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Evaluation of the inhibition of Neuroinflammation by Quassin

By

Oyinkansola Deborah Adegbola

A thesis submitted to the University of Huddersfield in partial fulfilment of the requirements for the degree Master by research in Pharmaceutical Science

The University of Huddersfield

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Acknowledgements

"Commit to the LORD, whatever you do, and he will establish your plans"

- (Proverbs 16:3)

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Abstract

The over-activation of microglia is a common feature of neuroinflammation. This is associated with neuronal damage and the release of inflammatory mediators within the CNS in response to an injury or infection. The release of pro-and anti-inflammatory cytokines, chemokines and other mediators plays a vital role in the pathogenesis of neurodegenerative diseases. Studies have demonstrated the use of natural compounds possessing anti-inflammatory and neuroprotective properties in inhibiting neuroinflammation. Quassin is a bitter constituent derived from the bark of the *quassia amara* tree; it is used as a traditional medicine in several cultures. Several studies have reported quassin to possess antiulcerogenic, antiplasmodial, antileishmanial and anti-malarial properties. However, there is limited research on the anti-inflammatory properties of quassin. This study therefore aimed at investigating the inhibition of neuroinflammation by quassin through evaluating the actions of quassin on underlying mechanisms of neuroinflammation in LPS-stimulated Bv2 microglia. The inhibitory effects of quassin (10, 20, 40 and 80 μM) against neuroinflammation were investigated in LPS (100ng/ml) stimulated BV2 microglia.

Griess assay was used to measure the levels of nitrite in BV2 microglia cells. The levels of pro-inflammatory cytokines TNFα, IL-1β and IL-6; chemokines CCL2 and CCL5; and anti-inflammatory cytokine IL-10 were measured using ELISA kits. COX-2 and iNOS protein expressions were measured using western blotting. Luciferase reporter gene assay and ELISA were used to measure quassin's activity on NF-κB -phospho-p65 and NF-κB promoter.

Results from this study demonstrated that quassin inhibited IL-1β, and IL-6 at a concentration of 80 μM. Additionally, quassin suppressed NO's production and the expression of iNOS protein at 20 and 80 μM. The anti-inflammatory effects of quassin were not sufficient at inhibiting the production of TNFα, CCL2, CCL5, and COX-2 protein expression, alongside increasing the release of IL-10 in activated microglia. On the other hand, quassin was able to inhibit the production of pro-inflammatory cytokines IL-1β and IL-6 released in the NF-κB signalling pathway. Western blotting and Luciferase reporter gene assay showed that quassin did not inhibit phosphorylation p65 and NF-κB dependent gene expression. Findings from this study suggest that quassin's inhibitory effects on IL-1β, IL-6, NO and the expression of iNOS

are not mediated by targeting the phosphorylation of NF-κB -phospho-p65 subunit in LPS-stimulated BV2 microglia.

In conclusion, this study has contributed to our understanding of the effect of quassin in inhibiting neuroinflammation mediation in neurodegenerative diseases. Quassin showed promising results through the inhibition of proinflammatory cytokines IL-1β, and IL-6 and also in the suppression of NO production. Further investigations need to be carried out in order to determine the anti-inflammatory activity of quassin.

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Abbreviations

Aβ: Amyloid-beta

AD: Alzheimer's disease

ALS: Amyotrophic lateral sclerosis

BBB: Blood-brain barrier

BSA: Bovine serum albumin

CCL2: Chemokine ligand 2

CCL5: Chemokine ligand 5

CCR2: Chemokine ligand receptor -2

CCR5: Chemokine ligand receptor - 5

CNS: Central Nervous system

COX: Cyclooxygenase

COX-1: Cyclooxygenase 1

COX-2: Cyclooxygenase 2

DA: dopamine

ERK: Extracellular signal-regulated kinase

eNOS: Endothelial nitric oxide synthase

IFNγ: Interferon gamma

iNOS: inducible nitric oxide synthase

IL-10: Interleukin- 10

IL-1β: Interleukin- 1 beta

IL-6: Interleukin- 6

IκB: Inhibitors of Kappa B

IKK: inhibitor of NF-kappa B kinase

JNK: c-jun N-terminal kinase

LBs: Lewy bodies

LPS: Lipopolysaccharide

NADPH: Nicotinamide adenine dinucleotide phosphate

nNOS: neuronal nitric oxide synthase

NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells

NO: Nitric oxide

MAPK: mitogen activated protein kinase

p38 MAPK: p38 mitogen activated protein kinase

p65: Transcription factor p65 (RelA)

PD: Parkinson's disease

PGE_2: Prostaglandin E2

PNS: Peripheral Nervous system

PRR: Pathogen recognition receptors

TLR4: Toll-like receptor 4

TNFα: Tumour necrosis factor alpha

TNFR: tumour necrosis factor receptor

TGF-β: Transforming growth factor-beta

ROS: reactive oxygen species

oC: Celsius degree

μM: Micro molar

ng/ml: nanogram/millilitre

Chapter 1. General Introduction

Inflammation is a defence process initiated by the immune system that controls pathogenic and microbial invasion within the immune system (Gogoleva, et al., 2019). An acute or chronic inflammation response provides immediate protection against the effects of tissue injury and foreign proteins. A normal inflammatory response is beneficial as it is crucial to healthy well-being and repairs tissue injury. Inflammation within the immune system can be triggered by various factors such as damaged cells, pathogens and toxic compounds. Inflammation can either be acute or chronic. Acute inflammation is a rapid, short-term response that rapidly defends the injury or tissue damage. A cellular and molecular cascade of events and interactions effectively minimize impending injuries or infection (Matsuda, et al., 2019). Acute inflammation that has not been eliminated or injuries sustained at tissue level can lead to chronic inflammation. Chronic inflammation involves a response that occurs over a prolonged period, which can be detrimental to the central nervous system (CNS) and peripheral nervous system (PNS) (Gogoleva, et al., 2019). This type of prolonged inflammatory response is damaging and contributes to various chronic inflammatory diseases (Chen, et al., 2018). Neuroinflammation occurs within nervous tissues, which is a common feature of several neurodegenerative diseases (Matsuda, et al., 2019).

1.1 Immune responses within the central nervous system (CNS)

The nervous system encompasses a complex network of nerves and neurones that function by transmitting signals between various parts of the body. The nervous system is divided into two main parts; the central nervous system (CNS), which comprises the brain and spinal cord; and the peripheral nervous system (PNS), which contains nerves outside the CNS. The blood-brain barrier (BBB) is a selectively permeable membrane that protects the CNS and communicates signals between the PNS and CNS. The primary function of the nervous system is to control and coordinate operations of other systems through the collection of sensory information. It further processes, interprets and integrates the information and initiates a response throughout the body. The immune system comprises a network of cells and proteins that defends the body against infections. These infections are caused by pathogens such as bacteria, fungi, parasites and viruses. The immune system responds to harmful stimuli through inflammation, which eliminates damaging stimuli and induces the healing process (Clark & Kodadek, 2016).

Microglia is a brain resident macrophage that is part of the innate immune system located in the parenchyma of the CNS (Gogoleva, et al., 2019). During an inflammatory response, this

cell type produces inflammatory mediators such as cytokines and chemokines that alert the immune system. Activation of microglia acts to protect the CNS against damaging immune responses mediated by neuroinflammation, which occurs through the transduction of pathogen recognition receptors (PRR). There is evidence to support the over-activation of microglia leading to overexpression or dysregulation of pro-inflammatory mediators, which play a role in the pathogenesis of neurodegenerative disorders (Tang &Le, 2016; Gogoleva, et al., 2019).

1.2 Microglia cells

Glial and neurones are two types of cells that perform functions within the nervous system. Neurones are nerve cells that act as information carriers sending signals within the brain and communicating these signals to target cells. Glial cells are non-neuronal cells that support and protect the central nervous system (CNS). Additionally, research has demonstrated glial cells to be involved in intracellular signalling in which they communicate with other glial cells through intracellular waves of calcium via chemical messengers (Shaheen, 2021). Microglia, astrocytes and oligodendrocytes are glial cells within the CNS. Microglia are specialised cells that makeup 10% of the population within the CNS and function as macrophages, removing damaged neurones and infections whilst maintaining a healthy microenvironment. They are responsible for initiating immune responses within the brain and helping with the clearance of debris and dead neurones from nervous tissues through a process known as phagocytosis, thus protecting neurones in the CNS (Dionisio-Santos, et al., 2019). Microglia activation occurs during acute and chronic inflammation or when encountering a stimulus or pathogen (Dionisio-Santos, et al., 2019). Microglia have been identified to trigger the release of pro-inflammatory mediators, contributing to the progression of neurodegenerative disorders when hyperpolarised. Microglia secretes pro-inflammatory mediators that actively participates in neurodegeneration and are vital components of molecular and cellular events that have neurodegenerative consequences. (Griffin, et al., 1998).

Resting microglia have a small soma and long, highly branched proteases, and when activated microglia become larger. During an inflammatory response resting, microglia transform to activated microglia; it secretes cytotoxic substances such as reactive oxygen species (ROS), cytokines and chemokines that kill the foreign species or bacteria. Dependent on the specific activation or interaction with other molecules, microglia can transform into different morphological phenotypes. As shown in figure 1, the diversity in the actions exhibited by microglia may be due to their ability to form different activation stages; classical (M1) and alternative (M2) (Porro, et al., 2020). M1 phenotype have been demonstrated to be triggered

by various stimuli's such as, Interferon (IFN), β-amyloid (Aβ), α-Synuclein and Lipopolysaccharide (LPS) (Subhramanyam, et al., 2019). Additionally, when activated M1 phenotype of microglia is characterised by the production of pro-inflammatory mediators, including pro-inflammatory cytokines (IL-1β, IL-6, and TNFα), chemokines (CCL2, CCL5, CXCL1), ROS, nitric oxide (NO) and prostaglandins (Bachiller, et al., 2018). M2 phenotype, the alternative pathway is induced by Interlukin-4 (IL-4) and Interleukin-13 (IL-13) (Subhramanyam, et al., 2019). The M2 phenotype is considered to be neuroprotective and is characterised by the expression of IL-10, IL-4 alongside transforming growth factor-beta (TGF-β) and arginase (Lively & Schlichter, 2018). Lipopolysaccharide (LPS) is a Toll-like receptor- 4 (TLR-4) ligand that is primarily expressed in microglia cells. LPS induced activation on microglia cells results in the production of proinflammatory mediators such as TNFα, IL-1β, PGE_2 and NO. The inflammatory cytokines released are key mediators of neuroinflammation (Zhao, et al., 2019). The acute response of microglia to inflammation within the regular immune system is protective and helps avoid further injuries and induces tissue repair. Amplified or chronic activation of microglia cells provokes the intense activation and accumulation of dead cells at the injury site, causing neuronal damage. Hence, microglia cells are cellular targets instead of neurones in understanding neuroinflammatory mechanisms.

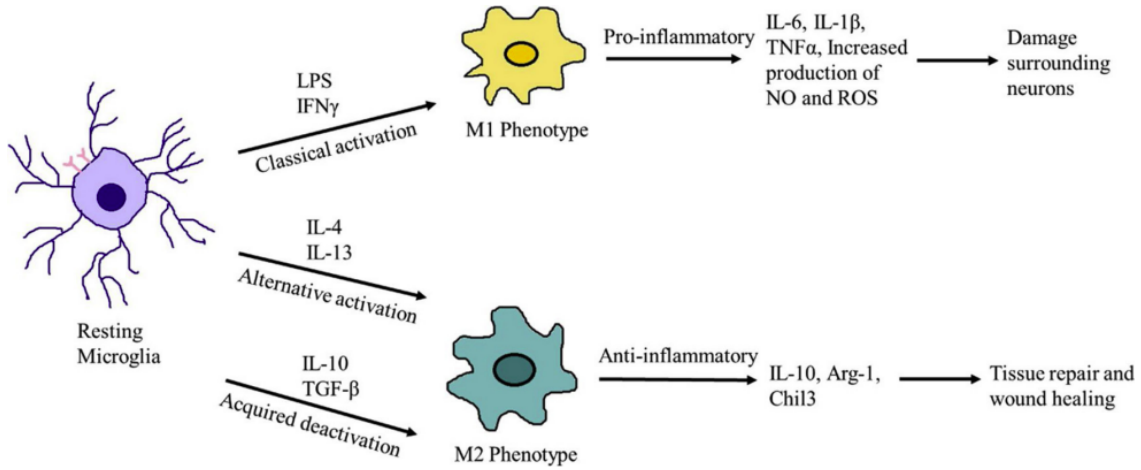


Figure 1: The activation phenotypes of microglia

Activation of distinct phenotype and the release of inflammatory mediators is dependent on the inflammatory stimuli used to induce inflammation. M1 phenotype; secrete pro-inflammatory cytokines and inflammatory mediators. The activation of the inflammatory mediators is induced by LPS and IFNγ. M2 phenotype; secretes anti-inflammatory cytokines. The activation of anti-inflammatory mediators is induced by IL-13 and IL-4 (Subhramanyam, et al., 2019).

1.3 Neuroinflammation

Neuroinflammation is defined as the ability of the central nervous system to mount an inflammatory response within the innate immune response during a pathological event (Mendiola & Cardona, 2018). It is characterised by the activation of resident macrophage glial cells, committed to the CNS immune surveillance, through the release of cytokines, chemokines and other inflammatory mediators, which recruit peripheral cells, including lymphocytes, monocytes and neutrophils (Porro, et al., 2020). Neuroinflammation is a dominant feature of neurodegenerative diseases, with chronic inflammation being the driving force of many (Velagapudi, et al., 2019). Various mechanisms within the brain have been found to activate neuroinflammation, including microglia and pro-inflammatory mediators that are released. Microglia is considered one of the hallmark effector cells involved in mounting inflammatory responses to injuries or infections. Highly destructive and pathological neuroinflammation is coupled with microglia hyper-activation, increased production of cytokines and chemokines, increasing blood-brain barrier (BBB) permeability and break down and infiltration of peripheral immune cells. Examples of neuroinflammatory cytokine production that act on other cells, influencing cellular mechanisms and pathology, exhibit how signals are communicated within or to the CNS during an immune response. The mechanism of neuroinflammation during an immune response remains challenging to define summarily, as it is extensive. Currently, there is no cure for neurodegenerative conditions such as alzheimer disease (AD) and parkinson's disease (PD). Targeting neuroinflammation is a critical approach for slowing down the progression of neurodegenerative diseases.

1.3.1 Neuroinflammation in neurogenerative diseases

Neurodegenerative diseases are associated with the progressive loss of neurones, cognitive and motor functions (Dugger & Dickson, 2017). The pathology of neurogenerative diseases involves the accumulation of protein aggregates in the neurones, which is caused by mutations and protein conformation. The collection of protein aggregates within the neurones elicit an immune response from within the neurones, leading to neuronal damage. The pathology of neurogenerative diseases is not well understood; however, studies have investigated the role of the inflammatory process as a cause of neurogenerative diseases. Neuroinflammation within the brain is one variable that elicits these responses to these diseases. Understanding and regulating the interactions between the immune and central nervous systems is an important target to delay the onset or slow down the progression of neurodegenerative diseases. Neuroinflammatory responses that results in the alteration of microglial cells as a consequence

of activation, is a critical feature in the pathogenesis of neuronal CNS-related diseases such as AD, PD and amyotrophic lateral sclerosis (ALS) (Tang & Le, 2016; Guzman-Martinez, et al., 2019) (Figure 2). Therapeutic strategies targeting the regulation of inflammatory factors and hyper-activated microglia can be used to prevent or slow down the pathology of these diseases (Timmerman, et al., 2018). Progressive neuronal dysfunction such as proteotoxic and oxidative stress and programmed cell death is also a common feature shared by several neurodegenerative diseases (Dugger & Dickson, 2017). Extensive research has shown that activation of microglia within the CNS is the principal component of neuroinflammation, providing a defensive immune response to injury or the disease (Tang & Le, 2016).

Alzheimer's disease is a major cause of dementia; the pathogenesis of this disease is characterised by intracellular accumulation of hyperphosphorylated tau protein, neurofibrillary tangles and extracellular deposition of amyloid-beta (Aβ) in senile plaques. (Timmerman, et al., 2018). Neurodegeneration is a feature of this chronic disease, coupled with impaired memory and cognition (Mendiola & Cardona, 2018). In AD patients, plaques accumulate because over-activated microglia cannot undergo phagocytosis of accumulated amyloid beta (Aβ). The build-up of these amyloid plaques induces activation of primary microglia and stimulates the production of the inflammatory mediator, NO. The production of pro-inflammatory cytokine IL-1β in LPS-activated microglia suppresses the phagocytosis of fibrillar Aβ peptides.

Parkinson's disease is another neurodegenerative disease, defined by the progressive loss of dopaminergic (DA) neurones in the substantia nigra (SN) (Tang & Le, 2016). The pathology of PD is characterised by the formation of Lewy bodies (LBs) within DA neurones (Subhramanyam, et al., 2019). The reduction of DA neurones is a causative factor for the motor symptoms experienced by patients with PD. Microglia-mediated neuroinflammation is a significant component in the progression of PD, which is provoked by misfolded proteins and pathogenic toxins. Previous research has established that the release of aggregated α-synuclein, a prevalent gene in PD directly stimulates the activation of the M1 microglia phenotype from dead DA neurones.

Through the activation of NADPH oxidase and NF-κB pathways an increase in the production of pro-inflammatory cytokines and reactive oxygen species (ROS) is initiated (Subhramanyam, et al., 2019). Treatment with LPS has been reported to cause extensive neuronal death in dopaminergic neurones *in vivo* and *in vitro* neuroinflammation models. L-dopa is a well-

established therapeutic that is currently administered to patients with PD to increase dopamine levels in the basal ganglia; despite improving motor symptoms, it does not halt the neurodegenerative symptoms that occur.

