AMAURODERMA RUGOSUM (BLUME & T. NEES) TORREND ATTENUATES THE PATHOPHYSIOLOGICAL CASCADE OF INFLAMMATORY EVENTS IN EPILEPTOGENESIS

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ABSTRACT

Amauroderma rugosum has been used as a remedy to prevent epileptic episodes, incessant crying by babies and cancer, reduce inflammation, and treat diuresis and upset stomach by the indigenous people in Malaysia and the Chinese in China. To date, the medicinal properties and traditional beliefs of *A. rugosum* are yet to be explored and verified. Hence, the aims of this study are to show the successful cultivation of *A. rugosum* and evaluate the antioxidant, anti-inflammatory, anti-neuroinflammatory, and anti-epileptic activities using *in vitro* and *in vivo* approaches. Based on the results obtained, mycelium of *A. rugosum* has good source of nutrients. Besides that, hexane fraction (HF) from mycelium, ethanolic extracts of wild (WB) and domesticated (DB) basidiocarps of *A. rugosum* showed significant antioxidant, anti-inflammatory, and anti-neuroinflammatory activities. HF was selected for in-depth study on the modulation of neuroinflammatory pathways and anti-epileptic activity. HF was the most effective nitric oxide (NO) scavenger and NO production inhibitor in lipopolysaccharide (LPS)-stimulated macrophage (RAW264.7) and microglia (BV-2) cells. The inhibition of NO productions was mediated through the down-regulation of inducible nitric oxide synthase (iNOS) expression. Besides, HF significantly suppressed the inflammatory cytokine such as tumour necrosis factor alpha (TNF-α) and up-regulated anti-inflammatory cytokine, interleukin-10 (IL-10) in macrophage cells. Furthermore, HF was found to down-regulate proinflammatory cytokines (IL-1α, IL-1β, and IL-6) and other proinflammatory (Traf1, Traf2, and Cd14) gene expressions in microglial cells. In addition, HF up-regulated anti-inflammatory genes such as Nfkbia and Nfkbie. *In vivo* assessment in rats revealed that HF could ameliorate epileptic seizure behaviours such as wet dog shakes, facial myoclonia, and paw tremor. Two major compounds were detected in HF which includes ethyl linoleate and ergosterol. In conclusion, HF

exhibited antioxidant activity and attenuated proinflammatory mediators and epileptic seizure behaviours. Therefore, *A. rugosum* may serve as a potential therapeutic agent in the management of inflammation leading to epilepsy.

ABSTRAK

Amauroderma rugosum adalah sejenis cendawan liar yang kerap digunakan sebagai ubat oleh orang-orang asli di Malaysia dan orang Cina di China untuk mengelakkan episod epilepsi, menangani bayi menangis malam dan kanser, mengurangkan keradangan, dan merawat diuretik dan masalah perut. Setakat ini, ciri-ciri perubatan cendawan liar ini belum diterokai dan kepercayaan tradisionalnya belum disahkan. Oleh itu, matlamat kajian ini adalah untuk menjayakan kultivasi *A. rugosum* dan menilai aktiviti-aktiviti seperti antioksidan, anti-radang, anti-radang otak, dan anti-epileptik dengan menggunakan pendekatan *in vitro* dan *in vivo*. Berdasarkan keputusan eksperimen yang diperolehi daripada kajian ini, miselium *A. rugosum* mempunyai kandungan nutrien yang baik. Selain itu, pecahan heksana (HF) daripada miselium, dan ekstrak etanol daripada cendawan liar dan cendawan yang dikultivasi menunjukkan aktiviti antioksidan yang signifikan serta aktiviti anti-radang dan aktiviti anti-radang otak yang berkesan. HF telah dipilih untuk kajian yang mendalam mengenai modulasi laluan radang otak dan aktiviti anti-epilepsi. HF merupakan ekstrak yang paling berkesan di antara ekstrak-ekstrak yang dikaji dalam perencatan nitrik oxida (NO) dimana HF dapat menyekat produksi NO daripada makrofaj dan mikroglia yang dirangsang oleh lipopolisakarida (LPS). Penyekatan produksi NO oleh HF adalah melalui supresi ekspresi gen “inducible nitric oxide synthase” (iNOS). Di samping itu, HF dapat menyekat sitokin radang seperti tumor nekrosis faktor alpha (TNF-α) dan anti-sitokin radang, interleukin-10 (IL-10) yang dihasilkan oleh sel-sel makrofaj. Tambahan pula, HF didapati boleh menyekat ekpresi gen-gen sitokin radang seperti IL-1α, IL-1β dan IL-6, dan gen-gen radang yang lain seperti Traf1, Traf2 dan Cd14. Selain itu, HF dapat mengawal gen anti-radang seperti Nfkbia dan Nfkbie. Dalam kajian anti-epileptik yang menggunakan pendekatan *in vivo*, HF didapati mempunyai aktiviti anti-epileptik

yang dapat memperbaiki tingkah laku epilepsi seperti “wet dog shake”, “facial myoclonia”, dan “paw tremor”. Dua komponen utama yang telah dikesan dalam HF termasuk etil linoleate dan ergosterol. Kesimpulannya, HF mempunyai aktiviti antioxidant, anti-radang, anti-radang otak, dan anti-epileptik. Oleh itu, *A. rugosum* berpontensi digunakan sebagai agen terapeutik dalam pergurusan aktiviti radang yang membawa kepada epilepsi.

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"Family is the most important thing in the world!" – Princess Diana

Chan Pui Mun
2016

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LIST OF SYMBOLS AND ABBREVIATIONS

Symbols/Abbreviations	Description
%	Percentage
µm	micrometer
4-HNE	4-hydroxynonenol
A	Absorbance
AACC	American Association of Cereal Chemists
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
ACTB	Beta-actin
ACTH	adrenocorticotropic hormone
AEDs	Anti-epileptic drugs
AF	Aqueous fraction
ALD	X-linked adrenoleukodystrophy
Anova	One-way analysis of variance
AOAC	Association of Analytical Communities/Association of Official Agricultural Chemist
AP-1	Activator protein-1
A-SMase	Acidic sphingomyelinase
ATCC	American Type Culture Collection
B.C.	Before Christ
B.C.E	Before the common era
BBB	Blood-brain barrier
BHT	Butylated hydroxytoluene
bNOS / NOSI	Brain nitric oxide synthase

BUN	Blood urea nitrogen
C/EBPβ	CCAAT/enhancer-binding protein beta
C3	Complement component 3
Ca	Calcium
$CaCO_3$	Calcium carbonate
Casp1	Caspase 1
CAT	Catalase
CD14	Cluster of differentiation 14
CD-4	Cluster of differentiation-4
cDNA	Complementary deoxyribonucleic acid
cm	Centimeter
CNS	Central nervous system
CO_2	Carbon dioxide
COX-2	Cyclooxygenase-2
COX-2	Cyclooxygenase-1
CTCF	Corrected total cell fluorescence
Cu	Copper
DAPI	4',6-diamidino-2-phenylindole
DB	Ethanol extract of domesticated basidiocarp
Ddit3	DNA damage-inducible transcript 3
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Nucleoside triphosphate
DPPH	2,2-diphenyl-1-picrylhydrazyl
EAF	Ethyl acetate fraction

EC_{50}	half maximal effective concentration
EDTA	Ethylenediaminetetraacetic acid
EE	Crude ethanol extract of mycelium
EEG	Electroencephalogram
ELISA	Enzyme-linked immunosorbent assay
EMG	Electromyogram
eNOS / NOSIII	Endothelial nitric oxide synthase
ERK/MAPK	Extracellular signal-regulated kinase/mitogen-activated protein kinase
eV	Electrovolt
FAM	Fluorescein
FAO	Food and Agriculture Organization of the United Nations
FBS	Foetal bovine serum
FDA	Food and Drug Administration
Fe	Iron
FITC	Fluorescein isothiocyanate
g	Gram
g	Gravity
$GABA_A$	γ-aminobutyric acid receptor
GC-MS	Gas chromatography–mass spectrometry
GOT	Aminotransferase aspirate
GPCRs	G-protein-coupled receptors
GPT	Aminotransferase alanine
GPx	Glutathione peroxidase
GSH	Glutathione
H_2O_2	Hydrogen peroxide

H_3PO_4	Phosphoric acid
HF	Hexane fraction
HMGB1	high-mobility group box 1
HPLC	High performance liquid chromatography
HSP	Heat shock protein
IACUC	Institutional Animal Care and Use Committee
IC_{50}	half maximal inhibitory concentration
ICE/caspase-1	Inhibition of interleukin-converting enzyme
ICP-OES	Inductively coupled plasma optical emission spectrometry
ID	Identity
IFN-γ	interferon-γ
IgG	Immunoglobulin G
IKK	Inhibitory of kappa B kinase
IL-1	Interleukin-1
IL-10	Interleukin-10
IL-12p40	Subunit beta of interleukin-12 or cytotoxic lymphocyte maturation factor 2, p40
IL-1α	Interleukin-1 alpha
IL-1β	Interleukin-1 beta
IL-2	Interleukin-2
IL-6	Interleukin-6
IL-8	Interleukin-8
iNOS	Inducible nitric oxide synthase
IVIg	Intravenous immunoglobulin
IκB-α	inhibitory of kappa B alpha

JNK	c-Jun N-terminal Kinase
K	Potassium
$K_2S_2O_8$	Potassium persulphate
KA	Kainic acid
kg	Kilogram
L	Liter
L-NAME	N_{ω}-nitro-l-arginine-methyl ester
LPS	Lipopolysaccharide
Lt Cx	EEG recordings in the left sensorimotor cortex
MDA	malondialdehyde
MES	Maximal electro shock
MF	Molecular formula
Mg	Magnesium
mg	Milligram
MGB	Minor groove binder
min^{-1}	Revolution per minute
mL	Milliliter
mM	Millimolar
Mm	*Mus musculus*
Mn	Manganese
mol^{-1}	Per mole
mRNA	Messenger ribonucleic acid
MS	Multiple sclerosis
ms	Millisecond
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

MW	Molecular weight
n	number
N.D.	Not detectable
Na	Sodium
$NaCO_3$	Sodium carbonate
$NaHCO_3$	Sodium bicarbonate
$NaNO_2$	Sodium nitrite
NCBI	National Center for Biotechnology Information
Nfkbia/IκB-α	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
Nfkbie/IκBε	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, epsilon
NF-κB	Necrosis factor kappa B
NGF	Nerve growth factor
nm	Nanometer
NO	Nitric oxide
No.	Number
NOS / NOSII	Nitric oxide synthase
NSAIDs	non-steroidal inflammatory drugs
N-SMase	Neutral sphingomyelinase
$O_2^{\cdot -}$	Superoxide anion
^oC	Degree celcius
$OH^{\cdot}$	Hydroxyl radical
P	Phosphorus
p38 MAPK	p38 mitogen-activated protein kinases
PBS	Phosphate buffer saline

PBST	Phosphate buffer saline-Triton X-100
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PDB	Potato dextrose broth
PGE_2	Prostaglandin E_2
PI3K-Akt-mTOR	Phosphatidylinositol-3-kinase - protein kinase B - mammalian target of rapamycin
PUFAs	Polyunsaturated fatty acids
p-value	Marginal significance within a statistical hypothesis test
PVL	Periventricular leukomalacia
R_2	Correlation
RDA	Recommended daily allowance
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
rpm	Revolutions per minute
RT	Reverse transcription
RT	Retention time
Rt Cx	EEG recordings in the right sensorimotor cortex
S.D.	Standard deviation
S100β	S100 calcium-binding protein beta
Se	Selenium
SEA	Single Experiment Analysis
Ser32	Serine 32
Ser36	Serine 36

SOD	Superoxide dismutase
sp.	Species
SPSS	Statistical Product and Service Solutions
Stmn1	Stathmin 1
TBHQ	tertiary butyl hydroquinone
TLR	Toll-like receptor
TLR-4	Toll-like receptor-4
TNF-α	Tumour necrosis alpha
TPC	Total phenolic content
Traf1	TNF receptor-associated factor 1
Traf2	TNF receptor-associated factor 2
U.S	United states
UV-VIS	Ultraviolet-visible
VA	Valproic acid
w/v	Weight per volume
WB	Ethanol extract of wild basidiocarp
Zn	Zinc
α	Alpha
β	Beta
Δ	Delta
κ	Kappa
μ	Micro
μg	Microgram
μL	Microliter
μM	Micromolar

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CHAPTER 1

GENERAL INTRODUCTION

Oxidative stress is caused by the imbalance between production of reactive oxygen species (ROS), and the ability of biological systems to detoxify reactive intermediates. This imbalance causes damage to important biomolecules and organs with potential impacts on the entire organism (Ďuračková, 2010). Most ROS are generated in cells by the mitochondrial respiratory chain, which is largely modulated by the rate of electron flow through the respiratory chain complexes. The biological reduction of molecular oxygen in aerobic cells produce ROS such as superoxide anion ($O_{2}^{\cdot -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\cdot}$) and organic peroxides, and the excess production of these radicals can oxidise and damage proteins, nucleic acids and lipids (Poyton, Ball, & Castello, 2009). Hence, ROS are often associated with chronic inflammation and a wide variety of cancers (Reuter, Gupta, Chaturvedi, & Aggarwal, 2010).

Inflammation is the reaction of tissue to irritation, injury or infection, characterised by pain, redness and swelling (Wen et al., 2011). It is a protective process of the body that functions to destroy invading organisms or repair tissues after injury (Lee, Jung, Bang, Chung, & Kim, 2012). However, sustained or excessive inflammation may predispose the host to various chronic inflammatory diseases such as epilepsy, arthritis, asthma, multiple sclerosis and atherosclerosis (Lee et al., 2012; Reuter et al., 2010; Vezzani, 2005; Wen et al., 2011). During inflammation, activated macrophages secrete several inflammatory mediators such as nitric oxide (NO), tumour necrosis factor alpha (TNF-α), and interleukins (IL-6, IL-1α, IL-1β) (Dudhgaonkar, Thyagarajan, & Sliva, 2009;

Sautebin, 2000; Yang, Yim, Song, Kim, & Hyun, 2009; Yu, Weaver, Martin, & Cantorna, 2009). Overproduction of these proinflammatory mediators can result in detrimental and pathogenic effects to the host.

Brain inflammation or neuroinflammation has been implicated as one of the main factors that cause epilepsy (Ravizza et al., 2008; Vezzani, Aronica, Mazarati, & Pittman, 2013; Vezzani, 2005). Chronic neuroinflammation causes the loss of blood-brain barrier (BBB) function and increases the vascular permeability to circulate leukocytes and inflammatory mediators. Thus, this affects the neuronal activity in the brain and eventually causes recurrent seizures that lead to the development of epileptic disorders (Marchi, Granata, & Janigro, 2014).

Considering the undesirable side effects of anti-inflammatory and anti-epileptic drugs available in the market, natural products/herbal medicines have gained significant interest as a source of new effective therapeutic agents. Mushrooms have been consumed by humans as a component of their normal diet since ancient times. The increased interest in scientific studies on mushrooms is attributed to the significant amounts of bioactive compounds produced by the fruiting body and the mycelium biomass (Ferreira, Barros, & Abreu, 2009). In Malaysia, selected mushrooms are used by the locals and indigenous people as a home remedy (Sabaratnam, Wong, Naidu, & David, 2011). *Amauroderma rugosum* is a basidiomycete with a stipe that is black with white pore underneath that bruises to a blood red colour when touched (Zhishu, Guoyang, & Taihui, 1993). *Amauroderma* sp., which is also known as the "epileptic child mushroom" or "cendawan budak sawan" in the Malay language, is worn around the neck by the indigenous people in Malaysia to prevent fits and incessant crying by babies (Azliza, Ong, Vikineswary, Noorlidah, & Haron, 2012; Chang & Lee, 2004). Fits

or epilepsy has been linked with inflammation and its development is termed epileptogenesis (Walker & Sills, 2012). Besides, the Chinese people in China use this mushroom to reduce inflammation, treat diuresis and upset stomach, and prevent cancer (Dai & Yang, 2008).

Justification of study

Of the many diseases that threaten human lives, chronic inflammatory diseases are a common epidemic. Inflammation is inherent to the pathogenesis of a variety disease such as cancer, cardiovascular disorders, asthma, and diseases of central nervous systems. Besides, inflammation is one of the major causes of epilepsy (Vezzani, 2005, 2014). Thus, targeting inflammation may be a strategy towards finding a therapeutic treatment for epilepsy (Walker & Sills, 2012).

Although many drugs have been used to treat inflammatory related disorders, most of them have limited potential as their side effects cause serious damage and suffering to patients (Ong, Lirk, Tan, & Seymour, 2007). One of the alternative possible strategies to suppress inflammation is the use of natural compounds such as botanicals, bioactive food components or functional foods (Jedinak et al., 2011). Today, people are diverting their attention to herbal medicines because they have vast popularity as self-medication products and are easily available (Harvey, Edrada-Ebel, & Quinn, 2015).

Several studies have proven that mushrooms possessed good nutritional value and health-promoting effect to human beings. Also, researchers have discovered possible functional properties in mushrooms, which could be effective in the treatment of inflammatory diseases (Elsayed, Enshasy, Wadaan, & Aziz, 2014). Mushrooms can be added to the diet and used orally, without the need to go through clinical trials as an

ordinary medicine. Hence, it is important to perform scientific investigations on mushrooms to discover possible functional properties, which could be efficient in possible treatments of inflammatory and chronic diseases.

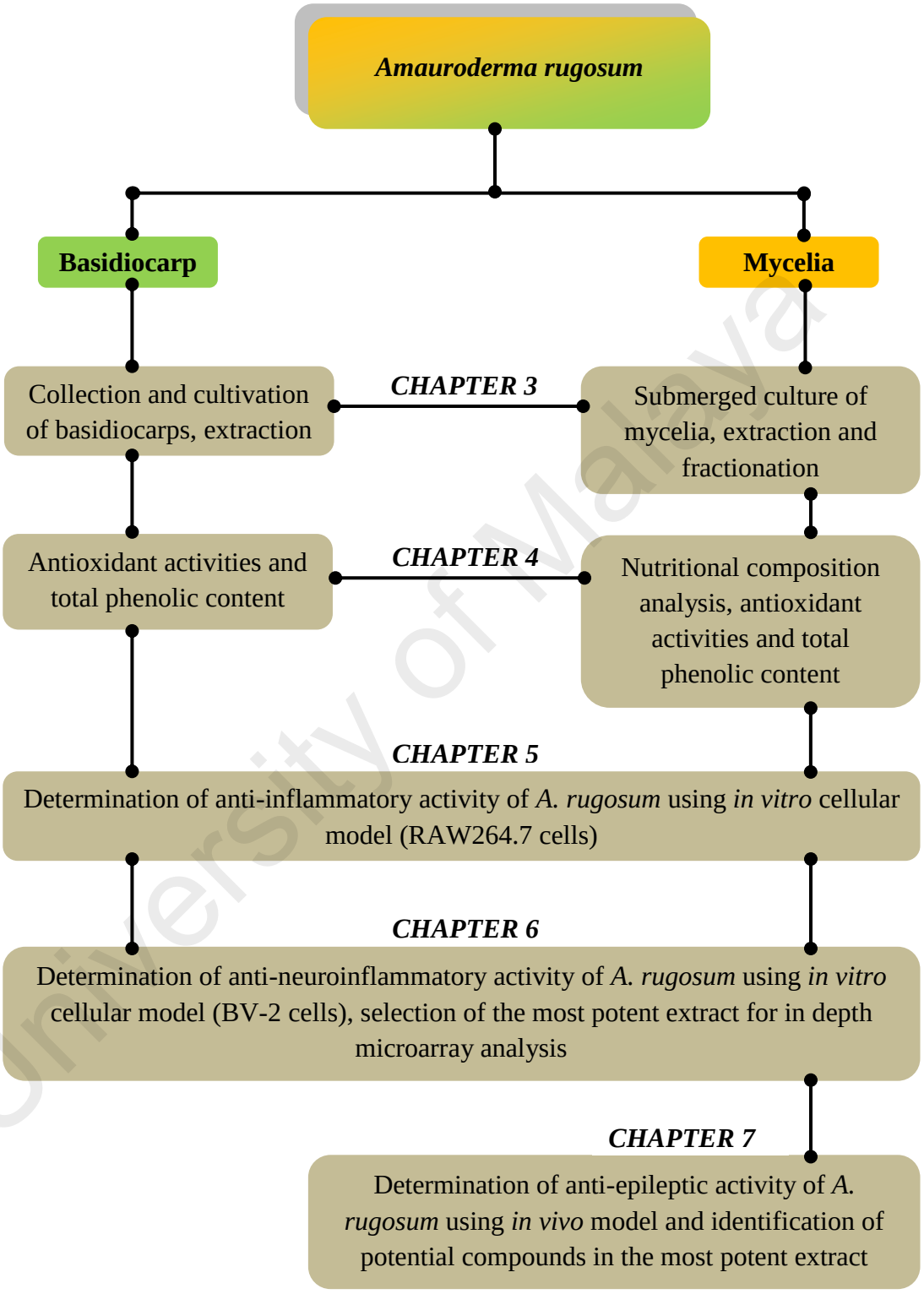
The study of antioxidant potential, anti-inflammatory, anti-neuroinflammatory, and anti-epileptic effects of *A. rugosum* was initiated based on the traditional aboriginal beliefs that this mushroom can reduce or prevent fit episodes and inflammation. Therefore, it is imperative that the claims on the potential anti-inflammatory and anti-neuroinflammatory activities in the prevention of epilepsy by *A. rugosum* be validated.

Objectives

The objectives of this study were:

1) to collect and cultivate the basidiocarps of *Amauroderma rugosum*, culture mushroom mycelia using submerged culture technique, and extract and fractionate chemical components from basidiocarps and mycelia of *A. rugosum*.
2) to identify the nutritional composition of freeze-dried mycelia and determine the antioxidant and total phenolic content of *A. rugosum* extracts
3) to evaluate the *A. rugosum* extracts for anti-inflammatory activity at biochemical and molecular levels using *in vitro* cellular model – murine macrophage, RAW264.7 cells
4) to evaluate the *A. rugosum* extracts for anti-neuroinflammatory activity at biochemical and molecular levels using *in vitro* cellular models – murine microglial, BV-2 cells
5) to determine the anti-epileptic activity of *A. rugosum* extract using *in vivo* model.

Flow chart of experimental design



CHAPTER 2

LITERATURE REVIEW

2.1 Mushrooms

For many years, mushrooms have been known for their health benefits and are great source of nutrients and medicinal properties. Mushrooms provide multiple benefits to human kind such as food, health tonics and medicine, feed and fertilizers. The most common species of mushrooms used in medicine are shiitake (*Lentinus edodes*), reishi (*Ganoderma lucidum*), turkey tail (*Trametes versicolor*), split gill (*Schizophyllum commune*), mulberry yellow polypore (*Phellinus linteus*), and chaga or cinder conk (*Inonotus obliquus*) (Wasser, 2002).

Mushrooms have significant pharmacological effects and physiological properties such as immune enhancement, maintenance of homeostasis and regulation of biorhythm, cure of various diseases, and prevention from life threatening diseases such as cancer, cerebral stroke and cardiovascular diseases (Ajith & Janardhanan, 2007). In Malaysia, some local communities and aborigines use mushrooms as a source of food and also as medicine without knowing the active components that contribute to the medicinal effect (Chang & Lee, 2004). Studies have shown that mushrooms consist of effective substances that have antioxidant, antifungal, anti-inflammatory, anti-tumour, anti-viral, anti-bacterial and anti-diabetic properties (Vishwakarma, Bhatt, & Gairola, 2011; Yu et al., 2009).

Although there are approximately 10,000 known mushroom species and 2,000 are safe for human consumption, all medicinal properties of edible wild mushrooms are yet to be

fully explored (Joseph, Sabulal, George, Smina, & Janardhanan, 2009). Edible mushrooms can be added to the diet and used orally without the need to go through clinical trials as an ordinary medicine. It is safe and useful new approach for disease prevention and treatment (Lull, Wichers, & Savelkoul, 2005).

2.1.1 Diversity of mushrooms with medicinal benefits

Mushrooms have great potential as polypharmaceutic drugs due to the variety of bioactivities (Phan, David, Naidu, Wong, & Sabaratnam, 2014). Medicinal mushrooms are known to possess approximately 130 medicinal functions which include antioxidant, anti-cancer, anti-fungal, anti-diabetic, anti-inflammation, anti-parasitic, hepatoprotective, and detoxification (Wasser, 2014). In addition, numerous bioactive compounds from mushrooms have been reported and documented to be effective in enhancing human quality of life (Lindequist, Niedermeyer, & Jülich, 2005).

A handful of mushrooms have been reported to possess anti-inflammatory properties. Yu, Weaver, Martin, & Cantorna (2009) concluded that edible mushrooms such as *Agaricus bisporous, Grifola frondosa, L. edodes,* and *Pleurotus eryngii* possessed anti-inflammatory activities which can regulate production of cytokines by macrophage and T-cell. The ethanolic extract of *G. lucidum* was found to inhibit inflammatory response by suppressing the NF-κB and toll-like receptor pathways in LPS-stimulated BV-2 microglial cells (Yoon et al., 2013). Besides, a study by Queiroz et al. (2010) suggested that glucans from *Caripia montagnei* contains polysaccharides with immunomodulatory properties and may be a potential anti-inflammatory agent.

The mushroom *Pleurotus sajor-caju* is rich in β-glucan and has anti-diabetic, lipid lowering effect, and antioxidant potential (Kanagasabapathy et al., 2013). Besides,

Pleurotus ostreatus was found to have significant hypoglycaemic effect in diabetic mice and improved hyperlipidemia and impaired kidney functions in alloxan-induced diabetic mice (Ravi, Renitta, Prabha, Issac, & Naidu, 2013). Ma, Chen, Dong, & Lu (2013) reported that ergosterol, ergosterol peroxide, and trametenolic acid isolated from *Inonotus obliquus* have anti-inflammatory effects. The same group of researchers also found that ergosterol peroxide and trametenolic acid have cytotoxic effects on human prostatic carcinoma cell PC3 and breast carcinoma MDA-MB-231 cell which suggest the potential effect of *I. obliquus* as an anti-cancer agent.

2.2 Overview of *Amauroderma rugosum*

Amauroderma rugosum (Blume and T. Nees) Torrend is a basidiomycete with stipe, ravous to black pileus, and white pore underneath which bruises to blood red when it is touched (Figure 2.1) (Zhishu et al., 1993). It is commonly known as *"Jia Zhi"* in China. The Chinese use *A. rugosum* to reduce inflammation, prevent cancer, and treat diuresis and upset stomach (Dai & Yang, 2008). Besides, *A. rugosum* is also known as epileptic child mushroom or "cendawan budak sawan" in Malay language. It is a belief of the Temuan people in Malaysia that the fresh mushroom stipe which is diced, strung and worn as necklace can prevent epileptic episodes and incessant crying by babies (Azliza et al., 2012; Chang & Lee, 2004).

Figure 2.1: *Amauroderma rugosum*.

2.2.1 Medicinal benefits of *Amauroderma* sp.

To date, this study is the first report on the medicinal effect of *A. rugosum* (Chan, Kanagasabapathy, Tan, Sabaratnam, & Kuppusamy, 2013; Chan, Tan, Chua, Sabaratnam, & Kuppusamy, 2015). Other studies have also found that *Amauroderma* sp. displayed medicinal effects. *Amauroderma rude* is also known as *"Xuezhi"* in China, which means "bloody mushroom". Jiao et al. (2013) reported that *A. rude* exhibited anti-cancer activity which can inhibit cancer cell survival and induce apoptosis. On the other hand, ergosterol purified from *A. rude* was found to inhibit cancer growth *in vitro* and *in vivo* effectively by up-regulating multiple tumour suppressors. Thus, ergosterol from this medicinal mushroom may serve as a potential alternative for cancer therapy (Li et al., 2015). Besides, purified polysaccharides from *A. rude* possessed multiple effects on regulating the immune system at molecular and cellular level (Pan, Han, Huang, Yu, & Jiao, 2015). Other *Amauroderma* species such as *Amauroderma subresinosum* was found to be rich in fatty acids such as 11-octadecaenoic, lignoceric acid, 14-methylpentadecanoic, 8,11-octadecaenoic, and 8-octadecenoic (Quang, Nga, & Tham, 2011).

2.3 Bio-prospect of mushroom

Mushrooms have been consumed by human since ancient time. There are more than 2,000 species of mushrooms that exist in nature but only 25 species are being accepted as food and are commercially cultivated (Valverde et al., 2015). Mushrooms are regarded as a functional food with high nutritional and medicinal values. It contains multiple health benefits such as anti-inflammation (Dudhgaonkar et al., 2009), antioxidant (Cheung, Cheung, & Ooi, 2003), anti-diabetic (Kanagasabapathy et al., 2012), enhanced neurite outgrowth activity (Eik et al., 2012), and anti-cancer (Chen et al., 2005). Besides that, there is increasing number of research being done on mushrooms to investigate the medicinal properties. Many studies on the biochemical mechanisms of the therapeutic activities of mushrooms have been carried out in recent years with focus on the advancement of the biotechnology prospective. Also, new technologies and production techniques have been constantly developed as the demand for mushroom supply increases (Gregori, Švagelf, & Pohleven, 2007).

2.4 An overview of cultivation of mushroom

During 600 A.D in Greek, mushrooms were perceived as the "Food of the Gods" and Romanian warriors consumed mushrooms as a source of energy. In China, mushroom is considered as the "elixir of life" due to the richness in medicinal properties (Valverde et al., 2015). Currently, China is the world's largest mushroom producer (Table 2.1) with 1.6 million metric ton mushroom production in the year 2007 (Aida, Shuhaimi, Yazid, & Maaruf, 2009). Mushroom industry is ranked the fifth largest in the agriculture sector in China (Zhang, Geng, Shen, Wang, & Dai, 2014).

Table 2.1: Worldwide production of mushroom. (Source: Aida et al. (2009))

Country	Production		Percentage (%)
	1997	2007	
China	562,194*	1,605,000	65.0*
United States	366,810	390,000	5.9*
Canada	68,020	81,500	16.5*
India	9,000*	48,000	81.3*
Indonesia	19,000*	30,000	36.7*
Republic of Korea	13,181	28,500	53.8*
Islamic Republic of Iran	10,000*	28,000	64.3
Vietnam	10,000*	18,000	44.4*
Thailand	9,000*	10,000	10*
Israel	1,260	9,500	86.7*
Jordan	500	700	28.6*
Kazakhstan	-	500	100*
Singapore	-	10	100*

*Food and Agriculture Organization of the United Nations (FAO) estimate.

Mushroom cultivation has gained significant interest by many farmers as a complimentary vegetable supply for many decades. The production of mushroom has been continuously increased over time due to the overwhelming demand and awareness of health benefits of mushrooms by the consumers (Aida et al., 2009). In addition, mushroom cultivation requires short duration of growth and it is land saving. Therefore, it is mostly welcomed by the poor farmers (Shah, Ashraf, & Ishtiaq, 2004). According to Valverde et al. (2015), *A. bisporus* is the most cultivated mushroom worldwide, followed by *L. edodes*, *Pleurotus spp*., and *Flammulina velutipes*. These mushrooms require simple cultivation techniques and are easily produced. Mushroom cultivation has become food security as mushroom industry provides employment, livelihood, food, nutrition, and ecological viability (Singh & Singh, 2014).

2.5 Moving towards mass production using submerged culture technique

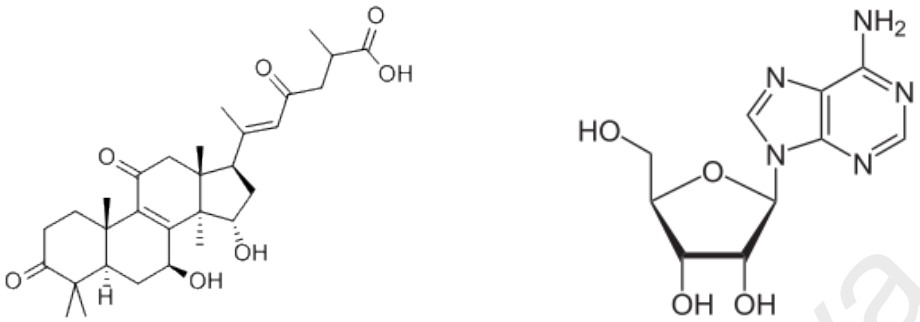
In recent years, submerged cultivation has gained popularity as a new promising alternative for productions of mycelium and bioactive metabolites. This is because the

production of fruiting bodies of the medicinal mushrooms is time consuming and the quality of the end product is difficult to control (Elisashvili, 2012). On the other hand, submerged cultivation can increase the product yield in a short period of time. However, currently there are only 15% of all products that are based on extracts from mycelia and the remaining 80-85% is mainly derived from the fruiting bodies. In addition, there are small percentages of mushroom products that are obtained from culture filtrates (Lindequist et al., 2005). The success of submerged cultivation of medicinal mushrooms is dependent on the demand of production and development of novel production systems that overcome the problems associated with mushroom cultivation (Elisashvili, 2012). Although there are increasing number of research being conducted on the submerged cultivation in production of mycelia and bioactive metabolites, the physiological and engineering understanding of this method is still lacking. Therefore, the contribution of research and development in the production of mycelia through submerged cultivation is much needed.

2.6 The pharmacological potential of medicinal mushrooms

In order to uncover the bioactive compounds in mushrooms, researchers often employ extraction and fractionation techniques. Besides, for further identification and purification, techniques such as gas-chromatography, ion-exchange chromatography, gel filtration and high performance liquid chromatography are used (Zhu, Kim, & Chen, 2008). In recent years, there are increasing evidences which demonstrate mushrooms as having great potential for production of bioactive metabolites. Lee, Kim, & Kim (2015) showed that dichloromethane fraction of *Innotus obliquus* induced cell cycle arrest at G_1 phase and inhibited HT-29 colon cancer cell proliferation. Besides, hot water extract of *G. lucidum* which is rich in ganoderic acid (**1**) and adenosine (**2**) (Figure 2.2) was found

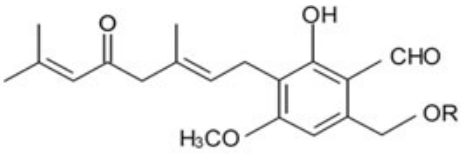
to increase the *in vivo* antioxidant capacity and reduce the oxidative damage during wound healing in diabetic rats (Cheng et al., 2013).



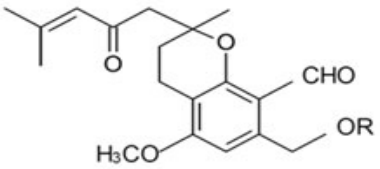
(**1**) (**2**)

Figure 2.2: Structures of ganoderic acid and adenosine.

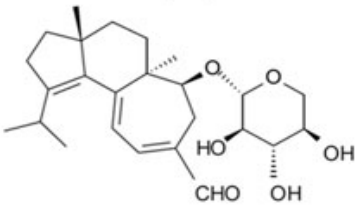
Lau et al. (2014) reported that mycelium and culture broth of *Lignosus rhinocerotis* showed higher or comparable antioxidant capacities, low cytotoxic effect, and consists of low-molecular-weight chemical constituents and thus, serves as the potential substitutes for the sclerotium. A study conducted by Kashiomoto et al. (2010) showed that the water-soluble extract from cultured medium of *G. lucidum* mycelia attenuated the small intestinal injuries that were caused by cancer treatments such as radiotherapy and chemotherapy. Hence, the mushroom extract might be used to improve the quality of life of cancer patients.



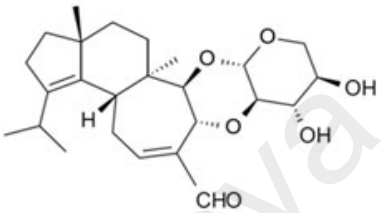
(**3**) R = palmytoyl
(**4**) R = stearoyl
(**5**) R = linoleoyl



(**6**) R = palmytoyl
(**7**) R = stearoyl
(**8**) R = linoleoyl



(**9**)



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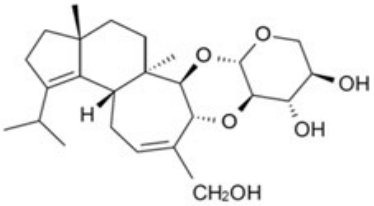

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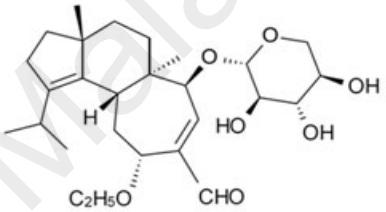

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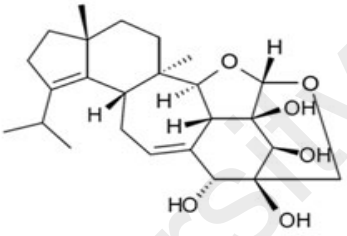
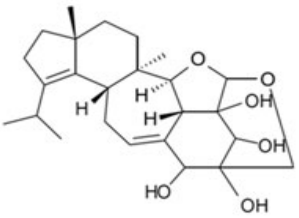


(**13**)



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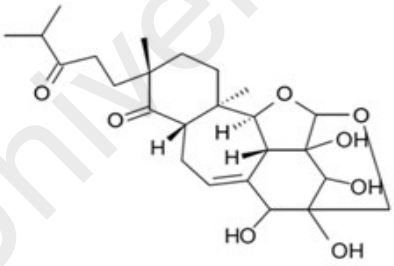

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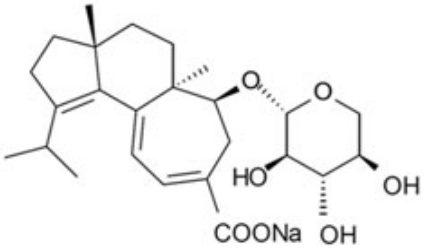

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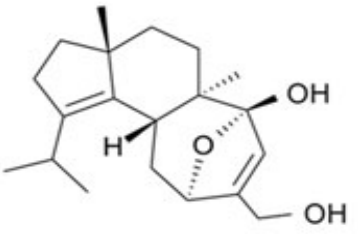
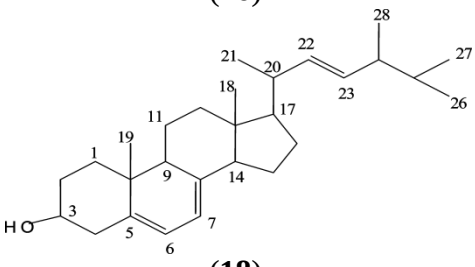


(**17**)



(**18**)

Figure 2.3: Structures of Hericinones, erinacines, and ergosterol.

Based on a review done by Phan, David, Naidu, Wong, & Sabaratnam (2014), which found that hericenones C (**3**), D (**4**), E (**5**), F (**6**), G (**7**), and H (**8**) (Figure 2.3) isolated from fruiting bodies of *H. erinaceus* induced biosynthesis of nerve growth factor (NGF) *in vitro* and erinacines A – I (**9-17**; Figure 2.3) from mycelia of *H. Erinaceus* stimulated the biosynthesis of NGF *in vitro* and *in vivo*. On the other hand, Kanagasabapathy, Malek, Kuppusamy, & Vikineswary (2011) found that ergosterol (**18**; Figure 2.3) is the major component in *P. sajor-caju* which serves as the precursor of ergocalciferol and it is extensively used as dietary supplement for hypocalcinaemia and osteoporosis patients.

2.7 Nutritional value of mushrooms

From the nutritional point of view, mushrooms are gaining popularity and becoming more important in our diet (Ayaz et al., 2011). Mushrooms have a great deal of nutritional value due to their high content of protein with essential amino acids, fiber, vitamins and minerals. Besides, mushrooms have low fat content (Valverde et al., 2015). A study done by Phan, Wong, David, Naidu, & Sabaratnam (2012) found that the fruiting bodies of *Pleurotus giganteus* have high carbohydrate, dietary fibre, and potassium. The same research team postulated that the high potassium content in *P. giganteus* may enhance neurite extension. The edible mushrooms grown in Vietnam such as *P. ostreatus*, *Volvariella volvacea*, and *L. edodes* were found to be good sources of protein and have high ash content (Hung & Nhi, 2012). Besides, wild edible mushrooms from Turkey Trabzon-Macka District which include *Craterellus cornucopioides, Armillaria mellea, Sarcodon imbricatus, Lycoperdon perlatum, Lactarius volemus, Ramaria flava, Cantharellus cibarius,* and *Hydnum repandum* were rich in protein and carbohydrates but low in fat content (Colak, Faiz, & Sesli, 2009).

