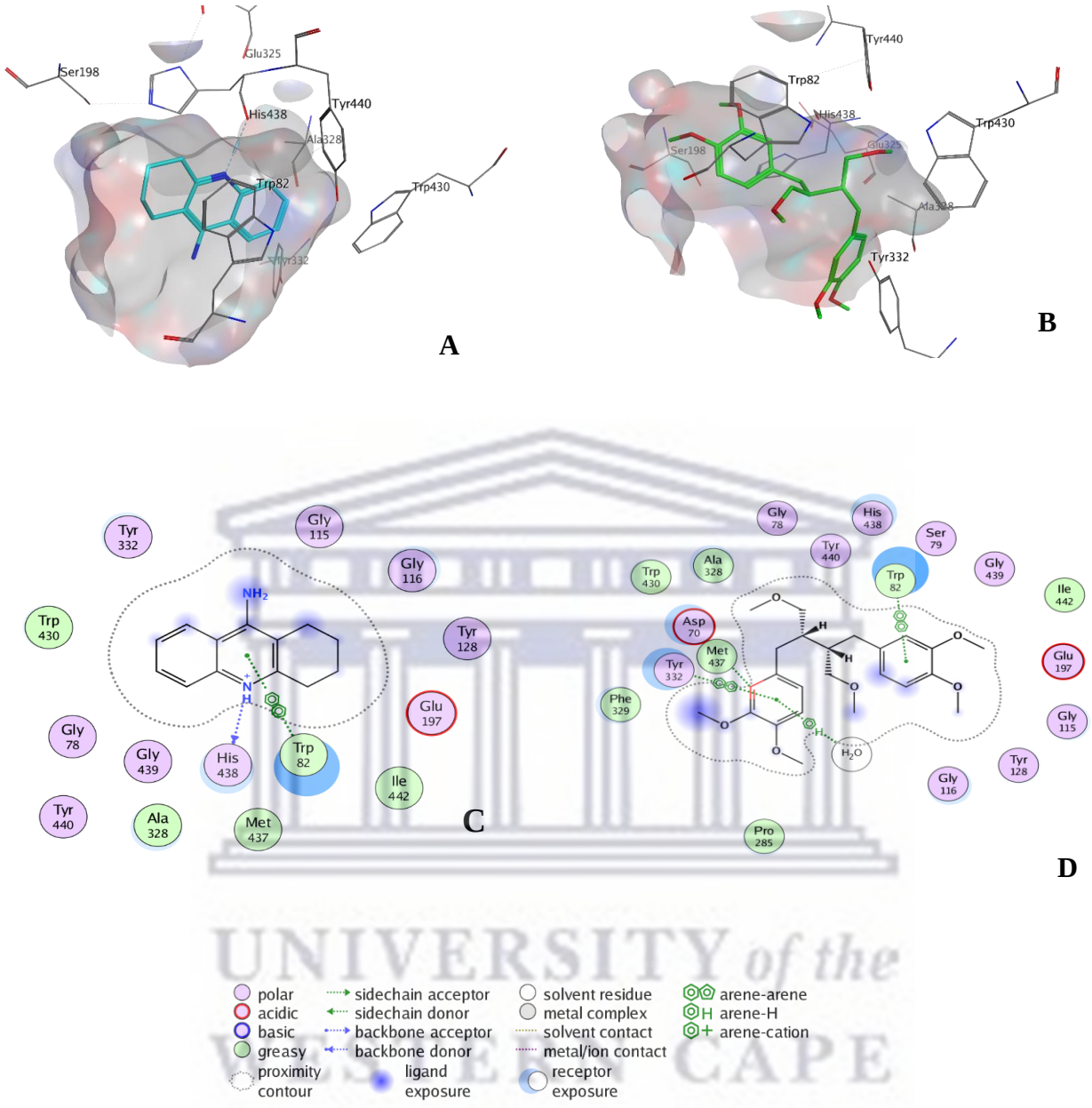

Figure 12: The three-dimensional representations of the binding orientations of tacrine (A) and phyllanthin (B) within the BuChE active site and the two-dimensional binding interaction map of tacrine (C) and phyllanthin (D) within the BuChE active site using the MOE 2020 software suite *(Molecular Operating Environment, 2020). Phyllanthin was docked along the BuChE active site wall resulting in multiple sections of its chemical structure exposed to the external environment. The two-dimensional binding interaction map of tacrine (C) and phyllanthin (D) within the BuChE active site. The dimethoxy benzene moieties Phyllanthin orientated within the PAS and entrance of the BuChE active site formed a hydrogen bond interaction with Asp70 and π-π stacking interactions with Trp82 (PAS) and Tyr 332. Both B and D representation shows that the phyllanthin does not fit within the BuChE active site and thus minimal experimental BuChE inhibitory activity should be expected.*

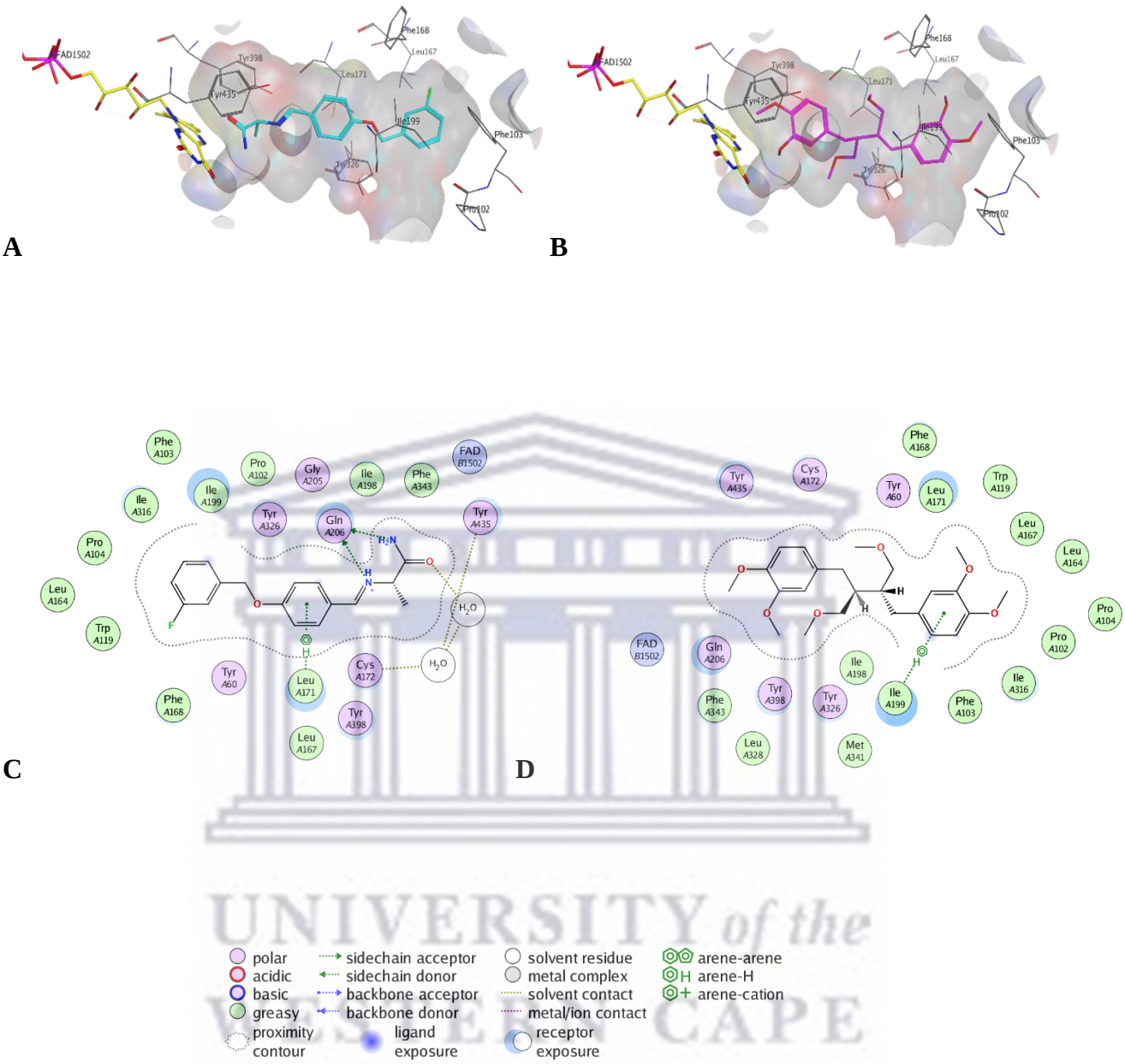

Figure 13: The three-dimensional representations of the binding orientations of safinamide (A) and phyllanthin (B) within the MAO-B active site and the two-dimensional binding interaction map of safinamide (C) and phyllanthin (D) within the MAO-B active site, using the MOE 2020 software suite (Molecular Operating Environment, 2020). *Phyllanthin was docked across both the entrance and substrate cavities of the MAO-B active site. The two-dimensional binding interaction map of safinamide (C) and phyllanthin (D) within the MAO-B active site. The dimethoxy benzene Phyllanthin moiety oriented within the entrance cavity formed a hydrogen-π interaction with boundary residue Ile199 found between the two cavities of the MAO-B active site.*

CHAPTER FIVE

Discussion

5.1 Introduction

As the life expectancy of the population increases, so does the prevalence of age-related disorders, such as PD. This increase puts extra strain on the healthcare system and sparks the need for new treatment options which include therapies capable of showing the progression of PD. Currently, levodopa is the most effective treatment available for PD. However, levodopa therapy is faced with a number of complications that may accelerate the progression of PD (Fahn, 1996). To develop a truly effective therapy, improvement of our current understanding of the cellular and molecular mechanisms underlying PD pathogenesis and progression is crucial (Xicoy, Wieringa and Martens, 2017).

Over the years, numerous PD models have been used to investigate the complex mechanisms involved in PD. However, J. William Langston's pioneering research in the 1980's paved the way for the extensive use of MPTP as an *in-vivo* neurotoxin that replicates some aspects of the neuronal loss observed in PD. Today MPP^+, the toxic metabolite of MPTP, treated SH-SY5Y cells have become a vital *in-vitro* model for investigating the mechanisms of neurodegeneration in PD (Hare et al., 2013). MPP^+ induces a syndrome clinically and pathologically similar to PD by disrupting mitochondrial function (Xicoy, Wieringa and Martens, 2017). The mitochondria is responsible for many vital cellular functions, such as is the production of energy, the regulation of calcium homeostasis and PCD. Additionally, defects in mitochondrial respiration have long been implicated in the etiology and pathogenesis of PD. Taken together, mitochondrial dysfunction holds promise as a potential therapeutic target to slow the progression of neurodegeneration in PD (Perrier and Villa, 2012).

