Evaluation of the therapeutic potential of Green rooibos (*Aspalathus linearis*) extract in neurological disease

by

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Declaration

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Abstract

Background: The intricate relationship between oxidative stress and neuroinflammation has been demonstrated in the aetiology of most neurodegenerative diseases. Current pharmacological treatments of these diseases have limitations and side effects. This has led to extensive investigations of novel therapeutic approaches to modify the course of these conditions. Globally, phytochemicals, especially polyphenols, have been investigated *in vitro* and *in vivo* for their antioxidant and anti-inflammatory properties. *Aspalathus linearis* (Rooibos) is one such plant. Although unfermented (green) rooibos is well known for its antioxidant capacity, no information is available on its neuroprotective capacity.

Aim: This study aimed to establish the neuroprotection potential of unfermented rooibos extract in neuronal cells and astrocytes. In addition, an evaluation of the antioxidant and anti-inflammatory capacity, as well as potential mechanism of action of rooibos in neuroprotection was done *in vivo* in a zebrafish larval model.

Method: Antioxidant properties of unfermented rooibos were quantified using cell-free systems (DPPH, ORAC and xanthine/xanthine oxidase). Green rooibos extract (Green oxithin^{TM}) neuroprotective capacity was assessed in mouse neuroblastoma (Neuro-2a) cells exposed to different concentrations of hydrogen peroxide (250 or 125 µM) after a 24-hour treatment with different rooibos extract concentrations (12.5, 25, 50 and 100 µg/mL), as well as in human astrocytes treated with varying concentrations of rooibos extract (800, 400, 200, 100 and 50 µg/ml) and exposed to 20 µg/mL of *E.coli* lipopolysaccharide (LPS). MCP-1 secretion by astrocytes were determined by ELISA and XTT assay was used to assess cell viability. Intracellular ROS was quantified as a parameter of cellular redox status and MAO-A inhibition as further indicators of neuroprotection. Moreover, an *in vivo* PTZ-induced seizure model was employed to probe GABA receptor modulation as potential mechanism of action of rooibos.

Results: Green rooibos scavenged free radicals of both exogenous and endogenous origin in a dose dependent manner with a similar profile activity to vitamins C or E. Green rooibos extract protected N2a cells, predominantly at the assault of a lethal concentration (250 µM) of H_2O_2 where up to 15% ($p<0.0001$) cell survival was observed. Intracellular ROS levels were significantly reduced ($p<0.0001$) at both 250 and 125 µM H_2O_2. In a dose dependent way, MCP-1 production decreased significantly in cells pre-treated with rooibos ($p<0.0024$). Finally, current data

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generated in zebrafish do not support a GABA receptor-associated mechanism of action rooibos.

Conclusion: Green rooibos extract modulated the production of MCP-1 by astrocytes and mildly inhibited MAO-A.

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Opsomming

Agtergrond: Die komplekse verhouding tussen oksidatiewe stress en neuroinflammasie word geïllustreer deur die etiologie van meeste neurodegeneratiewe siektes. Huidige farmakologiese behandeling van hierdie siektes het beperkings en newe-effekte. Daarom word uitgebreide ondersoeke gedoen na nuwe terapeutiese aanslae om hierdie toestande aan te spreek. Fitochemikalieë en veral polifenole word wyd ondersoek, beide in vitro en in vivo, vir hul anti-oksidant en anti-inflammatoriese effekte. *Aspalathus linearis* (rooibos) is een so ‘n plant. Alhoewel ongegisde (groen) rooibos bekend is vir sy anti-oksidant aktiwiteit, is geen inligting bekend oor die neurobeskermende kapasiteit van rooibos nie.

Doel: Ons doel was om die neurobeskermende eienskappe van groen rooibos te bepaal, in neurone en astrosiete. Die anti-oksidant en ensiem inhiberende eienskappe van rooibos is ook ondersoek. Laastens is ‘n evaluering van ‘n moontlike meganisme van aksie van rooibos in ‘n *in vivo* zebravis larwe model gedoen.

Metodes: Anti-oksidant effek van groen rooibos is gekwantifiseer deur gebruik te maak van selvrye sisteme (DPPH, ORAC en xanthine/xanthine oksidase). Die neurobeskermende effek van groen rooibosekstrak (Green oxithin^{TM}) is bepaal in muis neuroblastoomselle (Neuro-2a), deur die selle bloot te stel aan 250 of 125 µM waterstofperoksied, na ‘n 24-uur kondisionering met verskillende rooibosekstrakkonsentrasies (12.5, 25, 50 en 100 µg/mL). Anti-inflammatoriese effekte van rooibos is ook bepaal in menslike astrosiete wat behandel is met verskillende konsentrasies van rooibos (800, 400, 200, 100 en 50 µg/ml) asook 20 µg/mL E.coli lipopolisakkaried (LPS). MCP-1 sekresie is bepaal met ELISA. Sel oorlewing (XTT) en intersellulêre RSS vlakke is ook bepaal, asook die vermoë van rooibos om MAO-A te inhibeer. Laastens in ‘n *in vivo* zebravis PTZ model gebuik om te ondersoek of die kalmerende effekte van rooibos via GABA reseptors plaasvind.

Resultate: Groen rooibos het vrye radikale, beide eksogeen en endogen in oorsprong – op ‘n dosis-afhanklike manier geabsorbeer, soortgelyk aan die effek van vitamien C en E. In die teenwoordigheid van 250 µM H_2O_2 het groen rooibos effektief seldood verminder ($p<0.0001$), terwyl intrasellulêre RSS verlaag ($p<0.0001$) is by beide 250 en 125 µM H_2O_2. MCP-1 sekresie deur astrosiete was ook beduidend laer ($p<0.0024$) in selle wat met rooibos behandel is. Laastens het data in zebravisse nie op ‘n GABA reseptor-geassosieerde meganisme van rooibos gedui nie.

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Gevolgtrekking: Huidige data suggereer dat groen rooibos wel ‘n neurobeskermende rol kan speel via die modulering van MCP-1 produksie en die inperking van MAO-A aktiwiteit.

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Contents

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List of Acronyms & Abbreviations

A. linearis	*Aspalathus linearis*
AD	Alzheimer's disease
ATP	Adenosine Triphosphate
BBB	Blood Brain Barrier
BDNF	Brain Derived Neurotrophic Factor
CNS	Central Nervous System
COX	Cyclooxygenases
CSF	Cerebral Spinal Fluid
DMEM	Dulbecco's Modified Eagle's medium
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's phosphate buffered saline
DPPH	2,2-diphenylpicrylhydrazyl EGCG: Epigallocatechin gallate
E. coli	*Escherichia coli*
eNOS	Endothelial Nitric oxide synthase
FBS	Foetal Bovine Serum
GABAA	γ-aminobutyric acid type A
GABAA R	GABAA Receptor
GFAP	Glial Fibrillary Acidic Protein
GSH	Glutathione
GSSG	Glutathione disulfide-reduced form
GST	Glutathione-S-transferase
H_2O_2	Hydrogen peroxide
HPLC	High Performance Liquid Chromatography

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IFN-γ	Interferon gamma
IL	Interleukin
IL-1α	Interleukin 1 alpha
IL-1β	Interleukin 1 beta
IL-6	Interleukin 6
IL-8	Interleukin 8
iNOS	Inducible nitric oxide synthase
LPS	Lipopolysaccharide
MAPK	Mitogen activated protein kinase
MBN	Methyl benzyl nitrosamine
MCP-1	Monocyte Chemoattractant Protein 1
mg	Milligram
MHC II	Major Histocompatibility Complex II
mL	Millilitre
mM	Millimolar
MOA	Monoamine Oxidase
MS	Multiple Sclerosis
NADPH	Nicotinamide adenine dinucleotide phosphate-reduced form
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
ng	Nanogram
NGF	Nerve Growth Factor
NLRs	NOD-like receptors
NMDA	N-methyl-D-aspartate
NMDAR	NMDA Receptor

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NO	Nitric oxide
P450	Cytochrome P450 enzyme
PD	Parkinson’s disease
PI3/Akt	Phosphatidylinositol-4,5-bisphosphate 3-kinase/Protein Kinase B
ROS	Reactive Oxygen Species
RPM	Revolutions per minute
TNF	Tumour Necrosis Factor
µg	Microgram
µL	Microlitre
µM	Micromolar

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List of Figures

Stellenbosch University https://scholar.sun.ac.za

List of Tables

Stellenbosch University https://scholar.sun.ac.za

Chapter 1 – Introduction

Since time immemorial, plants and their derivatives have been known to possess biological activity. According to the latest World Health Organisation (WHO) report on traditional and complementary medicine (T&CM), 88% of member states (170 countries) have acknowledged use of T&CM (World Health Organization 2019). Furthermore, traditional, and complementary medicine are regarded as having a significant contribution to the WHO sustainable development goal 3 (SDG3) - *ensuring healthy lives and promoting well-being for all at all ages.* In addition, Kennedy & Wightman (2011) estimated that half of all licensed drugs that were registered worldwide in the 25-year period prior to 2007 were natural products or their synthetic derivatives. The launch of the Traditional Medicine Strategy: 2014-2023 by WHO has further heightened the global shift towards natural products (Smith & Swart 2018). Thus, numerous scientific studies aimed at harnessing both the nutritional and biological properties of plants have been carried out (Jabeur *et al.*, 2019)

The bioactive properties of plants demonstrated by a huge amount of scientific literature has been associated with several classes of phytochemical compounds such as phenolic compounds, vitamins, alkaloids, terpenoids, and natural pigments(Ahmed *et al*., 2017; Barba *et al*., 2017; Gollo *et al*., 2020). Furthermore, Ajuwon *et al*., (2015) reported that attention is being focused mostly on phenolic compounds which are considered potent antioxidant and anti-inflammatory compounds, even though their modes of action are still not fully understood. Garg *et al.*, (2019) reported that more than 8000 polyphenols have been identified in various plants and have been grouped into severally types, namely, phenolic acids, flavonoids, flavanols, flavones, flavanones, anthocyanidins, lignans and stilbenes. Flavonoids make up about 60% of the known polyphenols. (Bueno *et al.*, 2012)

Flavonoids have been found to possess strong antioxidant, anti-atherosclerotic, anti-inflammatory, antimutagenic, antitumor and antiviral activities (McKay & Blumberg 2007). In addition, they have been demonstrated to act as neuroprotectants. Some of the mechanisms of neuroprotection include targeting key proteins involved in Alzheimer's diseases and acting against beta-amyloid toxicity (Bastianetto *et al.,*

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2006). Furthermore they have been documented as inducers of cytoprotective proteins; interacting with neurotransmitter systems such as adenosine (Alexander 2006), dopamine, GABA (Hanrahan et al., 2015; Saaby 2011; Moghbelinejad et al., 2017) and glycine that may be involved in neuroprotection. The anti-inflammatory property of flavonoids has been extensively studied in the context of neurodegenerative diseases. (Spagnuolo *et al*., 2018; Wang *et al.*, 2016; Jaeger *et al., 2018*; Dourado *et al.*, 2020; Iriti *et al.*, 2010; Solanki et al., 2015; Li, *et al*., 2020).

Neurodegeneration is the slow, progressive and irreversible loss of neurons in specified regions of the brain and spinal cord resulting in physical and mental impairments (Spagnuolo *et al*., 2018; Dourado *et al*., 2020; Sucheck *et al*., 2020). It is the main pathologic characteristic of many neurodegenerative diseases such as Alzheimer's disease (AD); multiple sclerosis (MS), Parkinson disease (PD), Huntington disease (HD), amyotrophic lateral sclerosis (ALS). The main causes of these diseases is multifactorial making it difficult to place a finger on a single causative agent. Sucheck *et al*., (2020) revealed some of the cellular hallmarks that are a common feature in these diseases: inflammation, oxidative stress, excitotoxicity, mitochondrial dysfunction, and chronic activation of the innate immune cells within the CNS. While admitting to the complex mechanisms involved in aging, Petersen & Smith, (2016) suggested that it is one of the conditions directly associated with neurodegenerative diseases (NDs) via cumulative oxidative stress and chronic inflammation. Furthermore, neuroinflammation has been well documented as pivotal in neurodegeneration (Erickson *et al.*, 2012; Hyochol Ahn 2017; Guzman-Martinez *et al*., 2019; Jha *et al*., 2012; Kempuraj *et al*., 2017; Luca *et al*., 2018; Müller, 2018; Ramana *et al*., 2006; Ransohoff *et al*., 2015; Reardon, 2018; Streit *et al*., 2004; Yang & Zhou, 2019)

Currently, the drugs available for the treatment of NDs only treat the symptoms rather than preventing or curing neurodegeneration (Akhtar *et al*., 2020; Hossain *et al*., 2019; Kabir *et al*., 2019; Rowinska-Zyrek *et al*., 2015). Furthermore, Fremova *et al*., (2017) opined the few number of drugs available that have the capability to reach levels of therapeutic efficacy within the brain and be tolerated for prolonged treatment periods. There is therefore still a great need for effective therapeutic agents to treat NDs. In this regard it is important to develop more and novel therapeutic models for curbing

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NDs. As already mentioned, the WHO has since strongly recognised the role played by natural medicines. Moreover, the modern consumer has a bias for neutraceuticals. This trend has also been ostensibly observed with the advent of Covid-19 where trials for such plants as the *Artemisia* species are being done and scientific opinions on herbal medicine as a cure for Covid-19 have already been published (Asdadi *et al.*, 2020; Haq *et al.*, 2020; Jahan and Rahmatullah, 2020; Kapepula *et al.*, 2020; Law, Leung and Xu 2020; Pandey *et al.*, 2020; Robertson and Sc 2020). Given such a bias for natural medicine, this study focussed on the neuroprotective effects of green rooibos extract in the context of neurodegenerative diseases.

Rooibos tea, scientifically known as *Aspalathus linearis* (Brum f) Dahlg. (Family Fabaceae; Tribe Crotolarieae) is a shrubby legume that is indigenous to the mountainous area of Clanwilliam in the Western Cape Province of South Africa (Ajuwon *et al.*, 2013). It is low in tannins and alkaloids- and is famously known to be caffeine-free (Canda *et al*., 2014) and has been traditionally used as herbal tea by the Khoi-San people in the Cape region (Van Wyk and Gorelik 2017). By 2015 its production had reached more than 2,000 metric tonnes, serving both local and increasing international market demand (Ajuwon *et al*., 2015). The potency of rooibos lies in its unique polyphenolic compounds. Smith & Swart, (2018) reported that rooibos is richly endowed with such polyphenolic compounds as flavones, flavonols, monomeric and oligomeric flavan-3-ols, lignans, hydroxycinnamic acid and derivatives, phenolic carboxylic acids, dihydrochalcones and flavanones.

Furthermore, green rooibos has been demonstrated to be rich in the unique dihydrochalcone glucoside, aspalathin, which is oxidized to the flavanones dihydro-iso-orientin and dihydroorientin during fermentation. It is also laden with the cyclic dihydrochalcone, aspalalinin; the rare 3-dehydroxy dihydrochalcone glucoside, nothofagin; the C-glycosyl flavones orientin, isoorientin, vitexin, and isovitexin; and the flavones hemiphlorin and chrysoeriol, luteolin and luteolin-7-O-glucoside (Marnewick, 2014). Numerous studies on the efficacy of rooibos as a nutraceutical have been done (Mckay and Blumberg 2007; Breiter *et al.*, 2011; Marnewick *et al.*, 2011; Ajuwon *et al.*, 2013; Ajuwon, Oguntibeju and Marnewick, 2014; Canda, Oguntibeju and Marnewick, 2014; Herbst, Ed and Cur, 2014; Hong, Lee and Kim 2014; Ajuwon *et al.*, 2015; Van der Merwe *et al.*, 2015; Smith and Swart 2016, 2018; Van Wyk and Gorelik 2017;

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Akinrinmade *et al.*, 2017; Hostetler *et al.*, 2017; Sasaki, Nishida *et al*., 2018; Smith 2018; Piek *et al.*, 2019), however, there is paucity of information regarding the neuroprotective capacity of green rooibos.

The aim of this study therefore was to establish the bioactive properties of green rooibos in terms of cytoprotective activity in neuronal cells and in astrocytes as well as antioxidant and enzyme inhibitory properties that could explain observed effects in the cells. Furthermore, the study aimed at probing a potential mechanism of action of rooibos in neuroprotection using an *in vivo* zebrafish model.

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Chapter 2 – Literature Review

Neurodegenerative diseases have been inextricably linked to neuroinflammation and the dysregulation of the CNS homeostasis. Because neuroinflammation has a negative connotation and is viewed as inherently deleterious and maladaptive, a substantial amount of research has focussed on its pathological aspects. This chapter will therefore start with a discussion on neuroinflammation, the main glial cells involved and its link with oxidative stress. Following which, a review on antioxidants both endogenous and dietary will be done. Additionally, the flavonoids which are the main constituents of rooibos will be reviewed in the context of neuroinflammation. Moreover, rooibos, the main subject matter of the present study will be discussed in the context of its demonstrated therapeutic efficacies.

Because of the rise of zebrafish in studies such as toxicology, neurobiology, regenerative medicine, and oncology; the efficacy of the zebrafish model will be reviewed in the context of the present study. The point of departure for this literature review will be a review of the literature on neuroinflammation.

2.0. Neuroinflammation

Inflammation is generally defined as a physiological response, involving innate immunity, following exposure to various insults such as tissue injury, microbial pathogen infection, chemical toxins, or ultraviolet light radiation. (Bengmark 2004). It can either be acute or chronic. On one hand acute inflammation involves the immediate and early response to an insult and constitutes a defensive response that would necessarily lead to the repair of the damaged site. (Streit *et al.,* 2004). On the other hand, chronic inflammation is a result of persistent stimuli.

The focus of this discussion is on neuroinflammation which is not too different from general inflammation except that it occurs within the brain or spinal cord (DiSabato *et al.,* 2016). The uniqueness and perhaps the enigmatic part about neuroinflammation is its inimitable cell types and their communication mechanisms. This has subsequently led to substantial neuroinflammation research interest in recent years. (Yang and Zhou 2018). In addition, emerging evidence suggests strong positive correlation between neuroinflammation and the pathogenesis of many neurodegenerative diseases (Morales *et al*., 2016)

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Neuroinflammation can be more specifically defined as an inherent host-defence mechanism to protect and restore the normal structure and function of the brain against infection and injury. (Kempuraj *et al*., 2017)

As Norbert, (2018) further explains, inflammation is a necessary response to infection, harmful chemicals and tissue damage. The process thereof is very complex, involving signalling cascades, activation of transcription factors, increase in inflammatory enzymes, release of various oxidants and pro-inflammatory molecules. (Bengmark 2004). Furthermore, inflammation is mediated by the production of cytokines, chemokines, reactive oxygen species and secondary messengers (DiSabato *et al*., 2016). These mediators are released by resident CNS glia (microglia and astrocytes), endothelial cells, and peripherally derived immune cells. There are immune, physiological, biochemical, and psychological consequences of these neuroinflammatory responses.

The consequences of neuroinflammation are a strange case of Jekyll and Hyde. On the one hand neuroinflammation induces and/or aggravates neurodegeneration in the central nervous system (CNS), while on the other hand, it favours the recovery of the injured neurons (Kempuraj *et al.,* 2017). Therefore the consequences of neuroinflamation can be said to be neuroprotective or neurotoxic (Norbert 2018). Additionally, these contrasting effects depend on interactions between environmental factors and various components of the inflammatory response of which genetic variation plays a major role in the latter. Alagan *et al*., (2019) concurred with many researchers (Radtke *et al*., 2017; Kempuraj *et al*., 2016; Wood 2003;) on the fact that neuroinflamation is the major instigator of neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), and multiple sclerosis (MS) and of neuropsychiatric disorders such as major depressive disorder, schizophrenia, and bipolar disorder. To treat or manage these diseases, Wood, (2003) revealed that, microglia have been identified for pharmacological approaches to halt or prevent slowly progressing neurodegenerative diseases.

According to Guzman-Martinez and coworkers, (2019), microglia do not act in solitude in the inflamatory and neuro-immunomodulatory process. They regulate the innate immune functions of astrocytes. This induces the transduction of intracellular signals of the astrocytes. Thereafter a cascade of reactions occur, culminating in the

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permiability of the Blood Brain Barrier (BBB) and its subsequent breaching by the immune cells in the brain parenchyma (Guzman-Martinez *et al*., 2019). Moreover the simple innate immune response becomes amplified. It therefore seems logical to first understand the actions of microglia and astrocytes in order to treat neurodegenerative diseases. Furthermore, potential drugs should aim at inhibiting, with minimal side effects, the exaggerated neuroinflammation that is observed in all these diseases. In the next section, focus will be placed on what is already known about the glial cells and their role in neuroinflammation.

2.1. Glial cells and neuroinflamation

“Glia”, (Greek: Glue) refers to the non-neuronal cells of the nervous system found in the peripheral (Schwann cells, satelite glia, perineural glia) and central (astrocytes, oligodendrites, microglia and perivascular glia) nervous system. (Jha *et al*., 2012). Glial cells can make up as much as 70% of the total cell population in the brain and spinal cord, yet it is only in recent years that their importance in physiological and pathophysiological processes has been appreciated. Furthermore, many researchers now refer to the ‘neuronal-glial complex’ as the structural and functional unit of the brain, rather than just the neuron (Jha *et al*., 2012).

2.1.1. The Major Players: Microglia

Among all the glial cells the role of microglia in initiating or promoting inflammatory processes has been well documented (DiSabato *et al*., 2016; Zhang and An 2007; Ransohoff *et al*., 2015; Streit et al., 2004; Takeuchi, 2013; Sochocka *et al*., 2016). It is therefore imperative to make them the focal point for any discussion of neuroinflammation.

Microglia comprise of 10-20% of the glial CNS cell population (Wood 2003; DiSabato *et al*., 2016). They develop from myeloid precursor cells in the embryonic yolk sac during embryogenesis. According to DiSabato *et al*., (2016), the microglia then move to the area of the CNS around day 8.5. Furthermore, they are said to be long-lived cells that have limited turnover from the myeloid cells of the bone marrow over the course of a lifetime (DiSabato *et al*., 2016). However, DiSabato *et al*., (2016) asserted that when depleted, microglia can still be produced by a progenitor source within the CNS although the rate of this turnover is still unclear and appears to be very low. Such

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a low turnover rate would essentially expose them to pro-inflammatory effects of ageing, injury or stress.

The functions of microglia include acting the same way as macrophages in dealing with invasions by infectious agents and tumors. Furthermore, two photon imaging studies have shown microglia using their processes to actively survey their micro environment (Cianciulli *et al.*, 2020). Microglia normally present a ramified shape with elongated pseudopodia and suppressed genomic activity. However, when stimulated, they downregulate surface-bound keratan sulphate proteoglycans and assume an amoeboid shape. (Wood 2003). Furthermore, in this state, genomic upregulation is initiated and results in the production of potentially neurotoxic mediators. (Wood 2003). Additionally, these mediators activate astrocytes, another group of glial cells involved in neuroinflammation. Together microglia and astrocytes produce several signaling molecules, thereby setting up an autocrine feedback system (Guzman-Martinez *et al*., 2019).

When the glial cells (mainly microglia and astrocytes) are activated, they regulate the expression of different surface markers, such as the molecular pattern recognition receptors (PRRs), major histocompatibility complex II (MHC-II). This subsequently results in the production of cytokine proinflammatory drugs such as Interleukin (IL) 1 beta (IL-1β), IL-6; il-12; Interferon (IFN), gamma (IFN-γ), and tumor necrosis factor (TNF) alpha (TNF-α) (Guzman-Martinez *et al*., 2019). These cytokines have been observed to have an effect on the brain endothelial cells leading to either the disruption or modulation of the restrictive properties of the blood brain barrier. (Erickson *et al*., 2012). In addition, the actin cytoskeleton and tight junction expressions are altered in the process. Moreover, many of these cytokines are able to cross the BBB, thereby enabling a direct link between the circulating and CNS compartments of the immune system (Erickson *et al*., 2012). Figure 2.1 shows how the BBB is breached during neuroinflammation.

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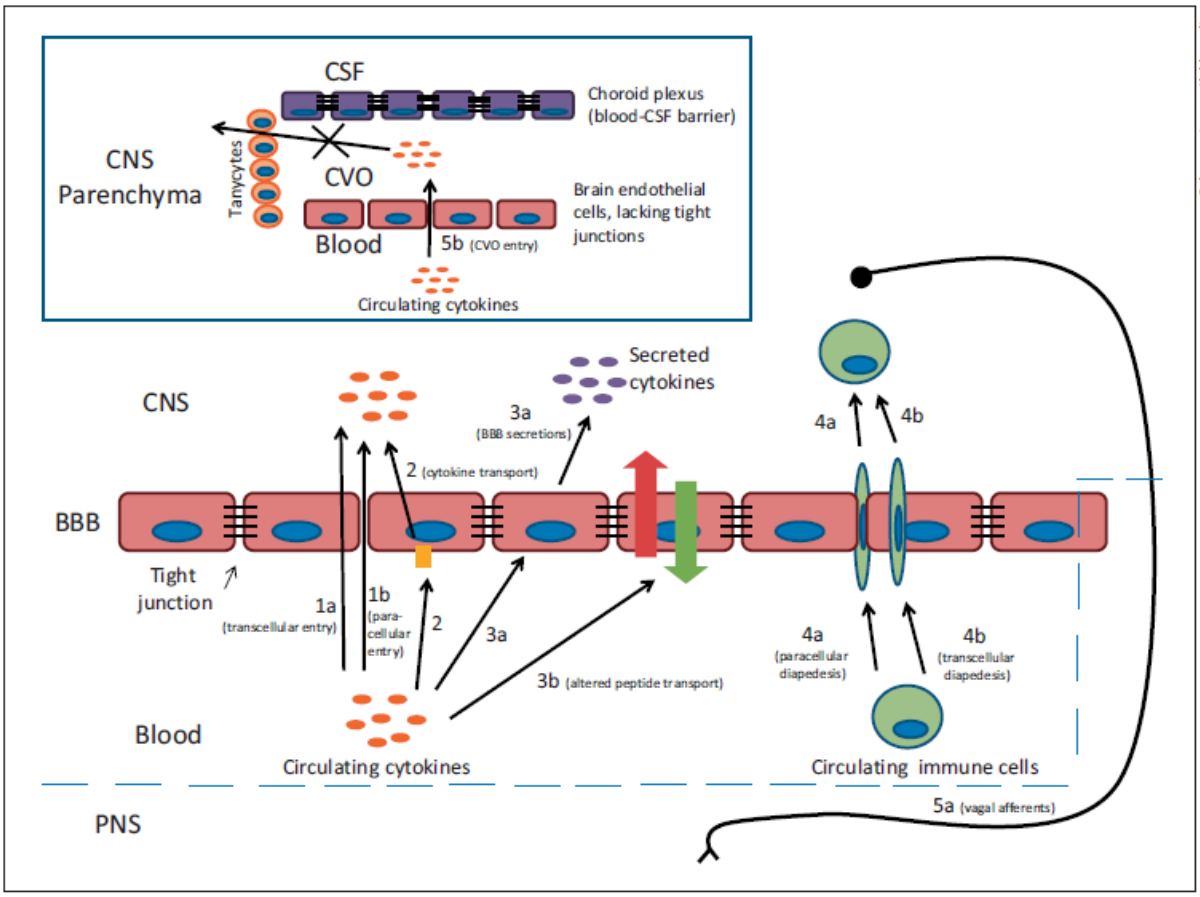

Figure 2-1 An endocentric view of neuroinflammation (Erickson et al, 2012)

In addition to the the production of cytokines during neuroinflammation, cellular adhesion molecules, chemokines (e.g. Monocyte chemoattractant protein-1, MCP-1), reactive oxygen species and surface antigens are also produced, thereby further enhancing the CNS immune cascade (Jha *et al.*, 2012). Under homeostatic circumstances, the activation of microglial and the increased expression of cytokines are intended to protect the CNS and benefit the host organism (Reardon 2018). The neurotoxicity of mediators produced during neuroinflammation are normally buffered by a diverse array of inactivation and cytoprotective measures (Zhang *et al.*, 2007). For example the anti-inflammatory cytokines (IL-4; IL-10; IL-11; and IL-13) act antagonistically to the proinflammatory cytokines. However, amplified, exaggerated or chronic activation of microglia will potentially lead to serious pathological changes. (Guzman-Martinez *et al.*, 2019)

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2.1.2. The Microglial neuroinflammation process

Microglial cells are implicated in the whole cascade of reactions culminating into neuroinflammation. As already stated, they have the ability to sense microenvironmental changes within the brain that are caused by pathogens, injuries or chemicals. Due to these insults, microglia become activated . Aguilera *et al*., (2018) described this as a process through which they rapidly change their resting ramified morphology to an amoeboid shape. With such a phenotype microglia are enabled to proliferate and migrate to the lesioned site where they phargocytise and destroy pathogens while removing damaged cells. (Lawson *et al*., 1990).

