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INVESTIGATION OF THE Nrf2-MEDIATED INHIBITION OF NEUROINFLAMMATION BY DIOSGENIN

A thesis submitted to the University of Huddersfield in partial fulfilment of the requirements for the degree of Doctor of Philosophy

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December 2017
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Abstract

Chronic activation of microglia results in neuroinflammation and subsequently neurodegeneration. Diosgenin is a plant-derived steroidal saponin found in fenugreek and roots of yam. Some studies have shown that diosgenin possess anti-inflammatory effect in various experimental models. However, little is known about the anti-neuroinflammatory effect of diosgenin in the microglia. In this study, the anti-neuroinflammatory effect of diosgenin (5, 10 and 20 µM) was investigated in LPS-activated BV2 microglia. Nitrite and PGE₂ levels were measured using a Griess assay and an enzyme immunoassay (EIA), respectively. In addition, levels of the cytokines TNFα, IL-6, IL-1β and IL-10 were measured using specific mouse ELISA. Further, LPS-induced ROS generation in BV2 microglia was tested using a DCFDA assay. Protein levels of COX-2, iNOS, the main targets in NF-κB pathway as well as the levels of p38 MAPK and Akt were measured with immunoblotting. Additionally, the effect of diosgenin on the activity of NF-κB promoter was examined using a luciferase reporter gene assay in HEK293 cells. NF-κB binding to the DNA was investigated using EMSA. The role of Nrf2 in the anti-neuroinflammatory effect of diosgenin was investigated in BV2 microglia by western blot analysis of HO-1, NQO1 and Nrf2. BV2 microglia were transfected with siRNA for Nrf2, followed by stimulation with LPS in order to investigate the role of Nrf2 in the anti-inflammatory activity of diosgenin. The effect of diosgenin on neuroinflammation-induced HT22 neuronal toxicity was also evaluated using conditioned medium obtained from LPS-activated BV2 microglia.

Results show that diosgenin reduced the production of NO and PGE₂ through inhibition of iNOS and COX-2 expression, respectively in LPS-stimulated BV2 microglia. In addition, diosgenin reduced the secretion of other pro-inflammatory factors including IL-6, IL-1β, TNFα and ROS. By contrast, the compound increased IL-10 release in LPS-stimulated BV2 cells. Further results show that the anti-neuroinflammatory effect of diosgenin is mediated through the inhibition of NF-κB signalling pathway. Furthermore, the compound also attenuated Akt signalling, but did not inhibit p38 MAPK signalling in LPS-stimulated BV2 cells. Additionally, diosgenin treatment resulted in an up-regulation of the expression
of the antioxidant proteins HO-1 and NQO1, and GSH in BV2 microglia, suggesting that diosgenin activates Nrf2 signalling. In addition, the compound increased the nuclear translocation of Nrf2 and its binding to ARE in BV2 microglia. Diosgenin down-regulated the level of the inhibitory protein Keap1, indicating that diosgenin activates Nrf2/ARE pathway by down-regulation of Keap1. Transfection of BV2 microglia with Nrf2 siRNA resulted in the loss of anti-neuroinflammatory effect of diosgenin. Diosgenin produced a neuroprotective effect through the inhibition of neuroinflammation-induced neurotoxicity and ROS generation in HT22 mouse hippocampal neurons. Taken together, these results demonstrate that diosgenin inhibits LPS-mediated neuroinflammation through interference with NF-κB signalling. The anti-neuroinflammatory effect of diosgenin is dependent on the activation of Nrf2 signalling.

The results obtained from this study suggest that diosgenin inhibited NF-κB-mediated neuroinflammation through molecular mechanisms that are possibly closely linked to Nrf2/ARE antioxidant protection system in BV2 microglia cells. It was demonstrated that diosgenin is neuroprotective in both neuroinflammation and oxidative stress-mediated neuronal damage, an action that is important in neurodegeneration. The effects of the compound on processes linked to oestrogen receptors suggests a potential oestrogenic activity which needs further investigation. Activation of microglial AMPK is a property which further demonstrates that diosgenin may be producing inhibition of neuroinflammation through activation of endogenous systems which block the transcriptional activity of NF-κB.

Keywords: Microglia, neuroinflammation, Nrf2, diosgenin
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<tbody>
<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
</tr>
<tr>
<td>AGE</td>
<td>Advanced glycation end product</td>
</tr>
<tr>
<td>Akt</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>ALS</td>
<td>Amyotrophic lateral sclerosis</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine monophosphate</td>
</tr>
<tr>
<td>AMPK</td>
<td>Adenosine monophosphate-activated protein kinase</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>ARE</td>
<td>Antioxidant responsive element</td>
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<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>Aβ</td>
<td>Amyloid-beta</td>
</tr>
<tr>
<td>BACE1</td>
<td>β-site APP cleaving enzyme 1</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood brain barrier</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>bFGF</td>
<td>Basic fibroblast growth factor</td>
</tr>
<tr>
<td>BH4</td>
<td>Tetrahydrobiopterin</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CAT</td>
<td>Catalase</td>
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<tr>
<td>CCR1-7</td>
<td>CC chemokine receptor 1-7</td>
</tr>
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<td>CD14</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
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<td>Cyclooxygenase-1</td>
</tr>
<tr>
<td>COX-2</td>
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<tr>
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<td>CXC chemokine receptor 1-5</td>
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<td>4',6-diamidino-2-phenylindole dihydrochloride</td>
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<td>DCFH</td>
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<td>DMEM</td>
<td>Dulbecco’s modified Eagles medium</td>
</tr>
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<td>DMSO</td>
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<td>Ethylenediaminetetraacetic</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EIA</td>
<td>Enzyme immunoassay</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EMSA</td>
<td>Electrophoretic mobility shift assay</td>
</tr>
<tr>
<td>EP1-4</td>
<td>E-type prostanoid 1-4</td>
</tr>
<tr>
<td>ER</td>
<td>Oestrogen receptor</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FcγRI-III</td>
<td>Fc gamma receptor I-III</td>
</tr>
<tr>
<td>GluR1-7</td>
<td>Glutamate receptor 1-7</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>NEMO</td>
<td>NF-κB Essential Modulator</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor-kappa B</td>
</tr>
<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NO$_2^-$</td>
<td>Nitrite</td>
</tr>
<tr>
<td>NOX</td>
<td>NADPH oxidase</td>
</tr>
<tr>
<td>NQO1</td>
<td>(NADPH): quinone oxidoreductase 1</td>
</tr>
<tr>
<td>Nrf2</td>
<td>Nuclear factor-erythroid 2-related factor 2</td>
</tr>
<tr>
<td>NSAIDs</td>
<td>Non-steroidal anti-inflammatory drugs</td>
</tr>
<tr>
<td>NT-3/4</td>
<td>Neurotrophin-3/4</td>
</tr>
<tr>
<td>O$_2$</td>
<td>Molecular oxygen</td>
</tr>
<tr>
<td>O$_2^-$</td>
<td>Superoxide</td>
</tr>
<tr>
<td>p75NTR</td>
<td>p75 neurotrophin</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson's disease</td>
</tr>
<tr>
<td>PGE2</td>
<td>Prostaglandin E2</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>RAGE</td>
<td>Receptor for advanced glycation end products</td>
</tr>
<tr>
<td>RNA</td>
<td>Reactive nitrogen species</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>SCARA-1</td>
<td>Scavenger receptor class A type 1</td>
</tr>
<tr>
<td>SCARB-2</td>
<td>Scavenger receptor class B type 2</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of mean</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>TAK1</td>
<td>Transforming growth factor-β-activated kinase 1</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris-Borate-EDTA</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris-buffered saline with Tween 20</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>Transforming growth factor-β1</td>
</tr>
<tr>
<td>TGF-β1R</td>
<td>Transforming growth factor-β1 receptor</td>
</tr>
<tr>
<td>TLR4</td>
<td>Toll-Like receptor 4</td>
</tr>
<tr>
<td>TNFR1/2</td>
<td>TNFα receptor type 1/2</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>TRAF6</td>
<td>TNF-receptor-associated factor 6</td>
</tr>
<tr>
<td>Trk-B1</td>
<td>Truncated tropomyosin-related kinase-B-T1 receptor</td>
</tr>
</tbody>
</table>
Chapter 1
General Introduction
1.1. Introduction

1.1.1. The immune response in the central nervous system

The human nervous system is a complex network of nerves and neurons that transmit information in the form of signals between various parts of the body. Structurally, the nervous system is divided into two parts; the central nervous system (CNS) and the peripheral nervous system. The CNS is made up of two parts the brain and the spinal cord, and is the most important part of the nervous system (Noback et al., 2005, Mai and Paxinos, 2011).

The CNS is an important part for survival due to the presence of, for example, about 100 billion neurons in the brain. It is necessary for the CNS and for neurons to be well protected due to a limited regenerative capacity against harmful influences. In general, there are various types of cells in the CNS including neurons, epithelial cells, oligodendrocytes, microglia and astrocytes. Microglia are the primary immune cells in the brain (Tian et al., 2015, Gundersen et al., 2015). For decades, it was thought that the basic function of the CNS was only to regulate most bodily functions such as signal transmission regulation. In fact, the CNS is a highly immunologically-active organ and plays an essential role in the regulation of normal immune and inflammatory responses due to the presence of some specific immune cells named microglia that have some important pattern recognition receptors (Lampron et al., 2013, Schwartz et al., 2013).

The immune response in the CNS can be defined as a biological response to foreign invaders such as damaged cells and microorganisms (Ransohoff and Brown, 2012, Bilbo and Schwarz, 2012). In the presence of insults, microglia are activated to protect all the components of the CNS, especially neurons against excessive immune response-mediated neuroinflammation through transduction of microglial pattern recognition receptors (Kraft and Harry, 2011).

Findings from several studies have demonstrated that microglia play a key role in the modulation of the immune and inflammatory responses in the CNS (Tian et al., 2009). The CNS environment is monitored or scanned by microglia to recognise and then to remove the physical and chemical insults such as pathogens (Olson and Miller, 2004). For example, a bacterial infection is identified
as one of the most important inducer of an immune response due to the presence of microglia in the CNS (Buchanan et al., 2010). Numerous studies have suggested that this biological response is either a defence response to repair the host or a pathological response that completely damages the cells depending on the duration of microglial activation (Gomes-Leal, 2012). However, several studies have been carried out to understand the pathophysiologic mechanisms of neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS) (Cameron and Landreth, 2010, Taylor et al., 2013, Brites and Vaz, 2014). These authors have suggested that an excessive immune response in a form of uncontrolled neuroinflammation is the most common factor that results in neurodegenerative disorders (Block and Hong, 2005, Kim et al., 2015).

### 1.1.2. Cell types of the central nervous system

In general, the three glial cells microglia, astrocytes and oligodendrocytes as well as neurons are the main components of the CNS. The glial cells and neurons represent 90% and 10% of the cells, respectively in the CNS. Microglial cells constitute 10% to 20% of the total population of glial cells in the adult brain (Gomez-Nicola and Perry, 2015, Ginhoux et al., 2013). As shown in Table 1, the non-neuronal glial cells play an important role to support and protect neurons (Verkhratsky and Butt, 2007, Mai and Paxinos, 2011, Tian et al., 2015, Gundersen et al., 2015).

**Table 1 Types of glial cells in the nervous system**

<table>
<thead>
<tr>
<th>Types of glial cells</th>
<th>Basic functions</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>1- Central nervous system</strong></td>
<td></td>
</tr>
<tr>
<td>A- Microglia</td>
<td>Regulate the immune and neuroinflammatory responses</td>
</tr>
<tr>
<td>B- Astrocytes</td>
<td>Form blood brain barrier and synapses</td>
</tr>
<tr>
<td>C- Oligodendrocytes</td>
<td>Form the myelin sheaths around the axons of neurons</td>
</tr>
<tr>
<td>D- Ependymal cells</td>
<td>Produce and circulate in the cerebrospinal fluid</td>
</tr>
<tr>
<td><strong>2- Peripheral nervous system</strong></td>
<td></td>
</tr>
<tr>
<td>A- Schwann cells</td>
<td>Form the myelin sheaths around the axons of neurons</td>
</tr>
<tr>
<td>B- Satellite cells</td>
<td>Cover the surface of nerve cell bodies</td>
</tr>
</tbody>
</table>
1.1.2.1. Neurons
Approximately one hundred billion neurons are located in the human brain. The structure of the specialised cell neuron is not similar to that of other cells in the body because it consists of a dendrite, an axon and a cell body. Unlike others cells, neurons are never replaced when they die (Sherwood et al., 2010, Kempuraj et al., 2016). Electrical massages or information are transmitted by neuron-neuron communication through different electrochemical processes. Due to the importance of neurons in the regulation of most bodily functions, these cells are considered as the most important cell type in the humans. Therefore, neurons have been extensively studied in a wide variety of scientific fields including neuroscience and pharmacology. In the brain, the neurons are protected by glial cells such as microglia as well as the blood brain barrier (BBB) (Abbott et al., 2010, Lampron et al., 2013). Normal activation of microglia is required to protect neurons against viral and bacterial products (Graeber et al., 2011). However, long-term microglial over-activation is identified as a hallmark of neuroinflammation, because long exposure of surrounding healthy neurons to microglial neurotoxic mediators results in neuronal loss and death (Rojo et al., 2014).

1.1.2.2. Microglia
1.1.2.2.1. Nature of microglia
Microglia are the primary innate immune cells in the CNS (Graeber et al., 2011). Microglia are a type of glial cells present in a resting state. The morphology of microglial cells is ramified with a small cell body and extended multiple branches in all directions. They are derived from the myeloid progenitors of the embryonic yolk sac (Michell-Robinson et al., 2015). Microglia are the only cell population in the CNS that originate outside the brain. Microglia are identified as macrophages of the brain because both microglia and peripheral macrophages share the same properties and roles in the immune response (Perry and Teeling, 2013). These mononuclear phagocytic cells are found in all areas of the brain so they form part of the CNS structure. Microglia have been reported to play a central role in the activation of the immune response in the CNS (Ransohoff and Brown, 2012). As shown in Table 2, microglia are important immune effectors due to the presence of a diverse variety of receptors on their surfaces that play essential roles in
neuroinflammation (Rock and Peterson, 2006, Pocock and Kettenmann, 2007, Sierra et al., 2008, Kettenmann et al., 2011, Sierra et al., 2013, Murugan et al., 2013, Murer et al., 2001). These receptors are expressed in microglia and play detrimental roles in microglial activation-mediated neuroinflammation (Mead et al., 2012).

### Table 2 Types of microglial cell membrane receptors

<table>
<thead>
<tr>
<th>Receptor type</th>
<th>Examples of receptor subtypes</th>
<th>Examples of ligand(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytokines</td>
<td>IFN-γ-R, M-CSF-R, TNFR1/2, IL-1R1/2, IL-10R</td>
<td>IL-1α/β, IL-6, IL-10, TNFα, IFN-γ</td>
</tr>
<tr>
<td>Chemokines</td>
<td>CXCR1-5, CCR1-7, MCP-1-R</td>
<td>MIP-1/2, MCP-1, IP-10, Fractalkine</td>
</tr>
<tr>
<td>Neurotrophin</td>
<td>p75NTR, Trk-B1, TGF-β-R</td>
<td>NGF, BDNF, bFGF, NT-3/4, EGF, TGF-β1</td>
</tr>
<tr>
<td>Toll-like</td>
<td>TLR1-9</td>
<td>LPS, LTA, Zymosan</td>
</tr>
<tr>
<td>Complement</td>
<td>C1qR, C3aR, C5aR</td>
<td>C1q, C3a, C5a</td>
</tr>
<tr>
<td>Oestrogen</td>
<td>ERα, ERβ</td>
<td>17β-oestradiol</td>
</tr>
<tr>
<td>Glutamatergic</td>
<td>GluR1-7, mGluR1-8, KA1/2R,</td>
<td>Glutamate, Kainate</td>
</tr>
<tr>
<td>Scavenger</td>
<td>SCARA-1/-2, SCARB-1/-2,</td>
<td>LDL, HDL, Thrombospondin, Aβ</td>
</tr>
<tr>
<td>Immunoglobulins</td>
<td>FcyRI-III, RAGE</td>
<td>AGE, MHC-I/II, ICAM-1, Immunoglobulins</td>
</tr>
<tr>
<td>superfamily</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucocorticoid</td>
<td>Glucocorticoid receptor</td>
<td>Cortisol, Corticosterone, Dexamethasone</td>
</tr>
</tbody>
</table>

It is well-known that microglia exert several roles in the CNS including antigen presentation, production of many pro-inflammatory and anti-inflammatory factors such as nitric oxide (NO), cytokines, reactive oxygen species (ROS), complement components and glutamate as well as phagocytosis. Microglia are activated in order to initiate an inflammatory response, so it is necessary to understand the phenotypic states of microglia during neuroinflammation in order to understand the pathophysiological roles of microglia in the brain. Several studies have demonstrated that the phenotypes of activated microglia can be divided into two subtypes M1 and M2 (Walker and Lue, 2015).

**1.1.2.2.2. Phenotypes of activated microglia**

In the healthy brain, the fundamental role of microglia is to protect neurons against pathological invaders including bacterial infection, dead cells (e.g. dying
neurons), and brain debris (Graeber et al., 2011, Tang and Le, 2016). Under physiological conditions or in the absence of inflammatory stimulus, the morphology of resting microglia is ramified. Microglia are morphologically and functionally changed into an active cells to produce various types of active mediators in the presence of pathological assaults such as a bacterial infection, brain injury, and brain trauma (Varnum and Ikezu, 2012). Several studies have demonstrated that the phenotypes of activated microglia can be divided into two subtypes M1 and M2 (Walker and Lue, 2015). The M1 phenotype of activated microglia is responsible for the induction of the early stage of the inflammatory response against pathogen invasion and dead cells. As shown in Figure 1.1, in the existence of an inflammatory stimulus such as the bacterial product LPS; a wide variety of endogenous pro-inflammatory and oxidative mediators including tumour necrosis factor-α (TNFα), interleukin-6 (IL-6), interleukin-1β (IL-1β), prostaglandin E2 (PGE2), NO, and ROS are secreted from M1-classically activated microglia (Hu et al., 2015, Tang and Le, 2016).

After that, M1 microglia are transformationally changed into amoeboid/phagocytic form (M2 microglia) to phagocytise the detected insult (Brown and Neher, 2010, Fu et al., 2014). M2 microglia are characterised by the production of anti-inflammatory and neuroprotective factors such as interleukin 10 (IL-10) and transforming growth factor-β1 (TGF-β1). This results in the clearance of dead cells and pathogens by phagocytosis and then the modulation of neuroinflammation. M2 microglia are considered as important reactive phagocytic cells in the regulation of the immune response (Walker and Lue, 2015). Thus, the detected damaged cells, cell debris, and pathogens can be removed by microglia using secreted pro-inflammatory mediators as well as phagocytosis (Brown and Neher, 2010).
Figure 1.1 Phenotypes of activated microglia.

Resting microglia are activated by various inflammatory stimuli such as lipopolysaccharide (LPS) and brain injury. The M1 phenotype of the activated microglia is responsible for the production of several pro-inflammatory and oxidative factors such as nitric oxide (NO), reactive oxygen species (ROS), cytokines, and chemokines. This results in neuroinflammation. After that, activated microglia are transformed into M2 phenotype which is responsible for the secretion of anti-inflammatory and neuroprotective factors such as interleukin 10 (IL-10) and transforming growth factor-β1 (TGF-β1) that protect neurons (Hu et al., 2015, Xia et al., 2015, Rojo et al., 2014).

1.1.2.2.3. Microglia-mediated neuroinflammation

Many studies have reported that the inflammatory response of reactive microglia is either neuroprotective or neurotoxic, depending on the intensity of both microglial cell activation and neuroinflammation. It is well known that sustained hyper-activation of microglia has been associated with the neuronal loss and neuronal death (Xu et al., 2016). Several lines of evidence have suggested that long-term microglial over-activation due to the presence of inflammatory insults such as LPS are more likely to cause neuroinflammation and neurotoxicity (Graeber et al., 2011). Healthy (viable) neurons and glial cells that are located
close to the damaged neurons or dead cells are affected during neuroinflammatory response due to chronic exposure of these healthy cells to high levels of both pro-inflammatory and neurotoxic factors and microglial phagocytosis (Xu et al., 2016, Graeber et al., 2011).

According to numerous experimental studies, synaptic dysfunction and neuronal cell death have been associated with over-activated microglia and neuroinflammation (Suzumura, 2013, Viviani et al., 2014). For example, neuronal death due to the protein amyloid-beta (Aβ) is mediated by highly phagocytic activity of microglia on neurons (Neniskyte et al., 2011). Another study showed that LPS-induced neuronal death due to microglial phagocytosis and neuroinflammation is mediated by the protein Milk-fat globule EGF factor-8 (Fricker et al., 2012). In addition, hyper-activation of microglia results in dysfunction of the BBB due to a direct contact of the BBB with microglial pro-inflammatory and neurotoxic mediators (Sumi et al., 2010). Furthermore, over-activated microglia play synergistic role with astrocytes in the induction of neuronal death (Liddelow et al., 2017).

Regarding the dual role of microglia in AD pathology, microglial cells are found in close association with Aβ deposits in the AD brain. Increased numbers of reactive microglia within and around Aβ plaques have been detected in a wide variety of AD models and in AD patients (Combs, 2009). It was shown that microglia are shifted to a more pro-inflammatory and less phagocytic state during chronic activation due to the presence of Aβ in AD (Hickman et al., 2008, Krabbe et al., 2013). Several reports have also established that microglia are implicated in the progression of ALS (Frakes et al., 2014, Gerber et al., 2012).

1.1.2.2.4. Microglia as a target for the modulation of neuroinflammation

In recent years, the pathophysiological roles of microglia have been extensively studied in cell culture studies due to their vital roles in defence and/or pathology of the CNS (Chew et al., 2006, Schwartz et al., 2013). A timeline of the main tools and methods that have revolutionised and critically contributed to elucidate microglial cells identity, ontogeny, and function is summarised in Figure 1.2 (Sousa et al., 2017).
Figure 1.2 The history of microglia research


It is well established that neuroinflammation might be inhibited or progressively slowed by a number of effective approaches. For example, inhibiting the
production of numerous pro-inflammatory factors from hyper-reactive microglia by drug treatment is one of the effective strategies against neuroinflammation (McCarty, 2006, Cartier et al., 2014). Activation of microglial cell lines such as murine primary microglia or BV2 cells by potent inflammatory stimuli such as LPS (Bachstetter et al., 2011), TNFα (Syed et al., 2007), or Aβ (Pan et al., 2011) have been used as experimental models to study the pathophysiological roles of microglia and to find new compounds active against neuroinflammation (Choi et al., 2011). Most of recent studies have focused on how to control chronic microglial activation-mediated neuroinflammation by using test compounds such as natural products in the experimental models (Figuera-Losada et al., 2014). The main purpose of experimental studies is to find effective drugs that might be used for preventing or treating CNS diseases such as AD and PD because numerous CNS disorders are thought to be mediated by over-activation of microglia (Karunaweera, 2015, Minter et al., 2016).

In addition, systemic inflammation plays a critical role in the etiology of neuroinflammation (Perry et al., 2007). For example, activation of microglia and subsequent neuroinflammation can be induced by an intravenous injection of LPS in rat brain (Qin et al., 2007, Hoogland et al., 2015). It has also been observed that the peripheral pro-inflammatory mediator TNFα can pass the BBB to induce neuroinflammation (Qian et al., 2010). Thus, it is necessary to protect neurons against neuroinflammation in the CNS or induced by either systemic inflammation through the modulation of microglial activity.

1.1.3. Defining neuroinflammation
The immune response is one of the most important lines of defence against damaged cells, microorganisms, and brain injury. A neuroinflammatory response is induced to protect both neuronal and non-neuronal cells in the CNS (Lyman et al., 2014). In fact, neuroinflammation is not easy to define because it is protective and/or destructive (Graeber et al., 2011, O’callaghan et al., 2008). Neuroinflammation can be defined as an active defensive process or response by microglia against brain injury and brain infection as part of the innate immune system (Zipp and Aktas, 2006, Patro et al., 2016). There are many factors that control neuroinflammation, including the time (duration) and the intensity (rate) of neuroinflammatory response. Neuroinflammation can be divided into two classes;
an acute and chronic neuroinflammation. An acute neuroinflammation is started rapidly and become severe in a short period during brain injury, in which activated microglia are rapidly moved to the site of brain injury to repair the damage. In contrast to acute neuroinflammation, chronic neuroinflammation is a prolonged and persistent neuroinflammation that lasts for several months or years and that progressively causes neurodegeneration. For example, long-term neuroinflammation has been associated with common CNS disorders such as AD, PD, and ALS (Graeber et al., 2011).

The pathological mechanisms of neuroinflammation have been incompletely defined because it is mainly mediated by complex pathological factors such as microglial over-activation (Glass et al., 2010). Microglial over-activation is considered as an initial event of the pathophysiological process neuroinflammation (Khandelwal et al., 2011). Several in vivo and in vitro experiments have been performed to understand the mechanisms of neuroinflammation using several ligands that are known to produce neuroinflammation. A neuroinflammatory stimulus can be defined as a physical or chemical insult that cause neuroinflammation in the CNS. Numerous inflammatory stimuli including brain injury, LPS, and Aβ are known to produce neuroinflammation (Sondag et al., 2009, Fan et al., 2015).

1.1.4. Role of neuroinflammation in neurodegenerative diseases

It is well known that normal activation of resting microglia and thereby, a normal immune response is responsible for protecting all types of cells in the CNS. On the other hand, long-term immune response due to chronic microglial over-activation and excessive neuroinflammation is implicated in neurodegenerative diseases (Yuste et al., 2015). As shown in Figure 1.3, the most common neurodegenerative disorders that are associated with neuroinflammation are AD, PD, and ALS (Morales et al., 2016).
Figure 1.3 Model of neuroinflammation and neurodegeneration cycle.

Ramified microglia are activated in the presence of a damage signal. Excessive production of microglial pro-inflammatory and neurotoxic mediators results in neuroinflammation and thereby, neurodegenerative diseases. In addition, neurodegenerative neurons release mediators that activate microglia to produce more pro-inflammatory and neurotoxic mediators (Morales et al., 2016, Solito and Sastre, 2012, Rocha et al., 2015, Brites and Vaz, 2014).

In the last few years, several cell culture, animal, and human studies have demonstrated that neuronal cell death and synaptic dysfunctions in neurodegenerative diseases are associated with over-inflammatory response (Morales et al., 2016). During neuroinflammatory response, many microglial pro-inflammatory and neurotoxic mediators produce direct or indirect deleterious effects on neurons in various models of AD, PD, and ALS (Solito and Sastre, 2012, Rocha et al., 2015, Brites and Vaz, 2014). In general, these
neurodegenerative diseases are characterised by selective death of certain group of neurons in the CNS due to an increase in the expression and levels of mediators. For example, it has been suggested that NO produced by microglial inducible nitric oxide synthase (iNOS) during neuroinflammation is responsible for neuronal death because NO inhibits cytochrome oxidase in competition with molecular oxygen (O2) and release glutamate that cause excitotoxicity (Brown and Neher, 2010). TNFα as one of the potent pro-inflammatory mediators induce neuronal loss that is mediated by excessive microglial phagocytosis (Neniskyte et al., 2014). Neuronal loss induced by neuroinflammation is also mediated by up-regulation of the microglial factor cathepsin H (Fan et al., 2015). In addition, the total number of microglial cells increase in CNS diseases (Fu et al., 2014). Thus, neuroinflammation is identified as an essential target to prevent the pathogenesis of these disorders.

Neuroinflammation has been implicated in the pathogenesis of AD. This disease is an irreversible, progressive neurodegenerative disorder characterised by problems in memory, thinking, and behaviour (Cameron and Landreth, 2010, Perl, 2010). Neuroinflammation is known to play an essential role in Aβ- and tau-mediated neurodegeneration. In different experimental models, production of the AD marker, Aβ plaques as well as phosphorylation of tau tangles are increased due to over-activated microglia-mediated neuroinflammation (Bronzuoli et al., 2016, Metcalfe and Figueiredo-Pereira, 2010). The expression and levels of the pro-inflammatory mediators such as IL-1β, TNFα and IL-6, cyclooxygenase-2 (COX-2), and complement component q1 increased in many animal models of AD (Solito and Sastre, 2012, De Felice and Lourenco, 2015). In addition, neuroinflammation has been associated with tau-mediated neurodegeneration in a mouse model (Jaworski et al., 2011).

Secondly, PD is a neurodegenerative movement disorder characterised by the progressive loss of dopaminergic neurons in the substantia nigra and the deposition of the protein α-synuclein. Degeneration of nigrostriatal connections is responsible for motor, cognitive, and psychiatric symptoms (Taylor et al., 2013). It is noted that the number of microglial cells are increased in PD patients (Rocha et al., 2015). Based on biochemical analyses, levels of TNFα, IL-1β are increased in the midbrain of PD patients (Wang et al., 2015b). In addition, neurotoxicity
induced by α-synuclein is also mediated by neuroinflammatory response of microglia (Wang et al., 2015a).

ALS is another neurodegenerative disease that is characterised by progressive loss of motor neurons in the brain and the spinal cord. It results in progressive muscle atrophy, paralysis, and then death due to respiratory failure (Brites and Vaz, 2014, Komine and Yamanaka, 2015). Several findings have suggested that neuroinflammation is involved in the pathological features of ALS (Bowerman et al., 2013). The expression and levels of secreted microglial chemokines such as MCP-1 and IL-8 are high in the cerebrospinal fluids of patients with ALS (Kuhle et al., 2009). In addition, the expression of TNFα receptors as well as TNFα level have been significantly elevated in ALS patients (Poloni et al., 2000).

In addition, several findings have demonstrated that a number of biological processes are affected by excessive neuroinflammatory response. For example, the differentiation of neuronal progenitor cells to neurons (neurogenesis) in the adults is impaired due to excessive neuroinflammation (Fuster-Matanzo et al., 2013, Ekdahl et al., 2009). It has been suggested that basal hippocampal adult neurogenesis induced by tissue damage associated with LPS infusion is impaired due to neuroinflammation (Gomes-Leal, 2012).

Disruption of the BBB by hyper-activated microglia is also associated with excessive neuroinflammation (da Fonseca et al., 2014, Banks et al., 2015). Physiologically, the brain is protected by the BBB against systemic inflammation that induced by peripheral inflammatory attacks such as bacterial infection (Abbott et al., 2010, Lampron et al., 2013). Neuroinflammation has been proposed to play a critical role in the BBB breakdown due to the toxic effects of microglial pro-inflammatory and neurotoxic factors on the BBB that also results in phagocytes infiltration and subsequently more neuroinflammation in the brain (Abbott et al., 2010). Several ligands can be used experimentally to understand the association between neuroinflammation and dysfunction of the BBB (Qin et al., 2007, Ransohoff and Brown, 2012). For example, LPS as a potent bacterial product disrupt the BBB by microglia-induced neuroinflammation due to generation of ROS (Banks and Erickson, 2010). Therefore, neuroinflammation is
not only associated with the pathogenesis of neurodegenerative diseases, but also with the BBB degradation.

1.1.5. Roles of neuroinflammatory mediators in neuroinflammation
Recent findings have demonstrated that various types of microglial pro-inflammatory and oxidative factors are associated with the pathogenesis of many chronic neurodegenerative diseases (Brown and Neher, 2010). Microglial cells are the main source of neurodegenerative mediators in the brain. Microglia have been shown to be the most important target for the therapeutic agents that might be used for recovery of CNS diseases (Liu and Hong, 2003). It is important to understand the pathological and the molecular roles of neuroinflammatory and oxidative mediators in order to understand the pathogenesis of neurodegenerative diseases.

