# *IN VITRO* ANTI-NEUROINFLAMMATORY AND NEURITOGENIC STIMULATORY EFFECTS OF A MEDICINAL MUSHROOM *LIGNOSUS RHINOCEROTIS* (COOKE) RYVARDEN

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**FACULTY OF SCIENCE**
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Field of Study: FUNGAL BIOTECHNOLOGY

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## ABSTRACT

Neuroinflammation and neurite degeneration are contributing factors leading to the progression of neuronal loss and age-related neurodegenerative diseases e.g Alzheimer's and Parkinson's disease. Current drug therapy for neurodegenerative diseases is ineffective with many side effects and it is palliative and manages only the symptoms of the diseases. In recent years, the attention of researchers has been inclined towards the alternative and complementary approaches, such as dietary supplementations and functional foods which have minimal side effects. Numerous neuroactive substances from natural sources as preventive and therapeutic agents for neurodegenerative diseases by promoting anti-neuroinflammatory and neuritogenic stimulatory potential have received extensive attention. Among the natural sources explored for anti-neuroinflammatory and neuritogenic stimulatory properties, medicinal mushrooms have shown huge potential. In the present study, four medicinal mushrooms were evaluated for cytotoxic, inhibition of nitric oxide (NO) production and neuritogenic stimulatory activities in murine BV2 microglial and rat pheochromocytoma (PC-12) cells. The preliminary results showed that the hot aqueous extract of *Lignosus rhinocerotis* sclerotium significantly ($p < 0.05$) inhibited NO production (75.57%) in lipopolysaccharides (LPS)-stimulated BV2 microglia and stimulated 20.99 ± 1.01% of neurite bearing cells in PC-12 cells that comparable to the positive control, 50 ng/ml of nerve growth factor (NGF). The hot aqueous extract of *L. rhinocerotis* sclerotium was not cytotoxic to BV2 and PC-12 cells after 48 hours of exposure. The hot aqueous extract of *L. rhinocerotis* sclerotium was further fractionated into three solvent fractions: ethyl acetate, n-butanol and aqueous fractions. The anti-neuroinflammatory and neuritogenic stimulatory effects of the solvent fractions and the underlying mechanisms were investigated. The results demonstrated that ethyl acetate and n-butanol fractions (125 and 250 µg/ml) inhibited the production of pro-inflammatory mediators and cytokines, including NO, prostaglandin $E_2$ ($PGE_2$), tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), and the expressions of inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), TNF-α, IL-1β and IL-6 in LPS-stimulated BV2 microglia. The anti-neuroinflammatory effects of ethyl acetate and n-butanol fractions are mediated through the suppression of the toll-like receptor 4 (TLR4), mitogen-activated protein kinases (MAPKs) (extracellular signal-regulated kinases1/2 (ERK1/2), stress-activated protein kinases/jun amino-terminal kinases (SAPK/JNK) and p-38 MAPK), protein kinase B (AKT) and nuclear factor-kappaB (NFκB) signaling pathways with the inhibition of the transcription factors, cyclic adenosine monophosphate (cAMP) response element-binding protein (CREB), activator protein 1 (AP-1) and NFκB. The ethyl acetate and n-butanol fractions (10 µg/ml) significantly ($p < 0.05$) stimulated a higher percentage of neurite bearing cells compared to NGF (50 ng/ml) without stimulating the production of NGF in PC-12 cells. The ethyl acetate and n-butanol fractions mimicked the neuritogenic activity of NGF by targeting the tropomyosin receptor kinase A (TrkA) receptor and activated the mitogen-activated protein kinase kinase (MEK)/ERK1/2 and phosphoinositide 3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) signaling pathways with the phosphorylation of CREB, and leading to the increased expressions of neuritogenesis biomarker, the growth associated protein 43 (GAP43), tubulin alpha 4A (TUBA4A) and tubulin beta 1 (TUBB1) in PC-12 cells. In conclusion, the present findings demonstrated that *L. rhinocerotis* sclerotium mitigated neuroinflammation and stimulated neuritogenesis in BV2 and PC-12 cells, respectively.

## ABSTRAK

Keradangan neuro dan degenerasi neurit merupakan faktor-faktor yang mengakibatkan kemerosotan dan kehilangan sel saraf serta penyakit-penyakit neurodegenerasi seperti penyakit Alzheimer's dan Parkinson's. Terapi ubat-ubatan semasa bagi penyakit-penyakit neurodegenerasi kurang berkesan, mempunyai kesan sampingan dan ia adalah paliatif di mana ia hanya menguruskan gejala-gejala penyakit-penyakit tersebut. Sejak kebelakangan ini, perhatian para penyelidik cenderung kepada pendekatan alternatif dan pelengkap, seperti suplemen makanan dan makanan berfungsi yang mempunyai kesan sampingan yang minimum. Terdapat banyak bahan-bahan neuroaktif daripada sumber semulajadi yang berpotensi mencegah keradangan neuro dan merangsang pengunjuran neurit telah mendapat perhatian yang meluas sebagai ejen pencegahan dan terapeutik untuk penyakit neurodegenerasi. Antara calon-calon daripada sumber semulajadi, cendawan ubatan telah menunjukkan potensinya dalam aktiviti mencegah keradangan neuro dan merangsang pengunjuran neurit. Dalam kajian ini, empat jenis cendawan ubatan telah dipilih untuk penilaian tahap ketoksian, kesan menghalang penghasilan nitrik oksida dan merangsang pengunjuran neurit dengan menggunakan sel BV2 mikroglia dan sel pheochromocytoma (PC-12). Hasil awalan menunjukkan bahawa ekstrak akueus panas sclerotium *Lignosus rhinocerotis* berupaya menghalang 75.57% penghasilan nitrik oksida (NO) dari BV2 mikroglia yang dirangsang oleh lipopolisakarida (LPS) secara signifikan ($p < 0.05$) dan meningkatkan peratus pengunjuran neurit sel PC-12 sebanyak 20.99 ± 1.01% yang setanding dengan kontrol positif, iaitu 50 ng/ml faktor pertumbuhan saraf (NGF). Ekstrak akueus panas sclerotium *L. rhinocerotis* tidak menunjukkan kesan toksik terhadap sel-sel BV2 mikroglia dan PC-12 selepas pendedahan selama 48 jam. Ekstrak akueus panas sclerotium *L. rhinocerotis* telah difraksinasikan kepada tiga jenis fraksi pelarut, iaitu fraksi etil asetat, fraksi n-butanol dan fraksi akueus. Kesan anti keradangan neuro dan rangsangan pengunjuran neurit fraksi-fraksi tersebut serta mekanisme yang mendasari kesan-kesan ini turut dikaji. Hasil kajian menunjukkan bahawa fraksi-fraksi etil asetat dan n-butanol (125 dan 250 µg/ml) berupaya menghalang penghasilan mediator-mediator pro radang dan sitokin, termasuk NO, prostaglandin $E_2$ ($PGE_2$), alfa nekrosis faktor tumor (TNF-α) dan interleukin-1β (IL-1β) dan juga ekspresi-ekspresi iNOS (inducible nitric oxide synthase), COX-2 (cyclooxygenase-2), TNF-α, IL-1β dan IL-6 dari BV2 mikroglia yang dirangsang oleh LPS. Kesan anti keradangan neuro bagi fraksi-fraksi etil asetat dan n-butanol adalah bertindak melalui penghalangan reseptor TLR4 (toll like receptor 4) serta isyarat-isyarat laluan seperti pengaktifan mitogen protein kinase (MAPKs) (isyarat luaran sel (ERK1/2), pengaktifan stress protein kinase/jun amino terminal kinase (SAPKJNK) dan p-38 MAPK), protein kinase B (AKT) dan faktor nuclear kappa B (NFκB) dengan penindasan factor-faktor transkripsi cyclic adenosine monophosphate (cAMP) response element-binding protein (CREB), protein pengaktif 1 (AP-1) dan NFκB. Fraksi-fraksi etil asetat dan n-butanol (10 µg/ml) merangsang lebih tinggi peratus pengunjuran neurit berbanding dengan NGF (50 ng/ml) secara signifikan ($p < 0.05$) tanpa merangsang penghasilan NGF dalam sel PC-12. Fraksi-fraksi etil asetat dan n-butanol meniru aktiviti pengunjuran neurit NGF dengan mensasarkan reseptor TrkA (tropomyosin receptor kinase A) dan mengaktifkan isyarat-isyarat laluan seperti pengaktifan mitogen protein kinase kinase (MEK)/ERK1/2, phosphoinositide 3-kinase (PI3K)/AKT/sasaran mammalian rapamycin (mTOR) dan meningkatkan pemfosforilan CREB dengan seterusnya meningkatkan ekspresi-ekspresi penanda bio pengunjuran neurit seperti protein pertumbuhan 43 (GAP43), tubulin alfa 4A (TUBA4A) dan tubulin beta 1 (TUBB1) dalam sel PC-12. Kesimpulan, hasil kajian ini mencadangkan bahawa sclerotium *L. rhinocerotis* dapat mengurangkan keradangan neuro dan merangsang pengunjuran neurit.

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## ABBREVIATIONS AND SYMBOLS

| | |
|---|---|
| % | Percentage |
| X | Times |
| µg | Microgram |
| µl | Microlitre |
| µm | Micrometre |
| µM | Micromolar |
| Abs | Absorbance |
| AKT | Protein kinase B |
| Amyloid-β | Amyloid beta |
| AP-1 | Activator protein 1 |
| Aspirin | Acetylsalicylic acid |
| ATCC | American type culture collection |
| BBB | Blood-brain barrier |
| BDNF | Brain-derived neurotrophic factor |
| BNB | Blood-nerve barrier |
| cDNA | Complementary deoxyribonucleic acid |
| CNS | Central nervous system |
| $CO_2$ | Carbon dioxide |
| COX-2 | Cyclooxygenase-2 |
| CREB | cyclic adenosine monophosphate (cAMP)-response element binding protein |
| DAPI | 4'-6-diamidino-2-phenylindole |
| DMEM | Dulbecco's modified eagle's medium |
| DMSO | Dimethyl sulfoxide |

| | |
|---|---|
| EDTA | Ethylenediaminetetraacetic acid |
| ELISA | Enzyme-linked immunosorbent assay |
| eNOS | Endothelial nitric oxide synthase |
| ERK | Extracellular-signal regulated kinases |
| F-12 K | Kaighn's modification of Ham's F-12 |
| FBS | Fetal bovine serum |
| FGF | Fibroblast growth factor |
| FITC | Fluorescein isothiocyanate |
| g | Gram |
| GAP43 | Growth associated protein 43 |
| GDNF | Glial-derived neurotrophic factor |
| HPLC | High performance liquid chromatography |
| HRP | Horseradish peroxidase |
| $IC_{50}$ | Half maximal inhibitory concentration |
| IgG | Immunoglobulin G |
| IκB | Inhibitor of nuclear factor kappa B |
| IKK | Inhibitor of nuclear factor kappa B kinase |
| iNOS | Inducible nitric oxide synthases |
| JNK | c-Jun N-terminal kinase |
| kg | Kilogram |
| l | Litre |
| LDH | Lactate dehydrogenase |
| IFN-γ | Interferon-gamma |
| IL | Interleukin |
| IL-1β | Interleukin 1 beta |
| L-NAME | Nω-Nitro-L-arginine methyl ester |

| | |
|---|---|
| LPS | Lipopolysaccharide |
| m | Metre |
| MAPK | Mitogen-activated protein kinase |
| MEK | Mitogen-activated protein kinase |
| mg | Milligram |
| ml | Millilitre |
| mm | Millimetre |
| mM | Millimolar |
| MMP-3 | Matrix metalloproteinase-3 |
| mTOR | Mammalian target of rapamycin |
| MTS | 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium |
| MTT | 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide |
| MyD88 | Myeloid differentiation primary response gene 88 |
| NADPH | Nicotinamide adenine dinucleotide phosphate |
| NFκB | Nuclear factor kappa B |
| ng | Nanogram |
| NGF | Nerve growth factor |
| nm | Nanometre |
| nM | Nanomolar |
| nNOS | Neuronal nitric oxide synthase |
| No. | Number |
| NO | Nitric oxide |
| NSAID | Non-steroidal anti-inflammatory drug |
| NT | Neurotrophin |
| $^{o}$C | Degree Celsius |

| | |
|---|---|
| OD | Optical density |
| PBS | Phosphate buffered saline |
| PC-12 | Pheochromocytoma cells |
| pg | Pico gram |
| $PGE_2$ | Prostaglandin E2 |
| PI3K | Phosphatidylinositol-3 kinase |
| PMSF | Phenylmethylsulfonyl fluoride |
| PNS | Peripheral nervous system |
| qRT-PCR | Quantitative reverse transcriptase polymerase chain reaction |
| RNA | Ribonucleic acid |
| RNS | Reactive nitrogen species |
| ROS | Reactive oxygen species |
| SAPK | Stress-activated protein kinase |
| SD | Standard deviation |
| TGF-β | Transforming growth factor-beta |
| TLR4 | Toll like receptor 4 |
| TMB | 3,3′,5,5′-tetramethylbenzidine |
| TNF-α | Tumor necrosis factor alpha |
| TrkA | Tropomyosin receptor kinase A |
| TUBA4A | Tubulin alpha 4A |
| TUBb1 | Tubulin beta 1 |
| UV | Ultraviolet |
| v/v | Volume per volume |
| w/v | Weight per volume |
| w/w | Weight per weight |
| WHO | World Health Organization |

# CHAPTER I: GENERAL INTRODUCTION

## 1.1 INTRODUCTION

Neurodegenerative diseases are an increasing public health concern with respect to global burden of disease in terms of human suffering and economic cost. The morbidity and mortality of neurodegenerative diseases is increasing. However, current drug therapy for neurodegenerative diseases is ineffective with many side effects and it is palliative and manages only the symptoms of the diseases (Nowacek, Kosloski, & Gendelman, 2009; Robichaud, 2006). Dementia is a clinical syndrome under the umbrella term neurodegenerative disease, which includes Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, and multiple sclerosis. Alzheimer's disease is the world's most common and most feared form of dementia that gradually gets worse over time. It affects memory, thinking, and behaviour (Burns & Iliffe, 2009). According to the World Health Organization (WHO), in 2010 nearly 35.6 million people worldwide lived with dementia. The number is expected to double (65.7 million) by 2030 and more than triple (115.4 million) by 2050 (Acosta & Wortmann, 2009). In Malaysia, the population stands at over 28 million people. According to the Alzheimer Disease International report, the prevalence of dementia in Malaysia in 2005 was 0.063% and the annual incidence rate was 0.020% (≥ 560,000 people) (Economics, 2006). It is projected that this figure will increase to 0.126% and 0.454% in 2020 and 2050, respectively (Economics, 2006). However, the countries in the Asia Pacific region including Malaysia may not be well prepared to provide quality health care services for the people with dementia and their caregivers (Economics, 2006).

Current pharmacological approaches for neurodegenerative diseases are limited in their ability to modify significantly the course of the disease (Nowacek et al., 2009).

It has been shown that long-term administration of synthetic drug has the potential for significant side effects (Fosbøl et al., 2009; Dugowson & Gnanashanmugam, 2006). As alternative approaches, there has been a global spotlight on the neurosciences research focus on the study of neurodegenerative processes and discovery of potential agents from natural sources for the management of neurodegenerative diseases.

Increasing evidences showed that glial-mediated neuroinflammation (Frank-Cannon, Alto, McAlpine, & Tansey, 2009; Kim & Joh, 2006) and neurite or axon degeneration (Wang, Medress, & Barres, 2012; Bjartmar, Wujek, & Trapp, 2003) are among the main contributors to the pathogenesis of neurodegenerative diseases. Glial-mediated neuroinflammation results from persistent activation of microglia triggered by various injurious or inflammatory stimuli (Frank-Cannon et al., 2009). Uncontrolled and prolonged activation of microglia triggers sustained release of various pro-inflammatory mediators and cytokines that are toxic to neuronal cells and central nervous system (CNS), including nitric oxide (NO), prostaglandins E2 ($PGE_2$), inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β) and interleukin-6 (IL-6) (Ramesh, MacLean, & Philipp, 2013; Ramesh, Philipp et al., 2013). The sustained release of these pro-inflammatory mediators and cytokines may trigger neuronal and glial dysfunction, neurite or axon degeneration, neurodegeneration and brain destruction (Ramesh, MacLean et al., 2013; Ramesh, Philipp et al., 2013).

Neurite or axon degeneration is an early event that leads to the progressive neuronal cell loss in neurodegenerative diseases (Wang, Medress, & Barres, 2012; Bjartmar, Wujek, & Trapp, 2003). Development and regeneration of the network of neurites (neuritogenesis) establishes a strategy for the prevention and treatment of neurodegenerative diseases (Santos et al., 2015). Evidences revealed the ability of neurotrophins, exemplified by nerve growth factor (NGF) acts as stimulatory substances

to triggers regeneration of neurite of damaged neurons (Pesavento, Capsoni, Domenici, & Cattaneo, 2002; McKerracher, 2001). However, the neuroactivity and therapeutic application of NGF is restricted by its high molecular weight polypeptide structure (Granholm et al., 1998; Poduslo & Curran, 1996). It is unstable and unable to cross the blood-brain barrier (BBB) and blood-nerve barrier (BNB) (Granholm et al., 1998; Poduslo & Curran, 1996). Thus, discovery of smaller substances from natural or dietary sources with low molecular weight that mimic and/or enhance the neuritogenic stimulatory activity of NGF is mandatory.

Reducing glial-activated neuroinflammation and promoting neurite regeneration may be preventive and therapeutic strategies for neurodegenerative diseases. Neuroactive components from natural sources including dietary supplements and functional foods as preventive and therapeutic agents for neurodegenerative diseases by promoting anti-neuroinflammatory (Karunaweera, Raju, Gyengesi, & Münch, 2015) and neuritogenic potential (Phan, David, Naidu, Wong, & Sabaratnam, 2014; Tohda, Kuboyama, & Komatsu, 2005) have received extensive attention. As compared to the synthetic drugs currently available for neurodegenerative diseases, natural products or functional foods serve a better choice as alternative and complementary medicine for the management of neurodegenerative diseases (Karunaweera et al., 2015; Phan, David, Naidu, et al, 2014).

From ancient to modern times, natural products and functional foods have been used as alternative and complementary medicine to manage and treat numerous ailments. It is believed that natural products and functional foods that possess anti-neuroinflammatory and neuritogenic stimulatory effects may play a role in neuronal health maintenance. Among them, culinary and medicinal mushrooms are been shown to have potential as functional food for neuronal health maintenance (Xu & Beelman, 2015; Phan, David, Naidu, et al., 2014).

Mushrooms are widely consumed as food by many societies throughout the world and are appreciated for their texture, flavor, high nutritional values (Chang & Miles, 1989), and pharmacological properties (Lindequist, Niedermeyer, & Jülich, 2005). Mushrooms are reported as good sources of mycomedicines. Further, the medicinal mushrooms have a well-established history of use in traditional oriental therapies (Wasser & Weis, 1999; Hobbs, 1995). Moreover, edible mushrooms should not be considered only as simple food, as some of them have been shown to be rich in bioactive components. They are shown to be beneficial in prevention and/or treatment of several ailments (Wasser, 2010). A total of 126 medicinal functions are attributed to medicinal mushrooms, including anti-tumor, immunomodulating, antioxidant, radical scavenging, anti-hypercholesterolemia, and anti-diabetic effects (Wasser, 2010). The term 'mushroom nutriceuticals' was introduced by Chang & Buswell (1996) for the bioactive components that are extracted from either the mushroom mycelium or basidiocarps (fruiting body).

Recently, discovery of novel nutriceuticals that are neuroactive from medicinal mushrooms has attracted intense interest. Numerous neuroactive components isolated from several mushrooms have been shown to be potent anti-neuroinflammatory (Jayasooriya et al., 2011; Jeong et al., 2010) and had neuritogenic stimulatory activities (Xu & Beelman, 2015; Phan, David, Naidu, et al., 2014). However, many medicinal mushrooms are largely unexplored. In Malaysia, many wild or cultivated mushrooms are claimed to have medicinal usage by the local and indigenous communities. Medicinal mushrooms such as *Lignosus* spp. (Tan et al., 2012; Chang & Lee, 2004), *Amauroderma* sp. (Chan, Tan, Chua, Sabaratnam, & Kuppusamy, 2015; Chang & Lee, 2004), *Hericium erinaceus* (Wong et al., 2007), *Cordyceps sinensis* (Chang & Lee, 2004), *Ganoderma lucidum* (Chang & Lee, 2004), *Ganoderma neo-japonicum* (Tan et al., 2015), and *Tremella fuciformis* (Chang & Lee, 2004) have been used as general

tonic to boost immunity and to treat variety ailments by indigenous communities and traditional oriental medicine practitioners in Malaysia. Though the usage of these mushrooms has a long history of use as folk medicine, the nutritional and medicinal properties are scanty and poorly explored in Malaysia (Chang & Lee, 2004).

## 1.2 RESEARCH OBJECTIVES

This study aimed to identify medicinal mushrooms that possess both anti-neuroinflammatory and neuritogenic stimulatory effects *in vitro*. Four medicinal mushrooms, namely *Ganoderma lucidum* (Curtis : Fr.) P. Karst, *Ganoderma neo-japonicum* Imazeki, *Grifola frondosa* (Dicks.) Gray and *Lignosus rhinocerotis* (Cooke) Ryvarden were selected for the study.

The objectives of this study were to:

i. screen and identify the extracts of the selected medicinal mushrooms for their cytotoxic, inhibition of nitric oxide production and neuritogenic stimulatory activities.
ii. study the fractions of the extract of the selected medicinal mushroom for their cytotoxic and inhibitory effects on the production and expressions of pro-inflammatory mediators and cytokines in lipopolysaccharides-stimulated murine BV2 microglial cell line.
iii. elucidate the anti-neuroinflammatory signaling pathways of the selected medicinal mushroom fractions in lipopolysaccharides-stimulated murine BV2 microglial cell line.
iv. study the neuritogenic stimulatory effects of the selected medicinal mushroom fractions on rat pheochromocytoma (PC-12) cell line by using nerve growth factor (NGF) as positive or comparison control.

v. elucidate the involvement of NGF responsive signaling pathways of neuritogenesis stimulated by the selected medicinal mushroom fractions in PC-12 cells.

# CHAPTER II: LITERATURE REVIEW

## 2.1 NEURODEGENERATIVE DISEASES

The term "neuro-" refers to neuronal or nerve cells, while "-degeneration" refers to progressive loss or damage (Terry, 2008; Przedborski, Vila, & Jackson-lewis, 2003). Neurodegeneration is an established term applied to the progressive loss of nerve structure and function in central nervous system (CNS). This deterioration is slow and progressive neuronal dysfunction that gradually leads to a loss of cognitive abilities, such as memory and decision making (Burns & Iliffe, 2009). Neuronal degeneration is the key pathological characteristic of various age-related neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease, Huntington's disease and multiple sclerosis (Amor, Puentes, Baker, & van der Valk, 2010; Jellinger & Stadelmann, 2001). Alzheimer's disease is the most common neurodegenerative disease worldwide (Burns & Iliffe, 2009). It is one form of dementia, a brain function syndrome characterized by a cluster of symptoms and signs manifested by difficulties in memory, thinking, disturbances in language, psychological and psychiatric changes, and impairments in activities of daily living (Burns & Iliffe, 2009). Parkinson’s disease is the second most common neurodegenerative diseases, after Alzheimer’s disease (Tanner & Goldman, 1996). Clinically, it is a chronic and progressive movement disorder that is characterized by tremor, rigidity, bradykinesia and postural instability, which result from the progressive loss of dopaminergic neurons in the substantia nigra (Warner & Schapira, 2003; Spillantini, Crowther, Jakes, Hasegawa, & Goedert, 1998). The prevalence of neurodegenerative diseases is increasing rapidly (Acosta & Wortmann, 2009). Neurodegenerative diseases have been identified as the major clinical problem in the developed countries (Ross & Tabizi, 2011; Hung, Chen, Hsieh, Chiou, & Kao, 2010)

and major economic burdens for health care systems (Olesen, Gustavsson, Svensson, Wittchen, & Jonsson, 2012). Neurodegenerative disease is going to become increasingly important as the population ages. It has been demonstrated that neuroinflammation (Glass et al., 2010; Frank-Cannon, Alto, McAlpine, & Tansey, 2009) and neurite or axon degeneration (Wang, Medress, & Barres, 2012; Bjartmar, Wujek, & Trapp, 2003) are contributing factors leading to the progression of neurodegenerative diseases. Therefore, effective anti-neuroinflammatory and neuritogenic agents may be benefits for the management, prevention and treatment of neurodegenerative diseases.

## 2.2 NEUROINFLAMMATION

The inflammatory response is an early and non-specific immune reaction to tissue damage or pathogen invasion. Neuroinflammation refers to a local tissue inflammatory response of the CNS and is characterized by activation of innate immune system, release of pro-inflammatory mediators and vascular permeability (Tansey, McCoy, & Frank-Cannon, 2007). Acute neuroinflammatory response is an immediate and short lived activation of resident glial cells such as microglia and astrocytes, trigger by various injurious or inflammatory stimuli such as infections, trauma, stroke and toxins within the CNS (Popovich & Longbrake, 2008; Crutcher et al., 2006). Acute neuroinflammatory response is basically a defensive response and usually beneficial to the CNS, where it promotes the reparation of damaged tissue and reduces further injury (Frank-Cannon, et al., 2009; Streit, Mrak, & Griffin, 2004). In contrast, chronic neuroinflammation is a long-standing neuroinflammatory response results from persists long after an initial injury or insult by stimuli. In chronic neuroinflammation, long-standing and self-perpetuating activation of microglia triggers sustained release of various pro-inflammatory mediators and cytokines that are toxic to neuronal cells and CNS (Figure 2.1) (Frank-Cannon et al., 2009; Streit et al., 2004). The sustained release

of pro-inflammatory mediators and cytokines continue to trigger the extension and repetition of the inflammatory cycle by persistent activation of microglia. Prolonged activation of microglia resulting in further release of pro-inflammatory mediators such as nitric oxide (NO), cytokines, chemokines, prostaglandins, reactive oxygen and nitrogen species (ROS/RNS), which result in neurites or axon degeneration, neuronal dysfunction, neuronal loss (necrosis or apoptosis), and eventually leads to neurodegenerative diseases (Figure 2.1) (Frank-Cannon, et al., 2009; Streit et al., 2004).

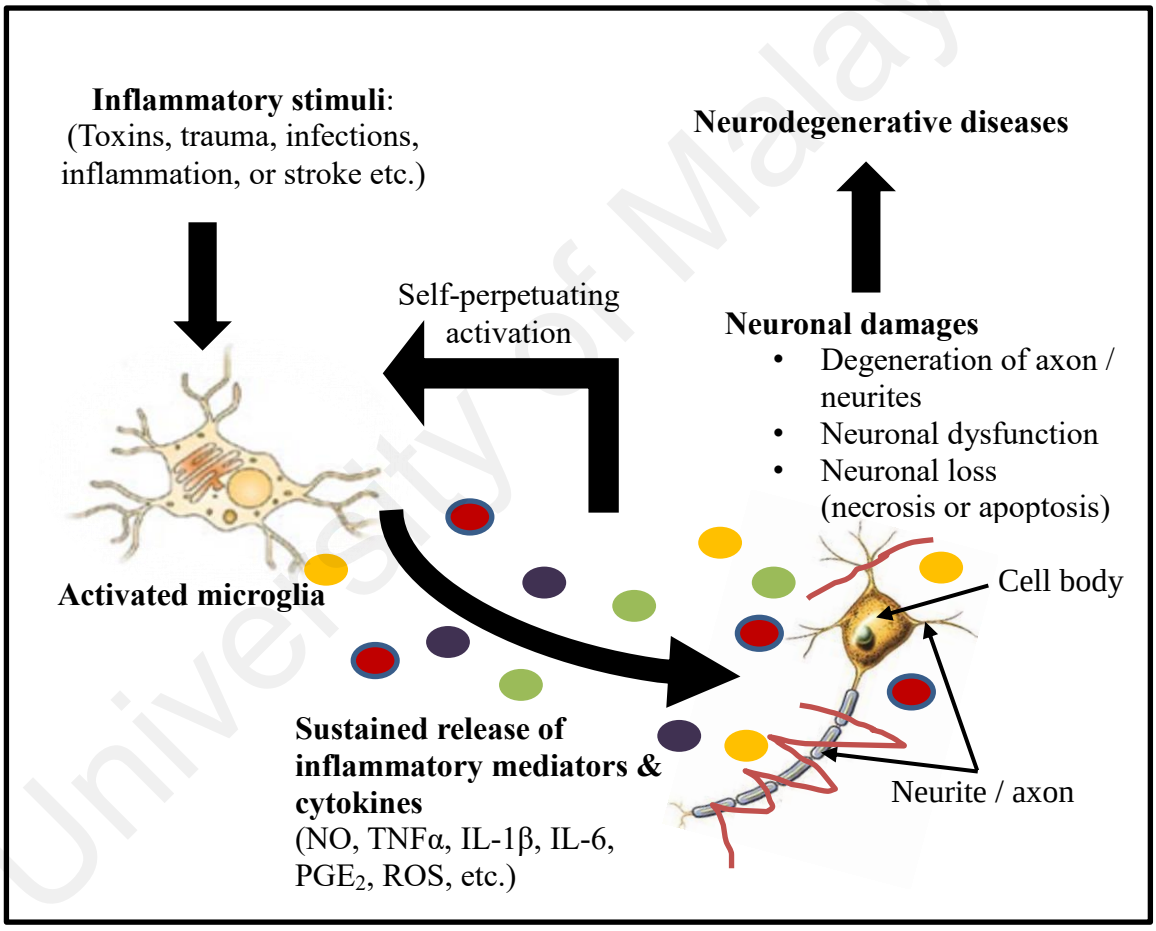



**Figure 2.1: Chronic neuroinflammation process.** Chronic neuroinflammation is a long-standing neuroinflammatory response results from persists long after an initial injury or insult by stimuli. Long-standing and self-perpetuating activation of microglia triggers sustained release of various pro-inflammatory mediators and cytokines that continue trigger the extension and repetition of the inflammatory cycle. The overproduction of pro-inflammatory mediators and cytokines may resulted in neurites or axon degeneration, neuronal dysfunction, neuronal loss, and eventually lead to neurodegenerative diseases.

### 2.2.1 Neuroinflammation and neurodegenerative diseases

Accumulating evidence pointed to neuroinflammation as an active participant in the progression of neurodegenerative diseases (Glass et al., 2010). It has been demonstrated that Alzheimer's disease (Heneka et al., 2015; Eikelenboom et al., 2002), Parkinson's disease (More, Kumar, Kim, Song, & Choi, 2013; Kim & Joh, 2006), multiple sclerosis (Yiangou et al., 2006), Huntington's disease (Möller, 2010; Sapp et al., 2001), amyotrophic lateral sclerosis (Yiangou et al., 2006; Sargsyan, Monk, & Shaw, 2005), and tauopathies (Yoshiyama et al., 2007) are associated with chronic neuroinflammation and elevated levels of several cytokines. Neuropathological and neuroradiological studies have been indicated that neuroinflammatory responses maybe take part prior to a substantial loss of the neuronal cells in the progression of neurodegenerative diseases (Frank-Cannon et al., 2009). Unfortunately, there is still no effective neuroprotective therapy, nor a definitive medical diagnosis (Burns & Iliffe, 2009).

The relationship between neuroinflammation and Alzheimer's disease has been extensively studied (Heneka et al., 2015; Eikelenboom et al., 2002). Accumulation of microglia at amyloid plaques in Alzheimer's disease brain have been reported (McGeer, Itagaki, Tago, & McGeer, 1987). Activation of microglia is an early pathogenic event in Alzheimer's disease (Block et al., 2007; Eikelenboom et al., 2002). Activation of microglia is an early pathogenic event in Alzheimer's disease (Block et al., 2007; Eikelenboom et al., 2002). Amyloid-β has been reported as one of the inflammatory stimuli that trigger the microglia activation and sustained release of pro-inflammatory mediators and cytokines that cause neurotoxicity to the neurons in Alzheimer's disease (Block et al., 2007; Eikelenboom et al., 2002). It has also been reported that activated microglial cells are found in the area of degenerating dopaminergic neurons in the substantia nigra, putamen, hippocampus, transentorhinal cortex, cingulate cortex and temporal cortex of patients with Parkinson’s disease (Imamura et al., 2003; Langston et

al., 1999). Neurotoxic factors released by damaged dopaminergic neurons were reported to stimulate the production of ROS by prolonged activated microglia in Parkinson's disease (Zhang, Wang, et al., 2005). Several reports demonstrated that neurodegenerative diseases increase in prevalence with age. Prolonged activation of microglia and chronic neuroinflammation are associated with age-related neurodegenerative diseases (Orellana et al., 2015; Blasko et al., 2004). Therefore, aging is also an important factor contributes to microglia-mediated neuroinflammation and the progression of neurodegenerative diseases.

### 2.2.2 Microglia-mediated neuroinflammation

Microglia-mediated neuroinflammation is an important mechanism responsible for the initiation and progression of neurodegenerative diseases (Frank-Cannon et al., 2009). Microglial cells are the resident macrophages of the CNS and play important roles in brain immune systems (Barron, 1995) and neuroinflammation (Ransohoff & Perry 2009; Hanisch & Kettenmann 2007). Numbers of studies validated the involvement of microglia in both acute and chronic neurological diseases (Sugama et al., 2009; Hanisch & Kettenmann 2007). In acute neuroinflammatory response, activated microglia able to release cytoprotective and neuroprotective agents, include anti-inflammatory cytokines such as interleukin-10 (IL-10), interleukin-4 (IL-4), and transforming growth factor-beta (TGF-β) (Figure 2.2) (Ramesh, MacLean, & Philipp, 2013; Ramesh, Philipp, Vallières, MacLean, & Ahmad, 2013), and neurotrophic factors such as nerve growth factor (NGF), neurotrophin-3 (NT-3), neurotrophin-4/5 (NT-4/5), glial-derived neurotrophic factor (GDNF) and fibroblast growth factor (FGF), to maintain and promote neuronal survival and regeneration (Ramesh, MacLean et al., 2013; Nakajima, Tohyama, Maeda, Kohsaka, & Kurihara, 2007). However, in chronic neuroinflammation, uncontrolled or prolonged activation of microglia causes excessive production of pro-inflammatory

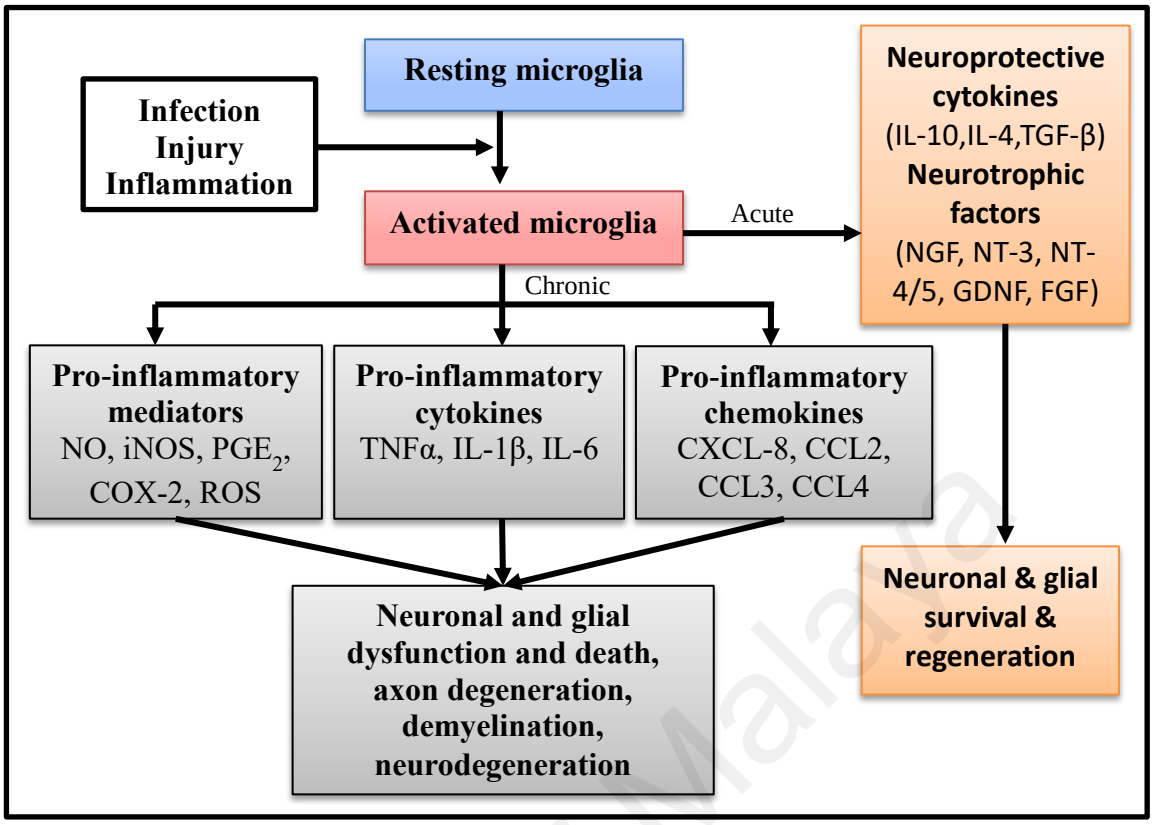



**Figure 2.2: The mechanism of microglia-mediated neuroinflammation.** Resting microglia may be activated by infection, injury or inflammation. In acute neuroinflammation, activated microglia may release cytoprotective and neuroprotective agents (anti-inflammatory cytokines and neurotrophic factors) to maintain and promote neuronal survival and regeneration. In chronic neuroinflammation, uncontrolled or prolonged activation of microglia causes excessive production of pro-inflammatory mediators, cytokines and chemokines that are neurotoxic, leading to neuronal and glial dysfunction and death, axon degeneration, demyelination, and neurodegeneration.

mediators and cytokines that are neurotoxic, such as NO, prostaglandins $E_2$ ($PGE_2$), inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), interleukin-6 (IL-6), chemokines (CXCL-8, CCL2, CCL3, CCL4), ROS and RNS (Ramesh, MacLean et al., 2013; Ramesh, Philipp et al., 2013), which results in neuronal and glial dysfunction and death, axon degeneration, demyelination, neurodegeneration and brain destruction (Figure 2.2) (Ramesh, MacLean et al., 2013; Ramesh, Philipp et al., 2013).

A review by Block, Zecca, & Hong (2007) suggested that environmental factors (infectious agents, pesticides and heavy metals) and environmental toxins

(lipopolysaccharide (LPS), paraquat and rotenone) are the source of inflammatory stimuli which stimulate microglia activation that is associated in the etiology of neurodegenerative diseases. Other common inflammatory stimuli that involved in microglia activation are chemokines (CCL5, CCL2, and CXCL10) (Möller, Hanisch, & Ransom, 2000), matrix metalloproteinase-3 (MMP-3) (Stone, Reynolds, Mosley, & Gendelman, 2009), endogenous disease proteins, and neuronal injury (Block & Hong, 2005). Inflammatory stimuli such as LPS, amyloid-β, interferon-gamma (IFN-γ), and TNF-α have often been used in current *in vitro* and *in vivo* studies of microglia-mediated neuroinflammation (Block et al., 2007). Lipopolysaccharide, the polysaccharide component of the cell walls of gram-negative bacteria is the most accepted and widely used inflammatory stimulus to stimulate microglia activation and neuroinflammatory response in *in vivo* (Herrera, Castaño, Venero, Cano, & Machado, 2000; Willard, Hauss-Wegrzyniak, & Wenk, 1999) and *in vitro* (Ko et al., 2010; Horvath, McMenemy, Alkaitis, & DeLeo, 2008) models. Treatment with LPS in cell culture or animals has been used as a useful model system for the study of Alzheimer's disease (Bester, Soma, Kell, & Pretorius, 2015) and Parkinson's disease (Carvey, Chang, Lipton, & Ling, 2003). Upon exposure of LPS, microglia is activated causing chronic neuroinflammation with overproduction of pro-inflammatory mediators and cytokine, and eventually leads to neuronal damage and death (Bester et al., 2015; Horvath et al., 2008).

### 2.2.3 Pro-inflammatory mediators and cytokines

Microglia activation in the CNS is associated with the release of pro-inflammatory mediators such as NO, $PGE_2$, ROS, inflammatory enzymes (iNOS, COX-2), and cytokines (TNF-α, IL-1β and IL-6), which are responsible as crucial factors for chronic neuroinflammation amplification. Elevated levels of pro-inflammatory mediators and

cytokines are observed at all stages of Alzheimer's disease (Mrak & Griffin, 2005). Increased expression of pro-inflammatory mediators and cytokines released by activated microglia were also found in Parkinson's disease (Tansey et al., 2007).

### 2.2.3.1 Nitric oxide (NO) and inducible nitric oxide synthase (iNOS)

Nitric oxide is an important intercellular molecule involved in the regulation of diverse physiological and pathophysiological mechanisms in cardiovascular, nervous and immunological systems (Aktan, 2004). Nitric oxide is endogenously generated enzymatically by nitric oxide synthases (NOS), from L-arginine and molecular oxygen utilizing nicotinamide adenine dinucleotide phosphate (NADPH) to L-citrulline and NO (Figure 2.3) (Tejero et al., 2011).

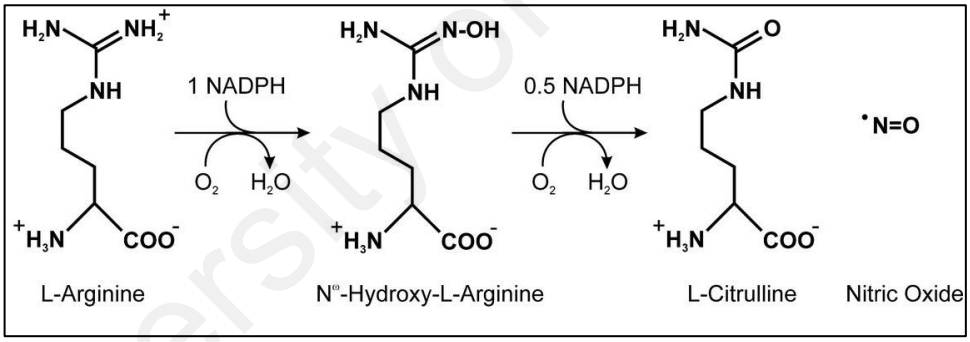



**Figure 2.3: Biosynthesis of NO by NOS enzymes** (Tejero et al., 2011).

There are three major isoforms of NOS: (i) NOS I, neuronal nitric oxide synthase (nNOS), (ii) NOS II, inducible nitric oxide synthase (iNOS) which present in various cell types upon inflammatory stimulation, and (iii) NOS III, endothelial nitric oxide synthase (eNOS) (Michel & Feron, 1997). Of these, iNOS has been regarded as an important enzyme involved in the regulation of inflammation (Vane et al., 1994) and neuroinflammation (Brown, 2010, 2007). In normal conditions, NO acts as an important biological messenger molecule with neuronal immunomodulatory effect (MacMicking, Xie, & Nathan, 1997). However, increased expression of iNOS with excessive release

of NO by activated microglia in a sustained manner is cytotoxic to the neuronal cells, causing inhibition of neurogenesis and neuritogenesis (Kim & Joh, 2006; Liu, 2003), neuronal cell death (Nakamura, Cho, & Lipton, 2012; Brown, 2010) and neuroinflammation, that correlates with the progression of neurodegeneration (Brown, 2010, 2007; Salerno, Sorrenti, Di Giacomo, Romeo, & Siracusa, 2002). Therefore, controlling and/or inhibiting the iNOS expression and production of NO may be a beneficial approach for the prevention and treatment of neuroinflammatory-associated disorders and neurodegenerative diseases.

#### 2.2.3.2 Prostaglandin $E_2$ ($PGE_2$) and cyclooxygenase-2 (COX-2)

Prostaglandin is a key inflammatory mediator that plays an important role in the generation of inflammatory response (Ricciotti, Garret, & FitzGerald, 2011). There are four major form of prostaglandins: (i) prostaglandin $E_2$ ($PGE_2$), (ii) prostacyclin ($PGI_2$), (iii) prostaglandin $D_2$ ($PGD_2$), and (iv) prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) that act as autocrine and paracrine lipid mediators to maintain local homeostasis in the body (Ricciotti et al., 2011). Generally, $PGE_2$ production is relatively low in uninflamed tissues, but will be boosted drastically starting in the early stage of inflammation (Ricciotti et al., 2011). Increased levels of $PGE_2$ in various neuroinflammatory and neuropathological conditions supported the hypothesis that $PGE_2$ leads to neurodegeneration (Shimizu & Wolfe, 1990). Prostaglandins are synthesized from arachidonic acid by cyclooxygenase (COX) (Figure 2.4) (Trappe & Liu, 2013) and their biosynthesis is significantly increased in inflammatory response (Ricciotti et al., 2011).

There are two isoforms of COX: cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) (Smith, DeWitt, & Garavito, 2000). Cyclooxygenase-1 is expressed in normal physiological functions while COX-2 is expressed in response to

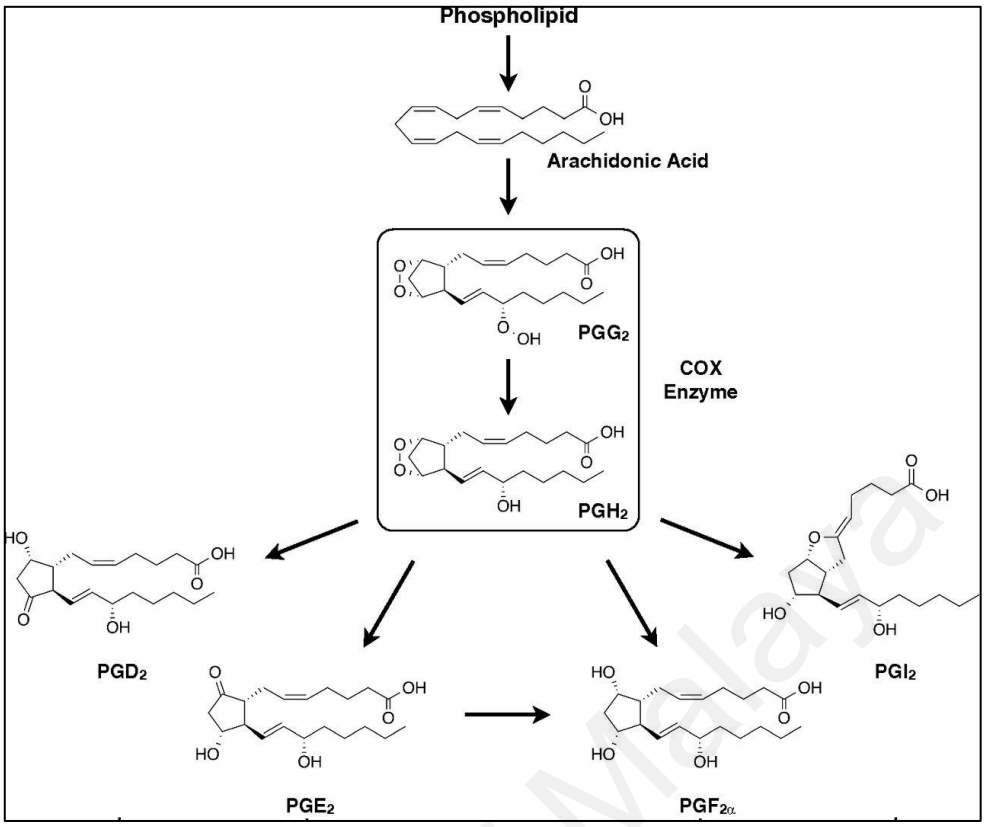



**Figure 2.4: Biosynthesis of prostaglandins by cyclooxygenase enzyme** (Trappe & Liu, 2013).

inflammatory stimuli such as cytokines and LPS (Dubois et al., 1998). Cyclooxygenase-2 is known to produce large amount of prostaglandin $E_2$, which induces inflammation (Mitchell, Larkin, & Williams, 1995) and neuroinflammation (Bauer et al., 1997; Kaufmann, Andreasson, Isakson, & Worley, 1997). Cyclooxygenase-2 plays a major role in $PGE_2$ biosynthesis in inflammatory cells and in CNS, where synthesis of $PGE_2$ is a key factor in the development of neuroinflammation (Kalinski, 2012; Ricciotti et al., 2011; Rajakariar, Yaqoob, & Gilroy, 2006). It has been demonstrated that COX-1 and COX-2 are the targets of nonsteroidal anti-inflammatory drugs (NSAIDs). The NSAIDs act as selective COX-2 and $PGE_2$ inhibitors are designed for the treatment of chronic inflammatory diseases (Kalinski, 2012; Vane, 1971) and neurodegenerative diseases, such as Alzheimer's disease (Smith, Garavito, & DeWitt, 1996; Andersen et al., 1995).

#### 2.2.3.3 Pro-inflammatory cytokines

Cytokines, a group of multifunctional substances produced by resident and migrating cells, are demonstrated to involve in inflammatory response. To date, there are more than 100 members of cytokines and their specific receptors have been identified (Hopkins, 2003; Haddad, 2002). In the CNS, cytokines act as brain's immuno-modulators and neuro-modulators which regulate the neurodevelopment, neuroinflammation, and synaptic transmission (Ramesh, MacLean, & Philipp, 2013; Benveniste, 1992). Cytokines are divided into two groups, anti- and pro-inflammatory, depending on their action during inflammation episode. The anti-inflammatory cytokines such as IL-4, IL-6, IL-10, IL-11, and IL-13 are immune-regulatory substances that control the pro-inflammatory cytokine response (Opal & DePalo, 2000). In contrast, the pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 are involved in the initiation and amplification of the inflammatory process (Dinarello, 2000). Evidences showed that pro-inflammatory cytokines are involved in the activation of the neuroinflammatory signaling pathways and transcription factors such as mitogen-activated protein kinases (MAPKs), protein kinase C (PKC), nuclear factor kappa B (NFκB), and activator protein 1 (AP-1) (Kracht & Saklatvala, 2002; Haddad, 2002). Neurodegeneration is concomitant with continual activation of microglia and overproduction of pro-inflammatory cytokines that amplify and perpetuate the inflammatory response, and eventually results in neuronal injury, damage and loss (Ramesh, MacLean et al., 2013; Frank-Cannon et al., 2009). A review by Frank-Cannon et al. (2009) presented clear evidences of the initiation and amplification roles of pro-inflammatory cytokines in various neuroinflammatory-associated neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease, Huntington's disease and amyotrophic lateral sclerosis.

**Tumor necrosis factor alpha (TNF-α)**

Tumor necrosis factor alpha (TNF-α) exerts both homeostatic and pathophysiological roles in the CNS (Montgomery & Bowers, 2012). In pathological conditions, TNF-α is considered as neurodegenerative cytokine that induces neurotoxicity and neuroinflammatory response in CNS that is associated with several neurological disorders (Frankola, Greig, Luo, & Tweedie, 2011; McCoy & Tansey, 2008). Synthesis of TNF-α by microglia is regulated and triggered by bacterial and viral pathogens, injury and immunological stimuli such as cytokines, complement factors, and immune complexes (Montgomery & Bowers, 2012). The TNF-α synthesis may also be triggered by self-perpetuating activation via binding to its associated receptors, TNF-RI and TNF-RII that initiate the neuroinflammatory cascades causing sustained release of pro-inflammatory mediators and cytokines (Montgomery & Bowers, 2012). It has been demonstrated that the IFN-γ (Mangano et al., 2012; Hanisch, 2002), amyloid-β (Chong, 1997) and LPS (Lee, Liu, Dickson, Brosnan, & Berman, 1993; Sawada, Kondo, Suzumura, & Marunouchi, 1989) are potent stimulus of TNF-α expression in microglia. Elevation of TNF-α is a hallmark of traumatic brain injury (Goodman, Robertson, Grossman, & Narayan, 1990), Alzheimer's disease (Álvarez, Cacabelos, Sanpedro, García-Fantini, & Aleixandre, 2007; Fillit et al., 1991), Parkinson's disease (Boka et al., 1994; Mogi et al., 1994), multiple sclerosis (Rieckmann et al., 1995; Sharief & Hentges, 1991), and amyotrophic lateral sclerosis (Babu et al., 2008; Poloni et al., 2000). In drug discovery and development for neurodegenerative diseases, TNF-α inhibitors have shown positive effects for the treatment of Alzheimer's disease patients with substantial cognitive and behavioral improvements (Tobinick & Gross, 2008; Tobinick, Gross, Weinberger, & Cohen, 2006). Therefore, natural substances that act as TNF-α inhibitors may bestow the alternative choice for the management of neurodegenerative diseases.

**Interleukin-1 beta (IL-1β)**

Interleukin-1 beta (IL-1β) is one of the 11 representatives of the IL-1 family (Allan et al., 2005). It is considered as one of the key pro-inflammatory cytokines involved in the pathogenesis of neuroinflammation and neurodegenerative diseases (Matousek et al., 2012; Allan et al., 2005). Evidences show that IL-1β is participated in traumatic brain injury-stimulated hippocampal neuronal cell damage (Lu et al., 2005) and dopaminergic cell death in LPS-stimulated microglia (Long-Smith et al., 2010). Abnormally elevated levels of IL-1β are found in Alzheimer's and Parkinson's diseases patients (Alam et al., 2015; Allan et al., 2005). The production of IL-1β by microglia is known to be upregulated by stimuli such as LPS (Peng et al., 2014) and amyloid-β (Wu et al., 2013; Meda et al., 1999). It has been demonstrated that IL-1β is the major inducer of COX-2 and $PGE_2$ biosynthesis (Samad et al., 2001; Molina-Holgado, Ortiz, Molina-Holgado, & Guaza, 2000). Thus, IL-1β inhibitors have been proposed as a therapeutic target in many neuronal disorders (Liu & Chan, 2014; Basu, Krady, & Levison, 2004).

**Interleukin-6 (IL-6)**

Interleukin 6 (IL-6) demonstrated both neurodegenerative (pro-inflammatory) (Morales, Farias, & Maccioni, 2010) and neuroprotective (anti-inflammatory) (Peng, Qiu, Lu, & Wang, 2005; Godbout & Johnson, 2004) activities. However, IL-6 that produced by activated microglia and astrocytes is mainly pro-inflammatory and neurodegenerative (McGeer & McGeer, 2001; Tilg, Dinarello, & Mier, 1997). Both *in vitro* (Lee, Liu, Dickson, Brosnan, & Berman, 1993) and *in vivo* (Burton, Sparkman, & Johnson, 2011; Henry, Huang, Wynne, & Godbout, 2009) studies showed that LPS is one of the major stimuli that able to boost hyperactive IL-6 response in microglia. It has also been demonstrated that IL-6 expression is induced by TNF-α and IL-1β *in vitro* (Gadient & Otten, 1997; Ringheim, Burgher, & Heroux, 1995). Overproduction of IL-6 associated

with TNF-α and IL-1β is resulted in neurological disorders and neurodegenerative diseases (Tonelli & Postolache, 2005; McGeer & McGeer, 2001). Evidences showed that IL-6 expression is up-regulated in Alzheimer's disease patients' brain (Helmy, Naseer, Shafie, & Nada, 2012; Licastro et al., 2000) and senescent brain (Ye & Johnson, 1999).

### 2.2.4 Intrinsic mechanisms and signaling pathways of LPS-stimulated neuroinflammation

Activation of toll-like receptor 4 (TLR4) by LPS plays crucial roles in microglia-mediated neuroinflammation (Lu, Yeh, & Ohashi, 2008). Activation of TLR4 leads to an induction of a series of downstream signaling proteins and transcription factors, including those responsible for the regulations of pro-inflammatory mediators and cytokines expressions in neuroinflammatory process (Lu et al., 2008).

#### 2.2.4.1 Toll-like receptor 4 (TLR4)

The mammalian toll-like receptors (TLRs) are germline-encoded receptors expressed by the innate immune cells that are stimulated by bacteria, viruses and fungi (Akira, Uematsu, & Takeuchi, 2006; Janeway & Medzhitov, 2002). Members of the TLR family play critical roles as regulators of innate and adaptive immune responses (Aravalli, Hu, & Lokensgard, 2008; Aravalli, Peterson, & Lokensgard, 2007). To date, there are 11 human TLRs and 13 murine TLRs have been identified (Okun et al., 2009). The TLR interaction is known to stimulate the expression of pro-inflammatory cytokines as well as the functional maturation of antigen (Lee & Iwasaki, 2007; Akira et al., 2006). Toll-like receptor 4 (TLR4) is an important member of TLR family and is highly expressed on macrophages and microglia upon stimulation by LPS (Glass et al., 2010). It has been demonstrated that TLR4 is an important sensor for LPS (Poltorak et

al., 1998). According to Block, Zecca, & Hong (2007), TLR4 was found upregulated in LPS-stimulated brain inflammation in both *in vivo* and *in vitro* studies. Upon LPS stimulation, TLR4 undergoes oligomerization and recruits its downstream adaptors through interactions with the toll-interleukin-1 receptor (TIR) domain adaptor protein, myeloid differentiation primary response gene 88 (MyD88) (Lu et al., 2008; O'Neill & Bowie, 2007) and phosphatidylinositol-3 kinase/protein kinase B (PI3K/AKT) (Kogut, Chiang, Swaggerty, Pevzner, & Zhou, 2012; Liu et al., 2008). The MyD88-dependent pathway was shown to be responsible for pro-inflammatory cytokine expression by activates the downstream NFκB and MAPKs signaling pathways (Kogut et al., 2012; Sato et al., 2005) and leads to the induction of the transcription factors, NFκB, AP-1 and cyclic adenosine monophosphate (cAMP) response element binding protein (CREB) (Figure 2.5) (Glass et al., 2010; Lu et al., 2008).

#### 2.2.4.2 Nuclear factor kappa B (NFκB)

Nuclear factor kappa B is demonstrated as the primary pathway involved in the activation of pro-inflammatory genes (Karin & Delhase, 2000; Baldwin, 1996). Activation of NFκB by LPS in microglia promotes the upregulation of pro-inflammatory cytokines that are involved in the regulation of neuroinflammation responses (Kaminska, Mota, & Pizzi, 2015; Surh et al., 2001; Karin & Delhase, 2000). Upon LPS binding to TLR4, the MyD88-dependent signaling is being activated, leading to the activation of transforming growth factor-β-activated kinase 1 (TAK1) and IκB kinase complex (IKK: IKK-α, -β, -γ). The IκBs are phosphorylated upon IKK signal activation, leading to ubiquitination and degradation of IκB-α, and allowing NFκB dimers (p65 and p50) to translocate into the nucleus where it regulates the transcription of its target genes, leading to the transcription of pro-inflammatory genes, such as inducible enzymes, iNOS, COX-2, and pro-inflammatory cytokines, including TNF-α,

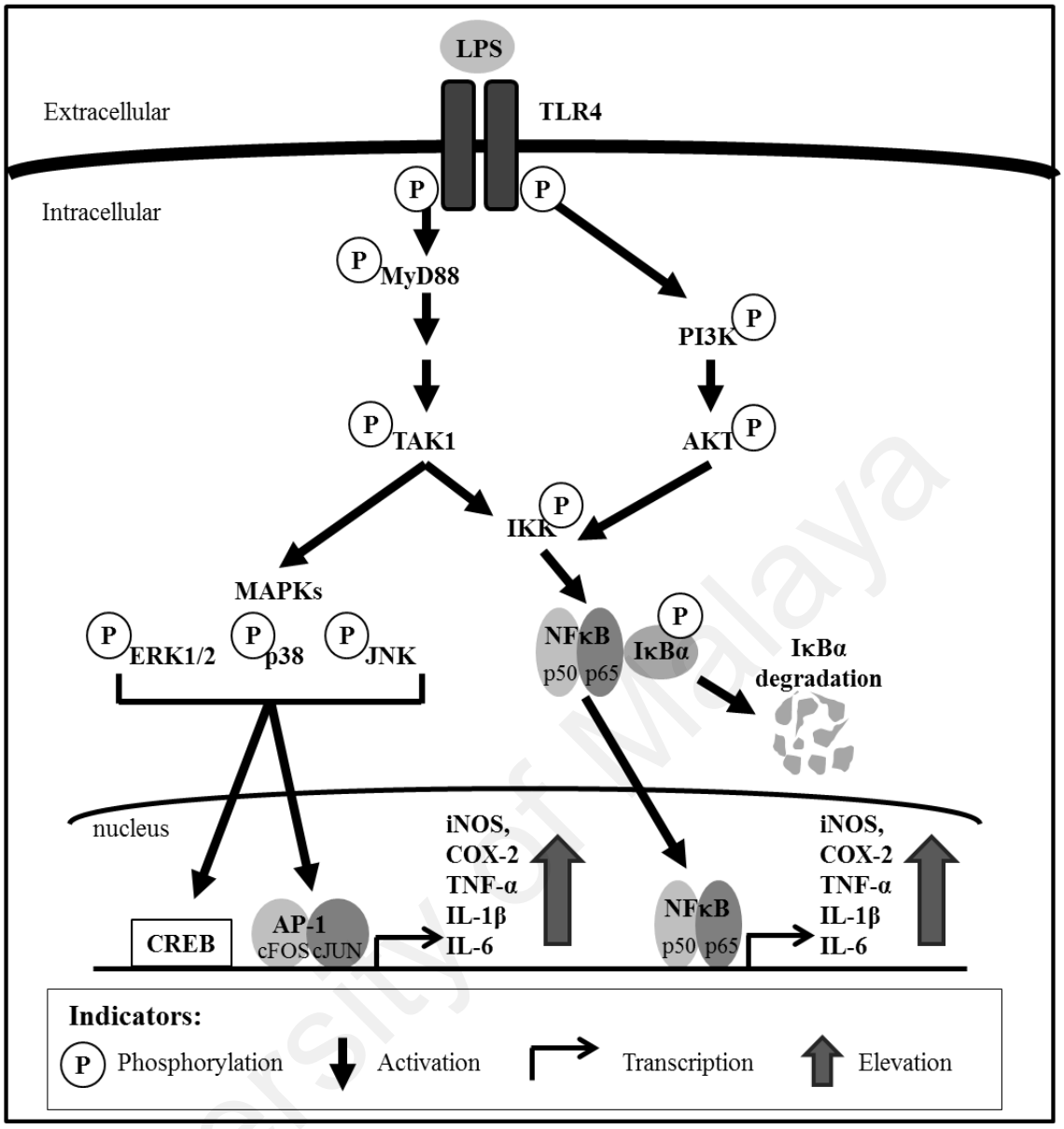



**Figure 2.5: Schematic illustration of LPS-stimulated TRL4-mediated signaling pathways.** Toll-like receptor 4 (TLR4) is activated by lipopolysaccharides (LPS). Activation of TLR4 leads to the phosphorylation of myeloid differentiation primary response gene 88 (MyD88) and phosphatidylinositol-3 kinase/protein kinase B (PI3K/AKT) pathways. Activation of the MyD88-dependent pathway leads to the phosphorylation of transforming growth factor-β-activated kinase 1 (TAK1) and activates the downstream nuclear factor kappa B (NFκB) and mitogen-activated protein kinase (MAPKs): extracellular signal-regulated kinase 1/2 (ERK1/2), p38 MAPK, and Jun amino-terminal kinases (JNK) signaling pathways. Activation of NFκB and MAPKs lead to the induction of the transcription factors, NFκB, cyclic adenosine monophosphate (cAMP) response element binding protein (CREB), and activator protein 1 (AP-1), respectively. Activation of these transcription factors may upregulate the expressions of various pro-inflammatory enzymes (iNOS and COX-2) and cytokines genes (TNF-α, IL-1β and IL-6).

IL-1β and IL-6 (Figure 2.5) (Kaminska et al., 2015; Surh et al., 2001). The NFκB transcription factor plays an important role in the production of pro-inflammatory cytokines and it is believed to be a promising target for the treatment of neuroinflammatory diseases (Kaminska et al., 2015).

#### 2.2.4.3 Phosphatidylinositol-3 kinase/protein kinase B (PI3K/AKT)

Increasing evidence demonstrated the involvement of the PI3K/AKT pathway in TLR4-mediated signaling (Kogut et al., 2012; Liu et al., 2008). The PI3K/AKT signaling pathway has been shown to participate in the regulation of gene expression of iNOS and COX-2 via activation of NFκB cascade in microglia activated by LPS (Figure 2.5) (Lee, Kwon et al., 2012; Nam, Son, Park, & Lee, 2008). The phosphorylation of PI3K/AKT signaling pathway is directly involved in the NFκB cascade through inducing the IκB-degradation (Lee et al., 2006; Jang et al., 2005). Study by Jang et al. (2005) showed that the PI3K inhibitor, LY294002 significantly blocked the phosphorylation of AKT in catalase-stimulated BV2 microglia and concomitantly inhibited IκB-α degradation and downregulated the NFκB expression. The study suggested that PI3K/AKT are the upstream signaling molecules of NFκB signaling, which response to the upregulation of pro-inflammatory genes in activated BV2 microglia (Jang et al., 2005). Hence, inhibition of PI3K/AKT signaling may be an alternative anti-neuroinflammatory approach to inhibit the transcription of NFκB and pro-inflammatory genes.

#### 2.2.4.4 Mitogen-activated protein kinase (MAPKs)

The MAPK superfamily of serine/threonine kinases consist of three distinct members: (i) extracellular signal-regulated kinase 1/2 (ERK1/2), (ii) p38, and (iii) stress-activated protein kinases/Jun amino-terminal kinases (SAPK/JNK) that play a major role in cellular activation of a variety of cell types (Roux & Blenis, 2004). Stimulation of TLRs

results in the downstream activation of MyD88, TAK1 and phosphorylation of MAPK superfamily cascade leading to the activation of the transcription factors, including AP-1 and cyclic adenosine monophosphate (cAMP) response element binding protein (CREB), which upregulate the expressions of pro-inflammatory enzymes (iNOS and COX-2) and cytokine genes (Figure 2.5) (Lu et a., 2008; Kogut, Genovese, He, & Kaiser, 2008). In microglia, ERK1/2, p38 and JNK pathways are known to play important roles in neuroinflammatory processes through the activation of TLR4 by LPS (Lu et al., 2008; Jung, Chung, Kim, & Park, 2007). Furthermore, stimulation of microglia by LPS activates MAPKs signal transduction pathways and induces apoptotic death of neurons in co-culture (Xie, Smith, & Van Eldik, 2004). Thus, inhibition of these MAPKs signaling molecules may have great potential for preventing and treating neuroinflammatory-associated disorders.

## 2.3 NEURITOGENESIS

Neuroregeneration is includes endogenous neuroprotection leading to neuroplasticity and neuritogenesis or neurite outgrowth (Enciu et al., 2011). Neuritogenesis is a term form by "neurite-" referring to the branching of axons and dendrites of neuronal cells, and "-genesis" referring to formation, generation and development, illustrating the formation of neurite in the neuronal cells (Da Silva & Dotti, 2002). Neuritogenesis is an important and complex morphogenetic process in the brain development associated with neuronal differentiation, sprouting and extensions of neurites to form a functional and communications network within the neuronal cells (Da Silva & Dotti, 2002; Spencer, Shao, & Andres, 2002). It is a fundamental process underlying the establishment and plasticity of neuronal networks (Da Silva & Dotti, 2002). The sprouting of neurites, which will later become axons and dendrites, is an important event in early neuronal differentiation (Da Silva & Dotti, 2002).

As shown in Figure 2.6, neuritogenesis includes at least three stages: (i) neurite initiation (Sebok et al. 1999), (ii) neurite maintenance and elongation (Tucker, Meyer, & Barde, 2001; Sebok et al., 1999), and (iii) neurite network formation (Spencer et al., 2002), which is crucial for the proper functioning of the nervous system. Neuritogenic activity is essential for the maintenance and regeneration of the neuronal communications network (Da Silva & Dotti, 2002; Spencer et al., 2002). It has become one of the focuses of study in the search for preventive and therapeutic agents for neurodegenerative diseases (Phan, David, Naidu, Wong, & Sabaratnam, 2014; Williams & Dwyer, 2009).

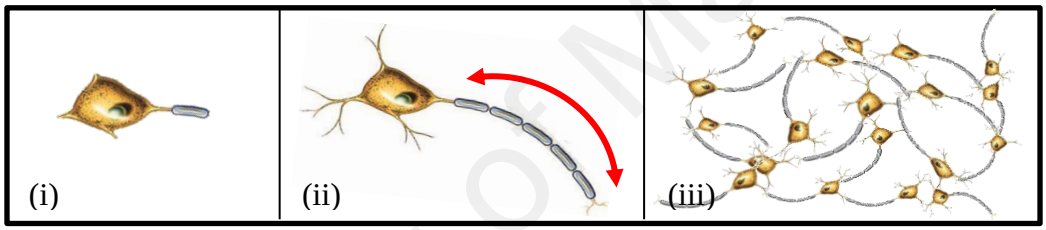



**Figure 2.6: Stages of neuritogenesis.** (i) Neurite initiation, (ii) Neurite maintenance and elongation, and (iii) Neurite network formation.

### 2.3.1 Neuritogenesis in neurodegenerative diseases

Neurite or axon degeneration is an early event that leads to the progressive neuronal cell loss in neurodegenerative diseases (Wang, Medress, & Barres, 2012; Bjartmar, Wujek, & Trapp, 2003). Neuritogenesis is associated with the development of the nervous system and contrary, dysfunction of neurite is associated with pathogenesis of the nervous system (Hagg, 2009). Recent findings showed that synapse degeneration, axon and dendrite loss in hippocampus and cortex is strongly correlated with cognitive decline in Alzheimer's disease (Evans et al., 2008; DeKosky & Scheff, 1990). The loss of dopaminergic axonal processes that eventually leads to the loss of neuron cell bodies is an early characteristic of Parkinson's disease (Abeliovich & Beal, 2006). Axonal degeneration has been identified as the major factor of irreversible neurological

disability in patients with multiple sclerosis (Bjartmar et al., 2003). Accumulative evidence has implicated that the production of neurotoxic factors in nervous system, such as amyloid-β from amyloid precursor protein (APP), and pro-inflammatory mediators and cytokines from activated glial cells during neuroinflammation are contributing factors that lead to neurite damage, synaptic failure and neuronal death (Christianson & Lo, 2015; Puttfarcken, Manelli, Neilly, & Frail, 1996). It has been shown that amyloid-β triggers synaptic degeneration, neurite damage and cell death in neuronal cell cultures (Christianson & Lo, 2015; Evans et al., 2008). Besides, diminution and dysfunction of neurotrophins or neurotrophic factors has been shown to be involved in the pathogenesis of Alzheimer's and Parkinson's diseases (Siegel & Chauhan, 2000; Connor & Dragunow, 1998).

Development and regeneration of the network of neurites establish a strategy for the prevention and treatment of neurodegenerative diseases (Santos et al., 2015). Tohda, Kuboyama, & Komatsu (2005) suggested that generation of neurites and restoration of the neuronal networks in injured brain is crucial for the recovery of brain functions. A recent report by Chen, Yin, Zhang, & Liao (2015) illustrated that successful neuronal regeneration involved in three processes, (i) injured neurons receive accurate and timely information about the location and extent of axonal damage, (ii) injured neurons trigger an appropriate extent of axonal regeneration, and (iii) the axons of injured neurons regrow through the lesion and reconnect with the target organ to restore the function. Evidences have revealed the ability of neurotrophins, exemplified by nerve growth factor acts as stimulatory substances to trigger regeneration of neurite and axon of damaged neurons (Pesavento, Capsoni, Domenici, & Cattaneo, 2002; McKerracher, 2001).

### 2.3.2 Neurotrophins

Neurotrophins or neurotrophic factors, including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4) (Figure 2.7) play distinctive roles in promoting neuronal survival, proliferation, development, neuritogenesis and maintaining the neurons functions (Thoenen, 1995; Kromer, 1987). It has been demonstrated that reduction in the levels of neurotrophins lead to major declines in brain cell performance (Connor & Dragunow, 1998; Thoenen, 1995). Neurotrophins, remarkably NGF is the key regulator of neuritogenesis (D'Ambrosi et al., 2000).

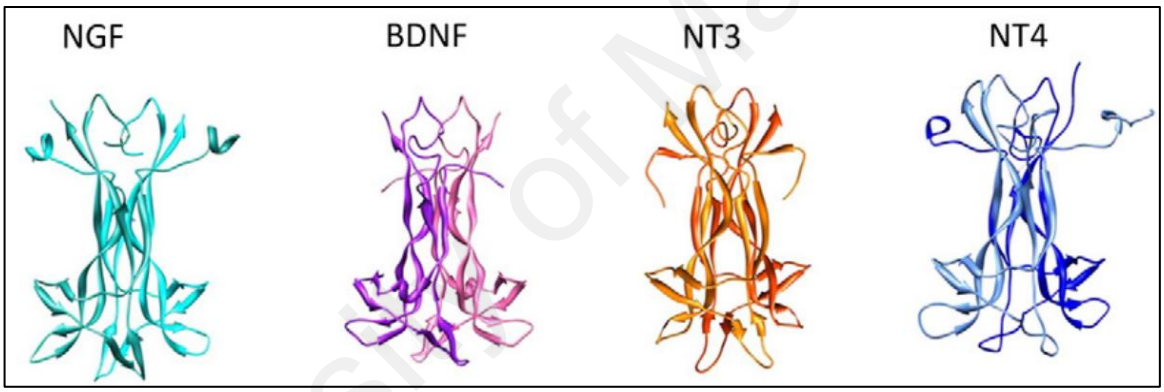



**Figure 2.7: The X-ray crystal structures of the neurotrophins.** Nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4) (Allen, Watson, Shoemark, Barua, & Patel, 2013).