Furthermore, microglia can be activated by subtle changes in intracellular ion influx/efflux balance or structural arrangement. Even extracellular ion changes are a stimulus to microglia as they possess purinergic, cholinergic and adrenergic receptors (Kreutzberg 1996).

Microglial activation

In addition to monitoring the homeostasis of the CNS environment, glial cells also act as resident innate immune cells. Yang and Zhou (2018) revealed that it is microglial cells that are always activated before any other glial cells while astrocytes, although the most abundant appear passive. The recognition of pathogens and other deleterious molecules in the CNS is mediated by pattern recognition receptors (PRRs).

The PRRs recognise two types of molecules: 1) pathogen-associated molecular patterns (PAMPS) which are microbial biproducts and 2) damage- associated molecular patterns (DAMPS) which are molecules associated with cell components or proteins that are released during cell damage or death (Frank *et al*., 2016; Czirr & Wyss-Coray 2012; Cunningham 2013; Morales *et al*., 2016; Yang and Zhou 2018)). Such damage or cell death is caused by an onset of traumatic conditions or interaction with some neurotransmitters such as glutamate, GABA and acetylcholine. (Tohidpour et al., 2017).

A family of receptors known as the toll-like receptors (TLRs) are the primary form of PRRs (Yang and Zhou 2018). TLRs are highly conserved membrane or cytosolic

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proteins. They act as signal transducers via a family of cytosolic toll adapter protein linking to downstream signaling cascades (Frank *et al*., 2016)

There are many of these TLRs which recognise PAMPS and DAMPS. For example , TLR2 recognises a spectrum of PAMPS originating from bacteria, viruses, fungi and parasites, while TLR3 engages double stranded RNAs. Furthermore, TLR4 is activated by Lipopolysaccaride (LPS) from gram-negative bacteria and endogenous DAMPS, such as high- mobility group box-1 (HMGB-1) heat shock proteins released from injured tissues(Laird *et al.*, 2014). In addition TLR 4 also engages accumulated mis-folded proteins, such as α-synuclein or prion.

The binding of TLR family with PAMPS or DAMPS results in the activation of microglia Frank *et al*., (2016) presented a mechanism for the priming of microglia for activation. They proposed that the HMBG-1, a ubiquitous nuclear DNA binding protein, is released into the intracellular millieu where it serves as an inflammatory signal mediated by TLR2 and TLR4. In addition, Yang and Zhou (2018) showed that the chemotactic function of HMGB-1 is mediated by the recepter for advanced glycation end products (RAGE) and the chemokine receptor CXCR4.

A cascade of reactions follow the TLR 4–HMGB-1 binding. The result being the formation and secretion of interleukin 1 beta (IL-1β), this is considered a critical step in the neuroinflammatory cascade. IL-1β is produced as a larger pro-hormone, pro IL-1β which is then cleaved by caspace–1 to give a biologically active mature form of IL-1β. An inflammasome called NLRP3 (nucleotide-binding domain and leucine rich repeat containing family, pyrin domain containing 3) mediates the maturation of IL-1β by activating caspace-1. In addition, the signaling at TLR4 initiates an intracellular signaling cascade to activate the immune related transcription factor NF-κB (nuclear factor of kappa light polypeptide gene enhancer in B-cells) which is key to the inflammatory effects of TLR ligation (Salminen *et al.*, 2008). Figure 2.2 summarises the complicated cascade of reactions in the activation of microglia.

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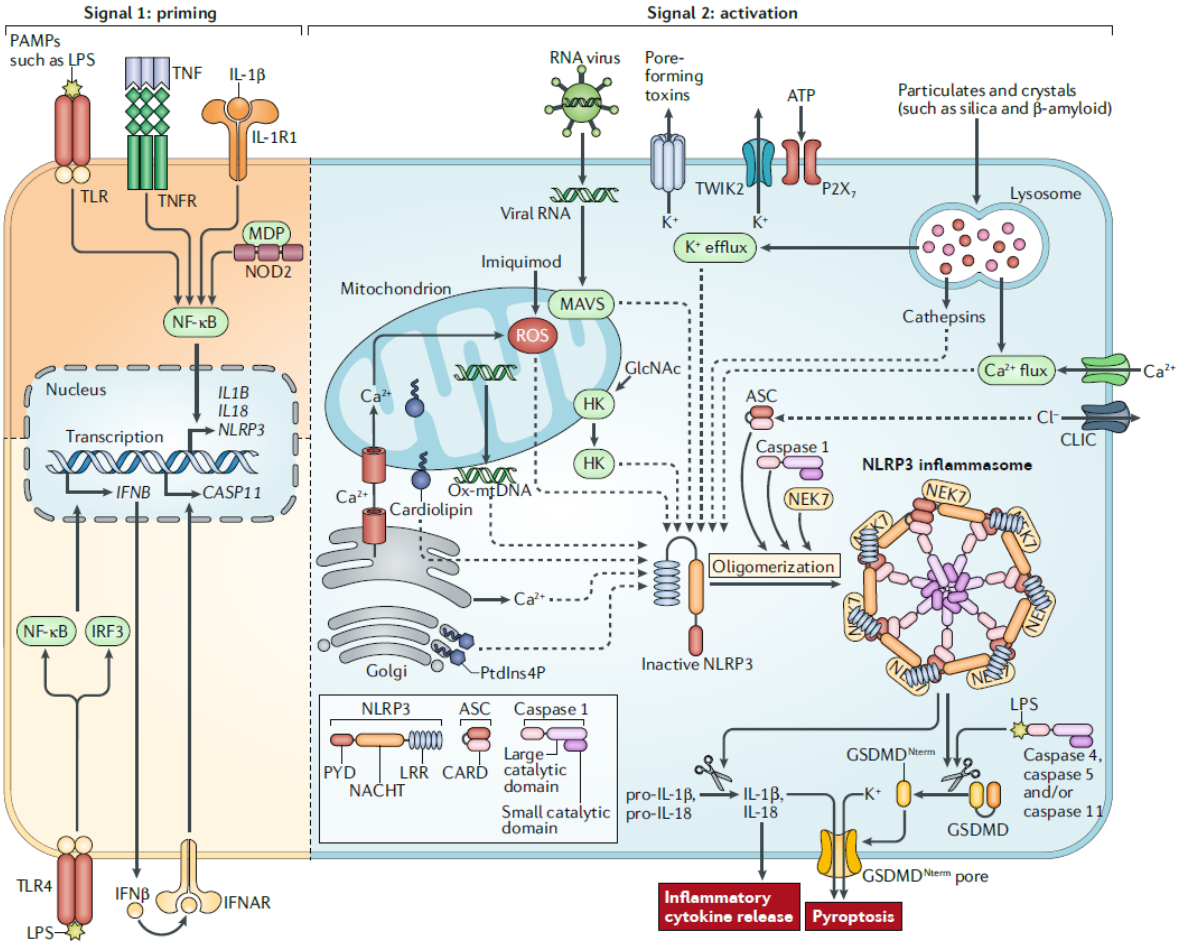

Figure 2-2. Summary of the cascade of reactions in the activation of microglia (Salminen et al., 2008)

According to Chang *et al*., (2009), when activated, microglia differentiate into three classes: 1) the resting state with many processes and small cell body size; 2) semi-activated state with thick and short processes and a cell body that is large; 3) the fully activated state with very thick and short processes and a very large amoeboid-like cell body. This can be seen in figure 2.3.

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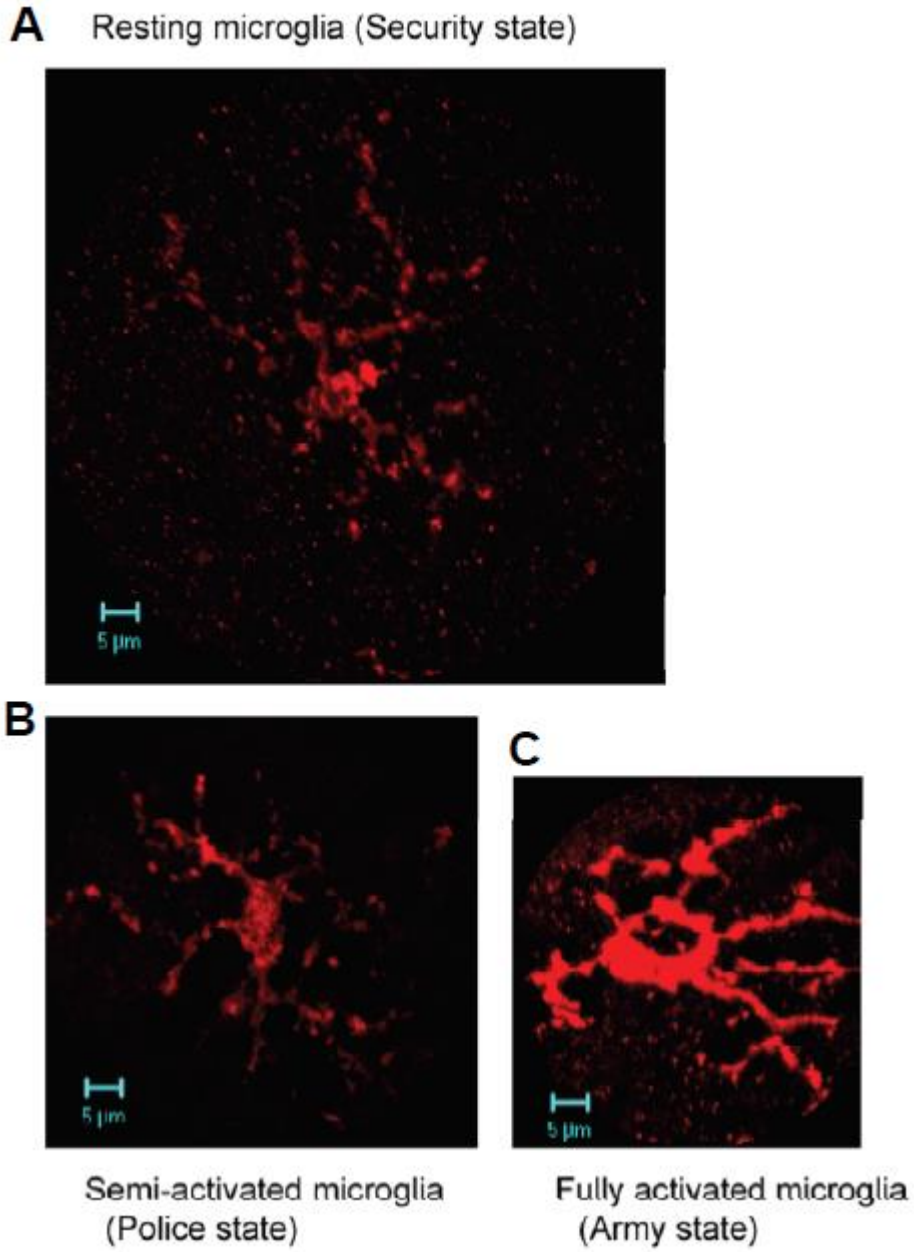

Figure 2-3 Activated microglia states: C resting state with many processes and small cell body size; B semi-activated state with thick and short processes and a cell body that is large; C the fully activated state with very thick and short processes (Chang et al, 2009).

Many other researchers, however have shown that microglia have a spectrum of activation morphologies but two are usually emphasized, that is, the neuroprotective or anti-inflammatory (M2) and the neurotoxic or proinflammatory (M1).

The M1 microglia secrete pro-inflammatory cytokines and chemokines (TNF-α; IL-6; IL-1β; IL-18; IL-23; INF-γ; NO and ROS) (Tohidpour *et al*., 2017; Lurie 2018; Yang and

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Zhou 2018). Furthermore the neurotoxic phenotype of microglia is characterised by the activation of purinergic receptors P2x7 subtype (activated by ATP), promoting inflamation and the destruction of cells through the formation of membrane pores (Tohidpour *et al*., 2017). In addition, cytokines (IL-1β, TNF-α and C1q) secreted by activated microglia induce toxic astrocytes which contribute to the death of neurons and oligodendrocytes and eventually breaching the blood brain barrier (Yang and Zhou 2018).

The pro-inflammatory microglia are not necessarily harmful. They are responsible for defending against invading pathogens and subsequently clear cellular debris to promote tissue repair and recovery of the CNS following injury or some other short term challenge. Aguilera and colleagues (Aguilera *et al.*, 2018) opined the upregulation of other molecules such as vascular cell adhesion molecule VCAM-1, lymphocyte function-associated antigen 1 (LFA-1), intracellular adhesion molecule (ICAM-1/CD54)- that comes as a result of microglial activation.

Microglia are not always pro-inflammatory. Their neuroprotective phynotype which occurs when they are semi-activated expresses anti-inflammatory cytokines (Interleukin 4, 10 and 13); cell growth factors (VEGF, NGF, PDGF, BDNF, TGF- β, GDNF) and exert anti-inflammatory effects ((Chang *et al.,* 2009; Yang and Zhou, 2018).

As has already been stated microglial activation is more on the grey scale instead of a simplistic black and white (M1/M2) phenomenon (Bachiller *et al.*, 2018). However the important question to ask is: what determines whether the inflammatory response will result in protective or neurodegenerative effect? Morales et al., (2016) reported that acute neuroinflammatory response is usually beneficial to the CNS. It minimises damage and helps repair damaged tissue. For example , microglial cells are able to remove glutamate, a well known neurotoxic substance which binds on the NMDA receptors on neurons and can lead to neuronal death (Morales *et al*., 2016). It is the continuous microglial stimulation that causes a chronic neuroinflammatory response

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which is almost always harmful and damaging to the neurons. In general the merits and demerits of neuroinflammation depend primarily on the length of the inflammatory response given by the microglial cells.

The role of MCP-1

Monocyte chemoattractant protein (CCL2) is well known for the regulation of the migration of microglia and that of monocytes, memory T lymphocytes, and natural killer (NK) cells and their infiltration through the BBB.(Deshmane *et al.*, 2009). It is among the most studied members of the chemokine family, and has been reported to be a potential therapeutic point of intervention for the treatment of various degenerative diseases such as multiple sclerosis (Sørensen *et al.*, 2004), rheumatoid arthritis (Hayashida *et al.*, 2001), atherosclerosis(Kusano *et al.*, 2004), and insulin-resistant diabetes (Panee 2012; Sartipy & Loskutoff, 2003). Different studies have shown that mitigation against production of MCP-1 or of its effects can be protective against different injuries or diseases (Hinojosa *et al.*, 2011). Anti-inflammatory drugs such as reboxetine (Gutiérrez *et al.*, 2018) which is marketed as an antideppressant, and neutraceuticals like Gyejibokryeong-Hwan (Park *et al.*, 2019); tetramethylpyrazine (Kao *et al.*, 2006); berberine (Wang *et al.*, 2018) and tanshinone IIA (Zhang *et al.*, 2018) have been reported to inhibit MCP-1.

However, Gutiérrez *et al.*, (2019) opined the complete suppression of CCL2/MCP-1 activity as potentially having deleterious consequences. Moreover, a number of reports have demonstrated the neuroprotective effects of MCP-1 (Chiu *et al.*, 2010; Eugenin *et al.*, 2003; Godefroy *et al.*, 2012; Hinojosa *et al.*, 2011; Madrigal *et al.*, 2009). The dual effects reported about MCP-1 need not be interpreted as contradictory. In fact according to existing data, the maintanance of MCP-1 concentrations seems to be the issue when it comes to the mediation of an appropriate inflammatory response by MCP-1 (Gutiérrez *et al.*, 2018). Both an excess and a deficiency of MCP-1 can be detrimental.

The excessive production of MCP-1 in the pathophysiology of many neurogenerative diseases has been well documented (Kusano *et al.*, 2004; Yu *et al.*, 2016; Semple et al., 2010; Hayashida *et al.*, 2001; Gutiérrez *et al.*, 2019; Park *et al.*, 2019).

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In summary, the importance of understanding the pathways that result in the activation and migration of microglia can not be over-emphasised. It is paramount in the development of drugs to combat neuroinflammation. For example Lurie (2018) reported that interventions could also be done through the process which activates NF-κB, a transcription factor that plays a key role in neuroinflammation. It is linked to depression-like symptoms and has become the focus of studies looking into factors that activate this pathway (Lurie 2018)

2.2. Astrocytes and neuroinflammation

Research extensively points to microglia as the hallmarks of neuroinflammation, however other glial cells such as astrocytes and oligodendrites are recruited into the fray and their participation can not be ignored especially that of astrocytes. This is mainly because astrocytes are the most abundant highly specialised and heterogenous type of glial cell with a morphology that changes depending on their developmental stage and locality (Morales *et al.,* 2016). They arise from a common lineage of neural progenitor cells within the neuroectoderm (Aguilera *et al.*, 2018).

Astrocytes have an important role in the homeostasis and function of the CNS. They serve as energy storage and generation cells; they have detoxification mechanisms and immune system modulation (Sharma *et al.*, 2007). Furthermore, they are involved in the water extracellular ions and pH homeostasis, clearance and extracellular modulation of neurotransmitters (especially glutamate); synaptic genesis; modulation of information processing and signal transmission; regulation of neural and synaptic plasticity and they provide trophic and metabolic support for neurons (Aguilera *et al.*, 2018, Jha *et al*., 2018). In addition, Kwon & Koh, (2020) reported that astrocytes also regulate blood flow and maintain the blood brain barrier (BBB).

A variety of cell surface receptors to cytokines are expressed on astrocytes so that they can respond to inflammatory stimuli. Aguilera et al., (2018) listed IL-1β; IL -6; IL-13; IFN γ; TGF-β; TNF-α and protein CD40L (CD154) as some of the receptors found on astrocytes. They also express TLR2, TLR3 and TLR4 to triger the inflammatory response to DAMPS and PAMPS molecules (Sofroniew 2014; Anderson *et al*., 2014).

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Activation of Astrocytes

Just like microglia, astrocytes change their morphology upon activation. They increase the expression of the fibrillary acidic protein (GFAP) which is a recognised marker of astrocyte reactivity (Chow *et al.*, 2010). In addition, YKL-40 (Chitinase 3-like 1) and D-Serine also serve as CSF biomarkers while GFAP and S100β are assessed as blood biomarkers (Kwon & Koh 2020). Astrocytes are activated by physiological changes in the CNS homeostasis and pathological insults in a general and disease specific mechanism (Pekny *et al*., 2019). Furthermore, it has been reported that astrocytes engage in a cross talk with microglia and play an important role in the acute and sub-acute tissue response to injury- a process known as gliosis or astrogliosis (Sharma *et al., 2007*; Sofroniew, 2014; Siegert *et al*., 2013; Norden *et al*., 2014; (Hao *et al*., 2001; Tarassishin *et al*., 2014). Reactive gliosis is a term used for the activation of astroglial cells regardless of the inducing agent which may be stroke, trauma, growth of tumor, or neurodegenerative disease.

In summary, from the literature consulted microglia and astrocytes are clearly major cellular role players in neuroinflammation. The mechanisms of activation of these cells have implications on research into therapeutic interventions in neurodegenerative diseases. A substantial number of researches use lipopolysaccharide (LPS) as an insult on microglia and astrocytes in order to create a neuroinflammatory environment for purposes of finding novel therapeutic interventions (Boonen 2018; Oh *et al.*, 2020; Chiu *et al*., 2010; Tarassishin et al., 2014; Park *et al.*, 2019). It will therefore be beneficial to the present study to understand the mechanism of LPS as one of the PAMPS in the induction of neuroinflammation.

2.3 LPS induced neuroinflammation

Lipopolysaccharide (LPS) is a gram-negative bacterial cell-wall component that represents one of the most potent natural adjuvants eliciting strong pro-inflammatory responses that can eventually cause a fatal sepsis syndrome in humans (Triantafilou *et al*., 2002; McAleer & Vella, 2008; Hamann *et al*., 2005). LPS has been reported to be a thermostable amphiphilic molecule consisting of 3 regions: lipophilic lipid A, hydrophilic polysaccharides or oligosaccharide core, and O-antigen (Mazgaeen and

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Gurung, 2020). Of these three regions, the lipid A portion of LPS has been shown to be the immunostimulatory moiety of LPS (Mazgaeen and Gurung 2020).

The most recognised sensor for LPS is TLR4 (Santalucia & Planas, 2013; Henneberger & Steinhäuser, 2016; Park *et al.*, 2019). However, recent reports have implicated two TLR4-independent LPS recognition systems, namely the transient receptor potential (TRP) channel-dependent sensing extracellular LPS and caspase-4/5/11-dependent sensing of extracellular LPS (Mazgaeen and Gurung 2020). The following section is a discussion of the literature that explain these LPS mechanisms that drive inflammatory responses in the CNS.

2.3.1. LPS recognition by TLR4

In an experiment involving human astrocytes Park et al., (2017) demonstrated the expression of functional TLR4 in astrocytes. Furthermore they showed that TLR4 requires CD14 to recognise LPS. Initiation of LPS recognition starts with its association with the LPS binding protein which then traffics it to CD14 (glyosylphosphatidyl inositol-anchored protein) (McAleer and Vella 2008).

However since since CD14 is devoid of transmembrane domain and does not signal in the presence of LPS, it takes it to TLR4 which then initiates the signaling process (McAleer and Vella, 2008). TLR4 then recruits an additional molecule, MD-2 which complexes with the extracellular domain of TLR4 (Triantafilou *et al*., 2002) to form a TLR4-MD-2-LPS complex which induces dimerisation of cytoplasmic toll-interleukin-1 Receptor (TIR) (Park *et al.*, 2017). Subsequently, the downstream adaptor protein MyD88 (myeloid differentiation primary response gene 88) and TIRAP (TIR-domain containing adaptor proteins) bind to the dimerised TIR domain structure forming Myddosome (a protein complex) and many other serine-threonine kinases of the IRAK family (Malp- *et al.*, 2002). Myddosome eventually mediates the activation of the transcription factor kβ (NF-κβ) which leads to the release of pro-inflammatory cytokines such as IL-6; IL-1β and TNF-α as shown in figure 2.4..

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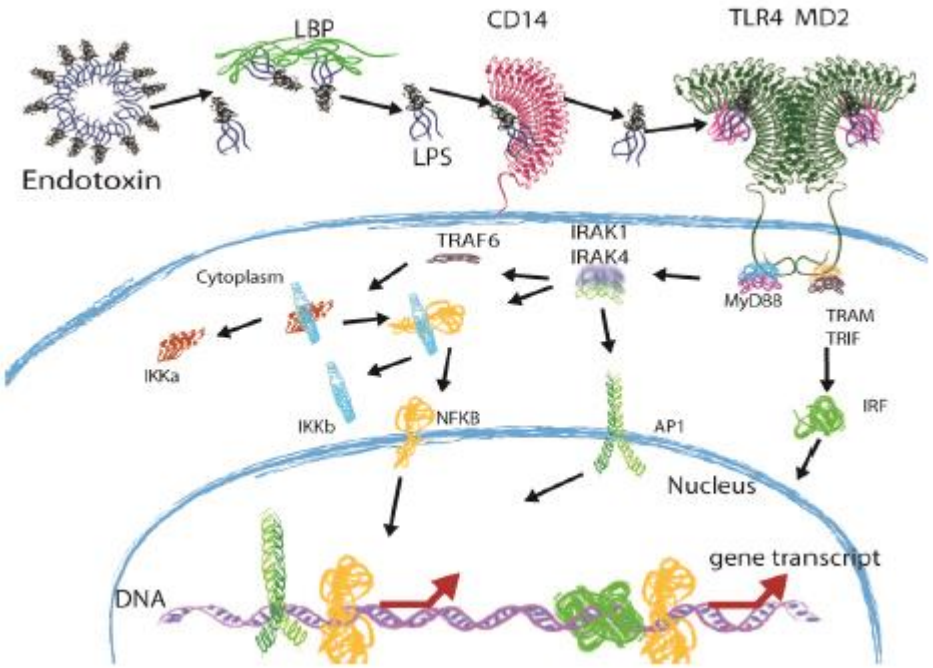

Figure 2-4 TLR4 LPS Signalling pathway (Nijland et al, 2014)

2.3.2. TRP Channel dependent Sensing

Transient receptor potential (TRP) cation channels belong to a superfamily of ion channel proteins mostly found in sensory neurons and epithelial cells (Mazgaeen and Gurung 2020). In addition to cellular sensing of several environmental and intracellular stimuli, TRP channels are important in the maintanance of cellular homeostasis. Their activation leads to depolarisation of resting membrane potential and subsequently activating voltage dependent non-selective ion channels which inadvertantly disrupts intracellular Ca^{2+} concentration (Mazgaeen and Gurung 2020). Meseguer *et al*., (2014) demonstrated that the neurogenic inflammation as a result of LPS relied on the activation of one TRP channel TRPA1 via an induction of calcium inflax. Furthermore Boonen *et al*., (2018) reported a similar mechanism through the activation of another TRP channel TRPV4. Many more studies have provided evidence that TRP ion channel such as TRPA1 and TRPV4 recognise LPS and initiate acute inflammatory responses (Startek *et al*., 2018; Boonen *et al*., 2018; Mickle *et al*., 2016.;Hajna *et al.,* 2020; Lagomarsino *et al*., 2021).

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2.3.3. Intracellular LPS Recognition by caspace-4/11

The TRP channels and TLR4 have been successfully demonstrated to recognise LPS independently leading an initiation of non-redundant signaling pathways. However both these receptors are membrane-bound and consequentially recognise extracellular or endosome-contained LPS. Recent reports have demonstrated how LPS is recognised through caspace-inflammasomes.

Mazgaeen & Gurung (2020) reported that LPS accesses the cytosol upon the disruption of the phagolysosome or via the HMGB1-facilitated uptake of LPS-containing outer membrane vesicles (OMV) released by live bacteria and their subsequent lysis. Huang *et al*., (2019) opined the abundance of LPS in OMVs. Furthermore, Meunier *et al*., (2014) demonstrated that host cells could, via endocytosis take in gram-negative bacteria through pathogen containing vesicles. This suggests multiple ways for the entry of LPS.

Once LPS is in the cytosol, caspase-4/-11 senses it, leading to its oligomerisation (Mazgaeen and Gurung 2020). In humans murine caspase 11 is duplicated as caspase -4 and -5-both of which have been demonstrated to detect cytosolic LPS (Oh *et al*., 2020). After oligomerisation, caspase -4 and -5 then cleaves the pyroptosis effector protein, GSDMD rendering the membrane porous leading to pottasium efflux and triggering the NLRP3 inflammasome. This leads to the synthesis and conversion of pro-IL-1β to IL-1β as well as IL-18. Caspace-11 and GSDMD are widely expressed in hematopoetic and non hematopoetic cells including macrophages, neutrophils, epithelial and endothelial cells (Oh *et al*., 2020). Moreover Kajiwara *et al*., (2016) observed its prevalence in human microglial cells and implicated it in the mediation of proinflammatory processes especially in AD.
Due to its unique characteristics caspace-11 inflammation pathway is designated non-canonical. This is compared to the canonical pathway where NLRP3 inflammasome activation depends on caspace-1 (Yi 2017).