2.8 Nutraceuticals of mushrooms

Nutraceutical is a substance that may be considered as food or part of a food which provides medicinal benefits such as prevention and treatment of diseases. Nutraceuticals include isolated nutrients, dietary supplements, genetically engineered foods, herbal products, and processed food such as cereals, soups, and beverages (Barros, Cruz, Baptista, Estevinho, & Ferreira, 2008). Mushrooms have enormous nutraceutical benefits and have been extensively studied to search for potential new therapeutic alternative. In general, mushrooms have abundant of amino acids such as leucine, valine, glutamine, glutamic and aspartic acid, and low fat content with fatty acids such as linoleic (C18:2), oleic (C18:1), and palmitic (C16:0) fatty acids. Besides, chitin, glycogen, trehalose, mannitol, fiber, β-glucans, hemicelluloses, and pectic substances were found in mushrooms (Valverde et al., 2015).

Beta-glucans are among the main polysaccharides found in mushrooms (Valverde et al., 2015). Kanagasabapathy et al. (2013) reported that β-glucan-rich extract from *P. sajor-caju* can prevent obesity, hyperglycemia, and oxidative stress. Besides, ergosterol and ergosterol peroxide purified from *Sarcodon aspratus* showed promising anti-inflammatory potential with the ability to suppress LPS-induced inflammatory responses through inhibition of NF-κB and C/EBPβ transcriptional activity, and phosphorylation of MAPKs (Kobori, Yoshida, Ohnishi-Kameyama, & Shinmoto, 2007). *G. lucidum, Morchella esculenta, L. edodes,* and *H. erinaceus* were found to be rich in phenolic compounds such as gallic acid, catechin, quercetin, and *p*-coumaric, which are beneficial for the prevention and treatment of a variety of diseases which include hypertension, diabetes, hepatitis, and cancer (Yildiz, Can, Laghari, Şahin, & Malkoç, 2015).

2.9 Oxidative stress

Oxidative stress is caused by the imbalance between the production of reactive oxygen species (ROS), and the ability of the biological system to detoxify the reactive intermediates. This imbalance causes damage of important biomolecules and organs with potential impact on the whole organism (Ďuračková, 2010). Most ROS are generated in cells by the mitochondrial respiratory chain and it is modulated largely by the rate of electron flow through respiratory chain complexes. The biological reduction of molecular oxygen in aerobic cells produce ROS such as superoxide anion ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\cdot}$) and organic peroxides. These free radicals also participate in a large number of subsequent reactions which produce other reactive metabolites (Figure 2.4) (Reuter et al., 2010). The excess production of these radicals can oxidize and damage proteins, nucleic acids, polysaccharides and lipids (Poyton et al., 2009). Hence, ROS are often associated with chronic inflammation and a wide variety of cancers (Reuter et al., 2010).

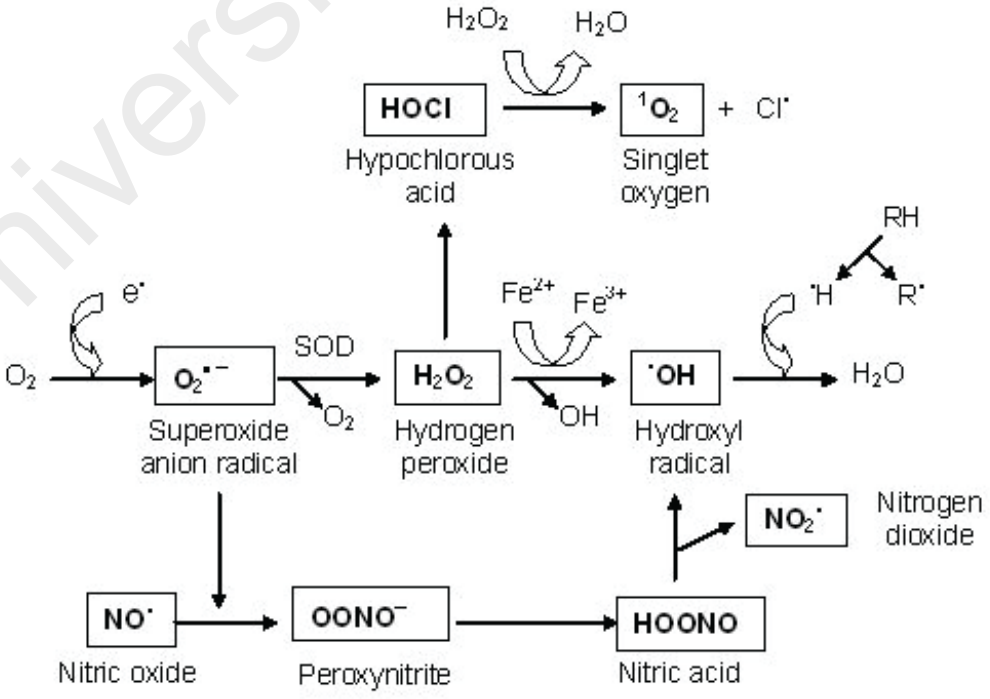


Figure 2.4: Association between free radicals and their reactive metabolites. (Source: Reuter et al. (2010))

2.9.1 Comorbidities of oxidative stress

Overproduction of free radicals can cause damage to DNA, protein and lipids through the utilisation of three main pathways. First, the lipid peroxidation triggers the arachidonic-acid cascade which stimulates the production of eicosanoids and promotes cell proliferation. Second, the by-products of arachidonic-acid pathway such as malondialdehyde (MDA) and 4-hydroxynonenol (4-HNE) are also DNA-damaging agents. Third, free radicals can modify enzyme systems which are involved in DNA repair and alter cell death signalling through modification of caspases and modulation of cell survival pathways which are regulated by key molecules such as JNK and p38 MAP kinase (Roberts et al., 2010). Hence, the damaging effect caused by excess ROS has been implicated in aging and a number of diseases, including cancer, diabetes mellitus, ischemia/reperfusion injury, inflammatory diseases, and neurodegenerative disorders (Valko et al., 2007).

It has been found that cellular redox imbalance leads to oxidative stress. The oxidative damage on DNA involves single- or double-stranded DNA breaks, purine, pyrimidine, or deoxyribose modifications, and DNA cross-links. These can result in arrest or induction of transcription, induction of signal transduction pathways, replication errors and genomic instability. Thus, the damage on DNA caused by high concentration of ROS may eventually lead to carcinogenesis (Valko, Rhodes, Moncol, Izakovic, & Mazur, 2006).

The increased formation of ROS may also cause oxidative stress in cardiac and vascular myocytes, which eventually leads to various cardiovascular diseases such as atherosclerosis, ischemic heart disease, hypertension, cardiomyopathies, cardiac hypertrophy and congestive heart failure (Dhalla, Temsah, & Netticadan, 2000).

Oxidative stress has been implicated as well in various coronary disease risk factors such as high blood pressure, diabetes and cigarette smoking, and this contributes to the pathogenesis of heart failure (Pashkow, 2011).

Damage from free radicals is also seen in rheumatoid arthritis patients. The formation of free radicals at the site of inflammation is linked predominantly with the pathogenesis of this disease. Many investigators have suggested that rheumatoid arthritis patients are prone to lipid peroxidation (Desai, Manjunath, Kadi, Chetana, & Vanishree, 2010). During lipid peroxidation, polyunsaturated fatty acids (PUFAs) are oxidised to lipid peroxyl radicals. Further oxidation of PUFA in perpetuating chain reaction can lead to cell membrane damage. Also, the reactive oxygen species are able to degrade matrix components in the skeletal joint by direct action or indirect activation of latent collagenases (Vasanthi, Nalini, & Rajasekhar, 2009).

2.10 Antioxidant defences

Excess free radicals such as superoxide anion, hydroxyl radical, hydrogen peroxide and nitric oxide can cause harm to body's cells and tissue through damage on proteins, DNA and lipids (Poyton et al., 2009). Many clinical studies have demonstrated that therapeutic applications of antioxidants may slow down or reverse injury caused by oxidative stress (Brambilla et al., 2008). Antioxidants are substances which are capable of neutralizing or inhibiting the action of free radicals (Devasagayam et al., 2004). There are two types of antioxidant defences, which are enzymatic and non-enzymatic antioxidant defences. Enzymatic antioxidants include superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT). On the other hand, non-enzymatic antioxidants comprise of ascorbic acid (Vitamin C), α-tocopherol (Vitamin E), glutathione (GSH), carotenoids and flavonoids (Valko et al., 2007). Maintaining the

equilibrium between free radicals in the body and cellular antioxidant defences is essential for the survival of organisms and their health. Although there is presence of endogenous defence mechanism to fight against overproduction of free radicals, consuming antioxidants in the diet is also vital to act as protective agents to reduce oxidative damage (Ferreira et al., 2009).

2.10.1 Antioxidant activity of mushrooms

Mushrooms are known as functional food and a source of beneficial medicinal properties. Studies have been extensively conducted on antioxidant properties of medicinal mushrooms such as *G. lucidum, Ganoderma tsugae, Coriolus versicolor*(Mau, Lin, & Chen, 2002), *Grifola frondosa* (Lee et al., 2003), *Phellinus linteus* (Song et al., 2003), *P. ostreatus* (Jayakumar, Thomas, & Geraldine, 2009), *Lactarius deliciosus* and *Sarcodon imbricatus* (Ferreira, Baptista, Vilas-Boas, & Barros, 2007). Many antioxidant compounds such as phenolic compounds, tocopherols, ascorbic acid and carotenoids have been extracted from mushrooms.

Phenolic compounds are the major antioxidants found in mushrooms (Ferreira et al., 2009). It is normally present in diet and well known for its antioxidant properties. Phenolics have been found to be beneficial for human health, protecting oxidative stress and related diseases such as cancers, inflammatory disorders, cardiovascular and neurodegenerative diseases (Sergent, Piront, Meurice, Toussaint, & Schneider, 2010). The bioactivity of phenolic compounds may be attributed to the reducing capacity by donating hydrogen and quenching singlet oxygen (Ferreira et al., 2009).

Studies have shown that tocopherols are involved in scavenging free radicals and play a vital role in protecting the body against degenerative malfunctions, mainly cancer and

cardiovascular diseases (Lee et al., 2005). Tocopherol-α and tocopherol-γ, which exhibit anti-inflammatory activities are found in mushrooms such as *Auricularia mesenterica, Auricularia polytricha, Auricularia fuscosuccinea, Tremella fuciformis* (Mau, Chao, & Wu, 2001), *Boletus edulis, Calocybe gambosa, Cantharellus cibarius, Craterellus cornucopioides,* and *Marasmius oreades* (Barros et al., 2008).

Ascorbic acid, which is also known as vitamin C, is thought to exert a protective role against various oxidative stress-related diseases such as heart disease, stroke and cancer (Barros, Cruz, Baptista, Estevinho, & Ferreira, 2003). Vitamin C is found in mushrooms such as *P. ostreatus* (Jayakumar et al., 2009), *Boletus edulis, Calocybe gambosa, Cantharelus cibarius, Craterellus cornucopioides, Marasmius oreades* (Barros et al., 2008) and *Hypsizigus marmoreus* (Lee, Yen, & Mau, 2007). Vitamin C is also known to be effective against superoxide, hydroxyl radical, hydrogen peroxide, peroxyl radical and singlet oxygen. Besides, it can protect biomembranes against lipid peroxidation damage through elimination of peroxyl radicals in the aqueous phase before the latter can initiate lipid peroxidation (Ferreira et al., 2009).

Carotenoids are pigments synthesized by plants and microorganisms but not animals. It is mainly found in fruits and vegetables but particularly, β-carotene was found in several mushroom species such as *P. ostreatus* (Jayakumar et al., 2009), *Hypsizigus marmoreus* (Lee et al., 2007), *A. bisporus, Polyporus squamosus, Lepista nuda, Russula delica* and *Verpa conica* (Elmastas, Isildak, Turkekul, & Temur, 2007). Studies have shown that β-carotene has antioxidant and anti-inflammatory activities (Bai et al., 2005) and the combination of lycopene, lutein, β-carotene and carnosic acid was able to produce a synergistic anti-inflammatory effect through redox-based inhibition of nuclear factor-kappa B (NF-kB) signalling (Hadad & Levy, 2012).

2.11 Inflammation

Inflammation is the reaction of tissue to irritation, injury or infection, characterised by pain, redness and swelling (Wen et al., 2011). It is a protective process of the body that functions to destroy invading organisms or repair tissues after injury (Lee et al., 2012). However, sustained or excessive inflammation may predispose the host to various chronic inflammatory diseases such as arthritis, asthma, multiple sclerosis and atherosclerosis (Reuter et al., 2010). There are two types of inflammation; acute and chronic inflammation. Acute inflammation is often characterised by the four classical signs which are redness, heat, swelling, and loss of function. Monocytes extravasate from blood vessels into the injured site and transform into macrophages. Macrophages play a vital role in inflammatory response in the initiation, maintenance and resolution of inflammation. However, if the cause of inflammation cannot be eliminated, inflammation will prolong and vary in intensity over time (Hakansson & Molin, 2011). Prolonged or chronic inflammation may contribute to detrimental outcomes such as chronic inflammatory diseases.

2.11.1 Comorbidities of inflammation

Acute inflammation is a vital process that acts as the first defense system to destroy the invading organism and repair tissues after injury. However, persistent or dysregulation of mechanisms in chronic inflammation may lead to the progression of a range of diseases such as atherosclerosis (Libby, Ridker, & Hansson, 2009), arthritis (Matsuno et al., 2002), and chronic neurological diseases such as Alzheimer's disease (Morales, Guzmán-Martínez, Cerda-Troncoso, Farías, & Maccioni, 2014) and epilepsy (Walker & Sills, 2012).

Atherosclerosis involves both innate and adaptive immune system. At early stage of atherogenesis, monocytes are recruited and attached to activated endothelial cells by leukocyte adhesion molecules. The continuous adhesion of monocytes at the atherosclerotic lesions causes the high expression of pro-inflammatory cytokines and other macrophage mediators. Prolonged inflammation can lead to chronic inflammation and studies have shown that it is linked to the formation of thrombosis (Libby et al., 2009).

Inflammation is the hallmark of rheumatoid arthritis. The overproduction of pro-inflammatory cytokines such as TNF-α, IL-1, and IL-6 by the inflammatory cells such as macrophages causes synovial inflammation and cartilage degradation (Kahlenberg & Fox, 2011). In addition, histamines, bradykinins, serotonin, prostaglandins, and other inflammatory factors released at the local inflamed area adding to the continuous destruction of the capsule and synovium Thus, patients with rheumatoid arthritis have intense pain which may be triggered by even gentle stimulation or slight movement at the affected joint (Laar et al., 2012).

In a normal adult, the central nervous system (CNS) has low or undetectable levels of inflammatory cell subsets and inflammatory cytokines. However, infiltrating innate and adaptive immune cells, activated microglia, and inflammatory secretions such as cytokines, chemokines, free radical species and proteases are found in the CNS in patients with neurological disorders such as Alzheimer's or Parkinson disease (Yong, 2010). Studies have shown that neuroinflammation is closely linked with most of the neurological conditions (Hirsch, Vyas, & Hunot, 2012; Morales et al., 2014; Naegele & Martin, 2014; Vezzani, 2005).

2.11.2 Inflammatory pathways

NF-κB plays an important role in the inflammatory response. The influence of NF-κB on cell survival depends on the types, development stages of cells, and pathophysiological conditions. It can be protective or destructive (Qin, Tao, & Chen, 2007). The activation of NF-κB has been found in many pathophysiological disorders such as cancer (Reuter et al., 2010), arthritis (Simmonds & Foxwell, 2008), and neurological disorders (Baker, Hayden, & Ghosh, 2011). There are two NF-κB activation pathways, which are the classical or canonical pathway and the non-canonical pathway. In the classical or canonical pathway, activated inhibitory kappa B kinase (IKK) complex causes the phosphorylation of Ser32 and Ser36 in inhibitory of kappa B alpha (IκB-α) which leads to ubiquitination and degradation by 26S proteasome. This causes the release of p65 and p50. On the other hand, the non-canonical pathway involves the activation of p100-RelB complex through phosphorylation of IKK-α homodimer which generates p-52-RelB. Hence, p65, p50, and p-52-RelB can then enter the nucleus to activate the target gene expression (Figure 2.5) (Qin et al., 2007).

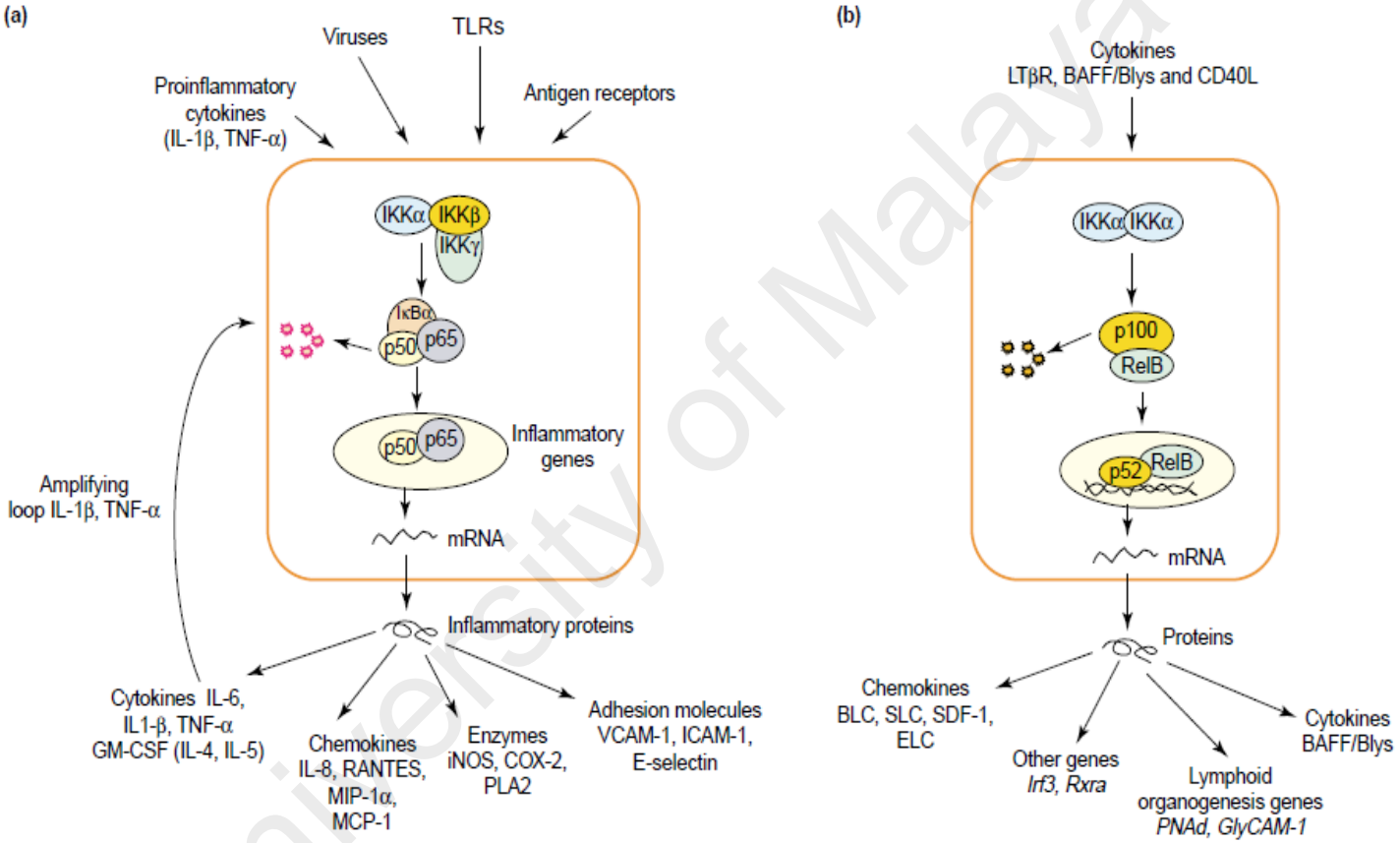


Figure 2.5: Schematic illustration of the NF-κB activation. (Source: Bonizzi & Karin (2004))

The NF-κB is involved in the regulation of a series of gene expression that are responsible for immune responses. These targeted genes include proinflammatory cytokines (e.g. IL-1, IL-2, IL-6, TNF-α), anti-inflammatory cytokines (e.g. IL-10), chemokines (e.g. IL-8), adhesion molecules (e.g. endothelial leukocyte adhesion molecule, vascular cell adhesion molecule and intercellular adhesion molecule), and inducible enzymes (e.g. iNOS and COX-2) (Caamano & Hunter, 2002; Liang, Zhou, & Shen, 2004).

NF-κB plays a vital role in regulating the nitric oxide synthase (NOS) expression. Nitric oxide is produced by a specific enzyme called NOS through the precursor L-arginine (Figure 2.6). There are three types of NOS namely neuronal or brain NOS (bNOS or NOS I), endothelial NOS (eNOS or NOS III), and inducible NOS (iNOS or NOS II). The third isoform, which is the iNOS is commonly expressed in tissues such as lung epithelium and synthesised in response to the inflammatory mediators (Cirino, Distrutti, & Wallace, 2006). Besides, the iNOS gene contains several binding sites for transcriptional factors such as NF-κB, activator protein 1 (AP-1), and various members of the CCAAT/enhancer-binding protein family (Hatziieremia, Gray, Ferro, Paul, & Plevin, 2006). Studies have shown that NO is associated with pathophysiological conditions and higher NO level is thought to be harmful to the organism as it amplifies the inflammatory response (Aktan, 2004; Sautebin, 2000).

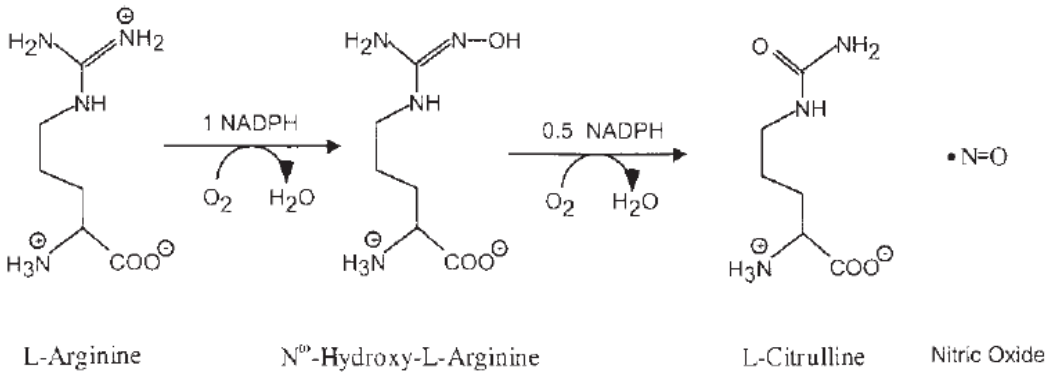


Figure 2.6: The NO synthesis process. (Source: Stuehr (2004))

2.11.3 Anti-inflammatory activity

Inflammation is a normal response to infection. However, prolonged inflammation may result in inflammatory disorders, neurodegenerative disease or cancer (Rodriguez-Vita & Lawrence, 2010). Over the years, anti-inflammatory drugs have been developed to reduce pain, inflammation and fever. The currently available anti-inflammatory therapies are steroids, non-steroidal anti-inflammatory drugs (NSAIDs) and anti-histamines, which act by inhibiting the synthesis or action of inflammatory mediators (Ward, 2008).

Aspirin, which is the most common anti-inflammatory drug, inhibits COX-1 and COX-2 enzymes to prevent the synthesis of inflammatory mediators such as prostaglandins and thromboxanes (Dinarello, 2010). Besides, anti-TNF-α drugs such as infliximab, adulimumab, cetolizumab and etanercept serve the function to reduce TNF-α activity. It is commonly used to treat patients with rheumatoid arthritis, Crohn's disease and psoriasis (Dinarello, 2010). Toculizumab, which is an anti-IL-6 receptor, is used to block the IL-6 receptor, decrease the IL-21 production by activated/memory CD4 T cells and reduce serum levels of IgG4-specific autoantibodies in rheumatoid arthritis and juvenile arthritis patients (Carbone et al., 2013).

Although biological agents and therapies have been designed to target and inhibit the action of inflammatory response which associate with detrimental diseases, most of the biopharmaceutical approaches have shortcomings (Ward, 2008). For example, short-term corticosteroid use is associated with mild side effects such as cutaneous effects, electrolyte abnormalities, hypertension, hyperglycemia, pancreatitis, hematologic, immunologic and neuropsychologic effects. Long-term usage of corticosteroid can cause osteoporosis, aseptic joint necrosis, adrenal insufficiency, gastrointestinal, hepatic and ophthalmologic effects, hyperlipidemia, growth suppression and possible congenital malformations (Buchman, 2001). Besides, non-steroidal anti-inflammatory drugs (NSAID) such as aspirin, flurbiprofen, naproxen, diclofenac and ibuprofen have serious ulcerogenic effects in gastrointestinal tract which limit their therapeutic usefulness (Keeble & Moore, 2002). Hence, considering the undesirable side effects of anti-inflammatory drugs available in the market, natural products or herbal medicines have gained significant interest as a source of new effective therapeutic agents.

2.11.4 Anti-inflammatory activity of mushrooms

Mushrooms have been consumed by humans as part of normal diet since ancient times. The increased interest in scientific studies on mushrooms is attributed to the significant amounts of bioactive compounds produced by the fruiting body and the mycelium liquid culture (Ferreira et al., 2009). Many studies have shown that mushrooms have anti-inflammatory properties.

Ganoderma lucidum which is a medicinal mushroom, also known as *"lingzhi"* in Chinese, has been used in traditional Chinese medicine for many years to prevent and treat a variety of diseases. The triterpene extract from *G. lucidum* has shown anti-inflammatory and anti-proliferative effects on macrophages through the inhibition of

NF-κB and AP-1 signalling pathways (Dudhgaonkar et al., 2009). Besides, oyster mushroom extract from *P. ostreatus* exhibited anti-inflammatory effect through the suppression of TNF-α, IL-6 and IL-12p40 and inhibited the production of PGE_2 and NO through the down-regulation of expression of COX-2 and iNOS genes, respectively (Jedinak, Dudhgaonkar, Wu, Simon, & Sliva, 2011).

The glucan extract from *Geastrum saccatum* also possessed anti-inflammatory properties. It mediated the anti-inflammatory effect through the inhibition of NOS and COX (Guerra et al., 2007). Methanolic extracts of edible mushrooms such as *A. bisporus, Cantherellus cibarius* and *Lactarius deliciosus* demonstrated anti-inflammatory activities through the inhibition of iNOS, IL-1β and IL-6 messenger RNAs (mRNAs) expression in response to LPS stimulation of macrophage cells (Moro et al., 2012).

2.12 Neuroinflammation

Neuroinflammation is the inflammation in the nervous tissue. It differs substantially from inflammation in other tissues (Xanthos & Sandkühler, 2013). Figure 2.7 illustrates the difference of innate immunity in the periphery and CNS. In the CNS, innate response does not initiate adaptive immunity directly. During neuroinflammation reaction, the perivascular macrophages and vascular pericytes replace the functions of mature dendritic cells in the CNS. Besides, astrocytes, microglia, and mast cells infiltration take place at the affected area. The permeability of microvessels for extravasation of large molecules and blood cells is reduced which creates a "blood-CNS" barrier (Xanthos & Sandkühler, 2013). Glial cells such as microglia and astrocytes are notably accumulated after CNS injury which then releases inflammatory factors. The sustained cycles of injury and response causes neurodestruction and this

worsens the disease process leading to a phenomenon called chronic neuroinflammation. Chronic neuroinflammation often contributes to neuronal dysfunction, injury, and loss which leads to pathologic sequelae (Streit, Mrak, & Griffin, 2004).

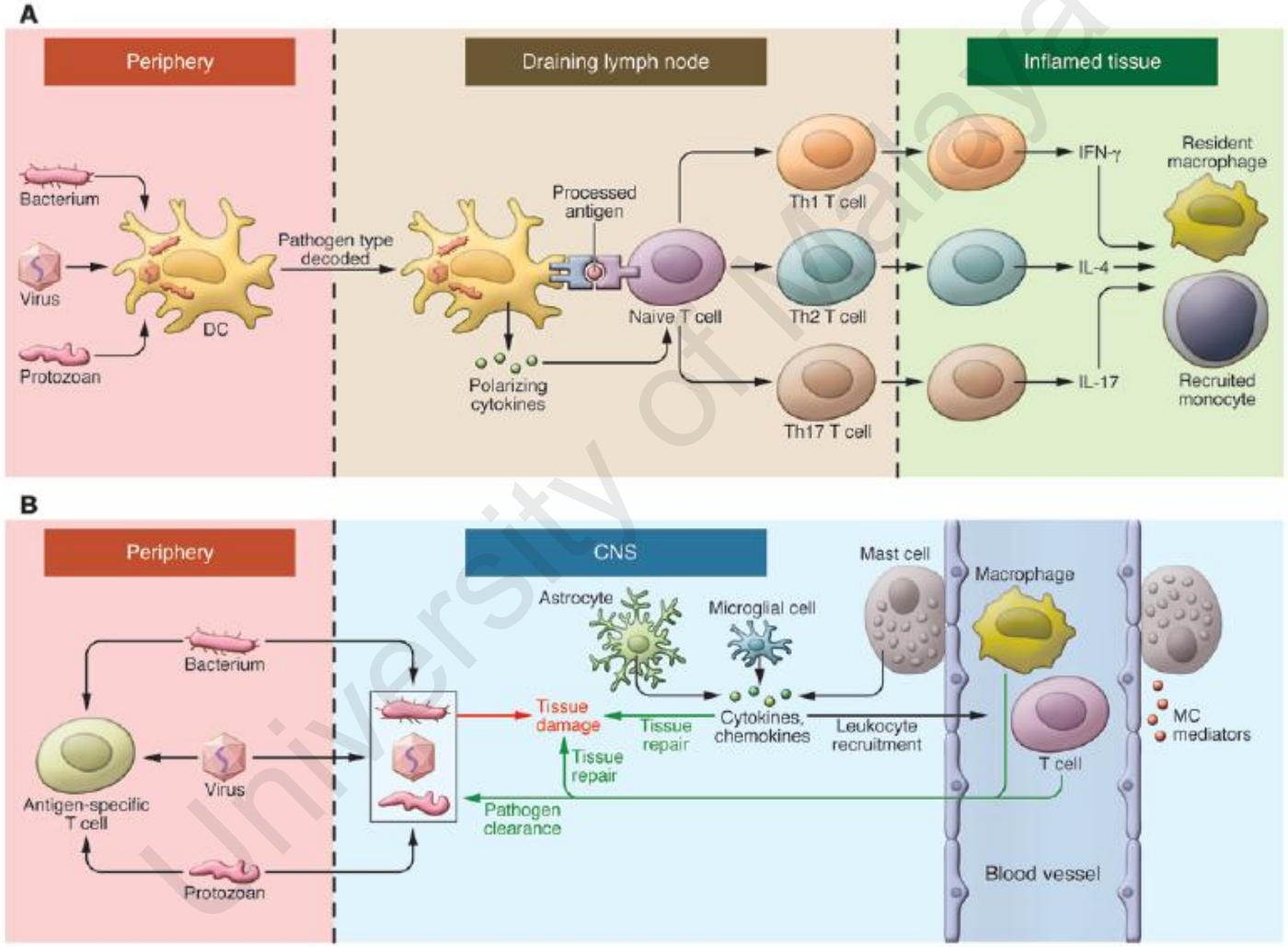


Figure 2.7: (A) Innate immunity in the periphery; (B) Innate immunity in the CNS. (Source: Ransohoff & Brown (2012))

2.12.1 Neuroinflammation in neurological diseases

Chronic neuroinflammation or persistent inflammation in the brain often causes detrimental effects on the organism. The breakdown of blood-CNS barrier during excessive inflammatory activity in the brain causes extreme extravasations of large molecules and immune cells into the CNS parenchyma that can damage the neuronal network (Xanthos & Sandkühler, 2013).

Parkinson's disease is a neurodegenerative disorder which involves the progressive loss of dopaminergic neurons in the *substantia nigra pars compacta* and presence of intraneuronal inclusions of the protein α-synuclein or also known as Lewy bodies (Rocha, Miranda, & Teixeira, 2015). Among the factors that cause Parkinson's disease, neuroinflammation is one of the triggers that cause neurodegeneration. Studies have shown that Parkinson's disease patients have hallmarks of neuroinflammation such as the presence of activated microglia and reactive astrocytes in the parenchyma of the CNS, increased production of cytokines, chemokines, prostaglandins, complement proteins, and reactive oxygen and nitrogen species (ROS/RNS) which disrupt the blood-brain barrier (BBB) and affect the neuronal activity (More, Kumar, Kim, Song, & Choi, 2013; Rocha et al., 2015).

Alzheimer's disease is the deposition of amyloid-β plaques in the brain parenchyma and neurofibrillary tangles within neurons that affects memory, thinking, and behaviour in human being (Morales et al., 2014). In early stage of Alzheimer's disease, microglial activation is observed. Besides, the cyclooxygenase (COX) expression and prostaglandin E_2 (PGE_2) levels were reported to be elevated (Yermakova & Banion, 2001). However, this inflammatory processes only predominates at early stage of Alzheimer's disease and eventually disappears over time (Krause & Müller, 2010).

Epilepsy is a brain disorder characterised by an enduring predisposition to generate seizures, emotional and cognitive dysfunction (Duncan, Sander, Sisodiya, & Walker, 2006). There are increasing evidences on the relevance of inflammation in the pathophysiology of epilepsy (Vezzani & Granata, 2005; Vezzani, Masa, & Baram, 2011; Walker & Sills, 2012). Overproduction of inflammatory mediators such as IL-1β, TNF, IL-6 and prostaglandin E2 (PGE2) has been found to promote seizure generation and exacerbation (Xiong, Qian, Suzuki, & Mcnamara, 2003).

2.12.2 Pathophysiological cascade of inflammation leading to epilepsy

Maroso et al. (2010) have found that activation of TLR4 and release of HMGB1 cause generation of seizures that lead to chronic epilepsy. Upon activation of TLR4, the seizure threshold decreases and this was found to cause increase in hippocampal neuronal network excitability (Galic et al., 2008). On the other hand, increased expression and secretion of HMGB1 by glial cells during seizure cause continuous neuronal hyperexcitability and sustained inflammation through activation of NF-κB and IL-1β (Maroso et al., 2010).

Besides, studies have found that overproduction of proinflammatory cytokines such as IL-1β, IL-6, and TNF-α from microglia and astrocytes trigger alteration of the neuronal excitability and increase generation of seizures. This may lead to neuronal cell loss, astrogliosis, and BBB damage. As this process persists, it increases the frequency of spontaneous seizures in the organism (Ravizza et al., 2008). Figure 2.8 shows the pathophysiological cascade of the inflammatory events leading to epilepsy.

Activation of the kinase family such as the extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) signalling pathway has been

implicated in epileptic disorder (Nateri et al., 2007). ERK kinases are found to be highly expressed in the CNS in response to various physiological stimuli that are associated with synaptic activity and brain plasticity such as calcium influx and neurotrophins during the pathological events in epilepsy (Pearson et al., 2001).

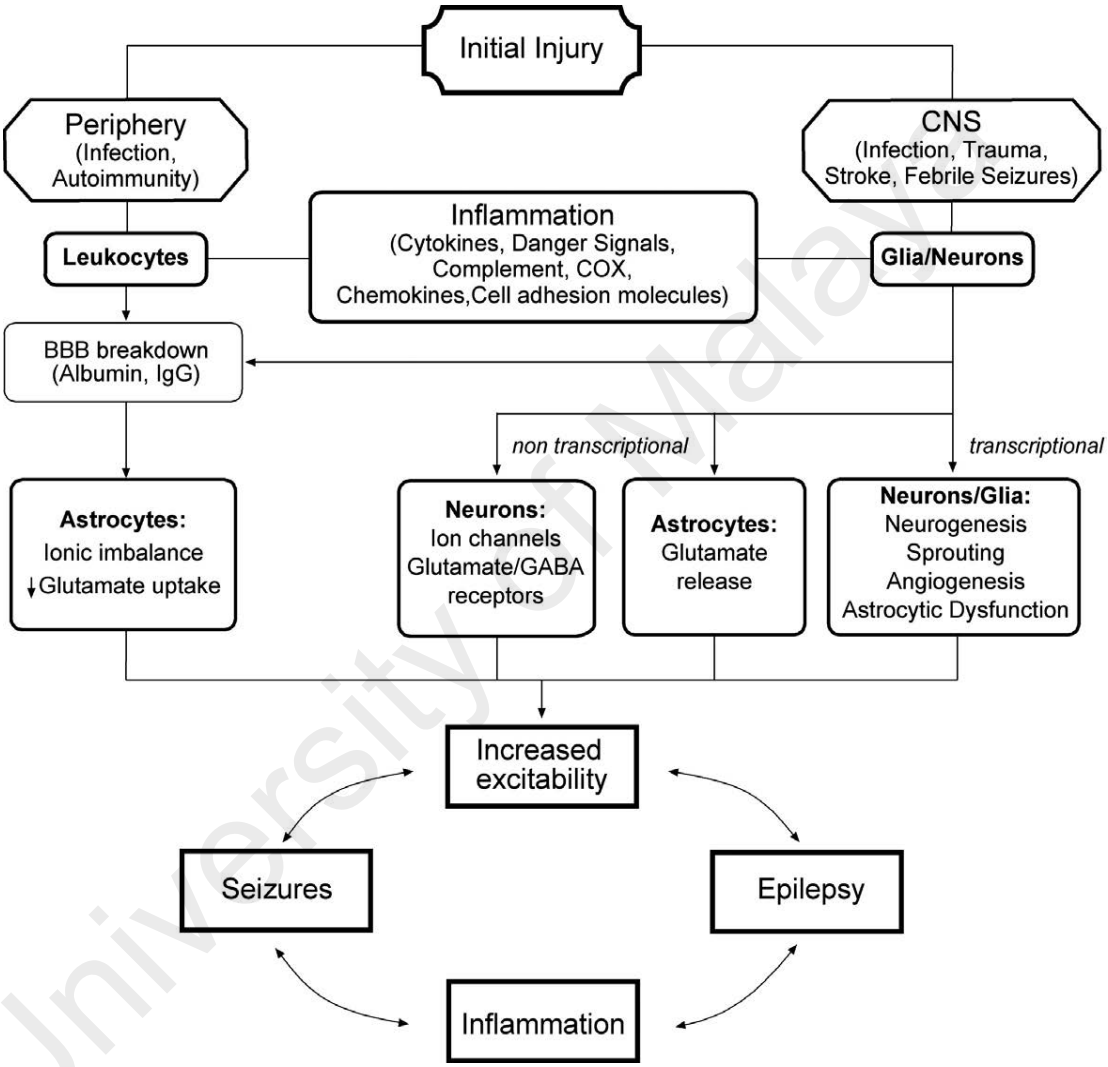


Figure 2.8: Illustration of the pathophysiological cascade of inflammation leading to epilepsy. (Source: Vezzani (2014))

2.12.3 Anti-neuroinflammatory activity

The CNS has its own resident immune system which helps to defend the CNS from pathogens and repair it after injury. However, if the inflammation progresses, it results in disease (Craft, Watterson, & Van Eldik, 2005). Therefore, research pertaining to therapeutic application and to find the potential targets to inhibit neuroinflammation has been on going.