Amyotrophic lateral sclerosis is a neurodegenerative disease caused by a loss of upper and lower motor neurones (Dugger & Dickson, 2017). The degeneration of the upper and lower motor neurones is responsible for the spasticity, modest and disabling weakness patients with ALS experience. Like other neurodegenerative diseases, ALS is characterised by the build-up of dysfunctional proteins within neurones, coupled with protein-rich cytoplasmic inclusion in motor neurones of the spinal cord (Tang & Le, 2016). Neuroinflammation characterised by microglial activation is identified as a standard feature shared by familial and sporadic ALS patients. The function of microglia in ALS can be both neuroprotective and cytotoxic, dependent on the activation intensity.

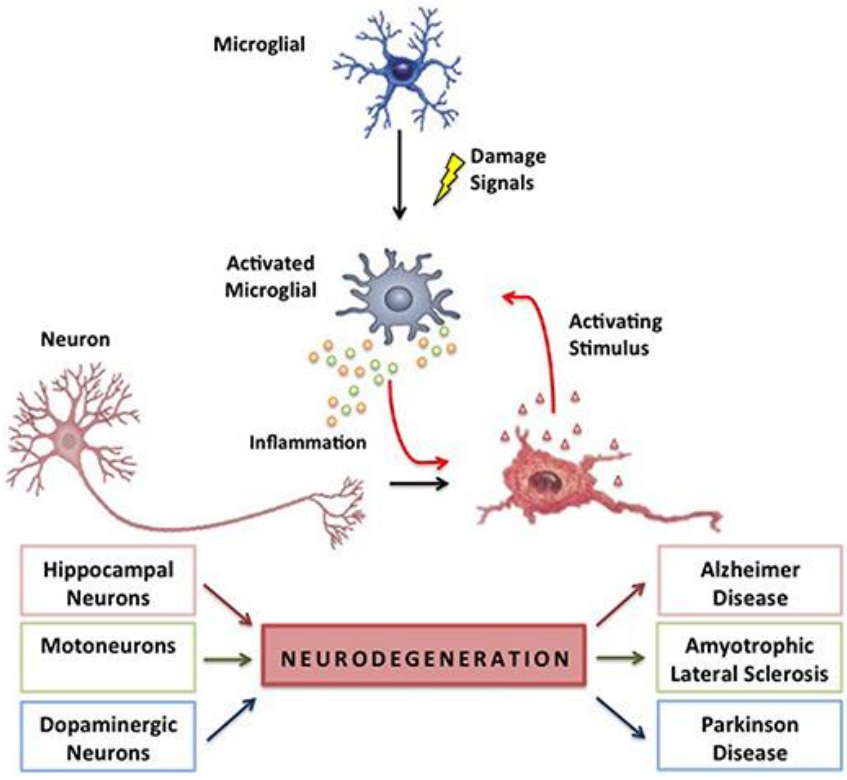


Figure 2: Illustration of neuroinflammation resulting in neurodegeneration cycle.

Various factors induce inflammation within the central nervous system (CNS), sustained inflammation results in damaging signals that lead to hyper-activation of microglia. The release of inflammatory and neurotoxic factors drives the progression of neuronal damage associated with neurodegenerative diseases (Morales, et al., 2016).

1.4 Roles of pro-inflammatory cytokines and inflammatory mediators in neuroinflammation

Inflammatory cytokines act as signalling molecules and are involved in inflammation, cell growth and cell survival (Ramesh, et al., 2013). Cytokines are mainly secreted by brain cells such as microglia, neurones and astrocytes. Up to now, numerous studies have revealed the correlation between the imbalance of pro-and anti-inflammatory cytokines and the progression of neuroinflammation in neurodegenerative diseases (Sultani, et al., 2012). Pro-inflammatory cytokines are immunoregulatory cytokines that are secreted during an inflammatory immune response (Jeon & Kim, 2016). Activated microglia produces pro-inflammatory cytokines involved in the upregulation of inflammatory reactions (Wang, et al., 2015). Bacterial endotoxin LPS and pro-inflammatory cytokines TNFα and IFNγ commonly used inflammatory stimuli to induce the activation of microglia (Lively & Schlichter, 2018). . Furthermore, TNFα, IL-6 and IL-1β are pro-inflammatory cytokines and are widely studied in relation to activated-microglia, inflammatory processes and neuroinflammation (Kim, et al., 2016). Neuroinflammation under normal physiological conditions is beneficial to the brain, with the acute response regulating the microenvironment in the CNS. It is now well established that excessive production of pro-inflammatory cytokines during pathogenic invasion or infection plays a crucial role in neuroinflammation, contributing to the pathogenesis of neurogenerative diseases (Ramesh, et al., 2013). Understanding the signalling pathways of pro-inflammatory cytokines involved in regulating diseases such as AD, PD, and ALS is essential in developing therapeutic strategies.

1.4.1Tumour necrosis factor-alpha (TNFα)

Tumour necrosis factor-alpha (TNFα) is a pro-inflammatory cytokine that mediates the inflammatory process within the immune system and is a crucial regulator of the host response to microbial challenges (Hop, et al., 2017; Lima, et al., 2019). Various cell types produce TNFα, however, macrophages are the primary type of monocytic lineage cells that produce TNFα (Lima, et al., 2019). During an inflammatory response, microglia secretes TNFα, initiating the production of additional pro-inflammatory cytokines. Hyper-activated microglia have been found to release excess amounts of TNFα, which is detrimental to the brain, and high levels of this cytokine have been associated with neurodegenerative diseases (Lladó, 2014). TNFα signalling occurs through the binding of TNFR1 and TNFR2 transmembrane receptors, with both being distinctive in their function and binding affinity (Page, et al., 2018). Activation of TNFR1 receptor produces a pro-inflammatory response involved in the

transduction of various intrinsic transduction pathways such as NF-κB, p38 MAPK, JNK and ERK.

On the other hand, activation of the TNFR2 receptor produces an anti-inflammatory response and promotes cell growth. Suppression of TNFR1 signalling is a critical target in slowing down the progression of neurodegenerative diseases. TNFα has been reported to control the secretion of pro-inflammatory mediators such as inducible NO synthase (iNOS), reactive oxygen species (ROS) and cyclooxygenase-2 (COX-2) through the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signalling in macrophages. This activation occurs through the stimulation of NF-κB inhibitor, IκB (Hop, et al., 2017). An investigation into TNFα activation on NF-κB in Raw 264.7 macrophage-like cells, found that blocking NF-κB signalling pathway inhibited TNFα induced effect on Brucella abortus clearance (Hop, et al., 2017). Suppressing the release of TNFα production in LPS-stimulated microglia is important in slowing down TNFα signalling that is mediated during neuroinflammation.

1.4.2 Interleukins

Interleukins (ILs) are a group of immunomodulatory cytokines that elicit immune responses in cells and tissues. Majority ILs are secreted by macrophages, monocytes, T-lymphocytes and endothelial cells. Based on the effects on leucocyte function, they are classified as either pro-inflammatory or anti-inflammatory. Pro-inflammatory ILs are involved in the upregulation of inflammatory reactions and are produced primarily by macrophages. Anti-inflammatory ILs play a role in counterbalancing pro-inflammatory responses of various infectious diseases. Glial cells, astrocytes and neurones are brain cells that synthesise ILs. The most studied ILs are IL-6, IL-1β and IL-10. The concentrations of interleukins increase rapidly in response to pathogenic invasion within the CNS and also treatment with exogenous LPS, IFNγ and TNFα.

1.4.3 Interleukin- 6 (IL-6)

Interleukin-6 (IL-6) is a potent pro-inflammatory cytokine produced in response to neuronal damage, and at high concentrations, plays a role in neurogenerative diseases (Dionisio-Santos, et al., 2019). It is secreted by macrophages in response to microbial stimuli's and has been found to mediate neuroinflammation during acute phase response. IL-6 is neuroprotective within the CNS; however, elevated levels can be pro-inflammatory and neurotoxic. The expression of IL-6 is enhanced at the sire of inflammation and with signalling occurring either via the classical or trans IL-6 signalling pathway. IL-6 mediates classical Il-6 signalling to the

IL-6 receptor, which is then associated with the cellular membrane-bound gp130. The pro-inflammatory response of IL-6 is associated with trans signalling, which is mediated with IL-6 bound to soluble IL-6 receptor and then attaches to gp130. Studies have identified IL-6 and IL-6 receptors in both glial and neuronal cells. Following stimulation with bacterial endotoxin-LPS, in BV2 microglia, IL-6 secretion occurs. Matsumoto et al. (2017), reported that IL-6 derived from pericytes enhanced BV2 microglia activation. Under normal physiological conditions, IL-6 exerts a neuroinflammatory immune response that is beneficial. During chronic neuroinflammation, IL-6 has been found to play a detrimental role in neurodegeneration diseases. Studies have reported that the expression of IL-6 during an inflammatory response associated with neuroinflammation and blood-brain barrier (BBB) disruption leading to cognitive impairment, a common symptom shared in patients with AD and PD (Furutama, et al., 2020). Over-expression of IL-6 has been established in neurodegenerative diseases and microglia models of neuroinflammation. Therefore, it is an essential target in therapeutic strategies to combat the neuropathology of these diseases.

1.4.4 Interleukin-1 Beta (IL-1β)

Interleukin-1 beta (IL-1β) is an inducible pro-inflammatory cytokine part of the interleukin-1 (IL-1) family and plays a role in normal immune and neuroinflammatory responses. IL-1β is expressed in brain cells, including microglia and neurones and is secreted through the mediation of Interleukin-1 type 1 and type 2 receptors (Mendiola & Cardona, 2018). IL-1β partakes in the regulation of immune responses, haematopoiesis, and inflammatory reactions. It exerts a pro-inflammatory response by signalling via interleukin 1 receptor type 1 (IL-1R1) and initiating acute neuroinflammatory processes (Mendiola & Cardona, 2018). Studies have shown after treatment with LPS, a significant increase in the secretion of IL-1β in brain-resident cells compared to untreated cells (Sheppard, et al., 2019). In another study investigating neuroinflammation in AD, infected brains from AD patients revealed elevated levels of IL-1β (Lopez-Rodriguez, et al., 2021). Additionally, Gao, et al. (2020) reported the neutralisation of IL-1β prevented PD-like pathological changes. The inhibition of IL-1β signalling has shown to be beneficial in inflammatory diseases, making IL-1β a promising target.

1.4.5 Anti-inflammatory cytokine: Interleukin-10 (IL-10)

Anti-inflammatory cytokine IL-10 is an immunoregulatory signalling molecule that is involved in initiating an immune response against pathogens, thus preventing neuronal damage (Ip, et al., 2017). Various anti-inflammatory cytokines have been acknowledged in the literature, including IL-1ra, IL-4, IL-10, TGF-β, and different soluble cytokine receptors. Interleukin 10 (1L-10) is an anti-inflammatory cytokine that plays an essential role in the immune response during neuroinflammation (Porro, et al., 2020). IL-10 is produced by immune cells and has an anti-inflammatory, inhibitory and self-regulating effect against inflammation. It is synthesised from microglia cells via TLR-4 activation and has been shown to reduce the production of pro-inflammatory cytokines, TNFα, IL-1β and IL-6. IL-10 elicits immunosuppressive effects both at cellular and humoral levels and during a damaging inflammatory response, has been shown to inhibit the activation of macrophages such as microglia into the site of injury (Steen, et al., 2020). IL-10 signalling is mediated through the binding to IL-10R, which is formed of a tetramer complex. A deficiency in the production of IL-10 can lead to excessive inflammation and chronic infection (Iyer & Cheng, 2012). Upregulation of IL-10 is a target to counteract the anti-inflammatory effects elicited by this pro-inflammatory cytokine. Therefore, increasing the secretion of IL-10 as a target in preventing neuroinflammation may lessen the development of neurodegenerative diseases. Evidence from AD mouse models reported that secreting IL-10 significantly reduced neuroinflammation and enhanced neurogenesis, further improving spatial cognitive dysfunction (Guillot-Sestier, et al., 2015).

1.5 Roles of Pro-Inflammatory chemokines in neuroinflammation

Chemokines are also known as chemotactic cytokines, secreted within the cytokine family and are involved in inducing inflammation through the recruitment of leukocytes (Ramesh, et al., 2013). Several chemokines have neuroprotective roles and function as pro- or anti-inflammatory mediators (Cartier, et al., 2005). Examples of chemokines include CCL5, CCL2, CCL7 and TSLP. Understanding the role of pro-inflammatory chemokines released in neurodegenerative disease is an essential aspect of this research. Chemokines are produced by various immune cells in response to cytokines and growth factors. Their function includes the movement of cells during inflammation, specific recruitment of lymphocytes, dendritic cells and macrophages. (Marques, et al., 2013). Based on the site of expression, chemokines can either be inflammatory (CCL2 and CCL5) or haemostatic (CXCl12 and CCL19) (Borsig, 2013;2014). Pro-inflammatory chemokines are involved in an inflammatory-mediated immune response through the recruitment of immune cells to the site of infection or injury (Liu, et al.,

2014). Pro-inflammatory chemokines and their receptors are both neuroprotective; however elevated levels can elicit neurotoxicity seen in neurodegenerative diseases.

1.5.1 Chemokine Ligand 2 (CCL2)

Chemokine ligand 2 (CCL2) is a pro-inflammatory chemokine involved in the recruitment of monocytes, memory T cells and dendritic cells to sites of inflammation during an inflammatory response. CCL2 is expressed in glial cells and, during peripheral inflammation, is elevated in the CNS. Furthermore, it is produced in response to LPS-induced brain inflammation (Le Thuc, et al., 2016). Activated microglia has also been found to express CCL2, as well as neurones and astrocytes. CCL2 signalling is mediated through the binding of the CCR2 receptor and has been reported to be implicated in various inflammatory and neurodegenerative diseases, with high levels found secreted by activated microglia (Hao, et al., 2020). Studies have reported increased levels of CCL2 in the prodromal stage of AD, in brain tissue and cerebral spinal fluid (Azizi, et al., 2014). Overexpression of CCL2 is associated with an increase in amyloid deposition in transgenic mice, as detected in the pathogenesis of AD. (Azizi, et al., 2014). CCL2/CCR2 signalling complex is involved in neuroinflammatory pathways, with the inhibition of this pro-inflammatory chemokine being a new target in reducing the underlying pathology observed in neurodegenerative diseases (Joly-Amado, et al., 2020).

1.5.2 Chemokine Ligand 5 (CCL5)

Chemokine ligand 5 (CCL5) is a chemoattractant pro-inflammatory chemokine that plays an active role in cerebral and peripheral inflammation and recruitment of leukocytes to inflammatory sites (Škuljec, et al., 2011). CCL5 binds to specific chemokine receptors, CCR1, CCR3 and CCR5. Microglia cells within the CNS express CCL5 and CCR5 receptors during an inflammatory response. CCL5 has been found to increase the inflammatory mediator nitric oxide (NO) in activated microglia (Škuljec, et al., 2011). The signalling cascade is associated with the polarisation and translocation of NF-κB, resulting in the increased phagocytic ability, cell survival and transcription of pro-inflammatory genes. Studies have reported in models investigating infections within CNS, the secretion of CCL5 in activated microglia. Additionally, neuronal degeneration was mediated when activated by astrocyte derived CCL5 (Marques, et al., 2013). There is limited research into the effects of pro-inflammatory CCL5 in the mediation of neuroinflammation; hence investigation into the inhibition of CCL5 in LPS-stimulated microglia is beneficial in slowing down CCL5 signalling.

1.6 Role of other inflammatory mediators in neuroinflammation

In recent years, there has been an increasing interest in the association of various types of microglial inflammatory mediators linked with the pathogenesis of chronic inflammation prevalent in neurodegenerative diseases (Abdulkhaleq, et al., 2018). Activated microglia cells initiate an inflammatory response in the CNS in response to pathogenic invasion or infection causing the release of inflammatory mediators. Nitric oxide (NO) and COX-2 are inflammatory mediators commonly generated in response to microglia activation and phagocytosis (Carniglia, et al., 2017). LPS activates microglial cells, which alters the expression of various inflammatory mediators (Batista, et al., 2019). Microglia are cellular targets for understanding and developing therapeutics against CNS diseases (Carniglia, et al., 2017). Further research is required to establish the pathological and molecular roles of inflammatory mediators in the pathogenesis of neurogenerative diseases.

1.6.1 Nitric Oxide (NO)/inducible nitric oxide synthase (iNOS)

Nitric oxide (NO) is an essential reactive nitrogen species (RNS) and a signalling molecule that is mediated during neuroinflammation (Yuste, et al., 2015). NO biologically functions as a vasodilator, inflammatory mediator and neuromodulator and is synthesised by immune cells such as microglia. NO within the CNS can be neuroprotective or neurotoxic, dependent on the concentration of NO expressed. An imbalance of NO production during an inflammatory immune response is a feature of over-activated microglia. Upregulation of NO and inducible nitrogen oxide synthase (iNOS) is linked to the pathology of neurodegenerative diseases (Park, 2004). Animal models of neuroinflammation have established the role of NO being coupled with excitotoxicity processes induced by activation of microglia and accumulation of glutamate (Jin, et al., 2021). Excessive NO produced by activated microglia during neuroinflammatory conditions blocks the re-uptake of glutamate, instigating neuronal death. Hyper-active microglia produce excessive amounts of pro-inflammatory mediators, iNOS resulting in excessive NO production.

