The effect of selected medicinal plants on rotenone-induced toxicity in SH-SY5Y neuroblastoma cells

by

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Subject of the work: The effect of selected medicinal plants on rotenone-induced toxicity in SH-SY5Y neuroblastoma cells

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6. My friends for believing and trusting in me that I can actually make it through.

“Gratitude is the memory of the heart”

Jean Baptiste Massieu
Abstract

Parkinson's disease (PD) is the second most common chronic neurodegenerative disease characterized by dopamine decrease in the substantia nigra. Currently, there is no promising cure for PD and this has resulted in extensive research into alternative medicines. The aim of this study was to investigate the effect of methanol and ethyl acetate extracts of *Lannea schweinfurthii* (Engl. Engl) (Anacardiaceae), *Zanthoxylum capense* (Thunb. Harv) (Rutaceae), *Scadoxus puniceus* (([L.] Friis & Nordal) (Amaryllidaceae) and *Crinum bulbispermum* (Burm. f.) Milne-Redh. & Schweick) (Amaryllidaceae) on rotenone-induced toxicity in SH-SY5Y neuroblastoma cells. The latter which mimics PD symptoms *in vitro*.

Cytotoxicity of the plant extracts was assessed using sulforhodamine B (SRB) assay. Intracellular reactive oxygen species (ROS) were measured fluorometrically with the use of the fluorescent dye 2’,7’-dichlorodihydrofluorescein diacetate (H₂DCF-DA). Intracellular glutathione content was measured fluorometrically after staining with monochlorobimane (MCB). Fluorescent dye 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide (JC-1) was used to assess the mitochondrial membrane potential (MMP) status of cells. Apoptosis was assessed by determining caspase-3 activity through detection of 7-amino-4-methylcoumarin (AMC) which is a product of caspase-3 substrate, acetyl-Asp-Glu-Val-Asp 7-amino-4-methylcoumarin (Ac-DEVD-AMC), cleaved by the caspase-3 enzyme.

Rotenone was used as an *in vitro* model to induce PD-like symptoms. Cytotoxicity studies for methanol extract of *Zanthoxylum capense* revealed the highest IC₅₀ value of 121.3 µg/mL, indicating low toxicity. The ethyl acetate extract of *Crinum bulbispermum* was observed to have no effect on the normal proliferation of the SH-SY5Y cells and produced an IC₅₀ value >100 µg/mL. The calculated IC₅₀ value obtained from rotenone cytotoxicity studies was 112
Zanthoxylum capense and Scadoxus puniceus plant extracts were observed to be neuroprotective against rotenone-induced toxicity.

A decrease in intracellular glutathione content as well as MMP was also observed in cells exposed to rotenone alone (50 nM). There was no intracellular ROS generation observed in cells exposed to rotenone alone (50 nM) after 24 h and 72 h. However, apoptotic cell death was observed in cells treated with rotenone (50 nM).

Intracellular ROS production was observed to be elevated by methanol and ethyl acetate extracts of C. bulbispermum. Methanol extracts of Z. capense was observed to increase intracellular glutathione content. MMP was increased effectively following treatment with ethyl acetate extract of C. bulbispermum. Moreover, both methanol and ethyl acetate plant extracts were found to decrease caspase-3 activity significantly (p<0.05), in cells exposed to 50 nM rotenone. Z. capense methanol extract reduced caspase-3 activity the most effectively.

Treatment with plant extracts was protective and decreased cell death. Furthermore, L. schweinfurthii, Z. capense, S. puniceus and C. bulbispermum, demonstrated strong antioxidant and anti-apoptotic effects against rotenone-toxicity, making them potential agents in developing therapies for treating PD.

**Keywords:** apoptosis, dopamine, glutathione, medicinal plants, mitochondrial membrane potential, neurodegeneration, oxidative stress, Parkinson’s disease (PD), rotenone, SH-SY5Y
# Table of Contents

Declaration.............................................................................................................................................. i  
Acknowledgements .............................................................................................................................. ii  
Abstract.................................................................................................................................................. iii  
Table of Contents .................................................................................................................................. v  
List of Abbreviations ........................................................................................................................... vi  
List of Figures ....................................................................................................................................... xi  
List of Tables ....................................................................................................................................... xiii  

## CHAPTER 1 - LITERATURE REVIEW .......................................................................................... 1  
1.1 Neurodegeneration .................................................................................................................... 1  
1.2 Parkinson’s disease ................................................................................................................... 1  
1.3 Types of PD ................................................................................................................................... 2  
  1.3.1 Sporadic PD ........................................................................................................................... 4  
  1.3.2 Familial PD ............................................................................................................................ 4  
1.4 Pathophysiology of PD ............................................................................................................. 6  
  1.4.1 Central dopamine pathways and their physiological roles .................................................. 6  
  1.4.2 Dopamine ............................................................................................................................. 8  
  1.4.3 Alpha-synuclein protein and Lewy body formation ............................................................ 12  
  1.4.4 Alpha-synuclein and neurotoxicity ....................................................................................... 14  
1.5 Clinical symptoms of PD ........................................................................................................ 16  
1.6 Risk factors of neurodegeneration in PD .................................................................................. 17  
  1.6.1 Oxidative stress .................................................................................................................... 17  
  1.6.2 The electron transport chain (ETC) and oxidative stress .................................................. 19  
  1.6.3 Mitochondrial dysfunction and cell death ........................................................................... 21  
  1.6.4 Neuroinflammation ............................................................................................................... 24  
  1.6.5 Proteosomal dysfunction ...................................................................................................... 24  
1.7 Neurotoxins used in in vitro models to investigate PD pathogenesis and pathology .......... 25  
1.8 Current PD therapeutic interventions ...................................................................................... 29  
1.9 The use of herbal remedies as PD therapeutics ...................................................................... 30  
1.10 Study Aim .................................................................................................................................. 32  
1.11 Study Objectives ..................................................................................................................... 32  
1.12 Project Overview ...................................................................................................................... 33
<table>
<thead>
<tr>
<th>CHAPTER 2 - MATERIALS AND METHODS</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1 Plant collection and extract preparation</td>
<td>35</td>
</tr>
<tr>
<td>2.2 Cell culture and maintenance</td>
<td>35</td>
</tr>
<tr>
<td>2.3 Cytotoxicity determination</td>
<td>37</td>
</tr>
<tr>
<td>2.4 Effect of plant extracts on rotenone toxicity</td>
<td>38</td>
</tr>
<tr>
<td>2.5 Determination of intracellular ROS levels</td>
<td>38</td>
</tr>
<tr>
<td>2.6 Measurement of intracellular glutathione content</td>
<td>39</td>
</tr>
<tr>
<td>2.7 Measurement of mitochondrial membrane potential</td>
<td>40</td>
</tr>
<tr>
<td>2.8 Caspase-3 activity as a measure of apoptosis</td>
<td>41</td>
</tr>
<tr>
<td>2.9 Statistical analyses</td>
<td>41</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>CHAPTER 3 - RESULTS AND DISCUSSION</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1 Cytotoxicity studies</td>
<td>43</td>
</tr>
<tr>
<td>3.1.1 Effect of rotenone and plant extracts alone on SH-SY5Y cell viability</td>
<td>43</td>
</tr>
<tr>
<td>3.1.2 Effect of the plant extracts on rotenone- induced SH-SY5Y cytotoxicity</td>
<td>46</td>
</tr>
<tr>
<td>3.2 Antioxidant activity</td>
<td>51</td>
</tr>
<tr>
<td>3.2.1 Reactive oxygen species generation</td>
<td>51</td>
</tr>
<tr>
<td>3.2.2 Intracellular glutathione content</td>
<td>56</td>
</tr>
<tr>
<td>3.3 Mitochondrial membrane potential</td>
<td>59</td>
</tr>
<tr>
<td>3.4 Apoptosis</td>
<td>62</td>
</tr>
<tr>
<td>3.5 Possible mechanism(s) of action of the plant extracts</td>
<td>65</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>CHAPTER 4 - CONCLUSION</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>SUMMARY</td>
<td>69</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>71</td>
</tr>
<tr>
<td>APPENDIX A</td>
<td>102</td>
</tr>
<tr>
<td>APPENDIX B</td>
<td>108</td>
</tr>
<tr>
<td>APPENDIX C</td>
<td>109</td>
</tr>
</tbody>
</table>
# List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAPH</td>
<td>2,2’-azobis-2-methyl-propanimidamide dihydrochloride</td>
</tr>
<tr>
<td>Ac-DEVD-AMC</td>
<td>Acetyl-Asp-Glu-Val-Asp 7-amino-4-methylcoumarin</td>
</tr>
<tr>
<td>AIF</td>
<td>Apoptosis inducing factor</td>
</tr>
<tr>
<td>AMC</td>
<td>7-amino-4-methylcoumarin</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>Calcium ions</td>
</tr>
<tr>
<td>CHAPS</td>
<td>3-[(3-cholamidopropyl)dimethylammonio]-1propanesulfonate</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>COMT</td>
<td>Catechol-O-methyltransferase</td>
</tr>
<tr>
<td>COX-2</td>
<td>Cyclooxygenase-2</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>DA-R</td>
<td>Dopamine receptors</td>
</tr>
<tr>
<td>DAQ</td>
<td>Dopamine quinones</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ETC</td>
<td>Electron transport chain</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>FADH₂</td>
<td>Flavin adenine dinucleotide</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
</tr>
<tr>
<td>g</td>
<td>Gram</td>
</tr>
<tr>
<td>g</td>
<td>Gravitational force</td>
</tr>
<tr>
<td>GABA</td>
<td>Gamma-aminobutyric acid</td>
</tr>
<tr>
<td>Glu/KA</td>
<td>Glutamate-kainate receptor complex</td>
</tr>
<tr>
<td>GluR</td>
<td>Glutamate receptor</td>
</tr>
<tr>
<td>GSH</td>
<td>Reduced glutathione</td>
</tr>
<tr>
<td>GSH-Px</td>
<td>Glutathione peroxidase</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione-S-transferase</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>H₂DCF-DA</td>
<td>2',7'-dichlorodihydrofluorescein diacetate</td>
</tr>
<tr>
<td>IC₅₀</td>
<td>Concentration that results in inhibition of 50% of biological activity</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Interleukin-1-beta</td>
</tr>
<tr>
<td>JC-1</td>
<td>5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
</tr>
<tr>
<td>L-DOPA</td>
<td>L-3,4-dihydroxyphenylalanine</td>
</tr>
<tr>
<td>MAO-B</td>
<td>Monoamine oxidase B</td>
</tr>
<tr>
<td>MCB</td>
<td>Monochlorobimane</td>
</tr>
<tr>
<td>Symbol</td>
<td>Definition</td>
</tr>
<tr>
<td>--------</td>
<td>------------</td>
</tr>
<tr>
<td>min</td>
<td>Minute</td>
</tr>
<tr>
<td>mL</td>
<td>Milliliter</td>
</tr>
<tr>
<td>mM</td>
<td>Millimolar</td>
</tr>
<tr>
<td>MMP</td>
<td>Mitochondrial membrane potential</td>
</tr>
<tr>
<td>MPP*</td>
<td>1-methyl-4-phenyl pyridinium</td>
</tr>
<tr>
<td>MPT</td>
<td>Mitochondrial membrane permeability transition</td>
</tr>
<tr>
<td>MPTP</td>
<td>1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NEM</td>
<td>N-ethylmaleimide</td>
</tr>
<tr>
<td>NFκβ</td>
<td>Nuclear factor kappa-beta</td>
</tr>
<tr>
<td>nM</td>
<td>Nanomolar</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-Methyl-D-aspartate</td>
</tr>
<tr>
<td>NSAIDs</td>
<td>Non-steroidal anti-inflammatory drugs</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson’s disease</td>
</tr>
<tr>
<td>pH</td>
<td>Negative logarithm of the hydrogen ion concentration</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>PUFAs</td>
<td>Polyunsaturated fatty acids</td>
</tr>
<tr>
<td>p53</td>
<td>Tumor protein 53</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Term/Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>----------------</td>
</tr>
<tr>
<td>RNS</td>
<td>Reactive nitrogen species</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SANBI</td>
<td>South African National Botanical Institute</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>SRB</td>
<td>Sulforhodamine B</td>
</tr>
<tr>
<td>TCA</td>
<td>Trichloroacetic acid</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor-alpha</td>
</tr>
<tr>
<td>VMAT2</td>
<td>Vesicular monoamine transporter 2</td>
</tr>
<tr>
<td>WHO</td>
<td>World health organization</td>
</tr>
<tr>
<td>°C</td>
<td>Degrees Celsius</td>
</tr>
<tr>
<td>µL</td>
<td>Microliter</td>
</tr>
<tr>
<td>µM</td>
<td>Micromolar</td>
</tr>
<tr>
<td>%</td>
<td>Percentage</td>
</tr>
</tbody>
</table>
List of Figures

Figure 1. Sagittal section of the brain showing the location of substantia nigra and ventral tegmental area ................................................................. 3

Figure 2. Schematic diagram illustrating the production and degradation of dopamine in the dopaminergic presynaptic neuron in the brain ......................................................... 9

Figure 3. Schematic diagram illustrating the production, release and action of dopamine on the dopamine receptors of a dopaminergic postsynaptic neuron in the brain ..................... 10

Figure 4. A Lewy body in a dopaminergic neuron in a Parkinson's disease brain ........... 13

Figure 5. Diagram illustrating possible mechanisms of action of alpha-synuclein protein toxicity leading to dopaminergic cell death in Parkinson's disease .............. 15

Figure 6. An illustration depicting the process of ROS production by the electron transport chain and its scavenging by the antioxidant defence mechanisms ......................... 20

Figure 7. Mitochondrial events that occur when the mitochondria are dysfunctional .. 23

Figure 8. Chemical structure of A) 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine, B) paraquat, C) 6-hydroxydopamine and D) rotenone (Blandini & Armentero, 2012) ............... 26

Figure 9. Mechanistic model depicting possible parameters involved in neurodegeneration. ........................................................................................................ 34

Figure 10. Effect of A) rotenone, B) methanol and C) ethyl acetate plant extracts on the survival of SH-SY5Y cells after a 72 h exposure period using the sulforhodamine B assay 45

Figure 11. Effect of methanol plant extracts on the survival of SH-SY5Y cells after 72 h exposure to rotenone at A) 10 nM, B) 50 nM and C) 100 nM, using the sulforhodamine B assay ........................................................................................................ 48
Figure 12. Effect of ethyl acetate plant extracts on the survival of SH-SY5Y cells after 72 h exposure to rotenone at A) 10 nM, B) 50 nM and C) 100 nM, using the sulforhodamine B assay ................................................................................................................................ .. 49

Figure 13. ROS generation in SH-SY5Y cells exposed to rotenone at 50 nM after pre-treatment with with A) methanol and B) ethyl acetate plant extracts following 24 h exposure period ................................................................................................................................ .. 53

Figure 14. ROS generation in SH-SY5Y cells exposed to rotenone at 50 nM after pre-treatment with A) methanol and B) ethyl acetate plant extracts following 72 h exposure period ................................................................................................................................ .. 54

Figure 15. Intracellular glutathione content in SH-SY5Y cells exposed to rotenone at 50 nM after pre-treatment with A) methanol and B) ethyl acetate plant extracts following 24 h exposure period ................................................................................................................................ .. 57

Figure 16. Mitochondrial membrane potential (MMP) in SH-SY5Y cells exposed to rotenone at 50 nM after pre-treatment with A) methanol and B) ethyl acetate plant extracts following 24 h exposure period ................................................................................................................................ .. 61

Figure 17. Caspase-3 activity in SH-SY5Y cells exposed to rotenone at 50 nM after pre-treatment with A) methanol and B) ethyl acetate plant extracts following 24 h exposure period ................................................................................................................................ .. 63

Figure 18. Possible mechanism (s) of action of the studied plant extracts in counteracting rotenone-induced toxicity. ................................................................................................................................ .. 66
List of Tables

Table 1. Implicated gene loci and genes in the pathogenesis of familial Parkinson’s disease and their mode of inheritance………………………………………………………………………………..5

Table 2. The genus and species, family, plant part and medicinal use (related to neurodegenerative disorders) of the plants investigated in the present study……………….. 36

Table 3. Summary of the effect of cells pre-treated with plant extracts compared to those treated with rotenone alone (50 nM) on the parameters tested. ................................. 67
Chapter 1 - Literature Review

1.1 Neurodegeneration

Neurodegeneration is the progressive degeneration of the central nervous system (CNS) resulting in loss of structure and/or function of neurons with subsequent cellular death\(^1\). Neurodegenerative diseases such as Parkinson’s, Huntington’s and Alzheimer’s are prominent in the elderly population\(^2\). It is estimated that more than 15 million people worldwide, above 60 years of age, are affected by neurodegeneration and with an aging population the number is expected to increase\(^3\).