Ravizza et al. (2006) reported that inhibition of interleukin-converting enzyme (ICE/caspase-1) or deletion of the caspase-1 gene can effectively suppress the brain production of the inflammatory cytokine, IL-1β. Besides, selective antagonists of HMGB1 and TLR4 showed anti-neuroinflammatory activity in chronic kainate model (Maroso et al., 2010). Also, prostaglandin E_2 (PGE_2) has been found to promote reactive astrogliosis and exhibit inflammatory functions. Therefore, inhibition of PGE_2 production may be a good target for anti-neuroinflammatory approach (Shimada, Takemiya, Sugiura, & Yamagata, 2014).

Recently, usage of natural products as an alternative medicine to treat neuroinflammation and related diseases has been increasing. Cui et al. (2010) found that citrus nobiletin had anti-neuroinflammatory potential where it suppressed the microglial activation in LPS-stimulated BV-2 cells. Besides, Elmann et al. (2011) reported that the phytochemicals of *Achillea fragrantissima* had anti-neuroinflammatory activities which effectively inhibited NO and ROS production and down-regulated IL-1β, TNF-α, COX-2, iNOS, and MMP-9 expressions in activated microglial cells. He, Chen, Qian, Chen, & Buzby (2010) showed that curcumin alleviated neuroinflammation *in vitro* and *in vivo* by inhibiting iNOS expression and NOX activation.

2.12.4 Anti-neuroinflammatory acitivity of mushrooms

Natural products such as mushrooms have shown great potential as anti-neuroinflammatory agents. Ethanol extract of *G. lucidum* showed therapeutic potential in inhibiting the inflammatory mediators and cytokines in LPS-stimulated BV-2 microglia cells (Yoon et al., 2013). A medicinal mushroom, *Phellinus igniarius* was found to contain biologically active compounds that can inhibit proliferation of lymphocytes and secretion of interferon-γ in lymph node of autoimmune encephalomyelitis mice. This suggests the potential of *P. igniarius* to amelioriate multiple sclerosis progression (Li et al., 2014). Besides, ergosterol of *Cordyceps militaris* was found to inhibit LPS-stimulated NO production in BV-2 microglia cells suggesting the potential anti-neuroinflammatory effect (Nallathamby et al., 2015). Also, cordycepin isolated from *Cordyceps militarisa* was reported to inhibit TNF-α and IL-1β productions and down-regulated iNOS and COX-2 expressions in LPS-stimulated microglia cells (Peng, Wang, Ge, Qu, & Jin, 2015).

2.13 Epilepsy

Epilepsy is a brain disorder characterised by an enduring predisposition to generate seizures, emotional and cognitive dysfunction (Duncan et al., 2006). This disorder affects more than 50 million people worldwide and it is one of the most common neurological disorders (Vezzani et al., 2011). There are two types of seizures, partial (focal) and generalised seizures. Partial or focal seizure refers to seizure that begins in a local area of the brain. This type of seizure is divided into two subtypes which are simple partial and complex partial seizures. Simple partial seizure does not involve alteration in consciousness. However, complex partial seizure involves alteration of consciousness. On the other hand, generalised seizure affects the entire brain

simultaneously. Generalised seizure includes symptoms such as absence, myoclonic, tonic-clonic, atonic, tonic, and clonic (Banerjee, Filippi, & Hauser, 2009).

Clinical and experimental evidences have shown strong support to the hypothesis that inflammatory processes within the brain might constitute a common and crucial mechanism in the pathophysiology of seizures and epilepsy (Vezzani & Granata, 2005; Vezzani & Baram, 2007). In addition, overexpression of proinflammatory cytokine (interleukin-1 beta (IL-1β)) and danger signals (high mobility group box 1 (HMGB1) and S100 calcium-binding protein beta (S100β)) were observed in epileptic patients (Vezzani, 2014).

2.13.1 Therapeutic strategy in epilepsy

Cyclooxygenase-2 (COX-2) has been implicated in epileptogenesis. Thus, inhibition of COX-2 may reduce the activation of microglia cells, leukocyte infiltration, suppress proinflammatory cytokines and oxidative stress mediators, and prevent neurodegeneration (Jung et al., 2006). Celecoxib is the first generation of non-steroidal anti-inflammatory drug (NSAID) that inhibits COX-2 resulting in decrease of the seizure frequency and duration, reduce hippocampal neurodegeneration and microglial activation, and inhibit generation of ectopic granule cells in hilus and new glia in CA1 of the hippocampus (Pitkänen & Lukasiuk, 2011).

Carbamazepine is widely used to treat simple and complex seizures and tonic-clonic seizures secondary to a focal discharge (Callaghan, O'Callaghan, Duggan, & Feely, 1978). However, it can also cause development of acute liver disease. Hence, carbamazepine has to be withdrawn immediately if acute liver disease develops or liver dysfunction worsens (Dawda & Ezewuzie, 2010). Besides, some older anti-epileptic

drugs (AEDs) such as phenobarbital, primidone, sodium valproate, and phenytoin are effective in treating tonic-clonic and partial seizures. However, these drugs have side effects such as fever, sore throat, rash, mouth ulcers, agitation, depression, and behavioural change (Dawda & Ezewuzie, 2010).

There are many different approaches towards treatment of epilepsy such as gene therapies (galanin gene therapy, neuropeptide Y (NPY) gene therapy, neurotropic factor-based gene therapy, and adenosine-based gene therapy), stem cell-based therapy, transplantation of GABA releasing cells, transplantation of adenosine-releasing cells, and molecular-genetic approaches (Sørensen & Kokaia, 2013). Clinical trials and evaluations have been on-going to find more possible alternative approaches for epilepsy treatment as most of the treatment or AEDs have side effects.

2.13.2 Natural products approaches to epilepsy

Enhancing efficacy and safety of AEDs are vital to provide better treatment for epileptic disorders. Thus, investigation of natural products has gained interest as a potential replacement or adjuvant for AEDs (Ekstein & Schachter, 2010). In fact, some natural products have shown potential anti-convulsive effects. For example, 15 days administration of poly herbal extract from Indian medicinal plants such as *Withania somnifera, Bacopa monnieri, Chlorophytum borivillianum, Glycyrrhiza glabra, Curcuma longa,* and *Terminalia argina* was found to be effective in increasing seizure threshold in maximal electro shock (MES)-induced rats through the inhibition of prostaglandin synthesis and monoamine oxidase activity and decrease in influx of calcium ions (Balamurugan, Dharan, & Rajan, 2008).

Ethanolic extracts of Malian medicinal plants such as *Alchornea cordifolia, Balanites aegyptiacus, Burkea Africana, Bridelia ferruginea, Orzoroa insignis, Pseudocedrala kotschyi, Flueggea virosa, Terminalia macroptera, Pteleopsis suberosa, Guiera senegalensis,* and *Psorospermum senegalense* were tested for anti-epileptic activities using [^{3}H]-flumazenil binding assay and the results showed potential anticonvulsant properties *in vitro.* Ethanolic extracts of *F. virosa* and *P. senegalense* had the most potent effect and highest binding affinity to benzodiazepine binding site on $GABA_A$ receptor which was similar to the effects exhibited by existing AEDs (Pedersen et al., 2009).

A type of traditional Chinese medicine, *Uncaria rynchophylla* and its active compound exhibited anti-epileptic effects in KA-induced rats through the regulation of toll-like receptor (TLR) and neurotrophin signalling pathways. In addition, it inhibited IL-1β and brain derived neurotrophic factor (BDNF) gene expression (Ho, Tang, Hsiang, & Hsieh, 2014).

2.13.3 Anti-epileptic potential of mushrooms

To date, there is limited information regarding anti-epileptic potential of mushrooms. Based on the literature review, *G. lucidum* is the only mushroom that is well-studied for its anti-convulsive potential. Wang et al. (2014) have found that the polysaccharides of *G. lucidum* could inhibit the intracellular calcium accumulation and stimulate CaMKII α expression in epileptic hippocampal neurons. Besides, *G. lucidum* spores could inhibit the expression of N-cadherin which indirectly prevents mossy fiber sprouting and thus inhibit neural circuit formation (Wang et al., 2013). Hence, this provides a mechanism for the anti-epileptic effects. In addition, *G. lucidum* spores could stimulate the expression of neurotrophin-4 which promotes neuronal survival and prevents injuries of

postictal hippocampal neurons. This eventually enhances the anti-epileptic effects (Wang et al., 2013).

2.14 Inflammation, neuroinflammation, and epilepsy – how are they linked?

Inflammation is one of the main factors that contribute to epilepsy. Neuroinflammation is well-known to cause epilepsy (Shimada et al., 2014; Vezzani et al., 2013). In addition, systemic inflammation also interrupts the neuronal excitability and increases seizure susceptibility (Riazi, Galic, & Pittman, 2010). Studies have revealed that systemic inflammatory conditions cause decrease in seizure threshold. For example, induction of intestinal inflammation in mice model by administration of croton oil causes decrease in clonic seizure threshold (Riazi et al., 2004). Another study found that some of the inflammatory disorders such as colitis, arthritis, and granuloma are associated with increased susceptibility to seizure. When rats with arthritis were treated with a synthetic drug known as thalidomide, the seizure scoring was reduced and proinflammatory cytokine tumour necrosis factor alpha (TNF-α) level was attenuated (Rao et al., 2008).

Figure 2.9 shows the illustration of the hypothetical cascade of events of peripheral inflammation leading to increase in seizure susceptibility. The activation of peripheral inflammation by foreign substance such as lipopolysaccharide (LPS) or stimulation of systemic inflammation, leads to the increased production of inflammatory mediators which can worsen or increase the over activation of the immune system. As the inflammatory response progresses, the inflammatory response within the central nervous system (CNS) is triggered. A mirror inflammatory response is mediated by the inflammatory mediators such as TNF-α, interleukins (IL-1β, IL-6), and oxidative stress. Besides, the blood-brain barrier (BBB) becomes vulnerable and increased in

susceptibility towards leukocyte infiltration and glial cell migration. The increased immune response in the brain may change the neuronal excitability and eventually decrease the seizure threshold and recurrent seizures causes epilepsy (Riazi et al., 2010).

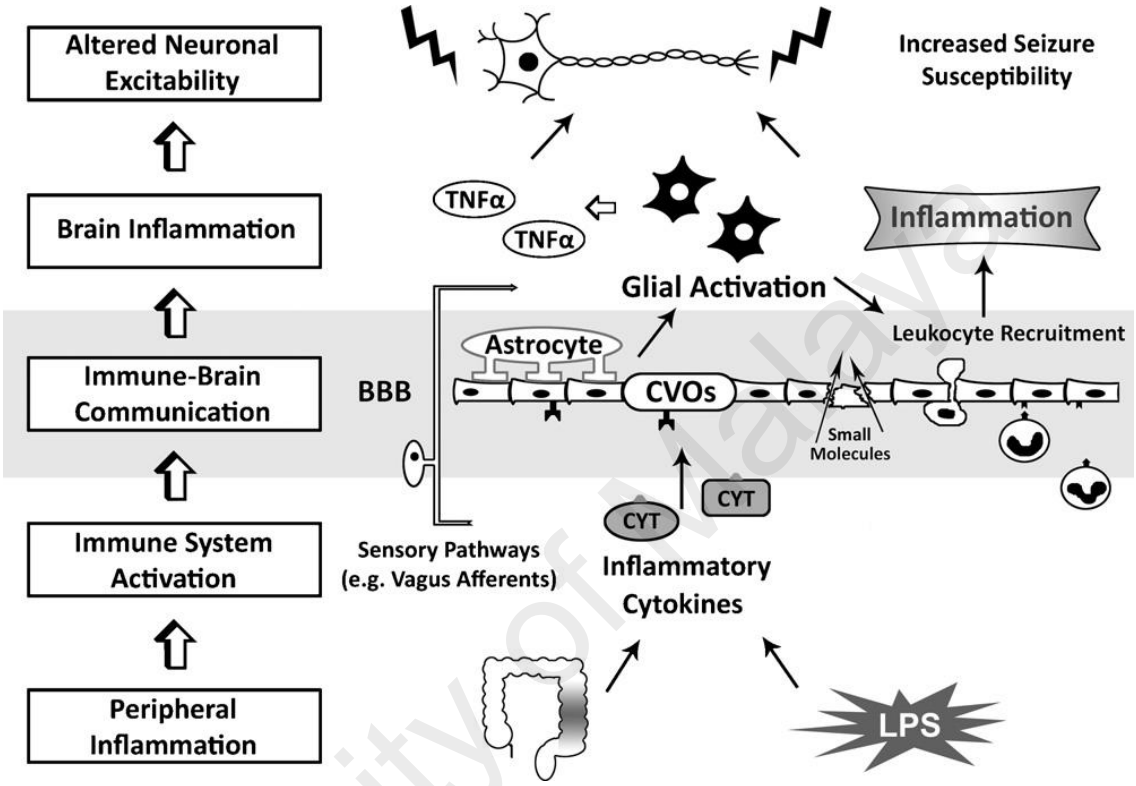


Figure 2.9: Schematic representation of peripheral inflammation leading to increased seizure susceptibility.

CHAPTER 3

COLLECTION AND CULTIVATION OF BASIDIOCARPS, PRODUCTION OF MYCELIUM USING SUBMERGED CULTURE TECHNIQUE, AND EXTRACTION AND FRACTIONATION

3.1 Introduction

Health awareness has increased worldwide and more people have turned towards healthy lifestyle and diet to prevent risk of disease. In recent years, mushroom has gained significant interest as medicinal food due to its various therapeutic properties. For instance, *Ganoderma lucidum* which is well-known as *Ling zhi*, has been used as Chinese medicine for many years as it is believed to promote health and longevity (Wachtel-Galor, Tomlinson, & Benzie, 2004). Therefore, mushroom cultivation has been expanding over the years due to the increase in market demand.

As the market demand for the mushroom production increases, it becomes harder to meet the market requirement due to time constraint. Hence, submerged cultivation has become an alternative production technique as it can be produced in larger amount in a shorter period of time. Studies have also found that mycelia and fruiting bodies have similar bioactive compounds (Lin et al., 2013) and beneficial biological activities such as antioxidant (Carvajal et al., 2012), neuroprotection (Wang et al., 2012), anti-inflammation (Elsayed et al., 2014).

Besides, there is increasing interest in research on discovering pharmacological potential of mushrooms. Many bioactive substances have been found in mushrooms and these include secondary metabolites (acids, terpenoids, polyphenols, sesquiterpenes,

alkaloids, sterols, metal chelating agents, nucleotide analogs, and vitamins), glycoproteins, and polysaccharides (mainly, β- glucan) (Valverde, Hernández-Pérez, & Paredes-López, 2015). Also, novel proteins such as lectins, lignocellulolytic enzymes, protease inhibitors, and hydrophobins have been found in mushrooms. These novel proteins have the ability to mitigate medical and biotechnological problems such as microbial drug resistance, low crop yields, and demands for renewable energy (Erjavec, Kos, Ravnikar, Dreo, & Sabotič, 2012).

Although there are more than 2,000 species of mushrooms found in nature, only about 1.3% are well-studied and being accepted as food and medicine (Hung & Nhi, 2012). Thus, exploration of other potential medicinal mushrooms should be done to unmask the possible therapeutic properties that may be beneficial to humankind. *Amauroderma rugosum* is a wild medicinal mushroom that is commonly used by the Chinese to treat diuresis and upset stomach, reduce inflammation, and prevent cancer (Dai & Yang, 2008). Besides, the Temuan (indigenous group) people in Malaysia believe that wearing the stipe of this mushroom can prevent fits episodes and incessant crying by babies (Azliza et al., 2012). To date, there are no reports on the cultivation of *A. rugosum*. Therefore, it is crucial to document on the mushroom cultivation and biomass production by means of submerge cultivation for specific application. In this chapter, the collection and cultivation of basidiocarps of *A. rugosum*, culture of mushroom mycelia using submerged culture technique, and extraction and fractionation of basidiocarps and mycelia of *A. rugosum* are presented. The mushroom powder extraction and fractionation are important for subsequent testing of specific biological activities such as antioxidant (Chapter 4), anti-inflammation (Chapter 5), anti-neuroinflammation (Chapter 6), and anti-epilepsy (Chapter 7).

3.2 Methodology

3.2.1 Chemicals and consumables

Table 3.1 shows the list of chemicals and consumables used in this study.

Table 3.1: List of chemicals and consumables.

Chemicals/Consumables	Company
Potato dextrose agar (PDA)	Difco™, BD, New Jersey, USA
Potato dextrose broth (PDB)	Difco™, BD, New Jersey, USA
Calcium carbonate ($CaCO_3$)	Systerm, Selangor, Malaysia
Whatman filter No. 4	Whatman International Ltd., Maidstone, England
Ethanol	Systerm, Selangor, Malaysia
Hexane	Fisher Scientific Inc., New Hampshire, USA
Ethyl acetate	Fisher Scientific Inc., New Hampshire, USA

3.2.2 Basidiocarp collection

The wild basidiocarps (KLU-M 1369) of *A. rugosum* were collected from the Royal Belum Forest, Perak, Malaysia (N05°28'58.80 E101°20'24.72; Figure 3.1) and authenticate identified by Mushroom Research Centre, University of Malaya, Kuala Lumpur, Malaysia, through morphology and molecular studies. The fresh basidiocarps were carefully wrapped in clean aluminium foil after removing the adhering soil and debris. The fresh basidiocarps were air-dried and maintained at moderate temperature during the transportation to the laboratory. The basidiocarps were sliced into smaller pieces and freeze-dried.

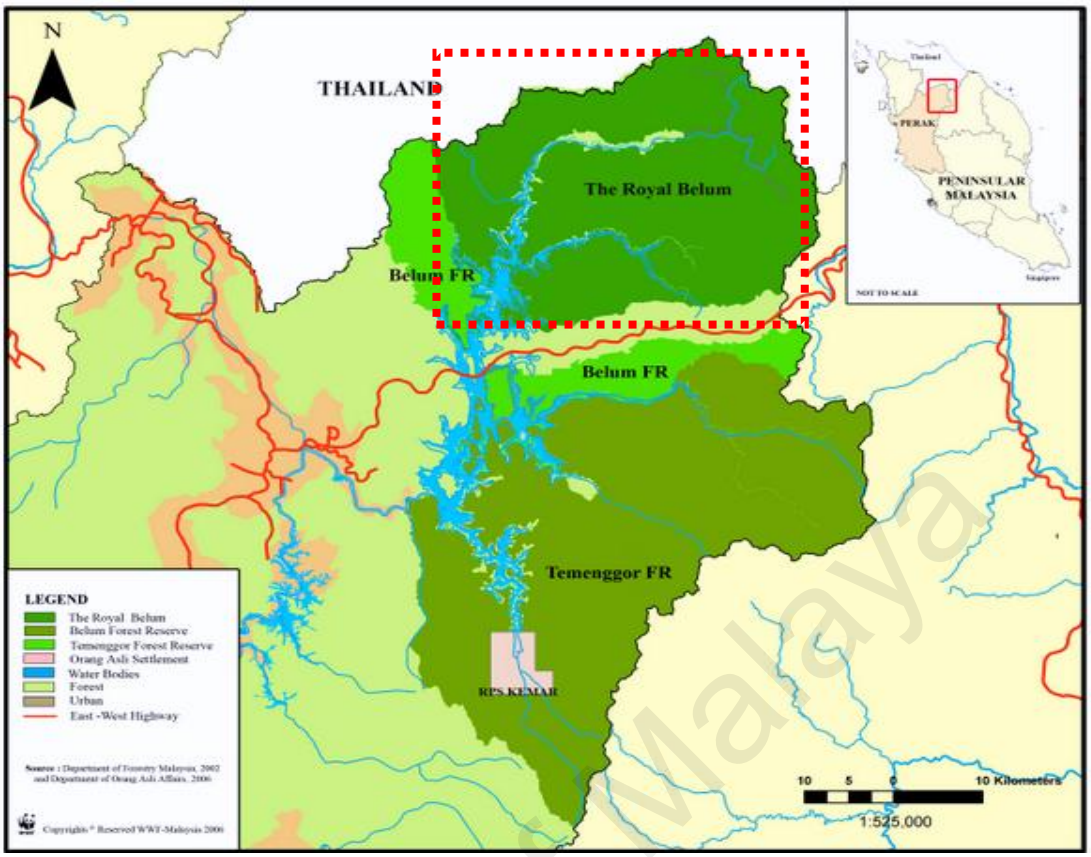


Figure 3.1: The location of Belum Forest, Perak, Malaysia. (Source from: http://whoaadventures.com/package/discover-temenggor-forest)

3.2.3 Production of mycelium

The mycelium pure culture (KUM 61131) of *A. rugosum* was obtained by tissue culture of the fresh basidiocarp. Mycelium of *A. rugosum* was maintained on PDA (Appendix A (A)) medium. Seven-day old mycelium grown on PDA was used as inoculum (Figure 3.2A). Ten plugs from the periphery of the colony were transferred into 500 mL Erlenmeyer flasks with baffle containing 100 mL of PDB (Appendix A (B)) medium and incubated in an incubator shaker at 100 rpm, 27ºC for 14 days. The mycelium broth was then freeze-dried and stored at 4ºC for further use (Figure 3.2B).

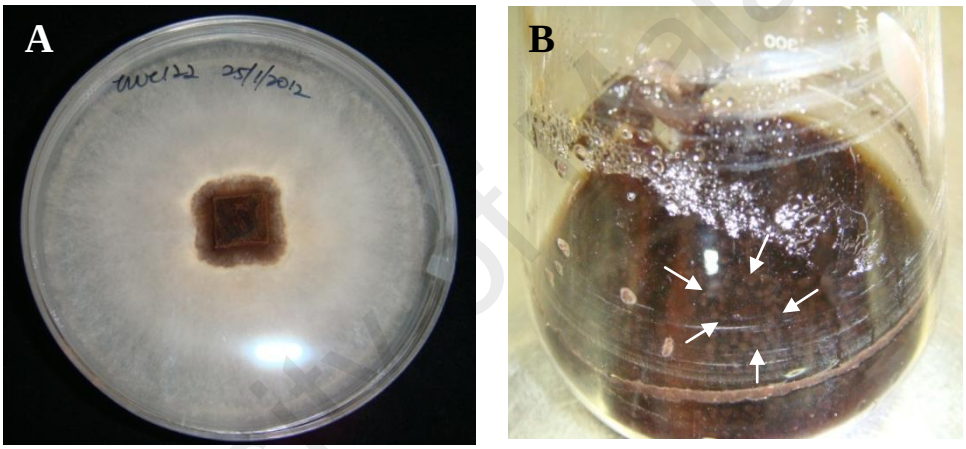


Figure 3.2: Mycelium of *A. rugosum* (A) On PDA plate; (B) On PDB (mycelium indicated by arrows).

3.2.4 Cultivation of basidiocarps

The wheat grain was cleaned and soaked in tap water overnight. Then, the soaked grains were drained and autoclaved at 121ºC for 15 minutes. The sterilised grains were allowed to cool for 24 hours before inoculating with mycelium culture of *A. rugosum*. After that, the inoculated grains were incubated at room temperature for 14 days until the mycelium completely covered the grains (spawn). Each mushroom bag contained 300 g of substrate comprising $CaCO_3$ (1%), rice bran (10%), rubberwood saw dust (89%), and water (70%). The bags filled with the substrates were autoclaved at 121ºC for 15 minutes to sterilise them. Then, the sterilised bags were left to cool for 24 hours

before inoculating them with the prepared spawn. The bags were stored in the mushroom house (University of Malaya, Kuala Lumpur, Malaysia) under indirect sun light at 26ºC with 80% to 85% relative humidity. After harvesting the basidiocarps, they were sliced into smaller pieces and freeze-dried. Biological efficiency of the domesticated basidiocarps was calculated as follows:

$$\text{Biological efficiency (\%)} = \frac{\text{Fresh weight of basidiocarp (g)}}{\text{Dry weight of substrate (g)}} \text{ X } 100$$

3.2.5 Preparation of *A. rugosum* extracts

The freeze-dried mycelium grown in submerged culture, wild, and domesticated basidiocarps of *A. rugosum* were extracted using ethanol at a ratio of 1:10 (w/v) for 2 days at room temperature. The solvent-containing extract was decanted and filtered through Whatman filter paper No. 4. The extraction process was repeated five times, filtrates were combined, and excess solvent was evaporated using rotary evaporator. The dried crude ethanol extract (EE) of mycelium and ethanolic extracts of the wild (WB) and domesticated (DB) basidiocarps of *A. rugosum* was kept at 4ºC until further use. The crude ethanolic extract (EE) of mycelia of *A. rugosum* grown in submerged culture was subjected to further extraction with hexane at a ratio of 1:10 (w/v) to give hexane-soluble fraction (HF) and hexane-insoluble residue. The hexane-insoluble residue was further partitioned between ethyl acetate and water (1:2) to give ethyl acetate-soluble fraction (EAF) and water-soluble fraction (AF). All extracts were kept in 4ºC for further use.

3.3 Results

3.3.1 Growth stages

The growth stages of *A. rugosum* basidiocarps were observed (Table 3.2). The mycelia fully colonised the 300 g substrate in 15 days. In about 25 days, pinhead started emerging and they grew into mature basidiocarps after 1-2 weeks. The domesticated basidiocarps of *A. rugosum* (9.6 ± 3.8 g; Figure 3.3A) showed a higher average fresh weight compared to the basidiocarps which grown in the wild (7.6 ± 3.9 g; Figure 3.3B).

Table 3.2: The colonisation of sawdust bags and basidiocarps formation by *A. rugosum*.

Growth stages	Domesticated basidiocarp
Mycelia colonisation (days)	15
Mycelia growth rate (cm/2 days)	1.9 ± 0.1
Pinhead emergence (days)	25.7 ± 3.5
Fresh weight (g)	9.6 ± 3.8
Biological efficiency (%)*	5.4 ± 2.2

*Based on one harvest

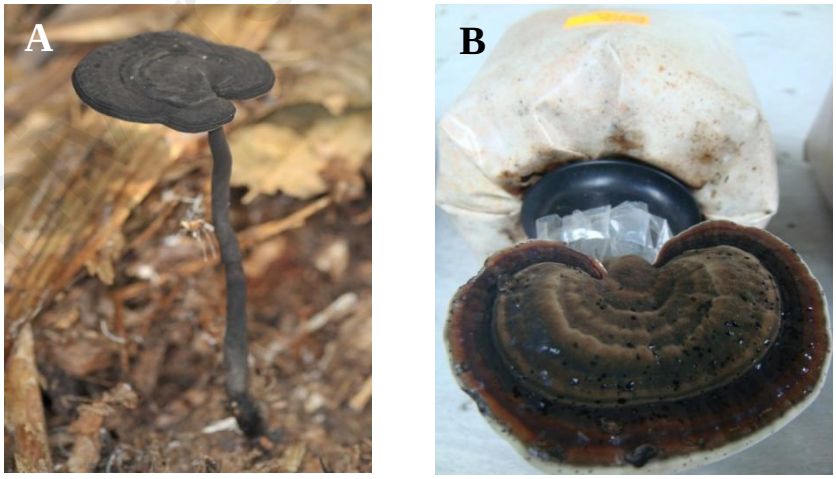


Figure 3.3: (A) Wild basidiocarp and (B) domesticated basidiocarp of *A. rugosum*.

3.3.2 Extraction yield of basidiocarps and mycelium of *A. rugosum* grown in submerged culture

Table 3.3 shows the total yield of ethanolic extracts of mycelium, wild and domesticated basidiocarp, and fractions of mycelium using different solvents (hexane, ethyl acetate, and water). Total yield of freeze-dried product from 10 flasks of mycelium was 24.4 ± 2.1 g.

Table 3.3: Total yield of *A. rugosum* extracts.

Product	Yield (g)
Wild basidiocarp (WB)	1.6
Domesticated basidiocarp (DB)	0.6
Crude ethanol extract (EE)	7.7 ± 0.8
Hexane fraction (HF)	0.2 ± 0.1
Ethyl acetate fraction (EAF)	0.3 ± 0.1
Aqueous fraction (AF)	6.3 ± 1.5

Total yield of extracts of mycelia, wild and domesticated basidiocarps are expressed as mean ± standard deviation (S.D), n = 3. HF, EAF, and AF were fractionated from 10 g of crude ethanolic extract.

3.4 Discussion

Mushroom cultivation is a profitable agribusiness. The development and innovation in mushroom cultivation is important for food security, reduction of poverty, and improvement of economic status of small farmers (Shah, Ashraf, & Istiaq, 2004). Cultivation of basidiocarps can also ensure constant supply of mushrooms and reduce over harvesting of wild mushrooms in the forests (Tan et al., 2015). Besides, mushrooms are sources of food and contain useful medicinal properties against diabetes (Silva, Rapior, Hyde, & Bahkali, 2012), cancer (Chen et al., 2005), and heart disorder (Martin, 2010). Hence, the rapid growth of demand for mushroom and interest in mushroom research encourages mushroom farming and expansion of research and development of new products.

In this study, the mycelium of *A. rugosum* was obtained from tissue culture and maintained on PDA medium. Overall, the growth rate of the fruiting bodies of *A. rugosum* was fast as compared to the other mushrooms reported previously (Table 3.4). The size of the mushroom bag prepared was 300 g and only 1 flush was observed. The biological efficiency was noticeably low. Studies have shown that factors such as genotype of mushroom strains, type of substrate, and environmental susceptibility, may affect the growth and fructification of mushrooms (Abdullah, Haimi, Lau, & Annuar, 2013; Moonmoon, Uddin, Ahmed, Shelly, & Khan, 2010).

Solvent extraction was chosen as a mode of extraction in this study. According to the traditional application of *A. rugosum* by the Temuan people in Malaysia, the stipe of the fruiting body is diced, strung, and worn as necklace to prevent epileptic episodes and incessant crying by babies (Azliza et al., 2012). Therefore, this has raised the interest in the investigation on the potential volatile compounds that may be present in *A. rugosum.*

Table 3.4: Comparison of spawn run and yield of selected domesticated or cultivated mushrooms.

Mushroom	Substrate	Bag size (g)	Spawn run (Days)	Pinhead emergence (Days)	No. of harvest	Biological efficiency (%)
A. rugosum (This study)	Sawdust, rice bran	300	15	26	1	5.4
Oudemansiella tanzanica (Magingo et al., 2004)	Sawdust, rice bran	1000	19	2	20	101.9
Volvariella spp. (Tripathy et al., 2011)	50% Wheat, 50% rice bran	-	12	6	3	13.6
Pleurotus ostreatus (Pathmashini et al., 2008)	Paddy	800	32	50	5	12.0
Pleurotus ostreatus (Pathmashini et al., 2008)	Sorghum	800	23	36	5	25.4
Pleurotus ostreatus (Pathmashini et al., 2008)	Kurakkan	800	21	35	5	30.8
Pleurotus ostreatus (Pathmashini et al., 2008)	Maize	800	22	45	5	16.6
Pleurotus ostreatus (Shah et al., 2004)	Sawdust, wheat straw	1000	17	25	11	43.6
Pleurotus ostreatus (Shah et al., 2004)	Sawdust	1000	17	24	22	64.7
Lentinus squarrosulus (Adesina et al., 2011)	*Spondias mombin*, rice bran	-	28	7	12	10.3
Ganoderma neo-japonicum (Tan et al., 2015)	Sawdust	500	38	58	-	-

3.5 Conclusion

In conclusion, fruiting bodies and submerged cultivation can be successfully applied for production of *A. rugosum*. Further optimisation on the fruiting bodies cultivation is important to achieve a higher biological efficiency. Different approaches on the extraction and fractionation process can be done according to the aspect of interest of the study.

CHAPTER 4

NUTRITIONAL COMPOSITION ANALYSIS, ANTIOXIDANT ACTIVITIES AND TOTAL PHENOLIC CONTENT OF *AMAURODERMA RUGOSUM*

4.1 Introduction

Plants have a long history in drug discovery but there are still limited studies on other natural products such as mushrooms. Recently, mushrooms have gained significant interest as a new source of therapeutics (Chan et al., 2013). Mushrooms have been found to possess good antioxidant activity and great nutritional value (Ferreira et al., 2009; Mattila, Suonpää, & Piironen, 2000). However, the nutritional and antioxidant properties of *A. rugosum* are not known.

Therefore, the aim of this chapter was to identify the nutritional composition of freeze-dried mycelia and determine the potential antioxidant activities and total phenolic content of *A. rugosum* extracts. The extracts of *A. rugosum* (EE: ethanol extract; HF: hexane fraction; EAF: ethyl acetate fraction; AF: aqueous fraction; WB: wild basidiocarp extract; DB: domesticated basidiocarp extract) as described in Chapter 3 were used to test for antioxidant activities and total phenolic content.

4.2 Methodology

4.2.1 Chemicals and reagents

The list of chemicals and reagents used in this study is shown in Table 4.1.

Table 4.1: List of chemicals and reagents.

Chemicals/Reagents	Company
2,2-diphenyl-1-picrylhydrazyl (DPPH)	Sigma-Aldrich, St. Louis, MO, USA
Ethanol	Systerm, Selangor, Malaysia
Trolox	Sigma-Aldrich, St. Louis, MO, USA
Ascorbic acid	Sigma-Aldrich, St. Louis, MO, USA
Butylated hydroxytoluene (BHT)	Sigma-Aldrich, St. Louis, MO, USA
2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS)	Calbiochem, Merck, Milipore, Darmstadt, Germany
Potassium persulphate ($K_2S_2O_8$)	Merk & Co., New Jersey, USA
Folin-ciocalteau phenol	Merk & Co., New Jersey, USA
Sodium carbonate ($NaCO_3$)	Merk & Co., New Jersey, USA
Gallic acid	Sigma-Aldrich, St. Louis, MO, USA

4.2.2 Nutritional composition analysis

Two hundred grams of freeze-dried mycelia of *A. rugosum* grown in submerged culture was sent to Consolidated Laboratory (M) Sdn. Bhd. for nutritional analysis. Table 4.2 shows the test parameters and methods used in the nutritional analysis.

Table 4.2: List of test parameters and methods used in nutritional analysis.

No.	Test parameter	Test method
1	Energy	By calculation (based on Method of Analysis for Nutrition Labelling, AOAC, 1993, page 106 and 105)
2	Total fat	In-house method, CL-TM-01-028, based on AOAC 989.05, 2005
3	Carbohydrate	By calculation (based on Method of Analysis for Nutrition Labelling, AOAC, 1993)
4	Protein	In-house method, CL-TM-01-018, based on AACC 46-12 Vol. II 9th Edition
5	Cholesterol	In-house method, CL-TM-01-022, based on JAOAC Vol. 64, No. 1, 1981 (HPLC)
6	Dietary fibre	AOAC 985.29, 2005
7	Magnesium (as Mg)	In-house method, CL-TM-01-020, based on AOAC 985.01 and AOAC 922.02. 2005; AACC 40-70, Vol. II 9th Edition (ICP-OES)
8	Iron (as Fe)	In-house method, CL-TM-01-020, based on AOAC 985.01 and AOAC 922.02. 2005; AACC 40-70, Vol. II 9th Edition (ICP-OES)
9	Zinc (as Zn)	In-house method, CL-TM-01-020, based on AOAC 985.01 and AOAC 922.02. 2005; AACC 40-70, Vol. II 9th Edition (ICP-OES)
10	Phosphorus (as P)	In-house method, CL-TM-01-020, based on AOAC 985.01 and AOAC 922.02. 2005; AACC 40-70, Vol. II 9th Edition (ICP-OES)
11	Potassium (as K)	In-house method, CL-TM-01-020, based on AOAC 985.01 and AOAC 922.02. 2005; AACC 40-70, Vol. II 9th Edition (ICP-OES)
12	Copper (as Cu)	In-house method, CL-TM-01-020, based on AOAC 985.01 and AOAC 922.02. 2005; AACC 40-70, Vol. II 9th Edition (ICP-OES)
13	Manganese (as Mn)	In-house method, CL-TM-01-020, based on AOAC 985.01 and AOAC 922.02. 2005; AACC 40-70, Vol. II 9th Edition (ICP-OES)
14	Selenium (as Se)	In-house method, CL-TM-01-020, based on AOAC 985.01 and AOAC 922.02. 2005; AACC 40-70, Vol. II 9th Edition (ICP-OES)
15	Sodium (as Na)	In-house method, CL-TM-01-020, based on AOAC 985.01 and AOAC 922.02. 2005; AACC 40-70, Vol. II 9th Edition (ICP-OES)
16	Calcium (as Ca)	In-house method, CL-TM-01-020, based on AOAC 985.01 and AOAC 922.02. 2005; AACC 40-70, Vol. II 9th Edition (ICP-OES)

4.2.3 Antioxidant activities

4.2.3.1 Scavenging of 2,2-diphenyl-1-picrylhydrazyl (DPPH)

(A) Principle

The free radical DPPH radical is used to estimate the scavenging activity of antioxidants. It measures the compounds that are radical scavengers. DPPH has a deep violet colour in solution that can be detected at 515 nm. Upon mixing of DPPH with a substance that can donate a hydrogen atom, the violet colour will change into pale yellow that gives rise to the reduced form of DPPH (Figure 4.1) (Molyneux, 2004).

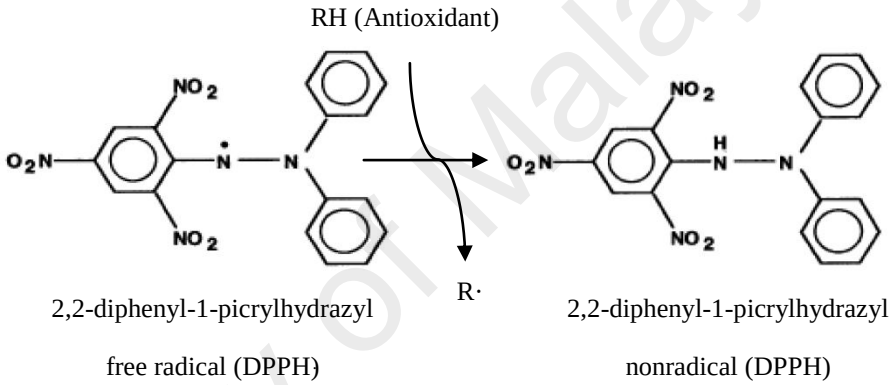


Figure 4.1: Conversion of DPPH radical to DPPH nonradical by antioxidant compound.

(B) Method

The DPPH assay was performed according to the method by Brand-Williams, Cuvelier, & Berset (1995). Five microliters of *A. rugosum* extract (EE, HF, EAF, AF, WB, or DB) was added into a 195 µL reaction mixture which consisted of DPPH radical in ethanol solution. The mixture was incubated for 3 hours in the dark and the absorbance was measured at 515 nm using a spectrophotometer (BioTek, USA). Ascorbic acid, trolox and BHT served as positive controls. The values were expressed as EC_{50}. EC_{50} is defined as the amount of antioxidant required to scavenge 50% of the DPPH radicals.

4.2.3.2 Scavenging of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS)

(A) Principle

The ABTS radical cation is commonly used for the measurement of total antioxidant activity of water-soluble and lipid-soluble antioxidants, pure compounds, and food extracts by spectrophotometric method. The reaction between ABTS and potassium persulfate leads to the production of blue/green $ABTS^{\cdot+}$ chromophore. This can be detected at the wavelength of 734 nm (Re et al., 1999).

(B) Method

The ABTS assay was used to measure the antioxidant capacity of the *A. rugosum* extracts. This assay was carried out based on the method by Re et al. (1999). Briefly, 7 mM ABTS was mixed with 2.45 mM $K_2S_2O_8$ and incubated in the dark at room temperature for 12-16 hours to allow the production of ABTS radical cation ($ABTS^{\cdot+}$). The $ABTS^{\cdot+}$ solution was further diluted with ethanol to an absorbance of 0.70 (± 0.02) at 734 nm and equilibrated at 30°C. Then, 100 µL of $ABTS^{\cdot+}$ solution was added to 10 µL of *A. rugosum* extract (EE, HF, EAF, AF, WB, or DB) and the absorbance at 734 nm was measured after 1 minute. Ascorbic acid, trolox and BHT were used as positive controls. The values were expressed as EC_{50}.

4.2.4 Total phenolic content

(A) Principle

The Folin-Ciocalteau colorimetric assay is widely used to measure the total concentration of phenolic hydroxyl groups in plant and mushroom extracts. Folin-Ciocalteau reagent reacts with polyphenols to form a blue complex that can be measured at 750 nm (Blainski, Lopes, & Mello, 2013).