**Nerve growth factor (NGF)**

Nerve growth factor was the first neurotrophin discovered by Dr. Rita Levi-Montalcini in the 1950s (Levi-Montalcini 1987; Levi-Montalcini, 1966). Nerve growth factor is mandatory for the development and maintenance of sympathetic and parasympathetic nervous system (Furukawa & Furukawa, 1990; Levi-Montalcini, 1966). Nerve growth factor is produced in the neocortex and hippocampus (Schindowski, Belarbi, & Buée, 2008) to maintain the cholinergic neurons of the forebrain (Koliatsos et al., 1991; Furukawa & Furukawa, 1990). Studies proven that the NGF has been targeted as a

potential therapeutic agent for the treatment of neurodegenerative diseases (Connor & Dragunow, 1998; Koliatsos et al., 1991). Deprivation of NGF may affect the cholinergic neurons, causing neuronal atrophy, memory impairments, and further leads to neurodegenerative diseases (Hefti & Weiner, 1986; Capsoni et al., 2000). Study by Hefti & Weiner (1986) reported that a decrease in NGF in the brain is believed to be the main cause of neuronal dysfunction and neurodegenerative diseases, remarkably Alzheimer's disease. Further, Capsoni et al. (2000) presented the evidence that a decrease in NGF in mice's brain led to neurodegeneration and Alzheimer's-like symptoms. Learning ability and memory were improved in aged anti-nerve growth factor transgenic mice after administration of NGF (Capsoni et al., 2000). However, according to Granholm et al. (1998) and Poduslo & Curran (1996), the neuroactivity and therapeutic application of NGF is restricted by its high molecular weight polypeptide structure, with approximately molecular weight of 29,000 (Angeletti & Bradshaw, 1971; Angeletti, Bradshaw, & Wade, 1971). Peripheral administration of NGF does not significantly penetrate the blood-brain barrier (BBB) and blood-nerve barrier (BNB) (Granholm et al., 1998; Poduslo & Curran, 1996). Hence, smaller substances from natural or dietary sources with low molecular weight that mimic and/or enhance the NGF activities have become the core focus in the search for preventive and therapeutic agents for neurodegenerative diseases (Williams & Dwyer, 2009).

### 2.3.3 Intrinsic mechanisms and signaling pathways underlying NGF-stimulated neuritogenesis

The neuritogenic effect of NGF is transduced by its high affinity tyrosine receptor, the tropomyosin receptor kinase A (TrkA) and stimulated by the NGF responsive signaling pathways (Patapoutian and Reichardt, 2001). Activation of TrkA by NGF leads to phosphorylation of several downstream signaling proteins, transcription factors and

neuritogenic biomarkers that are responsible for the neuritogenesis (Limpert, Karlo, & Landreth, 2007; Frade & Barde, 1998).

#### 2.3.3.1 Tropomyosin receptor kinase A (TrkA)

Tropomyosin receptor kinase A (TrkA) is a cell surface transmembrane receptor for NGF (Patapoutian & Reichardt, 2001; Kaplan & Stephens, 1994). The neuritogenic activity of NGF is revealed by its promotion of neuronal differentiation and axon elongation (Greene, & Tischler, 1976). Nerve growth factor-mediated activation of TrkA is the earliest step leading to signal transduction of downstream proteins (Kaplan & Stephens, 1994). Activated TrkA is critical for the activation of several signaling pathways via intracellular signaling molecules that include Ras, Raf (Qiu & Green, 1991), ERK (Vaudry, Stork, Lazarovici, & Eiden, 2002) and PI3K (Tyson, Larkin, Hamai, & Bradshaw, 2003; Jackson et al., 1996), to kick start neuritogenesis (Figure 2.8) (Limpert et al., 2007; Frade & Barde, 1998). The activation of TrkA has been suggested as a therapeutic strategy for Alzheimer's disease (Longo & Massa, 2013).

#### 2.3.3.2 Mitogen-activated protein kinase kinase/extracellular signal-regulated kinase1/2 (MEK/ERK1/2)

Nerve growth factor was found to activate the Ras/mitogen-activated protein kinase (Ras/MAPK) signaling pathway that mediates the phosphorylation including the MEK/ERK pathway (Kao, Jaiswal, Kolch, & Landreth, 2001). In MEK/ERK1/2 cascade, proteins of the activated MAPKKs (MAP kinase kinases) namely MEKs (MAPK or ERK kinases) will phosphorylate and activate MAPKs (MAP kinases) namely ERKs (Vaudry et al., 2002; Kao et al., 2001). The MAPK/ERK1/2 pathway is a chain of proteins in the cell that participate by Ras, Raf, MEK1/2 and ERK1/2 proteins. The MAPK/ERK1/2 cascade is a signal transduction pathway that involves a large

variety of processes such as differentiation, proliferation, apoptosis, cell cycle progression, cell migration, and metabolism (Roskoski, 2012). The MEK/ERK1/2 signaling cascade is indeed crucial in both *in vitro* (Vaudry et al., 2002) and *in vivo* (Wong, Naidu, David, Bakar, & Sabaratnam, 2012; Naidu, David, Asher, & Fawcett, 2009) for neuronal cell survival, neuritogenesis, and axonal regeneration in mouse dorsal root ganglia neurons. The MEK/ERK1/2 signaling pathway is believed to be the major cascade for NGF to stimulate neuritogenesis in neuronal cell culture, such as PC-12 cells (Figure 2.8) (Vaudry et al., 2002).

#### 2.3.3.3 Phosphatidylinositol-3-kinase/protein kinase B (PI3K/AKT)

Phosphatidylinositol-3-kinase/protein kinase B (PI3K/AKT) signaling pathway is another major downstream cascade of NGF-stimulated neuritogenesis and neuronal survival (Figure 2.8) (Naidu et al., 2009; Ditlevsen et al., 2003). Previous studies have suggested that PI3K/AKT pathway promotes neurite differentiation and elongation in NGF-stimulated PC-12 cells (Kita et al., 1998; Jackson et al., 1996) and sympathetic neurons (Kuruvilla, Ye, & Ginty, 2000). Study by Kim, Seger, Suresh Babu, Hwang, & Yoo (2004) showed that beside ERK pathway, the PI3K/AKT signaling pathway is also strongly involved in NGF-stimulated neuritogenesis in PC-12 cells. Namikawa et al. (2000) reported that the activation of AKT is involved in the neuritogenic activity and motor axon regeneration in both *in vitro* (PC-12 cells) and *in vivo* (wistar rats) models. In addition, PI3K/AKT pathway is required for distal axon growth and regeneration (Kuruvilla et al., 2000) and axon growth in mouse embryonic sensory neurons (Markus, Zhong, & Snider, 2002).

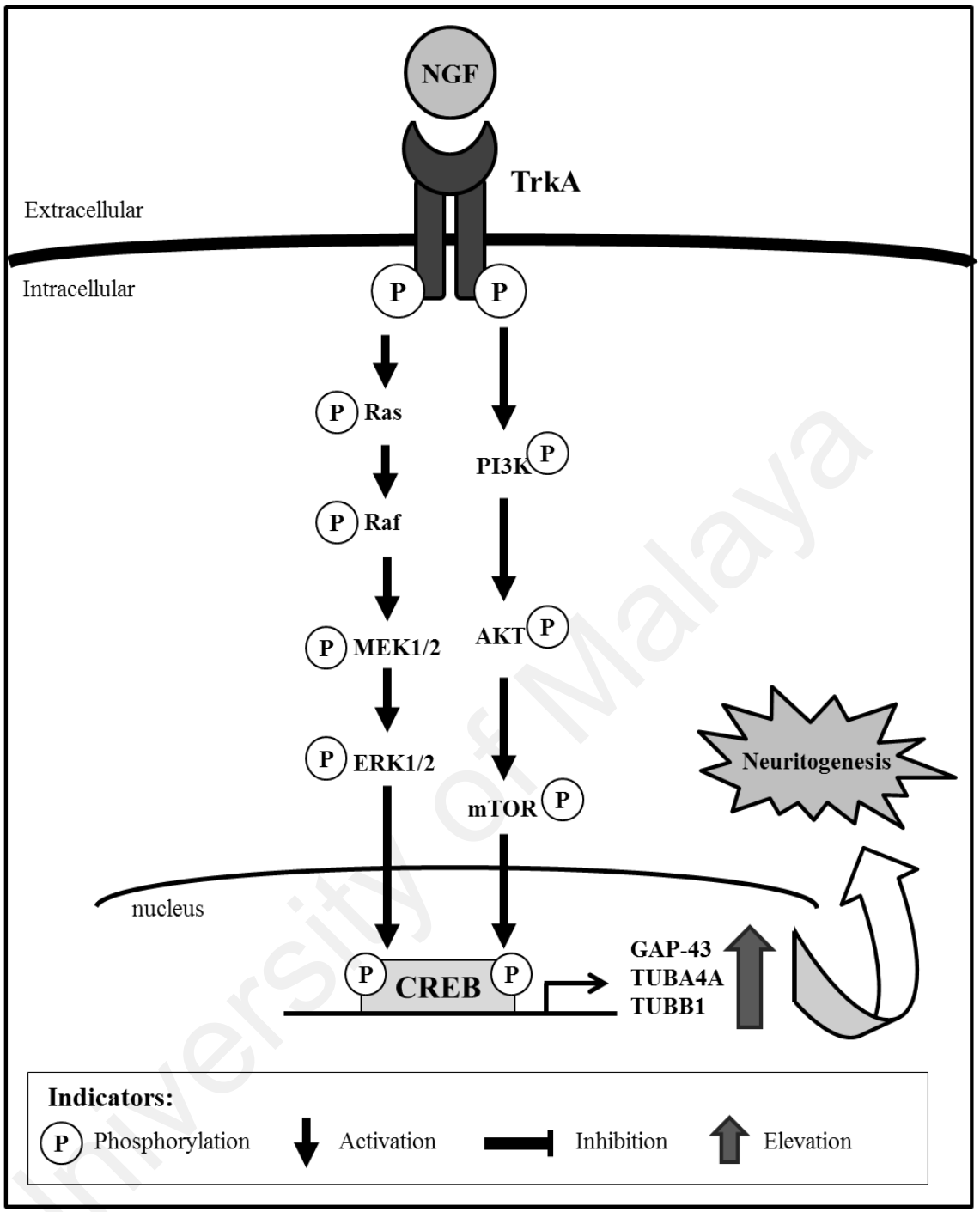





**Figure 2.8: Schematic illustration of NGF-stimulated TrkA signaling pathways.** The neuritogenic effect of NGF is transduced by its high affinity tyrosine receptor, the tropomyosin receptor kinase A (TrkA). Activated TrkA leads to the phosphorylation of Ras/Raf/mitogen-activated protein kinase kinase1/2/extracellular signal-regulated kinase1/2 (Ras/Raf/MEK1/2/ERK1/2) and phosphatidylinositol-3-kinase/protein kinase B/mammalian target of rapamycin (PI3K/AKT/mTOR) signaling pathways. Activation of ERK1/2 and mTOR leads to the phosphorylation of the transcription factor, cyclic adenosine monophosphate (cAMP) response element binding protein (CREB), and sequentially stimulates the upregulation of downstream genes, the growth associated protein 43 (GAP43), tubulin alpha (TUBA4A), and tubulin beta (TUBB1), which then initiates neuritogenesis.

#### 2.3.3.4 Cyclic adenosine monophosphate (cAMP) response element binding protein (CREB)

The transcription factor, cyclic adenosine monophosphate (cAMP) response element binding protein (CREB) is a mediator of the nuclear response to neurotrophins (Finkbeiner et al., 1997; Bonni, Ginty, Dudek, & Greenberg, 1995) and also a critical mediator of NGF-dependent gene expression in neuronal cells (Ahn et al., 1998; Ginty, Bonni, & Greenberg, 1994). Treatment with NGF leads to the activation of MEK1/2/ERK1/2 and AKT/mammalian target of rapamycin (mTOR), and sequentially stimulates the phosphorylation of CREB protein (Ser133) which then initiates the neuronal differentiation and outgrowth (Figure 2.8) (Read & Gorman, 2009; Xing, Kornhauser, Xia, Thiele, & Greenberg, 1998). Upon phosphorylation of Ser133, CREB recruits its transcriptional activator CREB-binding protein (CBP), to activates a specific set of gene transcriptions through binding to the CRE element (CRE-regulated genes) that are involved in neurogenesis and neuritogenesis (Spencer, Vauzour & Rendeiro, 2009; Montminy, Sevarino, Wagner, Mandel, & Goodman, 1986).

#### 2.3.3.5 Neuritogenic biomarkers

The subsequent phosphorylation of CREB lead to an up-regulation of downstream genes, including growth associated protein 43 (GAP43) and microtubules that are relevant to nerve regeneration (Ambron & Walters, 1996; Hüll & Bahr, 1994), neuronal growth and neuritogenesis of PC12 cells (Figure 2.8) (Phan, David, Wong, Naidu, & Sabaratnam, 2015; Jap Tjoen San et al., 1991). Expression of the neuron-specific protein, GAP-43 is enhances with the onset of neuronal development demonstrated by the growth of axons (Costello, Meymandi, & Freeman, 1990). The GAP-43 is identified as the regulator of cytoskeleton dynamics to promote neurite outgrowth (Skene, 1989). In the study by Jap Tjoen San and co-workers (1991 & 1992) further showed the

relationship between the expression levels of GAP-43 and axonal outgrowth in PC-12 cells. Thereafter, GAP-43 was suggested as a useful indicator of the differentiation status of PC-12 cells (Jap Tjoen San, Schmidt-Michels, Oestreicher, Schotman, & Gispen, 1992; Jap Tjoen San et al., 1991).

Microtubules are composed of globular proteins called tubulin, formed by heterodimers of alpha and beta tubulin, such as tubulin alpha 4A (TUBA4A) and tubulin beta 1 (TUBB1) (Conde & Cáceres, 2009). Microtubules are one component of the cytoskeleton and are crucial for the assembly, organization and dynamics in axons and dendrites (Conde & Cáceres, 2009). It has been reported that neuritogenesis of PC-12 cells stimulated by NGF require transcriptionally-dependent microtubules stabilization (Greene et al., 1984). Microtubules in PC-12 cells neurites serve as compressive supports for neuritogenesis and neurite elongation (Lamoureux et al., 1990). Removal of NGF from PC-I2 cells cultured for 5 days in the presence of NGF causes neurite retraction, microtubule disassembly, and reduction in tubulin (Drubin, Kobayashi, Kellogg, & Kirschner, 1988). Therefore, GAP-43 and microtubules (tubulin alpha and tubulin beta) genes have been introduced as key neuronal biomarkers for neurite extension (neuritogenic biomarkers) in neuronal cells (Lamoureux et al., 1990; Skene, 1989).

## 2. 4 *IN VITRO* CELL CULTURES

Cell culture refers to a culture derived from dispersed cells taken from original tissue, from a primacy culture or from a cell line or cell strain by enzymatic, mechanical or chemical disaggregation (Freshney, 2005). Cell culture has been used extensively after the availability of defined cell culture media with supplements, which provide a controlled environment for the cells. The advantages of the application of cell culture including (i) control of the physiochemical environment (pH, temperature, osmotic

pressure, oxygen $O_2$ and carbon dioxide $CO_2$ levels), (ii) characterization and homogeneity of the samples, (iii) economy, scale and mechanization of culture, (iv) *in vitro* modeling of *in vivo* conditions, and (v) may avoid animal experiments (Freshney, 2005).

### 2.4.1 *In vitro* cell cultures for neuroinflammatory study

*In vitro* and *in vivo* experimental models with reactive glial cells have been extensively employed in neuroprotective, neuroinflammatory and anti-neuroinflammatory studies. As alternative models to *in vivo* study, the *in vitro* approaches were established and utilized as powerful tools to study the inhibitory effects of anti-neuroinflammatory candidates on glia activation, expression of pro-inflammatory mediators and cytokines, and the underlying mechanisms in response to known stimuli (Stansley, Post, & Hensley, 2012; Henn et al., 2009). Presently, there are numerous types of microglia and microglia-like cultures used to investigate the neuroinflammatory and anti-neuroinflammatory phenomena, including primary microglial cells isolated from the cortex of a rat or mouse before or early after birth, and immortalized microglial cell cultures, such as retroviral immortalized microglia (BV2 and N9), human immortalized microglia (HMO6) and spontaneously immortalized microglia (EOC and HAPI) (Stansley et al., 2012).

The immortalized murine BV2 microglial cell line was reported to exhibited both phenotypic and functional properties of reactive microglial cells (Bocchini et al., 1992). BV2 microglial cell line is generated by infecting the cells with retrovirus to yield a larger number of cells quickly compared to the primary cultures (Stansley et al., 2012). The BV2 microglial cell line has been used extensively in research related to neuroinflammatory and neurodegenerative disorders (Stansley et al., 2012). A study by Horvath, McMenemy, Alkaitis, & DeLeo (2008) showed the comparison results of the

primary rat microglial and BV2 microglial cell line, mainly on the activation markers, viability and pro-inflammatory mediators and cytokines production. The study demonstrated the similarities of both primary and BV2 microglia cultures, where BV2 microglia expressed Iba-1, a microglia activation marker and released substantial amounts of pro-inflammatory mediators and cytokines upon LPS stimulation that were comparable to the primary microglia (Horvath et al., 2008). Furthermore, Henn et al. (2009) suggested that the BV2 microglial cell line is an appropriate model alternative to the primary cultures. In response to LPS, BV2 microglia expressed 90% of inflammatory genes that also expressed by primary microglia (Henn et al., 2009). Beside LPS, amyloid-β also serves as an effective stimulus to activate BV2 microglia to generate an *in vitro* model to study the expressions of pro-inflammatory mediators and cytokines in neuroinflammatory-associated Alzheimer's disease (Park, Kim et al., 2015; Dhawan & Combs, 2012). The BV2 microglial cell line serves as a useful *in vitro* model for the search of potential anti-neuroinflammatory agents for the management of neuroinflammatory-associated neurodegenerative diseases (Stansley et al., 2012; Henn et al., 2009). Thus, BV2 microglial cell line is an appropriate *in vitr*o model for the present study, to study the anti-neuroinflammatory activity and the underlying mechanisms of the selected mushrooms.

### 2.4.2 *In vitro* cell cultures for neuritogenesis study

*In vitro* neuritogenic stimulatory assay has been used generally in neurobiology and neuroscience researches to study the neurotrophic effect of neurotrophins and neuritogenic substances. Several cell lines (primary cell cultures and cell lines) were established and employed for the study of neuritogenesis and the underlying mechanisms. Commonly used primary cell cultures for neuritogenic stimulatory study are the rat or mouse hippocampal neurons (Sarma et al., 2015; Fujiwara et al., 2003),

cortical neurons (Kubo, 2015; Nejatbakhsh et al., 2011), and dorsal root ganglion neurons (Ng, Cartel, Roder, Roach, & Lozano, 1996). There are also many neuronal or neuronal-like cell lines available as *in vitro* models for neuritogenesis studies, such as rat pheochromocytoma (PC-12) (Krawczyk, Twarog, Kurowska, Klopotowska, & Matuszyk, 2015; Tan, Luan et al., 2011), mouse neuroblastoma-rat glioma hybrid (NG108-15) (Campanha, Carvalho, Schlosser, 2014; Wong, Sabaratnam, Abdullah, Naidu, & Keynes, 2007), Neuro2a mouse neuroblastoma (N2a) (Phan et al., 2015; Wu & Ledeen, 1991), human neuroblastoma (SHSY-5Y) (Gouarné et al., 2015; Sharma, Sharma, & Pant, 1999), rat neuroblastoma (B50) (Wu & Ledeen, 1991), and rat neuroblastoma (B103) (Mook-Jung et al., 1997).

The PC-12 cell line is established from rat adrenal pheochromocytoma (adrenal medullary tumor) (Greene & Tischler, 1976). It has been used extensively as an *in vitr*o model system for the study of the actions of NGF (Teng, Angelastro, Cunningham, & Greene, 2006; Greene & Tischler, 1976) and NGF mimics (Williams & Dwyer, 2009). The PC-12 cell line is also used to study the NGF responsive signaling pathways (Vaudry et al., 2002; Greene et al., 1984) for neuronal differentiations, proliferation and survival. The PC-12 cells respond to neurotrophins, such as NGF by differentiating into sympathetic neuron-like phenotypes characterized by neurite outgrowth and expression of several neuronal specific proteins (Guroff, 1984; Tischler & Greene, 1975). Addition of nanomolar amounts of NGF to PC-12 cells will lead to cells mitotic arrest, differentiation into a sympathetic-like neuronal phenotype, extend axon-like outgrowth and increase the expression of neuronal proteins (Greene & Tischler, 1976). The molecular signaling pathways involved in PC-12 cells differentiation have been vigorously explored over the past decade (Vaudry et al., 2002). Therefore, PC-12 cell line is an appropriate *in vitr*o model for the present study, to study the neuritogenic

stimulatory activity, NGF-mimicking activity and the underlying mechanisms of the selected mushrooms.

## 2.5 NEUROACTIVE SUBSTANCES FROM NATURAL SOURCES

A renowned quote by Hippocrates, the father of medicine: "let food be thy medicine and medicine be thy food" imply that there are numerous bioactive substances that possess therapeutic potential available in human daily food. In recent years, neuroactive substances from natural sources have received extensive attention as preventive and therapeutic agents for neurodegenerative diseases by promoting anti-neuroinflammatory (Karunaweera et al., 2015) and neuritogenic potential (Phan, David, Naidu et al., 2014; More et al., 2012). As compared to the synthetic drugs currently available for neurodegenerative diseases, natural products or foods serve a better choice as alternative and complementary medicine for the management, prevention and treatment of neurodegenerative diseases (Karunaweera et al., 2015; Tohda et al., 2005).

### 2.5.1 Anti-neuroinflammatory substances from natural sources

The nonsteroidal anti-inflammatory drugs (NSAIDs) are commonly administrated drug to reduce inflammation. However, long-term administration of NSAIDs has the potential for significant side effects (Fosbøl et al., 2009; Dugowson & Gnanashanmugam, 2006). Therefore, discovery of anti-inflammatory substances from natural sources as potential and safe medicines without the harmful side effects of NSAIDs serve a promising alternative treatment for inflammatory-associated disorders (Karunaweera et al., 2015; Elsayed, Enshasy, Wadaan, & Aziz, 2014). Plant- and herbal-derived preparations have been used as anti-inflammatory (Bhagyasri, Lavakumar, Divya Sree, & Ashok Kumar, 2015; Prasad & Aggarwal, 2011) and anti-neuroinflammatory (Kulkarni, Kellaway, & Kotwal, 2005) agents since ancient times.

The evaluation of the effectiveness of anti-inflammatory substances from natural sources on brain and neuronal inflammation has been extensively explored by modern science recently. In recent years, mushroom-derived preparations are becoming increasing popular because of their effectiveness as mycomedicines for inflammatory-related disorders (Elsayed et al., 2014; Gunawardena et al., 2014). Many potent anti-neuroinflammatory compounds such as polyphenols and flavonoids have been isolated and purified from plants and mushrooms. A review by Gupta et al. (2014) showed the potential of many dietary polyphenols such as curcumin, resveratrol, ellagic acid, kaempferol and xanthohumol to downregulate various pro-inflammatory biomarkers *in vitro* and *in vivo*. It has been proven that many common foods such as garlic (*Allium sativum*), turmeric (*Curcuma longa*), ginger (*Zingiber officinale*), blueberry (*Vaccinium angustifolium*), grape (*Vitis vinifera*), green tea (*Camellia sinensis*), ginseng (*Panax ginseng* and *Panax pseudoginseng*), bitter melon (*Momordica charantia*), and culinary and/or medicinal mushrooms possess anti-neuroinflammatory properties (Table 2.1). These natural products have been employed as potent, natural, and safe anti-neuroinflammatory agents based on their ability to mitigate microglia activation, inhibit the production of pro-inflammatory mediators and cytokines, and downregulate the neuroinflammatory genes *in vitro* and *in vivo* (Table 2.1).

**Table 2.1: Natural substances from plants and mushrooms which possess anti-neuroinflammatory effects *in vitro* and *in vivo.***

| Natural Source | Extract/ Compound | Study Model | Stimulus | Anti-neuroinflammatory activity | References |
|---|---|---|---|---|---|
| **Plant** | | | | | |
| *Allium sativum* | Diallyl disulfide | BV2 microglia | LPS | NO ↓, iNOS ↓, $PGE_2$ ↓, COX-2↓, TNF-α ↓, IL-1β ↓, MCP-1 ↓ | Park et al., 2012 |
| *Artemisia argyi* | Sesquiterpene dimer | BV2 microglia | LPS | NO ↓, $PGE_2$ ↓, iNOS ↓, COX-2↓, TNF-α ↓, IL-1β ↓, IL-10 ↑ | Zeng, Wang, Dong, Jiang, & Tu, 2014 |
| *Camellia sinensis* | Epigallocatechin-3-gallate | Sprague-dawley rats (hippocampus) & primary microglia | Infrasound | TNF-α ↓, IL-1β ↓, IL-6 ↓, IL-18↓ | Cai et al., 2014 |
| *Camellia sinensis* | Epigallocatechin-3-gallate | EOC 13.31 microglia | Amyloid-β | iNOS ↓, TNF-α ↓, IL-1β ↓, IL-6 ↓ | Wei et al., 2015 |
| *Curcuma longa* | Curcumin | Wistar rats | Pentylenetetrazole | TNF-α ↓, IL-1β ↓, IL-2 ↓, IL-6 ↓, MCP-1 ↓ | Kaur, Patro, Tikoo, & Sandhir, 2015 |
| *Entada africana* | $CH_2Cl_2$/MEOH 5% fraction | N9 microglia | LPS | NO ↓, iNOS ↓, TNF-α ↓, IL-1β ↓, IL-6 ↓ | Owona, Njayou, Laufer, Schluesener, & Moundipa, 2013 |
| *Erigeron breviscapus* | Scutellarin | Primary & BV2 microglia | LPS | NO ↓, ROS ↓, iNOS ↓, TNF-α ↓, IL-1β ↓ | Wang et al., 2011 |

| Natural Source | Extract/ Compound | Study Model | Stimulus | Anti-neuroinflammatory activity | References |
|---|---|---|---|---|---|
| **Plant** | | | | | |
| *Isodon japonicus* | Isodojaponin D | BV2 microglia | LPS | iNOS ↓, COX-2↓, TNF-α ↓, IL-1β ↓, IL-6 ↓ | Lim et al., 2010 |
| | Kamebakaurin | BV2 microglia | LPS | NO ↓, iNOS ↓, COX-2 ↓ | Kim et al., 2011 |
| *Matricaria recutita* | Apigenin Luteolin | Primary & N9 microglia | IFN-γ and/or CD40 ligation | CD 40 ↓, TNF-α ↓, IL-6 ↓ | Rezai-Zadeh et al., 2008 |
| *Mesua kunstleri* | Mesuagenin C | BV2 microglia | LPS | NO ↓, $PGE_2$ ↓, iNOS ↓, COX-2↓, TNF-α ↓, IL-1β ↓, IL-2 ↓, IL-6 ↓, IFN-γ ↓, ROS ↓ | Kamarudin, Kadir, Chan, & Awang, 2013 |
| *Momordica charantia* | Juice | C57BL/6 female mice | High-fat diet | IL-16↓, IL-22 ↓, IL-17 ↓, glial activation ↓ | Nerurkar et al., 2011 |
| *Myagropsis myagroides* | Sargachromenol-rich ethanolic extract | BV2 microglia | LPS | NO ↓, $PGE_2$ ↓, TNF-α ↓, IL-1β ↓, IL-6 ↓, iNOS ↓, COX-2↓ | Kim et al., 2014 |
| *Panax ginseng* | Ginsenoside Rbl | Wistar rats | Aβ1-42 | COX-2 ↓, nNOS ↓, improve learning & memory behavior | Wang et al., 2011 |
| | Ginsenoside Rd | Primary mesencephalic neuron-glia | LPS | NO ↓, $PGE_2$ ↓, iNOS ↓, COX-2↓ | Lin, Zhang, Moldzio, & Rausch, 2007 |
| *Panax pseudoginseng* | Pseudoginsenoside -F11 | N9 microglia | LPS | NO ↓, ROS ↓, $PGE_2$ ↓, TNF-α ↓, IL-1β ↓, IL-6 ↓ | Wang et al., 2014 |

| Natural Source | Extract/ Compound | Study Model | Stimulus | Anti-neuroinflammatory activity | References |
|---|---|---|---|---|---|
| **Plant** | | | | | |
| *Rabdosia japonica* | Glaucocalyxin A | BV2 & primary microglia | LPS | NO ↓, iNOS ↓, COX-2↓, TNF-α ↓, IL-1β ↓, IL-6↓ | Kim et al., 2013 |
| *Stephania tetrandra* | Tetrandrine | BV2 microglia | Amyloid-β | TNF-α ↓, IL-1β ↓ | He et al., 2011 |
| *Scutellaria baicalensis* | Ethanol extract | BV2 microglia | LPS | NO ↓, $PGE_2$ ↓, iNOS ↓, COX-2↓ | Jeong et al., 2011 |
| *Vitis vinifera* | Resveratrol | Primary & N9 microglia | LPS | NO ↓, iNOS ↓, TNF-α ↓, IL-1β ↓, IL-6 ↓, MCP-1 ↓ | Lu et al., 2010 |
| *Vaccinium angustifolium* | Polyphenolic-enriched fraction | BV2 microglia | LPS | NO ↓, iNOS ↓, COX-2 ↓, TNF-α ↓, IL-1β ↓ | Lau, Joseph, McDonald, & Kalt, 2009 |
| *Zingiber officinale* | 6-shogaol | BV2 & primary microglia | LPS | NO ↓, $PGE_2$ ↓, TNF-α ↓, IL-1β ↓, iNOS ↓, COX-2↓ | Ha et al., 2012 |
| | | Astrocytes | LPS | iNOS ↓, COX-2↓ | Shim, Kim, Choi, Kwon, & Kwon, 2011 |
| | | Mesencephalic cells | $MPP^+$ | NO ↓, TNF-α ↓ | Park et al., 2013 |
| | | C57BL/6 mice | MPTP | iNOS ↓, COX-2↓ | Park et al., 2013 |

| Natural Source | Extract/ Compound | Study Model | Stimulus | Anti-neuroinflammatory activity | References |
|---|---|---|---|---|---|
| **Mushrooms** | | | | | |
| *Cordyceps militaris* | Cordycepin | BV2 microglia | LPS | NO ↓, $PGE_2$ ↓, iNOS ↓, COX-2 ↓, TNF-α ↓, IL-1β ↓ | Jeong et al., 2010 |
| | Ergosterol-enriched subfraction | BV2 microglia | LPS | NO ↓ | Nallathamby et al., 2015 |
| *Ganoderma lucidum* | Ethanol extract | BV2 microglia | LPS | NO ↓, $PGE_2$ ↓, TNF-α ↓, IL-1β ↓ | Yoon et al., 2013 |
| | Methanol extract | Co-cultures of dopaminergic neurons & microglia | LPS + MPP+ | NO ↓, TNF-α ↓, IL-1β ↓ | Zhang et al., 2011 |
| *Laetiporus sulphureus* | Exopolysaccharide | BV2 microglia | LPS | NO ↓, iNOS ↓, TNF-α ↓, $PGE_2$ ↓, COX-2 ↓ | Jayasooriya et al., 2011 |
| *Taiwanofungus camphoratus* | Methanol extract | EOC 13.31 microglia | LPS + IFN-γ, Amyloid-β | iNOS ↓, COX-2 ↓ | Liu et al., 2007 |

### 2.5.2 Neuritogenic substances from natural sources

Neuritogenic substances hold the promise of therapeutic efficacy in the treatment of neuronal injuries by the virtue of their ability to stimulate outgrowth of neurites (Phan, David, Naidu, et al., 2014; More et al., 2012). As discussed, the neuroactivities and therapeutic application of NGF are restricted by its high molecular polypeptide. Therefore, discovery of neuritogenic substance from natural sources with smaller molecular size that mimics and/or enhances the NGF activities or that may stimulates the production of NGF is a promising alternative treatment for neurodegenerative disease (Williams & Dwyer, 2009). A large number of natural products and their bioactive constituents have been demonstrated to stimulate neuritogenesis by acting alone or synergistically in combination with NGF. Table 2.2 summarizes different natural substances from plants or mushrooms which possess neuritogenic or NGF synthesis stimulatory effects *in vitro*. Studies have shown the potential of many common foods such as flat lemon (*Citrus depressa*), green tea (*Camellia sinensis*), turmeric (*Curcuma longa*), and culinary and/or medicinal mushrooms contain neuritogenic compounds that may mimic or enhance the neuritogenic effect of NGF *in vitro* (Table 2.2).

**Table 2.2: Natural substances from plants and mushrooms which possess neuritogenic or NGF synthesis stimulatory effects *in vitro*.**

| Natural Source | Extract / Compound | *In vitro* model | Neuritogenic activity | References |
|---|---|---|---|---|
| **Plant** | | | | |
| *Baccharis gaudichaudiana* | Clerodane diterpenes (compound 2,3,5) | PC-12D cells | Potentiate NGF (2 ng/ml)-stimulated neuritogenesis | Guo et al., 2007 |
| *Camellia sinensis* | Polyphenols | PC-12 cells | Potentiate NGF (2 ng/ml)-stimulated neuritogenesis | Gundimeda, McNeill, Schiffman, Hinton, & Gopalakrishna, 2010 |
| *Centella asiatica* | standardized extract, ECa 233 | IMR-32 | Stimulate neuritogenesis | Wanakhachornkrai et al., 2013 |
| *Citrus depressa* | Nobiletin | PC-12D cells | Stimulate neuritogenesis | Nagase et al., 2005 |
| *Curcuma longa* | Curcumin<br>Curcuminoids | PC-12 cells | Stimulate neuritogenesis | Liao et al., 2012; John, Wong, Naidu, Sabaratnam, & David, 2013 |
| *Gentiana rigescens* | Gentisides A and B | PC-12 cells | Stimulate neuritogenesis | Gao, Li, & Qi, 2010 |
| *Panax ginseng* | Ginsenoside Rb1<br>Ginsenoside Rg1 | Dopaminergic cells | Stimulate neuritogenesis | Radad et al., 2004 |
| *Polygonum multiflorum* | Emodin | N2a cells | Stimulate neuritogenesis | Park, Jin, et al., 2015 |
| *Pueraria montana* | Ethanol extract | Embryonic (E19) brain neurons | Stimulate neuritogenesis | Haque Bhuiyan et al., 2015 |

| Natural Source | Extract / Compound | *In vitro* model | Neuritogenic activity | References |
|---|---|---|---|---|
| **Plant** | | | | |
| *Smyrnium olusatrum* | Isofuranodiene | PC-12 cells | Stimulate neuritogenesis alone, potentiate NGF (50 nM)-stimulated neuritogenesis & increase neurite length | Mustafa et al., 2016 |
| *Withania somnifera* | Withanolide A, Withanoside IV & VI | Rat cortical neuron | Stimulate neuritogenesis | Kuboyama et al., 2002 |
| **Mushrooms** | | | | |
| *Cordyceps militaris* | Methanol extract | N2a cells | Stimulate neuritogenesis | Lee et al., 2011 |
| | Ethanol extract | N2a cells | Stimulate neuritogenesis | Phan, David, Naidu, Wong, & Sabaratnam, 2013 |
| *Dictyophora indusiata* | Dictyophorine A & B | Astroglial cells | Stimulate NGF synthesis | Kawagishi et al., 1997 |
| *Hericium erinaceus* | Hericenones (C-H) | Astroglial cells | Stimulate NGF synthesis | Kawagishi et al., 1991; Kawagishi & Ando, 1993; |
| | Erinacines (A-I) | Astroglial cells | Stimulate NGF synthesis | Kawagishi et al., 1994; Kawagishi, Shimada, Sakamoto, et al., 1996; Kawagishi, Simada, Shizuki et al., 1996; Lee et al., 2000 |
| *Lignosus rhinocerotis* | Hot aqueous extract (sclerotium) | PC-12 cells | Stimulate neuritogenesis | Eik et al., 2012 |
| | Hot aqueous extract (mycelium) | PC-12 cells | Stimulate neuritogenesis | John et al., 2013 |
| | Hot aqueous extracts (sclerotium & mycelium) | N2a cells | Stimulate neuritogenesis | Phan, David, Naidu, Wong, & Sabaratnam, 2013 |

| Natural Source | Extract / Compound | *In vitro* model | Neuritogenic activity | References |
|---|---|---|---|---|
| **Mushrooms** | | | | |
| *Pleurotus giganteus* | Hot aqueous & ethanol extracts | PC-12 cells | Stimulate neuritogenesis | Phan, Wong, David, Naidu, & Sabaratnam, 2012 |
| | Hot aqueous & ethanol extracts | N2a cells | Stimulate neuritogenesis | Phan, David, Naidu, Wong, & Sabaratnam, 2013 |
| | Uridine | N2a cells | Stimulate neuritogenesis | Phan, David, Wong, Naidu, & Sabaratnam, 2015 |
| *Sarcodon scabrosus* | Cyathane diterpenes | PC-12 cells | Potentiate NGF (20 ng/ml)-stimulated neuritogenesis | Shi, Liu, Gao, & Zhang, 2011 |
| *Termitomyces albuminosus* | termitomycesphins A-D | PC-12 cells | Stimulate neuritogenesis | Qi, Ojika, & Sakagami, 2000 |
| | termitomycesphins E-F | | | Qi, Ojika, & Sakagami, 2001 |
| *Tremella fuciformis* | Water extract | PC-12h cells | Stimulate neuritogenesis & increase neurite length | Kim et al., 2007 |
| *Tricholoma* sp. | Tricholomalides A-C | PC-12 cells | Stimulate neuritogenesis | Tsukamoto et al., 2003 |

## 2.6 MEDICINAL MUSHROOMS

Mushrooms have long been attracting a great deal of interest in many areas of food and biopharmaceuticals. Mushrooms are known as healthy food because they contain wide variety of nutritional components such as polysaccharides, protein, dietary fibers, lectins, minerals and low in calories and fat (with a high proportion of unsaturated fatty acids) (Ergönül, Akata, Kalyoncu, & Ergönül, 2013; Chang & Miles, 2008). In addition, mushrooms are a good source of phosphorus, iron and vitamins, including thiamine, riboflavin, ascorbic acid, ergosterol, and niacin (Barros et al., 2008). However, the awareness of mushrooms as functional foods, nutriceuticals and nutraceuticals with medicinal values has only recently emerged (Cheung, Cheung, & Ooi, 2003). Reports documented that mushrooms' extracts are rich in bioactive components such as polyphenols and flavonoids (Mishra, Pal, & Arunkumar, 2014; Radzki, Sławińska, Jabłońska-Ryś, & Gustaw, 2014) that possess pharmacological effects. In general, mushrooms or mushrooms' extracts that have preventive and therapeutic components for health-related conditions or ailments are known as medicinal mushrooms.

### 2.6.1 Medicinal mushrooms as mycomedicines and nutraceuticals

Medicinal mushrooms have a well-established history of use as mycomedicines in folk medicines and traditional oriental therapies (Wasser, 2010, 2002; Wasser & Weis, 1999). Mushroom-derived preparations have been used commonly as clinical practice in many countries, including China, Japan, Korea and other Asian countries for over the past two to three decades (Wasser & Weis, 1999; Hobbs, 1995). For over 3,000 years, the Chinese have revered and used medicinal mushrooms for health-maintaining and overall wellness, especially as tonics for the immune system (Hobbs, 1995). However, the medicinal efficacies of the mushrooms as mycomedicines and nutraceuticals were not clearly established by modern science until about two decades ago (Zhuang & Mizuno,

1999). The information is even scanty and poorly known in Malaysia (Chang & Lee, 2004). In Malaysia, many mushrooms have been cultivated or have long been utilized as food by indigenous folks. Selected species of *Pleurotus*, *Auricularia*, *Cookeina*, *Cyathus*, *Favolus*, *Lentinus*, *Pleurocybella*, *Schizophyllum* and *Termitomyces* are consumed as food (Chang & Lee, 2004). Further, species of *Lignosus*, *Pycnoporus*, *Lentinus*, *Daldinia*, *Ganoderma*, *Trametes*, *Tremella* and *Grifola* are used to treat various ailments or health related conditions (Chang & Lee, 2004; Wasser, 2002).

Many traditionally used medicinal mushrooms including extracts of the species of genus *Ganoderma*, *Hericium*, *Grifola*, *Auricularia*, *Flammulina*, *Lentinus*, *Pleurotus*, *Trametes*, *Tremella*, and *Schizophyllum* have been reported as potential nutraceuticals and mycomedicines (Wasser, 2002). Available evidences suggested that these edible and/or medicinal mushrooms expressed promising anti-oxidants (Cheung, Cheung, & Ooi, 2003; Jones, & Janardhanan, 2000), anti-tumor (Roupas, Keogh, Noakes, Margetts, & Taylor, 2012; Wasser 2002), anti-virus (Roupas et al., 2012; Wasser & Weis, 1999), anti-microbial (Roupas et al., 2012; Tidke & Rai, 2006), anti-inflammatory (Elsayed et al., 2014; Geng et al., 2014), anti-diabetic (Roupas et al., 2012), immunomodulating (Roupas et al., 2012; Wasser & Weis, 1999) activities and preventing hypercholesterolemia (Wasser & Weis, 1999), hyperlipidemia (Zhu et al., 2013), and hyperglycemia (Zhu et al., 2013). These functional activities are mainly contributed by their bioactive components, including phenols (Roupas et al., 2012; Cheung, Cheung, & Ooi, 2003), terpenoids (Roupas et al., 2012; Wasser & Weis, 1999), glucans (Smiderle et al., 2014; Vannucci et al., 2013), amino acids (Bernas, Jaworska, & Lisiewska, 2006), enzymes (Bernas et al., 2006), proteins, vitamins, minerals, and polysaccharides (Roupas et al., 2012; Bernas et al., 2006).

### 2.6.2 Medicinal mushrooms as anti-neuroinflammatory and neuritogenic agents for the management of neurodegenerative diseases

Extensive studies have demonstrated that medicinal mushrooms possess neuritogenic and neuroprotective effects *in vitro* and/or *in vivo* which are potential for the management of neurodegenerative diseases (Xu & Beelman, 2015; Phan, David, Naidu, et al., 2014). There is, however, to date, very limited information on the anti-neuroinflammatory activity of medicinal mushrooms. Recently, Phan, David, Naidu, et al. (2014) reviewed the therapeutic potential of culinary-medicinal mushrooms for the management of neurodegenerative diseases. The review presented various extracts and compounds from culinary-medicinal mushrooms that possessed neuritogenic and neuroprotective effects (Phan, David, Naidu, et al., 2014). Futher, Xu, & Beelman (2015) reviewed the protective potential of five medicinal mushrooms commonly used in Asian countries: *Hericium erinaceus*, *Termitomyces albuminosus*, *Ganoderma lucidum*, *Dictyophora indusiata*, and *Mycoleptodonoides aitchisonii* against neurodegenerative diseases. Hence, the potential of medicinal mushrooms have attracted intense interest in the search for mushroom nutriceuticals and nutraceuticals that may contribute to either preventive or therapeutic management of neurodegenerative diseases.

#### 2.6.2.1 *Hericium erinaceus* (Bull.: Fr.) Pers.

*Hericium erinaceus* (Bull.: Fr.) Pers. (Figure 2.9) is also known as monkey head mushroom, lion's mane mushroom, "Yamabushitake" (Japanese) or "Houtougu" (Chinese) (猴头菇).

*Hericium erinaceus* is a well-known medicinal mushroom in Asia, Europe, and North America (Mizuno, 1995). In recent years, *H. erinaceus* has been proven its pharmacological activities, notably on the management of neurological disorders. *In vivo* studies revealed the potential of *H. erinaceus* in peripheral nerve regeneration

Kingdom : Fungi
Phylum : Basidiomycota
Class : Agaricomycetes
Order : Russulales
Family : Hericiaceae
Genus : *Hericium*
Species : *erinaceus*

**Figure 2.9: Basidiocarps of *Hericium erinaceus* and its taxonomy.** Source: Mushroom Research Centre, University of Malaya.

following crush injury (Wong, Naidu, David, Bakar, & Sabaratnam, 2012; Wong et al., 2011). Animals (Mori, Obara, Moriya, Inatomi, & Nakahata, 2011) and human (Mori, Inatomi, Ouchi, Azumi, & Tuchida, 2009) studies showed that *H. erinaceus* may be appropriate for the prevention or treatment of dementia. Accumulating evidences showed that the hot aqueous extract of *H. erinaceus* stimulated neuritogenesis in NG108–15 cells, a hybrid neuronal cell line derived from mouse neuroblastoma and rat glioma (Lai, Naidu et al., 2013; Wong et al., 2007) and PC-12 cells (Zhang et al., 2015; Li, See, Moon, Yoo, & Lee, 2013). Stimulators of NGF synthesis may be potential therapeutic agents for degenerative neuronal disorder. The neuroactive compounds isolated from methanol extract of *H. erinaceus* basidiocarps (hericenones C to H) (Mori et al., 2008; Kawagishi & Ando, 1993; Kawagishi et al., 1991) and mycelium (erinacines A to I) (Shimbo, Kawagishi, & Yokogoshi, 2005; Lee et al., 2000; Kawagishi, Shimada, Sakamoto, et al., 1996; Kawagishi, Simada, Shizuki et al., 1996; Kawagishi et al., 1994) were reported to promote the biosynthesis of NGF *in vitro* and *in vivo* (Shimbo et al., 2005). Phan, Lee et al. (2014) reported that hericenone E isolated from *H. erinaceus* cultivated in Malaysia enhanced the NGF synthesis, and stimulated neuritogenesis in PC-12 cells via MEK/ERK and PI3K/AKT pathways. Recently, biosynthesis of gold nanoparticles (AuNPs) using the hot aqueous extract of

*H. erinaceus* was first reported by Raman et al. (2015). The study revealed the neuritogenic stimulatory potential of the myco-synthesized AuNPs from *H. erinaceus* on PC-12 cells (Raman et al., 2015). Raman et al. (2015) suggested that nanotechnology may be employed for myco-synthesized nanoparticles to deliver mushroom extracts to cross the BBB and trigger neuroactivities. It has been reported that *H. erinaceus* possessed anti-inflammatory effects in RAW 264.7 macrophage cells (Noh et al., 2014; Geng et al., 2014). Sterols (Li et al., 2015) and hericirine (alkaloid) (Li et al., 2014) from *H. erinaceus* were shown significant anti-inflammatory effects in LPS-stimulated RAW264.7 cells.

#### 2.6.2.2 *Pleurotus giganteus* (Berk.) Karunarathna & K. D. Hyde

*Pleurotus giganteus* (Berk.) Karunarathna & K. D. Hyde (Figure 2.10) is a culinary mushroom. *Pleurotus giganteus* characterized by its unbranched skeletal hyphae usually grows on buried woody substrates (Corner, 1981).

Kingdom : Fungi
Phylum : Basidiomycota
Class : Agaricomycetes
Order : Agaricales
Family : Pleurotaceae
Genus : *Pleurotus*
Species : *giganteus*

**Figure 2.10: Basidiocarps of *Pleurotus giganteus* and its taxonomy.** Source: Mushroom Research Centre, University of Malaya.

*Pleurotus giganteus* is formerly known as *Panus* or *Lentinus giganteus* (Karunarathna et al., 2012; Abdullah, Abdullah, Sabaratnam, & Grand, 2007) and also known in Malay as “seri pagi” (morning glory) or in Chinese “Zhudugu” (猪肚菇) or

swine's stomach (Deng et al., 2006). There is a confusion of scientific name of this mushroom. According to Deng et al. (2006), "Zhudugu" has been misused its scientific name as *Clitocybe maxima* in China. Data demonstrated that the characteristics of "Zhudugu" matched those of *Panus giganteus* (*Pleurotus giganteus*), and were clearly different from those of *C. maxima* (Deng et al., 2006).

In Malaysia, wild *P. giganteus* is a popular mushroom consumed by indigenous communities (Lee, Chang, & Noraswati, 2009). The nutritional composition of *P. giganteus* reported by Phan, Wong, David, Naidu, & Sabaratnam (2012), found that the basidiocarps contain high carbohydrate, dietary fibre, potassium, phenolic compounds and triterpenoids. In a later report, Phan, David, Tan et al. (2014) compared the chemical compositions of the commercial and domesticated wild strains of *P. giganteus*. The commercial strain presented higher carbohydrate, dietary fiber, total fat, and mono saturated fat content compared to the domesticated wild strain (Phan, David, Tan, et al., 2014). The aqueous and ethanol extracts of *P. giganteus* had shown anti-oxidant (Phan, David, Tan, et al., 2014), liver protection (Wong, Abdulla, et al., 2012), and anti-Candida activities (Phan, Lee et al., 2013). Today, there is growing evidence to support the potential of *P. giganteus* to mitigate neurodegenerative diseases. It has been shown that the hot aqueous and ethanol extracts of *P. giganteus* basidiocarps were non-cytotoxic towards PC-12 and N2a cells (Phan, David, et al., 2013), and stimulated neuritogenesis in PC-12 (Phan et al., 2012) and N2a cells (Phan, David, Tan, et al., 2014). The MEK/ERK and PI3K/AKT signaling pathways may be involved in *P. giganteus* extracts-stimulated neuritogenesis in PC-12 cells (Phan et al., 2012). Recently, a study by Phan et al. (2015) showed the neuritogenic stimulatory activity of chemical constituents from *P. giganteus*, including linoleic acid, oleic acid, cinnamic acid, caffeic acid, p-coumaric acid, succinic acid, benzoic acid, and uridine. Uridine (100 μM) stimulated neuritogenesis in N2a cells that was higher than NGF (50 ng/ml)-treated cells

by approximately 1.8-fold (Phan et al., 2015). Further, it was shown that the uridine-stimulated neuritogenic activity was mediated through the MEK/ERK1/2/CREB and PI3K/AKT/mTOR/CREB signaling pathways with enhancement of the expression of neuritogenesis biomarkers (GAP-43, tubulin alpha 4a, and beta) (Phan et al., 2015).

#### 2.6.2.3 *Cordyceps militaris* (L.: Fr.) Link

*Cordyceps* species, notably *Cordyceps militaris* (L.: Fr.) Link (Figure 2.11) have been known as an ethno-pharmacologically valuable mushroom and being the second most commercialized medicinal mushroom species in Korea, China, and Japan (Das, Masuda, Sakurai & Sakakibara, 2010; Zhou, Gong, Su, Lin, & Tang, 2009). *Cordyceps militaris* is known as the 'winter worm-summer grass' in Korea or "Dongchongxiacao" (冬虫夏草) in Chinese. It is a novel species of mushroom that grows inside the body of *Lepidoptera* larvae as a parasite (Zhou et al., 2009).

| | |
|---|---|
| Kingdom | : Fungi |
| Phylum | : Basidiomycota |
| Class | : Sardariomycetes |
| Order | : Hypocreales |
| Family | : Clavicipitaceae |
| Genus | : *Cordyceps* |
| Species | : *militaris* |

**Figure 2.11: Basidiocarps of *Cordyceps militaris* and its taxonomy.** Source: http://mushroaming.com/

*Cordyceps militaris* have long and rich history of usage as a tonic for longevity, endurance, and vitality, and as medicine to treat various inflammatory diseases, renal dysfunction, aged-related degenerative conditions, and cancers in traditional Oriental medicine (Won & Park, 2005). Previous reviews by Ng & Wang (2005) and Patel &

Ingalhalli (2013) have documented numerous pharmacological activities of *Cordyceps* species, including anti-oxidant, anti-tumor, immunomodulatory, anti-viral, anti-ageing, anti-diabetic, and anti-inflammatory. The ethanol extract of *C. militaris* presented topical anti-inflammatory activity in the croton oil-induced ear edema in mice (Won & Park, 2005). The hot aqueous extract of *C. militaris* exhibited anti-inflammatory effect by inhibiting the production of NO, TNF-α, and IL-6 in LPS-stimulated RAW 264.7 macrophage cells (Jo et al., 2010). A beta-(1→3)-D-glucan isolated from the alkaline extract of *C. militaris* showed anti-inflammatory effect by inhibiting the IL-1β, TNF-α, and COX-2 expression in LPS-stimulated human monocytic cell line (THP-1), and exhibited anti-nociceptive and anti-inflammatory activities against formalin-induced nociception and LPS-induced peritonitis in mice (Smiderle et al., 2014).

Recently, many studies have shown the potential of *C. militaris* as preventive and therapeutic agents for neurodegenerative diseases. The methanol extract of *C. militaris* was shown to increased choline acetyltransferase expression in N2a cells (Lee, Park et al., 2011). Simultaneously, administration of *C. militaris* methanol extract significantly reversed the scopolamine-induced deficit in rats' memory and learning ability (Lee, Park et al., 2011). The study suggested that *C. militaris* may be effective in protecting memory-related neuronal degeneration in brain and delaying the progression of memory deficits associated with various neurodegenerative diseases (Lee, Park et al., 2011). Cheng et al. (2011) reported that cordycepin, a neuroactive compound isolated from *C. militaris* protected the hippocampal neurons from ischemic injury *in vitro* and prevented post-ischemic neuronal degeneration and brain slice injury *in vivo*. An enriched ergosterol sub-fraction CE3 of ethyl acetate fraction of *C. militaris* was shown inhibited the production of NO in LPS-stimulated murine BV2 microglial cells (Nallathamby et al., 2015). Jeong et al. (2010) reported that cordycepin possessed anti-neuroinflammatory activity by inhibiting the production of NO, $PGE_2$ and the

expressions of iNOS, COX-2, TNF-α and IL-1β in LPS-stimulated murine BV2 microglia via the suppression of the NFκB, AKT, and MAPK signaling pathways (Jeong et al., 2010). There is, however, limited information on neuritogenic activity of *C. militaris*. Lee, Park et al. (2011) reported that pre-treatment with *C. militaris* methanol extract significantly stimulated neuritogenesis in N2a cells. Das et al. (2010) suggested that the mycelial extract of *C. militaris* NBRC 9787 and *C. militaris* G81-3 contain 106.8 and 45.4 mg/kg extract of uridine (a neuritogenic compound), respectively.

#### 2.6.2.4 *Grifola frondosa* (Dicks.: Fr.) SF Gray

*Grifola frondosa* (Dicks.: Fr.) SF Gray (Figure 2.12) is also known as 'hen of the woods' in English or "Maitake" in Japanese which means 'dancing mushroom' (舞茸). *Grifola frondosa* has been used as a health food for centuries in China and Japan (Mayell, 2001).

| | |
|---|---|
| Kingdom | : Fungi |
| Phylum | : Basidiomycota |
| Class | : Agaricomycetes |
| Order | : Polyporales |
| Family | : Fomitopsidaceae |
| Genus | : *Grifola* |
| Species | : *frondosa* |

**Figure 2.12: Basidiocarps of *Grifola frondosa* and its taxonomy.** Source: http://www.mycobank.org/

Maitake is a delicious culinary mushroom and also valued for its medicinal properties (Mayell, 2001). It has been cultivated for the uses as dietary supplement since two decades ago (Mayell, 2001). Polysaccharides from *G. frondosa*, significant by the beta-glucans (D- and MD-fraction) have shown their potential as immunomodulatory and anti-tumor agents (Mayell, 2001). *Grifola frondosa* also been

used as a remedy for pain and inflammation in Southeast Asia (Mayell, 2001). Agaricoglycerides, esters from *G. frondosa* showed potent anti-inflammatory activity by reducing the IL-1β, NFκB, ICAM-1, COX-2, and iNOS levels in animal models (Han & Cui, 2012). However, to date, no study has found on anti-neuroinflammatory activity of *G. frondosa*. For neuritogenic activity, the lysophosphatidylethanolamine, a neurotrophic compound isolated from *G. frondosa* significantly stimulated neuritogenesis and upregulated the neurofilament M expression in PC-12 cells via MEK/ERK1/2 signaling pathway (Nishina et al., 2006). Hot aqueous extract of *G. frondosa* at 75 µg/ml stimulated significant neuritogenesis in PC-12 cells via MEK/ERK1/2 and PI3K/AKT signaling pathways (Seow, Naidu, David, Wong, & Sabaratnam, 2013).

#### 2.6.2.5 *Ganoderma lucidum* (Curtis) P. Karst and *Ganoderma neo-japonicum* Imazeki

The genus *Ganoderma* is a popular medicinal mushroom, and has been used in traditional Chinese medicine (TCM) as a tonic and sedative in Asian countries, including China, Japan and Korea over the past two millennia (Paterson, 2006; Cheung, Hui, Chu, Chiu, & Ip, 2000). The *Ganoderma* species also named as "Lingzhi" (灵芝) in Chinese and "Reishi" in Japanese. According to "Shennong Ben Cao Jing"《神农本草经》also named as The Classic of Herbal Medicine, a Chinese book on agriculture and medicinal plants (300 BC – 200 AC), Lingzhi was classified into six categories based on color, which are red, yellow, black, white, green and purple. *Ganoderma lucidum* (Curtis) P. Karst (Figure 2.13a) is the most common red Lingzhi, and *Ganoderma neo-japonicum* Imazeki (Figure 2.13b) is categorized as purple Lingzhi.

**a**

Kingdom : Fungi
Phylum : Basidiomycota
Class : Agaricomycetes
Order : Polyporales
Family : Ganodermataceae
Genus : *Ganoderma*
Species : *lucidum*

**b**

Kingdom : Fungi
Phylum : Basidiomycota
Class : Agaricomycetes
Order : Polyporales
Family : Ganodermataceae
Genus : *Ganoderma*
Species : *neo-japonicum*

**Figure 2.13: Basidiocarps of (a) *Ganoderma lucidum* and its taxonomy. (b) *Ganoderma neo-japonicum* and its taxonomy.** Source: Mushroom Research Centre, University of Malaya.

*Ganoderma lucidum* (Curtis) P. Karst (Figure 2.13a) is one of the most commonly used medicinal mushrooms by TCM practitioners in Asia (Paterson, 2006). *Ganoderma lucidum* is widely cultivated in Malaysia due to the high humidity and temperature throughout the year (Cheng et al., 2013). The basidiocarps, mycelia and spores of *G. lucidum* contain approximately 400 different bioactive compounds, included polysaccharides, triterpenoids, nucleotides, sterols, steroids, fatty acids, proteins or peptides and trace elements (Sanodiya et al., 2009). Enormous studies revealed that the extracts of *G. lucidum* possessed significant anti-oxidant (Jones & Janardhanan, 2000), anti-tumor (Nonaka et al., 2006; Lin & Zhang, 2004), anti-diabetic (Ma, Hsieh, & Chen, 2015; Zhu et al., 2013), anti-inflammatory (Jones & Janardhanan, 2000), immunomodulating (Chen et al., 2004; Lin & Zhang, 2004) and wound healing

(Cheng et al., 2013) activities, and protects the cellular DNA from radiotherapy and accidental radiation exposure (Pillai, Savi, Maurya, Nair, & Janardhanan, 2006). Accumulating evidences showed that *G. lucidum* does not present cytotoxic effect and has demonstrated to be safe (Kim et al., 1986; Sugiura & Ito, 1977). *In vivo* study showed that the lethal dose to kill 50% of the studied subjects for a single intraperitoneal injection dose of *G. lucidum* extract in rodents was as high as 38 g/kg (Chang, 1995). Administration of aqueous extract of *G. lucidum* to mice (5 g/kg during 30 days) showed no changes in body weight, organ weight or hematological parameters (Wasser, 2005).

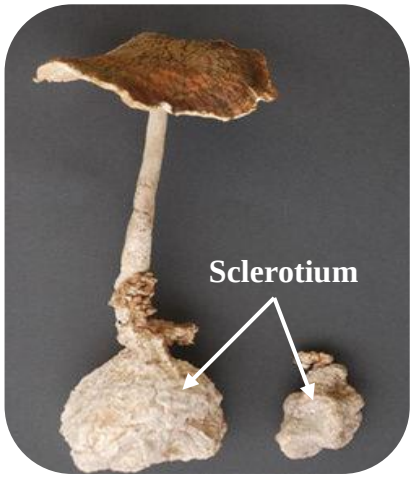
The potential use of *G. lucidum* for the prevention and treatment of neurodegenerative diseases has also been examined. It was shown that long-term consumption of *G. lucidum* may minimize the progression of Alzheimer's disease (Zhou et al., 2012; Lai, Yu et al., 2008) and Parkinson's disease (Zhang et al., 2011). Aqueous extract of *G. lucidum* significantly attenuated amyloid-β-induced synaptotoxicity and protects neuron from amyloid-β-induced toxicity (Lai, Yu et al., 2008). Pre-administration *of G. lucidum* improved the impairments of spatial learning and memory, protected hippocampus neurons from oxidative impairment in intra-cerebroventricular (ICV) injection of streptozotocin (STZ) rats (Zhou et al., 2012). Zhang et al. (2011) reported that *G. lucidum* protected the dopaminergic neuron degeneration through inhibition of microglial activation. Treatment with *G. lucidum* extracts significantly reduced the production NO, TNF-α and IL-1β and downregulated the TNF-α and IL-1β mRNA expressions in LPS-stimulated microglia (Zhang et al., 2011). Ethanol extract of *G. lucidum* was shown inhibited the production of pro-inflammatory mediators and cytokines in LPS-stimulated murine BV2 microglia via the inhibition of TLR4, MyD88, and NFκB expressions (Yoon et al., 2013). *Ganoderma lucidum* extracts may be a promising agent for the prevention and treatment of neurodegenerative diseases by

inhibiting inflammatory mediator responses in activated microglia (Yoon et al., 2013; Zhang et al., 2011). Furthermore, the aqueous extract of *G. lucidum* showed neuroprotective effects by promoting neuritogenesis (Seow et al., 2013; Cheung et al., 2000) and reduction of senescence of neuronal cells (Cheung et al., 2000). Cheung et al. (2000) demonstrated the presence of neuroactive compounds in *G. lucidum* by presenting the neuritogenic stimulatory activity in PC-12 cells. The study suggested that the neuritogenic effect of the aqueous extract of *G. lucidum* was mediated via the Ras/ERK and CREB signaling pathways (Cheung et al., 2000). Study by Seow et al. (2013) demonstrated that the hot aqueous extract of *G. lucidum* exhibited neuritogenic stimulatory activity that mimic NGF via MEK/ERK1/2 and PI3K/AKT signaling pathways. In addition, a lipophilic fraction of *G. lucidum* was shown to stimulate neuritogenesis in PC-12 cells (Zhang, Peng et al., 2005).

*Ganoderma neo-japonicum* Imazeki (Figure 2.13b) is found in Mainland China, Japan and Taiwan, and grows saprotrophically on dead hardwoods or bamboos (Hsieh & Yeh, 2004). In Malaysia, *G. neo-japonicum* grows in the forests and only on decaying clumps of tropical bamboo (*Schizostachyum brachycladium*) (Tan et al., 2015). A water infusion is used by the indigenous folks as food and medicine to treat fever and epilepsy, and tonic to strengthen the body (Tan et al., 2015). However, the scientific validation of the medicinal properties of *G. neo-japonicum* has not been fully investigated. Hence there is very few information on this mushroom in literature. Study by Seow et al. (2013) was the first to report the neuritogenic activity of *G. neo-japonicum* via MEK/ERK1/2 and PI3K/AKT signaling pathways. The hot aqueous extract of *G. neo-japonicum* (50 µg/ml) stimulated maximal percentage of neurite bearing cells by 14.22 ± 0.43%, which was higher than of 75 µg/ml of hot aqueous extract *G. lucidum* (12.61 ± 0.11%) and 50 ng/ml of NGF (11.94 ± 0.38%) in PC-12 cells (Seow et al., 2013). However, to date, no study has found on anti-neuroinflammatory activity of *G. neo-japonicum*.

#### 2.6.2.6 *Lignosus rhinocerotis* (Cooke) Ryvarden

*Lignosus rhinocerotis* (Cooke) Ryvarden is also known as the 'tiger's milk mushroom' in English, "cendawan susu rimau" in Malay or "Hurulingzhi" (虎乳灵芝) in Chinese (Figure 2.14).




Kingdom : Fungi
Phylum : Basidiomycota
Class : Agaricomycetes
Order : Polyporales
Family : Polyporaceae
Genus : *Lignosus*
Species : *rhinocerotis*

**Figure 2.14 *Lignosus rhinocerotis* and its taxonomy.** Source: Mushroom Research Centre, University of Malaya.

*Lignosus rhinocerotis* is native to the tropical rainforest in the region of South China, Thailand, Malaysia, Indonesia, Philippines and Papua New Guinea (Tan et al., 2010). *Lignosus rhinocerotis* is documented in The Diary of John Evelyn (publication dated 22 June 1664) as "*Lac tygridis*" (meaning tiger's milk) and later reported by Sir Henry Nicholas Ridley, 'the father of Malaya's rubber industry' (Ridley, 1890), as an important medicinal mushroom used by the indigenous communities. It is believed that the mushroom emerges at the spots where the milk of tigers has spilled on the ground (Tan, 2012). In Malaysia, it is hailed as a 'National Treasure' and it is the most popular medicinal mushroom used by the indigenous communities of Peninsular Malaysia (Lee et al, 2009). The benefits of its underground tuber or sclerotium (where most of the nutritional and medicinal components are deposited) (Figure 2.14) are well documented

compared to its basidiocarp (Lau, Abdullah, Aminudin, Lee, & Tan, 2015). According to the ethnopharmacological reports, the sclerotium of the mushroom is sliced, boiled and drank as a decoction by the indigenous communities as general tonic for overall wellness and also to treat several of ailments (Chang & Lee, 2004), including fever, cough, asthma, chronic hepatitis, gastric ulcer, cancer, food poisoning, (Lee et al., 2009; Chang & Lee, 2004). In China, the sclerotium of *L. rhinocerotis* is an expensive medicine used as a tonic by the TCM physicians to treat liver cancer, chronic hepatitis and gastric ulcer (Dai & Yang, 2008; Huang, 1999). Based on the traditional practice and scientific studies, Tan et al. (2012) proposed *L. rhinocerotis* as a potential functional ingredient to incorporate in functional foods, beverages and nutraceuticals.

*Lignosus rhinocerotis* was proved very difficult to cultivate and only available by collection from the jungle (Lee, Tan, Fung, Tan, & Ng, 2012). Hence, it is highly priced ranging from USD 15–25 per basidiocarp including the sclerotium (Abdullah, Dzul Haimi, Lau, & Annuar, 2013). Recently, Tan et al. (2012) reported a breakthrough in the cultivation of the *L. rhinocerotis* strain, LiGNO™ cultivar TM02 on agar, solid and spawn medium with high production yield. Thus, this breakthrough has overcome the supply problem and make possible for the research and investigation of the pharmacological and therapeutic activities of this mushroom.

Toxicology study of *L. rhinocerotis* extracts by using *in vitro* and *in vivo* approaches is necessary for the development of *L. rhinocerotis* as health supplements. The cold water extract of *L. rhinocerotis* sclerotium (TM02) was shown non-cytotoxic to the normal human breast (184B5) and lung (NL20) cells (Lee, Tan et al., 2012). The *L. rhinocerotis* sclerotium extracts were reported non-cytotoxic to the normal human colon (CCD-18Co), kidney (HEK-293), nasopharyngeal (NP 69), oral (OKF6), rat kidney (NRK-52E), and vero cell lines (Lau et al., 2014; Suziana Zaila et al., 2013). Phan, David et al. (2013) reported that the hot aqueous extract of *L. rhinocerotis*

sclerotium (TM02) was not cytotoxic towards N2a and embryonic fibroblast (3T3) cell lines. The ethanol extract of *L. rhinocerotis* sclerotium (TM02) was found non-cytotoxic towards murine BV2 microglia (Nallathamby et al., 2013). Recent study by Seow et al. (2015) documented that the hot aqueous and ethanol extracts, and crude polysaccharides of *L. rhinocerotis* sclerotium (TM02) did not exert any cytotoxic effect towards PC-12 cells after 48 hours of incubation. *In vivo* studies reported no treatment-related chronic toxicity in Sprague Dawley rats after a long term (180 days) oral administration of the *L. rhinocerotis* sclerotium freeze-dried powder (TM02) at daily dosage up to 1,000 mg/kg (Lee, Enchang, Tan, Fung, & Pailoor, 2013; Lee, Tan, Fung, Pailoor, & Sim, 2011). In addition, blood biochemical parameters related to toxicity were reported as normal, however, these studies did not estimate the levels of the mushroom in the blood (Lee, Enchang, et al., 2013; Lee, Tan, et al., 2011). Overall, the *in vitro* and *in vivo* studies showed that the cultivated *L. rhinocerotis* sclerotium (TM02) is safe for consumption. Moreover, Lai, Loo et al. (2013) documented that the wild *L. rhinocerotis* collected from Pahang, Malaysia contain low levels of heavy metals, such as mercury, cadmium and lead.

A number of studies revealed that the sclerotium of *Polyporus rhinocerus* or *Lignosus rhinocerus* (synonym of *L. rhinocerotis*) (Lau et al., 2015) demonstrated anti-inflammatory (Lee et al., 2014), anti-oxidant (Lau et al., 2014; Yap et al., 2013), anti-tumor (Lau, Abdullah, & Lee, 2013; Lee, Tan, et al., 2012), immunomodulating (Wong, Lai & Cheung, 2011, 2009; Guo, Wong, & Cheung, 2011), anti-microbial (Mohanarji, Dharmalingam, & Kalusalingam, 2012), anti-viral (Kavithambigai, Sabaratnam, & Thayan, 2013), fibrinolytic (Kho, 2014; Ahmad, Noor, & Ariffin, 2013), and anti-coagulant (Teo, 2014; Sabaratnam et al., 2013) activities. Lee et al. (2014) reported that the cold water, hot water and methanol extracts of *L. rhinocerotis* sclerotium (TM02) possessed anti-acute inflammatory activity *in vivo.* The cold water extract significantly

reduced carrageenan-induced paw edema and inhibited TNF-α production in LPS-stimulated RAW 264.7 macrophage cells (Lee et al., 2014). However, to date, no study has found on anti-neuroinflammatory activity of *L. rhinocerotis*.

Limited information is available on the neuronal activity of *L. rhinocerotis*. In Malaysia, the indigenous communities also drank the decoction of *L. rhinocerotis* sclerotium to increase their alertness during hunting (tacit knowledge). Tan et al. (2012) documented that the consumption of tiger's milk mushroom improved stamina and alertness in healthy people. Increment of the mental alertness is believed to be related to the neuroactivity and neuronal communication network in brain. The potential of *L. rhinocerotis* to stimulate neuritogenesis was first investigated by Eik, Naidu, David, Wong, & Sabaratnam (2011). The hot aqueous extract of *L. rhinocerotis* sclerotium (TM02) induced significantly higher percentage of neurite bearing cells compared to other mushrooms tested, including *Hericium erinaceus*, *Termitomyces* sp. and *Agrocybe* sp. (Eik et al., 2011). Later, Eik et al. (2012) and Phan et al. (2013) reported that 20 µg/ml of hot aqueous extract of *L. rhinocerotis* sclerotium (TM02) stimulated significantly higher percentage of neurite bearing cells than the NGF (50 ng/ml) in PC-12 and N2a cells, respectively. The hot aqueous extract of mycelium of *L. rhinocerotis* was also found promoting neuritogenesis in PC-12 (John et al., 2013) and N2a cells (Phan, David, et al., 2013). Recent study by Seow et al. (2015) demonstrated that the hot aqueous and ethanol extracts, and crude polysaccharides of *L. rhinocerotis* sclerotium (TM02) stimulated neuritogenesis in PC-12 cells, without stimulating the synthesis of NGF. The study further revealed that the hot aqueous extract of *L. rhinocerotis* mimic the NGF activity by stimulating neuritogenesis in PC-12 cells via NGF responsive pathway, the TrkA/MEK/ERK1/2 signaling pathway (Seow et al., 2015).

To date, the chemical investigations of *L. rhinocerotis* are scanty. Studies by Suziana Zaila et al. (2013) and Mohanarji, Dharmalingam, & Kalusalingam (2012) documented the presence of alkaloids, flavonoids, mucilage and gum in organic solvent extracts of *L. rhinocerotis*. Recent study by Nallathamby et al. (2016) reported that the ethyl acetate fraction of *L. rhinocerotis* sclerotium contained numbers of unsaturated fatty acids. The flame ionization detector (FID) and gas chromatography–mass spectrometry (GC–MS) analysis showed that the linoleic, ethyl linoleic and oleic acids were the major lipid constituents (Nallathamby et al., 2016). Based on the chemical analysis reports of ultra-high performance liquid chromatography-electrospray ionization tandem-mass spectrometry (UHPLC–ESI–MS/MS) and GC–MS, Lau et al. (2014) suggested that the aqueous extracts of *L. rhinocerotis* contained numerous low-molecular weight secondary metabolites, including fatty acids (linoleic acid, palmitic acid, stearic acid, and oleic acid), triterpenoids, sugar alcohols (arabinitol), amino acids (phenylalanine and tryptophan), organic acids (citric acid), and sterols (ergosterol). However, further confirmation, purification and characterization of these compounds have not been taken.

# CHAPTER III: SCREENING OF MUSHROOMS FOR CYTOTOXIC, NITRIC OXIDE INHIBITORY AND NEURITOGENIC STIMULATORY ACTIVITIES *IN VITRO*

## 3.1 INTRODUCTION

The potential of medicinal mushrooms as mycomedicines, functional foods and dietary supplements (nutriceuticals and nutraceuticals) has been widely explored (Zaidman, Yassin, Mahajna, & Wasser, 2005; Wasser, 2002). Today, the neuroactivities of medicinal mushrooms are under intense study and research. Many studies have revealed the preventive and therapeutic potential of medicinal mushrooms in management of neurodegenerative diseases (Xu & Beelman, 2015; Phan, David, Naidu, Wong, & Sabaratnam, 2014).