Endogenous nitrogen oxide synthase (NOS) synthesises NO through the conversion of L-arginine to NO and L-citrulline (Tse, 2017). Traditionally, NO is synthesised by 3 NOS isomers: inducible NO synthase (iNOS), endothelial NO synthase (eNOS) and neuronal NO synthase (nNOS) (Tse, 2017). The pathological effects are associated with the pathogenesis of several neurodegenerative diseases due to the high level of NO present in the CNS. Inducible NO synthase (iNOS) is a central downstream mediator of inflammation produced in various

cell types; the regulation of this isoform takes place at a transcriptional level. iNOS is the only isoform of NO synthesised from NOS induced by inflammatory ligands such as pro-inflammatory cytokines and bacterial endotoxin LPS. Evidence supports the role of inflammatory ligands in producing large amounts of iNOS and NO in microglia cell culture models. NO, and iNOS signalling pathway is a feature in mechanism in over-activated microglia. NF-κB and AP-1 transcriptional factors are implicated in the trans-activation of the iNOS gene. The expression of iNOS mediated by the activation of microglia within the NF-κB pathway initiates several intrinsic pathways linked to RNS formation, caspase and neuronal NOS signalling. Studies have demonstrated the inhibition of LPS-induced NF-κB signalling, mediated iNOS, and NO production will suppress microglia in degenerative neuronal disorders (Jin, et al., 2021). Excessive NO secretion in neuroinflammation is involved in disease pathology such as AD and PD (Liy, et al., 2021). Inhibition of NO salvages neuronal cell death caused by high levels of NO production in the CNS during neuroinflammation. Pharmacological interventions to rectify the imbalance of NO and iNOS signalling in neurodegenerative diseases in critical.

1.6.2 Cyclooxygenase-2 (COX-2)

Cyclooxygenase (COX) is an enzyme that catalyses the conversion of arachidonic acid to prostaglandins (PGs) (López & Ballaz, 2020). COX has two heme-containing isoforms; cyclooxygenase-1 (COX-1) and cyclooxygenase- 2 (COX-2). COX-1 is a ubiquitous enzyme that is expressed in most cells types is essential in normal physiological functions. Interestingly, COX-2 activation initiates inflammation and is expressed in brain synaptic dendrites and spinal cord neurones. COX-2 is the inducible isoform, expressed in several cell types such as microglia, neurones and astrocytes in response to cytokines and pro-inflammatory molecules (Minghetti, 2004; Mitchell & Kirby, 2019). The expression of COX-2 within the brain is associated with activities of inflammatory mediators, involved in neurodegenerative processes of several acute and chronic diseases. PGE_2 is a well-established metabolite of COX-2 that speeds up the conversion of arachidonic acid into PGE_2. COX-2/PGE_2 signalling cascade induces inflammation involved in the pathogenesis of various neurodegenerative diseases such as AD and PD.

There is growing evidence that neuroinflammation is triggered by the induction of glial COX-2 expression, which is markedly increased in microglia and astrocytes. LPS has been used as inflammatory stimuli in models of neuroinflammation; results showed selective inhibition of COX-2 elevated neuronal damage and glial activation in the expression of brain cytokines (Aïd

& Bosetti, 2011). Studies have reported the secretion of COX-2 being effective neuromodulators of inflammation in the brain, with pathological factors leading to excitotoxicity and neurodegeneration (López & Ballaz, 2020). The initiation of COX-2 signalling pathway in microglia cells has contributed to the progress of neuroinflammation in neurodegenerative diseases such as AD and cancer (Hashemi, et al.,2019). Blocking the expression of COX-2 protein remains a valuable target as a potential neuroprotective treatment strategy aimed at slowing or halting the progression of the disease, such as PD (Teismann, 2012). A study found an increase in hippocampal PGE2 levels was associated with a strong induction of COX-2 expression in PD patients, which increased disease progression and was localised explicitly in microglia cells (Minghetti, 2004). Identification of the specific roles of COX-2 in CNS inflammation and its coupling to downstream receptors could open new approaches for the treatment of neuroinflammatory conditions.

1.7 Nuclear factor-kappa B (NF-κB) signalling in neuroinflammation

Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF-κB) is a small family of inducible transcription factors that is a primary regulator of DNA transcription, cytokine production, cell survival, immune and inflammatory responses within the CNS (Zhou, et al., 2020). NF-κB plays a crucial role in several biological processes such as inflammation, cell survival and apoptosis. The NF-κB family consists of 5 protein subunits p65/RelA, RelB, c-Rel, p105/p50 (NF-kB1), and p100/p52 (NF-kB2), all forming homo/heterodimeric complexes that are transcriptionally distinct. NF-κB p65/p50 heterodimers are members of the NF-κB transcription family that are widely studied. Activation of microglia NF-κB during neuroinflammation mediates induction of various pro-inflammatory genes and regulates the transcription of many target genes against inflammatory ligands such as LPS and TNFα. Neuroinflammation is beneficial as it produces an acute response to the host and resolves the injury promptly. NF-κB signalling serves to be neuroprotective when it is tightly regulated to maintain homeostasis (Yu, et al., 2020). Extensive research has demonstrated NF-κB activation to be neuroprotective against excitotoxicity and oxidative stress in neurone cell bodies. Yu, et al. (2020), reported ubiquitination being involved in the positive regulation of NF-κB signalling. On the other hand, excessive activation of NF-κB signalling causes chronic inflammation (Shih, et al., 2015). Downregulation of microglia NF-κB can cause excessive tissues damages and contributes to the development of acute or chronic neurodegenerative diseases such as AD and PD. Studies have shown activation of NF-κB signalling, stimulated

with LPS to express inflammatory proteins COX-2 and PGE2. (Shih, et al., 2015). Furthermore, NF-κB mediates glial responses to pathogenic invasion through TLR signalling. NF-κB activated microglia triggers the release of pro-inflammatory cytokines such as TNFα and IL-1β and reactive oxygen species (Shih, et al., 2015).

NF-κB complex in the cytoplasm interacts with inhibitors kB proteins IκB kinase (IKK), initiating IκB's phosphorylation upon stimulation. This phosphorylation facilitates ubiquitination, allowing NF-κB dimers (p50 or p65) to be released from IκB after degradation. This enables NF-κBs to be translocated into the nucleus, eliciting transcriptional activity (Zhou, et al., 2020). NF-κB transcription factor recognises and binds to p65 responsive elements in the regulatory region of target genes; this leads to the transcriptional activation of TNFα, IL-1β and IL-6 (Subhramanyam, et al., 2019). NF-κB has two signalling pathways known as canonical (classical) and non-canonical (Alternative) (Figure 3). Despite the difference in NF-κB's signalling, together with canonical and non-canonical pathways, it regulates immune and inflammatory responses (Liu, et al., 2017). The canonical NF-κB signalling pathway involves both acute and chronic inflammatory reactions; pro-inflammatory cytokines and toll-receptor ligands induce this pathway. The canonical pathway mediates the activation of the IKK complex, which contains IKKα (IKK1), IKKβ (IKK2), and NF-κB essential modulator (NEMO) (Dorrington, et al., 2019). On the other hand, the activation of the non-canonical NF-κB signalling pathway can only be initiated by activation factor (BAFF), lymphotoxin β, CD40 ligand (CD40L), and receptor activator of nuclear factor kappa-B ligand (Liu, et al., 2017; Meyerovich, et al., 2018). NF-κB transcription factor is a recognised risk in AD, with pro-inflammatory molecules released when Aβ in secreted initiates the upregulation of NF-κβ expression (Jha, et al., 2019). The regulation of microglial NF-κB activity is critical in suppressing chronic inflammation that arises in neuroinflammation. An additional better understanding of the mechanism underlying the pathological activation of NF-κB in neurodegenerative disease is crucial for identifying effective therapeutics for treating neuroinflammatory disorders.

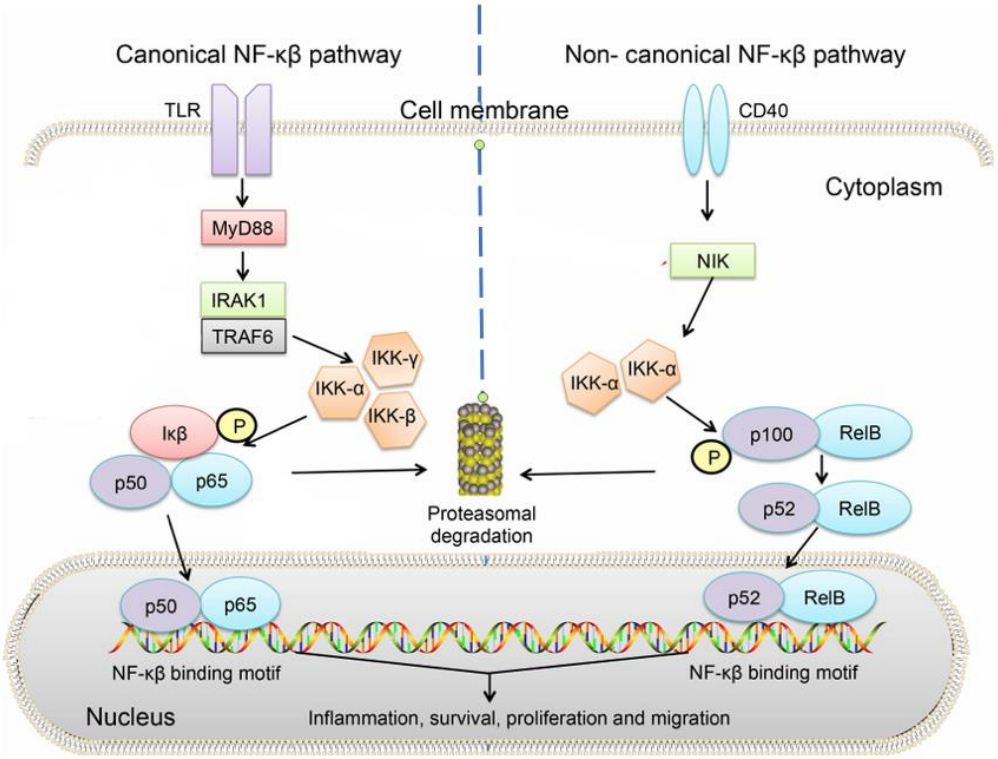


Figure 3: Adapted diagram of Canonical and Non-canonical NF-κB signalling pathway

Nuclear factor kappa-B (NF-κB) transcription factor in its inactive state is located in the cytoplasm, bound to inhibitor IκB. Translocation of NF-κB into the nucleus is a result of the degradation of IκB linked to the phosphorylation of the IKK complex. This initiates the release of inflammatory mediators. The canonical NF-κB pathway involves a rapid response to inflammation. Non-canonical NF-κB pathway initiates a slow response (Jha & Pan-Yun Ting, 2015).

1.8 p38 mitogen-activated protein kinase (MAPK) activation in neuroinflammation

Mitogen activated protein kinases (MAPKs) cascade via a toll-like receptor and TNF receptor families, further leading to the transcription of inflammatory genes such as iNOS, TNα and IL-1β. The suppression of MAPK is a strategy in neuroinflammatory conditions; it can reduce the transcription and synthesis of pro-inflammatory cytokines in microglia. MAPK are three cascades consisting of extracellular signal-related kinases ½ (ERK1/2), c-Jun N-terminal kinases 1/2 (JNK) and p38 MAPk, which the latter is activated by MKK3 and MKK6 and one of the most studied MAPKs. (Zhao, et al., 2018). P38 MAPk is a regulator of various pro-inflammatory cytokines in the pathogenesis of the neurodegenerative disease. P38 MAPK signalling plays a role in the activation and production of neurotoxic mediators in neuroinflammation. The activation of TLR4 with LPS triggers p38 MAPK signalling. P38

MAPK has been identified as an enzyme with an inflammatory role in immune cells, including microglia. P38 MAPK has been found to play a role in inflammation, cell death, cell proliferation and differentiation. LPS-induced microglia cells have been reported to activate p38 MAPK and are a signalling mechanism governing TNFα expression regulation in macrophages.

1.9 Lipopolysaccharide (LPS) as an inducer of neuroinflammation

Lipopolysaccharide (LPS) is a significant component of the outer membrane found on the cell wall of gram-negative bacteria, capable of inducing a strong response from the animal immune system (Zhao, et al., 2018). LPS is a bacterial product that is regularly used to induce inflammation, through toll-like receptor 4 (TLR4), *in vitro* models of neuroinflammation (Park & Lee, 2013). LPS released from infecting pathogens initiate a robust innate immune response. Toll-like receptors regulate immune-inflammatory responses against bacterial infection and bacterial products such as LPS. LPS is released from LPS binding protein (LBP), extracted from bacterial membranes and vesicles. LBP transmits LPS to the protein cluster of differentiation CD14. CD14 divides LPS aggregates into monomeric molecules, coupling LPS to TLR4 and myeloid differentiation TLR4-MD-2 complex. Aggregation of TLR4-MD-2 complex after binding of LPS leads to activation of signalling pathways such as NF- κB (Park & Lee, 2013). LPS is commonly used as inflammatory stimuli in neuroinflammation models, as it can initiate acute inflammatory responses and has pathological effects that can be studied in neuroinflammatory disorders (Sivaprakasam, et al., 2019). Studies have shown LPS to be involved in activating signalling pathways, NF-κB and p38 MAPK at a transcriptional level. Therefore, LPS has been identified as a suitable ligand for over-activated microglia-mediated neuroinflammation.

1.10 BV2 microglia cell line as an *in vitro* model for neuroinflammation

Microglia are resident macrophage cells within the CNS and plays a role in immunity, neuroinflammatory pathologies and toxicity. BV2 and N9 microglia cell lines are commonly used as they are easy to maintain and, due to their unrestricted proliferation capacity, produce abundant cells. BV2 microglial cells are generated from primary microglia with oncogenes v-raf/v-myc (J2 virus) and maintain microglia's morphological and functional characteristics (Timmerman, et al., 2018). Furthermore, this cell line shares the same properties to microglia over the body, concerning the phagocytic capacity, antigen profile and antimicrobial activity. BV2 microglia is commonly used as an alternative *in vitro* cell culture model to investigate

neuroinflammation and neurodegenerative diseases (Timmerman, et al., 2018). Activated BV2 microglia produces pro-inflammatory cytokines by inflammatory factors and oxidative stress. BV2 microglia cells are responsive to LPS, commonly used to induce inflammation in the BV2 microglial cell line alongside treatment with the compound or drug of interest. Stansley et al. (2012) reported LPS to induce 90% of inflammation-related genes when used as a stimulant for BV2 cells compared to primary rat microglia cells (Henn, et al., 2009). Chronic activation of microglia is a feature of neurodegenerative diseases followed by elevated pro-inflammatory cytokines, which further disrupts the regular CNS activity. On the other hand, N9 microglial cells are also widely studied microglia cell lines. Parallel to BV2 cells, they can produce and secrete similar pro-inflammatory mediators and are responsive to LPS (Timmerman, et al., 2018). Research on BV2 microglia has demonstrated this cell lines ability to function similarly to primary microglia cells within the body regarding cytokine secretion, synaptic plasticity and neuronal networking (Sivaprakasam, et al., 2019). The limitation to using BV microglia cell line as a model for neuroinflammation includes the limited proliferation capacity of the cells. Due to the risk of cell death BV2 cells have to be passage frequently and freshly prepared more often in comparison to other cell lines. However the benefits such as its stimulatory response, preparation time and feasibility between experiments makes it most suitable. Hence, the BV2 microglia cell line is suitable *in vitro* for studying neuroinflammatory mechanisms and inhibitory effects of novel compounds using LPS as an inflammatory stimulus (Sivaprakasam, et al., 2019). In the present study, to understand the effect of quassin in neuroinflammation, LPS-activated BV2 microglia were used as an *in vitro* model to investigate the neuroinflammatory effects.

1.11 Anti-inflammatory natural compounds

Natural compounds derived from plants display therapeutic potential against various chronic conditions, including inflammation and cancer (Diederich, 2020). For centuries, traditional medicine has used natural compounds to cure or alleviate the symptoms of a wide variety of diseases. Scientists have rediscovered natural compounds with anti-inflammatory potential, and there is extensive research to understand their mechanism of action. Natural compounds possess neuroprotective potentials against neuroinflammation by mediating antioxidant, anti-inflammatory and antiapoptotic properties, selectively promoting and inhibiting various molecular transduction pathways and modulating inflammatory responses involved in neurodegeneration (An, et al., 2020). Many studies have established the beneficial effects of natural compounds derived from plants, vegetables, dietary nutrients and endogenous

molecules on neuroinflammation (Azab, et al., 2016; Khadaka, 2020; Olajide, 2020). Neurodegenerative diseases are a significant health concern, with neuroinflammation being a fundamental process in the progression of various neurodegenerative diseases. Researchers have attempted to find potential therapeutics to inhibit neuroinflammation within the CNS using natural compounds.

Additionally, abnormal activation of microglia in LPS-induced inflammation is a feature of neuroinflammation, coupled with the release of pro-inflammatory cytokines and inflammatory mediators. Natural compounds place an advantage on synthetic drugs as they possess low side effects (Shal, et al., 2018). However, the pharmacological profiles are a limitation due to their low bioavailability and poor solubility and permeability through the blood-brain barrier (BBB). Thymoquinone, formononetin, flavonoids, alkaloids and paeonol are natural compounds that have been reported to exert anti-neuroinflammatory effects on LPS-activated microglia via inhibition of the NF-κB signalling pathway (An, et al., 2020). They also cause a decrease in the expression of inflammatory mediators (NO, iNOS and COX-2) and pro-inflammatory cytokines (IL-6, TNFα, IL-1β). In a study conducted by Cobourne-Duval et al. (2018), it was shown that treatment of thymoquinone in LPS/IFN-γ- activated BV2 microglial cells significantly decreased the expression of pro-inflammatory cytokines and target genes of the NF-κB pathway (Cobourne-Duval, et al., 2018). Formononetin is another example of a natural compound used in the inhibition of neuroinflammation by targeting NF-kB signalling pathways in BV2 microglia; formononetin reduced the expression of TNFα, IL-6, IL-1β, as well as iNOS and COX-2 (El-Bakoush & Olajide, 2018). α-Synuclein is a neuronal protein that contributes to the pathogenesis of parkinson's disease (PD); hydroxytyrosol, a primary metabolite derived from oleuropein, a phenolic bitter natural compound found in olive oil, this compound has been reported to possess anti-inflammatory activities. Results suggested hydroxytyrosol inhibited α-synuclein aggregation (Diederich, 2020; Siracusa, et al., 2020).