1.1.5.1. Nitric oxide
The bioactive free radical NO is classified as one of the most important reactive nitrogen species (RNS) that induce neuroinflammation (Ali et al., 2012). Several studies have suggested that NO is derived from three NOS isomers; iNOS, endothelial NO synthase, and neuronal NO synthase (Garry et al., 2015). NO is produced from various immune cells such as microglia against pathogens (Ghasemi and Fatemi, 2014). Figure 1.4 shows that microglial NO is formed by oxidation of L-arginine into L-citrulline by the enzyme iNOS, $O_2$, tetrahydrobipterin (BH4), and nicotinamide adenine dinucleotide phosphate hydrogen (NADPH). Under circumstances such as depletion of L-arginine and BH4; the anion superoxide ($O_2^{-}$), an initial product for the synthesis of ROS is also produced because of microglial iNOS coupling (Fürstermann and Sessa, 2011).
Figure 1.4 Synthesis of nitric oxide (NO) in microglia.

In uncoupled (normal) state, NO is formed due to the oxidation of L-arginine into L-citrulline in the presence of inducible nitric oxide synthase (iNOS), molecular oxygen (O$_2$) and a number of cofactors such as tetrahydrobiopterin (BH$_4$) and nicotinamide adenine dinucleotide phosphate hydrogen (NADPH). In coupled (dysfunctional) state, superoxide anions (O$_2^-$) are produced in the absence of L-arginine and BH$_4$ (Förstermann and Sessa, 2011, Yuste et al., 2015).

The fundamental effects of NO have been reported to be either neuroprotective and/or neurotoxic in the CNS; depending on its concentration. The physiological actions of NO are associated with the regulation of vasodilatation and neurotransmission in the CNS (Förstermann and Sessa, 2011, Ali et al., 2012). However, the pathological effects of NO are associated with the pathogenesis of neurodegenerative diseases due to the detrimental effects of NO at high level (Saha and Pahan, 2006, Ali et al., 2012). NO has direct and/or indirect toxic effects on neurons; depending on its levels. In the CNS, the formation of the highly reactive metabolite O$_2^-•$ is indicated as the first step in ROS generation. The secreted microglial NO reacts with O$_2^-•$ that is generated from other sources to form a highly RNS peroxynitrite (Zaki et al., 2005). It is well known that the toxic effects of peroxynitrite are lipid peroxidation, deoxyribonucleic acid (DNA) oxidation and protein nitration (Cobb and Cole, 2015). NO that is secreted from hyperactive microglia plays a key role in neuroinflammation and neuronal cell death (Brown and Neher, 2010).

It is documented that iNOS is a major important NOS isoform in the physiopathology of the CNS because iNOS is an important source of NO, ROS, and RNS in the brain. Unlike endothelial and neuronal isomers of NOS; iNOS is induced by many inflammatory ligands such as LPS and cytokines (Ghasemi and
Fatemi, 2014, Garry et al., 2015). Secondly, iNOS is an important source of both NO and \( \text{O}_2^- \) that interact with other free radicals to form RNA and ROS in the brain. Finally, amounts of NO and ROS produced by iNOS is 100-1000 times more than that produced by other NOS types (Garry et al., 2015).

Regarding the modulation of neuroinflammation, the protein iNOS is not constitutively expressed in microglial cells (Lei et al., 2014). It is induced by LPS alone or a combination of more than one stimulus. Many studies have demonstrated that several inflammatory ligands can produce large amounts of iNOS and NO in different cell culture models of microglia. In an in vivo study, the rates of both iNOS expression and NO production can be increased in LPS-activated microglia (Habashi et al., 2016). In addition, it is noted that iNOS-NO pathway is one of the most important mechanisms in hyperactive microglia-mediated neurotoxicity (Mander and Brown, 2005). However, the transcription of the enzyme iNOS is regulated by various transcription factors of the signalling pathways such as NF-κB (Do et al., 2010, Saha and Pahan, 2006).

1.1.5.2. Prostaglandin E2

Prostaglandins are lipid autacoids derived from arachidonic acid. The most important bioactive prostaglandins produced in vivo are PGE\(_2\), prostacyclin, prostaglandin D2, and prostaglandin F2 (Ricciotti and FitzGerald, 2011). PGE\(_2\) is one of the most abundant prostaglandin in almost all cell types of the CNS (Ricciotti and FitzGerald, 2011). A number of studies have demonstrated that PGE\(_2\) exert essential pathophysiological roles in the CNS through the activation of four distinct G-protein coupled receptors E-type prostanoid (EP), EP1-4 (Yagami et al., 2016). PGE\(_2\) is known to regulate both normal immune and neuroinflammatory responses in the CNS (Yagami et al., 2016). In addition, PGE\(_2\) is an active signal in the modulation of fever due to a bacterial infection (Kalinski, 2012). PGE\(_2\) production is dramatically increased in immune cells such as microglia in response to a wide variety of stimuli including LPS, and cytokines (Dai et al., 2011, De Oliveira et al., 2008). It has been reported that EP2 and EP3 are expressed in microglia (Bonfill-Teixidor et al., 2017). For example, up-regulation of several pro-inflammatory mediators and enzymes such as COX-2, iNOS, IL-6, and IL-1β induced by PGE2 are mediated by EP2 in LPS and IFN-γ-
activated rat microglia (Bonfill-Teixidor et al., 2017). Therefore, these receptors are associated with neuroinflammation.

Two types of cyclooxygenases have been identified in the body (Cudaback et al., 2014). Cyclooxygenase-1 (COX-1) is constitutively expressed in most cells because the end products of COX-1 are responsible for the modulation of normal physiological functions. In contrast, COX-2 is constitutively expressed in the cells of the brain including microglia and neurons (Minghetti, 2004, Kirkby et al., 2016). The end products of COX-2 are associated with the modulation of neuroinflammatory response. COX-2 is the common known source of the important prostaglandin, PGE$_2$ in cancer and inflammatory diseases (Giuliano and Warner, 2002, Yagami et al., 2016). Arachidonic acid is released from the plasma membrane phospholipids by the enzyme phospholipase A. Arachidonic acid is then catalysed by COX-2 into PGE$_2$ (Yagami et al., 2016).

It has been reported that the COX-2/PGE$_2$ cascade is involved in the etiology of various neurodegenerative diseases such as AD, and PD (Listi et al., 2010, Teismann, 2012). COX-2 overexpression has been detected in patients with AD due to over-activated microglia-mediated neuroinflammation (Hoozemans et al., 2002). In order to understand the role of microglial neurotoxic factors such as PGE$_2$ and signalling pathways in neuroinflammation-mediated neurodegenerative diseases, many inflammatory ligands such as LPS have extensively been used to induce neuroinflammation using models of cell cultures and animals. For example, LPS treatment increased PGE$_2$ level in primary microglia due to COX-2 expression induction (De Oliveira et al., 2008). Expression of COX-2 is regulated by signalling pathways including NF-κB and MAPK (Tsatsanis et al., 2006). It is reported that COX-2 expression and then PGE$_2$ production is regulated by NF-κB in LPS-stimulated microglia (Gong et al., 2008, Oh et al., 2010). Therefore, COX-2 is responsible for the production of microglial PGE$_2$ in LPS-stimulated microglia (Hoozemans et al., 2002, De Oliveira et al., 2008).

Non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin and ibuprofen are drugs that possess anti-inflammatory, antipyretic, and analgesic effects due to inhibition of COX-2/PGE$_2$ pathway (Brune and Patrignani, 2015). In addition,
several epidemiological studies have evaluated the activity of these drugs against neurodegenerative disorders such as AD (Moore et al., 2010). Thus, this cascade has been identified as one of the important cascades that regulate systemic inflammation and neuroinflammation. Many findings have shown that COX-2 inhibitors have beneficial activities such as anti-inflammatory and neuroprotective activities in the brain (Strauss, 2008). However, long-term use of NSAIDs are known to produce severe side effects such as stomach ulcers and bleeding due to non-competitive inhibition of COX isomers (Brune and Patrignani, 2015, Goldstein and Cryer, 2015). It is known that prostaglandins play protective role in maintaining gastric mucosal defence system. NSAIDs have been noted to produce stomach damage through inhibiting mucosal COX-1 activity.

1.1.5.3. Pro-inflammatory cytokines

In general, cytokines can be classified into two main groups; pro-inflammatory or anti-inflammatory cytokines. Cytokines are endogenous proteins that produce a wide range of functions including development of the CNS and immune response regulation (Deverman and Patterson, 2009). Under physiological condition or in the healthy CNS, gene encoding cytokines as well as cytokine receptors are constitutively expressed at low levels (Lucas et al., 2006). Cytokines play important roles in regulation of the pathophysiological environment of the CNS (Smith et al., 2012). A number of studies have showed that the actions of potent pro-inflammatory cytokines are either neuroprotective or neurodegenerative in the CNS, based on the intensity and duration of the neuroinflammatory response (Smith et al., 2012). The most common cytokines that play critical roles in both sustained neuroinflammation and the CNS disorders are TNFα, IL-6 and IL-1β (Wang et al., 2015b).

1.1.5.3.1. Tumor necrosis factor-α

Tumor necrosis factor-α (TNFα) is a potent pro-inflammatory cytokine and is the most important pro-inflammatory mediator of TNFα family in the CNS (McCoy and Tansey, 2008, Park and Bowers, 2010). It was first identified as a serum factor that can induce haemorrhagic necrosis of tumors (Van Hauwermeiren et al., 2011). The physiological and pathological actions of TNFα in the brain are not completely understood (Figiel, 2008, Clark et al., 2010). The main roles of TNFα have been associated with the normal immune response, neuroinflammation,
oxidative stress, and apoptosis (Fischer and Maier, 2015). Several findings have demonstrated that the major functions of TNF are mediated through the activation of two distinct receptors, TNF receptor type 1 and type 2 (TNFR 1/2) (Sedger and McDermott, 2014). TNFR1 is ubiquitously expressed on all cell types and is activated by the two forms of TNF, the membrane-bound and soluble one. TNFR2 is expressed in a more limited manner on some cells including endothelial cells, microglia, and neurons and is mainly activated by the membrane-bound form of TNF (Faustman and Davis, 2013). The main biological roles of TNF such as neuroinflammatory response and apoptosis are mediated by the activation of TNFR1 (Thommesen and Lægreid, 2005). In contrast to TNFR1, the biological responses of TNFR2-mediated TNF signalling is limited to include cell survival (Thommesen and Lægreid, 2005, Sedger and McDermott, 2014).

It is thought that TNFα-mediated signalling pathway is involved in the pathogenesis of several diseases including uncontrolled neuroinflammatory response-mediated neurodegenerative diseases (McCoy and Tansey, 2008). For example, TNFα level is found to be high in the neurodegenerative brains such as the brains of AD patients (Grammas and Ovase, 2001, Tarkowski et al., 2003). Based on the important role of microglial cytokines in the regulation of neuroinflammation, the pro-inflammatory ligand TNFα can be secreted from immune effectors such as microglia in response to numerous inflammatory stimuli (Lee et al., 2002, Welser-Alves and Milner, 2013). For instance, TNFα expression is increased in human microglia treated with LPS or Aβ (Lee et al., 2002). It is well known that TNFR1 is involved in hyper-activated microglia-produced motor neuronal death (Veroni et al., 2010). In addition, TNFα is identified as one of the most potent inducers that produce TNFα from microglia (Syed et al., 2007, Kuno et al., 2005). A study showed that this cytokine induced neurotoxicity through increasing the secretion of glutamate from microglia (Takeuchi et al., 2006).

However, it is necessary to regulate the neuroinflammatory response of microglia in order to prevent or treat TNFα-mediated CNS diseases such as neurodegenerative diseases (McCoy and Tansey, 2008, Chadwick et al., 2008). It could be mediated through inhibiting both over-activation of TNF signalling and decreasing the excessive production of microglial TNFα in the CNS. For example,
resveratrol inhibited the production of some pro-inflammatory mediators such as NO and TNFα in LPS-activated microglia (Bi et al., 2005).

1.1.5.3.2. Interleukin-6

The potent cytokine IL-6 is one of the most important members of the IL-6 family. The IL-6 family is a group of cytokines including IL-6, IL-11, ciliary neurotrophic factor, leukaemia inhibitory factor and others. In general, the actions of IL-6 are mediated through the activation of specific receptors (Scheller and Rose-John, 2006). Classic IL-6 signalling is mediated by the binding of IL-6 to the membrane bound IL-6 receptor. After that, this complex is associated with cellular membrane bound gp 130. This signalling is mainly regenerative and protective. IL-6 trans-signalling is another signalling that is mediated by binding of IL-6 to the soluble IL-6 receptor and then the complex is attached to gp 130. In contrast to classic IL-6 signalling, this signalling is involved in the regulation of a pro-inflammatory response (Schaper and Rose-John, 2015).

The IL-6 receptor is expressed in various brain cells including astrocytes, microglia, epithelial cells, and neurons (Ert et al., 2012, Aniszewska et al., 2015). Several studies have established that IL-6 receptors are expressed in different culture models of microglia (Sawada et al., 1993, Ert et al., 2012, Aniszewska et al., 2015). IL-6 is produced in high concentrations in response to potent stimuli such as pro-inflammatory ligands and brain injury. For example, IL-6 is one of the major important pro-inflammatory mediator that is secreted following LPS treatment of microglia (Minogue et al., 2012, Smith et al., 2012).

The pathophysiological actions of IL-6 in the CNS are neuroprotective, pro-inflammatory as well as neurotoxic, depending on its level. IL-6 is known to exert biological functions including the regulation of normal immune and neuroinflammatory responses at normal level (Scheller and Rose-John, 2006). However, many findings have suggested that IL-6 has a detrimental role in neurodegenerative diseases due to uncontrolled neuroinflammation. For example, the expression and level IL-6 are elevated in the cerebrospinal fluids of patients with AD and PD (Ert et al., 2012, Dursun et al., 2015). One of the neurotoxic actions of IL-6 is that it is involved in neuronal loss using developing cerebellar granule neurons (Conroy et al., 2004). In addition, chronic exposure of
neurons to IL-6 is noted to increase calcium influx in response to N-methyl-D-aspartate (NMDA) and cause neurodegenerative changes (Sallmann et al., 2000). Furthermore, the expression of TNFα, IL1β, and COX-2 as well as motor neuronal survival are reduced following IL-6 treatment in rat primary microglia (Krady et al., 2008).

1.1.5.3.3. Interleukin-1β

Interleukin-1β (IL-1β) is the most powerful membrane cytokine of the IL-1 family (Netea et al., 2010). IL-1β is known to play a central role in the regulation of normal immune and neuroinflammatory responses (Henry et al., 2009). In the presence of an brain infection or a brain injury, IL-1β is secreted as a host defence response (Van de Veerdonk et al., 2011). It is expressed in a variety of cell types in the CNS include microglia and neurons (Ferreira et al., 2010). Several evidences have shown that the biological actions of IL-1β are mediated by activation of two specific receptors; IL-1 receptor type 1 and type 2 (IL-1R1/2) (Weber et al., 2010).

Like other microglial cytokines, IL-1β signalling has been associated with the pathophysiological condition of cells in the CNS. High levels of both IL-1β coding gene and IL-1β significantly were detected in neurodegenerative diseases including AD and PD (Griffin et al., 2006, Dursun et al., 2015). Many experimental studies have indicated that this microglial cytokine plays a key role in the modulation of neuroinflammation and neurotoxicity because IL-1β treatment produces free radicals-induced neuronal death in rat mixed glial/neuronal co-culture (Thornton et al., 2006). For instance, neurotoxicity is mediated by IL-1β through increasing the synthesis of Aβ and Tau phosphorylation in AD (Shaftel et al., 2008). Furthermore, a recent study showed that IL-1β can induce neurotoxicity through activation of the apoptotic protein p53 (Rossi et al., 2014). It is known that the rate of production of the most important pro-inflammatory mediators can be dramatically increased by addition of the potent cytokine IL-1β in microglia. In various microglial cells cultures, the endotoxin LPS enhanced the expression of IL-1R1 and level of IL-1β (Pinteaux et al., 2002).
1.1.5.4. Reactive oxygen species

Oxidative stress is a pathological disturbance due to an imbalance between the rate of ROS production and rate of ROS metabolism (Schieber and Chandel, 2014). ROS are a class of wide variety of molecules that oxidise the main cellular macromolecules such as lipids, proteins, and DNA (Schieber and Chandel, 2014). The cellular biological functions of these target molecules are then negatively altered. The highly reactive molecules of ROS as well as RNS are listed in Table 4 (Rahman et al., 2012). Importantly, normal or low levels of ROS are secreted to regulate several biological processes such as cell signalling, metabolism, cell survival, migration, and proliferation. One of the physiological effects of ROS are associated with host defence against pathogens (Beckhauser et al., 2016). The major important ROS such as $O_2^\cdot -$ and $H_2O_2$ are synthesized by various enzymatic and non-enzymatic processes. The most important sources of ROS are NADPH oxidase, xanthine oxidase; cyclooxygenase, and iNOS. In addition, the mitochondrial electron transport chain is another important source of ROS in the mammalian CNS (Haslund-Vinding et al., 2017). It is well known that the substrate $O_2^\cdot -$ is an initial product for synthesis of ROS. It is formed due to the reduction of $O_2$ by the addition of one electron in the presence of ROS-generated enzymes or in mitochondria. It is well documented that several substances are known to act as either oxidative stressors or antioxidants in the body (Rahal et al., 2014).
Table 3 Examples of the main reactive oxygen species (ROS) and reactive nitrogen species (RNS)

<table>
<thead>
<tr>
<th></th>
<th>Reactive oxygen species (ROS)</th>
<th>Reactive nitrogen species (RNS)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Radicals</td>
<td>Non-radicals</td>
</tr>
<tr>
<td>1</td>
<td>Superoxide: O$_2^-$</td>
<td>Hydrogen peroxide: H$_2$O$_2$</td>
</tr>
<tr>
<td>2</td>
<td>Hydroxyl: OH$^-$</td>
<td>Hypochlorous acid: HOCl</td>
</tr>
<tr>
<td>3</td>
<td>Peroxyl: RO$_2^-$</td>
<td>Hypobromus acid: HOBr</td>
</tr>
<tr>
<td>4</td>
<td>Alkoxy: RO$^-$</td>
<td>Ozone: O$_3$</td>
</tr>
<tr>
<td>5</td>
<td>Hydroperoxyl: HO$_2^-$</td>
<td>Single oxygen: Δg</td>
</tr>
<tr>
<td></td>
<td>Radicals</td>
<td>Non-radicals</td>
</tr>
<tr>
<td>1</td>
<td>Nitric oxide: NO$^-$</td>
<td>Nitrogen dioxide: NO$_2$</td>
</tr>
<tr>
<td>2</td>
<td>Nitrous acid: HNO$_2$</td>
<td>Nitrosyl cation: NO</td>
</tr>
<tr>
<td>3</td>
<td>Nitrosyl anion: NO$^-$ · NO$^-$</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Dinitrogen tetroxide: N$_2$O$_4$</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Dinitrogen trioxide: N$_2$O$_3$</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Peroxynitrite: ONOO$^-$/</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Peroxinitrous acid: ONOOOH</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Alkylperoxynitriles: ROONO</td>
<td></td>
</tr>
</tbody>
</table>

However, in physiological conditions, a wide range of antioxidant enzymes are produced to detoxify and eliminate ROS (Valko et al., 2007). In inflammatory or oxidative conditions due to presence of ROS and RNS, healthy cells are not completely able to protect their cellular components such as proteins, lipids, and nucleic acids. This results in various molecular modifications that are responsible for disturbed biological functions as shown in Figure 1.5 (Lugrin et al., 2014).
Figure 1.5 Major molecular targets and biological consequences of oxidative stress.

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are toxic molecules that cause damage to the major cellular components such as proteins, lipids, and nucleic acids. This results in various molecular modifications that disturb biological functions (Lugrin et al., 2014, Schieber and Chandel, 2014, Haslund-Vinding et al., 2017).

Neuroinflammation-mediated oxidative stress has been linked to life-threatening diseases such as neurodegenerative disorders, cancer and aging. The pathological effects of ROS at high levels are associated with oxidative stress-induced cellular damage such as neuronal death (Kim et al., 2015). Direct or indirect chronic oxidative stress has been mainly implicated in neuronal loss and death because the biological functions of the neuronal components are damaged by ROS (Schieber and Chandel, 2014). ROS are markedly secreted at high rate in the pathological conditions such as neuroinflammation, brain injury and cancer (Olmez and Ozyurt, 2012). Based on the importance of both neuroinflammation and the role of microglial ROS in the neuroinflammatory and oxidative responses, several studies have suggested that excessive ROS levels are associated with the progression of neurodegenerative diseases (Hsieh and Yang, 2013). ROS
are identified as one of the major neurotoxic molecules in which elevated levels of ROS have been detected in the patients with AD and PD (Guidi et al., 2006, Kim et al., 2015). For instance, the elevated level of 4-hydroxy-2, 3-nonenal, which is an important breakdown product of lipid peroxidation has been observed in AD brains. In addition, DNA bases are hydroxylated due to ROS-mediated oxidative stress (Rahman et al., 2012). In addition, many inflammatory target proteins including matrix metalloproteinase-9 (MMP-9), cytosolic phospholipase A2 (cPLA2), COX-2, iNOS, intercellular adhesion (ICAM) are upregulated by various pro-inflammatory factors including cytokines, peptides infection products, peroxidants and others by a ROS signal-dependent manner in neuroglial cells such as microglia (Figure 1.6). This results in neuroinflammation and neuronal death (Hsieh and Yang, 2013).

Several findings have shown that microglia are one of the biological sources of ROS in the CNS that respond to pathogens. For example, H2O2 synergically increased LPS-induced increase in NO production in BV2 microglial cell (Eguchi et al., 2011). LPS increased the production rate of ROS that is followed by iNOS expression induction (Pawate et al., 2004). In addition, Aβ peptide-induced microglial over-activation is noted to be mediated by ROS (Kang et al., 2001). The biological roles of several signalling cascades including NF-κB, p38 MAPK, and PI3K/AKT that regulate many biological processes such as neuroinflammation are regulated, at least in part, by the second messengers ROS (Hensley et al., 2000, Ray et al., 2012). For example, the activity of NF-κB which is a sensitive transcriptional factor in the neuroinflammatory response and oxidative stress, in part, is regulated by ROS after addition of pro-inflammatory stimuli such as Aβ and LPS in microglial BV2 cell cultures (Kang et al., 2001, Park et al., 2015a).
Figure 1.6 Schematic representation of the redox signals due to ROS production and their role in the development of neuroinflammation and neuronal death.

Several inflammatory target proteins including matrix metalloproteinase-9 (MMP-9), cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), and intercellular adhesion (ICAM) can be upregulated by various pro-inflammatory factors. The most important factors include cytokines (e.g., interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor-α (TNFα), peptides (e.g., beta-amyloid (Aβ), infection (e.g., bacterial and virus), oxidants (e.g., hydrogen peroxide (H₂O₂) and others via a ROS signal-dependent manner in neuroglial cells (e.g., microglia). These inflammatory mediators can cause neuroinflammation and neuronal death (Hsieh and Yang, 2013, Vilhardt, 2005, Von Bernhardi et al., 2015, Polazzi and Monti, 2010).
However, one of the main purpose of extensive researches that have been carried out is to inhibit the neurotoxic effects of ROS in the body. For instance, the levels of pro-inflammatory and oxidative mediators such as NO and ROS can be reduced by synthetic and plant-derived natural compounds that suppress neuroinflammatory genes expression (Chung et al., 2010, Wu et al., 2012). Therefore, a normal balance between the cell signalling and ROS level in the CNS is an important factor for both cell death and cell survival.

It is well known that NADPH oxidase is an important source of ROS generation. It is involved in the regulation of immune response and host defence. NADPH oxidase is expressed in various CNS cell types such as neurons and microglia (Haslund-Vinding et al., 2017). The enzyme is highly expressed and extremely distributed in microglia (Chéret et al., 2008, Wilkinson and Landreth, 2006). Three isoforms of NADPH oxidase; NOX1, NOX2, and NOX4 have been expressed in microglia (Harrigan et al., 2008). However, this enzyme plays a critical role in the ROS-mediated neuroinflammatory and oxidative responses. For example, microglia-induced neurotoxicity is mediated by NADPH oxidase hyper-activation and ROS over-production (Qin et al., 2004). It is established that the enzyme NADPH oxidase is up-regulated in a variety of neurodegenerative disorders such as AD (Block, 2008, Gao et al., 2003). NADPH oxidase activity is regulated by signalling pathways (Chéret et al., 2008). NADPH-dependent ROS signalling is involved in LPS-induced increase in activation of signalling pathways and gene expression of pro-inflammatory factors such as TNFα and IL-6 in rat microglia (Pawate et al., 2004). In addition, several conventional and natural compounds have been reported to significantly attenuate LPS-induced increase in microglial NADPH oxidase activity (Maraldi, 2013, Huo et al., 2011). Overall, it is important to inhibit NADPH-dependent ROS signalling pathway in order to protect neurons (Block, 2008, Choi et al., 2012). In addition, it is necessary to maintain the cellular redox balance and to find effective substances that might be used to preventing the negative feedbacks of ROS on the survival of healthy mammalian cell (Poljsak et al., 2013).
1.1.6. Importance of IL-10 in neuroinflammation

Although reactive microglial cells are known to secret a wide spectrum of pro-inflammatory cytokines during the immune response, microglial IL-10 has been identified as the most important anti-inflammatory cytokine in the brain (Iyer and Cheng, 2012). It is produced to counteract cellular damage driven by an excessive neuroinflammatory response. The IL-10 receptor (IL-10R) is expressed by all CNS cell types such as neurons and microglia (Lobo-Silva et al., 2016). IL-10 has been associated with increased neuronal survival and the regulation of adult neurogenesis (Lobo-Silva et al., 2016). It is noted that IL-10 gene encoding and IL-10R is constitutively expressed in inactivated human and rat microglia (Ledeboer et al., 2002, Lee et al., 2002). Many experiments have been carried out to understand the molecular mechanism of the anti-neuroinflammatory action of IL-10 in the CNS. It is well established that IL-10 actions have been mostly mediated by IL-10 receptors (Lobo-Silva et al., 2016). Because of its cytokine nature, the gene encoding for IL-10 plays an important role in the regulation of host defence and neuroinflammatory processes (Kremlev and Palmer, 2005).

IL-10 has a role in the modulation of neuroinflammation, and this cytokine is known to be an active anti-inflammatory agent against neurotoxicity induced by potent toxic ligands such as LPS and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Qian et al., 2010). In a rat model of PD, 6-hydroxydopamine-induced neurotoxicity was inhibited by IL-10 (Johnston et al., 2008). In addition, polymorphism of the gene IL10 might be a risk factor for AD (Zhang et al., 2011). The anti-inflammatory and neuroprotective actions of IL-10 have been investigated in numerous experimental models (Qian et al., 2006b, Xin et al., 2011). For example, IL-10 inhibited neuroinflammation and neurotoxicity through inhibiting the expression of cytokine receptors and pro-inflammatory cytokines production in LPS-induced hyper-activated microglia (Zhu et al., 2015). IL-10 inhibited LPS-induced TNFα production in human microglia (Lee et al., 2002). In addition, IL-10 attenuated the secretion of TNFα, IL-1β, and ROS from primary microglia (Kremlev and Palmer, 2005, Qian et al., 2006a). IL-10 is endogenously expressed in microglia in order to inhibit LPS-induced neurodegeneration in the rat cerebral cortex (Park et al., 2007). IL-10 treatment inhibited NF-κB-mediated increase in IL-6 by LPS in microglia (Henry et al.,
IL-10 markedly reduced LPS-mediated neurotoxicity through inhibition of NADPH oxidase in primary midbrain cultures (Qian et al., 2006a). Inhibiting microglial hyper-activation and subsequent neurotoxicity by increasing the level of the target IL-10 in the brain is an effective strategy in preventing uncontrolled neuroinflammation (Asadullah et al., 2003).

1.1.7. Lipopolysaccharide as an activator of neuroinflammation

The bacterial product LPS is an inflammatory component that induces uncontrolled neuroinflammation through the activation of Toll-like receptors such as Toll-Like receptor 4 (TLR4). They are mammalian homologues of the Drosophila melanogaster Toll receptor protein (Buchanan et al., 2010). Toll-like receptors are the major important receptors among the signalling pattern recognition receptors that recognise microbial products (Lehnardt, 2010). TLR4 has been known to play a critical role in the regulation of normal immune and neuroinflammatory responses against bacterial infection and bacterial products such as LPS (Takeda and Akira, 2005). TLR4 is known to be highly expressed by the potent neuroinflammatory stimulus LPS as part of the pro-inflammatory response of the host (Yao et al., 2013). Microglial TLR4 is activated by LPS to induce signalling pathways that regulate the immunity in the CNS. Several lines of evidence have demonstrated that microglial TLR4 is responsible for recognising the endotoxin LPS in the brain in order to destroy and remove the bacterial product. This results in the protection of a wide variety of cells in the CNS (Molteni et al., 2016).

LPS is the major outer membrane component of the cell wall in Gram-negative bacteria (Buchanan et al., 2010). Several studies have suggested that the neuroinflammatory effects of LPS have been mediated through the activation of TLR4 signalling transduction, in which the soluble plasma protein LPS-binding protein is combined to the bacterial product LPS. Subsequently, the complex is associated with the soluble protein cluster of differentiation (CD14). LPS is then transferred to the receptor complex of TLR4 and myeloid differentiation 2 (MD-2) by CD14. Endocytosis of the TLR4/MD-2/LPS complex results in conformational changes of the receptor. The adaptor protein myeloid differentiation primary response gene 88 is then recruited to the receptor complex in the presence of TIR domain-containing adaptor protein. Then, interleukin-1 receptor-associated
kinase 1 and 4 and TNF-receptor-associated factor 6 (TRAF6) are recruited to the receptor complex. After that, transforming growth factor-β-activated kinase 1 (TAK1) is activated by the signal transducer TRAF6 (Glezer et al., 2007, Kaminska et al., 2016). The downstream IKK and MAPKs signalling pathways are activated by phosphorylated TAK1 that results in activation of NF-κB and activator protein-1, respectively. As shown in Figure 1.7, the LPS stimulatory action is mediated by the activation of multiple signalling pathways (Kacimi et al., 2011, Ostareck-Lederer et al., 2013). Several studies have shown that LPS is a potent inflammatory ligand for the activation of many signalling pathways such as NF-κB (Kacimi et al., 2011), MAPKs (Han et al., 2002, Xie et al., 2004), and Akt (Saponaro et al., 2012). These cascades are responsible for neuroinflammatory and neurotoxic product-mediated neuroinflammation.

Microglia are the major cell population in the CNS that respond to LPS via TLR4. Many cell-based studies have been performed to study the role TLR4 signalling pathway in neuroinflammation due to bacterial infection using different in vitro microglial cell lines such as BV2 microglia (Lehnardt, 2010). Several findings have suggested that several pro-inflammatory mediators are produced from immune cells such as microglia after application of LPS (Bachstetter et al., 2011).

It has also been suggested that activation of NADPH oxidase is regulated by TLR4 signalling (Haslund-Vinding et al., 2017). In LPS-stimulated microglia, NO-generated iNOS and O2•−-generated NADPH oxidase modulated peroxynitrite-mediated oligodendrocyte death (Li et al., 2005). Therefore, microglial TLR4 is identified as a major target for the in vitro specific binding of LPS and a good target for compounds that might be used to prevent hyper-active microglia-mediated neuroinflammation.
Figure 1.7 Proposed model for lipopolysaccharide (LPS)-induced microglial hyper-activation and neuronal death.