(B) Method

Total phenolic content of *A. rugosum* extract (EE, HF, EAF, AF, WB, or DB) was measured using Folin-Ciocalteu phenol reagent according to the method by Cheung, Cheung, & Ooi (2003) with slight modifications. First, 50 µL of 10% Folin-Ciocalteau phenol reagent was added to 50 µL of extract. The mixed solution was incubated in the dark at room temperature for 3 minutes. This was followed by the addition of 100 µL of 10% Na_2CO_3 and further incubation in the dark at room temperature for 1 hour. The absorbance was measured at 750 nm using a spectrophotometer. Gallic acid was used as a standard phenolic compound. All samples were analysed in triplicate and the total phenolic content was expressed as Gallic acid equivalents (GAEs/g of extract).

4.2.5 Statistical analysis

All values are expressed as means ± standard deviation (SD) of triplicate values. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Duncan's Multiple Comparison Test using Statistical Product and Service Solutions, SPSS® Statistics for Windows, Version 17.0 and $p < 0.05$ was denoted as being statistically significant. Effective concentrations (EC_{50}) were calculated using GraphPad Prism software version 5.0. The scatter plot and regression line for the correlation between TPC and antioxidant activities were plotted using GraphPad.

4.3 Results

4.3.1 Nutritional composition of mycelia of *A. rugosum* grown in submerged culture

The nutritional composition of freeze-dried mycelia of *A. rugosum* grown in submerged culture is depicted in Table 4.3. The freeze-dried mycelia grown in submerged culture contained 0.3% of the recommended daily allowance (RDA) of total fat, a non-detectable level of cholesterol, 25.5% of the RDA of carbohydrate, 16.6% of the RDA of protein, 38.4% of the RDA of dietary fibre, and were rich in minerals such as phosphorus (14.4% of the RDA), potassium (11.6% of the RDA) and sodium (25.4% of the RDA).

Table 4.3: Nutritional composition of freeze-dried mycelia of *A. rugosum* grown in submerged culture.

Component	Method	Composition/100g	Recommended daily allowance (RDA)
Energy	AOAC	341 kcal	-
Total Fat	AOAC 989.05	0.2 g	65 g
Carbohydrate	AOAC	76.5 g	300 g
Protein	AACC 46-12	8.3 g	50 g
Cholesterol	HPLC	N.D*	300 mg
Dietary Fibre	AOAC 985.29	9.6 g	25 g
Magnesium	ICP-OES	5.7 mg	400 mg
Iron	ICP-OES	1 mg	18 mg
Zinc	ICP-OES	0.8 mg	15 mg
Phosphorus	ICP-OES	144.4 mg	1000 mg
Potassium	ICP-OES	404.9 mg	3500 mg
Copper	ICP-OES	0.01mg***	2.0 mg
Manganese	ICP-OES	0.03 mg	2.0 mg
Selenium	ICP-OES	N.D**	70 µg
Sodium	ICP-OES	609.9 mg	2400 mg
Calcium	ICP-OES	4.7 mg	1000 mg

N.D: Not detectable; AOAC: Association of Analytical Communities/Association of Official Agricultural Chemist; AACC: American Association of Cereal Chemists; HPLC: high-performance liquid chromatography; ICP-OES: inductively coupled plasma optical emission spectrometry. *level of cholesterol < 0.001 mg/100 g; **level of selenium < 0.02 mg/kg; ***per kg

4.3.2 Antioxidant activities and total phenolic content of *A. rugosum*

Table 4.4 shows the antioxidant activity and total phenolic content of *A. rugosum* extracts. The antioxidant activity of *A. rugosum* extracts were evaluated based on DPPH and ABTS assays. Both WB and DB showed similar total phenolic content and DPPH scavenging activity. However, WB displayed a higher ABTS scavenging activity compared to DB.

Table 4.4: Antioxidant activity and total phenolic content of *A. rugosum* extracts.

	Total phenolic content (mg of GAEs / g of extract)	**DPPH (EC_{50} µg/mL)**	**ABTS (EC_{50} µg/mL)**
Ascorbic acid	-	2.5	8.1
BHT	-	6.6	20.7
Trolox	-	4.1	17.3
WB	47.9 ± 0.0	76.9	222.9
DB	46.4 ± 0.0	73.3	469.6
EE	93.6 ± 0.0	17.1	110
HF	154.1 ± 0.0	8.2	51.6
EAF	555.4 ± 0.0	2.3	18.3
AF	114.1 ± 0.0	10.1	52.6

DPPH and ABTS are expressed as half maximal effective concentrations (EC_{50}); TPC results are expressed as mean ± standard deviation (S. D.; n = 3). (EE: ethanol extract; HF: hexane fraction; EAF: ethyl acetate fraction; AF: aqueous fraction; WB: wild basidiocarp extract; DB: domesticated basidiocarp)

On the other hand, the DPPH scavenging activity of the mycelia extracts (EE: ethanol extract; HF: hexane fraction; EAF: ethyl acetate fraction; AF: aqueous fraction) in descending order of potency was EAF > HF > AF > EE, as shown in Table 4.4. The descending order of the ABTS scavenging potency of the extracts was EAF > HF > AF > EE (Table 4.4). Besides, EAF had the highest amount of phenolic compounds, followed by HF, AF and EE. Overall, EAF had the highest antioxidant activities and total phenolic content. The mycelia extracts of *A. rugosum* showed better antioxidant activities and higher total phenolic content compared to the fruiting bodies extracts.

The relationship between EC_{50} values of antioxidant activities and TPC of all extracts (WB, DB, EE, HF, EAF, and AF) are shown in Figures 4.2 and 4.3. It was found that TPC and EC_{50} values for DPPH and ABTS had a weak correlation (R_2 = 0.338 and R_2 = 0.29, $p > 0.05$, respectively).

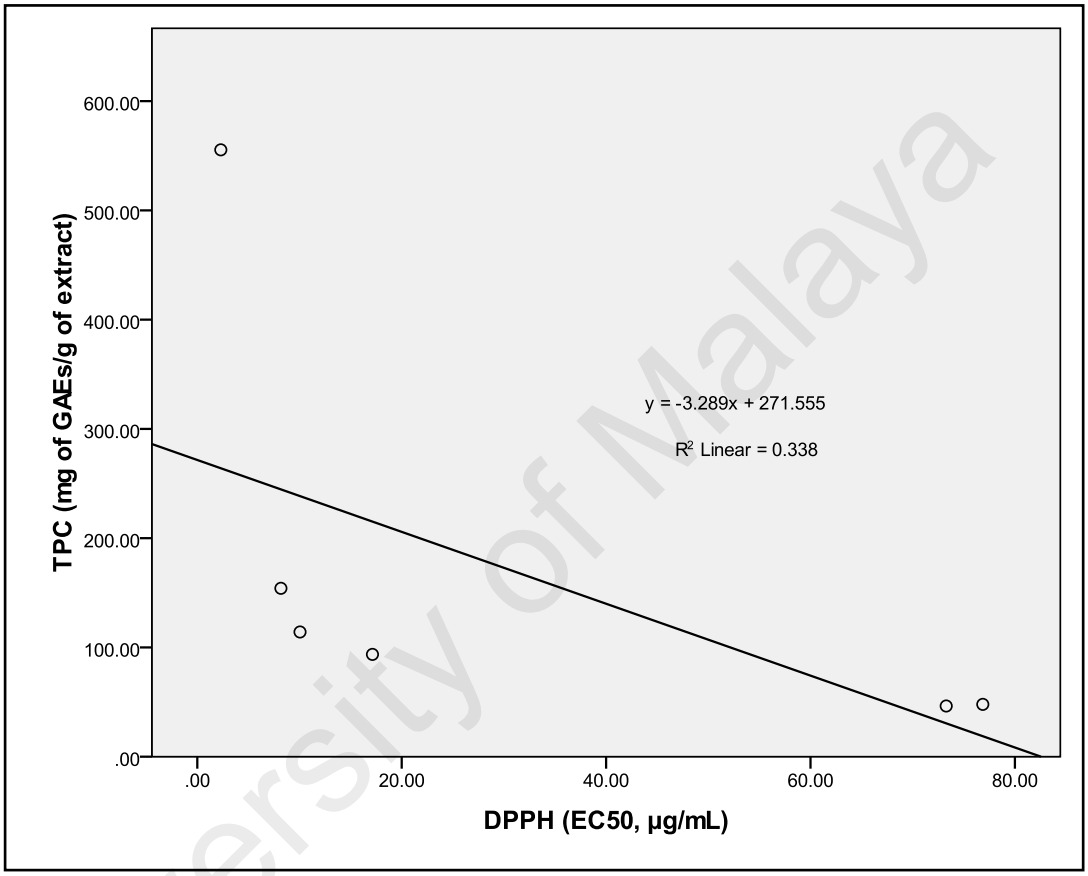


Figure 4.2: Correlation between EC_{50} value of DPPH and TPC.

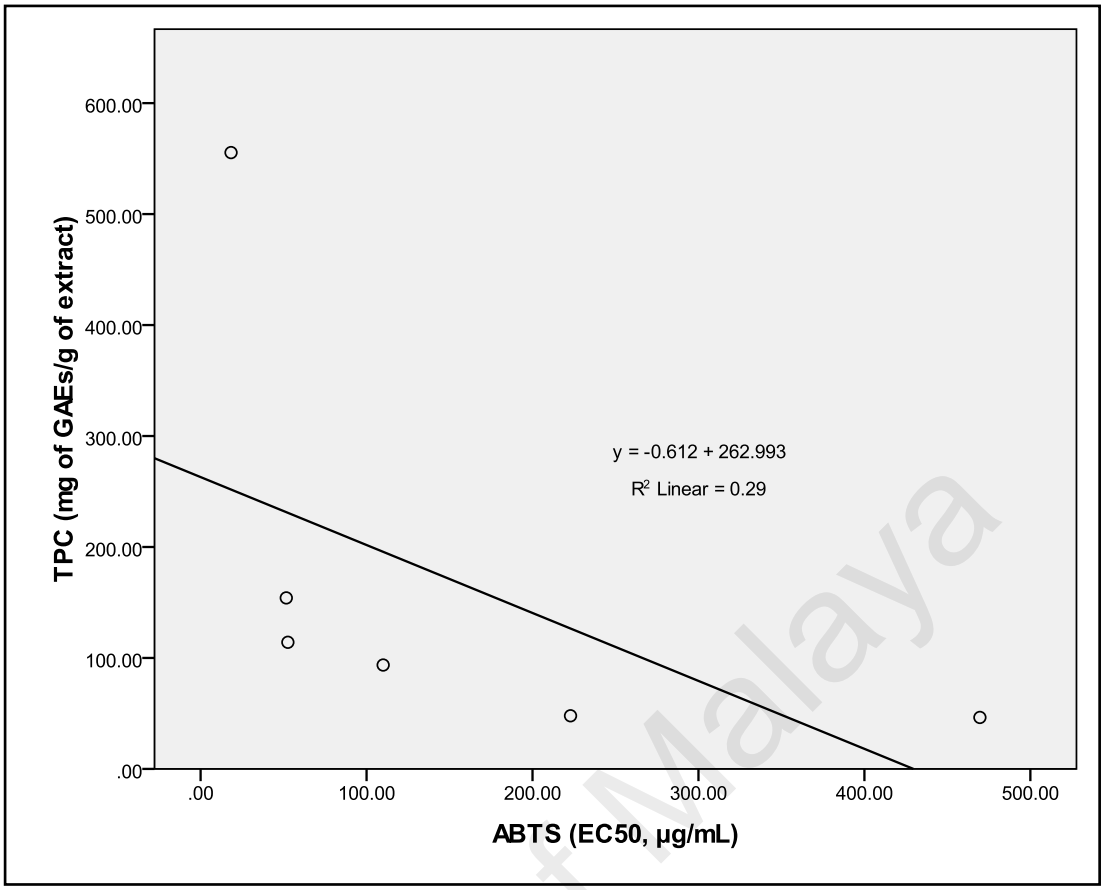


Figure 4.3: Correlation between EC_{50} value of ABTS and TPC.

4.4 Discussion

It has been reported that mushrooms are good sources of nutritional components such as proteins, minerals and vitamins. However, there are no existing data in the literature on the nutritional components of *A. rugosum*. The knowledge of the composition and nutritional value of wild mushrooms is limited compared to vegetables or culinary and medicinal mushrooms (Kalač, 2013). Thus, it is important to examine the medicinal properties and biologically active components of wild mushrooms that may benefit humankind (Lindequist et al., 2005). Moreover, increasing the understanding of the ethnomedicinal use of wild mushrooms is necessary for successful bioprospecting. According to the U.S. Food and Drug Administration (FDA), it is important to disclose the nutrient content of a dietary supplement to ensure its safety and effectiveness before and after a product is marketed. Generally, fruiting bodies of mushrooms have low fat content, high protein content (including essential amino acids) that range from 19 to 35% of the RDA, and large amounts of carbohydrate and fibre, ranging from 51 to 88% and from 4 to 20% of the RDA, respectively (Mattila et al., 2000). Omar, Abdullah, Kuppusamy, Abdulla, & Sabaratnam (2011) reported that, *Lentinus squarrosulus* mycelia extract contained 4.1% carbohydrate, 0.8% total fat and <0.1 g of the 100 g RDA of crude fibre. The present study showed that the mycelia of *A. rugosum* grown in submerged culture contained 6.2-times more carbohydrate and 96-times more fibre content than the *L. squarrosulus* mycelia extract. Furthermore, the mycelia of *A. rugosum* grown in submerged culture have lower total fat than the *L. squarrosulus* mycelia and a non-detectable level of cholesterol and are rich in minerals, such as phosphorus, potassium and sodium. Low total fat and cholesterol content are favourable as nutritional supplements for heart patients. Also, high levels of potassium help the body to regulate the amount of sodium, which lower blood pressure. Furthermore, phosphorus is one of the important minerals that combine with calcium to form calcium

phosphate, which gives strength and rigidity to bones and teeth (Chan et al., 2013). Due to the limited amount of basidiocarp samples, only mycelia of *A. rugosum* were analysed for nutritional compositions.

It is well known that the overproduction of ROS can adversely alter and damage lipids, proteins and DNA, and have been implicated in a number of diseases. Thus, extensive research has been actively conducted for the past few decades to search and identify antioxidants in natural products. Recently, mushrooms have gained significant interest as a new source of therapeutic agents. Many studies on antioxidant activity of mushrooms have been conducted (Ferreira, Barros, & Abreu, 2009; Gan, Amira, & Asmah, 2013; Kanagasabapathy, Malek, Kuppusamy, & Vikineswary, 2011; Kanagasabapathy et al., 2012). In the present study, WB and DB showed similar total phenolic content and DPPH scavenging activity. However, WB displayed a higher ABTS cation radical scavenging activity as compared to DB. Parrilla, Rosa, Martínez, & Aguilar (2007) showed that in general, wild mushrooms have higher phenolic content than cultivated ones. The same group of researchers also proved that the methanolic extract of wild *Agaricus bisporus* has higher total phenolic concentration than the commercial species. Conversely, the present study showed ethanolic extracts of wild and domesticated *A. rugosum* have similar total phenolic content. The discrepancy between the two studies may be due to the difference in the growth conditions and types of polyphenolic components present in the extracts. The EAF from mycelia of *A. rugosum* had the highest total phenolic content and antioxidant activity when compared with all the other solvent extracts. This finding is similar to the study reported by Öztürk et al. (2011), which showed that the ethyl acetate extract of *Agaricus bitorquis* had the highest total phenolic content when compared with all the extracts of that species. Moreover, the high phenolic content in EAF may contribute to its antioxidant

activity (Sim, Nurestri, & Norhanom, 2010). Omar et al. (2011) reported that the aqueous extract of *L. squarrosulus* mycelia exhibited DPPH scavenging activity with an IC_{50} value of 14.29 mg/mL. Furthermore, the DPPH scavenging activity of the ethanol extract and hot aqueous extract of *Pleurotus ferulae* mycelium showed EC_{50} values of 12.0 mg/mL and 4.3 mg/mL, respectively (Kim et al., 2012). According to Carvajal et al. (2012), the DPPH scavenging activity of the *Agaricus brasiliensis* fruiting bodies was better than the *A. brasiliensis* mycelium, but the ABTS cation radical scavenging ability analysis indicated that the mycelia had greater antioxidant activity than the fruiting bodies. In the present study, mycelia of *A. rugosum* displayed a better antioxidant activity than the fruiting bodies, based on both of ABTS cation radical and DPPH scavenging activities. Carvajal et al. (2012) suggested that the observed differences in results may be attributed to the types of polyphenols and non-phenolic components with antioxidant activities that are present in the various extracts.

Analysis of the relationship between the TPC and antioxidant activities of *A. rugosum* extracts revealed that there was no significant correlation between these parameters. According to Prior, Wu, & Schaich (2005), the Folin-Ciocalteau assay is used to estimate the TPC present in the extract, but free radical scavenging assays are not specific for polyphenols. Various phenolic compounds respond differently in the DPPH and ABTS assays, and the results of these assays depend on the number of phenolic groups present in the extracts (Singleton & Rossi, 1965). Therefore, the insignificant correlation between the TPC and antioxidant activity may be attributed to the presence of non-phenolic compounds with antioxidant activities (Sengul et al., 2009).

4.5 Conclusion

In conclusion, *A. rugosum* extracts contain good antioxidant activity. Both WB and DB showed similar total phenolic content and DPPH scavenging activity, but WB displayed higher ABTS cation scavenging activity than DB. On the other hand, among the mycelia extracts, EAF showed the highest antioxidant activity and total phenolic content, followed by HF, AF, and EE. It has been previously described in the Literature Review (Chapter 2) that reactive oxygen species (ROS) are associated with chronic inflammation and numerous of other diseases. Thus, antioxidants play a vital role in reducing ROS and therefore, prevent the pathogenesis of inflammatory disorders. In this chapter, the good antioxidant activity showed in *A. rugosum* extracts may be useful in slowing down or reversing the injuries caused by oxidative stress and may serve as a protective agent against related diseases such as inflammatory disorders.

CHAPTER 5

DETERMINATION OF ANTI-INFLAMMATORY ACTIVITY OF *AMAURODERMA RUGOSUM* USING *IN VITRO* CELLULAR MODEL (RAW264.7 CELLS)

5.1 Introduction

In the previous chapter (Chapter 4), *A. rugosum* extracts were found to possess good antioxidant activities. The introduction of the same chapter also mentioned that oxidative stress is one of the causes of inflammation. Therefore, it is pertinent to examine the potential anti-inflammatory effects of *A. rugosum*. Inflammation is a biological response of tissue in attempting self-protection against harmful stimuli such as pathogens, damaged cells and irritants. There are two types of inflammation; acute and chronic inflammation. Acute inflammation is often characterised in the four classical signs which are redness, heat, swelling, and loss of function. Monocytes extravasate from blood vessels into the injured site and transform into macrophages. Macrophages play a vital role in inflammatory response in the initiation, maintenance and resolution of inflammation. However, if the cause of inflammation cannot be eliminated, inflammation will prolong and vary in intensity over time (Hakansson & Molin, 2011). Prolonged or chronic inflammation may contribute to detrimental outcomes such as chronic inflammatory diseases.

Macrophages are activated by proinflammatory mediators such as lipopolysaccharide (LPS), interleukin-1β (IL-1β), and interferon-γ (IFN-γ) by binding to toll-like receptor-4 (TLR-4) which in turn activate the inflammatory signalling pathway component, nuclear factor kappa B (NF-κB) (Lee et al., 2012). This subsequently stimulates the

release of numerous proinflammatory mediators such as nitric oxide (NO), tumour necrosis factor alpha (TNF-α), and interleukin-6 (IL-6) (Lee et al., 2012). These proinflammatory mediators play a major role in the pathogenesis of various inflammatory disorders and serve as potent biomarkers for the assessment of the inflammatory process. In order to study the anti-inflammation potential of *A. rugosum*, RAW264.7 cell line (macrophage) was used as an *in vitro* model. The aim of this chapter was to assess the *A. rugosum* extracts for anti-inflammatory activity at biochemical and molecular levels using an *in vitro* approach (murine macrophage, RAW264.7 cells).

5.2 Methodology

5.2.1 Chemicals, reagents, consumables, and assay kits

Table 5.1 shows the list of chemicals, reagents, consumables, and kits used in this study.

Table 5.1: List of chemicals, reagents, consumables, and kits.

Chemicals/Reagents/Consumables/Kits	Company
Dulbecco's Modified Eagle's Medium (DMEM)	Sigma-Aldrich, St. Louis, MO, USA
Foetal bovine serum (FBS)	Sigma-Aldrich, St. Louis, MO, USA
Tryphan blue dye	Sigma-Aldrich, St. Louis, MO, USA
Penicillin-streptomycin	Biowest, MO, USA
L-glutamine	Sigma-Aldrich,St. Louis, MO, USA
Fungizone	Biowest, MO, USA
3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)	Calbiochem, Merck, Millipore, Darmstadt, Germany
Dimethyl sulfoxide (DMSO)	Fisher Scientific Inc., New Hampshire, USA
Sodium nitroprusside	Sigma-Aldrich,St. Louis, MO, USA
Sodium nitrite ($NaNO_2$)	
Phosphate buffer saline (PBS)	Oxoid Ltd., Thermo Scientific, Hampshire, UK.
Dexamethasone	Sigma-Aldrich,St. Louis, MO, USA
Aspirin	Sigma-Aldrich,St. Louis, MO, USA
Quercetin	Sigma-Aldrich,St. Louis, MO, USA
Sulphanilamide	Sigma-Aldrich,St. Louis, MO, USA
N-(1-napthy)ethylenediamine	Sigma-Aldrich,St. Louis, MO, USA
Phosphoric acid (H_3PO_4)	Sigma-Aldrich,St. Louis, MO, USA
Escherichia coli (O55:B5) lipopolysaccharide (LPS)	Sigma-Aldrich,St. Louis, MO, USA
N_ω-nitro-l-arginine-methyl ester (L-NAME)	Sigma-Aldrich,St. Louis, MO, USA
Paraformaldehyde	Sigma-Aldrich,St. Louis, MO, USA
Triton X-100	Sigma-Aldrich,St. Louis, MO, USA
NF-κB primary antibody	Cell Signalling Technology (CST), USA
iNOS primary antibody	Cell Signalling Technology (CST), USA
FITC-conjugated anti-rabbit IgG secondary antibody	Merck Milipore, Germany
Prolong® Gold Antifade reagent with DAPI	Thermo Scientific, USA
12-well chamber slide, removable, microscopy glass slide, sterile	ibidi GmbH, Germany
TNF-α enzyme-linked immunosorbent assay (ELISA) kit	Thermo Scientific, USA
IL-10 ELISA kit	Thermo Scientific, USA
RNAqueous®-4PCR kit	Ambion, USA
High Capacity cDNA Reverse Transcription kit	Applied Biosystems, USA

5.2.2 Cell culture

The murine macrophage cell line (RAW264.7 cells) from American Type Culture Collection (ATCC^{TM}, CAT #: TIB-71) was cultured in DMEM containing 10% FBS, 0.1% penicillin-streptomycin, 0.1% L-glutamine and 0.1% fungizone at 37°C in a humidified atmosphere containing 5% CO_2 (Figure 5.1). When RAW264.7 cell culture had reached 80-90% confluency, the cells were scraped to remove them from the culture flask and then centrifuged at 1000 *x g* at room temperature for 5 minutes. The cell viability was determined by trypan blue dye exclusion method and direct counting with a hemocytometer.

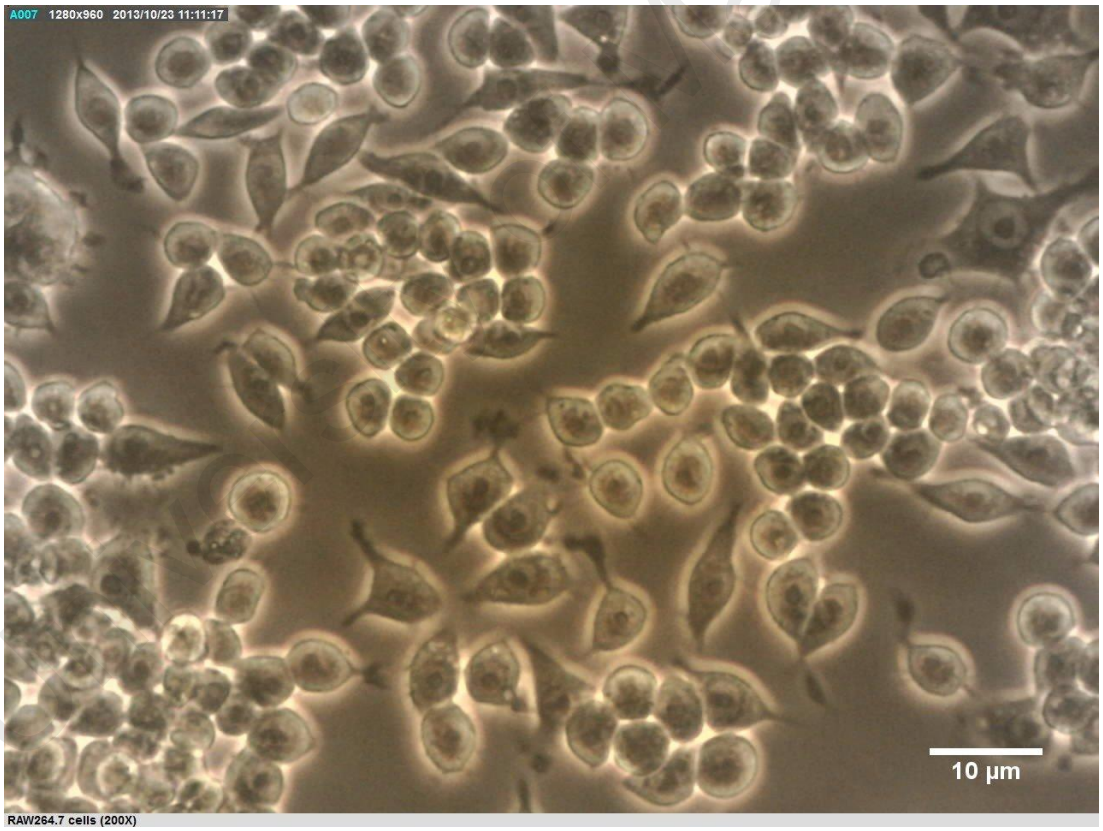


Figure 5.1: Morphology of *in vitro* cellular model for inflammatory study – RAW264.7 cells.

5.2.3 Cell viability

The effects of *A. rugosum* extracts on RAW264.7 cell viability were determined using the MTT assay as described by Weyermann, Lochmann, & Zimmer (2005). RAW264.7 cells (4000 cells/well) were seeded in 96-well plates and incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 24 hours. The attached cells were treated with *A. rugosum* extracts (EE: ethanol extract; HF: hexane fraction; EAF: ethyl acetate fraction; AF: aqueous fraction; WB: wild basidiocarp ethanolic extract; or DB: domesticated basidiocarp ethanolic extract) at different concentrations (0.01, 0.1, 1, 10, or 100 µg/mL). After 24 hours incubation, 5 mg/mL of MTT reagent (Appendix A (C)) was added to each well. The supernatant from each of the 96 wells (containing cells subjected to the various treatments) were removed after 4 hours incubation and the formazan salt formed was solubilised with 100% DMSO. The viable cells reduced the pale yellow substrate to a purple formazan product. The absorbance was measured at 560 nm and the percentage of viable cells was determined relative to the control group (untreated cells).

5.2.4 Nitric oxide scavenging assay

(A) Principle

In this assay, sodium nitroprusside is used to generate nitric oxide. Nitric oxide reacts with oxygen and forms nitrite. The nitrite ions diazotize with sulphanilamide acid and couple with naphthyl ethylenediamine to form a pink solution which can be measured at 540 nm (Boora, Chirisa, & Mukanganyama, 2014).

(B) Method

The nitric oxide radical scavenging assay was performed according to the method reported by Lee et al. (2012) with slight modifications. Briefly, 90 µL of sodium

nitroprusside (5 mM dissolved in PBS) solution was added to 10 µL of *A. rugosum* extract (EE: ethanol extract; HF: hexane fraction; EAF: ethyl acetate fraction; AF: aqueous fraction; WB: wild basidiocarp ethanolic extract; or DB: domesticated basidiocarp ethanolic extract). The plate was incubated for 90 minutes under exposure to light. Next, 100 µL of Griess reagent (1% sulphanilamide and 0.1% N-(1-naphthyl)ethylenediamine in 2.5% H_3PO_4) was added to the wells containing the mixture and the nitrite levels which correspond to the resulting pink solution was measured at 540 nm. Quercetin was used as a positive control. Each assay was carried out in triplicate and the results were expressed as percentage of NO production:

$$\text{Nitric oxide production (\%)} = \frac{A_{\text{sample}}}{A_{\text{control}}} \text{ x } 100$$

where $A_{control}$ = absorbance in no presence of *A. rugosum* extracts or standards and A_{sample} = absorbance in the presence of *A. rugosum* extracts or standards.

5.2.5 Nitric oxide assay

The nitric oxide (NO) was determined according to the method reported by Lee et al. (2012). RAW264.7 cells (4 X 10^5 cells/well) were seeded into 96-well plates and incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 24 hours. The attached cells were co-incubated with *A. rugosum* extracts (0.01, 0.1, 1, 10, or 100 µg/mL) and 1 µg/mL *Escherichia coli* (O55:B5) LPS for another 24 hours. The production of NO was determined by measuring the nitrite levels in the culture supernatant using Griess reagent (1% sulphanilamide and 0.1% N-(1-naphthyl)ethylenediamine in 2.5% H_3PO_4) at 540 nm as described in section 5.2.4 (B). The cell viability was determined using MTT assay. N-nitro-l-arginine-methyl ester (L-NAME) at a concentration of 250 µM was used as an iNOS inhibitor (positive control).

A standard curve generated with sodium nitrite (0-100 µM) was used to calculate the levels of nitrite produced.

5.2.6 Immunofluorescence staining of NF-κB and iNOS

RAW264.7 cells (5 X 10^4 cells/well) were seeded into a chamber slide and incubated overnight at 37°C in a humidified atmosphere containing 5% CO_2. The attached cells were co-incubated with *A. rugosum* extract (HF100: 100 µg/mL of hexane fraction; WB0.1: 0.1 µg/mL of wild basidiocarp ethanolic extract; DB: 0.1 µg/mL of domesticated basidiocarp ethanolic extract) and 1 µg/mL of *Escherichia coli* (O55:B5) LPS for 24 hours. After the cells were washed with phosphate-buffered saline (PBS), the cells were fixed immediately with 4% of paraformaldehyde for 20 minutes. Then, the cells were permeabilised with 0.5% of PBS-Triton X-100 (PBST) for 10 minutes. The slides were then incubated with primary antibody against NF-κB p65 or iNOS (1: 100 dilution; Appendix A (D)) for 2 hours at room temperature. Next, the slides were washed with 0.3% of PBST and incubated with fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG secondary antibodies at 1:80 dilutions (Appendix A (D)) in the dark at room temperature for another 2 hours. The slides were then washed again with 0.3% of PBST. The cells were mounted with Prolong® Gold Antifade Reagent with 4', 6-diamidino-2-phenylindole (DAPI). The slides were observed under fluorescence microscope with FITC and DAPI filters and images were captured using Nikon's Imaging Software, NIS-Elements.

5.2.7 Measurement of TNF-α and IL-10

The levels of TNF-α and IL-10 were measured using ELISA kits according to the manufacturer's instructions (Mouse TNF-α and IL-10 ELISA Kit Instructions, Thermo Scientific). Briefly, RAW264.7 cells (5 X 10^6 cells) were seeded into a 25 cm^3 tissue

culture flask and incubated overnight at 37°C in a humidified atmosphere containing 5% CO_2. The attached cells were co-incubated with *A. rugosum* extracts (HF100: 100 µg/mL of hexane fraction; WB0.1: 0.1 µg/mL of wild basidiocarp ethanolic extract; DB: 0.1 µg/mL of domesticated basidiocarp ethanolic extract) and 1 µg/mL LPS for 24 hours. Then, the supernatant was collected to determine the TNF-α and IL-10 levels by ELISA.

5.2.8 Gene expression study

RAW264.7 cells (5 X 10^6 cells) were seeded into a 25 cm^3 tissue culture flask and incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 24 hours. The attached cells were co-incubated with *A. rugosum* extracts (HF100: 100 µg/mL of hexane fraction; WB0.1: 0.1 µg/mL of wild basidiocarp ethanolic extract; DB: 0.1 µg/mL of domesticated basidiocarp ethanolic extract) and 1 µg/mL LPS at 37°C in a humidified atmosphere containing 5% CO_2 for another 24 hours. The RNA extraction was carried out using RNAqueous®-4PCR kit. The purity of the isolated RNA was determined based on the ratio of the absorbance at 260 nm and 280 nm (Appendix B (A)). Purified RNA with a A_{260}/A_{280} ratio between 1.8 and 2.0 was further used to synthesise complementary DNA (cDNA) by polymerase chain reaction (PCR) approach. The integrity of the RNA samples was assessed using an Agilent® 2100 Bioanalyzer. RNA samples with RNA integrity number (RIN) of 8 to 10 were used for the subsequent experiment (Appendix C (A)). High Capacity cDNA Reverse Transcription Kit which contains all reagents needed for reverse transcription (RT) of total RNA to single-stranded cDNA was used in this study. Generally, 10 µL High Capacity cDNA Reverse Transcription master mix (RT buffer, dNTP mix, randoms primers, Multiscribe reverse™ transcriptase enzyme and nuclease free water) was added to 10 µL RNA samples. The mixture was then loaded into a thermal cycler (Biorad,

USA) and PCR was carried out according to optimized thermal cycling conditions as recommended by the manufacturer. Table 5.2 shows the list of genes investigated in this study and the corresponding accession numbers. Mouse β-actin (ACTB) was used as the endogenous control in this study. All TaqMan® probes (Applied Biosystems, USA) used in this investigation were labelled with FAM™ reporter dye at the 5' end and MGB quencher at the 3' end. The fold change in the target genes normalised to ACTB and relative to LPS was calculated using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001).

Table 5.2: Genes investigated using quantitative reverse transcription PCR (qRT-PCR).

No.	Gene name and abbreviation	Assay ID	Accession number
1	Nuclear factor kappa B (NF-κB) p65	Mm00501346_m1	NM_009045.4
2	Inducible nitric oxide synthase (iNOS)	Mm 00440502_m1	NM_010927.3
3	Tumour necrosis factor alpha (TNF-α)	Mm 00443258_m1	NM_013693.2
4	Interleukin 10 (IL-10)	Mm 00439614_m1	NM_010548.2

General abbreviation of genes selected for this study and corresponding assay ID and accession number were obtained from the Applied Biosystems website and NCBI database. Assay ID refers to the Applied Biosystems Gene Expression Assays inventoried kits with proprietary primer and TaqMan® probe mix. Assay ID with "Mm" is referred to as *"Mus musculus"*. All Gene Expression Assay kits indicated are FAM/MGB probed.

5.2.9 Statistical analysis

All values are expressed as means ± standard deviation (SD) of triplicate values. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Duncan's Multiple Comparison Test using Statistical Product and Service Solutions, SPSS® Statistics for Windows, Version 17.0 and $p < 0.05$ was denoted as being statistically significant.

5.3 Results

5.3.1 Cell viability (MTT) assay

5.3.1.1 Optimization of cell number and incubation period of MTT assay for RAW264.7 cells

MTT colorimetric assay is a convenient and reliable method used to measure cell viability. However, the accuracy depends on the establishment of assay conditions that optimize accuracy and eliminate confounding factors that affect the MTT-specific activity. Hence, it is important to optimize the cell number and incubation period for MTT assay because the MTT reduction varies greatly between different cell types (Sylvester, 2011). Figure 5.2 shows the optimization of cell number and incubation period for MTT assay. Cell number ranging from 1 X 10^3 cells/well to 8 X 10^3 cells/well and incubation period from 12 to 60 hours were tested. The optimum cell number and incubation hour for MTT assay was determined based on the resulting optical density. Figure 5.2 shows that the optimum incubation period was 24 hours where the absorbance value ranged from 0.209 ± 0.015 A to 1.380 ± 0.014 A.

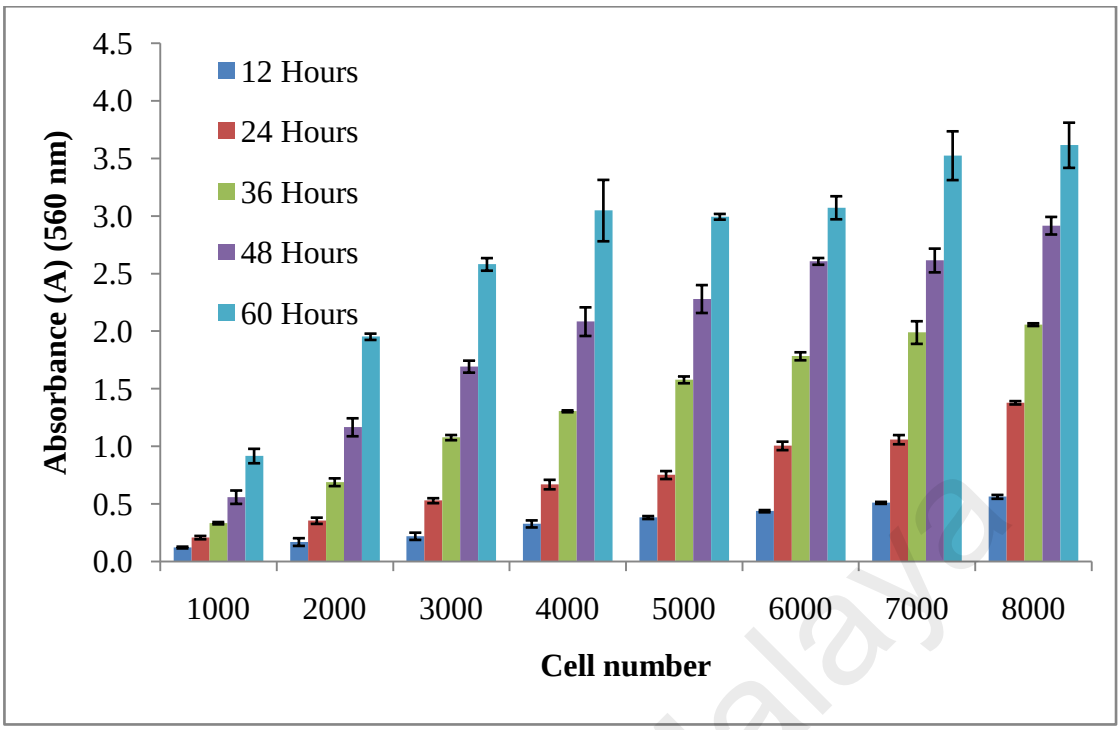


Figure 5.2: Cell number and time-course relationship with MTT assay. Various concentrations (1 X 10^3 cells/well to 8 X 10^3 cells/well) of cells were plated in triplicate into 96-well culture plate and allowed for attachment at 12, 24, 36, 48, and 60 hours, respectively. MTT was added into each well and incubated for 4 hours. Then, media was removed and 100% DMSO was added into each well to dissolve the formazan crystals. The absorbance was measured at 560nm. Values were expressed as mean ± standard deviation (S.D.; n = 3).

Figure 5.3 illustrates the linear plot relationship between the optical density and the number of cells per well for 24 hours incubation period. A continuous increase in absorbance value for the cell numbers ranging from 1 X 10^3 cells/well to 8 X 10^3 cells/well was observed. The optimum cell number was 4000 cells/well, where the absorbance value was 0.670 ± 0.041 A.

