# EXPLORING NEUROPROTECTION BY FAGARAMIDE AND THE CANNABINOID ARVANIL

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# ABSTRACT

Neuroinflammation is among the common pathophysiological process involved in neurodegenerative diseases' onset and progression. Several pharmacologically active compounds have been revealed to possess anti-neuroinflammatory and neuroprotection activities and thus attenuate disease progression. This research was designed to evaluate the neuroprotection activity of two distinct potential compounds, fagaramide and arvanil, on LPS- stimulated BV2 microglia and $H_2O_2$- induced SH-SY5Y neurotoxicity. BV2 microglia were treated with arvanil (0.1, 0.25 and 0.5 µM) or fagaramide (5, 10 and 20 µM) and then stimulated with LPS (100 ng/ml). The results revealed that both arvanil and fagaramide could significantly reduce the levels of the pro-inflammatory cytokines (TNF-α and IL-6). In addition, the protein expression of iNOS and COX-2 with their related mediators, NO and $PGE_2$, respectively, were attenuated significantly when LPS-activated BV2 cells and pre-treated with arvanil and fagaramide. For further investigation on the molecular mechanisms behind the anti-neuroinflammatory activities of arvanil and fagaramide, their modulated effects on the inflammatory signalling pathways have been examined. The results demonstrated that both compounds exerted their anti-neuroinflammatory effects independent of NF-κB, MAPKs and Akt signalling pathways in LPS-activated BV2 microglia. Furthermore, the activities of arvanil and fagaramide were not mediated by the activation of the Nrf2/HO-1/NQO1 antioxidant axis in BV2 microglia. The anti-neuroinflammatory activity of arvanil in LPS-stimulated microglia was not possibly mediated through binding to CB1, CB2 and TRPV1 receptors. Furthermore, results from the activity of arvanil and fagaramide on $H_2O_2$-induced apoptosis suggested that both compounds could not attenuate the neurotoxicity in differentiated SH-SY5Y neuroblastoma cells, which was induced by $H_2O_2$. Collectively, arvanil and fagaramide inhibited neuroinflammation independently from the modulation of NF-κB, MAPKs and Akt signalling pathways. The anti-neuroinflammatory activities of arvanil and fagaramide were not mediated by the upregulation of the Nrf2 pathway. Arvanil and fagaramide did not protect the SH-SY5Y neuroblastoma cells from the neurotoxicity of $H_2O_2$, suggesting that arvanil and fagaramide activities might be achieved by inhibiting neuroinflammation without reversing neurotoxicity.

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| AD | Alzheimer's Disease |
| AEA | Anandamide |
| ALS | Amyotrophic lateral sclerosis |
| ANOVA | Analysis of variance |
| AP-1 | Activator protein 1 |
| APP | Amyloid precursor protein |
| ARE | Antioxidant response elements |
| BBB | Blood-brain barrier |
| CB | Cannabinoid |
| CB1R | Cannabinoid receptor 1 |
| CB2R | Cannabinoid receptor 2 |
| CBD | Cannabidiol |
| CNS | Central nervous system |
| COX-2 | Cyclooxygenase 2 |
| CSF | Cerebrospinal fluid |
| CTFB | Complete Transcription Factor Binding Assay Buffer |
| DMSO | Dimethyl sulfoxide |
| DNA | Deoxyribonucleic acid |
| ECS | endocannabinoid system |
| EGF | Epidermal growth factor |
| ELISA | Enzyme-linked immunosorbent assay |
| ERK | Extracellular signal-regulated kinase |
| FADD | Fas-associated death domain protein |
| FBS | Foetal bovine serum |
| FGF | Fibroblast growth factor |
| GFAP | Glial fibrillary acidic protein |
| $H_2O_2$ | Hydrogen peroxide |
| HO-1 | Heme oxygenase-1 |
| IFNγ | Interferon gamma |
| IKK | IκB kinase |
| IL | Interleukin |
| iNOS | Inducible nitric oxide synthases |

| | |
|---|---|
| JNK | c-Jun N-terminal-activated protein kinase |
| Keap1 | Kelch-like erythroid cell-derived protein |
| LDH | Lactate dehydrogenase |
| LPS | Lipopolysaccharide |
| MAPII | Microtubule-associated protein II |
| MAPK | Mitogen-activated protein kinase |
| MS | Multiple sclerosis |
| NADPH | Nicotinamide adenine dinucleotide phosphate |
| NED | N-1-naphthyl ethylenediamine dihydrochloride |
| NF-κB | Nuclear factor kappa-light-chain-enhancer of activated B cells |
| NMDA | N-methyl-D-aspartate |
| NO | Nitric oxide |
| NOS | Nitric oxide synthases |
| NOX | NADPH oxidase |
| NQO1 | Nicotinamide adenine dinucleotide phosphate quinone oxidoreductase 1 |
| Nrf2 | Nuclear factor erythroid 2-related factor 2 |
| PBS | Phosphate buffered saline |
| PD | Parkinson's disease |
| PG | Prostaglandin |
| PI3K | Phosphatidylinositol-3-kinase |
| PMS | Phenazine methosulfate |
| PMSF | Phenylmethane sulfonyl fluoride |
| PPAR-γ | Peroxisome proliferator-activated receptor gamma |
| RNS | Reactive nitrogen species |
| ROS | Reactive oxygen species |
| SAPK | Stress-activated protein kinase |
| SIRT1 | Sirtuin 1 |
| SOD | Superoxide dismutase |
| STAT | signal transducer and activator of transcription |
| THC | Tetrahydrocannabinol |
| TLR | Toll-like receptor |
| TMB | Tetramethylbenzidine |
| TNF-α | Tumour necrosis factor-alpha |
| TRPV1 | Transient receptor potential vanilloid subtype 1 |

| | |
|---|---|
| WHO | World Health Organization |
| XTT | 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide |

# 1 Chapter One: Introduction

## 1.1 Background

Neurodegenerative diseases represent several age-related disorders that negatively affect memory and cognitive awareness, such as Alzheimer’s disease (AD), Huntington’s disease (HD), Parkinson’s disease (PD), amyotrophic lateral sclerosis (ALS) and multiple sclerosis (MS). The pathophysiology of these diseases is different, as some affect memory and cause cognitive impairments while others influence the motor functions such as moving, breathing and speaking (Abeliovich & Gitler, 2016; Gitler, Dhillon, & Shorter, 2017; Taylor, Brown, & Cleveland, 2016; Wyss-Coray, 2016). The available treatments for neurodegenerative diseases are to manage only the symptoms and slow the progression because the complete mechanisms behind the pathology of these diseases are still unclear. The World Health Organization (WHO) warned that neurodegenerative disorders would overtake cancer in 20 years and become the second reason for death after cardiovascular diseases (Durães, Pinto, & Sousa, 2018; Gitler *et al*., 2017). Although some neurodegenerative diseases are characterised by the deposition of Aβ peptide and tau protein, they share the same fundamental events that lead to neuronal dysfunction and disease progression. Oxidative stress and neuroinflammation are the major players in the pathogenicity of most neurodegenerative diseases, including PD, amyotrophic lateral sclerosis, Huntington’s disease and AD (Dugger & Dickson, 2017; J. Li, O, Li, Jiang, & Ghanbari, 2013).

## 1.2 Neuroinflammation mediated neurodegenerative diseases

Neuroinflammation is not only a process stimulated by abnormal aggregations such as neurofibrillary tangles and senile plaques; it also contributes to the disease pathogenesis as much as tangles and plaques themselves ( Zhang *et al*., 2013). During neuroinflammation, the major cellular players in the brain are astrocytes, monocytes, microglia, and macrophages. Neurons were also proposed to take part in the neuroinflammatory process by enhancing the expression and generation of inflammatory

molecules (Morales, Farías, Cortes, & Maccioni, 2016; Morales, Guzmán-Martínez, Cerda-Troncoso, Farías, & Maccioni, 2014).

### 1.2.1 Microglia

Microglia play a vital role in the plasticity maintenance of neuronal circuits and it contributes to the remodelling and protection of synapses (Ji, Akgul, Wollmuth, & Tsirka, 2013). To a certain degree, this action is intermediated by the emission of trophic factors as a brain-derived neurotrophic factor, which participates in the formation of memory (Parkhurst *et al*., 2013).

When microglia are activated via pathological triggers, like protein aggregates or neuronal death, it expands their activities to the injury site and start immigration to the lesion, where it exerts their immune response (Heneka *et al.*, 2015). However, microglia have the ability to link to protein aggregations in neurodegenerative disease by cell-surface receptors, like α6β1 integrin, SCARA1, CD36, CD47, CD14 and toll-like receptors (TLR9, TLR6, TLR4, and TLR2) and this reaction is essential to the inflammatory process (Heneka *et al*., 2015).

Generally, resting microglial cells maintain neuronal survival and development by secreting several neurotrophic factors, such as the basic fibroblast growth factor (bFGF) and nerve growth factor (NGF) (Ueno *et al.*, 2013). Microglia plays a dual role in neuroinflammation through the dynamic changes from the pr-oinflammatory M1 phenotypes to immunosuppressive M2 phenotypes. Several stimuli have been revealed to stimulate the classical microglial activation, which considers M1 phenotype, including interferon (IFN)-γ, lipopolysaccharide (LPS) and β-amyloid (Aβ) (Banati, 2003; Block, Zecca, & Hong, 2007). The classical activation of M1 microglia is associated with pro-inflammatory cytokines production, such as tumour necrosis factor- α (TNF-α), interleukin-1β (IL-1β), superoxide, nitric oxide (NO) and reactive oxygen species (ROS) which are generated from the upregulated NF-κB signalling cascade (Swarup *et al*., 2011; Vay *et al.*, 2018). However, activation of microglia can enhance the neurotoxicity (Figure ***1-1***) through the increased burden of ROS, oxidative stress and neuroinflammation (Cai, Hussain, & Yan, 2014; Mandrekar-Colucci & Landreth, 2010).

Microglia have been reported to express TLR4 abundantly, which play a prominent role in activating these cells in many neurodegenerative diseases such as AD (Lehnardt *et al.*, 2003). However, LPS; found in the outer membrane of Gram-negative bacteria and the viral envelope proteins; identifies and binds with LPS-binding protein (LBP) and glycosylphosphatidylinositol-anchored protein that generally associated with TLR4 and starts the pro-inflammatory signalling cascades activation (Subhramanyam, Wang, Hu, & Dheen, 2019).

On the other hand, microglia can switch to the M2 phenotype, triggered by IL-4 or IL-13, and produce anti-inflammatory cytokines such as IL-10 and a repair factors like arginase (Arg-1) and Chitinase-3 (Chil3) which are essential in wound healing and tissue repair (Cherry *et al*., 2014; Tang & Le, 2016). Therefore, the microglia must switch from the M1 pro-inflammatory activity to the anti-inflammatory M2 phenotype to prevent brain tissue damage resulting from excessive pro-inflammatory mediators' production, provide cellular debris phagocytosis and initiates the tissue repair process (Subhramanyam *et al*., 2019). The disruption in M1/M2 transformation has been studied in ischemia-reperfusion (I/R) injury model rats where NF-κB (p65) expression levels were significantly increased, accompanied by elevated expression levels of TNF-α, IL-18 and IL-6 (Shilun Yang *et al*., 2019).

**1.2.2 Monocytes**

Monocytes are essential in maintaining homeostasis and protecting against several germs attacks, such as bacteria, fungi, viruses and parasites (Lauvau & Hohl, 2015). Following injury, monocytes transform into macrophages and produce an inflammatory reaction. However, macrophages derived from bone marrow could infiltrate the CNS following particular circumstances and participate more effectively in removing amyloid deposits than microglia (London, Cohen, & Schwartz, 2013; Malm, Koistinaho, Muona, Magga, & Koistinaho, 2010). Alongside its beneficial effects, monocyte can play a crucial role in neuronal loss by differentiation to antigen-presenting cells, including macrophages and dendritic cells, leading to abnormal production of ROS and overproduction of different pro-inflammatory molecules (Hohsfield & Humpel, 2015). The NOD-like receptor family, pyrin domain

containing 3 (NLRP3) inflammasome, is recently recognized as a critical mediator of microglia/monocyte-induced neurotoxicity. It has been documented that fibrillar Aβ plays as an activator of inflammasomes, which stimulates caspase-1 signalling and results in the production of pro-inflammatory cytokines such as IL-1β, IL-18 and IL-1α (Sheedy *et al*., 2013)

#### 1.2.3 Astrocytes

Astrocyte represents the second prominent player in neuroinflammation during neurodegeneration diseases. The astrocyte supports the blood-brain barrier (BBB) endothelial cells, providing nutrients for neurons and maintaining ion balance. However, astrocytes have been suggested to play a part in the progression of neurodegenerative disorders (Bagyinszky *et al*., 2017)**.**

Reactive astrogliosis is a multistage, complex and pathologically specific reaction that represents the response of astrocytes to neuronal damage, while the remodelling of those cells is commonly aimed at recovery of injured tissue and in neuroprotection (Sofroniew, 2009; Sofroniew & Vinters, 2010). Besides activated microglia, reactive astrocytes have been found to be accumulated around senile plaques in post-mortem human tissue for patients who had AD (Medeiros & LaFerla, 2013) and also in AD animal models (Olabarria, Noristani, Verkhratsky, & Rodríguez, 2010). Interestingly, Reactive astrocyte is characterized by an increase in the expression of a glial fibrillary acidic protein (GFAP) (Olabarria, Noristani, Verkhratsky, & Rodríguez, 2011).

Furthermore, like microglia, astrocytes enhance the expression of Aβ degrading enzymes during exposure to native Aβ *in vivo* (Pihlaja *et al*., 2011). This Aβ exposure also has the ability to activate astrocytes which in turn lead to release interleukins, cytokines and nitric oxide and eventually cause exacerbation in neuroinflammatory response reactions (Agostinho, A Cunha, & Oliveira, 2010; Avila-Muñoz & Arias, 2014).

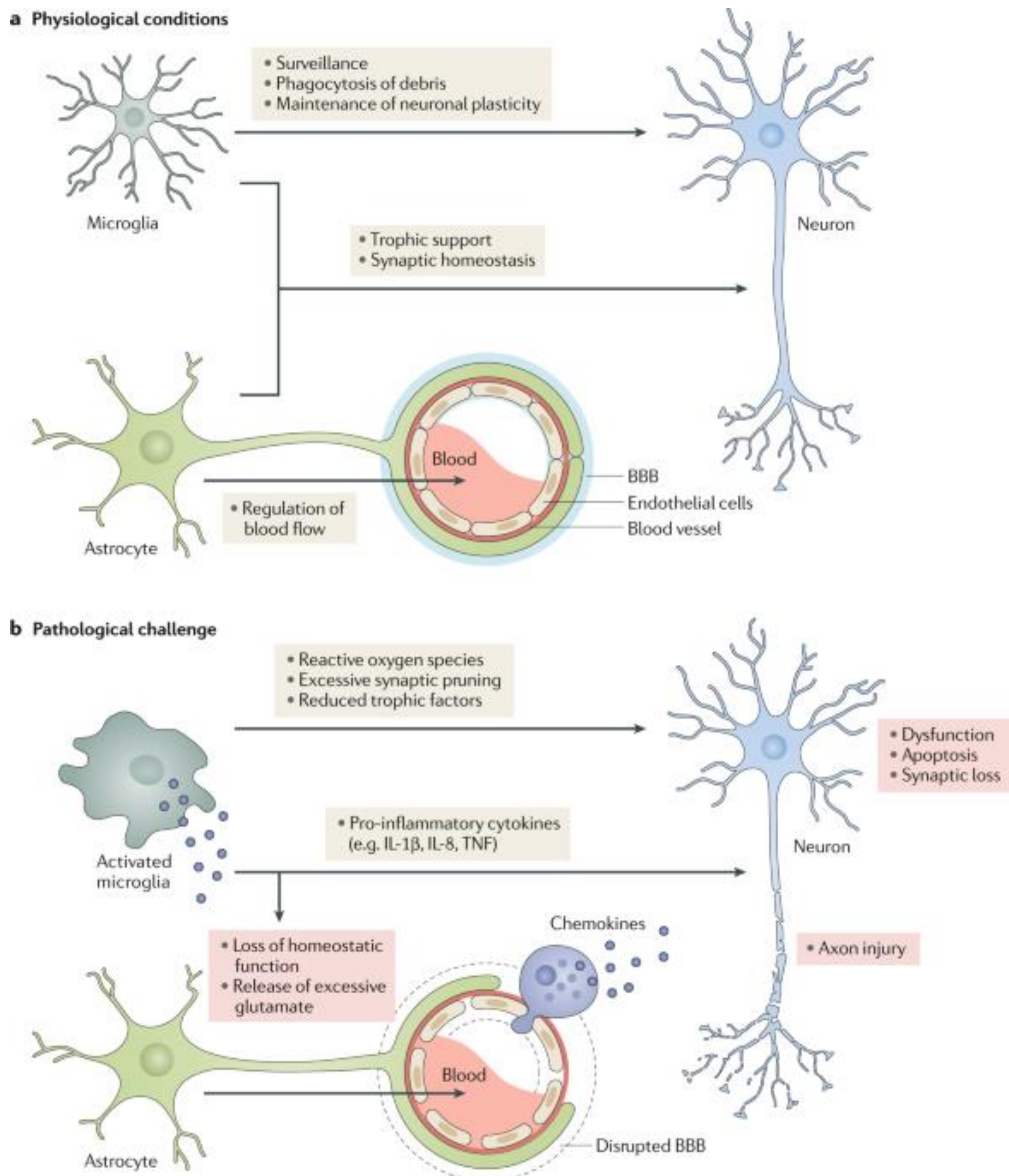



**Figure 1-1 Brain cells' activity status under (a) physiological conditions, (b) pathological challenge. (Leng & Edison, 2021).**

## 1.3 Mediators involved in neuroinflammation

### 1.3.1 Cytokines

Cytokines participate in almost all aspects of neuroinflammation, including anti-inflammatory and pro-inflammatory processes, neuronal injury and microglial response to tissue damage. Astrocytes and

Microglia are the sources of the main cytokines in neuroinflammatory processes (De Strooper & Karran, 2016). Elevated concentrations of pro-inflammatory cytokines like IL-1β, IL--6 and TNF-α are associated with increased concentrations of Aβ in ageing TgAPPsw and PSAPP transgenic mice (Patel *et al*., 2005). Exposure of microglia and astrocytes to pre-aggregated Aβ1–42 causes activation of these cells and lead to increased expression of pro-inflammatory cytokines (TNF-α, IL-6 and IL-1β) (Lue *et al*., 2001), ROS and potentially cytotoxic compounds which might cause neuronal damage and death ( Park, Sapkota, Kim, Kim, & Kim, 2011). In PD, exposure to the aggregation of misfolded synaptic protein α-synuclein will activate microglia, which consequently produce pro-inflammatory mediators (Alvarez-Erviti, Couch, Richardson, Cooper, & Wood, 2011; Couch, Alvarez-Erviti, Sibson, Wood, & Anthony, 2011). In chronic neuroinflammation, there is a persistent release of cytokines due to the continuous activation of glial cells, which may participate in the progression of neurodegenerative diseases (Lyman, Lloyd, Ji, Vizcaychipi, & Ma, 2014).

#### 1.3.1.1 Tumour Necrosis Factor α

TNF-α is a critical pro-inflammatory cytokine in the immune system that enhances and boosts inflammation, which may contribute to and accelerate neurodegenerative diseases during uncontrolled circumstances. TNF-α might activate death receptors (tumour necrosis factor receptor 1 (TNFR1/p55) or Fas (CD95/ APO-1)) and initiate a release of cytotoxic signals which induce cellular apoptosis (Kaushal & Schlichter, 2008). In neuroinflammation, TNF-α is an NF*κ*B pathway response gene, and, besides ROS, it has the ability to activate the NFκB signalling pathway, thereby augmenting the TNF/ROS/NFκB responses (Fischer & Maier, 2015) (Figure ***1-2***). This main role of NFκB, accompanied by the powerful cross-talk within pro-inflammatory cytokines, chiefly TNF-α and ROS/RNS, confirms the interrelation among oxidative stress and inflammation in neurodegeneration (Fischer & Maier, 2015). Upon release from the activated glial cells, TNF-α actions are usually accomplished by its binding to the TNFR1 or TNFR2 receptors to enhance extrinsic apoptotic death signalling pathway through Fas-associated death domain (FADD) or mediate inflammation through activation of nuclear factor kappa-B (NFκB) pathway, respectively (Muhammad, 2019; Sedger &

McDermott, 2014). TNF-α binding affinity was observed with TNFR1 more than with TNFR2 (Chang, Yee, & Sumbria, 2017; X. Cheng, Yang, He, Li, & Shen, 2010). The overexpression of TNFR1 is required for NF-κB signalling pathway activation (R. Li *et al*., 2004), whereas transgenic AD mice models that lack TNFR1 showed a reduced level of activated microglia and improved cognitive performance (P. He *et al*., 2007; Kinney *et al.*, 2018).

The study by Janelsins *et al.* (2008) proposed that overexpression of TNF-α signalling promotes the pathology of neurodegenerative diseases and affects neuronal viability (Janelsins *et al*., 2008). There are pieces of evidence suggesting an increase in the level of TNF-α in the cerebrospinal fluid and serum of MS, which affect the disease progression (Codarri, Fontana, & Becher, 2010; Göbel, Ruck, & Meuth, 2018; Sharief & Hentges, 1991). In the meantime, the cognitive deficits can be controlled via pharmacological management to inhibit TNF-α levels in the AD mice model (Shin *et al*., 2014). The results propose inhibiting neuronal TNF-α signalling might protect neurons throughout the disease progression. A study by Çelik *et al.* (2020) suggested that rat brain inflammation shown by increased levels of TNF-α and other mediators might be reversed by exposure to compounds that act directly on suppressing these mediators (Çelik *et al.*, 2020).

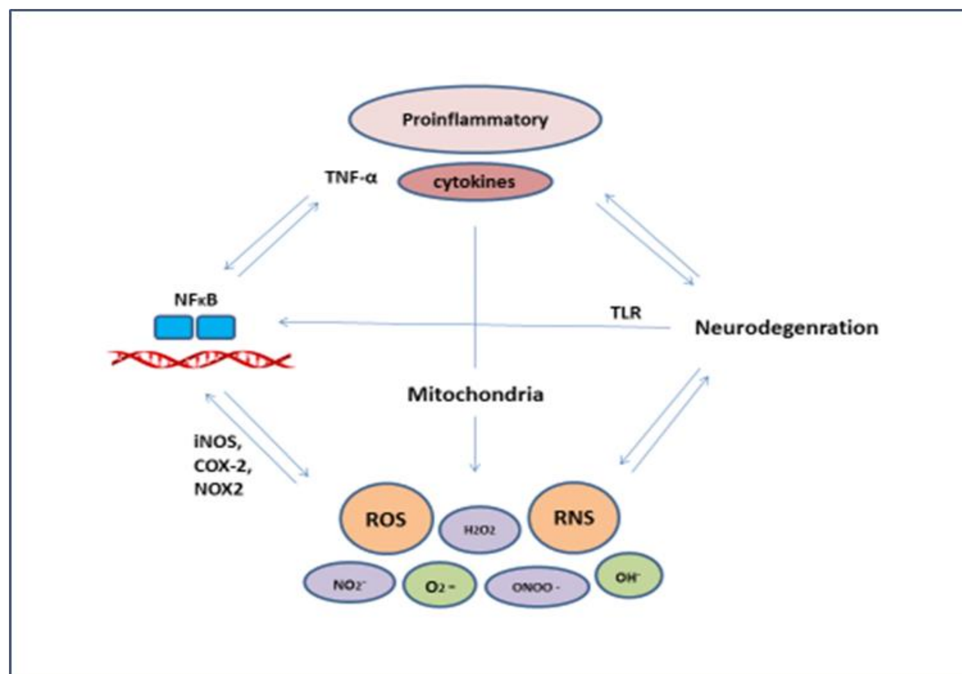



**Figure 1-2** ***The interrelation of inflammatory cytokines and ROS in neurodegeneration disease.***

#### 1.3.1.2 Interleukin-6

IL-6 is an inflammatory cytokine generated mainly from astrocytes and activated microglia in various brain regions (Fischer & Maier, 2015). It is also can be secreted from non-immune cells such as endothelial. IL-6 can provoke an acute phase reaction triggered by external stimuli and promotes the release of pro-inflammatory cytokines from the affected tissues (F. Zhang & Jiang, 2015). The cellular expression for the IL-6 receptor (IL-6R) is limited by leukocytes and microglia, where it is bound to its ligand IL-6. However, the biological effects of IL-6 are produced by two diverse processes: classical and trans-signalling. Regarding the classical way, IL-6 binds directly to the transmembrane IL-6 receptor and initiates an immune reaction. This mechanism occurred only in T cells, neutrophils, hepatocytes and monocytes (Scheller, Chalaris, Schmidt-Arras, & Rose-John, 2011). In contrast, the trans-signalling mechanism can be illustrated by the binding of IL-6 to the soluble IL-6R and forms a complex which would then bind to the cellular membrane and start the pro-inflammatory effects (Göbel *et al.*, 2018; Rothaug, Becker-Pauly, & Rose-John, 2016). However, IL-6 trans-signalling is the predominant mechanism in the brain responsible for IL-6 pro-inflammatory activity (Campbell *et al*., 2014). The classical pathway is mediated through the inflammatory pathways (JAK/STAT, AKT and MAPK). It produces cytokines that will have a feedback inhibition by suppressor of cytokine signalling (SOCS) (Figure ***1-3***), while the IL-6 trans-signalling deviates from such suppression effects (Hodes, Ménard, & Russo, 2016).

It has been demonstrated that IL-6 expression is induced by stimulation with IL-1β and TNF-α, which provokes more immune reactions and affects the disease progression (Matsumoto *et al*., 2018; Toldo *et al*., 2014). Several studies highlighted the increased level of IL-6 in the serum and cerebrospinal fluid of MS patients (Ashtari, Madanian, Shaygannejad, Zarkesh, & Ghadimi, 2019; Briken *et al*., 2016; Stelmasiak *et al*., 2000). Furthermore, IL-6 performs a complex role in cognitive function regulation in AD. Weaver *et al*. (2002) observed that elevated IL-6 levels are associated in humans with age-related cognitive deterioration (Weaver *et al.*, 2002). Afterwards, many researchers established that following inflammatory conditions, excessive production of IL-6 by activated microglia, caused by inflammation

or ageing, may have the ability to impair cognitive processes like memory and spatial learning (Dugan *et al.*, 2009). In a 10-year longitudinal study, high levels of peripheral IL-6 have been noticed during late midlife, which might cause a cognitive decline later in life (Singh-Manoux *et al.*, 2014).

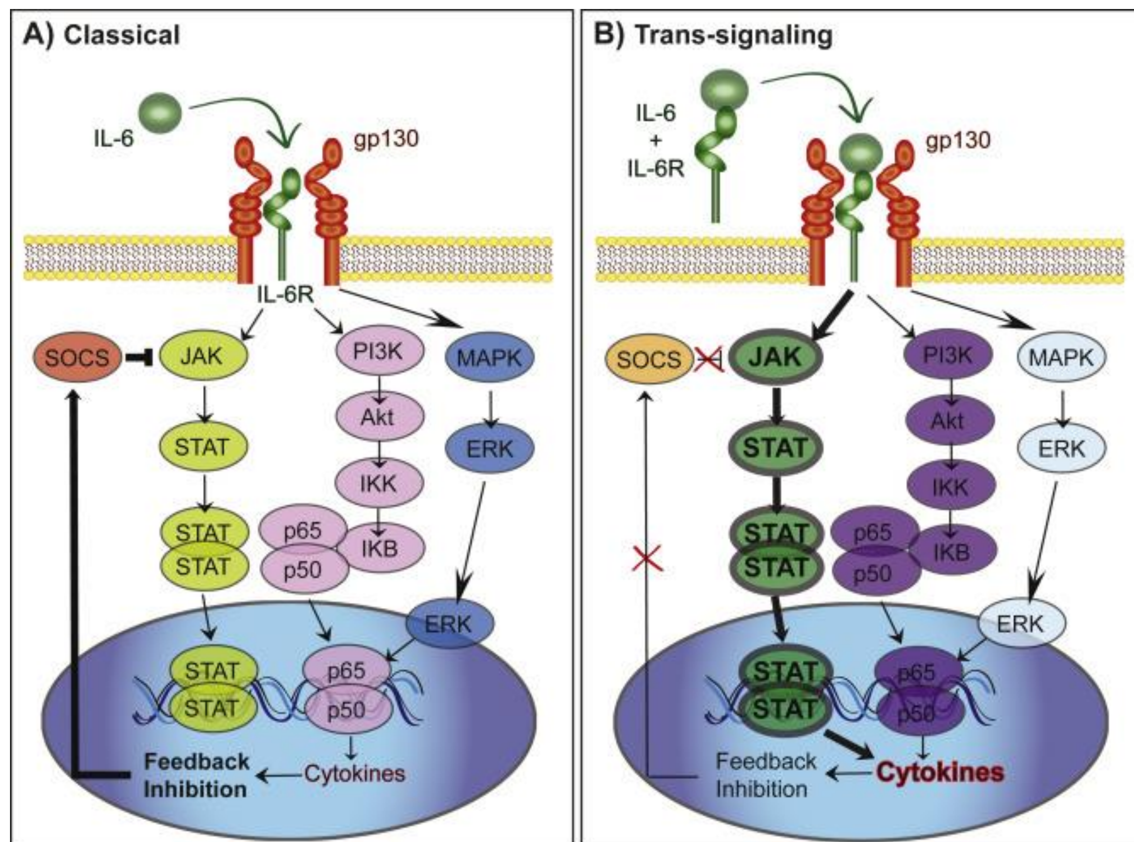



**Figure 1-3 A diagram for (A) Classical IL-6 pathway against (B) IL-6 trans-signalling pathway. (Hodes *et al.*, 2016).**

### 1.3.1.3 Prostaglandins

Cyclooxygenases (COX) are a set of heme-containing enzymes that participate in prostaglandins formation from arachidonic acid (AA) via molecular oxygen addition (Figure ***1-4***). There are two structurally different COX enzymes: COX-1 (constitutively present in all tissues) and COX-2 (founded mainly in the kidney and brain or induced during inflammation) (López & Ballaz, 2020).

During neuroinflammation, pro-inflammatory mediators (reactive nitrogen and oxygen species, cytokines) or other stimuli (such as LPS) have been suggested to increase COX-2 expression (Huang *et al.*, 2018). Inducible COX-2 produces prostaglandins (PGs) in the brain regions from arachidonic acid

(Ricciotti & FitzGerald, 2011). The resultant PGs usually activate the microglia and cause neuronal damage (Liu *et al*., 2017) since PG receptors are present predominantly on the membranes of microglia (Caggiano & Kraig, 1999). COX-2 produces various prostaglandins such as $PGE_2$, $PGF_2\alpha$, $PGD_2$, $PGA_2$ and $PGI_2$ (Foegh, 1998). $PGI_2$, $PGD_2$ and $PGE_2$ are observed in the CNS (Wolfe & Coceani, 1979). $PGE_2$ is principally produced by macrophages and microglia in the CNS and is connected to neuroinflammation (Wood, 2012). Further, the $PGE_2$ receptors (EP1-EP4) are chiefly located in the microglia (Caggiano & Kraig, 1999) and lead to further production of inflammatory mediators through binding with $PGE_2$ (Shi *et al.*, 2012). It has been reported that the high expression level of both COX-2 and $PGE_2$ correlated with dopaminergic neurons' destruction in PD (Teismann *et al*., 2003). While transgenic ablation of COX-2 was suggested to enhance dopaminergic neuron survival in *in vivo* model of PD (Feng *et al*., 2002). The expression of COX-2 and the level of $PGE_2$ are both elevated in the frontal cortex and CSF of AD patients (Combrinck *et al*., 2006). Additionally, COX-2 and $PGE_2$ with other pro-inflammatory mediators (IL 1β, TNFα, nitrite, ROS) may contribute to neuronal death in AD models (Arun Kumar *et al*., 2020; Rockwell, Martinez, Papa, & Gomes, 2004). A recent study revealed the LPS-mediated production of $PGE_2$ and upregulation of COX-2 in microglia, while their production was attenuated by blocking NF-κB/MAPK signalling pathway (Yao *et al*., 2019). Therefore, targeting COX-2/ $PGE_2$ signalling has been shown to have a reverse effect on neuronal damage of neurodegenerative diseases (Zhu *et al*., 2020).

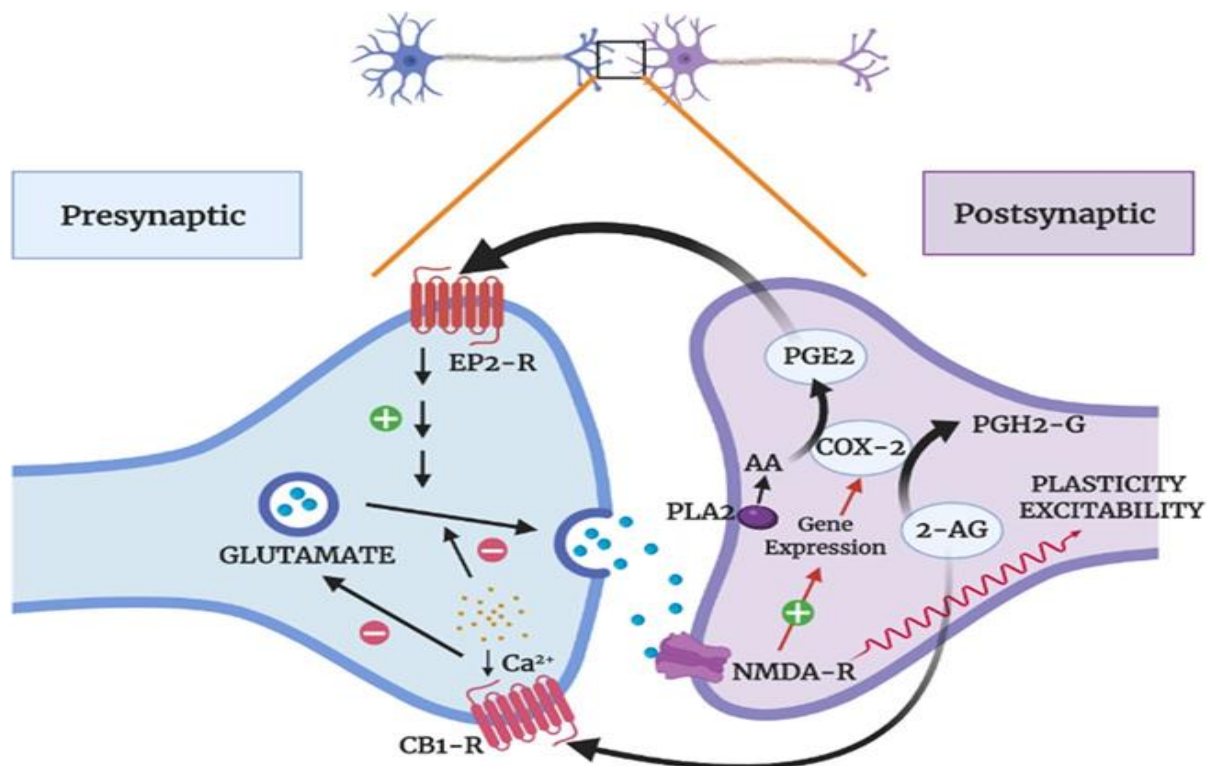



**Figure 1-4 Prostaglandins formation by the activity of cyclooxygenase enzymes.**

**(López & Ballaz, 2020)**

### 1.3.2 Reactive Oxygen and Nitrogen Species

Free radicals are chemical molecules with an unpaired electron generated intracellularly during physiological processes and play a crucial role in various cellular compartment activities such as cell cycle regulation, phagocytosis, and enzyme activation. The mitochondrial electron transport chain uses approximately 98% of molecular oxygen in the cytochrome oxidase system, and the residual oxygen molecules are reduced to both superoxide radicals and hydrogen peroxide (Aso, Ferrer, Duyckaerts, Delatour, & Potier, 2016). The significant ROS formation, enhanced by the electron transport system inside the mitochondria following stressful situations and in ageing, establishes a risk for developing AD when no effective antioxidant system is possible (Clarke *et al*., 2015).

Admittedly, many studies have confirmed mitochondria dysfunction as a critical part involved in the pathogenesis of neurodegenerative diseases due to the production of ROS (Ohta & Ohsawa, 2006; Selfridge, Lezi, Lu, & Swerdlow, 2013; Zhao & Zhao, 2013). However, AD patients' brains suffer from a significant range of oxidative damage which is correlated with the abnormal signed accumulation of

Aβ and the deposition of neurofibrillary tangles (Christen, 2000). In addition to instantly stimulating neuronal cell death, the overproduction of ROS promotes neuroinflammation by activating the inflammatory signalling pathways resulting in the release of pro-inflammatory mediators from microglia (Von Bernhardi, Eugenín-von Bernhardi, & Eugenín, 2015).

The nitric oxide molecule is the most crucial player in the oxidative hypothesis of neurodegenerative diseases (Zhao, 2005). NO is mainly generated from L-arginine by the action of nitric oxide synthase (NOS) enzymes, and it is mainly required in cell differentiation, regulation of blood flow, and neurotransmission (Vincent, Tilders, & Van Dam, 1998; Yun, Dawson, & Dawson, 1996). In the CNS, there is a continuous expression for two NOS enzymes: the endothelial (eNOS) and constitutive neuronal (nNOS) isoforms. When the brain is subjected to hypoxia, ischemia and other pathological stimulants such as LPS, TNF-α, interferon-gamma (IFN-γ) and IL-1β (Terazawa *et al*., 2013), another isoform is activated, which is known as inducible NOS (Yuan *et al*., 2015). iNOS generates a series of deterioration events, including excessive NO molecule generation and severe brain injury because of peroxidation reactions. Accumulating data proposes that all three NOS are implicated in an assortment of central nervous system diseases, including AD (Katusic & Austin, 2013; Silverman, 2009; Zhihui, 2013). To illustrate, nNOS is a calcium-dependent enzyme normally activated by access of calcium into the cell and leads to NO synthesis. In neurodegenerative diseases, calcium release from extracellular and intracellular origins is known to be dysregulated enhancing a compensatory reaction to increasing its level. However, the resultant high levels of calcium cause increased nNOS activation, which produces oxidative stress-associated macromolecule injury and leads to synaptic loss and, eventually, neuronal death (Asiimwe, Yeo, Kim, Jung, & Jeong, 2016; Chakroborty, Kim, Schneider, West, & Stutzmann, 2015). In brain regions like the hippocampus, NO is vital as a signalling molecule in memory formation and learning (Neitz *et al*., 2013). The primary NO source in typical physiological situations in the brain derives from eNOS, while any defects in the eNOS enzyme will impair cognitive function and increase neurodegenerative changes (Austin, Santhanam, & Katusic, 2010; Hopper & Garthwaite, 2006).

Under normal physiological conditions, iNOS is not expressed. However, its expression occurs as a response to released pro-inflammatory cytokines during disease progression, resulting in excess NO (Chao et al., 2013; Zhang, Li, Prabhakaran, Borowitz, & Isom, 2006). As shown in Figure ***1-5***, excess production of NO is involved in neurodegenerative processes such as inhibition of mitochondrial respiration, axonal and synaptic damage and provoke neuronal apoptosis via a set of mechanisms, including aggravation of oxidative stress (Ashutosh Kumar *et al*., 2014) and inhibiting the expression of Bcl-2 ( Lin *et al*., 2013). High levels of NO result in neuroinflammation by promoting microglia to release pro-inflammatory mediators, whereas attenuating the production of NO preserves the adjacent neurons and limits the neuroinflammation events (Cho *et al*., 2016; Kang, Choi, Moon, Kim, & Kim, 2013).

NADPH oxidases represent a source of free radical superoxide. These multi-subunit enzyme systems are located mainly in the cell membrane, which is characterised by their catalytic subunit NOX. Different NOX proteins are expressed in CNS cells, including microglia, astrocytes and neurons, with NOX2 being the principal form in astrocytes and microglia (Brandes, Weissmann, & Schröder, 2014; Cooney, Bermudez-Sabogal, & Byrnes, 2013). Under normal conditions, ROS that is generated by NADPH oxidases enzymes is implicated in tissue homeostasis and cell signalling (Brandes *et al*., 2014; Gao, Zhou, & Hong, 2012). During inflammation, NADPH oxidase activation is heavily increased, which results in a rise in ROS levels. This activation has been involved in oxidative stress-mediated neurodegenerative disease (Gao *et al*., 2012) .

Another important modulator of oxidative stress is reactive nitrogen species (RNS). One of the important RNS players is nitric oxide (NO) which mainly reacts with the superoxide; from NADPH oxidase; and forms peroxynitrite (Asiimwe *et al.*, 2016; Smith, Kapoor, & Felts, 1999). Consequently, $ONOO^{-}$ may react with $CO_2$ to form the highly reactive radicals $CO_3^{\cdot}$ and $NO_2^{\cdot}$ that causes neuronal damage (Fischer & Maier, 2015).

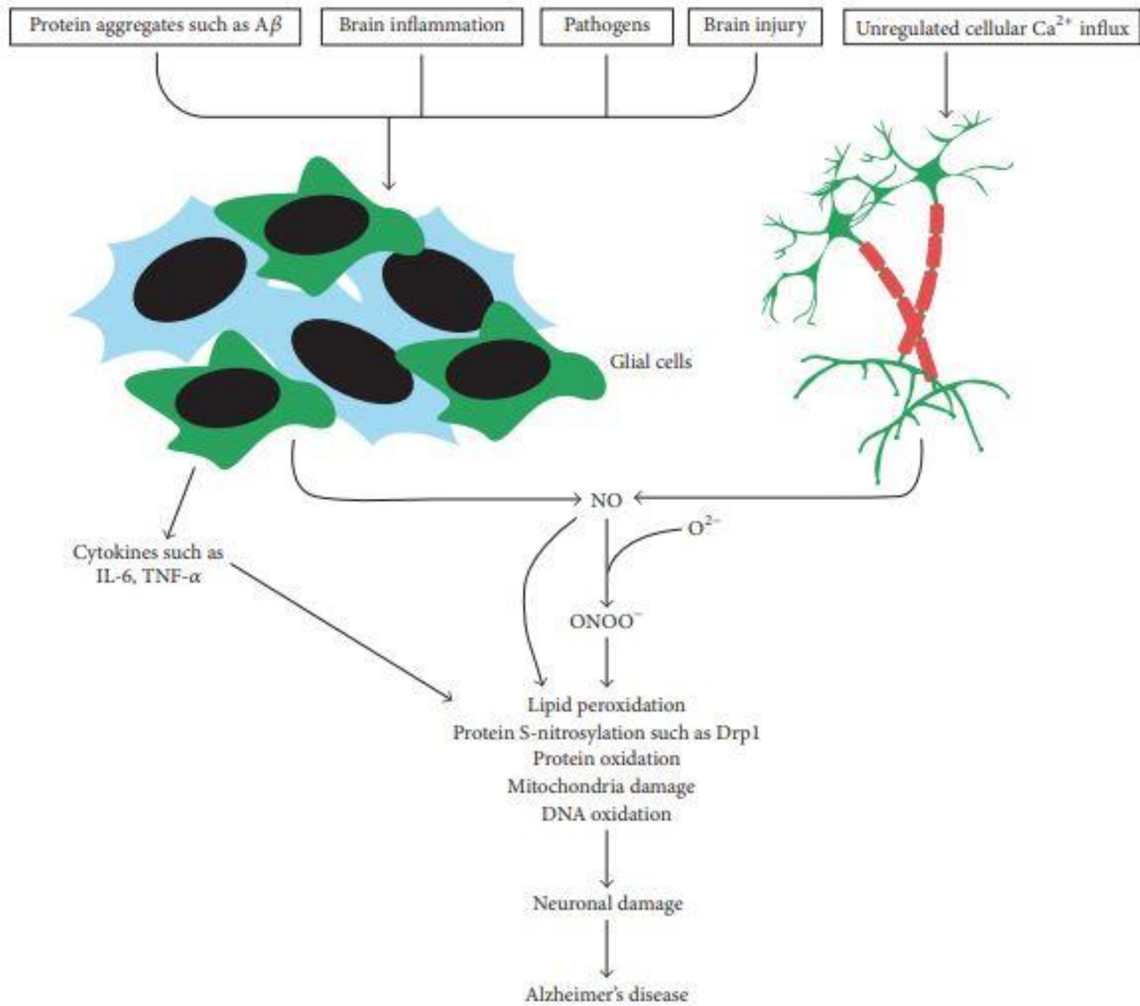



**Figure 1-5** ***Effects of nitric oxide production from activate glial cells.***

**(Asiimwe *et al.*, 2016)*.***

## 1.4 Signalling pathways involved in neuroinflammation

### 1.4.1 NF-κB signalling pathway

Constant activation of microglia accelerates neuroinflammation and neuronal damage (Heppner, Ransohoff, & Becher, 2015). NF-κB represents a key regulator for microglia-mediated inflammatory processes (Granic, Dolga, Nijholt, van Dijk, & Eisel, 2009). In the nervous system, NF-κB has been suggested to have an essential role by working as a transcription regulator in neuronal survival, neuroinflammation, apoptosis, differentiation, synaptic plasticity and neurite outgrowth (Kunz *et al.*, 2015). To regulate inflammation, NF-κB plays a dynamic role in releasing numerous inflammatory factors and molecules like chemokines, cytokines, pro-inflammatory enzymes and pro-inflammatory

mediators (Gupta *et al*., 2014). The expression of those molecules has been recognized in the tissue of the brain and cerebrospinal fluid of neurodegenerative cases (Emmanouil, Taoufik, Tseveleki, Vamvakas, & Probert, 2011; He *et al*., 2011).

In resting cells, NF-κB is a dimer redox transcription factor residing primarily in the cytoplasm in an inactive state. NF-κB signalling pathway mainly mediates the expression of genes which regulate several processes, such as cell survival, apoptosis, inflammation, immunity and control inducible expression of inflammatory response elements (Huang & Hung, 2013). Several family members of the NF-κB transcription factor have been classified in mammalian cells: p65 (RelA), c-Rel, RelB, p52/p100 (NF-κB2) and p50/p105 (NF-κB1) ( Zhang & Jiang, 2015). They are generally complex with the family members of inhibitory IκB proteins (IκBα, IκBβ, IκBγ) (Maqbool, Lattke, Wirth, & Baumann, 2013). Among all the components of the NF-κB family, only p65, c-Rel, and RelB have the ability to activate the transcription pathway of the target genes (Shi *et al*., 2016).

Under several environmental conditions or stress (such as ROS, LPS, Aβ or cytokines), activation of the IκB kinase (IKK) complex results in phosphorylation of IκB followed by degradation. As shown in Figure ***1-6***, the remaining NF-κB dimer complex is activated, phosphorylated and translocated to the nucleus, where the modulation of target genes expression takes place through binding with DNA sequence (Granic *et al*., 2009; Mincheva-Tasheva & Soler, 2013; Snow, Stoesz, Kelly, & Albensi, 2014). NF-κB is predominately activated as a response to pro-inflammatory stimuli, such as IL-1β or TNF-α (Hayden, West, & Ghosh, 2006) or through stimulation by LPS ( Zhao, Zhou, Xu, & Zhang, 2014). NF-κB activation was noticeably increased after exposure to neurotoxin, MPTP, in the PD mouse model (Lee *et al*., 2013). In MS, activated NF-κB, resulting from activated microglia, causes an increase in the production of pro-inflammatory mediators (Leibowitz & Yan, 2016). Additionally, the activated form is particularly observed in glial cells and neurons that surround Aβ plaques of AD brains (Kaltschmidt, Uherek, Volk, Baeuerle, & Kaltschmidt, 1997), which in turn contributes to cytokines production (Nakajima,

Matsushita, Tohyama, Kohsaka, & Kurihara, 2006) and protein expression of inducible nitric oxide synthase (Bhat, Feinstein, Shen, & Bhat, 2002).

### 1.4.2 Role of MAPKs signalling pathway in neuroinflammation

In neuroinflammatory responses, the mitogen-activated protein kinase (MAPK) signalling pathways perform a crucial role in neuronal degeneration after exposure to TNF-α, Aβ, lipopolysaccharide or stress stimulation (Bendotti, Tortarolo, & Borsello, 2006). These signalling pathways are usually preserved in eukaryotic cell regulation mechanisms. MAPKs sub-families include c-Jun N-terminal kinase (JNK1/2/3), extracellular signal-regulated kinase (ERK1/2) and p38 kinase (p38 α β γ δ) (Krishna & Narang, 2008; Xin Wang & Tournier, 2006) which are commonly activated after stress stimuli (Sugino *et al.*, 2000) via phosphorylation of both threonine and tyrosine residues (Dérijard *et al.*, 1994) (Figure ***1-6***).

#### 1.4.2.1 JNK

The c-Jun N-terminal kinase (JNK), also known as stress-activated protein kinase (SAPK), is expressed in parenchyma cells, connective tissues and immune cells such as astrocytes, microglia and oligodendrocytes (Mehan, Meena, Sharma, & Sankhla, 2011). Evidence shows that JNK is involved in pathological events like neuroinflammation via AP-1 (Jang, Kelley, & Johnson, 2008; Pocivavsek, Burns, & Rebeck, 2009) and physiological–regenerative events such as memory formation, brain development and repair (Figueiredo, Pais, Gomes, & Chatterjee, 2008; Raivich *et al.*, 2004). It has been reported that JNK is implicated in microglia enlargement and induction of pro-inflammatory cytokine genes that code for IL-6, TNF-α, and COX-2 (Waetzig & Herdegen, 2004). Indeed, studies propose that activation of JNK serves as an enhancer to microglia activation, which leads to further production of inflammatory mediators and enhanced proteins expression such as iNOS (Mehan *et al.*, 2011) and enhances apoptosis signals which lead to cell death (Yarza, Vela, Solas, & Ramirez, 2016). Furthermore, there was a noticeably high expression level of phosphorylated JNK accompanied by Aβ co-localization in the brains of patients who suffered from AD (Killick *et al.*, 2014).

#### 1.4.2.2 p38

The second most important MAPK protein is p38 which plays a critical role in neuroinflammation pathogenesis. Activated p38 MAPK pathway has been identified in animal models of AD and in the post-mortem brains of AD patients (Schnöder *et al*., 2016; A. Sun, Liu, Nguyen, & Bing, 2003).

The p38 MAPK is a polypeptide which exists in 4 isoforms (α β γ δ), and all, in the neuroinflammation, are activated through phosphorylation on Tyr182 and Thr180 residues (Ashwell, 2006). Data shows that p38 MAPK has the ability to regulate pro-inflammatory signalling systems by contributing to the biosynthesis of cytokines, such as IL-1β and TNF-α in immune cells (Yokota & Wang, 2016). Studies of a neuroinflammatory mechanism in post-mortem AD human brains revealed an increase in p38 MAPK activity, and the intensity of phospho-p38 MAPK immunoreactivity has been positively linked with the increased levels of neurofibrillary tangle and neuritic β-amyloid plaques in the neurons (Hensley *et al.*, 1999). Activation of p38 MAPK was observed to occur at a very early stage of neurodegenerative diseases (Pei *et al*., 2001; Sun *et al*., 2003).

Cell culture experiments revealed that the increased activity of MAPK was an influential contributor to neuroinflammation. Bhat *et al*. (1998) reported that p38 MAPK cascades play a key role in the LPS-activated glial cells by contributing to the transcriptional and post-translational regulation of TNF-α and iNOS gene expressions (Bhat, Zhang, Lee, & Hogan, 1998).

Following LPS stimulation of primary human and rat microglia, the p38 MAPK pathway was shown to be activated and mediate the production of NO and TNF-α (Xing, Xin, Hunter, & Bing, 2008). Furthermore, LPS-stimulated BV-2 mouse microglia were discovered to activate all three MAPK cascade pathways and were connected to IL-1β production (Kim, Smith, & Van Eldik, 2004). These results suggest that p38 MAPK is predominately involved in the activation of glial cells and the resulting neuroinflammation leads to neurotoxicity. Hence, p38 MAPK inhibition might have a promising therapeutic strategy for neuroinflammation (Munoz & Ammit, 2010).

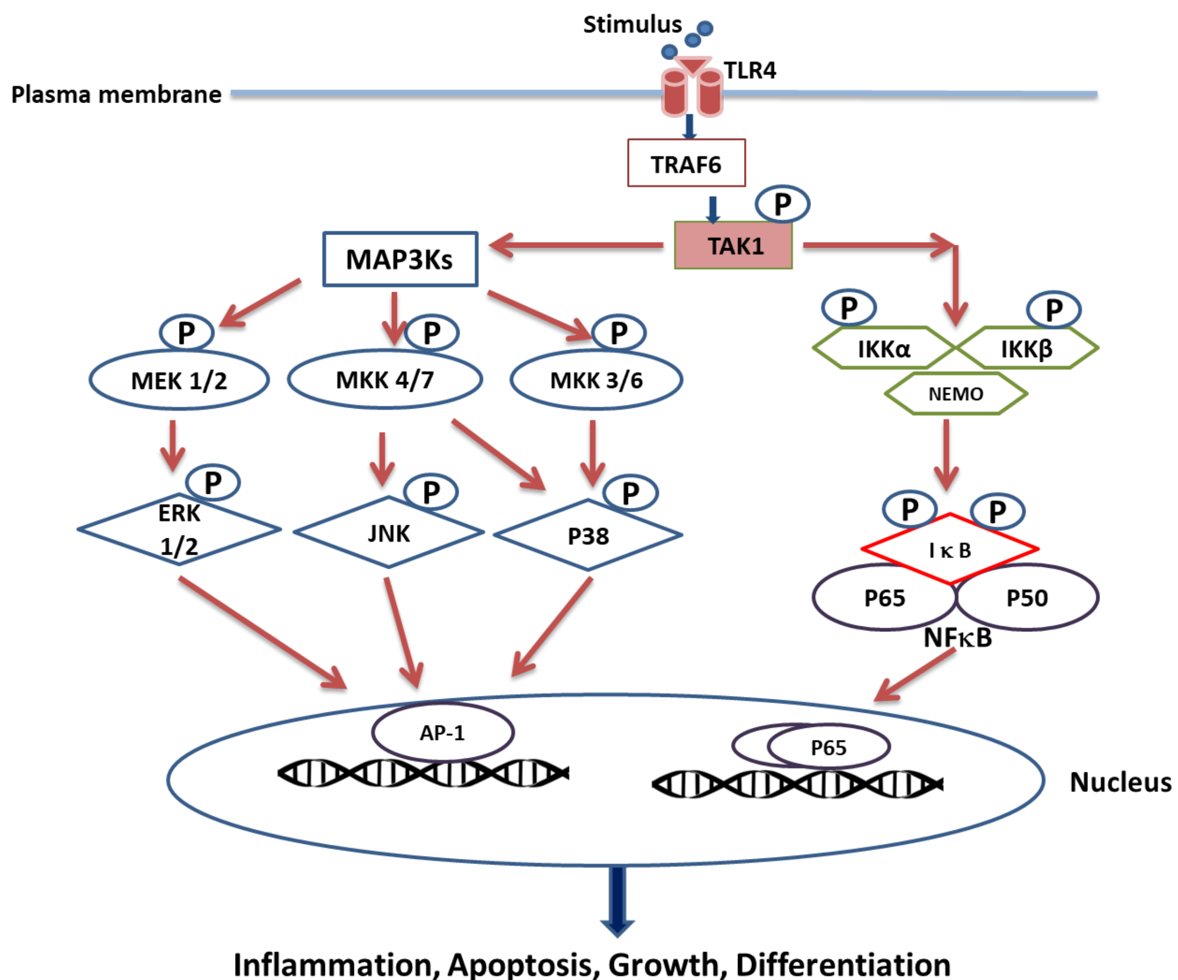



**Figure 1-6 NF-Kappa B and MAPKs signalling pathways.**

### 1.4.3 Roles of PI3K/Akt signalling pathway in neuroinflammation

The phosphoinositide 3-kinase/protein kinase B (PI3K/Akt) cascade is an essential intracellular signalling pathway that may interact with cellular migration, proliferation, metabolism, differentiation and apoptosis (Gao *et al.*, 2020; Xu *et al.*, 2016).

Recently, studies documented amelioration in neuroinflammation and brain injury after inhibiting the PI3K / Akt pathway by pharmacologically-active compounds, suggesting the pathway's involvement in neuroinflammation processes (Guo *et al.*, 2019; Jung *et al.*, 2017; Tang *et al.*, 2017).

Activation of the PI3K / Akt pathway may alter the cellular function and their responses to the triggered signals via mediation phosphorylation of serine and threonine for the downstream substrates. PI3K is activated and phosphorylated after binding the stimuli to the cell surface receptor,

leading to the phosphorylation of membrane lipids and yielding phosphatidylinositol (3,4,5)-trisphosphate (PIP3). The phosphorylated PIP3 could recruit Akt to the cell membrane, which undergoes activation and is translocated from the plasma membrane to the cellular cytoplasm, where it phosphorylates the targeted proteins. In neuroinflammation, the activated Akt (by PI3K or the mammalian target of rapamycin complex 2 mTORC2) could start phosphorylation of IκB kinase (IKK) complex where it represents the upstream activation of the NF-κB pathway (Hemmings & Restuccia, 2012). Experimental evidence revealed that blocking the PI3K/Akt cascade pathway and MAPKs pathway in the LPS stimulated microglia leads to the reduction of pro-inflammatory factors such as IL-6, TNF-α, COX-2, and iNOS (Bozic *et al*., 2015).

Nevertheless, several reports have indicated the neuroprotective ability of the compounds extracted from plants by inhibiting PI3K/Akt phosphorylation which deactivates NF-κB in neuroinflammation disease models (Liu *et al*., 2016; Tai *et al*., 2013). Jayasooriya *et al.* (2014) examined a compound called isobutyrylshikonin, extracted from the root of Lithospermum erythrorhizon, used to treat wounds and burns to modulate immune responses. Their results revealed that isobutyrylshikonin exerted anti-inflammatory effects by attenuating the expression of iNOS and COX-2 with their related products NO and $PGE_2$, respectively. The molecular mechanism was linked to the ability of the compound to mitigate LPS-induced phosphorylation of PI3K and Akt, which are upstream molecules of NF-κB, in LPS-activated BV2 microglial cells (Jayasooriya *et al*., 2014).

### 1.4.4 Nuclear factor erythroid 2-related factor 2 (Nrf2)

The nuclear factor erythroid 2-related factor 2 (Nrf2) belongs to a transcription factor called cap'N'collar (CNC) (Gan & Johnson, 2014) and functions as an oxidative stress sensor. In normal physiological conditions, Nrf2 is located in the cytoplasm associated with its inhibitor Kelch-like ECH-associated protein 1 (Keap1) (K. Chan, Han, & Kan, 2001). In neurodegenerative diseases, ROS production and oxidative damage are increased. As a result, the Nrf2 pathway is activated (Figure ***1-7***) with Nrf2 dissociating from Keap1 and translocated to the nucleus where it binds with the antioxidant

response element (ARE) to initiate modulation of the expression of genes encoding for antioxidant elements and enzymes (Jaramillo & Zhang, 2013; Prasad, 2016) such as heme oxygenase-1 (HO-1), NADPH quinone oxidoreductase 1 (NQO1), superoxide dismutase (SOD) and glutathione (GSH) (Qu, Sun, Zhang, Yu, & Zhuang, 2020).

Heme oxygenase-1 (HO-1), an inducible enzyme, works against oxidative stress by forming three enzymatic by-products from degrading heme molecules: carbon monoxide, biliverdin and free iron possess a crucial role in anti-inflammatory reactions. HO-1 expression is closely regulated by the nuclear binding of Nrf2 molecules to HO-1- AREs sites (Nguyen, Sherratt, & Pickett, 2003). The induction of HO-1 expression has been reported to protect the glial cells and upregulates the microglial anti-inflammatory cytokines after exposure to LPS stimulation (Parada *et al*., 2015).

NADPH quinone oxidoreductase I (NQO1) expression is tightly regulated by Nrf2 and is responsible for the catalysis of two-electron mediated reduction of endogenous and exogenous quinones (Ross & Siegel, 2017). NQO1, as a cytosolic flavoprotein, is a detoxifying enzyme which conserves the plasma membrane from lipid peroxidation and the effect of the free radical by the catalyst of reactive quinones to their stable and non-toxic hydroquinone forms (Atia & Abdullah, 2014). Several pieces of evidence suggest the anti-inflammatory role of NQO1 in the modulation of neuroinflammation disease models. For example, a recent study investigated the involvement of NQO1 in neuroinflammation. To illustrate, NQO1 was upregulated in BV2 cells by an active compound; nootkatone; while knockdown NQO1 resulted in an increase in the pro-inflammatory mediators' levels even though BV2 cells were treated with the same compound (Park *et al*., 2021).

It has been documented that activation of the Nrf2/ARE pathway can overcome oxidative damages related to neurodegenerative diseases (Lee, Park, Lee, & Jang, 2013; Xi *et al*., 2012; Zou *et al*., 2013). The Nrf2/HO-1 axis can combat oxidative damage by suppressing oxidation, inflammation, apoptosis and mitochondrial damage (Jeayeng *et al.*, 2017). Nowadays, several natural and synthetic compounds, such as linalool, emodin and sulforaphane, have been identified for their inhibitory

activity against neuroinflammation, which is mediated by activation of the Nrf2 signalling cascade (Y. Li *et al*., 2015;  Park, Jin, Ko, Park, & Choi, 2016; Subedi, Lee, Yumnam, Ji, & Kim, 2019)

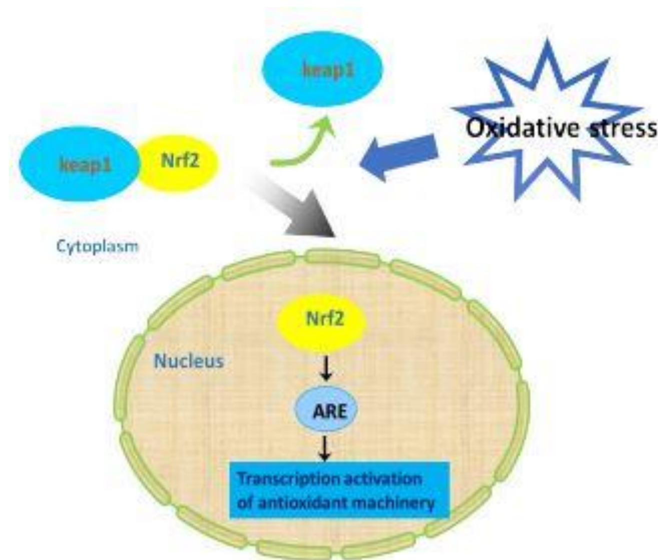



**Figure 1-7 Modulation of Nrf2 signalling pathway after exposure to the oxidative stress.**

**(Sajjad *et al*., 2019)**

## 1.5 Roles of cannabinoid receptors in neurodegeneration

### 1.5.1 The endocannabinoid system

The endocannabinoid system (ECS) comprises of endogenous ligands (endocannabinoids such as nandamide and 2-archidonoyl glyerol), receptors and metabolising enzymes such as fatty acid amide hydrolase and monoacylglycerol acid lipase. ECS has an essential function in several physiological and pathological activities. Synthetic cannabinoids (SCs), as well as phytocannabinoids, can associate with the components of the ECS and lead to a noticeable impact on disease progression (Anna *et al*., 2018; Atwood & Mackie, 2010; Lafaye, Karila, Blecha, & Benyamina, 2017; Onaivi, Ishiguro, Gu, & Liu, 2012; Papaseit *et al*., 2018).

Normally, cannabinoid molecules associate with cannabinoid receptors to exert their effects (Tampi, Young, & Tampi, 2018). Two distinct receptors have been cloned and identified from mammalian tissues; cannabinoid receptors type 1 (CB1 receptor) (Matsuda, Lolait, Brownstein, Young, & Bonner,

1990) and type 2 (CB2 receptor) (Munro, Thomas, & Abu-Shaar, 1993). The expression of the CB1 receptor is abundant in CNS and found in the peripheral area of nerve terminals and various extra-neuronal sites such as the eye, uterus, adipocytes, spleen, testis and vascular endothelial (Herkenham *et al*., 1991; A C Howlett, 2005; Allyn C Howlett, 2002; Porter & Felder, 2001).

The expression of CB1 receptors has been reported to be increased during the asymptomatic stages of some neurodegenerative diseases such as AD (Aso *et al*., 2012). These findings propose an effort of the CB1 receptor to overcome the synaptic impairment at the initial stages of AD. Certainly, pharmacological activation of CB1 receptors with synthetic or natural agonists led to reverse amyloid-β peptide (Aβ) neurotoxic effect in vitro and in vivo AD models (Aso *et al*., 2012; X Chen, Zhang, & Chen, 2011; Janefjord, Mååg, Harvey, & Smid, 2014). However, genetic deletion of CB1 receptors aggravates Alzheimer-like symptoms in the transgenic APP/PS1animal model (Aso, Andrés-Benito, & Ferrer, 2018).

On the other hand, the CB2 receptors are entirely represented in the immune system, which is involved in cellular immune responses (Klein, 2005). Intriguingly, upregulation of CB2 receptors in microglia arises in certain inflammation-related neuropathology, such as AD, PD and MS (Aso *et al*., 2016; Concannon, Okine, Finn, & Dowd, 2016). The neuroprotective activity of CB2 receptor agonists is known to be connected with the inhibition of microglia activation, resulting in the suppression of neurotoxic molecules release and alleviation of neuronal cell damage (Eljaschewitsch *et al*., 2006).

During the last decade, emerging evidence has revealed that CB receptors can act in the brain as both CB1-CB2 receptor heteromers (Callén *et al*., 2012). The expression of these heteromers was specified in a diversity of brain regions, like globus pallidus, pineal gland and the nucleus accumbens (Callén *et al*., 2012). Because of this strong interaction between CB1 and CB2 receptors, the response to drugs that work as agonists or antagonists might be changed when CB receptors are engaged as hetero-receptor complexes (Cassano *et al*., 2017).

