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INHIBITION OF NEUROINFLAMMATION BY THE METHANOLIC EXTRACT OF *ZANTHOXYLUM ZANTHOXYLOIDES* ROOT AND SKIMMIANINE

FOLASHADE ABIODUN OGUNRINADE

A THESIS SUBMITTED TO THE SCHOOL OF APPLIED SCIENCES AT THE UNIVERSITY OF HUDDERSFIELD, IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN PHARMACEUTICAL SCIENCES

THE UNIVERSITY OF HUDDERSFIELD

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Acknowledgements

Abstract

Studies have demonstrated the role of neuroinflammation in causing neurotoxicity and neuronal damage in neurodegenerative diseases and cerebral malaria (CM) patients. *Zanthoxylum zanthoxyloides* (*Z. Z*) is reputed for its anti-inflammatory activity. This study investigated the effect of Z.*Z.* and it's bioactive, skimmianine (SK) on neuroinflammation in BV2 microglia cells stimulated with lipopolysaccharide (LPS) and synthetic hemozoin (sHZ). Cultured mouse microglia (BV2) cells were stimulated with LPS (100 ng/ml) and sHZ (400 µg/ml) following pre-treatment with *Z. zanthoxyloides* (4, 6 and 8 µg/ml) and skimmianine (10, 20, and 30 µM). Levels of nitric oxide (NO), PGE_2, pro IL-1β, IL-1β, IL-6, TNFα and IL-10 were measured in cell culture supernatants. Protein expressions of iNOS, COX-2, NF-κB phospho-p65 subunit, phospho-IκBα, total IκBα, phospho-IKK, total IKK, caspase-1 and NLRP3 in extracts were investigated with western blotting. HEK293 cells were transfected with Cignal NF-κB (LUC) (CCS-013L) vector and reporter gene assays were used to investigate the effect of Z.*Z* and SK on luciferase activity of NF-κB in the nucleus. Isolation of SK was monitored with solid-phase-extraction (SPE), ELISA and HPLC. Structure elucidation of the isolated compound was determined by NMR analysis and mass spectrometry. Z.*Z* extract and SK reduced the levels of PGE_2, IL-1β, IL-6, TNFα IL-1β, pro-IL-1β, caspase-1 protein and NLRP3 protein and increased the levels of IL-10 in BV2 cells stimulated with LPS and sHZ. Further experiments showed that *Z. Z* and SK decreased the expression of iNOS, COX-2 protein and inhibited NF-κB and NLRP3signaling pathway in both LPS and sHZ-stimulated BV2 cells. These results suggest that Z.*Z* and SK inhibit neuroinflammation through NF-κB and NLRP3 signalling pathway in stimulated BV2 cells. This study suggests that bioactive compounds in *Z. zanthoxyloides* extract are potential phytochemical leads for treating neuroinflammation mediated neurodegenerative diseases and CM.

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Abbreviations

AD	Alzheimer's disease
ALS	Amyotrophic lateral scelorosis
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BBB	Blood brain barrier
BSA	Bovine serum albumin
CM	Cerebral malaria
CNS	Central nervous system
COX-2	Cyclooxygenase-2
DMSO	Dimethylsulfoxide
DTT	Dithiothreitol
ELISA	Enzyme linked immunoabsorbent assay
EMSA	Electrophilic mobility shift asay
Enos	Endothelial nitric oxide synthase
FBS	Fetal bovine serum
HMBC	Heteronuclear multiple-bond correlation spectroscopy
HPLC	High performance liquid chromatography
HSQC	Heteronuclear single quantum correlation
IFN-γ	Interferon gamma
IκB	Inhibitors of Kappa B
IKK	Inhibitors of NF-Kappa B kinase
IL-10	Interleukin-10
IL-1β	Interleukin- 1 beta
IL-6	Interleukin-6
iNOS	Inducible nitric oxide synthase
IUPAC	International Union of Pure and Applied Chemistry
LDS	Lithium Dodecyl Sulphate
LPS	Lipopolysaccharide

MeOH	Methanol
Mrna	Messenger ribonucleic acid
MS	Mass spectrometry
NEMO	NF-KB essential modofier
NF-κB	Nuclear factor κ light chain enhancer of activated B cells
ng/ml	nanogram per millilitre
NLR	Nod like receptor
NMR	Nuclear magnetic resonance spectroscopy
NO	Nitric oxide
P. falciparum	*Plasmodium falciparum*
p65	Transcription factor p65 (NF-κB)
PAMPs	Pathogen associated molecular patterns
PBS	Phosphate buffer saline
PD	Parkinson disease
PGE_2	Prostaglandin E2
PRBC	Parasitised red blood cell
PVDF	Polyvinylidene
RNA	Ribonuleic acid
ROS	Reactive oxygen species
Rpm	Revolutions per minute
RPMI medium	Roswell park memorial institute medium
sHZ	Synthetic hemozoin
SK	Skimmianine
SPE	Solid phase extraction
TAK1	Transforming growth factor-β-activated kinase
TBS	Tris- buffered saline
TBS-T	TBS plus tween 20
TLR	Toll like receptor
TLR-4	Toll like receptor-4
TNFR1	Tumor necrosis factor-alpha receptor-1

TNF-α	Tumor necrosis factor-alpha
TRAF6	TNF-α receptor associated factor 6
Z. Z	*Zanthoxylum zanthoxyloides*
µg	Microgram
µl	Microlitre
µM	Micromolar

1 GENERAL INTRODUCTION

1.1 Neuroinflammation and activation of microglia cells

Neuroinflammation occurs when the cells of the central nervous system (CNS) including microglia and macroglia such as oligodendrocytes and astrocytes give an immunological response to infection, neurotoxins and injury, hence protecting the CNS. (baruaChen, Zhang, & Huang, 2016; Shabab, Khanabdali, Moghadamtousi, Kadir, & Mohan, 2017). Microglia cells are macrophages resident in the CNS and are about 10% of the cell population in CNS (Timmerman, Burm, & Bajramovic, 2018). Microglia cells are described to be in 'M0 state' as they act as surveillance in the CNS environment under homeostatic conditions and not in a resting state as previously reported (Butovsky et al., 2014; Timmerman et al., 2018). Reports have further demonstrated that there is a change in the phenotypic state of microglia cell when stimulated with inflammatory stimuli including lipopolysaccharide (LPS). In addition, microglia cells were described to transform morphologically from "M0" state (ramified) to "M1" state (amoeboid) when stimulated. In neuroinflammation, microglia cells are important as they have been demonstrated to be a major source of excessive secretion of pro-inflammatory cytokines and chemokines when activated with inflammatory stimuli. In addition, activated microglia cells secrete inflammatory cytokines such as TNFα, IL-1β, IL-6, and chemokines such as macrophage inflammatory protein-2 (MIP-2) and monocyte chemoattractant protein-1 (MCP-1). Moreover, reports have described the role of microglia cells including BV2 microglia cell line stimulated by LPS as experimental models to investigate the activity of potential compounds with anti-neuroinflammatory effect (Saijo & Glass, 2011; Stansley, Post, & Hensley, 2012). Thus, investigations on therapeutic drugs or medicinal plant that inhibits neuroinflammation mediated by activated microglia cells

is important in the treatment of neurodegenerative diseases such as parkinson's disease (PD) and Alzheimer's disease (AD).

Furthermore, increased production of pro-inflammatory cytokines (IL-1β, IL-6 and TNFα) have been observed in studies on incubation of monocytes and macrophages with hemozoin (Dunst, Kamena, & Matuschewski, 2017; Olivier, Van Den Ham, Shio, Kassa, & Fougeray, 2015; Perkins et al., 2011). Hemozoin is a malaria pigment that is derived from *Plasmodium falciparum*, a parasite that initiates the development of malaria in human through the bite of Anopheles mosquito (Ghazanfari, Mueller, & Heath, 2018; Hora, Kapoor, Thind, & Mishra, 2016). Moreover, studies done in cultured cells have also demonstrated a significant increase in production of nitric oxide (NO) in macrophage cells when stimulated with hemozoin (Jaramillo, Maritza, Godbout, & Olivier, 2005). These studies suggest the significant role of hemozoin in neuroinflammation. Therefore, inhibition of neuroinflammation induced by hemozoin is essential in reducing the increased level of pro-inflammatory cytokines and inflammatory mediators in stimulated microglia cells.

1.1.1 Roles of pro -inflammatory mediators and inflammatory signalling pathways in neuroinflammation

1.1.1.1 Tumor necrosis factor α (TNFα)

Tumor necrosis factor-alpha (TNF-α) is a pro-inflammatory cytokine which increased level of its secretion from activated cells by inflammatory stimuli such as LPS plays a crucial role in neuroinflammation (Kempuraj et al., 2016; Y.-K.Kim, Na, Myint, & Leonard, 2016; J. A.Smith, A. Das, S. K. Ray, & N. L. J. B. r. b. Banik, 2012b). Microglia cells are the main source of cytokines released during chronic inflammation, especially in neurodegenerative diseases (Heneka, Kummer, & Latz, 2014; Kempuraj et al., 2016; Y.-K. Kim et al., 2016). In AD, amyloid-beta (Aβ) was reported to activate

microglia cells to potentiate the release of pro-inflammatory cytokines including TNFα, which could lead to loss of neurons and tau hyperphosphorylation (Von Bernhardi, Tichauer, & Eugenín, 2010; W.-Y. Wang, Tan, Yu, & Tan, 2015). Furthermore, numerous studies have demonstrated the production of TNF-α in LPS from stimulated microglia cells and further described its inhibition to play a vital role in reducing neuroinflammation (Graeber, Li, & Rodriguez, 2011; Y.-C. Lu, Yeh, & Ohashi, 2008; Sheppard, Coleman, & Durrant, 2019). The role of TNFR1 expression in most cell types and activation of NF-κB signalling pathway have been described to be important as it leads to neuroinflammation (Kempuraj et al., 2016; Smith et al., 2012b). TNF-α binds to the TNFR1 to cause activation of adaptor protein called TNF receptor-associated death domain (TRADD) (G. Olmos & J. Lladó, 2014; Shabab et al., 2017). Subsequently, the recruitment of receptor interacting protein (RIP) occurs to activate and phosphorylate IKK and IκB (Sedger & McDermott, 2014). This process leads to nuclear translocation of NF-κB to cause transcription of genes controlling the secretion of proinflammatory cytokines, COX-2 and iNOS which results to neuroinflammation. Research in the past has shown that excessive production of TNF-α from activated glia cells causes excitotoxicity and apoptosis (Shabab et al., 2017; Smith et al., 2012b).

In addition, studies have shown that TNF-α increases the level of glutamate in activated cells, a major excitatory neurotransmitter that causes excitotoxicity and also the secretion of TNF-α in excess (Olaniyan et al., 2016; Sedger & McDermott, 2014) However, excitotoxicity caused by glutamate release has been reported to be involved in the pathogenesis of neurodegenerative diseases including Parkinson's disease, Multiple sclerosis and Alzheimer's disease (Lull & Block, 2010; G. Olmos & J. J. M. o. i. Lladó, 2014). In addition, a previous study demonstrated that binding of TNF-α via

TNFR1 signalling pathway causes excitotoxicity by elevating the release of glutamate from microglia cells (Boeuf et al., 2012; H. Takeuchi et al., 2006). The role of excessive secretion of pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) in macrophages induced by hemozoin has been demonstrated to be involved in the progression of neuroinflammation (Dunst et al., 2017; Gimenez et al., 2003). Therefore, inhibiting the elevated level of TNFα production in brain macrophages stimulated by inflammatory stimuli such as LPS is essential in reducing neuroinflammation in activated microglia cells.

1.1.1.2 Interleukin-6 (IL-6)

Previous research on increased level of IL-6 production has been associated with the pathology of neurodegenerative diseases including Parkinson's diseases and Huntington's disease (Kempuraj et al., 2016; Tanaka, Narazaki, & Kishimoto, 2014). An elevated level of IL-6 was observed in brains of Alzheimer's disease and was reported to potentiate neuronal loss in AD brain (Baune & T, 2015; McManus & Heneka, 2017). Studies on microglia cells induced by LPS have demonstrated that elevated level of IL-6 secretion from activated microglia cells which may be involved in the progression of neurodegenerative diseases (Y.-K. Kim et al., 2016; Smith et al., 2012b; Tanaka et al., 2014). Moreover, previous studies have observed an increased level of IL-6 secreted in macrophages activated with hemozoin (Griffith, Sun, McIntosh, & Bucala, 2009). A different study on BV2 microglia cells induced by hemozoin observed a significant increase in the level of IL-6 secreted and described its inhibition as a therapeutic target in reducing neuroinflammation (Velagapudi, Kosoko, Olajide, & neurobiology, 2019). These studies shows the importance of excessive production of pro-inflammatory cytokines in hemozoin induced neuroinflammation. Therefore, the effect of elevated level of IL-6 in neuroinflammation

has made it an attractive cytokine to target by reducing its level of secretion from activated microglia cells.

1.1.1.3 Interleukin-10 (IL-10)

Interleukin-10 is an anti-inflammatory cytokine produced in neural tissues and immune cells. It has a molecular weight of 18kDa and has 160 amino acid residues in its polypeptide chain (Wojdasiewicz, Poniatowski, & Szukiewicz, 2014). In stimulated immune cells, an increased level of IL-10 is produced as a response to reduce the pro-inflammatory cytokine level of IL-6, IL-1β and TNF-α (Oliveira, Sakata, Issy, Gerola, & Salomão, 2011; Y. Sun et al., 2019). Furthermore, IL-10 secretion is blocked by elevated level of cytokines such as IL-13, IFNγ and IL-4 (Oliveira et al., 2011). This results in a low level of IL-10 production in neuroinflammation mediated by increase in pro-inflammatory cytokines. An *in vivo* study on IL-10 deficient mice observed a decline in cognitive function when injected with LPS in comparison to the wild type mice (Cameron, Brent, & Landreth, 2010). Furthermore, the reduced level of IL-10 production has been found to be associated with pathology of neurodegenerative diseases and also described to be involved in the advancement of these diseases (Meng et al., 2018). Therefore, increasing the level of IL-10 cytokine may be an important strategy in reducing neuroinflammation by inhibiting activation of microglia cells.

1.1.1.4 Nitric oxide

Studies have shown the role of excessive production of nitric oxide in the pathogenesis of neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, multiple sclerosis, and amyotrophic lateral sclerosis (Kempuraj et al., 2016; Shabab et al., 2017; Sharman et al., 2019; Tse, 2017). Investigations on activated microglia cells have established the synthesis of nitric oxide (NO) through the induction of nitric oxide synthase (NOS) (Hwang et al., 2017). Reports have shown that nitric oxide synthase occurs in three isoforms in the CNS: inducible NOS (iNOS), endothelial NOS (eNOS)

and neuronal NOS (nNOS) (Lind et al., 2017; Shabab et al., 2017; Yuste, Tarragon, Campuzano, & Ros-Bernal, 2015). Different from eNOS and nNOS, iNOS produces excessive amount of NO and are secreted when cells including microglia cell are induced by inflammatory stimuli such as LPS and TNF-α (Förstermann & Sessa, 2012). Furthermore, iNOS has been demonstrated as a significant form of NOS because it is a main source of NO production in the brain (D. Kim et al., 2018). Also, reaction of NO with O^{2-} further leads to generation of reactive nitrogen species (RNS) and reactive oxygen species (ROS), which causes neurotoxicity at increased levels (Asiimwe et al., 2016). Previous studies have also demonstrated an increased level of reactive nitrogen species (RNS) in neuroinflammation to cause apoptosis through nitrosative stress (Chen et al., 2016). Moreover, investigations done in vitro and in vivo have further showed upregulation of iNOS with elevated level of NO production in macrophages stimulated with ligand such as LPS and hemozoin (S. H. Lee et al., 2016; Olivier et al., 2015; Velagapudi et al., 2019). These studies have demonstrated the role of excessive release of NO and increased expression of iNOS in neuroinflammation. Consequently, inhibiting level of NO and iNOS expression in activated microglia cells is essential in reducing neuroinflammation.

1.1.1.5 Prostaglandin E_2

Prostaglandin E_2 (PGE_2) is derived from arachidonic acid has been reported to play an important role as a mediator in inflammatory processes (Stephenson, Nutma, van der Valk, & Amor, 2018). Cyclooxygenase-2 (COX-2) speeds up the conversion of arachidonic acid to PGH_2 (Prostaglandin H2). This, in turn, gets converted by PGES

(Prostaglandin E synthase) to PGE2 (S.-H. Kim et al., 2019; Shabab et al., 2017).
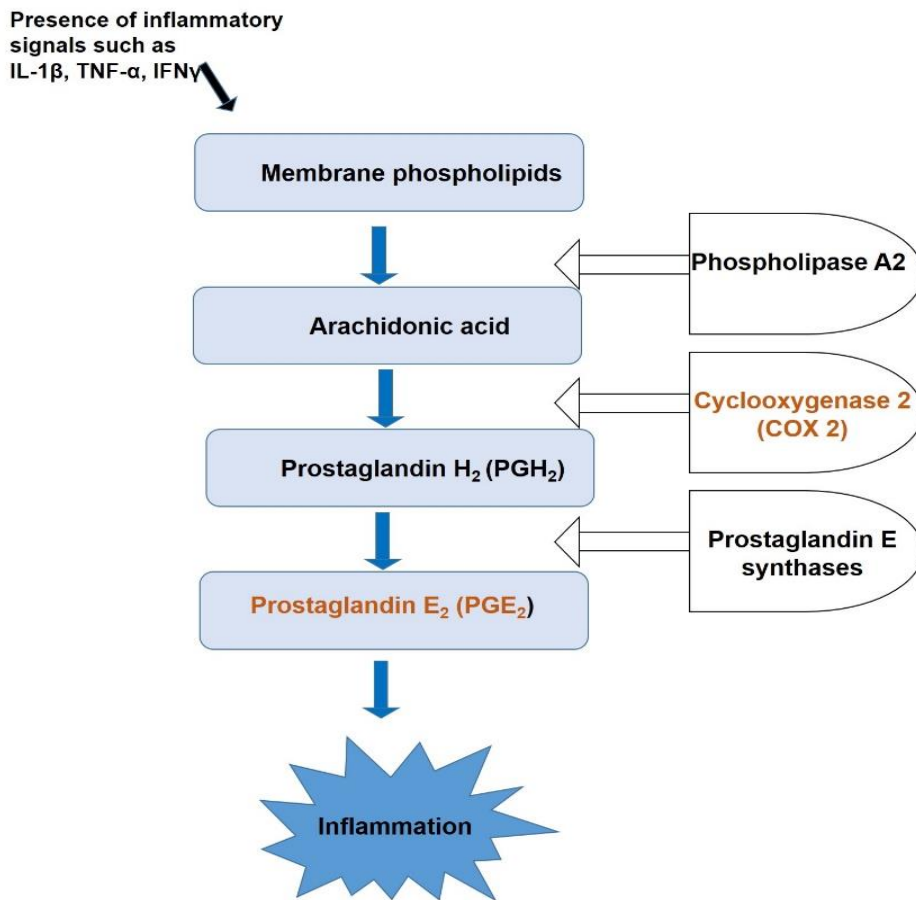
Figure 1-1: Prostaglandin E$_2$ synthesis pathway

Inflammatory stimuli including TNF-α hydrolyses the membrane phospholipids to secrete arachidonic acid. Thereafter, COX-2 converts arachidonic acid to PGH$_2$, which in turn is converted to PGE$_2$ (a pro-inflammatory mediator) through prostaglandinE synthases.

Previous studies have described an increased level of PGE$_2$ and expression of COX-2 protein in microglia cells stimulated with LPS (Ricciotti, FitzGerald, & biology, 2011; Sakai et al., 2017). This shows the importance of activated PGE$_2$/ COX-2 pathway in microglia cells contributing to the progress of neuroinflammation. In rat model of AD, over-expression of COX-2 enzyme was observed in brain of rats and was demonstrated to be involved in causing progression of neuroinflammation and increase cognitive dysfunction (Sil & Ghosh, 2016). The importance of PGE$_2$/ COX-2 pathway has been reported as non-steroidal anti-inflammatory drugs including aspirin

causes an inhibition of this pathway to have effect such as anti-inflammatory and analgesic.

Hence, the inhibition of excessive secretion of PGE_2 through reducing the expression of COX-2 protein might reduce neuroinflammation, which is crucial in pathology of neurodegenerative diseases.

1.1.1.6 NF-κB activation in neuroinflammation

Nuclear factor kappa B cells (NF-κB) is a transcription factor which plays a crucial role in the immune system by controlling the expression of genes that encodes pro-inflammatory cytokines, iNOS , COX-2 gene in addition to regulating cell proliferation, differentiation and survival (Biswas & Bagchi, 2016; Feng et al., 2017). NF-κB was discovered for over 30 years ago and has received a lot of attention due to its role in the pathology of numerous disorders (Zhang, Qian, Lenardo, & Baltimore, 2017). These include cardiovascular disorders (Gupta & Ashraf, 2018; Mangali et al., 2019), neurodegenerative diseases (Rather et al., 2018; Youssef, Ibrahim, Akashi, & Hossain, 2019), diabetes (Jayachandran, Vinayagam, Ambati, Xu, & Chung, 2018) and cancer (Patel, Horgan, McMillan, & Edwards, 2018; C. Xiao et al., 2018).

There are five NF-κB genes in mammals, which are related structurally: RelA, RelB, c-Rel, NF-κB 1(p105, p50) and NF-κB 2 (p100, p52) (Hayden & Ghosh, 2011; Serasanambati & Chilakapati, 2016). These proteins share a common Rel homology domain, which they can form heterodimers and homodimers (Liu, Zhang, Joo, & Sun, 2017). NF-κB dimers remain at resting state in the cytoplasm, because of its attachment to the inhibitory kappa-B (IκB) proteins (Biswas & Bagchi, 2016). Reports have demonstrated that NF-κB is activated by more than 150 powerful inducers including pro-inflammatory cytokines (TNF-α and IL-1β), bacterial components

(lipopolysaccharide), viral components and oxidative stress (Herrington, Carmody, & Goodyear, 2016; Serasanambati & Chilakapati, 2016).

Canonical and non-canonical pathways are the two main signalling pathways involved in NF-κB stimulation (Liu et al., 2017; Serasanambati & Chilakapati, 2016; S.-C. J. C. r. Sun, 2011). In canonical NF-κB pathway, receptors such as pattern recognition receptors (PRRs), TNF receptor, B-cell receptor and cytokine receptor bounds to by their respective ligands (Liu et al., 2017; Serasanambati & Chilakapati, 2016; H. Zhang, Sun, & bioscience, 2015). Binding of ligands to receptors leads to activation of TRAF6/ TAK1, which in turn activates the IKK complex (Durand & Baldwin, 2017). Afterwards, phosphorylation of IκB by IKK occurs, which leads to the degradation of IκB by proteasomes in the cytoplasm (Anchoori et al., 2010). Subsequently, there is nuclear translocation of NF-κB p65 subunit to cause transcription of inflammatory genes by binding of NF-κB to DNA of target genes (Underwood et al., 2013). Previous studies have demonstrated that NF-κB regulates the expression of iNOS, COX-2 and genes involved in the secretion of pro-inflammatory cytokines (Herrington et al., 2016). More investigations have shown that production of inflammatory mediators and pro-inflammatory cytokines are dependent on NF-κB transcription (Hayden & Ghosh, 2011; Liu et al., 2017). Observations from these studies confirms the importance of NF-κB transcription in neuroinflammation.

Studies have identified three main ways which NF-κB signalling pathway are inhibited to prevent its activation. They are: inhibiting the kinases (IκB, IKK) pathway in cytoplasm (Biswas & Bagchi, 2016; Herrington et al., 2016), blocking the ligand binding to receptor, which stops the signalling event at early stage (Molteni, Bosi, & Rossetti, 2018). In addition, inhibiting the binding of NF-κB to DNA prevents the transcription activity of NF-κB in the nucleus leading to a decrease in

neuroinflammation (Biswas & Bagchi, 2016; Herrington et al., 2016). These mechanisms of inhibition have been described to be essential target for inhibiting activated NF-κB signalling pathway, which is crucial in neuroinflammation and neurodegenerative diseases (Shabab et al., 2017).
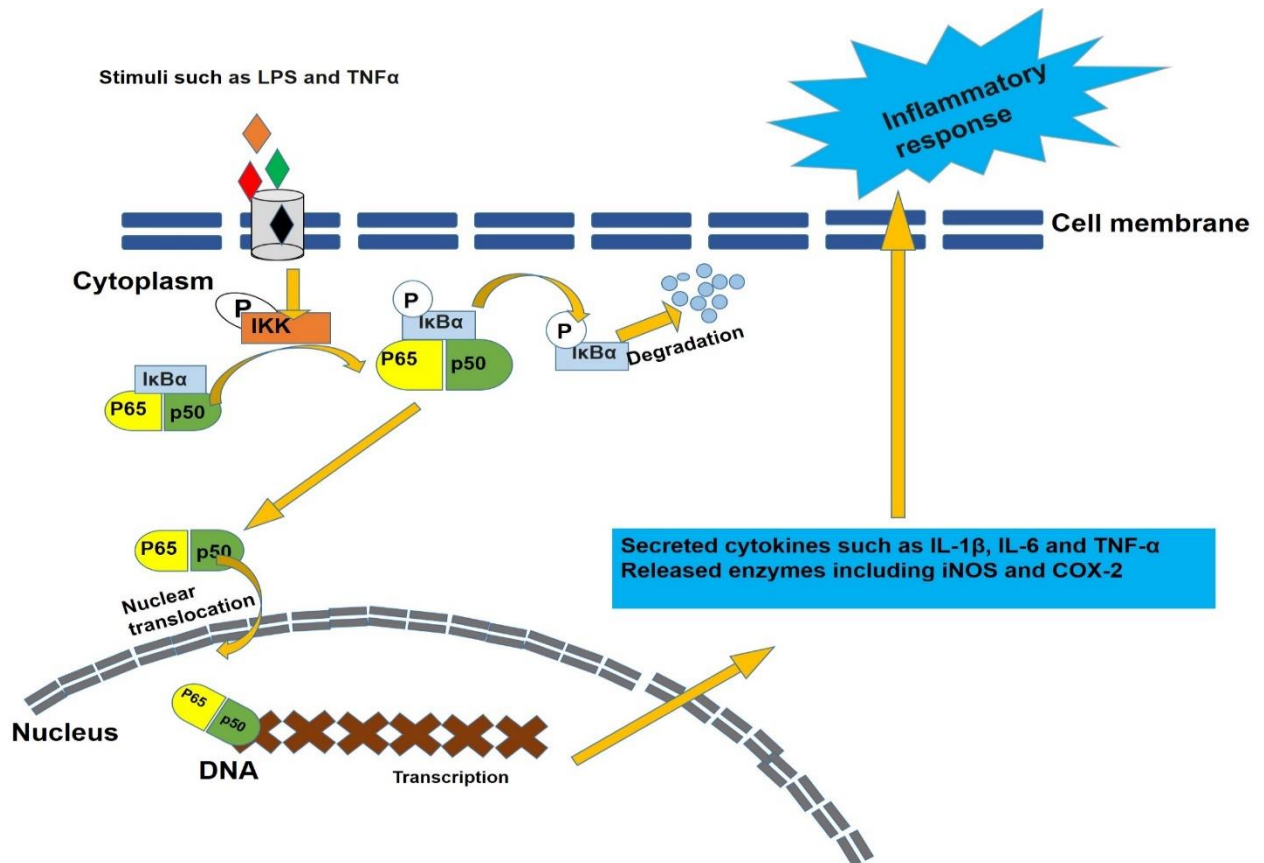
Figure 1-2: NF-κB pathway and inflammatory mediators

Ligands such as LPS and TNF-α binds to receptors to cause a conformational change and activates the IKK complex. Subsequently, phosphorylation of IκB by IKK occurs, which leads to the degradation of IκB by proteasomes in the cytoplasm. Afterwards, nuclear translocation of NF-κB p65 subunit occurs and causes transcription of inflammatory genes by binding of NF-κB to DNA of target genes. This event leads to secretion of pro-inflammatory factors.The family of IκB proteins includes IκBα, IκB β, IκBε, IκBζ, BCL-3 (B cell lymphoma 3) and IκBNS (Courtois, 2018; Durand & Baldwin, 2017). Studies have identified IκBα as the most abundant in its family and more importantly as they mainly controls the NF-*κ*B dimers (p65 and p50) by inhibiting NF-*κ*B until phosphorylation of IκBα occurs in the cytoplasm (Prescott & Cook, 2018;

Serasanambati & Chilakapati, 2016). Reports have shown that IκBα phosphorylation is done by a kinase called IKK (IκB kinase)(Durand & Baldwin, 2017). IKK is made of three subgroups namely: IKKα (IKK1), IKKβ (IKK2), IKKγ (NEMO- NF-κB essential modulator). IKKα and IKKβ are kinases that function as a catalyst while IKKγ has a regulatory function(Christian, Smith, & Carmody, 2016). Moreover, attenuating the level of IκB and IKK phosphorylation have been demonstrated as unique targets for pharmacological inhibition of NF-κB signalling pathway (Feng et al., 2017; Li et al., 2018; B. Wang et al., 2018). An example of IKKβ inhibitor is β-carboline, which acts as an analog of ATP and binds to ATP site on IKK molecule to cause inhibition (Wu, Clausen, Nielsen, & therapeutics, 2015). In addition, some IKK inhibitors such as BMS-345541 act by having allosteric effect because the more selective in inhibiting IKKβ over IKKα (Nadia Lampiasi & Giovanna Montana, 2016). This mechanism of action contributes to the reason some therapeutic agents could not target IKK phosphorylation. Furthermore, sulfasalazine, an inhibitor of IκB blocks NF-κB activation by preventing degradation of IκB in the cytoplasm (Wahl, Liptay, Adler, & Schmid, 1998; Y.-R. Wang et al., 2016).

1.1.1.7 Role of NLRP3 inflammasome in inflammation

Inflammasomes are protein complexes that were first identified and described in 2002 (Martinon, Burns, & Tschopp, 2002; Shao, Xu, Han, Su, & Liu, 2015). They include NLRP1, NLRP2, NLRP3, dsDNA (double stranded DNA), NLRC4 and AIM2 (absent in melanoma 2) (Shao, Cao, & Liu, 2018; Shao et al., 2015). Reports have shown that NLRP3 inflammasome is made up of NLRP3 protein, procaspase-1 and an adapter protein called ASC (apoptosis-associated speck-like protein) (Martinon et al., 2002; E. Ozaki, Campbell, & Doyle, 2015; Shao et al., 2018). NLRP3 inflammasome have been demonstrated to be activated by stimuli such as uric acid and hemozoin. (Eigenbrod

& Dalpke, 2015; Shao et al., 2018). Subsequently, there is assembly of NLRP3, procaspase-1 and an ASC into a complex. This complex then catalyses the conversion of procaspase-1 into an active caspase-1, leading to secretion of mature IL-1β from pro IL-1β.

In addition, activation of NLRP3 inflammasome have been shown to be involved in the secretion of IL-1β and IL-18 cytokines, which leads to pyroptotic cell death because of elevated, level these pro-inflammatory cytokines (Gaidt & Hornung, 2017; Shi, Gao, & Shao, 2017). In addition, previous studies have shown that activated NLRP3 Inflammasome controls the secretion of IL-1β in immune and inflammatory cells, which includes monocytes, dendritic cells and macrophages (S Jahan et al., 2017).
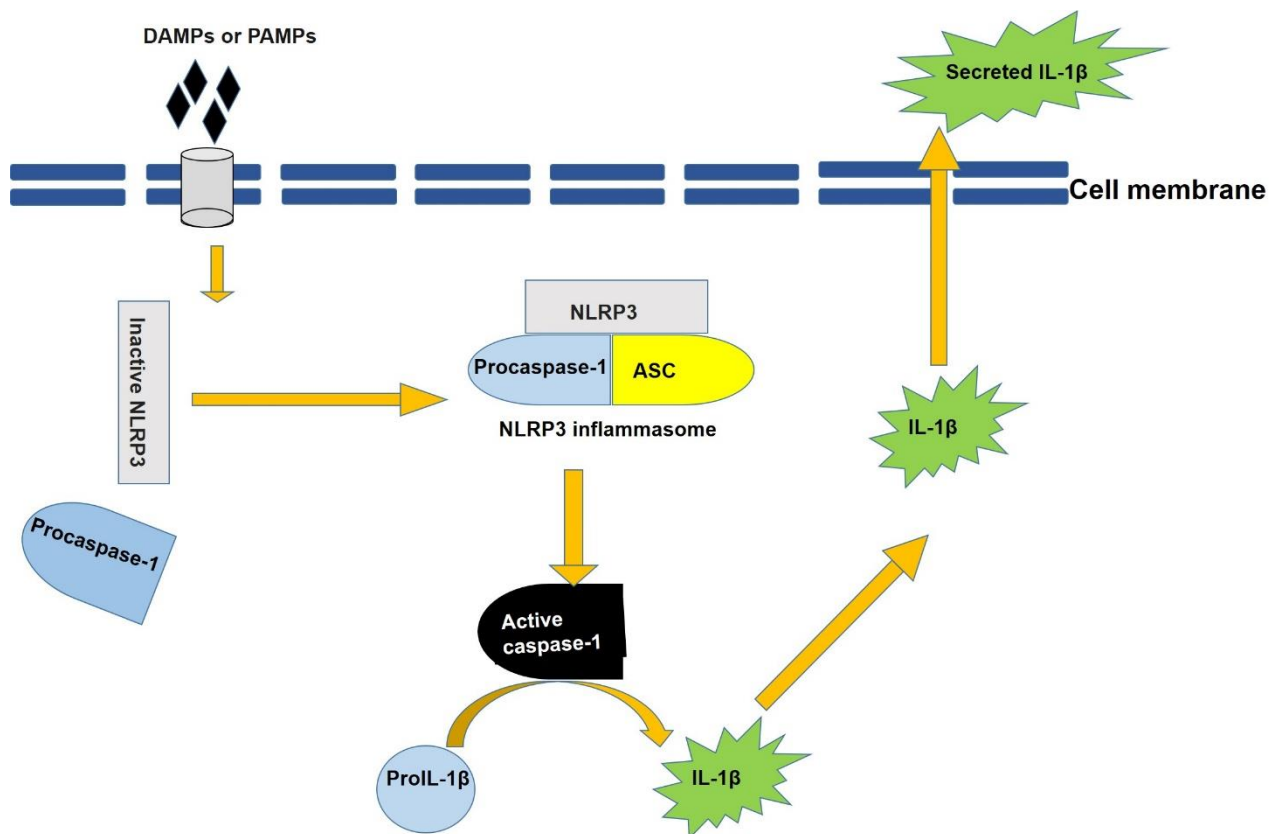
Figure 1-3: NLRP3 inflammasome pathway

Activation of NLRP3 inflammasome by DAMPS (Damage-associated molecular pattern such as uric acid and heparin sulfate) or PAMPs (pathogen-associated molecular pattern molecules such as LPS) causes the conversion of pro-caspase-1 to active caspase-1. Subsequently, there is maturation of Pro-IL-1 β to IL-1 β and this leads to secretion of IL-1 β, a pro-inflammatory cytokine.

IL-1β is one of the important pro-inflammatory cytokine produced in response to inflammation, pathogen and injury (Wojdasiewicz et al., 2014). IL-1 β has a predicted molecular weight ranging between 31-33 kDa. Pro IL-1 β is an inactive form of IL-1 β, it is converted by an enzyme called caspase-1 to IL-1 β and released as cytokines outside the cell (Dinarello, 2013; Piccioli & Rubartelli, 2013). Investigations have further shown that secretion of IL-1β are mainly from macrophages and monocytes of the innate immune system as well as endothelial cells and fibroblast that are non-immune cells (Dinarello, 2013; Piccioli & Rubartelli, 2013).Pathogen associated molecular patterns (PAMPs) act on the pattern recognition receptor (IL-1RI) in macrophages to secrete pro-IL-1β, hence regulating gene expression pathway (Lopez-Castejon & Brough, 2011; Piccioli & Rubartelli, 2013; O. Takeuchi & Akira, 2010; Wojdasiewicz et al., 2014). Investigations on overproduction of IL-1β in activated microglia cells have been suggested to contribute to the progression of neurotoxicity and neuroinflammation (Mendiola & Cardona, 2017). In addition, previous studies have observed excessive release IL-1β in neuroinflammation in LPS stimulated BV2 microglia cells (Matt, Lawson, & Johnson, 2016; Mendonca, Taka, Bauer, Cobourne-Duval, & Soliman, 2017). Similarly, more investigations have demonstrated over-production of IL-1β from macrophages and human monocytes stimulated with hemozoin, a malaria pigment (White, Turner, Medana, Dondorp, & Day, 2010). These studies indicate the involvement of IL-1β in the advancement of neuroinflammation, an important factor in the pathogenesis of neurodegenerative diseases. Therefore, targeting elevated level of IL-1β in neuroinflammation through inhibition of NLRP3/ caspase-1 activation and pro-IL-1 β is crucial in reducing the degree of IL-1β secretion in activated microglia cells.

1.2   Neuroinflammation in neurodegenerative diseases

The effect of neuroinflammation in neurodegenerative diseases including Parkinson's disease (PD), multiple sclerosis, Huntington's disease, amyotrophic lateral sclerosis (ALS) and Alzheimer's disease (AD) have been described to be an important factor that exacerbate the progression of these diseases (Chen et al., 2016; McManus & Heneka, 2017). However, the involvement of activated microglia cells are observed from postmortem examination done on patients with neurodegenerative diseases followed by damage and loss of neurons as the disease progresses (Bolós, Marta, Perea, & Avila, 2017; Ransohoff, 2016). In addition, secretion of pro-inflammatory cytokines (TNFα, IL-1β and IL-6), inflammatory mediators such as iNOS, COX-2 and reactive oxygen species (ROS) as a result of microglia cell activation have been demonstrated to cause neuroinflammation, exacerbating neurodegeneration (Park, Sapkota, Kim, Kim, & Kim, 2011; Shabab et al., 2017).
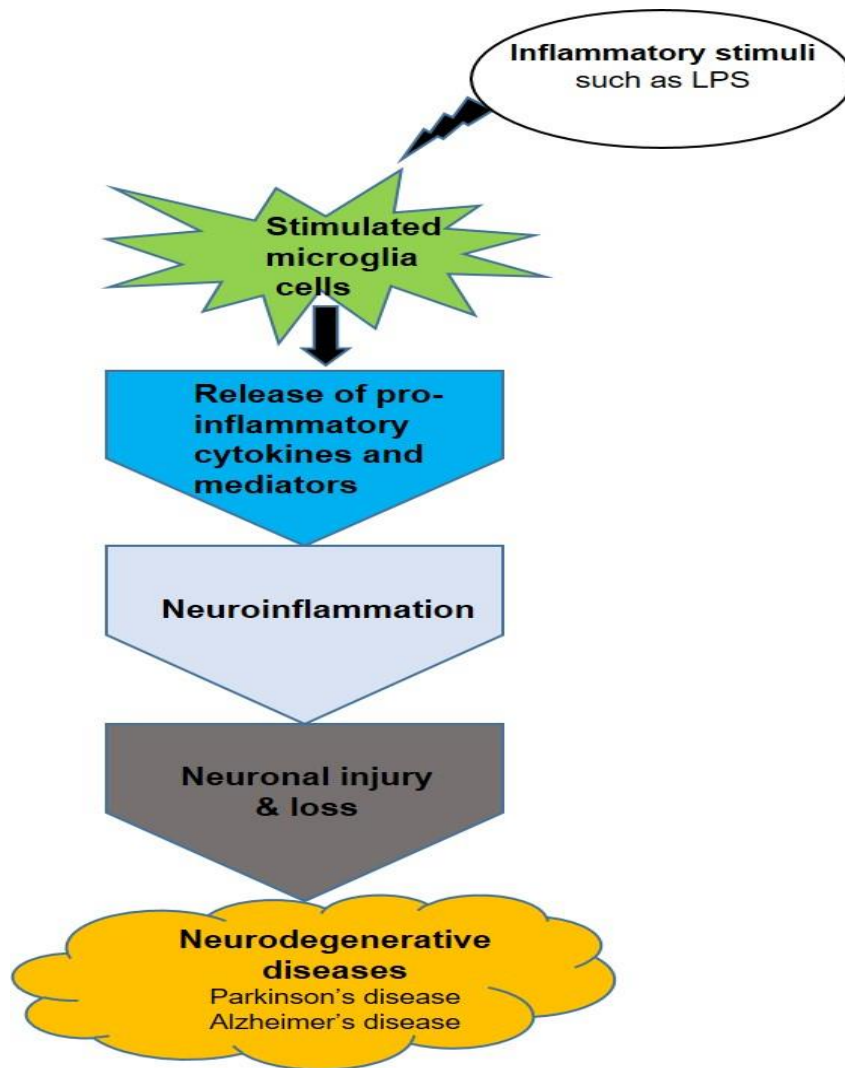
Figure 1-4: Neuroinflammation in neurodegenerative diseases

Activation of microglia cells by stimuli such as LPS causes the release of pro-inflammatory factors leading to neuroinflammation. Thereafter, prolonged neuroinflammation causes damage and loss to the neurons, and this exacerbates neurodegeneration.

Reports have shown an accumulation of amyloid beta (Aβ) plaque to be present in AD patient's brain using postmortem analysis (Kempuraj et al., 2016). Previous studies have demonstrated that Aβ deposit in the brain of AD patients triggers the activation of microglia cells to cause the excessive secretion of pro-inflammatory (McManus & Heneka, 2017) cytokines, which contributes to the progression of the disease. Furthermore, the activation of microglia cells by Aβ aggregates are described to be recognized by toll-like receptors (TLRs) in microglia cells (Sharman et al., 2019). An *in vitro* study demonstrated that the induction of microglia cells by Aβ resulted in secretion of reactive oxygen species (ROS) and reactive nitrogen species (RNS), causing neurotoxicity (Von Bernhardi, Eugenín-von Bernhardi, & Eugenín, 2015). These neurotoxic mediators were suggested to increase the progression of neurodegenerative diseases (W. J. Huang, Zhang, & Chen, 2016). The response of microglia cells to Aβ plaque by toll-like receptors are suggested to cause induction of neuroinflammation in AD (Sadigh-Eteghad et al., 2015). The use of anti-inflammatory therapy, nonsteroidal anti-inflammatory drugs (NSAIDs) such as acetic acid, oxicam and inhibitor of COX-2 enzyme have been shown to reduce the risk of AD progression (Rubio-Perez, Miguel, & Morillas-Ruiz, 2012). Conversely, due to the complexity in the association of AD pathology and innate immunity, NSAIDs do not show its use clinically in AD patient (Du, Wang, & Geng, 2018). Reports demonstrated that NSAIDS could not inhibit the neuroinflammation involved in the pathology of AD but rather it reduced the symptoms of the disease (Gao, Yu, Chen, & Zhou, 2016).

Studies have demonstrated the presence of activated microglia cells, increased level of iNOS, COX-2 and pro-inflammatory cytokines in the substantia nigra and striatum of Parkinson's disease patient (Chao, Yinxia, Wong, & Tan, 2014; De Virgilio et al., 2016; Halliday & Stevens, 2011; Tiwari & Pal, 2017). The loss of neuron in the basal

ganglia is associated with motor symptoms such as posture unsteadiness, stiffness, tremor, and slow movement (bradykinesia) in patient with PD (De Virgilio et al., 2016; Tiwari & Pal, 2017). Previous investigations on *in vivo* studies of CNS disorders including PD have further described that neuroinflammation increases the progression of neuronal damage. In neuroinflammation, increase in level of pro-inflammatory cytokines including TNF-α as a result of activated microglia cells have been shown to cause disruption of blood brain barrier (BBB) in neurodegenerative diseases. Increase in BBB permeability further contributes to the progression of neurodegenerative diseases by allowing the passage of cytokines and neurotoxic molecules. Moreover, previous investigations on cerebrospinal fluid of patients with PD have shown an elevated level of inflammatory mediators including reactive oxygen species (ROS), reactive nitrogen species (RNS) and TNF-α. In addition, post mortem examination on the tissue of patients with amyotrophic lateral sclerosis (ALS) revealed the presence of activated microglia cells (Frakes et al., 2014; Philips & Robberecht, 2011). An *in vivo* study on transgenic mice suggested that activation of microglia cells is involved in the death of motor neurons (Philips & Rothstein, 2015). These observations suggest the role of pro-inflammatory cytokines and inflammatory mediators causing neuroinflammation and neurotoxicity in neurodegenerative diseases. Drugs such as levodopa are available in the treatment of PD but are limited in their action as they only treat symptoms which includes slow movement but not inflammation involved in the pathology of PD (LeWitt, 2015; Poewe et al., 2017). The outcome of the failed clinical trial of NSAIDs led to investigations for the search of drugs with anti-inflammatory properties that could reduce neuroinflammation in neurodegenerative diseases.

Numerous research has identified the inhibitory activity of some medicinal plant on inflammation in both animal models and cell culture studies(Shakya, 2016; Tomlinson & Akerele, 2015). Investigations on medicinal plants with anti-inflammatory properties have shown to be promising in the treatment of neurodegenerative diseases (X.-S. Wang et al., 2017). *In vitro* studies on *Curcumin longa* are suggested to have a possible role in the treatment of AD by reducing inflammation induced by amyloid-beta (Aβ) (Hamaguchi, Ono, Yamada, & therapeutics, 2010). A report in a different study demonstrated that *Coriandum sativum* improved memory performance due to its anti-inflammatory activities and indicated to be a potential treatment in AD (Mani & Parle, 2009). Moreover, previous *in vivo* studies on *oxalis corniculata* showed its anti-inflammatory and anti-oxidant activity in Parkinson's disease model (Aruna, Rajeswari, & Sankar, 2016). Therefore, these studies suggest the importance of medicinal plant with anti-inflammatory activities. These medicinal plants could be a major source of drugs that could offer a potential treatment in reducing neuroinflammation in neurodegenerative diseases.

1.2.1 Gap in Knowledge

To date, investigations to determine the effect of *Zanthoxylum zanthoxyloides* extract and isolated bioactive compounds from this extract on neuroinflammation are unknown.

1.2.2 Aim and objectives of this study

This study was aimed to investigate whether *Zanthoxylum zanthoxyloides* extract and isolated bioactive compounds could modulate LPS-induced neuroinflammation in BV-2 microglia cells. In addition, there was a separate investigation on the effect of *Zanthoxylum zanthoxyloides* extract and isolated bioactive compounds on neuroinflammation stimulated by hemozoin in BV-2 microglia cells.

Hence, this research will categorically achieve the following objectives:

1. To investigate the inhibitory activity of *Z. zanthoxyloides* extract in LPS and hemozoin-induced neuroinflammation in BV-2 microglia.
2. To identify bioactive compounds in *Z. zanthoxyloides* extract
3. To evaluate the inhibitory activity of isolated bioactive compounds from this extract on LPS and hemozoin-induced neuroinflammation in BV-2 microglia cells.

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2 Chapter 2- Background information on medicinal plants in drug discovery

Medicinal plants have become an area of research interest in the past till date (Malviya & Malviya, 2017; Medhi, Deka, & Bhau, 2013). This is because of their wide use in traditional medicine and drug discovery (Patiño, Prieto, & Cuca, 2012). In drug discovery, medicinal plants are useful in the search of bioactive compounds as they are a rich source of numerous secondary metabolites. Morphine from opium poppy (Norn, Kruse, & Kruse, 2005), Vinblastine and Vincristine from *Catharanthus roseus* (Priyadarshini & Keerthi, 2012), Artemisinin from *Artemisia annua* (Miller & Su, 2011), Digoxin from *Digitalis lanata* (Chauhan, Ruby, & Dwivedi, 2012) are as a result of drug discovery from medicinal plants (Patiño et al., 2012; Shakya, 2016; Singh, 2015).