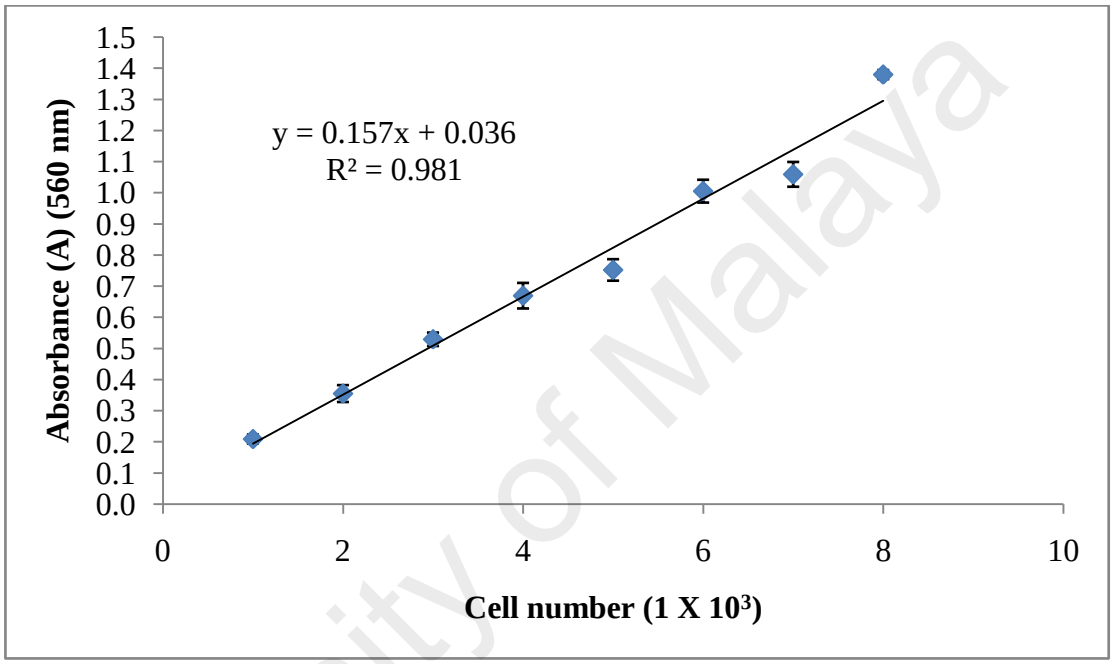


Figure 5.3: Linear plot relationship between the optical density and the number of cells per well for 24 hours incubation period. Various concentrations of cells (1 X 10^3 cells/well to 8 X 10^3 cells/well) were plated in triplicate into 96-well culture plate. The cells were allowed to attach for 24 hours. MTT was added into each well and incubated for 4 hours. Then, media was removed and 100% DMSO was added into each well to dissolve the formazan crystals. The absorbance was measured at 560nm. Values are expressed as mean ± S.D. (n = 3).

5.3.1.2 Effects of *A. rugosum* extracts on RAW264.7 cell viability

The MTT assay was used to determine the effect of *A. rugosum* extracts on RAW264.7 cell viability. The cell viability of the positive control (cells without any treatment) was denoted as 100%. All the extracts tested, except for AF, had no cytotoxic effects on RAW264.7 cells (Figure 5.4). AF treatment at 1 µg/mL caused a significant ($p < 0.05$) decrease in the number of viable cells. EE and HF promoted proliferation of cells at concentrations greater than 1 µg/mL.

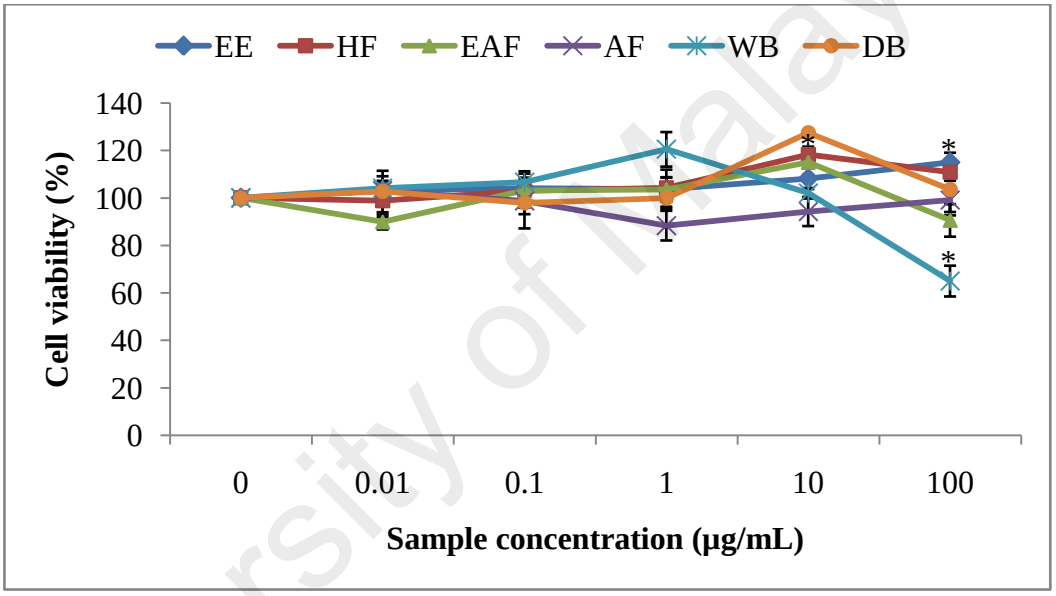


Figure 5.4: The effects of *A. rugosum* extracts on RAW264.7 cell viability. RAW264.7 cells were treated with *A. rugosum* extracts and cells without any treatment were expressed as 100%. The data presented represent mean ± S.D., n = 3 and *$p < 0.05$ compared to control 100%. (EE: ethanol extract; HF: hexane fraction; EAF: ethyl acetate fraction; AF: aqueous fraction; WB: wild basidiocarp ethanolic extract; DB: domesticated basidiocarp ethanolic extract)

5.3.2 Nitric oxide scavenging activity of *A. rugosum*

The *A. rugosum* extracts were tested for NO scavenging activity. In this assay, quercetin was used as a positive control. Quercetin is a flavonoid widely found in plants that is well known for its antioxidant and radical scavenging properties. Various concentrations of quercetin were prepared in two-fold dilution to determine the optimum concentration to be used as positive control. Quercetin concentration of 6.25 µg/mL which showed approximately 40-50% nitric oxide radical scavenging activity was selected as the concentration to be used as positive control (Figure 5.5).

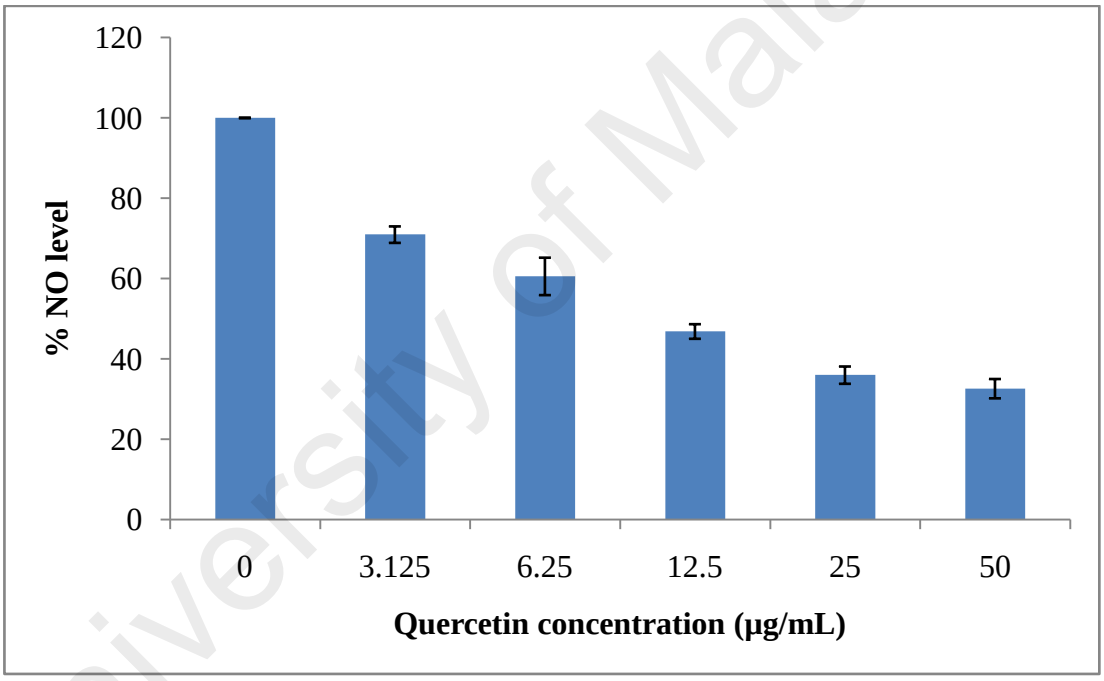


Figure 5.5: Selection of quercetin concentration to be used as positive control. Various concentrations of quercetin were prepared (two-fold dilution). Each assay was carried out in triplicate and the results were expressed as percentage of NO level.

The ability of *A. rugosum* extracts to scavenge NO radical was evaluated. Figure 5.6 shows that the *A. rugosum* extracts were able to scavenge the NO radical in a dose-dependent manner. At a concentration of 500 µg/mL, EAF, WB and DB were able to scavenge 46.7%, 50% and 54.6% of NO radical, respectively, which was comparable to Quercetin that scavenged 39.4% of NO radical at a similar concentration.

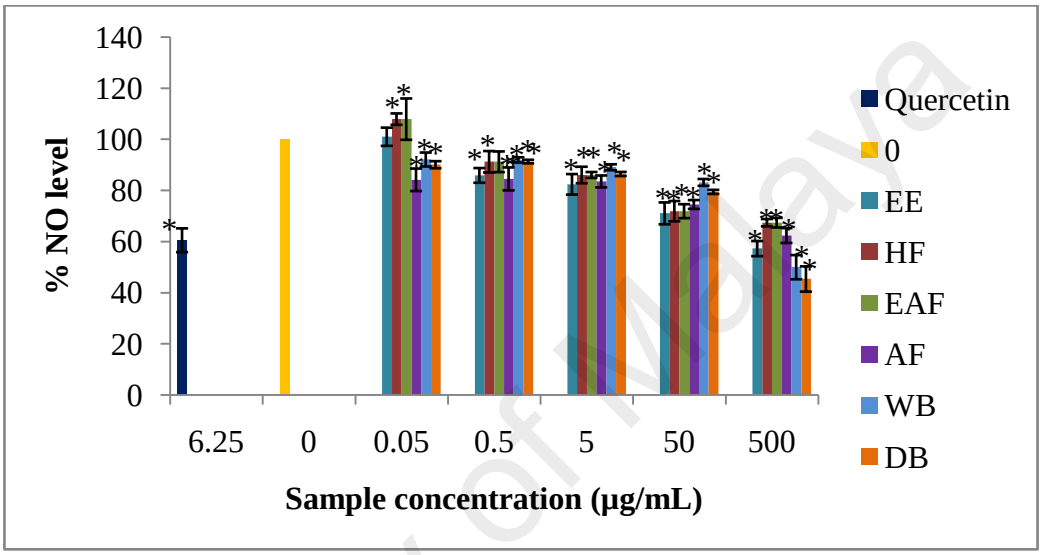


Figure 5.6: Effects of *A. rugosum* extracts on NO level by sodium nitropruside (SNP). *A. rugosum* extracts were co-incubated with SNP (5mM dissolved in PBS) solution for 90 minutes. Quercetin (6.25 mg/mL) was served as positive control. Results shown represent the mean ± S.D., n = 3 and * $p < 0.05$ versus SNP-produced NO alone. (EE: ethanol extract; HF: hexane fraction; EAF: ethyl acetate fraction; AF: aqueous fraction; WB: wild basidiocarp ethanolic extract; DB: domesticated basidiocarp ethanolic extract)

5.3.3 Nitric oxide (NO) assay

5.3.3.1 Optimization of cell number for NO assay

Nitric oxide (NO) was determined by measuring the amount of nitrite produced in cell culture supernatant. The nitrite concentrations in the supernatant were determined by comparison with sodium nitrite standard curve (Figure 5.7). Figure 5.8 illustrates the optimization of cell number for NO assay. Cell numbers ranging from 1 X 10^5 cells/well to 7 X 10^5 cells/well were tested. Figure 5.8 shows that the NO production elevated as the cell number increased from 1 X 10^5 cells per well to 4 X 10^5 cells/well. However, the NO production decreased as the cell number further increased from 5 X 10^5 cells/well to 7 X 10^5 cells/well. It was found that 4 X 10^5 cells/well exhibited the highest amount of NO (26.15 ± 0.002 µM). The selection of optimum cell number for NO assay was based on the highest amount of NO production. Hence, 4 X 10^5 cells/well was selected for NO inhibition assay.

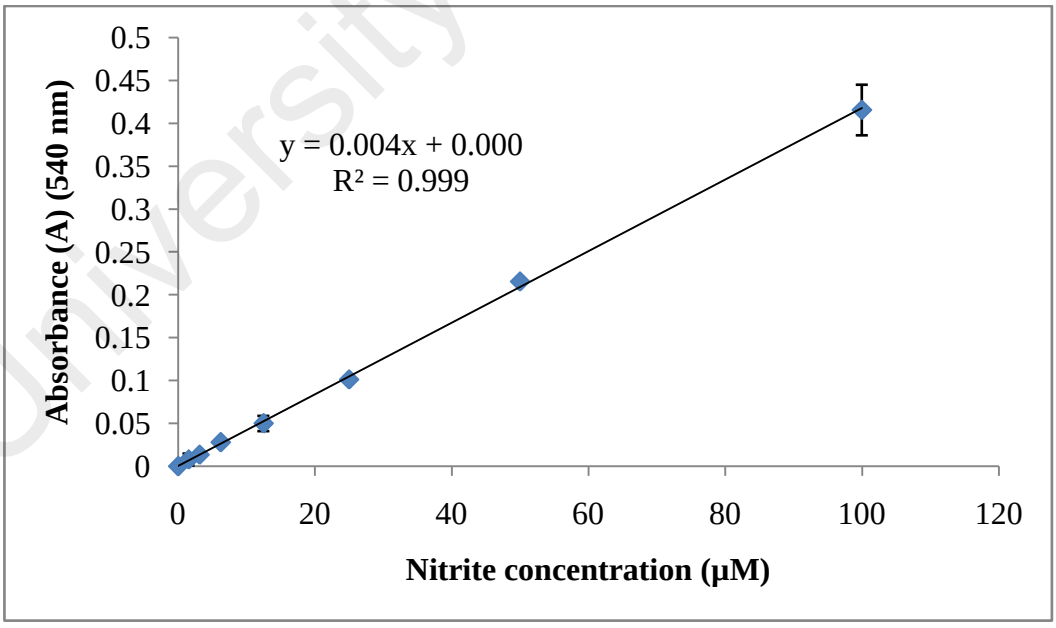


Figure 5.7: Sodium nitrite standard curve. All values were expressed as mean ± S.D. (n = 3).

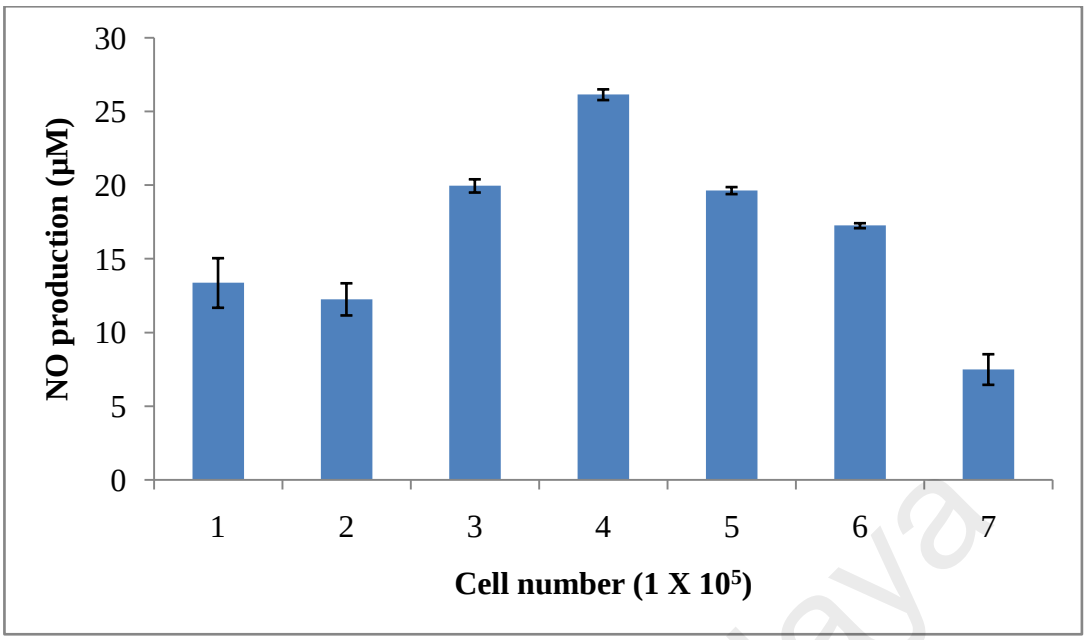


Figure 5.8: Optimization of cell number for NO assay. RAW264.7 cells ranging from 1 X 10^5 cells/well to 7 X 10^5 cells/well were stimulated with 1 µg/mL of LPS for 24 hours. Fifty microliters of cell culture supernatant was mixed with 50 µL of Griess reagent in 96-well plate. Absorbance was measured at 540nm.

5.3.3.2 Effects of *A. rugosum* extract on LPS-stimulated NO production and expression in RAW264.7 cells

Murine macrophage RAW264.7 cells were challenged with LPS to produce NO and the effect of *A. rugosum* extracts on NO inhibition was assessed. All *A. rugosum* extracts, except for AF, inhibited NO production in a dose-dependent manner (Figure 5.9). The unstimulated cells secreted NO at the basal level of 1.39 ± 0.17 µM, while the non-treated LPS-stimulated cells showed an increase in NO production (35.83 ± 2.18 µM; 0% inhibition). L-NAME, a standard NOS inhibitor was used as the positive control and it significantly ($p < 0.05$) inhibited NO (5.91 ± 2.26 µM; 61.1%) at 250 µM. Among the *A. rugosum* extracts tested, HF significantly ($p < 0.05$) inhibited NO production at all concentrations tested and complete inhibition was observed at 100 µg/mL (Figure 5.9) without affecting the cell viability of HF treated cells (cell viability: 104.8%; Figure 5.10). Besides, the inducible nitric oxide synthase (iNOS) gene in RAW264.7 cells was up-regulated upon stimulation with 1 µg/mL of LPS and was significantly down-regulated with the treatment of HF at a concentration of 100 µg/mL and WB and DB at a concentration of 0.1 µg/mL (Figure 5.11, 5.12, and 5.13).

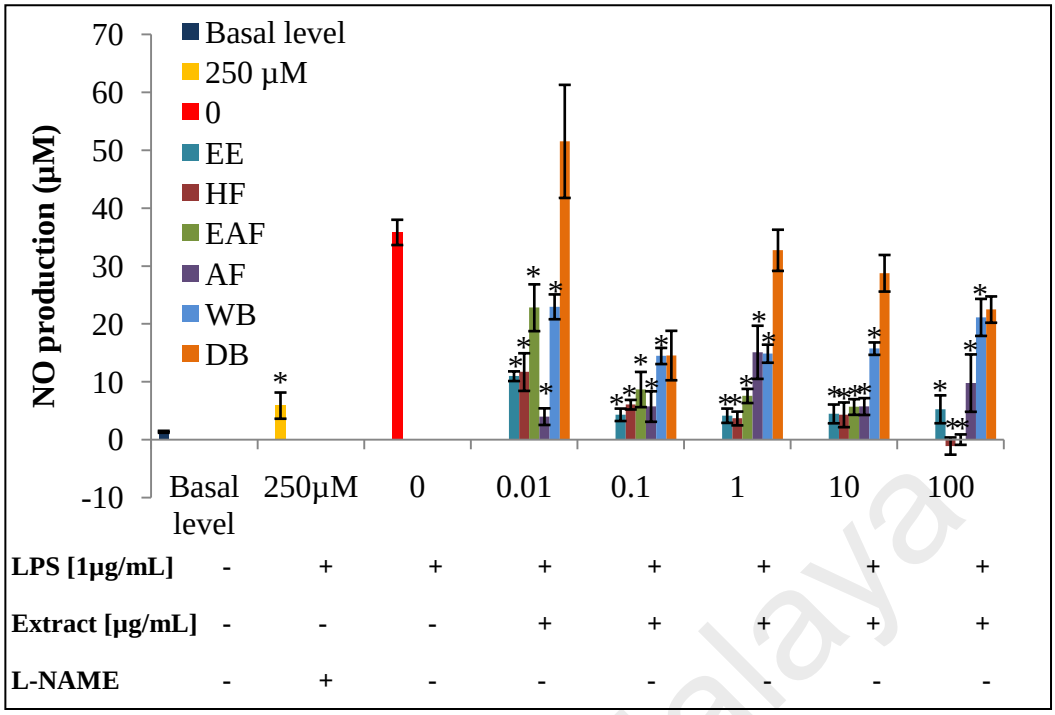


Figure 5.9: Effects of *A. rugosum* extracts on LPS-induced NO production by RAW264.7 cells. RAW264.7 cells were co-incubated with various concentrations of *A. rugosum* extracts and 1 µg/mL LPS for 24 hours. L-NAME (250 µM) served as positive control. Results shown represent the mean ± S.D., n = 3 and $^{*}p < 0.05$ versus LPS-induced NO level alone. (EE: ethanol extract; HF: hexane fraction; EAF: ethyl acetate fraction; AF: aqueous fraction; WB: wild basidiocarp ethanolic extract; DB: domesticated basidiocarp ethanolic extract)

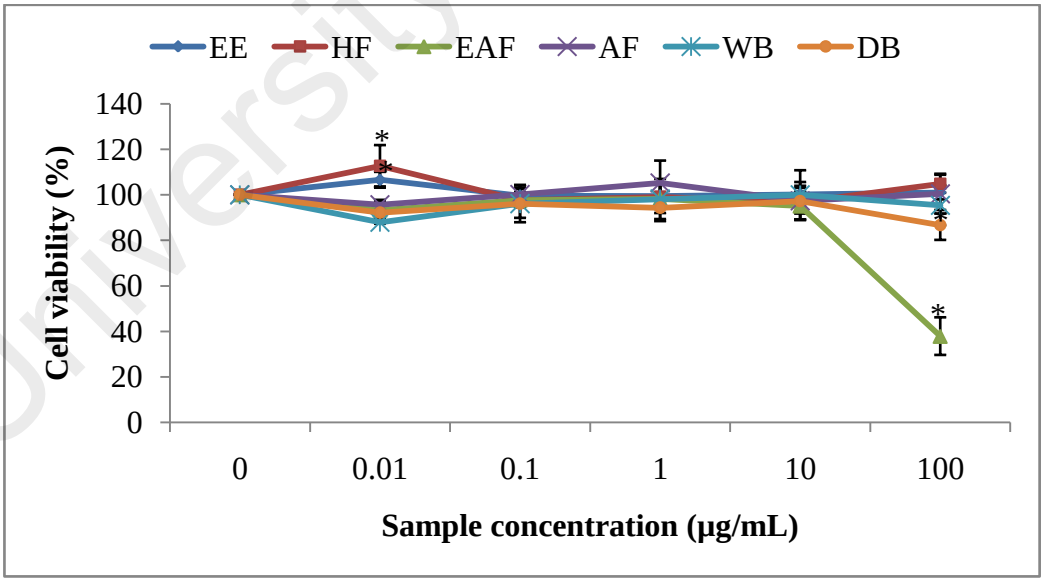


Figure 5.10: Effects of *A. rugosum* extracts on LPS-stimulated RAW264.7 cell viability. Cell viability of LPS-stimulated murine macrophage RAW264.7 cells were assessed using MTT method. Data shown represent the mean ± S.D., n = 3 and $^{*}p < 0.05$ compared to control 100%. (EE: ethanol extract; HF: hexane fraction; EAF: ethyl acetate fraction; AF: aqueous fraction; WB: wild basidiocarp ethanolic extract; DB: domesticated basidiocarp ethanolic extract)

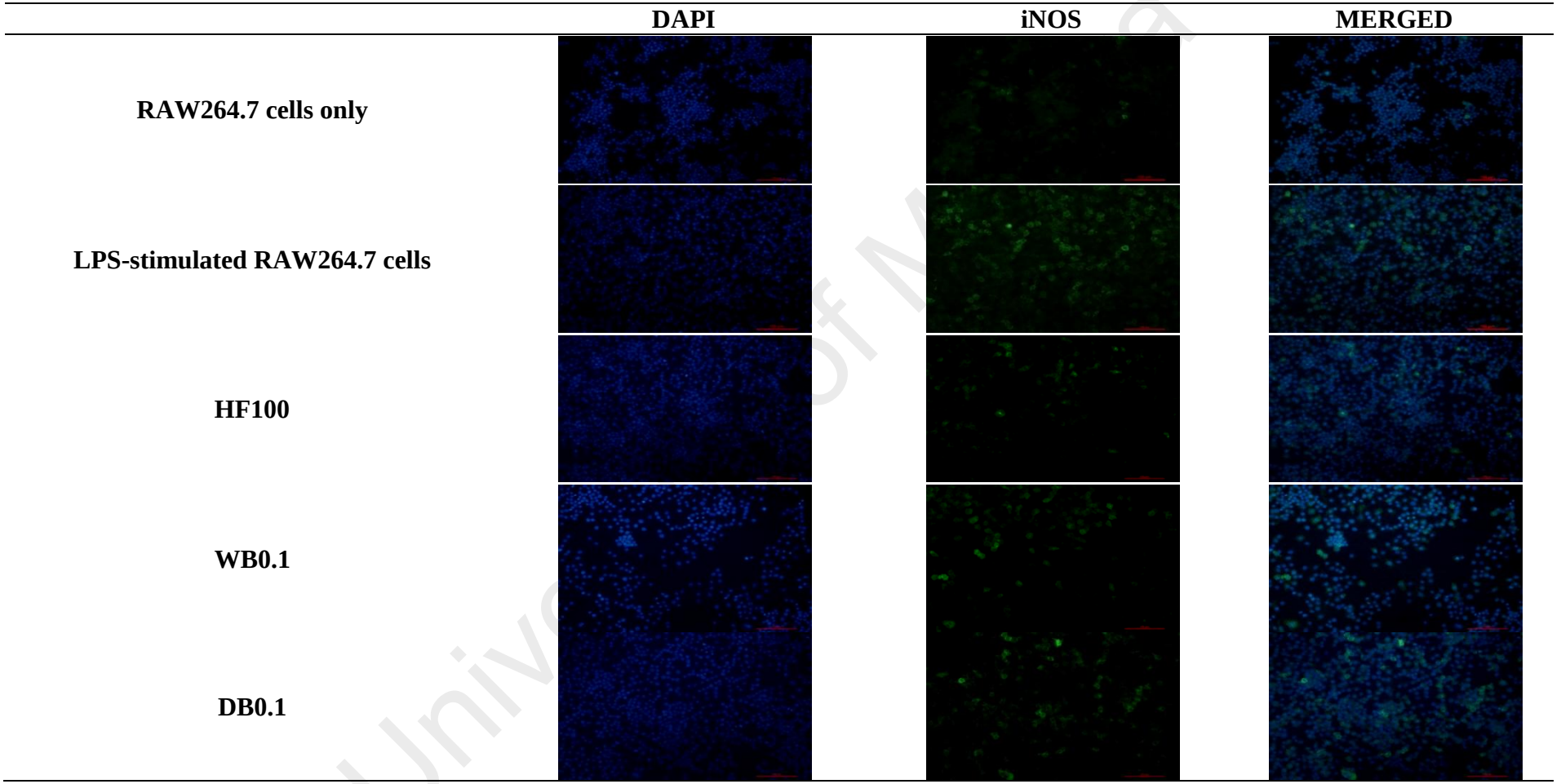


Figure 5.11: Effects of HF, WB, and DB on iNOS activity in LPS-stimulated RAW264.7 cells. iNOS was localised by fluorescence microscopy after immunofluorescence staining with iNOS antibody (green). Cells were stained with DAPI for visualization of nuclei (blue). (HF100: 100 μg/mL of hexane fraction; WB0.1: 0.1 μg/mL of wild basidiocarp ethanolic extract; DB: 0.1 μg/mL of domesticated basidiocarp ethanolic extract; iNOS: inducible nitric oxide synthase)

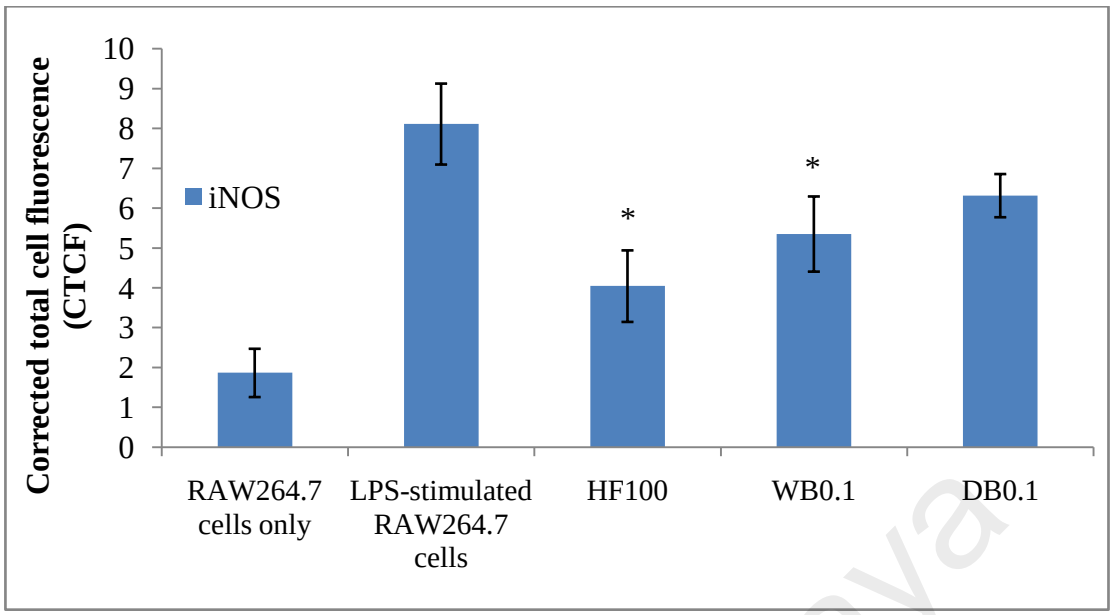


Figure 5.12: Quantification of fluorescence intensity. The fluorescence intensity was quantified using ImageJ and displayed in corrected total cell fluorescence (CTCF) Results shown represent the mean ± S.D., n = 3 and *p < 0.05 versus LPS-induced iNOS expression alone. (HF100: 100 µg/mL of hexane fraction; WB0.1: 0.1 µg/mL of wild basidiocarp extract; DB: 0.1 µg/mL of domesticated basidiocarp extract)

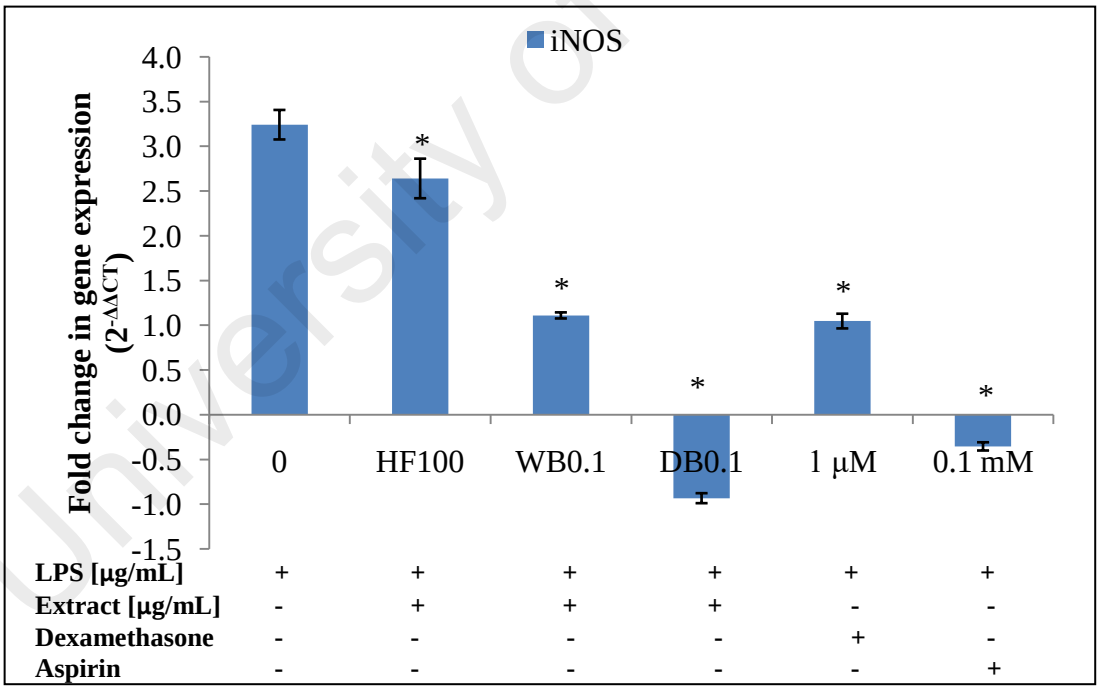


Figure 5.13: *In vitro* effect of *A. rugosum* extracts on LPS-stimulated iNOS expression in RAW264.7 cells. RAW264.7 cells were co-incubated with *A. rugosum* extracts (HF100: 100 µg/mL of hexane fraction; WB0.1: 0.1 µg/mL of wild basidiocarp ethanolic extract; DB0.1: 0.1 µg/mL of domesticated basidiocarp ethanolic extract) and 1 µg/mL LPS for 24 hours. Dexamethasone (1 µM) and aspirin (0.1 mM) served as positive controls. β-actin was used as loading control and normalised against the treated group. Results shown represent the mean ± S.D., n = 3 and $^{*}p < 0.05$ versus LPS-induced NO level alone.

5.3.4 Effects of *A. rugosum* on LPS-stimulated TNF-α and IL-10 levels and expressions in RAW264.7 cells

The HF, WB, and DB attenuated the level of TNF-α in LPS-stimulated RAW264.7 cells (Figure 5.14) which was due to the down-regulation of the TNF-α gene expression (Figure 5.15). Besides, HF, WB, and DB promoted the production of IL-10, an anti-inflammatory cytokine (Figure 5.16). Significant up-regulation of IL-10 expression by HF, WB, and DB was evident (Figure 5.17).

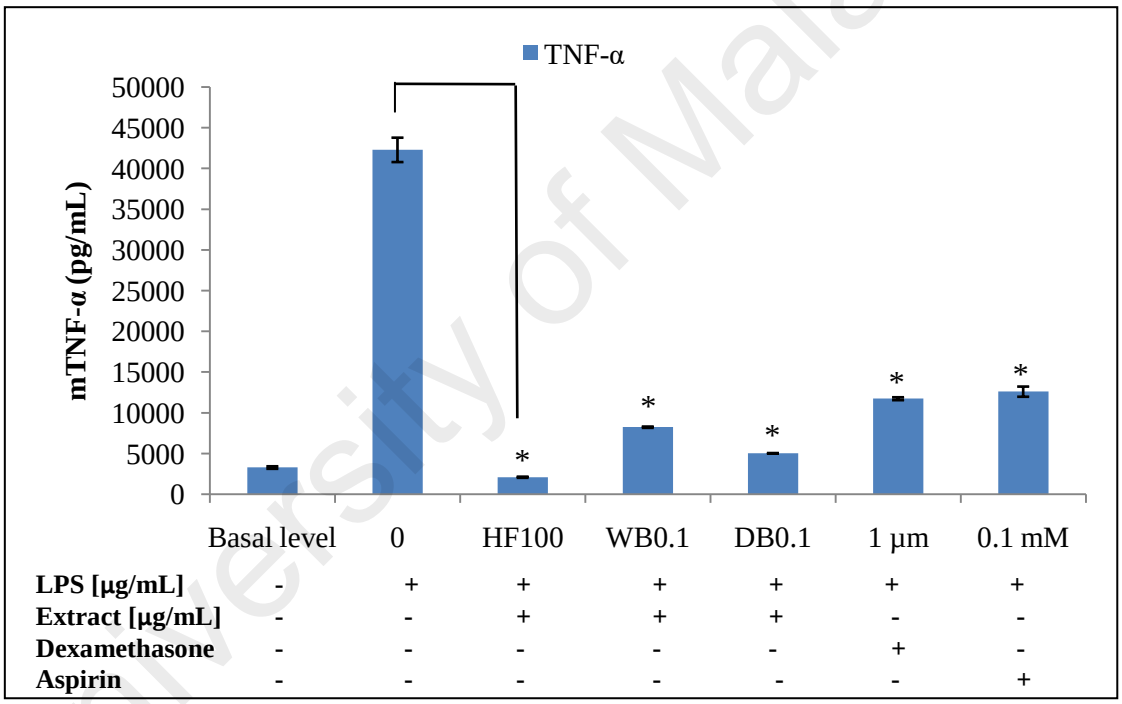


Figure 5.14: Effects of *A. rugosum* extracts on TNF-α cytokine level. RAW264.7 cells were co-incubated with *A. rugosum* extracts (HF100: 100 µg/mL of hexane fraction; WB0.1: 0.1 µg/mL of wild basidiocarp ethanolic extract; DB0.1: 0.1 µg/mL of domesticated basidiocarp ethanolic extract) and 1 µg/mL LPS for 24 hours. Dexamethasone (1 µM) and aspirin (0.1 mM) served as positive controls. Results shown represent the mean ± S.D., n = 3 and *p < 0.05 versus LPS induced NO level alone.

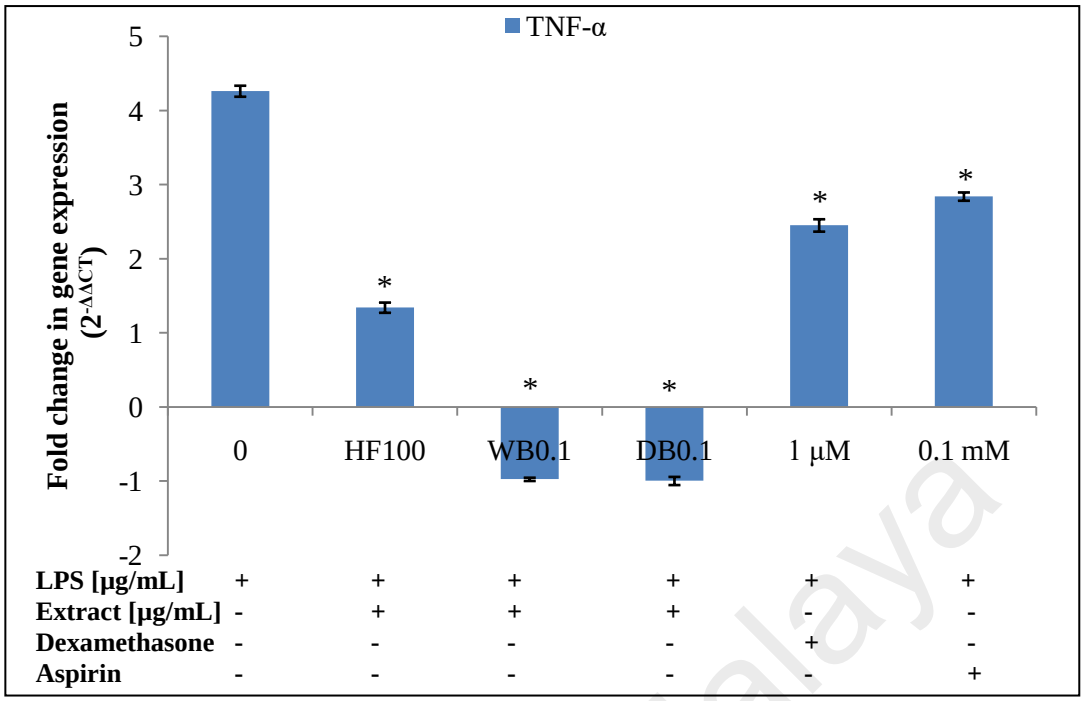


Figure 5.15: Effects of *A. rugosum* extracts on TNF-α gene expression. RAW264.7 cells were co-incubated with *A. rugosum* extracts (HF100: 100 µg/mL of hexane fraction; WB0.1: 0.1 µg/mL of wild basidiocarp ethanolic extract; DB0.1: 0.1 µg/mL of domesticated basidiocarp ethanolic extract) and 1 µg/mL LPS for 24 hours. Dexamethasone (1 µM) and aspirin (0.1 mM) served as positive controls. β-actin was used as loading control and normalised against the treated group. Results shown represent the mean ± S.D., n = 3 and *p < 0.05 versus LPS-induced NO level alone.