Neurodegenerative diseases are multifactorial, i.e. there is a complex interplay of parameters that cause their manifestation. Due to this, it is inherently difficult to develop medications for treatment of these diseases\(^4\). Research has shown that during their induction and progression, many neurodegenerative diseases share similar pathophysiological pathways. These include abnormal protein degradation pathways and excessive accumulation of intracellular/ extracellular mutated proteins, which induce neuronal cell death with subsequent loss of neuronal cell mass\(^5,6\).

1.2 Parkinson’s disease

Parkinson’s disease (PD), is the second most common progressive age-related neurological and movement disorder, and is currently estimated to affect more than 6 million people worldwide\(^7\). The prevalence of the disease is reported to be 1 - 2% amongst those who are over 65 years and about 4% amongst individuals who are older than 80 years. PD is expected to double in continents with large populations in the next twenty years\(^8,9\). It is a life-
threatening disease and a challenge for the future with old age being the greatest risk factor\textsuperscript{10,9}. 

PD is characterized by a marked selective loss of/ and degeneration of dopaminergic neurons in the brainstem regions, resulting in dopamine reduction\textsuperscript{11,12}. Specifically the substantia nigra and ventral tegmental area, located in the midbrain, and locus coeruleus, located in the pons, are of importance\textsuperscript{13,14} (Figure 1). Moreover, the abnormal accumulation of alpha-synuclein protein into dense inclusions called Lewy bodies in the remaining dopaminergic neurons of the substantia nigra is a prominent hallmark of this disease\textsuperscript{15}. 

PD results from the death of cells in the substantia nigra, as well as loss of dopamine and neuromelanin production by these cells. The substantia nigra, also called “the black substance” due to its dark appearance, is a structure found in the brainstem, specifically the midbrain (Figure 1). This part of the brain consists of two portions: the substantia nigra pars compacta, comprised of dopaminergic neurons, and the substantia nigra reticulate, consisting of gamma-aminobutyric acid-producing (GABA) or GABAergic neurons\textsuperscript{16}. The latter is involved in eye movements, whereas the substantia nigra pars compacta plays a key role in voluntary movements. The substantia nigra pars compacta forms part of the nigrostriatal pathway and disruption of this dopaminergic neural pathway has been indicated as the primary result of dopamine depletion in the brain\textsuperscript{17}. 

1.3 Types of PD 

Two types of PD are known: the sporadic and familial forms, which share neither known link nor etiology\textsuperscript{7}. They do, however, have common pathological, biochemical and clinical hallmarks\textsuperscript{19}. 

Figure 1. Sagittal section of the brain showing the location of substantia nigra and ventral tegmental area (Dopamine and serotonin pathways, http://www.drugabuse.gov/pubs/teaching/largegifs/slide-2). VTA = ventral tegmental area$^{18}$. 

**Dopamine Pathways**
- Frontal cortex
- Nucleus accumbens
- VTA

**Functions**
- Reward (motivation)
- Pleasure, euphoria
- Motor function (fine tuning)
- Compulsion
- Perseveration

**Serotonin Pathways**
- Striatum
- Substantia nigra
- Hippocampus
- Raphe nuclei

**Functions**
- Mood
- Memory processing
- Sleep
- Cognition
1.3.1 Sporadic PD

Approximately 95% of PD cases are sporadic\(^9\). Sporadic PD is a consequence of both environmental and genetic factors. Sporadic PD is also referred to as idiopathic PD because of its multifactorial and idiopathic in nature\(^{20,7}\). Consistent exposure to chemicals in remote areas and farms, as well as consumption of well water, has been found to be potential risk factors of sporadic PD\(^{21}\).

1.3.2 Familial PD

Familial PD is associated with autosomal recessive and dominant genetic mutations, the latter being most dominant. This type of PD is characterized with an early onset and accounts for 5 - 10% of PD cases\(^{22,23}\).

Research on the genetics involved in PD pathogenesis has provided insights into the molecular pathways involved in both familial and sporadic PD. Approximately 16 genetic loci and their associated genes, which are responsible for PD pathogenesis, have been identified to date. Amongst these genes are those considered susceptible factors in causing sporadic PD\(^{24}\). The most common genes that have been identified and extensively studied include \textit{alpha-synuclein}, \textit{parkin}, \textit{uch-l1}, \textit{pink1} and \textit{dj-1}, which are localized in loci PARK1, PARK2, PARK5, PARK6 and PARK7, respectively\(^{7,25}\) (Table 1).

The \textit{parkin} gene is located on chromosome 6q25.2-27 and mutations in this gene have been shown to result in the manifestation of an early onset of an autosomal recessive familial PD\(^{26,7}\). The \textit{uch-l1} gene (\textit{ubiquitin C-terminal hydrolase L1} gene) is found on chromosome 4p14\(^{26,7}\). A substitution point mutation in this gene has been shown to cause early onset of an autosomal dominant PD manifestation\(^{27}\). Both \textit{parkin} and \textit{uch-l1} genes are involved in proteosomal degradation pathways, which are responsible for the clearance of mutated, dysfunctional and unwanted proteins\(^{28,29}\).
Table 1. Implicated gene loci and genes in the pathogenesis of familial Parkinson’s disease and their mode of inheritance.

<table>
<thead>
<tr>
<th>Locus</th>
<th>Gene</th>
<th>Chromosome</th>
<th>Inheritance</th>
<th>Probable function</th>
</tr>
</thead>
<tbody>
<tr>
<td>PARK1 &amp; PARK4</td>
<td>α-synuclein</td>
<td>4q21</td>
<td>Dominant</td>
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Genetic mutations in the *pink1* gene have been reported to contribute to the manifestation of an autosomal recessive PD\(^{30}\). The *pink1* gene is localized on chromosome 1p35-36 and point mutations are common in this gene\(^7\). Certain cellular stress responses such as cytochrome C release are specifically regulated and controlled by a protein kinase encoded by the *pink1* gene. Failure of this protein kinase to regulate cellular stress responses in mitochondria makes cells more susceptible to cell death\(^{24}\).

Genetic alterations in the *dj-1* gene, found on chromosome 1p36, have been implicated in autosomal recessive familial PD\(^7\). The protein encoded by this gene is found in the mitochondrial matrix of astrocytes\(^31\). The *dj-1* gene has been reported to have a physiological role in the mitochondrial response to oxidative stress\(^{32,33}\). Deletion and point mutations, degradation or reduced expression of the *dj-1* gene can lead to familial PD pathogenesis, through the induction of oxidative stress\(^{34,35,36}\).

### 1.4 Pathophysiology of PD

#### 1.4.1 Central dopamine pathways and their physiological roles

Dopamine is a neurotransmitter primarily synthesized in the cytosol of the presynaptic dopamine axonal nerve terminal in the substantia nigra and adrenal glands in the medulla\(^{37}\). It is classified as a catecholamine, and is the most abundant neurotransmitter in the brain\(^{38}\). Distribution of dopamine from one brain region to another is made possible via dopaminergic neural pathways. The four major dopamine neural pathways are the mesolimbic, mesocortical, tuberoinfundibular and the nigrostriatal dopaminergic pathways.
(i) **Mesolimbic pathway**

The mesolimbic pathway transports dopamine from the ventral tegmental area to the nucleus accumbens and parts of the limbic area\(^ {38}\) (Figure 1). The ventral tegmental area is located in the midbrain, whereas the nucleus accumbens is in the limbic system (Figure 1). This pathway is implicated in controlled behavior and dysfunction of this pathway can produce mental disorders such as schizophrenia\(^ {39}\).

(ii) **Mesocortical pathway**

The mesocortical pathway originates in the ventral tegmental area and project to areas of the frontal cortex\(^ {38}\) (Figure 1). Like the mesolimbic pathway, the mesocortical pathway also plays a role in cognitive functions. Similarly, dysfunction of this neural pathway can result in behavioral disorders like schizophrenia\(^ {40}\).

(iii) **Tuberoinfundibular pathway**

The tuberoinfundibular pathway consists of neurons that convey dopamine from the arcuate nuclei of the hypothalamus to the pituitary gland\(^ {38}\). This pathway influences the secretion of the milk-producing hormone, prolactin\(^ {41}\).

(iv) **Nigrostriatal pathway**

The nigrostriatal pathway neurons originate from the substantia nigra and terminate in the striatum. The nigrostriatal pathway is intricately involved in the control of fine locomotion\(^ {42}\) (Figure 1). Dysfunction of this pathway is associated with movement disorders, like PD, and decreased production of dopamine from dopamine-producing cells in the substantia nigra pars compacta, enhances defects in the nigrostriatal pathway\(^ {43}\).
1.4.2 Dopamine

Approximately 80% of the brain catecholamine content is dopamine\textsuperscript{38,44}. The molecular structure of dopamine consists of an amine group, benzene ring, two hydroxyl groups and an ethyl side chain\textsuperscript{38} (Figure 2). Dopamine in the brain is implicated in several physiological functions including movement, cognition and learning functions, working memory and behavioral roles. Dopamine deficiency is known to be the primary cause of motor dysfunction\textsuperscript{14}.

(i) Dopamine synthesis and release

Tyrosine, which is the main precursor of dopamine, is transported to neurons by its amino acid transporters, where it is acted on by the enzyme tyrosine hydroxylase that converts tyrosine into L-3,4-dihydroxyphenylalanine (L-DOPA) (Figure 2). Decarboxylation of L-DOPA yields dopamine, which is then transported via vesicular monoamine transporter 2 (VMAT2) into synaptic vesicles. The synaptic vesicle pH is kept low so as to avoid auto-oxidation of dopamine in the presynaptic neuron, thus preventing oxidation damage to the neuron\textsuperscript{46}.

Dopamine in these vesicles is then released into the synaptic cleft through the aid of calcium. Under physiological conditions, dopamine in the synaptic cleft can be (1) taken back into the presynaptic neuron via dopamine transporter 1, where it is repackaged into vesicles, (2) broken down by the enzyme catechol-O-methyltransferase (COMT), monoamine oxidase (MAO) or dopamine hydroxylase, preventing the neurotransmitter from exerting its effect,\textsuperscript{46} or (3) can bind to dopamine postsynaptic receptors allowing the neurotransmitter to exert its physiological functions on dopamine postsynaptic receptors, leading to transmission of a signal to another nerve cell\textsuperscript{47} (Figure 3).
Figure 2. Schematic diagram illustrating the production and degradation of dopamine in the dopaminergic presynaptic neuron in the brain\textsuperscript{75}. COMT = catechol-O-methyltransferase; L-DOPA = L-3,4-dihydroxyphenylalanine; MAO = monoamine oxidase.
Figure 3. Schematic diagram illustrating the production, release and action of dopamine on the dopamine receptors of a dopaminergic postsynaptic neuron in the brain\textsuperscript{47}. COMT = catechol-O-methyltransferase; DA = dopamine; MAO = monoamine oxidase. (A) Storage of neurotransmitters in synaptic vesicles. (B) Binding of neurotransmitters to postsynaptic receptors of a postsynaptic neuron. (C) Re-uptake of neurotransmitters from the synapse back to presynaptic neuron for repackaging into synaptic vesicles. (D) Degradation of neurotransmitters by monoamine oxidase enzyme. (E) Degradation of neurotransmitters by catechol-O-methyltransferase enzyme.
(ii) Dopamine receptors

When dopamine is released into the synaptic cleft, it binds to dopamine postsynaptic receptors where it exerts its physiological functions\(^{48}\) (Figure 3). The structure of a dopamine receptor consists of seven alpha helices, anchored in the plasma membrane, an oligosaccharide extracellular loop and an intracellular loop\(^{49}\). The receptor is coupled to a G protein. There are five dopamine receptors (DA-Rs) namely D1, D2, D3, D4 and D5 receptors\(^{44}\). These receptors are subdivided into two groups, based on their biochemical properties and physiological roles. These are the D1-like and D2-like subfamilies. The D1 and D5 receptors belong to the D1-like subfamily while D2, D3 and D4 receptors belong to the D2-like subfamily\(^{50}\). These receptors are widely distributed in the brain. All DA-Rs are found in the cortex and limbic area\(^{51}\).

Additionally, the D1 and D2 receptors are also located in the striatum and the cardiovascular region such as the heart, with the D2 receptors also being found in the pituitary area\(^{52}\). Overstimulation of DA-Rs has been implicated in psychotic disorders, while under stimulation results in motor dysfunctions\(^{44}\). Regulation of dopamine levels in the brain is crucially important in preventing manifestations of severe neurological diseases.