The genus *Zanthoxylum* of the family *Rutaceae* is very important because of its wide range of uses in medicine and industry (Medhi et al., 2013; Patiño et al., 2012). *Zanthoxylum* is derived from a Greek word *Xanthoxylum*, which means ''yellow wood''(Patiño et al., 2012). It is made up of about 549 species which are majorly located in America (Mexico, Brazil, Argentina and Puerto Rico) (Groppo & Pirani, 2017), Asia (China, India, Vietnam and Malaysia) (Zhu, Huang, Ji, Su, & Zhou, 2016) and Africa (Kenya, Zimbabwe, Cameroon, Senegal, Rwanda and Nigeria) (Adesina, 2005; Tine, Diop, et al., 2017). *Zanthoxylum* species that are known especially in Africa are about thirty-five in numbers (Tine, Diop, et al., 2017). They include *Zanthoxylum gillettii* (Sinan, Zengin, Bene, & Mahomoodally, 2019), *Zanthoxylum rubescens* (Penali, Mulholland, Tano, Cheplogoi, & Randrianarivelojosia, 2007)*, Zanthoxylum bouetense* (Pan, 2010)*, Zanthoxylum tessmannii* (Tankeo et al., 2015) and *Zanthoxylum zanthoxyloides* (Kassim et al., 2015; Prempeh & Mensah-Attipoe, 2009).

2.1 Zanthoxylum zanthoxyloides

Zanthoxylum zanthoxyloides (*Z. zanthoxyloides*), a popular specie of *Zanthoxylum* genus is a spiny tree that is about 6- 12 meters tall (Iwu, 2014; Nwankwo, Chioma, Ofem, Amara, & Chijioke, 2017). In West Africa, the different parts of *Z. zanthoxyloides* tree such as the leaves, fruits, stem bark and root are popularly used in traditional medicine for the treatment of diseases which includes malaria (Gansané et al., 2010), toothache (Orafidiya, Akinkunmi, Oginni, & Oluwamakin, 2010) and sickle cell disease (Folashade & Omoregie, 2013; Kouri, 2004; Ouattara et al., 2009). In Nigeria, the root parts of *Z. zanthoxyloides* are used as chewing stick due to its antimicrobial and antibacterial activities against oral microbiota (Adesina, 2005; Kénou, Christel & Djossa, & Assogbadjo, 2018; Medhi et al., 2013; Orafidiya et al., 2010).

Studies have identified the use of methanolic extract of *Zanthoxylum zanthoxyloides* in the treatment of malaria (Gansané et al., 2010; Kassim et al., 2005; Odugbemi, Akinsulire, Aibinu, & Fabeku, 2007; Traoré et al., 2015; Wangensteen, An, Christopher, Diallo, & Karl, 2017). An *in vitro* study which used a multi-resistant strain of *Plasmodium falciparum* revealed that *Z. zanthoxyloides* extract has anti-plasmodial activity with an IC_{50} of 1.2μg/ml, hence supporting its wide use in treatment of malaria (Gansané et al., 2010). In addition, previous studies have shown the anti-inflammatory activity of methanolic extract of *Zanthoxylum zanthoxyloides* root (Fogang et al., 2012; Prempeh & Mensah-Attipoe, 2008, 2009). The study showed an inhibition of carrageenan-induced paw inflammation in rats and xylene-induced pinna inflammation in mice by methanolic extract of *Z. zanthoxyloides*.

Figure 2-1*: Zanthoxylum zanthoxyloides* tree

Table 2.1: Synonyms, trivial and local names of *Zanthoxylum zanthoxyloides*

Synonyms of *Zanthoxylum zanthoxyloides*	*Fagara zanthoxyloides*
Trivial/ English name	Candle wood Toothache bark
African names	Rapeko (Moore language from Burkina Faso) Guene gui deg (Wolof language from Gambia, Senegal and Mauritania)
Traditional Nigerian names	Orin-ata/ Igi-ata (Yoruba) Aga (Igbo) Fasa-kuwari (Hausa)

2.1.1 Compounds isolated from *Zanthoxylum zanthoxyloides*

Z. zanthoxyloides extract has been shown to contain secondary metabolites such as flavonoid (Azando et al., 2011; Ogunbolude et al., 2014a), alkaloid (Dupont et al., 2005; Michael, 2017; Wouatsa et al., 2013), aliphatic and aromatic amides (Adesina, 1986; Patiño et al., 2012). It also contains lignans (Adesina, 2005; Azando et al., 2011; Patiño, Prieto, & Cuca, 2008) and coumarins (Cho et al., 2012; Negi, Bisht, Bh, Singh, & Sundriyal, 2011; Tine, Yang, et al., 2017) as shown below.

Table 2.2: Example of compounds isolated from different parts of *Zanthoxylum zanthoxyloides*

Isolated compounds	Parts of *Z. zanthoxyloides*			References
	Roots	Stem	Fruits	
Flavonoids				(Ogunbolude et al., 2014b; Tine, Yang, et al., 2017)
Neohesperidin	+	+	+	''
Hesperidin	+	+	+	,,
Quercetin	+	+	+	,,
Neodiosmin				,,
Lignans				,,
Sesamin	+			(Chaaib et al., 2003)
Asarinin	+			(Adesina, 2005)
Coumarins				
Bergapten		+	+	(Adesina, 2005; Patiño et al., 2012)
Imperatonin		+		(Adesina, 2005; Patiño et al., 2012)
6,7,8-Trimethoxy Coumarin	+			(Patiño et al., 2012; Wangensteen et al., 2017)
4-Methoxycoumarin			+	(Adesina, 2005; Tine, Diop, et al., 2017)

Isolated compounds	Parts of *Z. zanthoxyloides*			References
	Roots	Stem	Fruits	
6-Methoxycoumarin			+	(Adesina, 2005; Tine, Renucci, Costa, Wélé, & Paolini, 2017)
Tripterpenes				
Squalene	+			(Adesina, 2005; Mbaze et al., 2007; Rasooli, 2012)
Lupeol	+		+	(Misra, Wouatsa, Kumar, Kumar, & Tchoumbougnang, 2013; Rasooli, 2012)
Campesterol	+			(Adesina, 2005; Rasooli, 2012)
β-Amyrin	+			(Adesina, 2005; Rasooli, 2012)
Alkamides				
Cis-fagaramide	+	+		(Adesina, 2005; Patiño et al., 2012)

Isolated compounds	Parts of *Z. zanthoxyloides*			References
	Roots	Stem	Fruits	
Pellitorine	+			(Adesina, 2005; Patiño et al., 2012)
*Trans-*fagaramide	+			(Adesina, 2005; Patiño et al., 2012)
(2*E*,4*E*)-*N*-Isobutyl tetradeca-2,4-dienamide			+	(Adesina, 2005; Patiño et al., 2012)
Benzophenanthridine alkaloids				
Fagaronine	+	+		(Adesina, 2005; Patiño et al., 2012)
Chelerythrine	+			(Adesina, 2005; Patiño et al., 2012)
Fagaridine		+		(Adesina, 2005; Patiño et al., 2012)
6-Hydroxy-5,6-Dihydro chelerythrine	+			(Adesina, 2005; Patiño et al., 2012; Wang-sheng et al., 2017)
Buesgenine	+			(Adesina, 2005; Patiño et al., 2012; Wang-sheng et al., 2017)

Isolated compounds	Parts of *Z. zanthoxyloides*			References
	Roots	Stem	Fruits	
Aporphine alkaloids				
Berberine	+			(Adesina, 2005; Patiño et al., 2012)
Tembetarine	+			(Adesina, 2005; Patiño et al., 2012)
Magnoflorine	+			(Adesina, 2005; Patiño et al., 2012)
*N, N-*Dimetylindicarpine	+			(Patiño et al., 2012; Queiroz et al., 2006a)
Quinoline alkaloids				
Dictamnine	+			(Patiño et al., 2012; Wangensteen et al., 2017)
Skimmianine	+			(Adesina, 2005; Patiño et al., 2012)
Fagarine	+			(Adesina, 2005; Patiño et al., 2012)
Acronycine	+		+	(Adesina, 2005; Patiño et al., 2012)

Neohesperidin

Hesperidin

Quercetin

Neodiosmin

Sesamin

Asarinin

Bergapten

Imperatonin

6, 7, 8-Trimethoxy coumarin

4-methoxycoumarin

6-methoxycoumarin

Squalene
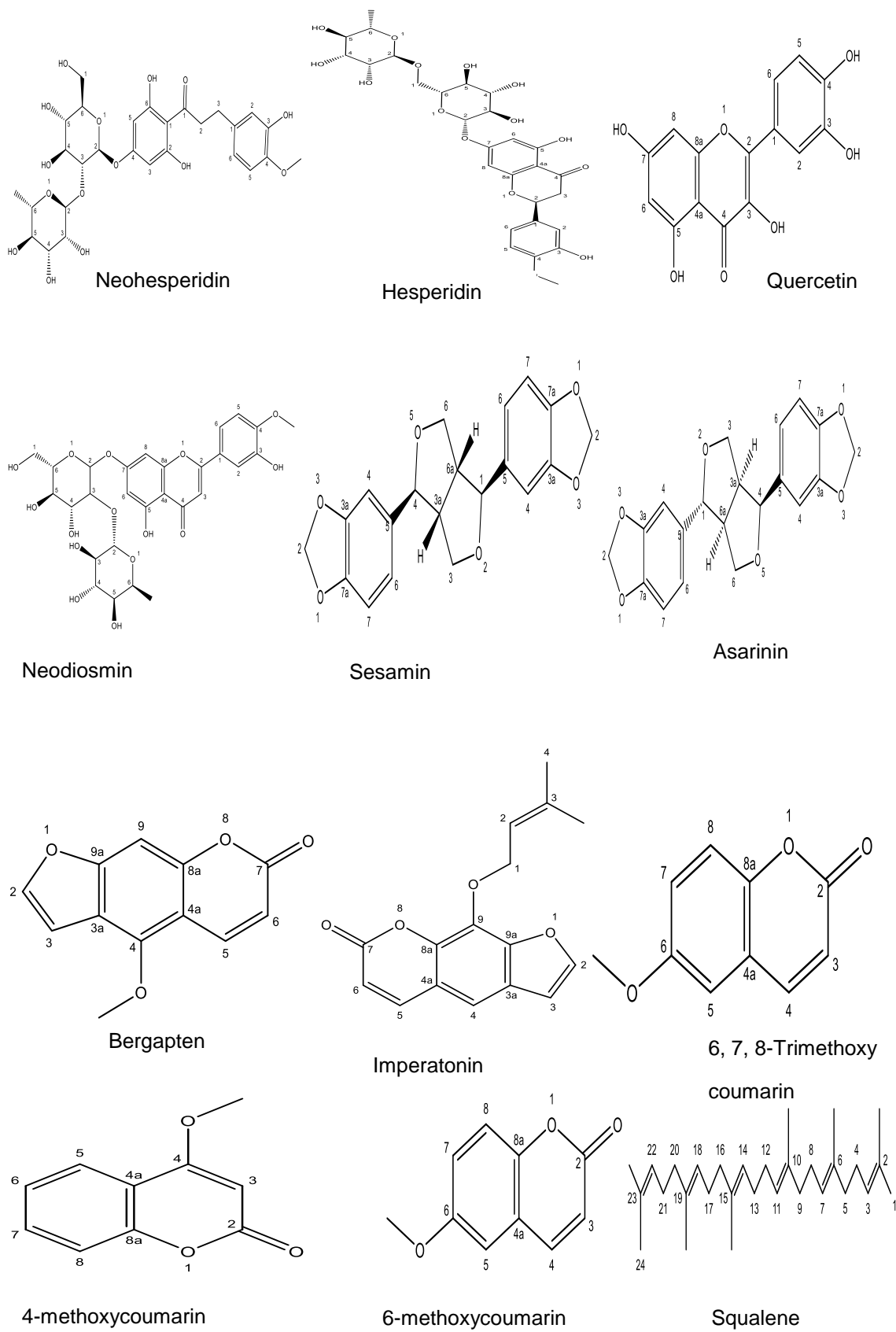
Figure 2-2: Chemical structures of compounds isolated from *Zanthoxylum zanthoxyloides*

lupeol

Campesterol

β- Amyrin

Fagaramide

Pellitorine

(2*E*,4*E*)-*N*-Isobutyl
tetradeca-2,4-dienamide

Fagaronine

Chelerythrine

Fagaridine

6-Hydroxy-5, 6

Dihydrochelerythrine

Buesgenine
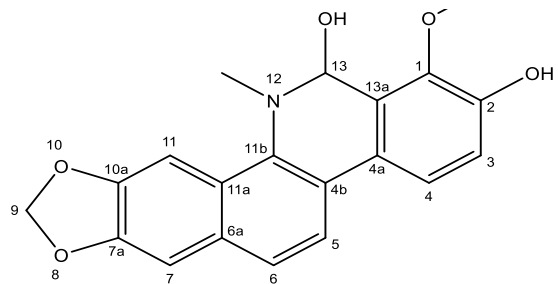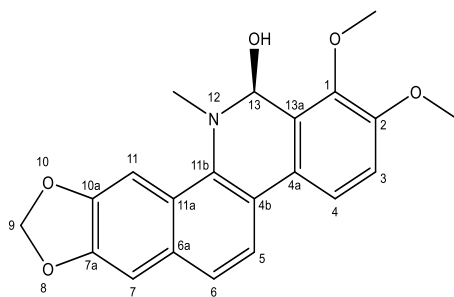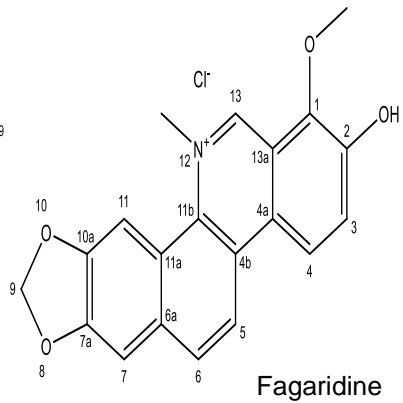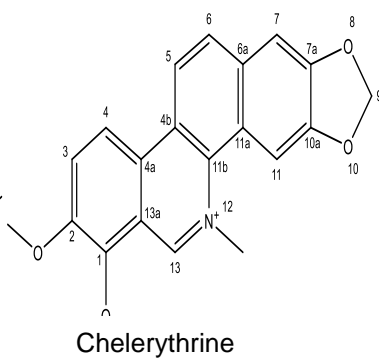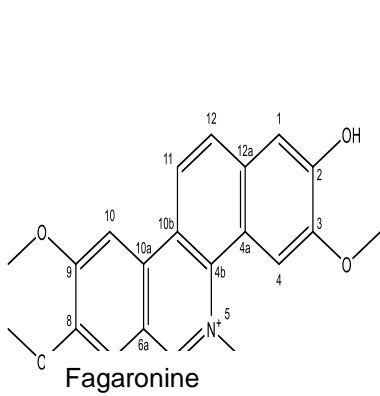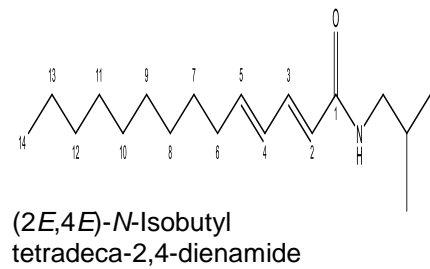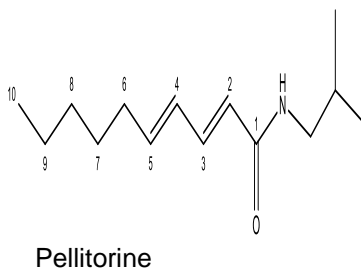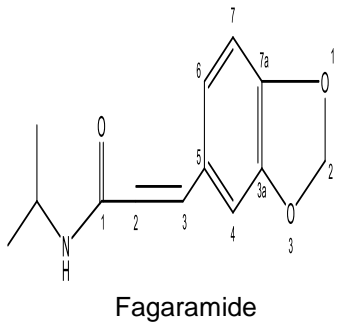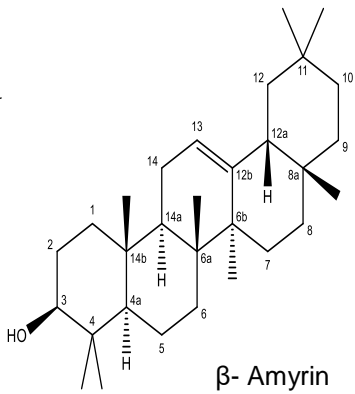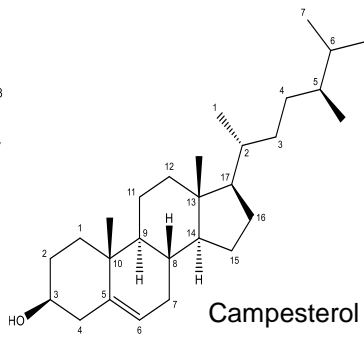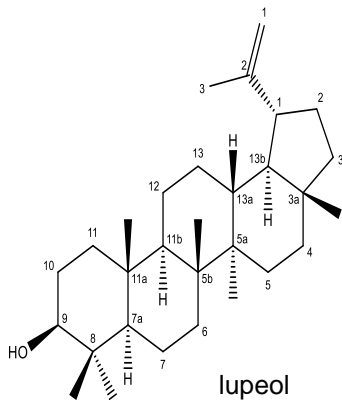
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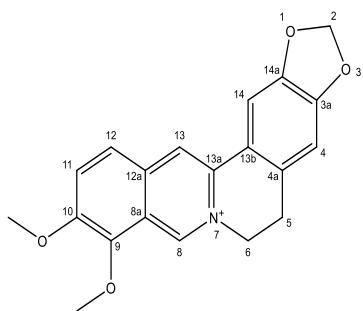
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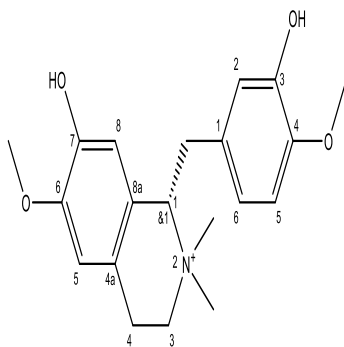
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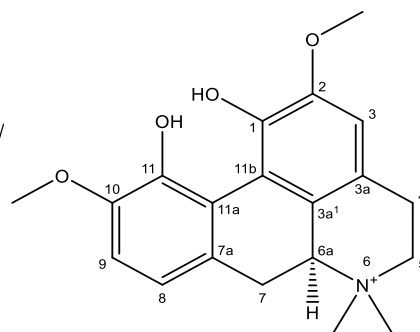
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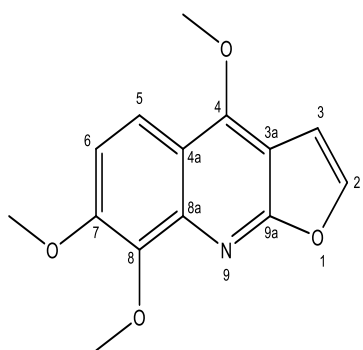
Dictamnine
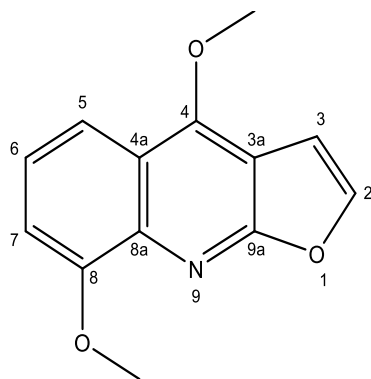
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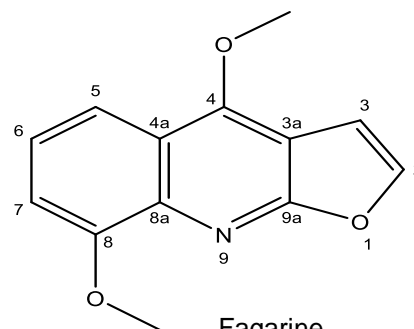
Fagarine
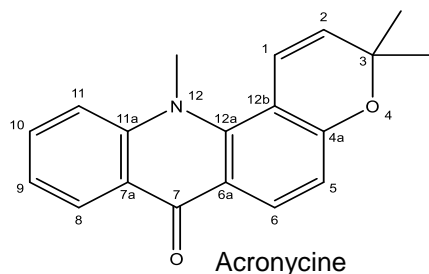
Acronycine

Figure 2-2 continued

2.2   Methodology

2.2.1   Collection of plant

Zanthoxylum zanthoxyloides roots were collected from the botanical garden of University of Ibadan, Nigeria in the month of June 2015. Mr D.P.O. Esimekhuai (a taxonomist) in the Department of Botany, University of Ibadan, Nigeria, authenticated the plant. A voucher specimen coded UIH 22474 was deposited in the University Herbarium.

2.2.2   Soxhlet extraction

Soxhlet extraction is a common technique in isolation of compounds that is useful in extraction of solid samples in a solvent, based on the polarity and phytochemical components in the plant (Azwanida, 2015; De Castro & Priego-Capote, 2010). Soxhlet extraction in hexane and dichloromethane (DCM) are necessary to remove the non-polar and semi-polar components in plants while extraction in methanol is important to obtain polar components from plants. This method requires putting of powdered sample of the extract in a filter paper made of cellulose and this is placed in the thimble chamber of the Soxhlet apparatus (Azwanida, 2015). In this study, solvents including hexane, dichloromethane (DCM) and methanol needed for the extraction were put in a round-bottom flask. The extraction solvent is heated, and the vapour condenses into the thimble chamber (where the extract is placed) and drips back into the bottom flask. This cycle is repeated until the extraction process is completed.

The powdered plant material of *Z. zanthoxyloides* root was extracted using a Soxhlet extractor as shown in

Figure *2-3*. The root sample was extracted in 800 ml each of *n*-hexane, dichloromethane and methanol sequentially. These extraction solvents were heated up between 50-60°C using an Electrothermal™ (Fisher Scientific, Loughborough,

Uk). The extraction process was cycled ten times for each solvent. The methanolic extracts was dried using a Cole palmer rotary evaporator (Stone UK).and then stored at 4°C for pharmacological assays. In this study, methanolic extract of *Zanthoxylum zanthoxyloides* was continued with and used for pharmacological assays because it has been reported in numerous in vivo studies to have anti-inflammatory activities (Fogang et al., 2012; Prempeh & Mensah-Attipoe, 2008, 2009).
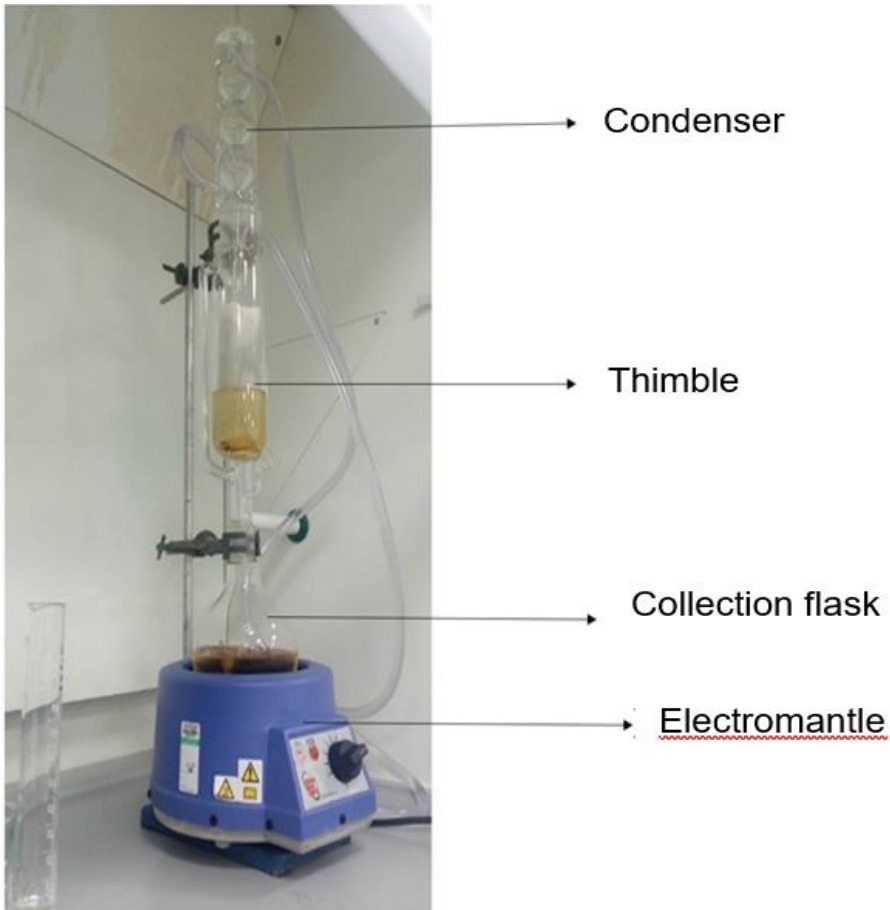
Figure 2-3: Extraction of *Z. zanthoxyloides* using a Soxhlet apparatus

2.2.3 Cell culture

2.2.3.1 Culture of BV2 microglia cells

BV2 mouse microglia cell line ICLCATL03001 was purchased from Interlab Cell Line Collection (Banca Biologica e Cell Factory, Italy) and cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Life Technologies) supplemented with 10% fetal bovine serum (Sigma), 1mM sodium pyruvate (Sigma), 100U/ml Penicillin and 100mg/ml Streptomycin (Sigma). Once the cells became confluent, cells were sub-cultured at 1:10 using trypsin/EDTA solution (Sigma) to detach adhered cells to the flask, as BV2 cells are semi-adherent cells. The cells were later maintained at 37° C in 5% CO_2 incubator after subculture. Cells were seeded in plates at a concentration of 2×10^5 cells/ ml, before experiments were carried out. Subsequently, the plated cells were being incubated until they reached confluence before experiments.

2.2.3.2 Culture of HEK 293 cells

Human Embryonic Kidney (HEK) cells (European collection of cell cultures) are adherent cells and they were cultured in MEM medium (ThermoFisher Scientific, UK). MEM medium was supplemented with 2mM l-glutamine (Sigma), 10% FBS (Sigma), 100U/ml Penicillin and 100mg/ml Streptomycin (Sigma). The cells were later maintained at 37° C in 5% CO_2 incubator. Cells were sub-cultured at 1:3 with trypsin/EDTA solution (Sigma), when they became confluence.

2.2.4 Sample preparation

Methanolic extract of *Zanthoxylum zanthoxyloides* extract was prepared in DMSO (Sigma, UK). Primary stock concentration was prepared and stored in -80°C. A stock concentration of 2, 3 and 6 mg/ml was prepared to reach a final or working concentration of 4, 6 and 8µg/ml of *Zanthoxylum zanthoxyloides* used for experiments. 1 mg/ml of lipopolysaccharide (LPS) (Sigma, UK) made from

Salmonella enterica serotype typhimurium was used in preparing a stock concentration of 100 µg/ml and kept in 4°C. BV2 cells were treated with methanolic extract of *Zanthoxylum zanthoxyloides* (4, 6 and 8µg/ml) for 30 minutes and stimulated with LPS at 100 ng/ml for time required by different experiments. In a different investigation, hemozoin (5 mg) (InvivoGen, USA) was dissolved in 1 ml of sterile PBS to make 5mg/ml. This mixture was then sonicated (Grant XUBA 1) to obtain a homogenous suspension. A working concentration of 400 µg/ml was used from the stock in experiments. BV2 microglia cells were treated with methanolic extract of *Z. zanthoxyloides* (4, 6 and 8 µg/ml) for 30 minutes and stimulated with hemozoin at 400 µg/ml for the time needed by each experiment.

2.2.5 Griess assay (Measurement of nitrite production)

Nitric oxide (NO) is an important mediator for regulating chronic inflammation in the central nervous system (CNS). Nitrite (NO_2^-) production level was measured in the cell culture supernatant as an indicator of NO production because NO_2^- is non-volatile and stable (Green et al., 1982; Tse, 2017; Tsikas, 2007). The principle of Griess reaction relies on two main steps: the reaction of sulphanilic acid with acidified NO_2^- containing sample to form diazonium ion and addition of N-(1-naphthyl) ethylenediamine to form chromophoric azo dye, which absorbs light at a wavelength of 540 nm. Cultured BV2 cells were seeded at density of 2 x 10^5 cells/ ml (1mL/well) in a 24-well plate and incubated until it reached confluence. At confluence, BV2 cells were treated with *Z. zanthoxyloides* (4, 6 and 8 µg/ml) and stimulated with LPS (100 ng/ml) for 24 hours. In a separate study, there was incubation of BV2 cells with *Z. zanthoxyloides* (4, 6 and 8 µg/ml) for 30 minutes, followed by stimulation with hemozoin (400 µg/ml) for 24 hours. Subsequently, cell culture supernatants were collected and centrifuged at 1500 rpm for 5 minutes. Nitrite concentration in the cell culture supernatant was measured

using the Griess assay kit (Promega, UK). Briefly, 50 µl of sulphanilamide was added to 50 µl of cell culture supernatants in a 96 well plate. The plate was then incubated in the dark for 10 minutes at room temperature. Afterwards, 50 µl of NED solution (N-1-napthylethylenediamine) was added and incubated in the dark for 10 minutes at room temperature. Absorbance was read at a wavelength of 540nm in a microplate reader (Infinite F50, Tecan).

2.2.6 Determination of TNFα, IL-6, IL-1β, IL-10 and Pro-IL-1β by Enzyme linked immunosorbent assay (ELISA)

ELISA is a plate-based assay technique that is designed for detecting and quantifying antigens in cell supernatants (Konstantinou, 2017; Lequin, 2005). Sandwich ELISA was used for this experiment because it is a very sensitive assay for detection of antigens in cell culture supernatant. Sandwich ELISA measures the antigens in a sample between two layers of antibodies: capture and detection antibodies. TNFα, IL-6 and IL-1β are pro-inflammatory cytokines that mediate chronic inflammation and elevated levels of their secretion in activated microglia cells have been shown to be involved in neuroinflammation (Becher & Spath, 2017). IL-10 is an anti-inflammatory cytokine as increased level of IL-10 has been reported to reduce the level of pro-inflammatory cytokines in LPS stimulated macrophage (Castillo, Patricia, & Kolls, 2016; Couper, Blount, & Riley, 2008). Pro IL-1β is a pro-form of IL-1β, a potent pro-inflammatory cytokine produced by cells of the innate immune system in response to pathogens. BV2 cells were seeded in 24-well plates at a concentration of $2x10^5$ cells/ml (1 mL/well) and incubated at $37^{\circ}C$ until they reached confluence. BV2 cells were treated with *Z. zanthoxyloides* (4, 6 and 8 µg/ml) for 30 min and then stimulated with LPS (100 ng/ml) for 24 hours. In a different investigation, BV2 microglia cells were

incubated with *Z. zanthoxyloides* (4, 6 and 8 µg/ml) for 30 min and then stimulated with hemozoin (400 µg/ml) for 24 hours.

In both studies, levels of TNFα, IL-6, IL-1β and IL-10 in cell supernatants were measured with a Biolegend, ELISA kit, UK while Pro IL-1β was measured with an Invitrogen ELISA kit.
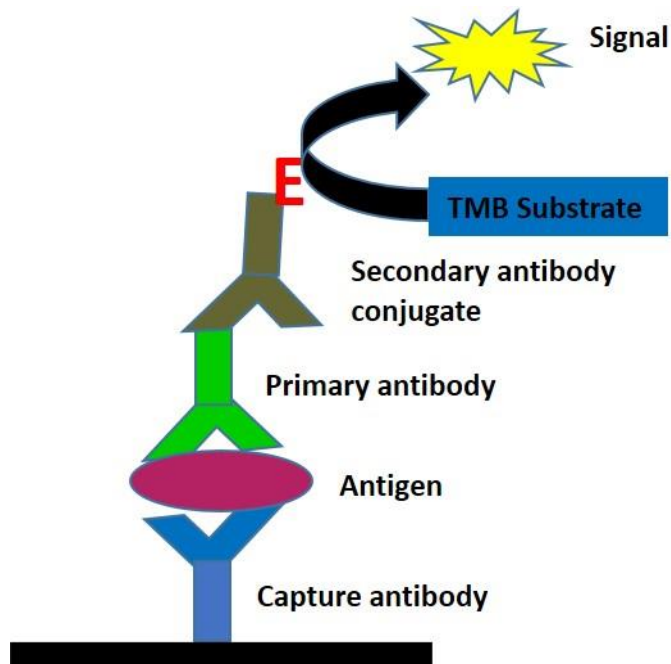
Figure 2-4: Sandwich ELISA

ELISA kits and samples were brought to room temperature from storage before the experiments. Microplates were coated with monoclonal capture antibody of cytokines to be detected overnight at 4°C fridge. The microplates were then washed with 1x wash buffer for four times to remove unbound antibodies. Subsequently, blocking buffer (assay diluent) was added to each well for 1 hour to cover the non-specific binding sites in the plates. Samples and standards were added to the appropriate wells and incubated at room temperature for 2 hours with shaking. Afterwards, the wells were washed for four times and 100µl of detection antibody was added to bind specifically to the target antigen. This was incubated for an hour further with shaking

at room temperature. Following incubation, the wells were washed four times and 100 µl of Avidin-HRP (horseradish peroxidase) was added to each wells for 30 minutes with shaking at room temperature. The addition of this enzyme was to amplify the signal that can be detected (colorimetric) by acting on the substrate to develop a colour. Subsequently, the plate was washed five times at the end of the incubation and 100µl of TMB (3, 3',5, 5'-Tetramethylbenzidine) substrate was added to each well. This was incubated at room temperature in the dark for 15 to 30 minutes. Lastly, the blue colour that developed after adding TMB substrate was stopped by adding 100µl of stop solution to each well. Absorbance was read at 450nm using a plate reader (Infinite F50, Tecan).

2.2.7 Caspase-1 Glo® inflammasome assay

Caspase-1 is part of the cysteine aspartic acid-specific protease (caspase) family (Kanneganti, 2018; Sollberger, Strittmatter, Garstkiewicz, Sand, & Beer, 2014). It is important for cytokine maturation (from pro-IL1β to IL-1β and pro-IL18 to IL-18) when induced by inflammatory stimuli (Flores et al., 2018; Sollberger et al., 2014). The Caspase-1 Glo® inflammasome assay is a bioluminescent method which selectively measures caspase-1 activity in cells. This principle is based on the binding of caspase 1 to a luminogenic substrate, Z-WEHD-aminoluciferin. This leads to a cell lysis which produces light, a luminescent signal that is proportional to caspase-1 activity in the cells. BV2 cells were seeded in a white, opaque 96 well plates (2×10^5/cells/ml) for luminescence signal to be measured. The plate was incubated at 37°C until they become confluent.
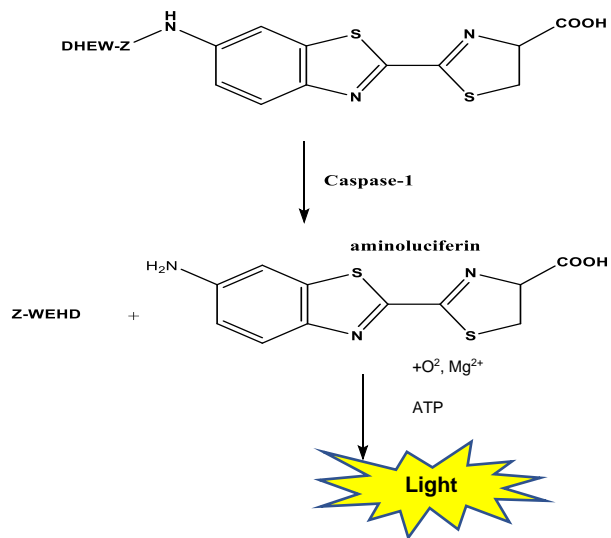
Figure 2-5: Reaction of Caspase-Glo® 1 inflammasome assay

BV2 cells were treated with *Zanthoxylum zanthoxyloides* (4, 6 and 8µg/ml) for 30 min and stimulated with hemozoin (400µg/ml) for 24 hours. Caspase-1 activity was measured using the Caspase-1 Glo® inflammasome assay kit (Promega, USA). The reagents in the assay kit were allowed to equilibrate to room temperature prior to the experiment. 100µl of prepared Caspase-Glo® 1 Reagent was added to the wells in the 96 well plate. This process was then followed by incubation at room temperature for 60 minutes to stabilize the luminescent signal. At the end of the incubation, luminescence was read with FLUOstar OPTIM reader (BMG LABTECH).

2.2.8 Prostaglandin E2 production using Enzyme Immunoassay (EIA)

Prostaglandin E_2 (PGE$_2$) is an abundant metabolite of arachidonic acid and a mediator of inflammation (S.-H. Kim et al., 2019; Shabab et al., 2017). It is catalysed by cyclooxygenase-2, an enzyme that converts arachidonic acid to prostaglandins (Shabab et al., 2017). Prostaglandin E2 level in the cell culture supernatants was measured by DetectX® PGE$_2$ Multi-Format ELISA kit (Arbor assays, USA). Enzyme immunoassay detects the PGE$_2$ amount in a sample by the binding of antigen to an antibody. BV2 cells were seeded in 24-well plates (2×10^5/1 ml/well) and incubated at 37°C until they become confluent. BV2 cells were treated with *Zanthoxylum zanthoxyloides* (4, 6 and 8 µg/ml) for 30 min and then stimulated with LPS (100 ng/ml) for 24 hours. Separately, microglia BV2 cells were treated with *Z. zanthoxyloides* (4, 6 and 8 µg/ml) for 30 min and then activated with hemozoin (400 µg/ml) for 24 hours. At the end of the incubation, cell culture supernatants were centrifuged and collected into an Eppendorf tube. The kits and samples were brought to room temperature from storage prior to experiment. 100µl of samples and prepared standards were added to the plates pre-coated with goat anti-mouse IgG. Subsequently, 25µl of PGE$_2$ conjugate and PGE$_2$ antibody was added. Then, this was incubated at room temperature for 2

hours with shaking at 250rpm. The wells were washed for four times after the incubation step to remove unbound proteins. Thereafter, 100 µl of TMB (3, 3', 5, 5'-Tetramethylbenzidine) substrate was added to each well and incubated for 30 minutes at room temperature without shaking. The incubation was stopped by a stop solution (1M of hydrochloric acid). Absorbance was measured in a plate reader (Infinite F50, Tecan) at wavelength of 450nm.

2.2.9 Extraction of cytoplasmic lysates from cultured BV2 cells

Protein extraction is an important step to isolate proteins from cultured cells, and an initial step in analytical techniques including western blotting. Cultured BV2 cells were washed with cold 1x PBS at the end of cell stimulation. Then, 25µl of RIPA (Radio immunoprecipitation assay buffer) lysis buffer (Cell signalling, UK), which contains 2Mm PMSF (Phenylmethylsulfonyl fluoride) was added to the wells and incubated on ice for 15 minutes. Thereafter, wells containing cells were scraped and the lysates were collected in an Eppendorf tube on ice. Then, the lysates were collected in epperndorf tubes and placed in a cold centrifuge for 15 minutes at 13500 rpm. Afterwards, the lysates were collected in new epperndorf tubes and stored in -80°C freezer until is needed.

2.2.10 Extraction of nuclear extracts from cultured BV2 cells

Nuclear extraction was done using Episeeker Nuclear Extraction Kit (Abcam, UK). At the end of BV2 cells incubation, these were placed on ice and washed with cold 1x PBS. Thereafter, 20µl of pre-extraction buffer (ENE1 buffer) (made up of protease inhibitor) and DTT were added to the cells for 10 minutes while incubated on ice. The cells were then centrifuged at 13000 rpm for 1 minute at 4°C. Then, 10µl nuclear extraction buffer (ENE 2 buffer) was added to the pellets and put on ice for 15 minutes with intermittent vortexing every 5 minutes. Subsequently, the samples were

centrifuged for 15 minutes at 13500 rpm at 4°C. Lastly, the nuclear extract was transferred to a new eppendorf tube and stored at -80°C.

2.2.11 Measurement of protein concentration

Concentration of protein in the cytoplasmic and nuclear cell extracts were measured using Pierce Coomassie (Bradford) protein assay kit (Thermo Scientific, UK). This assay is based on reaction between the coomassie dye and protein in an acidic environment which leads to development of colour change (colorimetric reaction) that can be read (Bradford, 1976; Grintzalis, Georgiou, & Schneider, 2015; Noble & Bailey, 2009). In this assay, the cytoplasmic and nuclear lysates were diluted 1:15 in deionised water. Then, 5µl of the lysates and BSA (bovine serum albumin) standards (125- 2000µg/ml) were added to wells in 96 well plate in duplicates. Thereafter, coomassie reagent (250µl) was added to all the wells and incubated at room temperature for 10 minutes. The absorbance was in a microplate reader (Infinite F50, Tecan) at a wavelength of 540nm.

2.2.12 Western blotting

Western blotting is a technique that detects presence of a target protein in a cell. It mainly involves separation of protein mixtures according to their molecular weight and specific detection of target protein by appropriate antibodies (Mahmood & Yang, 2012). Firstly, 5 µl of LDS (lithium dodecyl sulphate) (Life Technologies) and 2 µl of sample reducing agent (Life Technology) were added to 25 µg of protein (as described in section 2.2.9, 2.2.10 and 2.2.11). These steps are required to denature proteins, which breaks down the disulphide bonds between the tertiary structures of proteins. Denaturing of protein unfolds the tertiary structure of protein into linear forms, which helps to migrate proteins easily onto the Bis-Tris gel during electrophoresis. Then, lysates were heated with dry bath (Fisher Scientific) in LDS and sample

reducing agent at 70°C for 10 minutes to speed up the protein denaturing process. Thereafter, gel electrophoresis was performed which involves loading of samples into wells of a pre- cast polyacrylamide gels (made up of Bis-Tris gel 4-12%) (Life Technologies) and adding of NuPAGE® Antioxidant (ThermoFisher, UK) to the running buffer (Life Technologies) in a gel tank. Electrophoresis was important to separate the protein of interest from other proteins in the sample according to their molecular weight and this was ran at a constant voltage of 200V for 35 minutes. Subsequently, the separated protein was transferred from the gel onto a polyvinylidine fluoride (PVDF) membranes (Thermoscientific, UK) for 2 hours at 25V. This transfer process was required to immobilize proteins on a support membrane (PVDF) which is important for immuno-detection of target protein. PVDF membrane was used in this experiment because of its high affinity for protein binding and also the membrane can be stripped and re-probed for another target protein. Membranes were blocked at room temperature for 1 hour using Odyssey blocking buffer (LICOR Biosciences) so as to prevent the binding of non-specific proteins and thus increasing the specificity of target protein. Following the blocking step, the membrane was incubated with primary antibodies overnight at 4°C to bind to the target protein. On the next day, the membranes were washed thrice with 1x TBS-T (Tris-buffered saline and tween 20) (Chem Cruz ™) for 5 minutes each. Then, the membrane was incubated with secondary antibody (Alexa Fluor 680 goat anti-rabbit; 1:10000) (Invitrogen) in the dark for 1 hour at room temperature. After the incubation, the membrane was washed thrice with 1x TBS-T. The protein detection was scanned using LICOR Odssey imager. Protein expression was analysed using Image J software (National Institutes of Health).

Table 2.3: List of antibodies used in western blot experiments

Antibody	Source	Type	Dilution	Predicted molecular weight (kDa)
iNOS	Cell signalling	Rabbit Monoclonal	1:1000	130
COX-2	Abcam	Rabbit polyclonal	1:1000	69
Caspase-1	Santa Cruz	Rabbit polyclonal	1:500	45
NLRP-3	Abcam	Rabbit polyclonal	1:1000	114
p-Iκβ-α	Santa Cruz	Rabbit polyclonal	1:500	41
Total- Iκβ-α	Santa Cruz	Rabbit polyclonal	1:500	41
p-IKK-α	Santa Cruz	Rabbit polyclonal	1:500	85
Total- IKK-α	Santa Cruz	Rabbit polyclonal	1:500	85
p- NFκB-p65	Abcam	Rabbit polyclonal	1:500	65
Total NFκB-p65	Santa Cruz	Rabbit polyclonal	1:500	65
Beta-actin	Sigma Aldrich	Rabbit polyclonal	1:1000	42
Alexa Fluor 680	Invitrogen	Goat anti-rabbit	1:10000	Secondary Antibody (Not required)

2.2.13 NF-κB p65 transcription factor assay

NF-κB is a transcription factor that regulates gene expression and plays an important role in inflammation and immune responses (Liu et al., 2017). The excessive release of pro-inflammatory cytokines in neuroinflammation and neurodegenerative disorders has been associated with the activation of NF-κB transcription factor in stimulated microglia cells (E.-J. Lee et al., 2017; Ramaswami & Hayden, 2015). Hence, inhibiting the binding of DNA to NFκB in the nucleus is fundamental in reducing neuroinflammation. NF-κB transcription factor assay is an electrophoretic mobility shift assay (EMSA) that uses ELISA technique to study NF-κB to DNA binding in the nucleus (Serasanambati & Chilakapati, 2016). NF-κB p65 transcription factor kit (TransAM® NF-κB transcription factor Active Motif, Belgium) was used in this assay as it is sensitive, non- radioactive and can be measured quantitatively (E.-J. Lee et al., 2017; Ramaswami & Hayden, 2015). This kit has 96-well plate that contains oligonucleotide NF-κB binding site ('5-GGGACTTTCC-3') which has been immobilised in the wells of the plate. Figure describes the method showing the activated NF-κB p65 in the nuclear extract binding to the oligonucleotide containing binding site and addition of primary and secondary antibody for the detection of activated NF-κB transcription factor. Afterwards, absorbance was read at 450 nm of wavelength after adding developing solution and stop solution.
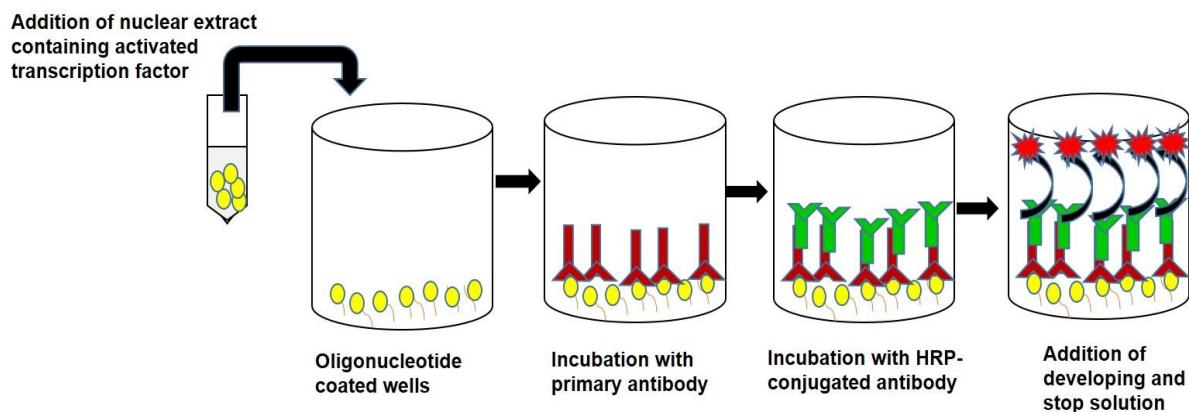
Figure 2-6: Diagram showing NFκB transcription factor assay principle (ELISA format)

In this study, effect of *Z. zanthoxyloides* on DNA binding activity of NF-κB in BV2 cells were carried out using an ELISA based TransAM® NF-κB transcription factor EMSA kit (Active Motif, Belgium). Cultured BV2 cells were treated with *Zanthoxylum zanthoxyloides* (4, 6 and 8 μg/ml) for 30 minutes prior to stimulation with LPS (100 ng/ml) for 1 hour. Differently, pre-treatment of cultured BV2 cells with *Zanthoxylum zanthoxyloides* (4, 6 and 8 μg/ml) for 30 minutes and stimulation with hemozoin (400 μg/ml) for an hour were also investigated. At the end of 1-hour incubation, nuclear extracts were collected in epperndorf tubes. Subsequently, 30 μl of complete binding buffer was added to the wells so as to increase the affinity between the activated NF-κB in nuclear extracts and the binding site (containing DNA oligonucleotide which has been immobilised). Then, 20 μg of nuclear lysates samples were added to the appropriate wells. Subsequently, the samples were incubated for 1 hour with shaking (100 rpm) at room temperature. The wells of the plate containing the samples were then washed for three times to remove the unbound proteins which results in background reduction and also increase the signal of the binding reaction. Thereafter, 100 μl of NF-κB p65 antibody (primary antibody at 1:1000 dilution factor) was added for 1 hour with shaking at room temperature. The addition of the primary antibody was important to detect the targeted NF-κB p65 in the nuclear lysates. At the end of the

incubation, the washing step was repeated and 100 μl/ well of anti -rabbit HRP-conjugated antibody (secondary antibody at 1:1000) was added for 1 hour at room temperature. Addition of secondary antibody was essential because it increases the sensitivity of NF-κB p65 detection by binding to its primary antibody. The washing step was repeated again after secondary antibody incubation. Furthermore, 100 μl /well of developing solution (TMB substrate) were added and incubated for 15 minutes in the dark. Addition of TMB substrate is important for detection of activated NF-κB p65 because it reacts with the HRP linked with the secondary antibody to form blue coloured products for colorimetric measurement. Reaction with TMB substrate was stopped by adding100 μl/ well of stop solution (H_2SO_4 solution). Absorbance was measured in a plate reader (Infinite F50, Tecan) at wavelength of 450nm.