5.2 Neuroprotective Effects of Phyllanthin Against MPP^+-induced Cytotoxicity and Morphological Damage to SH-SY5Y Cells

Phyllanthin has extensively been reported to be the primary active lignand in *P. amarus*, which has been implicated in the strengthening and recovery of peripheral nerves after injury (Wannannond et al., 2011). In the present study, the neuroprotective potential of phyllanthin, was

investigated in an MPP^+-induced neurotoxicity model, in SH-SY5Y cells. Cytotoxicity assessment of phyllanthin was carried out to establish the optimum concentrations used in the study. Findings from this study show that phyllanthin increased cell viability in the SH-SY5Y cells when compared to the control cells. Furthermore, when the viability of SH-SY5Y cells was assessed following exposure to the MPP^+ toxin and pre-treatment with phyllanthin, the findings showed that phyllanthin protected the cells from the toxicity induced by MPP^+. Furthermore, the images obtained after treatments appeared to show the restoration of cellular morphology. Taken together, these results support the neuroprotective potential of phyllanthin.

5.3 Neuroprotective Effects of Phyllanthin Against MPP^+-Induced Decrease in Mitochondrial Membrane Potential (MMP) in SH-SY5Y Cells

The maintenance of the MMP is vital for cell survival and also plays an important role in apoptosis, therefore, it is considered to be an accurate depiction of mitochondrial integrity (Osellame et al., 2012). In healthy mitochondria, the ability of the fluorescent probe, TMRE, to accumulate is illustrated by a high TMRE fluorescence intensity. The solubility and charge of the probe enables it to accumulate in the mitochondrial matrix. However, CCCP depolarizes the mitochondrial membrane, impairing the cell's ability to retain the fluorescent signal of TMRE and is therefore used as a positive control (Kwon et al., 2017). Results from the present study show that treatment with MPP^+ had no impact on MMP, likewise the pre-treatment with phyllanthin. Although previous studies have shown that MPP^+ reduces MMP (Chen et al., 2021, Enogieru et al., 2020, Kim et al., 2018, Zhong et al., 2018, Lee, Lee and Kim, 2011), in the present study, we were unable to demonstrate that. However, we showed that MPP^+ reduced cellular ATP which is also indicative of mitochondrial toxicity.

5.4 Neuroprotective Effects of Phyllanthin Against MPP^+-Induced Depletion of Cellular ATP in SH-SY5Y Cells

One of the expected outcomes of impaired mitochondrial function is bioenergetic failure due to a reduction in ATP production (Richardson et al., 2006). Reduced ATP levels halt's ETC activity in the mitochondria, resulting in the disruption of ionic pump systems, which causes an increase in intracellular Ca^{2+}, Na^+ and Cl- concentration and the efflux of K^+. These actions cause the depolarization of neurons, which leads to excess neurotransmitter release, resulting in neuronal

excitotoxicity (Benarroch, 2011). In the present study, exposure to MPP^+ caused a significant drop in ATP production. Supporting this view, MPP^+ has been found to cause a rapid and significant reduction of cellular ATP levels, in both brain synaptosomal preparations and whole mouse brain tissues (Chan et al. 1991, Scotcher et al. 1990). However, the pretreated samples in our study showed an increase in ATP production of at least 15% over the MPP^+ control. To support our findings, phyllanthin has been reported to improve ATP generation in various experimental models (Ranakrishna, Gopi and Setty, 2012).

5.5 Neuroprotective Effects of Phyllanthin Against MPP^+-Induced Apoptosis in SH-SY5Y Cells

Programmed cell death (PCD) is a physiological process whereby molecular programs, intrinsic to the cell, are activated, resulting in its own destruction. PCD is a fundamental property of all multicellular organisms and is essential for their development, tissue homeostasis, organ morphogenesis, and defense against mutated or infected cells. However, uncontrolled PCD can lead to many complications, one of which is neurodegeneration (Vila and Przedborski 2003). Apoptosis, the most common type of PCD, result from the activation of two distinct molecular cascades: the intrinsic (mitochondrial) and the extrinsic (death receptor) pathways. Both pathways converge at the mitochondrial level and involve the activation of initiator caspases (caspase 8 and 9) that facilitates the proteolytic maturation of executioner caspases (caspase 3 and 7) which are the final effectors of cell death (Perier and Vila 2012). In the present study, our results showed that the exposure to MPP^+ significantly increases the levels of caspase within the cells. This is in accordance with two respective studies that reported that MPP^+ elevates caspase activity (Ramachandian et al., 2006, Chu et al., 2005). However, our results also showed that all of the samples pretreated with phyllanthin have substantially produced less caspase 3/7 activity when compared to the MPP^+-treated cells. Similarly, it has been reported that phyllanthin successfully inhibited alcohol-induced cell death, by blocking caspase-3/7 activation in HepG2 cells (Yuan et al., 2021, Sukketsiri, Sawangjaroen and Tanasawet, 2016).

5.6 In vitro enzymatic activity and molecular docking of phyllanthin on ChE and MOA-B

In the present study the binding and interaction of phyllanthin with AChE, BuChE and MAO-B was investigated. Phyllanthin exhibited a π-π stacking interaction with important PAS residue

Trp286 within the AChE active site (figure 11B and 11D). The docked compound exhibited weak hydrophobic bond interactions with mid-section active site residues Phe295, Phe338, Tyr124, Tyr337. The compound formed weak hydrophobic interactions with important CAS residue Trp86 and catalytic triad residue (His447), found within the CAS, (Colletier et al., 2006, Zhang et al., 2002) which is essential in the hydrolysis of ACh by AChE. Phyllanthin at 100 µM was unable to inhibit AChE in the *in vitro* AChE assay. The compound only exhibited strong interactions with PAS residues. However, phyllanthin was unable to form strong binding interactions with the CAS residues where ACh catalysis took place. Due to the stereochemistry of the two methoxy substituents on the aliphatic chain of phyllanthin, the methoxy substituents were unable to orientate on either side of the narrow mid-section residues (figure 12B and 12D), Thus, resulting in the phyllanthin being positioned further within the PAS and increasing the distance between the dimethoxy benzene moiety positioned in the CAS and important CAS site residues. Therefore, it can be deduced that the compound was unable to block and/or alter the catalytic triad residues (Ser203, Glu334, His447) and in turn further explains the poor *in vitro* AChE inhibitory activity (Marakovic et al., 2016, Colletier et al., 2006).

Furthermore, the compound docked within the BuChE active site and exhibited hydrogen bond interactions with Asp70 (figure 13B and 13D). The docked ligand formed π-π stacking interactions with Trp82 and Tyr332. The docked ligand exhibited hydrophobic interactions with one of the catalytic triad residues His438 which is responsible for the hydrolysis of ACh (Boasak et al., 2018, Harkcom et al., 2007). In figure 13B, it can be observed that various sections of the ligand structure are exposed to the external environment of the binding pocket. Thus, it can be inferred that the poor *in vitro* BuChE inhibition result (table 1) obtained is due to the ligand being too large for the binding pocket and no strong binding interactions (hydrogen bond, hydrogen-π or/and π-π stacking interactions) were formed between the ligand and the catalytic triad residues. This observation is further supported by figure 13D, where a section of the dimethoxy benzene moiety is shown to break out of the active site and the structure is shown in red.

In addition, the MAO-B's active site contains two cavities namely the entrance cavity and hydrophobic substrate cavity. The substrate cavity contains two residues (Tyr398 and Tyr435) and co-factor flavin (FAD) that form aromatic cage-like interactions around a MAO-B substrate. The aromatic cage interactions around the substrate stabilize the substrate within the active site for

catalysis. (Binda et al., 2002) The mid-section separating the two cavities is lined with residues Phe168, Leu171, Ile199 and Tyr326. The orientation of Ile199 either allows access of substrates or inhibitors to the substrate cavity of the active site (Milezek et al., 2011, Binda et al., 2007). Phyllanthin's structure was docked across both the entrance and substrate cavities (figure 13B). The dimethoxy benzene moiety positioned in the entrance cavity exhibited a hydrogen-π interaction with Ile199 and formed weak hydrophobic interactions with mid-section residues Phe168, Leu171 and Tyr326 (figure 13B and 13D). The other dimethoxy benzene moiety exhibited weak hydrophobic interactions with the substrate cavity residues Tyr398, Tyr435 and co-factor FAD. The compound exhibited poor *in vitro* MAO-B inhibition activity (table 1) even though the compound exhibited a greater binding affinity score compared to safinamide (table 1). The poor *in vitro* MAO-B inhibition activity can be attributed to the formation of predominantly weak hydrophobic interactions especially in the substrate cavity residues and the co-factor FAD.

5.7 Conclusión

The results obtained in this study showed that phyllanthin was successful in attenuating the toxicity induced by MPP^+ in SH-SY5Y cells. Pretreatment with phyllanthin did not only improve cell viability/morphology but also reversed the mitochondrial imbalances brought upon by MPP^+ exposure, significantly increasing ATP production in the cells. In addition to an increase in ATP production, the results also showed that pretreatment with phyllanthin prevented elevated caspase 3/7 activities in the cells. The molecular docking results showed that phyllanthin was unable to form strong binding interactions (hydrogen bond, hydrogen-πand/or π-π stacking interactions) with important catalytic triad ChE residues responsible for the metabolism of the ACh neurotransmitter, resulting in the poor *in vitro* inhibitory activity obtained by phyllanthin. As there is a need for an alternative treatment to the current gold standard, levodopa, the neuroprotective effects of phyllanthin seen in this study makes it a promising novel natural product for the treatment of PD, with potentially minimal side effects. However, more studies are needed to further elucidate the true effects of Phyllanthin

5.8 Study Limitations

The inability to perform more assays to ascertain the potential mechanisms of action of phyllanthin was one of the limitations experienced in this study, mainly due to time and financial constraints.

Additionally, dopaminergic neurons are the cells primarily affected in PD. However, obtaining appropriate and sufficient amounts of human primary neurons is a major challenge considering ethical constraints, and the difficulty with which these cells are handled and cultured. These further limited the application of this cell type in the present study.

5.9 Future Recommendations

Future recommendations for this study would be:

1. Performing additional assays to ascertain the exact molecular mechanisms of action of phyllanthin.
2. Evaluating the *in vivo* pathophysiological effects of phyllanthin in animal models of PD to provide a better understanding of the possible neuroprotective effects of phyllanthin.