The mechanisms of LPS inflammation elucidated in this section provides opportunities for therapeutic intervention to mitigate neuroinflammation. Batista *et al.,* (2019) reviewed a large body of experimental protocols where LPS induced

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neuroinflammation has provided understanding into the complex molecular and cellular mechanisms underlying neurodegenerative diseases

2.4. Neuronal Environment and inflammation: therapeutic implications

The unique neuronal environment modulates immune responses that give the CNS an immune–previledged status to a certain extent. This section will therefore briefly review the most relevant literature on how homeostasis is maintained in the CNS environment and how such an understanding can be harnessed in the development of therapeutic interventions for neurodegenerative diseases.

Mechanisms that curb cerebral immune responses include different signals for immune suppression; neurotrophins; anti-inflammatory cytokines; Cell to cell contact via adhesion molecules and a unique CNS environment. These are briefly introduced in the next sections.

2.4.1. Signals for immune suppression

There are factors of the CNS that modulate the immune response in a unique way. These include the neurotransmitters and neurohormones which occur in abundance in the CNS milieu. These have been shown to have immune suppressive effect. For example, Frohman and co-workers (1988) observed that noradrinaline has inhibitory effects on the IFN-γ-induced MHC class II expression on astrocytes. Furthermore, norepinephrine (NE) was reported to significantly reduce nitric oxide and IL-β production in rat cortical microglia (Russo *et al.*, 2004). Other examples include the demonstrated inhibitory effects of vasoactive intestinal peptide (VIP) on MHC class II antigens on astrocytes. In addition, 17β-estradiol has been demonstrated to reduce LPS and phorbol ester-stimulated superoxide production from microglial cells (Chang, *et al*, 2019).

2.4.2. Neurotrophins

The neuroprotective nature of neurotrophins has been demostrated. For instance, BDNF has been shown to reduce LPS-stimulated production of microglial cells (Pöyhönen *et al*., 2019; Nakajima *et al.*, 2001; Lindholm *et al*., 1992; Lively *et al*., 2018). In addition to BDNF, Nakajima and coworkers (2001) demonstrated the concomittant production of NGF by microglia exposed to LPS. Furthermore, they

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established that the PKC-α associated signal transduction mechanism mediated the production of BDNF and NGF. Neurotrophins are therefore an important component of the CNS milieu that helps maintain homeostasis.

2.4.3. Anti-inflammatory cytokines

The maintainance of homeostasis is a highly dynamic process requiring a well calibrated system that swiftly responds to disturbances by galvanising all necessary mechanisms to restore equilibrium (Dokalis *et al*., 2019). Cytokines are an integral part of brain homeostasis and present an opportunity for therapeutic interventions in the treatment of neurodegenerative diseases. Kronfol & Remick (2000) described them as hormones because they exhibit similar properties to those of the classic hormones of the endocrine system. Because of their pleiotropic nature, and involvement in multiple biological activities, cytokine nomenclature is difficult to establish.

However they have been historically classified according to their major properties as: proinflammatory and anti-inflammatory cytokines; chemokines, lymphokines and growth factors. Other families were categorised differently and named interferons (antiviral proteins), interleukins (acting between leukocytes), or colony-stimulating factors (acting on hemopoietic cell growth). (Feldmann & Pusey 2006.). The over 200 identified cytokines exhibit autocrine, paracrine, and endocrine actions and mediate cellular intercommunication (Gulati *et al*., 2016). Furthermore, cytokines bind to specific membrane receptors and the cytokine–receptor affinity is so high that biological effects are produced even at picomolar concentrations of the cytokine (Gulati *et al*., 2016). In addition, cytokines have been shown to be complex and biphasic in nature- the same molecule can exhibit both beneficial and detrimental effects to the body. Therefore a delicate balance of cytokines in the CNS needs to be maintained in order to preserve the integrity of the CNS milieu. Neurodegenerative diseases occur when there is a disturbance to this homeostasis as depicted in figure 2.5.

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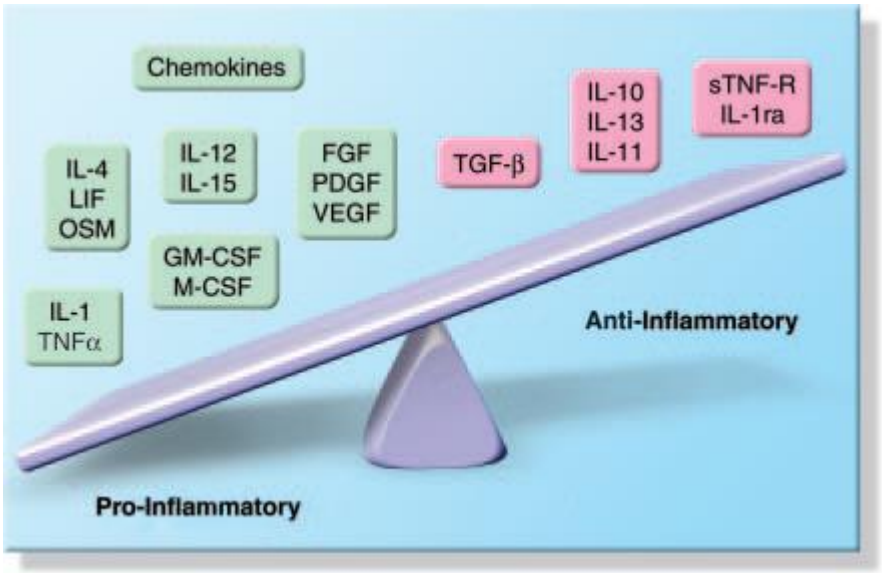

Figure 2-5 In neurodegenerative diseases both Pro and Anti-inflammatory cytokines are upregulated but the proinflammatory cytokines have an upper hand (Feldmann and Pusey, no date) (Feldmann & Pusey, 2006).

Substantial research targeting pro-and anti-inflammatory cytokines and their transcription factors regulating their expression in an effort to identify possible novel therapeutic approaches for the treatment of inflammation-associated neurodegeneration has been done (Safieh-Garabediana *et al*., 2004).

The approach to cytokine therapeutic intervention has been concentrated on blocking cytokines found to be elevated in disease states and supplement cytokines that are found to have possible neuroprotective or anti-inflammatory effects (Kusano *et al*., 2004; Azodi 2016). The mechanisms involved in cytokine inhibition and therapy were summerised by Safieh-Garabediana *et al*., (2004) in table 2.1.

Level	**Process**	**Mechanism**
Cytokine synthesis and secretion	Transcriptional regulation	Transcription factors
	mRNA stability	3'–AU–rich motifs
	Transcription/translation	Hormones (e.g. corticosteroids); Cytokines

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	Processing and release	Proteases (e.g. metalloproteases)
Expression of membrane cytokine receptors	Competition	Non-signaling receptors (e.g. IL–1R type II)
Interaction of cytokines with membrane receptors	Interference	Soluble cytokine receptors; Receptor antagonists (e.g. IL–1Ra); Non-receptor cytokine–binding protein (e.g. uromodulin); Anticytokine auto-antibodies

Table 2-1 The mechanisms involved in cytokine inhibition and therapy (Safieh-Garabediana, et al., 2004)

In the recent years, one of the cytokines that has received a lot of attention is TNF-α. Clark and Vissel (2019) reviewed the strategies around reducing rising TNF-α levels in the CNS during neuroparthogenesis. They reported mild success using specific anti-TNF drugs such as etanercept. Furthermore, the drug XPro1595 rapidly forms heterotrimers with native TNF resulting in a complex which is a dominant negative inhibitor of TNF. Another approach put across by Greig *et al.*, (2004) uses 3,6[1]-dithiothalidomide, a synthetised agent that readily enters the brain and decreases TNF RNA stability. In addition, research on how to reduce TNF has gone as far as the use of stem cells. Steinberg and co-workers, (2018) surgically implanted SB623 stem cells into brains of patients suffering from post-stroke syndromes. Among the many mechanisms that the stem cells reduced neuroimflammation, one of them was that it potentiated the release of anti-inflammatory cytokine IL-10 and, significantly, decreasing TNF levels (Steinberg *et al.*, 2018).

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Other cytokine therapies have been targeted on TGF-β which is expressed by microglial cells. It functions as an autocrine or paracrine signal to inhibit proliferation of astroglial and microglial cells (Lindholm *et al*., 1992; Lively *et al.*, 2018). Furthermore, Qian *et al*., (2008) reported that astrocyte-produced TGF-β reduces immune responsiveness of microglial cells exposed to LPS. Also Xiao *et al*., (1997) showed that TGF-β induces apoptosis of microglial cells – an occurrence that depletes their number and hence reduce neuroinflammation. Despite the positive effects of TGF-β, Laskowitz *et al*., (2001) opined the involvement of the cytokine in β-amyloid formation. Further research therefore has to be done.

2.4.4. Cell to cell contact via adhesion molecules

While intervention through soluble factors like neurotransmitters, neurohormones, neurotrophic factors and anti-inflammatory factors in the CNS presents therapeutic potential for the treatment of neurodegenerative diseases, some reports have explored the role played by cell to cell contacts. Chang *et al*, (2019) showed that interactions between neurons and glial cells via the NCAM-NCAM can restrict the responsiveness of glial cells to LPS. In addition, Hoek *et al*., (2000) demonstrated that the interaction of glia and neurons via CD200-CD200R decreases glial infammatory responses.

Together, the literature on neuroprotection therapies involving cytokine manipulation in order to maintain the delicate homeostasis of the CNS shows very wide research opportunities in that area. Furthermore, therapeutic approaches in the amelioration of inflammation associated with neurodegenerative diseases are almost inexhaustible. To remain relevant to the current thesis topic the discussion in the next section, will focus on interventions via neurotransmitter receptors, specifically, GABA receptors and through neuroenzymes with focus on MAO-A.

2.5. GABA receptors

Many research efforts have been done on γ-amino butyric acid (GABA) receptors (Wongsamitkul *et al*., 2017; Johnston *et al*., 2009; Möhler 2012; Rudolph & Mohler, 2006; Saaby et al., 2009; Hanrahan *et al*., 2011; Saaby 2011) The intention being to find intervention therapies for the treatment of neurodegenerative pathologies.

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GABA is the major inhibitory neurotransmitter in the mammalian nervous system and regulates neuronal transmission in the brain (Jembrek & Vlainić 2015). As a consequence, GABA levels mediate excitatory and inhibitory signals and are therefore implicated in neuropsychiatric disorders (Jembrek & Vlainić 2015). $GABA_A$ receptors are expected to provide novel pharmacological profiles (Rudolph & Mohler 2006). This is because of the widespread presence of the $GABA_AR$ subtypes-which are essentially involved in all CNS functions. The GABA receptor subtype depends on the neurocircuitry involved (Wallner *et al*., 2018). Research has focussed on the development and application of subtype-selective drugs that will not have deleterous side-effects while providing specific therapeutic benefits.

Furthermore these drugs can be either positive or negative allosteric modulators (PAMS and NAMS) (Wallner *et al*., 2018). For example, PAMS such as benzodiazepines (e.g. diazapam) have been employed in the treatment of generalised anxiety disorders, panic anxiety, sleep disturbances and epilepsy (Rudolph & Mohler 2006). In addition, the main property of many classes of therapeutic agents, including barbiturates act as positive allosteric modulators (PAMs) since they potentiate the action of GABA on $GABA_A$ R (Johnston *et al.,* 2009).

Due to the undesirable side effects of most synthetic drugs, a lot of focus has been on herbal products. Because of the great diversity of herbal plants, it seems this approach is a minefield that has only been scratched on the surface. As has been emphasised, the understanding of the mechanisms that maintain homeostasis in the CNS is the bedrock upon which therapeutic interventions are to be built. The myriad of enzymes involved in reaction cascades in the CNS also offers potential portals for therapeutic mediation. One such enzyme is MAO.

2.6. Monoamine oxidase

Monoamine oxidase (MAO) is a mitochondrial enzyme mostly expressed in gastro-intestinal, hepatic and neuronal tissues (Finberg *et a*l, 2016). It catalyses the oxidative deamination of biogenic and xenobiotic amines as well as neuroactive and vasoactive amines in the CNS and peripheral tissues (Cai 2014). Youdim, (2018) discussed two forms of MAO called MAO-A and MAO-B. MAO-A is responsible for oxidative deamination of noradrenaline and serotonin while MAO-B metabolises benzylamine

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and phenylethylamine (Youdim 2018). Due to MAO's involvement in modulating the function of neurotransmitters associated with various psychological conditions such as mood disorders, anxiety, depression, attention deficit hyperactivity, schizophrenia, sexual maturation, neurodegenerative diseases, attention has been given to this protein as a therapeutic target (Cai 2014). An increasing number of studies have linked MAO to neurodegenerative diseases including Parkingson's disease (PD) (Youdim & Bakhle 2006; Youdim 2018), Alzheimer's disease (Naoi *et al.*, 2006); dementia (Cummings 1995) and even depression (Cai 2014). Furthermore, it has also been reported that MAO is involved in neurodegenerative diseases via oxidative stress, neuroinflammation, apoptosis, activation of glia and non-clearance of aggregated protein (Cai 2014).

It has been demonstrated that inhibition of MAO exerts neuroprotective effects in patients with AD (Youdim 2018). Carradori *et al*, (2014) reported MAO-B activity to be generally higher in patients presenting neurodegenerative diseases like AD and PD. Many studies have demonstrated that the inhibition of MAO activity improves cognitive impairment (Soto *et al*, 1999; Entzeroth and Ratty 2017); via improvement in antioxidant activities (Weinreb *et al.*, 2011) and the potentiation of iron chelating activities. Furthermore, MAO -inhibition helps in the regulation of β-Amyloid precursor protein (APP) and Aβ expression processing (Weinreb *et al*., 2011) and also inhibits cholinesterase activity (Youdim *et al*., 2005). Many drugs such as phenelzine, pergline have been demonstrated to be potent MAO inhibitors that bind irreversibly with either MAO-A or MAO-B or both (Finberg *et al*, 2016). However, there are many phytochemicals that have been shown to also confer neuroprotective effects via MAO inhibition (Johnston *et al.*, 2009). Carradori *et al*., (2014) reported natural compounds such as β-carbolines, xanthine, alkaloids, flavonoids, xanthones, cannabinoids, miscellanea. Table 2.2 shows some of these phytochemical compounds.

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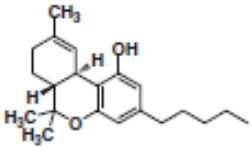
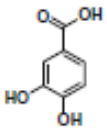
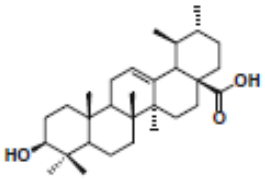
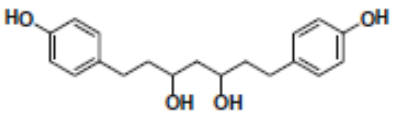
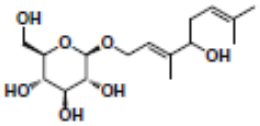
Natural compound	Stucture of the most active or selective MAO-B inhibitor	MAO-B inhibition value	Comments
Cannabinoids	 Tetrahydrocannabinol (THC)	IC_{50} pMAO-B = 22.6 μM	
Miscellanea	 Protocatechuic acid	IC_{50} rMAO-B = 300 μM	MAO-B inhibition could be attributed to different active principles in the phytocomplex which act synergistically
	 Ursolic acid	IC_{50} rMAO-B = 780 μM	
	 3,5-dihydroxy-1,7-bis (4-hydroxyphenyl)heptane	IC_{50} rMAO-B = 196 μM	
	 Rosiridin	hMAO-B inhibition = 83.8 %	

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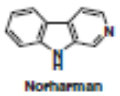
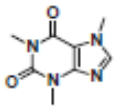
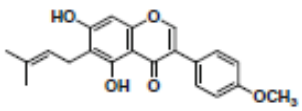
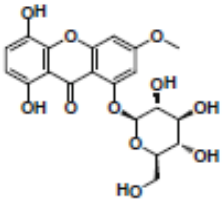
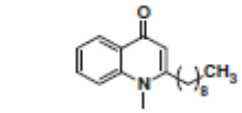
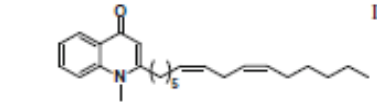
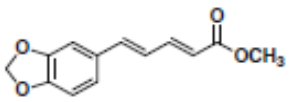
Natural compound	Stucture of the most active or selective MAO-B inhibitor	MAO-B inhibition value	Comments
β-Carbolines	 Norharman	IC_{50} hMAO-B = 4.68 μM, K_i hMAO-B = 1.12 μM	Harman, harmine and their corresponding synthetic derivatives are preferentially hMAO-A inhibitors
Xanthines	 Caffeine	K_i hMAO-B = 3.83 mM	Caffeine and its corresponding synthetic derivatives are potent dual hMAO-B inhibitors and hA_{2A} receptor antagonists used for the treatment of PD
Flavonoids	 Gancaonin A	IC_{50} rMAO-B = 0.8 μM	The most studied chemical class of natural compounds with regard toMAO inhibition, antioxidant and metal-chelating properties, and anti-inflammatory activity
Xanthones	 Swertianolin	hMAO-B inhibition = 93.6 %	Dual inhibitory activity against MAO and AChE
Alkaloids	 1-Methyl-2-nonyl-4(1*H*)-quinolone	IC_{50} rMAO-B = 2.3 μM	Structure-activity studies correlate MAO inhibitory property with the number of double bonds and the length of the lateral chain
	 1-Methyl-2-[(6*Z*,9*Z*)-6,9-pentadecadienyl] -4(1*H*)-quinolone	IC_{50} rMAO-B = 3.6 μM, K_i rMAO-B = 3.8 μM	
	 Methylpiperate	IC_{50} rMAO-B = 1.6 μM, K_i rMAO-B = 1.3 μM	

Table 2-2 Phytochemical compounds that effectively inhibit MAO (Carradori et al., 2014)

The neuroinflammation therapeutic interventions through GABA receptors, neuroenzymes and manipulation of cytokine balance and other mechanisms involved in maintaining CNS homeostasis have shown a measure of success. In addition to these inteventions, the use of antioxidants to alleviate oxidative stress which is intricately related to neuroinflammation, has also been demonstrated as another potent option. The next section will therefore review oxidative stress in terms of neuroinflammation.

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3.0. Oxidative stress

Oxidative stress refers to overproduction of reactive oxygen species (ROS) in the cells and tissues to the extent that the body's antioxidant system is unable to cope. (Hussain *et al.,* 2016). In another sense, oxidative stress is a condition in which the critical balance between free radical generation and antioxidant defenses is unfavourable (Lobo *et al.*, 2010). This is represented in Figure 2.7 below.

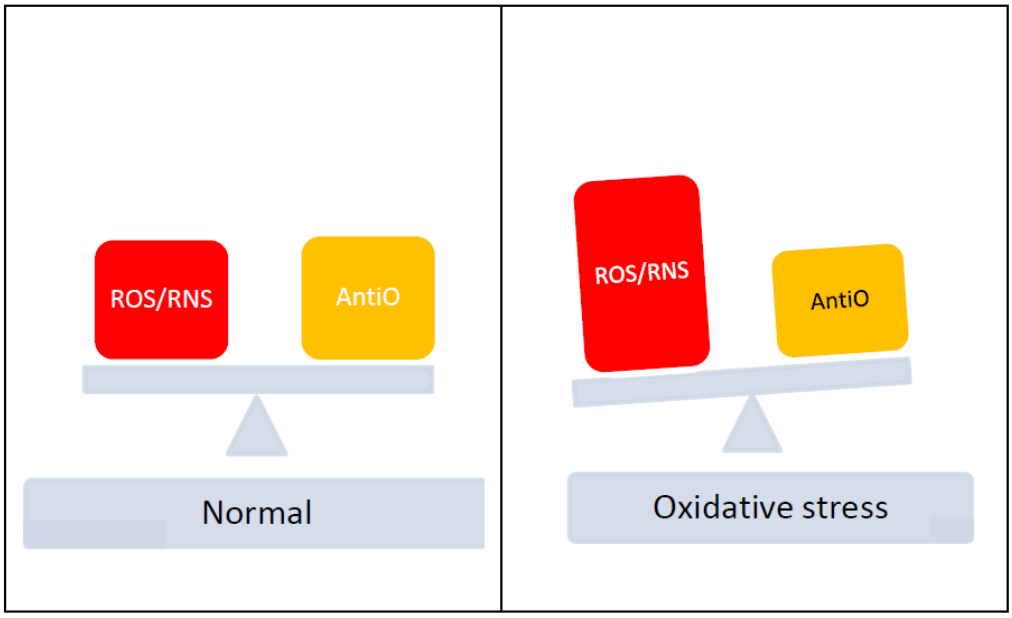

*Figure 2-6 Comparison between normal environments and a shift in the balance between ROS/RNS and AntiO. *ROS/RNS (Reactive oxygen/nitrogen species); AntiO (Antioxidants)*

The deleterious effects of oxidative stress, which include damage to a wide range of molecular species including lipids, proteins and nucleic acids, have been well documented (Lobo *et al.*, 2010).

The main players in oxidative stress are free radicals which are defined as independent, highly unstable and reactive molecular species containing an unpaired electron in an atomic orbital. As Petersen and Smith (2016) asserted, free radicals and other reactive oxygen and nitrogen species (RONS), are derived from normal essential metabolic processes in the body. Such processes may be non-enzymatic reactions of oxygen with organic compounds or maybe initiated by ionizing reactions (Lobo *et al*, 2010). Ebadi 2001 and Petersen & Smith 2016) listed some of the internally and externally generated free radical and RONS sources as follows:

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- Mitochondrial electron transport chain
- Enzyme systems such as cytochrome P450
- Xanthine oxidase
- NADH-oxidase complex
- Perioxisomes
- Cyclooxygenase
- Inflammation
- Phagocytosis
- Arachidonate pathways
- Exercise
- Ischemia/reperfucion injury
- Cigarette smoke
- Environmental pollutants
- Radiation
- Certain drugs, pesticides
- Industrial solvents
- Ozone
- Ageing

In addition to the above list, Hong, (2014) asserted that pyschological stress has also been linked to the production of oxidative species.

Most of the endogenously produced ROS in brain cells, come from the mitochondrially mediated pathways. Furthermore, up to 2% of the overall cellular mitochondrial O_2 consumption may be related to the production of ROS including O_2^-. (Kim *et al*, 2015), as will be explained in the next section.

3.1. Endogenous sources of ROS and RONS

The mitochondrial electron transport chain (ETC) is made up of five subunit complexes. Complex 1, which is made of NADH-Coenzyme Q reductase is responsible for ROS production of O_2^- and facilitates electron transfers from NADH to CoQ. Complex 2 which consists of succinate dehydrogenase also produces O_2^-, albeit at low levels. It is involved in the reduction of CoQ. Coenzyme Q-cytochrome c reductase makes up complex 3 which is involved in the generation of O_2^- in the

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intermembrane space. (Kim *et al.,* 2015). Complex 4 consists of cytochrome c oxidase while ATP Synthase makes up complex 5.

These complexes vary in their capacity to produce ROS and their relative predominance varies in different organs and disease conditions. For instance in healthy individuals complex 1 produces ROS at a rate that is half that of complex 3. However under pathological conditions ranging from accelerated aging to neurodegenerative diseases, complex 1 assumes the main role in ROS productions. (Kim *et al.*, 2015).

In addition to the mitochondrial ETC, the other identified sources of ROS include NADPH oxidases (NOx); endoplasmic reticulum (during protein folding and/or misfolding), peroxisomal metabolic activities such as fatty acid oxidation, amino acid catabolism and phospholipid biosynthesis. Furthermore in the presence of metal ions,iron in particular, hydrogen peroxide can generate the highly reactive hydroxyl (·OH) radical through the Fenton reaction (Betteridge 2000). Activated inflammatory cells, neutrophils, eosinophils produce superoxide (O_2^-), hydrogen peroxide (H_2O_2) and nitric oxide (NO_2^-) during respiratory burst.

Across the spectrum of oxidative stress research, the most studied ROS include superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (·OH), peroxynitrite (ONO_2^-) and nitric oxide (NO) (Petersen and Smith 2016). Furthermore it is important to also appreciate that the production of these free radicals is a necessity.

Free radicals are important for the normal activities of phagocytic cells; as signallling molecules, regulating cell growth and apoptosis, adhesion and differentiation; gene transcription and regulation of soluble guanylate cyclase activity (Petersen and Smith 2016; Uttara *et al*., 2009). In particular NO is an essential signalling molecule that regulates the relaxation and proliferation of vascular smooth muscle cells, leukocytes adhesion, plateletes aggregation, angiogenesis, thrombosis, vascular tone and hemodynamics (Uttara *et al.*, 2009). Physiological complications set in when the

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antioxidation systems that keep them in check become overwhelmed leading to chronic oxidative stress.

3.2. Deleterious effects of Oxidative stress

Overproduction of free radicals has been shown to instigate oxidative damage to biomolecules, (proteins, lipids and DNA) which subsequently leads to many chronic diseases such as atherosclerosis, cancer, diabetes, rheumatoid arthritis, and many neurodegenerative diseases (Uttare *et al.*, 2009).

3.2.1. Oxidative damage to protein and DNA

Proteins are susceptible to oxidative modification through specific amino acids, free radical mediated peptide cleavage, and formation of protein cross-linkage due to reactions with lipid peroxidation products (Lobo *et al*, 2010). Protein oxidation inevitably leads to the alteration of signal transduction mechanisms; enzyme activity, heat stability, and proteolysis susceptibility. According to Lobo *et al*., (2010), It is usually the free radical peroxyl that oxidises proteins.

3.2.2. Lipid peroxidation

Lipid peroxidation affects polyunsaturated fatty acids on cell membranes. The hydroxyl radical is thought to be involved in initiating ROS and remove a hydrogen atom, thus producing a lipid radical that acts as a secondary free radical which can react with other biomolecules (Lobo *et al.*, 2010). A propagation of chain reactions is therefore initiated. This becomes a source of other compounds such as alkans, malanoaldehyde, and isoprotanes which are used as markers in lipid peroxidation.

3.2.3. Oxidative damage to DNA

Many studies in oxidative stress concur on the susceptibility of DNA and RNA to oxidative damage (Hussain *et al*, 2016; Lobo *et al*, 2010; Petersen and Smith 2016). Furthermore, DNA is considered as a major target of pro oxidants. Lebo *et al*., (2010) reported that mitochondrial DNA are the most susceptible and the release of glycol, dTG, and 8-hydroxy-2-deoxyguanosine has been reported during oxidative damage to DNA, with the latter being suggested as a biological marker for oxidative stress.

If not regulated properly, oxidative stress can induce a variety of chronic and degenerative diseases, as well as the ageing process and chronic conditions such as

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cancer, pulmonary diseases, neurological diseases, neuropathy, ocular and cardiovascular diseases, and foetal complications. (Pham-Huy *et al*., 2008).

3.3. Oxidative stress and the brain

The brain is one of the most metabolically active organs in the body. It is the most sensitive target tissue to oxidative stress for anumber of reasons. Firstly the brain, comprises only 2% of body weight but consumes as much as 20% of oxygen supply in the body. This suggests the potential generation of large amounts of ROS during the oxidative phosphorylation to produce ATP. (Hong *et al.*, 2014). Secondly, the metals such as copper and iron, which occur in high concentrations in the brain, catalise the generation of ROS due to their redox-active nature. Thirdly, lipid peroxydation occurs in the brain cell membranes as a result of the high levels of polyunsaturated fatty acids it contains.