2.2.14 Immunofluorescence

The use of immunofluorescence technique has been described to be important in detection and expression of target proteins in cells (Donaldson & G, 2015). In IMF assay, antibodies are labelled with fluorescent dyes to visualise the antigen-antibody reaction under a fluorescent microscope. The technique can be a direct or an indirect antigen-antibody reaction. The indirect antigen-antibody reaction involves two antibodies: the primary antibody, which attaches directly to the target molecule and secondary antibody labelled with a florescent dye (fluorophore) to detect the primary antibody for visualising the target protein. In addition, counterstaining with (DAPI 4', 6 diamidino- 2-phenylindole dichydrochloride) in immunofluorescence is an essential process that stains the nucleus for assessing the cell structure and determine the cell numbers. In this technique, cultured BV2 cells were seeded out in 24-well plates ($2x10^5$/1 ml/well) until they were confluent. Cultured microglia BV2 cells were pre-treated with *Z. zanthoxyloides* (4, 6 and 8 μg/ml) for 30 minutes and further stimulated

with LPS (100 ng/ml) for 1 hour. In a separate experiment, BV2 microglia cells were incubated with *Z. zanthoxyloides* (4, 6 and 8 µg/ml) for 30 minutes and induced with hemozoin (400 µg/ml) for 60 minutes. At the end of 1 hour incubation, BV2 cells were brought out from the incubator, placed on ice and fixed with ice-cold methanol (100%) for 15 minutes in -20°C freezer. Fixation with methanol was required to immobilize antigens in cells for staining and visualization, preserve the intracellular structure and also permeabilize the cells for antibody-antigen binding in detecting the target protein. Subsequently, PBS was used in washing the cells after each incubation step to remove unbound antibodies which can cause poor signal to background ratio. Afterwards, the cells were incubated for an hour with blocking solution (5% BSA solution and 10% horse serum in TBS-T (1x)) at room temperature to prevent non-specific binding of antibodies to antigens that may interfere with the detection of protein of interest. Then, cells were incubated overnight at 4°C with primary antibody, Total-NFκB-p65 (Santa Cruz Biotechnology) diluted in 1:100. Following the overnight incubation, washing step was repeated and cells were incubated for further 2 hours with secondary antibody (Alexa Flour 488- conjugated donkey anti-rabbit IgG, Life Technologies) in the dark at dilution ratio of 1 to100. Lastly, cells were counterstained with 50nm of DAPI (Invitrogen) for 5 minutes. Fluorescent cell images were acquired using a fluorescent microscope, EVOS® Floid cell imaging (Life Technologies).

2.2.15 Reporter gene assay

Studies have reported that the binding of NF-κB to its binding site causes transcription of genes that controls the production of pro-inflammatory cytokines and inflammatory mediators in neuroinflammation. (Damoiseaux & Hasson, 2018). The reporter gene assay is important to study expression of target genes at the stage of transcription in the nucleus. Moreover, reporter gene assay specifically analyses the functional activity

of transcription factors in causing transcription of target genes. Cignal NFκB (LUC) (CCS-013L) (Cignal® SA Biosciences) vector used in this study depends on technology of dual luciferase (firefly and renilla) reporter enzymes to provide a sensitive, reproducible results and minimise experimental variables. Firefly luciferase acts as experimental reporter by observing modifications in expressions of target genes while renilla luciferase acts as control reporter to minimise variable factors such as cell number differences and transfection efficiency (Branchini et al., 2018; Clément et al., 2015). Dual-Glo® luciferase assay gives an advantage to analyse both firefly and renilla luciferase activity in the same sample (Branchini et al., 2018; Clément, Salone, & Rederstorff, 2015). Figure.2-7 shows the firefly luciferase reaction which involves oxidation of luciferin to oxyluciferin, catalysed by firefly luciferase in the presence of oxygen, magnesium and adenosine triphosphate (ATP). This reaction results in production of light that is measurable as luminescent intensity. Differently, renilla luciferase enzyme converts coelenterazine into coelenteramide in presence of oxygen to emit light.

Figure.2-7: Firefly luciferase reaction with substrate (Luciferin) to produce light

Luciferin $\longrightarrow$ Oxyluciferin + CO_2 + AMP + PPi + LIGHT

Firefly Luciferase (Mg^{2+}, O_2, ATP)
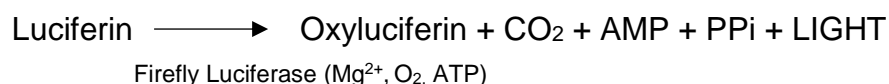
(CO_2 – Carbon dioxide, ATP- Adenosine triphosphate, AMP- Adenosine monophosphate, PPi- inorganic pyrophosphatase)
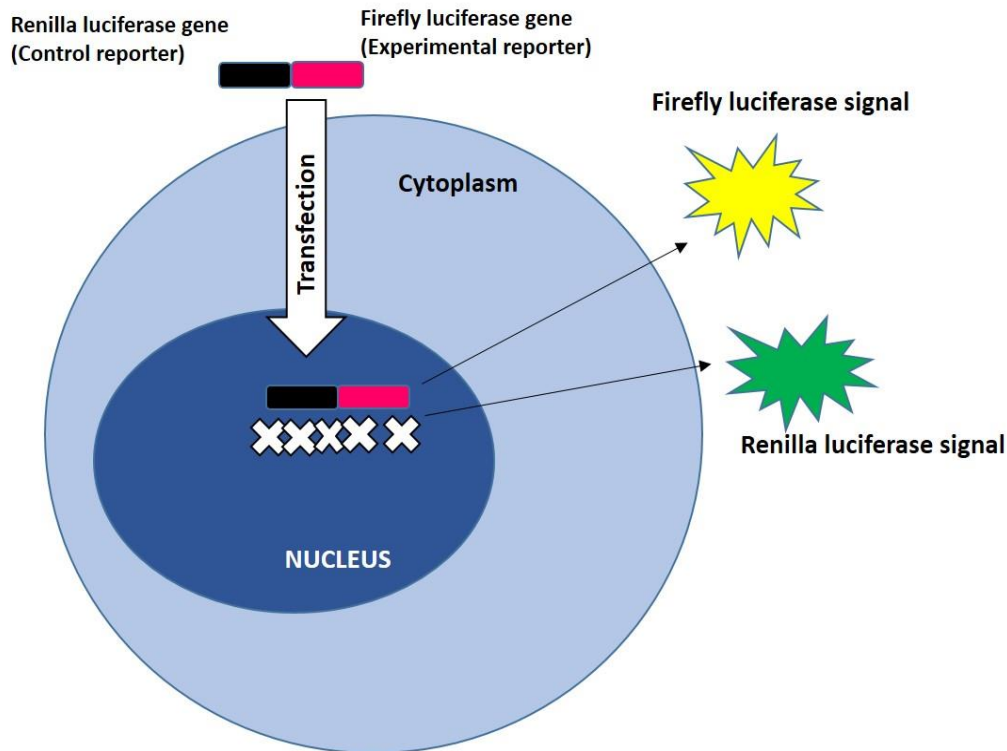
Figure 2-8: Schematic diagram of dual reporter assay

The Cignal NFκB (LUC) (CCS-013L) (Cignal® SA Biosciences) vector was used in this study to investigate the effect of *Z. zanthoxyloides* on NF-κB mediated transcriptional activity in HEK293 cells with the use of Dual-Glo® luciferase assay (Promega, UK). Cultured HEK293 cells were grown in a T-75 flask containing complete MEM medium (ThermoFisher, UK) with 5% FBS as supplement. Afterwards, the cells were sub-cultured when they reached confluence at a ratio 1:3 Subsequently, HEK293 cells were seeded out at a concentration of 4×10^5 cells/mL in a solid-white 96-well plate and re-suspended in Opti-MEM reduced serum medium (Gibco) with 5% FBS twenty-four hours before transfection. HEK293 cells were then transfected with Cignal NFκB (LUC) (CCS-013L) vector (1ng DNA/ μl) in addition to lipofectamine™ 2000 transfection reagent (ThermoFisher, UK) and subsequently incubated for 16 hours in 5% CO2 incubator at 37ºC. Thereafter, the media was changed to Opti-MEM® (without 5% FBS) and incubated for additional 8 hours after transfection with NF-κB vector. In order to elucidate effect of *Z. zanthoxyloides* extract on NF-κB driven gene

expression, transfected HEK293 cells were pre-incubated with *Z. zanthoxyloides* for 30 minutes and stimulated with TNF-α (1 ng/mL) for 8 hours. Afterwards, NF-kB-mediated gene expression was measured with Dual-Glo luciferase assay kit (Promega, UK) at the end of the 6 hours stimulation. 75µl of Dual-Glo® luciferase assay reagent was added to each well and incubated for 10 minutes at room temperature to measure firefly (experimental reporter) luminescence. After the incubation, 75µl of Dual-Glo® stop &Glo® Reagent was added to the wells to measure renilla (control reporter) luminescence. Luminescent intensity were measured using FLUOstar OPTIMA reader (BMG LABTECH).

2.2.16 MTT assay for cell viability

Cell viability assays measures the ratio of live to dead cells in a cell population (Riss et al., 2016; Van Meerloo, Kaspers, & Cloos, 2011). 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay is a viability assay that causes reduction by mitochondrial reductase, converting yellow tetrazolium salt to insoluble crystalline purple formazan product to indicate viable cells (Bahuguna, Ashutosh, Khan, Bajpai, & Kang, 2017; Riss et al., 2016; Van Meerloo et al., 2011). MTT assay was done to ensure that concentrations of *Z. zanthoxyloides* used in this study was not decreasing the viability of BV2 cells after treatment and the result obtained were not as a result of toxicity of the extract. Viability of BV2 cells were determined on stimulation of BV2 cells with LPS (100 ng/ml) or in a separate experiment with hemozoin (400 µg/ml) after pre-treatment with *Zanthoxylum zanthoxyloides* (4, 6 and 8µg/ml).
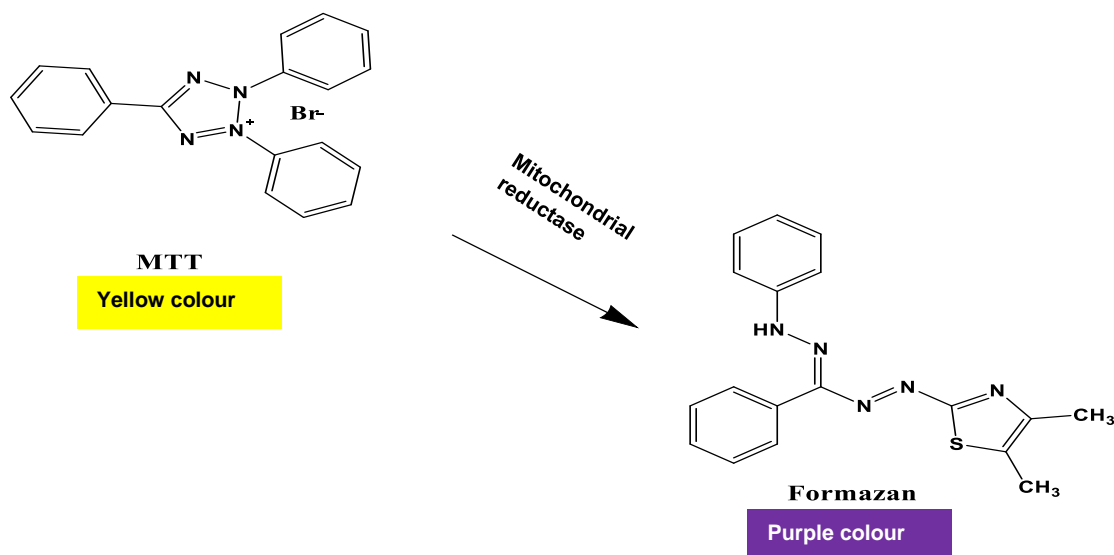
Figure 2-9: Principle of MTT assay

Cultured BV2 cells were seeded in 96 well plates (2×10^5/cells/ml) and incubated at 37°C until they become confluent. BV2 cells were stimulated with *Z. zanthoxyloides* (4, 6 and 8µg/ml) for 30 min and then stimulated with LPS (100 ng/ml)/ hemozoin (400µg/ml) for 24 hours. Twenty-four hours after stimulation, culture medium was replaced with MTT solution (5mg/ml) (Sigma) and incubated for 4 hours. MTT solution (150 ul) was replaced with DMSO and mixed thoroughly on a plate shaker to dissolve the crystals formed. Absorbance was read at 540nm with a plate reader (Tecan F50).

2.2.17 Statistical analysis

All experiments were performed independently at least three times. Data are expressed as mean ±SEM. Statistical analysis was performed using one-way analysis of variance (ANOVA) with post-hoc Student-Newman-Keuls test. ANOVA compared the mean values between independent groups to determine the statistical difference. As required for each experiment, data were converted to percentage in relative to untreated control, hemozoin control, LPS control or TNFα control. Statistical analysis was performed using Graph pad prism software version 5. Statistical difference of p value less than 0.05 ($p < 0.05$) were considered significant. Moreover, level of significance in experiments were shown as $^{\alpha\alpha\alpha}$ $p < 0.001$ in comparison with the untreated cells while * $p < 0.05$, **$p < 0.01$, ***$p < 0.001$ were compared with the LPS or hemozoin control.

2.3 `Results

2.3.1 Methanolic extract of *Zanthoxylum zanthoxyloides* decreased TNFα, IL-1β, IL-6 and elevated IL-10 production in LPS activated BV2 microglia

In neuroinflammation, prolonged exposure of LPS to microglia cells have been reported to secrete excessive pro-inflammatory cytokines (TNFα, IL-1β, IL-6), reactive nitrogen and oxygen species (Becher & Spath, 2017; Molteni, Gemma, & Rossetti, 2016). The effect of *Z. zanthoxyloides* extract on important pro-inflammatory cytokines were examined to investigate the anti-neuroinflammatory activity of this extract on elevated levels of secreted TNFα, IL-1β, IL-6 in LPS stimulated BV2 microglia cells. Result in Figure 2-10 shows that BV2 cells activated with LPS (100 ng/ml) caused a significant increase ($p < 0.001$) in the level of TNFα (~14.3-fold increase) elevation in comparison to untreated cells. Conversely, pre-treatment with *Z. zanthoxyloides* at 4 and 6 µg/ml significantly reduced ($p < 0.05$) the production of TNFα (~1.3-fold and ~1.5-fold respectively) when compared to LPS control while a further decrease ($p < 0.01$) was observed at 8 µg/ml of this extract (~2-fold reduction) in comparison to LPS stimulated cells. In addition, there was a marked increase ($p < 0.001$) in the level of IL-1β (~10-fold increase) in BV2 cells activated with LPS relative to unstimulated BV2 cells. The outcome of this investigation showed that *Z. zanthoxyloides* at 4 µg/ml significantly decreased ($p < 0.01$) the secretion of IL-1β (23% reduction) when compared to BV2 cells stimulated with LPS. Pre-treatment with 6 and 8 µg/ml of *Z. zanthoxyloides* further inhibited the secretion of IL-1β (65% and 33% decrease) respectively, when compared to LPS control. Similarly, the effect of *Z. zanthoxyloides* on IL-6 production were also determined. The extract at 4 µg/ml reduced ($p < 0.01$) the production of IL-6 (66.5%) while at 6 and 8 µg/ml, there was a further decrease ($p < 0.001$) (44% and 32%) respectively.

A reduced level of IL-10 (an anti-inflammatory cytokine) production has been detected during neuroinflammation caused by excessive release of pro-inflammatory cytokines in stimulated microglia cells (Meng et al., 2018). The effect of *Z. zanthoxyloides* extract on level of IL-10 production in LPS activated BV2 microglia cells were also investigated. Result in Figure 2-10d shows a significant decrease (p<0.001) in the level of IL-10 produced (~2.9-fold reduction) in BV2 cells treated with LPS in comparison to the untreated cells. On the other hand, pre-treatment with *Z. zanthoxyloides* (4, 6 and 8 µg/ml) significantly increased (p<0.001) the level of IL-10 (~1.5-fold, ~2.1-fold and ~2.6-fold respectively) when compared to LPS treated cells.
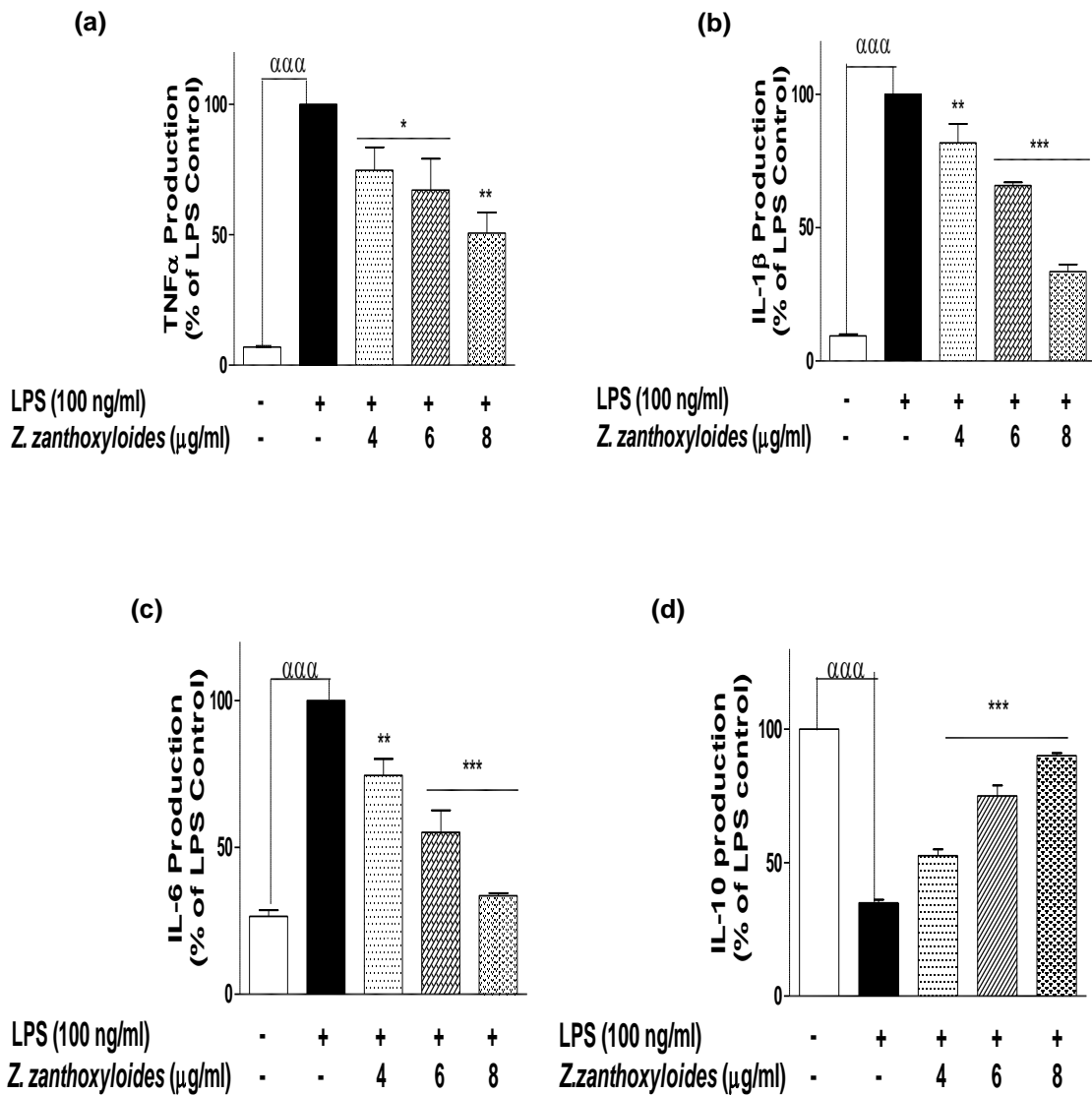
Figure 2-10: *Zanthoxylum zanthoxyloides* reduced TNFα, IL-1β, IL-6 and increased IL-10 production in BV2 microglia cells stimulated with LPS

Cultured BV2 cells were treated in presence or absence of *Z. zanthoxyloides* (4, 6 and 8 µg/ml) and stimulated with LPS (100 ng/ml) for 24 hours. At the end of the incubation period, cell culture supernatants were collected and cytokine levels of (a) TNFα, (b) IL-1β and (c) IL-6 (d) IL-10 were analysed using ELISA technique. Data are expressed as mean ± SEM for 3 separate experiments. Analysis was done using one-way ANOVA with post-hoc Student Newman-Keuls test (multiple comparisons ^{ααα} $p<0.001$ in comparison with the untreated cells and $*p<0.05$ $**p<0.01$, $***p<0.001$) in comparison with LPS control.

2.3.2 Methanolic extract of *Zanthoxylum zanthoxyloides* inhibited nitrite production through suppression of iNOS protein in LPS-activated BV2 microglia

Reports have associated the excessive release of nitric oxide (NO) mediated by inducible nitric synthase (iNOS) with neuroinflammation in stimulated microglia cells by stimuli such as LPS. (Dawson & Dawson, 2018; Yuste et al., 2015). Effect of *Z. zanthoxyloides* on the production of NO and expression of iNOS protein were investigated in this study. Result showed a significant increase ($p < 0.001$) in the levels of NO (~4-fold increase) in BV2 cells stimulated with LPS when compared to untreated cells. However, *Z. zanthoxyloides* at 4, 6 and 8 µg/ml significantly inhibited ($p < 0.001$) the production of NO production (~1.9-fold, ~2.3-fold and ~3.2-fold respectively) in a concentration- dependent manner in comparison to LPS stimulated BV2 cells Following the results on the inhibitory action of *Z. zanthoxyloides* on NO, western blot experiment was done to further investigate the effect of this extract on expression of iNOS. A significant elevation in the expression of iNOS protein was detected on stimulation with LPS (100 ng/ml) in comparison to unstimulated cells. Result revealed a marked reduction ($p < 0.001$) in the expression of iNOS protein by *Z. zanthoxyloides* at 4 (~1.3-fold), 6 (~1.8-fold) and 8 µg/m l (~2.2-fold) in BV2 cells stimulated with LPS when compared to cells stimulated with LPS alone. These results suggest that *Z. zanthoxyloides* (4, 6 and 8 µg/ml) reduced NO production through suppressing the expression of iNOS protein in LPS-activated BV2 cells.

(a)
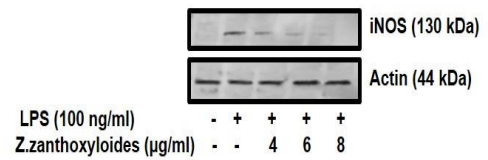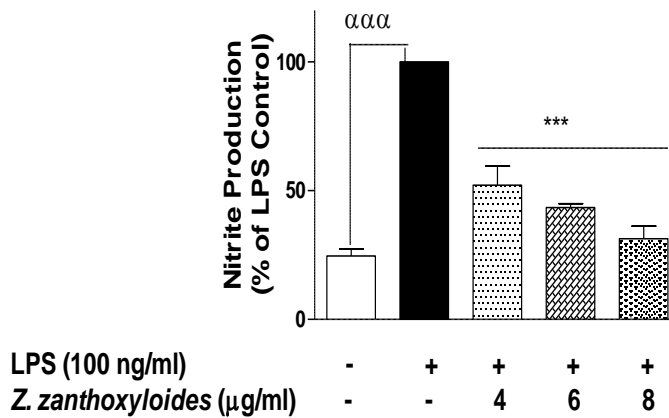
(b)
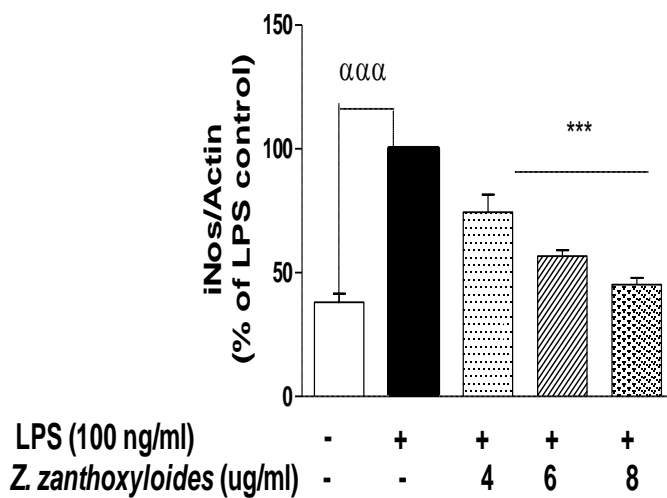
Figure 2-11: *Zanthoxylum zanthoxyloides* inhibited nitric oxide (NO) release (a) and inducible nitric oxide (iNOS) protein expression (b) in BV2 cells stimulated with LPS

Cultured BV2 cells were treated with or without *Zanthoxylum zanthoxyloides* (4, 6 and 8 µg/ml) and stimulated with LPS (100 ng/ml) for 24 hours. Thereafter, cell culture supernatants and cytoplasmic extract were collected and evaluated for production of NO and protein expression of iNOS. *Zanthoxylum zanthoxyloides* reduced nitric oxide (NO) release (A) through inhibiting inducible nitric oxide (iNOS) protein expression (B) in BV2 cells stimulated with LPS. Data are expressed as mean ±SEM for 3 separate experiments. Analysis was done using one-way ANOVA with post-hoc Student Newman-Keuls test [ααα p<0.001 in comparison with the untreated cells and (***p<0.001) in comparison with LPS control.

2.3.3 Methanolic extract of *Zanthoxylum zanthoxyloides* suppressed PGE_2 production by inhibiting COX-2 protein expression in LPS activated BV2 microglia

The excessive secretion of PGE_2 mediated by COX-2 have been described to contribute significantly to brain inflammation in neurodegenerative disorders (Sil & Ghosh, 2016; Weiwer, 2018). Therefore, inhibition of COX-2 gene in reducing the release of PGE_2 mediator is important for attenuating neuroinflammation in activated microglia cells. Investigations on the effect of *Z. zanthoxyloides* on the secretion of PGE_2 and expression of COX-2 protein were elucidated. Result showed a marked increase ($p < 0.001$) in the levels of PGE_2 (~4.4-fold increase) in cells treated with LPS when compared to BV2 cells that are untreated. Conversely, the level of PGE_2 was reduced at 6 (~1.3-fold decrease) and 8 µg/ml (~1.6-fold reduction) of this extract in LPS stimulated BV2 cells when compared to LPS activated cells only. *Z. zanthoxyloides* at 4 µg/ml did not significantly decrease the production of PGE_2 in relation to LPS control. Subsequently, to understand whether the inhibitory action of the extract on PGE_2 were mediated by COX-2, the effect of *Z. zanthoxyloides* on protein expression of COX-2 was investigated. Results in Figure 2-12 shows a significant elevation ($p < 0.001$) in the level of COX-2 expression (~2.7-fold increase) in LPS stimulated BV2 cells in relation to BV2 cells that were unstimulated. However, *Z. zanthoxyloides* at 4 µg/ml significantly reduced ($p < 0.01$) the expression of COX-2 (78.6%) while at 6 and 8ug/ml of the extract, there was a further marked reduction ($p < 0.001$) (58.4% and 42.6%) respectively, in LPS stimulated BV2 cells in relation to BV2 cells that were stimulated with LPS only.
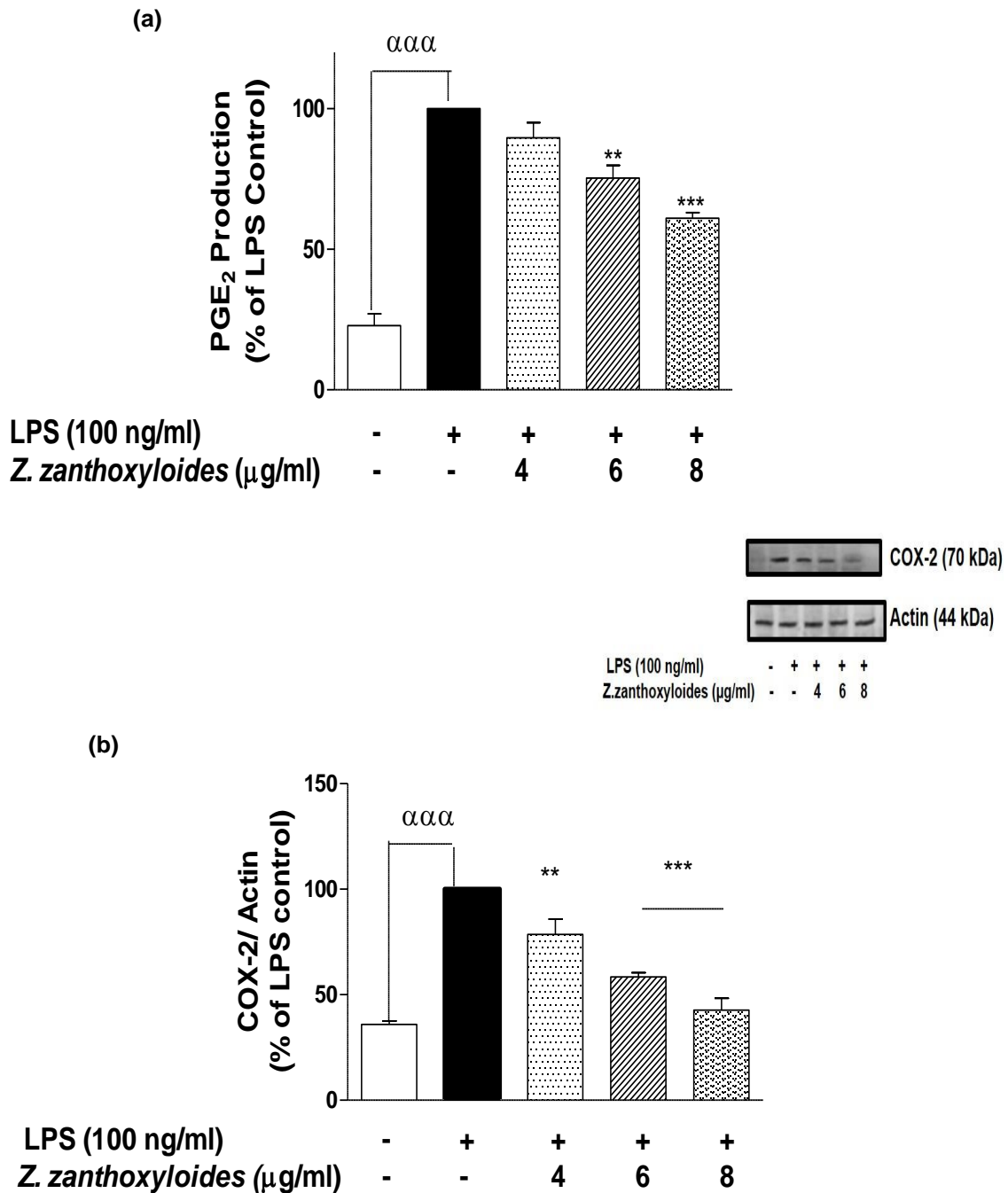
Figure 2-12: *Zanthoxylum zanthoxyloides* suppressed PGE2 production and COX-2 protein expression in LPS activated BV2 microglia

Cultured BV2 cells were treated with or without *Z. zanthoxyloides* (4, 6 and 8 µg/ml) and stimulated with LPS (100 ng/ml) for 24 hours. *Z. zanthoxyloides* suppressed PGE2 production (a) and COX-2 protein expression (b) in BV2 microglia. Data are expressed as mean ±SEM for 3 separate experiments Analysis of data was done on 3 separate experiments using one-way ANOVA with post-hoc Student Newman-Keuls test (multiple comparisons [ααα] $p < 0.001$ in comparison with the untreated cells and **$p < 0.01$, ***$p < 0.001$) in comparison with LPS control.

2.3.4 Methanolic extract of *Zanthoxylum zanthoxyloides* inhibited neuroinflammation through NFκB signaling pathway

The significant role of NFκB in controlling the secretion of pro-inflammatory cytokines and expression of inflammatory mediators such as iNOS and COX-2 have been reported in activated microglia cells (Biswas & Bagchi, 2016; El-Bakoush & Olajide, 2018; J. A. Smith, A. Das, S. K. Ray, & N. L. Banik, 2012a). Following results on the inhibitory effect of *Z. zanthoxyloides* on production of TNFα, IL-1β, IL-6 and protein expression of iNOS and COX-2, investigation on the effect of this extract on NFκB signalling pathway were further elucidated. Dual-Glo® luciferase assay was used in studying the effect of *Z. zanthoxyloides* on NF-κB mediated gene transcription. Result in Figure 2-13a displays a significant increase ($p<0.001$) in the activation of NF-κB dependent gene transcription increase on activation of transfected HEK293 cells with TNFα when compared to untreated cells. However, pre-treatment with *Z. zanthoxyloides* at 4 µg/ml resulted in reduction ($p<0.05$) of NF-κB mediated luciferase reporter gene expression (~1.24-fold decrease) in relation to HEK293 cells stimulated with TNFα. A further marked inhibition of NF-κB mediated gene transcription was observed on pre-treatment with *Z. zanthoxyloides* at 6 µg/ml (~1.5-fold reduction, $p<0.01$) and 8 µg/ml (~2.4-fold decrease, $p<0.001$).

Following the translocation of activated NF-κB into the nucleus, NF-κB binds to the promoter region of DNA for transcription of genes such as iNOS which are involved in inflammation. Hence, inhibiting the binding of NF-κB to DNA is crucial in blocking NF-κB from initiating gene transcription. Effect of *Z. zanthoxyloides* on DNA-binding activity of NF-κB were determined in BV2 microglia stimulated by LPS. Result in Figure 2-13b reveals a significant elevation ($p<0.001$) in the DNA binding of NF-κB (~2.7-fold increase) in relation to unstimulated BV2 cells. Conversely, there was a marked

reduction ($p<0.001$) in DNA binding of NF-κB on pre-treatment with *Z. zanthoxyloides* at 6 (~1.4-fold rise) and 8 µg/ml (~1.6-fold increase) in LPS –stimulated BV2 cells when compared to LPS stimulated BV2 cells alone. There was no significant reduction in DNA binding activity of NF-κB at 4 µg/ml of *Z. zanthoxyloides.*
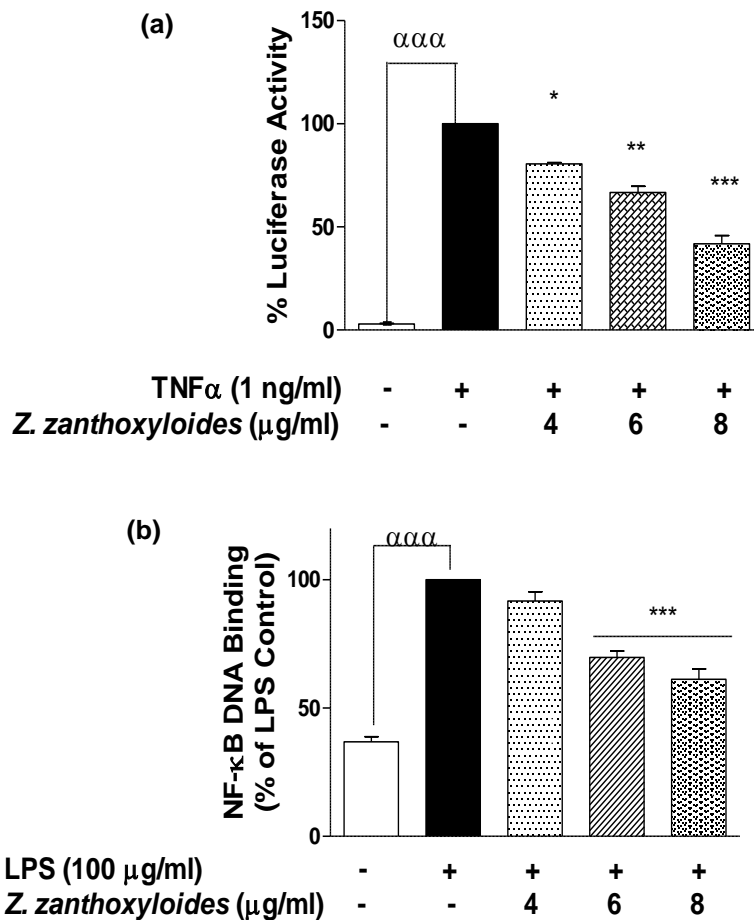
Figure 2-13: *Zanthoxylum zanthoxyloides* inhibited neuroinflammation through NFκB signaling pathway

(a) *Z. zanthoxyloides* concentration dependently inhibited activity of NFκB in transfected HEK293 cells induced by TNFα. Transfected HEK293 cells were stimulated with TNFα (1ng/ml) in presence or absence of *Z. zanthoxyloides* (4, 6 and 8 µg/ml) for 6 hours. (b) *Z. zanthoxyloides* at 6 and 8 µg/ml decreased DNA binding activity of NFκB in LPS stimulated BV2 microglia cells. Subsequently, luminescence was measured. Nuclear cell lysates were added to wells pre-coated with NFκB oligonucleotide, followed by addition of NFκB p65 antibody and secondary antibody. Thereafter, absorbance was measured on a microplate reader. Analysis of data was done on 3 separate experiments using one-way ANOVA with post-hoc Student Newman-Keuls test (multiple comparisons [ααα] $p<0.001$ in comparison with the untreated cells and *$p<0.05$**$p<0.01$, ***$p<0.001$) in comparison with TNFα control and LPS control.

Furthermore, its effect on phosphorylation of IKKα protein were elucidated. Result from this study in Figure 2-14a presents a significant increase (p<0.001) in the level of IKKα phosphorylation by ~3.4-fold in LPS-induced BV2 microglia but pre-treatment with *Z. zanthoxyloides* (4, 6 and 8 µg/ml) did not cause a reduction in IKK phosphorylation when compared to BV2 cells treated with LPS. Moreover, western blot experiments were done to investigate the effect of *Z. zanthoxyloides* extract on the expression of IκB and NFκB -p65 subunit in BV2 cells stimulated with LPS. As expected, western blot result in Figure 2-14b significantly increased (p<0.001) the phosphorylation of IκB by ~3-fold when compared to untreated cells. However, there was a marked reduction (p<0.001) in the level of IκB phosphorylation on pre-treatment with *Z. zanthoxyloides* at 4 (~1.2-fold), 6 (~1.6-fold) and 8 µg/ml (~2.4-fold) in LPS-stimulated BV2 cells when compared to LPS control. Furthermore, there was a significant degradation (p<0.001) of total IκBα by 30% in BV2 cells stimulated with LPS when compared to untreated cells. In contrast, *Z. zanthoxyloides* at 6 and 8 µg/ml in Figure 2-14c significantly inhibited the degraded total IκB and increased it by 14.3% (p<0.01) and 29.5% (p<0.001) respectively when compared to LPS control while there was no significant increase in the level of total IκBα at 4 µg/ml of *Z. zanthoxyloides.* Furthermore, effect of *Z. zanthoxyloides* on translocated NFκB p65 subunit in the nucleus were investigated. Result in Figure 2-14d shows a marked increase (p<0.001) in phosphorylation of p65 by ~2.8-fold on stimulation of BV2 cells with LPS in comparison to the unstimulated cells. However, a significant inhibition (p<0.001) was observed in the level of phosphorylated p65 on treatment of BV2 cells with *Z. zanthoxyloides* at 4 (~1.2-fold), 6 (~1.7-fold) and 8 µg/ml (~2.4-fold) in LPS-stimulated BV2 cells in relation to LPS control. Afterwards, immunofluorescence assay similarly showed a reduced expression of activated p65 subunit when treated with *Z. zanthoxyloides* (4, 6 and 8 µg/ml) in LPS activated microglia.
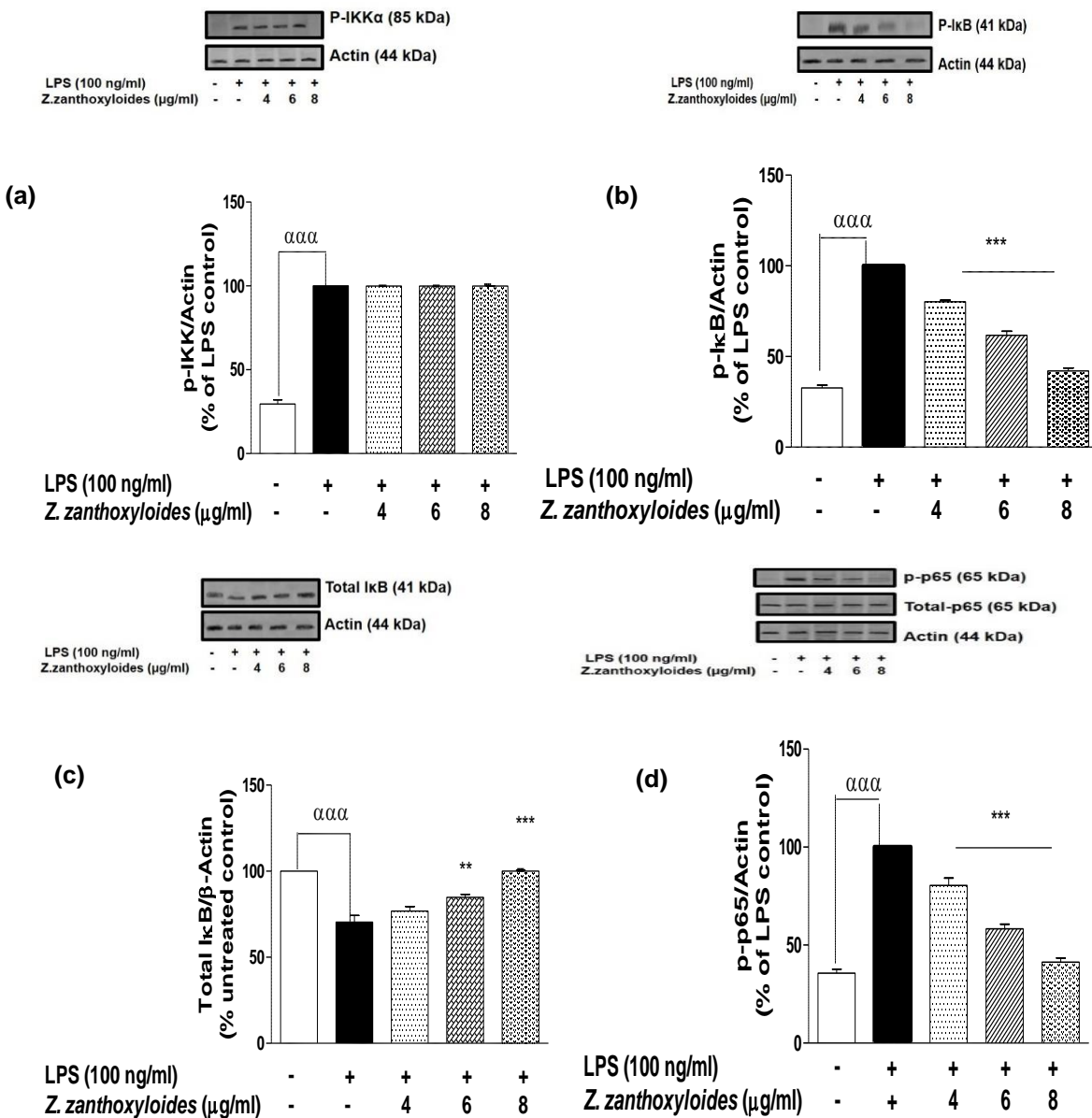
Figure 2-14: Effect of *Zanthoxylum zanthoxyloides* on phosphorylated IKK, IκB and nuclear translocation of NF-KB p65 in BV2 cells activated with LPS

Zanthoxylum zanthoxyloides did not significantly reduce phosphorylation of IKK. In contrast, *Zanthoxylum zanthoxyloides* significantly inhibited (B) phosphorylation of IκB, (C) degradation of IKB and (D) translocation of NF-KB p65 subunit in LPS activated BV2 cells. Cultured BV2 cells were stimulated with LPS (100ng/ml) in the presence or absence of *Zanthoxylum zanthoxyloides* (4, 6 and 8μg/ml) for 1 hour. At the end of the incubation, cytoplasmic or nuclear extract was collected and western blot was performed to analyse p65 subunit and IκB protein expression. Analysis was done using one-way ANOVA with post hoc Student Newman–Keuls test (multiple comparisons, ^{ααα} p<0.001 in comparison with the untreated cells and **p<0.01 ***p<0.001 in comparison with LPS control.

Figure 2-15: Effect of *Zanthoxylum zanthoxyloides* on p65 NF-κB protein expression in LPS activated microglia

Immunofluorescence assay was done to further confirm the western blot result on NF-KB p65 subunit. Zanthoxylum zanthoxyloides (4, 6 and 8 µg/ml) decreased the expression of NF-KB p65 in LPS induced BV2 cells. Activated microglia cells were labelled with NF-KB p65 antibody and counterstained with DAPI. Images were acquired using EVOS® Floid ® cell imaging and analysed using image J.