Studies showed that neuroinflammation (Frank-Cannon, Alto, McAlpine, & Tansey, 2009; Kim & Joh, 2006) and neurites or axon degeneration (Wang, Medress, & Barres, 2012; Bjartmar, Wujek, & Trapp, 2003) are involved in several neurodegenerative diseases. Discovery of anti-neuroinflammatory and neuritogenic stimulatory agents may be preventive and therapeutic strategies for neurodegenerative diseases. In recent years, neuroactive components that promote anti-neuroinflammatory (Karunaweera, Raju, Gyengesi, & Münch, 2015) and neuritogenic (Phan, David, Naidu, Wong, & Sabaratnam, 2014; More et al., 2012) activities from natural sources such as herbs, spices and mushrooms have become the core focus in the search for preventive and therapeutic agents for neurodegenerative diseases. Among the natural sources explored, medicinal mushrooms have shown huge potential (Xu & Beelman, 2015; Phan, David, Naidu, et al., 2014). Recent reports have shown that extracts from numerous mushrooms possess potent anti-neuroinflammatory and neuritogenic

stimulatory activity *in vitro* and/or *in vivo*. Extracts of *Taiwanofungus camphoratus* (Liu et al., 2007), ethanol extract of *Ganoderma lucidum* (Yoon et al., 2013; Zhang et al., 2011) and exopolysaccharide of *Laetiporus sulphureus* var. *miniatus* (Jayasooriya et al., 2011) have shown significant anti-neuroinflammatory activity *in vitro*, by inhibiting the production of pro-inflammatory mediators and cytokines in activated microglial cells. The medicinal mushroom, *Hericium erinaceus* (Bull.:Fr.) Pers. has neuritogenic and nerve regeneration potential in *in vitro* (Li, See, Moon, Yoo, & Lee, 2013; Wong et al., 2007) and *in vivo* (Wong, Naidu, David, Bakar, & Sabaratnam, 2012; Wong et al., 2011) studies. Further, the neuritogenic effects of several mushrooms have also been documented, including the sclerotium (Eik et al., 2012) and mycelium (John, Wong, Naidu, Sabaratnam, & David, 2013) of *Lignosus rhinocerotis* (Cooke) Ryvarden, extracts of *Pleurotus giganteus* (Berk.) Karunarathna & K.D. Hyde (Phan, Wong, David, Naidu, & Sabaratnam, 2012) and *Cordyceps militaris* (Lee et al., 2011). However, there are many other species of mushrooms known to have neuroactivities based on ethnopharmacological uses, but yet to validated scientifically.

The immortalized murine BV2 microglial cell line has been used extensively in the research related to neuroinflammatory and neurodegenerative disorders (Stansley et al., 2012). Studies by Horvath, McMenemy, Alkaitis, & DeLeo (2008) and Henn et al. (2009) showed that the BV2 microglial cell line is an appropriate model alternative to the primary cultures for the studies of microglia-mediated neuroinflammatory and the underlying mechanism. The BV2 microglia expressed microglia activation marker and released substantial amounts of pro-inflammatory mediators and cytokines upon LPS stimulation that were comparable to the primary microglia (Horvath et al., 2008). Furthermore, in response to LPS, BV2 microglia expressed 90% of inflammatory genes that also expressed by primary microglia (Henn et al., 2009). In the present study, the BV2 microglial cell line was utilized as an *in vitro* model system to investigate the

inhibitory effect of mushrooms extracts and crude polysaccharides on NO production (Chapter III). Further, the BV2 microglial cell line was used to investigate the anti-neuroinflammatory and the underlying mechanisms of the active fractions of selected mushroom (Chapter IV).

The PC-12 cell line has been used extensively as an *in vitr*o model system for the study of the actions of NGF (Teng, Angelastro, Cunningham, & Greene, 2006; Greene & Tischler, 1976) and NGF mimics (Williams & Dwyer, 2009). The PC-12 cell line is also used to study the NGF responsive signaling pathways (Vaudry et al., 2002; Greene et al., 1984) for neuronal differentiations, proliferation and survival. The PC-12 cells respond to neurotrophins, such as NGF by differentiating into sympathetic neuron-like phenotypes characterized by neurite outgrowth and expression of several neuronal specific proteins (Guroff, 1984; Tischler & Greene, 1975). In the present study, the PC-12 cell line was utilized as an *in vitro* model system to study the neuritogenic stimulatory effect of mushrooms extracts and crude polysaccharides (Chapter III). Further, the PC-12 cell line was used to investigate the NGF-mimicking activity and the underlying mechanisms of the active fractions of selected mushroom (Chapter V).

The aims of this chapter were to identify and select a potential mushroom extract based on its anti-neuroinflammatory and neuritogenic stimulatory effects *in vitro*. Four medicinal mushrooms were selected for screening, namely (i) *Ganoderma lucidum* (Curtis : Fr.) P. Karst, (ii) *Ganoderma neo-japonicum* Imazeki, (iii) *Grifola frondosa* (Dicks.) Gray, and (iv) *Lignosus rhinocerotis* (Cooke) Ryvarden. The identification and selection of a potential mushroom extract was based on three screening criteria, the (i) cytotoxic effect on BV2 microglia and PC-12 cells, (ii) inhibition of NO production in LPS-stimulated BV2 microglia, and (iii) neuritogenic stimulatory effect in PC-12 cells.

## 3.2 MATERIALS AND METHODS

### 3.2.1 Mushrooms samples

The *G. lucidum* basidiocarps (KLU-M 1233) were obtained from Ganofarm in Tanjung Sepat, Selangor. The *G. neo-japonicum* basidiocarps (KLU-M 1231) were collected from a forest in Ulu Grik, Perak. *Grifola frondosa* basidiocarps (KLU-M 1229) were purchased from a hypermarket in Selangor, Malaysia. The mushrooms were identified and authenticated by experts in the Mushroom Research Centre, University of Malaya. Voucher specimens are deposited in the University of Malaya Herbarium (KLU-M). The *L. rhinocerotis* sclerotium freeze-dried powder (LiGNO™ cultivar TM02) was purchased from Ligno Biotech Sdn. Bhd., Malaysia (Sabaratnam & Chang, 2013). Every batch of cultivar TM02 freeze-dried powder was identified and validated by the internal transcribed spacer regions of ribosomal RNA (Tan et al., 2010).

### 3.2.2 Preparation of mushrooms samples

The fresh basidiocarps of *G. frondosa* were sliced, weighed and freeze-dried while *G. lucidum* and *G. neo-japonicum* were air dried. The dried basidiocarps were then ground to powder by a Waring commercial blender, and the mushrooms powders were stored at –20 °C prior to extraction. Same batch of mushrooms basidiocarps was used throughout the assays.

#### 3.2.2.1 Hot aqueous extract

The hot aqueous extraction was carried out according to Wong et al. (2007). Briefly, the freeze-dried mushroom powder was weighed and soaked in distilled water at a ratio of 1:20 (w/v) and was agitated on a shaker at 150 rpm at room temperature for 24 hours. Then, the mixture was double boiled in a water bath for 30 minutes, and cooled to room temperature. The mixture was then centrifuged at 7,800 X g for 15 minutes, and the

supernatant was collected and filtered through a Whatman no. 4 filter paper. The resulting hot aqueous extract was freeze-dried at –50 ± 2 °C for 48 hours.

#### 3.2.2.2 Ethanol extract

The freeze-dried mushroom powder was weighted and soaked in 80% ethanol (v/v in distilled water) at a ratio of 1:10 (w/v) at room temperature for three days, and the process was repeated three times. The cocktails were then filtered through a Whatman no. 1 filter paper. Filtered supernatant was collected and the ethanol was evaporated using a rotary evaporator (Eyela N-1000, USA).

#### 3.2.2.3 Crude polysaccharides

The crude polysaccharides were extracted according to the alkaline extraction method described by Ojha, Chandra, Ghosh, & Islam (2010). Freeze-dried mushroom powder was weighted and soaked in 4% (w/v) sodium hydroxide (NaOH) to submerge the mushroom powder. The mixture was heated in 80 °C water bath for 45 minutes, and then the mixture was centrifuged at 7,800 X g for 45 minutes. Supernatant was collected and precipitated with absolute ethanol at a ratio of 1:5 (v/v). The mixture was kept for 12 hours at 4 °C. Then, the precipitated polysaccharides were centrifuged at 7,800 X g for 45 minutes. The residue was dialysed using a diethylaminoethyl (DEAE) cellulose bag for 4 hours. The crude polysaccharides were freeze-dried at –50 ± 2 °C for 48 hours.

The hot aqueous and ethanol extracts, and crude polysaccharides were stored in airtight bottles at –20 °C prior to use. All extracts and crude polysaccharides were prepared freshly in complete cell culture medium (supplemented cell culture medium), and sterilized with a micro-pore filter of size 0.2 microns (Sartorius, Germany) prior assays.

### 3.2.3 *In vitro* cell cultures

#### 3.2.3.1 Murine BV2 microglial cell line

Murine BV2 microglia were maintained in Dulbecco Modified Eagle medium (DMEM) supplemented with 1% (v/v) penicillin-streptomycin, 0.1% (v/v) gentamycin, 0.5% (v/v) fungizone, 5% (v/v) heat-inactivated fetal bovine serum (FBS), 1X non-essential amino acids (NEAA) (all purchased from Gibco, Life Technologies, USA) and 0.1% (v/v) bovine insulin (Sigma-Aldrich, USA) with final pH 7.2 - 7.4. Cells were incubated at 37 °C in a 5% $CO_2$-humidified incubator and were passaged every two to three days upon 80% confluent. Upon confluence, cells were harvested by treating with 0.25% (v/v) trypsin in 1 mM of ethylenediaminetetraacetic acid (EDTA) (Gibco, Life Technologies, USA) for five minutes at 37 °C. The cells are collected by centrifugation at 500 X g for five minutes and resuspended in complete DMEM.

#### 3.2.3.2 Rat pheochromocytoma cell line

Rat pheochromocytoma (PC-12) cell line was purchased from American Type Culture Collection (Cat#: CRL-1721.1) (ATCC, USA). PC-12 cells were maintained in ATCC-formulated Kaighn's Modification of Ham's F-12 Medium (F-12 K medium) (Sigma-Aldrich, USA), supplemented with 15% (v/v) of heat-inactivated horse serum (PAA Laboratories, Germany) and 2.5% (v/v) of heat-inactivated fetal bovine serum (FBS) (PAA Laboratories, Germany) at 37 °C in a 5% $CO_2$-humidified incubator. Cells were passaged every two to three days upon 80 % confluent. Upon confluence, cells were harvested by using cell scrapper.

### 3.2.4 Assessment of cytotoxicity of mushrooms extracts and crude polysaccharides

Cytotoxicity was assessed by the mitochondrial-dependent reduction of 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich, USA) to purple formazan. Assay was carried out according to the method described by Morgan (1998). Cells were plated in 96-well plates at a density of 1 X $10^4$ (PC-12 cells) or 5 X $10^4$ (BV2 microglia) cells per well and incubated overnight at 37 °C in a 5% $CO_2$-humidified incubator. Then, a dilution series of extracts or crude polysaccharides were added into the cells. After 48 hours of incubation, 20 µl of MTT (5 mg/ml) in phosphate buffered saline (PBS, pH 7.4) was added into each well and incubated at 37 °C for 4 hours. Subsequently, the supernatant was carefully discarded by aspiration, and 100 µl of dimethyl sulfoxide (DMSO) was then added into each well to dissolve the MTT formazan crystals, mixed thoroughly and incubated for 15 minutes. The extent of the reduction of MTT was determined by measurement of the absorbance at 540 nm with 690 nm as background absorbance using an enzyme-linked immunosorbent assay (ELISA) microplate reader (Sunrise, Tecan, Switzerland). The complete medium (supplemented cell culture medium) was the blank. Cells in complete medium only were considered as 100% of cell viability. The 50% inhibitory concentration ($IC_{50}$) was analyzed using Probit regression analysis of SPSS 17.0 (SPSS Science Inc., Chicago, IL).

### 3.2.5 Assessment of inhibition of nitric oxide production in LPS-stimulated BV2 microglia by mushrooms extracts and crude polysaccharides

#### 3.2.5.1 Treatment and stimulation of BV2 microglia

BV2 microglial cells were plated in 96-well plates at a density of 5 X $10^4$ cells per well and incubated overnight at 37 °C in a 5% $CO_2$-humidified incubator. Cells were then

pre-treated with increasing concentrations of extracts or crude polysaccharides for 24 hours and followed by stimulation with one μg/ml of lipopolysaccharide (LPS) from *Escherichia coli* 055:B5 (Sigma-Aldrich, USA) in complete DMEM without phenol red for 24 hours.

### 3.2.5.2 Griess and cell viability assays

Griess assay was performed to measure the concentration of nitrite (a stable oxidized product of NO) released into the culture supernatant. The cell culture supernatant (50 μl) was transferred to a 96-well plate, added with 50 μl of Griess reagent (1% (w/v) sulphanilamide and 0.1% (w/v) N-1-napthylethylenediamine dihydrochloride in 2.5% (v/v) phosphoric acid (all Sigma-Aldrich, USA). The absorbance was read at 530 nm with 690 nm as background absorbance. The blank was complete DMEM (supplemented cell culture medium) without phenol red, and the negative control was cells with complete DMEM without phenol red only. Nitrite oxide concentration was calculated following deduction of blank, with reference to a standard curve generated by serially diluted sodium nitrite (0 to 100 μM).

To exclude the possibility that the inhibition of NO production is due to the cytotoxic effect of the extracts or crude polysaccharides treatment, the cell viability was performed after the culture supernatant was transferred for Griess assay. Cell viability was assessed by the mitochondrial-dependent reduction of 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS), according to the protocol of Immunology Lab, University of Putra Malaysia. A total of 10 μl of MTS solution (CellTiter 96® AQueous One Solution Reagent) (Promega, USA) was added into each well and incubated for two hours. The quantity of formazan product was determined by measurement of the absorbance at 490 nm with 690 nm as background absorbance using an ELISA microplate reader. The complete DMEM

without phenol red medium was the blank. The viability of BV2 cells in complete DMEM without phenol red medium was considered as 100%.

### 3.2.6 Assessment of neuritogenic stimulatory effects of mushrooms extracts and crude polysaccharides in PC-12 cells

#### 3.2.6.1 Stimulation of neuritogenesis in PC-12 cells

Neuritogenesis stimulation assay was performed according to Smalheiser & Schwartz (1987). Cells were plated at a density of 5 X $10^3$ cells per well in 12-well plates and then treated with extracts or crude polysaccharides (25 to 100 µg/ml) in complete F-12 K medium. Eik et al. (2012) reported that 50 ng/ml (w/v) of NGF-7S was the optimum concentration for neuritogenesis in PC-12 cells. Thus, in the present study, cells treated with 50 ng/ml (w/v) of NGF-7S from murine submaxillary gland (Sigma-Aldrich, USA) served as a positive control. Cells in complete F-12 K medium without treatment served as a negative control. Assay plates were incubated for 48 hours at 37 ± 2 °C in a 5% $CO_2$-humidified incubator prior to scoring the neurite-bearing cells.

#### 3.2.6.2 Quantification of neurite bearing cells

Differentiated cells were counted by visual examination of the field by using a phase contrast microscope. A neurite-bearing cell was defined as a cell with one or more axon-like extension that was double or more the length of the cell body diameter (Smalheiser & Schwartz, 1987). Ten random microscopic fields with an average of 200 - 300 cells per well were assessed under an inverted microscope (Nikon Eclipse TS100) with 20X magnifications. The images were captured with a QImaging Go-3 color CMOS Camera (QImaging, Canada) and by the image processor system, Image-Pro Insight (MediaCybernetics, MD). The percentage of neurite-bearing cells was evaluated by scoring the proportion of neurite-bearing cells to the total number of cells in a well.

### 3.2.7 Statistical analysis

All the experimental data were expressed as the mean ± standard deviation (SD) of triplicate values of at least three independent experiments. Statistical differences between groups were assessed using one-way analysis of variance (ANOVA) of a minimum of three independent experiments and post-hoc analysis, Duncan's multiple range test (DMRT), $p < 0.05$ was considered to be significant. Software SPSS 17.0 (SPSS Science Inc., Chicago, IL) was used.

## 3.3 RESULTS

### 3.3.1 The extraction yield of mushrooms extracts and crude polysaccharides

The extraction yield of hot aqueous extracts of *G. lucidum*, *G. neo-japonicum*, *G. frondosa*, and *L. rhinocerotis*, ethanol extract and crude polysaccharides of *L. rhinocerotis* are summarized in Table 3.1.

**Table 3.1: Extraction yield from mushrooms studied by different extraction methods.**

| Mushrooms | Extract | Yield (%, w/w) |
|---|---|---|
| *Ganoderma lucidum* | Hot aqueous | 36.70 |
| *Ganoderma neo-japonicum* | Hot aqueous | 57.52 |
| *Grifola frondosa* | Hot aqueous | 35.44 |
| *Lignosus rhinocerotis* | Hot aqueous | 48.20 |
| *Lignosus rhinocerotis* | Ethanol | 14.30 |
| *Lignosus rhinocerotis* | Crude polysaccharides | 43.16 |

### 3.3.2 The cytotoxic effects of mushrooms extracts and crude polysaccharides on BV2 microglia

Cytotoxicity assay was done to identify the non-cytotoxic concentrations of extracts or crude polysaccharides for further studies. The cytotoxic effects of mushrooms extracts and crude polysaccharides on BV2 microglia are presented in Figure 3.1. The viability of BV2 microglial cells in complete DMEM medium was considered as 100%. The viable rates of treated cells decreased in a concentration-dependent manner for hot aqueous extracts of *G. lucidum*, *G. neo-japonicum* and *G. frondosa* (Figure 3.1a), and hot aqueous extract, ethanol extract and crude polysaccharides of *L. rhinocerotis* (Figure 3.1b), after 48 hours of incubation. A significant ($p < 0.05$) increased of viability was observed at the concentrations of 3.91 µg/ml and 7.81 µg/ml of hot aqueous extracts of *G. lucidum* (Figure 3.1a), and at 7.81 µg/ml of hot aqueous extract of *L. rhinocerotis* (Figure 3.1b). The percentage of viable cells decreased gradually as the concentration of the extracts and crude polysaccharides were increased. There was a significant ($p < 0.05$) increase of cytotoxicity starting from the concentrations of 31.25 µg/ml (*G. neo-japonicum*), 62.50 µg/ml (*G. frondosa*), 500 µg/ml (hot aqueous extract of *L. rhinocerotis*), 15.63 µg/ml (ethanol extract of *L. rhinocerotis*), and 250 µg/ml (crude polysaccharides of *L. rhinocerotis*), reducing the percentage of viability by 25.60%, 38.98%, 10.32%, 9.34%, and 9.37% respectively, compared to 100% viability. Interestingly, no significant ($p > 0.05$) cytotoxicity found in hot aqueous extract of *G. lucidum*-treated cells up to 1000 µg/ml. The hot aqueous extracts of *G. frondosa* and *G. neo-japonicum*, and ethanol extract of *L. rhinocerotis* were cytotoxic to BV2 microglia at higher concentrations. The inhibitions percentage of hot aqueous extracts of *G. frondosa* and ethanol extract of *L. rhinocerotis* were higher than 80%, at 125 µg/ml (80.04%) and 1000 µg/ml (85.75%), respectively. Over 95% of reduced viability was

found at the concentrations of 250 µg/ml and above in hot aqueous extract of *G. frondosa*-treated cells.

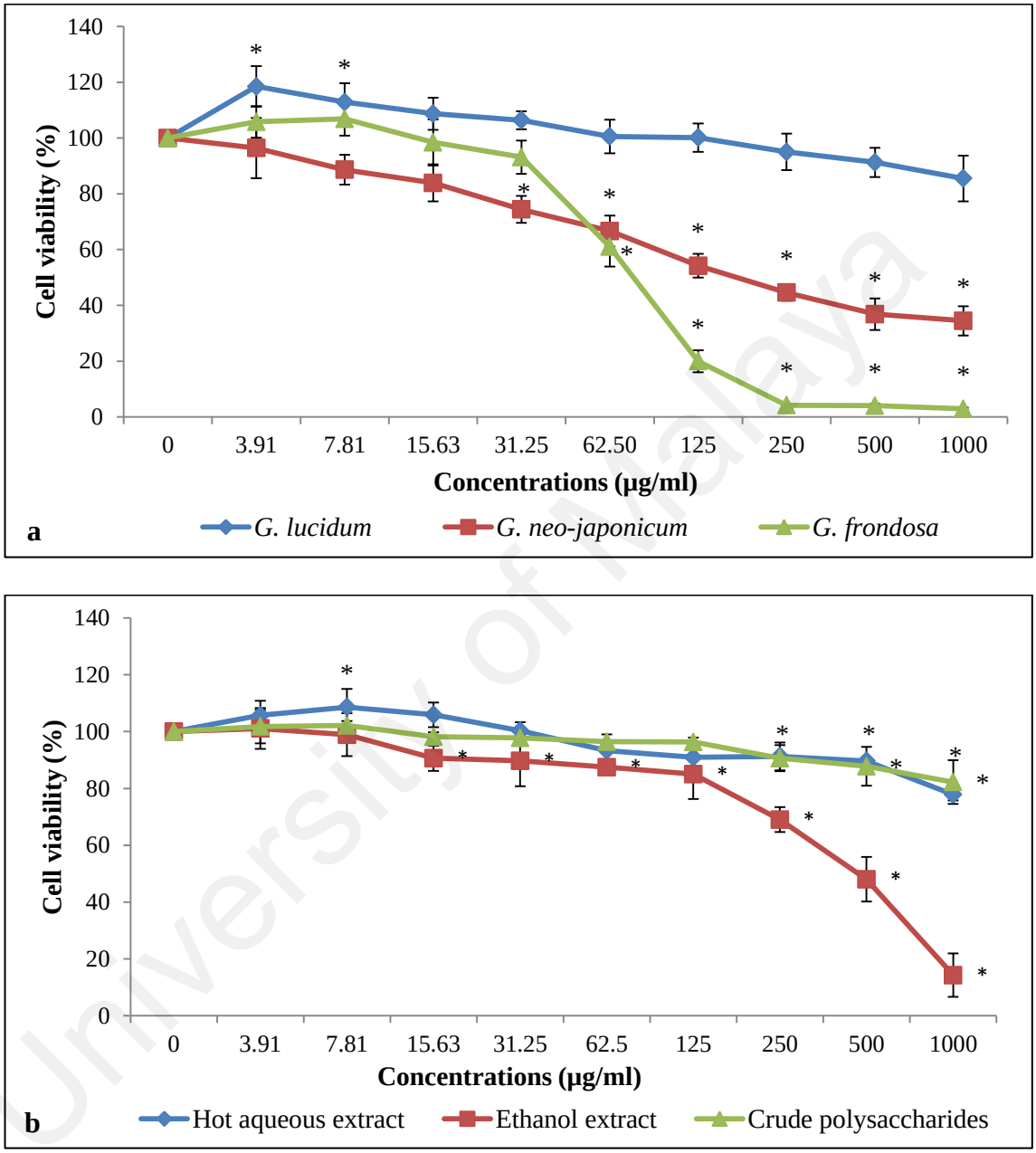



**Figure 3.1: The cytotoxic effects of mushrooms extracts and crude polysaccharides on BV2 microglia.** (a) Hot aqueous extract of *G. lucidum*, *G. neo-japonicum* and *G. frondosa*, (b) Hot aqueous extract, ethanol extract and crude polysaccharides of *L. rhinocerotis*. Cells were incubated with extracts or crude polysaccharides at concentrations from 0 to 1000 µg/ml for 48 hours and measured for viability by MTT assay. The mean absorbance obtained using complete DMEM medium with BV2 cells was designated as 100% viable. Data were expressed as means ± SD from three independent experiments carried out in triplicates. * $p < 0.05$ compared to BV2 cells only.

The required concentrations to inhibit the viability of BV2 microglia by 50% ($IC_{50}$) for all mushrooms extracts and crude polysaccharides at 48 hours are shown in Table 3.2. The hot aqueous extract of *L. rhinocerotis* has highest $IC_{50}$ value (20.01 ± 1.69 mg/ml), followed by crude polysaccharides of *L. rhinocerotis* (16.04 ± 1.03 mg/ml) and hot aqueous extract of *G. lucidum* (13.57 ± 1.81 mg/ml).

**Table 3.2: The 50% inhibitory concentration ($IC_{50}$) of mushrooms extracts and crude polysaccharides on BV2 microglia viability after 48 hours of incubation.**

| Mushrooms | Extract | $IC_{50}$ (mg/ml) on BV2 cells |
|---|---|---|
| *Ganoderma lucidum* | Hot aqueous | $13.57 \pm 1.81^{a,b}$ |
| *Ganoderma neo-japonicum* | Hot aqueous | $0.22 \pm 0.08^{a}$ |
| *Grifola frondosa* | Hot aqueous | $0.08 \pm 0.01^{a}$ |
| *Lignosus rhinocerotis* | Hot aqueous | $20.01 \pm 1.69^{b}$ |
| *Lignosus rhinocerotis* | Ethanol | $0.42 \pm 0.02^{a}$ |
| *Lignosus rhinocerotis* | Crude polysaccharides | $16.04 \pm 1.03^{a,b}$ |

The data represent the mean ± SD from three independent experiments carried out in triplicates. Means with different alphabets represent significant difference ($p < 0.05$).

### 3.3.3 The cytotoxic effects of mushrooms extracts and crude polysaccharides on PC-12 cells

The viability of PC-12 cells in complete F-12 K medium was considered as 100%. The viable rate of treated cells decreased in a concentration-dependent manner for hot aqueous extracts of *G. lucidum*, *G. neo-japonicum* and *G. frondosa* (Figure 3.2a), and hot aqueous extract, ethanol extract and crude polysaccharides of *L. rhinocerotis* (Figure 3.2b) after 48 hours of incubation.

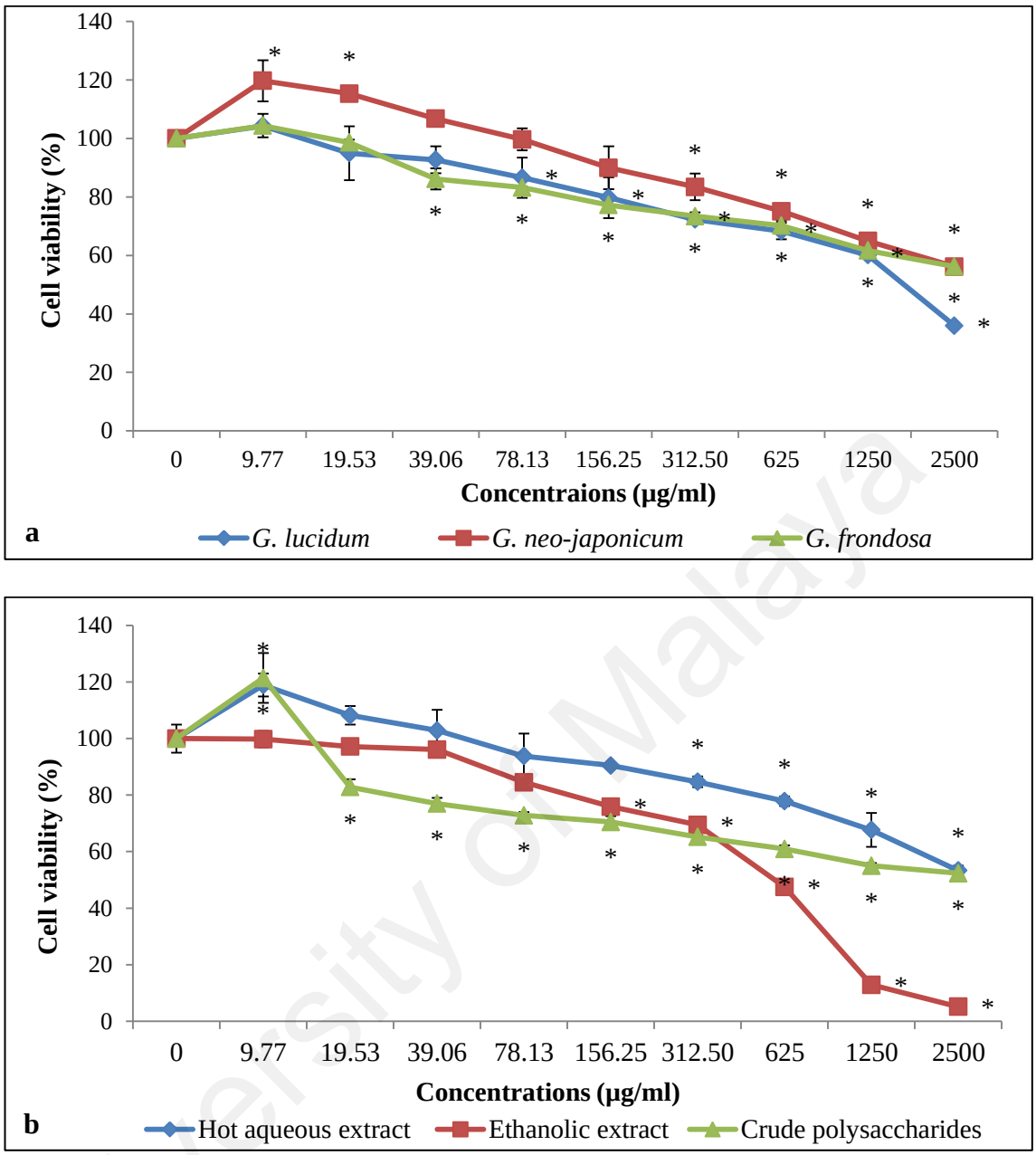



**Figure 3.2: The cytotoxic effects of mushrooms extracts and crude polysaccharides on PC-12 cells.** (a) Hot aqueous extract of *G. lucidum*, *G. neo-japonicum* and *G. frondosa*, (b) Hot aqueous extract, ethanol extract and crude polysaccharides of *L. rhinocerotis*. Cells were incubated with extracts or crude polysaccharides at concentrations from 0 to 2500 µg/ml for 48 hours and measured for viability by MTT assay. The mean absorbance obtained using complete F-12K medium with PC-12 cells was designated as 100% viable. Data were expressed as means ± SD from three independent experiments carried out in triplicates. * $p < 0.05$ compared to PC-12 cells only.

Hot aqueous extracts of *G. neo-japonicum* and *L. rhinocerotis*, and crude polysaccharides of *L. rhinocerotis* significantly ($p < 0.05$) increased in viability at low concentration, 9.77 μg/ml. The percentage of viable cells decreased gradually as the concentration of the extracts and crude polysaccharides were increased. There was a significant ($p < 0.05$) increase in cytotoxicity starting from the concentrations of 78.13 μg/ml (*G. lucidum*), 312.50 μg/ml (*G. neo-japonicum*), 39.06 μg/ml (*G. frondosa*), 312.50 μg/ml (hot aqueous extract of *L. rhinocerotis*), 156.25 μg/ml (ethanol extract of *L. rhinocerotis*) and 19.53 μg/ml (crude polysaccharides of *L. rhinocerotis*), reducing percentage of viability by 13.41%, 16.57%, 13.85%, 15.30%, 15.45%, and 17.16%, respectively, compared to 100% viability. Ethanol extract of *L. rhinocerotis* was found cytotoxic to PC-12 cells at higher concentrations, 1250 μg/ml (12.85% of viable cells) and 2500 μg/ml (5.13% of viable cells). The required concentrations to inhibit the viability of PC-12 cells by 50 % ($IC_{50}$) for all extracts and crude polysaccharides at 48 hours are shown in Table 3.3. The hot aqueous extract of *L. rhinocerotis* has highest $IC_{50}$ value (3.52 ± 0.33 mg/ml), followed by crude polysaccharides of *L. rhinocerotis* (3.27 ± 0.78 mg/ml), hot aqueous extracts of *G. frondosa* (3.11 ± 0.55 mg/ml) and *G. neo-japonicum* (3.04 ± 0.26 mg/ml).

**Table 3.3: The 50% inhibitory concentration ($IC_{50}$) of mushrooms extracts and crude polysaccharides on PC-12 cell viability after 48 hours of incubation.**

| Mushrooms | Extract | $IC_{50}$ (mg/ml) on PC-12 cells |
|---|---|---|
| *Ganoderma lucidum* | Hot aqueous | $1.50 \pm 0.12^{b}$ |
| *Ganoderma neo-japonicum* | Hot aqueous | $3.04 \pm 0.26^{c}$ |
| *Grifola frondosa* | Hot aqueous | $3.11 \pm 0.55^{c}$ |
| *Lignosus rhinocerotis* | Hot aqueous | $3.52 \pm 0.33^{c}$ |
| *Lignosus rhinocerotis* | Ethanol | $0.41 \pm 0.02^{a}$ |
| *Lignosus rhinocerotis* | Crude polysaccharides | $3.27 \pm 0.78^{c}$ |

The data represent the mean ± SD from three independent experiments carried out in triplicates. Means with different alphabets represent significant difference ($p < 0.05$).

### 3.3.4 The inhibition of nitric oxide production in LPS-stimulated BV2 microglia by mushrooms extracts and crude polysaccharides

To investigate the inhibition of NO production in LPS-stimulated BV2 microglia by the mushrooms extracts, the Griess assay was performed to measure the concentration of nitrite (a stable oxidized product of NO) released into the culture supernatant. The MTS assay was performed to exclude the possibility that the inhibition of NO production is due to the cytotoxicity of the extracts. Pre-treatment with all extracts of the mushrooms tested significantly ($p < 0.05$) inhibited NO production in LPS-stimulated cells, compared to LPS control (Figure 3.3). In LPS control, the LPS stimulated approximately 23-fold higher production of NO in BV2 microglia compared to the negative control (BV2 cells only) significantly ($p < 0.05$) after 24 hours (Figure 3.3). Hot aqueous extracts of *G. lucidum*, *G. neo-japonicum*, *G. frondosa* and *L. rhinocerotis*, and ethanol extract of *L. rhinocerotis* significantly ($p < 0.05$) inhibited NO production in LPS-stimulated cells compared to LPS control, with no cytotoxic effect (Figure 3.3a – 3.3e). However, no significant difference ($p > 0.05$) was observed in the treatment of crude polysaccharides of *L. rhinocerotis* at all concentrations tested compared to LPS control (Figure 3.3f), indicating that crude polysaccharides of *L. rhinocerotis* did not exhibit inhibitory effect on NO production in LPS-stimulated BV2 microglia. There was no significant difference ($p > 0.05$) between low concentrations of *G. lucidum* (3.91 µg/ml to 250 µg/ml) and LPS control, however, higher concentration of *G. lucidum* (500 µg/ml) significantly ($p < 0.05$) inhibited NO production in LPS-stimulated BV2 microglia (Figure 3.3a) by approximately 42%, compared to LPS control.

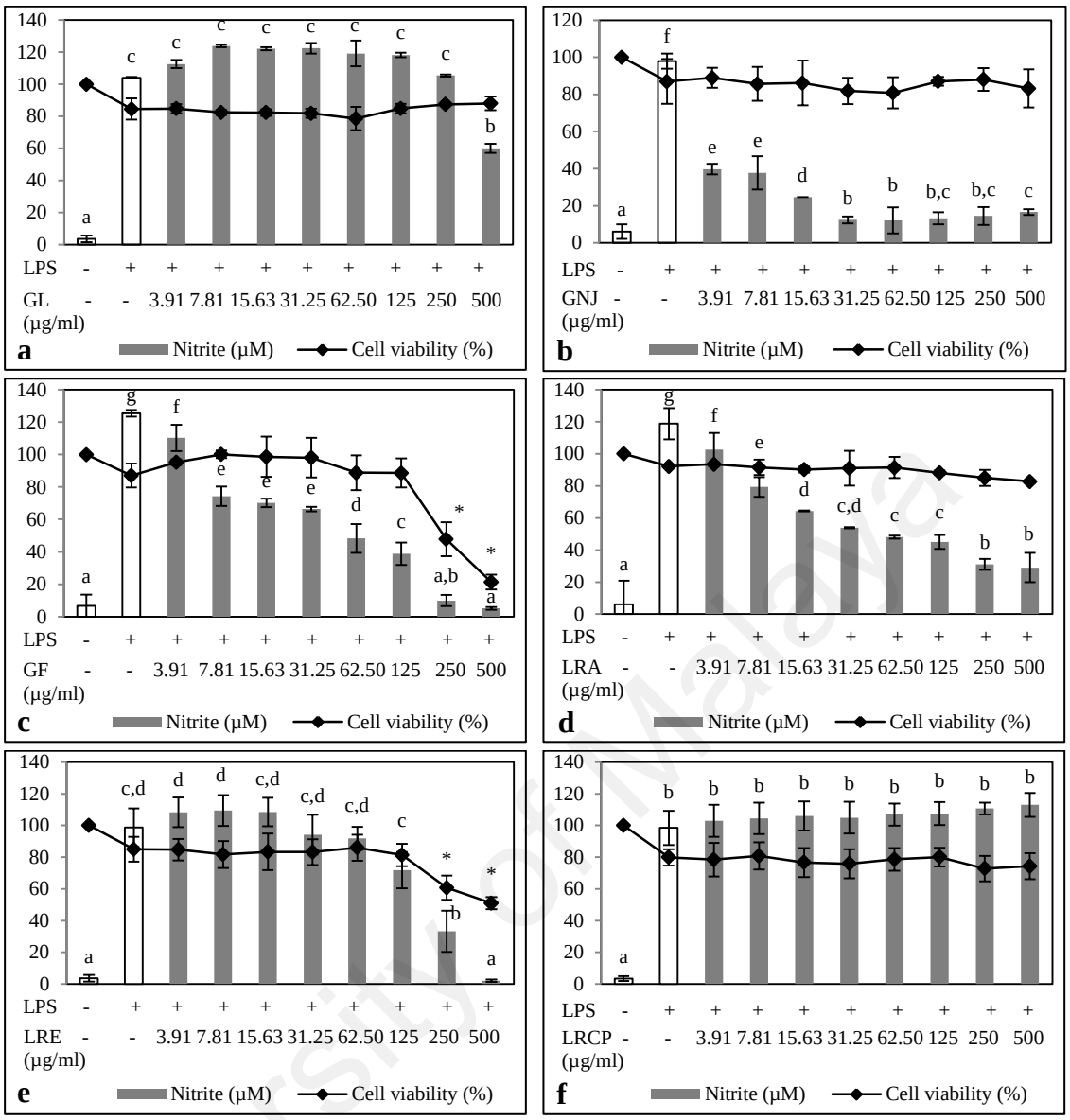



**Figure 3.3: The inhibition of NO production in LPS-stimulated BV2 microglia by mushrooms extracts and crude polysaccharides.** Cells were pre-treated with (a) hot aqueous extract of *G. lucidum* (GL), (b) hot aqueous extract of *G. neo-japonicum* (GNJ), (c) hot aqueous extract of *G. frondosa* (GF), (d) hot aqueous extract of *L. rhinocerotis* (LRA), (e) ethanol extract of *L. rhinocerotis* (LRE), and (f) crude polysaccharides of *L. rhinocerotis* (LRCP), at concentrations from 3.91 µg/ml to 500 µg/ml for 24 hours and then stimulated with one µg/ml LPS for 24 hours. Data presented the concentrations of nitrite (µM) in culture supernatant and percentage of cell viability (%), determined at 24 hours post-LPS stimulation by Griess and MTS assay, respectively. Cells in complete DMEM medium served as a negative control and considered as 100% viable. Cells stimulated with LPS without treatment served as a LPS control. Data were expressed as means ± SD from three independent experiments carried out in triplicates. Means with different alphabets represent significant difference ($p < 0.05$) for nitrite concentrations. * $p < 0.05$ percentage of cell viability compared to the respective LPS control.

Interestingly, hot aqueous extracts of *G. neo-japonicum* (Figure 3.3b) and *L. rhinocerotis* (Figure 3.3d) significantly ($p < 0.05$) inhibited the NO production in LPS-stimulated BV2 microglia at all concentrations tested (3.91 to 500 µg/ml), with no cytotoxic effect. Low concentrations (3.91 to 15.63 µg/ml) of *G. neo-japonicum* inhibited 60% and above of NO production while higher concentrations (31.25 to 500 µg/ml) of *G. neo-japonicum* successfully inhibited 80% and above of NO production, compared to LPS control. Hot aqueous extract of *L. rhinocerotis* inhibited NO production in concentration-dependent manner, from 13% of inhibition at 3.91 µg/ml to 76% of inhibition at 500 µg/ml. Hot aqueous extract of *G. frondosa* (Figure 3.3c) significantly ($p < 0.05$) inhibited NO production at all concentrations tested, however, the extract was cytotoxic at higher concentrations (250 µg/ml and above). Low concentrations (3.91 to 15.63 µg/ml) of ethanol extract of *L. rhinocerotis* (Figure 3.3e) stimulated approximately 10% of NO production, compared to LPS control. The NO production was inhibited starting from 31.25 µg/ml of ethanol extract of *L. rhinocerotis*, however, the extract was cytotoxic at higher concentrations (250 µg/ml and above). The maximum percentage of NO inhibition of each extract (without cytotoxic effect) was shown in the following order: hot aqueous extract of *G. neo-japonicum* (87.65% at 62.50 µg/ml) > hot aqueous extract of *L. rhinocerotis* (75.57% at 500 µg/ml) > hot aqueous extract of *G. frondosa* (69.05% at 125 µg/ml) > hot aqueous extract of *G. lucidum* (42.29% at 500 µg/ml) > ethanol extract of *L. rhinocerotis* (27.20% at 125 µg/ml).

### 3.3.5 The neuritogenic stimulatory effects of mushrooms extracts and crude polysaccharides in PC-12 cells

Neurite extension of PC-12 cells was regarded as an index of neuritogenesis. All the tested concentrations (25 to 100 µg/ml) of hot aqueous extracts of *G. lucidum*, *G. neo-japonicum*, *G. frondosa*, and hot aqueous extract, ethanol extract and crude polysaccharides of *L. rhinocerotis* stimulated neuritogenic effect in PC-12 cells after 48 hours of incubation (Figure 3.4).

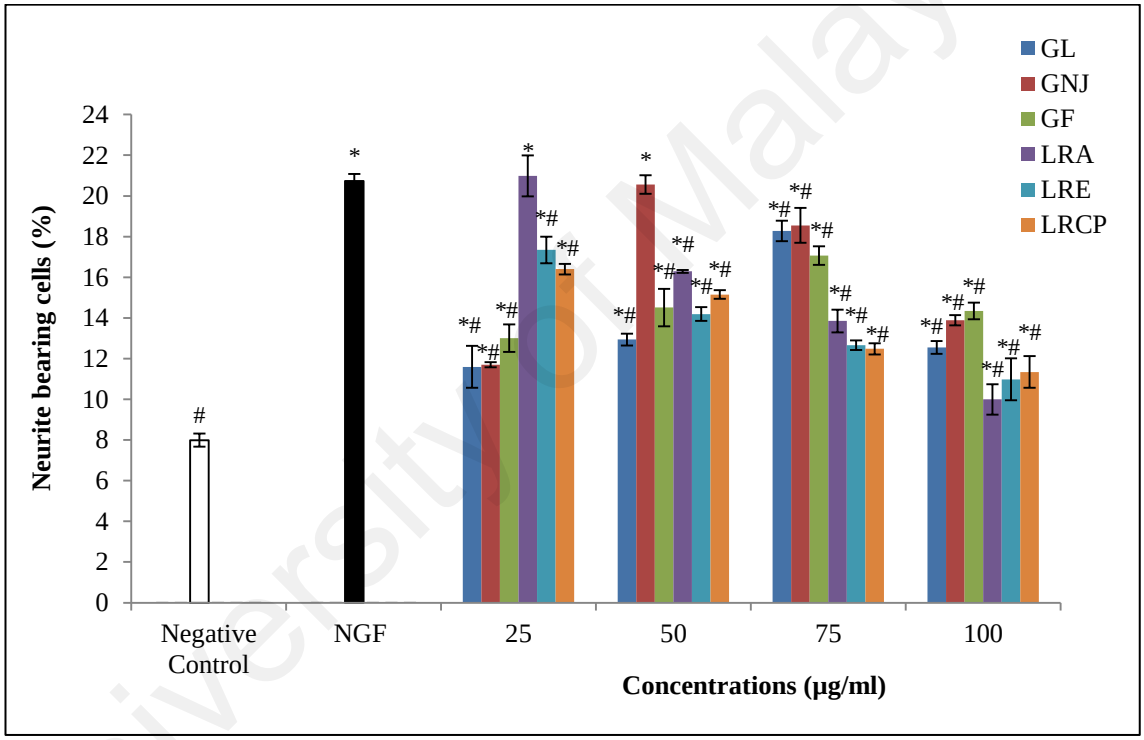



**Figure 3.4: The neuritogenic stimulatory effects of mushrooms extracts and crude polysaccharides in PC-12 cells.** Cells were incubated with NGF (50 ng/ml), hot aqueous extracts of *G. lucidum* (GL), *G. neo-japonicum* (GNJ), *G. frondosa* (GF), *L. rhinocerotis* (LRA), ethanol extract of *L. rhinocerotis* (LRE), and crude polysaccharides of *L. rhinocerotis* (LRCP) at concentrations from 25 µg/ml to 100 µg/ml for 48 hours. Cells in complete F-12 K medium served as a negative control. Cells treated with 50 ng/ml of NGF served as a positive control. Data were expressed as means ± SD from three independent experiments carried out in triplicates. * $p < 0.05$ compared to the negative control. # $p < 0.05$ compared to the positive control (NGF).

There were significant differences ($p < 0.05$) between the negative control (cells in complete F-12 K medium) and all concentrations of all extracts and crude polysaccharides tested. The percentage of neurite-bearing cells of hot aqueous extracts of *G. lucidum*-, *G. neo-japonicum*- and *G. frondosa*- treated cells were significantly ($p < 0.05$) increased in a concentration-dependent manner. However, the percentage of neurite-bearing cells was concentration-dependently decreased by increasing of the concentrations of hot aqueous and ethanol extracts, and crude polysaccharides of *L. rhinocerotis*. Maximum stimulation of neuritogenesis by hot aqueous extract of *L. rhinocerotis* was achieved at 20.99% of neurite-bearing cells at 25 μg/ml, followed by *G. neo-japonicum* (20.55%) at 50 μg/ml, *G. lucidum* (18.27%) at 75 μg/ml, ethanol extract of *L. rhinocerotis* (17.35%) at 25 μg/ml, *G. frondosa* (17.07%) at 75 μg/ml, and crude polysaccharides of *L. rhinocerotis* (16.40%) at 25 μg/ml. Nerve growth factor (50 ng/ml)-treated cells was used as a positive control. Interestingly, the percentage of neurite-bearing cells of hot aqueous extract of *G. neo-japonicum* and *L. rhinocerotis* at 50 μg/ml (20.55 ± 0.46%) and 25 μg/ml (20.99 ± 1.01%), respectively were comparable to the neuritogenic stimulation by NGF (20.73 ± 0.35%), with no significant difference ($p > 0.05$) .

## 3.4 DISCUSSION

All four medicinal mushrooms, namely *Ganoderma lucidum, Ganoderma neo-japonicum,* G*rifola. frondosa* and *Lignosus rhinocerotis* were screened for cytotoxicity, NO inhibitory and neuritogenic stimulatory effects by using two *in vitro* model system, the BV2 microglial and PC-12 cell lines.

The water soluble components from the basidiocarps of *G. lucidum, G. neo-japonicum, G. frondosa* and the sclerotium of *L. rhinocerotis* were extracted by hot aqueous extraction method. Hot aqueous extraction is the most commonly used method

by the indigenous communities and traditional Chinese medicine (TCM) physicians to prepare decoctions, tonics or essences from medicinal mushrooms (Stengler, 2005; Mizuno, Sakai, & Chihara, 1995). It is widely used to extract bioactive constituents from medicinal and edible mushroom (Nandan et al., 2008; Lin, Lin, Chen, Ujiie, & Takada, 1995). Hot aqueous extracts promised higher yield (approximately 65 to 70% higher) and greater proportions of water-soluble constituents in mushrooms compared to the ethanol extraction (Nwachukwu & Uzoeto, 2010; Stengler, 2005). In agreement with the previous studies by Nwachukwu & Uzoeto (2010) and Stengler (2005), in the present study, the hot aqueous extract of *L. rhinocerotis* gave a 70% higher yield compared to the ethanol extraction. In general, mushroom's polysaccharides are targeted as the active components in the hot aqueous extract (Mizuno, 1999). Aqueous extraction has been used for the isolation of bioactive polysaccharides from mushrooms such as glucans (Daba & Ezeronye, 2003; Wasser, 2002). Mushroom's polysaccharides such as α-glucans and β-glucans are known as effective anti-tumor and immunomodulatory agents (Vannucci et al., 2013). According to Cheung, Hui, Chu, Chiu, & Ip (2000), the extract of *Ganoderma* contained polysaccharides that are neuroactive. It had been reported that crude aqueous extract of *Tremella fuciformis* (white jelly mushroom) possessed neuritogenic stimulatory effect *in vitro* (Kim et al., 2007). According to Lin et al. (1995), treatment with the aqueous extract of *G. lucidum* and *G. neo-japonicum* showed antioxidant effect on free radical scavenging and hepatoprotective effects against carbon tetrachloride ($CCl_4$)-induced liver injury. To simulate the traditional application of the medicinal mushrooms by the indigenous communities and TCM physicians (Stengler, 2005; Huang, 1999), in the present study, the basidiocarps of *G. lucidum*, *G. neo-japonicum*, *G. frondosa*, and sclerotium of *L. rhinocerotis* were prepared by hot aqueous extraction method. To further identify the

potential preparation of *L. rhinocerotis*, the ethanol extract and crude polysaccharides were prepared.

Cytotoxicity of mushrooms extracts is a crucial preliminary analysis for both *in vitro* and *in vivo* studies. Aqueous extraction is believed to have lower cytotoxic effect compared to many of the organic solvent extracts (Faridur, Rezaul, Farhadul, Rowshanul, & Tofazzal, 2010). In addition, water is non-toxic to cells. In the present study, of all hot aqueous extracts, the *L. rhinocerotis* and *G. lucidum* has lower cytotoxic effect towards BV2 microglia compared to *G. neo-japonicum* and *G. frondosa*. Of all extracts and crude polysaccharides tested, the hot aqueous extract and crude polysaccharides of *L. rhinocerotis* sclerotium exhibited lowest cytotoxicity towards both BV2 microglia and PC-12 cells. The $IC_{50}$ value of hot aqueous extract and crude polysaccharides of *L. rhinocerotis* was higher than the ethanol extract of *L. rhinocerotis* by approximately 98% and 97% in BV2 microglia, respectively, and approximately 88% and 87% in PC-12 cells, respectively. The present data was in agreement with the ethnomedicinal preparation method of *L. rhinocerotis* sclerotium by the indigenous communities and TCM physicians, showing that the aqueous extract is safe to consume (Lee, Chang, & Noraswati, 2009; Lee & Chang, 2004). The *L. rhinocerotis* sclerotium extracts were reported non-cytotoxic to the normal human breast (184B5) and lung (NL20) cells, normal human colon (CCD-18Co), kidney (HEK-293), nasopharyngeal (NP 69), oral (OKF6), rat kidney (NRK-52E), and Vero cell lines (Lau et al., 2014; Suziana Zaila et al., 2013; Lee, Tan, Fung, Tan, & Ng, 2012). Low concentrations of ethanol extract of *L. rhinocerotis* sclerotium were found non-cytotoxic towards murine BV2 microglial cells (Nallathamby et al., 2013). According to Phan, David, et al. (2013), the hot aqueous extract of *L. rhinocerotis* sclerotium was less cytotoxic compared to other mushrooms extracts studied, including *Pleurotus pulmonarius*, *H. erinaceus*, *G.*

*lucidum*, *G. neo-japonicum* and *G. frondosa* in both Neuro2a mouse neuroblastoma (N2a) and embryonic fibroblast (3T3) cell lines.

In the present study, BV2 microglial cell line was utilized as an *in vitro* model system to investigate the inhibitory effect of mushrooms extracts and crude polysaccharides on NO production, stimulated by LPS. Lipopolysaccharide is a common inflammatory stimulus used to study neuroinflammatory activities in BV2 microglia (Horvath et al., 2008; Bocchini et al., 1992). Upon treatment of LPS, BV2 microglia will be activated, leading to *in vitro* neuroinflammatory responses mediated by the production of various pro-inflammatory mediators that are neurotoxic, including NO. Nitric oxide is a free radical which acts as an important mediator of inflammation in several animal models (Vane et al., 1994). It has been shown natural substances that inhibit the production of NO may be beneficial for the prevention and treatment of neuroinflammation-associated disorders. Medicinal mushrooms including *Taiwanofungus camphoratus* (Liu et al., 2007), *Cordyceps militaris* (Nallathamby et al., 2015; Jeong et al., 2010), *Ganoderma lucidum* (Yoon et al., 2013; Zhang et al., 2011) and *Laetiporus sulphureus* (Jayasooriya et al., 2011) have been reported to possess anti-neuroinflammatory activity *in vitro*, by inhibiting the production of NO in activated BV2 microglia. According to Yoon et al. (2013), 1000 µg/ml of ethanol extract of *G. lucidum* inhibited approximately 64% of NO in LPS-stimulated BV2 microglia. *Ganoderma lucidum* extract at 200 µg/ml significantly reduced the production of NO in LPS-stimulated microglia (Zhang et al., 2011). Exopolysaccharides of *L. sulphureus* at 2000 µg/ml inhibited approximately 64% of NO in LPS-stimulated BV2 microglia (Jayasooriya et al., 2011). Recent report by Nallathamby et al. (2015) showed that 10 µg/ml of ethyl acetate fraction and 10 µg/ml of ergosterol-enriched sub-fraction CE3 of ethyl acetate fraction of *C. militaris* inhibited 48.0% and 44.7% of NO production in LPS-stimulated BV2 microglia, respectively. Low concentration of cordycepin (7.5

µg/ml), a well-known compound of *C. militaris*, successfully inhibited approximately 75% of NO in LPS-stimulated BV2 microglia (Jeong et al., 2010). A study by Gunawardena et al. (2014) reported that the hot aqueous extracts of *Pleurotus ostreatus* (oyster mushroom), *Agaricus bisporus* (honey-brown mushroom) and *Agaricus bisporus* (button mushroom) inhibited the NO production in LPS plus IFN-γ-stimulated N11 microglia, with $IC_{50}$ values of 0.47 ± 0.28 mg/ml, 0.16 ± 0.13 mg/ml and 0.14 ± 0.01 mg/ml, respectively. In the present study, the extracts of *G. lucidum*, *G. neo-japonicum*, *G. frondosa* and *L. rhinocerotis* were found to inhibit the production of NO in LPS-stimulated BV2 microglia. It is noteworthy that all tested concentrations (3.91 to 500 µg/ml) of hot aqueous extracts of *G. neo-japonicum* and *L. rhinocerotis* successfully inhibited the NO production without any signs of cytotoxicity. The maximum percentage of NO inhibition of hot aqueous extracts of *G. neo-japonicum* and *L. rhinocerotis* was achieved at 62.50 µg/ml (87.65%) and at 500 µg/ml (75.57%), respectively. These findings suggest that the *G. neo-japonicum* basidiocarp and *L. rhinocerotis* sclerotium may contain active substance(s) that have anti-neuroinflammatory potential through the inhibition of NO production in LPS-stimulated BV2 microglia.

In the present study, PC-12 cell line was utilized as an *in vitro* model system to investigate the neuritogenic stimulatory effect of mushrooms extracts and crude polysaccharides. The results suggested that all mushrooms extracts and crude polysaccharides tested caused a marked stimulation of neuritogenesis in PC-12 cells. The hot aqueous extracts of *G. neo-japonicum* (50 µg/ml) and *L. rhinocerotis* (25 µg/ml) triggered maximal stimulation of neuritogenesis at a lower concentration compared to *G. lucidum* (75 µg/ml) and *G. frondosa* (75 µg/ml). From data obtained in this study, the $IC_{50}$ values of cytotoxicity of hot aqueous extracts of *G. lucidum*, *G. neo-japonicum*, *G. frondosa* and *L. rhinocerotis*, ethanol extract and crude polysaccharides of *L.*

*rhinocerotis* were approximately 20-, 61-, 41-, 140-, 16-, and 131-fold higher than their optimum concentration that stimulated maximal neuritogenesis. Interestingly, the hot aqueous extracts of *G. neo-japonicum* and *L. rhinocerotis* appeared to be comparably active with the neuritogenic effect of NGF in PC-12 cells. No significant difference found in the percentage of neurite-bearing cells between 50 ng/ml of NGF-, 25 μg/ml of hot aqueous extract of *L. rhinocerotis* and 50 μg/ml of hot aqueous extract of *G. neo-japonicum*-stimulated neuritogenesis. This is in agreement with the previous studies, reported that 20 μg/ml of hot aqueous extract of *L. rhinocerotis* sclerotium (Eik et al., 2012) and *L. rhinocerotis* mycelium (John et al., 2013) stimulated neuritogenesis in PC-12 cells that comparable to NGF. Neuritogenic activity of mushrooms other than *G. neo-japonicum*, *G. lucidum* and *G. frondosa* has also been reported. These included *Hericium erinaceus* (Li, See, Moon, Yoo, & Lee, 2013; Mori et al., 2008; Wong et al., 2007), *Sarcodon scabrosus* (Shi, Liu, Gao, & Zhang, 2011), *Sarcodon cyrneus* (Marcotullio et al., 2007, 2006), *Termitomyces albuminosus* (Qi, Ojika, & Sakagami, 2001, 2000) and *Cordyceps militaris* (Lee et al., 2011). The hot aqueous extract of *H. erinaceus* stimulated neuritogenic activity in PC-12h cells as the treatment dose increased from 0.01 mg/ml to 1 mg/ml (Li, See, Moon, Yoo, & Lee, 2013). According to Wong et al. (2007), the extracts of *H. erinaceus* basidiocarp and mycelium induced neuritogenesis in NG108-15. Mori et al. (2008) documented that the ethanol extract of *H. erinaceus* promoted neuritogenesis of PC-12 cells, enhanced the NGF mRNA expression, and stimulated the secretion of NGF from human astrocytoma (1321N1) cells. Shi et al. (2011) reported that cyathane diterpenoids isolated from *S. scabrosus* showed significant neuritogenic activity in combination with 20 ng/ml of NGF in PC-12 cells after 24 hours of treatment. The extract of *C. militaris* stimulated neuritogenesis, enhanced neuronal functions of N2a cells (*in vitro*) and improved cognitive behaviour that related to memory ability (*in vivo*) (Lee et al., 2011). In the present study, the hot

aqueous extracts of *G. neo-japonicum* and *L. rhinocerotis* stimulated neuritogenic activity that was comparable to the NGF in PC-12 cells. These findings suggest that the *G. neo-japonicum* basidiocarp and *L. rhinocerotis* sclerotium may possess NGF-like bioactive compounds that mimic the neuroactivity of NGF for neuronal survival, development and differentiation.

## 3.5 CONCLUSIONS

The extracts of basidiocarps of *G. lucidum, G. neo-japonicum, G. frondosa* and the sclerotium of *L. rhinocerotis* inhibited NO production in LPS-stimulated BV2 microglia and stimulated neuritogenesis in PC-12 cells. All the extracts and crude polysaccharides tested were not cytotoxic to BV2 microglia and PC-12 cells. Based on the findings, the hot aqueous extracts of *G. neo-japonicum* and *L. rhinocerotis* inhibited maximal percentage of NO production at lower concentrations without visible signs of cytotoxicity. The NO inhibitory activity of both *G. neo-japonicum* and *L. rhinocerotis* were observed for the first time. Further, the hot aqueous extracts of these two mushrooms stimulated maximal percentage of neurite bearing cells at lower concentrations that was comparable to NGF. The present findings showed that the hot aqueous extract of *L. rhinocerotis* exhibited highest $IC_{50}$ values of viability towards both BV2 microglia and PC-12 cells compared to other mushrooms tested. Furthermore, it is easier to obtain the sclerotium of *L. rhinocerotis* in larger quantity via commercial source, compared to the wild grown *G. neo-japonicum*. Therefore, the hot aqueous extract of *L. rhinocerotis* sclerotium was selected for further investigation.

# CHAPTER IV: ANTI-NEUROINFLAMMATORY EFFECT OF *LIGNOSUS RHINOCEROTIS* SCLEROTIUM AND THE UNDERLYING MECHANISMS

## 4.1 INTRODUCTION

Neuroinflammation is characterized by continuous and/or uncontrolled activation of microglia (Streit, 2006). It plays crucial roles in the pathogenesis of several neurodegenerative diseases such as Alzheimer's disease (Heneka et al., 2015; Eikelenboom et al., 2002) and Parkinson's disease (Tansey, McCoy, & Frank-Cannon, 2007; Kim & Joh, 2006). Prolonged activation of microglia leads to overproduction of various pro-inflammatory mediators that are neurotoxic to the CNS including nitric oxide (NO), prostaglandins $E_2$ ($PGE_2$), inducible nitric oxide synthase (iNOS), Cyclooxygenase-2 (COX-2), cytokines such as tumor necrosis factor alpha (TNFα), interleukin-1 beta (IL-1β) and interleukin-6 (IL-6), which may result in neuroinflammation (Ramesh, MacLean, & Philipp, 2013; Ramesh, Philipp, Vallières, MacLean, & Ahmad, 2013). Therefore, controlling and/or inhibiting the overproduction of pro-inflammatory mediators and cytokines may be an approach for the management of neuroinflammatory-associated disorders and neurodegenerative diseases.

In Chapter III, the hot aqueous extract of *Lignosus rhinocerotis* sclerotium exhibited significant NO inhibitory and neuritogenic stimulatory activities without cytotoxic effect, compared to hot aqueous extracts of other mushrooms tested, including *Ganoderma lucidum*, *Ganoderma neo-japonicum* and *Grifola frondosa*. Furthermore, the hot aqueous extract of *L. rhinocerotis* sclerotium exhibited lower cytotoxic effect, inhibited higher percentage of NO production, and stimulated higher percentage of neurite bearing cells when compared to the ethanol extract and crude polysaccharides of

*L. rhinocerotis* sclerotium. Therefore, in this chapter, the hot aqueous extract of *L. rhinocerotis* sclerotium was selected for further investigation.

The aims of this chapter were to (i) fractionate the hot aqueous extract of *L. rhinocerotis* sclerotium by using organic solvent to yield three fractions: ethyl acetate, n-butanol and aqueous fractions, (ii) investigate the cytotoxic effects of solvent fractions compared to the crude hot aqueous extract on BV2 microglia, (iii) investigate the inhibition of pro-inflammatory mediators (NO, iNOS, $PGE_2$, COX-2) and cytokines (TNF-α, IL-1β, IL-6) production and expressions in LPS-stimulated BV2 microglia by the active fraction(s), (iv) investigate the involvement of toll-like receptor 4 (TLR4), nuclear factor-kappaB (NFκB), protein kinase B (AKT) and mitogen-activated protein kinases (MAPKs), including extracellular signal-regulated kinase 1/2 (ERK1/2), p38 MAPK and stress-activated protein kinases/Jun amino-terminal kinases (SAPK/JNK) signaling pathways, and (v) investigate the involvement of downstream transcription factors that stimulated neuroinflammatory, NFκB, activator protein 1 (AP-1) and cAMP response element-binding protein (CREB) in the inhibitory activities of the active fraction(s) in LPS-stimulated BV2 microglia.

## 4.2 MATERIALS AND METHODS

### 4.2.1 General methodology

Preparation of the hot aqueous extract of *L. rhinocerotis* sclerotium is described in Section 3.2.2.1. The methods of BV2 microglia cell culture, treatment and stimulation of BV2 cells, cytotoxic assay, Griess and cell viability assays are described in Sections 3.2.3.1, 3.2.4, 3.2.5.1 and 3.2.5.2. Statistical analysis is described in Section 3.2.7.

### 4.2.2 Preparation of ethyl acetate, n-butanol and aqueous fractions

The hot aqueous extract of *L. rhinocerotis* sclerotium was fractionated using organic solvents in the order of ethyl acetate and n-butanol (Figure 4.1). The ethyl acetate and n-butanol fractions were evaporated using a rotary evaporator (Buchi Rotavapor R-210, Switzerland). The residual aqueous fraction was collected and freeze-dried at –50 ± 2 °C for 48 hours. All fractions were stored in airtight bottles and kept in −20 °C prior to use. All fractions were reconstituted in complete cell culture medium, and were passed through a micropore filter of size 0.2 microns (Sartorius, Germany) prior assays.

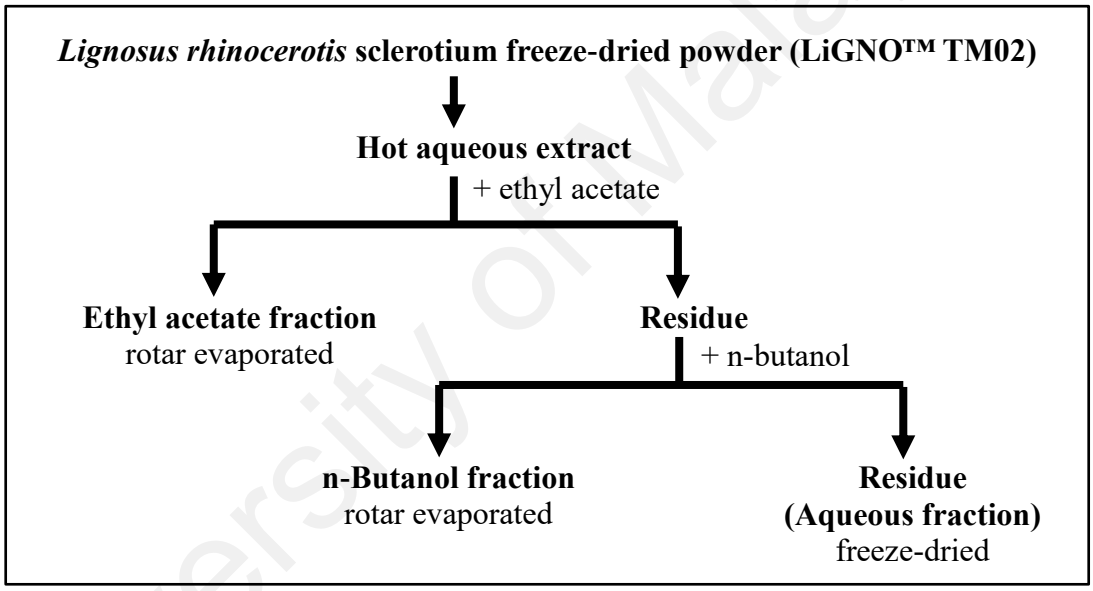



**Figure 4.1: Fractionation scheme of hot aqueous extract of *L. rhinocerotis* sclerotium.**

### 4.2.3 Preparation of positive controls

Acetylsalicylic acid (aspirin), a non-steroidal anti-inflammatory drug (NSAID) and Nω-Nitro-L-arginine methyl ester (L-NAME), a specific inhibitor of iNOS enzyme activity, were used as positive or comparison controls for the study of anti-neuroinflammatory activities of *L. rhinocerotis* sclerotium. Stock solution of aspirin (Sigma-Aldrich, USA) at one molar was prepared in DMSO and stored at –20 °C prior to use. Final concentration of one millimolar of aspirin was freshly prepared by diluting in complete

DMEM medium (without phenol red) and filtered sterilized before use. Stock solution of L-NAME (Sigma-Aldrich, USA) at two millimolar was prepared in complete DMEM medium (without phenol red), filtered sterilized and stored at 4 °C prior to use. Final concentration of 250 µM of L-NAME was freshly prepared by diluting in complete DMEM medium (without phenol red) before use.

### 4.2.4 Assessment of extracellular levels of prostaglandin $E_2$ ($PGE_2$), tumor necrosis factor alpha (TNF-α) and interleukins-1 beta (IL-1β) in LPS-stimulated BV2 microglia

The extracellular levels of $PGE_2$, TNF-α and IL-1β were measured by using Quantikine® immunoassay ELISA kits (R & D systems, Minneapolis) according to the manufacturer's protocol. The cell culture supernatant was collected, centrifuged at 1500 X g for 15 minutes and maintained at 0 – 4 °C prior to assay. For TNF-α and IL-1β immunoassay, the cell culture supernatant was incubated with respective assay diluent in microwells precoated individually with monoclonal antibody specific for mouse TNF-α and IL-1β, respectively. Then, the microwells plate was incubated for two hours at room temperature. After washing with wash buffer, 100 µl of mouse TNF-α conjugate or mouse IL-1β conjugate was added to each well, respectively and incubated for two hours at room temperature. After the washing steps, 100 µl of substrate solution (mixture of stabilized hydrogen peroxide and chromogen tetramethylbenzidine) was added to each well and incubated for 30 minutes at room temperature (protected from light). For $PGE_2$ immunoassay, 150 µl of cell culture supernatant was incubated with 50 µl of primary antibody solution (mouse monoclonal antibody for $PGE_2$) in microwells precoated with goat anti-mouse polyclonal antibody. Then, the microwells plate was incubated for one hour at room temperature on plate shaker. Without washing, 50 µl of $PGE_2$ conjugate was added to each well and incubated for two hours at room

temperature. After the washing steps, 200 µl of substrate solution was added to each well and incubated for 30 minutes at room temperature (protected from light). The reactions of all assays were stopped by adding 100 µl of stop solution (hydrochloric acid) to each well. The plate was read at absorbance 450 nm with 570 nm as background absorbance with an ELISA microplate reader (Sunrise, Tecan). The concentrations of TNF-α, IL-1β and $PGE_2$ were determined from a standard curve plotted with known concentrations of the respective standard (TNF-α, 10.90 to 700 pg/ml; IL-1β: 12.50 to 400 pg/ml; $PGE_2$, 39 to 2500 pg/ml).

### 4.2.5 Protein expression analysis of pro-inflammatory mediators and signaling pathways in LPS-stimulated BV2 microglia

#### 4.2.5.1 Preparation of whole cell protein lysates

BV2 microglial cells were seeded at a density of $1 \times 10^6$ cells in a 25 $cm^3$ flask and incubated overnight at 37 °C in a 5% $CO_2$-humidified incubator. Then, the cells were pre-treated with freshly prepared mushroom solvent fractions in complete DMEM medium for 24 hours. The culture supernatant was discarded and replaced with freshly prepared LPS and at one microgram per milliliter in complete DMEM for 24 hours. Then, the whole cell protein lysates were extracted according to the required protocol of each ELISA kit (as follow).

**Extraction of whole cell protein lysates for Cusabio sandwich ELISA kit (Cusabio Biotech)**

The medium was removed, and the cells were rinsed with ice-cold PBS. Then, the cells were scraped off and collected in a microcentrifuge tube. The cell suspension was diluted with PBS until cell concentration reached 100 million/ml and stored overnight at –20 °C. After two freeze-thaw cycles to break up the cell membranes, the cell lysates

were centrifuged for five minutes at 5000 X g, 2 – 8 °C. The supernatant (whole cell lysates) was collected, aliquoted and stored at –80 °C.

**Extraction of whole cell protein lysates for PathScan® sandwich ELISA kit (Cell Signaling Technology)**

The medium was removed, and the cells were rinsed with ice-cold PBS. The PBS solution was removed. A total of 0.5 ml of ice-cold 1X cell lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM disodium EDTA, 1 mM ethylene glycol tetraacetic acid (EGTA), 1% Triton, 20 mM sodium pyrophosphate, 25 mM sodium fluoride, 1 mM b-glycerophosphate, 1 mM Sodium orthovanadate ($Na_3VO_4$) and 1 µg/ml leupeptin) (Cell Signaling Technology) plus 1 mM of phenylmethylsulfonyl fluoride (PMSF) (Sigma-Aldrich, USA) was added into each flask, and incubated on ice for five minutes. Cells were then scraped off, sonicated on ice, and micro-centrifuged at 10,000 X g for 10 minutes at 4 °C. The supernatant (whole cell lysates) was collected, aliquoted and stored at –80 °C.

### 4.2.5.2 Quantification of protein (Bradford assay)

The protein concentration in each cell lysate was measured by Coomassie Plus (Bradford) assay kit (Thermo Fisher Scientific), compared to a protein standard. A total of 10 µl of bovine serum albumin (BSA) standard (0 to 2000 µg/ml) or cell lysate was added into the appropriate microplate wells. Then, 300 µl of the Coomassie Plus Reagent was added into each well and mixed on a plate shaker for 30 seconds. The plate was removed from shaker and incubated on benchtop for 10 minutes at room temperature. The absorbance was measured at 595 nm with a plate reader (Sunrise, Tecan). The protein concentration of cell lysate was calculated following deduction of blank, with reference to the BSA standard curve versus its concentration in µg/ml.

#### 4.2.5.3 Measurement of the expressions of inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX-2) proteins in LPS-stimulated BV2 microglia

The protein expressions of iNOS and COX-2 were measured by using Cusabio sandwich ELISA kit (Cusabio Biotech) according to the manufacturer's protocol. The cell lysates were incubated in microwells precoated individually with monoclonal antibody specific for mouse iNOS and COX-2 respectively. Then, the microwells plate was incubated for two hours at 37 °C. Without washing, 100 µl of 1X Biotin-antibody (biotin-conjugated antibody specific to iNOS or COX-2, respectively) was added to each well and incubated for one hour at 37 °C. After washing with wash buffer, 100 µl of 1X avidin conjugated Horseradish Peroxidase was added to each well and incubated for one hour at 37 °C. After washing, 90 µl of 3,3',5,5'-tetramethylbenzidine (TMB) substrate was added to each well and incubated for 20 minutes at 37 °C, in dark. The reaction was stopped by adding 50 µl of stop solution to each well. The plate was read at absorbance 450 nm with 570 nm as background absorbance with an ELISA microplate reader (Sunrise, Tecan). The concentrations of iNOS and COX-2 were determined from a standard curve plotted with known concentrations of the respective standard (iNOS, 0 to 500 IU/ml; COX-2, 0 to 2000 pg/ml).