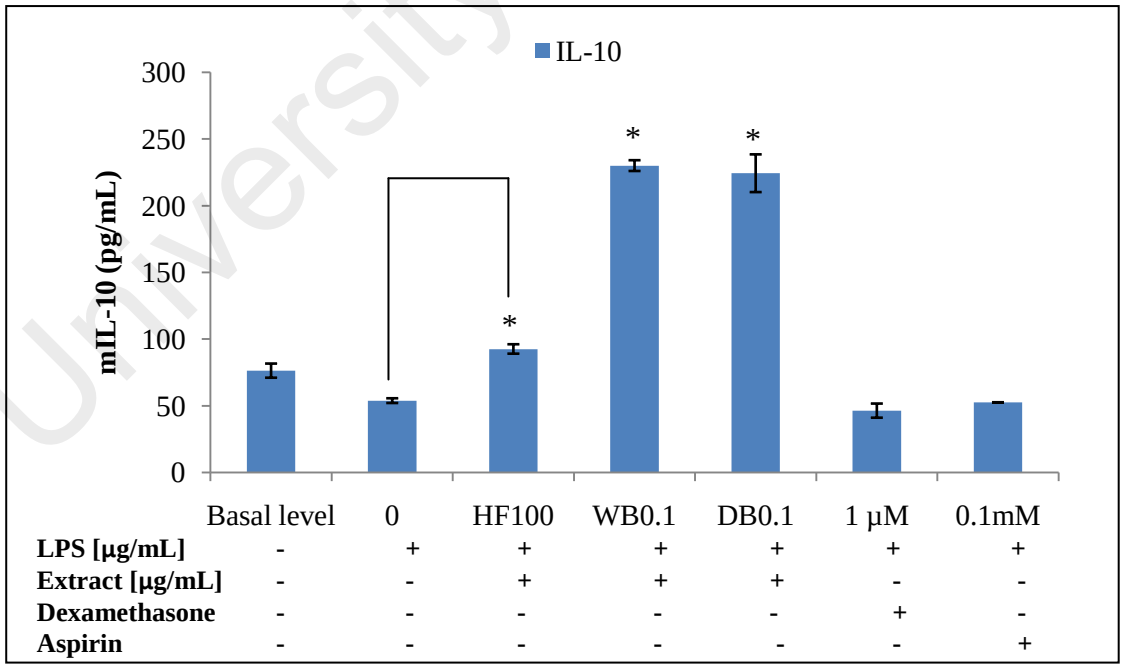


Figure 5.16: Effects of *A. rugosum* extracts on IL-10 cytokine level. RAW264.7 cells were co-incubated with *A. rugosum* extracts (HF100: 100 µg/mL of hexane fraction; WB0.1: 0.1 µg/mL of wild basidiocarp ethanolic extract; DB0.1: 0.1 µg/mL of domesticated basidiocarp ethanolic extract) and 1 µg/mL LPS for 24 hours. Dexamethasone (1 µM) and aspirin (0.1 mM) served as positive controls. Results shown represent the mean ± S.D., n = 3 and *p < 0.05 versus LPS-induced NO level alone.

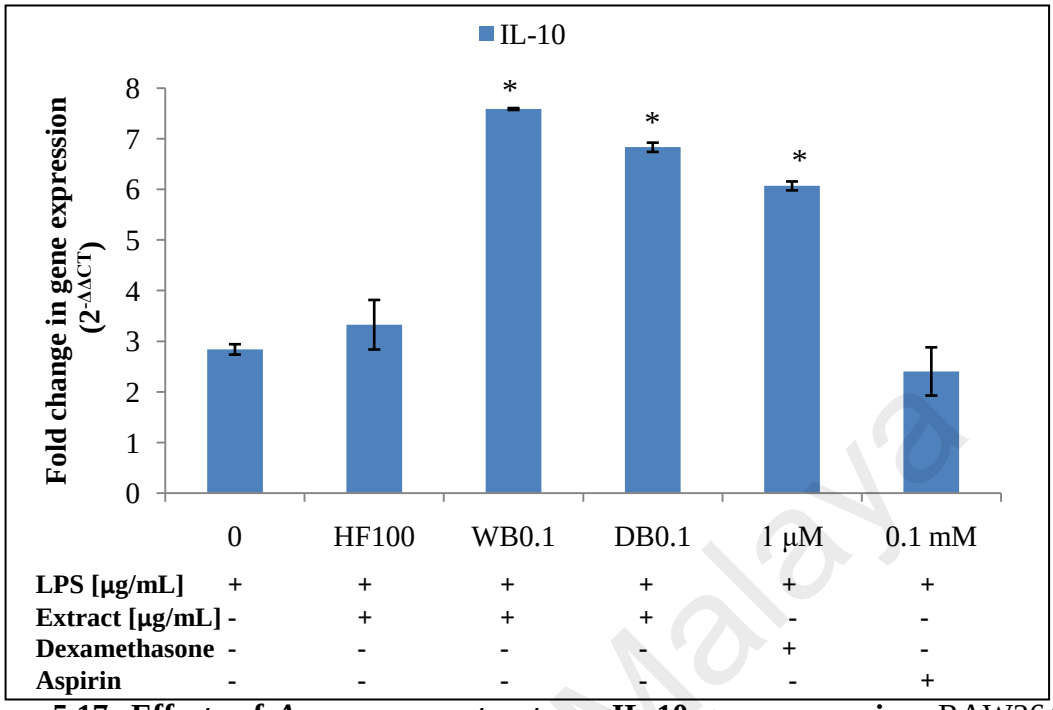


Figure 5.17: Effects of *A. rugosum* extracts on IL-10 gene expression. RAW264.7 cells were co-incubated with *A. rugosum* extracts (HF100: 100 µg/mL of hexane fraction; WB0.1: 0.1 µg/mL of wild basidiocarp ethanolic extract; DB0.1: 0.1 µg/mL of domesticated basidiocarp ethanolic extract) and 1 µg/mL LPS for 24 hours. Dexamethasone (1 µM) and aspirin (0.1 mM) served as positive controls. β-actin was used as loading control and normalised against the treated group. Results shown represent the mean ± S.D., n = 3 and *p < 0.05 versus LPS-induced NO level alone.

5.3.5 Effects of *A. rugosum* on LPS-stimulated NF-κB p65 expression in RAW264.7 cells

LPS-stimulated proinflammatory mediator production in RAW264.7 cells have been reported to be induced through NF-κB activation (Sharif, Bolshakov, Raines, Newham, & Perkins, 2007). Figure 5.18 and Figure 5.19 show the effect of HF, WB, and DB on the NF-κB p65 protein expression level in LPS-stimulated RAW264.7 cells. There was no significant effect of HF, WB, and DB on the NF-κB p65 subunit. Protein expression showed presence of NF-κB p65 activity in LPS-stimulated RAW264.7 cells (Figure 5.18 and 5.19). Dexamethasone (1 µM) and aspirin (0.1 mM) were used as positive controls. Figure 5.20 also shows similar results, where HF, WB, and DB had no significant effect on NF-κB p65 subunit gene expression.

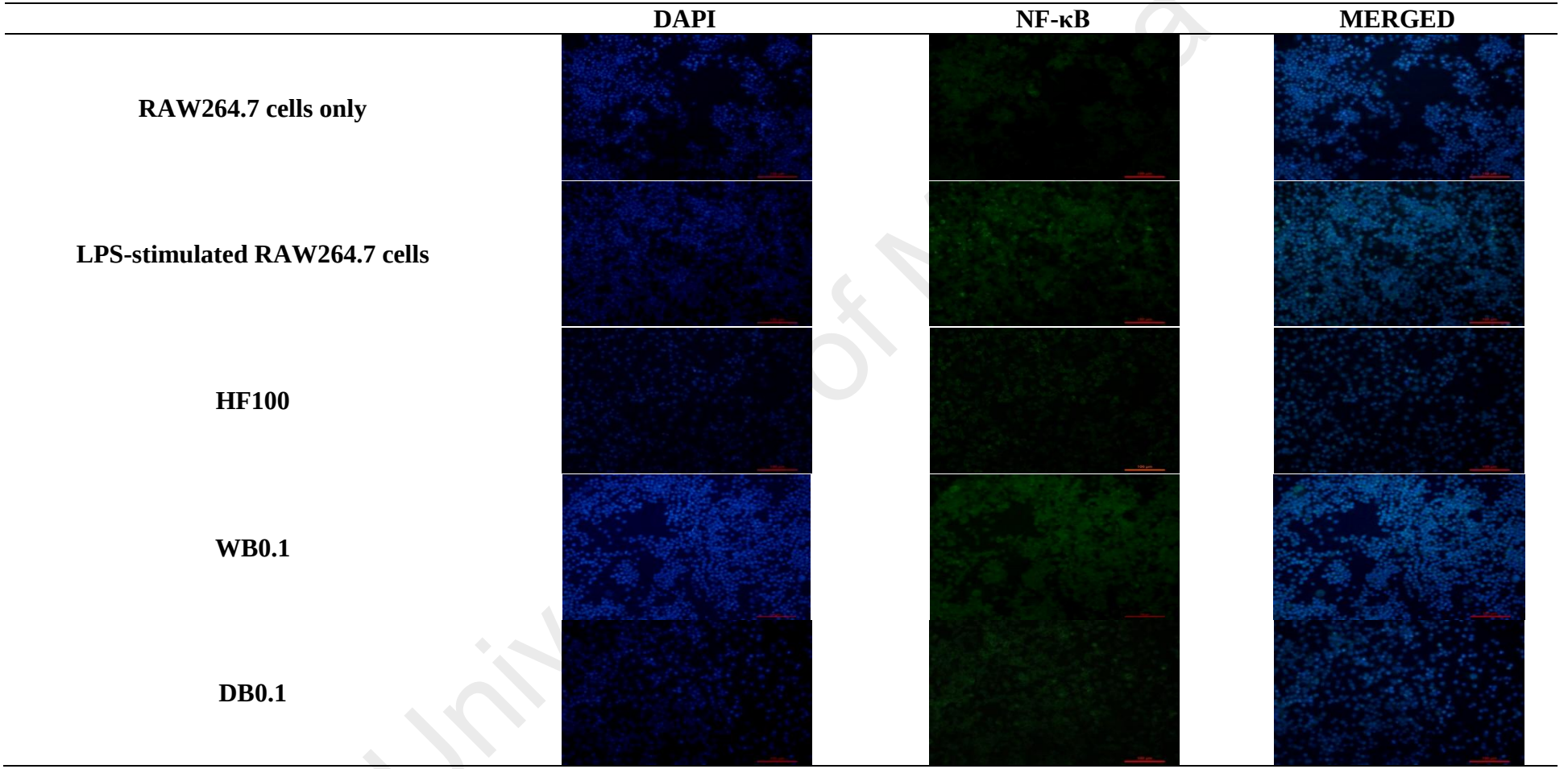


Figure 5.18: Effects of *A. rugosum* extracts on NF-κB activity in LPS-stimulated RAW264.7 cells. NF-κB p65 was localised by fluorescence microscopy after immunofluorescence staining with NF-κB p65 antibody (green). Cells were stained with DAPI for visualization of nuclei (blue). (HF100: 100 µg/mL of hexane fraction; WB0.1: 0.1 µg/mL of wild basidiocarp ethanolic extract; DB0.1: 0.1 µg/mL of domesticated basidiocarp ethanolic extract)

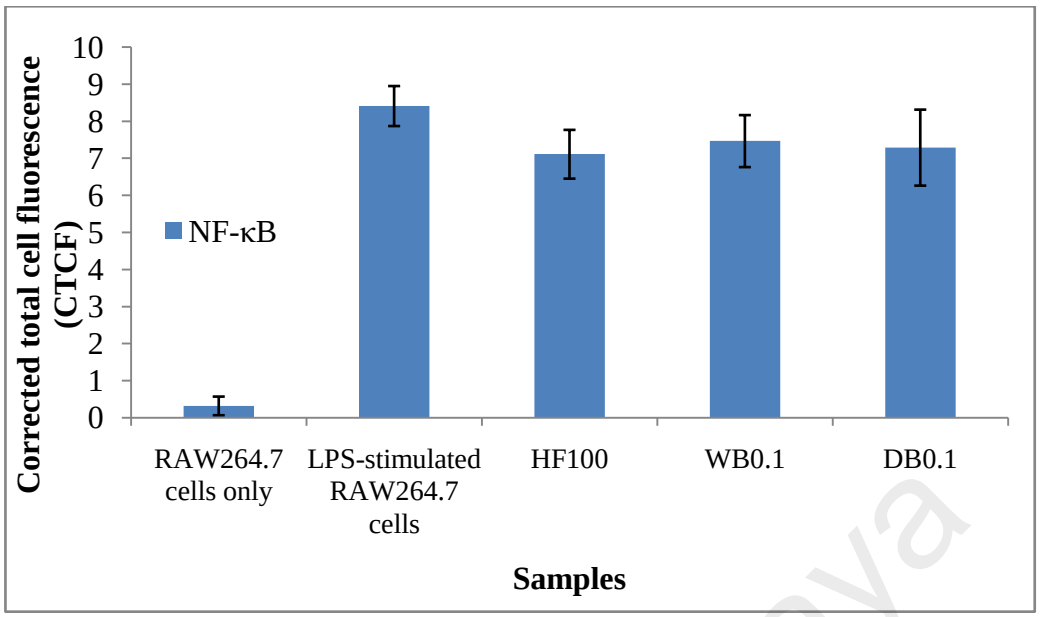


Figure 5.19: Quantification of fluorescence intensity. The fluorescence intensity was quantified using ImageJ and displayed in CTCF. Results shown represent the mean ± S.D., n = 3 and *p < 0.05 versus LPS-induced p65 NF-κB expression alone. (HF100: 100 µg/mL of hexane fraction; WB0.1: 0.1 µg/mL of wild basidiocarp extract; DB0.1: 0.1 µg/mL of domesticated basidiocarp extract)

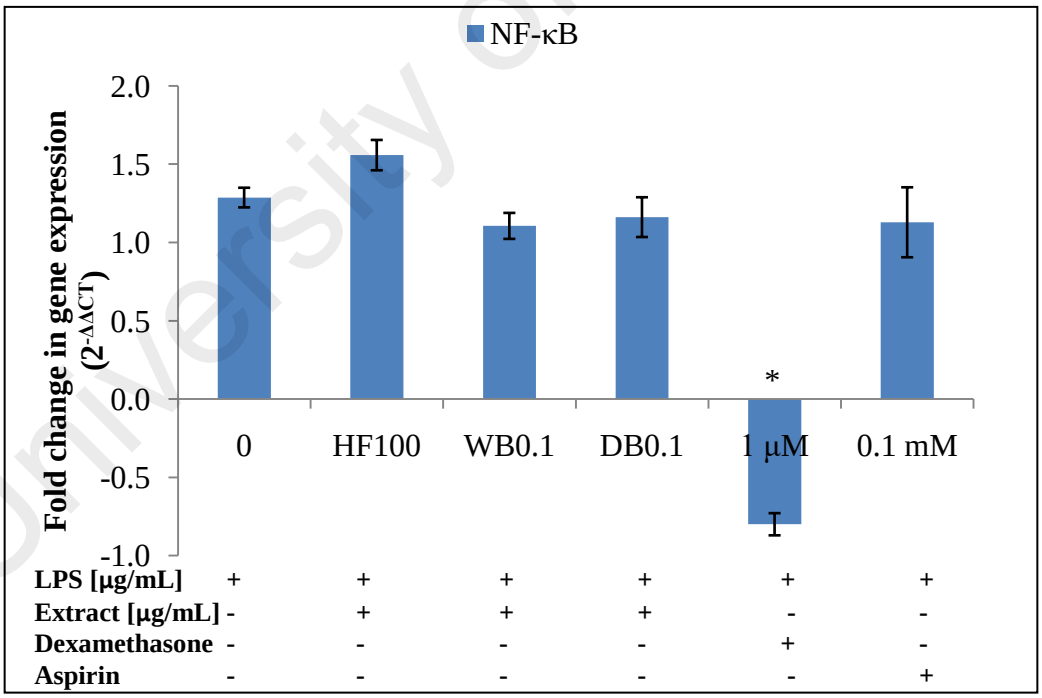


Figure 5.20-: Effects of *A. rugosum* extracts on NF-κB p65 gene expression. RAW264.7 cells were co-incubated with *A. rugosum* extracts (HF100: 100 µg/mL of hexane fraction; WB0.1: 0.1 µg/mL of wild basidiocarp extract; DB0.1: 0.1 µg/mL of domesticated basidiocarp extract) and 1 µg/mL LPS for 24 hours. Dexamethasone (1 µM) and aspirin (0.1 mM) served as positive controls. β-actin was used as loading control and normalised against the treated group. Results shown represent the mean ± S.D., n = 3 and *p < 0.05 versus LPS-induced NO level alone.

5.4 Discussion

In the present study, the MTT assay was used to evaluate the cytotoxic effect of *A. rugosum* extracts on RAW264.7 murine macrophage cells. It was found that *A. rugosum* extracts had no cytotoxic effects on RAW264.7 cells, except for the AF at a concentration of 1 µg/mL, which showed a significant ($p < 0.05$) but mild toxic effect. Moreover, the EE and HF significantly ($p < 0.05$) promoted proliferation of RAW264.7 cells at a higher concentration.

During inflammation, numerous inflammatory mediators such as NO, cytokines and prostaglandin E_2 are produced (Lee et al., 2012). However, excessive production of NO is harmful to living organisms. Griess assay was used to measure the accumulation of nitrite, which is the stable metabolite of NO. The quantity of nitrite in the culture medium was measured as an indicator of NO production. In the present study, the abilities of *A. rugosum* extracts to scavenge NO radicals and inhibit NO production in RAW264.7 murine macrophage cells *in vitro* were evaluated. It was found that all the *A. rugosum* extracts were able to scavenge NO radicals and reduce LPS-induced NO production in macrophages. Among the mycelia of *A. rugosum* extracts tested, the EAF and HF completely inhibited NO production at a concentration of 100 µg/mL. On the other hand, basidiocarps of *A. rugosum* extracts (WB and DB) reduced the NO production most effectively at 0.1 µg/mL. To determine whether the inhibition of NO was due to cell death or the down-regulation of iNOS expression, the MTT assay was performed after challenging the cells with LPS. It was found that the reduced NO level caused by EAF at 100 µg/mL was due to cell death. On the other hand, when treated with HF at 100 µg/mL, RAW264.7 cells remained viable, and their NO production was inhibited. Besides, the inhibition of LPS-stimulated NO production in macrophages by WB and DB was found to be not due to the reduction in the number of cells. In fact, *A.*

rugosum extracts (HF, WB, and DB) markedly down-regulated the expression of iNOS gene, which is responsible for the production of NO. In addition, the HF, WB, and DB were able to scavenge NO radicals in a dose-dependent manner. Jedinak et al. (2011) showed that oyster mushroom concentrate (*Pleurotus ostreatus*) markedly suppressed LPS-stimulated NO production in RAW264.7 cells at the highest concentration tested, which was 100 µg/mL. Lee et al. (2012) also reported that the curcumin derivative, 2,6-bis(2,5-dimethoxybenzylidene)-cyclohexanone, significantly inhibited LPS-stimulated NO production in RAW264.7 cells with an IC_{50} of 13.66 ± 0.61 µM. However, the same report indicated that the curcumin derivative did not scavenge NO radicals at any concentrations tested.

Although HF, WB and DB showed effective NO scavenging activity at 500 µg/mL, this concentration was not used in cell-based bioassay experiments, as concentration of extracts exceeding 100 µg/mL were cytotoxic to macrophage cells. A previous report on plants showed that high level of antioxidants is associated with genotoxic properties in human peripheral blood mononuclear cells (Wan-Ibrahim, Sidik, & Kuppusamy, 2010). The DB did not exert a dose-dependent effect on LPS-stimulated NO production in RAW264.7 cells. Unexpectedly, at the lowest concentration tested (0.01 µg/mL), DB caused significant elevation of NO production but in contrast, at higher concentrations this extract inhibited NO production effectively in LPS-stimulated RAW264.7 cells. This may in part be attributed to the "double-edged sword" effect that many natural compounds are known to exert on living systems and DB used in the present study is not an exception. One plausible explanation is that, low concentration of extract may promote inflammation as active compounds in the extract may be recognised as foreign substance by macrophages. However, at higher concentrations, the mixture of the compounds present in the extract may work synergistically to facilitate better

interactions in the signal transduction pathway to affect a favourable response by inhibiting NO production effectively. This suggests that critical dosage of DB is required to exhibit potent anti-inflammatory effect. In the initial screening of *A. rugosum* extracts, WB showed a slight toxic effect towards RAW264.7 cells (4 X 10^3 cells/well). However, the MTT assay carried out after the removal of spent media for NO assay, WB showed no toxic effect towards LPS-stimulated RAW264.7 cells (4 X 10^5 cells/well) which was possibly due to the significantly larger number of cells. Evidently, the concentration of the extract (based on extract w/v) was not sufficient to exert significant reduction in viability on the much higher concentration of cells.

Nuclear factor kappa B (NF-κB) is a transcription factor that has been found to be active in immune response, apoptosis and cellular growth (Liang et al., 2004). It is tightly regulated by interaction with inhibitory IκB proteins such as IκBα, IκBβ, IκBγ and IκBε (Gilmore, 2006). Activation of NF-κB pathway through the binding of stimuli to TLR-4 causes phosphorylation of IκB proteins by IκB kinase (IKK). The phosphorylated IκB followed by its ubiquitination and 26S proteosomal degradation leads to the dissociation of IκB and enables the free NF-κB to translocate into the nucleus and bind to the target genes (Beauparlant & Hiscott, 1996; May & Ghosh, 1998). The NF-κB regulates a series of gene expression that are responsible for immune responses. These targeted genes include proinflammatory cytokines (e.g. IL-1, IL-2, IL-6, TNF-α), anti-inflammatory cytokines (e.g. IL-10), chemokines (e.g. IL-8), adhesion molecules (e.g. endothelial leukocyte adhesion molecule, vascular cell adhesion molecule and intercellular adhesion molecule), and inducible enzymes (e.g. iNOS and COX-2) (Caamano & Hunter, 2002; Liang et al., 2004). However, overproduction of proinflammatory mediators often leads to fatal outcomes of chronic inflammatory diseases such as arthritis, asthma, multiple sclerosis and atherosclerosis (Lee et al.,

2012; Reuter et al., 2010; Wen et al., 2011). Therefore, blocking the activity of proinflammatory cytokines and specific inflammatory pathways such as NF-κB may serve as a useful strategy for treating inflammation.

The present study demonstrated that HF from mycelia of *A. rugosum* and WB and DB from basidiocarps of *A. rugosum* possessed a suppressive effect on downstream production of inflammatory mediators of nitric oxide and TNF-α, and promoted the production and expression of anti-inflammatory cytokine IL-10. This is similar to the study done by Jedinak et al. (Jedinak et al., 2011), which also demonstrated that the oyster mushroom concentrate markedly inhibited the expression of iNOS and TNF-α in LPS-stimulated macrophages. On the other hand, IL-10 is a potent anti-inflammatory cytokine which controls the inflammatory processes by suppressing various proinflammatory mediators (Asadullah, Sterry, & Volk, 2003). Numerous studies have suggested that the induction of IL-10 production might exert beneficial effects in reducing the chronic inflammatory response found in patients with inflammatory disorders (Barros et al., 2010; Hovsepian, Penas, Siffo, Mirkin, & Goren, 2013; Tsai et al., 2013). NF-κB plays a pivotal role in the immune response, where it controls the expression of various inflammatory cytokines. HF, WB, and DB did not show any inhibitory effect on the nuclear translocation of NF-κB p65. Therefore, it is possible that attenuation of proinflammatory mediators is attributed to direct inhibition. Alternatively, HF. WB, and DB may be effective in inhibiting other upstream inflammatory signalling molecules such as p38 MAPK and JNK signalling (Huang, Shi, & Chi, 2009; Kaminska, 2005). Hence, further studies are required to investigate the upstream mechanism of HF, WB, and DB on inflammatory pathways.

5.5 Conclusion

In conclusion, HF from mycelia and WB and DB from basidiocarps of *A. rugosum* were able to scavenge NO radicals and suppress LPS-stimulated NO production in RAW264.7 cells. The suppressive effect was associated with the down-regulation of the iNOS gene. Moreover, HF, WB, and DB demonstrated effective down-regulation of the proinflammatory cytokine, TNF-α and up-regulation of anti-inflammatory cytokine, IL-10. However, the nuclear translocation of NF-κB p65 was not blocked.

CHAPTER 6

DETERMINATION OF ANTI-NEUROINFLAMMATORY ACTIVITY OF *AMAURODERMA RUGOSUM* USING *IN VITRO* CELLULAR MODEL (BV-2 CELLS)

6.1 Introduction

Amauroderma rugosum is a basidiomycete with a black stipe and a white pored hymenium that bruises to a blood red colour when touched (Zhishu et al., 1993). *Amauroderma* sp., which is also known as the "epileptic child mushroom" or "cendawan budak sawan" in the Malay language, is worn around the neck by the indigenous people in Malaysia to prevent fits and incessant crying by babies (Azliza et al., 2012; Chang & Lee, 2004). The focus and interest of study of anti-neuroinflammation and anti-epileptic potential of *A. rugosum* was initiated based on the aboriginal belief that this mushroom can prevent fit episodes. In the previous chapters, the mycelium of *A. rugosum* was shown to be a good source of nutrients, antioxidants, (Chapter 3) and exhibited *in vitro* anti-inflammatory activities (Chapter 4). The hexane fraction (HF) showed good nitric oxide (NO) scavenging activity and inhibition of inflammatory mediators such as NO and tumour necrosis factor alpha (TNF-α) in LPS-stimulated macrophages (RAW264.7 cells) (Chapter 4).

Clinical and experimental evidences suggest that infection or inflammation may be the main contributor to seizure predisposition and occurrence, and other neurological disorders such as epilepsy (Galic et al., 2008). Several reports have also revealed that proinflammatory cytokines are involved in the pathophysiology of seizures and may be new targets for therapies against epilepsy (Bartfai et al., 2007; Somera-Molina et al.,

2007; Virta, Hurme, & Helminen, 2002). The wild mushroom (*A. rugosum*) used in this study has been traditionally used to control epileptic episodes in children suggesting potential use of this mushroom in the mitigation of inflammatory disorders leading to epilepsy. However, to our knowledge, the use of *A. rugosum* to mitigate neuroinflammation that causes epilepsy has not been studied. Therefore, it would be of great interest to validate the effect of *A. rugosum* extracts for potential anti-neuroinflammatory activity.

Inflammatory pathways are widely acknowledged to contribute to the pathogenesis of several neurodegenerative disorders such as multiple sclerosis, Parkinson's disease and Alzheimer disease. It is also known to be activated by neurologic infection, ischemic stroke and traumatic brain injury (Glass, Saijo, Winner, Marchetto, & Gage, 2010). In recent years, there are increasing evidence to show that inflammation might be a consequence as well as a cause of epilepsy (Vezzani et al., 2011). Clinical and experimental evidences strongly support the hypothesis that inflammatory processes within the brain might constitute a common and crucial mechanism in the pathophysiology of seizures and epilepsy (Vezzani & Granata, 2005; Vezzani & Baram, 2007).

Chronic brain inflammation which comprised of activation of microglia, astrocytes, endothelial cells of the blood-brain barrier (BBB) and peripheral immune cells, and the concomitant production of inflammatory mediators was first observed in patients with Rasmussen encephalitis (Rasmussen, Olszewski, & Lloyd-Smith, 1958). There are also evidence of immune system activation in some patients with seizure disorders and high incidence of seizures in autoimmune diseases, which led to the suggestion that immune

and inflammatory mechanisms have roles in some forms of epilepsy (Kan et al., 2012; Suleiman et al., 2011).

Overproduction of an array of inflammatory molecules such as interleukin-1 beta (IL-1β), TNF, interleukin-6 (IL-6) and prostaglandin E2 (PGE2) have been found to promote seizure generation and exacerbation (Xiong et al., 2003). The regulation of the synthesis of chemokines, cytokines, enzymes (such as COX-2) and receptors (such as toll-like receptors (TLRs), IL-1R1 and TNF p55 and p75 receptors) are mainly at the transcription factor necrosis factor kappa B (NF-κB) (Gilmore, 2006). This transcriptional pathway modulates the expression of genes involved in neurogenesis, cell death and survival and in molecular reorganization and plasticity which occur concomitantly with epileptogenesis (Vezzani et al., 2011). Besides, a protein called high-mobility group box 1 (HMGB1), which is endogenously released or produced by stressed or injured neurons and interacts with TLR-4 was found to promote seizure activity. Moreover, the induction of an additional wave of HMGB1 release from activated astrocytes and microglia was speculated to lead to a positive feedback cycles of seizures and inflammation that promote the development of epilepsy (Maroso et al., 2010). Figure 6.1 shows the schematic diagram of the pathophysiological cascade of inflammatory events in epilepsy. Therapies such as adrenocorticotropic hormone (ACTH), steroids, plasmapheresis and intravenous immunoglobulin (IVIg) have been used to treat epilepsy syndromes (Vezzani et al., 2011). ACTH has been proven to be an effective therapy for infantile spasms. However, the use of plasmapheresis, IVIg and steroids in treatment of epilepsy syndromes is still controversial (Vezzani et al., 2011).

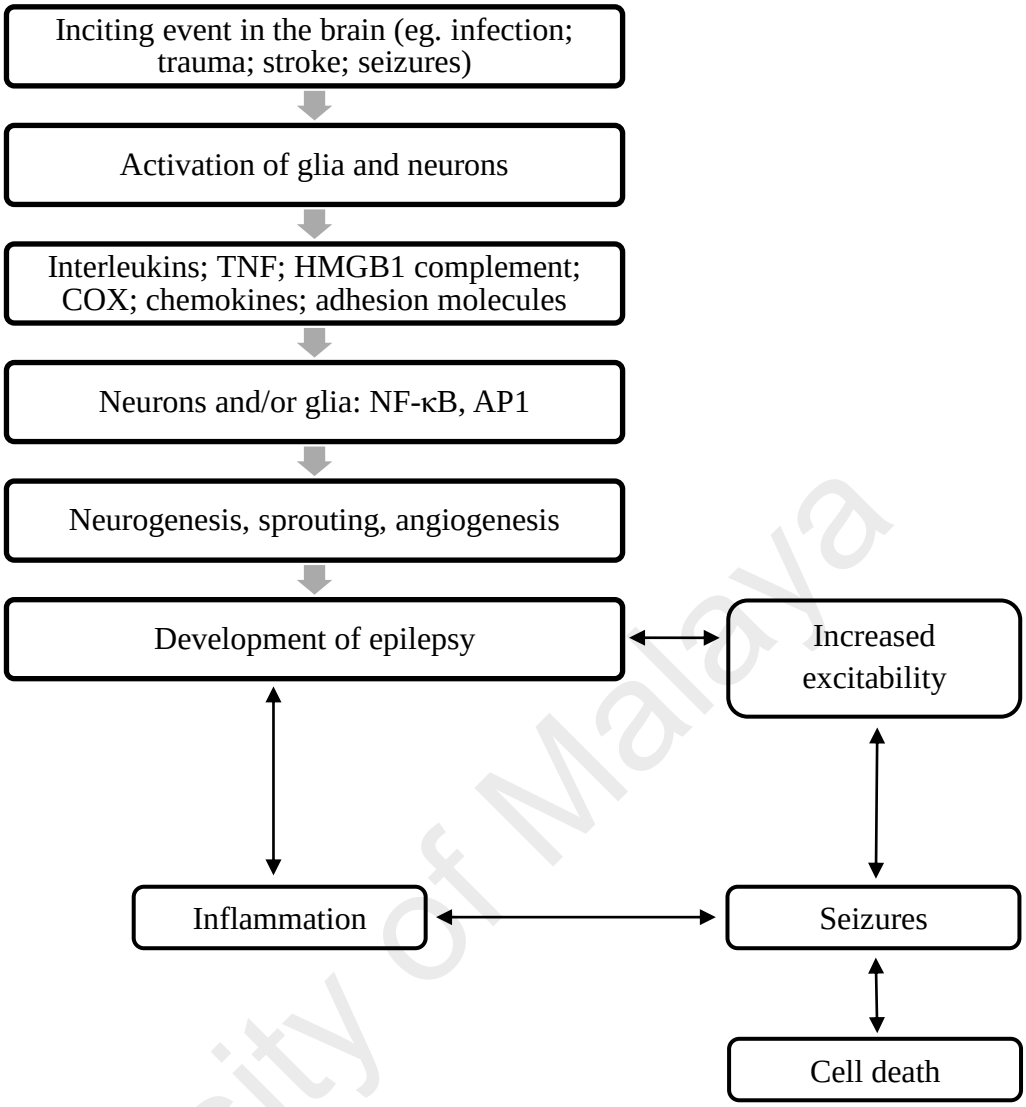


Figure 6.1: Pathophysiological cascade of inflammatory events in epilepsy. (Source: Vezzani et al., 2011)**.**

In order to aid the study of anti-neuroinflammation in mitigation of epilepsy, BV-2 cell line (microglial) was used as an *in vitro* model for this part of the study. As explained earlier, inflammation in brain disorders is characterized by the activation of glial cells (mainly comprised of microglia and astrocytes) which are the key producer of inflammatory mediators. Microglia are the resident immune cells of the central nervous system (CNS) which show increased inflammatory activity in response to infections, cerebral ischemia, traumatic brain injury or neuronal damage (Park, Koppula, Kim, & Jung, 2013). Therefore, BV-2 cell line (microglia) is useful as an *in vitro* model for the study of mechanisms related to injury-mediated inflammatory processes. The regulation

of microglial activation that leads to alleviation of neuroinflammatory diseases may be a crucial therapeutic target. Hence, the aim of the study presented in this chapter was to assess the *A. rugosum* extracts for anti-neuroinflammatory activity at biochemical and molecular levels using an *in vitro* approach (murine microglial, BV-2 cells).

6.2 Methodology

6.2.1 Chemicals, reagents, consumables, and kits

Table 6.1 shows the list of chemicals, reagents, consumables and kits used in this study.

Table 6.1: List of chemicals, reagents, consumables, and assay kits.

Chemicals/Reagents/Consumables/Kits	Company
Dulbecco's Modified Eagle's Medium (DMEM)	Gibco,Thermo Fisher Scientific Inc., USA
Foetal bovine serum (FBS)	Gibco,Thermo Fisher Scientific Inc., USA
Penicillin-streptomycin	Gibco,Thermo Fisher Scientific Inc., USA
Gentamycin	Gibco,Thermo Fisher Scientific Inc., USA
Fungizone	Gibco,Thermo Fisher Scientific Inc., USA
Trypsin-EDTA	Gibco,Thermo Fisher Scientific Inc., USA
Non-essential amino acids	Sigma-Aldrich, St. Louis, MO, USA
Insulin	Sigma-Aldrich, St. Louis, MO, USA
Sodium bicarbonate ($NaHCO_3$)	Merck, Germany
3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)	Calbiochem, Merck, Millipore, Darmstadt, Germany
Dimethyl sulfoxide (DMSO)	Fisher Scientific Inc., New Hampshire, USA
Escherichia coli (O55:B5) lipopolysaccharide (LPS)	Sigma-Aldrich, St. Louis, MO, USA
Sulphanilamide	Sigma-Aldrich, St. Louis, MO, USA
N-(1-napthy)ethylenediamine	Sigma-Aldrich, St. Louis, MO, USA
Phosphoric acid (H_3PO_4)	Sigma-Aldrich, St. Louis, MO, USA
Sodium nitrite ($NaNO_2$)	Sigma-Aldrich, St. Louis, MO, USA
N_ω-nitro-l-arginine-methyl ester (L-NAME)	Sigma-Aldrich, St. Louis, MO, USA
12-well chamber slide, removable, microscopy glass slide, sterile	ibidi GmbH, Germany
Phosphate buffer saline (PBS)	Oxoid Ltd., Thermo Scientific, Hampshire, UK.
iNOS primary antibody	Cell Signalling Technology (CST), USA
Triton X-100	Sigma-Aldrich,St. Louis, MO, USA
FITC-conjugated anti-rabbit IgG secondary antibody	Merck Milipore, Germany
Prolong® Gold Antifade reagent with DAPI	Thermo Scientific, USA
Absolutely RNA miniprep kit	Agilent, Santa Clara, California, USA
Fluorescent dye (Cy3) Low Input Quick Amp Labelling kit, One Colour	Agilent, Santa Clara, California, USA
Mus musculus Genome Oligo Microarray	Agilent, Santa Clara, California, USA

6.2.2 Cell culture

The murine microglial cell line (BV-2 cells; Figure 6.2) was obtained as a kind gift from Dr. Sharmili Vidyadaran (Universiti Putra Malaysia, UPM, Serdang, Malaysia). BV-2 cells were maintained in DMEM containing 5% FBS, 1% penicillin-streptomycin, 0.1% gentamycin, 0.5% fungizone, 1% non-essential amino acids, 0.1% insulin, and 1.5 g/L sodium bicarbonate at 37°C in a humidified atmosphere containing 5% CO_2. BV-2 cells were subcultured at 3 days interval and harvested by treating with 0.05% trypsin-EDTA for 5 minutes at 37°C.

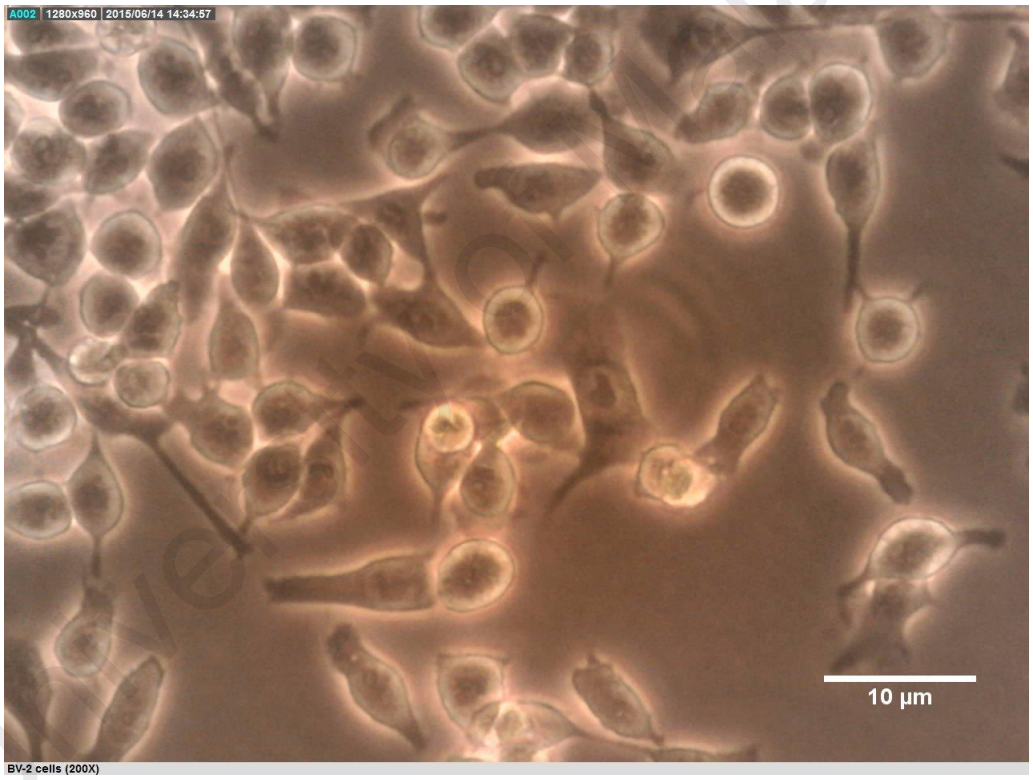


Figure 6.2: Morphology of *in vitro* cellular model of neuroflammatory study – BV-2.