2.3.5 Methanolic extract of *Zanthoxylum zanthoxyloides* did not reduce viability of LPS stimulated BV2 microglia cells

MTT assay was on done to assess the viability of LPS –induced BV2 microglia cells on pre-treatment with *Z. zanthoxyloides* at 4, 6 and 8 µg/ml. Results showed that there was no significant difference in viability of cells treated with *Z. zanthoxyloides* (4, 6 and 8 µg/ml) in LPS activated BV2 microglia cells when compared with control (untreated) cells.
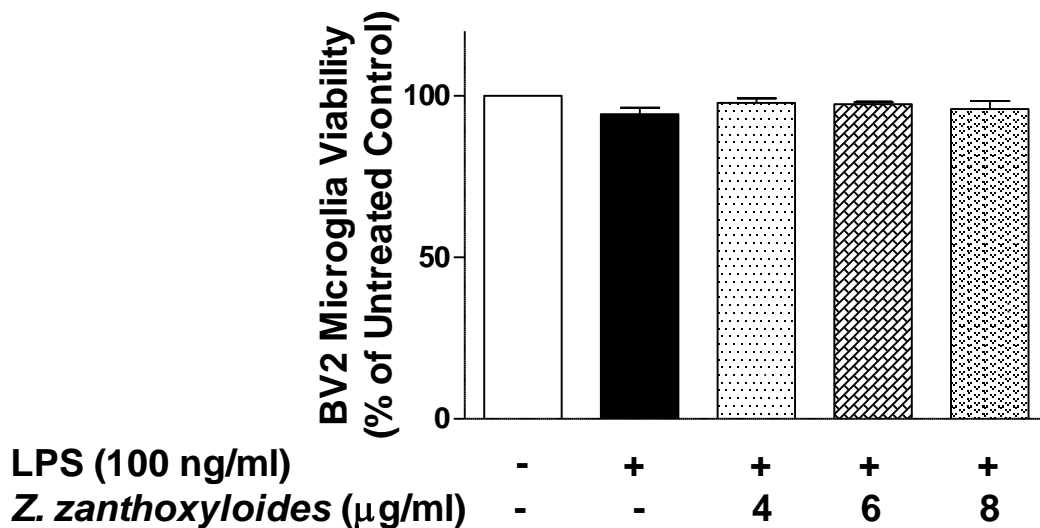
Figure 2-16: Z. zanthoxyloides did not affect BV2 cell viability

The viability of cultured BV2 cells pre-treated with *Z. zanthoxyloides* (4, 6 and 8 µg/ml) was not affected. BV2 cells were stimulated with LPS (100 ng/ml) in the presence or absence of *Z. zanthoxyloides* (4, 6 and 8 µg/ml) for 24 h. At the end of the incubation period, MTT assay was used to assess the viability of BV2 cells. All values are expressed as mean ± SEM for 3 separate experiments. Statistical analysis was performed using one-way ANOVA with post-hoc Student Newman-Keuls test in comparison with untreated cells.

2.3.6 Methanolic extract of *Zanthoxylum zanthoxyloides* reduced TNFα and IL-6 secretion and increased IL-10 in hemozoin-induced BV2 cells

Increase in secretion of pro-inflammatory cytokines (IL-6 and TNFα) and inflammatory mediators such as iNOS have been observed in studies on incubation of monocytes and macrophages with hemozoin, which results in neuroinflammation (Dunst et al., 2017; Olivier et al., 2015; Perkins et al., 2011). Therefore, inhibiting the level of excessive secretion of pro-inflammatory cytokines and inflammatory mediators may reduce neuroinflammation induced by hemozoin. Anti-inflammatory activity of *Z. zanthoxyloides* have been demonstrated *in vivo* to reduce inflammation induced by carrageenan and xylene (Prempeh & Mensah-Attipoe, 2008, 2009). However, studies to reveal the effect of *Z. zanthoxyloides* on neuroinflammation induced by synthetic hemozoin in BV2 microglia cells remains unknown. In subsequent studies, the effect of *Z. zanthoxyloides* on hemozoin-induced neuroinflammation stimulated with hemozoin in BV2 microglia cells were elucidated.

Effect of *Z. zanthoxyloides* on the degree of TNFα, IL-6 and IL-10 production in hemozoin induced BV2 cells were investigated in this study. The outcome of this study in Figure 2-17 displays a marked elevation ($p < 0.001$) in the levels of TNFα (~2.6-fold increase) and IL-6 (~6.7-fold increase) when compared to unstimulated BV2 cells. However, pre-treatment with *Z. zanthoxyloides* at 4 and 6 µg/ml in hemozoin induced BV2 cells significantly reduced ($p < 0.01$) the level of TNFα (~1.5-fold and ~1.9-fold, respectively) respectively in comparison to hemozoin-stimulated cells. A further significant decrease ($p < 0.001$) was observed on pre-treatment with 8 µg/ml of this extract (~2.6-fold reduction) in hemozoin-induced BV2 cells when compared to hemozoin-induced cells only. In addition, in hemozoin-induced BV2 cells, result also showed that *Z. zanthoxyloides* at 6 and 8 µg/ml significantly diminished the release of

IL-6 (55.2% and 33.5% respectively) relative to hemozoin stimulated cells. *Z. zanthoxyloides* (4 µg/ml) did not significantly reduce IL-6 secreted in hemozoin-induced BV2 cells.

Moreover, the effect of *Z. zanthoxyloides* on IL-10 production in BV2 cells activated with hemozoin were examined. Following stimulation of BV2 cells with hemozoin, there was a significant inhibition ($p < 0.001$) in the production of IL-10 (34% reduction) when compared to untreated cells. However, *Z. zanthoxyloides* at 4, 6 and 8 µg/ml markedly elevated ($p < 0.001$) the degree of IL-10 production (53%, 74% and 91.7% respectively) when compared to hemozoin induced BV2 cells.
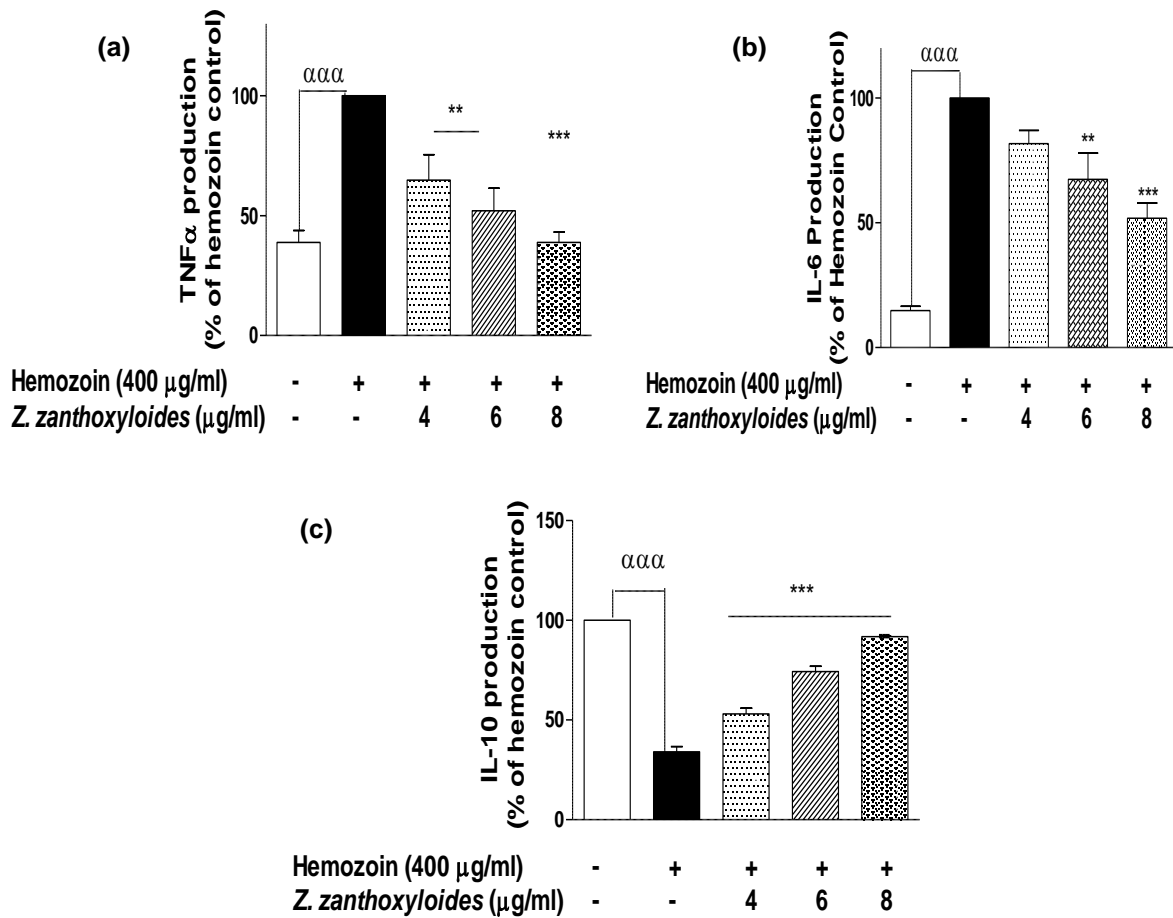
Figure 2-17: *Zanthoxylum zanthoxyloides* reduced TNFα, IL-6 and increased IL-10 release in hemozoin-activated BV2 microglia

Z. zanthoxyloides markedly reduced (a) TNFα and (b) IL-6 and increased (c) IL-10 production in hemozoin induced BV2 cells. Cultured BV2 cells were treated with or without *Z. zanthoxyloides* (4, 6 and 8µg/ml) and stimulated with hemozoin (400µg/ml) for 24 hours. At the end of the incubation period, cell culture supernatants were collected for ELISA measurements. Data are expressed as mean ±SEM for 3 distinct experiments. Analysis was done using one-way ANOVA with post-hoc Student Newman-Keuls test (multiple comparisons. αααα $p < 0.001$ in comparison with the untreated cells and **$p < 0.01$, ***$p < 0.001$) in comparison with hemozoin control.

2.3.7 Methanolic extract of *Zanthoxylum zanthoxyloides* inhibited nitrite production through suppression of iNOS protein in hemozoin-stimulated BV2 microglia

Excessive accumulation of NO in activated microglia cells have been demonstrated to cause neurotoxicity which may contribute to the progression of neurodegenerative diseases (Yuste et al., 2015). In addition, investigation showing stimulation of BV2 microglia cells with synthetic hemozoin resulted in a significant increase in levels of NO mediated by increased expression of iNOS protein (Velagapudi et al., 2019). Hence, inhibiting released NO and expressed iNOS enzyme could reduce neuroinflammation in neurodegenerative diseases. The effect of *Z. zanthoxyloides* on secretion of NO and expression of iNOS were determined.

Result in Figure 2-18 shows a significant elevation ($p < 0.001$) in the levels of NO released (~4.2-fold increase) in hemozoin stimulated BV2 cells in comparison to unstimulated cells. *Z. zanthoxyloides* at 4, 6 and 8 µg/ml markedly decreased) NO production (77.9%, 63.3% and 46% respectively) in hemozoin activated cells. Furthermore, western blot experiment was done to investigate the effect of *Z. zanthoxyloides* on protein expression of iNOS. *Z. zanthoxyloides* 4, 6 and 8 µg/ml significantly inhibited ($p < 0.001$) expressed iNOS protein (73%, 55% and 41% respectively) when compared to hemozoin-stimulated BV2 cells.

A
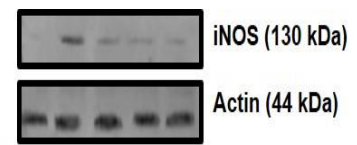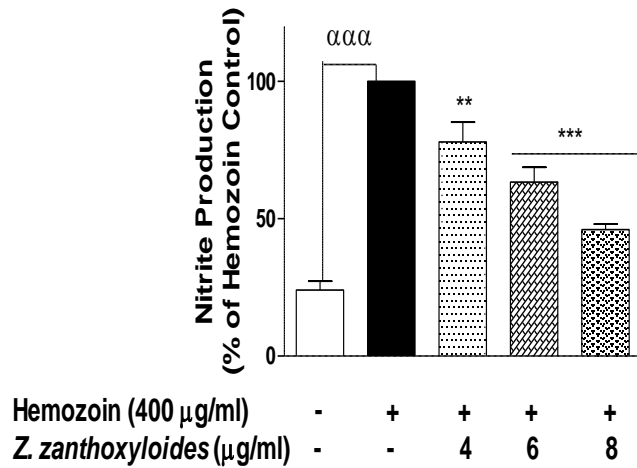
B
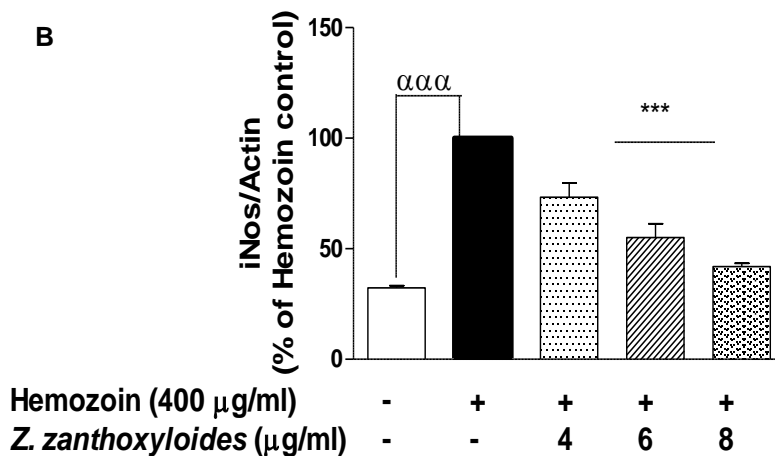
Figure 2-18: Effect of *Zanthoxylum zanthoxyloides* on nitric oxide (NO) release and inducible nitric oxide synthase (iNOS) protein expression in BV2 cells stimulated with hemozoin

Zanthoxylum zanthoxyloides inhibited NO production (A) in hemozoin induced BV2 microglia. Further investigation using immunoblotting showed that *Zanthoxylum zanthoxyloides* (4, 6 and 8µg/ml) produced significant suppression of iNOS protein expression (B). BV2 Cells were treated with or without *Zanthoxylum zanthoxyloides* (4, 6 and 8ug/ml) and stimulated with hemozoin (400µg/ml) for 24 hours. Data are expressed as mean ±SEM for 3 passages. Analysis was done using one-way ANOVA with post-hoc Student (multiple comparisons [ααα] $p < 0.001$ in comparison with the untreated cells and **$p < 0.01$ ***$p < 0.001$ in comparison with hemozoin control

2.3.8 Methanolic extract of *Zanthoxylum zanthoxyloides* suppressed PGE$_2$ production by inhibiting COX-2 protein expression in hemozoin induced BV2 microglia

The role of excessive secretion of PGE$_2$ catalysed by COX-2 enzyme have been reported to be involved in neuroinflammation (S.-H. Kim et al., 2019; Ricciotti et al., 2011). Hence, the inhibition of PGE$_2$ and COX-2 protein might reduce neuroinflammation induced by hemozoin. Results in Figure 2-19 shows the effect of *Z. zanthoxyloides* on released PGE$_2$ and expression of COX-2 in hemozoin induced BV2 cells. A significant increase ($p < 0.001$) in the secretion of PGE$_2$ (~9.8-fold increase) was detected in cells stimulated with hemozoin in comparison to untreated cells. However, pre-treatment with *Z. zanthoxyloides* at 4 and 6 µg/ml markedly suppressed ($p < 0.01$) the elevated level of PGE$_2$ (~1.3-fold and ~1.5-fold reduction respectively) in hemozoin-stimulated BV2 cells relative to hemozoin-stimulated cells. Moreover, a decrease was observed at 8 µg/ml of this extract (~1.9-fold). Thereafter, western blot experiments on expression of COX-2 was investigated to further explain the mechanism of PGE$_2$ secretion in hemozoin-induced BV2 cells. There was a significant increase ($p < 0.001$) in the level of COX-2 protein (~3.7-fold) in hemozoin-stimulated BV2 cells when compared to unstimulated BV2 cells. On the other hand, *Z. zanthoxyloides* (4, 6 and 8 µg/ml) caused a marked and concentration-dependent decrease in COX-2 expression (71.7%, 49.8% and 30.4% respectively) in comparison to cells treated with hemozoin.
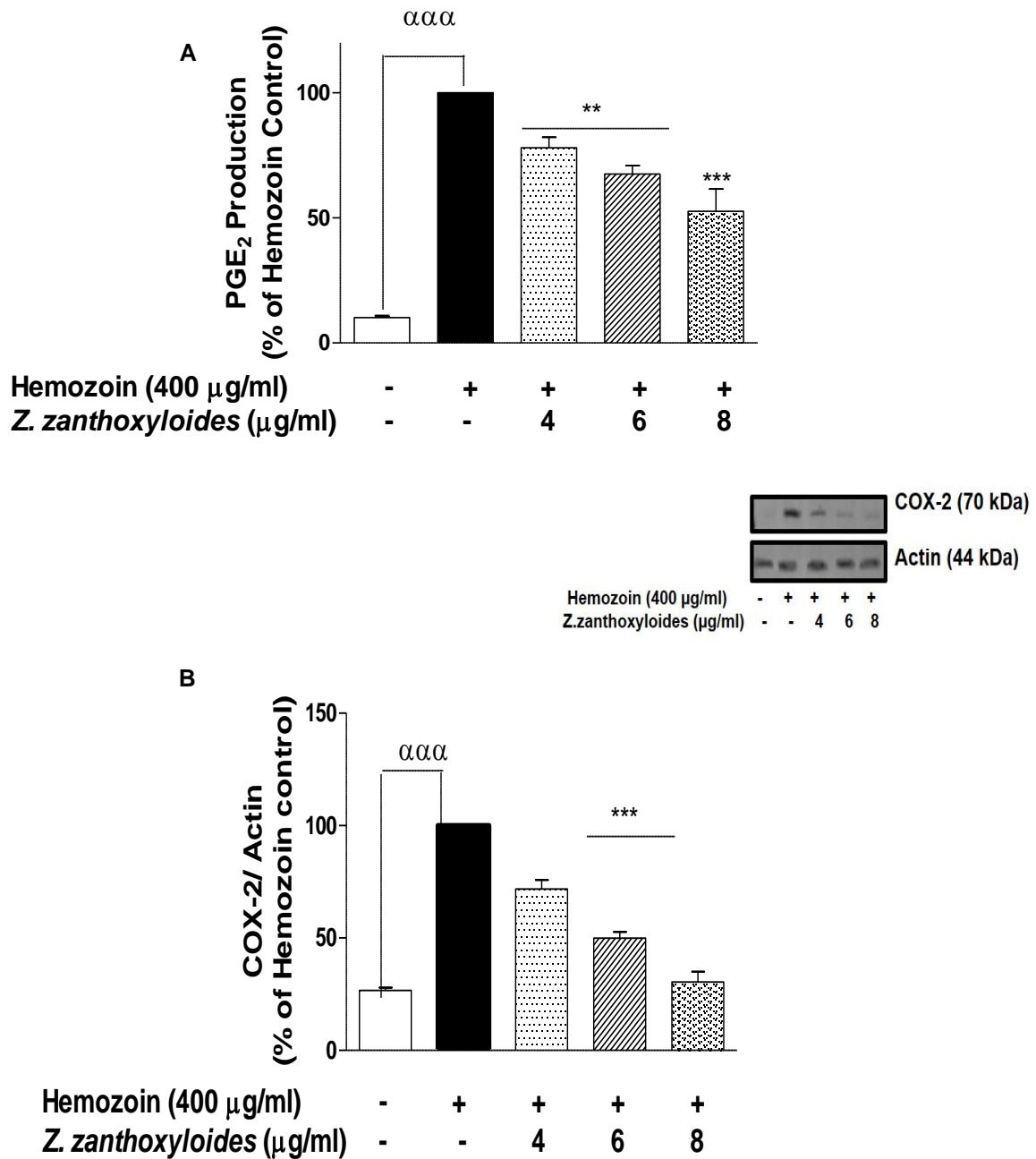
Figure 2-19: *Zanthoxylum zanthoxyloides* suppressed PGE2 production by inhibiting COX-2 protein expression in BV2 cells stimulated with hemozoin

BV2 Cells were treated with or without *Zanthoxylum zanthoxyloides* (4, 6 and 8ug/ml) and stimulated with hemozoin (400µg/ml) for 24 hours. *Zanthoxylum zanthoxyloides* (4, 6 and 8µg/ml) significantly reduced PGE2 production (A). Also, results in (B) show marked reduction of COX-2 protein in hemozoin induced cells. Data are expressed as mean ±SEM for 3 passages. Analysis was done using one-way ANOVA with post-hoc Student (multiple comparisons $^{\alpha\alpha\alpha}$ p<0.001 in comparison with the untreated cells and **p<0.01 ***p<0.001 in comparison with hemozoin control.

2.3.9 Methanolic extract of *Z. zanthoxyloides* inhibit neuroinflammation via NF-κB signaling pathway in hemozoin-activated BV2 microglia

The significant role of NF-κB in regulating the expression of cytokines and chemokines in cells induced by hemozoin have been demonstrated in a previous study to investigate the mechanism of hemozoin action in murine macrophages (Jaramillo et al., 2005). Furthermore, NF-κB p65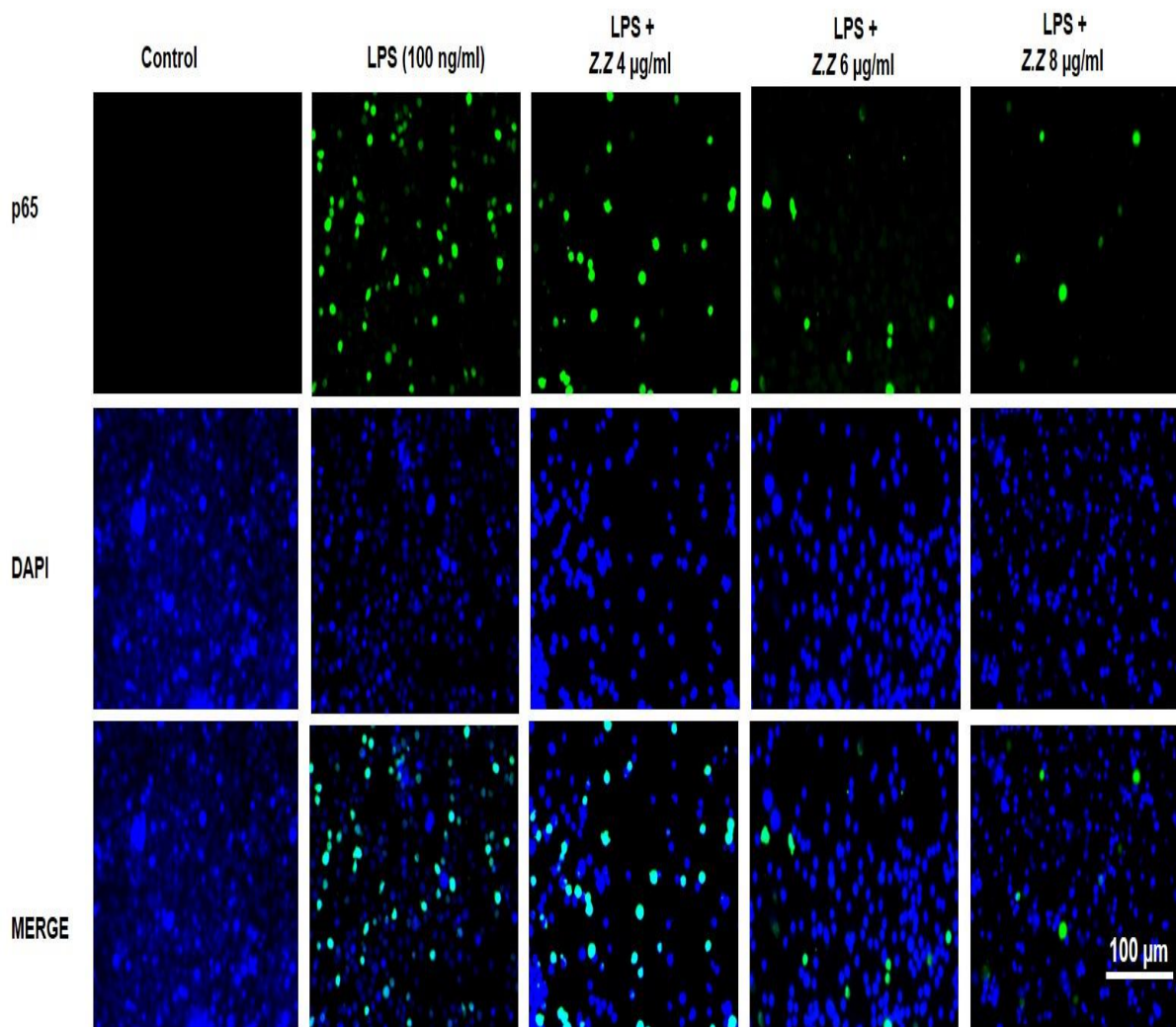 subunit binds to the DNA binding site in the nucleus after translocation to cause transcription of genes such as iNOS and COX-2 (Inukai, Kock, Bulyk, & development, 2017). Therefore, reducing the binding of NF-κB to DNA is essential to inhibit the transcriptional activity of NF-κB which secretes pro-inflammatory cytokines and inflammatory mediators. In this study, the effect of *Z. zanthoxyloides* on DNA binding activity of NF-κB in hemozoin stimulated BV2 cells were investigated. Result in Figure 2-20a display a significant elevation ($p < 0.001$) in the DNA binding activity of NF-κB (~2.1-fold increase) on stimulation of BV2 cells with hemozoin (400 µg/ml) in comparison to untreated cells. On the other hand, *Z. zanthoxyloides* at 6 and 8 µg/ml, markedly decreased the DNA binding activity of NF-κB (~1.3-fold and ~1.7-fold reduction respectively) while at 4 µg/ml of this extract, there was no significant difference when compared to BV2 cells stimulated with hemozoin.

Subsequently, western blots experiments were done to elucidate the effect of *Z. zanthoxyloides* on the expression of IκB and NF-κB -p65 subunit in BV2 cells stimulated with hemozoin. Results shows a marked increase ($p < 0.001$) in the level of p-IκB-α (~3-fold increase) in hemozoin treated cells when compared to untreated BV2cells. However, pre-treatment with *Z. zanthoxyloides* at 4 µg/ml significantly inhibited ($p < 0.01$) phosphorylation of p-IκB- α (~1.2-fold reduction) in hemozoin-induced BV2 cells in relation to cells stimulated with hemozoin only. A further marked

inhibition (p<0.001) was detected at 6 (~1.3-fold) and 8 μg/ml (~2.3-fold) of *Z. zanthoxyloides* extract.

A significant decrease (p<0.001) was observed in the level of total IκB-α (63.9%) in BV2 cells stimulated with hemozoin when compared to untreated cells. Furthermore, *Z. zanthoxyloides* at 4, and 6 μg/ml markedly elevated (p<0.01) the degraded total IκB-α (74.2% and 80.6% increase respectively) in hemozoin-stimulated BV2 cells when compared to hemozoin control. Furthermore, 8 μg/ml of this extract markedly restored (p<0.001) the level of total IκB-α (97% increase).

Thereafter, pre-treatment of *Z. zanthoxyloides* on nuclear translocation of NF-κB -p65 subunit in BV2 cells activated with hemozoin was investigated. Following stimulation of BV2 cells with hemozoin, there was a significant increase (p<0.001) in phosphorylation of p65 (~3.1-fold increase) in comparison to unstimulated cells. In contrast to this, *Z. zanthoxyloides* at 4 μg/ml markedly reduced (p<0.05) the degree of p-p65 (~1-fold decrease) while there was a further significant inhibition (p<0.001) at 6 (~1.6-fold reduction) and 8 μg/ml (~2.5-fold) of this extract in comparison to hemozoin stimulated cells. These results were further confirmed by immunofluorescence assay showing a reduced expression of NFκB -p65 when pre-treated with *Z. zanthoxyloides* (4, 6 and 8 μg/ml) in hemozoin activated microglia cells.
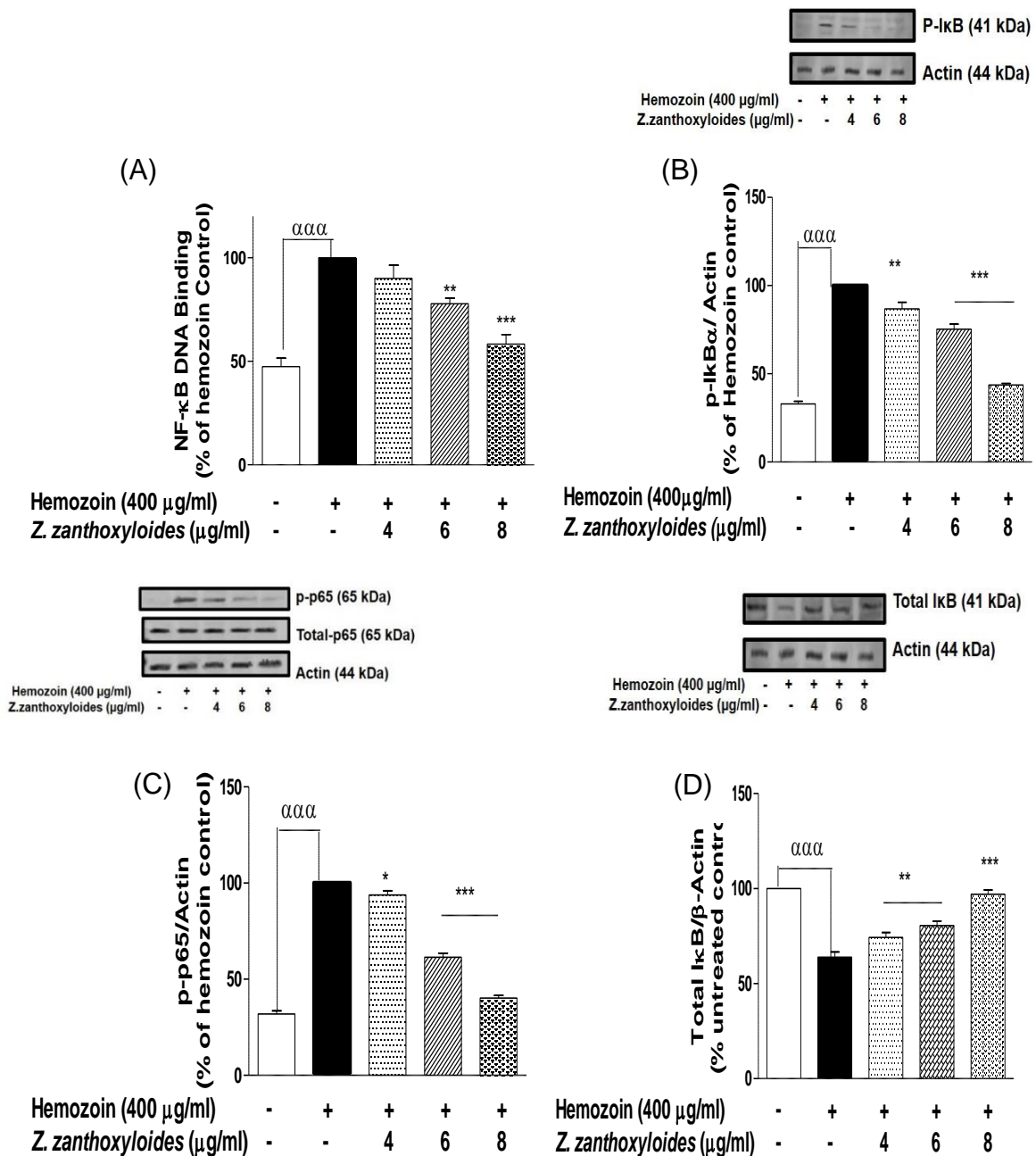
Figure 2-20: *Zanthoxylum zanthoxyloides* inhibits neuroinflammation via NFκB signalling pathway in hemozoin activated BV2 microglia

A) *Z. zanthoxyloides* at 6 and 8 µg/ml decreased DNA binding activity of NFκB in hemozoin stimulated BV2 microglia cells. (B) *Zanthoxylum zanthoxyloides* markedly reduced (B) phosphorylation of IκB, (C) degradation of IKB and (D) translocation of NF-KB p65 subunit in hemozoin induced BV2 cells. Cultured BV2 cells were stimulated with hemozoin (400µg/ml) in the presence or absence of *Zanthoxylum zanthoxyloides* (4, 6 and 8µg/ml) for 1 hour. At the end of the incubation, cytoplasmic or nuclear extract was collected and western blot was done to measure the level of p65 subunit and IκB protein expression. Analysis of data was done on 3 separate experiments using one-way ANOVA with post-hoc Student Newman-Keuls test (multiple comparisons ^{ααα} p<0.001 in comparison with the untreated cells and *p<0.05**p<0.01, ***p<0.001) in comparison with hemozoin control.
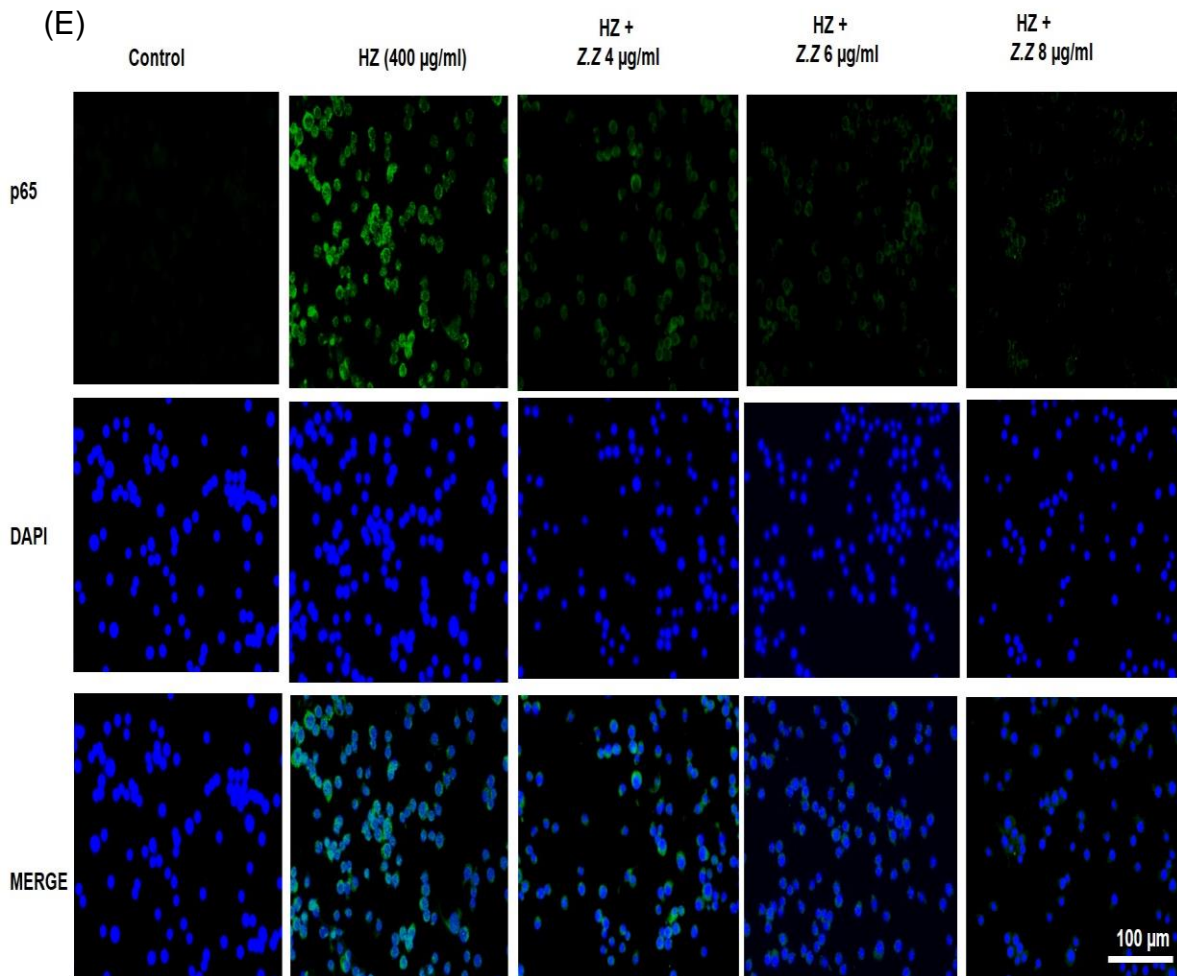
Figure 2-21: Effect of *Zanthoxylum zanthoxyloides* on p65 NF-κB protein expression in hemozoin induced BV2 microglia

Zanthoxylum zanthoxyloides (4, 6 and 8 µg/ml) decreased the expression of NF-ĸB p65 in hemozoin induced BV2 cells. Activated BV2 microglia cells were labelled with NF-ĸB p65 antibody and counterstained with DAPI. Images were acquired using EVOS® Floid ® cell imaging and analysed using image J

2.3.10 Methanolic extract of *Zanthoxylum zanthoxyloides* reduced pro-IL-1β and IL-1β secreted by inhibiting NLRP3 protein and caspase-1 activity in hemozoin induced BV2 microglia

The role of activated NLRP3 inflammasome and caspase-1 by pathogens such as hemozoin has been reported to be involved in the maturation of pro-IL-1β to active IL-1β, hence controlling the secretion of IL-1β (Jo, Kim, Shin, & Sasakawa, 2015; Primiano et al., 2016). Increase in release of IL-1β from activated microglia cells have been demonstrated to be involved in advancement of neuroinflammation (Jason et al., 2001; Oyegue-Liabagui et al., 2017). Hence, decreasing the level of pro-IL-1β and IL-1β production through inhibiting activated caspase-1 and NLRP3 protein is essential in reducing neuroinflammation in stimulated microglia cells. In Figure 2-22 a and b, results present a marked increase ($p < 0.001$) in the levels of pro-IL-1β (~2-fold) and IL-1β (~2.9-fold) in BV2 cells induced by hemozoin when compared to unstimulated cells. Contrarily, *Z. zanthoxyloides* at 4, 6 and 8 µg/ml significantly reduced ($p < 0.001$) elevated level of pro-IL-1β (~1.4-fold, ~1.6-fold and ~1.9-fold decrease respectively) when compared to hemozoin treated cells. Based on this result, there was a further investigation on the effect of *Z. zanthoxyloides* on IL-1β, the mature form of pro-IL-1β cytokine. *Z. zanthoxyloides* at 4 µg/ml significantly decreased ($p < 0.05$) the level of secreted IL-1β (~1.2-fold while at 6 and 8 µg/ml of this extract, there was a further inhibition ($p < 0.01$) in secretion IL-1β (~1.3-fold and ~1.5-fold decrease respectively) when compared to cells induced with hemozoin.

Furthermore, Figure 2-22 c and d show a significant increase ($p < 0.001$) in caspase-1 activity (~6.8-fold increase) measured by caspase-1 inflammasome and expression of NLRP3 protein (~2.9-fold increase) were observed on stimulation of BV2 cells with synthetic hemozoin in comparison with untreated cells. However, the elevated level of

caspase-1 activity was significantly inhibited (p<0.001) by *Z. zanthoxyloides* at 4 (~1.1-fold), 6 (~1.4-fold) and 8 μg/ml (~1.7-fold) when compared to BV2 cells induced by hemozoin. This result was supported by western blot experiments showing that *Z. zanthoxyloides* (4, 6 and 8 μg/ml) decreased expression of activated caspase-1 in hemozoin induced BV2 cells. Moreover, western blot experiment result shows that *Z. zanthoxyloides* at 4 μg/ml markedly reduced (p<0.05) the level of NLRP3 protein expression (~1.1-fold) and a further decrease (p<0.001) was detected at 6 μg/ml (~1.6-fold) and 8 μg/ml (~2.2-fold) when compared to hemozoin treated BV2 cells.

(a)
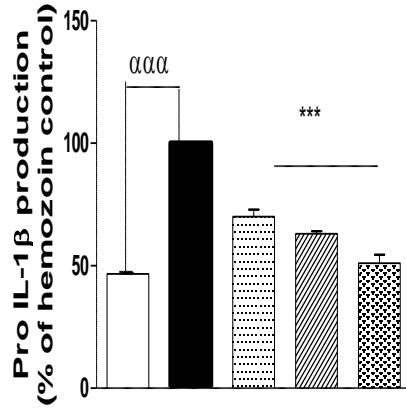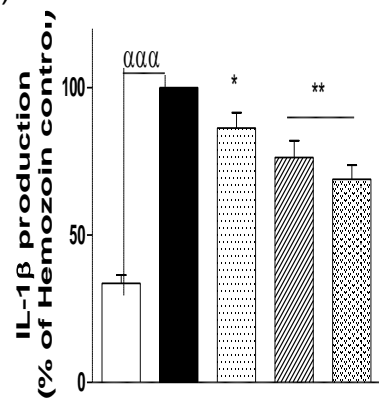
Pro IL-1β production (% of hemozoin control)

ααα   ***

Hemozoin (400 μg/ml)   -   +   +   +   +
Z. zanthoxyloides (μg/ml)   -   -   4   6   8

(b)

IL-1β production (% of Hemozoin control)

ααα   *   **

Hemozoin (400 μg/ml)   -   +   +   +   +
Z.zanthoxyloides (μg/ml)   -   -   4   6   8

Caspase 1 (45 kDa)
B-actin (44 kDa)

Hemozoin (400 μg/ml)   -   +   +   +   +
Z.zanthoxyloides (μg/ml)   -   -   4   6   8

(c)

Caspase-1 activation (% of Hemozoin control)

α α α   *   ***

Hemozoin (400 μg/ml)   -   +   +   +   +
Z. zanthoxyloides (μg/ml)   -   -   4   6   8

(d)

Caspase-1/Actin (% of Hemozoin control)

ααα   ***

Hemozoin (400 μg/ml)   -   +   +   +   +
Z. zanthoxyloides (μg/ml)   -   -   4   6   8

99

NLRP3 (106 kDa)

Actin (44 kDa)

Hemozoin (400 µg/ml)   -   +   +   +   +
Z.zanthoxyloides (µg/ml) -   -   4   6   8

e
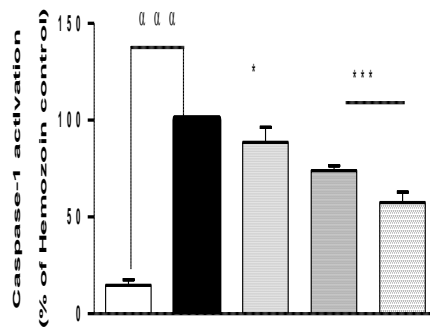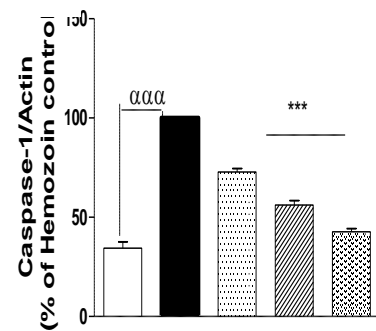
Hemozoin (400 µg/ml)   -   +   +   +   +
Z. zanthoxyloides (µg/ml)   -   -   4   6   8
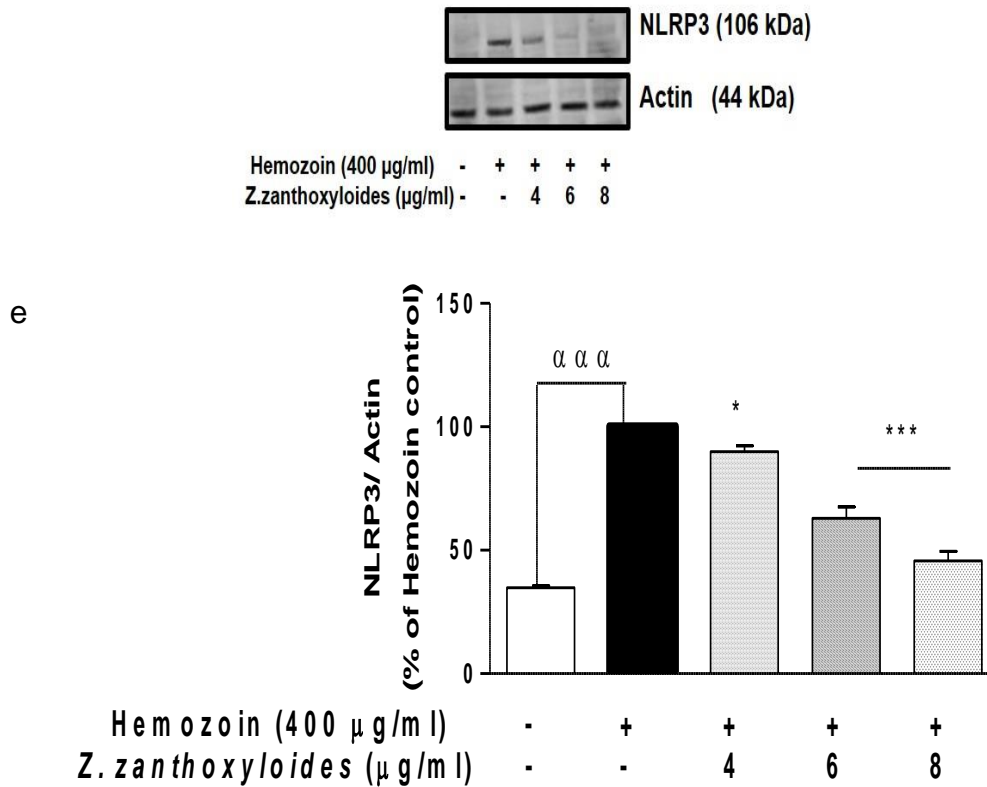
Figure 2-22: *Zanthoxylum zanthoxyloides* suppressed the levels of IL-1β and pro-IL-1β secretion by inhibiting NLRP3 protein and caspase-1 activity in hemozoin activated BV2 microglia

Zanthoxylum zanthoxyloides (4, 6 and 8 µg/ml) significantly inhibited (a) pro-IL-1β and (b) IL-1 β production (c) caspase-1 activation measured by caspase-1 Glo inflammasome assay in hemozoin induced BV2 microglia (d) expression of caspase-1 by immunoblotting experiment and (e) expression of NLRP3 protein. BV2 cells were stimulated with hemozoin (400 µg/ml) in the presence or absence of *Z. zanthoxyloides* (4, 6 and 8 µg/ml) for 24 hours. At the end of the incubation period, supernatants were collected for ELISA measurements. For western blot experiment, cytoplasmic extract was collected at the end of 24 hours' incubation and western blot was performed to measure protein expression of caspase-1 and NLRP3 protein. Data were expressed as mean ±SEM for 3 separate experiments. Analysis was done using one-way ANOVA with post-hoc Student Newman-Keuls test (multiple comparisons. ^{ααα} $p<0.001$ in comparison with the untreated cells and * $p<0.05$, **$p<0.01$, ***$p<0.001$) in comparison with hemozoin control.

2.3.11 Methanolic extract of *Zanthoxylum zanthoxyloides* did not decrease viability of BV2 cells induced by hemozoin

MTT assay was used in investigating the viability of BV2 cells pre-treated with *Z. zanthoxyloides* (4, 6 and 8μg/ml) and stimulated with hemozoin for 24 hours. Results showed no significant reduction in viability of cells in comparison to untreated BV2 cells.
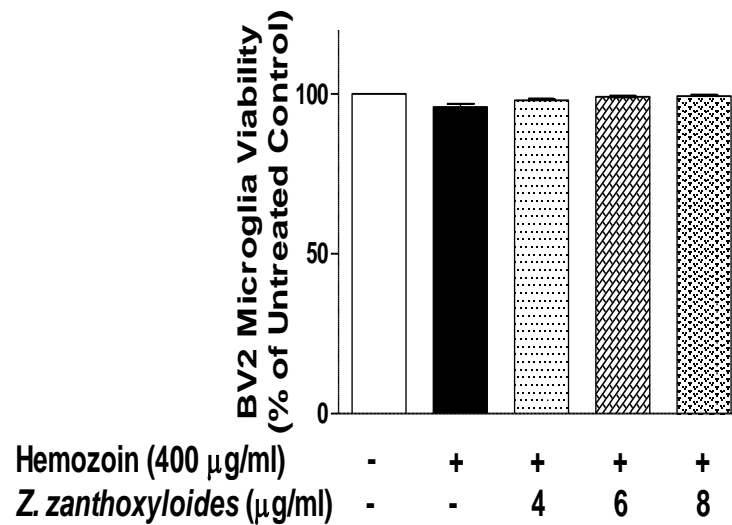
Figure 2-23: Z. zanthoxyloides did not decrease the viability of hemozoin induced BV2 microglia

Pre-treatment with Z. *zanthoxyloides* did not decrease the viability of BV2 microglia stimulated with hemozoin. Cells were stimulated with hemozoin (400 μg/ml) in the presence or absence of Z. zanthoxyloides (4, 6 and 8 μg/ml) for 24 h. At the end of the incubation period, MTT assay was done to investigate viability of BV2 cells. All values are expressed as mean ± SEM for 3 independent experiments. Analysis was done statistically using one-way ANOVA with post-hoc Student Newman-Keuls test in comparison with unstimulated cells

2.4 Discussion

Neuroinflammation occurs as a response of immune cells to stimuli such as LPS which activates toll like receptors to cause excessive secretion of pro-inflammatory cytokines and mediators (Cherry, Olschowka, & O'Banion, 2014; Lehnardt, 2010). Excessive production of pro-inflammatory cytokines in neuroinflammation has been shown to cause neurotoxicity which increases the progression of neurodegenerative diseases (Smith et al., 2012a; Wojdasiewicz et al., 2014). The inhibition of pro-inflammatory cytokines has been suggested to be a therapeutic strategy in reducing neuroinflammation, a contributing factor to the pathology of neurodegenerative diseases (El-Bakoush & Olajide, 2018; Rubio-Perez et al., 2012; Schain, Kreisl, & reports, 2017).The role of medicinal plants have been demonstrated in several studies to be significant in brain inflammation and development of anti-inflammatory drugs (Tomlinson & Akerele, 2015).