Toll-like receptors 4 (TLR4) are stimulated by LPS and this results in the activation of a variety of signalling molecules such as Akt, nuclear factor-kappa B (NF-κB), and p38 that regulate different signalling pathways in microglia. After that, several target genes such as inducible nitric oxide synthase (iNOS), cyclooxygenase (COX-2), and genes encoding for tumour necrosis factor-α (TNFα), interleukin-1β (IL-1β), and interleukin-6 (IL-6) are expressed in order to produce a variety of pro-inflammatory and oxidative mediators such as nitric oxide (NO), prostaglandin E₂ (PGE₂), TNFα, IL-1β, L-6, and reactive oxygen species (ROS). The healthy neuron is then damaged due to long-term of pro-inflammatory and oxidative mediators-mediated neuroinflammation and oxidative stress. In addition, the hyper-activated microglial cell and healthy neurons that are located close to the damaged neurons are also affected by neuronal death (Kaminska et al., 2016, Ostareck-Lederer et al., 2013, Kacimi et al., 2011).
1.1.8. Diosgenin
Several attempts have been made to discover novel oestrogen-like compounds, which specifically inhibit neuroinflammation, in order to avoid oestrogen deficiency-mediated menopausal symptoms and oestrogen-mediated breast cancer. Therefore, it is necessary to find natural or semi-synthetic oestrogens, for example phytoestrogens that lack the adverse effects of endogenous 17β-oestradiol for the treatment or prevention neuroinflammation-mediated neurodegeneration (Prokai and Simpkins, 2007, Cvoro et al., 2008).

1.1.8.1. Sources and chemistry of diosgenin
It is well known that steroidal saponins and their aglycones (steroidal sapogenins) as raw materials have economic and therapeutic values. They play some important roles in both the manufacture of various synthetic steroid hormonal drugs and the treatment of some diseases. All steroidal sapogenins possess a parent cholestane carbon skeleton (C27), having a perhydrocyclopentenophenanthrene (sterane) nucleus (rings A, B, C, and D), and the side chain of which undergoes cyclisation resulting in either a hexacyclic system (four carbocyclic and two heterocyclic rings) or a pentacyclic system (four carbocyclic and one heterocyclic ring). In a saponin containing a hexacyclic aglycone, such as diosgenin or tigogenin, the 3-OH group is usually decorated with an oligosaccharide chain. Spirostan-type saponins are the most common steroidal saponins in plants. Diosgenin is one of the most important sapogenins in the plant kingdom. Diosgenin [(25R)-5-spirosten-3β-ol] is a spirostanol saponin with a molecular weight of 414.627. The plant diosgenin has a ring structure similar to the chemical backbone of steroids such as 17β-oestradiol (Figure 1.8). The molecular formula of diosgenin is C27H42O3 and that of 17β-oestradiol is C18H24O2. In addition, the chemical structure of diosgenin is similar to that of other steroids including cholesterol, and dehydroepiandrosterone-the precursor to testosterone (Deshpande and Bhalsing, 2014, Masood-ur-Rahman and Ara, 2017, Basu and Sriramnroen, 2010, Hanson, 2010, Powell and D'Arcy, 2013, Hanson, 2005, Munafo Jr and Gianfagna, 2015).
Figure 1.8 Molecular structures of diosgenin and 17β-oestradiol (Raju and Rao, 2012, Patisaul and Jefferson, 2010).

Diosgenin is the major active ingredient found in plants such as *Trigonella foenum graecum* (Fenugreek) and many species of *Dioscorea* (e.g., Yam), and *Costus speciosus* (Koen) (Dangi et al., 2014, Yi et al., 2014) (Table 3). The root tubers of wild yam (*Dioscorea villosa*) and the seeds of fenugreek have been used as a major dietary source for the industrial production of diosgenin (Deshpande and Bhalsing, 2014, Jesus et al., 2016).
Table 4 List of medicinal plants containing diosgenin

<table>
<thead>
<tr>
<th>Genus</th>
<th>Species</th>
<th>Common name</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dioscorea</td>
<td><em>Dioscorea villosa</em></td>
<td>Wild Yam</td>
</tr>
<tr>
<td></td>
<td><em>Dioscorea alata</em></td>
<td>Greater Yam</td>
</tr>
<tr>
<td></td>
<td><em>Dioscorea nipponoca</em></td>
<td>Dioscorea nipponoca</td>
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<tr>
<td></td>
<td><em>Dioscorea colletti</em></td>
<td>Dioscorea Hypoglaucua</td>
</tr>
<tr>
<td>Trigonella</td>
<td><em>Trigonella foenum graecum</em></td>
<td>Fenugreek</td>
</tr>
<tr>
<td>Costus</td>
<td><em>Costus speciosus</em></td>
<td>Crape Ginger</td>
</tr>
<tr>
<td>Smilax</td>
<td><em>Smilax china</em></td>
<td>Smilax china</td>
</tr>
<tr>
<td></td>
<td><em>Smilax glabra</em></td>
<td>Smilax glabra</td>
</tr>
<tr>
<td>Asparagus</td>
<td><em>Asparagus officinalis</em></td>
<td>Wild Asparagus</td>
</tr>
<tr>
<td>Solanum</td>
<td><em>Solanum inanum</em></td>
<td>Grey bitter-apple</td>
</tr>
<tr>
<td></td>
<td><em>Solanum xanthocarpum</em></td>
<td>Yellow Berried Night Shade</td>
</tr>
</tbody>
</table>

1.1.8.2. Pharmacology of diosgenin

Diosgenin-containing medicinal plants have been used in folk medicine throughout the world. In recent years, several studies have been performed to study the activity of diosgenin as an isolated compound or as part of plant extracts (Patel et al., 2012, Venkata et al., 2017). Several findings have suggested that fenugreek, containing diosgenin, possess anti-inflammatory and antioxidant activities in various experiments (Sharififar et al., 2009, Gupta et al., 2010b, Uemura et al., 2010, Tripathi and Chandra, 2010, Tejaswini et al., 2012, Mandegary et al., 2012, Suresh et al., 2012, Belguith-Hadriche et al., 2013, Kumar and Bhandari, 2013, Khole et al., 2014, Abedinzade et al., 2015). Yam, containing diosgenin, produced anti-inflammatory activity in ovalbumin-induced food allergy in mice (Mollica et al., 2013). It was reported that diosgenin as an isolated compound produced numerous biological effects in a variety of cellular, animal and human models such as antioxidant (Son et al., 2007), anti-inflammatory (Ku and Lin, 2013, Tewtrakul and Itharat, 2007), antidiabetic (McAnuff et al., 2005), and antihyperlipidaemic (Gong et al., 2010).
1.1.8.2.1. Anti-inflammatory effects of diosgenin
Diosgenin has been evaluated for treating various types of metabolic and inflammatory diseases (Raju and Rao, 2012). For example, diosgenin from *Dioscorea villosa* extract was shown to produce an anti-inflammatory effect in a rodent model (Lima et al., 2013). Diosgenin from *Dioscorea membranacea* extract inhibited the production of NO in LPS-stimulated RAW 264.7 cells (Tewtrakul and Itharat, 2007). In addition, a number of studies have suggested that diosgenin, as an isolated active ingredient has anti-inflammatory and antioxidant activity in various *in vitro* inflammatory models. Further, diosgenin attenuated subacute intestinal inflammation in rats. In a murine model of food allergy, diosgenin inhibited allergen-induced intestinal inflammation immunoglobulin E secretion (Huang et al., 2010). It was shown that diosgenin inhibited palmitate-induced an increase in the secretion of pro-inflammation mediators such as TNFα and IL-6 in endothelial cells through inhibiting inhibitory kappa B kinase (IKK) and Nuclear factor-kappa beta (NF-κB) phosphorylation (Liu et al., 2012). In addition, diosgenin has been reported to exert anti-inflammatory activity against LPS-induced inflammation in mouse primary peritoneal macrophages (Singh et al., 2014), and in mouse lung injury (Gao et al., 2013). Furthermore, diosgenin inhibited the secretion of inflammatory mediators in co-culture model of 3T3-L1 adipocytes and RAW 264 macrophages (Hirai et al., 2010). Another study has shown that this compound inhibited LPS/interferon gamma (IFN-γ)-stimulated production of pro-inflammatory factors in Raw264.7 murine macrophages (Jung et al., 2010a).

1.1.8.2.2. Antioxidant effects of diosgenin
Some studies have indicated that diosgenin possess antioxidant activity in other organs. For example, diosgenin increased the levels of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX) as well as the antioxidant protein GSH in the liver, plasma and erythrocytes in high-cholesterol fed rats (Son et al., 2007). Furthermore, pre-treatment with diosgenin showed antioxidant effect on myocardial reperfusion injury in rat heart by increasing the activities of SOD and GPX (Badalzadeh et al., 2015). Diosgenin also attenuated the hydrogen peroxide (H₂O₂)-induced increase in ROS level and oxidative stress in human vein endothelial cells and in H92C cells.
(Gong et al., 2010, Jamshidi et al., 2016). It increased the activity of SOD, CAT, and GPX and thereby, reduced oxidative stress in adenine-induced chronic renal failure rats (Manivannan et al., 2015). Therefore, these findings suggest that diosgenin or herbal crude extracts containing diosgenin might be used as anti-inflammatory and anti-oxidant medication in traditional medicine.

1.1.8.2.3. Neuroprotective activity of diosgenin

Diosgenin has been experimentally identified as one of the natural products that produce neuroprotection (Venkatesan et al., 2015). In addition, the neuroprotective effect of diosgenin have been investigated in numerous experiments. For example, oral administration of diosgenin improved memory and inhibited galactose-induced oxidative stress in mice through increasing the activity of some antioxidant enzymes such as SOD and GPX (Chiu et al., 2011). A study showed that diosgenin improved memory and reduced axonal degeneration in an AD mouse model (Tohda et al., 2012). It has also been demonstrated that diosgenin treatment resulted in an improvement in cognitive functions in normal rats through the activation of steroid-binding receptors (Tohda et al., 2013). In a rodent model, diosgenin induced neuronal regeneration by increasing the level of nerve growth factor (NGF) and thus attenuated diabetic neuropathy that is characterised by axonal degeneration, demyelination, and atrophy (Kang et al., 2011). In addition, the compound stimulated the NGF production against Aβ-induced neuronal damage in mice (Koh et al., 2016). A diosgenin-rich yam extract was reported to enhance cognitive function in healthy humans (Tohda et al., 2017). It has also been reported that diosgenin attenuated mitochondrial dysfunction in the presence of dopamine in isolated rat synaptosomes (Kondeva-Burdina et al., 2007). Another study has showed that diosgenin protected neurons in vitro against Tat (a novel regulator of HIV transcription) plus morphine-induced neurotoxicity (Turchan-Cholewo et al., 2006). In addition, diosgenin prevented spinal cord injury-induced secondary injury by inhibiting the inflammatory response, repressing apoptosis, and promoting autophagy in perilesional tissues of rats (Zhang et al., 2017). In this study, diosgenin reduced the levels of pro-inflammatory levels of cytokines including TNFα, IL-1β, and IL-6 in spinal cord tissues. Diosgenin was also shown to attenuate the brain injury induced by transient focal cerebral ischemia-
reperfusion through reducing the levels of cytokines in blood serum of the ischemia-reperfusion treated rats (Zhang et al., 2016).

It inhibited neuronal damage through attenuating neuroinflammation and oxidative stress, and suppressed pentylenetetrazole-induced oxidative damage in brain tissues by increasing SOD, glutathione (GSH), and CAT levels in mice (Tambe et al., 2015). Diosgenin produced a neuroprotective activity in vitro against oxygen-glucose deprivation using primary cortical neuron culture (Chang et al., 2013). Some studies have showed that diosgenin might be used for the treatment of demyelinating diseases such as multiple sclerosis. For example, diosgenin produced a neuroprotective action in an attenuated experimental model of autoimmune encephalomyelitis in mice through activation of microglia (Liu et al., 2017). Further, diosgenin induced differentiation of oligodendrocyte progenitor cells without affecting the viability, proliferation, or migration of these cells in a purified rat culture model (Xiao et al., 2012). The effect was exerted through oestrogen receptors (ER)-mediated extracellular signal-regulated kinase 1/2 activation that accelerate remyelination. In addition, diosgenin glucoside produced neuroprotective effect through modulation of microglial polarization (Wang et al., 2017).

1.1.8.3. Effects of diosgenin on the signalling pathways

Finally, a number of studies have noted that diosgenin mediated its anti-inflammatory through down-regulation of signalling pathways such as NF-κB, mitogen-activated protein kinase (MAPK), and PI3K/AKT in different models (Jung et al., 2010a). For example, diosgenin attenuated NF-κB and MAPK pathways in LPS-induced lung injury in mice (Gao et al., 2013). Diosgenin reduced brain injury induced by transient focal cerebral ischemia-reperfusion in rats through inhibition of NF-κB (Zhang et al., 2016). Diosgenin down-regulated NF-κB and c-Jun N-terminal kinases in macrophage (Jung et al., 2010a).

Furthermore, it has been hypothesised that diosgenin produces oestrogen-like effects in humans because of its structural similarity to 17β-oestradiol. In addition, a number of studies have shown that diosgenin possess oestrogenic activity (Alcantara et al., 2011, Wu et al., 2015). Diosgenin reduced adipocyte differentiation through ERβ-induced Peroxisome proliferator-activated receptor γ
expression (Wang et al., 2015c). In addition, diosgenin increased the nuclear expression of ERβ (Wang et al., 2015c). Diosgenin induced hypoxia-inducible factor-1 activation and angiogenesis through ER-related Akt and p38 MAPK pathways in osteoblasts (Yen et al., 2005). Furthermore, diosgenin induced differentiation of oligodendrocyte progenitor cells in purified rat culture model through ER (Xiao et al., 2012).

1.2. Gap in knowledge
To date, the anti-neuroinflammatory activity of diosgenin against hyper-activated microglia-mediated neuroinflammation has not yet been studied. In addition, the molecular mechanisms involved in the neuroprotective effect of diosgenin have not been elucidated.

1.3. Aim and Objectives
This research was aimed at investigating whether diosgenin modulates neuroinflammation in LPS-activated BV2 cells. Therefore, this study sought to address the following specific objectives:

i. To determine the anti-neuroinflammatory effect of diosgenin in LPS-stimulated microglial BV2 cells.
ii. To identify the molecular mechanism underlying the anti-neuroinflammatory effect of diosgenin in LPS-activated BV2 cells
iii. To investigate whether antioxidant protective mechanisms are associated with the anti-neuroinflammatory effect of diosgenin.
iv. To determine the neuroprotective effect of diosgenin against neuroinflammation-induced HT22 neuronal death.

1.4. Thesis Structure
It is a good idea to summarise the next chapters as the following:

Chapter 2: This chapter will provide the significant results that demonstrates the anti-neuroinflammatory effect of diosgenin in LPS-stimulated microglial BV2 cells as well as the mechanism of the anti-neuroinflammatory activity of this compound against NF-κB in LPS-activated BV2 cells.

Chapter 3: The goal of this chapter is to provide the data that suggests the effect of diosgenin on the antioxidant defence system (Nrf2/ARE mechanism) in
unstimulated BV2 microglia. In addition, it is aimed to show the results that investigate the important role of Nrf2 signalling pathway in the modulation of the anti-neuroinflammatory effect of diosgenin in LPS-activated BV2 cells.

Chapter 4: The chapter 4 of the thesis describes the results that support the important role of diosgenin’s anti-neuroinflammatory in neuroinflammation-mediated neurodegeneration through activation of some important signalling cascades including AMPK and ER in BV2 cells.

Chapter 5: This chapter will provide a general summary about the results of the previous chapters.
Chapter 2
Diosgenin inhibited NF-κB-mediated neuroinflammation in LPS-activated BV2 microglia
2.1. Introduction

2.1.1. Microglial NF-κB signalling pathway

Nuclear factor-kappa beta (NF-κB), as a nuclear transcription factor, is a primary regulator of immune and/or inflammatory responses in the CNS (Shih et al., 2015). It was first identified as a nuclear factor κ-light-chain-enhancer of activated B cells. As seen in Figure 2.1, the NF-κB p65/p50 heterodimer is one of the five members of mammalian NF-κB transcription factor family (Christian et al., 2016, Schmukle and Walczak, 2012). The most widely studied form of NF-κB heterodimers are the p50 and p65 subunits because these subunits have been known as potent inducers of pro-inflammatory gene transcription during the immune response. All of these family members have a Rel-homology domain that is essential for DNA binding and dimerisation. The members of the NF-κB family are regulated by a wide variety of the inhibitory kappa B (IκB) and the IKK families. Several studies have demonstrated that NF-κB plays some key roles in the regulation of numerous biological processes including cell survival, apoptosis, and neuroinflammation (Mincheva-Tasheva and Soler, 2013, Ledoux and Perkins, 2014, Serasanambati and Chilakapati, 2016). Regarding neuroinflammation, microglial NF-κB is involved in the regulation of the transcription of a large number of target genes against a wide variety of inflammatory stimuli and insults such as brain infection and brain injury (Serasanambati and Chilakapati, 2016).
Figure 2.1 Members of the nuclear factor-kappa B (NF-κB), Inhibitory kappa B (IκB), and Inhibitory kappa kinase (IKK) families.

(A) The five members of the NF-κB family are RelA (p65), RelB, c-Rel, p105 (p50), and p100 (p52). (B) The IκB family of proteins consists of four members: IκBα, IκBβ, IκBε and BCL-3. (C) The three members of IKK complex: NF-κB Essential Modulator (NEMO or IKKγ), IκB kinase α, (IKKα or IKK1) and IκB kinase β (IKKβ or IKK2) (Schmukle and Walczak, 2012, Hoesel and Schmid, 2013).

Findings from several studies have shown that microglial NF-κB signalling pathway is associated with normal inflammatory response and neuroinflammation (Hoesel and Schmid, 2013). Normal activation of this cascade results in the synthesis and then production of pro-inflammatory mediators such as cytokines and ROS against the attack in the CNS. NF-κB signalling can be activated, for example, by LPS, TNFα, and IL-1β, through the activation of TLR4, TNFα receptor and IL-1β receptor, respectively in spite of the structural differences among these receptors (Shabab et al., 2017, Kuno et al., 2005).
most potent inducers that activate microglial NF-κB p50/p65 signalling pathway are LPS, Aβ, and TNFα (Kuno et al., 2005, Capiralla et al., 2012). This activation results in neuroinflammatory response against LPS or other abnormal products. NF-κB is present as an inactive in the cytoplasm because it is attached to the inhibitory subunit IkB-α (Figure 2.2).

Upon activation of microglial TLR4 by LPS, the IKK complex is phosphorylated and the IkB-α subunit is then phosphorylated by the phosphorylated IKK. After that, the phosphorylated IkB-α is polyubiquitinated and rapidly degraded by the proteasome enzyme. The free active NF-κB dimers are rapidly translocated into the nucleus, and then attached to specific sequences of DNA called κB sites that are located in the promoters of a large number of pro-inflammatory genes such as iNOS, COX-2, and genes encoding for TNFα, IL-1β, and IL-6 (Hayden and Ghosh, 2012). It is noted that NF-κB/DNA complex is responsible for the regulation of transcription of several target genes that have protective and/or neurotoxic effects on neurons (Vega and de Andres Martin, 2008).
Figure 2.2 Proposed model for lipopolysaccharide (LPS)-induced nuclear factor-kappa B (NF-κB) in microglial cell.

NF-κB is present as an inactive complex because it is attached to the subunit Inhibitory kappa B (IκB-α) in the cytoplasm. Upon activation of Toll-like receptor 4 (TLR4) with LPS, the Inhibitor kappa B kinase (IKK) complex is phosphorylated and the IκB-α subunit is then phosphorylated by the phosphorylated IKK. After that, the free active NF-κB is translocated into the nucleus, and attached to specific sequences of deoxyribonucleic acid (DNA) that are located in the promoters of pro-inflammatory and oxidative genes. The expression of target genes such as inducible nitric oxide synthase (iNOS), cyclooxygenase (COX-2), and genes encoding for tumour necrosis factor-α (TNFα), interleukin-1β (IL-1β), and interleukin-6 (IL-6) results in the production of a variety of pro-inflammatory and oxidative mediators such as nitric oxide (NO), prostaglandin E2 (PGE₂), TNFα, IL-1β, IL-6, and reactive oxygen species (ROS). This results in neuroinflammation- and oxidative stress-mediated neurodegenerative diseases (Vega and de Andres Martin, 2008, Spencer et al., 2012, O’Neill and Kaltschmidt, 1997).
2.1.2. Role of NF-κB signalling in neuroinflammation

Several studies have shown that chronic activation or dysregulation of the microglial NF-κB signalling pathway is implicated in a wide variety of chronic diseases such as cancer, diabetes mellitus, and neurodegenerative diseases (Tornatore et al., 2012, Hoesel and Schmid, 2013). In the CNS, NF-κB plays a potential role in the regulation of several genes such as COX-2, iNOS and those encoding TNFα, IL-1β, and IL-6 that regulate the secretion of pro-inflammatory and neurotoxic mediators including NO, cytokines, and ROS (Hoesel and Schmid, 2013). Findings from several studies have shown that the NF-κB signalling pathway is associated with the excessive neuroinflammatory and oxidative responses (Shih et al., 2015). This critical hypothesis has been confirmed because down-regulation of NF-κB-mediated transcriptional activity results in the suppression of NF-κB signalling-mediated neuroinflammation (Wan and Lenardo, 2010).

Moreover, NF-κB has been involved in the pathogenesis of AD (Zhang and Jiang, 2015). Elevated levels of β-site APP cleaving enzyme 1 (BACE1) were found in the brains of AD-suffering patients; increased BACE1 expression is responsible for the production of the toxic protein Aβ mediated by NF-κB activation (Chen et al., 2012). In addition, increasing NF-κB activation results in neuronal apoptosis in the hippocampus of Tg2576 transgenic mice model of AD (Niu et al., 2010). Furthermore, the activation of NF-κB has been noted in substantia nigra of AD patients and in animal models of PD. Zhang and co-workers showed that inactivation of the NF-κB cascade by inhibiting microglial IKK activity resulted in the protection of dopaminergic neurons against LPS-induced neurotoxicity (Zhang et al., 2010). It is noted that NF-κB activation is induced in the substantia nigra of PD patients and MPTP-treated mice. Deactivation of this microglial cascade by inhibiting IKK protected the dopaminergic neurons from MPTP-induced nigral degredation in a mouse model (Ghosh et al., 2007). In addition, NF-κB activity increased by MPTP in the substantia nigra of hemi-parkinsonian monkeys. Inhibition of NF-κB activation, and subsequently iNOS expression resulted in neuronal protection and locomotor activity improvement (Mondal et al., 2012). NF-κB activation has been associated with the pathology of ALS. For example, microglia induced motor neuron death due to NF-κB activation in
mutant SOD mice (Frakes et al., 2014). Thus, regulating microglial NF-κB activity is necessary to modulate uncontrolled neuroinflammation involved in neurodegenerative diseases such as AD and PD.

2.2. Specific Aim and Objectives
This research was aimed at investigating whether diosgenin modulates neuroinflammation in LPS-activated BV2 cells. Therefore, this study sought to address the following specific objectives:

I. To determine the anti-neuroinflammatory effect of diosgenin in LPS-stimulated microglial BV2 cells.
II. To identify the molecular mechanism underlying the anti-neuroinflammatory effect of diosgenin in LPS-activated BV2 cells

2.3. Methods
2.3.1. BV2 cell culture
BV2 microglia are a transformed cell line that are used instead of human or animal primary microglia for studying microglia-mediated neuroinflammation (Henn et al., 2009, Stansley et al., 2012). BV2 cells were generated by infecting mouse primary microglia with a v-raf/v-myc oncogene-carrying retrovirus (Blasi et al., 1990) BV2 mouse microglia cell line ICLC ATL03001 was purchased from Interlab Cell Line Collection (Banca Biologica e Cell Factory, Italy). BV2 cells used in this study were routinely cultured in T75 cm² cell culture flask containing Roswell Park Memorial Institute 1640 (RPMI 1640) medium (Life Technologies), supplemented with 10% heat-inactivated fetal bovine serum (FBS; Sigma), 2 mM L-glutamine (Sigma), 1 mM sodium pyruvate (Sigma), 100 U/mL of penicillin (Sigma), and 100 μg/mL of streptomycin (Sigma). The flask containing BV2 cells was incubated in a humidified atmosphere of 5% CO₂ and 95% O₂ at 37°C.

The culture medium was replaced by fresh complete RPMI 1640 medium approximately every 48 hours. The cells were sub-cultured when they reached 70-80% confluence. In general, BV2 cells were sub-cultured twice a week. Once confluent, the medium was poured into a sterile tube, and the flask was washed with 5 mL of Dulbecco’s phosphate-buffered saline (PBS; Life Technologies). Next, 0.05% trypsin-ethylenediaminetetraacetic (EDTA) (Sigma) was added to the flask in order to dissociate and disaggregate the cells, and incubated at 37°C.
for 2 minutes. 8 mL of RPMI 1640 medium was added to inactivate trypsin, and then poured into the tube that contained the spent medium. After centrifugation at 1200 g for 5 minutes, the supernatant was removed, and the pellet was resuspended in fresh RPMI 1640 medium. Next, the cells were again cultured into a new flask containing RPMI 1640 medium, and incubated under the same conditions for further subculture. Cells were counted by using a haemocytometer and diluted to the desired density in order to seed out the cells in a plate.

2.3.2. HEK293 cell culture
HEK293 cells are experimentally transformed cells derived from human embryonic kidney cells. It is an excellent cell line for transfection experiments because of their reliable growth and propensity for transfection (Thomas and Smart, 2005). HEK293 cells were obtained from HPA Cultures (Salisbury, UK). HEK293 cells were cultured in Minimum Essential Media (MEM)-Eagle’s medium (Life technologies), supplemented with 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 40 U/mL penicillin, and 40 μg/mL streptomycin. The flask was incubated in a humidified atmosphere of 5% CO₂ and 95% O₂ at 37°C. The medium was replaced by complete MEM-Eagles medium approximately every 48 hours. The cells were typically sub-cultured when they reached 70-80% confluence. In general, cells were passaged twice a week. Once confluent, cells were split to a ratio of 1:10 using trypsin/EDTA.

2.3.3. HT22 cell culture
The HT22 neuronal cell line was derived from HT4 cells that were originally immortalised from a primary mouse hippocampal neuronal culture. HT22 cells were a kind gift from Dr Jeff Davis. They were routinely maintained and grown in T75 cm² cell culture flasks containing Dulbecco’s modified Eagles medium (DMEM; Life Technologies), supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U/mL of penicillin, and 100 μg/mL of streptomycin. The flask was incubated in a humidified atmosphere of 5% CO₂ and 95% O₂ at 37°C. The culture medium was replaced by complete DMEM approximately every 48 hours using sterile tips. The cells were sub-cultured when they reached 75-80% confluence. Once confluent, the cells were washed with 5 mL of PBS. After removing PBS, 0.05% trypsin-EDTA was added to the flask, and incubated at 37°C for 2 minutes. 8 mL of complete DMEM was added to the
flask to inactivate trypsin, and then poured off into the tube containing the spent medium. The cell suspension was centrifuged at 1200 rpm for 5 minutes. The supernatant was aspirated, and the pellet was re-suspended in complete DMEM. HT22 cells concentration was counted using a haemocytometer. HT22 cells suspension was diluted with complete DMEM to get the required density of cells that to be seeded out in plates.

2.3.4. Treatment of cell culture
BV2 cells were seeded out at the required density in a cell culture plate, and then incubated for approximately 48 hours. Subsequently, the cultured medium was replaced with serum- and phenol red-free RPMI 1640 medium. After 2 hours’ incubation, BV2 cells were treated as follows; BV2 cells were left untreated in RPMI 1640 medium as a negative-control. Cells were stimulated with 100 ng/mL of LPS as a positive-control. BV2 cells were treated with 5, 10, and 20 µM of diosgenin for 30 minutes, followed by addition of 100 ng/mL of LPS. After that, the plate was incubated for the indicated time period, according to each experiment, as shown in the text. Diosgenin (≥ 93 %) (Sigma) was dissolved in dimethyl sulfoxide (DMSO; Sigma) to prepare a stock solution of 10 mM and then stored as small aliquots at -80 °C for short term future use. LPS (Sigma) was derived from Salmonella enterica serotype typhimurium SL1181. Aβ (human fragment 1-42) (Tocris Bioscience) at 2 µM was used as a pro-inflammatory ligand instead LPS in order to test whether diosgenin has anti-neuroinflammatory activity against Aβ-activated BV2 cells. Cells were treated as shown above and the positive-control was Aβ-treated BV2 microglia. The cells were counted using a haemocytometer and diluted to the desired density in order to seed out the cells in a plate.

HEK293 cells were used to study the effect of diosgenin on NF-κB-mediated transcriptional activity. 1 ng/mL of TNFα was used as an inflammatory ligand instead of LPS to test whether diosgenin produce anti-neuroinflammatory effect against TNFα-induced transcriptional activity in HEK293 cells. Therefore, the positive-control was TNFα-treated BV2 cells.
2.3.5. Cell viability assay

The 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) assay is a colorimetric assay that is widely used to evaluate cell viability in cell culture after drug or stimuli treatment. In this assay, the yellow compound MTT is reduced to a purple coloured formazan dye by the enzyme mitochondrial dehydrogenase in viable cells (Figure 2.3) (Aula et al., 2015).

The MTT assay was used to evaluate whether diosgenin causes any cytotoxic effect on BV2 microglial cells at concentrations used in subsequent studies. BV2 cells were seeded out at a concentration of $2.0 \times 10^5$ cells/mL (200 µL/well) in a 96-well plate and incubated for 48 hours. The medium was then replaced with serum-free RPMI 1640 medium for 2 hours. The cells were treated with 5, 10, and 20 µM of diosgenin for 30 minutes, and subsequently stimulated with 100 ng/mL of LPS. After incubation for 24 hours, the medium was carefully removed and the cells were incubated with 200 µL of MTT (0.5 mg/mL (Sigma) in PBS) at 37°C. After 4 hours, the medium was carefully removed and the formed insoluble purple formazan crystals were solubilized with 150 µL of DMSO. The plate was incubated at room temperature for ten minutes with shaking to increase the solubility of crystals. After that, the absorbance was measured at 540 nm using a microplate reader (Tecan Infinite F50) (Mosmann, 1983). The relative cell viability was expressed as a percentage relative to the untreated control cells.
Figure 2.3 Principle of MTT assay

The compound 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) is intracellularly reduced to a purple coloured formazan dye by the enzyme mitochondrial dehydrogenase in living cells (Aula et al., 2015, van Meerloo et al., 2011, Bahuguna et al., 2017).

2.3.6. Griess assay

Nitric oxide (NO) secreted from microglia has been identified as one of the most important pro-inflammatory factors involved in microglia-mediated neuroinflammatory response and neurotoxicity in high concentrations (Habib and Ali, 2011, Rojo et al., 2014). Thus, the Griess assay is used to estimate the accumulated level of nitrite (NO$_2^-$) after pro-inflammatory ligand treatment in cell culture. It is also used to evaluate anti-inflammatory effect of compound in cell cultures in the existence of pro-inflammatory ligand such as LPS. It is important
to know that NO\textsuperscript{2-} is measured as an indicator for the production of NO, because NO\textsuperscript{2-} is a stable end product of NO in the cell culture medium. Initially, sulfanilamide is quantitatively converted to a diazonium salt by reaction with NO\textsuperscript{2-} in acid solution. The diazonium salt is then coupled to N-[1-naphthyl]-ethylenediamine dihydrochloride (NED) to form an azo compound (Figure 2.4). Sulfanilamide and NED compete for NO\textsuperscript{2-} in the Griess reaction.