(iii) Dopamine toxicity

Studies revealed detrimental events caused by high dopamine levels in the cytosol of neurons\(^{37}\). During pathophysiological conditions, dopamine can form a complex with protein cysteine residues resulting in the formation of S-cysteinyldopamine on these proteins, which irreversibly alters the protein structure and function\(^{46}\). Moreover, increased cytosolic dopamine is transformed into reactive dopamine analogues, termed dopamine quinones (DAQ), that have the potential to cause the subsequent formation of oxidative radicals like superoxide and hydrogen peroxide\(^{53}\).
Apart from promoting an oxidative state, DAQs have also been implicated in activating microglial cells that initiate inflammatory responses and fibrillization of alpha-synuclein protein\(^{54,55,56}\). Alpha-synuclein protein in the presynaptic nerve terminal can form a complex with a dopamine transporter, alpha-synuclein-dopamine transporter complex, that catalyses dopaminergic neuronal apoptotic induction\(^{57}\). The complex is also reported to aid the re-uptake of the dopamine from the synaptic cleft back into the vesicles of presynaptic neurons, thus decreasing the availability of dopamine in the synaptic cleft and aiding in the formation of DAQs in the presynaptic nerve terminal\(^{37,58}\). Therapeutic interventions promoting dopamine synthesis as well as the presence of dopamine in the synaptic cleft by inhibiting the actions of COMT enzyme and dopamine re-uptake could ameliorate severe PD symptoms, improving the quality of life of PD patients\(^{37}\).

1.4.3 Alpha-synuclein protein and Lewy body formation

The most common pathological hallmark of sporadic PD is the presence of Lewy bodies in the brain\(^{59}\) (Figure 4). In the familial PD form, however, Lewy bodies are absent\(^{60}\). Lewy bodies are cytoplasmic inclusions that are spherical in shape and consist of a dense nucleus and a pale surrounding area, the corona, with fibers radiating from the nucleus\(^{7}\). The ultrastructure of Lewy bodies has revealed the presence of loosely arranged aggregated proteins including ubiquitin and alpha-synuclein proteins\(^{11}\).The exact mechanism of Lewy bodies formation, as well as the contribution of Lewy bodies in the pathogenesis of PD is still poorly understood\(^{61}\).

During dopaminergic neuronal degeneration, Lewy body lesion development has been shown to correspond with PD severity and progression. In the early stages of PD development, Lewy body lesions begin to develop in the lower parts of the brainstem, specifically the medulla oblongata.
Figure 4. A Lewy body in a dopaminergic neuron in a Parkinson's disease brain\textsuperscript{63}. 
As the disease progresses the lesions start to develop in the cerebral parts of the brain. During the last stages of the disease the lesions also start appearing in the neocortex.

Studies have shown that over-production of alpha-synuclein protein in presynaptic nerve terminals predispose subjects to the early onset of PD development. Under normal physiological conditions, alpha-synuclein is a small coiled cytosolic protein present in the presynaptic nerve terminals that constitutes < 1% of the total cytosolic protein content. The protein is known to play a role in the storage and release of neurotransmitters and modulation of synaptic function.

The gene encoding alpha-synuclein is commonly mutated and a dominant causal factor in both sporadic and familial PD forms. The presence of mutations in the alpha-synuclein gene, overproduction of alpha-synuclein and abnormal metabolism of alpha-synuclein protein, all lead to the accumulation of alpha-synuclein proteins and the formation of Lewy bodies.

Mutated alpha-synuclein proteins usually aggregate, and are phosphorylated and fibrillated proteins. During aggregation, monomeric forms of alpha-synuclein proteins self-aggregate to form oligomers, which are soluble. Soluble oligomers self-aggregate to form an insoluble fibril that has been shown to take on a beta sheet structure. When this happens, proteolytic processes are inhibited, leading to accumulation of alpha-synuclein mutated proteins with possible toxicity (Figure 5).

1.4.4 Alpha-synuclein and neurotoxicity

Several hypotheses have been proposed to explain how alpha-synuclein protein aggregates contribute to dopaminergic cell death. These are summarized in Figure 5. One hypothesis states that alpha-synuclein proteins form clusters near the plasma membrane resulting in pore formation, thus altering plasma membrane integrity.
DEATH OR DAMAGE OF DOPAMINE NEURONS

The hallmark pathology of Parkinson’s disease is the damage and death of dopamine-producing neurons in the brain. Dopamine plays a role in controlling movement, cognition, learning, and mood, and dysfunction of dopamine transmitting neurons contributes to the clinical features of Parkinson’s disease. In one study, 60% of worms died when the dopamine neurotransmitter levels were decreased. The removal of dopamine from the worm’s body results in a host of therapeutic treatments. 

**Figure 5.** Diagram illustrating possible mechanisms of action of alpha-synuclein protein toxicity leading to dopaminergic cell death in Parkinson’s disease. 

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When this happens, an influx of calcium ions into the cell through the pore continues until toxic levels of these ions are reached inside the cell\textsuperscript{14,77}. Elevated intracellular calcium is known to cause excitotoxicity, a condition that contributes to making neuronal cells susceptible to cell death (Figure 5).

Overproduction of non-mutant and mutant alpha-synuclein protein in the presynaptic nerve terminal has the potential to reduce dopamine release into the synaptic cleft\textsuperscript{78,79}. When this occurs, dopamine levels in the presynaptic nerve terminal increase to toxic levels where they are oxidized to form DAQs\textsuperscript{80}, causing oxidative damage to the dopaminergic neuronal cell\textsuperscript{81}. Alternatively, DAQs can bind to mutated alpha-synuclein proteins enhancing aggregation of soluble alpha-synuclein proteins into insoluble fibrils that are toxic and resistant to proteolysis\textsuperscript{53,54} (Figure 5).

Another hypothesis suggests that attenuation of mitochondrial complex I function in dopaminergic neurons might be the result of overexpression of alpha-synuclein proteins. This is due to the fact that these proteins are situated in mitochondrial membranes of neuronal cells\textsuperscript{14,82}. Also, mitochondria being one of the primary sites of reactive oxygen species (ROS) production could indicate oxidative stress in causing neuronal damage and death\textsuperscript{83,84}.

1.5 Clinical symptoms of PD

The clinical features of PD are mainly abnormal motor symptoms collectively called Parkinsonism\textsuperscript{85}. The latter includes tremors, bradykinesia, muscle rigidity and postural instability, which develop after 70 - 80\% of dopaminergic neurons have been lost in the substantia nigra pars compacta\textsuperscript{12,85,86}. Tremors are involuntary back and forth muscle movements that are usually affect body parts like the lower limbs, tongue and the jaw\textsuperscript{85,87}. Bradykinesia refers to a reduction in motion during prolonged body movements such as
decreased eye movements\textsuperscript{65,88}. Increased stiffness in muscles refers to muscle rigidity, which usually occurs during passive movements\textsuperscript{89}. Postural instability is characterized by diminished balanced with resultant unsteadiness, due to loss of postural flexes\textsuperscript{90}. In addition to these clinical symptoms, non-motor symptoms that occur during disease progression include effects on cognition, learning, working memory and behavioral roles, ultimately resulting in dementia\textsuperscript{14}.

### 1.6 Risk factors of neurodegeneration in PD

Both \textit{in vivo} and \textit{in vitro} studies have been advantageous in highlighting factors that are involved in PD pathogenesis, which could be targeted for possible neuroprotective interventions\textsuperscript{91}. These include oxidative stress, mitochondrial dysfunction, mitochondrial-mediated apoptosis, neuro-inflammation, proteosomal dysfunction and excitotoxicity, amongst others. Oxidative stress and defects in mitochondrial functioning are thought to be the main factors contributing to neurodegeneration in PD\textsuperscript{7}.

#### 1.6.1 Oxidative stress

Reactive oxygen species (ROS), which are highly reactive molecules, are by-products of normal cellular respiration\textsuperscript{92}. An imbalance between production of these reactive molecules and the body’s innate oxidant neutralizing mechanisms, results in a state of oxidative stress\textsuperscript{93}. Such a state has the potential to cause damage to tissues and macromolecules including proteins, lipids, nucleic acids and DNA, ultimately resulting in cell death\textsuperscript{94}. Apoptosis is a fundamental process of cell death that occurs via activation of distinct signalling pathways and contributes to neuronal degeneration\textsuperscript{95}. It has been suggested that oxidative DNA damage induced by ROS is the most prominent signal that results in apoptotic cell death in dopaminergic neurons\textsuperscript{96}.
The normal metabolism of dopamine in neurons is a highly oxidative process that has been reported to induce an oxidative state in neurons of the nigrostriatal pathway of PD patients, specifically the substantia nigra\(^9\). In addition to this, the by-products of dopamine metabolism can polymerize to form neurotoxins such as neuromelanin in the brain, which aggravates the oxidative stress induced by dopamine metabolism\(^9\).

The human brain contains the second highest content of lipids in the body, after adipose tissue\(^8\). Amongst these lipids are polyunsaturated fatty acids (PUFAs) that constitute the main components of cellular membranes\(^7\). PUFAs contain numerous unsaturated double bonds, separated by a methylene bridge\(^6\). The omega-3 and -6 PUFAs are considered essential fatty acids due to the fact that they are mainly obtained from the diet\(^9\). PUFAs play important roles in fluidity and permeability of cellular membranes. Apart from their structural roles in membranes, they also act as sources of energy, second messengers and substrates for inflammatory mediators, in addition to being implicated in neuronal growth, apoptosis and the release of neurotransmitters from the synaptic vesicles\(^6,9\).

Neurons contain PUFA-rich membranes that are sensitive to oxidative stress, making them susceptible to lipid peroxidation. Reports have shown that lipid peroxidation markers are prominent in the brains of PD patients\(^1\). Oxidative stress in dopaminergic neurons can cause lipid peroxidation\(^10\), which can reduce PUFA content in cellular membranes. When this happens, physiological functions of the membrane are affected, leading to dysfunctional receptor activities and alterations in enzymatic properties\(^1\). Besides direct oxidative damage to membrane lipids, \textit{in vitro} studies have demonstrated a correlation between oxidative stress and mitochondrial dysfunction, using neurotoxin models\(^12\).

Brain mitochondria lack the complete scavenging antioxidant defence system present in most other organs\(^13\). The potential effects of free radicals, especially hydrogen peroxide (H\(_2\)O\(_2\)) and superoxide anions (O\(_2^-\)), are eradicated through the actions of innate antioxidant enzymes like glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD)\(^14\) (Figure
6. GSH-Px requires the coenzyme, reduced glutathione (GSH), in order to convert hydrogen peroxide into water molecules, in this way neutralizing free radicals. GSH itself can also act as a protective agent by directly scavenging free radicals\textsuperscript{105} (Figure 6).

*In vivo* studies have reported a marked reduction in the activity and levels of GSH in the brain mitochondria of PD patients and a prominent elevation in the levels of oxidized glutathione\textsuperscript{106,107}. Overproduction of ROS in astrocytes is reported to be enhanced by defects in the cellular antioxidant status\textsuperscript{108}. Depletion of the innate antioxidants may be a possible primary event leading to dopaminergic neuronal loss, in this way, contributing to the development and progression of PD\textsuperscript{2}.

### 1.6.2 The electron transport chain (ETC) and oxidative stress

The energy that is supplied and used to maintain physiological functions performed by neurons, such as in the signal transduction, is mainly generated from the mitochondrial electron transport chain (ETC), during oxidative phosphorylation\textsuperscript{110,111}. Energy in the mitochondria is generated in the form of ATP\textsuperscript{110}. The ETC consists of five complexes, complex I, II, III, IV and V\textsuperscript{110}. Electron transport starts with the hydrogen carriers, nicotinamide adenosine dinucleotide (NADH) and flavin adenine dinucleotide (FADH\textsubscript{2}), which donate their electrons to complex I and complex II, respectively\textsuperscript{112}. Electrons are then transferred to complex III through the aid of coenzyme Q, then to complex IV through the aid of cytochrome C and finally to complex V, where ATP is generated\textsuperscript{112}. During this process, H\textsuperscript{+} ions are transferred through the inner mitochondrial membrane into the inter-membraneous space, creating an electrochemical gradient that is used for ATP production in complex V\textsuperscript{110}. Disruptions in the ETC result in the reduction in ATP production, a reduction in mitochondrial membrane potential (MMP), generation of free radicals and ultimately cell death\textsuperscript{92,94}. 

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Figure 6. An illustration depicting the process of ROS production by the electron transport chain and its scavenging by the antioxidant defence mechanisms. ETC = electron transport chain; Fe+ = ferrous ion; GRD = glutathione reductase; GSH = reduced glutathione; GSH Px = glutathione peroxidase; GSSH = oxidized glutathione; H2O = water molecule; H2O2 = hydrogen peroxide; OH = hydroxyl radical; O2 = molecular oxygen; SOD = superoxide dismutase.
ETC functioning is often disrupted at complex I. Free electrons then exit the ETC and are accepted by unpaired oxygen molecules to generate oxygen-containing free radicals\textsuperscript{94,113}. The latter has the ability to cause damage to macromolecules such as mitochondrial DNA, lipids and proteins\textsuperscript{92,94}. Deformities in complex I of the ETC results in ATP depletion that may lead to ROS generation\textsuperscript{114}. These elevated ROS levels affect mitochondrial functioning through the induction of mechanisms that make cells susceptible to death, such as reduction in mitochondrial membrane potential (MMP) and activation of apoptosis\textsuperscript{115,116}.

Furthermore, astrocytes contain reduced transition metals such as ferrous ions, which can react with hydrogen peroxide to form the very reactive, hydroxyl radical that has the ability to cause damage to cellular structures such as mitochondrial DNA, proteins and lipids\textsuperscript{104}. Due to this, the significant impact of mitochondrial dysfunction in the pathogenesis of PD has been reported\textsuperscript{102}. Therefore, maintenance of the ETC in the mitochondria is beneficial in preventing PD development\textsuperscript{92}.

### 1.6.3 Mitochondrial dysfunction and cell death

Mitochondria are eukaryotic cellular organelles, well-known for their energy producing functions\textsuperscript{111}. Other biological functions of mitochondria include the maintenance of calcium homeostasis and regulation of signals modulating the survival/death of cells\textsuperscript{117,118,119}.

Neuronal activities including synaptic transmission, axonal transport, and activity of the calcium ion pump, require sufficient energy in order to function efficiently\textsuperscript{120}. The brain and its neurons are highly energy dependent, thus disruptions in mitochondrial functioning greatly impacts neuronal activity and thus brain function\textsuperscript{120}.

DNA damage caused by free radicals occurs more frequently in mitochondria as opposed to nuclear DNA\textsuperscript{19}. This is due to the absence of the histone proteins in mitochondrial DNA that provide protection against damage caused by free radicals\textsuperscript{2}.
In addition to this, the repairing system in mitochondrial DNA is also absent\(^2\). The inner mitochondrial membrane, which is the main site for free radical production, is situated close to the mitochondrial DNA\(^{121}\). As a result, mitochondria are regarded as the potential site for initiating cellular stress signals that cause dopaminergic neuronal cell death\(^{122}\).

Opening of the mitochondrial membrane permeability transition (MPT) pore is a key event in mitochondrial-mediated apoptosis\(^{123}\). Disruptions in mitochondria, such as increased mitochondrial membrane permeability, can result in the release of intra-mitochondrial pro-apoptotic proteins such as Bax, Bid and Bak, as well as other pro-apoptotic factors like apoptosis-inducing factor (AIF) and cytochrome C, into the cytosol\(^{124}\). Since the release of cytochrome C from the mitochondrial space into the cytosol is irreversible, activation of the caspase cascade occurs\(^4\) (Figure 7).