In the current study, a significant increase in the level of pro-inflammatory cytokines (TNFα, IL-1β and IL-6) and reduced level of IL-10 was observed on stimulation of BV2 microglia cells with LPS. However, methanolic extract of *Z. zanthoxyloides* significantly decreased the level of pro-inflammatory cytokines and elevated the IL-10 level in BV2 cells stimulated with LPS This suggest that *Z. zanthoxyloides* extract inhibited neuroinflammation by reducing the secretion of pro-inflammatory cytokines (TNFα, IL-1β and IL-6) and elevating the production of anti-inflammatory cytokine (IL-10) to attenuate neuroinflammatory response. *Zanthoxylum acanthopodium*, a closely related species in *Zanthoxylum* genus was reported to reduce the level of pro-inflammatory cytokines (IL-6 and TNFα), inflammatory mediators (iNOS and COX-2) in LPS induced macrophage cells (Yanti, Nuriasari, & Juliana, 2011). Moreover, an *in vivo* study on LPS induced mice has demonstrated that *Zanthoxylum alatum*

significantly reduced the production of pro-inflammatory cytokines (IL-1β, IL-6, IL-2 and TNFα) and increased the level of IL-10, hence suggesting its anti-inflammatory effect (Barua et al., 2018). Observations from these previous studies demonstrates the inhibitory effect of species in *Zanthoxylum* genus on cytokines in both *in vivo* and in monocytes. This further support the results from this current study done in BV2 microglia cells. These studies indicate the anti-inflammatory activity of species of *Zanthoxylum* genus in different models of inflammation.

Furthermore, the effect of *Z. zanthoxyloides* on NO production and expression of iNOS were investigated. Result from this study showed that *Z. zanthoxyloides* markedly suppressed the release of NO production by inhibiting the expression of iNOS enzyme in LPS induced BV2 cells. This result shows a first indication that *Z. zanthoxyloides* could inhibit neuroinflammation by reducing the secretion of NO through reducing expression of iNOS enzyme. In addition, observation from this current study confirms the role of excessive release of nitric oxide (NO) mediated by iNOS (inducible nitric oxide synthase) in neuroinflammation and in pathology of neurodegenerative diseases which has been investigated previously (Asiimwe et al., 2016; Yuste et al., 2015). Moreover, *Z. zanthoxyloides* evidently reduced the level of secreted PGE$_2$ and the protein expression of COX-2. Similarly, a previous *in vivo* study reported the inhibitory effect of *Z. zanthoxyloides* on PGE$_2$ concentration in rat induced with carrageenan (Prempeh & Mensah-Attipoe, 2008). This observation suggests the anti-inflammatory activity of *Z. zanthoxyloides* extract in peripheral inflammatory cells and this current study provides first evidence that this extract could inhibit neuroinflammation through PGE$_2$/ COX-2 pathway in BV2 microglia activated with LPS.

Numerous studies have established the role of NF-κB in regulating the gene expression of COX-2, iNOS and genes that encodes pro-inflammatory cytokines which

releases IL-6, TNFα, NO and PGE$_2$ (Weiwer, 2018; Yu, Lao, & Zheng, 2016). In addition, binding of NF-κB to the promoter site in DNA of target genes is important for transcription of inflammatory genes such as COX-2 (Ang & Wernig, 2018). Moreover, investigations have shown an association between activated NF-κB signalling pathway and neurodegenerative diseases including PD and AD (Baune & T, 2015). Hence, inhibiting the activation of NF-κB in neuroinflammation is important in reducing the secretion of pro-inflammatory cytokines and inflammatory mediators. In this study, the effect of *Z. zanthoxyloides* on NF-κB signalling pathway were elucidated. Result showed that *Z. zanthoxyloides* significantly inhibited the transcriptional activity of NF-κB by decreasing NF-κB mediated luciferase activity in transfected HEK293 cells stimulated by TNFα. The outcome of this study implies that *Z. zanthoxyloides* may have inhibitory effect on activated NF-κB signalling pathway. Moreover, there was a reduction in the binding activity of NF-κB to DNA by *Z. zanthoxyloides* in LPS – stimulated BV2 cells. This result suggests that the anti-inflammatory activity of *Z. zanthoxyloides* on IL-6, IL-1β, TNFα, NO, PGE$_2$, iNOS and COX-2 may be due to the inhibitory effect of this extract on NF-κB signalling pathway.

NF-κB p65 and p50 are heterodimer subunits in NF-κB family members, are demonstrated to play an important role in transcription of inflammatory genes (Christian et al., 2016). In resting cells, NF-κB is bound to IκB and remain inactive until inflammatory stimuli such as LPS activate the cells. This leads to the phosphorylation of IKK, which in turn phosphorylates IκB and subsequent degradation of IκB. This event leads to the translocation of NF-κB to the nucleus which then binds to DNA of inflammatory genes for the purpose of transcription. Consequently, the effect of *Z. zanthoxyloides* on phosphorylation of p65 in LPS-stimulated BV2 microglia cells were elucidated. Result shows that *Z. zanthoxyloides* inhibited p65 phosphorylation in a

concentration dependent manner. Observation from these results suggest that *Z. zanthoxyloides* reduce neuroinflammation by exerting its inhibitory effect on NF-κB signalling pathway in BV2 microglia cells. Following these results, studies of *Z. zanthoxyloides* on kinases that are upstream in NF-κB signalling pathway were investigated. Contrarily, *Z. zanthoxyloides* did not inhibit the phosphorylation of IKK in BV2 cells stimulated with LPS. Taking into account that most IKK inhibitors have been reported to act by competing with ATP molecule on binding to sites on IKK to exert their action (Nadia Lampiasi & Giovanna Montana, 2016). This may cause interference reducing the activity of IKK inhibitors. In addition to this, some IKK inhibitors also act by having allosteric effect on IKK (Tian et al., 2015). For example, an IKK inhibitor can significantly reduce IKKβ phosphorylation and not inhibit IKKα. This may also contribute to the insignificant inhibition of IKKα phosphorylation by *Z. zanthoxyloides* extract in LPS stimulated BV2 microglia cells. Furthermore, result from this study shows inhibition of NF-κB signalling pathway by reducing the expression of phosphorylated p-IκBα and depletion of IκBα. Taken together, observations from these results suggest that *Z. zanthoxyloides* extract inhibit neuroinflammation through targeting IκBα/NF-κB signalling pathway in LPS-stimulated BV2 microglia cells. This current study provides first indication on the inhibitory effect of *Z. zanthoxyloides* on neuroinflammation through NF-κB signalling pathway in LPS-stimulated BV2 microglia cells. The inhibitory action of *Z. zanthoxyloides* in this study is important and might be helpful in treatment of neuroinflammation mediated neurodegenerative diseases.

Investigations have identified the role of neuroinflammation induced by hemozoin to be significant in pathologies such as cerebral malaria (Wassmer & Grau, 2017). Furthermore, previous studies have shown elevated level of of pro-inflammatory cytokines (IL-1β, IL-6 and TNFα) secretion on stimulation of monocytes and

macrophages with hemozoin (Dunst et al., 2017; Olivier et al., 2015; Perkins et al., 2011; Velagapudi et al., 2019). Therefore, inhibition of neuroinflammation by reducing the level of pro-inflammatory cytokines and inflammatory mediators in hemozoin-stimulated cells could be a potential therapeutic target for neuroinflammatory disorders. The effect of *Z. zanthoxyloides* on IL-6 and TNFα were investigated in this study. The outcome of this experiment showed an increase in the level of IL-6 and TNFα in BV2 cells induced by synthetic hemozoin. In addition, the increased level of TNFα and IL-6 were significantly decreased on pre-treatment with *Z. zanthoxyloides* in hemozoin activated BV2 cells. Moreover, a reduced level of IL-10 was detected on stimulation of BV2 cells with hemozoin. *Z. zanthoxyloides* increased the level of IL-10, suggesting the anti-inflammatory activity of this extract. The outcome of these investigations is significant and suggest that *Z. zanthoxyloides* can attenuate neuroinflammatory response by hemozoin to inhibit pro-inflammatory cytokines and increasing anti-inflammatory cytokines in brain macrophage cells. Observations from this current study proposes a new mechanism that *Z. zanthoxyloides* extract could reduce hemozoin-induced neuroinflammation in BV2 microglia cell line.

Excessive release of NO have been suggested to contribute to the development of neuroinflammation in malaria (Dunst et al., 2017). In this current study, a significant increase in level of NO mediated by iNOS was observed in BV2 cells stimulated with hemozoin. In addition, *Z. zanthoxyloides* suppressed the level of PGE_2 secreted through inhibition of COX-2 expression in BV2 cells induced by hemozoin. These results suggest the effect of *Z. zanthoxyloides* on PGE_2 and NO production through suppression of iNOS and COX-2 proteins, respectively. These results provide first proof of anti-inflammatory activity of *Z. zanthoxyloides* on neuroinflammation through reducing the production of NO and PGE_2 with their mediators.

Research in the past have demonstrated the activation of NF-κB by hemozoin through toll-like receptor 2 (TLR-2) and toll like receptor-9 (TLR-9) (Coban et al., 2006; Kawai & Akira, 2011; Polimeni et al., 2012). The activation of NF-κB through TLR2 and TLR9 receptors have been shown to be important in immune responses to infections which includes *P.falciparum*, a malaria parasite (Wagner, 2010). These studies suggest the involvement of activated TLRs and NF-κB signalling pathway in neuroinflammation induced by hemozoin. Hence, attenuation of hemozoin-induced neuroinflammation through inhibition of activated NF-κB pathway may help target neuroinflammatory related disorders. The current investigation explored the effect of *Z. zanthoxyloides* on NF-κB signalling pathway in order to elucidate the mechanism of action of this extract in hemozoin induced BV2 cells. In this study, an increased level of DNA binding activity of NF-κB, phospho-p65 and phosphorylation of IκB were observed in hemozoin induced BV2microglia cells. These results suggest that hemozoin was able to activate NF-κB signalling pathway in BV2 cells microglia. A similar result was reported revealing an increase in the phosphorylation of NF-κB p65 subunit and DNA binding activity of NF-κB in hemozoin-stimulated BV2 microglia cells (Velagapudi et al., 2019).

Z. zanthoxyloides significantly inhibited the DNA binding activity of NFκB, phosphorylation of NF-κB p65 subunit, phosphorylation of p-IκBα and degradation of IκBα in BV2 cells activated with hemozoin. These results indicate that Z. *zanthoxyloides* could inhibit neuroinflammation through blocking of IκBα/NF-κB pathway in hemozoin-induced BV2 microglia cells. In addition, the outcome of this investigation further indicates that Z. *zanthoxyloides* could reduce the binding of NF-κB to DNA, resulting in inhibition of gene transcription involved in inflammation such as iNOS and COX-2. This is the first study suggesting the general effect of *Z. zanthoxyloides* on NFκB signalling pathway in hemozoin-stimulated BV2 microglia

cells. Therefore, the inhibitory role of *Z. zanthoxyloides* on hemozoin-induced neuroinflammation though NFκB signalling pathway is important as it may be a potential therapeutic strategy in treatment of neuroinflammatory disorders such as CM.

Previous studies have demonstrated that hemozoin induces the release of IL-1β cytokine from pro-1β by activating NLRP3 inflammasome with further increased caspase-1 activity in macrophage cells and in BV2 microglia cells (Ozaki, Ema, & Campbell, 2015; Shio et al., 2009; Velagapudi et al., 2019). Observations from these studies suggest the role of hemozoin on inflammation through NLRP3 inflammasome pathway in peripheral blood cells and in the brain's resident macrophage cells. In this current research, similar increase in levels of NLRP3 with caspase-1 activity, pro-IL-1β and IL-1β cytokine were observed on stimulation of BV2 cells with hemozoin in comparison to untreated BV2 cells. *Z. zanthoxyloides* inhibited the expression of NLRP3 protein with subsequent reduction of caspase-1 activity (using both caspase-1 inflammasome assay and western blot experiments as detection technique) in hemozoin-stimulated BV2 microglia cells. In addition, *Z. zanthoxyloides* elevated level of pro-IL-1β and IL-1β. These results provide the first proof showing the inhibitory action of *Z. zanthoxyloides* on NLRP3 inflammasome and caspase-1 activity in BV2 cells activated with hemozoin. Results from these investigations suggest that *Z. zanthoxyloides* reduced hemozoin-induced neuroinflammation through NLRP3 pathway and this is significant as it may be a promising therapeutic agent in treatment of neuroinflammation in severe complications of malaria infections.

In summary, investigations on methanolic extract of *Z. zanthoxyloides* in this current study have been demonstrated to inhibit neuroinflammation by targeting NFκB and NLRP3 signalling pathway in BV2 cells induced by LPS or hemozoin in a separate study. The observations from these investigations have presented the first evidence

of anti-neuroinflammatory effect of methanolic extract of *Z. zanthoxyloides* in BV2 microglia cells. .

3 CHAPTER 3- Bioassay guided isolation of bioactive compounds from *Z. zanthoxyloides* and their anti-neuroinflammatory activities

3.1 Background of this chapter

Medicinal plants have been shown in several studies to be a primary source of discovering new drugs and the process includes isolation of bioactive compounds and phytochemical studies such as Mayer's test to detect the presence of alkaloids in a mixture (Shakya, 2016; Tomlinson & Akerele, 2015). Subsequently, the identification and elucidation of compound structure are investigated and pharmacological activities of the known compound are then determined (Atanasov et al., 2015; Latif & Sarker, 2012). In addition, isolated compounds from medicinal plants have been described to provide background information which includes size, molecular weight and functional groups which is necessary in synthesising more compounds chemically (Nothias et al., 2018). In order to isolate compounds from medicinal plants, bioassay guided fractionation is considered as first stage in drug discovery to detect the presence of compounds with biological activity in an extract during isolation process (Malviya & Malviya, 2017; Waksmundzka-Hajnos & Sherma, 2010). The process of compound Isolation are continuously repeated until a pure bioactive compound is derived from plants extract (Kabera, Semana, Mussa, & He, 2014). Examples of drugs isolated from plants includes: the isolation of aspirin, an anti-inflammatory drug from bark of *Salix alba* tree (Rainsford, 2013), isolation of quinine, an anti-malaria drug from the bark of *Cinchona succirubra* (Thanacoody, 2016) and isolation of paclitaxel (anti-cancer drug) from *Taxus brevifolia* tree (Priyadarshini & Keerthi, 2012). In addition, studies have also reported the isolation of artemisinin from *Artemisia annua* which is used in malaria treatment (Miller & Su, 2011). Moreover, isolation of galanthamine from *Huperzia*

serrata have been reported for its benefit in treatment of early phase of Alzheimer's disease (Zhan et al., 2016). Consequently, isolation of compounds from medicinal plants are essential as they are potential sources of therapeutic drugs.

3.2 Isolation of bioactive compounds from *Z. zanthoxyloides*

3.2.1 Solid phase extraction

Solid phase extraction (SPE) is a process that separates compounds between two phases.(Płotka-Wasylka, Justyna, Szczepańska, de la Guardia, & Namieśnik, 2016) In SPE technique, the compounds to be extracted are separated between a liquid and solid phase. SPE was used to further fractionate methanolic extract of *Z. zanthoxyloides* that was initially obtained through soxhlet extraction process (as described in 2.2.2). A commercially available SPE catridge was used for fractionation of the *Z. zanthoxyloides* extract (Reid & Sarker, 2012). Two grams (2 g) of the methanolic extract was used for solid phase extraction using a Strata C-18 cartridge 20g (Phenomenex, California, USA). The cartridge was previously washed with 50 mL of MeOH and followed by running 100 mL of water. Thereafter, the extract was dissolved in 10ml of 10% methanol (MeOH) in water. The cartridge was eluted with a gradient of methanol to water (MeOH-H_2O) mixture obtaining four fractions (F1, 20:80; F2, 50:50; F3, 80:20 and F4, 100:0). This procedure resulted in 200ml each of the fraction. The fractions were dried using a rotary evaporator and a freeze-dryer. Afterwards, fractions were kept in the 4°C fridge for further experiments.

3.2.2 Analytical and Preparative HPLC

The role of high performance liquid chromatography (HPLC) has been shown as an important technique in the process of isolation because of its use in separation and identification of different components in plants extract (Thammana, 2016). Results from HPLC technique is represented on a graph called chromatogram, which shows peaks representing the presence of compounds in a sample. Analytical and preparative HPLC are useful to separate, quantify, detect and isolate natural products or mixture of compounds. The use of prep-HPLC technique has been shown to

separate wide classes of compounds from crude extract in large quantities (Latif &

Sarker, 2012). In addition, solvents used in HPLC have the ability to dissolve the

sample, having high purity, transparent and not interfering with compound of interest

and have low viscosity (Huang, Tung-Yung, Chi, & Chien, 2018; Latif & Sarker, 2012).

Analytical HPLC was used in this experiment to screen the presence of secondary

metabolites in the fractions while prep HPLC was used in purifying and isolating the

compound. Twenty-six (26) mg of fraction 3 (the bioactive fraction in *Z. zanthoxyloides*

extract) was purified on an Agilent 1260 infinity HPLC system equipped with an Agilent

1260 DAD and an Ace-5 C18 column (150 × 21.2 mm, 5 µm particle size, Hichrom

Ltd). Preparative HPLC was performed using a gradient of mobile phase A (water

with 0.1% trifluoroacetic acid) and mobile phase B (methanol with 0.1% trifluoroacetic

acid) with the following time schedule: 30% B, 0–35 min; 100% B, 35-37min; 100 %

B, 37-39 min; 30% B, 39-42 min. The flow rate was maintained at 10.0 mL/min and

UV absorbance was recorded. Furthermore, Figure 3-8 shows the prep-HPLC that

was used to purify fraction 3 and the chromatogram showed the peak detection of

skimmianine at a retention time of 17.596 minutes.

3.2.3 Nuclear magnetic resonance (NMR) and Mass spectrometry

The use of NMR spectroscopy has been reported to be significant in elucidation of

compound structure by providing structural information about components in a

molecule. Studies have shown that proton NMR (^{1}H-NMR) detects the number of

hydrogen present in a compound and describes how they are connected to each

other while carbon NMR (^{13}C NMR) provides information about the number of

carbons in a compound. Hence, the use of ^{1}H -NMR and ^{13}C NMR are both

important in structure elucidation of unknown compounds, especially isolated

compounds from medicinal plants. The structure of skimmianine was determined

using spectroscopic analyses which includes proton (^{1}H) and carbon (^{13}C) NMR while 2D NMR experiments are ^{1}H-^{1}H COSY (Correlation Spectroscopy), HMBC (^{1}H-^{13}C Heteronuclear Multiple Bond Correlation Spectroscopy) and HSQC (^{1}H-^{13}C Heteronuclear Single Quantum correlation). Experiments were performed on either a Bruker AMX 600 or a Bruker AMX 300 instrument (Bruker, Germany). Chemical shifts (δ) are given in part by millions (ppm) and the coupling constants J in Hertz (Hz). Mass spectrometry (MS) is a technique that provides information about the molecular weight and fragmentation pattern of a compound (De Hoffmann & Edmond, 2000). MS was used in confirming and to provide more information from result obtained in NMR spectroscopy. High resolution mass spectra (HR-MS) were recorded at the National Mass Spectrometry Facility, Swansea, UK on a LTQ Orbitrap XL1 spectrometer. The analytes were prepared in methanol and the mass spectrum was analysed relating their mass to charge (m/z) ratio.
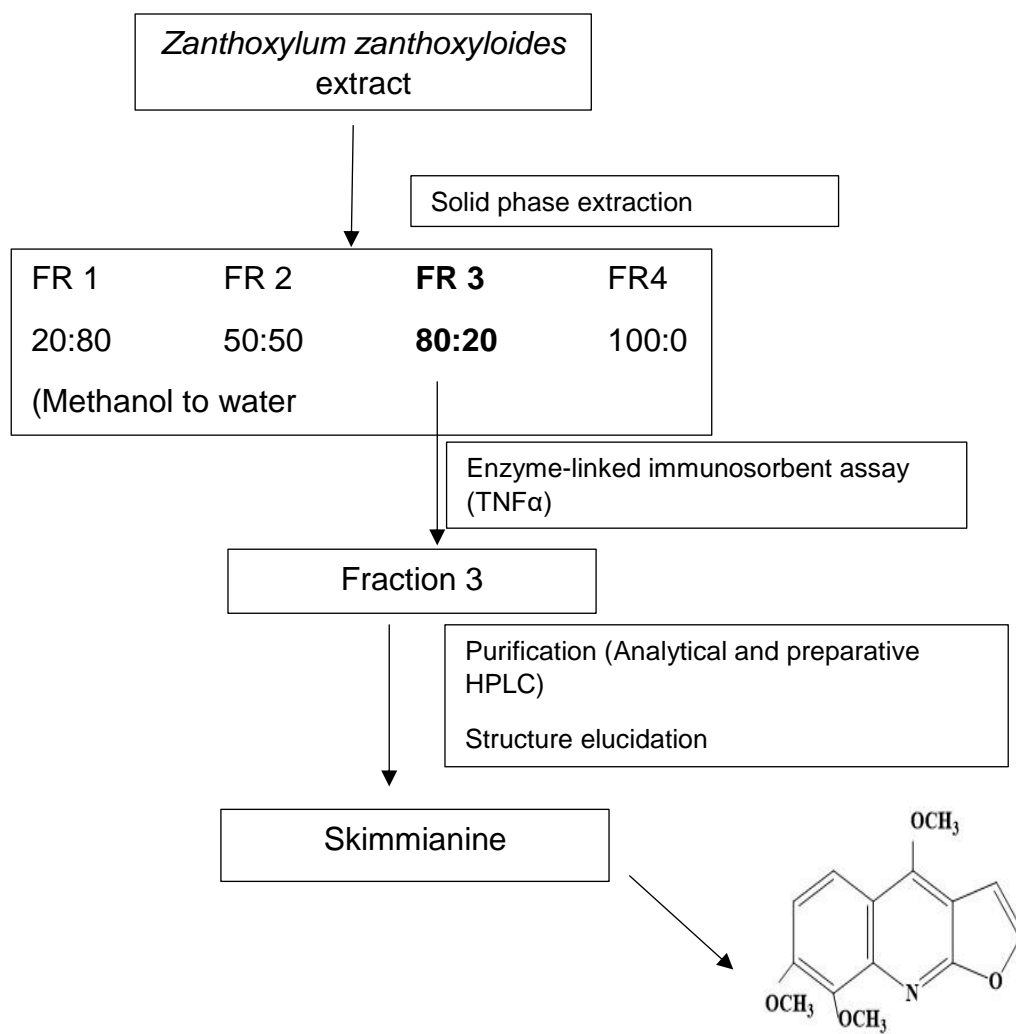
Figure 3-1: Isolation of bioactive compound (skimmianine) from fraction 3 of Z.

zanthoxyloides extract

3.2.4 Skimmianine

Previous Investigations on *Z. zanthoxyloides* extract in this current study showed an inhibitory activity of this extract on neuroinflammation. This further led to interest of isolating and identifying compounds in this extract that has inhibitory effect on neuroinflammation in BV2 microglia cells. In order to isolate and identify compounds from this extract that has anti-neuroinflammatory activity, bioassay guided fractionation was required to achieve this aim. Figure 3-1*:* summarises the process adopted in bioassay guided fractionation to isolate and identify bioactive compounds from *Z. zanthoxyloides* extract. Firstly, solid phase extraction (SPE) technique was used to separate this extract into four fractions (F1, F2, F3 and F4). Afterwards, an ELISA technique was used in investigating the inhibitory activity of F1, F2, F3 and F4 on the level of TNFα production in LPS-stimulated BV2 microglia cells. The outcome of this investigation showed fraction 3 as the most active by significantly reducing the elevated level of TNFα in LPS- activated BV2 microglia cells. Subsequently, purification of fraction 3 was done using HPLC and a major pure compound was detected as peak in fraction 3 which was significant from the chromatogram. Thereafter, structure elucidation of this compound was determined by nuclear magnetic resonance (NMR) and its molecular weight were obtained by mass spectrophotometer. Observation from this bioassay guided fractionation showed skimmianine as the main compound isolated in fraction 3 of *Z. zanthoxyloides* extract.

Skimmianine is the most abundant furoquinoline alkaloid that is isolated from *Zanthoxylum* genus (Phuyal, Jha, Raturi, & Rajbhandary, 2019; Queiroz et al., 2006b). Furoquinoline alkaloids are true alkaloids as they are organic nitrogen-containing compounds that serve as an important pool for discovery of drugs (Adamska-Szewczyk, Aldona, Glowniak, & Baj, 2016). Skimmianine (also known as beta-fagarine

or chloroxylonine) has an IUPAC name called 4, 7, 8-Trimethoxyfuro [2, 3-*b*] quinoline.

The chemical structure of skimmianine is shown below with a molecular formula of

$C_{14}H_{13}NO_4$ and molecular weight of 259.261 g/mol.
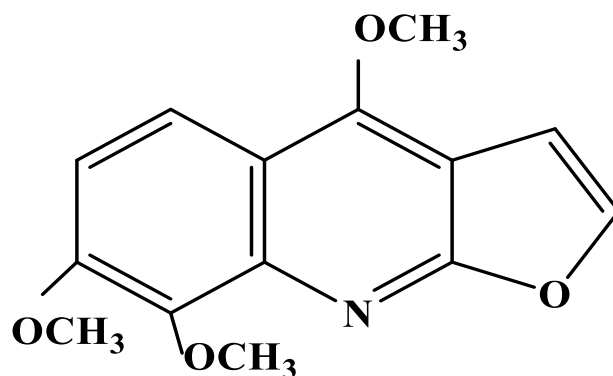
Figure 3-2: Chemical structure of skimmianine

Reports have shown previous isolation of skimmianine from stem bark of *Melicope*

madagascariensis (Rasamison et al., 2016), leaves of *Oricia renieri* and *Oricia*

suaveolens (Nouga et al., 2016), *Zanthoxylum buesgenii (Sandjo, Kuete, Tchangna,*

Efferth, & Ngadjui, 2014) and leaves of Evodia lepta (Sichaem et al., 2014). Examples

of furoquinoline alkaloids that are isolated from *Zanthoxylum* are kokusaginine,

dictamnine and skimmianine (Aniszewski, 2015; Patiño et al., 2012).

3.2.4.1 Pharmacological activities of skimmianine

Previous studies have demonstrated that skimmianine isolated from stems of

Esenbeckia leioncarpa showed an anti-acetylcholinesterase activity (Cardoso-Lopes

et al., 2010). Similarly, isolated skimmianine from *Zanthoxylum nitidum* was

demonstrated to have anti-cholinesterase activity by increasing the level of

acetylcholine (Yang, Zhang, Ren, & Yang, 2012). Furthermore, a study by Ratheesh

et al (2013) reported an anti-inflammatory activity of isolated skimmianine from *Ruta*

graveolens by significantly reducing the levels of pro-inflammatory cytokines (IL-6 and

TNFα), nitrite, prostaglandin E$_2$ and COX-2 expression in carrageenan induced rat (Ratheesh, Sindhu, & Helen, 2013). Furthermore, isolated skimmianine from root barks of *Dictamnus dasycarpus* has been reported to attenuate the increased level of nitric oxide produced in LPS-stimulated BV2 cells (Yoon et al., 2012) .However, this study did not further investigate the effect of isolated skimmianine on expression of iNOS enzyme, which is an important mediator for secretion of NO in LPS stimulated BV2 cells. Moreover, investigations of skimmianine isolated from *Zanthoxylum avicennae* and *tetradium ruticarpum* showed an inhibitory activity on elevated level of secreted superoxide anion in human neutrophils (Cho et al., 2012; T. Y. Wang et al., 2010). This study indicates the anti-inflammatory effect of skimmianine by reducing one of the reactive oxygen metabolites (superoxide anion) produced in inflammatory cells. However, studies to show the effect of skimmianine on important inflammatory mediators such as COX-2 and pro-inflammatory cytokines including IL-6 and TNFα in LPS-activated BV2 microglia cells are yet to be determined. In addition, investigations to elucidate the effect of skimmianine on NF-κB activation is unknown. Hence, effect of skimmianine on neuroinflammation in LPS-stimulated BV2 microglia cells were investigated.

3.3 Investigations on the effect of Fractions 1, 2, 3 and 4 of *Z. zanthoxyloides* extract and skimmianine on neuroinflammation induced by LPS in BV2 microglia cells

3.3.1 Drug preparation and treatment

Fractions 1, 2, 3 and 4 of *Z. zanthoxyloides* extract were prepared in DMSO (Sigma, UK). A stock concentration of 2, 3 and 6 mg/ml of these fractions were prepared separately to working concentrations of 4, 6 and 8 µg/ml prior to experiments. Skimmianine was dissolved in DMSO to a stock concentration of 0.1M. BV2 microglia cells were treated with skimmianine (10, 20 and 30 µM) for 30 minutes and stimulated with LPS at 100 ng/ml. Subsequently, further quantities of skimmianine (PhytoLab, Germany) were purchased for further investigations on effect of skimmianine on neuroinflammation. NMR spectroscopy was used to authenticate this compound before experiments. Lipopolysaccharide (LPS) at 1 mg/ml, derived from *Salmonella enterica* serotype typhimurium (Sigma, UK) was used in preparing a working concentration of 100 µg/ml and kept in 4°C.

3.3.2 Cell culture

3.3.2.1 BV2 Cells

BV2 microglia cell line ICLCATL03001 (Banca Biologica e Cell Factory, Italy) were cultured in RPMI 1640 medium (Life Technologies, UK) supplemented with 10% fetal bovine serum (Sigma), 1mM sodium pyruvate (Sigma), 100U/ml Penicillin and 100mg/ml Streptomycin (Sigma). Prior to experiments, the cells were sub-cultured, maintained in the incubator and seeded out in plates as described in 2.2.3.1.

3.3.2.2 HEK293 Cells

HEK293 cells were cultured in Minimum Essential Media (MEM) (ThermoFisher Scientific, UK) and generally maintained as shown in text 2.2.3.2. HEK293 cells

seeded out in plates once they became confluent for the purpose of transfection experiments. In this study, the effect of skimmianine on NF-κB mediated gene expression were investigated in TNFα-stimulated HEK293 cells.

3.3.2.3 HT22 neuronal cell culture

HT22 cells are immortalised cell line from culture of primary mouse hippocampal neuron and was derived from HT4 cells (Davis & Maher, 1994). HT22 neuronal cell line were kindly provided by Dr Jeff Davis (University of Swansea, UK). HT22 cells were maintained and cultured in a cell culture flask (T75 cm^2) that contains DMEM (Life Technologies, UK), with addition of 1mM sodium pyruvate, 2mM L-glutamine, 10% FBS (heat inactivated), 100 μg/ml of streptomycin and 100 U/mL of penicillin as supplements. T75 flask containing HT22 cells were kept in a 5% CO_2 incubator at a temperature of 37° C. Cultured cells were sub-cultured at 70 - 75% confluence. PBS (5 mls) was added to wash the cells in the flask and aspirated. There was addition of 2 mL of 0.25% trypsin-EDTA solution to dissociate HT22 cells in flask. Trypsinization of cells were terminated by adding 8 mL of DMEM complete medium to the flask. Afterwards, the dissociated cells were collected in a tube and centrifuged at 1200 rpm for 5 minutes. The supernatant was aspirated, followed by re-suspension of cell pellets in 10 mL of new complete DMEM. HT22 cells were counted with haemocytometer and subsequently seeded out in cell culture plates at a concentration of 2×10^5 cells/ml in preparation for experiments.

3.3.3 MTT assay for cell viability

MTT assay (as previously described in 2.2.16) was performed to assess the viability of cells after pre-treatment with skimmianine (10, 20 and 30 μM) and stimulated with LPS (100 ng/ml).

3.3.4 Measurement of Nitrite (Griess assay)

Cultured BV2 cells were pre-treated with skimmianine (10, 20 and 30 µM) for 30 minutes and then stimulated with LPS (100 ng/ml) for 24 hours. After incubation, cell culture supernatants were collected and centrifuged at 1500 rpm for 5 minutes. Nitrite concentration in the cell culture supernatant was measured using the Griess assay (earlier described in section 2.2.5).

3.3.5 Determination of TNFα, IL-6, IL-1β, IL-10 and Pro-IL-1β by Enzyme linked immunosorbent assay (ELISA

Skimmianine (10, 20 and 30 µM) was incubated with cultured BV2 cells and then, stimulated with LPS (100 ng/ml) for 24 hours. Afterwards, cytokine levels of TNFα, IL-1β, IL-6 and IL-10 were analysed in the cell culture supernatants using ELISA technique (as previously demonstrated in section 2.2.6).

3.3.6 Prostaglandin E2 measurement using enzyme immunoassay (EIA)

Cultured microglia BV2 cells were treated with skimmianine (10, 20 and 30 µM) for 30 minutes and subsequently activated with LPS (100 ng/ml) for 24 hours. At the end of incubation, Prostaglandin E2 level in the cell culture supernatants was measured (as initially described in section 2.2.8).

3.3.7 Extraction of cytoplasmic cell lysates from cultured BV2 cells

BV2 cells were treated with skimmianine (10, 20 and 30 µM) for 30 minutes and further activating with LPS (100 ng/ml) for the time required for each experiment, cytoplasmic cell lysates were extracted as explained in section 2.2.9.

3.3.8 Extraction of nuclear cell lysates from cultured BV2 cells

Cultured microglia BV2 cells were treated with skimmianine (10, 20 and 30 µM) for 30 minutes and subsequently activated with LPS (100 ng/ml) for duration required for each investigation. Nuclear cell lysates were extracted as described in section 2.2.10.

3.3.9 Measurement of protein concentration

After cytoplasmic and nuclear extraction, concentrations of proteins were determined (as shown in text 2.2.11) by Pierce Coomassie (Bradford) protein assay kit (Thermo Scientific, UK) before western blot experiments

3.3.10 Western blotting

Western blotting (as described in 2.2.12) is a technique that is useful in detection of target protein according to their molecular weights. This technique mainly involves separation of proteins in lysates by gel electrophoresis, transfer of proteins from gel onto membrane blot and detection of target proteins based on their molecular weights. Following the determination of protein concentration in cell lysates, western blotting technique were used to investigate the effect of skimmianine on expression of iNOS, COX-2, NF-κB p65, phospho-IκB-α, IκB-α.

3.3.11 NF-κB p65 transcription factor assay

NF-κB transcription factor assay (as described in 2.2.13) is an assay that investigates the binding of NF-κB to the consensus binding site on the DNA. In this study, the effect of skimmianine (10, 20 and 30 µM) on binding of NFκB-DNA binding in LPS-stimulated BV2 microglia cells were determined.

3.3.12 Immunofluorescence technique

Immunofluorescence is an imaging technique (described in 2.2.14) that is important in detection of antigen by a specific antibody and this reaction is observed under fluorescence microscope. In this study, the effect of skimmianine (10, 20 and 30 µM) on expression of NF-κB p65 were investigated in LPS-activated BV2 microglia cells

3.3.13 Reporter gene assay

As described in 2.2.15, HEK293 cells were transfected with Cignal NF-κB (LUC) (CCS-013L) vector to investigate the effect skimmianine on luciferase activity of NF-κB in the nucleus.

3.3.14 BV2 microglia conditioned medium)

Studies have shown that increased levels of inflammatory stimuli including TNFα and IL-6 from activated microglia cells cause neurotoxicity to surrounding neurons in CNS, which could increase the progression of neurodegenerative diseases(Kraft, Harry, & health, 2011). Cultured BV2 cells were seeded in a 6-well plate at a concentration of 2×10^5 cells/ml and put in the incubator at 37° C. After the cells had reached confluence, they were treated with skimmianine at 10, 20 and 30 µM for 30 minutes and then stimulated with LPS (1µg/ml) for 24 hours. At the end of the incubation, supernatants were collected from the cells in plate, transferred to an eppendorf tube and centrifuged for 5 minutes at 1200 rpm in order to remove cell debris from the conditioned medium. Following centrifugation, the conditioned medium was made into aliquot in eppendorf tube and then stored in -80° C freezer. Afterwards, levels of NO and TNFα were analysed in the conditioned medium to verify if these inflammatory mediators had been secreted from LPS induced BV2 cells.

3.3.15 Viability studies on HT22 hippocampal cells in conditioned medium from LPS-activated BV2 cells

MTT assay was used in assessing the viability of HT22 cells on exposure to conditioned medium from LPS activated microglia cells. Firstly, HT22 cells were seeded out in a 96 well plate at a concentration of 2×10^5 cells/ml (200 µl/ in each well) and incubated until the cells reached confluence. At the end of the incubation, cultured medium was aspirated carefully from each well and replaced with 200 µl of conditioned medium (from activated BV2 cells) and then incubated for 24 hours. Thereafter, MTT assay was used in determining the viability of HT22 neuronal cells.

3.3.16 Detection of MAP2 protein in conditioned medium–treated HT22 cells by immunofluorescence

Investigations have associated a reduced expression levels of microtubule-associated protein 2 (MAP2) with activated microglia cells to cause neuronal toxicity (Froger et al., 2010). MAP2 protein plays an important role in neuronal growth and stability and reduced expression of MAP2 have been shown to contribute to the progression of neurodegenerative diseases (Avila, Pallas, Bolós, Sayas, & Hernandez, 2016). The effect of skimmianine on expression of MAP2 protein in conditioned medium – activated HT22 cells were observed by immunofluorescence (described in 2.2.14).

3.4 RESULTS

3.4.1 Effect of fractions 1, 2, 3 and 4 on TNFα production in LPS-activated BV2 microglia

The role of excessive secretion of TNFα from activated microglia cells has been shown to be involved in neuroinflammation, a contributing factor to the progression of neurodegenerative diseases (Probert, 2015; Sedger & McDermott, 2014). Hence, inhibiting overproduction of TNFα from stimulated microglia cells is important in reducing the level of TNFα in neuroinflammation. Inhibitory effect of *Z. zanthoxyloides* extract on pro-inflammatory cytokines (TNFα, IL-1β and IL-6) and inflammatory mediators (COX-2, iNOS) and NF-κB proteins led to further studies in isolating compounds present in this extract. Subsequently, *Z. zanthoxyloides* extract was subjected to solid phase extraction (SPE) to obtain four fractions: FR1, FR2, FR3 and FR4. Subsequently, effects of FR1, FR2, FR3 and FR4 on elevated levels of TNFα in LPS activated BV2 cells were investigated to guide the isolation of compounds from the bioactive fraction(s). Result in Figure 3-3 shows a significant reduction ($p < 0.001$) in the levels of TNFα secreted when pre-treated with 4 µg/ml of FR3 (~1.2-fold reduction), 6 µg/ml of FR3 (~1.4-fold) and 8 µg/ml of FR3 (~1.6-fold) when compared to LPS stimulated cells Moreover, there was no significant reduction in the elevated levels of TNFα produced on treatment of LPS-stimulated cells with FR1, FR2 and FR4 at these concentrations (4, 6 and 8 µg/ml) in comparison to LPS control.
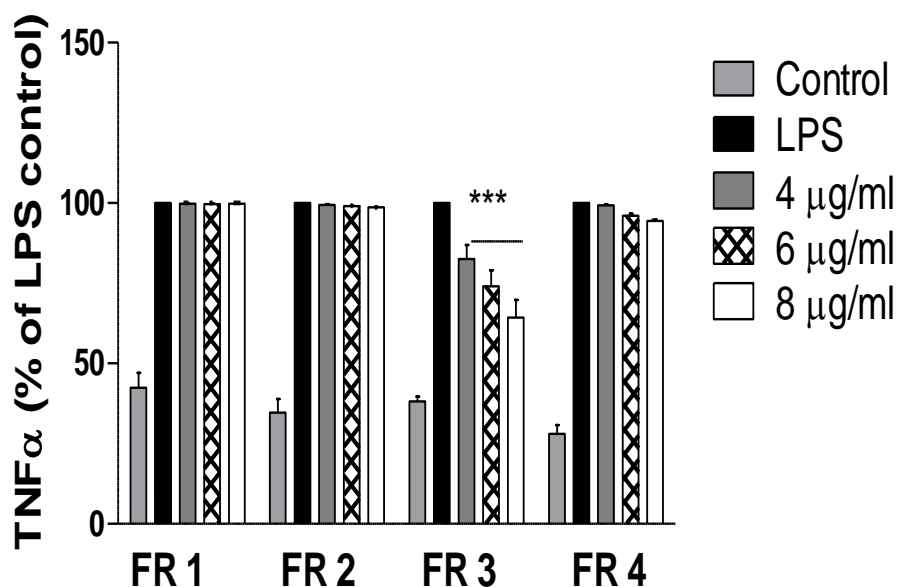
Figure 3-3: Effect of fraction 1, 2, 3 and 4 on TNFα in LPS activated BV2 microglia

ELISA (TNFα) experiment was done on all the fractions. The result showed that fraction 3 significantly reduced TNFα cytokine level. This was done to guide and determine the fraction with biological that activity before further purification. Cells were treated with or without fraction 1, 2, 3, 4 and stimulated with LPS (100 ng/ml) for 24 hours. At the end of the incubation period, supernatants were collected for ELISA measurements. Data are expressed as mean ±SEM for 3 passages. Analysis was done using one-way ANOVA with post-hoc Student Newman-Keuls test (***p<0.001) in comparison with LPS control.

3.4.2 Fractions (1, 2, 3 and 4) from *Z. zanthoxyloides* extract did not affect viability of BV2 cells

The viability of BV2 cells pre-treated with the fractions (FR 1, FR 2, FR 3 and FR 4) isolated from *Z. zanthoxyloides* extract were investigated. Results showed that there was no significant difference in viability of cells treated with FR 1, FR 2, FR 3 and FR 4 in LPS-stimulated cells when compared with untreated cells.
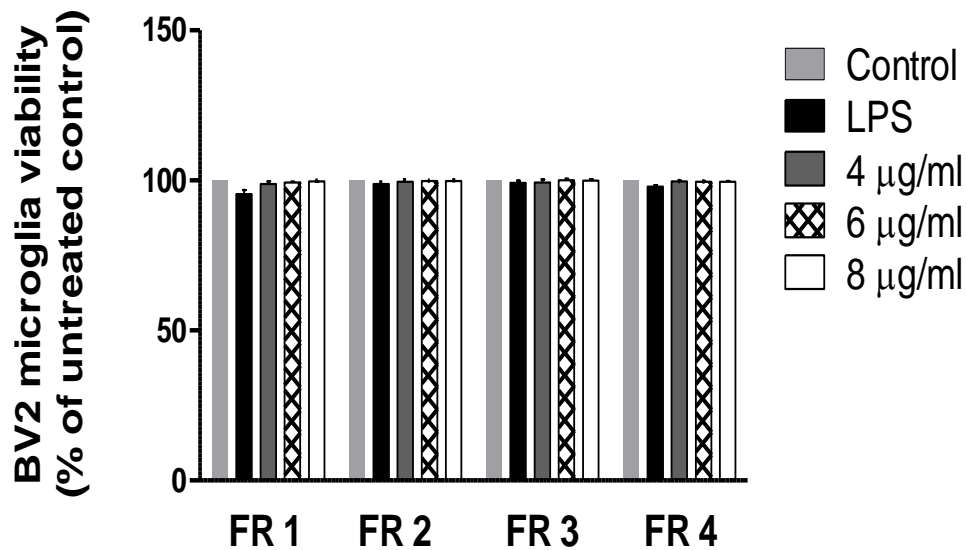
Figure 3-4: Effect of fraction 1, 2, 3 and 4 on BV2 cell viability

Pre-treatment with fraction 1, 2, 3 and 4 did not affect the viability of BV2 microglia stimulated with LPS. Cells were stimulated LPS (100 ng/ml) in the presence or absence of all the fractions for 24 h. At the end of the incubation period, MTT assay was carried out on cells. All values are expressed as mean ± SEM for 3 independent experiments. Statistical analysis was performed using one-way ANOVA with post-hoc Student Newman-Keuls test relative to untreated control.