In 1992, arachidonoylethanolamide (anandamide, AEA) was the first endocannabinoids discovered. It is followed by 2-arachidonoylglycerol (2-AG) (Allyn C Howlett, 2002). Generally, under physiological conditions, the endocannabinoid system has numerous roles. In CNS, endocannabinoids interfere with controlling emotions and cognitive functions in neuronal circuits of the cortex, amygdala, and hippocampus and strengthen abuse substances in the mesolimbic system (Gerdeman, Partridge, Lupica, & Lovinger, 2003). Endocannabinoids also control posture and movement of the human body (Mario van der Stelt & Di Marzo, 2003), gastrointestinal activity (Maccarrone & Wenger, 2005), pain perception regulation (Iversen & Chapman, 2002) and cardiovascular events (Randall, Harris, Kendall, & Ralevic, 2002). Under pathological conditions, endocannabinoid signalling was affected by dramatic tissue changes. However, in neurodegenerative experimental models like PD and AD, higher endocannabinoid levels have been found (Di Marzo & Petrosino, 2007) and might contribute to protection against disease deterioration (Figure ***1-8***).

### 1.5.2 Anti-inflammatory activity of CB receptor agonists

Mixed or selective CB2 receptor agonists were reported to decrease microglial response and production of a pro-inflammatory molecule following the introduction of Aβ into animals' brains (Esposito *et al*., 2007; Fakhfouri *et al*., 2012; Ramírez *et al.*, 2005; Van der Stelt *et al*., 2006; Wu *et al*., 2013). Similarly, selective CB2 receptor agonists reduced levels of pro-inflammatory cytokines and the amount of activated microglial cells that accumulate around Aβ deposition in two APP transgenic AD models (Aso, Juvés, Maldonado, & Ferrer, 2013; Martín-Moreno *et al*., 2012). Additionally, the administration of Sativex, a natural mixture of THC and CBD, also attenuated the reactivity of microglia in a genetic tauopathy model (Casarejos *et al*., 2013). THC is a critical CB1 partial agonist that might also play a part in inflammatory responses. Furthermore, a recent study illustrated the treatment with ACEA, a selective CB1 receptor agonist, chronically led to a decrease in the pro-inflammatory cytokine interferon-γ, which was expressed by astrocytes in APP/PS1 transgenic mice (Aso *et al.*, 2012).

Similarly, CBD decreased nitric oxide (NO) and iNOS expression levels, which represents one of the crucial enzymes in synthesising NO molecules (Giuseppe Esposito *et al*., 2006, 2011).

CB2 receptors expression is elevated in the PD model, while activation of those receptors will modulate the inflammatory responses by decreasing the pro-inflammatory cytokines (Gómez-Gálvez, Palomo-Garo, Fernández-Ruiz, & García, 2016) and upregulating the anti-inflammatory mediators (Molina-Holgado *et al*., 2003). Moreover, the knocking down CB2 receptors in the PD mouse model caused microglia activation and enhanced inflammatory reactions (He *et al*., 2020). Treatment of rotenone-induced neurotoxicity rat model of PD with CB2 receptor agonist, β-caryophyllene, attenuated the inflammatory reaction by decreasing the level of pro-inflammatory mediators (IL-1β, IL-6 and TNF-α), while pre-treatment with AM630, CB2 receptor antagonist, diminished the anti-inflammatory effects of β-caryophyllene (Javed, Azimullah, Haque, & Ojha, 2016).

Multiple sclerosis is an inflammatory demyelinated neurodegenerative disease. Studies revealed that effective immunomodulating therapeutics could limit disease progression. A recent finding showed the potential anti-inflammatory activity of CB2 receptor agonist, (−)-β-caryophyllene (BCP), in a murine model of MS through attenuation of the activated microglia and reduced the production of the pro-inflammatory cytokines, while this immunomodulation effect was reversed by pre-treatment with CB2 receptor antagonist, AM630 (Alberti, Barbosa, Vieira, Raposo, & Dutra, 2017).

It has been documented that cannabinoid receptors are highly expressed in Huntington's disease model and activation of those receptors with specific agonists caused a reduction in brain oedema and neuroinflammation, while genetic ablation of CB2 receptors resulted in microglial activation and

aggravated the disease symptoms, suggesting the role of cannabinoid receptors in attenuating the progression of the disease (Palazuelos *et al*., 2009).

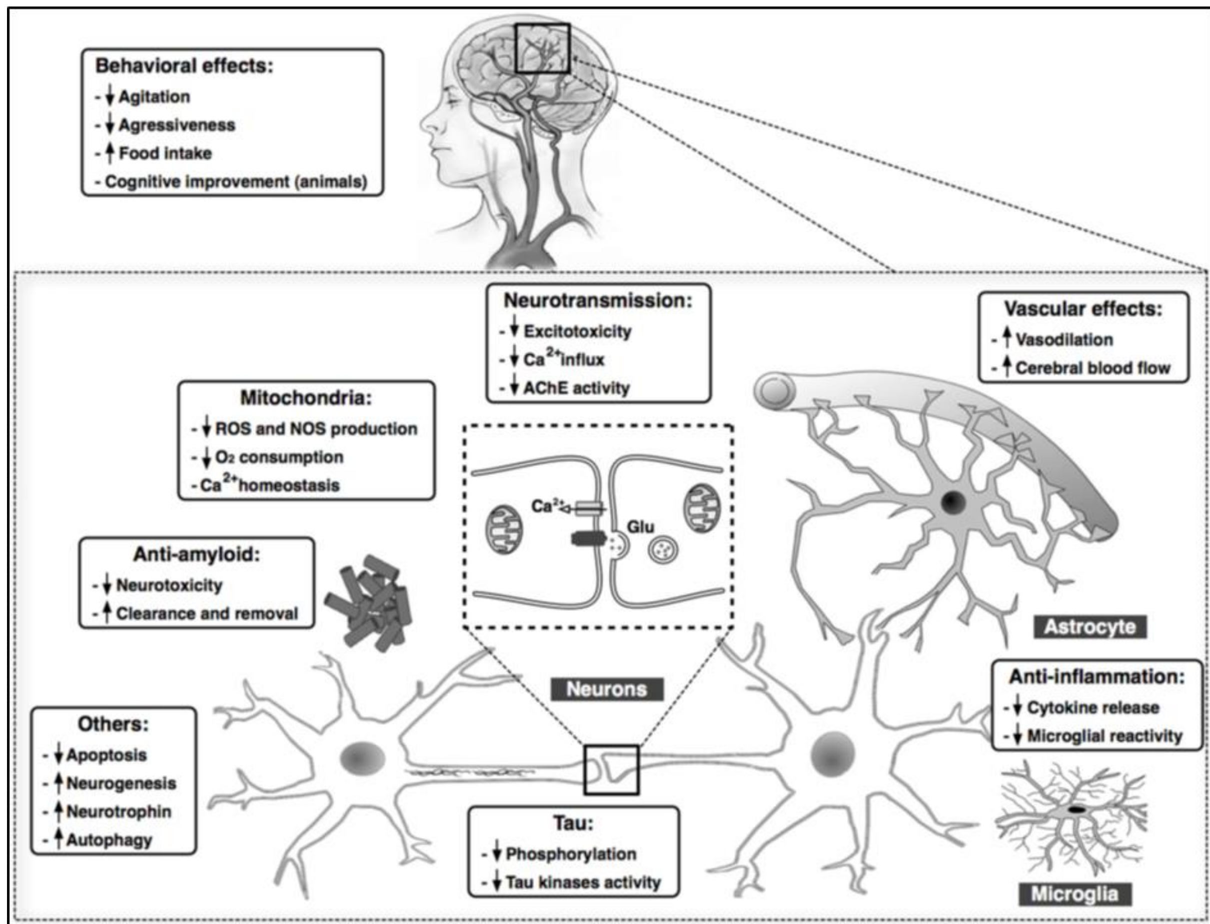



**Figure 1-8 Summary of the major findings confirming useful influences of cannabinoid receptor agonists.**

**(Aso & Ferrer, 2014).**

### 1.5.3 Impact of cannabinoid receptor agonists on disrupted mitochondrial activity

The link of mitochondria with neurons is more significant than with other cells since neurons are extremely energy-demanding cells principally based on aerobic oxidative phosphorylation. The antioxidant features of CBD were shown in cell cultures with toxic glutamate exposure (Hampson, Grimaldi, Axelrod, & Wink, 1998; Aidan John Hampson *et al*., 2000). In parallel with those findings, CBD blocked lipid peroxidation and ROS production in Aβ-PC12 neuronal cells. Also, CBD reduced caspase-3 levels, resulting in decreased apoptosis process and preventing the Aβ-induced elevation in the concentration of intracellular calcium (Iuvone *et al*., 2004). A recent study demonstrated the

protection activity of oleamide, an amide with activity on cannabinoid receptors, against the mitochondrial toxicity induced in the rat cortical slices. Oleamide restored mitochondrial function and increased antioxidant activity, while its effect was mediated by both CB1 and CB2 receptors (Reyes-Soto *et al*., 2022). Induction of APP/PS1 mice by selective CB2 receptor agonist, JWH-133, decreased lipid peroxidation and improved the levels of superoxide dismutase (Aso *et al*., 2013). A reduced level of free radicals and the activity of mitochondria was also illustrated in a tauopathy mouse model after exposure to chronic treatment with CB1/CB2 receptors agonist (Casarejos *et al.*, 2013). Table ***1-1*** illustrates some CBR agonists' anti-inflammatory, antioxidant and neuroprotective abilities in neuroinflammation and neurodegeneration disease models.

**Table 1-1 Summary of some synthetic CB receptor agonists on neuroinflammation and neurodegeneration disease models.**

| Disease model | Compound | CB receptors involved | Effect | References |
|---|---|---|---|---|
| microglial cells activated by IFN-γ | JWH-015 | CB2 agonist | -Decreased in TNFα and NO production<br>-Inhibition of microglial phagocytosis ability | (Ehrhart *et al*., 2005) |
| Microglial and neuronal rat culture stimulated by Aβ $_{1–40}$ | HU-210<br>WIN55,212-2<br>JWH-133 | CB1 agonist<br>CB1/CB2 agonist<br>CB2 agonist | -Prevent cognitive impairment<br>-Decrease TNFα level<br>-Prevent microglial activation | (Ramírez *et al.*, 2005) |

| Disease model | Compound | CB receptors involved | Effect | References |
|---|---|---|---|---|
| | | | -Prevent the loss of neuronal marker | |
| Aβ $_{1-40}$ model of AD | $\Delta^9$-THC | CB1/CB2 agonist | -Prevent Aβ aggregation<br>-Inhibit AChE | (Eubanks *et al.*, 2006) |
| Aβ in THP1 human macrophages and U373 human astrocytoma | JWH-015 | CB2 agonist | -Increase the removal of Aβ plaque<br>-Increase Aβ Phagocytosis | (Tolón *et al.*, 2009) |
| LPS in BV2 cell | CBD | CB1/CB2 agonist & TRPV1 receptor agonist | -Anti-inflammatory impact by blocking LPS-induced STAT1 activation | (Kozela *et al*., 2010) |
| LPS in mice model | CBD | CB1/CB2 agonist & TRPV1 | blocked LPS-induced rise in TNFα, COX-2 and disruption in the blood-brain barrier | (Ruiz-Valdepeñas *et al*., 2011) |

| Disease model | Compound | CB receptors involved | Effect | References |
|---|---|---|---|---|
| | | receptor agonist | | |
| LPS/IFNγ in Oligodendrocyte progenitor cells (OPC) | CBD | CB1/CB2 agonist & TRPV1 receptor agonist | -Decrease production of ROS<br>-Exert anti-apoptotic effect by decrease induction of caspase3 | (Mecha *et al.*, 2012) |
| *In vitro* model of the BBB. | CB13 | CB1/CB2 agonist | -Increase Aβ clearance across the BBB. | (Bachmeier, Beaulieu-Abdelahad, Mullan, & Paris, 2013) |
| Aβ in SH-SY5Y Cells | CBD | CB1/CB2 agonist & TRPV1 receptor agonist | - Selective activation of PPARγ<br>-increased cell survival by reducing the apoptotic rate | (Scuderi, Steardo, & Esposito, 2014) |
| APPxPS1 transgenic mice | CBD | CB1/CB2 agonist & TRPV1 | -Reversed social and recognition memory deficits | (D. Cheng, Low, Logge, Garner, & Karl, 2014) |

| Disease model | Compound | CB receptors involved | Effect | References |
|---|---|---|---|---|
| | | receptor agonist | | |
| Transgenic APP-2576 mice (a model of β-amyloidosis) | JWH-133<br>WIN55,212-2 | CB2 agonist<br>CB1/CB2 agonist | -enhance glucose uptake in the brain | (Köfalvi *et al.*, 2016) |
| AD rats model induced by Okadaic acid | JWH-133 | CB2 agonist | -decrease in Caspase-3, Aβ, IL-1β and TNF-α levels | (Çakır *et al.*, 2019) |
| AD lymphoblasts model by infection with the Epstein–Barr virus and SH-SY5Y cells model of AD | PGN33 | CB2 agonist | -prevent the activation of the PI3K/Akt pathway<br>- inhibit the neuronal death by β-amyloid | (Del Cerro *et al.*, 2018) |
| Familial Alzheimer's Disease PSEN1 E280A Cholinergic-Like Neurons | CP55940 | CB1 agonist | -inhibit ROS generation and tau phosphorylation<br>-block cellular apoptosis | (Soto-Mercado, Mendivil-Perez, Jimenez-Del-Rio, & Velez-Pardo, 2020) |

### 1.6 Arvanil

Arvanil is a cannabinoid CB1 and vanilloid TRPV1 agonist that showed vasodilatory, analgesic and anti-inflammatory *in vivo*. A recent study has reported the anticonvulsant activity of arvanil by increasing the electroconvulsive threshold when injected intraperitoneally in mice before applying the maximal electroshock seizure threshold test (Tutka *et al.*, 2018). However, there is a lack of published data about arvanil neuroprotection activity, which need more investigations.

### 1.7 Fagaramide

Several compounds originally extracted from plant parts, such as dry ginger extract and Satureja Cuneifolia phenolic contents, have shown promising neuroprotective effects *in vivo* and *in vitro* neurodegenerative diseases' model (Apetz, Munch, Govindaraghavan, & Gyengesi, 2014; Mathew & Subramanian, 2014; Taslimi *et al*., 2020). Fagaramide is a compound obtained from the stem bark of the plant *Zanthoxylum zanthoxyloides* in Africa, whereas most of its extracts are used in the malaria treatment, cancer as well as cardiac palpitations (Sandberg, Perera-Ivarsson, & El-Seedi, 2005; Zirihi, Mambu, Guédé-Guina, Bodo, & Grellier, 2005). Compounds extracted from the *Zanthoxylum zanthoxyloides* plant showed promising anti-inflammatory activities that attenuate neuroinflammatory conditions (Ogunrinade *et al*., 2021). However, the biological activities of fagaramide are not studied yet; therefore, it is interesting to investigate its anti-inflammatory and neuroprotective activities.

### 1.8 BV2 microglia as a model of neuroinflammation

Neurodegenerative diseases affect brain cells and cause neuronal damage; hence, it is impossible to isolate the brain tissue for disease hallmarks examination and investigate the neuroprotective activity of new pharmacological agents. As mentioned, microglia represent an essential player in the neuroinflammation pathological events; therefore, a model system of microglia has been invented that biologically mimics the isolated human glial cells. Initially, researchers used primary cell culture

from an animal model by isolating around 30 rodent brains to have a microglia culture enough for a limited number of simple experiments (Henn *et al*., 2009).

The cost of the freshly prepared model, due to a limitation of their proliferation capacity; and the need for high animal consumption, led the science to build an alternative cell line system that would be more suitable, cheaper and less time-consuming. Using human-origin microglia (such as HMO6) is rare due to the difficulties in the isolation from human embryos, while it has legal and ethical consequences (Stansley, Post, & Hensley, 2012). The immortalized mouse-derived microglia (BV2 microglia cells) have been used as a model in research related to neuroinflammation due to the low cost of obtaining and easily immortalized by transfection of primary mouse microglia with v-raf/v-myc oncogene (Blasi, Barluzzi, Bocchini, Mazzolla, & Bistoni, 1990). In addition, BV2 microglial cells express a high level of TLR-4 on their surface, which are necessary for activating those cells (Lehnardt *et al*., 2003). Lipopolysaccharide (LPS) is used to activate BV2 microglia; with a concentration of 100ng/ml, binding to TLR-4 and leading to exert inflammatory reactions by releasing cytotoxic molecules from the activated cells such as TNF-α and IL-1β through NF-κB pathway activation (Dai *et al*., 2015).

Most of the studies in the neuroinflammation platform use 100ng/ml of LPS to stimulate BV2 microglia cells while it is used as an *in vitro* model for investigating the anti-inflammatory and neuroprotection effects of several novel compounds such as platinum nanoparticles, curcumin and neurotropin (Elmazoglu *et al*., 2021; Jiawei Zhang *et al*., 2019; Yuqiu Zheng *et al*., 2018).

## 1.9 Hydrogen peroxide-induce SH-SY5Y neuroblastoma cells as a neurotoxicity model

Brain tissues are usually characterized by a high metabolic rate with high polyunsaturated fatty acids; therefore, it is more susceptible to oxidative stress challenges. Reactive oxygen species overload, resulting from oxidative stress exposure, is the primary aetiology of neuronal damage through protein denaturation, disrupting cellular integrity and lipid peroxidation, which leads to cellular apoptosis (Deng, Su, Ivins, Van Houten, & Cotman, 1999). However, neuronal death may contribute to the

progression of several neurodegenerative diseases such as PD, AD and Huntington's disease (Everse & Coates, 2009; M. H. Yan, Wang, & Zhu, 2013).

Therefore, hydrogen peroxide has been used in this research to introduce neurotoxicity due to its ability to cross the cellular membrane and easily react with most cellular molecules; after being converted to highly reactive hydroxyl radical, it eventually induces cellular apoptosis (Halliwell & Aruoma, 1991). In neuroscience, there is a lack of an *in vitro* model that is similar to primary mature neurons with their axons, synapse and ability to express human proteins (Carolindah, Rosli, Adam, & Nordin, 2013; Gu, Li, Cui, Zhao, & Song, 2017). Accordingly, there was a need to investigate a cell line able to provide an efficient platform to study the neuronal damage which accompanied neurodegenerative diseases.

Due to the lack of human protein expression, primary rodent neurons are limited (Kovalevich, Santerre, & Langford, 2021). In addition, 3D human neural cell lines usually are costly and consume more time to be ready for investigation, while human stem cells usually are more prone to mutations (Choi *et al.*, 2014). As a result, when differentiated, neuronal-like cell lines could be used to conquer these challenges. The human neuroblastoma cell line SH-SY5Y is originally subcloned from parental neuroblastoma cell line SK-N-SH, isolated in 1970 from a bone marrow biopsy of a four-year-old female who has neuroblastoma (Biedler, Helson, & Spengler, 1973). It has been frequently used to study neurodegenerative diseases due to its ability to develop mature neuron-like markers, formation of neurites and express human neuron-specific proteins upon differentiation which make them phenotypically more similar to human primary neurons (Belda, Thompson, Sinha, Prabhu, & Heuvel, 2012; Constantinescu, Constantinescu, Reichmann, & Janetzky, 2007; Shipley, Mangold, & Szpara, 2016).

To induce SH-SY5Y neuroblastoma cells differentiation, retinoic acid (RA) is added to the culture medium to promote neurite outgrowth, inhibit proliferation rate and enhance neuronal markers expression (Constantinescu *et al*., 2007; Kovalevich *et al*., 2021; Shipley *et al.*, 2016); such as neuron-

specific β-III tubulin (Kunzler *et al*., 2017). Recently, cumulative studies have used $H_2O_2$ to induce oxidative stress in SH-SY5Y neuroblastoma cells, providing a convenient *In vitro* model to study neuronal damage and neurotoxicity (Hu *et al*., 2015; A. Y. Lee *et al*., 2018; H. R. Park *et al*., 2015; X. Zhao *et al*., 2019).

## 1.10 Background to the experimental methods

### 1.10.1 XTT assay

XTT assay is a colourimetric assay that measures the activity of the viable cells by changing the reagents' colour. XTT assay was used in this study due to its accuracy, high sensitivity and ease of use, especially with semi-adherent cells, as there was no need to remove the medium. Unlike the MTT assay that used dimethyl sulfoxide (DMSO) to solubilize the crystal dye, which may be related to some safety hazards (Aslantürk, 2018). "XTT reagent (2, 3-bis (2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) sodium salt is used to identify metabolically viable cells due to its sensitivity to cellular redox activity. PMS (Phenazine methosulfate), an electron transporter, is used as an electron-coupling activator to increase the consistency and assay sensitivity. Metabolically live cells have the ability to reduce water-soluble XTT reagent to an orange-coloured Tetrazolium Red Formazan (1-phenyl2-[phenyl (2-phenylhydrazinylidene) methyl] diazene) as shown in Figure ***1-9***.

### 1.10.2 Non-Radioactive CytoTox 96® assay

The CytoTox 96® non-radioactive cytotoxicity assay measures lactate dehydrogenase (LDH), a cytosolic enzyme which is released during cell lysis. The released LDH is measured in culture supernatant via coupled enzymatic assay, which leads to the conversion of a tetrazolium salt into a red formazan product. The yielded colour is proportionate to the lysed cell number (Figure ***1-9***). The half-life of released LDH from cells to the medium is around 9 hours. This assay is simple, reliable, fast and used to confirm the irreversible membrane damage and cellular death (Fotakis & Timbrell, 2006).

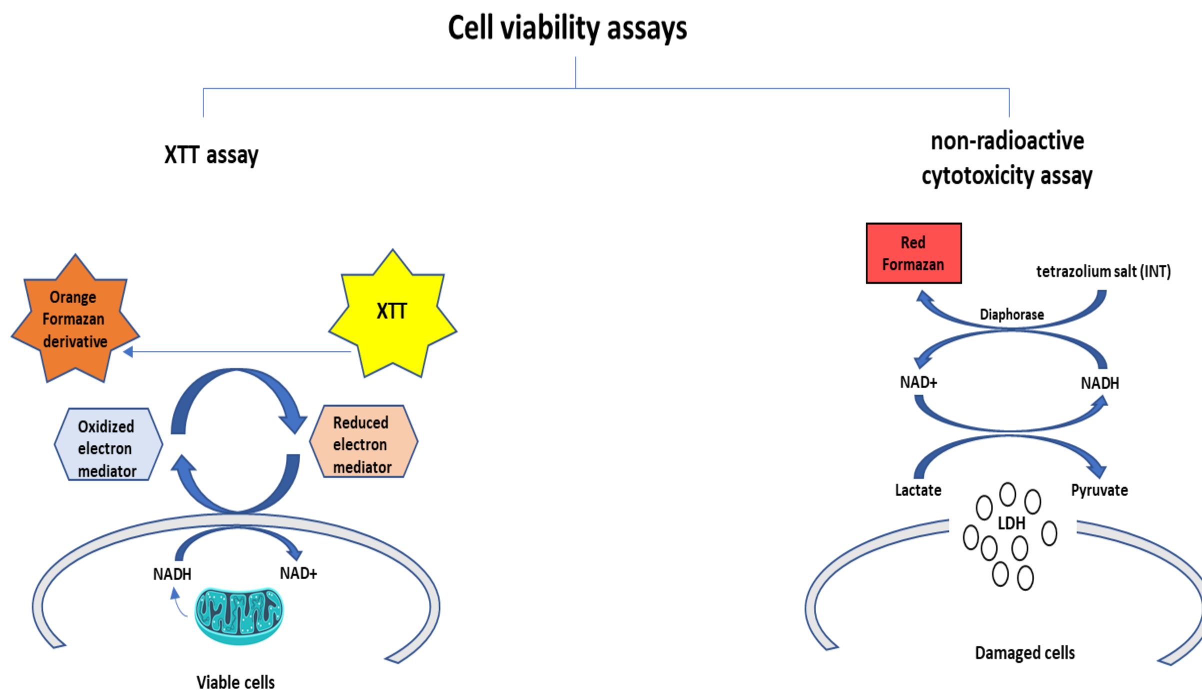



**Figure 1-9 The mechanisms of the used cell viability assays in this study.**

### 1.10.3 Enzyme-Linked Immunosorbent Assay (ELISAs)

Increased expression of pro-inflammatory cytokines from prolonged activated microglia contributes to neurodegenerative diseases progression and neuronal damage (Jiang, Yu, & Tan, 2012; Wang, Tan, Yu, & Tan, 2015). To detect and quantify cytokines' levels, an enzyme-linked immunosorbent assay is used as it is a sensitive and high-specificity procedure, as well as is a simple and highly efficient technique (Sakamoto *et al.*, 2018). Primarily, ELISA was developed in 1971 and was based on the antigen-antibody reaction concept (Engvall & Perlmann, 1971; Van Weemen & Schuurs, 1971). Later, the ELISA technique was developed in 1973 by enclosing the target antigen from the supernatant between the capture and detection antibodies in a method called sandwich system (Belanger, Sylvestre, & Dufour, 1973).

### 1.10.4 Western blot

Western blot is a widely used multi-step technique to detect and identify specific proteins among a mixture of proteins in the sample based on their molecular weight. Western blotting was presented

in 1979 (Towbin, Staehelin, & Gordon, 1979), and since that time, it has become a popular method in cell and molecular biology research. Briefly, the steps of western blot start from protein extraction, protein quantification, gel electrophoresis, membrane electrotransfer, membrane blocking and antibody incubation.

### 1.10.5 NF-**-κB** p65 transcription factor assay

NF-κB p65 transcription factor assay kit is a sensitive, colourimetric and non-radioactive technique for evaluating the DNA binding activity of NF-κB p65 in nuclear extracts. Each well of a 96-well plate has been pre-coated with specific double-stranded DNA (dsDNA) sequence carrying the NF-κB response element for binding to the NF-κB (p65) in nuclear extracts. This binding is detected by adding a specific primary antibody and then a secondary antibody, conjugated to HRP, and the reaction stops, and the absorbance reads at 450nm.

### 1.10.6 Transient transfection and luciferase reporter gene assays

In order to study the activation of transcription factors, luciferase-based reporter gene assays might be considered. Fundamentally, a reporter gene usually consists of a coding DNA sequence linked to a specific promoter sequence that regulates gene transcription in an expression vector introduced inside the cells by a transfection reagent. If the transcription is activated, the firefly luciferase enzyme catalyses the chemical reaction to convert the reporter protein (luciferin) to oxyluciferin. The energy released from this reaction is represented as light. For internal control, the activity of Renilla luciferase, which catalyses coelenterate-luciferin (coelenterazine) oxidation to produce light, is measured by adding a Renilla detection reagent.

### 1.10.7 Immunofluorescence

Immunofluorescence is an imaging method that relies on an antibody-antigen reaction to reveal and visualise the expression of target proteins or molecules in biological samples. Immunofluorescence was first defined in 1942; then, it has been developed over time to involve more steps yielding more accurate assay (Betterle & Zanchetta, 2012). The system uses 4', 6 diamidino2-phenylindole

dihydrochlorides (DAPI) fluorescent stain, which is associated with adenine–thymine-rich sites in dsDNA. DAPI stain is used after labelling the cells with the desired antibody, followed by visualisation with a fluorescence microscope.

## 1.11 Gap in knowledge

So far, no investigation has examined the neuroprotection and the molecular mechanism for the activity of arvanil and fagaramide. Accordingly, this research, for the first time, focuses on the neuroprotection activity of cannabinoid receptor1 agonist, arvanil, and fagaramide against neuroinflammation and neurotoxicity challenges.

## 1.12 Aim of the study

1. To Investigate the anti-inflammatory activity of arvanil in LPS- activated BV2 microglia cells.
2. To evaluate the effects of fagaramide on inflammatory responses in BV-2 microglia.
3. To determine the potential roles of NF-κB and MAPK activation in the anti-inflammatory effects of both compounds.
4. To elucidate the effects of arvanil and fagaramide on the Nrf2 antioxidant mechanism in the microglia.
5. To investigate the effects of arvanil and fagaramide on hydrogen peroxide-induced neurotoxicity.
6. To determine whether the anti-inflammatory effect of arvanil is mediated through cannabinoid or TRPV-1 receptors.

# 2 Chapter Two: Materials and Methods

## 2.1 Cell culture

Two different cell lines were used in investigations: BV2 mouse microglia cells and SH-SY5Y Human neuroblastoma cells.

### 2.1.1 BV2 microglia cells

RPMI medium (21875-034; Gibco) for culturing BV2 cells was supplemented with 10% fetal bovine serum FBS (F9665; Sigma Aldrich) and 1mM sodium pyruvate (11360-070; Gibco). The medium was warmed in a water bath at 37 °C before use.

BV2 cells were obtained from (ICLC ATL03001) Interlab Cell Line Collection, Banca Biological Cell Factory, Italy. To thaw the cells, a cryovial was taken from liquid nitrogen and thawed quickly in a water bath at 37°C. Thereafter, cell suspensions were removed from the cryovial to a centrifuge tube containing 9 ml of complete medium and centrifuged at 1200 rpm for 5 minutes. After that, the supernatant was removed, and the pellet was re-suspended with a 10 ml fresh complete medium and transferred to a T75 flask, where it was placed in the incubator (Air 95%; $CO_2$ 5%; Temperature 37℃).

At 70-80% confluence, the medium from the flask containing cells was removed, and the cells were washed with 5 ml PBS (14190-144; Gibco). Thereafter, 3 ml of TrypLE Express (12604-021; Gibco) was added to the flask and left in the incubator for 2-3 minutes to detach the cells. Then, 7-8 ml of complete medium was added after all the cells had been detached. The cell suspension was transferred to a 50-ml falcon tube and centrifuged at 1200 rpm for 5 minutes. The supernatant was then removed, and the pellet was re-suspended in a fresh complete medium. The desired amount of cell suspension was added to a new T75 culture flask with the complete medium. The medium was changed every 24 hours until the cells were 70% confluent and were ready for sub-culturing and seeding out in the desired plate at a concentration of $4 \times 10^4$ cells/ml.

### 2.1.2 SH-SY5Y cell line culturing and differentiation

The cells were purchased from European Culture Collections and cultured in the complete Minimum Essential Medium MEM (11095-080; Gibco): F12 (11765-054; Gibco) (1:1) supplemented with 1mM sodium pyruvate, 1% Non-Essential Amino Acids (11140-050; Gibco) + 15% Fetal Bovine Serum (FBS). For thawing SH-SY5Y cells, the same procedure was followed in 2.1.1 for BV2 cells. After reaching 60-80% confluence, the medium was removed from the flask, washed once with PBS then the cells were incubated with 3ml of TrypLE Express for 3 minutes. Then 7 ml of growth media was added, and the cells were collected in A 50 ml centrifuge tube to centrifuge for 5 minutes at 1200 rpm. Thereafter, the cells were cultured with a fresh complete growth medium and incubated at 37°C in a 5% $CO_2$ incubator. The medium was changed every 48 hours until the cells were 60-80% confluent and passaged or seeded out. For differentiation, SH-SY5Y cells were seeded out in the desired plate at a concentration of $4 \times 10^4$ cells/ml. After reaching 60% confluence, the medium was changed to a freshly prepared differentiation medium (F12: EMEM (1:1), 1% FBS and 10µM retinoic acid) to initiate the differentiation process in 37 °C, 5% $CO_2$ incubator. The differentiation medium was changed every 48 hours, and then after seven days of differentiation, the cells were examined under the microscope to evaluate neurite outgrowth (Figure ***2-1***).

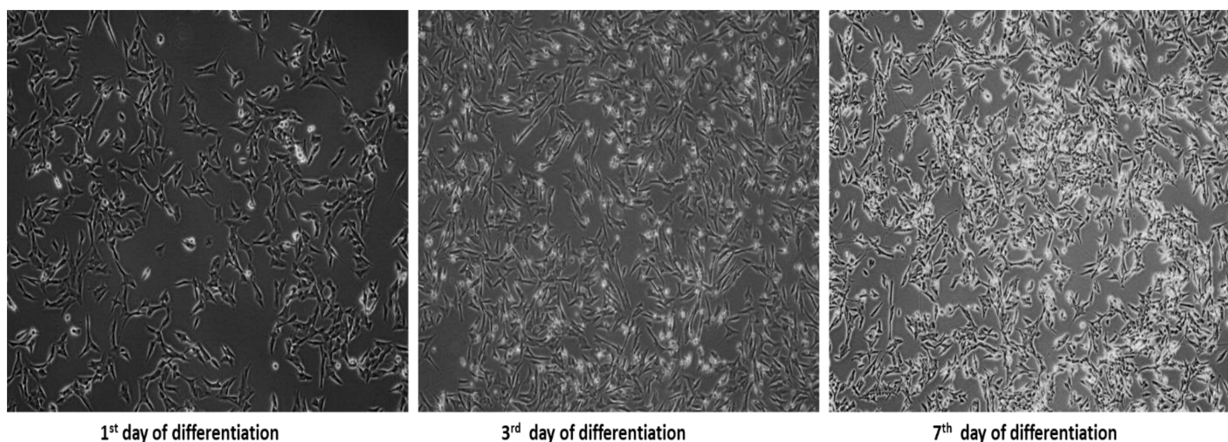



**Figure 2-1 Morphological appearance of undifferentiated and differentiated SH-SY5Y cells.**

## 2.2 Assessment of cell viability

To evaluate the viability of the cells, XTT and LDH assays were used.

### 2.2.1 XTT assay

BV2 cells were cultivated in a 96-well plate at $4 \times 10^4$ cells/ml density with 200µl of culture medium per well and incubated at 37°C. At 80% confluence, the medium was changed to serum-free RPMI medium for 1 hour (trying to exclude any experimental variability). Then, the cells treated with the compounds and stimulated with 100ng/ml LPS for 24 hours at 37°C.

XTT solution was freshly prepared by dissolving 4 mg of XTT powder (X6493; Thermo Fisher) in 4 ml of pre-warmed serum-free RPMI. 10 µl of 10 mM PMS was added to the XTT solution directly before labelling the cells. 25 µl of XTT/PMS solution was added to each well that contains 100 µl of cell culture. The plate was incubated for 2 hours in a $CO_2$ incubator, and the absorbance was read at a wavelength of 450 nm on a plate reader (Infinite F50, Tecan).

On the other hand, SH-SY5Y cells were seeded out in a 96-well plate at $4 \times 10^4$ cells/ml with 200µl of culture medium per well and then differentiated as indicated in 2.1.2. After treatment, the experiment stopped by adding freshly prepared 25 µl of XTT/PMS solution mixture, and after 2 hours of incubation, the absorbance was read at 450 nm on a plate reader (Infinite F50, Tecan).

### 2.2.2 Non-Radioactive CytoTox 96® assay

BV2 cells were seeded out at $4 \times 10^4$ cells/ml in each well of a 96-well plate. At confluence, the medium was changed to serum-free medium for 1 hour, and cells were treated with the compounds, stimulated with 100ng/ml of LPS and incubated for 9 hours.

CytoTox 96 Reagent was prepared freshly before each experiment by dissolving one vial of substrate mix with 12ml of Assay Buffer (G1780; Promega). Thereafter, 20 µl of 10X lysis solution was added to the wells of vehicle-only cells 45 minutes before adding CytoTox 96 Reagent to obtain a Maximum LDH Release Control. Then, the supernatant was collected and centrifuged at 13500 rpm for 5 minutes,

and 50μl taken from each sample, Maximum LDH Release Control, and non-cells control were transferred to a fresh 96-well flat clear bottom plate in duplicate. 50μl of the CytoTox 96 Reagent was added to each sample aliquot; incubated for 30 minutes at room temperature in the dark. Afterwards, 50μl of Stop Solution was dispensed to each well, and the absorbance was recorded at 490nm with Infinite F50, Tecan software.

To calculate the percentage cytotoxicity, the average value of the culture medium background was subtracted from all values of experimental wells. Then the corrected value was used to obtain the percent of cytotoxicity as follows:

Percentage of cytotoxicity= Experimental LDH Release/ Maximum LDH Release ×100

## 2.3 Measurement of nitrite production

Nitric oxide is released from microglia and macrophages upon activation and initiates an inflammatory cascade (C. C. Chao, Hu, Molitor, Shaskan, & Peterson, 1992). NO is a pro-inflammatory marker for oxidative challenges' involvement in several neurodegenerative diseases such as AD and PD. Generally, nitric oxide is converted to stable and non-volatile product nitrite in the culture medium, and the latter is detected by using the Griess reagent system.

BV2 cells were seeded in a 96-well plate at $4 \times 10^4$ cells/ml in 200 μl of culture medium in each well and kept in 37°C incubator until confluence. Later, the medium for each well was changed to serum-free medium for 1 hour, and the cells were treated with the compounds and stimulated with 100ng/ml of LPS for 24 hours. Thereafter, supernatants were collected and centrifuged at 13500 rpm for 5 minutes. Griess assay kit was obtained from Promega (G2930). A nitrite standard reference curve was prepared for each assay to determine the samples' concentrations of ($NO_2^-$). Aliquots of 50 μl/well from each experimental sample and standards were transported to a fresh 96-well plate in duplicate and incubated with 50 μl/well of sulphanilamide solution for 10 minutes in the dark at room temperature. This was followed by dispensing 50μl of NED (N-1-naphthyl ethylenediamine

dihydrochloride) solution for 10 minutes at room temperature in the dark. Absorbance was measured at 540nm within 30 minutes using a microplate reader (Infinite F50, Tecan software).

## 2.4 ELISAs

Mouse TNF-α and IL-6 ELISA kits were supplied by Biolegend (430904 and 431304, respectively). BV2 cells were cultured in a 24-well plate at $4 \times 10^4$ cells/ml and incubated at 37°C until 80% confluence. Afterwards, the culture medium was changed to serum-free RPMI medium for 1 hour and then treated and stimulated with LPS (100ng/ml) for 24 hours. At the end of the stimulation, the supernatant was collected and centrifuged for 5 minutes at 13500 rpm.

A day prior to running the experiment, 100 µl/well of capture antibody was used to coat a 96-well plate; in duplicate, the plate was incubated at 4°C overnight for 16-18 hours. On the next day, the plate was washed three times with 300 µl/well of washing buffer and then incubated with 200 µl/well 1X assay diluent for 1 hour at room temperature with shaking at 300rpm; to block non-specific binding. The serial dilutions of standard (500 to 0 pg/ml) were prepared with 1X assay diluent to generate the standard curve. To run the TNF-α ELISA experiment, the samples were diluted 1:9 with 1X assay diluent, while the samples for IL-6 ELISA were used without dilution.

The Prepared samples and the standard dilutions (100 µl/well) were added to the plate after washing with 300 µl/well of washing buffer and incubated for 2 hours on 300 rpm shaker at room temperature. The plate was then washed three with 300 µl/well washing buffer followed by incubation with 100 µl/well 1X diluted detection antibody for 1 hour, also at room temperature while shaking at 300rpm. The washing process was repeated 100 µl/well of the diluted Avidin-HRP was added, and the plate was incubated at room temperature with shaking for 30 minutes. The plate at this step was washed five times for 30 seconds in each wash to minimize the background staining. 100 µl of TMB Substrate Solution was suspended into each well, and the plate was incubated at room temperature without shaking in the dark place for 15 minutes (Figure ***2-2***). To terminate the experiment at this stage, 100

µl/well of stop solution was suspended sequentially, and the absorbance was read at 450nm with an Infinite F50, Tecan software.

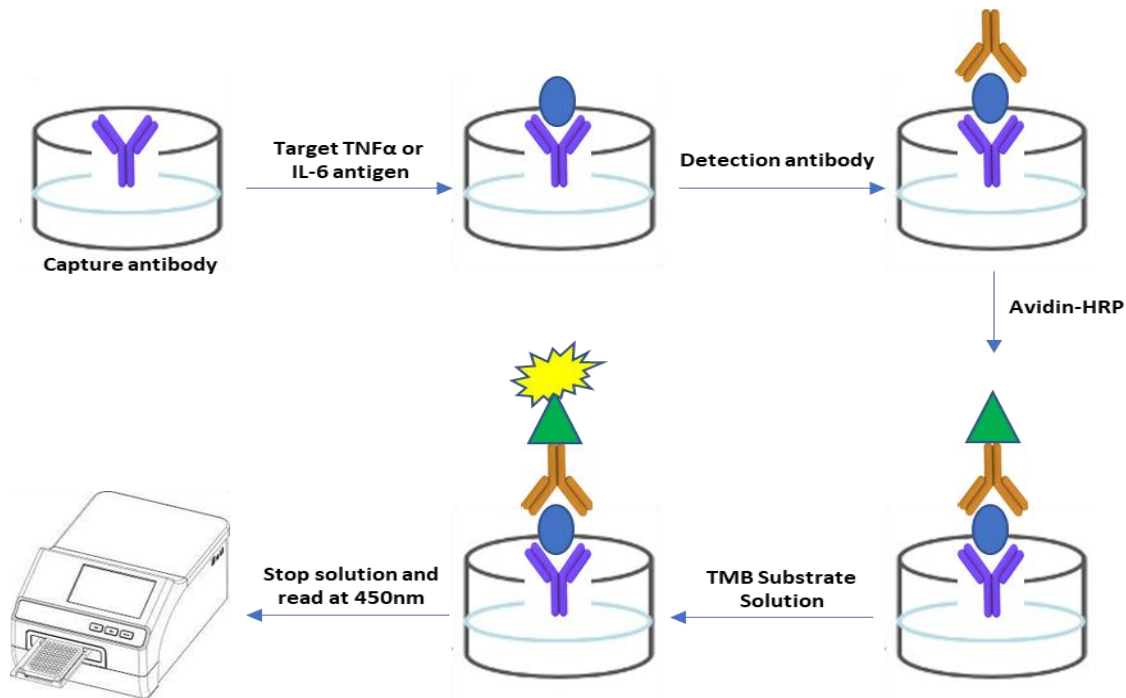



**Figure 2-2 ELISA schematic diagram.**

## 2.5 Measurement of prostaglandin E2

Another mediator released from microglia upon activation is $PGE_2$, which is produced from the over-expression of the COX-2 enzyme in many neurodegenerative diseases (Nagano *et al.*, 2021). To detect the presence of $PGE_2$ in culture medium, enzyme immunoassay (EIA) is used. The basic principle for EIA is detecting antigen-antibody complex through using specific enzyme-coupled secondary antibodies to quantify the presence of the antigen in the reaction. Then a specific substrate is used for signal detection by producing apparent colour changes, which can be read at a specific wavelength.

BV2 microglia cells were seeded in a 24-well plate at $4 \times 10^4$ cells/ml concentration and incubated at 37°C. At 80% confluence, the cells were treated and stimulated with LPS (100ng/ml). After 24 hours of incubation, supernatants were collected and centrifuged at 13500 rpm for 5 minutes. An enzyme immunoassay $PGE_2$ kit (K051-H1; Arbor Assays) was used to detect the concentration of $PGE_2$ in the supernatant, which was provided with a pre-coated 96-well strip plate. The serial dilutions of standard

(1000 to 0 pg/ml) were prepared freshly with 1X assay buffer to generate the standard curve. In addition, the samples were prepared in a 1:4 ratio with 1X assay buffer; 100 µl from the prepared samples or the standards were pipetted into each well in duplicate, as well as 25µl of DetectX prostaglandin $E_2$ conjugate and 25µl of DetectX prostaglandin $E_2$ antibody. The plate was then incubated at room temperature for two hours while shaking at 300rpm. Thereafter, the reagents were aspirated, and each well was washed with 300µl wash buffer four times. The plate was then incubated with 100µl/well of TMB substrate in the dark without shaking at room temperature for 30 minutes. To stop the reaction, 50µl of the stop solution was added to each well. The absorbance was read at 450 nm using an Infinite F50 microplate reader and the yielded absorbances were converted to a concentration's values. The concentrations of $PGE_2$ were calculated from the standard curve after subtracting the mean of each sample from the mean of the non-specific binding value.

### 2.6 Isolation of cytoplasmic lysates

BV2 microglia cells were seeded out in a 6-well plate at a concentration of $4 \times 10^4$ cells/ml and incubated at 37°C until 80% confluent; then, the cells were treated and stimulated with LPS (100ng/ml). At the end of incubation, the plate was placed on ice, the medium was discarded from each treated well and the cells were washed using 500µl/well ice-cold PBS. However, 20 µl of 1X lysis buffer (Cell Signaling) containing 1:100 phosphatase inhibitor cocktail (Thermo Scientific) and 1mM phenylmethane sulfonyl fluoride (PMSF) (Roche) was added to each well and the plate was incubated on ice for 10 minutes. Subsequently, cells were scraped and then sonicated briefly. The cell lysates were collected in microfuge tubes and centrifuged at 4°C for 15 minutes at 13500 rpm. The supernatants, cytoplasmic extraction, were collected and stored at -80°C freezer.

### 2.7 Isolation of nuclear extracts

Nuclear extracts were prepared to determine levels of nuclear proteins. BV2 microglia cells were seeded in a 6-well plate at a concentration of $4 \times 10^4$ cells/ml and incubated at 37°C until 80% confluent, and then the cells were treated and stimulated. At the end of incubation, the plate was

placed on ice and the cells were washed using 1 ml of ice-cold PBS. Nuclear extraction kit was purchased from Abcam (ab113474). The cells were incubated on ice with 1X pre-extraction buffer containing DTT with protease inhibitor cocktail (PIC) in a 1:1000 ratio for 10 minutes. The cells were centrifuged at 4°C for 1 minute at 12000 rpm. The supernatants were discarded and 10 µl of freshly prepared nuclear extraction buffer were used to resuspend the pellet. Thereafter, the extracts were incubated on ice for 15 minutes and sonicated for 3 x10 seconds to increase nuclear protein extraction. The extracted suspensions were centrifuged for 10 minutes at 14000 rpm at 4°C, and the nuclear extractions (the supernatants) were transferred into a new microcentrifuge vial and stored at -80°C freezer.

### 2.8 Protein quantification

In order to determine protein concentrations in cytoplasmic and nuclear extracts, the Bradford assay was performed. Serial dilutions (125-2000 µg/ml) of bovine serum albumin standards (Thermo Fisher) were prepared with de-ionized distilled water to create a standard curve, which served as an absorbance reference for estimating protein concentration. Each lysate sample was prepared in a 1:12 ratio with de-ionised distilled water in a new microcentrifuge vial, vortexed and spined for 10 seconds. 250 µL of Coomassie protein assay reagent (Thermo Fisher) was added to each well of a 96-well plate that contained 5µL of either standards or samples and incubated in the dark for 10 minutes at room temperature. The absorbance was measured at 540 nm using a microplate reader (Infinite F50, Tecan software) and the mean concentration for each sample was calculated depending on the standard curve values.

### 2.9 InstantOne ELISA for detection of phosphorylated NF-κB p65

Nuclear factor-κB p65 is one of the NF-κB subunits that heterodimerizes with the other subunits (p50 or p52). In response to a variety of stimuli, NF-κB p65 can be phosphorylated in both cytoplasm and nucleus and result in an expansion of neuroinflammation conditions, whereas controlling the phosphorylation of the p65 subunit represents a strategy in anti-inflammatory drug discovery

(Giridharan & Srinivasan, 2018). Therefore, it was demanded to detect the level of phosphorylated NF-κB p65 when BV2 cells were treated with the compounds and stimulated with 100ng/ml LPS.

Firstly, a time-point experiment was done to determine the optimal expression time for phosphorylation of p65 subunit following stimulation with LPS. To achieve this, BV2 cells were seeded out in a 6-well plate at a concentration of $4 \times 10^4$ cells/ml and incubated at 37°C. At 80 % confluence, the medium was changed to serum-free RPMI medium, and cells were stimulated with 100 ng/ml of LPS for 15, 30, 45, 60 and 90 minutes. Cell lysates were collected, and the experiment proceeded using the InstantOne ELISA as described below.

BV2 cells were seeded a in 6-well plate at a concentration of $4 \times 10^4$ cells/ml in 2 ml of culture medium and incubated at 37°C. At 80% confluence, cells were treated with the compounds and stimulated with LPS (100ng/ml) for 60 minutes. Then, cytoplasmic lysates were collected and proteins quantified as described early in 2.6 and 2.8, respectively.

NF-κB p65 (Phospho) [pS536] InstantOne ELISA Kit (85-86082-11; Invitrogen) was used to measure phospho-p65 level in cell cytoplasmic lysate. The antibodies cocktail was prepared 1:1 ratio of capture antibody and detection antibody Reagents. After samples preparation at a concentration of 40 µg with 1X cell lysis mix, 50 µl from each sample and 50 µl from the antibodies cocktail were suspended in each well and incubated for 1 hour at room temperature at 300 rpm. Subsequently, the plate was washed with 200 µL/well of 1X washing buffer 3 times and was incubated with 100 µL/well of the detection reagent at room temperature for ~20 minutes with shaking at 300 rpm. To stop the reaction, 100 µL of Stop Solution was added to each well, and the absorbance was measured using a microplate reader at 450 nm with an Infinite F50, Tecan software.

## 2.10 Immunoblotting

Following protein quantification (as described in Section 2.8), samples were prepared at a concentration of 40 µg, then 5 µl of 4X LDS sample buffer (Invitrogen) and 2 µl of 10X sample reducing agent (Invitrogen) were added to have 20 µl final volume. The samples were ready to be loaded into

the NuPAGE Bis-Tris gel (NP0321; Invitrogen) after denaturation at 70$^0$C for 10 minutes. 5 µl of precision plus protein dual colour standards (Bio-Rad Laboratories) was loaded into each gel for molecular weight estimation. For gel electrophoresis, the loaded gels were run in the tank using 1X running buffer (Invitrogen) at 200 V, 160 mA for 35 minutes. An antioxidant (Invitrogen) was added to prohibit sample re-oxidation and preserve it in a reduced state.

Subsequently, separated proteins were transferred from the gel to an Immobilon-FL PVDF Membrane (IPFL00010; Millipore) at 20V, 390 mA for 1 hour. Membranes were washed once for 5 minutes with 1X Tris buffered saline with tween 20 and blocked with a blocking buffer (927-60001; Licor Biosciences) for one hour at 20 rpm. The membranes were washed three times with TBS-T for 5 minutes and incubated with primary antibodies overnight at 4°C. Next day, the membranes were washed for three times/5 minutes each with TBS-T and incubated for 1 hour with secondary antibodies (Table ***2-1***) in the dark at room temperature. The membranes were washed three times with TBS-T, and the blots were scanned with the Licor 9120 Odyssey infrared imaging system. Molecular weight detection was estimated by comparing the observed bands to the Precision Plus Protein dual colour standards. The membranes were incubated with either anti-actin (for cytoplasmic proteins) or Lamin B (for nuclear proteins) antibodies as loading controls to normalise levels of detected proteins. The bands of western blots were processed and quantified by the Image J programme.

**Table 2-1 Antibodies used in western blot analysis.**

| Antibodies | Supplier | Catalog No. | Isotype | Dilution |
|---|---|---|---|---|
| iNOS | Cell Signaling Technology | 95423S | Rabbit IgG | 1:1000 |
| COX-2 | Abcam | ab15191 | Rabbit IgG | 1:1000 |
| Beta-actin | Sigma Aldrich | A5060 | Rabbit IgG | 1:1000 |
| p-IκB-α | Santa Cruz Biotechnology | sc-101713 | Rabbit IgG | 1:500 |
| Total-IκB-α | Santa Cruz Biotechnology | sc-371 | Rabbit IgG | 1:500 |

| | | | | |
|---|---|---|---|---|
| P-SAPK/JNK | Cell Signaling Technology | 4668S | Rabbit IgG | 1:1000 |
| SAPK/JNK | Cell Signaling Technology | 9252S | Rabbit IgG | 1:1000 |
| P-p38 MAPK | Cell Signaling Technology | 9215S | Rabbit IgG | 1:1000 |
| Total p38 | Santa Cruz Biotechnology | sc-535 | Rabbit IgG | 1:500 |
| P-Akt | Cell Signaling Technology | 4058S | Rabbit IgG | 1:1000 |
| Akt | Santa Cruz Biotechnology | sc-8312 | Rabbit IgG | 1:500 |
| HO-1 | Santa Cruz Biotechnology | sc-10789 | Rabbit IgG | 1:500 |
| NQO1 | Santa Cruz Biotechnology | sc-25591 | Rabbit IgG | 1:500 |
| Nrf2 | Santa Cruz Biotechnology | sc-365949 | Mouse IgG | 1:500 |
| Lamin B | Santa Cruz Biotechnology | sc-20682 | Rabbit IgG | 1:500 |
| CB1 Receptor | Cell Signaling Technology | 93815S | Rabbit IgG | 1:1000 |
| TRPV1 Receptor | Abcam | ab203103 | Mouse IgG | 1:1000 |
| CB2 Receptor | Santa Cruz Biotechnology | sc-293188 | Mouse IgG | 1:500 |
| MAP-2 | Santa Cruz Biotechnology | sc-20172 | Rabbit IgG | 1:500 |
| Rabbit Anti-Mouse IgG H&L (Alexa Fluor 680) | Abcam | ab186701 | Mouse IgG | 1/10000 |
| Goat Anti-Rabbit IgG H&L (Alexa Fluor 680) | Abcam | ab175773 | Rabbit IgG | 1/10000 |

### 2.11 NF-κB p65 transcription factor assay

BV2 cells were seeded at 4 × $10^4$ cells/ml. At 80% confluence, the cells were treated and stimulated for 60 minutes with 100ng/ml LPS. Thereafter, nuclear extracts were collected, and the protein was quantified (as detailed in sections 2.7 and 2.8, respectively). NF-κB p65 transcription factor assay kit was obtained from Abcam (ab133112). 10 µl of nuclear lysates samples were prepared at a

concentration of 40 µg and were suspended in each well after adding 90 µl of CTFB, 100 µl of CTFB was added to a non-specific binding well, and the plate was incubated at room temperature for 1 hour. The plate was then washed with 200 µl/well of 1X wash buffer five times. Subsequently, 100 µl of diluted NF-κB (p65) primary antibody was added to each well and the plate was incubated at room temperature for 1 hour. The washing step was then repeated, and 100 µl of diluted secondary antibody was added to each well and incubated at room temperature for 1 hour. Then, the washing process was repeated and the plate was incubated with 100 µl/well of transcription factor developing solution for 30 minutes in the dark at room temperature (Figure ***2-3***). To stop the reaction, 100 µl/well of stop solution was used, and the absorbance was read at 450 nm using a microplate reader with an Infinite F50, Tecan software.

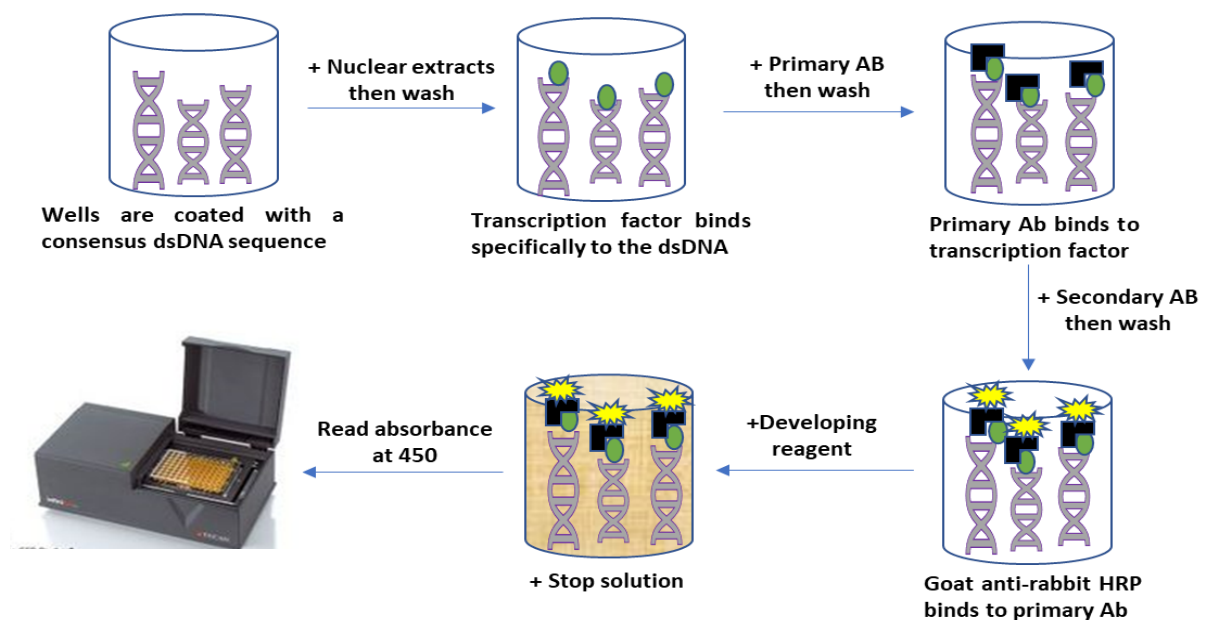



**Figure 2-3 Flowchart of NF-κB p65 Transcription Factor Assay.**

## 2.12 Transient transfection and luciferase reporter gene assays

BV2 cells were seeded into a 24-well plate at 4 × $10^4$ cells/ml concentration and incubated at 37°C. At 80% confluence, the culture medium was changed to 450 µl/well Opti-MEM (Gibco) and incubated at 37°C for 2 hours. In the meantime, Glial-Mag/DNA complexes were prepared by using 0.6 µl of Glial-Mag (GL00250; OZ Biosciences) and 2 µl of NF-κB reporter DNA (336841; QIAGEN) or ARE reporter

DNA (336841; QIAGEN) was diluted in 50 µl Opti-MEM medium. The prepared complex of Glial-Mag/DNA was incubated at room temperature for 30 minutes and added dropwise to the cells, followed by adding 5 µl of Glial-Boost. Thereafter, the cells were incubated at 37°C on a magnetic plate for 30 minutes in the incubator and then incubated for 2 hours without the magnetic plate. Later, 500 µl of Opti-MEM medium was added to each well and the plate was incubated for 24 hours at 37°C. Subsequently, the Opti-MEM medium was changed to serum-free RPMI, and the cells were treated and stimulated with 100ng/ml LPS.

A Dual-Glo Luciferase assay (E2920; Promega) system was used to analyse the Firefly and Renilla luciferase activity. After the desired incubation time, 925 µl of the medium was removed from each well, and 75 µl of Dual-Glo Luciferase assay reagent was added to induce cell lysis and the cells were incubated at room temperature for 20 minutes. The firefly luminescence was measured in a FLUOstar OPTIM reader (BMG LABTECH). Thereafter, 75 µl of Dual-Glo stop & Glo reagent was added, and the plate was incubated for 10 minutes, followed by measurement of Renilla activity. The relative ratio of firefly: Renilla luminescence was calculated for each well.

### 2.13 Immunofluorescence

BV2 cells were seeded at a concentration of $4 \times 10^4$ cells/ml into a 24-well plate and incubated at 37°C. At 80% confluence, cells were treated and stimulated with 100 ng/ml LPS.

SH-SY5Y cells were seeded into a 24-well plate at $4 \times 10^4$ cells/ml concentration. At 60% confluence, the medium was changed to a complete medium and 10µM retinoic acid (to initiate differentiation). Seven days after differentiation was initiated, the cells were treated.

Cells were washed once with PBS and then fixed with 200µl/well of cold methanol (100%) for 10 minutes at -20°C. Then, the cells were washed once with PBS and blocked with 200µl/well of blocking buffer (sc-516214; Santa Cruz) for 60 minutes at room temperature. The cells were incubated with 200µl/well Primary antibodies (Table ***2-2***) overnight at 4°C. Next day, the primary antibodies were removed, and the cells were washed with PBS once and then incubated with 200µl/well of Alexa Fluor

488-conjugated donkey anti-rabbit IgG (A-21206; Invitrogen) secondary antibody (1:500) in the dark for 2 hours. After washing once with PBS, 200 µl from 300 nM of DAPI (D1306; Invitrogen) were added for 1 minute at room temperature, followed by rinsing with PBS and images acquired with EVOS® FLoid® cell Imaging System (scale bar=100 µm) and processed by image J programme.

**Table 2-2 Antibodies used in immunofluorescence assays.**

| Antibody | Supplier | Catalog no. | Isotype | Dilution |
|---|---|---|---|---|
| Phospho-NF-κB p65 | Cell Signaling Technology | 3033S | Rabbit IgG | 1:1600 |
| Nrf2 | Santa Cruz Biotechnology | sc-365949 | Conjugated mouse IgG | 1:50 |
| MAP-2 | Santa Cruz Biotechnology | sc-20172 | Rabbit IgG | 1:50 |
| Tubulin β III | Biolegend | 801203 | Conjugated mouse IgG | 1:100 |

## 2.14 Detection of cellular reactive oxygen species (ROS)

Activated microglia are a potential source of ROS production in neuroinflammation. However, excessive ROS generation may place the microglia in a self-reinforcing cycle which causes further microglial stimulation and, in turn, produce more pro-inflammatory mediators that disparately influence the disease progression by affecting the neuronal health and viability (Spencer, Schilling, Miralles, & Eder, 2016). Attenuating ROS production by pharmacologically active compounds has been reported to modulate microglial-immunological responses and suppress LPS- induce production of pro-inflammatory cytokines (G. Y. Sun et al., 2016).

In order to detect ROS intracellularly, 2’, 7’-dichlorofluorescein diacetate DCFDA-cellular reactive oxygen species detection assay kit (ab113851; Abcam) was used. The assay is based on the ability of DCFDA dye to diffuse into the cell, where it is converted to a non-fluorescent compound by cellular

esterase. Cellular ROS then oxidises the resultant into 2', 7' –dichlorofluorescein (DCF) which is highly fluorescent and can be visualised by an immunofluorescence microscope.

BV2 cells were seeded at $4 \times 10^4$ cells/ml concentration into 24-well plate and incubated at 37°C until confluence. Later, cells were treated with the compounds and stimulated with 100ng/ml of LPS for 24 hours. Subsequently, the medium was removed, and the cells were washed twice with 500µl/well of 1X Buffer. Then the cells were stained by adding 500µl/well of 20µM DCFDA solution and incubated at 37°C for 45 minutes in the dark. Lastly, the cells were washed with 500µl/well of 1X Buffer for two times and images acquisition with EVOS® FLoid® cell Imaging System (scale bar=100 µm) and processed by the Image J programme.

### 2.15 Statistical analysis

Data were converted into % values of stimulant or negative control, then expressed as mean ±SEM and analysed by Graph Pad Prism statistical software version 7 (San Diego, USA) with one-way analysis of variance (one-way ANOVA) followed by Dunnett's Multiple Comparison Test (version 5.0, Prism software, Graph Pad, USA). Three separate experiments were done for each analysis (n=3). Values of $p<0.05$ were taken as being statistically significant.

# 3 Chapter Three: Investigation on The Neuroprotective Activity of Arvanil

## 3.1 Introduction

### 3.1.1 Background

One of the main causes of neurodegenerative diseases is neuroinflammation which leads to memory impairments, disease progression and neuronal damage. Neuroinflammation has been involved in the pathogenicity and progression of neurodegenerative diseases, which happens by cross-interaction between brain immunological cells, such as microglia and astrocytes with neurons. In a resting state, the microglia are characterised by ramified appearance, which expresses few surface receptors for antigen interaction purposes; while upon stimulation, the microglia activated, and their features change from a highly ramified morphology into an ameboid shape (Kreutzberg, 1996) and could exert either neurotoxic, by adapt M1 phenotype, or neuroprotective effect, the immunosuppressive M2 phenotype (Y. Tang & Le, 2016). Once activated, microglia produce various pro-inflammatory mediators such as TNFα, IL-6 and IL-1β besides the production of NO and ROS, as a first-line defensive platform (Cherry *et al*., 2014). Consequently, an overproduction of these factors can induce neuronal cells impairment and damage. The microglia are usually activated after exposure to external stimuli (such as LPS), which bind to TLR receptors and result in the activation of inflammatory cascade pathways that are responsible for pro-inflammatory cytokines generation. The master activated pathway is the IκB/NF-κB signalling cascade, which starts with the upstream activation of IκB kinase (IKK) after cellular exposure to external stimuli (such as LPS) that, in turn, lead to degradation of total IκB and phosphorylation of IκB (Subhramanyam *et al*., 2019). As a result of this phosphorylation, the cytoplasmic NF-κB dimers, p50/p65, are phosphorylated, released from the NF-κB/IκB complex and translocated into the nucleus (Kawai & Akira, 2007). Thereafter, the phosphorylated NF-κB can recognise and bind to its responsive elements in the regulatory sites of the inflammatory target genes in the nucleus, where it is used to regulate the transcriptional activation of pro-inflammatory genes such as TNF-α, IL-6, IL-1β, COX-2 and iNOS (Subhramanyam *et al*., 2019).

Besides NF-κB pro-inflammatory cascade pathway activation, MAPKs are also activated, resulting in increased expression of pro-inflammatory cytokines. Generally, the MAPK signalling cascade is essential in several cellular functions such as differentiation, proliferation and apoptosis (Kim & Choi, 2015). The MAPK signalling system is composed of three subfamilies p38 MAPK, c-Jun terminal kinase (JNK) and extracellular signal-regulated kinase (ERK 1/2) (Plotnikov, Zehorai, Procaccia, & Seger, 2011), among that p38 MAPK with JNK are related to cellular apoptosis while ERK1/2 is involved in cellular survival and proliferation (Kim & Choi, 2015). Studies suggest that p38 and JNK are linked to the activation of microglia in stroke and neurodegenerative diseases and mediate the transcription of several pro-inflammatory cytokines such as IL-1 β and TNF-α (Bachstetter *et al*., 2011; Yang *et al*., 2020). Whereas, selective inhibition of p38 MAPK may attenuate LPS-mediated neuroinflammation in BV2 microglia and LPS-injected mouse model (Gee *et al.*, 2018). Additionally, activated microglia by LPS can cause activation of the JNK pathway, which may provoke inflammation by increasing the expression of different AP-1 target genes, including IL-6, TNF-α, NO and COX-2 (You *et al*., 2018; Youssef, Ibrahim, Akashi, & Hossain, 2019). Both p38 and JNK pathways may also stimulate ROS production in activated microglia (H.-N. Sun *et al.*, 2008).