The Griess assay was used to investigate the anti-neuroinflammatory effect of diosgenin in LPS-stimulated BV2 cells. BV2 cells were seeded out at a concentration of 2.0 \times 10^5 cells/mL (2 mL/well) in a 6-well plate and then incubated for 48 hours. The cultured medium was replaced by serum-free RPMI 1640 medium for 2 hours. The cells were treated with 5, 10, and 20 µM of diosgenin, and incubated for 30 minutes and subsequently stimulated with 100 ng/mL of LPS for 24 hours. LPS-control BV2 cells were incubated with 100 ng/mL of LPS for 24 hours. In addition, BV2 cells were left untreated as negative control. The media were centrifuged at 1200 g for 5 minutes at 4°C to remove debris. The supernatants were stored at -80°C for short term future use. The concentrations of NO\textsuperscript{2-} in the culture supernatants of BV2 cells were determined using Griess reagent system (Promega), according to the manufacturer's instructions. 50 µl of each supernatant was mixed with 50 µl of 1% sulfanilamide in 96-well plate. The plate was then incubated at room temperature for 10 minutes in the dark. After that, 50 µl of 0.1% NED was added to each well and incubated for additional 10 minutes at room temperature in the dark. Absorbance was measured at 540 nm using a microplate reader (Tecan Infinite F50) (Griess, 1879).
Initially, sulfanilamide is quantitatively converted to a diazonium salt by reaction with NO$_2^-$ in acid solution. The diazonium salt is then coupled to N-[1-naphthyl]-ethylenediamine dihydrochloride (NED) to form an azo compound (Griess, 1879, Bryan and Grisham, 2007, Sun et al., 2003).

**2.3.7. Enzyme-linked immunosorbent assay (ELISA)**

Pro-inflammatory and anti-inflammatory cytokines are endogenous proteins that produce a wide range of actions in the CNS including immune response regulation (Deverman and Patterson, 2009). It was necessary to measure the levels of cytokines in BV2 cell culture by enzyme-linked immunosorbent assay (ELISA) since higher concentrations of pro-inflammatory cytokines are implicated in neuroinflammation and neurotoxicity (Smith et al., 2012, Wang et al., 2015b). ELISA is an immunochemical technique that is widely used for detection and quantification of antigens such as pro-inflammatory cytokines and transcription factors in cell culture samples.
The principle of one of the most common ELISA types, sandwich ELISA is summarised in Figure 2.5 (Aydin, 2015, Engvall and Perlmann, 1971). The target antigen molecule to be measured is sandwiched between two different primary antibodies the solidified capture antibody and the detection antibody. Next, an enzyme-conjugated secondary antibody is attached to the capture antibody. A chromogenic substrate is hydrolysed by the enzyme into a coloured product that is measured in order to quantify the antigen of interest. The rate of colour formation is directly proportional to the amount of antigen to be measured in the sample.

**Figure 2.5 Types and basic principle of enzyme-linked immunosorbent assay (ELISA).**

In sandwich ELISA, the target antigen molecule to be measured is bound between two different primary antibodies the solidified capture antibody and the detection antibody. Next, an enzyme-conjugated secondary antibody is attached to the capture antibody. A chromogenic substrate of the enzyme is then changed to a coloured product that is measured in order to quantify the antigen of interest (Aydin, 2015, Cox et al., 2014).

BV2 microglia were seeded out at a concentration of $2.0 \times 10^5$ cells/mL (2 mL/well) in a 6-well plate for 48 hours. The cultured medium was replaced by serum-free RPMI 1640 medium for 2 hours. The cells were treated with various concentrations of diosgenin (5, 10, and 20 µM) for 30 minutes and subsequently
stimulated with 100 ng/mL of LPS for 24 hours. LPS-control BV2 cells were incubated with LPS for 24 hours and the cells left untreated were indicated as untreated control. The media were centrifuged at 1200 g for 5 minutes at 4°C to collect the supernatants that were stored at -80°C for short term future use to measure the levels of the released pro-inflammatory factors using commercially available ELISA kits. The absorbance was measured at 450 nm using a microplate reader (Tecan Infinite F50). All samples were assayed in duplicate. If the samples needed to be diluted prior to the assay, then the concentrations were determined after multiplication by an appropriate dilution factor.

2.3.7.1. Determination of TNFα production in LPS-stimulated BV2 microglia

Several studies have suggested that TNFα is a potential signal in the modulation of immune and neuroinflammatory responses in the CNS. TNFα is a neurotoxic factor in high concentrations in the CNS (Fischer and Maier, 2015). Thus, it was important to measure the level of TNFα in BV2 cell cultures activated with LPS. The concentration of the pro-inflammatory factor TNFα in the BV2 cells supernatant was determined using mouse TNFα DuoSet ELISA Development System kit (R & D Systems) according to the manufacturer’s protocol.

After treatment of BV2 cells, 96-well plate was coated with 100 μL of TNFα capture antibody, and the sealed plate was incubated overnight at room temperature. The plate was washed with 400 μL of wash buffer (0.05% Tween-20 in PBS) to remove any unbound antibody. Next, the plate was blocked with 300 μL of a reagent diluent (1% bovine serum albumin; BSA) at room temperature for 1 hour to block non-specific binding. After washing, 100 μL of diluted samples were added, and the plate was incubated at room temperature for 2 hours. After washing, 100 μL of TNFα detection antibody was added and incubated at room temperature for 2 hours. 100 μL of streptavidin linked to horseradish-peroxidase (HRP) was added after washing and the plate was then incubated at room temperature for 20 minutes in the dark. After washing, 100 μL of substrate solution was added and the plate was incubated at room temperature for 20 minutes in the dark. 50 μL of a stop solution (2 N sulphuric acid) was added. The plate was gently tapped to ensure thorough mixing and the absorbance of the colour change was measured at 450 nm using a microplate reader (Tecan Infinite F50) (Engvall and Perlmann, 1971, Favre et al., 1997).
2.3.7.2. Determination of IL-6 production in LPS-stimulated BV2 microglia

Interleukin-6 (IL-6) is a well-known pro-inflammatory cytokine that plays some potential roles in neuroinflammation (Schaper and Rose-John, 2015). A Mouse IL-6 DuoSet ELISA Development System kit (R & D Systems) was used to determine the concentrations of the pro-inflammatory factor IL-6 in the supernatants of BV2 cell cultures, according to the manufacturer’s protocol. The method used for estimation of IL-6 levels in the samples is described in section 2.2.7.1.

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

The role of the pro-inflammatory cytokine IL-1β in the pathophysiology of the CNS is important in the immune and pro-inflammatory responses in the brain (Henry et al., 2009, Van de Veerdonk et al., 2011). A Mouse IL-1β ELISA MAX™ Standard Set kit (BioLegend) was used to determine the concentration of IL-1β in BV2 cell cultures, according to the manufacturer’s instruction. The method for the determination of the pro-inflammatory cytokine IL-1β concentration in the samples is described in section 2.2.7.1.

2.3.7.4. Determination of IL-10 production in LPS-stimulated BV2 microglia

Although microglia are known to produce a wide spectrum of pro-inflammatory cytokines during the immune response as well as neuroinflammation, microglial IL-10 is the most important anti-inflammatory cytokine in the brain (Kremlev and Palmer, 2005, Iyer and Cheng, 2012). The concentration of the anti-inflammatory factor IL-10 was determined by ELISA assay using a mouse IL-10 ELISA MAX™ Standard Set kit (BioLegend) according to the manufacturer’s protocol. The procedure for the determination of IL-10 concentration was described in section 2.2.7.1.

2.3.8. PGE₂ enzyme immunoassay

Several studies have suggested that the level of PGE₂ is increased during neuroinflammation-mediated neurotoxicity (Cimino et al., 2008). Therefore, PGE₂ is known to regulate both normal immune and neuroinflammatory responses in the CNS (Yagami et al., 2016). An Enzyme immunoassay (EIA), a competitive immunoassay was used for the detection and quantification of PGE₂ in the samples.
Concentrations of PGE$_2$ were measured using a DetectX PGE$_2$ enzyme immunoassay Kit (Arbor Assays) according to the manufacturer’s instructions. After treatment of BV2 cells and collection of supernatants as described in section 2.2.7, 100 μL of supernatants were added into the wells using an antibody (goat anti-mouse IgG)-coated 96 well plate. Secondly, 125 μL of an assay buffer was added into the non-specific binding wells. After that, 100 μL of an assay buffer was added into the wells to act as maximum binding wells. Next, 25 μL of the PGE$_2$-peroxidase conjugate was added to each well. 25 μL of PGE$_2$ antibody was added to each well, except the non-specific binding wells. The sealed plate was then incubated at room temperature for 2 hours with shaking. The plate was washed with 300 μL of wash buffer. 100 μL of substrate was added and the plate was incubated at room temperature for 30 minutes without shaking. After addition of 50 μL of a stop solution, the absorbance of the colour change was measured at 450 nm using a microplate reader (Tecan Infinite F50) (Schuurs and Van Weemen, 1980, Yalow, 1978).

2.3.9. Generation of intracellular ROS

The physiological effects of ROS at low level are associated with the host defence system. However, high levels of intracellular ROS are known to be associated with neuroinflammation and oxidative stress-induced cellular damage such as neuronal death (Kim et al., 2015). The fluorogenic dye 2’, 7’-dichlorofluorescin diacetate (DCFDA) diffuses into cells, and then is deacetylated to a non-fluorescent intermediate called 2’, 7’-dichlorofluorescin (DCFH) by intracellular esterases. DCFH is then rapidly oxidised by ROS into a highly fluorescent product called 2’, 7’-dichlorofluorescein (DCF) that is detected by fluorescence spectroscopy (Figure 2.6) (Held, 2012, LeBel et al., 1992).

Levels of ROS were measured to evaluate the effect of diosgenin on LPS-induced ROS generation in BV2 microglia using a DCFDA-cellular ROS detection assay kit (Abcam). BV2 cells were seeded out at a concentration of 2.5 × 10$^5$ cells/mL (100 μL/well) in a 96-well plate and then incubated overnight. The cells were washed with PBS, and then stained with 20 μM of DCFDA (Abcam) and incubated at 37°C for 30 minutes in the dark. The cells were washed with PBS, and then phenol red-free RPMI 1640 medium was added into each well. The cells were treated with 5, 10, 20 μM of diosgenin for 30 minutes, followed by stimulation with
LPS for 6 hours. The fluorescence intensity was measured with an excitation and emission wavelengths of 485 and 535 nm, respectively using a FLUOstar OPTIMA plate reader (BMG LABTECH) (Keston and Brandt, 1965). The fluorescence intensity of DCF is directly proportional with the level of intracellular ROS generated. All samples and standards were assayed in duplicate.

**Figure 2.6 Principle of ROS detection**

The fluorogenic dye 2', 7'-dichlorofluorescin diacetate (DCFDA) diffuses into cells, and is deacetylated to the non-fluorescent intermediate 2', 7'-dichlorofluorescin (DCFH) by cellular esterases. After that, DCFH is rapidly oxidized by ROS into a highly fluorescent product 2', 7'-dichlorofluorescein (DCF) that is detected by fluorescence spectroscopy (LeBel et al., 1992, Held, 2012, Dikalov and Harrison, 2014, Keston and Brandt, 1965).

**2.3.10. NADPH Assay**

The cofactor NADPH is responsible for the generation of NO and ROS in host defence and neuroinflammation and oxidative stress (Maghzal et al., 2012). In
addition, NADPH is an important cofactor in the maintenance of GSH in the reduced form GSH that is needed to prevent ROS toxicity (Vilhardt et al., 2017, Calkins et al., 2009). The reduced form, NADPH is used as a cofactor for many redox enzymes (Liu and Wang, 2007, Ying, 2008). The NADPH assay is a colorimetric assay used to measure the concentration of NADPH in various cells and tissues. It is used to study metabolic and redox state of cells and tissues as well as cell signalling in various cells.

Levels of NADPH were measured in LPS-activated BV2 cells after diosgenin treatment using a NADPH assay kit (Abcam). BV2 cells were seeded out at a concentration of 2.0 x 10^5 cells/mL (100 µl/well) in a 96-well plate and incubated for 48 hours. After changing the medium, the cells were treated with 5, 10, and 20 µM of diosgenin for 30 minutes followed by stimulation with LPS for 6 hours. After removing the medium, the cells were lysed with lysis buffer and incubated at room temperature for 15 minutes. Next, the lysate was centrifuged at 14000 g at 4°C for 5 minutes to collect the supernatant. 50 µl of sample was added to each well using 96-well plate, followed by addition of 50 µl of the NADPH reaction mixture. The plate was sealed and incubated at room temperature for 15 minutes in the dark. The absorbance was measured at 460 nm using a microplate reader (Tecan Infinite F50) (Kupfer and Munsell, 1968, Zhang et al., 2000). The cell lysates were prepared based on the total protein concentration (Bradford, 1976).

### 2.3.11. Preparation of cytoplasmic and nuclear protein lysates

Cytoplasmic and nuclear extraction are processes used in collecting the cytoplasmic and nuclear extracts from cultured cells, respectively. Cytoplasmic and nuclear extraction have been widely used in a variety of applications including studying gene expression and quantify protein level by western blotting. In the present study, the cytoplasmic and nuclear lysates were prepared to study the nuclear translocation of the transcription factors such as NF-κB. Nuclear extracts were prepared to study the activity of transcription factor inside the nucleus such as protein-DNA binding activity. Cytoplasmic lysates were prepared in order to determine the levels of proteins. Cell lysates were prepared to study the effect of diosgenin on LPS on activity of several transcription factors including NF-κB, p38 MAPK, and Akt in LPS-activated BV2 cells. Cytoplasmic and nuclear lysates were prepared to study the nuclear translocation of NF-κBp65 after
treatment of BV2 cells with diosgenin and LPS. In addition, cytoplasmic extracts were collected to measure the levels of pro-inflammatory enzymes iNOS and COX-2 and kinases including IκB-α, IKK, p38 and Akt that regulate NF-κB signalling. Furthermore, nuclear extracts were prepared to study the DNA-binding activity of NF-κB.

The EpiSeeker Nuclear Extraction kit (Abcam) was used to collect both cytoplasmic and nuclear extracts, according to the manufacturer’s instructions. After treatment of BV2 cells, the cells were washed with ice-cold PBS to remove the cell culture media. Next, the cells were scraped with ice-cold PBS and then centrifuged to get the pellets. After that, 20 µL of a mixture containing ice-cold ENE1 (10X Pre-Extraction Buffer), dithiothreitol (DTT), and a protease inhibitor cocktail was added to each cell pellet and, incubated on ice for 10 minutes. Thereafter, the cell extracts were vortexed for ten seconds and centrifuged at 12000 g for one minute at 4°C. The supernatants containing cytoplasmic protein lysates were collected carefully and stored at -80°C until use for immunoblotting.

Next, 10 µl of a mixture containing ENE2 (Extraction Buffer), DTT, and protease inhibitor cocktail was added to each pellet, and incubated on ice for 15 minutes with mixing every 3 minutes. The cell extracts were centrifuged at 14000 g for 15 minutes at 4°C. The supernatants containing nuclear proteins lysates were collected and stored at -80°C until use for immunoblotting.

In addition, a Cell Lysis Buffer (Cell Signaling) was used to collect protein lysates that were used to measure the levels of proteins in the cytoplasm. The plate was washed with ice-cold PBS, and 20 µL of ice-cold Cell Lysis Buffer containing 1 mM phenylmethylsulfonyl fluoride (Sigma) was then added into each well for 10 minutes on ice. Next, the cells were scraped to collect the cell extracts that were centrifuged at 13500 g for 10 minutes at 4°C to pellet the cellular debris. The supernatants containing protein lysates were collected and stored at -80°C until use for immunoblotting.

2.3.12. Determination of protein concentration
The Bradford protein assay is a simple colorimetric assay used to measure total protein concentration in a solution. The procedure is based on the binding of the dye Coomassie G-250 to primarily basic and aromatic amino acids residues of
the proteins under acidic conditions. The formation of a protein-dye complex results in a colour change from brown to blue (Figure 2.7) (Bradford, 1976). The procedure is based on the colour change of the dye in response to various concentration of proteins.

The protein concentrations in the cytoplasmic and nuclear lysates were quantified using a Coomassie Plus Assay Kit (Thermo Scientific) according to the manufacturer’s instructions. The concentrations of proteins in the lysates were quantified and then subjected for Western blotting applications. BV2 cell lysates were diluted with distilled water at a 1:10 ratio to obtain a sufficient concentration of protein. Next, 5 µL of both samples and seven known protein concentrations of BSA standard (125 to 2000 µg/mL) were added in duplicate into a 96-well plate. After that, 250 µL of Coomassie reagent was added and incubated at room temperature for 10 minutes. The absorbance was measured at 595 nm using a microplate reader (Tecan Infinite F50). The protein concentration in a test sample was determined using standard curve.
**Figure 2.7 Mechanism of Coomassie G-250 dye to protein.**

The colorimetric reaction is based on the binding of protein to Coomassie G-250 dye to form a protein-dye complex under acidic condition. This results in a colour change from brown to blue (Bradford, 1976, Brunelle et al., 2017).

**2.3.13. Western blotting**

Western blotting is an immunoblotting technique used to detect the existence of specific proteins in a sample based on their molecular weights. Firstly, the proteins in the sample are separated by gel electrophoresis. Following separation, the proteins are transferred from the gel onto a blotting membrane. The membrane is blocked to prevent nonspecific binding, and then incubated with a primary antibody, which specifically binds to the protein of interest. Next, the membrane is washed to remove any unbound primary antibody. The membrane is again incubated with a secondary antibody that specifically recognises and binds to the primary antibody that is linked to a reporter enzyme that produces colour (Towbin et al., 1979).
BV2 cells were seeded out at a concentration of $2.0 \times 10^5$ cells/mL (2 mL/well) in a 6-well plate and incubated for 48 hours. Cells were treated with 5, 10, and 20 µM of diosgenin for 30 minutes, and then stimulated with 100 ng/mL of LPS for the indicated time. After treatment and collection of the lysates, 20 µg of protein was mixed with NuPAGE LDS sample buffer (Invitrogen) and NuPAGE sample reducing agent (Invitrogen). The samples were heated at 70°C for 10 minutes. A total volume of 20 µL of each sample and 5 µL of protein standard were loaded onto a NuPAGE Novex 4-12% Bis-Tris gel (Invitrogen) with running buffer (Invitrogen). After that, the samples were separated by electrophoresis instrument at a constant voltage of 200 V for 35 minutes. The separated target proteins were then transferred onto polyvinylidene difluoride membrane (Immbilon-FL; Millipore) with transfer buffer (Invitrogen) at 25 V for 2 hours. The membrane was blocked with 5% non-fat dried milk (Cell Signaling) in Tris-Buffered Saline with Tween-20 (TBS-T; chemcruz Santa Cruz) at room temperature for 1 hour with shaking. The blot was then washed three times with TBS (Life technologies) at room temperature for 10 minutes with shaking. After that, it was incubated with the target primary antibodies at 4°C for overnight with shaking. The membrane was washed with TBS-T three times and incubated with the secondary antibody Alexa Fluor 680 goat anti-rabbit IgG (1:10000; Invitrogen) at room temperature for one hour in the dark with shaking. The blot was washed with TBS-T three times, and then the antigen-antibody complexes were detected using an Odyssey infrared imaging system (LI-COR, Bioscience) according to manufacturer’s instructions. β-actin (Sigma) was used as a loading control for the blotting of whole cell and cytosolic proteins and lamin B (Santa Cruz) was used for the same purpose but for the blotting of nuclear proteins. The determination of unknown proteins on the membrane was based on the values obtained for the bands of proteins in the molecular weight standard Precision Plus Protein unstained standard (Bio-Rad). The relative density of the protein expressions was measured using Image J (National Institutes of health). All antibodies were prepared in TBS-T at specific ratios.

The membrane was stripped using Restore Fluorescent Western Blot Stripping Buffer (Thermo scientific) to remove the primary and secondary antibodies from western blots. In brief, the membrane was incubated with the diluted stripping
buffer at room temperature for 20 minutes with constant shaking. After that, the membrane was rinsed with ultrapure water three times, and then washed with TBS-T three times. Western blotting was used to study the effect of diosgenin on the levels of proteins listed in Table 5.

**Table 5 List of primary and secondary antibodies used in Western blotting**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Host</th>
<th>Dilution Factor</th>
<th>Product Number</th>
<th>kDa</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>iNOS</td>
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<td>1:500</td>
<td>sc-650</td>
<td>130</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>COX-2</td>
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<td>sc-1747-R</td>
<td>72</td>
<td>Santa Cruz Biotechnology</td>
</tr>
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<td>sc-101713</td>
<td>41</td>
<td>Santa Cruz Biotechnology</td>
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<tr>
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<td>sc-371</td>
<td>35</td>
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</tr>
<tr>
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<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
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<td>85</td>
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</tr>
<tr>
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<td>65</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
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<td>1:500</td>
<td>sc-372</td>
<td>65</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
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<tr>
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<td>62</td>
<td>Santa Cruz Biotechnology</td>
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<tr>
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<td>67</td>
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<tr>
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<td>1:10000</td>
<td>A-21076</td>
<td></td>
<td>Life technologies</td>
</tr>
</tbody>
</table>

**2.3.14. NF-κB luciferase reporter gene assay**

The binding of transcription factors to their binding sites results in the transcriptional activation of several genes that regulate immune response and neuroinflammation (Vega and de Andres Martin, 2008, Hayden and Ghosh, 2012, Joshi and Johnson, 2012). The luciferase reporter gene assay was used to measure the specific ability of a transcription factor to activate the transcription of target genes and the transcription factor-dependent transcriptional activities. This assay has been used as a gene technology to study gene expression at the transcriptional level (Gorman et al., 1982). Firefly luciferases have been widely
used as experimental reporters to monitor changes in the expression of the genes of interest. *Renilla* luciferase can be used as a control reporter to normalise the results for any interfering factors such as differences in cell viability.

Mechanism of bioluminescent reactions that are catalysed by firefly and *Renilla* luciferases are shown in Figure 2.8. In the luciferase reaction, the substrate beetle luciferin is oxidised into oxyluciferin by the firefly luciferase in the presence of adenosine triphosphate (ATP), magnesium (Mg$^{2+}$) and O$_2$, which results in the production of extra energy in the form of light. In the *Renilla* luciferase reaction, coelenterazine is converted into coelenteramide by the *Renilla* luciferase in the presence of O$_2$ and results in the emission of light.
Figure 2.8 Mechanism of bioluminescent reactions catalysed by the firefly and Renilla luciferases.

In the luciferase reaction, the substrate beetle luciferin is oxidised into oxyluciferin by the firefly luciferase in the presence of adenosine triphosphate (ATP), magnesium (Mg$^{2+}$) and molecular oxygen (O$_2$), which results in the production of extra energy in the form of light. In the Renilla luciferase reaction, coelenterazine is converted into coelenteramide by Renilla luciferase only in the presence of O$_2$. This is also results in the emission of light (Thorne et al., 2010, Kaskova et al., 2016, Marques and Esteves da Silva, 2009).
Consequently, the NF-κB-dependent transcriptional activity was measured in cultured HEK293 cells using a luciferase reporter gene assay. HEK293 cell line was used to test the effect of diosgenin on NF-κB transcriptional activity using the ONE-Glo Luciferase Assay System (Promega), according to the manufacturer’s instructions. One day before transfection, HEK293 cells were seeded out at a concentration of $1.5 \times 10^5$ cells/mL (100 µL/well) in a 96-well plate, using complete MEM medium supplemented with 5% FBS. After 24 hours incubation, the complete spent MEM medium was replaced by Opti-MEM I reduced serum medium (Gibco). The cells were transfected with 100 ng pGL4.32[luc2P/NF-κB-RE/Hygro] vector (Promega) using a FuGENE 6 transfection reagent (Promega) at a 3:1 transfection reagent:DNA ratio and further incubated for 18 hours. The medium was replaced by Opti-MEM I reduced-serum medium and incubated for 6 hours. The transfected cells were treated with diosgenin for 30 minutes and then stimulated with 1 ng/mL TNFα. After 5 hours incubation, 100 µL of a mixture of ONE-Glo buffer and ONE-Glo substrate was added to each well and the luminescence was then measured with a FLUOstar OPTIMA plate reader (BMG LABTECH).

2.3.15. Immunofluorescence

Immunofluorescence is a cell imaging technique used to detect antigens in a sample using a specific primary antibody. There are two types of immunofluorescence; direct and indirect (Figure 2.9). Direct immunofluorescence is an assay in which a fluorochrome-conjugated primary antibody is attached to the antigen of interest. Indirect immunofluorescence is an assay in which the target protein in the sample is specifically bound to the unlabelled primary antibody during incubation. The plate is washed to remove any unbound primary antibody. Next, a secondary antibody is labelled with a fluorochrome and binds to the primary antibody. The complex is then visualised with fluorescence microscopy in order to detect the antigen of interest (Coons et al., 1955).
Figure 2.9 Types of immunofluorescence assays.

Direct immunofluorescence is an assay in which a fluorochrome-conjugated primary antibody is attached to the antigen of interest. Indirect immunofluorescence is an assay in which a fluorochrome-conjugated secondary antibody is attached to the primary antibody, and this results in the detection of the antigen of interest (Mohan et al., 2008, Odell and Cook, 2013).

The effect of diosgenin on nuclear accumulation of NF-κBp65 was detected using an indirect immunofluorescence assay. Microglia were seeded out at a concentration of 2.0 x 10^5 cells/mL (1 mL/well) in a 24-well plate and incubated for 48 hours. After changing the medium, the cells were treated with 5, 10, and 20 μM of diosgenin for 30 minutes and then stimulated with 100 ng/mL LPS for
one hour in order to detect nuclear accumulation of NF-κBp65. BV2 cells were either left untreated as a negative-control or stimulated with LPS (LPS-positive).

After treatment of the cells, the medium was aspirated and the cells were washed with PBS three times. The cells were fixed with ice-cold 100% methanol for 20 minutes at -20°C. After a wash with PBS, the fixed cells were blocked with 5% BSA containing 10% horse serum in 1X BPS for one hour at room temperature in order to block non-specific binding sites. Thereafter, the plate was incubated with the primary antibody against NF-κBp65 antibody (1:100 dilution; Santa Cruz) at 4°C for overnight. The cells were washed and then incubated with Alexa Fluor 488-conjugated donkey anti-rabbit immunoglobulin G secondary antibody (1:500 dilution; Life Technologies) at room temperature for 2 hours in the dark. After washing, the plate was incubated with 300 nM of 4′-6-diamidino-2-phenylindole dihydrochloride (DAPI; Invitrogen) for 5 minutes. After washing the stained cells, one drop of ProLong Gold Antifade Reagent (Invitrogen) was added to each well. All images were captured with EVOS FLoid cell imaging station (Life technologies).

**2.3.16. Electrophoretic mobility gel shift assay (EMSA)**

It is well known that NF-κB binds to the DNA at its specific binding site, and thereby regulates the production of several pro-inflammatory genes that play an important role in the regulation of immune and neuroinflammatory responses (Hayden and Ghosh, 2012). EMSA is used to study an interaction between proteins such as transcription factor and DNA. Solutions of protein and DNA are combined and the resulting mixture are subjected to electrophoresis through a non-denaturing polyacrylamide gel. After electrophoresis, the distribution of the protein-DNA complex is determined by autoradiography. In general, a protein-DNA complex migrates more slowly than the corresponding free DNA (Fried and Crothers, 1981).

EMSA was used to evaluate the effect of diosgenin on the DNA-binding activity of NF-κB. BV2 cells were seeded out at a concentration of 2.0 x 10^5 cells/mL (2 mL/well) in a 6-well plate and incubated for 48 hours. After changing the medium, the cells were treated with 5, 10, and 20 μM of diosgenin and then incubated with LPS for 1 hour. 5 μg/μL nuclear protein lysate was added into a mixture containing
ultra-pure water (Fisher), 10X Binding Buffer (100 mM Tris, 500 mM KCl, 10 mM DTT; pH 7.5), 25 mM DTT/2.5% Tween® 20, 1 μg/μL Poly (dI,dC) in 10 mM Tris, 1 mM EDTA; pH 7.5, 1 % NP-40 and 100 mM MgCl₂. 1 μL of double-stranded DNA oligonucleotide containing the consensus NF-κB DNA binding site (5’- AGT TGA GGG GAC TTT CCC AGG C -3’) end-labeled with IRDye 700 was added into the mixture. All components were mixed and incubated at room temperature for 30 minutes in the dark to allow NF-κB protein binding to DNA. 2 μL of 10X Orange Loading Dye (LI-COR-Bioscience) was added into the mixture. A total volume of 21 μL of each sample was loaded onto a 4-12% TBE gel (Life technologies) in Tris-Borate-EDTA (TBE) running buffer (Life technologies) and then electrophoresed at 100 V for one hour at room temperature in the dark. The gel was then dried, and the intensity of radioactive DNA/protein complex bands were detected using Odyssey infrared imaging system (LI-COR, Bioscience).

2.3.17. ELISA for DNA-binding of NF-κB
The TransAM format is a colorimetric assay used to study the binding of transcription factors to a consensus-binding site oligonucleotide. Firstly, the activated transcription factor in the nuclear extract binds to the consensus-binding site on the oligonucleotide immobilized in the well. After that, the plate is incubated with the primary and secondary antibodies in order to quantify the amount of activated transcription factor (Figure 2.10).(Engvall and Perlmann, 1971, Cox et al., 2014)
Figure 2.10 Flow chart of the TransAM process.

An activated transcription factor in the cell nuclear extract binds to the consensus-binding site on the oligo immobilized in the well. The plate is incubated with the primary and secondary antibodies in order to quantify the amount of activated transcription factor (Aydin, 2015, Cox et al., 2014, Engvall and Perlmann, 1971).

It was necessary to assess the effect of diosgenin on LPS-induced increase in DNA-binding activity of NF-κB in BV2 cells. The effect of diosgenin on the DNA-binding of NF-κBp65 was evaluated using the TransAM NF-κBp65 kit (Active Motif), according to the manufacturer’s instructions.

BV2 cells were seeded out at a concentration of $2.0 \times 10^5$ cells/mL (2 mL/well) in a 6-well plate and incubated for 48 hours. The medium was replaced by serum-
free RPMI 1640 medium for 2 hours. The cells were treated with 5, 10, and 20 µM of diosgenin, for 30 minutes, and then stimulated with 100 ng/mL of LPS for one hour. Untreated cells were used as negative control and the cells stimulated with 100 ng/mL LPS for one hour were used as positive-control. Nuclear extracts containing the activated transcription factors were incubated in a 96-well plate coated with a specific double-stranded oligonucleotides containing the NF-κB consensus site (5′-GGGACTTTCC-3′).

30 µL of Complete Binding Buffer AM3 (dithiothreitol, Herring sperm DNA, and Binding Buffer AM3) was added, followed by the addition of 20 µg of nuclear extract diluted in 20 µL of Complete Lysis Buffer. The sealed plate was incubated at room temperature for one hour with shaking. The plate was washed with 200 µL of Wash Buffer three times. The sealed plate was incubated with 100 µL of the primary antibody for one hour without shaking. The sealed plate was washed and then incubated with the HRP-conjugated secondary antibody for a further one hour. After the last wash, 100 µL of a developing solution was added to each well for 5 minutes in the dark. Next 100 µL of a stop solution was added to stop the colorimetric reaction. After 3 minutes, the absorbance was measured at 450 nm using a microplate reader (Tecan Infinite F50). The dilution factor was 1:1000 for the preparation of antibodies.

2.3.18. BV2 microglia conditioned media-induced neurotoxicity

It is well known that a direct contact between microglia and neurons is involved in the pathogenesis of neurodegenerative diseases due to uncontrolled neuroinflammation and neurotoxicity (Gresa-Arribas et al., 2012). Excessive production of pro-inflammatory mediators from microglia is responsible for neuronal death (Xu et al., 2016). For example, microglial pro-inflammatory factors such as NO, TNFα, and ROS are neurotoxic molecules at high concentrations in the CNS (Takeuchi et al., 2006, Ali et al., 2012). Therefore, it was necessary to assess the neuroprotective activity of diosgenin in microglia conditioned medium-induced neurotoxicity. For this purpose, BV2 microglial conditioned medium was prepared in order to evaluate the neuroprotective effect of diosgenin against BV2 microglial activation-induced HT22 neuronal death. BV2 cells were seeded out at a concentration of 2.0 x 10^5 cells/mL (2 mL/well) in a 6-well plate and incubated for 48 hours. After changing the medium, the cells were treated with 5, 10, and
20 µM of diosgenin for 30 minutes and then incubated with LPS (1 µg/mL) for 24 hours. The culture medium was centrifuged at 1200 g at 4°C for 5 minutes to remove cell debris to obtain the conditioned medium containing the pro-inflammatory mediators. The existence of pro-inflammatory mediators (NO, TNFα, IL-6, and PGE₂) in the conditioned medium was confirmed by measuring the levels of these factors.