3.4.3 Structure elucidation of compound isolated from *Z. zanthoxyloides* extract

Investigations on effect of fraction 1, 2, 3 and 4 (isolated from *Z. zanthoxyloides* extract) on elevated TNFα level in stimulated microglia cells showed that fraction 3 significantly inhibited TNFα produced from activated microglia cells (as shown in Figure 3-3). Consequently, fraction 3 was further separated and purified by HPLC technique leading to isolation of a main compound from fraction 3. NMR spectroscopy have been used in previous studies for structural elucidation of unknown compounds from plants extracts and their isolates (Akitt & Mann, 2017) . Structure of isolated compound from fraction 3 was elucidated by NMR spectroscopy. In Figure 3-5, proton (^{1}H) NMR spectrum of isolated compound shows a pair of doublets at $δ$ 7.28 and $δ$ 7.75 (each 1H, J = 2.6 Hz), characteristic of the H-1' and H-2' furan ring protons, and a downfield 3H singlet at $δ$ 4.39, characteristic of the methoxy group at C-4 in the structure of isolated compound. Another pair of doublets at $δ$ 8.05 and $δ$ 7.36 (each 1H, J = 9.4 Hz), characteristic of H-5 and H-6 of the quinolone ring respectively. The signals of two other methoxy group at $δ$ 3.92 and $δ$ 3.87 corresponding to 7-OCH_3- and 8-OCH_3. The carbon (^{13}C) NMR spectrum of isolated compound in Figure 3-614 shows signals corresponding to three methoxyl groups at $δ$ 58.4 (4-OCH_3), 55.8 (7-OCH_3 and 60.3 (8-OCH_3); four methines and seven quaternary carbons. The NMR results showed that the structure of isolated compound from fraction 3 is skimmianine with a molecular formula, $C_{14}H_{13}NO_4$. Furthermore, HR-MS mass spectrum was used to confirm and determine the molecular weight of skimmianine. Result in

Figure *3-7* shows the pseudomolecular ion peak at m/z 260 [M+H$^+$] - corresponding to the molecular formula, $C_{14}H_{13}NO_4$.
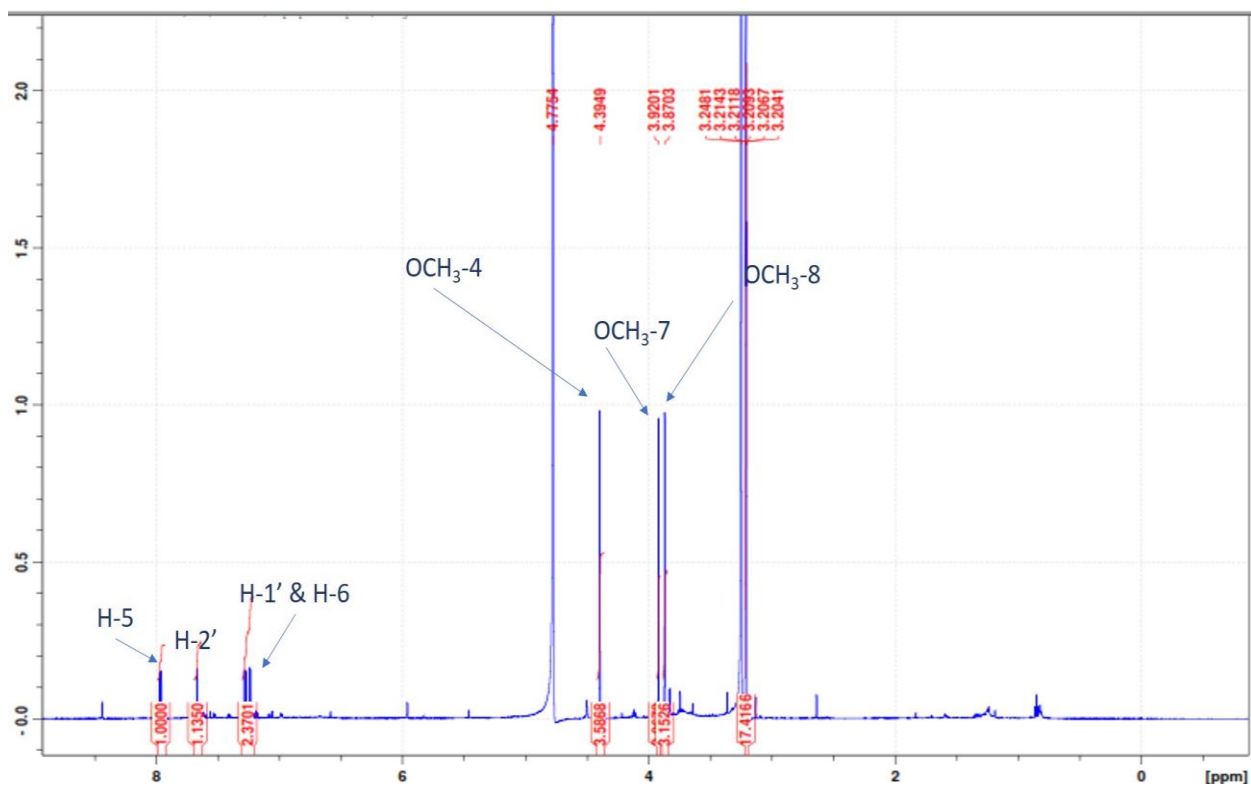
Figure 3-5: Proton NMR of skimmianine
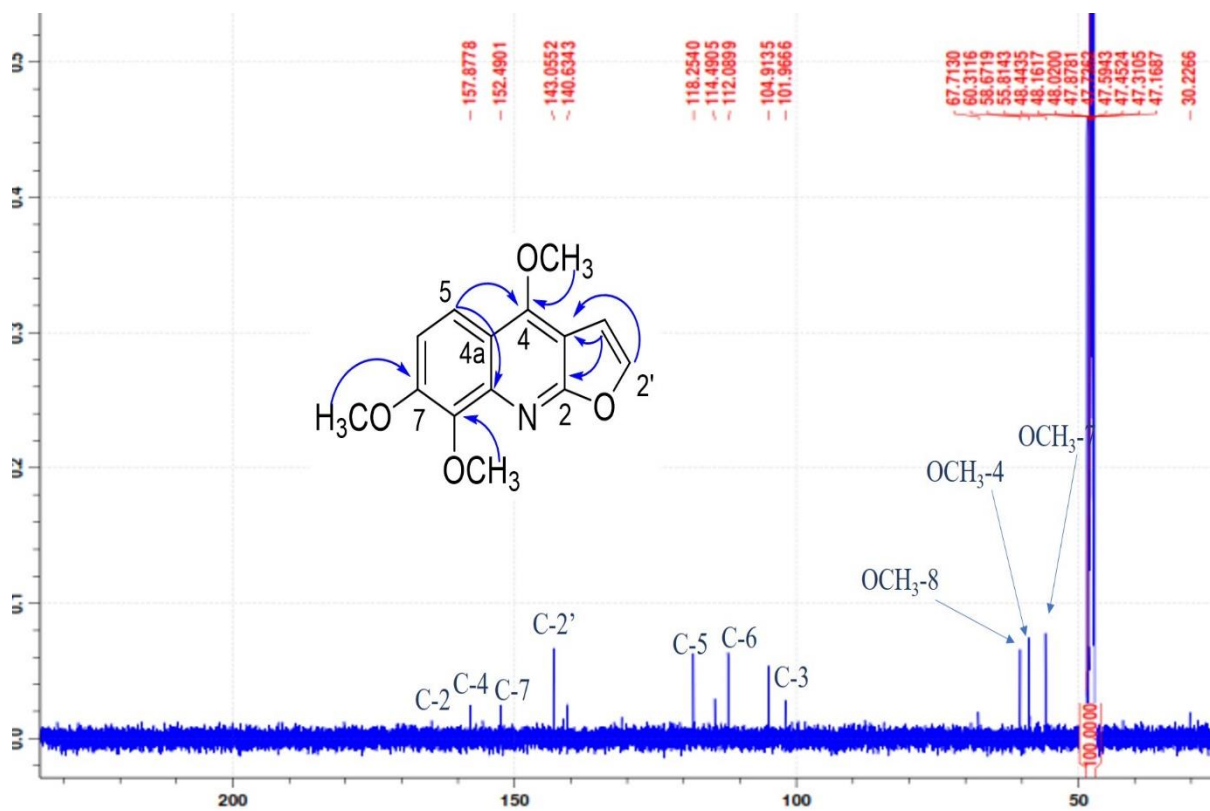
Figure 3-6: Carbon NMR of skimmianine
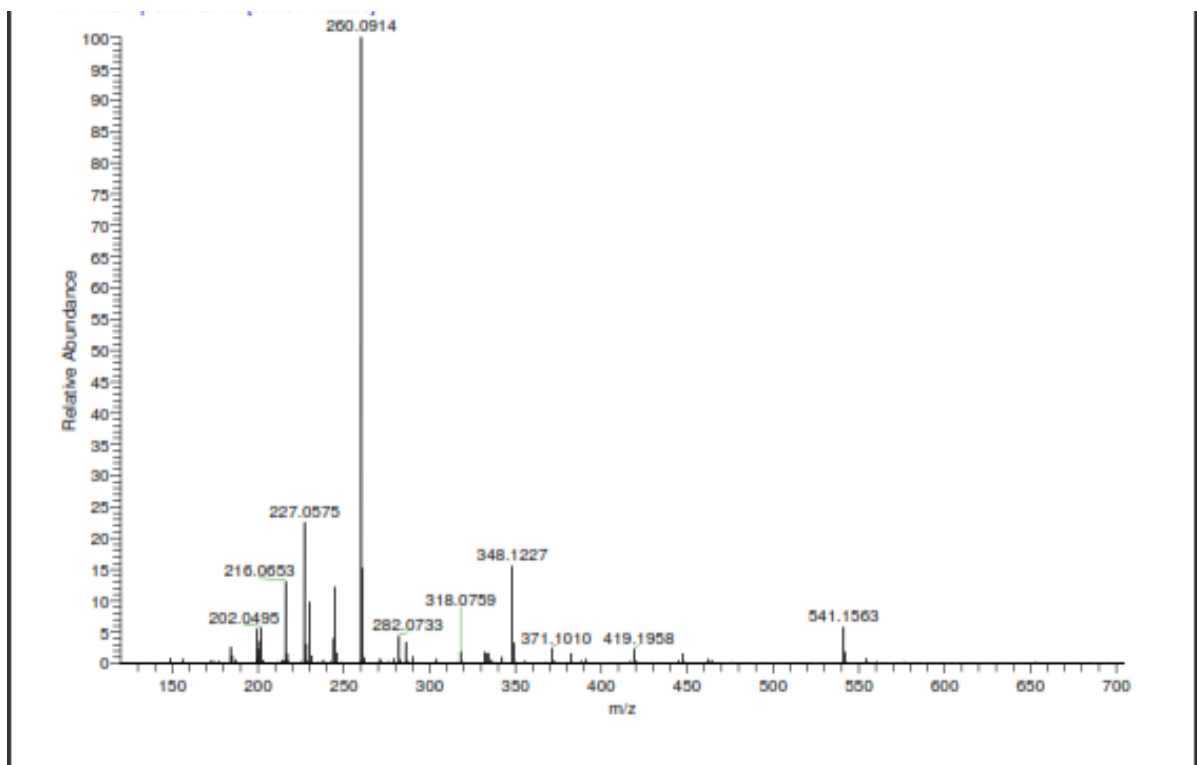
Figure 3-7: Mass spectra of skimmianine
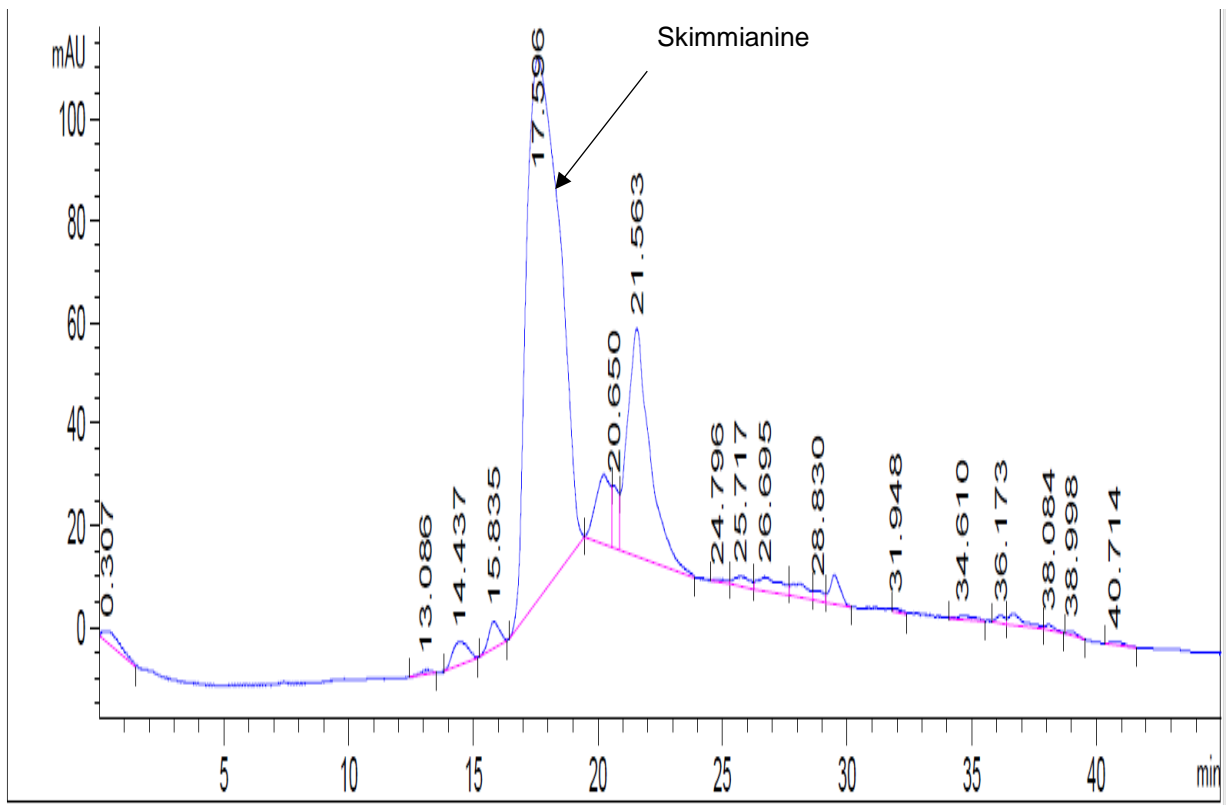
Figure 3-8: Chromatogram of fraction 3 (Preparative HPLC)

3.4.3.1 Skimmianine reduced TNFα, IL-1β and IL-6 production and increased IL-10 secretion in LPS activated BV2 microglia

Excessive secretion of pro-inflammatory cytokines (TNFα, IL-1β and IL-6) have been reported to contribute to neuroinflammation in macrophage cells stimulated with stimuli such as LPS (Turner, Nedjai, Hurst, & Pennington, 2014). Therefore, inhibition of pro-inflammatory cytokines is important and may result in reducing neuroinflammation.

Effect of skimmianine on production of TNFα, IL-1β, IL-6 and IL-10 in LPS-induced BV2 microglia cells were investigated. Result showed that LPS-induced a significant increase ($p < 0.001$) in the production of TNFα (~14.8-fold) when compared to untreated cells. However, pre-treatment with skimmianine at 10 µM (~1.2-fold) reduced TNFα production significantly ($p < 0.01$) in comparison with LPS control. In addition, there was a further significant reduction ($p < 0.001$) in the level of TNFα released at 20 µM, (~1.5-fold reduction) and 30 µM (~2-fold decrease). Furthermore, there was a significant elevation in degree production of IL-6 (~11.2-fold increase) in LPS-stimulated BV2 cells with in comparison to untreated cells. In contrast, pre-treatment with skimmianine at 20 µM showed a significant decrease ($p < 0.05$) in the release of IL-6 (66.6% reduction) while at 30 µM (44.6% decrease) of this compound, there was a further decrease ($p < 0.001$) in comparison to LPS treated cells. Effect of skimmianine at 10 µM was insignificant on production of IL-6 secreted in LPS-stimulated BV2 cells. Moreover, there was a significant increase ($p < 0.001$) in the secretion of level of IL-1β (~1.6-fold increase) in LPS-stimulated BV2 cells. On the other hand, skimmianine (10 µM) significantly inhibited ($p < 0.01$) the level of production of IL-1β (~ 1.2-fold) in LPS-stimulated BV2 cells in relation to LPS stimulated cells alone. A further significant inhibition ($p < 0.001$) was observed at 20 µM (~1.4-fold) and 30 µM (~1.5-fold) of skimmianine.

The effect of skimmianine on the level of IL-10 secreted in LPS induced neuroinflammation were investigated. Result showed a significant decrease ($p < 0.001$) in the production of IL-10 (~0.6-fold decrease) in LPS-stimulated BV2 cells when compared to unstimulated cells. However, skimmianine at 10 µM significantly increased ($p < 0.01$) the secretion of IL-10 (~0.8-fold decrease) in LPS-induced BV2 cells in comparison to LPS control. Moreover, skimmianine exerted a further increase ($p < 0.001$) in the level of IL-10 produced at 20 µM and 30 µM. Collectively, these results show that skimmianine at 10, 20 and 30 µM reduced neuroinflammation by inhibiting the levels of pro-inflammatory cytokines (TNFα, IL-1β and IL-6) secretion and increasing the degree of IL-10 production in BV2 microglia cells stimulated with LPS.
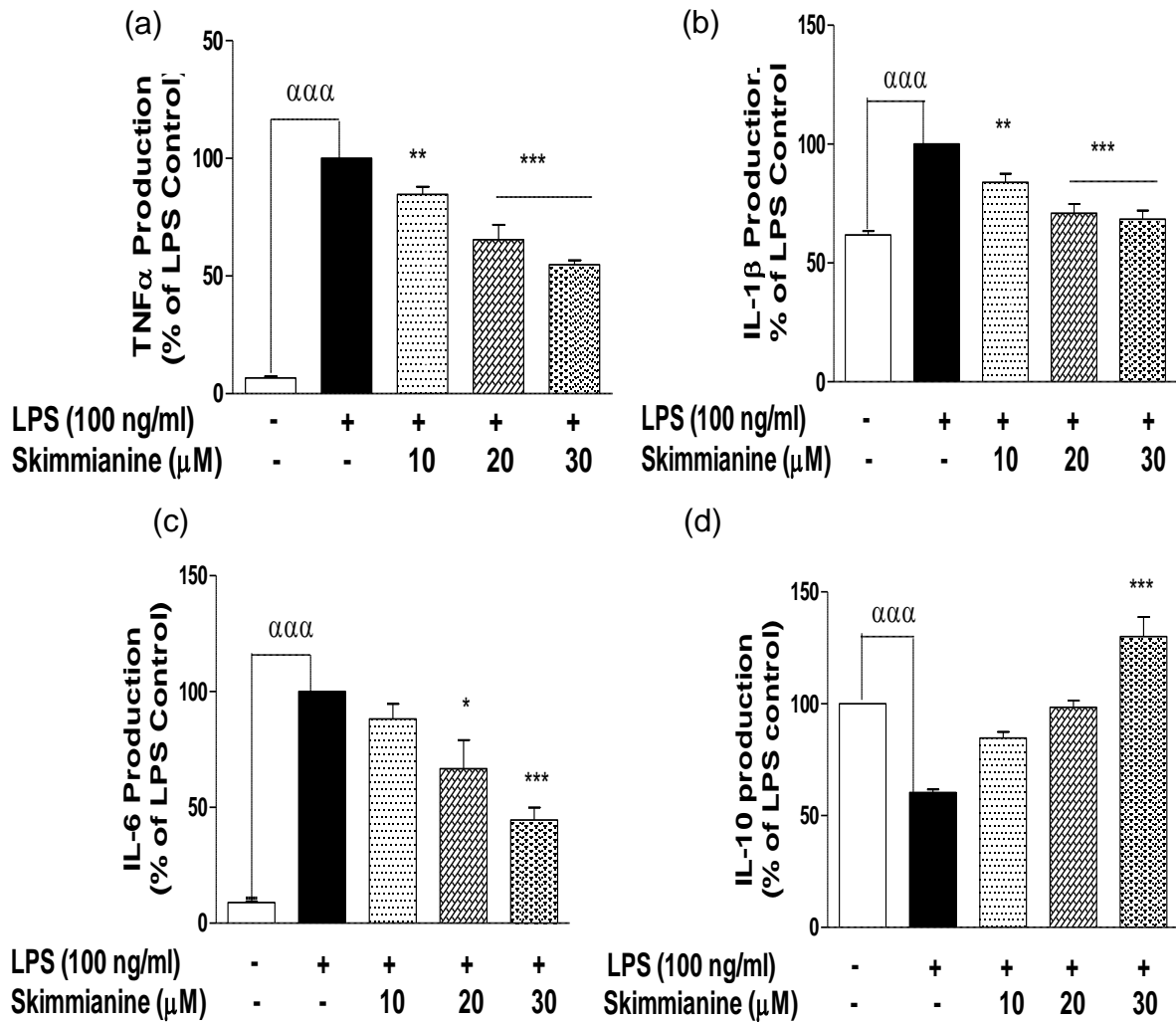
Figure 3.3-9: Skimmianine decreased TNFα, IL-1β and IL-6 production and increased IL-10 secretion in BV2 microglia cells stimulated with LPS

BV2 cells were treated with or without skimmianine (10, 20 and 30 µM) and then, stimulated with LPS (100 ng/ml) for 24 hours. Afterwards, cytokine levels of (a) TNFα, (b) IL-1β, (c) IL-6 and (d) IL-10 were analysed in the cell culture supernatants using ELISA technique. Three independents experiments were done and values obtained were calculated as mean ± SEM. Statistical analysis was done by post-hoc Student Newman-Keuls test with one-way ANOVA (multiple comparisons $^{\alpha\alpha\alpha}$ $p<0.001$ in comparison with the unstimulated cells and *$p<0.05$ **$p<0.01$, ***$p<0.001$) relative to LPS control.

3.4.3.2 Skimmianine reduced nitrite production and iNOS protein levels in LPS-activated BV2 microglia

Studies have shown an increased production of NO through iNOS from activated microglia cells in neuroinflammation (Asiimwe et al., 2016). In addition, excessive generation of NO and iNOS in activated cells have been shown to cause neurotoxicity and neuroinflammation (Yuste et al., 2015). Hence, inhibition of secreted NO and iNOS from activated microglia cells reduces their excessive production level in neuroinflammation. Effect of skimmianine on the levels of nitric oxide (NO) production in LPS activated BV2 cells were determined. The outcome of this study showed a significant increase ($p<0.001$) in the levels of NO (~16.3-fold increase) released from BV2 cells stimulated with LPS in comparison to untreated BV2 cells. Pre-treatment with skimmianine significantly reduced ($p<0.001$) the level of NO produced at a concentration of 10 µM (~1.4-fold decrease) in LPS activated BV2 cells when compared to LPS stimulated cells alone. A further significant reduction ($p<0.001$) was detected on treatment of LPS stimulated BV2 cells with skimmianine at 20 µM (~1.4-fold) and 30 µM (~1.8-fold). Following the inhibitory effect of skimmianine on NO, there was a further investigation on the effect of skimmianine on expression of iNOS protein using immunoblotting method. A marked increased ($p<0.001$) level of iNOS expression (~3.2-fold increase) was observed on treatment with LPS when compared to untreated cells. However, pre-treatment with skimmianine significantly inhibited ($p<0.01$) expression of iNOS at 10 µM (16% reduction) in LPS activated BV2 cells in relation to LPS induced cells alone. In addition, further significant inhibition ($p<0.001$) were observed at 20 and 30 µM of skimmianine (40.7% and 62.4% decrease respectively) when compared to BV2 cells treated with LPS only. These results

suggest the inhibitory role of skimmianine on increased level of NO and iNOS protein expression in BV2 cells stimulated with LPS.
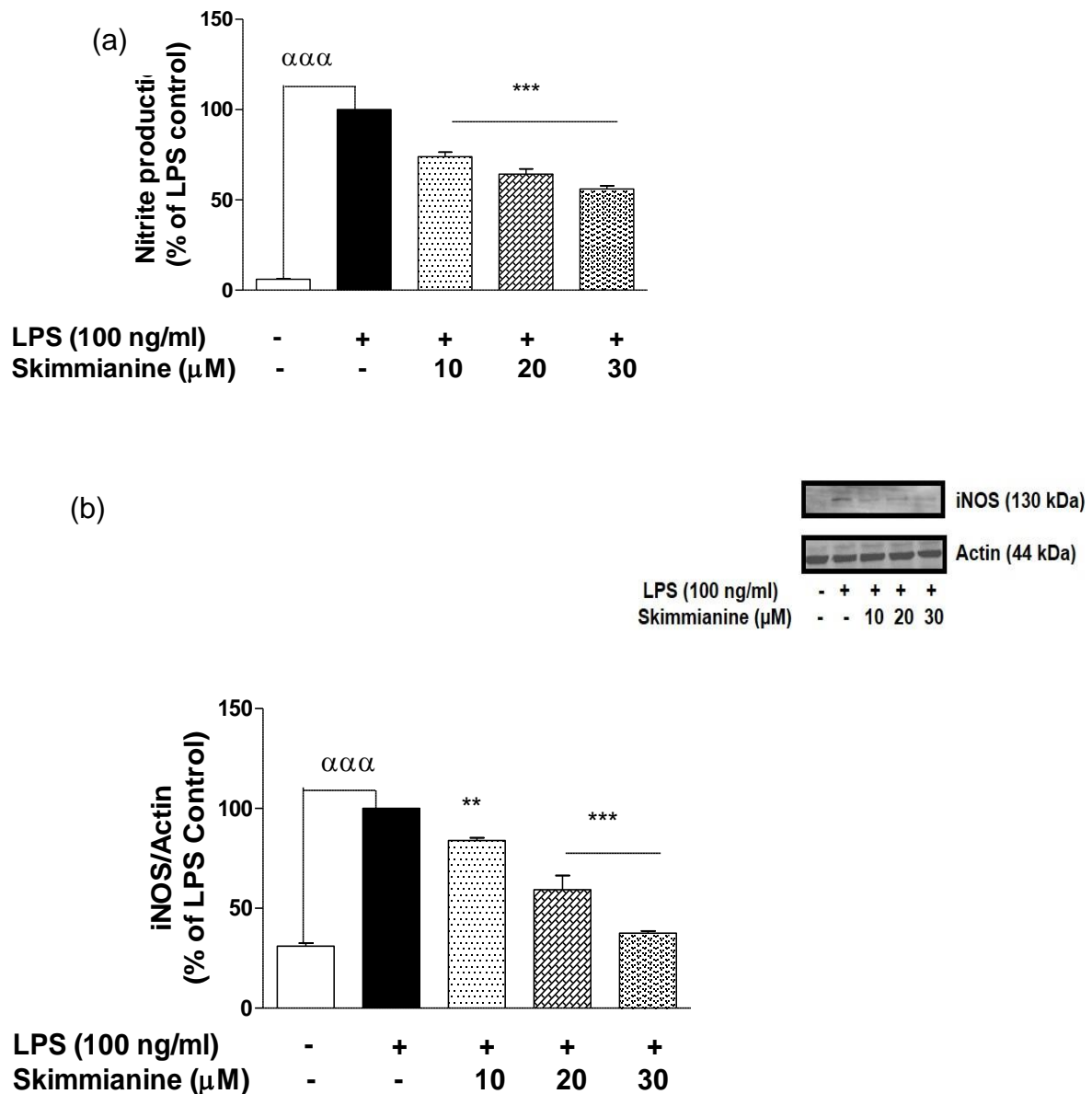
Figure 3.3-10: Skimmianine suppressed nitric oxide (NO) release (a) and inducible nitric oxide (iNOS) protein expression (b) in cultured BV2 cells activated with LPS

Skimmianine (10, 20 and 30 μM) was used to pre-treat BV2 cells for 30 minutes and then stimulated with LPS (100 ng/ml) for 24 hours. Subsequently, cell culture supernatants and cytoplasmic extract were collected and analysed for production of NO and protein expression of iNOS. Skimmianine inhibited nitric oxide (NO) production (a) through inhibiting inducible nitric oxide (iNOS) protein expression (b) in BV2 cells stimulated with LPS. Values are expressed as mean ±SEM for 3 separate experiments. Analysis was done using one-way ANOVA with post-hoc Student Newman-Keuls test ($^{\alpha\alpha\alpha}$ $p<0.001$ in comparison with the untreated cells and (**$p<0.01$,***$p<0.001$) in relation with LPS control.

3.4.3.3 Skimmianine reduced PGE$_2$ production and inhibited COX-2 protein expression in LPS activated BV2 microglia

Reports have shown the involvement of PGE$_2$/ COX-2 pathway in activated microglia cells to induce neuroinflammation in neurodegenerative diseases (Sil & Ghosh, 2016; Weiwer, 2018). As a result, inhibition of secreted PGE$_2$ level through reducing expression of COX-2 protein in activated microglia cells is crucial in regulating neuroinflammatory responses. Effect of skimmianine on production of PGE$_2$ and COX-2 expression in LPS stimulated BV2 cells were investigated in this study.

Result shows that in BV2 cells, LPS induced a significant increase (p<0.001) in the level of PGE$_2$ produced (~5.2-fold increase) relative to untreated cells. However, there was a marked reduction (p<0.01) on treatment of LPS stimulated BV2 cells with skimmianine at 10 μM (~ 1.2-fold decrease) in comparison to LPS stimulated cells alone. A further significant decrease (p<0.001) was observed at 20 (~1.5-fold) and 30 μM (~2-fold reduction) of this compound. Afterwards, immunoblotting experiments were done on COX-2 protein in explaining the mechanism by which PGE$_2$ were secreted. Observation from this result revealed a significant increase (p<0.001) in expression of COX-2(~3.8-fold). On the other hand, skimmianine at 10 μM inhibited COX-2 significantly (p<0.01) (16% reduction), while 20 and 30 μM of skimmianine showed a further significant inhibition (p<0.001) of COX-2 expression (38% and 62% decrease respectively), in relation to LPS control. Observed results indicates that skimmianine decreased of PGE$_2$ production by suppressing the expression of COX-2 protein in LPS stimulated BV2 microglia cells.
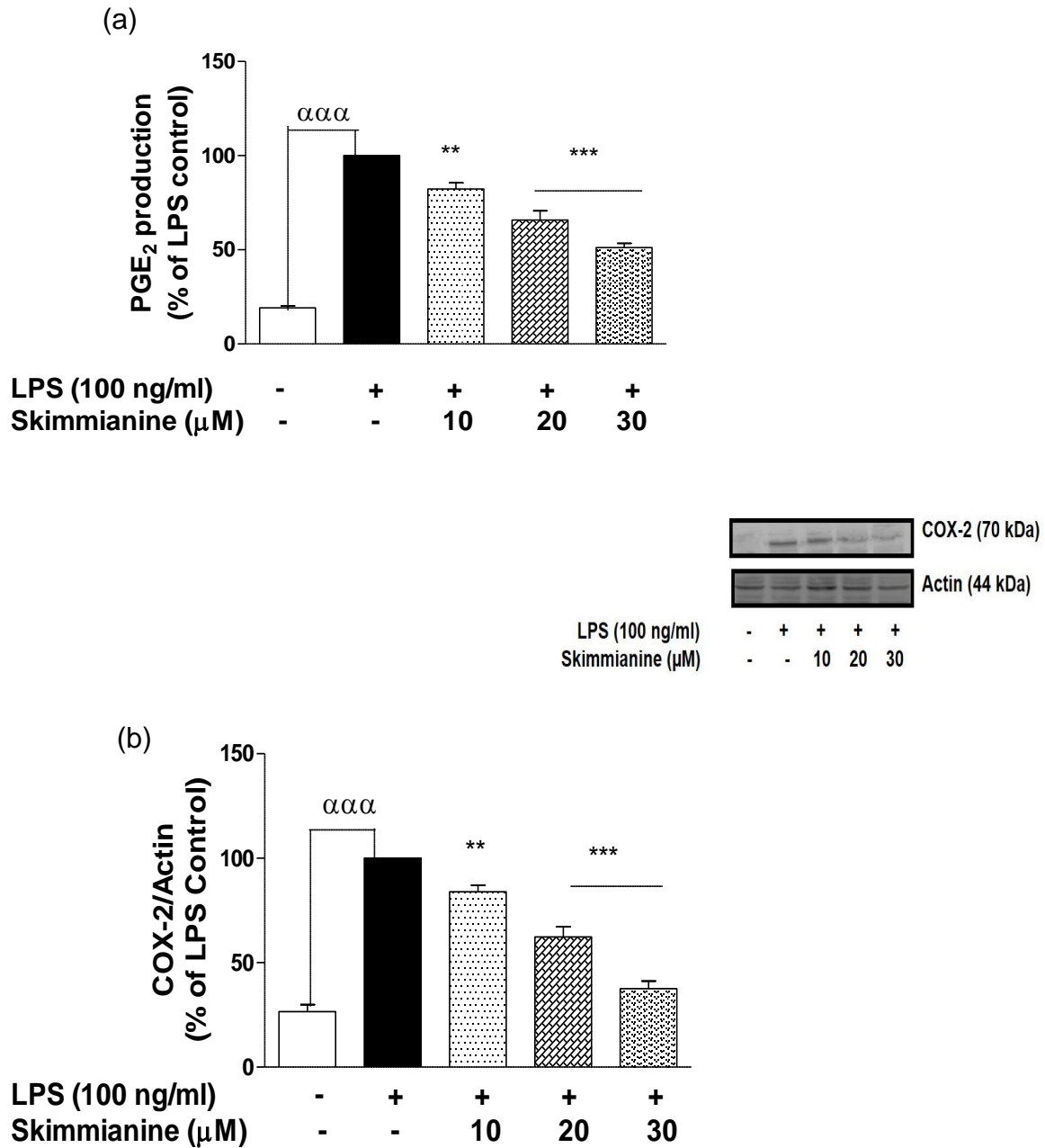
Figure 3.3-11: Skimmianine attenuated PGE2 production and COX-2 protein expression in LPS activated BV2 microglia

Cultured microglia BV2 cells were incubated with or without skimmianine (10, 20 and 30 μM) for 30 minutes and subsequently stimulated with LPS (100 ng/ml) for 24 hours. Skimmianine attenuated PGE2 production (a) and COX-2 protein expression (b) in BV2 microglia. Values are calculated as mean ±SEM for 3 separate experiments Statistical analysis was done using one-way ANOVA with post-hoc Student Newman-Keuls test (multiple comparisons $\alpha\alpha\alpha$ $p<0.001$ in comparison with the untreated cells and **$p<0.01$, ***$p<0.001$) in relation to LPS control.

3.4.3.4 Skimmianine inhibited activated NF-KB signaling pathway

NF-κB has been described to play an important role to control the expression of pro-inflammatory cytokines and enzymes such as iNOS, COX-2, TNFα and IL-6 (Biswas & Bagchi, 2016). Following the inhibitory effect of skimmianine on the increased level of COX-2, iNOS, and pro-inflammatory cytokines (TNFα, IL-1β and IL-6), a luciferase reporter gene assay was used to elucidate the effect of skimmianine on NF-κB-dependent gene transcription. Result in Figure 3.3-12a shows a significant activation ($p < 0.001$) of NF-κB-driven gene transcription (~5.5-fold increase) in transfected HEK293 cells stimulated with TNFα in comparison to untreated cells. However, there was a marked reduction ($p < 0.01$) of NF-κB mediated luciferase reporter gene by skimmianine at 10 μM (~1.2-fold reduction) and a further significant inhibition ($p < 0.001$) at 20 μM (~1.4-fold decrease) and 30 μM (~2.1-fold decrease) of this compound when compared to HEK293 cells treated with TNFα. Moreover, translocation of NF-κB into the nucleus after degradation of IκB causes activation of genes with DNA binding sites for NF-κB including, genes that encodes for pro-inflammatory cytokines, iNOS and COX-2 to regulate their production(Biswas & Bagchi, 2016; Feng et al., 2017). Hence, inhibiting the binding of NF-κB to DNA is important in reducing transactivation of NF-κB. The effect of skimmianine on the DNA-binding activity of NF-κB was further elucidated in BV2 microglia stimulated by LPS. Investigation in Figure 3.3-12b indicates an increase in the DNA binding of NF-κB (~2.2-fold increase) significantly ($p < 0.001$) in comparison to unstimulated BV2 cells. Conversely, skimmianine at 20 μM significantly decreased ($p < 0.01$) the DNA binding of NF-κB (21% decrease) while there was a further marked reduction ($p < 0.001$) at 30 μM of this compound (42% reduction) in relation to LPS control BV2 cells.
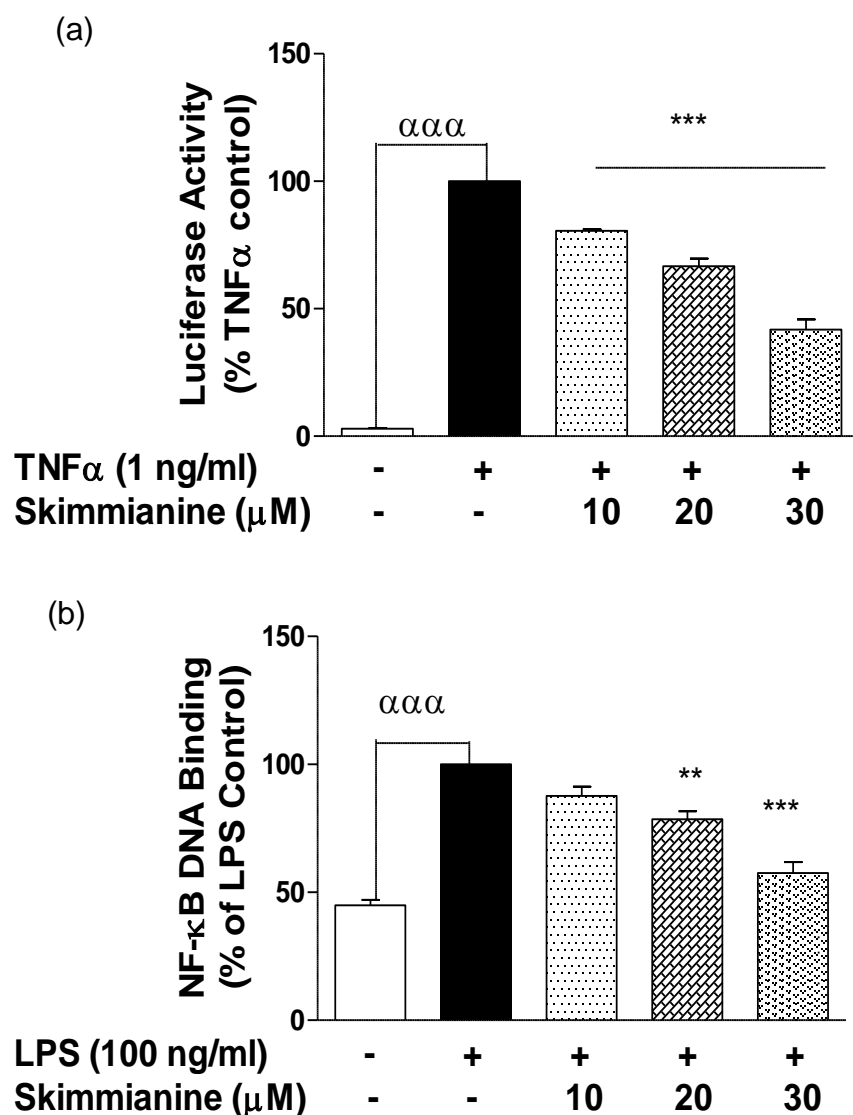
Figure 3.3-12: Skimmianine inhibited neuroinflammation through NF-κB signaling pathway

(A) Skimmianine significantly inhibited activity of NF-κB in transfected HEK293 cells induced by TNFα. Transfected HEK293 cells were stimulated with TNFα (1ng/ml) in presence or absence of skimmianine (10, 20 and 30 µM) for 6 hours. (B) Skimmianine at 20 and 30 µM decreased DNA binding activity of NFκB in LPS stimulated BV2 microglia cells. Subsequently, luminescence was measured. Nuclear cell lysates were added to wells pre-coated with NFκB oligonucleotide, followed by addition of NFκB p65 antibody and secondary antibody. Thereafter, absorbance was measured on a microplate reader. Analysis of data was done on 3 independent experiments using one-way ANOVA with post-hoc Student Newman-Keuls test (multiple comparisons ^{ααα} $p < 0.001$ in relation to untreated cells and **$p < 0.01$, ***$p < 0.001$) in comparison with TNFα control and LPS control.

3.4.3.5 Skimmianine inhibits nuclear translocation of NF-κB p65, phosphorylation and degradation of IκB in LPS-activated BV2 cells

Based on the inhibitory effect of skimmianine on transcriptional activity of NF-κB, investigation was further done to elucidate whether anti-neuroinflammatory effect of skimmianine was mediated by interfering with the NF-κB signalling pathway. In the cytoplasm, inactive NF-κB remains bound to the inhibitor of κB (IκB) until an inflammatory stimulus such as LPS causes an activation to phosphorylate IκB kinase (IKK). Subsequently, IKK phosphorylates IκB leading to dissociation and translocation of active NF-κB-p65 subunit into the nucleus to initiate the transcription of inflammatory genes including iNOS and COX-2. Also, proteasomal degradation of IκBα occurred in the cytoplasm.

Firstly, western blot experiments were done to elucidate the effect of skimmianine on IKK and IκB phosphorylation in LPS-activated microglia cells. On stimulation of BV2 cells with LPS, result in Figure 3.3-13a reveal a significant ($p<0.001$) increase in the level of IKK phosphorylation (~5-fold increase) in comparison to unstimulated cells. However, pre-treatment with skimmianine at 10, 20 and 30 μM did not significantly reduce the increased level of IKK phosphorylation when compared to LPS control. Furthermore, results inFigure 3.3-13b shows a significant increase ($p<0.001$) in the level of IκBα phosphorylation (~2.8-fold increase) was observed on stimulation of BV2 cells with LPS in relation to untreated cells. In addition, there was a marked level ($p<0.001$) of total IκB degradation (~1.8-fold decrease) in BV2 cells induced by LPS when compared to unstimulated cells. On the other hand, there was a marked reduction ($p<0.05$) in the phosphorylation of IκBα (1.1-fold decrease) on treatment of BV2 cells with 10 μM of skimmianine. In addition, skimmianine at 20 μM (~1.3-fold) and 30 μM (~1.8-fold) inhibited phosphorylation of IκB significantly ($p<0.001$) in

comparison to LPS stimulated cells. Moreover, Figure 3.3-13c shows that skimmianine at 10, 20 and 30 μM increased the level of total IκB (88%, 91% and 94% respectively) in LPS-stimulated BV2 cells when compared to untreated cells. Observations from the inhibitory activity of skimmianine on phosphorylation and degradation of IκBα led to further studies in determining the effect of skimmianine on translocation of NF-κB-p65 subunit into the nucleus. Cultured BV2 cells were treated with skimmianine for 30 minutes and followed by stimulation with LPS (100 ng/ml) for 1 hour. Figure 3.3-13d shows results from immunoblotting experiments and revealed a significant increase in the level ($p < 0.001$) of NF-κB p65 phosphorylation (~4-fold increase) in LPS stimulated BV2 cells when compared to unstimulated cells. However, the levels of phosphorylated p65 were markedly inhibited ($p < 0.01$) on pre-treatment with skimmianine 10 μM (15.9% reduction) in relation to LPS treated cells. Additionally, skimmianine showed a marked inhibition of phosphorylated p65 ($p < 0.001$) at 20 and 30 μM (37% and 60.1% decrease respectively) when compared to untreated cells. Afterwards, immunofluorescence analysis was performed to further investigate the effect of skimmianine on levels of phosphorylated p65 in BV2 cells. Results showed an increase in the expression of NF-κB p65 in LPS induced BV2 cells when compared to untreated cells. In contrast, skimmianine (10, 20 and 30 μM) reduced the expression levels of phosphorylated p65 in a concentration-dependent manner.
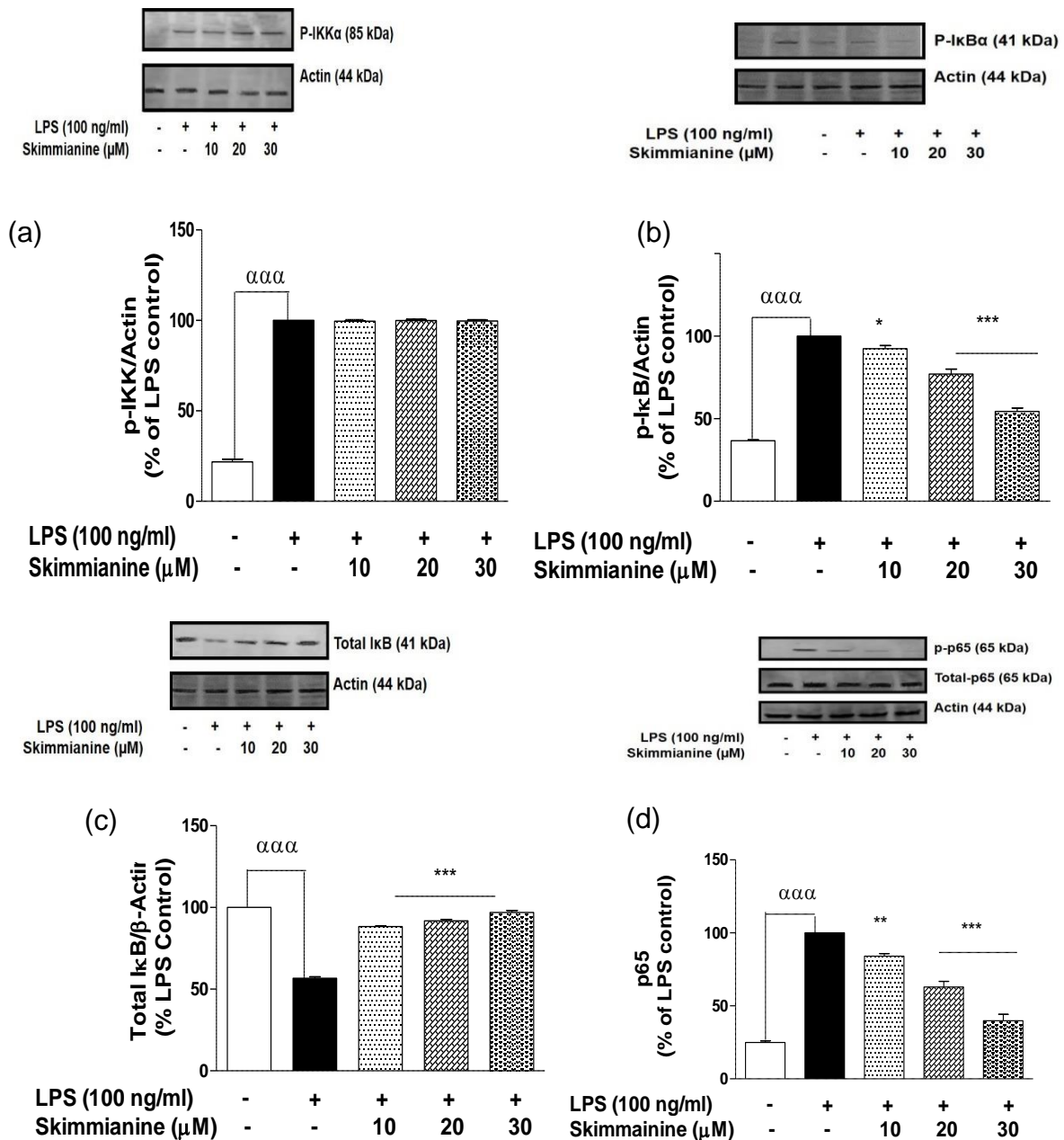
Figure 3.3-13: Skimmianine inhibits IκB phosphorylation, degradation and nuclear translocation of NF-ΚB p65 in BV2 cells activated with LPS

(a) Skimmianine did not significantly reduce phosphorylation of IKK. In contrast, skimmianine significantly inhibited (b) phosphorylation of IκB, (c) degradation of IKB and (d) translocation of NF-KB p65 subunit in LPS activated BV2 cells. Cultured BV2 cells were stimulated with LPS (100 ng/ml) in the presence or absence of skimmianine (10, 20 and 30 µM)) for 1 hour. At the end of the incubation, cytoplasmic or nuclear extract was collected and western blot was performed to analyse p65 subunit and IκB protein expression. Analysis was done using one-way ANOVA with post hoc Student Newman–Keuls test (multiple comparisons, ^{ααα} $p<0.001$ in comparison with the untreated cells and **$p<0.01$ ***$p<0.001$ in comparison with LPS control.

Figure 3-3-14: Effect of skimmianine on p65 NF-κB protein expression in LPS activated microglia

Immunofluorescence experiments was done to evaluate the effects of skimmianine (10, 20 and 30 μM) on p-p65 NF-κB protein expression in LPS activated microglia at 1 hour. Activated microglia cells were labelled with p-p65 antibody and counterstained with DAPI. Images were acquired using EVOS® Floid ® cell imaging and analysed using image J

3.4.3.6 Skimmianine reduced neuroinflammation-mediated neurotoxicity in HT22 hippocampal neuronal cells

Numerous studies have associated neuroinflammation as a result of activated microglia cells activation with the pathogenesis of neurodegenerative diseases (Bronzuoli, Rosanna, Iacomino, Steardo, & Scuderi, 2016). In addition, excessive production of pro-inflammatory cytokines from activated microglia cells have been reported to cause neurotoxicity and neuronal death (Schain et al., 2017). Considering the inhibitory effect of skimmianine on LPS-activated neuroinflammation, further study was done to investigate the neuroprotective effect of skimmianine on HT22 cells induced by conditioned medium from LPS-induced BV2 microglia cells. BV2 cells were treated with skimmianine (10, 20 and 30 µM) for 30 minutes and subsequently stimulated with LPS (1µg/ml) for 24 hours. Afterwards, the levels of NO and TNFα were analysed in conditioned medium from LPS-stimulated BV2 microglia cells prior to experiments on the neuroprotective effect of skimmianine. This was done to confirm LPS stimulation resulted in neuroinflammation and also to show the inhibitory effect of skimmianine on neuroinflammation before HT22 cells were exposed to the conditioned medium. Results showed a significant ($p < 0.001$) elevation in levels of NO (~4.5-fold increase) and TNFα (~4.6-fold increase) in LPS induced BV2 microglia cells in comparison to untreated cells. However, pre-treatment with skimmianine at 10, 20 and 30 µM showed a significant reduction ($p < 0.001$) in the levels of NO (20.4%, 42.1% and 62% inhibition, respectively) in comparison to LPS stimulated cells. In addition, the elevated levels of TNFα was significantly ($p < 0.01$) inhibited by skimmianine at 10 µM (~1.2-fold decrease) and a further marked reduction ($p < 0.001$) was observed at 20 (~1.5-fold) and 30 µM (~2.3-fold) of this compound when compared to LPS-activated BV2 cells.