6.2.3 Cell viability assay

MTT assay was performed according to the method described in Chapter 5 (Section 5.2.3, pg 71) with slight modifications on the cell number. BV-2 cells (5 X 10^4 cells/well) were seeded into a 96-well plate as previously mentioned by Tan, Ramasamy, Abdullah, & Vidyadaran (2011). The attached cells were treated with *A.*

rugosum extracts (EE: ethanol extract; HF: hexane fraction; EAF: ethyl acetate fraction; AF: aqueous fraction; WB: wild basidiocarp ethanolic extract; or DB: domesticated basidiocarp ethanolic extract) for 24 hours.

6.2.4 Nitric oxide assay

The nitric oxide (NO) assay on BV-2 cells (5 X 10^4 cells/well) was performed according to the method as described in Chapter 5 (Section 5.2.5, pg 72-73). The attached cells were co-incubated with *A. rugosum* extracts (EE: ethanol extract; HF: hexane fraction; EAF: ethyl acetate fraction; AF: aqueous fraction; WB: wild basidiocarp ethanolic extract; or DB: domesticated basidiocarp ethanolic extract) and 1 µg/mL of *Escherichia coli* (O55:B5) LPS for 24 hours.

6.2.5 Immunofluorescence staining of iNOS

The immunofluorescence staining of iNOS on BV-2 cells (5 X 10^4 cells/well) was performed according to the method as described in Chapter 5 (Section 5.2.6, pg 73). The attached cells were co-incubated with *A. rugosum* extract (HF100: 100 µg/mL of hexane fraction; WB0.1: 0.1 µg/mL of wild basidiocarp ethanolic extract; DB: 0.1 µg/mL of domesticated basidiocarp ethanolic extract) and 1 µg/mL of *Escherichia coli* (O55:B5) LPS for 24 hours.

6.2.6 Microarray

6.2.6.1 RNA isolation

BV-2 cells (2.5 X 10^6 cells) were seeded in 25cm^3 flask and allowed to attach overnight at 37°C in humidified atmosphere containing 5% CO_2. The cells were then treated with 100 µg/mL of HF and co-incubated with or without 1 µg/mL of LPS for 24 hours. Subsequently, the cells were harvested and the RNA was isolated using Absolutely

RNA Miniprep Kit according to the protocol provided by the manufacturer (Agilent). The purity of the isolated RNA was determined based on the ratio of the absorbance at 260 nm and 280 nm (Appendix B (B)). The preparation with a ratio between 1.8 and 2.0 for A_{260}/A_{280} was further used to determine the integrity of the RNA samples using an Agilent® 2100 Bioanalyzer. Samples with the RNA integrity number (RIN) between 8 and 10 were used for microarray (Appendix C (B)).

6.2.6.2 Microarray hybridization

The labelling and hybridization of cRNA was performed at Science Valley Sdn. Bhd., Selangor, Malaysia. Briefly, 25 ng of total RNA from each sample was amplified and labelled with fluorescent dye (Cy3, Low Input Quick Amp Labelling Kit, One Colour) according to the manufacturer's protocol (One-Color Microarray-Based Gene Expression Analysis, Low Input Quick Amp Labelling Version 6.7 Protocol). The amount of the resulting fluorescently labelled cRNA was quantified with the NanoDrop ND-1000 UV-VIS Spectrophotometer version 3.2.1 (Appendix D). The Cy3-labelled samples were hybridized with the microrray (*Mus musculus* Genome Oligo Microarray), comprising over 60,000 *mus musculus* genes and transcripts, for 17 hours at 65°C before washing and scanning. The scanning was done using an Agilent G4900DA SureScan Microarray Scanner. Data were extracted from resulting images (Feature Extraction Software; Agilent).

6.2.6.3 Data analysis

The Agilent GeneSpring GX 13.0 was used for analysis of the datasets. Biological replicate samples were sorted according to four conditions: cells only (control), cells induced with 1 µg/mL of LPS, cells treated with 100 µg/mL of HF, and cells induced with 1 µg/mL of LPS and treated with 100 µg/mL of HF. Filtering of the datasets was

performed (filter by flags present in all samples) by selecting only genes that are detectable in all samples (Appendix E). Fold change differences between control and induced and/or treated samples were calculated using cut-offs of 2-fold with significant values of $p < 0.05$. To analyse the four conditions, one-way analysis of variants (ANOVA) followed by Tukey's HSD (honest significant difference) test were used.

6.2.6.4 Pathway analysis

The Single Experiment Analysis (SEA) was employed to identify the matching pathways for the experiment. The normalised data containing the significant genes were compared with the curated pathway (WikiPathways).

6.2.7 Statistical analysis

Statistical analysis was performed using Statistical Product and Service Solutions (SPSS®) for Windows, version 17.0. One-way analysis of variance (ANOVA) followed by Duncan's Multiple Comparison Test was used to analyse the differences between group means. *P*-values less than 0.05 were considered as statistically significant and exact *p*-values were shown unless $p < 0.001$. All values are expressed as means ± standard deviation (SD) of triplicate values.

6.3 Results

6.3.1 Effects of *A. rugosum* extract on BV-2 cell viability

The MTT assay was used to determine the effect of *A. rugosum* extracts on BV-2 cell viability. The cell viability of the positive control (cells without any treatment) was denoted as 100%. None of the *A. rugosum* extracts tested showed toxicity on BV-2 cells at doses ranging from 0.01 µg/mL to 100 µg/mL and they promoted proliferation of these cells (Figure 6.3). These extracts caused an increase of 40% in the BV-2 cell

viability even at the lowest dose (0.01 µg/mL) tested. Hexane fraction (HF) and aqueous fraction (AF) promote the proliferation of BV-2 cells at all concentrations tested.

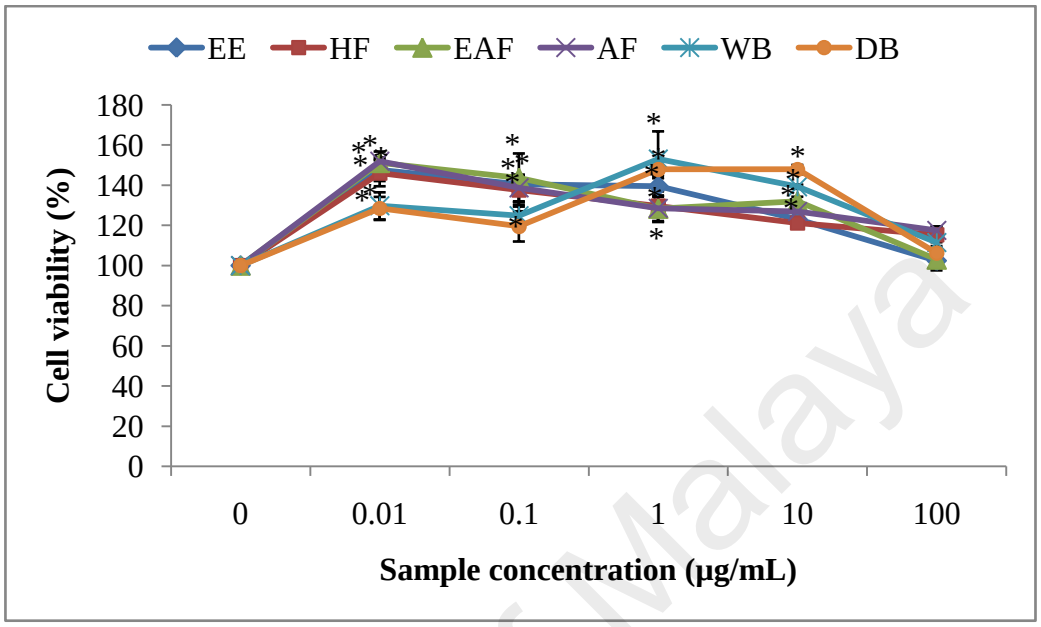


Figure 6.3: The effects of *A. rugosum* extracts on BV-2 cell viability. BV-2 cells were treated with *A. rugosum* extracts and cells without any treatment were expressed as 100%. The data presented represent mean ± standard deviation (S.D.), n = 3 and *p < 0.05 compared to control 100%. (EE: ethanol extract; HF: hexane fraction; EAF: ethyl acetate fraction; AF: aqueous fraction; WB: wild basidiocarp; DB: domesticated basidiocarp)

6.3.2 Effects of *A. rugosum* extract on LPS-stimulated NO production and expression in BV-2 cells

BV-2 cells were co-incubated with 1 µg/mL of LPS and *A. rugosum* extracts at respective concentrations for 24 hours. Figure 6.4 shows the effect of *A. rugosum* extracts on LPS-stimulated NO production in BV-2 cells. All the *A. rugosum* extracts were able to inhibit the NO production by the LPS-stimulated BV-2 cells in a dose-dependent manner. A significant inhibition of LPS-stimulated NO production in BV-2 cells was observed at 100 µg/mL of *A. rugosum* extracts (EE, HF, EAF, AF, WB, DB) tested. However, low NO production seen in AF-treated BV-2 cells were merely due to decline in cell viability (only viable cells produce NO) (Figure 6.5). On the other hand, HF, WB, and DB were able to inhibit LPS-stimulated NO production in BV-2 cells

without affecting the cell viability. The inhibition of LPS-stimulated NO production in BV-2 cells by HF, WB, and DB were due to suppression of the iNOS protein expression (Figure 6.6 and 6.7).

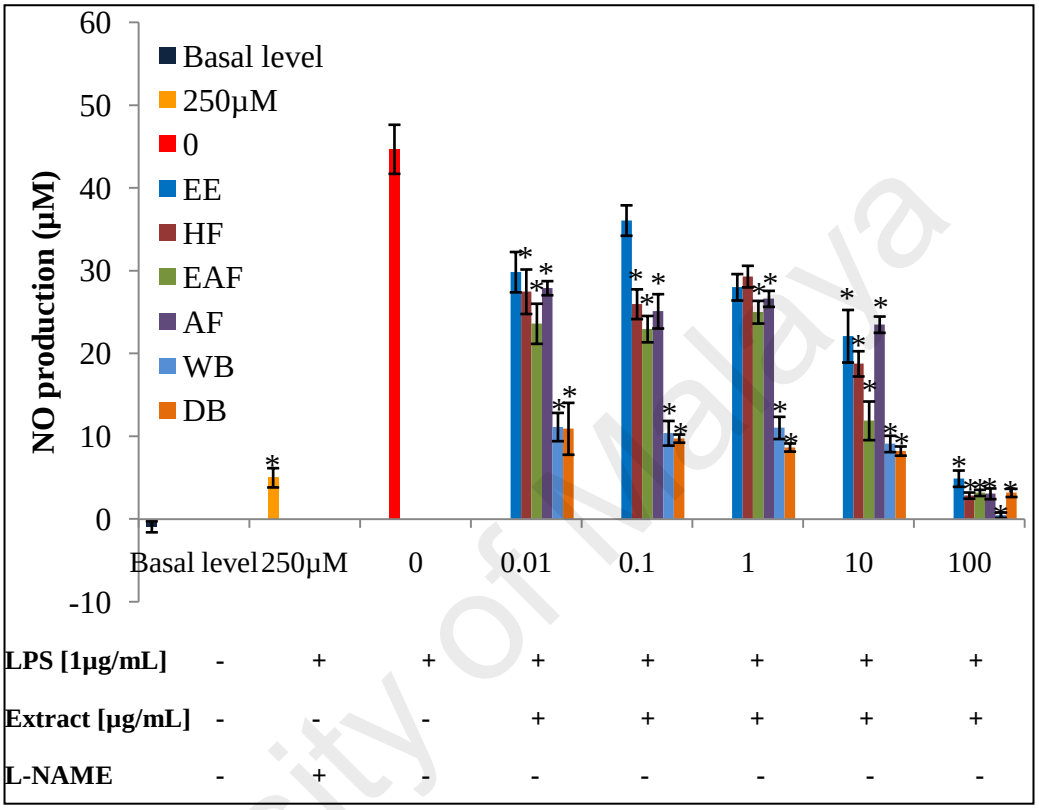


Figure 6.4: The effect of *A. rugosum* extracts on LPS-stimulated NO production in BV-2 cells. L-NAME at concentration of 250 µM, served as a positive control in this experiment. The data are presented in mean ± S.D. and *p < 0.05 as compared to the untreated LPS-stimulated BV-2 cells. (EE: ethanol extract; HF: hexane fraction; EAF: ethyl acetate fraction; AF: aqueous fraction; WB: wild basidiocarp; DB: domesticated basidiocarp)

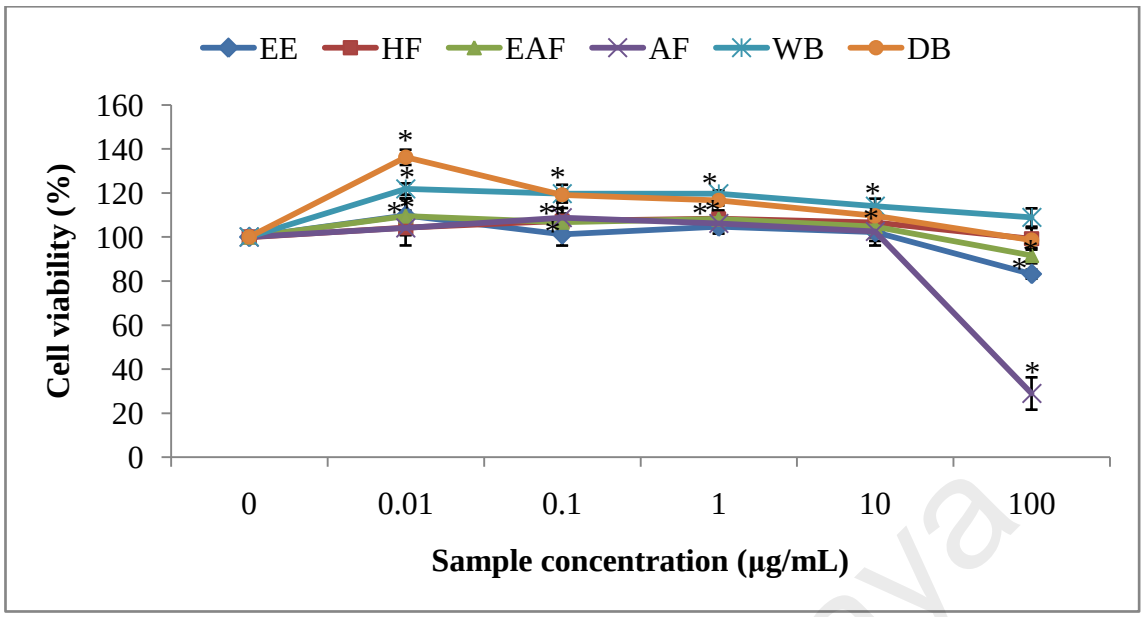


Figure 6.5: The effect of *A. rugosum* extracts on LPS-stimulated BV-2 cell viability. The data are displayed in percentage and untreated LPS-stimulated BV-2 cells were denoted as 100%. All data presented represent mean ± S.D. and *p < 0.05 as compared to untreated cells. (EE: ethanol extract; HF: hexane fraction; EAF: ethyl acetate fraction; AF: aqueous fraction; WB: wild basidiocarp; DB: domesticated basidiocarp)

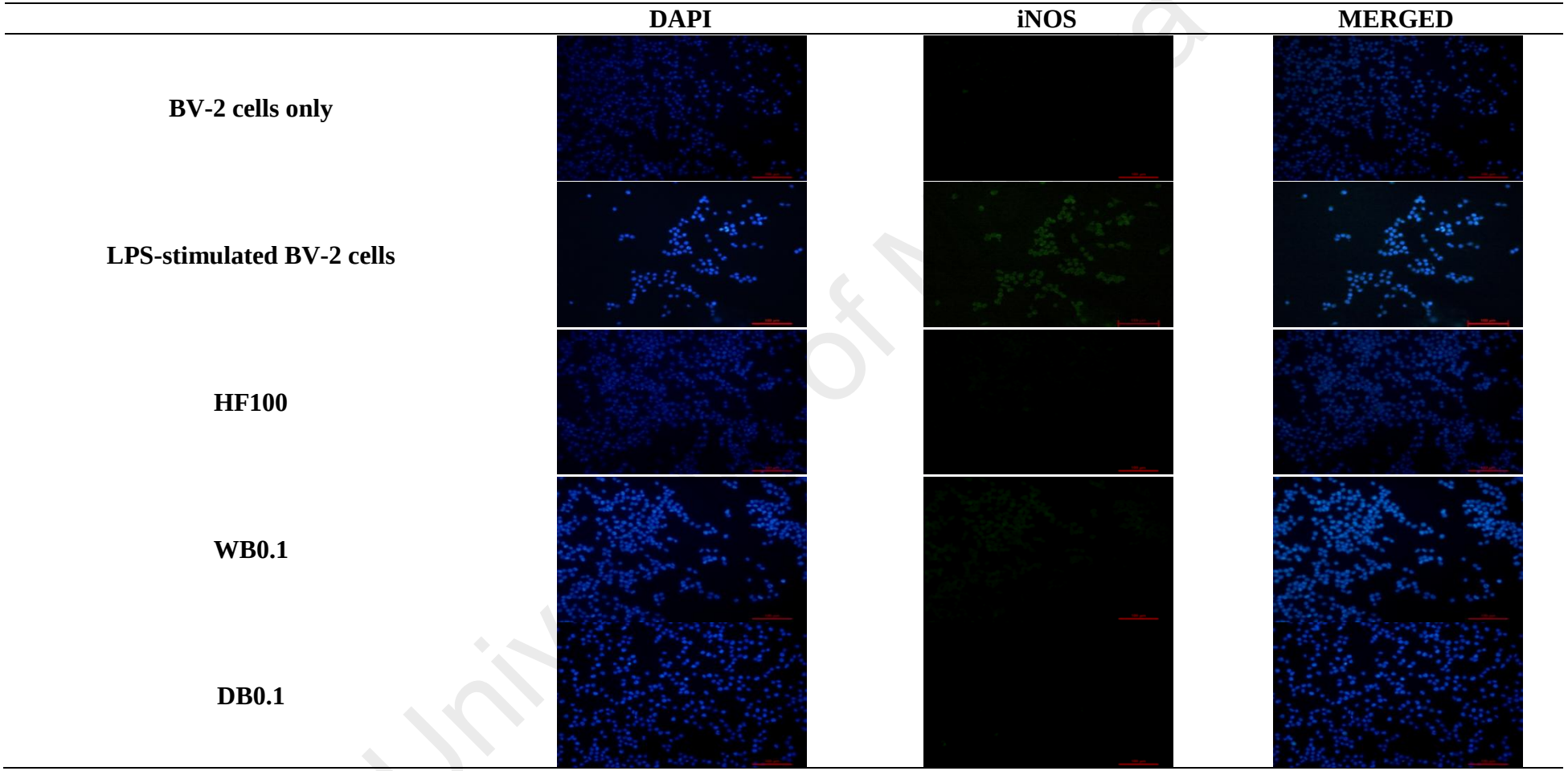


Figure 6.6: Effects of HF, WB, and DB on iNOS activity in LPS-stimulated BV-2 cells. iNOS was localised by fluorescence microscopy after immunofluorescence staining with iNOS antibody (green). Cells were stained with DAPI for visualization of nuclei (blue). (HF100: 100 µg/mL of hexane fraction; WB0.1: 0.1 µg/mL of wild basidiocarp extract; DB0.1: 0.1 µg/mL of domesticated basidiocarp extract)

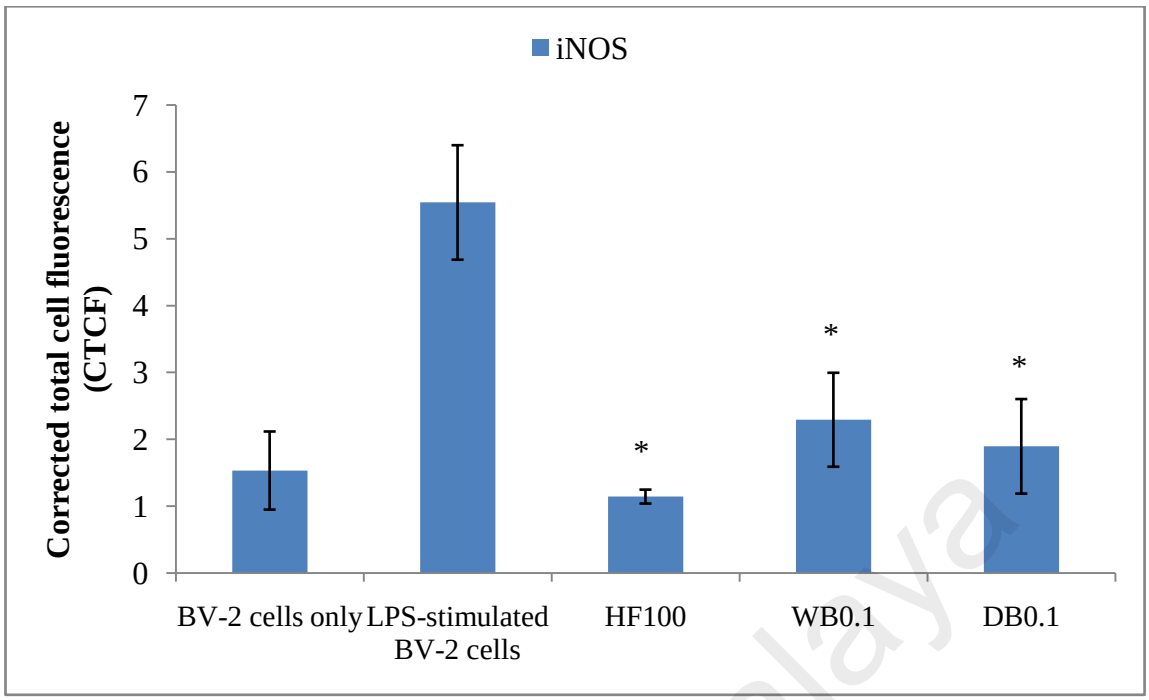


Figure 6.7: Quantification of fluorescence intensity. The fluorescence intensity was quantified using ImageJ and displayed in corrected total cell fluorescence (CTCF) Results shown represent the mean ± SD, n = 3 and *p < 0.05 versus LPS-induced iNOS expression alone. (HF100: 100 µg/mL of hexane fraction; WB0.1: 0.1 µg/mL of wild basidiocarp extract; DB: 0.1 µg/mL of domesticated basidiocarp extract)

6.3.3 Modulation of LPS-stimulated BV-2 cells gene expression profiles by *A. rugosum* extract

6.3.3.1 Gene expression in BV-2 microglial cells was reproducibly affected by different treatments

Figure 6.8 shows the 3D scatterplot of principle component analysis of BV-2 cells subjected to 4 different conditions. The biological replicates of each condition were closely grouped together. Besides, there was a clear stratification of samples compared to the control (cells only). The difference between the control group and the samples indicated detectable differences in the gene transcription patterns of these 4 conditions.

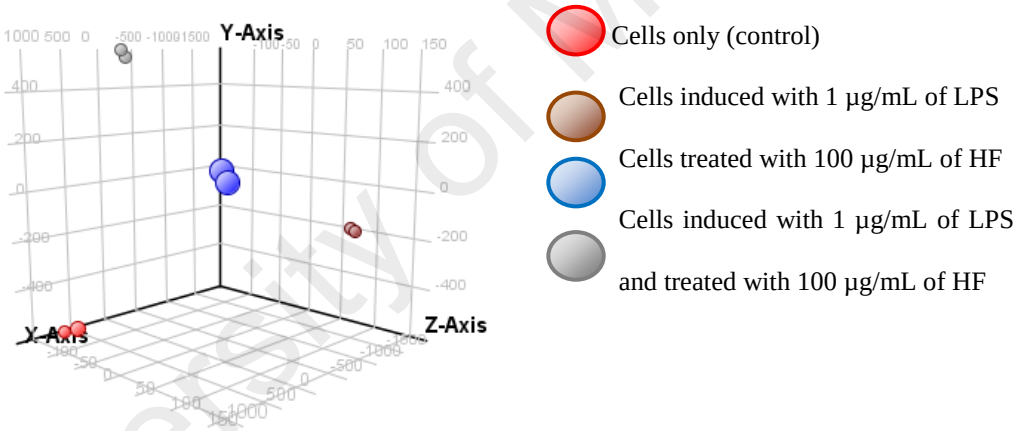


Figure 6.8: Principle component analysis (PCA) of the BV-2 cells in different treatment conditions. RNA was extracted from each sample and gene expression values were measured using *Mus musculus* Genome Oligo Microarray. The samples were stratifed according to 4 treatment conditions – cell only (control; red), cells induced with 1 µg/mL of LPS (brown), cells treated with 100 µg/mL of HF (blue), and cells induced with 1 µg/mL of LPS and treated with 100 µg/mL of HF (grey). Component % variance was; Component 1 = 67.9%, Component 2 = 23.7%, Component 3 = 7.4%, Component 4: 1.1%.

6.3.3.2 Hexane fraction regulates important neuroinflammatory pathways in LPS-stimulated BV-2 cells

WikiPathway is an open public database of biological pathways and it was used to identify the significant pathways within the set of 615 differentially expressed genes. A total of 31 pathways were regulated by HF (Table 6.2; Appendix F). The results obtained were based on the relative ratios of genes expressed in LPS-stimulated BV-2 cells with or without HF treatment. Table 6.3 shows the significantly regulated neuroinflammatory pathways.

Table 6.2: Pathways analysis within the set of differentially expressed genes.

Pathway Name	*P*-value	Significant genes / Pathway genes
mRNA processing	2.7×10^{-3}	15/551
Macrophage markers	3.4×10^{-4}	3/10
Eicosanoid synthesis	3.1×10^{-2}	2/19
Fatty acid biosynthesis	3.8×10^{-3}	3/22
Apoptosis	3.4×10^{-6}	9/83
G protein signalling pathways	4.4×10^{-2}	4/91
Chemokine signalling pathway	3.7×10^{-4}	10/193
MAPK signalling pathway	0	18/159
IL-1 signalling pathway	1.5×10^{-5}	6/37
Adipogenesis genes	2.5×10^{-5}	10/133
Oxidative damage	1.7×10^{-4}	5/41
Cytokines and inflammatory response	6.9×10^{-4}	4/30
Integrin-mediated cell adhesion	1.5×10^{-2}	5/101
Type II interferon signaling	7.3×10^{-6}	6/34
FAS pathway and stress induction of HSP regulation	1.8×10^{-2}	3/38
Amino acid conjugation of benzoic acid	1.5×10^{-2}	1/2
Phase I biotransformations non-P450	2.9×10^{-2}	1/8
Toll-like receptor signaling	1.2×10^{-2}	3/33
PluriNetWork	2.7×10^{-2}	9/291
XPodNet protein-protein interactions in the podocyte expanded by STRING	5.5×10^{-3}	22/836
p38 MAPK signalling pathway	1.3×10^{-2}	3/34
Complement activation classical pathway	2.2×10^{-2}	2/17
Triacylglyceride synthesis	4.4×10^{-2}	2/23
GPCRs Class B secretin-like	4.0×10^{-2}	2/22
MicroRNAs in cardiomyocyte hypertrophy	3.3×10^{-2}	4/104
Complement and coagulation cascades	1.9×10^{-3}	5/62
TNF-α NF-κB signalling pathway	1.8×10^{-5}	12/184
Focal adhesion PI3K-Akt-mTOR signalling pathway	2.3×10^{-3}	12/322
Urea cycle and metabolism of amino groups	3.3×10^{-2}	2/37
Toll-like receptor signalling pathway	1.4×10^{-7}	11/97
Androgen receptor signalling pathway	2.3×10^{-2}	5/112

MAPK: mitogen-activated protein kinase; IL-1: Interleukin-1; HSP: heat shock protein; GPCRs: G-protein-coupled receptors; TNF-α: tumour necrosis factor-alpha; NF-κB: nuclear factor-kappa B; PI3K-Akt-mTOR: Phosphatidylinositol-3-kinase - protein kinase B - mammalian target of rapamycin

Table 6.3: Analysis of neuroinflammatory pathways within the set of differentially expressed genes.

Pathway Name	*P*-value	Significant genes / Pathway genes
Macrophage markers	3.4×10^{-4}	3/10
MAPK signalling pathway	0	18/159
IL-1 signalling pathway	1.5×10^{-5}	6/37
Oxidative damage	1.7×10^{-4}	5/41
Cytokines and inflammatory response	6.9×10^{-4}	4/30
Toll-like receptor signalling	1.4×10^{-7}	11/97
p38 MAPK signalling pathway	1.3×10^{-2}	3/34
Complement activation classical pathway	2.2×10^{-2}	2/17
Complement and coagulation cascades	1.9×10^{-3}	5/62
TNF-α NF-κB signalling pathway	1.8×10^{-5}	12/184

Genome wide expressions were analysed in duplicates and highly significant changes are shown. Data are combined from companion of cells induced by LPS compared to cells treated with HF or cells induced with LPS and treated with of HF. MAPK: mitogen-activated protein kinase; IL-1: Interleukin-1; TNF-α: tumour necrosis factor-alpha; NF-κB: nuclear factor-kappa B; HF: hexane fraction

6.3.3.3 Expression levels of HF-regulated genes in neuroinflammatory pathways

In order to investigate the pharmacological and molecular mechanisms of HF, the expression levels of HF-regulated genes in neuroinflammatory pathways were examined. Table 6.4 shows HF down-regulated neuroinflammatory and up-regulated anti-neuroinflammatory genes in LPS-stimulated BV-2 cells.

Table 6.4: Fold change of related genes regulated by HF in LPS-stimulated BV-2 cells.

WikiPathway	Gene symbol	Fold change (FC)		
		HF-treated BV-2 cells	LPS-stimulated BV-2 cells	HF-treated LPS-stimulated BV-2 cells
Macrophage markers				
Cluster of differentiation 14	Cd14	-1.5	3.8	2.0
MAPK signalling pathway				
Cluster of differentiation 14	Cd14	-1.5	3.8	2.0
Tumour necrosis factor	Tnf	1.0	6.3	14.8
Stathmin 1	Stmn1	1.1	2.1	-1.1
Caspase 1	Casp1	1.0	2.3	1.7
TNF receptor-associated factor 2	Traf2	1.2	2.5	1.8
DNA damage-inducible transcript 3	Ddit3	-1.4	12.2	-1.1
Interleukin-1 beta	Il-1β	1.0	70.8	14.1
Interleukin-1 alpha	Il-1α	-2.2	31.4	13.4
IL-1 signalling pathway				
Interleukin-1 beta	Il-1β	1.0	70.8	14.1
Interleukin-1 alpha	Il-1α	-2.2	31.4	13.4
Sequestosome-1	Sqstm1	-1.0	4.5	1.4
Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	Nfkbia	2.0	5.9	13.8
Oxidative damage				
Tumour necrosis factor	Tnf	1.0	6.3	14.8
TNF receptor-associated factor 1	Traf1	1.1	36.9	15.1
TNF receptor-associated factor 2	Traf2	1.2	2.5	1.8
Cytokines and inflammatory response				
Tumour necrosis factor	Tnf	1.0	6.3	14.8
Interleukin-1 beta	Il-1β	1.0	70.8	14.1
Interleukin-1 alpha	Il-1α	-2.2	31.4	13.4
Interleukin 6	Il-6	-1.2	184.1	22.9

Table 6.4, continued.				
p38 MAPK signalling pathway				
DNA damage-inducible transcript 3	Ddit3	-1.4	12.2	-1.1
TNF receptor-associated factor 2	Traf2	1.2	2.5	1.8
Complement activation classical pathway				
Complement component 3	C3	1.2	7.6	3.7
TNF-α NF-κB signalling pathway				
Tumour necrosis factor	Tnf	1.0	6.3	14.8
Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	Nfkbia	2.0	5.9	13.8
TNF receptor-associated factor 2	Traf2	1.2	2.5	1.8
TNF receptor-associated factor 1	Traf1	1.1	36.9	15.1
Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, epsilon	Nfkbie	1.8	3.4	5.2

HF: Hexane fraction

6.4 Discussion

The data presented in the previous chapters have shown that *A. rugosum* extracts possessed potential antioxidant and anti-inflammatory activities. Particularly the HF showed the best antioxidant activity (Chapter 4) and potential anti-inflammatory property (Chapter 5) (Chan et al., 2013). In this study, the results showed that LPS activated the major neuroinflammatory pathways and HF was able to inhibit the LPS-activated neuroinflammatory pathways. These results suggest that HF has potential anti-neuroinflammatory effects.

Neuroinflammation plays a major role in the pathophysiology of neurological disorders (Yong, 2010). When the CNS is injured, several neuroinflammatory pathways will be activated. Microglia and other innate immune cells respond quickly to injury and stimulate the production of cytokines, chemokines, free radical species, proteases, and other mediators of injury (Amor, Puentes, Baker, & Valk, 2010). Nitric oxide (NO) is a bioactive free radical that is often known to possess a "double-edged sword" effects. It acts as a neuromodulator and neurotransmitter in the brain. However, excess production of NO by glial cells may cause harm to the neuronal function. Overproduction of NO has been observed in many neurolopathological and neuroinflammatory conditions such as periventricular leukomalacia (PVL), Krabbe's disease, X-linked adrenoleukodystrophy (ALD), and multiple sclerosis (MS) (Ghasemi & Fatemi, 2014). The increased production of NO in BV-2 cells was significant after LPS-stimulation and treatment with *A. rugosum* extracts attenuated the NO production. Among the extracts tested, HF showed the best inhibition of NO in LPS-stimulated BV-2 cells. Therefore, HF was selected for in depth study to unveil the mechanism of action via pathway analysis.

Lipopolysaccharide (LPS), an endotoxin used in this study is known to stimulate the activation of microglia which possibly mediates the induction of toll-like receptor (TLR) and subsequently activates the neuroinflammatory pathways(Kacimi, Giffard, & Yenari, 2011). Cluster of differentiation 14 (CD14), which is the co-receptor of TLR was found to be highly activated in LPS-stimulated microglial cells. Highly elevated expression of CD14 is commonly found in neurological disorders. It has been reported that CD14 may contribute to the overall neuroinflammatory response in the brain (Zhou, Wang, Yang, & Wang, 2013). In the present study, HF of *A. rugosum* was found to downregulate the CD14 expression in LPS-stimulated microglial cells by 1.9-fold. Besides, the production of proinflammatory cytokines such as IL-1β, IL-1α, and IL-6 were increased after LPS stimulation. However, they were significantly reduced upon treatment with HF (IL-1β: 17.3-fold; IL-1α: 2.3-fold; IL-6: 8-fold). IL-1β is commonly produced in activated microglia in the injured CNS (Yong, 2010). This proinflammatory cytokine has a critical role in enhancing nociceptive transmission in spinal cord inflammation. The overproduction of IL-1β in the CNS has been identified as the crucial mechanism in persistent pain (Ren & Torres, 2009). Besides that, IL-1β is commonly found to be highly expressed in epileptic patients (Ho et al., 2014). In this study, HF was found to significantly down-regulate IL-1β in the LPS-stimulated microglial cells.

Activation of MAPK signalling pathway in the CNS has been associated in neurological diseases (eg. Epilepsy) to be linked with neuroinflammation (Ramesh, 2014). MAPKs consist of 3 distinct groups which are p38 MAPK, ERKs, and JNKs (Cuadrado & Nebreda, 2010). The p38 MAPK has a major role in mediating cellular stress and inflammatory responses. It is involved in regulating the production of TNF, IL-6, and several other cytokines that contribute to inflammation. Studies have shown that the

inhibition of MAPK pathways may ameliorate neuroinflammation-related neurological diseases (Kaminska, 2005; Kim, Kim, & Sharma, 2004; Kim, Yu, Piao, Kim, & Lee, 2004). From the microarray data, it is evident that the HF regulated MAPK signalling pathways. MAPK-associated genes such as Stmn1, Casp1, Traf2, Ddit3, Cd14, and interleukins (IL-1β and IL-1α) were up-regulated upon LPS stimulation and were down-regulated by HF.

In the CNS, binding of inflammatory stimuli to receptors of the cell surface also triggers intracellular signalling transduction pathway such as nuclear factor kappa B (NF-κB) pathway (Ramesh, 2014). NF-κB plays an important role in the CNS as it is involved in many important processes such as synaptic processes, neurotransmission, and neuroprotection (Kaltschmidt & Kaltschmidt, 2009). However, dysregulation of NF-κB is commonly found in neurological disorders (Qin et al., 2007). The binding of stimuli that induce the NF-κB activity cause phosphorylation of I-kappa-B (IκB) and therefore, activates the NF-κB complex. Nfkbia/IκB-α and Nfkbie/IκBε are IκB proteins which function to inactivate the NF-κB complex by preventing the translocation of p65 and p50 subunits into the nucleus (Qin et al., 2007). Studies have demonstrated that the increased expression of IκB proteins or the prevention of IκB phosphorylation can attenuate inflammation (Lee et al., 2011; Lee et al., 2008). Both Nfkbia/IκB-α and Nfkbie/IκBε were highly up-regulated by HF in the LPS-stimulated microglial cells. Tumour necrosis factor (TNF) is a proinflammatory cytokine that is involved in many crucial physiological processes such as synaptic plasticity, learning and memory, sleep, food and water intake, and astrocyte-induced synaptic strengthening (Olmos & Lladó, 2014). Besides, TNF stimulates the activation of signal transduction pathways such as NF-κB, extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), p38 MAPK, acidic sphingomyelinase (A-SMase), and neutral sphingomyelinase (N-

SMase) pathways (Olmos & Lladó, 2014). In this study, HF treatment caused the level of TNF mRNA in LPS-stimulated BV-2 cells to increase 2.3-fold by HF treatment. A study done by Yu et al. (2009) showed that edible mushrooms such as white button, crimini, maitake, oyster, and shiitake extracts also induced the TNF-α production in LPS-stimulated macrophages. Although upregulation of TNF expression was observed in HF treated LPS-stimulated BV-2 cells, the signal transduction pathways such as NF-κB, MAPK, and IL-1 were suppressed. Hence, this suggests that the upregulation of TNF by HF did not affect the other proinflammatory genes.

Neuroinflammation has been postulated as one of the main causes of epilepsy (Vezzani et al., 2013, 2011). Clinical and experimental verification have proven that inflammation causes prolongation of seizure activities (Galic et al., 2008; Kiarash Riazi et al., 2010). Thus, mitigation of neuroinflammation is important to prevent or reduce the epileptic episodes. The efficacy of HF in suppressing the inflammatory mediators suggests its potential capability as anti-neuroinflammatory agent which could possibly ameliorate epileptic symptoms.

6.5 Conclusion

In conclusion, HF significantly reduced the NO level in LPS-stimulated BV-2 cells and regulated 10 neuroinflammatory pathways. HF suppressed the LPS-induced cytokines (IL-1α, IL-1β, and IL-6) and other proinflammatory gene expressions (Traf1, Traf2, Cd14), and up-regulated anti-inflammatory genes such as Nfkbia and Nfkbie. Therefore, it is pertinent to suggest that HF has the potential to serve as an anti-neuroinflammatory agent and may be useful in ameliorating seizures.

CHAPTER 7

DETERMINATION OF ANTI-EPILEPTIC ACTIVITY OF *AMAURODERMA RUGOSUM* USING *IN VIVO* MODEL AND IDENTIFICATION OF POTENTIAL COMPOUNDS

7.1 Introduction

Epilepsy is the recurrence of seizures. It affects more than 50 million people worldwide and 90% of them are from developing countries (Mukhopadhyay, Kandar, Das, Ghosh, & Gupta, 2012). This brain disorder has detrimental impact on all aspects of quality of life (Figure 7.1). The recurrence of seizures is often unpredictable and dangerous as it increases the risk of injury, hospitalization, and mortality. Moreover, it adversely affects a patient's mental health and often causes anxiety, depression, or cognitive impairment (Kerr, 2012).