#### 4.2.5.4 Measurement of the expressions of neuroinflammatory signaling proteins in LPS-stimulated BV2 microglia

The protein expressions of phosphorylated nuclear factor kappa B p65 subunit (p-NFκB p65), phosphorylated inhibitor kappa B-alpha (p-IκBα), phosphorylated stress-activated protein kinases/Jun amino-terminal kinases (p-SAPK/JNK), phosphorylated p38 mitogen-activated protein kinases (p-p38 MAPK), phosphorylated extracellular-signal regulated kinase1/2 (p-ERK1/2), phosphorylated protein kinase B (p-AKT), and phosphorylated cAMP-response element binding protein (p-CREB) was measured by

using PathScan® sandwich ELISA kits (Cell Signaling Technology) according to the manufacturer's protocol. The cell lysates were incubated in microwells precoated individually with p-NF-κB p65 (Ser536), p-IκBα (Ser32), p-SAPK/JNK (Thr183/Tyr185), p-p38 MAPK (Thr180/Tyr182), p-ERK1/2 (p44/42) (Thr202/Tyr204), p-AKT (Thr308), and p-CREB (Ser133) antibodies. Then, the microwells plate was incubated overnight at 4 °C. After washing with wash buffer, 100 µl of reconstituted detection antibody was added to each well and incubated for one hour at 37 °C. After the washing steps, 100 µl of reconstituted horseradish peroxidase (HRP)-linked secondary antibody was added to each well and incubated for 30 minutes at 37 °C. After the washing steps, 100 µl of TMB substrate was added to each well and then incubated for 10 minutes at 37 °C, in dark. The reaction was stopped by adding 100 µl of stop solution to each well. The plate was read at absorbance 450 nm with an ELISA microplate reader (Sunrise, Tecan). The magnitude of absorbance for the developed color is proportional to the quantity of the p-NF-κB p65, p-IκBα, p-SAPK/JNK, p-p38 MAPK, p-ERK1/2, p-AKT, and p-CREB expressed by BV2 microglia.

### 4.2.6 Immunocytofluorescence analysis of iNOS and COX-2 in LPS-stimulated BV2 microglia

BV2 microglial cells were seeded at a density of $5 \times 10^4$ cells per well in a 12-well micro-chamber (ibidi, Martinsried, Germany), incubated overnight at 37 ± 2 °C in a 5% $CO_2$-humidified incubator. The cells were pre-treated with freshly prepared mushroom solvent fractions in complete DMEM medium for 24 hours. Then, the culture supernatant was removed and replaced with freshly prepared LPS and at one microgram per milliliter in complete DMEM for 24 hours. The cell culture supernatant was removed and the cells were washed twice with PBS. The cells were then fixed with 4% (w/v) paraformaldehyde (in PBS) for 20 minutes. After washing with PBS, the cells

were incubated with 0.5% (v/v) triton-X in PBS (PBST) for 10 minutes, and followed by incubation with primary antibody, mouse monoclonal antibody specific for iNOS (NOS2, C-11) or COX-2 (D-12) (1:200 in 5% goat serum blocking buffer, Santa Cruz Biotechnology) for two hours at room temperature. After washing three times with 0.3% of PBST, the cells were incubated with fluorophore-conjugated secondary antibody, goat anti-mouse IgG-fluorescein isothiocyanate (FITC) antibody (1:200 in 5% goat serum blocking buffer, Santa Cruz Biotechnology) for two hours at room temperature, in dark. After washing three times with 0.3% of PBST, the cells were mounted with ProLong® gold anti-fade reagent with 4-6-diamidino-2-phenylindole (DAPI) (Life Technologies Corporation). The slides were observed under fluorescence illumination using FITC and DAPI filters of Nikon Eclipse 80i fluorescent microscope and images were captured with Nikon's Imaging Software, NIS-Elements.

### 4.2.7 Gene expression analysis of inflammatory mediators and signaling pathways in LPS-stimulated BV2 microglia

#### 4.2.7.1 Preparation of total Ribonucleic acid (RNA) extract

BV2 microglial cells were seeded at a density of $1 \times 10^6$ cells in a $25cm^3$ flask and incubated overnight at 37 °C in a 5% $CO_2$-humidified incubator. The cells were pre-treated with freshly prepared mushroom solvent fractions in complete DMEM medium for 24 hours. Then, the culture supernatant was removed and replaced with freshly prepared LPS and at one microgram per milliliter in complete DMEM for 24 hours. Total RNA was isolated from treated and non-treated cells using the RNAqueous-4PCR kit (Ambion, USA) according to the manufacturer's protocol. The culture supernatant was discarded and the cells were rinsed with PBS. A total of 500 µl of lysis/binding solution (solution containing guanidinium thiocyanate) was added into each flask. Then, the cells were scraped off, collected in microcentrifuge tube and vortex vigorously to

lyse cell pellets. The sample lysate was then mixed with 500 µl of 64% ethanol solution, mixed gently and applied to a silica-based filter which selectively and quantitatively binds mRNA and the larger ribosomal RNAs (very small RNAs such as tRNA and 5S ribosomal RNA are not quantitatively bound). The filter cartridge (attached to a collection tube) was then centrifuged at 15,000 X g for 30 seconds allowing the mixture to pass through the filter. The flow-through liquid was discarded. The above steps were repeated with maximum filtration of 700 µl aliquots each time until all of the samples have been drawn though the filter. Then, the filter was washed with wash solution #1 and wash solution #2/3 to remove residual DNA, protein, and other contaminants. A total of 700 µl of wash solution #1 was added to the filter cartridge and the flow-through liquid was discarded, followed by two washes with 500 µl of wash solution #2/3. The RNA was then eluted in 70-80 °C elution solution (nuclease-free water containing a trace amount of EDTA). The collected total RNA was stored at −80 °C in single-use aliquots.

#### 4.2.7.2 Analysis of purity, integrity and concentration of RNA extract

The purity of RNA extract (1.5 µl) was determined by measuring its absorbance at 260 nm and 280 nm using a NanoDrop spectrophotometer. The RNA purity was measured at the ratio of $A_{260}$ to $A_{280}$ values. The pure RNA reading was in the range of 1.8 to 2.1. The RNA integrity and concentration were assessed with the RNA 6000 Nano Chip kit using the Bioanalyzer 2100 (Agilent Technologies, Germany). A total of one µl of RNA extract was loaded to each of the 12 sample wells per chip according to manufacturer's protocol. Nine microliter of gel-dye mix was loaded into the RNA Nano chip in the assigned well and pressurized for 30 seconds. Each well were loaded with six microliter of RNA 6000 Nano marker followed by loading one microliter of RNA ladder (ladder well) and one microliter of RNA extract (sample well). The chip was placed into the

Agilent 2100 bioanalyzer and RNA 6000 Nano assay was run. RNA is detected by fluorescence of the intercalating dye in the gel-dye mix. A successful total RNA run was observed with one marker peak and two ribosomal (18S, 28S) peaks showed in an electropherogram. The RNA concentration (ng/µl) and RNA integrity number (RIN) were implemented with 2100 expert software version B.02.02. The RIN obtained for each sample was based on the ratio of ribosomal bands and the presence/absence of degradation products. In the present study, only RNA extracts with RIN of 9 to 10 were used for complementary deoxyribonucleic acid (cDNA) synthesis.

#### 4.2.7.3 Synthesis of complementary DNA (cDNA)

The High Capacity RNA-to-cDNA Kit (Applied Biosystems, USA) was used for reverse transcription (RT) of total RNA to single-stranded complementary DNA (cDNA) on StepOnePlus™ Systems (Applied Biosystems). A total of 20 µl per reaction of RT reaction mix was prepared on ice, consisted of 10 µl of 2X RT buffer, one µl of 20X RT enzyme mix and nine µl of two µg of total RNA (diluted in nuclease-free water). Each RT reaction mix was mixed gently, loaded into each well of MicroAmp™ Optical 8-Tube Strip (0.1 ml) (Applied Biosystems) and sealed with MicroAmp™ Optical 8-Cap Strip (Applied Biosystems). The tubes were then centrifuged to spin down the contents and to eliminate air bubbles. The RT reaction was performed on StepOnePlus™ Systems, by incubating the reaction at 37 °C for 60 minutes. The reaction was stopped by heating to 95 °C for five minutes and held at 4 °C. The cDNA was stored at −80 °C in single-use aliquots.

#### 4.2.7.4 Real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Real-time quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) was

performed using TaqMan primer probes and TaqMan Fast Advanced Master Mix on StepOnePlus™ Systems using assays-on-demand and chemistries as recommended by the manufacturer (all Applied Biosystems). TaqMan gene expression assays (Applied Biosystems) is built on 5' nuclease chemistry and consist of a FAM™ dye-labeled TaqMan MGB probe, and two unlabeled PCR primers. All components are quality control (QC) tested and formulated into a single 20X mix. The PCR protocol consisted of 40 cycles of Taq DNA polymerases at 95 °C for 20 seconds, followed by denaturation at 95 °C for one second, and finally 60 °C for 20 seconds to allow for extension and amplification of the target sequence. Mouse β-actin (ACTB) (Mm00607939_s1) was used as the endogenous control. The expression levels (fold change) in the target messenger RNA (mRNAs) were normalized to ACTB and relative to untreated cells (negative control) was calculated using the 2-ΔΔCT (comparative CT) method by using StepOne software v2.1. Table 4.1 shows the list of selected mRNAs investigated in this study.

**Table 4.1: Target mRNAs investigated using real-time quantitative reverse transcription PCR (qRT-PCR).**

| No | Gene name and abbreviation Nuclear | Assay ID | Accession No. |
|---|---|---|---|
| 1 | Inducible nitric oxide synthase 2 (iNOS) | Mm00440502_m1 | NM_010927.3 |
| 2 | Prostaglandin-endoperoxide synthase 2 (COX-2 / PTGS2) | Mm00478374_m1 | NM_011198.3 |
| 3 | Tumor necrosis factor alpha (TNF-α) | Mm00443258_m1 | NM_013693.3 |
| 4 | Interleukin 1 beta (IL-1β) | Mm00434228_m1 | NM_008361.3 |
| 5 | Interleukin 6 (IL-6) | Mm00446190_m1 | NM_031168.1 |
| 6 | Toll-like receptor 4 (TLR4) | Mm00445273_m1 | NM_021297.2 |
| 7 | Jun proto-oncomRNA (cJUN) | Mm00495062_s1 | NM_010591.2 |
| 8 | FBJ osteosarcoma oncomRNA (cFOS) | Mm00487425_m1 | NM_010234.2 |
| 9 | Nuclear factor of kappa B (NFkB p65) | Mm00501346_m1 | NM_009045.4 |
| 10 | Nuclear factor of kappa B 1 (NFkB p50) | Mm00476361_m1 | NM_008689.2 |

General abbreviation of mRNAs, corresponding assay ID and accession number were obtained from the Applied Biosystems website and NCBI database. Assay ID refers to the Applied Biosystems mRNA Expression Assays inventoried kits with proprietary primer and TaqMan Fast Advanced Master Mix. Assay ID with “Mm” is referred to as “Mus musculus”. All mRNA Expression Assay kits indicated are FAM/MGB probed.

## 4.3 RESULTS

### 4.3.1 The extraction yield of solvent fractions of *L. rhinocerotis* sclerotium

The extraction yield of solvent fractions from hot aqueous extract of *L. rhinocerotis* sclerotium is summarized in Table 4.2. The aqueous fraction obtains the highest yield from its crude hot aqueous extract, followed by n-butanol fraction and ethyl acetate fraction.

**Table 4.2: Extraction yield of hot aqueous extract of *L. rhinocerotis* sclerotium and its solvent fractions.**

| Extract / Fractions | Yield (%, w/w) |
|---|---|
| Hot aqueous extract | 48.20 |
| Ethyl acetate fraction | 0.13 |
| n-Butanol fraction | 0.45 |
| Aqueous fraction | 62.5 |

### 4.3.2 The cytotoxic effects of hot aqueous extract and solvent fractions of *L. rhinocerotis* sclerotium on BV2 microglia

The cytotoxic effects of hot aqueous extract and its solvent fractions, ethyl acetate, n-butanol and aqueous fractions on BV2 microglia are presented in Figure 4.2. The viability of BV2 microglial cells in complete DMEM medium was considered as 100%. The viable rate of treated cells decreased in a concentration-dependent manner for hot aqueous extract, ethyl acetate, n-butanol and aqueous fractions after 48 hours of incubation. A significant ($p < 0.05$) increased of viability was observed at the concentrations of 1.96 μg/ml and 3.91 μg/ml of hot aqueous extract. The percentage of viable cells decreased gradually as the concentration of the hot aqueous extract and solvent fractions were increased.

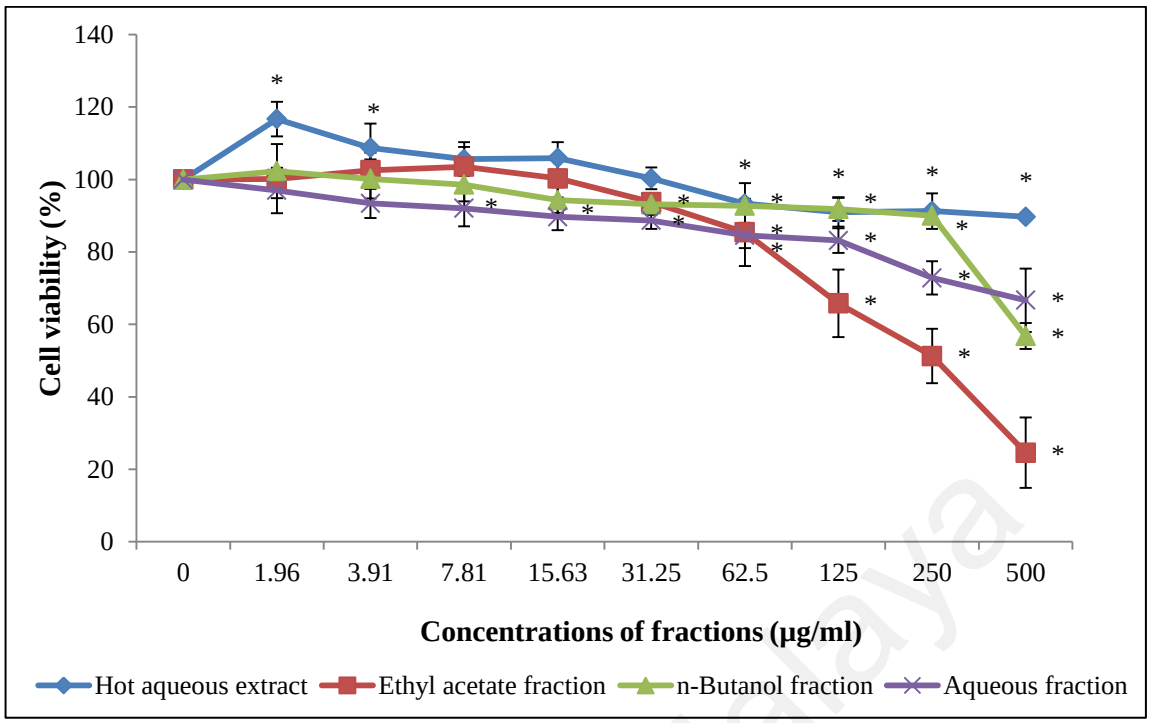



**Figure 4.2: The cytotoxic effects of hot aqueous extract and solvent fractions of *L. rhinocerotis* sclerotium on BV2 microglia.** Cells were incubated with hot aqueous extract, ethyl acetate, n-butanol or aqueous fractions at concentrations from 0 to 500 µg/ml for 48 hours. The mean absorbance obtained using complete DMEM medium with BV2 cells was designated as 100% viability. Data were expressed as means ± SD from three independent experiments carried out in triplicates. * $p < 0.05$ compared to BV2 cells only.

There was a significant ($p < 0.05$) increase in cytotoxicity starting from the concentration of 125 µg/ml (hot aqueous extract), 62.50 µg/ml (ethyl acetate fraction), 31.25 µg/ml (n-butanol fraction), and 7.81 µg/ml (aqueous fraction), reducing the percentage of viability by 9.08%, 14.60%, 6.86%, and 7.95% respectively, compared to 100% viability. The ethyl acetate, n-butanol and aqueous fractions were cytotoxic to BV2 cells at higher concentrations. The required concentration to inhibit the viability of BV2 microglia by 50% ($IC_{50}$) for hot aqueous extract, ethyl acetate, n-butanol and aqueous fractions at 48 hours are shown in Table 4.3. The hot aqueous extract has highest $IC_{50}$ value (20.01 ± 1.69 mg/ml), and followed by aqueous fraction (15.02 ± 1.21 mg/ml).

**Table 4.3: The 50% inhibitory concentration ($IC_{50}$) of hot aqueous extract and solvent fractions of *L. rhinocerotis* sclerotium on BV2 microglia viability after 48 hours of incubation.**

| Extract / fractions | $IC_{50}$ (mg/ml) on BV2 microglia |
|---|---|
| Hot aqueous extract | $20.01 \pm 1.69^{d}$ |
| Ethyl acetate fraction | $0.74 \pm 0.45^{a}$ |
| n-Butanol fraction | $7.09 \pm 1.78^{b}$ |
| Aqueous fraction | $15.02 \pm 1.21^{c}$ |

The data represent the mean ± SD from three independent experiments carried out in triplicates. Means with different alphabets represent significant difference ($p < 0.05$).

### 4.3.3 The inhibition of nitric oxide production in LPS-stimulated BV2 microglia by hot aqueous extract and solvent fractions

To investigate the inhibition of NO production in LPS-stimulated BV2 microglia by the hot aqueous extract and solvent fractions, the Griess assay was performed to measure the concentration of nitrite (a stable oxidized product of NO) released into the culture supernatant. The MTS assay was performed to exclude the possibility that the inhibition of NO production is due to the cytotoxicity of the extract or fractions. Pre-treatment with hot aqueous extract, ethyl acetate and n-butanol fractions of *L. rhinocerotis* sclerotium significantly ($p < 0.05$) inhibited NO production in LPS-stimulated cells in concentrations-dependent manner (Figure 4.3). In LPS control, the LPS stimulated approximately 98% higher production of NO in BV2 microglia compared to the negative control (cells only) significantly ($p < 0.05$) after 24 hours. Pre-treatment with hot aqueous extract (Figure 4.3a), ethyl acetate (Figure 4.3b) and n-butanol (Figure 4.3c) fractions significantly ($p < 0.05$) inhibited NO production in LPS-stimulated cells, compared to LPS control. The hot aqueous extract inhibited NO production in LPS-stimulated cells significantly ($p < 0.05$) at all concentrations tested, with no cytotoxic effect. However, no significant difference ($p > 0.05$) was observed in the treatment of aqueous fraction (Figure 4.3d) at all concentrations tested compared to LPS control,

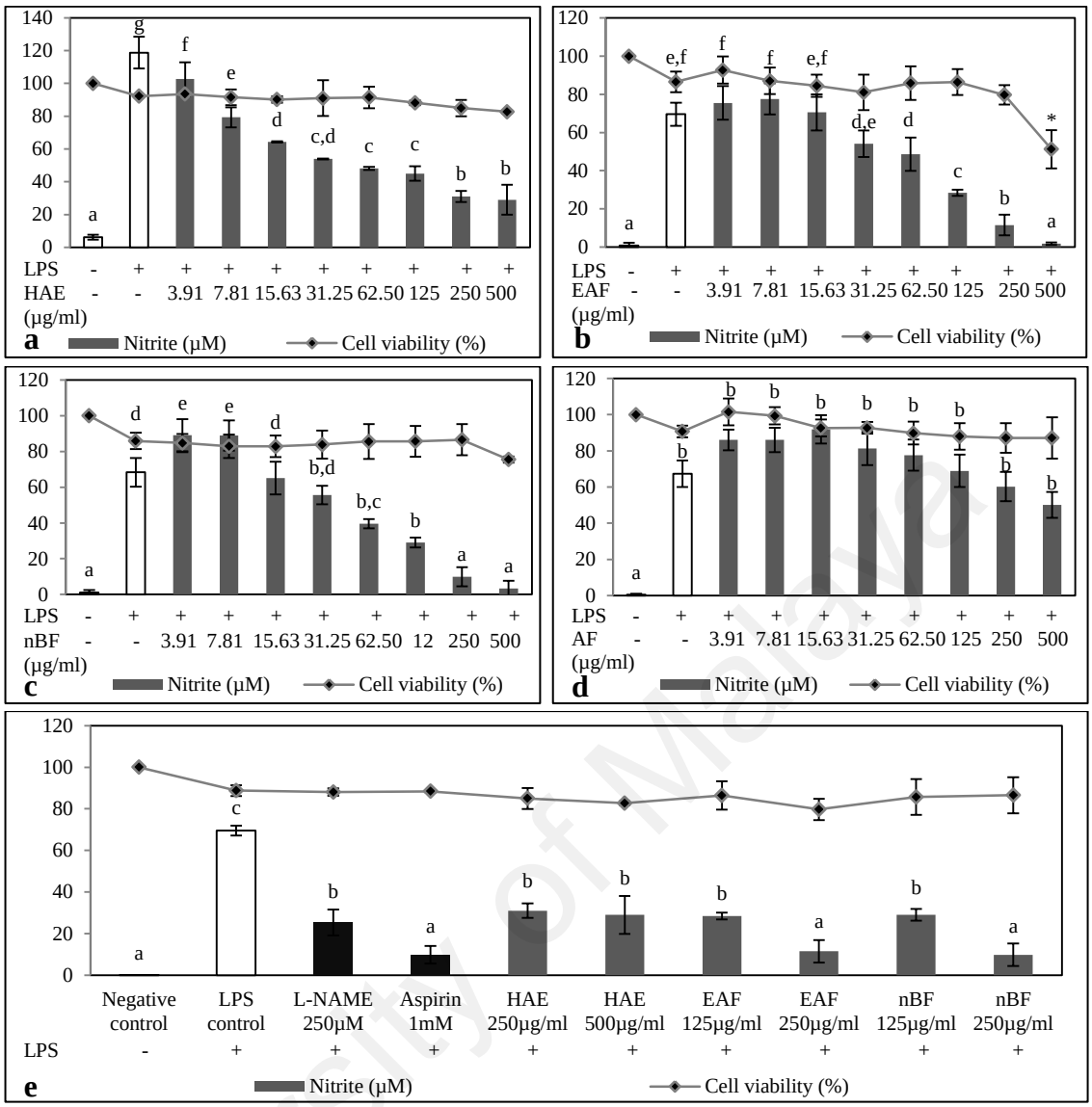



**Figure 4.3: The inhibition of NO production in LPS-stimulated BV2 microglia by hot aqueous extract and solvent fractions.** Cells were pre-treated with (a) hot aqueous extract (HAE), (b) ethyl acetate fraction (EAF), (c) n-butanol fraction (nBF), and (d) aqueous fraction (AF), at concentrations from 3.91 to 500 µg/ml, (e) 250 µM of L-NAME, 1 mM of aspirin, and optimum concentrations of HAE, EAF and n-BF, for 24 hours and then stimulated with one µg/ml of LPS for 24 hours. Data presented the concentration of nitrite (µM) in culture supernatant and percentage of cell viability (%), determined at 24 hours post-LPS stimulation by Griess and MTS assay, respectively. BV2 cells in complete DMEM medium served as a negative control and considered as 100% viability. Cells stimulated with LPS without treatment served as a LPS control. Data were expressed as means ± SD from three independent experiments carried out in triplicates. Means with different alphabets represent significant difference ($p < 0.05$) for nitrite concentrations. * $p < 0.05$ percentage of cell viability compared to the respective LPS control.

indicating that aqueous fraction did not exhibit inhibitory effect on NO production in LPS-stimulated cells. Low concentrations of n-butanol fraction (3.91 to 7.81 µg/ml) significantly ($p < 0.05$) increased NO production compared to LPS control. There was no significant ($p > 0.05$) reduction of NO production at low concentrations of ethyl acetate fraction (3.91 to 15.63 µg/ml) and n-butanol fraction (15.63 to 31.25 µg/ml), compared to LPS control. Interestingly, higher concentrations (62.50 to 250 µg/ml) of ethyl acetate and n-butanol fractions significantly ($p < 0.05$) inhibited 30% or higher percentage of NO production in LPS-stimulated cells compared to LPS control, with no cytotoxic effect. However, 500 µg/ml of ethyl acetate fraction was cytotoxic towards BV2 microglia. The maximum percentage of NO inhibition (without cytotoxic effect) was shown in the following order: n-butanol fraction (85.56% at 250 µg/ml) > ethyl acetate fraction (83.47% at 250 µg/ml) > hot aqueous extract (75.57% at 500 µg/ml). The positive controls, Aspirin at 1 mM and L-NAME at 250 µM significant ($p < 0.05$) inhibited the NO production in LPS-stimulated cells by approximately 86% and 64% respectively, compared to LPS control (Figure 4.3e). Interestingly, 250 µg/ml and 500 µg/ml of hot aqueous extract, 125 µg/ml of ethyl acetate and n-butanol fractions inhibited NO production that were comparable to the L-NAME, whereas, 250 µg/ml of ethyl acetate and n-butanol fractions inhibited NO production that were comparable to the aspirin.

### 4.3.4 The inhibition of iNOS protein and mRNA expressions in LPS-stimulated BV2 microglia by solvent fractions

Nitric oxide is endogenously generated enzymatically by inducible nitric oxide synthase (iNOS). Determination of iNOS expressions was aimed to confirm the NO inhibitory activity by the solvent fractions. Pre-treatment with ethyl acetate and n-butanol fractions significantly ($p < 0.05$) inhibited the iNOS protein and mRNA expressions in LPS-

stimulated BV2 microglia (Figure 4.4). The iNOS protein expression in LPS-stimulated BV2 microglia was significantly ($p < 0.05$) increased by approximately 0.5-fold, compared to the negative control (cells only) (Figure 4.4a). Pre-treatment with L-NAME (250 µM), aspirin (1 mM), ethyl acetate and n-butanol fractions (125 and 250 µg/ml) significantly ($p < 0.05$) inhibited the iNOS protein expression to the basal level. n-Butanol fraction at 250 µg/ml significantly ($p < 0.05$) inhibited the iNOS protein expression that lower than the basal level. The LPS also significantly ($p < 0.05$) upregulated the iNOS mRNA expression in BV2 microglia by approximately 2.2-fold, compared to the negative control (Figure 4.4b). Pre-treatment with L-NAME (250 µM), aspirin (1 mM), ethyl acetate and n-butanol fractions (125 and 250 µg/ml) significantly ($p < 0.05$) downregulated the iNOS mRNA expression that lower than the negative control. These data show that ethyl acetate and n-butanol fractions inhibited the NO production by inhibiting the protein and mRNA expressions of iNOS.

### 4.3.5 The inhibition of $PGE_2$ production, and COX-2 protein and mRNA expressions in LPS-stimulated BV2 microglia by solvent fractions

Prostaglandin $E_2$ is endogenously generated enzymatically by COX-2. Determination of COX-2 expressions was aimed to confirm the $PGE_2$ inhibitory activity by the solvent fractions. Pre-treatment with ethyl acetate and n-butanol fractions significantly ($p < 0.05$) inhibited $PGE_2$ production (Figure 4.5a), COX-2 protein (Figure 4.5b) and mRNA (Figure 4.5c) expressions in LPS-stimulated BV2 microglia. Stimulating BV2 microglia with LPS resulted in a significant ($p < 0.05$) increase in the extracellular $PGE_2$ level by approximately 60%, compared to the negative control (Figure 4.5a).

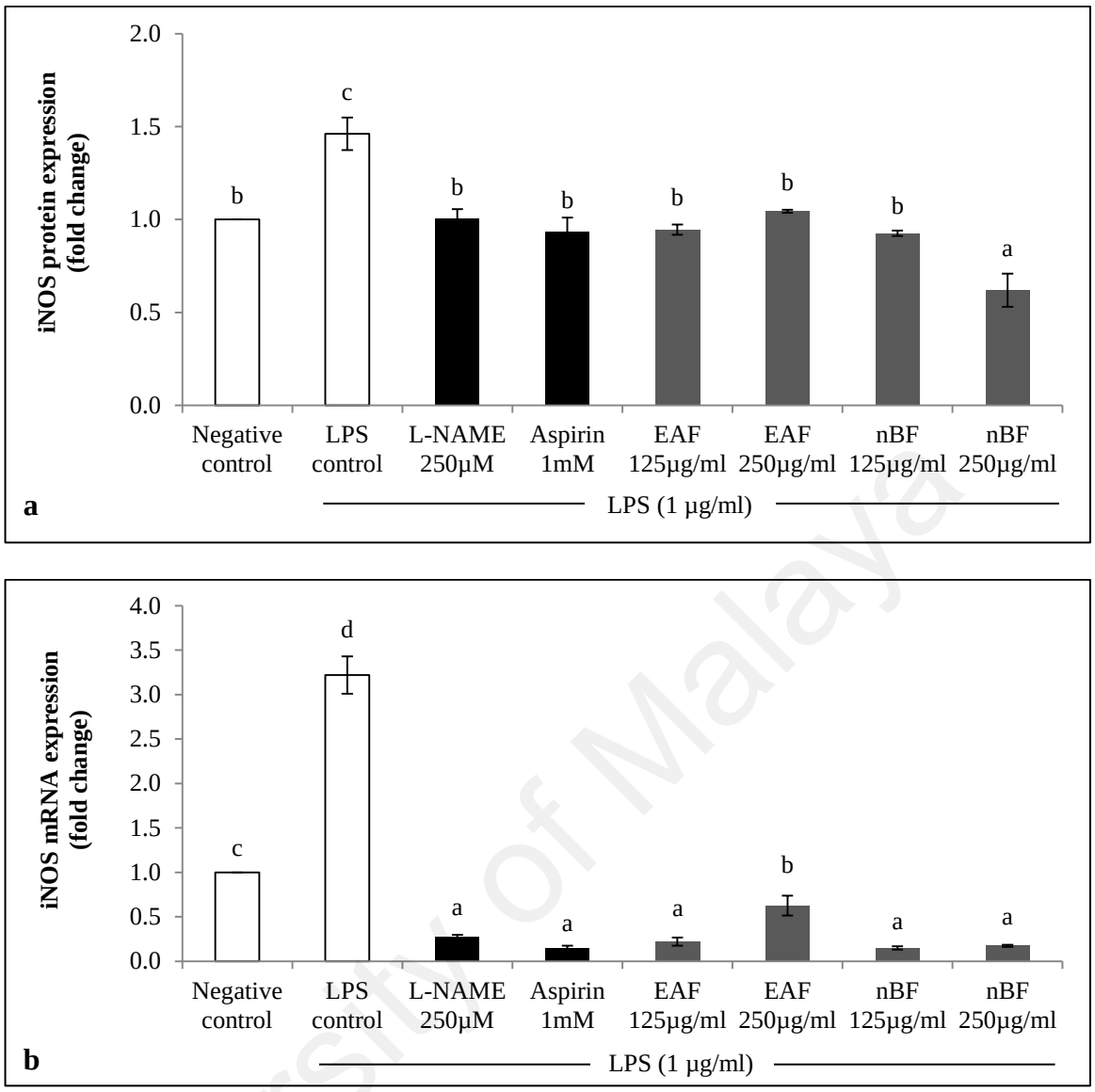



**Figure 4.4: The inhibition of iNOS protein and mRNA expressions in LPS-stimulated BV2 microglia by solvent fractions.** Cells were pre-treated with L-NAME (250 µM), aspirin (1 mM), ethyl acetate fraction (EAF) (125 and 250 µg/ml), and n-butanol fraction (nBF) (125 and 250 µg/ml) for 24 hours, and then stimulated with one µg/ml of LPS for 24 hours. Protein and RNA extracts for the detection of (a) iNOS protein expression by ELISA, and (b) iNOS mRNA expression by qRT-PCR were extracted at 24 hours post-LPS stimulation. Cells in complete DMEM medium served as a negative control. Cells stimulated with LPS without treatment served as a LPS control. Data were expressed as means ± SD from three independent experiments carried out in triplicates. Means with different alphabets represent significant difference ($p < 0.05$).

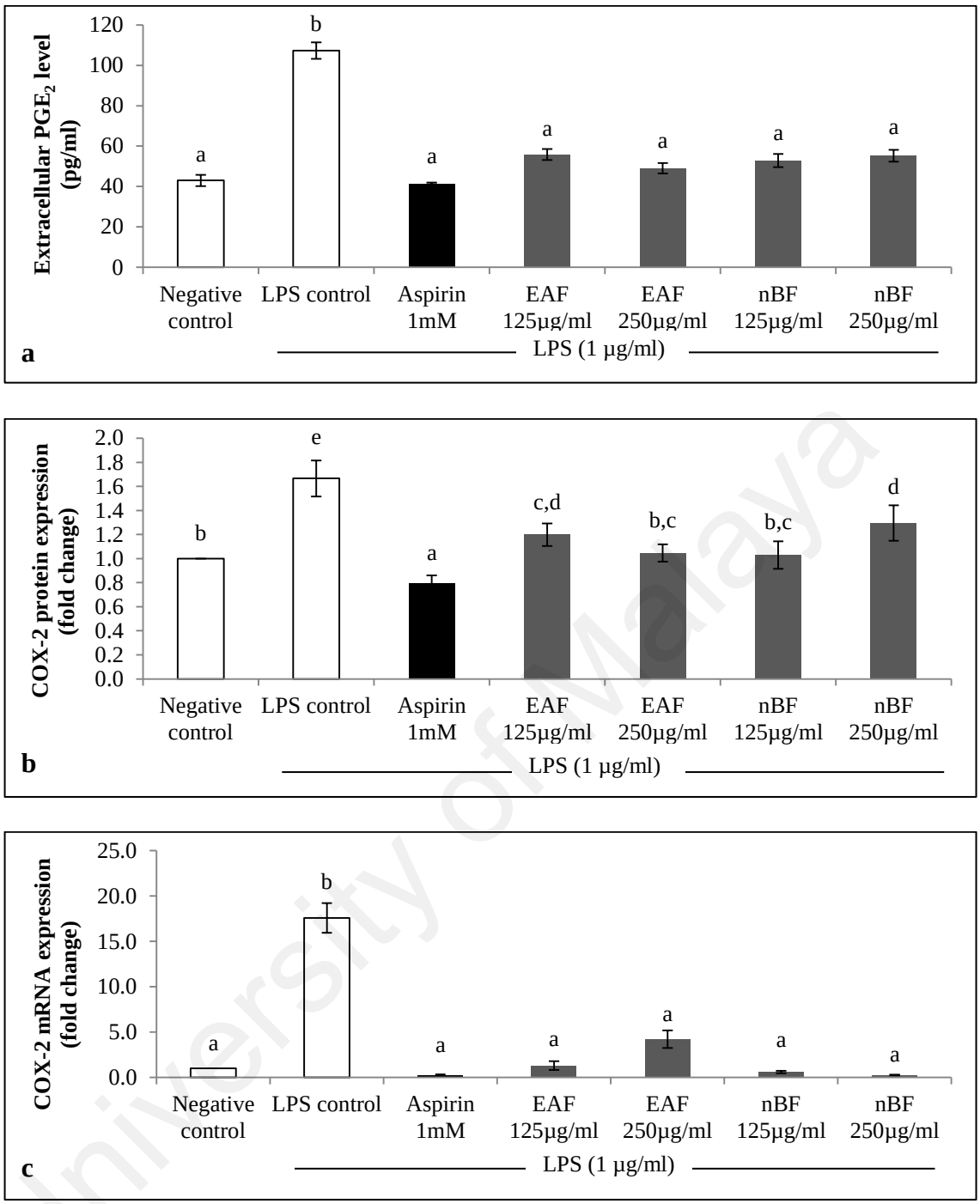



**Figure 4.5: The inhibition of $PGE_2$ production, COX-2 protein and mRNA expressions in LPS-stimulated BV2 microglia by solvent fractions.** Cells were pre-treated with aspirin (1 mM), ethyl acetate fraction (EAF) (125 and 250 µg/ml), and n-butanol fraction (nBF) (125 and 250 µg/ml) for 24 hours, and then stimulated with one µg/ml of LPS for 24 hours. Culture supernatant, protein and RNA extracts for the detection of (a) Extracellular level of $PGE_2$ by ELISA, (b) COX-2 protein expression by ELISA, and (c) COX-2 mRNA expression by qRT-PCR were collected at 24 hours post-LPS stimulation. Cells in complete DMEM medium served as a negative control. Cells stimulated with LPS without treatment served as a LPS control. Data were expressed as means ± SD from three independent experiments carried out in triplicates. Means with different alphabets represent significant difference ($p < 0.05$).

Pre-treatment with aspirin (1 mM) significantly ($p < 0.05$) inhibited the $PGE_2$ production in LPS-stimulated cells, by approximately 61%, compared to the LPS control (Figure 4.5a). Pre-treatment with 125 and 250 µg/ml of ethyl acetate and n-butanol fractions significantly ($p < 0.05$) inhibited the $PGE_2$ production that were comparable to aspirin. The COX-2 protein expression in LPS-stimulated BV2 microglia was significantly ($p < 0.05$) increased by approximately 0.67-fold, compared to the negative control (Figure 4.5b). Pre-treatment with aspirin (1 mM), ethyl acetate and n-butanol fractions (125 and 250 µg/ml) significantly ($p < 0.05$) inhibited the COX-2 protein expression in LPS-stimulated cells. The LPS significantly ($p < 0.05$) upregulated the mRNA expression of COX-2 in BV2 microglia by approximately 17-fold, compared to the negative control. Pre-treatment with aspirin (1 mM), ethyl acetate and n-butanol fractions (125 and 250 µg/ml) significantly ($p < 0.05$) downregulated the COX-2 mRNA expression to the basal level. These data show that aspirin, ethyl acetate and n-butanol fractions inhibited the $PGE_2$ production by the attenuation of the protein and mRNA expressions of COX-2.

### 4.3.6 Immunocytofluorescence study of solvent fractions on iNOS and COX-2 expressions in LPS-stimulated BV2 microglia

Immunocytofluorescence staining of iNOS and COX-2 (stained green by FITC) and counter-stained by DAPI are depicted in Figure 4.6 and Figure 4.7, respectively. Negative control (cells only) exhibited negligible iNOS and COX-2 expression. BV2 microglial cells stimulated by LPS expressed significant increment of iNOS and COX-2 expressions, by exhibiting increased of green fluorescence intensity around the cells. Pre-treatment with L-NAME (250 µM), ethyl acetate and n-butanol fractions (125 and 250 µg/ml) markedly decreased the iNOS expression (Figure 4.6) when compared to the LPS control. Whereas, pre-treatment with aspirin (1 mM), ethyl acetate and n-butanol

fractions (125 and 250 µg/ml) markedly decreased the COX-2 expression (Figure 4.7), compared to the LPS control.

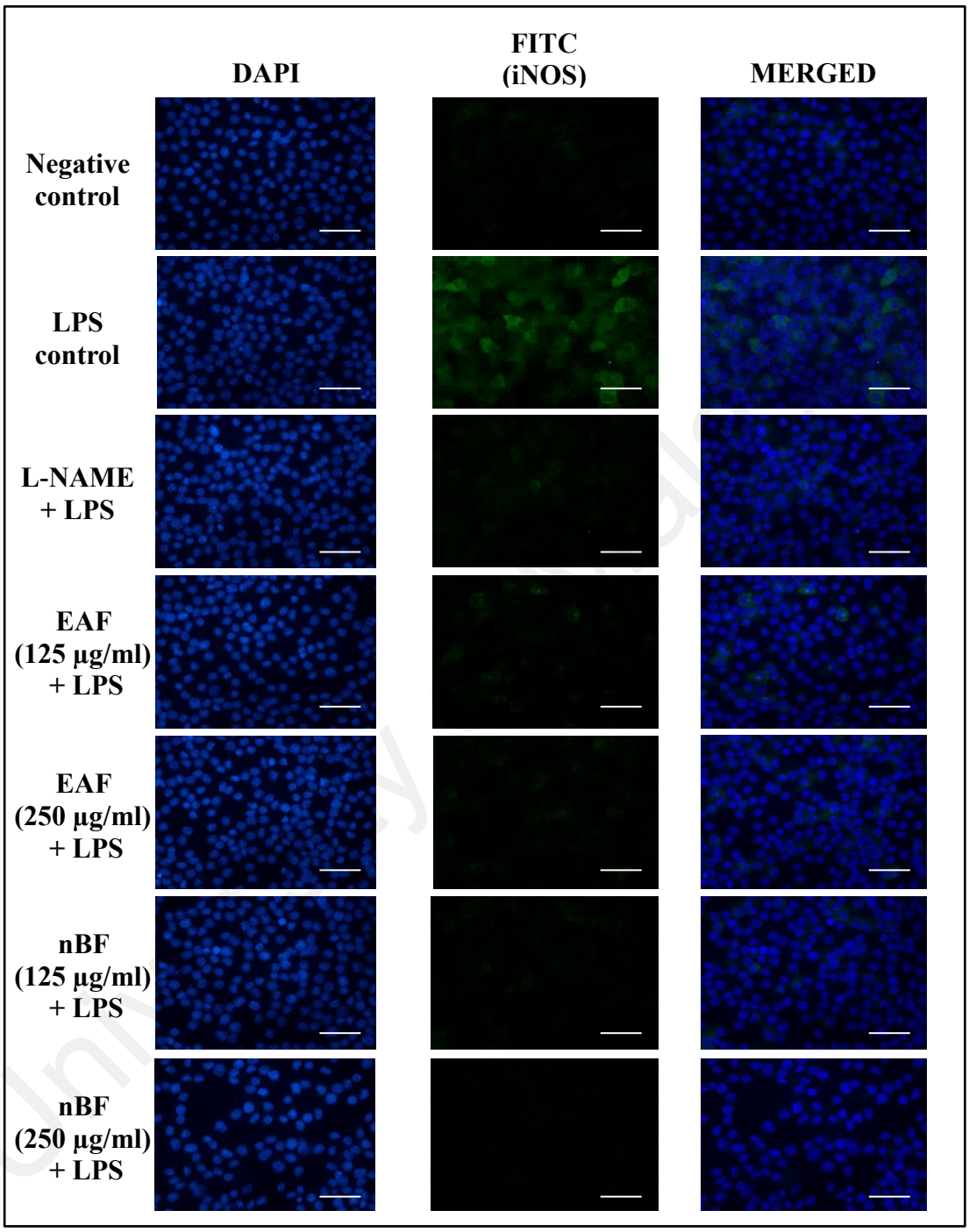



**Figure 4.6: Immunocytofluorescence analysis of iNOS expression in LPS-stimulated BV2 microglia.** Cells were pre-treated with L-NAME (250 µM), ethyl acetate fraction (EAF) (125 and 250 µg/ml), and n-butanol fraction (nBF) (125 and 250 µg/ml) for 24 hours, and then stimulated with one µg/ml of LPS for 24 hours. Cells in complete DMEM medium served as a negative control (exhibited negligible iNOS expression). Cells stimulated with LPS without treatment served as a LPS control. Nuclei stained blue and iNOS expression stained green. LPS control showed significant increment of iNOS expression compared to negative control. Cells pre-treated with L-NAME, EAF and nBF markedly decreased the iNOS expression compared to LPS control. Scale bars represent 50 µm.

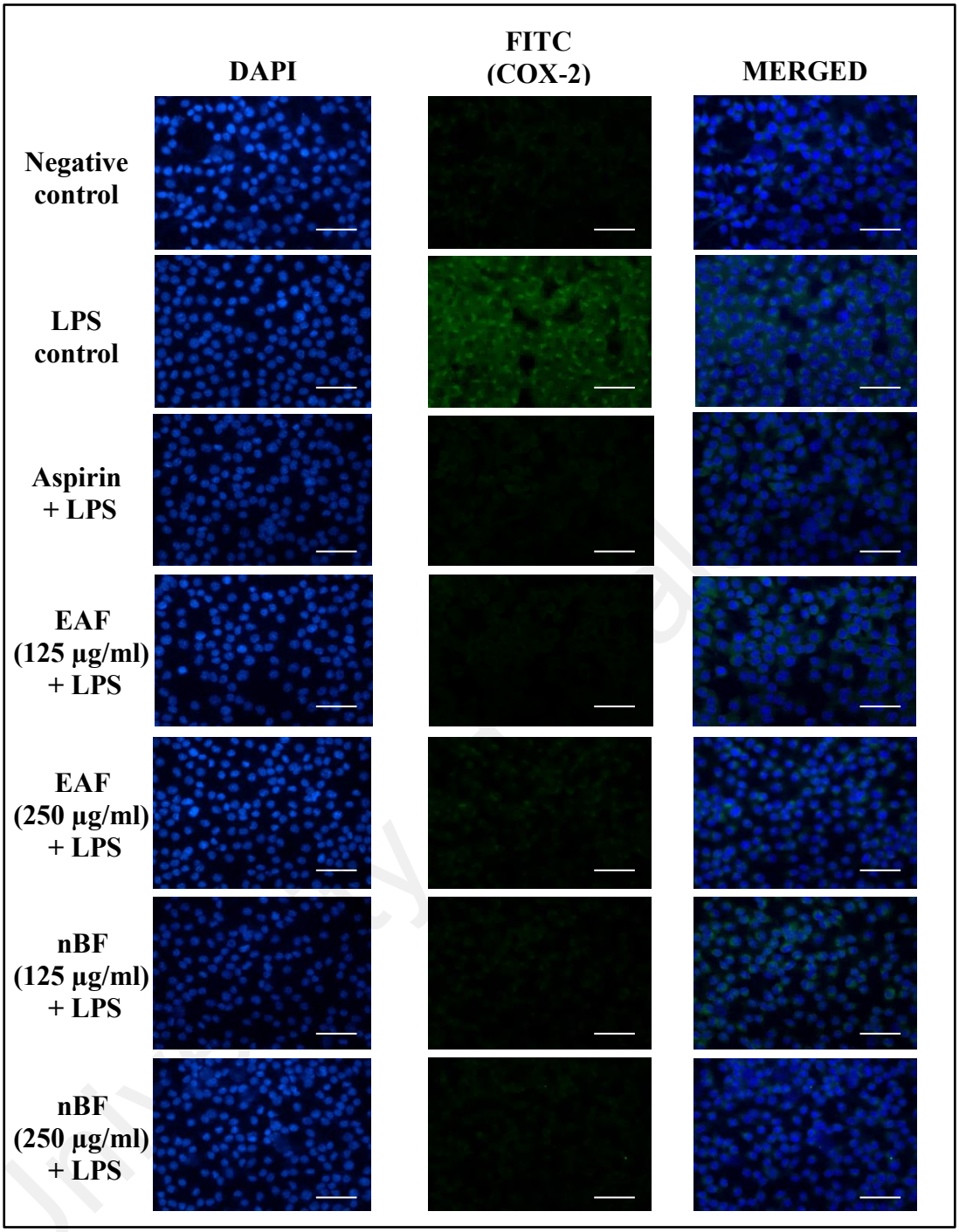



**Figure 4.7: Immunocytofluorescence analysis of COX-2 expression in LPS-stimulated BV2 microglia.** Cells were pre-treated with L-NAME (250 µM), ethyl acetate fraction (EAF) (125 and 250 µg/ml), and n-butanol fraction (nBF) (125 and 250 µg/ml) for 24 hours, and then stimulated with one µg/ml of LPS for 24 hours. Cells in complete DMEM medium served as a negative control (exhibited negligible COX-2 expression). Cells stimulated with LPS without treatment served as a LPS control. Nuclei stained blue and COX-2 expression stained green. LPS control showed significant increment of COX-2 expression compared to negative control. Cells pre-treated with L-NAME, EAF and nBF markedly decreased the COX-2 expression compared to LPS control. Scale bars represent 50 µm.

### 4.3.7 The inhibition of pro-inflammatory cytokines production and mRNA expression in LPS-stimulated BV2 microglia by solvent fractions

The production of pro-inflammatory cytokines in BV2 microglia is induced by the upregulation of its specific gene at the transcriptional level through the stimulation of LPS. Elevated levels of pro-inflammatory cytokines (TNF-α, IL-1β and IL6) have been linked to neuroinflammation. The ability of solvent fractions to inhibit the production and mRNA expressions of TNF-α, IL-1β and IL6 were determined. Pre-treatment with ethyl acetate and n-butanol fractions significantly ($p < 0.05$) inhibited the production of TNF-α and IL-1β (Figure 4.8), and the mRNA expressions of TNF-α, IL-1β and IL6 (Figure 4.9) in LPS-stimulated BV2 microglia. The extracellular concentration of TNF-α (Figure 4.8a) in LPS-stimulated BV2 microglia was detected at 930.70 ± 17.43 pg/ml, which was approximately 84% increase compared to the negative control (cells only). Pre-treatment with aspirin (1 mM), ethyl acetate and n-butanol fractions (125 and 250 µg/ml) significantly ($p < 0.05$) inhibited the TNF-α production in LPS-stimulated cells by approximately 23% (aspirin), 38% (125 and 250 µg/ml of ethyl acetate fraction, and 125 µg/ml of n-butanol fraction), and 43% (250 µg/ml of n-butanol fraction). The extracellular concentration of IL-1β (Figure 4.8b) in LPS-stimulated BV2 microglia was detected at 128.16 ± 13.04 pg/ml, which was approximately a 51% increase when compared to the negative control. Pre-treatment with aspirin (1 mM), ethyl acetate and n-butanol fractions (125 and 250 µg/ml) significantly ($p < 0.05$) inhibited the IL-1β production in LPS-stimulated cells that were comparable to the negative control.

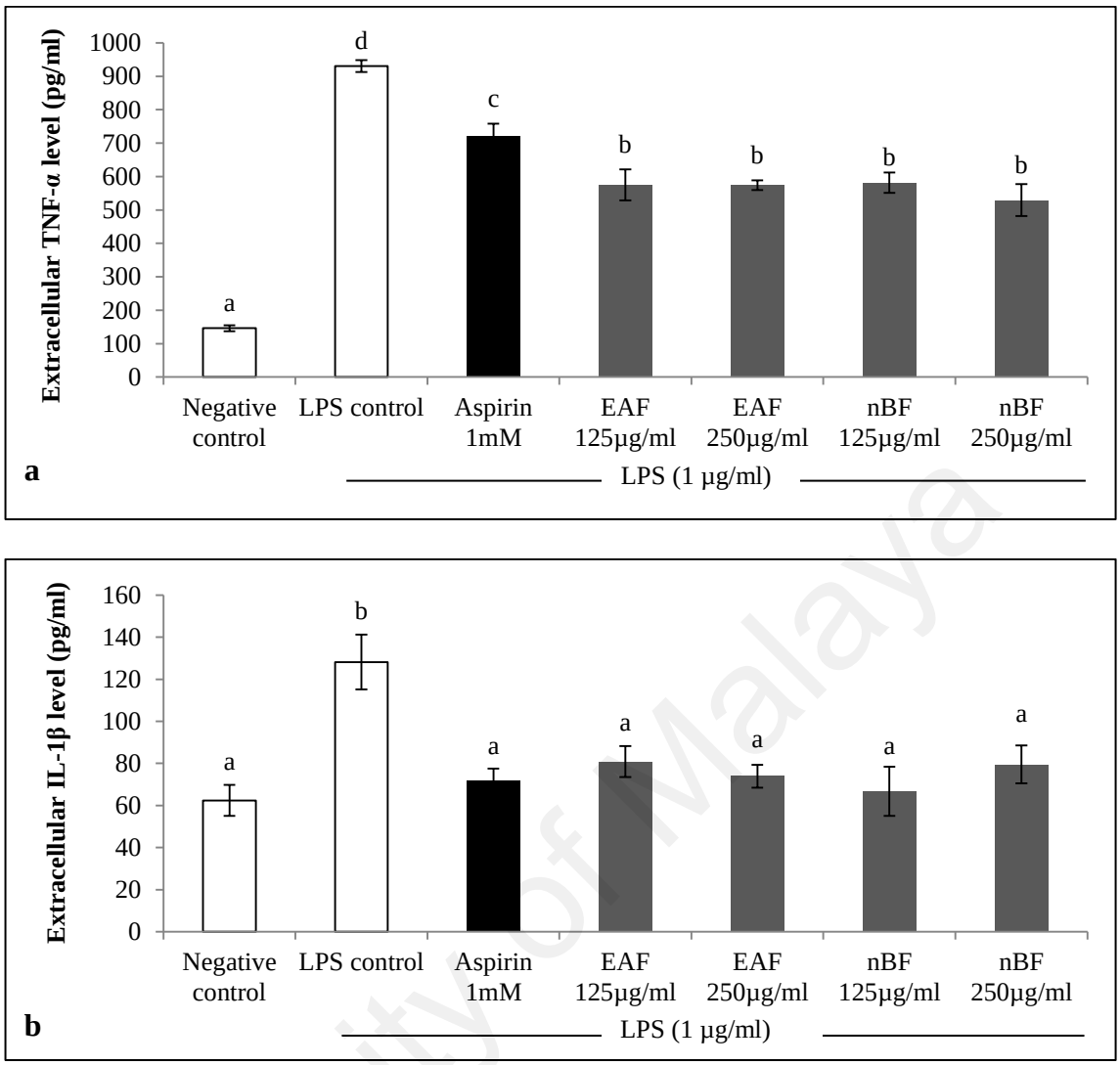



**Figure 4.8: The inhibition of TNF-α and IL-1β production in LPS-stimulated BV2 microglia by solvent fractions.** Cells were pre-treated with aspirin (1 mM), ethyl acetate fraction (EAF) (125 and 250 µg/ml), and n-butanol fraction (nBF) (125 and 250 µg/ml) for 24 hours, and then stimulated with one µg/ml of LPS for 24 hours. Culture supernatant for the detection of extracellular level of (a) TNF-α and (b) IL-1β were collected at 24 hours post-LPS stimulation. Cells in complete DMEM medium served as a negative control. Cells stimulated with LPS without treatment served as a LPS control. Data were expressed as means ± SD from three independent experiments carried out in triplicates. Means with different alphabets represent significant difference ($p < 0.05$).

The LPS significantly ($p < 0.05$) upregulated the mRNA expressions of TNF-α (Figure 4.9a), IL-1β (Figure 4.9b) and IL-6 (Figure 4.9c) in BV2 microglia by approximately 17.3-, 1.4-, and 2.5-fold, respectively, compared to the respective negative control. Pre-treatment with aspirin (1 mM), ethyl acetate and n-butanol fractions (125 and 250 µg/ml) significantly ($p < 0.05$) downregulated the TNF-α and IL-1β mRNA expressions to the basal level, without significant difference ($p > 0.05$) compared to negative control. Pre-treatment with n-butanol fraction (125 and 250 µg/ml) significantly ($p < 0.05$) downregulated the IL-6 mRNA expression to the level that comparable to the negative control, while pre-treatment with aspirin (1 mM) and ethyl acetate fraction (125 and 250 µg/ml) significantly ($p < 0.05$) downregulated the IL-6 mRNA expression which lower than the negative control. These data demonstrate that aspirin, ethyl acetate and n-butanol fractions inhibited the production of TNF-α, IL-1β and IL-6 by regulating the TNF-α, IL-1β and IL-6 mRNA expression at the transcriptional level.

### 4.3.8 The inhibition of toll-like receptor 4 (TLR4) mRNA expression in LPS-stimulated BV2 microglia by solvent fractions

Toll-like receptor 4 (TLR4) is highly expressed on microglia upon stimulation by LPS. Activation of TLR4 leads to the activation of downstream NFκB, AKT and MAPKs signaling pathways which are responsible for neuroinflammation. Pre-treatment with ethyl acetate fraction significantly ($p < 0.05$) downregulated the TLR4 mRNA expression in LPS-stimulated BV2 microglia, but not for n-butanol fraction (Figure 4.10). Treatment with LPS significantly ($p < 0.05$) upregulated the TLR4 mRNA expression in BV2 microglia by approximately 4-fold, compared to the negative control. Pre-treatment with aspirin (1 mM) and ethyl acetate fraction (125 and 250 µg/ml) significantly ($p < 0.05$) downregulated the TLR4 mRNA expression in LPS-stimulated cells.

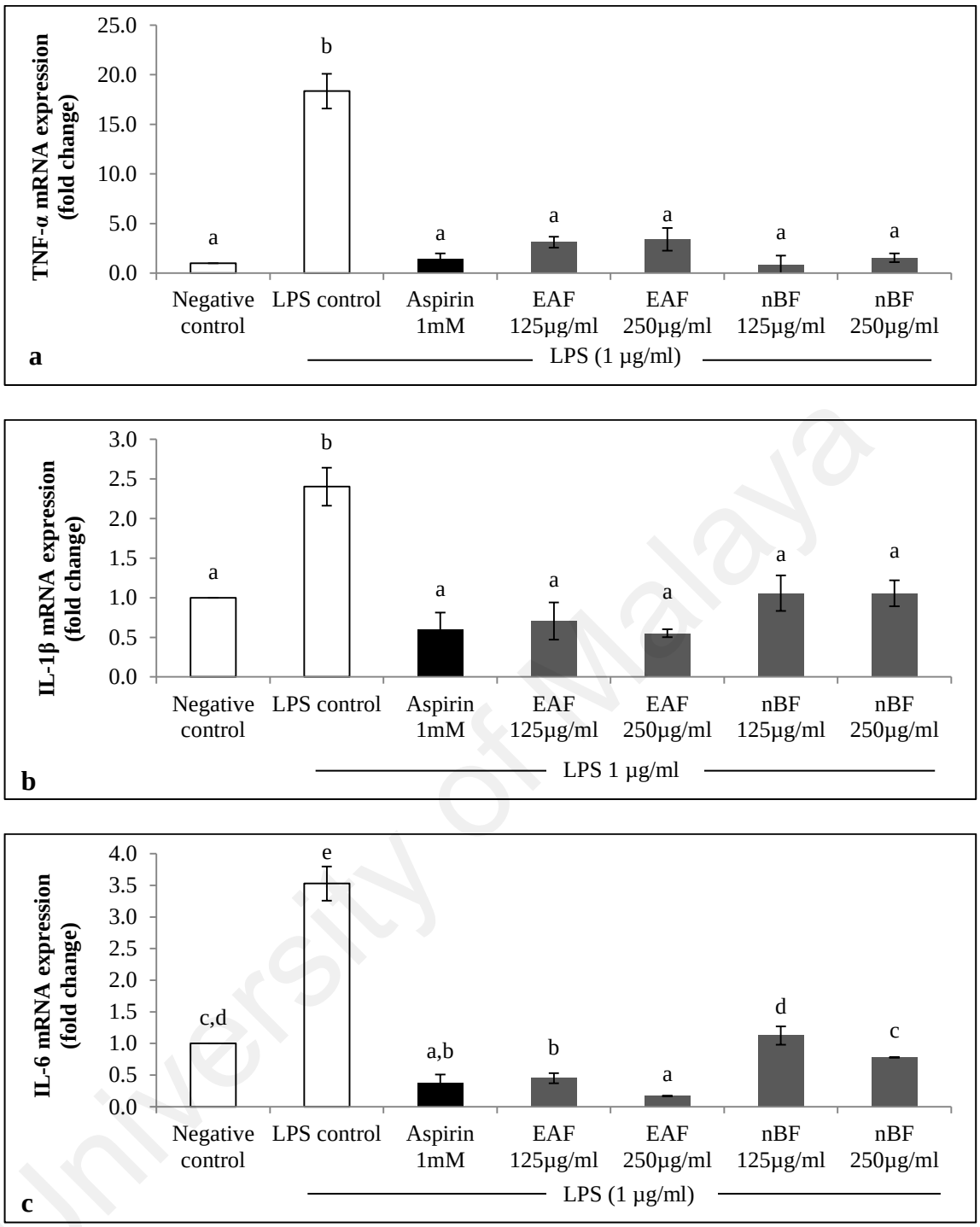



**Figure 4.9: The inhibition of TNF-α and IL-1β mRNA expressions in LPS-stimulated BV2 microglia by solvent fractions.** Cells were pre-treated with aspirin (1 mM), ethyl acetate fraction (EAF) (125 and 250 µg/ml), and n-butanol fraction (nBF) (125 and 250 µg/ml) for 24 hours, and then stimulated with one µg/ml of LPS for 24 hours. RNA extracts for the detection of mRNA expression of (a) TNF-α, (b) IL-1β and (c) IL-6 were collected at 24 hours post-LPS stimulation. Cells in complete DMEM medium served as a negative control. Cells stimulated with LPS without treatment served as a LPS control. Data were expressed as means ± SD from three independent experiments carried out in triplicates. Means with different alphabets represent significant difference ($p < 0.05$).

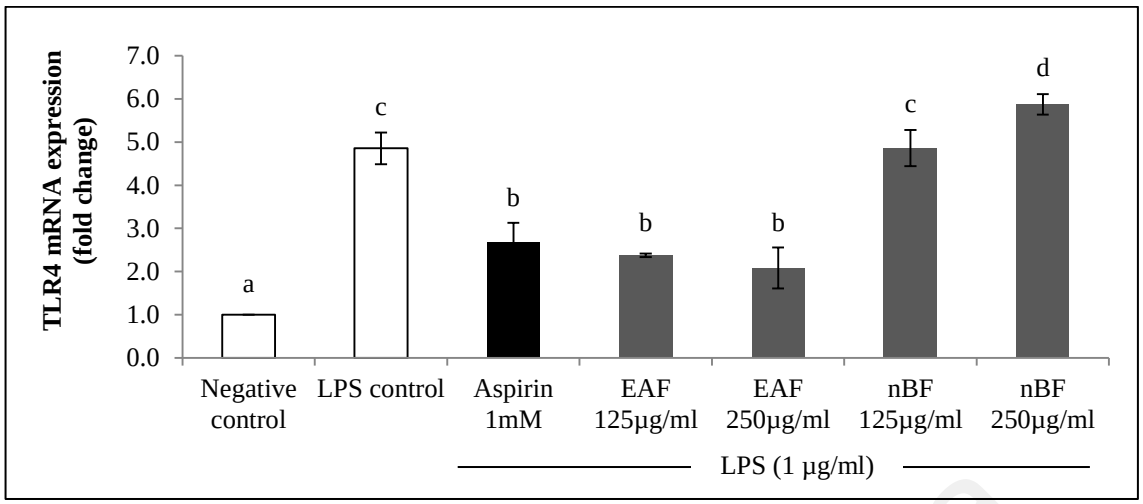



**Figure 4.10: The inhibition of TLR4 mRNA expression in LPS-stimulated BV2 microglia by solvent fractions.** Cells were pre-treated with aspirin (1 mM), ethyl acetate fraction (EAF) (125 and 250 µg/ml), and n-butanol fraction (nBF) (125 and 250 µg/ml) for 24 hours, and then stimulated with one µg/ml of LPS for 24 hours. RNA extracts were collected at 24 hours post-LPS stimulation. Cells in complete DMEM medium served as a negative control. Cells stimulated with LPS without treatment served as a LPS control. Data were expressed as means ± SD from three independent experiments carried out in triplicates. Means with different alphabets represent significant difference ($p < 0.05$).

However, n-butanol fraction did not downregulated the TLR4 mRNA expression (Figure 4.10). Pre-treatment with 250 µg/ml of n-butanol fraction upregulated the TLR4 mRNA expression that was significant ($p < 0.05$) higher than LPS control. No significant difference ($p > 0.05$) found between 125 µg/ml of n-butanol fraction and LPS control. These findings suggest that aspirin and ethyl acetate fraction may inhibit the downstream cascade of LPS-stimulated neuroinflammation by inhibiting the activation of TLR4.

### 4.3.9 The inhibition of p-IκB-α and p-NFκB p65 protein expressions in LPS-stimulated BV2 microglia by solvent fractions

The involvement of the NFκB signaling pathway activated by TLR4 in LPS-stimulated BV2 cells was investigated. Phosphorylation of IκB-α and NFκB p65 will lead to the transcription of NFκB p65 and NFκB p50 genes which then upregulate the transcription

of pro-inflammatory genes. The ability of solvent fractions to inhibit the phosphorylation of IκB-α and NFκB p65 was measured by ELISA. The protein expressions of phosphorylated IκB-α (Ser32) and NFκB p65 (Ser536) in LPS-stimulated BV2 microglia were significantly ($p < 0.05$) inhibited by ethyl acetate and n-butanol fractions (Figure 4.11). The LPS significantly ($p < 0.05$) increased the expressions of phosphorylated IκB-α (Figure 4.11a) and NFκB p65 (Figure 4.11b). Pre-treatment with aspirin (1 mM) significantly ($p < 0.05$) decreased the LPS-stimulated protein expression of p-IκB-α to the basal level, without significant difference ($p > 0.05$) compared to the negative control. Pre-treatment with ethyl acetate and n-butanol fractions (125 and 250 µg/ml) also significantly ($p < 0.05$) decreased the LPS-stimulated the p-IκB-α expression, compared to the LPS control (Figure 4.11a). Pre-treatment with aspirin, ethyl acetate and n-butanol fractions (125 and 250 µg/ml) significantly ($p < 0.05$) decreased the LPS-stimulated protein expression of p-NFκB p65 to the basal level, without significant difference ($p > 0.05$) compared to negative control (figure 4.11b). These findings showed that aspirin, ethyl acetate and n-butanol fractions may inhibit the NFκB signaling pathway by inhibiting the phosphorylation of IκB-α and NFκB p65.

### 4.3.10 The inhibition of NFκB p65 and NFκB p50 mRNA expression in LPS-stimulated BV2 microglia by solvent fractions

The investigation of NFκB p65 and NFκB p50 mRNA expressions were aimed to confirm the inhibition of translocation of p-NFκB p65 and p-NFκB p50 into the nucleus by the solvent fractions. Pre-treatment with ethyl acetate and n-butanol fractions significantly ($p < 0.05$) downregulated the NFκB p65 (Figure 4.12a) and NFκB p50 (Figure 4.12b) mRNA expressions in LPS-stimulated BV2 microglia.

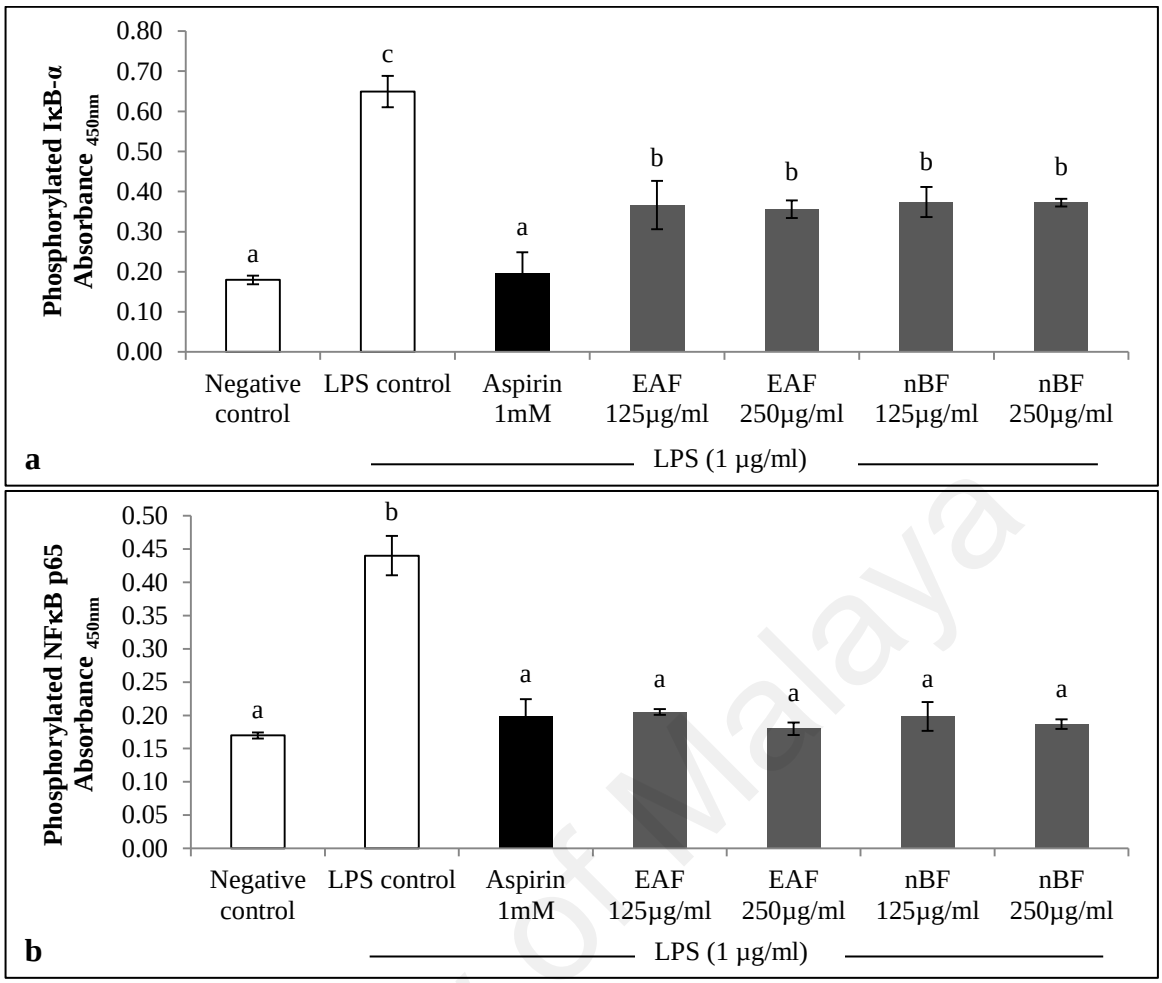



**Figure 4.11: The inhibition of p-IκB-α (Ser32) and p-NFκB p65 (Ser536) protein expressions in LPS-stimulated BV2 microglia by solvent fractions.** Cells were pre-treated with aspirin (1 mM), ethyl acetate fraction (EAF) (125 and 250 µg/ml), and n-butanol fraction (nBF) (125 and 250 µg/ml) for 24 hours, and then stimulated with one µg/ml of LPS for 24 hours. Protein extracts for the detection of phosphorylated (a) IκB-α and (b) NFκB p65 expressions were collected at 24 hours post-LPS stimulation. Cells in complete DMEM medium served as a negative control. Cells stimulated with LPS without treatment served as a LPS control. Phosphorylated IκB-α and NFκB p65 protein level was measured by determination of the absorbance at 450 nm. Data were expressed as means ± SD from three independent experiments carried out in triplicates. Means with different alphabets represent significant difference ($p < 0.05$).

Treatment with LPS significantly ($p < 0.05$) upregulated the NFκB p65 (Figure 4.12a) and NFκB p50 (Figure 4.12b) mRNA expression in BV2 microglia by approximately 2.4- and 3.2-fold respectively, compared to the respective negative control. Pre-treatment with aspirin (1 mM), ethyl acetate and n-butanol fractions (125 and 250 µg/ml) significantly ($p < 0.05$) downregulated the LPS-stimulated NFκB p65

and NFκB p50 mRNA expressions to the basal level, without significant difference ($p > 0.05$) compared to negative control. These data demonstrate that aspirin, ethyl acetate and n-butanol fractions inhibited the activation of the major neuroinflammatory signaling pathway, the NFκB and its transcription factors, NFκB p65 and NFκB p50 in LPS-stimulated BV2 microglia.

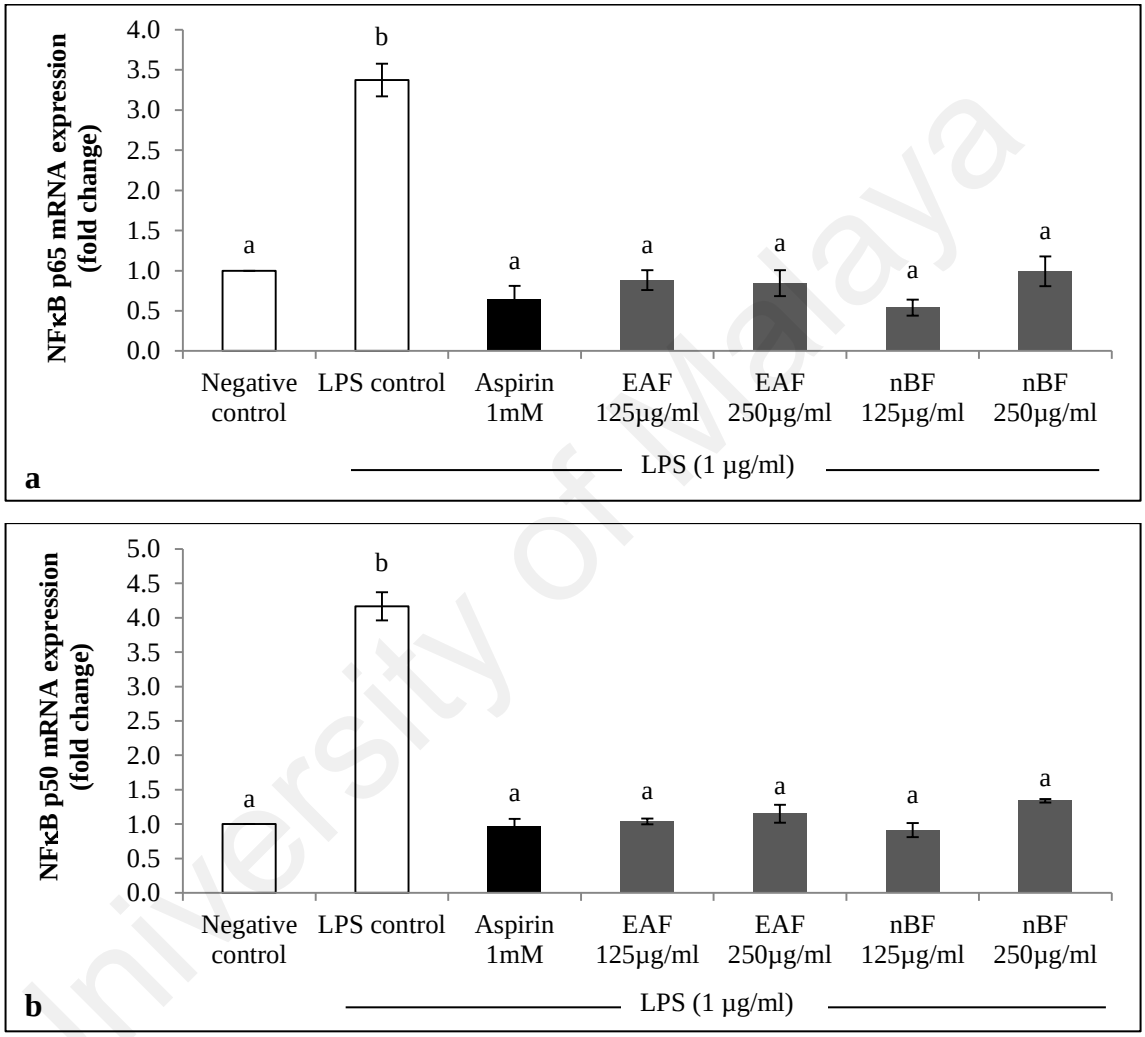



**Figure 4.12: The inhibition of NFκB p65 and NFκB p50 mRNA expression in LPS-stimulated BV2 microglia by solvent fractions.** Cells were pre-treated with aspirin (1 mM), ethyl acetate fraction (EAF) (125 µg/ml and 250 µg/ml), and n-butanol fraction (nBF) (125 µg/ml and 250 µg/ml) for 24 hours, and then stimulated with 1 µg/ml of LPS for 24 hours. RNA extracts for the detection of mRNA expression of (a) NFκB p65, and (b) NFκB p50 were collected at 24 hours post-LPS stimulation. Cells in complete DMEM medium served as a negative control. Cells stimulated with LPS without treatment served as a LPS control. Data were expressed as means ± SD from three independent experiments carried out in triplicates. Means with different alphabets represent significant difference ($p < 0.05$).