Subsequently, investigations to assess the viability of HT22 cells (as an indication for neurotoxicity) induced by conditioned medium were determined by MTT assay. Results showed a significant ($p<0.001$) 45.4% decrease in viability of HT22 cells induced by conditioned medium from LPS stimulated BV2 cells. In contrast to this, pre-treatment with skimmianine at 10 µM in conditioned medium from BV2 significantly increased ($p<0.01$) HT22 cell viability (68.6%. increase) In addition, concentrations of skimmianine at 20 and 30 µM elevated ($p<0.001$) the viability of HT22 cells (77.5% and 85.3% increase respectively) when compared to LPS treated cells in conditioned medium. In neuroinflammation, reduced level of microtubule associated protein 2 (MAP2) have been associated with neurotoxicity (Karalija, Kelk, Wiberg, & Kingham, 2016; Kaushal, Kumar, Mehra, & Dhar, 2018). Investigation was done to show the expression of MAP2 in HT22 cells after treatment with conditioned medium from LPS-activated BV2 cells. Results from immunofluorescence assay reveals a decrease in the expression of MAP2 protein in HT22 cells treated with conditioned medium from LPS activated BV2 cells. On the other hand, skimmianine (10, 20 and 30 µM) treated BV2 cells in the conditioned medium was observed to increase the expression of MAP2 protein in HT22 hippocampal cells.
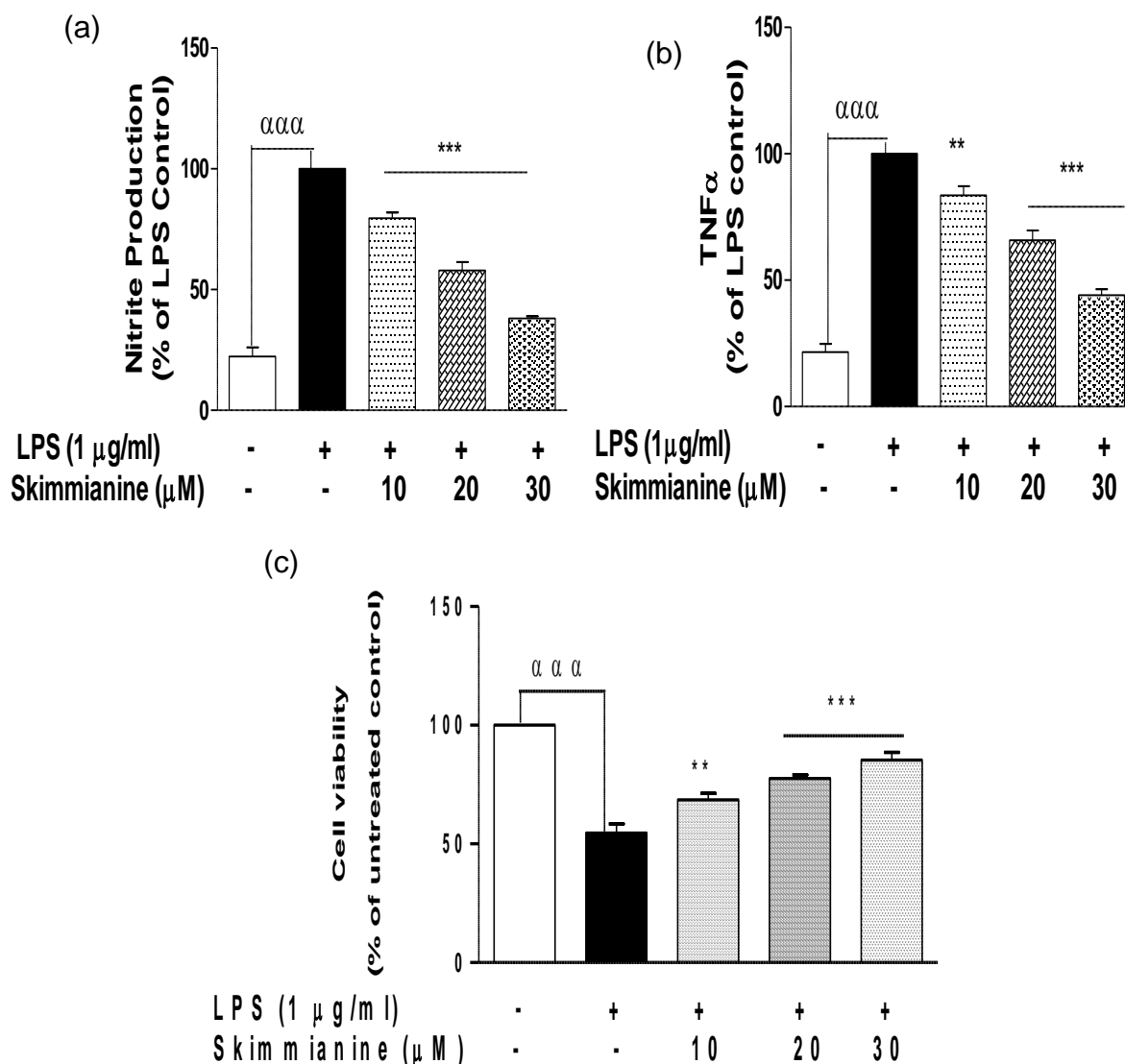
Figure 3.3-15: Skimmianine reduced neuroinflammation-mediated neurotoxicity in HT22 hippocampal neuronal cells

Cultured BV2 cells were pre-incubated with skimmianine for 30 minutes and activated with LPS (1μg/ml) for 24 hours. Subsequently, determination of the levels of (a) NO by Griess assay and (b) TNFα by ELISA technique were analysed in the conditioned medium in LPS stimulated BV2 cells. Furthermore (c) MTT assay was used to assess the viability of HT22 cells stimulated by conditioned medium from LPS activated BV2 cells. Analysis was done using one-way ANOVA with post hoc Student Newman–Keuls test (multiple comparisons, [ααα] $p < 0.001$ in comparison with the untreated cells and **$p < 0.01$ ***$p < 0.001$ in relation to LPS treated cells.
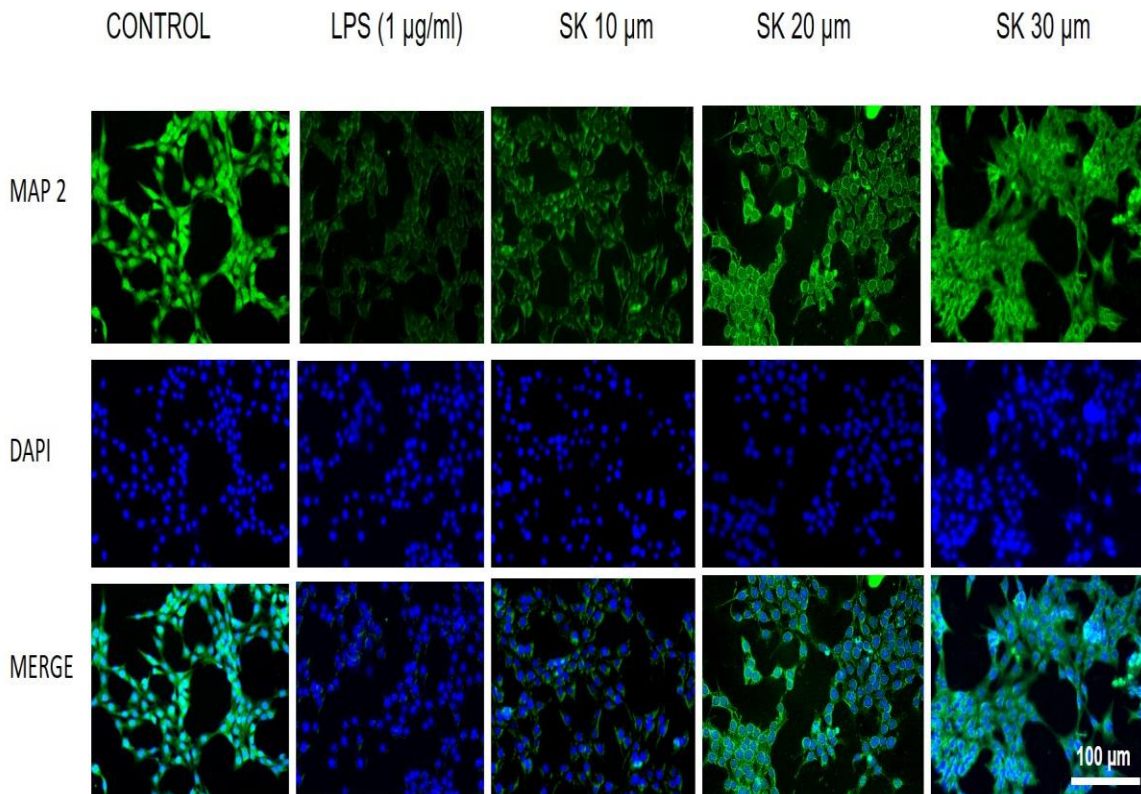
Figure 3-16: Effect of skimmianine on p65 NF-κB protein expression in LPS induced BV2 microglia

Immunofluorescence assay were performed to elucidate the effect of conditioned medium from LPS activated microglia cells on MAP2 protein in HT22 cells. Skimmianine (10, 20 and 30 μM) on p-p65 NF-κB protein expression in LPS (1 μg/ml) activated microglia at 1 hour. Activated microglia cells were labelled with p-p65 antibody and counterstained with DAPI. Images were acquired using EVOS® Floid ® cell imaging and analysed using image J

3.4.3.7 Skimmianine did not affect the viability of BV2 cells

MTT assay was used to elucidate the viability of BV2 microglia cells on pre-treatment with skimmianine (10, 20 and 30 μM) for 30 minutes and then, stimulation with LPS (100 ng/ml) for 24 hours. Observation from the result shows that skimmianine did not affect the viability of BV2 cells at the concentrations used in this experiment.
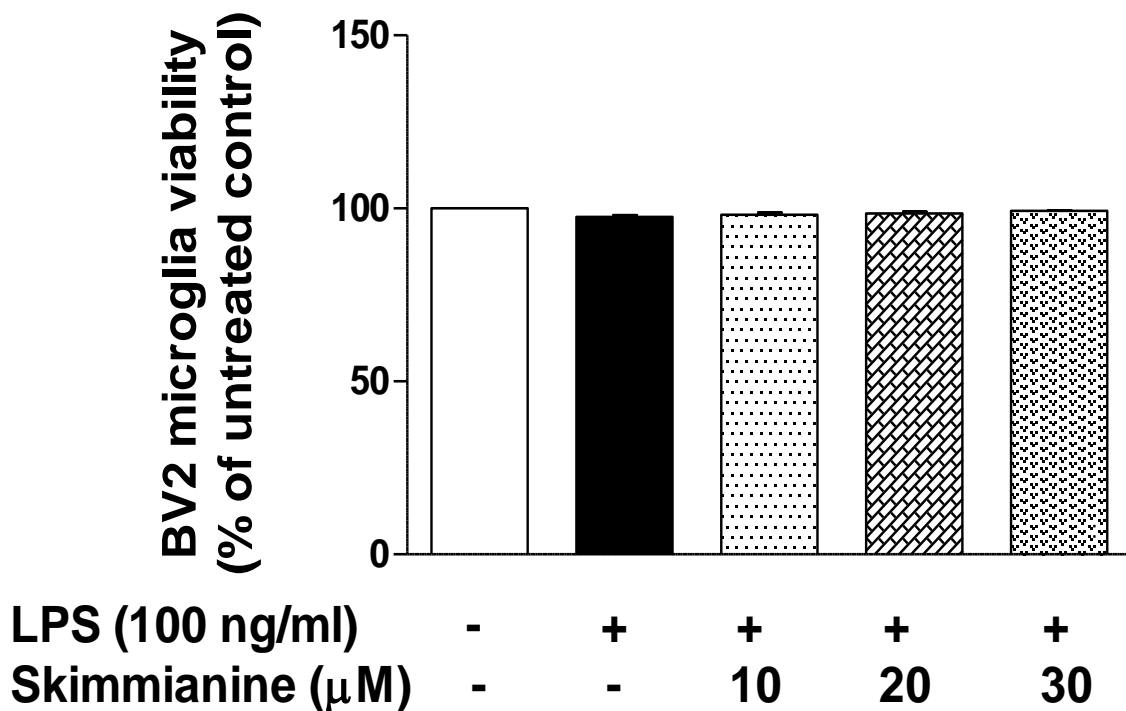
Figure 3-17: Skimmianine did not decrease the viability of BV2 microglia cells

Pre-treatment with skimmianine at concentration of 10, 20 and 30 μM did not decrease the viability of BV2 cells when stimulated with LPS (100 ng/ml) for 24 hours. MTT assay was used to determine the viability of BV2 cells after treatment. All values are expressed as mean ± SEM for 3 independent experiments. Statistical analysis was performed using one-way ANOVA with post-hoc Student Newman-Keuls test in comparison with untreated cells

3.5 Discussion

Research in the past have identified the involvement of activated microglia cells in neuroinflammation (Kempuraj et al., 2016). The role of neuroinflammation has been described to be a contributing factor to the progression of neurodegenerative diseases such as Parkinson's disease, Multiple sclerosis and Alzheimer's diseases (Chen et al., 2016; Schain et al., 2017).In neuroinflammation, studies have shown activated microglia cells to cause excessive production of pro-inflammatory cytokines, which results in neurotoxicity (Schain et al., 2017). Therefore, reducing the elevated level of pro-inflammatory cytokines in neuroinflammation is crucial in treating neuro-inflammatory related disorders. It is well-established that isolated compounds from medicinal plants are important in drug discovery and are beneficial in reducing inflammation in the CNS (Subedi, Gaire, Do, Lee, & Kim, 2016; Tomlinson & Akerele, 2015).

Results from bioassay guided fractionation of *Z. zanthoxyloides* extract showed the isolation of skimmianine from fraction 3 of *Z. zanthoxyloides* extract. Previous studies on *Z. zanthoxyloides* extract have reported the isolation of skimmianine and the proton and carbon NMR were similar to the results obtained in this current study (Adamska-Szewczyk et al., 2016; Guetchueng et al., 2018). Skimmianine is a member of furoquinoline alkaloids isolated from *Z. zanthoxyloides* extract and its isolation is important in the development of drugs for therapeutic purpose.

Investigations to determine the anti-neuroinflammatory activity of skimmianine are still unknown. In this current research, inhibitory effect of isolated skimmianine from *Z. zanthoxyloides* on neuroinflammation was elucidated in BV2 cells stimulated with LPS. Results in this study revealed that skimmianine reduced the level of significant pro-inflammatory cytokines which includes IL-6, TNFα and IL-1β secreted from LPS-

stimulated BV2 cells. In addition, a reduced level of IL-10, an anti-inflammatory cytokine was observed in BV2 cells activated with LPS. However, skimmianine elevated the level of IL-10 produced in LPS-activated microglia cells Observations from this results suggest that skimmianine inhibited neuroinflammation through reducing the secreted level of pro-inflammatory cytokines and increasing the degree of anti-inflammatory cytokine in LPS stimulated BV2 microglia cells. A previous *in vivo* study by Ratheesh et al; 2013 reported that isolated skimmianine from *Ruta graveolens* reduced the mRNA levels of IL-6 and TNFα in carrageenan induced rats (Ratheesh et al., 2013), hence supporting the outcome of this current study. The results in this study provides new evidence on the inhibitory effect of skimmianine on important pro-inflammatory cytokines (IL-6, TNFα and IL-1β) and anti-inflammatory cytokine (IL-10) using an in vitro model in BV2 microglia cells (brain resident macrophage cells).

The role of excessive production of NO and increased expression of iNOS in neuroinflammation have been reported to cause neurotoxicity and play a major role in the pathology of neurodegenerative diseases (Yuste et al., 2015). Furthermore, elevated levels of nitric oxide (NO) and inducible nitric oxide synthase (iNOS) were observed on stimulation of BV2 cells with LPS in this study. Skimmianine significantly reduced the production of NO by inhibiting the expression of iNOS enzyme in LPS-activated BV2 cells. These results indicate inhibitory effect of skimmianine on neuroinflammation by reducing the level of released NO and expressed iNOS enzyme in LPS-stimulated microglia cells. In other studies, skimmianine has been reported to inhibit the increased level of nitric oxide released in LPS-stimulated BV2 cells (Yoon et al., 2012). In addition, elevated level of NO in carrageenan induced rats was significantly inhibited by skimmianine (Ratheesh et al., 2013). Collectively, these

results suggest the inhibitory effect of skimmianine on NO production in neuroinflammation.

Moreover, skimmianine suppressed the production of PGE$_2$ by reducing the expression of COX-2 in BV2 cells stimulated with LPS. These results suggest skimmianine could suppress neuroinflammation in activated microglia cells by inhibiting COX-2 enzyme and its released PGE$_2$ mediator. Observations from these results were also reported in a study by Ratheesh et al; 2013, that skimmianine caused a significant reduction in the concentration of PGE$_2$ and activity of COX-2 in carrageenan induced rats. Hence, these studies suggest the inhibitory activity of skimmianine on neuroinflammation by reducing the level PGE$_2$ and COX-2 enzyme.

NF-κB, an essential transcription factor in neuroinflammation, has been well known to regulate the gene expression of COX-2, iNOS and genes that encodes pro-inflammatory cytokines which includes IL-6 and TNFα (Serasanambati & Chilakapati, 2016; Q. Zhang, Lenardo, & Baltimore, 2017). Investigations on the important role of NF-κB signalling pathway in neuroinflammation has made its inhibition a promising target, especially in treatment of neurodegenerative diseases. Following the result showing the inhibitory effect of skimmianine on produced pro-inflammatory cytokines, NO/iNOS and PGE$_2$/COX-2 released from activated microglia cells, a further study showing the effect of skimmianine on translocation of NF-κB p65 and DNA binding were investigated. In neuroinflammation, there is translocation of activated NF-κB into the nucleus and binding of NF-κB to the DNA of inflammatory genes to mediate the transcription of inflammatory cytokines and mediators (Biswas & Bagchi, 2016). Outcome of this study showed that skimmianine inhibited NF-κB mediated luciferase transcription in HEK293 cells stimulated with TNFα. Furthermore, skimmianine reduced the binding of NF-κB to DNA in the nucleus. These results suggest that

skimmianine inhibited neuroinflammation by reducing NF-κB-DNA binding activity and transcription of activated NF-κB in the nucleus.

As a result of the inhibitory role of skimmianine on activated NF-κB in BV2 cells, effect of skimmianine on upstream kinases of NF-κB signalling pathway were subsequently targeted. Result showed that skimmianine did not inhibit the phosphorylation of IKK. Reports have demonstrated one of the ways through which IKK inhibitors exerts its effect is by acting on adenosine triphosphate (ATP) binding site found on IKK molecule (Herrington et al., 2016). However, some IKK inhibitors have been reported not to be effective because other kinases cross react and compete with the binding site on IKK molecule (Prescott & Cook, 2018). Consequently, these reasons may explain why skimmianine could not inhibit IKK phosphorylation. Further investigation on the direct effect of skimmianine on IKK using kinase enzyme assays was not done in this study.

Afterwards, the effect of skimmianine on IκB-α and NF-κB p65 subunit was determined. Result showed that skimmianine significantly blocked p-IκB-α and NF-κB p65 subunit phosphorylation. In addition, skimmianine restored the degradation of IκB-α in LPS activated microglia cells. Taken together, these results showed that skimmianine did not exert its inhibitory effect on upstream of IκB-α but it attenuates neuroinflammation by targeting IκB-α/ NF-κB pathway in BV2 cells stimulated with LPS. These results are presented for the first time indicating the inhibitory activity of skimmianine on activated NF-κB signalling pathway in LPS-induced neuroinflammation in BV2 cells. Results from this study presents the first evidence in LPS-stimulated BV2 microglia cells suggesting that skimmianine reduces neuroinflammation by targeting IκB/ NF-κB signalling pathway but skimmianine was not effective on the upstream of IκB-α at the concentrations used in this study.

Furthermore, the neuroprotective effect of skimmianine on HT22 hippocampal neuronal cells were investigated. In neuroinflammation, the role of pro-inflammatory cytokines and mediators including NO, TNFα and reactive oxygen species (ROS) have been demonstrated to be neurotoxic. Results in this study revealed that skimmianine increased the viability of HT22 cells and also elevated the expression of MAP2 by reducing the increased levels of NO and TNFα in conditioned medium from LPS activated BV2 cells. The outcome of this study is presented for the first time suggesting the neuroprotective effect of skimmianine on HT22 cells.

In summary, results from this study showed the isolation of skimmianine from *Z. zanthoxyloides* extract and previous studies have confirmed the isolation of this compound in same extract. Moreover, anti-neuroinflammatory activity of skimmianine on pro-inflammatory cytokines and inflammatory mediators in BV2 microglia cells were investigated. Also, neuroprotective effects of skimmianine on HT22 cells were determined. Previous studies on anti-inflammatory activity of skimmianine were done using *in vivo* model. This current study was done in BV2 microglia cell line and further investigations were done on the mechanisms involving the anti-neuroinflammatory activity of skimmianine through NF-κB signalling pathway.

4 Investigations on the effect of skimmianine on neuroinflammation induced by hemozoin in BV2 microglia cells

4.1 Background of this study

Previous studies have shown an increased level of pro-inflammatory cytokines (IL-1β, IL-6 and TNFα) production on incubation of monocytes and macrophages with hemozoin (Dunst et al., 2017; Olivier et al., 2015; Perkins et al., 2011). Hemozoin is a malaria pigment derived from *Plasmodium falciparum*, a parasite that initiates the development of malaria in human through the bite of *Anopheles* mosquitoes (Ghazanfari et al., 2018; Hora et al., 2016). In addition, macrophages have been described to be the main site of cytokine secretion leading to increase in neuroinflammation observed in cerebral malaria (CM), a neurological complication of severe *Plasmodium falciparum* infections (Schofield & Grau, 2005; Urquhart, 1994). These reports suggest a role for neuroinflammation as one of the factors involved in the pathology of cerebral malaria. Studies conducted *in vitro* have also demonstrated a significant increase in production of nitric oxide (NO) in macrophage cells stimulated with hemozoin (Maritza Jaramillo, Gowda, Radzioch, & Olivier, 2003; Maneerat et al., 2000). This suggests that hemozoin contributes to the progression of inflammation by increasing the level of NO production. Research in the past have also reported that hemozoin stimulates macrophages to increase the release of IL-1β cytokine and pro-IL-1β production by activating NLRP3 and caspase-1 enzyme (Shao et al., 2018). This implies that hemozoin elevated the level of IL-1β production in inflammation through activation of NLRP3 pathway.

The role of hemozoin on brain macrophage cells has also been reported to cause an increase in pro-inflammatory factors, suggesting the effect of hemozoin in increasing

neuroinflammation in cells. A marked increase in the level of NO and pro-inflammatory cytokines (IL-1β, IL-6 and TNFα) was reported to be released by BV2 microglia cells stimulated with synthetic hemozoin (Velagapudi et al., 2019). Furthermore, previous studies have shown that hemozoin causes inflammation by stimulating TLR 2 and TLR 9 which in turn activates NF-κB signalling pathway (Bafica et al., 2006; Coban et al., 2005). This suggests the involvement of hemozoin in inflammation through activation of TLR/NF-κB signalling pathway. Moreover, an increase in phosphorylation of IκBα and p65 subunit of NF-κB was observed in human monocytes stimulated with hemozoin (M. Jaramillo et al., 2009; Olivier et al., 2015). Similarly, in hemozoin-activated BV2 cells, an elevation in the phosphorylation of NF-κB p65 subunit and DNA binding activity of NF-κB were detected (Velagapudi et al., 2019). Observation from these studies indicate that hemozoin could increase the production of inflammatory cytokines and mediators in neuroinflammation through activation of NF-κB signalling pathway in peripheral and brain macrophage cells. Therefore, inhibition of NF-κB and NLRP3 pathway in hemozoin activated microglia cells may reduce the level of inflammatory cytokines and mediators produced in neuroinflammation, an important factor involved in the pathogenesis of cerebral malaria.

So far, investigation to determine the effect of skimmianine on hemozoin-induced neuroinflammation in BV2 microglia cells is unknown. This study therefore aims to investigate the effect of skimmianine on neuroinflammation induced by hemozoin in BV2 microglia cells with these specific objectives:

1. To determine the effect of skimmianine on pro-inflammatory cytokines, NO and PGE$_2$, iNOS and COX-2 in hemozoin-stimulated BV2 microglia cells

2. To determine effects of skimmianine on NF-κB activity in BV2 microglia cells stimulated with hemozoin

3. To examine the role of skimmianine on NLRP3 inflammasome activation in hemozoin activated BV2 microglia cells

4.2   Methodology

4.2.1   Drug preparation and treatment

Skimmianine was purchased from PhytoLab GmbH & Co.KG, Germany and dissolved in DMSO to a stock concentration of 0.1M. BV2 microglia cells were treated with skimmianine (10, 20 and 30 µM) for 30 minutes and then stimulated with hemozoin (400 µg/ml) for 24 hours or 1 hour, depending on each experiment.

4.2.2   Cell culture

4.2.2.1   Culture of BV2 cells

BV2 microglia cell line ICLCATL03001 (Banca Biologica e Cell Factory, Italy) were cultured in RPMI 1640 medium (Life Technologies, UK) supplemented with 10% fetal bovine serum (Sigma), 1mM sodium pyruvate (Sigma), 100U/ml Penicillin and 100mg/ml Streptomycin (Sigma). For the purpose of experiments, the cells were sub-cultured, maintained in the incubator and seeded out in plates as described in 2.2.3.1.

4.2.2.2   MTT assay for cell viability

The viability of BV2 microglia cells pre-treated with skimmianine (10, 20 and 30 µM) and stimulated with hemozoin (400 µg/ml) were analysed by MTT assay (described in 2.2.16)

4.2.3   Measurement of Nitrite (Griess assay)

Once cultured BV2 microglia cells reached confluence, the cells were pre-treated with skimmianine (10, 20 and 30 µM) for 30 minutes and then stimulated with hemozoin (400 µg/ml) for 24 hours. After incubation, cell culture supernatants were collected in epperndorf tubes and centrifuged at 1500 rpm for 5 minutes. Griess assay as described in 2.2.5, was used to measure nitrite concentration in cell culture supernatant.

4.2.4 ELISA assay

In order to measure the levels of pro- and anti-inflammatory cytokine production in cell culture supernatants, BV2 cells were treated with skimmianine (10, 20 and 30 µM) and then, stimulated with hemozoin (400 µg/ml) for 24 hours. After incubation, levels of TNFα, IL-1β, IL-6 and IL-10 were measured in cell culture supernatants using ELISA technique as described in 2.2.6.

4.2.5 Prostaglandin E2 measurement using enzyme immunoassay (EIA)

Prostaglandin E2 levels in BV2 cell culture supernatants were measured (as described in 2.2.8) after treatment of BV2 microglia cells with skimmianine (10, 20 and 30 µM) for 30 minutes and activation with hemozoin (400 µg/ml) for 24 hours.

4.2.6 Extraction of cytoplasmic cell lysates

Prior to western blot experiments, extraction of cytoplasmic lysates (described in 2.2.9) was a step required to isolate lysates in the cytoplasm at the end of incubation of hemozoin-stimulated BV2 cells treated with skimmianine (10, 20 and 30 µM).

4.2.7 Extraction of nuclear cell lysates

Following incubation of hemozoin-stimulated BV2 cells treated with skimmianine (10, 20 and 30 µM), nuclear extraction was performed as described in 2.2.10.

4.2.8 Measurement of protein concentration

After cytoplasmic and nuclear extraction, concentrations of proteins were determined (as shown in text 2.2.11) by Pierce Coomassie (Bradford) protein assay kit (Thermo Scientific, UK) before western blot experiments.

4.2.9 Western blotting

Western blotting (as described in 2.2.12) is a technique that is useful in detection of target protein according to their molecular weights. This technique mainly

involves separation of proteins in lysates by gel electrophoresis, transfer of proteins from gel onto membrane blot and detection of target proteins based on their molecular weights. Following the determination of protein concentration in cell lysates, western blotting technique were used to investigate the effect of skimmianine on expression of iNOS, COX-2, NF-κB p65, phospho-IκB-α, IκB-α, NLRP3 and caspase-1 protein.

4.2.10 NF-κB p65 transcription factor assay

NF-κB transcription factor assay (as described in 2.2.13) is an assay that investigates the binding of NF-κB to the consensus binding site on the DNA. In this study, the effect of skimmianine (10, 20 and 30 μM) on binding of NFκB-DNA binding in hemozoin-stimulated BV2 microglia cells were determined.

4.2.11 Immunofluorescence technique

Immunofluorescence is an imaging technique (described in 2.2.14) that is important in detection of antigen by a specific antibody and this reaction is observed under fluorescence microscope. In this study, the effect of skimmianine (10, 20 and 30 μM) on expression of NF-κB p65 were investigated in hemozoin-activated BV2 microglia cells.

4.3   Results

4.3.1   Skimmianine inhibited IL-6 and TNFα production and increased IL-10 secretion in hemozoin-stimulated BV2 cells

An increase in the production of pro-inflammatory cytokines (TNFα and IL-6) has been associated with neuroinflammation causing reduced neurocognitive function in cerebral malaria. Hence, reducing the levels of pro-inflammatory cytokines is crucial in targeting hemozoin-induced neuroinflammation. In this investigation, effect of skimmianine on level of TNFα in hemozoin-stimulated BV2 microglia cells were determined. Results in **Error! Reference source not found.** shows a significant elevation ($p<0.001$) in production of TNFα (~4.4-fold-increase) in hemozoin-stimulated BV2 microglia cells when compared to untreated cells. However, skimmianine at 10 µM significantly reduced ($p<0.01$) the secretion of TNFα (74.3% decrease) in hemozoin-stimulated BV2 microglia cells when compared to hemozoin-stimulated BV2 cells. A further significant inhibition ($p<0.001$) was observed in the level of TNFα in hemozoin-activated cells treated with 20 and 30 µM of skimmianine (56.7% and 49.3% inhibition respectively), in comparison to hemozoin stimulated BV2 cells. Furthermore, hemozoin (400 µg/ml) produced a marked elevation in the level of IL-6 (~5-fold increase) in BV2 microglia cells. However, results show that skimmianine (10 µM) produced a decrease in IL-6 production (~1.2-fold reduction) in hemozoin-stimulated BV2 microglia cells when compared to hemozoin activated cells alone. Further significant reduction ($p<0.001$) was detected in hemozoin-stimulated BV2 microglia cells when treated with 20 µM and 30 µM of skimmianine (~1.4-fold and ~1.9-fold decrease respectively), in comparison with BV2 cells stimulated with hemozoin only. Moreover, the effect of skimmianine on secretion of IL-10 was investigated in hemozoin-induced BV2 cells. The outcome of the investigation shows a ~0.54-fold

reduction in IL-10 production from hemozoin stimulated BV2 cells when compared to unstimulated cells. However, skimmianine at 10 µM significantly raised (p<0.01) the level of IL-10 (~1.3-fold increase) when compared with hemozoin stimulated cells. Further significant upregulation (p<0.001) of IL-10 production were detected in hemozoin stimulated cells when treated with skimmianine at 20 and 30 µM (~1.6-fold and ~2-fold increase) in comparison to BV 2 cells activated with hemozoin.
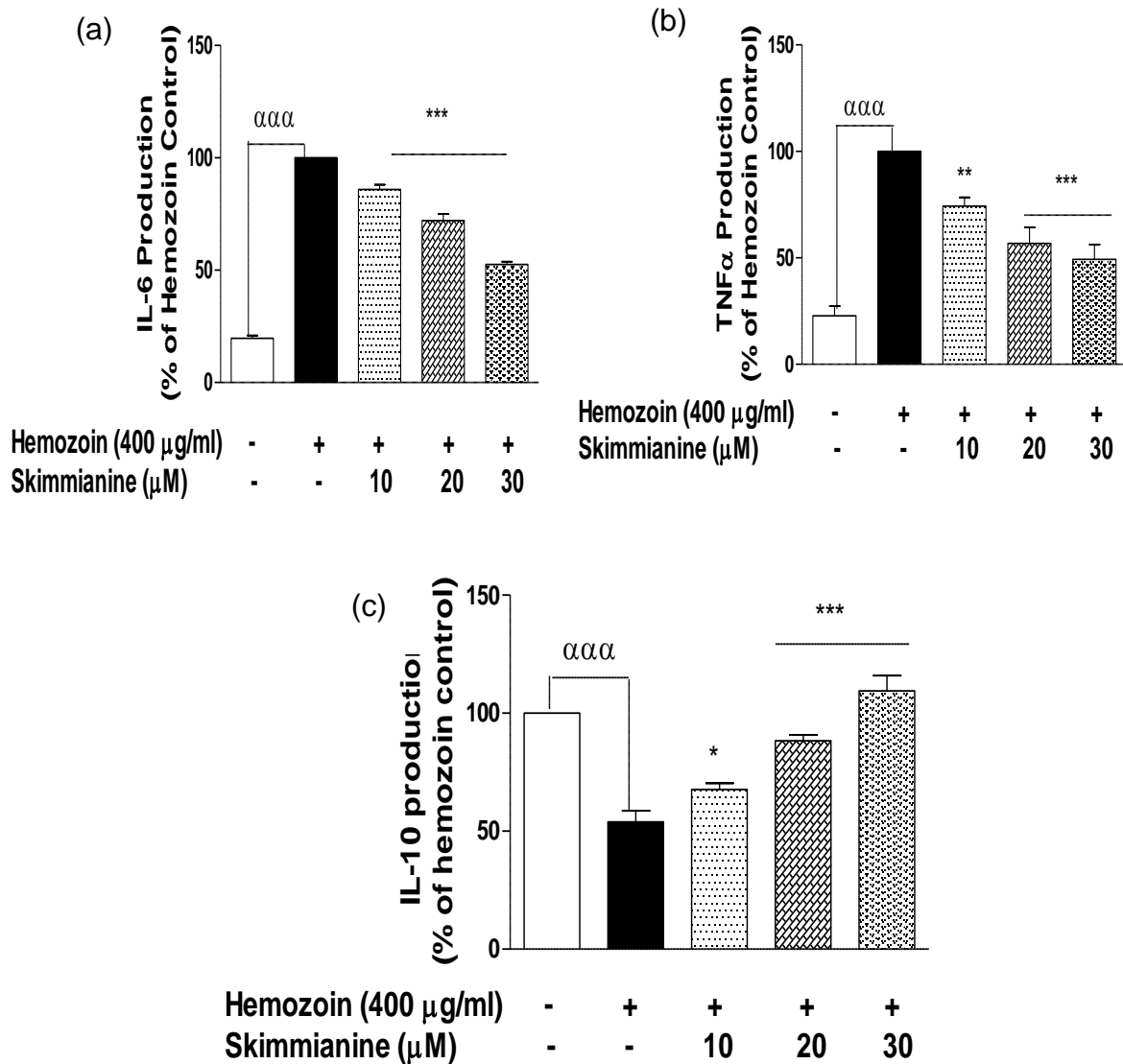
Figure 4-1: Skimmianine inhibited IL-6 and TNFα secretion and increased IL-10 production in BV2 microglia cells induced by synthetic hemozoin

Cultured BV2 cells were treated with skimmianine (10, 20 and 30 μM) for 30 minutes and then, induced by hemozoin (400 μg/ml) for 24 hours. Thereafter, cytokine levels of (a) TNFα, (b) IL-6 and (c) IL-10 were measured in the cell culture supernatants by ELISA technique. Data were plotted as mean ± SEM for three separate experiments. Student Newman-Keuls test followed by one-way ANOVA were used for statistical analysis (multiple comparisons, ^{ααα} p<0.001 in comparison with the unstimulated cells *p<0.05, **p<0.01, ***p<0.001) in relation to hemozoin-induced cells.

4.3.2 Skimmianine inhibited nitrite production by reducing the expression of iNOS protein in hemozoin-stimulated BV2 microglia cells

Reports have shown that increased secretion of nitric oxide (NO) caused by hemozoin leads to neurotoxicity which has been associated with cognitive dysfunction observed in cerebral malaria patients. (Sahu et al., 2015). This suggests a role for nitric oxide in neuroinflammation detected in pathogenesis of cerebral malaria. Therefore, inhibition of NO is important in reducing neuroinflammation, especially in neurological sequelae of malaria infection. Results of this study revealed that incubation of hemozoin with BV2 microglia cells resulted in a significant increase ($p < 0.001$) in NO production. However, secretion of NO was reduced at 10 µM of skimmianine (~1.2-fold decrease) in hemozoin-stimulated BV2 cells in comparison to hemozoin stimulated cells only. In addition, there was a further significant decrease in the levels of NO by skimmianine at 20 µM (~1.7-fold decrease) and 30 µM (~2.1-fold decrease). Thereafter, studies were carried out to investigate the effect of skimmianine on expression of iNOS protein by western blot experiment. Results show a significant increase ($p < 0.001$) in the level of iNOS protein in BV2 cells stimulated with hemozoin when compared to untreated cells. In contrast, a significant reduction ($p < 0.05$) in the level of iNOS protein was observed at 10 µM of skimmianine in hemozoin induced BV2 cells when compared to hemozoin treated cells. Further significant inhibition of iNOS protein expression was detected in hemozoin stimulated BV2 cells treated with 20 µM and 30 µM of skimmianine (52.3% and 40.53% respectively) in relation to hemozoin stimulated cells.
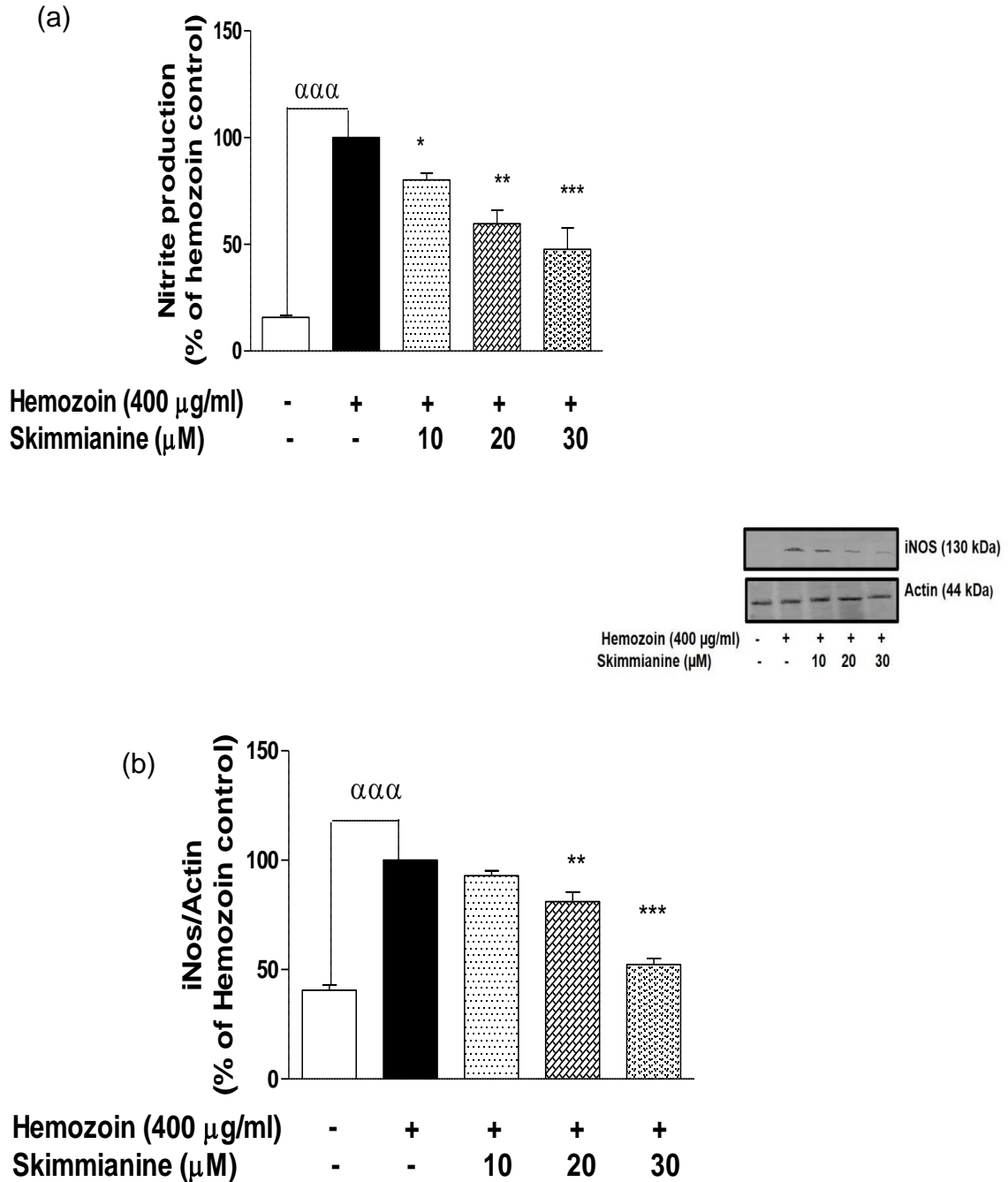
Figure 4-2: Skimmianine decreased nitric oxide (NO) release (a) and inducible nitric oxide (iNOS) protein expression (b) in hemozoin-induced BV2 cells

Cultured BV2 cells were pre-treated with skimmianine (10, 20 and 30 µM) for 30 minutes and subsequently stimulated with hemozoin (400 µg/ml) for 24 hours. Then, cell culture supernatants and cytoplasmic extract were collected and analysed for secretion of NO and protein expression of iNOS. Skimmianine inhibited (a) nitric oxide (NO) production through inhibiting (b) inducible nitric oxide (iNOS) protein expression in BV2 cells stimulated with hemozoin. Values are represented as mean ±SEM for 3 separate experiments. Analysis was done using one-way ANOVA with post-hoc Student Newman-Keuls test ($\alpha\alpha\alpha$ $p<0.001$ in comparison with the untreated cells and (*$p<0.05$, **$p<0.01$,***$p<0.001$) shows significance relative to .hemozoin treated cells.

4.3.3 Skimmianine inhibited PGE$_2$ production by reducing COX-2 protein expression in hemozoin-stimulated BV2 microglia cells

The role of COX-2 protein expression in the brain have been reported to play an important role in murine model of CM infected with *plasmodium berghei* ANKA to increase the level of PGE$_2$ in neuroinflammation (Ball, MacDougall, McGregor, & Hunt, 2004; Dunst et al., 2017) . This suggests the involvement of neuroinflammation in the pathogenesis of cerebral malaria. Therefore, inhibition of PGE$_2$ secretion and COX-2 protein expression may be beneficial in reducing neuroinflammation observed in CM. This study investigates the effect of skimmianine on level of PGE$_2$ and COX-2 protein in hemozoin-activated BV2 cells. Stimulation of BV2 cells with hemozoin resulted in a marked increase ($p<0.001$) of PGE$_2$ production (~7-fold increase) in comparison to unstimulated BV2 cells. However, pre-treatment with skimmianine at 10 μM significantly decreased ($p<0.01$) the level of PGE$_2$ (74.4% decrease) in hemozoin-stimulated cells in comparison to hemozoin-activated cells. A further significant reduction in PGE$_2$ secretion was detected at 20 and 30 μM of skimmianine (53.3% and 57.2% reduction respectively) in hemozoin-stimulated cells in relation to hemozoin-activated cells only. Thereafter, effect of skimmianine on level of COX-2 protein were determined with western blots experiments. Result showed an elevated level ($p<0.001$) of COX-2 protein (~3.2-fold increase~) in hemozoin treated cells in relation to untreated cells. In contrast, 10 μM of skimmianine significantly ($p<0.05$) reduced the expression of COX-2 protein (1.1-fold decrease) in hemozoin-induced BV2 cells when compared to BV2 cells stimulated with hemozoin alone. Moreover, in hemozoin stimulated cells, there was a further inhibition of expression of COX-2 protein at 20 and 30 μM of skimmianine (1.4-fold and ~2.1-fold decrease, respectively), in relation to hemozoin activated cells. These results suggest that skimmianine inhibited PGE$_2$

secretion by reducing the expression of COX-2 protein in hemozoin stimulated BV2 microglia cells.

(a)

(b)
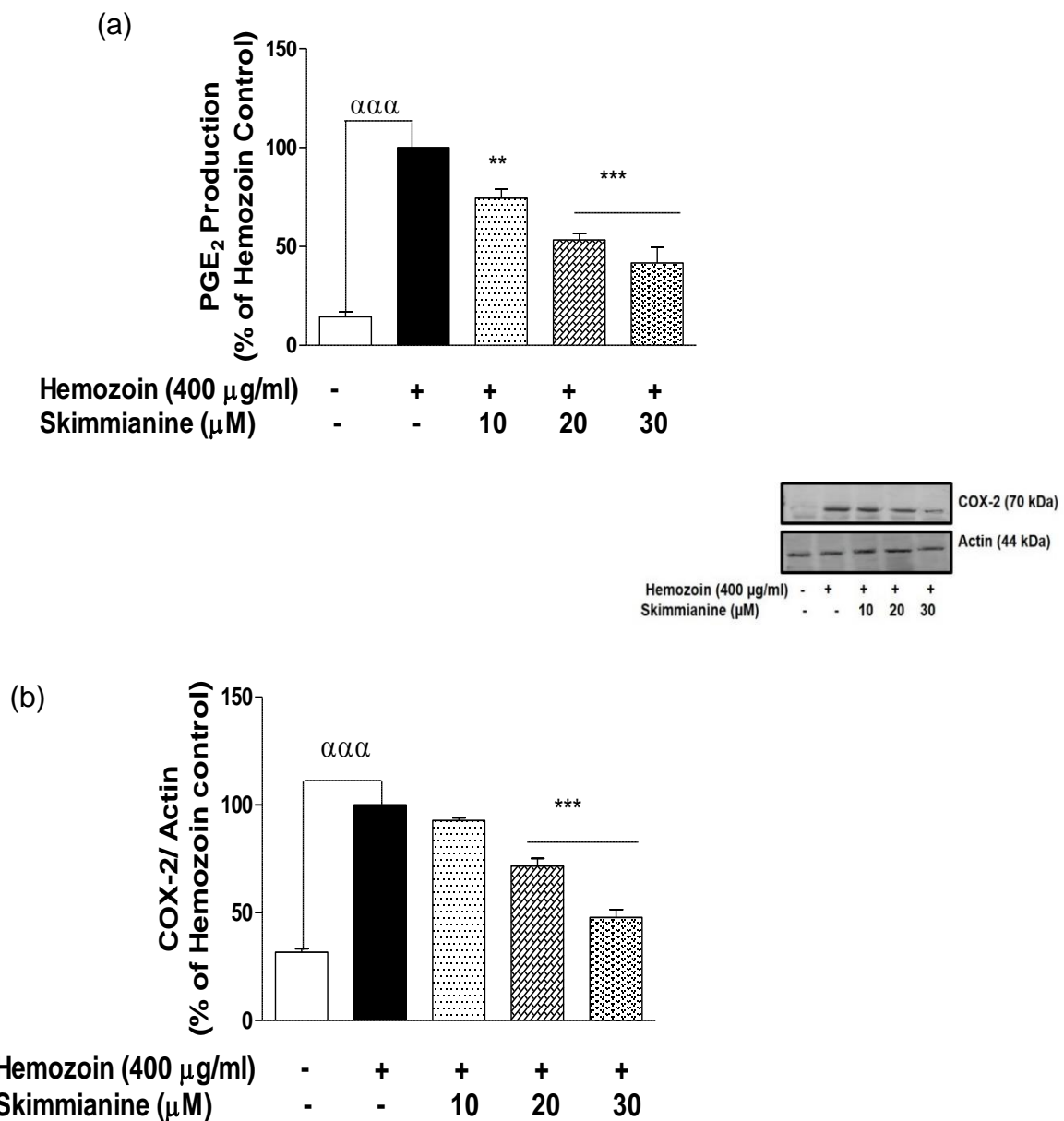
Figure 4-3: Skimmianine reduces PGE2 production and COX-2 protein expression in hemozoin induced BV2 cells

BV2 cells were treated with or without skimmianine (10, 20 and 30 μM) for 30 minutes and activated with hemozoin (400 μg/ml) for 24 hours. Skimmianine inhibited PGE2 production (a) and COX-2 protein expression (b) in BV2 microglia. Values are presented as mean ±SEM for 3 separate experiments Statistical analysis was done using one-way ANOVA with post-hoc Student Newman-Keuls test (multiple comparisons ^{ααα} $p < 0.001$ in comparison with the untreated cells and (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) in relation to hemozoin control

4.3.4 Skimmianine suppressed pro-IL-1β and IL-1β secretion through inhibition of NLRP3 protein and caspase-1 activity in hemozoin activated BV2 microglia

In the CNS, NLRP3 inflammasome has been demonstrated to be involved in progression of neuroinflammation by causing regulating the secretion of cytokines including IL-1β (Shao et al., 2018). Activation of NLRP3 inflammasome by stimuli such as hemozoin causes activation of pro-caspase -1 to caspase-1 and this further leads to conversion of pro-IL-1β to IL-1β (Mendiola & Cardona, 2017). This study determines the effect of skimmianine on pro-IL-1β and IL-1β in hemozoin activated BV2 microglia cells. The outcome of this study shows in Figure 4-4: Skimmianine suppressed the levels of IL-1β and pro-IL-1β secretion by inhibiting NLRP3 protein and caspase-1 activity in hemozoin activated BV2 microglia

a significant increase ($p<0.001$) in the secretion of pro-IL-1β and IL-1β (~2.7-fold and ~4.2-fold increase, respectively) in BV2 cells stimulated with hemozoin when compared to unstimulated cells. However, there was a marked decrease ($p<0.01$) in the degree of pro-IL-1β when treated with 10 and 20 µM of skimmianine (~1.1-fold and ~1.4-fold reduction respectively) in hemozoin activated BV2 microglia cells when compared to hemozoin stimulated cells. Further significant reduction ($p<0.001$) of pro-IL-1β secretion was detected at 30 µM of skimmianine (~1.8-fold decrease). Furthermore, in hemozoin activated BV2 microglia cells, the level of IL-1β reduced significantly ($p<0.01$) on pre-treatment with 10 and 20 µM of skimmianine (87.2%.and 82.9% respectively) in relation to hemozoin stimulated cells. Moreover, 30 µM of skimmianine produced a more significant inhibition ($p<0.001$) of secretion of IL-1β (72.4% decrease) in hemozoin stimulated BV2 microglia cells when compared to hemozoin activated cells.