References

- Abd El-Aziz, E.R., 2012. The effect of Ginkgo biloba extract on parkinsonism-induced biochemical changes in brain of irradiated rats.
- Abhinav, K., Stanton, B., Johnston, C., Hardstaff, J., Orrell, R.W., Howard, R., Clarke, J., Sakel, M., Ampong, M.A., Shaw, C.E. and Leigh, P.N., 2007. Amyotrophic lateral sclerosis in South-East England: a population-based study. *Neuroepidemiology*, *29*(1-2), pp.44-48.
- ACD/ChemSketch, version 2020.2.1, Advanced Chemistry Development, Inc., Toronto, ON, Canada, www.acdlabs.com, 2021.
- Adewusia, E.A., Foucheb, G. and Steenkamp, V., 2013. Effect of four medicinal plants on amyloid-β induced neurotoxicity in SHSY5Y cells. *African Journal of Traditional, Complementary and Alternative Medicines*, *10*(4), pp.6-11.
- Alagan, A., Jantan, I., Kumolosasi, E. and Azmi, N., 2019. Phyllanthus amarus protects against spatial memory impairment induced by lipopolysaccharide in mice. *Bioinformation*, *15*(8), p.535.
- Alagan, A., Jantan, I., Kumolosasi, E., Ogawa, S., Abdullah, M.A. and Azmi, N., 2019. Protective effects of Phyllanthus amarus against lipopolysaccharide-induced neuroinflammation and cognitive impairment in rats. *Frontiers in pharmacology*, *10*, p.632.
- Alonso, A.D.C., Li, B., Grundke-Iqbal, I. and Iqbal, K., 2008. Mechanism of tau-induced neurodegeneration in Alzheimer disease and related tauopathies. *Current Alzheimer Research*, *5*(4), pp.375-384.
- Andersen, J.K., 2004. Oxidative stress in neurodegeneration: cause or consequence?. *Nature medicine*, *10*(7), pp.S18-S25.
- Araki, W., Kitaguchi, N., Tokushima, Y., Ishii, K., Aratake, H., Shimohama, S., Nakamura, S. and Kimura, J., 1991. Trophic effect of β-amyloid precursor protein on cerebral cortical neurons in culture. *Biochemical and biophysical research communications*, *181*(1), pp.265-271.
- Aruna, K., Rajeswari, P.D.R. and Sankar, S.R., 2017. The effect of Oxalis corniculata extract against the behavioral changes induced by 1-methyl-4-phenyl-1, 2, 3, 6-

tetrahydropyridine (MPTP) in mice. *Journal of Applied Pharmaceutical Science*, *7*(3), pp.148-153.

- Bala, K., Tripathy, B.C. and Sharma, D., 2006. Neuroprotective and anti-ageing effects of curcumin in aged rat brain regions. *Biogerontology*, *7*(2), pp.81-89.
- Balunas, M.J. and Kinghorn, A.D., 2005. Drug discovery from medicinal plants. *Life sciences*, *78*(5), pp.431-441.
- Bardien, S., Keyser, R., Yako, Y., Lombard, D. and Carr, J., 2009. Molecular analysis of the parkin gene in South African patients diagnosed with Parkinson's disease. *Parkinsonism & related disorders*, *15*(2), pp.116-121.
- Bastianetto, S., Yao, Z.X., Papadopoulos, V. and Quirion, R., 2006. Neuroprotective effects of green and black teas and their catechin gallate esters against β-amyloid-induced toxicity. *European Journal of Neuroscience*, *23*(1), pp.55-64.
- Bates, G.P., Dorsey, R., Gusella, J.F., Hayden, M.R., Kay, C., Leavitt, B.R., Nance, M., Ross, C.A., Scahill, R.I., Wetzel, R. and Wild, E.J., 2015. Huntington disease. *Nature reviews Disease primers*, *1*(1), pp.1-21.
- Beal, M.F., 2003. Bioenergetic approaches for neuroprotection in Parkinson's disease. *Annals of Neurology: Official Journal of the American Neurological Association and the Child Neurology Society*, *53*(S3), pp.S39-S48.
- Beal, M.F., 2005. Mitochondria take center stage in aging and neurodegeneration. *Annals of Neurology: Official Journal of the American Neurological Association and the Child Neurology Society*, *58*(4), pp.495-505.
- Ben-Shlomo, Y., Whitehead, A.S. and Smith, G.D., 1996. Parkinson's, Alzheimer's, and motor neurone disease. *BMJ*, *312*(7033), p.724.
- Bhattacharya, D. and Cheng, J., 2013. 3Drefine: Consistent protein structure refinement by optimizing hydrogen bonding network and atomic-level energy minimization. *Proteins: Structure, Function, and Bioinformatics*, *81*(1), pp.119-131.
- Biernat, J., Gustke, N., Drewes, G. and Mandelkow, E., 1993. Phosphorylation of Ser262 strongly reduces binding of tau to microtubules: distinction between PHF-like immunoreactivity and microtubule binding. *Neuron*, *11*(1), pp.153-163.

- Binda, C., Mattevi, A. and Edmondson, D.E., 2002. Structure-function relationships in flavoenzyme-dependent amine oxidations: a comparison of polyamine oxidase and monoamine oxidase. *Journal of biological chemistry*, *277*(27), pp.23973-23976.

- Binda, C., Wang, J., Pisani, L., Caccia, C., Carotti, A., Salvati, P., Edmondson, D.E. and Mattevi, A., 2007. Structures of human monoamine oxidase B complexes with selective noncovalent inhibitors: safinamide and coumarin analogs. *Journal of medicinal chemistry*, *50*(23), pp.5848-5852.
- Binder, L.I., Frankfurter, A. and Rebhun, L.I., 1985. The distribution of tau in the mammalian central nervous system. *The Journal of cell biology*, *101*(4), pp.1371-1378.
- Blennow, K., Mattsson, N., Schöll, M., Hansson, O. and Zetterberg, H., 2015. Amyloid biomarkers in Alzheimer's disease. *Trends in pharmacological sciences*, *36*(5), pp.297-309.
- Bosak, A., Ramić, A., Šmidlehner, T., Hrenar, T., Primožič, I. and Kovarik, Z., 2018. Design and evaluation of selective butyrylcholinesterase inhibitors based on Cinchona alkaloid scaffold. *PLoS One*, *13*(10), p.e0205193.
- Bower, J.H., Diop, A.G., Gouider, R. and Schmutzhard, E., 2014. Addressing neurologic needs in sub–Saharan Africa: An opportunity for multisociety cooperation. *Neurology*, *83*(13), pp.1207-1209.
- Bronstein, J.M., Tagliati, M., Alterman, R.L., Lozano, A.M., Volkmann, J., Stefani, A., Horak, F.B., Okun, M.S., Foote, K.D., Krack, P. and Pahwa, R., 2011. Deep brain stimulation for Parkinson disease: an expert consensus and review of key issues. *Archives of neurology*, *68*(2), pp.165-165.
- Buée, L., Bussière, T., Buée-Scherrer, V., Delacourte, A. and Hof, P.R., 2000. Tau protein isoforms, phosphorylation and role in neurodegenerative disorders. *Brain Research Reviews*, *33*(1), pp.95-130.
- Buttiglione, M., Vitiello, F., Sardella, E., Petrone, L., Nardulli, M., Favia, P., d'Agostino, R. and Gristina, R., 2007. Behaviour of SH-SY5Y neuroblastoma cell line grown in different media and on different chemically modified substrates. *Biomaterials*, *28*(19), pp.2932-2945.

- Callio, J., Oury, T.D. and Chu, C.T., 2005. Manganese superoxide dismutase protects against 6-hydroxydopamine injury in mouse brains. *Journal of Biological Chemistry*, *280*(18), pp.18536-18542.
- Campbell, M.C. and Tishkoff, S.A. (2008) "African genetic diversity: Implications for human demographic history, modern human origins, and complex disease mapping," *Annual Review of Genomics and Human Genetics*, 9(1), pp. 403–433. Available at: https://doi.org/10.1146/annurev.genom.9.081307.164258.
- Carr, A.C., McCall, M.R. and Frei, B., 2000. Oxidation of LDL by myeloperoxidase and reactive nitrogen species: reaction pathways and antioxidant protection. *Arteriosclerosis, thrombosis, and vascular biology*, *20*(7), pp.1716-1723.
- Castino, R., Fiorentino, I., Cagnin, M., Giovia, A. and Isidoro, C., 2011. Chelation of lysosomal iron protects dopaminergic SH-SY5Y neuroblastoma cells from hydrogen peroxide toxicity by precluding autophagy and Akt dephosphorylation. *Toxicological Sciences*, *123*(2), pp.523-541.
- Chan, C.S., Gertler, T.S. and Surmeier, D.J., 2010. A molecular basis for the increased vulnerability of substantia nigra dopamine neurons in aging and Parkinson's disease. *Movement disorders*, *25*(S1), pp.S63-S70.
- Chan, C.S., Guzman, J.N., Ilijic, E., Mercer, J.N., Rick, C., Tkatch, T., Meredith, G.E. and Surmeier, D.J., 2007. 'Rejuvenation'protects neurons in mouse models of Parkinson's disease. *Nature*, *447*(7148), pp.1081-1086.
- Chan, P., DeLanney, L.E., Irwin, I., Langston, J.W. and Di Monte, D., 1991. Rapid ATP loss caused by 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine in mouse brain. *Journal of neurochemistry*, *57*(1), pp.348-351..
- Charles, C., 2019. *How to use Phyllanthus amarus to cure hepatitis B*. Available at: https://www.mightyceeblog.com/2019/03/how-to-use-phyllantus-amarus-to-cure.html (Accessed: 11 May 2023).
- Chen, Y., Chen, C., Song, D., Liu, T. and Cheng, O., 2021. Dexmedetomidine protects SH-SY5Y cells against MPP+-induced declining of mitochondrial membrane potential and cell cycle deficits. *European Journal of Neuroscience*, *54*(1), pp.4141-4153.

- Cheung, J., Rudolph, M.J., Burshteyn, F., Cassidy, M.S., Gary, E.N., Love, J., Franklin, M.C. and Height, J.J., 2012. Structures of human acetylcholinesterase in complex with pharmacologically important ligands. *Journal of medicinal chemistry*, *55*(22), pp.10282-10286.
- Choi, Y.T., Jung, C.H., Lee, S.R., Bae, J.H., Baek, W.K., Suh, M.H., Park, J., Park, C.W. and Suh, S.I., 2001. The green tea polyphenol (−)-epigallocatechin gallate attenuates β-amyloid-induced neurotoxicity in cultured hippocampal neurons. *Life sciences*, *70*(5), pp.603-614.
- Colletier, J.P., Fournier, D., Greenblatt, H.M., Stojan, J., Sussman, J.L., Zaccai, G., Silman, I. and Weik, M., 2006. Structural insights into substrate traffic and inhibition in acetylcholinesterase. *The EMBO journal*, *25*(12), pp.2746-2756.
- Commenges, D., Scotet, V., Renaud, S., Jacqmin-Gadda, H., Barberger-Gateau, P. and Dartigues, J.F., 2000. Intake of flavonoids and risk of dementia. *European journal of epidemiology*, *16*(4), pp.357-363.
- Crocker, S.J., Smith, P.D., Jackson-Lewis, V., Lamba, W.R., Hayley, S.P., Grimm, E., Callaghan, S.M., Slack, R.S., Melloni, E., Przedborski, S. and Robertson, G.S., 2003. Inhibition of calpains prevents neuronal and behavioral deficits in an MPTP mouse model of Parkinson's disease. *Journal of Neuroscience*, *23*(10), pp.4081-4091.
- Cruz-Vicente, P., Passarinha, L.A., Silvestre, S. and Gallardo, E., 2021. Recent developments in new therapeutic agents against Alzheimer and Parkinson diseases: in-silico approaches. *Molecules*, *26*(8), p.2193.
- Davie, C.A., 2008. A review of Parkinson's disease. *British medical bulletin*, *86*(1), pp.109-127.
- De Vos, K.J., Grierson, A.J., Ackerley, S. and Miller, C.C., 2008. Role of axonal transport in neurodegenerative diseases. *Annu. Rev. Neurosci.*, *31*, pp.151-173.
- Dekker, M.C., Coulibaly, T., Bardien, S., Ross, O.A., Carr, J. and Komolafe, M., 2020. Parkinson's disease research on the African continent: obstacles and opportunities. *Frontiers in neurology*, *11*, p.512.
- deSouza, R.M., Moro, E., Lang, A.E. and Schapira, A.H., 2013. Timing of deep brain stimulation in Parkinson disease: a need for reappraisal?. *Annals of neurology*, *73*(5), pp.565-575.