Another pathway that may be modulated during microglial cell activation is PI3K/Akt signalling cascade pathway. Phosphoinositide 3-kinases (PI3Ks) are enzymes that regulate inflammatory responses after cellular exposure to several factors such as cytokines, growth factors as well as lipopolysaccharide (LPS) and generate phospholipids by signals transducing into intracellular messages (Cianciulli *et al*., 2020). These phospholipids cause activation and reposition of several signalling proteins, such as serine/threonine kinase, which permits the protein kinase movement into the cytoplasm and then towards the nucleus where it regulates several signalling proteins such as NF-κB which, in turn, modulate the cellular response (Cianciulli *et al*., 2020). However, Akt is considered a major mediator in the PI3Ks signalling pathway, and thus the activation of this cascade is crucial when the microglia are activated upon exposure to the stimulus, which may contribute to the neuroinflammation (Saponaro *et al*., 2012; Yan Zhu, Chen, Liu, Peng, & Qiu, 2016).

Generally, exposure to a high level of ROS during neuroinflammation will lead to the upregulation of antioxidant defence mechanisms to counteract neuronal damage and degenerative progression. One of the main intrinsic protective systems that are activated against cellular oxidative damage is the Nrf2 pathway (Sanjiv Singh, Nagalakshmi, Sharma, & Ravichandiran, 2021). In the resting state, the transcription factor (Nrf2) is located in the cytoplasm and associated with a sensor protein called kelch-like ECH-associated protein1 (Keap-1). Under oxidative stress, the inhibitory Keap-1 protein undergoes modification and promotes the inhibitory complex dissociation, which leads to the nuclear translocation of Nrf2 molecules (Deshmukh, Unni, Krishnappa, & Padmanabhan, 2017). At the nuclear level, Nrf2 binds to specific DNA sites called antioxidant response elements (ARE) and provokes the transcription of several cytoprotective genes such as superoxide dismutase (SOD), heme oxygenase-1 (HO-1), glutathione (GSH), NADPH quinine oxidoreductase (NQO1), catalase (CAT) and Uridine 50-diphospho-glucuronosyltransferase (UDP-glucosyltransferase) (M. Sandberg, Patil, D'Angelo, Weber, & Mallard, 2014; Tonelli, Chio, & Tuveson, 2018). Activation of the Nrf2 signalling pathway usually occurs to counteract neuroinflammatory damage. Nrf2 activation was identified after treatment of oxygen-glucose deprivation/reperfusion (OGDR)-activated BV2 microglia and LPS-induced M1 phenotype microglia with potential Nrf2 activators' where the expression of antioxidant downstream genes that scavenge ROS and nitric oxide are induced (Kwon, Cheon, Park, Song, & Lee, 2017; X. Xu *et al.*, 2018). Another recent study revealed that enhancing Nrf2 nuclear accumulation and transcriptional activity would increase the expression levels of both HO-1 and NQO1, which, in turn, deter LPS-stimulated ROS generation in BV2 microglial cells (Liao *et al.*, 2020). During neuroinflammation, the imbalance between Nrf2 and NF-κB signalling pathways develops and may aggravate the disease's pathogenicity. The crosstalk between both pathways usually occurs at the nuclear level, where the NF-κB p65 subunit competes with Nrf2 to interact with CREB-binding protein (CBP), and the result will be the inactivation of one of these pathways with upregulation of the second one (Liu, Qu, & Shen, 2008). When the NF-κB pathway is activated, the pro-inflammatory mediators are expressed and the transcription of genes that are under the control of Nrf2 is attenuated (Nair,

Doh, Chan, Kong, & Cai, 2008). Therefore, modulation of the Nrf2 signalling pathway and its associated antioxidant mediators will be a promising strategy to hinder oxidative stress and attenuate the neuroinflammation pathogenicity.

Neurons are the main cells which are affected by oxidative stress due to their dependency on oxidative-phosphorylation reactions for energy production, rich in polyunsaturated fatty acids that tend to oxidation; as well as the concentrations of antioxidant enzymes required for free radicals scavenge are relatively low (Chen, Guo, & Kong, 2012; Finkel & Holbrook, 2000). Oxidative stress is believed to be a feature of several neurodegenerative diseases, such as AD and PD where neuronal function and structure are affected dramatically (Uttara, Singh, Zamboni, & Mahajan, 2009). Reactive species, including hydroxyl radical, peroxynitrite, nitric oxide and hydrogen peroxide, act as harmful or useful mediators depending on their levels (Carvalho *et al*., 2009). ROS/RNS- mediated neuronal injuries have been reported in the brain and CSF of patients who suffered from neurodegeneration, where they were found to enhance lipid peroxidation and protein damage (Castegna *et al*., 2003; Niedzielska *et al*., 2016; Sultana & Butterfield, 2010). Mitochondria is the main source of reactive oxygen species (Wang *et al*., 2014). Mitochondrial dysfunction is linked to an increase in ROS production, which leads to the activation and release of pro-apoptotic factors such as cytochrome c and apoptosis-inducing factor (AIF), causing cellular apoptosis (Webster, 2012). Additionally, a previous finding suggested that the disrupted mitochondria produce abnormal levels of $H_2O_2$ and result in Aβ accumulation intracellularly which is a key pathogenic factor in AD development (Aleardi *et al*., 2005). Interestingly, a recent study detected the mitochondrial $H_2O_2$ level in the brain of a live AD rat model by using a modified fluorescence imaging technique (Li *et al*., 2020). Therefore, the significant association of oxidative stress with neuronal death in neurodegeneration proposed the use of potential neuroprotective agents as a therapeutic approach to scavenging ROS and limiting cytotoxicity-mediated neuronal damage.

### 3.1.2 Cannabinoid system in CNS

Cannabinoid receptors, CB1R and CB2R, are part of the endocannabinoid system, which are distributed in most tissues. Whereas, CB1Rs are predominately expressed in brain regions such as the hippocampus, hypothalamus, amygdala, and cerebellum (Mackie, 2005). In addition, CB1R participates in synaptic transmission because it is expressed in the neurons as well as, to a lower extent, in microglia, oligodendrocytes, and astrocytes (Castillo, Younts, Chávez, & Hashimotodani, 2012; S. Zou & Kumar, 2018). On the other hand, CB2Rs are generally expressed in the immune cells and peripheral tissues, including the GI tract, cardiovascular system, bone, reproductive system and to a lower extent in the brain tissue (Gong *et al.*, 2006; Zou & Kumar, 2018). Endogenous and exogenous cannabinoids might act on cannabinoid receptors to exert antioxidant, anti-inflammatory, and neuroprotective effects in the central nervous system (Braun *et al.*, 2018; Jia *et al*., 2014; Sahu *et al*., 2019). Activation of CBRs by cannabinoid agonists results in anti-inflammatory effects through attenuation of pro-inflammatory cytokines and elevation of anti-inflammatory cytokines levels in LPS-stimulated microglial cells (Haspula & Clark, 2020; Malek, Popiolek-Barczyk, Mika, Przewlocka, & Starowicz, 2015). CB2R agonists were also documented to effectively mitigate microglial activation after being triggered by neuroinflammatory stimuli (Ma *et al*., 2015), restrict the inflammatory reactions by lowering the expression of the tight junction protein in microvascular endothelial cells at BBB (Ramirez *et al*., 2012), and stimulate Aβ clearance (Wu *et al*., 2013a). Recently, a novel CB2R agonist, NTRX-07, is under Phase 1 trials to be used in AD treatment because of its ability to relieve neuroinflammatory conditions (Naguib & Giordano, 2019). NTRX-07 has been shown to reduce the pro-inflammatory cytokines by inhibiting the NF-κB signalling pathway.

### 3.1.3 Arvanil

Arvanil [N-(3-methoxy-4-hydroxy-benzyl)-arachidonoylamide] is a hybrid metabolically stable molecule between capsaicin and anandamide (Figure ***3-1***). This hybridisation qualified it as a ligand for CB1/TRPV1 receptors (Di Marzo *et al.*, 2002). Hence, arvanil is named from the arachidonoyl moiety of anandamide and vanillyl molecule of capsaicin. Arvanil appears to be a strong agonist for CB1

receptors and has the ability to activate TRPV1 receptors (Melck *et al*., 1999). In addition, arvanil acts as a potent inhibitor for anandamide membrane transporter (AMT) (Di Marzo *et al.*, 2002) and for the enzyme which is responsible for hydrolysing AEA and other endocannabinoid ligands, FAAH (Glaser *et al*., 2003).

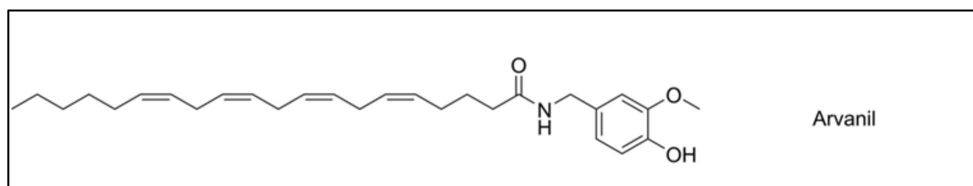



**Figure 3-1 Chemical structure of arvanil.**

**(Schubert *et al*., 2019).**

Arvanil exhibited various biological activities such as anticonvulsant (Tutka *et al.*, 2018), as well as anti-tumour by activation of caspase-8, which, in turn, induces apoptosis (Sancho, Calzado, Di Marzo, Appendino, & Muñoz, 2003). Interestingly, arvanil has been demonstrated to have some neuroprotective activity against ouabain-induced rats' excitotoxicity through both CB1 and VR1 receptors (Veldhuis *et al*., 2003).

A study conducted by Marquez N. *et al*. (2006) showed that arvanil inhibits T-cell activation by affecting calcium mobilisation, IL-2 gene transcription and blocking TNF-induced NF-κB activation through inhibition of NF-κB binding to DNA, IκBα degradation and NF-κB-dependent transcription (Márquez *et al*., 2006). The neuroprotective activity of arvanil has not been investigated on brain cells. Therefore, this chapter presents the ability of arvanil to modulate the neuroinflammatory condition and examines its neuroprotective effects.

## 3.2 Methodology

### 3.2.1 Preparation of compounds and stimulants

Arvanil and SB366791 were purchased from Tocris Bioscience, Rimonabant hydrochloride (SR-141716A) and $H_2O_2$ were purchased from Sigma Aldrich, SR144528 was purchased from Abcam, lipopolysaccharide - LPS derived from Salmonella typhimurium, S-form (was purchased from Innaxon

Biosciences), while Dimethyl Sulfoxide DMSO and DNase, RNase free sterile water from Fisher Scientific. All compounds were prepared in DMSO at stock concentrations of 0.1 M, aliquoted and stored in a -80 °C freezer. Prior to each experiment, a working concentration was prepared from the stock aliquots by dilution with DMSO to achieve the desired concentrations. For BV2 stimulation, 100 ng/ml LPS was prepared freshly prior to each experiment from the stock concentration (1mg/ml) by dilution with sterile PBS.

### 3.2.2 Treatment of BV2 cells with arvanil

BV2 cells were seeded at a concentration of $4 \times 10^4$ cells/ml in 96-well, 24-well and 6-well plates in 200 µl for 96-well, 1 ml for 24-well and 2 ml for 6-well plate of complete RPMI. When the cells were 80% confluent, the medium was changed to a serum-free RPMI for one hour. Afterwards, the cells were pre-incubated with different concentrations of arvanil for 30 minutes, followed by stimulation with 100 ng/ml LPS and incubated for a specific duration.

### 3.2.3 Treatment of BV2 cells with receptors antagonists

BV2 cells were seeded at a concentration of $4 \times 10^4$ cells/ml in a 24-well plate with 1 ml of complete RPMI. When the cells were 80% confluent, the medium was changed to a serum-free RPMI for one hour. Subsequently, the cells were pre-treated with different concentrations of SB366791 (TRPV1 antagonist), SR-141716A (CB1 receptor antagonist) and SR144528 ($CB_2$ receptor inverse agonist) for 30 minutes, followed by treatment with 0.5 µM of arvanil for 30 minutes and then stimulated with 100 ng/ml LPS for 24-hr.

### 3.2.4 Treatment of SH-SY5Y cells with arvanil

SH-SY5Y cells were seeded in at concentration of $4 \times 10^4$ cells/ml in 96-well, 24-well and 6-well plates in 200 µl for 96-well, 1 ml for 24-well and 2 ml for 6-well plate and differentiated as indicated in 2.1.2. DNase, RNase free sterile water was used to prepare 30, 50, 100, 200 µM $H_2O_2$ from stock concentration (9.8 M) which was used to induce SH-SY5Y cytotoxicity. After seven days of

differentiation, the cells were treated with different concentrations of arvanil for 30 minutes, followed by exposure to $H_2O_2$ and the plates were incubated in the incubator for 24 hours.

### 3.2.5 Assessment of cell viability

XTT assay and Non-Radioactive CytoTox 96® Assay were performed as described in 2.2.1 and 2.2.2, respectively.

### 3.2.6 Griess assay

BV2 cells were seeded in a 96-well plate at $4 \times 10^4$ cells/ml in 200 µl of culture medium in each well and kept in 37°C incubator until confluence. Later, the cells were treated with arvanil and stimulated with 100ng/ml of LPS, as indicated in 3.2.2. After 24-hours of incubation, the nitric oxide level was measured by Griess assay as described in 2.3.

### 3.2.7 ELISAs

BV2 cells were cultured in a 24-well plate at $4 \times 10^4$ cells/ml and incubated at 37°C until 80% confluence. Afterward, the culture medium was changed to serum-free RPMI medium for 1 hour, then treated with arvanil and stimulated with LPS (100ng/ml) as indicated in 3.2.2 for 24 hours.

In addition, BV2 cells were treated with receptor antagonists and arvanil and stimulated with LPS, as described in section 3.2.3. Subsequently, the TNF-α and IL-6 levels were measured as detailed in section 2.4.

### 3.2.8 $PGE_2$ level

BV2 microglia cells were seeded in a 24-well plate at a concentration of $4 \times 10^4$ cells/ml and incubated at 37°C. At 80% confluence, the cells were treated with arvanil and stimulated with LPS (100ng/ml) as indicated in 3.2.2 for 24 hours. The concentrations of $PGE_2$ were measured as described in section 2.5.

### 3.2.9 Western blotting

BV2 cells were treated and stimulated as described in 3.2.2, while SH-SY5Y cells were treated as indicated in section 3.2.4. Isolations of cytoplasmic and nuclear lysates and protein quantification

were performed as described in sections 2.6, 2.7 and 2.8, respectively. Then western blot analysis was performed as detailed in chapter two, section 2.10.

### 3.2.10 Reporter gene assay

BV2 cells were seeded into a 24-well plate at a concentration of $4 \times 10^4$ cells/ml and incubated at 37°C. At 80% confluence, the cells were transfected, as indicated in section 2.12. The cells were then treated with arvanil and stimulated with 100 ng/ml LPS, and luminescence was measured as detailed in section 2.12 using Dual-Glo Luciferase assay (Promega).

### 3.2.11 Detection of phosphorylated NF-κB p65

BV2 cells were seeded in a 6-well plate at a concentration of $4 \times 10^4$ cells/ml in 2 ml of culture medium and incubated at 37°C. At 80% confluence, cells were treated with the arvanil and stimulated with LPS (100ng/ml) as detailed in 3.2.2 for 60 minutes. NF-κB p65 (Phospho) InstantOne ELISA Kit (Invitrogen) was used as described in chapter two, section 2.9.

### 3.2.12 Immunofluorescence

BV2 cells were seeded at a concentration of $4 \times 10^4$ cells/ml into a 24-well plate and incubated at 37°C. At 80% confluence, cells were treated with arvanil and stimulated with 100 ng/ml LPS, as detailed in section 3.2.2.

SH-SY5Y cells were seeded into a 24-well plate at a concentration of $4 \times 10^4$ cells/ml. At 60% confluence, the medium was changed to the complete medium and 10µM retinoic acid (to initiate differentiation). Seven days after differentiation was initiated, the cells were treated as indicated in section 3.2.4. An immunofluorescence assay was performed, as detailed in chapter two, section 2.13.

### 3.2.13 ROS immunofluorescence

BV2 cells were seeded at a concentration of $4 \times 10^4$ cells/ml into a 24-well plate and incubated at 37°C until confluence. Later, cells were treated with arvanil and stimulated with 100ng/ml of LPS, as

indicated in section 3.2.2, for 24 hours. DCFDA Cellular ROS Detection Assay (Abcam) was used to detect ROS and the experiment has been described in chapter two section 2.14.

### 3.2.14 NF-κB p65 transcription factor assay

BV2 cells were seeded at a concentration of $4 \times 10^4$ cells/ml. At 80% confluence, the cells were treated with 0.1, 0.25 and 0.5 µM arvanil and stimulated for 60 minutes with 100ng/ml LPS. Thereafter, nuclear extracts were collected, and the protein was quantified (as detailed in sections 2.7 and 2.8, respectively). NF-κB p65 transcription factor assay kit (Abcam) was used to evaluate the DNA binding activity of NF-κB p65, as detailed in chapter two section 2.11.

### 3.2.15 Statistical analysis

Data were converted into % values of stimulant or negative control, then expressed as mean ±SEM and analysed by Graph Pad Prism statistical software version 7 (San Diego, USA) with one-way analysis of variance (one-way ANOVA) followed by Dunnett's Multiple Comparison Test (version 5.0, Prism software, Graph Pad, USA). Three separate experiments were done for each analysis (n=3). Values of $p<0.05$ were taken as being statistically significant.

## 3.3 Results

### 3.3.1 Concentrations of arvanil used did not affect the BV2 microglia viability

To evaluate the effects of various concentrations of Arvanil (0.1, 0.25, 0.5, 1, 2 µM) on the viability of BV2 microglia, XTT and non-radioactive cytotoxicity assays were used. Mitochondrial function (XTT reduction) and membrane damage (LDH leakage) were used as cytotoxicity endpoints and are frequently used to determine the effective concentrations of novel compounds that do not affect cell viability. Culture cells without compounds were taken as the negative control.

BV2 cells treated with 0.1, 0.25 and 0.5 µM arvanil alone and with LPS stimulation showed a non-significant change in viability compared with negative control. On the contrary, when the concentration was increased to 1 µM, arvanil caused a significant ($p< 0.01$) reduction (~1.6-fold reduction) in cell viability in LPS-stimulated cells compared to untreated cells. The same was noticed when the cells were treated with 2 µM arvanil; with and without LPS; BV2 microglia viability was significantly decreased ($p< 0.001$) (~2.3-fold reduction) and ($p< 0.01$) (~1.6-fold decrease) when compared to control negative cells in absence and presence of LPS, respectively (Figure ***3-2***).

LDH levels were not significantly changed when BV2 cells were pre-treated with 0.1, 0.25 and 0.5 µM arvanil and stimulated with 100 ng/ml LPS compared to LPS-stimulated cells. However, pre-treatment with 1 µM and 2 µM of arvanil showed a significant increase (1µM; ~3.9-fold increase and 2µM; ~7.2-fold increase) in LDH release.

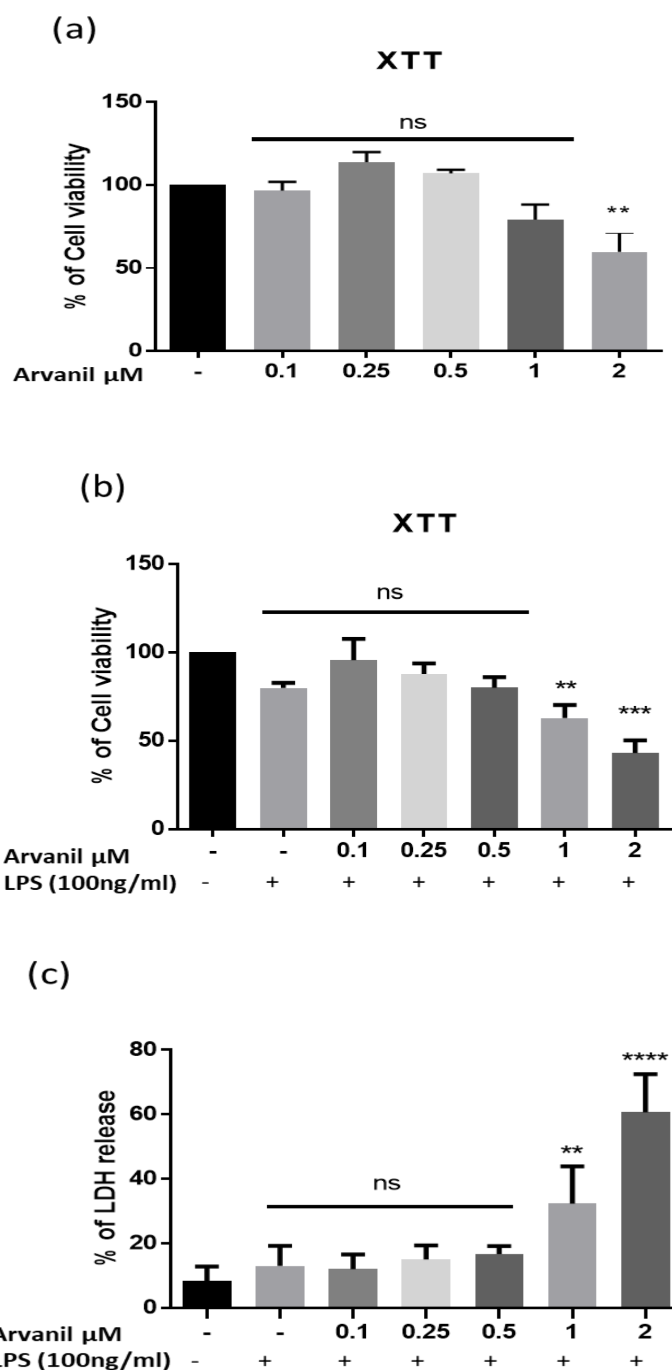



**Figure 3-2 Microglia viability did not change when pre-treated with 0.1, 0.25 and 0.5 µM arvanil with or without stimulation with LPS by using XTT and cytotoxicity assays.**

(a) BV2 microglia incubated with various concentrations of arvanil for 24hr and then 25 µl of XTT/PMS was added to stop the experiments. (b) XTT assay was performed on BV2 microglia cells that were pre-treated with different concentrations of arvanil for 30 min and stimulated with 100ng/ml of LPS for 24 hr. (c) cytotoxicity assay was used to reveal the level of LDH released from damaged BV2 cells when pre-treated with various arvanil concentrations for 30 min followed by stimulation with LPS for 9 hr. Data for three independent experiments were presented as mean ± SEM and were analysed using one-way ANOVA followed by Dunnett's Multiple Comparison Test. *p<0.05, **p<0.01, *** p< 0.001 and **** p< 0.0001 versus untreated control cells for XTT assay, whereas * p< 0.05, **p<0.01 and *** p< 0.001 versus LPS-stimulated BV2 cells for non-radioactive cytotoxicity assay.

### 3.3.2 Pre-treatment of bv2 microglia with arvanil resulted in suppression of LPS-induced production of pro-inflammatory cytokines

Activated microglial is considered a significant source of cytokines molecules in the neuroinflammatory condition (Perry, Nicoll, & Holmes, 2010). When activated by LPS, BV2 microglia produce pro-inflammatory cytokines such as IL-6 and TNF-α (Horváth *et al*., 2021; J. Yan, Du, Qin, & Gao, 2020). ELISA technique was used to determine the potential effect of arvanil on the production of pro-inflammatory cytokines (TNF-α and IL-6) in the supernatant. BV2 cells were treated and stimulated as indicated in 3.2.2. LPS-stimulated cells showed a significant elevation ($p < 0.0001$) in TNF-α when compared with untreated cells. Interestingly, arvanil, 0.1, 0.25 and 0.5 µM, treatment prior to 100ng/ml LPS stimulation produced a significant suppression (0.1; ~1.6-fold, 0.25; ~1.9-fold and 0.5; ~2.6-fold reduction) in TNF-α level when compared with the cells that stimulated by 100ng/ml of LPS (Figure ***3-3***). Regarding IL-6, LPS-stimulated cells showed a significant elevation ($p < 0.0001$) in IL-6 level when compared with untreated cells. As expected, arvanil (0.1, 0.25 and 0.5 µM) produced significant ($p < 0.0001$) inhibitory effect (0.1; ~1.4-fold, 0.25; ~1.8-fold and 0.5; ~2.4-fold reduction) on IL-6 production in LPS-stimulated microglia.

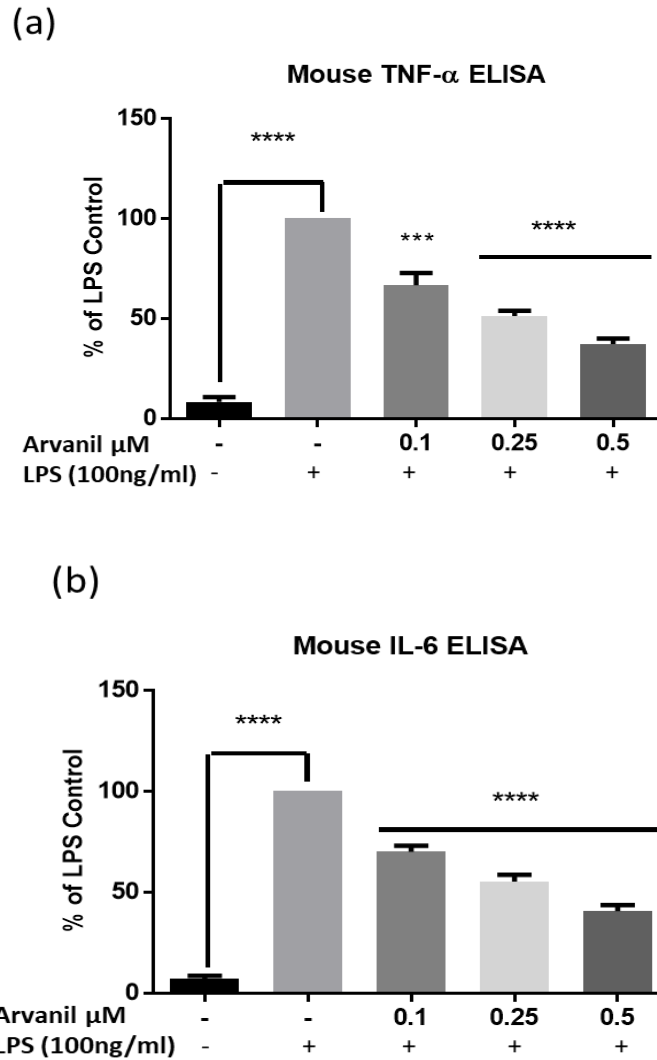



**Figure 3-3 Arvanil reduced the level of TNF-α and IL-6 in LPS- stimulated BV2 microglia.**

ELISA assay was used to determine TNF-α and IL-6 levels in the supernatant after treatment with 0.1, 0.25 and 0.5 µM arvanil for 30 min and then stimulated with LPS for 24 hr. (a) Cells were incubated with 0.1, 0.25 and 0.5 µM arvanil exhibited a dramatic reduction in TNF-α level. (b) arvanil pre-treatment prevented the production of IL-6 caused by LPS. Data for three independent experiments were presented as mean ± SEM and were analysed using one-way ANOVA followed by Dunnett's Multiple Comparison Test. *p<0.05, **p<0.01, *** p< 0.001 and **** p< 0.0001 compared to LPS-stimulated BV2 cells.

### 3.3.3 Arvanil inhibited iNOS protein expression and reduced increased NO production in LPS-stimulated BV2 microglia

The study examined the effect of arvanil on iNOS expression and NO production in activated BV2 cells. The level of NO-mediated by iNOS expression was reported to be increased during BV2 microglia activation by LPS and cause cellular damage (Sierra *et al*., 2014), while inhibition of their expression proved to reduce their cytotoxic effects in CNS (Streyczek, Apweiler, Sun, & Fiebich, 2022). The Griess assay was used to determine the level of NO in cell supernatants following treatment of BV2 cells with 0.1, 0.25 and 0.5 µM of arvanil for 30 minutes followed by stimulation with 100ng/ml of LPS for 24 hr as indicated in 3.2.2. Results show that compared to the unstimulated cells, nitrite production was significantly elevated ($p<0.001$) in BV2 cells stimulated with 100ng/ml LPS (~4.3-fold increase). Nitrite production was significantly decreased in the cells that were treated with 0.25 µM ($p<0.01$) (~2.2-fold reduction) and 0.5 µM ($p<0.001$) (~2.4-fold reduction) of arvanil and stimulated with 100 ng/ml LPS compared with LPS-stimulated cells, while treatment with 0.1 µM arvanil has not revealed a significant suppression effect on nitrite level (Figure ***3-4***a).

As nitrite level is proportion directly with iNOS protein expression following an inflammatory stimulus, the cytoplasmic extractions were collected after 24 hr of BV2 cells stimulation by 100 ng/ml LPS and pre-treated with arvanil. The western blot was used, and the results revealed that iNOS expression was significantly ($p<0.0001$) suppressed (0.25 µM; ~4.5-fold, 0.5 µM; ~9.5-fold reduction) when the cells were treated with 0.25 and 0.5 µM of arvanil followed by stimulation with LPS. Pre-treatment with 0.1µM arvanil caused a ~2.1-fold reduction of iNOS expression level (Figure ***3-4***b).

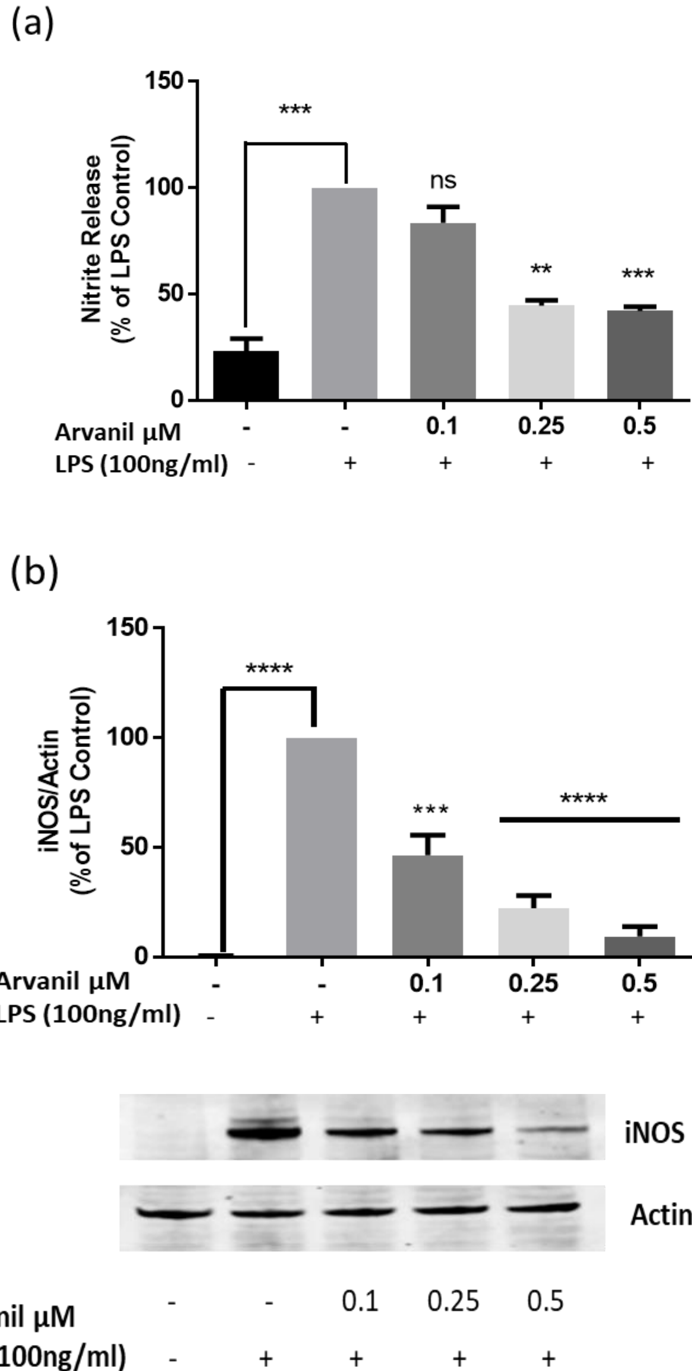



**Figure 3-4 Arvanil reduced Nitrite level and iNOS protein expression in LPS-induced BV2 microglia.**

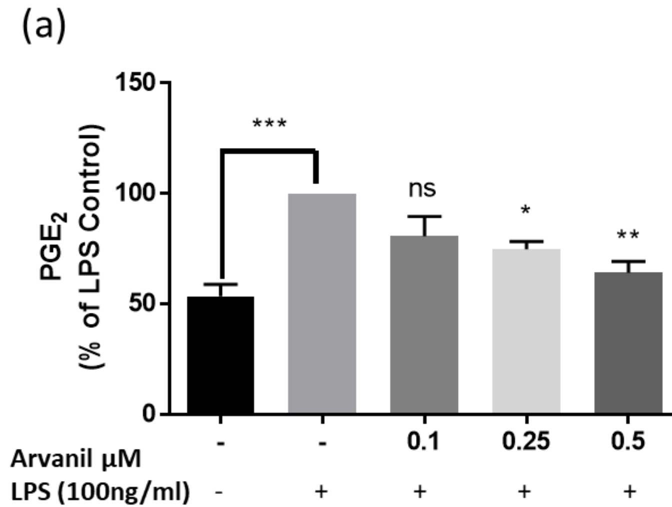
BV2 cells were pre-treated with 0.1,025, 0.5 µM arvanil for 30 min and stimulated with 100ng/ml LPS for 24 hr. (a) The analysis of nitrite level in the supernatant showed a marked decreased when BV2 cells were treated with 0.25 and 0.5 µM arvanil. (b) Arvanil significantly inhibited the expression of iNOS in the activated BV2 cells by using the cytoplasmic extraction in the western blot analysis. Actin was used as an internal control. Data for three independent experiments were presented as mean ± SEM and were analysed using one-way ANOVA followed by Dunnett's Multiple Comparison Test. *p<0.05, **p<0.01, *** p< 0.001 and **** p< 0.0001 compared to LPS-stimulated BV2 cells.

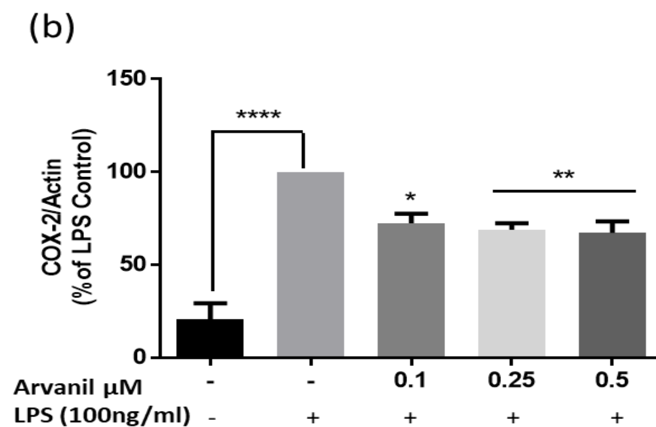
### 3.3.4 Arvanil inhibited $PGE_2$ production and COX-2 protein expression in in LPS-activated BV2 microglia

The research has evaluated arvanil impact on COX2- mediated $PGE_2$ production. Glial cells, when activated, increase the expression of COX-2 and its products; $PGE_2$, and in turn exacerbate neuroinflammation and neurotoxicity (Guan & Wang, 2019; Wang *et al*., 2014).

BV2 cells were treated and stimulated, as indicated in section 3.2.2. at the end of the experiment, LPS-stimulated BV2 cells showed an increase in $PGE_2$ production (~1.9-fold increase) compared with unstimulated cells. $PGE_2$ levels were dramatically decreased in LPS-stimulated BV2 cells when treated with 0.25 µM (~1.3-fold reduction) and 0.5 µM (~1.5-fold reduction) of arvanil. However, treatment with 0.1 µM did not change the $PGE_2$ level significantly (Figure ***3-5***a).

As COX-2 enzyme is responsible for generating $PGE_2$, COX-2 was further assessed, and the results showed that activated BV2 microglia expressed a high level of COX-2 (~4.8-fold increase) compared with non-stimulated cells. However, arvanil produced a significant inhibitory effect on COX-2 protein expression from LPS-activated BV2 cells at concentrations of 0.1 µM (~1.4-fold reduction), 0.25 µM (~1.44-fold reduction) and 0.5 µM (~1.5-fold reduction) compared to stimulated cells (Figure ***3-5***b).



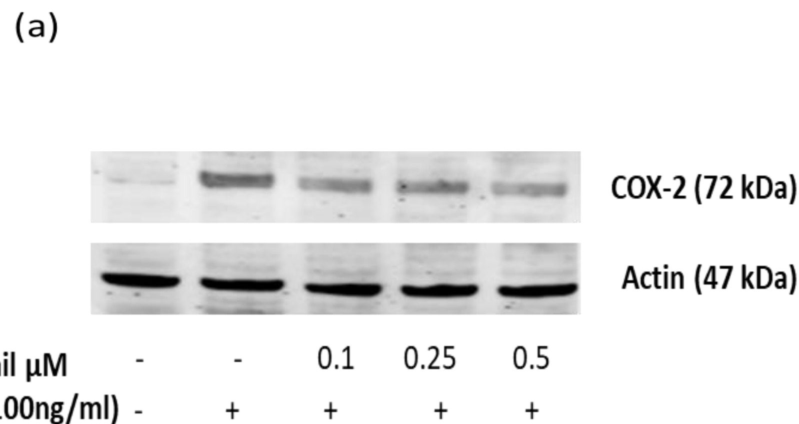




**Figure 3-5 Arvanil reduced $PGE_2$ level and COX-2 protein expression in LPS-induced BV2 microglia.**

BV2 cells were treated with 0.1, 0.25 and 0.5 µM arvanil for 30 min and stimulated for 24hr with 100ng/ml LPS. The supernatant and cytoplasmic extractions were collected to obtain $PGE_2$ level and COX-2 expression with western blot, respectively. (a) arvanil could significantly decrease $PGE_2$ production in LPS-activated BV2 cells. (b) the inhibitory effect of arvanil on COX-2 was observed when the cells were incubated with the compound and then stimulated with LPS. Actin was used as a loading control. Data for three independent experiments were presented as mean ± SEM and were analysed using one-way ANOVA followed by Dunnett's Multiple Comparison Test. *$p<0.05$, **$p<0.01$, *** $p< 0.001$ and **** $p< 0.0001$ compared to LPS-stimulated BV2 cells.

### 3.3.5 Arvanil did not attenuate ROS production in LPS- stimulated BV2 microglia cells

Under physiological conditions, ROS represents an intracellular messenger involved in several cellular processes, such as differentiation and cellular growth (Murphy *et al.*, 2011; Jixiang Zhang *et al*., 2016). Upon stimulation, microglia are activated and produce pro-inflammatory cytokines, as well as ROS which aggravates neuroinflammation condition and activates the inflammatory pathways (Block *et al.*, 2007; Simpson & Oliver, 2020). To evaluate the effects of arvanil on LPS- induced ROS production in BV2 microglia, the cells were pre-treated with 0.1, 0.25 and 0.5 µM arvanil and stimulated with LPS for 24hr, as indicated in section 3.2.2. The DCFDA-fluorescent experiment was performed using fluorescence microscopy to visualize the ROS generation. The images showed that LPS markedly generated ROS when compared with untreated BV2 cells. Pre-treatment with arvanil had no inhibitory effects on ROS levels in LPS-challenged BV2 cells (Figure ***3-6***).

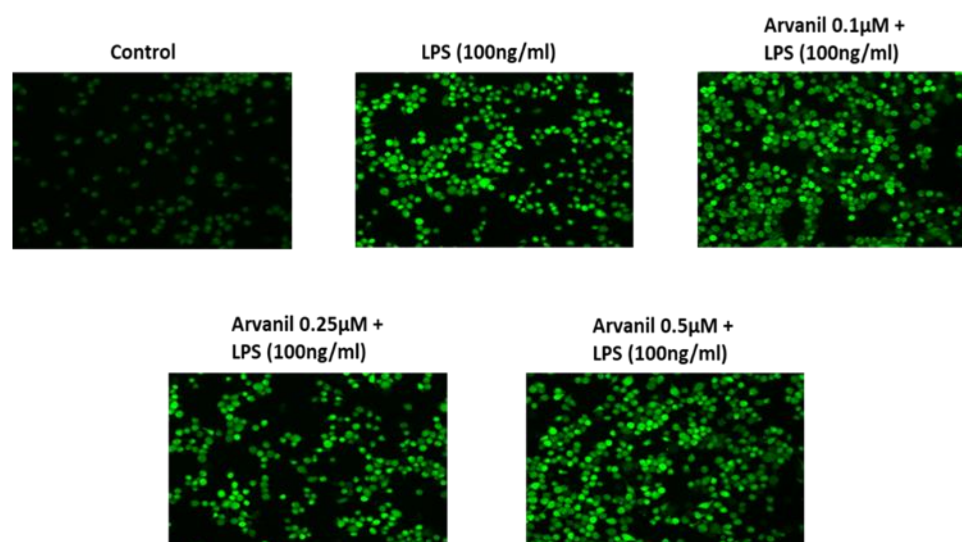



**Figure 3-6 The generations of reactive oxygen species were not attenuated by pre-treatment of LPS-stimulated microglia with arvanil.**

BV2 microglia were treated with 0.1, 0.25 and 0.5 µM arvanil for 30min before stimulation with 100 ng/ml of LPS for 24hr. ROS production in BV2 microglia was measured by using a DCFDA-assay kit. The cells were stained by adding 500µl/well of 20µM DCFDA solution and incubated at 37°C for 45 minutes in the dark, and the fluorescent were read with EVOS® FLoid® Cell Imaging System. Pre-treatment with arvanil could not inhibit ROS generation in LPS-stimulated cells. Images were processed by image J analysis software, and the scale bar was 100 µm.

### 3.3.6 The effect of arvanil on NF-κB signalling pathway in LPS-activated BV2 microglia

NF-κB is a key transcriptional factor responsible for the generation of pro-inflammatory mediators. When microglia cells are stimulated, NF-κB is released from IκBα via phosphorylation and degradation of IκBα (Karin & Delhase, 2000). The ability to inhibit the NF-κB activation was evaluated to explore the potential molecular mechanisms for the anti-inflammatory effects of arvanil.

Firstly, the time course of NF-κB activation was determined by activating BV2 cells with 100ng/ml LPS for 15, 30, 45, 60 and 90 minutes. Cell lysates were analysed with NF-κB p65 (phospho) ELISA, and the results showed that the maximum p-p65 NF-κB expression was observed at 60 minutes compared with unstimulated cells (Figure ***3-7***a). Based on these results, BV2 cells were stimulated with LPS for 60 minutes in the absence or presence of different concentrations of arvanil, and cell lysates were analysed. As depicted in Figure ***3-8***a, phosphorylated p65 was significantly increased in LPS-stimulated cells (~2.6-fold increase) compared with unstimulated cells. However, pre-incubation with arvanil at 0.1, 0.25 and 0.5 µM did not affect protein levels of phosphorylated p65 in stimulated BV2 microglia. To confirm these findings, immunofluorescence experiments were carried out and Figure ***3-8***b showed a high expression of p-p65 in the LPS-challenged BV2 cells compared to unstimulated cells. Nevertheless, pre-treated cells with arvanil did not inhibit p-p65 expression in the stimulated cells.

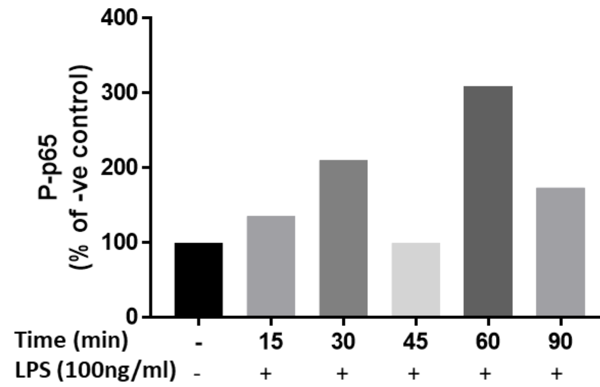
For further investigation, luciferase reporter gene assay and ELISA-based enzyme-linked immunosorbent assay have been used to study the nuclear transactivation of NF-κB and NF-κB -DNA binding activity, respectively.

For reporter gene assay, transfection with a vector comprising NF-κB regulated luciferase reporter has been achieved and among 2, 4, 6, 8 hr time-course for 100ng/ml LPS stimulation, the 6-hr activation showed the maximum expression of NF-κB modulated luciferase reporter gene when compared to unstimulated transfected BV2 cells (Figure ***3-7***b). There was a significant increase in the expression of the NF-κB modulated luciferase reporter gene ($p<0.01$) in the transfected cells (~6.6-fold increase) that were stimulated with 100 ng/ml LPS when compared to unstimulated transfected BV2 cells,

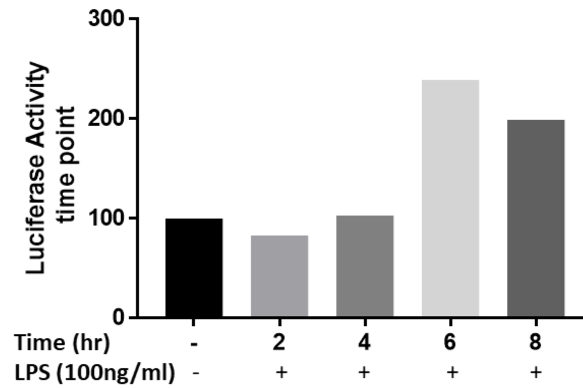
whereas pre-incubation of transfected cells with 0.1, 0.25 and 0.5 μM of arvanil followed by 6-hr stimulation with LPS declared a nonsignificant alteration when compared to LPS stimulated transfected cells (Figure ***3-9***a).

On the other hand, BV2 cells were treated with arvanil followed by stimulation with 100 ng/ml LPS for 60 min and then the nuclear extracts were used to study the NF-κB -DNA binding activity by enzyme-linked immunosorbent assay. The results demonstrated that LPS stimulation activated (~3-fold increase) the DNA binding of NF-κB significantly ($p<0.05$) compared to unstimulated BV2 cells, while this DNA binding activity was not altered significantly when BV2 cell pre-incubated with 0.1, 0.25 and 0.5 μM of arvanil (Figure ***3-9***b).

(a)




(b)




(c)

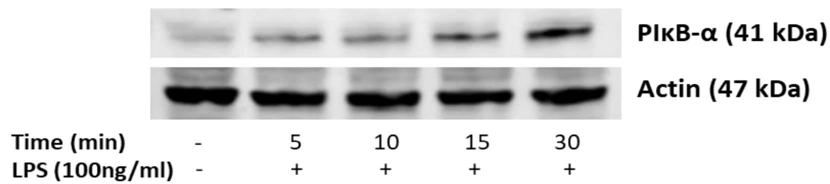
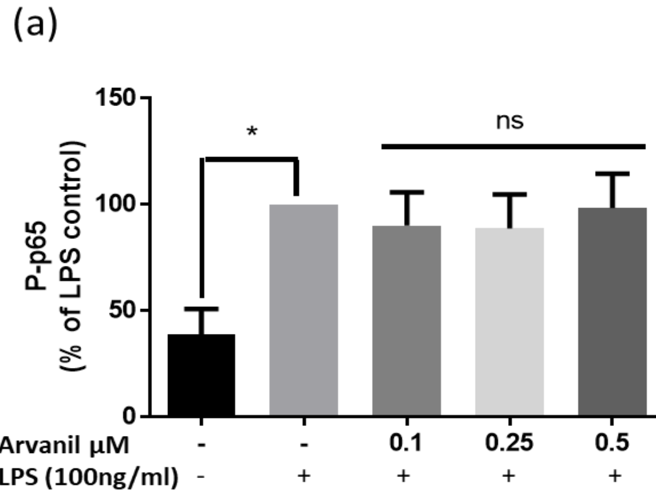



**Figure 3-7 Time-course experiments for LPS induced activation of NF-κB pathway in BV2 microglia.**

(a) BV2 microglia were stimulated with 100 ng/ml LPS for 15, 30, 45, 60 and 90 min and the maximum phosphorylated p65 was observed at 60 min in the cytoplasmic extractions by using p-p65 NF-κB ELISA assay. (b) NF-κB luciferase reporter transfected BV2 cells were challenged with 100 ng/ml LPS for 2, 4, 6 and 8 hr. The maximum expression of NF-κB modulated luciferase reporter gene was at 6-hr. (c) Western blot analysis for cytoplasmic extractions showed the time required for 100 ng/ml LPS to produce the highest level of phosphorylated IκBα was 30 min, actin used as a loading control.

(a)



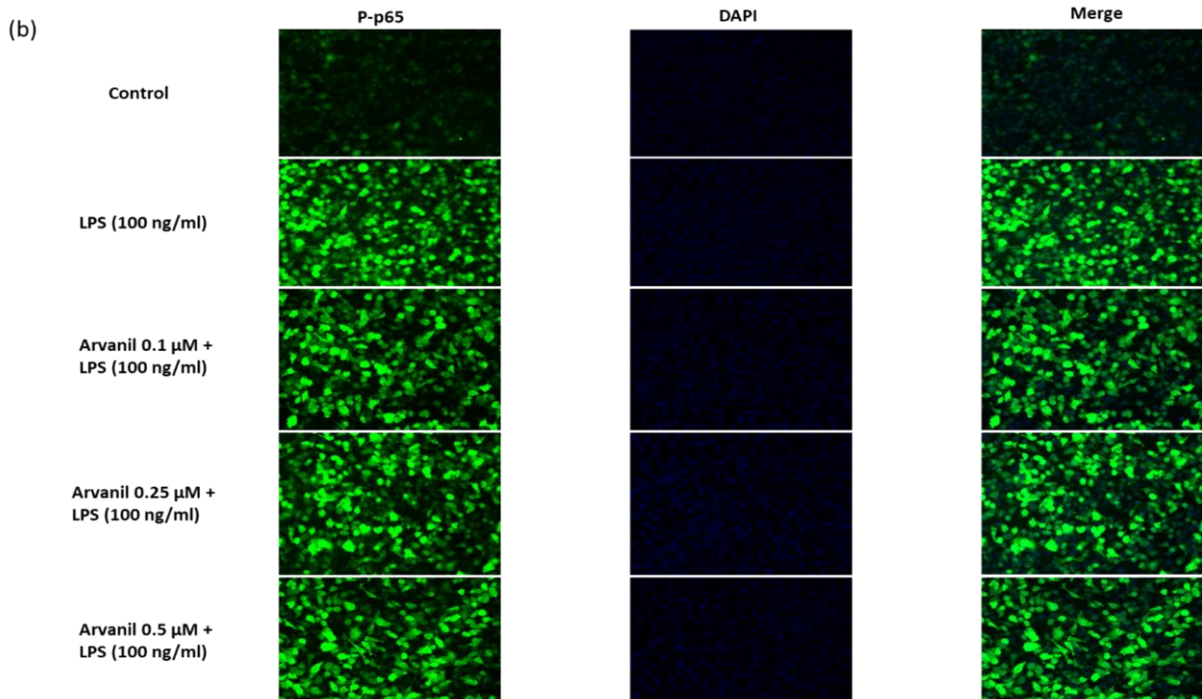




**Figure 3-8 Arvanil pre-treatment did not change LPS-induced expression of phosphorylated p65 in BV2 microglia.**

(a) Cytoplasmic extractions were collected after treatment of BV2 cells with arvanil and stimulation with LPS for 60 min. p-p65 NF-κB ELISA analysis revealed that arvanil pre-treatment did not affect the LPS-induced expression of p-p65. (b) BV2 cells were treated with arvanil and stimulated with LPS for 60 min; then p-p65 antibody was used to label the cells followed by incubation with DAPI. The fluorescence images confirmed that arvanil concentrations could not inhibit the expression of p-p65 induced by LPS. Images were processed by image J analysis software, and the scale bar=100 µm. Data for three independent experiments were presented as mean ± SEM and were analysed using one-way ANOVA followed by Dunnett's Multiple Comparison Test. *p<0.05, **p<0.01, *** p< 0.001 and **** p< 0.0001 compared to LPS-stimulated BV2 cells.

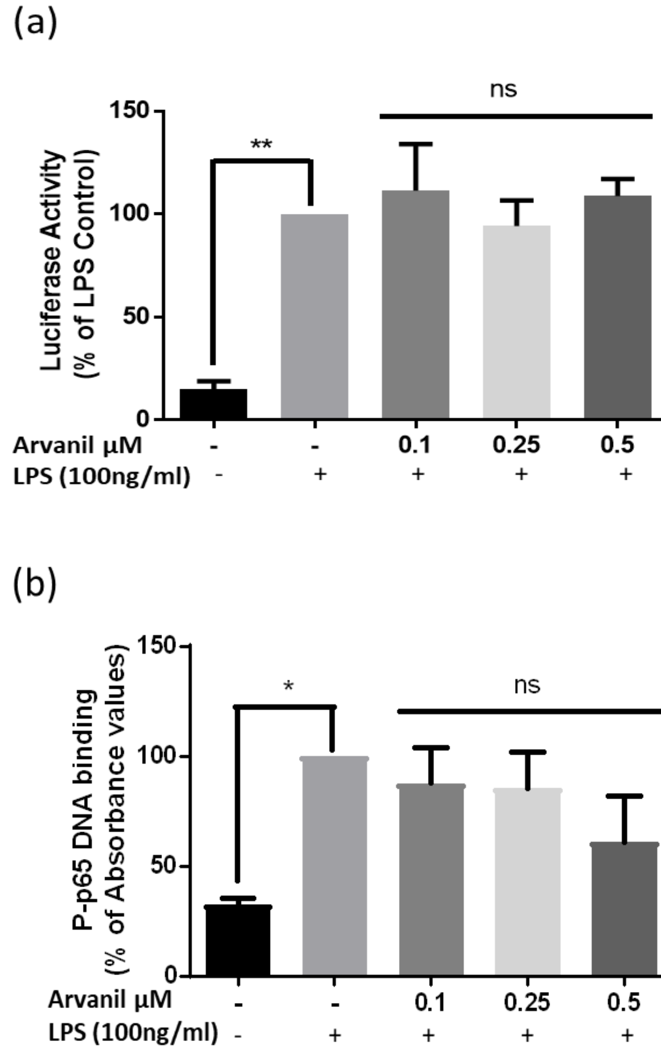



**Figure 3-9 Pre-treatment with arvanil did not affect NF-κB signalling pathway at nucleus level in the LPS-activated BV2 cells.**

(a) BV2 cells were transfected with NF-κB luciferase reporter, then treated with arvanil and stimulated with LPS. After 6-hr of stimulation, luciferase Dual Glo was used to read the luminescence reaction. Arvanil did not inhibit NF-κB activity induced by LPS in transfected BV2 cells. Renilla luciferase was used as an internal control. (b) The nuclear extractions from BV2 cells pre-treated with arvanil and stimulated with LPS for 60 min have been collected and subjected to ELISA-based enzyme-linked immunosorbent assay to study NF-κB -DNA binding activity. The LPS-induced DNA binding capacity of p65 was not decreased by arvanil in BV2 microglia. Data for three independent experiments were presented as mean ± SEM and were analysed using one-way ANOVA followed by Dunnett's Multiple Comparison Test. *p<0.05, **p<0.01, *** p< 0.001 and **** p< 0.0001 compared to LPS-stimulated BV2 cells.

### 3.3.7 Arvanil activity on IκBα level in LPS stimulated BV2 microglia

Generally, NF-κB is sequestered in the cellular cytoplasm as an inactive state by binding with IκBα. Once the cells are activated, IκBα is phosphorylated and degraded, leaving the NF-κB-p65 subunit to be activated and translocated to the nucleus. An experimental attempt was carried out to investigate whether arvanil pre-treatment impacts the phosphorylation and degradation of kappa B protein.

Firstly, a time-point western blotting experiment was done to determine the time the IκBα subunit was phosphorylated and degraded. The stimulated cells showed significant upregulation of p-IκBα expression (~1.9-fold increase) at 30 minutes compared to unstimulated BV2 cells (Figure ***3-7***c). However, this expression was not inhibited significantly when BV2 cells pre-incubated with 0.1, 0.25 and 0.5 µM of arvanil and stimulated with 100ng/ml LPS for 30 min (Figure ***3-10***a). Interestingly, unstimulated BV2 cells displayed a high level of total-IκBα protein (~2-fold increase) compared to LPS-stimulated microglia, while pre-incubation with 0.5 µM arvanil inhibited total-IκBα degradation significantly (~2.4-fold increase). However, pre-treatment with 0.1 and 0.25 µM did not inhibit total-IκBα degradation in the stimulated microglia (Figure ***3-10***b).

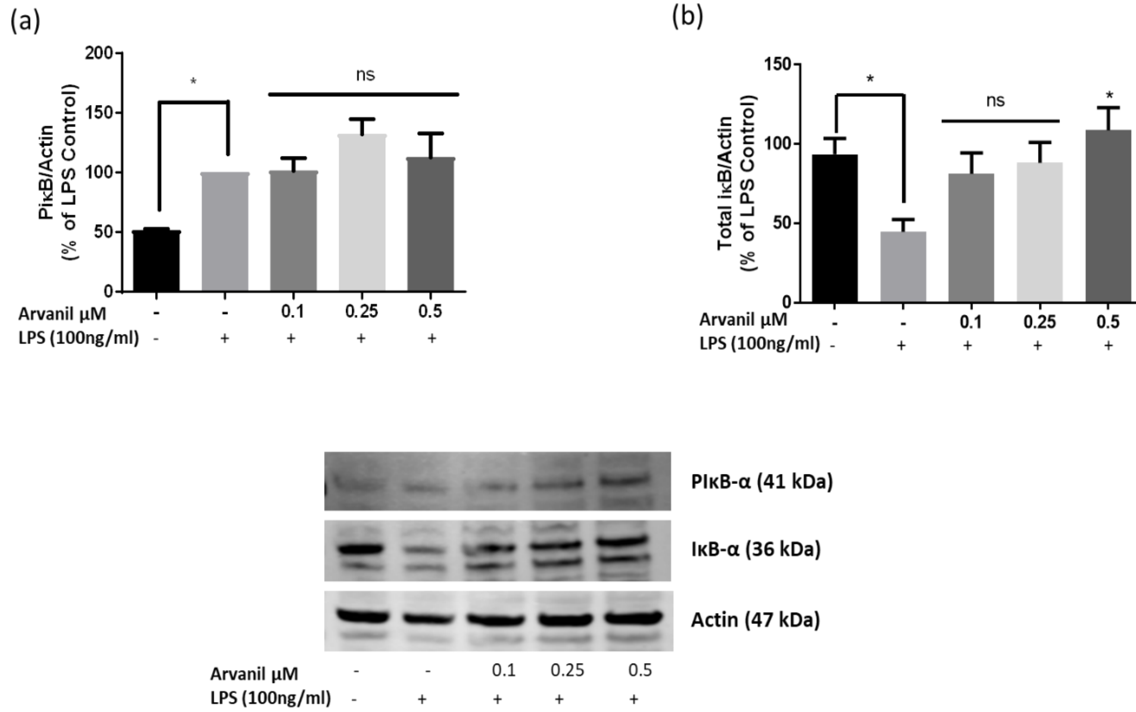



**Figure 3-10 Arvanil pre-treatment inhibited the degradation of IκBα in LPS stimulated BV2 microglia.**

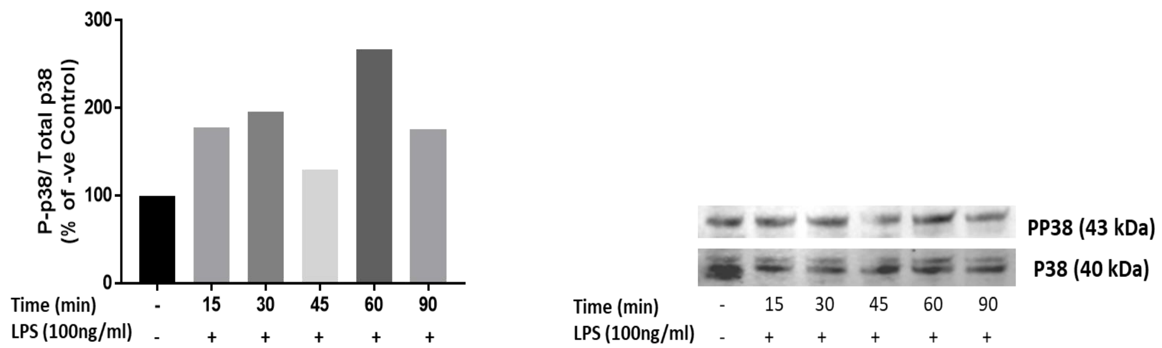
The microglia were pre-treated with different concentrations of arvanil for 30min and stimulated with 100ng/mL of LPS for 30 min, then the cytoplasmic extractions were collected and analysed by western blot. (a) Arvanil was not causing any change in the phosphorylated protein expression of IκBα induced by LPS. (b) pre-treatment with 0.5 µM arvanil inhibited IκBα degradation in the LPS-stimulated BV2 cells. Actin was used as a loading control. Data for three independent experiments were presented as mean ± SEM and were analysed using one-way ANOVA followed by Dunnett's Multiple Comparison Test. *p<0.05, **p<0.01, *** p< 0.001 and **** p< 0.0001 compared to LPS-stimulated BV2 cells.

### 3.3.8 The anti-inflammatory activity of arvanil is not mediated through MAPK signalling pathway in LPS-activated BV2 microglia

Research suggested that dysregulation in p38 MAP kinase and c-Jun N-terminal kinase (JNK) were associated with the pathology of neurodegenerative diseases (Kaminska, Gozdz, Zawadzka, Ellert-Miklaszewska, & Lipko, 2009; Yeung, Aziz, Guerrero-Castilla, & Arguelles, 2018)**.** Furthermore, the induction of the MAPK signalling pathway in the activated microglia promotes releasing of pro-inflammatory mediators such as IL-6, TNF-α, IL-1β and COX-2 which will aggravate the neuroinflammation condition (Ahmed *et al*., 2020; Espinosa-Oliva *et al*., 2011). Arvanil's pro-inflammatory activity was investigated to determine whether it is mediated by inhibiting the MAPKs signalling pathway.

In the beginning, time point experiments were performed to identify the time required for maximum activation of phospho p38 MAP kinase and phospho JNK MAP kinase. BV2 microglia were stimulated with 100ng/ml LPS for 15, 30, 45, 60 and 90 minutes; cell lysates were collected and analysed by western blot. The results revealed that the maximum phosphorylation of p38 and JNK was observed at 60 minutes post-stimulation (Figure ***3-11***). To investigate whether arvanil has an inhibitory effect on p-p38 protein level, BV2 cells were stimulated with LPS for 60 minutes, which caused an increase (~2.6-fold increase) in p38 phosphorylation ($p<0.01$) when compared to untreated cells, whereas pre-incubated with arvanil were insignificantly affect the p-p38 level in LPS- stimulated microglia (Figure ***3-12***a).

Additionally, the level of phosphorylated JNK has been studied in activated microglia by western blot to investigate whether the anti-inflammatory activity of arvanil is mediated through JNK MAP kinase signalling pathways. LPS-stimulated microglia demonstrated an upregulation (~2.3-fold increase) of p-JNK protein level ($p<0.05$) when compared to unstimulated cells. However, arvanil did not inhibit the increased p-JNK protein levels in the LPS-activated BV2 cells (Figure ***3-12***b).



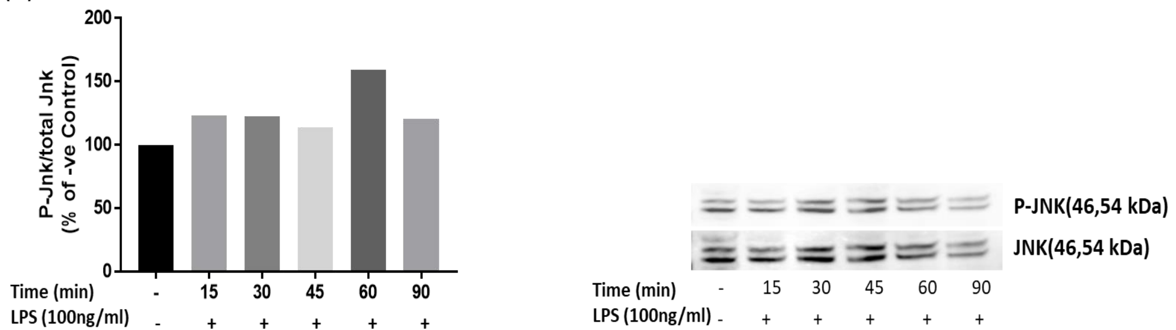




**Figure 3-11 Time-point experiments for LPS induced phosphorylation of p38 and JNK in BV2 microglia.**

BV2 cells were stimulated with 100 ng/ml of LPS for 15, 30, 45, 60 and 90 min; the cytoplasmic extractions were then collected and analysed by western blot. (a) the membrane was incubated with p-p38 overnight, and total p38 was used as an internal control. A high expression level of p-p38 was observed at 60 min when compared to untreated cells. (b) the membrane was incubated with p-JNK and total JNK used as an internal control; p-JNK expression was at the highest level when the cells were stimulated for 60 min. The protein levels were quantified by ImageJ analysis software.