1.12 Quassinoids

Quassinoids are triterpenoids derived from the Simaroubaceae family. They are bitter compounds from the *quassia amara* tree. The name quassnoids was derived from quassin, the first compound to be structurally identified and isolated from the *quassia amara* tree. They possess a wide range of biological activities such as anti-tumour, anti-malarial, insecticidal, amoebicidal and anti-inflammatory (Bhattacharjee, et al., 2008; Lia, et al., 2019). Over the past few decades, research in natural resources for new drug discovery have research plants rich in quassinoids. They are structurally unique and complex natural products and exhibit a

wide range of interesting chemical properties and biological. Chemically quassinoids are degraded triterpenoids derived from tetracyclic triterpenes. Furthermore, oxygenated lactones with variable hydroxyl groups, hydroxyl esterified, carbonyl, methoxy and carbomethoxyl (Lia, et al., 2019). As shown in figure 4, the six main categories of quassinoids are: C_{26}, C_{25}, C_{18}, C_{22}, C_{19} and C_{20}. The latter C_{20} is the largest interest group and exhibits lower cytotoxicity than other classes (Duan, et al., 2021). To date, there are only several studies that have investigated the neuroprotective and anti-inflammatory activities of quassinoids, in particular on macrophage cell line models. A recent study by Guo, et al. (2019) investigated the neuroprotective effects of EtOAc extracts. EtOAc extracts showed neuroprotective activities by suppressing neuroinflammation and reducing amyloid β deposition in L-glutamate-stimulated PC12 and Aβ25-35-stimulated SH-SY5Y cell models (Guo, et al., 2019). Isobrucein B is another quassinoid that reported anti-inflammatory effects, and treatment with this natural compound reduced the production of pro-inflammatory cytokines TNFα and IL-1β in LPS-stimulated macrophages (Silva, et al., 2015). Despite limited literature on the cytotoxicity of quassinoids against glial and neuronal cells, the cytotoxicity of quassinoids has been established against cancer cell lines; human breast cancer cell line (MCF-7) and human melanoma cells (HM3kO) (Tee & Azimahtol, 2005; Tran, et al., 2014; Tung, et al., 2017).

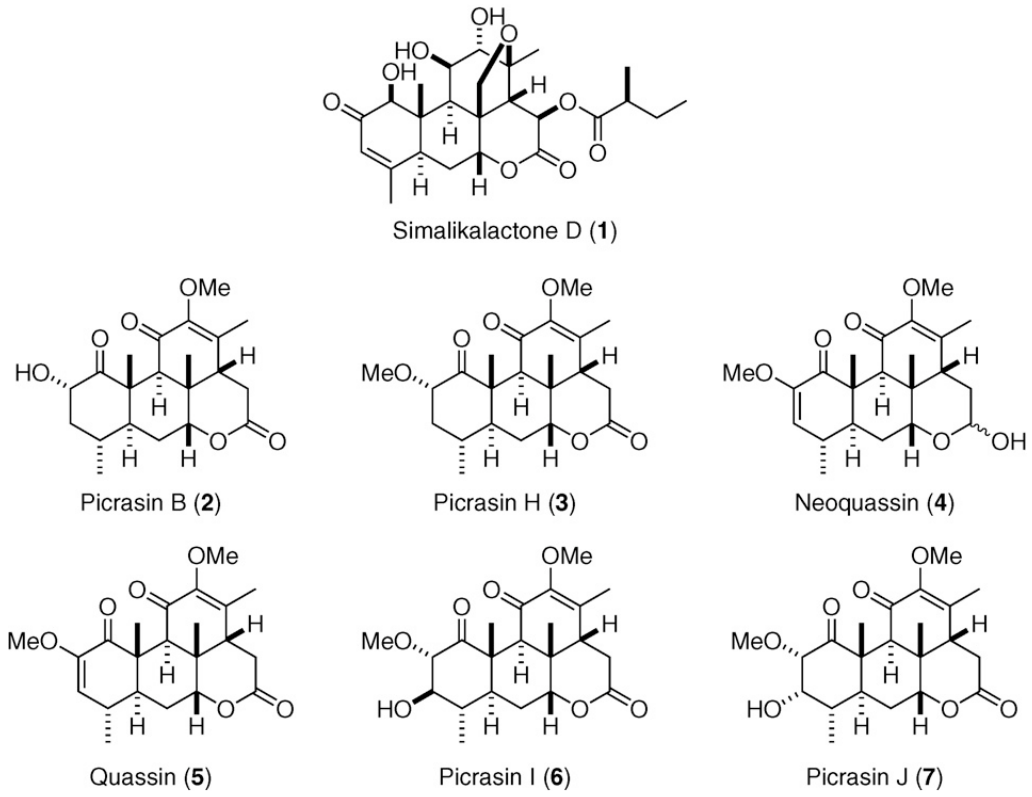


Figure 4: Chemical structures of isolated quassinoids from *quassia* Amara. *(Houël, et al., 2009)*

1.12.1 Quassin

Quassin is a white bitter, crystalline substance from the quassionid family (figure 5); it is a natural product extracted from the bark of *Quassia amara* and is used traditionally in Chinese herbal medicine (Bhattacharjee, et al., 2008; Clark, 1937). Quassin has been reported to have antiulcerogenic, antiplasmodial, antileishmanial, anti-malarial, anti-diabetic activities (Lang'at-Thoruwas, et al., 2003; Bhattacharjee, et al., 2008; Husain, 201; Faisal & Akbarsha, 2020). A study by Bhattacharjee et al. (2008) found quassin at a dose of 25µg/ml exhibited low levels of cytotoxicity on murine peritoneal macrophages. Additionally, this study reported quassins ability to suppress the levels of NO and upregulation of pro-inflammatory cytokines IL-12 and TNFα in L. donovani-infected macrophages (Bhattacharjee, et al., 2008). Despite the reported properties of quassin, its ability to inhibit neuroinflammation has not yet been investigated. As part of continuing research to discover biologically active natural products, the anti-inflammatory activity of quassin in targeting neuroinflammation was investigated. By determining effects on the release of pro-inflammatory mediators, pro-inflammatory cytokines and chemokines, anti-inflammatory cytokine and transcription activity on the NF-κB signalling pathway in BV2 LPS-stimulated microglia.

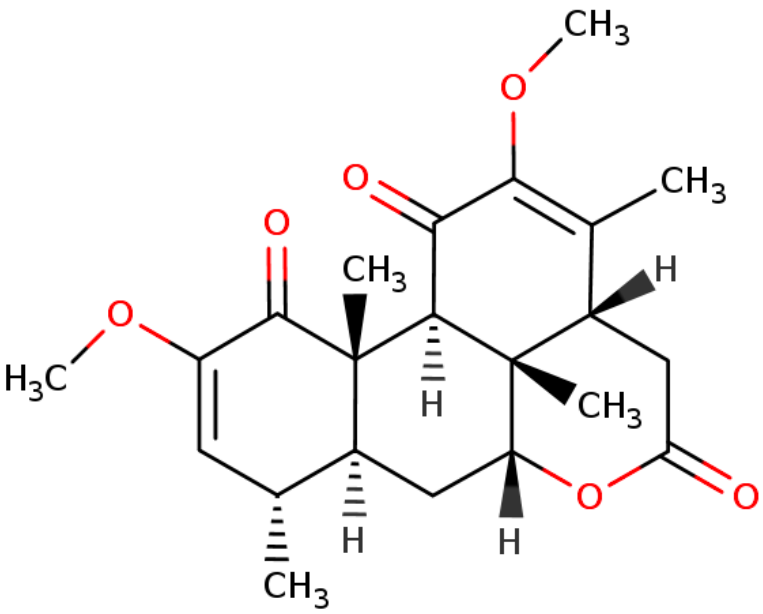


Figure 5: Chemical structure of quassin (Chambers, 2022)

1.13 Gap in knowledge

To date there are limited studies that have investigated the anti-inflammatory effects of quassin. Furthermore, the mechanism of action of quassin in inhibiting neuroinflammation is not known.

1.14 Aim of this study

The overall aim of this research is to evaluate quassin's inhibitory effects on neuroinflammation in LPS-stimulated BV-2 microglia cells.

1.7.1 Specific Objectives

2. To examine the effects of quassin on the release of pro-inflammatory cytokines in LPS-stimulated BV-2 microglia.
3. To examine the effects of quassin on the release of pro-inflammatory chemokines in LPS-stimulated BV-2 microglia.
4. To evaluate whether inhibitory actions of quassin on LPS induced neuroinflammation are due to the interference with NF-kB signalling in microglia.

Chapter 2: Materials and Methods

2.1 BV-2 Microglia

Mouse microglia are widely used in *in vitro* studies due to their unrestricted proliferation capacity and response to external stimuli such as lipopolysaccharide (LPS) (Asraf, et al., 2018). BV-2 mouse microglia have been used over the years to study neuroinflammation and neurodegenerative diseases (Asraf, et al., 2018). In this research, BV2 microglia cell line (ICLC ATLO3001) was obtained from Interlab Cell Line Collection, Banca Biologicae cell factory, Italy. The cells were cultured in Roswell Park Memorial Institute Medium (RPMI) 1640 with L-glutamine (2mM), supplemented with 10 % Foetal Bovine Serum (FBS) (Sigma), 100 mg/ml Penicillin/Streptomycin (Sigma) and 100mM sodium pyruvate (Sigma).

Cells were passaged every 48 hours in a 75cm2 filter-capped vented flask and cultured in a 5% CO2 incubator at 37°C. Once confluent cells were washed with 5 ml phosphate-buffered saline (PBS) and 0.25% trypsin-EDTA solution (2 ml). Fresh RPMI medium (8 ml) was added to terminate the action of trypsin. Cells were then transferred into a 50ml centrifuge tube and

centrifuged at 1200 rpm for 5 minutes. The cell pellet was then re-suspended in a 10 ml complete RPMI medium. Cell count was performed, and cells were seeded out at a concentration of 1 x 10^5 cells/ml in the respective cell culture plates required. The volume of cell suspension that was added into each well was dependent on the cell culture plate used for the respective experiments.

2.1.1 Quassin and Treatment with bacterial lipopolysaccharide (LPS)

Quassin (PhytoLab, Germany) was dissolved in DMSO (Fisher) to form a primary stock of 0.1M and stored in small aliquots at -80℃. Lipopolysaccharide (LPS) is derived from salmonella. Typhimurium, s-type TLRpure Sterile Solution (100 ng/ml) (Innaxon), was used to stimulate BV2 microglia for all experiments. LPS was prepared freshly before treatment with sterile water (Water for Injection) (GibcoTM) for all experiments. For all experiments, the working concentrations of quassin (10, 20, 40 and 80 µM) were prepared freshly, in DMSO (Vehicle). All prepared aliquots were vortexed before use.

2.2 MTS: Cell Viability Assay

The MTS assay (Promega) is a cell viability assay that uses a colorimetric method to quantify the number of viable cells in culture supernatants. MTS was used in this study to establish that the concentrations used did not affect cell viability. It indicated that any reduction in signals that might occur was not due to cell death. The advantage of the MTS assay compared to other cell viability and cytotoxic assays are that it only involves one step and is fast and reliable (Riss, et al., 2016). MTS assay involves the breakdown of a tetrazolium (5-(3-carboxymethoxyphenyl)-2-(4,5-dimethyl-thiazoly)-3-(4-sulfophenyl), a salt that produces coloured formazan that is soluble in cell culture medium (Figure 6). Viable cells contain NADH dependent dehydrogenase enzymes that break down the tetrazolium to produce formazan. The formazan dye is quantified by measuring the absorbance at 490-500 nm.

BV2 microglial cells were seeded at a density of 1×10^5 cells/ml in a 96-well cell culture plate, a volume of 200 µl was added into each well and incubated at 37℃ for 24 hours. Once cells were 70% confluent, cells were then stimulated with and without LPS in the absence and presence of quassin (10,20,40 and 80µM) and incubated for 24 hours at 37℃. After 24 hours, 100µl of cell culture medium was removed from each well and replaced with 20µl of MTS solution. The plate was then further incubated for 3 hours at 37℃, and the colorimetric formazan solution was read at 490nm with a microplate reader (Tecan, Infinite F50).

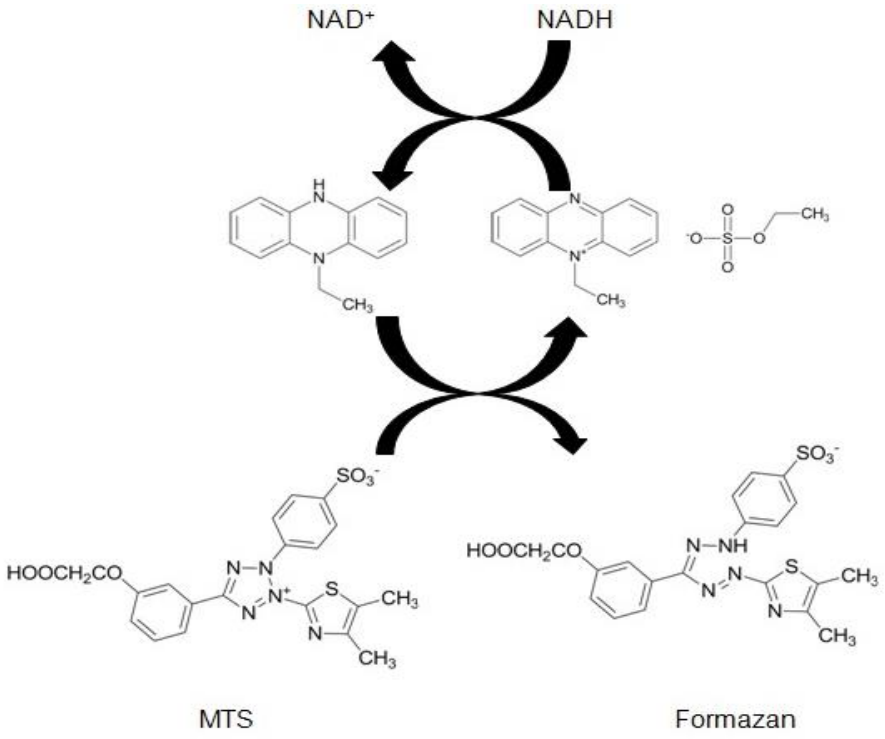


Figure 6: Reaction of MTS tetrazolium forming formazan product *(Riss, et al., 2016)*

2.3 Nitrite Production (Griess Assay)

Nitric oxide (NO) is a mediator that plays an important role in neuroinflammation, it is released from activated BV2 microglia cells following neurotoxic or inflammatory damage (Yoshioka, et al., 2016; Liy, et al., 2021). Studies have reported glial cells within the brain generating excessive NO synthesis during neuroinflammation. This excess release leads to tissue damage and neuronal cell death (Albaayit, et al., 2019 Liy, et al., 2021). Nitric oxide (NO) on its own is unstable, however in the human body it reacts with other molecules to become stable. Nitric oxide is produced by enzyme nitric oxide synthase (NOS), from amino acid L-arginine (Tse, 2017) Therefore, regulation of NO production in BV2 microglia cells is important for maintaining homeostasis within the brain and neuronal survival (Yoshioka, et al., 2016). Nitrite (NO_2^-) an inducible form of nitric oxide is measured with the use of Griess assay. Griess assay is a colorimetric assay used to determine the nitrite production released in cells, in this case in BV2 microglial culture supernatants. As seen in figure 7, this Assay involves a two-step diazotization reaction in which NO reacts with sulphanilamide to form a diazonium salt. The next step involves coupling with N-(1-naphthyl) ethylenediamine dihydrochloride (NNED)

under acidic conditions which produce a chromophobic azo product, the pink colour which is measured for absorbance (Váradi, et al., 2019) (Figure 1).

The reagents used for Griess assay was prepared prior to the experimental assay. To achieve a 1% solution of sulphanilamide, 0.5g of sulphanilamide (Aldrich) was dissolved in 5ml phosphoric acid and 47.5ml deionised water. Furthermore, to achieve 0.1% of NNED, 0.05g of NNED (Sigma-Aldrich) was dissolved in 50ml deionised water. Reagents prepared were vortexed and stored at 4℃. BV2 cells were seeded out at a density of 1×10^5 cells/ml in a 24 well plate cell culture plate and a volume of 1000µl was added to each well. . Once confluent, the cells were pre-treated with quassin (10, 20, 40 and 80 µM) for 30 minutes, then stimulated with LPS (100 ng/ml) in the continued presence of quassin. After 24 hours of stimulation, culture supernatants were collected and centrifuged for 5 minutes at 13500 rpm. Then 50 µl of culture supernatants were added to a 96 well culture plates in duplicates, followed by the addition of 50 µl of Sulphanilamide solution. The plate was covered and incubated in the dark for 10 minutes at room temperature. Thereafter, 50 µl of NNED was added, the well plate was further incubated for 10 minutes in the dark. The absorbance was measured within 30 minutes at 540nm in a microplate reader (Tecan, Infinite F50).