Moreover, the effect of skimmianine on caspase-1 and NLRP3 protein in hemozoin activated BV2 microglia cells were also determined. Caspase-1 activity was measured by caspase-1 inflammasome assay and immunoblotting technique. Stimulation of BV2 cells with hemozoin resulted in a significant elevation ($p < 0.001$) in caspase-1 activity (~7.6-fold increase) in comparison to unstimulated BV2 cells. However, skimmianine at 10, 20 and 30 µM significantly ($p < 0.001$) reduced caspase-1 activity (~1.3-fold, ~1.9-fold and ~2.9-fold decrease respectively) in hemozoin stimulated cells in relation to hemozoin-activated cells. Afterwards, skimmianine (10, 20 and 30 µM) significantly reduced the expression of caspase-1 protein.by western blotting technique in hemozoin stimulated cells when compared to hemozoin stimulated cells. Moreover, in hemozoin-stimulated BV2 cells, an elevated ($p < 0.001$) level of NLRP3 protein (~2.5-fold increase) was observed when compared to unstimulated BV2 cells. However, a decrease in the level of NLRP3 protein was detected in hemozoin –stimulated BV2 cells treated with 10 µM of skimmianine (~1.2-fold reduction) in comparison to hemozoin-stimulated BV2 cells. Additionally, in hemozoin –stimulated BV2 cells, further inhibition of NLRP3 protein expression was observed in BV2 microglia cells when treated with 20 and 30 µM of skimmianine (1.3-fold and ~1.8-fold reduction, respectively) in comparison with hemozoin-stimulated cells.
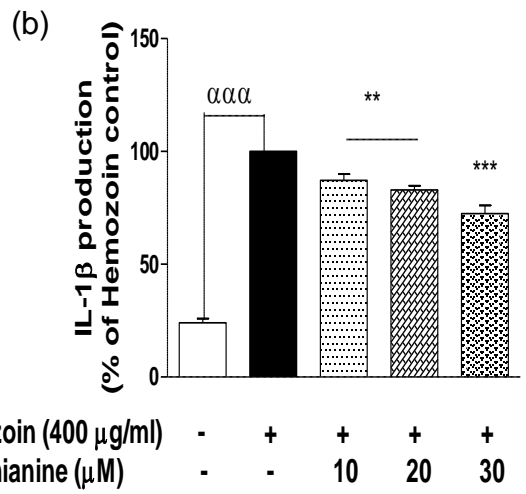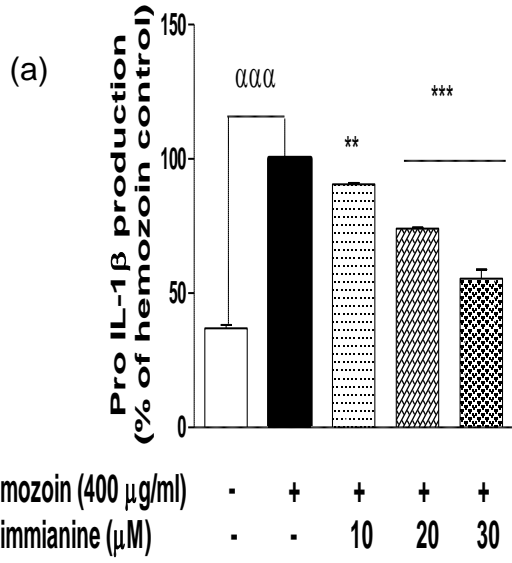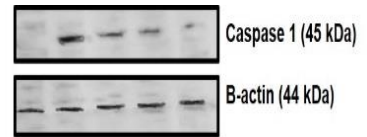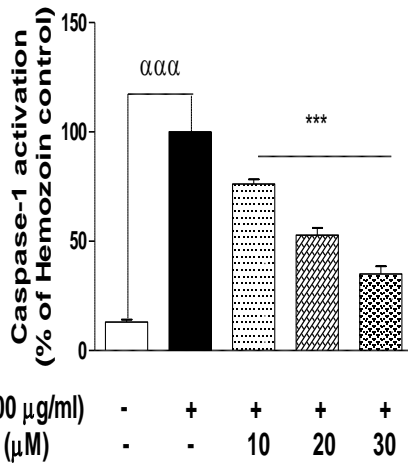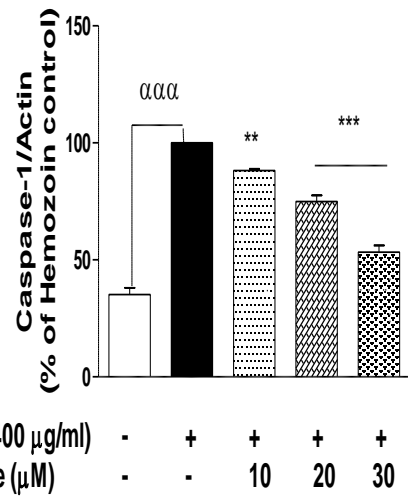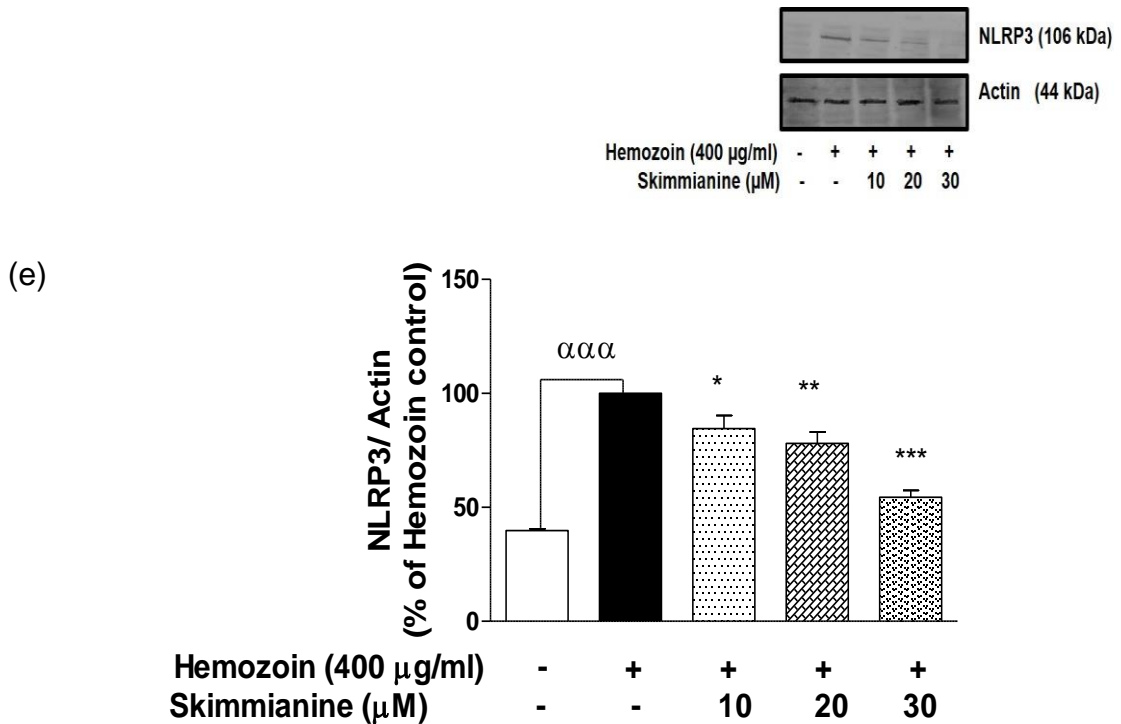
(a)

(b)

(c)

(d)

Figure 4-4: Skimmianine suppressed the levels of IL-1β and pro-IL-1β secretion by inhibiting NLRP3 protein and caspase-1 activity in hemozoin activated BV2 microglia

Skimmianine (10, 20 and 30 µM) significantly inhibited (a) pro-IL-1β and (b) IL-1 β production (c) caspase-1 activation measured by caspase-1 Glo inflammasome assay in hemozoin induced BV2 microglia (d) expression of caspase-1 by immunoblotting experiment and (e) expression of NLRP3 protein. BV2 cells were stimulated with hemozoin (400 µg/ml) in the presence or absence of Skimmianine (10, 20 and 30 µM) for 24 hours. At the end of the incubation period, supernatants were collected for ELISA measurements. For western blot experiment, cytoplasmic extract was collected at the end of 24 hours' incubation and western blot was performed to measure protein expression of caspase-1 and NLRP3 protein. Data were expressed as mean ±SEM for 3 separate experiments. Analysis was done using one-way ANOVA with post-hoc Student Newman-Keuls test (multiple comparisons. $^{\alpha\alpha\alpha}$ $p<0.001$ in comparison with the untreated cells and * $p<0.05$, **$p<0.01$, ***$p<0.001$) in comparison with hemozoin control.

4.3.5 Skimmianine reduces neuroinflammation through NF-κB signaling pathway in hemozoin-stimulated BV2 microglia cells

Previous studies have shown the role of hemozoin on activation of NF-κB signalling pathway by toll-like receptor 2 (TLR-2) and toll like receptor-9 (TLR9) (Bafica et al., 2006; Wagner, 2010). This suggests that there is an involvement of hemozoin in neuroinflammation through NF-κB signalling pathway. Reports have further demonstrated that activation of NF-κB leads to the translocation of NF-κB p65 into the nucleus and subsequently binds to the DNA for transcription of genes including iNOS. The production of NO, IL-6 and TNFα as a result of activated NF-κB have been reported to increase neuroinflammation, which is involved in cognitive dysfunction observed in CM patients. Hence, inhibition of activated NF-κB pathway is important and may be a potential target in reducing neuroinflammation in cerebral malaria. Effect of skimmianine on DNA binding activity of NF-κB in hemozoin stimulated BV2 cells were determined in this study

Result in Figure *4-5* shows a significant increase ($p<0.001$) in the DNA binding of NF-κB (~ 1.9-fold) in BV2 microglia cells stimulated with hemozoin when compared to unstimulated BV2 cells. In contrast, skimmianine at 10 μM significantly reduced ($p<0.05$) the DNA binding activity to NF-κB (~ 1.1-fold decrease) in hemozoin stimulated BV2 cells when compared to hemozoin stimulated cells. Moreover, in hemozoin stimulated cells, skimmianine at 20 and 30 μM resulted in a further inhibition ($p<0.001$) of DNA binding activity to NF-κB (~ 1.2-fold and ~ 1.4-fold respectively) when compared to hemozoin stimulated cells. Furthermore, the effect of skimmianine on expression of IκB and NF-κB -p65 subunit in BV2 cells stimulated with hemozoin were investigated. Result showed a significant increase ($p<0.001$) in the phosphorylation of NF-κB p65 subunit (~3.4-fold increase) in hemozoin stimulated BV2

cells when compared to unstimulated cells. However, skimmianine at 10 µM significantly (p<0.05) inhibited phosphorylation of NF-κB p65 (~1.1-fold decrease) in hemozoin-stimulated BV2 cells in comparison to hemozoin-activated cells. Moreover, in hemozoin-stimulated cells, skimmianine.at 20 and 30 µM significantly reduced (p<0.001) the phosphorylation of NF-κB p65 subunit (~1.3-fold and ~2.1-fold decrease respectively) in comparison to hemozoin-stimulated cells.

Thereafter, immunoblotting experiments were done to investigate the effect of skimmianine on p-IκBα phosphorylation and IκBα degradation in BV2 cells stimulated by hemozoin. The outcome of this experiment shows a significant increase (p<0.001) in the level of p-IκBα phosphorylation (~2.3-fold increase) in hemozoin-induced BV2 cells when compared to unstimulated BV2 cells. However, pre-treatment with skimmianine at 10 µM resulted in a marked reduction (p<0.01) of p-IκBα phosphorylation (~1.1-fold decrease) in hemozoin-stimulated BV2 cells in comparison to hemozoin-stimulated cells. Furthermore, 20 and 30 µM of skimmianine caused a significant inhibition (p<0.001) of IκBα phosphorylation (~1.2-fold and ~2-fold reduction respectively) in relation to BV2 cells treated with hemozoin alone. Moreover, in hemozoin-stimulated BV2 cells, there was a marked degradation (p<0.001) of total IκB (44.6% decrease) in comparison to unstimulated cells. In contrast to this, **Error! Reference source not found.**d present that skimmianine (10, 20 and 30 µM) significantly increased (p<0.001) total IκB (68%, 85% and 93% respectively) in hemozoin stimulated BV2 cells when compared to unstimulated BV2 cells.
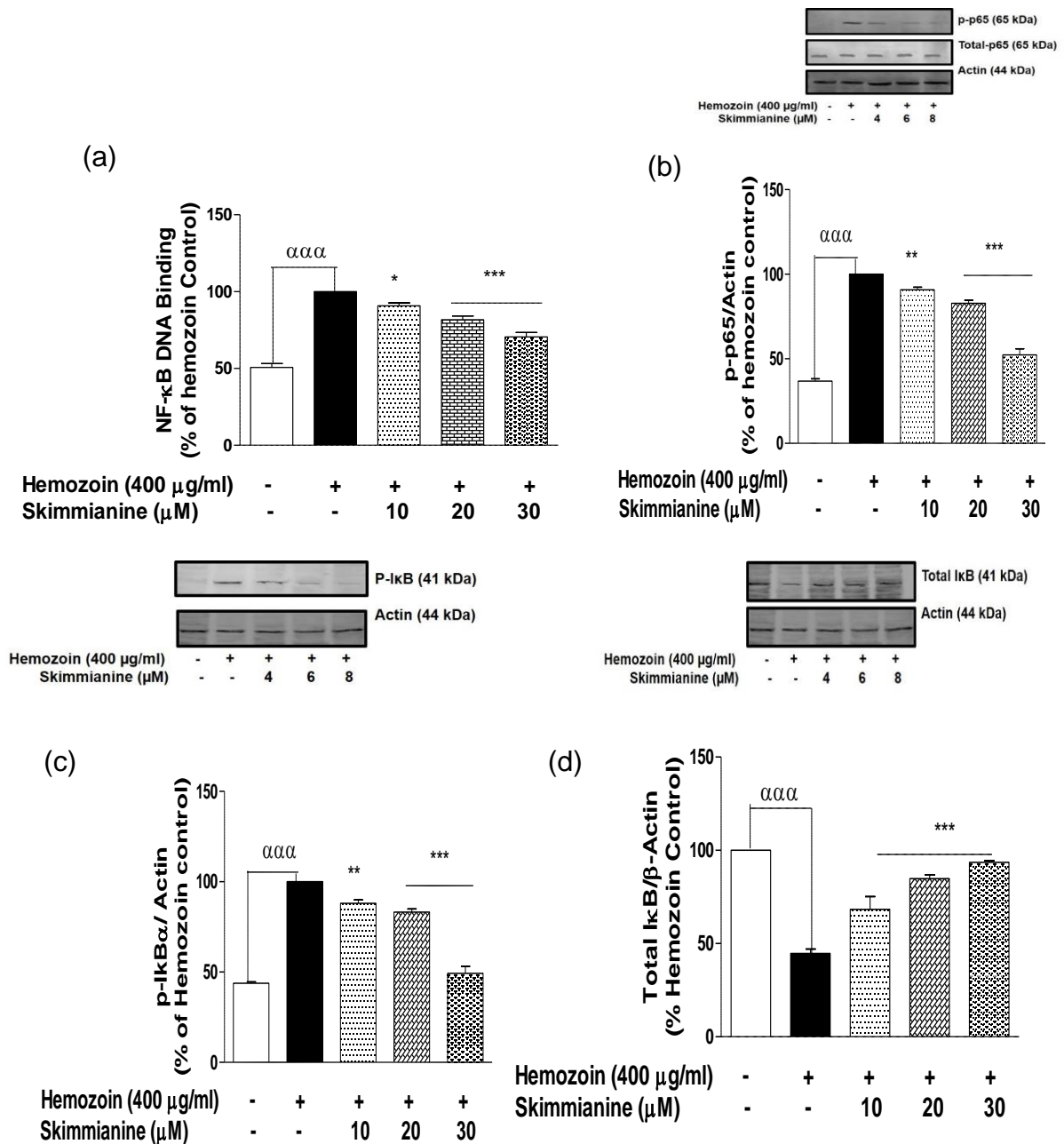
Figure 4-5: Skimmianine inhibited neuroinflammation through NF-κB signaling pathway in hemozoin activated BV2 microglia cells

Skimmianine (10, 20 and 30 µM) (a) decreased DNA binding activity of NFκB in hemozoin stimulated BV2 microglia cells. (b) translocation of NF-KB p65 subunit (c) phosphorylation of IκB, (d) degradation of IKB and in hemozoin stimulated BV2 cells. Cultured BV2 cells were stimulated with hemozoin (400 µg/ml) in the presence or absence of skimmianine (10, 20 and 30 µM)) for 1 hour. At the end of the incubation, cytoplasmic or nuclear extract was collected and western blot was performed to analyse p65 subunit and IκB protein expression. Analysis of data was done on 3 independent experiments using one-way ANOVA with post-hoc Student Newman-Keuls test (multiple comparisons αααα $p<0.001$ in relation to untreated cells and *$p<0.05$, **$p<0.01$, ***$p<0.001$) in comparison with hemozoin control.

4.3.6 Pre-treatment with skimmianine did not affect the viability of BV2 cells in hemozoin-stimulated BV2 cells

The viability of BV2 microglia cells were assessed when pre-treated with skimmianine (10, 20 and 30 µM) for 30 minutes and then stimulated with hemozoin (400 µg/ml) for 24 hours. Results in Figure 4-6: Skimmianine did not suppress the viability of BV2 microglia cells in hemozoin-activated BV2 microglia cells

shows that skimmianine did not affect the viability of BV2 cells at the concentrations used in this experiment.
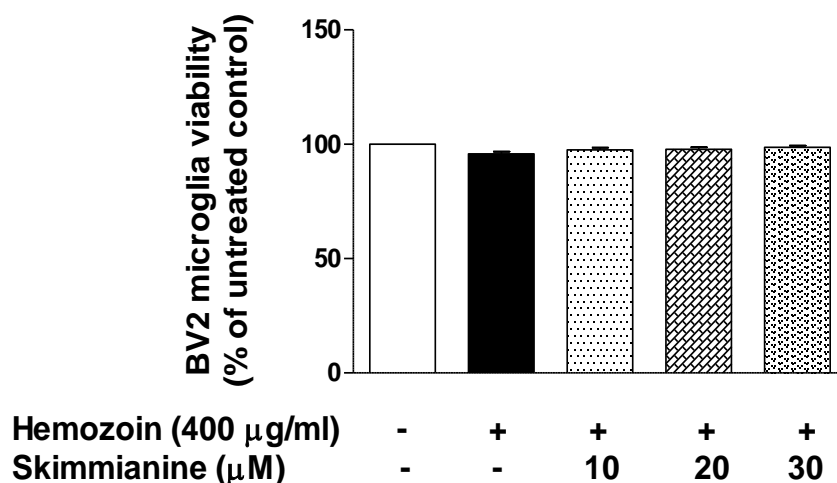
Figure 4-6: Skimmianine did not suppress the viability of BV2 microglia cells in hemozoin-activated BV2 microglia cells

Skimmianine (10, 20 and 30 µM) did not reduce the viability of BV2 cells when stimulated with hemozoin (400 µg/ml) for 24 hours. MTT assay was used to determine the viability of BV2 cells after treatment. All values are expressed as mean ± SEM for 3 separate experiments. Statistical analysis was performed using one-way ANOVA with post-hoc Student Newman-Keuls test in comparison with untreated cells

4.4 Discussion

Investigations on the role of neuroinflammation in cerebral malaria have demonstrated a decline in neurocognitive function of CNS (Song, Pei, Yao, Wu, & Shang, 2017). Studies have further reported an association between hemozoin and secretion of pro-inflammatory cytokines in post-mortem analysis done on brain tissue of CM patients (Dunst et al., 2017). In BV2 microglia cells, effect of skimmianine on hemozoin induced neuroinflammation were investigated in this study.

Skimmianine significantly reduced the level of TNFα and IL-6 in hemozoin-stimulated BV2 microglia cells. In addition, skimmianine evidently increased the secretion of IL-10 in hemozoin-induced BV2 microglia cells. The outcome of this finding is reported for the first time in this study. Other studies have identified the potential role of a related quinolone alkaloid, berberine in inhibition of inflammation in severe plasmodium falciparum infection (Kumar, Chopra, Mukherjee, Pottabathini, & Dhull, 2015). Excessive production of pro-inflammatory cytokines in neuroinflammation have been demonstrated to be involved in causing cognitive dysfunction in CM patients (Kihara, Carter, & Newton, 2006; Tapajós et al., 2019). Consequently, this has made inhibition of TNFα and IL-6 an attractive target to reduce neuroinflammation in cerebral malaria. Observation from the inhibition of pro-inflammatory cytokines and mediators in hemozoin activated cells by skimmianine may play a possible role in the treatment of CM.

Furthermore, skimmianine inhibited NO production by reducing the expression of iNOS in hemozoin-stimulated BV2 microglia cells. These results suggest that skimmianine could inhibit hemozoin-activated neuroinflammation through inhibiting the release of NO and iNOS protein. Result in this investigation proposes a new mechanism of anti-inflammatory activity of skimmianine in hemozoin-activated cells.

Increase in production of NO has been reported to cause coma in cerebral malaria patients, hence suggesting the importance of inhibiting NO and iNOS in order to reduce neuroinflammation in CM (Grau & Craig, 2012). The inhibitory effect of skimmianine in this study has indicated its possible action in treatment of CM. Furthermore, skimmianine suppressed PGE_2 production through inhibiting the expression of COX-2 enzyme in hemozoin-stimulated BV2 microglia cells. This study suggests the inhibitory effect of skimmianine on hemozoin-stimulated neuroinflammation through reduction of PGE_2 / COX-2 production. Reports have shown that increase in the level of COX-2 protein and PGE_2 is involved in the pathology of murine cerebral malaria (Ball et al., 2004; Xiao, Patterson, Yang, Lal, & hygiene, 1999). As a result, these studies suggest the importance of inhibiting PGE_2 / COX-2 in neuroinflammation and this may indicate the promising role of skimmianine in treatment of CM.

This study has also established that skimmianine reduced the production of pro-IL-1β and IL-1β through inhibition of NLRP3 protein and caspase-1activation in hemozoin-stimulated BV2 microglia cells. These results suggest that skimmianine reduced neuroinflammation through NLRP3 pathway in hemozoin-activated BV2 microglia cells. Previous studies have shown berberine to reduce neuroinflammation in BV2 microglia cells and in experimental cerebral malaria (D. Y. Lu, Tang, Chen, & Wei, 2010; D. Xiao et al., 2018). The activation of NLRP3 infalmmasome by hemozoin has been reported to increase the secretion of IL-1β in neuroinflammation to cause neurotoxicity, which has been linked to neurocognitive disorder in CM (Kalantari et al., 2014; Olivier et al., 2015). Therefore, it is crucial to reduce the activation of NLRP3 pathway in neuroinflammation. The potential role of alkaloids in the *Z. zanthoxyloides*

extract including skimmianine on neuroinflammation is significant in treatment of neuroinflammatory disorder such as cerebral malaria.

Moreover, skimmianine evidently produced inhibition of hemozoin-induced neuroinflammation in BV2 microglia by targeting NF-κB p65 signalling pathway. Reports have shown the importance of NF-κB activation in excessive production of pro-inflammatory cytokines and mediators, which induces neuroinflammation in CM (Punsawad, Maneerat, Chaisri, Nantavisai, & Viriyavejakul, 2013). Therefore, the role of skimmianine in inhibition of NF-κB signalling pathway is significant in reducing neuroinflammation and may alter the progression of cerebral malaria. Collectively, skimmianine has shown a significant inhibitory role on neuroinflammation induced by hemozoin in BV2 microglia cells. This may be implicated in therapeutic approach of treating cerebral malaria.

5 Chapter 5- General discussion and conclusion

5.1 General discussion

Neuroinflammation has been shown to play a key role in the pathogenesis of neurodegenerative diseases and cerebral malaria (Grau & Craig, 2012; Hora et al., 2016). Reports have suggested that prolonged process of neuroinflammation may lead to neurotoxicity and neuronal damage, which can cause progression of neurodegenerative diseases (Balez, Rachelle, Ooi, & Lezanne, 2016). In addition, neuroinflammation mediated neurotoxicity has been associated with neurocognitive dysfunction which is observed in patients having cerebral malaria, a neurological sequelae of malaria (Bangirana et al., 2015). Moreover, studies have reported an increase in production of pro-inflammatory cytokines and molecules such as IL-6, TNFα and NO in activated microglia cells which may initiate neuroinflammation that is detected in neurological complication of *plasmodium falciparum* infection and in neurodegenerative diseases (Schain et al., 2017; Tapajós et al., 2019).

Several studies have reported the use of medicinal plants in altering the progress of neurodegeneration (Shakya, 2016; Tomlinson & Akerele, 2015). The role of *Zanthoxylum* genus has been reported in numerous studies of its potential in reducing neuroinflammation and ameliorating cognitive impairment (Barua et al., 2018; Kiraithe, N, Nguta, Mbaria, & Kiama, 2016; Saikia et al., 2018). *Zanthoxylum zanthoxyloides* (*Z. zanthoxyloides*) tree is one of the popular species of *Zanthoxylum* genus (family-*Rutaceae)* because of its extensive use in traditional medicine (Adesina, 2005; Wangensteen et al., 2017). Reports have shown that the different parts of *Z. zanthoxyloides* tree including root and leaves are beneficial in reducing inflammatory diseases locally (Patiño et al., 2012). Furthermore, reports have shown the common use of *Z. zanthoxyloides* root as chewing stick in western part of Nigeria for the treatment of dental caries because it possess anti-inflammatory activities (Orafidiya et

al., 2010). Moreover, the leaves of *Z. zanthoxyloides* have been shown to be beneficial in the treatment of malaria, suggesting its anti-plasmodial activities (Kénou et al., 2018). These reports indicate the potential role of *Z. zanthoxyloides* in neuroinflammation and neurological complication of severe malaria. Furthermore, previous investigation on *Z. zanthoxyloides* root extract demonstrated the anti-inflammatory activity of this extract on carrageenan-induced paw inflammation in rats (Prempeh & Mensah-Attipoe, 2009). This suggest the anti-inflammatory effect of *Z. zanthoxyloides* extract in peripheral cells.

In this study, the effect of *Z. zanthoxyloides* root extract were investigated on neuroinflammation in LPS and separately in hemozoin-stimulated BV2 microglia cells. *Z. zanthoxyloides* extract reduced the production of fundamental pro-inflammatory cytokines (TNFα, IL-1β and IL-6), NO, PGE$_2$, expression of COX-2 and iNOS protein in LPS or hemozoin stimulated BV2 microglia cells. In addition, *Z. zanthoxyloides* extract increased the level of IL-10 (an anti-inflammatory cytokine) secretion in LPS and also in hemozoin-stimulated BV2 microglia cells. These results indicate that *Z. zanthoxyloides* extract could attenuate neuroinflammation through its inhibitory effect on important interleukins and mediators in BV2 microglia cells. Previous studies on *Z. zanthoxyloides* extract suggested its inhibitory activity on PGE$_2$ concentration in rat induced with carrageenan (Prempeh & Mensah-Attipoe, 2008). Furthermore, reports on *Zanthoxylum acanthopodium* (Yanti et al., 2011) and *Zanthoxylum armatum* (Sati, Sati, Raturi, Badoni, & Singh, 2011)*,* which are related species of same genus (*Zanthoxylum*) with *Z. zanthoxyloides* have evidently reduced the secretion of pro-inflammatory cytokines and inflammatory mediators (iNOS and COX-2) in animal and cell culture studies. These investigations suggest anti-inflammatory activity of species of *Zanthoxylum* in diverse models of inflammation.

Sustained activation of microglia cells by inflammatory inducers to secrete pro-inflammatory mediators and cytokines are of importance in the progression of neurodegenerative diseases (Cameron et al., 2010; Sheppard et al., 2019). The excessive release of neuroinflammatory factors including TNFα and NO in activated microglia cells have been shown to cause neurotoxicity and damage to the surrounding neurons (Asiimwe et al., 2016; D. Kim et al., 2018). In addition, elevated level of pro-inflammatory cytokines including IL-1β have been linked with neurocognitive decline in CM patients (Tapajós et al., 2019). As a result, reduction of pro-inflammatory cytokines and mediators by *Z. zanthoxyloides* extract in this study has shown the possible role of this extract in treatment of neurodegenerative diseases and cerebral malaria.

Moreover*, Z. zanthoxyloides* extract inhibited p-IκB phosphorylation, total IκB degradation and phosphorylation of NF-κB p65 in LPS and separately in hemozoin-stimulated BV2 microglia cells. These results provide new evidence and indicate that anti-inflammatory effect of *Z. zanthoxyloides* on pro-inflammatory cytokines and inflammatory mediators may be through the inhibitory action of this extract on NF-κB signalling pathway in BV2 microglia cells. Furthermore, *Z. zanthoxyloides* did not inhibit the phosphorylation of IKK in BV2 cells stimulated with LPS. Studies have reported that there is cross talking of other kinases including Jun-N-terminal kinase and p38 which may interfere with the inhibition of IKK phosphorylation in the upstream part of NF-κB signalling pathway (Hoesel & Schmid, 2013; Tian et al., 2015). Also, inhibitors of IKK phosphorylation act through the mechanism by competing with ATP binding site on IKK or having allosteric effect on IKKα/β (Nadia Lampiasi & Giovanna Montana, 2016; Prescott & Cook, 2018). Consequently, *Z. zanthoxyloides* extract at these concentrations (4, 6 and 8 μg/ml) may not be acting through this mechanism to

inhibit IKK phosphorylation in LPS stimulated BV2 microglia cells. Previous investigation have reported the suppressive effect of *Zanthoxylum rhesta* (a related specie to *Z .zanthoxyloides*) on expression of COX-2 and iNOS protein through NF-κB signalling pathway.in LPS-stimulated raw macrophage cells (Thu et al., 2010). This finding confirms the current report on the anti-inflammatory effect of *Z. zanthoxyloides* extract, thus explaining the application of this extract in traditional medicine

Studies have identified the involvement of NF-κB transcriptional activity in activated microglia cells as it controls the expression of genes including COX-2 and iNOS to cause an increase in neuroinflammation (Biswas & Bagchi, 2016; Feng et al., 2017). Reports have further shown that neuroinflammation is a crucial factor that contributes to the progression of neurodegenerative diseases such as multiple sclerosis and AD (Stephenson et al., 2018). Investigations have further revealed the significant role of inhibiting activated NF-κB signalling pathway.by drugs to reduce neuroinflammation in neurodegenerative diseases (Chen et al., 2016). A different report has further demonstrated the effect of activated NF-κB in malaria infection to increase the level of pro-inflammatory genes leading to neuroinflammation observed in pathology of CM (Pais & Penha-Gonçalves, 2018). Therefore, inhibition of activated NF-κB signalling pathway by *Z. zanthoxyloides* extract is essential in reducing neuroinflammation and has implication in the treatment of neurodegenerative diseases and cerebral malaria.

In hemozoin-induced BV2 microglia cells, *Z. zanthoxyloides* extract reduced the level of pro-IL-1β, IL-1β, NLRP3 protein and caspase-1 activity. A previous investigation has shown that the anti-inflammatory activity of *Zanthoxylum bungeanum* extract, (a related specie to *Z. zanthoxyloides* in genus of *Zanthoxylum*) on activated NLRP3 inflammasome in mice (Z. Zhang et al., 2017). This indicates the role of *Z. zanthoxyloides* extract on NLRP3 inflammasome pathway. The significant role of

NLRP3 inflammasome activation have made the NLRP3 pathway an attractive target in the CNS to regulate the secretion of IL-1β, which is implicated in exacerbation of neuroinflammation. More investigations have revealed that the activation of NLRP3 inflammasome by ligands including hemozoin causes over production of IL-1β leading to neuroinflammation and neurotoxicity, which has been associated with neurocognitive impairment in CM patients (De Miranda et al., 2015; Pais & Penha-Gonçalves, 2018). Consequently, inhibition of NLRP3 inflammasome activation by *Z. zanthoxyloides* extract is important reducing the level of IL-1β in neuroinflammation and may be beneficial in slowing the progress of cognitive dysfunction in cerebral malaria.

The use of bioassay guided fractionation has been demonstrated to be an important strategy in isolating most active fraction and compounds in plant (Weller, 2012). It also minimizes the risk of losing the active fraction or compound in plants extract. Based on the inhibitory activity of *Z. zanthoxyloides* extract on neuroinflammation in, it became necessary to isolate and identify compound (s) in this extract by bioassay guided fractionation that could be responsible for anti-neuroinflammatory effect observed in this extract. Four fractions (F1, F2, F3 and F4) were isolated from *Z. zanthoxyloides* extract by solid phase extraction (SPE) technique. Investigations on the fractions revealed F3 as the most active fraction due to its significant reduction of the levels of TNFα in LPS-activated BV2 microglia cells. Taken together, it is suggested that fraction 3 may contain the active constituents responsible for the anti-neuroinflammatory activity of *Z. zanthoxyloides* extract.

Further chemical and pharmacological analyses revealed the bioactive compound is 4, 7, 8-Trimethoxyfuro [2, 3-*b*] quinolone, also known as skimmianine. Similar isolation of skimmianine has been reported for *Z. zanthoxyloides* fruit extract (Guetchueng et

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al., 2018), hence confirming the presence of skimmianine in fraction 3 of *zanthoxyloides* extract in this study. In addition, other studies have also reported the isolation of skimmainine from other related species of *Zanthoxylum* including *Zanthoxylum buesgenii* (Sandjo et al., 2014), *Zanthoxylum nitidum* (Yang et al., 2012).

Further investigations on the effects of skimmianine on neuroinflammation showed a reduction in the levels of TNFα, IL-6, NO, PGE$_2$, iNOS protein and COX-2 protein in BV2 cells activated separately with LPS and synthetic hemozoin. A previous report demonstrated that skimmianine inhibited the release of NO in LPS-stimulated BV2 cells (Yoon et al., 2012). However, this study did not determine the effect of skimmianine on the iNOS, which is critical to NO production in inflammation. Moreover, studies have shown that berberine, (a related alkaloid to skimmianine in *Z. zanthoxyloides*), inhibits secretion of TNFα, IL-6 and IL-1β in LPS-stimulated BV2 microglia cells (Z. Zhang, Li, Li, & An, 2016). This may suggest that there are other anti-inflammatory alkaloids present in of *Z. zanthoxyloides* in BV2 cells. The important role of increased production of pro-inflammatory factors including TNFα, IL-6, NO in CNS inflammation has been reported (Becher & Spath, 2017). Moreover, the prolonged exposure of neurons to pro-inflammatory cytokines and mediators have been demonstrated to result in neurotoxicity and thus, advancement of neurodegeneration (Balez et al., 2016; G. Olmos & J. Lladó, 2014). As a result, the anti-neuroinflammatory effect of skimmianine is significant in reducing neuroinflammation and suggest its potential role towards treatment of neurodegenerative diseases such as AD and also in cerebral malaria.

Further investigations revealed that, skimmianine reduced the secretion of pro-IL-1β and IL-1β cytokine through inhibition of NLRP3 protein expression and caspase-1 activity in hemozoin-stimulated BV2 microglia cells. Previous investigations have

identified the inhibitory role of berberine on activation of NLRP3 inflammasome to reduce the secretion of IL-1β in murine macrophage cells (C.-G. Li et al., 2017). In addition, therapeutic targeting of activated NLRP3 pathway has been reported to be an important strategy in reducing neuroinflammation in cerebral malaria (Sadaf Jahan et al., 2017; Kordes, Matuschewski, & Hafalla, 2011) . Reports have further shown an association between NLRP3 activation and long term cognitive dysfunction in children with cerebral malaria (Pais & Penha-Gonçalves, 2018; Song et al., 2017). Therefore, the inhibitory role of skimmianine on secretion of IL-1β in neuroinflammation through NLRP3 pathway is essential and may be promising in the treatment of cerebral malaria.

In neuroinflammation, several studies have established the role of NF-κB signalling pathway activation in increasing the expression of genes such as COX-2 and iNOS gene (Biswas & Bagchi, 2016; Feng et al., 2017). In addition, reports have further demonstrated that inhibition of activated NF-κB is crucial in reducing the level of pro-inflammatory cytokines and mediators in neuroinflammation, which is involved in the pathology of neurodegenerative diseases and cerebral malaria (Nebl, De Veer, & Schofield, 2005; Verzola et al., 2017). Skimmianine inhibited the NF-κB signalling pathway in BV2 cells activated with hemozoin and LPS. Interestingly, similar results have been reported for berberine, which suppressed NF-κB activation in BV2 microglia cells and in murine primary microglia (Jia et al., 2012; Nam et al., 2010).

Generally, this study has clearly demonstrated that inhibition of neuroinflammation by *Z. zanthoxyloides* extract may possibly be due to the presence of mixture of phytochemical compounds acting in synergy in this extract, which may contribute to the activity of *Z. zanthoxyloides* extract. This study has further established that one of such anti-neuroinflammatory compounds is skimmianine. Furthermore, reports have

shown other compounds isolated from *Z. zanthoxyloides* extract to inhibit inflammation *in vivo* and *in vitro*. Berberine, a compound isolated from *Z. zanthoxyloides* extract was shown to reduce neuroinflammation by activating AMPK in BV2 microglia (Lu, Dah-Yuu, Tang, Chen, & Wei, 2010) and also inhibit NF-κB pathway in macrophage cells (H. Zhang et al., 2017). In addition, pellitorine, one of the isolates in *Z. zanthoxyloides* extract was also reported to have anti-inflammatory activity on LPS induced- monocytes and in mice (W. Lee et al., 2014). These studies imply that the anti-inflammatory activities of other compounds isolated from *Z. zanthoxyloides* extract are significant in different models of inflammation. Therefore, it would be interesting to further investigate the anti-neuroinflammatory activity of other phytochemical compounds in *Z. zanthoxyloides* extract. This might provide more information on the anti-inflammatory activity of compounds isolated from *Z. zanthoxyloides* extract and identify compounds that act in synergy in the extract to produce inhibitory effect on inflammation.
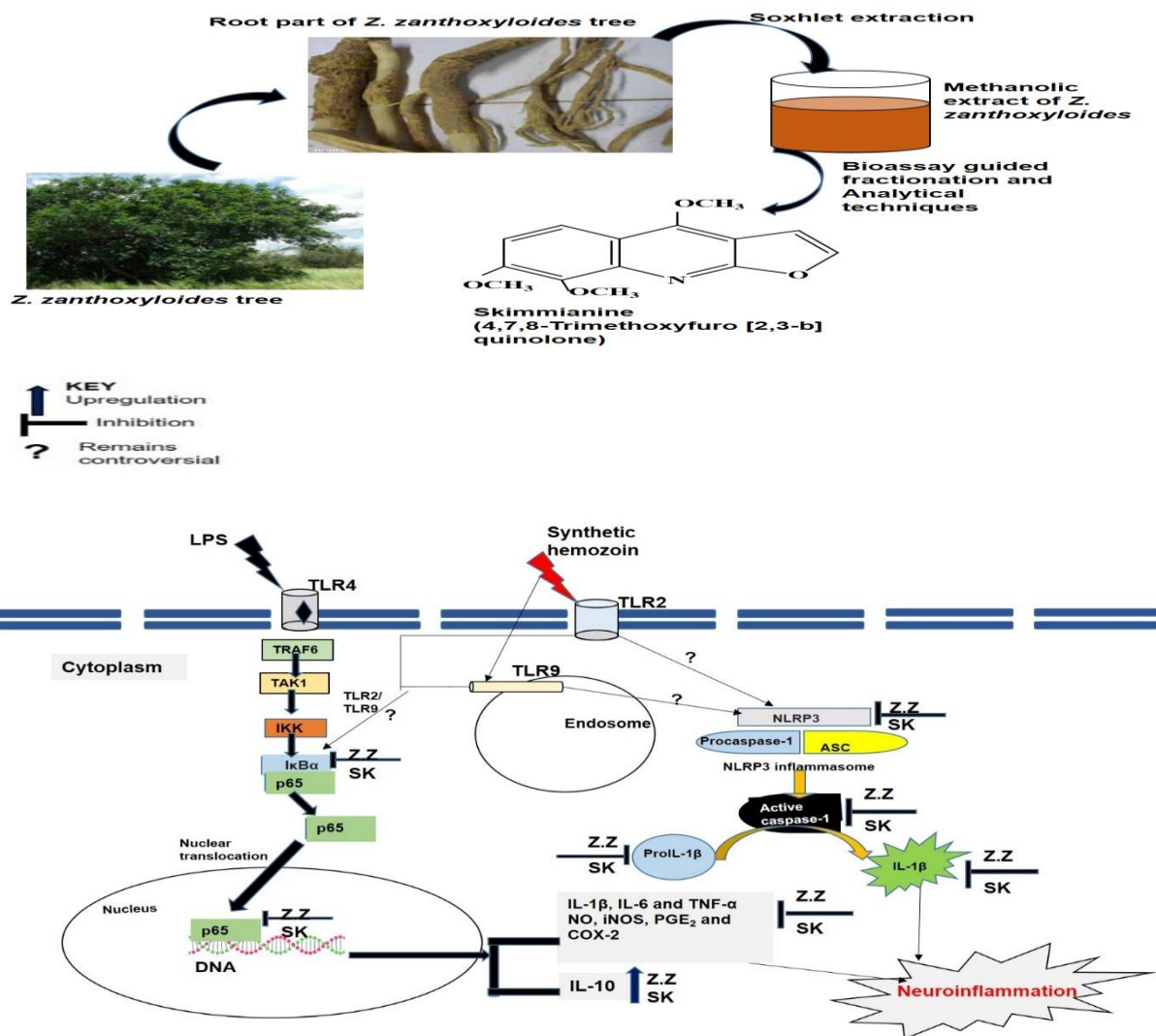
Figure 5-1: Isolation of skimmianine from *Zanthoxylum zanthoxyloides* extract and effect of *Zanthoxylum zanthoxyloides* and skimmianine in BV2 microglia cells stimulated with LPS or synthetic hemozoin

Z.Z: *Zanthoxylum zanthoxyloides*, SK: Skimmianine, TLR4: Toll like receptor-4, TLR9: Toll like receptor-9, TLR2: Toll like receptor 2, LPS: Lipopolysaccharide, IL-1β: Interleukin- 1 beta, IL-6: Interleukin-6, IL-10: Interleukin-10, TNFα: Tumor necrosis factor-alpha, iNOS: Inducible nitric oxide synthase , NO: Nitric oxide, PGE_2: Prostaglandin E_2, COX-2: Cyclooxygenase-2, NLRP3: Nod like receptor protein3, ASC: associated speck-like protein containing a caspase activating recruitment domain, DNA: Deoxyribonucleic acid, , IκB: Inhibitor of kappa B, IKK: IκB kinase. Figure 5.1 shows that In LPS activated microglia cells, *Zanthoxylum zanthoxyloides* (Z.Z) and skimmianine (SK) targeted IκB/NF-κB signalling pathway to reduce production of IL-1β, IL-6, TNFα, NO, PGE_2, expression of COX-2 and iNOS enzyme. Also, an increase in production of IL-10 was observed on treatment of LPS-stimulated BV2 cells with Z. zanthoxyloides extract and skimmianine. Reports have shown that hemozoin activates NLRP3 inflammasome to cause excessive secretion of IL-1 β but the role of TLR2 or TLR9 on NLRP3 pathway remains controversial (Olivier et al., 2015). This figure also shows that Z. zanthoxyloides extract and skimmianine inhibited the secretion of IL-1β by reducing the level of pro-IL-1β, caspase-1 activity and expression of NLRP3 protein. Studies have demonstrated that binding of hemozoin toTLR2 and TLR9 causes activation of NF-κB signalling pathway (M. Jaramillo et al., 2009; Y. Li et al., 2017). In this study, Z. zanthoxyloides extract and skimmianine reduced hemozoin induced neuroinflammation through inhibition of IκB-α/ NF-κB pathway in BV2 microglia cells.

5.2 Conclusion

The outcome of this study has shown the isolation of anti-neuroinflammatory skimmianine from *Z. zanthoxyloides* extract. Results in these findings have further demonstrated that *Z. zanthoxyloides* extract and skimmianine inhibited neuroinflammation through NF-κB signalling pathway in BV2 microglia cells stimulated with LPS. More investigations revealed that *Z. zanthoxyloides* extract and isolated skimmianine suppressed hemozoin induced neuroinflammation by targeting NF-κB and NLRP3 pathway.

5.3 Recommendations for future studies

Further investigations are needed to determine the role of *Z. zanthoxyloides* extract and skimmianine.in neuroinflammation. Studies on the effect skimmianine in animal models of neurodegenerative diseases such as Alzheimer's disease. This is an important phase in drug development and will further explain the pharmacokinetic properties of skimmianine. The role of BBB (blood-brain barrier) in the CNS is a major factor to be considered for drugs to act in the CNS. Hence, it is important to investigate the permeability of skimmianine across the BBB. Similarly, synthesising an analogue of skimmianine is recommended to increase the lipophilicity of skimmianine such as addition of a non-polar group to the structure of skimmianine. This may contribute to the transport of skimmianine across the CNS.

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