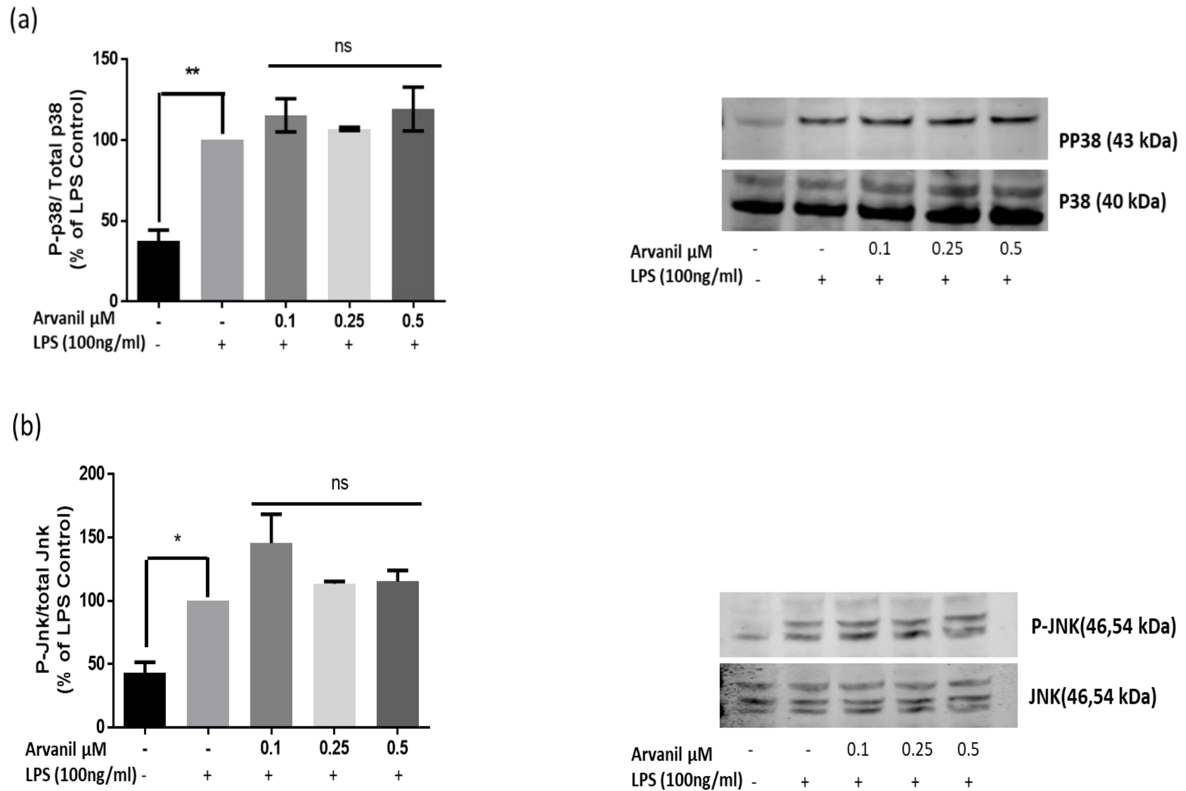



**Figure 3-12 Arvanil pre-treatment has an insignificant impact on p-p38 and p-JNK protein levels in LPS-stimulated microglia.**

BV2 microglia were pre-incubated with 0.1, 0.25 and 0.5 µM arvanil for 30 min, then stimulated with 100ng/ml LPS for 60 min. To stop the stimulation, cytoplasmic lysates were collected and processed by western blot analysis. The membranes were incubated with (a) p-p38 antibody and then with total-p38 as an internal control; the analysed results showed that the inhibition effect of arvanil on p-p38 protein expression was insignificant in LPS-induced neuroinflammation. (b) p-JNK incubation showed an insignificant change in its protein level when cells were pre-treated with arvanil and stimulated with LPS, total JNK was used as an internal control. Data for three independent experiments were presented as mean ± SEM and were analysed using one-way ANOVA followed by Dunnett's Multiple Comparison Test. *$p<0.05$, **$p<0.01$, *** $p< 0.001$ and **** $p< 0.0001$ compared to LPS-stimulated BV2 cells.

### 3.3.9 The anti-inflammatory activity of arvanil is not mediated through inhibition of Akt protein in LPS-activated BV2 microglia

Evidence suggested the involvement of PI3K/Akt signalling pathway in LPS-induced microglia and resulted in increase in the expression of pro-inflammatory mediators (Saponaro *et al*., 2012; Zhong, Qiu, Yu, Chen, & Yan, 2020; Zhou, Wang, Li, Li, & Geng, 2015). Akt could enhance the phosphorylation of the IKK enzyme complex, which, in turn, enhances the activation of the NF-κB signalling pathway that ends up with the production of the pro-inflammatory mediators. Further investigation on the possibility of involving Akt protein in arvanil-mediated protection against LPS-induced neuroinflammation was conducted.

Initially, a time point experiment was performed to identify the time required for maximum phosphorylation of Akt protein upon stimulation with LPS. 100 ng/ml LPS stimulated BV2 cells for 15, 30, 45, 60 and 90 min and the western blot analysis lysates suggested maximum phosphorylation of Akt protein occurred at 60 minutes post-stimulation (Figure ***3-13***a). However, LPS-activated BV2 cells showed a significant increase (~1.6-fold increase) in p-Akt protein expression ($p < 0.05$) when compared to unstimulated cells. Interestingly, the inhibition effect of 0.1, 0.25 and 0.5 µM arvanil on Akt phosphorylation level was nonsignificant in LPS-activated BV2 cells (Figure ***3-13***b).

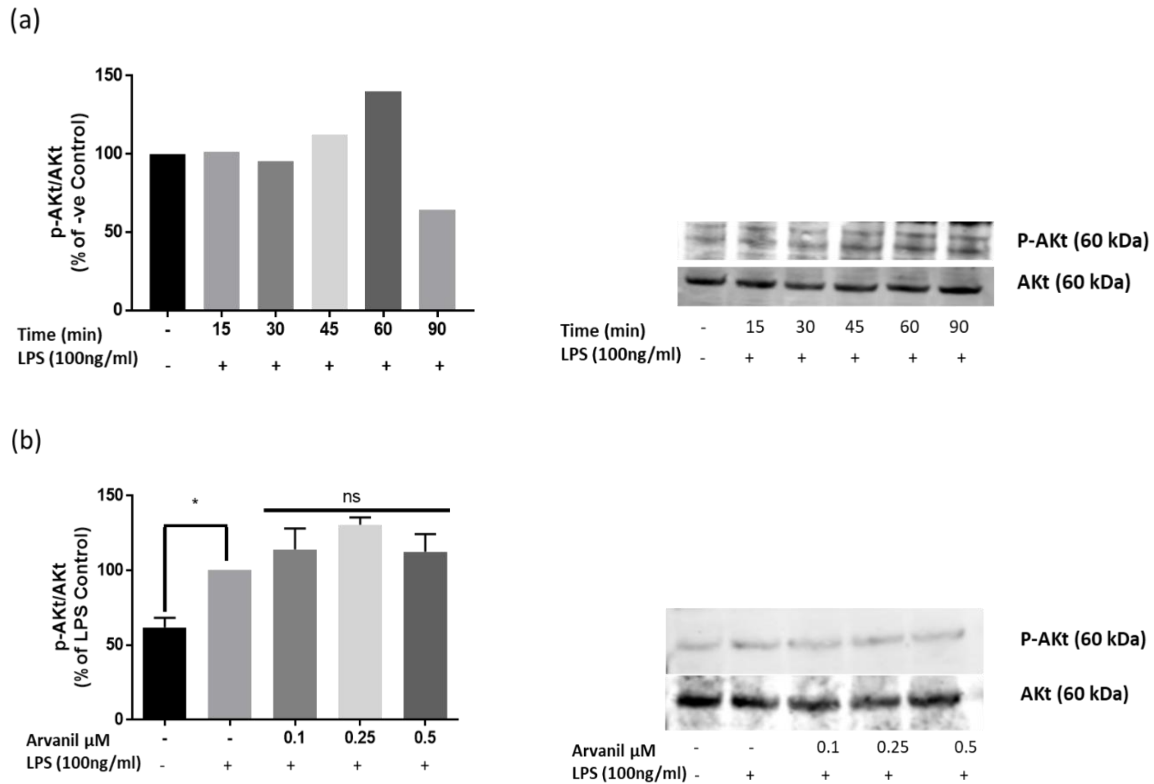



**Figure 3-13 Arvanil pre-treatment had no inhibitory effect on phosphorylation level of Akt protein in LPS-stimulated microglia.**

(a) BV2 cells were stimulated with 100 ng/ml LPS for 15, 30, 45, 60 and 90 min, and then the cytoplasmic extraction was analysed by western blot. The maximum phosphorylation of Akt protein occurred at 60 min. (b) cells were pre-incubated with 0.1, 0.25 and 0.5 µM for 30 min and stimulated with LPS for 60 min. The inhibition activity of arvanil on p-Akt was nonsignificant in LPS-stimulated cells. Total Akt was used as an internal control. Data for three independent experiments were presented as mean ± SEM and were analysed using one-way ANOVA followed by Dunnett's Multiple Comparison Test. *p<0.05, **p<0.01, *** p< 0.001 and **** p< 0.0001 compared to LPS-stimulated BV2 cells.

### 3.3.10 BV2 microglia expressed cannabinoid receptor 1 and cannabinoid receptor 2

BV2 microglia, like other body cells, express surface receptors that interact with signalling substances to exert physiological or pathological actions. Generally, microglia express TLRs, which normally mediate various non-immune responses such as neurogenesis and brain development (Trotta, Porro, Calvello, & Panaro, 2014). Arvanil is a cannabinoid CB1 agonist. In order to investigate whether it exerts its anti-inflammatory activities through binding to CB1R or CB2R, BV2 microglia were examined for CB1 and CB2 expression by western blot analysis and the result revealed, interestingly, that the cells expressed both CB1R and CB2R as shown in Figure ***3-14***.

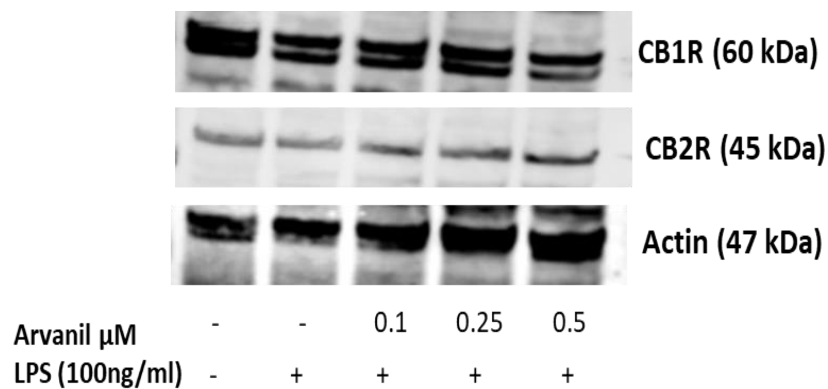



**Figure 3-14 LPS-stimulated BV2 microglia cells were expressed CB1R and CB2R.**

BV2 cells were treated with 0.1, 0.25 and 0.5 µM of arvanil for 30 minutes and then stimulated with 100 ng/ml LPS for 24 hr. To stop the stimulation, the cytoplasmic extracts were collected and analysed by western blot. The results showed that the cells expressed CB1R and CB2R. Actin was used as a loading control, and the protein levels were quantified by ImageJ analysis software.

### 3.3.11 Arvanil inhibited LPS-induced neuroinflammation in microglia without binding to cannabinoid receptor 1

As CB1 receptors were expressed in the BV2 microglia, an attempt was made to discover whether the anti-neuroinflammatory activity of arvanil was achieved through binding to CB1 receptors by using SR141716 as a selective CB1R antagonist. Using of cannabinoid receptor antagonists has been demonstrated in several studies to investigate whether the activity of synthetic cannabinoid agonists is mediated by cannabinoid receptors (Liu *et al*., 2021; Marques *et al*., 2020).

The inhibitory effect of 0.5 µM arvanil on IL-6 and TNF-α cytokines has been examined with 0.05 µM SR141716 on LPS-stimulated BV2 cells. The results in Figure ***3-15*** demonstrated that, in comparison with LPS-stimulated cells, the SR141716 antagonist significantly decreased the level of TNF-α (~4-fold reduction) and IL-6 (~1.3-fold reduction) when the cells were treated with 0.05 µM SR141716 followed by LPS induction. Treatment with LPS alone showed a significant increase in the level of both IL-6 and TNF-α, while treatment of BV2 cells with arvanil prior to LPS caused a significant reduction in those cytokines. However, treatment with 0.05 µM SR141716 for 30 min followed by 0.5 µM arvanil for another 30 min prior to activation by LPS for 24 hr demonstrated a significant reduction in the level of TNF-α ($p < 0.0001$) (~3.8-fold reduction) and IL-6 ($p < 0.05$) (~1.2-fold reduction) when compared with the LPS-stimulated cells.

Different concentrations of SR141716 (CB1R antagonist) were tested on BV2 viability with XTT assay, and the results in Figure ***3-16*** show that 0.005, 0.01 and 0.05 µM of SR141716 have insignificant effects on the viability of BV2 cells. Then, the XTT assay was conducted to study the viability of BV2 cells after pre-treatment with 0.05 µM SR141716 and 0.5 µM arvanil and stimulated with 100 ng/ml LPS for 24 hr (as indicated in 3.2.3). The results revealed that the BV2 viability was not affected significantly after being pre-treated with 0.05 µM SR141716 followed by 0.5 µM arvanil.

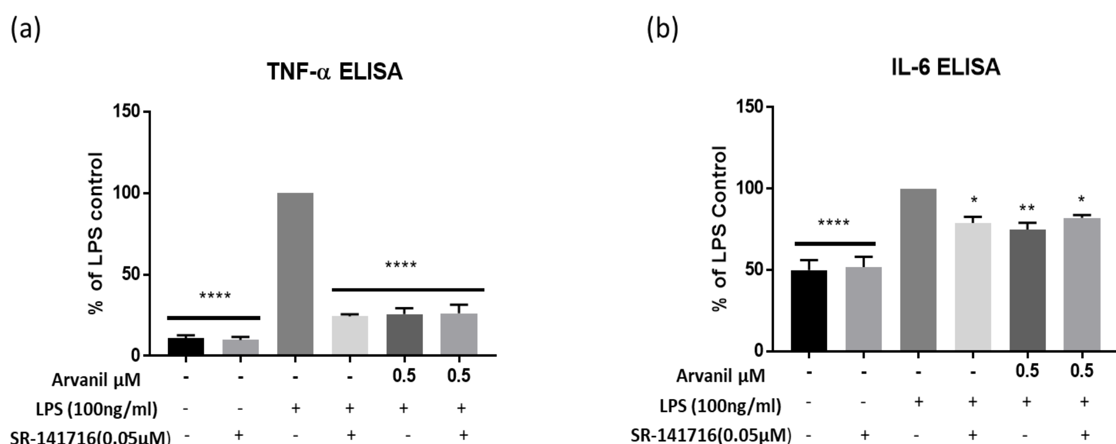



**Figure 3-15 Pre-treatment with both SR141716 and arvanil decreased the elevated pro-inflammatory cytokines in LPS-stimulated microglia.**

BV2 cells were pre-treated with 0.05 µM SR141716 for 30 min, then incubated with 0.5 µM arvanil for another 30 min followed by stimulation with 100 ng/ml LPS. After 24 hr of stimulation, the experiment ended by collecting the supernatant to examine the level of (a) TNF-α and (b) IL-6. Pre-incubation with SR141716 in the presence or absence of arvanil reduced the level of both cytokines in LPS-stimulated cells. Data for three independent experiments were presented as mean ± SEM and were analysed using one-way ANOVA followed by Dunnett's Multiple Comparison Test. *p<0.05, **p<0.01, *** p< 0.001 and **** p< 0.0001 compared to LPS-stimulated BV2 cells.

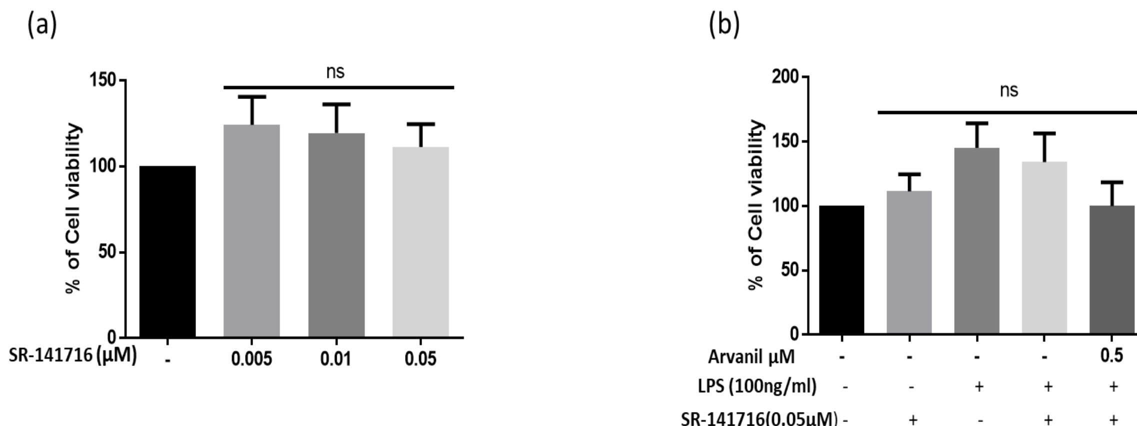



**Figure 3-16 SR141716 did not affect the viability of BV2 microglia.**

(a) BV2 cells were treated with 0.005, 0.01 and 0.05 µM of SR141716 for 24 hr, and then 25 µl of XTT/PMS was added to stop the experiments. The results showed that SR141716 did not decrease the viability of BV2 cells significantly. (b) BV2 cells were pre-treated with 0.05 µM of SR141716 for 30 min followed by incubation with 0.5 µM arvanil for another 30 min, then the cells were stimulated with 100 ng/ml. The stimulation was stopped after 24 hr by adding 25 µl of XTT/PMS. The result revealed that the used concentration of SR141716 (0.05 µM) did not affect the viability of stimulated BV2 cells when incubated with arvanil. Data for three independent experiments were presented as mean ± SEM and were analysed using one-way ANOVA followed by Dunnett's Multiple Comparison Test. *p<0.05, **p<0.01, *** p< 0.001 and **** p< 0.0001 versus untreated control cells for XTT assay.

### 3.3.12 The anti-neuroinflammation activity of arvanil in LPS-stimulated microglia is not mediated through binding to cannabinoid receptor 2

As BV2 microglia in this study expressed cannabinoid receptor 2 (CB2R) besides the expression of CB1R, there was an interest to know if arvanil inhibited neuroinflammation through binding with CB2R. The study was performed by using CB2R antagonists (SR144528) to block CB2 receptors and examine the anti-neuroinflammation activity of arvanil afterwards.

TNF-α and IL-6 levels were measured after incubation of BV2 cells with 0.5 μM of SR144528, followed by treatment with 0.5 μM of arvanil and then stimulation with LPS. Results in Figure ***3-17*** revealed that 0.5 μM of SR144528 treatment followed by LPS- activation caused a significant reduction in TNF-α (~1.3-fold reduction) and IL-6 level (~1.8-fold reduction) compared with stimulated cells. However, pre-treatment of stimulated cells with 0.5 μM arvanil showed a significant reduction in IL-6 (~2.4-fold reduction) and TNF-α (~2.1-fold reduction) protein levels. Similarly, levels of those pro-inflammatory cytokines (TNF-α and IL-6) were reduced significantly (IL-6; ~3.1-fold reduction and TNF-α; ~3.1-fold reduction) when the BV2 cells were pre-incubated with SR144528 and arvanil then stimulated with LPS. The significant differences were compared with LPS-stimulated cells.

SR144528 was tested for its cellular cytotoxicity, and the results in Figure ***3-18***a showed that the concentrations 0.05, 0.1 and 0.5 μM had insignificant alterations in the viability of BV2 cells compared to untreated cells. Furthermore, the selected SR144528 concentration (0.5 μM) was tested with arvanil on the viability of LPS-stimulated cells and the result revealed that the used concentration did not change the viability of BV2 cells compared to untreated cells by using XTT assay (Figure ***3-18***b).

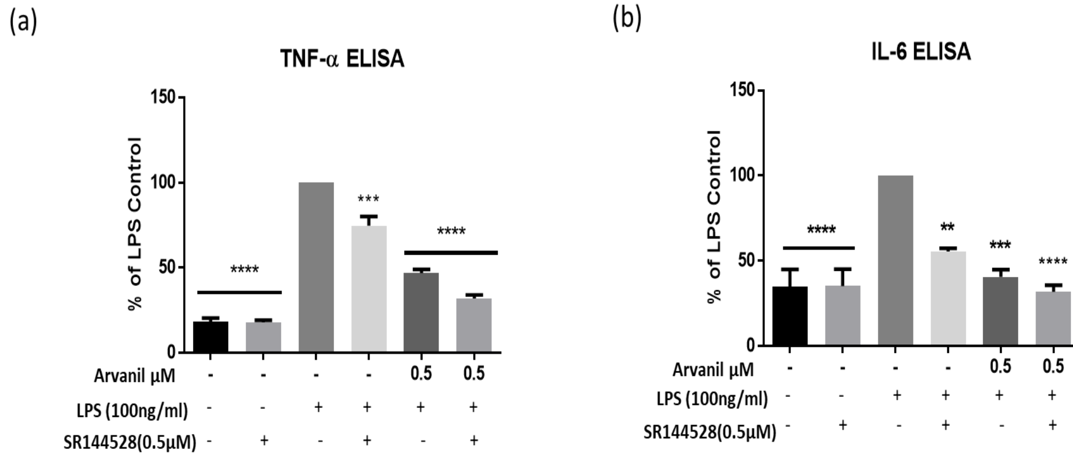



**Figure 3-17 Pre-incubation with both SR144528 and arvanil reduced the elevated pro-inflammatory cytokines levels in LPS-stimulated microglia.**

ELISA assay for obtaining TNF-α (a) and IL-6 (b) levels for BV2 cells that were treated with 0.5 µM CB2 receptor antagonist (SR144528) for 30 min and 0.5 µM arvanil for another 30 min followed by LPS stimulation for 24 hr. Stimulated cells that were incubated with 0.5 µM SR144528 and 0.5 µM arvanil exhibited a dramatic reduction in TNF-α and IL-6 levels, similar to that obtained when the stimulated cells were treated with arvanil alone. Data for three independent experiments were presented as mean ± SEM and were analysed using one-way ANOVA followed by Dunnett's Multiple Comparison Test. *p<0.05, **p<0.01, *** p< 0.001 and **** p< 0.0001 compared to LPS-stimulated BV2 cells.

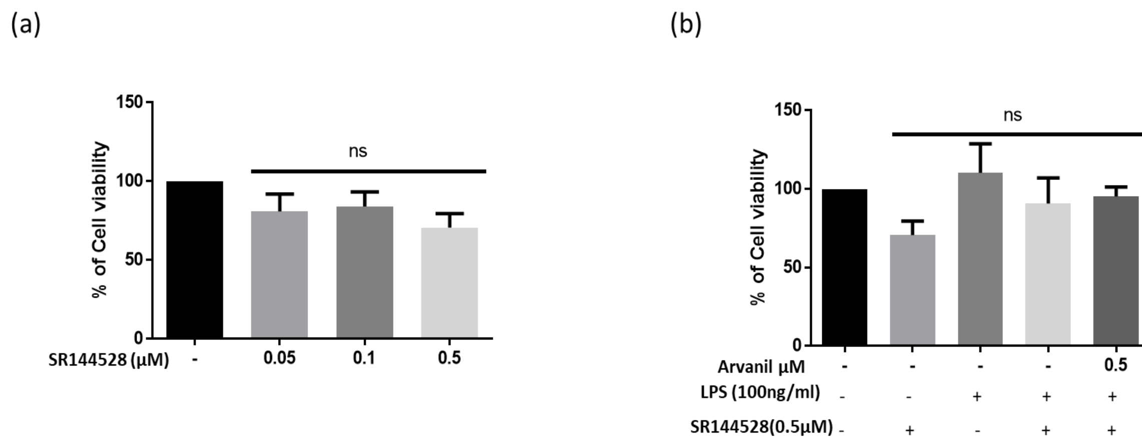



**Figure 3-18 The viability of BV2 microglia has not been affected when incubated with cannabinoid receptor 2 antagonist (SR144528).**

(a) BV2 cells were treated with 0.05, 0.1 and 0.5 µM of SR144528 for 24 hr, and then 25 µl of XTT/PMS was added to stop the reaction. The results showed that SR144528 did not decrease the viability of BV2 cells significantly. (b) BV2 cells were pre-treated with 0.5 µM of SR144528 for 30 min followed by incubation with 0.5 µM arvanil for another 30 min; then the cells were stimulated with 100 ng/ml LPS. The stimulation was stopped after 24 hr by adding 25 µl of XTT/PMS. The result revealed that the used concentration of SR144528 (0.5 µM) did not affect the viability of stimulated BV2 cells when incubated with arvanil. Data for three independent experiments were presented as mean ± SEM and were analysed using one-way ANOVA followed by Dunnett's Multiple Comparison Test. *p<0.05, **p<0.01, *** p< 0.001 and **** p< 0.0001 versus untreated control cells for XTT assay.

### 3.3.13 BV2 microglia expressed TRPV1 receptors

In addition to CB1R, Arvanil is a TRPV1 receptor agonist and in order to investigate whether its anti-inflammatory activities were mediated through TRPV1 receptors, BV2 microglia were examined for TRPV1 receptors expression. The result from western blot analysis showed that BV2 cells expressed TRPV1 receptors, as shown in Figure ***3-19***.

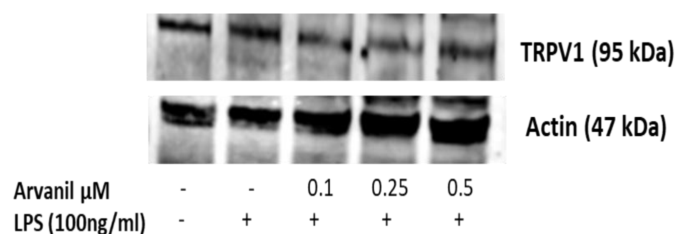



**Figure 3-19 LPS-stimulated BV2 microglia cells were expressed TRPV1 receptors.**

BV2 cells were treated with 0.1, 0.25 and 0.5 µM of arvanil for 30 minutes and then stimulated with 100 ng/ml LPS for 24 hr. To stop the stimulation, the cytoplasmic extracts were collected and analysed by western blot. The results showed that the cells expressed TRPV1 receptors. Actin was used as a loading control, and the protein levels were quantified by ImageJ analysis software.

### 3.3.14 Arvanil did not bind to TRPV1 receptors to exert its anti-neuroinflammatory activity in LPS-activated microglia

TRPV1 is an ion channel that is found to be mainly presented in sensory nerve cells. Interestingly, recent studies demonstrated that TRPV1 is expressed in immune cells such as macrophages, dendritic cells and T lymphocytes (Bujak, Kosmala, Szopa, Majchrzak, & Bednarczyk, 2019; Wang & Siemens, 2015). Several findings have revealed that treatment with TRPV1 agonists may attenuate inflammatory responses and lowered cytokines and chemokines production through TRPV1 activation (J. Chen et al., 2018; Youping Wang et al., 2017). Arvanil is a TRPV1 agonist and was examined in this study to discover whether its anti-neuroinflammatory activity has been achieved through binding and activating TRPV1 receptors by using TRPV1 receptors antagonist (SB366791).

The anti-neuroinflammatory effect of arvanil has been investigated after blocking TRPV1 receptors with SB366791 by measuring TNF-α and IL-6 levels. BV2 cells were treated and stimulated, as indicated in section 3.2.3. The results showed that the arvanil did not lose its anti-neuroinflammatory effects when TRPV1 receptors were blocked. TNF-α (~1.5-fold reduction) and IL-6 levels (~1.6-fold reduction) were significantly reduced when BV2 cells were treated with 1.25 µM SB366791 and then with 0.5 µM arvanil followed by LPS stimulation compared to LPS-activated cells. However, the inhibitory effects of 1.25 µM SB366791 on TNF-α and IL-6 protein levels were not significant in LPS-activated BV2 cells (Figure ***3-20***).

The viability of BV2 cells was examined by XTT assay after pre-incubation with 1.25, 2.5, 5, 10, 20 µM of SB366791 for 24 hr and the results in Figure ***3-21***a demonstrated that among all the used concentrations, 1.25 µM did not decrease the viability of BV2 cells significantly when compared to untreated cells. The same concentration (1.25 µM) has been examined in the presence of 0.5 µM arvanil in LPS-stimulated BV2 cells to investigate the viability of the cells, and the results showed that pre-incubation with SB366791 and arvanil had insignificant effects on stimulated cells compared to untreated ones (Figure ***3-21***b).

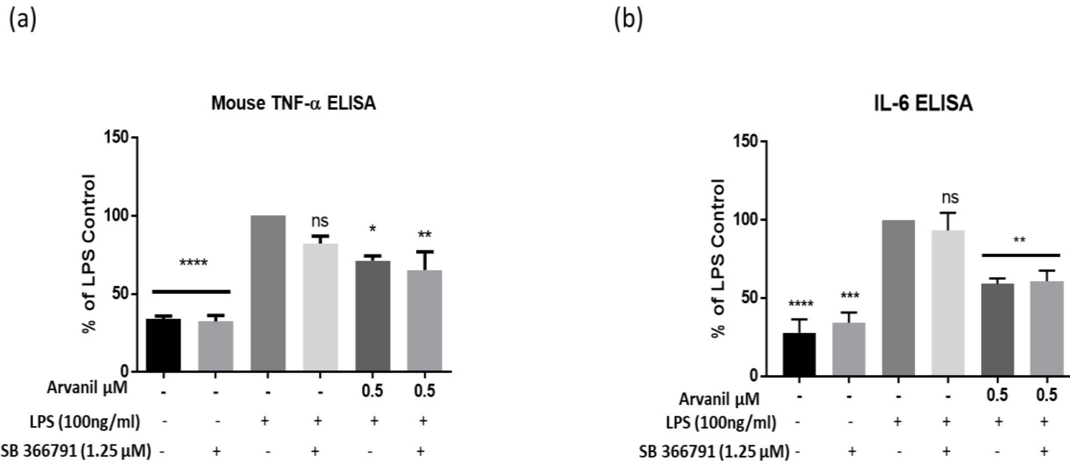



**Figure 3-20 Blocking TRPV1 receptors with their antagonist did not affect arvanil inhibitory effects on the cytokines' levels in LPS-activated microglia.**

Cells were pre-incubated with 1.25 µM of SB366791 for 30 min followed by 0.5 µM of arvanil for 30 min, then stimulated with LPS for 24 hr. The supernatants were collected, and ELISA assays were performed to detect the protein levels of (a) TNF-α and (b) IL-6. The results revealed that the inhibitory effects of arvanil on both cytokines did not change when TRPV1 receptors were blocked with SB366791. Data for three independent experiments were presented as mean ± SEM and were analysed using one-way ANOVA followed by Dunnett's Multiple Comparison Test. *p<0.05, **p<0.01, *** p< 0.001 and **** p< 0.0001 compared to LPS-stimulated BV2 cells.

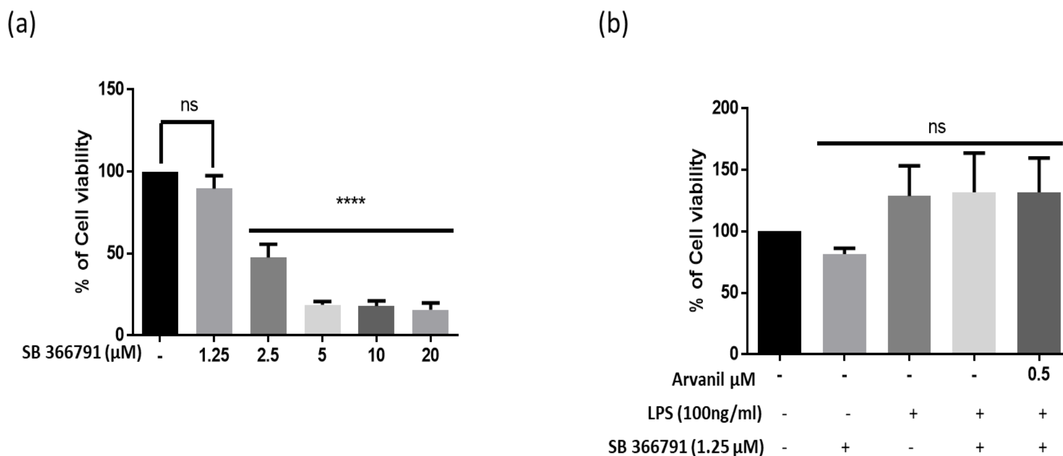



**Figure 3-21 The viability of BV2 microglia has not been affected when incubated with TRPV1 receptors antagonist.**

(a) BV2 cells were treated with 1.25, 2.5, 5, 10 and 20 µM of SB366791 for 24 hr, then 25 µl of XTT/PMS was added to stop the reaction. The results showed that only 1.25 µM SB366791 did not decrease the viability of BV2 cells significantly. (b) BV2 cells were pre-treated with 1.25 µM of SB366791 for 30 min followed by incubation with 0.5 µM arvanil for another 30 min; then the cells were stimulated with 100 ng/ml LPS. The stimulation has been stopped after 24 hr by adding 25 µl of XTT/PMS. The result revealed that the used concentration of SB366791 (1.25 µM) did not affect the viability of stimulated BV2 cells when incubated with arvanil. Data for three independent experiments were presented as mean ± SEM and were analysed using one-way ANOVA followed by Dunnett's Multiple Comparison Test. *p<0.05, **p<0.01, *** p< 0.001 and **** p< 0.0001 versus untreated control cells for XTT assay.

### 3.3.15 Arvanil did not increase the protein levels of HO-1 and NQO1 in BV2 microglia cells

One of the main antioxidant pathways activated during neuroinflammation is Nrf2 which mediates the production of HO-1 and NQO1 antioxidant elements (Jayaram & Krishnamurthy, 2021; Qu *et al*., 2020). The anti-inflammatory activity of the Nrf2 signalling pathway is well-defined by several studies. For example, it has been reported that upregulation of HO-1 and NQO1 expression caused a transcriptional depression of pro-inflammatory mediators (IL-6, TNF-α, IL-1β) in microglia, astrocytes and monocytes (Kobayashi *et al*., 2016; Quinti *et al*., 2017). As a result, arvanil was investigated in BV2 cells to examine its ability to activate HO-1 and NQO1 antioxidant elements. Western blot analysis for the lysates from BV2 microglia treated with arvanil (0.1, 0.25 and 0.5 µM) was used to explore the modulation activity of arvanil on HO-1 and NQO1 levels. The levels of HO-1 were not increased significantly when BV2 cells were treated with 0.1, 0.25 and 0.5 µM of arvanil. Additionally, the expression levels of NQO1 were not upregulated significantly when BV2 cells were treated with 0.1, 0.25 and 0.5 µM of arvanil compared with untreated cells (Figure ***3-22***).

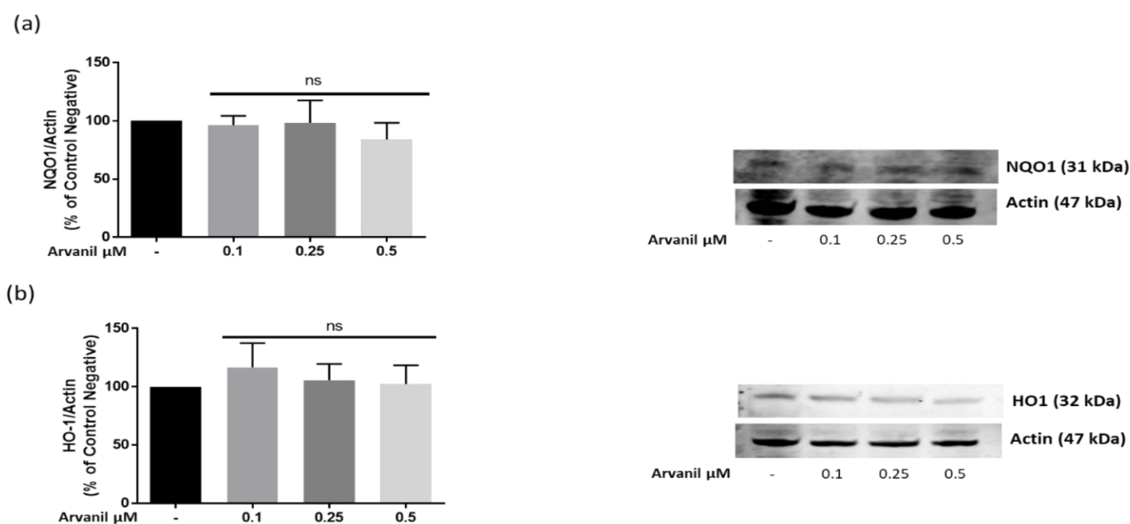



**Figure 3-22 Arvanil could not increase the expression of NQO1 and HO-1 in BV2 microglia.**

BV2 microglia were treated with 0.1, 0.25 and 0.5 µM arvanil for 24hr, and then the cytoplasmic extractions were collected and analysed by western blot. The results revealed that arvanil did not raise the protein expressions of (a)NQO1 and (b) HO-1 versus untreated cells. Actin was used as a loading control. Data for three independent experiments were presented as mean ± SEM and were analysed using one-way ANOVA followed by Dunnett's Multiple Comparison Test. $*p<0.05$, $**p<0.01$, $***\ p< 0.001$ and $****\ p< 0.0001$ compared to untreated cells.

### 3.3.16 Arvanil did not activate Nrf2/ARE signalling pathway in BV2 microglia cells

Several studies suggested that targeting the Nrf2 signalling pathway is associated with neuroinflammation inhibition (Park, Kim, & Park, 2015; Velagapudi *et al*., 2017). However, the Nrf2 pathway is tightly regulated at different levels, including cytoplasmic, nuclear, transcriptional, post-transcriptional or translational levels; therefore, arvanil effects on Nrf2 activation at the nuclear level were examined.

Firstly, the result of the time-point western blot experiment showed that at 24 hours, arvanil was able to produce the highest Nrf2 expression. Western blot analysis of nuclear extracts from BV2 cells treated with 0.1, 0.25 and 0.5 µM arvanil for 24hr showed that the level of Nrf2 protein expression did not change significantly compared to untreated cells (Figure ***3-23***a).

Furthermore, a luciferase reporter gene assay was done to determine if arvanil could enhance the activation of antioxidant responsive elements (ARE). A time-point experiment was performed to investigate the time required for arvanil to achieve maximum activation for ARE luciferase reporter. BV2 microglia were treated with 0.5 µM arvanil for 2, 4, 6, 8 and 24hr and the maximum ARE activation was detected at 24hr treatment. As indicated in Figure ***3-23***b, all the used concentrations of arvanil did not increase the ARE-luciferase activity in microglia. To confirm these findings, an immunofluorescence experiment was performed. The results showed that arvanil did not upregulate the expression of Nrf2 levels when compared to untreated BV2 cells (Figure ***3-23***c).

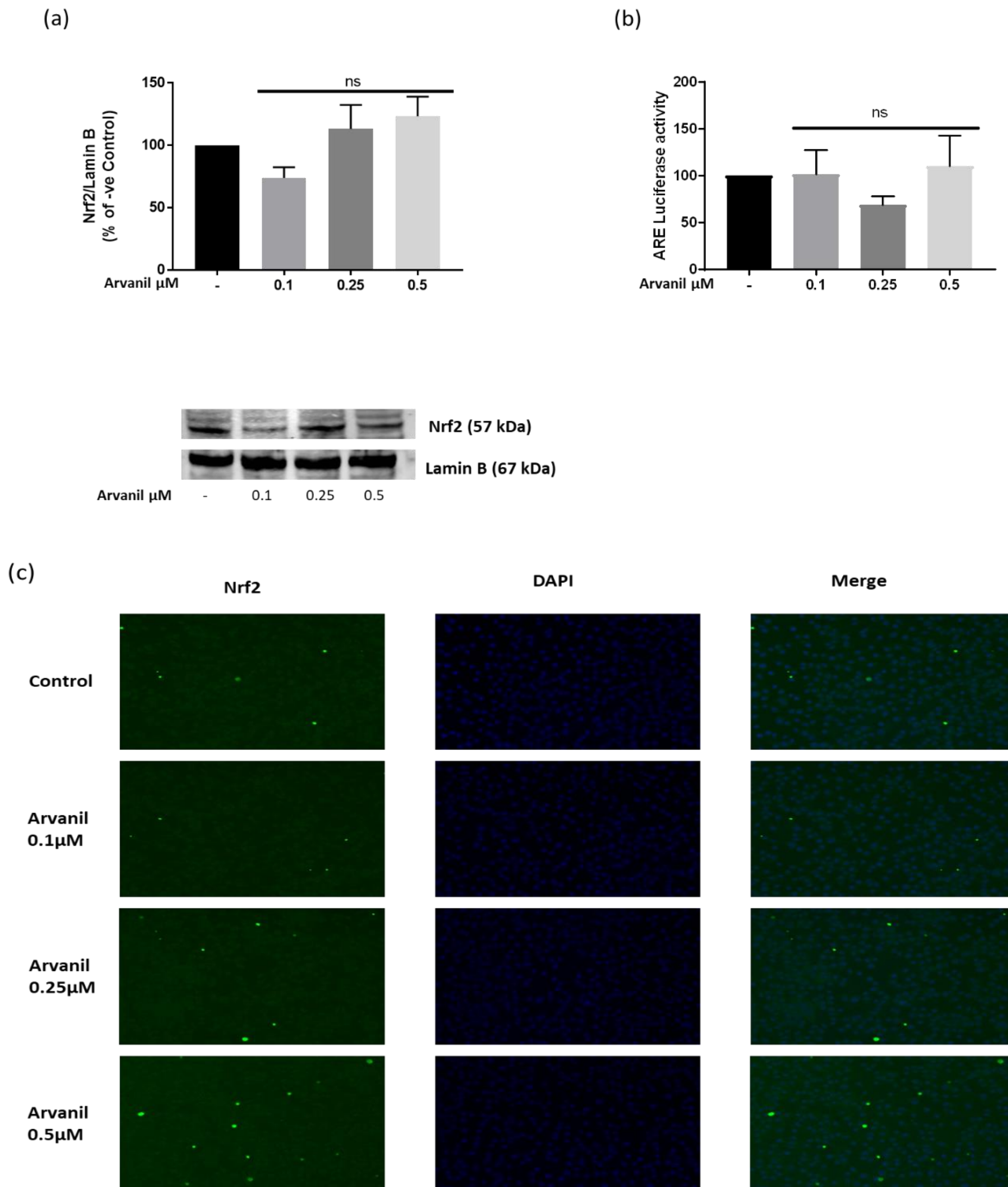



**Figure 3-23 Arvanil did not up-regulate the Nrf2/ARE antioxidant axis in microglia.**

BV2 cells were treated with 0.1, 0.25 and 0.5 µM of arvanil for 24hr. (a) nuclear extractions were collected and subjected to western blot analysis. The membranes were incubated with an Nrf2 antibody, and the results showed that arvanil could not increase the expression of Nrf2 when compared to untreated cells. (b) BV2 cells were transfected with ARE luciferase reporter then treated with arvanil for 24hr, the luciferase Dual Glo was used to read the luminescence reaction and renilla luciferase was used as an internal control. The results revealed that Arvanil did not increase ARE activity compared to untreated transfected cells. (c) Nrf2 antibody was used to label the cells, followed by incubation with DAPI. The fluorescence images confirmed that arvanil could not up-regulate the expression of Nrf2. Images were processed by image J analysis software and the scale bar=100 µm. Lamin B was used as a loading control in western blot experiments. Data for three independent experiments were presented as mean ± SEM and were analysed using one-way ANOVA followed by Dunnett's Multiple Comparison Test. *$p<0.05$, **$p<0.01$, *** $p< 0.001$ and **** $p< 0.0001$ compared to untreated cells.

### 3.3.17 Optimisation of neurotoxic concentration of $H_2O_2$ in SH-SY5Y cells

Several studies have employed $H_2O_2$ to induce neuronal damage in SH-SY5Y neuroblastoma cells (Song & Tao, 2020; Tian *et al.*, 2019) and, thus, will provide a useful model system in investigating the neuroprotective potential of therapeutics. In this study, various concentrations of $H_2O_2$ were selected to induced oxidative damage, and XTT assay was employed to establish the concentration of $H_2O_2$ that induce maximum toxicity to differentiated SH-SY5Y. Incubation of the cells with 50, 100 and 200 µM $H_2O_2$ exhibited a significant reduction in viability after 24 hours. However, $H_2O_2$ (100 and 200 µM) showed a more significant (~3.5-fold decrease) reduction ($p < 0.0001$) in the viability (Figure ***3-24***) than 50 µM ($p < 0.01$) (~1.6-fold decrease) when compared with the control group. Based on this finding, the concentration of 200 µM $H_2O_2$ was used in experiments to study the effects of arvanil on $H_2O_2$-induced neurotoxicity.

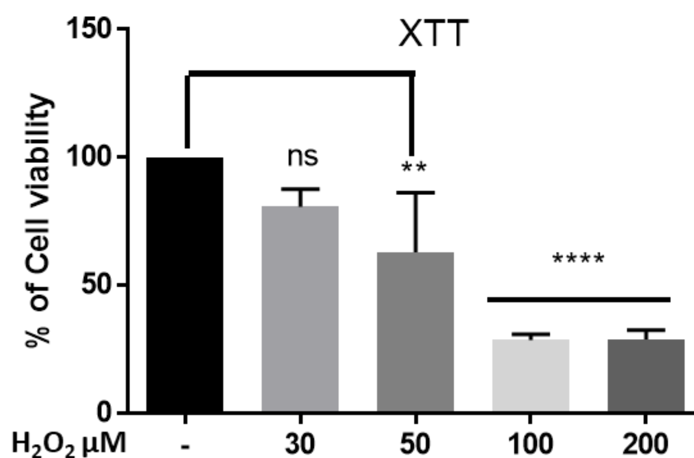



**Figure 3-24 $H_2O_2$ induce cytotoxicity by reducing the viability of SH-SY5Y cells.**

SH-SY5Y cells were incubated with various concentrations of $H_2O_2$ for 24hr and, then 25 µl of XTT/PMS was added to terminate the reaction. The results showed that incubation with 100 and 200 µM of $H_2O_2$ had the maximum neuronal damage compared with untreated cells. Data for three independent experiments were presented as mean ± SEM and were analysed using one-way ANOVA followed by Dunnett's Multiple Comparison Test. *p<0.05, **p<0.01, *** p< 0.001 and **** p< 0.0001 versus untreated control cells for XTT assay.

### 3.3.18 Pre-treatment with arvanil did not prevent $H_2O_2$-induced damage to SH-SY5Y neuronal cells

The viability of differentiated SH-SY5Y cells was measured with XTT assay after incubation with 0.1, 0.25 and 0.5 µM arvanil. Results showed non-significant changes in the cell viability when treated with arvanil alone compared with untreated cells. Differentiated SH-SY5Y cells were treated with arvanil and exposed to $H_2O_2$, as detailed in section 3.2.4. Cellular viability was significantly ($p < 0.001$) reduced (~2.5-fold decrease) when SH-SY5Y cells were exposed to 200 µM $H_2O_2$. Pre-incubation of differentiated SH-SY5Y cells with 0.1, 0.25 and 0.5 µM arvanil did not prevent the neurotoxic effects of 200 µM $H_2O_2$ (Figure ***3-25***).

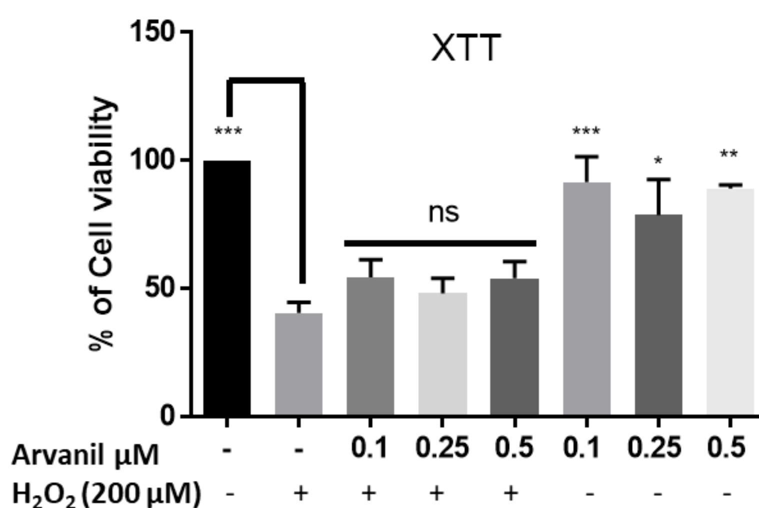



**Figure 3-25 Pre-incubation with arvanil did not increase the viability of $H_2O_2$-induced SH-SY5Y cytotoxicity.**

Differentiated SH-SY5Y neuroblastoma cells were treated with 0.1, 0.25 and 0.5 µM of arvanil with and without exposure to 200 µM $H_2O_2$ for 24hr. The experiments were terminated by adding 25 µl of XTT/PMS solution. The results exhibited that arvanil was not a cytotoxic compound when the cells were treated with it alone. However, arvanil did not appear to be a neuroprotective agent when applied to $H_2O_2$-induced cytotoxicity. Data for three independent experiments were presented as mean ± SEM and were analysed using one-way ANOVA followed by Dunnett's Multiple Comparison Test. *$p<0.05$, **$p<0.01$, *** $p< 0.001$ and **** $p< 0.0001$ versus $H_2O_2$-treated cells for XTT assay.

### 3.3.19 Pre-treatment with arvanil did not modulate the reduced expression of neuronal markers in the $H_2O_2$-induced SH-SY5Y cells neuronal damage

Generally, mature neurons express high levels of neuronal proteins such as Microtubule-associated proteins-2 (MAP-2) and βIII-Tubulin, which are used widely as markers of neuronal maturity (Dehmelt & Halpain, 2005; Katsetos *et al*., 1993; Thomson *et al*., 2021). When the neurons are exposed to high levels of oxidative stress or toxins, neuronal damage occurs and is accompanied by changes in the cellular morphology and plasticity with reduced expression of neuronal markers (A. Singhal, Morris, Labhasetwar, & Ghorpade, 2013).

Immunofluorescent stainings of neuronal protein MAP-2 and βIII-Tubulin were performed in differentiated SH-SY5Y to confirm the effect of arvanil visually. Differentiated SH-SY5Y cells were treated with arvanil and exposed to $H_2O_2$, as detailed in 3.2.4. The results from the western blot showed that $H_2O_2$ significantly decreased the expression (~1.4-fold reduction) of MAP-2 protein ($p< 0.01$) compared with untreated cells, whereas arvanil pre-treatment could not enhance MAP-2 expression noticeably (Figure ***3-26***a). Overall, $H_2O_2$-treated cells exhibited a marked decrease in MAP-2-positive cells with contracted cell bodies and the disappearance of neuronal dendrites compared to untreated cells. Pre-incubation with 0.5 µM arvanil did not reverse the $H_2O_2$-damaged effects on MAP-2 expression (Figure ***3-26***b). Furthermore, an immunofluorescence experiment was performed to investigate the expression of βIII-Tubulin in the differentiated SH-SY5Y cells when exposed to oxidative stress by $H_2O_2$ and pre-treated with 0.5 µM arvanil. Figure ***3-26***c shows that treatment with 200 µM $H_2O_2$ resulted in decreased βIII-Tubulin positive cells compared with untreated cells. However, 0.5 µM arvanil could not preserve βIII-Tubulin protein expression in the $H_2O_2$- treated SH-SY5Y cells.

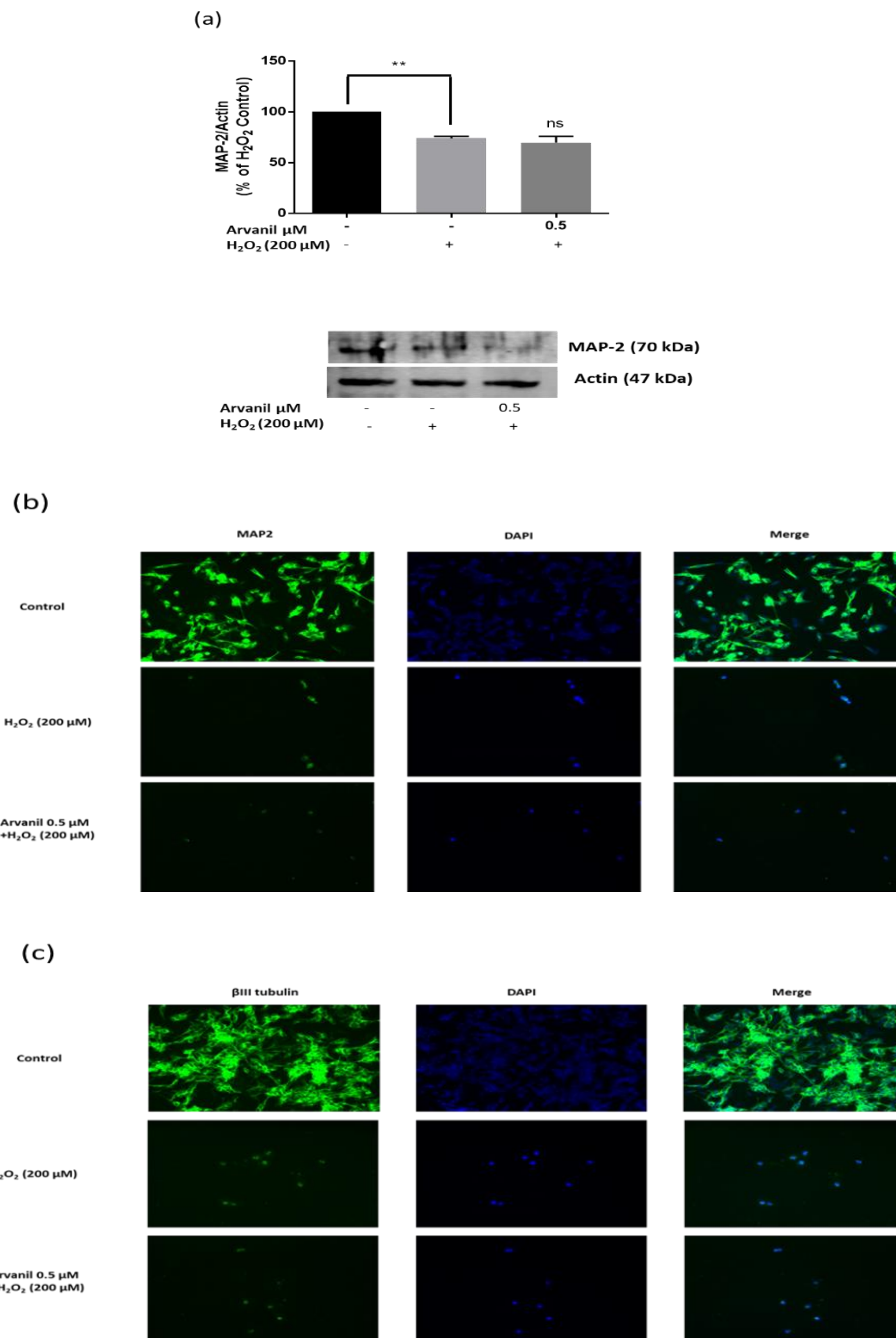



**Figure 3-26 Arvanil could not protect the SH-SY5Y cells from the $H_2O_2$-induce neuronal markers reduction.**

SH-SY5Y cells were pre-treated with 0.5 µM arvanil for 30 minutes, and then 200 µM of $H_2O_2$ was added and incubated for 24hr. (a) Cytoplasmic extractions were collected and the proteins were subjected to western blot analysis. Arvanil could not inhibit the cytotoxicity effects of $H_2O_2$ on MAP-2 expression. (b) MAP-2 antibody was used to label the cells, followed by incubation with DAPI. The fluorescence images confirmed that arvanil could not reverse the damage effects of 200 µM $H_2O_2$. (c) Cells were stained with βIII-Tubulin antibody overnight, then counterstained with DAPI and the images revealed that arvanil could not increase the expression of βIII-Tubulin in the $H_2O_2$-treated cells. Images were processed by image J analysis software and the scale bar=100 µm. Actin was used as a loading control in western blot experiments. Data for three independent experiments were presented as mean ± SEM and were analysed using one-way ANOVA followed by Dunnett's Multiple Comparison Test. *p<0.05, **p<0.01, *** p< 0.001 and **** p< 0.0001 compared to $H_2O_2$-treated cells.

## 3.4 Discussion

Studies have proposed that inflammation plays a central role in disease pathogenesis; hence monitoring and understanding the interactions between the nervous system and the immune system may represent a significant key for delaying or preventing many late-onset CNS diseases. Inflammation and oxidative stress are the master hypotheses in the progression of several neurodegenerative diseases such as AD and PD (Quintanilla, Orellana, & von Bernhardi, 2012). In neurodegenerative diseases, the endocannabinoid system goes through significant changes, particularly with diseases that have inflammatory responses. For instance, both CB1R and CB2R are nitrosylated in AD brains, which may participate in the reduced coupling of these receptors with their downstream signalling molecules (Ramírez *et al.*, 2005). In addition, FAAH, the endocannabinoid metabolizing enzyme, is usually up-regulated in neuritic plaque-associated glia (Benito *et al.*, 2003) and also in mononuclear cells of peripheral blood (D'Addario *et al*., 2012). Such elevation can contribute to the increase in the degradation of AEA in the senile plaque. However, FAAH overexpression could have harmful outcomes in disease progressions, such as limitation in the availability of neuronal AEA and a rising in the pro-inflammatory molecules produced by AEA metabolites like arachidonic acid (Calder, 2006).

Since the inflammatory process may contribute to the development of neurodegeneration diseases, the pro-inflammatory factors are implicated in the progression of brain cell damage (Valles *et al*., 2010). Those pro-inflammatory molecules initially have beneficial impacts since they participate in the tissue protection process, limiting the proliferation of the cells exposed to harmful elements (Allan & Rothwell, 2003). Nevertheless, a sustained inflammatory response might lead to neuronal damage or cell death (Burkert, Moodley, Angel, Brooks, & Graham, 2012; S. S. Choi, Lee, Lim, Satoh, & Kim, 2014). The outcome of this study indicated that arvanil prevented the increase of inflammatory mediators, TNF-α and IL-6 induced by LPS in BV2 microglia. These observations are consistent with those previously described by other studies that proposed the involvement of cannabinoid receptor agonists in attenuating pro-inflammatory cytokines in neuroinflammatory disease models (G Esposito, Scuderi, Valenza, Togna, & Latina, 2011; Martín-Moreno *et al*., 2012). Marquez *et al*. (2006) demonstrated in

their study that arvanil inhibited the gene transcription of IL-2 and its receptor expression, which eventually decreased the inflammatory response mediated by inhibition in the late events of T-cell activation (Márquez *et al.*, 2006).

Accumulating data from both *in vivo* and *in vitro* studies suggest that cannabinoid receptor agonists alleviate neural cell activation in the inflammatory response produced by neurotoxic agents, thereby reducing the levels of pro-inflammatory mediators such as IL-6, IL-1β and TNF-α (Giuseppe Esposito *et al*., 2011; Scuderi *et al*., 2011). Moreover, cannabinoid receptors activation constringe the release of those molecules from microglial cells (Ehrhart *et al*., 2005; Facchinetti, Del Giudice, Furegato, Passarotto, & Leon, 2003). It is proposed that arvanil may produce its anti-inflammatory activity through mechanisms involving these receptors. Fakhfouri *et al*. (2012) showed a relationship between CB1 as well as CB2 receptors agonists, as well as PPAR-γ, in the neuroprotective ability of cannabinoid agonist WIN 55,212 2 (which has a non-selective affinity for CB1 and CB2 receptors) against neuroinflammation. They showed that WIN 55,212 2 could minimise neuroinflammation by attenuating Aβ-induced pro-inflammatory cytokines, especially TNF-α production (Fakhfouri *et al.*, 2012). Moreover, a study conducted by Martín-Moreno *et al*. (2011) on the Aβ-mice model, with a rise in both IL-6 and TNF-α expression, has shown that pre-treatment with WIN 55,212 2 and CBD suppressed the increased levels of IL-6 and partly diminished TNF-α levels (Martin-Moreno *et al*., 2011).

Enhanced NO levels, released as a result of increased iNOS expression by activated microglia and astrocytes following a neuronal challenge, increase protein nitration of the neurons (Coma *et al*., 2005) and produce an obvious acceleration in tau protein hyper-phosphorylation, executing a crucial role in the disease pathology progression. There is evidence showing that the expression of iNOS is up-regulated in the cases of neurodegeneration, and its over-expression is known to contribute to the process of neuroinflammatory-neurodegenerative progression (Togo, Katsuse, & Iseki, 2004). Therefore, targeting iNOS expression and blockage of NO production has been assumed to exert

valuable effects in neuroinflammation-related diseases (Nathan *et al*., 2005). Based on this, the effect of arvanil on iNOS and NO levels was examined. Results showed that LPS induced an elevation in both NO and iNOS levels when compared with control cells. However, arvanil, caused a significant reduction of iNOS protein in LPS-stimulated microglia cells. Similarly, NO levels were also significantly reduced with 0.25 and 0.5 µM arvanil in the stimulated cells.

It has been proposed that CB1 receptor agonists may have the ability to inhibit iNOS expression and attenuate NO production through the activation of CB1 receptors. This assumption is based on two studies. The first one was reported by Waksman *et al*. (1999); they showed, for the first time, the effect of CB1R on the production of NO. The CB1 agonist, (-)-CP55940, mediates suppression of nitric oxide production, and this was mediated at the level of iNOS expression in LPS/IFN-γ-activated rat microglial cells. Pre-treatment with SR141716A (CB1-selective antagonist) caused a reversal of the (-)-CP55940 action on the cells, which reflected the involvement of the CB1 receptor in this action (Waksman, Olson, Carlisle, & Cabral, 1999). The second study by Esposito *et al*. (2006) explicated that ACEA, through CB1receptor, produced inhibition of β-amyloid-induced iNOS protein expression that in turn reduced NO production in C6 rat glioma cells, implicating the role of CB1 receptors in the decrease level of inflammation(Giuseppe Esposito *et al*., 2006).

ROS productions were also examined, representing an indication of oxidative damage. modulating ROS generation attenuated disease progressions and alleviated inflammatory damages in LPS-activated microglia (Sousa *et al*., 2020; Songwei Yang *et al*., 2020). The ROS assay results demonstrated that arvanil could not inhibit LPS-induced ROS production in BV2 microglia. Prostaglandins, produced within the metabolic pathway of arachidonic acid (AA), participate in neuroinflammation (Bagyinszky *et al*., 2017). COX-1 enzyme is expressed constitutively in the brain, while COX-2 expression is generally induced in neurodegenerative diseases both in glial cells and neurons (Wyss-Coray & Rogers, 2012). Elevated levels of prostaglandins generated by COX-2 have been observed in the frontal cortex of AD sufferers and with consistent activation of astrocytes and microglia in senile plaques (Akiyama *et al*.,

2000; Calsolaro & Edison, 2016). The results of this study have demonstrated that the expression of COX-2 and its by-product, $PGE_2$, was elevated in LPS-activated BV2 cells. Furthermore, pre-treating microglia with arvanil attenuated the effects of LPS by inhibiting the production of $PGE_2$ and suppressing COX-2 expression. These results are consistent with an earlier study showing that COX-2 and iNOS protein expression were decreased when astrocytes were pre-treated with CBR agonist, WIN 55,212-2, following incubation with Aβ1–42 (Aguirre-Rueda *et al*., 2015). Collectively, arvanil inhibited neuroinflammation through reducing pro-inflammatory mediators' levels.

NF-κB plays a crucial role in the regulation of neuroinflammation mediated by the microglia (F. Gao *et al*., 2013). Therefore, blocking the activity for NF-κB can lead to reducing the expression of several pro-inflammatory mediators genes such as TNF-α, $PGE_2$, IL-1β and NO (Chantong, Kratschmar, Nashev, Balazs, & Odermatt, 2012; Dalal *et al*., 2012; C. Zhu *et al*., 2012) and regulate the inflammatory response. Therefore, an attempt has been made to explore the molecular mechanism behind the anti-neuroinflammation activity of arvanil in LPS-activated microglia. This study showed that LPS stimulation of BV2 cells resulted in NF-κB p65 subunit phosphorylation but this elevation was not inhibited by pre-treatment with arvanil.

An attempt was made to investigate the upstream cytoplasmic mechanisms of the NF-κB signalling pathway that involve the IκB system. Interestingly, 0.5 µM of arvanil demonstrated an inhibition in the degradation of IκB, which is an essential upstream target in the NF-κB signalling pathway but did not inhibit the phosphorylation of IκB in LPS-stimulated BV2 cells. IκB pool in the cells is composed of a major pool of IκB, which is bounded to NF-κB (Scott, Fujita, Liou, Nolan, & Baltimore, 1993) and a minor pool that remains free. The free IκB is a poorer substrate for IKK than NF-κB-bound IκB (Mathes, O'dea, Hoffmann, & Ghosh, 2008). When the cell is stimulated, IKK is phosphorylated, which, in turn, phosphorylates and degrades NF-κB-bound IκB. Arvanil did not decrease the phosphorylation of IκB, suggesting that it may not work on NF-κB-bound IκB. However, arvanil inhibited IκB degradation,

indicating that it may inhibit the proteasomal degradation of free IκB and not NF-κB-bound IκB (which is involved mainly in the activation of the NF-κB pathway).

However, the complexity of the NF-κB pathway may allow the inhibitors to target and interfere with downstream nuclear mechanisms to inhibit this pathway without affecting upstream events. Therefore, the DNA binding affinity of pp65 to inflammatory genes and the nuclear transactivation of NF-κB were studied. The outcomes of this study showed that arvanil had no inhibitory effects on LPS-mediated NF-κB–DNA and did not reduce NF-κB transcriptional activity. These observations are inconsistent with a previously reported study by Marquez *et al*. (2006) demonstrated that arvanil inhibits p65 phosphorylation and NF-κB DNA binding in TNF-α stimulated Jurkat T cells (Márquez *et al*., 2006). Altogether, arvanil anti-neuroinflammatory activity is NF-κB-independent.