Lipid peroxidation results in the formation of complex reactive aldehyde species that are cytotoxic through their ability to react with proteins, DNA and lipids (Hong *et a*l., 2014). Fourthly, there are low levels of GSH (Tripeptide Glutathione), a compound involved in the endogenous antioxidant system (Hong *et al.*, 2014; Kim *et al.,* 2015). The brain is therefore susceptible to ROS attack especially on the terminally differentiated post-mitotic neuronal cells.

4.0. Antioxidants

The human body has several mechanisms to counteract oxidative stress by producing antioxidants, which are either naturally produced in situ, or externally supplied through foods and/or supplements (Pham-Huy *et al.*, 2008).

Antioxidants are substances that inhibit the process of oxidation, even at relatively small concentrations- (Tegeli *et al.*, 2014). They do this by donating an electron to a rioting free radical thereby neutralising it or by removal of RONS triggers by quenching chain-initiating catalysts. Furthermore, antioxidants may act on biological systems via different mechanisms that include electron donation, metal ion chelation, co-antioxidants, or by gene expression regulation thus reducing its capacity to damage

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(Lebo *et al.*, 2010). Antioxidants are therefore important in halting the deleterious effects of RONS.

Antioxidants can be classified as endogenous compounds or exogenous, which can be natural or synthetic.

4.1. Endogenous Antioxidants

The endogenous antioxidants are classified as enzymatic and non-enzymatic. The main enzymes directly involved in the neutralisation of ROS and RNS are: superoxide dismutase (SOD), Catalase (CAT) and Glutathione peroxidase (GPx). (Pham-Huy *et al*, 2008). SOD is one of the most potent antioxidant enzyme and it forms the first line of defense (Hong *et al.*, 2014). In fact, superoxide dismutates are a group of enzymes endowed with the ability to convert highly reactive superoxide radicals (dismutation) into hydrogen peroxide and molecular oxygen (Aguilar *et al.*, 2016).

Five SODs have been identified: of which SOD1 is found in humans and is relevant to the present study. SOD1 requires Copper and Zinc for its biological activity and a depletion in Copper ions leads to its complete inactivation. Consequently this Cu^{2+} deficiency causes multiple diseases (Aguilar *et al.,* 2016).

CAT is a tetrameric porphyrin containing enzyme located in peroxisomes. Its main function is to catalyse the decomposition of hydrogen peroxide to water and oxygen. (Hong *et al*., 2014; Aguilar *et al,* 2016). A report by Hong *et al.,* (2014) revealed that chronic stress decreases the activities of SOD and CAT (Aguilar *et al*., 2016). GPx acts in the presence of GSH to catalyse the reduction of hydrogen peroxide (H_2O_2) or organic peroxide (ROOH) to water or alcohol. The process results in the oxidation of GSH to GSSG (Oxidised Glutathione) (Aguilar *et al*., 2016). GSH is the most abundant thiol compound in mammalian cells. It is therefore important in the protection of the polyunsaturated fatty acids found in cell membranes from lipid peroxides (ROOH) formed by lipid peroxidation reactions. (Hong *et al*., 2014; Tegeli *et al*., 2014).

Other antioxidant enzymes that are important in reducing oxidative stress are Glutathione reductase (GR) and Glutathione peroxidase (GPx). GR catalyses the

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conversion of GSSG to GSH via NADPH as an electron donor whereas GPx, catalyses the reduction of peroxides by making use of glutathione as the electron donor (Hong *et al*., 2014).

The GSH/GSSH ratio has been reported as having an impact on signal transduction and cell control and should therefore be closely regulated (Herrlich& Bohmer 2000). The concentration of GSH is 10-100 times more than GSSH, thus even small changes in GSH will result in significant shifts in the GSH/GSSG ratio and consequently, redox balance. (Schafer & Buettner 2001). This therefore emphasises the importance of glutathione reductase in regulating the GSH/GSSH ratio. A decrease in GSH concentration has been linked to an induction in mitochondria-dependent apoptosis. Two studies by the same group (Ghibelli *et al*., 1995 and Ghibelli *et al*., 1999) have validated this when they showed that apopstosis can be blocked by a restoration of GSH concentration.

Romeu *et al*, (2010) proposed what they called a score for oxidative stress based on the analysis of several biomarkers. The enzymes glutathione S-transferase (GST), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), and both the reduced and oxidized glutathione (GSH, GSSG) were considered as important biomarkers for oxidative stress.

The following schematic diagram is a summary of the protective effects of the antioxidants:

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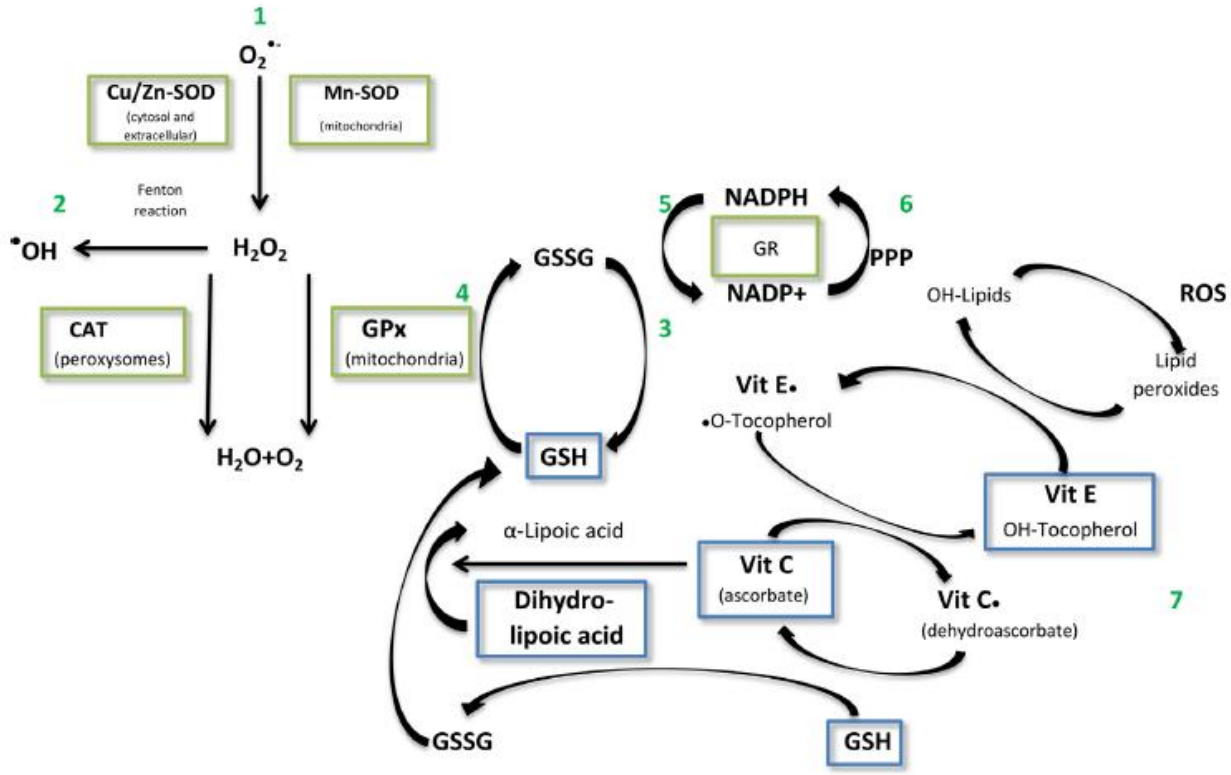

Figure 2-7 The mechanism of antioxidant Protection (Mironczuk-Chodakoska et al., 2018)

1. Formation of Superoxide radical (O_2^{*-}) by a single-electron reduction of oxygen. In a reaction catalysed by superoxide dismutase (Cu/Zn-SOD or Mn-SOD), the superoxide radical picks up an electron to form H_2O_2 which is then reduced to H_2O and O_2 by the catalyses of CAT and GPx
2. Fenton's reaction-catalysed by transition metals transforms H_2O_2 to the hydroxyl radical (HO^*) which further participates in the free radical chain reactions.
3. GSH, due to the presence of the thiol group of cysteine reacts with free radicals of proteins or other macromolecules, restoring them to the reduced form.
4. H_2O_2 is reduced by GSH in the reaction catalysed by Glutathione peroxide resulting in GSSG which then oxidises thiols of proteins.
5. GR reduces Glutathione disulphide via NADPH forming NADP+.
6. The first oxidative phase of the Pentose Phosphate pathway produces NADPH.
7. GSSG is changed back to GSH in a reaction buttressed by e.g. Vitamin C and α-lipoic acid. Vitamin E scavenges lipid peroxides and terminates oxidative chain reactions as a hydrogen donor. Non-oxidized form of vitamin E can be recycled back by vitamin C and glutathione. (Mironczuk-Chodakoska et al., 2018)

The mechanisms shown are a) preventive-through the halting of the deleterious reactions of free radicals with biomolecules in the body: b) Reparative-as they interrupt radical oxidation reactions and c) they inactivate the products of free radical reaction and their derivatives, by repairing or eliminating structural damage (Mironczuk-Chodakoska *et al*., 2018).

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4.3. Endogenous non-protein antioxidants

In addition to GSH the other non-protein antioxidants include:

Alpha-lipoic acid which exerts its antioxidant activities in both non polar and polar media in any subcellular compartment of the body (Adwas *et al*., 2019).

CoQ which is a benzoquinone derivative found in the mitochondrial respiratory chain and in other internal membranes. CoQ is well known for its activity in energy transduction and production of ATP via oxidative phosphorylation. It is considered as an endogenously synthesised lipid soluble antioxidant with a protective effect that stretches to lipids, proteins and DNA.

Ferritin is an iron-binding protein whose main function is to limit Iron (II) concentrations in the blood hence reducing oxygen free radicals. Furthermore, ferritin is thought to cause gene and protein alterations that help limit oxidant toxicity (Moussa *et al.,* 2019)

Uric acid is produced intracellularly as an intermediate product of the purine degradation pathway. Together with albumin, urate exerts its antioxidant activity in human plasma by scavenging oxygen radicals, thereby protecting erythrocyte membrane from lipid oxidation. In addition it protects cardiac, vascular and neural cells from oxidative injury. Protection of the later would decrease chances of neurodegenerative diseases such as multiple sclerosis and Parkinson's Disease (Mironczuk-Chodakoska *et al*., 2018; Hong *et al*., 2014) However uric acid can only exerts antioxidant effects in hydrophilic environment which is its major limitation (Kurutas 2016).

Bilirubin is the end product of heme degradation. Its role in the prevention of ischemic injury in isolated hearts by attenuation of oxidative damage has been reported in cultured cells. It is also involved in neuroprotection against hydrogen peroxide assault in which a redox cycle between beliverdin and bilirubin increases this protective effect (Pham-Huy *et al*., 2008; Kurutas 2016).

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4.4. Dietary antioxidants

Antioxidants from our diet are paramount in aiding endogenous antioxidant systems (Pham-Huy 2008; Carocho *et al*., 2018). One of the major causes of numerous chronic and degenerative diseases is a lack of these dietary antioxidants. They include Vitamin E (Tocopherol), Vitamin C (ascorbic acid), Beta-carotene (which can be converted to Vitamin A), Lycopene, Selenium, Omega 3 and Omega 6 fatty acids, as well as phytochemicals (Pham-Huy 2008).

The major source of dietary antioxidants is agricultural products and medicinal plants have been shown to be reliable sources of these antioxidants. They include fruits and vegetables, cereals, animal protein, oils, nuts, spices and teas (Carocho *et al*., 2018). It is worth noting that there has been a heightened global recognition and interestin biologically active compounds from plants (Compean & Ynalvez 2014). This has resulted in the development of many commercial medicines such as quinine (anti-malarial), galantanine (Alzheimers disease) and morphine (anti-cancer) (Van Wyk *et al*., 2009). Figure 2.9 is a flow chart showing a classification of dietary phytochemicals.

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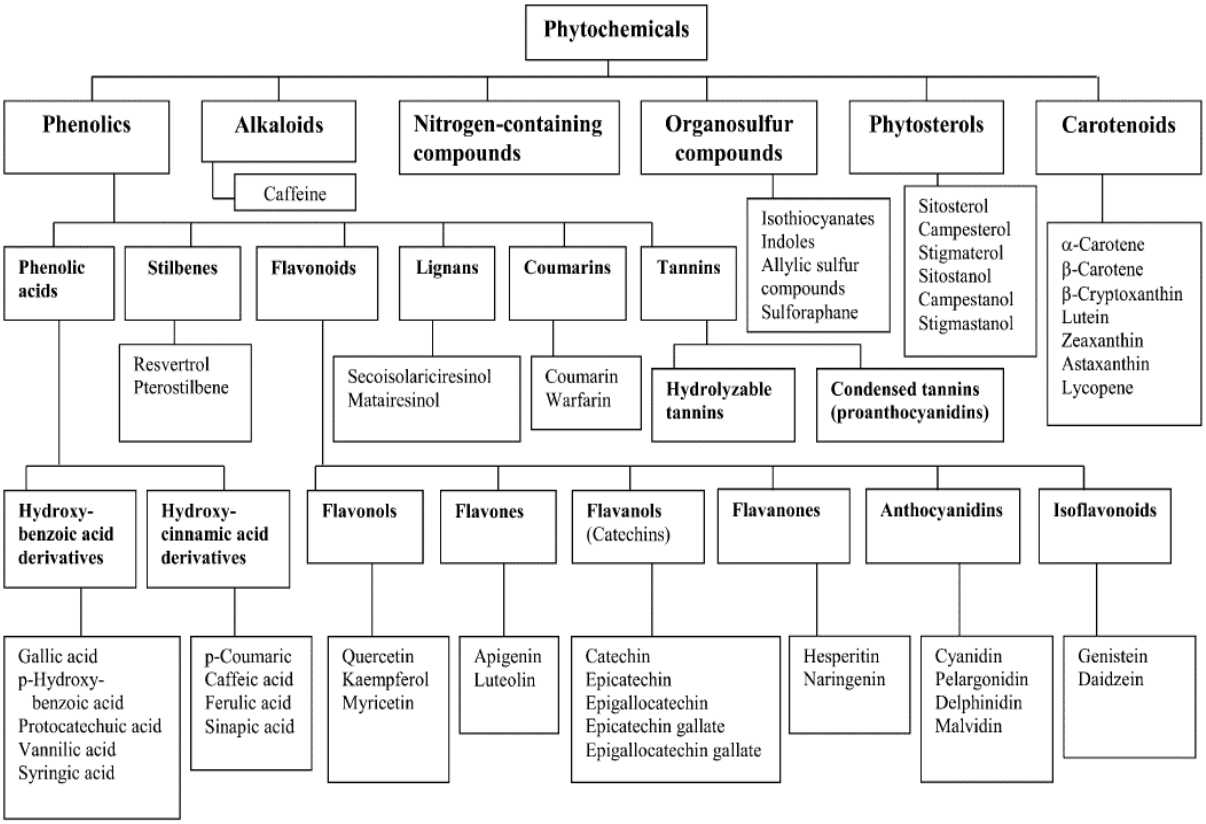

Figure 2-8 Classification of Dietary Phytochemicals (Liu, 2013)

It is evident that phytochemical reseach is far from being exhausted, judging from the number of phytochemicals that have been identified. Taiz. & Zeiger (2010) reported that more than 2000 phytochemicals have been identified. According to Edeoga *et al*., (2005), the most impotant phytochemicals (also refered to as 'Phytonutrients', 'Nutraceuticals', 'Functional ingredients' or 'Bioactive molecules') are alkaloids, tannins, flavonoids, and phenolic compounds. For the purpose of this thesis I will focus on the flavonoids found in rooibos extract and their possible neuroprotective properties.

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5.0. Flavonoids

5.1. Origin and biosynthesis

Flavonoids have attracted enormous interest in the recent years because of their multidimensional health effects on both humans and animals. According to Samanta *et al.,* 2011) 6500 flavanoids have been identified. They have been involved in neutraceutical, pharmaceutical, medicinal and cosmetic applications. This is because of their proven antioxidative, anti-infammatory, antimutagenic and anti-carcinogenic properties, which at least in part result from their capacity to modulate key cellular enzyme functions (Panche *et al.*, 2015).

5.2. Structure and classification of flavonoids

Flavonoids have a very wide variety of chemical structures made up of 15 carbon atoms in their basic skeletons with a C_6-C_3-C_6 framework constituted by two aromatic rings (Awonatack *et al.,* 2017; Groetewold 2006; Panche 2016; Kamboh *et al*., 2015). The term flavonoids refers to the compounds having the C_6-C_3-C_6 and was coined by Geissman and Hereiner in the 1950's. They mainly consist of two benzene rings called A and B linked via a heterocyclic pyrene ring called ring C as shown in Figure 2.10 (Kumar and Panday 2013)

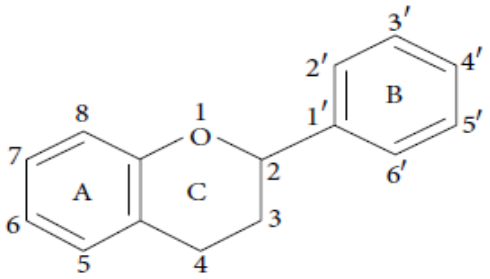

Figure 2-9 Basic Flavonoid structure (Kumar & Pandey, 2013)

Flavonoids can further be subdivided using a criterion based on three structural differences: (1) Saturation of the heteroatomic ring C; (2) the overall hydroxylation patterns (Groenwold 2006); (3) The carbon of the C ring on which the B ring is attached i.e C-2 or C-3 or C-4. Flavonoids in which the B ring is linked on position 2 can be further subdivided into flavones, flavonols, flavonones, flavonols or catechins, anthocyanins and chalcones as shown in table 2.3. (Panche *et al*.,2016).

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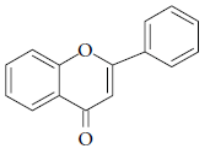
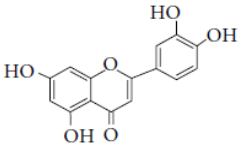
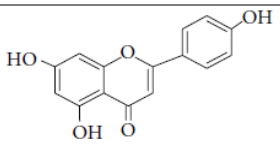
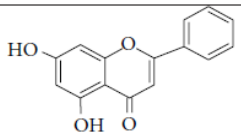
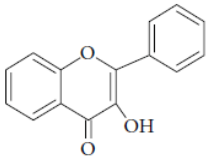
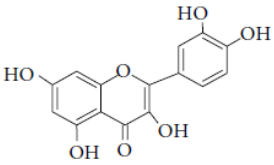
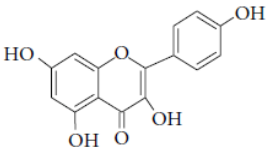
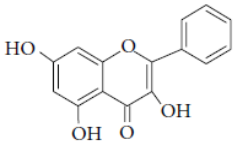
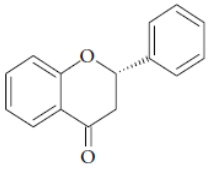
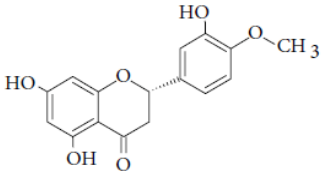
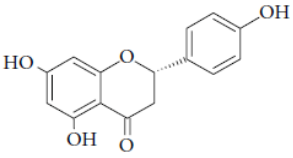
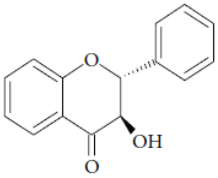
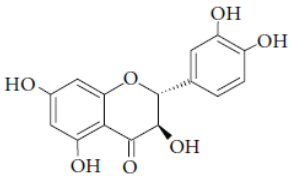
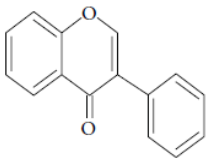
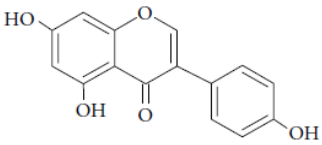
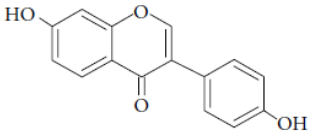
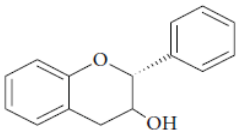
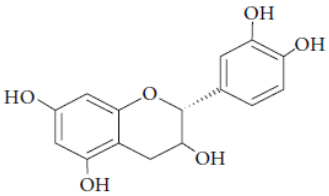
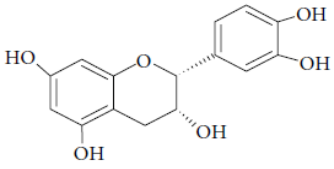
Group of flavanoid	Structure backbone	Examples		
Flavones		 Luteolin	 Apigenin	 Chrysin
Flavonols		 Quercetin	 Kaempferol	 Galangin
Flavanones		 Hesperetin	 Naringenin	
Flavanonol		 Taxifolin		
Isoflavones		 Genistein	 Daidzein	
Flavan-3-ols		 Catechin	 Epicatechin	

Table 2-3 Structures of different flavonoid Families (Kumar & Pandey, 2013)

Groenwold (2006) described how hydroxylation, methoxylation, O-glycosylation of hydroxyl groups as well as C glycosylation directly to carbon atom of the flavonoid skeleton results in the different sub groups of flavonoids. Figure 2.11 below shows these sub-groups and and their natural sources.

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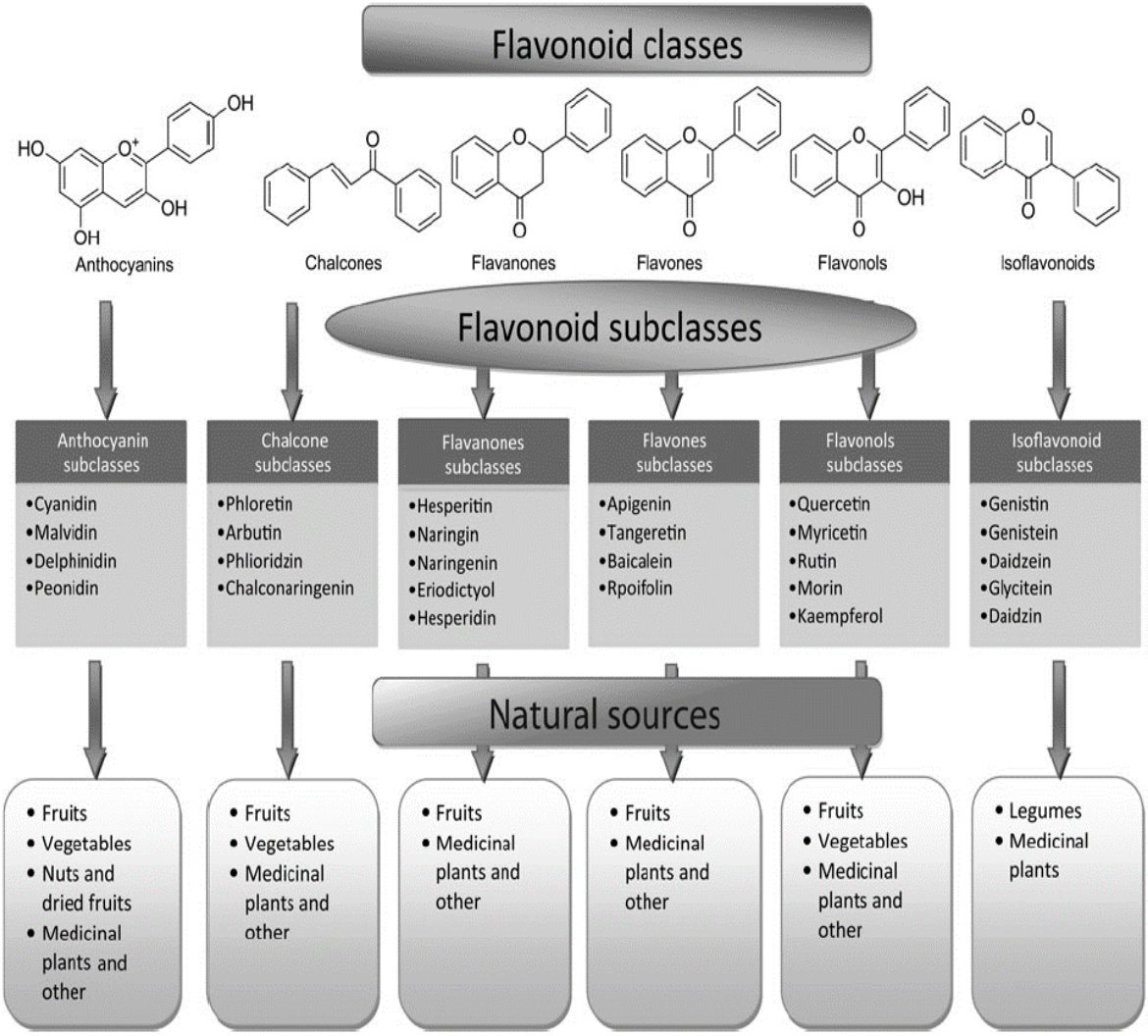

Figure 2-10 Flavonoid classes, sub-classes, and natural sources (Panche et al, 2016)

5.4. Chemical Properties and Biological Activity

Flavonoids are the most abundant group of polyphenols and are the most studied (Camara *et al.,* 2013). A plethora of research papers and books have shown flavonoids to have antioxidant, anti-inflamatory, anti-anatogenic, antibacterial, antiviral hepatoprotective, antiallergic in addition to immunomodulatory activities in a number of in vitro and animal models (Panche 2016; Kamboh 2015). This section will deal with some of the important biological activities of flavonoids.

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5.4.1. Antioxidant Activities

The antioxidant activity mechanisms of these compounds is largely influenced by the configuration, substitution and total number of hydroxyl groups. These factors greatly influence the metal chelating and radical scavenging ability (Kumar 2015). The configuration of the B ring hydroxyl is the most potent determinant of capacity for scavenging ROS and/or RNS.

Flavonoids action involves most of the antioxidant mechanisms such as: 1. Suppression of ROS formation by enzyme inhibition or chelation of trace elements that are involved in free radical generation; 2. Scavenging ROS and boosting or protection of antioxidant defences (Kumar 2015). Panche (2016) reported that, Hanasatei et al discovered that flavonoids such as epicatechin and rutin have inhibitory activity on the enzyme XO. In support of this, Kumar, (2016) showed that flavonoids can inhibit the enzymes such as microsomal monooxygenase, glutathione S-transferare, mitochondrial succinoxidase, NADH oxidase which catalyse ROS generation. Furthermore, it has been reported by Kerrey and Abbey, as cited in Panche *et al*, (2016) that flavonoids can inhibit LDL oxidation in *in vitro* studies; an action which can potentially protect LDL particles and mitigate the onset of atherosclerosis.

5.4.2. Metal Chelating

Metal ions can result in the formation of hydroxyl radicals by reducing hydrogen peroxide. However the lower redox potentials of flavonoids (F1-OH) enable them to reduce highly oxidizing free radicals (e.g superoxide, peroxyl, alkoxyl and hydroxyl radicals) formed by metal ions. They do this by donating a hydrogen atom (Kumar 2015).

In addition, flavonoids can inhibit the radical generating action of these marauding metal ions by chelating them. In the core structure of flavonoids there are specific coordination sites where the metal ions bind. These sites are between the 5 Hydroxyl and 4 carbonyl group in the A –ring and between the 3, 4 hydroxyl group in the B ring (Symonowicz and Kolonek 2012) as shown in figure 2.12.