### 4.3.11 The inhibition of p-ERK1/2, p-p38 MAPK, p-SAPK/JNK, p-AKT and p-CREB protein expressions in LPS-stimulated BV2 microglia by solvent fractions

The involvement of the MAPKs (ERK1/2, p-38 MAPK and SAPK/JNK) and AKT signaling pathways activated by TLR4 in LPS-stimulated BV2 microglia were investigated. The ability of solvent fractions to inhibit the phosphorylation of ERK1/2, p-38 MAPK, SAPK/JNK, AKT and the transcription factor, CREB proteins were measured by ELISA. Pre-treatment with ethyl acetate and n-butanol fractions significantly ($p < 0.05$) decreased the protein expression of phosphorylated MAPKs downstream target proteins, p-ERK1/2 (p44/p42) (Thr202/Tyr204) (Figure 4.13), p-p38 MAPK (Thr180/Tyr182) (Figure 4.14) and p-SAPK/JNK (Thr183/Tyr185) (Figure 4.15). The LPS significantly ($p < 0.05$) increased the expression of phosphorylated ERK1/2 (p44/p42) (Thr202/Tyr204) (Figure 4.13), p-p38 MAPK (Thr180/Tyr182) (Figure 4.14) and p-SAPK/JNK (Thr183/Tyr185) (Figure 4.15). Pre-treatment with aspirin (1 mM), ethyl acetate and n-butanol fractions (125 and 250 µg/ml) significantly ($p < 0.05$) decreased the LPS-stimulated protein expression of p-ERK1/2 (Figure 4.13). Interestingly, ethyl acetate (250 µg/ml) and n-butanol (125 and 250 µg/ml) fractions significantly ($p < 0.05$) decreased the LPS-stimulated p-ERK1/2 expression lower than that of aspirin. There was no significant difference ($p > 0.05$) in p-ERK1/2 expression between 250 µg/ml of ethyl acetate fraction-treated cells and negative control. Pre-treatment with aspirin (1 mM), ethyl acetate and n-butanol fractions (125 and 250 µg/ml) significantly ($p < 0.05$) decreased the LPS-stimulated protein expressions of p-p38 MAPK (Figure 4.14) and p-SAPK/JNK (Figure 4.15) that were comparable to the negative control.

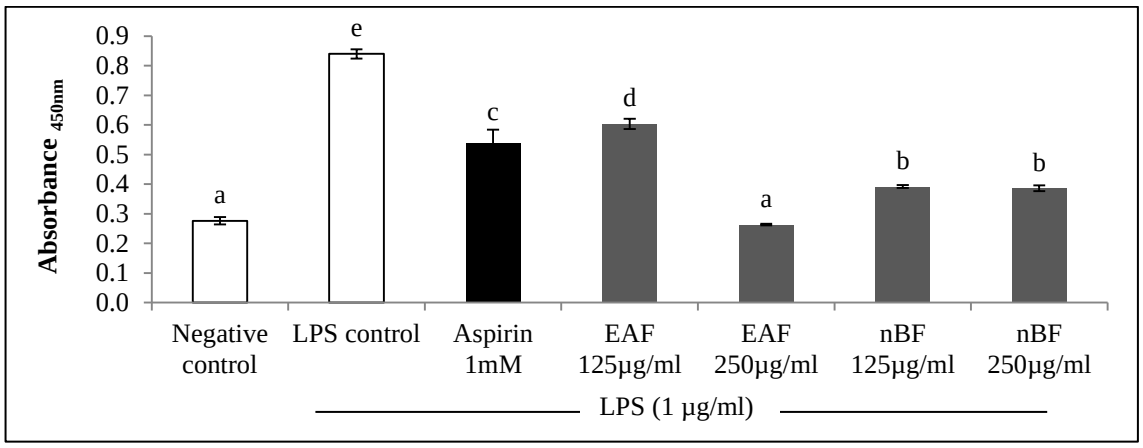



**Figure 4.13: The inhibition of p-ERK1/2 (p44/42) (Thr202/Tyr204) protein expression in LPS-stimulated BV2 microglia by solvent fractions.** Cells were pre-treated with aspirin (1 mM), ethyl acetate fraction (EAF) (125 and 250 µg/ml), and n-butanol fraction (nBF) (125 and 250 µg/ml) for 24 hours, and then stimulated with one µg/ml of LPS for 24 hours. Protein extracts were collected at 24 hours post-LPS stimulation. Cells in complete DMEM medium served as a negative control. Cells stimulated with LPS without treatment served as a LPS control. Phosphorylated ERK1/2 protein level was measured by determination of the absorbance at 450 nm. Data were expressed as means ± SD from three independent experiments carried out in triplicates. Means with different alphabets represent significant difference ($p < 0.05$).

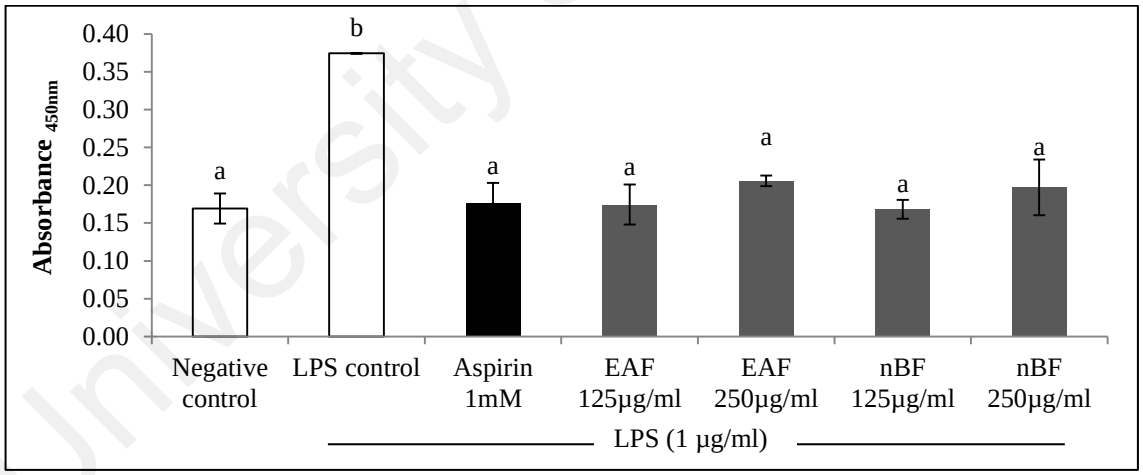



**Figure 4.14: The inhibition of p-p38 MAPK (Thr180/Tyr182) protein expression in LPS-stimulated BV2 microglia by solvent fractions.** Cells were pre-treated with aspirin (1 mM), ethyl acetate fraction (EAF) (125 and 250 µg/ml), and n-butanol fraction (nBF) (125 and 250 µg/ml) for 24 hours, and then stimulated with one µg/ml of LPS for 24 hours. Protein extracts were collected at 24 hours post-LPS stimulation. Cells in complete DMEM medium served as a negative control. Cells stimulated with LPS without treatment served as a LPS control. Phosphorylated p38 MAPK protein level was measured by determination of the absorbance at 450 nm. Data were expressed as means ± SD from three independent experiments carried out in triplicates. Means with different alphabets represent significant difference ($p < 0.05$).

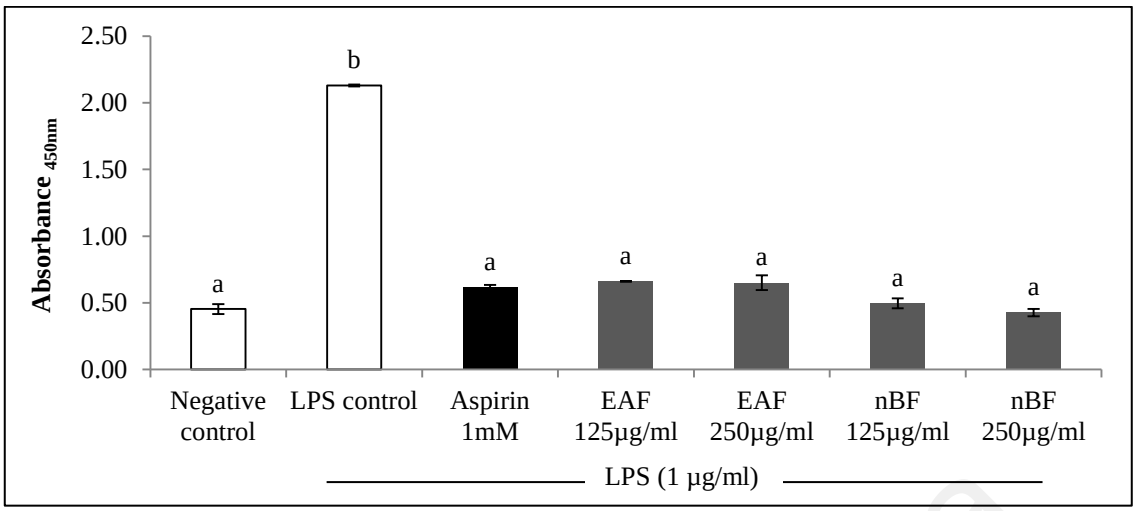



**Figure 4.15: The inhibition of p-SAPK/JNK (Thr183/Tyr185) protein expression in LPS-stimulated BV2 microglia by solvent fractions.** Cells were pre-treated with aspirin (1 mM), ethyl acetate fraction (EAF) (125 and 250 µg/ml), and n-butanol fraction (nBF) (125 and 250 µg/ml) for 24 hours, and then stimulated with one µg/ml of LPS for 24 hours. Protein extracts were collected at 24 hours post-LPS stimulation. Cells in complete DMEM medium served as a negative control. Cells stimulated with LPS without treatment served as a LPS control. Phosphorylated SAPK/JNK protein level was measured by determination of the absorbance at 450 nm. Data were expressed as means ± SD from three independent experiments carried out in triplicates. Means with different alphabets represent significant difference ($p < 0.05$).

The LPS significantly ($p < 0.05$) increased the protein expression of phosphorylated AKT (Thr308) (Figure 4.16). The phosphorylation of LPS-stimulated p-AKT was significantly ($p < 0.05$) decreased in aspirin-, ethyl acetate and n-butanol fractions-treated cells. Treatment of BV2 microglia with LPS also significantly ($p < 0.05$) increased the expression of phosphorylated CREB (Ser133) (Figure 4.17). Pre-treatment with aspirin, ethyl acetate and n-butanol fractions (125 and 250 µg/ml) significantly ($p < 0.05$) decreased the LPS-stimulated protein expressions of p-CREB. The present findings demonstrate that the aspirin, ethyl acetate and n-butanol fractions inhibited the activation of the neuroinflammatory signaling pathways, the ERK1/2, p-38 MAPK, SAPK/JNK and AKT, and the transcription factor, CREB in LPS-stimulated BV2 microglia.

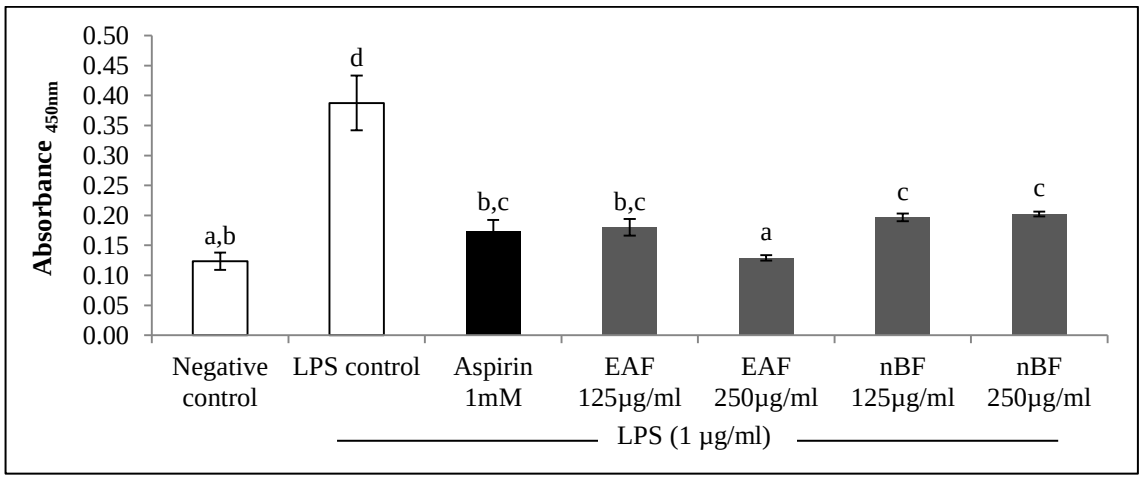



**Figure 4.16: The inhibition of p-AKT (Thr308) protein expression in LPS-stimulated BV2 microglia by solvent fractions.** Cells were pre-treated with aspirin (1 mM), ethyl acetate fraction (EAF) (125 and 250 µg/ml), and n-butanol fraction (nBF) (125 and 250 µg/ml) for 24 hours, and then stimulated with one µg/ml of LPS for 24 hours. Protein extracts were collected at 24 hours post-LPS stimulation. Cells in complete DMEM medium served as a negative control. Cells stimulated with LPS without treatment served as a LPS control. Phosphorylated AKT protein level was measured by determination of the absorbance at 450 nm. Data were expressed as means ± SD from three independent experiments carried out in triplicates. Means with different alphabets represent significant difference ($p < 0.05$).

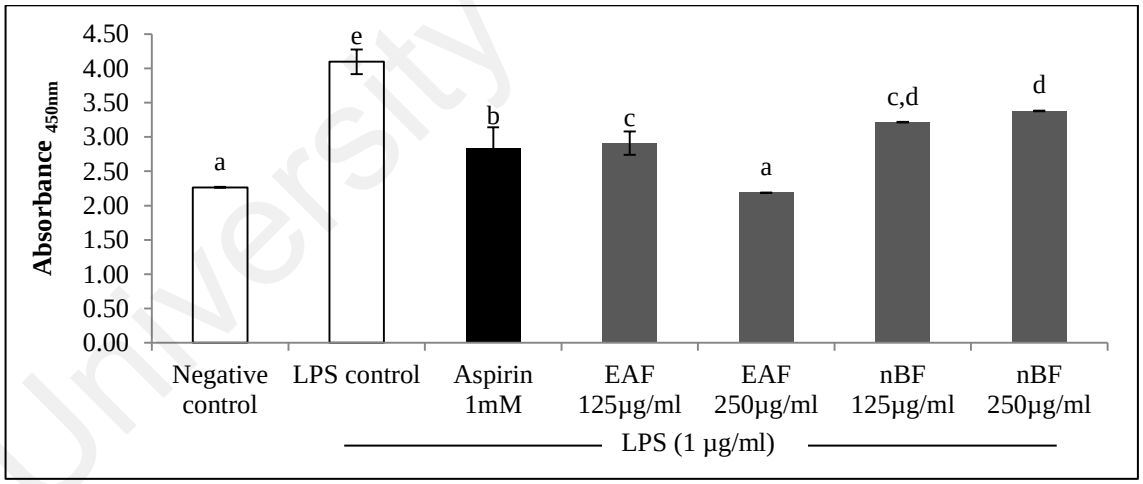



**Figure 4.17: The inhibition of p-CREB (Ser133) protein expression in LPS-stimulated BV2 microglia by solvent fractions.** Cells were pre-treated with aspirin (1 mM), ethyl acetate fraction (EAF) (125 and 250 µg/ml), and n-butanol fraction (nBF) (125 and 250 µg/ml) for 24 hours, and then stimulated with one µg/ml of LPS for 24 hours. Protein extracts were collected at 24 hours post-LPS stimulation. Cells in complete DMEM medium served as a negative control. Cells stimulated with LPS without treatment served as a LPS control. Phosphorylated CREB protein level was measured by determination of the absorbance at 450 nm. Data were expressed as means ± SD from three independent experiments carried out in triplicates. Means with different alphabets represent significant difference ($p < 0.05$).

### 4.3.12 The inhibition of cFOS and cJUN mRNA expressions in LPS-stimulated BV2 microglia by solvent fractions

Phosphorylation of ERK1/2, p-38 MAPK, SAPK/JNK signaling pathways will lead to the activation of the transcription factors, CREB and AP-1 (cFOS and cJUN), which subsequently upregulate the expressions of pro-inflammatory genes. The ability of solvent fractions to inhibit the transcription of cFOS and cJUN was investigated. Pre-treatment with ethyl acetate and n-butanol fractions significantly ($p < 0.05$) downregulated the cFOS (Figure 4.18a) and cJUN (Figure 4.18b) mRNA expressions in LPS-stimulated BV2 microglia. Treatment with the LPS significantly ($p < 0.05$) upregulated the cFOS and cJUN mRNA expressions in BV2 microglia by approximately 1- and 3-folds, compared to the negative control. Pre-treatment with aspirin (1 mM), ethyl acetate and n-butanol fractions (125 and 250 µg/ml) significantly ($p < 0.05$) downregulated the cFOS (Figure 4.18a) and cJUN (Figure 4.18b) mRNA expression in LPS-stimulated cells. Interestingly, 125 µg/ml of ethyl acetate fraction significantly ($p < 0.05$) downregulated the LPS-stimulated cFOS and cJUN mRNA expression lower than that of the respective negative control. The present data demonstrate that the aspirin, ethyl acetate and n-butanol fractions inhibited the activation of the neuroinflammatory signaling pathways, the ERK1/2, p-38 MAPK, and SAPK/JNK, and their transcription factors, CREB (Figure 4.17) and AP-1 (cFOS and cJUN) in LPS-stimulated BV2 microglia.

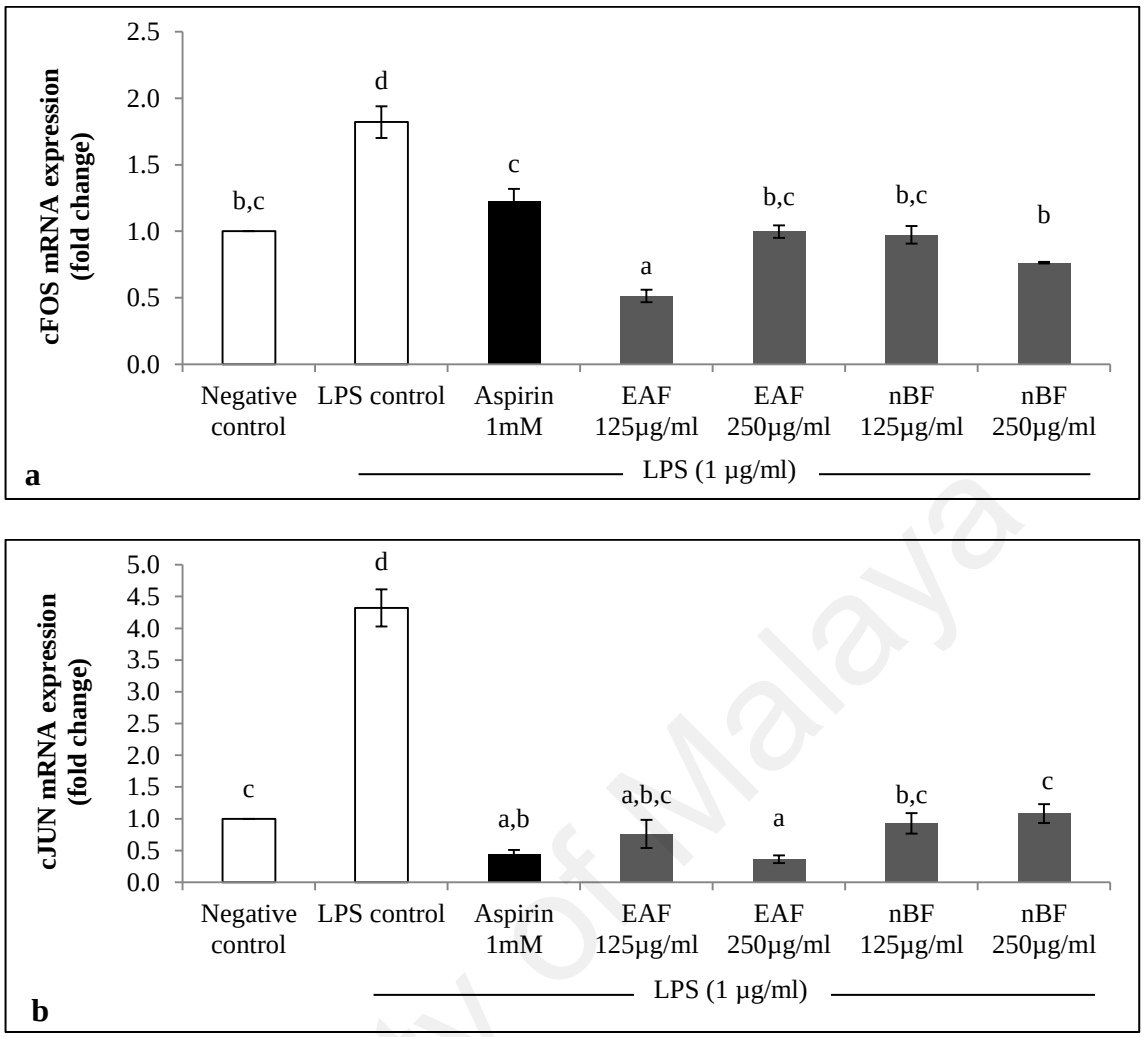



**Figure 4.18: The inhibition of cFOS and cJUN mRNA expressions in LPS-stimulated BV2 microglia by solvent fractions.** Cells were pre-treated with aspirin (1 mM), ethyl acetate fraction (EAF) (125 and 250 µg/ml), and n-butanol fraction (nBF) (125 and 250 µg/ml) for 24 hours, and then stimulated with one µg/ml of LPS for 24 hours. RNA extracts for the detection of mRNA expression of (a) cFOS and (b) cJUN were collected at 24 hours post-LPS stimulation. Cells in complete DMEM medium served as a negative control. Cells stimulated with LPS without treatment served as a LPS control. Data were expressed as means ± SD from three independent experiments carried out in triplicates. Means with different alphabets represent significant difference ($p < 0.05$).

## 4.4 DISCUSSION

The ethyl acetate, n-butanol and aqueous fractions of hot aqueous extract of *L. rhinocerotis* sclerotium were not cytotoxic to BV2 microglia. The data show that pre-treatment of BV2 microglia with ethyl acetate, and n-butanol fractions for 24 hours prior to LPS stimulation successfully mitigated the neuroinflammatory event in LPS-stimulated BV2 microglia by regulating the neuroinflammatory signaling pathways.

The hot aqueous extract of *L. rhinocerotis* sclerotium exhibited significant ($p < 0.05$) lower cytotoxic effect towards BV2 microglia when compared to the ethanol extract and crude polysaccharides (Chapter III, pp. 66). Similar to the hot aqueous extract, the aqueous fraction exhibited significant ($p < 0.05$) lower cytotoxic effect towards BV2 microglia, compared to ethyl acetate and n-butanol fractions. The $IC_{50}$ value of the cytotoxic effect of hot aqueous extract, ethyl acetate, and n-butanol fractions were approximately 40-, 4-, and 28-fold higher than the optimum concentration that inhibited NO, respectively.

Treatment of LPS will activate BV2 microglial cells, leading to *in vitro* inflammatory responses mediated by the production of various pro-inflammatory mediators and cytokines including NO, $PGE_2$, TNF-α, IL-1β, and IL-6 that are neurotoxic to the neuronal cells,. As discussed, NO is an important pro-inflammatory mediator (Vane et al., 1994). Overproduction of NO by increased expression of iNOS is closely related to neuroinflammatory responses and neurodegenerative diseases (Brown, 2010, 2007; Salerno, Sorrenti, Di Giacomo, Romeo, & Siracusa, 2002). Nω-Nitro-L-arginine methyl ester (L-NAME) is a specific inhibitor of iNOS enzyme. L-NAME has been widely used as positive control which inhibit NO production in both *in vivo* Singh et al., 2005; (Lautenschlager et al., 2000) and *in vitro* (Chow et al., 2012; Tan, Ramasamy, Abdullah, & Vidyadaran, 2011) studies. Animals pre-treated with L-NAME exhibited complete protection against behavioral deficits induced by intra-striatal LPS

injection (Singh et al., 2005). Administration of L-NAME significantly inhibited the loss of dopaminergic neurons stimulated by LPS (Arimoto & Bing, 2003). L-NAME at 250 μM was reported to have optimal reduction of NO production in interferon-gamma (IFN-γ)/LPS- (Chow et al., 2012) or LPS alone- (Tan, Ramasamy, et al., 2011) stimulated BV2 microglia. Another positive control used in the present study, the acetylsalicylic acid also known as aspirin, is a non-steroidal anti-inflammatory drugs (NSAID) that widely used as a positive or drug control in many inflammatory-related studies *in vivo* (Tanne et al., 2008; Paul-Clark, Van Cao, Moradi-Bidhendi, Cooper, & Gilroy, 2004) and *in vitro* (Xu et al., 2015; Wang, Wu, et al., 2011).

In the present study, LPS alone significantly ($p < 0.05$) stimulated the NO production in BV2 microglia. The hot aqueous extract, ethyl acetate, and n-butanol fractions (but not aqueous fraction) significantly ($p < 0.05$) inhibited the production of NO in LPS-stimulated BV2 microglia, without cytotoxicity. L-NAME at 250 μM and aspirin at 1 mM significantly ($p < 0.05$) inhibited the production of NO in LPS-stimulated BV2 microglia by approximately 64% and 86%, respectively. It is noteworthy that the hot aqueous extract (250 and 500 μg/ml), ethyl acetate (125 μg/ml) and n-butanol (125 μg/ml) fractions inhibited the LPS-stimulated NO production that were comparable to the L-NAME. Moreover, 250 μg/ml of ethyl acetate and n-butanol fractions inhibited NO productions that were comparable to the aspirin (Figure 4.3e). Furthermore, ethyl acetate and n-butanol fractions suppressed iNOS expressions at protein and mRNA levels that were comparable to the L-NAME and aspirin (Figure 4.4). These findings suggest that the significant reduction in LPS-stimulated NO production is correlated with the inhibition of iNOS expression. Based on the present findings, ethyl acetate and n-butanol fractions may mimic the activity of L-NAME and aspirin as iNOS inhibitors.

Studies showed that inflammation-associated enzymes, iNOS and COX-2 induce neurodegeneration in CNS and brain (Teismann et al., 2003; Hunot, Hartmann, & Hirsch, 2001). It has been demonstrated that iNOS and COX-2 were identified post-mortem in Alzheimer's and Parkinson's disease brains (Collins, Toulouse, Connor, & Nolan, 2012; Zilka et al., 2012). Cyclooxygenase is a key enzyme in the conversion of arachidonic acid to prostaglandins, which involved in neuroinflammation (Vlad, Miller, Kowall, & Felson, 2008; Hernan, Logroscino, & Garcia Rodriguez, 2006). It is mainly induced in response to LPS (Farooqui, Horrocks, & Farooqui, 2007). Studies showed that COX-2 is markedly upregulated and $PGE_2$ production is elevated in rodent brain microglia after LPS treatment (Minghetti, Polazzi, Nicolini, Greco, & Levi, 1998; Bauer et al., 1997). Aspirin is a common drug prescribed as an anti-inflammatory medication for number of inflammatory conditions (acute and chronic), including fever, pain and rheumatism in human (Eccles, 1995; Beaver, 1988). It has been demonstrated that aspirin exert it anti-inflammatory effects through inhibition of COX via NFκB signaling pathway (Sairam, Saravanan, Banerjee, Mohanakumar, 2003; Vane, 1971). However, due to its adverse effects reported, the search for COX-2 inhibitors from natural sources has become focus in the discovery and development of anti-inflammatory agents (Surh et al., 2011; Kang et al., 2004). Several bioactive components from natural sources have been reported possess COX-2 inhibitory activity that may act as potential preventive and therapeutic agent for inflammatory-related diseases. Reports documented that dietary polyphenols such as curcumin from turmeric (Kang et al., 2004; Kim, Park, Joe & Jou, 2003) and blueberry polyphenols (Lau, Joseph, McDonald, & Kalt, 2009) may act as COX inhibitors, which possess *in vitro* anti-neuroinflammatory effects by inhibiting the COX expression and prostaglandin synthesis in LPS-stimulated BV2 microglia. In the present study, ethyl acetate and n-butanol fractions (125 and 250 μg/ml) markedly decreased the production of $PGE_2$, and inhibited the protein and mRNA

expressions of COX-2 in LPS-stimulated BV2 microglia (Figure 4.5). Moreover, the inhibitory effects of ethyl acetate and n-butanol fractions on $PGE_2$ production and COX-2 expressions were comparable to that of aspirin (1 mM). These data suggest that the ethyl acetate and n-butanol fractions may possess aspirin-like anti-neuroinflammatory effects, potentially act as natural COX-2 inhibitor.

Prolonged activation of microglial cells leads to elevated production of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6, and subsequently leads to neuronal dysfunction and loss (Amor, Puentes, Baker, & van der Valk, 2010; Rojo, Fernández, Maccioni, Jimenez, & Maccioni, 2008). Pro-inflammatory cytokines are stimulators and/or co-stimulators of iNOS and COX-2 gene expression and they play major roles in various neuroinflammatory and neurodegenerative diseases (Rojo et al., 2008; Hofmann et al., 2007). Therefore, inhibition of pro-inflammatory cytokines may also beneficial to the prevention and treatment of neuroinflammatory and neurodegenerative diseases. Park et al. (2012) suggested that diallyl disulfide (DADS), a lipid-soluble compound of *Allium sativum* (garlic) is a potential novel therapeutic candidate for the treatment of various neurodegenerative diseases. Pre-treatment with DADS significantly inhibited the production of pro-inflammatory cytokines including IL-1β and TNF-α in LPS-stimulated BV2 microglia by suppressing the mRNA expressions of these proteins (Park et al., 2012). Curcumin promotes anti-neuroinflammatory effects by suppressing the production and transcription of IL-1β, IL-6, and TNF-α in LPS-stimulated BV2 microglia (Jin, Lee, Park, Choi, & Kim, 2007). In the present study, ethyl acetate and n-butanol fractions (125 and 250 μg/ml) successfully decreased the production of TNF-α and IL-1β (Figure 4.8). Furthermore, the ethyl acetate and n-butanol fractions (125 and 250 μg/ml) downregulated the mRNA expressions of TNF-α, IL-1β and IL-6 in LPS-stimulated BV2 microglia that were comparable to the aspirin (Figure 4.9). These findings suggest that ethyl acetate and n-

butanol fractions inhibited the production of pro-inflammatory cytokines by regulating the gene expressions at transcriptional level, which is involved in the microglia-mediated neuroinflammatory process.

Several reports documented that extracts or components from medicinal mushrooms including *Taiwanofungus camphoratus* (Liu et al., 2007), *Cordyceps militaris* (Jeong et al., 2010), and *Ganoderma lucidum* (Yoon et al., 2013; Zhang et al., 2011) exhibited anti-neuroinflammatory activity *in vitro*, by inhibiting the production of pro-inflammatory mediators and cytokines in activated microglia. Liu et al. (2007) documented that the methanol extract of *T. camphoratus* basidiocarps, a well-known valuable medicinal mushroom in Taiwan, possess significant anti-neuroinflammatory effect by inhibiting the iNOS, COX-2, and TNF-α expression in LPS/IFN γ-activated microglia. Cordycepin isolated from *C. militaris* significantly inhibited the excessive production of NO, $PGE_2$, and pro-inflammatory cytokines (TNF-α and IL-1β) in LPS-stimulated BV2 microglia (Jeong et al., 2010). Methanol extract of *G. lucidum* inhibited the production of pro-inflammatory mediators and cytokines, including NO, TNF-α, IL-1β and downregulated the mRNA expressions of TNF-α and IL-1β. The present study revealed the anti-neuroinflammatory effect of ethyl acetate and n-butanol fractions of *L. rhinocerotis* sclerotium by inhibiting the production of pro-inflammatory mediators and cytokines, including NO, $PGE_2$, TNF-α and IL-1β, and downregulated the protein and/or mRNA expressions of iNOS, COX-2, TNF-α, IL-1β and IL-6.

Toll-like receptor 4 (TLR4) is a major pattern-recognition receptor (PRR) expressed by microglial cells (Okun et al., 2009). The TLR4 activation is responses to the binding with LPS, and initiates the signal transduction pathways, MAPKs (ERK1/2, p-38 MAPK, SAPK/JNK), AKT and NFκB cascades that lead to diverse transcriptional responses that responsible for neuroinflammation in microglial cells (Verstak et al., 2009; Block, Zecca, & Hong, 2007). Thus, inhibiting the activation of microglia

mediated by TLR4 in neuroinflammatory responses deserve a potentially neurodegeneration treatment strategy. Studies showed that the expression of TLR4 stimulated by LPS was time-dependent and significantly increased after treatment of LPS for 24 hours in BV2 microglia (Yoon et al., 2013; Park, Sapkota, Kim, Kim, & Kim, 2011). In agreement with these studies, in the present study, the TLR4 mRNA expression was significantly ($p < 0.05$) upregulated by approximately 4-fold after 24 hours of LPS treatment, compared to the negative control (cells only). Pre-treatment with aspirin and ethyl acetate fraction (125 and 250 μg/ml) significantly ($p < 0.05$) downregulated the TLR4 mRNA expression in LPS-stimulated BV2 microglia (Figure 4.10). It has been reported that ethanol extract of *G. lucidum* significantly inhibited the excessive production of NO, $PGE_2$, IL-1β and TNF-α through the inhibition of TLR4 expression and the suppression of NFκB translocation and transcriptional activity in LPS-stimulated BV2 microglia (Yoon et al., 2013). Recent report by Wang et al. (2014) showed that pseudoginsenoside-F11, ginsenoside isolated from leaves of *Panax pseudoginseng* (Himalayan ginseng) exerts anti-neuroinflammatory effect on LPS-stimulated microglia by inhibiting TLR4-mediated NFkB, AKT and MAPKs signaling pathways.

Nuclear factor-kappa B signaling pathway has been implicated in the pathogenesis of neuroinflammation (Kaminska, Mota, & Pizzi, 2015; Karin & Delhase, 2000). It has been demonstrated that LPS increased the activation of NFκB subunit, through phosphorylation, ubiquitination, degradation and translocation IκB-α, NFκB p50 and p65 subunits (Kaminska et al., 2015; Karin & Delhase, 2000). Translocation of NFκB p65 subunit will regulates the transcription factor NFκB, leading to the transcription of pro-inflammatory genes, such as inducible enzymes, iNOS, COX-2, and pro-inflammatory cytokines, including TNF-α, IL-1β and IL-6, that are involve in the regulation of neuroinflammation responses (Kaminska et al., 2015; Surh et al., 2001). In

the present findings, LPS significantly ($p < 0.05$) increased the phosphorylation of p65 subunit of NFκB (Ser536) and IκB-α (Ser32) at endogenous level and upregulated the nuclear translocation and DNA binding of the p65 and p50 subunit of NFκB in BV2 microglia. Pre-treatment with aspirin (1 mM), ethyl acetate and n-butanol fractions (125 and 250 μg/ml) successfully attenuated the LPS-stimulated NFκB activity by inhibiting the phosphorylation of NFκB p65 and IκB-α (Figure 4.11), and downregulated the transcription of NFκB p65 and NFκB p50 mRNA expressions (Figure 4.12) in BV2 microglia. These data suggest that the inhibitory effects of ethyl acetate and n-butanol fractions of *L. rhinocerotis* sclerotium on the production and expressions of pro-inflammatory mediators and cytokines may be correlated with the inhibition of NFκB expression in LPS-stimulated BV2 microglia.

The phosphorylation of PI3K/AKT signaling pathway is directly involved in the activation of NFκB through IκB-α degradation and the regulation of the expression of pro-inflammatory genes in LPS-stimulated BV2 microglia (Lee et al., 2006; Jang et al., 2005). Recent studies have shown the potential of natural compounds such as catechin (Kamarudin, Kadir, & Raflee, 2013), mesuagenin C (Kamarudin, Kadir, Chan, & Awang, 2013), ginsenoside Rg5 (Lee, Park, et al., 2013) and pseudoginsenoside-F11 (Wang et al., 2014) mitigated LPS-stimulated neuroinflammation in microglia via inhibition of AKT signaling pathway. In the present study, the LPS significantly ($p < 0.05$) increased phosphorylation of AKT (Thr308). The increased phosphorylation of AKT by LPS was inhibited by pre-treatment of aspirin, ethyl acetate and n-butanol fractions (125 and 250 μg/ml) (Figure 4.16). The present results reveal that the inhibition of pro-inflammatory mediators and cytokines expressions by ethyl acetate and n-butanol fractions in LPS-stimulated BV2 microglia may be mediated through downregulation of AKT phosphorylation.

Beside TLR4/IκB-α/NFκB and TLR4/PI3K/AKT/NFκB pathways, accumulating evidence showed that MAPKs (ERK1/2, p38 MAPK, SAPK/JNK) signaling pathways also play important roles in the expressions of pro-inflammatory genes in activated microglia (Oh et al., 2009; Bhat, Zhang, Lee, & Hogan, 1998). Several signaling molecules of MAPKs are phosphorylated in response to the exposure of LPS in BV2 microglia, including ERK1/2 (p44/p42) (Thr202/Tyr204), p38 MAPK (Thr180/Tyr182) and SAPK/JNK (Thr183/Tyr185), leading to the activation of inflammation-associated transcription factors, AP-1 (cFOS and cJUN) (Jeong, Hyun, Kim Van Le, Kim, & Kim, 2013; Park et al., 2012) and CREB (Brautigam, Frasier, Nikodemova, & Watters, 2005; Ajmone-Cat, De Simone, Nicolini, & Minghetti, 2003). The present results demonstrated that the ethyl acetate and n-butanol fractions (125 and 250 μg/ml) inhibited the phosphorylation of ERK1/2 (Figure 4.13), p38 MAPK (Figure 4.14) and SAPK/JNK (Figure 4.15), and concomitantly inhibited the mRNA expression of CREB (Figure 4.17) and AP-1 (cFOS and cJUN) (Figure 4.18) in LPS-stimulated BV2 microglia. These data reveal that the MAPKs (ERK1/2, p38 MAPK, and SAPK/JNK) signaling pathways and the transcription factors, AP-1 and CREB are involved in the anti-neuroinflammatory activity of ethyl acetate and n-butanol fractions.

Numerous studies have demonstrated that the anti-neuroinflammatory effects of natural substances were mediated through downregulation of NFκB, AKT and MAPKs signaling pathways. A recent report by Kim et al. (2014) documented that the sargachromenol-rich ethanol extract of *Myagropsis myagroides* (brown algae) (MME) inhibited the mRNA and protein expressions of iNOS and COX-2, resulting in reduction of NO and $PGE_2$ production in LPS-stimulated BV2 microglia. The sargachromenol-rich MME inhibited the activation and translocation of NFκB by preventing IκB-α degradation and inhibited the phosphorylation of ERK and JNK (Kim et al., 2014). The study suggested that the anti-neuroinflammatory effect of sargachromenol-rich MME in

LPS-stimulated BV2 microglia is mainly regulated by the inhibition of IκB-α/NF-κB and ERK/JNK pathways (Kim et al., 2014). Ginsenoside Rg5 isolated from the rhizome of *Panax ginseng* suppressed NO and TNF-α production and inhibited the mRNA expressions of iNOS, TNF-α, IL-β and COX-2 through the inhibition of PI3K/AKT, ERK, p38 MAPK and JNK phosphorylation and the DNA binding activities of NFκB and AP-1 (cFOS and cJUN) in LPS-stimulated BV2 microglia (Lee, Park, Jung, Kim, & Kim, 2013). Isodojaponin D, a new diterpene isolated from *Isodon japonicus* significantly inhibited the pro-inflammatory mediators (iNOS, NO, COX-2, PGE2), and cytokines (TNF-α, IL-β, Il-6) in LPS-stimulated BV2 microglia by inhibiting the NFκB and MAPKs (ERK1/2, p38, and JNK) signaling pathways (Lim et al., 2010). Study by Oh et al. (2009) documented that oleic acid (monounsaturated omega-9 fatty acid) a major component of olive oil, which also found abundantly in mushrooms, inhibited the productions and/or expressions of pro-inflammatory mediators (NO, ROS, $PGE_2$, iNOS and COX-2) in LPS-stimulated BV2 microglia through the inhibition of AKT/IKK/NFκB, p38 MAPK and NFκB signaling pathways, but not ERK. Liu et al. (2007) reported that the methanol extract of *T. camphoratus* basidiocarps inhibited the iNOS, COX-2, and TNF-α expression in LPS/IFN γ-activated microglia through the phosphorylation of ERK, JNK and NFκB. Cordycepin isolated from *C. militaris* inhibited the production of pro-inflammatory mediators in LPS-stimulated BV2 microglia is associated with the suppression of NFκB translocation by blocking IκB-α degradation and inhibited the phosphorylation of AKT, ERK1/2, JNK, and p38 kinase (Jeong et al., 2010).

Based on the present findings, the ethyl acetate and n-butanol fractions mitigated neuroinflammation in LPS-stimulated BV2 microglia by inhibiting the NFκB, MAPKs and AKT signaling pathways (Figure 4.19). The ethyl acetate and n-butanol fractions inhibited the production and expressions of pro-inflammatory mediators (NO and $PGE_2$),

inducible enzymes (iNOS and COX-2) and cytokines (TNF-α, IL-1β and IL-6) in LPS-stimulated BV2 microglia by suppressing the TLR4-mediated signaling pathways, NFκB, MAPKs (ERK1/2, p38 MAPK, and JNK), and AKT. Furthermore, the transcription factors, NFκB, CREB, and AP-1 were inhibited by ethyl acetate and n-butanol fractions. Taken together, the present findings suggest that the ethyl acetate and n-butanol fractions of *L. rhinocerotis* may contain anti-neuroinflammatory components that mitigate neuroinflammation *in vitro*.

## 4.5 CONCLUSIONS

The ethyl acetate and n-butanol fractions possessed anti-neuroinflammatory effects by modulating microglia activation. Both ethyl acetate and n-butanol fractions inhibited LPS-stimulated production of pro-inflammatory mediators and cytokines, including NO, $PGE_2$, TNF-α and IL-1β and expressions of iNOS, COX-2, TNF-α, IL-1β and IL-6 in BV2 microglia. The anti-neuroinflammatory effect of ethyl acetate was mediated through downregulation of the TLR4 receptor in LPS-stimulated BV2 microglia. The ethyl acetate and n-butanol fractions inhibited the expressions of pro-inflammatory mediators and cytokines through the inhibition of MAPKs (ERK1/2, p-38 MAPK and SAPK/JNK), AKT and NFκB signaling pathways with the suppression of the transcription factors, CREB, AP-1 and NFκB. This may be the first report documenting the *in vitro* cytotoxic and anti-neuroinflammatory effects, and the underlying mechanisms of ethyl acetate and n-butanol fractions of hot aqueous extract of *L. rhinocerotis* sclerotium.

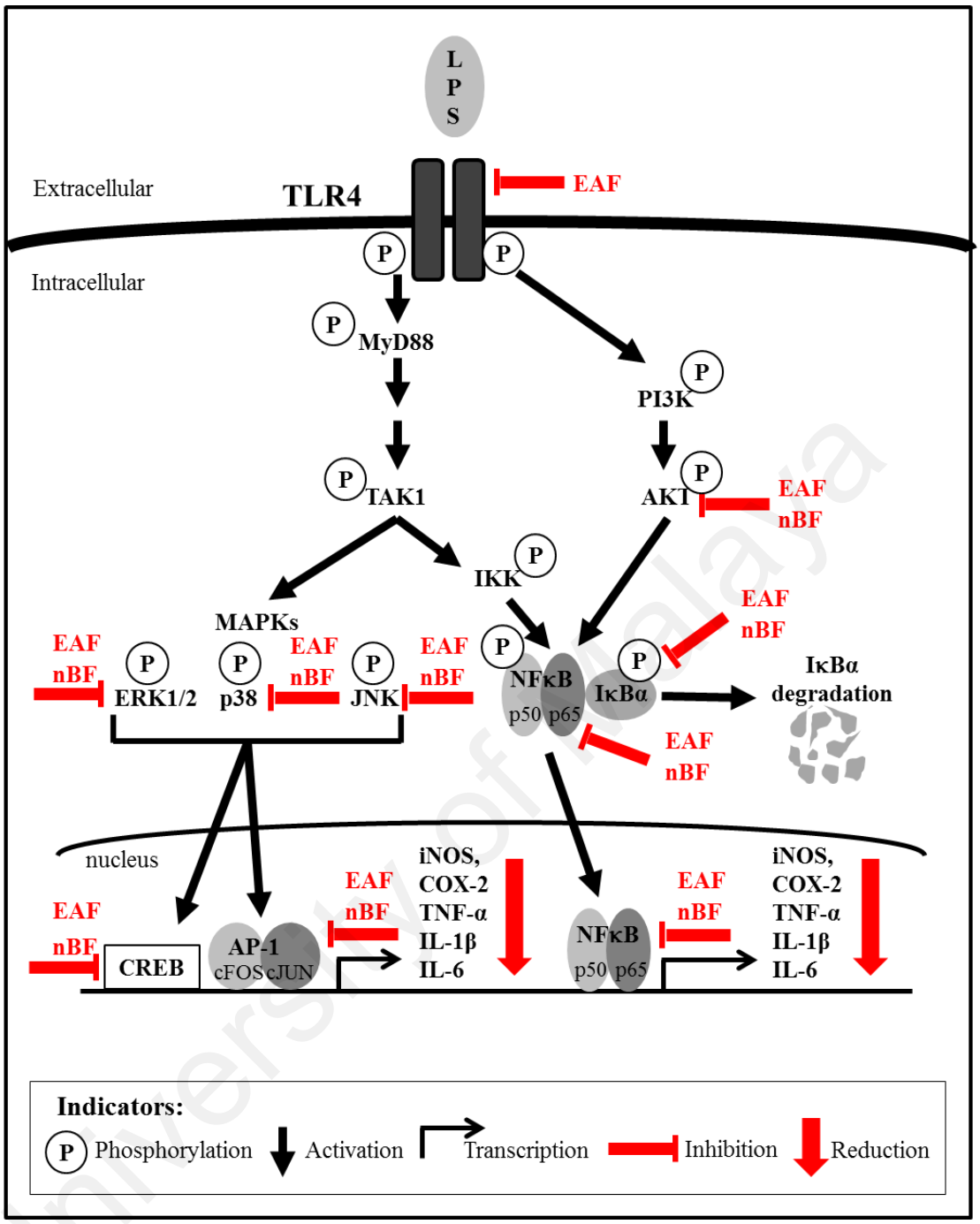



**Figure 4.19: Hypothetic mechanism of anti-neuroinflammatory activity of ethyl acetate and n-butanol fractions in LPS-stimulated signaling pathways.** Based on the present findings, the activation of toll-like receptor 4 (TLR4) by lipopolysaccharides (LPS) is inhibited by ethyl acetate fraction (EAF). The phosphorylation of TLR4-mediated signaling pathways, extracellular signal-regulated kinase 1/2 (ERK1/2), p38 MAPK, and Jun amino-terminal kinases (JNK), protein kinase B (AKT) and nuclear factor kappa B (NFκB) were inhibited by EAF and n-butanol fraction (nBF). The EAF and nBF inhibited the transcription of cyclic adenosine monophosphate (cAMP) response element binding protein (CREB), activator protein 1 (AP-1) and NFκB, and concomitantly inhibited the mRNA expressions of pro-inflammatory enzymes (iNOS and COX-2) and cytokines genes (TNF-α, IL-1β and IL-6).

# CHAPTER V: NEURITOGENIC STIMULATORY EFFECT OF *LIGNOSUS RHINOCEROTIS* SCLEROTIUM AND THE UNDERLYING MECHANISMS

## 5.1 INTRODUCTION

As discussed, neuritogenesis is crucial for the maintenance and regeneration of the neuronal communications network (Da Silva & Dotti, 2002; Spencer et al., 2002). It has become one of the focuses of study in the search for preventive and therapeutic agents for neurodegenerative diseases (Phan, David, Naidu et al., 2014; Williams & Dwyer, 2009). The neurotrophin, NGF play distinctive roles in promoting and maintaining the neuronal health (Thoenen, 1995; Kromer, 1987). It has been identified as a potential therapeutic agent for the treatment of neurodegenerative diseases (Koliatsos et al., 1991). However, due to its large molecular polypeptide structure, NGF is unstable and unable to cross the BBB (Granholm et al., 1998). Smaller molecules that mimic and/or enhance the NGF activities have become the core focus in the search for preventive and therapeutic agents for neurodegenerative diseases (Williams & Dwyer, 2009). Among the natural or dietary sources explored for NGF mimics, medicinal mushrooms have shown huge potential (Xu & Beelman, 2015; Phan, David, Naidu, et al., 2014).

Cultured PC-12 cell line is widely used as an *in vitro* model system to investigate the neuritogenic activity of NGF (Teng, Angelastro, Cunningham, & Greene, 2006; Tischler & Greene, 1975) and NGF mimics (Williams & Dwyer, 2009), and the NGF responsive signaling pathways (Vaudry, Stork, Lazarovici, & Eiden, 2002; Greene et al., 1984). To understand the thorough performance of the potential of NGF mimics, study of the intrinsic mechanisms of NGF mimics-stimulated neuritogenesis *in vitro* is

important. Treatment of NGF in PC-12 cells is associates with the expression of TrkA receptor (Kaplan & Stephens, 1994) and initiation of two predominant signaling pathways, the MAPK/MEK/ERK and PI3K/AKT/mTOR pathways, which eventually lead to neuritogenesis (Vaudry et al., 2002; Kaplan & Miller, 1997). Activation of these signaling pathways will further induced the phosphorylation of the transcription factor, CREB (Ginty, Bonni & Greenberg, 1994), and lead to the activation of numerous downstream genes including GAP-43 (Jap Tjoen San, Schmidt-Michels, Oestreicher, Schotman, & Gispen, 1992; Jap Tjoen San et al., 1991) and microtubules (Drubin, Kobayashi, Kellogg, & Kirschner, 1988; Greene et al., 1984) to regulate neuritogenesis in PC-12 cells.

In Chapter III, the hot aqueous extract of *L. rhinocerotis* sclerotium showed its potential as neuritogenic stimulant in PC-12 cells that comparable to the NGF. In the present chapter, three solvent fractions fractionated from hot aqueous extract of *L. rhinocerotis* sclerotium: ethyl acetate, n-butanol and aqueous fractions were used to further investigate the neuritogenic stimulatory effect of *L. rhinocerotis* sclerotium. The aims of the present chapter were to investigate (i) the cytotoxic and neuritogenic stimulatory effects of the solvent fractions compared to the crude hot aqueous extract, (ii) whether the hot aqueous extract and solvent fractions stimulate the biosynthesis of NGF, (iii) the involvement of TrkA receptor and NGF responsive signaling pathways, MEK/ERK1/2/CREB and PI3K/AKT/mTOR/CREB, and (iv) the involvement of downstream gene GAP-43, TUBA4A and TUBB1 that stimulate neuritogenesis in solvent fractions-stimulated neuritogenesis in PC-12 cells.

## 5.2 MATERIALS AND METHODS

### 5.2.1 General methodology

Preparation of the hot aqueous extract, ethyl acetate, n-butanol and aqueous fractions are described in Section 3.2.2.1 and 4.2.2. The methods of PC-12 cell culture, stimulation of neuritogenesis, and quantification of neurite bearing cells are described in Sections 3.2.3.2, 3.2.6.1 and 3.2.6.2. Cytotoxicity assay is described in Section 3.2.4. Statistical analysis is described in Section 3.2.7.

### 5.2.2 Immunocytofluorescence analysis of neurofilament

Immunocytofluorescence assay was carried out according to Schimmelpfeng, Weibezahn, & Dertinger (2004). PC-12 cells were seeded at a density of $5 \times 10^3$ cells per well in 12-well micro-chamber (ibidi, Martinsried, Germany). The cells were treated with freshly prepared NGF or mushroom hot aqueous extract or solvent fractions for 48 hours at 37 ± 2 °C in a 5% $CO_2$-humidified incubator. The cell culture supernatant was removed and the cells were washed twice with PBS. The cells were then fixed with 4% (w/v) paraformaldehyde (in PBS) for 20 minutes. After two washes with PBS, the cells were incubated with anti-neurofilament 200 antibody produced in rabbit (1:80 in 10% sheep serum blocking buffer; Sigma-Aldrich, USA) for one hour at room temperature, followed by fluorophore-conjugated secondary antibody, anti-rabbit IgG-fluorescein isothiocyanate (FITC) antibody produced in sheep (1:80 in 10% sheep serum blocking buffer; Sigma-Aldrich, USA) for two hours at room temperature in dark. After three washes with PBS, the cells were mounted with ProLong® gold antifade reagent with 4-6-Diamidino-2-phenylindole (DAPI) (Life Technologies Corporation). The slides were observed under fluorescence illumination using FITC and DAPI filters of Nikon Eclipse 80i fluorescent microscope and images were captured with Nikon's Imaging Software, NIS-Elements.

### 5.2.3 Assessment of extracellular concentration of NGF

Cells were plated at a density of 1 X $10^4$ cells per well in 96-well plates. The cells were treated with freshly prepared NGF or mushroom hot aqueous extract or solvent fractions in complete F-12 K medium for 48 hours. The cell culture supernatant was collected, centrifuged at 1500 × g for 15 minutes and maintained at 0 – 4 °C prior to assay. The samples were diluted with sample diluent at a ratio of 1:2 (v/v). The concentration of NGF in culture supernatant was measured by using ChemiKine™ nerve growth factor sandwich enzyme-linked immunosorbent assay (ELISA) kit (Chemicon® International Inc.) according to the manufacturer's protocol. A total of 100 µl of culture supernatant was added to each well of the ChemiKine NGF strip and incubated at 4 °C overnight. After washing with wash buffer, 100 µl of the diluted anti-mouse NGF monoclonal antibody was added to each well and incubated at room temperature for two hours on plate shaker. After washing steps, 100 µl of the diluted peroxidase conjugated donkey anti-mouse IgG polyclonal antibody was added to each well and incubated at room temperature for two hours on plate shaker. After washing steps, 100 µl of 3,3',5,5'-tetramethylbenzidine in a proprietary buffer with enhancer (TMB/E substrate) to each well and incubated at room temperature for 10 minutes. The reaction was stopped by adding 100 µl of stop solution (hydrogen chloride solution) to each well. The plate was read immediately at absorbance 450 nm with an ELISA microplate reader (Sunrise, Tecan). The extracellular concentration of NGF was calculated following deduction of blank, with reference to a standard curve generated by serially diluted NGF Standard (Mouse) (0 to 1000 pg/ml).

### 5.2.4 Assessment of the involvement of TrkA receptor and NGF responsive signaling pathways with specific inhibitors

The inhibitors for Trk receptor (K252a), MEK/ERK1/2 (U0126, PD98059), and PI3K (LY294002) (all Sigma-Aldrich, USA) were used. Stock solutions (10 mM) of the inhibitors were prepared in DMSO and stored at −20 °C in the dark. Final concentrations of 100 nM of K252a, 10 μM of U0126, 40 μM of PD98059 and 30 μM of LY294002 were freshly prepared by diluting in complete F-12 K medium before use. Cells were pre-incubated either with or without the inhibitor for one hour at 37 ± 2 °C in a 5% $CO_2$-humidified incubator, respectively before the treatment with 50 ng/ml of NGF or 10 μg/ml of ethyl acetate fraction or 10 μg/ml of n-butanol fraction. Cells were then incubated for 48 hours prior to scoring the neurite-bearing cells.

### 5.2.5 Protein expression analysis of NGF responsive signaling pathways

#### 5.2.5.1 Preparation of whole cell protein lysates

PC-12 cells were seeded at a density of $5 \times 10^5$ cells in 25 $cm^3$ flask and incubated overnight at 37 °C in a 5% $CO_2$-humidified incubator. Then, the cells were treated with freshly prepared NGF or solvent fractions in complete F-12 K medium for 48 hours. The extraction method of whole cell protein lysates for PathScan® sandwich ELISA kit (Cell Signaling Technology) is described in Section 4.2.5.1. The protein concentration was quantified by Bradford assay described in Section 4.2.5.2.

#### 5.2.5.2 Measurement of the expressions of neuritogenic signaling proteins

The protein expressions of phosphorylated mitogen-activated protein kinase kinase (p-MEK1/2), phosphorylated extracellular-signal regulated kinase1/2 (p-ERK1/2), phosphorylated protein kinase B (p-AKT), phosphorylated mammalian target of rapamycin (p-mTOR), and phosphorylated cAMP-response element binding protein (p-

CREB) in NGF- or solvent fractions-treated PC-12 cells were measured by using PathScan® sandwich ELISA kits (Cell Signaling Technology) according to the manufacturer's protocol described in Section 4.2.5.4.

#### 5.2.5.3 Measurement of growth associated protein 43 (GAP-43), tubulin alpha 4A (TUBA4A), and tubulin beta 1 (TUBB1)

The protein expression of GAP-43, TUBA4A and TUBB1 in NGF- or solvent fractions-treated PC-12 cells were measured by using sandwich ELISA kits (Cloud-Clone Corp., USA) according to the manufacturer's protocol. The cell lysates were incubated in microwells and incubated for two hours at 37 °C. Then, the liquid in each well was removed without washing. A total of 100 µl of Detection Reagent A working solution was added to each well and incubated for one hour at 37 °C. After washing with wash buffer, 100 µl of Detection Reagent B working solution was added to each well and incubated for 30 minutes at 37 °C. After washing steps, 90 µl of substrate solution was added to each well and incubated for 20 minutes at 37 °C. The reaction was stopped by adding 50 µl of stop solution to each well. The plate was read at 450 nm with an ELISA microplate reader (Sunrise, Tecan). The concentrations of GAP-43, TUBA4A and TUBB1 were determined from a standard curve plotted with known concentrations of the respective proteins (GAP-43, 0 to 20 ng/ml; TUBA4A, 0 to 40 ng/ml; TUBA4A, 0 to 80 ng/ml).

## 5.3 RESULTS

### 5.3.1 The cytotoxic effects of hot aqueous extract and solvent fractions of *L. rhinocerotis* sclerotium on PC-12 cells

The viability of PC-12 cells in complete F-12 K medium was considered as 100%. The viable rate of treated cells decreased in a concentration-dependent manner for hot

aqueous extract, ethyl acetate, n-butanol and aqueous fractions of *L. rhinocerotis* sclerotium (Figure 5.1), after 48 hours of incubation.

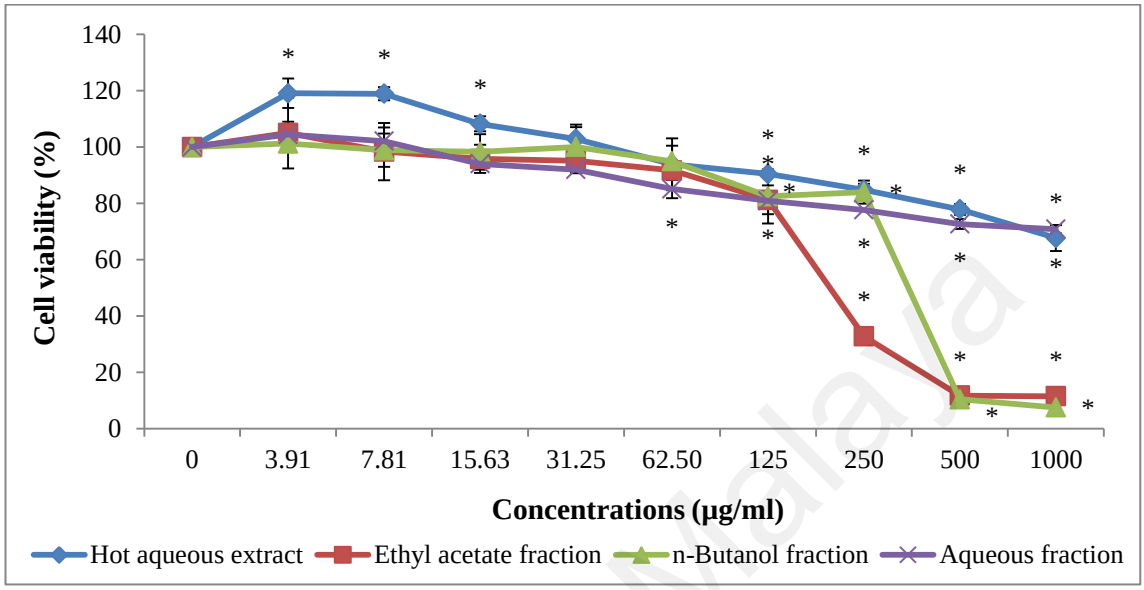



**Figure 5.1: The cytotoxic effects of hot aqueous extract and solvent fractions of *L. rhinocerotis* sclerotium on PC-12 cells.** Cells were incubated with hot aqueous extract, ethyl acetate, n-butanol or aqueous fractions at concentrations from 0 to 1000 µg/ml for 48 hours. The mean absorbance obtained using complete F-12 K medium with PC-12 cells was designated as 100% viable. Data were expressed as means ± SD from three independent experiments carried out in triplicates. * $p < 0.05$ compared to the respective 100% of viability.

The hot aqueous extract significantly ($p < 0.05$) increased the cell viability at low concentrations (3.91 to 15.63 µg/ml). The cytotoxic rate increased gradually as the concentrations of the hot aqueous extract and solvent fractions were increased. The percentage of viable cells in hot aqueous extract-, ethyl acetate and n-butanol fractions-treated cells were reduced significantly ($p < 0.05$) starting from 125 µg/ml, compared to 100% viability. Whereas, the viability of aqueous fraction-treated cells was reduced significantly ($p < 0.05$) starting from 62.50 µg/ml, compared to 100% viability. The percentage of viable cells in ethyl acetate and n-butanol fractions-treated cells decreased significantly ($p < 0.05$) to a cytotoxic level (below 50% viability) at 250 µg/ml (32.78 ± 2.31%) and 500 µg/ml (10.45 ± 0.83%), respectively. The required concentrations to

inhibit the viability of PC-12 cells by 50 % ($IC_{50}$) for hot aqueous extract, ethyl acetate, n-butanol and aqueous fractions after 48 hours of incubation are shown in Table 5.1. The aqueous fraction and hot aqueous extract had higher $IC_{50}$ value compared to the ethyl acetate and n-butanol fractions.

**Table 5.1: The 50% inhibitory concentration ($IC_{50}$) of hot aqueous extract and solvent fractions of *L. rhinocerotis* sclerotium on PC-12 cells viability after 48 hours of incubation.**

| Extract / fractions | $IC_{50}$ (mg/ml) on PC-12 cells |
|---|---|
| Hot aqueous extract | $3.52 \pm 0.33^{b}$ |
| Ethyl acetate fraction | $0.19 \pm 0.02^{a}$ |
| n-Butanol fraction | $0.28 \pm 0.01^{a}$ |
| Aqueous fraction | $6.12 \pm 1.99^{c}$ |

The data represent the mean ± SD from three independent experiments carried out in triplicates. Means with different alphabets represent significant difference ($p < 0.05$).

### 5.3.2 The neuritogenic effects of hot aqueous extract and solvent fractions of *L. rhinocerotis* sclerotium in PC-12 cells

Neurite extension of PC-12 cells was regarded as an index of neuritogenesis. The increase in the percentage of neurite bearing cells showed the ability of tested extract or fraction to induce neuritogenesis in PC-12 cells without the presence of NGF. The hot aqueous extract, ethyl acetate, n-butanol and aqueous fractions of *L. rhinocerotis* sclerotium stimulated neuritogenesis in PC-12 cells after 48 hours of incubation (Figure 5.2).

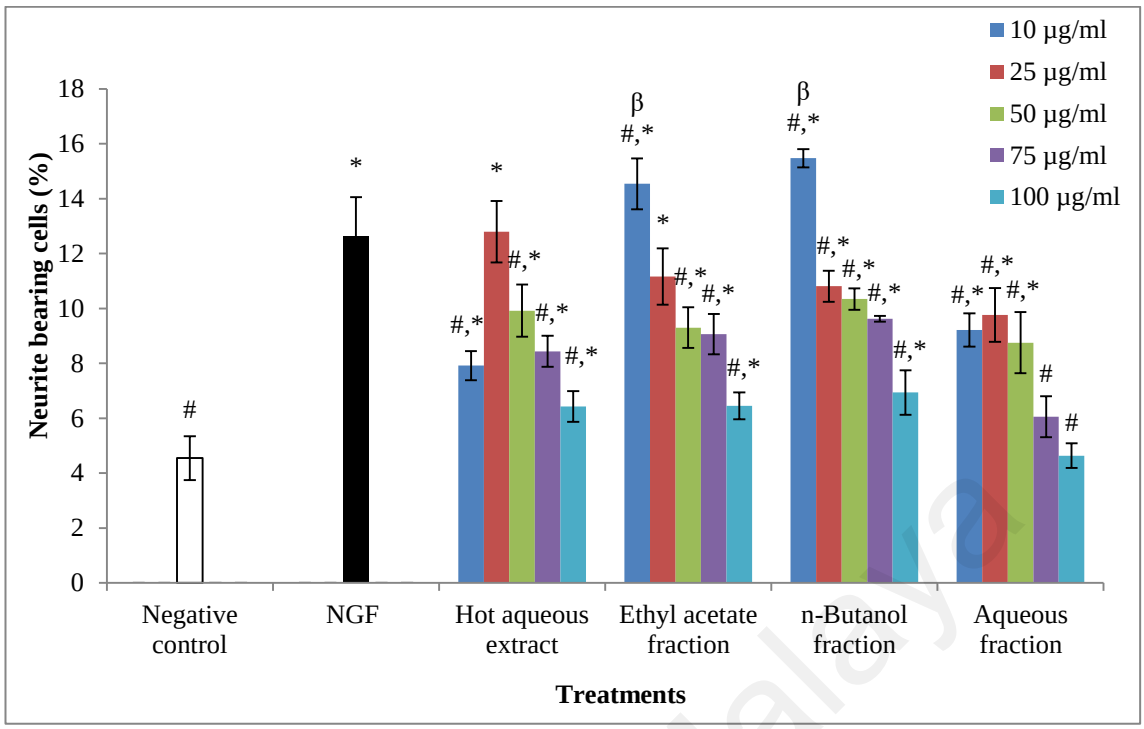



**Figure 5.2: The neuritogenic effects of hot aqueous extract and solvent fractions of *L. rhinocerotis* sclerotium in PC-12 cells.** Cells were incubated with NGF (50 ng/ml), hot aqueous extract, ethyl acetate, n-butanol or aqueous fractions at 10 µg/ml to 100 µg/ml for 48 hours. Cells in complete F-12 K medium served as a negative control. Cells treated with 50 ng/ml of NGF served as a positive control. Data were expressed as means ± SD from three independent experiments carried out in triplicates. * $p < 0.05$ compared to the negative control. $^{\#}$ $p < 0.05$ compared to NGF. $^{\beta}$ $p < 0.05$ higher value compared to NGF.

The percentage of neurite bearing cells at all tested concentrations of hot aqueous extract- and solvent fractions-treated cells increased significantly ($p < 0.05$) compared to the negative control (cells in complete F-12 K medium only), except for 75 µg/ml and 100 µg/ml of aqueous fraction. The percentage of neurite bearing cells decreased in concentration-dependent for hot aqueous extract and aqueous fraction at 25 to 100 µg/ml, and for ethyl acetate and n-butanol fractions at 10 to 100 µg/ml. There was an initial increase of the percentage of neurite bearing cells on increasing concentrations of hot aqueous extract and aqueous fraction, followed by a decrease at 25 µg/ml and above. The hot aqueous extract and aqueous fraction stimulated maximal neuritogenesis in PC-12 cells at 25 µg/ml, while ethyl acetate and n-butanol fractions

stimulated maximal neuritogenesis at a lower concentration, 10 μg/ml. The percentage of neurite bearing cells in ethyl acetate and n-butanol fractions-treated cells decreased in a concentration-dependent manner as the concentrations of the fractions were increased higher than 10 μg/ml,. The hot aqueous extract at 25 μg/ml stimulated neuritogenesis that was comparable to the positive control, 50 ng/ml of NGF. Interestingly, 10 μg/ml of ethyl acetate and n-butanol fractions significantly ($p < 0.05$) stimulated higher percentage of neurite bearing cells compared to NGF (12.61 ± 1.44%). The n-butanol fraction stimulated the highest percentage of neurite bearing cells (15.47 ± 0.33%) at 10 μg/ml, followed by the ethyl acetate fraction (14.54 ± 0.93%) at 10 μg/ml, hot aqueous extract (12.80 ± 1.12%) at 25 μg/ml, and aqueous fraction (9.77 ± 0.98%) at 25 μg/ml.

### 5.3.3 The morphology of PC-12 cells stained with anti-NF-200 antibody in hot aqueous extract- and solvent fractions-stimulated neuritogenesis

Immunocytofluorescence staining of neurofilaments confirmed the extension of neurites in PC-12 cells was stimulated by NGF, hot aqueous extract and solvent fractions of *L. rhinocerotis* sclerotium (Figure 5.3). PC-12 cells nuclei were stained blue by DAPI and neurofilaments were stained green by anti-NF-200 antibody labelled with FITC. In negative control, the cells are relatively small and rounded (Figure 5.3a). The cells were larger and elongated with significant neurite extensions that were double or more the length of cell body diameter in 50 ng/ml of NGF- (Figure 5.3b), 25 μg/ml of hot aqueous extract- (Figure 5.3c), 10 μg/ml of ethyl acetate fraction- (Figure 5.3d), 10 μg/ml of n-butanol fraction- (Figure 5.3e), and 25 μg/ml of aqueous fraction- (Figure 5.3f) treated cells.

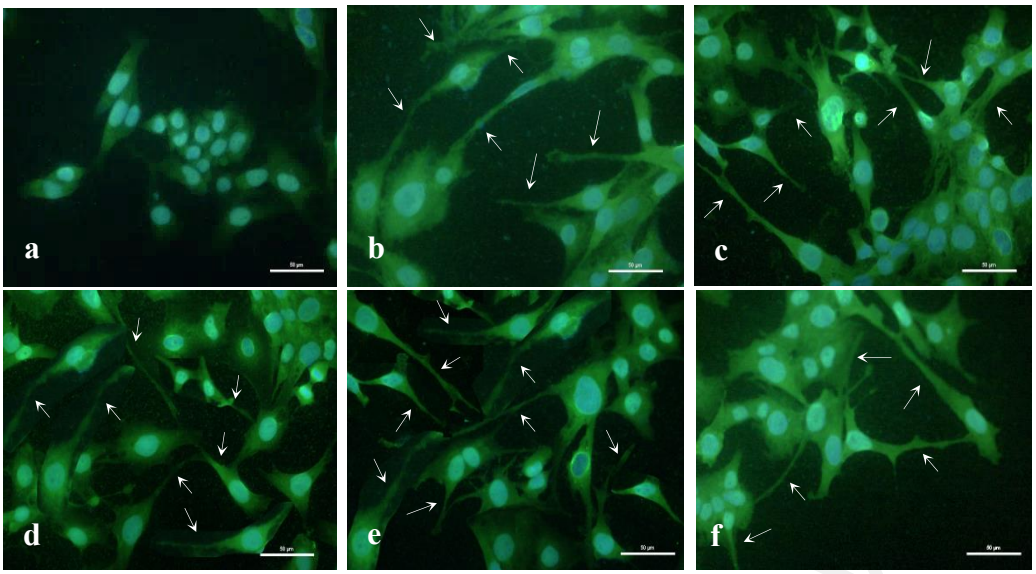



**Figure 5.3: Morphology of PC-12 cells stained with anti-NF-200 antibody.** Cells were incubated with (a) cells only, (b) NGF (50 ng/ml), (c) hot aqueous extract (25 µg/ml), (d) ethyl acetate fraction (10 µg/ml), (e) n-butanol fraction (10 µg/ml) and (f) aqueous fraction (25 µg/ml) for 48 hours. Cells in complete F-12 K medium served as a negative control. Cells treated with 50 ng/ml of NGF served as a positive control. Nuclei stained blue and neurofilaments stained green. Scale bars represent 50 µM. Arrows indicate neurite outgrowth (double or more the length of cell body diameter).