Activated MAPKs are important in inflammation-related processes, which in turn cause the release of inflammatory cytokines by up-regulating the pro-inflammatory gene expression (Velagapudi, Aderogba, & Olajide, 2014; Yongqun Zhu *et al.*, 2007). Earlier studies have described the implication of MAPKs in the regulation of LPS-induced production of inflammatory mediators (Jung *et al*., 2016; Song, Qu, Zhu, Zhang, & Ma, 2012). This study observed that p38 and JNK MAPKs pathways were activated by LPS stimulated BV-2 cells. However, incubation with arvanil did not inhibit the phosphorylation of p38 and JNK resulting from LPS stimulation, suggesting that arvanil anti-neuroinflammatory activity is MAPKs-independent. The result, regarding p38, may be consistent to a limited extent with a previous study which demonstrated that anandamide, an agonist for both the CB1 receptor and TRPV1 receptor as an arvanil, did not seem to interpose with the p38 pathway since the phosphorylation level was not inhibited by anandamide in LPS/IFNγ stimulated microglial cells (Correa *et al*., 2009). However, arvanil may affect the MAPKs pathway at the nuclear level; therefore, the AP-1 downstream transcription factor of MAPKs can be investigated.

Phosphoinositide 3-kinases (PI3Ks)/Akt signalling pathway usually regulates a wide range of physiological actions such as signal transduction, cytoskeletal reorganisation and vesicular traffic

(Cianciulli *et al.*, 2020; Deane & Fruman, 2004; Vanhaesebroeck, Guillermet-Guibert, Graupera, & Bilanges, 2010). However, the involvement of PI3Ks/Akt signalling cascade in the regulation of inflammatory responses to external stimuli has been reported (Vanhaesebroeck, Whitehead, & Piñeiro, 2016) through interaction with events that start from IKKβ and end with NF-κB activation, which results in transcription of pro-inflammatory genes in activated microglia. Several studies suggested the participation of PI3Ks and phosphorylation of its downstream signalling molecules, Akt, in the releasing of inflammatory molecules during LPS stimulation of microglia, while down-regulation of this pathway results in the alleviation of the inflammatory reactions (Cianciulli *et al.*, 2016; Lee, Park, Jung, Kim, & Kim, 2013; Zhong *et al.*, 2020). Arvanil has been studied to investigate whether its anti-neuroinflammatory effects were achieved through modulation of the Akt signalling molecule. Therefore, the phosphorylation of Akt has been investigated in this study as downstream effector molecules which might reflect the activation of the whole pathway. Activated microglia by LPS has been shown to induce Akt phosphorylation, and arvanil pre-treatment did not attenuate this phosphorylation significantly. These results indicate that Akt, the central signalling molecule of PI3Ks/Akt pathway, may not be involved in the mechanism behind the inhibitory activity of the arvanil on LPS-induced pro-inflammatory cytokines production. Accordingly, Arvanil's anti-inflammatory action is Akt-independent.

It is well-known that arvanil is an agonist for CB1 receptors and has the ability to activate TRPV1 receptors (Melck *et al.*, 1999). Growing evidence suggests that the expression of TRPV1 in brain cells such as microglia and astrocytes exerts both neuronal and non-neuronal regulations. Formerly, neuroscientists determined that the major role of TRPV1 is in the nociceptive neuronal sensory transmission that occurs in the peripheral nervous system (PNS) (Ho, Ward, & Calkins, 2012; Martins, Tavares, & Morgado, 2014). Lately, TRPV1 has been identified to be distributed widely in the CNS, which might contribute to an atypical neurotransmission system and be implicated in numerous functional activities via mediating the modulation of the neurons and glial cells (Kong, Peng, & Peng, 2017; Martins *et al.*, 2014). This study showed the expression and localisation of TRPV1 in the BV2

microglia. TRPV1 expressions were documented previously in rodent and human microglial cells, which are located in several brain compartments such as the cortex, cerebellum, hippocampus, hypothalamus and olfactory system (Huang *et al.*, 2015; W. Huang, Min, Liu, He, & Peng, 2014; Kong *et al*., 2017). Many studies have suggested the involvement of TRPV1 during the activation of microglia. Activation of the microglial TRPV1 channel enhanced mitochondrial cytochrome c production which leads to microglia death through autophagic or apoptotic pathways (Kim, Kim, Oh, & Jin, 2006). Furthermore, a report suggested that activation of TRPV1 might induce mitochondrial disruption events, which result in MAPK activation and ROS production (Miyake, Shirakawa, Nakagawa, & Kaneko, 2015). However, TRPV1 activation caused an increase in the pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 in the brain of the febrile seizure mouse model (Huang *et al.*, 2015). This study reported that pre-treatment with TRPV1 antagonist SB366791 did not reverse the inhibitory effect of arvanil on pro-inflammatory cytokines in LPS-activated BV2 cells, indicating that arvanil might exert its anti-neuroinflammatory activity independent of interaction with TRPV1 receptors.

As observed in previous studies, CB1 receptors are expressed in microglia in a constitutive manner, while CB2 receptors are in an inducible way (Carayon *et al*., 1998; Waksman *et al.*, 1999), which suggested that both receptors have distinct functions in the microglia. Accordingly, it has been shown that rat primary microglia express CB1 receptors that cause NO production when it's activated (Cabral, Harmon, & Carlisle, 2002), whereas CB2 receptors are expressed predominantly on several *in vitro* immune cells, including BV2 and primary microglia (Walter *et al*., 2003). The result of this study demonstrated that both CB1 and CB2 receptors are expressed in BV2 microglia. To determine if the anti-inflammatory effects of arvanil were mediated by acting on CB1 or CB2 receptors, BV2 cells were treated with selective CB1 (SR141716) and CB2 (SR144528) receptor antagonists before incubation with arvanil and LPS. Interestingly, the results showed that the inhibitory effects of arvanil on LPS-induced elevated levels of TNF-α and IL-6 were not reversed by either the CB1 or CB2 receptor

antagonist. Instead, the CB1 (SR141716) and CB2 (SR144528) receptor antagonists reduced the LPS-induced production of TNF-$\alpha$ and IL-6.

To explain these results, two different studies are considered. The first by Kozela E. *et al*. 2010 suggested that pre-treatment with CB1 or CB2 receptor antagonists did not affect THC or CBD inhibitory impact on LPS-stimulated IL-1β production from BV2 cells. They also proposed that neither CB1 nor CB2 antagonists inhibit the LPS-induced IL-1β release when BV2 cells incubated with those antagonists alone, followed by LPS stimulation (Kozela *et al.*, 2010). The second study by Ribeiro *et al*. 2013 had a different perspective. They showed that inhibitory the effects of a cannabinoid agonist on LPS-induced ROS production and iNOS expression were not reversed by pre-treatment with CB1 and CB2 receptor antagonists. Moreover, they found that treatment with CB1 and CB2 receptor antagonists (such as SR141716A and SR144582, respectively) alone resulted in the reduction of ROS generation, iNOS induction, ERK1/2 phosphorylation, and activation of NF-κB in LPS-stimulated BV2 cells. Hence, they proposed that CB1 and CB2 cannabinoid receptor agonists and antagonists may block microglia activation through CB1 and CB2 receptor-independent systems (Ribeiro, Wen, Li, & Zhang, 2013). The outcome of this study was consistent with the second study's results as they focused on the effect of CBR agonists and antagonists on the generation of various pro-inflammatory mediators and the possibility of different pathways' involvement in their actions. Both arvanil and CB receptor antagonists possibly exert their effects through mechanisms independent of interaction with cannabinoid receptors.

It has been reported that activating the antioxidant Nrf2 pathway and its regulated proteins resulted in inhibition of neuroinflammation (Velagapudi *et al.*, 2017). Principally, activation of Nrf2/ARE signalling by enhancing nuclear localisation, raising the transcriptional activity of Nrf2, as well as increasing the expression levels of HO-1 occurs concomitantly with significant mitigation of the LPS-induced production of pro-inflammatory mediators such as NO, iNOS, COX-2, $PGE_2$, IL-1β, and TNF-α (Chan *et al*., 2017; C. Li *et al*., 2018). As shown earlier in this study, arvanil exhibited a significant

inhibitory activity on LPS-induced neuroinflammation by decreasing the protein levels of TNF-α, IL-6, NO, $PGE_2$, iNOS and COX-2. Therefore, more investigation was required on the possibility of involvement Nrf2/HO-1/NQO1 antioxidant axis in arvanil anti-neuroinflammatory activity.

This study showed that the level of HO-1 and NQO1 were not increased when BV2 cells were treated with arvanil. To confirm these results at nuclear level, the expressions of Nrf2 were measured as it is the primary regulator of antioxidant proteins HO-1 and NQO1 (Loboda, Damulewicz, Pyza, Jozkowicz, & Dulak, 2016). The western blot analysis of the nuclear extraction showed that arvanil could not up-regulate the nuclear accumulation of Nrf2. It is well-known that Nrf2 binds to the antioxidant response elements ARE in the nucleus to mediate the transcription of antioxidant genes (Motohashi & Yamamoto, 2004). However, arvanil could not increase the transcriptional activity of antioxidant responsive element (ARE) in BV2 cells. Altogether, arvanil anti-neuroinflammatory activity is not mediated by Nrf2/HO-1/NQO1 antioxidant axis.

Several factors may involve in the progression of neurodegeneration; oxidative stress-induced neuronal damage is one of the main contributors to diseases' pathogenesis (Ferreiro *et al*., 2012). Post-mortem analysis detected elevated levels of oxidative damage to DNA, proteins, lipids and neurons in neurodegenerative disorders (Niedzielska *et al*., 2016). $H_2O_2$ is the most important reactive oxygen species produced during oxidative stress and has been used widely to examine neuronal damage in neurodegenerative *in vitro* models. Several studies utilised $H_2O_2$ to induce oxidative stress and neuronal damage in the human neuroblastoma SH-SY5Y cells as a model to screen the neuroprotective effects of various pharmacologically active compounds such as trehalose, glucomoringin-isothiocyanate and artemisinin (Gao *et al*., 2018; Jaafaru *et al*., 2019; Zhao *et al.*, 2019). Cell viability results exhibited that exposure to 200μM of $H_2O_2$ was sufficient to induce oxidative damage to the SH-SY5Y cells. Surprisingly, arvanil in this study did not inhibit apoptosis in $H_2O_2$-induced neurotoxicity of SH-SY5Y cells and did not enhance the expression of neuronal markers (MAP-2 and

βIII-Tubulin), suggesting that arvanil possesses an anti-neuroinflammatory activity through inhibiting pro-inflammatory mediators without reversing neurotoxicity.

Generally, there are controversial results from studies on the neuroprotection capacity of cannabinoid receptor agonists. The cannabinoid $\Delta^9$-tetrahydrocannabinol ($\Delta^9$-THC) showed neuroprotection against oxidative stress in SH-SY5Y cells through the restoration of mitochondrial contents involved in its biogenesis (Zeissler *et al*., 2016). Furthermore, the CB1 receptor agonist arachidonyl-2-chloroethylamide (ACEA) significantly reverses the reduction in cell viability and reduced lactate dehydrogenase (LDH) release that results from oxygen-glucose deprivation/reoxygenation (OGD/R)-induced neuronal injuries (Shuai Yang *et al.*, 2020). On the other hand, JWH-133, a selective cannabinoid CB2 receptor agonist, induced neuronal damage in neuroblastoma SH-SY5Y cells by decreasing the cells' viability and proliferation rate in a concentration-dependent manner (Wojcieszak, Krzemień, & Zawilska, 2016). A recent study revealed that a synthetic CB1 and CB2 cannabinoid receptor agonist, JWH-018, produced oxidative stress in SH-SY5Y cells by reducing the level of antioxidant enzymes, which may be an underlying mechanism of JWH-018 neurotoxicity (Sezer, Jannuzzi, Huestis, & Alpertunga, 2020).

In summary, arvanil significantly inhibited the LPS-induced production of pro-inflammatory mediators. Arvanil exerted its anti-neuroinflammatory activity independent of the NF-κB signalling pathway. Moreover, arvanil did not reduce MAPKs pathway activation and did not inhibit Akt phosphorylation level. Additionally, arvanil's anti-neuroinflammatory activity was Nrf2/HO-1/NQO1 antioxidant axis-independent. The anti-neuroinflammatory effects of arvanil were not possibly mediated through binding to CB1, CB2 and TRPV1 receptors. The activity of arvanil was possibly mediated through inhibiting neuroinflammation without counteracting neurotoxicity. Arvanil may exert its anti-neuroinflammatory activity by direct action on post-transcriptional, translational or post-translational levels of pro-inflammatory mediators' production, or arvanil may act on another pathway that could modulate neuroinflammation such as SIRT1.

# 4 Chapter Four: Investigation on The Neuroprotective Activity of Fagaramide

## 4.1 Introduction

### 4.1.1 Background

Neuroinflammation is one of the hallmarks of several neurodegenerative disorders, such as AD, PD and ALS, where the pathogenesis of those disorders is partially related to the excessive production of inflammatory mediators by activated microglia (Guzman-Martinez *et al*., 2019; Stephenson, Nutma, van der Valk, & Amor, 2018; Von Bernhardi & Eugenín, 2012). Several signalling pathways are involved when the microglia are activated, such as IκB/NF-κB, MAPKs and PI3K/Akt signalling pathways which result in the overproduction of inflammatory mediators.

Various plant-origin compounds have shown anti-neuroinflammatory activity, which is mediated by inflammatory signalling pathways. A flavonoid from citrus fruit peels, tangeretin, exerted anti-inflammatory activity by reducing TNF-α, IL-6, IL-1β, NO and $PGE_2$ levels and inhibiting protein expression of iNOS and COX-2 in LPS-stimulated microglia through modulating NF-κB signalling pathway. The mechanism of tangeretin's action was explained by distinctly attenuating the LPS-induced IκB-α and IKK-β phosphorylation and inhibiting the nuclear translocation of the NF-κB subunit, p65 (Shu *et al*., 2014). Z. Li *et al*. (2019) demonstrated that a flavonoid compound found in most vegetables and fruits, called chrysin, had the ability to alleviate LPS-induced neuroinflammation in BV2 cells, primary microglial cells and mice by reducing NO, IL-6 and TNF-α production that mediated by targeting NF-κB pathway (Li *et al.*, 2019).

The molecular mechanism behind the anti-neuroinflammatory activity of some plant-derived compounds has been shown to be related to their effects on MAPKs signals besides other inflammatory pathways. Pterostilbene, found in grapes and berries, proved to reduce the production of IL-6, TNF-α and suppressed iNOS protein expression and its related products, NO, in LPS-activated microglia through targeting MAPK signalling pathways (Hou *et al*., 2015). Furthermore, a herbal

compound called scutellarin proved to attenuate the inflammatory responses in the rats' activated microglia/brain macrophage (AM/BM) by reducing iNOS, TNF-α, and IL-1β protein levels and the molecular mechanisms were associated with the ability of scutellarin to markedly decrease the expression of p-p38, p-JNK levels (H.-L. Chen *et al.*, 2020). Another compound derived from Elephantopus scaber aromatic herb, deoxyelephantopin, also produced anti-inflammatory activity against LPS-stimulated BV-2 cells through modulating MAPKs and PI3K/Akt-dependent NF-κB signalling pathways (Andy, Chan, & Kadir, 2017).

Moreover, a recent study investigated the neuroprotection activity of some bioactive flavonoids derived from natural products as it revealed that these agents could alleviate cyclophosphamide-induced SH-SY5Y cells death and apoptosis through decreasing the lipid peroxidation level and up-regulating the expression of anti-apoptotic proteins with downregulating the expression of proapoptotic ones (Ayna, Özbolat, & Darendelioglu, 2020).

### 4.1.2 Zanthoxylum zanthoxyloides

Medicinal plants and their derived compounds served for decades as a good source of therapeutic purposes for the treatment of several diseases, including diabetes mellitus, neurodegenerative diseases and cancer (A. K. Singhal, Naithani, & Bangar, 2012; Upadhyay, Panjwani, & Yadav, 2014). The genus *Zanthoxylum*, belonging to the *Rutaceae* family, is an important medicinal plant used worldwide. There are more than thirty-nine *Zanthoxylum* species identified, including *Zanthoxylum nitidum (Lu et al.*, 2020), *Zanthoxylum armatum* (Paul, Kumar, Singh, & Choudhary, 2018), *Zanthoxylum gilletii* (Sinan, Zengin, Bene, & Mahomoodally, 2019), *Zanthoxylum zanthoxyloides* (Tine, Yang, *et al*., 2017) and *Zanthoxylum heitzii* (Goodman *et al*., 2016).

*Zanthoxylum zanthoxyloides* is a popular *Zanthoxylum* species growing in Africa and enriched with several phytochemical compounds, including coumarins, alkaloids, fluoride, flavonoids, terpenes, oxylipin and zantholic acid (Dofuor *et al*., 2020; Wouatsa *et al*., 2013). Those extracted phytocompounds are responsible for the biological activity of *Zanthoxylum zanthoxyloides* against

malaria, cancer, cardiac palpitations, syphilis as well as many types of pain (Acheampong, Baffour, Addo, Essuman, & Boye, 2021; Misra, Wouatsa, Kumar, Kumar, & Tchoumbougnang, 2013; Tine, Diop, *et al*., 2017).

An *in vitro* study revealed the antidiabetic activity of *zanthoxyloides* root extracts against alloxan-induced diabetes in rat models (Amah *et al*., 2022). In addition, Leaf and trunk bark extracts of *Z. zanthoxyloides* showed anti-oxidant activities when tested for their radical scavenging activities using ABTS assay (Tine, Yang, *et al*., 2017). A recent study documented the anti-neuroinflammatory activities of *Zanthoxylum zanthoxyloides* root extracts in LPS-stimulated BV2 cells. The extracts were able to inhibit the pro-inflammatory cytokines and up-regulate anti-inflammatory mediators (Ogunrinade *et al*., 2021).

### 4.1.3 Fagaramide

The known extracted compounds from *Zanthoxylum zanthoxyloides* are trans-fagaramide (Figure ***4-1***) , iso-γ-fagarine ,arnottianamide, arctigenin methyl ether, iso-skimmianine, savinin, (+)-sesamin and (+)-eudesmin (Chaaib, Queiroz, Ndjoko, Diallo, & Hostettmann, 2003; Mbaze *et al*., 2007). All of these compounds exerted a non-cytotoxic effect when tested on PC3 Caucasian prostate adenocarcinoma cell line; also, they showed an obvious suppressive impact on phagocytosis response on the whole blood which activated with serum-opsonized zymosan (Mbaze *et al*., 2009). Most of them, including fagaramide, showed antimycobacterial activity against TB strains which will open the research gate to investigate the possibility of presenting them as promising TB drugs (Oloya *et al.*, 2021).

*In vivo* study demonstrated that fagaramide had a weak anti-inflammatory effect (Oriowo, 1982a). Recently, a study conducted by Nna *et al*. (2019) indicated the antifungal and antibacterial activities of fagaramide toward clinical and plant pathogens (Nna, Tor-Anyiin, & Igoli, 2019).

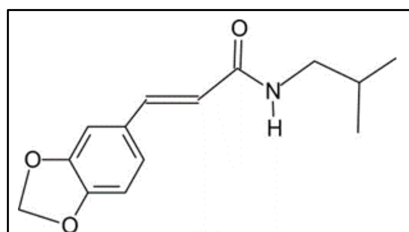



**Figure 4-1 The chemical structure of fagaramide.**

**(Bohle & Fotie, 2006).**

Skimmianine, structurally related to fagaramide and extracted from the same plant, exhibited a promising anti-inflammatory property and has a prospective therapeutic efficacy in inflammatory diseases. Interestingly, it was demonstrated to decrease the pro-inflammatory mediators' production, suppression of neutrophil infiltration, inhibition of histamine release and show some antioxidant effects by stimulating the activity of antioxidant enzymes (Garcia-Argaez, Apan, Delgado, Velázquez, & Martínez-Vázquez, 2000; Ratheesh, Sindhu, & Helen, 2013).

The information available on the neuroprotection activity of fagaramide is insufficient and needs more investigation. Accordingly, this chapter examined the anti-neuroinflammatory properties of fagaramide against inflammatory triggers using LPS-activated BV2 microglial cells. The molecular mechanisms of fagaramide were investigated on NF-κB, MAPKs (p38 and JNK) and Akt. The effect of fagaramide on the Nrf2/HO-1/NQO1-antioxidant axis was also studied. Additionally, fagaramide neuroprotective effects against $H_2O_2$- induced apoptosis in SH-SY5Y neuroblastoma cells were also highlighted.

## 4.2 Methodology

### 4.2.1 Preparation of compound and stimulant

Fagaramide was purchased from Sigma Aldrich. Fagaramide was prepared in DMSO at stock concentrations of 0.1 M, aliquoted and stored in -80 °C freezer. Prior to each experiment, a working concentration was prepared from the stock aliquots by dilution with DMSO to achieve the desired concentrations. For BV2 stimulation, 100 ng/ml LPS was prepared freshly prior to each experiment from the stock concentration (1mg/ml) by dilution with sterile PBS.

### 4.2.2 Treatment of BV2 cells with fagaramide

BV2 cells were seeded at a concentration of $4 \times 10^4$ cells/ml in a 96-well, 24-well and 6-well plates in 200 µl for 96-well, 1 ml for 24-well and 2 ml for 6-well plate of complete RPMI. When the cells were 80% confluent, the medium was changed to a serum-free RPMI for one hour. Afterwards, the cells were pre-incubated with different concentrations of fagaramide for 30 minutes, followed by stimulation with 100 ng/ml LPS and incubated for a preferred specific duration.

### 4.2.3 Treatment of SH-SY5Y cells with fagaramide

SH-SY5Y cells were seeded at concentration of $4 \times 10^4$ cells/ml in 96-well, 24-well and 6-well plates in 200 µl for 96-well, 1 ml for 24-well and 2 ml for 6-well plate and differentiated, as indicated in 2.1.2. DNase, RNase-free sterile water was used to prepare 200 µM $H_2O_2$ from stock concentration (9.8 M), which was used to induce SH-SY5Y cytotoxicity. After seven days of differentiation, the cells were treated with different concentrations of fagaramide for 30 minutes, followed by exposure to $H_2O_2$ and the plates were incubated in the incubator for 24 hours.

### 4.2.4 Assessment of Cell Viability

XTT assay and Non-Radioactive CytoTox 96® Assay were performed as described in 2.2.1 and 2.2.2, respectively.

### 4.2.5 Griess Assay

BV2 cells were seeded in a 96-well plate at $4 \times 10^4$ cells/ml in 200 µl of culture medium in each well and kept in 37°C incubator until confluence. Later, the cells were treated with fagaramide and stimulated with 100ng/ml of LPS, as indicated in 4.2.2. After 24-hours of incubation, the nitric oxide level was measured by Griess assay as described in 2.3.

### 4.2.6 ELISAs

BV2 cells were cultured in a 24-well plate at $4 \times 10^4$ cells/ml and incubated at 37°C until 80% confluence. Afterwards, the culture medium was changed to serum-free RPMI medium for 1 hour and

then treated with fagaramide and stimulated with LPS (100ng/ml) as indicated in 4.2.2 for 24 hours. Subsequently, the TNF-α and IL-6 levels were measured as detailed in section 2.4.

### 4.2.7 $PGE_2$ Level

BV2 microglia cells were seeded a in 24-well plate at a concentration of $4 \times 10^4$ cells/ml and incubated at 37°C. At 80% confluence, the cells were treated with fagaramide and stimulated with LPS (100ng/ml) as indicated in 4.2.2 for 24 hours. The concentrations of $PGE_2$ were measured, as described in section 2.5.

### 4.2.8 Western Blotting

BV2 cells were treated and stimulated as described in 4.2.2, while SH-SY5Y cells were treated as indicated in section 4.2.3. Isolations of cytoplasmic and nuclear lysates and protein quantification were performed as described in sections 2.6, 2.7 and 2.8, respectively. Then western blot analysis was performed as detailed in chapter two, section 2.10.

### 4.2.9 Reporter Gene Assay

BV2 cells were seeded into a 24-well plate at a concentration of $4 \times 10^4$ cells/ml and incubated at 37°C. At 80% confluence, the cells were transfected, as indicated in section 2.12. The cells were then treated with fagaramide and stimulated with 100 ng/ml LPS, and luminescence was measured as detailed in section 2.12 using Dual-Glo Luciferase assay (Promega).

### 4.2.10 Detection of Phosphorylated NF-κB p65

BV2 cells were seeded in a 6-well plate at a concentration of $4 \times 10^4$ cells/ml in 2 ml of culture medium and incubated at 37°C. At 80% confluence, cells were treated with fagaramide and stimulated with LPS (100ng/ml), as detailed in 4.2.2, for 60 minutes. NF-κB p65 (Phospho) InstantOne ELISA Kit (Invitrogen) was used as described in chapter two section 2.9.

### 4.2.11 Immunofluorescence

BV2 cells were seeded at a concentration of 4 × $10^4$ cells/ml into a 24-well plate and incubated at 37°C. At 80% confluence, cells were treated with fagaramide and stimulated with 100 ng/ml LPS, as detailed in section 4.2.2.

SH-SY5Y cells were seeded into a 24-well plate at a concentration of 4 × $10^4$ cells/ml. At 60% confluence, the medium was changed to the complete medium and 10µM retinoic acid (to initiate differentiation). Seven days after differentiation, the cells were treated, as indicated in section 4.2.3. Immunofluorescence assay was performed as detailed in chapter two section 2.13.

### 4.2.12 ROS Immunofluorescence

BV2 cells were seeded at a concentration of 4 × $10^4$ cells/ml into a 24-well plate and incubated at 37°C until confluence. Later, cells were treated with fagaramide and stimulated with 100ng/ml of LPS, as indicated in section 4.2.2 for 24 hours. DCFDA Cellular ROS Detection Assay (Abcam) was used to detect ROS, and the experiment has been described in chapter two section 2.14.

### 4.2.13 NF-κB p65 Transcription Factor Assay

BV2 cells were seeded at a concentration of 4 × $10^4$ cells/ml. At 80% confluence, the cells were treated with 0.1, 0.25 and 0.5 µM fagaramide and stimulated for 60 minutes with 100ng/ml LPS. Thereafter, nuclear extracts were collected, and the protein was quantified (as detailed in sections 2.7 and 2.8, respectively). NF-κB p65 transcription factor assay kit (Abcam) was used to evaluate the DNA binding activity of NF-κB p65, as detailed in chapter two section 2.11.

### 4.2.14 Statistical Analysis

Data were converted into % values of stimulant or negative control, then expressed as mean ±SEM and analysed by Graph Pad Prism statistical software version 7 (San Diego, USA) with one-way analysis of variance (one-way ANOVA) followed by Dunnett's Multiple Comparison Test (version 5.0, Prism software, Graph Pad, USA). Three separate experiments were done for each analysis (n=3). Values of $p<0.05$ were taken as being statistically significant.

## 4.3 Results

### 4.3.1 Concentrations of fagaramide used in the experiments did not decrease the BV2 microglia viability

XTT assay demonstrated that fagaramide treatment at concentrations of 1, 2.5, 5, 10 and 20 µM alone did not change the viability of BV2 microglia (Figure ***4-2***a). In addition, the viability was not affected significantly compared with untreated cells when BV2 cells were treated with those concentrations prior to stimulation with 100 ng/ml of LPS (Figure ***4-2***b). Similarly, concentrations of 1, 2.5, 5, 10 and 20 µM of fagaramide showed insignificant alteration in LDH release to the cell culture medium compared with LPS-stimulated BV2 cells (Figure ***4-2***c).

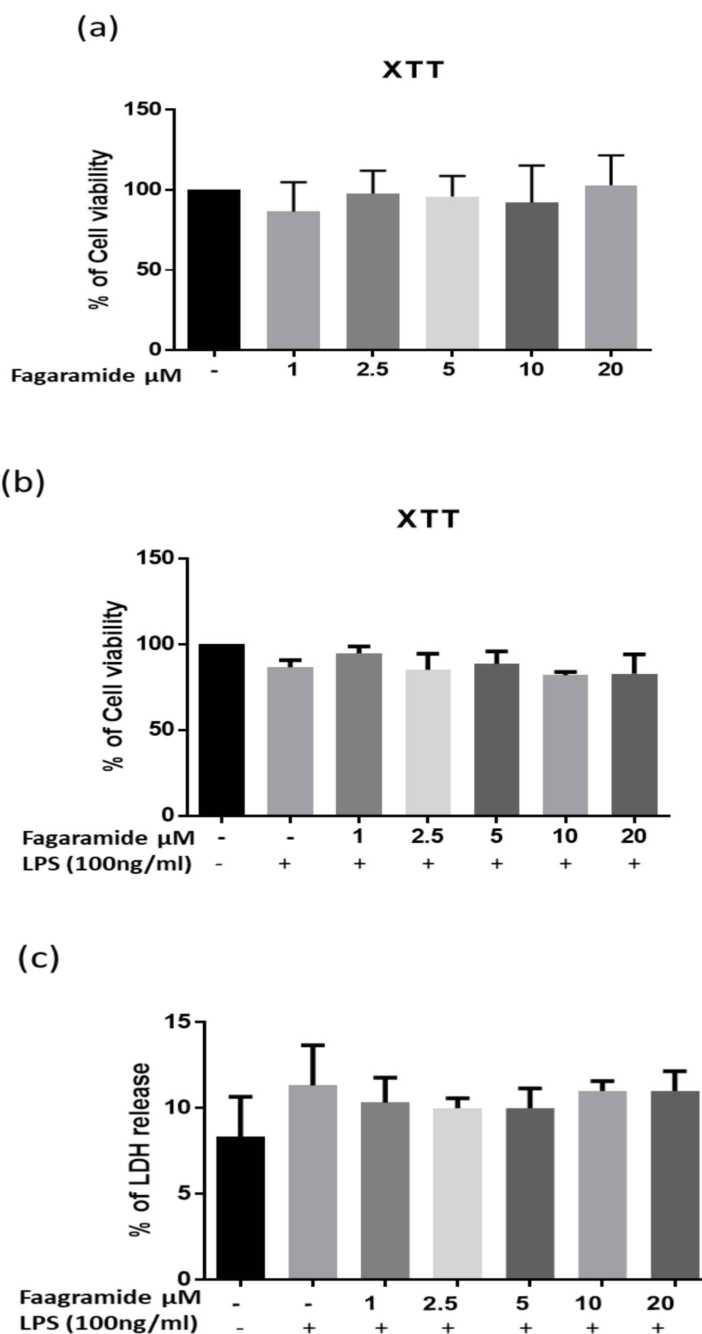


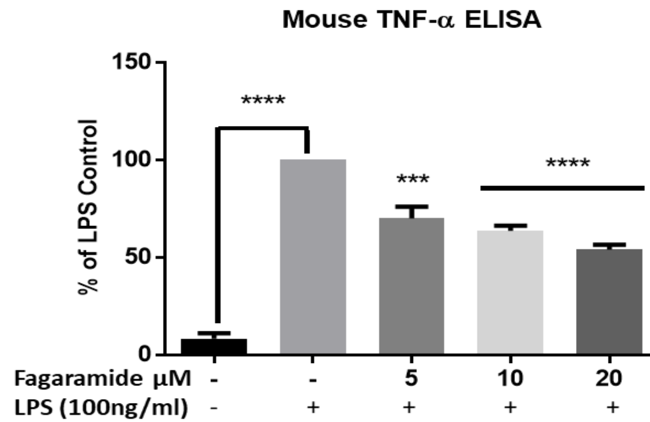

**Figure 4-2 Microglia viability did not change when pre-treated with fagaramide in the presence or absence of LPS by using XTT and cytotoxicity assays.**

(a) BV2 microglia were incubated with various concentrations of Fagaramide for 24hr, and then 25 µl of XTT/PMS was added to stop the experiments. (b) XTT assay was performed on BV2 microglia cells that were pre-treated with different concentrations of fagaramide for 30 min and then stimulated with 100ng/ml of LPS for 24 hr. (c) cytotoxicity assay was used to reveal the level of LDH released from damaged BV2 cells when pre-treated with various fagaramide concentrations for 30 min followed by stimulation with LPS for 9 hr. Data for three independent experiments were presented as mean ± SEM and were analysed using one-way ANOVA followed by Dunnett's Multiple Comparison Test. *p<0.05, **p<0.01, *** p< 0.001 and **** p< 0.0001 versus untreated control cells for XTT assay, whereas * p< 0.05 **p<0.01 and *** p< 0.001 versus LPS-stimulated BV2 cells for non-radioactive cytotoxicity assay.

### 4.3.2 Pre-treatment with fagaramide reduced level of pro-inflammatory cytokines in LPS-activated BV2 microglia

It has been well reported that activated microglia in neuroinflammation release several pro-inflammatory mediators such as TNF-α and IL-6 while attenuating their production by pharmacologically active compounds could alleviate the disease condition (Cianciulli, Salvatore, Porro, Trotta, & Panaro, 2016; Subedi *et al*., 2019). The levels of TNF-α and IL-6, produced from LPS-stimulated BV2 microglia pre-treated with fagaramide, were measured in the supernatant using an ELISA assay. A significant increase ($p< 0.0001$) in both TNF-α and IL-6 levels has been noticed in LPS-stimulated BV2 cells when compared with untreated cells (Figure ***4-3***). The results revealed that the level of TNF-α was significantly ($p< 0.0001$) decreased in LPS-stimulated cells after treatment with 10µM (~1.6-fold reduction) and 20 µM (~1.9-fold reduction) of fagaramide, whereas pre-treatment with 5µM caused ~1.4-fold reduction when compared with LPS-stimulated cells (Figure ***4-3***a). However, pre-treatment of activated BV2 microglia with 10 µM (~1.2-fold reduction) and 20 µM (~1.3-fold reduction) of fagaramide produced a significant reduction ($p< 0.05$) and ($p< 0.001$) in IL-6 production, respectively. Pre-treatment with 5 µM displayed a non-significant inhibitory impact on IL-6 level (Figure ***4-3***b).

(a)




(b)

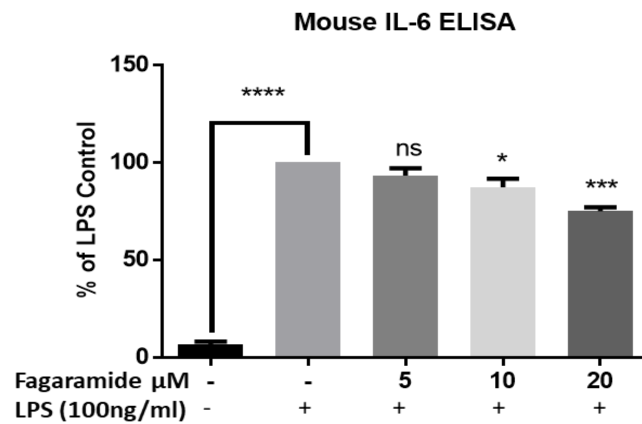



**Figure 4-3 Fagaramide decrease the production of TNF-α and IL-6 from LPS-activated microglia.**

The supernatant levels of (a) TNF-α and (b) IL-6 were determined using ELISA. BV2 microglia were treated with 5, 10 and 20 µM for 30 min followed by 100 ng/ml LPS stimulation for 24 hr. Fagaramide inhibited TNF-α and IL-6 production in LPS-stimulated BV2 microglia. Data for three independent experiments were presented as mean ± SEM and were analysed using one-way ANOVA followed by Dunnett's Multiple Comparison Test. *p<0.05, **p<0.01, *** p< 0.001 and **** p< 0.0001 compared to LPS-stimulated BV2 cells.

### 4.3.3 Fagaramide inhibited nitrite level and iNOS protein expression in LPS-activated BV2 microglia

Studies showed that LPS-activated microglia increased the expression of iNOS and its resultant nitric oxide (Habashi, Sabouni, Moghimi, & Majd, 2016; Yang *et al*., 2020). Therefore, the effects of fagaramide on the level of NO-mediated iNOS expression in LPS-stimulated microglia were highlighted in this section. BV2 microglia were treated with 5, 10 and 20 µM fagaramide for 30 minutes and stimulated with 100ng/ml LPS for 24 hr; then the supernatant was collected for Griess assay and the cytoplasmic extraction for western blot. The results demonstrated that both nitrite level (~5-fold increase) and iNOS expression were increased dramatically ($p < 0.001$) when BV2 cells were stimulated with 100ng/ml LPS compared with untreated cells. Nitrite productions were decreased when BV2 cells were treated with 5 µM (~1.2-fold reduction), 10 µM (~1.4-fold reduction) and 20 µM (~1.7-fold reduction) prior to LPS stimulation (Figure ***4-4***a). In addition, treatment with fagaramide significantly attenuated, 10 µM ($p<0.01$) (~1.7-fold reduction) and 20 µM ($p < 0.001$) (~2.9-fold reduction), LPS-induced iNOS protein expression (Figure ***4-4***b).

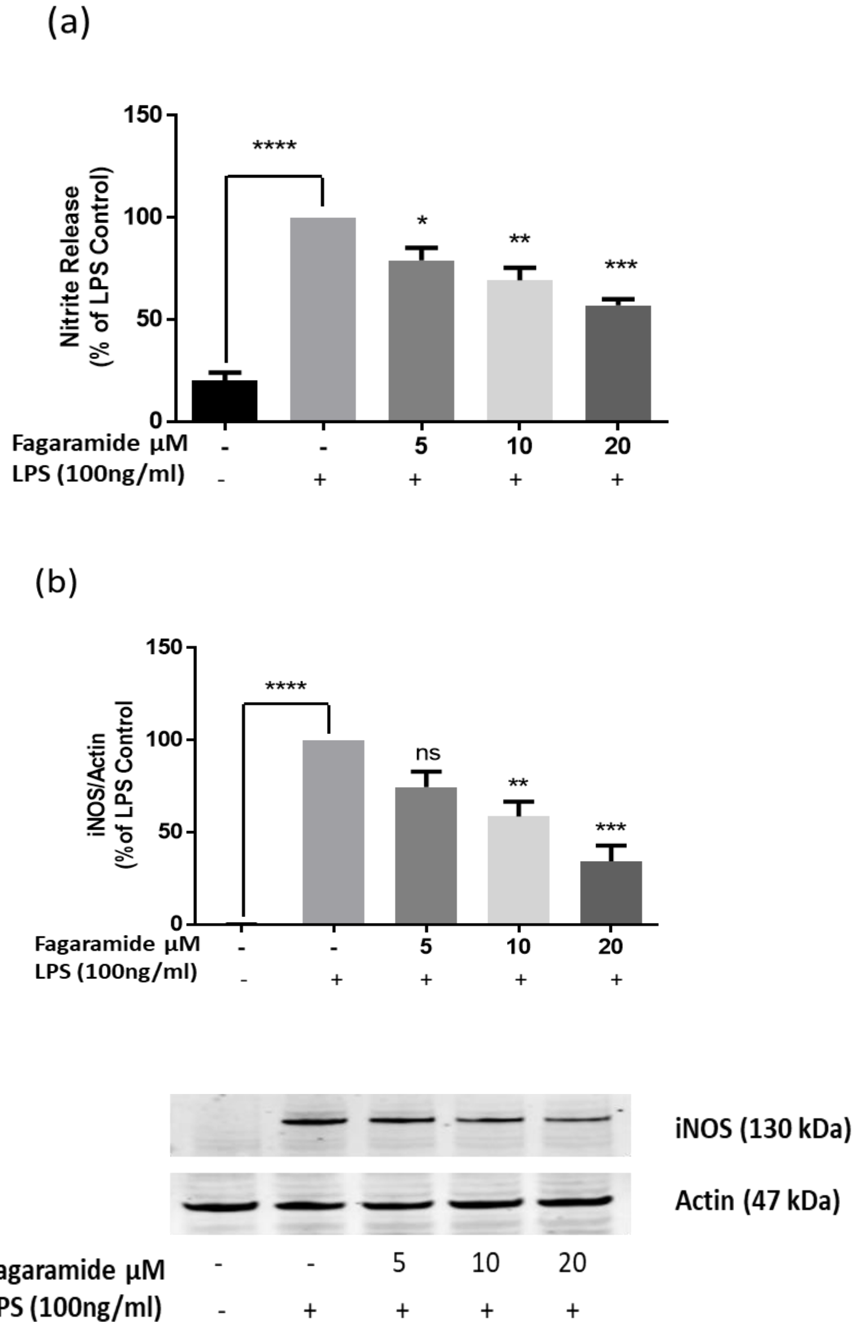



**Figure 4-4 Inhibition of iNOS expression and nitrite production from activated BV2 microglia after treatment with fagaramide.**

(a) nitrite released level was determined by Griess assay after 24 hr incubation with fagaramide in the stimulated BV2 cells, and its level was decreased when compared with LPS-stimulated BV2 cells. (b) BV2 cells treated with fagaramide and stimulated with LPS showed a marked reduction in iNOS protein expression compared with stimulated cells using the cytoplasmic extraction in the western blot analysis. Actin was used as an internal control. Data for three independent experiments were presented as mean ± SEM and were analysed using one-way ANOVA followed by Dunnett's Multiple Comparison Test. *$p<0.05$, **$p<0.01$, *** $p< 0.001$ and **** $p< 0.0001$ compared to LPS-stimulated BV2 cells.

### 4.3.4 Fagaramide inhibited production of $PGE_2$ and COX-2 protein expression in LPS-activated BV2 microglia

For further understanding of fagaramide impacts on activated microglia, COX-2 protein expression and its metabolic products $PGE_2$ were investigated. Generally, microglia increase the expression levels of COX-2 and $PGE_2$ when activated by external stimuli such as LPS (Yao *et al*., 2019; Zhu *et al.*, 2018). The results revealed that LPS-activated cells produced a significant ($p < 0.0001$) increased level of $PGE_2$ (~5-fold increase) compared to unstimulated cells. When BV2 microglia cells were treated with 20 µM fagaramide and then stimulated with LPS, the $PGE_2$ production was decreased (~1.4-fold reduction) significantly ($P < 0.05$), while at 5µM and 10µM the inhibitory impact was not significant compared to activated cells (Figure ***4-5***a).

The COX-2 protein expression was assessed by using cytoplasmic extractions with western blot, and the results of this study exhibited a significant ($p < 0.001$) increase in the expression of COX-2 (~2.2-fold increase) in LPS-stimulated BV2 cells compared to unstimulated cells. In addition, pre-incubation of stimulated BV2 cells with 20 µM fagaramide decreased the expression level of COX-2 dramatically ($P < 0.05$) (~1.4-fold reduction). While at 5µM and 10µM fagaramide, the COX-2 expression was not decreased significantly compared to LPS-activated BV2 cells (Figure ***4-5***b).

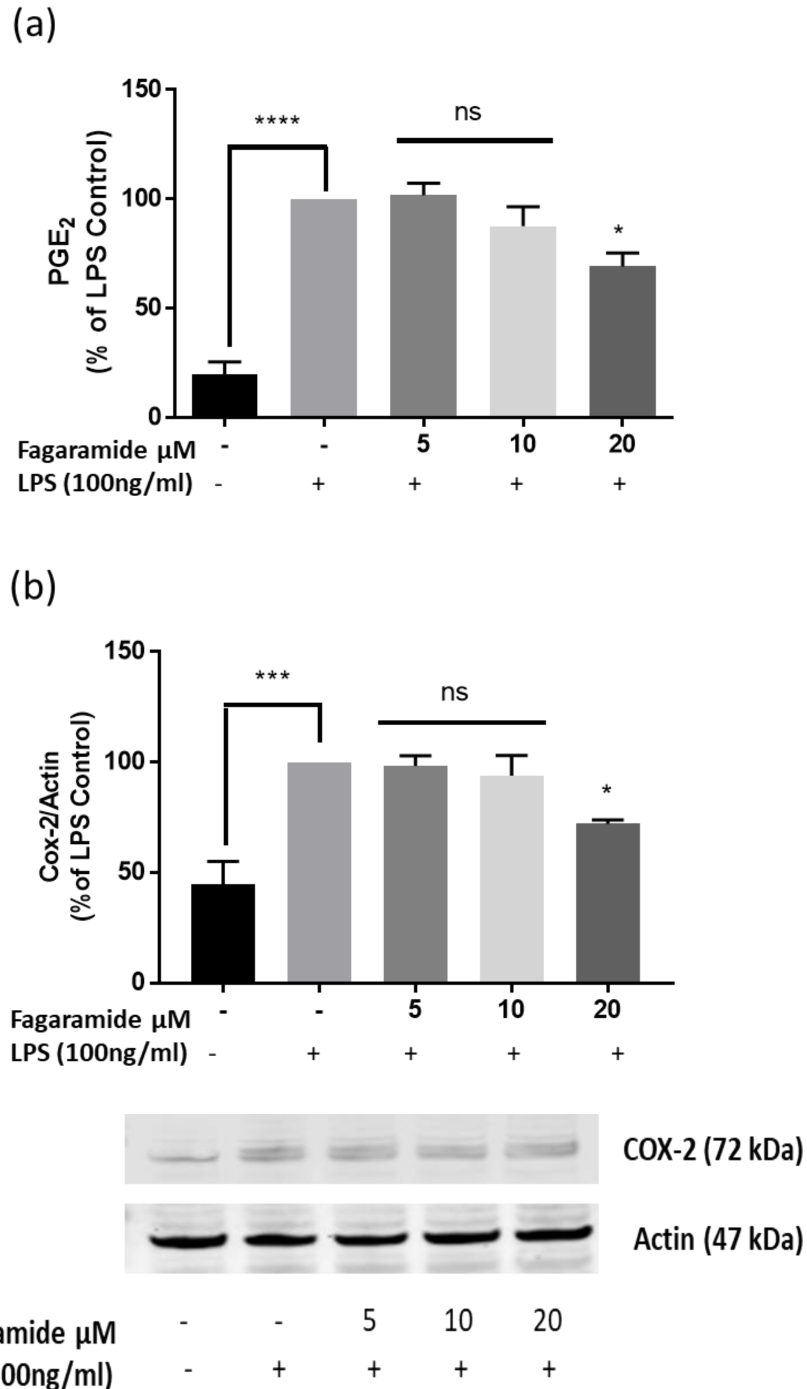



**Figure 4-5 Fagaramide reduced $PGE_2$ level and COX-2 protein expression in LPS-activated BV2 microglia.**

BV2 cells were treated with 5, 10 and 20 µM fagaramide for 30 min and stimulated for 24hr with 100ng/ml LPS. The supernatant and cytoplasmic extractions were collected to obtain $PGE_2$ and COX-2 expression with western blot, respectively. (a) fagaramide at 20 µM could significantly decrease $PGE_2$ production in LPS-activated BV2 cells. (b) the inhibitory effect of fagaramide on COX-2 was observed when the cells were incubated with 20 µM of the compound and then stimulated with LPS. Actin was used as an internal control. Data for three independent experiments were presented as mean ± SEM and were analysed using one-way ANOVA followed by Dunnett's Multiple Comparison Test. *$p<0.05$, **$p<0.01$, *** $p< 0.001$ and **** $p< 0.0001$ compared to LPS-stimulated BV2 cells.

### 4.3.5 Pre-treatment with fagaramide did not decrease ROS production in LPS-stimulated BV2 cells

Progressive impairment of mitochondrial function in neurodegenerative diseases mediated excessive generation of ROS that participated in the disease pathogenicity (Misrani, Tabassum, & Yang, 2021; Selfridge *et al*., 2013; Tobore, 2019). Several pieces of evidence suggested that maintaining ROS at a low level and controlling its production attenuate neuronal death and alleviate disease progression (Kim & Song, 2016; Xie *et al.*, 2017). It has been reported that the pharmacologically active compounds with anti-neuroinflammatory activity may show anti-oxidant effects by reducing ROS levels (Peng *et al*., 2020; You *et al*., 2018); therefore, the effect of fagaramide on ROS was examined in this study. The DCFDA-fluorescent experiment was performed using fluorescent microscopy to visualize the generation of ROS in LPS-stimulated BV2 cells and the result showed that LPS increased the production of ROS when compared to untreated cells (Figure ***4-6***). However, pre-incubation with 5, 10 and 20μM of fagaramide had no inhibitory effects on LPS-induced ROS generation in BV2 cells.

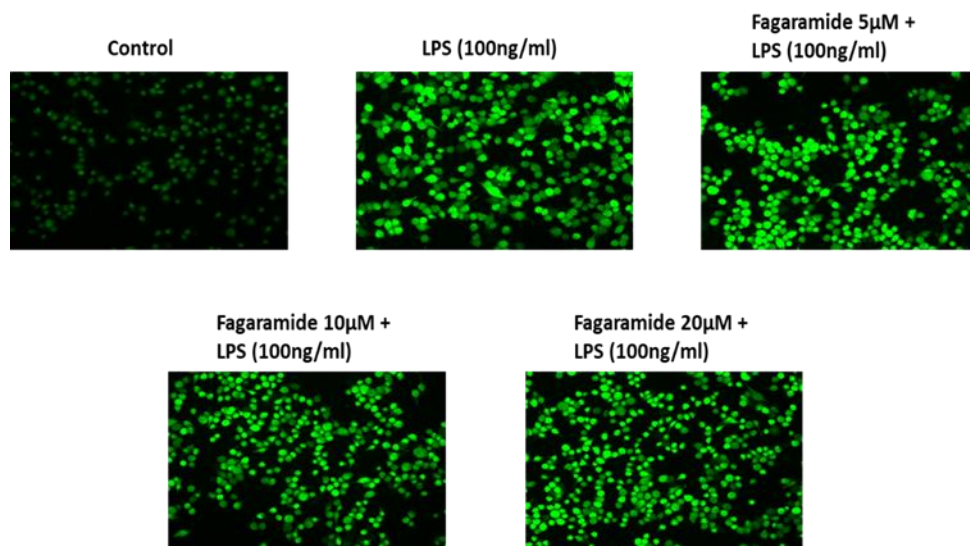



**Figure 4-6 Fagaramide did not decrease the level of reactive oxygen species in LPS-stimulated microglia.**

BV2 microglia were treated with 5, 10 and 20 μM fagaramide for 30min before stimulation with 100 ng/ml of LPS for 24hr. ROS production in BV2 microglia was measured by using a DCFDA assay kit. The cells were stained by adding 500μl/well of 20μM DCFDA solution and incubated at 37°C for 45 minutes in the dark, and the fluorescent were read with EVOS® FLoid® Cell Imaging System. Pre-incubation with fagaramide could not inhibit ROS generation in LPS-stimulated cells. Images were processed by image J analysis software, and the scale bar=100 μm.

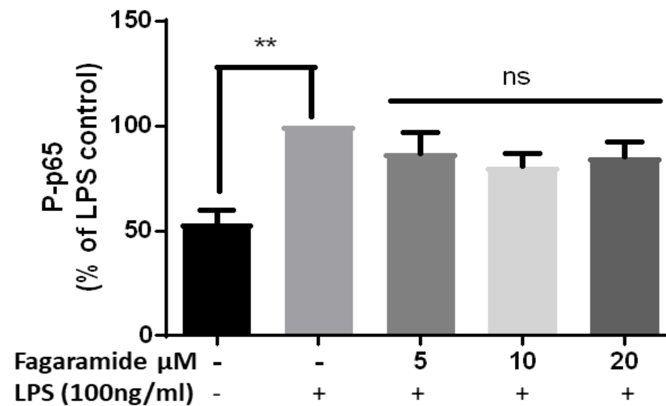
### 4.3.6 The effect of fagaramide on NF-κB signalling pathway in LPS-activated BV2 microglia

When microglia are stimulated, they generate pro-inflammatory mediators by involving various inflammatory signalling pathways; however, the NF-κB signalling cascade is the predominant engaged one (Ju Hwang, Choi, Park, & Hong, 2019; Shih, Wang, & Yang, 2015). Therefore, the effects of fagaramide on the activity of this pathway were investigated in this section.

Firstly, the impact of fagaramide on p-p65 NF-κB activation has been studied with NF-κB phospho-p65 ELISA assay using cytoplasmic extractions. The time needed for LPS stimulation was 60 minutes, as concluded in section 3.3.6. BV2 cells were stimulated with 100ng/ml LPS for 60 minutes in the absence or presence of different concentrations of fagaramide and the cytoplasmic extracts were analysed. The phosphorylated p65 level was remarkably ($p<0.01$) increased in LPS-stimulated BV2 cells (~1.9-fold increase) compared to unstimulated cells. Interestingly, pre-treatment with fagaramide at 5, 10, and 20 µM did not show an inhibitory effect on the phosphorylation of p65 in the stimulated BV2 microglia (Figure ***4-7***a). Immunofluorescence experiments have been accomplished to emphasise these findings. The results in Figure ***4-7***b indicated a high expression level of p-p65 in the LPS-challenged BV2 cells compared to unstimulated cells, whereas the pre-treated cells with fagaramide did not inhibit p-p65 expression in the stimulated cells.

Luciferase reporter gene assay has been used for further analyse fagaramide effects on the NF-κB signalling pathway through mediating the nuclear transactivation of NF-κB. Transfection with a vector comprising NF-κB regulated luciferase reporter was achieved, and the 6-hr activation showed the maximum expression of the NF-κB modulated luciferase reporter gene compared to unstimulated transfected BV2 cells. The results in Figure ***4-8***a indicated a significant ($p<0.01$) increase (~3.3-fold increase) in the expression of the NF-κB modulated luciferase reporter gene in the transfected LPS-stimulated cells compared to unstimulated transfected BV2 cells. Whereas pre-incubation of transfected cells with 5, 10 and 20 µM of fagaramide for 6-hr displayed a nonsignificant change compared to LPS-stimulated transfected cells. Additionally, BV2 cells were treated with fagaramide

followed by stimulation with 100 ng/ml LPS for 60 minutes to evaluate the NF-κB -DNA binding activity by enzyme-linked immunosorbent assay. The results demonstrated that LPS stimulation activated (~2.2-fold increase) the DNA binding of NF-κB significantly ($p<0.001$) compared to unstimulated BV2 cells, while this DNA binding activity was not inhibited significantly when BV2 cell pre-incubated with 5, 10 and 20 μM of fagaramide compared to stimulated BV2 cells (Figure ***4-8***b).



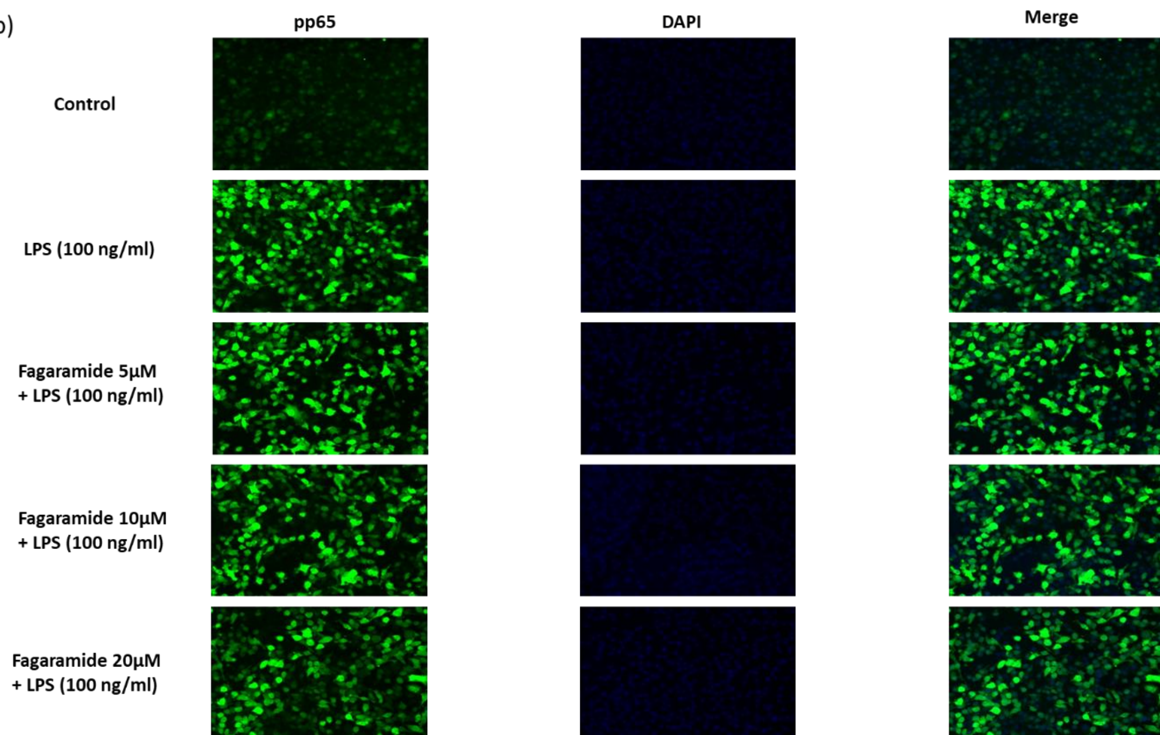




**Figure 4-7 Fagaramide pre-treatment did not change LPS-induced expression of phosphorylated p65 in BV2 microglia.**

(a) Cytoplasmic extractions were collected after incubating BV2 cells with fagaramide and stimulation with LPS for 60 min. p-p65 NF-κB ELISA analysis revealed that fagaramide pre-treatment did not affect the LPS-induced expression of p-p65. (b) BV2 cells were treated with fagaramide and stimulated with LPS for 60 min; then p-p65 antibody was used to label the cells, followed by incubation with DAPI. The fluorescence images confirmed that fagaramide concentrations could not inhibit the expression of p-p65 induced by LPS. Images were processed by image J analysis software and the scale bar=100 µm. Data for three independent experiments were presented as mean ± SEM and were analysed using one-way ANOVA followed by Dunnett's Multiple Comparison Test. *p<0.05, **p<0.01, *** p< 0.001 and **** p< 0.0001 compared to LPS-stimulated BV2 cells.

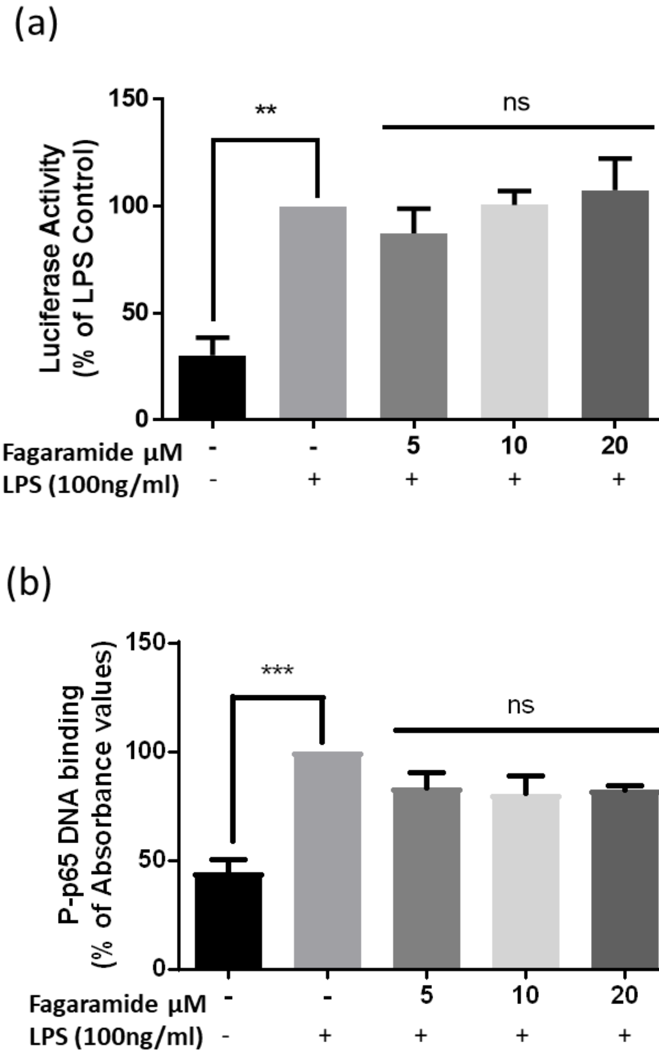



**Figure 4-8 Pre-treatment with fagaramide did not affect NF-κB signalling pathway at nucleus level in the LPS-activated BV2 cells.**

(a) BV2 cells were transfected with NF-κB luciferase reporter, then treated with fagaramide and stimulated with LPS. After 6-hr of stimulation, luciferase Dual Glo was used to read the luminescence reaction. Fagaramide pre-treatment did not inhibit NF-κB activity induced by LPS in transfected BV2 cells. Renilla luciferase was used as an internal control. (b) The nuclear extractions from BV2 cells pre-treated with fagaramide and stimulated with LPS for 60 min have been collected and analysed by ELISA-based enzyme-linked immunosorbent assay to study NF-κB -DNA binding activity. The LPS-induced DNA binding capacity of p65 was not decreased by fagaramide in BV2 microglia. Data for three independent experiments were presented as mean ± SEM and were analysed using one-way ANOVA followed by Dunnett's Multiple Comparison Test. *p<0.05, **p<0.01, *** p< 0.001 and **** p< 0.0001 compared to LPS-stimulated BV2 cells.

### 4.3.7 Fagaramide activity on IκBα level in LPS stimulated BV2 microglia

To investigate the effect of fagaramide on upstream proteins in the NF-κB signalling pathway, the phosphorylation and degradation of IκBα were studied after pre-treatment of stimulated BV2 cells with fagaramide. The results showed the stimulated cells significantly ($p<0.05$) upregulated phospho-IκBα expression (~2.5-fold increase) when compared to unstimulated BV2 cells. However, this expression was not inhibited significantly when BV2 cells pre-incubated with 5, 10 and 20 µM of fagaramide and stimulated with 100ng/ml LPS for 30 minutes (Figure ***4-9***a).

On the other hand, the total-IκBα level was measured by analysis of the cytoplasmic extraction with western blot. Figure ***4-9***b revealed that the unstimulated BV2 cells displayed a high level of total-IκBα protein (~1.6-fold increase) when compared to LPS-stimulated microglia ($p<0.05$). Interestingly, pre-incubation with 10 µM and 20 µM of fagaramide inhibited total-IκBα degradation significantly ($p<0.05$ and $p<0.01$ respectively) (10 µM; ~1.7-fold, 20 µM; ~1.9-fold increase). However, pre-treatment with 5 µM of fagaramide did not inhibit total-IκBα degradation in the stimulated microglia.

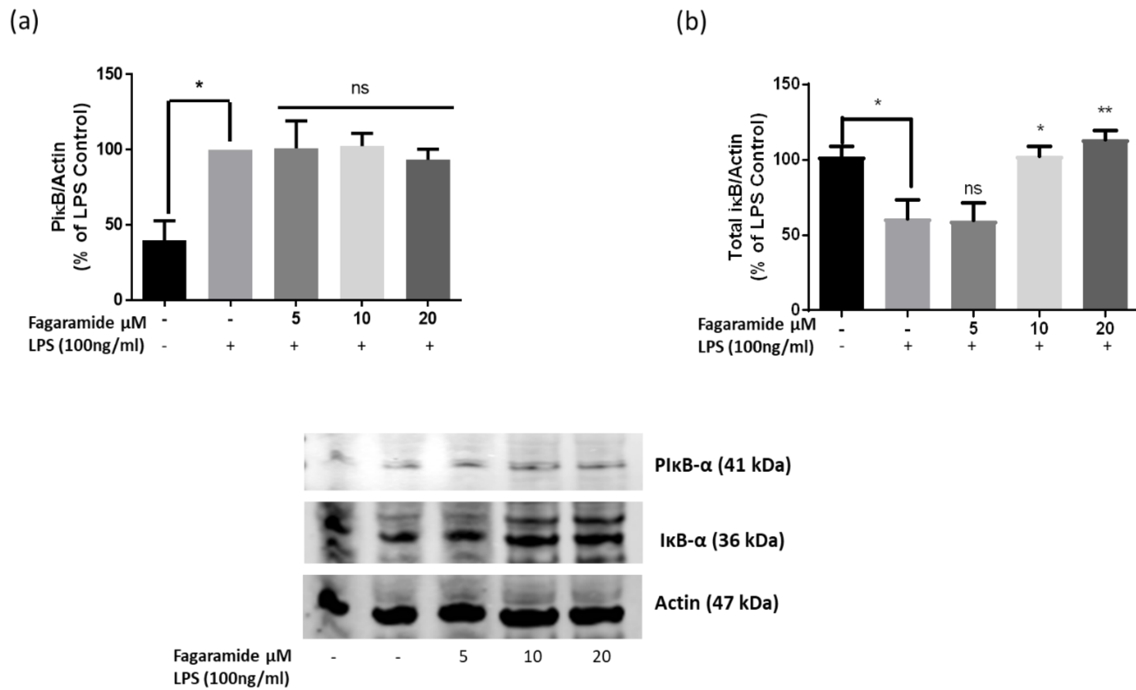



**Figure 4-9 Fagaramide decreased IκBα degradation in LPS stimulated BV2 microglia.**

The microglia were pre-treated with different concentrations of fagaramide for 30min and stimulated with 100ng/mL of LPS for 30 min; then, the cytoplasmic extractions were collected and analysed by western blot. (a) Fagaramide did not inhibit the phosphorylated protein expression of IκBα induced by LPS. (b) pre-treatment with 10 and 20 µM fagaramide inhibited the IκBα degradation in the LPS-stimulated BV2 cells. Actin was used as a loading control. Data for three independent experiments were presented as mean ± SEM and were analysed using one-way ANOVA followed by Dunnett's Multiple Comparison Test. *p<0.05, **p<0.01, *** p< 0.001 and **** p< 0.0001 compared to LPS-stimulated BV2 cells.