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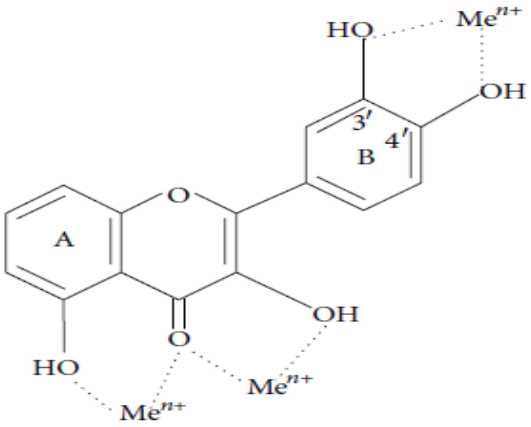

Figure 2-11 Binding sites for trace metals where Me^{n+} indicates metal ions (Kumar & Pandey, 2013).

Kostyuk *et al*, (2001) were able to show that the flavonoid-metal complexes generally have higher radical scavenging ability which depended on the oxidation potential (ORP) or electron transfer potential of the particular complex (Flaga *et al*., 2010).

5.4.3. Anti–inflammatory Activities

Many researchers have been able to demonstrate the anti-inflammotory activities of flavonoids (Korak 2019) The well researched flavonoids: hesperidin, luteolin and quercetin are known to modulate inflammatory activities via the enzyme systems involved in the generation of pro- inflammatory substances (Karak 2019; Tiwari & Husain 2017).

The ability of flavonoids to inhibit inflammatory processes by disabling the enzymes involved, has been linked by many reseachers to the prevention of neurodegenerative diseases. This has been shown in numerous reports about inhibitory effects on enzymes such as aldose reductase XO, phosphodiesterase, Cu^{2+} ATPase, lipoxygenase and COX (Panche *et al*, 2015; Jager & Saaby 2011; Hu *et al*., 2009; Khan *et al*., 2009; Shimmyo *et al*., 2008).

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6.0. Aspalathus linearis (rooibos)

6.1. General introduction and Background

Medicinal plants have played an important role in the socio-cultural, spiritual and medicinal area of Africans, especially those in the rural areas. (Doughari, 2012). One plant in particular is *Aspalathus linearis* (rooibos) which is popular globally as a herbal tea, although it is also available in guises such as creams, hair lotions, soaps and other skin care products (Smith & Swart 2018; Joubert *et al*., 2011).

Rooibos belongs to the genus *Aspalathus* (Fabaceae, Tribe Crotalarieae) which is made up of 270 species most which are endemic to the arid Western Cape of South Africa mainly in the mountains of Cederberg near Cape Town (Joubert *et al*., 2011; Reynolds & Ngcwangu 2010; Kawakami *et al.*, 1993).

Rooibos is a 1.5 metres high shrub made up of bright green needle-shaped leaves and pea-shaped yellow flowers (figure 2.13) and was traditionally consumed by the Khoi people. *A. linearis* is colloquially called rooibos (Afrkaans for “red bush”) due to the red colour of the dried leaves (Hoosen 2019).

Rooibos growing in the Cedarberg region of South Africa. Photo ©2003 Rooibos Ltd./SunnRooibos

Figure 2-12 Rooibos plants in Cedarberg (Erickson, 2003)

The map in Figure 2.13 shows the area in South Africa where rooibos is endemic.

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Figure 2-13 Map from Medicinal Plants of South Africa, 2002 The red shade shows Cedarberg region (Erickson, 2003)

The ever-surging popularity and demand of rooibos is attributed to its health promoting properties which are ascribed to its low tannin content, zero caffeine and potent antioxidant and anti-inflammatory properties (Hoosen 2019). Moreover, rooibos has shown no cytotoxic effects at all concentrations tested *in vitro* using whole cell cultures (Hoosen 2019) These health benefits of rooibos are because of its rich and unique polyphenol content, particularly flavonoids. This section will focus on the chemical composition and health benefits of rooibos.

6.2. Characterisation of *Aspalathus linearis* (rooibos)

Many phenolic compounds that include dihydrochalcones, flavanones, flavones (hemiphlorin and chrysoeriol, luteolin and luteolin-7-O-glucoside), flavonols (quercetin and its O-linked glycosides quercetin-3-robinobioside, hyperoside, isoquercitrin, and rutin, with phenolic acids), monomeric and oligomeric flavan-3-ols, lignans, hydroxycinnamic acid and derivatives, phenolic carboxylic acids have been identified in rooibos (Smith and Swart 2018; Marnewick 2014).

In addition to the flavonoids, rooibos also contains lignans (vladinol E, secoisolariciresinol, secoisolarici resinol glucoside) and phenolic acids (caffeic acid, ferulic acid, p-coumaric acid, p-hydroxybenzoic acid, vanillic acid, protocatechuic acid) (Hoosen 2019). Table 2.16 shows structures of the flavonoids found in rooibos.

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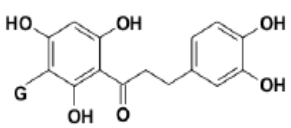
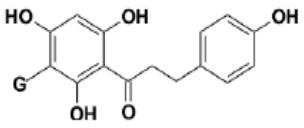
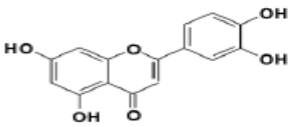
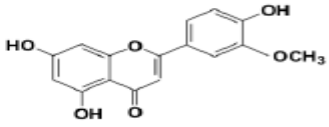
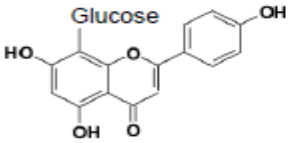
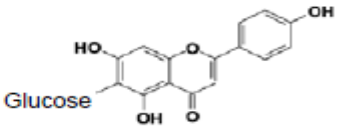
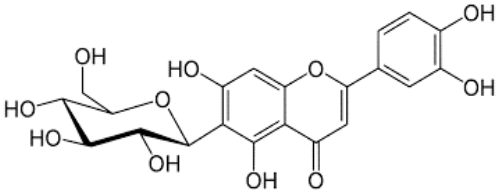
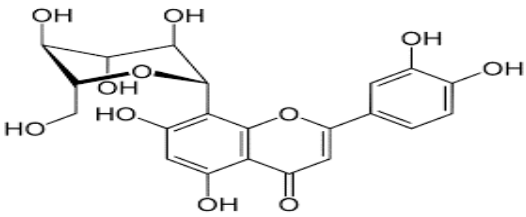
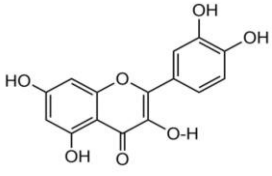
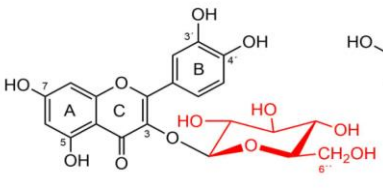
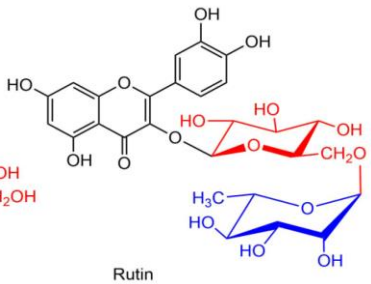
Flavonoids	Structure
Dihydrochalcones	 Aspalathin  Nothofagin
Flavones	 Luteolin  Chrysoeriol
	 Vitexin  Isovitexin
	 Orientin
	 Isoorientin
Flavonols	 Quercetin  Isoquercitrin  Rutin

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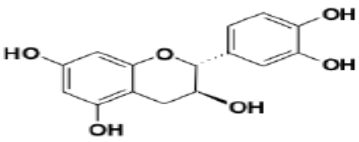
Flavanones	 (+)-Catechin

Table 2-4 Major phenolic compounds and oxidized derivatives in rooibos

Out of all the flavonoids of rooibos, two hydrochalcones: aspalathin and nothofagin, stand out as unique to rooibos. Joubert *et al.,* (2011), and other researchers agree that rooibos is the only natural source of aspalathin. Furthermore, Schulz *et al.,* (2003) and Von Gadow (1997) demonstrated that 43% of the total antioxidant capacity of rooibos can be attributed to aspalathin and nothofagin from acquous unfermented rooibos.

Rooibos is usually fermented by bruising it and leaving it in open air to allow it to undergo oxidation. This helps develop its characteristic colour, aroma and flavour. Consequently, the rooibos that has not undergone fermentation is called ‘green’ or unfermented rooibos. The process of fermentation, agricultural processes and processing methods in the manufacture of rooibos, notwithstanding genetic variation of the seeds used for propagation, impact the amount and type of flavonoids in rooibos. (Marnewick 2014). These flavonoids are the major contributers to the antioxidant, anti-inflammatory and possibly neuroprotective properties of rooibos and are thought to be the foundation for its health promoting properties (Snijman *et al*., 2009).

A review paper by Smith & Swart (2018), showed the results of an analysis of rooibos constituents done by Walters *et al.,* (2017). The report demonstrated the significant effect of fermentation on the content of phenolic compounds of rooibos. In the review, the greatest decrease was in the amount of aspalathin which was reported to significantly decrease in the order: unfermented; semi-fermented and fermented rooibos.

The fermentation process causes aspalathin to be converted to the eriodictyol-glucopyranoside isomers which are flavanones. Further oxidation of these isomers

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produces the flavone derivatives, orientin and iso-orientin (Smith & Swart 2018). Furthermore it was discovered that the fermantation process significantly reduced the oxidation of nothofagin to vitexin and isovitexin while Phenylpropenoic Acid Glucoside (PPAG) remained unaffected (Smith & Swart 2018). The present study used the unfermented rooibos extract.

6.3. Bioactivity and Antioxidant properties of rooibos

Numerous *in vitro* studies and reviews on the antioxidant activity of various types of rooibos extracts have been done (Grotewold 2006; Hoosen 2019; Jager & Saaby 2011; Kawakami *et al,* 1993; Smith & Swart 2018). The results have shown that it possesses potent antioxidant properties. The antioxidant activity of rooibos has been investigated since von Gadow *et al.,* (1997) showed that it has higher α, α-diphenyl-β-picrylhydrazyl (DPPH) radical scavenging activity than black and oolong teas.

Furthermore, rooibos tea has been shown to possess potent antimutagenic (Marnewick *et al.,* 2004; Marnewick 2014), cancer-modulating (Marnewick *et al.,* 2009) and antioxidant activities via the regeneration of coenzyme Q10 (Kucharska *et al*., 2004).

In an *in vivo* study on the antioxidative effects of rooibos in immobilization- induced oxidative stress on rat brain, Hong *et al.,* (2014) concluded that rooibos tea has the ability to (i) reverse the increase in stress-related metabolites (5-HIAA and FFA), (ii) prevent lipid peroxidation, (iii) restore the stress-induced protein degradation, (iv) regulate glutathione metabolism (GSH and GSH/GSSG ratio), and (v) modulate changes in the activity of antioxidant enzymes (SOD and CAT). In addition, other studies on the antioxidative effects of rooibos have gone further to show the action of particular flavonoids in reducing the effects of oxidative stress, as described in the following section.

6.3.1. Aspalathin and Nothofagin

Aspalathin has been shown to have antioxidant activity similar to α-tocopherol (Vitamin E), BHA (Butylhydroxyanisole) and BHT (Butylhydroxytoluene) (von Gadow et al,

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1997). It has been demonstrated using quantitative real-time PCR results that aspalathin has the ability to reduce endogenous intracellular level of ROS by targeting stress and ageing related genes (Hoosen 2019). Not much is known of nothofagin, however it has a similar structure to aspalathin and may have similar antioxidant capabilities (Erickson 2003). In a review paper, Smith and Swart (2018) reported that nothofagin and aspalathin have anti-inflamatory properties. They (Aspalathin and Nothofagin) were shown to reduce levels of TNF-α, IL-6 and NF-kB.

6.3.2. Orientin and Rutin

These are two of the most abundant monomeric flavonoids in rooibos:

Orientin is a strong free radical scavenger (Erickson 2003). In a research by Vrinda & Devi (2001), human blood was exposed to radiation and orientin was shown to reduce the number of cancer-associated changes on blood cells by half. In addition, another study showed that orientin inhibited lipid peroxidation in the liver of mice exposed to radiation. The same study also demonstrated the reduction of damage to the bone marrow and gastrointestinal tract by orientin (Devi *et al.,*1999; Devi *et al.,* 2000). Smith and Swart (2018) cited a study of rat model of colorectal cancer which showed that orientin, was linked to the lowering of TNF-α, IL-6 and NF-kB, as well as inflammatory enzymes iNOS and COX-2.

Rutin on the other hand seems to help maintain the strength of capillary walls. Supplements of rutin (o-(beta-Hydroxylethyl)-rutoside) have been used to treat hemarrhoids, vericose veins, and the lower leg edema associated with venous insufficiency and venous hypertension (Erickson 2003).

6.3.3. Quercetin and Luteolin

These compounds are potent antioxidants which have been shown to initiate apoptosis in cancer cells. Quercetin has particularly been shown to decrease primary tumor growth and stopped the metastasis of a model of pancreatic cancer (Mouria *et al.,* 2002). Furthermore luteolin and quercetin have been able to inhibit the proliferation of thyroid and colon cancer cells, respectively, albeit, *in vitro.* Qurcetin's mode of action seems to involve inhibition of COX-2 (Mutoh *et al.,* 2000). Both flavonoids are potent inhibitors of the formation of lipid peroxides. (Erickson 2003).

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The antioxidation and anti-inflammatory properties of flavonoids have already been established. rooibos being one of the plants with great amounts of flavonoids has been shown through numerous studies to have positive health benefits. The potency of Rooibos is used in multiple target mechanisms: via antioxidant, anti-inflammatory and anti-diabetic effects, as well as modulation of the immune system, adrenal steroidogenesis and lipid metabolism (Smith and Swart 2018). Therefore, rooibos has the potential of mitigating neurodegenerative diseases via modulation of neuroinflammation. It therefore can provide neuroprotection- which refers to the strategies and relative mechanisms able to defend the central nervous system (CNS) against neuronal injury due to both acute (e.g. stroke or trauma) and chronic neurodegenerative disorders (e.g. Alzheimer's disease and Parkinson's disease). The present study seeks to evaluate the therapeutic potential of rooibos. Table 2.5 is a summery of some of the published works on rooibos. The foregoing discussion of rooibos the table below show that most of the researches on rooibos are centered mostly on its antioxidant and anti-inflammatory nature. There is therefore a paucity of research on its neuroprotective actions.

Type of investigation	**Demonstrated effects**	**Reference**
Observational Studies	Rooibos preparation affects polyphenol content, and few consumers consume the ‘optimal rooibos cup’	(Piek *et al.*, 2019)
	Aqueous rooibos extract attenuated LPS-induced liver injury via inhibition of pro-inflammatory. cytokines and oxidative stress	(Ajuwon, Oguntibeju and Marnewick, 2014)
	mixtures of *Camellia sinensis* and *Aspalathus linearis* and soybean were compounded from Ginkgo. Only the tea and rooibos formula were the most efficacious at wrinkle reduction	(Chuarienthong et al, 2010)
Traditional and folk use	Traditionally rooibos in South Africa is used for the treatment of infantile colic, allergies, skin	(Van Der Merwe *et al.*, 2015)

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	problems, asthma Colds and influenza, stomach ailments, bitter tonic, analgesic, anthelmintic, traditional inhalant for a blocked nose Antispasmodic, traditional milk, health drink	(Joubert *et al.*, 2008) (Wyk and Gorelik, 2017) (McGaw, Steenkamp and Eloff, 2007)
Animal Studies	In an *ex vivo* experiment, rat kidneys perfused with physiological saline solution, were injected with nothofagin which was demonstrated to protect against systemic hypertension via the direct relaxation of renal arteries.	(Marques *et al.*, 2020)
	In an *in vitro* experiment, aspalathin showed cytoprotective effects on rat pancreatic β cells exposed to streptozotocin, hydrogen peroxide, or chronic high glucose. This effect was associated with increased translocation of NRF2 and expression of its antioxidant target genes Hmox1, Nqo-1 and Sod1.	(Moens *et al.*, 2020)
	Male adult rats were injected subcutaneously with nicotine and then treated with rooibos and melatonin. The co-treatment exerted beneficial vascular effects in the rats. This was associated with increased antioxidant enzyme activity.	(Schalkwyk et al, 2020)
	Aspalathus linearis extract pre-treatment protected Wistar rats exposed to methanol extract of diesel exhaust particles, against cardiovascular toxicity.	(Lawal *et al.*, 2019)
	Rooibos lowered cholesterol levels in control rats while co-treatment reduced ART-induced cardiovascular effects in isolated rat hearts.	(Webster *et al.*, 2019)

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	Afriplex GRT™ (an aspalathin-rich green rooibos extract)was shown to be anti-inflammatory on mice subjected to a high-fat diet.	(Layman *et al.*, 2019)
	Vervet monkeys were put on a 28-day treatment with Afriplex GRT. This resulted in the amelioration of hyperglycemia, lowered LDL cholesterol in diabetics, raised HDL cholesterol in non-diabetics, and reduced oxidative stress overall	(Orlando *et al.*, 2019)
	Long-term oral treatment of male rats with a fermented rooibos infusion improved their long-term spatial memory tested in a Morris water maze. The mechanism of rooibos in this context was associated with increases in striatal dopamine and its metabolite 3-MT levels.	(Pyrzanowska *et al.*, 2019)
	Treatment of mice with aspalathin and nothofagin provided protection against sepsis-triggered renal injury and reduced lethality	(Yang *et al,* 2018)
	Brain edema and neuronal apoptosis was significantly reduced after long-term consumption of fermented rooibos herbal tea by Wistar rats	(Akinrinmade *et al.*, 2017)
	Boar semen was supplemented with four concentrations of both fermented and unfermented rooibos extracts during 96 h of liquid storage at 17˚C. The extract enhanced sperm velocity, protected the acrosome structure, and preserved the membrane integrity during boar semen storage	(Ros-santaella and Pintus 2017)
	Orientin for rooibos was shown to have anti-proliferative and anti-inflammatory action against colorectal cancer in rats fed a high-fat diet and exposed to 1,2-dimethyl hydrazine. The mechanism is via the reduction of pro-	(Kalaiyarasu and Manju 2017)

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	inflammatory NF-κB expression and cytokines (TNF-α and IL-6)	
	Rooibos showed anti-inflammatory effects on LPS-exposed male Wistar rat, at systemic level, and when combined with palm oil, it exhibited an enhanced anti-inflammatory effect in the myocardial tissue.	(Ajuwon *et al.*, 2013)
Pharmacodynamic	Rooibos was analysed together with other plants both *in vitro* and *in vivo* to ameliorate against pathologies associated with oxidative stress (proliferation of cancer cells).	(Dabulici *et al.,* 2020)
	Infusions of rooibos and black tea showed free radical scavenging activities in vitro, increased GSH, SOD, and CAT activities in oxidative hepatic injury, and inhibited intestinal glucose absorption and α-amylase activities, and elevated muscle glucose uptake	(Xiao and Erukainure 2020) (Waisundara and Yian 2015)
	Rooibos tea reversed the increase in 5-HIAA and FFA, prevented lipid peroxidation, restored stress-induced protein degradation, regulated glutathione metabolism and changed SOD and CAT activities in immobilization-induced oxidative stressed rats showed that	(Hong *et al.,* 2014)
Literature Reviews	Rooibos as a socio-economically viable strategy to prevent and/or mitochondrial damage that results in fumonisin B1-induced generation of ROS	(Marnewick 2020)
	Antidiabetic potential of herbal tisanes	(Ajuwon *et al.*, 2018)
	Rooibos as therapeutic potential in the context of cardiovascular diseases	(Smith and Swart 2018)
	The potential cardioprotective properties of flavonoids and a phenylpropenoic acid found in	(Dludla *et al.*, 2017)

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	rooibos against diabetes-induced oxidative injury was reviewed

Table 2-5 Various research articles on rooibos

7.0. Methodological considerations: Zebrafish.

The prominence of zebrafish as an experimental animal has been growing rapidly since its establishment as a vertebrate model of development nearly 40 years ago (Phillips & Westerfield 2014). The zebrafish model has been described by Cassar, *et al.,* (2020) as a bridge between *in vitro* assays and mammalian *in vivo* studies. This section will summarise the merits and demerits of the model; explore the development of the model over the years and finally review scientific reports that have utilised the model thus far.

7.1. General description of Zebrafish

Zebrafish are a member of the *Cyprinidae* family which is comprised of more than 2000 species (Bozkurt 2020). Formerly it was called *Brachydanio rerio* but was later changed to *Danio rerio* in 1981 mainly because of the strong similarities between the two genera (Fang 2003). Zebrafish are small tropical freshwater fish found in Nepal, India, Bhutan, Northern Pakistan and South Asia (Bozkurt 2020). According to Hill, et al., (2005), zebrafish take 2-3 months to reach maturity and can produce 200-300 fertilised eggs weekly. Furthermore, they complete embryogenesis in 72 hours with pigmentation starting at about 30-72 hours post-fertilization. Moreover, adults of zebrafish reach about 2.5–4 cm in length, and have a transparent larval stage. Bozkurt, (2020) further describes the adult stage as having a stripe along the length of its body, and has a blue hue. Males are slender and torpedo-shaped, usually with a pink or yellow color.

7.2. Advantages of using Zebrafish

It has been established that zebrafish possess fully intergrated vertebrate organ system with a diverse repertoire of biological processes. This entails a much wider range of phenotypes can be assayed in zebrafish in contrast to cultured cells. (MacRae & Peterson 2015). Such disease phenotypes as pain, tumour metastasis,

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vascular tone are obsvervable in zebrafish but not in cell culture models. According to Phillips & Westerfield, (2014) zebrafish are as available and proliferative as cultured cells but present an *in vivo* and intricate vertebrate model system. Furthermore, the use of these fish reduces the usage of higher vertebrates in toxicity studies (Jayasinghe & Jayawardena 2019).

Besides offering what may be a reasonable compromise between physiological complexity and throughput, zebrafish have a fully characterised genome which revealed conserved functions of many genes that show orthologues to ~82% of disease-related genes in humans (Khan *et al.,* 2017; Phillips & Westerfield 2014) Santana *et al*., 2012). Furthermore, the larva-adult dual availability of zebrafish merit them for use in a wider spectrum of neurodegenerative-related phenotypes. (Santana *et al*., 2012).

When compared to mice, zebrafish develop faster, have a longer lifespan and are cheaper to buy and maintain. In addition, Cassar *et al.,* (2020) reported that young zebrafish are transparent, therefore making it easy for noninvasive examination of organ development and toxic ends. Moreover, they can be genetically modified much easier than rodents, thereby making them a better choice for studying mechanisms underlying neurodegenerative diseases (Santana *et al.,* 2012). Furthermore, notwithstanding lingering controversies, the use of zebrafish laevae under 5 days post-fertilization (dpf) is not regulated under animal studies hence it does not require ethical clearance. Therefore zebrafish become the best alternative to animal testing. (Cassar *et al.,* 2020)

The zebrafish brain organisation shows high conservation of the basic human brain. It has similar key neuronanatomical and neurochemical pathways to the human brain.This phylogenetical proximity makes zebrafish recognizable as a dependable model of human behaviour, neural circuitry and neural disease. (Santana *et al.,* 2012). For example the medial pallium of the zebrafish contains structures that are homologous to the mammalian amygdala-a structure at the centre of affective

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processing and emotionality in humans (Khan *et al.,* 2017). This has placed the zebrafish at a meritorious position in the study of social anxiety disorders and drug abuse. (Stein *et al.,* 2002).

In addition, the main neurotransmitter circuits found in humans, such as the glutamatergic and GABAergic have been shown to occur in zebrafish. Moreover, neurotransmitters such as GABA, dopamine, serotonin, histamine, glutamate and acetylcholine have also been reported (Santana *et al.,* 2012).

Also, present in zebrafish are the mamallian cellular types like: microglia (Avila *et al.*, 2007), oligodendrocytes, motor neurons (Westerfield *et al.,* 1986), astrocytes, myelin (Yoshida & Macklin 2005),and purkinje neurons (Koulen *et al.,* 2000). Again and interestingly, zebrafish possess a tight junction based blood-brain barrier, with considerable, and extremely controlled macromolecule permeability, a fact essential to the search for novel neuroprotective compounds (Santana *et al.,* 2012).

7.3. Challenges of zebrafish as an animal model

The most popular method of treating zebrafish is by solubilising chemicals in water (in-water-dosing). According to Cassar *et al.,* (2020), this poses two issues: 1) Chemicals with poor absorption or solubility will have to be injected into the fish thereby limiting the pool of chemicals that can be tested in high thoroughput screening; 2) the method may yield unique exposures as compared to typical mammalian routes due to the fish being immersed in the treatment solution. The following table summerises the advantages and disadvantages of using zebrafish in pharmacology assays. (Ali *et al.,* 2011)

Advantages and Disadvantages of the Use of Zebrafish in Biomedical Research	
Feature	**Benefit/Drawback**
Advantages	
Easy maintenance	Low housing costs

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Year round spawning	Research can run continuously
High fecundity (300–600 by single female at one time)	Low cost per assay
Optical transparency of early stages	Real-time (live) imaging of developmental processes and easy selection of precise developmental stages (in contrast to mammals)
Swimming begins at hatching (48–72 hpf) and more complex behaviour (food seeking) at 5 dpf	Behavioural studies can be made on very early stage
Very rapid development	Large number of experiments possible in short time period
Fertilization is external	Embryos accessible noninvasively, can be continuously imaged; there is no placental barrier or maternal compartment to influence drug experiments
Minimal parental care	Reduced epigenetic parental influence on experimental outcome
Mutants available, genome sequenced, morpholino knockdowns possible	Genetic basis of teratogenesis can be investigated
Animal protection laws often less stringent for zebrafish embryos than for mammals	Fewer legal restrictions on research
Eggs develop in nonsterile, simple buffers	Easy to raise and maintain embryos
Genome has important similarities to human (e.g., nearly all mammalian genes have a zebrafish counterpart; high conservation of key developmental genes with the human)	Common molecular pathways can be studied

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Very small size of early embryos	Only very low quantities of expensive test drugs and staining reagents needed; suitable for high throughput screening in 96 and 384 multiwall plates
Small egg size and external fertilization	Very precise control of drug delivery and dosage
Early embryo is permeable to small compounds	Suitable for drug testing
Disadvantages	
Last common ancestor with humans was 445 million years ago	Far more remote from humans than other animal models such as rodents (which have a 96-million-year divergence time from humans)
Exothermic (cold-blooded)	Physiology not identical to humans
Anatomical differences with human(e.g., lack of heart septation synovial joints, cancellous bone, limbs, and lungs).	Several human ethanol disorders are difficult or impossible to model in this species (e.g., cardiac septation defects)
Genome duplication	Many genes present as two copies, creating extra work to determine functional roles
Presence up to 48 hpf of the chorion	Interference with drug diffusion

Table 2-6 summary of the advantages and disadvantages of using zebrafish in pharmacology assays. (Ali et al., 2011)

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Despite the few challenges faced in zebrafish experimental models, a literature survey done by (Cassar *et al.*, 2020) shows a steady growth in scientific publications mentioning zebrafish. Publications have grown 4 fold in the past 10 years as presented in the figure 2.15 below.