- Dickson, D.W., 2001. α-Synuclein and the Lewy body disorders. *Current opinion in neurology*, *14*(4), pp.423-432.
- Dickson, D.W., Kouri, N., Murray, M.E. and Josephs, K.A., 2011. Neuropathology of frontotemporal lobar degeneration-tau (FTLD-tau). *Journal of Molecular Neuroscience*, *45*(3), pp.384-389.
- Dixit, R., Ross, J.L., Goldman, Y.E. and Holzbaur, E.L., 2008. Differential regulation of dynein and kinesin motor proteins by tau. *Science*, *319*(5866), pp.1086-1089.
- Dotchin, C.L., Msuya, O. and Walker, R.W., 2007. The challenge of Parkinson's disease management in Africa. *Age and ageing*, *36*(2), pp.122-127.
- Du Toit, N., Van Coller, R., Anderson, D.G., Carr, J. and Bardien, S., 2019. Frequency of the LRRK2 G2019S mutation in South African patients with Parkinson's disease. *neurogenetics*, *20*(4), pp.215-218.
- Du, X.X., Xu, H.M., Jiang, H., Song, N., Wang, J. and Xie, J.X., 2012. Curcumin protects nigral dopaminergic neurons by iron-chelation in the 6-hydroxydopamine rat model of Parkinson's disease. *Neuroscience bulletin*, *28*(3), pp.253-258.
- Dugger, B.N. and Dickson, D.W., 2017. Pathology of neurodegenerative diseases. *Cold Spring Harbor perspectives in biology*, *9*(7), p.a028035.
- Dugger, B.N., Adler, C.H., Shill, H.A., Caviness, J., Jacobson, S., Driver-Dunckley, E., Beach, T.G. and Arizona Parkinson's Disease Consortium, 2014. Concomitant pathologies among a spectrum of parkinsonian disorders. *Parkinsonism & related disorders*, *20*(5), pp.525-529.
- Dugger, B.N., Serrano, G.E., Sue, L.I., Walker, D.G., Adler, C.H., Shill, H.A., Sabbagh, M.N., Caviness, J.N., Hidalgo, J., Saxon-LaBelle, M. and Chiarolanza, G., 2012. Presence of striatal amyloid plaques in Parkinson's disease dementia predicts concomitant Alzheimer's disease: usefulness for amyloid imaging. *Journal of Parkinson's disease*, *2*(1), pp.57-65.
- Enemali, F., Iteire, K.A., Uwejigho, R.E. and Ogunberi, B., 2023. Aqueous leaf extract of Phyllanthus amarus protects against oxidative stress and misfiring of dopaminergic neurons in Paraquat-induced Parkinson's disease-like model of adult Wistar rats.
- Enogieru, A. B.;Omoruyi, S. I. & Ekpo, O. E. 2020. Aqueous leaf extract of Sutherlandia frutescens attenuates ROS-induced apoptosis and loss of mitochondrial membrane

potential in MPP^+-treated SH-SY5Y cells. *Tropical Journal of Pharmaceutical Research*, 19**,** 549-555.

- Eve, D.J., Nisbet, A.P., Kingsbury, A.E., Hewson, E.L., Daniel, S.E., Lees, A.J., Marsden, C.D. and Foster, O.J., 1998. Basal ganglia neuronal nitric oxide synthase mRNA expression in Parkinson's disease. *Molecular brain research*, *63*(1), pp.62-71.
- Exner, N., Lutz, A.K., Haass, C. and Winklhofer, K.F., 2012. Mitochondrial dysfunction in Parkinson's disease: molecular mechanisms and pathophysiological consequences. *The EMBO journal*, *31*(14), pp.3038-3062.
- Fahn, S. and Sulzer, D., 2004. Neurodegeneration and neuroprotection in Parkinson disease. *NeuroRx*, *1*(1), pp.139-154.
- Fährrolfes, R., Bietz, S., Flachsenberg, F., Meyder, A., Nittinger, E., Otto, T., Volkamer, A. and Rarey, M., 2017. Proteins Plus: a web portal for structure analysis of macromolecules. *Nucleic Acids Research*, *45*(W1), pp.W337-W343.
- Fan, J., Fu, A. and Zhang, L., 2019. Progress in molecular docking. *Quantitative Biology*, *7*(2), pp.83-89.
- Fukushima, T., Gao, T., Tawara, T., Hojo, N., Isobe, A. and Yamane, Y., 1997. Inhibitory effect of nicotinamide to paraquat toxicity and the reaction site on complex I. *Archives of toxicology*, *71*(10), pp.633-637.
- Furukawa, K., Barger, S.W., Blalock, E.M. and Mattson, M.P., 1996. Activation of K+ channels and suppression of neuronal activity by secreted β-amyloid-precursor protein. *Nature*, *379*(6560), pp.74-78.
- Gandhi, S. and Abramov, A.Y., 2012. Mechanism of oxidative stress in neurodegeneration. *Oxidative medicine and cellular longevity*, *2012*.
- Gandhi, S., Wood-Kaczmar, A., Yao, Z., Plun-Favreau, H., Deas, E., Klupsch, K., Downward, J., Latchman, D.S., Tabrizi, S.J., Wood, N.W. and Duchen, M.R., 2009. PINK1-associated Parkinson's disease is caused by neuronal vulnerability to calcium-induced cell death. *Molecular cell*, *33*(5), pp.627-638.
- Ghuman, S. and Coopoosamy, R.M., 2011. Crude sample preparation, extraction and in vitro screening for antimicrobial activity of selected wound healing medicinal plants in KwaZulu-Natal, South Africa: A review. *J Med Plants Res*, *5*, pp.3572-6.

- Giacobini, E., 2004. Cholinesterase inhibitors: new roles and therapeutic alternatives. *Pharmacological research*, *50*(4), pp.433-440.
- Giasson, B.I., Forman, M.S., Higuchi, M., Golbe, L.I., Graves, C.L., Kotzbauer, P.T., Trojanowski, J.Q. and Lee, V.M.Y., 2003. Initiation and synergistic fibrillization of tau and alpha-synuclein. *Science*, *300*(5619), pp.636-640.
- Gibb, W.R.G., Luthert, P.J. and Marsden, C., 1989. Corticobasal degeneration. *Brain*, *112*(5), pp.1171-1192.
- Gier, M.L., 2018. The potential neuroprotective effects of two South African plant extracts in hydrogen peroxide-induced neuronal toxicity.
- Gil-Lozano, C., Davila, A.F., Losa-Adams, E., Fairén, A.G. and Gago-Duport, L., 2017. Quantifying Fenton reaction pathways driven by self-generated H2O2 on pyrite surfaces. *Scientific reports*, *7*(1), pp.1-11.
- Giugni, J.C. and Okun, M.S., 2014. Treatment of advanced Parkinson's disease. *Current opinion in neurology*, *27*(4), p.450.
- Gleichmann, M. and Mattson, M.P., 2011. Neuronal calcium homeostasis and dysregulation. *Antioxidants & redox signaling*, *14*(7), pp.1261-1273.
- Gnanaraj, C., Sekar, M., Fuloria, S., Swain, S.S., Gan, S.H., Chidambaram, K., Rani, N.N.I.M., Balan, T., Stephenie, S., Lum, P.T. and Jeyabalan, S., 2022. In Silico Molecular Docking Analysis of Karanjin against Alzheimer's and Parkinson's Diseases as a Potential Natural Lead Molecule for New Drug Design, Development and Therapy. *Molecules*, *27*(9), p.2834.
- Goetz, C.G., 2000. Amyotrophic lateral sclerosis: early contributions of Jean-Martin Charcot. *Muscle & Nerve: Official Journal of the American Association of Electrodiagnostic Medicine*, *23*(3), pp.336-343.
- Goetz, C.G., Poewe, W., Rascol, O., Sampaio, C., Stebbins, G.T., Counsell, C., Giladi, N., Holloway, R.G., Moore, C.G., Wenning, G.K. and Yahr, M.D., 2004. Movement Disorder Society Task Force report on the Hoehn and Yahr staging scale: status and recommendations the Movement Disorder Society Task Force on rating scales for Parkinson's disease. *Movement disorders*, *19*(9), pp.1020-1028.
- Goetz, C.G., Tilley, B.C., Shaftman, S.R., Stebbins, G.T., Fahn, S., Martinez-Martin, P., Poewe, W., Sampaio, C., Stern, M.B., Dodel, R. and Dubois, B., 2008. Movement Disorder

Society-sponsored revision of the Unified Parkinson's Disease Rating Scale (MDS-UPDRS): scale presentation and clinimetric testing results. *Movement disorders: official journal of the Movement Disorder Society*, *23*(15), pp.2129-2170.

- Gomez-Crisostomo, N., 2011. Selva Rivas-Arancibia, Cesar Gallegos-Ríos, Nancy Gomez-Crisostomo, Ever Ferreira-Garcidueñas, Dulce Flores Briseño, Luz Navarro and Erika Rodríguez-Martínez. *Neurodegenerative Diseases: Processes, Prevention, Protection and Monitoring*, p.53.
- Goto, K., Mochizuki, H., Imai, H., Akiyama, H. and Mizuno, Y., 1996. An immuno-histochemical study of ferritin in 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP)-induced hemiparkinsonian monkeys. *Brain research*, *724*(1), pp.125-128.
- Govender, K., Girdwood, S., Letswalo, D., Long, L., Meyer-Rath, G and Milot., 2021. Primary healthcare seeking behaviour of low-income patients across the public and private health sectors in South Africa. *BMC Public Health*, *21*(1). pp.1-1Gray, N.E., Sampath, H., Zweig, J.A., Quinn, J.F. and Soumyanath, A., 2015. Centella asiatica attenuates amyloid-β-induced oxidative stress and mitochondrial dysfunction. *Journal of Alzheimer's Disease*, *45*(3), pp.933-946.
- Hammes, Gordon G. "Multiple conformational changes in enzyme catalysis." *Biochemistry* 41, no. 26 (2002): 8221-8228.
- Hantraye, P., Brouillet, E., Ferrante, R., Palfi, S., Dolan, R., Matthews, R.T. and Beal, M.F., 1996. Inhibition of neuronal nitric oxide synthase prevents MPTP–induced parkinsonism in baboons. *Nature medicine*, *2*(9), pp.1017-1021.
- Hardy, J., Duff, K., Hardy, K.G., Perez-Tur, J. and Hutton, M., 1998. Genetic dissection of Alzheimer's disease and related dementias: amyloid and its relationship to tau. *Nature neuroscience*, *1*(5), pp.355-358.
- Hare, D.J., Adlard, P.A., Doble, P.A. and Finkelstein, D.I., 2013. Metallobiology of 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine neurotoxicity. *Metallomics*, *5*(2), pp.91-109.
- Harkcom, W.T. and Bevan, D.R., 2007. Molecular docking of inhibitors into monoamine oxidase B. *Biochemical and biophysical research communications*, *360*(2), pp.401-406.
- Heikkila, R.E., Manzino, L., Cabbat, F.S. and Duvoisin, R.C., 1984. Protection against the dopaminergic neurotoxicity of 1-methyl-4-phenyl-1, 2, 5, 6-tetrahydropyridine by monoamine oxidase inhibitors. *Nature*, *311*(5985), pp.467-469.