### 4.3.8 The anti-inflammatory activity of fagaramide is independent on MAPK signalling pathways

Fagaramide exerted its anti-neuroinflammatory independence on the NF-κB signalling pathway; therefore, an attempt was made to discover whether the anti-neuroinflammatory effects were mediated by another signalling pathway involved in neuroinflammation, such as MAPKs signalling cascade. To evaluate the effects of fagaramide on the p38 MAP kinase pathway, BV2 microglia were treated with fagaramide for 30 minutes; then stimulated with 100 ng/ml LPS for 60 minutes. The results displayed that the LPS-stimulated cells expressed a remarkably high ($p< 0.05$) protein level (~2.8-fold increase) of phospho-p38 compared to non-stimulated cells. However, pre-incubation with 5, 10 and 20 µM of fagaramide did not inhibit LPS-induced high levels of phospho-p38 (Figure ***4-10***a). Another family member of MAPK, such as JNK, might involve in neuroinflammation pathogenicity and triggered by various stress stimuli (such as LPS) to produce pro-inflammatory mediators from activated microglia (Anfinogenova, Quinn, Schepetkin, & Atochin, 2020; Waetzig *et al*., 2005; L. Yan *et al*., 2013). Accordingly, LPS-stimulated BV2 cells expressed a significant ($p< 0.001$) high protein level of phospho-JNK (~3-fold increase) compared to non-stimulated cells. Whereas pre-incubations with 5, 10 and 20 µM of fagaramide were not decreasing the phospho-JNK protein level induced by LPS exposure (Figure ***4-10***b).

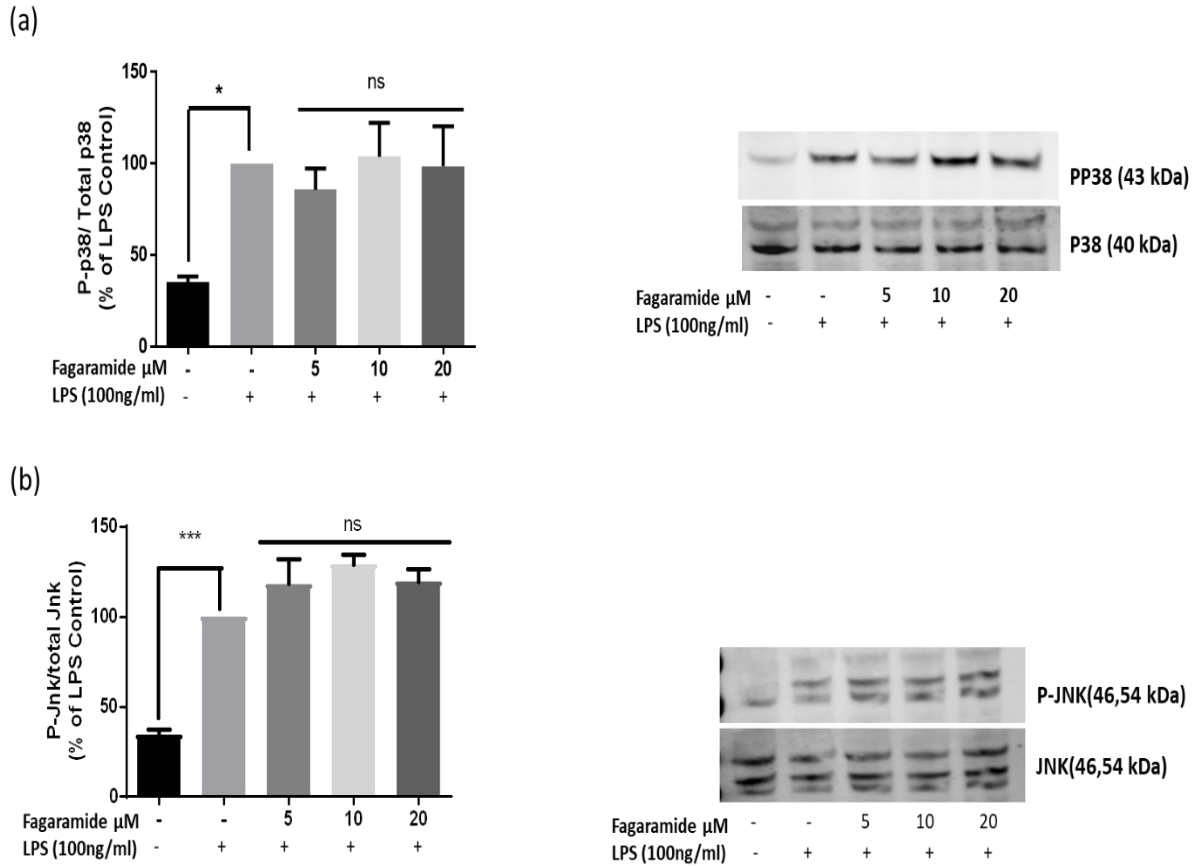



**Figure 4-10 Fagaramide pre-incubation of activated BV2 microglia did not affect the phosphorylation of p38 and JNK protein levels.**

BV2 cells were treated with different concentrations of fagaramide and stimulated with LPS for 60 min; then, the cytoplasmic extractions were collected and analysed with western blot. (a) the inhibition effects of fagaramide on p-p38 protein level were insignificant in LPS-stimulated cells. Total p38 was used as an internal control. (b) LPS-induced a high level of p-JNK was not attenuated by fagaramide pre-treatment. The total JNK was used as an internal control. Data for three independent experiments were presented as mean ± SEM and were analysed using one-way ANOVA followed by Dunnett's Multiple Comparison Test. *p<0.05, **p<0.01, *** p< 0.001 and **** p< 0.0001 compared to LPS-stimulated BV2 cells.

### 4.3.9 The anti-inflammatory activity of fagaramide is not mediated through inhibition of Akt protein in LPS-activated BV2 microglia

The molecular mechanism behind the anti-neuroinflammatory activity of fagaramide is still unclear; therefore, the inhibition effect of fagaramide on the Akt phosphorylation level has been investigated in this study. It has been reported that LPS increased the production of inflammatory mediators from microglia by activation of the PI3K/Akt signalling pathway, while this effect was inhibited when the cells were treated with powerful anti-inflammatory compounds (Gao *et al.*, 2020; Jung *et al.*, 2017). BV2 cells that were stimulated with LPS for 60 minutes showed a significant (p< 0.05) increase in the protein level of phospho-Akt (~1.8-fold increase) compared to untreated cells. However, there were insignificant changes in the expression of p-Akt level when the cells were pre-treated with 5, 10 and 20 µM fagaramide and stimulated with 100 ng/ml LPS (Figure ***4-11***). As a result, further investigation into this signalling pathway was unnecessary as fagaramide-mediated protection against LPS-induced neuroinflammation was independent of Akt proteins modulation.

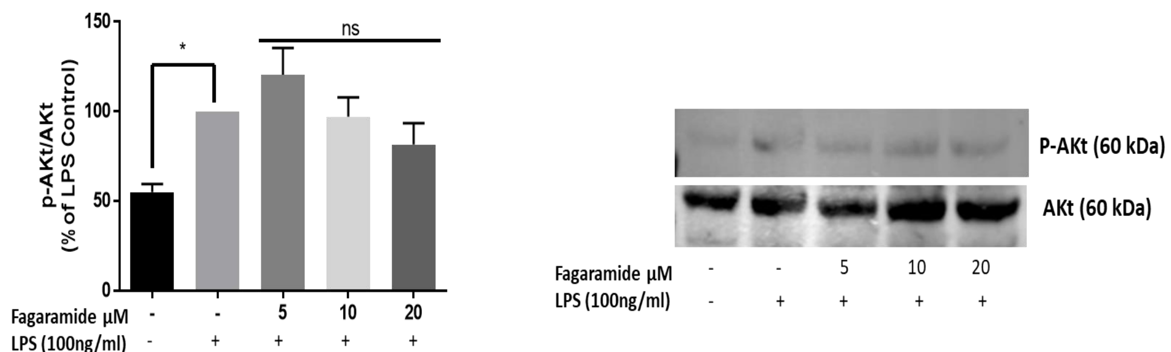



**Figure 4-11 Fagaramide has a nonsignificant activity on the LPS-stimulated phosphorylation of Akt protein.**

BV2 microglia were pre-treated with 5, 10 and 20 µM Fagaramide for 30 min followed by stimulation with 100 ng/ml LPS for 60 min, then the cytoplasmic extractions were collected and processed with western blot. The results revealed that fagaramide could not suppress the elevated level of phospho-Akt induced by LPS. Total Akt was used as an internal control. Data for three independent experiments were presented as mean ± SEM and were analysed using one-way ANOVA followed by Dunnett's Multiple Comparison Test. *p<0.05, **p<0.01, *** p< 0.001 and **** p< 0.0001 compared to LPS-stimulated BV2 cells.

### 4.3.10 Fagaramide did not ameliorate the protein levels of HO-1 and NQO1 in BV2 microglia cells

The level of antioxidant proteins HO-1 and NQO1 are maintained low under normal physiological conditions, whereas their expressions are increased either by the action of pharmacologically active compounds or as a defence protective barrier against oxidative challenges (Bi *et al*., 2018; Chen *et al*., 2017; Loboda, Damulewicz, Pyza, Jozkowicz, & Dulak, 2016). Decreased levels of antioxidant proteins can enhance the over-production of pro-inflammatory mediators such as iNOS and COX-2, while up-regulation of these antioxidants has been documented to produce anti-inflammation as well as the antioxidant effects (Chen *et al*., 2017; Lv *et al*., 2016). There is a crosstalk relation between the increased level of antioxidant proteins and the decreased level of pro-inflammatory molecules. Therefore, this study examined whether the neuroinflammation inhibitory effects of fagaramide are mediated through the upregulation of antioxidant elements; HO-1 and NQO1. Western blot analysis of cytoplasmic extracts revealed that fagaramide could not increase the expression of HO-1 and NQO1 in BV2 microglia cells (Figure ***4-12***).

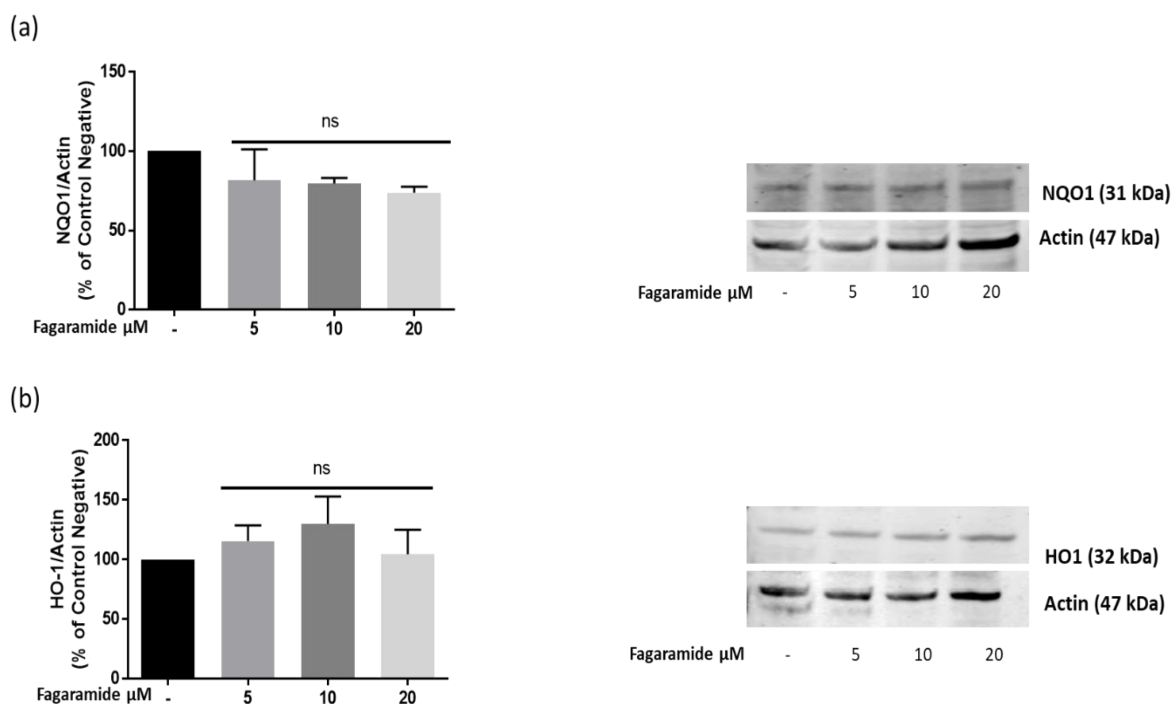



**Figure 4-12 The expressions of NQO1 and HO-1 were not increased after treatment of BV2 microglia with fagaramide.**

BV2 microglia were treated with 5, 10 and 20 µM fagaramide for 24hr; then, the cytoplasmic extractions were collected and analysed by western blot. The results revealed that fagaramide did not raise the protein expressions of (a)NQO1 and (b) HO-1 compared to untreated cells. Actin was used as a loading control. Data for three independent experiments were presented as mean ± SEM and were analysed using one-way ANOVA followed by Dunnett's Multiple Comparison Test. *$p<0.05$, **$p<0.01$, *** $p< 0.001$ and **** $p< 0.0001$ compared to untreated cells.

### 4.3.11 Fagaramide did not up-regulate Nrf2/ARE signalling pathway in BV2 microglia cells

It has been well-reported that there is a crosstalk between the Nrf2 signalling pathway and pro-inflammatory mediators' production at the transcriptional level during neuroinflammation. To illustrate, the up-regulation of the Nrf2/ARE axis attenuates the production of pro-inflammatory by modulation of the NF-κB pathway (Liu *et al*., 2018; Wardyn, Ponsford, & Sanderson, 2015). Even though fagaramide did not increase the level of antioxidant elements, it might target the Nrf2 pathway at the transcriptional level, which can, in turn, suppress pro-inflammatory mediators' production. Fagaramide ability to activate Nrf2/ARE axis has been highlighted in this study. Firstly, the result of the time-point experiment showed that; at 24 hours, fagaramide (20 µM) was able to produce the highest Nrf2 expression. Nuclear extracts from fagaramide- treated BV2 cells revealed that Nrf2 expressions were not up-regulated by fagaramide compared to untreated cells (Figure ***4-13***a). The results from the Nrf2 immunofluorescent assay confirm that fagaramide could not increase the expression of Nrf2 in BV2 cells (Figure ***4-13***c). The ARE-luciferase activity was investigated to determine whether fagaramide interfered with the transcriptional activity of Nrf2 at the nuclear level. Firstly, the time-course experiment was performed by treating BV2 cells with 20µM fagaramide for 2, 4, 6, 8 and 24hr, and the results showed that the maximum activation was achieved at 24hr. The results of ARE luciferase reporter assay revealed that fagaramide did not significantly enhance the activation of ARE in BV2 cells (Figure ***4-13***b).

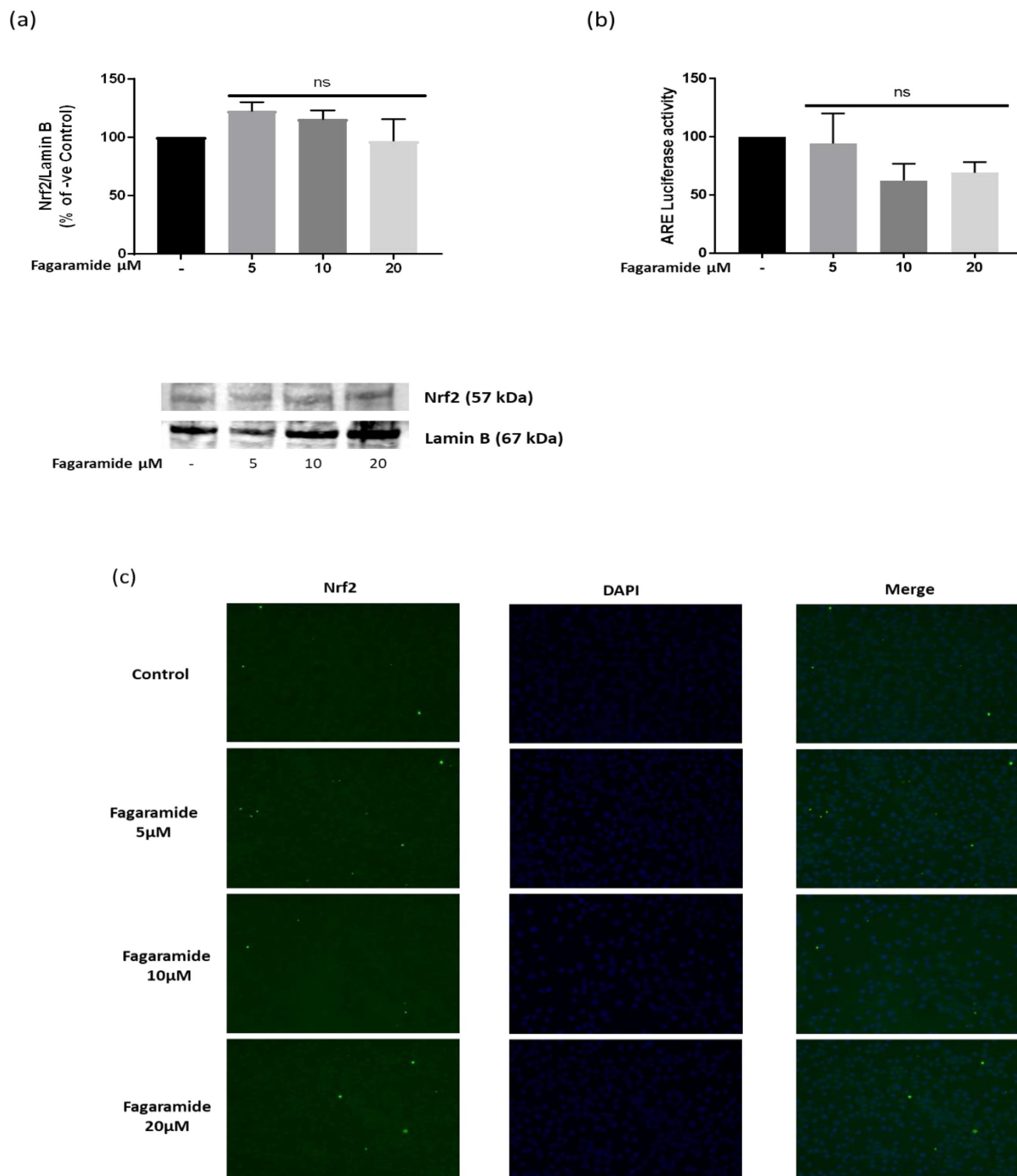



**Figure 4-13 Fagaramide could not up-regulate the Nrf2/ARE antioxidant axis in microglia.**

BV2 cells were treated with 5, 10 and 20 µM of fagaramide for 24hr. (a) nuclear extractions were collected and subjected to western blot analysis. The membranes were incubated with an Nrf2 antibody, and the results showed that fagaramide could not increase the expression of Nrf2 compared to untreated cells. (b) BV2 cells were transfected with ARE luciferase reporter and then treated with fagaramide for 24hr, the luciferase Dual Glo was used to read the luminescence reaction and Renilla luciferase was used as an internal control. The results revealed that fagaramide did not increase ARE activity compared to untreated transfected cells. (c) Nrf2 antibody was used to label the cells, followed by incubation with DAPI. The fluorescence images confirmed that fagaramide could not up-regulate the expression of Nrf2. Images were processed by image J analysis software and the scale bar=100 µm. Lamin B was used as a loading control in western blot experiments. Data for three independent experiments were presented as mean ± SEM and were analysed using one-way ANOVA followed by Dunnett's Multiple Comparison Test. *p<0.05, **p<0.01, *** p< 0.001 and **** p< 0.0001 compared to untreated cells.

### 4.3.12 Fagaramide pre-treatment did not prevent $H_2O_2$-induced damage to SH-SY5Y cells

To examine the neuroprotective activity of fagaramide in *in vitro* neuronal model, differentiated SH-SY5Y neuroblastoma were pre-incubated with 5, 10 and 20 µM fagaramide with and without exposure to 200 µM $H_2O_2$ and XTT assay was performed. The results in Figure ***4-14*** exhibited that treatment with fagaramide alone did not induce neurotoxicity to SH-SY5Y cells. $H_2O_2$- treated cells showed a significant reduction in cellular viability (~2.5-fold reduction) compared to untreated cells. Nevertheless, the cells pre-incubated with 5, 10 and 20 µM fagaramide and treated with $H_2O_2$ did not reverse the neurotoxicity effects of 200 µM $H_2O_2$.

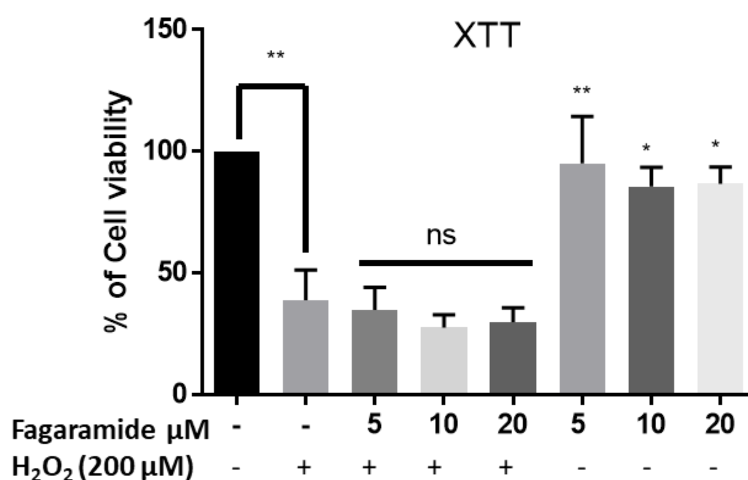



**Figure 4-14 Pre-treatment of SH-SY5Y cells with fagaramide did not increase the viability of $H_2O_2$-induced cytotoxicity.**

After differentiation of SH-SY5Y cells for 7 days, the cells were treated with 5, 10 and 20 µM of fagaramide with and without exposure to 200 µM $H_2O_2$ for 24hr. The experiments were terminated by adding 25 µl of XTT/PMS solution. The results showed that fagaramide did not decrease viability when the cells were treated with it alone. However, cells exposed to $H_2O_2$ had a noticeably decreased viability which was not reversed by fagaramide pre-treatment. Data for three independent experiments were presented as mean ± SEM and were analysed using one-way ANOVA followed by Dunnett's Multiple Comparison Test. *$p<0.05$, **$p<0.01$, *** $p< 0.001$ and **** $p< 0.0001$ versus untreated control cells for XTT assay.

### 4.3.13 Pre-incubation with fagaramide did not increase the expression of neuronal markers in the $H_2O_2$-induced SH-SY5Y neuronal damage

Morphologically, the viable differentiated SH-SY5Y neuroblastoma cells are like primary neurons with long processes and display augmented expression of neuronal markers such as βIII-tubulin and microtubule-associated protein-2 (MAP-2) (Kovalevich *et al*., 2021). However, neuronal death can be recognised by extensive loss of neuronal markers. Therefore, the neuronal markers MAP-2 and βIII-Tubulin were investigated in this research for the effect of fagaramide on $H_2O_2$-induced neuronal death. The cells were treated with 20 µM of fagaramide for 30min then incubated with 200 µM of $H_2O_2$ for 24hr, and the cytoplasmic extractions were collected and analysed by western blot. The results showed that $H_2O_2$ significantly decreased the expression of MAP-2 protein (~1.5-fold reduction) when compared with untreated cells, whereas fagaramide pre-treatment could not boost the expression of MAP-2 (Figure ***4-15***a). Immunofluorescent staining of neuronal protein MAP-2 was performed in differentiated SH-SY5Y to confirm the results obtained from the western blot. Principally, $H_2O_2$-treated cells displayed a considerable reduction in MAP-2-positive cells compared to untreated differentiated cells. Pre-incubation with 20 µM fagaramide did not reverse the damaging effects introduced by $H_2O_2$ on MAP-2 expression (Figure ***4-15***b). Similarly, an immunofluorescence experiment was conducted to examine βIII-Tubulin expression in the differentiated SH-SY5Y cells, which were pre-treated with 20 µM fagaramide and exposed to $H_2O_2$. Treatment of SH-SY5Y cells with 200 µM $H_2O_2$ decreased the βIII-Tubulin protein expression compared with untreated cells, while pre-incubation with 20 µM fagaramide did not reverse the damaging effect of $H_2O_2$ on βIII-Tubulin protein expression (Figure ***4-15***c).

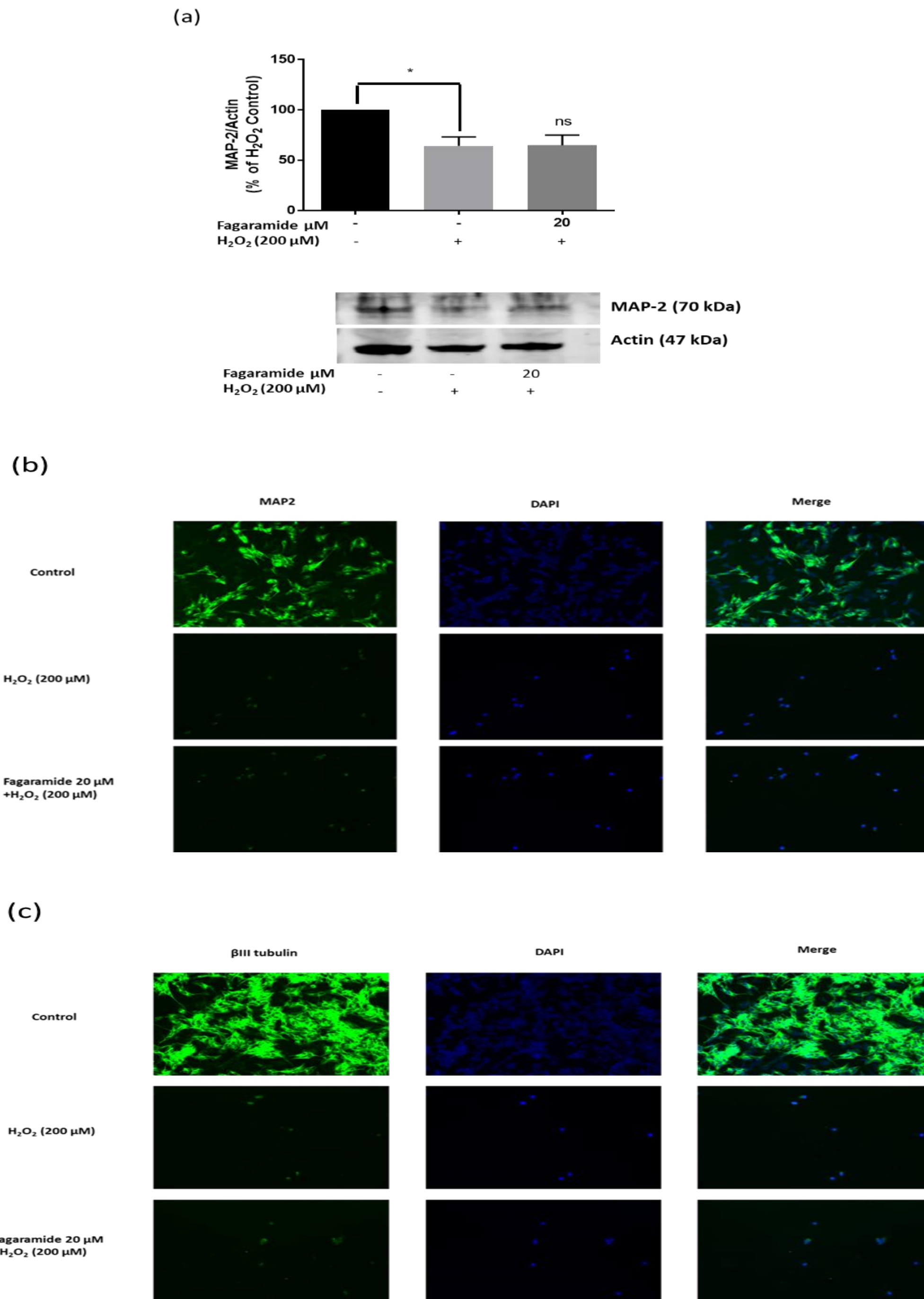



**Figure 4-15 Fagaramide did not attenuate $H_2O_2$-induced oxidative damage on neuronal markers.**

Differentiated SH-SY5Y cells were pre-treated with 20 µM fagaramide and 200 µM $H_2O_2$ for 24hr. (a) Cytoplasmic extractions were analysed by western blot, and the results showed that fagaramide could not alleviate the cytotoxicity effects of $H_2O_2$ on MAP-2 protein expression. (b) MAP-2 antibody was used to label the cells, followed by incubation with DAPI. The fluorescence images confirmed that fagaramide could not preserve MAP-2 protein from the oxidative damage effects of 200 µM $H_2O_2$. (c) Cells were stained with βIII-Tubulin antibody overnight, then counterstained with DAPI and the images displayed that fagaramide could not improve the expression of βIII-Tubulin in the $H_2O_2$-treated cells. Images were processed by image J analysis software, and the scale bar=100 µm. Actin was used as a loading control in western blot experiments. Data for three independent experiments were presented as mean ± SEM and were analysed using one-way ANOVA followed by Dunnett's Multiple Comparison Test. *$p<0.05$, **$p<0.01$, *** $p< 0.001$ and **** $p< 0.0001$ compared to $H_2O_2$-treated cells.

## 4.4 Discussion

Neuroinflammation is a chronic process that involves innate immune cells: microglia, and astrocytes and causes overproduction of pro-inflammatory cytokines such as TNF-α, IL-6, IL-1β, NO and $PGE_2$ that aggravate the disease and lead to neurodegeneration (Albornoz, Woodruff, & Gordon, 2018; Lyman *et al*., 2014). Recently, several *in vitro* studies have highlighted the neuroinflammation processes targeting compounds deriving from plants' parts could help in relieving the disease's neuropathological events and effectively attenuating its progression through mitigating the production of the pro-inflammatory cytokines (Fan *et al*., 2017; Kim, Quang, Oh, & Kim, 2017; Pan *et al.*, 2017).

In the current study, fagaramide reduced the level of TNF-α, IL-6, NO and $PGE_2$ in LPS-activated microglia, demonstrating inhibition of neuro-inflammation by this compound. Similar alkaloids to fagaramide have been reported to show anti-inflammatory activity; for example, skimmianine. Skimmianine, *Zanthoxylum zanthoxyloides* extracts, was shown to produce anti-inflammatory activity against carrageenan-induced acute inflammation. Skimmianine treatment produced a reduction in the mRNA levels of IL-6 and TNF-α (Ratheesh *et al*., 2013). Interestingly, this study showed that fagaramide treatment caused a reduction in COX-2 and iNOS expression in LPS-stimulated BV2 microglia. This observation is in agreement with a recent study that reported the anti-inflammatory activity of *Zanthoxylum zanthoxyloides* extracts in LPS-stimulated BV2 cells by reducing the expression levels of TNF-α, IL-6, NO, $PGE_2$, iNOS and COX-2 (Ogunrinade *et al*., 2021). An earlier study showed the ability of fagaramide to decrease the inflammatory response *in vitro* via the inhibition of prostaglandin synthesis (Oriowo, 1982b). This study agrees partially with the findings in the Oriowo study because only PG synthesis was assessed. To my knowledge, the current study reported the anti-inflammatory property of fagaramide involving other mediators for the first time.

The effect of fagaramide on ROS generation was investigated in this study by using a DCFDA stain. The outcome showed that fagaramide could not decrease ROS generation in LPS-stimulated microglia.

Originally, fagaramide is an extract from *Zanthoxylum zanthoxyloides* representing one of *Zanthoxylum* species. *Zanthoxylum heitzii* belongs to Zanthoxylum species, and some of its extracts revealed beneficial biological activity. *Fagara heitzii*, an extract from *Zanthoxylum heitzii*, has been reported to reduce the concentration of free radicals by applying DPPH radical scavenging activity assay (Chaaib *et al*., 2003; Masuku, 2013). Another study by Ntchapda F. *et.al* (2015) demonstrated the activity of *Zanthoxylum heitzii* stem bark aqueous extract by reducing liver oxidative stress markers in the diet-induced hypercholesterolemic rat models (Ntchapda, Maguirgue, Adjia, Etet, & Dimo, 2015). However, the antioxidant activities of those extracts were not examined directly on ROS generation by using *in vitro* model like fagaramide; therefore, it is unlikely to compare their activity to fagaramide effects on ROS generation.

Based on the ability of fagaramide to reduce the production of pro-inflammatory mediators in BV2-stimulated cells, the mechanisms behind fagaramide anti-inflammatory activity were investigated. NF-κB is a major transcription factor that regulate transcription of genes encoding production of pro-inflammatory mediators. Therefore, targeting the NF-κB signalling pathway may affect neuroinflammation-mediated neurodegenerative diseases. Effect of fagaramide on LPS- induced phosphorylation of p65 was investigated, and the outcomes showed that fagaramide did not inhibit the phosphorylation of p65.

NF-κB activation could be inhibited by interference with upstream mechanisms, including IKK phosphorylation, IκB phosphorylation and degradation. Therefore, an effort was made to study the effect of fagaramide on the phosphorylation and degradation of IκB. Interestingly, fagaramide inhibited LPS-mediated degradation of IκB in BV2 cells but had no inhibitory effect on the phosphorylation level of the IκB subunit. These observations suggested that fagaramide may inhibit the proteasomal degradation of the free IκB and not the NF-κB-bound IκB, as the latter is mainly involved in the neuroinflammation-mediated NF-κB activation and undergoes both phosphorylation and degradation by IKK (Mathes *et al*., 2008).

The activity of NF-κB is not only affected by the phosphorylation of upstream molecules, but also by interaction with complex networks at the nuclear level. Therefore, NF-κB signalling may be targeted at the downstream sites in the pathway to exert the activity, for example affecting the NF-κB –DNA binding (Shin *et al*., 2004) or the NF-κB dependent transactivation without targeting other signals of this pathway. Thus, fagaramide effects on NF-κB–DNA binding and NF-κB transcriptional activity were conducted. The outcomes of this study showed that fagaramide had no inhibitory effects on the binding activity of NF-κB to DNA in LPS-stimulated cells. Additionally, the results observed that fagaramide did not inhibit the transcriptional activity of NF-κB as it did not decrease NF-κB mediated luciferase activity in LPS-stimulated transfected cells. These results indicate that fagaramide inhibited neuroinflammation independently on the NF-κB pathway.

Another major inflammatory signal pathway is MAPK which play a vital role in extracellular signals transduction. This signalling pathway is involved in inflammatory responses by modulating the expression of inflammatory genes. Both *in vitro* and *in vivo* investigations indicated that activation of MAPKs subfamilies, as a response to stimuli, are essential for a number of inflammatory reactions by leading to further production of inflammatory mediators such as TNF-α, IL-6, NO and COX-2 (Raingeaud *et al.*, 1995; Zhao *et al*., 2014). The outcomes of this study indicated that fagaramide treatment did not inhibit phosphorylation of p38 and JNK in LPS-activated BV2 cells, suggesting that fagaramide anti-inflammatory activity is independent of MAPKs. On the contrary, Ogunrinade *et al*., 2021 reported that *Z. zanthoxyloides* extract, a related compound to fagaramide, exerted its anti-neuroinflammatory activities by inhibiting LPS-induced phospho-p38 expression. This variation in the activity between two compounds extracted from the same plant species may be related to the presence of different functional groups in their chemical structures, which might affect their pharmacological activity.

In neuroinflammation, PI3K/Akt pathway may participate in signalling transduction and cause activation and phosphorylation of Akt protein which may provoke the generation of pro-inflammatory

mediators (Cantley, 2002; Fukao & Koyasu, 2003). It has been reported that PI3K/Akt pathway promotes the expression of iNOS and COX-2 in LPS-activated microglia (Hsieh, Deng, Chang, & Huang, 2018). Therefore, the ability of fagaramide to prevent Akt phosphorylation was investigated. Pre-incubation with fagaramide did not inhibit the increased level of Akt phosphorylation in LPS-stimulated BV2 cells. Akt is a major signalling mediator of the PI3K/Akt pathway, and fagaramide did not show any inhibitory effect on its phosphorylation level, suggesting that fagaramide anti-neuroinflammatory activity is Akt-independent.

Recently, the role of Nrf2 signalling in neuroinflammation has been well documented. Activation of the Nrf2 pathway resulted in the amelioration of neuroinflammation and was involved in the anti-neuroinflammatory responses (Chuan Li, Zhang, & Frei, 2016; Yaxin Zheng *et al*., 2019).

This study attempted to investigate whether the anti-neuroinflammatory effects of fagaramide were mediated by the Nrf2 antioxidant mechanism. The results of this study demonstrated that fagaramide was not able to increase the expression of NQO1 and HO-1 protein levels in BV2 microglia. Nrf2 activity can be regulated at multiple levels. Therefore, fagaramide activity was further investigated on Nrf2 activity at the nuclear level, and the results revealed that fagaramide did not up-regulate the protein level of Nrf2 in microglia. In addition, fagaramide did not enhance the transcriptional activity of antioxidant responsive element (ARE), suggesting that fagaramide anti-inflammatory activity is Nrf2-independent. On the contrary, Hydroethanolic extract from one of the *Zanthoxylum* species, *Zanthoxylum alatum,* demonstrated anti-inflammatory activity by up-regulation of Nrf2 and HO-1 levels in rat hippocampal tissues (Saikia *et al*., 2018). In addition, an extract from *Zanthoxylum bungeanum;* one of *Zanthoxylum* species; was reported to have neuroprotection effects by enhancing protein levels of HO-1 and upregulation of Nrf2 in AD mice model (Y. Liu *et al*., 2022). The outcomes of the current study suggested that fagaramide could not up-regulate antioxidant elements as other compounds extracted from the same plant species, *Zanthoxylum* species. This might

be related to the difference in the chemical structures between the extracts, which may result in different pharmacological behaviours.

Neuronal damage has been associated with the pathology of several neurodegenerative diseases like AD and PD (Brieger, Schiavone, Miller Jr, & Krause, 2012; Niranjan, 2014). Several studies have shown the ability of $H_2O_2$ to induce neuronal damage when exposed to cell cultures (Chen *et al*., 2015; Hu *et al*., 2014); therefore, $H_2O_2$ was used to introduce neurotoxicity. The result of this study indicated that fagaramide did not exert a neurotoxicity effect on SH-SY5Y cells and at the same time, it did not ameliorate the neuronal damage that resulted from exposure of differentiated SH-SY5Y cells to $H_2O_2$. In addition, both MAP-2 and βIII-Tubulin expression was kept low when $H_2O_2$-damaged cells were pre-treated with fagaramide. These findings suggested, for the first time, that fagaramide possesses anti-neuroinflammatory activity possibly by reducing pro-inflammatory mediators and has no effects on neurotoxicity.

In summary, the anti-neuroinflammatory activity of fagaramide on pro-inflammatory mediators was investigated in LPS-induced BV2 cells. The NF-κB, MAPKs, Nrf2 and Akt signalling pathways were studied to investigate the molecular mechanism behind the anti-neuroinflammatory activity of fagaramide. In addition, the neuroprotective effects of fagaramide on $H_2O_2$-induced apoptosis of differentiated SH-SY5Y cells were examined.

This is the first time multiple pathways have been examined to investigate the molecular mechanisms of fagaramide anti-inflammatory properties. The anti-neuroinflammatory activity of fagaramide was NF-κB, MAPKs, Akt and Nrf2-independent. Fagaramide may act on the post-transcriptional level of pro-inflammatory mediators' production processes. Also, the anti-inflammatory effects of fagaramide might be correlated to the inhibition of the downstream transcription factor (AP-1) of the MAPKs pathway, which needed to be investigated.

# 5 Chapter Five: General Discussion and Conclusion

## 5.1 General discussion

Several factors may contribute to the pathogenesis of neurodegenerative diseases, including environmental, genetic, and endogenous factors. However, neurodegenerative pathological mechanisms can be ranged from abnormal protein aggregation, mitochondrial dysfunction, oxidative stress with reactive oxygen species, DNA damage, and neuroinflammatory events (Jellinger, 2010). Generally, neuroinflammation is a defence process that protects the central nervous system compartments by removing or hindering pathological stimuli (H. S. Kwon & Koh, 2020; Wyss-Coray & Mucke, 2002). While this inflammatory reaction can have beneficial outcomes by facilitating tissue restoration and clearing cellular debris, persistent inflammatory responses are destructive as they inhibit regeneration and lead to sustained neuronal damage (Kempuraj *et al*., 2016; Russo & McGavern, 2016). The prominent players in the neuroinflammatory reactions are the microglia cells. Microglia are the innate immune cells of the central nervous system, which play roles in tissue maintenance, pathogen defence and response to injury (Colonna & Butovsky, 2017; Nayak, Roth, & McGavern, 2014).

Microglia are involved in the neuroinflammation-induced neurodegeneration pathogenicity by the production of inflammatory mediators, including TNF-α, IL-6 and IL-1β after exposure to internal triggers or external stimuli (Hansen, Hanson, & Sheng, 2018). However, suppressing pro-inflammatory cytokines during inflammation by pharmacologically active compounds has been related to alleviating disease conditions and enhancing cellular survival (Yitong Wang, Ge, Yu, & Cheng, 2021; Z. Yao *et al.*, 2021).

Arvanil is a potent agonist at CB1 receptors and has the ability to activate TRPV1 receptors (Melck *et al*., 1999). Arvanil has shown some anti-inflammatory effects by inhibiting early events in T-cell receptor-mediated T-cell activation (Márquez *et al*., 2006). However, information is lacking about the neuroprotection activities of arvanil; therefore, this study was designed to investigate the

neuroprotection activity of this compound. Results of this study revealed that arvanil reduced the level of TNF-α, IL-6, NO and $PGE_2$ and inhibited the protein expression of iNOS and COX-2 in LPS-stimulated BV2 cells. Accumulating data from both *in vivo* and *in vitro* studies suggest that cannabinoid receptor agonists alleviate neural cell activation in the inflammatory response produced by neurotoxic agents, thereby reducing the levels of pro-inflammatory mediators such as IL-6, IL-1β and TNF-α (Borgonetti *et al*., 2022; Giuseppe Esposito *et al*., 2011; Ma *et al.*, 2015; Scuderi *et al*., 2011).

As arvanil revealed anti-neuroinflammatory activity by reducing the pro-inflammatory mediators; therefore, this study was directed to explore the mechanisms that are responsible for this activity. The primary regulator for inflammation is the NF-κB signalling pathway. Several studies have indicated that targeting NF-κB during inflammation causes decreased levels of inflammatory mediators (Gao, Shen, Zhao, Hao, & Yang, 2019; Lee, Park, Lee, Ahn, & Kim, 2019). Therefore, arvanil has been investigated in this study for its activity on the NF-κB pathway. LPS-induced NF-κB p65 phosphorylation was not inhibited by pre-treatment with arvanil. The upstream effectors in the NF-κB p65 pathway were also investigated by examining phosphorylation and degradation levels of IκB. Interestingly, 0.5 µM of arvanil reduced the degradation of IκB but did not inhibit the phosphorylation of IκB in LPS-stimulated BV2 cells, suggesting that arvanil might possibly affect the free pool of IκB and not the NF-κB-bound IκB pool. NF-κB is a complex pathway that involves reactions and undergoes modulations at different levels, which allows for being targeted at any stage of activation; therefore, the downstream of NF-κB p65 at the nuclear level was investigated. Arvanil did not reduce NF-κB p65-DNA binding or block NF-κB reporter gene transcription, suggesting that arvanil anti-neuroinflammatory activity is NF-κB-independent.

Accordingly, the molecular mechanism of the anti-neuroinflammatory activity of arvanil was further investigated on the MAPKs signalling pathway, which is commonly involved in neuroinflammatory responses (Velagapudi *et al.*, 2014; Yongqun Zhu *et al*., 2007). This study observed that arvanil did not inhibit the phosphorylation of p38 and JNK, indicating that arvanil anti-inflammatory activity is

independent of the MAPKs pathway. The result, regarding p38, may be consistent to a limited extent with a previous study which demonstrated that anandamide, an agonist for both the CB1 receptor and TRPV1 receptor as an arvanil, does not seem to interpose with the p38 pathway since the phosphorylation level showed the same profiles of activation in the absence or presence of anandamide in LPS/IFNγ stimulated microglial cells (Correa *et al*., 2009). Further investigation on nuclear downstream MAPKs signals, such as AP-1 transcriptional activity, is suggested as it may be related to the anti-neuroinflammatory action of arvanil.

PI3Ks/Akt pathway could regulate the production of pro-inflammatory molecules through Akt protein molecules (Dan *et al*., 2008). The direct target for Akt kinase on the NF-κB pathway is phosphorylation of the IKK complex, which causes activation of this pathway (Hoesel & Schmid, 2013). Therefore, Arvanil has been studied to investigate whether its anti-neuroinflammatory effects were achieved by the action of Akt protein. Arvanil did not decrease LPS-induced Akt phosphorylation in BV2 cells significantly. Akt protein level was not changed in the presence of arvanil, meaning that the anti-inflammatory activity of arvanil is Akt-independent.

As arvanil is a potent agonist for CB1 receptors and TRPV1 receptors; therefore, arvanil neuroinflammatory activity was examined if it is mediated through cannabinoid or TRPV1 receptors. The results indicated that CB1, CB2 and TRPV1 receptors were expressed in the BV2 microglia. Many studies have suggested the involvement of TRPV1 during the activation of microglia. Some of them revealed that treatment with TRPV1 agonists might attenuate inflammatory responses and lowered cytokines and chemokines production through TRPV1 activation (J. Chen *et al.*, 2018; Youping Wang *et al*., 2017). However, this study reported that pre-treatment with TRPV1 antagonist did not reverse the inhibitory effect of arvanil on pro-inflammatory cytokines in LPS-activated BV2 cells, indicating that arvanil activities might be achieved independent of TRPV1 receptors.

The investigations were directed to determine if the anti-inflammatory effects of arvanil were mediated by acting on CB1 or CB2 receptors. Interestingly, the results showed that the inhibitory

effects of arvanil on LPS-induced elevated levels of TNF-α and IL-6 were not antagonised by either the CB1 or CB2 receptor antagonist. Instead, the CB1 and CB2 receptor antagonists reduced the LPS-induced production of TNF-α and IL-6. These observations suggest that arvanil anti-neuroinflammatory activity might not be mediated through binding to CB1 and CB2 receptors. This result is consistent with a study delivered by Ribeiro *et al*. (2013), which proposed that CB1 and CB2 cannabinoid receptor agonists and antagonists may block microglia activation through CB1 and CB2 receptor-independent system, respectively (Ribeiro *et al*., 2013).

As a response to the increased level of pro-inflammatory mediators, cellular compensatory reactions are usually activated to counteract neuroinflammatory reactions. Thus, one of the main intrinsic protective systems that are activated during inflammation is the Nrf2 pathway (Sanjiv Singh *et al*., 2021). It has been reported that the levels of pro-inflammatory mediators were suppressed by the activity of up-regulated Nrf2 pathway (Alvi *et al*., 2021; Okorji, Velagapudi, El-Bakoush, Fiebich, & Olajide, 2016). Therefore, the arvanil effect on the Nrf2 antioxidant system was investigated. The results demonstrated that arvanil did not increase the protein levels of both HO-1 and NQO1 in BV2 microglia. In addition, arvanil could not increase the expression of Nrf2 at the nuclear level and did not increase the transcriptional activity of antioxidant responsive element (ARE) in BV2 cells. These observations suggest that arvanil anti-neuroinflammatory activity is not mediated through Nrf2 pathway activation.

Several factors may involve in the progression of neurodegeneration diseases; oxidative stress-induced neuronal damage is one of the main contributors to their pathogenesis (Ferreiro *et al*., 2012). Arvanil showed anti-neuroinflammatory activity; therefore, the study was directed to examine its ability to reduce neurotoxicity by using *in vitro* neuronal cell culture. The results showed that pre-treatment with arvanil could not reverse SH-SY5Y cell damage introduced by $H_2O_2$ exposure, suggesting that arvanil is an anti-neuroinflammatory compound through inhibiting pro-inflammatory mediators without activity against neurotoxicity.

Several compounds originally extracted from plant parts (such as dry ginger extract) have shown promising neuroprotective effects in *in vivo* and *in vitro* disease models (Apetz *et al*., 2014; Mathew & Subramanian, 2014; Taslimi *et al*., 2020). Fagaramide is a compound obtained from the stem bark of *Zanthoxylum zanthoxyloides* in Africa whereas most of its extracts are used in malaria treatment, cancer, as well as cardiac palpitations (F. Sandberg *et al*., 2005; Zirihi *et al*., 2005). However, the neuroprotective effects of fagaramide were not yet studied. Therefore, this study was designed to investigate whether fagaramide can inhibit neuroinflammation in LPS-stimulated BV2 cells and if so, the possible molecular mechanisms responsible for this activity would be studied.

Neuroinflammation represents the chronic process of the brain diseases' progression and causes overproduction of pro-inflammatory cytokines such as TNF-α, IL-6, IL-1β, NO and $PGE_2$ that aggravate the disease and lead to neurodegeneration (Albornoz *et al*., 2018; Lyman *et al*., 2014). However, pharmacological inhibition of inflammatory cytokines is reported to reduce the inflammatory response in neurodegenerative diseases (Walters, Phillips, Zheng, Biju, & Kuruvilla, 2016). Results of this study displayed that pre-treatment with fagaramide led to reducing the levels of TNF-α, IL-6, NO and $PGE_2$ in LPS-activated microglia. The results also showed that fagaramide treatment caused a reduction in COX-2 and iNOS expression in LPS-stimulated BV2 microglia, demonstrating inhibition of neuroinflammation by this compound. Therefore, the mechanism behind fagaramide anti-neuroinflammatory effects was investigated in this study.

As highlighted early, NF-κB is a major signalling pathway that participates in the inflammatory responses against several stimuli (such as LPS and TNF-α) and leads to producing pro-inflammatory mediators (Shareen Singh & Singh, 2020). Therefore, fagaramide effects on NF-κB activity was studied. Fagaramide did not reduce the phosphorylation level of the NF-κB p65 subunit and had no inhibitory effect on the phosphorylation of IκB; however, fagaramide reduced the degradation of IκB. Fagaramide effects on the nuclear mechanism of the NF-κB pathway were investigated, and the results demonstrated that it did not block the DNA binding affinity of NF-κB p65 to inflammatory genes

and had no inhibitory effects on NF-κB reporter gene transcription. These findings suggest that the anti-neuroinflammatory effect of fagaramide is not mediated by inhibition of the NF-κB pathway.

The anti-neuroinflammatory activity of fagaramide might be mediated by another major inflammatory signal pathway known MAPK; therefore, fagaramide effects on p38 and JNK activation were examined. Fagaramide did not inhibit the phosphorylation of p38 and JNK, indicating that the anti-neuroinflammatory action of fagaramide is MAPKs-independent. A recent study demonstrated that the anti-neuroinflammatory activity of root extract of *Zanthoxylum zanthoxyloides* was mediated by inhibiting NF-κB and p38 MAPKs pathways *(Ogunrinade et al*., 2021) which is inconsistent with the findings of this study regarding the mechanisms of action. There may be different functional groups in the chemical structures of fagaramide and the other extracts of *Zanthoxylum zanthoxyloides*, which caused different pharmacological actions.

It has been reported that phosphorylation of Akt protein in the PI3K/Akt pathway could trigger a cascade of signals that promote expressions of inflammatory mediators, whereas inhibition of Akt activity in the stimulated microglia leads to the reduction of pro-inflammatory factors production (Han *et al*., 2016; X. Shi *et al*., 2017). However, pre-incubation with fagaramide did not inhibit the increased level of Akt phosphorylation in LPS-stimulated BV2 cells, suggesting the anti-neuroinflammatory activity of fagaramide is independent of Akt protein.

Recent reports proposed that the transcriptional inhibition of pro-inflammatory mediators (TNF-α, IL-6, IL-1β and MCP-1) in monocytes, microglia, astrocytes and macrophages was mediated by activation of the Nrf2 signals pathway (Eri H Kobayashi *et al*., 2016; Quinti *et al*., 2017). The molecular mechanism of the anti-neuroinflammatory activity of fagaramide is still unclear; therefore, the study was directed to investigate whether this effect was mediated by activation of Nrf2 antioxidant pathway. Fagaramide did not enhance the expression of NQO1 and HO-1 in BV2 microglia. Its activity was further investigated on the Nrf2 level, and the results revealed that fagaramide could not up-regulate the nuclear protein level of Nrf2 in the microglia and did not enhance the transcriptional activity of

antioxidant responsive element (ARE). These observations suggested that the anti-neuroinflammatory activity of fagaramide is not mediated by the activation of the Nrf2/HO-1/NQO1 antioxidant axis.

As fagaramide in this study showed anti-neuroinflammatory activity, so further research on the effect of fagaramide blocking neurotoxicity was conducted. Therefore, fagaramide neuroprotection effect was studied on $H_2O_2$-induced SH-SY5Y neuroblastoma toxicity. Fagaramide did not inhibit the neuronal damage that resulted from exposure of differentiated SH-SY5Y cells to 200 µM of $H_2O_2$, indicating that fagaramide activity was achieved by inhibiting neuroinflammation without reversing neurotoxicity.

## 5.2 Conclusion

Arvanil and fagaramide demonstrated anti-neuroinflammatory activities in LPS-stimulated BV2 cells. However, the molecular mechanisms behind their effects unclear yet as their anti-neuroinflammatory effects were not mediated through inhibition of NF-κB, MAPKs and Akt and not mediated through activation of the Nrf2 pathway. Arvanil's anti-neuroinflammatory activity was not possibly mediated by binding to CB1, CB2 and TRPV1 receptors. Both compounds did not protect SH-SY5Y neuroblastoma cells from toxicity damage of $H_2O_2$.

## 5.3 Recommendations for future studies

As it is not clear which signalling pathways mediated the anti-neuroinflammatory activity of arvanil and fagaramide, research on another possible involved mechanism may be considered. Both compounds may be inhibited pro-inflammatory mediators' production on post-transcription, translation or post-translational levels, therefore more investigations on the pro-inflammatory inhibition activity of arvanil and fagaramide at these levels are encouraged.

Activation of sirtuins 1 (SIRT1) has been shown to reduce the activity of the NF-κB inflammatory pathway through deacetylating of the p65 subunit, resulting in a decrease in the production of pro-inflammatory mediators (H. Yang *et al.*, 2012). Therefore, it would be reasonable to investigate the effects of arvanil and fagaramide on SIRT1 activation.

The existence of the blood-brain barrier (BBB) is a big challenge during the development or investigation of medicines or compounds that target CNS diseases. Accordingly, the ability of fagaramide and arvanil to cross the BBB should be studied.

Microglia express TLR4, which play important roles in cellular response against stimuli. It has been proven that TLR4 undergo activation when the microglia are stimulated with LPS and, in turn, mediates activation of inflammatory pathways. Therefore, arvanil and fagaramide should be investigated for their effects on TLR4 activation and expression.

## References

Abeliovich, A., & Gitler, A. D. (2016). Defects in trafficking bridge Parkinson's disease pathology and genetics. *Nature*, *539*(7628), 207–216.

Acheampong, D. O., Baffour, I. K., Addo, J. K., Essuman, M. A., & Boye, A. (2021). Zanthoxylum zanthoxyloides alkaloidal extract improves ccl4-induced hepatocellular carcinoma-like phenotypes in rats. *Evidence-Based Complementary and Alternative Medicine*, *2021*.

Agostinho, P., A Cunha, R., & Oliveira, C. (2010). Neuroinflammation, oxidative stress and the pathogenesis of Alzheimer's disease. *Current Pharmaceutical Design*, *16*(25), 2766–2778.

Aguirre-Rueda, D., Guerra-Ojeda, S., Aldasoro, M., Iradi, A., Obrador, E., Mauricio, M. D., … Valles, S. L. (2015). WIN 55,212-2, agonist of cannabinoid receptors, prevents amyloid β1-42 effects on astrocytes in primary culture. *PLoS One*, *10*(4), e0122843.

Ahmed, T., Zulfiqar, A., Arguelles, S., Rasekhian, M., Nabavi, S. F., Silva, A. S., & Nabavi, S. M. (2020). Map kinase signaling as therapeutic target for neurodegeneration. *Pharmacological Research*, *160*, 105090.

Akiyama, H., Barger, S., Barnum, S., Bradt, B., Bauer, J., Cole, G. M., … Fiebich, B. L. (2000). Inflammation and Alzheimer's disease. *Neurobiology of Aging*, *21*(3), 383–421.

Alberti, T. B., Barbosa, W. L. R., Vieira, J. L. F., Raposo, N. R. B., & Dutra, R. C. (2017). (−)-β-Caryophyllene, a CB2 receptor-selective phytocannabinoid, suppresses motor paralysis and neuroinflammation in a murine model of multiple sclerosis. *International Journal of Molecular Sciences*, *18*(4), 691.

Albornoz, E. A., Woodruff, T. M., & Gordon, R. (2018). Inflammasomes in CNS diseases. In *Inflammasomes: Clinical and Therapeutic Implications* (pp. 41–60). Springer.

Aleardi, A. M., Benard, G., Augereau, O., Malgat, M., Talbot, J. C., Mazat, J. P., … Rossignol, R. (2005). Gradual alteration of mitochondrial structure and function by β-amyloids: importance of

membrane viscosity changes, energy deprivation, reactive oxygen species production, and cytochrome c release. *Journal of Bioenergetics and Biomembranes*, *37*(4), 207–225.

Allan, S. M., & Rothwell, N. J. (2003). Inflammation in central nervous system injury. *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences*, *358*(1438), 1669–1677.

Alvarez-Erviti, L., Couch, Y., Richardson, J., Cooper, J. M., & Wood, M. J. A. (2011). Alpha-synuclein release by neurons activates the inflammatory response in a microglial cell line. *Neuroscience Research*, *69*(4), 337–342.

Alvi, A. M., Al Kury, L. T., Alattar, A., Ullah, I., Muhammad, A. J., Alshaman, R., … Li, S. (2021). Carveol attenuates seizure severity and neuroinflammation in pentylenetetrazole-kindled epileptic rats by regulating the Nrf2 signaling pathway. *Oxidative Medicine and Cellular Longevity*, *2021*.

Amah, C. C., Joshua, P. E., Ekpo, D. E., Okoro, J. I., Asomadu, R. O., Obelenwa, U. C., & Odiba, A. S. (2022). Ethyl acetate fraction of Fagara zanthoxyloides root-bark possess antidiabetic property against alloxan-induced diabetes and its complications in Wistar rat model. *Journal of Ethnopharmacology*, *293*, 115259.

Andy, S. N., Chan, C. K., & Kadir, H. A. (2017). Deoxyelephantopin from Elephantopus scaber modulates neuroinflammatory response through MAPKs and PI3K/Akt-dependent NF-κB signaling pathways in LPS-stimulated BV-2 microglial cells. *Journal of Functional Foods*, *38*, 221–231.

Anfinogenova, N. D., Quinn, M. T., Schepetkin, I. A., & Atochin, D. N. (2020). Alarmins and c-Jun N-terminal kinase (JNK) signaling in neuroinflammation. *Cells*, *9*(11), 2350.

Anna, S., Premoli, M., Tambaro, S., Kumar, A., Maccarinelli, G., Memo, M., & Mastinu, A. (2018). Cannabis sativa : A comprehensive ethnopharmacological review of a medicinal plant with a long history. *Journal of Ethnopharmacology*, *227*(May), 300–315. https://doi.org/10.1016/j.jep.2018.09.004

Apetz, N., Munch, G., Govindaraghavan, S., & Gyengesi, E. (2014). Natural compounds and plant

extracts as therapeutics against chronic inflammation in Alzheimer's disease–a translational perspective. *CNS & Neurological Disorders-Drug Targets (Formerly Current Drug Targets-CNS & Neurological Disorders)*, *13*(7), 1175–1191.

Ashtari, F., Madanian, R., Shaygannejad, V., Zarkesh, S. H., & Ghadimi, K. (2019). Serum levels of IL-6 and IL-17 in multiple sclerosis, neuromyelitis optica patients and healthy subjects. *International Journal of Physiology, Pathophysiology and Pharmacology*, *11*(6), 267.

Ashwell, J. D. (2006). The many paths to p38 mitogen-activated protein kinase activation in the immune system. *Nature Reviews Immunology*, *6*(7), 532–540.

Asiimwe, N., Yeo, S. G., Kim, M.-S., Jung, J., & Jeong, N. Y. (2016). Nitric oxide: exploring the contextual link with Alzheimer's disease. *Oxidative Medicine and Cellular Longevity*.

Aslantürk, Ö. S. (2018). In vitro cytotoxicity and cell viability assays: principles, advantages, and disadvantages. *Genotoxicity-A Predictable Risk to Our Actual World*, *2*, 64–80.

Aso, E., Andrés-Benito, P., & Ferrer, I. (2018). Genetic deletion of CB1 cannabinoid receptors exacerbates the Alzheimer-like symptoms in a transgenic animal model. *Biochemical Pharmacology*, *157*, 210–216.

Aso, E., & Ferrer, I. (2014). Cannabinoids for treatment of alzheimer's disease: Moving toward the clinic. *Frontiers in Pharmacology*, *5 MAR*(March), 1–11. https://doi.org/10.3389/fphar.2014.00037

Aso, E., Ferrer, I., Duyckaerts, C., Delatour, B., & Potier, M. C. (2016). CB2 Cannabinoid Receptor As Potential Target against Alzheimer's Disease. *Frontiers in Neuroscience*, *118*(1), 1–10. https://doi.org/10.3389/fnins.2016.00243

Aso, E., Juvés, S., Maldonado, R., & Ferrer, I. (2013). eCB2cannabinoid receptor agonist ameliorates alzheimer-like phenotype in AβPP/PS1 mic. *Journal of Alzheimer's Disease*, *35*(4), 847–858. https://doi.org/10.3233/JAD-130137

Aso, E., Palomer, E., Juvés, S., Maldonado, R., Muñoz, F. J., & Ferrer, I. (2012). CB 1 agonist ACEA protects neurons and reduces the cognitive impairment of AβPP/PS1 mice. *Journal of Alzheimer's Disease*, *30*(2), 439–459.

Atia, A., & Abdullah, A. (2014). Tocotrienols: the other half of natural vitamin E. *Res J Pharm Biol Chem Sci. 2014; 5 (2): 533*, *43*.

Atwood, B. K., & Mackie, K. (2010). CB2: a cannabinoid receptor with an identity crisis. *British Journal of Pharmacology*, *160*(3), 467–479.

Austin, S. A., Santhanam, A. V, & Katusic, Z. S. (2010). Endothelial nitric oxide modulates expression and processing of amyloid precursor protein. *Circulation Research*, *107*(12), 1498–1502.

Avila-Muñoz, E., & Arias, C. (2014). When astrocytes become harmful: functional and inflammatory responses that contribute to Alzheimer's disease. *Ageing Research Reviews*, *18*, 29–40.

Ayna, A., Özbolat, S. N., & Darendelioglu, E. (2020). Quercetin, chrysin, caffeic acid and ferulic acid ameliorate cyclophosphamide-induced toxicities in SH-SY5Y cells. *Molecular Biology Reports*, *47*(11), 8535–8543.

Bachmeier, C., Beaulieu-Abdelahad, D., Mullan, M., & Paris, D. (2013). Role of the cannabinoid system in the transit of beta-amyloid across the blood–brain barrier. *Molecular and Cellular Neuroscience*, *56*, 255–262.

Bachstetter, A. D., Xing, B., de Almeida, L., Dimayuga, E. R., Watterson, D. M., & Van Eldik, L. J. (2011). Microglial p38α MAPK is a key regulator of proinflammatory cytokine up-regulation induced by toll-like receptor (TLR) ligands or beta-amyloid (Aβ). *Journal of Neuroinflammation*, *8*(1), 1–12.

Bagyinszky, E., Van Giau, V., Shim, K., Suk, K., An, S. S. A., & Kim, S. (2017). Role of inflammatory molecules in the Alzheimer's disease progression and diagnosis. *Journal of the Neurological Sciences*, *376*, 242–254.

Banati, R. B. (2003). Neuropathological imaging: in vivo detection of glial activation as a measure of

disease and adaptive change in the brain. *British Medical Bulletin*, *65*(1), 121–131.

Belanger, L., Sylvestre, C., & Dufour, D. (1973). Enzyme-linked immunoassay for alpha-fetoprotein by competitive and sandwich procedures. *Clinica Chimica Acta*, *48*(1), 15–18.

Belda, B. J., Thompson, J. T., Sinha, R., Prabhu, K. S., & Heuvel, J. P. Vanden. (2012). The dietary fatty acid 10E12Z-CLA induces epiregulin expression through COX-2 dependent PGF2αsynthesis in adipocytes. *Prostaglandins and Other Lipid Mediators*, *99*(1–2), 30–37. https://doi.org/10.1038/s41593-018-0175-4

Bendotti, C., Tortarolo, M., & Borsello, T. (2006). Targeting stress activated protein kinases, JNK and p38, as new therapeutic approach for neurodegenerative diseases. *Central Nervous System Agents in Medicinal Chemistry (Formerly Current Medicinal Chemistry-Central Nervous System Agents)*, *6*(2), 109–117.

Benito, C., Núñez, E., Tolón, R. M., Carrier, E. J., Rábano, A., Hillard, C. J., & Romero, J. (2003). Cannabinoid CB2 receptors and fatty acid amide hydrolase are selectively overexpressed in neuritic plaque-associated glia in Alzheimer's disease brains. *Journal of Neuroscience*, *23*(35), 11136–11141.

Betterle, C., & Zanchetta, R. (2012). The immunofluorescence techniques in the diagnosis of endocrine autoimmune diseases. *Autoimmunity Highlights*, *3*(2), 67–78.

Bhat, N. R., Feinstein, D. L., Shen, Q., & Bhat, A. N. (2002). p38 MAPK-mediated Transcriptional Activation of Inducible Nitric-oxide Synthase in glial cells roles of nuclear factors, nuclear factor κB, cAMP response element-binding protein, ccaat/enhancer-binding protein-β, and activating transcription factor-2. *Journal of Biological Chemistry*, *277*(33), 29584–29592.