2.3.18.1. Measurement of intracellular ROS in HT22 cells
The pathological effects of microglial ROS are associated with neuroinflammation and oxidative stress-induced neuronal death (Olmez and Ozyurt, 2012, Kim et al., 2015). The effect of diosgenin on the level of intracellular ROS in HT22 cells induced by the conditioned media of LPS-activated microglia was evaluated. HT22 cells were seeded out at a concentration of 2.5 × 10⁵ cells/mL (100 µL/well) in a 96-well plate and incubated for 48 hours. After that, HT22 cells were washed with PBS, and then stained with 20 µM of DCFDA and, incubated at 37°C for 30 minutes in the dark. The cells were washed with PBS, and then phenol red-free RPMI 1640 medium was added into each well. Next, the cells were treated with the 100 µL of conditioned medium for 6 hours. Fluorescence intensity was then measured with an excitation and emission wavelengths of 485 and 535 nm, respectively using a FLUOstar OPTIMA plate reader (BMG LABTECH).

2.3.18.2. Determination of HT22 neuronal viability
The conditioned medium prepared from LPS-activated BV2 cells was used to evaluate the effect on viability of HT22 cells using an MTT assay. After preparation of the conditioned media, HT22 cells were seeded out at a concentration of 2.0 × 10⁵ cells/mL (200 µl/well) in a 96-well plate and incubated for 24 hours. The cultured medium in each well was replaced with 200 µl of conditioned medium and the plate was then incubated for 24 hours. After that, an MTT assay was carried out on HT22 cells as described in section 2.2.5 in order to determine the viability of HT22 cells.

2.3.19. Statistical Analysis
Values are expressed as the mean ± SEM of three independent experiments. Data were analysed using one-way ANOVA for multiple comparisons with post-hoc Student Newman-Keuls test. The ANOVA compares the mean between
various groups and determines whether those means are significantly different from each other. To determine the specific groups that are significantly different from each other, the results were evaluated by with post-hoc Student Newman-Keuls test with multiple comparisons. Calculations for statistical analysis were performed with Graph Pad Prism software version 5. Differences were significant at $p<0.05$. For the neuroinflammation experiments, designations include $\&p<0.05$, $\&\&p<0.01$, $\&\&\&p<0.001$ compared with untreated control; and $^*p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ compared to LPS-treated control.
2.4. Results

2.4.1. Diosgenin did not affect the viability of BV2 cells

The MTT assay was used to determine whether diosgenin do not produce cytotoxic effect on BV2 cells and to select, at least three various concentrations of needed to perform the present study. Microglial BV2 cells were treated with various concentrations (1, 5, 10, 20, 25, 30, 35, and 40 µM) of diosgenin for 30 minutes and then stimulated with 100 ng/ml of LPS for 24 hours. It was important to know that various concentration (25, 50, 100, 500 and 1000 ng/mL) of LPS were tested to determine the effective concentration that induce microglial activation using Griess assay. The result showed that the concentrations of LPS at 100 ng/ml and 500 ng/ml were the effective ones and subsequently the lower concentration of LPS (100 ng/ml) was selected to evaluate the anti-neuroinflammatory effect of diosgenin. MTT assay result showed that the concentrations of diosgenin at 1, 5, 10, and 20 µM did not affect the viability of BV2 cells, compared to untreated BV2 cells (Figure 2.1). The cell viability in the untreated BV2 microglia is shown as 100%. Further, this assay indicated that LPS at 100 ng/ml did not affect viability of BV2 cells. Therefore, this finding suggest that diosgenin at 5, 10, and 20 µM had no cytotoxicity effect on BV2 cells and subsequently, these three concentrations were selected for performing the further experiments of the present research.
Figure 2.11 Effect of diosgenin on the viability of LPS-stimulated BV2 cells.

BV2 cells were treated for 30 minutes with diosgenin, and then stimulated with 100 ng/mL of LPS for 24 hours. Data are expressed as the mean ± SEM of three independent experiments. Data were analysed using one-way ANOVA for multiple comparisons with post hoc Student Newman-Keuls test. $^p<0.05$, $^{\alpha\&\alpha}p<0.01$, $^{\alpha\&\alpha\alpha}p<0.001$ compared with untreated control; and $^*p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ compared to LPS-treated control.

2.4.2. Diosgenin attenuated NO production by suppressing iNOS expression in LPS-stimulated BV2 cells

It is well known that the NO/iNOS system plays an important role in hyperactivated microglia-mediated neuroinflammation (Ghasemi and Fatemi, 2014, Garry et al., 2015). Thus, the effect of diosgenin in LPS-activated NO/iNOS mechanism in BV2 microglia was investigated. Results show that the stimulation of BV2 cells with LPS for 24 hours produced a marked increase (14.3 ± 5.1 µM) ($p<0.001$) in the production of nitrite (a measure of NO production), compared to the unstimulated-control (Figure 2.12). The observed increase in the level of nitrite in LPS-activated BV2 cells is defined as 100%. Treatment of cells with 5, 10, and 20 µM of diosgenin significantly ($p<0.001$) reduced the production of nitrite, compared to LPS-stimulated BV2 cells (Figure 2.12). Pre-treatment of the cells with 20 µM of diosgenin resulted in 50% of NO production, compared to the LPS-control.
Figure 2.12 Effect of diosgenin on the NO production of LPS-stimulated BV2 cells.

BV2 cells were treated for 30 minutes with diosgenin, and then stimulated with 100 ng/mL of LPS for 24 hours. The levels of NO were measured as described above. Data are expressed as the mean ± SEM of three independent experiments. Data were analysed using one-way ANOVA for multiple comparisons with post hoc Student Newman-Keuls test. *p<0.05, **p<0.01, ***p<0.001 compared with untreated control; and *p<0.05, **p<0.01, ***p<0.001 compared to LPS-treated control.

To determine whether diosgenin reduced the level of nitrite (NO) in LPS-stimulated BV2 cells through modulation of the NO/iNOS pathway, the levels of iNOS were detected and quantified using western blotting. The level of iNOS protein was significantly increased by LPS (p<0.001) after 24 hours, compared to that of untreated cells (Figure 2.13). The observed increase in the level of this protein by LPS is expressed as 100%. However, LPS-induced increases in iNOS expression was significantly reduced (p<0.001) with all concentrations of diosgenin used (Figure 2.13). It was observed that 48.5% and 26.5% of iNOS proteins were produced in the presence of 10 µM and 20 µM of diosgenin, respectively when compared to the LPS-control.
Figure 2.13 Effect of diosgenin on the iNOS expression in LPS-stimulated BV2 cells.

BV2 cells were treated for 30 minutes with diosgenin, and then stimulated with 100 ng/mL of LPS for 24 hours. The levels of iNOS were measured as described above. Data are expressed as the mean ± SEM of three independent experiments. Data were analysed using one-way ANOVA for multiple comparisons with post hoc Student Newman-Keuls test. *p<0.05, **p<0.01, ***p<0.001 compared with untreated control; and *p<0.05, **p<0.01, ***p<0.001 compared to LPS-treated control.

2.4.3. Diosgenin reduced the secretion of PGE₂ by reducing COX-2 expression in LPS-stimulated BV2 cells

Like NO, the microglial pro-inflammatory mediator PGE₂ plays an important role in the regulation of the immune system and the neuroinflammatory response in the CNS (Yagami et al., 2016). As expected, LPS stimulation of cells with LPS resulted in a marked increase (960.5 ± 84.3 pg/mL) (p<0.001) in PGE₂ levels, compared to unstimulated cells (Figure 2.14). The degree of the inflammatory response induced by LPS in the LPS-control is shown as 100%. On the other hand, diosgenin treatment significantly (p<0.001) reduced LPS-induced PGE₂
production, compared to the LPS-control (Figure 219). In the presence of diosgenin (10 µM and 20 µM), PGE\(_2\) release was 21% and 12.3%, respectively when compared to the cells treated with only LPS (Figure 2.14)

![Graph showing PGE\(_2\) levels with different concentrations of diosgenin and LPS](graph.png)

**Figure 2.14 Effect of diosgenin on the production of PGE\(_2\) in LPS-stimulated BV2 cells.**

BV2 cells were treated for 30 minutes with diosgenin, and then stimulated with 100 ng/mL of LPS for 24 hours. The levels of PGE\(_2\) were measured as described above. Data are expressed as the mean ± SEM of three independent experiments. Data were analysed using one-way ANOVA for multiple comparisons with post hoc Student Newman-Keuls test. *\(p<0.05\), **\(p<0.01\), ***\(p<0.001\) compared with untreated control; and *\(p<0.05\), **\(p<0.01\), ***\(p<0.001\) compared to LPS-treated control.

It is well established that COX-2 is responsible for PGE\(_2\) production in microglia (Bonfill-Teixidor et al., 2017). Western blotting was used to determine whether diosgenin-induced decrease in PGE\(_2\) levels in LPS-stimulated BV2 cells was associated with the down-regulation of LPS-induced increase in the COX-2 protein. Compared to the negative control, LPS produced a significant increase (\(p<0.01\)) in the COX-2 level (100%) (Figure 2.15). However, pre-treatment with 5, 10, and 20 µM of diosgenin significantly reduced (\(p<0.001\)) the level of COX-2, compared to that in LPS-stimulated BV2 cells (Figure 2.15). Pre-treatment with 20 µM diosgenin resulted in the expression of 45.5% of COX-2 protein, in comparison with LPS-stimulation of BV2 microglia.
Figure 2.15 Effect of diosgenin on the expression of COX-2 in LPS-stimulated BV2 cells.

BV2 cells were treated for 30 minutes with diosgenin, and then stimulated with 100 ng/mL of LPS for 24 hours. The levels of COX-2 were measured as described above. Data are expressed as the mean ± SEM of three independent experiments. Data were analysed using one-way ANOVA for multiple comparisons with post hoc Student Newman-Keuls test. \&p<0.05, \&\&p<0.01, \&\&\&p<0.001 compared with untreated control; and *p<0.05, **p<0.01, ***p<0.001 compared to LPS-treated control.

2.4.4. Diosgenin reduced the levels of the pro-inflammatory cytokines TNFα, IL-6 and IL-1β in LPS-stimulated BV2 microglia

Reactive microglia secrete a wide variety of pro-inflammatory cytokines that play a critical role in neuroinflammation (McCoy and Tansey, 2008, Fischer and Maier, 2015). To assess whether the anti-neuroinflammatory action of diosgenin is associated with the modulation of the production of pro-inflammatory cytokines, the effect of diosgenin on the LPS-induced production of TNFα, IL-6, and IL-1β in BV2 cells was investigated.
The activation of cells with LPS resulted in a significant increase (p<0.001) in the release of TNFα (448.0 ± 13.0 pg/mL) from BV2 microglia (Figure 2.16a). This increase was significantly (p<0.001) reduced by diosgenin (5-20 µM). It was observed that 35% of TNFα level was detected in the supernatant of cells pre-treated with 20 µM diosgenin, compared to the LPS-control (Figure 2.16a).

The pro-inflammatory cytokine IL-6 is another important mediator in microglia-mediated neuroinflammatory response (Smith et al., 2012, Minogue et al., 2012, Schaper and Rose-John, 2015). As expected, the stimulation of microglia with 100 ng/mL LPS showed a marked increase (435.2 ± 29.8 pg/mL) (p<0.001) in the production of IL-6, compared to untreated microglia (Figure 2.16b). However, the LPS-induced increase in IL-6 release was significantly diminished with all the concentrations of diosgenin tested. As shown in Figure 2.16b, 37% of IL-6 was produced (p<0.001) by 20 µM diosgenin, compared to LPS-control. IL-1β is also involved in microglia-mediated excessive neuroinflammatory (Henn et al., 2009, Van de Veerdom et al., 2011). Exposure of BV2 cells to LPS led to a significant elevation (322.2 ± 20.0 pg/mL) (p < 0.001) in the IL-1β level, compared with untreated BV2 cells (Figure 2.16c). However, the elevated level of IL-1β induced by LPS was significantly reduced with 5, 10, and 20 µM of diosgenin (p<0.001) (Figure 2.16c). The level of this cytokine was 53.5% in LPS-activated BV2 cells pre-treated with 20 µM diosgenin.
Figure 2.16 Effect of diosgenin on the production of TNFα (a), IL-6 (b) and IL-1β (c) production in LPS-stimulated BV2 cells.

BV2 cells were treated for 30 minutes with diosgenin, and then stimulated with 100 ng/mL of LPS for 24 hours. The levels of TNFα, IL-6 and IL-1β were measured as described above. Data are expressed as the mean ± SEM of three independent experiments. Data were analysed using one-way ANOVA for multiple comparisons with post hoc Student Newman-Keuls test. &p<0.05, &&p<0.01, &&&p<0.001 compared with untreated control; and *p<0.05, **p<0.01, ***p<0.001 compared to LPS-treated control.
2.4.5. Diosgenin increased the production of the anti-inflammatory cytokine IL-10 in LPS-stimulated BV2 cells

Microglia produce anti-inflammatory cytokines such as IL-10 during the host defence (Kremlev and Palmer, 2005, Iyer and Cheng, 2012), in order to bring about resolution of inflammation. The effect of diosgenin on the secretion of IL-10 after addition of LPS was also evaluated. The result showed that LPS stimulation caused a significant reduction (20.5 ± 10.5 pg/mL) (p<0.001) in the IL-10 level, compared to the untreated-control BV2 cells (Figure 2.17). The IL-10 level (246.9 ± 20.4 pg/mL) measured in the untreated control is taken as 100%. However, the LPS-induced reduction in IL-10 level was significantly inhibited with increasing concentrations of diosgenin (p<0.01) (Figure 2.17).

![Figure 2.17](image)

**Figure 2.17 Effect of diosgenin on the production of IL-10 in LPS-stimulated BV2 cells.**

BV2 cells were treated for 30 minutes with diosgenin, and then stimulated with 100 ng/mL of LPS for 24 hours. The levels of IL-10 were measured as described above. Data are expressed as the mean ± SEM of three independent experiments. Data were analysed using one-way ANOVA for multiple comparisons with post hoc Student Newman-Keuls test. *p<0.05, **p<0.01, ***p<0.001 compared with untreated control; and *p<0.05, **p<0.01, ***p<0.001 compared to LPS-treated control.
2.4.6. Diosgenin reduced the intracellular ROS level in LPS-stimulated BV2 microglia

Since microglial ROS play a critical role in the pathogenesis of CNS inflammation (Hsieh and Yang, 2013), the effect of diosgenin on ROS generation in the activated cells was also examined. There was a significant increase in the ROS level following activation of microglia with LPS (Figure 2.18). However, diosgenin significantly inhibited the generation of ROS in LPS-activated BV2 cells (Figure 2.18). Compared to the LPS-control, the level of ROS in LPS-stimulated BV2 cells pre-treated with 20 µM diosgenin was 55% (p<0.001).

![Graph showing the effect of diosgenin on ROS production](image)

**Figure 2.18** Effect of different concentrations of diosgenin on the production of ROS in LPS-stimulated BV2 cells.

BV2 cells were treated for 30 minutes with diosgenin, and then stimulated with 100 ng/mL of LPS for 6 hours. The levels of ROS were measured as described above. Data are expressed as the mean ± SEM of three independent experiments. Data were analysed using one-way ANOVA for multiple comparisons with post hoc Student Newman-Keuls test. *p<0.05, **p<0.01, ***p<0.001 compared with untreated control; and *p<0.05, **p<0.01, ***p<0.001 compared to LPS-treated control.
2.4.7. Diosgenin did not affect NADPH level in LPS-stimulated BV2 cells

The cofactor NADPH is responsible for the cellular generation of NO and ROS that are regulated by iNOS and NADPH oxidase, respectively (Maghzal et al., 2012, Rojo et al., 2014). However, NADPH is an important cofactor in the maintenance of the reduced form of GSH that inhibit ROS toxicity (Calkins et al., 2009, Vilhardt et al., 2017). This experiment was performed to determine whether diosgenin would affect the level of NADPH in BV2 microglia. In comparison to the unstimulated cells, the pro-inflammatory agent LPS significantly (p<0.001) lowered the level of NADPH (Figure 2.19). However, diosgenin treatment did not significantly increase the level of NADPH in the cells stimulated by LPS (Figure 2.19).

![Figure 2.19 Effect of different concentrations of diosgenin on the NADPH level in LPS-activated BV2 cells.](image)

BV2 cells were treated for 30 minutes with diosgenin, and then stimulated with 100 ng/mL of LPS for 6 hours. The levels of NADPH were measured as described above. Data are expressed as the mean ± SEM of three independent experiments. Data were analysed using one-way ANOVA for multiple comparisons with post hoc Student Newman-Keuls test. &p<0.05, &amp;p<0.01, &amp;&amp;p<0.001 compared with untreated control; and *p<0.05, **p<0.01, ***p<0.001 compared to LPS-treated control.
2.4.8. Diosgenin reduced the transcriptional activity of NF-κB in TNFα-stimulated HEK293 cells

The previous results showed that diosgenin down-regulated the expression, and consequently production of a number of pro-inflammatory mediators that are regulated by NF-κB in neuroinflammation. Therefore, it was interesting to examine whether diosgenin produced anti-neuroinflammatory effect via inhibiting NF-κB signalling pathway. First of all, the inhibitory effect of diosgenin on NF-κB-mediated gene expression in general was investigated using an NF-κB-dependent reporter gene assay. TNFα stimulation of HEK293 cells induced a marked increase (p<0.001) in the transcriptional activity of NF-κB, in comparison with the untreated-control (Figure 2.20). The rate of transcriptional activity mediated by TNFα is shown as 100%. Treatment with 5, 10, and 20 μM significantly (p<0.05) attenuated TNFα-induced increase in the transcriptional activity of NF-κB, compared to TNFα-control. Pre-treatment with 20 μM of diosgenin reduced by 40 % the TNFα-induced NF-κB-transcriptional activity (Figure 2.20).
Figure 2.20 Effect of diosgenin on the transcriptional activity of NF-κB in TNFα-stimulated HEK293 cells.

Transfected HEK293 cells were treated for 30 minutes with diosgenin, and then stimulated with 1 ng/mL of TNFα for 6 hours. The intensity of luciferase activity was measured as described above. Data are expressed as the mean ± SEM of three independent experiments. Data were analysed using one-way ANOVA for multiple comparisons with post hoc Student Newman-Keuls test. *p <0.05, &&p<0.01, &&&p<0.001 compared with untreated control; and *p<0.05, **p<0.01, ***p<0.001 compared to TNFα-treated control.

2.4.9. Diosgenin did not inhibit LPS-induced IKK phosphorylation in LPS-stimulated BV2 cells

Since diosgenin reduced the transcriptional activity of NF-κB in the transfected HEK293 cells, it was necessary to further explore whether the anti-neuroinflammatory effect of diosgenin was mediated by inhibiting NF-κB signalling pathway in BV2 microglia. The inhibitory effect of diosgenin on the IKK phosphorylation in LPS-stimulated BV2 cells was firstly investigated using western blotting. As expected, the result showed that LPS produced a significant elevation in the level of the phosphorylated form of IKK (p-IKK), compared to untreated cells (Figure 2.21). However, this LPS-induced increase in IKK phosphorylation was not affected in the cells pre-treated with diosgenin (5-20 μM) (Figure 2.21).
Figure 2.21 Effect of diosgenin on the IKK phosphorylation in LPS-stimulated BV2 cells.

BV2 cells were treated for 30 minutes with diosgenin, and then stimulated with 100 ng/mL of LPS for 30 minutes. The levels of p-IKK and total IKK were measured as described above. Data are expressed as the mean ± SEM of three independent experiments. Data were analysed using one-way ANOVA for multiple comparisons with post hoc Student Newman-Keuls test. \( ^{\text{a}}p<0.05, ^{\text{ab}}p<0.01, ^{\text{abc}}p<0.001 \) compared with untreated control; and \( ^{*}p<0.05, ^{**}p<0.01, ^{***}p<0.001 \) compared to LPS-treated control.

2.4.10. Diosgenin treatment prevented the phosphorylation and degradation of IkB-α in LPS-stimulated BV2 cells

Since diosgenin had no any inhibitory effect on LPS-induced IKK phosphorylation, the next step was to examine whether diosgenin attenuated phosphorylation of IkB-α induced by LPS stimulation. Expectedly, LPS significantly induced marked phosphorylation of IkB-α, and thereby elevation of the p-IkB-α protein, compared to untreated cells (Figure 2.22a). The elevated level of p-IkB-α protein induced by LPS alone is taken as 100%. In addition, LPS activation of BV2 cells resulted in the degradation of IkB-α (Figure 2.22b).
However, LPS-induced increase in phosphorylation and degradation of IκB-α was significantly blocked by pre-treatment with diosgenin (5-20 µM) as observed in Figure 2.22a. Diosgenin restored total IκB-α depletion induced by LPS (Figure 2.22b). For example, pre-treatment with diosgenin at the concentration of 20 µM resulted in 35% of p-IκB-α level (p<0.001), when compared to the LPS control.
Figure 2.22 Effect of diosgenin on the IkB-α phosphorylation (a) and IkB-α degradation (b) in LPS-activated BV2 microglia.

BV2 cells were treated for 30 minutes with diosgenin, and then stimulated with 100 ng/mL of LPS for one hour. The levels of IkB-α (a) and total IkB-α (b) were measured as described above. Data are expressed as the mean ± SEM of three independent experiments. Data were analysed using one-way ANOVA for multiple comparisons with post hoc Student Newman-Keuls test. $^\text{ap}<0.05$, $^{\text{app}}p<0.01$, $^{\text{appp}}p<0.001$ compared with untreated control; and $^\text{p}<0.05$, $^{\text{pp}}p<0.01$, $^{\text{ppp}}p<0.001$ compared to LPS-treated control.
2.4.11 Diosgenin inhibited the nuclear translocation of NF-кB in LPS-stimulated BV2 cells

Following observations showing that diosgenin interfered with LPS-induced phosphorylation and degradation of p-κB-α, further investigations were conducted to determine whether diosgenin would affect the nuclear translocation of NF-κB in LPS-treated BV2 microglia. As shown in Figure 2.23, the cytoplasmic level of total NF-κBp65 was significantly (p<0.01) lowered by LPS, compared to untreated cells. The total level of p65 in the untreated BV2 microglia is expressed as 100%. However, compared to the LPS-control, treatment of the cells with diosgenin significantly increased the levels of total p65 in LPS-stimulated BV2 cells (Figure 2.23). It was observed that the level of the non-phosphorylated form of NF-κB in the activated cells pre-treated with 20 μM was 95%, compared to the untreated cells.
Figure 2.23 Effect of diosgenin on the cytoplasmic level of total NF-κBp65 in LPS-treated BV2 cells.

BV2 cells were treated for 30 minutes with diosgenin, and then stimulated with 100 ng/mL of LPS for one hour. The levels of total NF-κBp65 (p65) were measured as described above. Data are expressed as the mean ± SEM of three independent experiments. Data were analysed using one-way ANOVA for multiple comparisons with post hoc Student Newman-Keuls test. &p<0.05, &&p<0.01, &&&p<0.001 compared with untreated control; and *p<0.05, **p<0.01, ***p<0.001 compared to LPS-treated control.

Since diosgenin inhibited LPS-induced reduction in cytoplasmic p65 in BV2 microglia, it was expected that the compound reduced the level of the phosphorylated form of NF-κB (p-p65) in the nucleus of cells activated by LPS. The result showed that the nuclear level of p-p65 in LPS-stimulated BV2 cell was significantly higher than that in the untreated control (Figure 2.24). On the other hand, the increased level of p-p65 induced by LPS response was significantly (p<0.001) reduced by diosgenin treatment (Figure 2.24). Pre-treatment of BV2 cells with 20 µM diosgenin resulted in detection of 31% p-p65 protein, when compared to the LPS control.
Figure 2.24 Effect of diosgenin on the nuclear level of p-NF-κBp65 in LPS-treated BV2 cells.

BV2 cells were treated for 30 minutes with diosgenin, and then stimulated with 100 ng/mL of LPS for one hour. The levels of p-NF-κBp65 (p-p65) were measured as described above. Data are expressed as the mean ± SEM of three independent experiments. Data were analysed using one-way ANOVA for multiple comparisons with post hoc Student Newman-Keuls test. *p<0.05, **p<0.01, ***p<0.001 compared with untreated control; and *p<0.05, **p<0.01, ***p<0.001 compared to LPS-treated control.

The inhibitory impact of diosgenin on the nuclear translocation of NF-κB in BV2 microglia observed in western blotting was confirmed with indirect immunofluorescence staining. The immunofluorescence analysis showed the cytoplasmic distribution of p65 in untreated BV2 cells (Figure 2.25). When BV2 cells were stimulated with LPS, the nuclear translocation of NF-κB was increased, compared to that of untreated control (Figure 2.25). In comparison to the LPS-control, diosgenin attenuated the nuclear translocation of NF-κB in a concentration dependent effect.
Figure 2.25 Effect of diosgenin on the nuclear translocation of NF-κB in LPS-treated BV2 cells after immunofluorescence staining.

The BV2 cells were either left untreated in the negative control group or stimulated with 100 ng/mL of LPS for one hour (LPS-positive group). In addition, the BV2 cells were pre-treated with the indicated concentrations of diosgenin for 30 minutes before 100 ng/mL of LPS stimulation. The green fluorescence represented the location and level of NF-κBp65 in the cytoplasmic and nuclear zones. BV2 cells were stained with the counterstain DABI for visualization of DNA (blue fluorescence) (Scale bar= 100 µm).
2.4.12. Diosgenin inhibited the LPS-induced DNA-binding activity of NF-κB in LPS-stimulated BV2 cells

In this study, the effect of diosgenin on the DNA-binding activity of NF-κB in LPS-activated BV2 microglia was investigated. Results show that there was a marked increase in the DNA-binding activity of NF-κB in cells stimulated with LPS (Figure 2.26). In the presence of diosgenin (5-20 μM) however, the DNA-binding activity of NF-κB was significantly reduced, compared to the LPS-control. The position of the NF-κB/DNA complex and that of the free probe are shown in Figure 2.26.
**Figure 2.26 Effect of diosgenin on the DNA-binding activity of NF-κB in LPS-stimulated BV2 cells.**

BV2 cells were treated for 30 minutes with diosgenin, and then stimulated with 100 ng/mL of LPS for one hour. Results are presented as mean percentage ± SEM of three independent experiments. None is a control without nuclear extract and the negative control is the nuclear extract of untreated BV2 cells. Data were analysed using one-way ANOVA for multiple comparisons with post hoc Student Newman-Keuls test. $^\&$$^p<0.05$, $^\&\&$$^p<0.01$, $^\&\&\&$$^p<0.001$ compared with untreated control; and *$p<0.05$, **$p<0.01$, ***$p<0.001$ compared to LPS-treated control.
2.4.13. Diosgenin did not affect the LPS-induced p38 phosphorylation in LPS-stimulated BV2 cells

As diosgenin reduced the levels of several pro-inflammatory mediators, it was important to determine whether diosgenin exhibited an inhibitory effect on p38 MAPK signalling pathway that is activated during neuroinflammation (Bachstetter et al., 2011, Ramesh, 2014). Western blotting analysis showed that 100 ng/mL of LPS stimulated the cells to activate p38 signalling. A marked increase in the level of p-p38 was observed after stimulation of BV2 cells with LPS, compared to the untreated cells (Figure 2.27). However, treatment with 5, 10 and 20 µM of diosgenin did not produce inhibition of LPS-induced p38 phosphorylation, compared to LPS-control (Figure 2.27).
Figure 2.27 Effect of diosgenin on the p38 phosphorylation in LPS-treated BV2 microglia.

Effects of diosgenin on the p38 phosphorylation in LPS-treated BV2 cells. BV2 cells were treated for 30 minutes with diosgenin, and then stimulated with 100 ng/mL of LPS for one hour. The levels of p38 and total p38 were measured as described above. Data are expressed as the mean ± SEM of three independent experiments. Data were analysed using one-way ANOVA for multiple comparisons with post hoc Student Newman-Keuls test. \( ^{\&}p<0.05, \^{\&\&}p<0.01, \^{\&\&\&}p<0.001 \) compared with untreated control; and \( ^{*}p<0.05, \^{**}p<0.01, \^{***}p<0.001 \) compared to LPS-treated control.

2.4.14. Diosgenin inhibited the phosphorylation of Akt in LPS-stimulated BV2 cells

The signalling pathway PI3K/AKT plays a potential role in the pathogenesis of neuroinflammation-mediated neurodegenerative diseases (Maiese et al., 2012, Cohen, 2013). Akt is the main target that regulate the activity of this pathway in the brain. Several studies have indicated that NF-κB signalling is regulated, at least in part by PI3K/Akt signalling (Madrid et al., 2000, Dan et al., 2008). Since diosgenin reduced the levels of pro-inflammatory molecules such as NO and
TNFα and attenuated NF-κB activity in LPS-activated BV2 microglia, the effect of diosgenin on Akt signalling was investigated. In response to LPS stimulation, an elevated level of the phosphorylated form of Akt (p-Akt) was observed in BV2 microglia (Figure 2.28). However, the LPS-induced increase in Akt phosphorylation was significantly (p<0.001) inhibited by 5, 10, and 20 μM of diosgenin, compared to the LPS-control. Pre-treatment of stimulated BV2 microglia with diosgenin (20 μM) resulted in the expression of 20% of p-Akt level, compared to the LPS-control (Figure 2.28).

Figure 2.28 Effect of diosgenin on the Akt phosphorylation in LPS-activated BV2 cells.

BV2 cells were treated for 30 minutes with diosgenin, and then stimulated with 100 ng/mL of LPS for one hour. The levels of Akt and total Akt were measured as described above. Data are expressed as the mean ± SEM of three independent experiments. Data were analysed using one-way ANOVA for multiple comparisons with post hoc Student Newman-Keuls test. §p<0.05, §§p<0.01, §§§p<0.001 compared with untreated control; and *p<0.05, **p<0.01, ***p<0.001 compared to LPS-treated control.
2.4.15. Diosgenin exerted weak anti-inflammatory activity in Aβ-activated BV2 cells

Many studies have shown that exposure to Aβ resulted in microglial hyperactivation and neuroinflammation (Minter et al., 2016). This is known to increase the production of several pro-inflammatory and oxidative mediators such as TNFα, IL-1β, and ROS (Jekabsone et al., 2006). In this research, the anti-neuroinflammatory effect of diosgenin against Aβ-activation of BV2 microglia was also investigated. Results show that 5, 10, and 20 µM of diosgenin did not affect BV2 viability, compared to untreated cells (100% cell viability) (Figure 2.29). In addition, Aβ at this concentration did not reduce cell viability, when compared to the untreated cells (Figure 2.29).

![Figure 2.29 Effect of diosgenin on the viability of Aβ-stimulated BV2 cells.](image)

BV2 cells were treated for 30 minutes with diosgenin, and then stimulated with 2 µM of Aβ for 24 hours. Data are expressed as the mean ± SEM of three independent experiments. Data were analysed using one-way ANOVA for multiple comparisons with post hoc Student Newman-Keuls test. &p<0.05, &&p<0.01, &&&p<0.001 compared with untreated control; and *p<0.05, **p<0.01, ***p<0.001 compared to Aβ-treated control.