The caspase cascade consists of a family of cysteine proteases that are the effectors of apoptosis and that cause degradation of various cellular components, leading to loss of DNA repairing enzymes\(^{125}\). Caspase-3 activation executes apoptosis\(^{126}\). These proteins and factors are responsible for the breakdown of the cellular cytoskeleton, mitochondrial DNA and DNA-associated proteins leading to neuronal cell death via mitochondrial-mediated apoptotic pathways\(^{124}\) (Figure 7).

From *in vivo* studies, extensive deposits of Lewy bodies in the brains of PD patients, which induced the presence of apoptosis-inducing factors such as activated caspase-3, have been noted\(^95\). Increased expression of pro-apoptotic factors or proteins has also been reported in cortical regions of the brains of PD patients\(^{127,128}\). Dopaminergic neurodegeneration has been shown to be triggered by an increase in ROS production, which is induced by Lewy body inclusions with resultant mitochondrial-mediated apoptotic pathway\(^{94}\).
Figure 7. Mitochondrial events that occur when the mitochondria are dysfunctional\textsuperscript{129}. ATP = adenosine triphosphate; Ca\textsuperscript{2+} = calcium ions; COX-2 = cyclooxygenase-2; Glu/KA = glutamate-kainate receptor complex; GluR = glutamate receptor; LDH = lactate dehydrogenase; MTT = 3 - (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; RNS = reactive nitrogen species; ROS = reactive oxygen species.
Suppression of apoptosis at the level of cytochrome C release might be an effective therapeutic strategy for preventing dopaminergic neurodegeneration\textsuperscript{130} and may be achieved through targeting the anti-apoptotic proteins, Bcl-2 and Bcl\textsubscript{XL}, which prevent mitochondrial membrane permeability. Modulating the pro-apoptotic / anti-apoptotic balance would be advantageous in preventing dopaminergic cell death via apoptosis in PD\textsuperscript{4}.

\textbf{1.6.4 Neuro-inflammation}

Neuro-inflammation is associated with aging and correlates with oxidative stress, which initiates signals leading to the activation of specific genes such as tumor necrosis factor-alpha (TNF-\textgreek{a}), nuclear factor kappa B (NF\textkappa{B}) and interleukin-1-beta (IL-1\textbeta) that aid in inflammation\textsuperscript{131,132}. Elevated levels of these genes have been reported to be present in the brains of both Alzheimer's disease and PD patients leading to activation of astrocytes and microglia with subsequent neuronal cell death\textsuperscript{133}.

It is thought that lipid metabolism may be involved in the pathogenesis of PD. Conversion of PUFA, arachidonic acid, into prostaglandins is reported to be elevated in the substantia nigra of PD patients\textsuperscript{134}. Prostaglandins act as chemical mediators of the inflammatory response. The conversion of PUFAs into prostaglandins is made possible by an inflammatory enzyme, cyclooxygenase-2 (COX-2), which is found to be elevated in the substantia nigra of PD patients\textsuperscript{135}. COX-2 enzyme is implicated in the oxidation of PUFAs and dopamine, thus enhancing lipid peroxidation and DAQs formation in neurons\textsuperscript{66,136}.

\textbf{1.6.5 Proteosomal dysfunction}

Under physiological conditions, normal, mutated or misfolded proteins are attacked by two distinct pathways for degradation and clearance. These are the ubiquitin-proteosomal
pathway and the heat-shock protein chaperones that are responsible for clearing unwanted intracellular proteins\textsuperscript{60,137}.

Degradation via the heat-shock protein chaperones requires energy in the form of ATP. This protein degradation pathway is less common and involves binding of heat-shock proteins to unwanted proteins, degrading them using lysosomal and proteosomal mechanisms\textsuperscript{137}. The ubiquitin-proteosomal pathway is a non-lysosomal route of degradation, commonly used by cells to clear unwanted proteins\textsuperscript{138}. Lysine residues of misfolded, mutated proteins are targeted with ubiquitin for proteolytic degradation\textsuperscript{60}. The latter is achieved through 26S proteasome, an enzymatic complex\textsuperscript{60,139}. Protein oxidation, protein aggregation, misfolded proteins and the presence of protein adducts in the substantia nigra of PD brains are all evidence of inappropriate protein handling, leading to dysfunctional and impaired proteosomal activity\textsuperscript{139}.

### 1.7 Neurotoxins used in \textit{in vitro} models to investigate PD pathogenesis and pathology

\textit{In vitro} models used to study PD pathogenesis involve the induction of PD-like characteristics in different neuronal cell lines. Two cell lines often used in these studies are PC12 and SH-SY5Y neuroblastoma cells. To induce PD-like characteristics, neurotoxins such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), 6-hydroxydopamine, or other environmental pesticides and herbicides like rotenone and paraquat are regularly used\textsuperscript{66,102} (Figure 8). These neurotoxins have been shown to cause selective dopaminergic neuronal cell death toxicity \textit{in vivo}, making them suitable to be used to investigate PD pathogenesis/ pathophysiology \textit{in vitro}\textsuperscript{140}. 
Figure 8. Chemical structure of A) 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine, B) paraquat, C) 6-hydroxydopamine and D) rotenone\textsuperscript{140}.
(i) MPTP

MPTP is a chemical compound that was accidentally discovered to cause Parkinsonism in 1982, after intravenous administration by young drug users\(^{141}\) (Figure 8A). The compound has a chemical structure that resembles that of diamorphine (heroin) and produces PD-like symptoms in drug users\(^{140}\). MPTP is a lipophilic compound that rapidly crosses the blood-brain barrier, producing the 'rush' that drug users crave\(^9\). After its entry into the brain, MPTP is metabolised by microglial monoamine oxidase B (MAO-B), converting MPTP to its active, toxic metabolite, 1-methyl-4-phenyl pyridinium (MPP\(^+\)).

It has been reported that MPP\(^+\) interferes with normal mitochondrial functioning by directly binding to the ETC complex I, causing irreversible ETC complex I inhibition that results in ATP depletion and elevated ROS production\(^9\). Furthermore, this compound is reported to be involved in the activation/release of pro-apoptotic proteins, namely the BcL-2 family proteins, cytochrome C and caspases that are involved in apoptosis\(^{140}\). Due to these cellular events, apoptotic cell death, selectively of dopaminergic neurons occurs\(^{140}\).

(ii) Paraquat

Paraquat (Figure 8B) is a pesticide that produces selective cytotoxicity in dopaminergic neurons. It is closely related to MPP\(^+\), but has a different mechanism of action\(^{140}\). This compound is highly charged, which restricts its movement across the blood-brain barrier, therefore its entry is achieved by transport via a neutral amino acid transporter into the brain\(^{142}\).

Once inside the brain, a sodium-dependent transport mechanism facilitates the entry of paraquat into dopaminergic neurons. Once in the neurons, paraquat impairs mitochondrial functioning by disrupting the antioxidant system\(^{140}\). It disrupts the conversion of oxidized glutathione to reduced glutathione, the latter playing a pivotal role in the scavenging of free
radicals, thus resulting in uncontrolled free radical production\textsuperscript{142}. In addition, paraquat directly contributes to the intracellular levels of ROS by disrupting the redox cycling of cells, causing a high generation of superoxide free radicals. Thus, this herbicide impairs mitochondrial homeostasis mainly through oxidative stress. Unlike MPP\textsuperscript{+}, paraquat has been shown to have a low affinity to the ETC complex I\textsuperscript{140}. However, the mode of cell death also appears to follow the mitochondrial apoptotic pathway.

(iii) 6-hydroxydopamine

The hydroxylated dopamine derivative, 6-hydroxydopamine (Figure 8C), was one of the earliest PD models to be developed\textsuperscript{143}. This compound is not lipophilic and shares structural similarities with other neurotransmitters, thus it can be transported into the dopaminergic neurons by both dopamine and noradrenaline transporters\textsuperscript{144}. Due to this, the compound can accumulate in both dopamine and noradrenaline neuronal cells limiting its specificity for dopaminergic neuronal cell death\textsuperscript{19}.

Once 6-hydroxydopamine is in the substantia nigra, it accumulates in the mitochondria and specifically impairs the ETC complex I activity, reducing ATP production and causing increased free radicals, ultimately leading to excessive and rapid dopaminergic cell death\textsuperscript{59,145}.

(iv) Rotenone

Rotenone is a pesticide commonly used in the United States and United Kingdom\textsuperscript{146} (Figure 8D). Biochemically, rotenone is lipophilic and readily crosses the blood brain barrier where high concentrations can be found\textsuperscript{147}. In neuronal cells, including dopaminergic cells, rotenone also crosses membranes sequestrating different subcellular compartments such as
mitochondria, where it exerts its inhibitory mechanism by disrupting the oxidation phosphorylation pathway in the ETC

In vivo, rotenone causes PD symptoms through its specific inhibition of complex I\textsuperscript{114, 149}. This happens when rotenone forms a complex with mitochondrial complex I ATP production inhibitors, resulting in disruptions in the production of ATP\textsuperscript{148}.

1.8 Current PD therapeutic interventions

There is no PD cure thus far\textsuperscript{150}. Current therapies are aimed at increasing dopamine levels, enhancing dopaminergic cell survival and attenuating the clinical PD symptoms\textsuperscript{151, 152}.

Levodopa, or L-DOPA, a dopamine precursor, is the principal drug used in treating PD symptoms\textsuperscript{153}. It crosses the blood-brain barrier and once in the CNS, is converted to dopamine by L-DOPA decarboxylase, in this way elevating dopamine levels\textsuperscript{24}. Levodopa is administered with a decarboxylase inhibitor, carbidopa or benserazide, in order to prevent its early degradation, thereby increasing the amount of L-DOPA that reaches the CNS\textsuperscript{24}. Though Levodopa is known to be a classic PD treatment, it is associated with severe adverse effects such as motor complications and psychiatric problems later in the diseased state\textsuperscript{62}.

Enhancement of dopamine in the brain is achieved by administration of dopamine agonists such as bromocriptine, cabergoline, pramipexole and ropinirole\textsuperscript{24}. These drugs activate the postsynaptic dopamine receptors facilitating dopamine binding to these receptors. Unfortunately the efficacy of dopamine agonists is poor when it comes to treating motor complications\textsuperscript{154}.

Inhibitors of dopamine degradation enzymes, COMT and monoamine oxidase B (MAO-B), are yet another therapeutic approach to treating PD symptoms\textsuperscript{154}. COMT inhibitors, like entacapone, prevent degradation of dopamine in the CNS, thereby enhancing dopamine
Selegiline is an MAO-B inhibitor that inhibits oxidative dopamine degradation, thus promoting dopamine availability to neurons. However, these drugs are also associated with motor complications\textsuperscript{154}.

Other therapeutic interventions include \textit{N}-Methyl-\textit{D}-aspartate antagonists (NMDA antagonists), statins, glutamate modulators and anti-inflammatory therapy such as non-steroidal anti-inflammatory drugs (NSAIDs)\textsuperscript{155}. The use of these therapies is thought to delay the advancement of the disease, though they do not cure the disease\textsuperscript{156}. NMDA antagonists such as amantadine are administered in the late stages of PD and are useful in improving motor complications caused by Levodopa treatment. NSAIDs may contribute to attenuating neuro-inflammation. Glutamate modulators, on the other hand, aid in regulating mitochondrial processes such as oxidative phosphorylation, and also antagonize the NMDA receptor complex\textsuperscript{157}.

Adverse drug effects such as hair loss, stomach pains and ulcers are associated with statins, toxicity to the kidneys and ears with NSAIDs, whereas muscle soreness and headache is associated with glutamate modulators. Also, results have been unsatisfactory when using these therapies\textsuperscript{158}. Therefore, there is a need for therapies that will minimize the adverse drug effects\textsuperscript{155}.

1.9 The use of herbal remedies as PD therapeutics

The limitations of PD therapies have resulted in extensive research into complementary and alternative medicines\textsuperscript{159,160}. Statistics on PD patients have shown that in the United States, United Kingdom and Korea, approximately 40\%, 39\% and 76\% of patients, respectively, used herbal treatments for neurodegenerative diseases that resulted in improved memory and cognitive functions\textsuperscript{159,161}. 

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Due to the presence of a variety of chemical compounds in plants, each having specific pharmacological properties, they possess various therapeutic benefits such as anti-hypnotic, anti-psychotic, anti-oxidant and anti-inflammatory effects, some of which can prove to be beneficial in treating patients with neurodegenerative diseases. Studies have been conducted on plants in the hope of discovering new therapies that possess beneficial curative effects with minor side effects.

Amongst these studies, Chinese herbs such as *Panax ginseng* C.A Meyer (Ginseng), *Yi-Gan San* (Yukukansan), *Gastrodia elata* Blume (White Dragon Grass) and *Radix Paeoniae alba* (White Peony Root) have been shown to be protective against deleterious effects of MPP⁺ neurotoxin. The aqueous, root extract of *Panax ginseng* has been reported to protect SH-SY5Y neuroblastoma cells from MPP⁺ toxicity mainly through its anti-oxidative and anti-apoptotic properties. *Yi-Gan San*, a polyherbal medicine consisting of nine plants, has long been used to treat neurodegenerative diseases. It possesses anti-apoptotic effects and has been shown to promote dopaminergic neuronal survival, demonstrating its neuroprotective effect in SH-SY5Y neuroblastoma cells when exposed to MPP⁺.

The neuroprotective effect of ethanol extract of the dried roots of *Gastrodia elata* on MPP⁺-induced neurotoxicity in human dopaminergic SH-SY5Y cells, was investigated by An et al. (2010). This extract was found to inhibit the action of caspase-3 activity, the production of free radicals and also inhibit loss of neuronal cell viability in the SH-SY5Y cells. *Gastrodia elata* is used to treat epilepsy, vertigo and dizziness.

The natural compound, Paeoniflorin, a glycoside isolated from *Radix Paeoniae alba*, has been used to treat Alzheimer’s disease, PD and epilepsy. A study conducted by Cao et al. (2010) investigated the protective effect of this compound on MPP⁺-induced toxicity in PC12 cells. It was observed that Paeoniflorin inhibited accumulation of misfolded proteins through the induction of the autophagic pathway and also inhibited apoptotic cell death.
Neuroblastoma cells are widely used to investigate the neuroprotective effect of plants against neurotoxins \textit{in vitro}, the reason being that these cells share many similarities with innate neurons\textsuperscript{114}. \textit{In vitro} studies include pretreatment with plant extracts followed by induction of neurotoxicity using a neurotoxin. Studies like these have yielded promising results in support of using plants for the discovery and development of possible PD therapeutic interventions\textsuperscript{165}.

1.10 Study Aim

The aim of this study was to determine the effects of methanol and ethyl acetate extracts of four South African medicinal plants on rotenone-induced neuronal toxicity using the SH-SY5Y neuroblastoma cell line.