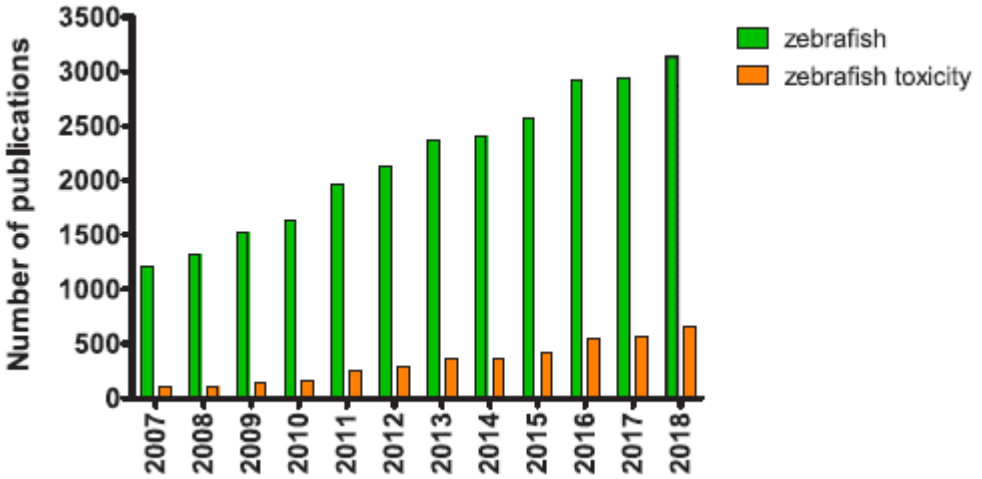

Figure A

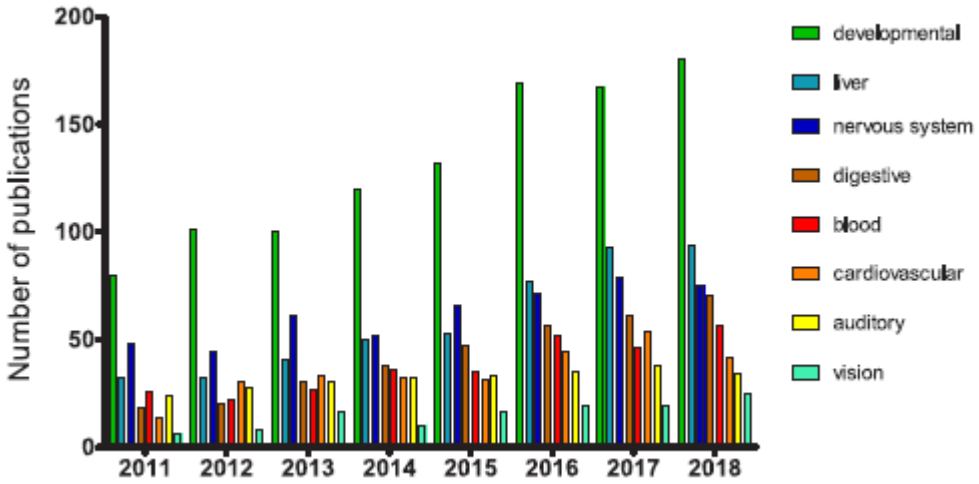

Figure B

Figure 2-14: A shows the steady growth of publications on zebrafish for toxicology. B shows various systems used for toxic end points and the number of such publications has been on an upward trend (Cassar et al., 2020)

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The arguments for the use of zebrafish model in understanding various disease phenomena are overwhelming. In the current study, the interest is in neurodegenerative diseases.

8.0. Conclusion

Based on the literature reviewed, the intricate relationship between neurodegenerative diseases and neuroinflammation is overwhelming. Moreover, oxidative stress has been implecated as one of the potential common etiology in various neurodegenerative diseases causing neuronal damage. Impairment of the DNA repair system and mitochondrial dysfunction. Also from the review, it can be gathered that polyphenols act as antioxidants and have cytoprotective effects.

Rooibos has been widely demostrated as one of the herbal plants endowed with flavonoids and a substantial number of articles have been published with regards to its antioxidant potential. However, current literature does not give information on the neuroprotective capacity of rooibos.

8.1. Problem Statement

Rooibos is arguably one of the most researched herb and substantial evidence exists demonstrating its efficacious effects in various disease etiologies – especially as an antioxidant in the context of neurodegenerative diseases – a paucity exists in literature on its capacity in neuroprotection and the mechanisms involved. It will be greatly beneficial to harness the therapeutic potency of rooibos in the treatment of neurodegenerative diseases.

8.2. Hypothesis

It is hypothesised that green rooibos extract with its rich endowment of flavanoids plays a neuroprotective role in the development and progression of neuroinflammation.

8.3. Aim

Given the paucity of information regarding the neuroprotective capacity of rooibos, we aimed at evaluating the neuroprotective effects of rooibos and the possible mechanisms involved.

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8.4. Objectives

To achieve the aim, the following objectives were formulated

1. To characterise green rooibos in terms of its antioxidant properties (DPPH, ORAC or X/XO assays
2. To evaluate the neuroprotective potential of green rooibos extract in rat neuroblastoma cells against hydrogen peroxide-induced toxicity using the mitochoTdrial activity (XTT) and intracellular ROS production assays
3. To assess the neuroprotective effects of green rooibos extract in astrocytes exposed to LPS by use of a cytokine (MCP-1) ELISA test.
4. To investigate the neuroactive potential of rooibos in terms of its capacity to inhibit key neuroenzymes, namely MAO-A
5. To probe a potential anxiolytic mechanism of of action for green rooibos extract in an *in vivo* zebrafish model.

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Chapter 3 – Materials and Methods

Introduction

Aspalathus linearis contains several flavonoids including the two unique antioxidants the dihydrochalcone-*C*-glucosides, aspalathin and nothofagin, (Snijman *et al*., 2009). Furthermore, it also contains some phenolic compounds of which some of them are the flavones, orientin, isoorientin, vitexin and isovitexin as well as the flavonols, rutin and isoquercitrin. The potential of *A. linearis* to exhibit neuroprotective properties was evaluated through a series of experiments. Firstly, preliminary experiments were done. The aim being to:

- Assess the antioxidant capacity of rooibos by comparison to known antioxidants. To achieve this, the ORAC, DPPH, and Superoxide radical scavenging assays were employed.
- Evaluate the risk of cytotoxicity of the rooibos extract on a neuronal (Neuro-2a) cell line and in the process identify the optimal concentration of rooibos that is not cytotoxic to cells.
- Investigate the neuroprotective capacity of the rooibos extract on Neuro-2a cells after exposure to hydrogen peroxide (a cytotoxic agent) via the XTT assay.
- Probe the potential mechanisms by which the extract may confer cytoprotection. This was inferred by employing the DCF method for the detection of intracellular ROS and the monoamine oxidase A (MAO-A) activity assay.

The results from the experiments above were then used to inform the assessment of the neuroprotective capacity of rooibos extract on human astrocytes exposed to LPS. Furthermore, a measurement of cytokines was done on the supernatant using the enzyme-linked immunosorbent assay (ELISA).

Moreover, to further confirm the *invitro* assessments, an *in vivo* Zebra fish model was used to investigate the neuroprotective capacity of the rooibos extract.

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3.1. Characterisation of the rooibos extract

The Green rooibos extract (Green OxithinTM) was generously donated by Professor Roy van Brummelen (Institute for Pharmaceutical Services (Pty) Ltd, Pretoria, South Africa). A proprietary method was employed to extract the unfermented *Aspalathus linearis* leaves in 65% ethanol by a natural product extraction specialist company (Brenn-O-Kem (Pty) Ltd, Wolseley, South Africa). All solvents were subsequently removed using appropriate industrial methods and the product was tested to confirm compliance to industry standards. The moisture content of the extract was 0.1 % and total polyphenol content was 41.1 % according to the Folin method (Singleton 1985).

3.1.0. Antioxidant capacity:

3.1.1. ORAC Assay

The oxygen radical absorbance capacity (ORAC) assay is an evaluation of the radical chain breaking capacity of antioxidants by monitoring the inhibition of peroxyl radical–induced oxidation of a florescent probe. The method measures hydrogen atom donating ability of antioxidants, and is, therefore, a hydrogen atom transfer (HAT)-based method. A free radical initiator is used to produce the peroxyl radicals which result in a loss of fluorescent intensity of a florescent probe over time. Antioxidants present in the assay prevent the peroxyl radical oxidation of the fluorescent probe until the antioxidant activity in the sample is used up. The remaining peroxyl radicals quench the fluorescence of the fluorescent probe. Typically, the reactions are allowed to run to completion and a fluorescence decay curve, usually represented as the area under the curve (AUC), is then obtained and correlated to the antioxidant capacity of the sample (Brescia 2012). The AUC is employed to quantify the total peroxyl radical antioxidant activity in a sample and is compared to an antioxidant standard curve of the water-soluble vitamin E analog Trolox™.

The ORAC assay employed to measure the capacity of *Aspalathus linearis* extract was one according to the method described by Davalos *et al.,* (2004). In brief, the reaction was perfomed in 75 mM phosphate buffer (pH 7.4). A final assay mixture of 200 µl was made. Antioxidant (20 *µ*l) and fluorescein (120 *µ*l) solution, with a final concentration of 70nM was used as the oxidizable substrate while 2,2'-Azobis (2-

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aminopropane) dihydrochloride (AAPH) (60μl, 12 mM final concentration) was used as the oxygen radical generator.

The antioxidant and fluorescein mixture was placed into a microplate and incubated for 15 minutes at 37°C, following which AAPH was rapidly added using a multichannel pipet. The microplate was then immediately placed in the reader and the fluorescence recorded every minute for 80 minutes with intermittent automatic shaking of the microplate prior to each reading. A phosphate buffer was used as a blank (control) and eight calibration solutions using Trolox (1-8 μM, final concentration) as antioxidant were also carried out in each assay. All the reaction mixtures were prepared in duplicate and at least three independent runs were performed. Fluorescent measurements were normalized to the curve of the blank (no antioxidant).

3.1.2. DPPH

The antioxidant activity of the plant extracts was determined using a method commonly known as the DPPH Antioxidant assay, mainly because DPPH (2, 2 – Diphenyl – picrylhydrazyl) is stable and can be used to determine the antioxidant capacity of several matrices (Molyneux 2004). The protocol employed was described by López *et al.,(*2007) where ascorbic acid was the positive control while methanol added to distilled water served as a blank control.

The method employs 2,2-diphenyl-picrylhydrazyl (DPPH), a stable free radical which has a delocalized spare electron over the whole molecule preventing it from dimerization as observed in most free radicals. (Kedare *et al*., 2011). This delocalisation results in a deep violet colour that absorbs at 520 nm in ethanol solution. Upon mixing DPPH with a reducing agent (Hydrogen donor), its colour changes to pale yellow (reduced form) and then can be spectrophotometrically measured at 517 nm (Santos *et al.*, 2017).

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The protocol in brief: One hundred and fifty microlitres of the *A. linearis* methanol extract, concentrations were mixed with 150 μL of a DPPH methanolic solution (0.04 mg/ml). Following which, absorbance was measured at 517 nm after 30 min of incubation at room temperature. The scavenging capacity was given as percent (%) DPPH scavenged, calculated as:

$$\frac{optical\ density\ of\ Control - \ optical\ density\ of\ compound}{optical\ density\ of\ control}\ X\ 100$$

The ability of *A. linearis* extract to scavenge DPPH• was compared with ascorbic acid.

3.1.3. Superoxide radical Scavenging Assay

The superoxide radical scavenging activity of *A. linearis* was measured by the reduction of NBT (Nitroblue tetrazolium) according to a previously reported method (Rodríguez-Chávez *et al.,* 2015). The system xanthine oxidase was employed as a superoxide radical (O_2^-) generator. A concoction of 90 μM xanthine, 16 mM Na_2CO_3, 22.8 μM NBT, and 18 mM phosphate buffer (pH 7.0) with a final volume of 800 μL was mixed with different concentrations of *A. linearis.* To start the reaction, 100 μL of xanthine oxidase (168 U/L) was added. The absorbance at 295 nm (for uric acid production) and 560 nm (for O_2^- in the assay system) was measured against an appropriate blank to determine the quantity of formazan generated. Vitamin E was used as the control.

3.2. *In vitro* assessments:

3.2.1. Cytoprotective activity in Neuro-2a cells

Cell culture

Rat neuroblastoma cells (Neuro-2a) were acquired from the ATCC (ATCC® CCL-131™) and were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% gentamicin, and maintained in a 100% relative humidified atmosphere of 5% CO2 at 37°C. Neuro-2a cells were seeded in flasks for stock maintenance and in 96-well plates for experiments. DMEM was regularly replaced every two or three days and cultures split once they reached 80% confluence.

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Cell treatments

Cells were treated with green rooibos extract in a wide range of concentrations from 0-1000 µg/mL for 24h in order to detect cytotoxic and non-cytotoxic concentrations. After this, cells were pre-treated with 4 different concentrations of extract (12.5-25-50-100 µg/mL) for 24 h prior to hydrogen peroxide exposure (125 or 250 µM) for 24 h. Rooibos extract was dissolved in DMEM with 1 % FBS and filtered for experiments. The potential protective effects were explored between 12.5-100 µg/mL because these concentrations were not toxic to the cells and represented an amount of flavonoids that could be physiologically relevant.

Cell viability: MTT assay

The MTT assay was used as a predictor of cell survival and mitochondrial activity (Mosmann, 1983). After treatments, cells were incubated with MTT solution (2 mg/mL) for 3 h at 37 °C. Successively, the medium was replaced with DMSO in each well to dissolve formazan crystals. Finally, absorbance was measured at 550 nm. All experiments were performed in triplicate and results expressed as percentage of control (100%).

3.2.2. Monoamine Oxidase Activity Assay

Two different concentrations of hydrogen peroxide were used to induce oxidative stress on neuro-2a (mouse neuroblastoma) cells in the presence or absence (negative control) of rooibos extract. The concentrations of H_2O_2 employed were 125 uM (only mildly toxic) and 250 uM (highly toxic). Thereafter cell survival was assessed using the XTT assay, which measures mitochondrial reductive capacity and therefore indirectly estimates cellular viability. Furthermore, the ability of the extract to inhibit monoamine oxidase A (MAO-A) activity, was also assessed. In the assay, MAO reacts with p-tyramine, a substrate for both MAO-A, resulting in the formation of H_2O_2, which is determined by a fluorometric method (λ_{ex}= 530/λ_{em}= 585 nm).

3.2.3. ROS Measurement

Measurements of intracellular ROS levels in neuro-2a cells were made using 2′,7′-dichlorodihydrofluoroscein diacetate (DCFH-DA) (Barzegar & Moosavi-Movahedi,

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2011). DCFH-DA is a fluorescent dye with the ability to readily diffuse across the cell membrane and become enzymatically hydrolysed by endogenous esterases and effectively remains trapped inside the cell. The de-esterified product (DCFH) of this hydrolysis is then oxidised by intracellular ROS mainly H_2O_2, $HO^{\bullet}$, $ROO^{\bullet}$, $NO^{\bullet}$ and $ONOO^{-}$ resulting in fluorescent DCF which stains the cells, hence providing a semi-quantitative measure of ROS generation. The method employed for this assay was described by LeBel *et al.*,(1992)

The mouse neuroblastoma cell samples were incubated in the presence of 10 mM $DCFH_2$-DA in phosphate buffered saline (PBS) at 37°C for 30 min, following which they were then washed twice with PBS and centrifuged at 1200 rpm to get rid of the extracellular $DCFH_2$-DA. The intensity of the trapped fluorescent dye (DCF) inside the cells is an index of intracellular ROS levels. It can be determined by fluorescence spectrophotometry values obtained by excitation and emission at wavelengths of 498 nm and 530 nm respectively.

3.3. Neuroprotective activity in Astrocytes

3.3.1. Cell culture

The study made use of human astrocytes, of low passage (< 10) obtained from Life Technologies (Cat#: N7805-100).

Figure 3.1 gives a time line from the thawing, propagation and general maintanance of cells:

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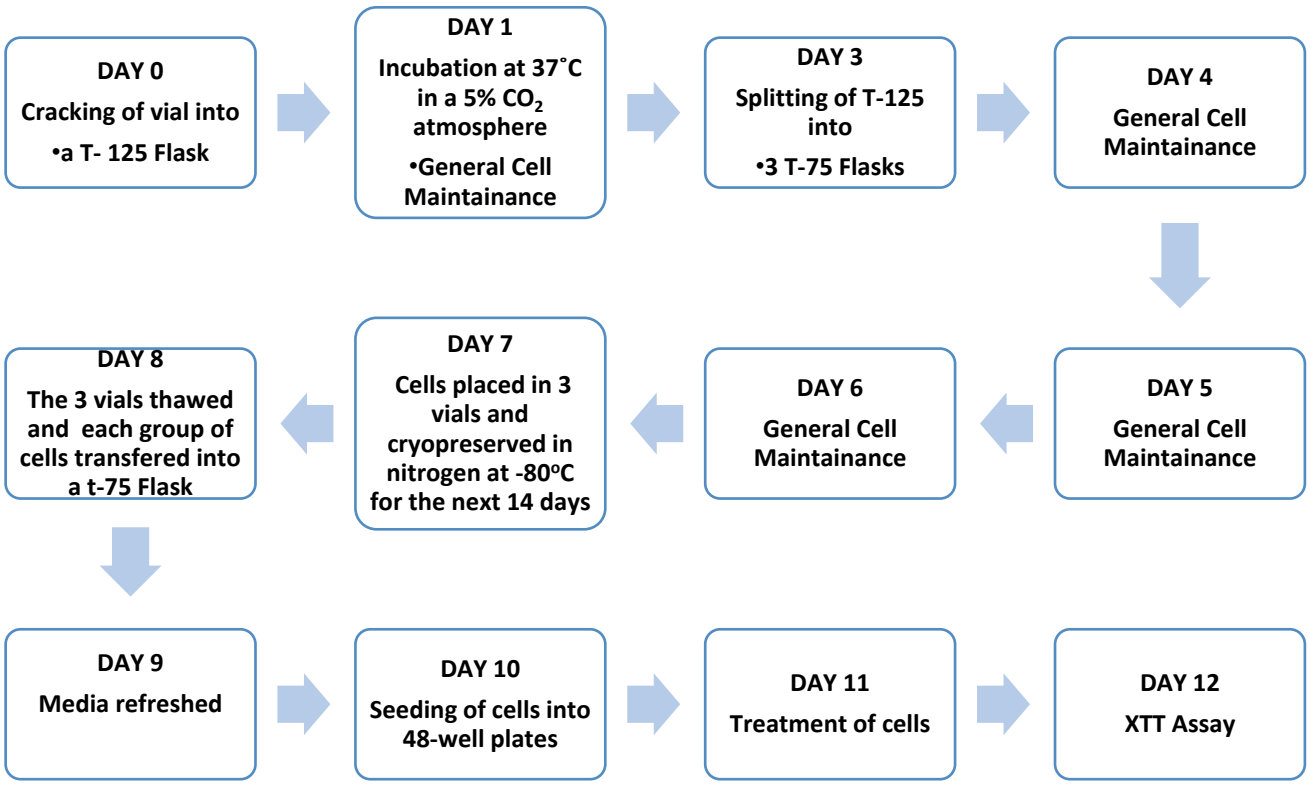

Figure 3-1 Cell culture maintenance timeline

To thaw the cells a volume of 4 ml of complete Dulbecco's Modified Eagle Medium (DMEM), containing 10% Foetal bovine serum (FBS) and 1% Penicillin-Streptomycin was preheated to 37^0C in a bead bath. Following this, the vial containing approximately 1 x 10^6 cells was removed from long-term liquid nitrogen storage and immersed in a pre-heated water bath (at 38^0C) until ice of about 2mm was visible in the vial. After spraying the vial with 70% alcohol the cell suspension was removed from the vial with a 1 ml pipette and slowly added into the pre-warmed medium in a T-75 flask. The cells were then incubated at 37˚C in a 5% CO_2 atmosphere for 24 hours to fully adhere to the plate.

To maintain cultures, cells were propagated to a confluence of 80% prior to experimental procedures. For the duration of the experiment, the growth medium was replaced every 48 hours. This was done by removing the media and washing the cells with 2ml phosphate buffer saline (PBS) before adding 2 ml of 0.25% trypsin to the flask

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and incubating for 5 minutes at 37 °C in a 5% CO_2 atmosphere. New media was added at a ratio of 2:1 (Media: Trypsin) to inactivate the trypsin before using a microscope to check for clumping. Any clumps observed were dissociated by gently pipetting about six times up and down using a 1000 µl micropipette. Each time when media was changed a 200 µl sample was taken and placed in a microcentrifuge tube for counting. While counting, the cells were transferred into a 15 ml tube and centrifuged at 800 rpm (100 g) for 5 minutes and the supernatant was aspirated using a Pasteur pipette. Thereafter the cell pellet was resuspended by gently pipetting up and down a few times in 10 ml of pre-warmed culture medium before transferring into a new T-75 flask.

When the cells reached 80% confluency, the three T-75 flasks were seeded into two 48 well-culture plates at a density of 1,5 x 10^4 cells/well in growth media and incubated for 24 hours to allow for full adherence to the plate.

3.3.2. Preparation of treatment solutions

Rooibos: A stock solution of a proprietary ethanol extract of *Aspalathus linearis* (Green Oxithin™) was prepared in dimethyl sulfoxide (DMSO) and vortexed for 5 minutes. This mixture was then diluted with prewarmed media and a serial dilution was done to give final working concentrations of treatments that were not more than 0.5% v/v DMSO. The final concentrations made were as follows: 800 µg/ml; 400 µg/ml; 200 µg/ml; 100 µg/ml; 50µg/ml.

Lipopolysaccharide

Hank's Buffered Salt Solution (HBSS) was used to prepare *E. coli* (L4391, Sigma-Aldrich) lipopolysaccharide (LPS) mixed with the media and the respective concentrations of rooibos extracts resulting in a final concentration of 20 µg/mL, for astrocyte inflammatory stimulation. This was determined to be the optimal concentration of LPS from a prior experiment done by Cásedas *et al.,* (2018).

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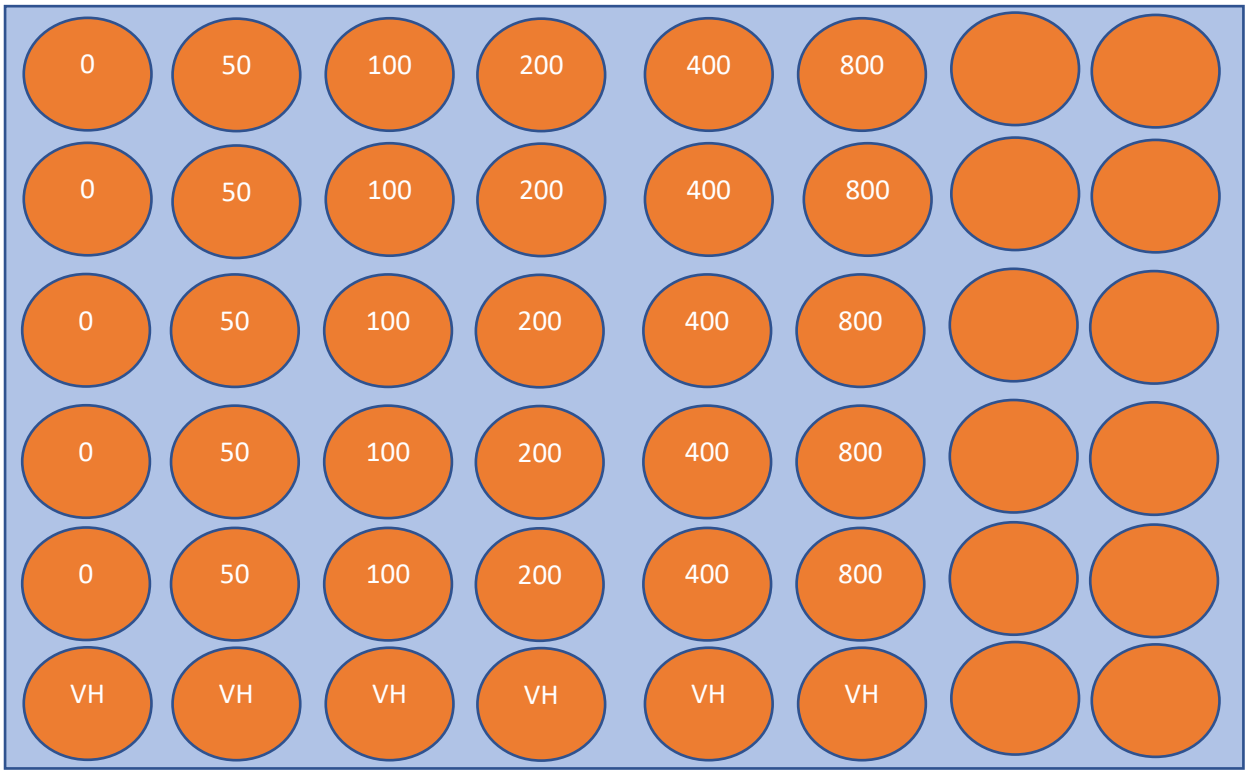
3.3.3. Rooibos extract treatment intervention

The astrocyte cell culture supernatant was aspirated from each of the 48-well plates. Thereafter, a 30-minute pre-treatment period was initiated, which involved the addition of the different treatments of rooibos to the respective wells. (Growth media only was added to the control and LPS-control groups). After 30 minutes, cells treated with the culture medium (0.5% DMSO) in the presence of LPS (20µg/ml) as illustrated in Figure 3.2. The plates were then placed into the incubator for a further 23.5 h. The supernatant was removed and at the end of the incubation period and stored at -80 °C for subsequent cytokine quantification. In addition, an XTT mitochondrial reductive capacity assay was performed on the fresh cells immediately after the end of the 24-hour incubation period.

a)



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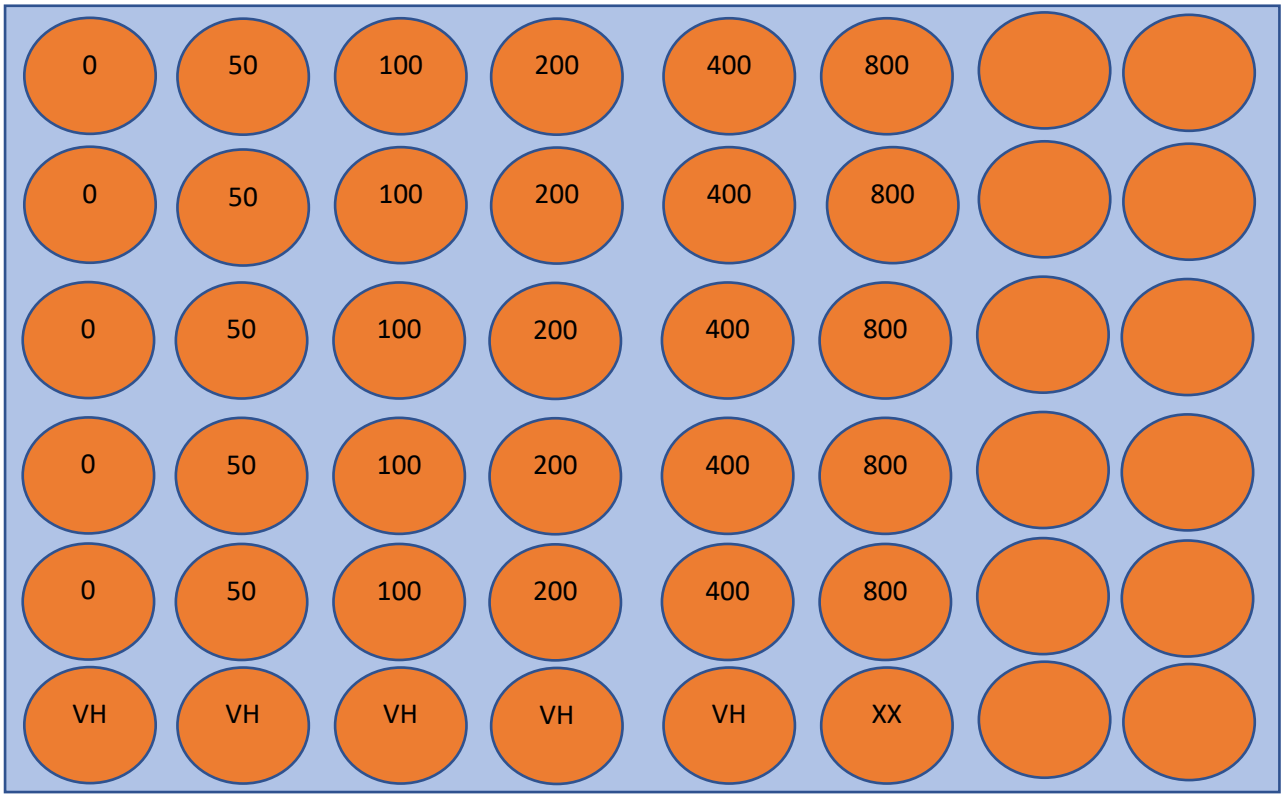

Figure 3-2 a) 48-plate with only rooibos and no LPS. b). 48 plate with rooibos at various concentrations in the presence of LPS at 20 µg/ml concentration. VH = Vehicle with media and LPS.