### 5.3.4 The concentrations of extracellular NGF in hot aqueous extract- and solvent fractions-treated cells

The increase in extracellular NGF in cell supernatant showed the ability of extract or fraction tested to induce NGF production by PC-12 cells. The concentration of extracellular NGF in PC-12 cells without treatment (negative control) was detected at 91.65 ± 3.54 pg/ml (Figure 5.4). The concentration of extracellular NGF in 50 ng/ml of NGF-treated cells (positive control) was significantly ($p < 0.05$) increased to 388.15 ± 15.26 pg/ml, compared to negative control. However, there was no significant difference ($p > 0.05$) in the concentration of extracellular NGF between hot aqueous extract (25 µg/ml), ethyl acetate fraction (10 µg/ml), n-butanol fraction (10 µg/ml), aqueous fraction (25 µg/ml) and the negative control.

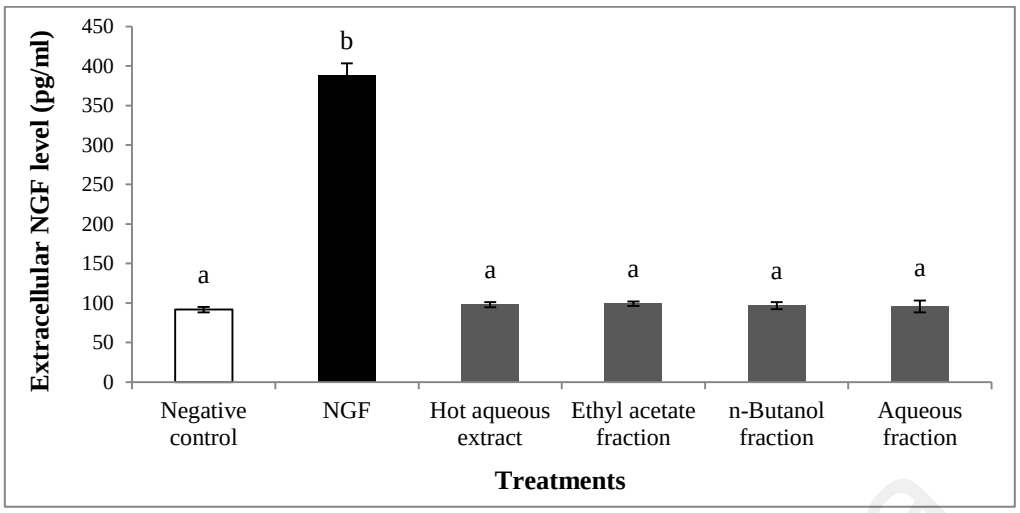



**Figure 5.4: Extracellular NGF concentrations in supernatants of NGF-, extract- or solvent fractions-treated PC-12 cells.** Cells were incubated with NGF (50 ng/ml), hot aqueous extract (25 μg/ml), ethyl acetate fraction (10 μg/ml), n-butanol fraction (10 μg/ml) or aqueous fraction (25 μg/ml) for 48 hours. Cells in complete F-12 K medium served as a negative control. Cells treated with 50 ng/ml of NGF served as a positive control. Data were expressed as means ± SD from three independent experiments carried out in triplicates. Means with different alphabets represent significant difference ($p < 0.05$).

### 5.3.5 The effects of specific inhibitors of TrkA receptor and NGF responsive signaling pathways in solvent fractions-stimulated neuritogenesis

The NGF high affinity receptor, TrkA and NGF responsive pathways, MEK/ERK1/2 and PI3K/AKT were selected as the targeted cascades for neuritogenic activity in PC-12 cells. The inhibitors of Trk (K252a), MEK (U0126 and PD98059) and PI3K (LY294002) significantly ($p < 0.05$) blocked the NGF-, ethyl acetate and n-butanol fractions-stimulated neuritogenesis (Figure 5.5). Inhibitors K252a, U0126, PD98059 and LY294002 decreased the percentage of neurite bearing cells by approximately 82.13%, 86.15%, 91.56% and 68.72% in NGF-treated cells, 83.46%, 88.98%, 88.47% and 80.43% in ethyl acetate fraction-treated cells, and 85.39%, 92.69%, 91.74 and 85.86% in n-butanol fraction-treated cells, respectively, compared to the respective control (without inhibitor). The significant ($p < 0.05$) reduction of neuritogenesis was also observed in the negative control with the addition of the inhibitors.

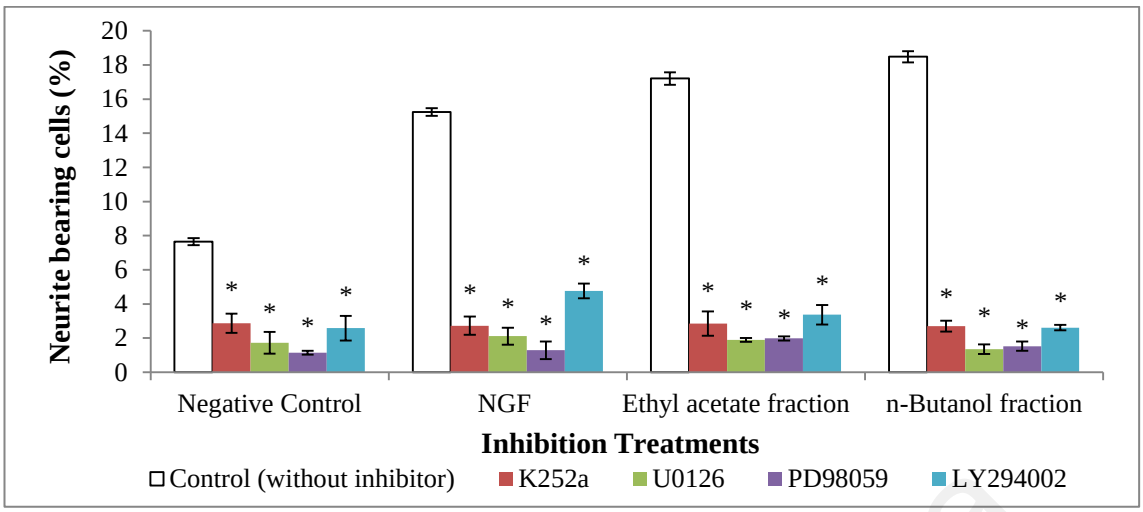



**Figure 5.5: The effects of specific inhibitors of TrkA receptor and NGF responsive signaling pathways in solvent fractions-stimulated neuritogenesis.** Cells were pre-treated with inhibitors K252a, U0126, PD98059 or LY294002 for one hour before the treatment with NGF (50 ng/ml), ethyl acetate fraction (10 µg/ml) or n-butanol fraction (10 µg/ml). Cells in complete F-12 K medium served as a negative control. Cells treated with 50 ng/ml of NGF served as a positive control. A control (without inhibitor) was used in each treatment group. Data were expressed as means ± SD from three independent experiments carried out in triplicates. * $p < 0.05$ compared to the respective controls.

### 5.3.6 The effects of solvent fractions on the protein expressions of p-MEK1/2, p-ERK1/2, p-AKT, p-mTOR and p-CREB

The involvement of TrkA receptor and NGF responsive signaling pathways (MEK/ERK1/2 and PI3K/AKT) in solvent fractions-stimulated neuritogenesis was revealed by using specific inhibitors. The ability of solvent fractions to activate the specific protein kinases involved in the NGF responsive signaling pathways was further investigated. The phosphorylation of the downstream target proteins was measured by ELISA. The magnitude of absorbance for the developed color is proportional to the quantity of target protein expressed by PC-12 cells. The expressions of phosphorylated proteins, p-MEK1/2 (Ser217/221) (Figure 5.6), and p-ERK1/2 (p44/p42) (Thr202/Tyr204) (Figure 5.7) in PC-12 cells treated with NGF (50 ng/ml), ethyl acetate (10 µg/ml) and n-butanol (10 µg/ml) fractions were significantly ($p < 0.05$) higher compared to the negative control, respectively. There were no significant difference ($p >$

0.05) in protein expression between NGF-, ethyl acetate and n-butanol fractions-treated cells for both p-MEK1/2 and p-ERK1/2. However, the phosphorylation of MEK1/2 and ERK1/2 in NGF-, ethyl acetate and n-butanol fractions-treated cells were significantly ($p < 0.05$) suppressed in the presence of MEK inhibitor, U0126.

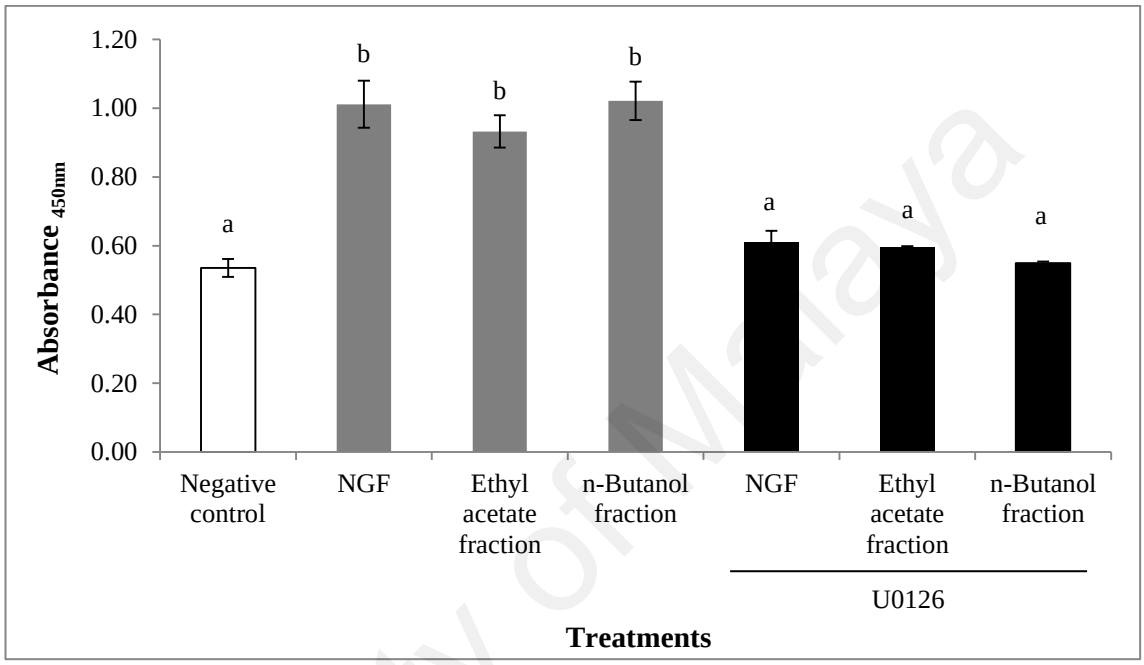



**Figure 5.6: Protein expression of phosphorylated MEK1/2 (Ser217/221) in NGF- and solvent fractions-treated PC-12 cells.** Cells were incubated with NGF (50 ng/ml), ethyl acetate fraction (10 µg/ml) or n-butanol fraction (10 µg/ml) for 48 hours. For inhibitor treatment, cells were pre-treated with U0126 (MEK inhibitor) one hour prior to the treatment of NGF or solvent fractions. Cells in complete F-12 K medium served as a negative control. Phosphorylated MEK1/2 protein level was measured by determination of the absorbance at 450 nm. Data were expressed as means ± SD from three independent experiments carried out in triplicates. Means with different alphabets represent significant difference ($p < 0.05$).

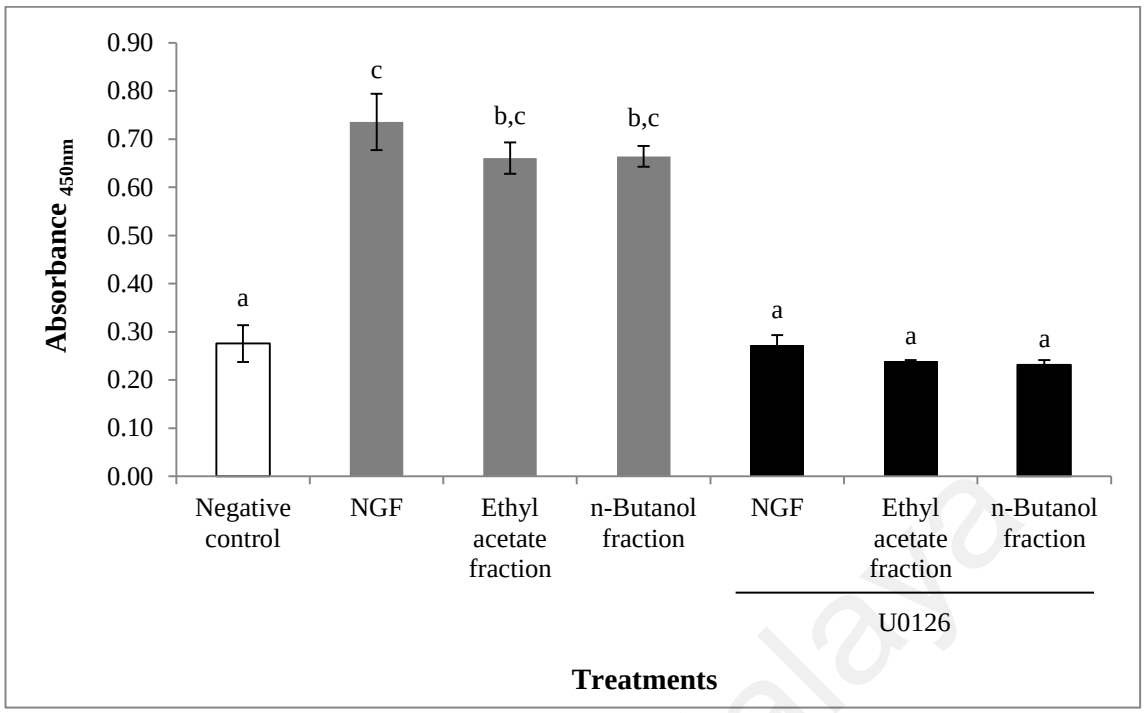



**Figure 5.7: Protein expression of phosphorylated ERK1/2 (p44/42) (Thr202/Tyr204) in NGF- and solvent fractions-treated PC-12 cells.** Cells were incubated with NGF (50 ng/ml), ethyl acetate fraction (10 µg/ml) or n-butanol fraction (10 µg/ml) for 48 hours. For inhibitor treatment, cells were pre-treated with U0126 (MEK inhibitor) one hour prior to the treatment of NGF or solvent fractions. Cells in complete F-12 K medium served as a negative control. Phosphorylated ERK1/2 protein level was measured by determination of the absorbance at 450 nm. Data were expressed as means ± SD from three independent experiments carried out in triplicates. Means with different alphabets represent significant difference ($p < 0.05$).

The NGF, ethyl acetate and n-butanol fractions also triggered significantly ($p < 0.05$) higher p-AKT (Thr308) protein expression when compared to the negative control (Figure 5.8). However, the phosphorylation of AKT in NGF-, ethyl acetate and n-butanol fractions-treated cells was significantly ($p < 0.05$) suppressed in the presence of PI3K inhibitor, LY294002.

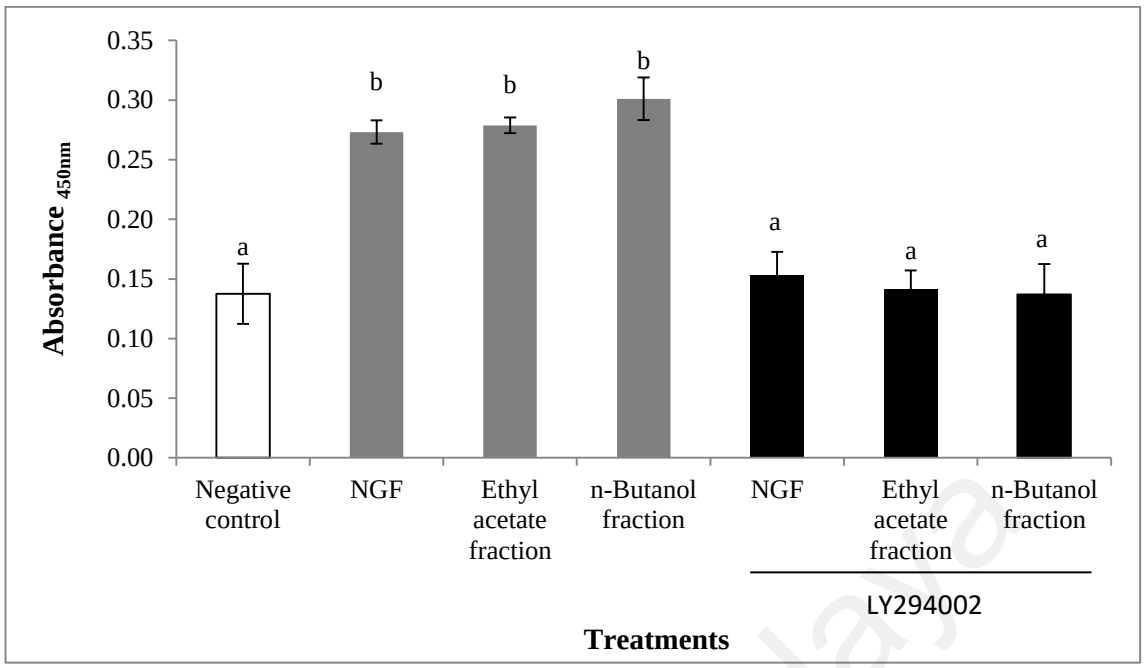



**Figure 5.8: Protein expression of phosphorylated AKT (Thr308) in NGF- and solvent fractions-treated PC-12 cells.** Cells were incubated with NGF (50 ng/ml), ethyl acetate fraction (10 µg/ml) or n-butanol fraction (10 µg/ml) for 48 hours. For inhibitor treatment, cells were pre-treated with LY294002 (PI3K inhibitor) one hour prior to the treatment of NGF or solvent fractions. Cells in complete F-12 K medium served as a negative control. Phosphorylated AKT protein level was measured by determination of the absorbance at 450 nm. Data were expressed as means ± SD from three independent experiments carried out in triplicates. Means with different alphabets represent significant difference ($p < 0.05$).

PC-12 cells treated with NGF, ethyl acetate and n-butanol fractions also exhibited significantly ($p < 0.05$) higher phosphorylation of mTOR (Ser2448) when compared to the negative control (Figure 5.9). The cellular transcription factor, CREB is activated by the downstream proteins of ERK1/2 and mTOR. Therefore, the phosphorylation of CREB (Ser133) was investigated. Treatment of PC-12 cells with ethyl acetate and n-butanol fractions triggered significantly ($p < 0.05$) higher phosphorylation of CREB, compared to the negative control and NGF (Figure 5.10).

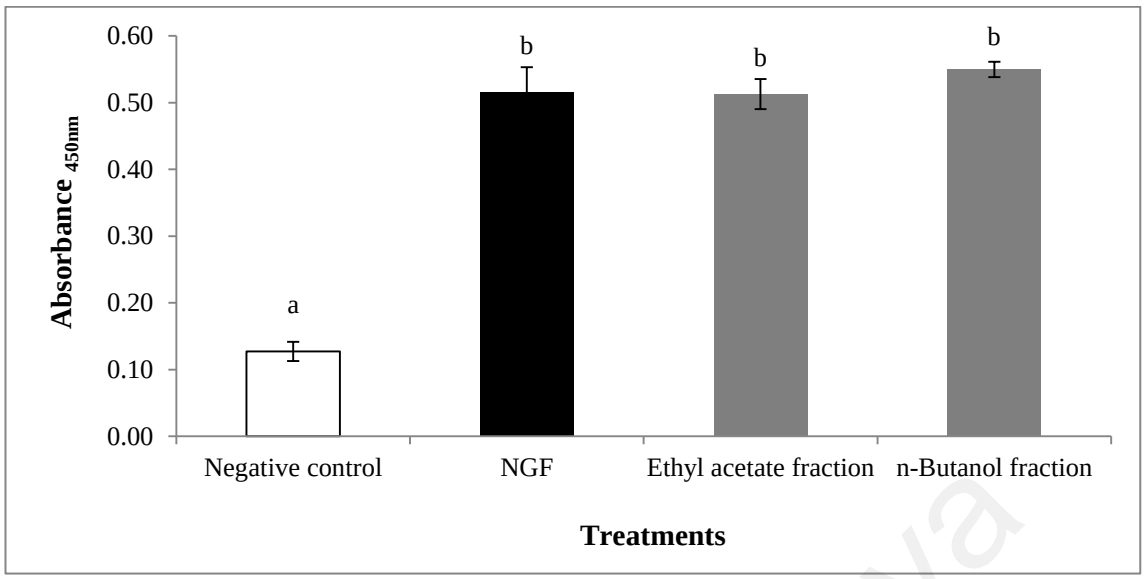



**Figure 5.9: Protein expression of phosphorylated mTOR (Ser2448) in NGF- and solvent fractions-treated PC-12 cells.** Cells were incubated with NGF (50 ng/ml), ethyl acetate fraction (10 µg/ml) or n-butanol fraction (10 µg/ml) for 48 hours. Cells in complete F-12 K medium served as a negative control. Phosphorylated mTOR protein level was measured by determination of the absorbance at 450 nm. Data were expressed as means ± SD from three independent experiments carried out in triplicates. Means with different alphabets represent significant difference ($p < 0.05$).

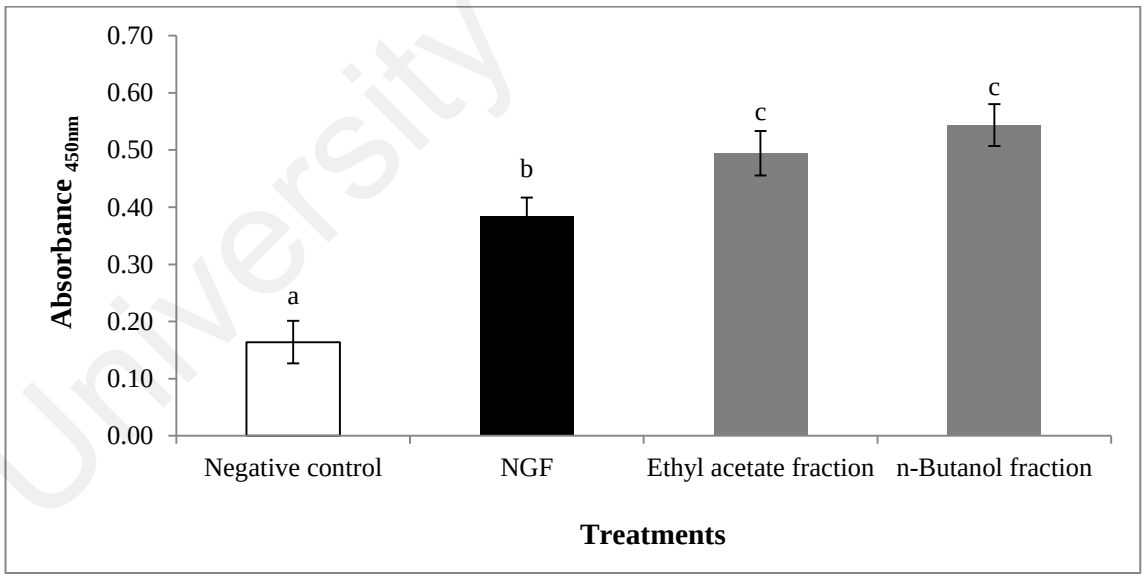



**Figure 5.10: Protein expression of phosphorylated CREB (Ser133) in NGF- and solvent fractions-treated PC-12 cells.** Cells were incubated with NGF (50 ng/ml), ethyl acetate fraction (10 µg/ml) or n-butanol fraction (10 µg/ml) for 48 hours. Cells in complete F-12 K medium served as a negative control. Phosphorylated CREB protein level was measured by determination of the absorbance at 450 nm. Data were expressed as means ± SD from three independent experiments carried out in triplicates. Means with different alphabets represent significant difference ($p < 0.05$).

### 5.3.7 The effects of solvent fractions on the protein expressions of GAP-43, TUBA4A and TUBB1

Phosphorylation of CREB may lead to the activation of numerous downstream genes including GAP-43, TUBA4A and TUBB1 to promote neuritogenesis. The protein concentrations of GAP-43 (Figure 5.11a), TUBA4A (Figure 5.11b) and TUBB1 (Figure 5.11c) in PC-12 cells treated with NGF (50 ng/ml), ethyl acetate (10 μg/ml) and n-butanol (10 μg/ml) fractions were significantly ($p < 0.05$) higher compared to the negative control. There are no significant difference ($p > 0.05$) in protein expression between NGF-, ethyl acetate and n-butanol fractions-treated cells for GAP-43, TUBA4A and TUBB1.

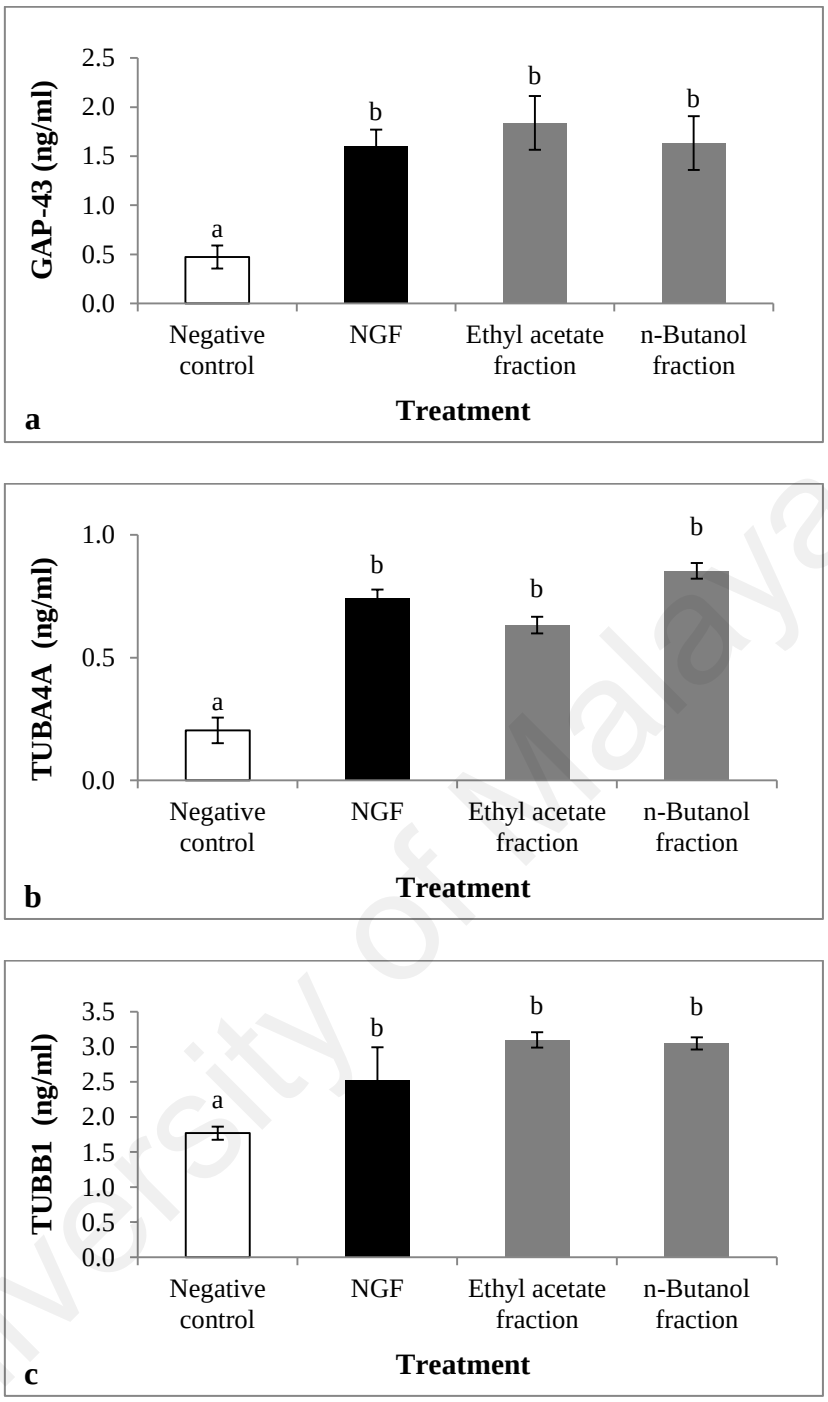



**Figure 5.11: Protein concentration of GAP-43, TUBA4A and TUBB1 in NGF- and solvent fractions-treated PC-12 cells.** Cells were incubated with NGF (50 ng/ml), ethyl acetate fraction (10 μg/ml) or n-butanol fraction (10 μg/ml) for 48 hours. Cells in complete F-12 K medium served as a negative control. Protein concentrations of (a) GAP-43, (b) TUBA4A, and (c) TUBB1 were determined at 48 hours post-treatment of NGF or solvent fractions. Data were expressed as means ± SD from three independent experiments carried out in triplicates. Means with different alphabets represent significant difference ($p < 0.05$).

## 5.4 DISCUSSION

The hot aqueous extract of *L. rhinocerotis* sclerotium exhibited significant ($p < 0.05$) lower cytotoxic effect towards PC-12 cells when compared to the ethanol extract and crude polysaccharides (Chapter III, pp. 66). Further, the cytotoxic effect of three fractions of hot aqueous extract of *L. rhinocerotis* sclerotium, the ethyl acetate, n-butanol and aqueous fractions on PC-12 cells was investigated. Similar to the hot aqueous extract, aqueous fraction exhibited significant ($p < 0.05$) lower cytotoxic effect towards PC-12 cells, compared to ethyl acetate and n-butanol fractions. Anyhow, the $IC_{50}$ value of the cytotoxic effect of the hot aqueous extract, ethyl acetate, n-butanol and aqueous fractions were approximately 141-, 19-, 28- and 245- fold higher than their optimum concentrations that stimulated neuritogenesis, respectively.

The hot aqueous extract at 25 μg/ml stimulated maximal percentage of neurite bearing cells in PC-12 cells that was comparable to the positive control, 50 ng/ml of NGF. Interestingly, both ethyl acetate and n-butanol fractions of hot aqueous extract significantly ($p < 0.05$) stimulated higher percentage of neurite bearing cells compared to their crude hot aqueous extract and NGF (Figure 5.2). These findings suggest that the ethyl acetate and n-butanol fractions of *L. rhinocerotis* may contain low-molecular weight secondary metabolites that possess neuritogenic effect. To date, there are many neuroactive or neuritogenic components have been isolated from mushrooms. Phan, David, Naidu, et al. (2014) recorded the *in vitro* neuritogenic potential of numerous extracts and secondary metabolites isolated from different culinary-medicinal mushrooms which may be targeted as preventive and therapeutic agents for the management of neurodegenerative diseases. Secondary metabolites isolated from the Chinese mushroom *Sarcadon* species, including cyrneine A and B, glaucopine C (Marcotullio et al., 2007, 2006), scabronine A-G (Waters, Tian, Li, Danishefsky, 2005; Obara et al., 1999) shown to stimulate neuritogenesis or promote NGF biosynthesis *in*

*vitro*. Cyathane diterpenoids from *Sarcodon scabrosus*, the sarcodonins G and A at 25 μM showed significant neuritogenic activity mediated with 20 ng/ml of NGF (Shi, Liu, Gao, & Zhang, 2011). Tricholomalides A-C at 100 mM isolated from mushroom *Tricholoma sp.* significantly stimulated neuritogenesis in PC-12 cells (Tsukamoto et al., 2003). Neuroactive compounds isolated from *Hericium erinaceus*, including hericinones (Phan, Lee, et al., 2014; Kawagishi et al., 1991) and erinacines (Shimbo, Kawagishi, & Yokogoshi, 2005; Kawagishi et al., 1994) showed significant stimulation of production or biosynthesis of NGF *in vitro*.

Increment level of extracellular NGF in cell supernatant showed the ability of tested extracts or compounds to induce NGF production by PC-12 cells (Phan, Lee, et al., 2014). In the present study, the concentration of extracellular NGF in NGF-treated cells (positive control) was approximately four-fold increase compared to the negative control. Based on the concentration of extracellular NGF measured and percentage of neurite bearing cells observed in NGF-treated cells, neuritogenesis in PC-12 cells may be NGF-dependent. Number of studies documented the potential of extracts or compounds of medicinal mushrooms to stimulate the biosynthesis and production of NGF *in vitro* (Lai, Naidu et al., 2013; Marcotullio et al., 2007). Cyrneine A (200 μM) stimulated significantly ($p < 0.05$) higher percentage of neurite-bearing cells compared to 100 ng/ml of NGF in PC-12 cells with the stimulation of NGF production and gene expression in human astrocytoma cells (1321N1) (Marcotullio et al., 2007). According to Lai, Naidu et al. (2013), hot aqueous extract of *H. erinaceus* stimulated the production of NGF in NG108-15 cells. The concentration of the extracellular NGF in NG108-15 cells treated with 50 μg/ml of hot aqueous extract of *H. erinaceus* was 21.4% higher, compared to 20 ng/ml of NGF-treated cells (Lai, Naidu et al., 2013). Compounds from *H. erinaceus*, including hericenone C-E (Kawagishi et al., 1991), erinacine A-C (Kawagishi et al., 1994) stimulated biosynthesis of NGF and exhibited

neuritogenesis in astroglial cells. A recent study by Phan, Lee, et al. (2014) reported that hericenone E potentiated NGF-stimulated neuritogenesis in PC-12 cells by stimulating the production of NGF that was almost two times higher than that of positive control (50 ng/ml of NGF). Taken together, these studies suggested that the neuritogenic effect of the stated compounds were performed through the stimulation of production of NGF by neuronal cells. However, in the present study, the hot aqueous extract and solvent fractions of *L. rhinocerotis* sclerotium stimulated the neuritogenic activity without stimulating the production of NGF in PC-12 cells (Figure 5.4). There was no significant increment of the concentration of extracellular NGF in hot aqueous extract-, ethyl acetate, n-butanol and aqueous fractions-treated cells, compared to the negative control. These findings showed that the hot aqueous extract and solvent fractions may contain NGF-like compound(s) (NGF mimics or substitute for NGF) that mimic the neuritogenic activity of the NGF.

Neurofilament staining by immunofluorescence is a useful indicator of PC-12 cell differentiation and it served as a firm support to the observation that NGF stimulated neuritogenesis in PC-12 cells (Schimmelpfeng, Weibezahn, & Dertinger, 2004). Neurofilaments are neuron specific intermediate filament proteins (8-10 nm) that are located in axons, a major component of the cytoskeleton that supporting the axon cytoplasm, and found specifically in most matures neurons (Lee, Xu, Wong, & Cleveland, 1993). Neurofilaments are composed predominantly of distinct subunits, namely neurofilament light (NF-L), medium (NF-M) and heavy (NF-H) (Liem, Yen, Salomon, & Shelanski, 1978). The anti-NF-200 antibody recognizes both phosphorylated and non-phosphorylated forms of heavy neurofilament subunit NF-H at 180-220 kDa. In the present study, the morphology of PC-12 cells treated with NGF, hot aqueous extract, ethyl acetate, n-butanol and aqueous fractions of *L. rhinocerotis* sclerotium showed significant neurofilaments stained by anti-NF-200 antibody (Figure

5.3). These findings confirmed the neuritogenesis in PC-12 cells was stimulated by NGF, hot aqueous extract, ethyl acetate, n-butanol and aqueous fractions of *L. rhinocerotis* sclerotium.

Nerve growth factor binds to its high affinity catalytic receptor, TrkA to initiates the NGF responsive pathways, including the MEK/ERK1/2 and PI3K/AKT signaling pathways, to kick start neuritogenesis in PC12 cells (Vaudry et al., 2002). Once the TrkA is phosphorylated, it became a scaffolding structure and recruits proteins that ultimately propagate the MEK/ERK (Egea et al., 2000) and PI3K/AKT (Jackson et al., 1996) signaling pathways. Specific inhibitors of protein kinase served as powerful tool to study the kinase activities in selected signaling pathway (Phan, Lee, et al., 2014). The K252a acts as a specific and potent inhibitor of Trk receptor, inhibits the phosphorylation of NGF-induced TrkA, and selectively blocks the activities of NGF in PC-12 cells (Koizumi et al., 1988). In the present study, the neuritogenic activity of ethyl acetate and n-butanol fractions was blocked 83.46% and 85.39%, respectively by K252a, which parallel to the inhibition effect of K252a towards NGF-treated cells (82.13%) (Figure 5.5). These findings showed that the NGF-, ethyl acetate and n-butanol fractions-stimulated neuritogenesis were TrkA-dependent in PC-12 cells.

Activation of NGF responsive pathways by mushroom extract is crucial in the preliminary search of neuroactive compound(s) that may mimic the neuritogenic activity of NGF. The present study illustrated the involvement of two predominant cellular signaling pathways in ethyl acetate and n-butanol fractions-stimulated neuritogenesis, namely MEK/ERK1/2/CREB and P13K/AKT/mTOR/CREB which are important in regulating growth and differentiation of PC-12 cells. The MEK/ERK1/2/CREB (Vaudry et al., 2002; Creedon, Johnson, Lawrence, 1996) and PI3K/AKT/mTOR/CREB (Markus, Zhong, Snider, 2002; Jackson et al., 1996) signaling pathways are activated by NGF to stimulate neurite extension and branching of neuronal

cells. To understand the NGF mimicking activity of mushroom solvent fractions in PC-12 cells, inhibitors of specific proteins related to NGF signaling pathways were used. Specific inhibitors of MEK and P13K could attenuate the ability of NGF and solvent fractions to stimulate neuritogenesis in PC-12 cells. Inhibition of MEK/ERK (Vaudry et al., 2002) and PI3K/AKT (Higuchi, Onishi, Masuyama, & Gotoh, 2003) in PC-12 cells will avert NGF-stimulated neurite elongation, cell protective effect and cell survival (Klesse, Meyers, Marshall, & Parada, 1999). Both U0126 and PD98059 are selective and potent inhibitors of MEK 1 and MEK 2 (Favata et al., 1998), while LY294002 is a selective and potent inhibitor of PI3K (Vlahos, Matter, Hui, & Brown, 1994). Priming PC-12 cells with MEK1/2 inhibitors (U0126 and PD98059) and PI3K inhibitor (LY294002) lead to the inhibition of phosphorylation and activation of MEK/ERK1/2 and PI3K/AKT, respectively, and eventually diminish the interrelated cellular processes including cell differentiation and neuritogenesis (Kim, Seger, Suresh Babu, Hwang, Yoo, 2004; Kawamata, Yamaguchi, Shin-ya, & Hori, 2003).

In the present study, the neuritogenic activity of ethyl acetate and n-butanol fractions was blocked by U0126, PD98059 and LY294002 inhibitors (Figure 5.5). There are numerous studies reported that the MEK/ERK1/2 and PI3K/AKT-dependent signaling pathways play a crucial role in the neuritogenic effect of edible and/or medicinal mushrooms. Previous studies documented that MEK/ERK1/2 and PI3K/AKT signaling pathways were involved in neuritogenesis stimulated by extracts of *Pleurotus giganteus* (Phan et al., 2012) and hot aqueous extracts of *Ganoderma lucidum*, *Ganoderma neo-japonicum* and *Grifola frondosa* (Seow, Naidu, David, Wong, & Sabaratnam, 2013). Nishina et al. (2006) demonstrated that the activation of MAPK by lysophosphatidylethanolamine, a neuroactive compound extracted from *G. frondosa* was suppressed by U0126, but not by K252a. The study suggested that the MEK/ERK1/2 signaling pathway was involved in lysophosphatidylethanolamine-

stimulated neuritogenesis in PC12 cells, but was not through the activation of TrkA receptor (Nishina et al., 2006). Phan, Lee, et al. (2014) reported that the neuritogenic activity potentiated by hericenone E was found to be partially blocked (46%) by K252a and almost completely blocked by U0126, PD98059, and LY294002. The study suggested that hericenone E potentiated NGF-stimulated neuritogenesis in PC12 cells was partially mediated by TrkA, but was MEK/ERK1/2 and PI3K/AKT dependent (Phan, Lee, et al., 2014). In the present study, all four inhibitors (K252a, U0126, PD98059 and LY294002) successfully attenuated the NGF-, ethyl acetate and n-butanol fractions-stimulated neuritogenesis in PC-12 cells, showed that the ethyl acetate and n-butanol fractions mimicked the NGF neuritogenic activity by binding to the TrkA receptor and activated the NGF responsive pathways, MEK/ERK1/2 and PI3K/AKT in PC-12 cells.

Protein expression analysis of the target proteins in MEK/ERK1/2/CREB and P13K/AKT/mTOR/CREB signaling pathways was performed to validate the findings of inhibitors screening. Persistent phosphorylation and activation of MEK/ERK1/2 (Barrie, Clohessy, Buensuceso, Rogers, & Allen, 1997; Marshall 1995) and PI3K/AKT (Nusser, Gosmanova, Zheng, & Tigyi, 2002) signaling pathways are associated with several cellular processes including proliferation, survival, neuritogenesis, and regeneration. The phosphorylation of Ser217/221 on MEK1/2 leading to the phosphorylation of Thr202/Tyr204 on ERK1/2, and translocate into the nucleus to phosphorylate the transcription factor, CREB, thereby regulating several cellular processes (Peyssonnaux & Eychène, 2001). Activation of PI3K leading to the phosphorylation of Thr308 on AKT (Anderson, Coadwell, Stephens, & Hawkins, 1998), mTOR (Ser2448) and CREB (Read & Gorman, 2009). CREB is a major transcription factor for neurite extension by the phosphorylation of Ser133 (Yamamoto, Gonzalez, Biggs, & Montminy, 1988). It is required for axon growth induced by NGF (Lonze, Riccio, Cohen, & Ginty, 2002). The

NGF responsive signaling leads to the phosphorylation of CREB at Ser133 (Ginty, Bonni & Greenberg, 1994) which sequentially leads to the activation of neuritogenesis biomarkers, GAP-43 (Jap Tjoen San et al., 1992, 1991; Skene, 1989) and microtubules (Drubin et al., 1988; Greene et al., 1984) which regulate neuronal growth and neuritogenesis. Therefore, activation of MEK/ERK1/2/CREB and PI3K/AKT/mTOR/CREB signaling pathways leading to the activation of the downstream genes, GAP-43 and microtubules (alpha tubulin TUBA4A and beta tubulin TUBB1) provided the biochemical evidence for neuritogenesis and the presence of neurite-stimulating agent(s) in natural products. Recent study by Won et al. (2015), documented that DA-9801, a plant extract from a mixture of *Dioscorea japonica* and *Dioscorea nipponica* stimulated neuritogenesis in PC-12 cells through ERK1/2/CREB pathway. Treatment of DA-9801 markedly enhanced the phosphorylation of ERK1/2 and CREB in PC-12 cells (Won et al., 2015). Claulansine F, a carbazole alkaloid isolated from the stem of wampee, *Clausena lansium* (Lour) Skeels was found stimulated neuritogenesis in PC-12 cells via activation of MAPK/ERK pathway and subsequently up-regulated the GAP-43 expression (Ma et al., 2013). Phan et al. (2015) reported that uridine (100 μM), a neuroactive compound in mushroom *P. giganteus* stimulated neuritogenesis in N2a cells via MEK/ERK1/2/CREB and PI3K/AKT/mTOR/CREB signaling pathways. Uridine successfully increased the protein levels of phosphorylated ERK1/2, AKT, mTOR, and further induced the phosphorylation of CREB and enhanced the expression of GAP43, TUBA4A and TUBB1 in N2a cells (Phan et al., 2015). In agreement with the study by Phan et al. (2015), the present study showed that NGF, ethyl acetate and n-butanol fractions of *L. rhinocerotis* sclerotium significantly ($p < 0.05$) increased the levels of phosphorylated MEK1/2 (Ser217/221), ERK1/2 (Thr202/ Tyr204), AKT (Thr308), mTOR (Ser2448),

and further induced the phosphorylation of CREB (Ser133) and enhanced the expression of GAP43, TUBA4A and TUBB1 in PC-12 cells.

Based on the present findings, ethyl acetate and n-butanol fractions mimicked the neuritogenic activity of NGF by activating the NGF responsive signaling pathways in PC-12 cells (Figure 5.12). The neuritogenic stimulatory activity of ethyl acetate and n-butanol fractions was mediated through the NGF high affinity receptor, TrkA and the MEK1/2/ERK1/2 and PI3K/AKT/mTOR signaling pathways. Furthermore, the phosphorylation of transcription factor, CREB and the gene expressions of neuritogenesis biomarkers, GAP43, TUBA4A, and TUBB1 were upregulated, which then initiated neuritogenesis in PC-12.

## 5.5 CONCLUSIONS

The hot aqueous extract of *L. rhinocerotis* sclerotium and its solvent fractions, ethyl acetate, n-butanol and aqueous fractions stimulated neuritogenesis in PC-12 cells. All the concentrations of hot aqueous extract and solvent fractions tested for neuritogenic activity were not cytotoxic to PC-12 cells. The ethyl acetate and n-butanol fractions (10 µg/ml) stimulated higher percentage of neurite bearing cells compared to that of NGF (50 ng/ml). The hot aqueous extract and solvent fractions stimulated neuritogenic activity without stimulating the production of NGF in PC12 cells. Based on the present findings, ethyl acetate and n-butanol fractions of *L. rhinocerotis* mimicked the neuritogenic activity of NGF by targeting the TrkA receptor and regulated the MEK/ERK1/2 and PI3K/AKT/mTOR signaling pathways with the activation of the transcription factor, CREB, leading to the elevation of GAP43, TUBA4A and TUBB1 expressions in PC-12 cells. The present findings suggest that the *L. rhinocerotis* sclerotium may contain NGF-like neuroactive compound(s) (NGF mimics or substitute for NGF) that mimic the neuritogenic activity of NGF, and stimulated neuritogenesis in

PC-12 cells via NGF responsive pathways, MEK/ERK1/2/CREB and PI3K/AKT/mTOR/CREB.

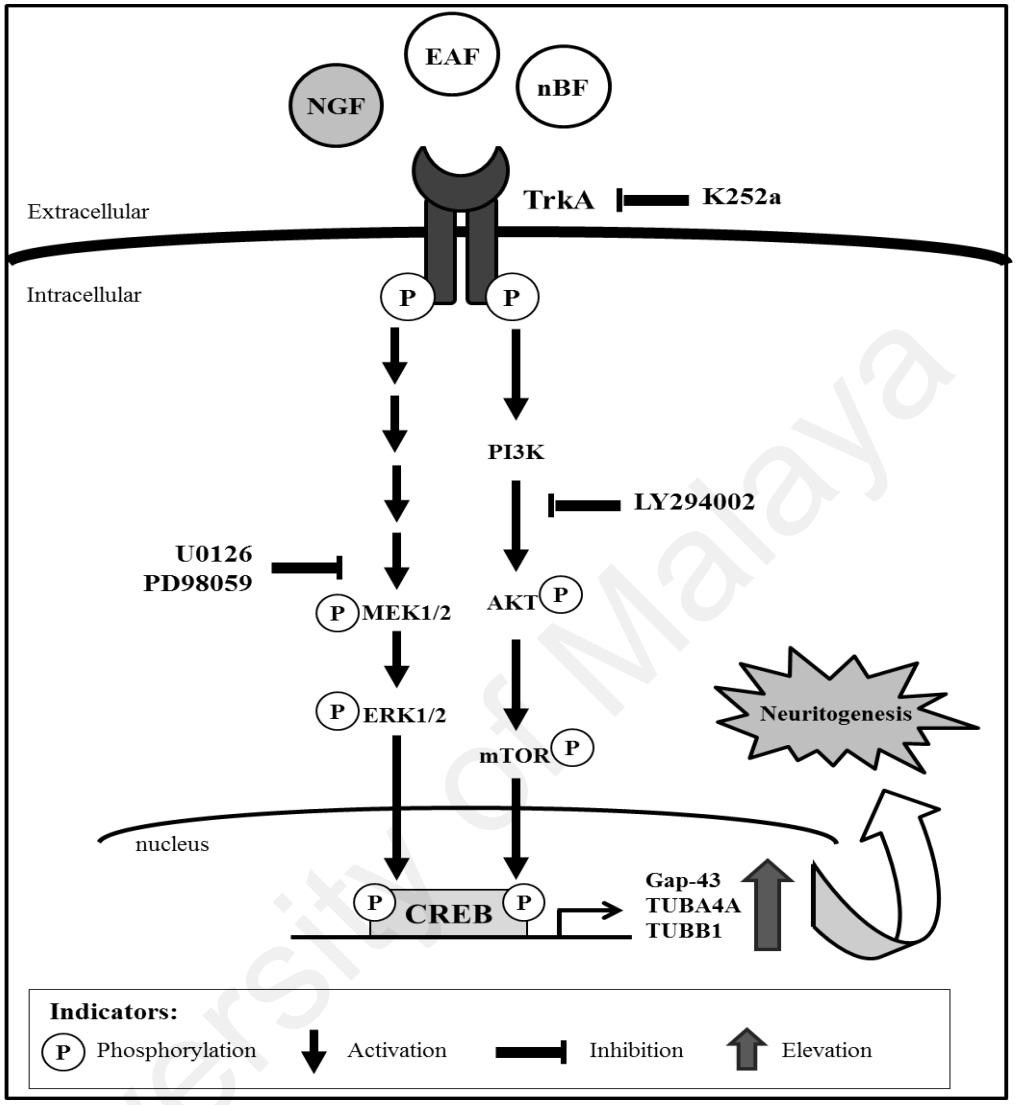



**Figure 5.12: Hypothetic mechanism of NGF, ethyl acetate and n-butanol fractions in stimulating neuritogenesis in PC-12 cells.** Based on the present findings, the neuritogenic effect of NGF, ethyl acetate (EAF) and n-butanol (nBF) fractions is mediated through the tropomyosin receptor kinase A (TrkA). Activated TrkA leads to the phosphorylation of mitogen-activated protein kinase kinase1/2/extracellular signal-regulated kinase1/2 (MEK1/2/ERK1/2) and phosphatidylinositol-3-kinase/protein kinase B/mammalian target of rapamycin (PI3K/AKT/mTOR) signaling pathways. Activation of ERK1/2 and mTOR leads to the phosphorylation of the transcription factor, cyclic adenosine monophosphate (cAMP) response element binding protein (CREB), and sequentially stimulates the upregulation of downstream genes, the growth associated protein 43 (GAP43), tubulin alpha (TUBA4A), and tubulin beta (TUBB1), which then initiates neuritogenesis. The Trk inhibitor (K252a), MEK/ERK1/2 inhibitors (U0126, PD98059) and PI3K/AKT inhibitor (LY294002) blocked the neuritogenic activity of NGF, ethyl acetate and n-butanol fractions. Thus, based on the present findings, ethyl acetate and n-butanol fractions mimicked the neuritogenic activity of NGF by binding to the TrkA receptor and activated the NGF responsive signaling pathways.

# CHAPTER VI: GENERAL DISCUSSION, PROPOSED FUTURE STUDIES AND CONCLUSIONS

## 6.1 GENERAL DISCUSSION

This study demonstrated the *in vitro* anti-neuroinflammatory and neuritogenic stimulatory effects of a medicinal mushroom, *Lignosus rhinocerotis* – Malaysia's treasure mushroom. In chapter III (pp. 66), four medicinal mushrooms, namely *Ganoderma lucidum*, *Ganoderma neo-japonicum*, *Grifola frondosa* and *L. rhinocerotis* were selected to investigate their preliminary anti-neuroinflammatory and neuritogenic stimulatory effects by using murine BV2 microglial and PC-12 cell lines, respectively. Of these four medicinal mushrooms, *L. rhinocerotis*, the tiger's milk mushroom showed its significant potential as NO inhibitor and neuritogenic stimulator compared to three other mushrooms tested. Of three different preparations of *L. rhinocerotis* sclerotium, the hot aqueous extract showed lowest cytotoxic effect towards both BV2 microglia and PC-12 cells, inhibited highest percentage of NO in LPS-stimulated BV2 microglia, and stimulated highest percentage of neuritogenesis in PC-12 cells when compared to the ethanol extract and crude polysaccharides.

Consequently, the hot aqueous extract of *L. rhinocerotis* sclerotium was fractionated by using organic solvent to yield ethyl acetate, n-butanol and aqueous fractions. Of these fractions, the ethyl acetate and n-butanol fractions showed significant anti-neuroinflammatory (Chapter IV, pp. 91) and neuritogenic stimulatory (Chapter V, pp. 139) effects *in vitro*. As potent anti-neuroinflammatory agents, the ethyl acetate and n-butanol fractions significantly ($p < 0.05$) inhibited NO production and iNOS expression that were comparable to positive controls, the iNOS inhibitor, L-NAME and the NSAID, aspirin. The ethyl acetate and n-butanol fractions also significantly ($p <$

0.05) inhibited the $PGE_2$, COX-2, TNF-α, IL-1β, IL-6 productions and expressions that were comparable to the aspirin in LPS-stimulated BV2 microglia. Simultaneously, as potent neuritogenic stimulatory agents, the ethyl acetate and n-butanol fractions stimulated higher percentage of neuritogenesis compared to the NGF, without stimulating the production of NGF in PC-12 cells. These findings suggest that the ethyl acetate and n-butanol fractions may contain chemical constituents, i.e secondary metabolites that are neuroactive. This hypothesis is supported by the anti-neuroinflammatory and neuritogenic stimulatory findings, showed that the ethyl acetate and n-butanol fractions exhibited higher ability to inhibit the production of pro-inflammatory mediators, and stimulated higher percentage of neuritogenesis compared to their crude hot aqueous extract and polysaccharides. According to Lau et al. (2013), besides carbohydrates and proteins, the hot aqueous extract of *L. rhinocerotis* sclerotium contained a higher percentage of phenolics than the cold aqueous extract. Lau et al. (2013) further suggested that secondary metabolites such as triterpenes and alkaloids may be present in *L. rhinocerotis* sclerotium extracts. In the present study, the hot aqueous extract of *L. rhinocerotis* sclerotium was further fractionated by organic solvents to target on the secondary metabolites or bioactives components with neuroactive potential. Solvent extraction and fractionation method has been demonstrated as the most frequently used method for the discovery of natural substances or secondary metabolites from plants (Kumar, Dhanani, & Shah, 2014). The substances are obtained depending on the solvent properties and solubility of the substances in the respective solvent (Kumar et al., 2014; Steven & Russell, 1993). Bioactive components such as polyphenols (Sangeetha & Vijayalakshmi, 2011; Steven & Russell, 1993), alkaloids (Anyasor et al., 2014; Steven & Russell, 1993), triterpenes (Anyasor et al., 2014) are usually the targeted secondary metabolites soluble in volatile

solvents, include ethyl acetate (Sangeetha & Vijayalakshmi, 2011; Steven & Russell, 1993) and n-butanol (Anyasor et al., 2014; Steven & Russell, 1993).

Recently, there is an increasing effort to discover the natural secondary metabolites from medicinal mushrooms. The ongoing challenge for researchers is to continue to develop and explore new or novel bioactive components from medicinal mushrooms, remarkably from those that have long been used as mycomedicines by the indigenous or traditional medicine practitioners. Nallathamby et al. (2015) found that sub-fraction CE3 from ethyl acetate fraction of *Cordyceps militaris* was enriched with ergosterol, a bioactive compound commonly available in mushrooms that possess pharmacological effects including anti-tumor (Bok et al., 1999) and anti-inflammatory (Kuo, Hsieh, & Lin, 2011; Akihisa et al., 2007).

Today, numerous secondary metabolites have been successfully isolated from different medicinal mushrooms. These secondary metabolites were reported to promote anti-neuroinflammatory (Table 2.1, pp. 39-42) and/or neuritogenic stimulatory (Table 2.2, pp. 44-46) effects *in vitro* and/or *in vivo*, including cordycepin (Jeong et al., 2010), hericenones (Mori et al, 2008; Kawagishi et al., 1991), erinacines (Kawagishi et al., 1994), scabronions (Obara et al., 1999), termitomycesphins (Qi et al., 2000), tricholomalides (Tsukamoto et al., 2003), and cyrneines (Marcotullio et al., 2006, 2004). However, to date, the chemical investigations of *L. rhinocerotis* are scanty. The earlier studies of *L. rhinocerotis* focused on the nutritional attributes (Yap et al., 2013; Lau, Abdullah, Aminudin, & Lee, 2013) and pharmacological activities (Seow et al., 2015; Lee et al., 2014; Lau et al., 2013) of the freeze-dried powder, crude extracts or polysaccharides of *L. rhinocerotis* sclerotium. To date, no compound has been successfully isolated or characterized from *L. rhinocerotis* yet. Studies by Suziana Zaila et al. (2013) and Mohanarji, Dharmalingam, & Kalusalingam (2012) documented the presence of alkaloids, flavonoids, mucilage and gum in organic solvent extracts of *L.*

*rhinocerotis*. Based on the chemical analysis reports of ultra-high performance liquid chromatography-electrospray ionization tandem-mass spectrometry (UHPLC–ESI–MS/MS) and gas chromatography–mass spectrometry (GC–MS), Lau et al. (2014) suggested that the aqueous extracts of *L. rhinocerotis* contained numerous low-molecular weight secondary metabolites, including fatty acids (linoleic acid, palmitic acid, stearic acid, and oleic acid), triterpenoids, sugar alcohols (arabinitol), amino acids (phenylalanine and tryptophan), organic acids (citric acid), and sterols (ergosterol). However, further confirmation, purification and characterization of these compounds have not been taken. Recent study by Nallathamby et al. (2016) reported that the ethyl acetate fraction of *L. rhinocerotis* sclerotium contained numbers of unsaturated fatty acids. The flame ionization detector (FID) and GC–MS analysis showed that the linoleic, ethyl linoleic and oleic acids were the major lipid constituents (Nallathamby et al., 2016). Among these lipid components, linoleic acid significantly ($p < 0.05$) reduced the NO production and downregulated the iNOS and COX-2 gene expressions in LPS-stimulated BV2 microglia (Nallathamby et al., 2016). Thus, by referring to the chemical profiling results by Lau et al. (2014) and study by Nallathamby et al. (2016), in the present study, two active fractions of *L. rhinocerotis*, the ethyl acetate and n-butanol fractions may contain low-molecular weight volatile neuroactive compounds such as phenolic, flavonoids, fatty acids and sterols that possess *in vitro* anti-neuroinflammatory and neuritogenic stimulatory effects which may beneficial for the management of neurodegenerative diseases. Further analysis of these fractions by identification, isolation, purification and characterization of the potential compounds should be taken.

## 6.2 PROPOSED FUTURE STUDIES AND CONCLUSIONS

The present study may be the first report revealing the *in vitro* anti-neuroinflammatory and neuritogenic stimulatory effects and the underlying mechanisms of ethyl acetate and n-butanol fractions of hot aqueous extract of *L. rhinocerotis* sclerotium. Future studies and research directions listed below are recommended for the exploration and development of *L. rhinocerotis* as pledging dietary supplements (nutraceuticals or nutriceuticals) and mycomedicine, notably with anti-neuroinflammatory and neuritogenic stimulatory effects:

(i) To further study the relationships and correlation between anti-neuroinflammatory and neuritogenic stimulatory activities of *L. rhinocerotis*, an *in vitro* model with *in vivo* conditions of CNS is needed. Establishment of a co-culture model of microglial and neuronal cells is suggested. It is worthy to study the (a) responses of both microglial and neuronal cells upon stimulation by inflammatory stimuli, (b) progression of microglial and neuronal cells degeneration, (c) neuroprotective effects of *L. rhinocerotis* on microglial cells by inhibiting the microglia activation, neuroinflammatory process and microglia degeneration, (d) neuroprotective effects of *L. rhinocerotis* on neuronal cells and neurites by inhibiting the production of neurotoxic substances produced by activated microglia, (e) neuritogenic stimulatory ability of *L. rhinocerotis* on neuronal cells in microglia-mediated neuroinflammatory condition, and (f) the underlying mechanisms of the activities stated, by using the co-culture model.

(ii) To identify, isolate, purify and characterize the potential neuroactive compounds presence in ethyl acetate and n-butanol fractions of *L. rhinocerotis* sclerotium. To date, no compound with significant pharmacological activity has been isolated from *L. rhinocerotis* yet. Beside

anti-neuroinflammatory and neuritogenic stimulatory properties, there are huge potential to isolate and identify unknown or novel compounds from *L. rhinocerotis* that may have other pharmacological activities.

(iii) To have solid knowledge on the anti-neuroinflammatory and neuritogenic effects of *L. rhinocerotis* and also to answer the hypothesis of the present *in vitro* study, animal studies with dose-response design are necessary to establish the efficacies and mechanisms of action of *L. rhinocerotis in vivo*.

(iv) To assess the safety of the consumption of *L. rhinocerotis* by using *in vivo* approach is necessary for the development of *L. rhinocerotis* as dietary or health supplements. The *in vivo* subacute toxicity of freeze-dried powder of *L. rhinocerotis* sclerotium has been studied previously. However, the *in vivo* subacute toxicity of extracts, fractions and compounds of *L. rhinocerotis* sclerotium are yet to be investigated. The *in vivo* pharmacokinetics and bioavailability of *L. rhinocerotis* also must be investigated.

In conclusion, this study indicates prospects of *L. rhinocerotis* sclerotium for commercial utilization as mycomedicine and dietary or health supplements for the management of neuroinflammatory- and neurite degenerative-associated disorders by inhibiting neuroinflammation and promotes neuritogenesis *in vitro*. The main findings of this study were:

(i) *Lignosus rhinocerotis* was identified as a potential medicinal mushroom that exhibited anti-neuroinflammatory and neuritogenic stimulatory effects *in vitro*. The hot aqueous extract of *L. rhinocerotis* sclerotium exhibited the highest $IC_{50}$ of BV2 microglia and PC-12 cell viability, inhibited significantly ($p < 0.05$) higher percentage of NO in LPS-stimulated BV2 microglia, and stimulated significantly ($p < 0.05$) higher percentage of

neurite bearing cells in PC-12 cells, compared to other mushrooms (*Ganoderma lucidum*, *Ganoderma neo-japonicum*, and *Grifola fromdosa*) and other preparations (ethanol and polysaccharides) of *Lignosus rhinocerotis* sclerotium studied.

(ii) The ethyl acetate and n-butanol fractions of hot aqueous extract of *L. rhinocerotis* sclerotium exhibited anti-neuroinflammatory effect in LPS-stimulated BV2 microglia. The ethyl acetate and n-butanol fractions (125 and 250 µg/ml) significantly ($p < 0.05$) inhibited the production and expressions (protein and mRNA) of pro-inflammatory mediators and cytokines, including NO, iNOS, $PGE_2$, COX-2, TNF-α, IL-1β and IL-6 in LPS-stimulated BV2 microglia, without cytotoxicity. Ethyl acetate fraction significantly ($p < 0.05$) blocked the expression of LPS-stimulated TLR4 receptor at the first place. Both ethyl acetate and n-butanol fractions inhibited the LPS-mediated neuroinflammatory signaling pathways, the NFκB, AKT, and MAPKs (ERK1/2, p38 MAPK, and SAPK/JNK). The ethyl acetate and n-butanol fractions further suppressed the expressions of the transcription factors involved in neuroinflammation, the NFκB, CREB and AP-1 (cFOS and cJUN). The anti-neuroinflammatory activity of ethyl acetate and n-butanol fractions were comparable to the NSAID control, aspirin (1 mM).

(iii) The ethyl acetate and n-butanol fractions of hot aqueous extract of *L.rhinocerotis* sclerotium stimulated neuritogenesis in PC-12 cells, without cytotoxicity. The ethyl acetate and n-butanol fractions (10 µg/ml) significantly ($p < 0.05$) stimulated higher percentage of neurite bearing cells compared to the positive control, 50 ng/ml of NGF. The ethyl acetate and n-butanol fractions stimulated neuritogenic activity but did not stimulate the

production of NGF in PC-12 cells. The ethyl acetate and n-butanol fractions stimulated neuritogenic activity by targeting the high affinity receptor of NGF, TrkA receptor and activated the NGF responsive signaling pathways, MEK/ERK1/2 and PI3K/AKT/mTOR with the activation of the transcription factor CREB, leading to the elevation of neuritogenesis biomarkers, GAP43, TUBA4A and TUBB1 expressions in PC-12 cells.

(iv) The ethyl acetate and n-butanol fractions may contain neuroactive compounds that mimic the activities of aspirin as anti-neuroinflammatory agent and of NGF as neuritogenic stimulatory agent.

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## LIST OF PUBLICATIONS AND PRESENTATIONS

### Journal articles

1. **Seow, S. L. S.**, Eik, L. F., Naidu, M., David, P., Wong, K. H., &Sabaratnam, V. (2015). *Lignosus rhinocerotis* (Cooke) Ryvarden mimics the neuritogenic activity of nerve growth factor via MEK / ERK1 / 2 signaling pathway in PC-12 cells. *Scientific Reports*, *5*, 16349. doi:10.1038/srep16349. **(I.F. 5.578)**

2. **Seow, S. L. S.**, Naidu, M., David, P., Wong, K. H., Sabaratnam, V. (2013). Potentiation of neuritogenic activity of medicinal mushrooms in rat pheochromocytoma cells. *BMC Complementary and Alternative Medicine*, *13*(1), 157. doi:10.1186/1472-6882-13-157. **(I.F. 1.877)**

### Oral Presentations

1. **Seow, S. L. S.**, Naidu, M., Sabaratnam, V. (2015). *Lignosus rhinocerotis (Cooke) Ryvarden: Medicinal mushroom that mitigates neuroinflammation in vitro.* Presented at the Postgraduate Seminar, Institute of Biological Sciences, Faculty of Science, University of Malaya, Malaysia, 24 June.

2. **Seow, S. L. S.**, Naidu, M., David, P., Wong, K. H., Sabaratnam, V. (2013). *Lignosus rhinocerotis (Cooke) Ryvarden induces neuritogenesis via extracellular signal-regulated kinase (ERK) pathway in rat pheochromocytoma cells.* Presented at the 7th International Medicinal Mushroom Conference, Beijing, China, 26-29 August(pp. 177-180).

**Poster Presentations**

1. **Seow, S. L. S.**, Naidu, M., Vidyadaran, S., Sabaratnam, V. (2016).*Tiger's milk mushroom – Mycomedicine that mitigates neuroinflammation in vitro*. Presented at the 3rdInternational Conference on Pharma and Food, Shizuoka, Japan, 16-18November (pp. 126).

2. **Seow, S. L. S.**, Naidu, M., David, P., Wong, K. H., Sabaratnam, V. (2013). *Tiger's milk mushroom – Nature's hidden treasure that promotes neuro health*. Presented at the International Functional Food Conference, Cyberjaya, Malaysia, 18-20 August (pp. 38). (Won the first place for poster presentation, student category).

2. **Seow, S. L. S.**, Naidu, M., David, P., Wong, K. H., Sabaratnam, V. (2012). *Neurite stimulation activity in rat pheochromocytoma cells (PC-12Adh) by medicinal mushrooms*. Presented at the National Postgraduate Seminar, Microbes: Diversity in Life, Diversity in Learning, Connecting Disciplines, University of Malaya, Malaysia, 11 July (pp. 103). (Won the best poster award).

# APPENDIX

## A: CELL CULTURE MEDIA

### 1. Nutrient Mixture F-12 Ham Kaighn's Modification (F-12 K) for rat pheochromocytoma cells (PC-12 cells)

With L-glutamine

Without sodium bicarbonate

Product Number: N3520 (Sigma-Aldrich)

| **Component** | **g/L** |
|---|---|
| L-Arginine | 0.4214 |
| L-Alanine | 0.017818 |
| L-Asparagine·$H_2O$ | 0.03002 |
| L-Aspartic acid | 0.02662 |
| L-Cysteine·HCl·$H_2O$ | 0.07024 |
| L-Glutamic acid | 0.02942 |
| L-Glutamine | 0.2922 |
| Glycine | 0.015014 |
| L-Histidine·HCl·$H_2O$ | 0.04192 |
| L-Isoleucine | 0.007872 |
| L-Lysine·HCl | 0.07304 |
| L-Leucine | 0.02624 |
| L-Methionine | 0.008952 |
| L-Proline | 0.06906 |
| L-Phenylalanine | 0.009912 |
| L-Serine | 0.02102 |
| L-Tryptophan | 0.004084 |
| L-Tyrosine | 0.010872 |
| L-Threonine | 0.02382 |
| L-Valine | 0.02342 |
| D-Biotin | 0.00007329 |
| Choline chloride | 0.01396 |
| Folic acid | 0.0013242 |
| Hypoxanthine | 0.004083 |
| myo-Inositol | 0.01802 |
| Niacinamide | 0.00003663 |
| D-Pantothenic acid·1/2 Ca | 0.000477 |
| Putrescine·2HCl | 0.0003222 |
| Pyridoxine·HCl | 0.00006168 |
| Riboflavin | 0.00003764 |
| Thiamine·HCl | 0.0003373 |
| Thymidine | 0.0007266 |
| Vitamin B12 | 0.0013554 |

| | |
|---|---|
| D+-Glucose | 1.260 |
| Pyruvic acid·Na | 0.220 |
| Phenol Red·Na | 0.00331806 |
| DL-6,8-Thioctic acid | 0.00020630 |
| Calcium chloride·Anhydrous | 0.10207 |
| Cupric sulfate·$5H_2O$ | 0.0000025 |
| Ferrous sulfate·$7H_2O$ | 0.000834 |
| Magnesium chloride [anhydrous] | 0.0495092 |
| Magnesium sulfate | 0.19264 |
| Potassium chloride | 0.28329 |
| Potassium phosphate monobasic | 0.058523 |
| Sodium phosphate dibasic | 0.11502 |
| Sodium chloride | 7.5972 |
| Zinc sulfate·$7H_2O$ | 0.00014375 |

**Preparation Instructions**

1. Measure out 90% of final required volume of water. Water temperature should be 15-20 °C.
2. While gently stirring the water, add the powdered medium. Stir until dissolved. Do NOT heat.
3. Rinse original package with a small amount of water to remove all traces of powder. Add to solution in step 2.
4. To the solution in step 3, add 2.5 g sodium bicarbonate or 33.3 ml of sodium bicarbonate solution [7.5%w/v] for each liter of final volume of medium being prepared. Stir until dissolved.
5. While stirring, adjust the pH of the medium to 0.1-0.3 pH units below the desired pH since it may rise during filtration. The use of 1N HCl or 1N NaOH is recommended.
6. Add additional water to bring the solution to final volume.
7. Sterilize immediately by filtration using a membrane with a porosity of 0.22 microns.
8. Aseptically dispense medium into sterile container.

**2. Dulbecco's Modified Eagle Medium (DMEM) for BV2 microglial cells**

With high glucose, L-glutamine and phenol red

Without sodium pyruvate, HEPES and sodium bicarbonate

Product Number: GIBCO 12100-046 (Thermo Fisher Scientific Inc.)

| **Component** | **mg/L** |
|---|---|
| Glycine | 30.0 |
| L-Arginine hydrochloride | 84.0 |
| L-Cystine 2HCl | 63.0 |
| L-Glutamine | 584.0 |
| L-Histidine hydrochloride-$H_2O$ | 42.0 |
| L-Isoleucine | 105.0 |
| L-Leucine | 105.0 |
| L-Lysine hydrochloride | 146.0 |
| L-Methionine | 30.0 |
| L-Phenylalanine | 66.0 |
| L-Serine | 42.0 |
| L-Threonine | 95.0 |
| L-Tryptophan | 16.0 |
| L-Tyrosine disodium salt dihydrate | 104.0 |
| L-Valine | 94.0 |
| Choline chloride | 4.0 |
| D-Calcium pantothenate | 4.0 |
| Folic Acid | 4.0 |
| Niacinamide | 4.0 |
| Pyridoxine hydrochloride | 4.0 |
| Riboflavin | 0.4 |
| Thiamine hydrochloride | 4.0 |
| i-Inositol | 7.2 |
| Calcium Chloride ($CaCl_2$) (anhyd.) | 200.0 |
| Ferric Nitrate ($Fe(NO_3)3"9H_2O$) | 0.1 |
| Magnesium Sulfate ($MgSO_4$) (anhyd.) | 97.67 |
| Potassium Chloride (KCl) | 400.0 |
| Sodium Chloride (NaCl) | 6400.0 |
| Sodium Phosphate monobasic | 125.0 |
| D-Glucose (Dextrose) | 4500.0 |
| Phenol Red | 15.0 |

**Preparation Instructions**

1. To a mixing container that is as close to the final volume as possible, add 950 mL of distilled water.
2. Add powdered medium to room temperature (15°C to 30°C) water with gentle stirring. Do not heat the water.
3. Rinse the inside of package to remove all traces of powder.
4. Add 3.7 g/L sodium bicarbonate to medium.
5. Adjust the pH to 0.2 and 0.3 units below the desired final working pH (7.0–7.4) by slowly adding, with stirring, 1 N NaOH or 1 N HCl. The pH may rise 0.1 to 0.3 units upon filtration.
6. Adjust the final volume (1 L) with distilled water.
7. Process the medium immediately into sterile containers by membrane filtration with a 0.2-µm filter using a positive-pressure system.

**3. Storage and stability of cell culture media**

Powdered media are hygroscopic and should be protected from moisture. The entire contents of each package should be used immediately after opening. Preparing a concentrated solution of medium is not recommended as precipitates may form. Supplements can be added prior to filtration or introduced aseptically to sterile medium. Store the dry powdered medium at 2-8 °C under dry conditions and liquid medium at 2-8 °C in the dark. Deterioration of the powdered medium may be recognized by any or all of the following: [1] color change, [2] granulation/clumping, [3] insolubility. Deterioration of the liquid medium may be recognized by any or all of the following: [1] pH change, [2] precipitate or particulates, [3] cloudy appearance [4] color change. The nature of supplements added may affect storage conditions and shelf life of the medium. Product label bears expiration date.