However, 2 µM of Aβ stimulated the cells to release a significant levels of nitrite (17.6 ± 1.1 µM) (Figure 2.30a), PGE₂ (536.8 ± 25.7 pg/mL) (Figure 2.30b), TNFα (802.2 ± 11.5 pg/mL) (Figure 2.30c) and IL-6 (463.6 ± 25.7 pg/mL) (Figure 2.30d), compared to unstimulated control. The level of each pro-inflammatory mediator
produced from Aβ-stimulated BV2 microglia is taken as 100%. However, in the presence of 20 µM of diosgenin, there was a significant reduction in the levels of nitrite (Figure 2.30a) and IL-6 (Figure 2.30d) released from the cells, compared to the Aβ-control. At 20 µM, diosgenin did not reduce the levels of PGE₂ (Figure 2.30b) and TNFα (Figure 2.30c), compared to the Aβ-control. It was observed that treatment with 5 µM and 10 µM diosgenin did not result in the reduction of the levels of all pro-inflammatory mediators in Aβ-stimulated BV2 cells (Figure 2.30).

**Figure 2.30 Effects of diosgenin on the Aβ-induced production of NO, PGE₂, TNFα and IL-6 in BV2 cells.**

BV2 cells were treated for 30 minutes with diosgenin, and then stimulated with 2 µM of Aβ for 24 hours. The levels of NO, PGE₂, TNFα and IL-6 were measured as described above. Data are expressed as the mean ± SEM of three independent experiments. Data were analysed using one-way ANOVA for multiple comparisons with post hoc Student Newman-Keuls test. Δp<0.05, ΔΔp<0.01, ΔΔΔp<0.001 compared with untreated control; and *p<0.05, **p<0.01, ***p<0.001 compared to Aβ-treated control.
2.4.16. Diosgenin inhibited the LPS-mediated indirect neurotoxicity in HT22 neuronal cells

Several studies have demonstrated that the hyper-activation of microglia and subsequent neuroinflammation are associated with neuronal death (Viviani et al., 2014, Xu et al., 2016). Since diosgenin attenuated LPS-induced neuroinflammation, it was hypothesised that diosgenin would prevent neuroinflammation-induced neuronal death. Therefore, the neuroprotective effect of diosgenin against microglial pro-inflammatory factors-induced HT22 neuronal death was examined. To identify and confirm whether LPS-induced neuroinflammation and also whether diosgenin attenuated this neuroinflammation and indirect neuronal death by inhibiting the production of microglial pro-inflammatory mediators, the levels of these factors (NO, PGE$_2$, TNFα and IL-6) in the conditioned media were firstly measured. The results showed that the levels of NO (19.3 ± 3.0 µM) (Figure 2.31a), PGE$_2$ (667.6 ± 68.5 pg/mL) (Figure 2.31b), TNFα (847.5 ± 22.2 pg/mL) (Figure 2.31c), and IL-6 (605.3 ± 53.8 pg/mL) (Figure 2.31d) in the conditioned medium prepared from LPS-treated BV2 were significantly (p<0.001) increased, compared to the untreated control. However, the levels of pro-inflammatory mediators were significantly reduced due to diosgenin treatment, compared to the LPS-control (Figure 2.31).
Figure 2.31 Effects of diosgenin on the production of NO, PGE$_2$, TNF$_\alpha$, and IL-6 in the conditioned medium of BV2 cells.

BV2 cells were treated for 30 minutes with diosgenin, and then stimulated with 1µg/mL of LPS for 24 hours. After that, the conditioned media were collected and the levels of NO, PGE$_2$, TNF$_\alpha$, and IL-6 were measured as described above. Data are expressed as the mean ± SEM of three independent experiments. Data were analysed using one-way ANOVA for multiple comparisons with post hoc Student Newman-Keuls test. *p<0.05, **p<0.01, ***p<0.001 compared with untreated control; and *p <0.05, **p<0.01, ***p<0.001 compared to LPS-treated control.

Based on these results, the direct LPS stimulation of BV2 microglia resulted in increased levels of NO, PGE$_2$, TNF$_\alpha$, IL-6 and ROS that are known to induce neuroinflammation-mediated neuronal damage (Dumont and Beal, 2011, Schieber and Chandel, 2014, Kim et al., 2015, Xu et al., 2016). Thus, the effect of conditioned medium obtained from stimulated microglia on the ROS generation in HT22 cells was investigated. The observation showed that the ROS level in HT22 neuronal cells treated with the conditioned medium of LPS-stimulated BV2 microglia was significantly (p<0.001) increased, compared to HT22 cells incubated with the conditioned medium of control BV2 microglia (Figure 2.32). However, the intracellular level of ROS in HT22 cells treated with the conditioned medium derived from BV2 microglia pre-treated with diosgenin was significantly
reduced, compared to the positive-control HT22 cells (Figure 2.37). Diosgenin at 10 µM and 20 µM showed 70% and 57.3% of ROS levels, respectively, compared to the control (Figure 2.32).

Figure 2.32 Effect of diosgenin on the ROS production in conditioned medium-treated HT22 cells.

HT22 cells were treated for 30 minutes with diosgenin, and then stimulated with conditioned medium obtained from 1 µg/mL LPS-activated BV2 microglia for 6 hours. The levels of ROS were measured as described above. Data are expressed as the mean ± SEM of three independent experiments. Data were analysed using one-way ANOVA for multiple comparisons with post hoc Student Newman-Keuls test. &p <0.05, &&p<0.01, &&&p<0.001 compared with untreated control; and *p<0.05, **p<0.01, ***p<0.001 compared to LPS-treated control.

An MTT assay was used to establish whether diosgenin exerted a neuroprotective effect against conditioned medium-induced HT22 neuronal cell death. The conditioned medium derived from BV2 cells stimulated with LPS resulted in a significant (p<0.01) reduction (44 %) in HT22 neuronal viability, compared to the untreated-control (Figure 2.33). However, the conditioned media obtained from BV2 microglia pre-treated with 10 µM and 20 µM of diosgenin significantly reduced conditioned medium-induced neurotoxicity (Figure 2.33). Treatment with 20 µM of diosgenin resulted in 90% HT22 cell viability, compared to that of untreated control. In addition, the conditioned medium obtained from untreated BV2 cells did not affect HT22 cell viability.
Figure 2.33 Effect of diosgenin on LPS-induced HT22 neuronal cell death.

BV2 cells were treated for 30 minutes with diosgenin, and then stimulated with 1 µg/mL of LPS for 24 hours. After that, the conditioned media were collected and HT22 cells were then incubated with the conditioned media. Viability of HT22 cells were determined by MTT assay. Data are expressed as the mean ± SEM of three independent experiments. Data were analysed using one-way ANOVA for multiple comparisons with post hoc Student Newman-Keuls test. *p<0.05, **p<0.01, ***p<0.001 compared with untreated control; and *p<0.05, **p<0.01, ***p<0.001 compared to LPS-treated control.
2.5. Discussion

It is well established that the microglia are associated with the pathogenesis of neuroinflammation and neuronal death (Lyman et al., 2014, Patro et al., 2016). Chronic activation of microglia and thereby, uncontrolled neuroinflammation results CNS inflammation. An excessive production of pro-inflammatory and neurotoxic factors such as NO, PGE$_2$, TNF$\alpha$, and ROS from microglia has been implicated in neurodegenerative diseases (Glass et al., 2010, Rojo et al., 2014). Thus, an inhibition of overproduction of these mediators during neuroinflammation is a key point for treatment of neuroinflammatory disorders (Sarris et al., 2011). It is well known that plant-based natural compounds play an important role in the drug discovery and CNS inflammation (Butler, 2008). In this study, the anti-neuroinflammatory effect of diosgenin was investigated in LPS-activated BV2 microglia.

Results obtained from this research show that diosgenin prevented the production of NO from BV2 microglia activated by LPS. In addition, diosgenin inhibited iNOS expression, an outcome which clearly suggests that the inhibitory effect of diosgenin on NO secretion was due to down-regulating the expression of iNOS protein. This is an interesting outcome, as iNOS has been reported to be involved in the pathogenesis of neuroinflammation-mediated neurodegenerative diseases (Ghasemi and Fatemi, 2014, Garry et al., 2015). In addition, diosgenin exhibited anti-neuroinflammatory activity against A$\beta$-induced NO production. This finding suggests that diosgenin inhibits neuroinflammatory processes which are closely linked to neurodegenerative diseases associated with NO/iNOS mechanism. Like NO, PGE$_2$ as well as the enzyme COX-2 play critical roles in microglial activation and CNS inflammation (Yagami et al., 2016, Teismann, 2012, Listi et al., 2010). In the present study, diosgenin inhibited PGE$_2$ production through reduction of the expression of COX-2 in LPS-activated BV2 cells. Thus, diosgenin could block neuroinflammation through suppressing microglial PGE$_2$/COX-2 pathway. Several studies have suggested that pro-inflammatory cytokines have been involved in the physiopathological features of neurodegenerative illnesses (McCoy and Tansey, 2008, Chadwick et al., 2008, Henry et al., 2009, Van de Veerdonk et al., 2011, Smith et al., 2012, Schaper and Rose-John, 2015). The results of this study showed that diosgenin attenuated the
production of the most important cytokines including TNFα, IL-6, and IL-1β from LPS-activated BV2 microglia. Compared to microglial pro-inflammatory cytokines, IL-10 as a microglial anti-inflammatory cytokine is produced in order to counteract neuroinflammatory responses (Iyer and Cheng, 2012, Lobo-Silva et al., 2016). It was observed that diosgenin increased the production of IL-10 in LPS-stimulated BV2 cells. This observation demonstrates that diosgenin shifts M1 (pro-inflammatory) phenotype of microglia into M2 (anti-proinflammatory) phenotype and thereby, reduces the rate of neuroinflammatory response. Similarly, microglial ROS are known as pro-neuroinflammatory and neurotoxic products (Kim et al., 2015, Schieber and Chandel, 2014). In this study, diosgenin treatment also abrogated the intracellular ROS generation in LPS-stimulated BV2 cells. This evidence suggests that the inhibition of microglial ROS generation might be one of the important effects that is responsible for mediating the anti-neuroinflammatory and antioxidant effects of diosgenin. Regarding the importance of the physiopathological role of NADPH in the microglia, diosgenin did not affect NADPH homeostasis in LPS-activated BV2 cells. The findings of this study have therefore clearly demonstrated that diosgenin produces an anti-neuroinflammatory effect against LPS-activated BV2 microglial cells, but possesses a weak anti-neuroinflammatory effect against Aβ-stimulated BV2 cells.

Diosgenin has been reported to possesses similar anti-inflammatory action in other immune and non-immune cells, thereby supporting the outcome of this study. For example, studies have shown that diosgenin inhibited palmitate-induced production of TNFα and IL-6 in endothelial cells (Liu et al., 2012). In addition, diosgenin exerted anti-inflammatory activity against LPS-induced increase in the production of some cytokines in mouse primary peritoneal macrophages (Singh et al., 2014), and in mouse lung injury (Gao et al., 2013). A study reported that diosgenin attenuated the production of several inflammatory mediators (e.g., TNFα, and NO) in co-culture model of 3T3-L1 adipocytes and RAW 264 macrophages (Hirai et al., 2010). Furthermore, diosgenin inhibited the LPS/interferon gamma (IFN-γ)-stimulated production of IL-6, NO, and ROS in RAW 264.7 murine macrophages (Jung et al., 2010a). The anti-trachea inflammatory effect of diosgenin was shown to be mediated by suppressing cytokines production (Junchao et al., 2017). This study provides the first evidence
on the action of diosgenin in reducing brain inflammation and the resulting damage to neurons. The outcome of this research also provides further information on the spectrum of anti-inflammatory activity of diosgenin and shows that its anti-inflammatory activity in brain-resident macrophages is consistent with the activity observed in other cells in the periphery.

It is well known that NF-κB regulates the expression of iNOS, COX-2 and genes encoding pro-inflammatory cytokines that secret different pro-inflammatory mediators (e.g NO, PGE₂, and TNFα) (Vega and de Andres Martin, 2008). Several findings showed that NF-κB is a potential target for the prevention and/or treatment of neuroinflammation-mediated neurodegenerative disorders (Tornatore et al., 2012, Hoesel and Schmid, 2013). Since diosgenin attenuated the expression of iNOS, COX-2 and the production of these mediators, the inhibitory effect of diosgenin on NF-kappa B activation was examined in LPS-activated BV2 microglia. Diosgenin was shown in this study to interfere with the transcriptional activity of NF-κB through the reduction in NF-κB-mediated luciferase expression in TNFα-activated HEK293 cells. Furthermore, diosgenin attenuated IκB-α phosphorylation and IκB-α degradation. Interestingly, the compound did not affect the upstream step involving IKK phosphorylation in LPS-treated BV2 microglia. Since the majority of IKK inhibitors act as ATP-competitive molecules or have allosteric effect to limit the activity of IKK, the reason for the inability of diosgenin to target the IKK step may be lacking of one of these inhibition mechanisms (Gupta et al., 2010a, Gamble et al., 2012).

Further downstream of IκB-α phosphorylation and degradation, diosgenin inhibited the nuclear translocation and DNA-binding activity of the p65 subunit of NF-κB in BV2 cells activated by LPS. Targeting the NF-κB cascade by diosgenin was consistent with previous observations. A study showed that diosgenin exhibited an immunosuppressive effect via NF-κB deactivation in murine macrophages (Jung et al., 2010a). In another study, diosgenin suppressed LPS-induced NF-κB activity in mouse lung injury (Gao et al., 2013). A review by Gupta et al. suggested that IκB-α phosphorylation was the main target of diosgenin in order to inhibit NF-κB activity (Gupta et al., 2010a). This hypothesis probably explains the lack of effect by diosgenin on IKK phosphorylation in this study, as
this step occurs upstream of IκB-α in LPS-induced activation of NF-κB signalling in most cells. This study did not use a kinase activity assay to further establish whether disogenin could produce a direct inhibition of IKK. For the first time, this research has demonstrated that disogenin inhibits neuroinflammation by targeting the molecular signalling pathway involving the IκB-α/NF-κB signalling pathway in LPS-activated BV2 microglia. The study showed that the compound did not have an effect upstream of IκB-α.

Several studies have suggested that p38 MAPK and Akt play a critical role in neuroinflammation and the pathogenesis of CNS diseases (Griffin et al., 2006, Munoz and Ammit, 2010). In this study, disogenin blocked Akt phosphorylation, but did not inhibit p38 phosphorylation in LPS-stimulated BV2 microglia. These findings demonstrate that the anti-neuroinflammatory activity of disogenin might be mediated in part via interference with Akt signalling. A number of studies have supported the results of this research. For example, disogenin suppresses NF-κB activation but not p38 MAPK activity in the peripheral immune cells macrophages (Jung et al., 2010a). Furthermore, investigations in a variety of cell types (Dan et al., 2008, Supriady et al., 2015), showed that the inhibitory effect of disogenin on IκB-α phosphorylation may be associated with inhibition of Akt phosphorylation. A similar activity has been reported for caffeine and kaempferol, which inhibited Akt-dependent NF-κB activation in microglia (Kang et al., 2012, Park et al., 2011).

Microglial pro-inflammatory mediators such as NO and ROS contribute significantly to neuroinflammation and neurotoxicity (Schieber and Chandel, 2014, Xu et al., 2016). Results of this research show that disogenin protected HT22 neuronal cells against neuronal death and ROS generation induced by conditioned medium obtained from LPS-activated BV2 microglia, through its ability to inhibit excessive production of NO, PGE₂, TNFα, IL-6.

In summary, these results demonstrate the anti-neuroinflammatory and neuroprotective effects of disogenin in BV2 microglia for the first time. In addition, the mode of action of the in vitro anti-neuroinflammatory activity of disogenin is proposed to be through the interference with microglial NF-κB activation by blocking IκB-α phosphorylation and thereby; NF-κB translocation.
Chapter 3
Nrf2 activation contributes to inhibition of neuroinflammation by diosgenin
3.1. Introduction

3.1.1. Nrf2/ARE signalling pathway in neuroinflammation

The transcription factor nuclear factor-erythroid 2-related factor 2 (Nrf2) is the most important antioxidant transcription factor in the defence system (Zhang et al., 2013). Nrf2 plays an important role in mitigating against uncontrolled neuroinflammation and oxidative stress (Innamorato et al., 2008, Sandberg et al., 2014). The fundamental role of Nrf2 is to regulate the balance between cellular damage and cellular host defence due to the pathogenicity of neuroinflammation and oxidative stress (Valko et al., 2007, Buendia et al., 2016). The pro-inflammatory and neurotoxic molecules such as cytokines, NO, and ROS that induce neuroinflammation and oxidative stress are chemically converted into less or harmless molecules by the up-regulation of Nrf2-dependent antioxidant enzymes and products (Kensler et al., 2007). This defence system is mediated by the production of direct antioxidant enzymes (e.g., heme oxygenase-1; HO-1), and detoxifying enzymes (e.g., (NADPH): quinone oxidoreductase 1; NQO1) as well as glutation and thiol homeostasis (Buendia et al., 2016).

Several studies have demonstrated that a Nrf2 signal has antioxidant and neuroprotective effects against neuroinflammation (Joshi and Johnson, 2012, Xiong et al., 2015). Furthermore, it has been shown that inhibition of the Nrf2 signal is responsible for the production of pro-inflammatory and oxidative mediators (Wardyn et al., 2015). For example, the levels of microglial inflammatory mediators such as iNOS, COX-2, IL-6, and TNFα are markedly elevated and that of anti-inflammatory markers such as interleukin 4 are reduced in Nrf2-deficient mice (Innamorato et al., 2008). Nrf2 knockout has also been shown to be responsible for increasing the expression of microglial pro-inflammatory factors such as iNOS, IL-6, and TNFα as well as the number of microglial cells due to neuroinflammation-induced LPS in mice (Innamorato et al., 2008). Regarding the role of Nrf2 in the pathology of AD, the Nrf2 target protein p62 is significantly reduced in the frontal cortex of AD patients (Salminen et al., 2012). Moreover, Nrf2 and Nrf2-dependent target genes such as NQO1 are lowered in APP/PS1 transgenic mice upon accumulation of Aβ deposits in the hippocampal neurons (Kanninen et al., 2008). These reports suggest that Nrf2 plays an important role in the regulation of neuroinflammation and oxidative
stress. In addition, Nrf2 might be an attractive therapeutic target for treating and/or preventing a number of CNS disorders that are induced by microglia-mediated uncontrolled neuroinflammation and oxidative stress (Innamorato et al., 2008, Joshi and Johnson, 2012).

3.1.1.1. Nrf2 activation
The antioxidant mechanism of action of Nrf2 signalling is summarised in Figure 3.1. In the basal condition, Nrf2 is inactivated because is combined to the negative regulator Kelch-like ECH associating protein 1 (Keap 1) in the cytoplasm of microglial cell. Once activated it is disassociated from Keap1 and translocated to the nucleus. Thereafter, Nrf2 is attached into the antioxidant responsive element (ARE) in the promoter regions of several target genes encoding phase II detoxifying and antioxidant enzymes. The most important anti-inflammatory and antioxidant proteins that are up-regulated by Nrf2 signalling including HO-1, NQO1, glutathione reductase (GR), GPX, SOD, and CAT (Joshi and Johnson, 2012). This result in the activation of a Nrf2-dependent antioxidant defence response against neuroinflammatory and oxidative assaults that exert cellular damage and neurotoxicity (Joshi and Johnson, 2012, Kim et al., 2010). Invasion of the brain by pathogens such as the bacterial product LPS results in the activation of the microglial Nrf2 pathway to activate host defence and then to eliminate the detrimental effects of the attack. For example, it is noted that a LPS-induced over-activation of microglia is significantly inhibited by over-expression of Nrf2 (Koh et al., 2011).
Figure 3.1 Proposed model for the activation of an Nrf2 signalling pathway by a Nrf2 activator in microglial cells.

Under normal conditions, microglial Nrf2 is inactivated because it is combined to the negative regulator Kelch-like ECH associating protein 1 (Keap 1) in the cytoplasm. In the presence of an Nrf2 activator, Nrf2 is disassociated from Keap1 and then translocated to the nucleus. After that, Nrf2 is attached to an antioxidant responsive element (ARE) in the promoter regions of target genes encoding phase II detoxifying and antioxidant enzymes. Subsequently, several enzymes such as heme oxygenase-1 (HO-1), nicotinamide adenine dinucleotide phosphate plus hydrogen (NAD(P)H):quinone oxidoreductase 1 (NQO1), glutathione reductase (GR), glutathione peroxidase (GPX), superoxide dismutase (SOD), and catalase (CAT) are expressed. This results in the modulation of an Nrf2-dependent antioxidant defence response (Joshi and Johnson, 2012, Kim et al., 2010, Murphy and Park, 2017).
3.1.1.2. Enzymatic and non-enzymatic antioxidants that are regulated by Nrf2

There are several enzymatic and non-enzymatic antioxidants that are up-regulated by Nrf2 in the CNS. The most important enzymes that play an important role in the antioxidant defence system are SOD, CAT, GPX, GR, HO-1, and NQO1 (Joshi and Johnson, 2012). O$_2^-$ is generated from a broad spectrum of sources including mitochondria and ROS-generated enzymes such as NADPH oxidase. As shown in Figure 3.2, O$_2$ is reduced to the primary product O$_2^-$ due to the addition of one electron by NADPH oxidase in which the electron donor NADPH is converted to NADP$^+$ and H$. After that, O$_2^-$ is rapidly reduced to more stable molecule H$_2$O$_2$ by the enzyme SOD. H$_2$O$_2$ is further converted to water (H$_2$O) by either CAT or GPX in presence of the antioxidant protein GSH as a reducing agent. The oxidized GSH (GSSG) is recycled to GSH by GR (Rojo et al., 2014, Maghzal et al., 2012). Thus, the neurotoxic effects of O$_2^-$ are reduced and/or suppressed and this results in the protection of neurons against neuroinflammation.

HO-1 is one of the most important phase II detoxifying enzyme that is highly expressed in microglia (Min et al., 2006). In addition, HO-1 expression is up-regulated in neurodegenerative diseases such as AD and PD (Schipper, 2000). HO-1 expression is induced by the stressor to prevent oxidative stress and then cellular damage. The primary function of this enzyme is to metabolize heme into bilirubin, which is subsequently converted into biliverdin, in which bilirubin and biliverdin are potent free radical scavengers in the CNS (Syapin, 2008). A variety of studies have suggested that HO-1 is a potent anti-inflammatory protein because it is responsible for the enzymatic degradation of the pro-inflammatory free heme as well as for production of some anti-inflammatory products such as bilirubin and carbon monoxide (Paine et al., 2010). These products play an essential role in the modulation of neuroinflammation and oxidative stress (Ryter et al., 2002). Increasing HO-1 expression in microglia by pharmacological agents is known to protect the brain against harmful attacks that induce neuroinflammation and oxidative stress (Lee and Suk, 2007). For example, some natural products inhibited LPS-induced inflammatory responses by increasing the expression of HO-1 in microglia (Jung et al., 2010b).
Several studies have documented that NQO1 is an important protective enzyme against inflammatory and oxidative responses in the CNS (Keum, 2012). NQO1 which is a broadly distributed cytoplasmic flavoprotein is reported to catalyse the two electrons reduction of a wide variety of substrates including quinones, quinone-imines, nitroaromatics, and azo compounds in order to prevent ROS generation (Stringer et al., 2004). Two and four-electron reductions of endogenous and exogenous quinones are catalysed to their hydroquinone forms by NQO1 in order to protect the cells (Ross et al., 2000). NQO1 is strongly expressed in many cells such as microglia (Lee et al., 2013, Lee et al., 2011). The anti-inflammatory and cytoprotective activities of NQO1 is regulated by the Keap1/Nrf2/ARE pathway (Atia et al., 2014).

![Figure 3.2 Schematic representation of the antioxidant defence system for detoxifying ROS.](image)

Molecular oxygen (O₂) is reduced to the initial product superoxide (O₂⁻) due to the addition of one electron by ROS-generated enzymes such as NADPH oxidase (NOX). The electron donor NADPH is converted to NADP⁺ and H⁺. After that, O₂⁻ is reduced to H₂O₂ by superoxide dismutase (SOD). H₂O₂ is further converted to water (H₂O) by either catalase (CAT) or glutathione peroxidase (GPX) in the presence of the antioxidant peptide glutathione (GSH) as a reducing agent. The oxidized GSH (GSSG) is recycled to GSH by glutathione reductase (GR) (Maghzal et al., 2012, Rojo et al., 2014, Di Meo et al., 2016).
3.1.2. Crosstalk between Nrf2 and NF-κB pathways

The transcription factor Nrf2 is not only an antioxidant protein that regulates the expression of detoxifying enzymes against oxidative stress, but also is an anti-inflammatory factor that controls NF-κB-mediated anti-inflammatory processes. A variety of studies have suggested that there is a cross-talk between Nrf2 and NF-κB signalling pathways in a wide variety of cells such as microglia (Buelna-Chontal and Zazueta, 2013, Bryan et al., 2013, Wardyn et al., 2015, Okorji et al., 2016). For example, Nrf2 signalling inhibits LPS-induced NF-κB activation in hyper-activated BV2 cells (Koh et al., 2011). In addition, several natural products have been shown to activate Nrf2 and inhibit NF-κB simultaneously in BV2 cells and primary microglia (Lee et al., 2011, Kang et al., 2013, Jayasooriya et al., 2014, Park et al., 2015b).

3.2. Specific Aim and Objectives

This part of the research was aimed to evaluate whether the Nrf2 signalling pathway played any role in the anti-neuroinflammatory effect of diosgenin. Therefore, this study sought to address the following specific objectives:

i. To evaluate the effect of diosgenin on Nrf2/ARE pathway in BV2 microglia
ii. To identify whether this antioxidant mechanism is involved in the anti-neuroinflammatory effect of diosgenin in LPS-activated BV2 cells

3.3. Methods

3.3.1. BV2 cell culture

Microglial BV2 cells were cultured as described in section 2.2.1. BV2 cells were left untreated in RPMI 1640 medium as a negative-control. In addition, BV2 cells were treated with 5, 10, and 20 µM of diosgenin for 30 minutes. After that, the plate was incubated for the indicated time, according to each experiment, as shown in the text.

3.3.2. HEK293 cell culture

HEK293 cells were used to study the effect of diosgenin on Nrf2 transcriptional activity. HEK293 cells were cultured as described in section 2.2.2.
3.3.3. Preparation of cytoplasmic and nuclear protein lysates

Cytoplasmic and nuclear extracts were prepared as described in section 2.2.11 to study the nuclear translocation of the transcription factor Nrf2. In addition, cytoplasmic extracts were collected to measure the amounts of HO-1 and NQO1 after diosgenin treatment. Furthermore, nuclear extracts were prepared to study the DNA-binding activity of Nrf2. BV2 cells were seeded out at a concentration of 2.0 x 10^5 cells/mL (2 mL/well) in a 6-well plate and then incubated for 48 hours. The culture medium was replaced by serum-free RPMI 1640 medium for 2 hours. Cells were treated with 5, 10, and 20 µM of diosgenin and then incubated for the indicated times.

3.3.4. Western blotting

Western blotting as described in section 2.2.13 was used to study the effect of diosgenin on the levels of Nrf2 and its antioxidant proteins (Table 6).

Table 6 List of primary and secondary antibodies used in Western blotting

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Host</th>
<th>Dilution Factor</th>
<th>Product Number</th>
<th>kDa</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>HO-1</td>
<td>Rabbit</td>
<td>1:500 WB</td>
<td>sc-10789</td>
<td>32</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>NQO-1</td>
<td>Rabbit</td>
<td>1:500 WB</td>
<td>sc-25591</td>
<td>31</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>Nrf2</td>
<td>Rabbit</td>
<td>1:500 WB</td>
<td>sc-722</td>
<td>61</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>Keap1</td>
<td>Rabbit</td>
<td>1:500 WB</td>
<td>sc-33569</td>
<td>69</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>Lamin B1</td>
<td>Rabbit</td>
<td>1:1000 WB</td>
<td>sc-20682</td>
<td>67</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>β-actin</td>
<td>Rabbit</td>
<td>1:1000 WB</td>
<td>A5060</td>
<td>42</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Goat anti-Rabbit IgG</td>
<td>Goat</td>
<td>1:10000 WB</td>
<td>A-21076</td>
<td>Life technologies</td>
<td></td>
</tr>
</tbody>
</table>

3.3.5. Measurement of GSH levels

The reduced form GSH is a potent non-enzymatic antioxidant that plays a key role in the regulation of microglial redox signalling and neuroinflammation (Rojo et al., 2014). GSH is known to protect neurons against the pathogenesis of neuroinflammation and oxidative stress (Aoyama and Nakaki, 2013, Lee et al.,
The amount of GSH in BV2 microglial cells was determined using GSH-Glo glutathione assay kit (Promega), according to the manufacturer’s instructions. The luminogenic substrate luciferin derivative (Luc-NT) is converted into luciferin by the enzyme glutathione S-transferase (GST) in the presence of glutathione. After that, luciferin is oxidized by Ultra-Glo luciferase in the presence of ATP, Mg$^{2+}$ and O$_2$, and resulted in production of extra energy in form of light (Figure 3.3). Cells were seeded out at a concentration of $1.0 \times 10^5$ cells/mL ($100 \mu$l/well) in a 96-well plate and incubated overnight. BV2 cells were then treated with 5, 10, and 20 µM of diosgenin for 24 hours. GSH-Glo reagent was prepared by adding Luciferin-NT and GST to the GSH-Glo reaction buffer. Carefully, the medium was replaced with GSH-Glo reagent 1X and the plate was incubated at room temperature for 30 minutes with shaking. Next, 100 µL of Luciferin detection reagent was added and incubated at room temperature for 15 minutes with shaking. Luminescence was measured using a FLUOstar OPTIM reader (BMG LABTECH) (Romero and Mueller-Klieser, 1998, Mourad et al., 2000).

![Figure 3.3 Chemical reactions involved in the measurement of glutathione (GSH).](image)

The luminogenic substrate luciferin derivative (Luc-NT) is converted to luciferin by the enzyme glutathione S-transferase (GST) in the presence of GSH. After that, luciferin is oxidized by luciferase in the presence of adenosine triphosphate (ATP), magnesium (Mg$^{2+}$) and molecular oxygen O$_2$, and resulted in the production of extra energy in the form of light (Li et al., 2013, Gorman et al., 1982).

### 3.3.6. Immunofluorescence

The effect of diosgenin on the nuclear accumulation of Nrf2 was detected using an indirect immunofluorescence assay as described in section 2.2.15. BV2 cells were seeded out at a concentration of $2.0 \times 10^5$ cells/mL (1 mL/well) in a 24-well plate and incubated for 48 hours. After changing the medium, the cells were treated with 5, 10, and 20 µM of diosgenin for 24 hours in order to investigate the
Nrf2 level. Untreated cells were used as negative control. The cells were incubated with a primary antibody against Nrf2 at 4°C for overnight.

**3.3.7. Luciferase reporter gene assay**

Binding of Nrf2 to DNA at its binding site results in the transcriptional activation of target genes that regulate the immune response and the neuroinflammatory process (Vega and de Andres Martin, 2008, Joshi and Johnson, 2012). Therefore, the effect of disogenin on Nrf2-dependent transcriptional activity was evaluated in cultured HEK293 cells using the antioxidant response element (ARE) luciferase reporter gene assay. HEK293 cells were seeded out as described in section 2.2.2. The cells were transfected with 100 ng total DNA of pGL4.37[luc2P/ARE/Hygro] (Promega) and pGL4.75 [hR luc/CMV] Renilla luciferase vectors (Promega), respectively FuGENE 6 transfection reagent (Promega) at a 3:1 transfection reagent:DNA ratio, and further incubated for 18 hours. Thereafter, the medium was replaced by Opti-MEM I reduced-serum medium and then incubated for 6 hours. The transfected cells were treated with 5, 10, 20 µM of diosgenin, and further incubated for 18 hours. 100 µL of Dual-Glo Luciferase reagent (Promega, UK) containing Dual-Glo Luciferase buffer and Dual-Glo Luciferase substrate was added into each well. After 10 minutes, firefly luminescence was measured using FLUOstar OPTIMA plate reader (BMG LABTECH). 100 µL of Dual-Glo stop & Glo reagent was added for 10 minutes and Renilla luminescence was then measured using the plate reader. The amounts of firefly luciferase activity values were normalized to Renilla luciferase activity values.