- Hirsch, E.C., Breidert, T., Rousselet, E., Hunot, S., Hartmann, A. and Michel, P.P., 2003. The role of glial reaction and inflammation in Parkinson's disease. *Annals of the New York Academy of Sciences*, *991*(1), pp.214-228.
- Huang, W.J., Zhang, X.I.A. and Chen, W.W., 2016. Role of oxidative stress in Alzheimer's disease. *Biomedical reports*, *4*(5), pp.519-522.
- Hunot, S., Boissiere, F., Faucheux, B., Brugg, B., Mouatt-Prigent, A., Agid, Y. and Hirsch, E.C., 1996. Nitric oxide synthase and neuronal vulnerability in Parkinson's disease. *Neuroscience*, *72*(2), pp.355-363.
- Hutton, M., Lendon, C.L., Rizzu, P., Baker, M., Froelich, S., Houlden, H., Pickering-Brown, S., Chakraverty, S., Isaacs, A., Grover, A. and Hackett, J., 1998. Association of missense and 5′-splice-site mutations in tau with the inherited dementia FTDP-17. *Nature*, *393*(6686), pp.702-705.
- Ibitoye, O.B., Aliyu, N.O. and Ajiboye, T.O., 2020. Protective influence of Phyllanthus muellarianus on ciprofloxacin-induced neurotoxicity in male rats. *Journal of Dietary Supplements*, *17*(3), pp.321-335.
- Ibrakaw, A.S., Omoruyi, S.I., Ekpo, O.E. and Hussein, A.A., 2020. Neuroprotective activities of Boophone haemanthoides (Amaryllidaceae) extract and its chemical constituents. *Molecules*, *25*(22), p.5376.
- Ismail, E.N., Jantan, I., Vidyadaran, S., Jamal, J.A. and Azmi, N., 2020. Phyllanthus amarus prevents LPS-mediated BV2 microglial activation via MyD88 and NF-κB signaling pathways. *BMC complementary medicine and therapies*, *20*(1), pp.1-13.
- Jackson-Lewis, V. and Przedborski, S., 2007. Protocol for the MPTP mouse model of Parkinson's disease. *Nature protocols*, *2*(1), pp.141-151.
- Jadiya, P., Khan, A., Sammi, S.R., Kaur, S., Mir, S.S. and Nazir, A., 2011. Anti-Parkinsonian effects of Bacopa monnieri: insights from transgenic and pharmacological Caenorhabditis elegans models of Parkinson's disease. *Biochemical and biophysical research communications*, *413*(4), pp.605-610.
- Jahromy, M.H., Jalili, M., Mohajer, A.J., Poor, F.K. and Dara, S.M., 2014. Effects of Nigella sativa seed extract on perphenzine-induced muscle rigidity in male mice. *World Journal of Neuroscience*, *2014*.

- Jankovic, J., 2008. Parkinson's disease: clinical features and diagnosis. *Journal of neurology, neurosurgery & psychiatry*, *79*(4), pp.368-376.
- Jansen, R.L.M., Brogan, B., Whitworth, A.J. and Okello, E.J., 2014. Effects of five Ayurvedic herbs on locomotor behaviour in a Drosophila melanogaster Parkinson's disease model. *Phytotherapy Research*, *28*(12), pp.1789-1795.
- Jenner, P. and Olanow, C.W., 2006. The pathogenesis of cell death in Parkinson's disease. *Neurology*, *66*(10 suppl 4), pp.S24-S36.
- Jenner, P., 2003. Oxidative stress in Parkinson's disease. *Annals of Neurology: Official Journal of the American Neurological Association and the Child Neurology Society*, *53*(S3), pp.S26-S38.
- Jiang, J., Wang, W., Sun, Y.J., Hu, M., Li, F. and Zhu, D.Y., 2007. Neuroprotective effect of curcumin on focal cerebral ischemic rats by preventing blood–brain barrier damage. *European journal of pharmacology*, *561*(1-3), pp.54-62.
- Johnson, M.E. and Bobrovskaya, L., 2015. An update on the rotenone models of Parkinson's disease: their ability to reproduce the features of clinical disease and model gene–environment interactions. *Neurotoxicology*, *46*, pp.101-116.
- Josephs, K.A., Tsuboi, Y., Cookson, N., Watt, H. and Dickson, D.W., 2004. Apolipoprotein E ε4 is a determinant for Alzheimer-type pathologic features in tauopathies, synucleinopathies, and frontotemporal degeneration. *Archives of neurology*, *61*(10), pp.1579-1584.
- Joshi, H. and Parle, M., 2007. Evaluation of the antiamnesic effects of Phyllanthus amarus in mice. *Colombia medica*, *38*(2), pp.132-139.
- Joshi, H. and Parle, M., 2007. Pharmacological evidences for antiamnesic potentials of Phyllanthus amarus in mice. *African Journal of Biomedical Research*, *10*(2).
- Kahle, P.J., 2008. α-Synucleinopathy models and human neuropathology: similarities and differences. *Acta neuropathologica*, *115*(1), pp.87-95.
- Kang, J., Lemaire, H.G., Unterbeck, A., Salbaum, J.M., Masters, C.L., Grzeschik, K.H., Multhaup, G., Beyreuther, K. and Müller-Hill, B., 1987. The precursor of Alzheimer's disease amyloid A4 protein resembles a cell-surface receptor. *Nature*, *325*(6106), pp.733-736.

- Kay, C., Fisher, E., and Hayden, M. 2014. “Huntington’s Disease chapter 7,” in Handbook of Clinical Neurology, eds G. P. Bates, S. J. Tabrizi, and L. Jones Oxford: Oxford University Press.
- Kim, H.-Y.;Jeon, H.;Kim, H.;Koo, S. & Kim, S. 2018. Sophora flavescens Aiton Decreases MPP(+)-Induced Mitochondrial Dysfunction in SH-SY5Y Cells. *Frontiers in aging neuroscience,* 10**,** 119-119.
- Kim, G.H., Kim, J.E., Rhie, S.J. and Yoon, S., 2015. The role of oxidative stress in neurodegenerative diseases. *Experimental neurobiology*, *24*(4), p.325.
- Kori, M., Aydın, B., Unal, S., Arga, K.Y. and Kazan, D., 2016. Metabolic biomarkers and neurodegeneration: a pathway enrichment analysis of Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis. *Omics: a journal of integrative biology*, *20*(11), pp.645-661.
- Kotzbauer, P.T., Giasson, B.I., Kravitz, A.V., Golbe, L.I., Mark, M.H., Trojanowski, J.Q. and Lee, V.M.Y., 2004. Fibrillization of α-synuclein and tau in familial Parkinson's disease caused by the A53T α-synuclein mutation. *Experimental neurology*, *187*(2), pp.279-288.
- Kumar, N., Abichandani, L.G., Thawani, V., Gharpure, K.J., Naidu, M.U.R. and Venkat Ramana, G., 2016. Efficacy of standardized extract of Bacopa monnieri (Bacognize®) on cognitive functions of medical students: a six-week, randomized placebo-controlled trial. *Evidence-Based Complementary and Alternative Medicine*, *2016*.
- Kutzelnigg, A. and Lassmann, H., 2014. Pathology of multiple sclerosis and related inflammatory demyelinating diseases. *Handbook of clinical neurology*, *122*, pp.15-58.
- Langston, J.W., Ballard, P., Tetrud, J.W. and Irwin, I., 1983. Chronic Parkinsonism in humans due to a product of meperidine-analog synthesis. *Science*, *219*(4587), pp.979-980.
- Lantos, P.L., 1998. The definition of multiple system atrophy: a review of recent developments. *Journal of neuropathology and experimental neurology*, *57*(12), p.1099.
- Lee, D.H., Kim, C.S. and Lee, Y.J., 2011. Astaxanthin protects against MPTP/MPP^+-induced mitochondrial dysfunction and ROS production in vivo and in vitro. *Food and chemical toxicology*, *49*(1), pp.271-280.
- Lee, D.J., Lozano, C.S., Dallapiazza, R.F. and Lozano, A.M., 2019. Current and future directions of deep brain stimulation for neurological and psychiatric disorders: JNSPG 75th Anniversary Invited Review Article. *Journal of neurosurgery*, *131*(2), pp.333-342.

- Lee, V.M., Goedert, M. and Trojanowski, J.Q., 2001. Neurodegenarative tauopathies. *Annual review of neuroscience*, *24*, p.1121.
- Lepetsos, P. and Papavassiliou, A.G., 2016. ROS/oxidative stress signaling in osteoarthritis. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease*, *1862*(4), pp.576-591.
- Leuner, K., Schütt, T., Kurz, C., Eckert, S.H., Schiller, C., Occhipinti, A., Mai, S., Jendrach, M., Eckert, G.P., Kruse, S.E. and Palmiter, R.D., 2012. Mitochondrion-derived reactive oxygen species lead to enhanced amyloid beta formation. *Antioxidants & redox signaling*, *16*(12), pp.1421-1433.
- Levites, Y., Amit, T., Mandel, S. and Youdim, M.B., 2003. Neuroprotection and neurorescue against Aβ toxicity and PKC-dependent release of non-amyloidogenic soluble precursor protein by green tea polyphenol (-)-epigallocatechin-3-gallate. *The FASEB Journal*, *17*(8), pp.1-23.
- LeWitt, P.A., 2008. Levodopa for the treatment of Parkinson's disease. *New England Journal of Medicine*, *359*(23), pp.2468-2476.
- Li, M., Binda, C., Mattevi, A. and Edmondson, D.E., 2006. Functional role of the "aromatic cage" in human monoamine oxidase B: structures and catalytic properties of Tyr435 mutant proteins. *Biochemistry*, *45*(15), pp.4775-4784.
- Liberatore, G.T., Jackson-Lewis, V., Vukosavic, S., Mandir, A.S., Vila, M., McAuliffe, W.G., Dawson, V.L., Dawson, T.M. and Przedborski, S., 1999. Inducible nitric oxide synthase stimulates dopaminergic neurodegeneration in the MPTP model of Parkinson disease. *Nature medicine*, *5*(12), pp.1403-1409.
- Lin, M.T. and Beal, M.F., 2006. Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases. *Nature*, *443*(7113), pp.787-795.
- Lindsay, J., Laurin, D., Verreault, R., Hébert, R., Helliwell, B., Hill, G.B. and McDowell, I., 2002. Risk factors for Alzheimer's disease: a prospective analysis from the Canadian Study of Health and Aging. *American journal of epidemiology*, *156*(5), pp.445-453..
- Logroscino, G., Traynor, B.J., Hardiman, O., Chiò, A., Mitchell, D., Swingler, R.J., Millul, A., Benn, E. and Beghi, E., 2010. Incidence of amyotrophic lateral sclerosis in Europe. *Journal of Neurology, Neurosurgery & Psychiatry*, *81*(4), pp.385-390.