Bhat, N. R., Zhang, P., Lee, J. C., & Hogan, E. L. (1998). Extracellular signal-regulated kinase and p38 subgroups of mitogen-activated protein kinases regulate inducible nitric oxide synthase and tumor necrosis factor-α gene expression in endotoxin-stimulated primary glial cultures. *Journal*

*of Neuroscience*, *18*(5), 1633–1641.

Bi, W., He, C., Li, X., Zhou, L., Liu, R., Zhang, S., … Zhang, P. (2018). Ginnalin A from Kujin tea (Acer tataricum subsp. ginnala) exhibits a colorectal cancer chemoprevention effect via activation of the Nrf2/HO-1 signaling pathway. *Food & Function*, *9*(5), 2809–2819.

Biedler, J. L., Helson, L., & Spengler, B. A. (1973). Morphology and growth, tumorigenicity, and cytogenetics of human neuroblastoma cells in continuous culture. *Cancer Research*, *33*(11), 2643–2652.

Blasi, E., Barluzzi, R., Bocchini, V., Mazzolla, R., & Bistoni, F. (1990). Immortalization of murine microglial cells by a v-raf/v-myc carrying retrovirus. *Journal of Neuroimmunology*, *27*(2–3), 229–237.

Block, M. L., Zecca, L., & Hong, J.-S. (2007). Microglia-mediated neurotoxicity: uncovering the molecular mechanisms. *Nature Reviews Neuroscience*, *8*(1), 57–69.

Bohle, D. S., & Fotie, J. (2006). 3-(1, 3-Benzodioxol-5-yl)-N-(2-methylpropyl) prop-2-enamide (fagaramide). *Acta Crystallographica Section E: Structure Reports Online*, *62*(8), o3537–o3538.

Borgonetti, V., Benatti, C., Governa, P., Isoldi, G., Pellati, F., Alboni, S., … Manetti, F. (2022). Non-psychotropic Cannabis sativa L. phytocomplex modulates microglial inflammatory response through CB2 receptors-, endocannabinoids-, and NF-κB-mediated signaling. *Phytotherapy Research*, *36*(5), 2246–2263.

Bozic, I., Savic, D., Laketa, D., Bjelobaba, I., Milenkovic, I., Pekovic, S., … Lavrnja, I. (2015). Benfotiamine attenuates inflammatory response in LPS stimulated BV-2 microglia. *PLoS One*, *10*(2), e0118372.

Brandes, R. P., Weissmann, N., & Schröder, K. (2014). Nox family NADPH oxidases: molecular mechanisms of activation. *Free Radical Biology and Medicine*, *76*, 208–226.

Braun, M., Khan, Z. T., Khan, M. B., Kumar, M., Ward, A., Achyut, B. R., … Baban, B. (2018). Selective activation of cannabinoid receptor-2 reduces neuroinflammation after traumatic brain injury via

alternative macrophage polarization. *Brain, Behavior, and Immunity*, *68*, 224–237.

Briken, S., Rosenkranz, S. C., Keminer, O., Patra, S., Ketels, G., Heesen, C., … Gold, S. M. (2016). Effects of exercise on Irisin, BDNF and IL-6 serum levels in patients with progressive multiple sclerosis. *Journal of Neuroimmunology*, *299*, 53–58.

Bujak, J. K., Kosmala, D., Szopa, I. M., Majchrzak, K., & Bednarczyk, P. (2019). Inflammation, cancer and immunity—implication of TRPV1 channel. *Frontiers in Oncology*, 1087.

Burkert, K., Moodley, K., Angel, C. E., Brooks, A., & Graham, E. S. (2012). Detailed analysis of inflammatory and neuromodulatory cytokine secretion from human NT2 astrocytes using multiplex bead array. *Neurochemistry International*, *60*(6), 573–580.

Cabral, G. A., Harmon, K. N., & Carlisle, S. J. (2002). Cannabinoid-mediated inhibition of inducible nitric oxide production by rat microglial cells: evidence for CB 1 receptor participation. In *Neuroimmune Circuits, Drugs of Abuse, and Infectious Diseases* (pp. 207–214). Springer.

Caggiano, A. O., & Kraig, R. P. (1999). Prostaglandin E Receptor Subtypes in Cultured Rat Microglia and Their Role in Reducing Lipopolysaccharide-Induced Interleukin-1 β Production. *Journal of Neurochemistry*, *72*(2), 565–575.

Cai, Z., Hussain, M. D., & Yan, L.-J. (2014). Microglia, neuroinflammation, and beta-amyloid protein in Alzheimer's disease. *International Journal of Neuroscience*, *124*(5), 307–321.

Çakır, M., Tekin, S., Doğanyiğit, Z., Erden, Y., Soytürk, M., Çiğremiş, Y., & Sandal, S. (2019). Cannabinoid type 2 receptor agonist JWH-133, attenuates Okadaic acid induced spatial memory impairment and neurodegeneration in rats. *Life Sciences*, *217*, 25–33.

Calder, P. C. (2006). Polyunsaturated fatty acids and inflammation. *Prostaglandins, Leukotrienes and Essential Fatty Acids*, *75*(3), 197–202.

Callén, L., Moreno, E., Barroso-Chinea, P., Moreno-Delgado, D., Cortés, A., Mallol, J., … Lluis, C. (2012). Cannabinoid receptors CB1 and CB2 form functional heteromers in brain. *Journal of Biological*

*Chemistry*, *287*(25), 20851–20865.

Calsolaro, V., & Edison, P. (2016). Neuroinflammation in Alzheimer's disease: current evidence and future directions. *Alzheimer's & Dementia*, *12*(6), 719–732.

Campbell, I. L., Erta, M., Lim, S. L., Frausto, R., May, U., Rose-John, S., … Hidalgo, J. (2014). Trans-signaling is a dominant mechanism for the pathogenic actions of interleukin-6 in the brain. *Journal of Neuroscience*, *34*(7), 2503–2513.

Cantley, L. C. (2002). The phosphoinositide 3-kinase pathway. *Science*, *296*(5573), 1655–1657.

Carayon, P., Marchand, J., Dussossoy, D., Derocq, J.-M., Jbilo, O., Bord, A., … Pénarier, G. (1998). Modulation and functional involvement of CB2 peripheral cannabinoid receptors during B-cell differentiation. *Blood, The Journal of the American Society of Hematology*, *92*(10), 3605–3615.

Carolindah, M. N., Rosli, R., Adam, A., & Nordin, N. (2013). An overview of in vitro research models for Alzheimer's disease (AD). *Regen Res*, *2*(2), 8–13.

Carvalho, C., Correia, S. C., Santos, R. X., Cardoso, S., Moreira, P. I., Clark, T. A., … Perry, G. (2009). Role of mitochondrial-mediated signaling pathways in Alzheimer disease and hypoxia. *Journal of Bioenergetics and Biomembranes*, *41*(5), 433–440.

Casarejos, M. J., Perucho, J., Gomez, A., Muñoz, M. P., Fernandez-Estevez, M., Sagredo, O., … Mena, M. A. (2013). Natural cannabinoids improve dopamine neurotransmission and tau and amyloid pathology in a mouse model of tauopathy. *Journal of Alzheimer's Disease*, *35*(3), 525–539.

Cassano, T., Calcagnini, S., Pace, L., De Marco, F., Romano, A., & Gaetani, S. (2017). Cannabinoid receptor 2 signaling in neurodegenerative disorders: from pathogenesis to a promising therapeutic target. *Frontiers in Neuroscience*, *11*, 30.

Castegna, A., Thongboonkerd, V., Klein, J. B., Lynn, B., Markesbery, W. R., & Butterfield, D. A. (2003). Proteomic identification of nitrated proteins in Alzheimer's disease brain. *Journal of Neurochemistry*, *85*(6), 1394–1401.

Castillo, P. E., Younts, T. J., Chávez, A. E., & Hashimotodani, Y. (2012). Endocannabinoid signaling and synaptic function. *Neuron*, *76*(1), 70–81.

Çelik, H., Kucukler, S., Çomaklı, S., Özdemir, S., Caglayan, C., Yardım, A., & Kandemir, F. M. (2020). Morin attenuates ifosfamide-induced neurotoxicity in rats via suppression of oxidative stress, neuroinflammation and neuronal apoptosis. *Neurotoxicology*, *76*, 126–137.

Chaaib, F., Queiroz, E. F., Ndjoko, K., Diallo, D., & Hostettmann, K. (2003). Antifungal and antioxidant compounds from the root bark of Fagara zanthoxyloides. *Planta Medica*, *69*(04), 316–320.

Chakroborty, S., Kim, J., Schneider, C., West, A. R., & Stutzmann, G. E. (2015). Nitric oxide signaling is recruited as a compensatory mechanism for sustaining synaptic plasticity in Alzheimer's disease mice. *Journal of Neuroscience*, *35*(17), 6893–6902.

Chan, C.-K., Tan, L. T.-H., Andy, S. N., Kamarudin, M. N. A., Goh, B.-H., & Kadir, H. A. (2017). Anti-neuroinflammatory activity of Elephantopus scaber L. via activation of Nrf2/HO-1 signaling and inhibition of p38 MAPK pathway in LPS-induced microglia BV-2 cells. *Frontiers in Pharmacology*, *8*, 397.

Chan, K., Han, X.-D., & Kan, Y. W. (2001). An important function of Nrf2 in combating oxidative stress: detoxification of acetaminophen. *Proceedings of the National Academy of Sciences*, *98*(8), 4611–4616.

Chang, R., Yee, K.-L., & Sumbria, R. K. (2017). Tumor necrosis factor α inhibition for Alzheimer's disease. *Journal of Central Nervous System Disease*, *9*, 1179573517709278.

Chantong, B., Kratschmar, D. V, Nashev, L. G., Balazs, Z., & Odermatt, A. (2012). Mineralocorticoid and glucocorticoid receptors differentially regulate NF-kappaB activity and pro-inflammatory cytokine production in murine BV-2 microglial cells. *Journal of Neuroinflammation*, *9*(1), 260.

Chao, C. C., Hu, S., Molitor, T. W., Shaskan, E. G., & Peterson, P. K. (1992). Activated microglia mediate neuronal cell injury via a nitric oxide mechanism. *The Journal of Immunology*, *149*(8), 2736–2741.

Chao, X. D., Ma, Y. H., Luo, P., Cao, L., Lau, W. B., Zhao, B. C., … Su, N. (2013). Up-regulation of heme oxygenase-1 attenuates brain damage after cerebral ischemia via simultaneous inhibition of superoxide production and preservation of NO bioavailability. *Experimental Neurology*, *239*, 163–169.

Chen, H.-L., Jia, W.-J., Li, H.-E., Han, H., Li, F., Zhang, X.-L.-N., … Wu, C.-Y. (2020). Scutellarin exerts anti-inflammatory effects in activated microglia/brain macrophage in cerebral ischemia and in activated BV-2 microglia through regulation of MAPKs signaling pathway. *Neuromolecular Medicine*, *22*(2), 264–277.

Chen, J.-Y., Zhu, G.-Y., Su, X.-H., Wang, R., Liu, J., Liao, K., … Liu, L. (2017). 7-deacetylgedunin suppresses inflammatory responses through activation of Keap1/Nrf2/HO-1 signaling. *Oncotarget*, *8*(33), 55051.

Chen, J., Hamers, A. J. P., Finsterbusch, M., Massimo, G., Zafar, M., Corder, R., … Ahluwalia, A. (2018). Endogenously generated arachidonate-derived ligands for TRPV1 induce cardiac protection in sepsis. *The FASEB Journal*, *32*(7), 3816–3831.

Chen, X., Guo, C., & Kong, J. (2012). Oxidative stress in neurodegenerative diseases. *Neural Regeneration Research*, *7*(5), 376.

Chen, X., Zhang, J., & Chen, C. (2011). ENDOCANNABINOID 2-ARACHIDONOYLGLYCEROL PROTECTS NEURONS AGAINST NL -AMYLOID INSULTS. *NSC*, *178*, 159–168. https://doi.org/10.1016/j.neuroscience.2011.01.024

Cheng, D., Low, J. K., Logge, W., Garner, B., & Karl, T. (2014). Chronic cannabidiol treatment improves social and object recognition in double transgenic APPswe/PS1ΔE9 mice. *Psychopharmacology*, *231*(15), 3009–3017. https://doi.org/10.1007/s00213-014-3478-5

Cheng, X., Yang, L., He, P., Li, R., & Shen, Y. (2010). Differential activation of tumor necrosis factor receptors distinguishes between brains from Alzheimer’s disease and non-demented patients.

*Journal of Alzheimer's Disease*, *19*(2), 621–630.

Cherry, J. D., Olschowka, J. A., & O'Banion, M. K. (2014). Neuroinflammation and M2 microglia: the good, the bad, and the inflamed. *Journal of Neuroinflammation*, *11*(1), 1–15.

Cho, N., Moon, E. H., Kim, H. W., Hong, J., Beutler, J. A., & Sung, S. H. (2016). Inhibition of nitric oxide production in BV2 microglial cells by triterpenes from tetrapanax papyriferus. *Molecules*, *21*(4), 459.

Choi, S. H., Kim, Y. H., Hebisch, M., Sliwinski, C., Lee, S., D'Avanzo, C., … Muffat, J. (2014). A three-dimensional human neural cell culture model of Alzheimer's disease. *Nature*, *515*(7526), 274–278.

Choi, S. S., Lee, H. J., Lim, I., Satoh, J., & Kim, S. U. (2014). Human astrocytes: secretome profiles of cytokines and chemokines. *PloS One*, *9*(4), e92325.

Christen, Y. (2000). Oxidative stress and Alzheimer disease 1 , 2. *The American Journal of Clinical Nutrition*, *71*, 621S–9S.

Cianciulli, A., Calvello, R., Porro, C., Trotta, T., Salvatore, R., & Panaro, M. A. (2016). PI3k/Akt signalling pathway plays a crucial role in the anti-inflammatory effects of curcumin in LPS-activated microglia. *International Immunopharmacology*, *36*, 282–290.

Cianciulli, A., Porro, C., Calvello, R., Trotta, T., Lofrumento, D. D., & Panaro, M. A. (2020). Microglia mediated neuroinflammation: focus on PI3K modulation. *Biomolecules*, *10*(1), 137.

Cianciulli, A., Salvatore, R., Porro, C., Trotta, T., & Panaro, M. A. (2016). Folic acid is able to polarize the inflammatory response in LPS activated microglia by regulating multiple signaling pathways. *Mediators of Inflammation*, *2016*.

Clarke, J. R., Lyra e Silva, N. M., Figueiredo, C. P., Frozza, R. L., Ledo, J. H., Beckman, D., … De Felice, F. G. (2015). Alzheimer-associated A  oligomers impact the central nervous system to induce peripheral metabolic deregulation. *EMBO Molecular Medicine*, *7*(2), 190–210.

https://doi.org/10.15252/emmm.201404183

Codarri, L., Fontana, A., & Becher, B. (2010). Cytokine networks in multiple sclerosis: lost in translation. *Current Opinion in Neurology*, *23*(3), 205–211.

Colonna, M., & Butovsky, O. (2017). Microglia function in the central nervous system during health and neurodegeneration. *Annual Review of Immunology*, *35*, 441.

Coma, M., Guix, F. X., Uribesalgo, I., Espuna, G., Sole, M., Andreu, D., & Munoz, F. J. (2005). Lack of oestrogen protection in amyloid-mediated endothelial damage due to protein nitrotyrosination. *Brain*, *128*(7), 1613–1621.

Combrinck, M., Williams, J., De Berardinis, M. A., Warden, D., Puopolo, M., Smith, A. D., & Minghetti, L. (2006). Levels of CSF prostaglandin E2, cognitive decline, and survival in Alzheimer's disease. *Journal of Neurology, Neurosurgery & Psychiatry*, *77*(1), 85–88.

Concannon, R. M., Okine, B. N., Finn, D. P., & Dowd, E. (2016). Upregulation of the cannabinoid CB2 receptor in environmental and viral inflammation-driven rat models of Parkinson's disease. *Experimental Neurology*, *283*, 204–212.

Constantinescu, R., Constantinescu, A. T., Reichmann, H., & Janetzky, B. (2007). Neuronal differentiation and long-term culture of the human neuroblastoma line SH-SY5Y. In *Neuropsychiatric disorders an integrative approach* (pp. 17–28). Springer.

Cooney, S. J., Bermudez-Sabogal, S. L., & Byrnes, K. R. (2013). Cellular and temporal expression of NADPH oxidase (NOX) isotypes after brain injury. *Journal of Neuroinflammation*, *10*(1), 917.

Correa, F., Docagne, F., Mestre, L., Clemente, D., Hernangómez, M., Loría, F., & Guaza, C. (2009). A role for CB2 receptors in anandamide signalling pathways involved in the regulation of IL-12 and IL-23 in microglial cells. *Biochemical Pharmacology*, *77*(1), 86–100.

Couch, Y., Alvarez-Erviti, L., Sibson, N. R., Wood, M. J. A., & Anthony, D. C. (2011). The acute inflammatory response to intranigral α-synuclein differs significantly from intranigral

lipopolysaccharide and is exacerbated by peripheral inflammation. *Journal of Neuroinflammation*, *8*(1), 1–14.

D'Addario, C., Di Francesco, A., Arosio, B., Gussago, C., Dell'Osso, B., Bari, M., … Mari, D. (2012). Epigenetic regulation of fatty acid amide hydrolase in Alzheimer disease. *PloS One*, *7*(6), e39186.

Dai, X., Li, N., Yu, L., Chen, Z., Hua, R., Qin, X., & Zhang, Y.-M. (2015). Activation of BV2 microglia by lipopolysaccharide triggers an inflammatory reaction in PC12 cell apoptosis through a toll-like receptor 4-dependent pathway. *Cell Stress and Chaperones*, *20*(2), 321–331.

Dalal, N. V, Pranski, E. L., Tansey, M. G., Lah, J. J., Levey, A. I., & Betarbet, R. S. (2012). RNF11 modulates microglia activation through NF-κB signalling cascade. *Neuroscience Letters*, *528*(2), 174–179.

Dan, H. C., Cooper, M. J., Cogswell, P. C., Duncan, J. A., Ting, J. P.-Y., & Baldwin, A. S. (2008). Akt-dependent regulation of NF-κB is controlled by mTOR and Raptor in association with IKK. *Genes & Development*, *22*(11), 1490–1500.

De Strooper, B., & Karran, E. (2016). The cellular phase of Alzheimer's disease. *Cell*, *164*(4), 603–615.

Deane, J. A., & Fruman, D. A. (2004). Phosphoinositide 3-kinase: diverse roles in immune cell activation. *Annu. Rev. Immunol.*, *22*, 563–598.

Dehmelt, L., & Halpain, S. (2005). The MAP2/Tau family of microtubule-associated proteins. *Genome Biology*, *6*(1), 1–10.

Del Cerro, P., Alquézar, C., Bartolomé, F., González-Naranjo, P., Pérez, C., Carro, E., … Martín-Requero, Á. (2018). Activation of the cannabinoid type 2 receptor by a novel indazole derivative normalizes the survival pattern of lymphoblasts from patients with late-onset Alzheimer's disease. *CNS Drugs*, *32*(6), 579–591.

Deng, G., Su, J. H., Ivins, K. J., Van Houten, B., & Cotman, C. W. (1999). Bcl-2 facilitates recovery from DNA damage after oxidative stress. *Experimental Neurology*, *159*(1), 309–318.

Dérijard, B., Hibi, M., Wu, I.-H., Barrett, T., Su, B., Deng, T., … Davis, R. J. (1994). JNK1: a protein kinase

stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain. *Cell*, *76*(6), 1025–1037.

Deshmukh, P., Unni, S., Krishnappa, G., & Padmanabhan, B. (2017). The Keap1–Nrf2 pathway: promising therapeutic target to counteract ROS-mediated damage in cancers and neurodegenerative diseases. *Biophysical Reviews*, *9*(1), 41–56.

Di Marzo, V., Griffin, G., De Petrocellis, L., Brandi, I., Bisogno, T., Williams, W., … Razdan, R. K. (2002). A structure/activity relationship study on arvanil, an endocannabinoid and vanilloid hybrid. *Journal of Pharmacology and Experimental Therapeutics*, *300*(3), 984–991.

Di Marzo, V., & Petrosino, S. (2007). Endocannabinoids and the regulation of their levels in health and disease. *Current Opinion in Lipidology*, *18*(2), 129–140.

Dofuor, A. K., Ayertey, F., Bolah, P., Djameh, G. I., Kyeremeh, K., Ohashi, M., … Gwira, T. M. (2020). Isolation and antitrypanosomal characterization of furoquinoline and oxylipin from zanthoxylum zanthoxyloides. *Biomolecules*, *10*(12), 1670.

Dugan, L. L., Ali, S. S., Shekhtman, G., Roberts, A. J., Lucero, J., Quick, K. L., & Behrens, M. M. (2009). IL-6 mediated degeneration of forebrain GABAergic interneurons and cognitive impairment in aged mice through activation of neuronal NADPH oxidase. *PLoS One*, *4*(5), e5518.

Dugger, B. N., & Dickson, D. W. (2017). Pathology of neurodegenerative diseases. *Cold Spring Harbor Perspectives in Biology*, *9*(7), a028035.

Durães, F., Pinto, M., & Sousa, E. (2018). Old drugs as new treatments for neurodegenerative diseases. *Pharmaceuticals*, *11*(2), 44.

Ehrhart, J., Obregon, D., Mori, T., Hou, H., Sun, N., Bai, Y., … Shytle, R. D. (2005). Stimulation of cannabinoid receptor 2 (CB 2) suppresses microglial activation. *Journal of Neuroinflammation*, *2*(1), 29.

Eljaschewitsch, E., Witting, A., Mawrin, C., Lee, T., Schmidt, P. M., Wolf, S., … Nitsch, R. (2006). The

endocannabinoid anandamide protects neurons during CNS inflammation by induction of MKP-1 in microglial cells. *Neuron*, *49*(1), 67–79.

Elmazoglu, Z., Kayhan, H., Santamaría, A., Rangel-López, E., Uğur, P. K., Ceylan, A., … Karasu, Ç. (2021). Platinum nanoparticles Protect Against Lipopolysaccharide-Induced Inflammation in Microglial BV-2 Cells via Decreased Oxidative Damage and Increased Phagocytosis. *Neurochemical Research*, 1–17.

Emmanouil, M., Taoufik, E., Tseveleki, V., Vamvakas, S.-S., & Probert, L. (2011). A role for neuronal NF-κB in suppressing neuroinflammation and promoting neuroprotection in the CNS. In *Advances in TNF Family Research* (pp. 575–581). Springer.

Engvall, E., & Perlmann, P. (1971). Enzyme-linked immunosorbent assay (ELISA) quantitative assay of immunoglobulin G. *Immunochemistry*, *8*(9), 871–874.

Espinosa-Oliva, A. M., De Pablos, R. M., Villarán, R. F., Argüelles, S., Venero, J. L., Machado, A., & Cano, J. (2011). Stress is critical for LPS-induced activation of microglia and damage in the rat hippocampus. *Neurobiology of Aging*, *32*(1), 85–102.

Esposito, G., De Filippis, D., Steardo, L., Scuderi, C., Savani, C., Cuomo, V., & Iuvone, T. (2006). CB1 receptor selective activation inhibits β-amyloid-induced iNOS protein expression in C6 cells and subsequently blunts tau protein hyperphosphorylation in co-cultured neurons. *Neuroscience Letters*, *404*(3), 342–346.

Esposito, G., Iuvone, T., Savani, C., Scuderi, C., De Filippis, D., Papa, M., … Steardo, L. (2007). Opposing control of cannabinoid receptor stimulation on amyloid-β-induced reactive gliosis: in vitro and in vivo evidence. *Journal of Pharmacology and Experimental Therapeutics*, *322*(3), 1144–1152.

Esposito, G., Scuderi, C., Valenza, M., Togna, G. I., & Latina, V. (2011). *Cannabidiol Reduces Ab-Induced Neuroinflammation and Promotes Hippocampal*.

Esposito, G., Scuderi, C., Valenza, M., Togna, G. I., Latina, V., de Filippis, D., … Steardo, L. (2011).

Cannabidiol reduces Aβ-induced neuroinflammation and promotes hippocampal neurogenesis through PPARγ involvement. *PLoS ONE*, *6*(12). https://doi.org/10.1371/journal.pone.0028668

Eubanks, L. M., Rogers, C. J., Beuscher IV, A. E., Koob, G. F., Olson, A. J., Dickerson, T. J., & Janda, K. D. (2006). A molecular link between the active component of marijuana and Alzheimer's disease pathology. *Molecular Pharmaceutics*, *3*(6), 773–777.

Everse, J., & Coates, P. W. (2009). Neurodegeneration and peroxidases. *Neurobiology of Aging*, *30*(7), 1011–1025.

Facchinetti, F., Del Giudice, E., Furegato, S., Passarotto, M., & Leon, A. (2003). Cannabinoids ablate release of TNFα in rat microglial cells stimulated with lypopolysaccharide. *Glia*, *41*(2), 161–168.

Fakhfouri, G., Ahmadiani, A., Rahimian, R., Grolla, A. A., Moradi, F., & Haeri, A. (2012). WIN55212-2 attenuates amyloid-beta-induced neuroinflammation in rats through activation of cannabinoid receptors and PPAR-γ pathway. *Neuropharmacology*, *63*(4), 653–666. https://doi.org/10.1016/j.neuropharm.2012.05.013

Fan, H.-H., Zhu, L.-B., Li, T., Zhu, H., Wang, Y.-N., Ren, X.-L., … Zhang, X. (2017). Hyperoside inhibits lipopolysaccharide-induced inflammatory responses in microglial cells via p38 and NFκB pathways. *International Immunopharmacology*, *50*, 14–21.

Feng, Z.-H., Wang, T.-G., Li, D.-D., Fung, P., Wilson, B. C., Liu, B., … Hong, J.-S. (2002). Cyclooxygenase-2-deficient mice are resistant to 1-methyl-4-phenyl1, 2, 3, 6-tetrahydropyridine-induced damage of dopaminergic neurons in the substantia nigra. *Neuroscience Letters*, *329*(3), 354–358.

Ferreiro, E., Baldeiras, I., Ferreira, I. L., Costa, R. O., Rego, A. C., Pereira, C. F., & Oliveira, C. R. (2012). Mitochondrial-and endoplasmic reticulum-associated oxidative stress in Alzheimer's disease: from pathogenesis to biomarkers. *International Journal of Cell Biology*, *2012*.

Figueiredo, C., Pais, T. F., Gomes, J. R., & Chatterjee, S. (2008). Neuron-microglia crosstalk up-regulates neuronal FGF-2 expression which mediates neuroprotection against excitotoxicity via JNK1/2.

*Journal of Neurochemistry*, *107*(1), 73–85.

Finkel, T., & Holbrook, N. J. (2000). Oxidants, oxidative stress and the biology of ageing. *Nature*, *408*(6809), 239–247.

Fischer, R., & Maier, O. (2015). Interrelation of oxidative stress and inflammation in neurodegenerative disease: role of TNF. *Oxidative Medicine and Cellular Longevity*, *2015*.

Foegh, M. L. (1998). The eicosanoids: prostaglandins, thromboxanes, leukotrienes, and related compounds. *Basic and Clinical Pharmacology*.

Fotakis, G., & Timbrell, J. A. (2006). In vitro cytotoxicity assays: comparison of LDH, neutral red, MTT and protein assay in hepatoma cell lines following exposure to cadmium chloride. *Toxicology Letters*, *160*(2), 171–177.

Fukao, T., & Koyasu, S. (2003). PI3K and negative regulation of TLR signaling. *Trends in Immunology*, *24*(7), 358–363.

Gan, L., & Johnson, J. A. (2014). Oxidative damage and the Nrf2-ARE pathway in neurodegenerative diseases. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease*, *1842*(8), 1208–1218.

Gao, F., Ding, B., Zhou, L., Gao, X., Guo, H., & Xu, H. (2013). Magnesium sulfate provides neuroprotection in lipopolysaccharide-activated primary microglia by inhibiting NF-κB pathway. *Journal of Surgical Research*, *184*(2), 944–950.

Gao, F., Shen, J., Zhao, L., Hao, Q., & Yang, Y. (2019). Curcumin alleviates lipopolysaccharide (LPS)-activated neuroinflammation via modulation of miR-199b-5p/IκB kinase β (IKKβ)/nuclear factor kappa B (NF-κB) pathway in microglia. *Medical Science Monitor: International Medical Journal of Experimental and Clinical Research*, *25*, 9801.

Gao, H.-M., Zhou, H., & Hong, J.-S. (2012). NADPH oxidases: novel therapeutic targets for neurodegenerative diseases. *Trends in Pharmacological Sciences*, *33*(6), 295–303.

Gao, Y., Cui, M., Zhong, S., Feng, C., Nwobodo, A. K., Chen, B., … Wang, Y. (2020). Dihydroartemisinin

ameliorates LPS-induced neuroinflammation by inhibiting the PI3K/AKT pathway. *Metabolic Brain Disease*, *35*(4), 661–672.

Gao, Z., Wang, H., Zhang, B., Wu, X., Zhang, Y., Ge, P., … Liang, J. (2018). Trehalose inhibits H2O2-induced autophagic death in dopaminergic SH-SY5Y cells via mitigation of ROS-dependent endoplasmic reticulum stress and AMPK activation. *International Journal of Medical Sciences*, *15*(10), 1014.

Garcia-Argaez, A. N., Apan, T. O. R., Delgado, H. P., Velázquez, G., & Martínez-Vázquez, M. (2000). Anti-inflammatory activity of coumarins from Decatropis bicolor on TPA ear mice model. *Planta Medica*, *66*(03), 279–281.

Gee, M. S., Kim, S.-W., Kim, N., Lee, S. J., Oh, M. S., Jin, H. K., … Lee, J. K. (2018). A novel and selective p38 mitogen-activated protein kinase inhibitor attenuates LPS-induced neuroinflammation in BV2 microglia and a mouse model. *Neurochemical Research*, *43*(12), 2362–2371.

Gerdeman, G. L., Partridge, J. G., Lupica, C. R., & Lovinger, D. M. (2003). It could be habit forming: drugs of abuse and striatal synaptic plasticity. *Trends in Neurosciences*, *26*(4), 184–192.

Giridharan, S., & Srinivasan, M. (2018). Mechanisms of NF-κB p65 and strategies for therapeutic manipulation. *Journal of Inflammation Research*, *11*, 407.

Gitler, A. D., Dhillon, P., & Shorter, J. (2017). Neurodegenerative disease: models, mechanisms, and a new hope. *Disease Models & Mechanisms*, Vol. 10, pp. 499–502. The Company of Biologists Ltd.

Glaser, S. T., Abumrad, N. A., Fatade, F., Kaczocha, M., Studholme, K. M., & Deutsch, D. G. (2003). Evidence against the presence of an anandamide transporter. *Proceedings of the National Academy of Sciences*, *100*(7), 4269–4274.

Göbel, K., Ruck, T., & Meuth, S. G. (2018). Cytokine signaling in multiple sclerosis: Lost in translation. *Multiple Sclerosis Journal*, *24*(4), 432–439.

Gómez-Gálvez, Y., Palomo-Garo, C., Fernández-Ruiz, J., & García, C. (2016). Potential of the

cannabinoid CB2 receptor as a pharmacological target against inflammation in Parkinson's disease. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, *64*, 200–208.

Gong, J.-P., Onaivi, E. S., Ishiguro, H., Liu, Q.-R., Tagliaferro, P. A., Brusco, A., & Uhl, G. R. (2006). Cannabinoid CB2 receptors: immunohistochemical localization in rat brain. *Brain Research*, *1071*(1), 10–23.

Goodman, C. D., Austarheim, I., Mollard, V., Mikolo, B., Malterud, K. E., McFadden, G. I., & Wangensteen, H. (2016). Natural products from Zanthoxylum heitzii with potent activity against the malaria parasite. *Malaria Journal*, *15*(1), 1–8.

Granic, I., Dolga, A. M., Nijholt, I. M., van Dijk, G., & Eisel, U. L. M. (2009). Inflammation and NF-κB in Alzheimer's disease and diabetes. *Journal of Alzheimer's Disease*, *16*(4), 809–821.

Gu, H., Li, L., Cui, C., Zhao, Z., & Song, G. (2017). Overexpression of let-7a increases neurotoxicity in a PC12 cell model of Alzheimer's disease via regulating autophagy. *Experimental and Therapeutic Medicine*, *14*(4), 3688–3698.

Guan, P.-P., & Wang, P. (2019). Integrated communications between cyclooxygenase-2 and Alzheimer's disease. *The FASEB Journal*, *33*(1), 13–33.

Guo, L.-T., Wang, S.-Q., Su, J., Xu, L.-X., Ji, Z.-Y., Zhang, R.-Y., … Ma, S.-P. (2019). Baicalin ameliorates neuroinflammation-induced depressive-like behavior through inhibition of toll-like receptor 4 expression via the PI3K/AKT/FoxO1 pathway. *Journal of Neuroinflammation*, *16*(1), 1–21.

Gupta, S. C., Tyagi, A. K., Deshmukh-Taskar, P., Hinojosa, M., Prasad, S., & Aggarwal, B. B. (2014). Downregulation of tumor necrosis factor and other proinflammatory biomarkers by polyphenols. *Archives of Biochemistry and Biophysics*, *559*, 91–99.

Guzman-Martinez, L., Maccioni, R. B., Andrade, V., Navarrete, L. P., Pastor, M. G., & Ramos-Escobar, N. (2019). Neuroinflammation as a common feature of neurodegenerative disorders. *Frontiers in Pharmacology*, 1008.

Habashi, S. A., Sabouni, F., Moghimi, A., & Majd, S. A. (2016). Modulation of lipopolysaccharide stimulated nuclear factor kappa B mediated iNOS/NO production by bromelain in rat primary microglial cells. *Iranian Biomedical Journal*, *20*(1), 33.

Halliwell, B., & Aruoma, O. I. (1991). DNA damage by oxygen-derived species Its mechanism and measurement in mammalian systems. *FEBS Letters*, *281*(1–2), 9–19.

Hampson, A. J., Grimaldi, M., Axelrod, J., & Wink, D. (1998). Cannabidiol and (−) Δ9-tetrahydrocannabinol are neuroprotective antioxidants. *Proceedings of the National Academy of Sciences*, *95*(14), 8268–8273.

Hampson, A. J., Grimaldi, M., Lolic, M., Wink, D., Rosenthal, R., & Axelrod, J. (2000). Neuroprotective Antioxidants from Marijuana a. *Annals of the New York Academy of Sciences*, *899*(1), 274–282.

Han, M. H., Lee, W. S., Nagappan, A., Hong, S. H., Jung, J. H., Park, C., … Jung, J. (2016). Flavonoids Isolated from Flowers of Lonicera japonica Thunb. Inhibit Inflammatory Responses in BV2 Microglial Cells by Suppressing TNF-α and IL-β Through PI3K/Akt/NF-kb Signaling Pathways. *Phytotherapy Research*, *30*(11), 1824–1832.

Hansen, D. V, Hanson, J. E., & Sheng, M. (2018). Microglia in Alzheimer's disease. *Journal of Cell Biology*, *217*(2), 459–472.

Haspula, D., & Clark, M. A. (2020). Cannabinoid receptors: an update on cell signaling, pathophysiological roles and therapeutic opportunities in neurological, cardiovascular, and inflammatory diseases. *International Journal of Molecular Sciences*, *21*(20), 7693.

Hayden, M. S., West, A. P., & Ghosh, S. (2006). NF-κ B and the immune response. *Oncogene*, *25*(51), 6758–6780.

He, F.-Q., Qiu, B.-Y., Zhang, X.-H., Li, T.-K., Xie, Q., Cui, D.-J., … Gan, H.-T. (2011). Tetrandrine attenuates spatial memory impairment and hippocampal neuroinflammation via inhibiting NF-κB activation in a rat model of Alzheimer's disease induced by amyloid-β (1–42). *Brain Research*, *1384*, 89–96.

He, P., Zhong, Z., Lindholm, K., Berning, L., Lee, W., Lemere, C., … Shen, Y. (2007). Deletion of tumor necrosis factor death receptor inhibits amyloid β generation and prevents learning and memory deficits in Alzheimer's mice. *The Journal of Cell Biology*, *178*(5), 829–841.

He, X., Yang, L., Huang, R., Lin, L., Shen, Y., Cheng, L., … Zhu, R. (2020). Activation of CB2R with AM1241 ameliorates neurodegeneration via the Xist/miR-133b-3p/Pitx3 axis. *Journal of Cellular Physiology*, *235*(9), 6032–6042.

Hemmings, B. A., & Restuccia, D. F. (2012). Pi3k-pkb/akt pathway. *Cold Spring Harbor Perspectives in Biology*, *4*(9), a011189.

Heneka, M. T., Carson, M. J., El Khoury, J., Landreth, G. E., Brosseron, F., Feinstein, D. L., … Ransohoff, R. M. (2015). Neuroinflammation in Alzheimer's disease. *The Lancet Neurology*, *14*(4), 388–405.

Henn, A., Lund, S., Hedtjärn, M., Schrattenholz, A., Pörzgen, P., & Leist, M. (2009). The suitability of BV2 cells as alternative model system for primary microglia cultures or for animal experiments examining brain inflammation. *ALTEX: Alternatives to Animal Experimentation*, *26*(2), 83–94.

Hensley, K., Floyd, R. A., Zheng, N., Nael, R., Robinson, K. A., Nguyen, X., … Markesbery, W. R. (1999). p38 kinase is activated in the Alzheimer's disease brain. *Journal of Neurochemistry*, *72*(5), 2053–2058.

Heppner, F. L., Ransohoff, R. M., & Becher, B. (2015). Immune attack: the role of inflammation in Alzheimer disease. *Nature Reviews Neuroscience*, *16*(6), 358–372.

Herkenham, M., Lynn, A. B., Johnson, M. R., Melvin, L. S., de Costa, B. R., & Rice, K. C. (1991). Characterization and localization of cannabinoid receptors in rat brain: a quantitative in vitro autoradiographic study. *Journal of Neuroscience*, *11*(2), 563–583.

Ho, K. W., Ward, N. J., & Calkins, D. J. (2012). TRPV1: a stress response protein in the central nervous system. *American Journal of Neurodegenerative Disease*, *1*(1), 1.

Hodes, G. E., Ménard, C., & Russo, S. J. (2016). Integrating Interleukin-6 into depression diagnosis and

treatment. *Neurobiology of Stress*, *4*, 15–22.

Hoesel, B., & Schmid, J. A. (2013). The complexity of NF-κB signaling in inflammation and cancer. *Molecular Cancer*, *12*(1), 1–15.

Hohsfield, L. A., & Humpel, C. (2015). Migration of blood cells to β-amyloid plaques in Alzheimer's disease. *Experimental Gerontology*, *65*, 8–15.

Hopper, R. A., & Garthwaite, J. (2006). Tonic and phasic nitric oxide signals in hippocampal long-term potentiation. *Journal of Neuroscience*, *26*(45), 11513–11521.

Horváth, G., Horváth, A., Reichert, G., Böszörményi, A., Sipos, K., & Pandur, E. (2021). u. *BMC Complementary Medicine and Therapies*, *21*(1), 1–14.

Hou, Y., Li, N., Xie, G., Wang, J., Yuan, Q., Jia, C., … Wang, B. (2015). Pterostilbene exerts anti-neuroinflammatory effect on lipopolysaccharide-activated microglia via inhibition of MAPK signalling pathways. *Journal of Functional Foods*, *19*, 676–687.

Howlett, A. C. (2002). The cannabinoid receptors. *Prostaglandins & Other Lipid Mediators*, *68*, 619–631.

Howlett, A. C. (2005). Cannabinoid receptor signaling. In *Cannabinoids* (pp. 53–79). Springer.

Hsieh, Y.-H., Deng, J.-S., Chang, Y.-S., & Huang, G.-J. (2018). Ginsenoside Rh2 ameliorates lipopolysaccharide-induced acute lung injury by regulating the TLR4/PI3K/Akt/mTOR, Raf-1/MEK/ERK, and Keap1/Nrf2/HO-1 signaling pathways in mice. *Nutrients*, *10*(9), 1208.

Hu, X.-L., Niu, Y.-X., Zhang, Q., Tian, X., Gao, L.-Y., Guo, L.-P., … Zhao, Q.-C. (2015). Neuroprotective effects of Kukoamine B against hydrogen peroxide-induced apoptosis and potential mechanisms in SH-SY5Y cells. *Environmental Toxicology and Pharmacology*, *40*(1), 230–240.

Huang, M.-Y., Tu, C.-E., Wang, S.-C., Hung, Y.-L., Su, C.-C., Fang, S.-H., … Huang, Y.-W. (2018). Corylin inhibits LPS-induced inflammatory response and attenuates the activation of NLRP3 inflammasome in microglia. *BMC Complementary and Alternative Medicine*, *18*(1), 1–9.

Huang, W.-C., & Hung, M.-C. (2013). Beyond NF-κB activation: nuclear functions of IκB kinase α. *Journal of Biomedical Science*, *20*(1), 3.

Huang, W.-X., Yu, F., Sanchez, R. M., Liu, Y.-Q., Min, J.-W., Hu, J.-J., … Liu, W.-H. (2015). TRPV1 promotes repetitive febrile seizures by pro-inflammatory cytokines in immature brain. *Brain, Behavior, and Immunity*, *48*, 68–77.

Huang, W., Min, J., Liu, Y., He, X., & Peng, B. (2014). Expression of TRPV1 in the C57BL/6 mice brain hippocampus and cortex during development. *Neuroreport*, *25*(6), 379–385.

Iuvone, T., Esposito, G., Esposito, R., Santamaria, R., Di Rosa, M., & Izzo, A. A. (2004). Neuroprotective effect of cannabidiol, a non-psychoactive component from Cannabis sativa, on β-amyloid-induced toxicity in PC12 cells. *Journal of Neurochemistry*, *89*(1), 134–141. https://doi.org/10.1111/j.1471-4159.2003.02327.x

Iversen, L., & Chapman, V. (2002). Cannabinoids: a real prospect for pain relief. *Current Opinion in Pharmacology*, *2*(1), 50–55.

Jaafaru, M. S., Nordin, N., Rosli, R., Shaari, K., Saad, N., Noor, N. M., & Razis, A. F. A. (2019). Neuroprotective effects of glucomoringin-isothiocyanate against H2O2-Induced cytotoxicity in neuroblastoma (SH-SY5Y) cells. *Neurotoxicology*, *75*, 89–104.

Janefjord, E., Mååg, J. L. V, Harvey, B. S., & Smid, S. D. (2014). Cannabinoid effects on β amyloid fibril and aggregate formation, neuronal and microglial-activated neurotoxicity in vitro. *Cellular and Molecular Neurobiology*, *34*(1), 31–42.

Janelsins, M. C., Mastrangelo, M. A., Park, K. M., Sudol, K. L., Narrow, W. C., Oddo, S., … Bowers, W. J. (2008). Chronic neuron-specific tumor necrosis factor-alpha expression enhances the local inflammatory environment ultimately leading to neuronal death in 3xTg-AD mice. *The American Journal of Pathology*, *173*(6), 1768–1782.

Jang, S., Kelley, K. W., & Johnson, R. W. (2008). Luteolin reduces IL-6 production in microglia by

inhibiting JNK phosphorylation and activation of AP-1. *Proceedings of the National Academy of Sciences*, *105*(21), 7534–7539.

Jaramillo, M. C., & Zhang, D. D. (2013). The emerging role of the Nrf2–Keap1 signaling pathway in cancer. *Genes & Development*, *27*(20), 2179–2191.

Javed, H., Azimullah, S., Haque, M. E., & Ojha, S. K. (2016). Cannabinoid type 2 (CB2) receptors activation protects against oxidative stress and neuroinflammation associated dopaminergic neurodegeneration in rotenone model of Parkinson's disease. *Frontiers in Neuroscience*, *10*, 321.

Jayaram, S., & Krishnamurthy, P. T. (2021). Role of microgliosis, oxidative stress and associated neuroinflammation in the pathogenesis of Parkinson's disease: The therapeutic role of Nrf2 activators. *Neurochemistry International*, *145*, 105014.

Jayasooriya, R. G. P. T., Lee, K.-T., Kang, C.-H., Dilshara, M. G., Lee, H.-J., Choi, Y. H., … Kim, G.-Y. (2014). Isobutyrylshikonin inhibits lipopolysaccharide-induced nitric oxide and prostaglandin E2 production in BV2 microglial cells by suppressing the PI3K/Akt-mediated nuclear transcription factor-κB pathway. *Nutrition Research*, *34*(12), 1111–1119.

Jeayeng, S., Wongkajornsilp, A., Slominski, A. T., Jirawatnotai, S., Sampattavanich, S., & Panich, U. (2017). Nrf2 in keratinocytes modulates UVB-induced DNA damage and apoptosis in melanocytes through MAPK signaling. *Free Radical Biology and Medicine*, *108*, 918–928.

Jellinger, K. A. (2010). Basic mechanisms of neurodegeneration: a critical update. *Journal of Cellular and Molecular Medicine*, *14*(3), 457–487.

Ji, K., Akgul, G., Wollmuth, L. P., & Tsirka, S. E. (2013). Microglia actively regulate the number of functional synapses. *PloS One*, *8*(2), e56293.

Jia, J., Ma, L., Wu, M., Zhang, L., Zhang, X., Zhai, Q., … Xiong, L. (2014). Anandamide protects HT22 cells exposed to hydrogen peroxide by inhibiting CB1 receptor-mediated type 2 NADPH oxidase. *Oxidative Medicine and Cellular Longevity*, *2014*.

Jiang, T., Yu, J.-T., & Tan, L. (2012). Novel disease-modifying therapies for Alzheimer's disease. *Journal of Alzheimer's Disease*, *31*(3), 475–492.

Ju Hwang, C., Choi, D.-Y., Park, M. H., & Hong, J. T. (2019). NF-κB as a key mediator of brain inflammation in Alzheimer's disease. *CNS & Neurological Disorders-Drug Targets (Formerly Current Drug Targets-CNS & Neurological Disorders)*, *18*(1), 3–10.

Jung, J.-S., Choi, M.-J., Lee, Y. Y., Moon, B.-I., Park, J.-S., & Kim, H.-S. (2017). Suppression of lipopolysaccharide-induced neuroinflammation by morin via MAPK, PI3K/Akt, and PKA/HO-1 signaling pathway modulation. *Journal of Agricultural and Food Chemistry*, *65*(2), 373–382.

Jung, Y. S., Park, J. H., Kim, H., Kim, S. Y., Hwang, J. Y., Hong, K. W., … Shin, H. K. (2016). Probucol inhibits LPS-induced microglia activation and ameliorates brain ischemic injury in normal and hyperlipidemic mice. *Acta Pharmacologica Sinica*, *37*(8), 1031–1044.

Kaltschmidt, B., Uherek, M., Volk, B., Baeuerle, P. A., & Kaltschmidt, C. (1997). Transcription factor NF-κB is activated in primary neurons by amyloid β peptides and in neurons surrounding early plaques from patients with Alzheimer disease. *Proceedings of the National Academy of Sciences*, *94*(6), 2642–2647.

Kaminska, B., Gozdz, A., Zawadzka, M., Ellert-Miklaszewska, A., & Lipko, M. (2009). MAPK signal transduction underlying brain inflammation and gliosis as therapeutic target. *The Anatomical Record: Advances in Integrative Anatomy and Evolutionary Biology: Advances in Integrative Anatomy and Evolutionary Biology*, *292*(12), 1902–1913.

Kang, C.-H., Choi, Y. H., Moon, S.-K., Kim, W.-J., & Kim, G.-Y. (2013). Quercetin inhibits lipopolysaccharide-induced nitric oxide production in BV2 microglial cells by suppressing the NF-κB pathway and activating the Nrf2-dependent HO-1 pathway. *International Immunopharmacology*, *17*(3), 808–813.

Karin, M., & Delhase, M. (2000). The IκB kinase (IKK) and NF-κB: key elements of proinflammatory

signalling. *Seminars in Immunology*, *12*(1), 85–98. Elsevier.

Katsetos, C. D., Frankfurter, A., Christakos, S., Mancall, E. L., Vlachos, I. N., & Urich, H. (1993). Differential localization of class III β-tubulin isotype and calbindin-D28k defines distinct neuronal types in the developing human cerebellar cortex. *Journal of Neuropathology & Experimental Neurology*, *52*(6), 655–666.

Katusic, Z. S., & Austin, S. A. (2013). Endothelial nitric oxide: protector of a healthy mind. *European Heart Journal*, *35*(14), 888–894.

Kaushal, V., & Schlichter, L. C. (2008). Mechanisms of microglia-mediated neurotoxicity in a new model of the stroke penumbra. *Journal of Neuroscience*, *28*(9), 2221–2230.

Kawai, T., & Akira, S. (2007). Signaling to NF-κB by Toll-like receptors. *Trends in Molecular Medicine*, *13*(11), 460–469.

Kempuraj, D., Thangavel, R., Natteru, P. A., Selvakumar, G. P., Saeed, D., Zahoor, H., … Zaheer, A. (2016). Neuroinflammation induces neurodegeneration. *Journal of Neurology, Neurosurgery and Spine*, *1*(1).

Killick, R., Ribe, E. M., Al-Shawi, R., Malik, B., Hooper, C., Fernandes, C., … Furney, S. (2014). Clusterin regulates β-amyloid toxicity via Dickkopf-1-driven induction of the wnt–PCP–JNK pathway. *Molecular Psychiatry*, *19*(1), 88–98.

Kim, D.-C., Quang, T. H., Oh, H., & Kim, Y.-C. (2017). Steppogenin isolated from cudrania tricuspidata shows antineuroinflammatory effects via NF-κB and MAPK pathways in lps-stimulated bv2 and primary rat microglial cells. *Molecules*, *22*(12), 2130.

Kim, E. K., & Choi, E.-J. (2015). Compromised MAPK signaling in human diseases: an update. *Archives of Toxicology*, *89*(6), 867–882.

Kim, J., & Song, J.-H. (2016). Thioxanthenes, chlorprothixene and flupentixol inhibit proton currents in BV2 microglial cells. *European Journal of Pharmacology*, *779*, 31–37.

Kim, S. H., Smith, C. J., & Van Eldik, L. J. (2004). Importance of MAPK pathways for microglial pro-inflammatory cytokine IL-1β production. *Neurobiology of Aging*, *25*(4), 431–439.

Kim, S. R., Kim, S. U., Oh, U., & Jin, B. K. (2006). Transient receptor potential vanilloid subtype 1 mediates microglial cell death in vivo and in vitro via Ca2+-mediated mitochondrial damage and cytochrome c release. *The Journal of Immunology*, *177*(7), 4322–4329.

Kinney, J. W., Bemiller, S. M., Murtishaw, A. S., Leisgang, A. M., Salazar, A. M., & Lamb, B. T. (2018). Inflammation as a central mechanism in Alzheimer's disease. *Alzheimer's & Dementia: Translational Research & Clinical Interventions*, *4*, 575–590.

Klein, T. W. (2005). Cannabinoid-based drugs as anti-inflammatory therapeutics. *Nature Reviews Immunology*, *5*(5), 400.

Kobayashi, E. H., Suzuki, T., Funayama, R., Nagashima, T., Hayashi, M., Sekine, H., … Nakayama, K. (2016). *Nrf2 suppresses macrophage inflammatory response by blocking proinflammatory cytokine transcription. Nat Commun 7: 11624*.

Kobayashi, E. H., Suzuki, T., Funayama, R., Nagashima, T., Hayashi, M., Sekine, H., … Nakayama, K. (2016). Nrf2 suppresses macrophage inflammatory response by blocking proinflammatory cytokine transcription. *Nature Communications*, *7*(1), 1–14.

Köfalvi, A., Lemos, C., Martín-Moreno, A. M., Pinheiro, B. S., García-García, L., Pozo, M. A., … Rodrigues, R. J. (2016). Stimulation of brain glucose uptake by cannabinoid CB2 receptors and its therapeutic potential in Alzheimer's disease. *Neuropharmacology*, *110*, 519–529.

Kong, W.-L., Peng, Y.-Y., & Peng, B.-W. (2017). Modulation of neuroinflammation: role and therapeutic potential of TRPV1 in the neuro-immune axis. *Brain, Behavior, and Immunity*, *64*, 354–366.

Kovalevich, J., Santerre, M., & Langford, D. (2021). Considerations for the Use of SH-SY5Y Neuroblastoma Cells in Neurobiology. In *Neuronal Cell Culture* (pp. 9–23). Springer.

Kozela, E., Pietr, M., Juknat, A., Rimmerman, N., Levy, R., & Vogel, Z. (2010). Cannabinoids Δ9-

tetrahydrocannabinol and cannabidiol differentially inhibit the lipopolysaccharide-activated NF-κB and interferon-β/STAT proinflammatory pathways in BV-2 microglial cells. *Journal of Biological Chemistry*, *285*(3), 1616–1626.

Kreutzberg, G. W. (1996). Microglia: a sensor for pathological events in the CNS. *Trends in Neurosciences*, *19*(8), 312–318.

Krishna, M., & Narang, H. (2008). The complexity of mitogen-activated protein kinases (MAPKs) made simple. *Cellular and Molecular Life Sciences*, *65*(22), 3525–3544.

Kumar, A., Behl, T., Jamwal, S., Kaur, I., Sood, A., & Kumar, P. (2020). Exploring the molecular approach of COX and LOX in Alzheimer's and Parkinson's disorder. *Molecular Biology Reports*, 1–18.

Kumar, A., Chen, S.-H., Kadiiska, M. B., Hong, J.-S., Zielonka, J., Kalyanaraman, B., & Mason, R. P. (2014). Inducible nitric oxide synthase is key to peroxynitrite-mediated, LPS-induced protein radical formation in murine microglial BV2 cells. *Free Radical Biology and Medicine*, *73*, 51–59.

Kunz, L., Schröder, T. N., Lee, H., Montag, C., Lachmann, B., Sariyska, R., … Messing-Floeter, P. C. (2015). Reduced grid-cell–like representations in adults at genetic risk for Alzheimer's disease. *Science*, *350*(6259), 430–433.

Kunzler, A., Zeidán-Chuliá, F., Gasparotto, J., Girardi, C. S., Klafke, K., Petiz, L. L., … de Bittencourt Pasquali, M. A. (2017). Changes in cell cycle and up-regulation of neuronal markers during SH-SY5Y neurodifferentiation by retinoic acid are mediated by reactive species production and oxidative stress. *Molecular Neurobiology*, *54*(9), 6903–6916.

Kwon, H. S., & Koh, S.-H. (2020). Neuroinflammation in neurodegenerative disorders: the roles of microglia and astrocytes. *Translational Neurodegeneration*, *9*(1), 1–12.

Kwon, Y.-W., Cheon, S. Y., Park, S. Y., Song, J., & Lee, J.-H. (2017). Tryptanthrin suppresses the activation of the LPS-treated BV2 microglial cell line via Nrf2/HO-1 antioxidant signaling. *Frontiers in Cellular Neuroscience*, *11*, 18.

Lafaye, G., Karila, L., Blecha, L., & Benyamina, A. (2017). Cannabis, cannabinoids, and health. *Dialogues in Clinical Neuroscience*, *19*, 309–317.

Lauvau, G., & Hohl, T. M. (2015). Monocyte-mediated defense against bacteria, fungi, and parasites. *Seminars in Immunology*, *27*(6), 397–409. Elsevier.

Lee, A. Y., Choi, J. M., Lee, M. H., Lee, J., Lee, S., & Cho, E. J. (2018). Protective effects of perilla oil and alpha linolenic acid on SH-SY5Y neuronal cell death induced by hydrogen peroxide. *Nutrition Research and Practice*, *12*(2), 93–100.

Lee, C., Park, G. H., Lee, S.-R., & Jang, J.-H. (2013). Attenuation of-amyloid-induced oxidative cell death by sulforaphane via activation of NF-E2-related factor 2. *Oxidative Medicine and Cellular Longevity*, *2013*.

Lee, G., Park, J.-S., Lee, E.-J., Ahn, J.-H., & Kim, H.-S. (2019). Anti-inflammatory and antioxidant mechanisms of urolithin B in activated microglia. *Phytomedicine*, *55*, 50–57.

Lee, K.-W., Im, J.-Y., Woo, J.-M., Grosso, H., Kim, Y.-S., Cristovao, A. C., … Fernandez, J. R. (2013). Neuroprotective and anti-inflammatory properties of a coffee component in the MPTP model of Parkinson's disease. *Neurotherapeutics*, *10*(1), 143–153.

Lee, Y. Y., Park, J.-S., Jung, J.-S., Kim, D.-H., & Kim, H.-S. (2013). Anti-inflammatory effect of ginsenoside Rg5 in lipopolysaccharide-stimulated BV2 microglial cells. *International Journal of Molecular Sciences*, *14*(5), 9820–9833.

Lehnardt, S., Massillon, L., Follett, P., Jensen, F. E., Ratan, R., Rosenberg, P. A., … Vartanian, T. (2003). Activation of innate immunity in the CNS triggers neurodegeneration through a Toll-like receptor 4-dependent pathway. *Proceedings of the National Academy of Sciences*, *100*(14), 8514–8519.

Leibowitz, S. M., & Yan, J. (2016). NF-κB pathways in the pathogenesis of multiple sclerosis and the therapeutic implications. *Frontiers in Molecular Neuroscience*, *9*, 84.

Leng, F., & Edison, P. (2021). Neuroinflammation and microglial activation in Alzheimer disease: where

do we go from here? *Nature Reviews Neurology*, *17*(3), 157–172.

Li, C., Chen, T., Zhou, H., Zhang, C., Feng, Y., Tang, F., … Lee, S. M.-Y. (2018). Schisantherin A attenuates neuroinflammation in activated microglia: role of Nrf2 activation through ERK phosphorylation. *Cellular Physiology and Biochemistry*, *47*(5), 1769–1784.

Li, C., Zhang, W.-J., & Frei, B. (2016). Quercetin inhibits LPS-induced adhesion molecule expression and oxidant production in human aortic endothelial cells by p38-mediated Nrf2 activation and antioxidant enzyme induction. *Redox Biology*, *9*, 104–113.

Li, J., O, W., Li, W., Jiang, Z.-G., & Ghanbari, H. A. (2013). Oxidative stress and neurodegenerative disorders. *International Journal of Molecular Sciences*, *14*(12), 24438–24475.

Li, R., Yang, L., Lindholm, K., Konishi, Y., Yue, X., Hampel, H., … Shen, Y. (2004). Tumor necrosis factor death receptor signaling cascade is required for amyloid-β protein-induced neuron death. *Journal of Neuroscience*, *24*(7), 1760–1771.

Li, S., Wang, P., Feng, W., Xiang, Y., Dou, K., & Liu, Z. (2020). Simultaneous imaging of mitochondrial viscosity and hydrogen peroxide in Alzheimer's disease by a single near-infrared fluorescent probe with a large Stokes shift. *Chemical Communications*, *56*(7), 1050–1053.

Li, Y., Lv, O., Zhou, F., Li, Q., Wu, Z., & Zheng, Y. (2015). Linalool inhibits LPS-induced inflammation in BV2 microglia cells by activating Nrf2. *Neurochemical Research*, *40*(7), 1520–1525.

Li, Z., Chu, S., He, W., Zhang, Z., Liu, J., Cui, L., … Chen, N. (2019). A20 as a novel target for the anti-neuroinflammatory effect of chrysin via inhibition of NF-κB signaling pathway. *Brain, Behavior, and Immunity*, *79*, 228–235.

Liao, S., Wu, J., Liu, R., Wang, S., Luo, J., Yang, Y., … Song, J. (2020). A novel compound DBZ ameliorates neuroinflammation in LPS-stimulated microglia and ischemic stroke rats: Role of Akt (Ser473)/GSK3β (Ser9)-mediated Nrf2 activation. *Redox Biology*, *36*, 101644.

Lin, M. T., & Beal, M. F. (2006). Mitochondrial dysfunction and oxidative stress in neurodegenerative

diseases. *Nature*, *443*(7113), 787.

Lin, Y.-S., Lin, C.-H., Huang, L.-D., Chao, T., Kuo, C.-D., Hung, L.-C., … Fu, S.-L. (2013). The suppression of thoc1 in cancer cell apoptosis mediated by activated macrophages is nitric oxide-dependent. *Biochemical Pharmacology*, *86*(2), 242–252.

Liu, A., Lin, L., Xu, W., Gong, Z., Liu, Z., & Xiao, W. (2021). l-Theanine regulates glutamine metabolism and immune function by binding to cannabinoid receptor 1. *Food & Function*, *12*(13), 5755–5769.

Liu, B., Yu, H., Baiyun, R., Lu, J., Li, S., Bing, Q., … Zhang, Z. (2018). Protective effects of dietary luteolin against mercuric chloride-induced lung injury in mice: involvement of AKT/Nrf2 and NF-κB pathways. *Food and Chemical Toxicology*, *113*, 296–302.

Liu, G.-H., Qu, J., & Shen, X. (2008). NF-κB/p65 antagonizes Nrf2-ARE pathway by depriving CBP from Nrf2 and facilitating recruitment of HDAC3 to MafK. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research*, *1783*(5), 713–727.

Liu, H.-S., Shi, H.-L., Huang, F., Peterson, K. E., Wu, H., Lan, Y.-Y., … Du, M. (2016). Astragaloside IV inhibits microglia activation via glucocorticoid receptor mediated signaling pathway. *Scientific Reports*, *6*(1), 1–14.

Liu, N., Zhuang, Y., Zhou, Z., Zhao, J., Chen, Q., & Zheng, J. (2017). NF-κB dependent up-regulation of TRPC6 by Aβ in BV-2 microglia cells increases COX-2 expression and contributes to hippocampus neuron damage. *Neuroscience Letters*, *651*, 1–8.

Liu, Y., Meng, X., Sun, L., Pei, K., Chen, L., Zhang, S., & Hu, M. (2022). Protective effects of hydroxy-α-sanshool from the pericarp of Zanthoxylum bungeanum Maxim. On D-galactose/AlCl3-induced Alzheimer's disease-like mice via Nrf2/HO-1 signaling pathways. *European Journal of Pharmacology*, *914*, 174691.

Loboda, A., Damulewicz, M., Pyza, E., Jozkowicz, A., & Dulak, J. (2016). Role of Nrf2/HO-1 system in development, oxidative stress response and diseases: an evolutionarily conserved mechanism.

*Cellular and Molecular Life Sciences*, *73*(17), 3221–3247.

London, A., Cohen, M., & Schwartz, M. (2013). Microglia and monocyte-derived macrophages: functionally distinct populations that act in concert in CNS plasticity and repair. *Frontiers in Cellular Neuroscience*, *7*, 34.

López, D. E., & Ballaz, S. J. (2020). The role of brain cyclooxygenase-2 (COX-2) beyond neuroinflammation: neuronal homeostasis in memory and anxiety. *Molecular Neurobiology*, *57*(12), 5167–5176.

Lu, Q., Ma, R., Yang, Y., Mo, Z., Pu, X., & Li, C. (2020). Zanthoxylum nitidum (Roxb.) DC: Traditional uses, phytochemistry, pharmacological activities and toxicology. *Journal of Ethnopharmacology*, *260*, 112946.

Lue, L., Rydel, R., Brigham, E. F., Yang, L., Hampel, H., Murphy Jr, G. M., … Shen, Y. (2001). Inflammatory repertoire of Alzheimer’s disease and nondemented elderly microglia in vitro. *Glia*, *35*(1), 72–79.

Lv, H., Ren, W., Zheng, Y., Wang, L., Lu, G., Yi, P., & Ci, X. (2016). Tenuigenin exhibits anti-inflammatory activity via inhibiting MAPK and NF-κB and inducing Nrf2/HO-1 signaling in macrophages. *Food & Function*, *7*(1), 355–363.

Lyman, M., Lloyd, D. G., Ji, X., Vizcaychipi, M. P., & Ma, D. (2014). Neuroinflammation: the role and consequences. *Neuroscience Research*, *79*, 1–12.

Ma, L., Jia, J., Liu, X., Bai, F., Wang, Q., & Xiong, L. (2015). Activation of murine microglial N9 cells is attenuated through cannabinoid receptor CB2 signaling. *Biochemical and Biophysical Research Communications*, *458*(1), 92–97.

Maccarrone, M., & Wenger, T. (2005). Effects of cannabinoids on hypothalamic and reproductive function. In *Cannabinoids* (pp. 555–571). Springer.

Mackie, K. (2005). Distribution of cannabinoid receptors in the central and peripheral nervous system. *Cannabinoids*, 299–325.

Malek, N., Popiolek-Barczyk, K., Mika, J., Przewlocka, B., & Starowicz, K. (2015). Anandamide, acting via CB2 receptors, alleviates LPS-induced neuroinflammation in rat primary microglial cultures. *Neural Plasticity*, *2015*.

Malm, T., Koistinaho, M., Muona, A., Magga, J., & Koistinaho, J. (2010). The role and therapeutic potential of monocytic cells in Alzheimer's disease. *Glia*, *58*(8), 889–900.

Mandrekar-Colucci, S., & Landreth, G. E. (2010). Microglia and inflammation in Alzheimer's disease. *CNS & Neurological Disorders-Drug Targets (Formerly Current Drug Targets-CNS & Neurological Disorders)*, *9*(2), 156–167.

Maqbool, A., Lattke, M., Wirth, T., & Baumann, B. (2013). Sustained, neuron-specific IKK/NF-κB activation generates a selective neuroinflammatory response promoting local neurodegeneration with aging. *Molecular Neurodegeneration*, *8*(1), 40.

Marques, A. M., Macena, M. V, Cardoso, A. R., Hammes, C. S. O., Pinheiro, F. M. L., Castro, N. G., & Neves, G. A. (2020). Effects of combined 5-HT2A and cannabinoid receptor modulation on a schizophrenia-related prepulse inhibition deficit in mice. *Psychopharmacology*, *237*(6), 1643–1655.