**3.3.8. DNA binding activity of Nrf2**

It is well known that Nrf2 binds to the DNA at its specific binding site to regulate the expression of several antioxidants and Phase II detoxification genes that play an important role in the regulation of the immune response, neuroinflammation, and oxidative stress (Kim et al., 2010, Joshi and Johnson, 2012). The effect of diosgenin on the DNA-binding activity of Nrf2 was studied as part of its antioxidant activity. The effect of diosgenin on the DNA-binding activity of Nrf2 was evaluated using the TransAM Nrf2 kit (Active Motif), according to the manufacturer’s instructions. BV2 cells were seeded out at a concentration of 2.0 x 10^5 cells/mL (2 mL/well) in a 6-well plate and incubated for 48 hours. The medium was
replaced by serum-free RPMI 1640 medium for 2 hours. The cells were treated with 5, 10, and 20 µM of diosgenin, and then incubated for one hour. Untreated cells were used as negative control. After that, nuclear extracts were prepared as described in section 3.3.3. Nuclear extracts containing activated transcription factors were incubated in a 96-well plate coated with a specific double-stranded oligonucleotides containing ARE consensus-binding site (5′-GTCACAGTGACTCAGCAGAA-TCTG-3′). The cells were incubated with Nrf2 antibody for one hour.

**3.3.9. Nrf2 siRNA transfection**

The purpose of RNA interference (RNAi) was to determine whether the anti-neuroinflammatory effect of diosgenin is dependent on Nrf2 activity. RNAi is the process by which the expression of a target gene is effectively silenced or knocked down by the selective inactivation of its corresponding mRNA by double-stranded RNA (dsRNA) molecule (Figure 3.4) (Fire et al., 1998). RNAi as a post-transcriptional gene silencing is a process to block the expression of specific protein such as Nrf2. It is important to note that the absence of or the low level of the target protein can be detected by western blotting analysis.

BV2 cells were seeded out at a concentration of 2.0 x 10⁵ cells/mL in a 6-well plate. The plate was incubated until the cells achieved approximately 60-70% confluence. For each transfection, 2 µL of 100 nM Nrf2 siRNA duplex (Santa Cruz Biotechnology) and 2 µL of siRNA transfection reagent (Santa Cruz Biotechnology) were diluted in Opti-MEM I reduced serum medium, and incubated for 30 minutes at room temperature. BV2 cells transfected with 100 nM control siRNA (Santa Cruz Biotechnology) were used as control siRNA-transfected cells. After washing, Opti-MEM I reduced serum medium was added to each transfection complex, and then 200 µL of siRNA transfection reagent mixture was added, and the plate incubated for 6 hours. The medium was replaced with RPMI 1640 medium, and the plate incubated for an additional 18 hours. After that, transfected BV2 cells were treated with 20 µM of diosgenin for 30 minutes followed by LPS stimulation for 24 hours. Gene knockdown of Nrf2 protein expression was confirmed by immunoblotting using lysates of control siRNA-transfected and Nrf2 siRNA-transfected BV2 cells. The effects of 20 µM of diosgenin on NO, TNFα, IL-6, and PGE₂ production in LPS-stimulated control
siRNA-transfected and Nrf2 siRNA-transfected BV2 cells were investigated as described in sections 2.2.6, 2.2.7.1, 2.2.7.2, and 2.2.8, respectively.

The levels of the phospho-NF-κBp65 in the nuclear extracts of control siRNA-transfected and Nrf2 siRNA-transfected BV2 cells were determined using phospho-NFκB p65 (Ser536) InstantOne ELISA Kit (Biolegend) according to the manufacturer’s protocol. 50 μL of nuclear extract was added to each of the wells in a 96-well plate. 50 μL of antibody cocktail containing 25 μL of capture antibody reagent and 25 μL of detection antibody reagent was added and the plate was then incubated at room temperature for one hour with shaking at 300 g. The wells were washed with 200 μL of wash buffer. Next, 100 μL of a detection reagent was added and the plate was incubated at room temperature for 30 minutes with shaking at 300 g. 100 μL of stop solution was added to stop the reaction and the absorbance was then measured at 450 nm using a microplate reader (Tecan Infinite F50).

The effect of Nrf2 silencing on the inhibitory effect of diosgenin on LPS-induced DNA-binding activity of NF-κB in BV2 cells was also investigated. The nuclear lysates of both control siRNA-transfected and Nrf2 siRNA-transfected BV2 cells were collected to measure NF-κB/DNA binding activity. The protocol of this experiment was described as showed in section 3.3.3.
Figure 3.4. Model of the RNA interference pathway.

The long double-stranded RNA molecule is cleaved to small interfering RNA (siRNA) by Dicer. One of the siRNA strands is cleaved and dissociated from the complex upon RISC activation. The other strand remains in the complex, and the activated RISC is attached to the target mRNA. This resulted in cleavage and then degradation of mRNA (Petrova et al., 2013, Meister and Tuschl, 2004, Aagaard and Rossi, 2007).
3.3.10. Statistical Analysis

Values are expressed as the mean ± SEM of three independent experiments. Data were analysed using one-way ANOVA for multiple comparisons with post-hoc Student Newman-Keuls test. The ANOVA compares the mean between the groups and determine whether those means are significantly different from each other. To determine the specific groups that are significantly different from each other were evaluated by with post-hoc Student Newman-Keuls test with multiple comparisons. Calculations for statistical analysis were performed with Graph Pad Prism software version 5. Differences were significant at $p<0.05$. For Nrf2 experiments, designations include $^&p<0.05$, $^&&p<0.01$, $^&&&p<0.001$ compared with untreated control. For siRNA experiments: $^@p<0.05$, $^{&&}p<0.01$, $^{&&&}p<0.001$ as compared within the groups of untreated control. $^$p<0.05, $^{&&}p<0.01$, $^{&&&}p<0.001$ as compared within the groups of LPS-treated control. $^*p<0.05$, $^{##}p<0.01$, $^{###}p<0.001$ as compared within the groups of pre-treated with 20 µM of diosgenin.
3.4. Results

3.4.1. Diosgenin enhanced HO-1, NQO1, and GSH levels in BV2 microglia

The transcription factor Nrf2 plays an important role in the regulation of microglial dynamics and neuroinflammation through the expression of antioxidant proteins like HO-1, NQO1 and GSH (Joshi and Johnson, 2012, Rojo et al., 2014). Experiments were carried out to achieve the overall objective of establishing a role for Nrf2 in the anti-neuroinflammatory effect of diosgenin in BV2 microglia. In this part of the study, the effects of diosgenin on the levels of Nrf2-regulated antioxidant proteins HO-1, NQO1 and GSH are shown. Results of western blotting showed that treatment of cells with diosgenin resulted in a marked increase in the levels of HO-1 protein, when compared to control cells (Figure 3.5). The levels of HO-1 were increased by 1-, 5.0- and 6.5-folds after treatment of cells with 5, 10 and 20 μM of diosgenin, respectively, compared to a non-treated control (Figure 3.5).
Figure 3.5 Effect of diosgenin on HO-1 levels in BV2 cells.

BV2 cells were treated with diosgenin for 24 hours. The total levels of HO-1 were measured as described above. Data are expressed as the mean ± SEM of three independent experiments. Data were analysed using one-way ANOVA for multiple comparisons with post hoc Student Newman-Keuls test. *p<0.05, **p<0.01, ***p<0.001 compared with untreated control.

Next, the results showed that diosgenin at 10 µM and 20 µM produced a marked (p<0.001) increase in the NQO1 expression, compared to that of untreated cells (Figure 3.6). Compared to untreated cells, 3.5-fold and 6.0-fold increases in the NQO1 levels were observed with 10 µM and 20 µM of diosgenin, respectively. However, at 5 µM, diosgenin treatment did not produce any significant increase in NQO1 expression.
Figure 3.6 Effect of diosgenin on NQO1 levels in BV2 cells.

BV2 cells were treated with diosgenin for 24 hours. The total levels of NQO1 were measured as described above. Data are expressed as the mean ± SEM of three independent experiments. Data were analysed using one-way ANOVA for multiple comparisons with post hoc Student Newman-Keuls test. \(^{\&}p<0.05, \^{\&\&}p<0.01, \^{\&\&\&}p<0.001\) compared with untreated control.

Since diosgenin significantly induced the expression of HO-1 and NQO1 in BV2 microglia at concentrations of 10-20 µM, it was hypothesised that diosgenin elevated the levels of the antioxidant product GSH in the cells. GSH is an important antioxidant peptide in the cellular defence system because the activity of GSH is regulated by the transcription factor Nrf2 (Rojo et al., 2014, Vilhardt et al., 2017). As observed in Figure 3.7, diosgenin treatment induced a significant elevation (p<0.05) in GSH levels, compared to the control cells. Diosgenin treatment at 5, 10, and 20 µM caused 0.3-, 0.46- and 0.65-fold increases in the GSH levels, respectively, compared to untreated cells.
Figure 3.7 Effect of diosgenin on GSH levels in BV2 cells.

BV2 cells were treated with diosgenin for 24 hours. The levels of GSH were measured as described above. Data are expressed as the mean ± SEM of three independent experiments. Data were analysed using one-way ANOVA for multiple comparisons with post hoc Student Newman-Keuls test. \( ^{\&}p<0.05, ^{\&\&}p<0.01, ^{\&\&\&}p<0.001 \) compared with untreated control.

3.4.2. Diosgenin increased the nuclear translocation of Nrf2 in BV2 microglia

The main mechanism of the antioxidant defence system that regulates neuroinflammation and oxidative stress is the activation of Nrf2 (Sandberg et al., 2014). Western blotting results show that diosgenin significantly reduced (\( p<0.001 \)) the level of Nrf2 protein in the cytoplasm (Figure 3.8), compared to that of untreated cells. The cytoplasmic level of Nrf2 was lowered 7.0- and 8.0-fold with 10 \( \mu \)M and 20 \( \mu \)M of diosgenin, respectively, compared to the control BV2 microglia.
Figure 3.8 Effect of diosgenin on the cytoplasmic level of Nrf2 in BV2 cells.

BV2 cells were treated with diosgenin for one hour. The cytoplasmic levels of Nrf2 were measured as described above. Data are expressed as the mean ± SEM of three independent experiments. Data were analysed using one-way ANOVA for multiple comparisons with post hoc Student Newman-Keuls test. &p<0.05, &&p<0.01, &&&p<0.001 compared with untreated control.

Expectedly, when cells were incubated with diosgenin, we observed a significant increases in the nuclear levels of nuclear Nrf2 protein (suggesting an increase in nuclear translocation of Nrf2), in comparison with untreated BV2 cells (Figure 3.9). It was observed that 5, 10 and 20 μM of diosgenin induced 1.75-, 3.5- and 6.0-fold increases in the nuclear level of Nrf2, respectively.
Figure 3.9 Effect of diosgenin on the nuclear level of Nrf2 in BV2 cells.

BV2 cells were treated with diosgenin for one hour. The nuclear levels of Nrf2 were measured as described above. Data are expressed as the mean ± SEM of three independent experiments. Data were analysed using one-way ANOVA for multiple comparisons with post hoc Student Newman-Keuls test. &p<0.05, &&p<0.01, &&&p<0.001 compared with untreated control.

Furthermore, the diosgenin-induced increase in Nrf2 nuclear accumulation observed was confirmed with immunofluorescence analysis. This showed that diosgenin treatment significantly increased the nuclear translocation of Nrf2, compared to that of untreated cells (Figure 3.10).
Figure 3.10 Effect of diosgenin on Nrf2 expression in BV2 cells after immunofluorescence staining.

The BV2 cells were either left untreated (negative control) or pre-treated with the indicated concentrations of diosgenin for 24 hours. The green fluorescence represents the location and levels of Nrf2 in the cytoplasmic and nuclear zones. BV2 cells were stained with the counterstain DABI for visualization of DNA (blue fluorescence). (Scale bar= 100 µm).

3.4.3. Diosgenin inhibited Keap 1 activity in BV2 cells

Under basal conditions, the transcription factor Nrf2 is a constitutively inactivated protein in the cytoplasm because it is complexed to the inhibitory protein Keap1 (Kim et al., 2010, Joshi and Johnson, 2012). The previous results showed that diosgenin increased the accumulation of the protein Nrf2 in the nucleus of BV2 microglial cells. It was therefore important to investigate whether diosgenin-mediated Nrf2 nuclear translocation was achieved through a modulation of Keap 1 activity. Results indicated that diosgenin significantly reduced the level of Keap1
in the cytoplasm, compared to that of control BV2 cells (Figure 3.11). Treatment with 10 µM and 20 µM of diosgenin resulted in 5.2- and 6.0-fold down-regulation in expression of the Keap1 protein, respectively, compared to the control cells.

![Figure 3.11 Effect of diosgenin on Keap 1 levels in BV2 cells.](image)

BV2 cells were treated with diosgenin for 24 hours. The levels of Keap1 were measured as described above. Data are expressed as the mean ± SEM of three independent experiments. Data were analysed using one-way ANOVA for multiple comparisons with post hoc Student Newman-Keuls test. *p<0.05, **p<0.01, ***p<0.001 compared with untreated control.

3.4.4. Diosgenin increased the DNA-binding activity of Nrf2 in BV2 cells

Nrf2 possesses two important domains; a distinct transactivation and a DNA binding domain that are essential for its transcriptional activity. As observed above, diosgenin treatment increased the nuclear accumulation of Nrf2 in BV2 cells. Further experiments to determine whether diosgenin increase DNA-binding activity of Nrf2 in BV2 microglial cells showed that the compound produced a
significant increase in the DNA-binding activity of Nrf2, when compared to untreated control cells (Figure 3.12). Diosgenin at 10 µM and 20 µM resulted in 5.0- and 7.0-fold increase in the Nrf2/DNA binding activity, respectively, compared to that of control cells.

![Graph showing effect of diosgenin on Nrf2/DNA binding activity in BV2 cells.]

**Figure 3.12 Effect of diosgenin on Nrf2/DNA binding activity in BV2 cells.**

BV2 cells were treated with diosgenin for one hour. The Nrf2/DNA binding activity was measured as described above. Data are expressed as the mean ± SEM of three independent experiments. Data were analysed using one-way ANOVA for multiple comparisons with post hoc Student Newman-Keuls test. *p<0.05, **p<0.01, ***p<0.001 compared with untreated control.

### 3.4.5. Diosgenin increased the ARE-luciferase activity in HEK293 cells

Following nuclear translocation, the Nrf2 protein is bound to specific elements called antioxidant responsive element (ARE) that are located on the promoters of target genes encoding antioxidant enzymes. To determine whether diosgenin increase the transcriptional activity of Nrf2, ARE-dependent luciferase activity was measured in HEK293 cells. Results in Figure 3.13 show that ARE-luciferase activity was significantly increased (p<0.001) by diosgenin when compared to untreated cells. Treatment of HEK293 cells with 5, 10 and 20 µM of diosgenin induced a 1.6-, 2.3- and 3.4-fold increase in the ARE-dependent luciferase activity, respectively, in comparison to the untreated cells.
Figure 3.13 Effect of diosgenin on ARE-luciferase activity in HEK293 cells.

Transfected HEK293 cells were treated for 6 hours with diosgenin. The intensities of Firefly and Renilla luciferase activities were measured as described above. Data are expressed as the mean ± SEM of three independent experiments. Data were analysed using one-way ANOVA for multiple comparisons with post hoc Student Newman-Keuls test. *p<0.05, **p<0.01, ***p<0.001 compared with untreated control.

3.4.6. The Anti-neuroinflammatory effect of diosgenin is dependent on Nrf2 activity in LPS-stimulated BV2 microglia

It has been demonstrated that there is a cross-talk between Nrf2 and NF-κB signalling pathways (Buelna-Chontal and Zazueta, 2013, Wardyn et al., 2015). It has also been reported that the absence of an Nrf2 response is responsible for the production of several pro-inflammatory and oxidative mediators and thereby, neuroinflammation in neurodegenerative conditions (Wardyn et al., 2015, Buendia et al., 2016). Since diosgenin has been shown in this research to inhibit neuroinflammation, as well as as increasing direct activation of Nrf2 in BV2 microglia, siRNA was used to determine whether the anti-neuroinflammatory effect of diosgenin was dependent on the up-regulation of the Nrf2 gene.

Nrf2 siRNA duplex was transfected into BV2 cells, and then treated with 20 µM of diosgenin for 30 minutes followed by 100 ng/mL LPS for 24 hours. The effects diosgenin on the production of NO, PGE₂, TNFα and IL-6 in both LPS-stimulated control siRNA-transfected and Nrf2 siRNA-transfected BV2 cells were investigated. Compared to the unstimulated control, LPS stimulated the cells to
release significant amounts of nitrite (19.3 ± 2.5 and 18.0 ± 3.8 µM) (Figure 3.14a), PGE₂ (535.5 ± 95.2 and 543.5 ± 93.9 pg/mL) (Figure 3.14b), TNFα (711.8 ± 47.1 and 700.4 ± 21.2 pg/mL) (Figure 3.14c) and IL-6 (565.0 ± 69.9 and 580.3 ± 71.6 pg/mL) (Figure 3.14d) in control siRNA-transfected and Nrf2 siRNA-transfected BV2 cells, respectively. The level of each pro-inflammatory mediator produced by LPS in control siRNA-transfected BV2 microglia is taken as 100%. It was observed that 20 µM diosgenin caused a significant reduction (p<0.001) in the concentrations of NO (7.8 ± 2.5 µM) (Figure 3.14a), PGE₂ (250.3 ± 46.2 pg/mL) (Figure 3.14b), TNFα (373.5 ± 10.0 pg/mL) (Figure 3.14c) and IL-6 (323.9 ± 23.1 pg/mL) (Figure 3.14d) that were induced by LPS in the control siRNA-transfected BV2 microglia. However, silencing of Nrf2 gene reversed the inhibitory effect of diosgenin (20 µM) on the production of these pro-inflammatory factors, compared to control cells.
Figure 3.14 Effects of diosgenin on NO (a), PGE\(_2\) (b), TNFα (c) and IL-6 (d) production in LPS-stimulated control siRNA-transfected and Nrf2 siRNA-transfected BV2 cells.

Both control siRNA-transfected and Nrf2 siRNA-transfected BV2 cells were treated for 30 minutes with 20 µM of diosgenin, and then stimulated with 100 ng/mL of LPS for 24 hours. The levels of NO, PGE\(_2\), TNFα and IL-6 were measured as described above. Data are expressed as the mean ± SEM of three independent experiments. Data were analysed using one-way ANOVA for multiple comparisons with post hoc Student Newman-Keuls test. *p<0.05, **p<0.01, ***p<0.001 as compared within the groups of untreated control. $p<0.05, $$p<0.01, $$$p<0.001 as compared within the groups of LPS-treated control. #p<0.05, ##p<0.01, ###p<0.001 as compared within the groups of pre-treated with 20 µM of diosgenin.

Since diosgenin exerted an anti-neuroinflammatory effect via inhibiting NF-κB signalling in LPS-stimulated BV2 cells (Chapter 2), it became necessary to investigate whether Nrf2 was required for the inhibition NF-κB activity by diosgenin in these cells. Results in Figure 3.15 show that diosgenin treatment (20 µM) showed a significant reduction (p<0.01) in LPS-induced increase in protein levels of p-NF-κB p65 sub-unit in control siRNA-transfected BV2 microglia. On the other hand, Nrf2 silencing reversed the NF-κB inhibitory effect of diosgenin,
compared to the control cells, suggesting that an interaction with the Nrf2 gene contributes to the inhibition of NF-κB activity by diosgenin (Figure 3.15).

Figure 3.15 Effect of diosgenin on LPS-induced increase in p-p65 level in LPS-stimulated control siRNA-transfected and Nrf2 siRNA-transfected BV2 cells.

Both control siRNA-transfected and Nrf2 siRNA-transfected BV2 cells were treated for 30 minutes with 20 μM of diosgenin, and then stimulated with 100 ng/mL of LPS for one hour. The levels of p-p65 in the nuclear lysates were measured as described above. Data were analysed using one-way ANOVA for multiple comparisons with post hoc Student Newman-Keuls test. \( \$ p<0.05, \$\$ p<0.01, \$\$\$ p<0.001 \) as compared within the groups of untreated control. \( \# p<0.05, \#\# p<0.01, \#\#\# p<0.001 \) as compared within the groups of LPS-treated control. \( \#\#\# p<0.001 \) as compared within the groups of pre-treated with 20 μM of diosgenin.

Furthermore, the effect of diosgenin (20 μM) on the DNA-binding activity of NF-κB was examined in the absence of the Nrf2 gene. The compound significantly (p<0.001) inhibited the LPS-induced DNA-binding activity of NF-κB in control siRNA-transfected BV2 cells (Figure 3.16). In contrast, diosgenin did not inhibit the binding of NF-κB into DNA in LPS-stimulated Nrf2 siRNA-transfected BV2 microglia as observed in Figure 3.16.
Figure 3.16 Effect of diosgenin on LPS-induced increase in DNA-binding activity of NF-κB in LPS-stimulated control siRNA-transfected and Nrf2 siRNA-transfected BV2 cells.

Both control siRNA-transfected and Nrf2 siRNA-transfected BV2 cells were treated for 30 minutes with 20 µM of diosgenin, and then stimulated with 100 ng/mL of LPS for one hour. DNA-binding activity of NF-κB in the nuclear lysates were measured as described above. Data are expressed as the mean ± SEM of three independent experiments. Data were analysed using one-way ANOVA for multiple comparisons with post hoc Student Newman-Keuls test. *p<0.05, **p<0.01, ***p<0.001 compared with untreated control; and *p<0.05, **p<0.01, ***p<0.001 compared to LPS-treated control.
3.5. Discussion

The transcription factor Nrf2 plays an anti-neuroinflammatory role against harmful stimuli that induce neuroinflammation in the CNS (Zhang et al., 2013, Sandberg et al., 2014). A number of studies have found that Nrf2 has antioxidant and neuroprotective effects against neuroinflammation (Joshi and Johnson, 2012, Xiong et al., 2015). Numerous studies have observed that Nrf2 inhibits NF-κB-mediated neuroinflammation in various cell lines such as microglia (Lee et al., 2011, Kang et al., 2013, Jayasooriya et al., 2014, Park et al., 2015b). Innamorato et al., have also reported that Nrf2 knockout resulted in increases in the production of microglial pro-inflammatory mediators in mice treated with LPS (Innamorato et al., 2008). Therefore, Nrf2 is a therapeutic target against neurodegenerative diseases that are associated with microglia-induced neuroinflammation (Calkins et al., 2009, Joshi and Johnson, 2012).

Results of investigations presented in Chapter 2 show that diosgenin inhibits neuroinflammation through inactivation of NF-κB in LPS-stimulated BV2 microglia, the next focus of this research was to determine whether diosgenin reduce neuroinflammation via Nrf2-dependent mechanisms. It is important to note that the defence against neuroinflammation is mediated through the production of direct antioxidant enzymes such as HO-1, and detoxifying enzymes like NQO1, as well as GSH and thiol homeostasis (Rojo et al., 2014, Vilhardt et al., 2017, Buendia et al., 2016). Diosgenin increased levels of the antioxidant HO-1, an anti-inflammatory protein which is known to block neuroinflammation (Ryter et al., 2002, Paine et al., 2010). This finding thus seems to suggest that diosgenin may be reducing the intensity of neuroinflammation by inducing HO-1 expression in microglia. This cytoprotective mechanism has been observed with some natural products that inhibited LPS-induced inflammatory responses through increasing the expression of HO-1 in microglia (Jung et al., 2010b, Lee et al., 2011, Foresti et al., 2013). Similarly, diosgenin elevated the amount of NQO1, which has also been shown to be effective against inflammatory and oxidative responses (Keum, 2012). In addition, diosgenin increased the level of the non-enzymatic antioxidant peptide GSH in BV2 microglia. GSH is known to protect neurons against the pathogenesis of neuroinflammation (Lee et al., 2010, Aoyama and Nakaki, 2013). Taken together, it appears diosgenin induces gene
expression of HO-1, NQO1 and GSH in BV2 microglia and thereby activating protective mechanisms against neuroinflammation.

It is well known that Nrf2 is responsible for the regulation of HO-1 and NQO1 expression as well as GSH levels in microglia (Kim et al., 2010, Joshi and Johnson, 2012). Since diosgenin induced the expression of these antioxidant proteins, it was expected that Nrf2 might play an essential role in the ability of diosgenin to increase their levels in BV2 microglia. Nrf2, as a modular protein, has distinct transactivation and DNA-binding domains that are essential for its transcriptional activity in the nucleus. Firstly, the result of this study showed that diosgenin increased antioxidant responsive element (ARE)-mediated luciferase activity, suggesting that diosgenin elevated the levels of HO-1, NQO1, and GSH by induction of ARE-mediated gene expression. In addition, diosgenin increased nuclear translocation of Nrf2 and also the DNA-binding activity of Nrf2 in BV2 microglia. Under normal conditions, Nrf2 is an inactive protein because of a combination with the inhibitory protein Keap 1. Upon Nrf2 pathway induction, Nrf2 is disassociated from the Keap1 protein that is enzymatically degraded. This results in a rapid nuclear translocation of Nrf2 followed by binding to ARE sequences. This leads to the secretion of cytoprotective antioxidant products against neuroinflammation. The result showed that diosgenin down-regulated the expression of Keap 1 protein, thereby suggesting that disassociation of Nrf2 from the protein Keap 1 plays an important role in diosgenin-induced nuclear translocation and thereby activation of Nrf2 in BV2 microglia. However, this study did not establish whether diosgenin may be inhibiting Keap1 activity by non-covalent direct inhibition of the keap1-Nrf2 protein-protein interaction or by interacting with the sulfhydryl groups of cysteine residues in Keap 1 by oxidation or alkylation (Abed et al., 2015). The results of this study clearly show for the first time that diosgenin induce Nrf2/Keap1/ARE signalling pathway in BV2 microglia. This outcome may contribute to the suggestion that diosgenin possibly targets molecular mechanisms which mitigate against neuroinflammation-mediated neurodegenerative diseases.

Several studies have suggested that a cross-talk between NF-κB and Nrf2 pathways have been observed in various cell types including microglia (Buelna-Chontal and Zazueta, 2013, Bryan et al., 2013). It is established that activation of
Nrf2 results in inhibition of NF-κB, and subsequently neuroinflammation (Koh et al., 2011). For example, a number of compounds (e.g., xanthohumol, quercetin and β-hydroxyisovalerylshikonin) attenuated the chronic microglial activation-induced neuroinflammatory response by Nrf2-dependent NF-κB inhibition (Lee et al., 2011, Kang et al., 2013, Jayasooriya et al., 2014). To verify whether the inhibition of NF-κB activation, and subsequently the neuroinflammation by diosgenin is dependent on Nrf2, the signal of the latter was blocked by Nrf2 gene knockdown. Results show that diosgenin reduced the levels of microglia-derived pro-inflammatory mediators (NO, PGE₂, TNFα, and IL-6) in LPS-stimulated control siRNA-transfected BV2 cells. However, the inhibitory effect of diosgenin was reversed by silencing Nrf2 in LPS-stimulated Nrf2 siRNA-transfected BV2 cells. These findings suggest that the anti-neuroinflammatory effect of diosgenin was dependent on Nrf2 activation. Next, to clarify whether diosgenin did not inhibit neuroinflammatory process because of Nrf2 knockdown, the result showed that diosgenin did not inhibit NF-κB over-activation induced by LPS after Nrf2 knockdown, compared to control cells. This finding suggest that siRNA-mediated knockdown Nrf2 gene expression prevents the anti-neuroinflammatory effect of diosgenin in LPS-activated BV2 microglia. Therefore, the anti-neuroinflammatory activity of diosgenin is dependent on the activation of Nrf2 in microglial BV2 cells in order to block neuroinflammation. In summary, the present study demonstrates that diosgenin activates Nrf2/ARE defence mechanism in BV2 microglia. In addition, the anti-neuroinflammatory effect of diosgenin seems to be dependent on the activation of Nrf2.
Chapter 4
Miscellaneous pharmacological activities of diosgenin in BV2 microglia
4.1. Introduction
4.1.1. Role of oestrogens in neuroinflammation

Oestrogens are an entire class of related hormones including oestriol, 17β-oestradiol, and oestrone. It is well known that 17β-oestradiol is the most potent non-selective oestrogen in the body (Cui et al., 2013). 17β-oestradiol is responsible for the development and regulation of the female reproductive system and secondary sex characteristics (Santoro et al., 2016). In addition, it plays anti-neuroinflammatory, antioxidant and neuroprotective actions in both sexes (Spence and Voskuhl, 2012, Laredo et al., 2014). In general, the oestrogen’s physiological functions including their anti-neuroinflammatory effect are mediated through the activation of two classes of ER; ERβ and ERα (Laredo et al., 2014). Due to importance of ER in the development and functions of the CNS, ERα and ERβ are distributed in most regions of the CNS including hypothalamus, hippocampus and cortex (Cui et al., 2013). Specifically, ERα and ERβ are expressed in neurons and a variety of glial cells (Sierra et al., 2008, Mhyre and Dorsa, 2006). In the mammalian brain of both sexes, oestrogens are synthesised in neurons and astrocytes by aromatase (Garcia-Segura, 2008). However, 17β-oestradiol is not synthesised in microglia that express ERα and ERβ (Yague et al., 2010, Ishihara et al., 2015).

17β-oestradiol is an endogenous therapeutic hormone that can prevent and/or treat neurodegenerative diseases (Correia et al., 2010, Arevalo et al., 2015). Several findings have demonstrated that low level of oestrogens are associated with excessive inflammation and thereby, postmenopausal symptoms such as arthritis, osteoporosis, and AD (Yang et al., 2010, Islander et al., 2011, Lobo et al., 2014). A number of observations have reported that oestrogen replacement therapy is effective against postmenopausal symptoms and neurodegenerative disorders (Sherwin, 2006). However, a long-term use of oestrogen replacement therapy can result in breast cancer, suggesting the role of non-specific oestrogenic effects of oestrogens (Lai et al., 2013, Nilsson et al., 2001).

The anti-neuroinflammatory and neuroprotective activities of oestrogens in the CNS have been extensively studied but not completely understood. Oestrogens protect the neurons directly or indirectly by modulating neuroinflammation (Vegeto et al., 2006). Due to the induction role of the hyper-activated microglia in
the excessive neuroinflammatory response and thereby, neuronal death, one of the anti-neuroinflammatory roles of oestrogens is mediated by suppression of microglial over-activation (Vegeto et al., 2003, Habib and Beyer, 2015). For example, oestrogens through activation of microglial ER attenuated the hyper-activated microglia-mediated neuroinflammation and cell death (Liu et al., 2005, Smith et al., 2011). In addition, oestrogens and ER agonists up-regulated the expression of ERα and ERβ in rat primary microglia and attenuated LPS toxicity-induced cell death (Smith et al., 2011). Furthermore, the elevated enzymatic levels of iNOS and COX-2 induced by LPS attenuated by oestrogens through activation of ERβ in BV2 microglia (Baker et al., 2004). 17β-oestradiol reduced LPS-induced microglial activation through increasing the expression of ERα in the male and ovariectomised female rats (Tapia-Gonzalez et al., 2008). It inhibited the expression of pro-inflammatory mediators including MCP-1, MIP-2, and TNFα mediated by the ligand LPS in the AD model (Vegeto et al., 2006). Oestrogens increased the level of the anti-inflammatory cytokine IL-10 and reduced the levels of TNFα and IFN-γ in LPS-stimulated microglial N9 cells. Oestrogens inhibited LPS-induced an increase in IL-6 and IL-1β in the brains of ovariectomised mice through ERα/ERβ-dependent mechanism as it did not attenuate LPS-induced neuroinflammation in ERα knockout and ERβ knockout mice (Brown et al., 2010). The mechanism of the anti-inflammatory effect of oestrogens is mediated by the suppression of signalings including NF-κB in microglia (Baker et al., 2004, Kalaitzidis and Gilmore, 2005, Wu et al., 2013), MAPK (Kato et al., 1995) and Akt (Zhang et al., 2001). ERs regulate the anti-neuroinflammatory effect of oestrogen by down-regulating the expression of NF-κB-dependent target genes (Chadwick et al., 2005, Suuronen et al., 2005).