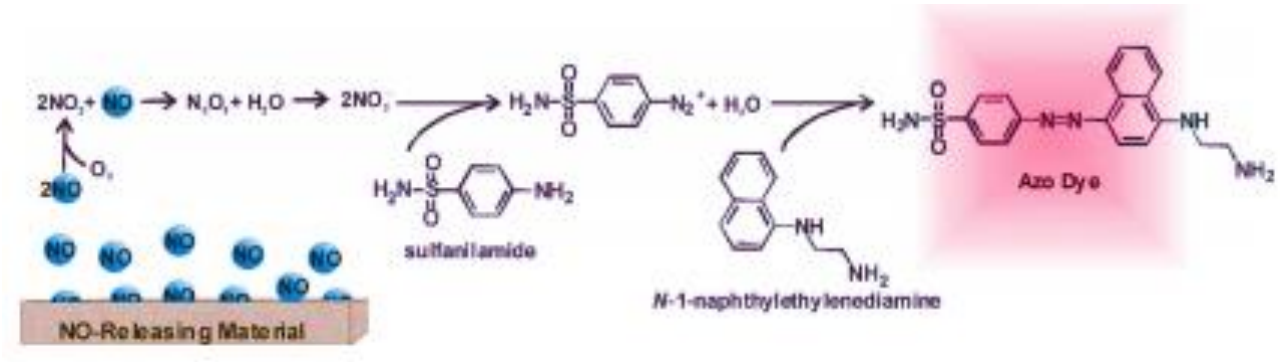


Figure 7: Chemical reaction of Griess Assay reagent measuring the production of nitrite
(Coneski & Schoenfisch, 2012)

2.4 Enzyme-linked immunosorbent assay (ELISA)

Enzyme linked immunosorbent assay (ELISA) is used to measure levels of cytokines, chemokines and proteins in culture supernatants. A sandwich ELISA was used to measure the antigens in the LPS-stimulated BV-2 microglial cells, between the capture and detection antibody (secondary antibody), as seen in figure 8. The capture antibody is immobilised to the surface of the microwell plate and specifically detected by an enzyme-labelled antibody (Terato, et al., 2016). A secondary antibody conjugate that binds to the detection antibody is

added and this causes a reaction with a substrate that produces a signal that is measurable at 450nm. Activated BV-2 microglia stimulated with LPS primarily produce excess pro-inflammatory cytokines, including TNFα, IL-1β and IL-6 (Yan, et al., 2020). Additionally, IL-10 is a key anti-inflammatory cytokine that is involved in neuroinflammation and under normal physiological conditions, plays a role in preventing inflammatory pathologies (Porro, et al., 2020). Pro-inflammatory chemokines are induced during an immune response to recruit cells within the immune system to sites of injury or infection (Shachar & Karin, 2013). CCL2 and CCL5 are widely studied chemokines that are formed during pathological conditions and actively participate in neuroinflammatory response (Grunz-Borgmann, et al., 2015). Therefore, inhibiting the release of these pro-inflammatory cytokines and chemokines and anti-inflammatory cytokines in LPS-stimulated BV-2 microglia is important in order to suppress neuroinflammation.

BV2 microglia cells were seeded out into a 24-well cell culture plate at a density of 1×10^5 cells/ml, a volume of 1000µl was added to each well and incubated at 37℃ for 24 hours. Once confluent, 1000ul of the culture medium was replaced with serum-free RPMI 1640 medium and incubated at 37℃ for 1 hour. After, cells were pre-treated with quassin (10,20,40 and 80 µM) for 30 minutes. Cells were then stimulated with LPS (100ng/ml) and incubated at 37℃ for a further 24 hours. Stimulation was terminated and the supernatants were collected and centrifuged for 5 minutes at 135000 rpm at 4°C. Culture supernatants were stored for short term use at -20℃ and Concentrations of TNFα, IL-1β, IL-6, IL-10, and CCL2 were measured with ELISA kits (Invitrogen). The levels of CCL5 pro-inflammatory chemokine were measured using DuoSet ELISA kit (R&D systems). Absorbance of all samples was measured at 450nm with microplate reader (Tecan Infinite F50).

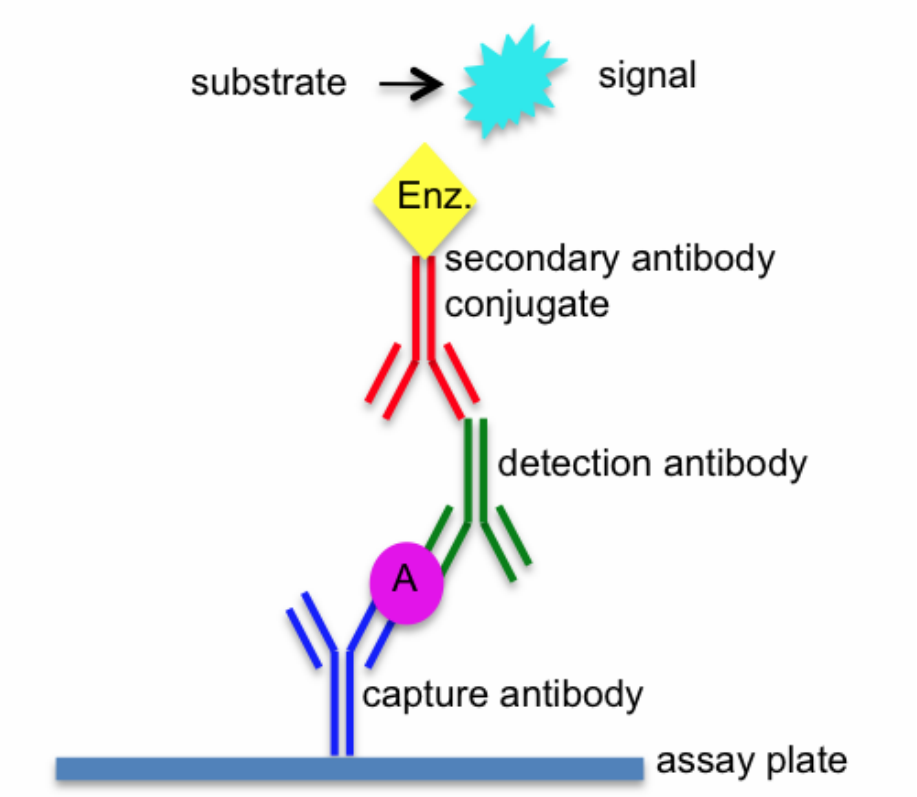


Figure 8: Principle of Sandwich enzyme-linked immunosorbent assay (ELISA) *(nordicbiosite, 2019)*

2.4.1 Determination of TNFα production in LPS-stimulated BV2 microglia

TNFα is a pro-inflammatory cytokine that is abundantly produced from activated microglia during an inflammatory mediated response (Olmos & Llado, 2014).Under physiological conditions levels of TNFα within the CNS is beneficial, however excess levels produced from activated microglia during pathological conditions can be harmful to the immune system (Raffaele, et al.,). Inhibiting the release of TNFα production in LPS-stimulated BV-2 microglia is crucial in regulating TNFα signalling during neuroinflammation (Jayasooriya, et al., 2011). The levels of pro-inflammatory cytokine TNFα was determined in LPS-stimulated BV-2 microglia cells using Mouse TNF alpha Uncoated ELISA kit (Invitrogen) the ELISA experiment was conducted according to the manufactures protocol.

After treatment with quassin (10,20,40 and 80 μM) and stimulation with LPS (100ng/ml), culture supernatants were centrifuged for 5 minutes at 135000 rpm at 4°C. The samples collected were used for the ELISA assay. Firstly, a 96-microplate was pre-coated with 100μl of 1:250 diluted specific target capture antibody in coating buffer to each well and incubated at 4℃ overnight. The next day, wells were washed with 250μl wash buffer and dried by tapping the 96 well plate on absorbent paper. Firstly, 200ul of reagent diluent was added to each well

to block the wells. The plate was then sealed and incubated at room temperature with shaking at 200rpm for 1 hour. After, contents of the wells were discarded, and the plate was washed once with 250µl of wash buffer. 100µl of freshly prepared standard dilutions were added, followed by 100µl of culture supernatants which were added in duplicates. The plate was sealed and incubated at room temperature for 2 hours at 200 rpm. After 2 hours, the contents of the wells were discarded, and the plate was washed 5 times with 250µl of wash buffer. Then, 100µl of detection antibody was added to the plate and incubated for 1 hour. The washing step was repeated and then 100µl of streptavidin- HRP was added to each well and left to incubate for 30 minutes. After, 100µl of TMB substrate was added and incubated for 15 minutes, 100µl of stop solution was added to stop the developing colour of TMB. The absorbance was measured at 450nm using a microplate reader (Tecan, Infinite F50).

2.4.2 Determination of IL-1β production in LPS-stimulated BV2 microglia

Pro-inflammatory cytokine IL-1β is an isoform from the Interleukin-1 family, sustained production of IL-1β contributes to neuroinflammation and pathophysiological processes of neurodegenerative diseases (Hewett, et al., 2012). Preclinical models of neurodegeneration and BV2 microglia LPS-stimulated have demonstrated blockade of IL-1β necessary for protection against damage caused by neuroinflammation (Newell, et al., 2018; Lee, et al., 2020). BV2 microglia cells were treated with quassin (10,20,40 and 80 µM) and stimulated with LPS (100 ng/ml) for 24 hours. Culture supernatants were collected and centrifuged for 5 minutes at 135000 rpm at 4°C. The levels of pro-inflammatory cytokine IL-1β was determined in LPS-stimulated BV-2 microglia cells using Mouse IL-1 beta Uncoated ELISA kit (Invitrogen), the ELISA experiment was conducted according to the manufactures protocol as described in section 2.4.1.

2.4.3 Determination of IL-6 production in LPS-stimulated BV2 microglia

Potent pro-inflammatory cytokine IL-6 is secreted from microglia cells following an injury of infection within the CNS (Erta, et al., 2012). Upon activation with LPS, BV2 microglia cells release elevated levels of IL-6, leading to neuronal damage (Minogue, et al., 2012). Studies have showed supressing the release of IL-6 in microglia cells has neuroprotective and anti-inflammatory effects (Recasens, et al., 2021). BV2 microglia cells were treated with quassin (10,20,40 and 80 µM) and stimulated with LPS (100 ng/ml) for 24 hours. Culture supernatants were collected and centrifuged for 5 minutes at 135000 rpm at 4°C. The levels of pro-inflammatory cytokine IL-6 was determined in LPS-stimulated BV-2 microglia cells using

Mouse IL-6 Uncoated ELISA kit (Invitrogen), the ELISA experiment was conducted according to the manufactures' protocol as described in in section 2.4.1.

2.4.4 Determination of IL-10 production in LPS-stimulated BV2 microglia

Anti-inflammatory cytokine IL-10 is an immunosuppressive molecule secreted by immune cells, having inhibitory effect against neuroinflammation and auto-immune pathologies (Lobo-Silva, et al., 2016; Porro, et al., 2020). Enhancing the levels of IL-10 in microglia cells is beneficial in counteracting the detrimental effects caused by reduced levels of IL-10 (Lobo-Silva, et al., 2016). BV2 microglia cells were treated with quassin (10,20,40 and 80 μM) and stimulated with LPS (100 ng/ml) for 24 hours. Culture supernatants were collected and centrifuged for 5 minutes at 135000 rpm at 4°C. The levels of pro-inflammatory cytokine IL-10 was determined in LPS-stimulated BV-2 microglia cells using Mouse IL-10 Uncoated ELISA kit (Invitrogen)the ELISA experiment was conducted according to the manufactures protocol as described in section 2.4.1.

2.4.5 Determination of CCL2 production in LPS-stimulated BV2 microglia

CCL2 is a pro-inflammatory chemokine expressed in glial cells, it is involved in the recruitment of monocytes and dendritic cells to sites of inflammation (Le Thuc, et al., 2016). The inhibition of CCL2 production in LPS- stimulated model of inflammation, has been successful in microglia cells following treatment with quassin receptor Erβ (Brown, et al., 2010). Regulating the release of chemokines such as CCL2 is a promising approach in the development of anti-inflammatory compounds in targeting neuroinflammation (Salvi, et al., 2017). BV2 microglia cells were treated with quassin (10,20,40 and 80 μM) and stimulated with LPS (100 ng/ml) for 24 hours. Culture supernatants were collected and centrifuged for 5 minutes at 135000 rpm at 4°C. The levels of pro-inflammatory cytokine CCL2 was determined in LPS-stimulated BV-2 microglia cells using MCP1/CCL2 Mouse Uncoated ELISA kit (Invitrogen), the ELISA experiment was conducted according to the manufactures protocol as described in insert in section 2.4.1.

2.4.6 Determination of CCL5 production in LPS-stimulated BV2 microglia

Pro-inflammatory chemokine CCL5 plays an important role in inflammation-mediated immune response by recruiting leukocytes to the site of injury (Škuljec, et al., 2011). Studies have found that microglia cells induced with LPS and IFN-γ, caused an increase in the production of CCL5 (Brown, et al., 2010; Marques, et al., 2013; Grunz-Borgmann, et al., 2015). BV2 microglia cells were treated with quassin (10,20,40 and 80 μM) and stimulated with LPS (100 ng/ml) for 24 hours. Culture supernatants were collected and centrifuged for 5 minutes at

135000 rpm at 4°C. A mouse CCL5/RANTES DuoSet ELISA kit (R&D systems) was used to determine the concentrations of CCL5 in LPS-stimulated BV-2 microglia. The method used for measuring the levels of CCL5 is as described in section in section 2.4.1.

2.5 Preparation of cytoplasmic lysates

To determine the levels of iNOS, COX-2 protein expression and NF-κB-phosho-p65 transcriptional factor LPS-stimulated BV-2 microglia, cytoplasmic lysates were collected. Stimulation ended with 6 well plate being placed on ice, culture supernatants were collected and centrifuged at 4℃ for 10 minutes at 13500 rpm. Cells were scraped and washed with ice cold PBS, PBS was collected and centrifuged for a further 10 minutes. Supernatants were discarded and 10 µl of lysis buffer (Cell signalling) that contained phenylmethylsulphonyl fluoride PMSF (0.1M) (Roche) was added to the cell pellet. Cells were incubated on ice for 15 minutes, sonicated for 5 minutes and further incubated on ice for 10 minutes. Lysates were further centrifuged at 4℃ for 15 minutes at 13500rpm. The cytoplasmic lysates (supernatants) were collected, quantified and stored at -20℃.

2.6 Protein Quantification (Bradford Assay)

Bradford assay is a colorimetric assay used to quantify protein concentrations within a sample (Rekowski, et al., 2021). As seen in figure 9, this assay involved the use of Coomassie protein assay reagent (Thermo fisher) which binds to proteins in cytoplasmic lysates and pre-diluted Bovine serum albumin standards (BSA) (125-2000 µg/ml) (Thermo fisher). Lysates were diluted with de-ionised double distilled water at a 1:11 ratio, 5µl of both samples and standards were pipetted into a 96-well microplate. Then 250µl of Coomassie reagent was added and the plate was incubated for 10 minutes at room temperature in the dark. Absorbance was measured at 540nm using microplate reader (Tecan Infinite F50). Protein concentrations of the samples are calculated from the standard curve. Protein concentrations of the cytoplasmic lysate samples collected, were calculated from the standard curve.

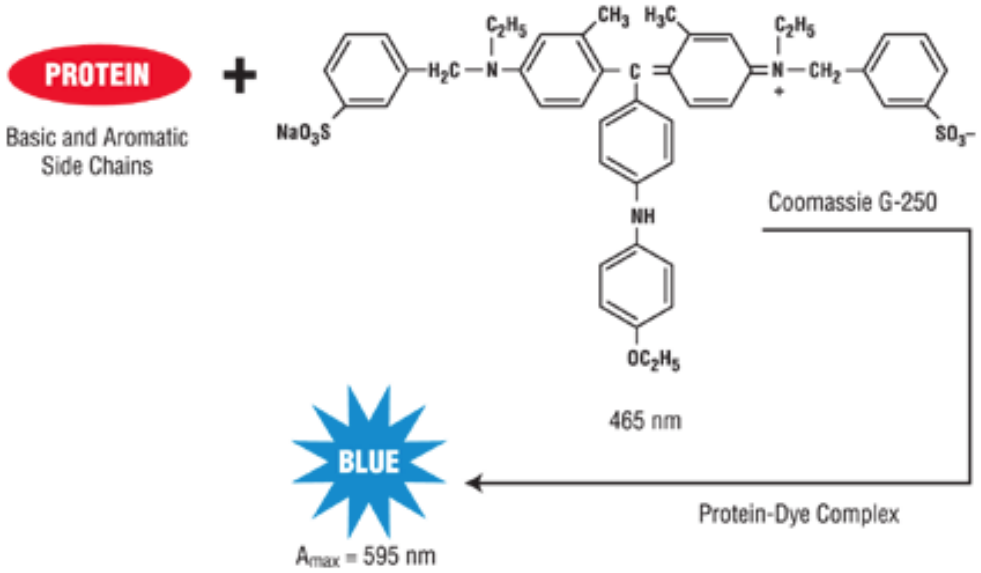


Figure 9: The colorimetric reaction of Coomassie brilliant blue dye used to quantify the number of proteins present in culture cytoplasmic lysates *(Qcbio, n.d.)*

2.7 Western Blot analysis

Western blotting is a technique that is used to identify the presence of specific proteins among a mixture of proteins. The detection of protein is based on antibodies that are designed to exclusively bind epitopes of the target protein of interest (Bass, et al., 2017). This technique involves the denaturing of proteins, which then undergoes electrophoresis and are separated according to their molecular weights. The proteins are transferred onto the membrane with the use of electric currents. Incubation of membrane with blocking buffer is carried out in order to stop antibodies form unspecific binding to the membrane and other proteins. Subsequently, membranes are incubated with a primary antibody which binds only to the protein of interest. The secondary antibody used is conjugated and is assigned to the primary antibody. Washing of the membrane in between incubation allows for unbound antibodies to be removed (Bass, et al., 2017).