Márquez, N., De Petrocellis, L., Caballero, F. J., Macho, A., Schiano-Moriello, A., Minassi, A., … Di Marzo, V. (2006). Iodinated N-acylvanillamines: potential "multiple-target" anti-inflammatory agents acting via the inhibition of t-cell activation and antagonism at vanilloid TRPV1 channels. *Molecular Pharmacology*, *69*(4), 1373–1382.

Martín-Moreno, A. M., Brera, B., Spuch, C., Carro, E., García-García, L., Delgado, M., … de Ceballos, M. L. (2012). Prolonged oral cannabinoid administration prevents neuroinflammation, lowers β-amyloid levels and improves cognitive performance in Tg APP 2576 mice. *Journal of Neuroinflammation*, *9*(1), 8. https://doi.org/10.1186/1742-2094-9-8

Martin-Moreno, A. M., Reigada, D., Ramirez, B. G., Mechoulam, R., Innamorato, N., Cuadrado, A., &

de Ceballos, M. L. (2011). Cannabidiol and Other Cannabinoids Reduce Microglial Activation In Vitro and In Vivo: Relevance to Alzheimer's Disease. *Molecular Pharmacology*, *79*(6), 964–973. https://doi.org/10.1124/mol.111.071290

Martins, D., Tavares, I., & Morgado, C. (2014). "Hotheaded": the role OF TRPV1 in brain functions. *Neuropharmacology*, *85*, 151–157.

Masuku, B. P. (2013). *Total Phenolic Composition, Antibacterial and Antioxidant Activities of Fagara heitzii Aubr & Pellegr Medicinal Plant of Gabon*.

Mathes, E., O'dea, E. L., Hoffmann, A., & Ghosh, G. (2008). NF-κB dictates the degradation pathway of IκBα. *The EMBO Journal*, *27*(9), 1357–1367.

Mathew, M., & Subramanian, S. (2014). *In vitro evaluation of anti-Alzheimer effects of dry ginger (Zingiber officinale Roscoe) extract*.

Matsuda, L. A., Lolait, S. J., Brownstein, M. J., Young, A. C., & Bonner, T. I. (1990). Structure of a cannabinoid receptor and functional expression of the cloned cDNA. *Nature*, *346*(6284), 561.

Matsumoto, J., Dohgu, S., Takata, F., Machida, T., Hatip, F. F. B., Hatip-Al-Khatib, I., … Kataoka, Y. (2018). TNF-α-sensitive brain pericytes activate microglia by releasing IL-6 through cooperation between IκB-NFκB and JAK-STAT3 pathways. *Brain Research*, *1692*, 34–44.

Mbaze, L. M., Lado, J. A., Wansi, J. D., Shiao, T. C., Chiozem, D. D., Mesaik, M. A., … Roy, R. (2009). Oxidative burst inhibitory and cytotoxic amides and lignans from the stem bark of Fagara heitzii (Rutaceae). *Phytochemistry*, *70*(11–12), 1442–1447.

Mbaze, L. M., Poumale, H. M. P., Wansi, J. D., Lado, J. A., Khan, S. N., Iqbal, M. C., … Laatsch, H. (2007). α-Glucosidase inhibitory pentacyclic triterpenes from the stem bark of Fagara tessmannii (Rutaceae). *Phytochemistry*, *68*(5), 591–595.

Mecha, M., Torrao, A. S., Mestre, L., Carrillo-Salinas, F. J., Mechoulam, R., & Guaza, C. (2012). Cannabidiol protects oligodendrocyte progenitor cells from inflammation-induced apoptosis by

attenuating endoplasmic reticulum stress. *Cell Death & Disease*, *3*(6), e331.

Medeiros, R., & LaFerla, F. M. (2013). Astrocytes: conductors of the Alzheimer disease neuroinflammatory symphony. *Experimental Neurology*, *239*, 133–138.

Mehan, S., Meena, H., Sharma, D., & Sankhla, R. (2011). JNK: a stress-activated protein kinase therapeutic strategies and involvement in Alzheimer's and various neurodegenerative abnormalities. *Journal of Molecular Neuroscience*, *43*(3), 376–390.

Melck, D., Bisogno, T., De Petrocellis, L., Chuang, H., Julius, D., Bifulco, M., & Di Marzo, V. (1999). Unsaturated long-chain N-acyl-vanillyl-amides (N-AVAMs): vanilloid receptor ligands that inhibit anandamide-facilitated transport and bind to CB1 cannabinoid receptors. *Biochemical and Biophysical Research Communications*, *262*(1), 275–284.

Mincheva-Tasheva, S., & Soler, R. M. (2013). NF-κB signaling pathways: role in nervous system physiology and pathology. *The Neuroscientist*, *19*(2), 175–194.

Misra, L. N., Wouatsa, N. A. V., Kumar, S., Kumar, R. V., & Tchoumbougnang, F. (2013). Antibacterial, cytotoxic activities and chemical composition of fruits of two Cameroonian Zanthoxylum species. *Journal of Ethnopharmacology*, *148*(1), 74–80.

Misrani, A., Tabassum, S., & Yang, L. (2021). Mitochondrial dysfunction and oxidative stress in Alzheimer's disease. *Frontiers in Aging Neuroscience*, *13*, 57.

Miyake, T., Shirakawa, H., Nakagawa, T., & Kaneko, S. (2015). Activation of mitochondrial transient receptor potential vanilloid 1 channel contributes to microglial migration. *Glia*, *63*(10), 1870–1882.

Molina-Holgado, F., Pinteaux, E., Moore, J. D., Molina-Holgado, E., Guaza, C., Gibson, R. M., & Rothwell, N. J. (2003). Endogenous interleukin-1 receptor antagonist mediates anti-inflammatory and neuroprotective actions of cannabinoids in neurons and glia. *Journal of Neuroscience*, *23*(16), 6470–6474.

Morales, I., Farías, G. A., Cortes, N., & Maccioni, R. B. (2016). Neuroinflammation and neurodegeneration. In *Update on Dementia*. InTech.

Morales, I., Guzmán-Martínez, L., Cerda-Troncoso, C., Farías, G. A., & Maccioni, R. B. (2014). Neuroinflammation in the pathogenesis of Alzheimer's disease. A rational framework for the search of novel therapeutic approaches. *Frontiers in Cellular Neuroscience*, *8*, 112.

Motohashi, H., & Yamamoto, M. (2004). Nrf2–Keap1 defines a physiologically important stress response mechanism. *Trends in Molecular Medicine*, *10*(11), 549–557.

Muhammad, M. (2019). Tumor necrosis factor alpha: a major cytokine of brain neuroinflammation. *Cytokines*, *861231*.

Munoz, L., & Ammit, A. J. (2010). Targeting p38 MAPK pathway for the treatment of Alzheimer's disease. *Neuropharmacology*, *58*(3), 561–568.

Munro, S., Thomas, K. L., & Abu-Shaar, M. (1993). Molecular characterization of a peripheral receptor for cannabinoids. *Nature*, *365*(6441), 61.

Murphy, M. P., Holmgren, A., Larsson, N.-G., Halliwell, B., Chang, C. J., Kalyanaraman, B., … Gems, D. (2011). Unraveling the biological roles of reactive oxygen species. *Cell Metabolism*, *13*(4), 361–366.

Nagano, T., Tsuda, N., Fujimura, K., Ikezawa, Y., Higashi, Y., & Kimura, S. H. (2021). Prostaglandin E2 increases the expression of cyclooxygenase-2 in cultured rat microglia. *Journal of Neuroimmunology*, *361*, 577724.

Naguib, M., & Giordano, T. (2019). DT-02-05: A NOVEL THERAPEUTIC (NTRX-07) TARGETING NEUROINFLAMMATION IN ALZHEIMER'S DISEASE IS UNDERGOING PHASE I TRIALS. *Alzheimer's & Dementia*, *15*, P1490–P1491.

Nair, S., Doh, S. T., Chan, J. Y., Kong, A.-N., & Cai, L. (2008). Regulatory potential for concerted modulation of Nrf2-and Nfkb1-mediated gene expression in inflammation and carcinogenesis.

*British Journal of Cancer*, *99*(12), 2070–2082.

Nakajima, K., Matsushita, Y., Tohyama, Y., Kohsaka, S., & Kurihara, T. (2006). Differential suppression of endotoxin-inducible inflammatory cytokines by nuclear factor kappa B (NFκB) inhibitor in rat microglia. *Neuroscience Letters*, *401*(3), 199–202.

Nathan, C., Calingasan, N., Nezezon, J., Ding, A., Lucia, M. S., La Perle, K., … Kwon, N. S. (2005). Protection from Alzheimer's-like disease in the mouse by genetic ablation of inducible nitric oxide synthase. *Journal of Experimental Medicine*, *202*(9), 1163–1169.

Nayak, D., Roth, T. L., & McGavern, D. B. (2014). Microglia development and function. *Annual Review of Immunology*, *32*, 367.

Neitz, A., Mergia, E., Imbrosci, B., Petrasch-Parwez, E., Eysel, U. T., Koesling, D., & Mittmann, T. (2013). Postsynaptic NO/cGMP increases NMDA receptor currents via hyperpolarization-activated cyclic nucleotide-gated channels in the hippocampus. *Cerebral Cortex*, *24*(7), 1923–1936.

Nguyen, T., Sherratt, P. J., & Pickett, C. B. (2003). Regulatory mechanisms controlling gene expression mediated by the antioxidant response element. *Annual Review of Pharmacology and Toxicology*, *43*(1), 233–260.

Niedzielska, E., Smaga, I., Gawlik, M., Moniczewski, A., Stankowicz, P., Pera, J., & Filip, M. (2016). Oxidative stress in neurodegenerative diseases. *Molecular Neurobiology*, *53*(6), 4094–4125.

Nna, P. J., Tor-Anyiin, T. A., & Igoli, J. O. (2019). Fagaramide and Pellitorine from the Stem Bark of Zanthoxylum zanthoxyloides and Their Antimicrobial Activities. *South Asian Research Journal of Natural Products*, 1–8.

Ntchapda, F., Maguirgue, K., Adjia, H., Etet, P. F. S., & Dimo, T. (2015). Hypolipidemic, antioxidant and anti—atherosclerogenic effects of aqueous extract of Zanthoxylum heitzii stem bark in diet—induced hypercholesterolemic rats. *Asian Pacific Journal of Tropical Medicine*, *8*(5), 359–365.

Ogunrinade, F. A., Guetchueng, S. T., Katola, F. O., Aderogba, M. A., Akande, I. S., Sarker, S. D., &

Olajide, O. A. (2021). Zanthoxylum zanthoxyloides inhibits lipopolysaccharide-and synthetic hemozoin-induced neuroinflammation in BV-2 microglia: roles of NF-κB transcription factor and NLRP3 inflammasome activation. *Journal of Pharmacy and Pharmacology*, *73*(1), 118–134.

Ohta, S., & Ohsawa, I. (2006). Dysfunction of mitochondria and oxidative stress in the pathogenesis of Alzheimer's disease: on defects in the cytochrome c oxidase complex and aldehyde detoxification. *Journal of Alzheimer's Disease*, *9*(2), 155–166.

Okorji, U. P., Velagapudi, R., El-Bakoush, A., Fiebich, B. L., & Olajide, O. A. (2016). Antimalarial drug artemether inhibits neuroinflammation in BV2 microglia through Nrf2-dependent mechanisms. *Molecular Neurobiology*, *53*(9), 6426–6443.

Olabarria, M., Noristani, H. N., Verkhratsky, A., & Rodríguez, J. J. (2010). Concomitant astroglial atrophy and astrogliosis in a triple transgenic animal model of Alzheimer's disease. *Glia*, *58*(7), 831–838.

Olabarria, M., Noristani, H. N., Verkhratsky, A., & Rodríguez, J. J. (2011). Age-dependent decrease in glutamine synthetase expression in the hippocampal astroglia of the triple transgenic Alzheimer's disease mouse model: mechanism for deficient glutamatergic transmission? *Molecular Neurodegeneration*, *6*(1), 55.

Oloya, B., Namukobe, J., Heydenreich, M., Ssengooba, W., Schmidt, B., & Byamukama, R. (2021). Antimycobacterial Activity of the Extract and Isolated Compounds From the Stem Bark of Zanthoxylum leprieurii Guill. and Perr. *Natural Product Communications*, *16*(8), 1934578X211035851.

Onaivi, E. S., Ishiguro, H., Gu, S., & Liu, Q.-R. (2012). CNS effects of CB2 cannabinoid receptors: beyond neuro-immuno-cannabinoid activity. *Journal of Psychopharmacology*, *26*(1), 92–103.

Oriowo, M. A. (1982a). Anti-inflammatory activity of piperonyl-4-acrylic isobutyl amide, an extractive from Zanthoxylum zanthoxyloides. *Planta Medica*, *44*(01), 54–56.

Oriowo, M. A. (1982b). Anti-inflammatory activity of piperonyl-4-acrylic isobutyl amide, and extractive from zanthoxylum zanthoxyloides. *Planta Medica*, *44*(1), 54–56. https://doi.org/10.1055/s-2007-971402

Palazuelos, J., Aguado, T., Pazos, M. R., Julien, B., Carrasco, C., Resel, E., … Azcoitia, I. (2009). Microglial CB2 cannabinoid receptors are neuroprotective in Huntington's disease excitotoxicity. *Brain*, *132*(11), 3152–3164.

Pan, L.-L., Xu, P., Luo, X.-L., Wang, L.-J., Liu, S.-Y., Zhu, Y.-Z., … Liu, X.-H. (2017). Shizukaol B, an active sesquiterpene from Chloranthus henryi, attenuates LPS-induced inflammatory responses in BV2 microglial cells. *Biomedicine & Pharmacotherapy*, *88*, 878–884.

Papaseit, E., Pérez-mañá, C., Pérez-acevedo, A. P., Hladun, O., Carmen, M., Muga, R., … Farré, M. (2018). Cannabinoids : from pot to lab. *International Journal of Medical Sciences*, *15*. https://doi.org/10.7150/ijms.27087

Parada, E., Buendia, I., Navarro, E., Avendaño, C., Egea, J., & López, M. G. (2015). Microglial HO-1 induction by curcumin provides antioxidant, antineuroinflammatory, and glioprotective effects. *Molecular Nutrition & Food Research*, *59*(9), 1690–1700.

Park, H. R., Lee, H., Park, H., Jeon, J. W., Cho, W.-K., & Ma, J. Y. (2015). Neuroprotective effects of Liriope platyphylla extract against hydrogen peroxide-induced cytotoxicity in human neuroblastoma SH-SY5Y cells. *BMC Complementary and Alternative Medicine*, *15*(1), 1–11.

Park, J.-E., Park, J.-S., Leem, Y.-H., Kim, D.-Y., & Kim, H.-S. (2021). NQO1 mediates the anti-inflammatory effects of nootkatone in lipopolysaccharide-induced neuroinflammation by modulating the AMPK signaling pathway. *Free Radical Biology and Medicine*, *164*, 354–368.

Park, S. E., Sapkota, K., Kim, S., Kim, H., & Kim, S. J. (2011). Kaempferol acts through mitogen-activated protein kinases and protein kinase B/AKT to elicit protection in a model of neuroinflammation in BV2 microglial cells. *British Journal of Pharmacology*, *164*(3), 1008–1025.

Park, S. Y., Jin, M. L., Ko, M. J., Park, G., & Choi, Y.-W. (2016). Anti-neuroinflammatory effect of emodin in LPS-stimulated microglia: involvement of AMPK/Nrf2 activation. *Neurochemical Research*, *41*(11), 2981–2992.

Park, S. Y., Kim, Y. H., & Park, G. (2015). Cucurbitacins attenuate microglial activation and protect from neuroinflammatory injury through Nrf2/ARE activation and STAT/NF-κB inhibition. *Neuroscience Letters*, *609*, 129–136.

Parkhurst, C. N., Yang, G., Ninan, I., Savas, J. N., Yates III, J. R., Lafaille, J. J., … Gan, W.-B. (2013). Microglia promote learning-dependent synapse formation through brain-derived neurotrophic factor. *Cell*, *155*(7), 1596–1609.

Patel, N. S., Paris, D., Mathura, V., Quadros, A. N., Crawford, F. C., & Mullan, M. J. (2005). Inflammatory cytokine levels correlate with amyloid load in transgenic mouse models of Alzheimer's disease. *Journal of Neuroinflammation*, *2*(1), 9.

Paul, A., Kumar, A., Singh, G., & Choudhary, A. (2018). Medicinal, pharmaceutical and pharmacological properties of Zanthoxylum armatum: A Review. *J Pharmacogn Phytochem*, *7*(4), 892–900.

Pei, J.-J., Braak, E., Braak, H., Grundke-Iqbal, I., Iqbal, K., Winblad, B., & Cowburn, R. F. (2001). Localization of active forms of C-jun kinase (JNK) and p38 kinase in Alzheimer's disease brains at different stages of neurofibrillary degeneration. *Journal of Alzheimer's Disease*, *3*(1), 41–48.

Peng, X., Li, C., Yu, W., Liu, S., Cong, Y., Fan, G., & Qi, S. (2020). Propofol attenuates hypoxia-induced inflammation in BV2 microglia by inhibiting oxidative stress and NF-κB/Hif-1α signaling. *BioMed Research International*, *2020*.

Perry, V. H., Nicoll, J. A. R., & Holmes, C. (2010). Microglia in neurodegenerative disease. *Nature Reviews Neurology*, *6*(4), 193.

Pihlaja, R., Koistinaho, J., Kauppinen, R., Sandholm, J., Tanila, H., & Koistinaho, M. (2011). Multiple cellular and molecular mechanisms are involved in human Aβ clearance by transplanted adult

astrocytes. *Glia*, *59*(11), 1643–1657.

Plotnikov, A., Zehorai, E., Procaccia, S., & Seger, R. (2011). The MAPK cascades: signaling components, nuclear roles and mechanisms of nuclear translocation. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research*, *1813*(9), 1619–1633.

Pocivavsek, A., Burns, M. P., & Rebeck, G. W. (2009). Low-density lipoprotein receptors regulate microglial inflammation through c-Jun N-terminal kinase. *Glia*, *57*(4), 444–453.

Porter, A. C., & Felder, C. C. (2001). The endocannabinoid nervous system: unique opportunities for therapeutic intervention. *Pharmacology & Therapeutics*, *90*(1), 45–60.

Prasad, K. N. (2016). Simultaneous activation of Nrf2 and elevation of antioxidant compounds for reducing oxidative stress and chronic inflammation in human Alzheimer's disease. *Mechanisms of Ageing and Development*, *153*, 41–47.

Qu, Z., Sun, J., Zhang, W., Yu, J., & Zhuang, C. (2020). Transcription factor NRF2 as a promising therapeutic target for Alzheimer's disease. *Free Radical Biology and Medicine*.

Quintanilla, R. A., Orellana, J. A., & von Bernhardi, R. (2012). Understanding risk factors for Alzheimer's disease: interplay of neuroinflammation, connexin-based communication and oxidative stress. *Archives of Medical Research*, *43*(8), 632–644.

Quinti, L., Dayalan Naidu, S., Träger, U., Chen, X., Kegel-Gleason, K., Llères, D., … Moniot, S. (2017). KEAP1-modifying small molecule reveals muted NRF2 signaling responses in neural stem cells from Huntington's disease patients. *Proceedings of the National Academy of Sciences*, *114*(23), E4676–E4685.

Raingeaud, J., Gupta, S., Rogers, J. S., Dickens, M., Han, J., Ulevitch, R. J., & Davis, R. J. (1995). Pro-inflammatory Cytokines and Environmental Stress Cause p38 Mitogen-activated Protein Kinase Activation by Dual Phosphorylation on Tyrosine and Threonine (∗). *Journal of Biological Chemistry*, *270*(13), 7420–7426.

Raivich, G., Bohatschek, M., Da Costa, C., Iwata, O., Galiano, M., Hristova, M., … Wolfer, D. P. (2004). The AP-1 transcription factor c-Jun is required for efficient axonal regeneration. *Neuron*, *43*(1), 57–67.

Ramírez, B. G., Blázquez, C., del Pulgar, T. G., Guzmán, M., & de Ceballos, M. L. (2005). Prevention of Alzheimer’s disease pathology by cannabinoids: neuroprotection mediated by blockade of microglial activation. *Journal of Neuroscience*, *25*(8), 1904–1913.

Ramirez, S. H., Haskó, J., Skuba, A., Fan, S., Dykstra, H., McCormick, R., … Zhang, M. (2012). Activation of cannabinoid receptor 2 attenuates leukocyte–endothelial cell interactions and blood–brain barrier dysfunction under inflammatory conditions. *Journal of Neuroscience*, *32*(12), 4004–4016.

Randall, M. D., Harris, D., Kendall, D. A., & Ralevic, V. (2002). Cardiovascular effects of cannabinoids. *Pharmacology & Therapeutics*, *95*(2), 191–202.

Ratheesh, M., Sindhu, G., & Helen, A. (2013). Anti-inflammatory effect of quinoline alkaloid skimmianine isolated from Ruta graveolens L. *Inflammation Research*, *62*(4), 367–376.

Reyes-Soto, C. Y., Villaseca-Flores, M., Ovalle-Noguez, E. A., Nava-Osorio, J., Galván-Arzate, S., Rangel-López, E., … Tinkov, A. A. (2022). Oleamide Reduces Mitochondrial Dysfunction and Toxicity in Rat Cortical Slices Through the Combined Action of Cannabinoid Receptors Activation and Induction of Antioxidant Activity. *Neurotoxicity Research*, 1–12.

Ribeiro, R., Wen, J., Li, S., & Zhang, Y. (2013). Involvement of ERK1/2, cPLA2 and NF-κB in microglia suppression by cannabinoid receptor agonists and antagonists. *Prostaglandins & Other Lipid Mediators*, *100*, 1–14.

Ricciotti, E., & FitzGerald, G. A. (2011). Prostaglandins and inflammation. *Arteriosclerosis, Thrombosis, and Vascular Biology*, *31*(5), 986–1000.

Rockwell, P., Martinez, J., Papa, L., & Gomes, E. (2004). Redox regulates COX-2 upregulation and cell death in the neuronal response to cadmium. *Cellular Signalling*, *16*(3), 343–353.

Ross, D., & Siegel, D. (2017). Functions of NQO1 in cellular protection and CoQ10 metabolism and its potential role as a redox sensitive molecular switch. *Frontiers in Physiology*, *8*, 595.

Rothaug, M., Becker-Pauly, C., & Rose-John, S. (2016). The role of interleukin-6 signaling in nervous tissue. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research*, *1863*(6), 1218–1227.

Ruiz-Valdepeñas, L., Martínez-Orgado, J. A., Benito, C., Millán, Á., Tolón, R. M., & Romero, J. (2011). Cannabidiol reduces lipopolysaccharide-induced vascular changes and inflammation in the mouse brain: an intravital microscopy study. *Journal of Neuroinflammation*, *8*(1), 5.

Russo, M. V, & McGavern, D. B. (2016). Inflammatory neuroprotection following traumatic brain injury. *Science*, *353*(6301), 783–785.

Sahu, P., Mudgal, J., Arora, D., Kinra, M., Mallik, S. B., Rao, C. M., … Nampoothiri, M. (2019). Cannabinoid receptor 2 activation mitigates lipopolysaccharide-induced neuroinflammation and sickness behavior in mice. *Psychopharmacology*, *236*(6), 1829–1838.

Saikia, B., Barua, C. C., Sarma, J., Haloi, P., Tamuli, S. M., Kalita, D. J., … Barua, A. G. (2018). Zanthoxylum alatum ameliorates scopolamine-induced amnesia in rats: behavioral, biochemical, and molecular evidence. *Indian Journal of Pharmacology*, *50*(1), 30.

Sakamoto, S., Putalun, W., Vimolmangkang, S., Phoolcharoen, W., Shoyama, Y., Tanaka, H., & Morimoto, S. (2018). Enzyme-linked immunosorbent assay for the quantitative/qualitative analysis of plant secondary metabolites. *Journal of Natural Medicines*, *72*(1), 32–42.

Sancho, R., Calzado, M. A., Di Marzo, V., Appendino, G., & Muñoz, E. (2003). Anandamide inhibits nuclear factor-κB activation through a cannabinoid receptor-independent pathway. *Molecular Pharmacology*, *63*(2), 429–438.

Sandberg, F., Perera-Ivarsson, P., & El-Seedi, H. R. (2005). A Swedish collection of medicinal plants from Cameroon. *Journal of Ethnopharmacology*, *102*(3), 336–343.

Sandberg, M., Patil, J., D'Angelo, B., Weber, S. G., & Mallard, C. (2014). NRF2-regulation in brain health

and disease: implication of cerebral inflammation. *Neuropharmacology*, *79*, 298–306.

Saponaro, C., Cianciulli, A., Calvello, R., Dragone, T., Iacobazzi, F., & Panaro, M. A. (2012). The PI3K/Akt pathway is required for LPS activation of microglial cells. *Immunopharmacology and Immunotoxicology*, *34*(5), 858–865.

Scheller, J., Chalaris, A., Schmidt-Arras, D., & Rose-John, S. (2011). The pro-and anti-inflammatory properties of the cytokine interleukin-6. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research*, *1813*(5), 878–888.

Schnöder, L., Hao, W., Qin, Y., Liu, S., Tomic, I., Liu, X., … Liu, Y. (2016). Deficiency of neuronal p38α MAPK attenuates amyloid pathology in Alzheimer disease mouse and cell models through facilitating lysosomal degradation of BACE1. *Journal of Biological Chemistry*, *291*(5), 2067–2079.

Schubert, D., Kepchia, D., Liang, Z., Dargusch, R., Goldberg, J., & Maher, P. (2019). Efficacy of cannabinoids in a pre-clinical drug-screening platform for Alzheimer's disease. *Molecular Neurobiology*, *56*(11), 7719–7730.

Scott, M. L., Fujita, T., Liou, H.-C., Nolan, G. P., & Baltimore, D. (1993). The p65 subunit of NF-kappa B regulates I kappa B by two distinct mechanisms. *Genes & Development*, *7*(7a), 1266–1276.

Scuderi, C., Esposito, G., Blasio, A., Valenza, M., Arietti, P., Steardo Jr, L., … Iuvone, T. (2011). Palmitoylethanolamide counteracts reactive astrogliosis induced by β-amyloid peptide. *Journal of Cellular and Molecular Medicine*, *15*(12), 2664–2674.

Scuderi, C., Steardo, L., & Esposito, G. (2014). Cannabidiol promotes amyloid precursor protein ubiquitination and reduction of beta amyloid expression in SHSY5YAPP+ cells through PPARγ involvement. *Phytotherapy Research*, *28*(7), 1007–1013.

Sedger, L. M., & McDermott, M. F. (2014). TNF and TNF-receptors: From mediators of cell death and inflammation to therapeutic giants–past, present and future. *Cytokine & Growth Factor Reviews*, *25*(4), 453–472.

Selfridge, J. E., Lezi, E., Lu, J., & Swerdlow, R. H. (2013). Role of mitochondrial homeostasis and dynamics in Alzheimer's disease. *Neurobiology of Disease*, *51*, 3–12. https://doi.org/10.1016/j.nbd.2011.12.057

Sezer, Y., Jannuzzi, A. T., Huestis, M. A., & Alpertunga, B. (2020). In vitro assessment of the cytotoxic, genotoxic and oxidative stress effects of the synthetic cannabinoid JWH-018 in human SH-SY5Y neuronal cells. *Toxicology Research*, *9*(6), 734–740.

Sharief, M. K., & Hentges, R. (1991). Association between tumor necrosis factor-α and disease progression in patients with multiple sclerosis. *New England Journal of Medicine*, *325*(7), 467–472.

Sheedy, F. J., Grebe, A., Rayner, K. J., Kalantari, P., Ramkhelawon, B., Carpenter, S. B., … Golenbock, D. T. (2013). CD36 coordinates NLRP3 inflammasome activation by facilitating intracellular nucleation of soluble ligands into particulate ligands in sterile inflammation. *Nature Immunology*, *14*(8), 812–820.

Shi, J., Wang, Q., Johansson, J. U., Liang, X., Woodling, N. S., Priyam, P., … Montine, T. J. (2012). Inflammatory prostaglandin E2 signaling in a mouse model of Alzheimer disease. *Annals of Neurology*, *72*(5), 788–798.

Shi, X., Cai, X., Di, W., Li, J., Xu, X., Zhang, A., … Fang, Y. (2017). MFG-E8 selectively inhibited Aβ-induced microglial M1 polarization via NF-κB and PI3K-Akt pathways. *Molecular Neurobiology*, *54*(10), 7777–7788.

Shi, Z.-M., Han, Y.-W., Han, X.-H., Zhang, K., Chang, Y.-N., Hu, Z.-M., … Hong, W. (2016). Upstream regulators and downstream effectors of NF-κB in Alzheimer's disease. *Journal of the Neurological Sciences*, *366*, 127–134.

Shih, R.-H., Wang, C.-Y., & Yang, C.-M. (2015). NF-kappaB signaling pathways in neurological inflammation: a mini review. *Frontiers in Molecular Neuroscience*, *8*, 77.

Shin, H.-M., Kim, M.-H., Kim, B. H., Jung, S.-H., Kim, Y. S., Park, H. J., … Kim, Y. (2004). Inhibitory action of novel aromatic diamine compound on lipopolysaccharide-induced nuclear translocation of NF-κB without affecting IκB degradation. *FEBS Letters*, *571*(1–3), 50–54.

Shin, J.-W., Cheong, Y.-J., Koo, Y.-M., Kim, S., Noh, C.-K., Son, Y.-H., … Sohn, N.-W. (2014). α-Asarone ameliorates memory deficit in lipopolysaccharide-treated mice via suppression of pro-inflammatory cytokines and microglial activation. *Biomolecules & Therapeutics*, *22*(1), 17.

Shipley, M. M., Mangold, C. A., & Szpara, M. L. (2016). Differentiation of the SH-SY5Y human neuroblastoma cell line. *Journal of Visualized Experiments: JoVE*, (108).

Shu, Z., Yang, B., Zhao, H., Xu, B., Jiao, W., Wang, Q., … Kuang, H. (2014). Tangeretin exerts anti-neuroinflammatory effects via NF-κB modulation in lipopolysaccharide-stimulated microglial cells. *International Immunopharmacology*, *19*(2), 275–282.

Sierra, A., Navascués, J., Cuadros, M. A., Calvente, R., Martín-Oliva, D., Ferrer-Martín, R. M., … Marín-Teva, J. L. (2014). Expression of inducible nitric oxide synthase (iNOS) in microglia of the developing quail retina. *PLoS One*, *9*(8), e106048.

Silverman, R. B. (2009). Design of selective neuronal nitric oxide synthase inhibitors for the prevention and treatment of neurodegenerative diseases. *Accounts of Chemical Research*, *42*(3), 439–451.

Simpson, D. S. A., & Oliver, P. L. (2020). ROS generation in microglia: understanding oxidative stress and inflammation in neurodegenerative disease. *Antioxidants*, *9*(8), 743.

Sinan, K. I., Zengin, G., Bene, K., & Mahomoodally, M. F. (2019). Chemistry and pharmacology of three antiplasmodial traditional medicinal plants from tropical Africa–A review. *South African Journal of Botany*, *126*, 265–276.

Singh-Manoux, A., Dugravot, A., Brunner, E., Kumari, M., Shipley, M., Elbaz, A., & Kivimaki, M. (2014). Interleukin-6 and C-reactive protein as predictors of cognitive decline in late midlife. *Neurology*, *83*(6), 486–493.

Singh, S., Nagalakshmi, D., Sharma, K. K., & Ravichandiran, V. (2021). Natural antioxidants for neuroinflammatory disorders and possible involvement of Nrf2 pathway: A review. *Heliyon*, *7*(2), e06216.

Singh, S., & Singh, T. G. (2020). Role of nuclear factor kappa B (NF-κB) signalling in neurodegenerative diseases: an mechanistic approach. *Current Neuropharmacology*, *18*(10), 918–935.

Singhal, A. K., Naithani, V., & Bangar, O. P. (2012). Medicinal plants with a potential to treat Alzheimer and associated symptoms. *International Journal of Nutrition, Pharmacology, Neurological Diseases*, *2*(2), 84.

Singhal, A., Morris, V. B., Labhasetwar, V., & Ghorpade, A. (2013). Nanoparticle-mediated catalase delivery protects human neurons from oxidative stress. *Cell Death & Disease*, *4*(11), e903–e903.

Smith, K. J., Kapoor, R., & Felts, P. A. (1999). Demyelination: the role of reactive oxygen and nitrogen species. *Brain Pathology*, *9*(1), 69–92.

Snow, W. M., Stoesz, B. M., Kelly, D. M., & Albensi, B. C. (2014). Roles for NF-κB and gene targets of NF-κB in synaptic plasticity, memory, and navigation. *Molecular Neurobiology*, *49*(2), 757–770.

Sofroniew, M. V. (2009). Molecular dissection of reactive astrogliosis and glial scar formation. *Trends in Neurosciences*, *32*(12), 638–647.

Sofroniew, M. V, & Vinters, H. V. (2010). Astrocytes: biology and pathology. *Acta Neuropathologica*, *119*(1), 7–35.

Song, Y., Qu, R., Zhu, S., Zhang, R., & Ma, S. (2012). Rhynchophylline attenuates LPS-induced pro-inflammatory responses through down-regulation of MAPK/NF-κB signaling pathways in primary microglia. *Phytotherapy Research*, *26*(10), 1528–1533.

Song, Z., & Tao, A. (2020). The neuroprotective effects of astragaloside IV against H2O2-induced damage in SH-SY5Y cells are associated with synaptic plasticity. *Journal of Chemistry*, *2020*.

Soto-Mercado, V., Mendivil-Perez, M., Jimenez-Del-Rio, M., & Velez-Pardo, C. (2020). Multi-Target

Effects of the Cannabinoid CP55940 on Familial Alzheimer's Disease PSEN1 E280A Cholinergic-Like Neurons: Role of CB 1 Receptor. *Journal of Alzheimer's Disease*, (Preprint), 1–20.

Sousa, N. A., Oliveira, G. A. L., de Oliveira, A. P., Lopes, A. L. F., Iles, B., Nogueira, K. M., … Ramos-Jesus, J. (2020). Novel ocellatin peptides mitigate LPS-induced ROS formation and NF-kB activation in microglia and hippocampal neurons. *Scientific Reports*, *10*(1), 1–16.

Spencer, N. G., Schilling, T., Miralles, F., & Eder, C. (2016). Mechanisms underlying interferon-γ-induced priming of microglial reactive oxygen species production. *PloS One*, *11*(9), e0162497.

Stansley, B., Post, J., & Hensley, K. (2012). A comparative review of cell culture systems for the study of microglial biology in Alzheimer's disease. *Journal of Neuroinflammation*, *9*(1), 1–8.

Stelmasiak, Z., Kozioł-Montewka, M., Dobosz, B., Rejdak, K., Bartosik-Psujek, H., Mitosek-Szewczyk, K., & Belniak-Legieć, E. (2000). Interleukin-6 concentration in serum and cerebrospinal fluid in multiple sclerosis patients. *Medical Science Monitor: International Medical Journal of Experimental and Clinical Research*, *6*(6), 1104–1108.

Stephenson, J., Nutma, E., van der Valk, P., & Amor, S. (2018). Inflammation in CNS neurodegenerative diseases. *Immunology*, *154*(2), 204–219.

Streyczek, J., Apweiler, M., Sun, L., & Fiebich, B. L. (2022). Turmeric Extract (Curcuma longa) Mediates Anti-Oxidative Effects by Reduction of Nitric Oxide, iNOS Protein-, and mRNA-Synthesis in BV2 Microglial Cells. *Molecules*, *27*(3), 784.

Subedi, L., Lee, J. H., Yumnam, S., Ji, E., & Kim, S. Y. (2019). Anti-inflammatory effect of sulforaphane on LPS-activated microglia potentially through JNK/AP-1/NF-κB inhibition and Nrf2/HO-1 activation. *Cells*, *8*(2), 194.

Subhramanyam, C. S., Wang, C., Hu, Q., & Dheen, S. T. (2019). Microglia-mediated neuroinflammation in neurodegenerative diseases. *Seminars in Cell & Developmental Biology*, *94*, 112–120. Elsevier.

Sugino, T., Nozaki, K., Takagi, Y., Hattori, I., Hashimoto, N., Moriguchi, T., & Nishida, E. (2000).

Activation of mitogen-activated protein kinases after transient forebrain ischemia in gerbil hippocampus. *Journal of Neuroscience*, *20*(12), 4506–4514.

Sultana, R., & Butterfield, D. A. (2010). Role of oxidative stress in the progression of Alzheimer's disease. *Journal of Alzheimer's Disease*, *19*(1), 341–353.

Sun, A., Liu, M., Nguyen, X. V, & Bing, G. (2003). P38 MAP kinase is activated at early stages in Alzheimer's disease brain. *Experimental Neurology*, *183*(2), 394–405.

Sun, G. Y., Li, R., Cui, J., Hannink, M., Gu, Z., Fritsche, K. L., … Simonyi, A. (2016). Withania somnifera and its withanolides attenuate oxidative and inflammatory responses and up-regulate antioxidant responses in BV-2 microglial cells. *Neuromolecular Medicine*, *18*(3), 241–252.

Sun, H.-N., Kim, S.-U., Lee, M.-S., Kim, S.-K., Kim, J.-M., Yim, M., … Lee, D.-S. (2008). Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-dependent activation of phosphoinositide 3-kinase and p38 mitogen-activated protein kinase signal pathways is required for lipopolysaccharide-induced microglial phagocytosis. *Biological and Pharmaceutical Bulletin*, *31*(9), 1711–1715.

Swarup, V., Phaneuf, D., Dupré, N., Petri, S., Strong, M., Kriz, J., & Julien, J.-P. (2011). Deregulation of TDP-43 in amyotrophic lateral sclerosis triggers nuclear factor κB–mediated pathogenic pathways. *Journal of Experimental Medicine*, *208*(12), 2429–2447.

Tai, W., Ye, X., Bao, X., Zhao, B., Wang, X., & Zhang, D. (2013). Inhibition of Src tyrosine kinase activity by squamosamide derivative FLZ attenuates neuroinflammation in both in vivo and in vitro Parkinson's disease models. *Neuropharmacology*, *75*, 201–212.

Tampi, R. R., Young, J. J., & Tampi, D. J. (2018). Cannabinoids for the treatment of behavioral and psychological symptoms of dementia receptors ". *Neurodegener. Dis.Manag.*, 20–23.

Tang, G., Yang, H., Chen, J., Shi, M., Ge, L., Ge, X., & Zhu, G. (2017). Metformin ameliorates sepsis-induced brain injury by inhibiting apoptosis, oxidative stress and neuroinflammation via the

PI3K/Akt signaling pathway. *Oncotarget*, *8*(58), 97977.

Tang, Y., & Le, W. (2016). Differential roles of M1 and M2 microglia in neurodegenerative diseases. *Molecular Neurobiology*, *53*(2), 1181–1194.

Taslimi, P., Köksal, E., Gören, A. C., Bursal, E., Aras, A., Kılıç, Ö., … Gülçin, İ. (2020). Anti-Alzheimer, antidiabetic and antioxidant potential of Satureja cuneifolia and analysis of its phenolic contents by LC-MS/MS. *Arabian Journal of Chemistry*, *13*(3), 4528–4537.

Taylor, J. P., Brown, R. H., & Cleveland, D. W. (2016). Decoding ALS: from genes to mechanism. *Nature*, *539*(7628), 197–206.

Teismann, P., Tieu, K., Choi, D.-K., Wu, D.-C., Naini, A., Hunot, S., … Przedborski, S. (2003). Cyclooxygenase-2 is instrumental in Parkinson’s disease neurodegeneration. *Proceedings of the National Academy of Sciences*, *100*(9), 5473–5478.

Terazawa, R., Akimoto, N., Kato, T., Itoh, T., Fujita, Y., Hamada, N., … Nozawa, Y. (2013). A kavalactone derivative inhibits lipopolysaccharide-stimulated iNOS induction and NO production through activation of Nrf2 signaling in BV2 microglial cells. *Pharmacological Research*, *71*, 34–43.

Thomson, A. C., Schuhmann, T., de Graaf, T. A., Sack, A. T., Rutten, B. P. F., & Kenis, G. (2021). The effects of serum removal on gene expression and morphological plasticity markers in differentiated SH-SY5Y cells. *Cellular and Molecular Neurobiology*, 1–11.

Tian, W., Zhao, J., Lee, J.-H., Akanda, M. R., Cho, J.-H., Kim, S.-K., … Park, B.-Y. (2019). Neuroprotective effects of Cornus officinalis on stress-induced hippocampal deficits in rats and H2O2-induced neurotoxicity in SH-SY5Y neuroblastoma cells. *Antioxidants*, *9*(1), 27.

Tine, Y., Diop, A., Diatta, W., Desjobert, J., Boye, C. S. B., Costa, J., … Paolini, J. (2017). Chemical diversity and antimicrobial activity of volatile compounds from Zanthoxylum zanthoxyloides Lam. according to compound classes, plant organs and Senegalese sample locations. *Chemistry & Biodiversity*, *14*(1), e1600125.

Tine, Y., Yang, Y., Renucci, F., Costa, J., Wélé, A., & Paolini, J. (2017). LC-MS/MS analysis of flavonoid compounds from Zanthoxylum zanthoxyloides extracts and their antioxidant activities. *Natural Product Communications*, *12*(12), 1934578X1701201213.

Tobore, T. O. (2019). On the central role of mitochondria dysfunction and oxidative stress in Alzheimer's disease. *Neurological Sciences*, *40*(8), 1527–1540.

Togo, T., Katsuse, O., & Iseki, E. (2004). Nitric oxide pathways in Alzheimer's disease and other neurodegenerative dementias. *Neurological Research*, *26*(5), 563–566.

Toldo, S., Mezzaroma, E., O'Brien, L., Marchetti, C., Seropian, I. M., Voelkel, N. F., … Abbate, A. (2014). Interleukin-18 mediates interleukin-1-induced cardiac dysfunction. *American Journal of Physiology-Heart and Circulatory Physiology*, *306*(7), H1025–H1031.

Tolón, R. M., Núñez, E., Pazos, M. R., Benito, C., Castillo, A. I., Martínez-Orgado, J. A., & Romero, J. (2009). The activation of cannabinoid CB2 receptors stimulates in situ and in vitro beta-amyloid removal by human macrophages. *Brain Research*, *1283*, 148–154.

Tonelli, C., Chio, I. I. C., & Tuveson, D. A. (2018). Transcriptional regulation by Nrf2. *Antioxidants & Redox Signaling*, *29*(17), 1727–1745.

Towbin, H., Staehelin, T., & Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proceedings of the National Academy of Sciences*, *76*(9), 4350–4354.

Trotta, T., Porro, C., Calvello, R., & Panaro, M. A. (2014). Biological role of Toll-like receptor-4 in the brain. *Journal of Neuroimmunology*, *268*(1–2), 1–12.

Tutka, P., Wlaź, A., Florek-Łuszczki, M., Kołodziejczyk, P., Bartusik-Aebisher, D., & Łuszczki, J. J. (2018). Arvanil, olvanil, AM 1172 and LY 2183240 (various cannabinoid CB1 receptor agonists) increase the threshold for maximal electroshock-induced seizures in mice. *Pharmacological Reports*, *70*(1), 106–109.

Ueno, M., Fujita, Y., Tanaka, T., Nakamura, Y., Kikuta, J., Ishii, M., & Yamashita, T. (2013). Layer V cortical neurons require microglial support for survival during postnatal development. *Nature Neuroscience*, *16*(5), 543–551.

Upadhyay, P., Panjwani, D., & Yadav, A. K. (2014). Neuropathology staging and treatment strategies of Alzheimer's disease: an update. *International Journal of Nutrition, Pharmacology, Neurological Diseases*, *4*(1), 28.

Uttara, B., Singh, A. V, Zamboni, P., & Mahajan, R. (2009). Oxidative stress and neurodegenerative diseases: a review of upstream and downstream antioxidant therapeutic options. *Current Neuropharmacology*, *7*(1), 65–74.

Valles, S. L., Dolz-Gaiton, P., Gambini, J., Borras, C., LLoret, A., Pallardo, F. V, & Viña, J. (2010). Estradiol or genistein prevent Alzheimer's disease-associated inflammation correlating with an increase PPARγ expression in cultured astrocytes. *Brain Research*, *1312*, 138–144.

van der Stelt, M., & Di Marzo, V. (2003). The endocannabinoid system in the basal ganglia and in the mesolimbic reward system: implications for neurological and psychiatric disorders. *European Journal of Pharmacology*, *480*(1–3), 133–150.

Van der Stelt, M., Mazzola, C., Esposito, G., Matias, I., Petrosino, S., De Filippis, D., … Iuvone, T. (2006). Endocannabinoids and β-amyloid-induced neurotoxicity in vivo: effect of pharmacological elevation of endocannabinoid levels. *Cellular and Molecular Life Sciences CMLS*, *63*(12), 1410–1424.

Van Weemen, B., & Schuurs, A. (1971). Immunoassay using antigen—enzyme conjugates. *FEBS Letters*, *15*(3), 232–236.

Vanhaesebroeck, B., Guillermet-Guibert, J., Graupera, M., & Bilanges, B. (2010). The emerging mechanisms of isoform-specific PI3K signalling. *Nature Reviews Molecular Cell Biology*, *11*(5), 329–341.

Vanhaesebroeck, B., Whitehead, M. A., & Piñeiro, R. (2016). Molecules in medicine mini-review: isoforms of PI3K in biology and disease. *Journal of Molecular Medicine*, *94*(1), 5–11.

Vay, S. U., Flitsch, L. J., Rabenstein, M., Rogall, R., Blaschke, S., Kleinhaus, J., … Schroeter, M. (2018). The plasticity of primary microglia and their multifaceted effects on endogenous neural stem cells in vitro and in vivo. *Journal of Neuroinflammation*, *15*(1), 1–21.

Velagapudi, R., Aderogba, M., & Olajide, O. A. (2014). Tiliroside, a dietary glycosidic flavonoid, inhibits TRAF-6/NF-κB/p38-mediated neuroinflammation in activated BV2 microglia. *Biochimica et Biophysica Acta (BBA)-General Subjects*, *1840*(12), 3311–3319.

Velagapudi, R., Kumar, A., Bhatia, H. S., El-Bakoush, A., Lepiarz, I., Fiebich, B. L., & Olajide, O. A. (2017). Inhibition of neuroinflammation by thymoquinone requires activation of Nrf2/ARE signalling. *International Immunopharmacology*, *48*, 17–29.

Veldhuis, W. B., Van der Stelt, M., Wadman, M. W., Van Zadelhoff, G., Maccarrone, M., Fezza, F., … Nicolay, K. (2003). Neuroprotection by the endogenous cannabinoid anandamide and arvanil against in vivo excitotoxicity in the rat: role of vanilloid receptors and lipoxygenases. *Journal of Neuroscience*, *23*(10), 4127–4133.

Vincent, V. A. M., Tilders, F. J. H., & Van Dam, A. M. (1998). Production, regulation and role of nitric oxide in glial cells. *Mediators of Inflammation*, *7*(4), 239–255.

Von Bernhardi, R., Eugenín-von Bernhardi, L., & Eugenín, J. (2015). Microglial cell dysregulation in brain aging and neurodegeneration. *Frontiers in Aging Neuroscience*, *7*, 124.

Von Bernhardi, R., & Eugenín, J. (2012). Alzheimer's disease: redox dysregulation as a common denominator for diverse pathogenic mechanisms. *Antioxidants & Redox Signaling*, *16*(9), 974–1031.

Waetzig, V., Czeloth, K., Hidding, U., Mielke, K., Kanzow, M., Brecht, S., … Hanisch, U. (2005). c-Jun N-terminal kinases (JNKs) mediate pro-inflammatory actions of microglia. *Glia*, *50*(3), 235–246.

Waetzig, V., & Herdegen, T. (2004). Neurodegenerative and physiological actions of c-Jun N-terminal kinases in the mammalian brain. *Neuroscience Letters*, *361*(1–3), 64–67.

Waksman, Y., Olson, J. M., Carlisle, S. J., & Cabral, G. A. (1999). The central cannabinoid receptor (CB1) mediates inhibition of nitric oxide production by rat microglial cells. *Journal of Pharmacology and Experimental Therapeutics*, *288*(3), 1357–1366.

Walter, L., Franklin, A., Witting, A., Wade, C., Xie, Y., Kunos, G., … Stella, N. (2003). Nonpsychotropic cannabinoid receptors regulate microglial cell migration. *Journal of Neuroscience*, *23*(4), 1398–1405.

Walters, A., Phillips, E., Zheng, R., Biju, M., & Kuruvilla, T. (2016). Evidence for neuroinflammation in Alzheimer's disease. *Progress in Neurology and Psychiatry*, *20*(5), 25–31.

Wang, H., & Siemens, J. (2015). TRP ion channels in thermosensation, thermoregulation and metabolism. *Temperature*, *2*(2), 178–187.

Wang, P., Guan, P., Wang, T., Yu, X., Guo, J., & Wang, Z. (2014). Aggravation of A lzheimer's disease due to the COX-2-mediated reciprocal regulation of IL-1β and A β between glial and neuron cells. *Aging Cell*, *13*(4), 605–615.

Wang, W.-Y., Tan, M.-S., Yu, J.-T., & Tan, L. (2015). Role of pro-inflammatory cytokines released from microglia in Alzheimer's disease. *Annals of Translational Medicine*, *3*(10).

Wang, X., & Tournier, C. (2006). Regulation of cellular functions by the ERK5 signalling pathway. *Cellular Signalling*, *18*(6), 753–760.

Wang, X., Wang, W., Li, L., Perry, G., Lee, H., & Zhu, X. (2014). Oxidative stress and mitochondrial dysfunction in Alzheimer's disease. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease*, *1842*(8), 1240–1247.

Wang, Y., Cui, L., Xu, H., Liu, S., Zhu, F., Yan, F., … Zhu, M. (2017). TRPV1 agonism inhibits endothelial cell inflammation via activation of eNOS/NO pathway. *Atherosclerosis*, *260*, 13–19.

Wang, Y., Ge, X., Yu, S., & Cheng, Q. (2021). Achyranthes bidentata polypeptide alleviates neurotoxicity of lipopolysaccharide-activated microglia via PI3K/Akt dependent NOX2/ROS pathway. *Annals of Translational Medicine*, *9*(20).

Wardyn, J. D., Ponsford, A. H., & Sanderson, C. M. (2015). Dissecting molecular cross-talk between Nrf2 and NF-κB response pathways. *Biochemical Society Transactions*, *43*(4), 621–626.

Weaver, J. D., Huang, M.-H., Albert, M., Harris, T., Rowe, J. W., & Seeman, T. E. (2002). Interleukin-6 and risk of cognitive decline: MacArthur studies of successful aging. *Neurology*, *59*(3), 371–378.

Webster, K. A. (2012). Mitochondrial membrane permeabilization and cell death during myocardial infarction: roles of calcium and reactive oxygen species. *Future Cardiology*, *8*(6), 863–884.

Wojcieszak, J., Krzemień, W., & Zawilska, J. B. (2016). JWH-133, a selective cannabinoid CB2 receptor agonist, exerts toxic effects on neuroblastoma SH-SY5Y cells. *Journal of Molecular Neuroscience*, *58*(4), 441–445.

Wolfe, L. S., & Coceani, F. (1979). The role of prostaglandins in the central nervous system. *Annual Review of Physiology*, *41*(1), 669–684.

Wood, H. (2012). Alzheimer disease: Prostaglandin E 2 signalling is implicated in inflammation early in the Alzheimer disease course. *Nature Reviews Neurology*, *8*(8), 411.

Wouatsa, V. N. A., Misra, L., Kumar, S., Prakash, O., Khan, F., Tchoumbougnang, F., & Venkatesh, R. K. (2013). Aromatase and glycosyl transferase inhibiting acridone alkaloids from fruits of Cameroonian Zanthoxylumspecies. *Chemistry Central Journal*, *7*(1), 1–15.

Wu, J., Bie, B., Yang, H., Xu, J. J., Brown, D. L., & Naguib, M. (2013a). Activation of the CB2 receptor system reverses amyloid-induced memory deficiency. *Neurobiology of Aging*, *34*(3), 791–804.

Wu, J., Bie, B., Yang, H., Xu, J. J., Brown, D. L., & Naguib, M. (2013b). Activation of the CB2receptor system reverses amyloid-induced memory deficiency. *Neurobiology of Aging*, *34*(3), 791–804. https://doi.org/10.1016/j.neurobiolaging.2012.06.011

Wyss-Coray, T. (2016). Ageing, neurodegeneration and brain rejuvenation. *Nature*, *539*(7628), 180–186.

Wyss-Coray, T., & Mucke, L. (2002). Inflammation in neurodegenerative disease—a double-edged sword. *Neuron*, *35*(3), 419–432.

Wyss-Coray, T., & Rogers, J. (2012). Inflammation in Alzheimer disease—a brief review of the basic science and clinical literature. *Cold Spring Harbor Perspectives in Medicine*, *2*(1), a006346.

Xi, Y.-D., Yu, H.-L., Ding, J., Ma, W.-W., Yuan, L.-H., Feng, J.-F., … Xiao, R. (2012). Flavonoids protect cerebrovascular endothelial cells through Nrf2 and PI3K from β-amyloid peptide-induced oxidative damage. *Current Neurovascular Research*, *9*(1), 32–41.

Xie, N., Wu, C., Wang, C., Cheng, X., Zhang, L., Zhang, H., & Lian, Y. (2017). Inhibition of the mitochondrial calcium uniporter inhibits Aβ-induced apoptosis by reducing reactive oxygen species-mediated endoplasmic reticulum stress in cultured microglia. *Brain Research*, *1676*, 100–106.

Xing, B., Xin, T., Hunter, R. L., & Bing, G. (2008). Pioglitazone inhibition of lipopolysaccharide-induced nitric oxide synthase is associated with altered activity of p38 MAP kinase and PI3K/Akt. *Journal of Neuroinflammation*, *5*(1), 4.

Xu, K., Wang, X., Zhang, Q., Liang, A., Zhu, H., Huang, D., … Ye, W. (2016). Sp1 downregulates proinflammatory cytokine-induced catabolic gene expression in nucleus pulposus cells. *Molecular Medicine Reports*, *14*(4), 3961–3968.

Xu, X., Zhang, L., Ye, X., Hao, Q., Zhang, T., Cui, G., & Yu, M. (2018). Nrf2/ARE pathway inhibits ROS-induced NLRP3 inflammasome activation in BV2 cells after cerebral ischemia reperfusion. *Inflammation Research*, *67*(1), 57–65.

Yan, J., Du, G., Qin, X., & Gao, L. (2020). Baicalein attenuates the neuroinflammation in LPS-activated BV-2 microglial cells through suppression of pro-inflammatory cytokines, COX2/NF-κB

expressions and regulation of metabolic abnormality. *International Immunopharmacology*, *79*, 106092.

Yan, L., Liu, S., Wang, C., Wang, F., Song, Y., Yan, N., … Sun, G. (2013). JNK and NADPH oxidase involved in fluoride-induced oxidative stress in BV-2 microglia cells. *Mediators of Inflammation*, *2013*.

Yan, M. H., Wang, X., & Zhu, X. (2013). Mitochondrial defects and oxidative stress in Alzheimer disease and Parkinson disease. *Free Radical Biology and Medicine*, *62*, 90–101.

Yang, H., Zhang, W., Pan, H., Feldser, H. G., Lainez, E., Miller, C., … Sweitzer, S. (2012). *SIRT1 activators suppress inflammatory responses through promotion of p65 deacetylation and inhibition of NF-κB activity*.

Yang, L., Zhou, R., Tong, Y., Chen, P., Shen, Y., Miao, S., & Liu, X. (2020). Neuroprotection by dihydrotestosterone in LPS-induced neuroinflammation. *Neurobiology of Disease*, *140*, 104814.

Yang, S., Chu, S., Ai, Q., Zhang, Z., Gao, Y., Lin, M., … Peng, Y. (2020). Anti-inflammatory effects of higenamine (Hig) on LPS-activated mouse microglia (BV2) through NF-κB and Nrf2/HO-1 signaling pathways. *International Immunopharmacology*, *85*, 106629.

Yang, S., Hu, B., Wang, Z., Zhang, C., Jiao, H., Mao, Z., … Zhao, J. (2020). Cannabinoid CB1 receptor agonist ACEA alleviates brain ischemia/reperfusion injury via CB1–Drp1 pathway. *Cell Death Discovery*, *6*(1), 1–11.

Yang, S., Wang, H., Yang, Y., Wang, R., Wang, Y., Wu, C., & Du, G. (2019). Baicalein administered in the subacute phase ameliorates ischemia-reperfusion-induced brain injury by reducing neuroinflammation and neuronal damage. *Biomedicine & Pharmacotherapy*, *117*, 109102.

Yao, C., Liu, X., Zhou, Z., Xiang, Y., Yuan, S., Xie, W., … Ji, A. (2019). Melatonin attenuates expression of cyclooxygenase-2 (COX-2) in activated microglia induced by lipopolysaccharide (LPS). *Journal of Toxicology and Environmental Health, Part A*, *82*(7), 437–446.

Yao, Z., Xu, L., Jin, L., Wang, B., Fu, C., Bai, Y., & Wu, H. (2021). κ-Carrageenan Oligosaccharides Inhibit

the Inflammation of Lipopolysaccharide-Activated Microglia Via TLR4/NF-κB and p38/JNK MAPKs Pathways. *Neurochemical Research*, 1–10.

Yarza, R., Vela, S., Solas, M., & Ramirez, M. J. (2016). c-Jun N-terminal kinase (JNK) signaling as a therapeutic target for Alzheimer's disease. *Frontiers in Pharmacology*, *6*, 321.

Yeung, Y. T., Aziz, F., Guerrero-Castilla, A., & Arguelles, S. (2018). Signaling pathways in inflammation and anti-inflammatory therapies. *Current Pharmaceutical Design*, *24*(14), 1449–1484.

Yokota, T., & Wang, Y. (2016). p38 MAP kinases in the heart. *Gene*, *575*(2), 369–376.

You, M.-M., Chen, Y.-F., Pan, Y.-M., Liu, Y.-C., Tu, J., Wang, K., & Hu, F.-L. (2018). Royal jelly attenuates LPS-induced inflammation in BV-2 microglial cells through modulating NF-κB and p38/JNK signaling pathways. *Mediators of Inflammation*, *2018*.

Youssef, M., Ibrahim, A., Akashi, K., & Hossain, M. S. (2019). PUFA-plasmalogens attenuate the LPS-induced nitric oxide production by inhibiting the NF-kB, p38 MAPK and JNK pathways in microglial cells. *Neuroscience*, *397*, 18–30.

Yuan, X., Guo, X., Deng, Y., Zhu, D., Shang, J., & Liu, H. (2015). Chronic intermittent hypoxia-induced neuronal apoptosis in the hippocampus is attenuated by telmisartan through suppression of iNOS/NO and inhibition of lipid peroxidation and inflammatory responses. *Brain Research*, *1596*, 48–57.

Yun, H.-Y., Dawson, V. L., & Dawson, T. M. (1996). Neurobiology of nitric oxide. *Critical Reviews™ in Neurobiology*, *10*(3–4).

Zeissler, M.-L., Eastwood, J., McCorry, K., Hanemann, C. O., Zajicek, J. P., & Carroll, C. B. (2016). Delta-9-tetrahydrocannabinol protects against MPP+ toxicity in SH-SY5Y cells by restoring proteins involved in mitochondrial biogenesis. *Oncotarget*, *7*(29), 46603.

Zhang, B., Gaiteri, C., Bodea, L.-G., Wang, Z., McElwee, J., Podtelezhnikov, A. A., … Dobrin, R. (2013). Integrated systems approach identifies genetic nodes and networks in late-onset Alzheimer's

disease. *Cell*, *153*(3), 707–720.

Zhang, F., & Jiang, L. (2015). Neuroinflammation in Alzheimer's disease. *Neuropsychiatric Disease and Treatment*, *11*, 243–256.

Zhang, J., Wang, X., Vikash, V., Ye, Q., Wu, D., Liu, Y., & Dong, W. (2016). ROS and ROS-mediated cellular signaling. *Oxidative Medicine and Cellular Longevity*, *2016*.

Zhang, J., Zheng, Y., Luo, Y., Du, Y., Zhang, X., & Fu, J. (2019). Curcumin inhibits LPS-induced neuroinflammation by promoting microglial M2 polarization via TREM2/TLR4/NF-κB pathways in BV2 cells. *Molecular Immunology*, *116*, 29–37.

Zhang, L., Li, L., Prabhakaran, K., Borowitz, J. L., & Isom, G. E. (2006). Trimethyltin-induced apoptosis is associated with upregulation of inducible nitric oxide synthase and Bax in a hippocampal cell line. *Toxicology and Applied Pharmacology*, *216*(1), 34–43.

Zhao, B. (2005). Nitric oxide in neurodegenerative diseases. *Frontiers in Bioscience-Landmark*, *10*(1), 454–461.

Zhao, H., Cheng, L., Liu, Y., Zhang, W., Maharjan, S., Cui, Z., … Nie, L. (2014). Mechanisms of anti-inflammatory property of conserved dopamine neurotrophic factor: inhibition of JNK signaling in lipopolysaccharide-induced microglia. *Journal of Molecular Neuroscience*, *52*(2), 186–192.

Zhao, M., Zhou, A., Xu, L., & Zhang, X. (2014). The role of TLR4-mediated PTEN/PI3K/AKT/NF-κB signaling pathway in neuroinflammation in hippocampal neurons. *Neuroscience*, *269*, 93–101.

Zhao, X., Fang, J., Li, S., Gaur, U., Xing, X., Wang, H., & Zheng, W. (2019). Artemisinin attenuated hydrogen peroxide (H2O2)-induced oxidative injury in SH-SY5Y and hippocampal neurons via the activation of AMPK pathway. *International Journal of Molecular Sciences*, *20*(11), 2680.

Zhao, Y., & Zhao, B. (2013). Review Article Oxidative Stress and the Pathogenesis of Alzheimer ' s Disease. *Oxidative Medicine and Cellular Longevity*, *2013*. https://doi.org/10.1155

Zheng, Y., Fang, W., Fan, S., Liao, W., Xiong, Y., Liao, S., … Liu, J. (2018). Neurotropin inhibits

neuroinflammation via suppressing NF-κB and MAPKs signaling pathways in lipopolysaccharide-stimulated BV2 cells. *Journal of Pharmacological Sciences*, *136*(4), 242–248.

Zheng, Y., Zhu, G., He, J., Wang, G., Li, D., & Zhang, F. (2019). Icariin targets Nrf2 signaling to inhibit microglia-mediated neuroinflammation. *International Immunopharmacology*, *73*, 304–311.

Zhihui, Q. (2013). Modulating nitric oxide signaling in the CNS for Alzheimer's disease therapy. *Future Medicinal Chemistry*, *5*(12), 1451–1468.

Zhong, J., Qiu, X., Yu, Q., Chen, H., & Yan, C. (2020). A novel polysaccharide from Acorus tatarinowii protects against LPS-induced neuroinflammation and neurotoxicity by inhibiting TLR4-mediated MyD88/NF-κB and PI3K/Akt signaling pathways. *International Journal of Biological Macromolecules*, *163*, 464–475.

Zhou, L., Wang, K., Li, L., Li, H., & Geng, M. (2015). Pinocembrin inhibits lipopolysaccharide-induced inflammatory mediators production in BV2 microglial cells through suppression of PI3K/Akt/NF-κB pathway. *European Journal of Pharmacology*, *761*, 211–216.

Zhu, C., Xiong, Z., Chen, X., Peng, F., Hu, X., Chen, Y., & Wang, Q. (2012). Artemisinin attenuates lipopolysaccharide-stimulated proinflammatory responses by inhibiting NF-κB pathway in microglia cells. *PloS One*, *7*(4).

Zhu, J., Li, S., Zhang, Y., Ding, G., Zhu, C., Huang, S., … Li, M. (2018). COX-2 contributes to LPS-induced Stat3 activation and IL-6 production in microglial cells. *American Journal of Translational Research*, *10*(3), 966.

Zhu, X., Yao, Y., Yang, J., Zhengxie, J., Li, X., Hu, S., … Gan, G. (2020). COX-2-PGE2 signaling pathway contributes to hippocampal neuronal injury and cognitive impairment in PTZ-kindled epilepsy mice. *International Immunopharmacology*, *87*, 106801.

Zhu, Y., Chen, X., Liu, Z., Peng, Y.-P., & Qiu, Y.-H. (2016). Interleukin-10 protection against lipopolysaccharide-induced neuro-inflammation and neurotoxicity in ventral mesencephalic

cultures. *International Journal of Molecular Sciences*, *17*(1), 25.

Zhu, Y., Li, H., Long, C., Hu, L., Xu, H., Liu, L., … Shao, F. (2007). Structural insights into the enzymatic mechanism of the pathogenic MAPK phosphothreonine lyase. *Molecular Cell*, *28*(5), 899–913.

Zirihi, G. N., Mambu, L., Guédé-Guina, F., Bodo, B., & Grellier, P. (2005). In vitro antiplasmodial activity and cytotoxicity of 33 West African plants used for treatment of malaria. *Journal of Ethnopharmacology*, *98*(3), 281–285.

Zou, S., & Kumar, U. (2018). Cannabinoid receptors and the endocannabinoid system: signaling and function in the central nervous system. *International Journal of Molecular Sciences*, *19*(3), 833.

Zou, Y., Hong, B., Fan, L., Zhou, L., Liu, Y., Wu, Q., … Dong, M. (2013). Protective effect of puerarin against beta-amyloid-induced oxidative stress in neuronal cultures from rat hippocampus: involvement of the GSK-3β/Nrf2 signaling pathway. *Free Radical Research*, *47*(1), 55–63.