1.11 Study Objectives

The study objectives using the SH-SY5Y cell line were to:

1. Determine the effect of the methanol, ethyl acetate plant extracts and rotenone on cell viability using the sulforhodamine B assay (SRB).
2. Determine the effect of rotenone on cell viability following pre-treatment of cells with methanol or ethyl acetate plant extracts.
3. Determine intracellular ROS production using fluorometry.
4. Determine intracellular glutathione content using fluorometry.
5. Determine the mitochondrial membrane potential using fluorometry.
6. Determine caspase-3 activity as a marker of apoptosis using fluorometry.
1.12 Project Overview

The complex interplay of various factors that causes the induction and progression of PD makes development of PD-targeted therapeutic interventions difficult\(^3\) (Figure 9). From literature, oxidative stress and mitochondrial dysfunction seem to play key roles in PD manifestation\(^7\). Oxidative stress is the result of excessive free radical levels that originate from dopamine metabolism and electron transfer in the ETC, during energy metabolism\(^93\). Excessive free radicals deplete innate antioxidant defences\(^104\). When this happens, mitochondrial processes are affected causing mitochondrial dysfunction\(^122\). The effect of the medicinal plants on factors involved in PD pathogenesis (see Figure 9) were determined in the hope of identifying plants with the promise to be developed into therapies for treatment of PD.
Figure 9. Mechanistic model depicting possible parameters involved in neurodegeneration. The coloured parameters are the ones that were assessed in the study. GSH = reduced glutathione; MMP = mitochondrial membrane potential; ROS = reactive oxygen species.
Chapter 2 - Materials and Methods

All the chemicals and reagents used in this study are provided in Appendix A. Equipment used in this study is provided in Appendix B. Ethical approval to carry out the study using the commercial cell line is provided in Appendix C.

2.1 Plant collection and extract preparation

The plants investigated in this study were obtained from the South African National Botanical Institute (SANBI, Tshwane) or collected in Venda, Limpopo (voucher numbers starting with ‘LT’) (Table 2). Voucher specimens of the plants are deposited at the SANBI herbarium.

Plant material was air-dried and ground to a fine powder. Plant material (1.5 g) was extracted with either 15 mL of methanol or ethyl acetate. Extracts were sonicated for 30 min and placed on a shaker for 2 h, after which they were incubated at 4°C for 20 h. Extracts were centrifuged at 1000 g for 10 min, syringe-filtered (0.22 µM) and dried using a rotary vacuum evaporator. Dried extracts were reconstituted in dimethylsulfoxide (DMSO). Gravimetric yields were determined and plant extracts were stored at -20°C until use. (The final exposure concentration of DMSO in cells never exceeded 0.5% v/v).

2.2 Cell culture and maintenance

SH-SY5Y neuroblastoma cells were cultured in 75 cm³ culture flasks at 37°C under an atmosphere of 5% CO₂ and humidified air.
Table 2. The genus and species, family, plant part and medicinal use (related to neurodegenerative disorders) of the plants investigated in the present study.

<table>
<thead>
<tr>
<th>Genus and species (common name)</th>
<th>Family</th>
<th>Plant part used</th>
<th>Voucher number</th>
<th>Medicinal Use</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Crinum bulbispermum</em> (Burm.f.) Milne-Redh. &amp; Schweick. (Orange river lily)</td>
<td>Amaryllidaceae</td>
<td>Bulb</td>
<td>SANBI</td>
<td>This species is reported to possess anticonvulsant properties&lt;sup&gt;173&lt;/sup&gt;.</td>
</tr>
<tr>
<td><em>Lannea schweinfurthii</em> (Engl.) Engl. (False marula)</td>
<td>Anacardiceae</td>
<td>Root bark</td>
<td>LT 19</td>
<td>Infusions obtained from roots are reported to enhance memory and are sedative&lt;sup&gt;174,175&lt;/sup&gt;.</td>
</tr>
<tr>
<td><em>Scadoxus puniceus</em> (L.) Friis &amp; Nordal. (Red paintbrush)</td>
<td>Amaryllidaceae</td>
<td>Bulb</td>
<td>SANBI</td>
<td>The species is implicated in CNS excitation and depression&lt;sup&gt;176&lt;/sup&gt;.</td>
</tr>
<tr>
<td><em>Zanthoxylum capense</em> (Thunb.) Harv. Small knobwood</td>
<td>Rutaceae</td>
<td>Root</td>
<td>LT 4</td>
<td>Decoctions from this species are used to diminish epilepsy&lt;sup&gt;177&lt;/sup&gt;.</td>
</tr>
</tbody>
</table>

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The cells were grown in Ham’s F12 medium supplemented with 10% heat-inactivated fetal calf serum (FCS) and 1% penicillin/streptomycin. The medium was replaced every 2-3 days as required.

Once cells reached a confluency of 80%, medium was discarded and cells were washed with phosphate buffered saline (PBS). Cells were detached using a 0.125% Trypsin/Versene solution and harvested by centrifugation at 200 g for 5 min. Cells were resuspended in 1 mL of medium and viable cells were counted using trypan blue staining (0.4% w/v in PBS).

### 2.3 Cytotoxicity determination

Cytotoxicity was determined using the sulforhodamine B assay (SRB) as described by Vichai & Kirtikara (2006). The SRB assay is one of the most commonly used assays for *in vitro* cytotoxicity screening. Sulforhodamine is a bright-pink dye that stains the protein contents of cells that are fixed to culture plates. In this enumeration assay, the amount of dye bound to fixed protein is directly proportional to the total cell mass.

Cells (100 μL, $1 \times 10^5$ cells/mL) were seeded into 96-well plates, followed by the addition of 80 μL of 2% FCS-supplemented medium and incubated for 24 h. An aliquot of 20 μL of either plant extracts (final exposure concentrations of 0.78 - 100 μg/ml) or rotenone (final exposure concentrations of $1.28 \times 10^{-4}$ - 50 μM) was added to separate wells. Wells with medium only served as blanks. Minocycline (10 μM), which is known to counteract rotenone toxicity, was used as positive control throughout the study to counteract the effects of rotenone.

After 72 h incubation, 100 μL of the supernatant was aspirated from all wells and replaced with 100 μL of cold trichloroacetic acid (TCA) solution (30% w/v), to fix cell monolayers. The plate was then incubated at 4°C for 1 h to fix cell monolayers. After incubation the plate was gently washed four times with slow-running water to remove any TCA. The plate was dried in
an oven, after which 100 μL of 0.057% (w/v) SRB solution was added to the wells to stain the cellular protein contents. The plate was incubated for 30 min at 4°C and washed twice with 200 μL of a 1% acetic acid solution (v/v) to remove excess unbound dye. The plate was allowed to dry, after which the dye-protein complex was dissociated using 200 μL of a 10 mM Tris-base solution (pH 10.5).

The plate was incubated for 30 min to allow the dye to dissolve completely, after which the absorbance was read at 540 nm with a reference wavelength of 630 nm, using an E_{LX} 800_{UV} Universal plate reader.

2.4 Effect of plant extracts on rotenone toxicity

To investigate the possible protective effect of the extracts against rotenone toxicity, pre-seeded 96-well plates were treated with four non-toxic concentrations of extracts, as determined from the cytotoxicity experiments. Thereafter, rotenone at concentrations of 10 nM, 50 nM and 100 nM was added and plates incubated for 72 h. Cell viability was then assessed using the SRB assay as described above (Section 2.3, page 37-38).

2.5 Determination of intracellular ROS levels

Intracellular ROS production was assessed using the method described by Shaykhalishahi et al. (2009)\textsuperscript{180} with slight modifications. This method uses a fluorescent dye, 2′,7′-dichlorodihydrofluorescein diacetate (H\textsubscript{2}DCF-DA). H\textsubscript{2}DCF-DA diffuses into cells passively, where the acetate groups are cleaved by intracellular esterases to yield dichlorodihydrofluorescein (DCF), which fluoresces upon reaction with intracellular oxidants like hydrogen peroxide\textsuperscript{181}. Assuming that the same number of cells are present and that all
cells absorbed the same amount of H$_2$DCF-DA, the fluorescence intensity of DCF positively correlates with the amount of intracellular ROS present$^{180}$.

Briefly, 20 µL (20 µM) of DCF-DA in PBS solution was added to both the treated cells (plant extracts and rotenone treatments) and controls after 24 h of exposure. The plate was incubated for 30 min at 37ºC and then washed once with 100 µL of PBS to remove excess DCF-DA solution. The fluorescence intensity was determined using a fluorescent plate reader equipped with excitation and emission wavelengths of 492 nm and 525 nm, respectively. The values are expressed as the mean relative fluorescence normalized to the percentage of the control value. A ROS-inducing agent, 2,2'-azobis-2-methylpropanimidamide dihydrochloride (AAPH) (150 µM), was included as an additional positive control to ensure that the assay worked as expected.

2.6 Measurement of intracellular glutathione content

Glutathione is mostly present intracellularly in a reduced form. The remainder is found in the oxidized form. Reduced glutathione is fundamental to cellular homeostasis and survival$^{182}$. To investigate the intracellular reduced glutathione status of cells, a fluorescent thiol probe, monochlorobimane (MCB), was used. MCB crosses the cell membrane easily where it binds to intracellular reduced glutathione to produce a significant fluorescence signal that can be measured and quantified. This reaction is catalyzed by the enzyme glutathione-S-transferase (GST)$^{183}$.

The test was performed according to Nair et al. (1991)$^{183}$. Following 24 h exposure of the treated cultured cells with plant extracts and rotenone, 20 µL (40 µM) of MCB in PBS solution was added to all the wells. The plates were incubated for 2 h at 37ºC followed by a washing step with PBS (100 µL). Fluorescence intensity of MBC was recorded at excitation and emission wavelengths of 360 nm and 460 nm, respectively, using a fluorescence

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microplate reader. The results were obtained by subtracting blank values from the obtained values, which was then normalized to a percentage of control cells. A glutathione depleting agent, \(N\)-ethylmaleimide (NEM) (10 \(\mu\)M), was used as an additional positive control to ensure that the assay worked as expected.

### 2.7 Measurement of mitochondrial membrane potential

To investigate the mitochondrial membrane potential (MMP) of cells, the fluorescent dye 5,5' ,6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide (JC-1) was used. Under physiological condition, the dye accumulates inside healthy mitochondria, forming aggregates that give off red fluorescence. JC-1 dye is unique in that it is a ratiometric dye and at lower concentrations, like that found in the cytosol, the dye exists in its monomeric form, which gives off a green fluorescence. The ratio of red/green fluorescence gives an indication of the status of the MMP\(^{184}\).

The MMP was determined according to Sternfeld \textit{et al.} (2007)\(^{185}\), with minor modifications. Briefly, JC-1 dye was dissolved in DMSO to a stock concentration of 1.5 mM. After treatment of cells for 24 h with plant extracts and rotenone, 100 \(\mu\)L of the supernatant was discarded and 20 \(\mu\)L (10 \(\mu\)M) of JC-1 dye diluted in PBS, was added and plates incubated for 30 min at 37°C and 5% \(CO_2\) in the dark. Following dye loading, the medium was removed and 100 \(\mu\)L PBS added to the wells. The fluorescence intensity of the dye was read using a fluorescence microplate reader at excitation wavelength of 485 nm. Filters with wavelengths of 520 nm and 590 nm were used to detect the green and red fluorescence signals, respectively. Valinomycin (20 \(\mu\)M), a mitochondrial uncoupler, was used as an additional positive control to ensure that the assay worked as expected.
2.8 Caspase-3 activity as a measure of apoptosis

Caspase-3 is activated during the early stages of apoptosis\(^{186}\). The principle of this assay is that the caspace-3 substrate, acetyl-Asp-Glu-Val-Asp 7-amino-4-methylcoumarin (Ac-DEVD-AMC), is cleaved by active caspase-3 enzyme, resulting in the release of the fluorochrome, 7-amino-4-methylcoumarin (AMC), which is detected fluorometrically. The fluorescence signal of this fluorochrome is proportional to the activity of caspase-3 in the cells.

The assay was conducted according to the method of van Tonder (2012)\(^{187}\). Briefly, following exposure, control and treated cells (plant extracts and rotenone treatments) were lysed with 25 µL of a cold lysis buffer [10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 2 mM ethylenediaminetetraacetic acid (EDTA), 5 mM 3-[(3-cholamidopropyl)dimethylammonio]-1propanesulfonate (CHAPS), 5 mM beta-mercaptopoethanol, 0.5 mM phenylmethylsulfonyl fluoride (PMSF)] and kept on ice for 30 min. Thereafter 100 µL of reaction buffer (20 mM HEPES, 2 mM EDTA, 5 mM beta-mercaptopoethanol, 0.5 mM PMSF, 2 mM caspase-3 substrate) was added to wells following an overnight incubation at 37ºC. Fluorescence was detected using a fluorescence microplate reader with excitation 360 nm and emission 460 nm. Staurosporine (3 µM), an apoptosis inducer, was used as positive control to ensure that the assay worked as expected.

2.9 Statistical analyses

For cell viability, the inhibitory concentration that kills 50% of cells (IC\(_{50}\) values) of the plant extracts and rotenone was determined on the SH-SY5Y cells by fitting the obtained results to a Hill equation with top = 100, bottom = 0 and a variable slope using GraphPad Prism 5.0 software. The IC\(_{50}\) values were obtained from the survival curves and are expressed as the mean ± SEM.
All experiments were carried out in triplicate on three different occasions. Depending on the normality of the data, either Mann-Whitney or Student’s t-tests was performed to test for significant mean differences amongst various groups, using GraphPad Prism 5.0 software. Results for endpoints assays are expressed as a percentage (%) of the mean control values, ± SEM. Significant differences from the untreated cell control are indicated by ■ for p value < 0.05. Significant differences between groups exposed to rotenone alone and groups treated with plant extracts followed by rotenone exposure, is indicated by * for p value < 0.05.
Chapter 3 - Results and Discussion

3.1 Cytotoxicity studies

PD remains a concern in the elderly population. Therapeutic interventions used to treat PD have limited therapeutic effect and cause severe adverse effects. For this reason, there remains a need to develop effective treatment options. Herbal remedies have been, and are still, used worldwide to treat neurodegenerative diseases.

Reports from the World Health Organization (WHO) have shown that approximately 80% of the world’s population make use of traditional medicine. Also, more than 80% of Africans and Asians solely depend on traditional medicine for primary health care.

Determining whether herbal remedies contain a specific bio-activity is made simple through the use of in vitro models. The use of SH-SY5Y neuroblastoma cells as an in vitro neuronal cell model dates back to the 1980’s. These cells share various biochemical and functional characteristics of innate neuronal cells, like the expression of dopamine and noradrenaline producing enzymes, tyrosine and dopamine-β-hydroxylase enzymes, acetylcholine, norepinephrine, and various growth factor receptors.