BV2 microglia cells were seeded out at a density of 1×10^5 cells/ml into a 6 well plate, a volume of 2000µl was added to each well and incubated for 48 hours at 37℃. Cells were treated with quassin (10, 20, 40 and 80 µM) for 30 minutes, followed by stimulation with LPS

(100 ng/ml) for 24 hours. After stimulation, cytoplasmic lysates were collected and the protein in the samples were quantified with the use of Bradford assay. Samples were prepared by adding 20µg of protein with 5µl lithium dodecyl sulphate (LDS)(Invitrogen) and 2ul of sample reducing agent (500mM dithiothreitol (DTT) (Invitrogen). Samples were denatured at 70℃ for 10 minutes. 20ul of each sample and 5µl of ladder (BioRad) were loaded onto NuPAGE™ 10%, Bis-Tris, Mini Protein gels (Invitrogen) with running buffer (Invitrogen). Electrophoresis was used to separate the proteins at a constant voltage of 160V for 35 minutes. Then proteins were transferred from the gel onto a polyvinylidene fluoride membrane (PVDF) (Immbilion-FL; Millipore) in transfer buffer (Invitrogen) for 1 hour 15 minutes at 20 V. The membrane was washed with Tris-buffered saline (TBS-T) once for 3 minutes, then blocked with intercept™ blocking buffer (Licor) in TBS at room temperature for 60 minutes with shaking. Membranes were then washed three times for 3 minutes with TBS-T with shaking. Thereafter membranes were incubated with primary antibodies overnight at 4℃, with shaking. Primary antibodies used are listed in Table 1. Membranes were washed with TBS-T three times for 3 minutes, with shaking. Then incubated with secondary antibody Alexa Fluor 680 goat anti-rabbit IgG (Invitrogen), for 60 minutes at room temperature in the dark, with shaking. The washing step was repeated once. The band detection was done using an Odyssey infrared imaging system (LI-COR). The molecular weight of each protein was done by comparing the bands on the blots to the Precision Plus Protein™ unstained standards (Bio Rad). The relative density of the protein bands was measured using Image J software (National Institutes of Health). All antibodies and buffers were prepared in TBS-T to reduce background signal.

Western blot membranes were stripped using Restore™ fluorescent western blot stripping buffer (Thermo scientific) to remove the primary and secondary antibodies. The membranes were incubated with diluted stripping buffer (1:4 ratio) for 15 minutes with shaking. Thereafter, deionised water was used to wash the membrane once, followed by washing with TBS-T three times. Beta-actin was used in this study as loading control to confirm the same amount of protein was loaded across the gel membranes. Western blot was used to study the effect of quassin on the expression of proteins and loading control listed in Table 1.

Table 1: Primary antibodies used in western blot analysis.

Antibodies	Host	Dilution Factor	kDa	Supplier
iNOS	Rabbit	1:1000	130	Abcam
COX-2	Rabbit	1:500	72	Abcam
Beta-actin (Loading control)	Rabbit	1:1000	42	Sigma

2.8 Phospho-NF-κB p65 ELISA

NF-κB is a transcription factor which plays an important role in inflammation. Phosphorylation of NF-κB subunits has been shown to results in the upregulation or downregulation of transcription target genes (Moles, et al., 2016). Levels of phosho-NF-κB p65 were measured with Sandwich ELISA antibody pair kit (Cell signalling).

BV2 microglia cells were seeded out at a density of 1×10^5 cells/ml into a 6 well plate and incubated for 48 hours at 37℃. Cells were treated with quassin (10, 20, 40 and 80 µM) for 30 minutes, followed by stimulation with LPS (100 ng/ml) for 24 hours. After stimulation, cytoplasmic lysates were collected and the protein in the samples were quantified with the use of Bradford assay. Firstly, 96-microplate was pre-coated with 100µl of 1:100 diluted specific target capture antibody in coating buffer to each well and incubated at 4℃ overnight. The next day, wells were washed with 200µl wash buffer and dried by tapping the 96 well plate on absorbent paper. Initially, 150 µl of blocking buffer was added to each well to block the wells. The plate was then sealed and incubated at room temperature with shaking at 200rpm for 2 hours. After contents of the wells were discarded and the plate was washed once with 200µl of washed buffer. 100µl cytoplasmic lysates which were added in duplicates. Plate was sealed and incubated at room temperature for 2 hours at 200 rpm. Thereafter, contents of wells were discarded, and the plate was washed 4 times with 200µl of wash buffer. Then 100µl of detection antibody was added to the plate and incubated for 1 hour. The washing step was repeated and then 100µl of anti-rabbit- HRP was added to each well and left to incubate for 30 minutes. After, 100µl of TMB substrate was added and incubated for 10 minutes, 100µl of stop solution was added to stop the developing colour of TMB. The absorbance was measured at 450nm using a microplate reader (Tecan, Infinite F50).

2.9 NF-κB luciferase reporter gene assay

NF-κB transcription factor has been shown in studies to regulate the gene expression of various inflammatory mediators that are released in response to neuroinflammation (Tiwari & Pal, 2017). Reporter gene assays are used to study gene expressions at a transcriptional level. In luciferase reporter gene assay, NF-κB is cloned into a vector with a reporter gene, a construct is then created which contains the reporter gene and a regulatory element of interest that is then used to transfect the cells. NF-κB transcriptional activity was measured in cultured BV2 microglia cells using Dual-glo Luciferase assay (Promega). Cignal NF-κB vector was used to study the NF-kB gene expression at a transcriptional level in BV2 microglia. This assay involves the luciferase reaction, whereby luciferin is oxidised to form oxyluciferin through activation in the presence of a firefly luciferase enzyme. Adenosine triphosphate (ATP), O_2 and magnesium (Mg^{2+}) are also present in this reaction. This reaction produces a stable bioluminescent signal that can be measured. The firefly luciferases used in this assay represents experimental reporters, which monitor changes that occur in gene expressions. The renilla luciferase acts as a control reporter, which normalises the results for any outlier factors (Ng, et al., 2018).

The effect of quassin on NF-κB transcriptional activity was investigated using Dual-glo Luciferase assay. Prior to transfection, cells were seeded out at a density of 1×10^5 cells/ml into a 24-well plate, using Roswell Park Memorial Institute Medium (RPMI) 1640 with L-glutamine (2mM), supplemented with 10 % foetal Bovine Serum (FBS) (Sigma), 100 mg/ml penicillin/streptomycin (Sigma) and 100mM sodium pyruvate (Sigma). BV2 cells were incubated for 24 hours at 37 ℃, till confluent. After 24 hours, complete RPMI was replaced with 450ul of Opti-MEM(Gibco) and incubated for 2 hours at 37 ℃. The cells were transfected with Cignal NF-κB vector (Qiagen) and Glial Mag/ DNA complex (OZ Biosciences). BV2 cells were then incubated on a magnetic plate (OZ Biosciences) and further incubated for 2 hours. After 2 hours, 500ul of Opti-MEM (Gibco) was added to each well and further incubated for 20 hours. The transfected cells were treated with quassin for 30 minutes and then stimulated with LPS (100 ng/ml) and incubated for 4 hours. Thereafter, 925 µl of the medium was replaced with 75µl of Dual-glo luciferase reagent and incubated for 15 minutes in the dark with shaking. Culture supernatants were then re-suspended into a white 96 well plate and firefly luminescence were measured with FLUOstar OPTIMA plate reader (BMG LABTECH). To

measure renilla luciferase activity (internal control), Dual-glo stop reagent and substrate were added at equal volumes to Dual-glo stop buffer (1:100 ratio), measurement was taken after 10 minutes of incubation. The firefly luciferase data was normalized to the renilla luciferase activity.

2.10 Statistical Analysis

Statistical analysis of all data collected was analysed by One-way analysis of variance (ANOVA) with Dunnett test for multiple comparisons. Graph Pad prism software 9 was used for statistical analysis calculations. Values are expressed as the mean ± SEM of three separate experiments. One-way ANOVA compares the mean between various groups and determines whether those means are significantly different from each other. Dunnett multiple comparison test was done to determine the specific groups that are significantly different from each other. Differences were considered significant at $p < 0.05$*, $p<0.01$**, $p<0.001$***, $p<0.0001$****.

Chapter 3: Results

3.1 Quassin did not affect BV2 cell viability

The cytotoxic effect of quassin was evaluated at various concentrations (10, 20, 40 and 80µM). BV2 microglia cells were pre-treated with quassin for 30 minutes before stimulation with LPS (100 ng/ml) and incubated for 24 hours at 37℃. Results showed that there was no significant difference in the viability of cells treated with quassin at various concentrations (10, 20, 40 and 80µM) when compared with untreated cells as shown in figure 10.

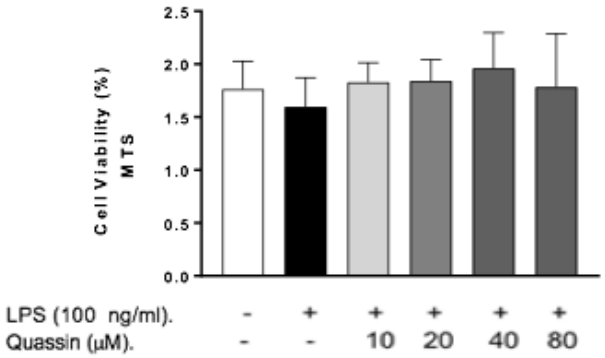


Figure 10: Effect of quassin on cell viability in LPS-activated BV2 microglia

BV2 microglia cells were stimulated with LPS (100 ng/ml) in the presence and absence of quassin (10, 20, 40 and 80µM) . MTS cell viability assay was carried out on the cells. Pre-treatment with quassin (10, 20, 40 and 80µM) did not affect the viability of BV2 microglia stimulated with LPS. Data was expressed as mean ± SEM for 3 separate experiments. Statistical analysis was done using one-way ANOVA and Dunnett test for multiple comparisons.

3.2Quassin reduced the production of some pro-inflammatory cytokines in BV2 microglia activated with LPS

Over-activated microglia have been found to release various pro-inflammatory cytokines including TNFα, IL-1β and IL-6 (Yan, et al., 2020). Excess production of these pro-inflammatory cytokines can lead to brain damage and neurodegeneration. Therefore, inhibition of these pro-inflammatory cytokines is important in targeting neuroinflammation (Yan, et al., 2020). The effect of quassin on pro-inflammatory cytokines were examined to investigate the anti-neuroinflammatory activity of this compound in LPS stimulated BV2 microglia cells. The levels of TNFα, IL-1β and IL-6 in culture supernatants were analysed using ELISA (Invitrogen). As seen in Figure 11(a), stimulation of BV-2 microglia cells with LPS (100 ng/ml) resulted in a significant increase ($p< 0.001$) in the secretion of TNFα (~ 14.6- fold increase) in comparison to unstimulated cells. Pre-treatment with quassin 10, 20, 40 and 80µM, showed a concentration dependent decrease in the production of TNFα (~ 1.1, ~1.2, ~1.2 and ~1.2 fold

decrease), however the results observed were not statistically significant in comparison to LPS untreated cells. Additionally, the production of IL-1β production in figure 11(b), showed a significant increase ($p< 0.05$) in the level of IL-1β (~1.8- fold increase) in LPS-stimulated BV-2 cells in comparison to unstimulated cells. There was reduction in the secretion of IL-1β following pre-treatment with 80µM (~2.2-fold reduction) of quassin, showing a significant decrease ($p < 0.01$) (45% decrease). Pre-treatment 10, 20 and 40 µM of quassin showed no marked significant decrease (64%, 66% and 64% decrease) in the production of L-1β (56%) when compared to LPS control. The outcome of this investigation showed a decrease in the secretion of IL-6 with treatment with quassin at 80 µM (~0.96-fold decrease), indicating a significant decrease ($p < 0.05$), when compared to LPS control. However, in BV2 cells activated with LPS (100 ng/ml), there was an observed decrease in the level of IL-6 (~1.2-fold decrease), in comparison to unstimulated BV-2 cells. As shown in figure 11(c) the level of IL-6 in unstimulated cells are higher than in LPS-stimulated cells. Similarly, there was a further decrease in production of IL-6, in BV-cells treated with quassin at 10, 20 and 40 µM (~ 0.8, ~ 0.9 and ~0.9- fold reduction), however it was not statistically significant (115%, 113% and 114%) respectively.

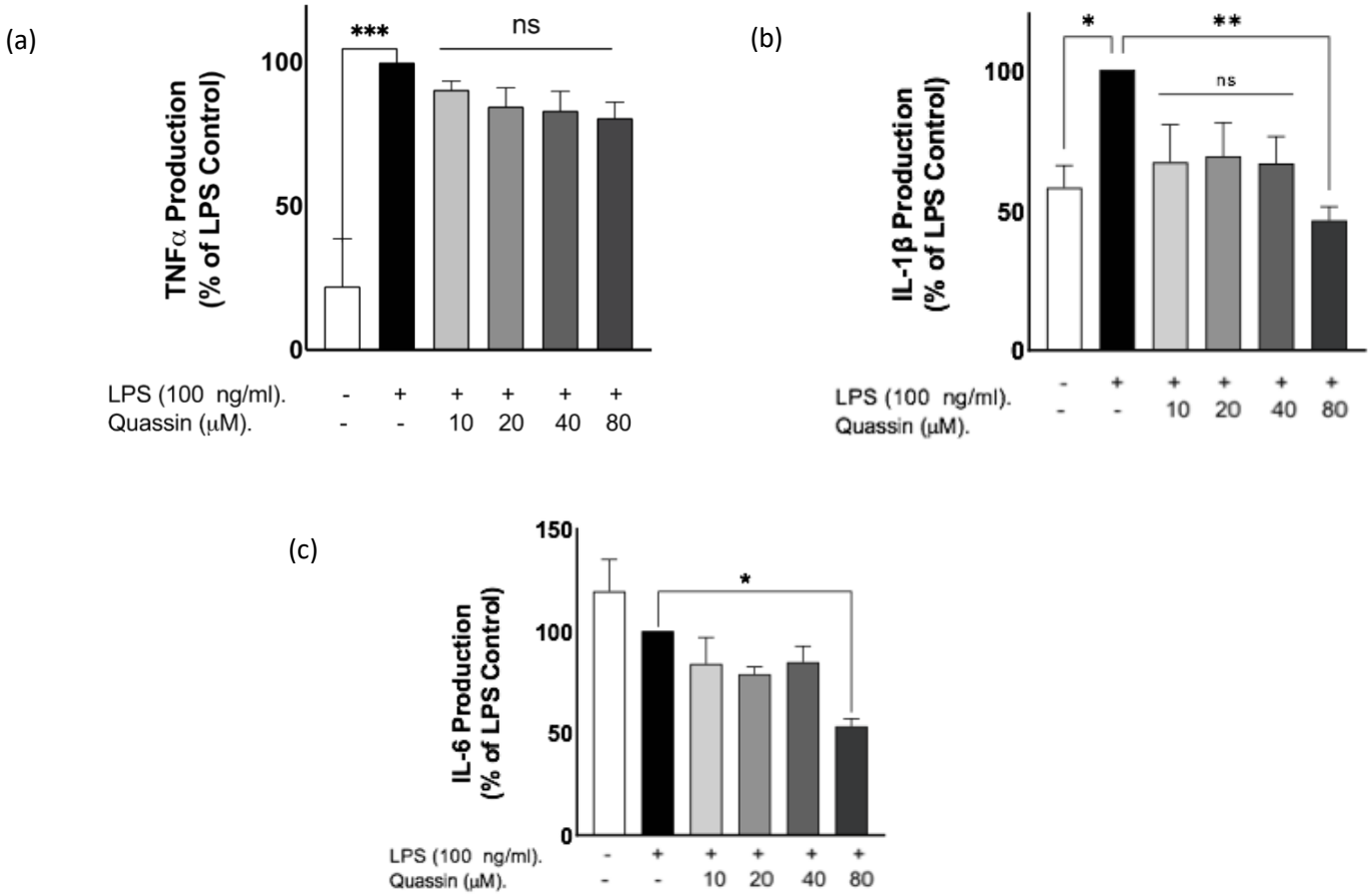


Figure 11: Effect of quassin on TNFα, IL-1β and IL-6 production in LPS activated BV2 microglia

BV2 microglia cells were treated with quassin (10, 20, 40 and 80μM) for 30 minutes and stimulated with LPS (100 ng/ml). The levels of (a) TNFα (b) IL1β and (c) IL-6 were measured with ELISA. Quassin reduced IL-1β production in LPS-activated BV2 microglia. However, quassin did not affect the production of TNFα and IL-6 in LPS-activated BV2 microglia. Data was expressed as mean ± SEM for 3 separate experiments. Statistical analysis was done using one-way ANOVA and Dunnett test for multiple comparisons.

3.3 Quassin did not increase the production of IL-10 in LPS activated BV2 microglia

Studies in animal models of neuroinflammation have shown the benefits of enhancing the release of anti-inflammatory cytokine IL-10 (Gao, et al., 2020). . The effect of quassin on the level of IL-10 production in LPS activated BV2 microglia cells was investigated. Results in figure 12 shows that there was a decrease (~0.8- fold decrease) in the level of IL-10 production in LPS activated BV-2 microglia cells, the reduction observed showed no statistical significance in comparison to untreated cells. In addition, pre-treatment with quassin (10, 20,

40 and 80 µM) shows that there was no significant increase in the level of IL-10 (~1.1-fold, ~1.2-fold, ~1-fold and ~1.1-fold increase), when compared to LPS treated cells.