3.3.4 XTT Assay

The XTT (2,3-bis[2-methoxy-4-nitro-5-sulfophenyl] −2H-tetrazolium-5-carboxanilide) is a colorimetric assay for the nonradioactive quantification of cellular proliferation, viability, and cytotoxicity. The assay indirectly analyses cell viability by assessing mitochondrial reductive capacity. (Kuhn *et al.*, 2003). Its main advantage over other similar assays is its reduced assay time and sample handling, while offering equivalent sensitivity. (Roehm *et al.,* 1991)

Mitochondrial dehydrogenases, succinoxidase and cytochrome P450 systems, as well as flavoprotein oxidases of viable cells cleave the tetrazolium ring of XTT, yielding orange formazan crystals which are insoluble in aqueous solutions. The resulting orange solution is calorimetrically measured. An increase or decrease in cell number

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results in a concomitant change in the amount of formazan formed, indicating the degree of cytotoxicity caused by the test material, or as a measure of cell quantity.

The XTT assay was done following the 24-hour incubation period, and the removal of the supernatant from each well, for later analyses. A concentration of 1mg/ml of XTT (X4626, Sigma Aldrich) was prepared by dissolving it in DPBS at 37°C and shaken in a shaking incubator also at 37 °C. Following which PMS [N-methyl-dibenzopyrazine-methylsulfate/phenazine methosulphate (P9625, Sigma Aldrich)] was added to the DPBS/XTT mixture at a ratio of 5 parts PMS is to 1 part DPBS/XTT. This mixture was then added to media prepared for each of the 48 well plates such that each well would have 70 µl of XTT solution. XTT is light sensitive hence during the whole experiment, the XTT test tube was covered with aluminium foil to protect it from light.

The astrocyte monolayer was then washed twice with DPBS to remove residual rooibos treatment, following which the XTT/DPBS/PMS/medium mixture was added to each well. After covering the plates with Aluminium foil, they were then incubated at 37 °C and 5% CO_2 for 4 hours.

Following incubation, optical densities were determined at 490-nm using a Universal Microplate Reader (Bio-Tek Instruments, Inc. EL800).

3.5. Cytokine measurement

An enzyme-linked immunosorbent assay (sandwich-ELISA) was performed to determine the amount of MCP-1 and IL-β in the supernatant preserved from the cell culture experiment. Both the MCP-1 and IL-β ELISA were conducted using the Human immunoassay kit (Elabscience, USA) and the manufacturer's protocol was followed. The detection range of the MCP-1 assay was 62.50 – 4000 pg/mL (sensitivity: 37.50 pg/mL) and that of IL-β was 7.81 – 500 pg/mL (sensitivity: 4.96pg/mL). Absorbances were measured at 450nm using the Perkin Elmer Victor Nivo system.

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3.6. In vivo models – Zebra Fish Larvae

3.6.1. Ethics Statement

All procedures performed on zebrafish were approved by the Stellenbosch University Animal Research Ethics Committee (SU AREC) (reference # ACU/2019-11820).

3.6.2 Animals

Adult longfin zebrafish (*Danio rerio*) were housed in groups of 15-20 in 60 L tanks at a temperature maintained at 28°C and a pH between 6.8-7.3. Furthermore, the tanks had a biological filter and a circulating water system, and constant aeration. In addition, the fish were fed at 5% body mass/day of semi-floating micropellets (Hikari). Eggs were obtained after spawning males and females in a 2.1 ratio. This procedure was performed by a SAVC-registered permanent staff member unit. Eggs were suspended in E3 media (refer to next section for preparation) and incubated at 28.5 °C 4 days, refreshing media daily. Hatching of the eggs took 2 to 3 days. At 112 hpf larvae were used for the PTZ assay, as described below.

3.6.3. Reagents

The medium used (E3) Embryo media (E3) was prepared by making a 5X stock made up of 14.16 g NaCl; 0.65 g KCl; 2.20 g CaCl2; 4.05 g MgSO4 in E3 to give a concentration of 20 uM and the final concentration in each well was 4 µM. Furthermore, a final pH of 7.4, was obtained after the addition of 50 µL of 0.5 M sodium bicarbonate. Thereafter, a dilution ratio of 1 part of the 50x stock to 49 parts double distilled water was done and 200 µl of 0.05% methylene blue was added and the mixture was stored in an incubator at 28.5° C.

Pentylenetetrazole (PTZ) was procured from Sigma-Aldrich (P6500). A fresh working stock of PTZ (50 mM) was prepared by dissolving PTZ in the E3 medium. For a final concentration of 10 mM of PTZ, 200 µL of E3 was pipetted into each well before adding 50 µL of PTZ. For control wells, an additional 50 µL of E3 was added.

Diazepam (DZP) (5 mg/mL [17.56 mM]) was purchased on prescription, from the Stellenbosch university campus pharmacy. For use in the assay, the diazepam was diluted in E3 to a final concentration of 4 µM.

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3.6.4. The zebrafish PTZ induced seizure model

To induce experimental epileptic like seizures, larvae (n= 12) bathed in E3 media were individually exposed to 10 mM of PTZ in 48 well plates (Sun *et al.*, 2019). A control group was exposed to both PTZ and diazepam (which is a $GABA_A$ positive allosteric modulator) while the experimental group was placed in media with PTZ and increasing concentrations of the green rooibos extract (5, 10, 15, 20 and 25 µg/ml). Furthermore, a positive control group was only exposed to PTZ dissolved in E_3 media. All treatments were performed in triplicates and the well plates were incubated for 24 hours at 28.5 °C.

The locomotive behaviour of the zebrafish larvae was then tracked at 25 frames/second for 40 minutes at 28.5 °C using a Noldus DanioVision behavioural monitoring system and Ethovision software. The parameters for the analysis were total distance moved and the average speed of the larvae.

3.7. Statistical analysis

All antioxidant experiments were performed in triplicate and repeated on three different days. Data are expressed as mean ± SE (standard error). GraphPad Prism v.6 was used for data analyses, figures, non-linear regression and statistical analysis. Cell experiments were analysed using ANOVA followed by Dunnett's or Tukey´s multicomparison test.

Prism 5 software was used to analyse the data and generate graphs. A one-way and a two-way ANOVA was performed for the *in vitro* and *in vivo* data followed by the Bonferroni's *post-hoc* test. In all analyses, the significance level was taken as $p \leq 0.05$.

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Chapter 4 - Results

4.1. Pharmacological Characterisation of antioxidant and neuroprotective potency

The oxygen radical absorbance capacity (ORAC) value for the green rooibos extract was 8.1 µmol. TE/mg.

It was able to neutralize both DPPH (Figure 4.1) and superoxide radicals (Figure 4.2) and compared favourably to the potency of the assay reference standards.

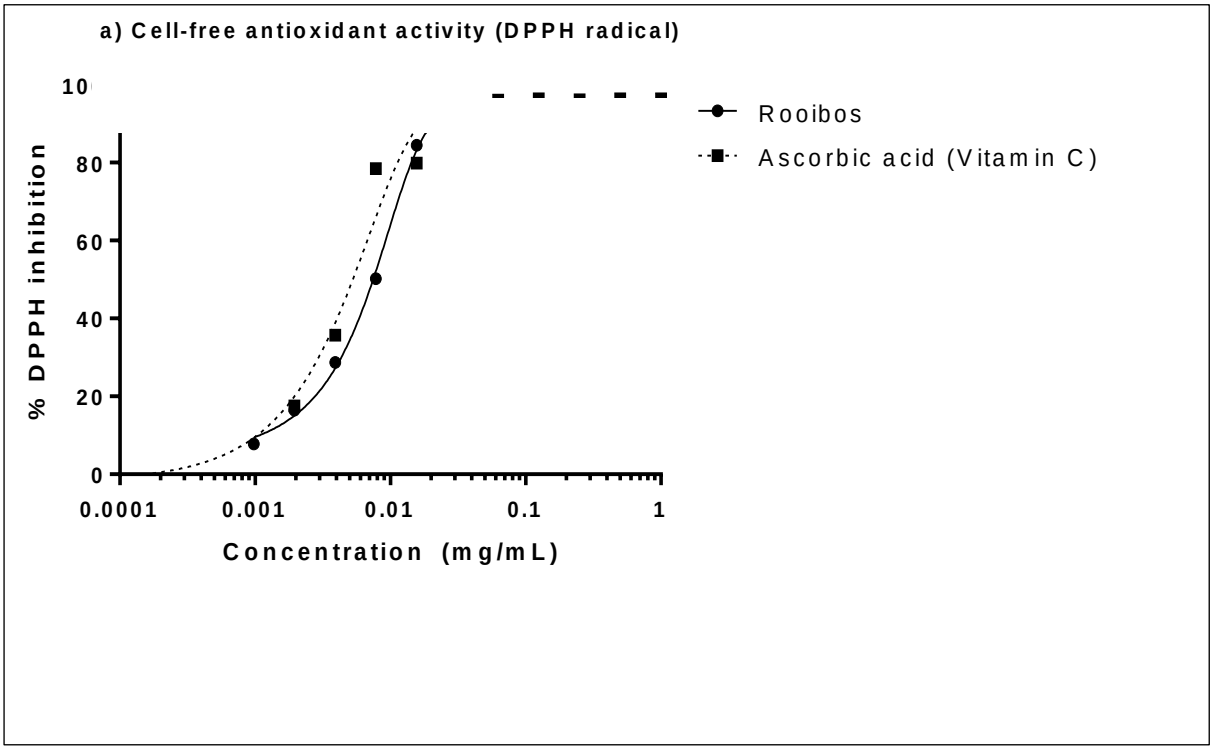

Figure 4-1 Antioxidant activity of green rooibos extract (in terms of DPPH scavenging capacity) compared to a reference, Vitamin C (Ascorbic acid)

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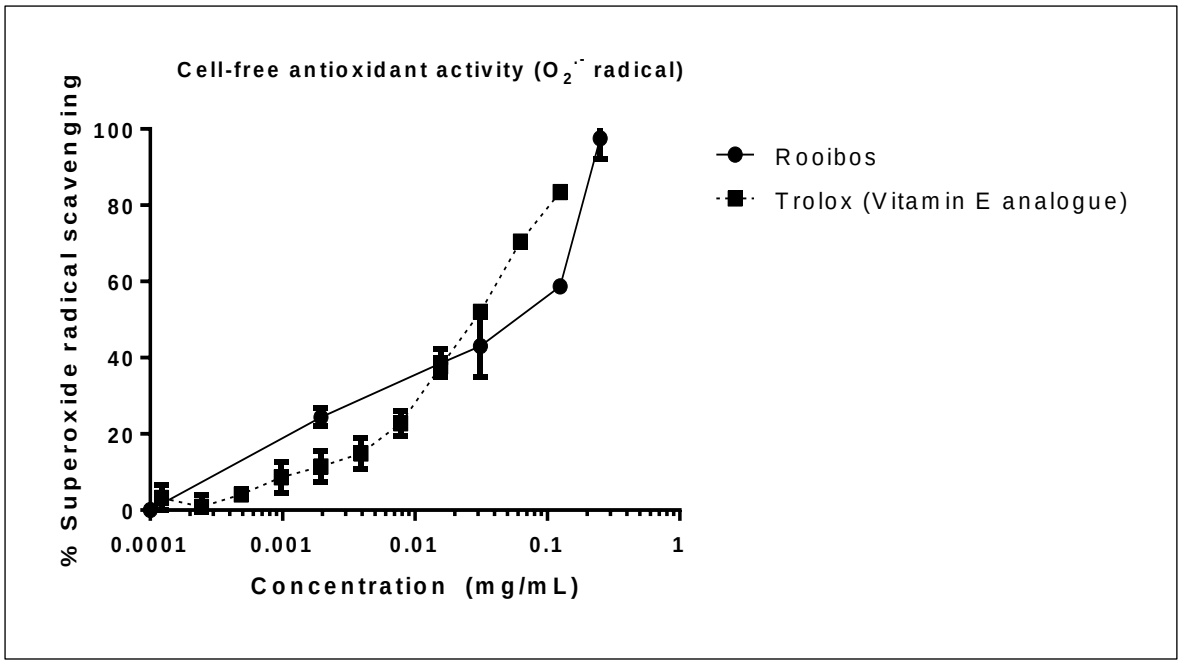

Figure 4-2 Antioxidant capacity of green rooibos extract in terms of superoxide quenching compared with Vitamin E analog (Trolox) (n=3) (mean ± SD/SEM)

In addition to the evaluation of the antioxidant capacity of rooibos extract, its neuroprotective potential was assessed in terms of its capacity to inhibit MAO-A. In this context, the extract showed a mild dose-dependent inhibition of MAO-A when compared to the reference selective inhibitor of MAO-A,clorgyline (Figure 4.3).

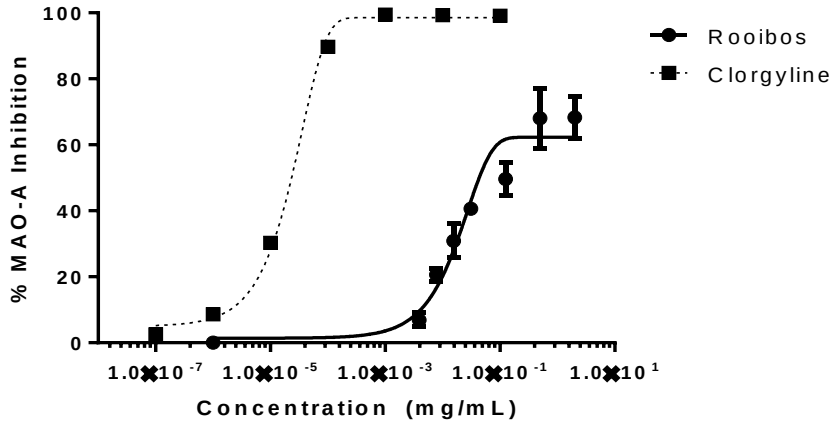

Figure 4-3. MAO-A inhibition by green rooibos extract compared to the reference inhibitor clorgyline

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4.2. In vitro results

4.2.1. Cytoprotective activity of rooibos extract in mouse neuroblastoma cells (Neuro-2a-**cells)**

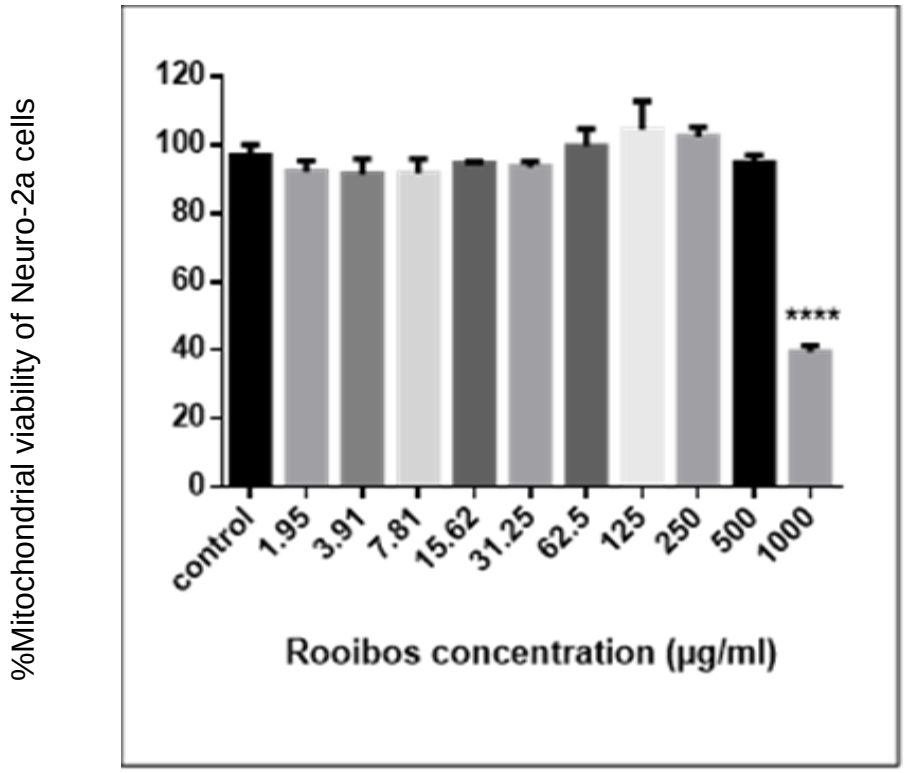

*Figure 4-3 Results of MTT assay showing cytotoxicity profile of rooibos extraction expressed as cell survival ****p<0.0001 (ANOVA and Dunnett's test)*

After 24 hours of exposure to green rooibos extract, Neuro-2a cells exhibited a (non-significant) dose-dependent increase in mitochondrial activity from 15,62 µg/ml, reaching a peak at 125 µg/ml. Thereafter a decrease occurred but was only significant at very high doses ($p<0.0001$) of the rooibos extract (1000 µg/ml) (Figure 4.4). Rooibos did not exhibit cytotoxicity except at very high doses (1000 µg/ml), where approximately 60% loss of viability was observed.

Furthermore, the XTT assay indicated significant cytoprotective effects of the green rooibos against an oxidative insult induced by hydrogen peroxide. (Figure 4.5). The cytoprotection conferred by rooibos was only statistically significant when the cells were pre-treated with 50 and 100 µg/ml of the rooibos extract for 24h with a lethal concentration of hydrogen peroxide (250 µM) (Figure 4.5A). However, no significant cytoprotective effects were exerted by the unfermented rooibos extract at a sub-lethal dose of 125 µM (Figure 4.4B).

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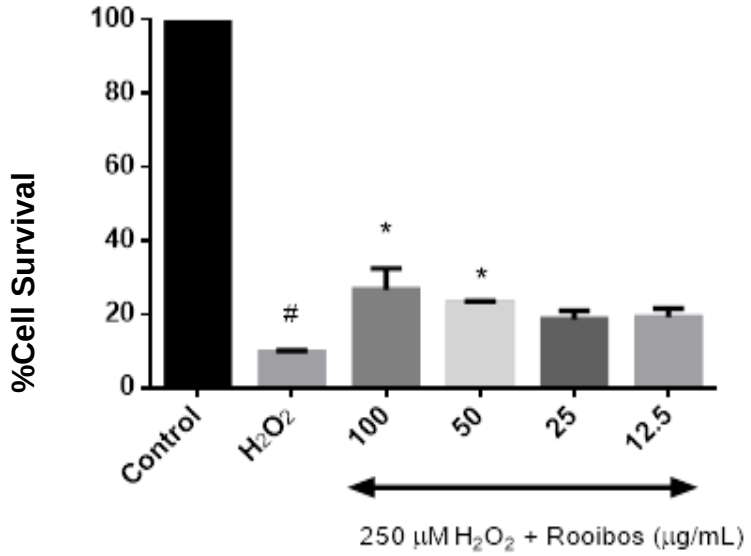

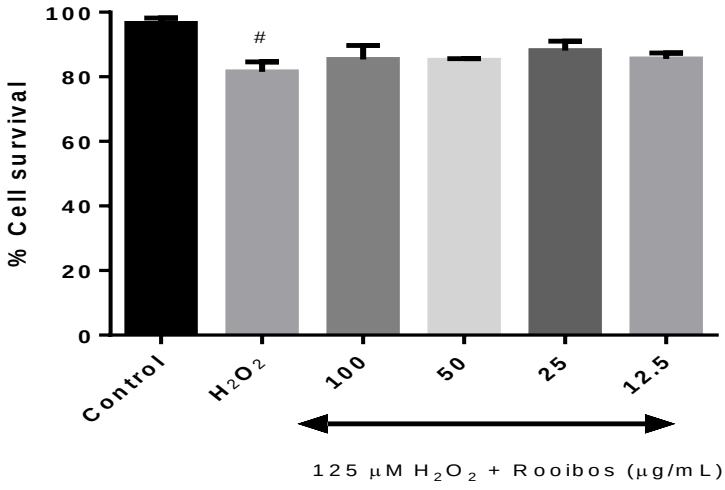

*Figure 4.4 Cytoprotective activity of green rooibos extract in mouse neuroblastoma (Neuro-2a) cells exposed to two different concentrations of hydrogen peroxide: a) a lethal oxidative dose of 250µM and b) sub-lethal dose of 125µM. Graph A shows that after 24 h of pre-treatment with rooibos extract cell survival increased from 9.7% to 26.7% at a rooibos concentration of 100 µg/mL and up to 23% at a concentration of 50 µg/mL (# $p < 0.0001$ versus control, * $p < 0.05$ versus hydrogen peroxide, ANOVA and Dunnett´s multicomparison test). Graph b shows no significant cytoprotection (from 81.5% in cells exposed to hydrogen peroxide to 85.3 and 85.1 % in cells pre-treated with 100 and 50 µg/mL of rooibos extract for 24 h)*

The cytoprotection mechanism of the green rooibos unfermented extract was evaluated in terms of ROS production in these cells. In the presence of both 125 and

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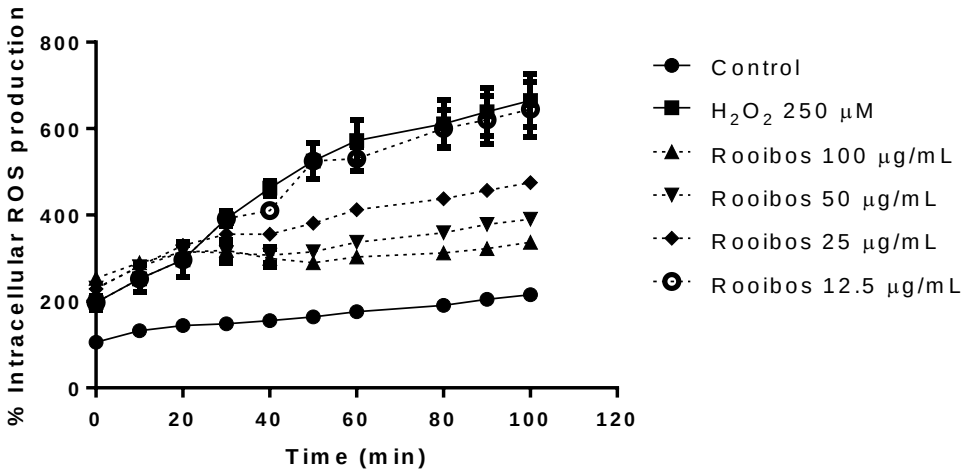
250 µM of H_2O_2 (Figure 4.5), rooibos exposure displayed a dose-dependent capability to limit intracellular ROS levels, suggesting this as potential cytoprotective mechanism in neuronal cells.

a)



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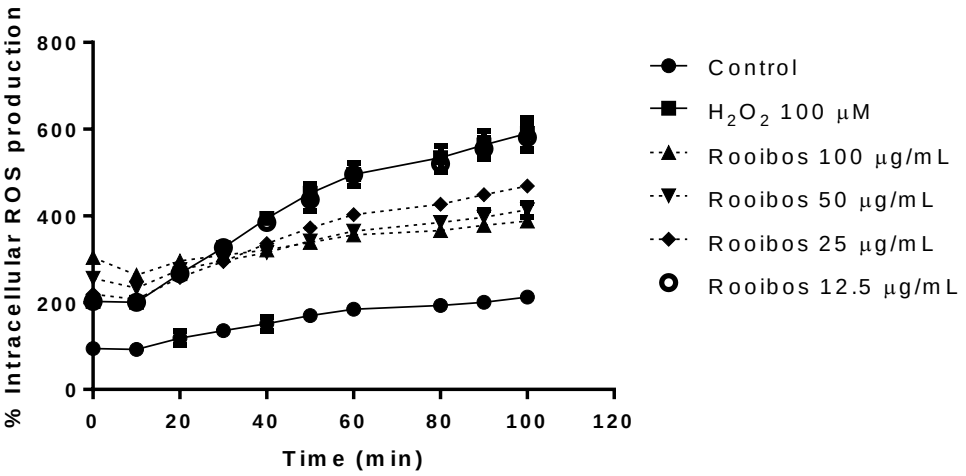

*Figure 4 5 The redox status of cells in terms of intracellular ROS production for Neuro-2a cells exposed to a) 125 µM or b) 250 µM H2O2 for 90 mins. Rooibos concentrations of 25; 50 and 100 µg/mL significantly reduced intracellular ROS production at 70; 80 and 90 min compared to cells treated with either 250 or 125 µM H_2O_2 only. (**** $p < 0.0001$, Two-way ANOVA and Tukey's multiple comparisons test).*

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4.2.2. Assessments on astrocytes

In terms of mitochondrial viability, in the absence of LPS, Rooibos did not elicit adverse effects at lower doses. The mitochondrial reductive capacity only significantly declined from a concentration of 400 µg/ml ($p<0.0001$) – an effect exacerbated by addition of LPS (Figure 4.6).

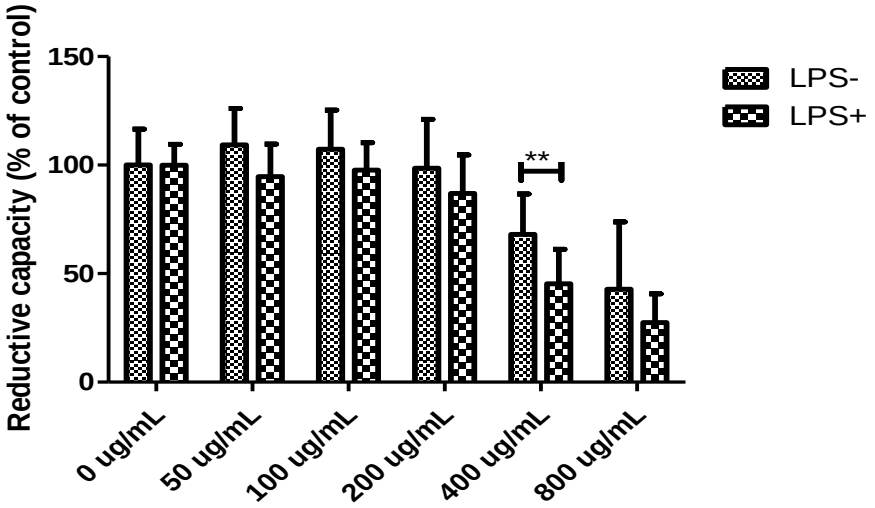

Figure 4-6 Cell viability determined by XTT assay, following treatment with different concentrations of green rooibos extract with or without LPS stimulation.

In terms of cytokine response, the results in Figure 4.8 illustrates a dose-dependent effect of rooibos to reduce the amount of MCP-1 produced in the absence of LPS. ($p<0.005$). A similar trend is also observed for the cells exposed to LPS.