## B: DATA AND STATISTICAL ANALYSIS

Figure 1: Calibration plot for sodium nitrite

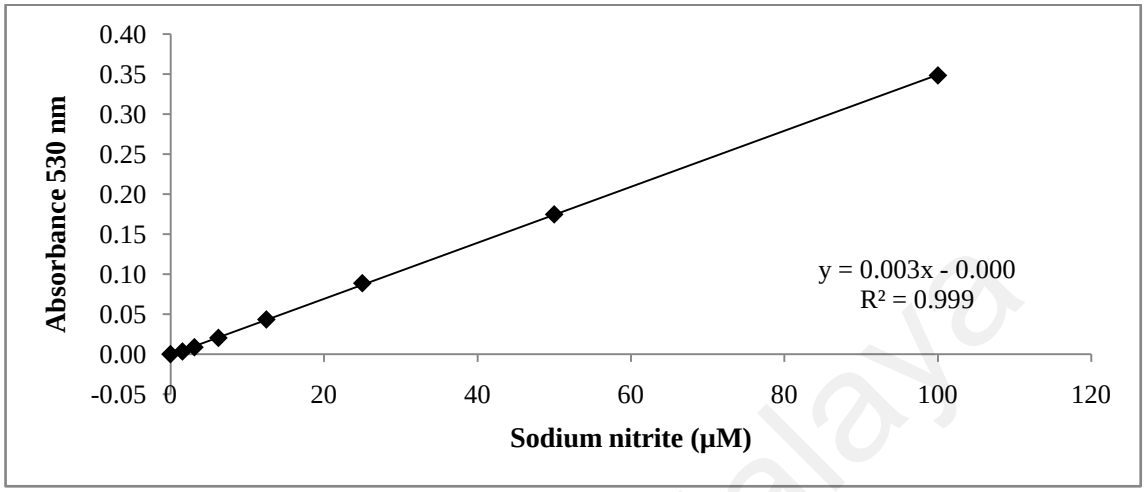


Figure 2: Calibration plot for protein concentration of BV2 microglial cells (Bradford assay)

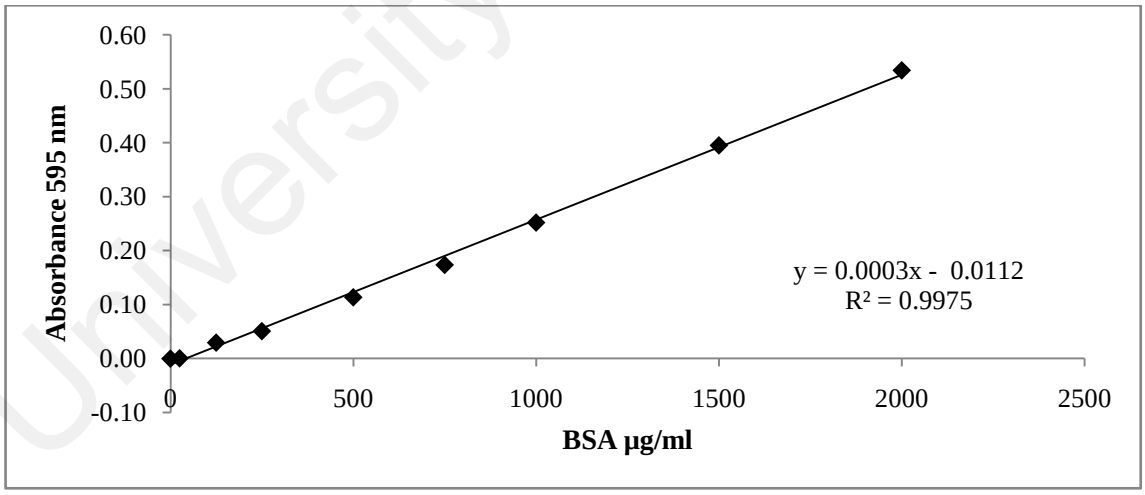

Figure 3: Calibration plot for protein concentration of PC-12 cells (Bradford assay)

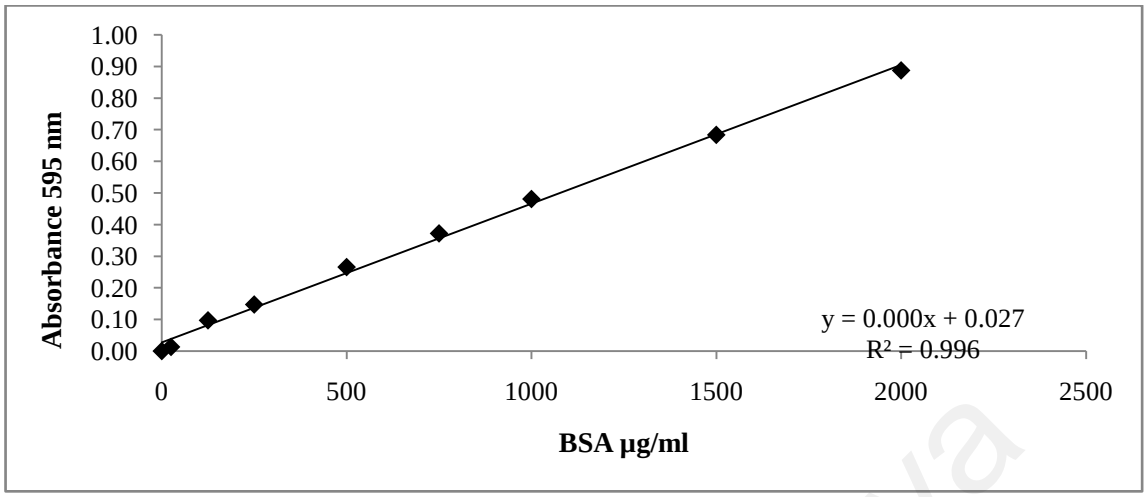


Figure 4: Calibration plot for TNF-α

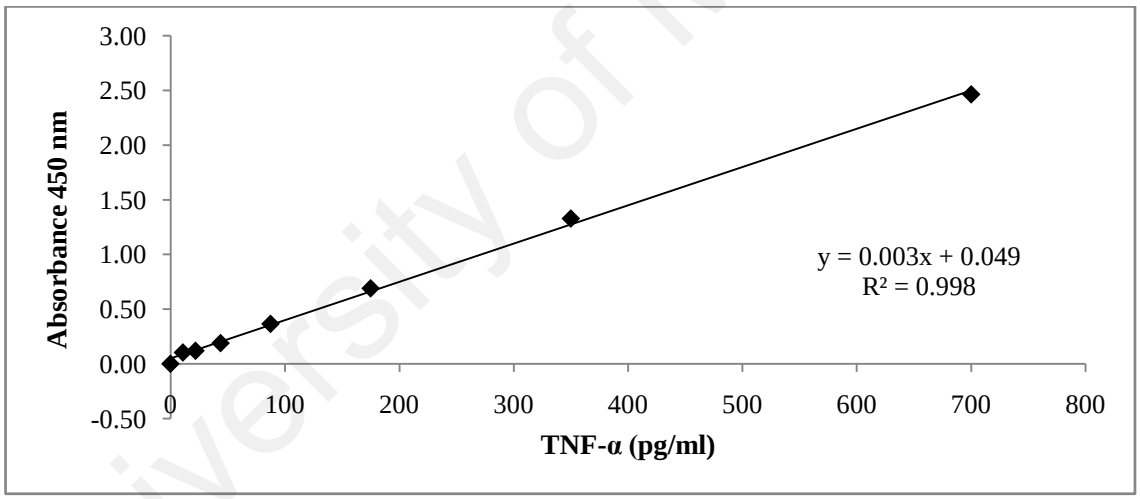

Figure 5: Calibration plot for IL-1β

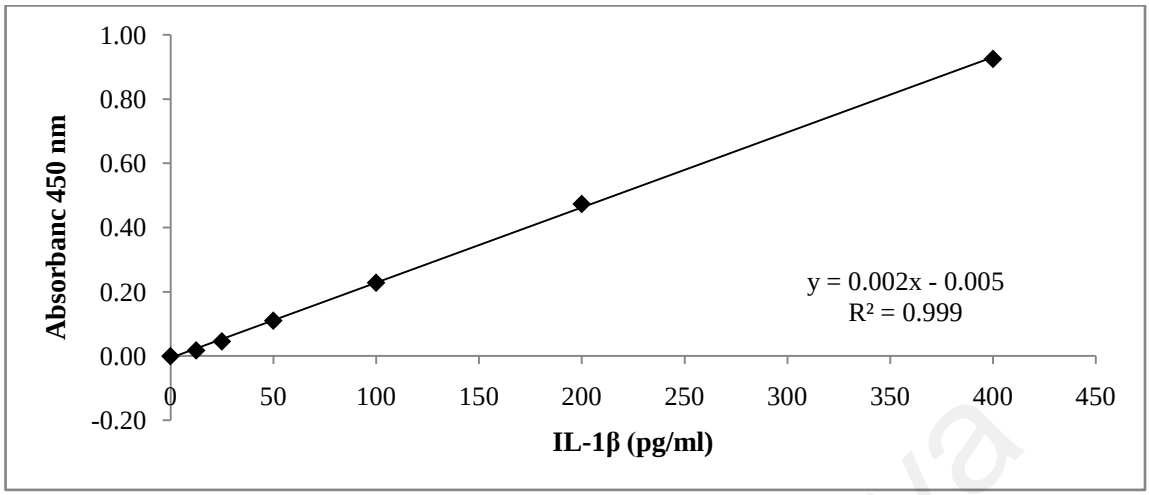


Figure 6: Calibration plot for iNOS

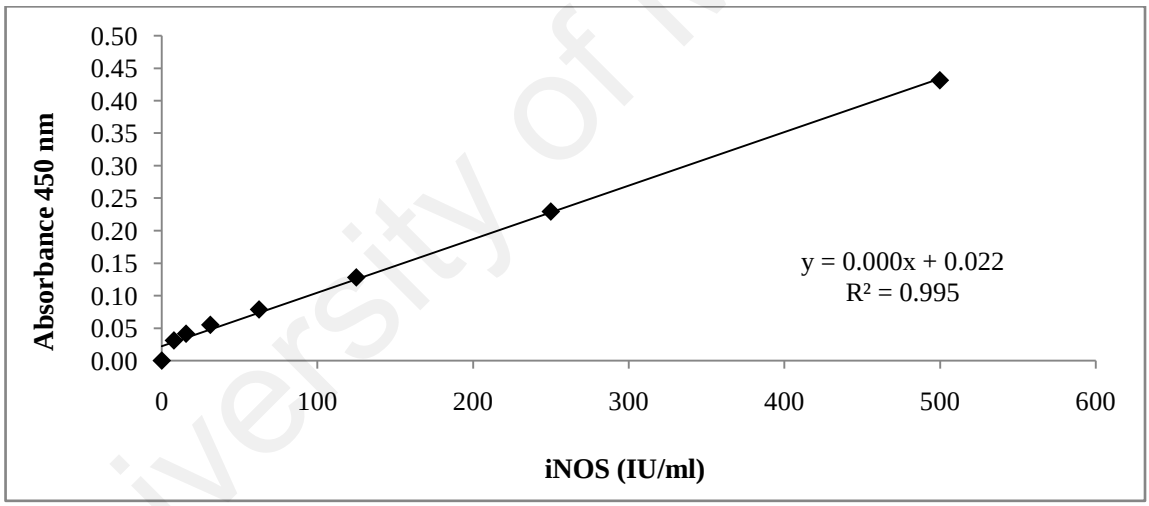

Figure 7: Calibration plot for COX-2

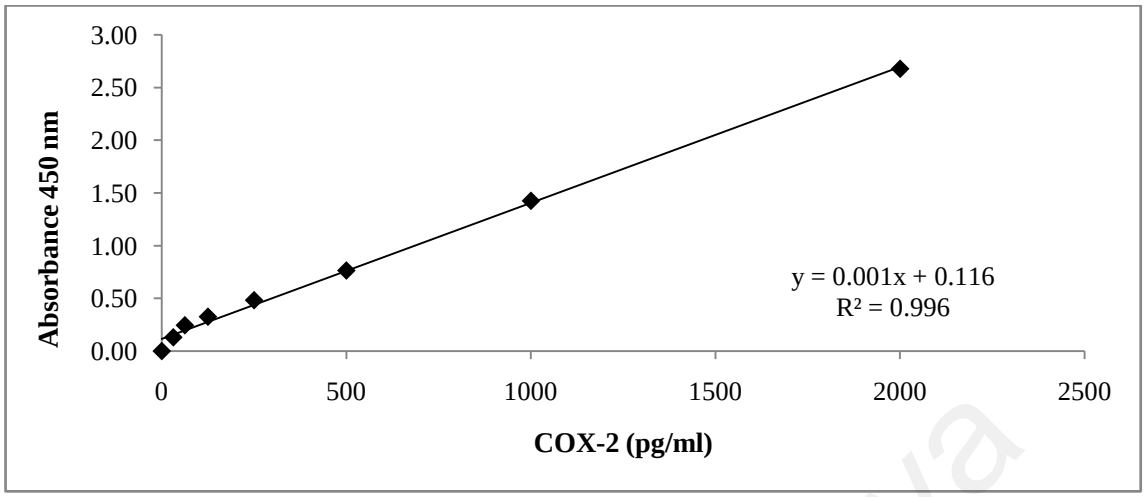


Figure 8: Calibration plot for NGF

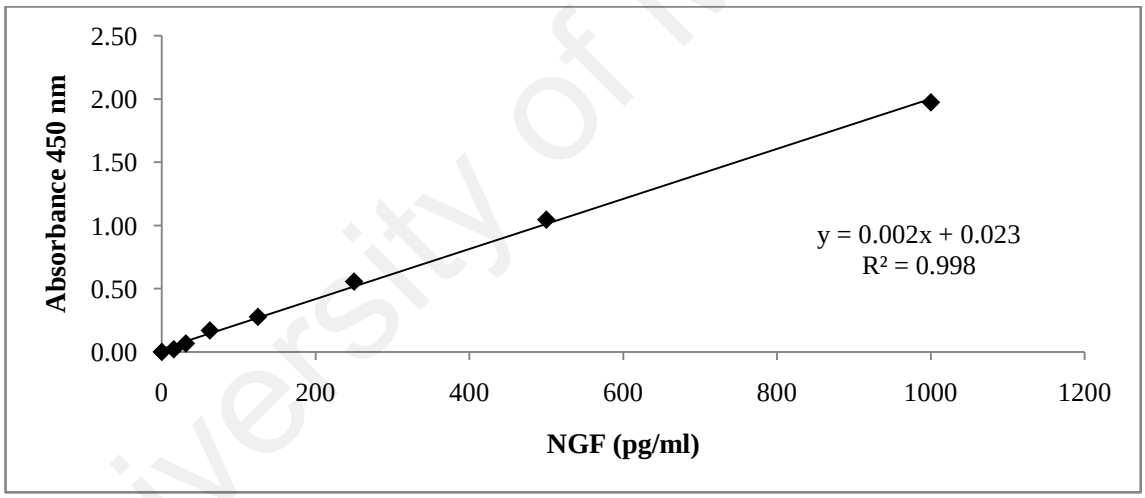

Figure 9: Calibration plot for GAP43

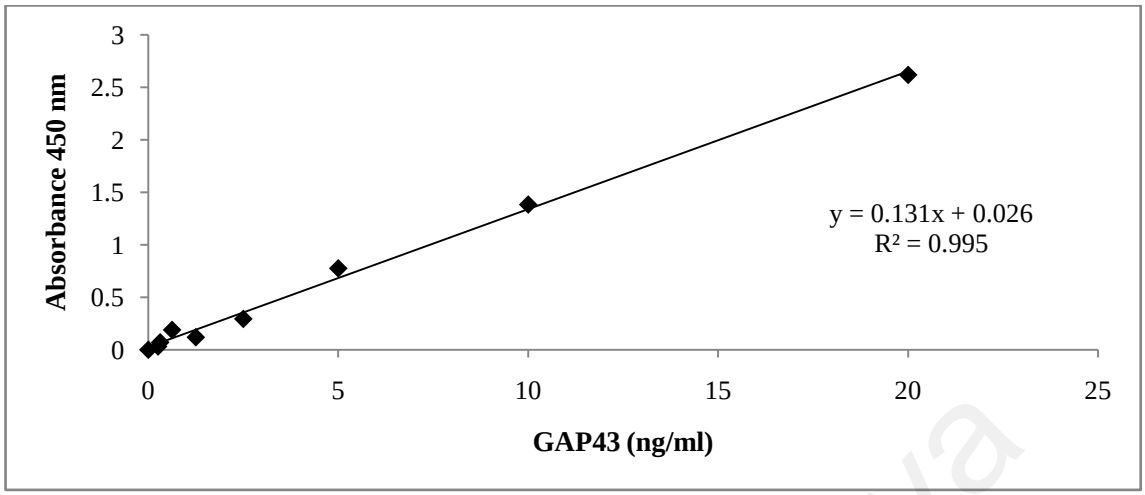


Figure 10: Calibration plot for TUBA4A

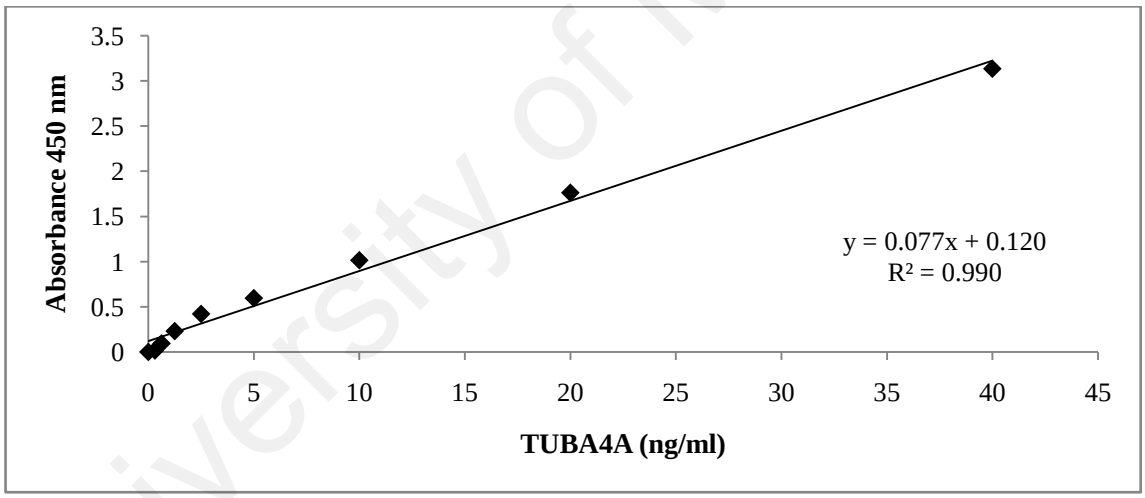

Figure 11: Calibration plot for TUBB1

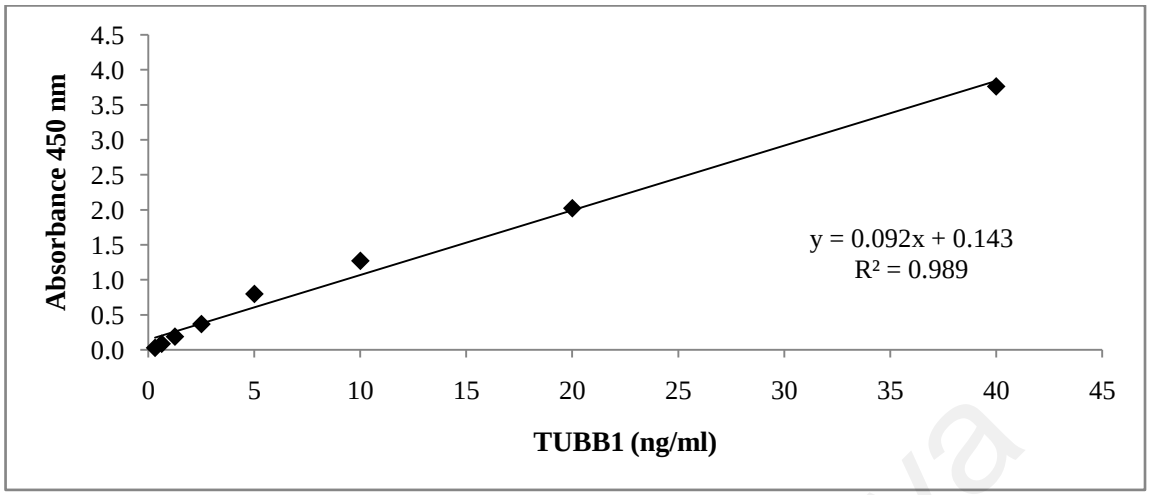

Table 1: One way analysis of variance (ANOVA): $IC_{50}$ of mushrooms extracts and crude polysaccharides on BV2 microglial cells viability after 48 hours of incubation.

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 1.2609 | 5 | 2.5208 | 3.049 | .053 |
| Within Groups | 9.9218 | 12 | 8.2677 | | |
| Total | 2.2529 | 17 | | | |

Table 1.1 Duncan Post Hoc Tests.

| | | Subset for alpha = 0.05 | |
|---|---|---|---|
| VAR00001 | N | 1 | 2 |
| *G. frondosa* hot aqueous extract | 3 | 79.9067 | |
| *G. neo-japonicum* hot aqueous extract | 3 | 218.0033 | |
| *L. rhinocerotis* ethanol extract | 3 | 41.6400 | |
| *G. lucidum* hot aqueous extract | 3 | 13566.4000 | 13566.4000 |
| *L. rhinocerotis* crude polysaccharides | 3 | 16041.8000 | 16041.8000 |
| *L. rhinocerotis* hot aqueous extract | 3 | | 20011.8933 |
| Sig. | | .073 | .425 |

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Table 2: One way analysis of variance (ANOVA): $IC_{50}$ of mushrooms extracts and crude polysaccharides on PC-12 cells viability after 48 hours of incubation.

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 2.2937 | 5 | 4585582.308 | 24.714 | .000 |
| Within Groups | 2226588.653 | 12 | 185549.054 | | |
| Total | 2.5157 | 17 | | | |

Table 2.1 Duncan Post Hoc Tests.

| | | Subset for alpha = 0.05 | | |
|---|---|---|---|---|
| VAR00001 | N | 1 | 2 | 3 |
| *L. rhinocerotis* ethanol extract | 3 | 410.5200 | | |
| *G. lucidum* hot aqueous extract | 3 | | 1500.6100 | |
| *G. neo-japonicum* hot aqueous extract | 3 | | | 3037.3467 |
| *G. frondosa* hot aqueous extract | 3 | | | 3107.5067 |
| *L. rhinocerotis* crude polysaccharides | 3 | | | 3270.5133 |
| *L. rhinocerotis* hot aqueous extract | 3 | | | 3515.1900 |
| Sig. | | 1.000 | 1.000 | .232 |

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Table 3: One way analysis of variance (ANOVA): $IC_{50}$ of hot aqueous extract and solvent fractions of *L. rhinocerotis* on BV2 microglial cells viability after 48 hours of incubation.

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 6.3268 | 3 | 2.1098 | 19.921 | .205 |
| Within Groups | 8.7828 | 8 | 1.0988 | | |
| Total | 1.5119 | 11 | | | |

Table 3.1 Duncan Post Hoc Tests.

| | | Subset for alpha = 0.05 | | | |
|---|---|---|---|---|---|
| VAR00001 | N | 1 | 2 | 3 | 4 |
| Ethyl acetate fraction | 3 | 740.0230 | | | |
| n-Butanol fraction | 3 | | 7090.1100 | | |
| Aqueous fraction | 3 | | | 15017.2267 | |
| Hot aqueous extract | 3 | | | | 20011.8933 |
| Sig. | | 1.000 | 1.000 | 1.000 | 1.000 |

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Table 4: One way analysis of variance (ANOVA): $IC_{50}$ of hot aqueous extract and solvent fractions of *L. rhinocerotis* on PC-12 cells viability after 48 hours of incubation.

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 7.3177 | 3 | 2.4397 | 23.942 | .000 |
| Within Groups | 8150150.685 | 8 | 1018768.836 | | |
| Total | 8.1327 | 11 | | | |

Table 4.1 Duncan Post Hoc Tests.

| | | Subset for alpha = 0.05 | | |
|---|---|---|---|---|
| VAR00001 | N | 1 | 2 | 3 |
| Ethyl acetate fraction | 3 | 187.9267 | | |
| n-Butanol fraction | 3 | 284.7100 | | |
| Hot aqueous extract | 3 | | 3515.1900 | |
| Aqueous fraction | 3 | | | 6120.8133 |
| Sig. | | .909 | 1.000 | 1.000 |

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Table 5: One way analysis of variance (ANOVA): Nitric oxide concentration of pre-treatment of ethyl acetate fraction in LPS-stimulated BV2 microglial cells.

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 24014.174 | 9 | 2668.242 | 42.091 | .000 |
| Within Groups | 1267.846 | 20 | 63.392 | | |
| Total | 25282.020 | 29 | | | |

Table 5.1 Duncan Post Hoc Tests.

| | | Subset for alpha = 0.05 | | | | | |
|---|---|---|---|---|---|---|---|
| VAR00001 | N | 1 | 2 | 3 | 4 | 5 | 6 |
| Negative control | 3 | .2433 | | | | | |
| 500 µg/ml | 3 | 1.5431 | | | | | |
| 250 µg/ml | 3 | | 16.8667 | | | | |
| 125 µg/ml | 3 | | | 33.1967 | | | |
| 62.50 µg/ml | 3 | | | | 48.1500 | | |
| 31.25 µg/ml | 3 | | | | 56.6833 | 56.6833 | |
| LPS control | 3 | | | | | 64.8900 | 64.8900 |
| 15.63 µg/ml | 3 | | | | | 70.5400 | 70.5400 |
| 3.91 µg/ml | 3 | | | | | | 75.4800 |
| 7.81 µg/ml | 3 | | | | | | 77.5767 |
| Sig. | | .844 | 1.000 | 1.000 | .204 | .056 | .087 |

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Table 6: One way analysis of variance (ANOVA): Nitric oxide concentration of pre-treatment of n-butanol fraction in LPS-stimulated BV2 microglial cells.

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 23404.754 | 9 | 2600.528 | 20.143 | .000 |
| Within Groups | 2582.026 | 20 | 129.101 | | |
| Total | 25986.780 | 29 | | | |

Table 6.1 Duncan Post Hoc Tests.

| VAR00001 | N | Subset for alpha = 0.05 | | | |
|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 |
| Negative control | 3 | .9367 | | | |
| 500 µg/ml | 3 | 4.3167 | | | |
| 250 µg/ml | 3 | 11.8100 | | | |
| 125 µg/ml | 3 | | 36.2667 | | |
| 62.50 µg/ml | 3 | | 40.0300 | 40.0300 | |
| 31.25 µg/ml | 3 | | | 56.6167 | 56.6167 |
| LPS control | 3 | | | | 62.8100 |
| 15.63 µg/ml | 3 | | | | 68.7333 |
| 3.91 µg/ml | 3 | | | | 76.3000 |
| 7.81 µg/ml | 3 | | | | 77.0933 |
| Sig. | | .281 | .689 | .089 | .059 |

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Table 7: One way analysis of variance (ANOVA): Nitric oxide concentration of pre-treatment of aqueous fraction in LPS-stimulated BV2 microglial cells.

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 19971.130 | 9 | 2219.014 | 3.126 | .016 |
| Within Groups | 14195.078 | 20 | 709.754 | | |
| Total | 34166.208 | 29 | | | |

Table 7.1 Duncan Post Hoc Tests.

| VAR00001 | N | Subset for alpha = 0.05 | |
|---|---|---|---|
| | | 1 | 2 |
| Negative control | 3 | .5159 | |
| 500 µg/ml | 3 | | 53.3997 |
| 250 µg/ml | 3 | | 65.8556 |
| LPS control | 3 | | 73.2427 |
| 125 µg/ml | 3 | | 75.7548 |
| 62.50 µg/ml | 3 | | 83.3423 |
| 3.91 µg/ml | 3 | | 86.0132 |
| 7.81 µg/ml | 3 | | 86.0831 |
| 31.25 µg/ml | 3 | | 87.9851 |
| 15.63 µg/ml | 3 | | 91.8563 |
| Sig. | | 1.000 | .140 |

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Table 8: One way analysis of variance (ANOVA): iNOS protein expression of solvent fractions in LPS-stimulated BV2 microglial cells.

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 1.113 | 7 | .159 | 12.046 | .000 |
| Within Groups | .211 | 16 | .013 | | |
| Total | 1.325 | 23 | | | |

Table 8.1 Duncan Post Hoc Tests.

| | | Subset for alpha = 0.05 | | |
|---|---|---|---|---|
| VAR00001 | N | 1 | 2 | 3 |
| nBF 250 µg/ml | 3 | .6200 | | |
| nBF 125 µg/ml | 3 | | .9233 | |
| Aspirin | 3 | | .9300 | |
| EAF 125 µg/ml | 3 | | .9433 | |
| Negative control | 3 | | 1.0000 | |
| L-NAME | 3 | | 1.0033 | |
| EAF 250 µg/ml | 3 | | 1.0433 | |
| LPS control | 3 | | | 1.4600 |
| Sig. | | 1.000 | .269 | 1.000 |

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Table 9: One way analysis of variance (ANOVA): iNOS mRNA expression of solvent fractions in LPS-stimulated BV2 microglial cells.

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 23.173 | 7 | 3.310 | 439.204 | .000 |
| Within Groups | .121 | 16 | .008 | | |
| Total | 23.294 | 23 | | | |

Table 9.1 Duncan Post Hoc Tests.

| | | Subset for alpha = 0.05 | | | |
|---|---|---|---|---|---|
| VAR00001 | N | 1 | 2 | 3 | 4 |
| nBF 125 µg/ml | 3 | .1500 | | | |
| Aspirin | 3 | .1507 | | | |
| nBF 250 µg/ml | 3 | .1740 | | | |
| EAF 125 µg/ml | 3 | .2207 | | | |
| L-NAME | 3 | .2770 | | | |
| EAF 250 µg/ml | 3 | | .6263 | | |
| Negative control | 3 | | | 1.0000 | |
| LPS control | 3 | | | | 3.2190 |
| Sig. | | .124 | 1.000 | 1.000 | 1.000 |

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Table 10: One way analysis of variance (ANOVA): COX-2 protein expression of solvent fractions in LPS-stimulated BV2 microglial cells.

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 1.403 | 6 | .234 | 21.905 | .000 |
| Within Groups | .149 | 14 | .011 | | |
| Total | 1.552 | 20 | | | |

Table 10.1 Duncan Post Hoc Tests.

| | | Subset for alpha = 0.05 | | | | |
|---|---|---|---|---|---|---|
| VAR00001 | N | 1 | 2 | 3 | 4 | 5 |
| Aspirin | 3 | .7967 | | | | |
| Negative control | 3 | | 1.0000 | | | |
| nBF 125 µg/ml | 3 | | 1.0300 | 1.0300 | | |
| EAF 250 µg/ml | 3 | | 1.0433 | 1.0433 | | |
| EAF 125 µg/ml | 3 | | | 1.2000 | 1.2000 | |
| nBF 250 µg/ml | 3 | | | | 1.2967 | |
| LPS control | 3 | | | | | 1.6700 |
| Sig. | | 1.000 | .634 | .075 | .271 | 1.000 |

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Table 11: One way analysis of variance (ANOVA): COX-2 mRNA expression of solvent fractions in LPS-stimulated BV2 microglial cells.

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 716.451 | 6 | 119.409 | 10.836 | .000 |
| Within Groups | 154.268 | 14 | 11.019 | | |
| Total | 870.719 | 20 | | | |

Table 11.1 Duncan Post Hoc Tests.

| | | Subset for alpha = 0.05 | |
|---|---|---|---|
| VAR00001 | N | 1 | 2 |
| Aspirin | 3 | .2460 | |
| nBF 250 µg/ml | 3 | .2547 | |
| nBF 125 µg/ml | 3 | .5883 | |
| Negative control | 3 | 1.0000 | |
| EAF 125 µg/ml | 3 | 1.4620 | |
| EAF 250 µg/ml | 3 | 4.2163 | |
| LPS control | 3 | | 17.5857 |
| Sig. | | .209 | 1.000 |

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Table 12: One way analysis of variance (ANOVA): TNF-α mRNA expression of solvent fractions in LPS-stimulated BV2 microglial cells.

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 816.356 | 6 | 136.059 | 4.575 | .004 |
| Within Groups | 624.488 | 21 | 29.738 | | |
| Total | 1440.844 | 27 | | | |

Table 12.1 Duncan Post Hoc Tests.

| | | Subset for alpha = 0.05 | |
|---|---|---|---|
| VAR00001 | N | 1 | 2 |
| nBF 125 µg/ml | 4 | .8585 | |
| Negative control | 4 | 1.0000 | |
| ASpirin | 4 | 1.4330 | |
| nBF 250 µg/ml | 4 | 1.5410 | |
| EAF 125 µg/ml | 4 | 3.1033 | |
| EAF 250 µg/ml | 4 | 3.4053 | |
| LPS control | 4 | | 17.0940 |
| Sig. | | .565 | 1.000 |

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 4.000.

Table 13: One way analysis of variance (ANOVA): IL-1β mRNA expression of solvent fractions in LPS-stimulated BV2 microglial cells.

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 7.208 | 6 | 1.201 | 3.714 | .020 |
| Within Groups | 4.528 | 14 | .323 | | |
| Total | 11.736 | 20 | | | |

Table 13.1 Duncan Post Hoc Tests.

| | | Subset for alpha = 0.05 | |
|---|---|---|---|
| VAR00001 | N | 1 | 2 |
| EAF 250µg/ml | 3 | .5510 | |
| Aspirin | 3 | .5943 | |
| EAF 125 µg/ml | 3 | .7047 | |
| Negative control | 3 | 1.0000 | |
| nBF 250 µg/ml | 3 | 1.0553 | |
| nBF 125 µg/ml | 3 | 1.0570 | |
| LPS control | 3 | | 2.4007 |
| Sig. | | .343 | 1.000 |

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Table 14: One way analysis of variance (ANOVA): IL-6 mRNA expression of solvent fractions in LPS-stimulated BV2 microglial cells.

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 23.486 | 6 | 3.914 | 230.305 | .000 |
| Within Groups | .238 | 14 | .017 | | |
| Total | 23.724 | 20 | | | |

Table 14.1 Duncan Post Hoc Tests.

| | | Subset for alpha = 0.05 | | | | |
|---|---|---|---|---|---|---|
| VAR00001 | N | 1 | 2 | 3 | 4 | 5 |
| EAF 250 µg/ml | 3 | .1673 | | | | |
| Aspirin | 3 | .3697 | .3697 | | | |
| EAF 125 µg/ml | 3 | | .4503 | | | |
| nBF 250 µg/ml | 3 | | | .7777 | | |
| Negative control | 3 | | | 1.0000 | 1.0000 | |
| nBF 125 µg/ml | 3 | | | | 1.1250 | |
| LPS control | 3 | | | | | 3.5290 |
| Sig. | | .078 | .461 | .055 | .260 | 1.000 |

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Table 15: One way analysis of variance (ANOVA): TLR4 mRNA expression of solvent fractions in LPS-stimulated BV2 microglial cells.

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 38.850 | 6 | 6.475 | 57.308 | .000 |
| Within Groups | .791 | 7 | .113 | | |
| Total | 39.641 | 13 | | | |

Table 15.1 Duncan Post Hoc Tests.

| | | Subset for alpha = 0.05 | | | |
|---|---|---|---|---|---|
| VAR00001 | N | 1 | 2 | 3 | 4 |
| Negative control | 3 | 1.0000 | | | |
| EAF 250 µg/ml | 3 | | 2.0825 | | |
| EAF 125 µg/ml | 3 | | 2.3775 | | |
| Aspirin | 3 | | 2.6845 | | |
| LPS control | 3 | | | 4.8545 | |
| nBF 125 µg/ml | 3 | | | 4.8590 | |
| nBF 250 µg/ml | 3 | | | | 5.8755 |
| Sig. | | 1.000 | .129 | .990 | 1.000 |

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Table 16: One way analysis of variance (ANOVA): p-IκB-α protein expression of solvent fractions in LPS-stimulated BV2 microglial cells.

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | .431 | 6 | .072 | 49.245 | .000 |
| Within Groups | .020 | 14 | .001 | | |
| Total | .451 | 20 | | | |

Table 16.1 Duncan Post Hoc Tests.

| | | Subset for alpha = 0.05 | | |
|---|---|---|---|---|
| VAR00001 | N | 1 | 2 | 3 |
| Negative control | 3 | .1797 | | |
| Aspirin | 3 | .1950 | | |
| EAF 250 µg/ml | 3 | | .3557 | |
| EAF 125 µg/ml | 3 | | .3663 | |
| nBF 250 µg/ml | 3 | | .3723 | |
| nBF 125 µg/ml | 3 | | .3740 | |
| LPS control | 3 | | | .6490 |
| Sig. | | .630 | .596 | 1.000 |

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Table 17: One way analysis of variance (ANOVA): p-NFκB p65 protein expression of solvent fractions in LPS-stimulated BV2 microglial cells.

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | .219 | 6 | .036 | 55.129 | .000 |
| Within Groups | .014 | 21 | .001 | | |
| Total | .233 | 27 | | | |

Table 17.1 Duncan Post Hoc Tests.

| | | Subset for alpha = 0.05 | |
|---|---|---|---|
| VAR00001 | N | 1 | 2 |
| Negative control | 4 | .1698 | |
| EAF 250 µg/ml | 4 | .1800 | |
| nBF 250 µg/ml | 4 | .1868 | |
| Aspirin | 4 | .1982 | |
| nBF 125 µg/ml | 4 | .1983 | |
| EAF 125 µg/ml | 4 | .2050 | |
| LPS control | 4 | | .4403 |
| Sig. | | .100 | 1.000 |

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 4.000.

Table 18: One way analysis of variance (ANOVA): NFκB p65 mRNA expression of solvent fractions in LPS-stimulated BV2 microglial cells.

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 15.047 | 6 | 2.508 | 26.473 | .000 |
| Within Groups | 1.326 | 14 | .095 | | |
| Total | 16.374 | 20 | | | |

Table 18.1 Duncan Post Hoc Tests.

| | | Subset for alpha = 0.05 | |
|---|---|---|---|
| VAR00001 | N | 1 | 2 |
| nBF 125 µg/ml | 3 | .5393 | |
| Aspirin | 3 | .6457 | |
| EAF 250 µg/ml | 3 | .8457 | |
| EAF 125 µg/ml | 3 | .8813 | |
| nBF 250 µg/ml | 3 | .9920 | |
| Negative control | 3 | 1.0000 | |
| LPS control | 3 | | 3.1937 |
| Sig. | | .122 | 1.000 |

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Table 19: One way analysis of variance (ANOVA): NFκB p50 mRNA expression of solvent fractions in LPS-stimulated BV2 microglial cells.

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 25.022 | 6 | 4.170 | 80.718 | .000 |
| Within Groups | .723 | 14 | .052 | | |
| Total | 25.746 | 20 | | | |

Table 19.1 Duncan Post Hoc Tests.

| | | Subset for alpha = 0.05 | |
|---|---|---|---|
| VAR00001 | N | 1 | 2 |
| nBF 125 µg/ml | 3 | .9137 | |
| Aspirin | 3 | .9717 | |
| Negative control | 3 | 1.0000 | |
| EAF 125 µg/ml | 3 | 1.0370 | |
| EAF 250 µg/ml | 3 | 1.1517 | |
| nBF 250 µg/ml | 3 | 1.3403 | |
| LPS control | 3 | | 4.1660 |
| Sig. | | .058 | 1.000 |

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Table 20: One way analysis of variance (ANOVA): p-ERK1/2 protein expression of solvent fractions in LPS-stimulated BV2 microglial cells.

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | .758 | 6 | .126 | 310.836 | .000 |
| Within Groups | .006 | 14 | .000 | | |
| Total | .763 | 20 | | | |

Table 20.1 Duncan Post Hoc Tests.

| | | Subset for alpha = 0.05 | | | | |
|---|---|---|---|---|---|---|
| VAR00001 | N | 1 | 2 | 3 | 4 | 5 |
| EAF 250 µg/ml | 3 | .2633 | | | | |
| Negative control | 3 | .2763 | | | | |
| nBF 250 µg/ml | 3 | | .3860 | | | |
| nBF125 µg/ml | 3 | | .3917 | | | |
| Aspirin | 3 | | | .5393 | | |
| EAF 125 µg/ml | 3 | | | | .6030 | |
| LPS control | 3 | | | | | .8397 |
| Sig. | | .443 | .736 | 1.000 | 1.000 | 1.000 |

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Table 21: One way analysis of variance (ANOVA): p-p38 MAPK protein expression of solvent fractions in LPS-stimulated BV2 microglial cells.

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | .132 | 6 | .022 | 3.702 | .011 |
| Within Groups | .125 | 21 | .006 | | |
| Total | .257 | 27 | | | |

Table 21.1 Duncan Post Hoc Tests.

| | | Subset for alpha = 0.05 | |
|---|---|---|---|
| VAR00001 | N | 1 | 2 |
| nBF 125 µg/ml | 4 | .1683 | |
| Negative control | 4 | .1693 | |
| EAF 125 µg/ml | 4 | .1745 | |
| Aspirin | 4 | .1760 | |
| nBF 250 µg/ml | 4 | .1973 | |
| EAF 250 µg/ml | 4 | .2060 | |
| LPS control | 4 | | .3745 |
| Sig. | | .547 | 1.000 |

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 4.000.

Table 22: One way analysis of variance (ANOVA): p-SAPK/JNK protein expression of solvent fractions in LPS-stimulated BV2 microglial cells.

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 8.968 | 6 | 1.495 | 7.302 | .000 |
| Within Groups | 4.299 | 21 | .205 | | |
| Total | 13.266 | 27 | | | |

Table 22.1 Duncan Post Hoc Tests.

| | | Subset for alpha = 0.05 | |
|---|---|---|---|
| VAR00001 | N | 1 | 2 |
| nBF 250 µg/ml | 4 | .4277 | |
| Negative control | 4 | .4535 | |
| nBF 125 µg/ml | 4 | .4965 | |
| EAF 250 µg/ml | 4 | .6523 | |
| EAF 125 µg/ml | 4 | .6605 | |
| Aspirin | 4 | 1.1157 | |
| LPS control | 4 | | 2.1290 |
| Sig. | | .069 | 1.000 |

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 4.000.

Table 23: One way analysis of variance (ANOVA): p-AKT protein expression of solvent fractions in LPS-stimulated BV2 microglial cells.

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | .154 | 6 | .026 | 28.322 | .000 |
| Within Groups | .013 | 14 | .001 | | |
| Total | .167 | 20 | | | |

Table 23.1 Duncan Post Hoc Tests.

| | | Subset for alpha = 0.05 | | | |
|---|---|---|---|---|---|
| VAR00001 | N | 1 | 2 | 3 | 4 |
| EAF 250 µg/ml | 3 | .1273 | | | |
| Negative control | 3 | .1420 | .1420 | | |
| Aspirin | 3 | | .1837 | .1837 | |
| EAF 125 µg/ml | 3 | | .1857 | .1857 | |
| nBF 125 µg/ml | 3 | | | .2143 | |
| nBF 250 µg/ml | 3 | | | .2180 | |
| LPS control | 3 | | | | .4067 |
| Sig. | | .561 | .113 | .218 | 1.000 |

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Table 24: One way analysis of variance (ANOVA): p-CREB protein expression of solvent fractions in LPS-stimulated BV2 microglial cells.

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 7.999 | 6 | 1.333 | 41.775 | .000 |
| Within Groups | .447 | 14 | .032 | | |
| Total | 8.446 | 20 | | | |

Table 24.1 Duncan Post Hoc Tests.

| | | Subset for alpha = 0.05 | | | | |
|---|---|---|---|---|---|---|
| VAR00001 | N | 1 | 2 | 3 | 4 | 5 |
| EAF 250 µg/ml | 3 | 2.2087 | | | | |
| Negative control | 3 | 2.2600 | | | | |
| Aspirin | 3 | | 2.6613 | | | |
| EAF 125 µg/ml | 3 | | | 2.9863 | | |
| nBF 125 µg/ml | 3 | | | 3.2053 | 3.2053 | |
| nBF 250 µg/ml | 3 | | | | 3.3803 | |
| LPS control | 3 | | | | | 4.0940 |
| Sig. | | .730 | 1.000 | .155 | .250 | 1.000 |

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Table 25: One way analysis of variance (ANOVA): cFOS mRNA expression of solvent fractions in LPS-stimulated BV2 microglial cells.

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 3.019 | 6 | .503 | 18.342 | .000 |
| Within Groups | .384 | 14 | .027 | | |
| Total | 3.403 | 20 | | | |

Table 25.1 Duncan Post Hoc Tests.

| | | Subset for alpha = 0.05 | | | |
|---|---|---|---|---|---|
| VAR00001 | N | 1 | 2 | 3 | 4 |
| EAF 125 µg/ml | 3 | .5127 | | | |
| nBF 250 µg/ml | 3 | .7633 | .7633 | | |
| nBF 125 µg/ml | 3 | | .9723 | .9723 | |
| EAF 250 µg/ml | 3 | | .9973 | .9973 | |
| Negative control | 3 | | 1.0000 | 1.0000 | |
| Aspirin | 3 | | | 1.2250 | |
| LPS control | 3 | | | | 1.8207 |
| Sig. | | .085 | .128 | .106 | 1.000 |

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Table 26: One way analysis of variance (ANOVA): cJUN mRNA expression of solvent fractions in LPS-stimulated BV2 microglial cells.

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 28.609 | 6 | 4.768 | 64.155 | .000 |
| Within Groups | 1.041 | 14 | .074 | | |
| Total | 29.650 | 20 | | | |

Table 26.1 Duncan Post Hoc Tests.

| | | Subset for alpha = 0.05 | | | |
|---|---|---|---|---|---|
| VAR00001 | N | 1 | 2 | 3 | 4 |
| EAF 250 µg/ml | 3 | .3633 | | | |
| Aspirin | 3 | .4430 | .4430 | | |
| EAF 125 µg/ml | 3 | .7600 | .7600 | .7600 | |
| nBF 125 µg/ml | 3 | | .9273 | .9273 | |
| Negative control | 3 | | | 1.0000 | |
| nBF 250 µg/ml | 3 | | | 1.0813 | |
| LPS control | 3 | | | | 4.0190 |
| Sig. | | .112 | .057 | .204 | 1.000 |

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Table 27: One way analysis of variance (ANOVA): Neuritogenic effect of hot aqueous extract and solvent fractions in PC-12 cells.

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 534.632 | 21 | 25.459 | 30.732 | .000 |
| Within Groups | 36.451 | 44 | .828 | | |
| Total | 571.083 | 65 | | | |

Table 27.1 Duncan Post Hoc Tests.

| | | Subset for alpha = 0.05 | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| VAR00001 | N | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| Neg. control | 3 | 4.5467 | | | | | | | | | | | |
| AF 100 µg/ml | 3 | 4.6367 | | | | | | | | | | | |
| AF 75 µg/ml | 3 | 6.0567 | 6.0567 | | | | | | | | | | |
| HAE 100 µg/ml | 3 | 6.0933 | 6.0933 | | | | | | | | | | |
| EAF 100 µg/ml | 3 | | 6.4533 | 6.4533 | | | | | | | | | |
| nBF 100 µg/ml | 3 | | 6.9367 | 6.9367 | 6.9367 | | | | | | | | |
| HAE 10 µg/ml | 3 | | | 7.9200 | 7.9200 | 7.9200 | | | | | | | |
| HAE 75 µg/ml | 3 | | | | 8.4400 | 8.4400 | 8.4400 | | | | | | |
| AF 50 µg/ml | 3 | | | | | 8.7567 | 8.7567 | 8.7567 | | | | | |
| EAF 75 µg/ml | 3 | | | | | 9.0633 | 9.0633 | 9.0633 | | | | | |
| AF 10 µg/ml | 3 | | | | | 9.2200 | 9.2200 | 9.2200 | 9.2200 | | | | |
| EAF 50 µg/ml | 3 | | | | | 9.3033 | 9.3033 | 9.3033 | 9.3033 | | | | |
| nBF 75 µg/ml | 3 | | | | | 9.6233 | 9.6233 | 9.6233 | 9.6233 | 9.6233 | | | |
| AF 25 µg/ml | 3 | | | | | | 9.7667 | 9.7667 | 9.7667 | 9.7667 | | | |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| HAE 50 µg/ml | 3 | | | | | | 9.9200 | 9.9200 | 9.9200 | 9.9200 | | | |
| nBF 50 µg/ml | 3 | | | | | | | 10.3467 | 10.3467 | 10.3467 | | | |
| nBF 25 µg/ml | 3 | | | | | | | | 10.8133 | 10.8133 | | | |
| EAF 25 µg/ml | 3 | | | | | | | | | 11.1633 | 11.1633 | | |
| NGF | 3 | | | | | | | | | | 12.6133 | 12.6133 | |
| HAE 25 µg/ml | 3 | | | | | | | | | | | 12.7967 | |
| EAF 10 µg/ml | 3 | | | | | | | | | | | | 14.5400 |
| nBF 10 µg/ml | 3 | | | | | | | | | | | | 15.4767 |
| Sig. | | .062 | .289 | .068 | .061 | .051 | .094 | .072 | .068 | .074 | .057 | .806 | .214 |

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Table 28: One way analysis of variance (ANOVA): Extracellular NGF concentration in hot aqueous extract- and solvent fractions-treated PC-12 cells.

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 206793.913 | 4 | 51698.478 | 887.899 | .000 |
| Within Groups | 582.256 | 10 | 58.226 | | |
| Total | 207376.169 | 14 | | | |

Table 28.1 Duncan Post Hoc Tests.

| | | Subset for alpha = 0.05 | |
|---|---|---|---|
| VAR00001 | N | 1 | 2 |
| Negative control | 3 | 91.6500 | |
| Aqueous fraction | 3 | 93.9833 | |
| n-Butanol fraction | 3 | 94.3167 | |
| Ethyl acetate fraction | 3 | 99.4433 | |
| NGF | 3 | | 388.3167 |
| Sig. | | .270 | 1.000 |

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Table 29: One way analysis of variance (ANOVA): The effect of specific inhibitors in solvent fractions-stimulated neuritogenesis in PC-12 cells.

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 2141.732 | 24 | 89.239 | 481.158 | .000 |
| Within Groups | 9.273 | 50 | .185 | | |
| Total | 2151.006 | 74 | | | |

Table 29.1 Duncan Post Hoc Tests.

| | | Subset for alpha = 0.05 | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| VAR00001 | N | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
| Neg. control PD98059 | 3 | 1.1400 | | | | | | | | | | | | | |
| NGF PD98059 | 3 | 1.2867 | 1.2867 | | | | | | | | | | | | |
| nBF U0126 | 3 | 1.3500 | 1.3500 | 1.3500 | | | | | | | | | | | |
| nBF PD98059 | 3 | 1.5267 | 1.5267 | 1.5267 | 1.5267 | | | | | | | | | | |
| Neg. control U0126 | 3 | 1.7300 | 1.7300 | 1.7300 | 1.7300 | | | | | | | | | | |
| EAF U0126 | 3 | 1.8967 | 1.8967 | 1.8967 | 1.8967 | 1.8967 | | | | | | | | | |
| EAF PD98059 | 3 | | 1.9833 | 1.9833 | 1.9833 | 1.9833 | | | | | | | | | |
| NGF U0126 | 3 | | | 2.1100 | 2.1100 | 2.1100 | 2.1100 | | | | | | | | |
| HAE U0126 | 3 | | | | 2.2500 | 2.2500 | 2.2500 | 2.2500 | | | | | | | |
| Neg. control LY294002 | 3 | | | | | 2.5800 | 2.5800 | 2.5800 | 2.5800 | | | | | | |
| HAE PD98059 | 3 | | | | | 2.6033 | 2.6033 | 2.6033 | 2.6033 | | | | | | |
| HAE LY294002 | 3 | | | | | 2.6033 | 2.6033 | 2.6033 | 2.6033 | | | | | | |
| nBF LY294002 | 3 | | | | | 2.6133 | 2.6133 | 2.6133 | 2.6133 | | | | | | |
| nBF K252a | 3 | | | | | 2.7000 | 2.7000 | 2.7000 | 2.7000 | | | | | | |
| NGF K252a | 3 | | | | | 2.7233 | 2.7233 | 2.7233 | 2.7233 | | | | | | |
| EAF K252a | 3 | | | | | | 2.8467 | 2.8467 | 2.8467 | | | | | | |
| Neg. control K252a | 3 | | | | | | 2.8700 | 2.8700 | 2.8700 | | | | | | |
| HAE K252a | 3 | | | | | | | 3.0633 | 3.0633 | | | | | | |
| EAF LY294002 | 3 | | | | | | | | 3.3667 | | | | | | |
| NGF LY294002 | 3 | | | | | | | | | 4.7667 | | | | | |
| Neg. control | 3 | | | | | | | | | | 7.6467 | | | | |
| NGF | 3 | | | | | | | | | | | 15.2367 | | | |
| HAE | 3 | | | | | | | | | | | | 16.0967 | | |
| EAF | 3 | | | | | | | | | | | | | 17.2067 | |
| nBF | 3 | | | | | | | | | | | | | | 18.4767 |
| Sig. | | .063 | .087 | .062 | .076 | .051 | .073 | .055 | .064 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 |

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Table 30: One way analysis of variance (ANOVA): p-MEK1/2 protein expression of solvent fractions in PC-12 cells.

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | .438 | 3 | .146 | 36.310 | .000 |
| Within Groups | .032 | 8 | .004 | | |
| Total | .471 | 11 | | | |

Table 30.1 Duncan Post Hoc Tests.

| | | Subset for alpha = 0.05 | |
|---|---|---|---|
| VAR00001 | N | 1 | 2 |
| Negative control | 3 | .5353 | |
| Ethyl acetate fraction | 3 | | .8990 |
| n-Butanol fraction | 3 | | .9877 |
| NGF | 3 | | 1.0113 |
| Sig. | | 1.000 | .071 |

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Table 31: One way analysis of variance (ANOVA): p-ERK1/2 protein expression of solvent fractions in PC-12 cells.

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | .397 | 4 | .099 | 60.077 | .000 |
| Within Groups | .017 | 10 | .002 | | |
| Total | .413 | 14 | | | |

Table 31.1 Duncan Post Hoc Tests.

| | | Subset for alpha = 0.05 | | |
|---|---|---|---|---|
| VAR00001 | N | 1 | 2 | 3 |
| Negative control | 3 | .2753 | | |
| Ethyl acetate fraction | 3 | | .6607 | .6607 |
| n-Butanol fraction | 3 | | .6643 | .6643 |
| NGF | 3 | | | .7353 |
| Sig. | | 1.000 | .346 | .057 |

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Table 32: One way analysis of variance (ANOVA): p-AKT protein expression of solvent fractions in PC-12 cells.

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | .050 | 3 | .017 | 49.058 | .000 |
| Within Groups | .003 | 8 | .000 | | |
| Total | .052 | 11 | | | |

Table 32.1 Duncan Post Hoc Tests.

| | | Subset for alpha = 0.05 | |
|---|---|---|---|
| VAR00001 | N | 1 | 2 |
| Negative control | 3 | .1377 | |
| NGF | 3 | | .2733 |
| Ethyl acetate fraction | 3 | | .2788 |
| n-Butanol fraction | 3 | | .3010 |
| Sig. | | 1.000 | .115 |

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Table 33: One way analysis of variance (ANOVA): p-mTOR protein expression of solvent fractions in PC-12 cells.

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | .240 | 3 | .080 | 92.635 | .000 |
| Within Groups | .003 | 4 | .001 | | |
| Total | .243 | 7 | | | |

Table 33.1 Duncan Post Hoc Tests.

| | | Subset for alpha = 0.05 | |
|---|---|---|---|
| VAR00001 | N | 1 | 2 |
| Negative control | 3 | .1275 | |
| Ethyl acetate fraction | 3 | | .5125 |
| NGF | 3 | | .5150 |
| n-Butanol fraction | 3 | | .5500 |
| Sig. | | 1.000 | .277 |

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Table 34: One way analysis of variance (ANOVA): p-CREB protein expression of solvent fractions in PC-12 cells.

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | .256 | 3 | .085 | 51.099 | .000 |
| Within Groups | .013 | 8 | .002 | | |
| Total | .270 | 11 | | | |

Table 34.1 Duncan Post Hoc Tests.

| | | Subset for alpha = 0.05 | | |
|---|---|---|---|---|
| VAR00001 | N | 1 | 2 | 3 |
| Negative control | 3 | .1640 | | |
| NGF | 3 | | .3847 | |
| Ethyl acetate fraction | 3 | | | .4942 |
| n-Butanol fraction | 3 | | | .5437 |
| Sig. | | 1.000 | 1.000 | .177 |

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Table 35: One way analysis of variance (ANOVA): GAP43 protein expression of solvent fractions in PC-12 cells.

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | .040 | 3 | .013 | 15.894 | .011 |
| Within Groups | .003 | 4 | .001 | | |
| Total | .043 | 7 | | | |

Table 35.1 Duncan Post Hoc Tests.

| | | Subset for alpha = 0.05 | |
|---|---|---|---|
| VAR00001 | N | 1 | 2 |
| Negative control | 3 | .0880 | |
| NGF | 3 | | .2365 |
| n-Butanol fraction | 3 | | .2405 |
| Ethyl acetate fraction | 3 | | .2675 |
| Sig. | | 1.000 | .349 |

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Table 36: One way analysis of variance (ANOVA): TUBA4A protein expression of solvent fractions in PC-12 cells.

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | .729 | 3 | .243 | 3.992 | .052 |
| Within Groups | .487 | 8 | .061 | | |
| Total | 1.216 | 11 | | | |

Table 36.1 Duncan Post Hoc Tests.

| | | Subset for alpha = 0.05 | |
|---|---|---|---|
| VAR00001 | N | 1 | 2 |
| Negative control | 3 | .2030 | |
| Ethyl acetate fraction | 3 | .6330 | .6330 |
| NGF | 3 | | .7420 |
| n-Butanol fraction | 3 | | .8537 |
| Sig. | | .065 | .324 |

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Table 37: One way analysis of variance (ANOVA): TUBB1 protein expression of solvent fractions in PC-12 cells.

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 3.537 | 3 | 1.179 | 4.189 | .047 |
| Within Groups | 2.251 | 8 | .281 | | |
| Total | 5.788 | 11 | | | |

Table 37.1 Duncan Post Hoc Tests.

| | | Subset for alpha = 0.05 | |
|---|---|---|---|
| VAR00001 | N | 1 | 2 |
| Negative control | 3 | 1.7700 | |
| Ethyl acetate fraction | 3 | | 3.0953 |
| n-Butanol fraction | 3 | | 3.0533 |
| NGF | 3 | | 2.8813 |
| Sig. | | 1.000 | .648 |

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

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RESEARCH ARTICLE Open Access

# Potentiation of neuritogenic activity of medicinal mushrooms in rat pheochromocytoma cells

Syntyche Ling-Sing Seow[1,2], Murali Naidu[1,3*], Pamela David[1,3], Kah-Hui Wong[1,3] and Vikineswary Sabaratnam[1,2]

## Abstract

**Background:** Senescence of the neurons is believed to be a focal factor in the development of age-related neurodegenerative diseases such as Alzheimer's disease. Diminutions in the levels of nerve growth factor (NGF) lead to major declines in brain cell performance. Functional foods, believed to mitigate this deficiency, will be reaching a plateau in the near future market of alternative and preventive medicine. In the search for neuroactive compounds that mimic the NGF activity for the prevention of neurodegenerative diseases, the potential medicinal values of culinary and medicinal mushrooms attract intense interest.

**Methods:** Cytotoxic effects of aqueous extracts of three medicinal mushrooms basidiocarps, *Ganoderma lucidum*, *Ganoderma neo-japonicum* and *Grifola frondosa* towards rat pheochromocytoma (PC-12) cells were determined by 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The potentiation of neuritogenic activity was assessed by neurite outgrowth stimulation assay. Involvement of cellular signaling pathways, mitogen-activated protein kinase kinase/extracellular signal-regulated kinase (MEK/ERK1/2) and phosphoinositide-3-kinase/protein kinase B (PI3K/Akt) in mushrooms-stimulated neuritogenesis were examined by using specific pharmacological inhibitors. Alteration of neuronal morphology by inhibitors was visualized by immunofluorescence staining of the neurofilament.

**Results:** All the aqueous extracts tested caused a marked stimulation of neuritogenesis with no detectable cytotoxic effects towards PC-12 cells. The aqueous extract of *G. neo-japonicum* triggered maximal stimulation of neurite outgrowth at a lower concentration (50 μg/ml) with 14.22 ± 0.43% of neurite-bearing cells, compared to *G. lucidum* and *G. frondosa* that act at a higher concentration (75 μg/ml), with 12.61 ± 0.11% and 12.07 ± 0.46% of neurite-bearing cells, respectively. The activation of MEK/ERK1/2 and PI3K/Akt signaling pathways were necessary for the NGF and aqueous extracts to promote neuritogenesis.

**Conclusions:** *Ganoderma lucidum*, *G. neo-japonicum* and *G. frondosa* may contain NGF-like bioactive compound(s) for maintaining and regenerating the neuronal communications network. The present study reports the first evidence of the neuritogenic effects of aqueous extracts of basidiocarps of *G. neo-japonicum in-vitro* and showed the involvement of MEK/ERK1/2 and P13K/Akt signaling pathways for neuritogenesis in PC-12 cells.

**Keywords:** *Ganoderma lucidum*, *Ganoderma neo-japonicum*, *Grifola frondosa*, Neuritogenesis, Neurodegenerative disease, Nerve growth factor, MEK/ERK signaling pathway, PI3K/Akt signaling pathway



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## Background

According to the World Health Organization (WHO), nearly 35.6 million people worldwide live with dementia in 2010. The number is expected to double by 2030 (65.7 million) and more than triple by 2050 (115.4 million) [1]. Dementia is a brain function syndrome characterized by a cluster of symptoms and signs manifested by difficulties in memory, disturbances in language, psychological and psychiatric changes, and impairments in activities of daily living. Alzheimer's disease is one form of dementia that gradually gets worse over time. It affects memory, thinking, and behaviour [2].

Neuritogenic activity is one of the focuses of the study on the preventive and therapeutic effects of neurodegenerative diseases. Neuritogenic substances hold the promise of therapeutic efficacy in the treatment of neuronal injuries by the virtue of their ability to stimulate outgrowth of neurites from neuronal cells [3]. Recent reports showed that many extracts or compounds from natural sources possessed significant neuritogenic activity *in vitro* and *in vivo*, included hericenones and erinacines from *Hericium erinaceus* (lion's mane mushroom) [4] and curcumin from *Curcuma longa* [5].

Nerve growth factor has potent biological activities such as promoting neuronal survival and neuritogenesis [6]. It is targeted as a potential therapeutic drug for the treatment of neurodegenerative disorders [7,8]. However, NGF is unstable and is unable to cross blood–brain barrier because of its high molecular polypeptide [9]. Hence, the potential medicinal values of culinary and medicinal mushrooms have attracted intense interest in the search for pharmacological compounds that mimic the NGF activity in the prevention of neurodegenerative diseases. Medicinal mushrooms have a long and rich history of use as mycomedicinals [10,11]. Extracts of medicinal mushrooms have long been an important part of traditional oriental medicines. Many studies reported that edible and medicinal mushrooms possessed neuritogenic effects. In the previous studies, the neuritogenic and nerve regeneration effects of *Hericium erinaceus* (Bull.:Fr.) Pers. in *in vitro* and *in vivo* [12-14], the sclerotium of *Lignosus rhinocerotis* (Cooke) Ryvarden (tiger's milk mushroom) [15] and *Pleurotus giganteus* (Berk.) Karunarathna & K.D. Hyde (morning glory mushroom) [16] were documented.

The genus *Ganoderma* is a popular medicinal mushroom, and is used in traditional Chinese medicine (TCM) as a tonic and sedative in Asian countries. For over two millennia its use is documented in countries including China, Japan and Korea [17,18]. *Ganoderma lucidum* (Curtis: Fr.) P. Karst, called "Lingzhi" in Chinese and "Reishi" in Japanese, is one of the most commonly used mushroom by TCM in Asia [17]. According to "Shennong Ben Cao Jing", a Chinese book on agriculture and medicinal plants (300 BC – 200 AC), Lingzhi is classified into six categories based on colour, which are red, yellow, black, white, green and purple. *Ganoderma lucidum* is the most common red Lingzhi and *Ganoderma neo-japonicum* Imazeki is categorized as purple Lingzhi. *Ganoderma neo-japonicum* is found in Mainland China, Japan and Taiwan, and grows saprotrophically on dead hardwoods or bamboos [19]. In Malaysia, *G. neo-japonicum* grows on bamboo. A water infusion is used by the indigenous folks as medicine and a tonic to strengthen the body (unpublished data). *Grifola frondosa* (Dicks.) Gray, also known by its Japanese name "Maitake" which means "dancing mushroom", has been used as a health food for centuries in China and Japan. Maitake is a delicious culinary mushroom and also valued for its medicinal properties. Studies have shown that *G. lucidum* [18] and *G. frondosa* [20] possessed neuritogenic effects in preventing and treating neurological disorders. However, no information is available on the neuronal effects of *G. neo-japonicum*.

The present work reports the study of neuritogenic effects of aqueous extracts of medicinal mushrooms basidiocarps, namely *H. erinaceus*, *G. lucidum*, *G. neo-japonicum* and *G. frondosa* on PC-12 cells. Furthermore, the effects of cellular signaling pathways, MEK/ERK1/2 and PI3K/Akt in the potentiation of neuritogenic activity in PC-12 cells by using specific pharmacological inhibitors were investigated.

## Methods

### Materials and chemicals

The *H. erinaceus* (KLU-M 1232) and *G. lucidum* (KLU-M 1233) basidiocarps were obtained from Ganofarm in Tanjung Sepat, Selangor. *Ganoderma neo-japonicum* (KLU-M 1231) basidiocarps were collected from a forest in Ulu Grik, Perak and *G. frondosa* basidiocarps (KLU-M 1229) were purchased from a hypermarket in Selangor, Malaysia. The mushrooms were identified and authenticated by experts in the Mushroom Research Centre, University of Malaya. Voucher specimens are deposited in the University of Malaya herbarium (KLU-M). Rat pheochromocytoma (PC-12Adh) cell line was purchased from American Type Culture Collection (ATCC; Rockville, MD, USA; Catalogue Number: CRL-1721.1). Kaighn's Modification of Ham's F-12 Medium (F-12 K medium), NGF-7S from murine submaxillary gland, 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), phosphate buffered saline (PBS), dimethyl sulfoxide (DMSO), MEK inhibitor (U0126, PD98059), PI3K inhibitor (LY294002), anti-neurofilament 200 (NF-200) antibody produced in rabbit and Anti-Rabbit IgG-Fluorescein isothiocyanate (FITC) antibody produced in sheep were obtained from Sigma Co. (St. Louis, MO, USA). ProLong® Gold Antifade Reagent with DAPI (4-6-Diamidino-2-phenylindole) was purchased from Life Technologies Corporation (California, USA). Fetal

bovine serum (FBS) and horse serum (HS) were purchased from PAA Laboratories (Cölbe, Germany).

### Preparation of aqueous extracts

The aqueous extracts were prepared according to Eik *et al.* [15]. Briefly, the fresh basidiocarps of *H. erinaceus* and *G. frondosa* were sliced, weighed and freeze-dried while *G. lucidum* and *G. neo-japonicum* were air dried. The dried basidiocarps were then ground into powder by a Waring commercial blender. The powder was then soaked in distilled water at a ratio of 1:20 (w/v) and 150 rpm at room temperature. After 24 h, the mixture was double boiled in a water bath for 30 min and after cooling was filtered through Whatman no. 4 filter paper. The resulting aqueous extracts were freeze-dried and kept at −20°C prior to use.

### *In vitro* cell culture

The rat pheochromocytoma (PC-12Adh) cells were sustained in ATCC formulated F-12 K medium and supplemented with 15% (v/v) of heat-inactivated HS and 2.5% (v/v) of heat-inactivated FBS with final pH 6.8 - 7.2. The cells were subcultured every 2 to 3 days and incubated at 37 ± 2°C in a 5% $CO_2$-humidified incubator.

### Cell viability and cytotoxicity assay

Cell viability was assessed by the mitochondrial-dependent reduction of MTT to purple formazan. PC-12 cells were plated in 96-well plates at a density of $5 \times 10^3$ cells/well and incubated overnight at 37°C in a 5% $CO_2$-humidified incubator. Then, the aqueous extracts (0–2500 μg/ml) were added into the cells. After 48 h of incubation, 20 μl of MTT (5 mg/ml) in PBS buffer (pH 7.4) was added into each well and incubated at 37°C for 4 h. Subsequently, the supernatant was carefully discarded by aspiration, and 100 μl of DMSO was then added into each well to dissolve the MTT formazan crystals, mixed thoroughly and incubated for 15 min. The extent of the reduction of MTT was determined by measurement of the absorbance at 540 nm with 690 nm as background absorbance with an ELISA microplate reader (Sunrise, Tecan, Austria). The complete F-12 K medium was the blank, and cells incubated in the medium only were denoted as the negative control.

### Neurite outgrowth stimulation assay

Cells were plated in 12-well plates at a density of $5 \times 10^3$ cells per well in complete F-12 K medium. The cells were treated with freshly prepared aqueous extracts at various concentrations ranged from 25 to 100 μg/ml (w/v). Eik *et al.* [15] reported that 50 ng/ml (w/v) of NGF-7S from murine submaxillary gland was the optimum concentration for neuritogenesis in PC-12 cells. In the present study, cells treated with 50 ng/ml (w/v) of NGF or 50 μg/ml (w/v) of *H. erinaceus* served as positive controls. Cells in complete F-12 K medium without treatment served as a negative control. Assay plates were incubated for 48 h at 37 ± 2°C in a 5% $CO_2$-humidified incubator.

### Quantification of neurite outgrowth

The cell morphology was assessed under an inverted microscope (Nikon Eclipse TS100). Neurite extension of PC-12 cells was regarded as an index of neuritogenesis. Neurite that was double or more the length of the cell body diameter was scored positive for a neurite-bearing cell [14]. The images were captured with a QImaging Go-3 color CMOS Camera (QImaging, Canada) and by the image processor system, Image-Pro Insight (MediaCybernetics, MD). The percentage of differentiated cells was evaluated by scoring the proportion of neurite-positive cells to total cells in randomly 10 selected microscopic fields per well, with an average of 200–300 cells per well.

### Treatment with specific inhibitors of signaling pathways

The MEK/ERK1/2 inhibitors (U0126, PD98059) and PI3K/Akt inhibitor (LY294002) were used in this study. Stock solutions (10 mM) of inhibitors were prepared in DMSO and stored at −20°C in the dark. Final concentrations of 10 μM of U0126, 30 μM of LY294002 and 40 μM of PD98059 were prepared by diluting in complete F-12 K medium just before use [16]. Cells were pre-incubated either with or without the inhibitor for 1 h at 37 ± 2°C in a 5% $CO_2$-humidified incubator, respectively before the treatment with 50 ng/ml (w/v) of NGF or the optimum concentration of each aqueous extract resulting in the neurite outgrowth stimulation assay. Cells were then incubated for 48 h prior to scoring the neurite-bearing cells.

### Immunofluorescence staining of neurofilament

Immunofluorescence assay was carried out according to Schimmelpfeng *et al.* [21] with some modifications. Briefly, cells were seeded in 12-well micro-chamber (ibidi, Martinsried, Germany) at a density of $5 \times 10^3$ cells per well in complete F-12 K medium. Then, the cells were pre-incubated either with or without the treatment of inhibitors. After 1 h, the cells were treated with the optimum concentration of each aqueous extract result in the neurite outgrowth stimulation assay for 48 h at 37 ± 2°C in a 5% $CO_2$-humidified incubator. Subsequently, the cells were fixed with 4% formalin (v/v) at room temperature for 20 min. After three washings with PBS, the cells were incubated with anti-NF-200 antibody produced in rabbit (1:80 dilution in blocking buffer) at room

temperature for 1 h. Then, the cells were incubated with fluorophore-conjugated secondary antibody, anti-Rabbit IgG-FITC antibody produced in sheep (1:80 dilution in blocking buffer) at room temperature for 1 h in the dark. Cells were mounted with aqueous mounting medium, ProLong® Gold Antifade Reagent with DAPI. Slides were observed under fluorescence illumination using FITC and DAPI filters and images were captured with Nikon's Imaging Software, NIS-Elements.

### Statistical analysis

All the experimental data were expressed as the mean ± standard deviation (SD). Statistical differences between groups were performed using one-way analysis of variance (ANOVA) of a minimum of three independent experiments and Duncan's multiple range tests (DMRT) $P < 0.05$ was considered to be significant.