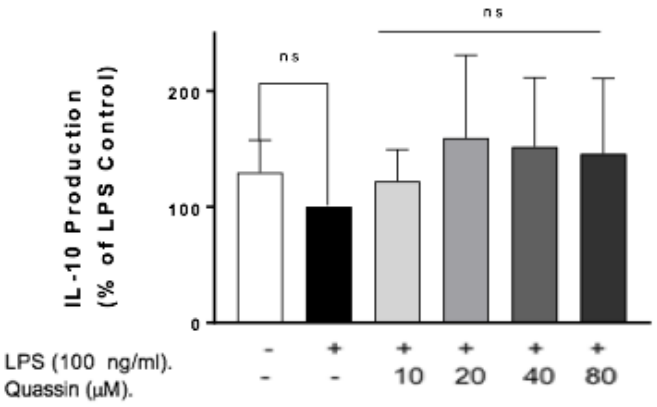


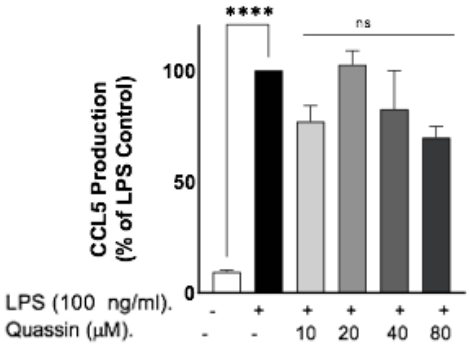
Figure 12: Quassin did not increase IL-10 production in LPS BV2 microglia

BV2 microglia cells were treated with quassin (10, 20, 40 and 80µM) and stimulated with LPS (100 ng/ml). Levels of IL-10 were measured with ELISA. Quassin did not affect the production of IL-10 in LPS-activated BV2 microglia Data was expressed as mean ± SEM for 3 separate experiments. Statistical analysis was done using one-way ANOVA and Dunnett test for multiple comparisons.

3.4 Quassin's effect on the production of pro-inflammatory chemokines, CCL2 and CCL5 in BV2 microglia activated with LPS

Pro-inflammatory chemokines are released by activated microglia during neuroinflammation, following the release of pro-inflammatory cytokines e.g., TNFα (Salvi, et al., 2017). Since quassin inhibited the production of IL-1β, the effect of the compound on pro-inflammatory chemokines CCL2 and CCL5 were also investigated in LPS stimulated BV2 microglia cells. Results in figure 13(a), revealed a marked increase in the production of CCL5 (~10.9- fold increase) in BV2 cells stimulated with LPS in comparison to untreated cells, the observed secretion showed a significant increase ($p<0.0001$). Pre-treatment with quassin at 10, 20, 40 80 µM showed that there was no statistical decrease in the levels of CCL5 production (~1.3- fold, ~ 1- fold,~1.3-fold and ~1.4-fold decrease). Investigation into the levels of CCL2 (~1.5-fold increase) in LPS-stimulated BV-2 cells, shows a significant increase ($p<0.01$) when compared to unstimulated cells. Pre-treatment with quassin (10, 20, 40 and 80 µM) did not inhibit the secretion of CCL2 (120%, 93%, 106% and 93% increase) when compared to untreated cells, results in figure 13 (b), shows that there was no statistically significant reduction.

(a)



(b)

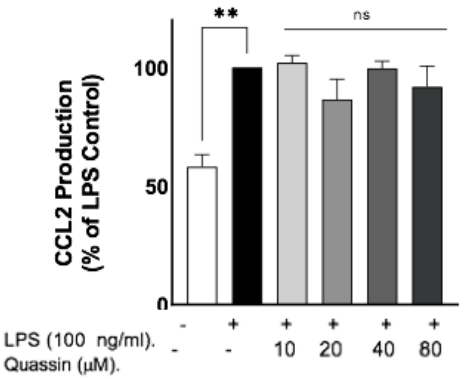


Figure 13: Effect of quassin on CCL5 and CCL2 production in LPS activated BV2 microglia

BV2 microglia cells were treated with of quassin (10, 20, 40 and 80μM) and stimulated with LPS (100 ng/ml). The levels of (a) CCL5 and (b) CCL2 were measured with ELISA. Quassin did not reduce CCL2 and CCL5 production in LPS-activated BV2 microglia. Data was expressed as mean ± SEM for 3 separate experiments. Statistical analysis was done using one-way ANOVA and Dunnett test for multiple comparisons.

3.5 Quassin inhibited nitrite production through suppression of iNOS protein in LPS-activated BV2 microglia

The suppression of pro-inflammatory mediators' nitric oxide (NO) and inducible nitic oxide synthase (iNOS) are critical targets in the inhibition of neuroinflammation (Okorji, et al., 2016). NO is an important mediator in the regulation of chronic inflammation. However, excessive release of NO has been reported to be neurotoxic. The effect of quassin on the production of NO and expression of iNOS protein were investigated in this study. The effect of quassin on nitrite production in LPS-stimulated BV2 microglia was evaluated with the use of Griess assay. Following stimulation with LPS (100 ng/ml), there was a significant increase ($p<0.0001$) in the production of NO (~7.2-fold increase) in comparison with unstimulated BV2 cells. However, quassin at 20 and 80μM significantly suppressed ($p <0.0001$) the release of NO production (~ 1.1-fold and ~1.5-fold decrease) in comparison to LPS-stimulated BV-2 cells (figure 14(a)). Further investigations into the expression of iNOS protein was carried out with western blot analysis. A significant increase ($p<0.01$) in the expression of iNOS (~9.2-fold) protein was observed on stimulation with LPS (100 ng/ml) in comparison to unstimulated BV-2 cells. Results in figure 14(b) revealed quassin at 20 and 80μM reduced (~3-fold and ~2.8-fold reduction) the expression of iNOS in BV-2 cells stimulated with LPS, the results observed shows a statistically significant decrease ($p<0.05$). Pre-treatment with quassin at 10 and 40 μM

shows a similar decrease (~2.3-fold and ~2.5-fold decrease) in the expression of iNOS , however the decrease observed showed no statistical significance. These results suggest that quassin supressed NO production in LPS activated microglia by reducing the levels of iNOS protein at 20 and 80µM concentrations.

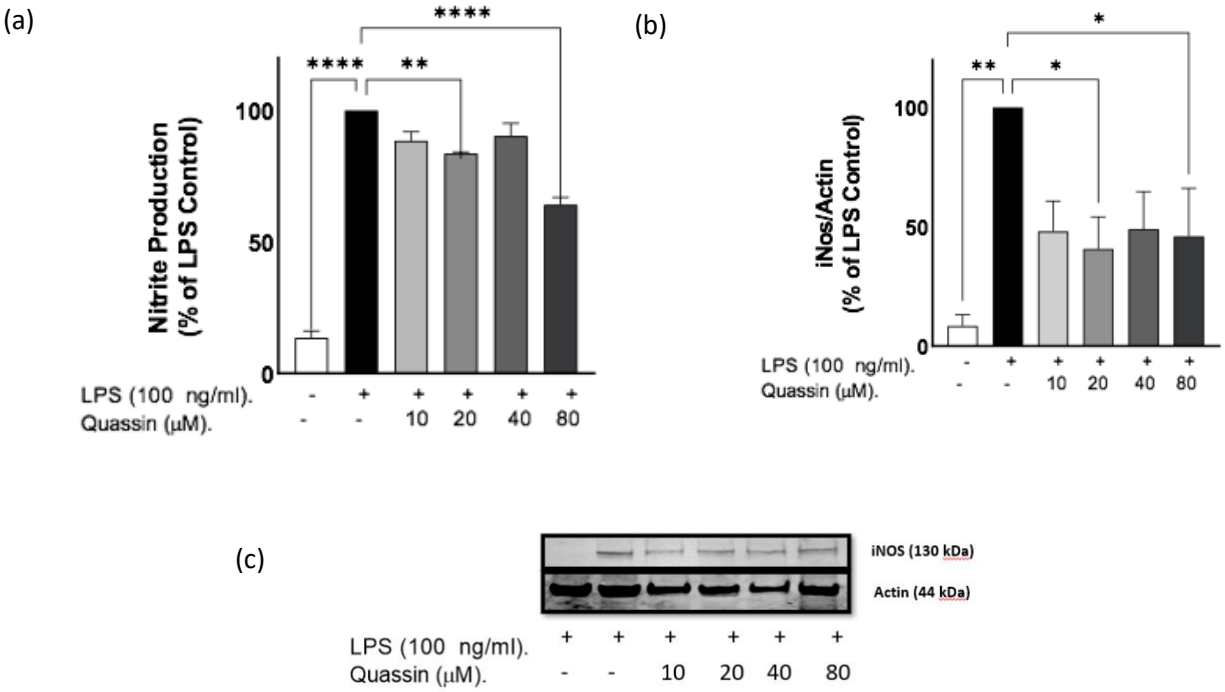


Figure 14: Quassin inhibited nitrite production and iNOS protein expression in LPS activated BV2 microglia.

BV2 microglia cells were treated with quassin (10, 20, 40 and 80µM) and stimulated with LPS (100 ng/ml). The levels of NO and iNOS protein expression were measured using Griess assay and western blot. (a) Quassin reduced NO in LPS activated BV2 cells for LPS control, 20 µM and 80 µM. (b) Quassin inhibited levels of iNOS protein in LPS activated BV2 cells for LPS control, 20 µM and 80 µM. (c) Cytoplasmic lysates were analysed for iNOS protein expression using western blot and actin was used as a loading control. Figure (b) is the quantification of the results obtained in figure (c). The western blot bands were quantified with the use of Image J software. Data was expressed as mean ± SEM for 3 separate experiments. Statistical analysis was done using one-way ANOVA and Dunnett test for multiple comparisons.

3.6 Quassin did not supress the levels of COX-2 protein in LPS-stimulated BV2 microglia

Pre-clinical studies have demonstrated inhibition of COX-2 expression in LPS-induced models of neuroinflammation as a useful target for neurodegenerative diseases (Ansari, 2010; Wu, 2018; Rezaei, 2019). The effect of quassin on the expression of COX-2 protein was

investigated to understand the inhibitory effect of the compound. Results in figure 15 show that there was a significant increase ($p<0.05$) in level of COX-2 expression (~2-fold increase) in LPS-stimulated BV-2 cells. The levels of COX-2 at 80 µM showed a marked reduction (~1.5-fold decrease), however the reduction shows no statistically significant reduction (66% decrease). Similarly, quassin at 10, 20 and 40 µM did not decrease (~1.2-fold, ~1.1-fold and ~1.2-fold reduction) the levels of COX-2, relative to LPS control(86%, 89% and 86% respectively).

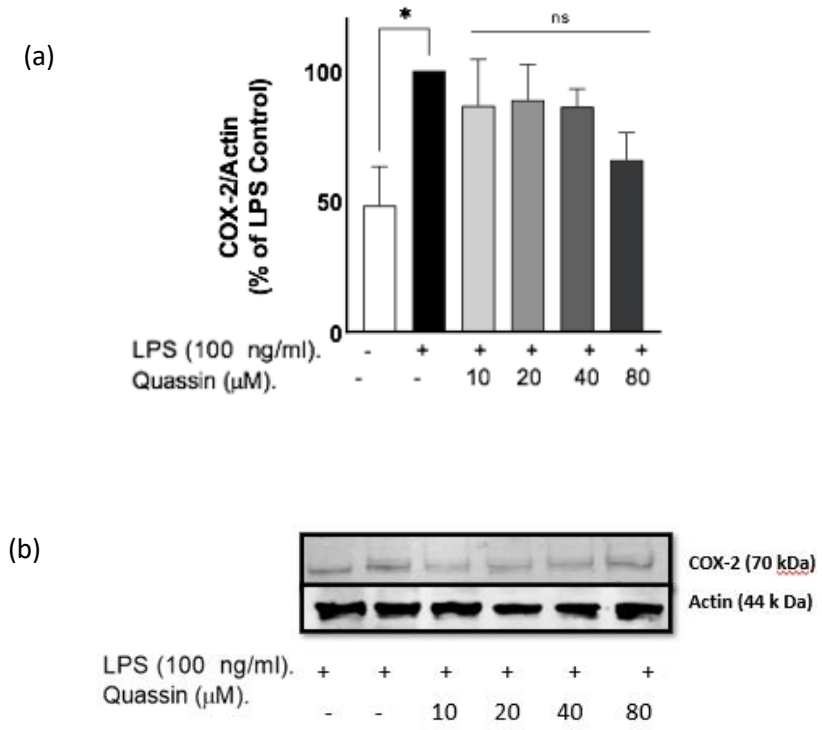


Figure 15: Quassin did not inhibit COX-2 protein expression in LPS activated BV2 microglia.

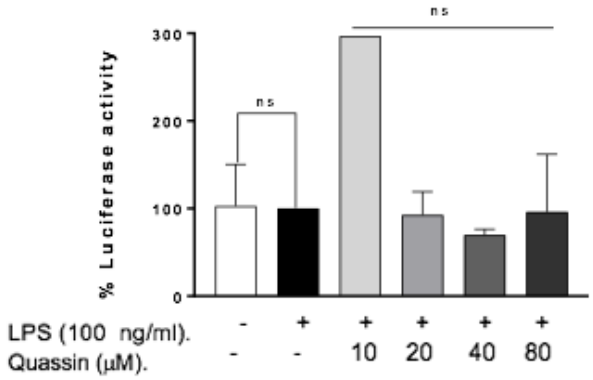
BV2 microglia cells were treated with quassin (10, 20, 40 and 80µM) and stimulated with LPS (100 ng/ml). The level of COX-2 protein expression was measured with the use of western blot. (a) Quassin did not inhibit the levels of COX-2 protein in LPS activated BV2 cells. (b) Cytoplasmic lysates were analysed for COX-2 protein expression using western blot and actin was used as a loading control. . Figure (a) is the quantification of the results obtained in figure (b). The western blot bands were quantified with the use of Image J software. Data was expressed as mean ± SEM for 3 separate experiments. Statistical analysis was done using one-way ANOVA and Dunnett test for multiple comparisons.

3.7 Quassin did not inhibit LPS activation of NF-kB in BV-2 microglia

Since quassin showed promising results in inhibiting the release of pro-inflammatory cytokines and levels of iNOS protein, its action of NF-kB signalling pathway was investigated in LPS stimulated BV2 microglia. To determine whether quassin shows any general effect on NF-kB mediated nuclear transactivation, Dual luciferase reporter gene assay was conducted. BV2 microglia cells were transfected with a vector containing NF-kB regulated luciferase reporter construct. Cells were then treated with quassin (10, 20, 40 and 80µM) and stimulated with LPS (100 ng/ml) to activate NF-κB gene transcription. Results from this study in figure 16(a) shows no significant difference (~0.2-fold respectively) in the activation of NF-κB dependent gene transcription on activation of transfected BV-2 cells stimulated with LPS, compared to untreated cells. In addition, pre-treatment with quassin at 10, 20, 40 and 80µM did not inhibit (~1.1-fold, ~0.8-fold, ~1.3-fold and ~2.7-fold change) NF-kB regulated luciferase gene transcription following stimulation with LPS.

Further experiments were carried out on the effects of quassin on LPS-induced phosphorylation of p65 in BV2 microglia. BV2 microglia cells were treated with quassin (10, 20, 40 and 80µM) for 30 minutes, followed by stimulation with LPS (100 ng/ml) for 60 minutes. After stimulation, lysates were collected and quantified with the use of bradford assay, followed by ELISA to detect phosphorylated p65. Results in figure 16(b) shows an increase in the phosphorylation of p65 (~1.8-fold), on stimulation of BV-2 cells with LPS in comparison to unstimulated cells. Despite the observed increase of 55%, there was no statistically significant increase between LPS and unstimulated cells. Furthermore, pre-treatment with quassin at 20 and 40 µM inhibited the level of phosphorylated p65 (~1.5-fold and ~2.3-fold change), as seen in figure 16(b) the there was no statistically significant inhibition in LPS-stimulated BV-2 cells, relative to LPS control. There was no significant inhibition of the level of phosphorylated p65 on treatment of BV-2 cells with quassin at 10 (~1.4-fold) and 80 µM (~1.2-fold) in LPS-stimulated-LPS BV-2 cells in comparison to LPS control.
These results suggest that quassin stimulated with LPS (100 ng/ml) did not inhibit NF-kB signalling in BV2 microglia.

(a)



(b)

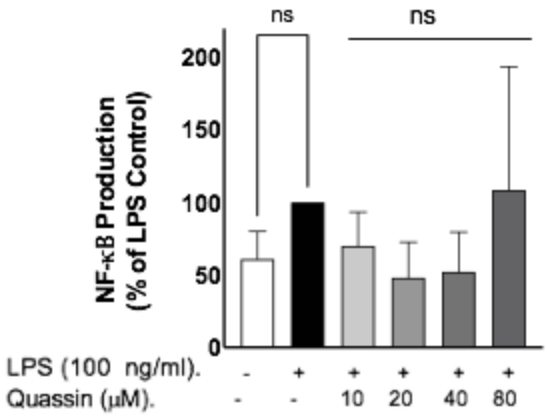


Figure 16: Quassin did not inhibit neuroinflammation by targeting NF-κB signalling in activated BV2 microglia

(a)Transfected BV2 cells treated with quassin (10, 20, 40 and 80μM), followed by stimulation with LPS (100 ng/ml). Quassin did not suppress NF-kB activity in transfected BV2 cells stimulated with LPS. (b) BV2 microglia cells were treated with quassin (10, 20, 40 and 80μM), and then stimulated with LPS (100 ng/ml). Quassin did not affect the phosphorylation of p65 in LPS-activate BV2 microglia

Chapter 4: General discussion and conclusion

Several studies have demonstrated the effects of quassin as an antiulcerogenic, antiplasmodial, antileishmanial, anti-malarial, anti-diabetic agent (Lang'at-Thoruwas, et al., 2003; Bhattacharjee, et al., 2008; Husain, 201; Faisal & Akbarsha, 2020). Quassin as a natural compound is extracted from the bark of the *quassia amara* tree that is traditionally used in Chinese herbal medicine (Bhattacharjee, et al., 2008). Reports have shown the beneficial use of natural compounds against neuroinflammation and slowing down the progression of neurodenegration through inhibiting various molecular signalling pathways (Shal, et al., 2018; Khadka, et al., 2020; Olajide & Sarker, 2020). The effects of quassin on inhibiting neuroinflammation in LPS-stimulated microglia involves complex underlying mechanisms which are not well established within literature. There is little published data on the anti-inflammatory activity of quassin in models of inflammation.