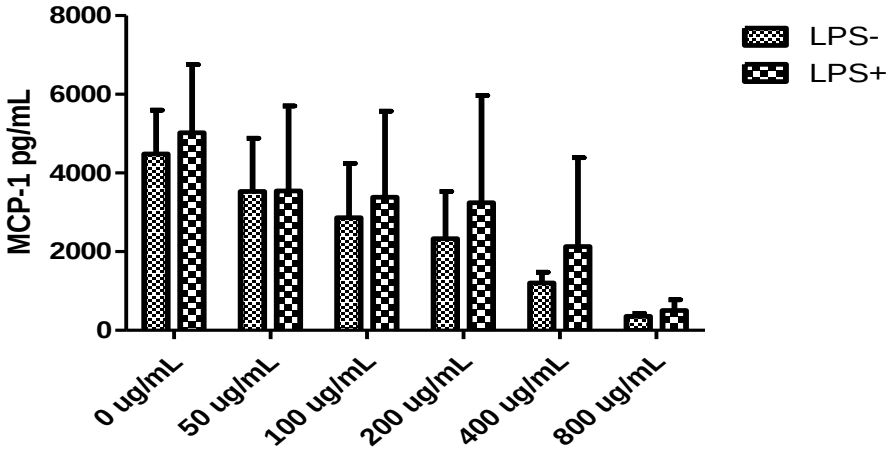

Figure 4-7 Effect of green rooibos extract with/without LPS stimulation, on pro-inflammatory cytokine (MCP-1 production by human astrocytes

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In terms of IL-1β secretion, all values were extremely low and deemed too close to the minimum detection threshold of the assay kit. Therefore, these results were not interpreted.

4.3. In vivo models – Zebrafish Larvae

To further probe potential neuroprotective mechanisms of green rooibos extract, a zebrafish PTZ (Pentylenetetrazol) model was used to assess effect of rooibos on gamma-aminobutyric acid (GABA) signalling.

In terms of model validation, the control PTZ-exposed larvae clearly exhibited the behavioural changes associated with experimentally induced epileptic-like seizures, as presented in terms of distance moved and the average velocities (Figures 4.9 and 4.10). In addition, the positive treatment control (Diazepam + PTZ) successfully prevented the action of PTZ. Together, this indicate that the model was correctly executed. Rooibos extract did not appear to have an effect in this model.

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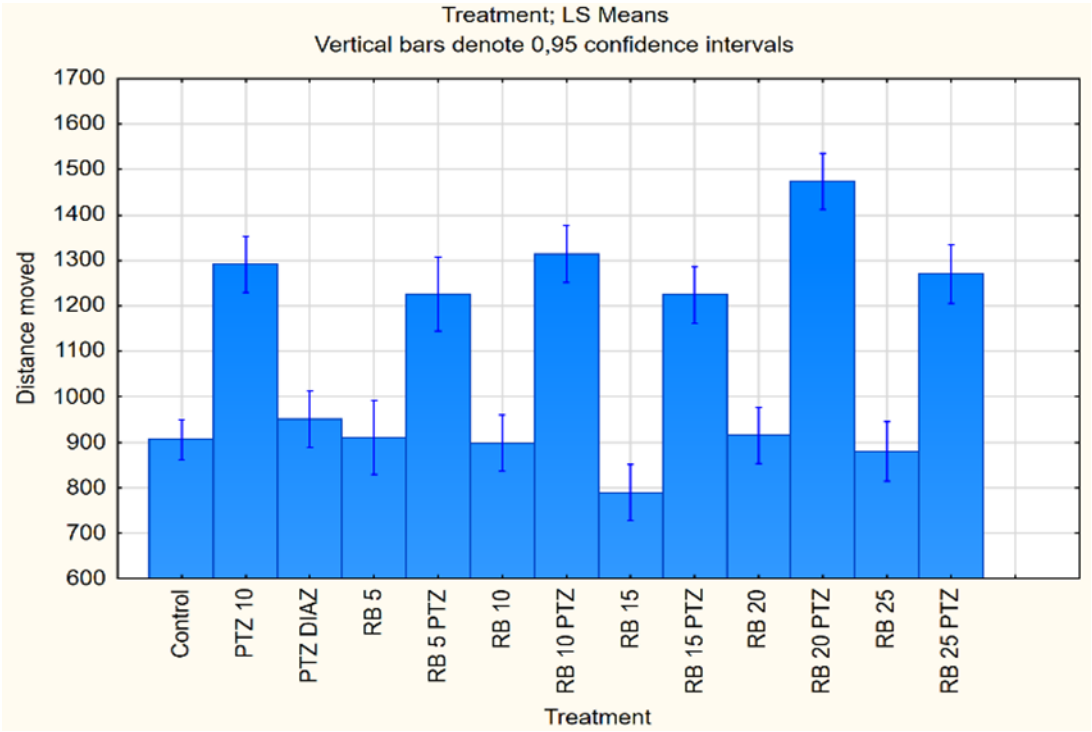

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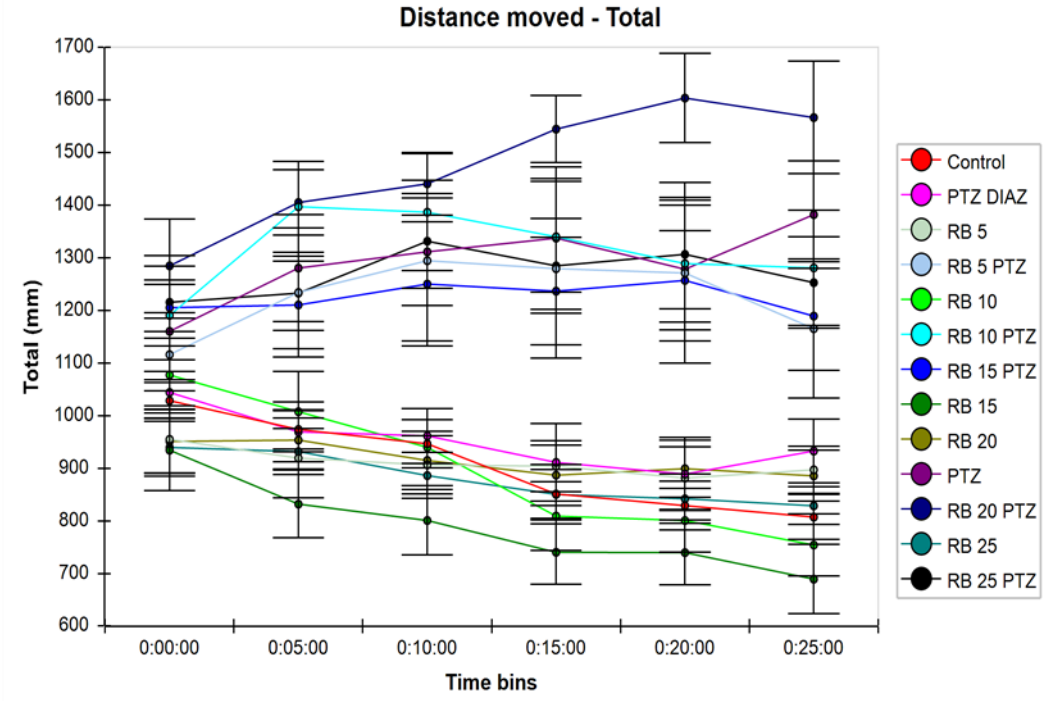

Figure 4-8. Movement of zebrafish treated with PTZ; PTZ/Diazepam and PTZ and increasing doses of green rooibos extract, A) in terms of distance B) Distance data divided time bins

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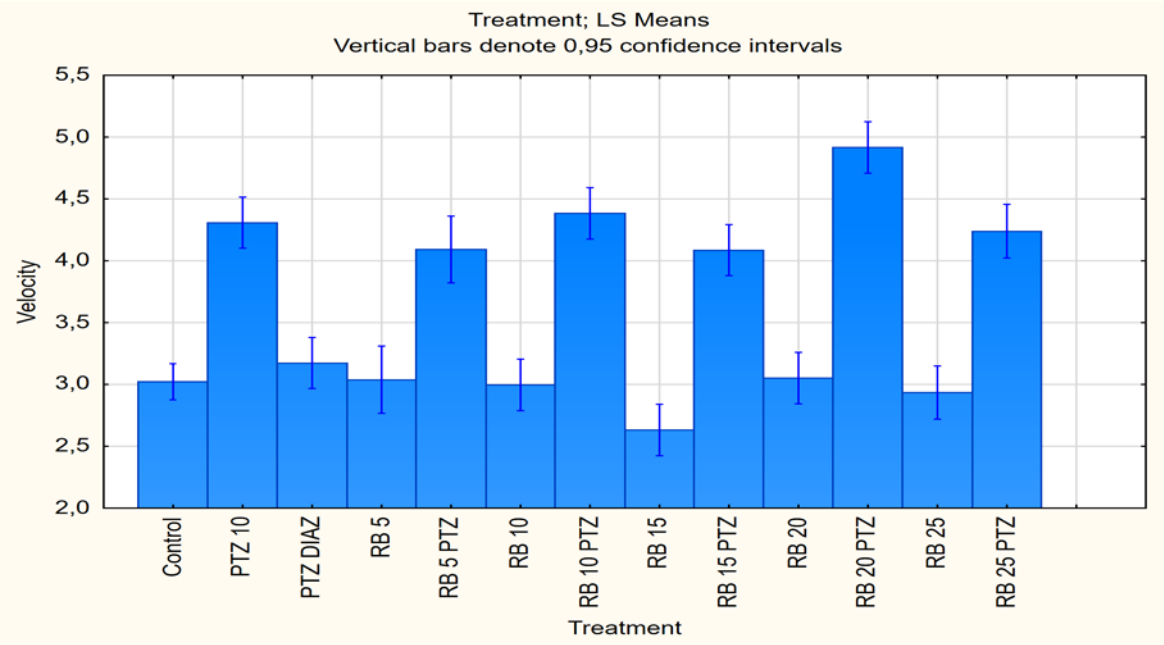

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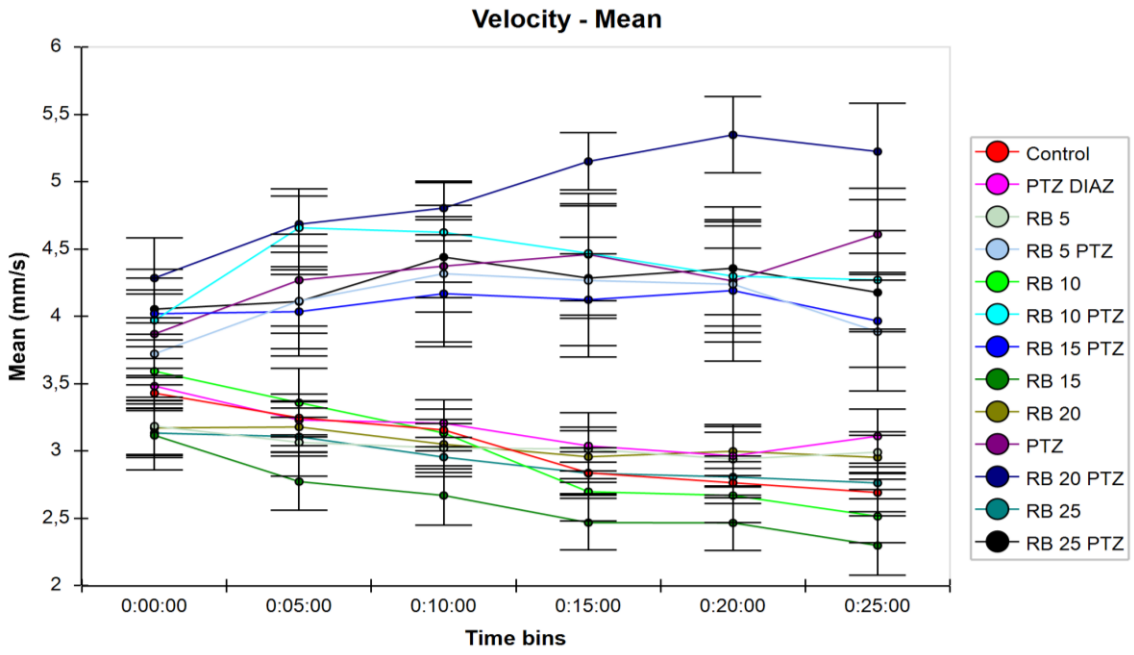

Figure 4-9 Movement of zebrafish treated with PTZ; PTZ/Diazepam and PTZ with increasing doses of green rooibos extract, in terms of A) velocity and B) Data divided time bins showing velocity

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Chapter 5 - Discussion

Neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease, Amyotrophic lateral sclerosis, Huntington's disease and other neuropsychiatric disorders like dementia, attention deficit hyperactivity disorder, Schizophrenia, bipolar disorder, and depression have for long been linked to neuroinflammation (DiSabato *et al.,* 2016; Erickson *et al.*, 2012; Guzman-Martinez *et al.,* 2019; Kempuraj *et al.,* 2016; Morales *et al.,* 2016). Furthermore, many molecular and cellular cascades that trigger neurodegenerative events, such as oxidative responses, buildup of protein aggregates, malfunctioning mitochondria, are entangled in a vicious and complicated cycle of neuroinflammation (Cianciulli *et al.*, 2020).

Despite the increase in the understanding of the pathophysiology and etiology of most of these diseases, Gorshkov *et al.,* (2018) opined that for the past more than 50 years the search for therapies has yielded a few viable treatment solutions. One of the appproaches that research has focussed on, is the concept of neuroprotection. This concept has evolved into three groups: (A) pharmacological intervention, (B) non-pharmacological intervention (e.g diet control, sleeping etc), and (C) cellular and genetic approaches (Chuen-Chung and Ho 2019). The current study focused on the pharmacological intervention through neutraceuticals, specifically unfermented green rooibos extract.

A myriad of published research papers on the anti-inflammatory and antioxidant effects of rooibos has generated data that firmly establishes rooibos as a potent medicinal plant. There is however no data on the potential neuroprotective effects of green rooibos. The data generated by the current research significantly adds to the literature on rooibos by exploring its neuroprotective potential through its capability to scavenge free radicals, modulation of the neuroenzyme monooxidase A, effect on inflammatory responses of astrocytes and its potential action on GABA receptors.

In terms of data obtained in cell-free systems, current data has demonstrated that green rooibos extract possesses similar antioxidant potency as ascorbic acid (Vitamin C) and alpha-tocopherol (Vitamin E). Furthermore, its ORAC value of approximately

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8.1 µmol TE/mg is significantly higher than that of blueberry, cranberry and cherry which have ORAC values of 0.85, 0.64, 0.60 µmol TE/mg respectively. This showed that rooibos is a powerful free radical scavenger. (Cásedas *et al.*, 2017).

These data also augured well with the neuronal cell culture model, where data suggests green rooibos extract to protect mouse neuroblastoma against loss of vialibity resulting from hydrogen peroxide-associated oxidative stress, at least in part due to its ability to limit ROS accumulation in cells. This potential is hit home further by the data in astrocytes, showing a relative anti-inflammatory effect of rooibos in the context of MCP-1 secretion, even in the presence of added (LPS-induced) oxidative stress.

The antioxidant/radical scavenging activity of rooibos has been thoroughly investigated in the periphery (Canda *et al*., 2014; Ajuwon *et al.*, 2013; Marnewick, 2014; Smith and Swart 2018; Sasaki *et al.*, 2018;) and the foregoing data confirms this.

Furthermore, rooibos extracts have been demonstrated to modulate steroid hormone biosynthesis thereby maintaining beneficial glucocorticoid levels (Smith and Swart 2018). The antioxidant and anti-inflammatory activities of rooibos have been proven as the mechanism for ameliorating the effects of chronic inflammatory diseases, diabetes and cardiovascular disease since these diseases are linked to oxidative stress and inflammation (Smith 2018). However, the current study is the first to demonstrate these effects in neuronal cells and astrocytes.

Another novel cytoprotection mechanism of action of the green rooibos extract demonstrated in the current data, was its inhibition of MAO-A. Monoamine oxidase is a mitochondrial enzyme which exists in two isoenzyme forms: MAO-A and MAO-B. It is important in catalyzing the oxidative deamination of a number of monoamines, both endogenous and exogenous, and has major roles in metabolizing released neurotransmitters, and in detoxification of a large variety of endogenous and exogenous amines(Finberg *et al.*, 2016). The current study showed that green rooibos extract has significant capacity to inhibit MAO-A.

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The involvement of MAO in neurodegenerative diseases has been extensively studied. (Mandel *et al.*, 2003; Zhang and Greenberg 2012; Youdim 2018). The mechanisms through which MAO exarcebates neurodegenerative diseases include failure of aggregated-protein clearance, triggering apoptosis, oxidative stress, inflammation and glial activation (Finberg *et al.*, 2016). Therefore the inhibition of MAO-A enzyme by the green rooibos extract is a beneficial and novel consequence of the green rooibos extract. Furthermore, Dhiman *et al.,* (2019); and Saaby *et al.,* (2009) reported the inhibition of MAO-A by flavonoids such as quercetin and/or quercitin derivatives- which are flavonoids found in the green rooibos extract.

In terms of safety, all cell types tolerated the rooibos well, except at extremely high doses. However, these high doses are unlikely to be consumed by an individual.
To put it to context one South African study done by Van Der Merwe *et al.*, (2015) discovered that on average people drink about three cups of fermented rooibos per day. Furthermore Marnewick *et al.*, (2011) did a clinical study to assess the possible health benefits of rooibos to humans through the modulation of oxidative stress in a population at risk for developing cardiovascular diseases (CVD). They demonstrated that drinking 6 cups of rooibos per day for six weeks did not cause any adverse effects. Six cups of rooibos per day was determined to contain 343 µg/ml of the polyphenols. However polyphenols are not completely absorbed to give 100% bioavailability, a fact demonstrated by Hostetler *et al*., (2017). This rate of consumption will yield a dose far less than the cytotoxic dose revealed by the current study. This therefore shows that the consumption of rooibos in physiologically feasible amounts carries no risk of cytotoxicity from the many polyphenols that it contains.

The absorption of the polyphenols *in vivo* was shown to be dependent on their interaction with other dietary ingredients and the manner in which they are consumed (Rein *et al.,* 2012).That is, they could be consumed as a constituent of a complex plant extract mixture, as a purified compound mixed into the feed (or food as in humans), in drinking water or through orogastric gavage of experimental animals (Muller *et al.*, 2018). The various factors affecting bioavailability wedge a significant difference in efficacy of results between *in vitro* and *in vivo* studies.

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In some *in vivo* studies on the bioavailability and metabolism of flavonoids from rooibos conducted on pigs, (Kreuz *et al.;* 2008) and humans (Courts & Williamson 2009; Stalmach *et al.,* 2009), it was demonstrated that the flavonoids are only absorbed poorly, with only trace quantities measurable in urine up to 24 hours after consumption. Furthermore, Bowles *et al.*, (2017) used the Caco-2 cell culture model system (lauded for its excellency for the determination of the absorption potential of drugs; their mechanisms of absorption and excretion and their metabolites) to confirm the low absorption of rooibos flavonoids. Additionally, Bowles and co-workers, further employed an *in vivo* model using mice and found aspalathin metabolites in the urine and not in the blood, suggesting high renal clearance and consequentially low bioavailability of the rooibos flavonoids. Notwithstanding the disadvantages of low absorption, these studies effectively demonstrated the safety of rooibos consumption and further reiterated the nearly impossible event of consuming rooibos at toxic levels.

In vitro models are done with well controlled antioxidant structures and concentrations for the evaluation of the antioxidant properties of single/complex compounds. They are good in providing data that probes possible mechanisms of action, but they do not always reflect the effects occurring *in vivo* (Haenen *et al.,* 2006). In addition, *in vitro* models do not account for vital variables such as metabolism, or the stability and activity of the putative metabolites and their bioavailability (Haenen *et al.,* 2006). It has therefore been suggested that a single assay (*in vitro* or *in vivo*) is insufficient in the determination of the bioactivity of a compound or mixture of compounds. Consequently, a multi-assay based on the specific research question to be addressed is imperative (Griffiths *et al.,* 2002; Collins 2005; Dilis and Trichopoulou 2010).

Therefore in order to evaluate the significance of the very favourable *in vitro* data for *in vivo* application, it is necessary to evaluate the effects of rooibos *in vivo*. For this reason, we performed a basic *in vivo* assessment using zebrafish, to not only assess tolerance of the rooibos, but also probe an additional potential mechanism of action of rooibos.

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The protocol for this evaluation was an *in-vivo* study of zebrafish lavae exposed to Pentylenetetrazol (PTZ) which instigates an epileptic-like seizure to the larvae. Green rooibos extract was ineffective in calming the zebrafish when compared to diazapam, a well known benzodiazepine drug that has sedative, anxiolytic, anticonvulsant, hypnotic, and muscle relaxant properties (Wongsamitkul *et al.*, 2017) via acting as an agonist to the neurotransimitter, GABA (γ-Aminobutyric acid).

Gamma-amino butyric acid is one of the major inhibitory neurotransmitters in the CNS that acts on receptors coupled to chloride channels. (Moghbelinejad *et al.,* 2017; Jembrek & Vlainić 2015). Disturbances in GABA levels aggravate disequlibrium between excitatory and inhibitory signals, resulting in the development of numerous neuropsychiatric disorders (Jembrek & Vlainić 2015). GABA exerts its effects through ionotropic and ($GABA_A$) and metabotropic ($GABA_B$) receptors and research has shown that the allosteric modulation of $GABA_A$ receptors mitigates many neurosychiatric diseases (Wongsamitkul *et al.*, 2017).

Drugs such as benzodiazepines (e.g. Diazapam), barbiturates, neuroactive steroids, intravenous and inhalational anesthetics, and ethanol have modulatory effects on the $GABA_A$ receptor complex. They are particularly used in the treatment of anxiety disorder, epilepsy, insomnia, spasticity, aggressive behaviour, and other pathophysiological conditions and disease (Jembrek & Vlainić 2015).

However, most of these psychoactive drugs especially benzodiazepines (which are the most prescribed), have important side effects that include sedation, myorelaxation, ataxia, amnesia, and ethanol and barbiturate potentiation and tolerance (Wasowski & Marder 2012). This has led to a growing interest in the development and application of subtype-selective drugs that will achieve specific therapeutic benefits without undesirable side effects (Jembrek & Vlainić 2015).

Flavonoids have been shown to have structural symilarities with benzodiazepines, such as diazepam, and have become the most widely studied positive modulators of $GABA_A$ receptors (Hanrahan *et al.,* 2015). Consequentially, flavonoids have been reported to act on the ionotropic receptors for GABA as positive or negative modulators, potentiating or dampening the effect of GABA (Jembrek & Vlainić 2015;

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Johnston 2015; Saaby 2011; Moghbelinejad *et al.,* 2017; Johnston *et al.*, 2009; Hanrahan et al, 2011). In addition, evidence shows that they can act as allosteric agonists and are able to modulate the action of other modulators (Johnston 2015).

In the context of the present study, rooibos is well known to be endowed with flavonoids, especially polyphenols (Smith and Swart 2018). Moreover, the green rooibos extract is rich in the flavonoid quercetin. In an *in vivo* model where kainic acid was used to induce epileptic seizures in mice, Moghbelinejad and co-workers, (2017) showed that quercetin was potent in reducing seizure severity via its action on $GABA_A$ α5 receptor gene. This literature provided the basis for our decision to investigate modulation of GABA receptor as potential mechanism of action.

Despite being endowed with flavonoids, the present study apparently showed no positive result for the green rooibos extract. This unexpected result can be attributed to three main reasons linked to the model used. Firstly, the zebra larvae may have completely metabolised the green rooibos extract during the 24 hour incubation period of the protocol implemented here and therefore by the time their behavior was assessed, the effect of rooibos may have been depleted already. A protocol using a more acute assessment may shed light on this possibility.

Secondly, the green rooibos extract was used in relatively low concentrations, as higher concentrations of the extract formed a precipitate in the wells, due to the poor solubility of high concentrations of the extract in a medium that is suitable as bathing medium for fish. Given the fact that larve at 4dpf spend much time lying on their sides at the bottom of the well, this precipitate may have confounded results by partial or total asphyxiation of the larvae. Therefore, the doses of the extract that could accurately be assessed in this model, may have been too low. An alternative option in terms of protocol design, would be microinjection of Rooibos instead, although this may not accurately mimick absorption and bioavailability of the extract.

Thirdly, the bioavailability of flavonoids from consumed rooibos in general is very poor (Breiter *et al.*, 2011; Hostetler *et al.,* 2017). More research is required to fully elucidate GABA signalling modulation as potential effect of Rooibos.

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5.1 Limitations of the study

In terms of potential limitations of the study, the choice of Neuro-2a cells may not have been ideal. The apparent resistance of Neuro-2a cells to hydrogen peroxidate-induced cell death (survival only decreased at 250 µM dose but not 125 µM) may be attributed to relatively high intracellular glutathione levels in neuro-2a cells compared to other neuronal cells types, for example that of PC12 cells (Calderón *et al.*, 1999). In addition, the neuro-2a cells express low voltage-gated sodium channels (20-fold lower than cerebellar granule neurons)(LePage *et al.*, 2005). These two characteristics may have afforded neuro-2a cells relatively greater resistance to H_2O_2-induced oxidative stress and neurotoxicity, making it perhaps a less ideal model in which to study treatment-associated modulation of redox, as endogenous antioxidant counters for the experimental stimuli may have prevented a more sensitive reflection of the effect of rooibos at lower levels of oxidative stress.

Furthermore, although we elucidated effects in terms of MAO-A inhibition, another potentially important cellular indicator of protection via redox moduation may be acetylcholinesterase (AChE). A study done on the human neuroblastoma cells (SH-SY5Y) found that H_2O_2 has an effect on the activity of AChE. (Garcimartín *et al.*, 2017). At high H_2O_2 concentrations, the levels of AChE were found to be much higher than when the H_2O_2 concentration was low. The research suggests that H_2O_2 acts as an allosteric activator of AChE and it affected more of the V_{max} than the K_m of the enzyme. (Garcimartín *et al.*, 2017). AChE is a well known indispensable enzyme that terminates acetylcholine-mediated neurotransmission at cholinergic synapses. Moreover its effect and mechanism in cell apoptosis and implications to neurodegenerative diseases has been well documented (Yang *et al.,* 2002; Calderón *et al.*, 1999; Shehadeh Masha'our *et al.*, 2012; Jiang and Zhang, 2008; Zhang *et al.*, 2002; Zhang and Greenberg 2012). Therefore, assessment of rooibos in terms of its potential to inhibit AChE may shed further light on its potential to protect against neurodegeneration.

Also, the study was limited to interpretation of only one cytokine, MCP-1. IL-1β was also assessed but either due to its short half life (Kuribayashi 2018); Gerhartz *et al.*,

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1994; Oda *et al.*, 2005), or low detection sensitivity of the assay kit, the data collected was not conclusive. Further research including more cytokines may provide a broader understanding of the anti-oxidative, anti-inflammatory and cytoprotective mechanisms of unfermented rooibos, as it pertains to the central compartment.

Lastly, the bioavailability of the flavonoids in vivo should be investigated. Given the known poor absorption of rooibos, this could potentially be improved by the use of bioenhancers.

5.2 Conclusion

In conclusion, current data presented suggests that the green rooibos ethanol extract employed here, is a rich source of polyphenols with antioxidant and neuroprotective potential. However, the bioavaialbity of different rooibos extracts remains to be elucidated. Also, more studies aimed at translation of *in vitro* data into *in vivo* models, are required to fully elucidate neuroprotective mechanism of rooibos.

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Chapter 6 References

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