- Logroscino, G., Traynor, B.J., Hardiman, O., Couratier, P., Mitchell, J.D., Swingler, R.J. and Beghi, E., 2008. Descriptive epidemiology of amyotrophic lateral sclerosis: new evidence and unsolved issues. *Journal of Neurology, Neurosurgery & Psychiatry*, *79*(1), pp.6-11.
- Lopes, F.M., Schröder, R., da Frota Júnior, M.L.C., Zanotto-Filho, A., Müller, C.B., Pires, A.S., Meurer, R.T., Colpo, G.D., Gelain, D.P., Kapczinski, F. and Moreira, J.C.F., 2010. Comparison between proliferative and neuron-like SH-SY5Y cells as an in vitro model for Parkinson disease studies. *Brain research*, *1337*, pp.85-94.
- Luthra, R. and Roy, A., 2022. Role of medicinal plants against neurodegenerative diseases. *Current Pharmaceutical Biotechnology*, *23*(1), pp.123-139.
- Mahdy, K., Shaker, O., Wafay, H., Nassar, Y., Hassan, H. and Hussein, A., 2012. Effect of some medicinal plant extracts on the oxidative stress status in Alzheimer's disease induced in rats. *Eur Rev Med Pharmacol Sci*, *16*(3), pp.31-42.
- Mahne, A.C., Carr, J.A., Bardien, S. and Schutte, C.M., 2016. Clinical findings and genetic screening for copy number variation mutations in a cohort of South African patients with Parkinson's disease. *South African Medical Journal*, *106*(6), pp.623-625.
- Malkus, K.A., Tsika, E. and Ischiropoulos, H., 2009. Oxidative modifications, mitochondrial dysfunction, and impaired protein degradation in Parkinson's disease: how neurons are lost in the Bermuda triangle. *Molecular neurodegeneration*, *4*(1), pp.1-16..
- Maraković, N., Knežević, A., Vinković, V., Kovarik, Z. and Šinko, G., 2016. Design and synthesis of N-substituted-2-hydroxyiminoacetamides and interactions with cholinesterases. *Chemico-Biological Interactions*, *259*, pp.122-132.
- Marek, K. and Jennings, D., 2009. Can we image premotor Parkinson disease?. *Neurology*, *72*(7 Supplement 2), pp.S21-S26.
- Marucci, G., Buccioni, M., Dal Ben, D., Lambertucci, C., Volpini, R. and Amenta, F., 2021. Efficacy of acetylcholinesterase inhibitors in Alzheimer's disease. *Neuropharmacology*, *190*, p.108352.
- Mathur, A., Verma, S.K., Purohit, R., Singh, S.K., Mathur, D., Prasad, G.B.K.S. and Dua, V.K., 2010. Pharmacological investigation of Bacopa monnieri on the basis of antioxidant, antimicrobial and anti-inflammatory properties. *J Chem Pharm Res*, *2*(6), pp.191-8.

- McConkey, B.J., Sobolev, V. and Edelman, M., 2002. The performance of current methods in ligand–protein docking. *Current Science*, pp.845-856.
- Meng, X.Y., Zhang, H.X., Mezei, M. and Cui, M., 2011. Molecular docking: a powerful approach for structure-based drug discovery. Curr Comput Aided Drug Des 7 (2): 146–157.
- Mesulam, M., Guillozet, A., Shaw, P. and Quinn, B., 2002. Widely spread butyrylcholinesterase can hydrolyze acetylcholine in the normal and Alzheimer brain. *Neurobiology of disease*, *9*(1), pp.88-93.
- Meyder, A., Nittinger, E., Lange, G., Klein, R. and Rarey, M., 2017. Estimating electron density support for individual atoms and molecular fragments in X-ray structures. *Journal of chemical information and modeling*, *57*(10), pp.2437-2447.
- Meziane, H., Dodart, J.C., Mathis, C., Little, S., Clemens, J., Paul, S.M. and Ungerer, A., 1998. Memory-enhancing effects of secreted forms of the β-amyloid precursor protein in normal and amnestic mice. *Proceedings of the national academy of sciences*, *95*(21), pp.12683-12688.
- Milczek, E.M., Binda, C., Rovida, S., Mattevi, A. and Edmondson, D.E., 2011. The 'gating'residues Ile199 and Tyr326 in human monoamine oxidase B function in substrate and inhibitor recognition. *The FEBS journal*, *278*(24), pp.4860-4869.
- Molecular Operating Environment MOE, Version 2020.09. http://www. chemcomp.com.
- Moradi, S.Z., Momtaz, S., Bayrami, Z., Farzaei, M.H. and Abdollahi, M., 2020. Nanoformulations of herbal extracts in treatment of neurodegenerative disorders. *Frontiers in bioengineering and biotechnology*, *8*, p.238.
- Morgan, S. and Orrell, R.W., 2016. Pathogenesis of amyotrophic lateral sclerosis. *British medical bulletin*, *119*(1), pp.87-97.
- Morimoto, T., Ohsawa, I., Takamura, C., Ishiguro, M. and Kohsaka, S., 1998. Involvement of amyloid precursor protein in functional synapse formation in cultured hippocampal neurons. *Journal of neuroscience research*, *51*(2), pp.185-195.
- Morishima, M. and Ihara, Y., 1994. Posttranslational modifications of tau in paired helical filaments. *Dementia and Geriatric Cognitive Disorders*, *5*(5), pp.282-288.

- Muller, F.L., Liu, Y. and Van Remmen, H., 2004. Complex III releases superoxide to both sides of the inner mitochondrial membrane. *Journal of Biological Chemistry*, *279*(47), pp.49064-49073.
- Muntha, P., 2016. Drug discovery & development–A Review. *Res. & Rev.: J. Pharm. Pharmaceut. Sci*, *5*(1), pp.135-142.
- Murphy, S., Simmons, M.L., Agullo, L., Garcia, A., Feinstein, D.L., Galea, E., Reis, D.J., Minc-Golomb, D. and Schwartz, J.P., 1993. Synthesis of nitric oxide in CNS glial cells. *Trends in neurosciences*, *16*(8), pp.323-328.
- Mythri, R.B., Harish, G., Dubey, S.K., Misra, K. and Srinivas Bharath, M.M., 2011. Glutamoyl diester of the dietary polyphenol curcumin offers improved protection against peroxynitrite-mediated nitrosative stress and damage of brain mitochondria in vitro: implications for Parkinson's disease. *Molecular and cellular biochemistry*, *347*(1), pp.135-143.
- Mzezewa, S.C., Omoruyi, S.I., Zondagh, L.S., Malan, S.F., Ekpo, O.E. and Joubert, J., 2021. Design, synthesis, and evaluation of 3, 7-substituted coumarin derivatives as multifunctional Alzheimer's disease agents. *Journal of Enzyme Inhibition and Medicinal Chemistry*, *36*(1), pp.1606-1620.
- Nachon, F., Carletti, E., Ronco, C., Trovaslet, M., Nicolet, Y., Jean, L. and Renard, P.Y., 2013. Crystal structures of human cholinesterases in complex with huprine W and tacrine: elements of specificity for anti-Alzheimer's drugs targeting acetyl-and butyryl-cholinesterase. *Biochemical Journal*, *453*(3), pp.393-399.
- Newhouse, K., Hsuan, S.L., Chang, S.H., Cai, B., Wang, Y. and Xia, Z., 2004. Rotenone-induced apoptosis is mediated by p38 and JNK MAP kinases in human dopaminergic SH-SY5Y cells. *Toxicological sciences*, *79*(1), pp.137-146.
- Nordberg, A., Ballard, C., Bullock, R., Darreh-Shori, T. and Somogyi, M., 2013. A review of butyrylcholinesterase as a therapeutic target in the treatment of Alzheimer's disease. *The primary care companion for CNS disorders*, *15*(2), p.26731.
- Obeso, J.A., Stamelou, M., Goetz, C.G., Poewe, W., Lang, A.E., Weintraub, D., Burn, D., Halliday, G.M., Bezard, E., Przedborski, S.J.M.D. and Lehericy, S., 2017. Past, present,

and future of Parkinson's disease: A special essay on the 200th Anniversary of the Shaking Palsy. *Movement Disorders*, *32*(9), pp.1264-1310.

- Ogawa, N., 1994. Levodopa and dopamine agonists in the treatment of Parkinson's disease: advantages and disadvantages. *European neurology*, *34*(Suppl. 3), pp.20-28.
- Omoruyi, S.I., Delport, J., Kangwa, T.S., Ibrakaw, A.S., Cupido, C.N., Ekpo, O.E. and Hussein, A.A., 2021. In vitro neuroprotective potential of Clivia miniata and Nerine humilis (Amaryllidaceae) in MPP+-induced neuronal toxicity in SH-SY5Y neuroblastoma cells. *South African Journal of Botany*, *136*, pp.110-117.
- Ovallath, S. and Deepa, P., 2013. The history of parkinsonism: descriptions in ancient Indian medical literature. *Movement Disorders*, *28*(5), pp.566-568.
- Ovallath, S. and Sulthana, B., 2017. Levodopa: History and therapeutic applications. *Annals of Indian Academy of Neurology*, *20*(3), p.185.
- Parker Jr, W.D., Parks, J.K. and Swerdlow, R.H., 2008. Complex I deficiency in Parkinson's disease frontal cortex. *Brain research*, *1189*, pp.215-218.
- Parkinson's Foundation. (2019). Stages of Parkinson's. [online] Available at: https://www.parkinson.org/Understanding-Parkinsons/What-is-Parkinsons/Stages-of-Parkinsons [Accessed 14 Nov. 2019].
- Perier, C., Bové, J., Vila, M. and Przedborski, S., 2003. The rotenone model of Parkinson's disease. *Trends in neurosciences*, *26*(7), pp.345-346.
- Picciotto, M.R., Higley, M.J. and Mineur, Y.S., 2012. Acetylcholine as a neuromodulator: cholinergic signaling shapes nervous system function and behavior. *Neuron*, *76*(1), pp.116-129.
- Poddighe, S., De Rose, F., Marotta, R., Ruffilli, R., Fanti, M., Secci, P.P., Mostallino, M.C., Setzu, M.D., Zuncheddu, M.A., Collu, I. and Solla, P., 2014. Mucuna pruriens (Velvet bean) rescues motor, olfactory, mitochondrial and synaptic impairment in PINK1B9 Drosophila melanogaster genetic model of Parkinson's disease. *PLoS One*, *9*(10), p.e110802.
- Polman, C.H., Reingold, S.C., Banwell, B., Clanet, M., Cohen, J.A., Filippi, M., Fujihara, K., Havrdova, E., Hutchinson, M., Kappos, L. and Lublin, F.D., 2011. O› Connor P., Sandberg-Wollheim M., Thompson AJ, Waubant E., Weinshenker B., Wolinsky.