3.1.1 Effect of rotenone and plant extracts alone on SH-SY5Y cell viability

To determine whether the medicinal plant extracts may have desirable effects in PD, PD-like symptoms need to be induced in the in vitro model. This is done using neurotoxins such as rotenone. Rotenone, a classic complex I inhibitor, is toxic in several cell lines. Exposure to 0.1, 1 and 10 µM of rotenone for 4 h was previously reported to decrease the viability of...
Neuro-2a mouse neuroblastoma cells by 86.78% ± 7.14%, 64.49% ± 3.41% and 50.11% ± 3.20%, respectively. Another study, which was carried out using SK-N-MC human neuroblastoma cells, demonstrated toxicity when cells were exposed to 10 nM, 100 nM and 1 µM of rotenone for 24 - 48 h.

From the Sherer et al. (2003) study, it was evident that the toxicity of rotenone depends on the concentration and the duration of exposure. Moreover, different cell lines and cell viability assays used can have an impact on the toxicity of rotenone. The effect of rotenone alone on the survival of SH-SY5Y cells is provided in Figure 10A. A calculated IC$_{50}$ value of 112 nM was determined from a fitted sigmoidal dose-response curve with a variable slope.

The effect of both methanol and ethyl acetate plant extracts on the viability of SH-SY5Y cells are presented in Figures 10B and 10C. The results are expressed as the percentage of cell viability, compared to the untreated cells. Both methanol and ethyl acetate plant extracts had a dose-dependent effect on the proliferation of SH-SY5Y cells across the concentration range tested. Cytotoxicity of plant extracts was assessed separately from rotenone to establish concentrations of the respective plant extracts that do not induce toxicity.

The effect of methanol and ethyl acetate plant extracts on the survival of SH-SY5Y cells was assessed over an incubation period of 72 h. An increase in extract concentration caused a decrease in cell viability (Figures 10B and 10C).

The methanol extract of *L. schweinfurthii* produced a calculated IC$_{50}$ value of 78.87 µg/mL while its ethyl acetate counterpart produced a calculated IC$_{50}$ value less than half of methanol extract (Figures 10B and 10C). A study conducted by Moshi et al. (2003) reported that the 20% aqueous ethanol extracts of *Lannea stuhlmanii* did not cause any significant toxicity in human cervical carcinoma, human colon adenocarcinoma or human skin carcinoma cells, with > 85% viability after 72 h exposure to 100 µg/ml. The difference in solvents used with different polarity might account for the difference in cytotoxicity.
Figure 10. Effect of A) rotenone, B) methanol and C) ethyl acetate plant extracts on the survival of SH-SY5Y cells after a 72 h exposure period using the sulforhodamine B assay.
Z. capense produced calculated IC\textsubscript{50} values of 121.3 and 90.18 µg/mL for its methanol and ethyl acetate extracts, respectively (Figures 10B and 10C). The methanol extract of this plant was the least toxic of all the plant extracts tested. Although the genus \textit{Zanthoxylum} has been reported to possess cytotoxic properties, the isolated compounds alone have been reported to have negligible or no cytotoxic effects\textsuperscript{197}. Da Silva \textit{et al.} (2007a)\textsuperscript{198} investigated the \textit{in vitro} cytotoxic effect of a volatile oil and α-humulene, β-caryophyllene, α-pinene and β-pinene terpenes, from \textit{Zanthoxylum rhoifolium} leaves in a tumor cell model. It was observed that both the volatile oil and β-caryophyllene exerted minor effect against proliferation of the cell, with α-humulene, α-pinene and β-pinene terpenes having no effect on the proliferation of cells.

\textit{S. puniceus} methanol extract had the lowest IC\textsubscript{50} value of all the plants tested, indicative of a high toxicity (Figure 10B). The Amaryllidaceae family is well-known for its high alkaloidal content\textsuperscript{199}. The potential cytotoxic effect caused by this species on SH-SY5Y cells, might be due to the presence of alkaloids.

The ethyl acetate extract of \textit{C. bulbispermum} produced a calculated IC\textsubscript{50} value of > 100 µg/mL, indicative of a low toxicity (Figure 10C). However, \textit{Crinum} species are also well-known for their high alkaloidal content\textsuperscript{199}, thus the low IC\textsubscript{50} value obtained for the methanol extract of \textit{C. bulbispermum} (Figure 10B), may be attributed to the presence of alkaloids.

**3.1.2 Effect of the plant extracts on rotenone-induced SH-SY5Y cytotoxicity**

Sub-toxic concentrations of the methanol and ethyl acetate plant extracts (Figures 10B and 10C) were selected to use in all subsequent cellular assays. The four sub-toxic concentrations selected for all the plants were 3.125, 6.25, 12.5 and 25 µg/mL. To determine the effects that the various plant extracts may have on rotenone-induced cytotoxicity, three
concentrations of rotenone were used (10, 50 and 100 nM) to induce cell death in SH-SY5Y cells following pre-treatment with selected sub-toxic concentrations of the plant extracts. Graphic illustrations depicting rotenone-induced cytotoxicity after pre-treatment with plant extracts are presented in Figures 11 and 12 for the methanol and ethyl acetate extracts, respectively.

Compared to the untreated cells, approximately 20% cell death was observed following treatment with 10 nM rotenone (Figures 11A and 12A). Viability in cells treated with 50 nM rotenone was reduced to 48%, when compared to the untreated cells (Figures 11B and 12B). Toxicity induced with 100 nM rotenone resulted in 80% cell death, when compared to the untreated cells (Figures 11C and 12C). Minocycline was observed to counteract rotenone toxicity (Figures 11 and 12), by increasing cell viability to 98%, 92% and 78% at 10, 50 and 100 nM rotenone concentrations.

Minocycline is a lipophilic tetracycline antibiotic that is known to possess anti-inflammatory and antioxidant activities, besides its anti-bacterial activity. This antibiotic has also been employed in animal models to study other neurological disorders such as Huntington's disease, stroke and PD. In PD animal model, MPTP was used as a neurotoxin. The neuroprotective effect of minocycline in these animal models was through the modulation of microglia activation, ROS production, apoptosis and the release of cytokines that promote inflammation. For this reason, minocycline may be beneficial in treating PD.

Both methanol and ethyl acetate extracts of Z. capense and S. puniceus, and the ethyl acetate extract of L. schweinfurthii, counteracted rotenone-induced toxicity at 10 nM concentration of rotenone (Figures 11 and 12). Moreover, cell viability was maintained when cells were pre-treated with concentrations of 3.125 and 6.25 µg/mL of both methanol and ethyl acetate extracts of Z. capense, S. puniceus and ethyl acetate extract of L. schweinfurthii at 10 nM of rotenone (Figures 11A and 12A).
Figure 11. Effect of methanol plant extracts on the survival of SH-SY5Y cells after 72 h exposure to rotenone at A) 10 nM, B) 50 nM and C) 100 nM, using the sulforhodamine B assay. Significant differences from the rotenone control are indicated by * representing p value < 0.05, while significant differences from untreated cells are indicated by ■, also representing p value < 0.05. PC = positive control (minocycline at 10 µM); Rot = rotenone; Untr = Untreated cells. (The pink dotted-line indicates 100% viability of the untreated cells while the black dotted-line is indicative of the level of cell death induced by the respective rotenone concentrations).
Figure 12. Effect of ethyl acetate plant extracts on the survival of SH-SY5Y cells after 72 h exposure to rotenone at A) 10 nM, B) 50 nM and C) 100 nM, using the sulforhodamine B assay. Significant differences from the rotenone control are indicated by * representing p value < 0.05, while significant differences from untreated cells are indicated by ■, also representing p value < 0.05. PC = positive control (minocycline at 10 µM); Rot = rotenone; Untr = Untreated cells. (The pink dotted-line indicates 100% viability of the untreated cells while the black dotted-line is indicative of the level of cell death induced by the respective rotenone concentrations).
The effect of plant extracts on viability, when toxicity was induced with 50 nM rotenone, was less pronounced than that observed at 10 nM, but greater than that at 100 nM (Figures 11B and 12B). None of the plant extracts were observed to be protective against toxicity induced with 100 nM rotenone (Figures 11C and 12C).

Dichloromethane and methanol extracts of *Lannea barteri* stem-bark and roots, were shown to contain polyphenolic compounds such as flavonoids, tannins, and sterols. The protective effect of *L. schweinfurthii* might be due to the presence of these polyphenols.

Cytotoxic activity of ethanol extract of *Lannea grandis* was shown in a study conducted by Roy *et al.* (2011). Phytochemical screening of *Lannea glandis* revealed the presence of alkaloids and glycosides such as saponins. These compounds are known for their cytotoxic properties, which may have been enhanced at high (100 nM) rotenone concentrations.

*Z. capense* was observed to exert protective effects against rotenone-induced toxicity in a dose-dependent manner (Figures 11 and 12). At 3.125 and 6.25 µg/mL this species was protective against toxicity induced with 10 nM of rotenone (Figures 11A and 12B). The *Zanthoxylum* genus is known to contain a variety of compounds with various biological activities. These compounds include alkaloids that possess anti-tumor properties, ligands that possess both anti-tumor and antioxidant properties, coumarins that inhibit activity of specific enzymes such as MAO, and flavonoids that possess antioxidant, anti-inflammatory and anti-tumor properties.

A decrease in extract concentrations of *S. puniceus*, resulted in increased cell viability at 10 nM of rotenone (Figures 11 and 12). The methanol extract of *S. puniceus* was observed to be less protective than its ethyl acetate extract (Figures 11 and 12). The protective effect of this species was observed at 3.125 and 6.25 µg/mL when toxicity was induced with 10 nM rotenone (Figures 11A and 12A). Low extract concentrations of this species offered protective effects in SH-SY5Y cells exposed to low concentrations of rotenone (Figures 11A and Figure 12A).
C. bulbispermum was observed to be the least protective plant against rotenone-induced toxicity. The toxicity of C. bulbispermum was observed to increase with increased rotenone toxicity for both types of solvents (Figures 11 and 12). Furthermore, the cytotoxic effect produced by this species was comparable to that of rotenone control (Figures 11A and 12A, 11C and 12C). The cytotoxic effect of this species could have been due to its reported high alkaloidal content\textsuperscript{213}.

Rotenone toxicity induced with 100 nM was observed to be too toxic for any of the plant extracts to show any protective effect, while 10 nM rotenone toxicity was unable to induce at least 50% toxicity in the cells. As toxicity induced with 50 nM rotenone produced close to 50% cell death, this concentration was selected to induce toxicity in all subsequent assays.

3.2 Antioxidant activity

3.2.1 Reactive oxygen species generation

ROS are implicated in the induction and progression of many diseases, including neurodegenerative diseases\textsuperscript{214,215}. There are two sources of ROS, the exogenous and endogenous sources\textsuperscript{215}. The exogenous sources are mostly acquired through the environment and rarely cause elevated ROS, these include radiation and smoking. Endogenous sources are reported to be the main sources of ROS in the body. The ETC, phagocytic respiratory burst, beta oxidation, lipid peroxidation, elevated intracellular calcium levels and auto-oxidation of catecholamines, such as dopamine and amino acids, are classified as endogenous sources of ROS generation\textsuperscript{214,216}.

Mitochondrial ETC complex I is the main site of ROS production from mitochondria\textsuperscript{217} and ROS production can be enhanced by a defective mitochondrial ETC complex I\textsuperscript{218}. Rotenone, a specific mitochondrial ETC complex I inhibitor, caused no intracellular ROS production in the present study. In contrast, ROS generation has been reported after treatment of both
undifferentiated and differentiated human stem cells with 8 μM rotenone after a 24 h exposure period\textsuperscript{219}. The rotenone concentration (50 nM) used in the present study, might have not been high enough to achieve similar results, regardless of the exposure time.

A significant (p < 0.05) intracellular ROS production was observed in cells exposed to AAPH, when compared to the untreated cells, indicating that the assay performed as expected (Figure 13). Compared to the untreated cells, there was no intracellular ROS production in cells exposed to rotenone alone for 24 h. Rather, rotenone exposure resulted in significant (p < 0.05) decreased intracellular levels of ROS. This decrease in intracellular ROS levels dissipated with exposure time, as can be seen from Figure 14, where no significant difference in ROS levels were observed after 72 h of exposure to the same concentration of rotenone. Minocycline, at a concentration of 10 μM, was able to counteract the effects of ROS after 24 h of exposure. However, minocycline caused a significant (p < 0.05) increase in intracellular ROS levels, when compared to the untreated cells, at both the 24 h and 72 h exposure times (Figures 13 and 14).

Both the methanol and ethyl acetate extracts of \textit{L. schweinfurthii} countered the decrease in intracellular ROS caused by rotenone after 24 h exposure (Figures 13A and 13B). However, after a 72 h exposure period, intracellular ROS levels were decreased to a greater extent than rotenone treatment alone, especially by the methanol extract (Figures 13A and 13B). A study conducted on the methanol and 80% ethanol extracts of \textit{Lannea velutina} (A. Rich.), showed that this species contains antioxidant compounds. Moreover, isolation of antioxidant proanthrocyanidins has been reported from various \textit{Lannea} species\textsuperscript{220}.

Phytochemical screening of three \textit{Lannea} species, \textit{Lannea acida} (A. Rich.), \textit{Lannea microcarpa} (Engl & Krause) and \textit{Lannea velutina} (A. Rich.), revealed high antioxidant contents in the plants\textsuperscript{221}. Methanol extracts were more potent at decreasing intracellular ROS levels than ethyl acetate extracts (Figures 13A and 14A).
Figure 13. ROS generation in SH-SY5Y cells exposed to rotenone at 50 nM after pre-treatment with A) methanol and B) ethyl acetate plant extracts following 24 h exposure period. Significant differences from the rotenone are indicated by * representing p value < 0.05, while significant differences from untreated cells are indicated by ■, also representing p value < 0.05. PC = positive control (minocycline at 10 µM); Rot = rotenone; Untr = Untreated cells. (The pink dotted-line indicates ROS levels in the untreated cells while the black dotted-line is indicative of ROS generation exposed to rotenone alone).
Figure 14. ROS generation in SH-SY5Y cells exposed to rotenone at 50 nM after pre-treatment with A) methanol and B) ethyl acetate plant extracts following 72 h exposure period. Significant differences from the rotenone are indicated by * representing p value < 0.05, while significant differences from untreated cells are indicated by ■, also representing p value < 0.05. PC = positive control (minocycline at 10 µM); Rot = rotenone; Untr = Untreated cells. (The pink dotted-line indicates ROS levels in the untreated cells while the black dotted-line is indicative of ROS generation in cells exposed to rotenone alone).
Anacardiaceae species are known to possess antioxidant activity\textsuperscript{222}. They might also contain other toxic compounds that increase intracellular ROS production as was observed for \textit{L. schweinfurthii}. Intracellular ROS production was increased after treatment with \textit{Z. capense} methanol and ethyl acetate extracts (Figures 13A and 13B). After 72 h, the extract significantly (\(p < 0.05\)) reduced intracellular ROS levels at the highest extract concentration tested (25 \(\mu\)g/mL) (Figures 14A and 14B). Phytochemical analysis of \textit{Z. capense} has revealed several bio-active compounds, including alkaloids, lignans, coumarins, amides, flavonoids and terpenes\textsuperscript{197,209,223}. The coumarins, 7,8-dihydroxy-4-methyl coumarin (DHMC) and 7,8-diacetoxy-4-methyl coumarin (DAMC), and flavonoids quercetin and quercetin penta-acetate, have been shown to be potential oxidants\textsuperscript{224}. Thus, the elevated intracellular ROS production seen at low test concentrations might be due to the presence of coumarins and flavonoids.