Neuroinflammation is a process of the innate immune system that mediates the release of cytokines, chemokines and other inflammatory mediators (Kwon, 2020). Neuroinflammation contributes to the pathogenesis and progression of neurodegenerative disorders such as alzheimer's disease (AD) and parkinson's disease (PD). Acute neuroinflammation involves a rapid and instant response to a pathogenic invasion or infection, in which inflammatory response functions to repair the site of injury. On the other hand, chronic inflammation an underlying feature of neuronal diseases results from this persistent and prolonged inflammatory response (Streit, 2004). Chronic activation of microglia cells is a component of neurodegenerative diseases, which results in neuronal damage and triggers the release of inflammatory cytokines, chemokines and other mediators during neuroinflammation. Excess levels of pro-inflammatory mediators IL-1β, TNF-α, IL-6, nitric oxide (NO), COX-2, CCL2 and CCL5 are released during an inflammatory response (Kwon, 2020). Prior studies have noted the importance of targeting the release of inflammatory mediators during a neuroinflammatory response.

Activation of NF-κB transcription factor in BV2 microglia is associated with the transcription of pro-inflammatory genes involved in the regulation of neuroinflammation. Additionally, sustained hyper-activation of this signalling pathway is linked to a wide range of degenerative neuronal disorders. NF-κB p65 subunit transcriptional activity is widely studied concerning the regulation of inflammatory responses. Bacterial endotoxin LPS, is an inflammatory

stimulus with the ability to initiate the phosphorylation of NF-κB p65 in BV-2 microglia cells. During neuroinflammation, transactivated NF-κB drives the binding of promoter sequences of the target DNA and undergoes phosphorylation to be translocated into the nucleus. The binding of the DNA region is reported to affect the expression of target genes such as inflammatory mediators (TNFα, IL-1β IL-6, iNOS and COX-2).

Research into the inhibition on neuroinflammation has shown an increased interest in the anti-inflammatory activities of natural compounds. This research was carried out to investigate the inhibitory effect of quassin on neuroinflammation in LPS activated BV2 microglia. In order to establish the anti-inflammatory effects of quassin in LPS stimulated BV2 microglia, the production inflammatory mediator's TNFα, IL-1β, IL-6, IL-10, CCL2, CCL5, NO and the expression of iNOS and COX-2 proteins were measured using ELISA, western blotting techniques and Griess assay. In addition, this study explored quassins ability to inhibit the phosphorylation of NF-κB-phospho-p65 and NF-kB dependent gene expression was established using Sandwich ELISA and Luciferase reporter gene assay.

Results from MTS assay showed that quassin did not affect the cell viability of BV-2 microglia cells. In this current research, pharmacologically relevant concentrations of quassin were used to assess the neuroinflammation inhibitory actions of quassin against LPS-stimulated BV2 microglia. Findings indicates that the inhibitory effects of quassin will be due to the actions of quassin alone and not due to the cytotoxicity of the compound. A previous study has established the cytotoxic effect of quassin in macrophages. Findings demonstrated that at a dose of 64.36 μM, the compound showed less cytotoxic properties (Bhattacharjee, et al., 2008).

In regard to the release of inflammatory mediators, stimulation of microglia with bacterial endotoxin lipopolysaccharide (LPS) (100 ng/ml) resulted in an increase in the production of pro-inflammatory cytokines TNFα and IL-1β; and pro-inflammatory chemokines CCL2 and CCL5. Moreover, stimulation with LPS increased the production of nitric oxide (NO), expression of iNOS and COX-2 protein in BV2 microglia. Various studies have demonstrated the ability of LPS to induce an increase in the production of inflammatory mediators in activated microglia (Muthumalage & Rahman, 2019) (Oliveira-Junior, et al., 2019) (Lopes, 2016). A study by Zhang et al, (2019), demonstrates LPS-induced neuroinflammation, causing an increase in the production of IL-1β, IL-6, NO and iNOS in activated BV2 microglia cells. These findings support the growing literature of LPS as an inflammatory stimulus used to induce neuroinflammation in microglia experimental models. Contrary to literature that have

investigated the release of IL-6 and IL-10 in LPS-stimulated microglia, the findings of this research following stimulation did not increase the production of IL-6 and reduced the levels of IL-10 in activated BV2 microglia. This research raises the question in regard to whether 100 ng/ml of LPS used to induce neuroinflammation was sufficient enough in IL6 and IL-10 production. One study showed that LPS at a low dose of 50 pg/ml increased the production of IL-6 and IL-10, as well as TNFα and NO in RAW 264.7 macrophage cells (Chae, 2018). LPS induces inflammatory-mediated response by binding to toll-like receptor 4 (TLR4) in activated microglia (Nam, et al., 2018). This interaction is associated with downstream signalling pathways that affects transcription factors such as NF-κB. Triggering a cascade events that initiates the release of pro-inflammatory mediators. Hence, LPS-induced neuroinflammation in BV2 microglia is important in understanding and inhibiting neuroinflammation in neuronal degenerative diseases.

Quassin suppressed the production of IL-1β in LPS-stimulated BV2 microglia at a concentration of 80 μM. Additionally there was a significant decrease in the production of IL-6 in LPS-stimulated BV2 microglia at 80 μM. It can be suggested that the due to the level of IL-6 in unstimulated cells being greater than that in LPS-stimulated cells (Figure 11 (c)), that the observed inhibition of IL-6 in 80 μM of quassin might be enhancing the effect of LPS stimulation on this occasion. Previous studies have recognised the inhibition of pro-inflammation cytokines IL-1β and IL-6 important in slowing down the pathology of inflammatory-mediated neuronal degeneration. A recent study by Lee et al. (2020) reported a decrease in IL-1β in LPS-stimulated BV microglia by *aquilariae lignum* fraction (ALF), a natural product. Similar to quassin, another compound Rehein used in traditional Chinese medicine, reduced the secretion of IL-1β in BV-2 microglia cells stimulated with LPS (Zheng, 2020). Furthermore, LPS-induced neuroinflammation in activated BV2 microglial cells has been reported to inhibit the production of IL-6 following treatment with TAK-242 (Fernández-Calle, 2017). Consequently, results from several studies confirms that the inhibition of IL-1β is therefore valuable in targeting to reduce neuroinflammation neurodegenerative disorders such as alzheimer's disease. Prior studies have recognised the implication of excess release of IL-1β by microglia cells associated accumulation of amyloid-beta (Aβ) seen in AD patients (Wang, et al., 2015; Mendiola & Cardona 2018). In accordance to results from previous studies the suppression of IL-6 has been demonstrated to be important in minimising the effects of neuroinflammation in neurodegenerative disorders. Results from this study suggest that the

effect of quassin on the levels of IL-6 in LPS-stimulated BV2 microglia may have an anti-inflammatory effect.

In this current research treatment with quassin (10, 20,40 and 80 μM) did not supress the production of TNFα, CCL2 and CCL5 in LPS activated BV2 microglia. These results indicated that quassin did not inhibit neuroinflammation through the inhibition TNFα, CCL2 and CCL5 production. Reports have shown the inhibition of TNFα, reduced neuronal dysregulation associated with neurofibrillary tangles in AD (Shamim, 2017). Excess production of TNFα in activated microglia cells is associated with the mediation of chronic inflammation and action of NF-κB signalling pathway (Kuno, 2005; Raffaele, et al., 2020). Despite an observed decrease in concentrations of quassin (10, 20,40 and 80 μM), there was no significant inhibition in the production of TNFα. Therefore, findings from this study suggest that quassin does not inhibit neuroinflammation through mediation of TNFα signalling. CCL5 and CCL2 are pro-inflammatory chemokines involved in neuroinflammation through the recruitment of leukocytes to sites of infection or injury.

There is growing attention into the inhibition of neuroinflammation by targeting CCL2 and CCL5. Evidence for prior research has demonstrated inhibition of CCL5 in LPS-induced neuroinflammation through the transcriptional activation of the NF-κB signalling pathway (Sanjeewa, 2020). Additionally, downstream activation of CCL2 receptor CCR2 is associated with reduced neuronal loss, a common feature of neurodegenerative disease such as AD (Tian, 2017; Svirsky, 2020). Targeting, CCL5 and CCL2/CCR2 signalling cascade provides a novel target for anti-inflammatory therapeutics (Marques, et al., 2013). Despite, literature indicating the inhibition of these pro-inflammatory chemokines beneficial in reducing neuroinflammation, findings from this study suggest that quassin (10, 20,40 and 80 μM) was not sufficient enough in supressing the production of CCL2 and CCL5 pro-inflammatory chemokines.

The upregulation of IL-10 is demonstrated in studies to reverse the effects of LPS-induced neuroinflammation in microglia cells (Cianciulli, 2015). In this study, quassin did not elevate the levels of IL-10 production in LPS-stimulated microglia. There was an observed decrease of IL-10 production in LPS-stimulated microglia; however, the reduction was not statistically significant even when treated with quassin at (10, 20,40 and 80 μM). Targeting the reduction of IL-10 in neuroinflammatory conditions has therapeutic potential in regulating neuronal dysfunction and reducing neuronal stress. Gravel et al, (2016), provided evidence for the role

of IL-10 in amplifying the microglia immune response in the early stages of neurodegenerative disease, amyotrophic lateral sclerosis (ALS). Results from this study does not align with published literature into the enhancement of IL-10 in immune cells, in a study investigating the antileishmanial activities of quassin, the production of IL-10 significantly increase upon stimulation with *l.donovani*, murine peritoneal macrophages (Bhattacharjee, et al., 2008). These results suggest that quassin did not inhibit neuroinflammation through inducing the production of IL-10 in LPS-stimulated BV2 microglia.

Quassin reduced NO's production by reducing the expression of iNOS protein at 20 and 80 μM in LPS-stimulated BV2 microglia. It is well established that LPS-induced neuroinflammation in BV2 microglia elevates the production of nitric oxide and the expression of iNOS protein. Additionally, evidence supporting the inhibition of neuroinflammatory NO signalling slows down neurodegeneration and cellular stress (Tapias, 2017; Cinelli, 2020; Bourgognon, 2021). Thus, these results, therefore, suggest another mechanism for inhibiting neuroinflammation in stimulated BV2 microglia, following treatment with quassin. The ability of quassin to suppress the production of NO and expression of iNOS2 is demonstrated in macrophage cells (Bhattacharjee, et al., 2008). In addition, corylin, a natural product also used in traditional Chinese medicine, suppressed the production of NO and the expression of iNOS in LPS-stimulated BV-2 microglia (Huang, 2018). Findings from this study suggest a potential mechanism that Quassin mediates its anti-inflammatory activities through the iNOS/NF-kB signalling pathway by upregulation of inflammatory cytokines.

Quassin did not reduce the expression of COX-2 protein in LPS-induced BV2 microglia cells. There was an observed decrease at 80 μM; however, the results were not statistically significant. These results indicate that the activity of quassin is not mediated through inhibiting the expression of COX-2 in LPS-stimulated microglia. COX-2 is expressed at high levels in the early stages of AD and is thought to have pleiotropic effects in the pathophysiology of the disease (Tyagi, 2020). Thus, targeting the blockades of COX-2 is beneficial in tackling the underlying pathways of neuroinflammation in neurodegenerative diseases. In contrast to previous studies, we did not inhibit quassin against COX-2 protein expression in BV2 activated microglia.

The effect of quassin on phosphorylated NF-κB p65 evaluated revealed that there was no significant increase in the production of NF-κB -phospho-p65 in LPS-stimulated microglia, despite an observed increase. Additionally, quassin did not inhibit the phosphorylation of NF-

κB -phospho-p65 in BV microglia. Luciferase reporter gene assay indicated that quassin had no effect on NF-kB mediated transcription in LPS-stimulated BV2 microglia cells. Results suggest that quassin was not capable of inhibiting the transactivation of NF-κB in activated microglia. During neuroinflammation, transcription of inflammatory genes is initiated upon NF-κB 's translocation into the nucleus. As the results attained from ELISA and luciferase reporter gene assay did not inhibit the binding of NF-κB to the DNA of inflammatory genes, the effect of quassin on NF-κB -DNA binding activity was not investigated further. Previous research investigation neuroinflammation in LPS activated BV2 microglia found that targeting NF-κB 's downregulation inhibits the NF-κB signalling pathway (Cobourne-Duval, et al., 2018). Findings from this study suggest that quassin did not inhibit the production of inflammatory mediators (IL-1β, IL-6, NO and iNOS) through the phosphorylation of downstream NF-κB -phospho-p65 and NF-κB dependent gene expression.

In contrast to findings from this study, quassinoids have been demonstrated to block the NF-κB signalling pathway in LPS-stimulated macrophages. Cui et al. (2020) and Silva et al. (2015) reported quassinoids yadanzigan and isobrucein B to inhibit the signalling of NF-κB in LPS-stimulated macrophages during an inflammatory-mediated response. However, the findings of the current study do not support the previous research. Nonetheless, quassin's inability to inhibit the transcription of NF-κB signalling via p65 does not eliminate the possibility of quassin inhibiting the phosphorylation IκBα and IKKα in LPS-stimulated microglia. Reports have demonstrated quassinoid, Eurycomanone, ability to inhibit IκBα phosphorylation by inhibiting NF-κB signalling (Hajjouli, et al., 2014). Therefore, further research should be undertaken to target the phosphorylation of these transcription factors, which could block the nuclear translocation of NF-κB. Moreover, it could offer a possible explanation to quassin's inhibitory effect in decrease the production of IL-1β, IL-6, NO and iNOS in LPS-stimulated BV2 microglia.

Another possible signalling pathway that could mediate quassins inhibition on neuroinflammation in LPS-stimulated BV2 microglia is the p38 MAPK signalling pathway. P38 MAPK is part of the mitogen-activated protein kinases (MAPKs), which has been identified to regulate the expression of inflammatory mediators in microglia cells. Research has found downregulation of the p38 MAPK signalling pathway as a potential target for inhibiting neuroinflammation. Furthermore, several studies have demonstrated LPS-stimulation triggering p38 MAPK and increasing inflammatory cytokines that mediate

neuroinflammation (Zhao, et al., 2018). Similarly, a study investigating the effect of quassinoid eurycomanol in suppressing inflammation by inhibiting NF-kB signalling pathway, phosphorylation of IkBα phosphorylation and MAPK upstream signalling (Hajjouli, et al., 2014). Hence, a proposed mechanism for the inhibition of neuroinflammation could be through targeting the inhibition of the phosphorylation of p38 and inflammatory cytokines in activated microglia. Previous literature has no direct evidence to support the quassins ability to inhibit neuroinflammation through the mediation of the p38 MAPK signalling pathway. However, studies investigating the effects of quassinoid bruceine A on pancreatic activity showed that Bruceine A inhibited phosphorylation of p38 in human MIA PaCa-2 pancreatic cancer cells (Lu, et al., 2021).

4.1 Limitations

The concentrations of quassin that inhibited pro-inflammatory cytokines IL-1 β and IL-6 at 80 μM in this current research to assess the inhibitory effects in BV2 LPS-activated microglia may not be physiologically achievable in *in vivo* experiments due to numerous factors. These factors includes pharmacokinetic and pharmacodynamic properties of the compound which was not investigated at length in this study. Additionally the efficacy of quassin is another factor which takes into consideration the bioavailability and the ability for quassin to cross the blood brain barrier. In order to be able to investigate the activity of quassin further *in vivo* future investigations would need to perform a wide range efficacy dose selection, in order to identify a lower concentration at which quassin can still exert its inhibitory effects. LPS-induced neuroinflammation in BV2 microglia cells was used a a single model to investigate the inhibitory effects of quassin during an inflammatory response. Although the *in vitro* model is a promising tool, the time consuming nature of maintaining the cell line presents as an ongoing constraint, which was the case in this research. Furthermore all experiments conducted in this study were repeated 3 times to reduce the likely hood of errors. From the results obtained, high standard deviation between experiments was observed. In order to have reduced the anomalies that presented in the results, experiments with high standard deviation should have been repeated more than 3 times.

4.2 Conclusion

In conclusion, the present study contributes to our understanding of the effect of quassin on inhibiting neuroinflammation in LPS-stimulated BV-2 microglia. The results of this investigation show that quassin blocked the progression of neuroinflammation by inhibiting

the production of pro-inflammatory cytokines IL-1 β and IL-6 at 80 μM in LPS-stimulated BV2 microglia. Additionally, at 20 and 80μM, quassin blocked the NO and expression of iNOS protein. However, quassin did not block the progression of pro-inflammatory cytokine TNFα, chemokines; CCL2, CCL5 and the expression of COX-2 and did not activate anti-inflammatory cytokine IL-10 in microglia. Furthermore, the inhibitory effects of quassin were found not to be mediated by inhibiting NF-κB-phospho-p65. Ambiguity remains over quassins effect on the NF-κB signalling pathway. Thus, more investigations on the anti-inflammatory activity of quassin on other inflammatory mediators, transcription factors and signalling pathways will aid in establishing the mechanism in which quassin inhibits neuroinflammation in LPS-stimulated BV-2 microglia cells. Findings from this study add to the growing literature supporting natural compounds such as quassin's effect on inhibiting neuroinflammation concerning neurodegenerative disorders.

4.3 Future directions

This study has opened up a new question about the potential anti-neuroinflammatory effect of quassin. Future investigations should investigate quassins ability to phosphorylate other components of the NF-κB and p38 MAPK signalling pathway. Additionally, future investigations should focus on the neuroprotective and antioxidant properties of quassin. Lastly, an investigation into the capability of quassin in crossing the BBB *in vitro* models using Human cells lines is crucial to confirm the anti-inflammatory activity of quassin within the central nervous system.

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