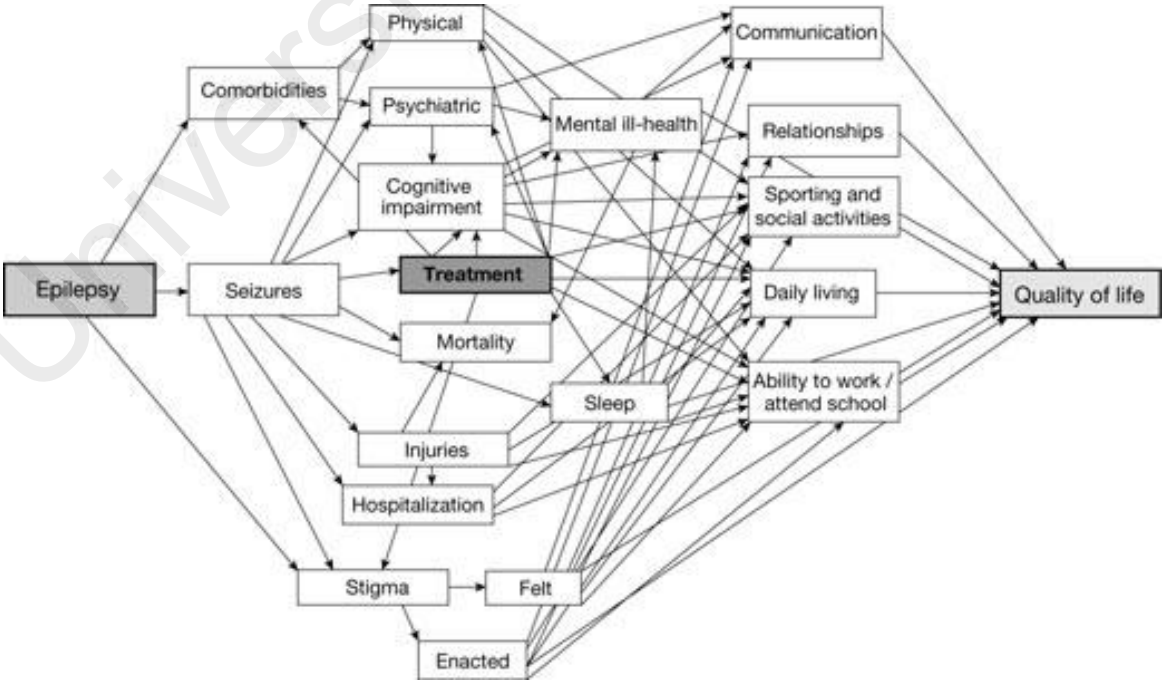


Figure 7.1: Impact of epilepsy on quality of life. (Source: Kerr (2012))

Anti-epileptic drugs (AEDs) are commonly associated with unfavourable side effects and these further impair the patients' quality of life (Kerr, Nixon, & Angalakuditi, 2011). For an example, carbamazepine is commonly used to treat simple and complex seizures. However, long-term usage of carbamazepine can damage the liver (Dawda & Ezewuzie, 2010). Sodium valproate is another type of AED which is use to control tonic-clonic seizures in primary generalised epilepsy. The side effects of sodium valproate include gastrointestinal pain, tremor, ataxia, sedation, hair loss, increase in appetite, and weight gain. Besides, phenytoin has been reported to be an effective AED for treating tonic-clonic and partial seizures. However, it has detrimental side effects such as hirsutism, gum hypertrophy, and aggravation of acne (Dawda & Ezewuzie, 2010). In general, AEDs can control seizures and reduce the adverse effects of epilepsy. Conversely, it has huge disadvantageous impact on the patients' quality of life. Therefore, constant research and exploration on potential treatments for epilepsy is clearly needed (Ekstein & Schachter, 2010).

The wild mushroom (*Amauroderma rugosum*) used in this study has been traditionally used to control epileptic episodes in children suggesting potential use of this mushroom in the mitigation of inflammatory disorders leading to epilepsy. However, to our knowledge, the use of *A. rugosum* to manage, prevent or treat epilepsy has not been studied. Besides that, results presented in the previous chapters showed that hexane fraction (HF) of *A. rugosum* has potential anti-inflammatory (Chapter 5) and anti-neuroinflammatory (Chapter 6) activities. Therefore, it would be of great interest to validate the effect of HF of *A. rugosum* for potential anti-epileptic activity. The aim of the study presented in this chapter was to validate the anti-epileptic activity of HF of *A. rugosum* using an *in vivo* approach.

7.2 Methodology

7.2.1 Chemicals

Table 7.1 shows the list of chemicals used in this study.

Table 7.1: List of chemicals.

Chemicals	Company
Kainic acid (KA)	Sigma-Aldrich, St. Louis, MO, USA
Valproic acid (VA)	Sigma-Aldrich, St. Louis, MO, USA
Phosphate buffer saline (PBS)	Oxoid Ltd., Thermo Scientific, Hampshire, UK.
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich, St. Louis, MO, USA
Chloral hydrate	Merck, Germany

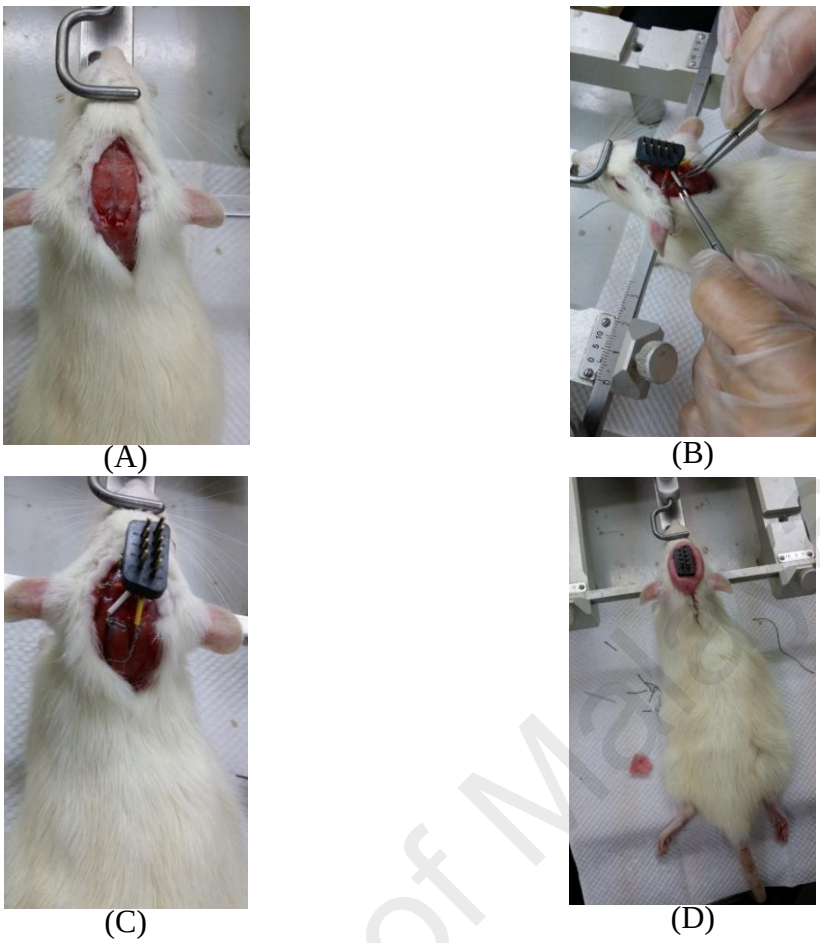
7.2.2 Animal and ethics statement

This study was carried out in China Medical University (CMU), Taichung, Taiwan and it was conducted in conformity with the policies and procedures of the Institutional Animal Care and Use Committee (IACUC) of CMU and Guidelines of the Chinese Society for Laboratory. The animal ethics approval was obtained from the IACUC, CMU (approval number: 104-341; Appendix G). Male Sprague-Dawley (SD) rats (200-300g) were purchased from BioLasco Taiwan Co., Ltd (Taipei, Taiwan) (Figure 7.2). The rats were housed in stainless steel wire mesh cages in a room at 22 ± 2ºC and 55 ± 5% humidity with 12-hour light/dark cycle. The rats had free access to food and water which were provided fresh every day.

Figure 7.2: Male sprague-dawley (SD) rats.

7.2.3 Electrodes preparation

A total of 36 rats were examined in this study. Electrodes were installed surgically on the head of the rat according to the method suggested by Ho, Tang, Hsiang, & Hsieh (2014) (Figure 7.3). The head of the rat was fixed to a stereotactic apparatus under anesthesia chloral hydrate (400 mg/kg, intraperitoneal; Appendix A (E)), at least 4 days prior to the electroencephalogram (EEG) and electromyogram (EMG) recordings. The stainless steel screws were implanted over the epidural region located on the bilateral sensory-motor cortices to serve as the recording electrodes and another electrode was fixed on the frontal sinus to serve as a reference for EEG recordings. In addition, two electrode wires were implanted around the neck muscle for EMG recording (Figure 7.4). These electrodes were plugged into a relay and connected to an EEG and EMG-recorder (MP100WSW, BIOPAC System Inc., Goleta, CA, USA).



(A) (B)

(C) (D)

Figure 7.3: Installation of electrode on the head of the rat. (A) Opening that exposes the epidural region of the brain; (B) installation of electrode on the bilateral sensory-motor cortices; (C) fixation of the two wires of the electrode to the neck muscle; (D) completion of installation of electrode to the head of the rat.

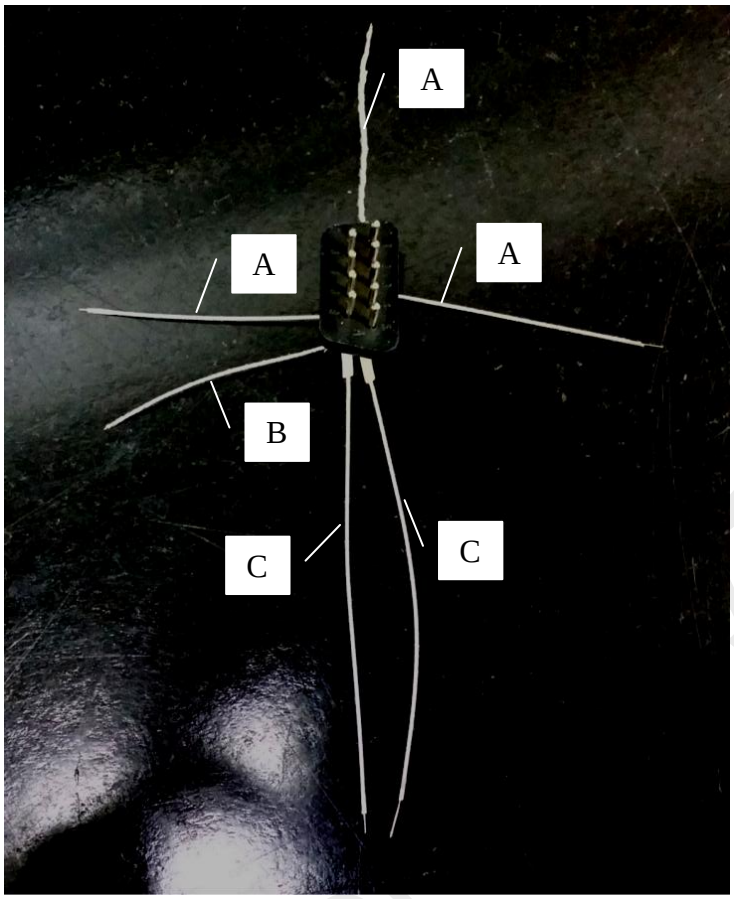


Figure 7.4: Electrode for EMG and ECG recordings. (A) Stainless steel screws were implanted over the epidural region located on the bilateral sensory-motor cortices to serve as the recording electrodes; (B) an electrode was fixed on the frontal sinus to serve as a reference for EEG recordings; (C) two electrode wires were implanted around the neck muscle for EMG recording.

7.2.4 Animal experiments

The rats were randomly assigned into 6 groups (n = 6) as tabulated in Table 7.2. The epileptic seizure behavioural observation and EEG and EMG recordings using brain wave software, AcqKnowledge Version 3.9.1.6, were performed 15 minutes before and during the first 3 hours after KA injection (Figure 7.5).

Table 7.2: Type of treatment for each group.

Groups	Treatment
Kainic acid (KA)	intraperitoneal injection of KA (12 mg/kg) only
Valproic acid (VA)	administration of valproic acid (250 mg/kg/day) 7 days and 15 minutes prior to KA injection
PBS-1% DMSO vehicle (PD)	administration of PBS-1% DMSO 7 days and 15 minutes prior to KA injection
HF (5 mg/kg)	administration of HF (5 mg/kg) 7 days and 15 minutes prior to KA injection
HF (8 mg/kg)	administration of HF (8 mg/kg) 7 days and 15 minutes prior to KA injection
HF (16 mg/kg)	administration of HF (16 mg/kg) 7 days and 15 minutes prior to KA injection

PBS: phosphate buffer saline; DMSO: dimethyl sulfoxide; HF: hexane fraction

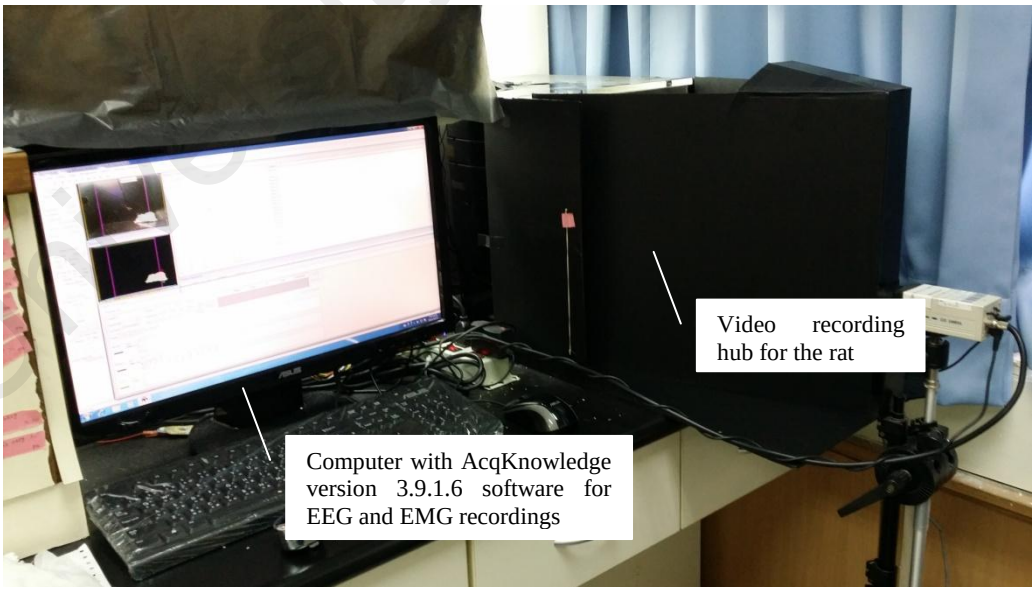


Figure 7.5: Station for the epileptic seizure behaviour recordings and observations.

7.2.5 Measurement of blood biochemistry – liver and kidney function tests

After 24 hours of the KA injection, the rat was sacrificed and 3 mL of whole blood was collected from the right femoral artery for blood biochemistry tests. The whole blood was centrifuged at 2405 *x g* for 10 minutes and the supernatant was sent out to Zhen Xing Enterprise Ltd., Taiwan (振杏企業有限公司) for aspirate aminotransferase (GOT), alanine aminotransferase (GPT), blood urea nitrogen (BUN), and creatinine level analysis.

7.2.6 GC-MS analysis

The GC-MS analysis of HF was performed with an Agilent Technologies 6890 N (United States) gas chromatography equipped with a 5975 inert mass selective detector (70eV direct inlet) and a HP-5 ms (5% phenylmethylpolysiloxane) capillary column (30 m X 250 µm X 0.25 µm film thickness). The GC using helium as the carrier gas at a flow rate of 1 mL/min was initially set at 100°C, and then programmed to increase to 300°C at a ramp rate of 5°C min^{-1} and was put on hold for 10 minutes at 300°C. The total ion chromatogram obtained was autointegrated by Chemstation and the constituents were identified by comparison to the mass spectral database (NIST 05 Mass Spectral Library, USA).

7.2.7 Statistical analysis

All values are expressed as means ± standard deviation (SD) of triplicate values. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Duncan's Multiple Comparison Test using Statistical Product and Service Solutions, SPSS® Statistics for Windows, Version 17.0 and $p < 0.05$ was denoted as being statistically significant.

7.3 Results

7.3.1 Effects of hexane fraction on kainic-induced epileptic seizures in Sprague Dawley rats

Upon KA injection, all rats developed epileptic seizures which were characterised by three epileptic behaviours - wet dog shakes, facial myoclonia, and paw tremor (Figure 7.6). Each epileptic behaviour has its own characteristic of EEG and EMG activities. Total counts of wet dog shakes, facial myoclonia, and paw tremor were reduced by HF treatment (Figure 7.7). At the concentration of 5 mg/kg, HF effectively reduced the epileptic behaviour in KA-induced epileptic seizures in rats. Valproic acid is an anti-convulsant drug that is commonly used to treat epilepsy and bipolar disorder. It was used as a positive control in this study with administration of 250 mg/kg/day, similar to the study done by Ho, Tang, Hsiang, & Hsieh (2014).

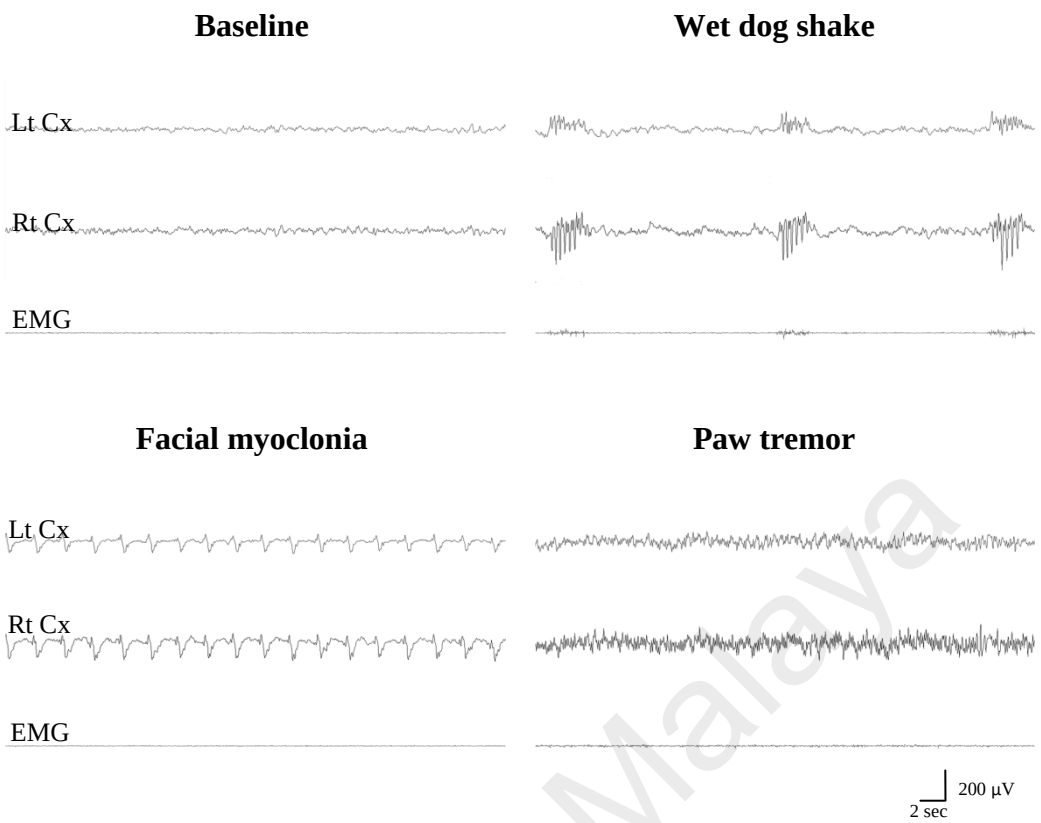


Figure 7.6: EEG and EMG recordings of KA-induced epileptic behaviours. Lt Cx: EEG recordings in the left sensorimotor cortex; Rt Cx: EEG recordings in the right sensorimotor cortex; EMG: EMG recordings in the neck muscles. Baseline recording of EEG and EMG was done prior to KA injection.

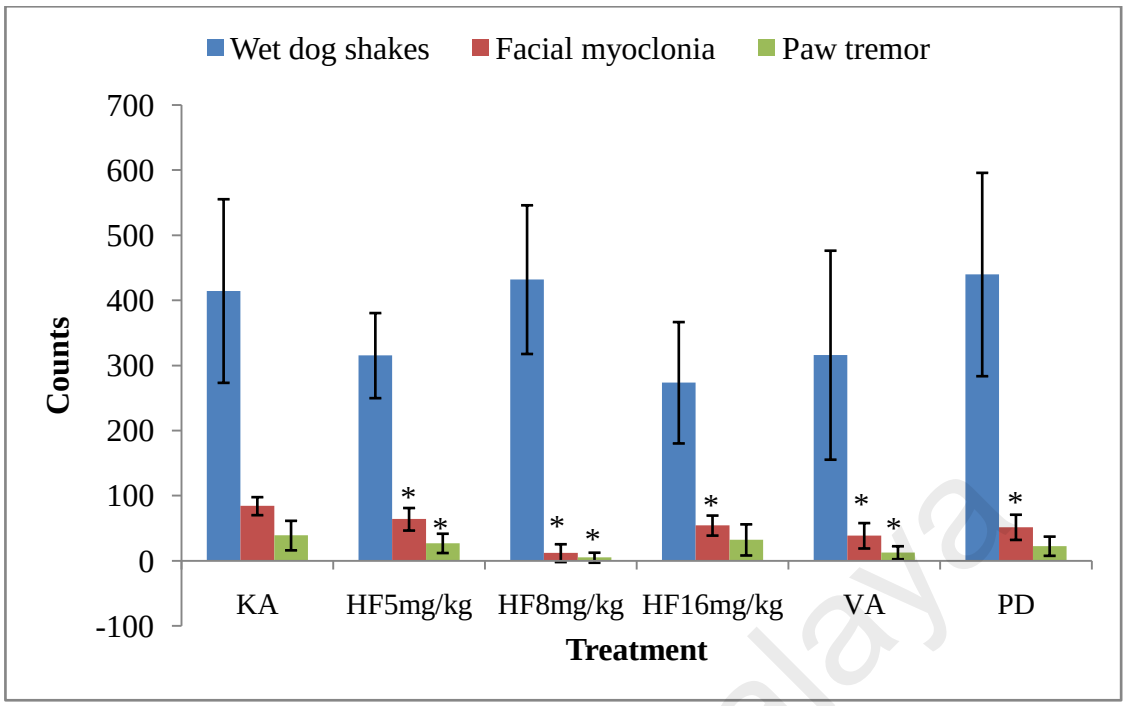


Figure 7.7: Effects of HF on KA-induced epileptic seizures in SD rats. Rats were administered with HF (5 mg/kg; 8 mg/kg; 16 mg/kg, respectively) 7 days prior to KA injection. Total counts of wet dog shakes, facial myoclonia, and paw tremor were observed for 3 hours after KA injection. Values are depicted in mean ± SD (n = 6/group) and *p < 0.05 compared to KA group. (KA: kainic acid group (negative control); HF5mg/kg: 5 mg/kg of hexane fraction; HF8mg/kg: 8 mg/kg of hexane fraction; HF16mg/kg: 16 mg/kg of hexane fraction; VA: valproic acid group (positive control); PD: PBS and 1% of DMSO vehicle group (normal group))

7.3.2 Effect of HF on blood biochemistry in KA-induced epileptic rats

Table 7.3 shows the effects of HF on blood biochemistry in KA-induced epileptic rats. There were no significant differences in levels of GPT, BUN, and creatinine between the samples. However, for GOT, there was significant difference between HF8mg/kg and HF16mg/kg administration.

Table 7.3: Effects of HF on blood biochemistry in KA-induced epileptic rats.

Group	GOT (U/L)	GPT (U/L)	BUN (mg/dL)	Creatinine (mg/dL)
KA	192.3 ± 61.3^{ab}	37.6 ± 11.0^{a}	26.3 ± 9.0^{a}	0.5 ± 0.3^{a}
HF5mg/kg	378.5 ± 234.1^{ab}	65.8 ± 75.8^{a}	21.8 ± 5.3^{a}	0.3 ± 0.1^{a}
HF8mg/kg	157.3 ± 87.2^{a}	25.2 ± 10.0^{a}	17.6 ± 6.2^{a}	0.3 ± 0.1^{a}
HF16mg/kg	422.1 ± 156.0^{b}	69.6 ± 25.4^{a}	32.1 ± 11.9^{a}	0.4 ± 0.2^{a}
VA	220.4 ± 81.3^{ab}	33.7 ± 10.7^{a}	24.7 ± 9.8^{a}	0.3 ± 0.1^{a}
PD	265.8 ± 118.4^{ab}	49.8 ± 21.3^{a}	20.0 ± 6.9^{a}	0.4 ± 0.1^{a}

Results are expressed in mean ± standard deviation (n=3). Different alphabets in the same column indicate significant difference between the samples ($p < 0.05$). GOT = aminotransferase aspirate, GPT = aminotransferase alanine, BUN = blood urea nitrogen. KA: kainic acid group (negative control); HF5mg/kg: 5 mg/kg of hexane fraction; HF8mg/kg: 8 mg/kg of hexane fraction; HF16mg/kg: 16 mg/kg of hexane fraction; VA: valproic acid group (positive control); PD: PBS and 1% of DMSO vehicle group (normal group).

7.3.3 Identification of compounds in HF

The chemical investigation of HF led to the identification of two major components, ethyl linoleate and ergosterol (Table 7.4, Figure 7.8).

Table 7.4: Chemical constituents of HF.

Chemical constituents	RT (min)	MW	MF	Peak Area (%)
Ethyl linoleate	24.81	308.27	$C_{20}H_{36}O_2$	4.30
Ergosterol	40.25	396.65	$C_{28}H_{44}O$	83.79

RT, retention time; MW, molecular weight (g mol^{-1}); MF, molecular formula.

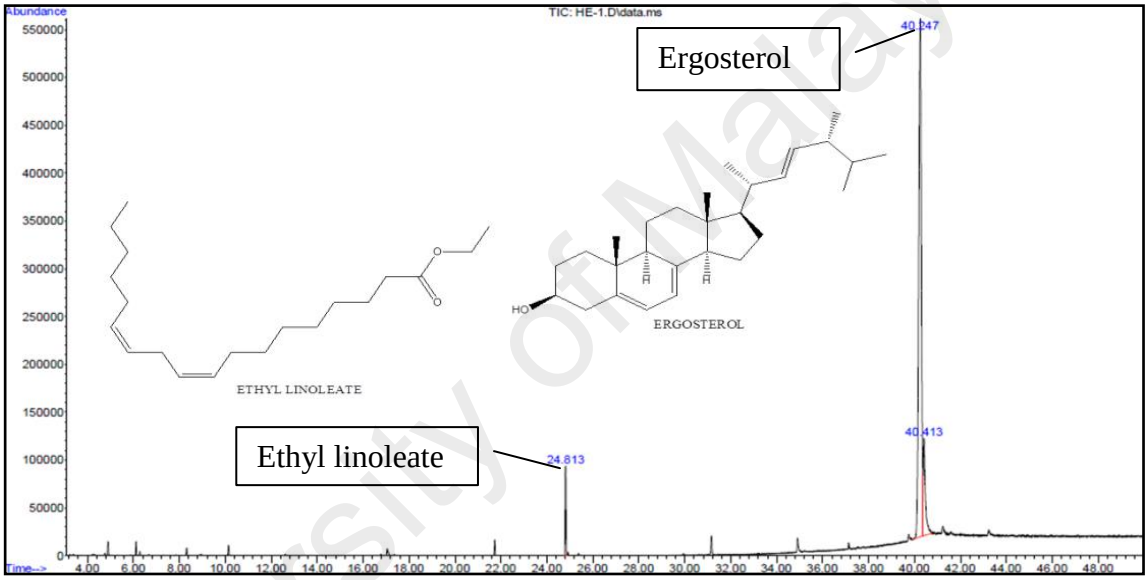


Figure 7.8: Chromatogram of HF of *A. rugosum*.

7.4 Discussion

Present study showed that the rats developed tonic-clonic seizures after KA intraperitoneal (i.p.) administration. The epileptic seizure behaviours include wet dog shake, facial myoclonia, and paw tremor as previously described by Hsieh et al. (1999). Wet dog shake began to appear about 20 minutes after KA i.p. administration and the maximal frequency was observed during the period from 60 to 80 minutes. Then, this characteristic progressed and was followed by the appearance of facial myoclonia and paw tremor. Intermittent polyspike-like EEG activity was seen in wet dog shakes and continuous spike activity in EEG was observed in paw tremor. Facial myoclonia was observed with asymmetrical continuous sharp wave in EEG. Similar observations were reported by Hsieh, Tang, Chiang, Hsieh, & Lin (1999).

Three different concentrations were tested on the rats which include 5 mg/kg, 8 mg/kg, and 16 mg/kg. Among the three concentrations tested, it was found that pre-treatment with 5 mg/kg of HF for 7 days was the most effective in suppressing the epileptic seizure behaviours. A similar study reported by Ho et al. (2014) showed that a traditional Chinese medicine, *Uncaria rhychophylla* and its bioactive compound, rhynchophylline exhibited anti-convulsive effects and significantly suppressed all the three epileptic seizure behaviours. Although 5 mg/kg of HF did not exhibit a potent effect in reducing the total counts of wet dog shakes as compared to the similar model described by Ho et al. (2014), it improved facial myoclonia and paw tremor behaviours. Thus, 5 mg/kg of HF may exhibit a partial anti-epileptic effect. Nevertheless, further examination on the pathohistology of the brain is warranted. On the other hand, 8 mg/kg of HF pre-treated rats had very low total counts of facial myoclonia and paw tremor. However, wet dog shake behaviour remained unchanged. It was observed that high counts of wet dog shakes in KA-induced epileptic seizure rats will have lower counts of

facial myoclonia and paw tremor. Therefore, the high counts of wet dog shakes observed in 8 mg/kg of HF pre-treated rats possibly affected the counts or frequency of facial myoclonia and paw tremor. Thus, further investigation on this postulation is vital in future studies. Based on the blood biochemistry results, short period of HF administration did not influence the liver and kidney functions.

Two major components were identified in HF, namely ethyl linoleate and ergosterol. Ethyl linoleate has been reported to alleviate inflammation and its combination with antioxidants such as α-1-histidine, α-tocopherol and tertiary butyl hydroquinone (TBHQ) can be used as a non-steroidal topical agent (Jelenko, Wheeler, Anderson, Callaway, & Mckinley, 1975). Ergosterol is abundant in many mushrooms and is known to be pro-vitamin D_2 (Kobori et al., 2007). The conversion of ergosterol to vitamin D_2 via UV radiation has a long history of commercial use of vitamin D_2 production as dietary supplements, pharmaceutical grade vitamin D preparations and food fortification (Phillips et al., 2011). A few studies have reported the successful conversion of ergosterol to vitamin D_2 in mushrooms via UV irradiation (Kalaras, Beelman, Holick, & Elias, 2012; Koyyalamudi, Jeong, Song, Cho, & Pang, 2009). According to Ma, Chen, Dong, & Lu (2013), ergosterol isolated from *Inonotus obliguus* was found to possess anti-inflammatory activities. Also, ergosterol purified from *Sarcodon aspratus* showed significant inhibition of LPS-induced inflammatory responses through nuclear factor-kappa B (NF-κB) and CAAT/enhancer binding protein-beta (C/EBPβ) and prevented the phosphorylation of p39, JNK and ERK MAPKs (Kobori et al., 2007). The Temuan indigenous people in Malaysia claimed that wearing the stipe of *A. rugosum* can prevent epileptic episodes (Azliza et al., 2012; Chang & Lee, 2004). The presence of these two compounds (ergosterol and ethyl linoleate) in HF may be responsible for the anti-epileptic activity demonstrated in this

study. However, further validation on the bioactivity of these two compounds is required. Besides, there may be presence of other volatile compounds in this mushroom thay may contribute to the bioactivity. In depth analysis on the potential bioactive compounds is vital for future studies.

7.5 Conclusion

In conclusion, HF was found to ameliorate the epileptic seizure behaviours. These results validated the traditional use of *A. rugosum* to prevent epileptic episodes by the Temuan indigenous population in Malaysia. Further in depth analysis on the histology and signalling pathways are crucial for better understanding on the mechanism of actions. Two major components were detected in HF, which were ethyl linoleate and ergosterol.

CHAPTER 8

CONCLUSION AND RECOMMENDATION

Amauroderma rugosum is a medicinal wild mushroom that is believed to be able to prevent cancer, epileptic episodes and incessant crying by babies, treat diuresis, and reduce inflammation. This mushroom is commonly used by the Chinese in China and Temuan people in Malaysia. However, the medicinal effects of *A. rugosum* that have been verbally transmitted by the indigenous people are not scientifically verified. To the best of our knowledge, this is the first scientific report which shows successful cultivation of *A. rugosum* and first description on the antioxidant, anti-inflammatory, anti-neuroinflammatory, and anti-epileptic activities.

In this study, *A. rugosum* basidiocarps have been successfully cultivated and it was found that submerged cultivation can be applied to increase the production of *A. rugosum*. However, further optimisation of the cultivation method is needed to achieve a higher biological efficiency and improve the quality of the fruiting bodies and mycelia of *A. rugosum*. Besides, different extraction and fractionation techniques and methods can be considered to increase the yield of extracts.

This study found that mycelia of *A. rugosum* were rich in nutrients and showed good antioxidant activities. The fruiting bodies (wild (WB) and cultivated (DB)) of *A. rugosum* showed similar total phenolic content and DPPH scavenging activity. However, WB had higher ABTS scavenging activity compared to DB. For the mycelia extracts, EAF displayed the highest antioxidant activities and total phenolic content,

followed by HF, AF, and EE. Further evaluation and tests can be done to confirm the potential antioxidant activity of *A. rugosum*.

Also, this study provides further evidences on the potential anti-inflammatory effect of *A. rugosum*. The hexane fraction (HF) from mycelia and WB and DB showed effective scavenging effect on nitric oxide (NO) radicals and suppression of lipopolysaccharide (LPS)-stimulated NO production in RAW264.7 macrophage cells. This suppressive effect was due to the down-regulation of the inducible nitric oxide synthase (iNOS) gene. Furthermore, HF, WB, and DB down-regulated the proinflammatory cytokine tumour necrosis factor alpha (TNF-α) and up-regulated anti-inflammatory cytokine interleukin-10 (IL-10). Conversely, nuclear translocation of necrosis factor kappa B (NF-κB) was not blocked. Further evaluation on the anti-inflammatory activities using different techniques such as western blot and electrophoretic mobility shift assay (EMSA) may be helpful to confirm the results of this study. Besides, further in depth evaluation on the protein and gene expressions may be carried out in future.

Both the basidiocarps and mycelia of *A. rugosum* extracts showed anti-neuroinflammatory activity. Due to the limited samples of the fruiting bodies, the best extract from the mycelia was selected for further in-depth study. The HF showed good antioxidant and the best anti-inflammatory activities. Thus, it was selected for further investigation on the modulation of neuroinflammatory pathways leading to epilepsy. It was found that the HF significantly reduced the LPS-stimulated NO production in BV-2 microglia cells and regulated 10 neuroinflammatory pathways. Besides, HF suppressed the proinflammatory cytokines such as IL-1α, IL-1β, and IL-6, and other proinflammatory genes such as Traf1, Traf2, and Cd14. Moreover, HF promoted the up-regulation of anti-inflammatory genes such as Nfkbia and Nfkbie. The anti-

inflammatory and anti-neuroinflammatory activities shown by HF strongly suggest its potential in mitigation of inflammation leading to epilepsy. Thus, HF was tested for anti-epileptic potential to provide a further assessment and evidence on its bioactivity.

This study discovered that HF had anti-epileptic potential which can ameliorate epileptic seizure behaviours such as wet dog shakes, facial myoclonia, and paw tremor. The inhibition of chronic inflammation may decrease seizure susceptibility. Hence, this further suggests that HF may have the ability to mitigate inflammation and reduce the epileptic seizure behaviours. However, further in-depth study on the histology, mechanism, and targeted signalling pathways are warranted. Two major compounds were detected in HF which includes ethyl linoleate and ergosterol. Both the compounds were reported to alleviate inflammation and neuroinflammation.

In summary, the above mentioned findings suggest that HF of *A. rugosum* may attenuate the pathophysiological cascade of inflammatory events and therefore ameliorate epilepsy. The bioactivity of *A. rugosum* demonstrated in this study validated the traditional use of this mushroom to reduce inflammation by the Chinese population and prevent epileptic episodes by the Temuan indigenous people.

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LIST OF SCHOLARLY CONTRIBUTIONS AND PROCEEDINGS

SCHOLARLY CONTRIBUTIONS

1) Chan, P. M., Kanagasabapathy, G., Tan, Y. S., Sabaratnam, V., & Kuppusamy, U. R. (2013). *Amauroderma rugosum* (Blume & T. Nees) Torrend: nutritional composition and antioxidant and potential anti-Inflammatory properties. *Evidence-Based Complementary and Alternative Medicine , 2013,* Article ID304713, 10 pages. doi:10.1155/2013/304713

2) Chan, P. M., Tan, Y. S., Chua, K. H., Sabaratnam, V., & Kuppusamy, U. R. (2015). Attenuation of inflammatory mediators (TNF-α and nitric oxide) and up-regulation of IL-10 by wild and domesticated basidiocarps of *Amauroderma rugosum* (Blume & T. Nees) Torrend in LPS-stimulated RAW264.7 Cells. *PLoS ONE, 10*(10), e0139593, 21 pages. doi:10.1371/journal.pone.0139593

PROCEEDINGS / ABSTRACTS

1) Chan, P. M., Tan, Y. S., Sabaratnam, V., & Kuppusamy, U. R. (2012). Antioxidant and anti-inflammatory properties of submerged culture mycelia extracts of *Amauroderma rugosum* Blume & T. Nees. *The 17th Biological Sciences Graduate Congress (17th BSGC), December 8-10, 2012, Chulalongkorn University, Bangkok, Thailand.*

2) Chan, P. M., Tan, Y. S., Sabaratnam, V., & Kuppusamy, U. R. (2013). The use of *Amauroderma rugosum* (Blume & T. Nees) Torrend as potential anti-inflammatory agent. *Asia-Korea Conference on Science and Technology (AKC2013), November 21-23, 2013, Resorts World Sentosa Convention Center, Singapore.*

3) Chan, P. M., Tan, Y. S., Sabaratnam, V., Chua, K. H., & Kuppusamy, U. R. (2014). Antioxidant and potential anti-inflammatory activity of basidiocarp of *Amauroderma rugosum* (Blume & T. Nees) Torrend. *The 18th Biological Sciences Graduate Congress (18th BSGC), January 6-8, 2014, University of Malaya, Kuala Lumpur, Malaysia.*

4) Chan, P. M., Tan, Y. S., Chua, K. H., Sabaratnam, V., & Kuppusamy, U. R. (2015). *Amauroderma rugosum* (Blume & T. Nees) Torrend: a potential antioxidant and anti-neuroinflammatory agent. *International Conference on Antioxidants and Degenerative Diseases (ICADD), June 3-4, 2015, Istana Hotel, Kuala Lumpur, Malaysia.*

5) Chan, P. M., Tan, Y. S., Chua, K. H., Sabaratnam, V., & Kuppusamy, U. R. (2015). Modulation of the neuroinflammatory pathways by *Amauroderma rugosum* (Blume & T. Nees) Torrend ergosterol-rich fraction. *2nd International Conference of Traditional and Complementary Medicine on Health 2015 (ICTMH2015), October 24-26, 2015, GIS NTU Convention, Taipei, Taiwan.*

6) Chan, P. M., Tan, Y. S., Chua, K. H., Sabaratnam, V., & Kuppusamy, U. R. (2015). Unlocking the anti-inflammatory potential of medicinal mushroom: *Amauroderma rugosum* (Blume & T. Nees) Torrend. *The 4th International Conference on Life Science & Biological Engineering, November 4-6, 2015, Ana Crowne Plaza Grand Court Nagoya, Nagoya, Japan.*