Finally, several observations have found that the chronic use of oestrogen replacement therapy results in deleterious effects including breast cancer because of the non-specific oestrogenic effects of oestrogens (Nilsson et al., 2001, Lai et al., 2013). Therefore, it is necessary to find and develop some safe oestrogen-like compounds, for instance phytoestrogens that lack the adverse impacts of endogenous 17β-oestradiol in order treat or to slow the progression of
neuroinflammation-mediated neurodegenerative disorders (Borrelli and Ernst, 2010). Finding synthetic or natural substances as specific ER agonists that possess an anti-neuroinflammatory effect, but lack the growth-promoting activity of oestrogen on the uterus and the mammary glands might be used against CNS inflammation such as AD and PD (Cvoro et al., 2008, Chakrabarti et al., 2014).

4.1.2. Role of AMPK signalling in neuroinflammation
Adenosine monophosphate (AMP)-activated protein kinase (AMPK), as a heterotrimeric protein kinase complex, is expressed in a wide variety of cells in the CNS including neurons and microglia (Carling et al., 2012). AMPK is involved in the regulation of cellular energy including the metabolism of glucose and lipids during the physiological and pathological states (Viollet et al., 2010). Canonically, it serves as a cellular energy sensor to restore cellular energy haemostasis in pathological conditions such hypoglycaemia, brain ischemia, as well as neuroinflammatory and oxidative responses (Hardie et al., 2006, Ronnett et al., 2009, Ramamurthy and Ronnett, 2012). AMPK is activated during the metabolic and oxidative stresses to protect the cells such as neurons against intracellular depletion of ATP by switching-off ATP-consuming biosynthetic pathways (Carling et al., 2012, Hardie et al., 2006). At high AMP level and low ATP level, AMPK is phosphorylated via upstream kinases and this results in the phosphorylation of down-stream targets that increase or balance the cellular level of ATP. The main AMPK-activating kinase is liver kinase B1, a protein expressed ubiquitously and recruited for AMPK phosphorylation after an elevation of the AMP/ATP ratio (Sid et al., 2013).

Many studies have suggested that AMPK play a central role in the modulation of inflammation by regulating inflammatory gene expression (Viollet et al., 2010, Sid et al., 2013). AMPK has been identified as an anti-inflammatory and neuroprotective protein in a wide variety of models. Regarding the role of microglia in neuroinflammation, a study showed that the activation of AMPK results in the inhibition of several IFN-γ-induced cytokines and chemokines in primary microglia (Meares et al., 2013). Several experiments have suggested that some compounds inhibit neuroinflammation through AMPK activation in LPS-activated microglial cells (Chen et al., 2014, Giri et al., 2004, Park et al., 2016, Lin et al., 2014a). In addition, AICAR protects hippocampal neurons against
glucose deprivation and glutamate excitotoxicity through the up-regulation of AMPK (Culmsee et al., 2001). However, AMPK activity has been involved in the pathology of neuroinflammation and neurodegenerative diseases such as AD (Cai et al., 2012). Several studies have shown that AMPK activation results in inhibiting amyloidogenesis in the neurons of AD models (Novikova et al., 2015). For example, Aβ production is increased in the cortical neurons of AMPK-knockout rat and the activation of AMPK results in the inhibition of Aβ deposition (Won et al., 2010). In the same study, AICAR is noted to decrease Aβ production through activating AMPK signalling. Furthermore, down-regulation of AMPK is responsible for inhibiting resveratrol-reduced Aβ accumulation in primary mouse neurons (Vingtdeux et al., 2010). Therefore, increasing the activity of AMPK to improve energy metabolism in the CNS is one of the strategies that is needed to prevent neurodegenerative disorders (Ronnett et al., 2009).

4.1.3. Role of hydrogen peroxide in neuroinflammation

Hydrogen peroxide (H₂O₂) is an important molecule in the host defence against pathogens and dead cells through induction of several antioxidant enzymes from microglia (Marinho et al., 2014, Veal et al., 2007). However, it is known that H₂O₂ is one of the major ROS that induce neuroinflammation and neurotoxicity (Eguchi et al., 2011, Jekabsone et al., 2006). Excessive production of H₂O₂ due to the over-activation of microglia is responsible for oxidative stress and neuroinflammation because H₂O₂ reacts with free iron to form more toxic molecules called hydroxyl radicals (Marinho et al., 2014). H₂O₂ is produced in response to inflammatory ligands such as LPS, Aβ and cytokines (Veal et al., 2007). It is noted that H₂O₂ is one of the ligand that induces neuronal apoptosis in the CNS. Therefore, it is necessary to attenuate the excessive production of H₂O₂ from hyper-activated microglia in the CNS. H₂O₂ has been used extensively in a variety of cell culture and animal models to study the association between neuroinflammation, oxidative stress and neurodegeneration. An increased iNOS expression and subsequently, NO production induced by LPS is significantly potentiated by H₂O₂ secreted from BV2 microglia (Eguchi et al., 2011).

4.2. Specific Aim and Objectives

This part of research was aimed at investigating whether diosgenin modulates neuroinflammation through activation of other important signalling pathways in
BV2 cells. Therefore, this study sought to address the following specific objectives:

I. To determine whether AMPK pathway plays a role in the modulation of the anti-neuroinflammatory activity of diosgenin in BV2 microglia.

II. To identify whether ER expression and activation are modulated by diosgenin in microglial BV2 cells.

4.3. Methods

4.3.1. BV2 cell culture
Microglial BV2 cells were cultured as described in section 2.2.1. BV2 cells were left untreated in RPMI 1640 medium as a negative-control. In addition, BV2 cells were treated with 5, 10, and 20 µM of diosgenin for 30 minutes. After that, the plate was incubated for the indicated time, according to each experiment, as shown in the text.

4.3.2. HT22 cell culture
HT22 cells were cultured as described in section 2.2.3.

4.3.3. Drug treatment
In order to investigate the effect of diosgenin on the amounts of ERβ and AMPK, BV2 cells were seeded out at a concentration of 2.0 x 10^5 cells/mL (2 mL/well) in a 6-well plate and then incubated for 48 hours. The culture medium was replaced by serum-free RPMI 1640 medium for two hours. BV2 cells were treated with 5, 10, and 20 µM of diosgenin and then incubated for 24 hours. The cells left untreated were used as a negative control.

To investigate whether diosgenin exerts an anti-neuroinflammatory effect by blocking ERβ in LPS-stimulated BV2 cells, the cells were seeded out at a concentration of 2.0 x 10^5 cells/mL (2 mL/well) in a 6-well plate and then incubated for 48 hours. Next, the cultured media were replaced by serum-free RPMI 1640 medium for two hours. The cells were treated with 20 µM of diosgenin for 30 minutes, and then stimulated with 100 ng/mL of LPS for 24 hours. In addition, the cells were pre-treated for 30 minutes with 100 mM of fulvestrant, followed by 20 µM of diosgenin for another 30 minutes, and then stimulated with 100 ng/mL of LPS for 24 hours. Further, the cells were stimulated with only 100 ng/mL of LPS for 24 hours and used as positive-control. Cells were left untreated...
in a serum- and phenol red-free RPMI 1640 medium as negative-control. After that, the cultured media were collected and centrifuged at 1200 g at 4°C for 5 minutes. The supernatants were stored at -80°C for short term future use to measure the levels of the released pro-inflammatory mediators NO, TNFα, IL-1β, and PGE₂ from the microglial cells using commercially available kits. The cells were treated in the same manner, but the incubation time of LPS was one hour in order to determine whether diosgenin attenuated LPS-induced p65 phosphorylation through activation of ERβ.

4.3.4. Griess assay
This assay was used to determine whether diosgenin exerts an anti-neuroinflammatory effect by blocking ERβ in LPS-stimulated BV2 cells. The level of NO in the cultured medium was determined as described in section 2.2.6.

4.3.5. Enzyme-linked immunosorbent assay
Like the Griess assay, ELISA method was used to establish whether diosgenin induced an anti-neuroinflammatory effect through a reduction in the levels of pro-inflammatory cytokines after activation of ERβ. The levels of microglial cytokines TNFα and IL6 in the cultured medium were measured as described in 2.2.7.

The effect of diosgenin on LPS-induced p65 phosphorylation in the absence and presence of ER antagonist in BV2 cells was determined by measuring the levels of phospho-NF-κBp65 in the nuclear extracts. The concentrations of phospho-NF-κBp65 in the nuclear extracts were determined using phospho-NF-κBp65 InstantOne ELISA Kit (Biolegend), according to the manufacturer’s protocol as described in section 3.3.9.

4.3.6. PGE₂ enzyme immunoassay
An EIA was used to investigate the anti-neuroinflammatory effect of diosgenin on ERβ in LPS-stimulated BV2 cells. The level of this pro-inflammatory factor was measured as described in section 2.2.8.

4.3.7. Preparation of cytoplasmic and nuclear protein lysates
After treatment of the cells, the cytoplasmic and nuclear lysates were prepared as described in section 2.2.11. Determination of protein concentrations in each sample was measured as showed in section 2.2.12.
4.3.8. Western blotting
A western blotting protocol was used as described in section 2.2.13. The cytoplasmic lysates were prepared to study the expression of ERβ and AMPK phosphorylation following diosgenin treatment. The nuclear lysates were prepared to observe the effect of diosgenin on NF-κB signalling after blocking of ERβ in LPS-activated BV2 microglia.

4.3.9. Cell viability
The neuroprotective effect of diosgenin against H₂O₂-induced HT22 neuronal death was investigated using an MTT assay. 50 µM of H₂O₂ (Sigma) was used in order to induce HT22 neuronal death. HT22 cells were seeded out at a concentration of 2.0 x 10⁵ cells/mL (200 µL/well) in a 96-well plate and incubated for approximately 48 hours. After changing the media, the cells were treated with 5, 10, and 20 µM of diosgenin and incubated for 30 minutes, and subsequently stimulated with 50 µM of H₂O₂ for 24 hours. The cells treated with 50 µM of H₂O₂ for 24 hours were used as a positive control and those left untreated were used as a negative control. The absorbance was measured to determine the cell viability of HT22 cells as previously described in section 2.2.5.

4.3.10. Statistical Analysis
Values were expressed as the mean ± SEM of three independent experiments. Data were analysed using one-way ANOVA for multiple comparisons with post-hoc Student Newman-Keuls test. The ANOVA compares the mean between the groups and determine whether those means are significantly different from each other. Specific groups that were significantly different from each other were evaluated with post-hoc Student Newman-Keuls test with multiple comparisons. Calculations for statistical analysis were performed with Graph Pad Prism software version 5. Differences were significant at p<0.05. *p<0.05, **p<0.01, ***p<0.001 compared with untreated control; and *p<0.05, **p<0.01, ***p<0.001 compared to LPS- or H₂O₂-treated control. @p<0.05, @@p<0.01, @@@p<0.001 as compared between the LPS + diosgenin-treated cells.
4.4. Results

4.4.1. Diosgenin increased AMPK phosphorylation in BV2 cells

As AMPK signalling plays a key role in the modulation of neuroinflammation (Hardie et al., 2006, Ronnett et al., 2009, Ramamurthy and Ronnett, 2012), the effect of diosgenin on AMPK phosphorylation was investigated in BV2 microglia. Compared to the untreated control, AMPK phosphorylation was significantly increased by diosgenin in BV2 cells. It was found that treatment with diosgenin at 5 µM and 10 µM enhanced the levels of p-AMPK (0.5- and 1.48-fold increase, respectively), when compared to control BV2 cells (Figure 4.1). In addition, 2.6-fold induction (p<0.001) in the p-AMPK level was detected with 20 µM of diosgenin, compared to the control (Figure 4.1).

![p-AMPK and β-actin Western blots](image)

**Figure 4.1 Effect of diosgenin on AMPK phosphorylation in BV2 cells.**

BV2 cells were treated with diosgenin for 24 hours. The levels of AMPK were measured as described above. Data are expressed as the mean ± SEM of three independent experiments. Data were analysed using one-way ANOVA for multiple comparisons with post hoc Student Newman-Keuls test. *p<0.05, **p<0.01, ***p<0.001 compared with untreated control.
**4.4.2. Diosgenin inhibited H₂O₂-mediated HT22 neuronal death**

Hydrogen peroxide (H₂O₂) is one of the microglial pro-inflammatory ROS molecules that induce neuroinflammation and neurotoxicity (Marinho et al., 2014). Therefore, the neuroprotective effect of diosgenin against H₂O₂-induced HT22 neuronal death was investigated. Results in Figure 4.2 show that exposure of HT22 neurons to H₂O₂ significantly (p<0.001) resulted in 74% neuronal death, compared to the control cells (Figure 4.2). However, it was observed that pre-treatment with 20 µM of diosgenin significantly produced an increase in the HT22 cell viability, compared to the H₂O₂ control (Figure 4.2).

![Graph showing Effect of diosgenin on H₂O₂-induced HT22 neuronal cell death.](image)

**Figure 4.2 Effect of diosgenin on H₂O₂-induced HT22 neuronal cell death.**

HT22 cells were treated for 30 minutes with diosgenin, and then stimulated with 50 µM of H₂O₂ for 24 hours. After that, the viability of HT22 cells were determined by an MTT assay. Data are expressed as the mean ± SEM of three independent experiments. Data were analysed using one-way ANOVA for multiple comparisons with post hoc Student Newman-Keuls test. §p<0.05, §§p<0.01, §§§p<0.001 compared with untreated control; and *p<0.05, **p<0.01, ***p<0.001 compared to H₂O₂-treated control.

**4.4.3. Diosgenin increased the levels of ERβ protein in BV2 microglia**

The ERβ subtype oestrogen receptor is highly expressed in BV2 microglial cells. A number of studies have indicated that estrogen receptors play an important role in the microglia-triggered neuroinflammatory response (Laredo et al., 2014, Spence and Voskuhl, 2012). Thus, this study examined the effect of diosgenin on
the expression of ERβ in BV2 cells. Immunoblotting analyses showed detectable levels of ERβ in untreated BV2 cells (Figure 4.3). Furthermore, a significant and concentration-dependent increase (p<0.001) in the levels of ERβ protein was reported with diosgenin (5-20 μM) treatment, compared to the untreated cells (Figure 4.3).

**Figure 4.3 Effect of diosgenin on ERβ levels in BV2 cells.**

BV2 cells were treated with diosgenin for 24 hours. The total levels of ERβ were measured as described above. Data are expressed as the mean ± SEM of three independent experiments. Data were analysed using one-way ANOVA for multiple comparisons with post hoc Student Newman-Keuls test. *p<0.05, **p<0.01, ***p<0.001 compared with untreated control.

**4.4.4. Diosgenin did not inhibit neuroinflammation in LPS-stimulated BV2 microglia pre-treated with fulvestrant**

Since diosgenin produced an anti-neuroinflammatory effect in LPS-stimulated BV2 microglia (Chapter 2) and up-regulated ERβ protein in untreated BV2 cells, it was next assessed whether the compound produced an ERβ-dependent inhibition of neuroinflammation in BV2 microglia. To achieve this, cells were
treated with the ER antagonist fulvestrant, then diosgenin (20 μM). After 24-hour incubation, the levels of pro-inflammatory mediators in the cultured media were measured. Results showed that LPS stimulation induced a significant increase (p<0.001) in the concentrations of the pro-inflammatory mediators (NO (11.1 ± 1.2 μM), PGE₂ (614.3 ± 92.8 pg/mL), TNFα (739.6 ± 106.4 pg/mL) and IL-6 (465.6 ± 30.4 pg/mL)) in LPS-stimulated BV2 microglial cells, in comparison with the untreated cells. However, diosgenin significantly reduced the microglial levels of these factors (NO (6.1 ± 0.2 μM), PGE₂ (269.6 ± 63.1 pg/mL), TNFα (330.5 ± 79.7 pg/mL) and IL-6 (185.4 ± 12.7 pg/mL)), compared to the LPS-control. However, Figures 4.4a-d show that diosgenin did not reduce the levels of pro-inflammatory mediators (NO (9.7 ± 1.2 μM), PGE₂ (553.1 ± 70.5 pg/mL), TNFα (694.7 ± 95.03 pg/mL) and IL-6 (448.0 ± 30.9 pg/mL)) in LPS-stimulated BV2 microglia in the presence of fulvestrant (100 nM), compared to the LPS-activated BV2 microglia pre-treated with diosgenin.
Figure 4.4 Effects of 20 µM of diosgenin on the LPS-induced an increase in production of NO, PGE₂, TNFα and IL-6 in BV2 cells in the absence or presence of fulvestrant.

The levels of NO, PGE₂, TNFα and IL-6 were measured as described above. Data are expressed as the mean ± SEM of three independent experiments. Data were analysed using one-way ANOVA for multiple comparisons with post hoc Student Newman-Keuls test. *p<0.05, **p<0.01, ***p<0.001 compared with untreated control; and *p<0.05, **p<0.01, ***p<0.001 compared to LPS-treated control. @p<0.05, @@p<0.01, @@@p<0.001 as compared between the LPS + diosgenin-treated cells.

Also, in comparison to untreated cells, LPS stimulation resulted in a significant increase in the levels of p-p65 (p<0.001) (Figure 4.5). However, pre-treatment of cells with diosgenin (20 µM) resulted in a significant reduction (p<0.05) in the p-p65 level, compared to the LPS-control (Figure 4.5). By contrast, diosgenin did not reduce the elevated level of p-p65 in LPS-activated cells in the presence of fulvestrant (100 nM), compared to LPS-activated BV2 microglia pre-treated with diosgenin (Figure 4.5).
Figure 4.5 Effect of 20 µM of diosgenin on the LPS-induced increase in the nuclear level of p-NF-κBp65 in BV2 cells.

The nuclear levels of p-NF-κBp65 (p-p65) were measured as described above. Data are expressed as the mean ± SEM of three independent experiments. Data were analysed using one-way ANOVA for multiple comparisons with post hoc Student Newman-Keuls test. *p<0.05, **p<0.01, ***p<0.001 compared with untreated control; and *p<0.05, **p<0.01, ***p<0.001 compared to LPS-treated control. @p<0.05, @@p<0.01, @@@p<0.001 as compared between the LPS + diosgenin-treated cells.
4.5. Discussion
Several signalling pathways play an important role in the pathogenesis of neuroinflammation. Each cellular cascade is regulated by molecule(s) or mediator(s) that inhibit or induce neuroinflammatory processes. For example, the estrogen, 17β-oestradiol and the protein AMPK are important endogenous substances that reduce microglia-mediated neuroinflammation. However, ROS including H$_2$O$_2$ are one of the major molecules that are known to induce neuroinflammation and therefore, neurotoxicity (Jekabsone et al., 2006, Eguchi et al., 2011). 17β-oestradiol as an anti-neuroinflammatory and neuroprotective hormone acts on two common receptors; ERα and ERβ that activate several target genes (Spence and Voskuhl, 2012, Smith et al., 2011, Laredo et al., 2014). AMPK is a protein that modulate the cellular metabolism and anti-neuroinflammatory effect in the CNS (Peixoto et al., 2017, Ronnett et al., 2009).

This study has demonstrated that diosgenin increased the phosphorylation of AMPK. A study has shown that diosgenin blocked the inflammation-mediated endothelial dysfunction by activating the AMPK pathway (Chen et al., 2016). In another study, diosgenin prevented the development of non-alcoholic fatty liver disease via AMPK phosphorylation (Cheng et al., 2017). However, this is the first report showing that disogenin activated AMPK in BV2 microglia. This observation demonstrates that the activation of AMPK possibly contributes to the inhibition of neuroinflammation by diosgenin (Ronnett et al., 2009, Ramamurthy and Ronnett, 2012). Further studies are however needed to determine whether the compound produce any modulatory effect on AMPK/sirtuin 1 survival mechanisms in the microglia, as part of its molecular mechanisms of action in activated microglia.

Diosgenin is classified as a phytoestrogen because its chemical structure is similar to that of steroids such as 17β-oestradiol (Bak et al., 2016, Scott et al., 2001). Consequently, it was hypothesised that diosgenin may, at least in part, mediate its anti-neuroinflammatory effect through interactions with the ERβ protein in BV2 microglia. Results showed that diosgenin increased the expression of ERβ in BV2 microglia, suggesting that diosgenin possibly plays a role in microglial ERβ-mediated gene expression and oestrogen signalling. This finding also provides evidence that ERβ is expressed in BV2 microglial cells as reported in other studies (Baker et al., 2004, Saijo et al., 2011). It is important to note that
elevated levels of the ERβ protein may suggest a possible involvement of a nonclassical effect of diosgenin on microglia as observed with 17β-oestradiol in another study (Liu et al., 2005). Oestrogens produce anti-inflammatory effects through the activation of ERβ in microglia (Baker et al., 2004). Thus, the increasing microglial ERβ protein expression by diosgenin may be partially involved in the mediation of its anti-inflammatory effect in this present study.

In order to determine whether the anti-neuroinflammatory activity of diosgenin is mediated at least in part, through ERβ, the microglial ERβ receptor was blocked with the ER antagonist fulvestrant. Results showed that diosgenin did not inhibit the LPS-induced neuroinflammation in LPS-activated BV2 cells pre-treated with fulvestrant. This seems to suggest a role for microglial ERβ in the inhibition of microglial pro-inflammatory mediator secretion induced by LPS. Similarly, diosgenin did not block NF-κB activity in LPS-stimulated BV2 cells pre-treated with fulvestrant. A number of studies have reported that diosgenin produced an oestrogenic effect (Rao and Kale, 1992, Alcantara et al., 2011, Wu et al., 2015), which may explain the observed role of oestrogen receptors in the effect of this compound. Interestingly, it has been reported that the membrane translocation and transcriptional activity of ER during proliferation of rat TM4 cells is activated by diosgenin (Wu et al., 2015).

Diosgenin has a neuroprotective effect in HT22 neuronal death induced by H2O2. Similar protective actions have been reported in other studies. For instance, diosgenin showed a protective mechanism against H2O2-induced oxidative stress in human vascular endothelial cells (Gong et al., 2010) as well as H9C2 cells (Jamshidi et al., 2016). This finding suggests that diosgenin inhibits neuronal damage due to an excessive generation of ROS such as H2O2.

In summary, it is possible that the activation of AMPK by diosgenin in the microglia could be another important anti-neuroinflammatory mechanism to support the previously observed anti-neuroinflammatory effects of this compound. In addition, an activation of ERβ signalling might be involved in the inhibitory effect of diosgenin in LPS-induced neuroinflammation. Further studies are needed to substantiate these preliminary results.
Chapter 5
General Discussion and Conclusion
5.1. General discussion

The steroidal saponin diosgenin is a natural product that exhibits numerous biological properties, such as antioxidant (Son et al., 2007), anti-inflammatory (Ku and Lin, 2013, Tewtrakul and Itharat, 2007) and antihyperlipidaemic (Gong et al., 2010) effects. The anti-inflammatory effects of the compound have been extensively investigated in a wide variety of inflammatory models. However, to date the anti-neuroinflammatory effect of diosgenin has not yet been investigated. In the current investigation, the anti-inflammatory effect of diosgenin was demonstrated in LPS-stimulated BV2 microglia.

Several studies have suggested that the microglial pro-inflammatory factors including NO, TNFα, IL-6, PGE₂ and ROS are involved in the pathogenesis of neurodegenerative diseases (González et al., 2014, Viviani et al., 2014). Over-activation of microglia results in an uncontrolled neuroinflammatory process that is responsible for neuronal loss and death (Lucas et al., 2006, Glass et al., 2010). This study has established that diosgenin inhibited the production of NO as well as the prostaglandin PGE₂ through the down-regulation of iNOS and COX-2, respectively in LPS-activated BV2 microglia. In the same culture model, diosgenin attenuated the production of the most important pro-inflammatory cytokines TNFα, IL-6 and IL-1β. In contrast, diosgenin increased the level of the anti-neuroinflammatory cytokine IL-10 in LPS-stimulated cells. Furthermore, diosgenin blocked the generation of ROS, but did not affect NADPH homeostasis in LPS-activated BV2 cells. Therefore, these results suggest that diosgenin produced an anti-neuroinflammatory effect against LPS-induced neuroinflammation in BV2 microglia. This outcome is consistent with the data obtained from other studies. For example, diosgenin induced anti-inflammatory activity against LPS-induced inflammation in mouse primary peritoneal macrophages (Singh et al., 2014), and in mouse lung injury (Gao et al., 2013). In addition, diosgenin attenuated the production of pro-inflammatory mediators in the co-culture model of 3T3-L1 adipocytes with RAW 264 macrophages (Hirai et al., 2010), and in RAW 264.7 murine macrophages (Jung et al., 2010a).

It is well known that the transcription factor NF-κB is the most important protein which controls the expression of a diverse variety of pro-inflammatory genes such as iNOS and COX-2 in microglia (Förstermann and Sessa, 2011, Habashi et al.,
2016, Yagami et al., 2016). In fact, the hyper-activation of microglial NF-κB cascade results in neuroinflammation and neuronal death, and subsequently neurodegenerative disorders (Hoesel and Schmid, 2013). Therefore, targeting the NF-κB signalling pathway is one of the most important strategy to slow the progression of microglia-induced CNS inflammation. NF-κBp65/p50 is one of the five members of mammalian NF-κB transcription factors family that regulates the immune and inflammatory responses (Lawrence, 2009). Upon stimulation of the microglial cell with the inflammatory ligand (e.g. LPS), IKK is phosphorylated in order to phosphorylate the inhibitory protein p-κB-α in the cytoplasm. Next, the dimer NF-κB is liberated from κB-α and translocated into the nucleus. After that, the phosphorylated subunit p65 binds to specific DNA sites on the promoter of the target genes to transcript pro-inflammatory mediators that regulate the normal immune response (Vega and de Andres Martin, 2008). The results of this research showed that diosgenin prevents the phosphorylation, nuclear translocation and then the DNA-binding activity of the subunit NF-κBp65 in LPS-activated BV2 microglia. Moreover, the inhibitory effect on LPS-induced NF-κB activation was mediated by inhibiting phosphorylation and degradation of κB-α. Diosgenin did not block the phosphorylation of the complex protein IKK. Based on these observations, it is proposed that diosgenin inhibits LPS-mediated neuroinflammation via targeting IκB/NF-κB cascade in BV2 microglial cells.

The p38 MAPK and Akt signalling cascades mediate microglial activation and neuroinflammatory responses (Bachstetter et al., 2011, Ramesh, 2014, Maiese et al., 2012, Cohen, 2013). Blocking the activity of these proteins is another potential stratgy for preventing neuroinflammation. This work shows that diosgenin attenuated LPS-induced Akt phosphorylation, but did not inhibit p38 phosphorylation due to LPS stimulation of BV2 microglia. A number of studies have supported these results. Diosgenin blocked NF-κB activity, but did not inhibit p38 MAPK activity in the peripheral immune cells macrophages (Jung et al., 2010a). In contrast, diosgenin attenuated p38 activation in LPS-induced lung injury (Gao et al., 2013). In addition, this compound interfered with death receptor-5 through p38 activation and Akt inactivation in colon cancer cells (Lepage et al., 2011). Lin et al. showed that diosgenin attenuated ROS generation via suppression of p38 and Akt pathways in the activated mouse neutrophils (Lin
et al., 2014b). The significant differences between these outcomes might be due to a number of factors including cell line type, culture environment and the concentrations of diosgenin as well as the pro-inflammatory ligands. Several studies have demonstrated that the antioxidant pathway of Nrf2 is important in the attenuation of neuroinflammation. Nrf2 is not only an antioxidant protein that regulates the expression of detoxifying enzymes, but also is anti-inflammatory factor to regulate NF-κB-mediated anti-inflammatory processes. Nrf2 plays important role in modulation of uncontrolled neuroinflammation and oxidative stress (Innamorato et al., 2008, Sandberg et al., 2014). In the present study, diosgenin increased the expression of the antioxidant enzymes HO-1 and NQO1, possibly due to its ability to activate the Nrf2/ARE signalling in BV2 microglia. This study also established that the presence of Nrf2 is a requirement for the NF-κB-mediated inhibition of neuroinflammation by diosgenin. A similar inhibitory mechanism has been reported for other compounds (Lee et al., 2011, Kang et al., 2013, Park et al., 2015b, Jayasooriya et al., 2014). An interesting outcome of this study is the observed activation of microglia AMPK by diosgenin. Considering the inhibitory role of AMPK in neuroinflammation (Ronnett et al., 2009, Ramamurthy and Ronnett, 2012), this is a line of investigation that deserves future attention. Diosgenin demonstrated neuroprotective effects in both neuroinflammation- and H2O2-induced HT22 neuronal death. This is a significant outcome, as there is a clear evidence indicating that diosgenin is a potential chemical scaffold for the identification of novel anti-inflammatory and neuroprotective compounds for neurodegenerative disorders. To fully exploit the therapeutic potential of diosgenin in this regard, it would be necessary to establish whether this compound or synthesised derivatives cross the BBB and acts in the CNS. In general, compounds cross the BBB by a wide variety of mechanisms including transmembrane diffusion and saturable transporters. It is well known that most of drugs cross the BBB by transmembrane diffusion because they are small (low molecular weight) molecules and soluble-lipid at the same time (Banks, 2009, Pajouhesh and Lenz, 2005). Regarding the importance of scaffolds in drug discovery, novel anti-neuroinflammatory compounds were obtained by assembling fragments derived from resveratrol (stilbene) and G1BH-130 (piperazinyl pyrimidine) using BV2 microglia (Fang et al., 2018).
The observed increase in the expression of ERβ in BV2 microglia by diosgenin is another novel outcome of this study that requires further pharmacological exploration. This assertion is further strengthened by results showing that the anti-inflammatory activity of diosgenin in BV2 microglia was lost in the presence of the oestrogen receptor antagonist fulvestrant. Therefore, in this study, the possible molecular mechanism of anti-neuroinflammatory action of diosgenin was mediated by inhibiting IκB-α phosphorylation/degradation of NF-κB cascade through ERβ-mediated activation of Nrf2.

5.2. General conclusion
The data obtained from this study suggest that diosgenin inhibited NF-κB-mediated neuroinflammation through molecular mechanisms that are possibly closely linked to Nrf2/ARE antioxidant protection system BV2 microglia cells. It was demonstrated that the compound is neuroprotective in both neuroinflammation and oxidative stress-mediated neuronal damage, an action that is important in neurogeneration. The effects of the compound on processes linked to estrogen receptor suggests a potential estrogenic activity which needs further investigation. Activation of microglial AMPK is a property which further demonstrates that diosgenin may inhibit neuroinflammation through activation of endogenous systems which block the transcriptional activity of NF-κB.

5.3. Future work
It is important to investigate the effects of diosgenin in transgenic animal models of neuroinflammation and in models of neurodegenerative disorders such as Alzheimer’s disease. The therapeutic benefit of compounds with potential in neurodegenerative disorders can only be applied if they are able to permeate the BBB and act in the CNS. Consequently, in vitro models employing hCMEC/D3 microvascular cells would be an excellent tool for investigating the BBB permeability of diosgenin. This study has opened up a new question about the potential oestrogenic effect of diosgenin. Studies are required to establish the potential pharmacological/therapeutic implications of this effect. Research questions need to focus on neuroprotection without peripheral actions such as proliferation of breast and ovarian cancer cells, a phenomenon that is common to many known synthetic and natural estrogenic compounds.
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