Diagnostic criteria for multiple sclerosis: 2010 revisions to the McDonald criteria. *JS Ann. Neurol*, *69*, pp.292-302.

- Polymeropoulos, M.H., Lavedan, C., Leroy, E., Ide, S.E., Dehejia, A., Dutra, A., Pike, B., Root, H., Rubenstein, J., Boyer, R. and Stenroos, E.S., 1997. Mutation in the α-synuclein gene identified in families with Parkinson's disease. *science*, *276*(5321), pp.2045-2047.
- Prusiner, S.B., Woerman, A.L., Mordes, D.A., Watts, J.C., Rampersaud, R., Berry, D.B., Patel, S., Oehler, A., Lowe, J.K., Kravitz, S.N. and Geschwind, D.H., 2015. Evidence for α-synuclein prions causing multiple system atrophy in humans with parkinsonism. *Proceedings of the National Academy of Sciences*, *112*(38), pp.E5308-E5317.
- Przedborski, S., Tieu, K., Perier, C. and Vila, M., 2004. MPTP as a mitochondrial neurotoxic model of Parkinson's disease. *Journal of bioenergetics and biomembranes*, *36*(4), pp.375-379.
- RajaSankar, S., Manivasagam, T., Sankar, V., Prakash, S., Muthusamy, R., Krishnamurti, A. and Surendran, S., 2009. Withania somnifera root extract improves catecholamines and physiological abnormalities seen in a Parkinson's disease model mouse. *Journal of Ethnopharmacology*, *125*(3), pp.369-373.
- Ramakrishna, V., Gopi, S. and Setty, O.H., 2012. Protective effect of Phyllanthus fraternus against bromobenzene-induced mitochondrial dysfunction in rat kidney. *Chinese Journal of Natural Medicines*, *10*(5), pp.328-333.
- Ratheesh, G., Tian, L. and Venugopal, J.R., 2017. Role of medicinal plants in neurodegenerative diseases. Biomanuf Rev 2: 2.
- Reddy, A.J., Dubey, A.K., Handu, S.S., Sharma, P., Mediratta, P.K., Ahmed, Q.M. and Jain, S., 2018. Anticonvulsant and antioxidant effects of Musa sapientum stem extract on acute and chronic experimental models of epilepsy. *Pharmacognosy Research*, *10*(1), p.49.
- Richardson, J.R., Quan, Y.U., Sherer, T.B., Greenamyre, J.T. and Miller, G.W., 2005. Paraquat neurotoxicity is distinct from that of MPTP and rotenone. *Toxicological sciences*, *88*(1), pp.193-201.
- Riederer, P., Danielczyk, W. and Grünblatt, E., 2004. Monoamine oxidase-B inhibition in Alzheimer's disease. *Neurotoxicology*, *25*(1-2), pp.271-277.

- Roengrit, T., Wannanon, P., Prasertsri, P., Kanpetta, Y., Sripanidkulchai, B.O. and Leelayuwat, N., 2014. Antioxidant and anti-nociceptive effects of Phyllanthus amarus on improving exercise recovery in sedentary men: a randomized crossover (double-blind) design. *Journal of the International Society of Sports Nutrition*, *11*(1), p.9.
- Ross, C.A., Aylward, E.H., Wild, E.J., Langbehn, D.R. and Jeffrey, D., 2014. Long, John H. *Warner, Rachael I. Scahill, Blair R. Leavitt, Julie C. Stout, Jane S. Paulsen, Ralf Reilmann, Paul G. Unschuld, Alice Wexler, Russell L. Margolis, and Sarah J. Tabrizi*, pp.204-216.
- Ryan, B.J., Hoek, S., Fon, E.A. and Wade-Martins, R., 2015. Mitochondrial dysfunction and mitophagy in Parkinson's: from familial to sporadic disease. *Trends in biochemical sciences*, *40*(4), pp.200-210.
- Schapira, A.H. and Jenner, P., 2011. Etiology and pathogenesis of Parkinson's disease. *Movement disorders*, *26*(6), pp.1049-1055.
- Schöning-Stierand, K., Diedrich, K., Fährrolfes, R., Flachsenberg, F., Meyder, A., Nittinger, E., Steinegger, R. and Rarey, M., 2020. Proteins Plus: interactive analysis of protein–ligand binding interfaces. *Nucleic acids research*, *48*(W1), pp.W48-W53.
- Schrag, A., Hovris, A., Morley, D., Quinn, N. and Jahanshahi, M., 2003. Young-versus older-onset Parkinson's disease: impact of disease and psychosocial consequences. *Movement disorders: official journal of the Movement Disorder Society*, *18*(11), pp.1250-1256.
- Schulz, J.B., Matthews, R.T., Muqit, M.M., Browne, S.E. and Beal, M.F., 1995. Inhibition of neuronal nitric oxide synthase by 7-nitroindazole protects against MPTP-induced neurotoxicity in mice. *Journal of neurochemistry*, *64*(2), pp.936-939.
- Selikhova, M., Williams, D.R., Kempster, P.A., Holton, J.L., Revesz, T. and Lees, A.J., 2009. A clinico-pathological study of subtypes in Parkinson's disease. *Brain*, *132*(11), pp.2947-2957.
- Sen, S., Chakraborty, R., Sridhar, C., Reddy, Y.S.R. and De, B., 2010. Free radicals, antioxidants, diseases and phytomedicines: current status and future prospect. *International journal of pharmaceutical sciences review and research*, *3*(1), pp.91-100.

- Seoposengwe, K., Van Tonder, J.J. and Steenkamp, V., 2013. In vitro neuroprotective potential of four medicinal plants against rotenone-induced toxicity in SH-SY5Y neuroblastoma cells. *BMC complementary and alternative medicine*, *13*(1), pp.1-11.
- Sethi, A., Joshi, K., Sasikala, K. and Alvala, M., 2019. Molecular docking in modern drug discovery: Principles and recent applications. *Drug discovery and development-new advances*, *2*, pp.1-21.
- Sheehan, J.P., Swerdlow, R.H., Parker, W.D., Miller, S.W., Davis, R.E. and Tuttle, J.B., 1997. Altered calcium homeostasis in cells transformed by mitochondria from individuals with Parkinson's disease. *Journal of neurochemistry*, *68*(3), pp.1221-1233.
- Sherzai, A., Edland, S.D., Masliah, E., Hansen, L., Pizzo, D.P., Sherzai, A. and Corey-Bloom, J., 2013. Spongiform change in dementia with Lewy bodies and Alzheimer disease. *Alzheimer Disease & Associated Disorders*, *27*(2), pp.157-161.
- Shulman, L.M., Gruber-Baldini, A.L., Anderson, K.E., Vaughan, C.G., Reich, S.G., Fishman, P.S. and Weiner, W.J., 2008. The evolution of disability in Parkinson disease. *Movement disorders*, *23*(6), pp.790-796.
- Singh, B., Pandey, S., Rumman, M., Kumar, S., Kushwaha, P.P., Verma, R. and Mahdi, A.A., 2021. Neuroprotective and neurorescue mode of action of Bacopa monnieri (L.) Wettst in 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine-induced Parkinson's disease: an in silico and in vivo study. *Frontiers in Pharmacology*, *12*, p.616413.
- Singh, B., Pandey, S., Verma, R., Ansari, J.A. and Mahdi, A.A., 2016. Comparative evaluation of extract of Bacopa monnieri and Mucuna pruriens as neuroprotectant in MPTP model of Parkinson's disease.
- Singh, B., Pandey, S., Yadav, S.K., Verma, R., Singh, S.P. and Mahdi, A.A., 2017. Role of ethanolic extract of Bacopa monnieri against 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP) induced mice model via inhibition of apoptotic pathways of dopaminergic neurons. *Brain research bulletin*, *135*, pp.120-128.
- Singh, S.K., Srivastav, S., Castellani, R.J., Plascencia-Villa, G. and Perry, G., 2019. Neuroprotective and antioxidant effect of Ginkgo biloba extract against AD and other neurological disorders. *Neurotherapeutics*, *16*(3), pp.666-674.

- Singhal, B., Lalkaka, J. and Sankhla, C., 2003. Epidemiology and treatment of Parkinson's disease in India. *Parkinsonism & Related Disorders*, *9*, pp.105-109.
- Small, D.H., Nurcombe, V., Reed, G., Clarris, H., Moir, R., Beyreuther, K. and Masters, C.L., 1994. A heparin-binding domain in the amyloid protein precursor of Alzheimer's disease is involved in the regulation of neurite outgrowth. *Journal of Neuroscience*, *14*(4), pp.2117-2127.
- Smith, M. and Modi, G., 2016. The clinical profile of idiopathic Parkinson's disease in a South African hospital complex-the influence of ethnicity and gender. *African Journal of Neurological Sciences*, *35*(1), pp.73-79.
- Soh, S.E., Morris, M.E. and McGinley, J.L., 2011. Determinants of health-related quality of life in Parkinson's disease: a systematic review. *Parkinsonism & related disorders*, *17*(1), pp.1-9.
- Song, S., Nie, Q., Li, Z. and Du, G., 2016. Curcumin improves neurofunctions of 6-OHDA-induced parkinsonian rats. *Pathology-Research and Practice*, *212*(4), pp.247-251.
- Spillantini, M.G. and Goedert, M., 2013. Tau pathology and neurodegeneration. *The Lancet Neurology*, *12*(6), pp.609-622.
- Spillantini, M.G., Schmidt, M.L., Lee, V.M.Y., Trojanowski, J.Q., Jakes, R. and Goedert, M., 1997. α-Synuclein in Lewy bodies. *Nature*, *388*(6645), pp.839-840.
- Spires-Jones, T.L., Stoothoff, W.H., de Calignon, A., Jones, P.B. and Hyman, B.T., 2009. Tau pathophysiology in neurodegeneration: a tangled issue. *Trends in neurosciences*, *32*(3), pp.150-159.
- Südhof, T.C. and Rizo, J., 2011. Synaptic vesicle exocytosis. *Cold Spring Harbor perspectives in biology*, *3*(12), p.a005637.
- Sullivan, P.G., Keller, J.N., Bussen, W.L. and Scheff, S.W., 2002. Cytochrome c release and caspase activation after traumatic brain injury. *Brain research*, *949*(1-2), pp.88-96.
- Sulzer, D. and Surmeier, D.J., 2013. Neuronal vulnerability, pathogenesis, and Parkinson's disease. *Movement Disorders*, *28*(6), pp.715-724.
- Sun, A.Y., Simonyi, A. and Sun, G.Y., 2002. The "French Paradox" and beyond: neuroprotective effects of polyphenols. *Free Radical Biology and Medicine*, *32*(4), pp.314-318.