\textit{S. puniceus} and \textit{C. bulbispermum} extracts were observed to elevate intracellular ROS levels, with methanol extracts for both species increasing intracellular ROS levels more than ethyl acetate extracts (Figures 13 and 14). \textit{S. puniceus} and \textit{C. bulbispermum} belong to the Amaryllidaceae family, which is well-known for its high alkaloidal content. Alkaloids found in this family include heamanthine, distichine and buphanine, which are known for their toxic effects\textsuperscript{177}. Due to the absence of rotenone-ROS production in this study, \textit{S. puniceus} and \textit{C. bulbispermum} species may possess potential oxidants that induced the observed intracellular ROS production.

To confirm no intracellular ROS generation due to rotenone exposure, experiments were repeated over a longer exposure period (72 h). Still no significant intracellular ROS production was observed in cells exposed to rotenone alone, compared to untreated cells (Figure 14). The positive control, minocycline, did cause intracellular ROS production, when compared to the untreated cells (Figure 14). AAPH also caused a significant (\(p < 0.05\)) increase in intracellular ROS production, when compared to the untreated cells, indicating that the assay performed as expected (Figure 14).
Rotenone is reported to induce intracellular ROS generation resulting in a reduction in the cellular antioxidant status\textsuperscript{225}. Vrablic \textit{et al.} (2001)\textsuperscript{226} conducted a study on hepatocytes where toxicity was induced using rotenone (50 nM). The authors reported a reduction in ROS production, which is similar to what was observed in the present study. Contrary to this, Gao \textit{et al.} (2003)\textsuperscript{227} have shown ROS production to be induced by 5 nM and 10 nM rotenone after 30 min incubation period in primary mesencephalic neuron glial cultures. The authors demonstrated that the generation of ROS from activated microglia induced by rotenone, was mediated by NADPH oxidase. The latter is a membrane-bound enzyme complex that generates superoxide radicals by transferring electrons from NADPH inside the cells to oxygen, across the membrane\textsuperscript{228}. Molina-Jimenez \textit{et al.} (2003)\textsuperscript{229} showed rotenone-induced ROS production in SH-SY5Y cells after 16 h treatment with 5 µM rotenone. It would appear as if rotenone-induced ROS generation \textit{in vitro} may be influenced by the type of cell line used, concentration of rotenone used and time of exposure to rotenone\textsuperscript{219}.

### 3.2.2 Intracellular glutathione content

Antioxidants are known to counteract the deleterious actions of ROS by scavenging, detoxifying and suppressing ROS formation, thus preventing cell damage\textsuperscript{214}. An increase in ROS production can result in depletion of the antioxidant status of the cell\textsuperscript{230}. Reduced glutathione acts as an antioxidant in dopaminergic cells and aids in combating ROS-induced cellular damage, thus protecting the cell against the deleterious effects of ROS\textsuperscript{231}.

In the present study no ROS generation was observed in cells treated with rotenone alone, when compared to the untreated cells (Figures 13 and 14). However, reduced intracellular glutathione content was observed in cells treated with rotenone alone and this effect was counteracted by minocycline (10 µM), which increased intracellular glutathione content (Figure 15).
Figure 15. Intracellular glutathione content in SH-SY5Y cells exposed to rotenone at 50 nM after pre-treatment with A) methanol and B) ethyl acetate plant extracts following 24 h exposure period. Significant differences from the rotenone control are indicated by * representing p value < 0.05, while significant differences from untreated cells are indicated by ■, also representing p value < 0.05. PC = positive control (minocycline); Rot = rotenone; Untr = Untreated cells. (The pink dotted-line indicates intracellular glutathione levels in the untreated cells while the black dotted-line is indicative of intracellular glutathione content in cells exposed to rotenone alone).
As rotenone exposure did not produce any ROS generation, it is possible that rotenone may have inhibited enzymes involved in glutathione synthesis or that the pesticide may have formed complexes with glutathione itself, decreasing free glutathione content. The positive control, N-ethylmaleimide (NEM) (10 µM), which decreases intracellular glutathione by conjugation, was observed to decrease intracellular glutathione content, indicating that the assay performed as expected (Figure 15).

Both solvent extracts of *L. schweinfurthii* increased the intracellular glutathione content when compared to rotenone alone. *L. schweinfurthii* (6.25 and 12.5 µg/mL) caused an increase in intracellular glutathione content, comparable/higher than that of the untreated cells (Figures 15A and 15B). Antioxidant activity has been reported by Adewusi & Steenkamp (2011)\textsuperscript{232}. Antioxidant activity of another species, *Lannea velutina* (A. Rich) has been shown and the antioxidant compounds identified as epicatechin and proanthrocyanidin\textsuperscript{220} in this species. Furthermore, the rotenone-countering effect of *L. schweinfurthii* was comparable, if not better, than that of minocycline at 10 µM concentration (Figure 15). Therefore, *L. schweinfurthii* appear to contain molecules that may be beneficial in treating PD.

*Z. capense* also increased intracellular glutathione content, with the methanol extract increasing glutathione levels more effectively than its ethyl acetate counterpart (Figures 15A and 15B). Though oxidants such as 7,8-dihydroxy-4-methyl (DHMC) and 7,8-diacetoxy-4-methyl coumarins (DAMC), and quercetin and quercetin penta-acetate flavonoids, are reported to be present in the genus *Zanthoxylum*\textsuperscript{224} antioxidants like lignans are also reported to be present in this genus\textsuperscript{209,212}. Two flavonoids, hyperoside and quercitrin have been isolated from the methanol extract of the fruits of *Z. piperitum*\textsuperscript{233}. At low doses the ethyl acetate extract resulted in an effect comparable to that of minocycline (Figure 15 B).

Both *S. puniceus* and *C. bulbispermum* resulted in intracellular glutathione content comparable to that observed in the untreated cells (Figures 15A and 15B). However, ethyl acetate extract of *S. puniceus* increased glutathione levels more than that of the untreated
cells at 25 µg/mL (Figure 15B). In the present study, a significant (p < 0.05) increase in intracellular glutathione content was observed for both *S. puniceus* and *C. bulbispermum* species. Methanol extract of *S. puniceus* produced a response comparable to that of minocycline at 3.125 µg/mL (Figure 15A). It is also possible that the studied plant extracts may contain precursors of glutathione like cysteine or could stimulate the synthesis of glutathione.

### 3.3 Mitochondrial membrane potential

Mitochondrial dysfunction has been implicated in the development of many disorders\(^2\). These disorders can be subdivided into acquired and inherited conditions\(^2,234\). Diabetes mellitus, cancer, bipolar disorder, schizophrenia, cardiovascular diseases, anxiety disorders, and neurodegenerative diseases such as Huntington’s disease, Alzheimer’s disease and PD, are all acquired conditions in which mitochondrial dysfunction may play a role\(^{235,236,237,238}\). Inherited conditions include neuropathy, myoclonic epilepsy and ragged-red fibers, mitochondrial encephalomyopathy and leber hereditary optic neuropathy, amongst others\(^{234}\). The latter can lead to damage of major organ systems such as the pancreas and liver, as observed in diabetes mellitus, the eyes in the case of optic neuropathy, the heart in the case of cardiomyopathy, and the brain and the nerves as observed in neurologic disorders\(^{234}\).

Therapeutic interventions aimed at treating mitochondrial-related disorders are still lacking. Moreover, there are certain limitations that make it difficult to diagnose mitochondrial dysfunctions\(^19\). These include the progression rate of the condition diagnosed, the onset of the condition at a certain age and inconsistency in symptoms observed\(^2,234\). *In vitro* models used to study mitochondrial dysfunction have been developed and provide insights into the biochemical pathways involved in disorders of the like\(^19\). In the present study, rotenone was used to induce mitochondrial dysfunction by uncoupling the MMP in the SH-SY5Y cells.
Compared to the untreated controls, cells exposed to rotenone alone showed a significant (p < 0.05) reduction in the MMP (Figure 16). Minocycline (10 µM) was not able to counteract the effect observed in cells exposed to rotenone alone (Figure 16). Valinomycin (20 µM) caused a significant (p < 0.05) reduction in the MMP, compared to the untreated cells, indicating that the assay performed as expected (Figure 16).

MMP levels were observed to be more effectively reduced by methanol extracts than ethyl acetate extracts (Figures 16A and 16B). Therefore, methanol did extract compounds that enhanced MMP reduction in SH-SY5Y cells (Figure 16A). Compared to rotenone, L. schweinfurthii significantly (p < 0.05) reduced MMP levels at all extract concentrations tested. The ethyl acetate extract of this species, was observed to have little effect on the reduction of MMP, when compared to the methanol extract (Figures 16A and 16B). Z. capense was also observed to decrease MMP levels (Figures 16A and 16B). S. puniceus and C. bulbispermum, where shown to decrease MMP levels at the highest concentration tested (25 µg/mL), with ethyl acetate extracts of these species being more potent than their methanol counterparts (Figures 16A and 16B).

MMP is critical for cell survival and homeostasis\textsuperscript{239}. Rotenone is known to inhibit the function of mitochondrial complex I, reducing ATP production from the ETC\textsuperscript{219,240}. Reduced ATP production depolarizes the inner mitochondrial membrane, causing the MMP to dissipate\textsuperscript{241}. Moreover, formation of an irreversible MPT pore on the inner mitochondrial membrane\textsuperscript{240,242} allows influx and efflux of ions and other solutes resulting in further MMP depolarization. Furthermore, loss of glutathione and increased intracellular cytosolic calcium can occur\textsuperscript{240}. All of these events ultimately contribute to apoptotic cell death\textsuperscript{241}. Therapeutic agents that can inhibit MPT pore formation would be beneficial in preventing MMP reduction and reduction in ATP production, thus suppressing cell death and promoting cell survival\textsuperscript{243}.  

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Figure 16. Mitochondrial membrane potential (MMP) in SH-SY5Y cells exposed to rotenone at 50 nM after pre-treatment with A) methanol and B) ethyl acetate plant extracts following 24 h exposure period. Significant differences from the rotenone control are indicated by * representing p value < 0.05, while significant differences from untreated cells are indicated by ■, also representing p value < 0.05. PC = positive control (minocycline at 10 µM); Rot = rotenone; Untr = Untreated cells; Valin = valinomycin. (The pink dotted-line indicates MMP in the untreated cells while the black dotted-line is indicative of MMP levels in cells exposed to rotenone alone).
The plant extracts did not prevent/reduce the MMP uncoupling effect of rotenone (Figure 16). This may be due to the irreversible formation of MPT pore that is reported to occur within 20 min of the rotenone apoptogenic effect, once mitochondrial dysfunction results. All of these events commit a cell to apoptotic cell death. Therefore, the time of rotenone exposure in cells is very critical in studying mitochondrial dysfunction. Moreover, MMP is not a static parameter. It is dynamic and fluctuates with the respiratory needs of the cell. If a cell requires more energy (high ATP utilization) the MMP will decrease as ATP production increases. If less energy is required, the opposite will happen. The fact that the plant extracts further decreased MMP may indicate that the extracts increased ATP utilization.

3.4 Apoptosis

Staurosporine, a general apoptosis inducer, caused a significant (p < 0.05) increase in caspase-3 activity, indicating that the assay performed as expected (Figure 17). Rotenone also up-regulated caspase-3 activity, when compared to the untreated controls. This action was counteracted by minocycline, which reduced caspase-3 activity (Figure 17). All the plant extracts were observed to significantly (p < 0.05) reduce rotenone-induced caspase-3 activity at all extract concentrations tested (Figures 17A and 17B).

However, methanol extracts were observed to reduce caspase-3 activity more efficiently than ethyl acetate extracts. Both the methanol and ethyl acetate extracts of L. schweinfurthii decreased caspase-3 activity in a dose-dependent manner (Figures 17A and 17B). Extracts of Z. capense were also observed to reduce caspase-3 activity (Figures 17A and 17B). Low concentrations of the methanol extract of Z. capense resulted in a response comparable to that of minocycline (Figure 17A). S. puniceus and C. bulbispermum extracts caused a significant (p < 0.05) reduction in caspase-3 activity (Figures 17A and 17B). The highest extract concentration tested (25 µg/mL) for both S. puniceus and C. bulbispermum methanol extracts, were more effective than minocycline at inhibiting caspase-3 activity (Figure 17A).
Figure 17. Caspase-3 activity in SH-SY5Y cells exposed to rotenone at 50 nM after pre-treatment with A) methanol and B) ethyl acetate plant extracts following 24 h exposure period. Significant differences from the rotenone control are indicated by * representing p value < 0.05, while significant differences from untreated cells are indicated by ■, also representing p value < 0.05. PC = positive control (minocycline at 10 µM); Rot = rotenone; Stauro = staurosporine; Untr = Untreated cells. (The pink dotted-line indicates caspase-3 activity in the untreated cells while the black dotted-line is indicative of caspase-3 activity in cells exposed to rotenone alone).
Apoptosis is a fundamental process used to regulate cell proliferation in multicellular organisms\(^2\). Rotenone induces apoptosis via mitochondrial dysfunction\(^2\). Within the mitochondria there are pro-apoptotic factors such as cytochrome C and other apoptosis-inducing factors\(^2\). Upon damage to the mitochondria, cytochrome C, which is normally found in the mitochondrial matrix, forms a complex with apoptosis-activation factor 1 (Apaf-1), which is present in the mitochondrial intermembrane space, and caspase-9. This newly formed complex, known as the apoptosome, activates the caspase-3 enzyme, in this way initiating apoptosis\(^2\). Caspase-3, the main executor of apoptosis, enhances proteolytic cleavage of cellular and nuclear material, leading to cell death\(^1\).

Rotenone-induced apoptosis occurs as a consequence of mitochondrial dysfunction, which is triggered by many factors including: disruption in ATP production, MMP uncoupling, increased intracellular calcium levels, elevated ROS generation and glutamate excitotoxicity, in neurons\(^2\). ATP depletion is implicated in depolarized MMP, with ultimate MPT pore formation in the inner mitochondrial membrane\(^2\). The pore allows increased intracellular calcium levels, as well as the irreversible release of cytochrome C into the cytosol\(^2\). Thus, rotenone-induced apoptosis observed in the present study, may be through the induction of MTP pore formation.

Minocycline has been reported to inhibit MPT pore formation thus preventing cytochrome C release, resulting in suppression of apoptosis\(^2\). In the present study, minocycline was observed to inhibit apoptosis, when compared to cells treated with rotenone alone (Figure 17). Cells undergoing apoptosis have been reported to have reduced MMP due to MTP pore formation\(^2\).