## Results

### The cells viability and cytotoxic effects of aqueous extracts on PC-12 cells

All aqueous extracts tested did not exert any detectable cytotoxic effect in PC-12 cells. The survival rates of the cells were decreased in a concentration-dependent manner, *G. lucidum* (Figure 1a,b), *G. neo-japonicum* (Figure 1c,d), and *G. frondosa* (Figure 1e,f). The negative control, cells in complete F-12 K medium only, was considered as 100% of cell viability. A significant ($p < 0.05$) stimulation of proliferation was observed at the concentration of 7.81 μg/ml and 15.63 μg/ml of *G. neo-japonicum*. The cell viability was significantly ($p < 0.05$) decreased at the concentration of 62.5 ug/ml (*G. lucidum*), 250 ug/ml (*G. neo-japonicum*) and 31.25 ug/ml (*G. frondosa*) with the percentage inhibitions of 13.41%, 16.57% and 13.85%, respectively, compared to the negative control. The reduction in the cell number could be a consequence of cell death or the

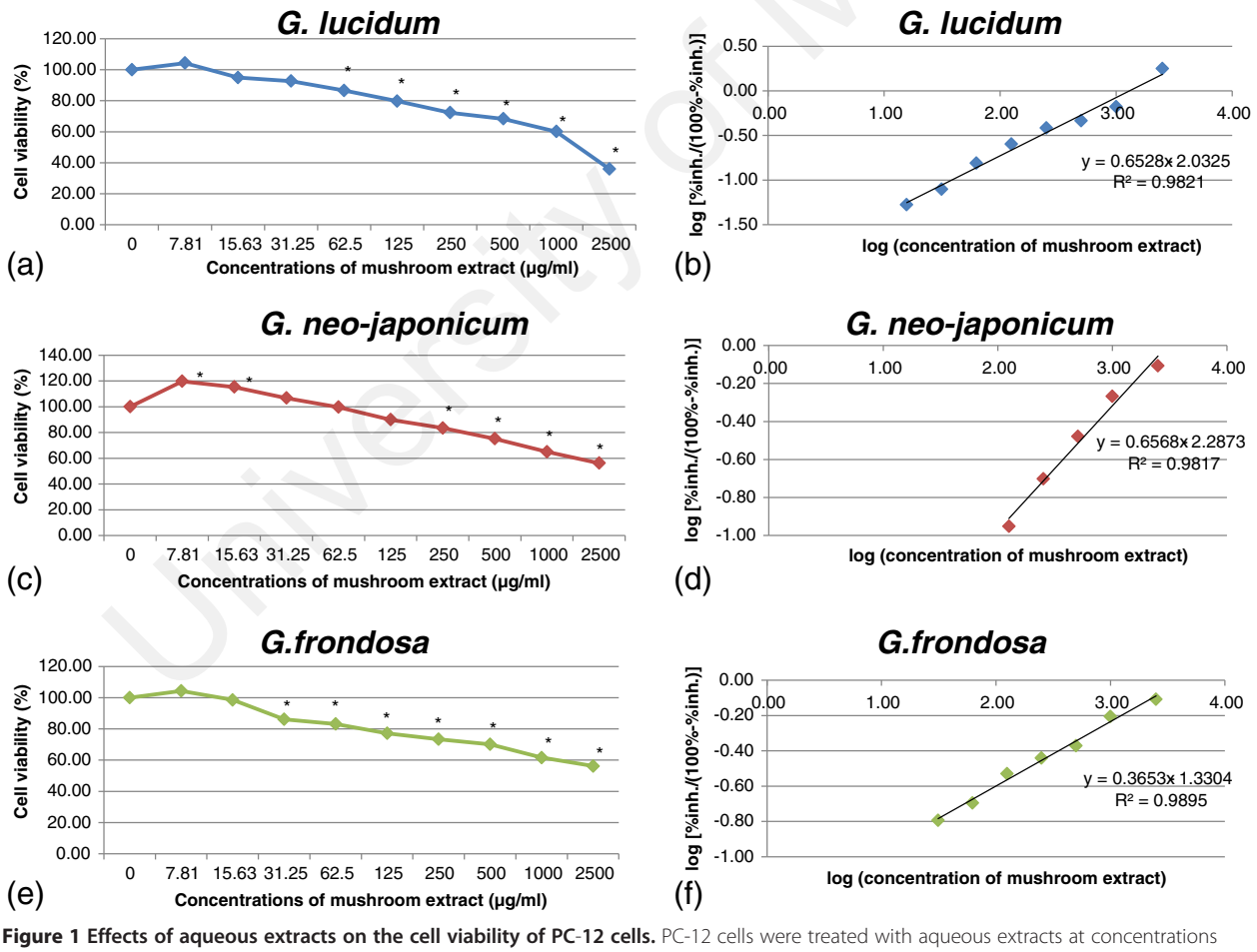


**Figure 1 Effects of aqueous extracts on the cell viability of PC-12 cells.** PC-12 cells were treated with aqueous extracts at concentrations from 0 to 2500 μg/ml for 48 h. **(a)** *Ganoderma lucidum*, **(c)** *Ganoderma neo-japonicum* and **(e)** *Grifola frondosa*. **(b)**, **(d)** and **(f)**: $IC_{50}$ was obtained from the intercept on the x-axis (y = 0) of the regression line using the linear part of the percentage inhibition (% inh.) curve. The mean absorbance was obtained using complete F-12 K medium with cells only was designated 100%. Results are shown as means ± standard deviation (n = 3). * $p < 0.05$ compared to the respective control 100%.

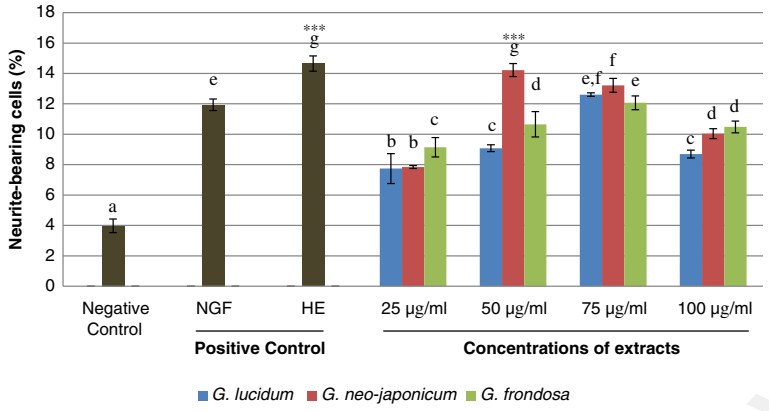


**Figure 2 Effects of aqueous extracts on the neuritogenic activity of PC-12 cells.** The percentage of neurite-bearing cells of PC-12 cells treated with various concentrations of aqueous extracts ranged from 25 to 100 µg/ml. Cells in complete F-12 K medium without treatment served as a negative control. Cells treated with 50 ng/ml of NGF or 50 µg/ml of *H. erinaceus* (HE) served as positive controls. Data were expressed as means ± standard deviation (n = 3). Means with different alphabets shows significant difference $p < 0.05$. *** $p < 0.001$ compared to NGF.

decrease in the cell division. The required concentration to inhibit the cell growth by 50% ($IC_{50}$) for aqueous extracts of *G. lucidum*, *G. neo-japonicum* and *G. frondosa* were 1298.71 ug/ml, 3037.32 ug/ml and 4384.68 ug/ml, respectively.

### The neuritogenic effect of aqueous extracts on PC-12 cells

All concentrations of aqueous extracts tested showed neuritogenic effects after 48 h of incubation (Figure 2). Nerve growth factor- and *H. erinaceus*-treated cells served as positive controls. The percentage of neurite-bearing cells of *G. lucidum*-, *G. neo-japonicum*- and *G. frondosa*- treated cells were significantly ($p < 0.05$) increased in a concentration-dependent manner. There were significant differences ($p < 0.05$) between the negative control and all concentrations of aqueous extracts tested. Interestingly, the percentage of neurite-bearing cells of aqueous extract of *G. neo-japonicum* at 50 µg/ml (14.22 ± 0.43%) was significantly higher ($p < 0.05$) compared to NGF and was comparable to neurite outgrowth stimulation by *H. erinaceus* (14.66 ± 0.5%). Maximum stimulation of neuritogenesis by aqueous extract of *G. neo-japonicum* was achieved at 50 µg/ml with 14.22% of neurite-bearing cells, followed by *G. lucidum* (12.61%) and *G. frondosa* (12.07%) at a higher concentration of 75 µg/ml. There was no significant difference in the percentage of neurite-bearing cells between 50 ng/ml of NGF (11.94 ± 0.38%) and 75 µg/ml of aqueous extract of *G. lucidum* (12.61 ± 0.11%) and *G. frondosa* (12.07 ± 0.46%).

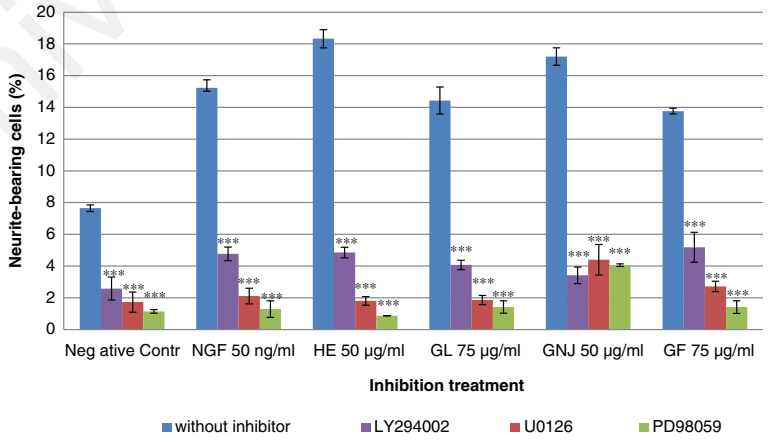


**Figure 3 Effects of the specific inhibitors of MEK/ERK1/2 and PI3K on aqueous extracts-stimulated neuritogenesis.** PC-12 cells were exposed to U0126, PD98059 and LY294002 for 1 h before the treatment of aqueous extracts of *G. lucidum* (GL), *G. neo-japonicum* (GNJ) and *G. frondosa* (GF). Cells in complete F-12 K medium without treatment served as a negative control. Cells treated with 50 ng/ml of NGF or 50 µg/ml of *H. erinaceus* (HE) served as positive controls. A control without inhibitor was used in each treatment group. Data were expressed as means ± standard deviation (n = 3). *** $p < 0.001$ compared to respective controls.

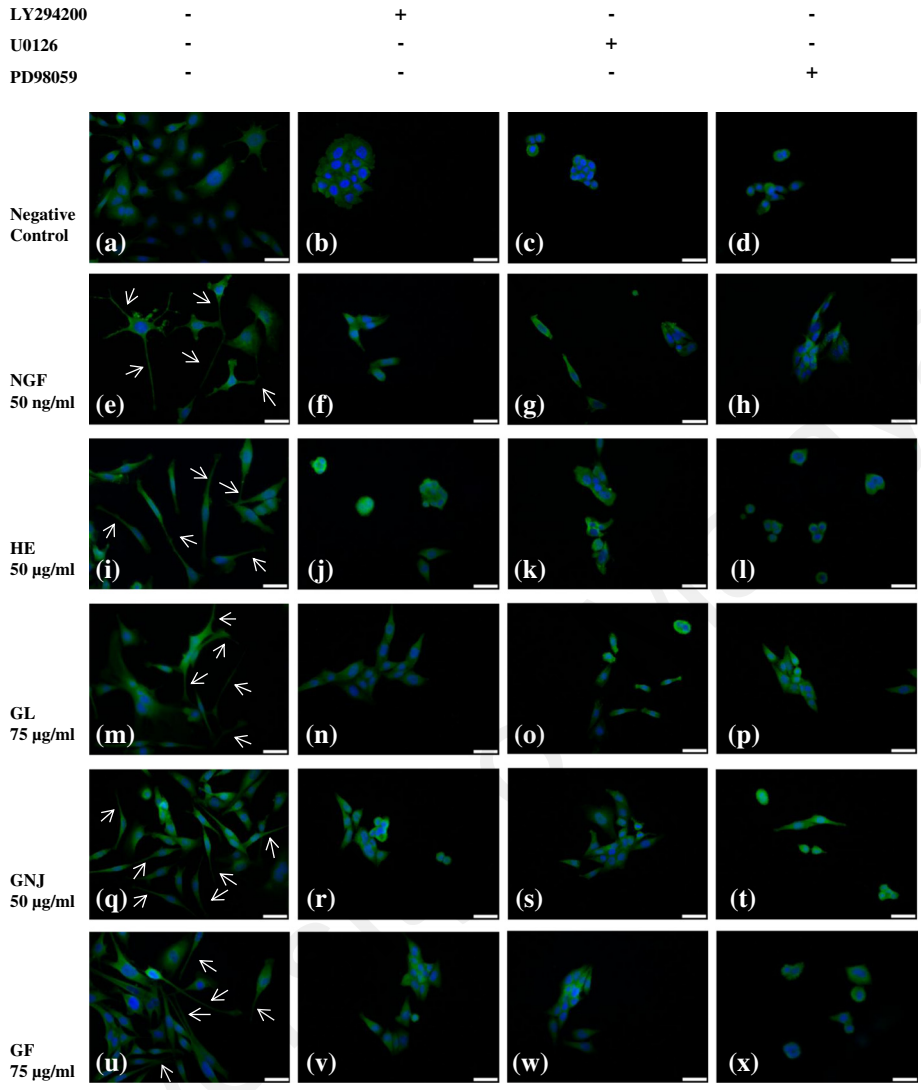


**Figure 4 Morphology of PC-12 cells in different treatment groups.** PC-12 cells were exposed to U0126 (10 μM), PD98059 (40 μM) and LY294002 (30 μM) for 1 h before the treatment of aqueous extracts. **(a)** to **(d)** negative control (complete F-12 K medium), **(e)** to **(h)** positive control (NGF; 50 ng/ml), **(i)** to **(l)** positive control (*H. erinaceus* (HE); 50 μg/ml), **(m)** to **(p)** 75 μg/ml of *G. lucidum* (GL), **(q)** to **(t)** 50 μg/ml of *G. neo-japonicum* (GNJ), **(u)** to **(x)** 75 μg/ml *of G. frondosa* (GF). Nuclei stained blue and neurofilaments stained green. Scale bars represent 50 μM. Arrows indicate neurite outgrowth.

### The involvement of MEK/ERK1/2 and PI3K/Akt signaling pathways in aqueous extracts-stimulated neuritogenesis

The MEK/ERK1/2 inhibitors, U0126 (10 μM) and PD98059 (40 μM) blocked the neuritogenic activity of aqueous extracts and NGF (Figure 3). The results showed that PD98059 decreased the percentage of neurite-bearing cells by approximately 90.16% in *G. lucidum*, 76.42% in *G. neo-japonicum* and 89.73% in *G. frondosa* treated cells compared to each individual control. In the presence of PI3K/Akt inhibitor, LY294002 (30 μM), the number of neurite-bearing cells were decreased significantly ($p < 0.001$). The significant ($p < 0.001$) reduction of neurite stimulation activities were also observed in the negative control-, NGF- and aqueous extracts of *H. erinaceus*- stimulated neuritogenesis with the addition of the inhibitors. These data suggest that activation of MEK/ERK1/2 and PI3K/Akt signaling pathways are involved in aqueous extracts-stimulated neuritogenesis in PC-12 cells.

### The effect of MEK/ERK1/2 and PI3K/Akt inhibitors on neuronal morphology visualized by immunofluorescence staining

To examine the pattern of neuritogenesis further, PC-12 cells were stained by immunofluorescence dyes incorporated with anti-NF-200 antibody. PC-12 cells

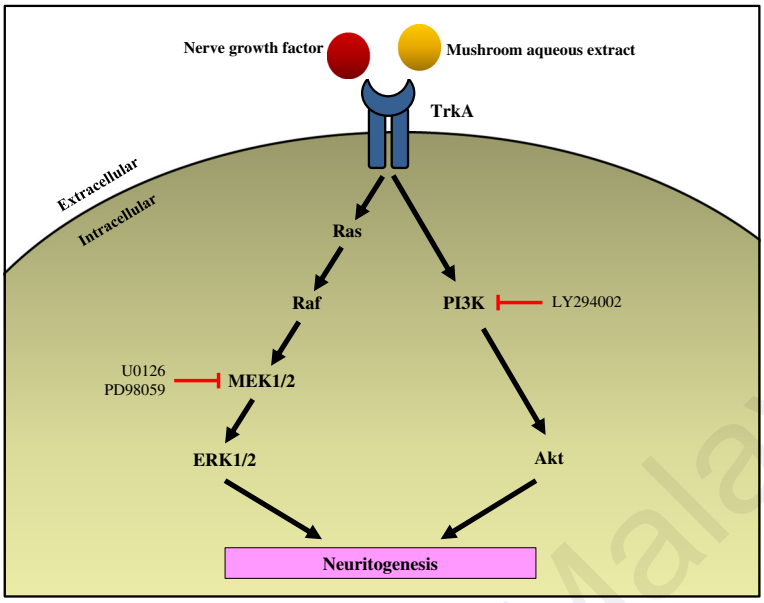


**Figure 5 Hypothetic mechanisms of NGF and mushroom aqueous extracts in promoting neuritogenesis in PC-12 cells.** Both nerve growth factor and aqueous extracts bind to tyrosine receptor, TrkA and initiate two major signaling pathways, the MEK/ERK1/2 and PI3K/Akt pathways. Activation of MEK/ERK1/2 and PI3K/Akt cascade promotes neuritogenesis of PC-12 cells. In the present study, the MEK/ERK1/2 inhibitors (U0126, PD98059) and PI3K/Akt inhibitor (LY294002) blocked the neuritogenic activity of NGF and aqueous extracts. Therefore, based on the present findings, the activation of MEK/ERK1/2 and PI3K/Akt signaling pathways are needed for the NGF and aqueous extracts in promoting neuritogenesis.

nuclei were stained blue by DAPI and neurofilaments were stained green by anti-NF-200 labeled with FITC. The cells were pre-treated, with or without specific inhibitors, prior to the addition of the aqueous extracts and incubated for 48 h. In the negative control, the cells are relatively small and rounded with few visible neurites (Figure 4a). With the treatment of 50 ng/ml of NGF, 50 μg/ml of *H. erinaceus*, 75 μg/ml of *G. lucidum*, 50 μg/ml of *G. neo-japonicum* and 75 μg/ml of *G. frondosa*, the cells were larger and elongated. Cells also exhibited neurite extensions that were double the length of the cell body diameter (Figure 4e, 4i, 4m, 4q, 4u). However, some morphological changes in neuronal differentiation were observed in the treatment of U0126, PD98059 and LY294002 inhibitors. The inhibitors blocked the neuritogenic activity of aqueous extracts and NGF and caused shrunken and rounded cell bodies without noticeable neurite extension. These results suggest that the activation of MEK/ERK1/2 and PI3K/Akt signaling pathways are needed for the NGF and aqueous extracts in promoting neuritogenesis.

## Discussion

In the present study, PC-12Adh cell line was utilized as a model system to investigate the cytotoxicity, neuritogenic activity and elucidate the underlying mechanisms of aqueous extracts of medicinal mushrooms basidiocarps, namely *G. lucidum*, *G. neo-japonicum* and *G. frondosa*. The PC-12 cell line is established from rat adrenal pheochromocytoma (adrenal medullary tumour) [22] and has been extensively used as a model to investigate the neuronal differentiation, proliferation and survival [23]. With the addition of NGF, PC-12 cells are able to differentiate into sympathetic neuron-like phenotypes characterized by neurite outgrowth and the expression of several neuron-specific proteins [22,24]. Nerve growth factor is crucial for the survival, developmental and differentiation of the central and peripheral neurons [25,26]. The neurotrophic effect of NGF is transduced by high affinity tyrosine receptor TrkA [27], the NGF receptor, and then it activates several signaling pathways via intracellular signaling molecules that include Ras [28], PI3K [26,29], ERK [30] and p38 MAPK [31].

Aqueous extraction has been the most commonly used method for the isolation of bioactive polysaccharides from mushrooms such as glucans [10,32]. According to Cheung *et al.* [18], the extract of *Ganoderma* contained polysaccharides that possessed neuroactivity. It had been reported that crude aqueous extract of *Tremella fuciformis* (white jelly mushroom) possessed neuritogenic effects *in vitro* and anti-amnesic effects *in vivo* [33]. According to Lin *et al.* [34], treatment with the water extract of *G. lucidum* and *G. neo-japonicum* showed antioxidant effect on free radical scavenging activity and hepatoprotective effect against CCl4-induced liver injury. Aqueous extraction is

believed to have lower cytotoxic effect compared to most of the organic solvent. In this study, medicinal mushrooms were extracted by water, in conjunction with the traditional use of mushrooms as part of TCM. In addition, water is non-toxic to cells. From data obtained in this study, the $IC_{50}$ value of cytotoxic activity of *G. lucidum*, *G. neo-japonicum* and *G. frondosa* were approximately 17-, 60- and 58- fold higher than their optimum concentration that stimulated neuritogenesis. Further, the results indicated that the aqueous extracts of all tested mushrooms were not cytotoxic to PC-12 cells.

The results suggested that all aqueous extracts tested caused a marked stimulation of neuritogenesis in PC-12 cells and they appeared to be comparably active with the neuritogenic effects *in vitro* of NGF. Therefore, the aqueous extracts of *G. lucidum*, *G. neo-japonicum* and *G. frondosa* may possess NGF-like bioactive compounds that mimic the neuroactivity of NGF for neuronal survival, development and differentiation. The aqueous extract of *G. neo-japonicum* triggered maximal stimulation of neuritogenesis at a lower concentration compared to *G. lucidum* and *G. frondosa* that act at a higher concentration. Neuritogenic activity of higher basidiomycetes other than *G. neo-japonicum*, *G. lucidum* and *G. frondosa* has also been reported. These included *H. erinaceus* [4,35], *Sarcodon scabrosus* [36], *Sarcodon cyrneus* [37,38], *Termitomyces albuminosus* [39,40] and *Cordyceps militaris* [41]. Shi *et al.* [36] reported that cyathane diterpenoids isolated from *S. scabrosus* showed significant neuritogenic activity in combination with 20 ng/mL of NGF in PC-12 cells after 24 h treatment. The extract of *C. militaris* stimulated neuritogenesis, enhanced neuronal functions of Neuro2A mouse neuroblastoma cells (*in vitro*) and improved cognitive behaviour that related to memory ability (*in vivo*) [41].

Our findings illustrated the potential cellular signaling pathways involved in aqueous extracts-stimulated neuritogenesis, namely MEK/ERK1/2 and P13K/Akt that are important in regulating growth and differentiation of PC-12 cells. Specific inhibitors of MEK/ERK1/2 and P13K/Akt could attenuate the ability of aqueous extracts to stimulate neuritogenesis in PC-12 cells. The MEK/ERK and PI3K/Akt signaling pathways can be activated by NGF to stimulate neurite extension and branching of neuronal cells [42-45]. Vaudry *et al.* [30] reported that the activation of MEK/ERK signaling pathway is necessary for neuritogenesis, in this case the neuronal differentiation in PC-12 cells by NGF. Inhibition of PI3K in PC-12 cells will avert NGF-stimulated neurite elongation [25], promote cell protective effect and cell survival [46].

In this study, the potentiation of aqueous extracts-stimulated neuritogenesis was blocked by U0126, PD98059 and LY294002. Therefore, the MEK/ERK and PI3K/Akt-dependent signaling pathways play a crucial role in the neuritogenic effect of medicinal mushrooms (Figure 5). This is in agreement with a previous study by Phan *et al.* [16], documented that MEK/ERK and PI3K/Akt signaling pathways were involved in neuritogenesis stimulated by extracts of *P. giganteus*. Some studies have shown the involvement of MAPK cascade in neuritogenesis. Extracts of *Ganoderma* [18] and lysophosphatidylethanolamine, a neuroactive compound isolated from *G. frondosa* [20] activated the MAPK cascade through the MEK/ERK1/2 phosphorylation of PC-12 cells.

Neurofilament staining by immunofluorescence served as firm support to the observation that aqueous extracts-stimulated neuritogenesis. Neurofilament is a neuron-specific protein that serves as a major component of the cytoskeleton that supporting the axon cytoplasm. It is a useful indicator of PC-12 cell differentiation [21]. The images showed clear morphological differences between the inhibitor-treated and non-inhibitor-treated groups. The addition of the MEK/ERK or PI3K/Akt inhibitors blocked the neuritogenesis of PC-12 cells and the neurite outgrowth of the NGF- and aqueous extracts-stimulated PC-12 cells.

Besides MEK/ERK1/2 and PI3K/Akt, other mechanisms may still be addressed for a comprehensive understanding of neuritogenic activity. The interaction between MEK/ERK and PI3K/Akt signaling pathways determined by flow cytometry or immunoblot analysis will be proposed for elucidation of mechanisms involved in the neuritogenic activity of the three selected mushrooms.

## Conclusions

Our findings suggested that all the medicinal mushrooms tested possessed neuritogenic activity without cytotoxic effect. The MEK/ERK1/2 and PI3K/Akt signaling pathways may play a role in the neuritogenic activity of the mushrooms. The precise mechanism underlying this activity remains to be investigated.

### Abbreviations

Akt: Protein kinase B; ERK: Extracellular signal-regulated kinase; MAPK: Mitogen activated protein kinase; MEK: Mitogen-activated protein kinase kinase; P13K: Phosphoinositide-3-kinase.

### Competing interests

The authors declare that they have no competing interests.

### Authors' contributions

SLSS carried out the experiment, drafted the manuscript, engaged in data acquisition and data interpretation. MN participated in the acquisition of funding and editing of the manuscript. PD and KHW were involved in the design of the study and manuscript editing. VS provided the grant, was involved in coordinating and monitoring of research, and manuscript editing. All authors read and approved the final manuscript.

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# *Lignosus rhinocerotis* (Cooke) Ryvarden mimics the neuritogenic activity of nerve growth factor via MEK/ERK1/2 signaling pathway in PC-12 cells

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**The traditional application of the sclerotium of *Lignosus rhinocerotis* (tiger's milk mushroom) by the indigenous folks as tonic and remedy to treat a variety of ailments has been documented in Malaysia. Indigenous communities claimed to have consumed the decoction to boost their alertness during hunting. Mental alertness is believed to be related to neuronal health and neuroactivity. In the present study, the cell viability and neuritogenic effects of *L. rhinocerotis* sclerotium hot aqueous and ethanolic extracts, and crude polysaccharides on rat pheochromocytoma (PC-12) cells were studied. Interestingly, the hot aqueous extract exhibited neuritogenic activity comparable to NGF in PC-12 cells. However, the extracts and crude polysaccharides stimulated neuritogenesis without stimulating the production of NGF in PC-12 cells. The involvements of the TrkA receptor and MEK/ERK1/2 pathway in hot aqueous extract-stimulated neuritogenesis were examined by Trk (K252a) and MEK/ERK1/2 (U0126 and PD98059) inhibitors. There was no significant difference in protein expression in NGF- and hot aqueous extract-treated cells for both total and phosphorylated p44/42 MAPK. The neuritogenic activity in PC-12 cells stimulated by hot aqueous and ethanolic extracts, and crude polysaccharides of *L. rhinocerotis* sclerotium mimicking NGF activity via the MEK/ERK1/2 signaling pathway is reported for the first time.**

Medicinal mushrooms and their extracts have a long and rich history of use in traditional oriental medicines as mycomedicines[1]. Increasingly, many are being regarded as functional foods and nutraceuticals. The neuroactivities of medicinal mushrooms are under intense study and research. Phan *et al.* (2014)[2] reviewed a number of studies of medicinal mushrooms, revealing the promises of medicinal mushrooms as useful therapeutic agents in the management and/or treatment of neurodegenerative disorders.

In Malaysia, *Lignosus rhinocerotis* (Cooke) Ryvarden is also known as 'tiger's milk mushroom' in English or 'cendawan susu rimau' in Malay. It is considered as a unique "National Treasure" that can only be found in a small geographic region in Southern China, Thailand, Malaysia, Indonesia, Philippines, Papua New Guinea, New Zealand and Australia[3]. In Malaysia, *L. rhinocerotis* is the most popular medicinal mushroom used by the indigenous communities of Peninsular Malaysia[4]. The benefits of its underground tuber or sclerotium (where most of the nutritional and medicinal components are deposited) compared to its basidiocarp are well documented (Table 1). According to the ethnopharmacological

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| Ethnopharmacological applications | | Users | References |
|---|---|---|---|
| Tonic for cough, fever, asthma, chronic hepatitis, gastric ulcer, liver and breast cancer, food poisoning | | Malays, Chinese and indigenous communities in Malaysia | Lee *et al.*, 2009 [4] |
| | | | Burkill *et al.*, 1966 [5] |
| Tonic for liver cancer, chronic hepatitis and gastric ulcer | | | Chang and Lee, 2004 [6] |
| | | Traditional Chinese medicine practitioners in China | Huang, 1999 [7] |
| Literature reported activities | Preparation methods | Research model | References |
| Anti-proliferative and anti-tumor | Hot aqueous-soluble polysaccharides of sclerotium | Human acute promyelocytic leukemic (HL-60), chronic myelogenous leukemia (K562) and human acute monocytic leukemia (THP-1) cell lines | Lai *et al.*, 2008 [27] |
| | Cold aqueous extract of sclerotium | Human breast carcinoma (MCF-7) and human lung carcinoma (A549) cell lines | Lee *et al.*, 2012 [28] |
| | Cold aqueous and hot aqueous extracts of sclerotium | human lung carcinoma (A549) | Lau *et al.* (2013) [29] |
| | | human hepatocellular carcinoma (Hep G2) | |
| | | human colorectal carcinoma (HCT 116) | |
| | | human nasopharyngeal carcinoma (HK1) | |
| | | human squamous carcinoma cells (HSC2) | |
| | | human breast adenocarcinoma (MCF7 and MDA-MB-231) | |
| | | human prostate adenocarcinoma cells (PC3) | |
| | | human acute promyelocytic leukaemia (HL-60) | |
| Immunomodulation | Hot aqueous-soluble polysaccharides and cold alkaline-soluble β-glucan | Human natural killer (NK-92MI, primary CD56+ NK) and monocyte cell lines | Wong *et al.*, 2009 [29] |
| | Sclerotium polysaccharides | BALB/c mice and athymic BALB/c nude mice | Wong *et al.*, 2011 [30] |
| | Hot aqueous extract of sclerotium | Murine macrophages (RAW 264.7) and murine primary macrophages (PMs) | Guo *et al.*, 2011 [31] |
| Antioxidant | Hot aqueous, cold aqueous and methanol extracts of sclerotium | – | Yap *et al.*, 2013 [32] |
| Neuritogenesis | Hot aqueous extract of sclerotium | Rat pheochromocytoma (PC-12) cells | Eik *et al.*, 2012 [33] |
| | Hot aqueous extract of mycelium | Rat pheochromocytoma (PC-12) cells | John *et al.*, 2013 [36] |

**Table 1. Summary of ethnopharmacological applications and literature reported activities of *Lignosus rhinocerotis*.**

reports, the sclerotium is sliced, boiled and drunk as an effective tonic for overall wellness and to treat several ailments including fever, cough, asthma, chronic hepatitis, gastric ulcer, cancer and food poisoning[4–7] (Table 1). Indigenous communities also drank this decoction to increase their alertness during hunting (tacit knowledge). Moreover, Tan *et al.* (2012)[3] documented that the consumption of tiger's milk mushroom improved stamina and alertness in healthy people. Increment of the mental alertness is believed to be related to the neuroactivity and neuronal communication network in brain. Based on the traditional practice as the basic for the scientific study, we explored *L. rhinocerotis*, the Malaysia's treasure mushroom, notably for the neuritogenic activity *in vitro*.

Neuritogenesis is a dynamic phenomenon associated with neuronal differentiation, where the neurons generate and extend their neurites to form a functional network[8]. Neuritogenic activity is important for the maintenance and regeneration of the neuronal communications network and has become one of the focuses of study in the search for preventive and therapeutic agents for neurodegenerative disorders. The neurotrophic factors (neurotrophins), exemplified by nerve growth factor (NGF), play distinctive roles in promoting neuronal survival[9], proliferation, development, neuritogenesis and maintaining the neurons functions[10]. Nerve growth factor was identified as a potential therapeutic agent for the treatment of neurodegenerative disorders[11]. A decrease in NGF in the brain is believed to be the main cause of neuronal dysfunction and neurodegenerative disorders, remarkably Alzheimer's disease[12]. Further, Capsoni

*et al.* (2000)[13] presented the evidence that a decrease in NGF in mice's brain led to neurodegeneration and Alzheimer's-like symptoms. Learning ability and memory were improved in aged anti-nerve growth factor transgenic mice after administration of NGF[13]. However, the neuroactivity of NGF is restricted due to its large molecular polypeptide structure. It is unstable and unable to cross the blood-brain barrier[14]. Smaller molecules that mimic and/or enhance the NGF activity have become the core focus in the search for preventive and therapeutic agents for neurodegenerative disorders[15]. Among the natural sources explored for NGF mimics, medicinal mushrooms have shown huge potential[2].

Nerve growth factor was found to activate the mitogen-activated protein kinase (MAPK) signaling pathway that mediates the phosphorylation including the mitogen-activated protein kinase kinase/extracellular signal-regulated kinase (MEK/ERK) pathway[16]. The MAPK/ERK pathway is a chain of proteins in the cell that participate by Ras, Raf, MEK1/2 and ERK1/2 proteins. The MAPK/ERK cascade is a signal transduction pathway that involves a large variety of processes such as differentiation, proliferation, apoptosis, cell cycle progression, cell migration, and metabolism[17]. The MEK/ERK1/2 signalling cascade is indeed crucial in both *in vitro*[18] and *in vivo* for neuronal cell survival, axonal regeneration[19] and neuritogenesis in extract-treated mouse dorsal root ganglia neurons[20].

The MEK/ERK1/2 signaling pathway is believed to be the major cascade for NGF to stimulate neuritogenesis in PC-12 cells[18]. The PC-12 cell line is widely used as an *in vitro* model system to investigate the neuritogenic activity of NGF and NGF mimics, and NGF responsive signaling pathways[15,18,21,22]. Nerve growth factor stimulates differentiation of PC-12 cells into a sympathetic neuronal-like phenotype and extend axon-like outgrowth[23]. PC-12 cells treated with NGF have been found to stop proliferating and differentiate into neuronal-like cells with neurite outgrowth[23]. *In vitro* neuritogenesis stimulated by hot aqueous extract of *Ganoderma* sp.[24], *Pleurotus giganteus* (Berk.) Karunarathna & K. D. Hyde[25] and *Ganoderma neo-japonicum* Imazeki[26] in PC-12 cells were mediated via MEK/ERK1/2 signaling pathway.

A number of studies revealed that the sclerotium of *Polyporus rhinocerus* (synonym of *L. rhinocerotis*) demonstrated anti-tumour[27–29], immunomodulating[30–32], and antioxidant[33] activities in *in vitro* (Table 1). However, very limited information is available on the neuronal activity of *L. rhinocerotis*. Our recent findings revealed that the hot aqueous extract of *L. rhinocerotis* sclerotium stimulated neuritogenesis in PC-12 cells[34]. To further investigate the neuritogenic activity of the *L. rhinocerotis* sclerotium, in the present study, we aimed to (i) compare the cell viability and neuritogenic effects of PC-12 cells of three different preparations, including hot aqueous and ethanolic extracts, and crude polysaccharides of *L. rhinocerotis* sclerotium, (ii) investigate whether the extracts and crude polysaccharides stimulate the production of NGF, and (iii) investigate the involvement of NGF responsive signaling pathway (MEK/ERK1/2) in *L. rhinocerotis* sclerotium-stimulated neuritogenesis in PC-12 cells.

## Results and Discussions

**The effects of hot aqueous and ethanolic extracts, and crude polysaccharides on viability of PC-12 cells.** The viability of cells in complete F-12 K medium was considered as 100%. The survival and proliferation of treated cells decreased in a concentration-dependent manner for hot aqueous extract (Fig. 1A), ethanolic extract (Fig. 1B), and crude polysaccharides (Fig. 1C). Hot aqueous extract and crude polysaccharides increased the cells proliferation significantly ($p < 0.05$) at low concentration, 9.77 μg/ml, compared to the negative control (cells in complete F-12 K medium only). The percentage of viable cells decreased gradually as the concentration of the extracts and crude polysaccharides were increased. The percentage of viable cells in hot aqueous and ethanolic extracts-treated cells were reduced significantly ($p < 0.05$) starting at 156.25 μg/ml compared to negative control, while the viability of crude polysaccharides-treated cells was reduced significantly ($p < 0.05$) starting at a low concentration, 19.53 μg/ml, compared to negative control. The required concentrations to inhibit the cell growth by 50% ($IC_{50}$) for hot aqueous, ethanolic extracts, and crude polysaccharides were 3223.98 μg/ml, 372.30 μg/ml and 2718.72 μg/ml, respectively. The cytoxicity of the hot aqueous extract was significantly ($p < 0.05$) lower in the *in vitro* PC-12 cell model when compared to the crude polysaccharides and ethanolic extract. In a study done by Lee *et al.* (2011, 2013)[35,36], it was reported that in the *in vivo* model, no treatment-related chronic toxicity was detected in Sprague Dawley rats after a long term (180 days) oral administration of the *L. rhinocerotis* sclerotium freeze-dried powder (cultivar TM02) at daily dosage up to 1,000 mg/kg. Further, blood biochemical parameters related to toxicity were reported as normal. In that study, however, Lee *et al.* (2011, 2013) did not estimate the levels of the mushroom in the blood.

**The neuritogenic effects of hot aqueous and ethanolic extracts, and crude polysaccharides in PC-12 cells.** Neurite extension of PC-12 cells was regarded as an index of neuritogenesis. All the tested concentrations of hot aqueous and ethanolic extracts, and crude polysaccharides stimulated neuritogenesis in PC-12 cells after 48 h of incubation (Fig. 2). The percentage of neurite-bearing cells in all the tested concentrations of extracts and crude polysaccharides treated cells were increased significantly ($p < 0.05$) compared to the negative control (cells in complete F-12 K medium only). The percentage of neurite-bearing cells was concentration-dependent (from 5 μg/ml to 100 μg/ml) of extracts and crude polysaccharides tested. There was an initial increase of the percentage of neurite bearing cells on increasing concentrations of extracts and crude polysaccharides, followed by a decrease at concentrations above 25 μg/ml. All the extracts and crude polysaccharides stimulated maximal neuritogenesis in PC-12 cells at 25 μg/ml. The hot aqueous extract stimulated highest percentage of neurite-bearing cells ($20.99 \pm 1.01\%$),

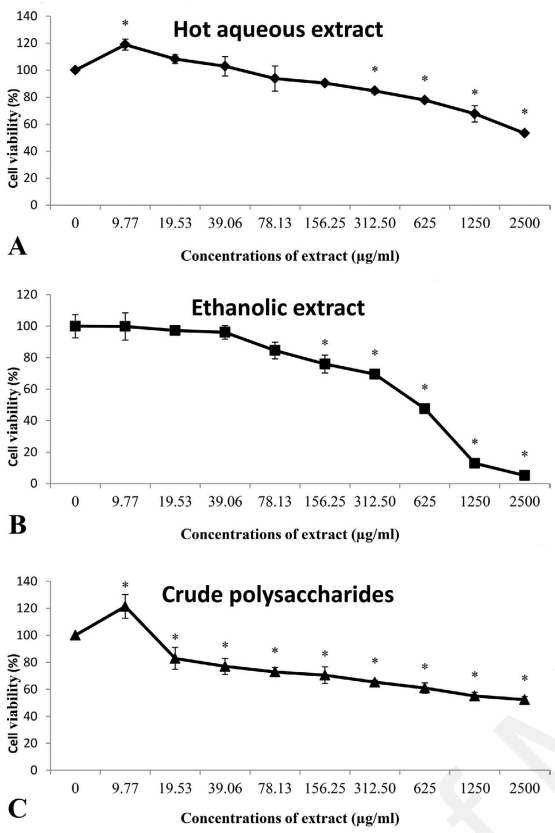


**Figure 1. The effects of hot aqueous and ethanolic extracts, and crude polysaccharides of *L. rhinocerotis* sclerotium on viability of PC-12 cells.** Cells were incubated with extracts or crude polysaccharides at concentrations from 0 to 2500 µg/ml for 48 h. (**A**) hot aqueous extract, (**B**) ethanolic extract, and (**C**) crude polysaccharides. The mean absorbance obtained using complete F-12 K medium with cells only (negative control) was designated 100% of cell viability. Results are shown as means ± standard deviation (n = 3). *$p < 0.05$ compared to the respective negative control 100%.

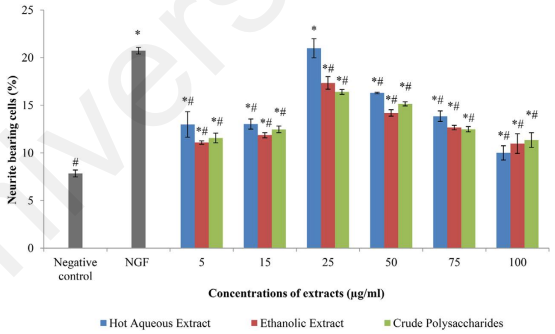


**Figure 2. The neuritogenic effects of hot aqueous and ethanolic extracts, and crude polysaccharides in PC-12 cells.** Cells were incubated with NGF (50 ng/ml), extracts or crude polysaccharides (5 to 100 µg/ml) for 48 h. Cells in complete F-12 K medium served as a negative control. Cells treated with 50 ng/ml of NGF served as a positive control. Data were expressed as means ± standard deviation (n = 3). *$p < 0.05$ compared to the negative control. #$p < 0.05$ compared to the positive control (50 ng/ml of NGF).

followed by the ethanolic extract (17.35 ± 0.66%) and crude polysaccharides (16.40 ± 0.26%), at 25 µg/ml. The $IC_{50}$ value of the cytotoxic activity of the hot aqueous extract, ethanolic extract and crude polysaccharides and was approximately 129-, 15- and 109- fold higher than their optimum concentration that stimulated neuritogenesis, 25 µg/ml. Our previous study[34] reported that 50 ng/ml of NGF was the optimum concentration for neuritogenesis in PC-12 cells. In the present study, cells treated with 50 ng/ml of NGF served as a positive control. Interestingly, there was no significant difference ($p > 0.05$) in the percentage of neurite-bearing cells between 50 ng/ml of NGF- and 25 µg/ml of hot aqueous extract-stimulated

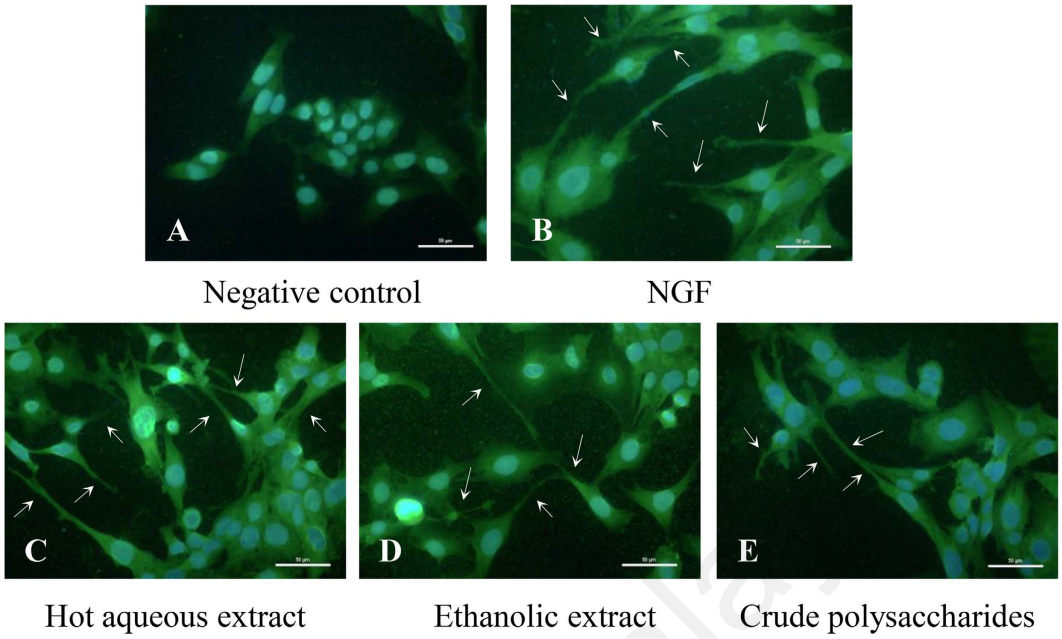


**Figure 3. Morphology of PC-12 cells stained with anti-NF-200 antibody.** Cells were incubated with or without NGF (50 ng/ml), hot aqueous extract (25 μg/ml), ethanolic extract (25 μg/ml) or crude polysaccharides (25 μg/ml) for 48 h. Cells in complete F-12 K medium served as a negative control. Cells treated with 50 ng/ml of NGF served as a positive control. Nuclei stained blue and neurofilaments stained green. Scale bars represent 50 μM. Arrows indicate neurite outgrowth.

neuritogenesis. This is in agreement with the previous studies, reported that 20 μg/ml of hot aqueous extract of *L. rhinocerotis* sclerotium[34] and *L. rhinocerotis* mycelium[37] stimulated neuritogenesis in PC-12 cells that comparable to NGF.

The hot aqueous extraction is the most commonly used method by the indigenous communities and traditional Chinese medicine (TCM) physicians to prepare decoctions, tonics or essences from medicinal mushrooms. In general, mushroom's polysaccharides are targeted as the active components in the hot aqueous extract[38]. Mushroom's polysaccharides include α-glucans and β-glucans, that known as effective anti-tumor and immunomodulatory agents[39]. Besides carbohydrates and proteins, Lau *et al.* (2013)[29] reported that the hot aqueous extract of *L. rhinocerotis* sclerotium contained a higher percentage of phenolics than the cold aqueous extract. Lau *et al.* (2013)[29] further suggested that secondary metabolites such as triterpenes and alkaloids may be present in *L. rhinocerotis* sclerotium extracts. Secondary metabolites isolated from different mushrooms that were reported to promote neuritogenesis *in vitro* and/or *in vivo*, include hericenones[40], erinacines[41], scabronions[42], termitomycesphins[43] and cyrneines[44]. These neuroactive compounds may be targeted as preventive and therapeutic agents for neurodegenerative disorders. In the present study, the hot aqueous extract of *L. rhinocerotis* sclerotium showed potent neuritogenic activity compared to the ethanolic extract and crude polysaccharides. These findings suggested that the hot aqueous extract containing chemical constituents that are neuroactive.

**The morphology of PC-12 cells stained with anti-neurofilament-200 antibody.** Neurofilaments are neuron specific intermediate filament proteins (8–10 nm) that are located in axons, found specifically in most mature neurons[45]. Neurofilaments are composed predominantly of distinct subunits, namely neurofilament light (NF-L), medium (NF-M) and heavy (NF-H)[46]. The anti-NF-200 antibody recognizes both phosphorylated and non-phosphorylated forms of heavy neurofilament subunit NF-H at 180–220 kDa. Immunostaining of neurofilaments confirmed the neuritogenesis was stimulated by NGF, hot aqueous, ethanolic and crude polysaccharides extracts (Fig. 3). PC-12 cells nuclei were stained blue by DAPI and neurofilaments were stained green by anti-NF-200 antibody labelled with FITC. Cells were elongated and exhibited significant neurite extensions in NGF-, hot aqueous extract-, ethanolic extract- and crude polysaccharides-stimulated neuritogenesis.

**The concentration of extracellular NGF of hot aqueous and ethanolic extracts-, and crude polysaccharides-treated cells.** The increase in extracellular NGF in cell supernatant showed the ability of tested compounds to induce NGF production by PC-12 cells[47]. The concentration of extracellular NGF of PC-12 cells without treatment (negative control) was detected at 65.64 pg/ml (Fig. 4). The concentration of extracellular NGF in 50 ng/ml of NGF-treated cells (positive control) was 353.42 pg/ml, which was approximately five fold increase compared to the negative control. However, there was no significant difference

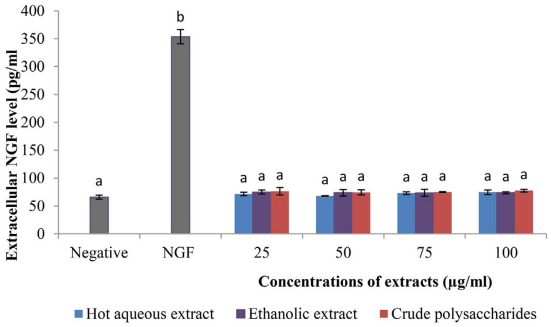


**Figure 4. Extracellular NGF concentration in supernatants of NGF-, extracts- or crude polysaccharides-treated PC-12 cells.** Cells were incubated with or without NGF (50 ng/ml), extracts or crude polysaccharides (25 to 100 μg/ml) for 48 h. Cells in complete F-12 K medium served as a negative control. Cells treated with 50 ng/ml of NGF served as a positive control. Data were expressed as means ± standard deviation (n = 3). Means with different alphabets show significant difference ($p < 0.05$).

($p > 0.05$) in the concentration of extracellular NGF between all the tested concentrations of the extracts and crude polysaccharides, and the negative control.

The present findings showed that the concentration of extracellular NGF in 50 ng/ml of NGF-treated cells increased by approximately 81% compared to the non-treated cells. Based on the amount of extracellular NGF measured and percentage of neurite bearing cells observed in positive control (NGF-treated cells), neuritogenesis in PC-12 cells may be NGF-dependent. Number of studies documented the potential of extracts and compounds of edible and medicinal mushrooms to stimulate the biosynthesis and secretion of NGF *in vitro*[40–42,44,47,48]. According to Lai *et al.* (2013)[48], hot aqueous extract of *Hericium erinaceus* (Bull.: Fr.) Pers. (lion's mane mushroom) stimulated the production of NGF in NG108–15 cells, a hybrid neuronal cell line derived from mouse neuroblastoma and rat glioma. The concentration of the extracellular NGF in NG108–15 cells treated with 50 μg/ml of hot aqueous extract of *H. erinaceus* was 21.4% higher, compared to the cells treated with 20 ng/ml of NGF[48]. Compounds from *H. erinaceus*, including hericenone C-E[40], erinacine A-C[41] stimulated biosynthesis of NGF and exhibited neuritogenesis in astroglial cells. In a recent study by Phan *et al.* (2014)[47] reported that hericenones E potentiated NGF-induced neuritogenesis in PC-12 cells by stimulating the production of NGF that was almost two times higher than that of positive control (50 ng/ml of NGF). However, in the present study, *L. rhinocerotis* sclerotium extracts and crude polysaccharides stimulated the neuritogenic activity without stimulating the production of NGF in PC-12 cells. These findings showed that the extracts might contain NGF-like compound(s) (NGF mimics or substitute for NGF) that mimic the neuritogenic activity of the NGF.

**The involvement of NGF responsive signaling pathway in hot aqueous extract-stimulated neuritogenesis.** The NGF responsive pathway, TrkA-MEK1/2-ERK1/2 was selected as the targeted cascade for neuritogenic activity in PC-12 cells. The Trk and MEK/ERK1/2 inhibitors, namely K252a, U0126 and PD98059 significantly ($p < 0.05$) blocked the NGF- and hot aqueous extract-stimulated neuritogenesis (Fig. 5). The K252a, U0126 and PD98059 decreased the percentage of neurite-bearing cells by approximately 82.13%, 86.15% and 91.56% in NGF-treated cells, and 80.97%, 86.68% and 84.59% in hot aqueous extract-treated cells, respectively. The significant ($p < 0.05$) reduction of neurite stimulation activity was also observed in the negative control with the addition of the inhibitors.

TrkA is a cell surface transmembrane receptor tyrosine kinase for NGF and activated TrkA is critical for activation of the Ras/MAPK signaling pathway[49,50]. Nerve growth factor binds to its high affinity receptor, TrkA to initiates the NGF responsive pathways, such as the MEK/ERK1/2 signaling pathway, to kick start neuritogenesis. Once the TrkA was phosphorylated, it became a scaffolding structure and recruits proteins that ultimately propagate the MEK/ERK signaling pathway[51]. Specific inhibitors of protein kinase served as powerful tool to study the kinase activities in selected signaling pathway[2]. K252a acts as a specific and potent inhibitor of Trk receptor, inhibits the phosphorylation of NGF-induced TrkA, and selectively blocks the activities of NGF in PC-12 cells[52]. In the present study, the neuritogenic activity of hot aqueous extract was blocked 80.97% by K252a, parallel to the inhibition effect of K252a (82.13%) towards NGF-treated cells. These findings showed that the hot aqueous extract-stimulated neuritogenesis was TrkA-dependant in PC-12 cells.

Both U0126 and PD98059 are selective and potent inhibitors of MEK 1 and MEK 2[53]. Priming PC-12 cells with U0126 and PD98059 will inhibit the phosphorylation and activation of MEK/ERK1/2, and eventually diminish cell differentiation and neuritogenesis[54]. Nishina *et al.* (2006)[55] demonstrated that the activation of MAPK by lysophosphatidylethanolamine, a neuroactive compound extracted from *Grifola frondosa* (maitake mushroom) was suppressed by U0126, but not by K252a. The study suggested that the MEK/ERK1/2 signaling pathway was involved in lysophosphatidylethanolamine-induced neuritogenesis in PC12 cells, but was not through the activation of TrkA receptor[55]. Phan *et al.* (2014)[47] reported

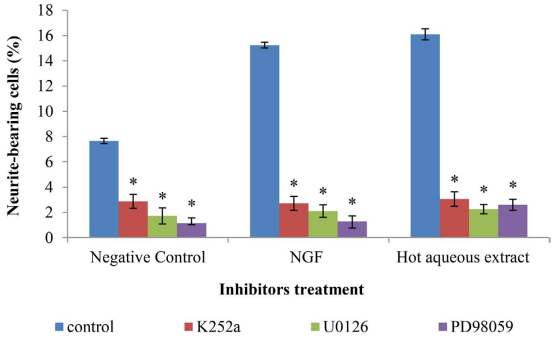


**Figure 5. The effects of the specific inhibitors of TrkA and MEK/ERK1/2 on NGF-, or hot aqueous extract-stimulated neuritogenesis.** Cells were pre-treated with K252a, U0126, PD98059 for one hour before the treatment with NGF (50 ng/ml) or hot aqueous extract (25 μg/ml). Cells in complete F-12 K medium served as a negative control. Cells treated with 50 ng/ml of NGF served as a positive control. A control (without inhibitor) was used in each treatment group. Data were expressed as means ± standard deviation (n = 3). *$p < 0.05$ compared to the respective controls.

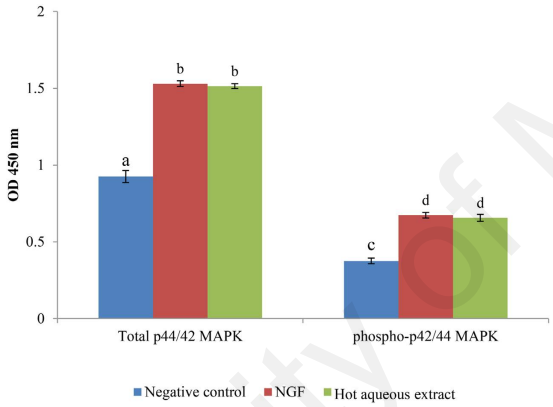


**Figure 6. Protein expression of total p44/42 MAPK (Erk1/2) and phosphorylated p44/42 MAPK (Thr202/Tyr204) activation in PC-12 after 48 h of incubation with NGF or hot aqueous extract.** Cells were incubated with or without NGF (50 ng/ml) or hot aqueous extract (25 μg/ml) for 48 h. Cells in complete F-12 K medium served as a negative control. Cells treated with 50 ng/ml of NGF served as a positive control. Total p44/42 and phosphorylated p44/42 protein level were measured by determination of the optical intensity (OD). Data were expressed as means ± standard deviation (n = 3). Different alphabets indicate significant difference ($p < 0.05$).

that the neuritogenic activity potentiated by hericenone E was found to be partially blocked (46%) by K252a and almost completely blocked by U0126 and PD98059, suggested that hericenone E potentiated NGF-stimulated neuritogenesis in PC12 cells was partially mediated by TrkA and MEK/ERK1/2 dependent. In the present study, all three inhibitors (K252a, U0126 and PD98059) successfully attenuated the NGF- and hot aqueous extract-stimulated neuritogenesis in PC-12 cells, showed that the hot aqueous extract of *L. rhinocerotis* sclerotium mimicked the NGF neuritogenic activity by binding to the TrkA receptor and activated the MEK/ERK1/2 signaling pathway in PC-12 cells.

**The protein expression of total p44/42 MAPK (ERK1/2) and phosphorylated p44/42 MAPK (Thr202/Tyr204) in hot aqueous extract-treated cells.** Endogenous level of total p44/42 MAPK (ERK1/2) and phosphorylated p44/42 MAPK (Thr202/Tyr204) were quantified by ELISA. The magnitude of absorbance for the developed colour is proportional to the quantity of MAPK protein expressed by PC-12 cells. The expression of both total and phosphorylated p44/42 MAPK protein in PC-12 cells treated with NGF and hot aqueous extract were significantly ($p < 0.05$) higher compared to the negative control (Fig. 6). Both total and phosphorylated p44/42 protein concentrations in NGF-treated cells were higher compared to the hot aqueous extract-treated cells and negative control ($p < 0.05$). However, there was no significant difference ($p > 0.05$) in protein expression between NGF- and hot aqueous extract-treated PC-12 cells for both total and phosphorylated p44/42, pERK1 and pERK2.

Activation of NGF responsive pathway by mushroom extract is crucial in the preliminary search of neuroactive compound(s) that may mimic the neuritogenic activity of NGF. Activation of MEK/ERK1/2 provided the biochemical evidence for neuritogenesis and the presence of neurite-stimulating agent(s) in the extract. Cheung *et al.* (2000)[24] reported that *G. lucidum* aqueous extract promoted neuritogenic and neuroprotective activity via Ras/ERK pathway in PC-12 cells, by demonstrating the phosphorylation of ERK1 and ERK2. In the present study, the endogenous level of total and phosphorylated p44 (ERK1) and p42 (ERK2) proteins were elevated, suggesting that the phosphorylation and activation of ERK1/2 were involved in the stimulation of neuritogenesis in PC-12 cells by the hot aqueous extract of *L. rhinocerotis* sclerotium.

**Immunofluorescence study demonstrated the protein expression of phosphorylated p44/42 MAPK (Thr202/Tyr204) in hot aqueous extract-treated cells.** Immunofluorescence staining was served as a visualize support to the protein expression of phosphorylated p44/42 MAPK (Thr202/Tyr204) (Fig. 7). The intensity of the immunofluorescence staining demonstrated the protein expression level of phosphorylated p44/42 protein in PC-12 cells. The intensity of immunofluorescence staining of phosphorylated p44/42 protein was higher in NGF- (Fig. 7B) and hot aqueous extract- (Fig. 7C) treated cells than the negative control (Fig. 7A). An MEK/ERK1/2 inhibitor, U0126 was used as a control to ensure the involvement of MEK/ERK1/2 signaling pathway in neuritogenesis. Pre-treatment with U0126 in PC-12 cells showed lower signal intensity (Fig. 7D–F). Results showed that neuritogenesis in PC-12 cells was dependent on the activation of MEK/ERK1/2 signaling pathway.

## Conclusions

The hot aqueous and ethanolic extracts, and crude polysaccharides of *L. rhinocerotis* sclerotium stimulated neuritogenesis in PC-12 cells. All the concentrations of the extracts and crude polysaccharides tested for neuritogenic activity were not cytotoxic to PC-12 cells. The hot aqueous extract (25 μg/ml) stimulated neuritogenic activity that was comparable to NGF (50 ng/ml). The extracts and crude polysaccharides stimulated neuritogenic activity but did not stimulate the production of NGF in PC12 cells. The neuritogenic activity of NGF- and hot aqueous extract may be mediated through the phosphorylation of TrkA receptor and ERK1/2 signaling pathway in PC-12 cells. *Lignosus rhinocerotis* sclerotium may contain neuroactive compound(s) that mimic the neuritogenic activity of NGF, and induce neuritogenesis in PC-12 cells via the NGF responsive pathway, TrkA-MEK1/2-ERK1/2 signaling pathway.

## Methods

**Preparation of hot aqueous and ethanolic extracts, and crude polysaccharides of *L. rhinocerotis* sclerotium.** *Lignosus rhinocerotis* sclerotium light brown and dry fluffy freeze-dried powder (LiGNO™ cultivar TM02) was purchased from Ligno Biotech Sdn. Bhd., Malaysia[56]. Every batch of cultivar TM02 freeze-dried powder is identified and validated by the internal transcribed spacer regions of ribosomal RNA[57]. The hot aqueous extraction was carried out according to Wong *et al.* (2007)[58] with modification. Briefly, the freeze-dried powder was soaked in distilled water at a ratio of 1:20 (w/v) and was agitated on a shaker at 150 rpm at room temperature, overnight. Then, the mixture was double boiled in a water bath for 30 min, and cooled to room temperature. The mixture was then centrifuged at 7,800 × g for 15 min, and the supernatant was collected and filtered through Whatman no. 4 filter paper. The resulting hot aqueous extract was freeze-dried and kept at −20 °C prior to use. For the ethanolic extract, the freeze-dried powder was soaked in 80% ethanol (v/v in distilled water) at room temperature for three days and the process was repeated three times. The ethanol was evaporated using a rotary evaporator (Eyela N-1000). The resulting ethanolic extract was kept in −20 °C prior to use. The crude polysaccharides were extracted according to the alkaline extraction method of Ojha *et al.* (2010)[59]. The freeze-dried powder was soaked in 4% (w/v) sodium hydroxide (NaOH) and heated in 80 °C water bath for 45 min, then the mixture was centrifuged at 7,800 × g for 45 min. Supernatant was collected and precipitated with absolute ethanol at a ratio of 1:5 (v/v). The mixture was kept for 12 h at 4 °C. The precipitated polysaccharides were centrifuged at 7,800 × g for 45 min. The residue was dialysed using diethylaminoethyl (DEAE) cellulose bag for 4 h. The crude polysaccharides were freeze-dried and kept at −20 °C prior to use.

***In vitro* cell culture.** The PC-12 cells (American Type Culture Collection, ATCC) were maintained in complete Kaighn's Modification of Ham's F-12 (F-12 K) Medium (Sigma) supplemented with 15% of heat-inactivated horse serum and 2.5% of heat-inactivated Fetal bovine serum (PAA Laboratories) at 37 ± 2 °C in a 5% $CO_2$-humidified incubator. The cells were passaged every 2 to 3 days upon 80% confluent.

**Assessment of the effects of hot aqueous and ethanolic extracts, and crude polysaccharides on viability of PC-12 cells.** Cells were plated at a density of $1 \times 10^4$ cells per well in 96-well plates and incubated overnight at 37 °C in a 5% $CO_2$-humidified incubator. Then, the supernatant was carefully replaced with freshly prepared extracts or crude polysaccharides (0–2500 μg/ml) in complete F-12 K medium. After 48 h of incubation, 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed as described previously[26]. The extent of the reduction of MTT was determined

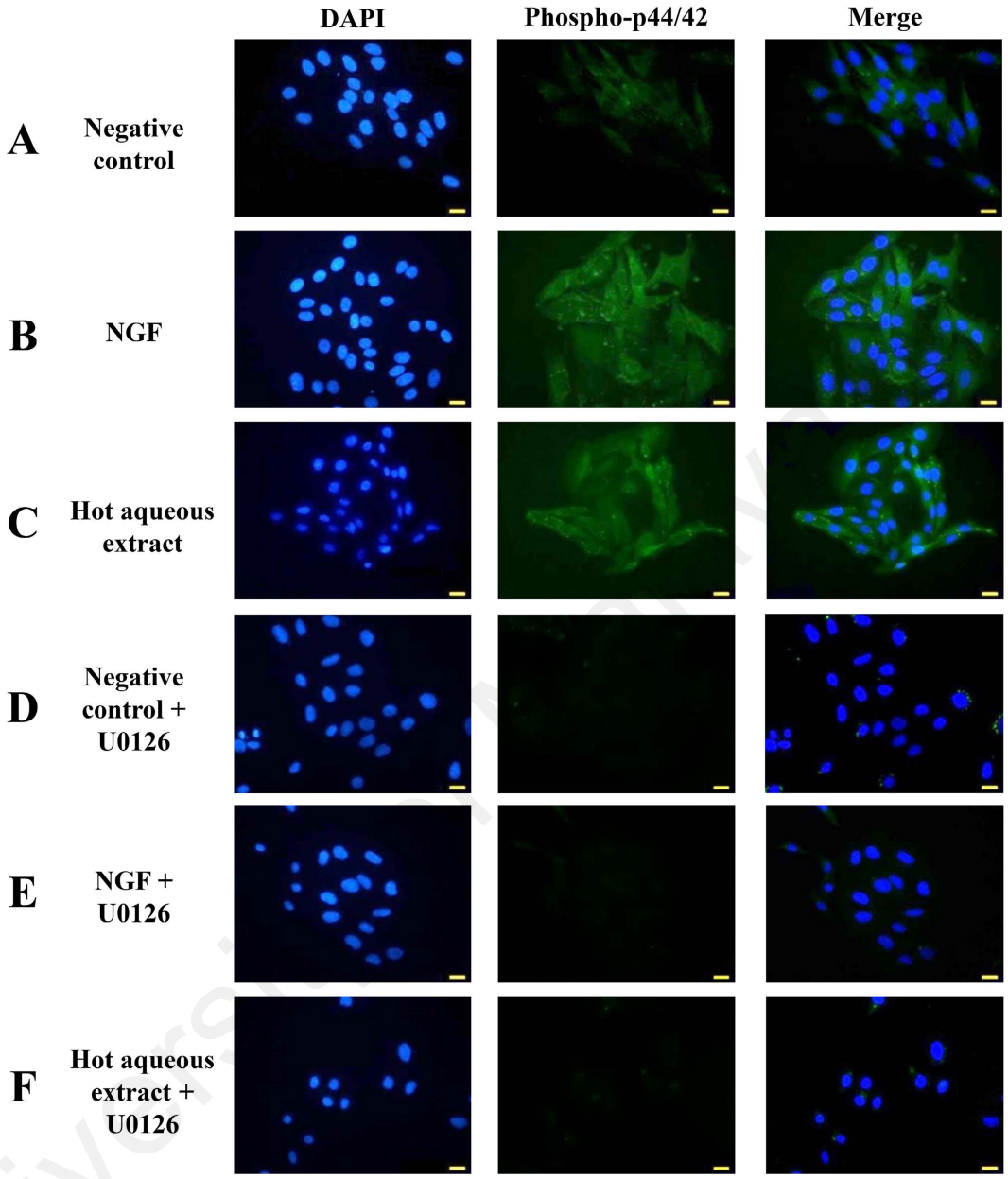


**Figure 7. Immunocytochemistry study of phosphorylated p44/42 MAPK (Thr202/Tyr204) in NGF- or hot aqueous extract-treated PC-12 cells after 48 h of incubation.** (**A**) Negative control, cells in complete F-12 K medium only. (**B**) Positive control, cells treated with 50 ng/ml of NGF. (**C**) Cells treated with 25 μg/ml of hot aqueous extract. (**D**–**F**) Cells were pre-treated with 10 μM U0126 prior to the treatment with NGF or hot aqueous extract. Phosphorylated p44/42 protein stained cell body in green. DAPI stained nuclei in blue. Scale bar = 50 μm.

by measurement of the absorbance at 540 nm with 690 nm as background absorbance with an ELISA microplate reader (Sunrise, Tecan). The complete F-12 K medium was the blank, and cells incubated in the medium only were denoted as the negative control. The 50% inhibitory concentration ($IC_{50}$) was interpolated from the response curve.

**Assessment of neuritogenic activity of hot aqueous and ethanolic extracts, and crude polysaccharides in PC-12 cells.** *Stimulation of neuritogenesis in PC-12 cells.* Cells were plated at a density of $5 \times 10^3$ cells per well in 12-well plates and then treated with freshly prepared extracts or crude polysaccharides (25 to 100 μg/ml) in complete F-12 K medium. Cells treated with 50 ng/ml of NGF (Sigma) served as a positive control, while cells in complete F-12 K medium without treatment served as a negative control. Assay plates were incubated for 48 h at 37 ± 2 °C in a 5% $CO_2$-humidified incubator prior to scoring the neurite-bearing cells.

*Quantification of neurite bearing-cells.* Differentiated cells were counted by visual examination of the field. A neurite-bearing cell was defined as a cell with one or more axon-like extension that was double or more the length of the cell body diameter[58]. Ten selected microscopic fields with an average of 200–300 cells per well were assessed under an inverted microscope (Nikon Eclipse TS100). The images were captured with a QImaging Go-3 color CMOS Camera (QImaging) and by the image processor system, Image-Pro Insight (MediaCybernetics). The percentage of neurite-bearing cells was evaluated by scoring the proportion of neurite-bearing cells to the total number of cells in a well.

*Immunofluorescence staining of neurofilaments.* Immunofluorescence assay was carried out according to Schimmelpfeng *et al.* (2004)[60]. Briefly, cells were seeded at a density of $5 \times 10^3$ cells per well in 12-well micro-chamber (ibidi). The cells were treated with the extracts or crude polysaccharides for 48 h at $37 \pm 2$ °C in a 5% $CO_2$-humidified incubator. After fixing with 4% paraformaldehyde, the cells were incubated with anti-neurofilament 200 antibody produced in rabbit (1:80; Sigma), and followed by fluorophore-conjugated secondary antibody, anti-rabbit IgG-fluorescein isothiocyanate (FITC) antibody produced in sheep (1:80; Sigma). Finally, the cells were mounted with ProLong® gold antifade reagent with 4-6-Diamidino-2-phenylindole (DAPI) (Life Technologies Corporation). The slides were observed under fluorescence illumination using FITC and DAPI filters and images were captured with Nikon's Imaging Software, NIS-Elements.

**Quantification of the concentration of extracellular NGF in cell culture supernatant.** Cells were plated at a density of $1 \times 10^4$ cells per well in 96-well plates. The cells were treated with freshly prepared extracts or crude polysaccharides (25 to 100 μg/ml) in complete F-12 K medium for 48 h. The cell culture supernatant was collected, centrifuged at $1500 \times$ g for 15 min and maintained at 0–4 °C prior to assay. The samples were diluted with sample diluent at a ratio of 1:2 (v/v). The amount of NGF in culture supernatant was measured by using ChemiKine™ nerve growth factor sandwich enzyme-linked immunosorbent assay (ELISA) kit (Chemicon® International Inc.) according to the manufacturer's protocol.

**Elucidation of the involvement of NGF responsive pathway in the hot aqueous extract-stimulated neuritogenesis in PC-12 cells.** *Treatment with specific inhibitors of signaling pathway.* The inhibitors for Trk receptor (K252a) and MEK/ERK1/2 signaling pathway (U0126, PD98059) (all Sigma, USA) were used. Stock solutions (10 mM) of the inhibitors were prepared in dimethyl sulfoxide (DMSO) and stored at −20 °C in the dark. Final concentrations of 100 nM of K252a, 10 μM of U0126 and 40 μM of PD98059 were prepared by diluting in complete F-12 K medium before use. Cells were pre-incubated either with or without the inhibitor for one hour at $37 \pm 2$ °C in a 5% $CO_2$-humidified incubator, respectively before the treatment with 50 ng/ml of NGF or 25 μg/ml of hot aqueous extract (the concentration that stimulated maximal percentage of neurite-bearing cells). Cells were then incubated for 48 h prior to scoring the neurite-bearing cells.

*Quantification of protein expression of total p44/42 MAPK (ERK1/2) and phosphorylated p44/42 MAPK (Thr202/Tyr204).* Cells were pre-incubated either with or without the MEK/ERK1/2 inhibitor, U0126 for one hour at $37 \pm 2$ °C in a 5% $CO_2$-humidified incubator, before the treatment with 50 ng/ml of NGF or 25 μg/ml of hot aqueous extract for 48 h. Cells were washed and harvested in PBS, and centrifuged at $10{,}000 \times$ g for 10 min at 4 °C. Cell pellets were resuspended in cell lysis and protein extraction buffer (Thermo Fisher Scientific Inc.) with protease inhibitor cocktail and 1 mM of phenylmethylsufonyl fluoride (Sigma) on ice, and vortex every 10 min for three times. Then, the extracted proteins were centrifuged and pelleted at $10{,}000 \times$ g for 10 min at 4 °C. Supernatants were aliquoted into clean micro centrifuge tubes and kept cool prior to assay. The protein expression was quantified by using the total p44/42 MAPK (ERK1/2) and phosphorylated p44/42 MAPK (Thr202/Tyr204) sandwich ELISA kits (Cell Signaling Technology) according to the manufacturer's protocol. The absorbance was recorded at 450 nm with an ELISA microplate reader (Sunrise, Tecan). Concentration of the protein antibodies of every treatment were quantified according to the standard graph and multiplied by the appropriate dilution factor.

*Immunofluorescence study of protein expression of phosphorylated p44/42 MAPK (Thr202/Tyr204).* Cells were pre-incubated either with or without the U0126 inhibitor for one hour at $37 \pm 2$ °C in a 5% $CO_2$-humidified incubator before the treatment with 50 ng/ml of NGF or 25 μg/ml of hot aqueous extract. Cells were then incubated for 48 h prior to immunofluorescence staining. Protein expression of phosphorylated p44/42 MAPK (Thr202/Tyr204) (1:100; Cell Signaling Technology) detection antibody was used as the primary antibody. The slides were observed under fluorescence illumination using FITC and DAPI filters and images were captured with Nikon's Imaging Software, NIS-Elements.

**Statistical analysis.** All the experimental data were expressed as the mean ± standard deviation (SD) of triplicate values. Statistical differences between groups were assessed using one-way analysis of

variance (ANOVA) of a minimum of three independent experiments and Duncan's multiple range test (DMRT), $p < 0.05$ was considered to be significant.

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## Author Contributions

S.L.-S.S. and L.-F.E. performed the experiments, and analysed the data; S.L.-S.S. drafted the manuscript; M.N., P.D. and K.-H.W. conceived and designed the experiments, and edited the manuscript. V.S. provided the grant, coordinated and monitored the research, and edited the manuscript. All authors reviewed and approved the final manuscript.

## Additional Information

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