- Surh, Y.J., 1999. Molecular mechanisms of chemopreventive effects of selected dietary and medicinal phenolic substances. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, *428*(1-2), pp.305-327.
- Surmeier, D.J., Guzman, J.N., Sanchez, J. and Schumacker, P.T., 2012. Physiological phenotype and vulnerability in Parkinson's disease. *Cold Spring Harbor perspectives in medicine*, *2*(7), p.a009290.
- Sussman, J.L., Harel, M., Frolow, F., Oefner, C., Goldman, A., Toker, L. and Silman, I., 1991. Atomic structure of acetylcholinesterase from Torpedo californica: a prototypic acetylcholine-binding protein. *Science*, *253*(5022), pp.872-879.
- Szabó, C., Ischiropoulos, H. and Radi, R., 2007. Peroxynitrite: biochemistry, pathophysiology and development of therapeutics. *Nature reviews Drug discovery*, *6*(8), pp.662-680.
- Talesa, V.N., 2001. Acetylcholinesterase in Alzheimer's disease. *Mechanisms of ageing and development*, *122*(16), pp.1961-1969.
- Tao, Z., Chun-Yan, H., Hua, P., Bin-Bin, Y. and Xiaoping, T., 2020. Phyllathin from phyllanthus amarus ameliorates epileptic convulsion and kindling associated post-Ictal depression in mice via inhibition of NF-κB/TLR-4 pathway. *Dose-Response*, *18*(3), p.1559325820946914.
- Taylor, J.P., Hardy, J. and Fischbeck, K.H., 2002. Toxic proteins in neurodegenerative disease. *science*, *296*(5575), pp.1991-1995.
- Thring, T.S.A., Springfield, E.P. and Weitz, F.M., 2007. Antimicrobial activities of four plant species from the Southern Overberg region of South Africa. *African Journal of Biotechnology*, *6*(15).
- Truelsen, T., Thudium, D. and Grønbæk, M., 2002. Amount and type of alcohol and risk of dementia: the Copenhagen City Heart Study. *Neurology*, *59*(9), pp.1313-1319.
- Uchikado, H., DelleDonne, A., Ahmed, Z. and Dickson, D.W., 2006. Lewy bodies in progressive supranuclear palsy represent an independent disease process. *Journal of Neuropathology & Experimental Neurology*, *65*(4), pp.387-395.
- Uchikado, H., Lin, W.L., DeLucia, M.W. and Dickson, D.W., 2006. Alzheimer disease with amygdala Lewy bodies: a distinct form of α-synucleinopathy. *Journal of Neuropathology & Experimental Neurology*, *65*(7), pp.685-697.

- Ueda, K., Fukushima, H., Masliah, E., Xia, Y.U., Iwai, A., Yoshimoto, M., Otero, D.A., Kondo, J., Ihara, Y. and Saitoh, T., 1993. Molecular cloning of cDNA encoding an unrecognized component of amyloid in Alzheimer disease. *Proceedings of the National Academy of Sciences*, *90*(23), pp.11282-11286.
- Uttara, B., Singh, A.V., Zamboni, P. and Mahajan, R., 2009. Oxidative stress and neurodegenerative diseases: a review of upstream and downstream antioxidant therapeutic options. *Current neuropharmacology*, *7*(1), pp.65-74.
- Valko, M., Leibfritz, D., Moncol, J., Cronin, M.T., Mazur, M. and Telser, J., 2007. Free radicals and antioxidants in normal physiological functions and human disease. *The international journal of biochemistry & cell biology*, *39*(1), pp.44-84.
- Van der Valk, J., Brunner, D., De Smet, K., Svenningsen, Å.F., Honegger, P., Knudsen, L.E., Lindl, T., Noraberg, J., Price, A., Scarino, M.L. and Gstraunthaler, G., 2010. Optimization of chemically defined cell culture media–replacing fetal bovine serum in mammalian in vitro methods. *Toxicology in vitro*, *24*(4), pp.1053-1063.
- Vidal, R., Frangione, B., Rostagno, A., Mead, S., Révész, T., Plant, G. and Ghiso, J., 1999. A stop-codon mutation in the BRI gene associated with familial British dementia. *Nature*, *399*(6738), pp.776-781.
- Vidal, R., Révész, T., Rostagno, A., Kim, E., Holton, J.L., Bek, T., Bojsen-Møller, M., Braendgaard, H., Plant, G., Ghiso, J. and Frangione, B., 2000. A decamer duplication in the 3′ region of the BRI gene originates an amyloid peptide that is associated with dementia in a Danish kindred. *Proceedings of the National Academy of Sciences*, *97*(9), pp.4920-4925.
- Vijayaraghavan, S., Karami, A., Aeinehband, S., Behbahani, H., Grandien, A., Nilsson, B., Ekdahl, K.N., Lindblom, R.P., Piehl, F. and Darreh-Shori, T., 2013. Regulated extracellular choline acetyltransferase activity—the plausible missing link of the distant action of acetylcholine in the cholinergic anti-inflammatory pathway. *PloS one*, *8*(6), p.e65936.
- Vila, M. and Przedborski, S., 2003. Targeting programmed cell death in neurodegenerative diseases. *Nature Reviews Neuroscience*, *4*(5), pp.365-375.
- Vincent, V.A.M., Tilders, F.J.H. and Van Dam, A.M., 1998. Production, regulation and role of nitric oxide in glial cells. *Mediators of Inflammation*, *7*(4), pp.239-255.

- Wang, X.J. and Xu, J.X., 2005. Possible involvement of Ca2+ signaling in rotenone-induced apoptosis in human neuroblastoma SH-SY5Y cells. *Neuroscience letters*, *376*(2), pp.127-132.
- Wannannond, P., Wattanathorn, J., Muchimapura, S., Thipkaew, C., Thukhummee, W., Leelayuwat, N. and Sripanidkulchai, B., 2012. Phyllanthus amarus facilitates the recovery of peripheral nerve after injury. *American Journal of Applied Sciences*, *9*(7), p.1000.
- Westermark, P., Benson, M.D., Buxbaum, J.N., Cohen, A.S., Frangione, B., Ikeda, S.I., Masters, C.L., Merlini, G., Saraiva, M.J. and Sipe, J.D., 2005. Amyloid: Toward terminology clarification report from the nomenclature committee of the international society of amyloidosis. *Amyloid*, *12*(1), pp.1-4.
- Wickremaratchi, M.M., Perera, D., O'Loghlen, C., Sastry, D., Morgan, E., Jones, A., Edwards, P., Robertson, N.P., Butler, C., Morris, H.R. and Ben-Shlomo, Y., 2009. Prevalence and age of onset of Parkinson's disease in Cardiff: a community based cross sectional study and meta-analysis. *Journal of Neurology, Neurosurgery & Psychiatry*, *80*(7), pp.805-807.
- Williams, D.R. and Lees, A.J., 2009. Progressive supranuclear palsy: clinicopathological concepts and diagnostic challenges. *The Lancet Neurology*, *8*(3), pp.270-279.
- Wirdefeldt, K., Adami, H.O., Cole, P., Trichopoulos, D. and Mandel, J., 2011. Epidemiology and etiology of Parkinson's disease: a review of the evidence. *European journal of epidemiology*, *26*(1), pp.1-58.
- World Health Organization, 2004. *Atlas: country resources for neurological disorders 2004: results of a collaborative study of the World Health Organization and the World Federation of Neurology*. World Health Organization.
- Wu, L.K., Agarwal, S., Kuo, C.H., Kung, Y.L., Day, C.H., Lin, P.Y., Lin, S.Z., Hsieh, D.J.Y., Huang, C.Y. and Chiang, C.Y., 2022. Artemisia Leaf Extract protects against neuron toxicity by TRPML1 activation and promoting autophagy/mitophagy clearance in both in vitro and in vivo models of MPP+/MPTP-induced Parkinson's disease. *Phytomedicine*, *104*, p.154250.

- Xicoy, H., Wieringa, B. and Martens, G.J., 2017. The SH-SY5Y cell line in Parkinson's disease research: a systematic review. *Molecular neurodegeneration*, *12*(1), pp.1-11.
- Xie, H.R., Hu, L.S. and Li, G.Y., 2010. SH-SY5Y human neuroblastoma cell line: in vitro cell model of dopaminergic neurons in Parkinson's disease. *Chinese medical journal*, *123*(08), pp.1086-1092.
- Xing, L.F., Guo, H.P., Wang, D.T., Sun, L.H. and Pan, S.Y., 2016. Protective effects and mechanisms of Ndfipl on SH-SY5Y cell apoptosis in an in vitro Parkinson's disease model. *Genet. Mol. Res*, *15*(2), pp.10-4238.
- Yadav, S.K., Prakash, J., Chouhan, S. and Singh, S.P., 2013. Mucuna pruriens seed extract reduces oxidative stress in nigrostriatal tissue and improves neurobehavioral activity in paraquat-induced Parkinsonian mouse model. *Neurochemistry international*, *62*(8), pp.1039-1047.
- Yiannopoulou, K.G. and Papageorgiou, S.G., 2013. Current and future treatments for Alzheimer's disease. *Therapeutic advances in neurological disorders*, *6*(1), pp.19-33.
- Yuan, H., Yang, Q., Yang, B., Xu, H., Nasif, O., Muruganantham, S. and Chen, J., 2021. Phyllanthin averts oxidative stress and neuroinflammation in cerebral ischemic-reperfusion injury through modulation of the NF-Kb and AMPK/Nrf2 pathways. *Journal of Environmental Pathology, Toxicology and Oncology*, *40*(1).
- Zarei, S., Carr, K., Reiley, L., Diaz, K., Guerra, O., Altamirano, P.F., Pagani, W., Lodin, D., Orozco, G. and Chinea, A., 2015. A comprehensive review of amyotrophic lateral sclerosis. *Surgical neurology international*, *6*.
- Zeng, X.S., Geng, W.S., Jia, J.J., Chen, L. and Zhang, P.P., 2018. Cellular and molecular basis of neurodegeneration in Parkinson disease. *Frontiers in aging neuroscience*, *10*, p.109.
- Zhang, Y., Kua, J. and McCammon, J.A., 2002. Role of the catalytic triad and oxyanion hole in acetylcholinesterase catalysis: an ab initio QM/MM study. *Journal of the American Chemical Society*, *124*(35), pp.10572-10577.
- Zhong, J.;Yu, H.;Huang, C.;Zhong, Q.;Chen, Y.;Xie, J.;Zhou, Z.;Xu, J. & Wang, H. 2018. Inhibition of phosphodiesterase 4 by FCPR16 protects SH-SY5Y cells against MPP^+-induced decline of mitochondrial membrane potential and oxidative stress. *Redox Biology*, 16**,** 47-58.

- Zhu, J. and Chu, C.T., 2010. Mitochondrial dysfunction in Parkinson's disease. *Journal of Alzheimer's Disease*, *20*(s2), pp.S325-S334.