Therapeutic targets aimed at inhibiting or suppressing MPT pore formation can be beneficial at inhibiting apoptosis and promoting dopaminergic neuron survival. Also, the possible mechanisms of the extracts in inhibiting apoptotic cell death may be through the inhibition or suppression of MPT pore formation and caspase-3 activity.
3.5 Possible mechanism(s) of action of the plant extracts

The proposed mechanisms of action of the studied plant extracts are depicted in Figure 18. Rotenone produced cytotoxic effects on the SH-SY5Y cells. This effect was counteracted by *Z. capense* and *S. puniceus*. The neuroprotective effect of these species may be attributed to their anti-apoptotic effect. The latter resulted due to inhibition/suppression of caspase-3 activity thus promoting cell survival. Moreover the pesticide reduced intracellular glutathione content, depolarization and up-regulated caspase-3 activity in the SH-SY5Y cells. *L. schweinfurthii* and *Z. capense* depolarized MMP further. MMP depolarization is known to correlate with increased cellular energy demands.

During cellular respiration, some electrons leak out of the ETC and are converted into free radicals that can enhance oxidative stress. Increased intracellular ROS generation produced by these species might be due to the uncoupling of MMP. Pre-treatment of the cells with *C. bulbispermum* produced cytotoxic effects following rotenone-induced toxicity, thus decreasing cell viability. This may be due to excessive intracellular ROS production induced by this species. However, all the species studies in the present study, preserved the intracellular glutathione content and inhibited caspase-3 activity potently. Suppression of caspase-3 activity by the extracts may be attributed to the inhibition of the MTP pore formation which in turn resulted in the prevention of intracellular glutathione leakage out of cells, thus preserving the cells.

A table summarizing the effect of cells pre-treated with plant extracts compared to those treated with rotenone alone (50 nM) on the parameters tested is shown in Table 3.
Figure 18. Possible mechanism(s) of action of the studied plant extracts in counteracting rotenone-induced toxicity. CB = *Crinum bulbispermum*; GSH = reduced glutathione; LS = *Lannea schweinfurthii*; MMP = mitochondrial membrane potential; ROS = reactive oxygen species; SP = *Scadoxus puniceus*; ZC = *Zanthoxylum capense*. Where ↓, ↑ represents: inhibition, decrease and increase, respectively.
Table 3. Summary of the effect of cells pre-treated with plant extracts compared to those treated with rotenone alone (50 nM) on the parameters tested.

<table>
<thead>
<tr>
<th>Extracts</th>
<th>Solvent</th>
<th>Viability</th>
<th>ROS</th>
<th>GSH</th>
<th>MMP</th>
<th>Apoptosis</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. schweinfurthii</em></td>
<td>Methanol</td>
<td>─</td>
<td>+</td>
<td>+</td>
<td>─</td>
<td>─</td>
</tr>
<tr>
<td></td>
<td>Ethyl acetate</td>
<td>─</td>
<td>+</td>
<td>+</td>
<td>n.c</td>
<td>─</td>
</tr>
<tr>
<td><em>Z. capense</em></td>
<td>Methanol</td>
<td>─</td>
<td>+</td>
<td>+</td>
<td>─</td>
<td>─</td>
</tr>
<tr>
<td></td>
<td>Ethyl acetate</td>
<td>n.c</td>
<td>+</td>
<td>+</td>
<td>n.c</td>
<td>─</td>
</tr>
<tr>
<td><em>S. puniceus</em></td>
<td>Methanol</td>
<td>─</td>
<td>+</td>
<td>+</td>
<td>─</td>
<td>─</td>
</tr>
<tr>
<td></td>
<td>Ethyl acetate</td>
<td>─</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>─</td>
</tr>
<tr>
<td><em>C. bulbispermum</em></td>
<td>Methanol</td>
<td>─</td>
<td>+</td>
<td>+</td>
<td>─</td>
<td>─</td>
</tr>
<tr>
<td></td>
<td>Ethyl acetate</td>
<td>─</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>─</td>
</tr>
</tbody>
</table>

Where: ─: decrease

+: increase

n.c: no change
Chapter 4 - Conclusion

This is the first study to investigate the effects of *L. schweinfurthii*, *Z. capense*, *S. puniceus* and *C. bulbispermum* species on the studied parameters. Limited literature is available on the protective mechanisms provided by these plants against pesticide-induced toxicity. Cytotoxicity studies using rotenone revealed a potent cytotoxic effect observed in SH-SY5Y cells. The cytotoxic effect of rotenone was dose-dependent. Rotenone caused no significant ROS production. However, a significant depolarization of the MMP and depletion of intracellular glutathione content were observed following exposure to rotenone (50 nM). In addition, caspase-3 activity was up-regulated in cells exposed to rotenone. These findings suggest rotenone causes mitochondrial dysfunction and mitochondrial-mediated induction of apoptosis, which is in accordance with literature.

Of the ethyl acetate plant extracts, *C. bulbispermum* did not show any cytotoxic effect in the SH-SY5Y cells. With regards to methanol extracts, *Z. capense* was shown to be the least cytotoxic to the neuroblastoma cells. *Z. capense* and *S. puniceus* plant extracts were observed to be protective against rotenone-induced toxicity. All four plants increased intracellular glutathione content in cells exposed to rotenone, and counteracted rotenone-induced caspase-3 activation. Although some plant extracts appeared to counteract the effects of rotenone on MMP, no significant change in MMP was observed.

Therefore, these plants demonstrated anti-apoptotic effects and restored the intracellular glutathione content against rotenone-induced toxicity, making them potential agents to be developed into therapies for treating PD. Isolating the active compounds using bio-activity guided fractionation of the extracts, can be the subject of future research. Research on the safety and efficacy of these plants is also required.
Summary

PD is a multifactorial disease with current therapies merely slowing the progression of the disease or alleviating symptoms. However, with the advancement of the disease, therapies become ineffective. For this reason there is a need to develop more effective treatment options for PD. This has resulted in extensive research into complementary and alternative medicines. Herbal remedies have and are still being used in developing countries to ameliorate and treat neurodegenerative diseases like PD.

The following plants were investigated in the present study: the root-bark of *Lannea schweinfurthii*, roots of *Zanthoxylum capense*, the bulbs of *Scadoxus puniceus* and *Crinum bulbispermum*. Rotenone was used to induce PD-like symptoms in the SH-SY5Y neuronal cell line. Cytotoxicity in the SH-SY5Y cells was assessed for both plant extracts alone and rotenone alone, using the SRB assay. The effects of the plant extracts on dopaminergic neuronal survival were assessed by pre-treating the cells with sub-toxic concentrations of the plant extracts prior to rotenone-induced toxicity. Intracellular ROS production was measured using a fluorescent dye H$_2$DCF-DA, while monochlorobimane was used to determine intracellular glutathione content. Mitochondrial membrane potential was assessed by staining the cells with the JC-1 dye. Caspase-3 activity, as marker of apoptosis, was determined using caspase-3 fluorescence assay.

Of all the plant extracts, *Z. capense* and *S. puniceus* showed the most promise for neuroprotective properties against rotenone-induced toxicity. No intracellular ROS production was observed in cells exposed to rotenone alone. However, intracellular ROS production was observed in cells treated with rotenone and the plant extracts, suggesting possible oxidant properties of the studied plant extracts, especially *S. puniceus* and *C. bulbispermum*. Though no significant intracellular ROS production was caused by rotenone,
the pesticide was observed to cause a reduction in intracellular glutathione content. All the studied plant extracts counteracted rotenone-induced glutathione depletion. The methanol extract of *Z. capense* (25 µg/mL) and ethyl acetate extract of *S. puniceus* (25 µg/mL) were the most potent of the plant extracts at increasing intracellular glutathione levels.

Excessive fluctuations in MMP which is indicative of mitochondrial dysfunction, was depolarized by rotenone. This effect was not significantly counteracted by plant extracts. Instead methanol extracts of *L. schweinfurthii* and *Z. capense* further depolarized MMP in these cells, suggesting possible toxicants present in these extracts that could contribute in reducing MMP levels in the cells. It is also possible that the plant extracts stimulated cellular metabolism, which would also cause depolarization of the MMP.

Apoptotic cell death is known to be up-regulated in neurodegeneration. Rotenone-induced caspase-3 activity was counteracted by the plant extracts. Moreover, *Z. capense*, *S. puniceus* and *C. bulbispermum* were observed to possess anti-apoptotic properties comparable to the positive control, minocycline. All four plants showed potential in restoring the intracellular glutathione content and anti-apoptotic effects against rotenone-induced toxicity, making them possible agents in developing therapies for treating PD.
References


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225. Swarnkar S, Singh S, Mathur R, Patro IK, Nath C. A study to correlate rotenone induced biochemical changes and cerebral damage in brain areas with neuromuscular coordination in rats. Toxicology. 2010a;272(1-3):17-22.


Appendix A: Chemicals/ Reagents

AAPH

2,2'-azobis-2-methyl-propanimidamide dihydrochloride, ROS-inducing agent, was purchased from Sigma Aldrich (Darmstadt, Germany).

Acetic acid

Acetic acid was purchased from Merck Chemicals (Wadeville, RSA).

Caspase-3

Caspase-3 substrate was obtained in powder form from Sigma Aldrich (Darmstadt, Germany).

CHAPS

3-[(3-cholamidopropyl)dimethylammonio]-1propanesulfonate, lysing reagent, was purchased from Sigma Aldrich (Darmstadt, Germany), and was obtained in powder form.

Culturing consumables

96-well transparent and white plates suitable for fluorescence were supplied by Nunc (Denmark).
DCF-DA

2',7'-dichlorofluorescein diacetate, fluorescent dye was purchased from Sigma Aldrich (Darmstadt, Germany).

DMSO

Dimethylsulfoxide was obtained from Merck Chemicals (Wadeville, RSA).

EDTA

Ethylenediaminetetraacetic acid, in its powder form, was purchased from Sigma Aldrich (Darmstadt, Germany).

Ethyl acetate

Ethyl acetate was supplied by Merck Chemicals (Wadeville, RSA).

FCS

Fetal calf serum was purchased from Highveld Biological, Johannesburg, and heat-inactivated before use at 56°C for 45 min.

Ham’s F12

Ham’s F12 medium was obtained from Sigma Aldrich (St Louis, USA) in powder form. A mass of 55.5 g of Ham’s F12 powdered medium was weighed and dissolved in 5 L sterile
deionized water. A mass of 11 g sodium bicarbonate was then added to the solution to adjust the pH. The solution was then filtered twice using sterile cellulose acetate filters (0.22 µM) then transferred into sterile 500 mL bottles, enriched with 1% penicillin/streptomycin and stored at 4°C. The medium was further enriched with 2% (v/v) FCS before use.

**HEPES**

4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, in its powder form was purchased from Sigma Aldrich (Darmstadt, Germany).

**JC-1**

5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide, fluorescent dye was purchased from Sigma Aldrich (Darmstadt, Germany).

**Methanol**

Methanol was purchased from Merck Chemicals (Wadeville, RSA).

**Minocycline**

Minocycline was obtained from Sigma Aldrich (Darmstadt, Germany).

**MCB**

Monochlorobimane was purchased from Sigma Aldrich (Darmstadt, Germany).
NEM

N-ethylmaleimide was purchased from Sigma Aldrich (Darmstadt, Germany).

PBS

Phosphate buffered saline was purchased from Becton and Dickinson Company (Sparks, USA), in powder form. A mass of 9.23 g was weighed and dissolved in 1 L deionized water. The solution was then filter sterilized and stored at 4°C before use.

Penicillin/streptomycin

Penicillin-streptomycin solution containing 10 000 U penicillin and 10 000 µg streptomycin, was procured from BioWhittaker (Walkersville, USA).

PMSF

Phenylmethylsulfonyl fluoride was purchased from Sigma Aldrich (Darmstadt, Germany), in powder form.

Rotenone

Rotenone was purchased from Sigma Aldrich (Darmstadt, Germany). Rotenone stock solution was prepared by dissolving 11.832 mg of the pesticide in 1 mL of DMSO (30 mM). The solution was vortexed and 20 µL aliquots were stored at -70°C until use.
SH-SY5Y

SH-SY5Y neuroblastoma cell line was purchased from the American tissue culture collection (ATCC no.CRL-2266) and was used as an in vitro model in the present study.

SRB

Sulforhodamine B was obtained from Sigma Aldrich (Darmstadt, Germany).

Staurosporine

Staurosporine was purchased from Sigma Aldrich (Darmstadt, Germany).

TCA

Trichloroacetic acid was purchased from Merck Chemicals (Wadeville, RSA).

Tris base

Tris base was purchased from Merck Chemicals (Darmstadt, Germany).

Trypan blue

Trypan blue for cell counting was obtained from BDH Laboratory Supplies (England).
Valinomycin

Valinomycin was purchased from Sigma Aldrich (Darmstadt, Germany).
Appendix B: Equipment

Plant material was ground using a Wiley Mill (Mode No.2, Philadelphia U.S.A). Plant extracts were dried using a rotary vacuum evaporator BUCHI Rotavapor R-200, LABOTEC.

A Beckman Coulter Allegra X22 centrifuge was used to spin cells while Reichert-Jung microscope was used for cell counting.

Spectrophotometrical readings were obtained using an ELX 800 UV Universal plate reader while fluorescence readings were obtained using BMG FluostarOptima.
**Appendix C: Ethical Approval**

The Research Ethics Committee, Faculty of Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.

- **FWA** 00002567, Approved dd 22 May 2002 and Expires 20 Oct 2016.
- **IRB** 0000 2235 IORG0001762, Approved dd 13/04/2011 and Expires 13/04/2014.

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### The use of a commercially purchased cell line was approved on 26/09/2012 by a properly constituted meeting of the Ethics Committee subject to the following conditions:

1. The approval is valid for 5 years period [till the end of December 2017], and
2. The approval is conditional on the receipt of 6 monthly written Progress Reports, and
3. The approval is conditional on the research being conducted as stipulated by the details of the documents submitted to and approved by the Committee. In the event that a need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

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### Members of the Research Ethics Committee:

- **Prof M J Bester**
- **Prof R Delport**
- **Dr NK Likibi**
- **Dr MP Mathebula**
- **Prof A Nienaber**
- **Mrs MC Nzeku**
- **Prof L M Ndle**
- **Snr Sr J Piastoli**
- **Dr R Reinders**
- **Dr T Ronsouw**
- **Dr L Schoeman**

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BSc(Health Promotion)/Postgraduate Dip (Health Promotion) – Community representative

Dr R Sommers

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Prof TJP Swart

BChD, MSc (Odont), MChD (Oral Path), PGCHE – School of Dentistry representative

Prof C W van Staden

MBChB, MMed (Psych); MD, FCPsych; FTCIL; UPLM - Chairperson

DR R SOMMERS; MBChB, MMed(Inf), MPHarmMed.
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